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# Chemo-enzymatic synthesis of glycosylated insulin using a GlcNAc tag

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ABSTRACT

Artificial insulin with an N-linked oligosaccharide was synthesized by a chemo-enzymatic method using endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M). GlcNAc-modified insulin was prepared by the reaction of the carboxymethyl glycoside of GlcNAc and 3 amino groups of bovine insulin using a dimethylphosphinothioic mixed anhydride (Mpt-MA) method. A transglycosylation reaction of the GlcNAc-modified insulin using Endo-M gave mono-transglycosylated insulin predominantly. We determined the transglycosylation site of the mono-transglycosylated insulin.



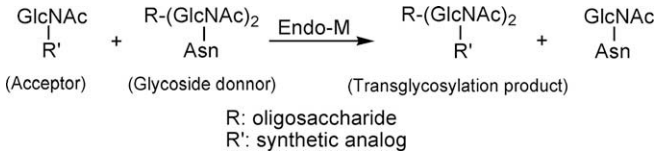
## 1. Introduction

Oligosaccharides in glycoproteins play important roles in biological processes such as cell–cell interactions and substrate–receptor recognition.[1–4] In order to study the functions of these oligosaccharides, it is important to synthesize the glycopeptides and their analogues. It was recently reported that the addition of carbohydrates could potentiate the effects of the drugs; for example, prolongation of the half-life of erythropoietin in vivo.[5–8]

The most common strategy for glycopeptides synthesis is to incorporate glycosyl amino acids as building blocks using a conventional solid-phase or solution-phase peptide synthesis method.[9–14] However, it remains difficult to synthesize a glycoprotein using this method.

Although artificial glycosylation of target proteins has been preformed using genetic expression in mammalian cells, glycosylation within mammalian cells cannot be controlled with respect to the structure of the carbohydrate and the glycosylation site. Recently, the synthesis of glycosylated insulin using these techniques was reported.[15]

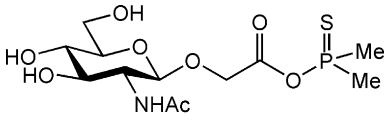
Endo-β-*N*-acetylglucosaminidases (EC 3.2.1.96) hydrolyze the glycosidic bond in the *N*,*N*-diacetylchitobiose moiety of N-linked sugar chains in glycoproteins.[16] This enzyme from *Mucor hiemalis* (Endo-M) transfers the oligosaccharide blocks of N-linked sugar chains, such as high-mannose-type, complex-type and hybrid-type oligosaccharides, from a glycopeptide or a glycoprotein to suitable acceptors having an *N*-acetylglucosamine (GlcNAc) residue (Scheme 1).[17–21]




**Scheme 1.** Transglycosylation reaction of Endo-M.

Endo-M was used to effectively reconstruct and remodel oligosaccharide derivatives. As examples using Endo-M, synthesis of glycosylated calcitonin and remodeling of RNAase were achieved.[22–25]

However, the chemo-enzymatic carbohydrate modification of a protein without a sugar chain using transglycosylation reaction has not been attempted. When a GlcNAc residue is added to a protein, the protein can be modified with a sugar chain using Endo-M. In order to connect a GlcNAc moiety and the amino groups of a protein by an amide linkage, we designed a dimethylphosphinothioic mixed anhydride (Mpt-MA)[26] of carboxymethylglycoside **1** for the linkage (Fig. 1). We designated the compound **1** a 'Sugaring tag'. In this study, bovine insulin was used as a model protein.




**1**

**Figure 1.** Structure of 'Sugaring tag'.

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## 2. Results and discussion

### 2.1. Synthesis of 'Sugaring tag'

First, we synthesized 'Sugaring tag **1**; as shown in Scheme 2. Reaction of 2-(benzyloxy)ethanol and oxazoline **2**[27] in the presence of 10-(*R*)-camphorsulfonic acid (CSA) gave the corresponding β-glycoside **3** in 82% yield. The benzyl group of **3** was removed by hydrogenation in the presence of 10% Pd/C to provide **4** in 78% yield. The corresponding carboxylic acid derivative **5** was obtained in 82% yield by a Jones oxidation.[28] Deprotection of the Ac groups of **5** using sodium methoxide quantitatively generated **6**. The reaction of the cesium salt of **6** and dimethylphosphinothioyl chloride (Mpt-Cl) in DMF gave the desired 'Sugaring tag **1**'. We used **1** without further purification.

