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Convenient synthesis of a glycopeptide analogue having a complex type disialyl-undecasaccharide

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Abstract—Described herein is a convenient synthesis of a glycopeptide analogue having an undecasaccharide and its stability towards peptide:N-glycosidase F (PNGase-F). To obtain the glycopeptide analogue, bromoacetamidyl undecasaccharide and undecapeptide containing a cysteine residue were synthesized and then a coupling reaction between haloacetamide and thiol was performed. The coupling reaction progressed smoothly at pH 7.0 in phosphate buffer and afforded the desired glycopeptide analogue. In addition, stability of the glycopeptide analogue thus obtained towards PNGase-F was investigated. The result indicated that the glycopeptide analogue was resistant to the enzymatic digestion.

Oligosaccharides on proteins and peptides play roles in a number of important biological events.[1] The functions of these oligosaccharides have been investigated, but it is difficult to evaluate the relationship between the oligosaccharide structure and its function. Heterogeneity, called glycoform, in the oligosaccharide structure hampers determination of what structure causes its bioactivity. Endeavors to reveal the relationship between the structure and its bioactivity now require a convenient and prompt preparation method of oligosaccharides as a pure form. In addition, since post-genome research will also require studies on the function of an oligosaccharide on the protein, synthetic research of peptides or proteins having pure oligosaccharides on their backbone is urgently desired.

Recently, genetic engineering methods for the preparation of glycoprotein have been developed by use of eukaryotic cells, such as the Chinese hamster ovary (CHO) cell not only for investigation of the function of oligosaccharides, but also for potential therapeutic reagents, such as erythropoietin (EPO). However, these methods can not regulate the heterogeneity in the oligosaccharide structure. To overcome this problem, a convenient synthetic method for preparation of neoglycoconjugate has been developed in order to attach pure oligosaccharides.[2] Flitsch added a monosaccharide onto the EPO by a haloacetamide method[3] and Boons coupled small oligosaccharides ranging from a mono- to penta-saccharides, with a thiol of cysteine at the 297 site of IgG1 by a disulfide formation.[4] Both individual proteins were genetically produced and their coupling reactions occurred between the thiol of cysteine and the anomeric position of the reducing end of the oligosaccharides activated. Although most oligosaccharide structures adopt a complex-type dibranched sialylundecasaccharide in the blood, the oligosaccharides used in their experiments are not entirely complete compared to that of the native structure. Therefore, preparation of native complex-type haloacetamidyl oligosaccharides is now very essential for the above protein engineering.[5] In order to have large oligosaccharides, extensive synthetic research has been reported,[6] but the methods require time-consuming and techniques for dealing with such complex molecules.

To cope with this problem, we have established a convenient semisynthetic method for the preparation of N-linked complex-type oligosaccharides from egg yolk on a large scale.[7] Therefore, we set out to apply our oligosaccharides to the synthesis of glycopeptide and glycoprotein analogues by use of the haloacetamide method. In this paper, we report the synthesis of a complex-type bromoacetamidyl sialyloligosaccharide, synthesis of a glycopeptide analogue by use of the haloacetamidyl oligosaccharide thus obtained and its stability towards peptide:N-glycosidase F (PNGase-F, EC3.5.1.52).[8]

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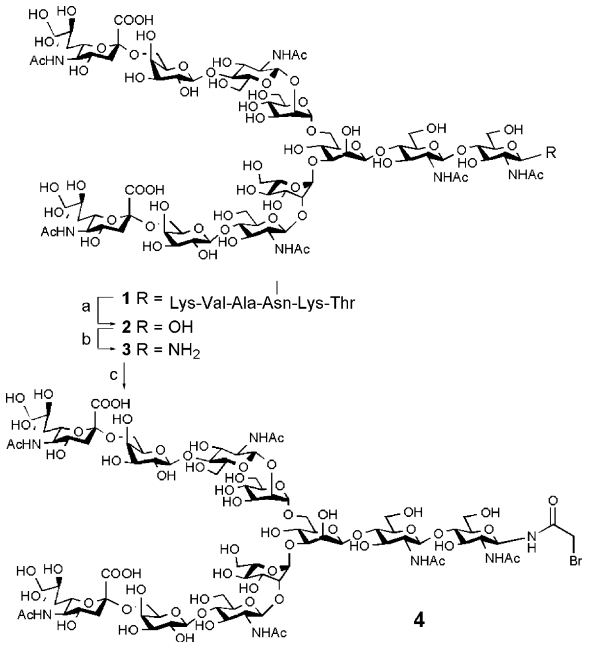


Scheme 1. Synthesis of bromoacetamidyl undecasaccharide **4**. Reagents and conditions: (a) 100 mM phosphate buffer (pH 7.5), PNGase-F; (b) satd NH_4HCO_3, H_2O; (c) $NaHCO_3$, H_2O then bromoacetic acid, DCC, DMF.

