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

Abhishek Santra,[a] Hai Yu,[a] Nova Tasnima,[a] Musleh M. Muthana,‡[a] Yanhong Li,[a] Jie Zeng,[ab] Nicholas J. Kenyon,[c] Angelique Y. Louie[d] and Xi Chen*[a]

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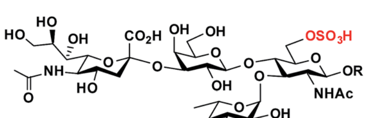
O-Sulfated sialyl Lewis *x* antigens play important roles in nature. However, due to their structural complexity, they are not readily accessible by either chemical or enzymatic synthetic processes. Taking advantage of a bacterial sialyltransferase mutant that can catalyze the transfer of different sialic 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.

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**), Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc6SβOR] 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 (Ca^{2+}-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 nodes[2] and in chronic inflammation.[3] It has also been shown that human sialic acid-binding immunoglobulin-like lectin[4] 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**), Neu5Acα2-3Gal6Sβ1-4(Fucα1-3)GlcNAcβOR] 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, Neu5Acα2-3Gal6Sβ1-4GlcNAcβOR),[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, Neu5Acα2-3Gal6Sβ1-4GlcNAcβ1-3Galβ1-4GlcβOR), was shown to bind to langerin,[13] a C-type (Ca^{2+}-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**) bound moderately



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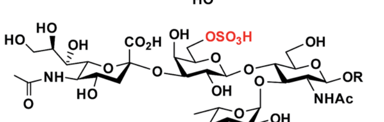

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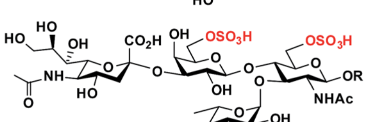

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Fig. 1 Structures of *O*-sulfated sialyl Lewis *x* including 6-*O*-sulfo-sLe^x (**1**), 6′-*O*-sulfo-sLe^x (**2**), and 6′, 6-di-*O*-sulfo-sLe^x (**3**).

[a]Department of Chemistry, University of California, Davis One Shields Avenue, Davis, CA 95616, USA. E-mail: xiichen@ucdavis.edu; Fax: +1-530-752-8995; Tel: +1-530-754-6037

[b]School of Food Science, Henan Institute of Science and Technology, Xinxiang, 453003, China

[c]Division of Pulmonary, Critical Care and Sleep Medicine, Department of Internal Medicine, University of California, Davis, CA 95616, USA

[d]Department of Biomedical Engineering, University of California, Davis, CA 95616, USA

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‡ Current address: Children's National Medical Center, Washington DC, 20010, USA.

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 α2-3-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 chemoenzymatically[27] 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**).[20] Several examples of the chemical[22,28] or chemoenzymatic[29] 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*-acetylneuraminic 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*-glycolylneuraminic 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 systems[36] containing an α2-3-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 α2-3-sialyltransferase vST3Gal-I[39] or a bacterial multifunctional sialyltransferase mutant, *Pasteurella*

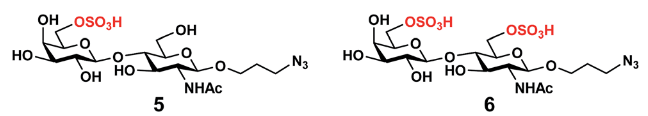
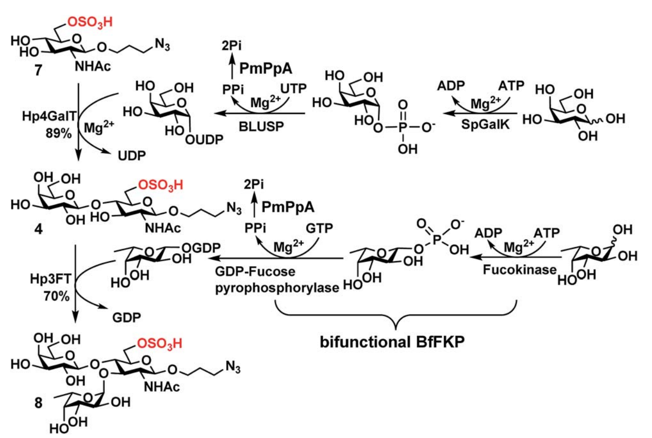
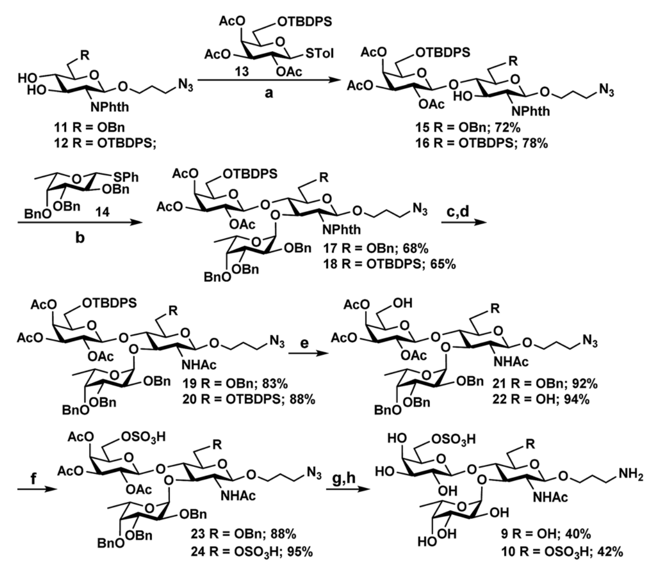


