Tyrosine Sulfation on a PSGL-1 Glycopeptide Influences the Reactivity of Glycosyltransferases Responsible for Synthesis of the Attached *O*-Glycan

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P-Selectin glycoprotein ligand-1 (PSGL-1) is the primary counter-receptor for P-selectin during leukocyte extravasation in the inflammatory response.[1] Previously, it has been determined that the *O*-glycan attached to threonine 16 and at least one site of tyrosine sulfation within the N-terminal 19 amino acids are required for optimal recognition of PSGL-1 by P-selectin.[2] In fact, the N-terminal glycosulfopeptide binds to P-selectin nearly as efficiently as the full-length dimeric PSGL-1, and the sulfated glycopeptide binds to the receptor approximately 10^5 times more tightly than does the unsulfated glycopeptide ligand (Figure 1a).[3] The *O*-linked glycan structure from PSGL-1 includes a terminal sialyl Lewis x (sLe^x) tetrasaccharide extended from a core 2 glycan (Figure 1b).[4]

To further examine PSGL-1/P-selectin recognition, as well as to investigate PSGL-1 biosynthesis, the chemoenzymatic synthesis of a binding determinant of PSGL-1 was undertaken. The target structure represents a minimal sequence containing a tyrosine sulfate and the glycosylated threonine residue, corresponding to amino acid residues 10−17 of the mature PSGL-1 protein. The synthesis combined solution- and solid-phase methods to arrive at a disaccharide-linked octapeptide in both sulfated and unsulfated forms. Glycosyltransferase-catalyzed elaboration of the glycan was then studied.[5] Results indicate that sulfation on tyrosine influences the reactivity of the glycosyltransferases responsible for the synthesis of sLe^x on the attached *O*-glycan.

The synthetic strategy involved incorporation of a protected disaccharide-linked threonine building block into solid-phase peptide synthesis (Scheme 1). Disaccharide−threonine conjugate **3** was obtained by reaction of **1**[6] with either glycosyl donor **2a**[7] under BF_3−OEt_2 catalysis or donor **2b**[8] with DMTST as the activating agent.[9] Conversion of **3** to **5** was accomplished by standard synthetic manipulations.

Building block **5** was incorporated into glycopeptide **6** utilizing a Rink Amide modified resin as the solid phase (Scheme 2). Following N-terminal acetylation, sequence **6** was treated with 95% TFA, H_2O, and ethane dithiol as a scavenger. These conditions caused simultaneous liberation of the sequence from the resin as the C-terminal amide and removal of the *t*Bu ester and ether protecting groups. The crude peptide obtained was initially purified by ether precipitation and small portions then further purified using RP-HPLC to give sequence **7**.[10]

Sulfation on the tyrosine residue of glycopeptide **7** was then accomplished with sulfur trioxide−pyridine complex. A workup protocol involving a methanol quench and immediate silica gel chromatography[11] allowed the sulfated sequence to be isolated in much higher yields than has been previously reported.[12] Saponification of the acetate esters then gave deprotected glycopeptide **8a** for the subsequent glycosyltransferase-catalyzed

