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# Efficient and systematic synthesis of a small glycoconjugate library having human complex type oligosaccharides

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ABSTRACT

To prepare a small library of homogeneous glycoconjugates with varying oligosaccharide structures, a combinatorial strategy was employed. The target glycopeptide was divided into two peptide segments (A and B) and both were prepared by solid phase peptide synthesis. These peptides, which can be coupled by native chemical ligation through an amide bond, were subsequently coupled to two kinds of human complex type oligosaccharides. This process systematically afforded the desired glycoconjugate library.

## 1. Introduction

Oligosaccharides on protein surfaces exhibit heterogeneous structures. The large number of glycosylation positions results in a remarkably heterogeneous pattern of post-translational modification. This has made it difficult to elucidate oligosaccharide function and decode the specific roles of the post-translational modifications formed by oligosaccharides. If glycoconjugates have two glycosylation sites in which the positions exhibit two different kinds of oligosaccharides (e.g., patterns such as acidic or neutral types) there are eight different kinds of glycoconjugate forms. Increasing the number of oligosaccharide patterns at each glycosylation site can synergistically bring about an increase in the number of glycoconjugates.

To investigate the function of the oligosaccharides of each glycoconjugate, individual homogeneous glycoconjugates of varying oligosaccharide structure need to be synthesized. For this purpose, we have employed a combinatorial strategy by means of the combined use of Native Chemical Ligation (NCL)[1,2] and efficient glycoconjugate formation. NCL is a potent peptide segment coupling method achieved through a native amide bond. The mechanism of NCL relies on the thiol exchange reaction between a peptide having an α-thioester at the C-terminal and another peptide having a cysteine residue at the N-terminal, and subsequent intramolecular acyl transfer to generate a native amide bond.[1] This simple combinatorial strategy using two types of oligosaccharides and two peptide segments of a target peptide can afford eight types of glycoconjugates with varying oligosaccharide structure and glycosylation positions. In terms of the incorporation of oligosaccharide into the peptide, we employed the haloacetamide method, which is a convenient coupling reaction between the thiol of the cysteine and the haloacetamide group at the reducing end of the oligosaccharide.[3]

We have examined the scope of this systematic synthesis of a small glycoconjugate library. The target glycoconjugate is a glycopeptide fragment in the AILIM/ICOS (Activation inducible lymphocyte immunomediately molecule/Inducible co-stimulator) on T-cells.[4] AILIM/ICOS has been identified as a member of the CD28/CTLA4 family, and activates T-cell by a pathway differing from the CD28 pathway. However, the detailed function has not been elucidated. AILIM/ICOS has an extracellular domain (21Glu–141Phe) including the signal-accepting domain (114Phe–119Phe) for the costimulatory signal from antigen presenting cells (APC) through a binding event between AILIM/ICOS on T-cells and B7h on APC. The AILIM/ICOS extracellular domain has two N-linked oligosaccharides close to the signal-accepting domain at the 89Asn and 110Asn positions. In the course of our ongoing research, the oligosaccharide at the 89Asn position seems to be an essential molecule for AILIM/ICOS activity. However, it is not clear what kind of glycoconjugate pattern is required for AILIM/ICOS activity, for example, acidic/neutral, or branched structures.

To investigate the relationship between AILIM/ICOS activity and oligosaccharide structure, we examined the synthesis of glycoconjugates (83Cys–135Leu) with varying types of oligosaccharide structures at the 89 and 110 positions. In addition, because AILIM/ICOS not possessing an oligosaccharide at position 89 appears to exhibit poor binding toward the B7h receptor on APC

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(Supplementary data), we selected the six candidates numbered **5**–**10** from eight glycoconjugates, which can be synthesized by this strategy. We have already demonstrated incorporation of N-linked disialyloligosaccharides into the peptide by a haloacetamide method.[3c] Therefore, the haloacetamide method was used for the synthesis of the AILIM/ICOS extracellular domain (83Cys–135Leu).

The AILIM/ICOS extracellular domain was mutated form Asn at the 89 and 110 positions to Cys for the introduction of oligosaccharides, and the other two Cys residues, at the 83 and 109 positions, were therefore protected by the acetamidomethyl (Acm) group during solid phase peptide synthesis (Fig. 1, **3** and **4**). To use NCL, the target glycoconjugate was divided into two segments (83Cys–109Cys **3** and 110Cys–135Leu **4**). In terms of the oligosaccharides, we selected the acidic and neutral forms, complex type disialyloligosaccharide **1** and its asialoform **2**, respectively. This choice was made because some protein–oligosaccharide binding events appear to be regulated by the topology of acidic functional groups in the oligosaccharide.[5] The acidic nature of oligosaccharides may result in dramatic change in the binding event. In this paper, we demonstrate the synthesis of small glycoconjugate library with varying oligosaccharide structures and glycosylation positions.

## 2. Results and discussion

### 2.1. Synthesis of bromoacetamidyl oligosaccharides

In our previous report, the preparation of bromoacetamidyl disialyloligosaccharide **1** employed an enzymatic reaction with PNGase-F to remove the asparagine residues from the oligosaccharide. Then, a bromoacetamide group was incorporated at the anomeric position.[3c] In the current experiment, we examined the synthesis of bromoacetamidyl oligosaccharides by means of a modified strategy (Scheme 1).

We used hydrazine monohydrate instead of PNGase-F on the Fmoc-asparaginyl disialyloligosaccharide **11**.[6] Fmoc-asparaginyl disialyloligosaccharide **11** was dissolved in hydrazine monohydrate and hydrazinolysis[7] was performed at 100 °C for 10 h. After the removal of hydrazine, N-acetylation using saturated $NaHCO_3$ solution and acetic anhydride afforded disialyloligosaccharyl hydrazide **12** in 51% yield. Subsequent hydrolysis of disialyloligosaccharyl hydrazide **12** afforded one of disialooligosaccharide **13**[3c] and asialooligosaccharide **14**,[8] selectively. The sialoside bond at the non-reducing end is more stable than the hydrazide group at the reducing end under acidic conditions. Thus, selective hydrolysis of the hydrazide group by the use of 10 M acetic acid solution afforded disialyloligosaccharide **13** in 92% yield. On the other hand, hydrolysis of both the hydrazide group and sialoside bond by the use of 40 mM HCl solution afforded asialooligosaccharide **14** in 89% yield. Kochetkov amination and haloacetamidation of oligosaccharides **13** and **14** were performed to obtain **1** and **2** by use of our previously reported conditions. As shown in Figure 2, this modified protocol afforded homogeneous bromoacetamidyl oligosaccharides **1** and **2**.[3c]

### 2.2. Incorporation of oligosaccharide into the peptide–thioester by the haloacetamide method and synthesis of the AILIM/ICOS glycopeptide fragments

