Studies toward chemical synthesis of protein: application of *N*-sulfanylethylanilide peptide as crypto-thioester

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Abbreviations

Acm	acetamidemethyl
Boc	*tert*-butoxycarbonyl
Bu	butyl
DIEA	*N,N'*-diisopropylethylamine
DIPCDI	*N,N'*-diisopropylcarbodiimide
DMF	*N,N'*-dimethylformamide
DNA	deoxyribonucleic acid
EDT	ethanedithiol
ESI-MS	electrospray ionization mass spectrometry
Fmoc	9-fluorenylmethoxycarbonyl
GM2AP	GM2 activator protein
Gn·HCl	guanidine hydrochloride
hANP	human atrial natriuretic peptide
HATU	*O*-(7-azabenzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HBTU	*O*-benzotriazole-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HEPPS	3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid
HexA	β-hexosaminidase A
HOBt	1-hydroxybenzotriazole
KCL	kinetically controlled ligation
MBHA	4-methylbenzhydrylamine
Me	methyl
MeCN	acetonitrile
MPPA	4-mercaptophenylacetic acid
NCL	native chemical ligation
Ph	phenyl
RP-HPLC	reversed-phase high-performance liquid chromatography
rt	room temperature
SEAlide	*N*-sulfanylethylanilide
SPPS	solid phase peptide synthesis
TCEP·HCl	tris(2-carboxyethyl)phosphine hydrochloride
Tf	trifluoromethanesulfonyl

TFA	trifluoroacetic acid
TMS	trimethylsilyl
Trt	triphenylmethyl

The commonly used one- and three-letter abbreviations for amino acids:

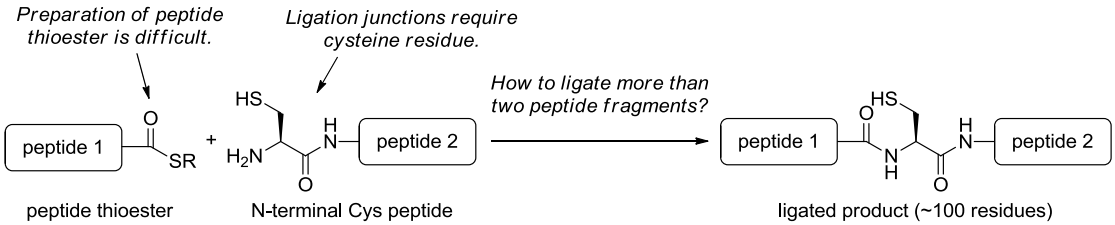
A	(Ala)	Alanine
C	(Cys)	Cysteine
D	(Asp)	Aspartic acid
E	(Glu)	Glutamic acid
F	(Phe)	Phenylalanine
G	(Gly)	Glycine
H	(His)	Histidine
I	(Ile)	Isoleucine
K	(Lys)	Lysine
L	(Leu)	Leucine
M	(Met)	Methionine
N	(Asn)	Asparagine
P	(Pro)	Proline
Q	(Gln)	Glutamine
R	(Arg)	Arginine
S	(Ser)	Serine
T	(Thr)	Threonine
V	(Val)	Valine
W	(Trp)	Tryptophan
Y	(Tyr)	Tyrosine

Contents

Preface

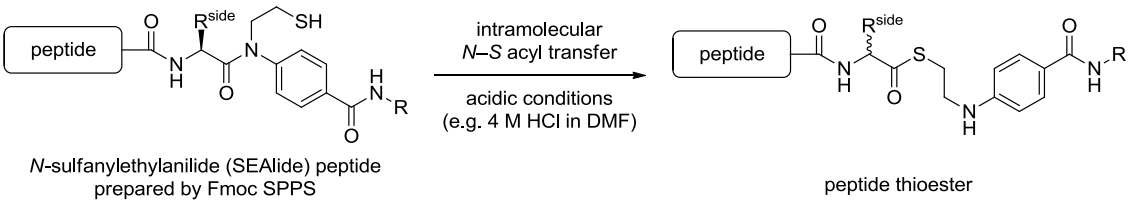
The homeostatic system relies on the appropriate functioning of a wide variety of proteins. There is a tremendous opportunity for protein therapeutics in the treatment of protein-related disorders.[1] Genetic engineering has allowed us to prepare naturally occurring proteins or protein therapeutics in medicine. Methods to strictly control of post-translational modifications such as glycosylation and introduce non-canonical amino acids can still be improved.

Chemical synthesis is an alternative route for preparation of functionalized proteins containing homogeneous post-translational modification or unnatural structural units. Native chemical ligation (NCL), featuring a chemoselective reaction between a peptide thioester and an N-terminal cysteinyl peptide, is among the most reliable ligation techniques in chemical protein synthesis (Scheme 1).[2] Although NCL has enabled construction of proteins consisting of around 100 residues with comparative ease, chemical synthesis of huge and complex proteins is still problematic due to the difficulty of preparing peptide thioesters, lack of a practical condensation protocol applicable to more than two peptides, and limitations on ligation sites.



Scheme 1. Native chemical ligation to assemble two unprotected peptides.

Recently, we developed *N*-sulfanylethylanilide (SEAlide) peptide as a useful chemical device that enables peptide thioester preparation through 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS) (Scheme 2).[3] Although the SEAlide peptide could be converted to the corresponding thioester under acidic conditions, the acidic treatment sometimes caused partial epimerization at the C-terminal amino acid.



Scheme 2. *N*-Sulfanylethylanilide peptide as a peptide thioester precursor.

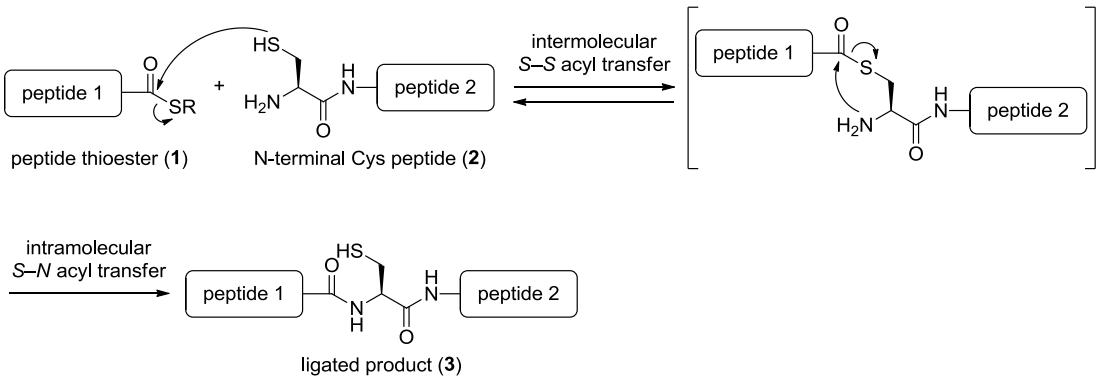
In this study, we disclose that the SEAlide peptide can participate in NCL under neutral phosphate buffer conditions without C-terminal epimerization and can be included in a practical one-pot multi-peptide ligation enabling the synthesis of a protein of over 100 residues. We also develop a novel technique to ligate peptides at the site of an *N*-glycosylated asparagine instead of a cysteine residue. This technique can be successfully applied to the total chemical synthesis of monoglycosylated GM2 activator protein (GM2AP) consisting of 162 residues.

In Chapter 1, discovery of an unexpected behavior of SEAlide peptides (i.e., direct participation in NCL under neutral conditions in the presence of phosphate salts) is described. A one-pot/three- or four-component ligation procedure using SEAlide peptides is also presented. Application of the multi-peptide ligation to synthesis of GM2AP analog with cysteine at the glycosylation site instead of asparagine is reported in Chapter 2. In Chapter 3, development of a novel ligation/desulfurization technique facilitating ligation at *N*-glycosylated asparagine junctions and its application to total chemical synthesis of native GM2AP is presented.

Chapter 1
Using *N*-sulfanylethylanilide peptide as a crypto-thioester in one-pot/sequential ligation

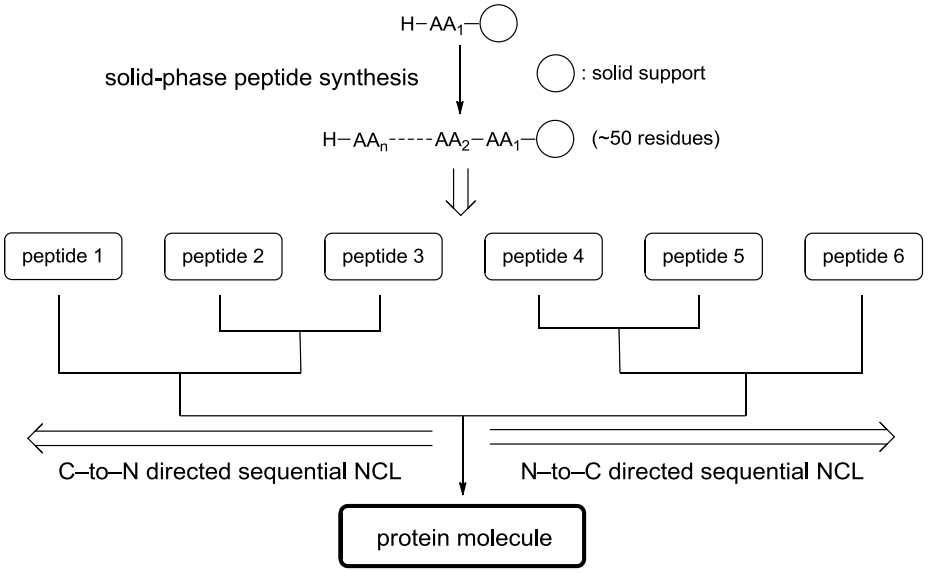
1.1 Sequential native chemical ligation for convergent chemical synthesis of proteins

In 1994, Kent and co-workers reported native chemical ligation (NCL), a simple technique to assemble two unprotected peptide fragments.[2] In the NCL protocol, a peptide thioester **1** chemoselectively reacts with an N-terminal cysteinyl peptide **2** through intermolecular *S*–*S* acyl transfer followed by intramolecular *S*–*N* acyl transfer to afford a ligated product **3** with a native peptide bond (Scheme 1.1). The feasibility of NCL has already been demonstrated through syntheses of numerous proteins, including enzymes,[4] glycoproteins possessing a homogeneous glycoform,[5] and proteins consisting of over 300 residues.[6] Therefore, NCL is recognized as among the most reliable ligation techniques available to chemically synthesize proteins.

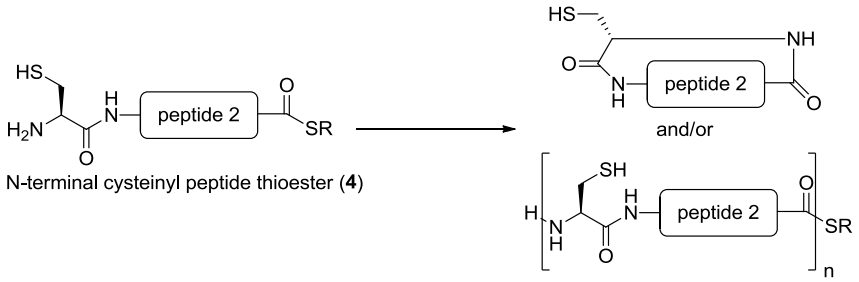


Scheme 1.1. Reaction mechanism of native chemical ligation.

Access to proteins over 100 residues frequently requires C–to–N and N–to–C directed sequential NCL protocols followed by convergent assembly because of the up to 50-residue limit on chain length achievable by solid-phase peptide synthesis (SPPS) (Scheme 1.2).[7] To achieve sequential NCLs between more than two peptides, an N-terminal cysteinyl peptide thioester fragment **4** is an indispensable intermediate; however, such an intermediate cannot be directly used without protecting amino and/or thiol functions on the N-terminal cysteine because of undesired inter- or intramolecular reactions of the N-terminal cysteine with the thioester moiety (Scheme 1.3).[8] For



Scheme 1.2. Sequential NCLs for convergent assembly of multi-peptide fragments.



Scheme 1.3. Side reactions of N-terminal cysteinyl peptide thioester.

sequential NCL leading to successful multi-peptide assembly in a C–to–N directed manner, several strategies using temporal protections on the cysteine have been reported (Figure 1.1).[9]

In contrast, N–to–C directed sequential NCL has remained a more challenging task due to the difficulty of adjusting the reactivity of thioesters. One pioneering approach to sequential ligation in an N–to–C directive manner is kinetically controlled ligation (KCL), reported by Kent and co-workers; it features the use of alkyl and aryl thioesters exhibiting sufficiently distinct reactivities (Scheme 1.4).[10] In this protocol, the cysteine moiety of the middle fragment **6** preferentially reacts with the more reactive aryl thioester **5** in an intermolecular manner to afford the ligated alkyl thioester **7**. The resulting alkyl thioester is then subjected to the next ligation step with C-terminal fragment **8** through *in situ* activation of the alkyl thioester to an aryl thioester by treatment with an aryl thiol. The consecutive reactions can be conducted in a one-pot manner preferred for saving time and resources while increasing chemical yield.

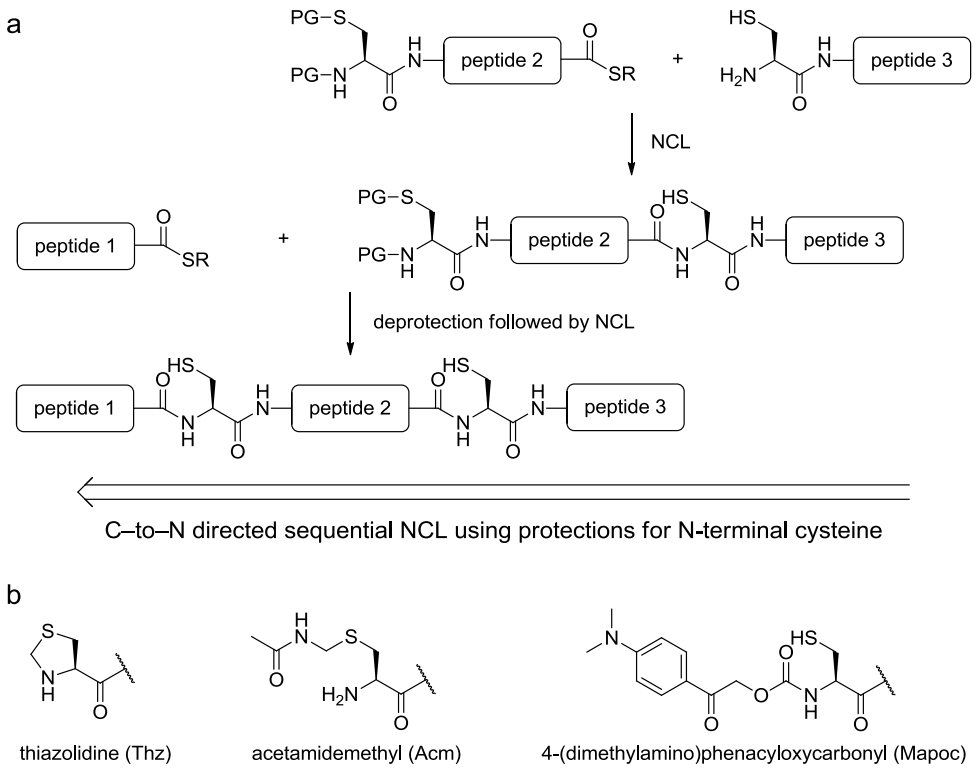
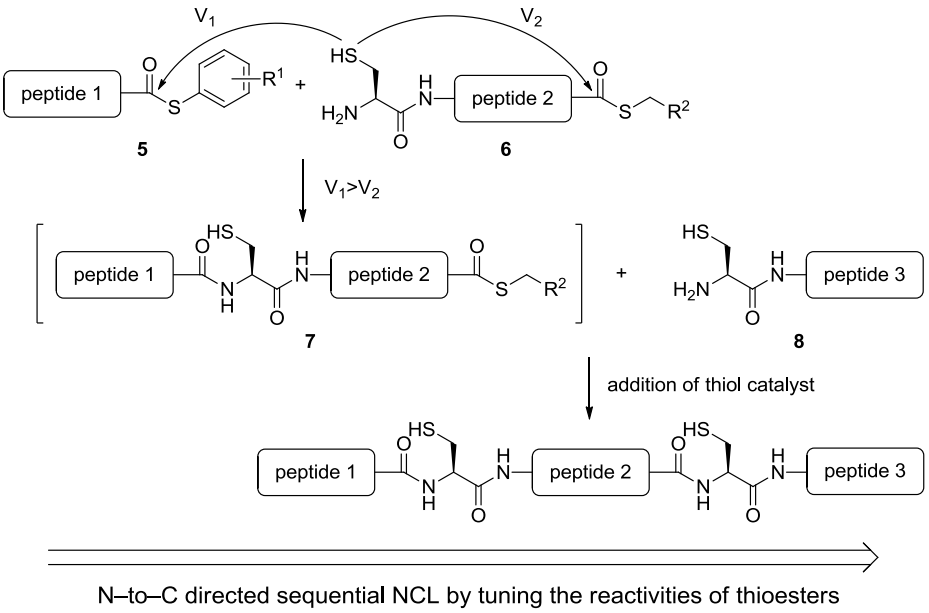
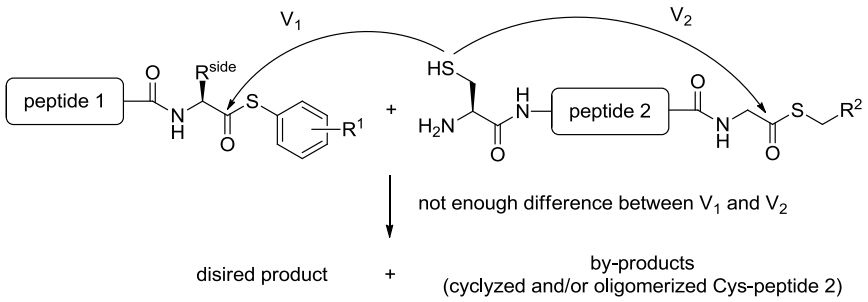


Figure 1.1. (a) C–to–N Directed sequential NCL with protections for N-terminal cysteine. (b) Examples of protections for N-terminal cysteine. PG: protecting group.



Scheme 1.4. Three-fragment assembly by using kinetically controlled ligation.

Although the KCL protocol has great potential to provide fully convergent synthetic routes, it is limited because the use of highly reactive amino acids such as glycine at the C-terminus of an alkyl thioester peptide causes side reactions due to the lack of reactivity differences between aryl and alkyl thioesters (Scheme 1.5).[11] Because of the limited number of ligation junctions, a practical and reliable N–to–C directed sequential NCL has been pursued.



Scheme 1.5. Side reactions of KCL with a highly reactive alkyl thioester.

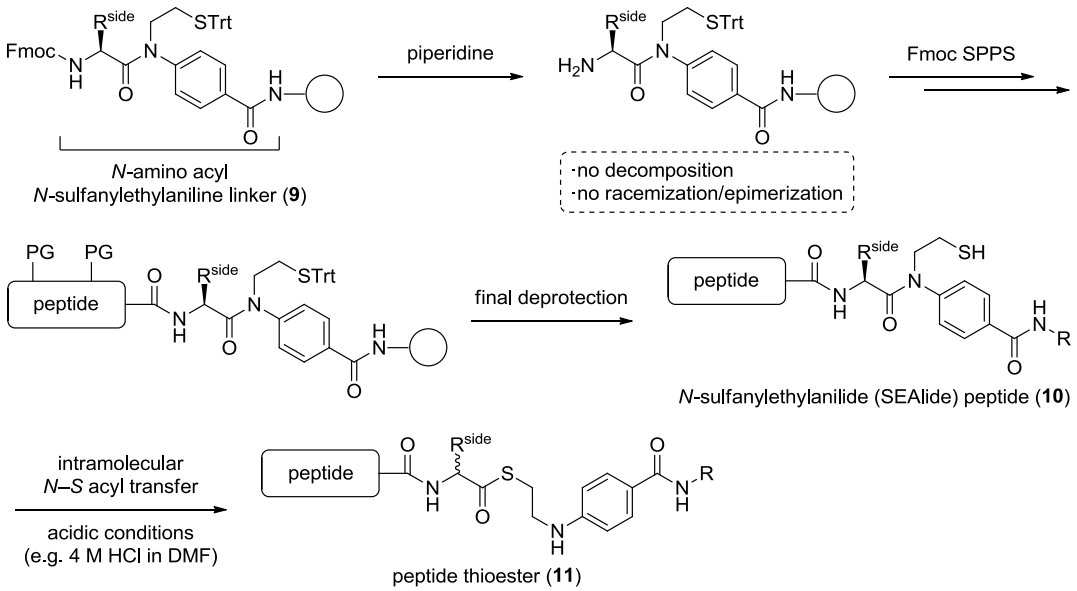
1.2 Use of N*-sulfanylethylanilide peptide as peptide thioester precursor: initial attempts at N–to–C directed sequential NCL*

The use of 9-fluorenylmethyloxycarbonyl (Fmoc)-based SPPS is preferred to *tert*-butyloxycarbonyl (Boc)-based SPPS because it is easier to perform and more compatible with modified peptides such as phosphopeptides and glycopeptides; however, the Fmoc protocol has seldom been used to prepare peptide thioesters directly because of their sensitivity to the basic reagents required for removal of the Fmoc group (Scheme 1.6).[12] Therefore, a methodology compatible with Fmoc chemistry to prepare peptide thioesters has been explored.[13, 14]

In the reported literature, procedures using *N–S* acyl transfer chemistry have shown potential utility in Fmoc SPPS-based thioester syntheses.[15, 16] In this context, our research group has also developed an *N*-sulfanylethylanilide (SEAlide) peptide that can be synthesized by Fmoc SPPS and used as a precursor to prepare peptide thioesters through intramolecular *N–S* acyl transfer.[3] The concept of peptide thioester synthesis based on the SEAlide peptide is summarized in Scheme 1.7. Peptide chain elongation by Fmoc SPPS on an *N*-amino acyl *N*-sulfanylethylaniline linker **9** followed by global deprotection and cleavage from the resin affords SEAlide peptides **10** possessing an amide bond at the C-terminus. The construction of SEAlide peptides having no thioester

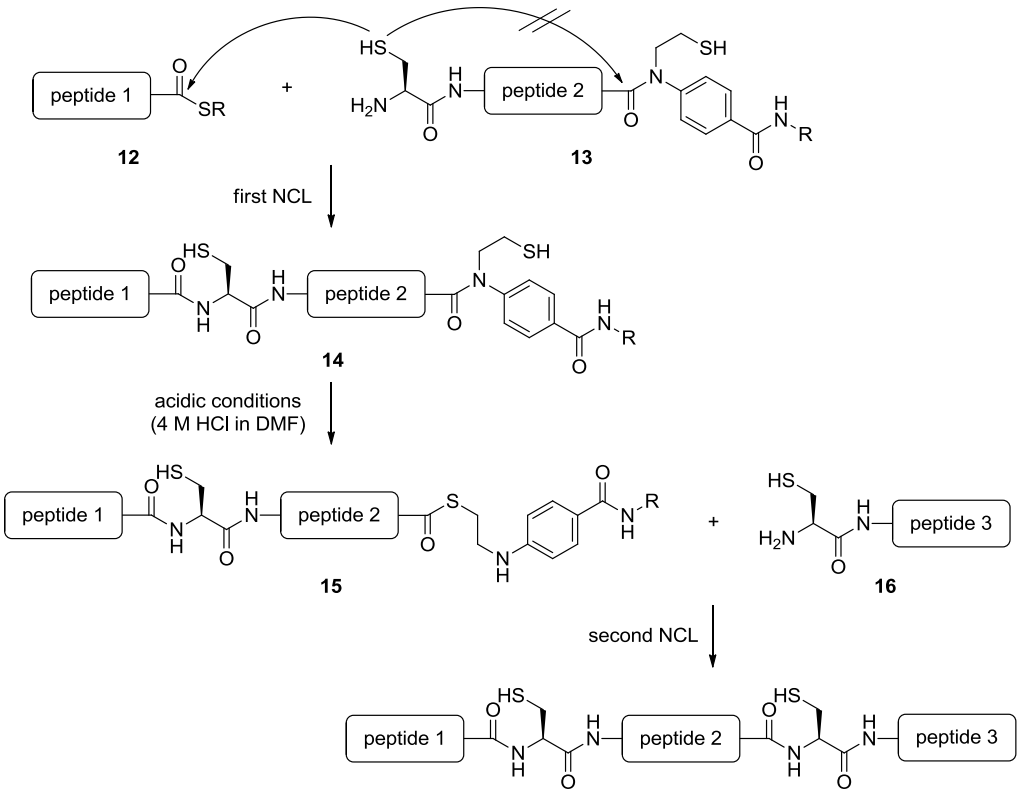
functionality does not cause side reactions, namely, decomposition and epimerization of the C-terminal residue. The resulting peptide can be converted to the corresponding thioester **11** under acidic conditions even though such acidic treatment sometimes causes epimerization at the C-terminal amino acid.

