This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Chemical Science

ARTICLE

Systematic Chemoenzymatic Synthesis of O-Sulfated Sialyl Lewis x Antigens

Abhishek Santraa, Hai Yua, Nova Tasnimaa, Musleh M. Muthanaa,b, Yanhong Lia, Jie Zengabc, Nicholas J. Kenyonbd, Angelique Y. Louiee, and Xi Chenf,∗

O-Sulfated sialyl Lewis x antigens play important roles in nature. However, due to their structural complexity, they are not readily accessible by either chemical or enzymatic synthetic processes. Taking advantage of a bacterial sialyltransferase mutant that can catalyze the transfer of different sialic acid forms from the corresponding sugar nucleotide donors to Lewis x antigens which are fucosylated glycans as well as an efficient one-pot multienzyme (OPME) sialylation system, O-sulfated sialyl Lewis x antigens containing different sialic acid forms and O-sulfation at different locations were systematically synthesized by chemoenzymatic methods.

Introduction

O-Sulfated sialyl Lewis x structures play important roles in immune regulation, inflammation, and cancer metastasis. For example, 6-O-sulfato-sialyl Lewis x (6-O-sulfo-sLe x) (1), Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAcβ1–3GlcNAcβ1–3GlcNAcβ1–3S[OR] with an O-sulfate group at the carbon-6 of the N-acetylgalcosamine (GlcNAc) residue is a well-known ligand for L-selectin, a C-type (Ca2+)-dependent carbohydrate-binding protein (lectin) expressed broadly on most leukocytes in the blood.1,2 The interaction of 6-O-sulfato-sLe x (1) and L-selectin plays critical roles in lymphocyte homing to the peripheral lymph nodes3 and in chronic inflammation.3 It has also been shown that human sialic acid-binding immunoglobulin-like lectin4 Siglec-9 binds strongly5,6 to 6-O-sulfato-sLe x but the biological importance of this interaction is less well understood.

On the other hand, 6-O-sulfato-sialyl Lewis x (6′-O-sulfo-sLe x) (2), Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAcβ1–4GlcNAcβ1–3S[OR] with an O-sulfate group at the carbon-6 of the galactose (Gal) residue (Scheme 1),7 in addition to 6′-O-sulfato-sialyl-N-acetyllactosamine (6′-O-sulfo-sLactNac, Neu5Acα2–3Galβ1–4GlcNAcβ1–4S[OR]),8 was shown by glycan microarray studies to be a preferred glycan ligand for Siglec-8 and for its paralog mouse Siglec-F.9 Siglec-8 is expressed on human allergic inflammatory cells including eosinophils, mast cells, and basophils.10–12 Reducing the number of eosinophils, such as by soluble 6′-O-sulfo-sLe x synthetic polymer induced apoptosis,13 has been suggested as an approach for asthma therapies.14 Furthermore, 6′-O-sulfo-sLe x (2) in addition to 6′-O-sulfo-sLacNac and 6′-O-sulfo-sialyl-lacto-N-neotetraose (6′-O-sulfo-sLNNt, Neu5Acα2–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc[OR]), was shown to bind to langerin,15 a C-type (Ca2+)-dependent lectin specific to Langerhans cells (immature antigen-presenting specific T cell immunity initiating dendritic cells of epidermis and mucosal tissues).14

Although less efficient than Neu5Acα2–8Neu5Acα2–3LacNac, both 6-O-sulfo-sLe x (1) and 6′-O-sulfo-sLe x (2) bind to human Siglec-7 moderately.16 Both are present in glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), an L-selectin ligand,17 with 6′-O-sulfo-sLe x (2) as the major sulfated form.16–18 Gal-6-O-sulfotransferase and GlcNAc-6-O-sulfotransferase have been found to synergistically produce L-selectin ligands. This indicates either the potential synergistic involvement of both 6-O-sulfo-sLe x (1) and 6′-O-sulfo-sLe x (2) or 6′,6-di-O-sulfo-sLe x (3) as O-sulfated groups at both Gal and GlcNAc residues in sLe x in L-selectin-binding.19 Human Siglec-7 and -8 have also been shown to bind stronger to 6′-di-O-sulfo-sLe x (3) than its mono-O-sulfated derivative (1) or (2) while mouse Siglec-F has been shown to bind similarly strongly to 6′,6-di-O-sulfo-sLe x (3) and 6′-O-sulfo-sLe x (2).6

