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Note

Multi-enzyme one-pot strategy for the synthesis of sialyl Lewis X-containing PSGL-1 glycopeptide

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Abstract—An enzymatic one-pot three-step glycosylation strategy was developed for the synthesis of sLe^x moiety of truncated PSGL-1 glycopeptide with and without sulfation. The method provided an efficient way to afford complex glycopeptides in a semi-preparative scale without further complicated and time-consuming purification process in each glycosylation step.

Keywords: PSGL-1; Sialyl Lewis X; Glycopeptide; One-pot synthesis; Glycosyltransferase

The selectin family of cell adhesion molecules (L-, E-, and P-selectins) are known to recognize with weak binding to the fucosylated, sialylated, and sulfated glycans, including those containing the sialyl Lewis X (sLe^x) tetrasaccharide determinant, NeuAcα2-3Galβ1-4(Fucα1-3)-GlcNAc-R, and related structures.[1] Among them, P-selectin glycoprotein ligand-1 (PSGL-1) has been well characterized as the ligand for P-selectin.[2] Previous investigations revealed that PSGL-1 is heavily decorated with N- and O-linked glycans containing sLe^x, and a cluster of three N-terminal tyrosine sulfate (Tyr-SO_3) groups. The binding of P-selectin to PSGL-1 requires the N-terminal fragments of PSGL-1 containing a core 2 O-linked glycan at Thr57 with a terminal sLe^x moiety and at least one of the sulfated tyrosine residues at Tyr46, Tyr48, and Tyr51.[3] It has also been shown that a truncated N-terminal PSGL-1 monomer exhibits high affinity with P-selectin.[4] Thus, the development of an efficient method for the synthesis of the truncated PSGL-1 and examination of its detailed recognition with P-selectin will facilitate the discovery of potential anti-inflammatory agents.

Although, in the past few decades, many efficient chemical methods for the synthesis of sLe^x and its derivatives have been developed,[5] the synthesis of complex carbohydrates still remains much more complicated in comparison with other biomolecules, such as DNA and protein. However, the use of enzymes in carbohydrate synthesis can dramatically reduce the protection–deprotection strategies and provide great stereo- and regioselectivity.[6] In addition, glycosyltransferase based one-pot systems with regeneration of sugar nucleotides in situ have been reported for the synthesis of Lewis X,[7] sLe^x,[8] 6′-SLN,[9] α-Gal epitope,[10] and hyaluronic acid polymer.[11]

The sulfation on the tyrosine residue of the truncated PSGL-1 glycopeptide has been achieved by using tyrosylprotein sulfotransferase (TPST)[12] or by using Fomc-Tyr-(SO_3H)-OH building block in the solid-phase peptide synthesis.[13] However, there are limitations with applying these methods in a micro-gram scale synthesis as TPST is not commercially available and the sulfate group is susceptible to cleavage under acid conditions. Herein, we employed the one-pot enzymatic glycosylation with β-1,4-galactosyltransferase (β-1,4-GalT), α-2,3-sialyltrans-