Based on the fact that the SEAlide peptides can be converted to the corresponding thioesters under acidic conditions, use of an N-terminal cysteinyl SEAlide peptide as a middle fragment was planned for N–to–C directed NCL (Scheme 1.8). The SEAlide peptide **13** possessing no thioester functions should react selectively with a thioester peptide **12**, affording the intermolecularly ligated product **14**. The resulting SEAlide peptide would participate in the next NCL step with N-terminal cysteinyl peptide **16** after conversion of the anilide moiety to the corresponding thioester **15** by acidic treatment.



Scheme 1.7. *N*-Sulfanylethylanilide peptide working as a thioester precursor. PG: protecting group.

The envisioned sequential NCL was attempted through the synthesis of CXCL14,[17] a member of the CXC family of chemokines consisting of 77 amino acids (Figure 1.2). Initially, the ligation between the N-terminal fragment **17** and the middle SEAlide fragment **18** was conducted in 6 M guanidine hydrochloride (Gn·HCl)-0.2 M sodium phosphate, pH 7.4, in the presence of tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) and 4-mercaptophenylacetic acid (MPAA) as a reducing reagent for disulfide bonds and a catalyst for NCL, respectively. The reaction progress was monitored by reversed-phase high-performance liquid chromatography (RP-HPLC)



Scheme 1.8. Strategy for N–to–C directed sequential NCL using SEAlide peptide.

as shown in Figure 1.2. After reaction at 37 ºC for 3.5 hours, decreased levels of the starting materials and formation of the ligated SEAlide peptide **19** were observed. Contamination of the cyclic material **20** was confirmed by mass spectrometry analysis of the peak eluted at 13.5 minutes.

The generation of the cyclized middle fragment **20** during NCL was in contradiction to preliminary results showing that the conversion of the SEAlide to the thioester occurs only under acidic conditions. If the formation of the cyclic peptide resulted from an intramolecular NCL, the SEAlide moiety should function as a thioester equivalent under neutral conditions. On the basis of these results, the synthesis of CXCL14 was discontinued and the investigation of the potential of the SEAlide peptides as a thioester equivalent, namely "a crypto-thioester", was begun.

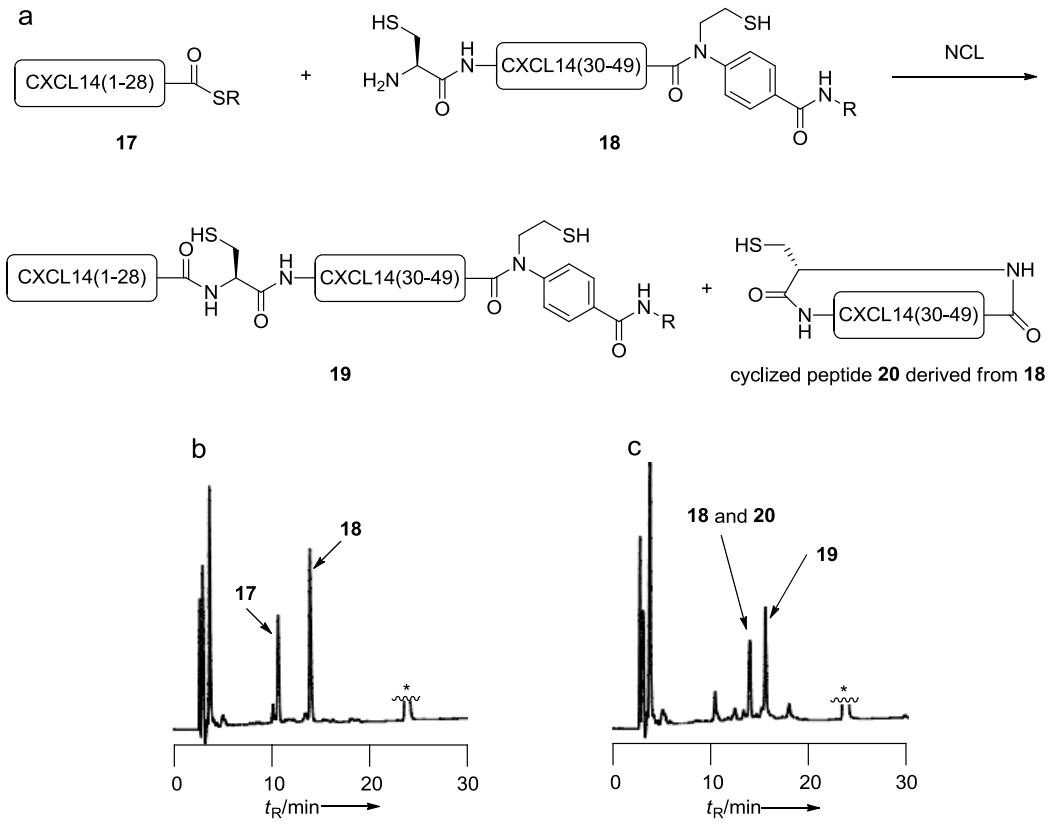
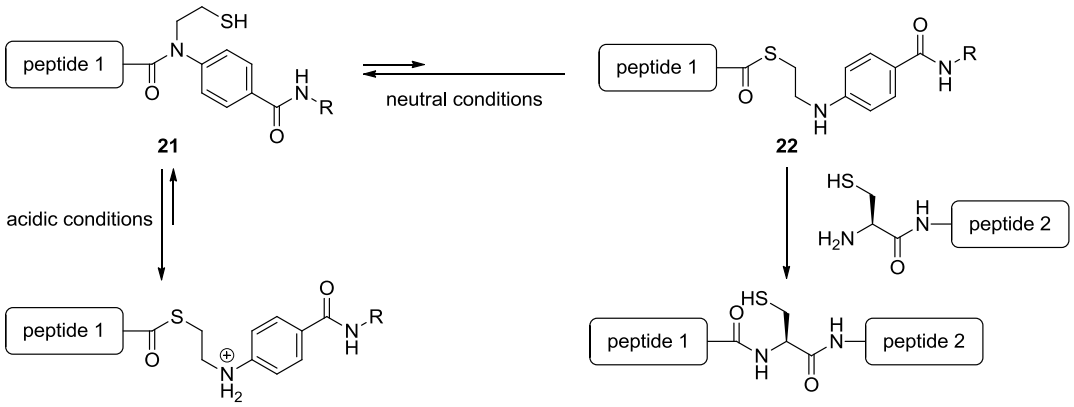


Figure 1.2. (a) NCL for synthesis of CXCL14 (^{28}Gly, ^{48}Gly). (b and c) HPLC monitoring of NCL between **17** and **18**. Fragments **17** (1 mM) and **18** (1 mM) were ligated in 0.2 M sodium phosphate buffer with 6 M Gn·HCl (pH 7.4) in the presence of 20 mM TCEP·HCl and 30 mM MPAA at 37 ºC. (b) After <5 min of incubation. (c) After 3.5 h of incubation. HPLC conditions: a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (15:85–45:55 over 30 min). *MPAA.

1.3 Evaluation of SEAlide peptide as crypto-thioester under usual NCL conditions

Based on the results described in the previous section, we speculated that the equilibrium between the anilide **21** and the thioester **22** would exist under neutral conditions. But the amount of thioester **22** is too small to be detected by RP-HPLC whereas stabilization of the thioester by protonation of the amino group is observed under acidic conditions (Scheme 1.9). In a neutral buffer containing an N-terminal cysteinyl peptide, the amount of thioester **22** is almost negligible; however, slightly formed **22** should be consumed by a reaction with the N-terminal cysteinyl peptide, thereby the equilibrium between **21** and **22** is shifted to thioester formation. To evaluate the feasibility of the SEAlide peptides as a crypto-thioester, a model reaction under usual



Scheme 1.9. SEAlide peptide as potential peptide thioester equivalent.

NCL conditions was attempted.

The model reaction of the SEAlide peptide **23a** with the N-terminal cysteinyl peptide **24** was conducted in 6 M Gn·HCl-0.2 M sodium phosphate in the presence of TCEP·HCl and MPAA, pH 7.3, 37 ºC (Figure 1.3). The reaction was almost complete within 48 hours and yielded the ligated peptide **25a** in 59% isolated yield. No intermediary thioester peptide was observed during the reaction. These results supported the hypothesis that SEAlide peptides can function as crypto-thioesters in the presence of N-terminal cysteinyl peptides and participate in NCL directly without pre-conversion to the corresponding thioesters.

Next, reactions of the SEAlide peptide with the N-terminal cysteinyl peptide were examined under various conditions (Table 1.1). The use of the two-fold amount of the SEAlide **23a** accelerated the NCL; however, the inverse ratio of the substrates did not affect the reaction rate (entries 1, 3, and 4). The NCL progress was completely suppressed by the use of an *S*-alkylated SEAlide peptide (entry 5). These results indicated that the intramolecular *N*–*S* acyl transfer of SEAlide peptides is an essential step in the reaction with cysteinyl peptides and a rate-determining step in the overall reaction. In a pH range of 6.0–8.2, no significant difference in the reaction progress was observed (entries 1, 6 and 7).

The reaction rate remarkably depended on buffer salts in the reaction media. Increasing the concentration of phosphate salts accelerated the NCL (entries 8–10). Unlike the phosphate salts, the use of 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid (HEPPS) or sodium acetate did not affect the reaction rate (entries 1, 11, and 12). Although imidazole is a potential additive to accelerate a ligation of peptide phenyl esters with N-terminal cysteinyl peptides,[18] the acceleration effect of imidazole buffer was lower than that of phosphate buffer (entry 13). The reaction suppression in

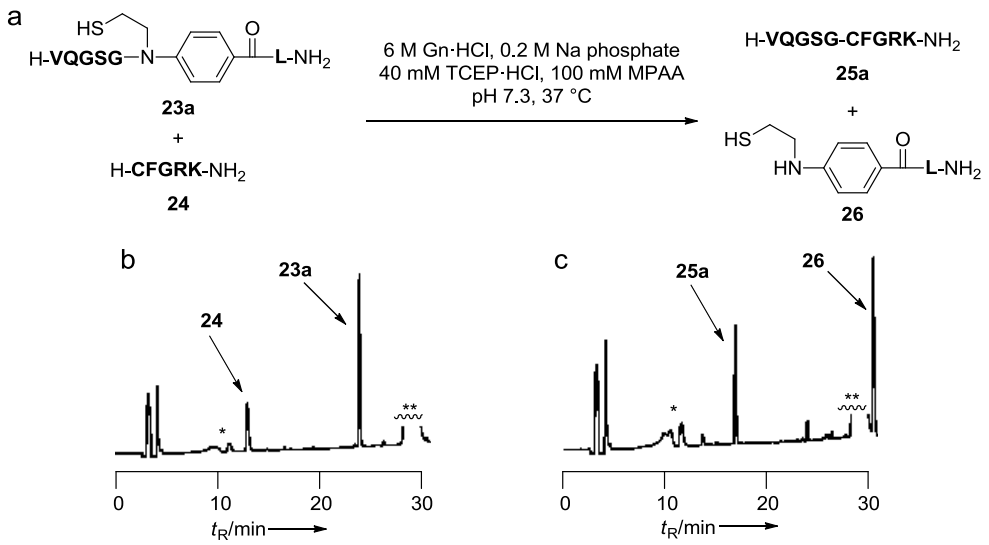
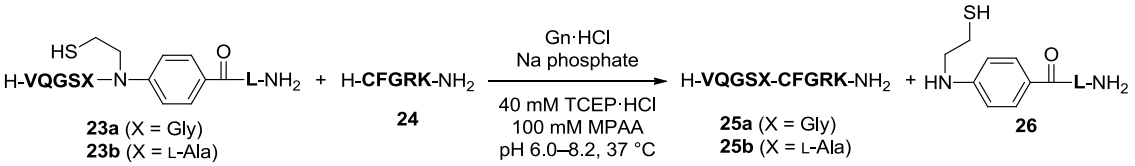


Figure 1.3. (a) NCL of SEAlide peptide **23a** without pre-conversion to thioester. (b, c) HPLC monitoring of NCL between **23a** and **24**. Fragments **23a** (1 mM) and **24** (1 mM) were ligated in 0.2 M sodium phosphate buffer with 6 M Gn·HCl (pH 7.3) in the presence of 40 mM TCEP·HCl and 100 mM MPAA at 37 ºC. (b) After <5 min of incubation. (c) After 48 h of incubation. HPLC conditions: a gradient curve 7 (Waters 600E) of 0.1% TFA-MeCN/0.1% TFA aq. (7:93–50:50 over 30 min). *Non-peptidic impurity. **MPAA.

HEPPS buffer was released by subsequent addition of phosphate salts, and the ligation yielded product efficiently (entry 14). While the effect was lesser than that of buffer salts, denaturant also affected the reaction rate (entries 1, 15, and 16). Addition of Gn·HCl might induce decrease in reaction rate through salt formation between phosphate and Gn, causing an effective concentration decrease of phosphate. Whereas acidic conversion of SEAlide peptide caused epimerization of a C-terminal chiral amino acid, the direct use of SEAlide peptide **21b** possessing alanine at the C-terminus did not cause the epimerization (entry 17).

These results indicated that the *N–S* acyl transfer of SEAlide peptides occurs even in neutral aqueous buffer in the presence of phosphate salts to form the corresponding thioesters and directly participates in NCL-based assembly with N-terminal cysteinyl peptides. Although the role of phosphate salts in the NCL has yet to be elucidated, one possible explanation is that phosphate salts might function as an acid-base catalyst to accelerate the intramolecular *N–S* acyl transfer of SEAlide peptides (Scheme 1.10). It is noteworthy that the use of SEAlide peptides without pre-conversion to the corresponding thioesters has advantages (namely, operational simplicity and no accompanying epimerization) over the previous method requiring acidic treatments.

Table 1.1. Model ligation of SEAlide peptides with N-terminal cysteinyl peptide.



23a (X = Gly)
23b (X = L-Ala)

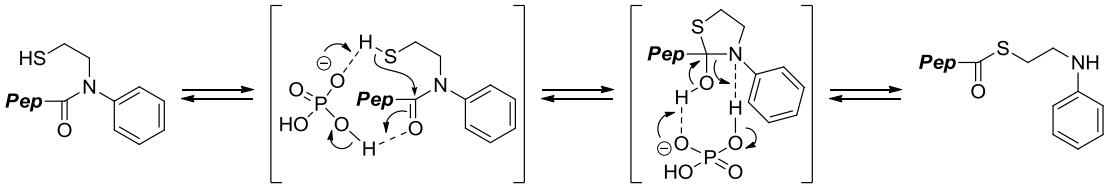
24

25a (X = Gly)
25b (X = L-Ala)

26

Entry	SEAlide peptide [mM]	Cys peptide **24** [mM]	Gn·HCl [M] (denaturant)	Na phosphate [M] (salt)	pH	Reaction time [h]	Fraction ligated[a] (integ. **25** / (integ. **24** + integ. **25**))
1	1.0 (**23a**)	1.0	6.0	0.2	7.3	24	0.76 (0.81[b])
2	1.0 (**23a**)	1.0	6.0	0.2	7.3	48	0.91
3	2.0 (**23a**)	1.0	6.0	0.2	7.3	24	1.00
4	1.0 (**23a**)	2.0	6.0	0.2	7.3	24	0.81[b]
5	1.0 (**23a**)[c]	1.0	6.0	0.2	7.3	24	0
6	1.0 (**23a**)	1.0	6.0	0.2	8.2	24	0.77
7	1.0 (**23a**)	1.0	6.0	0.2	6.0	24	0.86
8	1.0 (**23a**)	1.0	6.0	1.0	6.0	12	0.80
9	1.0 (**23a**)	1.0	6.0	0.2	6.0	12	0.60
10	1.0 (**23a**)	1.0	6.0	0.02	6.0	12	0.17
11	1.0 (**23a**)	1.0	6.0	0.2 (HEPPS)[d]	7.3	24	0.10
12	1.0 (**23a**)	1.0	6.0	0.2 (sodium acetate)[d]	7.3	24	0.22
13	1.0 (**23a**)	1.0	6.0	0.2 (imidazole·HCl)[d]	7.3	24	0.50
14[e]	1.0 (**23a**)	1.0	6.0	0.2 (HEPPS) + phosphate	7.3/6.0	48	0.82
15	1.0 (**23a**)	1.0	0	0.2	7.3	24	1.00
16	1.0 (**23a**)	1.0	6.0 (urea)	0.2	7.3	24	0.94
17	1.0 (**23b**)	1.0	6.0	0.2	7.3	48	0.93 (**25b**)[f]

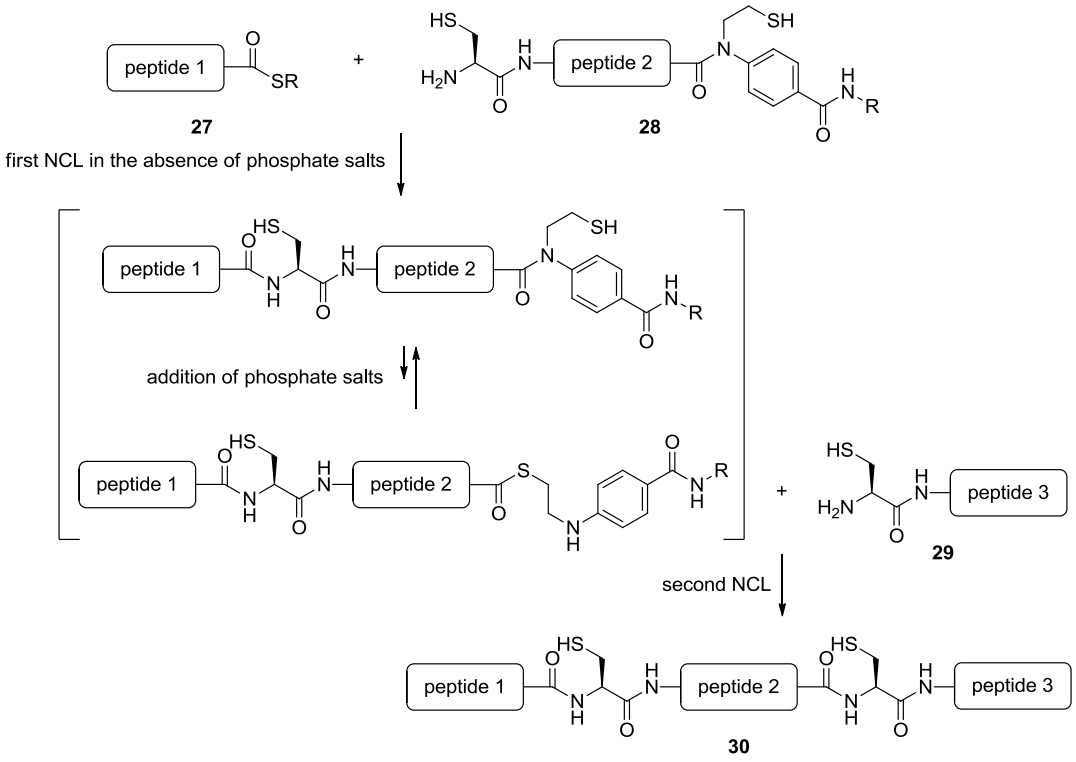
[a] The fraction ligated was determined by HPLC separation and integration of ligated product **25** (integ. **25**) detected at 220 nm as a fraction of the sum of the unreacted cysteine peptide (integ. **24**) + integ. **25**. [b] The fraction ligated was calculated from the equation (integ. **25** + integ. **26**) / (integ. **25** + integ. **26** + integ. **23**). [c] The thiol group was masked by a methyl group. [d] In the absence of phosphate salt. [e] After reaction for 24 h in the presence of 0.2 M HEPPS (entry 11), phosphate solution (6 M Gn·HCl-1 M Na phosphate [pH 4.5]) was added to the reaction mixture (final composition; 6 M Gn·HCl-0.25 M Na phosphate-0.15 M HEPPS, pH 6.0). In the aqueous buffer containing Gn·HCl at a concentration of 6 M, 1 M phosphate (NaH_2PO_4 [only], pH 4.5) can be completely dissolved. [f] No racemization at the L-Ala residue was detected during the ligation reaction.



Scheme 1.10. Putative mechanism of acceleration of *N–S* acyl transfer by phosphate salts. ***Pep***: peptide sequences.

1.4 Development of one-pot/N–to–C directed sequential NCL using SEAlide peptide

As mentioned in the previous section, it was clarified that the NCL using SEAlide peptides can be controlled by the presence or absence of phosphate salts. Based on the experimental results, an N–to–C directed multi-peptide ligation procedure was again designed using an N-terminal cysteinyl SEAlide peptide as a middle fragment (Scheme 1.11). In the envisioned ligation protocol, the N-terminal cysteine moiety of SEAlide peptide **28** should intermolecularly react with the thioester peptide **27** in the absence of the phosphate salts. Then addition of phosphate salts and N-terminal cysteinyl peptide **29** should allow the SEAlide moiety to function as thioester, yielding the ligated peptide **30** in a one-pot manner. To confirm the feasibility of this idea, synthesis of human atrial natriuretic peptide (hANP)[19] was attempted by the envisioned sequential NCL.



Scheme 1.11. Strategy for one-pot/three-fragment NCL using SEAlide peptide.

Initially, peptide fragments (**31**–**33**) covering an entire sequence of hANP were synthesized by means of Boc (**31**) or Fmoc SPPS (**32** and **33**). NCL reactions were monitored by RP-HPLC as shown in Figure 1.4. The first NCL of the thioester **31** (1 mM) with the cysteinyl SEAlide peptide **32** (1 mM) was conducted in 6 M Gn·HCl-0.2

M HEPPS in the presence of TCEP (30 mM) and MPAA (30 mM), pH 7.3 at 37 ºC. The reaction in the absence of phosphate salts went to completion within 3 hours to yield the desired ligated peptide **34** without a detectable amount of cyclic peptide derived from the middle fragment **32**. Then, successive addition of the C-terminal fragment **33** and phosphate salts into the reaction mixture allowed the second NCL to afford the reduced form hANP (**35**) successfully in a one-pot fashion.

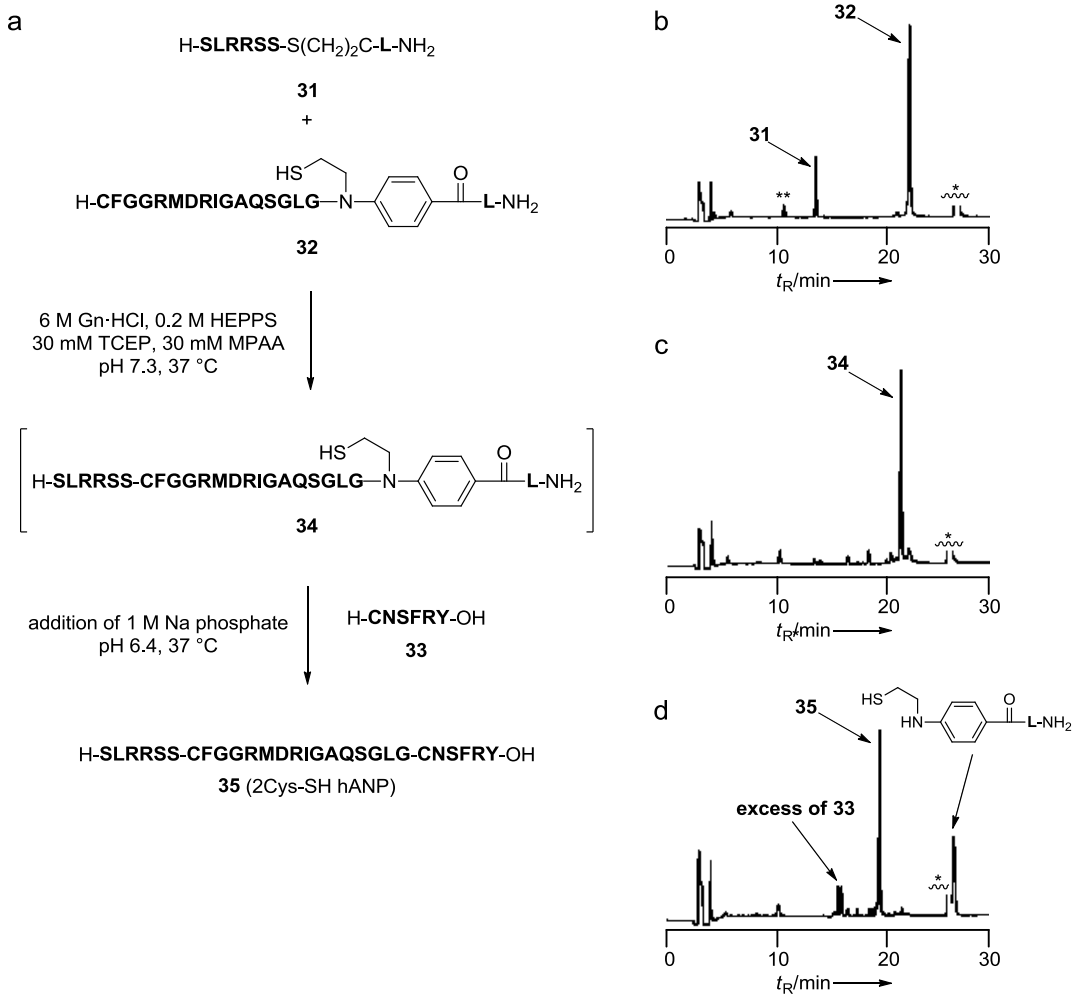


Figure 1.4. (a) Synthetic scheme for one-pot preparation of reduced form hANP **35**. (b–d) HPLC monitoring of NCLs. First NCL: fragments **31** (1 mM) and **32** (1 mM) were ligated in 0.2 M HEPPS buffer with 6 M Gn·HCl (pH 7.3) in the presence of 30 mM TCEP·HCl and 30 mM MPAA at 37 ºC. (b) After <5 min of the first NCL. (c) After 3 h of the first NCL. Second NCL: fragment **33** (1 mM) in 1 M Na phosphate (pH 6.4) was added to the reaction mixture and then incubated at 37 ºC. (d) After 24 h of the second NCL. Final concentrations: 0.5 mM each peptide in 0.1 M HEPPS-0.5 M Na phosphate buffer with 3 M Gn·HCl, 15 mM TCEP·HCl, and 15 mM MPAA. HPLC conditions: a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (5:95–45:55 over 30 min). *MPAA. **Non-peptidic impurity.