The biological importance of O-sulfated sLe x structures make them attractive synthetic targets. However, the structures of these compounds are relatively complex and include synthetically challenging α2–3-linked sialic acid which suffers from low stereoselectivity and high 2,3-elimination rate in chemical synthesis20–22 and acid labile O-sulfate group.23, 24 Chemically,20, 25, 26 or chemoenzymatically27 synthesized Neu5Acα2–3Gal building blocks have been used as effective synthons for constructing more complex sialosides including sLe x and 6′-O-sulfo-sLe x (1).28 Several examples of chemical28 or chemoenzymatic27 synthesis of 6′-O-sulfo-sLe x (1) as well as chemical synthesis of 6′-O-sulfo-sLe x (2),29 30 and 6′,6-di-O-sulfo-sLe x (3)31, 32 have been reported. All these
examples are, however, limited to compounds with the most abundant sialic acid form, N-acetyleneuraminic acid (Neu5Ac). Despite the presence of more than 50 different sialic acid forms identified in nature, O-sulfated sLe\(^{-}\) containing a sialic acid form other than Neu5Ac has not been synthesized.

We report here the development of efficient chemoenzymatic methods for systematic synthesis of O-sulfated sLe\(^{-}\) containing different sialic acid forms. The methods are demonstrated for representative examples of 6'-O-sulfo-sLe\(^{-}\) (1), 6-O-sulfo-sLe\(^{-}\) (2), and 6,6-di-O-sulfo-sLe\(^{-}\) (3) containing the most abundant Neu5Ac form and N-glycolyneuraminic acid (Neu5Gc), a sialic acid form commonly found in mammals other than human but can be incorporated into the human glycome from dietary sources.\(^{35}\)

One efficient approach for the synthesis of O-sulfated sLe\(^{-}\) with different sialic acid forms would be by direct sialylation of O-sulfated Le\(^{-}\) using one-pot multienzyme (OPME) sialylation systems\(^{37}\) containing an α2-3-sialyltransferase, a CMP-sialic acid synthetase (CSS),\(^{38}\) with or without a sialic acid aldolase.\(^{39}\) Such an approach has been successfully demonstrated for direct sialylation of non-sulfated Le\(^{-}\) for the synthesis of sLe\(^{-}\) containing a diverse array of naturally occurring and non-natural sialic acid forms by OMPE systems containing a recombinant viral α2-3-sialyltransferase vST3Gal)\(^{40}\) or a bacterial multifunctional sialyltransferase mutant, Pasteurella multocida α2-3-sialyltransferase 1 (PmST1 M144D).\(^{41}\) The latter with a high expression level (98 mg L\(^{-}\)1 culture, >1000-fold higher than that of vST3Gal-I) and high promiscuity in tolerating different modification on the sialic acid in the substrates is a superior choice for the synthesis.\(^{41}\) However, it was not clear whether O-sulfated Le\(^{-}\) structures could be used by PmST1 M144D as suitable acceptors in OPME sialylation process to produce desired O-sulfated sLe\(^{-}\) with different sialic acid forms.

