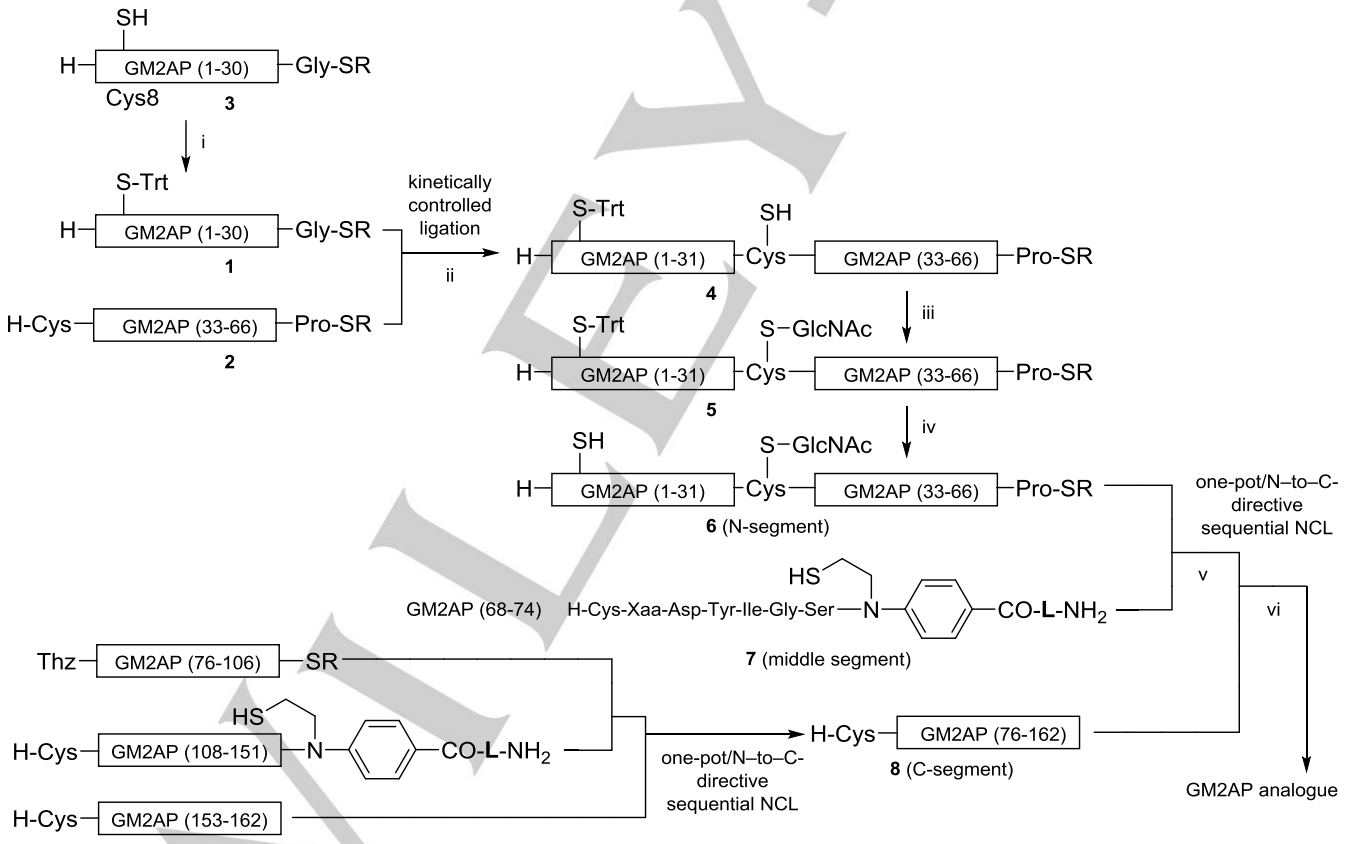
strategy required the synthesis of 43-residue fragments containing various replacements at the C-terminal portion corresponding to the 43-residue GM2AP (32–74), which proved to be laborious and time-consuming.[3] To address these issues, we planned to divide the previously utilized N-half segment into three fragments, including GM2APs (1–31), (32–67) and (68–74), and evaluated the condensation of the first two of these three fragments using NCL to afford an N-segment suitable for the second-generation synthesis. We envisioned that we could then use a one-pot/N–to–C-directed sequential NCL reaction involving three segments (GM2AP (1–67), (68–74) and (75–162)) to allow for the construction of the entire sequence of GM2AP (Scheme 1-ii). In this study, we decided to use N-terminal cysteinyl *N*-sulfanylethylanilide (SEAlide) peptide as the short middle segment for the one-pot/sequential ligation reaction. This decision was based on the idea that a short segment such as the SEAlide peptide could provide a robust and easy-to-use tailored synthetic platform for the construction of a GM2AP library, although NCL would be required to join the Pro67 thioester to Cys68. Although NCL reactions involving prolyl thioesters have been reported to be difficult to perform,[7] significant advances have recently been made in this area, allowing for the practical ligation of proline sites.[8,9] The success of an NCL reaction involving a prolyl thioester is highly dependent on an N-terminal side amino acid (Xaa) of the proline residue. The inclusion of a facile diketopiperazine-forming sequence results in the formation of two-residue (Xaa-Pro)-deleted NCL product.[8,10] In GM2AP, isoleucine is located at the N-terminal side of the proline residue. Given that the Ile-Pro sequence resists the formation of diketopiperazine and remains intact under NCL conditions,[8] we speculated that an NCL involving a proline thioester could be used to prepare GM2AP. Furthermore, it was expected that the considerable difference in the NCL reactivities of the prolyl and other amino acyl thioesters under conventional NCL conditions would allow the N-terminal cysteinyl prolyl thioester corresponding to GM2AP (32–67) to be used for the preparation of the N-segment, GM2AP (1–67). We planned to construct the monoglycosylated moiety of the N-segment by alkylating the thiol group of Cys32 in a manner identical to that used in the first strategy.[3a] Based on these considerations, we continued with our tailored strategy for the facile construction of a GM2AP library.