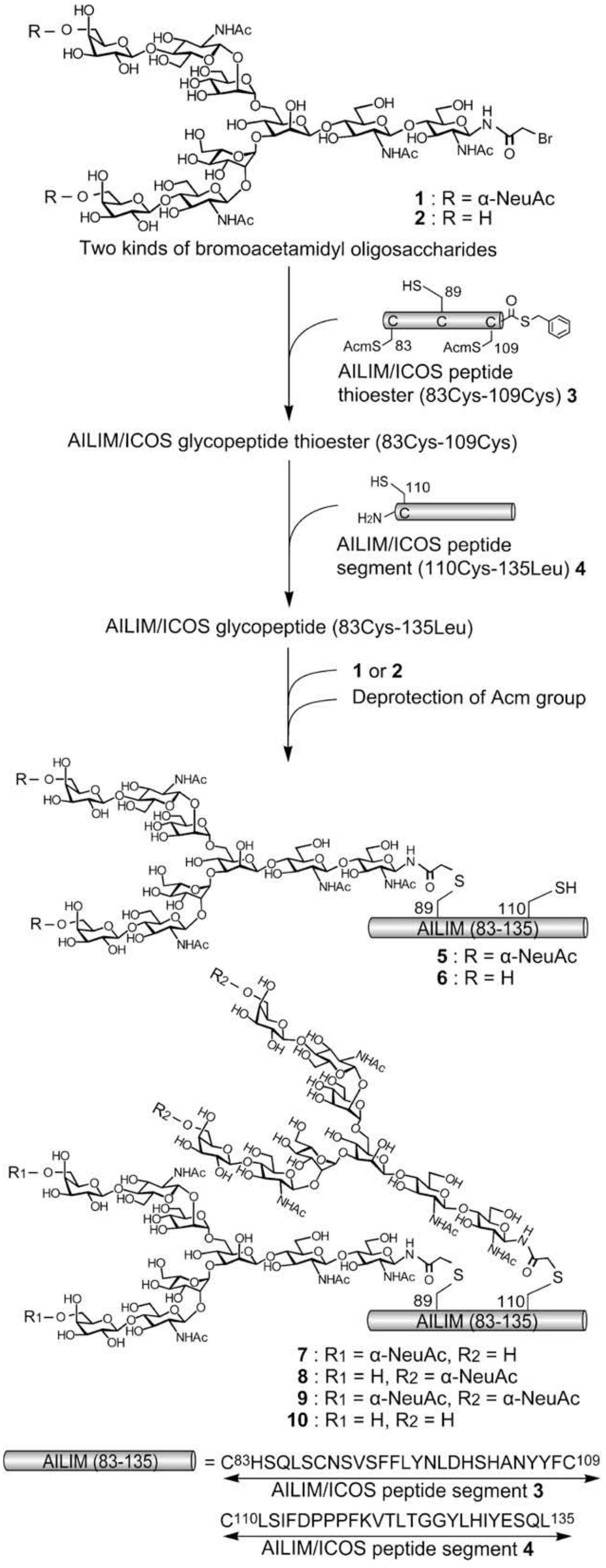
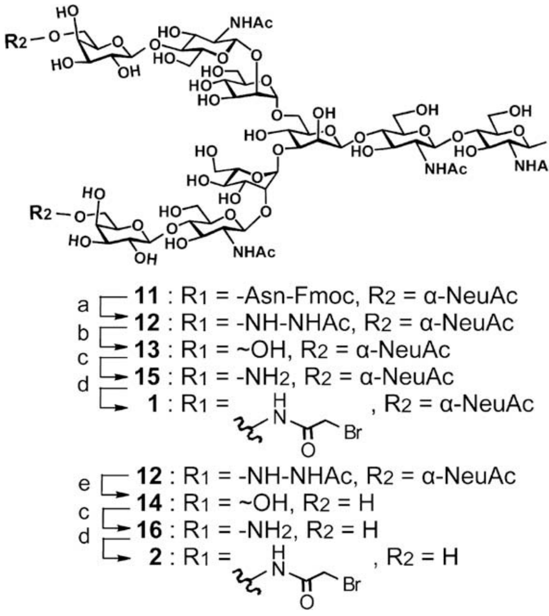


**Figure 1.** Synthetic strategy to AILIM/ICOS glycopeptide fragments.

In terms of the peptide-α-thioester **3** (83Cys–109Cys), the corresponding heptaicosa-peptide was synthesized by Fmoc SPPS using trityl resin with coupling of the amino acids employing 1-hydroxybenzotriazole (HOBt) and *N,N′*-diisopropylcarbodiimide (DIC) conditions (Scheme 2). After construction of the desired peptide sequence, the protected peptide was released from the resin by treatment with a solvent containing acetic acid, dichloromethane (DCM), and methanol. Thioesterification of the C-terminal carboxylic acid of this peptide was performed by use of benzyl thiol, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBop), and *N,N′*-diisopropylethylamine (DIPEA) followed by deprotection with 95% TFA solution containing TIPS to



**Scheme 1.** Synthesis of bromoacetamidyl oligosaccharides **1** and **2**. Reagents and conditions: (a) (i) hydrazine monohydrate, 100 °C; (ii) satd $NaHCO_3$, $Ac_2O$; (b) 10 M AcOH; (c) satd $NH_4HCO_3$, 37 °C; (d) bromoacetic acid, DIC, DMF, $NaHCO_3$, $H_2O$; (e) 40 mM HCl, 50 °C.

afford peptide-α-thioester **3**[9] (Scheme 2). Peptide **4**, having a cysteine at the N-terminus, was also prepared by Fmoc-SPPS.

For the preparation of peptide-α-thioester **3** incorporation of oligosaccharide was examined (Scheme 3). A coupling reaction of bromoacetamidyl asialooligosaccharide **2** with the thiol group at the 89Cys position was performed by our previously reported method in a solution of DMF–25 mM phosphate buffer (pH 7.5).[3c] This reaction afforded asialoglycopeptide-α-thioester **17** within 2 h in 50% yield (isolated yield), and the thioester group of this peptide was found to be stable during this conversion reaction as shown in Figure 3A–C. HPLC profiles indicate the smooth conversion of peptide-α-thioester **3** to the glycopeptide-α-thioester **17**.

In the case of the condensation reaction of disialyl type oligosaccharide **1** and peptide-α-thioester **3** (Scheme 3), the reaction was performed in a solution (200 mM phosphate buffer containing 6 M guanidine hydrochloride (pH 7.5, DMF = 1:1), because the disialyloligosaccharide was less soluble than asialooligosaccharide **2**. These conditions afforded the desired product, disialylglycopeptide-α-thioester **18**, in 70% yield. Using glycopeptide-α-thioesters **17** or **18** and peptide segment **4** (110Cys–135Leu) prepared by Fmoc SPPS (Scheme 2 and Supplementary data), the synthesis of the target glycoconjugates having a complex type oligosaccharide (**1** and **2**) was examined. The first target was a glycoconjugate having one of the oligosaccharides (**1** or **2**) at the 89Cys position. Asialoglycopeptide-α-thioester **17** and peptide segment **4** were coupled by NCL using 4-mercaptophenylacetic acid (MPAA),[10] and these conditions afforded asialoglycopeptide **19** in 45% yield (Scheme 3). Similarly, coupling of disialylglycopeptide-α-thioester **18** and peptide segment **4** by the same method afforded disialylglycopeptide **20** in 50% yield (Scheme 3). Because glycopeptides **19** and **20** have Acm groups at the 83Cys and 109Cys positions, deprotection of these by the use of AgOAc[11] was examined to afford target glycopeptides **5** and **6** having one of the oligosaccharides **1** and **2** (Scheme 3).

The synthesis of the AILIM/ICOS extracellular domain (83Cys–135Leu) having two different kinds of oligosaccharide at the 83Cys and 110Cys positions was then examined (Scheme 4). To prepare glycopeptides **19** and **20**, a second incorporation of oligosaccharide into the free thiol group at the 110Cys position, which was originally the N-terminal of peptide **4**, was required. Incorporation of disialyloligosacccharide **1** into 110Cys of glycopeptide **19** in phosphate buffer containing 6 M guanidine hydrochloride (pH 7.5) afforded glycopeptide **21** having an asialooligosaccharide and disialyloligosaccharide at the 89Cys and 110Cys positions, respectively (Fig. 3D–F). Deprotection of the Acm groups was performed by use of AgOAc[11] to afford **8**. Similarly, glycopeptides **22** and **7**, having a disialyloligosaccharide at the 89Cys position and an asialooligosaccharide at the 110Cys position, could be prepared by incorporation of asialooligosacccharide **2** to 110Cys of glycopeptide **20** followed by deprotection of the Acm groups.

For the synthesis of the AILIM extracellular domain (83Cys–135Leu) having the same two oligosaccharides at the 89Cys and 110Cys positions (Scheme 5), simultaneous incorporation of asialooligosaccharide **2** or disialyloligosaccharide **1** was performed on peptide **23**, prepared by NCL[10] between peptide-α-thioester **3** and peptide segment **4**, to afford glycopeptides **24** and **25**. Final deprotection of the Acm groups[11] afforded glycoconjugates **9** (40% yield) and **10** (67% yield).

Thus, we have succeeded in the synthesis of a small glycoconjugate library based on six varieties of the AILIM/ICOS extracellular domain (83Cys–135Leu) with varying oligosaccharide structure and glycosylation positions.

