Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 1017–1025

BIOORGANIC & MEDICINAL CHEMISTRY

Chemoenzymatic Synthesis of PSGL-1 Glycopeptides: Sulfation on Tyrosine Affects Glycosyltransferase-Catalyzed Synthesis of the *O*-Glycan

Kathryn M. Koeller, Mark E. B. Smith and Chi-Huey Wong*

Department of Chemistry, The Scripps Research Institute and Skaggs Institute for Chemical Biology, 10550 N. Torrey Pines Road, La Jolla, CA, 92037, USA

Received 27 October 1999; accepted 20 December 1999

Abstract—PSGL-1 is the primary glycoprotein ligand for P-selectin during the inflammatory response. Interestingly, the N-terminal sequence, containing both a site of tyrosine sulfation and an *O*-glycan, has been shown to bind to P-selectin with an affinity similar to full-length PSGL-1. To further characterize this system, the synthesis of glycopeptides from PSGL-1 was undertaken. The synthesis involved both solution- and solid-phase synthesis, as well as enzymatic transformations. During the synthesis, notable reactivity differences of the glycosyltransferases toward sulfated and unsulfated versions of the same glycopeptides were observed.

Introduction

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 ~5 orders of magnitude tighter than the unsulfated sequence.[3]

During protein biosynthesis, modifications such as *O*-linked glycosylation and sulfation occur within the Golgi apparatus. Although the order in which a given protein is decorated remains largely unknown, it has generally been reported that sulfation occurs as the final modification before exit from the Golgi.[4] In support of this idea, Cummings et al. have shown that tyrosylprotein sulfotransferase (TPST) can catalyze sulfation of the native glycosylated PSGL-1 N-terminal peptide, which contains a complete core 2 branched heptasaccharide.[3] However, it had been shown previously that TPST also accepts the non-glycosylated PSGL-1 sequence as a substrate.[5] Although it appears that TPST activity does not rely on the presence of an *O*-linked glycan, no previous studies have investigated the effect of sulfation on glycosyltransferase activity. Unlike phosphorylation, sulfation is most often accepted to be an irreversible protein modification in mammalian systems, as an appropriate protein sulfatase activity has not been identified.[4] It is, therefore, not clear whether the relationship between sulfation and glycosylation on PSGL-1 is of an on/off nature (analogous to phosphorylation), or whether it is associated with the construction of a high versus low affinity P-selectin ligand.

To further examine PSGL-1/P-selectin recognition, as well as to investigate PSGL-1 biosynthesis, a glycopeptide from the PSGL-1 N-terminus was selected as a synthetic target (Fig. 1b). Structures of the natural *O*-linked glycans from PSGL-1 have recently been elucidated, and contain a terminal sialyl Lewis x (sLe^x) tetrasaccharide extended off a core 2 glycan (Fig. 1a).[6] The target structure represents a minimal sequence containing tyrosine and the glycosylated threonine residue, corresponding to amino acid residues 10–17 of the mature PSGL-1 protein. The synthetic strategy combined chemical methods and solid-phase peptide synthesis to arrive at a disaccharide-linked octapeptide. Utilizing this strategy, access to both the sulfated and unsulfated disaccharide-linked peptides was possible. Glycosyltransferase-catalyzed elaboration of the glycan portion was then studied with respect to either the sulfated or unsulfated forms of the glycopeptides.[7] Results

*Corresponding author. Tel.: +1-619-784-2487; fax: +1-619-784-2409; e-mail: wong@scripps.edu

0968-0896/00/$ - see front matter
PII: S0968-0896(00)00041-9

indicate that sulfation on tyrosine at its semi-remote position within the peptide influences the reactivity of the glycosyltransferases responsible for the synthesis of sLe^x on the attached *O*-glycan.

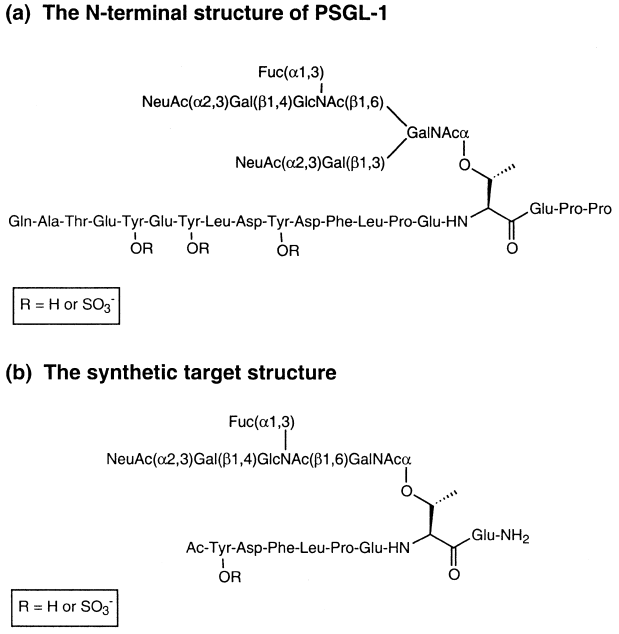
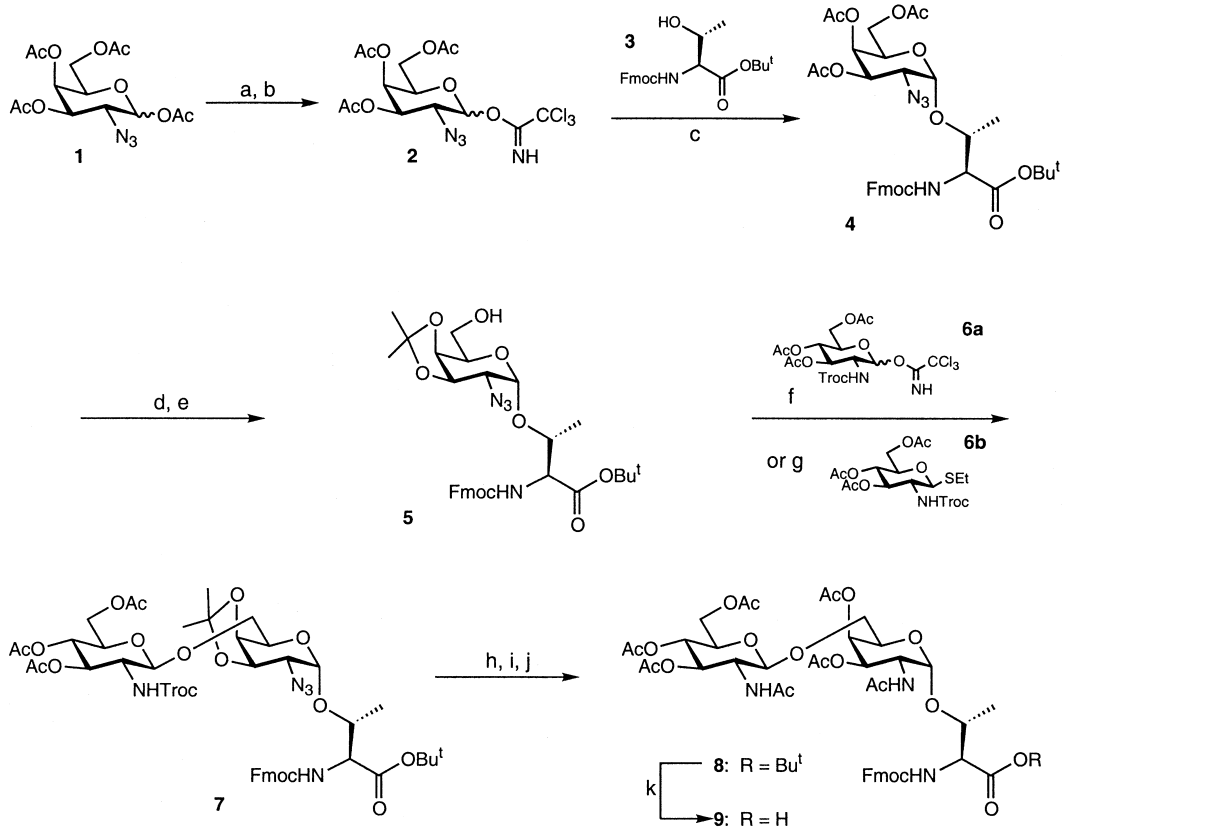


Figure 1. (a) The N-terminal structure of PSGL-1. (b) The synthetic target structure.