To compare the SEAlide peptide-mediated sequential NCL with conventionally used Kent's KCL, hANP synthesis was attempted by using aryl thioester **36** and N-terminal cysteinyl alkyl thioester **37** as alternatives to fragments **31** and **32**, respectively (Figure 1.5). The reaction between **36** and **37** was carried out in 6 M Gn·HCl-0.2 M sodium phosphate, pH 6.8 at room temperature, affording a complex mixture including the desired product **38**, cyclized middle fragment, and its derivatives because of the high reactivity of the glycyl alkyl thioester of the middle fragment **37**. Such side reactions were not observed in the reaction using the SEAlide peptide. These results indicated that SEAlide peptide-mediated sequential NCLs are a practical and reliable alternative ligation procedure to Kent's KCL protocol.

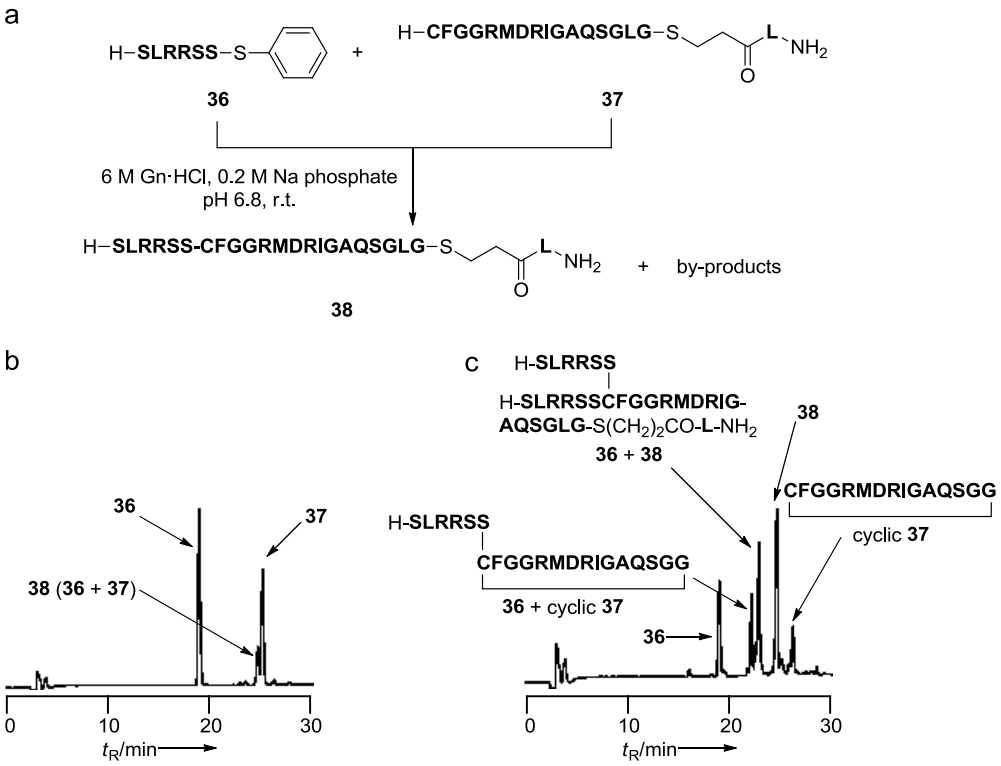


Figure 1.5. (a) KCL between aryl and Gly-alkyl thioester (**36** and **37**) under Kent's conditions. (b, c) HPLC monitoring of NCL between **36** and **37**. Fragments **36** (2 mM) and **37** (2 mM) were ligated in 0.2 M Na phosphate buffer with 6 M Gn·HCl (pH 6.8) at room temperature. (b) After <5 min of reaction. (c) After 1 h of reaction. HPLC conditions: a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (5:95–35:65 over 30 min).

From the point of view of simplifying synthesis and increasing chemical yield, one-pot NCL procedures using more components are preferred. Thus, we attempted a one-pot/four-component assembly by combining the SEAlide peptide-mediated

sequential NCL with Kent's KCL.

The one-pot/four-component NCL was attempted through the synthesis of an α-conotoxin ImI[20] derivative (4Cys-SH α-conotoxin ImI [^{11}Ala]) **45** as summarized in Figure 1.6. The reaction of glycyl aryl thioester **39** with N-terminal cysteinyl alkyl thioester **40** in 6 M Gn·HCl-0.2 M HEPPS, pH 6.8 at room temperature (Kent's conditions) gave the alkyl thioester **43** selectively. Successive addition of HEPPS buffer including the cysteinyl SEAlide peptide **41** and MPAA into the reaction mixture afforded the ligated SEAlide peptide **44**. Finally, addition of the cysteine amide **42** in phosphate buffer (1 M, pH 6.4) into the reaction solution allowed the SEAlide moiety to work as thioester, yielding the four-component-ligated product **45**. These results indicated that the combination of the sequential NCL using SEAlide peptide with the conventional KCL enables four-component assembly without multi-step purification. To our knowledge, this is the first example of one-pot/four-component assembly in the N–to–C direction.[21]

1.5. Conclusion

We disclosed that *N*-sulfanyethylanilide peptides, termed SEAlide peptides, efficiently function as a crypto-thioester peptide in the presence of phosphate salts even under neutral conditions. Furthermore, the one-pot/sequential NCL using SEAlide peptides was a practical and reliable alternative to traditional KCL. The feasibility of the novel sequential protocol was confirmed through the syntheses of hANP, α-conotoxin ImI (^{11}Ala) and CXCL14 (K. Tsuji et al. unpublished data).

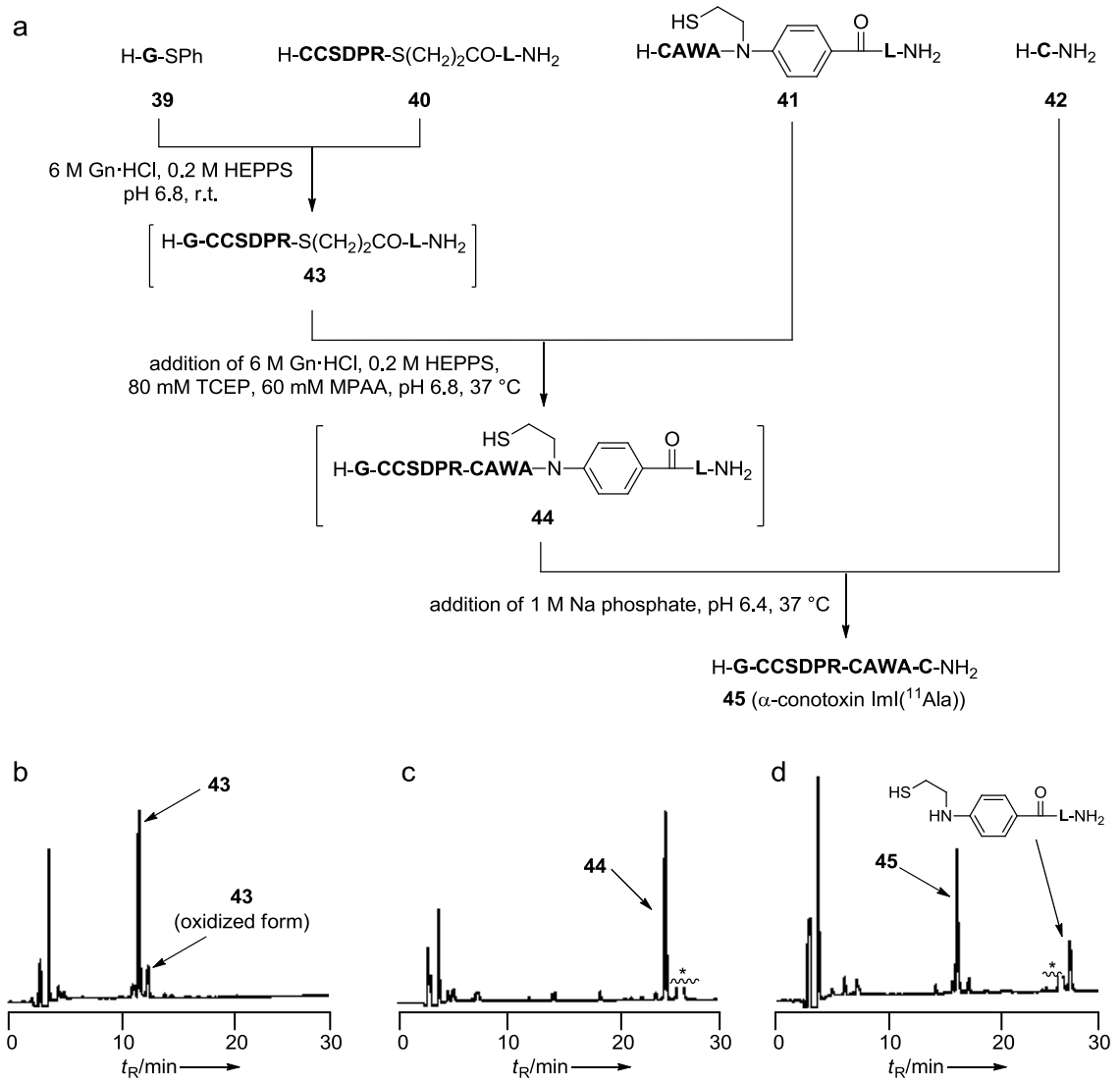


Figure 1.6. (a) Application of one-pot/four-component assembly to the synthesis of an α-conotoxin derivative. (b–d) HPLC monitoring of attempted reactions. First NCL: components **39** (2 mM) and **40** (2 mM) were ligated in 0.2 M HEPPS buffer with 6 M Gn·HCl (pH 6.8) at room temperature. (b) After 1 h of the first NCL. Second NCL: component **41** (2 mM) in 0.2 M HEPPS buffer with 6 M Gn·HCl, 80 mM TCEP·HCl and 60 mM MPAA (pH 6.8) was added to the reaction mixture and then incubated. Final concentrations: 1 mM each peptide in 0.2 M HEPPS buffer with 6 M Gn·HCl, 40 mM TCEP and 30 mM MPAA. (c) After 5 h of the second NCL. Third NCL: component **43** (1 mM) in 1 M Na phosphate buffer (pH 5.8) was added to the reaction mixture and then incubated at 37 °C. Final concentrations: 0.5 mM each peptide in 0.1 M HEPPS-0.5 M Na phosphate buffer with 3 M Gn·HCl, 20 mM TCEP and 15 mM MPAA. (d) After 20 h of the third NCL. HPLC conditions: a linear gradient of 0.1% TFA-MeCN/0.1% TFAaq (10:90–40:60 over 30 min). *MPAA

Chapter 2
Chemical synthesis of monoglycosylated GM2 activator protein analog

2.1 Lysosomal storage disease and protein replacement therapy

Lysosomes are membrane-enclosed organelles that digest biological polymers: proteins, nucleic acids, carbohydrates, and lipids. The organelles contain an array of hydrolases and co-factors to degrade a wide range of materials. Mutations in the genes encoding these proteins cause inherent deficiency or reduced activity of native proteins and accumulation of materials naturally degradable within lysosomes. Nowadays more than 30 of such inherited metabolic diseases, called lysosomal storage diseases (LSDs), are known.[22]

GM2 activator protein (GM2AP) is a lysosomal glycoprotein involved in degradation of GM2 ganglioside (GM2) by β-hexosaminidase A (HexA).[23] Mutations in *GM2A* (the gene encoding GM2AP) cause functional deficiency and excessive accumulation of GM2 mainly in nerve tissue, resulting in a fatal neurological disease known as the AB variant of GM2 gangliosidosis.

The major breakthrough for treating LSDs is protein replacement therapy.[24] This approach is based on periodic intravenous infusions of lysosomal proteins produced by recombinant DNA techniques. Working as an alternative to compensate for reduced activity of the defective protein, the functional recombinant proteins degrade the accumulated material. The clinical success of the approach has been shown in the treatment for several LSDs; however, its application to GM2 gangliosidosis is still more challenging because delivery of infused proteins into the central nervous system is difficult.[25] Thus, functionalization of proteins was envisioned as a potential solution to this problem.

Attempts to improve protein functionality mainly use genetic engineering technology to impart additional functions such as high activity, enhanced biostability, and tissue-specific targeting; meanwhile, the repertoire of proteins in protein libraries is fundamentally restricted by the limited number of coded amino acids that can be used for recombinant synthesis. Incorporating a wide range of unnatural structural units into protein molecules is an attractive way to extend the library of protein therapeutics. Total chemical synthesis of proteins is a useful strategy that enables rational incorporation of non-canonical structures into proteins. Thus we explored a convergent synthetic

platform to allow preparation of medicinal GM2AP derivatives for treatment of the AB variant of GM2 gangliosidosis.

2.2 Synthetic plan for total synthesis of GM2 activator protein analog

As mentioned in chapter 1, novel one-pot/multi-fragment ligations using SEAlide peptides were developed. These protocols prompted us to use SEAlide peptides in a convergent synthetic platform for GM2AP. Primary sequence of GM2AP consisting of 162 amino acid residues is shown in Figure 2.1. The sequence contains a glycosylation site at the 32nd asparagine (Asn32) and eight cysteine residues that form four disulfide bonds. Considering the NCL-based synthetic strategy, cysteine residues are indispensable at ligation junctions. Peptide fragments covering GM2AP, divided at the eight native cysteine residues, contain 7~60 residues. Because of the limitation on achievable chain length of peptides synthesized by SPPS (~50 residues), the longest fragment (60 residues) would seem to be almost impossible to synthesize in good yield and high purity. Therefore it was decided to replace the Asn32, the glycosylation site, with a cysteine residue. The substitution should provide a new ligation site on the longest peptide and an attachment site for sugar units by an alkylation protocol.[26]

Our convergent synthetic strategy for the monoglycosylated GM2AP analog **54** is shown in Scheme 2.1. To achieve the synthesis in a convergent fashion, the entire sequence of the GM2AP analog was divided into the monoglycosylated N-half fragment of the SEAlide peptide **51** and the N-terminal cysteinyl C-half fragment **52**. The N-half fragment **51** should be synthesized by standard NCL between the alkylthioester Fr 1 (**46**) and the N-terminal cysteinyl SEAlide peptide Fr 2 (**47**) followed by selective *S*-glycosylation. For the selective glycosylation, the peptides **46** and **47** should be protected by *S*-acetamidomethyl (Acm) protecting groups at Cys8 for **46**, and at Cys68

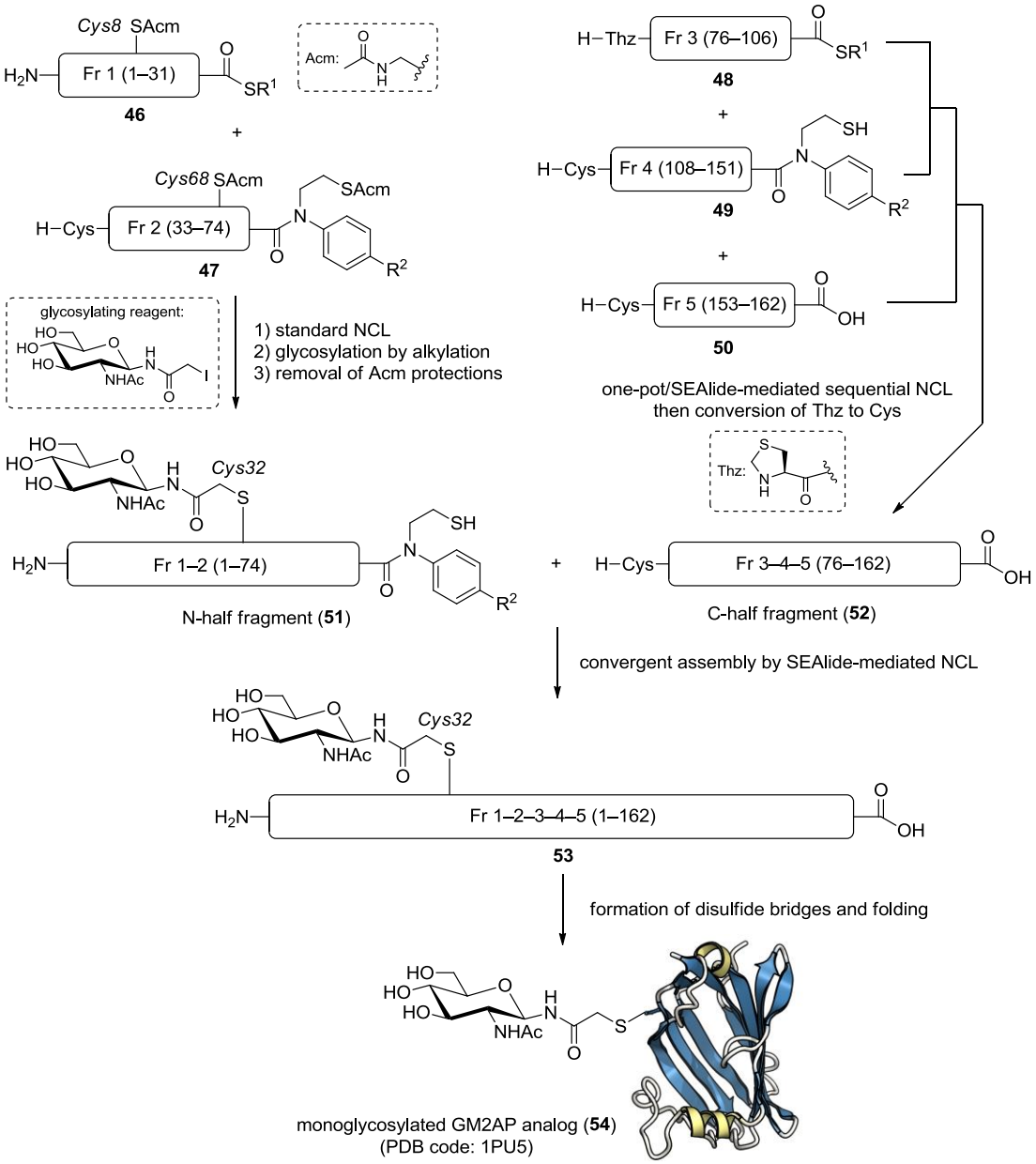
```
1           11          21          31          41          51
SSFSWDNCDE GKDPAVIRSL TLEPDPIIVP GNVTLSVMGS TSVPLSSPLK VDLVLEKEVA

61          71          81          91          101         111
GLWIKIPCTD YIGSCTFEHF CDVLDMLIPT GEPCPEPLRT YGLPCHCPFK EGTYSLPKSE

121         131         141         151          162
FVVPDLELPS WLTTGNYRIE SVLSSSGKRL GCIKIAASLK GI
```

Figure 2.1. Primary sequence of GM2AP. Underlines indicate ligation sites. Bold letters indicate cysteine residues. A bold and italic ***N*** represents an *N*-glycosylated residue that was replaced by *S*-glycosylated Cys in the GM2AP analog.

and the SEAlide moiety for **47**, respectively, except for Cys32. The Acm protections should be removed after the glycosylation to afford the requisite N-half fragment **51**. For the preparation of the C-half fragment **52**, one-pot/SEAlide-mediated sequential NCL was used. The first NCL of the alkyl thioester fragment Fr 3 (**48**) bearing a 1,3-thiazolidine-4-carbonyl (Thz) unit with the N-terminal cysteinyl SEAlide peptide Fr 4 (**49**) followed by the second NCL with the N-terminal cysteinyl peptide Fr 5 (**50**) should give the C-half fragment **52**. The Thz, a protected cysteine at the N-terminus of **48** used for prevention of undesired side reactions, should be converted to a cysteine

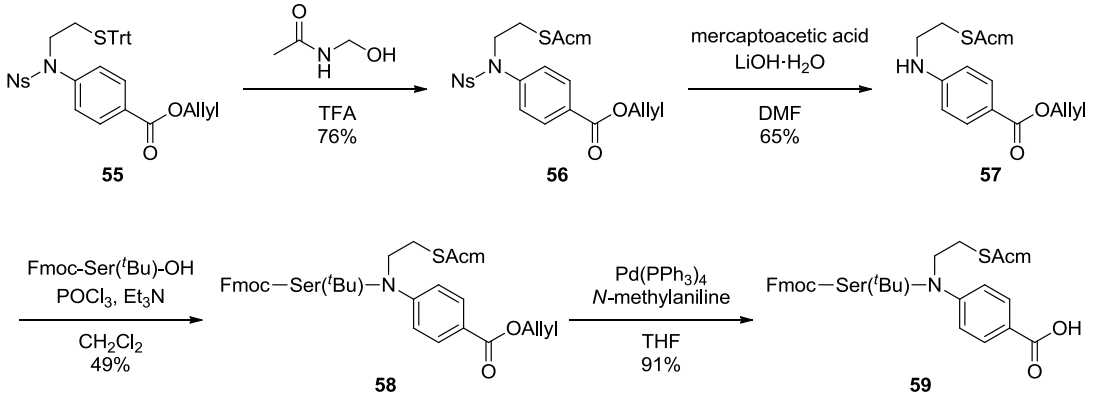


Scheme 2.1. Synthetic plan for total chemical synthesis of monoglycosylated GM2AP analog.

residue.[9d]

2.3 Chemical synthesis of monoglycosylated GM2AP analog

In the envisioned strategy, an Acm-protected *N*-sulfanylethylaniline linker is required, thus, synthesis of Acm-protected derivative **59** was attempted as shown in Scheme 2.2. Replacement of a triphenylmethyl (Trt) group on anilide derivative **55** with Acm followed by removal of the 2-nitrobenzenesulfonyl (Ns) group afforded the Acm-protected aniline derivative **57** in 49% yield over two steps. Protected serine derivative (Fmoc-Ser(^{t}Bu)-OH) corresponding to the Ser74 was incorporated by a $POCl_3$-Et_3N system without sodium hydride, which would cause deprotonation of the Acm group,[27] and the subsequent removal of the allyl group, successfully yielding the Fmoc-Ser(^{t}Bu)-incorporated linker **59** possessing the Acm protecting the sulfanyl group.



Scheme 2.2. Synthesis of Acm-protected *N*-sulfanylethylaniline linker.

All peptide fragments (**46**–**50**, Scheme 2.1) covering the entire sequence of GM2AP were prepared by manual SPPS. To achieve the selective *S*-glycosylation, thiol groups at the non-glycosylation sites in Fr 1 (**46**) and in Fr 2 (**47**) were protected with Acm groups. *In situ* neutralization protocols for Boc SPPS were used in syntheses of peptide thioesters Fr 1 (**46**) and Fr 3 (**48**).[28] SEAlide peptides Fr 2 (**47**) and Fr 4 (**49**) were synthesized on the Fmoc-Ser-incorporated *S*-Acm-protected aniline- and Fmoc-Gly-incorporated *S*-Trt-protected aniline-linked resin, respectively, using Fmoc SPPS. The SEAlide peptides were obtained without any problems; therefore, it was demonstrated that SEAlide peptides are applicable to long peptides (Fr 2: 43 residues, Fr 4: 45 residues). Peptide acid Fr 5 (**50**) was also successfully synthesized by Fmoc

SPPS.

