Synthetic Studies of GM2 Activator Protein (GM2AP) for Potential Therapeutic Agents of Lysosomal Storage Diseases

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Synthetic Studies of GM2 Activator Protein (GM2AP) for Potential Therapeutic Agents of Lysosomal Storage Diseases

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(Pharmaceutical Sciences)

Takahiro Nakamura

Abbreviations

Ala (**A**)	alanine
Arg (**R**)	arginine
Asn (**N**)	asparagine
Asp (**D**)	aspartic acid
Cys (**C**)	cysteine
Gln (**Q**)	glutamine
Glu (**E**)	glutamic acid
Gly (**G**)	glycine
His (**H**)	histidine
Ile (**I**)	isoleucine
Leu (**L**)	leucine
Lys (**K**)	lysine
Met (**M**)	methionine
Phe (**F**)	phenylalanine
Pro (**P**)	proline
Ser (**S**)	serine
Thr (**T**)	threonine
Trp (**W**)	tryptophan
Tyr (**Y**)	tyrosine
Val (**V**)	valine
Boc	*tert*-butoxycarbonyl
DIPEA	*N*,*N*-diisopropylethylamine
DIPCDI	*N*,*N'*-diisopropylcarbodiimide
DMF	*N*,*N*-dimethylformamide
EDT	1,2-ethanedithiol
Et_2O	diethyl ether
Fmoc	9-fluorenylmethyloxycarbonyl
GM2AP	GM2 activator protein
Gn·HCl	guanidine hydrochloride
HATU	*O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate

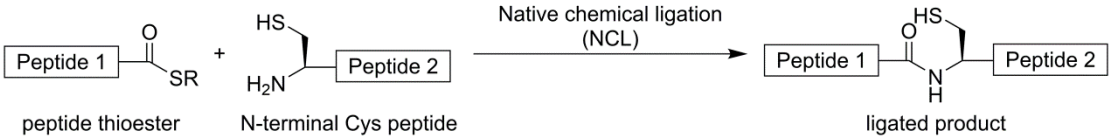
HEPPS	3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid
HexA	hexosaminidase A
HFIP	hexafluoro-2-propanol
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
MBHA	4-methylbenzhydrylamine
Me	methyl
MPAA	4-mercaptophenylacetic acid
NCL	native chemical ligation
rt	room temperature
SEAlide	*N*-sulfanylethylanilide
SPPS	solid phase peptide synthesis
TCEP·HCl	tris(2-carboxyethyl)phosphine hydrochloride
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
Thz	thiazolidine
TIPS	triisopropylsilane
TMS	trimethylsilyl
TOF	time of flight
Trt	triphenylmethyl

Contents

Preface

In recent years, proteins have been attracting increasing attention as potential agents for treating various diseases as well as target molecules for elucidating complex cellular processes within organisms.[1] The use of proteins as therapeutic agents requires a robust and reliable protein production system. Genetic engineering procedures represent successful applications for the production of naturally occurring proteins; however, the protocols still hold challenges, because both control of post-translational modifications such as glycosylation and incorporation of non-canonical amino acids into protein molecules are difficult.

Chemical synthesis is an attractive technique for preparation of proteins with homogeneous post-translational modifications or unnatural structural units. Native chemical ligation (NCL) is a chemical tactic to obtain such functionalized proteins (Scheme 1).[2] This approach features a chemoselective condensation between peptide thioesters and N-terminal cysteinyl peptides. Although NCL has greatly expanded the scope of chemically accessible proteins, its feasibility is still problematic due to the lack of a practical coupling protocol applicable to more than two peptides, and to limitations on ligation junctions.



Scheme 1. Native chemical ligation for assembly of two unprotected peptides.

Recent studies have indicated that the identity of amino acids at the C-terminus of thioesters strongly affected the rate of NCL reactions.[3] In particular, the thioesters containing proline at the C-terminus, namely “prolyl thioesters,” have been reported to hardly work under standard ligation conditions; thus, the use of this residue as a ligation site has been traditionally avoided.[4]

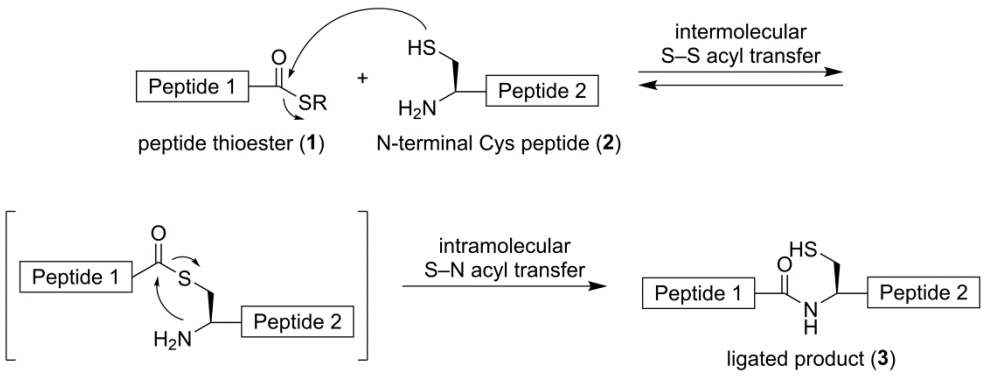
In this study, we disclose that prolyl thioesters can participate in NCL under suitable reaction conditions and can be utilized in practical one-pot multi-peptide ligation for the synthesis of proteins with over 100 residues. This proline-based coupling procedure can be successfully applied to the tailored synthesis of 162-residue monoglycosylated GM2 activator protein (GM2AP) analogs.

In Chapter 1, the use of prolyl thioesters as a reaction partner in peptide coupling reactions is examined. A one-pot/three-fragment ligation procedure using prolyl thioesters is also described. In Chapter 2, a novel platform for chemical access to a wide variety of GM2AP derivatives is presented.

Chapter 1
Use of prolyl thioesters for chemical synthesis of proteins

1.1 Recent studies of native chemical ligation using prolyl thioesters

In 1994, Kent and co-workers reported native chemical ligation (NCL), a simple technique to chemically prepare peptides or proteins by assembly of two unprotected peptide fragments.[2] The original conceptual framework of NCL is based on the chemoselective coupling of peptide thioesters **1** with N-terminal cysteinyl peptides **2** through intermolecular S–S acyl transfer followed by intramolecular S–N acyl transfer to give the corresponding ligated products **3**, as shown in Scheme 1.1. The development of NCL enabled chemical access to small- and medium-sized proteins. A number of NCL-mediated chemical syntheses of proteins, including enzymes and glycoproteins, have been reported.[5] Therefore, NCL has been shown to be an effective ligation method for chemical synthesis of proteins.



Scheme 1.1. Reaction mechanism of native chemical ligation.

Despite the broad utility of NCL in protein synthesis, some limitations remain. An examination of the reactivity of 20 proteinogenic amino acyl thioesters indicated that the rate of ligation was strongly dependent on the amino acids of the thioester parts.[3] In particular, prolyl thioesters have been reported to hardly participate in the ligation under standard NCL conditions.[4] Furthermore, recent studies have indicated that the poor reactivity of prolyl thioesters could be caused by electronic effects rather than steric effects; in a *trans* peptide bond configuration, the n→π* orbital interaction between the π*-orbital of the carbonyl group on the proline and the n-orbital of the

preceding $^{\alpha}$N carbonyl oxygen could reduce the electrophilicity of prolyl thioesters (Figure 1.1).[4]

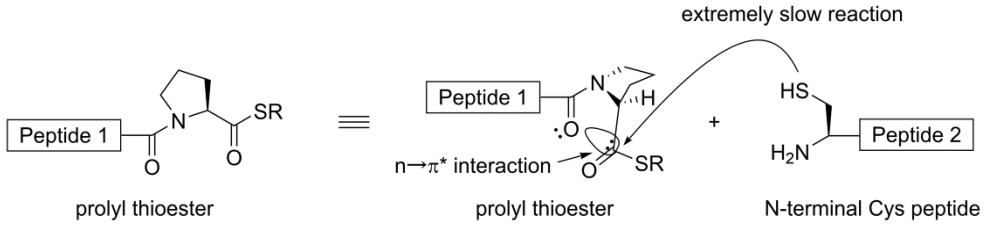
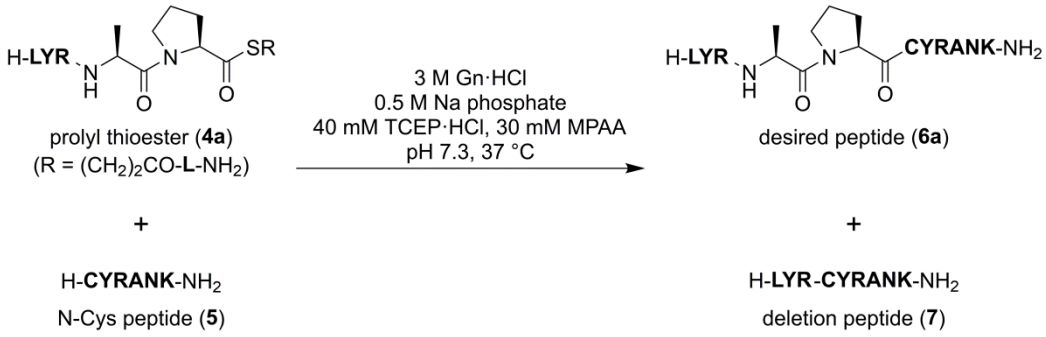


Figure 1.1. Origins of the low reactivity of prolyl thioesters in NCL reaction.

For practical use of prolyl thioesters in NCL, various approaches have been investigated.[6] Alewood and co-workers demonstrated that the use of selenoesters at the C-terminus enabled the proline-based coupling at the practical reaction rate; however, this protocol required an excessive amount of cysteinyl coupling partners for quantitative NCL conversion.[6c] Moreover, the preparation of C-terminal selenoesters was difficult and arduous due to their relatively low stability. In this study, we explored the suitable NCL conditions for the direct use of prolyl thioesters without conversion of thioester moiety to other units such as selenoesters.

1.2 Initial attempts in use of prolyl thioesters for chemical synthesis of proteins

Initially, to obtain negative control data concerning NCL using prolyl thioesters, a model prolyl thioester **4a** was subjected to NCL with N-terminal cysteinyl peptide **5** in 3 M guanidine hydrochloride (Gn·HCl)–0.5 M sodium phosphate, pH 7.3, at 37 °C, in the presence of 40 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) and 30 mM 4-mercaptophenylacetic acid (MPAA) as a reagent to reduce disulfide bonds and a catalyst for NCL, respectively (Scheme 1.2). However, contrary to our expectations, monitoring reaction by reversed-phase high-performance liquid chromatography (RP-HPLC) indicated that a considerable amount of the ligated peptide **6a** was generated after 24 h, with concomitant formation of the C-terminal two-amino acid deleted side-product **7** (Figure 1.2).



Scheme 1.2. Model NCL reaction of prolyl thioester with N-terminal cysteinyl peptide.

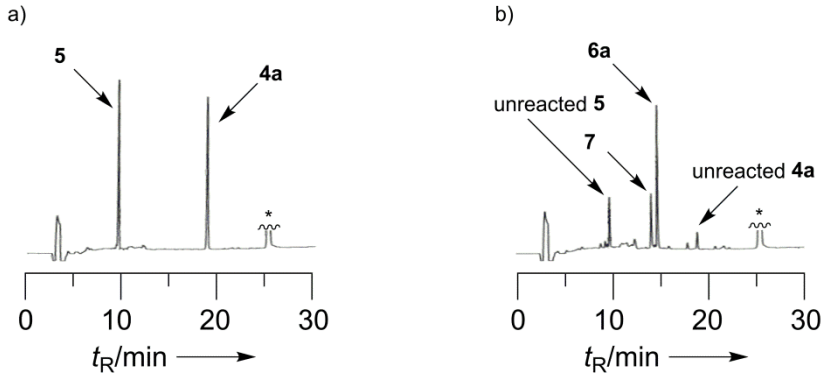
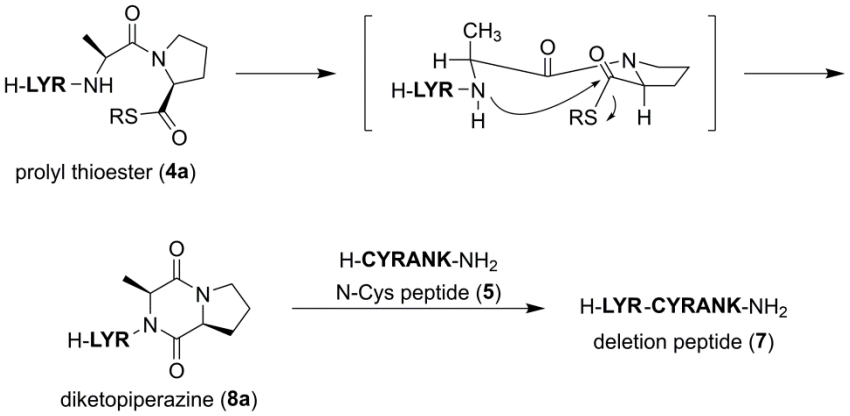


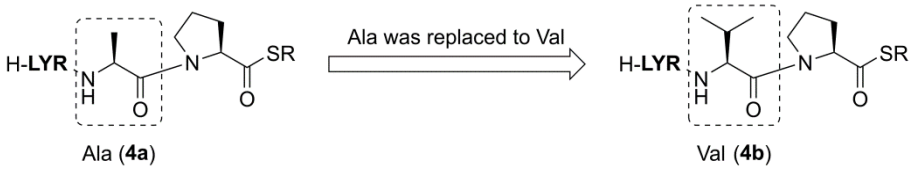
Figure 1.2. HPLC monitoring of model NCL between prolyl thioester **4a** and N-Cys peptide **5**. a) The NCL (t = 0 h): fragment **4a** (1 mM) and **5** (1 mM) were ligated in Gn·HCl [3 M, Na phosphate (0.5 M), pH 7.3] in the presence of TCEP·HCl (40 mM) and MPAA (30 mM) at 37 °C. b) The NCL (t = 24 h). Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II column (4.6×250 mm) with a linear gradient of CH_3CN (1–50% over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm. *MPAA.

This unexpected result encouraged us to search for suitable reaction conditions that would allow prolyl thioesters to function as a useful reaction partner in NCL. For the detailed examination of NCL conditions involving prolyl thioesters, however, selection of model thioester peptides that do not afford deletion peptides was required, because the formation of deleted products might complicate analysis of the experimental data. The generation of the deletion peptide **7** was probably caused by the formation of the diketopiperazine **8a** from prolyl thioester **4a** followed by nucleophilic attack of the N-terminal cysteinyl peptide **5**, as shown in Scheme 1.3. Since kinetic analysis of conversion of Xaa-Pro-*p*-nitroanilide (Xaa = Ala, Val, Gly, Phe, or Arg) to

the corresponding diketopiperazine indicated that Val-Pro peptide was insensitive to diketopiperazine formation,[7] we substituted Val for Ala in model peptide **4a**, as shown in Scheme 1.4.

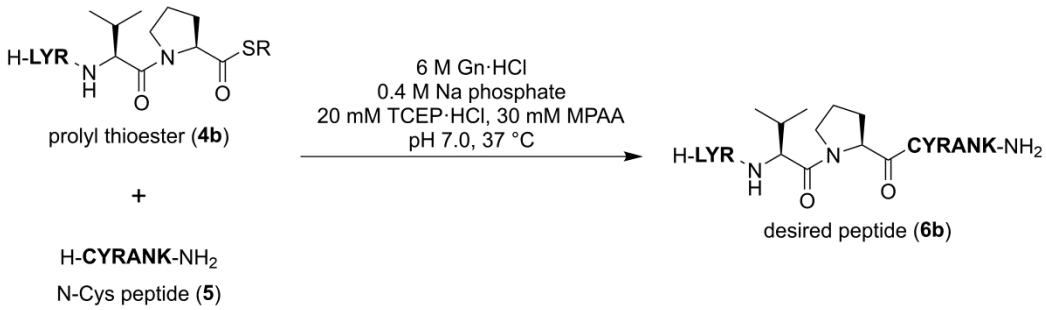


Scheme 1.3. Plausible route for formation of deletion peptide.



Scheme 1.4. Selection of model peptides suitable for examination of NCL using prolyl thioesters.

The ligation between the model peptide **4b** and N-terminal cysteinyl peptide **5** was conducted in 6 M Gn·HCl–0.4 M phosphate in the presence of 20 mM TCEP·HCl and 30 mM MPAA, pH 7.0 at 37 °C (Scheme 1.5). The reaction was monitored by RP-HPLC, as shown in Figure 1.3. As expected, this replacement suppressed the side reaction. Thus, the search for NCL conditions suitable for coupling of prolyl thioesters was attempted using this model peptide **4b**.



