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Systematic chemoenzymatic synthesis of *O*-sulfated sialyl Lewis x antigens†

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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.

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Introduction

O-Sulfated sialyl Lewis x structures play important roles in immune regulation, inflammation, and cancer metastasis.¹ For example, 6-*O*-sulfo-sialyl Lewis x [6-*O*-sulfo-*sLe*^x (**1**), Neu5Ac α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc6S β OR] with an *O*-sulfate group at the carbon-6 of the *N*-acetylglucosamine (GlcNAc) residue (Fig. 1) is a well known ligand for L-selectin, a C-type (Ca²⁺-dependent) carbohydrate-binding protein (lectin) expressed broadly in most leukocytes in the blood.^{1,2} The interaction of 6-*O*-sulfo-*sLe*^x (**1**) and L-selectin plays a critical role in lymphocyte homing to the peripheral lymph nodes² and in chronic inflammation.³ It has also been shown that human sialic acid-binding immunoglobulin-like lectin⁴ Siglec-9 binds strongly^{5,6} to 6-*O*-sulfo-*sLe*^x, but the biological importance of this interaction is less well understood.

On the other hand, 6'-*O*-sulfo-sialyl Lewis x [6'-*O*-sulfo-*sLe*^x (**2**), Neu5Ac α 2-3Gal6S β 1-4(Fuc α 1-3)GlcNAc β OR] with an *O*-sulfate group at the carbon-6 of the galactose (Gal) residue (Fig. 1),⁷ in addition to 6'-*O*-sulfo-sialyl-*N*-acetyllactosamine (6'-*O*-sulfo-*sLacNAc*, Neu5Ac α 2-3Gal6S β 1-4GlcNAc β 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 in human allergic inflammatory cells including eosinophils, mast cells, and basophils.^{5,10} Reducing the number of eosinophils, such as by soluble 6'-*O*-sulfo-*sLe*^x synthetic polymer induced apoptosis,¹¹ has been suggested as an approach for asthma therapies.¹² 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*, Neu5Ac α 2-3Gal6S β 1-4GlcNAc β 1-3Gal β 1-4Glc β OR), was shown to bind to langerin,¹³ 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 Neu5Ac α 2-8Neu5Ac α 2-3LacNAc, both 6-*O*-sulfo-*sLe*^x (**1**) and 6'-*O*-sulfo-*sLe*^x (**2**) bound moderately

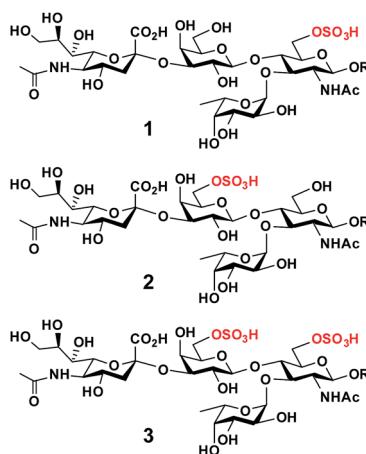


Fig. 1 Structures of *O*-sulfated sialyl Lewis x including 6-*O*-sulfo-*sLe*^x (**1**), 6'-*O*-sulfo-*sLe*^x (**2**), and 6',6-di-*O*-sulfo-*sLe*^x (**3**).

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to human Siglec-7.⁵ Both are present in glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), an L-selectin ligand,¹⁵ 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 the involvement of 6',6-di-O-sulfo-sLe^x (3) (Fig. 1) with O-sulfate groups at both the Gal and GlcNAc residues of sLe^x in L-selectin-binding.¹⁹ Human Siglec-7 and -8 have also been shown to bind more strongly to 6',6-di-O-sulfo-sLe^x (3) than their mono-O-sulfated derivatives (1 and 2), while mouse Siglec-F has been shown to bind with similar strength 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 makes them attractive synthetic targets. However, the structures of these compounds are relatively complex and include synthetically challenging α 2-3-linked sialic acid, which suffers from low stereoselectivity and a high 2,3-elimination rate in chemical synthesis,^{20–22} as well as the acid labile O-sulfate group.^{23,24} Chemically^{20,25,26} or chemoenzymatically²⁷ synthesized Neu5Ac α 2-3Gal building blocks have been used as effective synthons for constructing more complex sialosides including sLe^x and 6-O-sulfo-sLe^x (1).²⁰ Several examples of the chemical^{22,28} or chemoenzymatic²⁹ synthesis of 6-O-sulfo-sLe^x (1) as well as the chemical synthesis of 6'-O-sulfo-sLe^x (2)^{22,30,31} and 6',6-di-O-sulfo-sLe^x (3)³² have been reported. All these examples are, however, limited to compounds with the most abundant sialic acid form, *N*-acetylneurameric acid (Neu5Ac). Despite the presence of more than 50 different sialic acid forms

identified in nature,^{33,34} O-sulfated sLe^x containing a sialic acid form other than Neu5Ac has not been synthesized.