Primary sequence of GM2AP: SSFSWDNCDEGKDPAVIRSLTLEPDPIIVPGCVTLSVMGSTSVPLSSPLKVDLVLEKEVAGLWIKIPC***T***^{69}DYIGSCTFEHF CDVLDMLIPT GEPCPEPLRT YGLPCHCPFK EGTYSLPKSEFVVPDLELPSWLTTGNYRIESVLSSSGKRLGCIKIAASLKGI

Scheme 2. The tailored synthesis of the *S*-monoglycosylated GM2AP analogues. i) Trt-OH (3.3 eq.) in HFIP at r.t; ii) 6 M Gn·HCl-0.1 M phosphate buffer in the presence of 20 mM TCEP·HCl and 30 mM MPAA (pH 7.0) at 25 °C; iii) iodoacetyl-*N*-acetylglucosamine (5.0 eq.), 6 M Gn·HCl-0.1 M phosphate buffer (pH 7.4) at

37 °C; iv) TFA-TIPS-H_2O (95:2.5:2.5, (v/v) at 4 °C; v) 6 M Gn·HCl-0.1 M HEPPS buffer in the presence of 167 mM TCEP·HCl and 250 mM MPAA (pH 7.0) at 50 °C; vi) addition of **8** in 6 M Gn·HCl-0.4 M phosphate buffer in the presence of 40 mM TCEP·HCl and 60 mM MPAA (pH 7.0) at 37 °C, 3 days.

