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Chemo-enzymatic synthesis of eel calcitonin glycosylated at two sites with the same and different carbohydrate structures

Katsuji Haneda,[a,*] Midori Takeuchi,[a] Mizuka Tagashira,[b] Toshiyuki Inazu,[a,c] Kazunori Toma,[a,*] Yukihiro Isogai,[d] Masayuki Hori,[d] Kazuo Kobayashi,[e] Makoto Takeuchi,[e,✠] Kaoru Takegawa[f] and Kenji Yamamoto[g]

[a]*The Noguchi Institute, 1-8-1, Kaga, Itabashi, Tokyo 173-0003, Japan*
[b]*Research Center, Asahi Kasei Pharma Co., Izunokuni, Shizuoka 410-2321, Japan*
[c]*Department of Applied Chemistry, School of Engineering, Tokai University, Hiratsuka, Kanagawa 259-1292, Japan*
[d]*Laboratory for Pharmacology, Asahi Kasei Pharma Co., Izunokuni, Shizuoka 410-2321, Japan*
[e]*Central Laboratories for Key Technology, Kirin Brewery Co., Yokohama, Kanagawa 236-0004, Japan*
[f]*Department of Agriculture, Kagawa University, Kida-gun, Kagawa 761-0795, Japan*
[g]*Graduate School of Biostudies, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan*

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Abstract—Naturally occurring glycopeptides and glycoproteins usually contain more than one glycosylation site, and the structure of the carbohydrate attached is often different from site to site. Therefore, synthetic methods for preparing peptides and proteins that are glycosylated at multiple sites, possibly with different carbohydrate structures, are needed. Here, we report a chemo-enzymatic approach for accomplishing this. Complex-type oligosaccharides were introduced to the calcitonin derivatives that contained two *N*-acetyl-D-glucosamine (GlcNAc) residues at different sites by treatment with *Mucor hiemalis endo*-β-*N*-acetylglucosaminidase. Using this enzymatic transglycosylation reaction, three glycopeptides were produced, a calcitonin derivative with the same complex-type carbohydrate at two sites, and two calcitonin derivatives each with one complex-type carbohydrate and one GlcNAc. Starting from the derivatives with one complex-type carbohydrate and one GlcNAc, a high-mannose-type oligosaccharide was successfully transferred to the remaining GlcNAc using another *endo*-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae*. Thus, we were able to obtain glycopeptides containing not only two complex-type carbohydrates, but also both complex and high-mannose-type oligosaccharides in a single molecule. Using the resultant glycosylated calcitonin derivatives, the effects of di-N-glycosylation on the structure and the activity of calcitonin were studied. The effect appeared to be predictable from the results of mono-N-glycosylated calcitonin derivatives.

Keywords: Calcitonin; *endo*-β-*N*-Acetylglucosaminidase; Chemo-enzymatic synthesis; N-glycosylation; Multiple glycosylation sites

1. Introduction

Many proteins and peptides of higher living organisms are post-translationally modified by glycosylation. Naturally occurring glycoproteins and glycopeptides typically contain more than one glycosylation site. The carbohydrate structure of each glycosylation site can be different from one site to another, and is usually heterogeneous. Carbohydrate side chains are known to affect the three-dimensional structure, stability or biological activity of glycoproteins,[1] and multiple glycosylation may enhance such effects in a cooperative manner, which is often referred to as a cluster effect.[2]

We previously developed a chemo-enzymatic synthetic method for preparing glycopeptides,[3] which involves the

* Corresponding authors. Tel./fax: +81 3 5944 3212 (K.H.); tel./fax: +81 3 5944 3216 (K.T.); e-mail addresses: ha-kappa@mbp.nifty.com; toma@noguchi.or.jp
✠ Deceased.

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chemical synthesis of a peptide possessing an *N*-acetyl-D-glucosamine (GlcNAc) residue[4,5] and the subsequent enzymatic transglycosylation of a naturally occurring oligosaccharide to the GlcNAc moiety via catalysis by an *endo*-β-*N*-acetylglucosaminidase.[6] *endo*-β-*N*-Acetylglucosaminidase is an enzyme that hydrolyzes the glycosyl bond between the reducing terminal two GlcNAc residues of an N-linked oligosaccharide of a glycoprotein or glycopeptide, leaving only the reducing terminal GlcNAc residue attached to the amide nitrogen of an Asn residue. Some *endo*-β-*N*-acetylglucosaminidases, however, also exhibit transglycosylation activity[6,7] under certain conditions, and can be used to transfer an N-linked oligosaccharide en masse to an GlcNAc moiety. Thus, the transglycosylation reaction catalyzed by *endo*-glycosidase is one of the most powerful methods for the synthesis of glycopeptides.[8,9] Using a chemo-enzymatic approach, we were able to introduce an N-linked oligosaccharide not only onto an Asn residue of bioactive peptides,[10,11] but also onto a Gln residue, to which the biological addition of an oligosaccharide is impossible, only if a peptide possessing *N*-acetyl-D-glucosaminyl-L-asparagine (Asn(GlcNAc)) or *N*-acetyl-D-glucosaminyl-L-glutamine (Gln(GlcNAc)), respectively, were to be placed in the sequence.[12]

We employed two *endo*-β-*N*-acetylglucosaminidases in the transglycosylation reactions: one from *Mucor hiemalis* (Endo-M)[13,14] and the other from *Arthrobacter protophormiae* (Endo-A).[7] Endo-M hydrolyzes various types of N-linked oligosaccharides, but transglycosylates complex-type ones most efficiently. Endo-A, on the other hand, specifically hydrolyzes and transglycosylates high-mannose-type oligosaccharides.

The transglycosylation reaction enabled us to study the effect of glycosylation on the three-dimensional structure and biological activity of peptides. In our most recent experiments, eel calcitonin was used as a model peptide. Calcitonin is a hormone that regulates calcium metabolism, and is composed of 32 amino acids. Calcitonin and its synthetic derivatives are currently utilized as therapeutic agents in the treatment of hypercalcemia, Paget's disease and osteoporosis.[15] Although its third Asn residue is a potential N-glycosylation site, with the amino acid sequence Asn-Leu-Ser, calcitonin is not glycosylated in its major natural form. We introduced various oligosaccharide structures to several sites on calcitonin by a chemo-enzymatic method. In addition to Asn3, we introduced an N-linked oligosaccharide of either the complex-type or the high-mannose-type to Gln14, Gln20 and Asp26. In the case of Asp26, the residue was replaced with Asn. We successfully completed the chemo-enzymatic synthesis of N-glycosylated calcitonin derivatives possessing a single N-linked oligosaccharide onto either Asn3, Gln14, Gln20 or Asp26.[9–11,16–20] To gain a general perspective, we are currently compiling a structure–activity study of glycosylated calcitonin,[21] including data for O-glycosylated derivatives.[22,23]

We report here the chemo-enzymatic synthesis of di-N-glycosylated calcitonin derivatives that contain two oligosaccharides at two of the possible N-glycosylation sites selected among Asn3, Gln14, Gln20 and Asp26. The success of the efficient transglycosylation of two N-glycosylation sites permits us to investigate the effect of multiple glycosylation on the structure and activity of a bioactive peptide using structurally defined and chemically pure samples. Syntheses of glycopeptides that contain two different oligosaccharides on a single peptide have been achieved by a fully synthetic method,[24] a semi-synthetic method[25] and a chemo-enzymatic method using glycosyltransferases,[26] but, to the best of our knowledge, this may be the first example of the chemo-enzymatic introduction of two different oligosaccharides by transglycosylation reactions of *endo*-glycosidases.

