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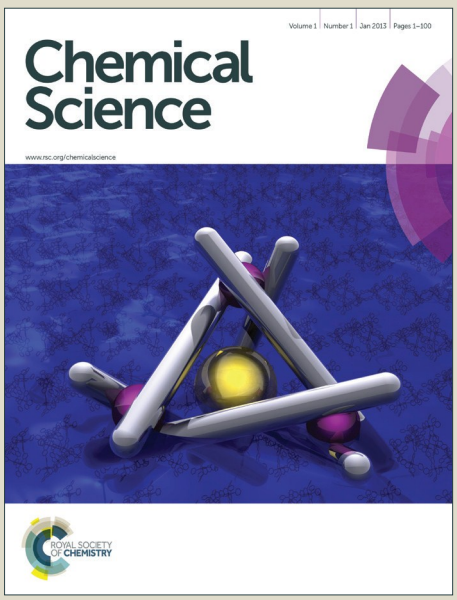

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ARTICLE

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.

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 is a well known ligand for L-selectin, a C-type (Ca^{2+}-dependent) carbonhydrate-binding protein (lectin) expressed broadly on most leukocytes in the blood.[1, 2] The interaction of 6-*O*-sulfo-sLe^x (**1**) and L-selectin plays critical roles 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 (Scheme 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 on human allergic inflammatory cells including eosinophils, mast cells, and basophils.[5, 10] Reducing the number of eosinophiles, 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 to human Siglec-7 moderately.[5] Both are presented 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 6',6-di-*O*-sulfo-sLe^x (**3**) with *O*-sulfate 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',6-di-*O*-sulfo-sLe^x (**3**) than its mono-*O*-sulfated derivative (**1**) or (**2**) while mouse Siglec-F has been shown to bound similarily 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 synthesis[20-22] and 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 chemical[28, 29] or chemoenzymatic[30] synthesis of 6-*O*-sulfo-sLe^x (**1**) as well as chemical synthesis of 6'-*O*-sulfo-sLe^x (**2**)[22, 31, 32] and 6',6-di-*O*-sulfo-sLe^x (**3**)[33] have been reported. All these

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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,[34, 35] *O*-sulfated sLe^x containing a sialic acid form other than Neu5Ac has not been synthesized.

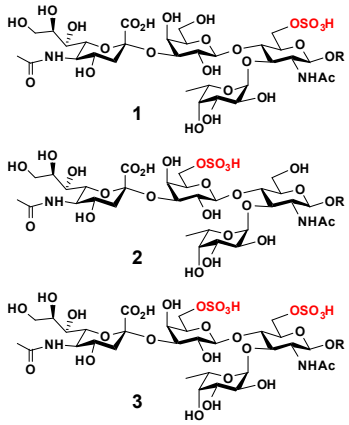


Fig. 1 Structures of *O*-sulfated sialyl Lewis x including 6-*O*-sulfo-sLe^x (**1**), 6'-*O*-sulfo-sLe^x (**2**), and 6', 6-di-*O*-sulfo-sLe^x (**3**).

We report here the development of efficient chemoenzymatic methods for 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 human but can be incorporated into the human glycome from dietary sources.[36]

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[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^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[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^x structures could be used by PmST1 M144D as suitable acceptors in OPME sialylation process to produce 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 test as a potential strategy. A one-pot three-enzyme (OP3E) α1–3-fucosylation system (Scheme 1)[40, 42] containing *Bacteroides fragilis* bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (BfFKP),[43] *Pasteurella multocida* inorganic pyrophosphorylase (PmPpA),[44] 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**) (Figure 2). LacNAcβProN$_3$[44] without any *O*-sulfate groups was used as a positive control.

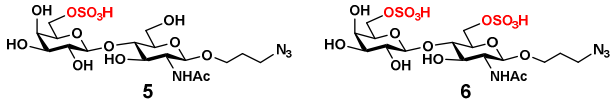
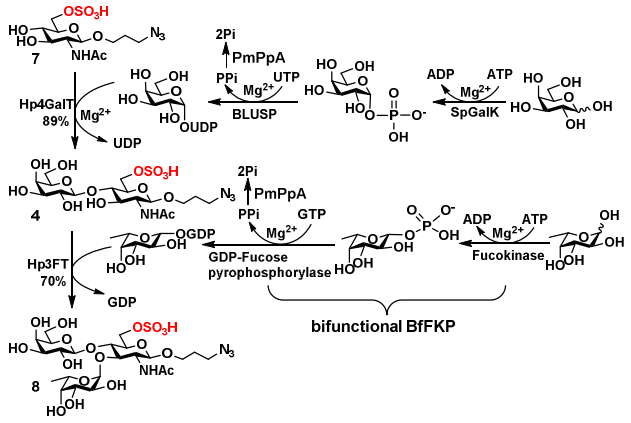


Fig. 2 Structures of chemically synthesized 6'-O-sulfo-LacNAcβProN$_3$ (5) and 6,6'-di-O-sulfo-LacNAcβProN$_3$ (6).



