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


Abstract: A synthetic protocol has been developed for the preparation of 162-residue S-monoglycosylated GM2-activator protein (GM2AP) analogues bearing various amino acid substitutions for Thr69. The facile incorporation of the replacements into the protein was achieved by a one-pot/N-to-C-directed sequential ligation strategy using readily accessible middle N-sulfanylethylanilide (SEAlide) peptide s consisting of seven amino acid residues. A kinetically-controlled ligation protocol was successfully applied to the assembly of three peptide segments covering the GM2AP. The native chemical ligation (NCL) reactivities of the SEAlide can be tuned by the presence or absence of phosphate salts. Furthermore, the NCL of the alkyl thioester fragment (GM2AP (1–31)) with the N-terminal cysteinyl prolyl thioester (GM2AP (32–67)) proceeded smoothly to yield the 67-residue prolyl thioester, with the prolyl thioester moiety remaining intact. This newly developed strategy enabled the facile synthesis of GM2AP analogues. Thus, we referred this synthetic protocol as "Tailored Synthesis" for the construction of a GM2AP library.

Introduction

The chemical synthesis of proteins has emerged as a fundamental research tool for elucidating the many different functions of proteins.[1] In this regard, native chemical ligation (NCL) represents an efficient strategy for the chemoselective condensation of peptide thioesters with N-terminal cysteinyl peptides to generate complex protein structures.[2] We recently achieved the NCL-mediated chemical syntheses of 162-residue S-monoglycosylated GM2-activator protein (GM2AP) using the N-sulfanylethylanilide (SEAlide) peptide as a crypto-thioester.[3,4] In these particular cases, the 162-residue sequence was divided into five peptide fragments, with the convergent assembly of the N- and C-half segments affording the desired protein molecule (Scheme 1-i). The requisite N-half segment (GM2AP (1–74)) was prepared by the coupling reaction of two N-terminal side fragments (GM2APs (1–31) and (32–74)). Notably, the synthetic GM2AP material assisted in the β-hexosaminidase A (Hex A)-catalyzed lysosomal hydrolysis of ganglioside GM2 to GM3, where the hydrolytic activity of Hex A is known to be dependent on the formation of a Hex A-GM2AP complex.[5] Computational analysis of this complex predicted that residues Cys68 to Ser74 would form a loop region, and that Thr69, in particular, could be critically involved in the formation of the stable complex.[6]

Scheme 1. Comparison between the previous and tailored synthetic strategy for preparation of S-monoglycosylated GM2-activator protein (GM2AP) analogues.

With this in mind, we hypothesized that the replacement of the Thr69 with some other suitable residues could result in the generation of GM2AP analogues with greater activity than the parent protein. However, our initial synthetic strategy for accessing the GM2AP was found to be unsuitable for the incorporation of different amino acids at this position. Notably, this

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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. 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.[8,9] 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.[9] Based on these considerations, we continued with our tailored strategy for the facile construction of a GM2AP library.

Primary sequence of GM2AP: SSF3WNCDEGKDRDPAVIRSLTLEPD0IPV0PQVLSVMGSTSVPSLPLVDELVELKEVAGLWIKPCRTYIQGCTFHERFCDVLMLIPTGEPCPPFRGKYGIFLPKSEPVDLELPWLTGNYRIGSVLSSSGKRLGC1K1A1RLG1

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-acetylgalactosamine (5.0 eq.), 6 M Gn-HCl-0.1 M phosphate buffer (pH 7.4) at
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 (Fr)s 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 HSCH2CH2CO-Leu-methylbenzhydrylamino (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-mercaptothiophenolacetic 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 glycol and prolyl alkyl thioesters, with the N-terminal cysteine of Fr 2 reacting selectively with the glycol thioester of Fr 1 in an intermolecular manner. In contrast, 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.[34] 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)-H2O 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.[35] 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 SEAilde peptides by Fmoc SPPS using an Fmoc-Ser(t-Bu)-N-sulfonylbenzylamine-linker-incorporated resin.[36] The use of the N-terminal cysteinyl SEAilde peptide is critical to the success of the one-pot/sequential ligation process. Although the amide-type SEAilde peptide does not function as a thioester in the absence of phosphate salts, the addition of phosphate salts triggers the participation of the SEAilde 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 SEAilde.
Sequential ligation

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). 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-monomolyslated 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 Waters MICROMASS® LCT PREMIER™ (ESI-TOF). For HPLC separations, a Cosmosil SCi™-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 SCi™-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 SCi™-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 HSCl2CH2CO-Leu-methyl benzhydrolamine (MBHA) resin (0.70 mmol amine/g, 1.0 g, 0.70 mmol). The resulting purified 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/v) at 4 °C for 2 h, and then the resin was filtered off. The filtrated resin was treated with 1 M trimethylsilylfluoromethanesulfonylamine (TMSOTf)-thioanisole in TFA (50 μL/1 mg resin)/m-cresol/EDT (100:5:5, v/v/v) at 4 °C for 2 h, and then the resin was filtered off. To the filtrate was added cooled EtO to give precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with EtO to afford crude 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 SCi™-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 SCi™-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 C17H18NS2O4S2 (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 protection protocols and subsequent HPLC purification afforded the desired peptide thioester 2 (Fr 2) (1.24 mg, 0.25 μmol).

