Efficient Synthesis of a Sialyl T-antigen-linked Glycopeptide by the Chemoenzymatic Method

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A sialyl T-antigen-linked tetrapeptide was prepared with the combined method of chemical synthesis and enzymatic synthesis. The GalNAc-linked peptide was first obtained by using a commercial peptide synthesizer, and then a galactose residue was attached with β-(1→3)-linkage by transglycosylating with a recombinant β-galactosidase from Bacillus circulans. The sialic acid residue was then connected with α-(2→3)-linkage with sialyltransferase from rat liver.

Key words: peptide synthesis; glycopeptide; mucin-type; β-galactosidase; sialyltransferase

The carbohydrate chains in glycoproteins and glycopeptides are expected to greatly mediate their biological properties and functions. The demand for easy synthesis of such peptides and proteins bearing sugar chains is thus increasing. The synthesis of N-linked glycopeptides has been performed by transglycosylating the entire structure of a high-mannose type or complex-type sugar chain to GlcNAc-linked peptides by utilizing endo N-acetylglucosaminidase A or M. In contrast, for the synthesis of O-linked glycopeptides, no versatile endo N-acetylgalactosaminidase is easily available at present. Therefore, organic chemical syntheses based on a solid-phase peptide synthesis are mainly employed. However, organic synthesis of the carbohydrate chain is time-consuming due to the repeated protection and deprotection of hydroxyl groups in the carbohydrate units. It is a challenge at present to establish an efficient and practical method for the synthesis of O-linked glycopeptides. We report here an efficient approach for the synthesis of glycopeptides carrying the O-linked sugar chain, NeuAcα(2→3)-Galβ(1→3)-GalNAc, which is called the sialyl T-antigen.

The key technique for the present method is the tertiaryglycosylation reaction using a recombinant β-galactosidase which can combine a galactose residue to GalNAc-peptide by β-(1→3)-linkage. As β-(1→3)-galactosyltransferase has not been cloned and expressed so far, only a β-galactosidase from bovine testes is known to transglycosylate a galactose residue with β-(1→3)-linkage. However, the source of the enzyme is an animal organ and therefore this enzyme is not appropriate for a large-scale synthesis. This is the reason why the chemoenzymatic approach to the synthesis of O-linked glycopeptides has not been developed so far. We have recently reported that β-galactosidase obtained by recombinant technology using cDNA of Bacillus circulans could give Galβ-(1→3)-GalNAc derivatives with high regioselectivity and a high reaction yield. We utilized this enzyme in the present study for the synthesis of glycopeptides bearing an O-linked sugar chain.

Although the final goal of our study is to elucidate the change in function of glycopeptides by varying the sugar chain structure, the purpose of the present study was to identify a new synthetic method for preparing glycopeptides. The model peptide synthesized is a fragment of the human fibroblast growth factor, but has no notable biological activity.

GalNAc-linked tetrapeptide 1 was synthesized by using a peptide synthesizer from Fmoc chemistry in the conventional manner with minor changes. Cbz-Gly, tetraacetylated GalNAc-linked Fmoc-Ser, and Fmoc-His were used in a molar ratio of 4:1:4 to 1 equivalent of Fmoc-Glu-resin. Cbz-Gly was used as a tag for isolating the galactosylated or sialylated products by reverse-phase HPLC in the subsequent reaction. The DCC-HOBt-activation method was selected for the condensation reaction, since racemization at the serine residue occurred when the HBTu-HOBut-activation method was used. No signals due to diastereomeric product were apparent in the 'H-NMR spectrum when the DCC-HOBt-activation method was employed. After treating according to the instructions for the peptide synthesizer and deacylating the GalNAc residue, glycopeptide 1 was isolated in a yield of 31% (Figure 1A).

In the transglycosylation reaction using recombinant β-galactosidase from B. circulans, 5 molar...
equivalents of Gal-β-pNP was used as a donor in the presence of 10% (V/V) of DMF. The reaction was monitored by HPLC, using an ODS column. Only one peak was observed in the region where the transglycosylated product was due to appear as can be seen in Figure 1B. The isolated yield of Galβ-(1→3)-GalNAc-tetrapeptide 2 was about 34%, and the structure was confirmed by an NMR analysis; namely, C-3 of the GalNAc residue was shifted from 72.3 ppm to 77.5 ppm in the 13C-NMR spectrum, suggesting that the galactose residue was attached to the C-3 hydroxyl group of the GalNAc residue.

The sialylation reaction used commercial sialyltransferase from rat liver\textsuperscript{15} ([EC 2.4.99.4], recombinant). The reaction product (Figure 1C) was isolated by HPLC, using an ODS column. The yield of isolated NeuAcα-(2→3)-Galβ-(1→3)-GalNAc-linked peptide 3 was about 62%. The α-(2→3)-linkage of the sialic acid residue was confirmed by the empirical rule for the chemical shifts of H-3ax (1.833 ppm) and H-3eq (2.752 ppm) in the 1H-NMR spectrum.\textsuperscript{16} There were no peaks around 1.71 ppm and 2.67 ppm which are characteristic for H-3ax and H-3eq of α-(2→6)-linked sialyloligosaccharides, respectively. The Cbz-group of the N-terminal glycine residue of 3 was quantitatively removed with H\textsubscript{2}/Pd(OH)\textsubscript{2}-black to give free trisaccharide-tetrapeptide 4 (Figure 1D).