In order to obtain a pure complex-type disialyl-undecasaccharide, we used sialylglycopeptide **1** as a soluble fraction from egg yolk.[9] This sialylglycopeptide **1** was treated with commercially available PNGase-F[10] to cleave the amide linkage between the reducing end amino sugar and the carbonyl group of the side chain of asparagine. This enzymatic cleavage afforded the desired disialyl-undecasaccharide **2** of which the reducing end sugar was a hemiacetal form. Then this oligosaccharide **2** was converted into amino sugar **3** by treatment with saturated ammonium bicarbonate.[11] This reaction afforded pure amino oligosaccharide **3** quantitatively.[12] After repetitive lyophilization to remove the remained excess ammonium bicarbonate, amino oligosaccharide **3** thus obtained showed 80% purity based on ^{1}H NMR analysis.[13] Bromoacetamidation with bromoacetic acid and dicyclohexylcarbodiimide (DCC) afforded activated oligosaccharide **4** in 77% yield[14] (Scheme 1). For the peptide having a cysteine residue, undecapeptide **5** (RDDNYCSTYRV), which is the Fc portion of IgG and cysteine is a substituent of Asn297, was prepared by conventional peptide synthesizer.[15] Coupling reaction of this peptide **5** with 0.5 equiv of a bromoacetamidyl oligosaccharide **4** was performed in phosphate buffer (100 mM, pH 7.0). Because oligosaccharide is valuable, 2-fold excess of peptide was used in this reaction (Scheme 2). Monitoring of this reaction was performed by HPLC. As shown in Figure 1, the HPLC profile clearly indicates that the reaction afforded the glycopeptide analogue immediately. As a result, the desired glycopeptide **6** was obtained in 64% yield.[16]

For the half-life of glycoprotein in the plasma, the presence of acidic oligosaccharide appears to be essential; therefore, resistant activity towards the metabolic

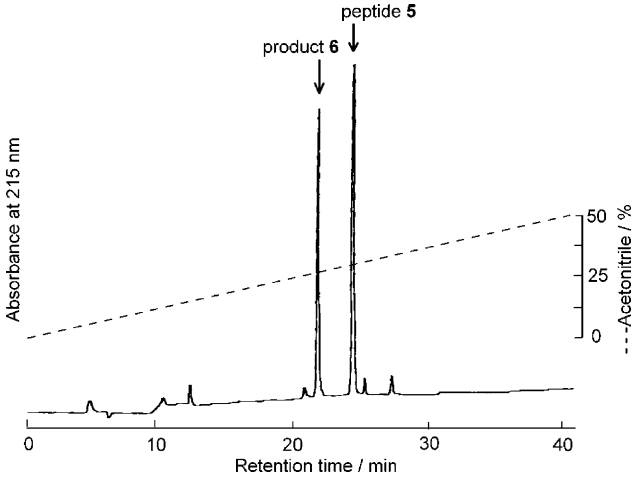
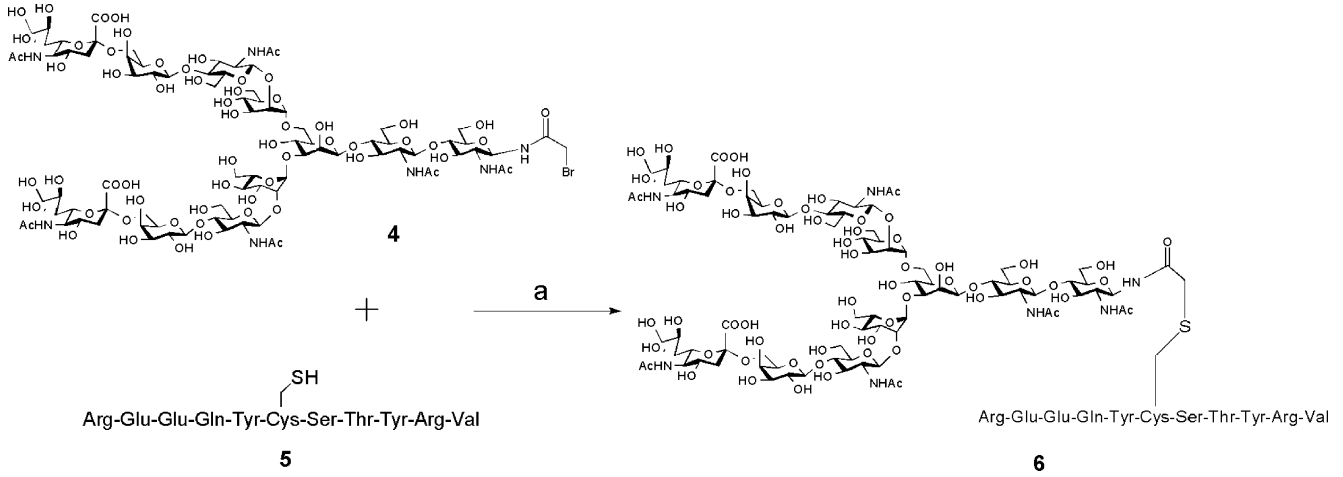