2. Experimental

2.1. Materials

Unless specifically described in the following sections, commercially available materials were used as received.

[Asn(GlcNAc)[3,26]]-calcitonin (CT(3-GlcNAc, 26-GlcNAc)), [Gln(GlcNAc),[20] Asn(GlcNAc)[26]]-calcitonin (CT(20-GlcNAc, 26-GlcNAc)) and [Gln(GlcNAc)[14,20]]-calcitonin (CT(14-GlcNAc, 20-GlcNAc)) were prepared by and purchased from Peptide Institute Inc. (Osaka, Japan).

Purified recombinant Endo-M cloned in *Candida boidinii*[9,27] and purified recombinant Endo-A cloned in *Escherichia coli*[28] were used for the transglycosylation reactions. The Endo-M enzyme solution (in 20 mM phosphate buffer at pH 7.0 containing 0.15 M NaCl) employed in this study showed 600 mU/mL of hydrolytic activity against dansyl-Asn[(Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$]-OH at pH 6.0 and 37 °C. The Endo-A enzyme solution (in 10 mM phosphate buffer of pH 7.0) showed 8.5 U/mL of hydrolytic activity against dansyl-Asn(Man$_6$-GlcNAc$_2$)-OH at pH 6.0 and 37 °C.

As a glycosyl donor for a disialo biantennary complex-type (STF: (Neu5Ac-Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$) oligosaccharide, a sialyl glyco-hexapeptide (SGP), H-Lys-Val-Ala-Asn[(Neu5Ac-Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$]-Lys-Thr-OH, derived from hen egg yolk[29] was purchased from Taiyo Kagaku Co. (Mie, Japan). As a glycosyl donor for a high-mannose-type (M6: Man$_6$-GlcNAc$_2$) oligosaccharide, asparagine having a M6 oligosaccharide, M6-Asn, was prepared from ovalbumin.[30]

2.2. Transglycosylation reaction of STF to CT(3-GlcNAc, 26-GlcNAc) with Endo-M

Three batch reactions were performed using 31.7 mg (8.22 μmol) of CT(3-GlcNAc, 26-GlcNAc). The mixture (0.12–0.3 mL) consisted of 10 mM CT(3-GlcNAc, 26-GlcNAc), 150 mM SGP and 120 mU/mL Endo-M in 60 mM potassium phosphate buffer (pH 6.25) was incubated at 37 °C for 30–45 min, and the reaction was terminated by the addition of an equal volume of a cold aqueous solution of 0.5% trifluoroacetic acid (TFA). The products were analyzed by reverse phase high performance liquid chromatography (RP-HPLC) using a ∅4.6 × 250 mm ODS column (Mightysil RP-18; Kanto Kagaku, Tokyo, Japan). The column was eluted with a linear gradient of acetonitrile concentration from 27.5% to 37.5% in an aqueous solution of 0.1% TFA over 40 min at a flow rate of 0.8 mL/min. The elution was monitored by measuring the absorbance at 214 nm. The transglycosylation yield (%) was calculated from the ratio of the peak area of the transglycosylation product against the initial peak area of the acceptor without consideration of the amount of the remaining acceptor. Three peaks, corresponding to transglycosylation products, were detected at 23.1 min (CT(3-STF, 26-STF)), 26.5 min (CT(3-STF, 26-GlcNAc)) and 30.2 min (CT(3-GlcNAc, 26-STF)), and the remaining acceptor at 34.0 min by RP-HPLC. The average transglycosylation yields for CT(3-STF, 26-STF), CT(3-STF, 26-GlcNAc) and CT(3-GlcNAc, 26-STF) were 38.1%, 20.6% and 23.7%, respectively, and 17.6% of unreacted acceptor remained. The transglycosylation products were isolated by preparative RP-HPLC using ∅20 × 250 mm ODS column (Mightysil RP-18; Kanto Kagaku, Tokyo, Japan) with a linear gradient condition (from 28% to 38% acetonitrile in an aqueous solution of 0.1% TFA over 40 min at a flow rate of 12 mL/min). The purified products were freeze-dried to give 15.23 mg (1.95 μmol, 23.7%) of CT(3-STF, 26-STF), 7.61 mg (1.31 μmol, 15.9%) of CT(3-STF, 26-GlcNAc) and 7.01 mg (1.20 μmol, 14.6%) of CT(3-GlcNAc, 26-STF). The structure of the products was confirmed by MALDI-TOF-MS in a negative ion mode using α-cyano-4-hydroxy-cinnamic acid as a matrix on a Voyager Biospectrometry Workstation (PerSeptive Biosystems, Framingham, USA). CT(3-STF, 26-STF): found *m/z* 7824.4, calcd for $C_{314}H_{514}N_{56}O_{168}S_2$ ($[M-H]^-$) 7824.9. CT(3-STF, 26-GlcNAc): found *m/z* 5821.5, calcd for $C_{238}H_{391}N_{51}O_{112}S_2$ ($[M-H]^-$) 5822.1. CT(3-GlcNAc, 26-STF): found *m/z* 5821.6, calcd for $C_{238}H_{391}N_{51}O_{112}S_2$ ($[M-H]^-$) 5822.1.

To identify the transglycosylation sites, the products were partially hydrolyzed using lysyl *endo*-peptidase (Lys-C; Sigma, Co., St. Louis, USA). A mixture (80 μL) of 40 nmol of the product or the starting material (final concentration: 0.5 mM) and Lys-C (2.5 U/mL) in pH 7.7 Tris–HCl buffer solution (25 mM) containing EDTA (1 mM) was incubated at 37 °C for 6 h, and the reaction was terminated by the addition of an equal volume of a cold aqueous solution of 0.5% TFA. The reaction mixture was analyzed by RP-HPLC using ∅4.6 × 250 mm ODS column (10–50% acetonitrile in an aqueous solution of 0.1% TFA over 40 min, 0.8 mL/min). Three peaks were observed for each glycopeptide. The peptide fragments were isolated, and analyzed by MALDI-TOF-MS. The retention time and the *m/z* values of each fragment are summarized in Table 1. From these data, the structure of the products

Table 1. Partial hydrolysis of transglycosylation products of CT(3-GlcNAc, 26-GlcNAc) by Lys-C, and RP-HPLC and MALDI-TOFMS analyses of the resulting fragments