**Results and discussion**

**Synthesis of O-sulfated disaccharides and O-sulfated Le\(^{-}\)**

In order to obtain O-sulfated Le\(^{-}\) as potential acceptor substrates for PmST1 M144D, enzyme-catalyzed α1–3-fucosylation of the corresponding O-sulfated disaccharides was test as a potential strategy. A one-pot three-enzyme (OP3E) α1–3-fucosylation system (Scheme 1)\(^{42,43}\) containing Bacteroides fragilis bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (BfFKP),\(^{44}\) Pasteurella multocida inorganic pyrophosphorylase (PmPPa),\(^{44}\) and Helicobacter pylori α1–3-fucosyltransferase (Hp1–3FT\(\Delta66\) or Hp3FT)\(^{45}\) was used for this purpose. The O-sulfated disaccharides tested were 6-O-sulfo-LacNAcβProN\(_2\) (4) (Scheme 1), 6'-O-sulfo-LacNAcβProN\(_2\) (5), and 6,6'-di-O-sulfo-LacNAcβProN\(_3\) (Figure 2). LacNAcβProN\(_4\) without any O-sulfate groups was used as a positive control.

![Fig. 2 Structures of chemically synthesized 6'-O-sulfo-LacNAcβProN\(_2\) (5) and 6,6'-di-O-sulfo-LacNAcβProN\(_3\) (6).](image)

**Scheme 1** Sequential OPME synthesis of 6-O-sulfo-Le\(^{-}\)βProN\(_2\) (8) from 6-O-sulfo-GlcNAcβProN\(_2\) (7) by OPME α1–4-galactosyl activation and transfer system for the formation of 6-O-sulfo-LacNAcβProN\(_2\) (4) followed by OPME α1–3-fucosyl activation and transfer system for the formation of 6-O-sulfo-Le\(^{-}\)βProN\(_2\) (8). Enzymes and abbreviations: SpGalK, Streptococcus pneumoniae TIGR4 galactokinase;\(^{45}\) BLUSP, Bifidobacterium longum UDP-sugar pyrophosphorylase;\(^{46}\) PmPPa, Pasteurella multocida inorganic pyrophosphorylase;\(^{44}\) Hp4GalT, Helicobacter pylori β1–4-galactosyltransferase;\(^{44}\) BfFKP, Bacteroides fragilis bifunctional L-fucokinase/GDP-fucose pyrophosphorylase;\(^{44}\) and Hp3FT, Helicobacter pylori α1–3-fucosyltransferase.\(^{40,42}\)

6-O-Sulfo-LacNAcβProN\(_2\) (4) was synthesized from 6-O-sulfo-GlcNAcβProN\(_2\) (7) using an improved OPME galactosyl activation and transfer system (Scheme 1) containing Streptococcus pneumoniae TIGR4 galactokinase (SpGalK),\(^{45}\) Bifidobacterium longum UDP-sugar pyrophosphorylase (BLUSP),\(^{46}\) PmPPa, and a Helicobacter pylori β1–4-galactosyltransferase (Hp4GalT).\(^{44}\) The EcGalK, BLUSP, and PmPPa allowed in situ formation of the donor substrate of Hp4GalT, uridine 5’-diphosphate-galactose (UDP-Gal), from monosaccharide galactose (Gal).\(^{46}\) It was previously shown that Hp4GalT, but not Neisseria meningitidis β1–4-galactosyltransferase (NmLgtB), was able to use 6-O-sulfated GlcNAc and derivatives as acceptor substrates for the
synthesis of β1–4-linked galactosides. The activity of Hp4GalT in synthesizing 6-O-sulfo-LacNacβProN3 (4) was confirmed again here using the improved OPME approach. An excellent 89% yield was obtained, compared favourably to the previous Hp4GalT-dependent OPME β1–4-galactosylation approach (70% yield) which used Escherichia coli K-12 glucose-1-P uridylyltransferase (EcGalU), Escherichia coli UDP-galactose-4-epimerase (EcGale), and PmpT/A to produce UDP-Gal in situ from glucose-1-phosphate. 6'-O-Sulfo-LacNacβProN3 (5) and 6,6'-di-O-sulfo-LacNacβProN3 (6) (Scheme 2) were chemically synthesized (see supporting information).