Results and Discussion

Synthesis of requisite peptide fragments

The requisite peptide fragments/segments used in our tailored strategy are summarized in Scheme 2, together with a schematic showing the ligation sequence. Fragments (Frs) **1** and **2**, corresponding to GM2APs (1–31) and (32–67), respectively, were prepared by *tert*-butyloxycarbonyl (Boc)-based solid-phase peptide synthesis (Boc-SPPS) using an *in situ* neutralization protocol[7a,11] on $HSCH_2CH_2CO$-Leu-methylbenzhydrylamine (MBHA) resin. For the preparation of Fr **1**, the completed resin was subjected to a two-step deprotection protocol consisting of sequential trimethylsilyl bromide (TMSBr[12]) and trimethylsilyltrifluoromethanesulfonate (TMSOTf[13]) treatments to afford the fully deprotected peptide **3**. The subsequent treatment of peptide **3** with triphenylmethyl alcohol (Trt-OH) under weakly acidic conditions (hexafluoro-2-propanol: HFIP) afforded Fr **1** bearing an *S*-Trt protecting group on Cys8.[14] Then, we envisioned that the NCL reaction of *S*-Trt protected Fr **1** with Fr **2** provided a monoglycosylation site (Cys32) for a regioselective *S*-alkylation reaction with iodoacetamide-*N*-acetylglucosamine.[15] The N-terminal cysteinyl prolyl thioester Fr **2** was obtained via an identical deprotection protocol to that used for Fr **1**. The NCL reaction of Fr **1** with Fr **2** was conducted in the presence of low concentration of two additives (30 mM 4-mercaptophenylacetic acid (MPAA[16]) and 20 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP[17])) at 25 °C in 6 M guanidine (Gn)·HCl-0.1 M phosphate buffer (pH 7.0) (Figure 1A and B). Pleasingly, this reaction exhibited good kinetic selectivity between the glycyl and prolyl alkyl thioesters, with the N-terminal cysteine of Fr **2** reacting selectively with the glycyl thioester of Fr **1** in an intermolecular manner. In contrast,

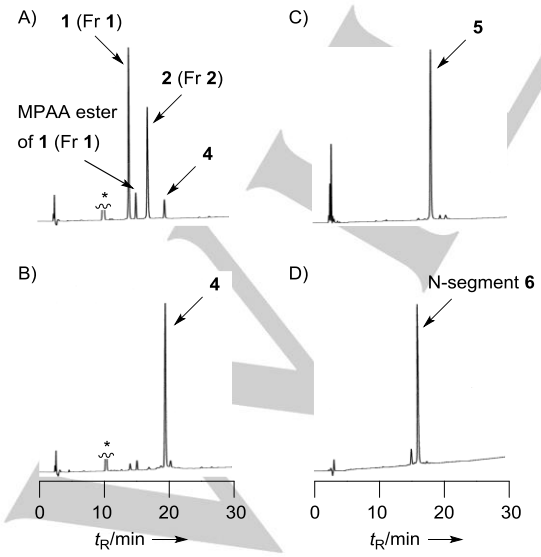


Figure 1. HPLC monitoring of reactions for the synthesis of the N-segment **6**. A) Ligation of **1** and **2** (t < 3 min). B) Ligation of **1** and **2** (t = 4 h). C) Alkylation of **4** (t = 2 h). D) Removal of the Trt protecting group (t = 0.5 h). *MPAA.

the prolyl thioester moiety of Fr **2** was left intact. This NCL reaction therefore provided efficient access to ligated peptide **4** bearing *S*-Trt protected and free cysteine residues at positions 8 and 32, respectively. The free cysteine residue was *S*-alkylated using iodoacetamide-*N*-acetylglucosamine to give the corresponding monoglycosylated peptide **5** (Figure 1C). Although naturally occurring GM2AP possesses an *N*-acetylglucosamine on its Asn residue at this position, the corresponding *S*-monoglycosylated molecule exhibits activity comparable to that of natural GM2AP.[3a] In fact, the substitution of this cysteine residue facilitated the preparation of the 67-residue monoglycosylated N-segment. Then, removal of the *S*-Trt protecting group with trifluoroacetic acid (TFA)-triisopropylsilane (TIPS)-H_2O afforded the N-segment **6**, which was required for the one-pot/N–to–C-directed sequential NCLs for the construction of the complete GM2AP sequence (Figure 1D). Computational analysis of the Hex A-GM2AP complex suggested that the replacement of Thr69 with Trp, His, Phe or Lys could lead to the formation of a stable complex.[6] Therefore, in addition to the native-type short middle segment **7a** (Xaa = Thr), we also prepared several similar derivatives bearing different amino acid replacements with Xaa (**7b–e**: Xaa = Trp, His, Phe and Lys, respectively) as the corresponding SEAlide peptides by Fmoc SPPS using an Fmoc-Ser(*t*-Bu)-*N*-sulfanylethylaniline-linker-incorporated resin.[18] The use of the N-terminal cysteinyl SEAlide peptide is critical to the success of the one-pot/sequential ligation process. Although the amide-type SEAlide peptide does not function as a thioester in the absence of phosphate salts, the addition of phosphate salts triggers the participation of the SEAlide peptide as a crypto-thioester in the NCL reaction.[4] The synthesis of the C-segment **8** consisting of 88 amino acid residues was achieved by the one-pot/N–to–C-directed sequential three fragment ligation using the SEAlide

