Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1303–1306

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Chemo-enzymatic synthesis of calcitonin derivatives containing *N*-linked oligosaccharides

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Received 11 March 1998; accepted 15 April 1998

Abstract Eel calcitonin derivatives containing various *N*-linked oligosaccharides were chemo-enzymatically synthesized by the transglycosylation reaction of *Mucor hiemalis* endo-β-*N*-acetylglucosaminidase (Endo-M) to a glycosylated calcitonin derivative [Asn(GlcNAc)^3]-CT in which *N*-acetyl-D-glucosamine (GlcNAc) is attached to the L-asparagine (Asn) residue of the peptide. © 1998 Elsevier Science Ltd. All rights reserved.

Key words: glycopeptides; enzyme and enzyme reactions; hormones; glycosylation

Oligosaccharides in complex glycopeptides or glycoproteins play important roles in biological processes such as cell-cell interaction, substrate-receptor recognition and stability[1]. The synthesis of glycopeptides and their mimics is therefore important to study these roles.

In this letter, we describe the chemo-enzymatic synthesis of eel calcitonin derivatives containing *N*-linked oligosaccharides.

Calcitonin is a calcium-regulating hormone composed of 32 amino acids existing as an amphiphilic α-helix conformation[2]. Both calcitonin and its synthetic derivative elcatonin[3] are widely used as therapeutic agents for hypercalcemia, Paget's disease and osteoporosis. Eel calcitonin contains no sugar chains, but does have a marker sequence triplet (Asn-Xaa-Ser/Thr as "Asn-Leu-Ser") for *N*-glycosylation. We attended to artificially add *N*-linked oligosaccharides to the Asn residue of the peptide by a chemo-enzymatic method, as a biological addition of oligosaccharides is impossible. We wished to investigate the effects of oligosaccharides on the biological activity and the tertiary structure of calcitonin.

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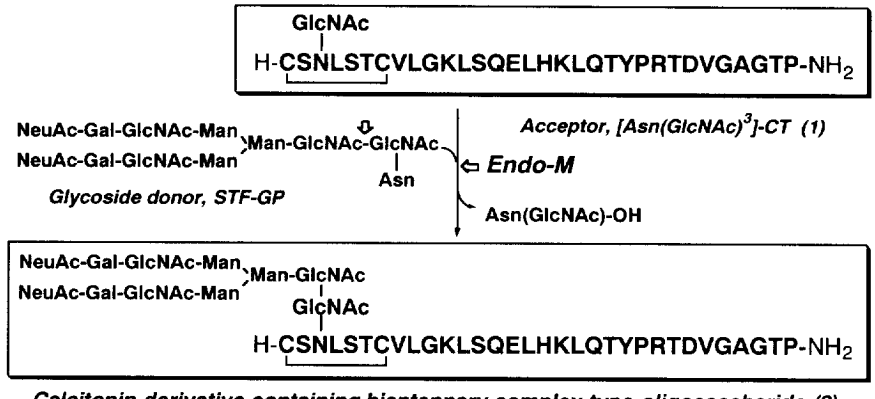
PII: S0960-894X(98)00209-1

Previously, we have prepared the glycopeptide analog of eel calcitonin, [Asn(GlcNAc)^3]-CT (**1**, abbreviated as CT-GlcNAc), in which GlcNAc is attached to the Asn residue of the peptide[4]. *N*-Glycopeptides containing an *N*-acetyl-D-glucosamine (GlcNAc) moiety have been made by a dimethylphosphinothioic mixed anhydride (Mpt-MA) method in which no protection of the sugar hydroxyl group was necessary[5]. The *N*-glycopeptides and their mimics are good glycoside acceptors in transglycosylation reactions catalyzed by microbial endo-β-*N*-acetylglucosaminidase[6,7] giving complex glycopeptide products containing natural oligosaccharides[8,9]. Then, following this method, eel calcitonin derivatives containing *N*-linked oligosaccharides were synthesized. This is the first report on the artificial addition of *N*-linked oligosaccharides to bioactive peptide hormones having no natural sugar chains.

Syntheses of eel calcitonin derivatives containing *N*-linked oligosaccharides were performed using Endo-M-facilitated transglycosylation reactions with [Asn(GlcNAc)^3]-CT (**1**)[4] as the glycoside acceptor. As a glycoside donor, disialo transferrin glycopeptide (STF-GP), Asn[(NeuAc-Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$]-OH, derived from human serum transferrin[10] was used (Scheme 1).



Scheme 1. Synthetic route for a eel calcitonin derivative containing disialo complex-type oligosaccharide.

The reaction mixture was composed of 25mM glycoside donor, 10mM [Asn(GlcNAc)^3]-CT (**1**) as an acceptor, 4mU/ml of Endo-M in the 60mM potassium phosphate buffer (pH 6.25) and 50mM EDTA[8]. The reverse-phase HPLC (RP-HPLC)[11] profile of the reaction mixture is shown in Fig. 1-I. After incubation for 6 hours at 37°C, a single new peak of a transglycosylation product corresponding to a calcitonin derivative containing disialo biantennary complex-type oligosaccharide, [Asn{(NeuAc-Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$}^{3}]-CT (**2**), was observed in 8.5% yield (to the acceptor added) and identified by mass spectrometry[12].