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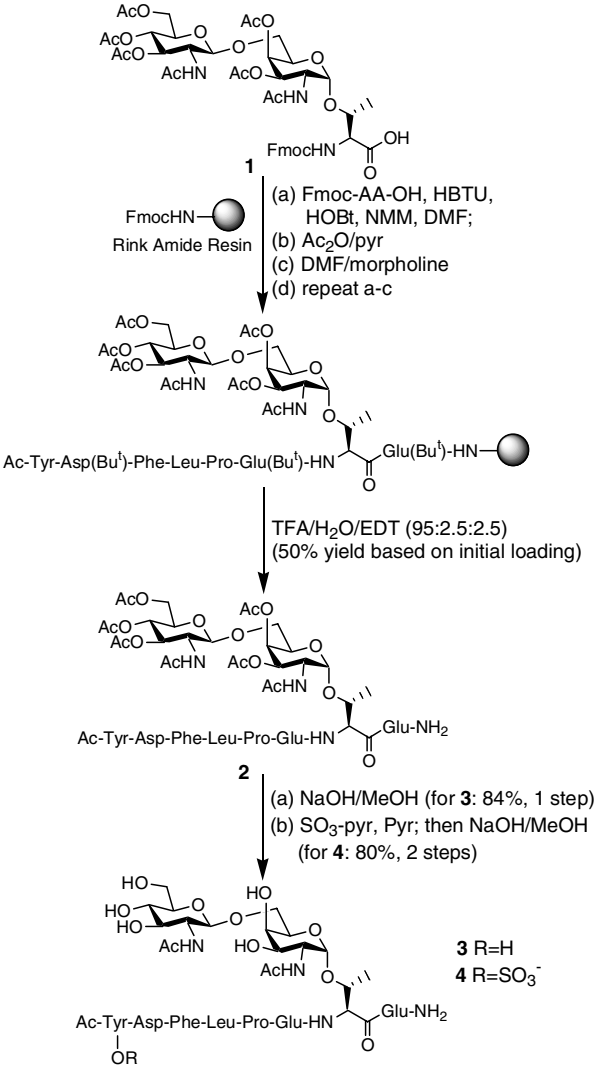
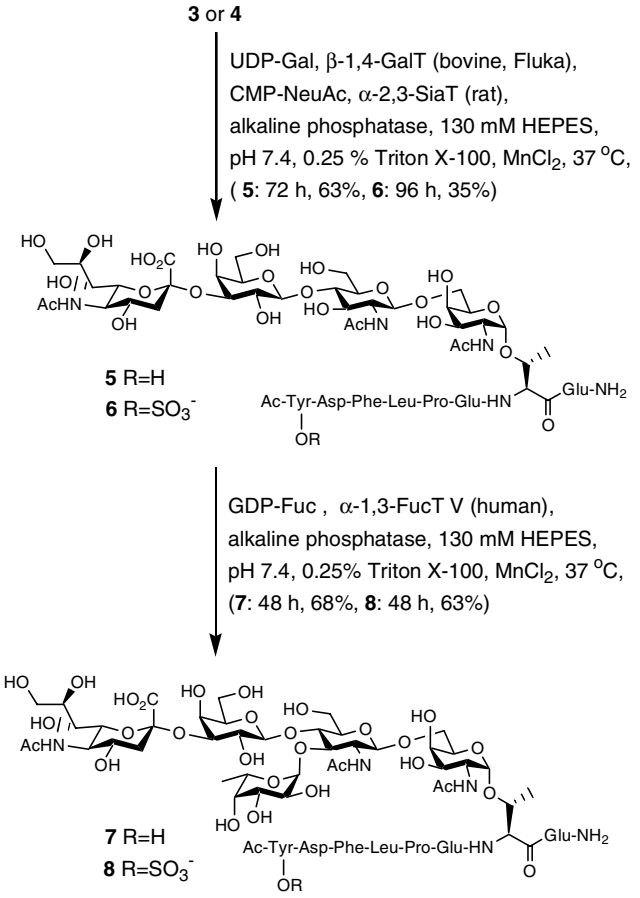
ferase (α-2,3-SiaT), and α-1,3-fucosyltransferase (α-1,3-FucT V) to construct the sLe^x moiety of truncated PSGL-1 octapeptide. Thus, the time-consuming and tedious purification steps can be greatly reduced.