Compound		Full length 1–32	Fragment 1–11	Fragment 12–18	Fragment 19–32
CT(3-GlcNAc, 26-GlcNAc)	HPLC (min)	36.0	25.1	10.1	17.4
	m/z Found	3822.5	1326.5	855.1	1677.3
	m/z Calcd	3821.3	1326.1	855.0	1677.9
	Ion form	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$
CT(3-STF, 26-STF)	HPLC (min)	32.8	23.5	10.0	15.5
	m/z Found	7824.4	3328.1	855.3	3675.2
	m/z Calcd	7824.9	3327.3	855.0	3678.7
	Ion form	$[M-H]^-$	$[M-H]^-$	$[M+H]^+$	$[M-H]^-$
CT(3-STF, 26-GlcNAc)	HPLC (min)	33.5	23.3	10.4	17.1
	m/z Found	5821.5	3326.1	855.1	1677.9
	m/z Calcd	5822.1	3327.3	855.0	1677.9
	Ion form	$[M-H]^-$	$[M-H]^-$	$[M+H]^+$	$[M+H]^+$
CT(3-GlcNAc, 26-STF)	HPLC (min)	34.2	24.9	10.5	15.5
	m/z Found	5821.6	1326.2	855.1	3682.4
	m/z Calcd	5822.1	1326.1	855.0	3680.7
	Ion form	$[M-H]^-$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$

HPLC condition: ∅4.6 × 250 mm; 10–50% CH_3CN in 0.1% TFA aq over 40 min; 0.8 mL/min.

Table 2. Partial hydrolysis of transglycosylation products of CT(20-GlcNAc, 26-GlcNAc) by Arg-C, and RP-HPLC and MALDI-TOFMS analyses of the resulting fragments

Compound		Full length	Fragment	
		1–32	1–24	25–32
CT(20-GlcNAc, 26-GlcNAc)	HPLC (min)	41.9	43.2	16.6
	m/z Found	3819.4	2919.5	917.3
	m/z Calcd	3819.3	2919.4	919.0
	Ion form	$[M-H]^-$	$[M-H]^-$	$[M+H]^+$
CT(20-STF, 26-STF)	HPLC (min)	38.9	41.1	13.4
	m/z Found	7824.1	4923.5	2922.4
	m/z Calcd	7824.9	4922.2	2921.8
	Ion form	$[M-H]^-$	$[M-H]^-$	$[M+H]^+$
CT(20-STF, 26-GlcNAc)	HPLC (min)	40.0	41.2	16.3
	m/z Found	5821.7	4925.5	919.1
	m/z Calcd	5822.1	4924.2	919.0
	Ion form	$[M-H]^-$	$[M+H]^+$	$[M+H]^+$
CT(20-GlcNAc, 26-STF)	HPLC (min)	40.7	43.1	13.4
	m/z Found	5822.9	2919.6	2920.4
	m/z Calcd	5822.1	2919.4	2921.8
	Ion form	$[M-H]^-$	$[M-H]^-$	$[M+H]^+$

HPLC condition: ∅4.6 × 250 mm; 5–50% CH_3CN in 0.1% TFA aq over 45 min; 0.8 mL/min.

were finally confirmed as CT(3-STF, 26-STF), CT(3-STF, 26-GlcNAc) and CT(3-GlcNAc, 26-STF).

2.3. Transglycosylation reaction of STF to CT(20-GlcNAc, 26-GlcNAc) with Endo-M

Using 12.93 mg (3.39 μmol) of CT(20-GlcNAc, 26-GlcNAc) (10 mM) as an acceptor, the transglycosylation reaction was performed in two batches in the same manner as was used for CT(3-GlcNAc, 26-GlcNAc). The reaction was performed for 30 or 45 min. Three peaks, corresponding to the transglycosylation products, were detected at 21.4 min (CT(20-STF, 26-STF)), 25.5 min (CT(20-STF, 26-GlcNAc)) and 28.1 min (CT(20-GlcNAc, 26-STF)), and the remaining acceptor at 32.4 min by RP-HPLC. The transglycosylation yields for CT(20-STF, 26-STF), CT(20-STF, 26-GlcNAc) and CT(20-GlcNAc, 26-STF) were 47.6%, 19.7% and 22.7%, respectively, and 10.0% of the acceptor remained. By preparative HPLC, 7.60 mg (0.971 μmol, 28.6%) of CT(20-STF, 26-STF), 2.69 mg (0.462 μmol, 13.6%) of CT(20-STF, 26-GlcNAc) and 3.23 mg (0.555 μmol, 16.4%) of CT(20-GlcNAc, 26-STF) were isolated. The products were identified by MALDI-TOF-MS. CT(20-STF, 26-STF): found *m/z* 7824.1, calcd for $C_{314}H_{514}N_{56}O_{168}S_2$ ($[M-H]^-$) 7824.9. CT(20-STF, 26-GlcNAc): found *m/z* 5821.7, calcd for $C_{238}H_{391}N_{51}O_{112}S_2$ ($[M-H]^-$) 5822.1. CT(20-GlcNAc, 26-STF): found *m/z* 5822.9, calcd for $C_{238}H_{391}N_{51}O_{112}S_2$ ($[M-H]^-$) 5822.1.

The transglycosylation sites were identified by partial hydrolysis using arginyl *endo*-peptidase (Arg-C; Sigma, Co., St. Louis, USA). A mixture (20 μL) of 20 nmol of glycopeptides (1.0 mM) and Arg-C (50 U/mL) in pH 8.0 potassium phosphate buffer solution (50 mM) was incubated at 32 °C for 30 h, and the reaction was terminated by the addition of an equal volume of a cold aqueous solution of 0.5% TFA. The reaction mixture was analyzed by RP-HPLC (5–50% acetonitrile in an aqueous solution of 0.1% TFA over 45 min, 0.8 mL/min). Two peaks were observed for each glycopeptide. From the HPLC retention time and the *m/z* value of each fragment, as summarized in Table 2, the structures of the products were confirmed to be CT(20-STF, 26-STF), CT(20-STF, 26-GlcNAc) and CT(20-GlcNAc, 26-STF).