### 2.2. Preparation of tri-GlcNAc insulin

Insulin is composed of two polypeptide chains (A and B) connected by disulfide bridges. Three amino groups are present in bovine insulin; two N-terminal sites and Lys B29.[29]

The reaction of bovine insulin and **1** was carried out in DMF–phosphate buffer with the pH maintained between 8.0 and 10.5 by the addition of 0.05 M NaOH (Scheme 3). Purification by gel filtration on Sephadex G-25 and affinity chromatography using wheat germ agglutinin (WGA) HPLC gave GlcNAc–modified insulins. When 1 equiv of **1** was used, only mono-GlcNAc insulin was produced in 30% ratio (Table 1). However, when 10 equiv of **1** was used, we obtained tri-GlcNAc insulin **7** as a major product. This was lyophilized to give tri-GlcNAc insulin **7**. The ratio was 89% as shown in Table 1. Characterization of these products used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS); Found: *m*/*z* 6515.92. Calcd: 6513.86.

### 2.3. Transglycosylation reaction of tri-GlcNAc insulin using Endo-M

Next, we attempted a transglycosylation of complex-type oligosaccharide blocks to tri-GlcNAc insulin **7** by Endo-M. Using tri-GlcNAc insulin **7** as the glycosyl acceptor, the transglycosylation of a complex-type oligosaccharide block [(NeuAc-Gal-GlcNAc-Man)$_2$-Man-GlcNAc-] from sialylglycopeptide SGP as a glycosyl donor in the presence of Endo-M was examined under the reaction conditions that were described previously.[30] The RP-HPLC profile of the transglycosylation from SGP to tri-GlcNAc insulin **7** for 8 h is shown in Figure 2. We assumed the 2 new products that appeared corresponded to mono-transglycosylated insulin **8** and di-transglycosylated insulin **9**. MALDI-TOF MS data indicated **8** (Found: *m*/*z* 8515.38. Calcd: 8513.53) and **9** (Found: *m*/*z* 10512.68. Calcd: 10515.22) could be clearly discriminated.

The time course of the transglycosylation reaction of tri-GlcNAc insulin **7** is shown in Figure 3. The peak corresponding to **8** appeared before that to **9**. The transglycosylation yield was based on the starting tri-GlcNAc insulin **7**. By the transglycosylation reaction using Endo-M, **8** (single isomer) was obtained in 41% yield after 4 h and **9** (mixture of three kinds of isomer) in 13% yield after 8 h.

Finally, we determined the transglycosylation site for mono-transglycosylated insulin **8**. Reduction of the disulfide bonds in modified insulin **8** with dithiothreitol (DTT)[31] and partial hydrolysis of the glycoprotein with trypsin (EC 3.4.21.4) gave the corresponding fragments. Characterizations of these fragments used MALDI-TOF MS spectroscopy (Figs. 4 and 5). MALDI-TOF MS spectroscopy of the fragments after DTT treatment indicated the transglycosylated B chain **10**. Trypsin digestion showed the transglycosylated the N-terminus of the A chain and B chain included fragment, **11** [A(1–21) + B(1–22)]. From these results, we found that the transglycosylation reaction using Endo-M proceeded at N-terminus of the B chain which seems to be the most flexible from the data of PDB ID 2A3G (Scheme 3).

## 3. Conclusion

We modified bovine insulin in order to introduce a natural N-glycan using a chemo-enzymatic approach. First, tri-GlcNAc insulin was prepared chemically by the reaction of 'Sugaring tag **1**' with bovine insulin. Next, a transglycosylation reaction of the tri-GlcNAc insulin using Endo-M gave transglycosylated insulin. Finally, we determined that the transglycosylation reaction had occurred at only the GlcNAc residue of N-terminus of the B chain giving a 41% yield.

