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Glycoinsulins: Dendritic Sialyloligosaccharide-Displaying Insulins Showing a Prolonged Blood-Sugar-Lowering Activity

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Abstract: Mono-, di-, and trisialyloligosaccharides were introduced to mutant insulins through enzymatic reactions. Sugar chains were sialylated by α2,6-sialyltransferase (α2,6-SiaT) via an accessible glutamine residue at the N-terminus of the B-chain attached by transglutaminase (TGase). Sia2,6-di-LacNAc-Ins(B-F1Q) and Sia2,6-tri-LacNAc-Ins(B-F1Q), displaying two and three sialyl-*N*-acetyllactosamines, respectively, were administered to hyperglycemic mice. Both branched glycoinsulins showed prolonged glucose-lowering effects compared to native or lactose-carrying insulins, showing that sialic acid is important in obtaining a prolonged effect. Sia2,6-tri-LacNAc-Ins(B-F1Q), in particular, induced a significant delay in the recovery of glucose levels.

Introduction

Insulin, which is composed of two polypeptide chains (A and B) joined by disulfide bridges, is the most important protein for the treatment of virtually all type 1 and many type 2 diabetic patients.[1−4] Its greatest weakness, however, is its short half-life in the circulatory system.[5] Commercial long-acting insulin formulations are designed to be less soluble in physiological solutions and thereby are able to dissolve more slowly at the injection site.[6−8] The disadvantages of these long-acting insulins are in the complex administration procedure,[9] in the difficulty in controlling circulating glucose levels,[10] and in the risk of anaphylactoid reactions.[11−13] Soluble long-acting derivatives, such as Co^{3+}−insulin,[14] fatty acid-acylated insulin,[9] PEG-conjugated insulin,[15] and so on, have been reported as potential candidates to overcome these shortcomings.[10]

In the case of other widely used protein-based drugs, such as erythropoietin, the introduction of sialic acid-containing carbohydrates into the protein has been reported to extend their half-life in vivo.[16,17]

In many cases, glycosylation of targeted proteins was carried out using an expression system in mammalian cells. However, glycosylation in mammalian cells cannot be controlled in terms of the structure of the glyco chain, glycosylation site, and number. Therefore, the preparation of controlled site-specific glycosylation may benefit from a chemical or chemoenzymatic

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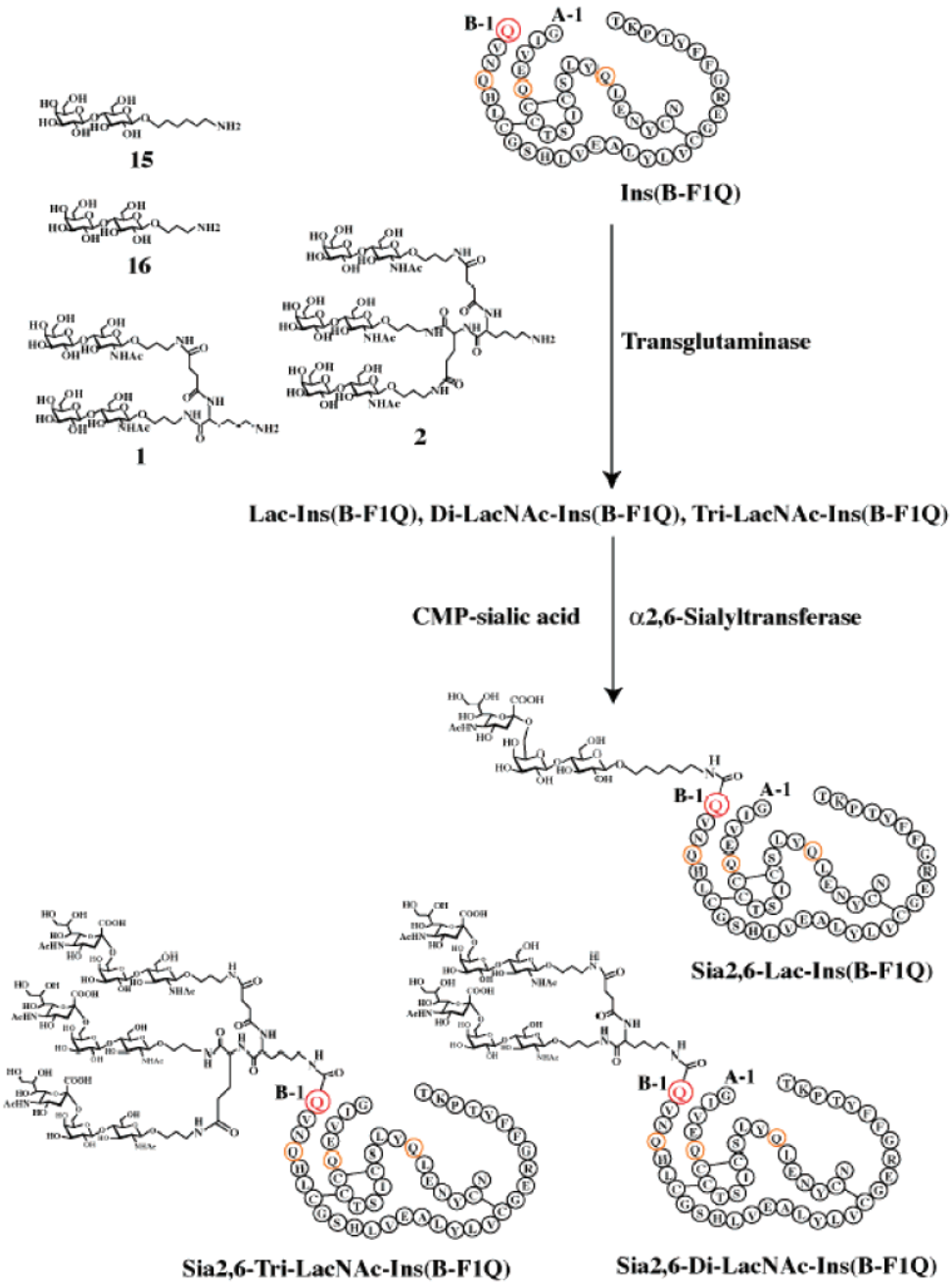
Scheme 1. Chemoenzymatic Syntheses of Sialooligosaccharide-Displaying Insulins



in vitro approach. Recently, Davis et al. have explored a chemical approach to creating a "glycodendriprotein" by the chemical attachment of galactose derivatives onto the inserted cysteine residue of target proteins.[18] Introduction of a sugar moiety to the glutamine (Q) residues using transglutaminase (TGase) was reported to be a very effective method for obtaining glycoconjugate peptides and proteins.[19−21]

We have reported that a glycosylated insulin using TGase could serve as a soluble long-acting option.[22] We had designed and synthesized a single-sialyllactose-displaying insulin, Sia2,6-Lac-Ins(B-F1Q), in which sialyllactose was enzymatically transferred to the glutamine residues (Q1 or Q4) of the insulin B-chain (Scheme 1). Sia2,6-Lac-Ins(B-F1Q) had increased solubility and prolonged biological potency. On the contrary, lactose-displaying insulin (Lac-Ins(B-F1Q)) showed glucose depression potencies similar to those of normal insulin (Ins-(WT)). We speculated that the sialic acid moiety of Sia2,6-Lac-Ins(B-F1Q) is an important factor in construction of long-acting insulins by protecting the insulin from decomposition and/or clearance in vivo.

In this study, aiming to obtain a further prolonged effect, we created divalent- and trivalent-sialyloligosaccharide-displaying insulins using an enzymatic approach. The glycosylation method

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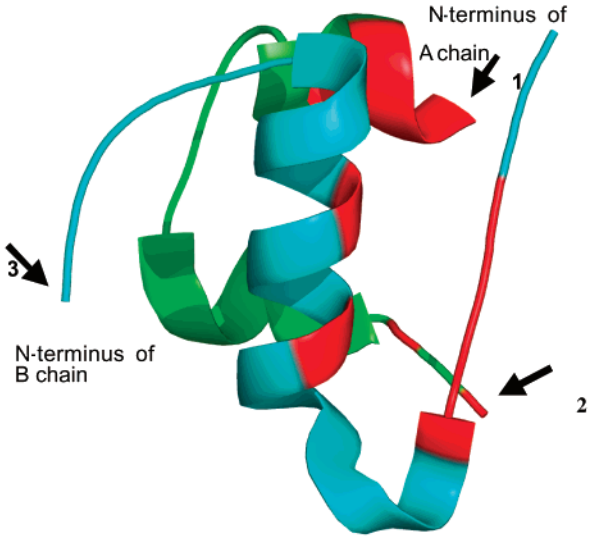

Figure 1. Glycosylation sites on the insulin structure: the 3D structure of human insulin in the T-state. This model is based on the crystal structure of Smith et al. (PDB ID 1G7A[23,25]). The A-chain is colored green, and the B-chain is colored cyan. The proposed receptor interaction sites (Gly A1, Ile A2, Val A3, Gln A5, Tyr A19, Asn A21, Val B12, Tyr B16, Gly B23, Phe B24, Phe B25, and Tyr B26) are colored red. The glycosylation sites are indicated by arrows: 1, N-terminus of the A-chain; 2, C-terminus of the A-chain; 3, N-terminus of the B-chain. This figure was prepared by PyMOL.[24]

through which the synthesized branched-sugar compounds were introduced to Ins(B-F1Q) was performed using TGase, and the transference of sialic acids from CMP-sialic acids to branched-sugar termini was performed by α2,6-sialyltransferase (α2,6-SiaT). Biological activities of these sugar-displaying insulins were described.