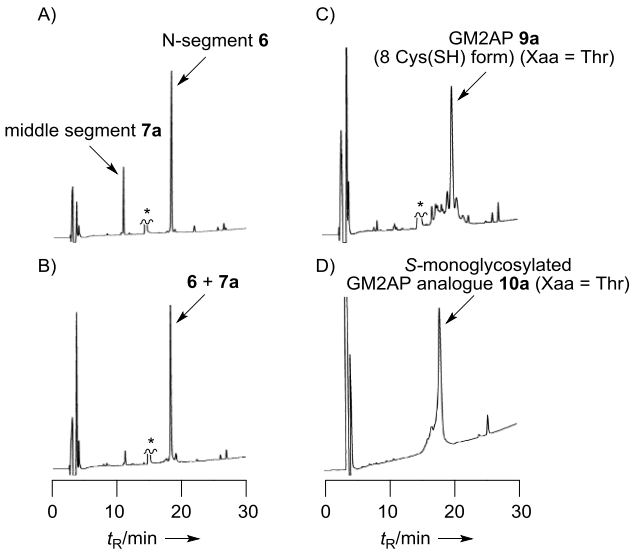


Figure 2. HPLC monitoring of reactions for the synthesis of the *S*-monoglycosylated GM2AP analogue **10a** (Xaa = Thr). A) First NCL (t < 3 min). B) First NCL (t = 24 h). C) Second NCL (t = 3 days). D) Folding reaction (t = 2 days).

chemistry, as previously reported for the first-generation synthesis.[3]

Sequential ligations

With the requisite peptide segments in hand, we performed the one-pot/N–to–C-directed sequential NCLs. The first NCL reaction between the N-segment **6** and the middle segment **7a** (Xaa = Thr), which was used as the SEAlide peptide, was conducted in 6 M Gn·HCl-0.1 M HEPPS buffer (pH 7.0) in the presence of high concentration of additives (250 mM MPAA and 167 mM TCEP·HCl) at elevated temperature (50 °C). These reaction conditions allowed the prolyl thioester moiety of **6** to be involved in the NCL.[8] When the reaction was conducted in the absence of phosphate salts, the SEAlide unit remained intact to efficiently form the ligated peptide within 24 h. Furthermore, we did not detect the formation of any of the cyclic peptide derived from the intramolecular NCL of the middle fragment during this reaction (Figure 2B). Upon completion of the first NCL, we added the C-segment **8** in 0.4 M phosphate buffer to the above reaction mixture to achieve the second NCL of the SEAlide unit with the N-terminal cysteine of **8**. The second NCL was conducted at 37 °C and proceeded to completion in 3 days to yield the fully ligated 162-residue protein molecule **9a** (Figure 2C). Several other middle segments (**7b**–**e**) were also incorporated into the protein molecule using an identical procedure (Figure 3A–D). After the isolation of the fully ligated protein molecules by HPLC, the resulting cysteine proteins were folded under literature conditions to give GM2AP analogues **10a**–**e** (Figure 2D and 3E–H).[3a] Preliminary biological evaluation of these GM2AP analogues indicated that they all assisted in the Hex A-catalyzed hydrolysis of GM2 to GM3 (Figure 3I).