Results and Discussion

Initially, a glycosylated threonine residue was required for use in solid-phase peptide synthesis. The strategy employed to obtain this construct is outlined in Scheme 1. Anomeric deacetylation of **1**[8] was followed by conversion to trichloroacetimidate **2**,[9] which was isolated as a mixture of anomers (α:β, 2:3). Treatment of **2** (α:β, 2:3) and **3**[10] with TMSOTf as activator in a dichloromethane:diethyl ether mixture (1:1) at −30 °C gave conjugate **4**[11] in excellent yield as predominantly the α-anomer (α:β, 6:1). Complete reaction at low temperature prevented any loss of the *tert*-butyl ester upon addition of TMSOTf. The reaction was quenched with diethylisopropylamine to avoid loss of the Fmoc group which was observed when triethylamine was utilized as the quenching reagent.

Deacetylation of **4** was then achieved with sodium methoxide in methanol. The pH was maintained at less than 8.5 to prevent Fmoc cleavage. Conversion to 3,4-isopropylidene **5** has been reported by Paulsen et al.[12] Attempts to repeat this procedure failed, due to precipitation of the 4,6-isopropylidene from solution during the course of the reaction. However, it was observed that using a mixed solvent of dichloromethane and 2,2-dimethoxypropane (1:1) was more beneficial. In this case, the 4,6-isopropylidene was again the predominant product early in the reaction, but did not precipitate.



Scheme 1. Synthesis of the glyco-threonine building block. Reagents and conditions: (a) H_2NNH_2-HOAc, DMF; (b) Cl_3CCN, 4Å MS, Cs_2CO_3, CH_2Cl_2, 76% in 2 steps; (c) **3**, TMSOTf, CH_2Cl_2/Et_2O, −30 °C, 91%; (d) NaOMe, MeOH; (e) 2,2-DMP, TsOH, 48% in 2 steps; (f) **6a**, BF_3-OEt_2, CH_2Cl_2, −30 °C, 100%; (g) **6b**, DMTST, CH_2Cl_2, 4Å MS, 0 °C, 75%; (h) AcOH/H_2O; (i) Ac_2O/pyr; (j) Zn, Ac_2O, 80% in 3 steps; (k) TFA/H_2O, 100%.

Over time, equilibration to 3,4-isopropylidene **5** occurred, and this intermediate was isolated in good yield.

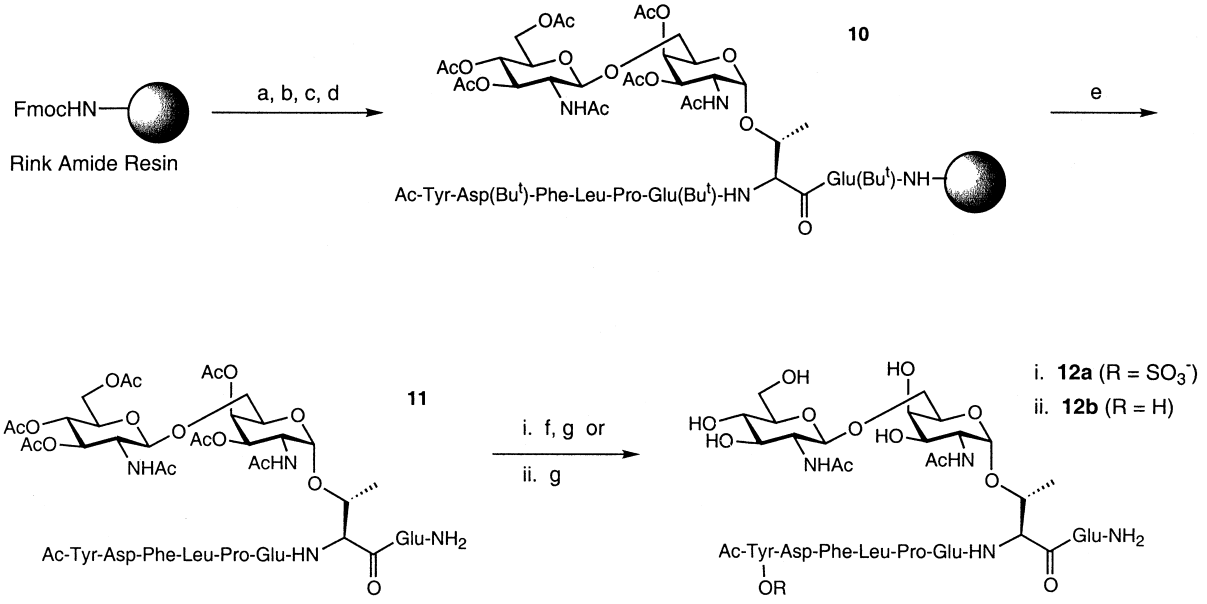
Formation of disaccharide-threonine conjugate **7** was then successfully achieved by using two separate glycosylation protocols. Combination of acceptor **5** and trichloroacetimidate donor **6a**[13] in dichloromethane at $-30\,^{\circ}C$ with BF_3-OEt_2 activation gave desired conjugate **7**. Alternatively, **7** was formed by reaction of **5** with thioglycoside donor **6b**[10] in dichloromethane at $0\,^{\circ}C$ using DMTST as the activating agent.[14] Treatment of **7** with AcOH:H_2O (4:1) at $45\,^{\circ}C$ then removed the isopropylidene group. Subsequent acetylation and conversion of the amino functionalities yielded intermediate **8**. Transformation of **8** to building block **9**, required for solid-phase peptide synthesis, was quantitatively performed by cleavage of the *tert*-butyl ester in a mixture of TFA:H_2O (95:5).

Building block **9** was initially incorporated into glycopeptide sequences on two different solid supports. The sequences were prepared to ascertain whether tyrosine sulfation could be best achieved during or post-SPPS. The sequence incorporating commercially available Fmoc-Tyr(OSO_3^-)-OH on solid phase was synthesized using a hyper acid labile Sieber Amide modified resin. Synthesis and cleavage from the resin proceeded without difficulty. However, all attempts to remove the *t*Bu protecting groups also resulted in cleavage of the sulfate ester.[15] As such, the sulfated sequence was never isolated in higher than trace yields.