Peptide thioester 2 (Fr 2): retention time = 23.3 min (analytical HPLC conditions: Cosmosil SCi™-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 SCi™-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 C16H19N4O2S3 (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(tert-butyloxycarbonyl)-L-lysine (HATU) (163 mg, 0.43 mmol) and L-lysine (0.98 mg, 0.24 mmol) to give a precipitate. A single peptide was purified by preparative HPLC to give the purified SEAlide peptide 7 (middle segment). The crude SEAlide peptide was purified by preparative HPLC to give the purified SEAlide peptide 7 (middle segment). The crude SEAlide peptide was purified by preparative HPLC to give the purified SEAlide peptide 7 (middle segment). The purified SEAlide peptide was employed for the construction of the protected peptide resin. The resulting purified SEAlide peptide 7 (middle segment) was treated with 1 M trimethylsilyltrifluoromethanesulfonate (TMSOTf) in situ (0.25 mmol) at room temperature. The resulting reaction mixture was treated with cooled EtO to give a precipitate. The precipitate was collected by centrifugation and thoroughly washed with EtO 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).

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 Gln-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 5C18-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 5C18-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 C90H155N92O70S4 (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 Gln-HCl (pH 7.4, 1.5 mL) was added iodoacetetyl-N-acetylgalactosamine (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 5C18-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 5C18-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 C90H158N92O70SiS (average isotopes) 7894.1, found 7894.0.

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-trisopropylsilane (TIPS)-H2O [95:2.5:2.5 (v/v/v)] at 4 °C. After 0.5 h, the reaction mixture was added cooled Et2O to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et2O 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 5C18-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 5C18-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 C90H158N92O70S (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 Gln-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 Gln-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 (0.02 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 5C18-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 C90H158N92O70S (average isotopes) 17838.4, found 17839.6.

Reduced GM2AP analogue 9b (Xaa = Trp): retention time = 21.4 min (analytical HPLC conditions: Cosmosil 5C18-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, 40% to 52% over 30 min); MS (ESI-TOF) calcd for C90H158N92O70S (average isotopes) 17923.6, found 17923.5.

Reduced GM2AP analogue 9c (Xaa = His): retention time = 18.5 min (analytical HPLC conditions: Cosmosil 5C18-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 C90H158N92O70S (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 C90H158N92O70S (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 C90H158N92O70S (average isotopes) 17865.5, found 17865.8.

Folding for Preparation of S-Monoglycosylated GM2AP Analog 10

Folding for preparation of S-monoglycosylated GM2AP analogue 10 was performed with a modified method of previously reported one.[9a] The reduced GM2AP analogue 9a (Xaa = Thr, 0.30 mg) was dissolved in 6 M Gln-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 A280 = ε280 C L. The A280 is the observed absorbance at 280 nm (A280 = 0.773), the ε280 (M−1 cm−1) is the molar extinction coefficient of GM2AP at 280 nm (ε280 = 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$_{60}$H$_{129}$N$_{17}$O$_{52}$S$_{7}$ (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$_{60}$H$_{129}$N$_{17}$O$_{52}$S$_{7}$ (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$_{60}$H$_{129}$N$_{17}$O$_{52}$S$_{7}$ (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$_{60}$H$_{129}$N$_{17}$O$_{52}$S$_{7}$ (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$_{60}$H$_{129}$N$_{17}$O$_{52}$S$_{7}$ (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.[6,10] Briefly, the GM2 ganglioside was incubated with recombinant human Hex A (2000 nmol h$^{-1}$ 4-methylumbelliferyl-$b$-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, Lys, respectively) in 10 mM sodium citrate buffer (pH 4.5) containing 0.01% bovine serum albumin (BSA), 0.01% 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 Cartridge. Aliquots of samples were spotted on a silica gel plate and developed with CHCl$_3$/MeOH/H$_2$O/0.2% (v/v) CaCl$_2$ (cq. 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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Keywords: native chemical ligation • prolyl thioester • N- sulfanylmethylamide • kinetically-controlled ligation • GM2AP

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 (SEAide) peptide consisting of seven amino acid residues.