Results and Discussion

A. Expression of Mutant Insulins Having an Accessible Glutamine Residue for Use as a Glycosylation Site. 1. Design of Sugar-Displaying Insulins. Our previous research[22] showed that native insulin was not a substrate of transglutaminase although it has three glutamine (Gln) residues. Therefore, accessible Gln residues for use as glycosylation sites needed to be inserted by genetic mutation. Although the details and mechanism of the insulin−insulin receptor (IR) interaction are still to be investigated, some residues on the insulin surface are known to be important to its interaction with the IR. The "classical binding surface" includes Gly A1, Gln A5, Tyr A19, Asn A21, Val B12, Tyr B16, Gly B23, Phe B24, Phe B25, and Tyr B26.[23] Additionally, internal residues Ile A2 and Val A3 are also believed to interact with the IR. These residues cluster on one side of the 3D structure of the insulin (Figure 1).[24,25] We prepared three mutant insulins with Gln substitutions on either the N-terminus of the A- or B-chains or the C-terminus of the A-chain. The addition of sugar chains to the Gln residues on either terminus of the insulin A-chain would be expected to weaken the interaction between the insulin and the IR, resulting in a slowing of the degradation rate of the insulin molecules. The addition of a sugar chain to the Gln residues on the N-terminus of the B-chain would be expected to have less effect on the insulin−IR interaction.

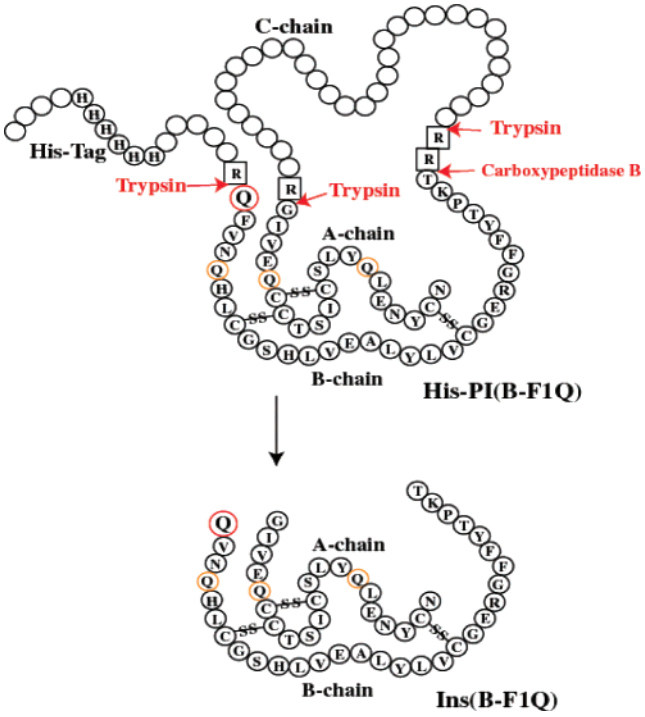

Figure 2. A single-step conversion from His−PI(B-F1Q) to Ins(B-F1Q). The His-tag and insulin C-chain are released concomitantly by trypsin and carboxypeptidase B.

2. Genetic Expression of Mutant Insulins. Mutagenic insulins Ins(B-F1Q), having a glutamine (Q) residue instead of phenylalanine (F) at the N-terminus of the insulin B-chain, Ins(A-G1Q), having a Q residue instead of glycine (G) at the N-terminus of the insulin A-chain, and Ins(A-N21Q), having a Gln residue instead of asparagine (N) at the C-terminus of the A-chain, were expressed as proinsulin, which is suitable for refolding after expression as an inclusion body in *Escherichia coli*.[26,27] In 1996, a single-step conversion method from IgG-binding domain (ZZ)-tagged proinsulin (ZZ-proinsulin) to insulin was reported by Per Jonasson et al.[28] This method was applied to the removal of the polyhistidine (His) tag fused to the N-terminus of proinsulins; the expressed His-fused proinsulins His−PI(WT), His−PI(B-F1Q), His−PI(A-G1Q), and His−PI(A-N21Q) had an arginine (R) residue between the His-tag and the proinsulin (Figure 2). It was revealed that trypsin cleaved at basic amino acid residues located in the C-terminus region during proinsulin processing, however, did not cleave at Arg-B22 and Lys-B29.[29] A single-step conversion from His−PI to insulin was carried out using trypsin and carboxypeptidase B in quantitative yield, and purification was performed by RP-HPLC.

B. Synthesis of Branched Oligosaccharides. The syntheses of divalent and trivalent *N*-acetyllactosaminyl (LacNAc) derivatives **1** and **2** are outlined in Schemes 2 and 3, respectively. First, N^{ϵ}-(benzyloxycarbonyl)-L-lysine (Lys(Z)) was acylated with succinic anhydride to afford dicarboxyl peptide **3** in 86%

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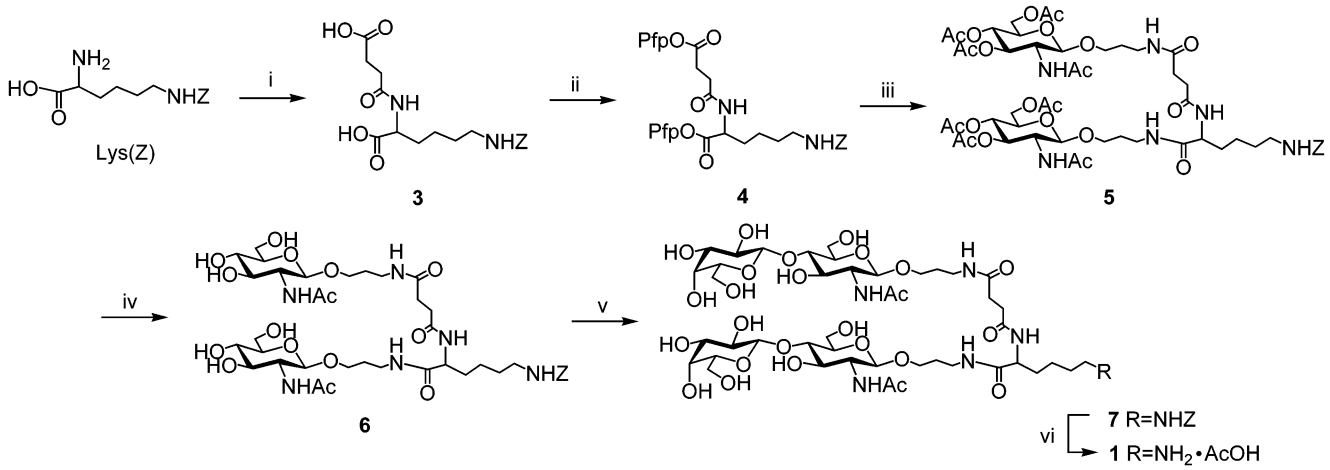
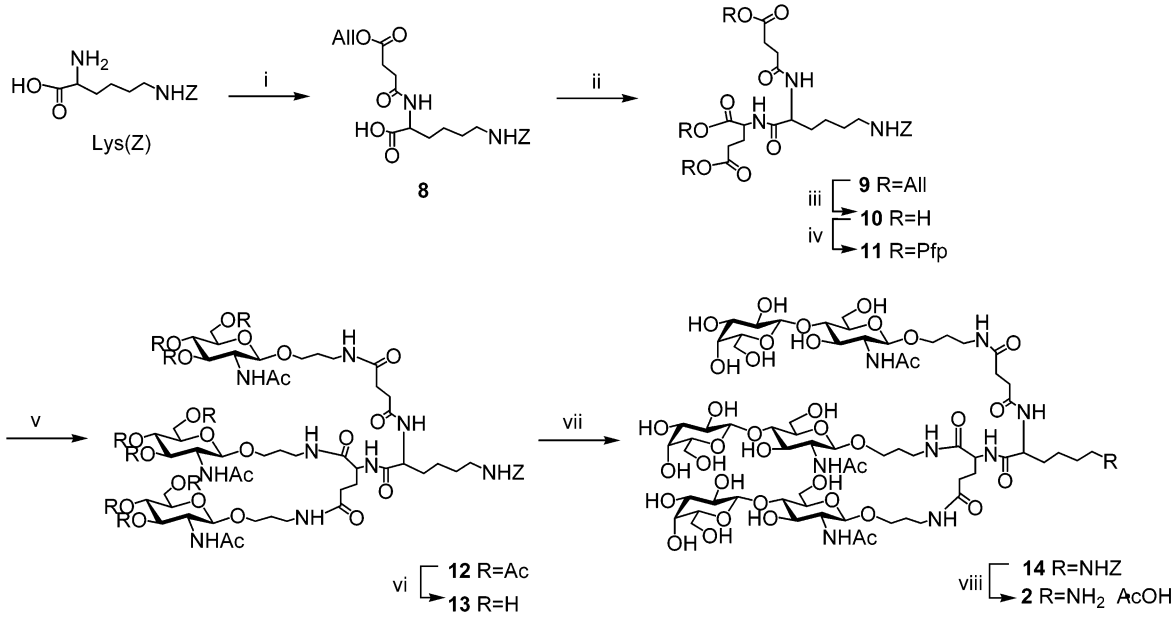
Scheme 2 [a]



[a] Reagents and conditions: (i) succinic anhydride, methanol, 40 °C, 12 h, 86%; (ii) pentafluorophenyl trifluoroacetate, pyridine, DMF, room temperature (rt), 1 h, 77%; (iii) 3-aminopropyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside, diisopropylethylamine, DMF, rt, 17 h, 84%; (iv) triethylamine, MeOH−H_2O, rt, 4 h, 90%; (v) UDP−galactose, β-1,4-galactosyltransferase, 50 mM HEPES buffer containing 10 mM $MnCl_2$ (pH 6.0), 37 °C, 48 h, 83%; (vi) H_2, 10% Pd−C, AcOH, MeOH−H_2O, rt, 6 h, 92%.

