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A Synthesis of a Glycopeptide Analogue of Eel Calcitonin

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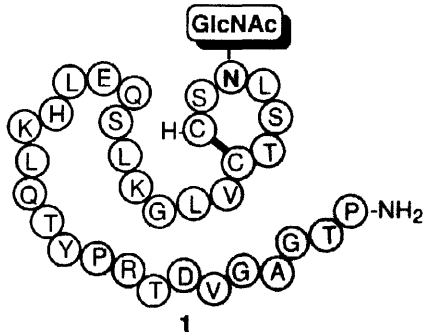
Abstract : The glycopeptide analogue of eel calcitonin, [Asn(GlcNAc)^3]-CT (**1**), in which *N*-acetylglucosamine (GlcNAc) is attached to the asparagine residue of the peptide was synthesized using a thioester method to build the polypeptide segment and a dimethylphosphinothioic mixed anhyride (Mpt-MA) method for the incorporation of the glycopeptide moiety.

Glycoproteins play an important role in biological processes, such as cell recognition, cell adhesion, immunogenic recognition and so on.[1] In order to study these roles, syntheses of glycopeptides and their mimics are important.

Several syntheses of glycopeptides containing a short oligo-peptide have been reported.[2] Recently we have described the solid-phase syntheses of glycopeptides by a dimethylphosphinothioic mixed anhyride (Mpt-MA) method in which no protection of the sugar hydroxyl group was necessary.[3] Furthermore, *N*-glycopeptides containing the *N*-acetyl-D-glucosamine (GlcNAc) moiety are good glycoside acceptors in transglycosylation reactions by endo-β-*N*-acetylglucosaminidase to give glycopeptides having natural sugar chains.[4,5] Therefore the importance of synthesis of glycopeptides containing a single GlcNAc residue is growing. Additionally, we developed a procedure in which partially protected peptide thioesters prepared *via* a solid-phase method were useful building blocks for protein synthesis (thioester method).[6,7]

In this letter, we describe a synthetic method of glycoprotein synthesis, using a Mpt-MA method for the introduction of Asn(GlcNAc) and a thioester method for building the protein moiety. The glycopeptide analogue of eel calcitonin (containing 32 amino acids), [Asn(GlcNAc)^3]-CT (**1**), in which GlcNAc is attached to the asparagine residue of the peptide was synthesized as a model compound.



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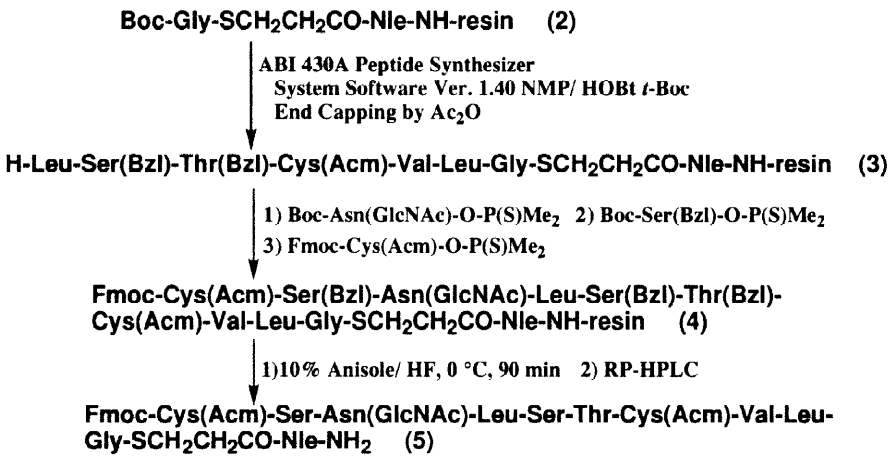
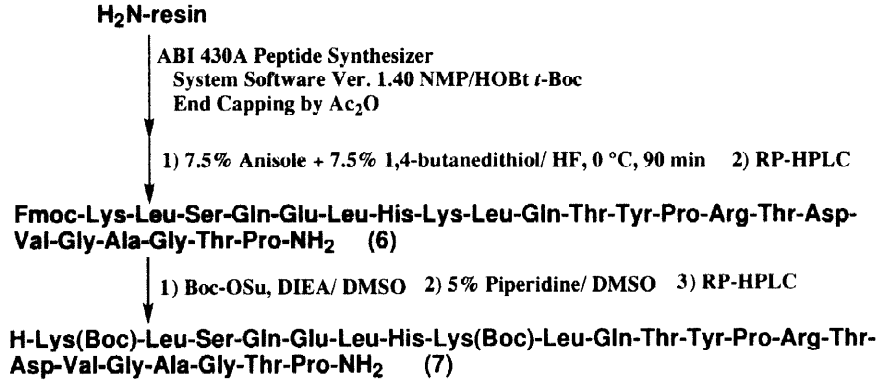
N^{α} - (*tert*-Butyloxycabonyl) -N^{ω} - (2-acetamido-2-deoxy-α-D-glucopyranosyl)-L-asparagine [Boc-Asn (GlcNAc)], which is a key-compound in this synthesis, was prepared by a similar method to that described in our previous report.[8]

