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Redox-Controlled Site-Specific α2−6-Sialylation

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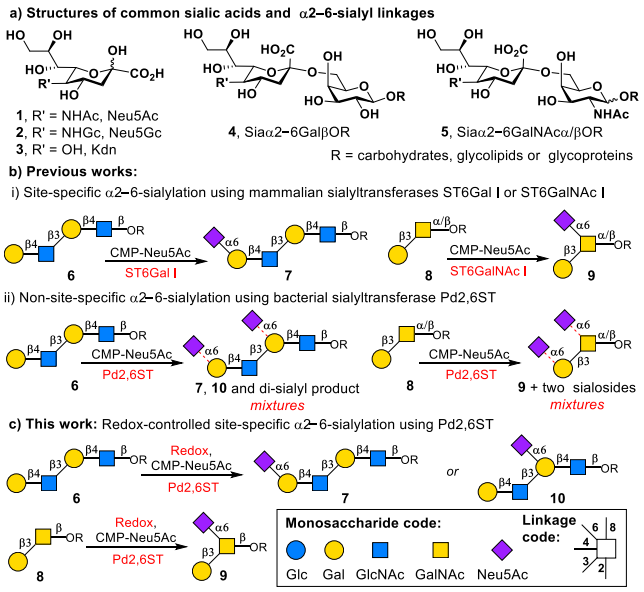
Supporting Information

ABSTRACT: The first bacterial α2−6-sialyltransferase cloned from *Photobacterium damselae* (Pd2,6ST) has been widely applied for the synthesis of various α2−6-linked sialosides. However, the extreme substrate flexibility of Pd2,6ST makes it unsuitable for site-specific α2−6-sialylation of complex substrates containing multiple galactose and/or *N*-acetylgalactosamine units. To tackle this problem, a general redox-controlled site-specific sialylation strategy using Pd2,6ST is described. This approach features site-specific enzymatic oxidation of galactose units to mask the unwanted sialylation sites and precisely controlling the site-specific α2−6-sialylation at intact galactose or *N*-acetylgalactosamine units.

Sialic acids (Sias) are the most common termini and among the most abundant monosaccharides of mammalian glycans.[1] As ubiquitous components of glycoproteins and glycolipids, three common sialic acids of *N*-acetylneuraminic acid (Neu5Ac, **1**), *N*-glycolylneuraminic acid (Neu5Gc, **2**) and 2-keto-3-deoxy-nonulosonic acid (Kdn, **3**) are found α2−6-linked to galactose (Gal) or *N*-acetylgalactosamine (GalNAc) residues (Scheme 1a). Owing to their remarkable structural diversity, sialic acid-containing glycans play important roles in many physiological and pathological processes, and most sialic acid-related biological processes require specific sialic acid forms, glycosidic linkage and defined underlying glycan chains.[2] For example, avian influenza viruses primarily bind to α2−3-linked sialic acid, whereas human influenza viruses preferentially recognize α2−6-linked sialic acid.[3] Recent studies also demonstrated that α2−6-sialylation is required for the anti-inflammatory activities of intravenous immunoglobulin (IVIG), and *in vitro* glycoengineered IVIG with uniform α2−6-sialylated *N*-glycans showed 10-fold enhancement in anti-inflammatory activities compared to unfractionated IVIG.[4] It is also well-known that the high expression levels of α2−6-sialylated glycans on a number of carcinomas are correlated with cancer progression and poor prognosis.[2b,5]

The past few decades have witnessed increasing attention on using glycosyltransferases for the synthesis of various glycans and glycoconjugates.[6] However, only very few recombinant sialyltransferases (SiaTs) from both mammals and bacteria have been widely applied for the construction of both α2−6-sialyl linkages **4** and **5** (Scheme 1a). ST6Gal I is a mammalian α2−6-SiaT which has been widely used for the α2−6-sialylation of terminal Gal residue, but it has very strict substrate specificity that can only use terminal type-2 glycan (Galβ1−4GlcNAc) as acceptor substrate (Scheme 1b, i).[7] ST6GalNAc I is another recombinant mammalian α2−6-SiaT which has been used for the α2−6-sialylation of GalNAc residue, but it can only use Tn antigen or T antigen as acceptor substrates (Scheme 1b, i).[8] The α2−6-sialylation of terminal Gal residues of type-1 glycan (Galβ1−3GlcNAc) and LacdiNAc (GalNAcβ1−4GlcNAc) have also been identified

Scheme 1. (a) Common Sialic Acid Forms and α2−6-Linkages; (b) Known and (c) Proposed Enzymatic α2−6-Sialylation Approaches