2.4. Transglycosylation reaction of STF to CT(14-GlcNAc, 20-GlcNAc) with Endo-M

Using 4.90 mg (1.20 μmol) of CT(14-GlcNAc, 20-GlcNAc) (10 mM) as an acceptor, the transglycosylation reaction was performed for 45 min in the same manner as was used for CT(3-GlcNAc, 26-GlcNAc). Three peaks, corresponding to the transglycosylation products, were detected at 25.1 min (CT(14-STF, 20-STF)), 29.1 min (CT(14-GlcNAc, 20-STF)) and 31.6 min (CT(14-STF, 20-GlcNAc)), and the remaining unreacted acceptor at 35.5 min by RP-HPLC. The transglycosylation yields for CT(14-STF, 20-STF), CT(14-GlcNAc, 20-STF) and CT(14-STF, 20-GlcNAc) were 32.7%, 29.5% and 21.0%, respectively, and 16.8% of the acceptor remained. By preparative HPLC, 2.47 mg (0.316 μmol, 26.3%) of CT(14-STF, 20-STF), 1.83 mg (0.314 μmol, 26.2%) of CT(14-GlcNAc, 20-STF) and 1.27 mg (0.218 μmol, 18.2%) of CT(14-STF, 20-GlcNAc) were isolated. The products were identified by MALDI-TOF-MS. CT(14-STF, 20-STF): found *m/z*

Table 3. Partial hydrolysis of transglycosylation products of CT(14-GlcNAc, 20-GlcNAc) by Lys-C, and RP-HPLC and MALDI-TOFMS analyses of the resulting fragments

Compound		Full length 1–32	Fragment 1–11	Fragment 12–18	Fragment 19–32
CT(14-GlcNAc, 20-GlcNAc)	HPLC (min)	37.6	27.3	10.3	18.9
	m/z Found	3821.4	1122.9	1057.6	1676.5
	m/z Calcd	3822.4	1123.3	1058.2	1678.9
	Ion form	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$
CT(14-STF, 20-STF)	HPLC (min)	34.8	27.6	6.9	17.4
	m/z Found	7831.6	1124.7	3059.4	3680.1
	m/z Calcd	7827.9	1123.3	3061.0	3681.6
	Ion form	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$
CT(14-STF, 20-GlcNAc)	HPLC (min)	36.3	27.1	6.4	18.6
	m/z Found	5827.8	1122.7	3061.4	1677.9
	m/z Calcd	5825.1	1123.3	3061.0	1678.9
	Ion form	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$
CT(14-GlcNAc, 20-STF)	HPLC (min)	35.8	27.2	11.1	17.1
	m/z Found	5826.8	1122.1	1058.1	3682.9
	m/z Calcd	5825.1	1123.3	1058.2	3681.6
	Ion form	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$

HPLC condition: ∅4.6 × 250 mm; 10–50% CH_3CN in 0.1% TFA aq over 40 min; 0.8 mL/min.

7831.6, calcd for $C_{314}H_{513}N_{55}O_{169}S_2$ ($[M+H]^+$) 7827.9. CT(14-STF, 20-GlcNAc): found *m/z* 5827.8, calcd for $C_{238}H_{390}N_{50}O_{113}S_2$ ($[M+H]^+$) 5825.1. CT(14-GlcNAc, 20-STF): found *m/z* 5826.8, calcd for $C_{238}H_{390}N_{50}O_{113}S_2$ ($[M+H]^+$) 5825.1.

The transglycosylation sites of the products were identified by partial hydrolysis using Lys-C. Using 20 nmol of the glycopeptides, the samples were digested with Lys-C (incubated for 8 h) and RP-HPLC analyses were performed in a similar manner as the glycosylation site analysis of the products from CT(3-GlcNAc, CT26-GlcNAc). Three peaks were observed for each glycopeptide. From the retention time and the *m/z* value of each fragment, as summarized in Table 3, the structures of the products were confirmed to be CT(14-STF, 20-STF), CT(14-STF, 20-GlcNAc) and CT(14-GlcNAc, 20-STF).

2.5. Transglycosylation reaction of M6 to CT(3-STF, 26-GlcNAc) with Endo-A

A mixture (21 μL) of 1.23 mg (0.211 μmol) of CT(3-STF, 26-GlcNAc) (10 mM), 1.51 mg (1.0 μmol) of M6-Asn (48 mM), and Endo-A (250 mU/mL) in pH 6.0 ammonium acetate buffer (25 mM) containing 30% dimethysulfoxide was incubated at 37 °C for 30 min, and the reaction was terminated by the addition of an equal volume of a cold aqueous solution of 0.5% TFA. The reaction mixture was analyzed by RP-HPLC using the same elution conditions as were used in the analysis of the Endo-M transglycosylation. A peak, corresponding to the transglycosylation product, was detected at 22.9 min, and the remaining unreacted acceptor at 25.9 min by RP-HPLC. The transglycosylation yield was 47.8%. By preparative HPLC, 0.21 mg (0.030 μmol, 14%) of CT(3-STF, 26-M6) was isolated, and identified by MALDI-TOF-MS: found *m/z* 6998.8, calcd for $C_{282}H_{464}N_{52}O_{147}S_2$ ($[M-H]^-$) 6998.2.

2.6. Transglycosylation reaction of M6 to CT(3-GlcNAc, 26-STF) with Endo-A

Using 1.11 mg (0.191 μmol) of CT(3-GlcNAc, 26-STF), the transglycosylation using Endo-A was performed in the same manner as stated above. A peak, corresponding to the transglycosylation product, was detected at 22.4 min, and the remaining unreacted acceptor at 28.9 min by RP-HPLC. The transglycosylation yield was 40.5%. By preparative HPLC, 0.20 mg (0.029 μmol, 15%) of CT(3-M6, 26-STF) was isolated, and identified by MALDI-TOF-MS: found *m/z* 6999.2, calcd for $C_{282}H_{464}N_{52}O_{147}S_2$ ($[M-H]^-$) 6998.2.

2.7. Neuraminidase treatment of CT(3-STF, 26-STF)

5.32 mg (0.68 μmol) of CT(3-STF, 26-STF) (4.9 mM) was treated with 1 U/mL of *Arthrobacter ureafaciens* neuramidase (Nacalai Tesque, Kyoto, Japan) in 60 mM acetate buffer of pH 5.0 at 37 °C for 60 min, and 2.90 mg (0.44 μmol, 64%) of desialylated product was isolated by preparative HPLC. The product was identified as CT(3-ASTF, 26-ASTF) (ASTF stands for asialo-STF) by MALDI-TOF-MS: found *m/z* 6657.9, calcd for $C_{270}H_{446}N_{52}O_{136}S_2$ ($[M-H]^-$) 6659.9.

2.8. Neuraminidase treatment of CT(20-STF, 26-STF)

1.77 mg (0.23 μmol) of CT(20-STF, 26-STF) (4.6 mM) was treated in the same manner as CT (3-STF,

26-STF), and 1.30 mg (0.20 μmol, 85%) of desialylated product was isolated by preparative HPLC. The product was identified as CT(20-ASTF, 26-ASTF) by MALDI-TOF-MS: found *m/z* 6659.6, calcd for $C_{270}H_{446}N_{52}O_{136}S_2$ ($[M-H]^-$) 6659.9.

2.9. Circular dichroism (CD) measurement

CD spectra of the calcitonin derivatives were obtained with a JASCO J-720 spectropolarimeter using aqueous solutions of 0%, 10%, 20%, 40% or 60% trifluoroethanol as previously reported.[18]

2.10. Hypocalcemic activity

The hypocalcemic activity of the calcitonin derivatives was measured by a method[31] using male rats, as previously described.[18] Although the data were obtained at two different concentrations, each with 10 animals, the activity values of each of the calcitonin derivatives were averaged and the values are expressed relative to that of calcitonin.