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

One efficient approach for the synthesis of O-sulfated sLe^x with different sialic acid forms would be by direct sialylation of O-sulfated Le^x using one-pot multienzyme (OPME) sialylation systems³⁶ containing an α 2-3-sialyltransferase and a CMP-sialic acid synthetase (CSS),³⁷ with or without a sialic acid aldolase.³⁸ Such an approach has been successfully demonstrated for direct sialylation of non-sulfated Le^x for the synthesis of sLe^x containing a diverse array of naturally occurring and non-natural sialic acid forms, using OPME systems containing a recombinant viral α 2-3-sialyltransferase vST3Gal-I³⁹ or a bacterial multifunctional sialyltransferase mutant, *Pasteurella*

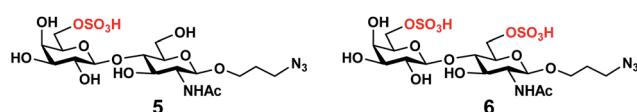
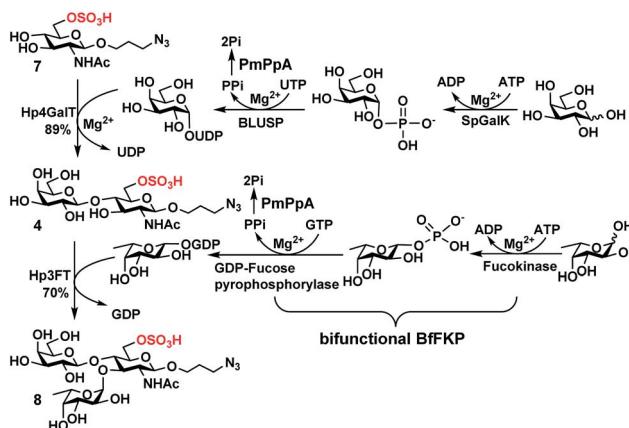
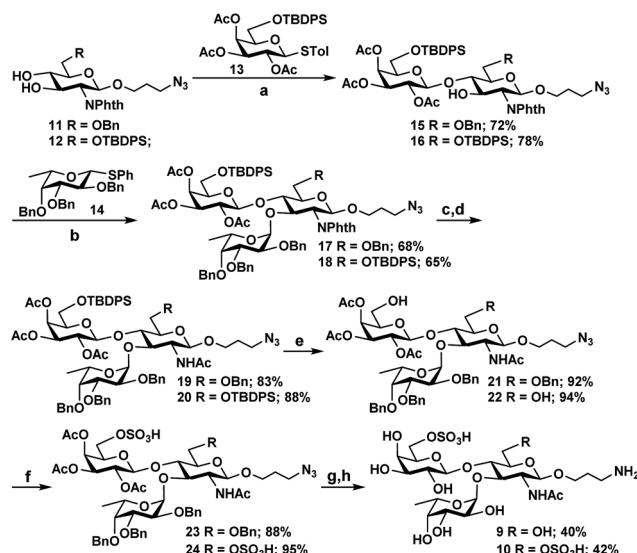


Fig. 2 Structures of chemically synthesized 6'-O-sulfo-LacNAc β ProN₃ (5) and 6,6'-di-O-sulfo-LacNAc β ProN₃ (6).