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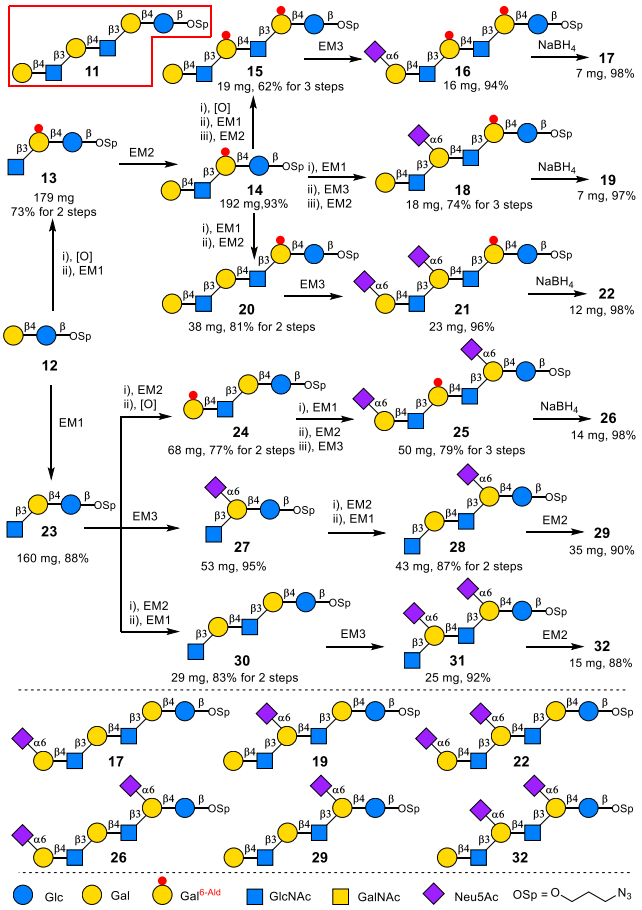
in a number of naturally occurring glycans;[9] however, the SiaTs responsible for the α2−6-sialylation modification are still unknown. Moreover, α-series cholinergic neuron-specific gangliosides, a subgroup of gangliosides with α2−6-sialylation on the GalNAc residue of extended type 1 glycan chain, play important roles in the development and regeneration of nervous system.[10] However, due to the substrate restriction and unavailability of siaT for modification of GalNAc residue, none of these complex sialoglycans have been enzymatically synthesized yet.[11]

In contrast to mammalian α2−6-SiaTs, the first recombinant bacterial α2−6-SiaT cloned from *Photobacterium damselae* (Pd2,6ST) can be overexpressed in a conventional *Escherichia coli* strain.[12] Owing to its remarkable activity and extreme substrate specificities, the Pd2,6ST has been extensively used for the construction of both Siaα2−6Gal and Siaα2−6GalNAc sequences for the synthesis of various *O*-glycans, *N*-glycans and human milk oligosaccharides.[7d,12a,13] Unfortunately, previous studies showed that the Pd2,6ST can recognize both internal and terminal Gal and GalNAc moieties of complex substrates, resulting a mixture of sialylated products (Scheme 1b, ii).[7d,13e−g] To overcome the limitations of both mammalian and bacterial α2−6-SiaTs, we describe herein a novel redox-controlled site-specific α2−6-sialylation approach to precisely control the reaction sites of Pd2,6ST for the synthesis of complex α2−6-linked sialosides (Scheme 1c).

