Synthesis of Sialyl T_N Glycopeptides – Enzymatic Sialylation by α2,6-Sialyltransferase from *Photobacterium damsela*

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Dedicated to Mr. Lee-Shang Chang who initiated this study but unfortunately passed away in April, 2002 because of colon cancer.

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Abstract: The α2,6-sialyltransferase from *Photobacterium damsela* was applied for the enzymatic sialylation of the T_N glycopeptide (APGSTA) with GalNAc α-linked to either the serine or threonine residue in the sequence. The enzyme preparation and reaction conditions were optimized prior to the application. In contrast to the mammalian sialyltransferases which recognize the moiety of GalNAcα(1,1)Thr only, this bacterial enzyme can accept GalNAcα(1,1)Thr as well as GalNAcα(1,1)Ser. Our study also introduced a 4-dimethylaminoazobenzene-4′-sulfonyl (dabsyl) chromophore to the N-terminus of the peptide backbone, which is suitable for glycoconjugate substrates without affecting the binding affinity

Keywords: antigens; enzyme catalysis; glycopeptides; glycosylation; sialic acids

Mucins are glycoproteins with oligosaccharide chains *O*-glycosidically linked to serine or threonine. Aberrant glycosylation of mucins has been observed during malignant transformation.[1] Carcinoma-associated mucins usually carry deviant or truncated carbohydrate sidechains as a result of incomplete assembly of the normal cell surface structures.[2] The abnormal sugar epitopes can be recognized by the immune system[3] and thus become targets of great therapeutic value.[4] Such premature synthesis of glycoconjugates in the cell membrane often leads to an exposure of core structures that would remain cryptic in normal cells due to further elongation. T_N [GalNAcα(1–1)Ser/Thr]- and T [Galα(1–3)GalNAcα(1–1)Ser/Thr]-antigens represent the most common core carbohydrates.[5] Sialylation of these O-antigens exists at low levels in normal tissues, but are highly involved in a number of human malignancies, such as ovary, stomach, lung, colon, breast, and pancreas adenocarcinomas.[2a,6] Expression of sialyl T_N (ST_N) has been linked to poor prognosis in cancer patients,[7] suggesting that the epitope may play an important role in metastatic cascades.[8] As one of the targets for active specific immunotherapy, ST_N antigen has been developed as the cancer vaccine by Biomira Inc., namely Theratope® vaccine.[9]

Despite the clinical significance of sialylated glycopeptides, their preparation still remains a great challenge and thus hampers further investigations. Chemical glycosylation of sialic acids[10] is often restricted to low yield and unsatisfactory stereoselectivity, in addition to tedious but necessary protection and deprotection steps.[11] Although enzymatic methods are able to avoid the aforementioned problems, the ultimate disadvantage arises from the availability of appropriate enzymes and high substrate specificity.[11] Most sialyltransferases are mammalian proteins. Among more than 20 mammalian sialyltransferases genes that have been cloned and characterized so far, only four sialyltransferases were found to exhibit GalNAc α2,6-sialyltransferase activity, including ST6GalNAc I–IV.[12] ST6GalNAc I and II have been shown to be a candidate as a sialyl T_N synthase for the expression of sialyl T_N antigens in native tissues.[13,14] Because mammalian sialyltransferases are membrane proteins that are usually obtained with difficulty owing to low solubility, the enzymes from bacterial sources may serve as the other alternative on account of their easy preparation in large quantity and relatively high activity.