Scheme 1.5. Model NCL reaction using Val-substituted peptide.

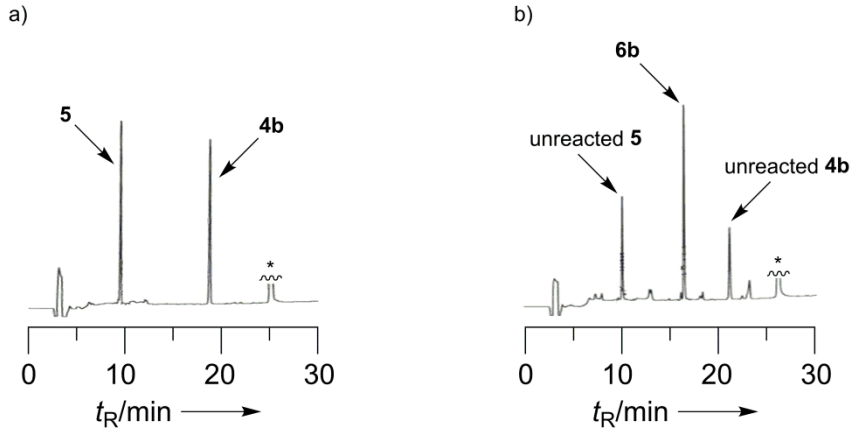


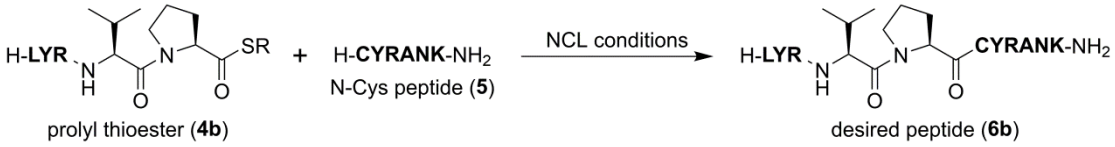
Figure 1.3. HPLC monitoring of model NCL between prolyl thioester **4b** and N-Cys peptide **5**. a) The NCL (t = 0 h): fragment **4b** (1 mM) and **5** (1 mM) were ligated in Gn·HCl [6 M, Na phosphate (0.4 M), pH 7.0] in the presence of TCEP·HCl (20 mM) and MPAA (30 mM) at 37 °C. b) The NCL (t = 24 h). Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II column (4.6×250 mm) with a linear gradient of CH_3CN (1–50% over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm. *MPAA.

1.3 Examination of NCL using prolyl thioesters

NCLs between equimolar amounts of **4b** and **5** were examined under various conditions (Table 1.1). A comparison of entries 3 and 4 indicated that the use of MPAA as a thiol catalyst further enhanced the progress of NCL involving prolyl thioesters compared to that of thiophenol. Furthermore, increasing the concentration of MPAA

further accelerated the NCL reaction from entries 5 vs 7. The reaction temperature was also a key factor for the acceleration of NCL (entries 4 vs 6 and 5 vs 9). The concentration of phosphate salts did not affect the reaction rate. These results supported the hypothesis that prolyl thioesters could serve as potential NCL reagents by an appropriate tuning of the reaction conditions, such as increasing concentration of MPAA and raising reaction temperature. Thus, the combination of adjustments of these two factors resulted in the dramatic enhancement of reaction rate to afford the desired ligated peptide **6b** in 74% isolated yield without epimerization at proline site (entry 10).

Table 1.1. Optimization of NCL conditions for prolyl thioesters.



prolyl thioester (**4b**) desired peptide (**6b**)

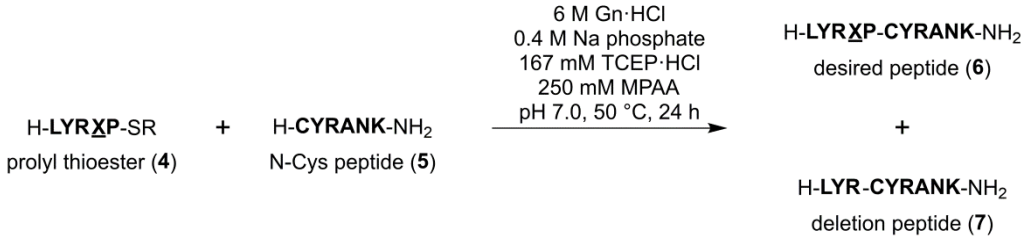
Entry	Conditions[a]	Fraction ligated[b]	Rate constants k (M^{-1} s^{-1})[c]
1	0.1 M Na phosphate, 4% benzylmercaptan, 4% thiophenol, 37 °C	0.07[d], 0.22[e]	4.70×10^{-3}
2	0.1 M Na phosphate, 4% thiophenol, 37 °C	0.06[d], 0.39[e]	4.30×10^{-3}
3	0.1 M Na phosphate, 4% thiophenol, 20 mM TCEP·HCl, 37 °C	0.08[d], 0.33[e]	7.10×10^{-3} [g]
4	0.1 M Na phosphate, 20 mM TCEP·HCl, 30 mM MPAA, 37 °C	0.20[d], 0.63[e]	7.30×10^{-3}
5	0.4 M Na phosphate, 20 mM TCEP·HCl, 30 mM MPAA, 37 °C	0.32[d], 0.77[e]	12.1×10^{-3}
6	0.1 M Na phosphate, 20 mM TCEP·HCl, 30 mM MPAA, 25 °C	0.06[d], 0.36[e]	3.10×10^{-3}
7	0.4 M Na phosphate, 20 mM TCEP·HCl, 250 mM MPAA, 37 °C	0.67[d]	55.5×10^{-3}
8	0.4 M Na phosphate, 20 mM TCEP·HCl, 250 mM MPAA, 50 °C	0.84[d]	143×10^{-3}
9	0.4 M Na phosphate, 20 mM TCEP·HCl, 30 mM MPAA, 50 °C	0.58[d], 0.93[e]	39.4×10^{-3}
10	0.4 M Na phosphate, 167 mM TCEP·HCl, 250 mM MPAA, 50 °C	0.85[d], 0.99[e]	158×10^{-3}
11	0.1 M Na phosphate, 167 mM TCEP·HCl, 250 mM MPAA, 50 °C	0.83[d], 0.98[e]	129×10^{-3}
12[f]	0.1 M Na phosphate, 20 mM TCEP·HCl, 30 mM MPAA, 37 °C	0.97[d], 0.98[e]	800×10^{-3}
13[f]	0.4 M Na phosphate, 167 mM TCEP·HCl, 250 mM MPAA, 37 °C	0.98[d], 1.00[e]	2650×10^{-3}

[a] All reactions were performed in the presence of 6 M Gn·HCl at pH 7.0 using equimolar amounts of peptides. [b] The fraction ligated was determined by integration of **6b** (integ. **6b**) as a fraction of the sum of the unreacted **5** (integ. **5**) + integ. **6b**. [c] Second order rate constants were derived from the integrated rate equation $1/[\mathbf{5}] = kt + 1/[\mathbf{5}]_0$. [d] 6 h reaction. [e] 24 h reaction. [f] Ala-substituted peptide (H-**LYRVA**-SR) was subjected to the model ligation. [g] Rate constants were calculated on the basis of disappearance of **5**. Presence of thiophenol resulted in unproductive consumption of peptide **5** although its reason is unclear. Therefore, the use of fraction ligated is inadequate for the comparison of entries 3 and 4.

As mentioned in the previous section, we observed the two-amino acid deleted side-products in the proline site ligation. To clarify the scope and limitations of the use of prolyl thioesters in NCL, we next examined the influence of amino acids adjacent to

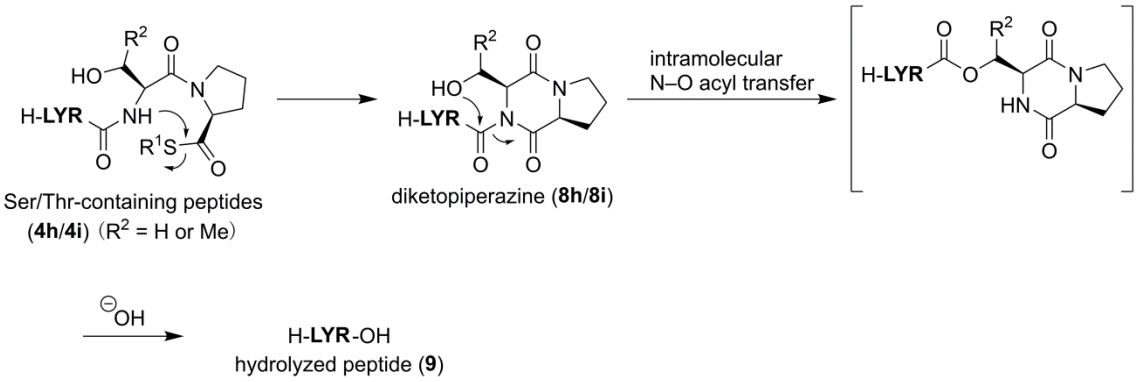
proline residue on formation of the deletion peptides (Table 1.2). The model prolyl thioesters, in which any of the 20 naturally occurring amino acids was contained at the position adjacent to the proline, were subjected to NCLs with N-terminal cysteinyl peptide **5** under the optimized conditions as mentioned before. The results indicated that most amino acids, except for Gly, Asn, Ser, Thr, and Cys, could be used as the amino acids adjacent to the proline. Ligation of Gly/Asn-containing peptides gave considerable amounts of the deletion peptide **7**. The Ser/Thr-containing peptides preferentially afforded the hydrolyzed peptide **9** instead of deletion peptide **7**. The hydrolyzed peptides probably have their origins in the formation of the diketopiperazines followed by nucleophilic attack of side-chain hydroxyl groups on serine or threonine (Scheme 1.6). The Cys-peptide was not obtained, because acidic deprotection of the corresponding resin afforded the deletion thioester (H-**LYR**-SR) as a major product instead of the requisite peptide. The generation of the deletion thioester was probably caused by a thiol-mediated reaction similar to that involved in Aimoto's cysteinyl prolyl ester.[8]

Table 1.2. Formation of the deleted peptides in ligations using prolyl thioesters.



Entry	X	Ratio[a]	Fraction ligated[b]	Entry	X	Ratio	Fraction ligated
1	Ala (**4a**)	0.24	0.75	11	Ile (**4k**)	0	0.92
2	Val (**4b**)	0	0.97	12	Met (**4l**)	0.05	0.84
3	Gly (**4c**)	2.3	0.29	13	Pro (**4m**)	0	0.96
4	Asp (**4d**)	0.33	0.73	14	Phe (**4n**)	0.04	0.94
5	Asn[c] (**4e**)	0.97	0.51	15	Tyr (**4o**)	0	0.82
6	Glu (**4f**)	0.07	0.92	16	Trp (**4p**)	0	0.84
7	Gln[c] (**4g**)	0.02	0.93	17	His (**4q**)	0.28	0.73
8	Ser[d] (**4h**)	0	0.29	18	Lys (**4r**)	0.03	0.81
9	Thr[d] (**4i**)	0	0.51	19	Arg (**4s**)	0	0.80
10	Leu (**4j**)	0	0.84	20	Cys[e]	---	---

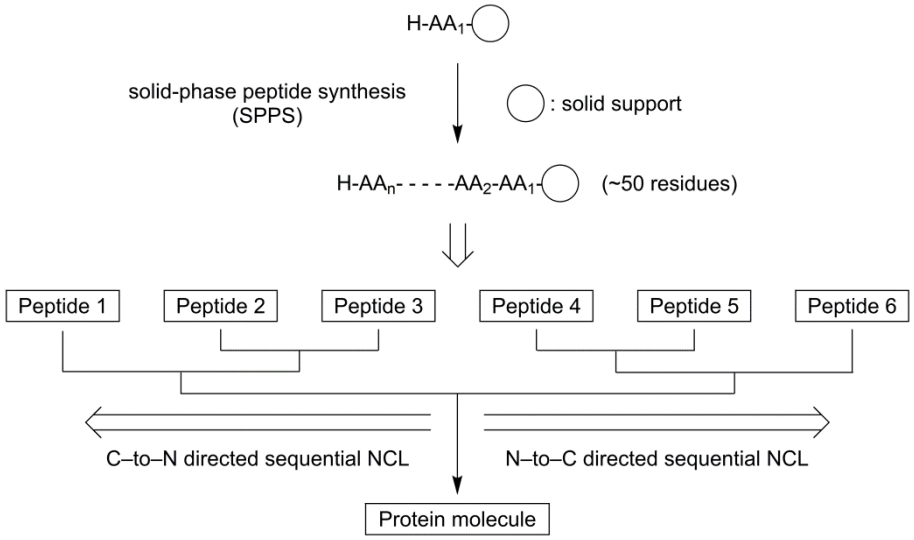
[a] Ratio was determined by HPLC separation and integration of the deletion peptide **7** (integ. **7**) detected at 220 nm as a ratio to the desired ligated peptide **6** (integ. **6**). [b] The fraction ligated was calculated from the equation (integ. **6**/(integ. **5** (unreacted) + integ. **6** + integ. **7**)). [c] Five-residue-extended peptide (H-**LRANK**-**LYRXP**-SR) was used for satisfactory resolutions in the HPLC analysis. [d] The deletion peptide **7** was not observed, but a hydrolyzed peptide (H-**LYR**-OH: **9**) was detected. [e] Requisite peptide (H-**LYRCP**-SR) was not obtained.



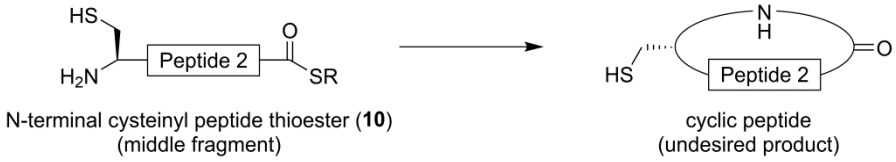
Scheme 1.6. Proposed mechanism of side reactions in Ser/Thr-containing peptides.

1.4 Recent studies of one-pot/N–to–C-directed sequential NCL

The discovery of NCL opened the opportunity for chemical access to small- and medium-sized proteins.[5] Chemical approach to proteins with over 100 residues generally requires C–to–N- and/or N–to–C-directed sequential NCL protocols followed by convergent assembly of synthesized peptide fragments, because of the up to 50-residue limitation on chain length of peptides available by solid-phase peptide synthesis (SPPS) (Scheme 1.7).[9] For success of sequential NCLs between more than two peptide fragments, the utilization of an N-terminal cysteinyl thioester fragment **10** as a middle fragment is necessary; however, such a middle fragment has an intrinsic tendency to be converted into an undesired cyclic peptide through the intramolecular NCL reaction of the N-terminal cysteine with the thioester moiety (Scheme 1.8).[10]

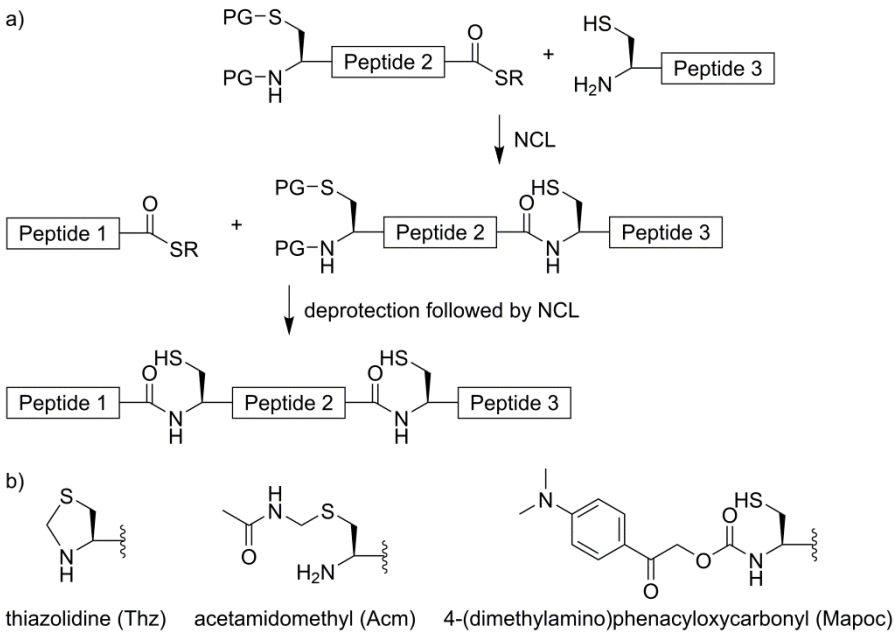


Scheme 1.7. Sequential NCLs for convergent assembly of multi-peptide fragments.