Conclusions

Easy access to middle fragments possessing seven amino acid residues as the crypto-thioester and their use in a one-pot/N–to–C-directive sequential NCLs put the preparation of an *S*-monoglycosylated GM2AP protein library within easy reach. We have accomplished the tailored synthesis of GM2AP using two different kinetically-controlled ligation protocols. A kinetic reaction based on the differences in the NCL reactivities of prolyl and conventional amino acyl thioesters was applied to the preparation of the N-segment **6**. A one-pot procedure was used to synthesize the C-segment **8** and the entire GM2AP molecule based on the SEAlide peptide-mediated kinetically-controlled

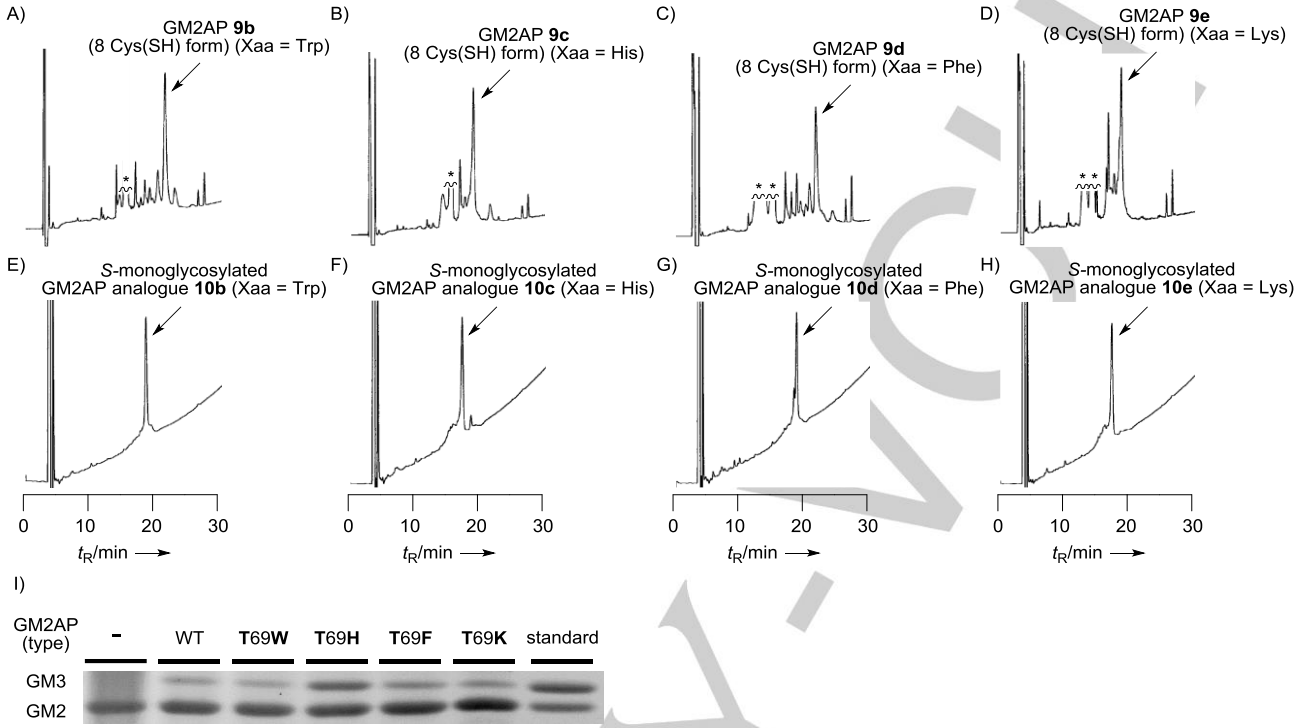


Figure 3. HPLC monitoring of reactions for the synthesis of the *S*-monoglycosylated GM2AP analogues **10b–e** (Xaa = Trp, His, Phe, Lys, respectively). A) Xaa = Trp; Second NCL (t = 3 days). B) Xaa = His; Second NCL (t = 3 days). C) Xaa = Phe; Second NCL (t = 3 days). D) Xaa = Lys; Second NCL (t = 3 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). (I) TLC monitoring of the degradation of GM2 to GM3 with Hex A in the presence of the synthesized GM2AP analogues. *MPAA.

ligation reaction. We believe that the results presented in this study will have a pronounced impact on the strategies used for the chemical synthesis of proteins. Although a detailed comparative evaluation of the activities of the GM2AP analogues prepared in this study has not yet been conducted, we are currently evaluating these analogues in our laboratory and will report our results in due course.