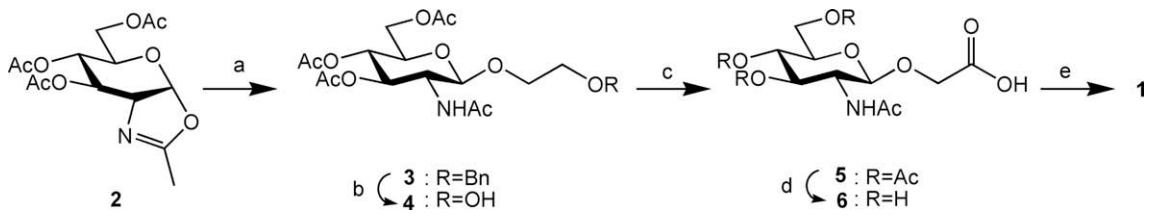
## 4. Experimental

### 4.1. General methods and materials

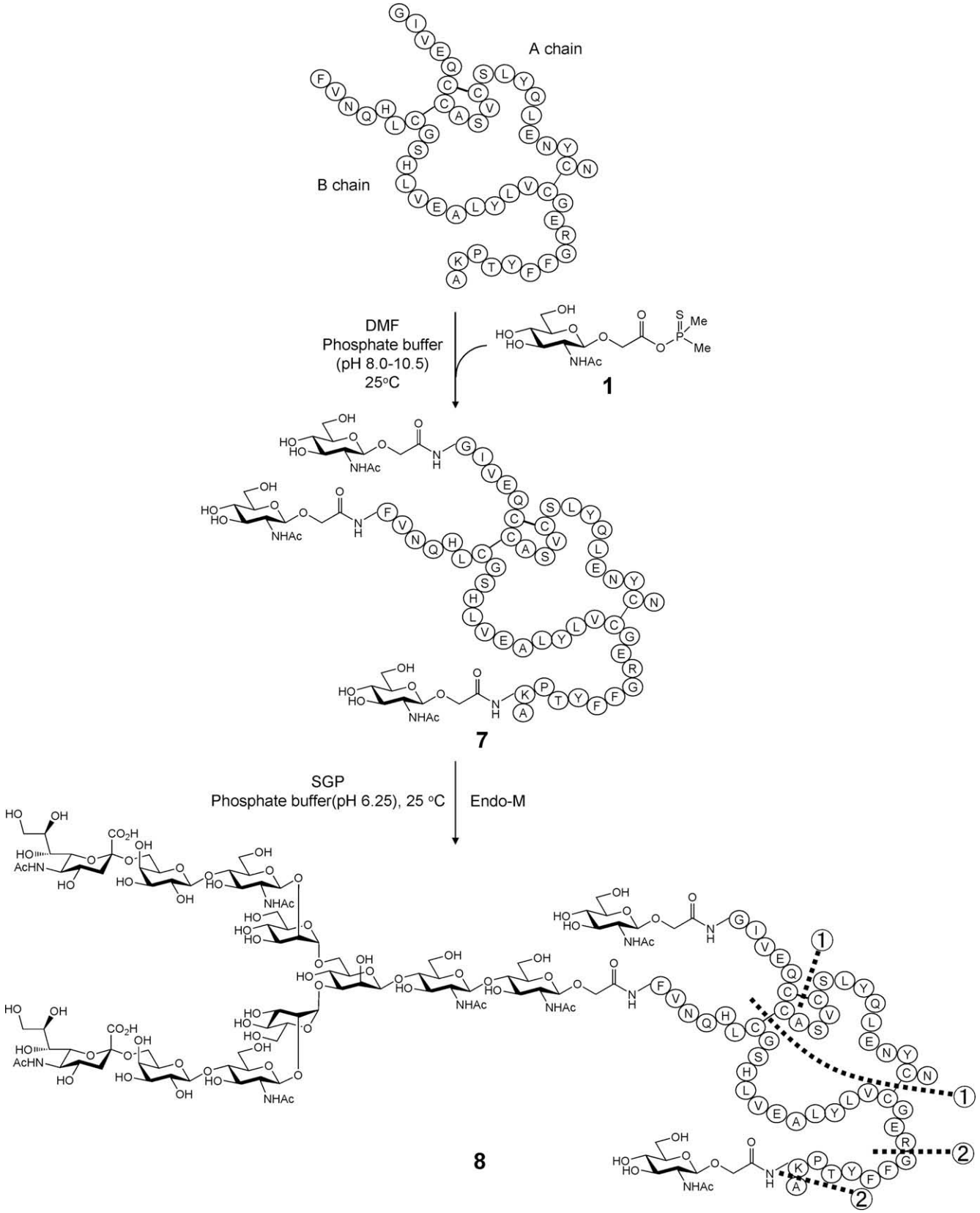
$^1$H NMR spectra were recorded at 400 MHz on a JEOL JNM-400. MALDI-TOF Mass spectra were obtained using a Daltonix-Bruker AUTOFLEX. The structure of the insulin derivatives were confirmed by MALDI-TOF MS in the linear and negative ion mode using 2,5-dihydroxy-benzoic acid as a matrix unless otherwise described. Reactions were monitored by thin-layer chromatography (TLC) on a pre-coated plate of Silica Gel 60F$_{254}$ (Merck) and/or RP-HPLC (Waters HPLC system equipped with a Delta 600 series interface). Flash chromatography was performed on silica gel (Silica Gel 60N, 40–50 μm; Kanto Chemical Co., Inc.). Bovine insulin was purchased from Sigma. Endo-M and the biantennary complex-type sialylglycopeptide (SGP),[32] Lys-Val-Ala-Asn[(NeuAc-Gal-GlcNAc-Man)$_2$ Man-GlcNAc$_2$]-Lys-Thr were from Tokyo Chemical Industry Co., Ltd. A wheat germ agglutinin (WGA) HPLC column was from Seikagaku Corporation.