The reaction train for the synthesis of the N-half fragment **51** is shown in Figures 2.2 and 2.3. NCL between the thioester **46** and the N-terminal cysteinyl SEAlide peptide **47** in 6 M Gn·HCl-0.2 M HEPPS (pH 7.4) in the presence of 3% (v/v) thiophenol yielded the 74-residue peptide **60** possessing Acm protections except for the cysteine at the ligation site. On the thiol function of Cys32, an *N*-acetylglucosamine unit was introduced by alkylation (Figure 2.3). The glycosylation of the partially protected peptide **59** with iodoacetyl-*N*-acetylglucosamine **61** in 6 M Gn·HCl-0.2 M HEPPS (pH 7.4) proceeded efficiently to afford the monoglycosylated peptide **62** in 90% isolated yield. The Acm groups on the resulting peptide **62** were removed by the action of AgOTf-anisole in TFA followed by incubation in the presence of dithiothreitol (DTT).[29] After RP-HPLC purification, the N-half fragment **51** was obtained in 42% isolated yield.

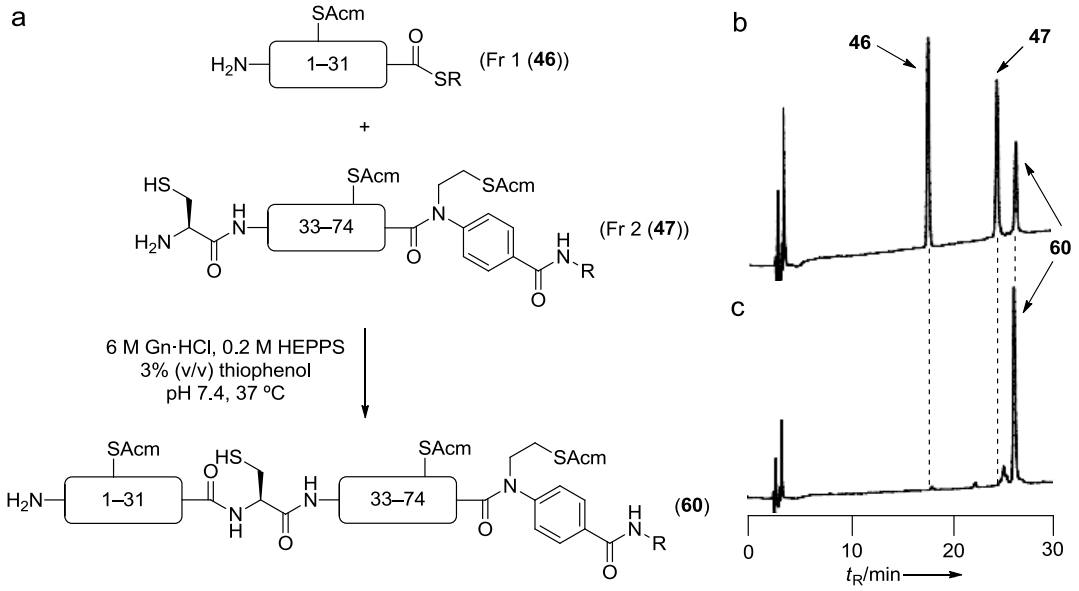


Figure 2.2. (a) NCL between Fr 1 (**46**) with Fr 2 (**47**). (b, c) HPLC monitoring of the NCL. Fragments **46** (1 mM) and **47** (1 mM) were ligated in 0.2 M HEPPS buffer with 6 M Gn·HCl (pH 7.4) in the presence of 3% (v/v) thiophenol at 37 ºC. (b) After <5 min of incubation. (c) After 3 h of incubation. HPLC conditions: a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (20:80–50:50 over 30 min).

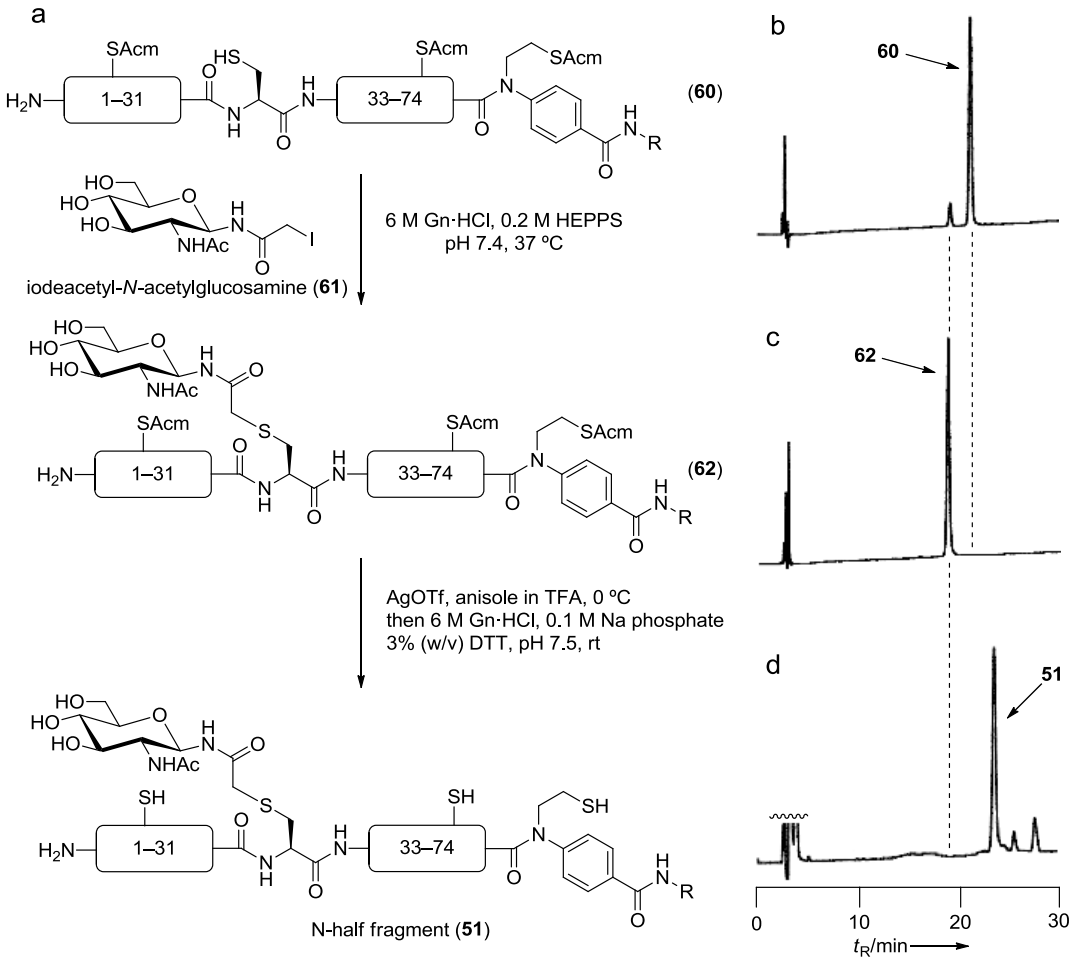


Figure 2.3. (a) Synthesis of N-half fragment **51**. (b–d) HPLC monitoring of glycosylation and Acm-deprotection. Glycosylation with **61**: fragment **60** (0.5 mM) was incubated in the presence of **61** (2.5 mM) in 0.2 M HEPPS buffer with 6 M Gn·HCl (pH 7.4) at 37 ºC. (b) After <5 min of glycosylation. (c) After 2 h of glycosylation. Removal of Acm groups: fragment **62** (4 mM) was treated with 400 mM AgOTf and 400 mM anisole in TFA at 4 ºC and then incubated in 0.1 M Na phosphate buffer with 6 M Gn·HCl (pH 7.5) in the presence of 3% (w/v) DTT at rt. (d) After 24 h of incubation in TFA. HPLC conditions: a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (30:70–50:50 over 30 min).

Next, the one-pot synthesis of the C-half fragment **52** was attempted (Figure 2.4). The first NCL between **48** and **49** in 6 M Gn·HCl-0.1 M HEPPS in the presence of 50 mM TCEP and 100 mM MPAA proceeded successfully to afford the ligated SEAlide peptide **63**. During the ligation, the SEAlide moiety remained intact because of the absence of phosphate salts in the reaction mixture. After confirmation of the reaction completion by RP-HPLC, a solution of **50** in 6 M Gn·HCl-0.4 M sodium phosphate was added to the reaction mixture to allow the resulting SEAlide peptide **63** to work as thioester and to participate in the second NCL with **50**. This reaction also proceeded smoothly to yield the Thz peptide **64** consisting of 88 amino acids. After checking completion of the second NCL by RP-HPLC, opening of the 1,3-thiazolidine ring at the N-terminus by addition of $MeONH_2$·HCl to the reaction mixture followed by RP-HPLC purification gave the C-half fragment **52** in 52% isolated yield. The sequence of reactions was successfully carried out to assemble three peptide fragments in a one-pot/N–to–C directive manner.

Convergent assembly between the N-half fragment **51** and the C-half fragment **52** by SEAlide-mediated NCL was attempted as shown in Figure 2.5. The NCL in 6 M Gn·HCl-0.5 M sodium phosphate in the presence of 50 mM TCEP and 50 mM MPAA went almost to completion within 24 hours to afford the monoglycosylated peptide **53** consisting of the entire sequence of GM2AP with substitution of Asn32. Meanwhile, a small amount of a material with a molecular weight identical to that of the ligation product **63** was obtained in the NCL reaction (denoted by **53'**). From previous investigations of SEAlide-mediated NCL,[27] partial epimerization of the Ser residue was observed during ligations using Ser-SEAlide peptides (~10%), similar to conventional NCL using Ser thioesters.[30] Therefore, the minor product was presumed to be the diastereomer of the desired peptide resulting from the epimerization of the serine residue at the ligation site. After RP-HPLC purification of the major product, the 162-residue peptide was obtained in its reduced form in 23% isolated yield. According to one report,[31] formation of four disulfide bridges of the resulting peptide **53** was performed in the presence of reduced and oxidized glutathione to give the folded monoglycosylated GM2AP analog **54**. These results clearly indicate that SEAlide peptides are applicable to ligation of large peptide fragments, considering the successful assembly of 74- and 88-residue peptides.

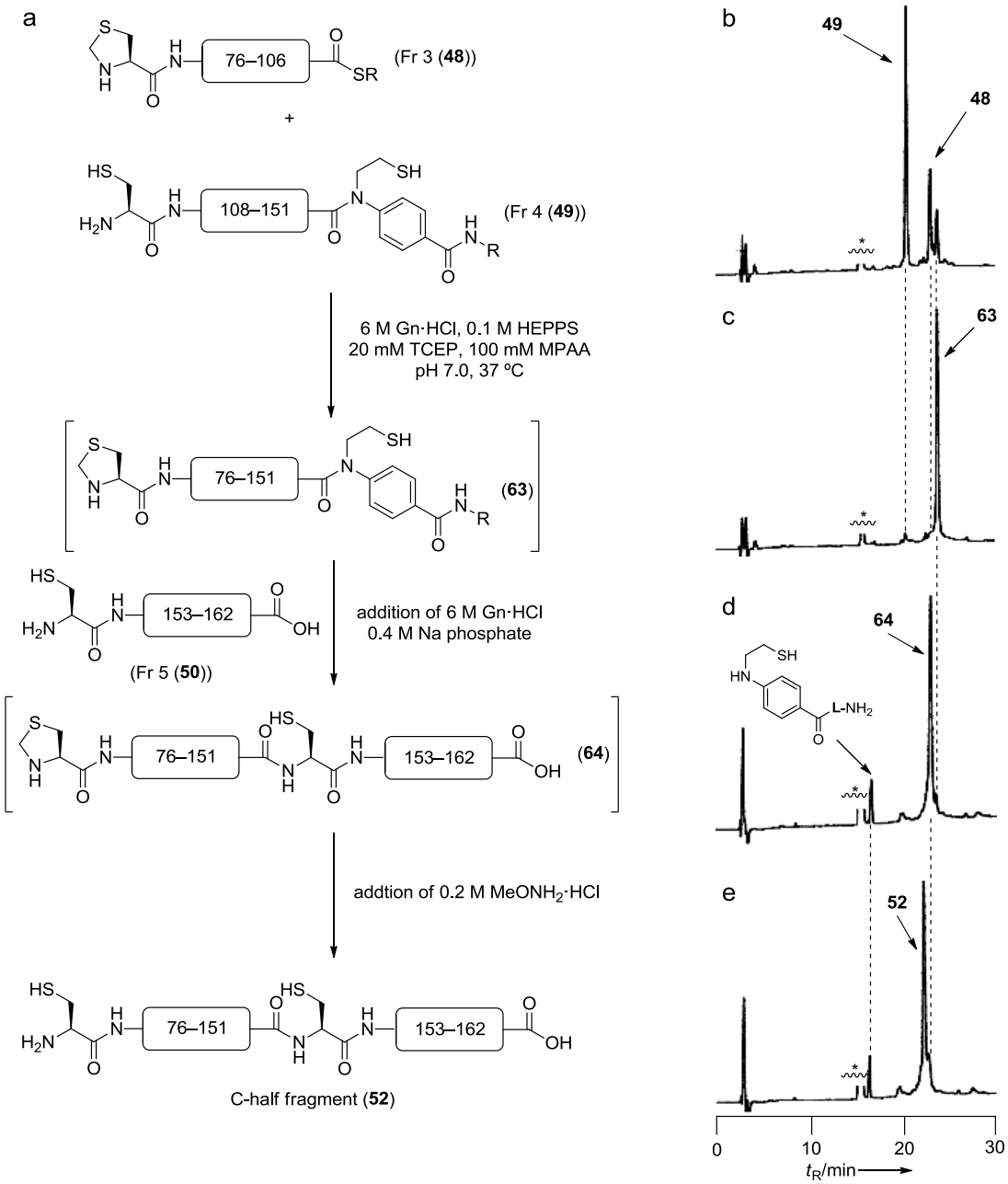


Figure 2.4. (a) One-pot preparation of the C-half fragment **52** using SEAlide chemistry. (b–e) HPLC monitoring of the reactions. First NCL: fragments **48** (2.0 mM) and **49** (2.0 mM) were ligated in 0.1 M HEPPS buffer with 6 M Gn·HCl (pH 7.0) in the presence of 50 mM TCEP·HCl and 100 mM MPAA at 37 ºC. (b) After <5 min of the first NCL. (c) After 3 h of the first NCL. Second NCL: to the reaction mixture (after the first NCL) was added fragment **50** (1.1 eq.) in 0.4 M Na phosphate buffer with 6 M Gn·HCl, and the mixture was then incubated at 37 ºC. (d) After 24 h of the second NCL. (final concentration: 6 M Gn HCl, 0.32 M Na phosphate, 20 mM MPAA, 10 mM TCEP, 0.4 mM peptide **48** and **49**) Opening of the 1,3-thiazoline ring: to the reaction mixture after the second NCL, solid form $MeONH_2$ HCl was added (final concentrations: 0.2 M). HPLC conditions: a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (20:80–50:50 over 30 min). *MPAA.

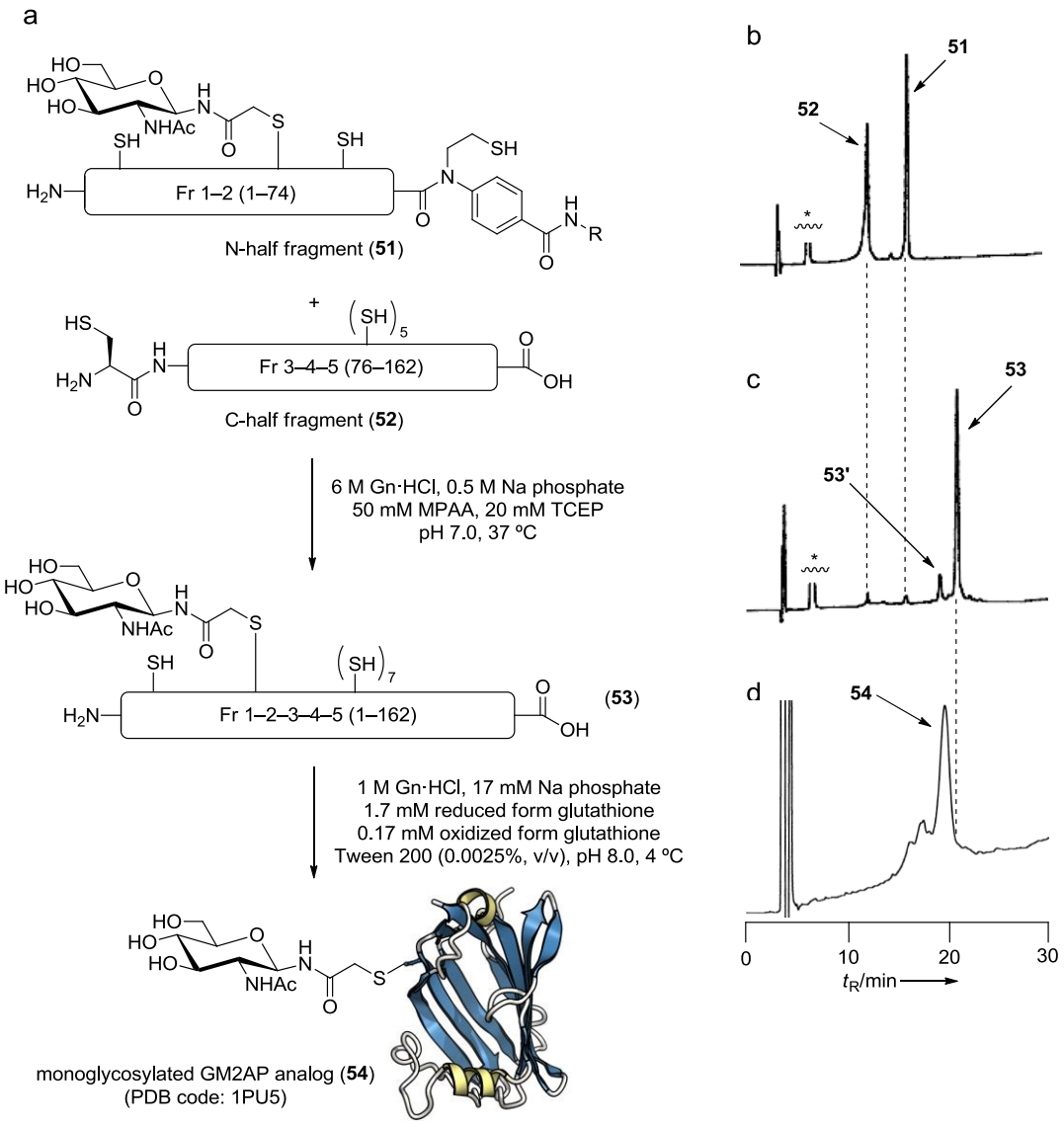


Figure 2.5. (a) Convergent assembly of N-half and C-half fragments (**51** and **52**). (b–d) HPLC monitoring of the NCL between **51** and **52** followed by folding. Components **51** (0.5 mM) and **52** (0.5 mM) were ligated in 0.5 M Na phosphate buffer with 6 M Gn·HCl (pH 7.0) in the presence of 50 mM MPAA and 20 mM TCEP at 37 ºC. (b) After <5 min of the NCL. (c) After 24 h of the NCL. Purified **53** (8 Cys[SH] form) was folded in 17 mM Na phosphate with 1 M Gn·HCl (pH 8.0, 0.08 mg/mL protein) in the presence of 1.7 mM reduced form glutathione, 0.17 mM oxidized form glutathione, and 0.0025% (v/v) Tween 20 at 4 ºC. (d) After 54 h of folding. HPLC conditions: Cosmosil Protein-R column (4.6 × 250 mm) with a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (35:65–55:45 over 30 min). *MPAA.

2.4 *Identification and bioactivity of chemically synthesized GM2AP analog*

Identification of the obtained protein was conducted by using mass spectrometry (MS) and circular dichroism (CD). Electrospray ionization- (ESI) MS analysis of the synthetic product indicated that its molecular weight was identical to that of the folded monoglycosylated GM2AP analog **54** (calcd [average isotopes] 17,830.4, obsd 17,830.3). Furthermore, the CD spectrum of the synthesized analog was similar to that reported in the literature.[32] Therefore we concluded that the chemically synthesized analog was folded into the correct structure.

Hydrolysis-assisting activity of the synthesized GM2AP analog in the conversion of GM2 into GM3 ganglioside (GM3) was then evaluated in the presence of hydrolase HexA. After incubation of GM2 with synthesized or *E. coli*-expressed GM2AP in the presence of HexA, GM2 and GM3 were separated and visualized on TLC by orcinol-H_2SO_4 as shown in Figure 2.7.[33] These results indicated that the synthetic protein **54** was more active than an *E. coli*-expressed carbohydrate-free protein. The carbohydrate-free GM2AP produced by *E. coli* is known to be at least as effective as the native glycosylated GM2AP purified from human tissues.[31]

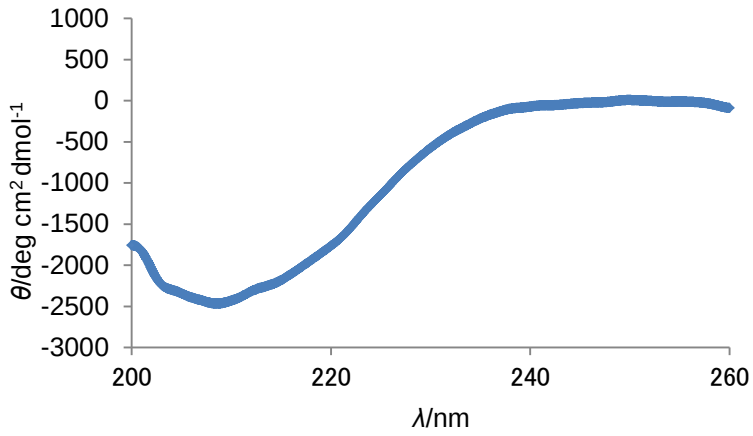


Figure 2.6. CD spectrum of the monoglycosylated GM2AP analog **54**.

2.5 *Conclusion*

Total chemical synthesis of the monoglycosylated GM2AP analog containing Cys instead of Asn32 was achieved. In this study, SEAlide peptides were successfully used in the one-pot preparation of the C-half fragment and the final convergent assembly.

The glycoconjugates containing a non-canonical linkage produced by *S*-alkylation was reported to be more stable than glycoconjugates containing the native linkage and metabolic enzymes,[34] which might suggest that the GM2AP analog has some advantages over the native protein. The established synthetic platform for the Cys32 GM2AP analog should enable the extension of GM2AP-based libraries, leading to medicinal-chemistry-based evaluation of protein therapeutics.

