# Synthesis of a Glycopeptide Containing Oligosaccharides: Chemoenzymatic Synthesis of Eel Calcitonin Analogues Having Natural N-Linked Oligosaccharides

**Mamoru Mizuno,[†] Katsuji Haneda,[†] Reiko Iguchi,[†] Ikuyo Muramoto,[†] Toru Kawakami,[‡] Saburo Aimoto,[‡] Kenji Yamamoto,[§] and Toshiyuki Inazu*,[†]**

*Contribution from the Noguchi Institute, 1-8-1, Kaga, Itabashi-ku, Tokyo 173-0003, Japan, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan, and Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*

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**Abstract:** We describe a novel chemoenzymatic synthesis of eel calcitonin (eCT) glycopeptide analogues having natural N-linked oligosaccharides, such as a disialo biantennary complex-type [(NeuAc-Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$], an asialo complex-type [(Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$], and a high-mannose type [Man$_6$-GlcNAc$_2$] as model compounds for glycoprotein synthesis. First, a glycoprotein containing *N*-acetylglucosamine (GlcNAc) was prepared by a chemical synthesis. Next, natural oligosaccharides were added to the prepared glycopeptide containing GlcNAc by a transglycosylation reaction using endo-$\beta$-*N*-acetylglucosaminidase (endo-$\beta$-GlcNAc-ase) from *Mucor hiemalis*.

## Introduction

Glycoproteins play an important role in biological processes, such as cell recognition, cell adhesion, immunogenic recognition, and so on. Moreover, the oligosaccharide moieties of the glycoprotein contribute to the solubility and thermal stability of proteins and to protection against proteolysis.[1] To study these roles of oligosaccharide moieties, proteins without the sugar chain of the original glycoprotein have been prepared by an enzymatic method using *N*-glycanase. Recently, recombinant proteins whose Asn residues containing oligosaccharides were replaced with other amino acids, such as Ala and Gln, have been prepared by genetic engineering. However, the artificial addition of oligosaccharides to the Asn residue in a protein having no sugar chains by genetic engineering is impossible. We then tried a chemoenzymatic method to transfer the sugar chain to an *N*-acetylglucosaminyl peptide, as a new strategy for glycopeptide synthesis.[2] Eel calcitonin is a calcium-regulating hormone that consists of 32 amino acid residues and has a consensus sequence of "Asn-Leu-Ser" for *N*-glycosylation but no sugar chains. The Asn residue at the position 3 exists in a ring structure formed by a disulfide bridge between two cysteine residues at positions 1 and 7. In this paper, we describe the artificial addition of N-linked oligosaccharides to the Asn residue of eel calcitonin by a chemoenzymatic method. We also studied the influence of the oligosaccharide attached to the Asn residue on the structure and on the biological activity of the eel calcitonin.

Our strategy in this study consisted of four steps. The first step was the synthesis of glycosylasparagine, which is the core unit of the *N*-glycopeptide, using the new simple method that we described previously.[3] The second was the preparation of the glycopeptide moiety containing the GlcNAc using the dimethylphosphinothioic mixed anhydride (Mpt-MA) method.[4] In a conventional glycopeptide synthesis, sugar hydroxyl functions were deprotected at the final stage by sodium methoxide, TMSOTf/TFA, hydrogenolysis, and so on.[5] However, several side reactions have been reported under these deprotection conditions.[5,6] To avoid the deprotection step of the sugar hydroxyl group, we described the solid-phase syntheses of glycopeptides containing GlcNAc by the Mpt-MA method, in which no protection of the sugar hydroxyl function was necessary.[4,7] The Mpt-MAs of amino acid derivatives have no reactivity with the hydroxyl function but have high reactivity with the amino function.[8] The third was a synthesis of the polypeptide chain using the thioester segment condensation method.[9−11] The thioester method is a minimum protection

* To whom all correspondence should be addressed. Tel: +81-3-3961-3255. Fax: +81-3-3964-4071. E-mail: LDI02534@nifty.ne.jp.
[†] The Noguchi Institute.
[‡] Osaka University.
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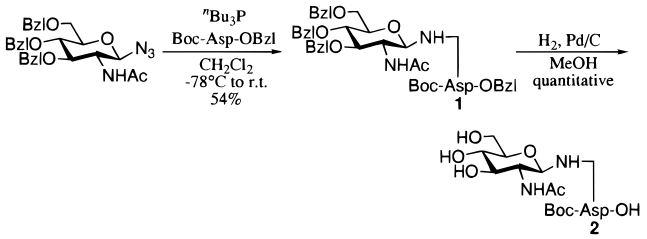
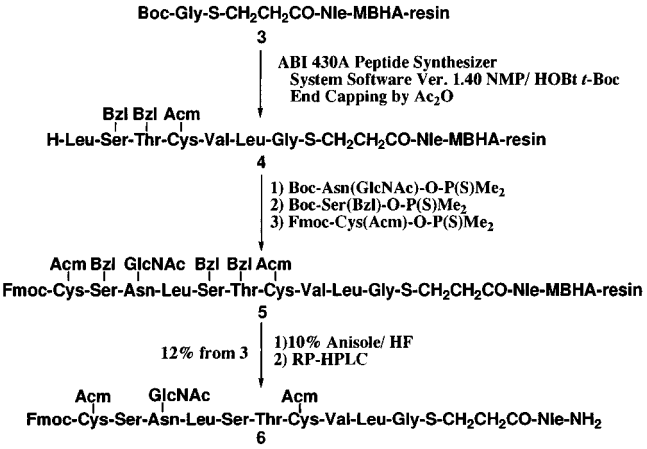
**Scheme 1.** Synthesis of Glycosylasparagine **2**




**Scheme 2.** Synthesis of Glycopeptide Thioester Segment **6** Using the Mpt-MA Method




method that requires only protection of side-chain amino groups and is very useful for protein synthesis. The last step was the transglycosylation reaction using endo-$\beta$-GlcNAc-ase from *Mucor hiemalis* (Endo-M).[12] Most of the endo-$\beta$-GlcNAc-ases can act only on high-mannose and hybrid-type oligosaccharides.[13] Endo-M has transglycosylation activity not only on high-mannose and hybrid-type oligosaccharides but also on complex-type oligosaccharides.