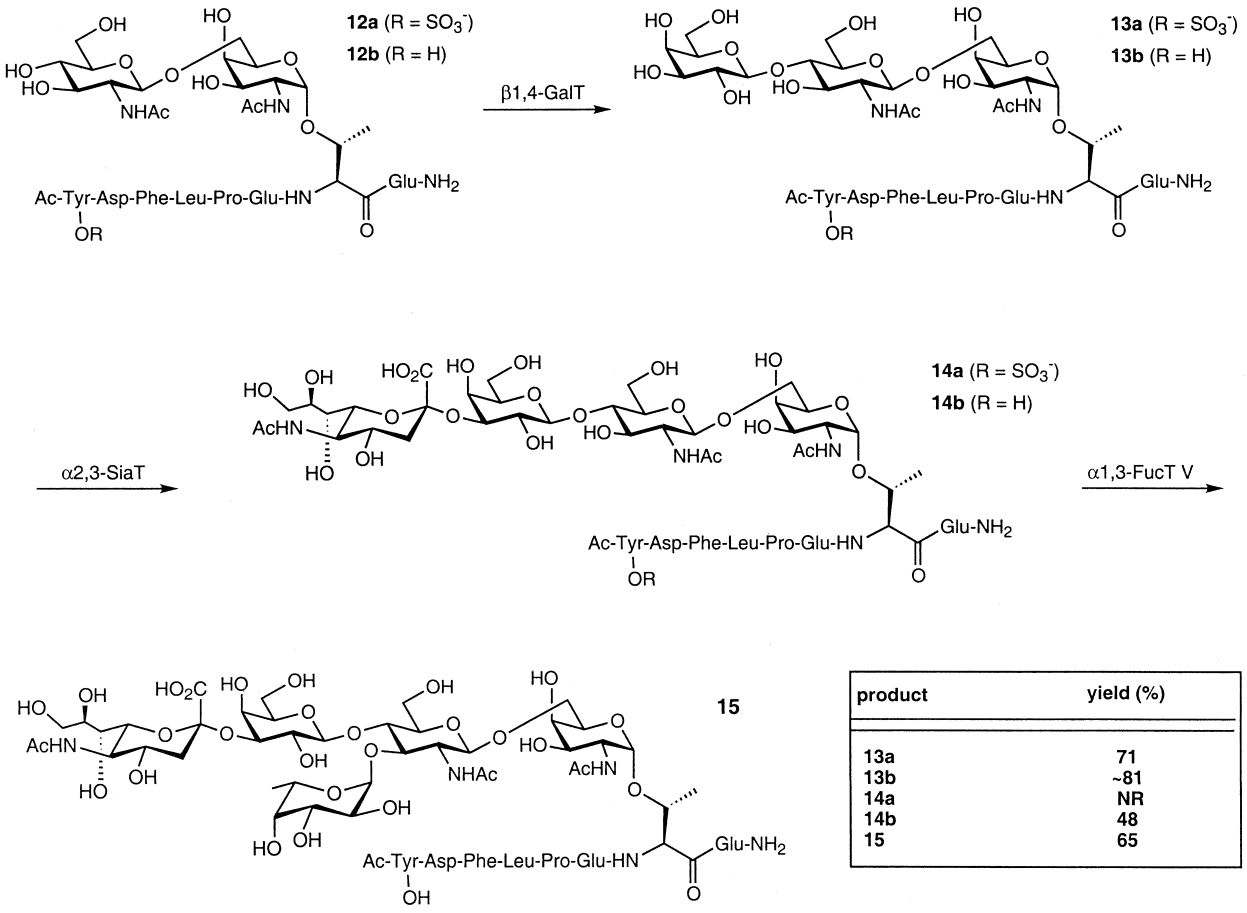
In contrast, non-sulfated sequence **10** was generated using a Rink Amide modified resin (Scheme 2). Following N-terminal acetylation, **10** was treated with 95% TFA, H_2O, and ethane dithiol as a scavenger. These conditions caused simultaneous liberation of the sequence from the resin as the C-terminal 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 **11**.

In order to sulfate tyrosine within the glycopeptide sequence, **11** was treated with sulfur trioxide-pyridine. An efficient work-up method was then sought, as attempts to repeat published procedures largely resulted in the isolation of the unsulfated starting material.[16] Fortunately, it was found that quenching the sulfating reagent with methanol, followed immediately by silica gel chromatography was an optimal work-up procedure.[17] This allowed the sulfated sequence to be isolated in high yield with minimal observed cleavage. Saponification of the acetate esters then gave deprotected glycopeptide **12a** for the subsequent glycosyltransferase-catalyzed glycosylations. Similarly, **11** was treated with sodium hydroxide in methanol to provide unsulfated **12b**. Thus, the procedure by which the glycopeptide was sulfated post-SPPS was optimal for synthesis of the desired sequence.

Glycosyltransferase-catalyzed glycosylations of the glycopeptides were then investigated (Scheme 3). Galactose was appended in a β1,4 linkage to GlcNAc in unsulfated sequence **12b** by treatment with β1,4-galactosyltransferase (β1,4-GalT) in the presence of donor substrate UDP-Gal to give **13b**. Similarly, addition of sialic acid was achieved by incubation of **13b** with α2,3-sialyltransferase (α2,3-SiaT) in the presence of CMP-NeuAc to obtain **14b**. Finally, the sLe^x structure was completed by the addition of branch point carbohydrate fucose in an α1,3 linkage to GlcNAc. This was accomplished by incubation of **14b** and GDP-Fuc with α1,3-fucosyltransferase V (α1,3-FucT V) to arrive at **15**. Alkaline phosphatase was added to all transferase reactions to reduce product inhibition.



Scheme 2. Solid-phase synthesis of glycopeptides. Reagents and conditions: (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% in 2 steps or (g) alone NaOH/MeOH 89%.



product	yield (%)
13a	71
13b	~81
14a	NR
14b	48
15	65

Scheme 3. Differences in glycosyltransferase reactivity with sulfated versus unsulfated glycopeptides.

In contrast, for sulfated glycopeptide sequence **12a**, the enzymatic reactions did not proceed as smoothly as for the unsulfated substrate. The β1,4-GalT reaction proceeded slowly, resulting in the difficult isolation of product **13a**. Proteolytic cleavage was also encountered at long reaction times. However, if the reaction was allowed to proceed until all starting material had been consumed, isolation of **13a** was possible. An even more pronounced variation of glycosyltransferase activity was observed with α2,3-SiaT. In this case, no formation of product **14a** was observed. It appears, therefore, that sulfation on tyrosine significantly interferes with the sialyltransferase reaction. This was unexpected, as the sulfate is not in close proximity to the glycan portion of the molecule.

To further characterize these observed differences, a kinetic study of the enzymatic glycosylations by β1,4-GalT was carried out. An assay that allowed the determination of kinetic parameters for the β1,4-GalT reaction was developed (Fig. 2, Table 1). Notably, the kinetic parameters determined from initial reaction rates differ for **12a** and **12b**. By examination of K_m, it appears that sulfation on tyrosine interferes with the binding of the substrate to the enzyme in some manner. However, this is compensated for by V_{max}, resulting in an overall value of k_{cat}/K_m that differs by ~3 fold for the sulfated and non-sulfated substrates.

In order to further investigate the cause of reactivity differences between the substrates, a structural study was undertaken.[18] Results indicate that there are no significant structural changes between glycopeptide sequences **12a** and **12b**. The 2-dimensional ROESY spectra show nearly identical overlap. It is assumed, then, that the differences in reactivity towards 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 peptides was with α2,3-SiaT. The sulfate appears to interfere with the transfer of sialic acid from CMP-NeuAc (Fig. 3).