2.11. Receptor binding assay

The receptor binding activity of the calcitonin derivatives was measured with a direct competition assay[32,33] using mouse osteoclast-like cells and ^{125}I-labeled elcatonin, as previously described.[18]

3. Results and discussion

3.1. Transglycosylation of STF with Endo-M

The amino acid sequence of eel calcitonin and the four artificial N-glycosylation sites are shown in Figure 1. Two of the four sites were replaced with the amino acid bearing an GlcNAc residue, and the transglycosylation reaction with Endo-M was carried out. As an example, the RP-HPLC profile of the transglycosylation from SGP to CT(3-GlcNAc, 26-GlcNAc) for a reaction time of 30 min is shown in Figure 2. Three new product peaks, corresponding to CT(3-STF, 26-STF), CT(3-STF, 26-GlcNAc) and CT(3-GlcNAc, 26-STF), appeared earlier than the remaining acceptor, CT(3-GlcNAc, 26-GlcNAc). The product that eluted first is predicted to be CT(3-STF, 26-STF), Because the transglycosylation of two STFs must make the resulting glycopeptide more hydrophilic. From the MALDI-TOF-MS data, a double STF product and single STF products can also be clearly discriminated. For the two single STF products, however, it is impossible to determine simply by MALDI-TOF-MS which GlcNAc residue had been transglycosylated. Therefore, we analyzed the transglycosylation site of each product by partial hydrolysis of the glycopeptides with Lys-C or Arg-C. The choice of *endo*-peptidase was determined based on the glycosylation sites. As shown in Figure 1, Arg-C was the choice for CT(20-STF, 26-GlcNAc) and CT(20-GlcNAc, 26-STF), because Arg24 is located between Gln20 and Asp26. Similarly, Lys-C was the choice for CT(14-STF, 20-GlcNAc) and CT(14-GlcNAc, 20-STF), because Lys18 is between Gln14 and Gln20. The RP-HPLC retention time of the (glyco)peptide fragments and the corresponding MALDI-TOF-MS data for the transglycosylation products of CT(3-GlcNAc, 26-GlcNAc), CT(20-GlcNAc, 26-GlcNAc) and CT(14-GlcNAc, 20-GlcNAc) are summarized in Tables

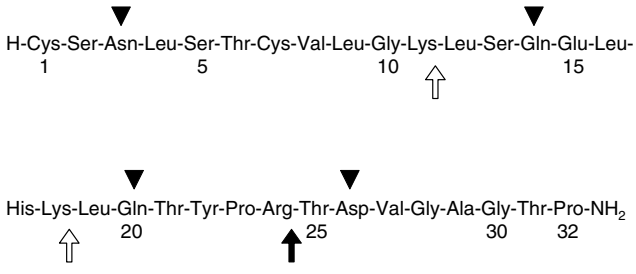


Figure 1. The amino acid sequence of eel calcitonin. Cys1 and Cys7 form a disulfide bond. Triangles indicate the artificial N-glycosylation sites employed in this study, white arrows indicate partial hydrolysis sites of Lys-C, and a black arrow that of Arg-C.

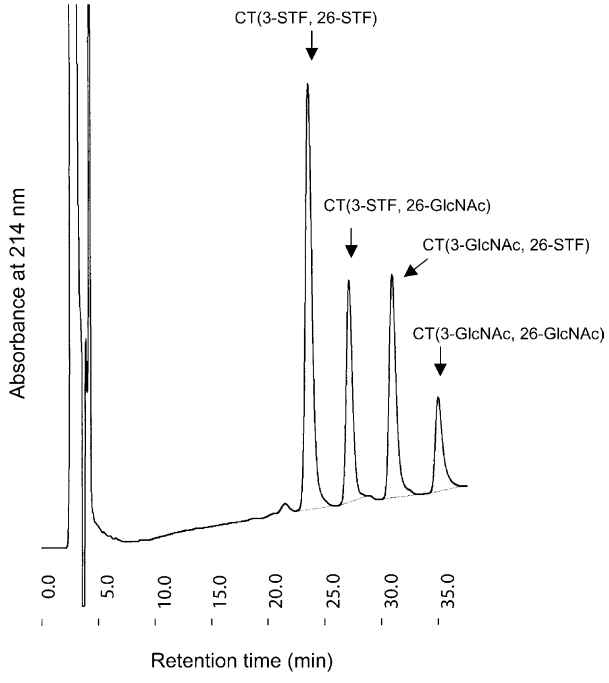


Figure 2. RP-HPLC profile of the Endo-M transglycosylation reaction. The transglycosylation to CT(3-GlcNAc, 26-GlcNAc) at the reaction time of 30 min detected by the absorbance at 214 nm. The experimental details are described in Section 2.

1–3, respectively. These transglycosylation sites were unambiguously determined from the results.

A time course for the transglycosylation reaction of CT(3-GlcNAc, 26-GlcNAc) is shown in Figure 3. Peaks corresponding to CT(3-STF, 26-GlcNAc) and CT(3-GlcNAc, 26-STF) appeared first, then the CT(3-STF, 26-STF) peak increased, eventually becoming the major product, and the remaining acceptor continued to decrease. The transglycosylation yield was based on the starting CT(3-GlcNAc, 26-GlcNAc), and 15-fold excess of SGP was used as a donor. The total transglycosylation yield of the Asn3 site, the sum of CT(3-STF, 26-STF) and CT(3-STF, 26-GlcNAc) yields, and that of the Asp26 site, the sum of CT(3-STF, 26-STF) and CT(3-GlcNAc, 26-STF) yields, increased monotonously with incubation time, reaching 65% (44% + 21%) and 68% (44% + 24%), respectively. These yields and the reaction rates were quite similar to those of the transglycosylation reaction of STF to CT with mono-GlcNAc at Asn3 (58%) or Asp26 (67%),[19] indicating that the transglycosylation to each site occurred independently and did not interfere with each other.

STF oligosaccharides were transglycosylated to CT(20-GlcNAc, 26-GlcNAc) as efficiently as CT(3-GlcNAc, 26-GlcNAc), producing CT(20-STF, 26-STF), CT(20-STF, 26-GlcNAc) and CT(20-GlcNAc, 26-STF) in transglycosylation yields of 48%, 20% and 23%, respectively. A time course is shown in Figure 4.

Using CT(14-GlcNAc, 20-GlcNAc), the transglycosylation of STF oligosaccharides by Endo-M was also performed in a similar manner, giving the products CT(14-STF, 20-STF), CT(14-GlcNAc, 20-STF) and CT(14-STF, 20-GlcNAc) in transglycosylation yields of 33%, 30% and 21%, respectively. A time course is shown in Figure 5. The transglycosylation yield of CT(14-STF, 20-STF) was slightly lower, and that of CT(14-GlcNAc, 20-STF) was slightly higher compared with the other Endo-M reactions.