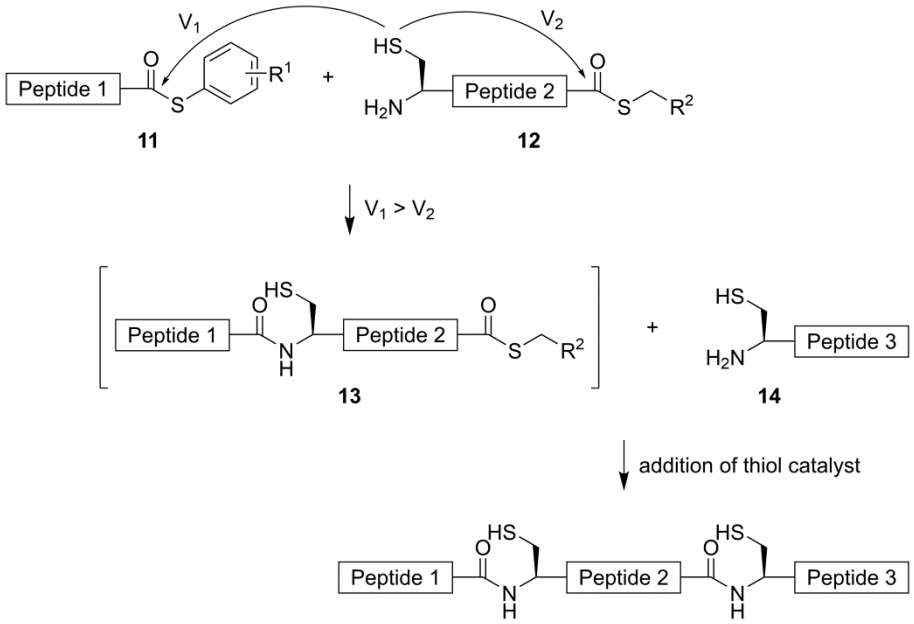
Scheme 1.8. Side reactions of N-terminal cysteinyl peptide thioesters.

The key to these multi-peptide assemblies is the use of a suitable protecting group for the N- or the C-terminus of a middle fragment, where these protecting groups are applied to C–to–N- or N–to–C-directed ligations, respectively. For a successful sequential ligation in the C–to–N direction, several N-terminal protection strategies have been reported, as shown in Scheme 1.9.[11]



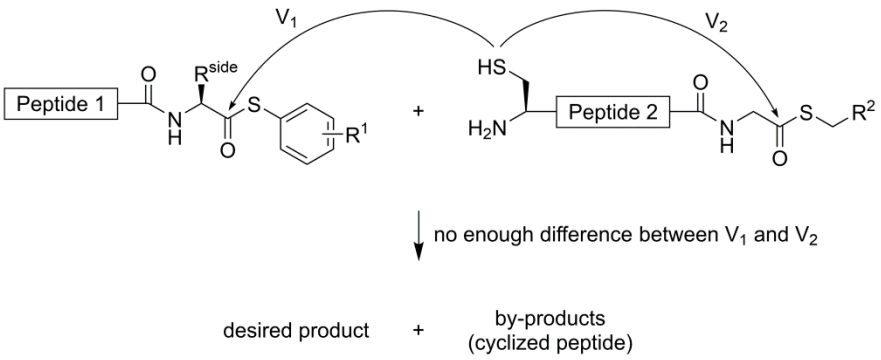
Scheme 1.9. (a) C–to–N-directed sequential NCL with protections for N-terminal cysteine. (b) Examples of protections for N-terminal cysteine. PG: protecting group.

In contrast, N–to–C-directed sequential NCL has remained a challenge due to the difficulty of designing masked thioesters that can be activated on demand. One pioneering strategy for sequential ligation in an N–to–C-directed manner is kinetically controlled ligation (KCL), reported by Kent's group (Scheme 1.10).[12] This approach relies on the different reactivity between alkyl and aryl thioesters. Generally, alkyl thioesters are sufficiently unreactive as not to participate in NCL reactions when competing aryl thioesters are present. Thus, the N-terminal cysteine moiety of the middle fragment **12** preferentially reacts with the more reactive aryl thioester **11** in an intermolecular manner to afford the ligated alkyl thioester **13**. The C-terminal alkyl thioester of the resulting peptide **13** is subsequently converted into an aryl thioester by treatment with an aryl thiol to participate in the next ligation step with C-terminal fragment **14**. The consecutive reactions can be performed in a straightforward and operationally simple one-pot manner.



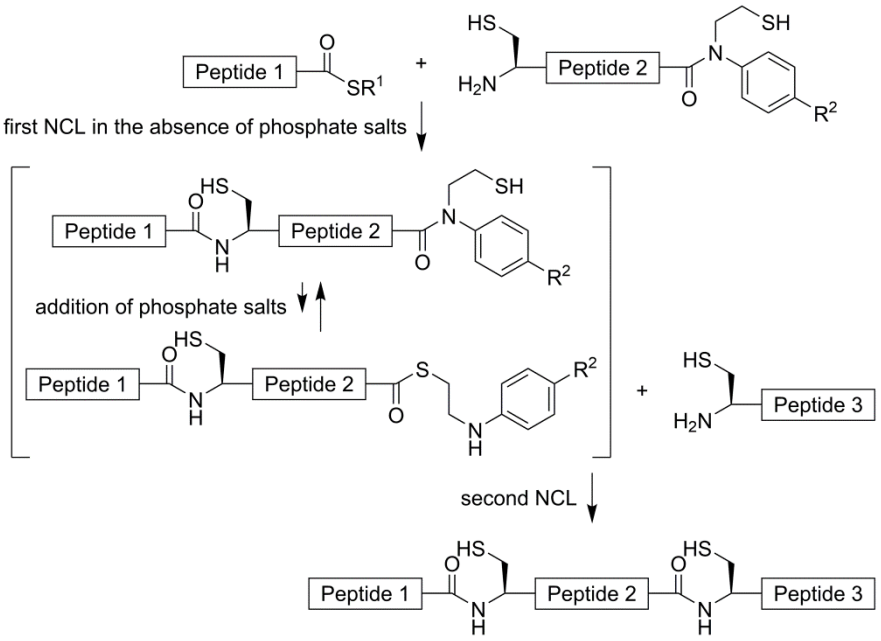
Scheme 1.10. Three-fragment assembly by kinetically controlled ligation.

Although the KCL protocol has afforded impressive success in N–to–C-directed synthetic routes, there remains one potential limitation. The use of significant reactive amino acids such as glycine at the C-terminus of an alkyl thioester does not show sufficient kinetic selectivity due to the lack of reactivity differences between alkyl and aryl thioesters (Scheme 1.11).[13] Therefore, a practical and reliable N–to–C-directed sequential NCL has been pursued.



Scheme 1.11. Side reactions of KCL with a highly reactive alkyl thioester.

Our group previously developed a novel one-pot/N–to–C-directed sequential NCL using *N*-sulfanylethylanilide (SEAlide) peptides (Scheme 1.12).[14] The SEAlide moiety generally remains electrophilically inert as the amide-form in the absence of phosphate salts; however, the addition of phosphate salts triggers the conversion of the amide-form to the corresponding thioester-form as an electrophilically active species. Thus, the use of the SEAlide peptides was extended to one-pot/N–to–C-directed sequential ligations due to its controllable reactivity. The feasibility of the SEAlide-mediated sequential ligations has already been demonstrated through the syntheses of several peptides and proteins.[14]

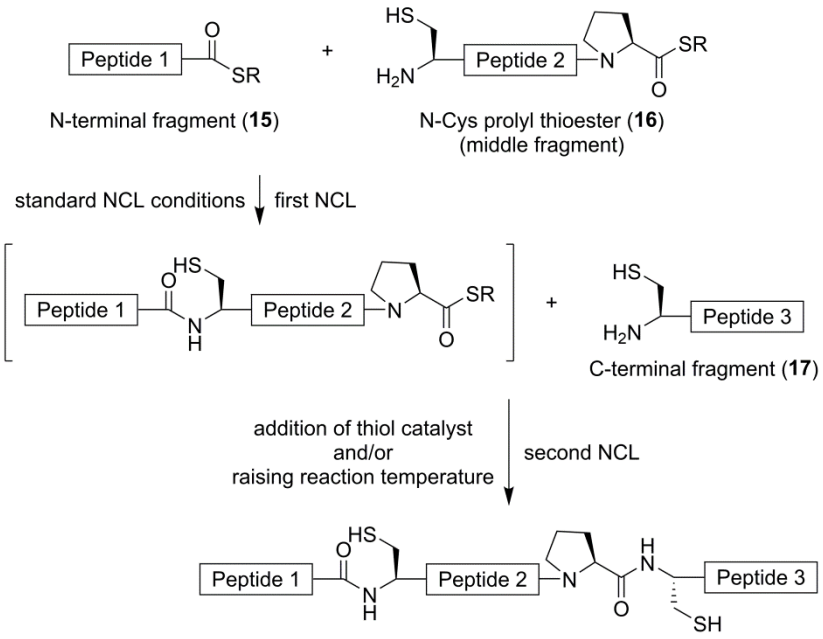


Scheme 1.12. Strategy for N–to–C-directed sequential NCL using SEAlide peptides.

1.5 Development of one-pot/N–to–C-directed sequential NCL using prolyl thioesters

As described in the previous section, it was clarified that the reactivity of prolyl thioesters could be controlled by tuning the concentration of thiol catalyst and the reaction temperature. Also, we found that NCL at the proline site proceeded a hundred times more slowly than that of alanine site under standard conditions (Table 1.1, entries 4 vs 12). These experimental results prompted us to envision that a novel one-pot/N–to–

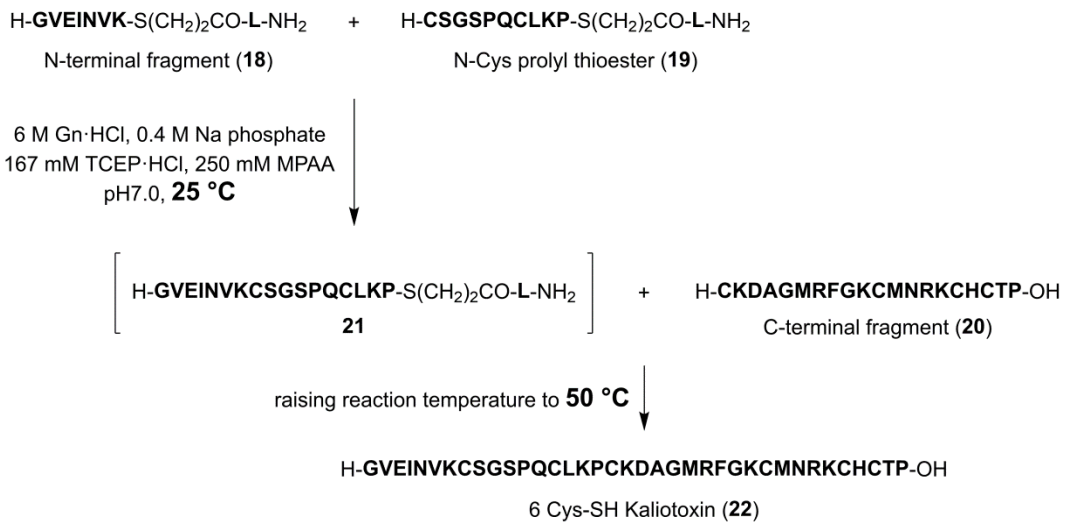
C-directed sequential ligation procedure could be designed using N-terminal cysteinyl prolyl thioesters as a middle fragment (Scheme 1.13). In the envisioned ligation protocol, the N-terminal cysteine moiety of middle fragment **16** should preferentially react with the thioester moiety of N-terminal fragment **15** in an intermolecular manner under standard NCL conditions. The enhancement of the reactivity of the proline moiety could then be achieved by increasing the MPAA concentration and/or raising the reaction temperature, allowing for the progress of the second ligation with C-terminal fragment **17** to afford ligated peptide in a one-pot manner. To verify the feasibility of this idea, syntheses of the 37-residue kaliotoxin were attempted through two protocols (Scheme 1.14 and 1.15).[15]



Scheme 1.13. Strategy for N–to–C-directed sequential NCL using prolyl thioesters.

Initially, peptide fragments (**18**–**20**) covering an entire sequence of kaliotoxin were prepared by Boc (**18** and **19**) or Fmoc (**20**) SPPS. NCL reactions of these fragments were monitored by RP-HPLC, as shown in Figures 1.4 and 1.5. In protocol 1, only a temperature adjustment was used to control the reactivity of proline moiety during the ligation (Scheme 1.14). The first NCL of the thioesters **18** (2 mM) with the N-terminal cysteinyl prolyl thioester **19** (2 mM) was conducted in 6 M Gn·HCl–0.4 M phosphate in the presence of 167 mM TCEP·HCl and 250 mM MPAA, pH 7.0 at 25 °C. The reaction under room temperature conditions went to completion within 3 h to yield

the desired ligated prolyl thioester **21**, even though a small amount of a cyclic peptide derived from the middle fragment **19** was detected. Successive addition of the C-terminal fragment **20** into the reaction mixture and subsequent raising of the reaction temperature (50 °C) then allowed the second NCL to afford the desired reduced-form kaliotoxin **22** in a one-pot manner in 30% isolated yield.



Scheme 1.14. Synthetic scheme for one-pot preparation of reduced-form kaliotoxin by means of only a temperature adjustment (protocol 1).

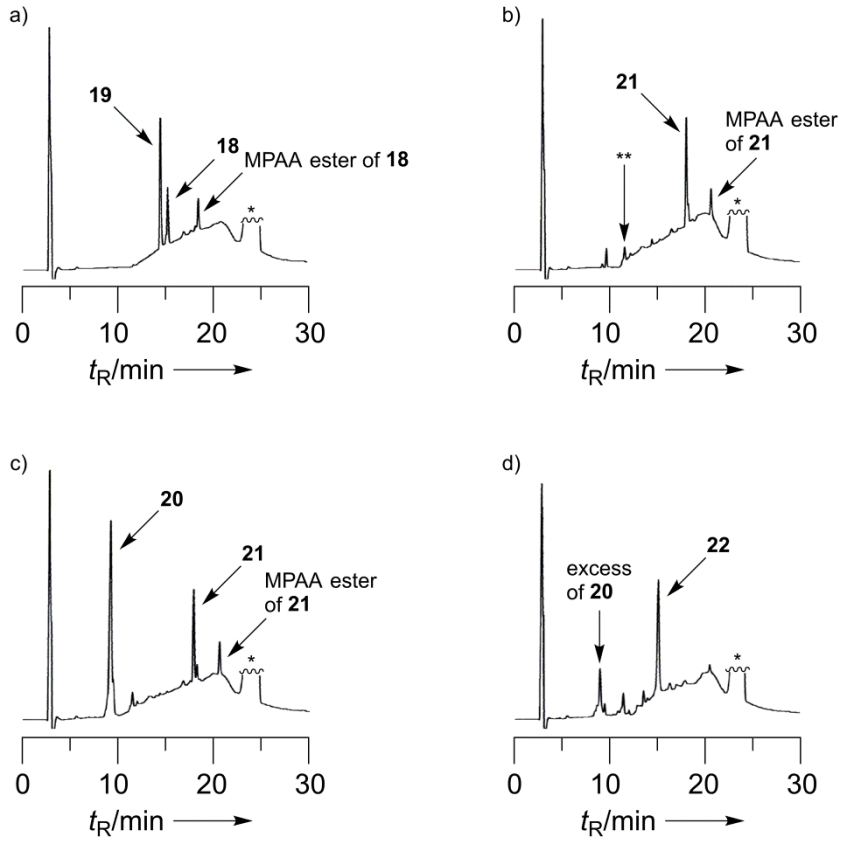
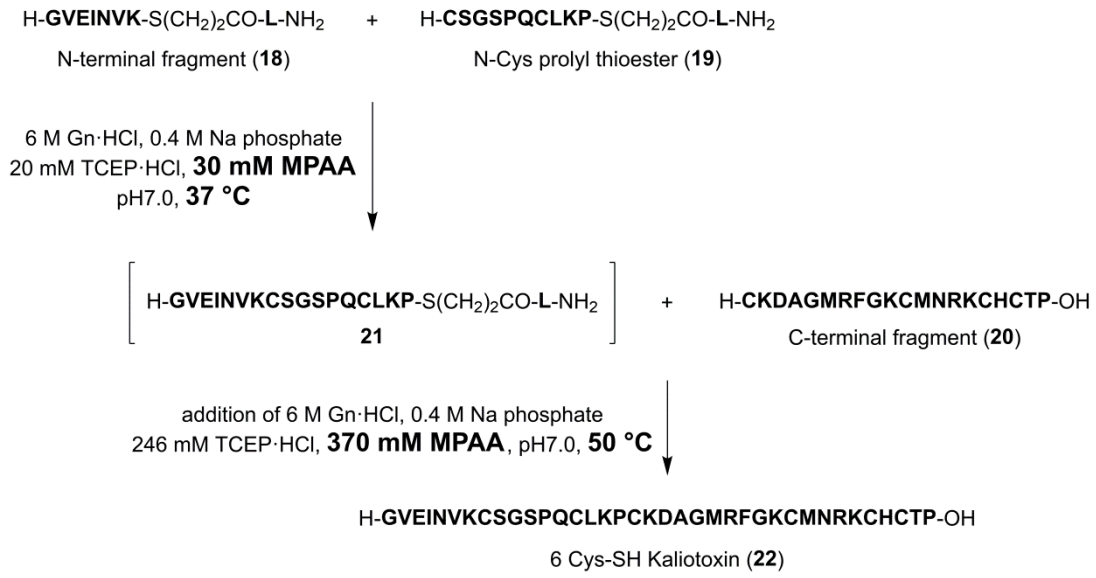


Figure 1.4. HPLC monitoring of one-pot/three-fragment NCL utilizing N-Cys prolyl thioester **19** (protocol 1). a) The first NCL (t = 0 h): fragment **18** (1 mM) and **19** (1mM) were ligated in Gn·HCl [6 M, Na phosphate (0.4 M), pH 7.0] in the presence of TCEP·HCl (167 mM) and MPAA (250 mM) at 25 °C. b) The first NCL (t = 3 h). c) The second NCL (t = 0 h): fragment **20** (1 mM) was added to the reaction mixture, and subsequent raise in the reaction temperature (50 °C). Final concentrations: 1 mM each peptide in Gn·HCl [6 M, Na phosphate (0.4 M), TCEP·HCl (167 mM), MPAA (250 mM), 50 °C, pH 7.0]. d) The second NCL (t = 24 h). Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II column (4.6×250 mm) with a linear gradient of CH_3CN (1–50% over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm. *MPAA. **The cyclic side product derived from **19**.