Conclusion

A combination of chemical and solid-phase synthesis provided access to sulfated and unsulfated peptides from the N-terminus of PSGL-1. Glycosyltransferase-catalyzed additions of galactose, sialic acid, and fucose to the glycopeptides were then studied. Current results indicate that sulfation on tyrosine has the potential to regulate the activity of the glycosyltransferases required for the synthesis of sLe^x attached to PSGL-1. This was unexpected, as the tyrosine residue does not appear to make contacts to the glycan portion of the molecule. Work is in progress to investigate any possible conformational

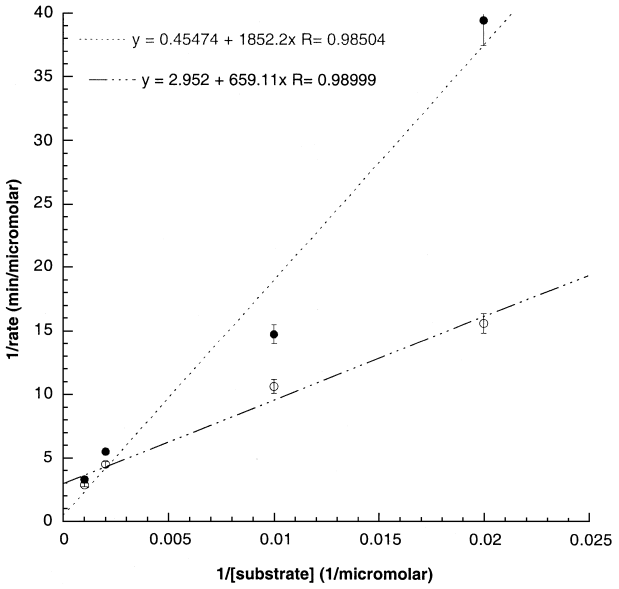


Figure 2. Lineweaver–Burk plot of β1,4-galactosyltransferase reactions: ●, for **12a** (Tyr-OSO_3^-); ○, for **12b** (Tyr-OH).

Table 1. Kinetic parameters for the β1,4-GalT reaction

Substrate	K_m (μM)	V_{max} (μM/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Sulfated **12a**	4073	2.190	0.2740	6.72×10^{-5}
Unsulfated **12b**	223	0.339	0.0425	1.90×10^{-4}

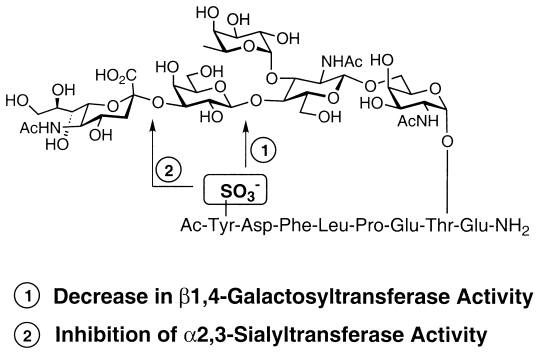


Figure 3. Effect of sulfation on enzymatic glycosylation.

change of the sulfated PSGL-1 glycopeptide upon galactosylation, and to study the glycosyltransferases from humans and other species. In any case, this study may have relevant implications in carbohydrate-mediated receptor recognition, as the sulfated PSGL-1 glycopeptide binds to P-selectin approximately five orders of magnitude more strongly than does the non-sulfated ligand.[3] Along with previous studies, the results discussed herein suggest that sulfation and glycosylation have the potential to act as a regulatory mechanism for the construction of PSGL-1 as either a low or high affinity P-selectin ligand.

Experimental

General

All non-aqueous reactions were run in oven-dried glassware under an inert Ar atmosphere. Reactions were monitored by thin-layer chromatography (TLC) utilizing *p*-anisaldehyde or cerium molybdate stain as the developing reagent. Unless otherwise noted, reagents and materials were obtained from commercial sources and used as provided. All non-aqueous solvents were distilled prior to use. 1D ^1H and ^{13}C NMR spectra were recorded on Bruker AMX-400, AMX-500, DRX-500, or DRX-600 MHz spectrometers, and were referenced to residual solvent peaks ($CDCl_3$: ^1H δ 7.24, ^{13}C δ 77.0; CD_3OD: ^1H δ 3.30, ^{13}C δ 49.0; D_2O: ^1H δ 4.76). 2D COSY, ROESY, and TOCSY spectra were recorded on a Bruker DRX-600 or DRX-500 spectrometer equipped with either a broadband or inverse probe for greater sensitivity. Solid-phase resins and Fmoc-amino acids were purchased from Novabiochem. UDP-Gal, CMP-NeuAc, GDP-Fucose, β1,4-galactosyltransferase from bovine, α2,3-sialyltransferase from rat liver, and α1,3-fucosyltransferase V from human were purchased from Calbiochem.

1,3,4,6-Tetra-*O*-acetyl-2-azido-2-deoxy-D-galactopyranoside (1). A solution of NaN_3 (8.94 g, 137 mmol) in H_2O (22 mL) was cooled to 0 °C and CH_2Cl_2 (36 mL) added. The mixture was stirred vigorously and Tf_2O (4.68 mL, 27.90 mmol) added over a period of 5 min, and the reaction stirred at 0 °C for 2 h. The organic phase was then separated and the aqueous phase washed twice with CH_2Cl_2. The combined organic phases were washed once with saturated Na_2CO_3 (sat) (75 mL). The total volume of TfN_3 in CH_2Cl_2 was 75 mL, and this reagent solution was used without further purification. $GalNH_2.HCl$ (3.0 g, 13.95 mmol) was dissolved in H_2O (45 mL) and treated with K_2CO_3 (2.88 g, 20.93 mmol) and $CuSO_4$ (21 mg, 132 μmol). MeOH (90 mL) was added followed by careful addition of the freshly prepared TfN_3 solution. More MeOH was added to achieve a homogeneous solution and the reaction was then stirred for 6 h at rt. The solvent was then removed under vacuum and the residue redissolved in pyridine (75 mL). After cooling to 0 °C, Ac_2O (45 mL) was added and the mixture stirred at rt for 2 h. Excess reagents were removed under vacuum and the residue dissolved in EtOAc (300 mL) before washing with saturated $CuSO_4$ (aq) (3×200 mL) and $NaHCO_3$ (aq) (200 mL). After drying over $MgSO_4$, the solvent was removed under vacuum to give a residue which was purified by flash chromatography (EtOAc:hexanes (3:7)) to give the title compound as a colorless syrup (5.20 g, 97%). Spectral analysis in agreement with ref 6.

***O*-(3,4,6-Tri-*O*-acetyl-2-azido-2-deoxy-D-galactopyranoside) trichloroacetimidate (2).** Azide **1** (8.97 g, 24.05 mmol) was dissolved in DMF (50 mL) and hydrazine acetate (2.21 g, 24.05 mmol) added. After 2 h, the reaction was diluted with EtOAc (300 mL) and washed once with H_2O (300 mL). After drying over $MgSO_4$, the solvent was removed and the residue purified by flash chromatography (EtOAc:hexanes (35:65)) to yield the