Scheme 3 [a]



[a] Reagents and conditions: (i) *N*-succinimidylsuccinic acid allyl ester, triethylamine, DMF, rt, 8 h, 98%; (ii) L-glutamic acid diallyl ester, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, DMF, rt, 15 h, 86%; (iii) $(Ph_3P)_4Pd$, *N*-methylaniline, THF, rt, 14 h, 74%; (iv) pentafluorophenyl trifluoroacetate, pyridine, DMF, rt, 1 h, 79%; (v) 3-aminopropyl 2-acetamido-3,4,6-tri-*O*-acetyl- 2-deoxy-β-D-glucopyranoside, diisopropylethylamine, DMF, rt, 17 h, 85%; (vi) triethylamine, MeOH−H_2O, rt, 4 h, quantitative yield; (vii) UDP−galactose, β-1,4-galactosyltransferase, 50 mM HEPES buffer containing 10 mM $MnCl_2$ (pH 6.0), 37 °C, 48 h, 96%; (viii) H_2, 10% Pd−C, AcOH, MeOH−H_2O, rt, 6 h, 84%.

yield. The coupling reaction of 3-aminopropyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (Ac_3-GlcNAc-propNH_2)[30] with the peptide **3** was carried out via pentafluorophenyl ester[31] **4** to give glycopeptide **5** in 84% yield. After de-*O*-acetylation of **5** by treatment with triethylamine, enzymatic elongation of the divalent GlcNAc derivative **6** was carried out using β1,4-galactosyltransferase with UDP−Gal in a HEPES buffer (50 mM, pH 6.0) for 48 h according to the conditions reported previously[32] to give divalent LacNAc peptide **7** in 83% yield. Finally, hydrogenation of **7** gave the desired glycopeptide **1** in 92% yield. On the other hand, glutamic acid was chosen as the branch point for the synthesis of trivalent LacNAc peptide (Scheme 3). Lys(Z) was treated with *N*-succinimidylsuccinic acid allyl ester to afford **8** in 98% yield. Condensation of the monocarboxyl derivative **8** with L-glutamic acid diallyl ester was carried out by DCC coupling in the presence of HOSu and gave triallyl ester **9** in 86% yield. Deallylation of **9** by *N*-methylaniline in THF catalyzed by $(Ph_3P)_4Pd(0)$[33] then gave the tricarboxyl derivative **10** in 74% yield. Intermediate **10** was activated by the pentafluorophenyl groups and condensed with Ac_3-GlcNAc-propNH_2. After de-*O*-acetylation, enzymatic ga-

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lactosylation of the trivalent GlcNAc derivative **13** proceeded smoothly to give trivalent LacNAc peptide **14** in 96% yield. Finally, hydrogenation of **14** gave the desired glycopeptide **2** in 84% yield. All spectral data (^{1}H and ^{13}C NMR) are described in the Experimental Section.

C. Glycoslylation of Mutant Insulins. Purified Ins(B-F1Q), Ins(A-G1Q), and Ins(A-N21Q) were tested for glycosylation with lactose derivatives **15** and **16** (Scheme 1). Ins(B-F1Q) and Ins(A-G1Q) were reacted with **15** to produce the monolactose-attached insulin in 50% yield, but Ins(A-N21Q) did not react with **15** (Scheme 1). Compound **16**, which has a shorter spacer compared to that of **15**, did not attach to any mutant insulins. The glycosylation site of Ins(B-F1Q) was determined to be at Q1 or Q4 of the insulin B-chain by MALDI-TOF analysis of the B-chain degraded from the N-terminus by aminopeptidase M.[22] The ^{1}H NMR spectrum of Ins(B-F1Q) showed that the chemical shift of the N-terminal region (B2−B5) of the insulin B-chain was changed (data not shown), suggesting the local structure of the insulin B-chain was changed. This change may explain why TGase glycosylation occurred at Q4 of the B-chain of Ins(B-F1Q). Since insulin is known to form stable hexamers in the presence of Zn^{2+},[6] the introduction of carbohydrate residues to this region may influence the formation of the insulin self-assembly.

Surprisingly, these divalent and trivalent sugar derivatives (**2** and **3**) were also effectively introduced to Ins(B-F1Q) using TGase. Sialylation of the lactose-attached insulins was almost quantitively carried out in the presence of sialyltransferase and CMP-sialic acid (Scheme 1). Synthesized divalent-sugar-displaying insulin (Sia2,6-di-LacNAc-Ins(B-F1Q)) and trivalent-sugar-displaying insulin (Sia2,6-tri-LacNAc-Ins(B-F1Q)) were purified by RP-HPLC, and their molecular weights were analyzed by MALDI-TOF mass spectroscopy (Figure 3). These mass spectra demonstrate that the sialyl groups were transferred by α2,6-SiaT to all termini of the branched sugars.

To investigate whether changes in the secondary/tertiary structure of the insulin molecule occurred because of mutation and glycosylation, far-UV CD spectra of the insulin derivatives were compared to that of Ins(WT) (Figure 4). The CD spectrum of Ins(A-G1Q) alone was different from that of Ins(WT), suggesting the α-helix structure of the N-terminal region of the insulin A-chain was changed by point mutation. The spectra of all insulin derivatives except for Ins(A-G1Q) were similar to that of Ins(WT).[15,34−37] Therefore, F1Q mutation and glycosylation at the N-terminus of the insulin B-chain had no effect on their overall structures on the basis of the CD spectra.

D. Biological Activity of Sugar-Displaying Insulins. 1. Blood-Glucose-Lowering Effects Were Measured Using Streptozotocin (STZ)-Mice. STZ is a drug that selectively destroys insulin-producing pancreatic β-cells, providing a model of type 1 diabetes.[38] STZ-mice are an appropriate model for testing the prolonged action of insulin derivatives.[5] Figure 5 shows the monitored blood-glucose levels of STZ-mice after subcutaneous administrations of Ins(WT), Ins(B-F1Q), Ins(A-G1Q), Lac-Ins(B-F1Q), Sia2,6-Lac-Ins(B-F1Q), Sia2,6-di-Lac-

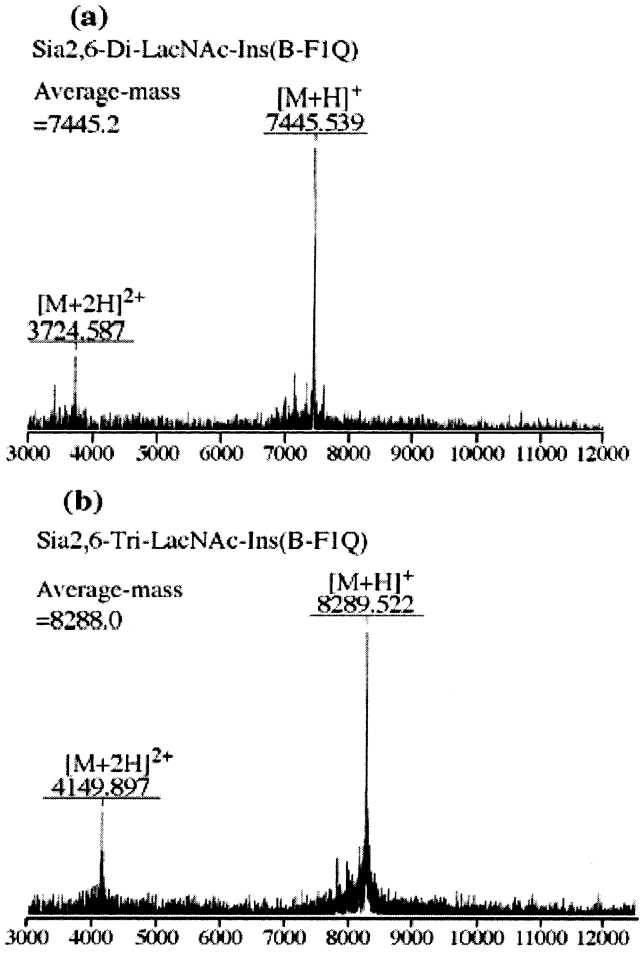

Figure 3. MALDI-TOF mass spectra of (a) Sia2,6-di-LacNAc-Ins(B-F1Q) and (b) Sia2,6-tri-LacNAc-Ins(B-F1Q).

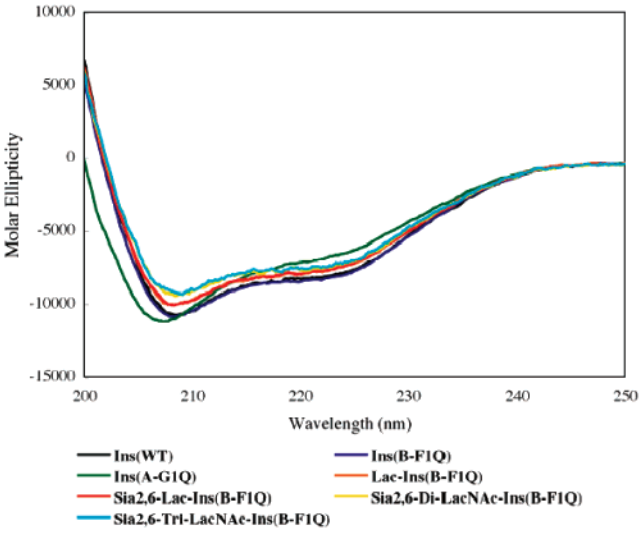

Figure 4. CD spectra of insulins and glycoinsulins.

NAc-Ins(B-F1Q), and Sia2,6-tri-LacNAc-Ins(B-F1Q). Three types of sialyloligosaccharide-displaying insulins showed an apparent prolonged glucose-lowering effect compared to the control Ins(WT). $T_{1/2}$ values of these compounds are listed in Table 1. Monovalent and divalent-sialyloligosaccharide-displaying insulin showed minor differences in glucose-lowering ability.