When an asialo transferrin glycopeptide (ASTF-GP), Asn[(Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$]-OH and a high-mannose type glycopeptide (M$_6$-GP), Asn(Man$_6$-GlcNAc$_2$)-OH, derived from ovalbumin[13] were used as the glycoside donor, calcitonin derivatives containing an

asialo complex-type oligosaccharide, [Asn{(Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$}^{3}]-CT (**3**)[14] (Fig. 1-II) and a high-mannose type oligosaccharide, [Asn(Man$_6$-GlcNAc$_2$)^3]-CT (**4**)[15] (Fig. 1-III, abbreviated as CT-M6), were successfully prepared.

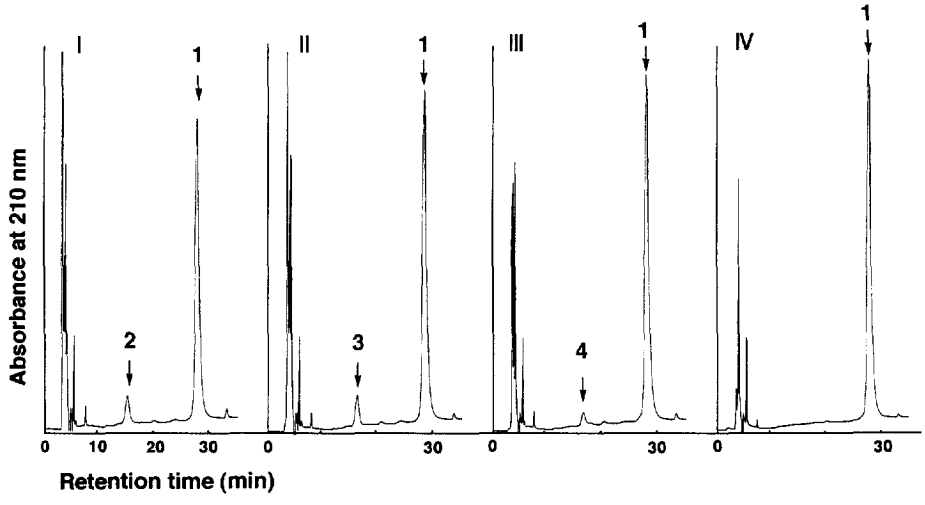


Fig. 1. RP-HPLC elution profiles of the reaction mixtures of transglycosylation reaction to [Asn(GlcNAc)^3]-CT catalyzed by Endo-M.

Every transglycosylation product **2**: [Asn{(NeuAc-Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$}^{3}]-CT, **3**: [Asn{(Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$}^{3}]-CT and **4**: [Asn(Man$_6$-GlcNAc$_2$)^3]-CT was formed in the reaction mixture containing STF-GP (I), ASTF-GP (II) and M$_6$-GP (III) as a glycoside donor. Only the acceptor [Asn(GlcNAc)^3]-CT (**1**) was detected in the reaction mixture without glycoside donor (IV).

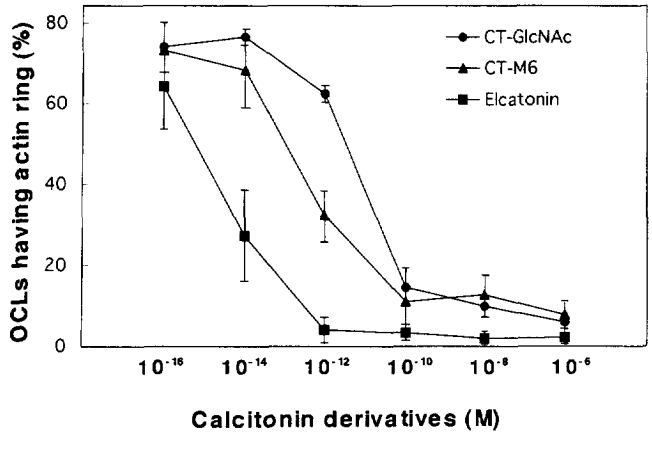


Fig. 2. Biological activity *in vitro* of glycosylated calcitonin derivatives[19].

Calcitonin inhibits osteoclastic bone resorption through the receptor abundantly expressed on the plasma membrane of osteoclasts[16]. Osteoclasts in a bone-resorbing state exhibit ringed structures of F-actin dots (actin rings). Elcatonin[17], a synthetic analog of eel calcitonin, disrupted actin rings and inhibited pit-forming activity of murine osteoclast-like multinucleated cells (OCLs) in a dose-dependent manner[18]. This calcitonin-induced change in the cytoskeleton is an indicator of the bone-resorbing activity of osteoclasts.