Experimental Section

General Methods

Exact mass spectra were recorded on a Water MICROMASS® LCT PREMIER™ (ESI-TOF). 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 MeCN (v/v, solvent B) was used for HPLC elution.

Preparation of *S*-Trt Protected Peptide Thioester 1 (Fr 1)

Unprotected peptide thioester **3** was prepared by Boc SPPS using *in situ* neutralization protocol on $HSCH_2CH_2CO$-Leu-methyl benzhydrylamine (MBHA) resin (0.70 mmol amine/g, 1.0 g, 0.70 mmol). The resulting completed resin (300 mg) was treated with 1 M trimethylsilyl bromide (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 trimethylsilyltrifluoromethanesulfonate (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 triphenylmethyl alcohol (Trt-OH) (3.3 eq.) in hexafluoro-2-propanol (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 **1** (Fr **1**). The crude peptide was purified by preparative HPLC to give the purified *S*-Trt protected peptide thioester **1** (Fr **1**) (0.98 mg, 0.24 μmol).

S-Trt protected peptide thioester **1** (Fr **1**): retention time = 21.2 min (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 = 27.7 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 for $C_{176}H_{259}N_{39}O_{51}S_2$ (average isotopes) 3801.3. found 3801.0

Preparation of Peptide Thioester 2 (Fr 2)

Peptide thioester **2** (Fr **2**) was prepared by the procedure similar to that described for Fr **1**. The two-step deprotection protocols and subsequent HPLC purification afforded the desired peptide thioester **2** (Fr **2**) (1.24 mg, 0.28 μmol).

Peptide thioester **2** (Fr **2**): retention time = 23.3 min (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 = 28.2 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 for $C_{183}H_{310}N_{42}O_{51}S_3$ (average isotopes) 4010.9, found 4010.4.

Preparation of SEAlide Peptide 7a–e (Middle Segment)

On NovaSyn® TGR resin (Rink amide type: 0.25 mmol amine/g, 0.90 g, 0.23 mmol) was coupled an Fmoc-Ser-incorporated *N*-sulfanylethylaniline linker (362 mg, 0.45 mmol) with the aid of *O*-(7-azabenzotriazol-l-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HATU) (163 mg, 0.43 mmol) and *N*,*N*-diisopropylethylamine (DIPEA) (149 μL, 0.86 mmol) to yield the SEAlide-linked resin. On the 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) for 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 **7** (middle segment). The crude SEAlide peptide was purified by preparative HPLC to give the purified SEAlide peptide **7** (middle segment).

SEAlide peptide **7a** (Xaa = Thr): retention time = 15.4 min (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 = 19.2 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 for $C_{46}H_{68}N_{10}O_{14}S_2$ ($[M + 2H]^{2+}$) 525.2, found 525.3.

SEAlide peptide **7b** (Xaa = Trp): retention time = 17.0 min (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 = 26.7 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 for $C_{53}H_{71}N_{11}O_{13}S_2$ ($[M + 2H]^{2+}$) 567.7, found 567.7.

SEAlide peptide **7c** (Xaa = His): retention time = 13.3 min (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 = 15.5 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 for $C_{48}H_{68}N_{12}O_{13}S_2$ ($[M + 2H]^{2+}$) 543.2, found 543.3.

SEAlide peptide **7d** (Xaa = Phe): retention time = 15.4 min (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 = 27.2 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 for $C_{51}H_{70}N_{10}O_{13}S_2$ ($[M + 2H]^{2+}$) 548.2, found 548.3.

SEAlide peptide **7e** (Xaa = Lys): retention time = 13.2 min (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 = 22.7 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 for $C_{48}H_{73}N_{11}O_{13}S_2$ ($[M + 2H]^{2+}$) 538.8, found 538.8.

Synthesis of Peptide Thioester 4

S-Trt protected peptide thioester **1** (Fr **1**) (4.6 mg, 1.2 μmol) and peptide thioester **2** (Fr **2**) (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 **4** (6.2 mg, 0.75 μmol, 64%).