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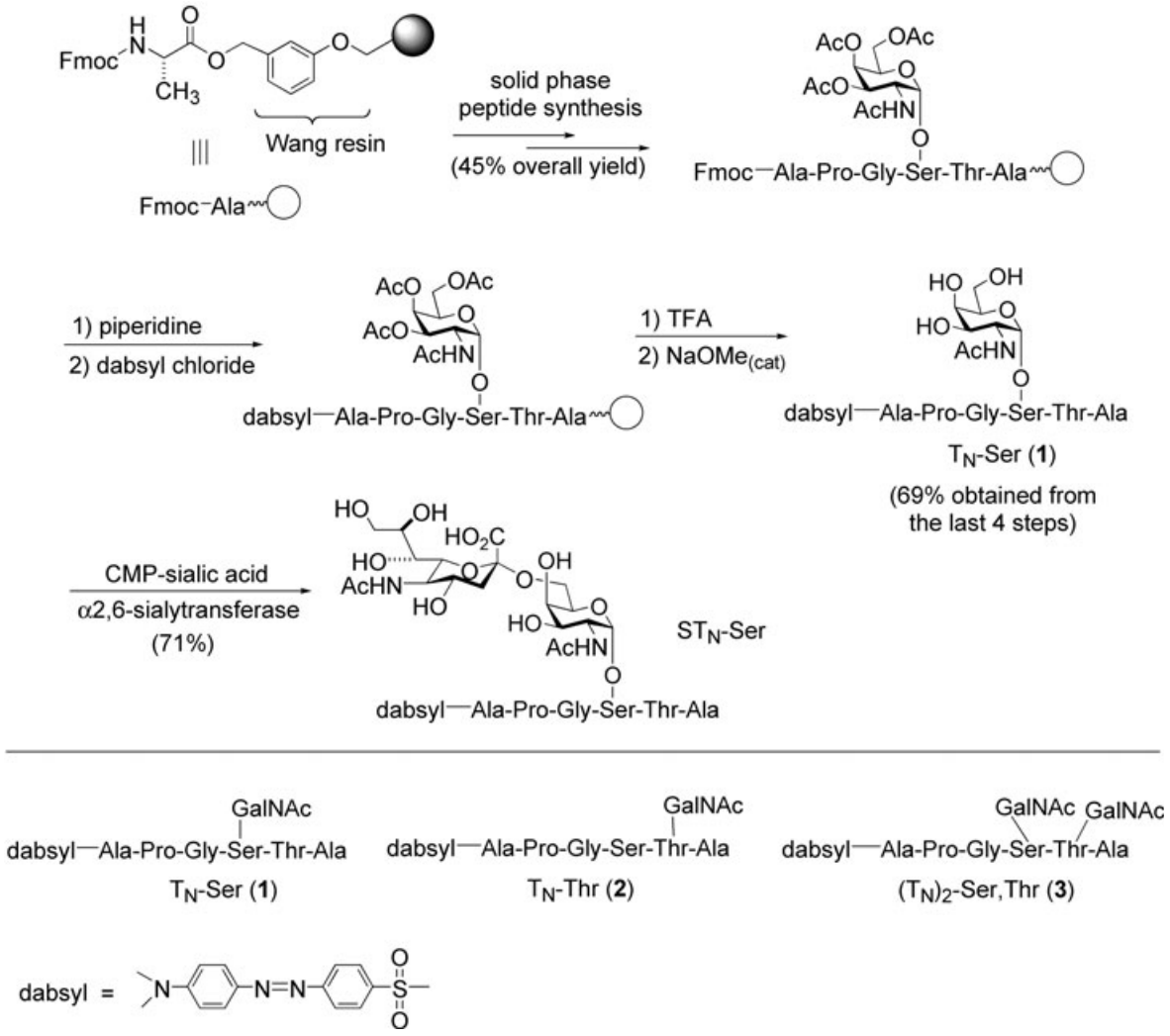
Several enzymes have been studied to catalyze the formation of the α2,6-sialyl glycosidic linkage and the synthesis of various sialylated glycoconjugates. The enzymatic synthesis of the sialyl T_N glycopeptide was initially established by Kihlberg et al. on the basis of a recombinant mouse ST6GalNAc I.[15] A successful high-yield expression of a chicken ST6GalNAc I and corresponding application for the synthesis of sialyl T and sialyl T_N antigens were achieved by Paulson and co-workers.[16] Herein we report the sialylation of T_N glycopeptide by using α2,6-sialyltransferase from *Photobacterium damsela*.