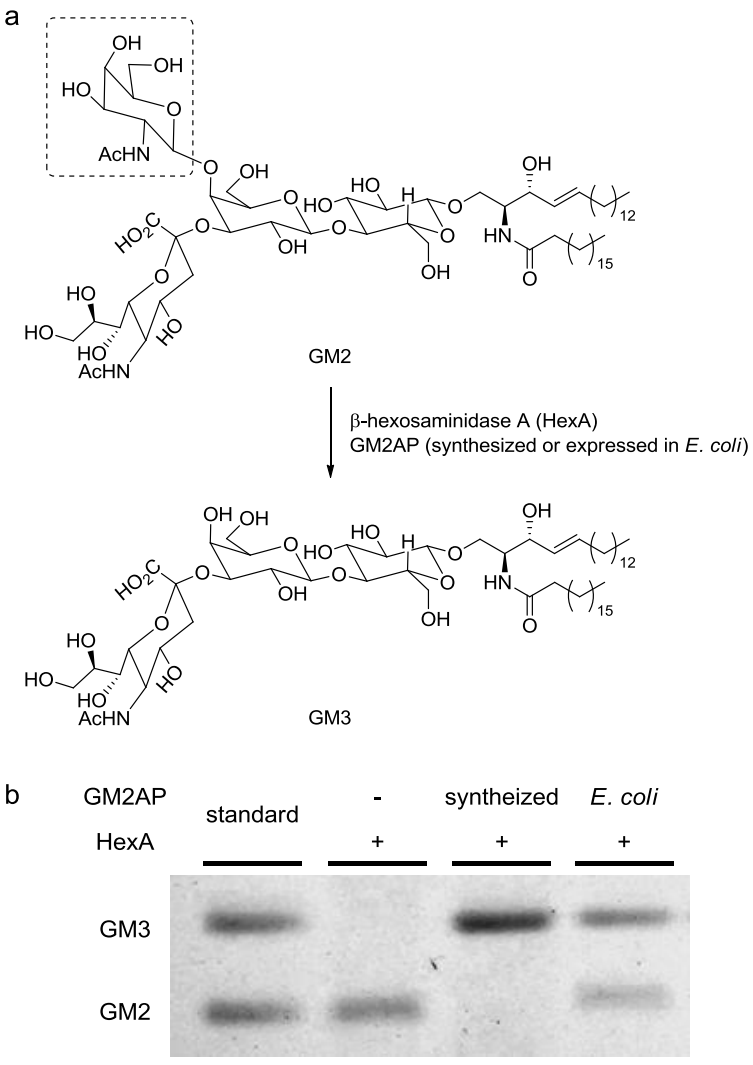


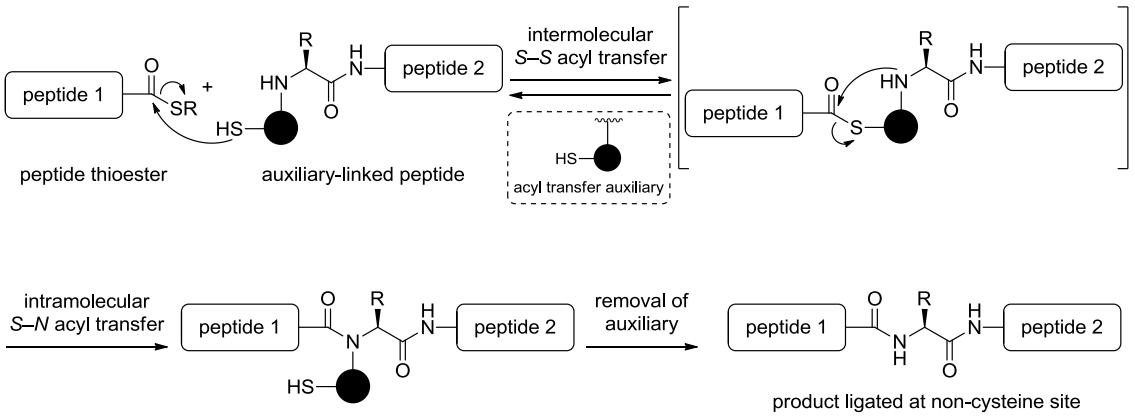
Figure 2.7. (a) Conversion of GM2 into GM3. (b) Comparable hydrolysis of GM2 with Hex A in the presence of 2.5 μg of synthesized **54** or *E. coli*-expressed non-glycosylated GM2AP. Hydrolysis was monitored by TLC of the reaction aliquots (developing solvent: $CHCl_3$/MeOH/0.2% (w/v) $CaCl_2$ aq. = 55:45:10, (v/v); detection reagent: orcinol reagent, 120 ºC, 5 min).

Chapter 3
Development of *N*-glycosylated asparagine site ligation with its application to total synthesis of native GM2 activator protein

3.1 Extended NCL-based approach to achieve peptide ligations at non-cysteinyl junctions

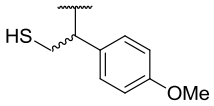
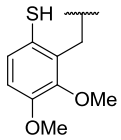
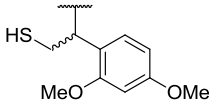
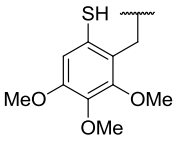
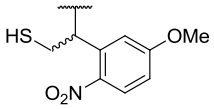
NCL is a remarkably powerful technique to enable chemical preparation of proteins; however, the fundamental requirement of cysteine residues at the ligation sites is often troublesome because cysteine is a rare amino acid (1.1% frequency in nature).[35] In many proteins, there are no cysteines or the cysteine residues are not in a position suitable for NCL. Therefore NCL-based methodologies enabling condensation at non-cysteinyl sites have been pursued.

One potential approach to this problem is the use of auxiliary-linked amino acids at an N-terminal residue instead of cysteine (Scheme 3.1).[36] The auxiliary moiety contains a thiol function which mimics the cysteinyl side chain and is linked to the α-amino group of the amino acid at a ligation junction. The principles underlying NCL are intermolecular capture of ligation partners and intramolecular transfer of acyl auxiliaries. In the auxiliary-mediated ligation, the thiol group captures a ligation partner like that of cysteines, and the captured peptide is irreversibly transferred to the secondary amino function. Several auxiliary groups removable under various conditions have been reported (Table 3.1).[37] However, auxiliary-mediated ligations are extremely limited to ligation sites containing at least one glycine residue because of the low



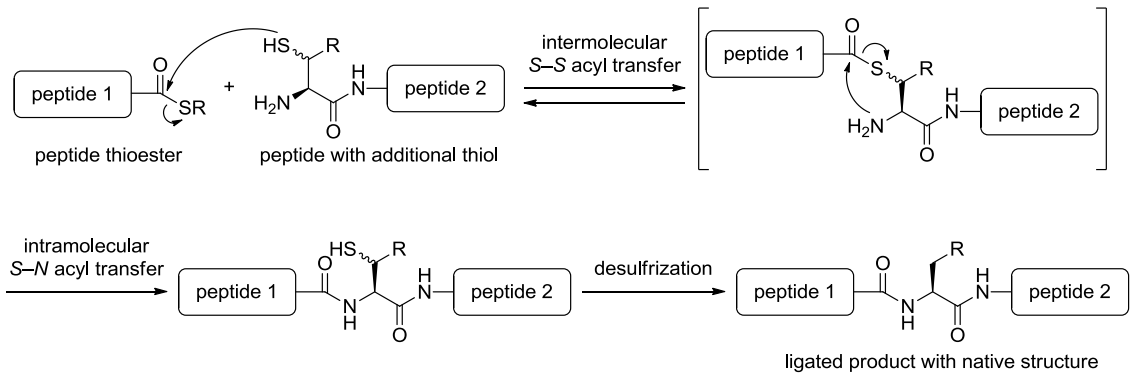
Scheme 3.1. NCL-based approach with acyl transfer auxiliary.

Table 3.1. Reported auxiliaries for ligations at non-cysteinyl junctions.

acyl transfer auxiliary	ligation site	acyl transfer auxiliary	ligation site
	Gly-Gly Phe-Gly Gly-Ala		His-Gly Gly-Gly Ala-Gly Lys-Gly Gly-Ala
	Gly-Gly Lys-Gly Leu-Gly Gly-Ala		Gly-Gly His-Gly Ala-Gly
	Lys-Gly Gly-Gly Gln-Gly Ala-Ala Ala-Gly		Gly-Gly Ala-Gly

reactivity of *N*-substituted amino acids and the bulky structures of the auxiliary groups.

Another approach to confer non-cysteinyl ligation sites is the ligation/desulfurization strategy (Scheme 3.2).[38, 39] In the alternative approach, a thiol function is linked to the side chain of an amino acid rather than to the α-amino group. After the linked-thiol-mediated ligation, the thiol functionality needs to be removed from the resulting ligation product. A radical-mediated mild desulfurization protocol, reported by Danishefsky and co-workers, has improved the applicability of the ligation/desulfurization chemistry.[40] Furthermore Payne and co-workers have developed a convenient one-pot ligation/desulfurization procedure.[41] This combined chemistry has initially been applied to the ligation at cysteine followed by conversion of the cysteine to alanine.[39a] Development of cysteine surrogates possessing thiol groups



Scheme 3.2. Ligation/desulfurization strategy with amino acids bearing thiol functions on their side chain.

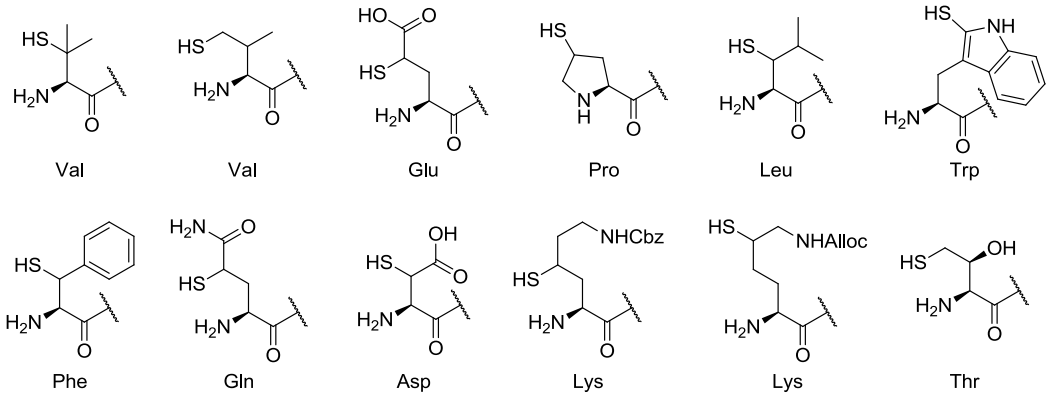
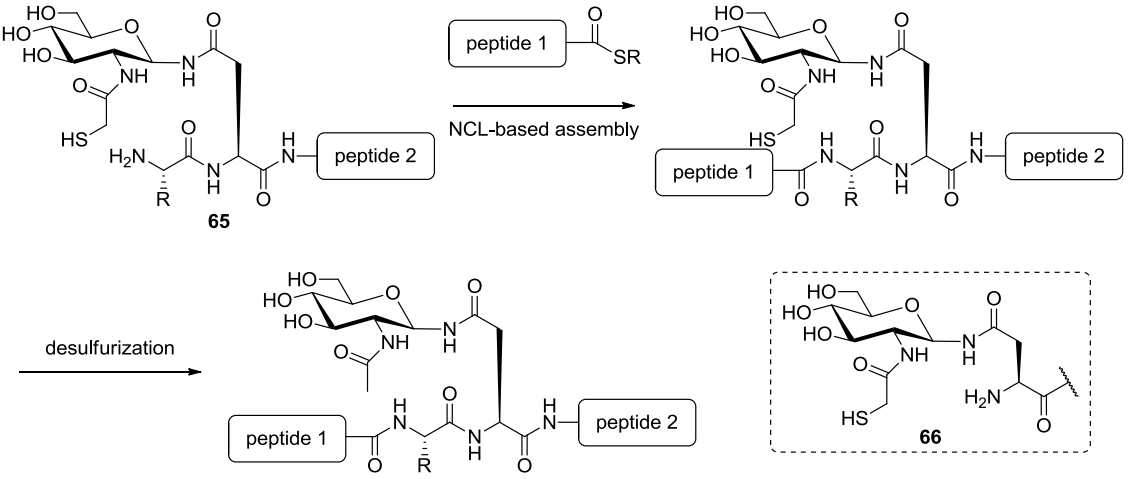


Figure 3.1. Reported cysteine surrogates.



Scheme 3.3. Sugar-assisted ligation to prepare glycopeptides.

on their side chain has expanded the feasibility of the ligation/desulfurization approaches that provide access to non-cysteinyl ligation sites (Figure 3.1).

Wong and co-workers have reported a sugar-assisted ligation based on the ligation/desulfurization approach (Scheme 3.3).[42] This ligation relies on a peptide thioester and a glycopeptide in which *N*-acetylglucosamine bears a thiol function. The thiol moiety mimics the cysteine side chain and enables the chemoselective assembly with a peptide thioester. Highest reaction rates were obtained for the glycopeptide **65** which had an extended amino acid at the N-terminus. The N-terminal placement of the glycosylated Asn derivative **66** caused slow and incomplete ligation.

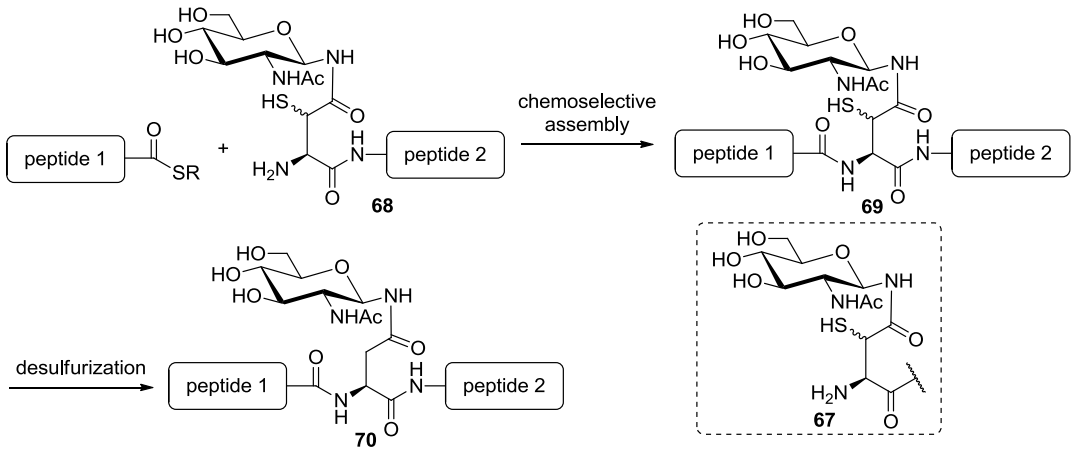
*3.2 Design and synthesis of β-mercapto-*N*-glycosylated asparagine derivative*

As mentioned in chapter 2, we have been interested in total synthesis of GM2AP. Although we synthesized GM2AP analog with replacement of an asparagine residue by a cysteine residue, total synthesis of native GM2AP is more challenging because cysteine is missing in a position suitable for NCL.

Sugar-assisted ligation seems to be applicable to our GM2AP synthesis. In this strategy, the ligation should be carried out at a Pro30-Gly31 junction. However, it is difficult to use a thioester fragment with a proline residue located at the C-terminus because of the low reactivity of the thioester.[43] In the GM2AP sequence, the N-terminus of the glycosylated Asn residue is glycine, which is more suitable for ligation junctions. To achieve chemical synthesis of native GM2AP, therefore, a decision was made to develop a novel ligation protocol that enables ligations at a position between an *N*-glycosylated Asn residue and its neighboring N-terminal amino acid.

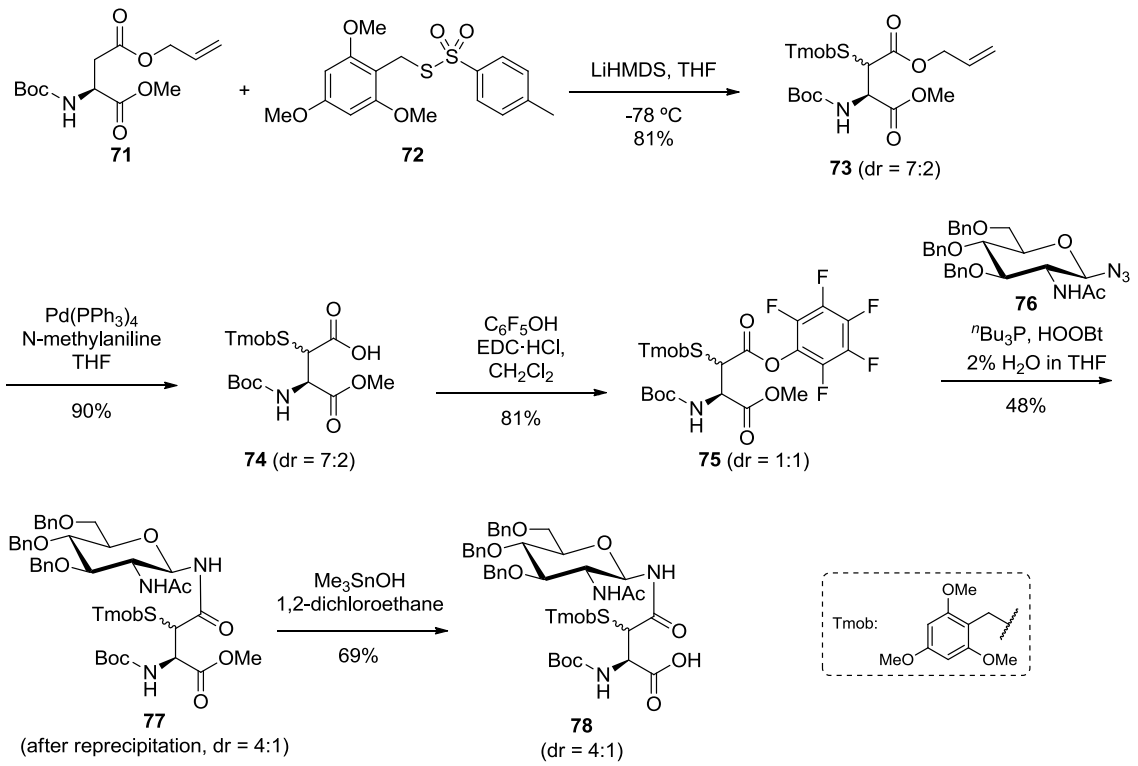
The envisioned strategy is shown in Scheme 3.4. A newly designed Asn derivative **67** bears a thiol group at a β-position. The thiol function should mimic a cysteine side chain to enable chemoselective assembly with a peptide thioester. The ligation of a peptide **68** possessing the β-mercapto-*N*-glycosylated Asn derivative with a peptide thioester should afford a ligated product **69** with an additional thiol function. Desulfurization of the additional thiol group would give a product **70** ligated between native *N*-glycosylated Asn and its neighboring N-terminal amino acid.

In the design strategy, access to the suitably protected β-mercapto Asn derivative



Scheme 3.4. Ligation/desulfurization using β-mercapto-*N*-glycosylated asparagine derivative.

78 by the protected amino acid Boc-Asp(OAllyl)-OMe **71** (Scheme 3.5) was envisioned. The 2,4,6-trimethoxybenzyl (Tmob) protected thiol group was introduced at the β-position using sulfenylating reagent **72**.[39m] The corresponding dianion, generated by treatment of **71** with two equivalents of lithium hexamethyldisilazide (LiHMDS) at –78 ºC, was reacted with **72** to afford the Tmob-protected β-mercapto Asp derivative **73** in 81% yield as a 7:2 diastereomeric mixture, determined by ^{1}H-NMR. These diastereoisomers would be eventually converted to the same desulfurized Asn residue; therefore, the diastereomeric mixture was used without separations. Palladium(0)-catalyzed deprotection of the allyl ester in the side chain followed by condensation with pentafluorophenol afforded the active ester **75** in 73% yield over two steps as a 1:1 diastereomeric mixture. The active ester was glycosylated with *O*-benzyl protected azide sugar **76** by the action of $^{n}Bu_3P$ to afford the glycosylated Asn derivative **77** in 48% yield as a 4:1 diastereomeric mixture after reprecipitation.[44] Finally, trimethyltin hydroxide-mediated deprotection of the methyl ester afforded the desired β-mercapto-*N*-glycosylated asparagine derivative **78** in 69% yield as a 4:1 diastereomeric mixture.[45]

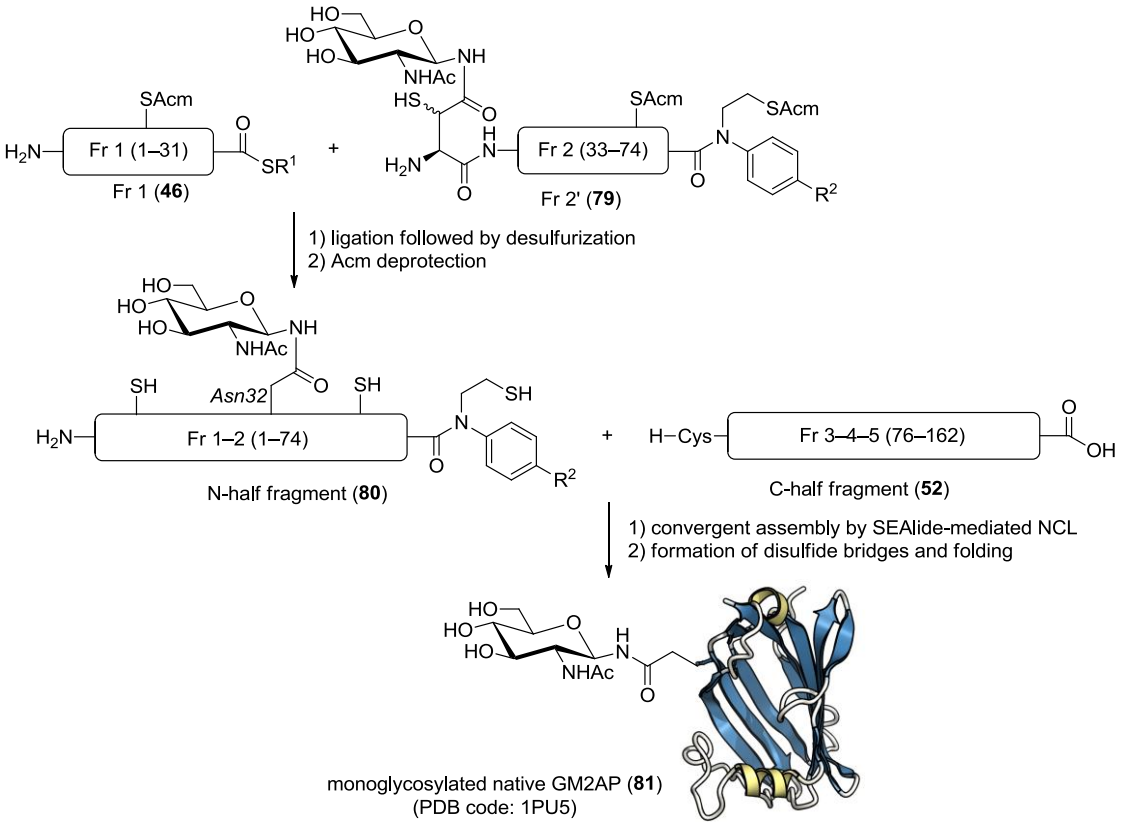


Scheme 3.5. Synthesis of β-mercapto-*N*-glycosylated asparagine derivative **78**.
dr: diastereomeric ratio.

3.3 Total chemical synthesis of monoglycosylated native GM2AP

To achieve total synthesis of native GM2AP, a synthetic plan using the β-mercapto Asn derivative **78** was envisioned as shown in Scheme 3.6. The synthetic plan is basically same as that for the GM2AP analog as mentioned in chapter 2. A newly requisite peptide fragment Fr 2' (**79**) was prepared as an Acm-protected SEAlide peptide by manual Fmoc SPPS. The Asn derivative **78** was successfully introduced into the N-terminal end of the resin-bound peptide chain by the use of benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate (PyBop)-$^{i}Pr_2EtN$ conditions.[46] After the introduction, global deprotection and cleavage from the solid phase was carried out with the aid of 1 M TMSBr-thioanisole in TFA.[47] Treatment with this cleavage cocktail, which enables benzyl groups to be simultaneously removed from the sugar moiety, afforded the partially Acm-protected SEAlide peptide **79** as an inseparable diastereomeric mixture.

The reaction progress for the synthesis of the native N-half fragment **80** is shown



Scheme 3.6. Plan to synthesize native GM2AP.

in Figure 3.2. Payne's one-pot ligation/desulfurization protocol using trifluoroethane thiol (TFET) as a thiol additive in ligations was attempted to prepare the N-half fragment **80**.[41] A chemoselective assembly between the thioester **46** and the β-mercapto Asn derivative-incorporated SEAlide peptide **79** in 6 M Gn·HCl-0.1 M sodium phosphate in the presence of 3% (v/v) TFET yielded the peptide **82**. After treatment of the reaction mixture with a nitrogen gas stream to remove excess TFET, desulfurization of the thiol function on the ligated peptide **82** was carried out. To the reaction mixture was added 6 M Gn·HCl, 400 mM TCEP·HCl aq., pH 7.0, together with reduced glutathione (in solid form) as a H-atom source and VA-044 (in solid form) as a radical initiator (final concentrations: 6 M, 200 mM, 40 mM, and 20 mM, respectively).[40] The desulfurization went to completion within 18 hours, yielding the Acm-protected SEAlide peptide **83** in 53% isolated yield over two steps. These reactions efficiently proceeded in a one-pot manner. The Acm groups on the resulting peptide **83** were removed by the action of AgOTf-anisole in TFA followed by incubation in the presence of DTT. After RP-HPLC purification, the native N-half fragment **80** was obtained in 54% isolated yield.

Next, SEAlide-mediated convergent assembly between the native N-half fragment **80** and the C-terminal fragment **52** (synthesized by the procedure used in the GM2AP analog synthesis) was attempted as shown in Figure 3.3. The NCL in 6 M Gn·HCl-0.5 M sodium phosphate in the presence of 20 mM TCEP and 50 mM MPAA went almost to completion within 30 hours to afford the monoglycosylated peptide **84**. After RP-HPLC purification, the glycosylated 162-residue peptide **84** consisting of the native entire sequence of GM2AP was obtained in its reduced form in 40% isolated yield. The four disulfide bonds of the resulting peptide were formed under the same synthesis conditions as used in GM2AP analog synthesis. After four days of folding, ESI-MS analysis of the major peak eluted at 24.2 minutes indicated that its molecular weight was identical to that of the folded monoglycosylated native GM2AP **81**. Although the folding conditions should be optimized, the developed Asn site ligation enabled total chemical synthesis of the homogeneously glycosylated GM2AP.