The peptide thioester resin **3** was prepared from **2**[9](Gly 0.34 mmol g^{-1}) by a Boc-strategy using the dicyclohexylcarbodiimide (DCC)-1-hydroxybenzotriazole (HOBt) coupling method.[10,11] The corresponding Mpt-MAs of Boc-Asn(GlcNAc)-OH, Boc-Ser(Bzl)-OH and Fmoc-Cys(Acm)-OH were one by one introduced into the heptapeptide thioester resin **3**.[10,12] The glycopeptide thioester resin **4** 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 **5**[13] was obtained in 12% yield by reversed-phase HPLC (RP-HPLC) (Scheme 1). During the synthesis of **5** from **2**, no significant side reactions were observed.



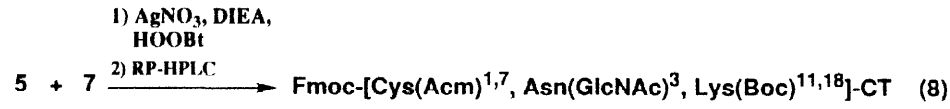
Scheme 1. Synthetic route for Fmoc-[Cys(Acm)[1,7], Asn(GlcNAc)[3]]-CT(1-10) (**5**)

The other peptide segment **6** was prepared by a Boc-strategy (Scheme 2).[10] For a thioester segment condensation, Boc groups were introduced to block side-chain amino groups of peptide segment **6** by treatment with *N-tert*-butyloxycarbonyloxysuccinimide (Boc-OSu) in the presence of $^{i}Pr_2NEt$ (DIEA), and *N*-terminal 9-fluorenylmethyloxycarbonyl (Fmoc) group was removed by treatment with 5% piperidine in DMSO.[7] After RP-HPLC purification, the partially protected peptide segment **7** was obtained in 19% yield, based on the amino group in the starting NH_2-resin.[9]



Scheme 2. Synthetic route for [Lys(Boc)[11,18]]-CT(11-32) (**7**)

Glycopeptide thioester segment **5** and partially protected peptide segment **7** 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 3).[7] The reaction mixture was stirred for 16h at room temperature. The RP-HPLC profile of the reaction mixture is shown in Fig. 1. Partially protected glycopeptide **8** was obtained in 78% yield after purification by RP-HPLC, followed by freeze-drying.



Scheme 3. Segment condensation of glycopeptide thioester **5** with peptide **7**.

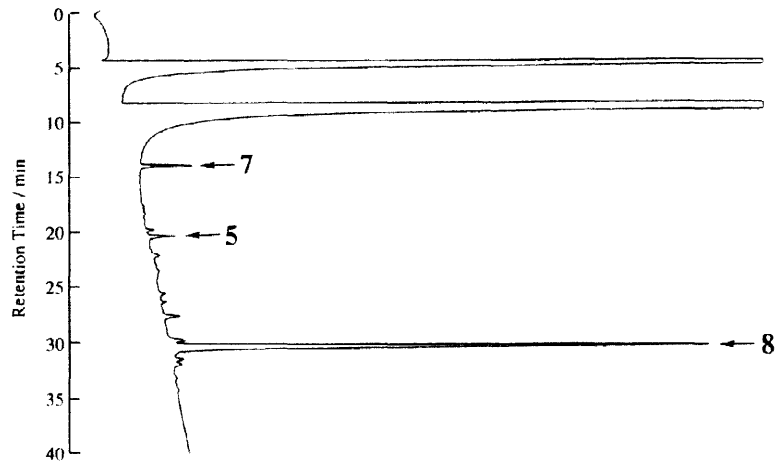
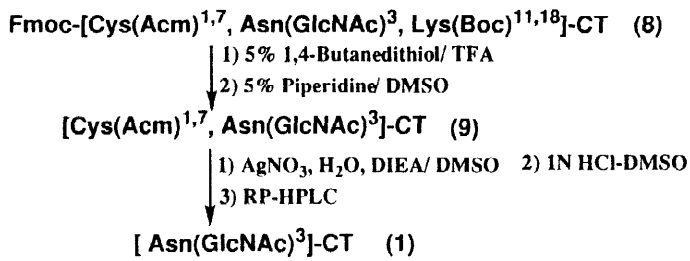