hemiacetal product as a white foam (7.90 g, 99%). (Spectral analysis in agreement with ref. 7). Then freshly powdered K_2CO_3 (1.67 g, 12.01 mmol), that had been flame-dried under vacuum immediately prior to the experiment, was added to a vigorously stirred solution of hemiacetal (5.00 g, 15.11 mmol) and fresh trichloroacetonitrile (3.33 mL, 33.23 mmol) in CH_2Cl_2 (70 mL). After 6 h (reaction not complete), the mixture was filtered through celite, concentrated under vacuum and the residue purified by flash chromatography (EtOAc: hexanes (3:7) to give the title compound as a white foam (5.55 g, 77%) (α:β, 1:2). Spectral analysis in agreement with ref 7.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-*O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine *tert*-butyl ester (4). Trichloroacetimidate **2** (3.42 g, 7.20 mmol) and threonine derivative **3** (1.91 g, 4.80 mmol) were combined and dried overnight under vacuum, then dissolved in a mixture of dichloromethane:diethyl ether (1:1) (50 mL). After cooling to −30 °C, TMSOTf (144 μL, 0.72 mmol) was added dropwise. The mixture was stirred at −30 °C for 30 min after which the reaction was quenched with Hunig's base (125 μL, 0.72 mmol). The solution was diluted with dichloromethane (100 mL) and washed once with 0.1 M HCl (100 mL). After drying over $MgSO_4$, the solution was concentrated and the crude product purified by flash chromatography (EtOAc:hexanes (3:7), to give the title compound as a white foam (3.10 g, 91%) (α:β, 6:1). Spectral analysis in agreement with ref 9.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-*O*-(2-azido-2-deoxy-3,4-*O*-isopropylidene-α-D-galactopyranosyl)-L-threonine *tert*-butyl ester (5). NaOMe (1% solution in MeOH) was added dropwise to a stirred solution of conjugate **4** (3.10 g, 4.37 mmol) at a rate such that pH was always between 8 and 8.5. After 6 h, deacetylation was complete and the reaction was quenched by the addition of AcOH until the pH was 6. The solvent was removed under vacuum to yield a residue that was purified using flash chromatography (EtOAc:hexanes, (9:1), to yield the triol as a white foam (1.71 g, 67%). (Spectral analysis in agreement with ref 9.) Triol (2.41 g, 4.13 g) was then dissolved in a mixture of dimethoxypropane: CH_2Cl_2 (1:1, 60 mL) and TsOH (cat.) added. The mixture was stirred for 12 h, diluted with CH_2Cl_2 (150 mL) and washed once with saturated $NaHCO_3$ (aq) (100 mL). After drying over $MgSO_4$, the solvent was removed under vacuum and the residue purified using flash chromatography (ether:hexanes (7:3), to give the title compound as a white foam (1.85 g, 72%). Spectral analysis in agreement with ref 10.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-*O*-{*O*-[3′,4′,6′-tri-*O*-acetyl-2′-deoxy-2′-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-(1′,6)-2-azido-2-deoxy-3,4-*O*-isopropylidene-α-D-galactopyranosyl}-L-threonine *tert*-butyl ester (7). Method A. Trichloroacetimidate **6a** (1.39 g, 2.36 mmol) and alcohol **5** (0.98 g, 1.57 mmol) were combined and dried overnight under vacuum, then dissolved in anhydrous dichloromethane (20 mL). After cooling to −30 °C, $BF_3.OEt_2$ (29 μL, 0.24 mmol) was added dropwise. Reaction was complete within 5 min and quenched by the addition of Hunig's base (42 μL, 0.24 mmol). The mixture was diluted with dichloromethane (100 mL) and washed once with 0.1 M HCl (aq) (100 mL). After drying over $MgSO_4$, the solution was concentrated and the crude product purified by flash chromatography (2:3 EtOAc:hexane) to give the title compound as a white foam in quantitative yield.

Method B. 4Å molecular sieves (1 g) was added to a stirred solution of donor **6b** (940 mg, 1.76 mmol) and acceptor **1** (880 mg, 1.41 mmol) in CH_2Cl_2 (5 mL). The suspension was cooled to 0 °C and freshly prepared DMTST (1.76 ml, 1 M stock solution in CH_2Cl_2) was added. The reaction was complete after 15 min and the reaction was quenched on addition of diisopropylethylamine (613 μL, 3.52 mmol), diluted with CH_2Cl_2 (100 mL) and washed once with 0.1 M HCl (100 mL). The acid wash dissolved the molecular sieves used in the reaction. After drying over $MgSO_4$, the solution was concentrated under vacuum to give a residue which was purified by flash chromatography (2:3 EtOAc:hexane) to give the title compound as a white foam (1.15 g, 75%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (2H, d, J = 7.5 Hz), 7.63 (2H, d, J = 7.5 Hz), 7.41 (2H, app t, J 7.5), 7.32 (2H, app t, J = 7.5 Hz), 5.58 (1H, d, J = 9 Hz), 5.50 (1H, d, J = 9 Hz), 5.31 (1H, t, J = 9.5 Hz), 5.07 (1H, t, J = 9.5 Hz), 4.95 (1H, d, J = 3.5 Hz), 4.88 (1H, d, J = 12.5 Hz), 4.76 (1H, d, J = 8.5 Hz), 4.52 (1H, d, J = 12 Hz.), 4.31 (1H, dd, J = 10, 7 Hz), 4.36 (2H, dd, J = 8, 5.5 Hz), 4.26 (4H, m), 4.15 (2H, m) 4.11 (1H, dd, J = 3.5 Hz), 4.08 (1H, dd, J = 11, 2.5 Hz), 3.80 (1H, dd, J = 11, 8.5 Hz), 3.69 (1H, m), 3.59 (1H, m), 3.36 1H, dd, J = 8, 3.5 Hz), 2.08, 2.02, 1.96 (9H, 3s), 1.50 (12H, bs), 1.35 (3H, s), 1.32 (3H, d, J = 6.5 Hz); ESIMS calcd for $C_{47}H_{58}N_5O_{18}Cl_3$ 1086/1088/1090, found 1086/1088/1090.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-*O*-{*O*-[2′-acetamido-3′,4′,6′-tri-*O*-acetyl-2′-deoxy-β-D-glucopyranosyl]-(1′,6)-2-acetamido-3,4-di-*O*-acetyl-2-deoxy-α-D-galactopyranosyl}-L-threonine *tert*-butyl ester (8). Acetonide **7** (4.33 g, 3.99 mmol) was dissolved in acetic acid:water (4:1, 100 mL) and stirred at 45 °C for 8 h. Addition of toluene and concentration under vacuum gave a residue which was dissolved in pyridine (50 mL). The solution was cooled to 0 °C and acetic anhydride (25 mL) added. After 5 min the ice bath was removed and the reaction stirred at room temperature for 3 h. Addition of toluene and concentration under vacuum gave a further residue which was dissolved in THF:AcOH:Ac_2O (3:2:1). Zinc dust (3 g), activated by stirring in a 2% solution of $CuSO_4$ (aq) (100 mL), was added to the THF mixture while still moist. After 30 min reaction was complete and the mixture was filtered through celite. Concentration of the solution gave a white solid which was redissolved in CH_2Cl_2 (100 mL) before washing with 0.1 M HCl (aq). After drying over $MgSO_4$, the solution was concentrated and the crude product purified by flash chromatography (neat EtOAc) to give the title compound as a white foam (3.10 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (2H, d, J = 7.5 Hz), 7.65 (2H, t, J = 7 Hz), 7.41 (2H, t, J = 7.5 Hz), 7.33 (2H, t, J = 7.5 Hz),

6.03 (1H, d, $J=9.5$ Hz), 5.73 (1H, d, $J=9$ Hz), 5.57 (1H, d, $J=8$ Hz), 5.9–5.33 (2H, m), 5.07 (1H, dd, $J=3$ Hz), 5.01 (1H, t, $J=9.5$ Hz), 4.88 (1H, d, $J=3$ Hz), 4.80 (1H, d, $J=8$ Hz), 4.58 (1H, dd, $J=10$, 2.5 Hz), 4.47–4.44 (2H, m), 4.32–4.25 (3H, m), 4.23–4.18 (2H, m), 4.06 (1H, d, $J=12$ Hz), 3.79 (1H, dd, $J=10.5$, 5 Hz), 3.69–3.66 (1H, m), 3.61 (2H, ABQ, $J=7.5$ Hz), 2.16 (3H, s), 2.08 (3H, s), 2.00–1.98 (12H, m), 1.94 (3H, s), 1.47 (9H, s), 1.33 (3H, d, $J=6.5$ Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8, 170.64, 170.58, 170.35, 170.3, 170.0, 169.4, 156.5, 143.9, 143.7, 141.3, 127.11, 127.08, 125.1, 120.01, 119.99, 100.8, 100.2, 99.4, 83.1, 76.4, 71.94, 71.89, 68.9, 68.5, 68.3, 67.7, 67.4, 67.3, 61.9, 58.8, 55.1, 47.4, 47.1, 28.1, 23.3, 23.2, 20.74, 20.68, 20.6, 20.5, 18.4. HRMS: calcd for $C_{71}H_{84}N_2O_{12}Cs$ 1146; found 1146.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-*O*-{*O*-[2′-acetamido-3′,4′,6′-tri-*O*-acetyl-2′-deoxy-β-D-glucopyranosyl]-(1′,6)-2-acetamido-3,4-di-*O*-acetyl-2-deoxy-α-D-galactopyranosyl}-L-threonine (9). Ester **8** (269 mg, 0.27 mmol) was dissolved in TFA:H_2O (95:5, 4 mL) and stirred at room temperature for 2 h. Addition of toluene and concentration under vacuum gave the title compound as a white solid in quantitative yield.