Figure 1. HPLC elution profile of the coupling reaction mixture. Elution condition; column: Mightysil RP-18 (10 × 250 mm) at a flow rate of 2.5 mL min^{-1}; eluent, aqueous acetonitrile containing 0.1% TFA.



Scheme 2. Synthesis of glycopeptide analogue **6**. Reagents and conditions: (a) 100 mM phosphate buffer (pH 7.0).

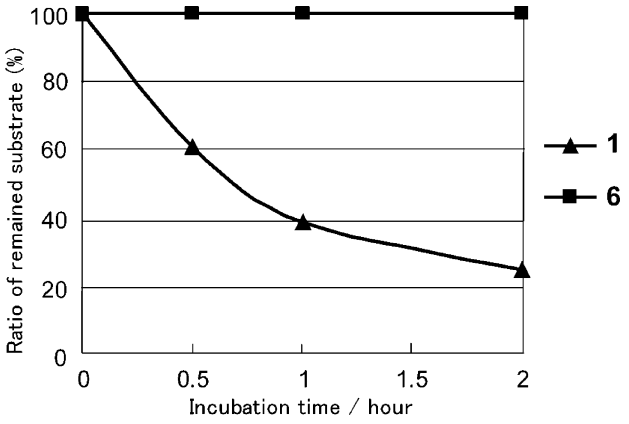


Figure 2. Digestion rate of sialylglycopeptide **1** and glycopeptide analogue **6** with PNGase-F. Assay condition: each substrate (1 mg) was incubated at 25 °C with PNGase-F (50 μU) at pH 7.5 in 100 mM phosphate buffer (1.7 mM). At the indicated times, 5 μL of reaction mixture was removed and digestion rate was quantitated using HPLC.

enzyme would be an essential function. We also investigated the stability of our glycopeptide analogue **6** towards a metabolic enzyme, PNGase-F, which is responsible for cleavage of an amide linkage at the side chain of asparagine. To glycopeptide analogue **6** was added the enzyme (50 μU); this digestion was monitored by HPLC,[17] and the results are summarized in Figure 2. Although 70% of natural glycopeptide **1** was easily cleaved within 2 h, synthetic glycopeptide analogue **6** was not digested. However, use of excess enzyme (5 mU) cleaved the amide linkage in the glycopeptide analogue **6**. This knowledge would be useful for the design of therapeutic reagents based on glycopeptide analogues.

Natural peptide consisting of amino acids ranging from 20 to 50 residues can be prepared by automatic peptide synthesizer. The feasible preparation of complex-type haloacetamidyl oligosaccharide enables us to synthesize glycopeptide analogues and to investigate the function of oligosaccharide on the peptide or protein.

In conclusion, we have performed the first synthesis of a complex-type haloacetamidyl sialyloligosaccharide and demonstrated that the glycopeptide analogue thus synthesized resisted PNGase-F digestion. Our synthesis can also be applied to the synthesis of a glycopeptide library for the high throughput system. Research is in progress to synthesize other glycopeptides by use of our haloacetamidyl oligosaccharide libraries.