In protocol 2, modulations of both the MPAA concentration and the reaction temperature were used for the enhancement of the reactivity of prolyl thioesters (Scheme 1.15). The first NCL between fragment **18** and **19** in 6 M Gn·HCl–0.4 M phosphate in the presence of 20 mM TCEP·HCl and 30 mM MPAA, pH 7.0 at 37 °C, followed by addition of a solution of peptide **20** in 6 M Gn·HCl–0.4 M phosphate in the

presence of 246 mM TCEP·HCl and 370 mM MPAA (final 133 mM and 200 mM, respectively), pH 7.0 and subsequent increase in reaction temperature (50 °C) gave the desired ligated peptide **22** in a one-pot manner in 29% isolated yield. Although no significant difference in reaction efficiency was observed between protocol 1 and 2, protocol 1 was preferred for the ease of operation.



Scheme 1.15. Synthetic scheme for one-pot preparation of reduced-form kaliotoxin by modulations of both thiol catalyst concentration and reaction temperature (protocol 2).

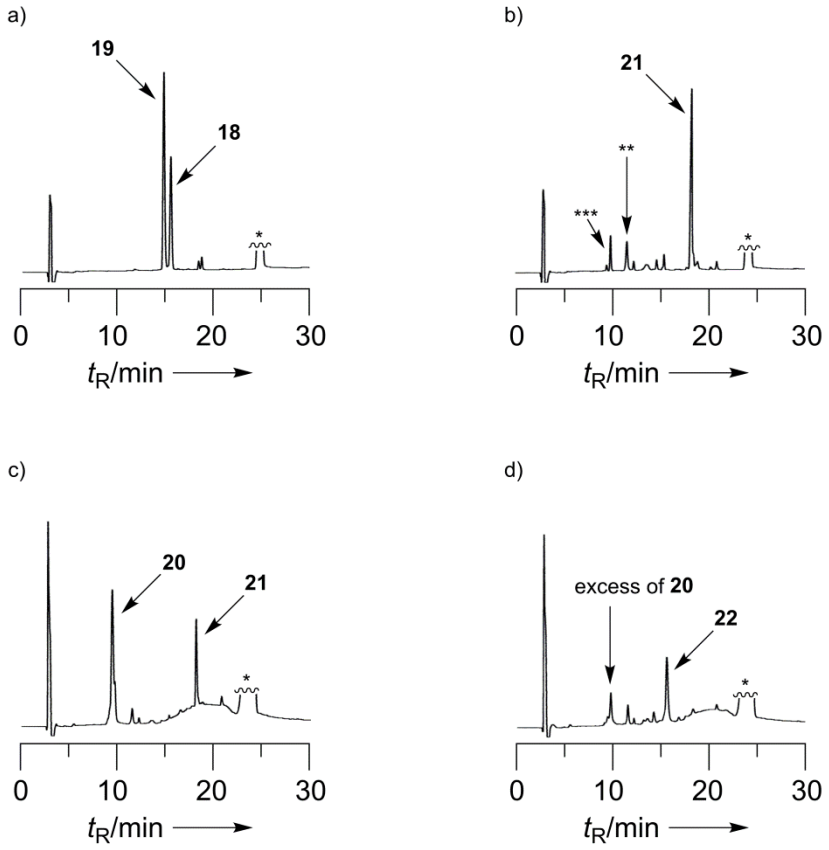


Figure 1.5. HPLC monitoring of one-pot/three-fragment NCL utilizing N-Cys prolyl thioester **19** (protocol 2). a) The first NCL (*t* = 0 h): fragment **18** (2 mM) and **19** (2 mM) were ligated in Gn·HCl [6 M, Na phosphate (0.4 M), pH 7.0] in the presence of TCEP·HCl (20 mM) and MPAA (30 mM) at 37 °C. b) The first NCL (*t* = 3 h). c) The second NCL (*t* = 0 h): fragment **20** (2 mM) in Gn·HCl [6 M, Na phosphate (0.4 M), pH 7.0] in the presence of TCEP·HCl (246 mM) and MPAA (370 mM) was added to the reaction mixture and subsequent raise in the reaction temperature (50 °C). Final concentrations: 1 mM each peptide in Gn·HCl [6 M, Na phosphate (0.4 M), TCEP·HCl (133 mM), MPAA (200 mM), 50 °C, pH 7.0]. d) The second NCL (*t* = 24 h). Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II column (4.6×250 mm) with a linear gradient of CH_3CN (1–50% over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm. *MPAA. **The cyclic side product derived from **19**. ***Non-peptidic impurity.

1.6 Conclusion

We disclosed that prolyl thioesters with an appropriate amino acid at the position adjacent to proline could be induced to participate in NCL by tuning the reaction conditions, such as the catalyst concentration and reaction temperature. Furthermore, our protocol for NCL using prolyl thioesters was extended to one-pot/N–to–C-directed sequential ligation due to their controllable reactivity. The feasibility of the novel protocol was confirmed through its use in the synthesis of the 37-residue kaliotoxin.

Chapter 2
Tailored synthesis of monoglycosylated GM2 activator protein analog

2.1 Lysosomal storage disease and protein replacement therapy

Lysosomal storage diseases (LSDs) comprise a group of more than 30 distinct disorders.[16] The common feature of these diseases is an inherent deficiency of lysosomal proteins, such as hydrolases and cofactors involved in the degradation of a wide range of materials in lysosomes. Such deficiency is caused by mutations in the genes encoding these proteins, resulting in the accumulation of substrates originally degradable within lysosomes.

GM2 activator protein (GM2AP) is a lysosomal glycoprotein required for the degradation of GM2 ganglioside (GM2) to GM3 by β-hexosaminidase A (HexA) through the formation of a HexA-GM2AP complex.[17] Loss of its functions derived from mutations in the gene encoding GM2AP causes excessive accumulation of undegraded GM2 in neuronal tissues, leading to a fatal lysosomal storage disease known as the AB variant of GM2 gangliosidosis.

The major approach for treatment of LSDs is protein replacement therapy, which features the use of functional lysosomal proteins produced by recombinant techniques.[18] These recombinant proteins are periodically infused into the vein of patients to compensate for reduced functions of the deficient proteins, resulting in significant improvement of the LSDs through the degradation of accumulated materials. Progressive improvement in the clinical course has been shown in the treatment for several LSDs; however, there are currently no effective treatments for GM2 gangliosidosis.[19]

The homogeneity of protein molecules prepared for treatment is an important factor for therapeutic effectiveness. Chemical synthesis is a useful approach to obtain such a homogeneous protein. Thus, we explored a synthetic platform for chemical access to various GM2AP derivatives to develop a medical treatment against the AB variant of GM2 gangliosidosis.

2.2 First synthetic plan, for total synthesis of GM2AP analog

Recently, our group achieved the NCL-mediated total chemical synthesis of GM2AP analog through previously developed SEAlide-chemistry.[20] GM2AP consists of 162 amino acid residues with a single N-linked glycosylation site at the 32nd asparagine (Asn32) and eight cysteine residues that form four disulfide bridges. The primary sequence is shown in Figure 2.1. Achieving NCL-based synthesis of this protein requires suitable cysteine ligation sites; however, the fragmentation at the native cysteine residues would offer a 67-residue peptide fragment (8–74), the preparation of which was not realistic because the chain length of peptides synthesizable by SPPS was limited up to approximately 50 residues. Therefore, we decided to replace the naturally glycosylated Asn32 with a cysteine residue. This substitution provided an additional ligation site for the longest fragment, and facilitated the incorporation of sugar units into the protein molecule through the alkylation of thiol group on the replaced cysteine.[21]

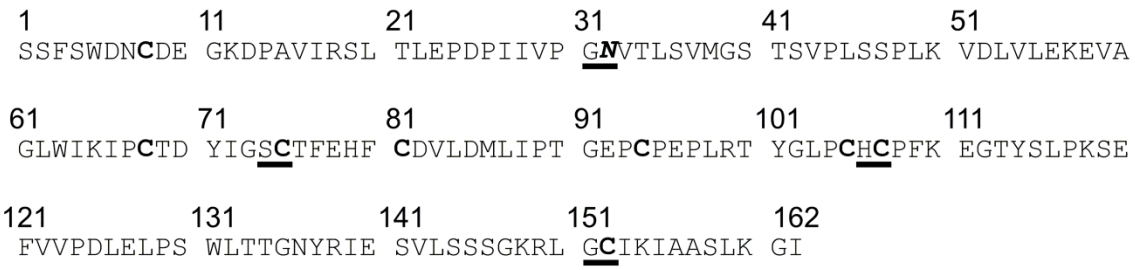
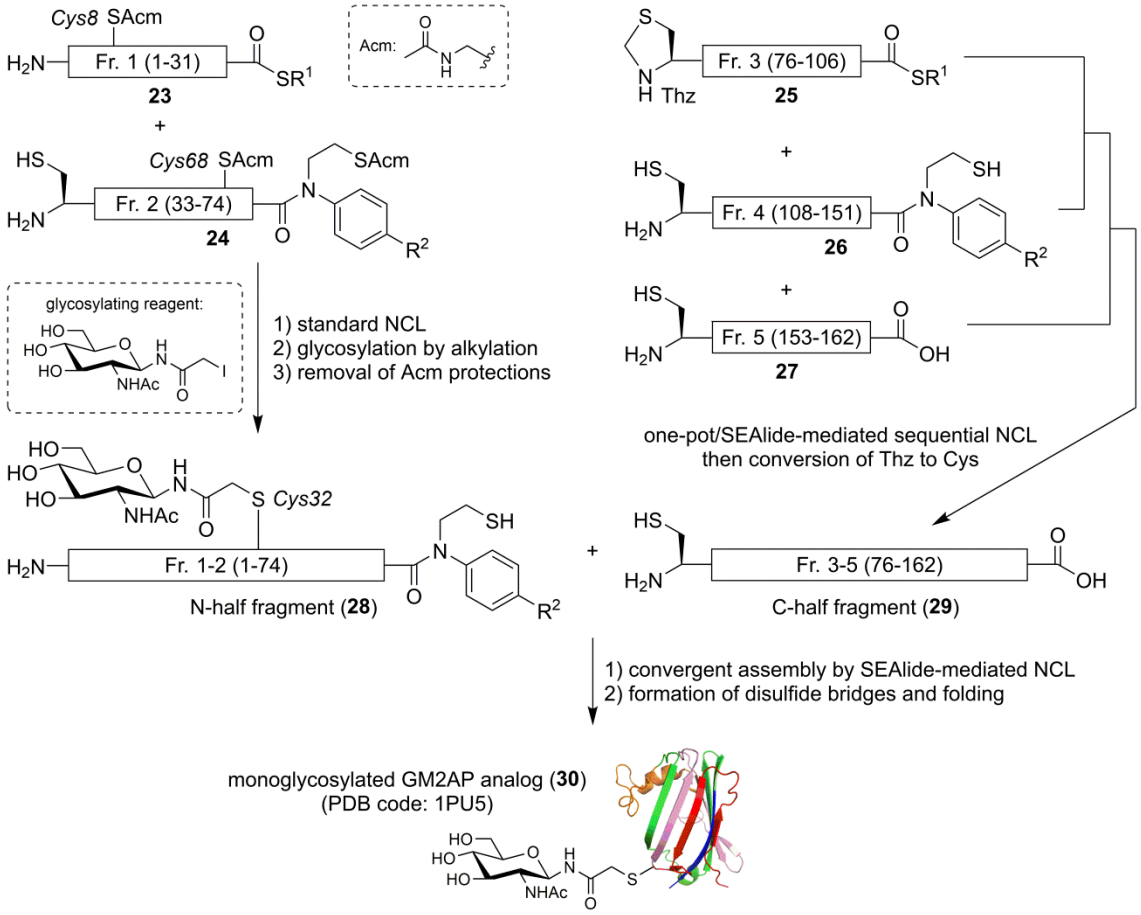


Figure 2.1. Primary sequence of GM2AP. Underlines indicate ligation sites. Bold letters indicate cysteine residues. The bold and italic *N* represents an *N*-glycosylated residue that was replaced by *S*-glycosylated Cys in the GM2AP analog.

Our first synthetic strategy for monoglycosylated GM2AP analog is shown in Scheme 2.1. From the point of view of simplicity and efficiency in the synthesis, the convergent assembly of multi-peptide fragments was planned. The entire sequence of GM2AP analog was divided into five peptide fragments. The N-half fragment **28** was prepared by coupling of the alkyl thioester **Fr. 1** (**23**) and the N-terminal cysteinyl SEAlide peptide **Fr. 2** (**24**) followed by selective *S*-glycosylation at the ligation site and subsequent removal of the acetamidomethyl (Acm) protection, a protecting group used for prevention of undesired glycosylation. The synthesis of the C-half fragment **29** was achieved through one-pot/SEAlide-mediated sequential NCL. The first NCL between the alkyl thioester fragment **Fr. 3** (**25**) containing an N-terminal

1,3-thiazolidine-4-carbonyl (Thz) unit and the N-terminal cysteinyl SEAlide peptide **Fr. 4** (**26**) in the absence of phosphate salts, followed by the second NCL with the N-terminal cysteinyl peptide **Fr. 5** (**27**) in the presence of phosphate salts, and subsequent opening of the thiazolidine ring gave the C-half fragment **29**. The convergent assembly of the N- and C-half fragments, **28** and **29** respectively, in the presence of phosphate salts and subsequent folding afforded the desired protein molecule.



Scheme 2.1. First synthetic plan, for total chemical synthesis of monoglycosylated GM2AP analog.

2.3 Second synthetic plan, for tailored synthesis of GM2AP analog

As mentioned in the previous section, GM2AP is an essential cofactor for GM2 degradation through the formation of a HexA-GM2AP complex. Computational

analysis of this complex predicted that the loop region, residues Cys68 to Ser74 in the GM2AP, was required for the interaction with HexA, and that Thr69, in particular, could be critically associated with the stabilization of this protein complex.[22] Furthermore, this computational assessment indicated that the replacement of Thr69 with some other suitable residues such as Trp, His, Phe, or Lys could lead to the enhancement of the degradative activity of GM2AP via the formation of more stable protein complexes. Thus, we explored GM2AP analogs with greater activity than the parent protein through the incorporation of different amino acids into the Thr69 position; however, our initial synthetic plan for accessing GM2AP was found to be inappropriate for the incorporation of these replacements into the whole protein, because the first protocol required the laborious and time-consuming preparation of the 43-residue fragment **Fr. 2** (**24**) bearing the replacement position at the C-terminus (Figure 2.2).