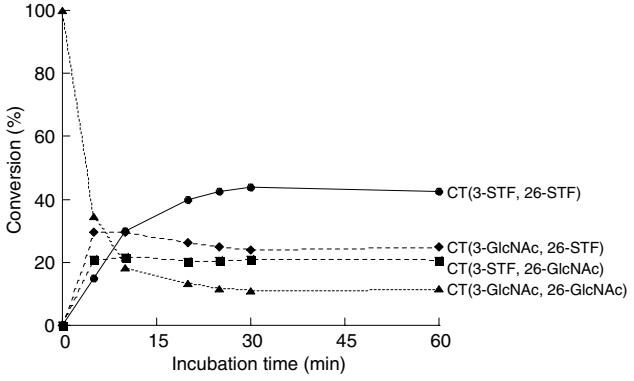


Figure 3. Time course for the Endo-M transglycosylation to CT(3-GlcNAc, 26-GlcNAc). HPLC yields of CT(3-STF, 26-STF) (●), CT(3-STF, 26-GlcNAc) (■), CT(3-GlcNAc, 26-STF) (◆), and CT(3-GlcNAc, 26-GlcNAc) (▲).

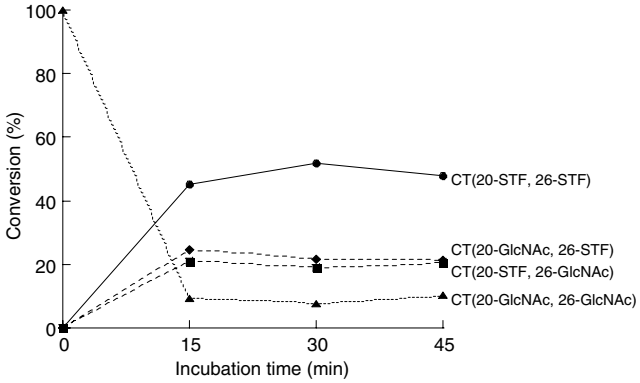


Figure 4. Time course for the Endo-M transglycosylation to CT(20-GlcNAc, 26-GlcNAc). HPLC yields of CT(20-STF, 26-STF) (●), CT(20-STF, 26-GlcNAc) (■), CT(20-GlcNAc, 26-STF) (◆), and CT(20-GlcNAc, 26-GlcNAc) (▲).

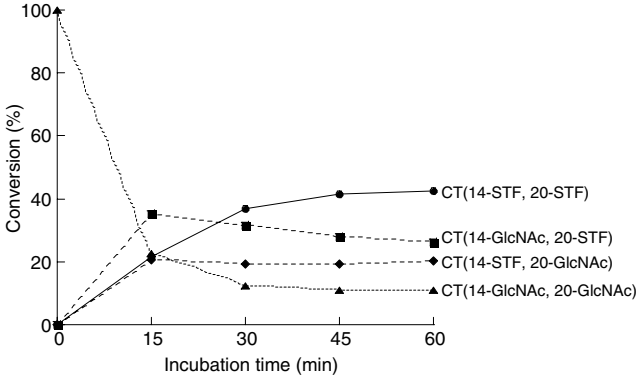


Figure 5. Time course for the Endo-M transglycosylation to CT(14-GlcNAc, 20-GlcNAc). HPLC yields of CT(14-STF, 20-STF) (●), CT(14-GlcNAc, 20-STF) (■), CT(14-STF, 26-GlcNAc) (◆), and CT(3-GlcNAc, 26-GlcNAc) (▲).

We had anticipated difficulties in the simultaneous transglycosylation of two sites, because the initially introduced oligosaccharide moiety of one site could interfere with the transglycosylation of the other site, and because the introduced oligosaccharide of one site may be hydrolyzed back to GlcNAc by Endo-M during the transglycosylation of the other site. The first possibility may not be the case, because all of the transglycosylation reactions proceeded quite satisfactorily and the apparent total transglycosylation yield at each site was comparable to that of the corresponding CT derivative with mono-GlcNAc. The second possibility may actually occur at a microscopic level, as enzymatic transglycosylation is a reversible reaction, but practically and macroscopically, the balance between the transglycosylation and the hydrolysis did not pose a problem. The slightly different outcome in the case of CT(14-GlcNAc, 20-GlcNAc) may reflect a site-dependent difference in this subtle balance.

In a preliminary small-scale experiment, we also examined the Endo-A transglycosylation of M6 to CT(3-GlcNAc, 26-GlcNAc). MALDI-TOF-MS analysis indicated the efficient production of glycosylated calcitonin derivatives containing M6 oligosaccharides, CT(3-M6, 26-M6), CT(3-M6, 26-GlcNAc) and CT(3-GlcNAc, 26-M6).

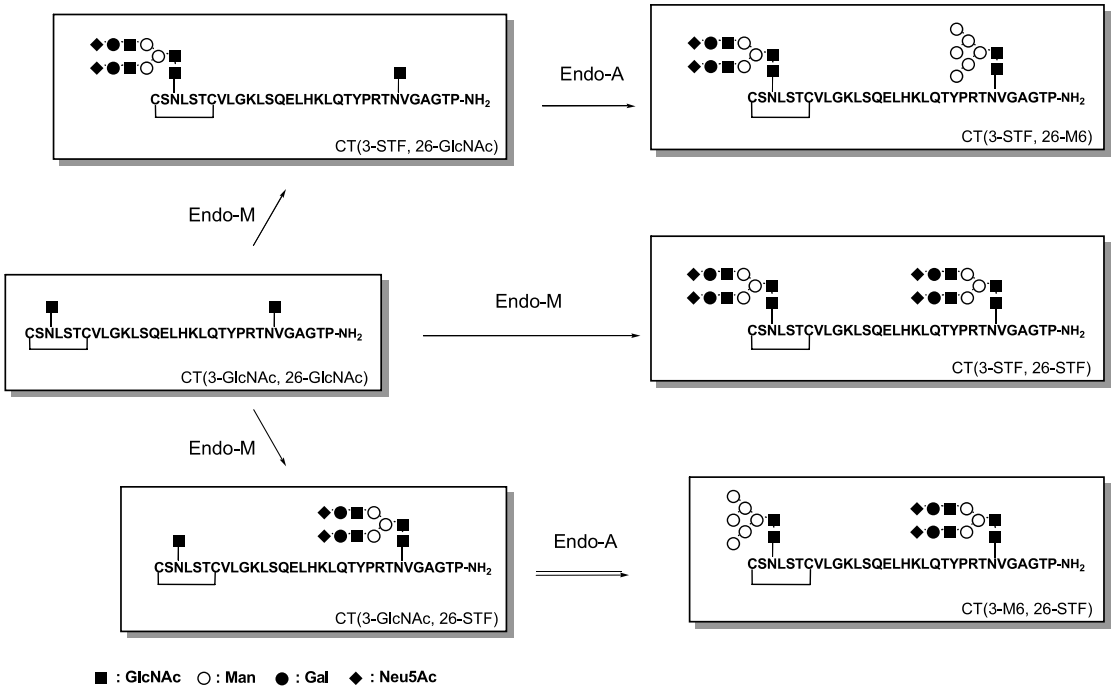
Enzymatic modification of oligosaccharide structures is a useful technique to introduce diversity into glycoforms. We previously demonstrated that transglycosylated oligosaccharide structures could be truncated into various structures by enzymatic digestion using mono-N-glycosylated calcitonin derivatives.[18,19] To show that the same procedure was applicable to the di-N-glycosylated derivatives, the removal of non-reducing terminal Neu5Ac residues by *A. ureafaciens* neuraminidase was carried out for CT(3-STF, 26-STF) and CT(20-STF, 26-STF) as a typical example. All of the Neu5Ac residues were removed within 60 min in good yields, giving CT(3-ASTF, 26-ASTF) and CT(20-ASTF, 26-ASTF), respectively.