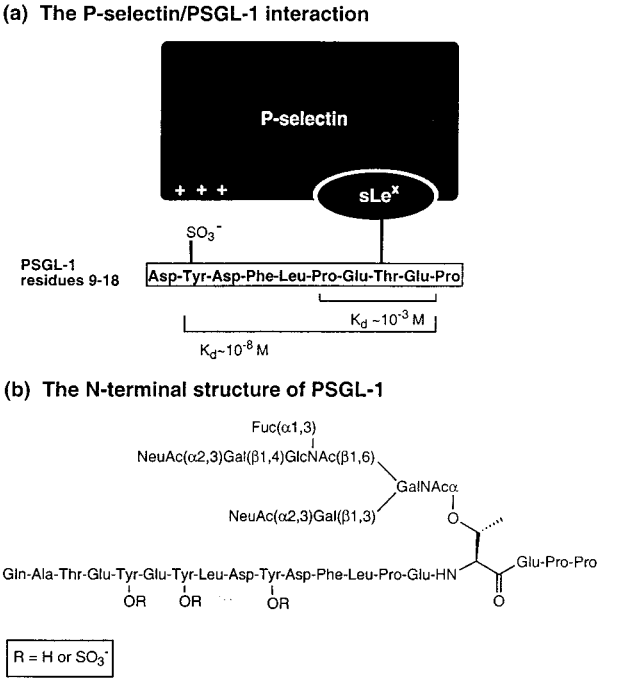
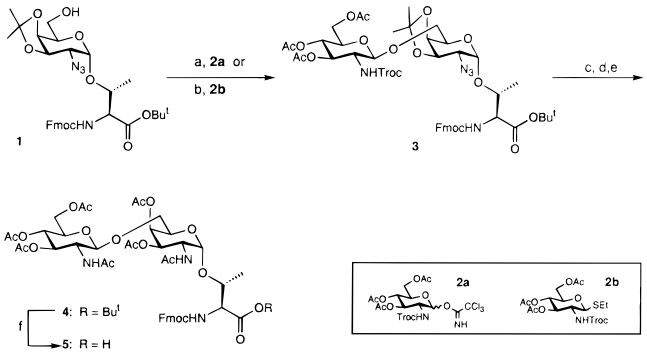


Figure 1. (a) The P-selectin/PSGL-1 interaction. (b) The N-terminal structure of PSGL-1.

Scheme 1. Synthesis of the glyco-Thr Building Block



[a] Conditions: (a) BF_3−OEt_2, −30 °C, CH_2Cl_2 (100%); (b) DMTST, CH_2Cl_2, 4 Å MS, 0 °C (75%); (c) $AcOH/H_2O$, 45 °C; (d) Ac_2O/Pyr; (e) Zn dust, THF/AcOH/Ac_2O (80%, 3 steps); (f) TFA/H_2O (95:5) (100%).

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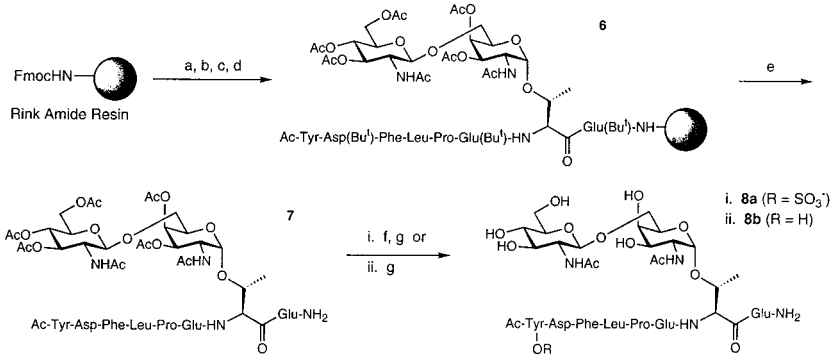
Scheme 2. Solid-Phase Synthesis of Glycopeptides



[a] (a) Fmoc−AA−OH, HBTU, HOBt, NMM, DMF; (b) Ac_2O/pyr; (C) DMF/morpholine; (d) repeat a−c; (e) TFA/H_2O/EDT (95:2.5:2.5) (68%, based on initial loading); (f) SO_3−pyr, Pyr, (g) NaOH/MeOH (81%, 2 steps); (g alone) NaOH/MeOH (89%, 1 step).

Scheme 3. Differences in Glycosyltransferase Reactivity with Sulfated vs Unsulfated Glycopeptides