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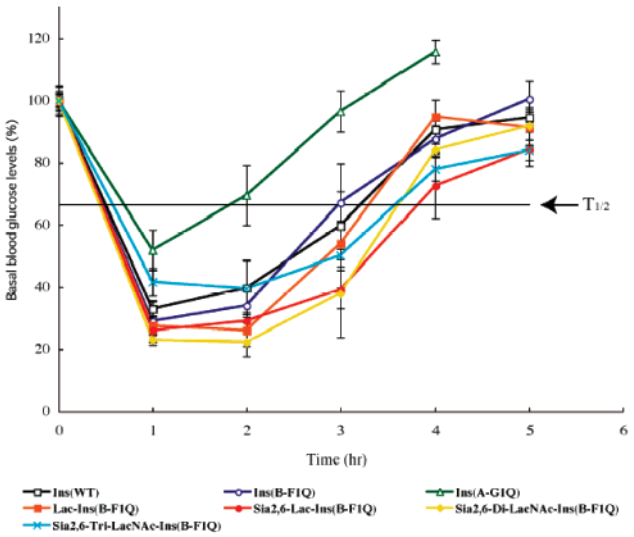

Figure 5. Blood-glucose levels in STZ-mice following subcutaneous injection (1 U/mouse) of insulins and glycoinsulins. Data are means ± SE, $n = 5$.

Table 1. Half-Life Values ($T_{1/2}$) of Ins(WT) and Glycoinsulins in Vivo

sample	$T_{1/2}$ (h) (±SE)
Ins(WT)	3.22 (0.27)
Ins(B-F1Q)	2.98 (0.24)
Ins(A-G1Q)	1.83 (0.28)
Lac-Ins(B-F1 Q)	3.31 (0.12)
Sia2,6-Lac-Ins(B-F1Q)	3.82 (0.22)
Sia2,6-di-LacNAc-Ins(B-F1Q)	3.62 (0.21)
Sia2,6-tri-LacNAc-Ins(B-F1Q)	3.59 (0.25)

The glucose-lowering effect of Sia2,6-tri-LacNAc-Ins(B-F1Q) was weak, but the recovery of glucose levels was slower than those of the other insulin preparations. On the contrary, lactose-displaying insulin, Lac-Ins(B-F1Q), showed rapid recovery of glucose levels. This might be due to the binding to the lactose receptor on the liver.

The behavior of Ins(B-F1Q) was quite close to that of Ins-(WT), but the glucose depression activity of Ins(A-G1Q) was significantly lower (Figure 5). The reason for the decreased activity was thought to be that the structure of Ins(A-G1Q) was disturbed by genetic mutation as supported by the CD study (Figure 4).

2. Evaluation of the Insulin Receptor Binding Capacities of the Glycoinsulins. The insulin receptor binding capacities of the glycoinsulins were analyzed by the procedure reported by Shechter et al.[5,39] Briefly, [^{125}I]insulin was used as a tracer, and the binding capacity of glycoinsulins was determined by calculation of the rate of displacement of [^{125}I]insulin from mouse adipocytes (Table 2). The binding capacity of Ins(WT) was taken as 100%. The binding capacities of Sia2,6-di-LacNAc-Ins(B-F1Q) and Sia2,6-tri-LacNAc-Ins(B-F1Q) were 89.9% and 74.4%, respectively. The N-terminal region of the insulin B-chain was located on the opposite site of the receptor binding domain.[23] However, the sugar-chain moieties of Sia2,6-di-LacNAc-Ins(B-F1Q) and, especially, Sia2,6-tri-LacNAc-Ins-(B-F1Q) were large, thereby inhibiting insulin receptor binding. The glucose depression curve of Sia2,6-di-LacNAc-Ins(B-F1Q) was similar to that of Sia2,6-Lac-Ins(B-F1Q) (receptor binding capacity 100.9%) as there was little loss of binding capacity. The loss of binding affinity of the sterically hindered Sia2,6-tri-LacNAc-Ins(B-F1Q) on the receptor may explain why it has a low glucose-lowering effect, and this seems to cause the delay in the recovery of the glucose level.[5,40]

Table 2. Insulin Receptor Binding Potency[a]

sample	binding potency (%)
Ins(WT)	100
Ins(B-F1Q)	100.5
Sia2,6-Lac-In s(B-F1Q)	100.9
Sia2,6-di-La cNAc-Ins(B-F1Q)	89.9
Sia2,6-tri-La cNAc-Ins(B-F1Q)	74.4

[a] The rate of displacement of [^{125}I]insulin was estimated from adipocytes. The binding potency of Ins(WT) is represented as 100%.

Conclusion

We synthesized mono-, di-, and trisialyloligosaccharide-displaying insulins using a chemoenzymatic approach. These sialic acid-displaying insulins showed prolonged blood-glucose-lowering activity in STZ-mice compared to native insulin. The glucose-lowering effect of Sia2,6-tri-LacNAc-Ins(B-F1Q) was mild and continued for a longer period than that of the other insulins tested. This suggests that the trivalent-sialyloligosaccharide-displaying insulin is useful in treating diabetes in that it may prevent insulin shock, which results from a sudden drop in glucose level after the injection of insulin. The prolonged effect may be due to the multivalent effects of the sialooligosaccharides on the stability of insulin in the blood stream and low affinity for the insulin receptor. This strategy can be widely applicable to the synthesis of various sugar-displaying protein drugs with increased half-lives in the circulatory system, since large amounts of nonnatural oligosaccharide structures can also be prepared by the combined chemical and enzymatic strategy described herein as well as partial structures of naturally occurring *N*- and *O*-glycan chains.

Experimental Section

General Methods. Unless otherwise stated, all commercially available solvents and reagents were used without further purification. Optical rotations were determined with a Perkin-Elmer 241 polarimeter for samples in a 10 cm cell at ambient temperature (22 ± 2 °C). ^{1}H and proton-decoupled carbon NMR spectra were recorded at 400 and 100.4 MHz, respectively, on a JEOL JNM-lambda-400 FT-NMR spectrometer. Ring-proton assignments in NMR were made by first-order analysis of the spectra and supported by HH-COSY experiments. Elemental analyses were performed with a Yanako CHN recorder MT-6. FAB, ESI, and MALDI-TOF mass spectra were obtained with a JEOL JMS-HX110 mass spectrometer, a JEOL JMS-700TZ mass spectrometer, and a Bruker REFLEX III mass spectrometer, respectively. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel 60F_{254} (layer thickness 0.25 mm; E. Merck, Darmstadt, Germany) and/or RP-HPLC (Hitachi HPLC system equipped with a D-7000 series interface). Flash chromatography was performed on silica gel (silica gel 60N, 40−50 μm; Kanto Chemical Industries Co. Ltd., Japan, and Iatro-beads, Iatron Laboratories Inc.). N^{ϵ}-(Benzyloxycarbonyl]-L-lysine was purchased from Nova Biochem.

N^{α}-(3-Carboxy-1-oxopropyl)-N^{ϵ}-(benzyloxycarbonyl)-L-lysine Diammonium Salt (3). To a suspension of N^{ϵ}-(benzyloxycarbonyl)-L-lysine (2.00 g, 7.13 mmol) in MeOH (80 mL) was added succinic

(39) Shechter, Y.; Maron, R.; Elias, D.; Cohen, I. R. *Science* **1982**, *216*, 542−545.

(40) Duckworth, W. C.; Bennett, R. G.; Hamel, F. G. *Endocr. Rev.* **1998**, *19*, 608−624.

anhydride (2.14 g, 21.4 mmol). The mixture was stirred for 12 h at 40 °C and concentrated. The residual syrup was dissolved in water and chromatographed on a DEAE-Sephacel column with 0.01 M NH_4HCO_3 as the eluant to give **3** (2.54 g, 86%): $[\alpha]_D$ +20.4° (*c* 0.264, MeOH); ^{1}H NMR (D_2O) δ 7.48−7.39 (m, 5H, aromatic), 5.10 (s, 2H, PhCH_2), 4.13 (dd, 1H, Lys-CH(α)), 3.12 (t, 2H, Lys-CH_2(ϵ)), 2.55−2.46 (br, 4H, COCH_2CH_2CO), 1.77 (m, 1H, Lys-CH_2(β)), 1.66 (m 1H, Lys-CH_2(β)), 1.48 (m, 2H, Lys-CH_2(δ)), 1.34 (m 2H, Lys-CH_2(γ)). Anal. Calcd for $C_{18}H_{22}N_2O_7\cdot(NH_4)_2$: C, 52.16; H, 7.30; N,13.52. Found: C, 52.64; H, 7.09; N, 13.03.

***N*$^{\alpha}$-(3-Carboxy-1-oxopropyl)-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysine Bis-(pentafluorophenyl) Ester (4).** To a solution of **3** (300 mg, 0.724 mmol) in DMF (5.0 mL) were added pyridine (140 μL, 1.74 mmol) and pentafluorophenyl trifluoroacetate (318 μL, 1.81 mmol). The reaction mixture was stirred for 1 h at room temperature and poured into ethyl acetate. The organic layer was successively washed with water, 0.1 N HCl(aq), water, 5% $NaHCO_3$(aq), and water, then dried, and concentrated. The obtained oil was purified by column chromatography with 1:1 (v/v) hexanes−ethyl acetate as the eluant to give **4** (440 mg, 77%): $[\alpha]_D$ −7.8° (*c* 0.155, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.36−7.26 (m, 5H, aromatic), 7.04 (d, 1H, NH(α)), 5.20 (brt, 1H, NH-(ϵ)), 5.08 (s, 2H, PhCH_2), 4.84 (dd, 1H, Lys-CH(α)), 4.20 (br, 2H, Lys-CH_2(ϵ)), 3.01 (m, 2H, COCH_2), 2.66 (m, 2H, COCH_2), 1.95 (m, 2H, Lys-CH_2(β)), 1.52 (m, 4H, Lys-CH_2(δ, γ)). Anal. Calcd for $C_{30}F_{10}H_{22}N_2O_7$: C, 50.57; H, 3.11; N, 3.93. Found: C, 50.84; H, 3.45; N, 4.08.