3.2. Transglycosylation of M6 to CT(3-STF, 26-GlcNAc) and CT(3-GlcNAc, 26-STF) using Endo-A

We were fortunate that the transglycosylation reaction with Endo-M gave, not only di-STF calcitonin, but also products in which one GlcNAc site was transglycosylated with STF with the other GlcNAc remaining intact. Using CT(3-STF, 26-GlcNAc) and CT(3-GlcNAc, 26-STF) as representative acceptors, the transglycosylation of M6 oligosaccharide to the remaining GlcNAc moiety was performed using Endo-A. Endo-A transglycosylated a high-mannose-type oligosaccharide to the GlcNAc residue of the acceptor CT(3-STF, 26-GlcNAc) or CT(3-GlcNAc, 26-STF) without hydrolyzing the STF oligosaccharide, producing CT(3-STF, 26-M6) or CT(3-M6, 26-STF), respectively. The site of attachment of STF and that of M6 were identified by Lys-C partial hydrolysis and MALDI-TOF-S analysis of the resulting (glyco)peptide fragments in the same manner as for the parent compounds.

Thus, we were able to obtain synthetic glycopeptides, which contain both complex biantenary and high-mannose-type oligosaccharides, two of the representative carbohydrate structures of *N*-glycans, in a single molecule. This methodology may be extended to the synthesis of peptides glycosylated at three or more sites with two or more carbohydrate structures. Although it may be difficult to control the ratio of each glycosylated derivative, the methodology would be useful in constructing glycopeptide libraries, because a few short steps are required to prepare the final products. The methodology also represents a practical approach to the synthesis of a single target glycopeptide, as undesirable glycosylation products can be recycled by digesting the unintended oligosaccharides with *endo*-glycosidases.

The order of the transglycosylation reactions is important, as Endo-M and Endo-A recognize the same GlcNAc structure as the glycosylation tag. As summarized in Scheme 1, we were able to obtain a glycopeptide with different oligosaccharides by employing Endo-M



Scheme 1. Chemo-enzymatic procedure for preparing calcitonin derivatives that are N-glycosylated at two sites.

and Endo-A in this order. Because Endo-M hydrolyzes not only STF but also M6, the transglycosylated M6 may be hydrolyzed by Endo-M, if the M6 oligosaccharide is introduced first by Endo-A.

3.3. Effect of di-N-glycosylation on the structure and biological activity of calcitonin

Calcitonin assumes a random coil in aqueous solution and forms an α-helical structure in a hydrophobic environment.[15,17] As evidenced by CD studies, di-N-glycosylation had almost no effect on the α-helical structure irrespective of the glycosylation sites or the carbohydrate structures present (data not shown). This result is consistent with data obtained from studies on a series of mono-N-glycosylation of calcitonin at Asn3, Gln14, Gln20 and Asp26, in that mono-N-glycosylation at any site did not change the conformation of the peptide backbone, although the possibility remains that glycosylation with large oligosaccharides at Gln20 may affect the helical structure of calcitonin.[19]

The hypocalcemic activity and receptor binding activity of the di-N-glycosylated calcitonin derivatives are summarized in Table 4. The hypocalcemic activity is known to be correlated with receptor binding activity.[15] We previously reported that the attachment of GlcNAc to calcitonin did not hinder the binding to calcitonin receptor,[20] and that the change in the biodistribution caused by the GlcNAc attachment actually enhanced its hypocalcemic activities.[18] The introduction of larger oligosaccharides, such as STF and M6, at a single site generally reduced the receptor binding activity, although the magnitude differed from site to site and was dependent on the carbohydrate structure.[18,19] The addition of STF to Asn3, however, had no effect on the hypocalcemic activity, and the addition of STF to Asp26 even enhanced the activity, mainly because of the altered biodistribution.[19] Introduction of a hydrophilic and negatively charged STF oligosaccharide to calcitonin significantly reduced the accumulation in the liver,[19] which is a main metabolic organ of hydrophobic peptides.[34]

Table 4. Hypocalcemic and receptor-binding activities of glycosylated calcitonin derivatives

Compound	Hypocalcemic activity[a]	Receptor-binding activity[b]
Calcitonin	1.00	2.18
[Asn^{26}]-Calcitonin	1.04	1.39
CT(3-GlcNAc, 26-GlcNAc)	1.79	1.11
CT(3-STF, 26-STF)	0.53	2.72×10^2
CT(3-STF, 26-GlcNAc)	0.99	1.25×10^2
CT(3-GlcNAc, 26-STF)	1.34	1.55×10
CT(3-STF, 26-M6)	0.40	1.52×10^2
CT(3-M6, 26-STF)	0.41	4.32×10

[a] Hypocalcemic activity is expressed as the relative value to that of calcitonin.

[b] Receptor-binding activity is expressed as IC_{50} (nM).

From these observations gained from the mono-N-glycosylated calcitonin derivatives, the biological activities of the di-N-glycosylated calcitonin derivatives which contain both STF and GlcNAc, CT(3-STF, 26-GlcNAc) and CT(3-GlcNAc, 26-STF), are predictable. They reduced their receptor binding activity, but retained or enhanced their hypocalcemic activity. The introduction of two large oligosaccharides, CT(3-STF, 26-STF), CT(3-STF, 26-M6) and CT(3-M6, 26-STF), further reduced the receptor binding activity. It seems that even changes in biodistribution, if any, did not compensate for the loss in the receptor binding, and their hypocalcemic activities became very low.

In summary, we report here the efficient synthesis of peptides N-glycosylated at two sites not only with the same carbohydrate structures but also with both complex-type and high-mannose-type carbohydrates in a single molecule using a short-step chemo-enzymatic procedure. Our method opens a practical route to study the effect of multiple glycosylation by structurally defined and chemically pure samples.