## 3. Conclusion

Repetitive use of the NCL method at the peptide N-terminus enables elongation of the polypeptide chain and the incorporation of additional oligosaccharides to the cysteine. In addition, we have

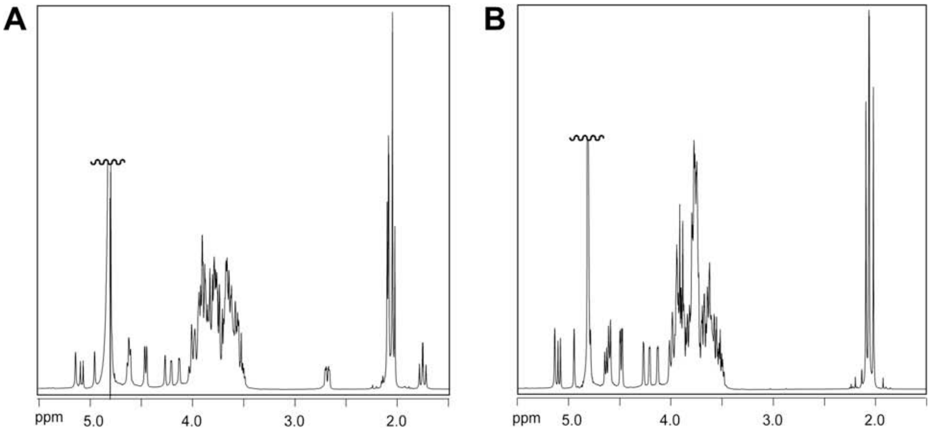
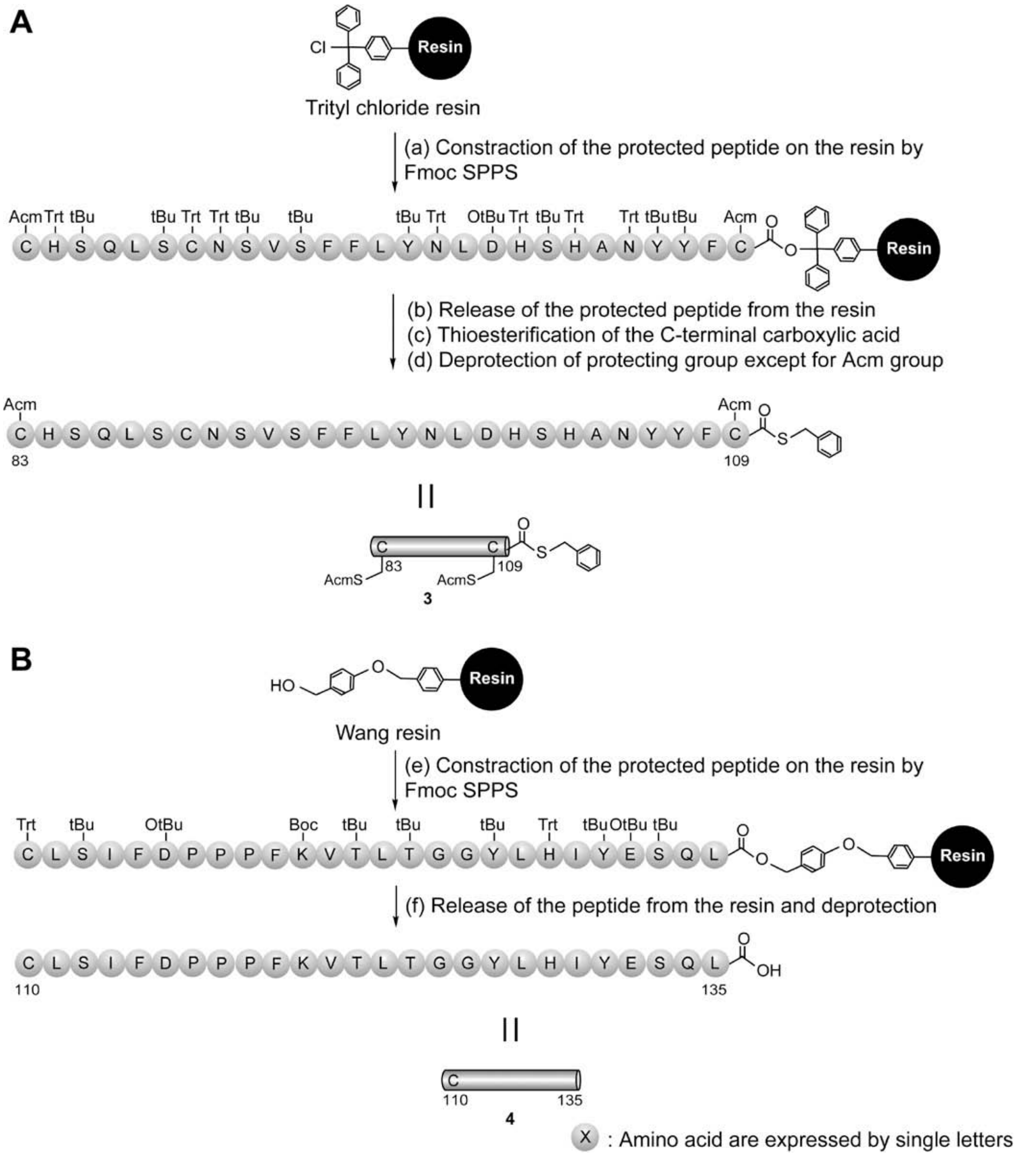


**Figure 2.** $^1H$ NMR spectra of bromoacetamidyl oligosaccharides. Spectrum A is bromoacetamidyl undecasaccharide **1** and spectrum B is bromoacetamidyl nonasaccharide **2**.



**Scheme 2.** Synthesis of peptide thioester **3** and peptide **4** by means of Fmoc SPPS and thioesterification. Reagents and conditions: (a) Fmoc-SPPS; (b) DCM, MeOH, AcOH; (c) BnSH, PyBOP, DIPEA, DMF, −20 °C; (d) 95% TFA solution containing triisopropylsilane; (e) Fmoc-SPPS; (f) 95% TFA solution containing triisopropylsilane and dithiothreitol.

also demonstrated convenient preparation of 24 kinds of diverse complex type oligosaccharides.[6] The combination of these methodologies could provide more complex patterns of varying oligosaccharide structures in glycoconjugate libraries. Furthermore, the thiol of the N-terminal cysteine can be used for immobilization of these glycoconjugates to chips having carboxymethyl group on its surface in a surface plasmon resonance (SPR) system. We demonstrate here a simple and practical synthetic method of a small glycoconjugate library, and suggest that this synthetic concept will help support efforts in glycobiology.