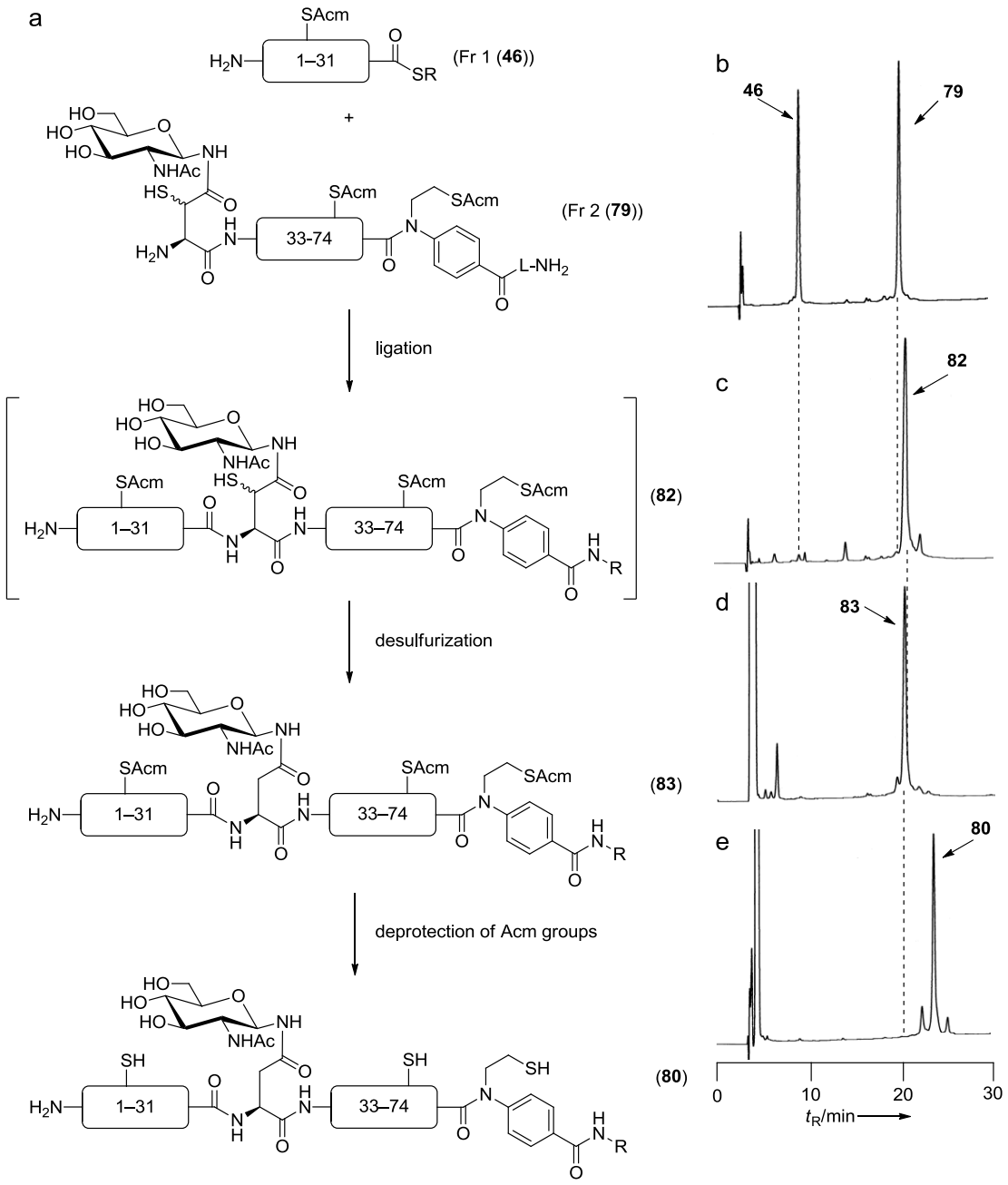


Figure 3.3. (a) Synthesis of native N-half fragment **79**. (b–e) HPLC monitoring of reactions. Fragments **46** (1 mM) and **79** (1 mM) were ligated in 0.1 M Na phosphate buffer with 6 M Gn·HCl (pH 7.3) in the presence of 3% (v/v) TFET at 37 ºC. (b) After <5 min of incubation. (c) After 8 h of incubation. After removal of TFET by N_2 stream, the mixture was incubated with 6 M Gn·HCl, 400 mM TCEP·HCl aq. (pH 7.0), reduced glutathione, and VA-044 at 37 ºC. (d) After 18 h of incubation. Fragment **83** (4 mM) was treated with 400 mM AgOTf and 400 mM anisole in TFA at 4 ºC and then incubated in 0.2 M HEPPS buffer with 6 M Gn·HCl (pH 6.1) in the presence of 5% (w/v) DTT at rt. (e) After 13 h of incubation in TFA. HPLC conditions: Cosmosil Protein-R column (4.6 × 250 mm) with a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (30:70–50:50 over 30 min).

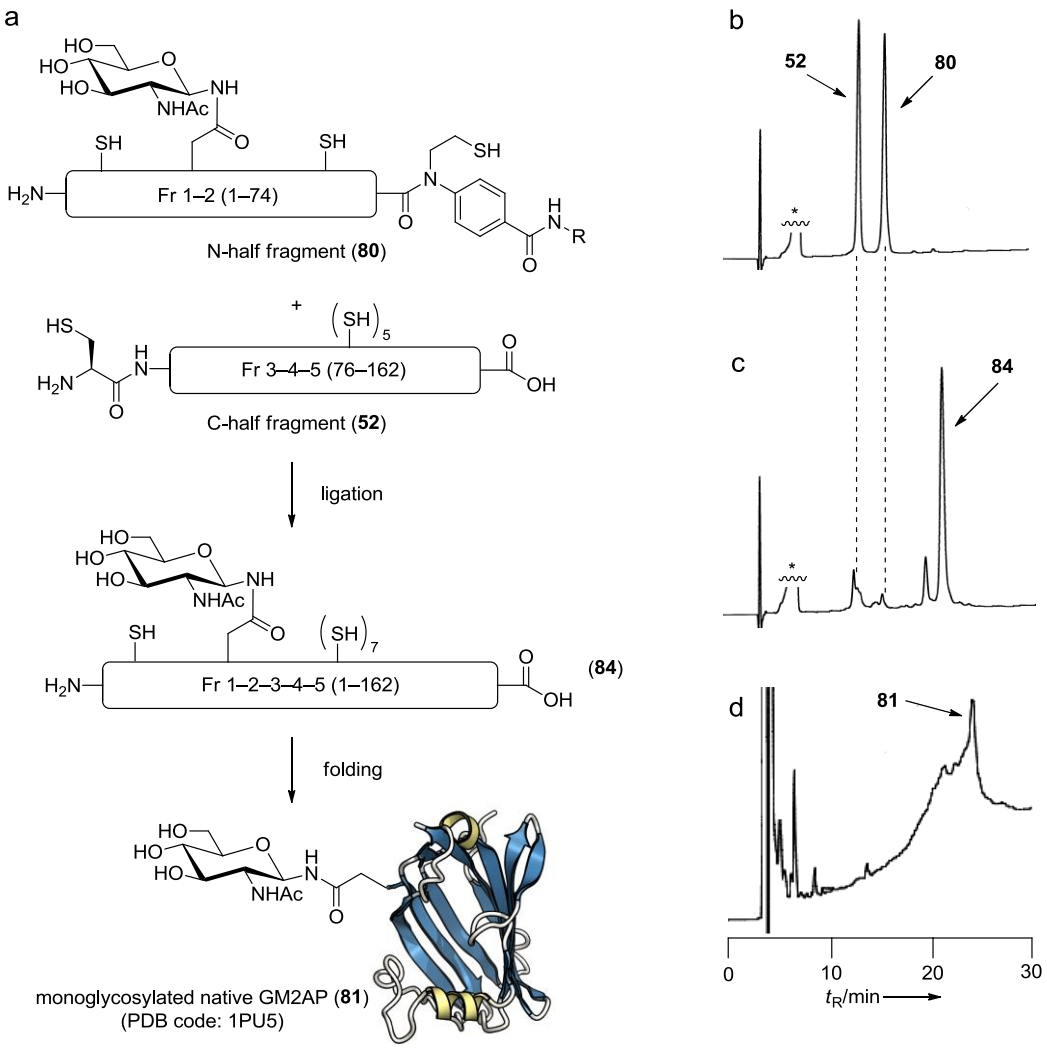


Figure 3.4. (a) Convergent assembly of N-half and C-half fragments (**80** and **52**). (b–d) HPLC monitoring of NCL between **80** and **52** followed by folding. Components **80** (0.5 mM) and **52** (0.5 mM) were ligated in 0.5 M Na phosphate buffer with 6 M Gn·HCl (pH 6.1) in the presence of 50 mM MPAA and 20 mM TCEP at 37 ºC. (b) After <5 min of NCL. (c) After 30 h of NCL. Purified **84** (8 Cys[SH] form) was folded in 17 mM Na phosphate with 1 M Gn·HCl (pH 8.0, 0.08 mg/mL protein) in the presence of 1.7 mM reduced glutathione, 0.17 mM oxidized glutathione and 0.0025% (v/v) Tween 20 at 4 ºC then rt. (d) After 4 days of folding. HPLC conditions: Cosmosil Protein-R column (4.6 × 250 mm) with a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (35:65–55:45 over 30 min). *MPAA.

3.4 Conclusion

A novel ligation/desulfurization procedure enabling *N*-glycosylated Asn site ligations was developed. This ligation procedure enabled the total synthesis of the monoglycosylated GM2AP **81**. Chemoenzymatic strategies, which feature enzymatic transfer of oligosaccharides to chemically synthesized monoglycosylated peptides, have received attention as an attractive way to synthesize glycoproteins using homogeneous oligosaccharides.[48] Combining the developed ligation method with the chemoenzymatic strategy should open new avenues for the preparation of glycoproteins possessing homogeneous glycoforms. The establishment of this synthetic route GM2AP monoglycosylation would provide GM2APs bearing various oligosaccharides, which might accelerate improvement in GM2AP functionality.

Chapter 4
Conclusions

1. The feasibility of using SEAlide peptides to act as crypto-thioesters under neutral conditions was discovered. Appropriate choice of additive in reaction media allowed SEAlide peptides to be used for one-pot/sequential NCL.

2. Total chemical synthesis of the 162-residue GM2AP analog containing Cys instead of Asn32 was achieved. In this study, the developed procedure using SEAlide peptides was successfully applied in the one-pot preparation of the C-half fragment and the final convergent assembly.

3. A novel ligation/desulfurization protocol enabling *N*-glycosylated Asn site ligations was developed. This ligation procedure enabled the total synthesis of the monoglycosylated GM2AP.

Taken together, our findings demonstrate that SEAlide peptides are a versatile reagent to achieve total chemical synthesis of proteins. The SEAlide peptides should provide a practical method to synthesize huge and complex proteins. Furthermore the established synthetic route for GM2APs would enable preparation of a wide variety of GM2AP derivatives. The search for additional applications of SEAlide peptides and a medicinal-chemistry-oriented examination of GM2APs for the development of protein therapeutics are currently underway in our laboratory.

Experimental section

General Methods

All reactions except for peptide synthesis were carried out under a positive pressure of argon. For column chromatography, silica gel (KANTO KAGAKU N-60) was employed. Mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™ (ESI-TOF) or Bruker Esquire200T (ESI-Ion Trap). NMR spectra were recorded using a Bruker AV400N at 400 MHz frequency for ^{1}H, and JEOL JNM-AL300 at 75 MHz frequency for ^{13}C. For HPLC separations, a Cosmosil $5C_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL min^{-1}), a Cosmosil Protein-R analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL min^{-1}), a $5C_{18}$-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min), a Cosmosil Protein-R semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL min^{-1}) or a Cosmosil $5C_{18}$-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL min^{-1}) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA aqueous solution (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution. Optical rotations were measured using a JASCO P-2200 polarimeter (concentration in g dL^{-1}). Measurement of absorbance at 280 nm was performed using a DU-650 spectrophotometer. CD spectrum was recorded with a JASCO J600 spectropolarimeter.

Chapter 1

On-resin thiolytic release of peptide thioester 17

On aminomethyl ChemMatrix resin (1.0 mmol amine/g, 300 mg, 0.30 mmol) was coupled Fmoc-Ala-OH (280 mg, 1.5 mmol) with the aid of DIPCDI (230 μL, 1.5 mmol) and HOBt·H_2O (230 mg, 1.5 mmol) in DMF at room temperature for 2 hours followed by Fmoc removal by 20% piperidine in DMF to give Ala-aminomethyl ChemMatrix resin. The resulting resin was treated with Fmoc-Gly-*N*-sulfanylethylaniline linker (430 mg, 0.60 mmol), HATU (220 mg, 0.57 mmol) and DIPEA (99 μL, 0.57 mmol) to yield the anilide-linked resin. On this resin, standard Fmoc SPPS (5.0 equiv. each of amino acid using DIPCDI (5.0 equiv.) and HOBt·H_2O (5.0 equiv.) in DMF (2 h) and Fmoc

removal with 20% piperidine in DMF (10 min)) was performed for the chain elongation to give protected peptide resin for peptide thioester **17**. This protected resin was treated with TFA-thioanisole-*m*-cresol-H_2O-1,2-ethandithiol (EDT) (80:5:5:5:5, (v/v)) at room temperature for 3.5 hours to give deprotected-peptide-attached resin. After complete wash of the resin with CH_2Cl_2, the resin was subjected to *N*–*S* acyl transfer reaction with 4 M HCl in DMF in the presence of TCEP·HCl (1%, (w/v)) at room temperature for 17 h followed by CH_2Cl_2 wash to yield the corresponding on-resin thioester peptide. The resulting thioester resin was reacted with sodium sulfanylethylsulfonate (3%, (w/v)) in 6 M Gn·HCl-0.2 M phosphate buffer (pH 7.0) at room temperature for 2 hours to yield the thiolytic released peptide thioester **17**.

17 Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 25% over 30 min, retention time = 11.0 min, MS (ESI-Ion Trap) *m*/*z* calcd ($[M + 3H]^{3+}$) 1126.9, found 1127.1.

Preparation of SEAlide peptide 18

On NovaSyn® TGR resin (Rink amide type: 0.20 mmol amine/g, 0.50 g, 0.10 mmol) was coupled an Fmoc-Gly-incorporating *N*-sulfanylethylaniline linker (140 mg, 0.20 mmol) with the aid of HATU (72 mg, 0.19 mmol) and DIPEA (33 µL, 0.19 mmol) to yield the SEAlide-linked resin. On this resin, such Fmoc SPPS as mentioned above was performed for the construction of the protected peptide resin. The resulting completed resin was treated with TFA-thioanisole-*m*-cresol-H_2O-EDT (80:5:5:5:5 (v/v), 50 µL/1 mg resin) at room temperature for 1.5 hours. The resin in the reaction mixture was filtrated off. To the resulting filtrate was added cooled Et_2O to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude SEAlide peptide **18**. The crude SEAlide peptide was purified by preparative HPLC (preparative HPLC conditions; linear gradient of solvent B in solvent A over 30 min: 20% to 30%) to give purified **18**.

18 Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 45% over 30 min, retention time = 13.4 min, MS (ESI-Ion Trap) *m*/*z* calcd ($[M + 2H]^{2+}$) 1296.6, found 1296.3.

Coupling of N-Cys-SEAlide peptide 18 with peptide thioester 17

NCL between peptide thioester **17** (1.0 mM) and N-Cys-SEAlide peptide **18** (1.0 mM) was performed in 6 M Gn·HCl-0.2 M phosphate buffer containing 20 mM TCEP and 30 mM MPAA (pH 7.4) at 37 °C. After 3.5 hours reaction, the reaction aliquot was analyzed by analytical HPLC. In Figure 1, arrow (**19**) indicates desired ligated product

(MS (ESI-TOF) *m*/*z* calcd ([M + 6H]$^{6+}$) 972.2, found 972.0); arrow (**20**) indicates cyclized byproduct (MS (ESI-TOF) *m*/*z* calcd ([M + 3H]$^{3+}$) 799.4, found 799.3).

Preparation of model SEAlide peptides 23

On 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 DIPCDI (48 μL, 0.30 mmol) and HOBt·H_2O (48 mg, 0.30 mmol) in DMF at room temperature for 2 hours followed by Fmoc removal by 20% piperidine in DMF to give Leu-NovaSyn® TGR resin. The resulting resin was treated with an Fmoc-Gly or Fmoc-Ala-incorporating SEA linker (0.30 mmol), DIPCDI (48 μL, 0.30 mmol) and HOBt·H_2O (48 mg, 0.30 mmol) to yield the SEAlide-linked resin. On these resins, standard Fmoc SPPS (3.0 equiv each of amino acid using DIPCDI (3.0 equiv) and HOBt·H_2O (3.0 equiv) in DMF (2 hours) and Fmoc removal with 20% piperidine in DMF (10 min)) were performed for the chain elongation to give protected peptide resins for SEAlide peptide **23a** and **23b**. The resulting completed resin was treated with TFA-Et_3SiH-H_2O (95:2.5:2.5 (v/v), 50 μL/1 mg resin) at room temperature for 1.5 hours. The resin in the reaction mixture was filtrated off. To the resulting filtrate was added cooled Et_2O to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude SEAlide peptide **23a** or **23b**. The crude SEAlide peptide was purified by preparative HPLC (preparative HPLC conditions; linear gradient of solvent B in solvent A over 30 min: 9% to 19% for **23a** or 15% to 25% for **23b**) to give purified **23a** or **23b**, respectively.

23a Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 18.4 min, MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 738.4, found 738.1.

23b Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 18.7 min, MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 752.4, found 752.2.

***S*-Alkylation of model SEAlide peptide 23a**

To a solution of **23a** (0.85 mg, 1.0 μmol) in Na phosphate buffer (pH 7.6, 20 mM, 1.0 mL) was added MeI (2 μL) at room temperature with additional stirring at 37 °C for 1 hour. Purification of the resulting reaction mixture on preparative HPLC (linear gradient of solvent B in solvent A, 13% to 23% over 30 min) yielded *S*-methylated **23a**.

Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 35% over 30 min, retention time = 23.1 min,

MS (ESI-Ion Trap) *m*/*z* calcd ($[M + H]^+$) 752.4, found 752.3.

Synthesis of peptide 24, 32, 33 and 41

For the preparations of peptide **24** (amide), SEAlide peptide **32**, peptide **33** (acid), and SEAlide peptide **41**, NovaSyn® TGR resin (Rink amide type: 0.25 mmol amine/g) or Rink Amide AM resin (0.62 mmol amine/g), Fmoc-Gly-SEAlide-linked resin (see; preparation of **23a**), and Fmoc-Tyr(O^{t}Bu)-Wang resin, and Fmoc-Ala-SEAlide-linked resin were used respectively. On these resins, peptides were elongated by using standard Fmoc-based protocols. After TFA cleavage followed by HPLC purifications, desired peptides were obtained.

24 Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 10.6 min, MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 609.3, found 609.2.

32 Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 20.2 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 958.5, found 958.0.

33 Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 15.9 min, MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 789.3, found 789.1.

42 Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 26.5 min, MS (ESI-Ion Trap) *m*/*z* calcd ($[M + H]^+$) 741.3, found 741.3.

Evaluation of coupling of SEAlide peptide 23 with N-terminal cysteinyl peptide 24

General Procedure: Before coupling reaction, quantity of peptide was determined by the data of amino acid analysis of hydrolysate (6 M HCl aq.) of the stock peptides. Direct ligation of SEAlide peptide **23** (1 mM) and cysteinyl peptide **24** (1 mM) was performed at 37 °C. At intervals 10 μL aliquots were withdrawn and the reaction was monitored by analytical HPLC. The extent of ligation (fraction ligated) was quantified by integration of peaks detected at 220 nm on HPLC analysis. Except for entry 4 (reaction of **23a** (1 mM) with **24** (2 mM)), fraction ligated was calculated from the equation (integration of product **25**/(integration of product **25** + integration of starting material **24**)) because the extinction coefficients of peptide **24** and **25** are very similar. For entry 4, following equation containing the correction term (integration of H-*SEA*-**L**-NH_2 **26**) was used due to the much difference extinction coefficients between the SEAlide peptide **23** and ligated peptide **25**: (integration of product **25** + integration

of H-*SEA*-**L**-NH_2 **26**)/(integration of **25** + integration of H-*SEA*-**L**-NH_2 **26** + integration of starting material **23**). Fraction ligated under several conditions as a function of reaction time was summarized below.

Fraction ligated under several conditions as a function of reaction time

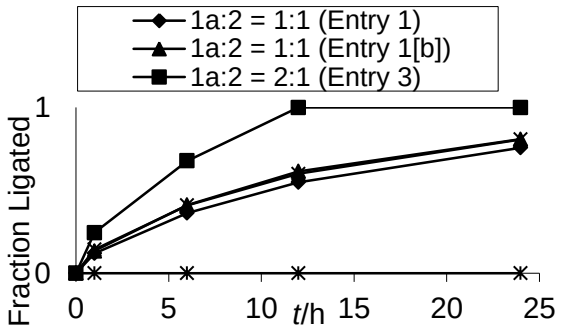


Figure S1. Influence of the ratio of **23a** to **24**.

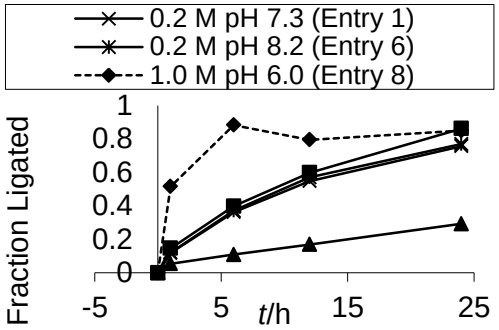


Figure S2. Influence of pH and concentration of phosphate salts.

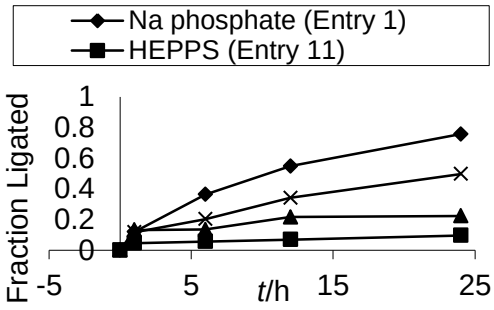


Figure S3. Influence of buffer salts.

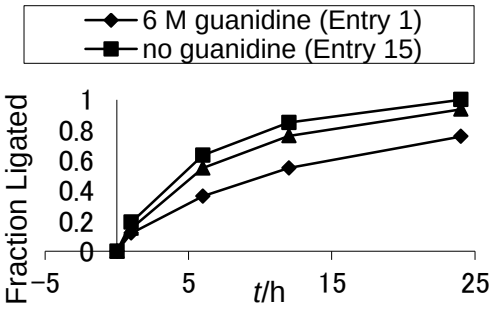


Figure S4. Influence of detergents.

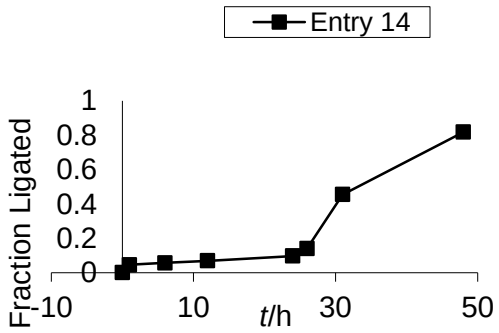


Figure S5. Ligation in the presence of HEPPS and subsequent addition of phosphate salt. Phosphate salt was added at the point of 25 h.

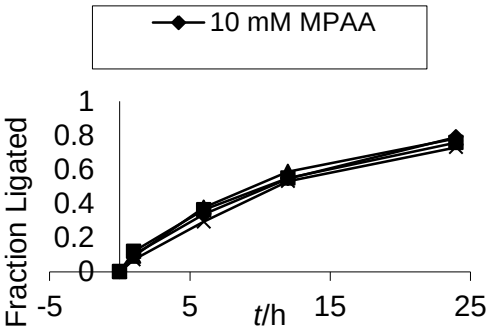


Figure S6. Influence of thiol additive.