Acknowledgements

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References

1. (a) Bertozzi, C. R.; Kiessling, L. L. *Science* **2001**, *291*, 2357–2364; (b) Helenius, A.; Aebi, M. *Science* **2001**, *291*, 2364–2369; (c) Rudd, P. M.; Elliott, T.; Cresswell, P.; Wilson, I. A.; Dwek, R. A. *Science* **2001**, *291*, 2370–2376.
2. Lee, R. T.; Lee, Y. C. *Glycoconjugate J.* **2000**, *17*, 543–551.
3. Haneda, K.; Inazu, T.; Yamamoto, K.; Kumagai, H.; Nakahara, Y.; Kobata, A. *Carbohydr. Res.* **1996**, *292*, 61–70.
4. Inazu, T.; Kobayashi, K. *Synlett* **1993**, 869–870.
5. Mizuno, M.; Muramoto, I.; Kobayashi, K.; Yaginuma, H.; Inazu, T. *Synthesis* **1999**, 162–165.
6. Yamamoto, K.; Kadowaki, S.; Watanabe, J.; Kumagai, K. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 244–252.
7. Takegawa, K.; Yamaguchi, S.; Kondo, A.; Iwamoto, H.; Nokoshi, M.; Kato, I.; Iwahara, S. *Biochem. Int.* **1991**, *24*, 849–855.
8. Yamamoto, K. *J. Biosci. Bioeng.* **2001**, *92*, 493–501.
9. Haneda, K.; Inazu, T.; Mizuno, M.; Yamamoto, K. In *Methods in Enzymology*; Lee, Y. C., Lee, R. T., Eds.; Academic Press: Oxford, 2003; Vol. 362, pp 74–85.

10. Mizuno, M.; Haneda, K.; Iguchi, R.; Muramoto, I.; Kawakami, T.; Aimoto, S.; Yamamoto, K.; Inazu, T. *J. Am. Chem. Soc.* **1999**, *121*, 284–290.
11. Haneda, K.; Inazu, T.; Mizuno, M.; Iguchi, R.; Yamamoto, K.; Kumagai, H.; Aimoto, S.; Suzuki, H.; Noda, T. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1303–1306.
12. Haneda, K.; Inazu, T.; Mizuno, M.; Iguchi, R.; Tanabe, H.; Fujimori, K.; Yamamoto, K.; Kumagai, H.; Tsumori, K.; Munekata, E. *Biochim. Biophys. Acta* **2001**, *1526*, 242–248.
13. Kadowaki, S.; Yamamoto, K.; Fujisaki, M.; Tochikura, T. *J. Biochem.* **1991**, *110*, 17–21.
14. Yamamoto, K.; Kadowaki, S.; Fujisaki, M.; Tochikura, T. *Biosci. Bioeng. Biochem.* **1994**, *58*, 72–77.
15. Azria, M. *The Calcitonins: Physiology and Pharmacology*; Karger: Basel, 1989.
16. Yamamoto, K.; Haneda, K.; Iguchi, R.; Inazu, T.; Mizuno, M.; Takegawa, K.; Kondo, A.; Kato, I. *J. Biosci. Bioeng.* **1998**, *87*, 175–179.
17. Hashimoto, H.; Toma, K.; Nishikido, J.; Yamamoto, K.; Haneda, K.; Inazu, T.; Valentine, K. G.; Opella, S. J. *Biochemistry* **1999**, *38*, 8377–8384.
18. Tagashira, M.; Tanaka, A.; Hisatani, K.; Isogai, Y.; Hori, M.; Takamatsu, S.; Fujibayashi, Y.; Yamamoto, K.; Haneda, K.; Inazu, T.; Toma, K. *Glycoconjugate J.* **2001**, *18*, 449–455.
19. Haneda, K.; Tagashira, M.; Yoshino, E.; Takeuchi, M.; Inazu, T.; Toma, K.; Iijima, H.; Isogai, Y.; Hori, M.; Takamatsu, S.; Fujibayashi, Y.; Kobayashi, K.; Takeuchi, M.; Yamamoto, K. *Glycoconjugate J.* **2004**, *21*, 377–386.
20. Tagashira, M.; Iijima, H.; Isogai, Y.; Hori, M.; Takamatsu, S.; Fujibayashi, Y.; Haneda, K.; Inazu, T.; Toma, K. In *Peptide Science 2000*; Shimonishi, T., Ed.; Protein Research Found: Japan, 2001; pp 125–128.
21. Tagashira, M.; Iijima, H.; Toma, K. *Trends Glycosci. Glycotechnol.* **2001**, *13*, 373–383.
22. Tagashira, M.; Iijima, H.; Isogai, Y.; Hori, M.; Takamatsu, S.; Fujibayashi, Y.; Yoshizawa-Kumagaye, K.; Isaka, S.; Nakajima, K.; Yamamoto, T.; Teshima, T.; Toma, K. *Biochemistry* **2001**, *40*, 11090–11095.
23. Tagashira, M.; Iijima, H.; Toma, K. *Glycoconjugate J.* **2002**, *19*, 43–52.
24. Warren, J. D.; Miller, J. S.; Keding, S. J.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2004**, *126*, 6576–6578.
25. Kajihara, Y.; Suzuki, Y.; Yamamoto, N.; Sasaki, K.; Sakakibara, T.; Juneja, L. R. *Chem. Eur. J.* **2004**, *10*, 971–985.
26. Takano, Y.; Hojo, H.; Kojima, N.; Nakahara, Y. *Org. Lett.* **2004**, *6*, 3135–3138.
27. Fujita, K.; Kobayashi, K.; Takeuchi, M.; Iwamatsu, A.; Yamamoto, K.; Kumagai, H. *Arch. Biochem. Biophys.* **2004**, *432*, 41–49.
28. Takegawa, K.; Yamabe, K.; Fujita, K.; Tabuchi, M.; Mita, M.; Izu, H.; Watanabe, A.; Asada, Y.; Sano, M.; Kondo, A.; Kato, I.; Iwahara, S. *Arch. Biochem. Biophys.* **1997**, *338*, 22–28.
29. Seko, A.; Koketsu, M.; Nishizono, M.; Enoki, Y.; Ibrahim, H. R.; Juneja, L. R.; Kim, M.; Yamamoto, T. *Biochim. Biophys. Acta* **1997**, *1335*, 23–32.
30. Tai, T.; Yamashita, K.; Ogata-Arakawa, M.; Koide, N.; Muramatsu, T.; Iwashita, S.; Inoue, Y.; Kobata, A. *J. Biol. Chem.* **1975**, *250*, 8569–8575.
31. Otani, M.; Kitazawa, S.; Yamauchi, H.; Meguro, T.; Orimo, H. *Horm. Metab. Res.* **1978**, *10*, 252–256.
32. Akatsu, T.; Tamura, T.; Takahashi, N.; Udagawa, N.; Tanaka, S.; Sasaki, T.; Yamaguchi, A.; Nagata, N.; Suda, T. *J. Bone Mineral Res.* **1992**, *7*, 1297–1306.
33. Tamura, T.; Takahashi, N.; Akatsu, T.; Sasaki, T.; Udagawa, N.; Tanaka, S.; Suda, T. *J. Bone Mineral Res.* **1993**, *8*, 953–960.
34. Ruwart, M. J. In *Peptide-Based Drug Design*; Taylor, M. D., Amidon, G. L., Eds.; American Chemical Society: Washington, DC, 1995; pp 249–262.