## Results and Discussion

**Synthesis of N-Terminal GlcNAc-peptide Thioester Segment (6) by Mpt-MA Method.** Boc-Asn(GlcNAc)-OH (**2**) which is a key compound in glycopeptide synthesis was prepared as shown in Scheme 1. Boc-Asn(GlcNAc(OBzl)$_3$)-OBzl (**1**) was synthesized using Boc-Asp-OBzl and 2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-$\beta$-D-glucopyranosyl azide in the presence of tri-*n*-butylphosphine.[3] Reduction of **1** via catalytic hydrogenation gave **2** in quantitative yield. The N-terminal GlcNAc-peptide thioester segment, Fmoc-[Cys(Acm),$^{1,7}$Asn(GlcNAc)$^3$]-eCT(1−10)-S-$CH_2CH_2CO$-Nle-$NH_2$ (**6**), was prepared as shown in Scheme 2. First, the protected heptapeptide **4** was prepared by a Boc solid-phase method using Boc amino acid derivatives and *N*,*N*-dicyclohexylcarbodiimide (DCC)−1-hydroxybenzotriazole (HOBt). By starting from an MBHA resin, we manually prepared Boc-Gly-S-$CH_2CH_2CO$-Nle-MBHA resin (**3**). This resin was used for the synthesis of **4** corresponding to the sequence of eCT(4−10). Next, the Asn(GlcNAc) residue was coupled by the Mpt-MA method using Boc-Asn(GlcNAc)-OH. Successively, the corresponding Mpt-MAs of Boc-Ser(Bzl)-OH and Fmoc-Cys(Acm)-OH were introduced one by one. The coupling of an Asn(GlcNAc) and coupling to an Asn(GlcNAc) residue require a lot of care because the reactivity of the Asn(GlcNAc) residue is not very high.[14] So we repeated the coupling reactions of Asn(GlcNAc), Ser(Bzl), and Cys(Acm) residues with the Kaiser test, which is a color test for the detection of free terminal amino groups.[15] The glycopeptide thioester resin **5** was treated with anhydrous HF containing 10% anisole to cleave the glycopeptide from the resin and remove the side-chain protecting groups. The glycopeptide thioester **6** was obtained in 12% yield by reversed-phase HPLC (RP-HPLC) (Figure 1). During the synthesis of **6** from **3**, no significant side reactions were observed.

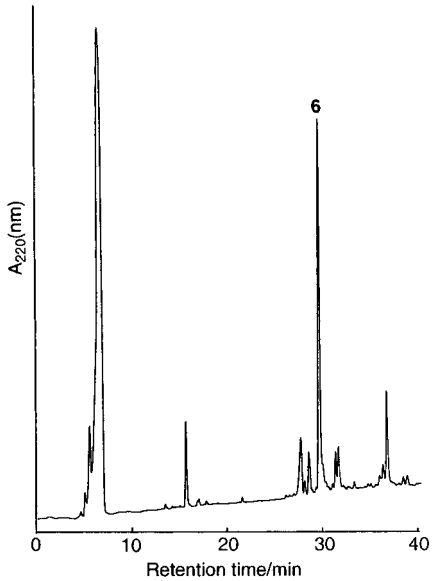



**Figure 1.** HPLC elution profile of crude glycopeptide thioester segment **6**: HPLC elution conditions; column: Cosmosil 5$C_{18}$AR (10 × 250 mm); linear increase of acetonitrile concentration from 30 to 70% in 0.1% aqueous trifluoroacetic acid over 40 min at a flow rate of 2.5 mL min$^{-1}$.

**Synthesis of C-Terminal Peptide Segment.** The other peptide segment [Lys(Boc)$^{11,18}$]-eCT(11−32) (**9**) was prepared by a Boc-solid-phase method as shown in Scheme 3. A protected peptide resin **7** corresponding to the sequence of eCT(11−32) was synthesized by starting from the MBHA resin. The peptide resin was treated with HF containing 7.5% anisole and 7.5% 1,4-butanedithiol to cleave the peptide from the resin and remove the side-chain protecting groups. The peptide segment **8** was obtained in 34% yield by RP-HPLC. For the thioester segment condensation, Boc groups were introduced to block the side-chain amino groups of the peptide segment **8** by treatment with *N*-(*tert*-butoxycarbonyloxy)succinimide (Boc-OSu) in the pres-

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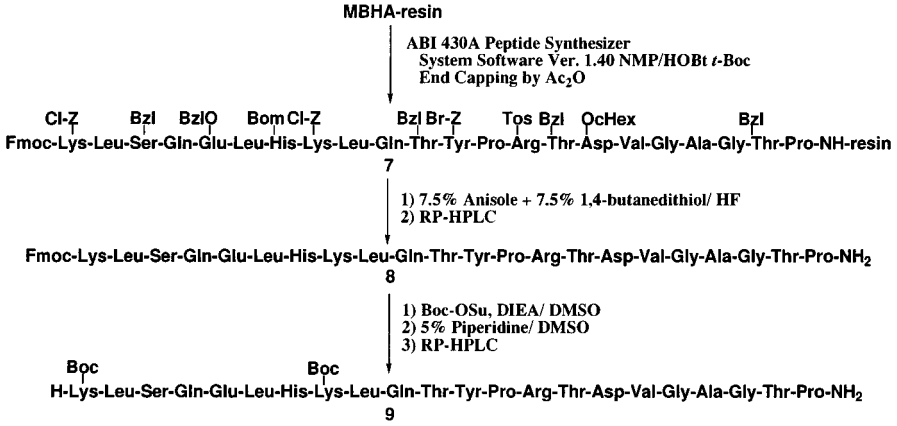
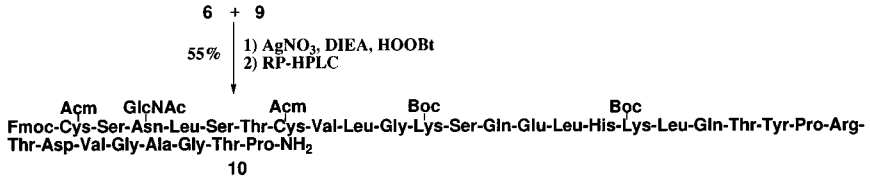
**Scheme 3.** Synthesis of Peptide Segment **9**




**Scheme 4.** Thioester Method for the Synthesis of **10**




ence of $^{i}Pr_2NEt$ (DIEA)[11] and an N-terminal 9-fluorenylmethyloxycarbonyl (Fmoc) group was removed by treatment with 5% piperidine in dimethyl sulfoxide (DMSO). After RP-HPLC purification, the partially protected C-terminal peptide segment **9** was obtained in 19% yield, based on the amino group in the starting resin.