The sialyltransferase gene from *Photobacterium damsela*, previously reported by Yamamoto et al., contained an open reading frame of 2028 base pairs encoding 676 amino acids.[17] It was found by the same group that a deletion of the C-terminal 178 amino acids led to significant improvements of enzyme solubility and activity.[18] Nevertheless, whether the deletion of the C-terminal domain causes any change of the reaction optimum (e.g., pH, temperature, and substrate specificity) has not been investigated previously.

The truncated enzyme corresponding to the amino acid residues from Met1 to Asp498 was cloned and overexpressed in *E. coli*. The deletion was formerly designed to eliminate the membrane binding as the C-terminus was suggested to play a role in such an interaction.[18] However, most of the protein still existed in an insoluble form after cell lysis in our hands. A modified procedure was thus carried out to greatly improve the protein solubility, including the precipitation with 4% polyethylene glycol and subsequent extraction with 2% Triton X-100.[19,20] The treatment produced a soluble form of the protein and afforded over 30% recovery of activity in the cell lysate. After purification steps by Q-Sepharose ion exchange and hydroxyapatite chromatography, SDS-PAGE analysis indicated that the enzyme of molecular mass 51 kDa has an apparent homogeneity greater than 90%. The activity assay was carried out



Scheme 1. Procedure to prepare sialyl T_N glycopeptides. The hexapeptide APGSTA with NeuAc(α2,6)GalNAc attached to Ser (**1**) is shown as an example. The bottom three molecules (**1–3**) were used as the substrates for the enzymatic sialylation.

by using HPLC,[21] the result of which was in agreement with the measurement by high-performance anion exchange chromatography (HPAEC). Some interesting features were disclosed as follows. The optimal pH was found to be 6.0 for the truncated protein, in contrast to pH 5.0 previously reported for the full-length form. The presence of a divalent metal ion is not essential to the enzyme catalysis as the addition of EDTA (10 mM) did not abolish the reaction. The rate of catalysis could be stimulated 2–3-fold by the presence of Mg^{2+}, Mn^{2+}, Ca^{2+} or Co^{2+}, but not Zn^{2+}, similar to the observation reported by Wakarchuk et al.[20] Moreover, the addition of Triton-X 100 was necessary for the enzyme preparation and activity, but the percentage of the detergent does not significantly affect the enzyme activity.

To apply this bacterial enzyme for the synthesis of sialyl T_N glycopeptide, three glycosyl hexapeptides APGSTA (**1–3**, as shown in Scheme 1) with GalNAc α-linked to serine or/and threonine, were derived from MUC1 tandem-repeat core (PDTRP**APGSTA**PPAHGVTSA). MUC1 mucin is a large transmembrane glycoprotein, of which the extracellular domain is densely glycosylated. As one of the normally cryptic carbohydrates, the ST_N epitope is exposed owing to abnormal and incomplete glycosylation as previously described.[22] An *in vitro* glycosylation study indicated the three possible glycosylation sites (underlined residues) in association with human tumor cell lines.[22a] For the purpose of sensitive detection, a 4-dimethylaminoazobenzene-4′-sulfonyl residue (dabsyl, $\varepsilon = 33{,}000\ cm^{-1}M^{-1}$ at 480 nm) was attached to N-terminus of these glycopeptides as a chromogenic tag.

Solid-phase peptide synthesis was applied to prepare the three T_N glycopeptides **1–3**,[23] including two monovalent T_N and one divalent T_N (Scheme 1), for the subsequent sialylation catalyzed by *P. damsela* α2,6-sialyltransferase. Pyridylaminated (PA)-lactose (a commonly used fluorogenic substrate) and lactose (the natural substrate) were also subjected to the enzymatic sialylation for a comparison. Table 1 lists their relative rates, indicating that compounds **1–3** and PA-lactose exhibit similar reaction rates though at about 1/10 level of lactose. In accordance with the previous study,[17] the enzyme showed comparable preference for either Gal or GalNAc. The presence of additional sugar residue enhanced the sialylation (lactose *vs.* PA-lactose). The enzyme had similar access to the serine and threonine in the sequence (**1** *vs.* **2**). The result is distinct from the studies of the chicken and mouse enzymes (ST6GalNAc I) that prefer threonine only.