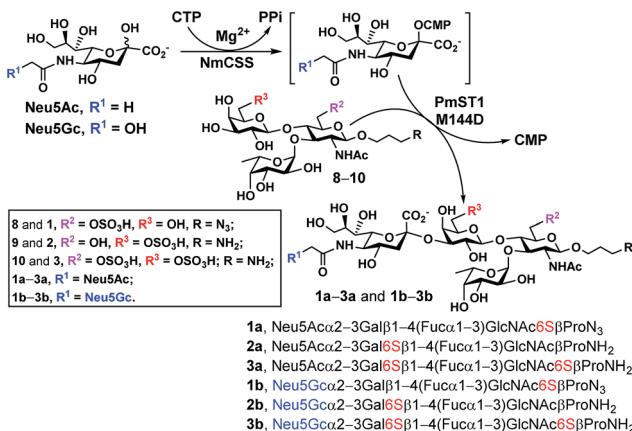


Scheme 1 Sequential OPME synthesis of 6-O-sulfo-Le^x β ProN₃ (8) from 6-O-sulfo-GlcNAc β ProN₃ (7) using an OPME β 1-4-galactosyl activation and transfer system for the formation of 6-O-sulfo-LacNAc β ProN₃ (4) followed by an OPME α 1-3-fucosyl activation and transfer system for the formation of 6-O-sulfo-Le^x β ProN₃ (8). Enzymes and abbreviations: SpGalK, *Streptococcus pneumoniae* TIGR4 galactokinase;⁴⁴ BLUSP, *Bifidobacterium longum* UDP-sugar pyrophosphorylase;⁴⁵ PmPpA, *Pasteurella multocida* inorganic pyrophosphorylase;⁴³ Hp4GalT, *Helicobacter pylori* β 1-4-galactosyltransferase;⁴³ BffKP, *Bacteroides fragilis* bifunctional L-fucokinase/GDP-fucose pyrophosphorylase;⁴² and Hp3FT, *Helicobacter pylori* α 1-3-fucosyltransferase.^{39,41}



Scheme 2 Chemical synthesis of 6'-O-sulfo-Le^x β ProNH₂ (9) and 6,6'-di-O-sulfo-Le^x β ProNH₂ (10). Reagents and conditions: (a) N-iodosuccinimide (NIS), TMSOTf, MS 4 Å, CH₂Cl₂, -40 °C, 30 min; (b) N-iodosuccinimide (NIS), TMSOTf, MS 4 Å, CH₂Cl₂-Et₂O (1:1), -18 °C, 45 min; (c) H₂N(CH₂)₂NH₂, 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, CH₃OH, 48 h.





Scheme 3 PmST1 M144D-mediated one-pot two-enzyme (OP2E) sialylation of O-sulfo analogues of Lewis x . Yields obtained for O-sulfated sLe x tetrasaccharides: **1a**, 85%; **1b**, 47%; **2a**, 82%; **2b**, 60%; **3a**, 64%; **3b**, 38%. Enzymes and abbreviations: NmCSS, *Neisseria meningitidis* CMP-sialic acid;³⁷ PmST1 M144D, *Pasteurella multocida* α 2-3-sialyltransferase 1 (PmST1) M144D mutant.⁴⁰

Pasteurella multocida α 2-3-sialyltransferase 1 (PmST1) M144D.⁴⁰ 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 modifications on the sialic acid in the substrates, is a superior choice for the synthesis.⁴⁰ However, it is not clear whether O-sulfated Le x structures could be used with PmST1 M144D as suitable acceptors in the OPME sialylation process to produce the desired O-sulfated sLe x with different sialic acid forms.

Results and discussion

Synthesis of O-sulfated disaccharides and O-sulfated Le x

In order to obtain O-sulfated Le x as potential acceptor substrates for PmST1 M144D, enzyme-catalyzed α 1-3-fucosylation of the corresponding O-sulfated disaccharides was tested as a potential strategy. A one-pot three-enzyme (OP3E) α 1-3-fucosylation system (Scheme 1)^{39,41} containing *Bacteroides fragilis* bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (BfFKP),⁴² *Pasteurella multocida* inorganic pyrophosphorylase (PmPpA),⁴³ and *Helicobacter pylori* α 1-3-fucosyltransferase (Hp1-3FT Δ 66 or Hp3FT) was used for this purpose. The O-sulfated disaccharides tested were 6-O-sulfo-LacNAc β ProN₃ (**4**) (Scheme 1), 6'-O-sulfo-LacNAc β ProN₃ (**5**), and 6,6'-di-O-sulfo-LacNAc β ProN₃ (**6**) (Fig. 2). LacNAc β ProN₃ (ref. 43) without any O-sulfate groups was used as a positive control.