### 4.2. 2-*O*-Benzyloxyethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (3)

10-(*R*)-Camphorsulfonic acid (5.65 g, 24.3 mmol) and 2-(benzyloxy)ethanol (32 mL, 225 mmol) were added to a stirred solution of 4′,5′-Dihydro-2′-methyloxazolo[5′,4′:1,2]-3,4,6-tri-*O*-acetyl-1,2-




**Scheme 2.** Preparation of 'Sugaring tag **1**'. Reagents and conditions: (a) HO(CH$_2$)$_2$OBn, CSA, CH$_2$Cl$_2$, MS 4 Å, 40 °C, 3 h, 82%; (b) H$_2$, 10% Pd–C, MeOH, rt, 23 h, 78%; (c) Jones reagent, acetone, rt, 13 h, 82%; (d) NaOMe (cat.), MeOH, rt, 18 h, quant; (e) Cs$_2$CO$_3$, water then Mpt-Cl, DMF.




**Scheme 3.** Synthesis of glycosylated insulin using Endo-M. (①: cleavage position by DTT, ②: hydrolysis position with trypsin).

dideoxy-α-D-glucopyranoside (7.28 g, 22.1 mmol) and powdered 4 Å molecular sieves (ca. 2 g) in $CH_2Cl_2$ (50 mL) at 40 °C. The resulting mixture was stirred for 3 h. The reaction mixture was cooled, then saturated aq $NaHCO_3$ and $CH_2Cl_2$ were added. The $CH_2Cl_2$ layers were washed with saturated aq $NaHCO_3$, brine, dried over anhydrous $Na_2SO_4$, and concentrated in vacuo. Purification by flash

**Table 1**
Reaction conditions using 'Sugaring tag **1**'

| Sugaring tag 1 (equiv) | Ratio of GlcNAc in insulin[a] (%) | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3[b] |
| 1 | 70 | 30 | 0 | 0 |
| 3 | 1 | 18 | 42 | 39 |
| 5 | 0 | 0 | 28 | 72 |
| 10 | 0 | 0 | 11 | 89 |

[a] The ratio of the GlcNAc tagged insulins were calculated from the ratio of the peaks of these GlcNAc tagged insulins. This was based on the assumption that the absorption wavelengths of these GlcNAc tagged insulins were approximately the same.
[b] Number of GlcNAc substitution.