Synthesis of thioester peptides 31, 37 and 40

Alkyl thioester peptides **31**, **37**, and **40** were prepared by Boc SPPS using *in situ* neutralization protocol,[28] on $HSCH_2CH_2CO$-Leu-4-methylbenzhydrylamine (MBHA) resin (0.70 mmol amine/g, 140 mg, 0.10 mmol).[9a] The resulting completed resin was treated with 1 M TMSOTf-thioanisole in TFA (50 μL/1 mg resin)/*m*-cresol (100/5, (v/v)) at 4 °C for 2 hours. The resin in the reaction was filtrated off. To the resulting filtrate was added cooled Et_2O to give precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude alkyl thioester peptide **31**, **37** or **40**. Crude peptide was purified by semi-preparative HPLC (semi-preparative HPLC conditions: $5C_{18}$-AR-II semi-preparative column with a linear gradient of solvent B in solvent A over 30 min: 5% to 35% for **31** or 15% to 30% for **37** or preparative HPLC conditions: $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A over 30 min: 7% to 17% for **40**) to give the purified **31**, **37** or **40**, respectively.

31 Analytical HPLC condition: $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 13.5 min, MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 905.5, found 905.1.

37 Analytical HPLC condition: $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 20.2 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 912.9, found 912.7.

40 Analytical HPLC condition: $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 15.2 min, MS (ESI-Ion Trap) *m*/*z* calcd ($[M + H]^+$) 880.3, found 880.5.

Conversion of alkyl thioester 31 to aryl thioester 36

Alkyl thioester **31** was dissolved at a concentration of 0.2 mM in 6.0 M Gn·HCl-0.2 M Na phosphate (pH 6.8) containing 0.2% (v/v) thiophenol. The reaction mixture was vigorously stirred for 12 hours with exclusion of air, and the reaction progress was monitored by analytical HPLC. The reaction mixture was acidified to pH 3 by addition of 6 M HCl aq.. After residual thiophenol and oxidized thiophenol were removed by extraction with Et_2O, the reaction mixture was purified by preparative HPLC ($5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 10% to 16% over 30 min) to give the aryl thioester peptide **36** (1.74 mg, 55%).

36 Analytical HPLC conditions: $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 15.7 min, MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 797.4, found 797.2.

Synthesis of hANP by using the SEAlide peptide

Ligation of alkyl thioestser peptide **31** (1.0 mM) and SEAlide peptide **32** (1.0 mM) was performed in 6 M Gn·HCl-0.2 M HEPPS buffer containing 30 mM TCEP and 30 mM MPAA (pH 7.3). The reaction was complete within 3 hours at 37 °C. After confirming the completion of the first NCL by HPLC analysis, 1.0 mM of peptide **33** in 1.0 M Na phosphate (pH 6.4) was added to the reaction mixture to yield the desired 2Cys-SH hANP **35** in a one-pot manner. Second NCL proceeded smoothly within 24 hours, and the generated peptide was purified by semi-preparative HPLC ($5C_{18}$-AR-II semi-preparative column with a linear gradient of solvent B in solvent A, 10% to 30% over 30 min) to give the desired peptide 2Cys-SH hANP **35** (2.14 mg, 0.57 mmol, 50%).

35 Analytical HPLC conditions: $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 5% to 45% over 30 min, retention time = 19.6 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 4H]^{4+}$) 771.1, found 770.9.

Kinetically controlled ligation using aryl thioester 36 and Alkyl Thioester 37[10]

Kinetically controlled ligation was performed in pH 6.8, 0.2 M Na phosphate buffer containing 6 M Gn·HCl, at a concentration of 2 mM for each peptide in the absence of thiophenol. After 1 hour reaction, the reaction aliquot was analyzed by analytical HPLC. In Figure 4, arrow (**37**) indicates cyclized byproduct cyclic-[Cys^7-Gly^{22}] (MS (ESI-TOF) *m*/*z* calcd ($[M+2H]^{2+}$) 803.9, found 803.7); arrow (**36 +** cyclic **37**) indicates another cyclic byproduct [Ser^1-Ser^6]-cyclic-[Cys^7-Gly^{22}] (MS (ESI-TOF) *m*/*z* calcd ($[M+3H]^{3+}$) 765.1, found 764.9); arrow (**36** + **38**) indicates the byproduct [Ser^1-Ser^6]-[Ser^1-Gly^{22}]-alkyl thioester (MS (ESI-TOF) *m*/*z* calcd ($[M+5H]^{5+}$) 640.3, found 640.3).

Synthesis of α-conotoxin ImI (^{11}Ala) by one-pot/four components coupling under kinetically conditions

Kinetically controlled ligation of glycine aryl thioester **39**[4] (2.0 mM) and alkyl peptide thioester **40** (2.0 mM) was performed in 6 M Gn·HCl-0.2 M HEPPS buffer (pH 6.8). The reaction was complete within 1 hour at room temperature. After confirming the completion of the first NCL by HPLC analysis, 2.0 mM of SEAlide peptide **41** in 6 M Gn·HCl-0.2 M HEPPS buffer containing 80 mM TCEP, 60 mM MPAA (pH 6.8) was added to the reaction mixture to yield the three fragments ligated SEAlide peptide (**44**) at 37 °C. Finally, cysteine amide **42** (1.0 mM) in 1 M Na phosphate buffer (pH 6.4) was added to the reaction mixture. Concentration of components was 0.5 mM each in 3 M

Gn·HCl-0.1 M HEPPS-0.5 M Na phosphate in the presence of 20 mM TCEP and 15 mM MPAA. The third NCL reaction proceeded smoothly within 20 hours at 37 °C, and the generated peptide was purified by semi-preparative HPLC ($5C_{18}$-AR-II semi-preparative column with a linear gradient of solvent B in solvent A, 17% to 27% over 30 min) to give the desired 4Cys-SH α-conotoxin ImI (^{11}Ala) **45** (0.4 mg, 0.27 μmol, 28%).

45 Analytical HPLC condition: $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 16.8 min, MS (ESI-Ion Trap) *m*/*z* calcd ($[M + 2H]^{2+}$) 635.7, found 635.8.

Check of the epimerization of C-terminal chiral amino acids

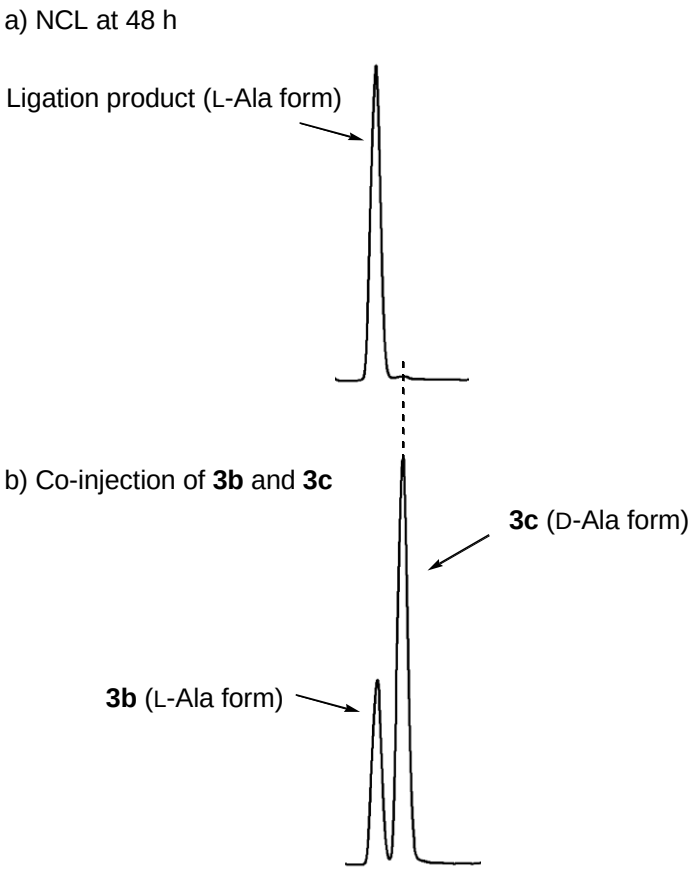
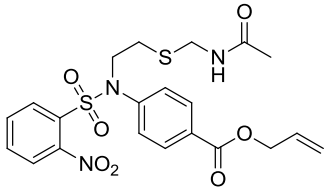


Figure S7. HPLC examination of epimerization of C-terminal chiral amino acid (L-Ala) during NCL: (a) NCL at 48 h HPLC conditions; (b) co-injection of **25b** and **25c**. Analytical HPLC conditions: $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 2% to 50% over 30 min. Only a critical retention time region of the HPLC charts was enlarged. Sequences of **25**: H-**VQGSX-CFGRK**-NH_2 (X = L-Ala (**25b**), D-Ala (**25c**)). D-Ala containing diastereomer peptide (**25c**) corresponding **25b** was synthesized by standard step-wise Fmoc SPPS protocol using Fmoc-D-Ala-OH.

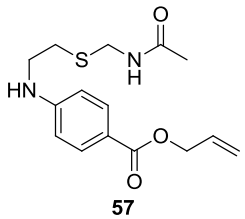
Chapter 2

Synthesis of Acm-protected *N*-sulfanylethyl aniline linker 59



56

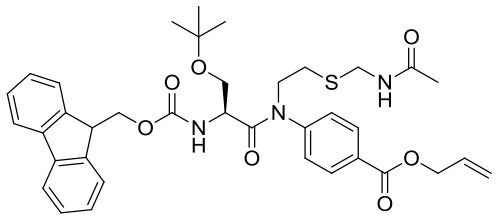
Compound **55**[3] (1.50 g, 2.26 mmol) was dissolved in 6.00 mL of TFA. After being stirred for 30 min, acetamidomethanol[49] (603 mg, 6.77 mmol) dissolved in 1.53 mL of TFA was added to the reaction mixture. The resultant mixture was stirred for additional 30 min. Then the TFA was evaporated, and the residue was neutralized by addition of 10% (w/v) Na_2CO_3 aq.. The resulting mixture was extracted with EtOAc. The organic phase was washed with saturated $NaHCO_3$ aq. and brine and dried over $MgSO_4$. After removal of the solvent under reduced pressure, chromatographic purification on silica gel (*n*-hexane/EtOAc = 1:2) gave Acm-protected compound **56** in 76% (852 mg) isolated yield as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ = 2.02 (3H, s), 2.72 (2H, t, J = 7.0 Hz), 4.06 (2H, t, J = 7.0 Hz), 4.40 (2H, d, J = 6.3 Hz), 4.82 (2H, ddd, J = 5.8, 1.5 and 1.3 Hz), 5.30 (1H, ddt, J = 10.5, 2.7 and 1.3 Hz), 5.41 (1H, ddt, J = 17.1, 2.7 and 1.5 Hz), 6.02 (1H, ddt, J = 17.1, 10.5 and 5.8 Hz), 6.18 (1H, br t, J = 6.3 Hz), 7.35 (2H, d, J = 8.7 Hz), 7.47-7.54 (2H, m), 7.62-7.70 (2H, m), 8.03 (2H, d, J = 8.7 Hz); ^{13}C NMR ($CDCl_3$) δ = 23.1, 30.6, 41.4, 51.7, 65.8, 118.6, 124.0, 129.1, 130.2, 130.9, 131.4, 131.5, 131.7, 131.8, 134.0, 141.6, 147.8, 165.1, 170.4; HRMS (ESI-TOF) *m*/*z* calcd for $C_{21}H_{23}N_3NaO_7S_2$ ($[M + Na]^+$): 516.0875, found 516.0884.



57

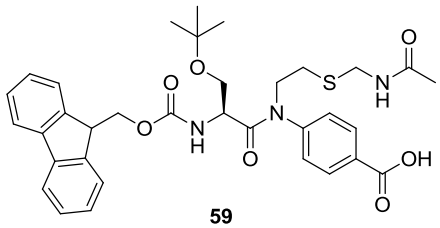
Compound **56** (235 mg, 0.547 mmol) in DMF (2.70 mL) was treated with $LiOH \cdot H_2O$ (248 mg, 6.02 mmol) and thioglycolic acid (228 μL, 3.28 mmol). The reaction mixture was stirred at room temperature for 1.5 hours, and then diluted with water. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with saturated $NaHCO_3$ aq. and brine, and then dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc = 1:2) to

give compound **57** (110 mg, 65%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ = 1.99 (3H, s), 2.85 (2H, t, *J* = 6.4 Hz), 3.46 (2H, t, *J* = 6.4 Hz), 4.42 (2H, d, *J* = 6.4 Hz), 4.77 (2H, ddd, *J* = 5.6, 1.6 and 1.2 Hz), 4.81 (1H, s), 5.25 (1H, ddt, *J* = 10.4, 1.6 and 1.2 Hz), 5.38 (1H, ddt, *J* = 17.2, 1.6 and 1.6 Hz), 6.02 (1H, ddt, *J* = 17.2, 10.4 and 5.6 Hz), 6.08-6.25 (1H, br m), 6.59 (2H, d, *J* = 8.8 Hz), 7.89 (2H, d, *J* = 8.8 Hz); ^{13}C NMR ($CDCl_3$) δ = 23.2, 30.0, 40.8, 42.9, 64.9, 111.6, 117.6, 118.4, 131.6, 132.7, 151.6, 166.5, 170.4; HRMS (ESI-TOF) *m*/*z* calcd for $C_{15}H_{20}N_2NaO_3S$ ($[M + Na]^+$) 331.1092, found 331.1103.



58

To a stirred mixture of aniline **57** (500 mg, 1.62 mmol) in CH_2Cl_2 (16.2 mL) were successively added Fmoc-Ser(^{t}Bu)-OH (3.11 g, 8.11 mmol), Et_3N (1.13 mL, 8.11 mmol) and $POCl_3$ (756 μL, 8.11 mmol) at 0 °C. After being stirred at same temperature for 13.5 hours, the reaction was quenched by the addition of saturated $NaHCO_3$ aq. The resulting mixture was extracted with EtOAc. The organic phase was successively washed with saturated $NaHCO_3$ aq. and brine, and then dried over $MgSO_4$. After removal of the solvent in vacuo, chromatographic purification on silica gel (*n*-hexane/EtOAc = 1:3) gave desired Fmoc-Ser(^{t}Bu)-incorporated linker **58** in 49% (538 mg) isolated yield as a pale yellow amorphousness: $[\alpha]^{21}_D$ 46.0 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ = 1.13 (9H, s), 2.01 (3H, s), 2.74 (2H, d, *J* = 6.8 Hz), 3.28-3.37 (1H, br m), 3.37-3.45 (1H, br m), 3.85-4.03 (2H, br m), 4.21 (1H, t, *J* = 6.8 Hz), 4.37 (2H, d, *J* = 6.8 Hz), 4.41 (2H, d, *J* = 4.7 Hz), 4.45 (1H, br m), 4.83 (2H, d, *J* = 5.6 Hz), 5.31 (1H, dd, *J* = 10.1 and 1.0 Hz) 5.41 (1H, br m), 5.42 (1H, dd, *J* = 17.1 and 1.0 Hz), 6.03 (1H, ddt, *J* = 17.1, 10.1 and 5.6 Hz), 7.00 (1H, br m), 7.31 (2H, dd, *J* = 7.4 and 7.3 Hz), 7.40 (2H, dd, *J* = 7.6 and 7.4 Hz), 7.41 (2H, br m), 7.58 (1H, d, *J* = 7.3 Hz), 7.59 (1H, d, *J* = 7.3 Hz), 7.78 (2H, d, *J* = 7.6 Hz), 8.14 (2H, d, *J* = 8.2 Hz); ^{13}C NMR ($CDCl_3$) δ = 23.1, 27.3, 29.0, 41.4, 47.1, 49.5, 51.8, 62.3, 65.8, 67.0, 73.7, 118.6, 120.0, 125.0, 125.0, 127.0, 127.7, 128.4, 130.2, 131.2, 131.9, 141.3, 143.7, 143.7, 144.9, 155.6, 165.1, 170.0, 171.3; HRMS (ESI-TOF) *m*/*z* calcd for $C_{37}H_{43}N_3NaO_7S$ ($[M + Na]^+$): 696.2719, found 696.2744.



59

Amino acid-incorporated linker **58** (300 mg, 0.445 mmol) in THF (4.50 mL) was treated with *N*-methylaniline (482 μL, 4.45 mmol) and $Pd(PPh_3)_4$ (51 mg, 0.045 mmol). After being stirred at room temperature for 2.5 hours, the solvent was removed in vacuo and the product was purified by silica gel chromatography ($CHCl_3$/MeOH = 95:5) to give the desired compound **59** (256 mg, 91%) as a pale yellow amorphousness: $[\alpha]^{21}_D$ 52.1 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ = 1.14 (9H, s), 2.03 (3H, s), 2.76 (2H, br t, *J* = 6.8 Hz), 3.30-3.41 (1H, br m), 3.41-3.50 (1H, br m), 3.86-4.05 (2H, br m), 4.20 (1H, t, *J* = 7.0 Hz), 4.31-4.39 (2H, br m), 4.39-4.44 (2H, br m), 4.46-4.57 (1H, br m), 5.69-5.80 (1H, br m), 6.96-7.13 (1H, br m), 7.28 (1H, t, *J* = 6.9 Hz), 7.30 (1H, t, *J* = 6.9 Hz), 7.39 (2H, dd, *J* = 7.4 and 6.9 Hz), 7.44 (2H, d, *J* = 8.2 Hz), 7.60 (1H, d, *J* = 6.9 Hz), 7.61 (1H, d, *J* = 6.9 Hz), 7.75 (2H, d, *J* = 7.4 Hz), 8.16 (2H, d, *J* = 8.2 Hz); ^{13}C NMR ($CDCl_3$) δ = 23.1, 27.3, 28.9, 41.4, 47.0, 49.7, 51.8, 62.3, 67.2, 73.8, 120.0, 125.1, 125.1, 127.0, 127.7, 128.5, 130.0, 131.7, 141.3, 143.7, 143.7, 145.0, 155.8, 168.6, 170.6, 171.6; HRMS (ESI-TOF) *m*/*z* calcd for $C_{34}H_{39}N_3NaO_7S$ ($[M + Na]^+$): 656.2406, found 656.2423.

Preparation of peptide thioesters 46 (Fr 1) and 48 (Fr 3)

Peptide thioesters **46** and **48** were prepared by Boc SPPS using *in situ* neutralization protocol on $HSCH_2CH_2CO$-Leu-MBHA resin (0.70 mmol amine/g, 0.43 g, 0.30 mmol). The resulting completed resin was treated with 1 M TMSOTf-thioanisole in TFA (50 μL/1 mg resin)/*m*-cresol/EDT (100/5/5, (v/v)) at 4 °C for 2 hours, and then the resin in the reaction mixture was filtrated off. To the resulting filtrate was added cooled Et_2O to give precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide thioesters **46** or **48**. The crude peptides were purified by preparative HPLC (Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A over 30 min: 25% to 35% for **46** or 36% to 42% for **48**) to give the purified **46** or **48**, respectively.

46 Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 20% to 50% over 30 min, retention time = 19.3 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 4H]^{4+}$) 907.9, found 907.5.

48 Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear

gradient of solvent B in solvent A, 20% to 50% over 30 min, retention time = 18.0 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 4H]^{4+}$) 962.2, found 961.1.

Preparation of SEAlide peptides 47 (Fr 2) and 49 (Fr 4)

On Rink amide ChemMatrix resin (0.54 mmol amine/g, 0.19 g, 0.10 mmol for **47**, or 0.56 g, 0.30 mmol for **49**) was coupled Fmoc-Leu-OH (3.0 equiv) with the aid of DIPCDI (3.0 equiv) and HOBt·H_2O (3.0 equiv) in DMF at room temperature for 2 hours. Then Fmoc removal was performed with 20% (v/v) piperidine in DMF to give a Leu-incorporated resin. The resulting resin was treated with an Fmoc-Ser(^{t}Bu)-incorporating SEA(SAcm) linker **59** (2.0 equiv) for **47** or an Fmoc-Gly-incorporating SEA(STrt) linker (2.0 equiv) for **49**, HATU (1.9 equiv), and DIEA (1.9 equiv) to yield the SEAlide-linked resins. On this resin, peptide was elongated by using standard Fmoc-based protocols as described above to give a protected peptide resin for SEAlide peptides **47** or **49**. The resulting completed resin was treated with TFA-*m*-cresol-thioanisole-EDT-H_2O (80:5:5:5:5 (v/v), 50 μL/1 mg resin) at room temperature for 2 hours. The resin in the reaction mixture was filtrated off. To the resulting filtrate was added cooled Et_2O to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude SEAlide peptide **47** or **49**. The crude SEAlide peptide was purified by preparative HPLC (Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A over 30 min: 37% to 38% for **47** or 34% to 35% for **49**) to give purified SEAlide peptide **47** or **49**, respectively.

47 Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 30% to 50% over 30 min, retention time = 17.4 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 5H]^{5+}$) 997.1, found 997.0.

49 Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 60% over 30 min, retention time = 20.6 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 6H]^{6+}$) 880.6, found 880.4.

Preparation of peptide 50 (Fr 5)

For the preparations of peptide **50**, Fmoc-Ile-Wang resin (0.58 mmol amine/g, 0.17 g, 0.10 mmol) was used. On this resin, peptide was elongated by using standard Fmoc-based protocols as described above. After TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-EDT-triethylsilane (80:5:5:5:2.5:2.5 (v/v), 50 μL/1 mg resin), 2 hours) followed by HPLC purification (Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A over 30 min: 17% to 27%),

desired peptide **50** was obtained.
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 60% over 30 min, retention time = 16.2 min, MS (ESI-Ion Trap) *m*/*z* calcd ($[M + H]^+$) 1116.7, found 1116.4.

Synthesis of Acm-protected Fr 1-2 60

Fr 1 **46** (7.6 mg, 1.9 μmol) and Fr 2 **47** (11 mg, 1.9 μmol) were dissolved in 1.9 mL of ligation buffer (6 M Gn·HCl, 0.2 M HEPPS, 3% (v/v) thiophenol, pH 7.4), and the solution was incubated at 37 °C. The reaction was completed within 3 hours. The crude peptide was purified by preparative HPLC (Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A over 30 min: 39% to 44%) to give purified Acm-protected Fr 1-2 **60** (11 mg, 1.2 μmol, 62%).
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 30% to 50% over 30 min, retention time = 22.0 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 8H]^{8+}$) 1049.8, found 1049.5.

Alkylation for synthesis of glycosylated Fr 1-2 62

To a solution of **60** (10 mg, 1.2 μmol) in 0.2 M HEPPS buffer with 6 M Gn·HCl (pH 7.4, 2.4 mL) was added iodoacetamide **61**[26] (2.3 mg, 5 equiv) at 37 °C and the resulting mixture was incubated at same temperature for 2 hours. Purification of the resulting reaction mixture on preparative HPLC (Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 39% to 44% over 30 min) yielded *S*-glycosylated peptide **62** (9.9 mg, 1.1 μmol, 90%).
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 30% to 50% over 30 min, retention time = 18.8 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 9H]^{9+}$) 962.2, found 962.0.

Acm removal of N-half fragment 51

The Acm-protected peptide **62** (16 mg, 1.7 μmol) was treated with AgOTf (43 mg, 0.17 mmol) and anisole (18 μL, 0.17 mmol) in TFA (420 μL, 4.0 mM peptide) at 4 °C. After 24 hours, to the reaction mixture was added cooled Et_2O to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O. The resulting crude peptide was dissolved in 10 mL solution of 6 M Gn·HCl-0.1 M Na phosphate buffer (pH 7.2) containing 3% (w/v) DTT. The suspension was stood at room temperature for 20 min and then filtrated. The filtrate was purified by preparative HPLC (Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent

A over 30 min: 39% to 40%) to afford the purified **51** (6.5 mg, 0.71 μmol, 42%)
Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 50% over 30 min, retention time = 24.0 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 9H]^{9+}$) 938.5, found 938.4.

One-pot synthesis of C-half fragment 52

Ligation of alkyl thioester peptide **48** (4.3 mg, 1.0 μmol) and SEAlide peptide **49** (6.0 mg, 1.0 μmol) was performed in 6 M Gn·HCl-0.1 M HEPPS buffer containing 50 mM TCEP and 100 mM MPAA (pH 7.0, 0.50 mL (2.0 mM each peptide)) at 37 °C. The reaction was completed within 3 hours. After confirming the completion of the first NCL by HPLC analysis, peptide **50** solution (1.1 equiv) in 6 M Gn·HCl-0.4 M Na phosphate buffer (pH 7.0, 2.0 mL) was added to the reaction mixture to yield the Thz-peptide in one-pot manner. The second NCL proceeded smoothly within 24 hours, and then to the reaction mixture was added $MeONH_2$·HCl (42 mg in solid form, final concentration 0.2 M) to open a 1,3-thiazolidine ring. After 3 hours, the crude material was purified by semi-preparative HPLC (Cosmosil Protein-R semi-preparative column with a linear gradient of solvent B in solvent A, 35% to 40% over 30 min) to give the desired C-half fragment **52** (5.2 mg, 0.47 μmol, 47%).
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 20% to 50% over 30 min, retention time = 22.3 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 8H]^{8+}$) 1213.4, found 1213.3.