6-O-Sulfo-LacNAc β ProN₃ (**4**) was synthesized from 6-O-sulfo-GlcNAc β ProN₃ (**7**)⁴³ using an improved OPME galactosyl activation and transfer system (Scheme 1) containing *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK),⁴⁴ *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP),⁴⁵ PmPpA, and a *Helicobacter pylori* β 1-4-galactosyltransferase (Hp1-4GalT or Hp4GalT).⁴³ 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).⁴⁵ It was previously shown that Hp4GalT, but not *Neisseria meningitidis* β 1-4-galactosyltransferase (NmLgtB), was able to use 6-O-sulfated GlcNAc and derivatives as acceptor substrates for the synthesis of β 1-4-linked galactosides.⁴³ The activity of Hp4GalT in synthesizing 6-O-sulfo-LacNAc β ProN₃ (**4**) was confirmed again here using the improved OPME approach.^{45,46} An excellent 89% yield was obtained, comparing favourably to the previous Hp4GalT-dependent OPME β 1-4-galactosylation approach (70% yield) which used *Escherichia coli* K-12 glucose-1-P uridylyltransferase (EcGalU), *Escherichia coli* UDP-galactose-4-epimerase (EcGalE), and PmPpA to produce UDP-Gal *in situ* from glucose-1-phosphate.⁴³ 6'-O-Sulfo-LacNAc β ProN₃ (**5**) and 6,6'-di-O-sulfo-LacNAc β ProN₃ (**6**) (Fig. 2) were chemically synthesized (see ESI†).

Among the three O-sulfated disaccharides tested, only 6-O-sulfo-LacNAc β ProN₃ (**4**) was a suitable acceptor for Hp3FT to produce the desired 6-O-sulfo-Le x βProN₃ (**8**). In contrast, 6'-O-sulfo-LacNAc β ProN₃ (**5**) and 6,6'-di-O-sulfo-LacNAc β ProN₃ (**6**) were not used efficiently by Hp3FT for the synthesis of the corresponding O-sulfated Le x derivatives. With the positive outcome in small scale reactions for fucosylation of 6-O-sulfo-LacNAc β ProN₃ (**4**), the preparative-scale synthesis of 6-O-sulfo-Le x βProN₃ (**8**) was carried out using the OP3E α 1-3-fucosyl activation and transfer system (Scheme 1). A yield of 70% was obtained. The combined sequential OPME β 1-4-galactosylation and OPME α 1-3-fucosylation (Scheme 1) was an effective approach for obtaining 6-O-sulfo-Le x βProN₃ (**8**) from a simple monosaccharide derivative 6-O-sulfo-GlcNAc β ProN₃ (**7**) in an overall yield of 62%.

As Hp3FT was not able to use 6'-O-sulfo-LacNAc β ProN₃ (**5**) or 6,6'-di-O-sulfo-LacNAc β ProN₃ (**6**) efficiently as acceptors for fucosylation to obtain the desired Le x trisaccharides, the target trisaccharides 6'-O-sulfo-Le x βProNH₂ (**9**) and 6,6'-di-O-sulfo-Le x βProNH₂ (**10**) were chemically synthesized (Scheme 2) from monosaccharide synthons **11**, **12**,²⁷ **13**, and **14**.²⁷ Notable features of the synthetic strategy include: (a) application of an efficient general protection strategy⁴⁷ for the synthesis of the two trisaccharides (*i.e.* similar protecting groups were used in the syntheses and the same reagents were used for their removal); (b) use of similar thioglycoside derivatives as glycosyl donors in all glycosylations; (c) high regio- and stereoselectivity in product formation; (d) one step removal of benzyl ethers and reduction of the azido group using 20% Pd(OH)₂/C (Pearlman's catalyst) and H₂.⁴⁸ 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*-butyldiphenylsilyl ether (TBDPS), respectively, were coupled stereoselectively with thioglycoside donor **13**, which was selectively protected with TBDPS at C6, in the presence of *N*-iodosuccinimide (NIS) and trimethylsilyl trifluoromethanesulfonate (TMSOTf)⁴⁹ 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 decreases the reactivity of the C-3 hydroxyl group. Therefore, glycosylation occurs regioselectively at the C-4 hydroxyl group.²⁷ Initial attempts to glycosylate acceptors **15** and **16** in



dichloromethane with 1.2 equivalents of thiophenyl fucoside **14** produced trisaccharides in alpha and beta mixtures. In contrast, stereospecific formation of trisaccharides was achieved when a mixed solvent of diethylether and dichloromethane (1 : 1)^{50,51} was employed. The 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: (a) conversion of the *N*-phthaloyl group to an acetamido group by removing the phthaloyl group using ethylenediamine, followed by *N*- and *O*-acetylation using acetic anhydride and pyridine; (b) HF-pyridine-mediated selective removal of the TBDS group;⁵² (c) *O*-sulfation of the primary hydroxyl group by SO_3 pyridine complex;^{52,53} (d) deacetylation by NaOMe in MeOH;⁵⁴ and (e) hydrogenation using $\text{Pd}(\text{OH})_2/\text{C}$ and H_2 (ref. 55) to obtain the desired 6'-*O*-sulfo- $\text{Le}^x\beta\text{ProNH}_2$ (**9**) and 6,6'-di-*O*-sulfo- $\text{Le}^x\beta\text{ProNH}_2$ (**10**).