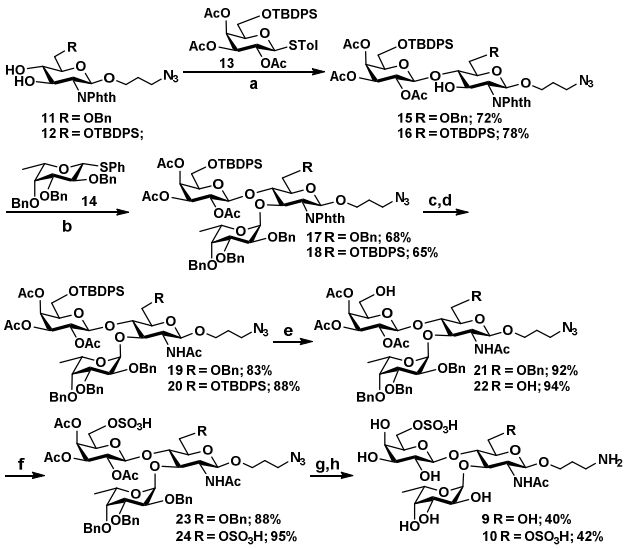
Scheme 1 Sequential OPME synthesis of 6-O-sulfo-Le^{x}βProN$_3$ (8) from 6-O-sulfo-GlcNAcβProN$_3$ (7) by OPME β1–4-galactosyl activation and transfer system for the formation of 6-O-sulfo-LacNAcβProN$_3$ (4) followed by OPME α1–3-fucosyl activation and transfer system for the formation of 6-O-sulfo-Le^{x}βProN$_3$ (8). Enzymes and abbreviations: SpGalK, *Streptococcus pneumoniae* TIGR4 galactokinase;[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;[43] and Hp3FT, *Helicobacter pylori* α1–3-fucosyltransferase.[40, 42]

6-*O*-Sulfo-LacNAcβProN$_3$ (**4**) was synthesized from 6-*O*-sulfo-GlcNAcβProN$_3$ (**7**)[44] 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 (Hp1–4GalT or 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.[44] The activity of Hp4GalT in synthesizing 6-*O*-sulfo-LacNAcβProN$_3$ (**4**) was confirmed again here using the improved OPME approach.[46, 47] 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 PmPpA to produce UDP-Gal *in situ* from glucose-1-phosphate.[44] 6'-*O*-Sulfo-LacNAcβProN$_3$ (**5**) and 6,6'-di-*O*-sulfo-LacNAcβProN$_3$ (**6**) (Scheme 2) were chemically synthesized (see supporting information).

Among 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**) nor 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[48] for the synthesis of two trisaccharides (i.e. similar protecting groups were used in the synthesis and same reagents were used for their removal); (b) use of similar thioglycosides 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 H$_2$.[49] 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** selectively protected with TBDPS at C6 in the presence of *N*-iodosuccinimide (NIS) and trimethylsilyl trifluoromethanesulfonate (TMSOTf)[50] 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.[27] Initial attempts to glycosylate acceptor **15** and **16** in dichloromethane with 1.2 equivalents of thiophenyl fucoside **14** produced trisaccharides of alpha and beta mixtures. In contrast, stereospecific formation of trisaccharides was achieved when a mixed solvent of diethylether and dichloromethane (1:1)[51, 52] 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;[53] (c) *O*-sulfation of the primary hydroxyl group by SO$_3$·pyridine complex;[53, 54] (d) deacetylation by NaOMe in MeOH;[55] and (e) hydrogenation using Pd(OH)$_2$/C and H$_2$[56] to obtain desired 6'-*O*-sulfo-Le^{x}βProNH$_2$ (**9**) and 6,6'-di-*O*-sulfo-Le^{x}βProNH$_2$ (**10**).

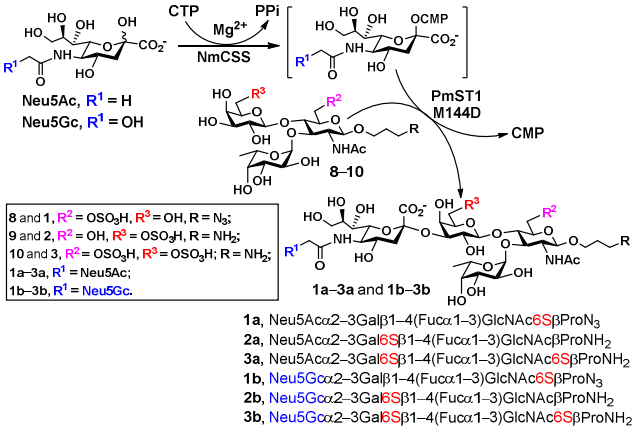


Scheme 2 Chemical synthesis of 6'-*O*-sulfo-Le^{x}βProNH$_2$ (**9**) and 6,6'-di-*O*-sulfo-Le^{x}βProNH$_2$ (**10**). Reagents and conditions: a) *N*-iodosuccinimide (NIS), TMSOTf, MS 4 Å, CH_2Cl_2, −40 °C, 30 min; b) *N*-iodosuccinimide (NIS), TMSOTf, MS 4 Å, CH_2Cl_2-Et_2O (1:1), -18 °C, 45 min; c) $H_2N(CH_2)_2NH_2$, *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.

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[41] in 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 of the wild-type enzyme. It was used efficiently in a one-pot three-enzyme (OP3E) sialylation systems for the synthesis of non-sulfated sLe^x tetrasaccharides containing diverse sialic acid forms from Le^x.[41] 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 by this

highly efficient one-pot two-enzyme system containing *Neisseria meningitidis* CMP-sialic acid (NmCSS)[38] and PmST1 M144D[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 **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**, **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[41] should be used.



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;[38] PmST1 M144D, *Pasteurella multocida* α2–3-sialyltransferase 1 (PmST1) M144D mutant.[41]

Conclusions

In conclusion, we have successfully developed an efficient chemoenzymatic method for 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, 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,[41] but also for producing biologically important but difficult-to-obtain *O*-sulfated sLe^x.

Acknowledgements

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