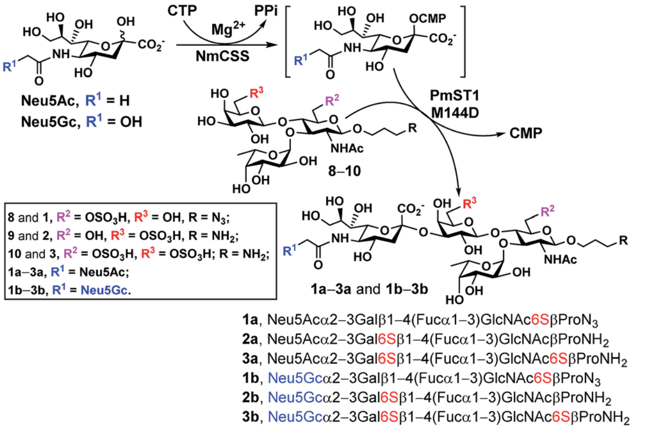
Fig. 2 Structures of chemically synthesized 6′-*O*-sulfo-LacNAcβProN$_3$ (**5**) and 6,6′-di-*O*-sulfo-LacNAcβProN$_3$ (**6**).



Scheme 1 Sequential OPME synthesis of 6-*O*-sulfo-Le^{x}βProN$_3$ (**8**) from 6-*O*-sulfo-GlcNAcβProN$_3$ (**7**) using an OPME β1-4-galactosyl activation and transfer system for the formation of 6-*O*-sulfo-LacNAcβProN$_3$ (**4**) followed by an OPME α1-3-fucosyl activation and transfer system for the formation of 6-*O*-sulfo-Le^{x}βProN$_3$ (**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* β1-4-galactosyltransferase;[43] BfFKP, *Bacteroides fragilis* bifunctional L-fucokinase/GDP-fucose pyrophosphorylase;[42] and Hp3FT, *Helicobacter pylori* α1-3-fucosyltransferase.[39,41]



Scheme 2 Chemical synthesis of 6′-*O*-sulfo-Le^{x}βProNH$_2$ (**9**) and 6,6′-di-*O*-sulfo-Le^{x}βProNH$_2$ (**10**). Reagents and conditions: (a) *N*-iodosuccinimide (NIS), TMSOTf, MS 4 Å, CH_2Cl_2, −40 °C, 30 min; (b) *N*-iodosuccinimide (NIS), TMSOTf, MS 4 Å, CH_2Cl_2–Et_2O (1 : 1), −18 °C, 45 min; (c) $H_2N(CH_2)_2NH_2$, *n*-BuOH, 90 °C, 8 h; (d) pyridine, Ac_2O, r.t., 10 h; (e) HF pyridine, 0 °C to r.t., overnight; (f) SO_3 pyridine, pyridine, 0 °C to r.t.; (g) 0.1 M NaOMe, MeOH, r.t., 3 h; (h) $Pd(OH)_2$/C, H_2, CH_3OH, 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* α2-3-sialyltransferase 1 (PmST1) M144D mutant.[40]

multocida α2-3-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 α1-3-fucosylation of the corresponding *O*-sulfated disaccharides was tested as a potential strategy. A one-pot three-enzyme (OP3E) α1-3-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* α1-3-fucosyltransferase (Hp1-3FTΔ66 or Hp3FT) was used for this purpose. The *O*-sulfated disaccharides tested were 6-*O*-sulfo-LacNAcβProN$_3$ (**4**) (Scheme 1), 6′-*O*-sulfo-LacNAcβProN$_3$ (**5**), and 6,6′-di-*O*-sulfo-LacNAcβProN$_3$ (**6**) (Fig. 2). LacNAcβProN$_3$ (ref. 43) without any *O*-sulfate groups was used as a positive control.