Among three O-sulfated disaccharides tested, only 6-O-sulfo-LacNacβProN3 (4) was a suitable acceptor for Hp3FT to produce the desired 6-O-sulfo-Le3βProN3 (8). In contrast, 6'-O-sulfo-LacNacβProN3 (5) and 6,6'-di-O-sulfo-LacNacβProN3 (6) were not used efficiently by Hp3FT for the synthesis of the corresponding O-sulfated Le3 derivatives. With the positive outcome in small scale reactions for fucosylation of 6-O-sulfo-LacNacβProN3 (4), the preparative-scale synthesis of 6-O-sulfo-Le3βProN3 (8) was carried out using the OP3E α1–3-fucosyl activation and transfer system (Scheme 1). A yield of 70% was obtained. The combined sequential OPME β1–4-galactosylation and OPME α1–3-fucosylation (Scheme 1) was an effective approach for obtaining 6-O-sulfo-Le3βProN3 (8) from a simple monosaccharide derivative 6-O-sulfo-GlcNAcβProN3 (7) in an overall yield of 62%.

As Hp3FT was not able to use 6'-O-sulfo-LacNacβProN3 (5) or 6,6'-di-O-sulfo-LacNacβProN3 (6) efficiently as acceptors for fucosylation to obtain the desired Le3 trisaccharides, the target trisaccharides 6'-O-sulfo-Le3βProN3H2 (9) and 6,6'-di-O-sulfo-Le3βProN3H2 (10) were chemically synthesized (Scheme 2) from monosaccharide synths 11, 12, 13, and 14. Notable features of the synthetic strategy include: (a) application of an efficient general protection strategy for the synthesis of two trisaccharids (i.e. similar protecting groups were used in the synthesis and same reagents were used for their removal); (b) use of similar thiglycosides derivatives as glycosyl donors in all glycosylations; (c) high regio and stereo selectivity in product formation; (d) one step removal of benzyl ethers and reduction of azido group using 20% Pd(OH)2/C (the Pearlman’s catalyst) and H2. More specifically, for the synthesis of 9 and 10, two N-phthalimide glucosamine derivatives 11 and 12 selectively protected at C6 with benzyl and tert-butylidiphenylsilyl ether (TBDBPS), respectively, were coupled stereoselectively with thiglycoside donor 13 selectively protected with TBDBPS at C6 in the presence of N-iodosuccinimide (NIS) and trimethylsilyl trifluoromethanesulfonate (TMSOTf) in dichloromethane. Disaccharide derivatives 15 and 16 were obtained in 72% and 78% yields, respectively. The bulky N-phthalimido protecting group in acceptors 11 and 12 provides steric hindrance to the neighboring C-3 hydroxyl group and enhances the reactivity of the C-4 hydroxyl group. Therefore, glycosylation occurs regioselectively at C-4 hydroxyl group. Initial attempts to glycosylate acceptors 15 and 16 in dichloromethane with 1.2 equivalents of thiofuran fucoside 14 produced trisaccharides of alpha and beta mixtures. In contrast, stereospecific formation of trisaccharides was achieved when a mixed solvent of diethyl ether and dichloromethane (1:1) was employed. Reaction of acceptors 15 and 16 with 1.2 equivalents of fucosyl donor 14 produced compounds 17 and 18 in 68% and 65% yields, respectively. Compounds 17 and 18 were then subjected to a series of synthetic transformations involving (a) conversion of N-phthaloyl group to acetamido group by removing phthaloyl group using ethylenediamine followed by N- and O-acetylation using acetic anhydride and pyridine; (b) HF-pyridine-mediated selective removal of TBDPS group; (c) O-sulfation of the primary hydroxyl group by SO3-pyridine complex; (d) deacetylation by NaOME in MeOH; and (e) hydrogenation using Pd(OH)2/C and H2 to obtain desired 6'-O-sulfo-Le3βProN3H2 (9) and 6,6'-di-O-sulfo-Le3βProN3H2 (10).