column chromatography (hexane–AcOEt, gradient elution 1:1–1:3) provided 2-*O*-benzyloxyethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (8.77 g, 82%) as a colorless amorphous solid. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 7.38–7.28 (5H, m, Ph), 5.60 (1H, d, *J* = 8.4 Hz, NH), 5.24 (1H, dd, *J* = 10.4, 9.3 Hz, H-3), 5.08 (1H, t, *J* = 9.3 Hz, H-4), 4.75 (1H, d, *J* = 8.4 Hz, H-1), 4.55 (2H, s, $CH_2$), 4.26 (1H, dd, *J* = 12.2, 4.9 Hz, H-$6_a$), 4.13 (1H, dd, *J* = 12.2, 2.4 Hz, H-$6_b$), 3.98 (1H, td, *J* = 11.6, 3.9 Hz, $CH_{2a}$), 3.91 (1H, dt, *J* = 10.4, 8.4 Hz, H-2) 3.80–3.75 (1H, m, $CH_{2b}$), 3.69 (1H, ddd, *J* = 9.3, 4.9, 2.4 Hz, H-5), 3.66–3.60 (2H, m, $CH_2$), 2.08 (3H, s, Ac), 2.02 (3H, s, Ac), 1.86 (3H, s, Ac), 1.76 (3H, s, Ac).

### 4.3. 2-Hydroxyethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (4)

2-*O*-Benzyloxyethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (15.0 g, 31.1 mmol) in MeOH (200 mL) was stirred under a $H_2$ atmosphere in the presence of 10% Pd/C (1.54 g) at room temperature for 23 h. The mixture was filtered through filter paper, and concentrated in vacuo. Purification by flash column chromatography ($CHCl_3$–MeOH, gradient elution 19:1–9:1) provided 2-hydroxyethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (24.2 g, 78%) as a colorless amorphous solid. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 5.86–5.80 (1H, br, NH), 5.25 (1H, dd, *J* = 10.8, 9.2 Hz, H-3), 5.06 (1H, dd, J = 10.2, 9.3 Hz, H-4), 4.71 (1H, d, *J* = 8.3 Hz, H-1), 4.25–4.11 (2H, m, H-6), 3.96–3.70 (6H, m, H-2, H-5, $CH_2\times2$), 2.85–2.80 (1H, br, OH), 2.10 (3H, s, Ac), 2.05 (3H, s, Ac), 2.04 (3H, s, Ac), 2.00 (3H, s, Ac).

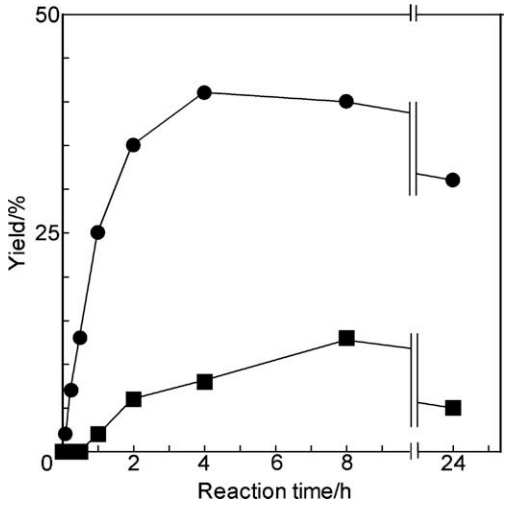



**Figure 3.** Time course of transglycosylation reaction using Endo-M. (●: mono-transglycosylated insulin **8**, ■: di-transglycosylated insulin **9**).