To validate the feasibility of our proposed redox-controlled site-specific α2−6-sialylation strategy, lacto-*N*-neohexaoside (LNnH) **11** containing three Gal residues (Scheme 2) was investigated first as a model substrate. LNnH potentially has seven α2−6-sialylated products, including three monosialylated heptasaccharides **17**, **19** and **29**, and three disialylated octasaccharides **22**, **26** and **32**. Galactose oxidase (GOase), a commercially available copper metalloenzyme, has been widely used in biosensor for detecting Gal or terminal Gal-containing glycans by selective oxidation of the C6-hydroxyl group of free galactose or terminal Gal residue into C6-aldehyde galactose (Gal$^{6\text{-Ald}}$).[14] The resulting C6-aldehyde group has also been extensively used as a chemical handle for further labeling and derivatization purposes.[14,15] We envisioned that the Gal$^{6\text{-Ald}}$ would act as a protected Gal residue and would not be sialylated by Pd2,6ST. Therefore, the site-specifically introduction of α2−6-linked sialic acid to the intact Gal residues could be achieved. To obtain monosialylated heptasaccharide **17** with an α2−6-linked sialic acid at nonreducing end of hexasaccharide **11**, both internal Gal residues of **11** need to be protected by oxidation, while the terminal Gal was left intact for site-specific α2−6-sialylation. As shown in Scheme 2, lactoside **12** was treated with oxidation module in the presence of GOase and peroxidase to convert the terminal Gal residue into Gal$^{6\text{-Ald}}$. The resulting disaccharide was then extended by a one-pot three-enzyme β1−3-*N*-acetylglucosaminylation module (EM1)[13b,d] to give trisaccharide **13** in 73% yields for 2 steps. Trisaccharide **13** was elongated by a β1−4-linked Gal using enzyme module 2 (EM2),[13b,d] and the nascent terminal Gal was also converted into Gal$^{6\text{-Ald}}$ by oxidation module, and then extended by sequential glycosylation with EM1 and EM2 to afford the hexasaccharide intermediate **15**. Although, all C6-aldehyde groups of oxidized Gal moieties exist as hydrated germinal diols, none of them could be utilized by Pd2,6ST in EM3[13b,d] as sialylation site. The monosialylated **16** was isolated as only product in 94% yield. Two Gal$^{6\text{-Ald}}$ residues of **16** can be reduced back to the Gal moieties by simply

Scheme 2. Redox-Controlled Site Specific α2−6-Sialylation of Lacto-*N*-neohexaoside (LNnH) 11[a]



[a]Reagents and conditions: [O], one-pot two enzyme oxidation module with commercial galactose oxidase and peroxidase; EM1, one-pot three-enzyme β1−3-*N*-acetylglucosaminylation module with BlNahK, EcGlmU and HpLgtA; EM2, one-pot three-enzyme β1−4-galactosylation module with EcGalK, BLUSP and NmLgtB; EM3, one-pot two-enzyme α2−6-sialylation module with NmCSS and Pd2,6ST, see Supporting Information for details.

treating with $NaBH_4$ in aqueous solution to provide monosialylated heptasaccharide **17** in 98% yield. The same redox-controlled site-specific α2−6-sialylation strategy was also successfully applied in the synthesis of monosialylated heptasaccharide **19**, disialylated octasaccharides **22** and **26** using corresponding Gal$^{6\text{-Ald}}$-containing glycans as intermediates. The syntheses of monosialylated heptasaccharide **29** and disialylated octasaccharide **32** can be realized by simply changing the glycosylation sequence of three same enzyme modules (EM1−EM3) without the need of oxidation module (Scheme 2).

The Siaα2−6Gal sequence is not only a common terminal component of various *N*-, *O*-glycans and glycolipids, but also has been identified as internal moieties in a number of naturally occurring glycans.[9d,16] However, the SiaT from either mammalian or bacteria source that can catalyze site-specific α2−6-sialylation at the internal Gal is still unknown. The redox-controlled α2−6-sialylation strategy provides a practical approach to harness the extreme substrate flexibility offered by readily available bacterial sialyltransferase Pd2,6ST, thus

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provides the first synthetic approach for on demand site-specific α2−6-sialylation of poly-LacNAc glycans.