The synthesis of three ST_N glycopeptides was carried out, the products of which were confirmed by ESI-MS analysis (Figure **1**). When the bacterial enzyme was used for the sialylation of the divalent T_N hexapeptide **3** (MW: 1195.2), the resulting mass spectrometric analysis exhibited the molecular ion (MW: 1485.4) corresponding to the addition of single sialic acid [1195.2 + 309.3 (NeuAc) − 18 (H_2O) = 1486.5]. The fragment peak of MW 1264.4 was contributed by the loss of GalNAc (MW: 211) because the product has one GalNAc residue exposed owing to incomplete sialylation. We propose that the enzymatic sialylation can occur at either Ser or Thr, as shown by the similar relative rates of glycopeptides **1** and **2**. Probably the second sialylation was prohibited by the steric hindrance, i.e., the proximity in space caused by the two neighboring glycosylation sites.

Table 1. Substrate specificity of the α2.6-sialyltransferase from *P. damsela*.[a]

Substrate	Activity [nmol/mg]	Relative rates [%]
Lactose	488.59	100.0
PA-lactose	43.35	8.9
T_N-Ser (**1**)	42.70	8.7
T_N-Thr (**2**)	59.30	12.1
$(T_N)_2$-Ser,Thr (**3**)	54.72	11.2

[a] All the reactions were carried out at 30 °C for 3 h and initiated by the addition of 20 μg of the enzyme to the mixture containing 80 mM sodium cacodylate buffer (pH 6.0), 0.5 mM substrate, 2.5 mM CMP-NeuAc, 10 mM $CaCl_2$ and 0.2% Triton X-100.

In summary, this report demonstrates the synthesis of sialyl T_N glycopeptide by the sialylation with a bacterial sialyltransferase. The same enzyme was recently used for the production of NeuAca(2,6)GalNAc that did not contain any peptide or amino acid.[24] The enzyme recognizes both the epitopes GalNAca(1,1)Thr and GalNAca(1,1)Ser, offering an advantage which cannot be carried out by mammalian enzymes. Furthermore, although glycosyltransferase assays usually rely on the use of chromophore- or fluorophore-containing substrates for higher sensitivity, most studies incorporate such a group to either the sugar donor (e.g., CMP-9-fluoresceinyl-NeuAc)[25] or sugar acceptor's reducing end (e.g., pyridylaminated sugar). Both methods inevitably change the structure and thus reduce the affinity with enzyme (i.e., increase K_m). The latter procedure takes advantage of the aldehyde at the reducing end for modification so that it is often not compatible to the study of glycoconjugate substrates. Our method thus provides an alternative solution by adding a chromophore to the peptide terminus. Further investigation to prepare other sialylated tumor-associated antigens is currently in progress.

Experimental Section

Enzyme Cloning, Overexpression, and Purification

The DNA corresponding to the protein sequence from Met1 to Asp498 was amplified from *P. damsela* genomic DNA by using