### Scheme 2

**Chemical synthesis of 6'-O-sulfo-Le3βProN3H2 (9) and 6,6'-di-O-sulfo-Le3βProN3H2 (10).** Reagents and conditions: a) N-iodosuccinimide (NIS), TMSOTf, 5 Å, CH2Cl2, -40 °C, 30 min; b) N-iodosuccinimide (NIS), TMSOTf, 5 Å, CH2Cl2-Et2O (1:1), -18 °C, 45 min; c) H2N(CH2)3NH2, n-BuOH, 90 °C, 8 h; d) pyridine, Ac2O, r.t., 10 h; e) HF pyridine, 0 °C to r.t., overnight; f) SO3-pyridine, pyridine, 0 °C to r.t.; g) 0.1 M NaOME, MeOH, r.t., 3 h; h) Pd(OH)2/C, H2, CH2OH, 48 h.

### Enzymatic synthesis of O-sulfated sLeα

With chemoenzymatically synthesized 6'-O-sulfo-Le3βProN3 (8) as well as chemically synthesized 6'-O-sulfo-Le3βProN3H2 (9) and 6,6'-di-O-sulfo-Le3βProN3H2 (10) in hand, a one-pot two-enzyme (OP2E) sialylation system (Scheme 3) was used to test the tolerance of PmST1 M144D in using these O-sulfated Le3 compounds as potential acceptor substrates. PmST1 M144D was previously engineered by protein crystal structure-assisted design. It has 20-fold reduced CMP-sialic acid (donor) hydrolysis activity and significantly (5588-fold) decreased CMP-sialic acid (donor) hydrolysis activity and (e) hydrogenation using Pd(OH)2/C and H2 to obtain desired 6'-O-sulfo-Le3βProN3H2 (9) and 6,6'-di-O-sulfo-Le3βProN3H2 (10).
highly efficient one-pot two-enzyme system containing Neisseria meningitidis CMP-sialic acid (NmCSS)\textsuperscript{35} and PmST1 M144D\textsuperscript{41} from the corresponding acceptors 8–10 and Neu5Ac or Neu5Gc, respectively. In general, Neu5Gc was used less efficiently by the OPME sialylation system, leading to lower yields for 1a–3a (38–60%) compared to their Neu5Ac-counterparts 1a–3a (64–85%). O-Sulfated sLe\textsuperscript{x} glycans with a propyl amine aglycone (compounds 2a, 3a, 2b, 3b) were found to be more challenging for column purification compared to the ones with a propyl azide aglycone (compounds 1a and 1b). When a desired sialic acid is readily available such as the case presented here, a one-pot two-enzyme (OP2E) system is sufficient. When only the 6-carbon precursors of the desired sialic acid forms are available, the one-pot three-enzyme (OP3E) sialylation system including an aldolase in addition to NmCSS and PmST1 M144D\textsuperscript{41} should be used.

\textbf{Conclusions}

In conclusion, we have successfully developed an efficient chemoenzymatic method for systematic synthesis of synthetically challenging O-sulfated sLe\textsuperscript{x} tetrasaccharides: 1a, 85%; 1b, 47%; 2a, 82%; 2b, 60%; 3a, 64%; 3b, 38%. Enzymes and abbreviations: NmCSS, Neisseria meningitidis CMP-sialic acid;\textsuperscript{35} PmST1 M144D, Pasteurella multocida α2–3-sialyltransferase 1 (PmST1) M144D mutant.\textsuperscript{35}

Acknowledgements

This work was supported by NIH grants R01GM094523 (to X.C.) and R21AI097354 (to A.Y.L.) as well as NSF grant CHE-1300449 (to X.C.). M.M.M. acknowledges UC-Davis and USDE for GAANN Fellowship (P200A120187). Bruker Avance-800 NMR spectrometer was funded by NSF grant DBIO-722538.

References