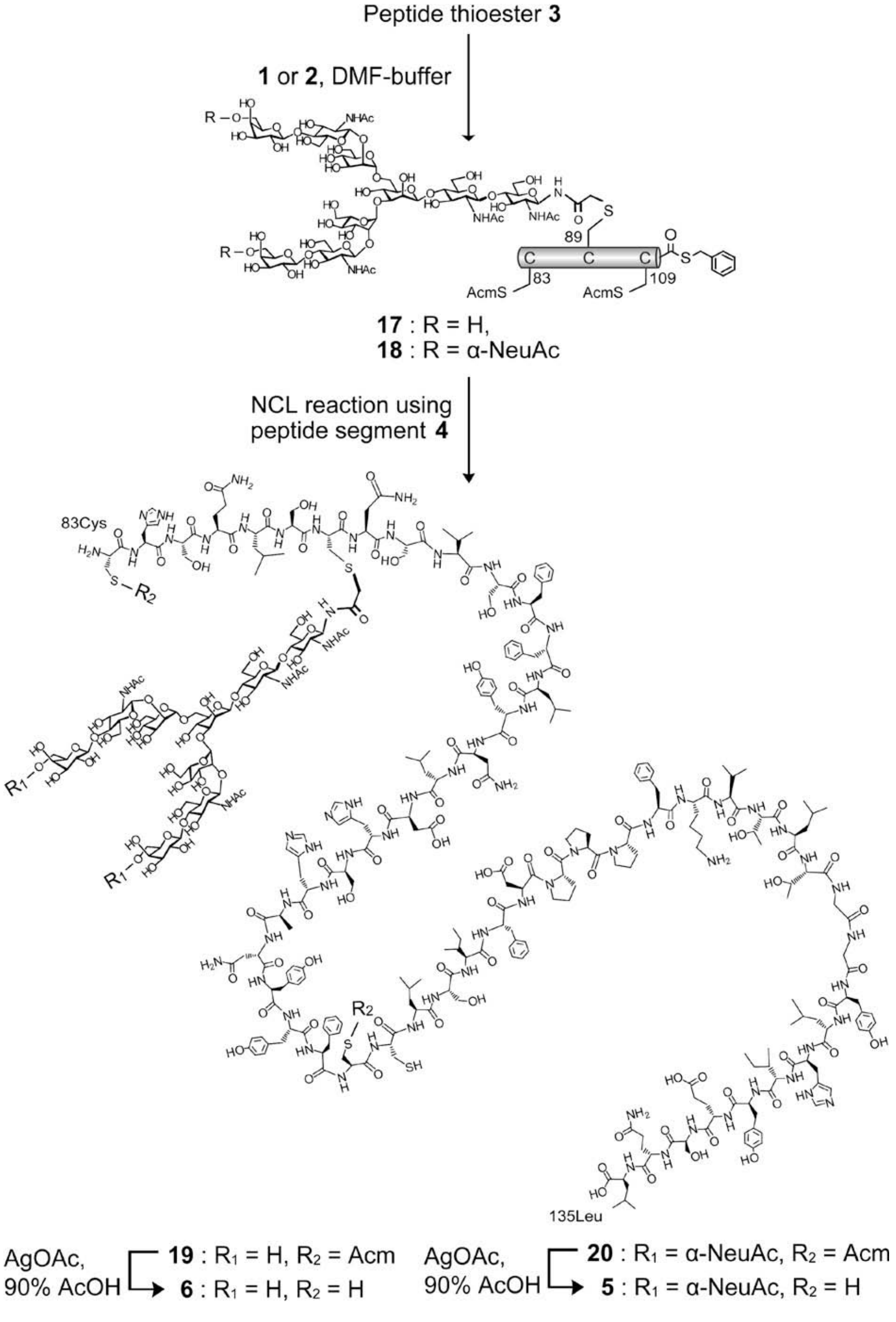
## 4. Experimental

### 4.1. Materials and methods

Homogeneous Fmoc-asparaginyl disialyloligosaccharides were prepared by the reported method.[6] Trityl resin, Wang resin and Fmoc-AA-OH derivatives were purchased from Merck (USA) and Peptide Institute, Inc. (Osaka, Japan). RP-HPLC analyses were carried out on a Waters HPLC system equipped with a photodiode array detector (Waters 2996) using Cadenza column (Intact Corp., 3 μm, 75 × 4.6 mm) or a Vydac C-18 (5 μm, 4.6 × 250 mm) unless otherwise indicated. ESI-MASS spectra were measured with a Bruker Daltonics Esquire 3000 plus. MALDI-HRMS spectra were measured with Bruker Daltonics Autoflex by the use of 2,5-dihydroxybenzoic acid as the matrix. $^1$H and $^{13}$C NMR spectra were measured with Bruker Avance 400 (For $^1$H NMR spectra; internal standard HOD = 4.81 ppm in $D_2O$ solution, for $^{13}$C NMR spectra; external standard (acetone) = 30.42 ppm in $D_2O$ solution) instrument.

### 4.2. Disialylundecasaccharyl hydrazide 12

Fmoc-asparaginyl disialyloligosaccharide **11** (70.0 mg, 27.4 μmol) was dissolved in hydrazine hydrate (3.0 mL) and was stirred at 100 °C for 10 h. After cooling to room temperature, hydrazine hydrate was removed under vacuum by use of desiccator equipped with concd $H_2SO_4$. Then, the residue was dissolved in satd $NaHCO_3$ solution (1.5 mL). To this solution was added $Ac_2O$ (150 μL) at 4 °C and this solution was stirred at room temperature. The reac-



**Scheme 3.** Synthesis of AILIM/ICOS glycopeptide fragments **5** and **6**.

tion was monitored by ESI-MS, and within 30 min ESI-MS spectrum indicated the complete acetylation. Then, the product of this reaction mixture was purified by gel permeation chromatography (Sephadex G-25, 0.03% $NH_3$ solution) and then preparative HPLC on a Waters preparative column (X-Bridge RP-18, 10 × 250 mm, 50 mM $NH_4OAc$–MeCN 99:1) at a flow rate 3.0 mL $min^{-1}$. The fractions containing a product were pooled and then lyophilized. Finally, the residue was desalted by gel permeation chromatography (Sephadex G-25, 0.03% $NH_3$ solution) to afford disialylundecasaccharyl hydrazide **12** in 51% yield (32.0 mg, 14.0 μmol). $^1H$ NMR ($D_2O$, 400 MHz): $\delta$ 5.12 (s, 1H, Man4-H-1), 4.94 (s, 1H, Man4′-H-1), 4.76 (s, 1H, Man3-H-1), 4.59 (br d, 3H, GlcNAc2,5,5′-H-1), 4.43 (d, 2H, *J* = 7.89 Hz, Gal6,6′-H-1), 4.25 (br d, 1H, Man3-H-2), 4.21 (d, 1H, *J* = 10.2 Hz, GlcNAc1-H-1), 4.19 (br d, 1H, Man4′-H-2), 4.11 (br d, 1H, Man4-H-2), 2.66 (br dd, 2H, NeuAc7,7′-H-3eq), 2.07 (s, 3H, Ac), 2.06 (s, 6H, Ac × 2), 2.02 (s, 6H, Ac × 2), 2.00 (s, 3H, Ac), 1.91 (s, 3H, Ac), 1.71 (dd, 2H, *J* = 12.6 Hz, NeuAc7,7′-H-3ax); $^{13}C$ NMR ($D_2O$, 100 MHz): 103.89 (Gal6,6′-C-1), 101.69, 99.68, 99.60 (GlcNAc2,5,5′-C-1), 100.81 (Man3-C-1), 100.50, 100.47 (NeuAc7,7′-C-2), 99.90 (Man4-C-1), 97.27 (Man4′-C-1), 88.48 (GlcNAc1-C-1), 76.74 (Man4′-C-2), 76.54 (Man4-C-2), 70.53 (Man3-C-2), 40.40 (NeuAc7,7′-C-3). MALDI-HRMS calcd for $C_{86}H_{142}N_8O_{62}Na$ $[M+Na]^+$ 2301.8097, found 2301.8100.

### 4.3. Disialylundecasaccharide 13

Disialylundecasaccharyl hydrazide **12** (30.0 mg, 13.2 μmol) was dissolved in 10 M AcOH solution (2.0 mL) and acid hydrolysis was performed at room temperature for 2 h. The reaction was monitored by ESI-MS. When the ESI-MS spectrum indicated the com-

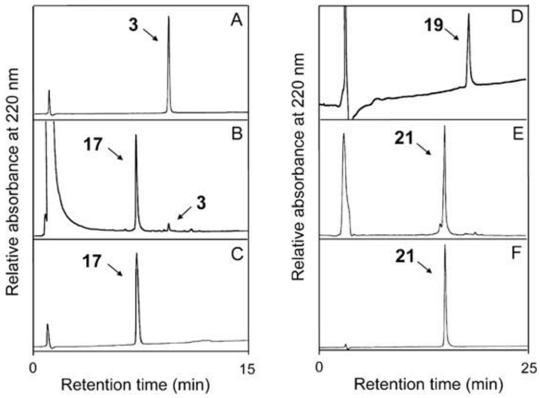


**Figure 3.** HPLC profiles of incorporation of bromoacetamidyl oligosaccharide to the peptide. (A) Purified peptide thioester **3** as a starting material, (B) reaction mixture of asialooligosaccharide **2** and peptide-α-thioester **3** after 3 h, (C) purified asialoglycopeptide-α-thioester **17**. HPLC elution condition: column, Cadenza CD-18 (3 μm, 4.6 × 75 mm) at a flow rate 1.0 mL $min^{-1}$. Linear gradient of 31.5–54% $CH_3CN$, containing 0.09% TFA in 0.1% TFA solution over 15 min, (D) A starting material: purified asialoglycopeptide **19**, (E) reaction mixture of disialyloligosaccharide **1** and asialoglycopeptide **19** after 3.5 h, and (F) purified glycopeptide **21**. HPLC elution condition: column, Vydac C-18 (5 μm, 4.6 × 250 mm) at a flow rate 1.0 mL $min^{-1}$. Linear gradient of 27–54% $CH_3CN$, containing 0.09% TFA in 0.1% TFA solution over 25 min.