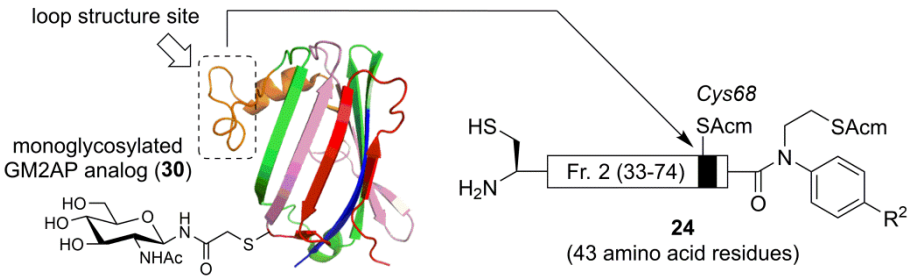
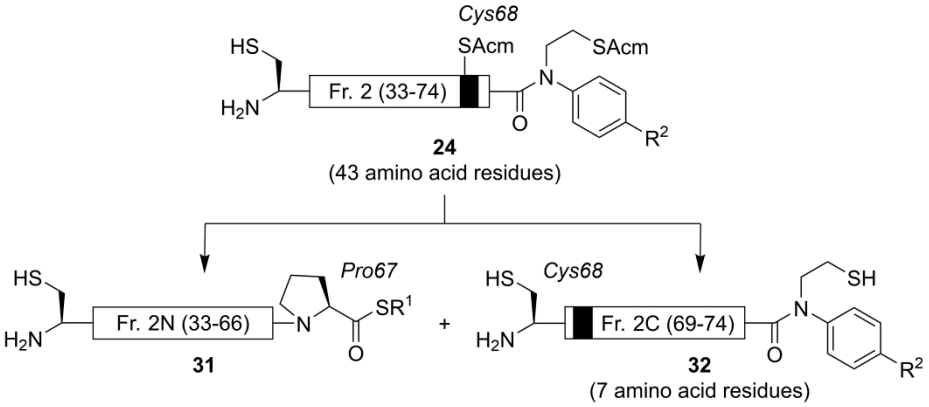


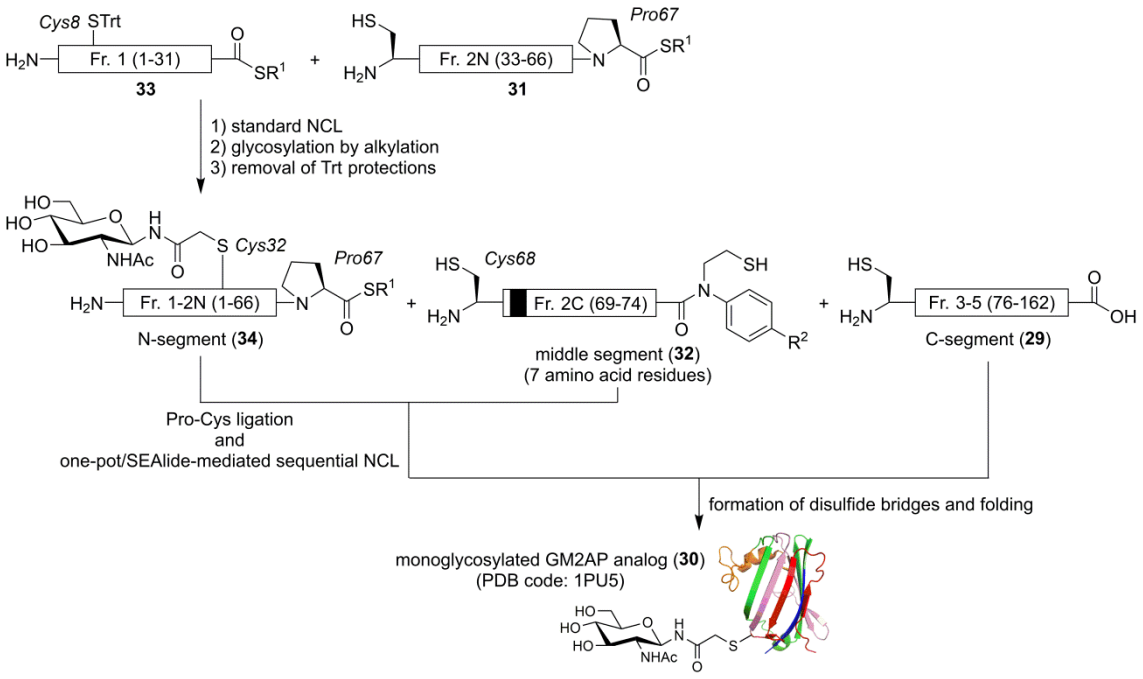
Figure 2.2. Loop structure site in GM2AP.

Our second synthetic strategy for facile access to a GM2AP library is shown in Scheme 2.2 and Scheme 2.3. To address the above-mentioned issue, we planned to use a readily accessible short peptide fragment containing the replacement position. Thus, the 43-residue **Fr. 2** (**24**) was divided into the two pieces, 36- and 7-residue peptide fragments (**Fr. 2N** (**31**) and **Fr. 2C** (**32**), respectively) (Scheme 2.2). This division provided an easily available short peptide **Fr. 2C** (**32**) as a Thr69-containing fragment. The use of a short peptide could overcome the difficulties mentioned above, and facilitate the construction of a GM2AP library through tailored synthesis of the proteins; however, our new synthetic route required a new ligation between Pro67 thioester and Cys68, where the reaction had been recognized to be unsuitable for practical use due to the low reactivity of prolyl thioesters under standard NCL conditions.[4] In this regard, we have recently disclosed that the coupling at the proline site could proceed smoothly under our optimized conditions. Therefore, we envisioned that our idea should be applicable to the preparation of various GM2AP derivatives.

In the envisioned strategy, the construction of the entire sequence of GM2AP should be achieved through the SEAlide-mediated ligation involving three materials: N-, middle-, and C-segments. The N-segment **34** should be synthesized by our previously developed kinetically controlled ligation between the alkyl thioester **Fr. 1** (**33**) and the N-terminal cysteinyl prolyl thioester **Fr. 2N** (**31**) followed by regioselective *S*-alkylation at the ligation site and subsequent removal of the *S*-Trt group introduced into the **Fr. 1** (**33**) with the aim to prevent undesired alkylation.[23] The preparation of the C-segment **29** should be achieved through a synthetic protocol identical to that used in our initial strategy.[20]



Scheme 2.2. Our idea for a second synthetic plan of GM2AP analog.



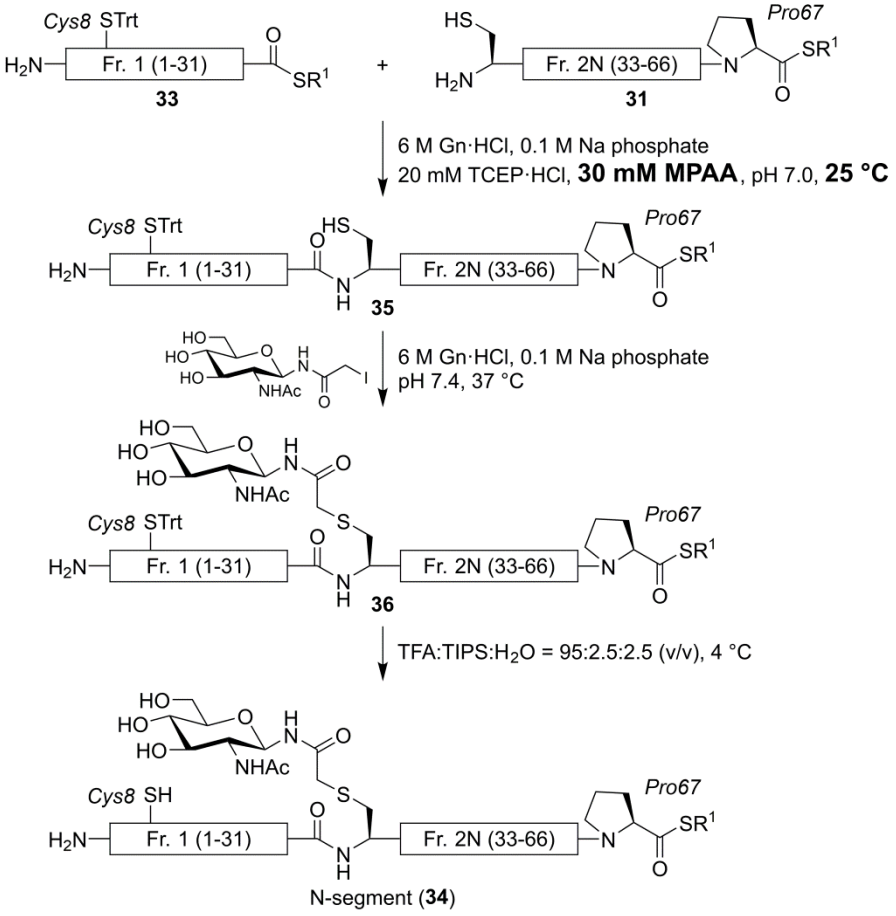
Scheme 2.3. Second synthetic plan, for preparation of GM2AP analogs bearing various amino acid substitutions for Thr69.

2.4 Tailored synthesis of GM2AP analog for construction of a protein library

The requisite peptide fragments (**25**–**27** and **31**–**33**) were prepared by means of Boc or Fmoc SPPS. To achieve the selective *S*-glycosylation, **Fr. 1** (**33**) was prepared as an *S*-Trt-protected fragment. As mentioned in the previous section, computational analysis of the HexA-GM2AP complex indicated that the replacement of Thr69 in GM2AP molecule with several different amino acids Xaa (Xaa = Trp, His, Phe, or Lys) could enhance the degradative activity of GM2AP through the formation of more stable protein complexes. Therefore, in addition to the native-type middle segment **32a** (Xaa = Thr), several analogs bearing these different amino acid replacements were prepared as the corresponding SEAlide peptides by Fmoc SPPS.

The reaction progress for the synthesis of the N-segment **34** is shown in Scheme 2.4 and Figure 2.3. NCL was conducted between the thioester **33** and the N-terminal cysteinyl prolyl thioester **31** in 6 M Gn·HCl–0.1 M phosphate in the presence of low concentrations of two additives (TCEP·HCl and MPAA) at 25 °C. Pleasingly, the N-terminal cysteine residue of **31** preferentially reacted with the more

reactive glycine moiety of **33** in an intermolecular manner to give the desired peptide possessing *S*-Trt protection, except for the cysteine at the ligation site. The unprotected cysteine residue was alkylated by the use of an *N*-acetylglucosamine unit to afford the corresponding monoglycosylated peptide **36**. The *S*-Trt group on the resulting peptide **36** was removed by treatment with TFA/TIPS/H_2O, and then HPLC purification, affording the desired N-segment **34** in 43% isolated yield. The synthesis of the C-segment was achieved through the procedure used in the first protocol.



Scheme 2.4. Synthetic route for the preparation of N-segment.

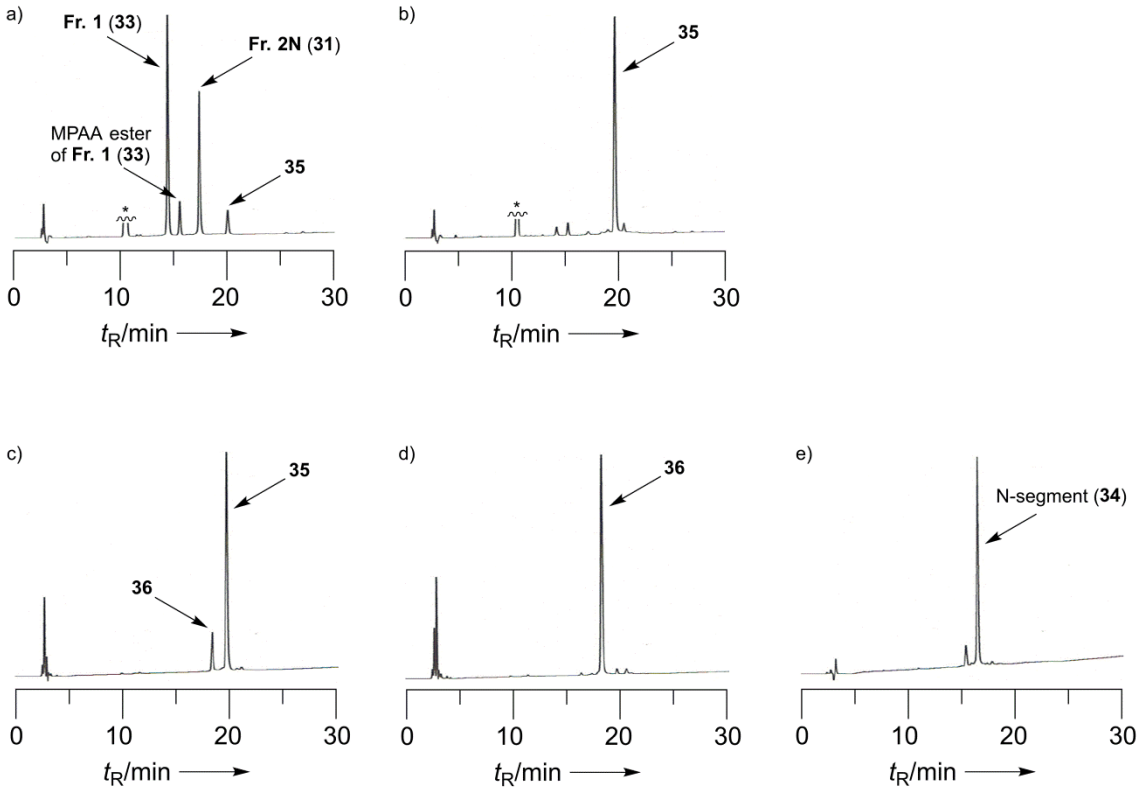
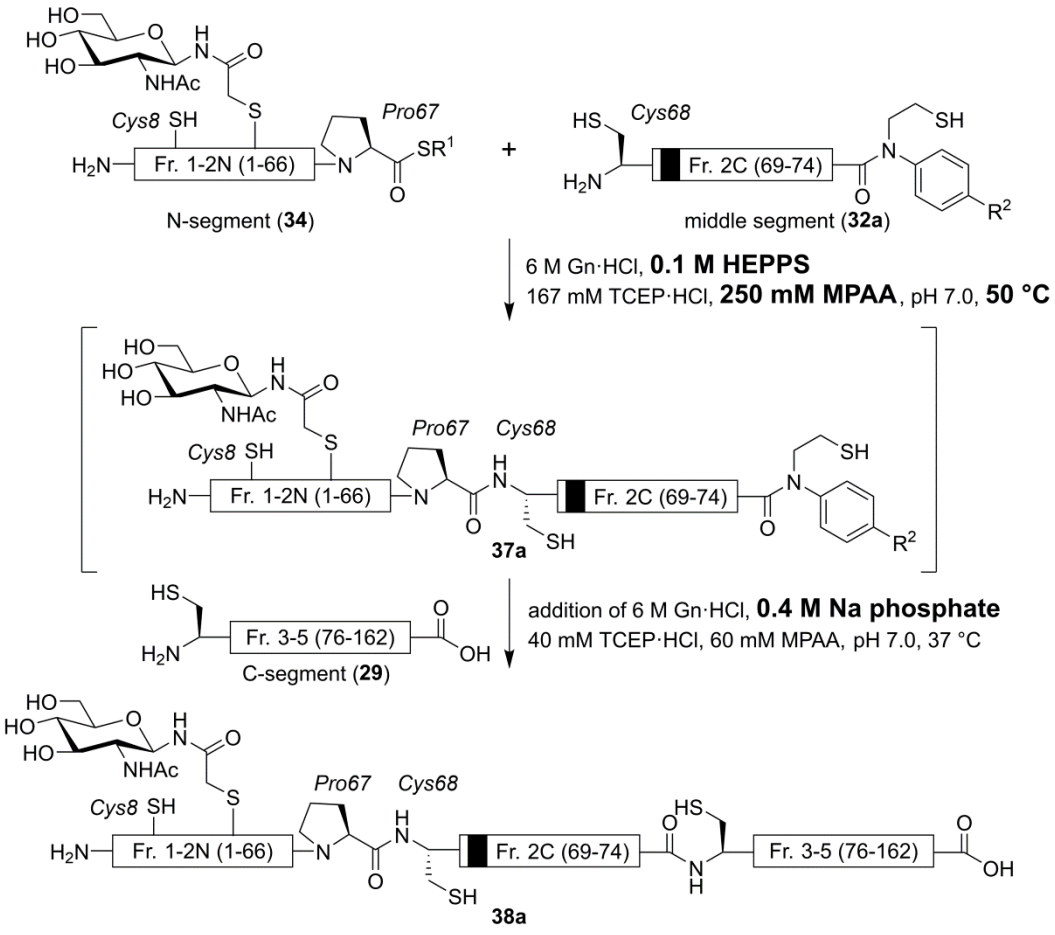


Figure 2.3. HPLC monitoring of NCL, alkylation and removal of Trt group for synthesis of N-segment (**34**). a) The NCL (t = 0 h): **Fr. 1** (**33**) (1 mM) and **Fr. 2N** (**31**) (1 mM) were ligated in Gn·HCl [6 M, Na phosphate (0.1 M), pH 7.0] in the presence of TCEP·HCl (20 mM) and MPAA (30 mM) at 25 °C. b) The NCL (t = 4 h). c) The alkylation (t = 0 h): peptide **35** (0.5 mM) was alkylated in Gn·HCl [6 M, Na phosphate (0.1 M), pH 7.4] in the presence of glycosyl iodoacetamide (2.5 mM). d) The alkylation (t = 2 h). e) The removal of Trt group (t = 0.5 h): peptide **36** (0.25 mM) was treated with TFA-TIPS-H_2O (95:2.5:2.5 (v/v)) at 4 °C for 0.5 h. Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II column (4.6×250 mm) with a linear gradient of CH_3CN (30–60% over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm. *MPAA.