Ac-Tyr-Asp-Phe-Leu-Pro-Glu-Thr(Ac_3GluNAcβ(1,6)Ac_2 GalNAcα)-Glu-NH_2 (11). Pretreatment of rink amide resin. Rink amide resin (loading 0.573 mmol/g, 440 mg) was shaken in DMF:morpholine (1:1, 10 mL) for 50 min, filtered and washed with DMF. **Fmoc removals**—resin bound peptide intermediates were shaken in DMF:morpholine (1:1, 10 mL) for 50 min, filtered and washed with DMF. **Couplings**—the resin was treated with a 5-fold excess of the Fmoc-amino acid (except for building block **9** which was employed in a 1.1-fold excess) in a 0.08 M solution in DMF, which contained 1.5 equiv of HOBt, 2.0 equiv of NMM and 1.0 equiv of HBTU per equivalent of Fmoc-amino acid. The mixture was shaken and coupling times were 5 h except for that of the building block **9** and the subsequent 3 couplings each of which were of an 18 h duration. The excess reactants were then removed by filtration and the support washed with DMF. **Capping and subsequent acetylation of the N-terminus**—the resin was suspended in Py/Ac_2O (3:1, 8 mL) and shaken for 10 min. The mixture was then filtered and the resin washed with DMF. **Peptide cleavage**—following acetylation of the N-terminus, the resin was washed with CH_2Cl_2 and then methanol, before drying under vacuum. The resin was then suspended in TFA:H_2O:EDT (95:2.5:2.5, 10 mL) and shaken for 4 h. The mixture was filtered and the resin washed with AcOH and DMF. The solution obtained was concentrated to leave a solution in DMF, and crude peptide obtained on ether precipitation. The precipitate was redissolved in MeOH and reprecipitated with ether to give a fluffy, white precipitate which was isolated by gravity filtration. The white solid obtained was dissolved in H_2O and lyophilized to give a white powder (346 mg). The partially purified glycopeptide was further purified in 30 mg portions using preparative RP-HPLC (C18; 0.1 M NH_4OAc/MeCN; 20–40% MeCN over 30 min; R_t 19.47 min) to give 25 mg (per portion) of the title sequence. Yield: 68% (based on initial loading of resin with amino acid). ^{1}NMR (500 MHz, CD_3OD) δ 7.27–7.15 (5H, m), 7.02 (1H, d, $J=9$ Hz), 6.69 (2H, d, $J=9$ Hz), 5.39 (1H, d, $J=3$ Hz), 4.64 (3H, m), 4.58 (2H, dd, $J=9$, 5 Hz), 4.52 (1H, d, $J=2$ Hz), 4.50 (2H, t, $J=6$ Hz), 4.45–4.34 (8H, m), 4.32–4.25 (3H, m), 4.20 (1H, dd, $J=6$, 2 Hz), 4.08 (1H, dd, $J=12$, 2 Hz), 3.84 (2H, dd, $J=10$, 9 Hz), 3.78–3.76 (4H, m), 3.61–3.57 (3H, m), 3.22 (1H, dd, $J=14$, 4 Hz), 3.00–2.94 (3H, m), 2.74 (1H, dd, $J=14$, 9 Hz), 2.59 (1H, dd, $J=16$, 6 Hz), 2.50 (1H, dd, $J=16$, 7 Hz), 2.41–2.32 (6H, m), 2.10 (3H, s), 2.04 (3H, s), 2.03 (3H, s), 1.99 (3H, s), 1.96 (3H, s), 1.92 (3H, s), 1.91 (3H, s), 1.90 (3H, s), 1.71–1.66 (2H, m), 1.57–1.54 (1H, m), 1.28 (3H, d, $J=6$ Hz), 0.95 (3H, d, $J=4$ Hz), 0.94 (3H, d, $J=4$ Hz); ES-MS (neg) calcd for $C_{75}H_{103}N_{11}O_{32}$ [M-H]$^-$1669.7, found 1670.

Ac-Tyr(SO_3^-)-Asp-Phe-Leu-Pro-Glu-Thr(GlcNAc(β1,6) GalNAcα)-Glu-NH_2 (12a). Compound **11** (29.0 mg, 17.4 μmol) was dissolved in dry pyridine (870 μL). Sulfur trioxide–pyridine complex was added, and the reaction stirred at ambient temperature for 5 h. MeOH (~1 mL) was added to quench the sulfating reagent. After stirring 5 min, the solution became clear and solvent was evaporated. The residue was immediately purified by silica gel chromatography (5:4:1 $CHCl_3$:MeOH:H_2O, $CHCl_3$ neutralized by passing through a plug of basic Al_2O_3). Fractions were collected, concentrated and used without further purification in the next reaction.

The solid residue was dissolved in MeOH (174 μL), and 1 M NaOH added until the solution had been adjusted to pH 8. The reaction was stirred at ambient temperature for 1.5 h, and solvent was evaporated. The resulting residue was purified by passing through a Sephadex G-25 size exclusion column, eluting with H_2O. Fractions containing product were lyophilized to yield **12a** (21.8 mg, 81%, 2 steps). ^{1}H NMR (600 MHz, D_2O) δ 7.35–7.34 (2H, m), 7.29–7.21 (7H, m), 4.86 (1H, d, $J=4$ Hz, GalNAc H-1), 4.62–4.52 (6H, m, Thr H-α, Leu H-α, Asp H-α, Tyr H-α, Phe H-α, GlcNAc H-1), 4.42–4.37 (2H, m, Glu H-α, Pro H-α), 4.26–4.22 (2H, m, Thr H-β, Glu H-α), 4.10–4.04 (3H, m, GalNAc H-2, H-4, GlcNAc H-5), 3.94–3.88 (3H, m, GlcNAc H-6_a, GalNAc H-6_a,H-6_b), 3.74–3.67 (4H, m, GlcNAc H-2, H-6_b, GalNAc H-3, Pro H-δ), 3.63–3.59 (1H, m, Pro H-δ), 3.52–3.49 (1H, m, GlcNAc H-3), 3.46–3.39 (2H, m, GlcNAc H-4, H-5), 3.11–3.06 (2H, m, Phe H-β), 3.01 (1H, dd, $J=14$, 8 Hz, Tyr H-β), 2.84 (1H, dd, $J=14$, 9 Hz, Tyr H-β), 2.56 (1H, dd, $J=16$, 5 Hz, Asp H-β), 2.48 (1H, dd, $J=16$, 8 Hz, Asp H-β), 2.42–2.37 (1H, m, Pro H-β), 2.31–2.21 (5H, m, Pro H-β, Glu H-γ), 2.04 (3H, s, AcO), 2.03 (3H, s, AcO), 1.91 (3H, s, AcO), 2.08–1.91 (6H, m, Pro H-γ, Glu H-β), 1.58–1.51 (3H, m, Leu H-β, H-γ), 1.23 (3H, d, $J=6$ Hz, Thr H-γ), 0.91 (3H, d, $J=6$ Hz, Leu H-δ), 0.88 (3H, d, $J=6$ Hz, Leu H-δ); ^{13}C NMR (100 MHz, D_2O/CD_3OD) d 151.06, 137.07, 135.31, 131.37, 130.20, 129.69, 128.16, 122.51, 102.17, 100.06, 77.89, 76.92, 75.09, 70.91, 70.70, 70.43, 69.71, 68.80, 61.76, 61.10, 58.09, 56.44, 55.76, 55.52, 54.38, 50.82, 50.61, 40.27, 37.79, 37.32, 30.39, 29.03, 25.58, 25.12, 23.44, 23.34, 23.27, 22.59, 21.78, 19.48; ES–MS (neg) calcd for $C_{65}H_{92}N_{11}O_{30}S$ [M−H]$^-$ 1538.6, found 1539.