6-*O*-Sulfo-LacNAcβProN$_3$ (**4**) was synthesized from 6-*O*-sulfo-GlcNAcβProN$_3$ (**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* β1-4-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* 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.[43] The activity of Hp4GalT in synthesizing 6-*O*-sulfo-LacNAcβProN$_3$ (**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 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 PmPpA to produce UDP-Gal *in situ* from glucose-1-phosphate.[43] 6′-*O*-Sulfo-LacNAcβProN$_3$ (**5**) and 6,6′-di-*O*-sulfo-LacNAcβProN$_3$ (**6**) (Fig. 2) were chemically synthesized (see ESI†).

Among the three *O*-sulfated disaccharides tested, only 6-*O*-sulfo-LacNAcβProN$_3$ (**4**) was a suitable acceptor for Hp3FT to produce the desired 6-*O*-sulfo-Le^{x}βProN$_3$ (**8**). In contrast, 6′-*O*-sulfo-LacNAcβProN$_3$ (**5**) and 6,6′-di-*O*-sulfo-LacNAcβProN$_3$ (**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-LacNAcβProN$_3$ (**4**), the preparative-scale synthesis of 6-*O*-sulfo-Le^{x}βProN$_3$ (**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-Le^{x}βProN$_3$ (**8**) from a simple monosaccharide derivative 6-*O*-sulfo-GlcNAcβProN$_3$ (**7**) in an overall yield of 62%.

As Hp3FT was not able to use 6′-*O*-sulfo-LacNAcβProN$_3$ (**5**) or 6,6′-di-*O*-sulfo-LacNAcβProN$_3$ (**6**) efficiently as acceptors for fucosylation to obtain the desired Le^x trisaccharides, the target trisaccharides 6′-*O*-sulfo-Le^{x}βProNH$_2$ (**9**) and 6,6′-di-*O*-sulfo-Le^{x}βProNH$_2$ (**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 strategy[47] 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 H$_2$.[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 TBDPS 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 $Pd(OH)_2$/C and H_2 (ref. 55) to obtain the desired 6′-*O*-sulfo-Le^{x}βProNH$_2$ (**9**) and 6,6′-di-*O*-sulfo-Le^{x}βProNH$_2$ (**10**).

Enzymatic synthesis of *O*-sulfated sLe^x

With chemoenzymatically synthesized 6-*O*-sulfo-Le^{x}βProN$_3$ (**8**) as well as chemically synthesized 6′-*O*-sulfo-Le^{x}βProNH$_2$ (**9**) and 6,6′-di-*O*-sulfo-Le^{x}βProNH$_2$ (**10**) in hand, a one-pot two-enzyme (OP2E) sialylation system (Scheme 3) was used to test the tolerance of PmST1 M144D[40] 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 α2-3-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-Neu5Acα2-3Le^{x}βProN$_3$ (**1a**, 80 mg, 85%), 6-*O*-sulfo-Neu5Gcα2-3Le^{x}βProN$_3$ (**1b**, 22 mg, 47%), 6′-*O*-sulfo-Neu5Acα2-3Le^{x}βProNH$_2$ (**2a**, 75 mg, 82%), 6′-*O*-sulfo-Neu5Acα2-3Le^{x}βProNH$_2$ (**2b**, 45 mg, 60%), 6,6′-di-*O*-sulfo-Neu5Acα2-3Le^{x}βProNH$_2$ (**3a**, 42 mg, 64%), and 6,6′-di-*O*-sulfo-Neu5Gcα2-3Le^{x}βProNH$_2$ (**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 M144D[40] 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 M144D[40] 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-Le^{x}βProN$_3$ (**8**) can be efficiently produced from a simple monosaccharide derivative 6-*O*-sulfo-GlcNAcβProN$_3$ (**7**) by a sequential OPME β1-4-galactosylation and OPME α1-3-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.

Acknowledgements

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