### 4.4. 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyloxyacetic acid (5)

Jones reagent (3.5 M, 18 mL) was added to a stirred solution of 2-hydroxyethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (12.5 g, 32.1 mmol) in acetone (200 mL) at 0 °C. After stirring for 13 h at room temperature, isopropanol was added to the reaction mixture. The mixture was evaporated in vacuo. Dichloromethane and brine were added to the residue. The organic layer was washed with brine, dried over anhydrous $Na_2SO_4$, and concentrated in vacuo. Recrystallization from AcOEt and hexane gave 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyloxyacetic acid (10.7 g, 82%) as a colorless crystal. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 6.61 (1H, d, *J* = 8.3 Hz, NH), 5.24 (1H, t, *J* = 9.8 Hz, H-3), 5.11 (1H, t, *J* = 9.8 Hz, H-4), 4.75 (1H, d, *J* = 8.3 Hz, H-1), 4.36 (2H, s, $CH_2$), 4.28 (1H, dd, *J* = 12.7, 4.4 Hz, H-$6_a$), 4.15 (1H, dd, *J* = 12.7, 2.4 Hz, H-$6_b$), 4.12–4.05 (1H, m, H-2), 3.72 (1H, ddd, *J* = 9.8, 4.4, 2.4 Hz, H-5), 2.10 (3H, s, Ac), 2.06 (3H, s, Ac), 2.03 (3H, s, Ac), 1.97 (3H, s, Ac). $^{13}$C NMR (400 MHz, $CDCl_3$) δ 172.1, 172.0, 171.0, 170.7, 169.3, 99.9, 72.6, 71.8, 68.4, 64.7, 61.9, 54.1, 22.9, 20.6, 20.6, 20.5.

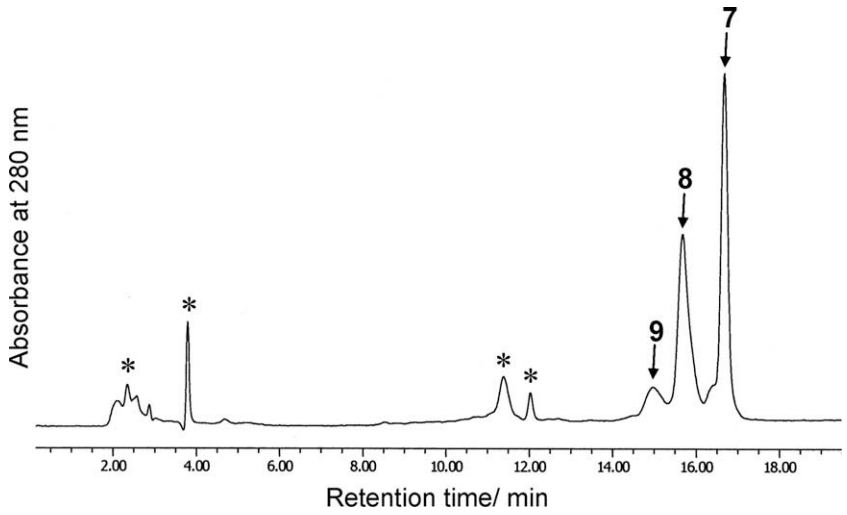



**Figure 2.** RP-HPLC profile of the Endo-M transglycosylation reaction. The transglycosylation to tri-GlcNAc insulin **7** at the reaction time of 8 h detected by the absorbance at 280 nm. *: Impurity from SGP.