***N*-[3-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyloxyl)propyl]-*N*$^{\alpha}$-[8-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyloxyl)-5-aza-1,4-dioxooctyl]-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysinamide (5).** To a solution of 3-aminopropyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (195 mg, 0.420 mmol) in DMF (5.0 mL) were added 4 (100 mg, 0.140 mmol) and diisopropylethylamine (73.2 μL, 0.420 mmol). The mixture was stirred for 17 h at room temperature and concentrated. The residual syrup was chromatographed on Iatro-beads with 8:1 (v/v) $CHCl_3$−MeOH as the eluant to give **5** (136 mg, 84%): $[\alpha]_D$ −11.0° (*c* 0.197, DMF); ^{1}H NMR (DMSO-d_6) δ 7.92 (dd, 1H, NH(α)), 7.89 (t, 2H, NH), 7.80 (dd, 1H, NH), 7.75 (dd, 1H, NH), 7.38−7.28 (m, 5H, aromatic), 7.19 (t, 1H, NH(ϵ)), 5.06 (t, 2H, $J_{2,3}$ = 10.0 Hz, H-3), 4.99 (s, 2H, PhCH_2), 4.82 (t, 2H, $J_{3,4}$ = 10.5 Hz, H-4), 4.59(d, 1H, $J_{1,2}$ = 8.2 Hz, H-1), 4.58(d, 1H, $J_{1,2}$ = 8.4 Hz, H-1), 4.18 (dd, 2H, $J_{5,6a}$ = 4.7 Hz and $J_{6a,6b}$ = 12.4 Hz, H-6a), 4.10 (m, 1H, Lys-CH(α)), 4.00 (dt, 2H, H-6b), 3.82 (m, 2H, H-5), 3.75−3.68 (m, 4H, OCH_2, H-2), 3.43 (m, 2H, OCH_2), 3.07 (m, 2H, NHCH_2), 3.02 (m, 2H, NHCH_2), 2.95 (m, 2H, Lys-CH_2(ϵ)), 2.38−2.28 (m, 4H, COCH_2), 2.01, 2.01, 1.96, 1.90, 1.77, 1.76 (all s, 24H, Ac), 1.66−1.55 (m, 5H, −CH_2−, Lys-CH_2(β)) 1.46 (m, 1H, Lys-CH_2(β)) 1.36 (m, 2H, Lys-CH_2(δ)) 1.27 (m, 1H, Lys-CH_2(γ)) 1.20 (m, 1H, Lys-γ). Anal. Calcd for $C_{52}H_{76}N_6O_{23}\cdot3H_2O$: C, 51.73; H, 6.85; N, 6.96. Found: C, 51.76; H, 6.65; N, 6.89.

***N*-[3-(2-Acetamido-2-deoxy-β-D-glucopyranosyloxyl)propyl]-*N*$^{\alpha}$-[8-(2-acetamido-2-deoxy-β-D-glucopyranosyloxyl)-5-aza-1,4-dioxooctyl]-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysinamide (6).** To a solution of 5 (40 mg, 34.7 μmol) in MeOH−water (3.0 mL, 2:1) was added triethylamine (232 μL, 1.66 mmol), and the mixture was stirred for 4 h at room temperature. After concentration, the residue was purified by gel filtration using Sephadex G-25 with H_2O as the eluant. The fraction was lyophilized to give **6** (28.0 mg, 90%): $[\alpha]_D$ −28.0° (*c* 0.314, H_2O); ^{1}H NMR (D_2O) δ 7.45−7.35 (m, 5H, aromatic), 5.03 (s, 2H, PhCH_2), 4.48 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1), 4.47 (d, 1H, $J_{1,2}$ = 8.8 Hz, H-1), 4.15 (br, 1H, Lys-CH(α)), 3.92 (dd, 2H, $J_{5,6a}$ = 4.2 Hz and $J_{6a,6b}$ = 13.4 Hz, H-6a), 3.88 (m, 2H, OCH_2), 3.75 (dd, 2H, $J_{5,6b}$ = 3.7 Hz, H-5), 3.70 (dd, 2H, $J_{2,3}$ = 10.1 Hz, H-2), 3.59 (m, 2H, OCH_2), 3.56 (m, 2H, H-3), 3.45 (m, 4H, H-4, H-6b), 3.30−3.15 (m, 4H, NHCH_2), 2.95 (t, 2H, Lys-CH_2(ϵ)), 2.55 (m, 2H, COCH_2), 2.50 (m, 2H, COCH_2), 1.93 (s, 6H, Ac), 1.83−1.73 (m, 5H, −CH_2−, Lys-CH_2(β)) 1.67 (m, 1H, Lys-CH_2(β)) 1.48 (m, 2H, Lys-CH_2(δ)) 1.33 (m, 2H, Lys-CH_2(γ)); ^{13}C NMR (D_2O) δ 177.4, 177.1, 176.8, 161.0, 139.2, 131.4, 131.0, 130.2, 103.6, 78.5, 76.5, 72.6, 70.4, 70.3, 69.4, 63.4, 58.2, 56.7, 42.7, 38.9, 38.8, 33.6, 33.5, 33.3, 31.0, 24.8. Anal. Calcd for $C_{40}H_{64}N_6O_{17}\cdot5H_2O$: C, 48.48; H, 7.52; N, 8.47. Found: C, 48.26; H, 7.14; N, 6.89.

***N*-{3-[2-Acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyloxyl]propyl}-*N*$^{\alpha}$-{8-[2-acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyloxyl]-5-aza-1,4-dioxooctyl}-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysinamide (7).** To a solution of 6 (30.0 mg, 33.3 μmol) and UDP−galactose (48.8 mg, 79.9 μmol) in 50 mM HEPES buffer (pH 6.0, 0.50 mL) (containing 10 mM manganese chloride) was added β-1,4-galactosyltransferase (1 U), and the reaction mixture was incubated for 48 h at 37 °C. The protein was removed by centrifugation in a 4 mL Microsep concentrator (10000 MW cutoff). The filtrate was evapolated in vacuo and subjected to a DEAE-Sephacel column (0.01 M NH_4HCO_3) for the removal of the nucleosides. The obtained residue was purified by gel filtration using Sephadex G-25 with water as the eluant to give **7** (34.0 mg, 83%): $[\alpha]_D$ −29.0° (*c* 0.172, H_2O); ^{1}H NMR (D_2O) δ 7.50−7.40 (m, 5H, aromatic), 5.14 (s, 2H, PhCH_2), 4.54 (d, 2H, $J_{1,2}$ = 7.2 Hz, H-1), 4.50 (d, 2H, $J_{1,2}$ = 7.9 Hz, H-1′), 4.18 (br t, 1H, Lys-CH(α)), 3.91 (br d, 2H, H-6a), 3.86 (d, 2H, H-4′), 3.83 (m, 2H, OCH_2), 3.76 (dd, 2H, $J_{5,6b}$ = 4.9 Hz and $J_{6a,6b}$ = 12.0 Hz, H-6b), 3.80−3.65 (m, 16H, H-2, H-3, H-4, H-5, H-3′, H-5′, H-6′a, H-6′b), 3.63−3.55 (m, 4H, OCH_2, H-2′), 3.36−3.20 (m, 4H, NHCH_2), 3.18 (br, 2H, Lys-CH_2(ϵ)), 2.57 (m, 2H, COCH_2), 2.54 (m, 2H, COCH_2), 2.06 (s, 6H, Ac), 1.80−1.75 (m, 5H, −CH_2−, Lys-CH_2(β)) 1.72 (m, 1H, Lys-CH_2(β)) 1.53 (m, 2H, Lys-CH_2(δ)) 1.37 (m, 2H, Lys-CH_2(γ)); ^{13}C NMR (D_2O) δ 177.3, 177.0, 176.7, 164.4, 161.0, 139.2, 131.4, 130.9, 130.2, 105.5, 103.6, 81.1, 77.9, 77.3, 75.1, 75.0, 73.5, 71.1, 70.3, 70.2, 69.3, 63.6, 62.7, 57.7, 56.6, 42.6, 38.8, 38.7, 33.5, 33.4, 33.2, 30.9, 24.8; HRMS-FAB *m*/*z* calcd for $C_{52}H_{85}N_6O_{27}$ $[M + H]^+$ 1225.5462, found 1225.5440.

***N*-{3-[2-acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyloxyl]propyl}-*N*$^{\alpha}$-{8-[2-acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyloxyl]-5-aza-1,4-dioxooctyl}-L-lysinamide, Monoacetic Acid (1).** To a solution of **7** (20 mg, 16.32 μmol) in MeOH−water (7.0 mL, 5:2 (v/v)) were added 10% Pd−C (10 mg) and AcOH (2.81 μL, 48.96 μmol). The suspension was stirred for 6 h under a hydrogen atmosphere and filtrated. After concentration, the residue was subjected to a CM-Sephadex C-25 column with 0.01−0.10 M NH_4OAc as the eluant. The fractions containing the product were purified by column chromatography using Sephadex G-25 with H_2O as the eluant and lyophilized to give **1** (17.5 mg, 92%): $[\alpha]_D$ −28.2° (*c* 0.294, H_2O); ^{1}H NMR (D_2O) δ 4.52 (d, 1H, $J_{1,2}$ = 7.6 Hz, H-1), 4.51 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 4.48 (d, 2H, $J_{1,2}$ = 7.7 Hz, H-1′), 4.19 (dd, 1H, Lys-CH_2(α)), 3.96 (br d, 2H, $J_{6a,6b}$ = 12.2 Hz, H-6a), 3.93 (d, 2H, $J_{3,4}$ = 3.5 Hz, H-4′), 3.91 (m, 2H, OCH_2), 3.84 (dd, 2H, $J_{5,6b}$ = 5.2 Hz and $J_{6a,6b}$ = 12.0 Hz, H-6b), 3.80−3.68 (m, 14H, H-2, H-3, H-4, H-5, H-5′, H-6′a, H-6′b), 3.67 (dd, 2H, $J_{2,3}$ = 9.9 Hz, H-3′), 3.61 (br, 2H, OCH_2), 3.54 (dd, 2H, $J_{2,3}$ = 10.1 Hz, H-2′), 3.30−3.15 (m, 4H, NHCH_2), 3.00 (t, 2H, Lys-CH_2(ϵ)), 2.60 (m, 2H, COCH_2), 2.54 (m, 2H, COCH_2), 2.04 (s, 6H, Ac), 1.93 (s, 3H, AcOH), 1.76 (m, 4H, −CH_2−), 1.69 (m, 4H, Lys-CH_2(β,δ)) 1.55−1.36 (m, 2H, Lys-CH_2(γ)); HRMS-ESI *m*/*z* calcd for $C_{44}H_{78}N_6O_{25}Na$ $[M + Na]^+$ 1113.4914, found 1113.4910.