Peptide thioester **4**: retention time = 19.7 min (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 = 23.7 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 for $C_{350}H_{551}N_{79}O_{100}S_4$ (average isotopes) 7593.8, found 7593.5.

Alkylation for the Synthesis of *S*-Glycosylated Peptide Thioester 5

To a solution of peptide thioester **4** (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 iodoacetyl-N-acetylglucosamine (1.5 mg, 5 eq.) at 37 °C and the resulting mixture was incubated at same temperature for 2 h. Purification of the resulting reaction mixture on semi-preparative HPLC yielded *S*-glycosylated peptide thioester **5** (3.3 mg, 0.38 μmol, 51%).

S-Glycosylated peptide thioester **5**: retention time = 18.1 min (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 = 21.0 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 for $C_{360}H_{567}N_{81}O_{106}S_4$ (average isotopes) 7854.1, found 7854.0.

Removal of Trt for the Synthesis of N-Segment 6

The *S*-Trt protected peptide thioester **5** (3.3 mg, 0.38 μmol) was treated with TFA-triisopropylsilane (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 **6**. The crude peptide was purified by semi-preparative HPLC to give purified N-segment **6** (1.4 mg, 0.16 μmol, 43%).

N-Segment **6**: retention time = 16.1 min (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 = 20.4 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 for $C_{341}H_{553}N_{81}O_{106}S_4$ (average isotopes) 7611.8, found 7611.3.

One-pot/N–to–C-Directed Sequential NCL Using N-Segment 6, Middle Segment 7 and C-Segment 8

Kinetically controlled ligation of N-segment **6** (0.74 mg, 0.09 μmol) and middle segment **7a** (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 **8** 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 was completed within 3 days, and then the crude material was purified by semi-preparative HPLC to give the desired reduced GM2AP analogue **9a** (Xaa = Thr) (0.33 mg, 0.017 μmol, 19%). One-pot/N–to–C-directed sequential NCL using other middle segments (**7b–e**) was also performed by the identical procedure to give desired reduced GM2AP analogue **9b–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 material in reaction using middle segment **7d**,**e** (Xaa = Phe, Lys) was performed by analytical HPLC.

Reduced GM2AP analogue **9a** (Xaa = Thr): retention time = 19.6 min (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 = 26.3 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 for $C_{800}H_{1262}N_{194}O_{243}S_{11}$ (average isotopes) 17838.4, found 17839.6.

Reduced GM2AP analogue **9b** (Xaa = Trp): retention time = 21.4 min (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 = 27.4 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 for $C_{807}H_{1265}N_{195}O_{242}S_{11}$ (average isotopes) 17923.6, found 17923.5.

Reduced GM2AP analogue **9c** (Xaa = His): retention time = 18.5 min (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 = 23.4 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 for $C_{802}H_{1262}N_{196}O_{242}S_{11}$ (average isotopes) 17874.5, found 17873.9.

Reduced GM2AP analogue **9d** (Xaa = Phe): retention time = 24.4 min (analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 20% to 70% over 30 min); MS (ESI-TOF) calcd for $C_{805}H_{1264}N_{194}O_{242}S_{11}$ (average isotopes) 17884.5, found 17884.1.

Reduced GM2AP analogue **9e** (Xaa = Lys): retention time = 20.3 min (analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 20% to 70% over 30 min); MS (ESI-TOF) calcd for $C_{802}H_{1267}N_{195}O_{242}S_{11}$ (average isotopes) 17865.5, found 17865.8.

Folding for Preparation of *S*-Monoglycosylated GM2AP Analogue 10

Folding for preparation of *S*-monoglycosylated GM2AP analogue **10** was performed with a modified method of previously reported one.[3a] The reduced GM2AP analogue **9a** (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 analogue **10a** (Xaa = Thr). Concentration of the *S*-monoglycosylated GM2AP analogue **10a** (Xaa = Thr) was determined as 0.60 mg/mL (0.05 mL, 11%) by measurement of absorbance at 280 nm and calculation using $A_{280} = \varepsilon_{280}\, c\, l$. 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[19]), the c (M) is concentration of a protein, and the l (cm) is the length of the optical path. Folding of other GM2AP molecules **9b–e** with substitution at the position was also performed by the identical procedure to give desired *S*-monoglycosylated GM2AP analogue **10b–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 analogue **10a** (Xaa = Thr): retention time = 18.2 min (analytical HPLC conditions: Cosmosil Protein-R analytical

column with a linear gradient of solvent B in solvent A, 30% to 70% over 30 min); MS (ESI-TOF) calcd for $C_{800}H_{1254}N_{194}O_{243}S_{11}$ (average isotopes) 17830.4, found 17830.6.