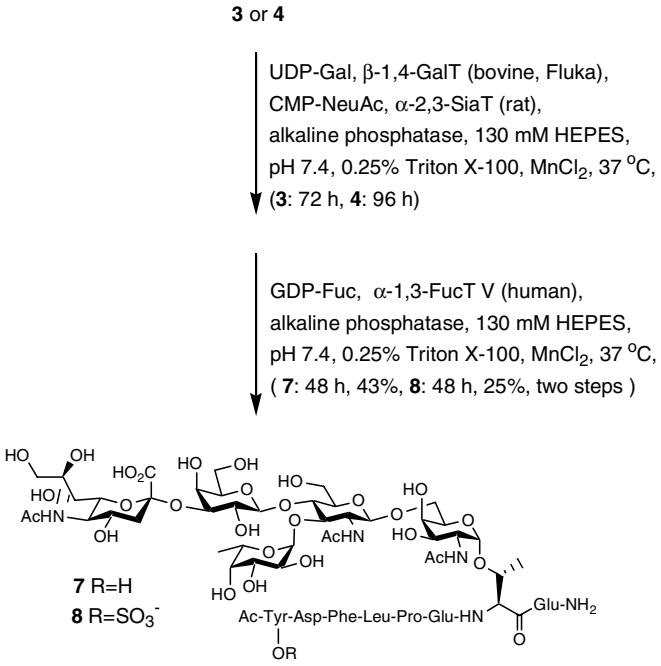
Initially, the disaccharide-linked threonine **1** was synthesized[14] and then incorporated into the solid-phase peptide synthesis utilizing a Rink Amide resin and Fmoc chemistry (Scheme 1). Following the N-terminal acetylation, cleavage from the resin and full deprotection, **2** was obtained with 50% overall yield. Basic hydrolysis of the acetate protecting groups of the saccharide moiety of **2** gave glycopeptide **3** (84% yield). Sulfation on the tyrosine residue of **2** was achieved by using sulfur trioxide–pyridine complex, followed by basic hydrolysis to yield sulfated glycopeptide **4** (80% yield for two steps). With the glycopeptides in hand, the one-pot synthesis of sLe^x by glycosyltransferase-catalyzed glycosylation was then investigated. Because LacNAc is a substrate for both SiaT and FucT, and Le^x is not a good substrate for SiaT, the sLe^x moiety was constructed by adding the galactose, sialic acid and fucose groups sequentially.[15] Because the optimal pH for the activities of GalT and SiaT is at 7.5, while FucT works more efficiently at pH 6.5, the sLe^x was constructed by two separated enzymatic reactions (Scheme 2). Glycopeptide **3** was treated with GalT and SiaT in the presence of donor substrates UDP-Gal and CMP-NeuAc in the HEPES buffer (pH 7.4) for 72 h to give **5** in 63% yield. However, the enzymatic glycosylation reaction of **4** did not proceed as smoothly as for the unsulfated substrate **3**. This may be due to the fact that sulfated glycopeptide **4** is not a good substrate for SiaT.[14a] After the addition of more glycosyltransferases (one portion more) and extension of the reaction time (for more 48 h), compound **6** was obtained in 35% yield. In contrast to the previous report, it was not necessary to add protease inhibitor cocktail to the reaction to give a reasonable product yield. Encouraged by the success of the one-pot two-step glycosylation approach, FucT and GDP-Fuc were incubated with **5** and **6**, respectively, in the HEPES buffer (pH 7.4). The complete sLe^x glycopeptides of truncated PSGL-1 with or without sulfation, **7** and **8**, were afforded in 68% and 63%, respectively. It should be noted that alkaline phosphatase was added to all of the men-



Scheme 1. Synthesis of unsulfated and sulfated glycopeptides **3** and **4**.



Scheme 2. Synthesis of unsulfated and sulfated glycopeptides **7** and **8**.



Scheme 3. Multi-enzyme one-pot synthesis of unsulfated and sulfated glycopeptides **7** and **8**.

tioned enzymatic reactions to reduce the product inhibition caused by the pyrophosphate group.

Finally, we utilized the aforementioned enzymatic reaction conditions to construct the sLe^x-containing glycopeptides using the multi-enzyme one-pot, three-step strategy (Scheme 3). The pH of the enzymatic reactions was fixed at 7.4. Substrates **3** and **4** were incubated with GalT and SiaT in the presence of UDP-Gal, CMP-NeuAc and alkaline phosphatase for 72 and 96 h, respectively. FucT and GDP-Fuc were then added in each reaction mixture and incubated for another 48 h. After purification, the truncated PSGL-1 with or without sulfation, **7** and **8**, were obtained with 43% and 25% yield, respectively.