Ac-Tyr-Asp-Phe-Leu-Pro-Glu-Thr(GlcNAc(β1,6)GalNAc α)-Glu-NH_2 (12b). Compound **11** (45.1 mg, 27.0 μmol) was dissolved in MeOH (270 μL). The pH was adjusted to ~8 with 1 M NaOH, and the reaction allowed to stir 4.5 h at ambient temperature. Solvent was evaporated, and the residue loaded onto a Sephadex G-25 size exclusion column, eluting with H_2O. Fractions containing product were lyophilized to give **12b** (35.1 mg, 89%). ^{1}H NMR (500 MHz, D_2O) δ 7.31–7.16 (5H, m, Phe aromatic H-2, H-3, H-4, H-5, H-6), 7.03 (2H, d, J = 8 Hz, Tyr aromatic H-2, H-6), 6.76 (2H, d, J = 8 Hz, Tyr aromatic H-3, H-5), 4.90 (1H, d, J = 4 Hz, GalNAc H-1), 4.53–4.47 (6H, m, Thr H-α, Leu H-α, Asp H-α, Tyr H-α, Phe H-α, GlcNAc H-1), 4.38–4.32 (2H, m, Glu H-α, Pro H-α), 4.23–4.17 (2H, m, Thr H-β, Glu H-α), 4.05–4.00 (3H, m, GalNAc H-2, H-4, GlcNAc H-5), 3.88–3.82 (3H, m, GlcNAc H-6_a, GalNAc H-6_a, H-6_b), 3.68–3.64 (4H, m, GlcNAc H-2, H-6_b, GalNAc H-3, Pro H-δ), 3.58–3.54 (1H, m, Pro H-δ), 3.45 (1H, t, J = 9 Hz, GlcNAc H-3), 3.38–3.35 (2H, m, GlcNAc H-4, H-5), 3.06–2.91 (3H, m, Phe H-β, Tyr H-β), 2.79–2.71 (1H, m, Tyr H-β), 2.52–2.40 (2H, m, Asp H-β), 2.30–2.21 (6H, m, Pro H-β, Glu H-γ), 1.99 (6H, s, AcO), 1.88 (3H, s, AcO), 2.08–1.88 (6H, m, Pro H-γ, Glu H-β), 1.54–1.45 (3H, m, Leu H-β, H-γ), 1.18 (3H, d, J = 6 Hz, Thr H-γ), 0.85 (3H, d, J = 5 Hz, Leu H-δ), 0.83 (3H, d, J = 5 Hz, Leu H-δ); ^{13}C NMR (100 MHz, D_2O) δ 155.36, 137.07, 131.48, 130.18, 129.69, 129.15, 128.14, 116.40, 102.17, 100.05, 76.91, 75.03, 70.90, 70.73, 70.46, 69.69, 61.75, 56.43, 56.16, 55.52, 54.42, 52.32, 48.79, 40.28, 39.13, 37.79, 37.11, 34.42, 30.38, 25.58, 25.10, 23.44, 23.31, 22.66, 21.80, 19.50; ES–MS (neg) calcd for $C_{65}H_{93}N_{11}O_{27}$ $[M-H]^-$ 1459, found 1459.

Ac-Tyr-Asp-Phe-Leu-Pro-Glu-Thr(Gal(β1,4)GlcNAc (β1,6)GalNAcα)-Glu-NH_2 (13b). Compound **12b** (6.0 mg, 4.1 μmol) and UDP-Gal (2.8 mg, 4.5 μmol) were dissolved in buffer (130 mM HEPES, pH 7.4, + 0.25% Triton X-100, 800 μL) containing a freshly prepared $MnCl_2$ solution (4.5 μmol). β1,4-GalT (60 μL, 41 mU) and alkaline phosphatase (0.4 μL, 400 mU) were added and the reaction shaken gently at ambient temperature for 2 days. The mixture was loaded onto a Biogel P-2 size exclusion column, eluting with water. The isolated product (5.4 mg, 81%) contained ~30% unreacted starting material. The mixture was carried on directly to the next transferase reaction without further purification. ES–MS (neg) calcd for $C_{71}H_{103}N_{11}O_{32}$ $[M-H]^-$ 1620.7, found 1621.

Ac-Tyr-Asp-Phe-Leu-Pro-Glu-Thr(NeuAc(α2,3)Gal(β1,4) GlcNAc(β1,6)GalNAcα)-Glu-NH_2 (14b). Compound **13b** (4.6 mg, 2.8 μmol), UDP-Gal (0.9 mg, 1.4 μmol), and CMP-NeuAc (2.0 mg, 3.1 μmol) were dissolved in buffer (130 mM HEPES, pH 7.4, + 0.25% Triton X-100, 560 μL) containing a freshly prepared $MnCl_2$ solution (5.7 μmol). β1,4-GalT (41 μL, 28 mU), α2,3-SiaT (27 μL, 28 mU), and alkaline phosphatase (0.3 μL, 300 mU) were added and the reaction shaken gently at ambient temperature for 2 days. The reaction was loaded onto a Biogel P-2 size exclusion column, eluting with water. Further purification was accomplished on a DEAE Sephadex A-25 anion exchange column (gradient 0–300 mM NH_4HCO_3), followed by an additional pass through Biogel P-2 for final desalting. This procedure gave the product **14b** (3.3 mg, 48%, 2 steps) as well as unreacted starting material **13b** (1.7 mg). ^{1}H NMR (500 MHz, D_2O) identifiable resonances δ 7.30–7.14 (5H, m, Phe aromatic), 7.00 (2H, d, J = 8 Hz, Tyr aromatic), 6.74 (2H, d, J = 8 Hz, Tyr aromatic), 2.65 (1H, dd, J = 12, 8 Hz, NeuAc H-3_{eq}), 1.96 (3H, s), 1.95 (3H, s), 1.94 (3H, s), 1.83 (3H, s), 1.72 (1H, t, J = 12 Hz, NeuAc H-3_{ax}), 1.15 (3H, d, J = 6 Hz, Thr -CH_3), 0.84 (3H, d, J = 6 Hz, Leu–CH_3), 0.81 (3H, d, J = 6 Hz, Leu–CH_3); ES-MS (neg) calcd for $C_{82}H_{120}N_{12}O_{40}$ $[M-H]^-$ 1911.8, found 1913.