**Glycopolypeptide Synthesis Using Thioester Fragment Condensation.** The N-terminal glycopeptide thioester segment **6** and the partially protected C-terminal peptide segment **9** were added to a mixture of $AgNO_3$, 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOObt), and DIEA in DMSO (Scheme 4). The reaction mixture was stirred overnight at room temperature. Dithiothreitol (DTT) was added to the reaction mixture to terminate the coupling reaction. The RP-HPLC profile of the reaction mixture is shown in Figure 2. Partially protected glycopeptide **10** was obtained in 55% yield after purification by RP-HPLC, followed by freeze-drying. The Boc group of **10** was removed by treating with TFA containing 5% 1,4-butanedithiol, and the Fmoc group was removed by treating with 5% piperidine in DMSO. After RP-HPLC purification, precursor **11** was obtained in 83% yield. The precursor **11** was treated with $AgNO_3$ and DIEA in aqueous DMSO, followed by 1 M HCl−DMSO at room temperature to remove the Acm groups and form a disulfide bond (Scheme 5).[11] After RP-HPLC purification of the reaction mixture, [Asn(GlcNAc)$^3$]-eCT (**12**) was obtained in 6% overall yield based on the amount of the amino group in the starting resin. The characterization of **12** was performed by MALDI-TOF MS and amino acid analysis.

**Enzymatic Transfer of Natural N-Linked Oligosaccharides.** The reaction mixture for transglycosylation was composed of glycosylasparagine having a disialo complex-type oligosaccharide from human transferrin as a glycoside donor, **12** as an acceptor, and Endo-M in the phosphate buffer, pH 6.25 (Scheme 6).[12] The reaction mixture after incubation at 37 °C for 6 h was analyzed by HPLC using an ODS column as shown in Figure 3. A single new peak (**13**−**15**) with an earlier retention time than that of the remaining **12** was observed. The fraction corresponding to the new peak of the reaction mixture was isolated by HPLC, freeze-dried, and subjected to ESI mass spectrometry. A triply charged mass ion $[M + 3H]^{3+}$ with *m*/*z* 1874.4 was detected (Figure 4). The molecular mass of 5620.2 Da calculated from this value was in accord with the theoretical value of [Asn{(NeuAc-Gal-GlcNAc-Man)$_2$-Man-(GlcNAc)$_2$}$^3$]-eCT, $C_{230}H_{377}O_{108}N_{49}S_2$ (5620.9 Da). After treatment of the isolated product with sialidase, the molecular ion $[M + H]^+$ measured by a MALDI-TOF mass spectrometer shifted to 5041.6 Da corresponding to the theoretical value of [Asn{(Gal-GlcNAc-Man)$_2$-Man-(GlcNAc)$_2$}$^3$]-eCT (5038.4 Da). On the basis of these data, the product was identified as the eCT analogue having disialo biantennary complex-type oligosaccha-

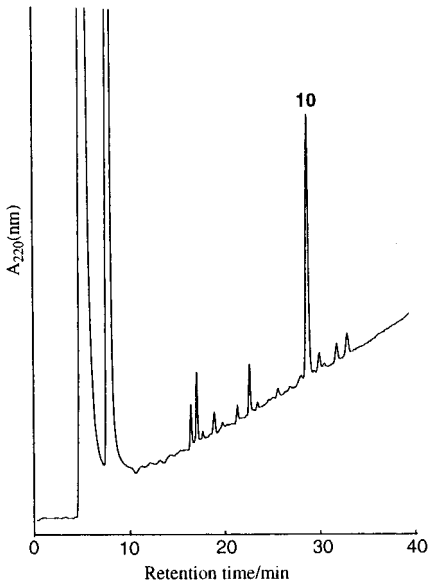



**Figure 2.** HPLC elution profile of the reaction mixture of the thioester method: HPLC elution conditions; column: Cosmosil 5$C_{18}$AR (10 × 250 mm); linear increase of acetonitrile concentration from 30 to 70% in 0.1% aqueous trifluoroacetic acid over 40 min at a flow rate of 2.5 mL min$^{-1}$.

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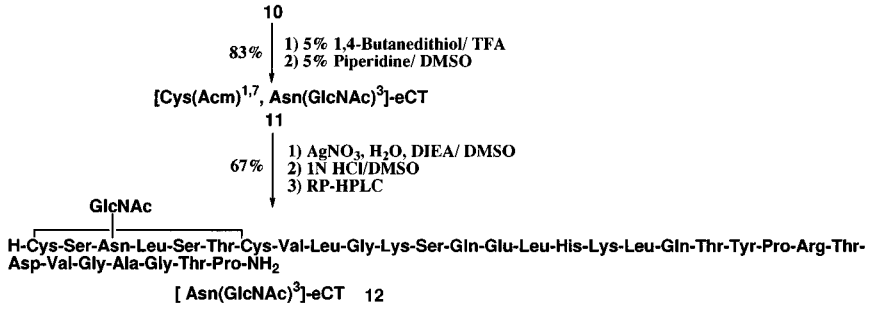
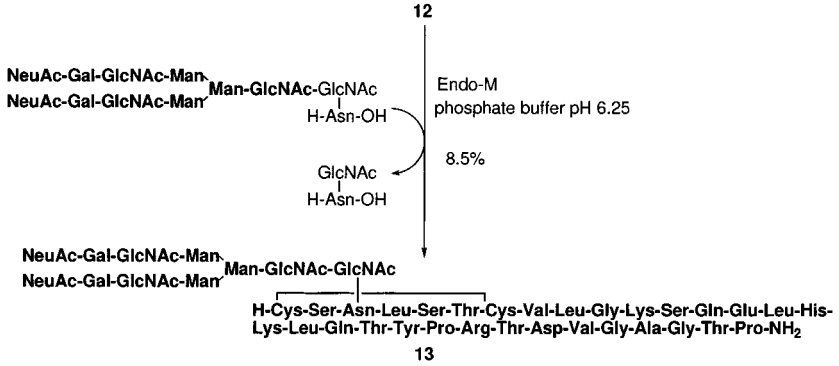
**Scheme 5.** Disulfide Bond Formation between $Cys^1$ and $Cys^7$ for the Synthesis of [Asn(GlcNAc)$^3$]-eCT **12**




**Scheme 6.** Transglycosylation of Disialo Complex-Type Oligosaccharide to **12** by Endo-M




ride **13**, and the resulting yield of transglycosylation was 8.5%. The yields of the eCT analogues having an asialo complex-type [Asn{(Gal-GlcNAc-Man)$_2$-Man-(GlcNAc)$_2$}$^3$]-eCT (**14**) and a high-mannose-type [Asn{(Man)$_6$-(GlcNAc)$_2$}$^3$]-eCT (**15**) were 7.5% and 3.5%, respectively.