In conclusion, we have successfully demonstrated a multi-enzyme one-pot, three-step glycosylation strategy for the synthesis of sLe^x-containing truncated PSGL-1 glycopeptides with or without sulfation. This method provides an efficient way to obtain complex glycopeptides with acceptable yields without further complicated and time-consuming purification in each glycosylation step. In the current study, we have obtained 7.0 and 2.0 mg for **7** and **8**, respectively, in a single multi-enzymatic reaction.

1. Experimental

1.1. General methods

General information: MALDI-Mass spectra were collected on a Voyager DE-PRO (Applied Biosystem, Houston, USA) equipped with nitrogen laser (337 nm) and operated in the delayed extraction reflector mode. ESI (Electrospray)-Mass Spectra: a Finnigan LCQ mass spectrometer (Thermo Finnigan, San Jose, CA, USA). UDP-Gal, CMP-NeuAc, GDP-Fucose, α-2,3-sialyltransferase (rat), and α-1,3-fucosyltransferase V (human) were purchased from Calbiochem. β-1,4-Galactosyltransferase (bovine) was purchased from Fluka.

The isolated compounds **1**–**8** have previously been characterized, and the NMR spectral data are in good agreement with the literature data.[14]

1.2. General procedure for the multi-enzyme one-pot synthesis strategy

1.2.1. Ac-Tyr-Asp-Phe-Leu-Pro-Glu-Thr(NeuAc(α2,3)-Gal(β1,4)GlcNAc(β1,6)GalNAcα)-Glu-NH_2 (5). Compound **3** (6.2 mg, 4.3 μmol), UDP-Gal (4.1 mg, 6.6 μmol), and CMP-NeuAc (4.8 mg, 7.3 μmol) were dissolved in buffer (130 mM HEPES, pH 7.4 with 0.25% Triton X-100, 800 μL) containing a freshly prepared $MnCl_2$ solution (8.0 μmol). β-1,4-GalT (7 mg, 61 mU), α-2,3-SiaT (16 μL, 60 mU), and alkaline phosphatase (6 μL, 600 mU) were added and the reaction mixture was shaken gently at 37 °C for 72 h. The solution was filtered on a syringe-driven filter (Millex-GV, 0.22 μm, Millipore) and then subsequently purified with a reverse phase C-18 HPLC column (Vydac, 218TP510) on an Agilent 1100 HPLC system by monitoring a UV absorbance peak which appeared at 220 nm. The following solvent system was used at a flow rate of 3 mL/min: 0–15 min, linear acetonitrile gradient from 0% to 5% in

water; 15–30 min, linear acetonitrile gradient from 5% to 30% in water (t_R 16.17 min). Fractions containing the product were obtained by lyophilizing to give **5** (5.1 mg, 63%). ESI-MS (neg) calcd for $C_{82}H_{120}N_{12}O_{40}$ $[M-H]^-$: 1911.8, found 1912.09.

1.2.2. Ac-Tyr(OSO_3^-)-Asp-Phe-Leu-Pro-Glu-Thr(NeuAc(α2,3)Gal(β1,4)GlcNAc(β1,6)GalNAcα)-Glu-NH_2 (6). Compound **4** (5 mg, 3.2 μmol), UDP-Gal (2.9 mg, 4.8 μmol), and CMP-NeuAc (4.2 mg, 6.4 μmol) were dissolved in buffer (130 mM HEPES, pH 7.4, with 0.25% Triton X-100, 600 μL) containing a freshly prepared $MnCl_2$ solution (6.5 μmol). β-1,4-GalT (8.1 mg, 70 mU), α-2,3-SiaT (19 μL, 70 mU), and alkaline phosphatase (6.9 μL, 690 mU) were added and the reaction mixture was shaken gently at 37 °C. After 48 h reaction time, β-1,4-GalT (8.1 mg, 70 mU) and α-2,3-SiaT (19 μL, 70 mU) were added again. After stirring for a total of 96 h, the solution was filtered on a syringe-driven filter (Millex-GV, 0.22 μm, Millipore) and was subsequently purified with the reverse phase C-18 HPLC column as described. The following solvent system was used at a flow rate of 3 mL/min: 0–25 min, linear acetonitrile gradient from 0% to 5% in water (t_R 8.09 min). Fractions containing the product were then isolated by lyophilizing to give compound **6** (2.4 mg, 35%). ESI-MS (neg) calcd for $C_{82}H_{119}N_{12}O_{43}S$ $[M-H]^-$: 1991.7, found 1990.0.