plete conversion of **12** to **13**, the reaction mixture was neutralized by 1 M NaOH solution at 4 °C and the product was purified by gel permeation chromatography (Sephadex G-25, 0.03% $NH_3$ solution) to afford the disialylundecasaccharide **13** in 92% yield (27.0 mg, 12.2 μmol). $^1H$ NMR ($D_2O$, 400 MHz): δ 5.20 (s, GlcNAc1α-H-1), 5.15 (s, 1H, Man4-H-1), 4.96 (s, 1H, Man4′-H-1), 4.79 (s, 1H, Man3-H-1), δ 4.71 (br d, *J* = 8.0 Hz, GlcNAc1β-H-1), 4.62 (br d, 3H, GlcNAc2,5,5′-H-1), 4.46 (d, 2H, Gal6,6′-H-1), 4.27 (br d, 1H, Man3-H-2), 4.21 (br d, 1H, Man4′-H-2), 4.13 (br d, 1H, Man4-H-2), 2.68 (dd, 2H, NeuAc7,7′-H-3eq), 2.09 (s, 3H, Ac), 2.08 (s, 6H, Ac × 2), 2.05 (s, 3H, Ac), 2.04 (s, 6H, Ac × 2), 1.74 (dd, 2H, *J* = 12.2 Hz NeuAc7,7′-H-3ax); $^{13}C$ NMR ($D_2O$, 100 MHz): 103.91 (Gal6,6′-C-1), 101.74, 99.71, 99.62 (GlcNAc2,5,5′-C-1), 100.84 (Man3-C-1), 100.47, 100.44 (NeuAc7,7′-C-2), 99.94 (Man4-C-1), 97.31 (Man4′-C-1), 90.79 (GlcNAc1-C-1), 76.76 (Man4′-C-2), 76.57 (Man4-C-2), 70.55 (Man3-C-2), 40.39 (NeuAc7,7′-C-3); MALDI-HRMS calcd for $C_{84}H_{138}N_6O_{62}Na$ $[M+Na]^+$ 2245.7722, found 2245.7716.

### 4.4. Nonasaccharide 14

Disialylundecasaccharyl hydrazide **12** (28.0 mg, 12.3 μmol) was dissolved in 40 mM HCl solution (2.5 mL) and the reaction mixture was stirred at 50 °C for 32 h. The reaction was monitored by ESI-MS. When the ESI-MS spectrum indicated the complete conversion of **12** to **14**, the reaction mixture was neutralized by 1 M NaOH solution at 4 °C and the product was purified by gel permeation chromatography (Sephadex G-25, $H_2O$), and anion exchange resin (AG 1-X8, 200–400 mesh, BIO-RAD) to afford the nonasaccharide **14** in 89% yield (18.0 mg, 11.0 μmol). $^1H$ NMR ($D_2O$, 400 MHz): δ 5.18 (br d, GlcNAc1α-H-1), 5.11 (s, 1H, Man4-H-1), 4.93 (s, 1H, Man4′-H-1), 4.76 (s, 1H, Man3-H-1), 4.69 (br d, *J* = 7.7 Hz, GlcNAc1β-H-1), 4.59 (m, 3H, GlcNAc2,5,5′-H-1), 4.47 (d, 2H, Gal6,6′-H-1), 4.25 (br d, 1H, Man3-H-2), 4.19 (br d, 1H, Man4′-H-2), 4.11 (br d, 1H, Man4-H-2), 2.08 (s, 3H, Ac), 2.04 (s, 6H, Ac × 2), 2.03 (s, 3H, Ac); $^{13}C$ NMR ($D_2O$, 100 MHz): 103.26 (Gal6,6′-C-1), 101.72, 99.77 (GlcNAc2,5,5′-C-1), 100.77 (Man3-C-1), 99.89 (Man4-C-1), 97.36 (Man4′-C-1), 95.15 (GlcNAc1β-C-1), 90.77 (GlcNAc1α-C-1), 76.72 (Man4′-C-2), 76.62 (Man4-C-2), 70.55 (Man3-C-2); MALDI-HRMS calcd for $C_{62}H_{104}N_4O_{46}Na$ $[M+Na]^+$ 1663.5814, found 1663.5829.

### 4.5. 1,β-Amino disialylundecasaccharide 15

Disialylundecasaccharide **13** (22.0 mg 10.0 μmol) was dissolved in satd $NH_4HCO_3$ solution (1.0 mL) and the amination was performed at 37 °C for 3 days. For monitoring of the reaction, an aliquot of the reaction mixture was N-acetylated by $Ac_2O$ and analyzed by ESI-MS. When the ESI-MS spectrum indicated the complete conversion of **13** to **15**, the product was purified by gel permeation chromatography (Sephadex G-25, 0.03% $NH_3$ solution) to afford the β-disialylundecasaccharyl amine **15** in 95% yield (21.0 mg, 9.5 μmol). $^1H$ NMR ($D_2O$, 400 MHz): δ 5.14 (s, 1H, Man4-H-1), 4.96 (s, 1H, Man4′-H-1), 4.61 (br d, 3H, GlcNAc2,5,5′-H-1), 4.45 (d, 2H, Gal6,6′-H-1), 4.26 (br d, 1H, Man3-H-2), 4.20 (br d, 1H, Man4′-H-2), 4.16 (d, 1H, *J* = 8.4 Hz GlcNAc1 H-1), 4.12 (br d, 1H, Man4-H-2), 2.68 (br dd, 2H, NeuAc7,7′-H-3eq), 2.09 (s, 3H, Ac), 2.07 (s, 6H, Ac × 2), 2.04 (s, 3H, Ac), 2.03 (s, 6H, Ac × 2), 1.73 (dd, 2H, *J* = 12.2 Hz, NeuAc7,7′-H-3ax); MALDI-HRMS calcd for $C_{84}H_{139}N_7O_{61}Na$ $[M+Na]^+$ 2244.7882, found 2244.7886.