***N*$^{\alpha}$-(3-Carboxy-1-oxopropyl)-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysine Allyl Ester (8).** To a suspension of *N*$^{\epsilon}$-[(phenylmethoxy)carbonyl]-L-lysine (1.30 g, 4.64 mmol) in DMF (80 mL) were added *N*-succinimidylsuccinic acid allyl ester (1.29 mL, 9.28 mmol) and triethylamine (1.29 mL, 9.28 mmol), and the mixture was stirred for 8 h at room temperature. After concentration, the residual oil was chromatographed on Iatro-beads with 40:1 (v/v) $CHCl_3$−MeOH as the eluant to give **8** (1.92 g, 98%): $[\alpha]_D$ +8.7° (*c* 0.282, MeOH); ^{1}H NMR ($CDCl_3$) δ 7.33 (br s, 5H, aromatic), 6.79 (br d, 1H, NH(α)), 5.85 (m, 1H, CH_2=CH−), 5.29−5.11 (m, 3H, CH_2=CH−, NH(ϵ)), 5.07 (s, 2H, PhCH_2), 4.60 (m, 1H, Lys-CH(α)),), 4.55 (d, 2H, −CH_2O), 3.18 (m, 2H, Lys-CH_2(ϵ)), 2.73 (m, 2H, COCH_2), 2.54 (m, 2H, COCH_2), 1.88 (m, 1H, Lys-CH_2-

(β)), 1.73 (m 1H, Lys-CH_2(β)), 1.50 (m, 2H, Lys-CH_2(δ)), 1.38 (m 2H, Lys-CH_2(γ)). Anal. Calcd for $C_{21}H_{28}N_2O_7$: C, 59.99; H, 6.71; N, 6.66. Found: C, 59.60; H, 6.81; N, 6.68.

***N*$^{\alpha}$-(3-Carboxy-1-oxopropyl)-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysyl-L-glutamic Acid Triallyl Ester (9).** A mixture of **8** (1.00 g, 2.38 mmol), *N*-hydroxysuccinimide (410 mg, 3.57 mmol), and dicyclohexylcarbodiimide (589 mg, 2.86 mmol) in DMF (10 mL) was stirred for 30 min at 0 °C. L-Glutamic acid diallyl ester (763 mg, 2.86 mmol) in DMF (5.0 mL) was then added to the reaction mixture. After being stirred for 15 h at room temperature, the solution was filtrated, diluted in ethyl acetate, washed with 1 N HCl(aq) and brine, then dried, and concentrated. The obtained residue was purified by column chromatography on silica gel with 80:1 (v/v) $CHCl_3$−MeOH as the eluant to give **9** (1.29 g, 86%): $[\alpha]_D$ −7.1° (*c* 0.272, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.36−7.26 (br, 6H, aromatic, NH(Glu)), 6.76 (br d, 1H, NH(Lys-CH_2(α)), 5.88 (m, 3H, CH_2═CH− × 3), 5.29−5.11 (m, 7H, CH_2═CH−, NH(Lys-ϵ)), 5.06 (s, 2H, PhCH_2), 4.60−4.48 (d, 8H, −CH_2O, Lys-CH(α), Glu-CH(α)), 3.17 (m, 2H, Lys-CH_2(ϵ)), 2.67 (m, 2H, COCH_2), 2.49 (m, 2H, COCH_2), 2.42 (m, 2H, Glu-CH_2(γ)), 2.20 (m, 1H, Glu-CH_2(β)), 2.01 (m, 1H, Glu-CH_2(β)), 1.85 (m, 1H, Lys-CH_2(β)), 1.66 (m 1H, Lys-CH_2(β)), 1.50 (m, 2H, Lys-CH_2(δ)), 1.39 (m 2H, Lys-CH_2(γ)). Anal. Calcd for $C_{32}H_{43}N_3O_{10}\cdot 0.5H_2O$: C, 60.17; H, 7.05; N, 6.58. Found: C, 60.52; H, 6.89; N, 6.89.

***N*$^{\alpha}$-(3-Carboxy-1-oxopropyl)-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysyl-L-glutamic Acid (10).** Tetrakis(triphenylphosphine)palladium(0) (138 mg, 0.119 mmol) and *N*-methylaniline (1.29 mL, 11.9 mmol) were added to a solution of **9** (500 mg, 0.704 mmol) in THF (10 mL), and the resulting solution was stirred for 14 h in the dark under a nitrogen atmosphere. After concentration, the residue was subjected to an ion exchange column (Dowex 50W-X8, H^+-form) and purified by a column of Iatro-beads with 15:1 (v/v) $CHCl_3$−MeOH as the eluant to give **10** (300 mg, 74%): $[\alpha]_D$ −7.4° (*c* 0.262, MeOH); ^{1}H NMR (CD_3OD) δ 7.34−7.25 (br, 5H, aromatic), 5.05 (s, 2H, PhCH_2), 4.42 (dd, 1H, Glu-CH(α)), 4.34 (dd, 1H, Lys-CH(α)), 3.11 (t, 2H, Lys-CH_2(ϵ)), 2.60 (m, 2H, COCH_2), 2.51 (m, 2H, COCH_2), 2.40 (t, 2H, Glu-CH_2(γ)), 2.19 (m, 1H, Glu-CH_2(β)), 1.97 (m, 1H, Glu-CH_2(β)), 1.83 (m, 1H, Lys-CH_2(β)), 1.66 (m 1H, Lys-CH_2(β)), 1.51 (m, 2H, Lys-CH_2(δ)), 1.42 (m 2H, Lys-CH_2(γ)). Anal. Calcd for $C_{23}H_{31}N_3O_{10}$: C, 54.22; H, 6.13; N, 8.25. Found: C, 53.98; H, 6.35; N, 8.13.

***N*$^{\alpha}$-(3-Carboxy-1-oxopropyl)-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysyl-L-glutamic Acid Tris(pentafluorophenyl) Ester (11).** To a solution of **10** (270 mg, 0.530 mmol) in DMF (5.0 mL) were added pyridine (141 μL, 1.75 mmol) and pentafluorophenyl trifluoroacetate (321 μL, 1.83 mmol). The reaction mixture was stirred for 1 h at room temperature and poured into ethyl acetate. The organic layer was successively washed with water, 0.1 N HCl(aq), water, 5% $NaHCO_3$(aq), and water, then dried, and concentrated. The obtained oil was purified by column chromatography with 1:1 (v/v) hexanes−ethyl acetate as the eluant to give **11** (420 mg, 79%): $[\alpha]_D$ −10.6° (*c* 0.254, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.49 (dd, 1H, NH(Glu)), 7.33−7.26 (br, 5H, aromatic), 6.86 (dd, 1H, NH(Lys-α)), 5.06 (s, 2H, PhCH_2), 5.05 (m, 1H, NH(Lys-ϵ)), 4.96 (m, 1H, Glu-CH(α)), 4.52 (dd, 1H, Lys-CH(α)), 3.16 (br, 2H, Lys-CH_2(ϵ)), 3.10−2.90 (m, 2H, COCH_2), 2.84 (m, 2H, Glu-CH_2(γ)), 2.63 (m, 2H, COCH_2), 2.47 (m, 1H, Glu-CH_2(β)), 2.22 (m, 1H, Glu-CH_2(β)), 1.92 (m, 1H, Lys-CH_2(β)), 1.74 (m 1H, Lys-CH_2(β)), 1.52 (m, 2H, Lys-CH_2(δ)), 1.41 (m 2H, Lys-CH_2(γ)). Anal. Calcd for $C_{41}F_{15}H_{28}N_3O_{10}$: C, 48.87; H, 2.80; N, 4.17. Found: C, 49.15; H, 2.96; N, 4.04.