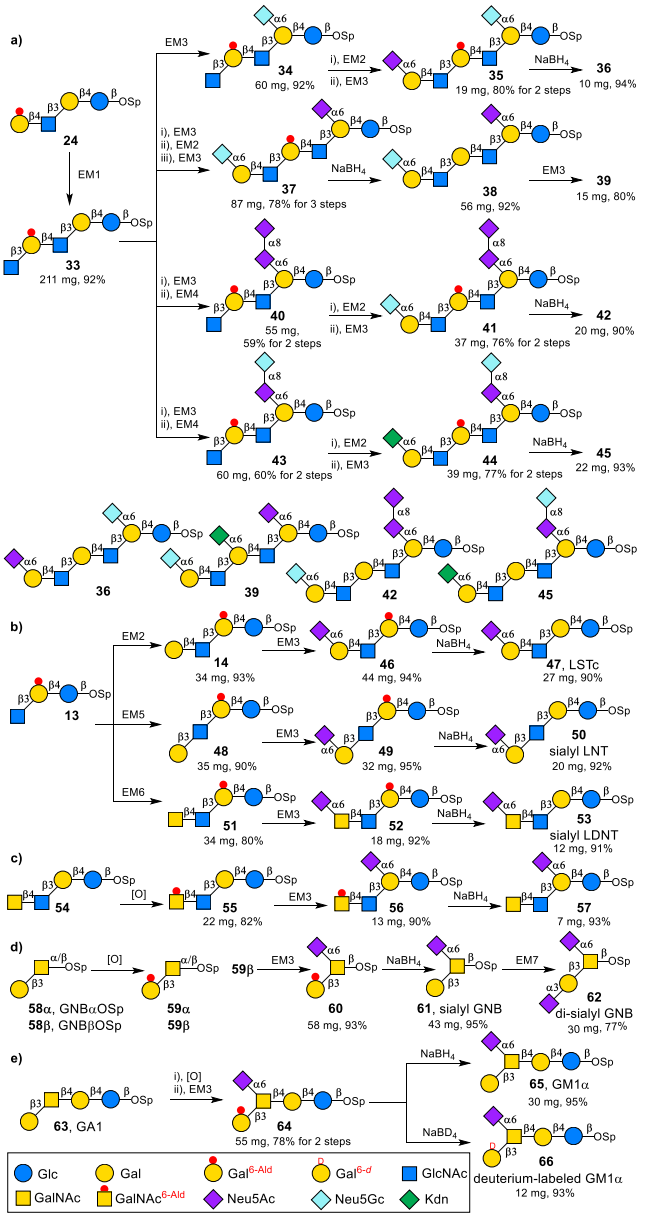
This redox-controlled site-specific α2−6-sialylation strategy also provides an easy access for the synthesis of complex sialosides bearing different α2−6-linked sialic acid forms. As shown in Scheme 3a, the tetrasaccharide **24** with a terminal Gal$^{6\text{-Ald}}$ was extended using EM1, then the innermost intact Gal was modified with an α2−6-linked Neu5Gc using EM3 in the presence of Neu5Gc as the donor precursor to give hexasaccharide **34**. The **34** was treated with EM2 to form a LacNAc termini which was further modified with an α2−6-linked Neu5Ac by EM3 in the presence of Neu5Ac as the donor precursor to afford intermediate **35**. The Gal$^{6\text{-Ald}}$ moiety of **35** was reduced back to Gal by $NaBH_4$ to give disialyl octasaccharide **36** containing hybrid sialic acid forms in excellent overall yields. Taking advantage of this redox-controlled site-specific sialylation strategy, octasaccharide **38** with two different sialic acid forms, and nonasaccharide **39** with three different sialic acid forms at designated positions were also achieved from **24** (Scheme 3a). Moreover, a one-pot two-enzyme α2−8-sialylation enzyme module (EM4) comprising a recombinant α2−8-sialyltransferase from *Campylobacter jejuni* (CjCstII)[17] was applied for the modification of innermost α2−6-linked Neu5Ac to produce nonasaccharide **42** and **45** in good overall yields, respectively (Scheme 3a, see Supporting Information for details).

Having established the redox-controlled site-specific α2−6-sialylation strategy for poly-LacNAc glycan receptors, the substrate scope and general applicability of the strategy was explored next. As shown in Scheme 3b, the terminal GlcNAc moiety of trisaccharide **13** was parallelly elaborated to type 2 chain by EM2 to give **14**, type 1 chain by EM5 to give **48**, and LacdiNAc by EM6 to give **51**, respectively. In addition to bacterial β1−4GalT NmLgtB in EM2, the recombinant β1−3GalT from *E. coli* (EcWbgO)[18] in EM5, and recombinant bovine GalT 1 mutant (GalT1 Y289L)[19] in EM6 all utilize trisaccharide **13** as receptor efficiently. As anticipated, the extreme substrate flexibility of Pd2,6ST in EM3 ensured the α2−6-sialylation at the terminal Gal of **14** and **48**, and terminal GalNAc of **51** furnishing pentasaccharides **46**, **49** and **52**, which were then treated with $NaBH_4$ to give the sialyl LNnT (LSTc) **47**, sialyl LNT **50** and sialyl LacdiNAc (sialyl LDNT) **53** in excellent yields, respectively (Scheme 3b). The GOase can also selectively oxidize the terminal GalNAc unit. Therefore, the selective oxidation of terminal GalNAc unit of LDNT **54** could achieve site-specific α2−6-sialylation at the internal Gal. After reduction, the monosialylated LDNT **57** was obtained in 69% overall yields for 3 steps (Scheme 3c).