The reason that the disialo complex-type oligosaccharide was a better glycoside donor for the transglycosylation reaction of Endo-M than an asialo complex type and a high-mannose type was described previously.[12] We have also studied the conformations of glycosylated-eCT using NMR, and the NMR data for **15** were obtained (Table 1). The detailed results will be described in the future.[16]

The potential biological activity of **15** was tested with murine osteoclast-like multinucleated cell (OCLs).[17] The calcitonin derivative **15** inhibited actin ring formation of OCLs at $10^{-14}$−$10^{-10}$ M concentration depending on the concentration of the drug administered. Concentrations of $10^{-12}$−$10^{-10}$ M of [Asn(GlcNAc)$^3$]-eCT and $10^{-16}$−$10^{-12}$ M of elcatonin, which is an analogue of eel calcitonin having a disulfide bond $Cys^1$-$Cys^7$ replaced by an ethylene linkage between residues 1 and 7, showed approximately the same strength of biological activity (relative strength is 0.79),[18] also inhibited actin ring formation of OCLs.[19]

## Conclusions

We have described a new strategy for the *N*-glycoprotein using the Mpt-MA method, the thioester method and the transglycosylation reaction of Endo-M. We made it possible to transfer various N-linked oligosaccharides to the *N*-acetylglucosaminyl peptide using the chemoenzymatic method. This strategy can be generally used for the preparation of natural *N*-glycopeptides and their analogues and neoglycopeptides. This is the first report on the artificial addition of N-linked oligosaccharides to the bioactive peptide having no natural sugar chains.

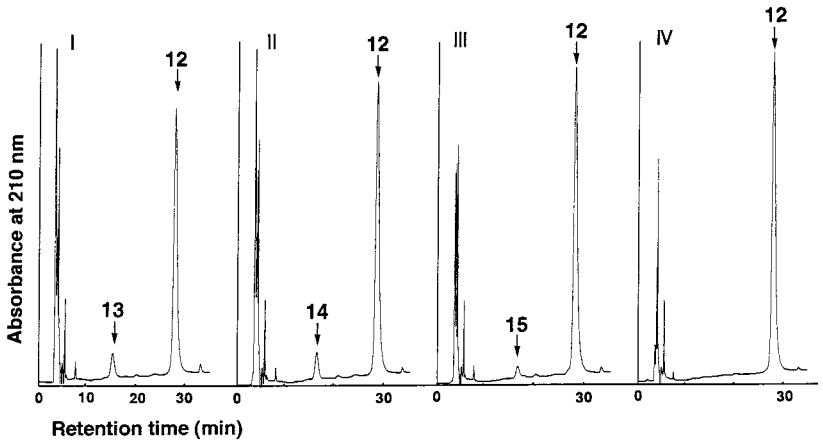



**Figure 3.** Transglycosylation reactions of natural N-linked oligosaccharides to **12** by Endo-M. The reaction mixtures were analyzed by HPLC using an ODS column and monitoring the UV absorption at 210 nm. A sample of each reaction mixture containing H-Asn[(NeuAc−Gal-GlcNAc-Man)$_2$-Man-(GlcAc)$_2$]-OH (I), H-Asn[(Gal-GlcNAc-Man)$_2$-Man-(GlcNAc)$_2$]-OH (II), or H-Asn[(Man)$_6$-(GlcNAc)$_2$]-OH (III) as a glycoside donor was analyzed by HPLC. The reaction mixture without a donor was also analyzed (IV).