Enzymatic synthesis of *O*-sulfated sLe^x

With chemoenzymatically synthesized 6-*O*-sulfo- $\text{Le}^x\beta\text{ProN}_3$ (**8**) as well as chemically synthesized 6'-*O*-sulfo- $\text{Le}^x\beta\text{ProNH}_2$ (**9**) and 6,6'-di-*O*-sulfo- $\text{Le}^x\beta\text{ProNH}_2$ (**10**) in hand, a one-pot two-enzyme (OP2E) sialylation system (Scheme 3) was used to test the tolerance of PmST1 M144D⁴⁰ for using these *O*-sulfated Le^x 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 α 2-3-sialidase activity compared to the wild-type enzyme. It was used efficiently in a one-pot three-enzyme (OP3E) sialylation system for the synthesis of non-sulfated sLe^x tetrasaccharides containing diverse sialic acid forms from Le^x .⁴⁰ To our delight, PmST1 M144D also tolerated *O*-sulfated Le^x containing *O*-sulfate at C-6, C-6', or both. In addition to *N*-acetylneuraminic acid (Neu5Ac), *N*-acetylneuraminic acid (Neu5Gc) was also successfully introduced to compounds **8–10**. *O*-Sulfated sLe^x tetrasaccharides 6-*O*-sulfo-Neu5Ac α 2-3 $\text{Le}^x\beta\text{ProN}_3$ (**1a**, 80 mg, 85%), 6-*O*-sulfo-Neu5Gc α 2-3 $\text{Le}^x\beta\text{ProN}_3$ (**1b**, 22 mg, 47%), 6'-*O*-sulfo-Neu5Ac α 2-3 $\text{Le}^x\beta\text{ProNH}_2$ (**2a**, 75 mg, 82%), 6'-*O*-sulfo-Neu5Ac α 2-3 $\text{Le}^x\beta\text{ProNH}_2$ (**2b**, 45 mg, 60%), 6,6'-di-*O*-sulfo-Neu5Ac α 2-3 $\text{Le}^x\beta\text{ProNH}_2$ (**3a**, 42 mg, 64%), and 6,6'-di-*O*-sulfo-Neu5Gc α 2-3 $\text{Le}^x\beta\text{ProNH}_2$ (**3b**, 40 mg, 38%) were successfully obtained using this highly efficient one-pot two-enzyme system containing *Neisseria meningitidis* CMP-sialic acid (NmCSS)³⁷ and PmST1 M144D⁴⁰ 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 **1b–3b** (38–60%) compared to their Neu5Ac-counterparts **1a–3a** (64–85%). *O*-Sulfated sLe^x glycans with a propyl amine aglycone (compounds **2a**, **3a**, **2b** and **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 in 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 M144D⁴⁰ should be used.

Conclusions

In conclusion, we have successfully developed an efficient chemoenzymatic method for the systematic synthesis of synthetically challenging *O*-sulfated sLe^x (**1a–3a** and **1b–3b**) containing different sialic acid forms (Neu5Ac or Neu5Gc) by direct sialylation of the corresponding *O*-sulfated Le^x structures **8–10** using an efficient one-pot two-enzyme (OP2E) system containing NmCSS and PmST1 M144D. The method can be extended to the synthesis of *O*-sulfated sLe^x structures containing other sialic acid forms. We have also shown here that a relatively complex trisaccharide 6-*O*-sulfo- $\text{Le}^x\beta\text{ProN}_3$ (**8**) can be efficiently produced from a simple monosaccharide derivative 6-*O*-sulfo-GlcNAc βProN_3 (**7**) by a sequential OPME β 1-4-galactosylation and OPME α 1-3-fucosylation process. PmST1 M144D has been demonstrated to be a powerful catalyst not only for synthesizing non-sulfated sLe^x structures as shown previously,⁴⁰ but also for producing biologically important but difficult-to-obtain *O*-sulfated sLe^x .

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