1.2.3. Ac-Tyr-Asp-Phe-Leu-Pro-Glu-Thr(NeuAc(α2,3)-Gal(β1,4)[Fuc(α1,3)]GlcNAc(β1,6)GalNAcα)Glu-NH_2 (7). Compound **3** (4.1 mg, 2.8 μmol), UDP-Gal (2.6 mg, 4.2 μmol), and CMP-NeuAc (3.2 mg, 4.8 μmol) were dissolved in buffer (130 mM HEPES, pH 7.4, with 0.25% Triton X-100, 600 μL) containing a freshly prepared $MnCl_2$ solution (6.0 μmol). β-1,4-GalT (4.7 mg, 40 mU), α-2,3-SiaT (11 μL, 40 mU), and alkaline phosphatase (4 μL, 400 mU) were added and the reaction mixture was shaken gently at 37 °C for 72 h. After 72 h reaction time, GDP-Fuc (1.9 mg, 3.1 μmol) and α-1,3-FucT V (58 μL, 28 mU) were added. After stirring for a total of 120 h, the solution was filtered on a syringe-driven filter and subsequently purified with the reversed phase C-18 HPLC column as described. The following solvent system was used at a flow rate of 3 mL/min: 0–15 min, linear acetonitrile gradient from 0% to 5% in water; 15–30 min, linear acetonitrile gradient from 5% to 30% in water (t_R 11.34 min). Fractions containing the product were then isolated by lyophilizing to give the product **7** (2.5 mg, 43%). ESI-MS (neg) calcd for $C_{82}H_{120}N_{12}O_{40}$ $[M-H]^-$: 2058, found 2056.

1.2.4. Ac-Tyr(OSO_3^-)-Asp-Phe-Leu-Pro-Glu-Thr(NeuAc(α2,3)Gal(β1,4)[Fuc(α1,3)]GlcNAc(β1,6)GalNAcα)-Glu-NH_2 (8). Compound **4** (5.3 mg, 3.4 μmol), UDP-Gal (3.1 mg, 5.1 μmol), and CMP-NeuAc (4.5 mg, 6.8 μmol) were dissolved in buffer (130 mM HEPES, pH 7.4, with 0.25% Triton X-100, 700 μL) containing a freshly prepared $MnCl_2$ solution (7.0 μmol). β-1,4-GalT (8.6 mg, 75 mU), α-2,3-SiaT (20 μL, 75 mU), and alkaline phosphatase (7.3 μL, 730 mU) were added and the reaction mixture was shaken gently at 37 °C. After 48 h β-1,4-GalT (8.6 mg, 75 mU) and α-2,3-SiaT (20 μL, 75 mU) were added again. After stirring for 48 h, GDP-Fuc (2.4 mg, 4.1 μmol) and α-1,3-FucT V (40 μL, 20 mU) were added. After stirring for a total of 144 h, the solution was filtered on a syringe-driven filter and subsequently purified with the reverse phase C-18 HPLC column as described. The following solvent system was used at a flow rate of 3 mL/min: 0–25 min, linear acetonitrile gradient from 0% to 5% in water (t_R 6.97 min). Fractions containing the product were then isolated by lyophilizing to give the product **8** (1.9 mg, 25%). ESI-MS (neg) calcd for $C_{82}H_{120}N_{12}O_{40}$ $[M-H]^-$: 2138, found 2136.

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