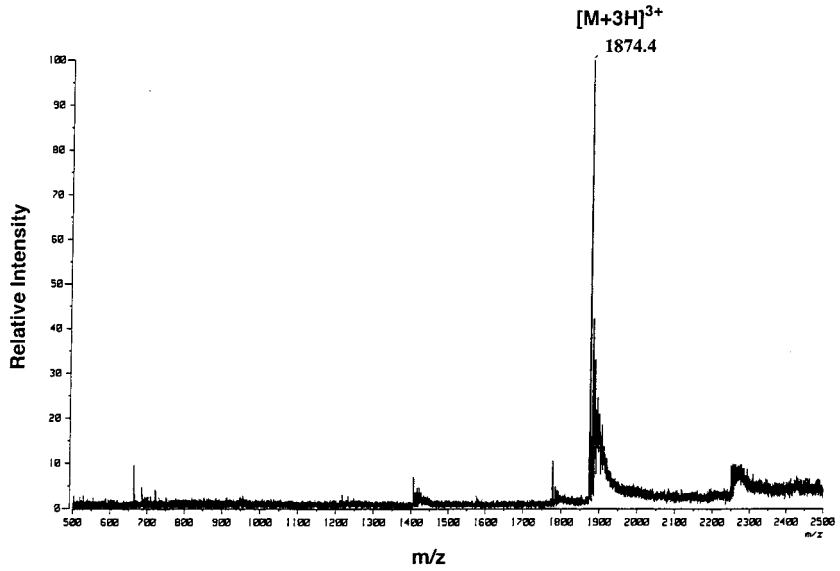



**Figure 4.** ESI mass spectrum of **13**.

**Table 1.** Assignment of $^1$H NMR of **15**[a]

| | chemical shifts (ppm) | | | |
|---|---|---|---|---|
| residue | α-NH | α-H | $\beta$-H$_2$ | others |
| Cys1 | | 4.41 | 3.65 | |
| Ser2 | 8.52 | 4.56 | 3.94, 3.82 | |
| Asn3 | 7.77 | 4.56 | 3.19 | |
| Leu4 | 8.61 | 4.04 | 1.77, 1.73 | $\gamma$ 1.66, $\delta$ 0.93 |
| Ser5 | 8.46 | 3.94 | 4.08 | |
| Thr6 | 7.89 | 4.24 | 4.27 | $\gamma$ 1.35 |
| Cys7 | 8.61 | 4.70 | 3.21 | |
| Val8 | 8.55 | 3.77 | 2.21 | $\gamma$ 1.06, $\delta$ 0.93 |
| Leu9 | 8.27 | 4.05 | 1.92, 1.85 | $\gamma$ 1.63, $\delta$ 0.93 |
| Gly10 | 8.14 | 4.04, 3.91 | | |
| Lys11 | 7.65 | 4.26 | 2.03, 1.92 | $\gamma$ 1.57, 1.52, $\delta$ 1.77, $\epsilon$ 2.99, $\epsilon$-NH$_2$ 7.45 |
| Leu12 | 8.33 | 4.03 | 1.89, 1.85 | $\gamma$ 1.62, $\delta$ 0.88 |
| Ser13 | 8.49 | 4.03 | 4.03 | |
| Gln14 | 7.85 | 4.13 | 2.33, 2.26 | $\gamma$ 2.58, 2.43 |
| Glu15 | 8.07 | 4.33 | 2.20, 2.13 | $\gamma$ 2.65, 2.35 |
| Leu16 | 8.46 | 4.03 | 1.71 | $\gamma$ 1.67, $\delta$ 0.88 |
| His17 | 8.01 | 4.55 | 3.37, 3.30 | C$_2$ 8.56, C$_4$ 7.33 |
| Lys18 | 7.85 | 4.12 | 2.02 | $\gamma$ 1.43, $\delta$ 1.76, 1.67, $\epsilon$ 2.99, $\epsilon$-NH$_2$ 7.36 |
| Leu19 | 7.91 | 4.16 | 1.91 | $\gamma$ 1.67, $\delta$ 0.94, 0.88 |
| Gln20 | 8.94 | 4.17 | 2.17 | $\gamma$ 2.53, 2.42 |
| Thr21 | 7.55 | 4.25 | 4.18 | $\gamma$ 1.11 |
| Tyr22 | 7.65 | 4.76 | 3.09, 3.05 | $\delta$ 7.14, $\epsilon$ 6.77 |
| Pro23 | | 4.43 | 2.20 | $\gamma$ 1.98, 1.94, $\delta$ 3.78, 3.40 |
| Arg24 | 8.10 | 4.35 | 1.95, 1.85 | $\gamma$ 1.74, 1.70, $\delta$ 3.23, NH 7.20 |
| Thr25 | 7.88 | 4.31 | 4.27 | $\gamma$ 1.19 |
| Asp26 | 8.23 | 4.78 | 2.99, 2.86 | |
| Val27 | 7.87 | 4.07 | 2.14 | $\gamma$ 0.95 |
| Gly28 | 8.30 | 3.99, 3.92 | | |
| Ala29 | 7.97 | 4.32 | 1.39 | |
| Gly30 | 8.23 | 3.97 | | |
| Thr31 | 7.92 | | | |
| Pro32 | | | | $\gamma$ 1.97, $\delta$ 3.81, 3.75 |

Furthermore, prepared glycosylated-eCT derivatives exhibited strong inhibitory activity on the actin ring formation of OCLs.

## Experimental Section

**Materials and Methods.** Abbreviations used are Acm, acetamidomethyl; Boc, *tert*-butoxycarbonyl; Boc-OSu, *N*-(*tert*-butoxycarbonyloxy)succinimide; Bom, benzyloxymethyl; Bzl, benzyl; Br-Z, 2-bromobenzyloxycarbonyl; cHex, cyclohexyl; Cl-Z, 2-chlorobenzyloxycarbonyl; DCC, *N*,*N*-dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; HOObt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; Mpt-Cl, dimethylphosphinothioyl chloride; MBHA resin, 4-methylbenzhydrylamine resin; TFA, trifluoroacetic acid; TMSOTf, trifluoromethanesulfonic acid trimethylsilyl ester; Tos, *p*-toluenesulfonyl.

**Preparation of GlcNAc-eCT.** RP-HPLC was performed on Cosmosil 5C18AR (10 × 250 mm) (Nakalai Tesque, Inc., Kyoto, Japan). Solvent system A: 0.1% TFA in water. Solvent system B: 0.1% TFA in acetonitrile. Detection was at 220 nm. Amino acids were analyzed on an L-8500 amino acid analyzer (Hitachi, Ltd., Tokyo, Japan) after hydrolysis with 6 M HCl or 4 M methanesulfonic acid at 110 °C for 24 h in an evacuated sealed tube. Peptide mass numbers were determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using a Voyage RP (PerSeptive Biosystems, Inc., Framingham, MA). Mass numbers were calculated as averages. MALDI-TOF mass spectrometry was performed in the positive ion mode using α-cyano-4-hydroxycinnamic acid (α-CHCA) as a matrix.

Solid-phase synthesis of peptide segment was carried out on a peptide synthesizer 430A (Applied Biosystems, Inc., Foster City, CA.). The 0.5-mmol standard protocol of system software version 1.40 NMP/HOBt *t*-Boc was employed. End capping by acetic anhydride was performed after each amino acid introduction reaction.

**Preparation of the Enzyme.** Endo-M was partially purified from the culture medium of *M. hiemalis*, as described by Kadowaki et al.[20] The enzyme preparation was free from other glycosidase activities but contained very little protease activity.

**Preparation of Substrates.** The glycosylasparagines having disialo complex-type H-Asn[(NeuAc-Gal-GlcNAc-Man)$_2$-Man-(NeuAc)$_2$]-OH and the asialo complex-type asparagine H-Asn[(Gal-GlcNAc-Man)$_2$-Man-(GlcNAc)$_2$]-OH, derived from human serum transferrin,[21] and the glycosylasparagine having high-mannose type oligosaccharide H-Asn-[(Man)$_6$-(GlcNAc)$_2$]-OH of ovalbumin were prepared as described previously.[13,22]

**Transglycosylation Reaction.** The yield of the transglycosylation product was calculated by the followimg equation: yield (%) = (area of the peak of the product/initial areas of the peak of the acceptor) × 100.[12] Electrospray (ESI) mass spectrometry was performed in the

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(21) Recently, we prepared it from egg yolk.[23]

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positive-ion mode on an Analytica Branford/JEOL JMS-SX 102A mass spectrometer (JEOL, Ltd., Tokyo, Japan). MALDI-TOF mass spectrometry was performed in the positive ion mode using 2,5-dihydroxybenzoic acid (DHB) as a matrix on a Finnigan Lasermat mass spectrometer (Finnigan Mat, U.K.).