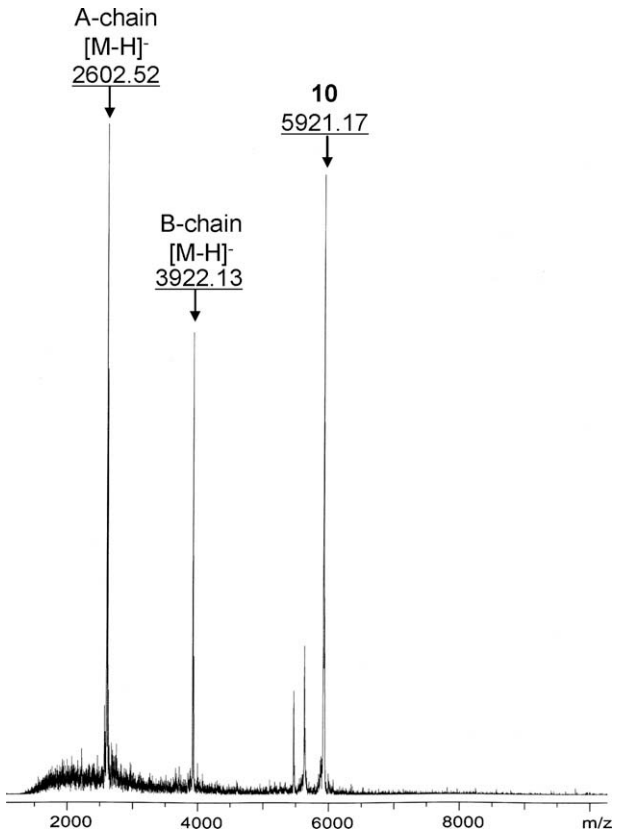



**Figure 4.** MALDI-TOF MS spectra after DTT treated mono-transglycosylated insulin **8**.

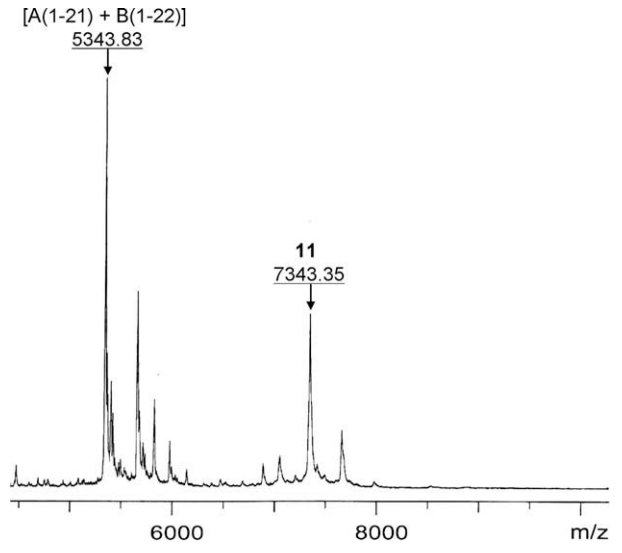



**Figure 5.** MALDI-TOF MS spectra after trypsin treated mono-transglycosylated insulin **8**.

### 4.5. 2-Acetamido-2-deoxy-β-D-glucopyranosyloxyacetic acid (6)

Sodium methoxide (91.0 mg, 1.68 mmol) was added to a stirred solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyloxyacetic acid (523 mg, 1.29 mmol) in MeOH (29 mL) at room temperature. The resulting mixture was stirred for 18 h and then neutralized with Amberlite IR 120 ($H^+$ form). The mixture was filtered and the filtrate was concentrated to give 2-acetamido-2-deoxy-β-D-glucopyranosyloxyacetic acid (355 mg, 99%) as a colorless amorphous solid. $^1$H NMR (400 MHz, $D_2O$): $\delta$ = 4.42 (1H, d, $J$ = 8.8 Hz, H-1), 4.03 (2H, s, $CH_2$), 3.76 (1H, dd, $J$ = 12.4, 5.2 Hz, H-6a), 3.62–3.54 (2H, m, H-2), 3.41–3.37 (1H, m,), 3.33–3.28 (2H, m,), 1.89 (3H, s, Ac). $^{13}$C NMR (400 MHz, $D_2O$) δ175.9, 174.7, 101.9, 77.0, 74.8, 70.8, 66.7, 61.7, 56.3, 23.2.

### 4.6. Tri-GlcNAc insulin 7

Cesium carbonate (49.0 mg, 150 μmol) was added to a stirred solution of 2-acetamido-2-deoxy-β-D-glucopyranosyloxyacetic acid (84.0 mg, 300 μmol) in water (1 mL). The water was removed azeotropically with EtOH and the cesium salt was dried over $P_2O_5$ in vacuo. Mpt-Cl (39.0 mg, 303 μmol) and 4 Å molecular sieves were added to a stirred solution of the cesium salt in DMF (2 mL) at 0 °C to room temperature for 3 h. The mixture was added to a stirred solution of bovine insulin (57.0 mg, 10 μmol) in DMF (2 mL) with 50 mM phosphate buffer (pH 9.5, 1 mL) at room temperature. The mixture was kept between pH 8.0 and 10.5 by the addition of 0.05 M NaOH. The solution was purified by gel filtration on Sephadex G-25 column with $H_2O$ as the eluant. The desired fractions were lyophilized to give a GlcNAc–insulin mixture (59.0 mg). The aliquot (10.0 mg) was loaded onto a WGA HPLC column (150 mm × 4.6 mm, 15.2 mg/mL gel, in 50 mM–Tris buffer [pH 7.3]). Elution was done using a linear gradient GlcNAc (0–120 mM) for 45 min at a flow rate of 0.5 mL/min and monitored by absorption at 254 nm. After desalting through a Sephadex G-25 column ($H_2O$), the desired fraction was lyophilized to give tri-GlcNAc insulin **7** (2.40 mg, 22% isolated yield based from the aliquot (10.0 mg) of GlcNAc–insulin mixture).