With the requisite three peptide segments in hand, the one-pot/N–to–C-directed sequential NCL was attempted as shown in Scheme 2.5. The first NCL, between the N-segment **34** and the native-type middle segment **32a** (Xaa = Thr), was conducted in 6 M Gn·HCl–0.1 M HEPPS in the presence of high concentrations of two additives (TCEP·HCl and MPAA) at elevated temperature (50 °C). These reaction conditions allowed the prolyl thioester moiety of N-segment **34** to participate in the ligation, with the SEAlide unit of middle segment **32a** remaining intact. This reaction went almost to

completion within 24 h to afford the ligated peptide **37a**. After confirmation of the reaction completion by RP-HPLC, a solution of the C-segment **29** in 6 M Gn·HCl–0.4 M phosphate buffer was added to the above reaction mixture to allow the resulting SEAlide peptide **37a** to function as the corresponding thioester to participate in the second NCL, with **29**. This reaction proceeded to completion in three days to afford the monoglycosylated peptide **38a** consisting of the entire sequence of GM2AP analogs (Figure 2.4). After RP-HPLC purification, the glycosylated 162-residue peptide **38a** was obtained as its reduced form in 19% isolated yield. The four disulfide bonds of the resulting peptide **38a** were formed under literature conditions to give the folded monoglycosylated GM2AP analog **30a** in 11% isolated yield (Figure 2.5).[24]



Scheme 2.5. SEAlide-mediated sequential NCL with N-, middle-, and C-segment.

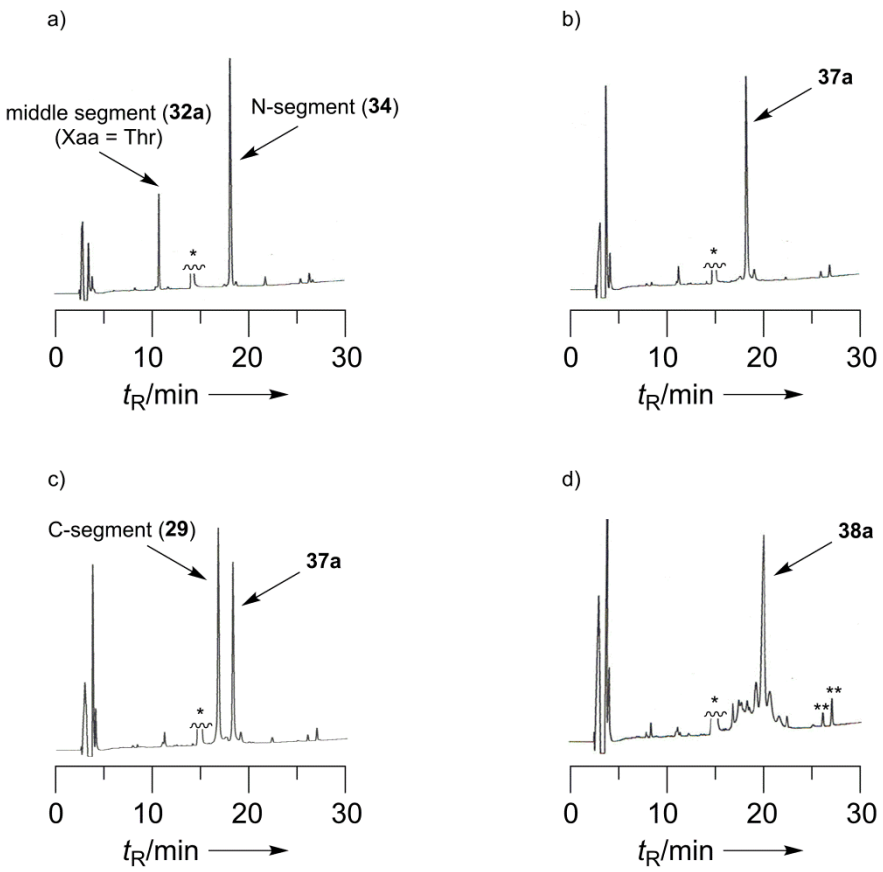
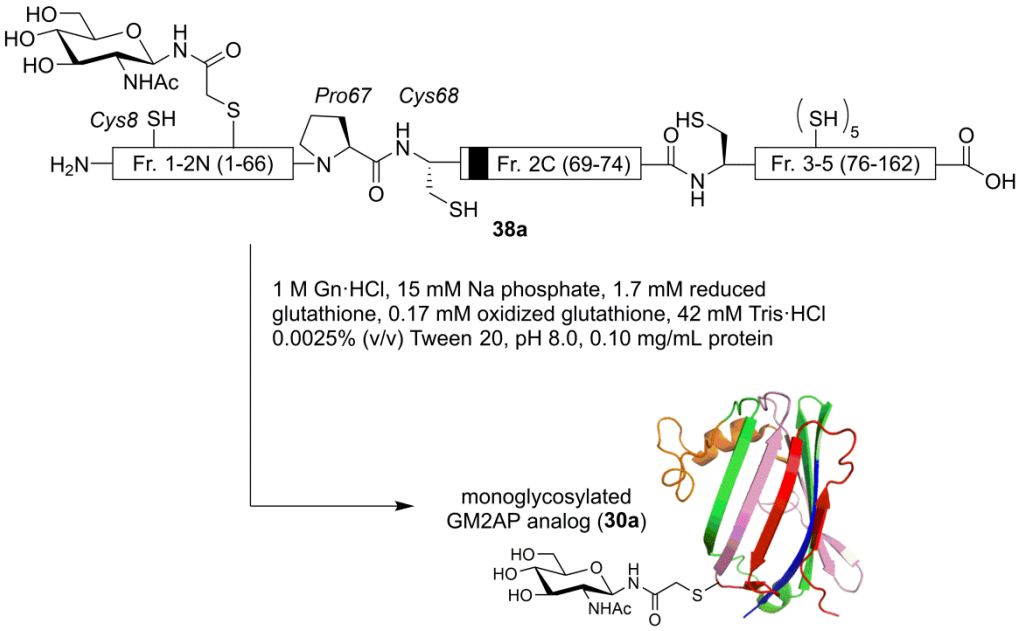


Figure 2.4. HPLC monitoring of SEAlide-mediated sequential NCL with N-, middle- and C-segment (**34**, **32a** and **29**, respectively). a) The first NCL (t = 0 h): N-segment (**34**) (1 mM) and middle segment (**32a**) (1 mM) were ligated in Gn·HCl [6 M, HEPPS (0.1 M), pH 7.0] in the presence of TCEP·HCl (167 mM) and MPAA (250 mM) at 50 °C. b) The first NCL (t = 24 h). c) The second NCL (t = 0 h): C-segment (**29**) (1 mM) in Gn·HCl [6 M, Na phosphate (0.4 M), pH 7.0] in the presence of TCEP·HCl (40 mM) and MPAA (60 mM) was added to the reaction mixture. Final concentrations: 0.5 mM each peptide in Gn·HCl [6 M, HEPPS (0.05), Na phosphate (0.2 M), TCEP·HCl (104 mM), MPAA (155 mM), 37 °C, pH 7.0]. d) The second NCL (t = 3 days). Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II column (4.6×250 mm) with a linear gradient of CH_3CN (20–70% over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm. *MPAA. **Non-peptidic impurity.



Scheme 2.6. Refolding reaction of reduced GM2AP analog.

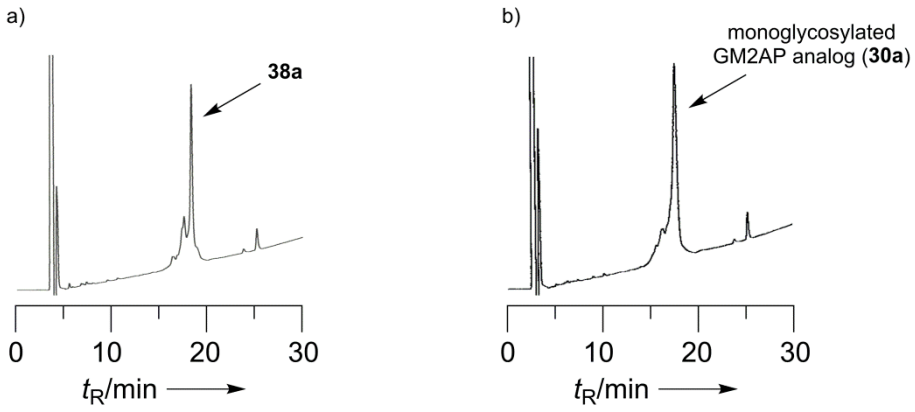


Figure 2.5. HPLC monitoring of refolding reaction of reduced GM2AP analog **38a** [8 Cys (SH) form]. a) The refolding reaction (t = 0 h): Reduced GM2AP analog **38a** [8 Cys (SH) form] was refolded in Gn·HCl [1 M, Na phosphate (15 mM), Tris·HCl (42 mM), reduced form glutathione (1.7 mM), oxidized form glutathione (0.17 mM), Tween 20 (0.0025%, v/v), pH 8.0, 0.10 mg mL^{-1} protein]. b) The refolding reaction (t = 2 days). Analytical HPLC conditions: Cosmosil Protein-R column (4.6×250 mm) with a linear gradient of CH_3CN (30－70% over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

The SEAlide-mediated NCLs with several other middle segments **32b**–**e** were conducted following an identical procedure, allowing for the incorporation of the replacements into the GM2AP molecule (Figure 2.6). After RP-HPLC purification of the fully ligated peptides, the formation of four disulfide bonds of the resulting peptides **38b**–**e** was performed in accordance with the above-mentioned method to give the folded monoglycosylated GM2AP analogs **30b**–**e** (Figure 2.6).

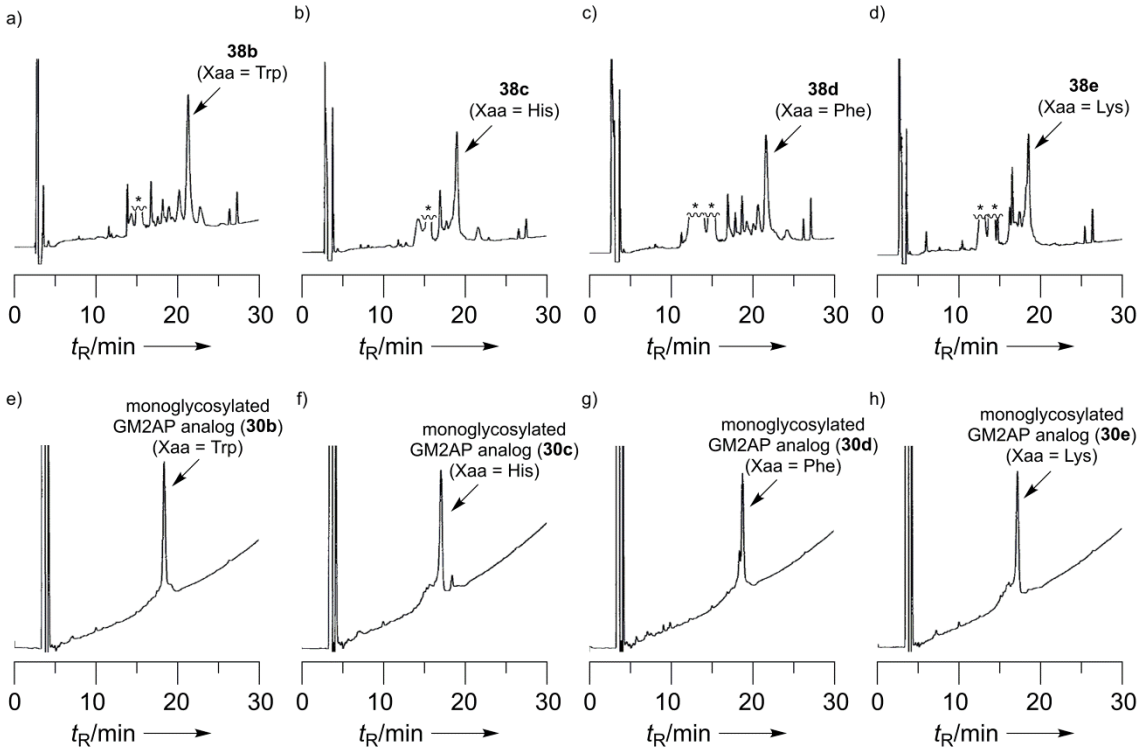


Figure 2.6. HPLC monitoring of reactions for the synthesis of the monoglycosylated GM2AP analogs **30b**–**e** (Xaa = Trp, His, Phe, or Lys, respectively). a) Xaa = Trp, second NCL (*t* = 3 days). b) Xaa = His, second NCL (*t* = 3 days). c) Xaa = Phe, second NCL (*t* = 3 days). d) Xaa = Lys, second NCL (*t* = 3 days). e) Xaa = Trp, folding reaction (*t* = 2 days). f) Xaa = His, folding reaction (*t* = 2 days). g) Xaa = Phe, folding reaction (*t* = 2 days). h) Xaa = Lys, folding reaction (*t* = 2 days). Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II column (4.6×250 mm) with a linear gradient of CH_3CN (20–70% or 30–70% over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm. *MPAA.

2.5 Biological evaluation of chemically synthesized GM2AP analog

Biological evaluations of the synthetic materials were performed using a GM2-degradation assay with HexA. After incubation of GM2 with synthesized samples in the presence of HexA, TLC monitoring of the degradation of GM2 was conducted using orcinol-H_2SO_4, as shown in Figure 2.7.[25] These results indicated that all synthetic proteins assisted in the HexA-catalysed degradation of GM2 to GM3. However, no significant difference in the activities between these samples was observed, in contrast to the prediction based on the computational assessment. Therefore, we are currently performing further detailed comparative evaluation of the activities of these proteins.

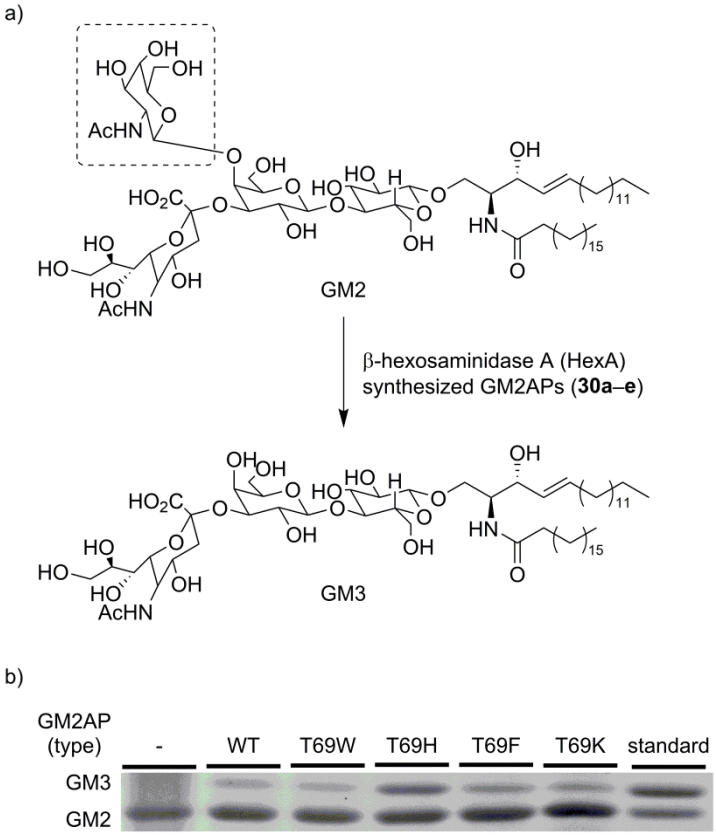


Figure 2.7. (a) Conversion of GM2 to GM3. (b) GM2-degradation assay with HexA in the presence of 5.0 or 6.7 μg of synthesized GM2APs **30a**–**e**. The degradation was monitored by TLC of the reaction aliquots (developing solvent: $CHCl_3$/MeOH/0.2% (w/v) $CaCl_2$ aq. = 60:40:9, (v/v); detection reagent: orcinol reagent, 120 °C, 5 min).