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10. PNGase-F was purchased from New England Biolabs, Inc.
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12. A solution of oligosaccharide **2** (30 mM) in saturated ammonium bicarbonate was stirred for 7 days at room temperature.
13. The rest material was a substrate **2**. The reverse reaction afforded substrate **2** by the loss of ammonia during the work up procedure.
14. Bromoacetic acid (6.2 mg, 45 μmol) and DCC (4.6 mg, 22 μmol) were dissolved in DMF (100 μL) and this mixture was stirred at room temperature for 1 h. Then, amino

oligosaccharide **3** (5 mg, 2.2 μmol) and $NaHCO_3$ (2.0 mg, 24 μmol) in H_2O (100 μL) were added to the solution and this mixture was stirred at room temperature. The reaction was monitored by TLC (2-propanol/1 M NH_4OAc = 2:1). After 1.5 h the mixture was neutralized by $NaHCO_3$ and evaporated in vacuo. Purification of the residue by gel permeation column (Sephadex G-25, ϕ1.5 cm × 30 cm, H_2O) afforded **4** (4 mg, 1.7 μmol) in 77% yield. ^{1}H NMR (400 MHz, HOD = 4.81) δ 5.22 (s, 1H, Man4-H-1), 5.16 (br d, 1H, GlcNAc1-H-1), 5.03 (s, 1H, Man4′-H-1), 4.86 (s, 1H, Man3-H-1), 4.70 (m, 3H, GlcNAc2,5,5′-H-1), 4.53 (d, 2H, Gal6,6′-H-1), 4.34 (br s, 1H, Man3-H-2), 4.28 (br d, 1H, Man4-H-2), 4.20 (br d, 1H, Man4′-H-2), 2.77 (br dd, 2H, NeuAc7,7′-H-3_{eq}), 2.17 (s, 3H, Ac), 2.15 (s, 6H, Ac × 2), 2.12 (s, 6H, Ac × 2), 2.10 (s, 3H, Ac), 1.80 (dd, 2H, NeuAc7,7′-H-3_{ax}); MALDI-TOF mass found: m/z 2340.8 calcd for $[M-H]^-$: 2341.7.

15. Undecapeptide **5** was synthesized by conventional Fmoc method (DCC/HOBt).
16. Analogue **6** was identified by NMR and mass spectroscopy. ^{1}H NMR (400 MHz, HOD = 4.81) δ 7.18 (4H, Ph), 6.89 (4H, Ph), 5.22 (s, 1H, Man4-H-1), 5.14 (br d, 1H, GlcNAc1-H-1), 5.04 (s, 1H, Man4′-H-1), 4.86 (s, 1H, Man3-H-1), 4.69–4.63 (m, 5H, GlcNAc2,5,5′-H-1, Tyr-αH, Cys-αH), 4.55–4.52 (m, 4H, Gal6,6′-H-1, Gln-αH, Ser-αH), 4.44–4.38 (m, 4H, Glu-αH × 2, Arg-Hα, Thr-αH), 4.34 (br s, 1H, Man3-H-2), 4.28 (m, 3H, Man4-H-2, Thr-βH), 4.23 (d, 1H, J = 5.9 Hz, Val-αH), 4.20 (br d, 1H, Man4′-H-2), 4.15 (1H, Arg-αH), 3.30, 3.25 (each 2H, Arg-CH_2), 3.14–2.99 (6H, Cys-βH, Tyr-βH × 2), 2.76 (br dd, 2H, NeuAc7,7′-H-3_{eq}), 2.57 (2H, Gln-γCH_2), 2.49, 2.35 (each 2H, Glu-γCH_2), 2.23–2.10 (m, 3H, Val-βH, Gln-βH), 2.16 (s, 3H, Ac), 2.15 (s, 6H, Ac × 2), 2.12 (s, 6H, Ac × 2), 2.10–1.98 (m, 6H, Glu-βH × 2, Arg-βH), 2.07 (s, 3H, Ac), 1.92–1.57 (m, 8H, NeuAc7,7′-H-3_{ax}, Arg-βH, Arg-γCH_2 × 2), 1.23 (d, 3H, Thr-γCH_3), 1.04 (d, 6H, Val-γCH_3); MALDI-TOF mass found: m/z 3694.3; calcd for $[M-H]^-$: 3694.4.
17. Elution condition; column: Develosil ODS HG-5 (0.3 × 150 mm); linear increase of acetonitrile concentration from 0% to 90% in 0.1% aqueous TFA over 60 min at a flow rate of 0.15 mL min^{-1}.