***N*$^{\alpha}$-[8-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyloxyl)-5-aza-1,4-dioxooctyl]-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysyl-*N*-bis[3-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyloxyl)-propyl]-L-glutamamide (12).** To a solution of 3-aminopropyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (207 mg, 0.446 mmol) in DMF (5.0 mL) were added **11** (100 mg, 99.2 μmol) and diisopropylethylamine (156 μL, 0.893 mmol), and the mixture was stirred for 17 h at room temperature. After concentration, the residual syrup was chromatographed on Iatro-beads with 7:1 (v/v) $CHCl_3$−MeOH as the eluant to give **12** (140 mg, 85%): $[\alpha]_D$ −14.4° (*c* 0.323, DMF); ^{1}H NMR (DMSO-d_6) δ 8.14 (br d, 1H, NH(α)), 7.96−7.88 (m, 4H, NH), 7.77 (m, 2H, NH), 7.53 (br t, 1H, NH), 7.38−7.28 (m, 5H, aromatic), 7.21 (t, 1H, NH(Lys-ϵ)), 5.07 (t, 3H, $J_{2,3}$ = 10.4 Hz and $J_{3,4}$ = 9.7 Hz, H-3), 5.00 (s, 2H, PhCH_2), 4.82 (t, 3H, $J_{4,5}$ = 9.8 Hz, H-4), 4.58(d, 3H, $J_{1,2}$ = 8.5 Hz, H-1), 4.21 (dd, 3H, $J_{5,6a}$ = 7.2 Hz and $J_{6a,6b}$ = 11.8 Hz, H-6a), 4.09 (m, 2H, Lys-CH(α), Glu-CH(α)), 4.01 (br d, 3H, H-6b), 3.82 (m, 3H, H-5), 3.77−3.64 (m, 6H, OCH_2, H-2), 3.43 (m, 3H, OCH_2), 3.15−2.92 (m, 8H, NHCH_2, Lys-CH_2(ϵ)), 2.50 (m, 2H, Glu-CH_2(γ)), 2.35 (m, 4H, COCH_2), 2.10 (m, 1H, Glu-CH_2(β)), 2.01, 2.01, 1.96, 1.91, 1.77, 1.77 (all s, 36H, Ac), 1.79 (m, 1H, Glu-CH_2(β)), 1.66−1.55 (m, 6H, −CH_2−, Lys-CH_2(β)), 1.39 (m, 2H, Lys-CH_2(δ)), 1.29 (m, 2H, Lys-CH_2(γ)). Anal. Calcd for $C_{74}H_{109}N_9O_{34}\cdot 2H_2O$: C, 52.13; H, 6.68; N, 7.39. Found: C, 51.93; H, 6.54; N, 7.00.

***N*$^{\alpha}$-[8-(2-Acetamido-2-deoxy-β-D-glucopyranosyloxyl)-5-aza-1,4-dioxooctyl]-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysyl-*N*-bis[3-(2-acetamido-2-deoxy-β-D-glucopyranosyloxyl)propyl]-L-glutamamide (13).** To a solution of **12** (50 mg, 30.0 μmol) in MeOH−water (6.0 mL, 2:1) was added triethylamine (150 μL, 1.08 mmol), and the mixture was stirred for 4 h at room temperature. After concentration, the residue was purified by gel filtration using Sephadex G-25 with H_2O as the eluant. The fraction was lyophilized to give **13** (38.7 mg, 100%): $[\alpha]_D$ −40.8° (*c* 0.159, H_2O); ^{1}H NMR (D_2O) δ 7.47−7.39 (m, 5H, aromatic), 5.11 (s, 2H, PhCH_2), 4.50 (d, 1H, $J_{1,2}$ = 8.8 Hz, H-1), 4.49 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1), 4.47 (d, 1H, $J_{1,2}$ = 8.6 Hz, H-1), 4.21 (br, 2H, Lys-CH(α), Glu-CH(α)), 3.92 (br d, 3H, H-6a), 3.88 (m, 3H, OCH_2), 3.74 (dd, 3H, $J_{6a,6b}$ = 9.7 Hz $J_{5,6b}$ = 4.2 Hz, H-5), 3.69 (t, 3H, $J_{2,3}$ = 10.2 Hz, H-2), 3.60 (m, 3H, OCH_2), 3.54 (m, 3H, H-3), 3.44 (m, 6H, H-4, H-6b), 3.30−3.10 (m, 8H, NHCH_2, Lys-CH_2(ϵ)), 2.54 (m, 4H, COCH_2), 2.29 (m, 2H, Glu-CH_2(γ)), 2.10 (m, 1H, Glu-CH_2(β)), 2.04 (s, 9H, Ac), 1.98 (m, 1H, Glu-CH_2(β)), 1.74 (br, 8H, −CH_2−, Lys-CH_2(β)), 1.51 (m, 2H, Lys-CH_2(δ)), 1.37 (br, 2H, Lys-CH_2(γ)); ^{13}C NMR (D_2O) δ 178.2, 177.8, 177.4, 177.3, 177.2, 175.8, 175.7, 161.2, 139.4, 131.6, 131.1, 130.3, 103.9, 78.7, 76.6, 72.7, 70.5, 70.3, 63.6, 58.4, 56.9, 56.1, 42.8, 39.1, 39.1, 39.0, 34.8, 33.6, 33.5, 33.2, 31.2, 31.1, 29.8, 25.0. Anal. Calcd for $C_{56}H_{91}N_9O_{25}\cdot 3H_2O$: C, 50.03; H, 7.27; N, 9.37. Found: C, 49.73; H, 6.82; N, 9.38.

***N*$^{\alpha}$-{8-[2-Acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyloxyl]-5-aza-1,4-dioxooctyl}-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysyl-*N*-bis{3-[2-acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyloxyl]propyl}-L-glutamamide (14).** To a solution of **13** (28 mg, 21.7μmol) and UDP−galactose (47.7 mg, 78.1 μmol) in 50 mM HEPES buffer (pH 6.0, 0.50 mL) containing 10 mM manganese chloride was added β-1,4-galactosyltransferase (1 U), and the reaction mixture was incubated for 48 h at 37 °C. The reaction mixture was treated as described for the preparation of **7** and gave **14** (37.0 mg, 96%): $[\alpha]_D$ −27.0° (*c* 0.166, H_2O); ^{1}H NMR (D_2O) δ 7.47−7.39 (m, 5H, aromatic), 5.11 (s, 2H, PhCH_2), 4.50 (br d, 3H, $J_{1,2}$ = 7.0 Hz, H-1), 4.47 (d, 3H, $J_{1,2}$ = 7.8 Hz, H-1′), 4.21 (m, 2H, Lys-CH(α), Glu-CH(α)), 3.98 (dd, 3H, $J_{6a,6b}$ = 12.2 Hz, $J_{5,6a}$ = 2.2 Hz, H-6a), 3.93 (d, 3H, $J_{3',4'}$ = 3.4 Hz, H-4′), 3.89 (m, 3H, OCH_2), 3.83 (dd, 3H, $J_{5,6b}$ = 4.9 Hz, H-6b), 3.80−3.68 (m, 21H, H-2, H-3, H-4, H-5, H-5′, H-6′a, H-6′b), 3.67 (dd, 3H, H-3′), 3.58 (m, 3H, OCH_2), 3.55 (dd, 3H, $J_{2',3'}$ = 9.9 Hz, H-2′), 3.30−3.10 (m, 8H, NHCH_2, Lys-CH_2(ϵ)), 2.55 (br, 4H, COCH_2), 2.29 (m, 2H, Glu-CH_2(γ)), 2.10 (m, 1H, Glu-CH_2(β)), 2.03 (s, 9H, Ac), 1.95 (m, 1H, Glu-CH_2(β)), 1.74 (br, 8H, −CH_2−, Lys-CH_2(β)), 1.51 (m, 2H, Lys-CH_2(δ)), 1.37 (br, 2H, Lys-CH_2(γ)); ^{13}C NMR (D_2O) δ 178.0, 177.7, 177.4, 177.1, 175.7, 161.1, 139.4, 131.5, 131.1, 130.3, 105.7, 103.8, 81.6, 78.1, 77.5, 75.4, 75.2, 73.8, 71.3, 70.6, 70.3, 69.5, 63.7, 63.0, 57.9, 56.8, 56.1, 42.9, 39.1, 39.1, 39.0, 34.8, 33.7, 33.6, 33.2, 31.2, 29.8, 25.0; HRMS-FAB *m*/*z* calcd for $C_{74}H_{121}N_9O_{40}Na$ $[M + Na]^+$ 1798.7609, found 1798.7600.

***N*$^{\alpha}$-{8-[2-Acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyloxyl]-5-aza-1,4-dioxooctyl}-L-lysyl-*N*-bis{3-[2-acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyloxyl]propyl}-**

L-glutamamide, Monoacetic Acid (2). The title compound (16.2 mg, 84%) was obtained by hydrogenation of **14** (20 mg, 11.26 μmol), as described for the preparation of **8**: $[\alpha]_D$ −30.0° (*c* 0.282, H_2O); ^{1}H NMR (D_2O) δ 4.52 (d, 2H, $J_{1,2}$ = 7.3 Hz, H-1), 4.51 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.48 (d, 3H, $J_{1',2'}$ = 7.8 Hz, H-1′), 4.23 (m, 2H, Lys-C*H*(α), Glu-C*H*(α)), 3.99 (br d, 3H, H-6a), 3.93 (d, 3H, $J_{3,4}$ = 3.4 Hz, H-4′), 3.91 (m, 3H, OCH_2), 3.84 (dd, 3H, $J_{5,6b}$ = 5.2 Hz and $J_{6a,6b}$ = 12.4 Hz, H-6b), 3.80−3.65 (m, 24H, H-2, H-3, H-4, H-5, H-3′, H-5′, H-6′a, H-6′b), 3.60 (br, 3H, OCH_2), 3.54 (dd, 3H, $J_{2,3}$ = 10.1 Hz, H-2′), 3.30−3.12 (m, 6H, NHCH_2), 3.01 (t, 2H, Lys-CH_2(ϵ)), 2.57 (m, 4H, COCH_2), 2.30 (m, 2H, Glu-CH_2(γ)), 2.12 (m, 1H, Glu-CH_2(β)), 2.04 (3s, 9H, Ac) 2.01 (m, 1H, Glu-CH_2(β)), 1.92 (s, 3H, AcOH), 1.76 (m, 6H, −CH_2−), 1.71 (m, 4H, Lys-CH_2(β,δ)), 1.43 (m, 2H, Lys-CH_2(γ)); HRMS-ESI *m*/*z* calcd for $C_{66}H_{115}N_9O_{38}Na$ $[M + Na]^+$ 1664.7241, found 1664.7279.

