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Highly Efficient Chemoenzymatic Synthesis of Naturally Occurring and Unnatural α2,6-Linked Sialosides: A *P. damsela* α2,6-Sialyltransferase with Extremely Flexible Donor Substrate Specificity**

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Keywords

carbohydrates; chemoenzymatic synthesis; enzyme catalysis; sialic acid; transferases

Sialic acids are a family of α-keto acids with a 9-carbon backbone. They have been predominantly found as terminal carbohydrate units on glycoproteins and glycolipids of vertebrates or as components of capsular polysaccharides and lipooligosaccharides of pathogenic bacteria.[1] Sialic acid-containing structures play pivotal roles in many physiologically and pathologically important processes, including cellular recognition and communication, bacterial and viral infection, and tumor metastasis, etc.[1] Currently, greater than 50 structurally distinct forms of sialic acids have been found in nature.[1] From which, more than 15 have been found on human red blood cell surfaces, saliva proteins, and gastrointestinal mucins.[2] Three basic forms of sialic acids (Scheme 1) are *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminc acid (KDN). Based on these three forms, single or multiple substitutions can occur at the hydroxyl group on C-4, C-5, C-7, C-8, and/or C-9 positions, including *O*-acetylation and the less frequent *O*-methylation, *O*-lactylation, *O*-sulfation, and *O*-phosphorylation (Scheme 2).[1]

Modifications of sialic acids and cell surface presentation of modified sialic acids are species- and tissue-specific. They are developmentally regulated and are believed to be closely related to their biological functions.[1] Nevertheless, a clear understanding of the mechanism and the significance of nature's sialic acid structural diversity is currently missing. This is mainly due to the difficulties in obtaining homogenous sialosides or sialylglycoconjugates, especially those contain diverse naturally occurring sialic acid modifications. These structures are extremely difficult to isolate in homogenous forms from natural sources[3] and chemical sialylation remains challenging.[4] Although sialyltransferase-catalyzed synthesis offers great advantages,[5] it suffers from the low expression level and the narrow substrate specificity of many sialyltransferases, especially those from mammalian sources.[6] Current chemical[4,7] and enzymatic[5,8] sialylation activities have been focusing on structures containing unnatural

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sialic acid derivatives and a limited number of natural sialic acid forms (e.g. Neu5Ac, Neu5Gc, KDN, Neu5Ac8Me, and Neu5,9Ac$_2$).[3–9] Further development of the synthetic methodology, thus, is required to obtain structurally defined naturally occurring sialosides for better understanding of their biological roles.

Sialic acid modifications, such as *O*-acetylation, *O*-methylation, *O*-lactylation, and *O*-sulfation, are believed to occur after the formation of sialylglycoconjugates or polysialic acids in mammals,[10] group C *meningococci*,[11] and *E. coli*.[12] There is indication, however, that *O*-acetylation may occur on free Neu5Ac in the biosynthesis of GBS (Group B *Streptococcus*) capsular polysaccharide.10b Only a few enzymes involved in the sialic acid modifications have been discovered, including 9-*O*-acetyltransferases from rat liver,10a bovine submandibular gland,[13] *E. coli* K1,[1b,14] and *C. jejuni*;[15] 4-*O*-acetyltransferases from guinea pig liver[16] and equine submandibular gland;[17] a sialic acid 8-*O*-methyltransferase from starfish *A. rubens*.[18] CMP-Neu5Ac hydroxylase[19] and a *C. jejuni* 9-*O*-acetyltransferase[15] are the only proteins in this category that have ever been cloned. Due to the unavailability of these sialic acid modifying enzymes, it is impractical to synthesize naturally occurring sialosides by totally following their biosynthetic pathways.

Instead of nature’s way of introducing sialic acid modifications after the oligosaccharide formation, we have established a highly efficient and convenient one-pot three-enzyme chemoenzymatic approach for the synthesis of sialosides containing naturally occurring as well as unnatural sialic acid modifications at C-5, C-7, C-8, and/or C-9. In this method, sialic acid modifications can be chemically introduced at the very beginning, onto the six carbon sugar precursors (ManNAc or mannose) of sialic acids. These ManNAc or mannose analogs can then be directly converted to the corresponding sialosides in one-pot using three enzymes, including a sialic acid aldolase, a CMP-sialic acid synthetase, and a sialyltransferase without the isolation of intermediates (Scheme 3). Depending on the type of the sialyltransferase used, α2,3- or α2,6- linked sialosides can be synthesized conveniently. Combining the diversity of organic synthesis and the highly efficient, regio- and stereo-selective enzymatic approaches, this chemoenzymatic system is an attractive synthetic method for complex sialic acid-containing structures.

Two major challenges for highly effective chemoenzymatic synthesis are i) availability and efficiency of biosynthetic enzymes; and ii) extent of substrate modification that can be tolerated by the enzymes. We have successfully found solutions for synthesizing complex sialosides by matching these two challenges.