**$N^{\alpha}$-(*tert*-butyloxycarbonyl)-$N^{\omega}$-(2-acetamido-2-deoxy-$\beta$-D-glucopyranosyl)-L-asparagine (2).** Tributylphosphine (2.5 mL, 10 mmol) was added to a dichloromethane (80 mL) solution of $N^{\alpha}$-(*tert*-butyloxycarbonyl)-L-aspartic acid α-benzyl ester (3.88 g, 12 mmol) and 2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-$\beta$-D-glucopyranosyl azide (5.17 g, 10 mmol) under an argon atmosphere at −78 °C. After being stirred for 22 h, the reaction mixture was diluted with chloroform and washed successively with aqueous $NaHCO_3$, water, and brine, and dried over anhydrous $Na_2SO_4$ and the solvent evaporated. The resulting precipitate was filtrated and washed with diethyl ether three times. Compound **1** (5.11 g, 6.4 mmol) was obtained as a white powder in 54% yield. Compound **1** (3.32 g, 4.2 mmol) was dissolved in $CH_2Cl_2$ (40 mL)−MeOH (100 mL) and hydrogenated over 10% Pd/C under atmospheric pressure. After 3.5 h, the mixture was filtered and the filtrate was evaporated in vacuo. The residue was washed with diethyl ether on a glass filter, and **2** (1.88 g, 4.2 mmol) was obtained as a white powder in quantitative yield. Mp: 210−213 °C. $[\alpha]^{27}_D$: 15.2 ° (*c* 1, DMSO). MALDI-TOF MS: found *m*/*z* $[M + Na]^+$ 458.3, calcd for $C_{17}H_{29}N_{13}O_{10}$ $[M + Na]^+$ 458.4. $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ = 8.10 (1H, d, $J$ = 8.8 Hz, 1-NH), 7.74 (1H, d, $J$ = 8.8 Hz, 2-NH), 6.62 (1H, d, $J$ = 8.3 Hz, Asn-α-NH), 4.79 (1H, t, $J$ = 9.28 Hz, 1-H), 4.21 (1H, m, Asn-α-CH), 3.66−3.05(6H, m, 2,3,4,5,6-H), 2.56−2.40 (2H, m, Asn-$\beta$-$CH_2$), 1.79 (3H, s, NAc), 1.35 (9H, s, *t*-Bu).

**Boc-Gly-S-$CH_2CH_2$CO-Nle-MBHA-Resin (3).** A mixture of Boc-Nle-OH (0.80 g, 3.46 mmol), 1.0 M HOBt in NMP (2.8 mL), and 1.0 M DCC in NMP (2.8 mL) was stirred for 1 h. The solution was added to a neutralized MBHA resin (2.4 g, $NH_2$: 1.85 mmol), and this mixture was shaken for 3 h. The resulting resin was washed with NMP (3 × 1 min), 50% methanol in $CH_2Cl_2$ (4 × 1 min and 2 × 3 min), and $CH_2Cl_2$ (3 × 1 min). The prepared resin was treated with 50% TFA in $CH_2Cl_2$ (1 × 5 min and 1 × 20 min), washed with $CH_2Cl_2$ (3 × 1 min), and treated with 5% DIEA in $CH_2Cl_2$ (1 × 15 min and 1 × 2 min) and 5% DIEA in NMP (3 × 1 min). Trt-S-$CH_2CH_2$COOH (0.90 g, 2.71 mmol), 1.0 M HOBt in NMP (2.7 mL) and 1.0 M DCC in NMP (2.7 mL), were mixed for 30 min, and the resulting solution was added to the resin. The suspension was shaken for 4 h. The resin was washed with NMP (3 × 3 min), 50% methanol in $CH_2Cl_2$ (3 × 3 min), and then $CH_2Cl_2$ (3 × 1 min) and finally dried in vacuo to give the Trt-S-$CH_2CH_2$CO-Nle-MBHA-resin (2.74 g, Nle: 0.52 mmol $g^{-1}$). An aliquot of the resin (0.79 g) was treated with 5% 1,2-ethanedithiol in TFA (4 × 10, 20, and 30 min), then washed with $CH_2Cl_2$ (3 × 1 min), treated with 5% DIEA in NMP (2 × 1 min), and washed with NMP (3 × 1 min). A mixture of Boc-Gly-OH (0.32 g, 1.81 mmol), 1.0 *M* HOBt in NMP (1.8 mL), and 1.0 M DCC in NMP (1.7 mL) was stirred for 1 h. This solution was added to the resin and the suspension was shaken for 16 h. The resin was washed with NMP (3 × 3 min), 50% methanol in $CH_2Cl_2$ (3 × 3 min), and then $CH_2Cl_2$ (3 × 1 min) and finally dried in vacuo to give the Boc-Gly-S-$CH_2CH_2$CO−Nle-MBHA-resin **3** (0.75 g, Nle: 0.55 mmol $g^{-1}$).