DNA Construction. The plasmid containing the human insulin gene (YG-HG294) was donated by the Japan Health Science Research Resources Bank. The normal human proinsulin (PI(WT)) gene[41] was amplified using primers PI, 5′-ACG GAT CCG GGT GGC CGC TTT GTG AAC CAA CAC-3′, and PI-R, 5′-CAG GTC GAC CTA GTT GCA GTA GTT CTC-3′. PCR was carried out using KOD-Plus polymerase (TOYOBO). The amplified PCR product was digested with restriction enzymes *Bam*HI and *Sal*I (TOYOBO), and inserted into the His-tag fusion protein expression vector pQE31 (Qiagen) previously digested with the same enzymes, resulting in plasmid His-PI(WT)/pQE31. Furthermore, a mutation of the insulin B-chain (from F1 to Q) was performed using the QuikChange mutagenesis kit (Stratagene) and primers B-F1Q, 5′-GGA TCC GGG TGG CCG CCA AGT GAA CCA ACA CCT C-3′, and B-F1Q-R, 5′-CAG GTG TTG GTT CAC TTG GCG GCC ACC CGG ATC C-3′, resulting in plasmid His−PI(B-F1Q)/pQE31. The primers used for the mutation of the insulin A-chain (from G1 to Q and from N21 to Q) were A-G1Q, 5′-GTC CCT GCA GAA GCG TCA AAT TGT GGA AGA ATG CTG-3′, A-G1Q−R, 5′-CAG CAT TGT TCC ACA ATT TGA CGC TTC TGC AGG GAC-3′, A-N21Q, 5′-GGA GAA CTA CTG CCA ATA GGT CGA CCT GC-3′, and A-N21Q-R, GCA GGT CGA CCT ATT GGC AGT AGT TCT CC-3′, resulting in plasmids His−PI(A-G1Q)/pQE31 and His−PI(A-N21Q)/pQE31, respectively.

Proinsulin Expression. *E. coli* cells (M15 bacteria) harboring His−PI(WT)/pQE31, His−PI(B-F1Q)/pQE31, His−PI(A-G1Q)/pQE31, or His−PI(A-N21Q)/pQE31 were grown overnight at 37 °C in 60 mL of LB medium containing ampicillin (100 μg/mL) and kanamycin (25 μg/mL). The overnight cultures were diluted 100-fold to 6 L in a Jarfermenter (BMS-10PI, ABLE) with the same medium and grown at 37 °C until OD_{600} reached approximately 0.7−0.8.[42] Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the culture was grown for an additional 5 h. Cells were harvested by centrifugation at 5000*g* for 10 min at 4 °C, and the bacterial pellet was resuspended in 300 mL of buffer A (20 mM Tris−HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole). Cellular membranes were disrupted by probe sonication (two cycles of 15 s on and 15 s off), and insoluble proteins were obtained by centrifugation at 5000*g* for 10 min at 4 °C.[43] The pellet was resuspended in 150 mL of buffer B (20 mM Tris−HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 8 M urea, 1 mM 2-mercaptoethanol) and shaken at room temperature for 30 min. This solution was then centrifuged at 10000*g* for 20 min at 4 °C, and the pellet was discarded. The supernatant was applied to a Superflow Ni−NTA resin column (bed volume 20 mL, Qiagen) equilibrated with buffer B at 4 °C and washed with buffer B, and then the bound proteins were eluted with buffer C (20 mM Tris−HCl, pH 8.0, 500 mM NaCl, 250 mM imidazole, 8 M urea, 1 mM 2-mercaptoethanol).

Proinsulin Refolding. The eluted proteins were precipitated by dialysis against H_2O. The sediment was dissolved and sulfonated in 10 mL/g (wet weight sediment) buffer D (20 mM Tris−HCl, pH 8.0, 100 mM Na_2SO_3, 10 mM $Na_2S_4O_6$, 8 M urea) at room temperature for 3 h.[43] This reaction was stopped by dialysis against 10 mM HCl, and the sulfonated proteins were collected as a white precipitate. The precipitate was dissolved in 50 mM glycine/NaOH, pH 10.5, at a final concentration of 0.1 mg/mL. Refolding was performed by adding 2-mercaptoethanol (1.5 mol/mol of cysteine) at 4 °C for 16 h.[43,44] To stop the reaction, the pH was adjusted to 3.0. Refolded products were concentrated and isolated by reversed-phase HPLC (RP-HPLC) using a semipreparative Vydac C4 column (10 × 250 mm). Elution conditions consisted of a linear gradient of acetonitrile (ACN) concentration from 25% to 45% containing 0.1% trifluoroacetic acid (TFA) for 25 min at a flow rate of 4 mL min^{-1}. The isolated products were then lyophilized.

Insulin Purification. The lyophilized His−PI fusion proteins were dissolved in buffer E (100 mM Tris/H_3PO_4, pH 7.5, 0.1% Tween 20) at a concentration of 2 mg/mL. Trypsin (sequencing grade, Roche) and carboxypeptidase B (sequencing grade, Roche) were added to the fusion protein at a ratio of 1:2:2000 (by mass).[28] The reaction was carried out at 37 °C for 30 min, and stopped by decreasing the pH to 3.0 by adding AcOH. The digestion products were purified by RP-HPLC using a semipreparative Vydac C4 column and analyzed by RP-HPLC using an analytical Vydac C4 column (4.6 × 250 mm). Elution conditions consisted of a linear gradient of ACN concentration from 25% to 35% containing 0.1% TFA for 25 min at a flow rate of 4 mL min^{-1} (semipreparative) or 1 mL min^{-1} (analytical).

Insulin Glycosylation. For synthesis of glycoinsulin, TGase (Oriental Yeast Co.) was used. A solution of Ins(B-F1Q) (0.6 mg, 104 nmol) in 20 mM Tris buffer (pH 7.5, 1 mL) containing 0.9 mM **1** (**2**, **8**, or **16**), 5 mM $CaCl_2$, and 4 U of TGase was incubated for 30 min at 37 °C.[45,46] An aliquot (20 μL) of the reaction mixture was withdrawn for analytical RP-HPLC. The reaction was stopped by decreasing the pH to 3.0. The mixture was applied to a semipreparative Vydac C4 column and eluted with a linear gradient of ACN from 25% to 30% for 30 min at a flow rate of 4 mL min^{-1}. The eluted products were nonreacted Ins(B-F1Q) (∼50%) and glycosylated Ins(B-F1Q), Lac-Ins(B-F1Q), di-Lac-Ins(B-F1Q), or tri-Lac-Ins(B-F1Q) (∼50%).

Sugar Elongation (Sialylation) Reaction of Lac-, Di-LacNAc-, or Tri-LacNAc-Ins(B-F1Q). A solution of Lac-, di-LacNAc-, or tri-LacNAc-Ins(B-F1Q) (80.5 nmol) in a 50 mM sodium cacodylate buffer (pH 7.5, 1 mL) containing 0.6 mM CMP−NeuAc, 0.2% BSA, 1.6 mM $MnCl_2$, 0.2% Triton CF54, 20 U of CIAP, and 150 mU of α2,6-SiaT was incubated for 2 h (Lac-Ins(B-F1Q)) or 30 min (di- or tri-LacNAc-Ins(B-F1Q)) at 37 °C.[47−49] The reaction procedure was checked by MALDI-TOF mass analysis. After the reaction was completed, purification of the products was performed by RP-HPLC as described above.

Circular Dichroism (CD) Studies. All insulin samples were dissolved in PBS (pH 7.4), and the protein concentrations were adjusted to 40 μM by UV absorption at 280 nm. CD measurements were performed on a Jasco-820 CD spectropolarimeter at 25 °C with a cell length of 0.1 cm. For the far-UV CD spectra, samples were scanned from 200 to 250 nm and accumulated 10 times at a resolution of 1.0 nm with a scanning speed of 100 nm/min. All the CD data were expressed as mean residue ellipticity.[50,51]

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Induction of Diabetes. We purchased C57BL/6J male mice at 5 weeks of age from Clea Japan Inc., and started experiments at 7−8 weeks of age.[52,53] The mice were fasted for 18−20 h and then injected intraperitoneally with a single dose of STZ (200 mg/kg of body mass, Wako) freshly dissolved in 0.05 M citrate buffer (pH 4.5).[54,55] At 48 h after STZ injection, blood samples were obtained from the tail vein, and blood-glucose levels were measured with a Glucocard (Kyoto Daiichi Kagaku Co.).[56] Mice with blood-glucose levels above 400 mg/dL were used as diabetic mice.[57]

Administration of Insulin Preparations. The dosage of insulin preparations was determined as 1 U by the absorbance at 280 nm. Solutions of insulin preparations in PBS (pH 7.4) were administered subcutaneously to STZ-treated diabetic mice (STZ-mice) using a disposable syringe (TERUMO). Blood sampling for glucose analysis was performed before administration and at 1, 2, 3, 4, and 5 h after administration. Groups consisted of five mice, and the data were presented as means ± SE.[10]

Cell Culture. 3T3-L1 fatty fibroblasts (Dainippon Seiyaku Co.) were cultured to confluence in 35 mm dishes in DMEM (Gibco) containing 10% fetal calf serum (FCS, Gibco) at 37 °C in a 5% CO_2 environment and were fed every 2 days. For differentiation of 3T3-L1 cells from adipocytes, cells were exposed to fresh medium (2 mL) containing 10% FCS, 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, and 1 μg/mL insulin. After 2 days, the medium was replaced with DMEM containing 10% FCS and insulin, and the cells were cultured for an additional 6 days.[58−60]

Insulin Binding Assay. Differentiated cells were washed three times with the binding buffer (100 mM Hepes, 120 mM NaCl, 1.2 mM $MgCl_2$, 5 mM KCl, 25 mM glucose, 10 mg/mL BSA, and 2 mg/mL Bacitracin)[60] and assayed for the displacement of ^{125}I-labeled insulin (Amersham) by glycoinsulins as described by Shechter et al.[5,38] Unbound insulins were removed, and the cells were washed three times with ice-cold buffer and lysed with 0.1% Triton X-100.[62] the radioactivity of the harvested cell was determined by γ-counter.

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