Figure 1. RP-HPLC elution profile of the reaction mixture of segment condensation of glycopeptide thioester **5** with **7**.[14]

The Boc group of **8** was removed by treating with TFA containing 5% 1,4-butanedithiol, and Fmoc group was removed by treating with 5% piperidine in DMSO. After RP-HPLC purification, precursor **9** was prepared. The precursor **9** was treated with $AgNO_3$ and DIEA in aqueous DMSO, followed by 1N HCl/DMSO at room temperature to remove the Acm groups and form a disulfide bond (Scheme 4).[7] After RP-HPLC purification of the reaction mixture, the glycopeptide analogue of eel calcitonin [Asn(GlcNAc)^3]-CT (**1**)[15] was obtained in 9% overall yield based on the amount of amino group in the starting NH_2-resin.[9] The characterization of **1**[16] was performed by MALDI-TOF MS and amino acid analysis.



Scheme 4. The preparation of [Asn(GlcNAc)^3]-CT(**1**) from **8**.

In summary, we have successfully developed a new synthetic method for glycoprotein synthesis using a Mpt-MA method for the incorporation of the glycopeptide moiety and a thioester method for building the peptide.

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9. 4-Methylbenzhydrylamine (MBHA) resin from Peptide Institute, Inc. was used.
10. Solid-phase synthesis in this study was performed *via* Boc-strategy. Amino acids were coupled as *N*-α- Boc derivatives by the following side chain protections : Benzyl (Bzl) for Glu, Ser, Thr; cyclohexyl (cHex) for Asp; 2-bromo-benzyloxcarbonyl (2-Br-Z) for Tyr; 2-chloro-benzyloxcarbonyl (2-Cl-Z) for Lys (Lys^{11} was coupled as Fmoc-Lys(Boc)); benzyloxymethyl (Bom) for His; p-toluenesulfonyl (Tos) for Arg; acetamidomethyl (Acm) for Cys.
11. Coupling reactions were carried out with a five-fold excess of Boc-protected amino acid except Asn(GlcNAc)^3, Ser^2 and Cys^1 residues.
12. Coupling reactions of each Mpt-MAs were carried out with a three-fold excess of Boc-Asn(GlcNAc)-OH and Boc-Ser(Bzl)-OH, Fmoc-Cys(Acm)-OH.
13. 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 (6M HCl, 110°C, 24h) : $Asp_{1.00}Thr_{0.70}Ser_{1.77}Gly_{1.07}Cys_{nd}Val+GlcNH_{21.36}Leu_{1.90}$. Cys was not observed, because *S*-Acm group is stable under the above conditions.
14. HPLC elution conditions ; Column : Cosmosil $5C_{18}AR$ (10 x 250 mm). Linear increase of acetonitrile concentration from 30 to 70% in 0.1% aq trifluoroacetic acid over 40 min at a flow rate of 2.5mL min^{-1}.
15. It will be described elsewhere that prepared **1** has been introduced into several kinds of natural oligosaccharide by a transglycosylation reaction catalyzed by endo-β-*N*-acetylglucosaminidase of *Mucor hiemalis* (Endo-M).[5]
16. MALDI-TOF MS. Found : m/z $[M+H]^+$ 3619.0, Calcd for $C_{154}H_{254}N_{44}O_{52}S_2$ $[M+H]^+$ 3619.1. Amino acid analysis (6M HCl, 110°C, 24h) : $Asp_{2.17}Thr_{3.89}Ser_{2.92}Glu_{3.44}Pro_{1.92}Gly_{3.00}Ala_{1.91}Cystine_{0.61}Val+GlcNH_{22.54}Leu_{5.37}Tyr_{1.10}Lys_{1.88}His_{0.91}Arg_{1.03}$.