**Fmoc-[Cys(Acm)$^{1,7}$,Asn(GlcNAc)$^3$]-eCT(1-10)-S-$CH_2CH_2$CO-Nle-$NH_2$ (6).** By starting from the Boc-Gly-S-$CH_2CH_2$CO-Nle-MBHA-resin (**3**) (0.75 g, 0.41 mmol), peptide chain elongation was carried out using a 430A peptide synthesizer to give 1.40 g of a protected peptide resin corresponding to the sequence of eel calcitonin(4-10), Boc-Leu-Ser-(Bzl)-Thr(Bzl)-Cys(Acm)-Val-Leu-Gly-S-$CH_2CH_2$CO-Nle-NH-resin (**4**). Further, 3-Asn(GlcNAc), 2-Ser(Bzl), and 1-Cys(Acm) residues were coupled manually by double coupling of the corresponding Mpt-MA. The Mpt-MAs of Boc-Asn(GlcNAc)-OH, Boc-Ser(Bzl)-OH, and Fmoc-Cys(Acm)-OH were prepared by mixing with 1.23 mmol of Boc-Asn-(GlcNAc), Boc-Ser(Bzl), and Fmoc-Cys(Acm) and 1 M Mpt-Cl in DMF (1.2 mL) and 2 M DIEA in NMP (0.62 mL) and NMP (1.0 mL), and after 30 min, 2 M DIEA in NMP (0.62 mL) was added to the solution of Mpt-MA. The reaction mixture of Mpt-MA was added to the protected peptide resin **4**, and the coupling time was 1 h. The terminations of these coupling reactions were determined by the Kisser test. An aliquot of the glycopeptide resin (400 mg) was treated with HF (9.0 mL) and anisole (1.0 mL) at 0 °C for 90 min. After evaporation of the HF, ether (10 mL) was added to the mixture and the obtained precipitate was washed with ether (10 mL × 2) and dissolved in TFA (10 mL). The TFA solution was filtered through a glass filter to remove the resin and poured into cold ether (300 mL). The resulting precipitation was isolated by decantation, and crude glycopeptide **3** (156 mg) was obtained. After purification by RP-HPLC [buffer A−buffer B, 20:80−40:60 (40 min)], Fmoc-[Cys(Acm),$^{1,7}$Asn(GlcNAc)$^3$]-eCT(1-10)-S-$CH_2CH_2$CO-Nle-$NH_2$ (**6**) was obtained (47 mg, 49 $\mu$mol, 12% based on Nle in the starting resin). MALDI-TOF MS: found *m*/*z* $[M + Na]^+$ 1787.4, calcd for $C_{77}H_{118}N_{16}O_{25}S_3$ $[M + Na]^+$ 1787.1. Amino acid analysis by hydrolysis with 6 M HCl at 110 °C for 24 h: $Asp_{1.00}Thr_{0.70}$-$Ser_{1.77}Gly_{1.07}Cys_{nd}Val$+$GlcNH_{2\ 1.36}Leu_{1.90}$. Cys was not observed, because the *S*-Acm group is stable under the above conditions.

**[Lys(Boc)$^{11,18}$]-eCT(11-32) (9).** By starting from MBHA-resin (1.00 g, 0.42 mmol, $NH_2$: 0.41 mmol $g^{-1}$), the chain elongation reaction was carried out and the peptide resin **7** (2.29 g) was obtained. An aliquot of the glycopeptide resin (500 mg) was treated with HF (8.5 mL), anisole (0.75 mL), and 1,4-butanedithiol (0.75 mL) at 0 °C for 90 min. After evaporation of the HF, ether (10 mL) was added to the mixture and the obtained precipitate was washed with ether (10 mL × 2), then extracted with 20% aqueous acetonitrile containing 5% acetic acid (20 mL) to give the crude peptide (178 mg) after freeze-drying. This was purified on RP-HPLC [buffer A−buffer B, 20:80−60:40 (40 min)] to yield Fmoc-eCT(11-32) (**8**) (96 mg, 30 $\mu$mol, 34% based on the amino group in the starting resin) after freeze-drying. MALDI-TOF MS: found *m*/*z* $[M + H]^+$ 2662.0, calcd for $C_{122}H_{186}N_{32}O_{35}$ $[M + H]^+$ 2662.0. Amino acid analysis by hydrolysis with 6 M HCl at 110 °C for 24 h: $Asp_{1.04}Thr_{2.98}Ser_{1.01}Glu_{3.03}Pro_{1.70}Gly_{2.14}Ala_{1.18}Val_{0.99}Leu_{3.17}Tyr_{1.00}Lys_{2.01}$-$His_{1.07}Arg_{1.03}$.

Boc-OSu (16 mg, 76 $\mu$mol) and DIEA (60 $\mu$L) were added to a solution of peptide **8** (26 mg, 7.6 $\mu$mol) in DMSO (2.0 mL), and the resulting solution was stirred for 7 h. Ether was added to the reaction mixture, and the resulting precipitation was washed with ether (10 mL × 3) and then dissolved in DMSO (2.0 mL). To the solution was added piperidine (0.1 mL), followed by stirring for 90 min. Ether was added to the reaction mixture, and the resulting precipitation was washed with ether (10 mL × 3). After purification by RP-HPLC [buffer A−buffer B, 20:80−65:35 (30 min)], [Lys(Boc)$^{11,18}$]-eCT(11-32) (**9**) was obtained (11 mg, 4.3 $\mu$mol, 56%). Overall yield based on the amino group in the starting resin was 19%. MALDI-TOF MS: found *m*/*z* $[M + H]^+$ 2639.7, calcd for $C_{117}H_{192}N_{32}O_{37}$ $[M + H]^+$ 2640.0. Amino acid analysis by hydrolysis with 6 M HCl at 110 °C for 24 h: $Asp_{1.05}Thr_{3.01}Ser_{1.00}$-$Glu_{3.18}Pro_{1.52}Gly_{2.10}Ala_{1.08}Val_{1.03}Leu_{3.23}Tyr_{0.99}Lys_{2.11}His_{1.09}Arg_{1.06}$.

**Fmoc-[Cys(Acm)$^{1,7}$,Asn(GlcNAc)$^3$,Lys(Boc)$^{11,18}$]-eCT (10).** A solution of $AgNO_3$ (0.9 mg, 5.4 $\mu$mol), HOObt (8.8 mg, 54 $\mu$mol), and DIEA (6.3 $\mu$L, 36 $\mu$mol) in DMSO (0.20 mL) was stirred for 1 h, and to the mixture was added a solution of glycopeptide thioester segment **3** (5.8 mg, 1.8 $\mu$mol) and peptide segment **9** (4.8 mg, 1.8 $\mu$mol) in DMSO (0.30 mL). After 16 h of stirring, DTT (2.5 mg, 16 $\mu$mol) was added to the reaction mixture to terminate the coupling reaction. A partially protected glycopeptide Fmoc-[Cys(Acm),$^{1,7}$Asn(GlcNAc)$^3$,Lys-(Boc)$^{11,18}$]-eCT (**7**) (4.3 mg, 1.0 mmol, 55%) was isolated by RP-HPLC [buffer A−buffer B, 30:70−70:30 (40 min)]. MALDI-TOF MS: found *m*/*z* $[M + H]^+$ 4185.4, calcd for $C_{185}H_{292}N_{46}O_{60}S_2$ $[M + H]^+$ 4185.8. Amino acid analysis by hydrolysis with 4 M methanesulfonic acid at 110 °C for 24 h: $Asp_{2.02}Thr_{3.57}Ser_{2.75}Glu_{3.06}Pro_{1.40}Gly_{3.00}Ala_{2.13}Cys_{nd}$-Val+$GlcNH_{2\ 2.38}Leu_{5.23}Tyr_{0.15}Lys_{1.94}His_{1.05}Arg_{0.96}$.