S-Monoglycosylated GM2AP analogue **10b** (Xaa = Trp): retention time = 18.1 min (analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 70% over 30 min); MS (ESI-TOF) calcd for $C_{807}H_{1257}N_{195}O_{242}S_{11}$ (average isotopes) 17915.5, found 17916.0.

S-Monoglycosylated GM2AP analogue **10c** (Xaa = His): retention time = 17.0 min (analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 70% over 30 min); MS (ESI-TOF) calcd for $C_{802}H_{1254}N_{196}O_{242}S_{11}$ (average isotopes) 17866.4, found 17866.7.

S-Monoglycosylated GM2AP analogue **10d** (Xaa = Phe): retention time = 18.6 min (analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 70% over 30 min); MS (ESI-TOF) calcd for $C_{805}H_{1256}N_{194}O_{242}S_{11}$ (average isotopes) 17876.5, found 17876.1.

S-Monoglycosylated GM2AP analogue **10e** (Xaa = Lys): retention time = 17.0 min (analytical HPLC conditions: Cosmosil Protein-R analytical column with a linear gradient of solvent B in solvent A, 30% to 70% over 30 min); MS (ESI-TOF) calcd for $C_{802}H_{1259}N_{195}O_{242}S_{11}$ (average isotopes) 17857.5, found 17858.5.

In Vitro GM2-degradation Assay of Synthesized GM2AP Analogue 10

In vitro GM2-degradation assay was performed as described previously.[20] Briefly, the GM2 ganglioside was incubated with recombinant human Hex A (2000 nmol h^{-1} 4-methylumbellifery-6-sulfo-β-D-glucosaminide potassium salt (4-MUGS)-degrading activity) in the presence or absence of 5 or 6.7 μg of synthesized GM2APs **10a–e** (Xaa = Thr, Trp, His, Phe, Lys, respectively) in 10 mM sodium citrate buffer (pH 4.5) containing 0.01% bovine serum albumin (BSA), 6% MeCN and 0.011% TFA 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 glycosphingolipids (GSLs) were isolated using a C18 Sep-Pak Cartrige. 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 GSLs, the thin-layer chromatography plate was sprayed with orcinol reagent and heated at 120 °C for 5 min.

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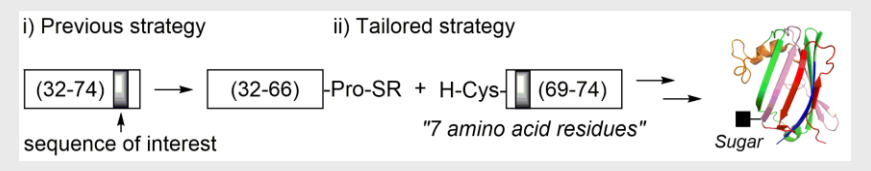

Tailored Synthesis: A synthetic protocol has been developed for the preparation of 162-residue *S*-monoglycosylated GM2-activator protein (GM2AP) analogues bearing various single amino acid substitutions for Thr69. The facile incorporation of these replacement into the whole protein was achieved by a one-pot/N–to–C-directed sequential ligation strategy using readily accessible short middle N-sulfanylethylanilide (SEAlide) peptide consisting of seven amino acid residues.

*Takahiro Nakamura, Kohei Sato, Naoto Naruse, Keisuke Kitakaze, Tsubasa Inokuma, Takatsugu Hirokawa, Akira Shigenaga, Kohji Itoh, Akira Otaka**

Tailored Synthesis of 162-Residue *S*-Monoglycosylated GM2-Activator Protein (GM2AP) Analogues that Allows Facile Access to Protein Library