### 4.6. 1,β-Amino nonasaccharide 16

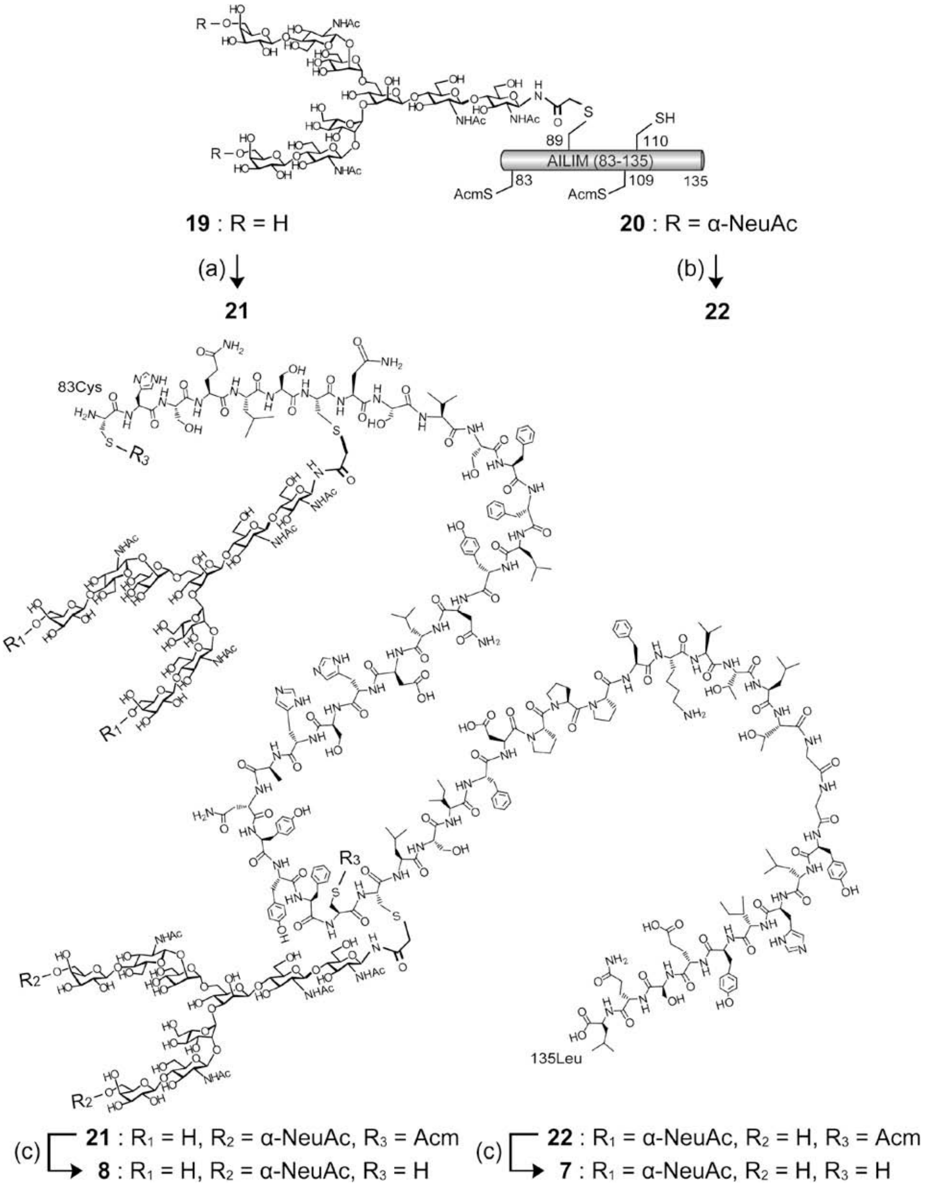
Nonasaccharide **14** (16.4 mg, 10.0 μmol) was dissolved in satd $NH_4HCO_3$ solution (1.0 mL) and the amination was performed at 37 °C for 2 days. After completion of the reaction, purification was performed by the same protocol mentioned in the preparation of **15**. The β-nonasaccharylamine **16** was obtained in 98% yield (16.0 mg, 9.8 μmol). $^1H$ NMR ($D_2O$, 400 MHz): δ 5.12 (s, 1H, Man4-H-1), 4.93 (s, 1H, Man4′-H-1), 4.77 (s, 1H, Man3-H-1), 4.60 (m, 3H, GlcNAc2,5,5′-H-1), 4.48 (d, 2H, Gal6,6′-H-1), 4.26 (br d, 1H, Man3-H-2), 4.20 (br d, 1H, Man4′-H-2), 4.15 (br d, 1H, *J* = 8.4 Hz, GlcNAc1-H-1), 4.12 (br d, 1H, Man4-H-2), 2.08 (s, 3H, Ac), 2.05 (s, 6H, Ac × 2), 2.04 (s, 3H, Ac); MALDI-HRMS calcd for $C_{62}H_{105}N_5O_{45}Na$ $[M+Na]^+$ 1662.5974, found 1662.5969.

### 4.7. Bromoacetamidyl disialylundecasaccharide 1

To solution of distilled $H_2O$ (700 μL) containing β-disialylundecasaccharyl amine **15** (15.0 mg, 6.8 μmol) and $NaHCO_3$ (17.0 mg) were added *N*,*N*′-diisopropylcarbodiimide (DIC: 83.0 μL, 540.0 μmol), and bromoacetic acid (28.0 mg, 204.0 μmol) in DMF (700 μL) at 4 °C. The reaction mixture was stirred at room temperature and was monitored by ESI-MS. When the ESI-MS spectrum indicated the complete conversion of **15** to **1**, this reaction mixture was added to an excess of acetone. This protocol led to the precipitation of the oligosaccharide from the reaction mixture. The precipitate was collected by centrifugation and the residue was purified by preparative RP-HPLC (X-Bridge RP-18, 10 × 250 mm, 50 mM $NH_4OAc$/MeCN 98:2; flow rate 3.0 mL $min^{-1}$), and then desalted by gel permeation chromatography (Sephadex G-25, $H_2O$) to afford the bromoacetamidyl disialylundecasaccharide **1** in 50% yield (8.0 mg, 3.4 μmol). $^1H$ NMR ($D_2O$, 400 MHz): δ 5.15 (s, 1H, Man4-H-1), 5.09 (d, 1H, *J* = 9.5 Hz, GlcNAc1-H-1) 4.96 (s, 1H, Man4′-H-1), 4.62 (m, 3H, GlcNAc2,5,5′-H-1), 4.46 (d, 2H, *J* = 8.1 Hz, Gal6,6′-H-1), 4.27 (br d, 1H, Man3-H-2), 4.21 (br d, 1H, Man4′-H-2), 4.13 (br d, 1H, Man4-H-2), 2.68 (br dd, 2H, NeuAc7,7′-H-3eq), 2.10 (s, 3H, Ac), 2.08 (s, 6H, Ac × 2), 2.04 (s, 6H, Ac × 2), 2.02 (s, 3H, Ac), 1.97 (s, 3H, Ac) 1.75 (dd, 2H, *J* = 12.3 Hz, NeuAc7,7′-H-3ax); ESI-MASS (negative mode) calcd for $C_{84}H_{140}BrN_7O_{62}$ $[M-2H]^{2-}$ 1171.0, found 1170.9.

### 4.8. Bromoacetamidyl nonasaccharide 2

Preparation of bromoacetamidyl nonasaccharide **2** was performed in the same manner as the preparation of **1**. β-Nonasaccharylamine **16** (13.0 mg, 8.0 μmol) was converted into **2** in 58% yield (8.0 mg, 4.6 μmol). $^1H$ NMR ($D_2O$, 400 MHz): δ 5.13 (s, 1H, Man4-



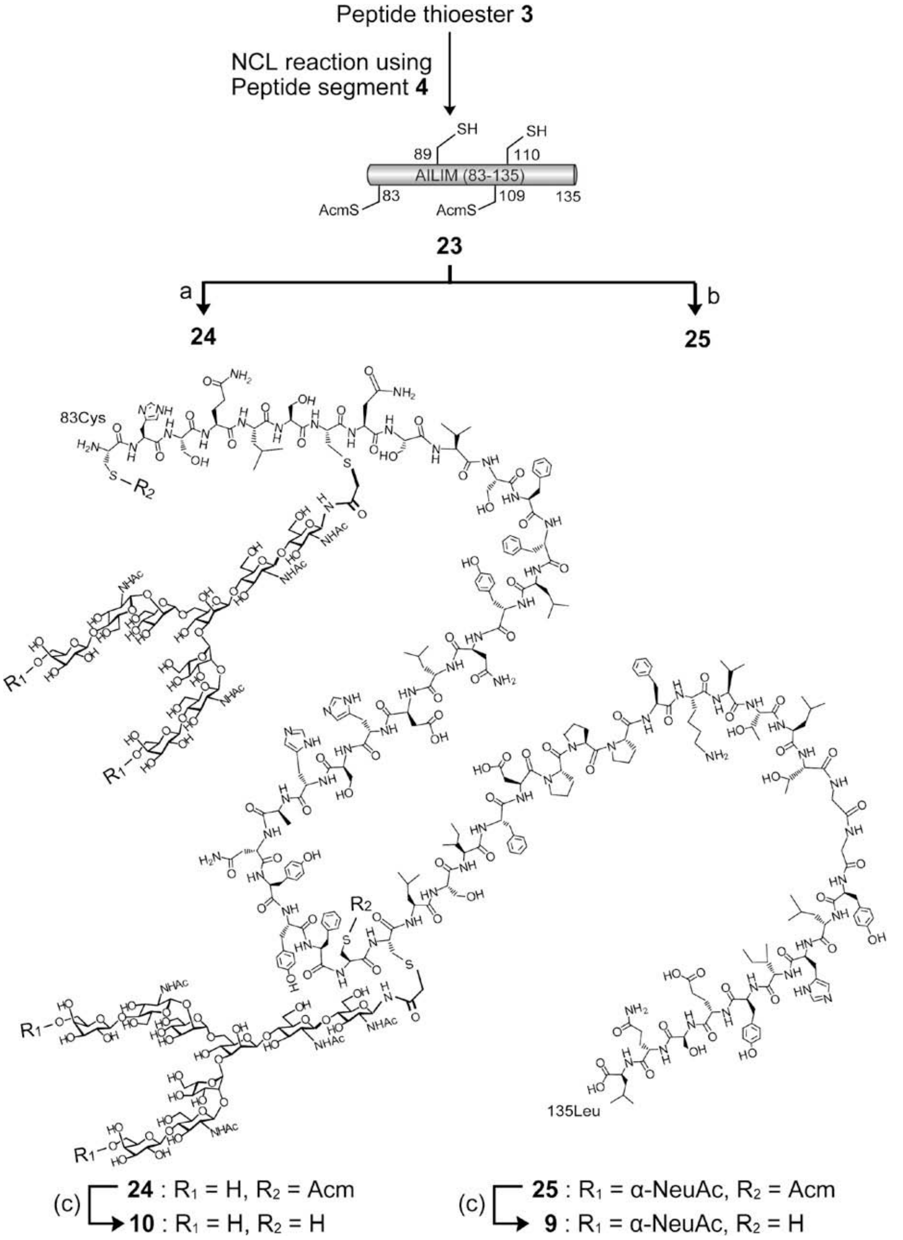
**Scheme 4.** Synthesis of AILIM/ICOS glycopeptide fragments **7** and **8**. Reagents and conditions: (a) **1**, 200 mM phosphate buffer; (b) **2**, 200 mM phosphate buffer; (c) AgOAc, 90% AcOH.