2.6 Conclusion

A novel synthetic platform for the preparation of the monoglycosylated GM2AP analogs containing various amino acid substitutions for Thr69 was developed. The facile incorporation of the replacements into the whole proteins was achieved by the use of readily accessible short peptide fragments consisting of seven amino acid residues. In this study, prolyl thioesters and SEAlide peptides were successfully used as versatile reagents in two different types of kinetically controlled ligation protocols. The established synthetic strategy should open opportunities for extension of a GM2AP-based library, and lead to the discovery of medicinal GM2AP agents for treatment of the AB variant of GM2 gangliosidosis.

Chapter 3
Conclusions

1. Suitable NCL conditions enabling prolyl thioesters to work as a useful coupling partner were discovered. Appropriate adjustments of the reaction conditions allowed prolyl thioesters to be used for one-pot/N–to–C-directed sequential ligation.

2. A novel synthetic platform for facile access to a GM2AP-based library was developed. In this study, two different procedures, using prolyl thioesters or SEAlide peptides, were successfully applied to the preparation of the N-segment or the C-segment and the entire GM2AP molecule, respectively.

Taken together, our findings demonstrate that prolyl thioesters are a versatile reagent for achieving total chemical synthesis of numerous proteins. Furthermore, the established synthetic route for GM2APs should open a new door for the extension of a GM2AP-based library through tailored synthesis of a wide variety of GM2AP derivatives, and could lead to the development of novel protein therapeutics against the AB variant of GM2 gangliosidosis.

Experimental section

General Methods

Mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™ (ESI-TOF) or a Bruker Esquire2000T (ESI-Ion Trap). For HPLC separations, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a Cosmosil Protein-R analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a Cosmosil 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) or a Cosmosil 5C_{18}-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL/min) 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 CH_3CN (v/v, solvent B) was used for HPLC elution.

Chapter 1

Preparation of prolyl thioesters **4a-d**, **f**, **h-s**

H-**LYRX<u>P</u>**-S$(CH_2)_2$CO-**L**-NH_2

4a-d, **f**, **h-s**

General procedure: Prolyl thioesters **4** were prepared by Boc SPPS using *in situ* neutralization protocol on $HSCH_2CH_2CO$-Leu-MBHA resin (0.70 mmol amine/g, 0.10 g, 0.070 mmol).[26] For the incorporation of amino acids on the prolyl thioester, preactivated Boc amino acid (Boc amino acid, DIPCDI, and HOBt·H_2O, 4 equiv. each in DMF for 30 min) was added to the resin. Then, 1 equiv. of DIPEA was added to the reaction mixture in four times every 30 minutes. Other amino acids were condensed according to the standard *in situ* neutralization protocol. The resulting completed resin (30 mg) 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 h, and then the resin was filtrated off. To the 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 prolyl thioesters **4**. The crude peptides were purified by preparative HPLC to give the purified prolyl thioesters **4**.

4a (**X** = Ala) (1.24 mg, 1.18 μmol, 6%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.2 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 12% to 25% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 819.5, found 819.3.

4b (**X** = Val) (1.27 mg, 1.18 μmol, 6%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 20.0 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 12% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 847.5, found 847.3.

4c (**X** = Gly) (1.37 mg, 1.33 μmol, 6%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.6 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 17% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 805.4, found 805.2.

4d (**X** = Asp) (1.01 mg, 0.93 μmol, 4%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 16.3 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 18% to 21% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 863.4, found 863.2.

4f (**X** = Glu) (1.33 mg, 1.20 μmol, 6%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.6 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear

gradient of solvent B in solvent A, 20% to 23% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 877.5, found 877.3.

4h (**X** = Ser) (1.38 mg, 1.30 μmol, 6%):
Analytical HPLC conditions: Cosmosil $5C_{18}$-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. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 18% to 21% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 835.5, found 835.3.

4i (**X** = Thr) (1.42 mg, 1.32 μmol, 6%):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.4 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 17% to 21% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 849.5, found 849.3.

4j (**X** = Leu) (1.21 mg, 1.11 μmol, 5%):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 22.3 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 26% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 861.5, found 861.3.

4k (**X** = Ile) (1.17 mg, 1.07 μmol, 5%):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 23.0 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 26% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 861.5, found 861.3.

4l (**X** = Met) (1.27 mg, 1.15 μmol, 6%):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 21.9 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 25% to 33% over 30 min. MS (ESI-TOF) *m*/*z* calcd

([M + H]$^+$) 879.5, found 879.2.

4m (**X** = Pro) (1.27 mg, 1.18 μmol, 6%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 18.2 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 17% to 25% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 845.5, found 845.3.

4n (**X** = Phe) (1.04 mg, 0.93 μmol, 4%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 23.2 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 26% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 895.5, found 895.2.

4o (**X** = Tyr) (1.58 mg, 1.39 μmol, 7%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 20.4 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 24% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 911.5, found 911.2.

4p (**X** = Trp) (1.20 mg, 1.03 μmol, 5%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 24.9 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 25% to 33% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 934.5, found 934.2.

4q (**X** = His) (1.17 mg, 1.05 μmol, 5%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 14.6 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 13% to 19% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 885.5, found 885.2.

4r (**X** = Lys) (1.47 mg, 1.21 μmol, 6%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 14.5 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 17% to 25% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 876.5, found 876.3.

4s (**X** = Arg) (1.55 mg, 1.24 μmol, 6%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 13.8 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 17% to 25% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 904.5, found 904.3.

Preparation of prolyl thioesters **4e** (**X** = Asn) and **4g** (**X** = Gln)

H-**LRANKLYRXP**-S$(CH_2)_2$CO-**L**-NH_2

4e and **4g**

Prolyl thioesters **4e** and **4g** were prepared in a manner similar to those for **4**.

4e (**X** = Asn) (1.42 mg, 0.75 μmol, 4%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 16.1 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 16% to 22% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 722.9, found 722.9.

4g (**X** = Gln) (1.57 mg, 0.82 μmol, 4%):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 15.9 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 13% to 22% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 729.9, found 729.9.

Preparation of N-terminal cysteinyl peptide **5**

H-**CYRANK**-NH_2

5

The peptide was elongated on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.60 g, 0.13 mmol) using standard Fmoc SPPS. The resulting complete resin (100 mg) was treated with TFA-*m*-cresol-thioanisole-H_2O-EDT (80:5:5:5:5 (v/v), 50 μL/1 mg resin) at room temperature for 2 h. After the resin was filtered off, cooled Et_2O was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with Et_2O and purified by preparative HPLC to give the desired N-terminal cysteinyl peptide **5** (1.96 mg, 2.00 μmol, 9%).

5:
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 15% over 30 min, retention time = 14.0 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 1% to 13% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 753.4, found 753.2.

Optimization of NCL conditions for prolyl thioester

Prolyl thioester **4b** (0.11 mg, 0.10 μmol) and N-terminal cysteinyl peptide **5** (0.11 mg, 0.10 μmol) were dissolved in 94 μL of various ligation buffers. After addition of 6 μL benzamide as internal standard, the reaction mixture was incubated at 25, 37, or 50 °C and the reaction progress was monitored by analytical HPLC. According to report by Kent[4a], reaction rates were estimated based on peak integration of the HPLC at reaction time = 0, 1, 2, 3 and 6 h. Second order rate constants (*k*) were derived from equation $1/[\mathbf{5}] = kt + 1/[\mathbf{5}]_0$.

6b:
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 16.2 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 691.4, found 691.3.

MPAA ester of 4b:

Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 23.0 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^{+}$) 797.4, found 797.3.

Influence of **X** residue on formation of deleted peptide

Prolyl thioester **4** (0.11 mg, 0.10 μmol) and N-terminal cysteinyl peptide **5** (0.11 mg, 0.10 μmol) were dissolved in 6 M Gn·HCl–0.4 M Na phosphate buffer containing 167 mM TCEP·HCl and 250 mM MPAA. The reaction mixture was incubated at 50 °C and the reaction progress was monitored by analytical HPLC.

6a (**X** = Ala):

Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 14.9 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 677.4, found 677.3.

6b (**X** = Val):

Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 16.1 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 691.4, found 691.3.

6c (**X** = Gly):

Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.4 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 670.4, found 670.3.

6d (**X** = Asp):

Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 14.7 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 699.4, found 699.3.

6e (**X** = Asn):

Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.7 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 3H]^{3+}$) 660.4, found 660.4.

6f (**X** = Glu):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 14.0 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 706.4, found 706.2.

6g (**X** = Gln):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.2 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 3H]^{3+}$) 665.0, found 665.4.

6h (**X** = Ser):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.1 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 685.4, found 685.3.

6i (**X** = Thr):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.4 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 692.4, found 692.3.

6j (**X** = Leu):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 17.7 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 698.4, found 698.3.

6k (**X** = Ile):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 17.2 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 698.4, found 698.3.

6l (**X** = Met):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 17.4 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 707.4, found 707.3.

6m (**X** = Pro):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.6 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 690.4, found 690.3.

6n (**X** = Phe):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 18.2 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 715.4, found 715.3.

6o (**X** = Tyr):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 16.2 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 723.4, found 723.2.

6p (**X** = Trp):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 18.5 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 734.9, found 734.8.

6q (**X** = His):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 13.5 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 710.4, found 710.3.

6r (**X** = Lys):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 13.1 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 705.9, found 705.8.

6s (**X** = Arg):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 13.8 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 719.9, found 719.8.

7:
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 14.2 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 593.3, found 593.3.

Deletion peptide derived from 4e or 4g (H-LRANKLYR-CYRANK-NH_2):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.9 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 3H]^{3+}$) 590.0, found 590.0.

Hydrolyzed peptide (H-LYR-OH) 9:
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 13.2 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^{+}$) 451.3, found 451.4.

Preparation of peptide thioesters **18** and **19**

H-**GVEINVK**-$S(CH_2)_2CO$-**L**-NH_2 H-**CSGSPQCLKP**-$S(CH_2)_2CO$-**L**-NH_2

18 **19**

Peptide thioesters **18** and **19** were prepared in a manner similar to those for **4**.

18 (2.61 mg, 2.20 μmol 3%):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 17.6 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 20% to 23% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^{+}$) 958.5, found 958.2.

19 (0.40 mg, 0.28 μmol 0.4%):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 16.9 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 20% to 23% over 30 min. MS (ESI-TOF) *m*/*z* calcd

([M + H]$^{+}$) 1219.6, found 1219.0.

Preparation of peptide **20**

H-**CKDAGMRFGKCMNRKCHCTP**-OH

20

The peptide was elongated on Fmoc-Pro-Wang resin (1.1 mmol amine/g, 0.20 g, 0.22 mmol) using standard Fmoc SPPS. The resulting complete resin (50 mg) was treated with TFA-*m*-cresol-thioanisole-H_2O-EDT (80:5:5:5:5 (v/v), 50 μL/1 mg resin) at room temperature for 2 h. After the resin was filtered off, cooled Et_2O was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with Et_2O and purified by preparative HPLC to give the desired peptide **20** (4.68 mg, 1.58 μmol, 3%).

20:
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 13.4 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 16% to 22% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + 3H]$^{3+}$) 762.7, found 762.5.

One-pot/N–to–C-directed sequential NCL for the synthesis of 6 Cys-SH kaliotoxin **22**

Protocol 1:
Ligation of peptide thioester **18** (1.78 mg, 1.50 μmol) and peptide thioester **19** (2.17 mg, 1.50 μmol) was performed in 6 M Gn·HCl–0.4 M Na phosphate buffer containing 167 mM TCEP·HCl and 250 mM MPAA (pH 7.0, 1.5 mL, 1.0 mM each peptide) at 25 °C. The reaction was completed within 3 h. After addition of peptide **20** (4.46 mg, 1.50 μmol) to the reaction mixture, temperature was elevated to 50 °C. The second NCL proceeded smoothly within 24 h. The crude material was purified by semi-preparative HPLC to give the purified 6 Cys-SH kaliotoxin **22** (2.23 mg, 0.451 μmol, 30%).

Protocol 2:

Ligation of peptide thioester **18** (1.78 mg, 1.50 μmol) and peptide thioester **19** (2.17 mg, 1.50 μmol) was performed in 6 M Gn·HCl–0.4 M Na phosphate buffer containing 20 mM TCEP·HCl and 30 mM MPAA (pH 7.0, 0.75 mL, 2.0 mM each peptide) at 37 °C. The reaction was completed within 3 h. After confirmation of the completion of the first NCL by HPLC analysis, peptide **20** (4.46 mg, 1.50 μmol) in 6 M Gn·HCl–0.4 M Na phosphate buffer containing 246 mM TCEP·HCl, 370 mM MPAA (pH 7.0, 0.75 mL) was added to the reaction mixture to yield the 6 Cys-SH kaliotoxin **22** in one-pot manner. The second NCL proceeded smoothly within 24 h. The crude material was purified by semi-preparative HPLC to give the purified 6 Cys-SH kaliotoxin **22** (2.17 mg, 0.439 μmol, 29%).

21:

Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 18.0 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 980.0, found 979.7.

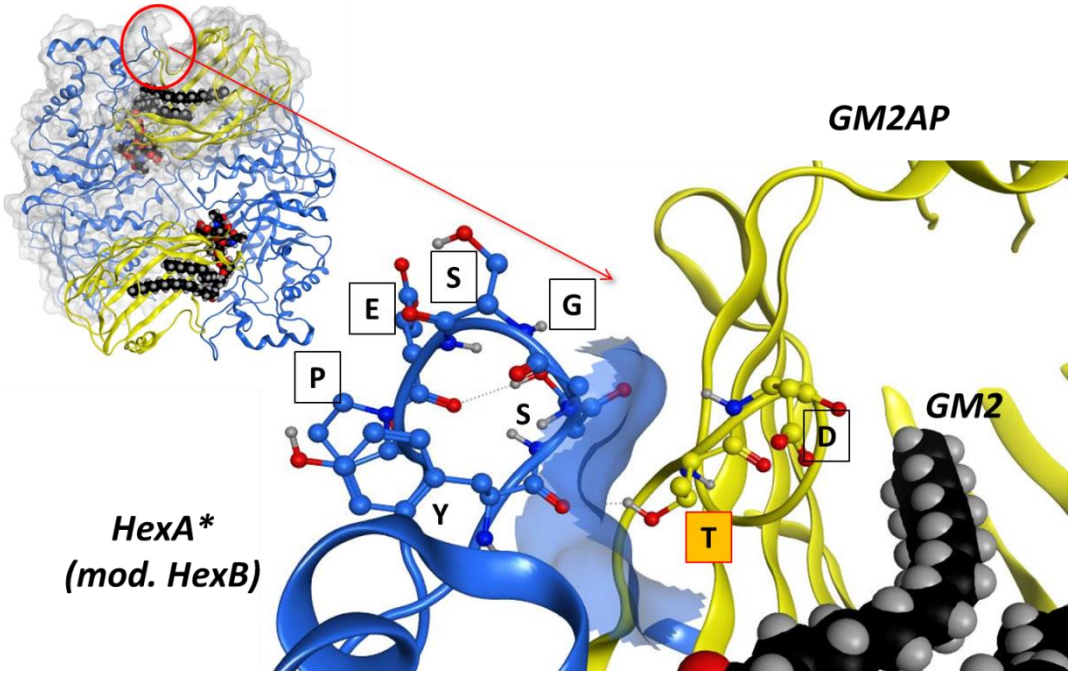
Cyclic peptide of 19:

Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 11.5 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^{+}$) 1001.5, found 1001.2.