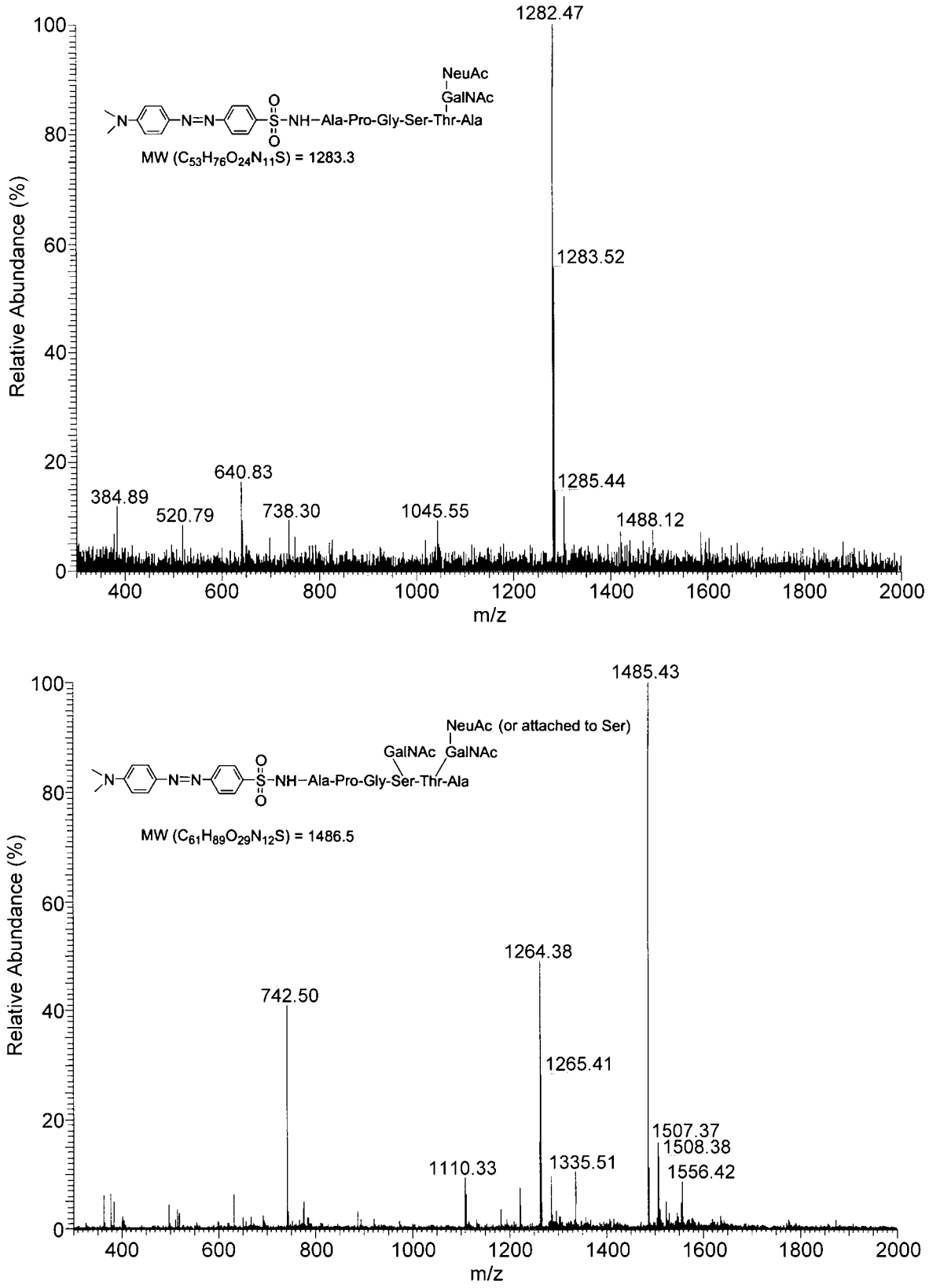


Figure 1. ESI-MS of the sialylation products of T_N glycopeptides **2** (*above*) and **3** (*below*).