H-1), 5.09 (d, 1H, $J = 9.8$ Hz, GlcNAc1-H-1), 4.94 (s, 1H, Man4′-H-1), 4.78 (s, 1H, Man3-H-1), 4.61 (m, 3H, $J = 8.0$ Hz GlcNAc2,5,5′-H-1), 4.48 (d, 2H, Gal6,6′-H-1), 4.26 (br d, 1H, Man3-H-2), 4.20 (br d, 1H, Man4′-H-2), 4.12 (br d, 1H, Man4-H-2), 2.09 (s, 3H, Ac), 2.06 (s, 3H, Ac) , 2.06 (s, 3H, Ac) , 2.02 (s, 3H, Ac); MALDI-HRMS calcd for $C_{64}H_{106}BrN_5O_{46}$ $[M+Na]^+$ 1782.5185, found 1782.5199.

### 4.9. Synthesis of AILIM/ICOS glycopeptides fragments. A typical procedure

#### 4.9.1. Peptide-α-thioester 3

The first amino acid, Fmoc-Cys-(Acm)-OH (410 mg, 990 μmol) was attached to trityl-chloride-resin (220 mg, 330 μmol) using *N,N′*-diisopropylethylamine (DIPEA: 470 μL, 2.6 mmol) in DCM (2.0 mL) and then peptide was elongated. Then each amino acid coupling reaction for peptide elongation was performed by treatment of corresponding Fmoc-amino acid derivatives (5.0 equiv), DIC (5.0 equiv), and 1-hydroxybenzotriazole (HOBt: 5.0 equiv) in DMF (3.3 mL) for 1 h. Deprotection of the Fmoc group was performed with 20% piperidine in DMF for 20 min. After construction of peptide, an aliquot of the resin (375 mg) was treated with acetic acid–methanol–DCM (2.4:0.36:1.92, 4.7 mL) for 3 h, and then the resin was filtered. Concentration of the filtrate afforded a crude peptide residue in which the peptide side chains were protected (85.0 mg). The residue was dissolved in benzene and then was concentrated by coevaporation three times. To the solution of this crude peptide in DMF (3.5 mL) was added benzylthiol (63.8 μL 518.5 μmol) and then this reaction mixture was stirred at −20 °C. After 15 min, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBop: 45.0 mg, 86.4 μmol), and DIPEA (14.7 μL, 86.4 μmol), were added and the reaction mixture was stirred at −20 °C. After 3 h, ethyl ether–hexane (1:1, 250 mL) was added. This process led to a precipitate of peptide thioester, which was then collected by filtration. The jelly type precipitate was then dissolved in DMF and concentrated. To this residue was added a solution containing 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS) and 2.5% $H_2O$ to remove protecting group for 2 h. Then the solution was concentrated in vacuo. Purifi-



**Scheme 5.** Synthesis of AILIM/ICOS glycopeptide fragments **9** and **10**. Reagents and conditions: (a) **2**, DMF–200 mM phosphate buffer; (b) **1**, DMF–200 mM phosphate buffer; (c) AgOAc, 90% AcOH.

cation of the residue by RP-HPLC (Vydac C-18: 10 × 250 mm, linear gradient of 27–54% $CH_3CN$, containing 0.09% TFA in 0.1% TFA solution over 25 min, flow rate 4.0 mL min$^{-1}$) afforded peptide-a-thioester **3** (9.0 mg, 2.6 μmol). ESI-MASS calcd for $C_{156}H_{211}N_{39}O_{43}S_4$ $[M+2H]^{2+}$ 1725.4, $[M+3H]^{3+}$ 1150.6, found 1725.2, 1150.5.

#### 4.9.2. Peptide fragment 4

This peptide segment was also prepared by use of solid phase synthesis. The first amino acid, Fmoc-Leu-OH (353 mg, 1.0 mmol) was attached to Wang resin (215 mg, 200 μmol) using 1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT: 296 mg, 1.0 mmol), *N*-methylimidazole (60 μL, 750 μmol) in DCM (4.0 mL). Each peptide elongation step was performed by treatment with the corresponding Fmoc-amino acid derivative (5.0 equiv), DIC (5.0 equiv), and HOBt (5.0 equiv) in DMF (4.0 mL) for 1 h. Deprotection of the Fmoc group was performed with 20% piperidine in DMF for 20 min. After construction of peptide, **4** was released from the resin (200 mg) by the use of a solution containing 95% TFA, 2.5% TIPS, 2.5% $H_2O$, and dithiothreitol (DTT, 5% v/v). This solution was added to ethyl ether (20.0 mL) to afford a precipitate of peptide **4**. The precipitate was collected by centrifugation. Purification of the residue by RP-HPLC (Vydac C-18: 10 × 250 mm, linear gradient of 27–54% $CH_3CN$, containing 0.09% TFA in 0.1% TFA solution over 25 min, flow rate 4.0 mL min$^{-1}$) afforded peptide **4** (12.3 mg). ESI-MASS calcd for $C_{139}H_{208}N_{30}O_{38}S$ $[M+H]^{+}$ 2938.5, $[M+2H]^{2+}$ 1469.8, $[M+3H]^{3+}$ 980.2, found 2939.2, 1470.3, 980.6.

### 4.10. Incorporation of bromoacetamidyl oligosaccharide to peptide

#### 4.10.1. Method A

To a solution of the peptide-α-thioester **3** (2.0 mg, 0.58 μmol) in DMF (240 μL) was added bromoacetamidyl asialooligosaccharide **2** (2.0 mg, 1.14 μmol) in 25 mM phosphate buffer (pH 7.5, 360 μL) at

4 °C and then reaction mixture was incubated at room temperature. The reaction was monitored by RP-HPLC (Cadenza CD-18: 4.6 × 75 mm, linear gradient of 31.5–54% $CH_3CN$, containing 0.09% TFA in 0.1% TFA solution over 15 min, flow rate 1.0 mL min$^{-1}$). After 3 h, direct purification of the reaction mixture by RP-HPLC (Vydac C-18: 4.6 × 250 mm, linear gradient of 27–54% $CH_3CN$, containing 0.09% TFA in 0.1% TFA solution over 25 min, flow rate 1.0 mL min$^{-1}$) afforded asialoglycopeptide-α-thioester **17** (1.5 mg, 0.29 μmol) in 50% yield. ESI-MASS calcd for $C_{220}H_{316}N_{44}O_{89}S_4$ $[M+2H]^{2+}$ 2565.7, $[M+3H]^{3+}$ 1710.8, $[M+4H]^{4+}$ 1283.3, found 2565.5, 1710.7, 1283.4.