MALDI-TOF MS (positive reflector mode) Calcd for $C_{284}H_{423}N_{68}O_{96}S_6$ $[M+H]^+$ 6513.86. Found: $m/z$ 6515.92.

### 4.7. Transglycosylation reaction with Endo-M

A transglycosylation reaction was performed with a reaction mixture comprised of 10 mM tri-GlcNAc insulin, 60 mM SGP and 0.2 U/mL of Endo-M in a total volume of 20 μL of 0.4 M phosphate buffer (pH 6.25) containing 30% DMSO (v/v). After incubation for 0–24 h at 25 °C, aliquots (2 μL) were added to 98 μL of 0.2% trifluoroacetic acid (TFA) solution to stop the reaction. Analyses of the transglycosylation products used RP-HPLC. Elution was done using a linear gradient of acetonitrile (20–50%) containing 0.1% aqueous TFA in 20 min at a flow rate of 1 mL/min. The reaction products were monitored by absorption at 280 nm. The yields of the transglycosylation products, based on the acceptors, were calculated from the ratio of the peaks of the transglycosylation products to the glycosyl acceptor (see Fig. 3). This was based on the assumption that the absorption wavelengths of the transglycosylation products and of the acceptor were approximately the same. The purification of the transglycosylation products used HPLC, which was the same as the analysis methods. The structure of the products was confirmed by MALDI-TOF MS. Mono-transglycosylated insulin **8**: MALDI-TOF MS Calcd for $C_{360}H_{543}N_{73}O_{152}S_6$ $[M-H]^-$ 8513.53. Found: $m/z$ 8515.38. Di-transglycosylated insulin **9**: MALDI-TOF MS Calcd for $C_{436}H_{667}N_{78}O_{208}S_6$ $[M-H]^-$ 10515.22. Found: $m/z$ 10512.68.

### 4.8. DTT treated mono-transglycosylated insulin 8

A solution of **8** in 6 M guanidine hydrochloride containing 0.5 M Tris buffer (pH 8.1) and 2 mM EDTA was placed in a Eppendorf centrifuge tube. After flushing with $N_2$ gas the centrifuge tube was stopped and heated at 50 °C for 30 min to completely denature the insulin. One milligram of DTT was added. The centrifuge tube was flushed once again with $N_2$ gas, and then the reaction was allowed to proceed at 50 °C for 4 h. After cooling, water was added to

the mixture and washed with ether. The aqueous phase was analyzed by MALDI-TOF MS. Transglycosylated B chain **10**: MALDI-TOF MS Calcd for $C_{253}H_{384}N_{47}O_{111}S_2$ $[M-H]^-$ 5920.53. Found: *m*/*z* 5921.17.

### 4.9. Trypsin treated mono-transglycosylated insulin 8

Trypsin digested at the carboxyl side of Arg B22 and Lys B29 in B chain. A solution of **8** in 2% trypsin containing 0.1 M Tris buffer (pH 7.8, 380 μL) was placed in an Eppendorf centrifuge tube at 37 °C for 3 h. The tube was stopped and heated at 100 °C for 30 min to completely denature the trypsin. After cooling, the reaction mixture was analyzed by MALDI-TOF MS. Transglycosylated [A(1–21) + B(1–22)] **11**: MALDI-TOF MS Calcd for $C_{303}H_{468}N_{63}O_{135}S_6$ $[M-H]^-$ 7341.00. Found: *m*/*z* 7343.35.

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