Ac-Tyr-Asp-Phe-Leu-Pro-Glu-Thr-(NeuAc(α2,3)Gal(β1,4) [Fucα1,3]GlcNAc(β1,6)GalNAcα)-Glu-NH_2 (15). Compound **14b** (2.3 mg, 1.2 μmol) and GDP-Fuc (0.8 mg, 1.3 μmol) were dissolved in buffer (100 mM MES, pH 6.0, + 0.25% Triton X-100, 240 μL) containing a freshly prepared $MnCl_2$ solution (1.3 μmol). α1,3-FucT V (24 μL, 12 mU) and alkaline phosphatase (0.1 μL, 100 mU) were added and the reaction shaken gently at ambient temperature for 2 days. The reaction was loaded onto a Biogel P-2 size exclusion column, eluting with water. Further purification was accomplished on a DEAE Sephadex A-25 anion exchange column (gradient 0–500 mM NH_4HCO_3), followed by an additional pass through Biogel P-2 for final desalting. The product was then isolated by lyophilization to give **14b** (1.6 mg, 65%). ^{1}H NMR (500 MHz, D_2O) identifiable resonances δ 7.38–7.27 (5H, m, Phe aromatic), 7.11 (2H, d, J = 8 Hz, Tyr aromatic), 6.84 (2H, d, J = 8 Hz, Tyr aromatic), 5.10 (1H, d, J = 3 Hz, Fuc H-1), 2.78 (1H, dd, J = 12, 9 Hz, NeuAc H-3_{eq}), 2.05 (3H, s), 2.04 (6H, s), 1.95 (3H, s), 1.81 (1H, t, J = 12 Hz, NeuAc H-3_{ax}), 1.25 (3H, d, J = 5 Hz, Fuc H-6), 1.17 (3H, d, J = 6 Hz, Thr -CH_3), 0.93 (3H, d, J = 6 Hz, Leu -CH_3), 0.90 (3H, d, J = 6 Hz, Leu -CH_3); ES–MS (neg) calcd for $C_{88}H_{130}N_{12}O_{44}$ $[M-H]^-$ 2058, found 2059.

Ac-Tyr(OSO_3^-)-Asp-Phe-Leu-Pro-Glu-Thr(Gal(β1,4)Glc-NAc (β1,6)GalNAcα)-Glu-NH_2 (13a). Compound **12a** (3.5 mg, 2.3 μmol) and UDP-Gal (1.4 mg, 2.3 μmol) were dissolved in buffer (130 mM HEPES, pH 7.4, + 0.25% Triton X-100, 460 μL) containing a freshly prepared $MnCl_2$ solution (2.3 μmol). β1,4-GalT (33 μL, 23 mU) and alkaline phosphatase (0.2 μL, 200 mU) were added and the reaction shaken gently at ambient temperature for 2 days. The mixture was loaded onto a Biogel P-2 size exclusion column, eluting with water. Further purification was accomplished on DEAE Sephadex A-25 (gradient 0–500 mM NH_4HCO_3) to give **13a** (2.8 mg, 71%). ^{1}H NMR (500 MHz, D_2O) identifiable resonances δ 7.37–7.25 (9H, m), 2.06 (3H, s), 1.96 (3H, s), 1.94 (3H, s), 1.59–1.54 (3H, m), 1.25 (3H, d), 0.93 (3H, d), 0.90 (3H, d); ES–MS (neg) calcd for $C_{71}H_{102}N_{11}O_{35}S$ $[M-H]^-$ 1700.6, found 1701.

HPLC assay

β1,4-galactosyltransferase reactions were set up for the sulfated and unsulfated glycopeptides at concentrations of 1000, 500, 100, 50 and 10 μM in 100 mM HEPES

buffer, pH 7.4 containing 100 μM $MnCl_2$, using conditions as described for **13a**. β1,4-GalT concentration for each reaction was 0.13 μM. Alkaline phosphatase was added in excess to each reaction tube. A 5 μL aliquot was removed from each reaction, diluted to 50 μL, and immediately frozen at the following timepoints: 0, 1, 3, 6, 23 h. Reaction samples were thawed immediately prior to injection on a C18 analytical column (A:H_2O, B: CH_3CN; gradient 15–20% B, 10 min). Product formation over the initial 6 h of reaction time was measured as the increase of uridine concentration over time. A calibration curve for uridine concentration was prepared with authentic sample, and measured peak areas calculated directly from the equation of the calibration plot. A Lineweaver–Burk plot was constructed to determine the kinetic parameters.

Acknowledgements

This research was supported by the N. I. H. (GM44154) K. M. K. thanks the American Chemical Society Organic Division for a fellowship and also Dr. Dee-Hua Huang and Dr. Laura Pasternack for aid in NMR experiments and analysis.

References and Notes

1. (a) Kansas, G. S. *Blood* **1996**, *88*, 3259. (b) Rosen, S. D.; Bertozzi, C. R. *Curr. Biol.* **1996**, *6*, 261.
2. (a) Wilkins, P. P.; Moore, K. L.; McEver, R. P.; Cummings, R. D. *J. Biol. Chem.* **1995**, *270*, 22677. (b) DeLuca, M.; Dunlop, L. C.; Andrews, R K.; Flannery, J. V. Jr.; Ettling, R.; Cumming, D. A.; Veldman, G. M.; Berndt, M. C. *J. Biol. Chem.* **1995**, *270*, 26734. (c) Sako, D.; Comess, K. M.; Barone, K. M.; Camphausen, R. T.; Cumming, D. A.; Shaw, G. D. *Cell* **1995**, *83*, 323. (d) Pouyani, T.; Seed, B. *Cell* **1995**, *83*, 333.
3. Leppanen, A.; Mehta, P.; Ouyang, Y.-B.; Ju, T.; Helin, J.; Moore, K. L.; van Die, I.; Canfield, W. M.; McEver, R. P.; Cummings, R. D. *J. Biol. Chem.* **1999**, *274*, 24838.
4. Huttner, W. B. *Ann. Rev. Physiol.* **1988**, *50*, 363.
5. Ouyang, Y.-B.; Lane, W. S.; Moore, K. L. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 2896.
6. Wilkins, P. P.; McEver, R. P.; Cummings, R. D. *J. Biol. Chem.* **1996**, *271*, 18732.
7. For a previous chemoenzymatic approach to glycopeptide synthesis, see Seitz, O.; Wong C.-H. *J. Am. Chem. Soc.* **1997**, *119*, 8766.
8. Alper, P. B.; Hung, S.-C.; Wong, C.-H. *Tetrahedron Lett.* **1996**, *37*, 6029.
9. Grundler, G.; Schmidt, R. R. *Liebigs Ann. Chem.* **1984**, 1826.
10. Schulz, M.; Kunz, H. *Tetrahedron Asymm.* **1993**, *4*, 1205.
11. Paulsen, H.; Adermann, K. *Liebigs Ann. Chem.* **1989**, 751.
12. Mathieux, N.; Paulsen, H.; Meldal, M.; Bock, K. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2359.
13. Dullenkopf, W.; Castro-Palomino; J. C.; Manzoni, L.; Schmidt, R. R. *Carbohydr. Res.* **1996**, *296*, 135.
14. Zhang, Z.; Ollmann, I. R.; Ye, X.-S.; Wischnat, R.; Baasov, T.; Wong, C.-H. *J. Am. Chem. Soc.* **1999**, *121*, 734.
15. (a) Yagami, T.; Shiwa, S.; Futaki, S.; Kitagawa, K. *Chem. Pharm. Bull.* **1993**, *41*, 376. (b) Kitagawa, K.; Futaki, S.; Yagami, T.; Sumi, S.; Inoue, K. *Int. J. Peptide Protein Res.* **1994**, *43*, 190.
16. Marseigne, I.; Roy, P.; Dor, A.; Durieuz, C.; Pelaprat, D.; Reibaud, M.; Blanchard, J. C.; Roques, B. P. *J. Med. Chem.* **1989**, *32*, 445.
17. The work up procedure employed was previously used for the sulfation of carbohydrate hydroxyls: Sanders, W. J.; Manning, D. D.; Koeller, K. M.; Kiessling, L. L. *Tetrahedron* **1997**, *53*, 16391.
18. For previous studies of the effect of tyrosine sulfation on peptide conformation see: (a) Durieux, C.; Belleney, J.; Lallemand, J.-Y.; Roques, B. P.; Fournie-Zaluski, M.-C. *Biochem. Biophys. Res. Comm.* **1983**, *114*, 705; (b) Fournie-Zaluski, M.-C.; Belleney, J.; Lux, B.; Durieux, C.; Gerard, D.; Gacel, G.; Maigret, B.; Roques, B. P. *Biochemistry* **1986**, *25*, 3778.