two primers, 5′-GTGCT TATCA TGAAG AAAAT ACTGA CAG-3′ (sense) and 5′-CTTTC TCGAG AGCCC AGAAC AGAAC ATC-3′ (anti-sense), to incorporate *Bsp*HI and *Xho*I sites (underlined), respectively. The PCR product was digested with the desired restricted enzymes and ligated with the vector pET-15b that had been treated with the enzymes *Nco*I and *Xho*I. The resulting plasmid was then subcloned to the vector pET-21b between the sites of *Xba*I and *Xho*I to give the plasmid pET21-phoST that was further transformed into *E. coli* NovaBlue (DE3) for overexpression. The clone harboring pET21-phoST was cultured in LB broth containing 100 μg/mL ampicillin at 25 °C for 36 h and subcultured to 500 mL of the same medium containing 0.02 mM isopropyl-1-thio-D-galactopyranoside (IPTG) at 25 °C for another 24 h. The culture was then harvested by centrifugation, lysed by 10 mL lysis buffer (50 mM HEPES, pH 7.0), disrupted using a French press (15,000 psi), and centrifuged at 14,000 *g*. The supernatant was treated with 4% polyethylene glycol (PEG, av. MW: 8,000) in 0.2 M NaCl by stirring at 4 °C for 30 min and centrifuged to collect the desired precipitated proteins. Extraction was carried out for the re-suspended pellets at least three times by vigorously vortexing with 1% Triton X-100 at 4 °C for 30 min, after which the supernatant was collected and applied to a HiTrap Q-Sepharose HP column (Pharmacia). The column had been pre-equilibrated with 50 mM Tris buffer (pH 7.0) containing 0.2% Triton X-100. After loading of the sample, the column was washed by the same buffer and proteins were eluted with a linear gradient of 0–500 mM NaCl. The desired fractions were collected, concentrated and applied to hydroxyapatite column (Bio-Rad), which had been pre-equilibrated with water containing 0.2% Triton X-100. After loading of the sample, the column was washed with a linear gradient of 0–500 mM potassium phosphate buffer (pH 7.0). The desired fractions were pooled and concentrated to yield the protein with homogeneity of more than 90%.

Synthesis of T_N Glycopeptides

The target glycopeptides (APGSTA) with GalNAc α-linked to serine or/and threonine were synthesized by solid phase peptide synthesis on Wang resin in conjunction with standard Fmoc chemistry. All the hydroxy groups of GalNAc, serine and/or threonine were protected by acetyl groups. The reaction progress was monitored by the ninhydrin test. Once the coupling reaction was complete, the resin-bound peptides were washed successively with DMF/DIPEA (95/5), DMF, dichloromethane and methanol (36–45% overall yield for **1–3**). The introduction of the chromogenic dabsyl group was achieved prior to TFA cleavage by the reaction of the resin-bound peptides with dabsyl chloride. Resin-bound peptides (0.5 mmol) in DMF/DIPEA (95/5) were added to 2 mmol of dabsyl chloride in the same solvent. The resin was then removed by treatment with TFA for 2 h. Final deprotection with a catalytic amount of NaOMe was carried out at room temperature. After lyophilization, the synthesized T_N glycopeptides were purified by reverse-phase HPLC as described above (65–69% yield for **1–3** in the last four steps) and further confirmed by ESI-MS.

Enzyme Reaction and Activity Assay

The reaction catalyzed by the α2,6-sialyltransferase from *P. damsela* was incubated at 30 °C for 3 h in 80 mM sodium cacodylate (pH 6.0) containing 5 mM substrate, 2 mM CMP-sialic acid, 10 mM $CaCl_2$, 0.2% Triton X-100, and 20 μg sialyltransferase. The reaction progress was monitored by TLC with 2-PrOH/H_2O/AcOH (10/3/1). After completion, the mixture was heated at 90 °C for 2 min, 500 μL $CHCl_3$ were added for extraction, and the aqueous layer was collected. The resulting solution was ready for further purification. The products of sialyllactose and sialyl-PA-lactose were subjected to ion exchange chromatography with a Mono Q column (Pharmacia). 0–25% 1 N NH_4HCO_3 was used as the eluent. Sialyl lactose was detected by UV at 210 nm. The detection of PA-lactose and its reaction product was carried out by the fluorescence emission at 380 nm using an excitation wavelength of 310 nm. T_N and sialyl T_N glycopeptides were purified by reverse-phase C18 column (ThermoQuest, Hyperprep HS-C18, 4.6 × 250 mm) with the eluent of CH_3CN/H_2O and monitored by the detection at 480 nm.

Both products resulting from the sialylation of lactose and PA-lactose were individually purified by HPLC and further subjected to HPAEC analysis using Dionex BioLC DX-600 system with a Carbopac PA-100 column (4 × 255 mm) and AminoTrap column (4 × 50 mm), where 100 mM sodium hydroxide (buffer A) and 1 M sodium acetate in 100 mM sodium hydroxide (buffer B) were used as the eluents. Both HPLC and HPAEC analyses were in good agreement.

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