Assembly of N-half fragment 51 and C-half fragment 52

The N-half fragment **51** (0.76 mg, 0.084 μmol) and the C-half fragment **52** (0.92 mg, 0.084 μmol) were dissolved in 6 M Gn·HCl-0.5 M Na phosphate buffer containing 50 mM TCEP and 50 mM MPAA (pH 6.0, 0.17 mL) and the mixture was incubated at 37 °C. After 24 hours, the reaction mixture was diluted with 6 M Gn·HCl-0.5 M Na phosphate buffer (pH 6.0, 1.0 mL), and then the crude peptide was purified by semi-preparative HPLC (Cosmosil Protein-R semi-preparative column with a linear gradient of solvent B in solvent A over 30 min: 40% to 45% over 30 min) to give purified reduced form of **53** (0.38 mg, 0.019 μmol, 23%).
Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 35% to 55% over 30 min, retention time = 21.5 min, MS (ESI-TOF) calcd (average isotopes) 17838.4, found 17837.9.

Folding for preparation of monoglycosylated GMAP analog 54

Folding for preparation of monoglycosylated GMAP analog **54** was performed with a modified method of previously reported one.[31] The reduced GM2AP analog **53** (0.84 mg) was dissolved in 6 M Gn·HCl-0.1 M Na phosphate buffer (pH 8.0, 1.3 mL). The resulting solution was added to 50 mM Tris·HCl buffer containing 2 mM reduced form glutathione, 0.2 mM oxidized form glutathione and 0.003% (v/v) Tween 20 (pH 8.0, 7.1 mL, final concentration of protein 0.10 mg mL^{-1}). After stored at 4 °C for a day and then at room temperature for additional one day, the folding buffer was exchanged to 15 mM Na phosphate buffer (pH 7.4) by using ultrafiltration. Concentration of the GM2AP analog **54** was determined as 0.18 mg mL^{-1} (3.7 mL, 67%) by measurement of absorbance at 280 nm and calculation using the equation $A_{280} = \varepsilon_{280}\, c\, l$. The A_{280} is the observed absorbance at 280 nm (A_{280} = 0.2058), the ε_{280} (M^{-1} cm^{-1}) is the molar extinction coefficient of GM2AP at 280 nm (ε_{280} = 22960, calculated as previously reported[50]), the c (M) is concentration of a protein, and the l (cm) is the length of the optical path. The resulting solution was directly used for measurement of CD spectrum and *in vitro* GM2-degradation assay, and then purified by analytical HPLC (Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A over 30 min: 35% to 55% over 30 min) to give GM2AP analog **54** (0.27 mg, 32 %).

Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 35% to 55% over 30 min, retention time = 20.0 min, MS (ESI-TOF) calcd (average isotopes) 17830.4, found 17830.3.

CD measurement of monoglycosylated GM2AP analog 54

CD spectrum in the range of 200 to 260 nm was recorded at room temperature in a 0.2 mm-path quartz cuvette. Molar mean-residue ellipticity θ was calculated as previously described.[32]

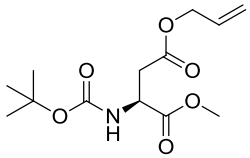
***In vitro* GM2-degradation assay of synthesized GM2AP analog 54**

In vitro GM2-degradation assay was performed as described previously.[33] Briefly, the GM2 was incubated with each recombinant human HexA (5000 nmol h^{-1} 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (4MUG)-degrading activity[51]) in the presence or absence of 2.5 μg of synthesized GM2AP analog **54** or *E. coli*-expressed GM2AP in 200 μL of 10 mM citrate buffer (pH 4.5) containing 0.1% bovine serum albumin (BSA) at 37 °C for 24 hours. After the incubation, the reaction was stopped by heating the tube with boiling water for 3 min, and then the GM2 and GM3 were isolated using a C_{18} Sep-Pak Cartridge. Aliquots of samples were spotted on a silica gel plate

and developed with $CHCl_3$/MeOH/0.2% (w/v) $CaCl_2$ aq. = 55:45:10, (v/v). To reveal the gangliosides, the thin-layer chromatography plate was sprayed with orcinol reagent and heated at 120 °C for 5 min.

Chapter 3

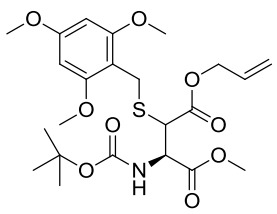
Synthesis of *N*-glycosylated asparagine derivative 78



71

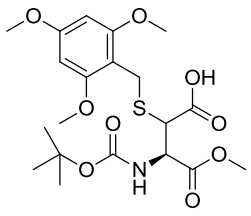
The protected asparagine derivative **71** was synthesized similarly to its enantiomer.[52] To ice-cold allyl alcohol (50 mL) was added acetyl chloride (7.10 mL, 100 mmol). After being stirred at the same temperature for 15 min followed by at rt for 1 hour, L-aspartic acid (3.33 g, 25.0 mmol) was added. The resulting mixture was stirred at rt for 23 hours and was poured into ice-cold Et_2O (250 mL) to give a precipitate. After being stirred at 0 ºC for 1 hour, the precipitate was collected by filtration and was washed with Et_2O to give the aspartic acid β-allyl ester as a white powder (4.05 g, 77%). To the solution of the allyl ester (3.50 g, 16.7 mmol) in water (25 mL) were added Boc_2O (4.37 g, 20.0 mmol) in dioxane (25 mL) and Et_3N (6.50 mL, 46.8 mmol), and then the solution was stirred at rt for 3 hours. After the reaction mixture was washed with Et_2O, the aqueous phase was acidified by 10% (w/v) citric acid aq. to pH 4–5 and then was extracted into EtOAc. The organic phase was successively washed with 10% (w/v) citric acid aq. and brine, and then dried over Na_2SO_4. Filtration of the mixture followed by concentration under reduced pressure gave the *N*-Boc-aspartate as a colorless oil (4.53 g, 99%). To the solution of the resulting compound (4.00 g, 14.6 mmol) in DMF (50 mL) was added K_2CO_3 (6.07 g, 43.9 mmol) and the reaction mixture was stirred at rt. After being stirred for 2 hours, methyl iodide (2.73 mL, 43.9 mmol) was added and the resultant mixture was stirred for additional 12 hours. Then the DMF was evaporated, and the residue was dissolved in EtOAc. The organic phase was washed with 1 M HCl aq., saturated $NaHCO_3$ aq., 5% (w/v) $Na_2S_2O_3$ and brine, and then dried over Na_2SO_4. After filtration and subsequent removal of the solvent under reduced pressure, chromatographic purification on silica gel (*n*-hexane/EtOAc = 5:1, (v/v)) gave the fully protected aspartate **71** in 86% isolated yield (3.62 g) as a colorless oil. The NMR spectrum was identical to that of the enantiomer: $[\alpha]^{18}_D$ -14.3 (*c* 0.96, MeOH); ^{1}H NMR ($CDCl_3$, 400

MHz) δ = 1.45 (9H, s), 2.85 (1H, dd, 17.0 and 4.9 Hz), 3.03 (1H, dd, 17.0 and 4.6 Hz), 3.76 (3H, s), 4.60 (2H, ddd, 5.8, 1.3 and 1.4 Hz), 4.52–4.67 (1H, m), 5.25 (1H, ddt, 10.4, 1.4 and 1.3 Hz), 5.32 (1H, ddt, 17.2, 1.4 and 1.4 Hz), 5.49 (1H, br d, 7.7 Hz), 5.90 (1H, ddt, 17.2, 10.4 and 5.8 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 28.2, 36.7, 49.9, 52.5, 65.5, 79.8, 118.4, 131.7, 155.2, 170.5, 171.4.



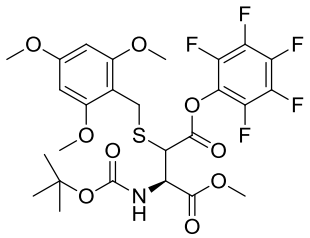
73

To a solution of **71** (3.26 g, 11.4 mmol) in THF (120 mL) at -78 °C was added LiHMDS (1 M in THF, 25.0 mL, 25.0 mmol) and the reaction mixture was stirred at the same temperature for 2 hours. Then a solution of *S*-(2,4,6-trimethoxybenzyl)toluenethiosulfonate **72**[41] (5.88 g, 16.0 mmol) in THF (60 mL) was added and the resultant was stirred for additional 3 hours at -78 °C. After quenching with saturated NH_4Cl aq., the THF was removed under reduced pressure, and the residue was extracted with EtOAc. The organic phase was successively washed with saturated NH_4Cl aq. and brine, and then dried over Na_2SO_4. After filtration followed by removal of the solvent in vacuo, Et_2O was added to give a precipitate. The precipitate was filtrated off and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc = 4:1 to 3:1, (v/v)) to give a diastereomeric mixture of **73** (7:2) as a pale yellow oil (4.64 g, 81%). The major diastereomer was isolated for characterization: $[\alpha]^{19}_{D}$ 57.7 (*c* 0.96, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.44 (9H, s), 3.70 (3H, s), 3.81 (9H, s), 3.90 (1H, br d, 12.8 Hz), 3.99 (1H, br d, 12.8 Hz), 4.12 (1H, br d, 4.3 Hz), 4.59 (2H, br d, 5.6 Hz), 4.82 (1H, br dd, 9.6 and 4.3 Hz), 5.23 (1H, dq, 10.6 and 1.1 Hz), 5.33 (1H, bq, 17.3 and 1.4 Hz), 5.66 (1H, br d, 9.6 Hz), 5.87 (1H, ddt, 17.3, 10.6 and 5.6 Hz), 6.11 (2H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 25.0, 28.4, 48.5, 52.6, 55.0, 55.4, 55.8, 55.8, 66.1, 80.0, 90.6, 90.6, 106.9, 118.4, 118.5, 131.7, 155.9, 159.0, 159.0, 160.7, 171.0, 171.5; HRMS (ESI-TOF) *m*/*z* calcd for $C_{23}H_{33}NNaO_9S$ ($[M + Na]^+$): 522.1774, found 522.1780.



74

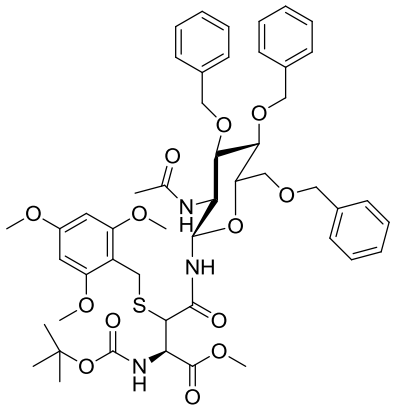
Allyl ester **73** (4.00 g, 8.01 mmol, diastereomeric mixture) in THF (30 mL) was treated with *N*-methylaniline (8.71 mL, 80.1 mmol) and $Pd(PPh_3)_4$ (185 mg, 0.160 mmol). After being stirred at room temperature for 2 hours, the solvent was removed in vacuo and the product was purified by silica gel column chromatography (*n*-hexane/EtOAc/AcOH = 2:1:0.05 to 3:2:0.05, (v/v)) to give a diastereomeric mixture of **74** (7:2) as a pale yellow amorphousness (3.31 g, 90%). The major diastereomer was isolated for characterization: $[\alpha]^{19}_{D}$ 41.6 (*c* 0.98, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.45 (9H, s), 3.72 (3H, s), 3.81 (3H, s), 3.82 (6H, s), 3.95 (2H, s), 4.12 (1H, br d, 3.7 Hz), 4.89 (1H, br dd, 9.9 and 3.7 Hz), 5.52 (1H, brd, 9.9 Hz), 6.13 (2H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 25.3, 28.4, 52.9, 55.0, 55.5, 55.9, 55.9, 80.3, 90.7, 106.6, 155.9, 159.0, 160.9, 160.9, 170.8, 170.8; HRMS (ESI-TOF) *m*/*z* calcd for $C_{20}H_{29}NNaO_9S$ ($[M + Na]^+$): 482.1461, found 482.1460.



75

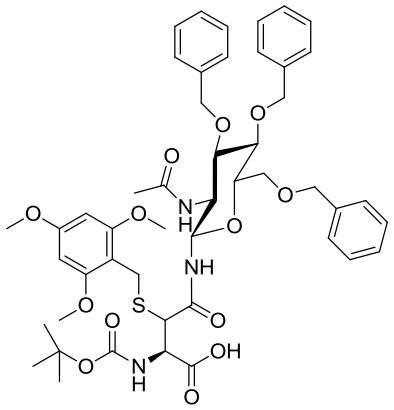
Compound **74** (2.01 g, 4.37 mmol, diastereomeric mixture) in CH_2Cl_2 (15.0 mL) was treated with pentafluorophenol (687 μL, 6.56 mmol) and EDC·HCl (1.01 g, 5.24 mmol). After being stirred at rt for 1 hour, the CH_2Cl_2 was removed in vacuo. EtOAc was added to the residue and the obtained solution was washed with water, saturated $NaHCO_3$ aq., saturated NH_4Cl aq. and brine. After drying over Na_2SO_4 followed by filtration, the organic layer was concentrated under reduced pressure. The obtained residue was subjected to chromatographic purification on silica gel (*n*-hexane/EtOAc = 3:1, (v/v)) to give a diastereomeric mixture of **75** (1:1) in 81% isolated yield (2.21 g) as a colorless amorphousness. Low polar diastereomer was isolated for characterization: $[\alpha]^{18}_{D}$ 108.4 (*c* 0.93, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.44 (9H, s), 3.74 (3H, s), 3.80 (3H, s), 3.83 (6H, s), 4.02 (2H, s), 4.39 (1H, br d, 4.8 Hz), 4.98 (1H, br dd, 10.1 and 4.8 Hz),

5.49 (1H, br d, 10.1 Hz), 6.12 (2H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 25.5, 28.4, 48.3, 52.9, 55.0, 55.4, 55.8, 55.8, 80.4, 90.6, 90.6, 106.5, 124.9, 138.0 (d, 246.6 Hz), 139.7 (d, 251.6 Hz), 141.3 (d, 246.6 Hz), 155.6, 159.1, 159.1, 160.9, 168.2, 170.2; HRMS (ESI-TOF) *m*/*z* calcd for $C_{26}F_5H_{28}NNaO_9S$ ($[M + Na]^+$): 648.1303, found 648.1292.



77

To a stirred solution of pentafluorophenyl ester **75** (1.75 g, 2.80 mmol, diastereomeric mixture), 2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl azide **76** (2.17 g, 4.20 mmol), and 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (456 mg, 2.80 mmol) in THF containing 2% (v/v) H_2O (28 mL) was added ^{n}Bu$_3$P (1.40 mL, 5.60 mmol) at 0 ºC. After being stirred at rt for 16 hours, the solvent was evaporated in vacuo. MeOH/CH_2Cl_2 (9:1, (v/v)) was added to the residue and the solution was washed with water and brine. The organic layer was then dried over Na_2SO_4, filtered, and concentrated in vacuo. The product was purified by silica gel column chromatography ($CHCl_3$) followed by reprecipitation from *n*-hexane/EtOAc to afford a 5:1 of diastereomeric mixture of **77** as a white amorphousness (1.26 g, 48%). Major diastereomer was isolated for characterization: $[\alpha]^{18}_D$ -6.7 (*c* 0.82, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.39 (9H, s), 1.89 (3H, s), 3.53–3.58 (1H, m), 3.59 (3H, s), 3.62–3.71 (2H, m), 3.71–3.77 (4H, m), 3.78 (9H, s), 3.87 (1H, d, 12.4 Hz), 4.00 (1H, t, 9.8 Hz), 4.51 (1H, d, 11.9 Hz), 4.57 (1H, d, 11.9 Hz), 4.61 (1H, d, 11.2 Hz), 4.67 (1H, brs), 4.70 (1H, d, 11.3 Hz), 4.77 (1H, d,11.2 Hz), 4.80 (1H, d, 11.3 Hz), 4.97, (1H, d, 9.8 Hz), 6.19 (1H, s), 7.16–7.42 (15H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 23.1, 26.0, 28.7, 51.7, 52.9, 55.2, 55.8, 56.3, 56.3, 56.8, 69.8, 74.4, 75.9, 76.2, 77.9, 79.4, 80.5, 84.7, 91.8, 91.8, 106.7, 128.7, 128.7, 128.7, 128.8, 129.0, 129.2, 129.4, 129.4, 139.4, 139.6, 139.9, 160.2, 160.2, 162.5, 172.2, 172.8, 174.0; HRMS (ESI-TOF) *m*/*z* calcd for $C_{49}H_{61}N_3NaO_{13}S$ ($[M + Na]^+$): 954.3823, found 954.3787.



78

Methyl ester **77** (0.30 g, 0.32 mmol) in 1,2-dichloroethane (3.0 mL) was treated with trimethyltin hydroxide (0.18 g, 0.97 mmol). After 10 hours reaction at 50 ºC, the solvent was removed under reduced pressure. The residue was dissolved in EtOAc and the solution was washed with 5% (w/v) $KHSO_4$ aq. and brine. After drying over Na_2SO_4 followed by filtration, the organic layer was concentrated in vacuo. The crude product was purified by silica gel column chromatography (EtOAc/MeOH/AcOH = 95:5:0.5, (v/v)) afforded **78** (dr = 5:1) in 69% isolated yield (0.20 g) as a colorless amorphousness. The obtained diastereomeric mixture was used to a subsequent reaction without separation: ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.48–1.49 (9H), 1.89 (3H, s), 3.48–4.01 (17H), 4.51 (1H, d, 11.8Hz), 4.59, (1H, d, 11.8 Hz), 4.61 (1H, d, 11.0 Hz), 4.65–4.83 (1H, m), 4.69 (1H, d, 11.3 Hz), 4.77 (1H, d, 10.5 Hz), 4.80 (1H, d, 11.0 Hz), 4.91–5.01 (1H, m), 6.18–6.22 (2 H, s), 7.10–7.45 (15H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 23.1, 26.3, 28.7, 52.1, 55.2, 55.8, 56.3, 56.3, 56.8, 69.8, 74.4, 75.9, 76.2, 77.9, 79.4, 80.6, 84.6, 91.9, 91.9, 106.8, 128.7, 128.7, 128.7, 129.0, 129.1, 129.4, 129.4, 129.4, 139.4, 139.6, 139.9, 160.2, 160.2, 162.4, 173.0, 173.4, 174.0; HRMS (ESI-TOF) *m*/*z* calcd for $C_{48}H_{59}N_3NaO_{13}S$ ($[M + Na]^+$): 940.3666, found 940.3653.

Preparation of SEAlide peptide 79 (Fr 2')

A peptide-bound resin was prepared (0.25 mmol) similarly to the synthesis of SEAlide peptide **47** (Fr. 2) described above. On the N-terminus of the resin, **78** (2.0 equiv) was coupled with the aid of PyBop (2.1 equiv) and DIEA (4.0 equiv) in DMF at rt for 3 hours. A portion of the resulting resin (500 mg) was treated with 1 M TMSBr-thioanisole in TFA (50 μL/1 mg resin)/*m*-cresol/EDT (100/5/5, (v/v)) at 4 °C for 2 hours. The resin in the reaction mixture was filtrated off. To the resulting filtrate was added cooled Et_2O to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude SEAlide peptide **79**.

The peptide was purified by preparative HPLC (Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A over 30 min: 34% to 40%) to afford purified SEAlide peptide **79** (16.3 mg from 500 mg of the protected resin).
79 Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 30% to 50% over 30 min, retention time = 15.8 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 4H]^{4+}$) 1307.7, found 1307.4.

One-pot ligation/desulfurization of 46 (Fr 1) and 79 (Fr 2')

Fr 1 **46** (11 mg, 2.7 μmol) and Fr 2 **47** (15 mg, 2.7 μmol) were dissolved in 2.7 mL of 6 M Gn·HCl-0.1 M Na phosphate, 3% (v/v) TFET, pH 7.3, and the solution was incubated at 37 °C. The reaction was completed within 8 hours. After treatment of the reaction mixture with nitrogen stream, to the reaction mixture were added 2.7 mL of aqueous solution of Gn·HCl (6 M) and TCEP·HCl (0.4 M), pH 7.0, reduced form glutathione (in solid form, 40 mM) and VA-044 (in solid form, 20 mM). After incubation at 37 ºC for additional 18 hours, the crude product was purified by semi-preparative HPLC (Cosmosil Protein-R semi-preparative column with a linear gradient of solvent B in solvent A over 30 min: 38% to 41%) to give purified Acm-protected Fr 1-2' **83** (13 mg, 1.4 μmol, 53%).
Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 50% over 30 min, retention time = 20.0 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 8H]^{8+}$) 1076.6, found 1076.6.

Acm removal of native N-half fragment 80

The Acm-protected peptide **83** (13 mg, 1.4 μmol) was treated with AgOTf (36 mg, 0.14 mmol) and anisole (15 μL, 0.14 mmol) in TFA (340 μL, 4.0 mM peptide) at 4 °C. After 13 hours, to the reaction mixture was added cooled Et_2O to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O. The resulting crude peptide was dissolved in 4 mL solution of 6 M Gn·HCl-0.2 M HEPPS buffer (pH 6.1) containing 5% (w/v) DTT. The suspension was incubated at 37 ºC for 30 min and then 4 mL of 6 M Gn·HCl aq. was added. After filtration, the filtrate was purified by semi-preparative HPLC (Cosmosil Protein-R semi-preparative column with a linear gradient of solvent B in solvent A over 30 min: 40% to 43%) to afford the purified **80** (7.0 mg, 0.77 μmol, 54%).
Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 50% over 30 min, retention time = 23.6 min, MS (ESI-TOF) *m*/*z* calcd ($[M + 8H]^{8+}$) 1049.9, found 1049.7.

Assembly of native N-half fragment 80 and C-half fragment 52

The native N-half fragment **80** (3.7 mg, 0.34 μmol) and the C-half fragment **52** (3.1 mg, 0.34 μmol) were dissolved in 6 M Gn·HCl-0.5 M Na phosphate buffer containing 50 mM TCEP·HCl and 50 mM MPAA (pH 6.1, 0.68 mL) and the mixture was incubated at 37 °C. After 30 hours, the reaction mixture was diluted with 3 mL of 6 M Gn·HCl aq. with 0.5% (w/v) TCEP·HCl, and then the crude peptide was purified by semi-preparative HPLC (Cosmosil Protein-R semi-preparative column with a linear gradient of solvent B in solvent A over 30 min: 43% to 46% over 30 min) to give purified reduced form of **84** (2.6 mg, 0.14 μmol, 40%).

Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 35% to 55% over 30 min, retention time = 20.9 min, MS (ESI-TOF) calcd (average isotopes) 17792.4, found 17793.4.

Folding for preparation of monoglycosylated GMAP analog 54

Folding for preparation of monoglycosylated native GMAP **81** (0.18 mg) was performed by the same buffer conditions described in the synthesis of GM2AP analog. After incubation at 4 ºC for one day, the reaction was stood at rt for four days. At this point, aliquot of the reaction mixture was analyzed by analytical HPLC. ESI-MS analysis revealed that the peak eluted at 24.2 min was indicated that its molecular weight was identical to that of the folded monoglycosylated GM2AP native form **81**.

Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 35% to 55% over 30 min, retention time = 24.2 min, MS (ESI-TOF) calcd (average isotopes) 17784.4, found 17783.7.

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List of publications

This study was published in the following papers.

1. *N*-Sulfanylethylanilide peptide as a crypto-thioester peptide
   Kohei Sato, Akira Shigenaga, Kohei Tsuji, Shugo Tsuda, Yoshitake Sumikawa, Ken Sakamoto and Akira Otaka
   ChemBioChem **2011**, *12*, 1840–1844.

2. One-pot/sequential native chemical ligation using *N*-sulfanylethylanilide peptide
   Akira Otaka, Kohei Sato, Hao Ding and Akira Shigenaga
   Chem. Rec. **2012**, *12*, 479–490.

3. Chemical synthesis of biologically active monoglycosylated GM2-activator protein analog using *N*-sulfanylethylanilide peptide
   Kohei Sato, Akira Shigenaga, Keisuke Kitakaze, Ken Sakamoto, Daisuke Tsuji, Kohji Itoh and Akira Otaka
   Angew. Chem. Int. Ed. **2013**, *52*, 7855–7859.

4. Chemical synthesis of proteins using *N*-sulfanylethylanilide peptides, based on N–S acyl transfer chemistry
   Akira Otaka, Kohei Sato and Akira Shigenaga
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