6 Cys-SH kaliotoxin 22:

Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 15.0 min. Semi-preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II semi-preparative column with a linear gradient of solvent B in solvent A, 18% to 26% over 30 min. MS (ESI-TOF) calcd (average isotopes) 4027.8, found 4027.3.

A)



B)

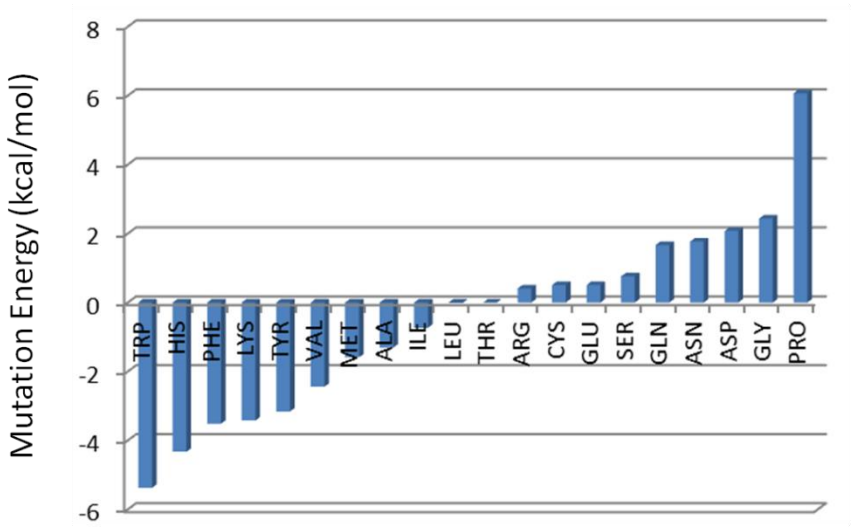


Figure S1. A) Computational analysis of Hex A-GM2AP complex. B) The computational analysis predicted that the substitution of several amino acid residues such as Trp, His, Phe, or Lys for Thr69 might be highly involved in formation of the stable complexes. *Loop region of amino acid sequence of modified HexB (mod. HexB) is identical to that of HexA.

Preparation of *S*-Trt-protected peptide thioester **Fr. 1** (**33**)

H-**SSFSWDNC**(Trt)**DEGKDPAVIRSLTLEPDPIIVPG**-S$(CH_2)_2$CO-**L**-NH_2

Fr. 1(**33**)

Unprotected peptide thioester **Fr. 1** (**33**) was prepared by Boc SPPS using *in situ* neutralization protocol on $HSCH_2CH_2CO$-Leu-MBHA resin (0.70 mmol amine/g, 1.0 g, 0.70 mmol). The resulting completed resin (300 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 h, and then the resin was filtrated off. The filtrated 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 h, and then the resin was filtrated off. To the filtrate was added cooled Et_2O to give precipitate. The formed precipitate was treated with Trt-OH (3.3 eq.) in HFIP at room temperature for 1 h. To the crude reaction mixture was added cooled Et_2O to give precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude *S*-Trt-protected peptide thioester **Fr. 1** (**33**). The crude peptide was purified by preparative HPLC to give the purified *S*-Trt-protected peptide thioester **Fr. 1**(**33**) (0.98 mg, 0.24 μmol, 0.1%).

Fr. 1 (**33**):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 65% over 30 min, retention time = 21.2 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 33% to 41% over 30 min. MS (ESI-TOF) calcd (average isotopes) 3801.3, found 3801.0.

Preparation of peptide thioester **Fr. 2N** (**31**)

H-**CVTLSVMGSTSVPLSSPLKVDLVLEKEVAGLWIKIP**-S$(CH_2)_2$CO-**L**-NH_2

Fr. 2N (**31**)

Peptide thioester **Fr. 2N** (**31**) was prepared by Boc SPPS using *in situ* neutralization protocol on $HSCH_2CH_2CO$-Leu-MBHA resin (0.70 mmol amine/g, 1.0 g, 0.70 mmol). The resulting completed resin (300 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 h, and then the resin was filtrated off. The filtrated 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 h, and then the resin was filtrated off. To the 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 thioester **Fr. 2N** (**31**). The crude peptide was purified by preparative HPLC to give the purified peptide thioester **Fr. 2N** (**31**) (1.24 mg, 0.28 μmol, 0.1%).

Fr. 2N (**31**):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 65% over 30 min, retention time = 23.3 min. Preparative HPLC conditions: Cosmosil 5C_{18}-AR-II preparative column with a linear gradient of solvent B in solvent A, 36% to 44 over 30 min. MS (ESI-TOF) calcd (average isotopes) 4010.9, found 4010.4.

Preparation of SEAlide peptide **Fr. 2C** (**32a**–**e**) (middle segments)

H-**C**-**Xaa**-**DYIGS**-SEAlide-**L**-NH_2

Fr. 2C (**32a**–**e**)

On NovaSyn® TGR resin (Rink amide type: 0.25 mmol amine/g, 0.90 g, 0.23 mmol) was coupled an Fmoc-Ser-incorporating *N*-sulfanylethylaniline linker (362 mg, 0.45 mmol) with the aid of HATU (163 mg, 0.43 mmol) and DIPEA (149 μL, 0.86 mmol) to yield the SEAlide-linked resin. On this resin, standard Fmoc SPPS was performed for the construction of the protected peptide resin. The resulting completed resin (50 mg) was treated with TFA-*m*-cresol-thioanisole-H_2O-EDT (80:5:5:5:5 (v/v), 50 μL/1 mg resin), 2 h, at room temperature. The resin in the reaction mixture was filtrated off. To the resulting filtrate was added cooled Et_2O to give a precipitate. The precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude SEAlide peptide **Fr. 2C** (**32a**–**e**). The crude SEAlide peptide was purified by preparative HPLC to give the purified SEAlide peptide **Fr. 2C** (**32a**–**e**).

Fr. 2C (**32a**) (Xaa = Thr):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 65% over 30 min, retention time = 15.4 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 25% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 525.2, found 525.3.

Fr. 2C (**32b**) (Xaa = Trp):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 65% over 30 min, retention time = 17.0 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 26% to 38% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 567.7, found 567.7.

Fr. 2C (**32c**) (Xaa = His):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 65% over 30 min, retention time = 13.3 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 24% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 543.2, found 543.3.

Fr. 2C (**32d**) (Xaa = Phe):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 65% over 30 min, retention time = 15.4 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 24% to 36% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 548.2, found 548.3.

Fr. 2C (**32e**) (Xaa = Lys):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 15% to 65% over 30 min, retention time = 13.2 min. Preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II preparative column with a linear gradient of solvent B in solvent A, 19% to 28% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 538.8, found 538.8.

Synthesis of peptide thioester **35**

S-Trt-protected peptide thioester **Fr. 1** (**33**) (4.6 mg, 1.2 μmol) and peptide thioester **Fr. 2N** (**31**) (5.2 mg, 1.2 μmol) were dissolved in 1.2 mL of ligation buffer (6 M Gn·HCl, 0.1 M Na phosphate, 20 mM TCEP·HCl, 30 mM MPAA, pH 7.0), and the solution was incubated at 25 °C. The reaction was completed within 4 h. The crude peptide was purified by semi-preparative HPLC to give purified peptide thioester **35** (6.2 mg, 0.75 μmol, 64%).

Peptide thioester **35**:
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 30% to 60% over 30 min, retention time = 19.7 min. Semi-preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II semi-preparative column with a linear gradient of solvent B in solvent A, 36% to 56% over 30 min. MS (ESI-TOF) calcd (average isotopes) 7593.8, found 7593.5.

S-Alkylation for the synthesis of **36**

To a solution of **35** (6.2 mg, 0.75 μmol) in 0.1 M Na phosphate buffer with 6 M Gn·HCl (pH 7.4, 1.5 mL) was added iodoacetamide (1.5 mg, 5 eq.) at 37 °C and the resulting mixture was incubated at same temperature for 2 h.[21] Purification of the resulting reaction mixture on semi-preparative HPLC yielded *S*-glycosylated peptide thioester **36** (3.3 mg, 0.38 μmol, 51%).

S-Glycosylated peptide thioester **36**:
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 30% to 60% over 30 min, retention time = 18.1 min. Semi-preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II semi-preparative column with a linear gradient of solvent B in solvent A, 39% to 51% over 30 min. MS (ESI-TOF) calcd (average isotopes) 7854.1, found 7854.0.

Removal of *S*-Trt protection for the synthesis of N-segment (**34**)

The *S*-Trt-protected peptide **36** (3.3 mg, 0.38 μmol) was treated with TFA-TIPS-H_2O [95:2.5:2.5 (v/v)] at 4 °C. After 0.5 h, to reaction mixture 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 N-segment (**34**)**.** The crude peptide was purified by

semi-preparative HPLC to give purified N-segment (**34**) (1.4 mg, 0.16 μmol, 43%).

N-segment (**34**):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 30% to 60% over 30 min, retention time = 16.1 min. Semi-preparative HPLC conditions: Cosmosil $5C_{18}$-AR-II semi-preparative column with a linear gradient of solvent B in solvent A, 35% to 55% over 30 min. MS (ESI-TOF) calcd (average isotopes) 7611.8, found 7611.3.

One-pot/ N–to–C-directed sequential NCL with N-segment **34**, middle segment **32**, and C-segment **29**

Kinetically controlled ligation of N-segment **34** (0.74 mg, 0.09 μmol) and middle segment **32a** (Xaa = Thr) (0.10 mg, 0.09 μmol) was performed in 6 M Gn·HCl–0.1 M HEPPS buffer containing 167 mM TCEP·HCl and 250 mM MPAA (pH 7.0, 90 μL (1.0 mM each peptide)) at 50 °C. The reaction was completed within 24 h. After confirming the completion of the first NCL by HPLC analysis, C-segment **29** solution (1.0 eq.) in 6 M Gn·HCl–0.4 M Na phosphate buffer containing 60 mM TCEP·HCl and 40 mM MPAA (pH 7.0, 90 μL) was added to the reaction mixture. The second NCL proceeded within 3 days, and then the crude material was purified by semi-preparative HPLC to give the desired reduced GM2AP analog **38a** (Xaa = Thr) (0.33 mg, 0.017 μmol, 19%). One-pot/N–to–C-directed sequential NCLs with use of the other middle segments (peptides **32b**–**e**) were also performed by an identical procedure to give the desired reduced GM2AP analogs **38b**–**e** [Xaa = Trp (0.33 mg, 0.017 μmol, 21%), His (0.030 mg, 0.0015 μmol, 2%), Phe (0.20 mg, 0.010 μmol, 10%), Lys (0.42 mg, 0.021 μmol, 22%), respectively], but purification of the crude materials in the cases of the reactions with middle segments **32d** and **32e** (Xaa = Phe, Lys) were performed by analytical HPLC.

Reduced GM2AP analog **38a** (Xaa = Thr):
Analytical HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column with a linear gradient of solvent B in solvent A, 20% to 70% over 30 min, retention time = 19.6 min. Semi-preparative HPLC conditions: Cosmosil Protein-R semi-preparative column with a linear gradient of solvent B in solvent A, 38% to 52% over 30 min. MS (ESI-TOF) calcd (average isotopes) 17838.4, found 17839.6.

Reduced GM2AP analog **38b** (Xaa = Trp):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 20% to 70% over 30 min, retention time = 21.4 min. Semi-preparative HPLC conditions: Cosmosil Protein-R semi-preparative column with a linear gradient of solvent B in solvent A, 39% to 53% over 30 min. MS (ESI-TOF) calcd (average isotopes) 17923.6, found 17923.5.

Reduced GM2AP analog **38c** (Xaa = His):
Analytical HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column with a linear gradient of solvent B in solvent A, 20% to 70% over 30 min, retention time = 18.5 min. Semi-preparative HPLC conditions: Cosmosil Protein-R semi-preparative column with a linear gradient of solvent B in solvent A, 40% to 52% over 30 min. MS (ESI-TOF) calcd (average isotopes) 17874.5, found 17873.9.

Reduced GM2AP analog **38d** (Xaa = Phe):
Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 20% to 70% over 30 min, retention time = 24.4 min. MS (ESI-TOF) calcd (average isotopes) 17884.5, found 17884.1.

Reduced GM2AP analog **38e** (Xaa = Lys):
Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 20% to 70% over 30 min, retention time = 20.3 min. MS (ESI-TOF) calcd (average isotopes) 17865.5, found 17865.8.

Folding for preparation of *S*-monoglycosylated GM2AP analogs **30**

Folding for preparation of *S*-monoglycosylated GM2AP analog **30** was performed with a modified method of previously reported one[24]. In the case of **30a**, the reduced GM2AP analog **38a** (Xaa = Thr) (0.30 mg) was dissolved in 6 M Gn·HCl–0.1 M Na phosphate buffer (pH 8.0, 0.46 mL), and 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, 2.5 mL, final concentration of protein 0.10 mg/mL). After being stored at 4 °C for one day and then at room temperature for additional one day, the crude material was purified by analytical HPLC to give the desired *S*-monoglycosylated GM2AP analog **30a** (Xaa = Thr). The concentration of the *S*-monoglycosylated GM2AP analog **30a** (Xaa = Thr) was determined as 0.60 mg/mL

(0.05 mL, 11%) by measurement of absorbance at 280 nm and calculation using the $A_{280} = \varepsilon_{280}\ c\ l$. The A_{280} is the observed absorbance at 280 nm (A_{280} = 0.773), the ε_{280} (M^{-1} cm^{-1}) is the molar extinction coefficient of GM2AP at 280 nm (ε_{280} = 22960, calculated as previously reported[27]), the c (M) is concentration of a protein, and the l (cm) is the length of the optical path. Folding of the other GM2AP molecules **38b**–**e** with substitution at the position was also performed by an identical procedure to give desired *S*-monoglycosylated GM2AP analogs **30b**–**e** [Xaa = Trp (0.42 mg/mL, 7%), His (0.43 mg/mL, 9%), Phe (0.23 mg/mL, 6%), Lys (0.36 mg/mL, 9%), respectively].

S-Monoglycosylated GM2AP analog **30a** (Xaa = Thr):
Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 70% over 30 min, retention time = 18.2 min. MS (ESI-TOF) calcd (average isotopes) 17830.4, found 17830.6.

S-Monoglycosylated GM2AP analog **30b** (Xaa = Trp):
Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 70% over 30 min, retention time = 18.1 min. MS (ESI-TOF) calcd (average isotopes) 17915.5, found 17916.0.

S-Monoglycosylated GM2AP analog **30c** (Xaa = His):
Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 70% over 30 min, retention time = 17.0 min. MS (ESI-TOF) calcd (average isotopes) 17866.4, found 17866.7.

S-Monoglycosylated GM2AP analog **30d** (Xaa = Phe):
Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 70% over 30 min, retention time = 18.6 min. MS (ESI-TOF) calcd (average isotopes) 17876.5, found 17876.1.

S-Monoglycosylated GM2AP analog **30e** (Xaa = Lys):
Analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 70% over 30 min, retention time = 17.0 min. MS (ESI-TOF) calcd (average isotopes) 17857.5, found 17858.5.

In vitro GM2-degradation assay of synthesized GM2AP analogs **30**

In vitro GM2-degradation assay was performed as described previously.[25] Briefly, the GM2 ganglioside was incubated with recombinant human HexA (2000 nmol/h 4-methylumbelliferyl-6-sulfo-β-D-glucosaminide potassium salts (4-MUGS)-degrading activity) in the presence or absence of 5 or 6.7 μg of synthesized GM2APs **30a**–**e** (Xaa = Thr, Trp, His, Phe, or Lys) in 10 mM sodium citrate buffer (pH 4.5) containing bovine serum albumin (BSA, 0.01%), CH_3CN (6%) and TFA (0.011%) at 37 °C for 16 h. 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 by use of 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. = 60:40:9, (v/v). To reveal the gangliosides, the thin-layer chromatography plate was sprayed with orcinol reagent and heated at 120 °C for 5 min.

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List of publications

This study was published in the following papers.

1. Examination of native chemical ligation using peptidyl prolyl thioesters
   Takahiro Nakamura, Akira Shigenaga, Kohei Sato, Yusuke Tsuda, Ken Sakamoto and Akira Otaka
   Chem. Commun. **2014**, *50*, 58–60.

2. Tailored Synthesis of 162-Residue *S*-Monoglycosylated GM2-Activator Protein (GM2AP) Analogues that Allows Facile Access to a Protein Library
   Takahiro Nakamura, Kohei Sato, Naoto Naruse, Keisuke Kitakaze, Tsubasa Inokuma, Takatsugu Hirokawa, Akira Shigenaga, Kohji Itoh and Akira Otaka
   ChemBioChem **2016**, *17*, 1986–1992.