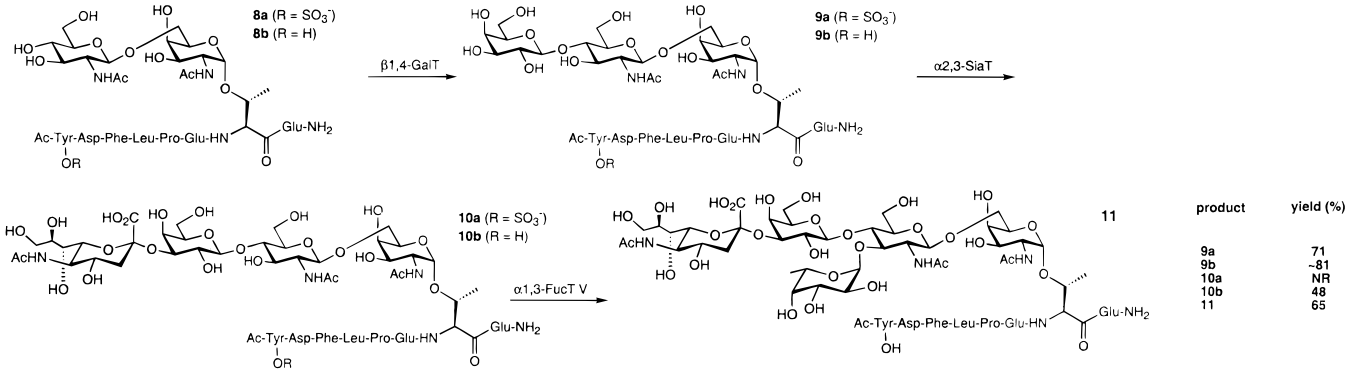


Table 1

substrate	K_m (μM)	V_{max} (μM/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
sulfated **8a**	4073	2.190	0.2740	6.72×10^{-5}
unsulfated **8b**	223	0.339	0.0425	1.90×10^{-4}

glycosylations. By this protocol, both sulfated **8a** and nonsulfated **8b** peptide sequences were readily accessible.

Initial attempts to append galactose in a β1,4-linkage to sulfated glycopeptide **8a** utilizing recombinant bovine β1,4-galactosyltransferase (β1,4-GalT) were only marginally successful (Scheme 3). However, product **9a** could be isolated if the reaction was driven to the disappearance of starting material. In contrast, no product formation was observed when **9a** was treated with CMP−NeuAc in the presence of recombinant rat α2,3-sialyltransferase (α2,3-SiaT).

For unsulfated glycopeptide **8b**, β1,4-GalT, α2,3-SiaT, and recombinant human α1,3-fucosyltransferase V (α1,3-FucT V) all accepted the glycopeptide substrates without difficulty. In this manner, the complete sLe^x tetrasaccharide was formed attached to the glycopeptide, yielding **11**.

To further characterize the observed reactivity differences, an assay was developed to determine the kinetic parameters for the β1,4-GalT reaction with both sulfated **8a** and nonsulfated **8b** (Table 1). Notably, sulfation of the tyrosine hydroxyl appears to interfere with the binding of the substrate to the enzyme (an 18-fold increase in K_m) and thus affects the reaction of β1,4-GalT at the remote GlcNAc acceptor site of the glycopeptide.

A structural study further indicated that there are no significant structural changes between glycopeptide sequences **8a** and **8b**. It is assumed then that the differences in reactivity toward β1,4-GalT may be caused by unfavorable electrostatic interactions of the sulfate with the enzyme. However, the most pronounced variation between the activity of sulfated and unsulfated glycopeptides was with α2,3-SiaT. The sulfate appears to significantly interfere with the transfer of sialic acid from CMP−NeuAc to the glycopeptide substrate, as no sialylated product was isolated. Whether sialyltransferases from other sources can effectively transfer NeuAc to the glycosulfopeptide is a topic of present investigation.

Current results indicate that sulfation on tyrosine can regulate the activity of the glycosyltransferases required for the synthesis of sLe^x attached to PSGL-1. Work is in progress to investigate the origin of these reactivity differences, and especially possible conformational changes of the sulfated PSGL-1 glycopeptide upon galactosylation. In any case, tyrosine sulfation may play an important regulatory role in carbohydrate-mediated receptor recognition. Although the order in which PSGL-1 is posttranslationally modified is currently unknown, previous studies have shown that both the glycosylated and nonglycosylated PSGL-1 sequences serve as substrates for the sulfotransferase.[3] Together with this study, these results suggest that enzymatic sulfation can occur at an early biosynthetic stage to prevent the formation of the sugar ligand, or alternatively, at the final stage to form the tight-binding glyco-sulfo-heterodimer of PSGL-1.

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Supporting Information Available: Experimental procedures and characterization data for **3**, **4**, **7, 8a**, **8b**, **10b**, and **11** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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