**[Cys(Acm)$^{1,7}$,Asn(GlcNAc)$^3$]-eCT (11).** A partially protected glycopeptide **10** (4.0 mg, 0.77 $\mu$mol) was treated with TFA containing 5% 1,4-butanedithiol (0.30 mL) at room temperature for 90 min. $H_2O$ (7.0 mL) was added to the reaction mixture, and the solution was washed with ether (3 mL × 5). After freeze-drying, crude glycopeptide was obtained. MALDI-TOF MS: found *m*/*z* $[M + H]^+$ 3985.4, calcd for $C_{175}H_{276}N_{46}O_{56}S_2$ $[M + H]^+$ 3985.5. This crude glycopeptide was treated with 5% piperidine in DMSO (0.40 mL) at room temperature for 2.5 h. Acetic acid (50 $\mu$L) was added to the solution for quenching. After purification by RP-HPLC [buffer A−buffer B, 20:80−60:40 (40 min)], [Cys(Acm),$^{1,7}$Asn(GlcNAc)$^3$]-eCT (**11**) was obtained (3.5 mg, 0.64 $\mu$mol, 83% based on **10**). MALDI-TOF MS: found *m*/*z* $[M + H]^+$ 3764.8, calcd for $C_{160}H_{266}N_{46}O_{54}S_2$ $[M + H]^+$ 3763.3. Amino acid

analysis by hydrolysis with 6 M HCl at 110 °C for 24 h: $Asp_{2.09}Thr_{3.55}Ser_{2.68}Glu_{3.16}Pro_{1.65}Gly_{3.00}Ala_{1.08}Cys_{1.39}Val+GlcNH_{2\ 1.22}Leu_{5.41}Tyr_{nd}Lys_{1.97}His_{1.02}Arg_{1.00.}$

**[Asn(GlcNAc)$^3$]-eCT (12).** Glycopeptide **11** (3.2 mg, 0.59 $\mu$mol) was dissolved in $H_2O$ (0.30 mL) to which was added a solution of $AgNO_3$ (0.5 mg, 3.0 $\mu$mol) and DIEA (1.5 $\mu$L, 8.9 $\mu$mol) in DMSO (100 $\mu$L). After 2 h of stirring, a mixture of 1 M hydrochloric acid and DMSO (1:1, v/v, 3.0 mL) was added to the solution, and the reaction mixture was stirred for 20 h. After purification by RP-HPLC [buffer A−buffer B, 80:20−50:50 (40 min)], [Asn(GlcNAc)$^3$]-eCT (**12**) was obtained (1.8 mg, 0.4 $\mu$mol, 67%). MALDI-TOF MS: found *m*/*z* [M + H]$^+$ 3618.9, calcd for $C_{154}H_{254}N_{44}O_{52}S_2$ [M + H]$^+$ 3619.1. Amino acid analysis by hydrolysis with 4 M methanesulfonic acid at 110 °C for 24 h: $Asp_{2.05}Thr_{3.87}Ser_{2.84}Glu_{3.26}Pro_{1.90}Gly_{2.98}Ala_{0.96}Cys_{0.81}Val+GlcNH_{2\ 2.55}Leu_{5.00}Tyr_{0.97}Lys_{1.96}His_{1.08}Arg_{1.03}$.

**Transglycosylation Reaction.** The transglycosylation reaction was carried out as reported previously.[12] The reaction mixture was composed of 250 nmol (final 25 mM) of a glycoside donor (H-Asn[(NeuAc-Gal-GlcNAc-Man)$_2$-Man-(GlcAc)$_2$]-OH, H-Asn[(Gal-GlcNAc-Man)$_2$-Man-(GlcNAc)$_2$]-OH or H-Asn[(Man)$_6$-(GlcNAc)$_2$]-OH), 100 nmol (final 10 mM) of an acceptor **12**, 40 $\mu$U (final 4 mU mL$^{-1}$) of Endo-M, 60 mM potassium phosphate buffer (pH 6.25), and 50 mM EDTA, which was added in order to avoid the action of the trace amount of protease contaminating the Endo-M preparation, in a total volume of 10 $\mu$L. After incubation for 6 h at 37 °C, the reaction mixture was mixed with 10 $\mu$L of 0.1% trifluoroacetic acid (TFA) to terminate the reaction, diluted to 200 $\mu$L with cold distilled water, and analyzed by HPLC. HPLC analysis of glycosylated-eCT derivatives was performed using a reverse-phase (ODS) column (6 × 250 mm, Mightysil RP-18, Kanto Chemical Co., Inc., Tokyo, Japan). Elution was carried out with a linear gradient of acetonitrile (30−35%) containing 0.1% TFA in 40 min at a flow rate of 1 mL min$^{-1}$. The reaction products were monitored by absorption at 210 nm. The disialo complex-type **13** was obtained in 8.5% yield. The yields of the eCT analogue having an asialo complex-type **14** and a high-mannose-type **15** were 7.5% and 3.5%, respectively.

**13**: found 5620.2, calcd from *m*/*z* [M + 3H]$^{3+}$ 1874.4, calcd for $C_{230}H_{377}N_{49}O_{108}S_2$ [M + H]$^+$ 5621.9. **14**: found *m*/*z* [M + H]$^+$ 5040.3, calcd for $C_{208}H_{343}N_{47}O_{92}S_2$ [M + H]$^+$ 5039.4. **15**: found *m*/*z* [M + H]$^+$ 4794.1, calcd for $C_{198}H_{327}N_{45}O_{87}S_2$ [M + H]$^+$ 4795.2.

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