### 4.10.2. Method B (using guanidine-HCl)

To a solution of the peptide (one of **3**, **19**, **20,** and **23**: 0.43 μmol) in DMF (120 μL) were added the bromoacetamidyl oligosaccharide **1** or **2** (1.1 μmol) in 200 mM phosphate buffer (pH 7.5, 180 μL) containing 6 M Gn·HCl at 4 °C and then reaction mixture was incubated at room temperature. The reaction was monitored by RP-HPLC (Cadenza CD-18: 4.6 × 75 mm, linear gradient of 31.5–54% $CH_3CN$, containing 0.09% TFA in 0.1% TFA solution over 15 min, flow rate 1.0 mL min$^{-1}$). After 2 h, direct purification of the reaction mixture by RP-HPLC (Vydac C-18: 4.6 × 250 mm, linear gradient of 27–54% $CH_3CN$, containing 0.09% TFA in 0.1% TFA solution over 25 min, flow rate 1.0 mL min$^{-1}$) afforded glycopeptide-α-thioester **18** or glycopeptides **21**, **22**, **24**, and **25**. Glycopeptide-α-thioester **18**: ESI-MASS calcd for $C_{242}H_{350}N_{46}O_{105}S_4$ $[M+2H]^{2+}$ 2856.9, $[M+3H]^{3+}$ 1905.0, $[M+4H]^{4+}$ 1429.0, $[M+5H]^{5+}$ 1143.4, found 2856.7, 1904.9, 1429.0, 1143.6. The experimental data (glycopeptide-α-thioester **18** or glycopeptides **17, 18, 21**, **22**, **24**, and **25)** are shown in detail in the Supplementary data.

## 4.11. Native chemical ligation

To a solution of corresponding peptide-α-thioester (**3**, **17,** and **18)** in 200 mM phosphate buffer (pH 7.5) containing 8 M Gn·HCl, 40 mM tris(2-carboxy-ethyl)phosphine hydrochloride (TCEP), 40 mM 4-mercapto phenyl acetic acid (MPAA) was added peptide segment **4** (2 equiv) and then reaction mixture was incubated at room temperature. The reaction was monitored by RP-HPLC (Cadenza CD-18: 4.6 × 75 mm, linear gradient of 31.5–54% $CH_3CN$, containing 0.09% TFA in 0.1% TFA solution over 15 min, flow rate 1.0 mL min$^{-1}$). After 4 h, direct purification of the reaction mixture by RP-HPLC (Vydac C-18: 4.6 × 250 mm, linear gradient of 27–54% $CH_3CN$, containing 0.09% TFA in 0.1% TFA solution over 25 min, flow rate 1.0 mL min$^{-1}$) afforded a corresponding peptide or glycopeptide. Glycopeptide **19**: ESI-MASS calcd for $C_{352}H_{516}N_{74}O_{127}S_4$ $[M+3H]^{3+}$ 2649.2, $[M+4H]^{4+}$ 1987.1, $[M+5H]^{5+}$ 1589.9, $[M+6H]^{6+}$ 1325.1, $[M+7H]^{7+}$ 1135.9, found 2648.6, 1986.9, 1589.8, 1325.0, 1135.8; Glycopeptide **20**: ESI-MASS calcd for $C_{374}H_{550}N_{76}O_{143}S_4$ $[M+3H]^{3+}$ 2843.4, $[M+4H]^{4+}$ 2132.8, $[M+5H]^{5+}$ 1706.4, $[M+6H]^{6+}$ 1422.2, $[M+7H]^{7+}$ 1219.2, found 2842.9, 2132.6, 1706.6, 1422.4, 1219.6.

## 4.12. Deprotection of Acm group

Glycopeptides having Acm groups were dissolved in 90% acetic acid solution (200 μL) containing 10 mM AgOAc. After stirring for 3 h, to this mixture was added dithiothreitol (DTT, 1.0 mg). After stirring for 1 h, the resultant precipitate was removed by centrifugation and the supernatant was subjected to purification by RP-HPLC (Vydac C-18: 4.6 × 250 mm, linear gradient of 27–54% $CH_3CN$, containing 0.09% TFA in 0.1% TFA solution over 25 min, flow rate 1.0 mL min$^{-1}$) to obtain glycopeptides **5**–**10**. Glycopeptide **5**: ESI-MASS calcd for $C_{368}H_{540}N_{74}O_{141}S_4$ $[M+3H]^{3+}$ 2796.0, $[M+4H]^{4+}$ 2097.2, $[M+5H]^{5+}$ 1678.0, $[M+6H]^{6+}$ 1398.5, found 2795.7, 2097.4, 1678.2, 1398.9; Glycopeptide **6**: ESI-MASS calcd for $C_{346}H_{506}N_{72}O_{125}S_4$ $[M+3H]^{3+}$ 2601.8, $[M+4H]^{4+}$ 1951.6, $[M+5H]^{5+}$ 1561.5, $[M+6H]^{6+}$ 1301.4, found 2601.6, 1951.6, 1561.5, 1301.4; Glycopeptide **21**: ESI-MASS calcd for $C_{438}H_{655}N_{81}O_{189}S_4$ $[M+4H]^{4+}$ 2552.9, $[M+5H]^{5+}$ 2042.5, $[M+6H]^{6+}$ 1702.3, $[M+7H]^{7+}$ 1459.2, found 2552.7, 2042.6, 1702.4, 1459.6; Glycopeptide **22**: ESI-MASS calcd for $C_{438}H_{655}N_{81}O_{189}S_4$ $[M+4H]^{4+}$ 2552.9, $[M+5H]^{5+}$ 2042.5, $[M+6H]^{6+}$ 1702.3, $[M+7H]^{7+}$ 1459.2, found 2552.4, 2042.4, 1702.2, 1459.2; Glycopeptide **7**: ESI-MASS calcd for $C_{432}H_{645}N_{79}O_{187}S_4$ $[M+4H]^{4+}$ 2517.4, $[M+5H]^{5+}$ 2014.1, $[M+6H]^{6+}$ 1678.6, $[M+7H]^{7+}$ 1438.9, found 2516.6, 2013.8, 1678.5, 1438.7; Glycopeptide **8**: ESI-MASS calcd for $C_{432}H_{645}N_{79}O_{187}S_4$ $[M+4H]^{4+}$ 2517.4, $[M+5H]^{5+}$ 2014.1, $[M+6H]^{6+}$ 1678.6, $[M+7H]^{7+}$ 1438.9, found 2517.0, 2014.1, 1678.6, 1439.1; Peptide **23**: ESI-MASS calcd for $C_{288}H_{411}N_{69}O_{81}S_4$ $[M+3H]^{3+}$ 2089.0, $[M+4H]^{4+}$ 1567.0, $[M+5H]^{5+}$ 1253.8, $[M+6H]^{6+}$ 1045.0, $[M+7H]^{7+}$ 895.9, found 2088.7, 1566.9, 1253.9, 1045.1, 895.9; Glycopeptide **24**: ESI-MASS calcd for $C_{416}H_{621}N_{79}O_{173}S_4$ $[M+4H]^{4+}$ 2407.3, $[M+5H]^{5+}$ 1926.0, $[M+6H]^{6+}$ 1605.2, $[M+7H]^{7+}$ 1376.0, found 2406.8, 1925.9, 1605.1, 1376.0; Glycopeptide **25**: ESI-MASS calcd for $C_{460}H_{689}N_{83}O_{205}S_4$ $[M+4H]^{4+}$ 2698.5, $[M+5H]^{5+}$ 2159.0, $[M+6H]^{6+}$ 1799.3 $[M+7H]^{7+}$ 1542.4, found 2698.0, 2158.8, 1799.4, 1542.3; Glycopeptide **9**: ESI-MASS calcd for $C_{454}H_{679}N_{81}O_{203}S_4$ $[M+4H]^{4+}$ 2663.0, $[M+5H]^{5+}$ 2130.6, $[M+6H]^{6+}$ 1775.7, found 2662.3, 2130.3, 1775.5; Glycopeptide **10**: ESI-MASS calcd for $C_{410}H_{611}N_{77}O_{171}S_4$ $[M+4H]^{4+}$ 2371.7, $[M+5H]^{5+}$ 1897.6, $[M+6H]^{6+}$ 1581.5, found 2371.3, 1897.5, 1581.6.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2009.02.013.

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