Kren and Thiem have reported a "one pot reaction" for the synthesis of the sialyl T-antigen by employing β-galactosidase from bovine testes in combination with sialyltransferase.\textsuperscript{17} Their study is based on the idea of avoiding hydrolysis of the galactose residue by \textit{in situ} sialylation with sialyltransferase as soon as the Galβ-(1→3)-GalNAc-linkage has been formed. The yield of the sialyl T-antigen was higher than that of corresponding glycopeptide 3 in the present report. However, their method seems to be valid only for the synthesis of sialic acid-terminating saccharides like the sialyl T-antigen. The present syn-
thetic method is widely applicable for various glycopeptides containing the Galβ-(1→3)-GalNAc-linkage even without a sialyl residue.

**Experimental**

**General.** β-Galactosidase activity was determined by using Gal-β-pNP as the substrate in a 0.1 M phosphate buffer (pH 6.0) at 37°C. One unit of enzymatic activity is defined as the amount of enzyme to liberate 1 μmol of para-nitrophenol per minute. Sialyltransferase from rat liver was purchased from Calbiochem-Novabiochem Co. (USA). One unit is defined as the amount of enzyme to transfer 1 μmol of sialic acid from CMP-NeuAc to N-acetyllactosamine per minute at 37°C and pH 7.4.

**Synthesis of the GalNAc-linked tetrapeptide (1).** A commercial preloaded HMP-resin of N-Fmoc-L-Glu (356.4 mg, 0.25 mmol) and cartridges of N-Fmoc-N-trityl-L-His (619.7 mg, 1 mmol), N-Fmoc-O-[2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl]-L-Ser (171.3 mg, 0.25 mmol), and N-Cbz-Gly (209 mg, 1 mmol) were used according to the standard synthesizer program (model 431A™, Perkin-Elmer Japan Co., Chiba, Japan) with the DCC-HOBt-activation method. The obtained resin-linked glycopeptide (511.5 mg) was suspended in 10 ml of a 95% TFA solution and the mixture stirred for 1.5 h. After filtration and concentration, the obtained syrup was dissolved in 10 ml of water. The solution was then stirred at room temperature by maintaining pH 10 with a 0.1 N NaOH solution. The degree of deacetylation was monitored by HPLC (Mightysil RP-18 column, Kanto Chemical Co., Tokyo) eluted with a gradient of 0~20% aqueous acetonitrile containing 0.1% TFA (40 min). Desired glycopeptide 1 was isolated by using a similar column of larger size (3.5 cm × 25 cm). After freeze drying, 30.0 mg of white powder was obtained (31% yield).

**Synthesis of Galβ-(1→3)-GalNAc-tetrapeptide (2).** GalNAc-linked tetrapeptide 1 (15.1 mg, 20 μmol) and Gal-β-pNP (30.1 mg, 100 μmol) were dissolved in 120 μl of a 0.1 M phosphate buffer (pH 6.0) containing 10% DMF. To the solution, 30.2 μl of a β-galactosidase solution (EC 3.2.0.23), recombinant, 25 units/ml was added. The solution was incubated at 37°C, and HPLC was measured at appropriate time intervals. After 3.0 h, the solution was heated at 100°C for 5 min to deactivate the enzyme. Isolation of the product by HPLC with Mightysil RP-18 enabled 6.5 mg (34% yield) of Galβ-(1→3)-GalNAc-tetrapeptide 2 to be obtained together with a 8.7 mg recovery of 1.

**Synthesis of NeuAca-(2→3)-Galβ-(1→3)-GalNAc-tetrapeptide (4).** Compound 2 (9.0 mg, 9.6 μmol) and CMP-NeuAc (13.4 mg, 19.2 μmol) were dissolved in 200 μl of a 50 mM cacodylate buffer (pH 6.0) containing of 0.1% triton X and 1 mg/ml of BSA. To this solution, 177 μl (338 μM) of sialyltransferase from rat liver was added. After incubating at 37°C for 3 h, the reaction was stopped. Isolation with a preparative Mighty sil column (3.5 cm × 25 cm) enabled 7.2 mg of sialylated compound 3 to be obtained (62% yield) and 0.5 mg of starting material 2 was recovered.

To a 5 ml aqueous solution of compound 3 (5.0 mg) was added 1 mg of palladium hydroxide, and the solution was stirred in a hydrogen gas atmosphere for 4 h. After filtering off the catalyst, the filtrate was freeze-dried to give 3.8 mg of 4 (93% yield). Selected 1H-NMR data (500 MHz, D2O-δ): 1.796 (t, 3J(H,H) = 12.0 Hz, 1H, H-6a(sia)); 2.021(s, 3H, N-Ac(GalNAc)); 2.030(s, 3H, N-Ac(sia)); 2.752(dd, 3J(H,H) = 4.51 Hz, 12.41 Hz, 1H, H-3a(sia)); 4.318(dd, 3J(H,H) = 3.66 Hz, 11.02 Hz, 1H, H-2(GalNAc)); 4.358(broad dd, 1H, Hα(Glu)); 4.551(d, 3J(H,H) = 7.80 Hz, 1H, H-1(Gal)); 4.664(t, 3J(H,H) = 5.26 Hz, 1H, Hø(Ser)); 4.720(dd, 3J(H,H) = 1.85 Hz, 7.14 Hz, 1H, Hø(His)); 4.892(d, 3J(H,H) = 3.72 Hz, 1H, H-1(GalNAc)); 7.344(broad s, 1H), 8.634(d, 3J(H,H) = 1.33 Hz, 1H). TOF-MS: calcld. for [M+H], 1085.4; found, 1085.7; calcld. for [M+N], 1107.4; found, 1107.3.

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