The redox-controlled sialylation strategy could also be applied for the site-specific α2−6-sialylation of internal GalNAc residue for the synthesis of sialyl GNB **61**, disialyl GNB **62** and α-series ganglioside glycan GM1α **65**. It was shown that the Pd2,6ST could modify both Gal and GalNAc residues of GNB **58*α*** or **58*β***, resulting a mixture of two monosialylated and one disialylated products for each of them (Scheme 1b, ii).[13f] For site-specific α2−6-sialylation at internal GalNAc residue, the terminal Gal of **58*α*** or **58*β*** was converted into Gal$^{6\text{-Ald}}$ by oxidation enzyme module to give **59*α*** or **59*β***. Both **59*α*** or **59*β*** were treated with α2−6-sialylation module EM3, however, only **59*β*** could be utilized by Pd2,6ST to afford the sialoside **60**. These results were consistent with previous report that the α-linked GalNAc is not a good substrate for Pd2,6ST.[12a] Sialoside **60** was reduced with $NaBH_4$ to afford sialyl GNB **61** in 73% yields for 3 steps. The **61** could be further elaborated to disialyl GNB **62** by an α2−3-sialylation enzyme module (EM7)[13b,20] (Scheme 3d).

As aforementioned, owing to the substrate restriction and unavailability of siaT for modification of GalNAc residue, none of α-series cholinergic neuron-specific gangliosides has been enzymatically synthesized yet. The first enzymatic synthesis of α-series ganglioside glycan GM1α **65** using redox-controlled

Scheme 3. Redox-Controlled Site Specific α2−6-Sialylation with Different Sialic Acid Forms and Substrate Scope and Application of Redox-Controlled Site-Specific α2−6-Sialylation Strategy[a]



[a]Reagents and conditions: EM4, one-pot two-enzyme α2−8-sialylation module with NmCSS and CjCstII; EM5, one-pot three-enzyme β1−3-galactosylation module with EcGalK, BLUSP and EcWbgO; EM6, one-pot three-enzyme β1−4-*N*-acetylgalactosaminylation module with BlNahK, EcGlmU and GalT1 Y289L; EM7, one-pot two-enzyme α2−3-sialylation module with NmCSS and PmST1, see Supporting Information for details.

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sialylation strategy was also explored. Starting from known asialo-GM1 (GA1) **63**,[21] a similar sequence involving enzymatic oxidation, selective α2−6-sialylation of internal GalNAc, and subsequent reduction provided ganglioside GM1α **65** in 74% yields for 3 steps (Scheme 3e). Interestingly, the Pd2,6ST in EM3 can only introduce α2−6-linked Neu5Ac at GalNAc residue to give pentasaccharide **64**, while sialylation of innermost Gal residue was not observed. The redox-controlled sialylation strategy utilized for the synthesis of GM1α **65** provides a novel approach for the synthesis of other α-series gangliosides. Besides, by replacing $NaBH_4$ with $NaBD_4$ in the reduction step, the $Gal^{6\text{-}Ald}$-containing intermediates in this study, such as **64**, can be transformed into deuterium labeled products (e.g., GM1α **66**), which could be used as probe in elucidating multiple biochemical processes (Scheme 3d, see Supporting Information for details).

The binding profiles of synthesized sialosides with sialic acid-recognition proteins were also examined using printed sialoglycan slides. The plant lectin *Sambucus nigra* agglutinin (SNA) is known to be able to specifically recognize α2−6-linked sialosides.[22] SNA exhibited very strong binding exclusively to all sialosides with a terminal α2−6-sialylated LacNAc moiety, while no significant binding was observed for internal α2−6-sialylated glycans (Figure S7). Chicken polyclonal anti-Neu5Gc antibody IgY (pChGc) bound to all Neu5Gc-containg glycans with a preference toward terminal α2−6-linked Neu5Gc (Figure S8). In contrast, human anti-Neu5Gc antibody rich serum only bound tightly with terminal α2−6-linked Neu5Gc (Figure S9). The His-tagged typhoid toxin (PltB-His) recognized all Neu5Ac modified glycans (Figure S10), while human sialic acid-binding lectin Siglec-9 (hSiglec-9-Fc) only bound tightly to terminal Neu5Ac-modified glycans and Neu5Acα2−8Neu5Acα2−6-linked glycan (Figure S11). Plant lectins *Maackia amurensis* lectin I and II (MAL-I, -II) were also examined. As expected only α2−3-linked sialoside **62** exhibited strong affinity to MAL-II (Figure S12−S13).[22]

In summary, a novel substrate engineering strategy[23] was developed to harness the extreme substrate flexibility of Pd2,6ST for site-specific α2−6-sialylation of both Gal and GalNAc residues of various substrates. This strategy overcomes the limitation of availability and substrate specificities of known mammalian and bacterial α2−6-sialyltransferases, thereby providing a general and concise approach for the synthesis of complex α2−6-sialylated glycans with a single bacterial α2−6-sialyltransferase Pd2,6ST.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b00044.

Detailed experimental procedures and product characterization (PDF)

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Notes

The authors declare no competing financial interest.

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