In preliminary experiments *in vitro*, the glycosylated calcitonin derivatives exhibited strong inhibitory activity on the actin ring formation of OCLs as shown in Fig. 2[19]. Calcitonin derivatives **4** (10^{-14} to 10^{-10} M), **1** (10^{-12} to 10^{-10} M) and elcatonin (10^{-16} to 10^{-12} M) were found to inhibit actin ring formation of OCLs dose dependently. We are investigating the effects of oligosaccharides on the biological activities of calcitonin derivatives (including compounds **2**, **3** and eel calcitonin itself) more precisely.

The influences of GlcNAc or *N*-linked oligosaccharides artificially attached to the Asn residue of the peptide on the conformation or tertiary structure of calcitonin are under investigation.

References and note

[1] [a] Varki A. Glycobiology 1993;3:97-130. [b] Blite DL. Trends Glycosci. Glycotechnol. 1993;5:81-88. [c] Dwek RA. Chem. Rev. 1996;96:683-720.

[2] (a) Ogawa K, Nishimura S, Doi M, Kyogoku Y, Hayashi M, Kobayashi Y. Eur. J. Biochem. 1994;222:659-666. (b) Doi M, Kobayashi Y, Kyogoku Y, Gohda K, Takimoto M, in River JE, Marshall GR(Eds.). Proceeding of the 11th American Ppeptide Symposium, ESCOM, 1989:45.

[3] Morikawa T, Munekawa E, Sakakibara S, Noda T, Otani M. Experimentia 1976;32:1104-1106.

[4] Mizuno M, Muramoto I, Kawakami T, Seike M, Aimoto S, Haneda K, Inazu T. Tetrahedron Lett. 1997;39:55-58.

[5] (a) Inazu T, Kobayashi K. Synlett 1993:869-870. (b) Inazu T, Mizuno M, Kohda Y, Kobayashi K, Yaginuma H, in Nishi N (Ed.). Peptide Chemistry 1995, Protein Research Foundation. Osaka, Japan. 1996:61-64.

[6] Yamamoto K, Kadowaki S, Watanabe J, Kumagai H. Biochem. Biophys. Res. Commun. 1994;203:244-252.

[7] Takegawa K, Yamaguchi S, Kondo A, Iwamoto H, Nakoshi M, Iwahara S. Biochem. Int. 1991;24:849-855.

[8] (a) Haneda K, Inazu T, Yamamoto K, Kumagai H, Nakahara Y, Kobata A. Carbohydr. Res. 1996;292:61-70. (b) Yamamoto K, Fujimori K, Haneda K, Mizuno M, Inazu T, Kumagai H, *ibid.* in press

[9] Wang L-X, Fan J-Q, Lee YC, Tetrahedron Lett. 1996;37:1975-1978.

[10] Kadowaki S, Yamamoto K, Fujisaki M, Izumi K, Tochikura T, Yokoyama T. Agric. Biol. Chem. 1990;54:97-106.

[11] HPLC elution conditions: Column Mightysil RP-18 (6 x 250mm, Kanto Chemical). Linear increase of aceronitrile concentration from 30 to 35% in 0.1% aq trifluoroacetic acid over 40 min at a flow rate of 1 mL min^{-1}.

[12] ESI-MS. Calcd: 5620.2 from m/z $[M+3H]^{3+}$ 1874.4, MW for $C_{230}H_{377}O_{108}N_{49}S_2$ 5620.9. MALDI-TOF MS. Found: m/z $[M+H]^+$ 5041.6, after treatment with sialidase corresponding to the desialylated compund (MW 5038.4).

[13] Tai T, Yamashita K, Ogata-Arakawa M, Koide N, Muramatsu T, Iwashita S, Inoue Y, Kobata A. J. Biol. Chem. 1975;250:8569-8575.

[14] Yield: 7.5%; MALDI-TOF MS. Found: m/z $[M+H]^+$ 5040.3, Calcd for $C_{208}H_{343}O_{92}N_{47}S_2$ $[M+H]^+$ 5039.4

[15] Yield: 3.5%; MALDI-TOF MS. Found: m/z $[M+H]^+$ 4794.1, Calcd for $C_{198}H_{327}O_{87}N_{45}S_2$ $[M+H]^+$ 4795.2

[16] (a) Nicholson GL, Moseley JM, Sexton PM, Mendelsohn FAO, Martin TJ. J. Clin. Invest. 1986;78:355-360. (b) Suda T, Takahashi N, Martin TJ. Endocr. Rev. 1992;13:66-80.

[17] An eel calcitonin analog having a disufide bond Cys^1-Cys^7 replaced by an ethylene linkage between residues 1 and 7.

[18] Suzuki H, Nakamura I, Takahashi N, Ikuhara T, Matsuzaki K, Isogai Y, Hori M, Suda T. Endocrinology 1996;137: 4685-4690

[19] Murine OCLs were prepared from the co-culture of mouse primary osteoclastic cells and bone marrow cells on collagen gel matrix. After they were treated with graded concentrations of calcitonin derivatives for 60 min, OCLs were stained for TRAP (tartrate-resistant acid phosphatase) positive, and the actin ring formation was visualized by rhodamin-conjugated phalloidin staining.