Previously, we reported the high level expression of a recombinant sialic acid aldolase from *E. coli* K-12 and a CMP-sialic acid synthetase from *N. meningitidis* (NmCSS).[20] Both enzymes have flexible substrate specificity. They have been used successfully in one-pot two-enzyme preparative synthesis of CMP-sialic acid derivatives[20] and one-pot three-enzyme synthesis of α2,3-linked sialoside libraries.[21] In order to prepare α2,6-linked sialosides with naturally occurring or unnatural sialic acid modifications, we investigated the donor substrate specificity of a recombinant *Photobacterium damsela* α2,6-sialyltransferase (Pd2,6ST) and explored its application in the one-pot three-enzyme synthesis.

Pd2,6ST was the first bacterial α2,6-sialyltransferase that has ever been cloned.[22] The isolation of this sialyltransferase was firstly reported in 1996.[23] Subsequently, its extremely flexible acceptor substrate specificity was described.[8c,24] No study, however, has been reported on the donor substrate specificity of this enzyme.

In order to facilitate the protein purification, a truncated Pd2,6ST encoding amino acid residues 16–497 of the full length protein was cloned into pET15b vector and expressed as an N-His$_6$-tagged protein. The recombinant Pd2,6ST was purified by a nickel-nitrilotriacetic acid (Ni-

NTA) agarose affinity column. The tolerance of donor substrate modification of the purified Pd2,6ST was firstly tested by thin-layer chromatography (TLC) analysis (EtOAc : MeOH : H_2O : HOAc = 4 : 2 : 1: 0.1) in the one-pot three-enzyme system shown in Scheme 3, in which CMP-sialic acid derivatives were generated in situ from sialic acid precursors catalyzed by the aldolase and NmCSS. For sialosides that do not contain *O*-acetyl or *O*-lactyl groups, reactions were typically performed in a Tris-HCl buffer at pH 8.5 for 2–10 h at 25 °C. When this condition was used for the synthesis of sialosides containing *O*-acetyl groups, de-*O*-acetylation was observed. Therefore, a Tris-HCl buffer (pH = 7.5) with lower pH value was used for synthesizing sialosides containing *O*-acetylated or *O*-lactylated sialic acid residues to avoid the hydrolysis of the esters. After observing the product formation on TLC, preparative scale syntheses were carried out. Sialoside products were purified by gel filtration chromatography and characterized by NMR and high resolution mass spectrometry (HRMS).

As shown in Table 1, using 3-azidopropyl lactoside **20** as an acceptor for Pd2,6ST, naturally occurring α2,6-linked sialosides containing Neu5Ac or its C5-substituted analogs (**26**–**32**) were firstly synthesized in 75–99% yields (entries **a**–**g**) from ManNAc, mannose, or their C2-modified analogs (**1**–**7**) as sialic acid precursors. 5-*O*-Acetyl-KDN (KDN5Ac) (the sialic acid form in **29**) has been found on the eggs of *Rana temporaria*, *Rana arvalis*, and *Pleurodeles waltii*;[2b] 5-*O*-methyl-KDN (KDN5Me) (the sialic acid form in **30**) has been observed in *Sinorhizobium fredii*;[25] *N*-acetylglycolyl-neuraminic acid (Neu5GcAc) (the sialic acid form in **31**) has been detected in rat peritoneal macrophages.[26]

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Naturally occurring α2,6-linked sialosides containing a C9-substituted Neu5Ac or KDN (**33**–**35**) were synthesized in 72–84% yields (entries **h**–**j**) from the corresponding C6-modified ManNAc or mannose (**8**–**10**). 9-*O*-Acetylation has been found in nearly all higher animals and certain bacteria and is believed to play a pivotal role in modulating various biological processes.[27] For example, 9-*O*-acetyl-*N*-acetylneuraminic acid ($Neu5,9Ac_2$) (the sialic acid form in **33**) is an essential determinant of the cell surface receptors for influenza C virus, but prevents the recognition of influenza A and B viruses.[28] It is also presented in an antigenic ganglioside found in developing rat embryonic cells.[29] 9-*O*-Lactyl-Neu5Ac (Neu5Ac9Lt) (the sialic acid form in **34**) has been found in bovine submandibular gland glycoprotein[30] and human RBC membranes.[2a] 9-*O*-Acetyl-KDN (KDN9Ac) (the sialic acid form in **35**) has found in *O. nerka adonis* polysialoglycoproteins.[31]

Naturally occurring α2,6-linked sialosides containing a di-substituted sialic acid residue **36** and **37** have also been synthesized successfully in 78% and 65% yields (entries **k** and **l**), respectively. 9-*O*-Acetyl-Neu5Gc (Neu9Ac5Gc) (the sialic acid form in **36**) has been found in bovine submandibular gland glycoprotein.[30] 5,9-Di-*O*-acetyl-KDN ($KDN5,9Ac_2$) (the sialic acid form in **37**) has also been found in nature.[1a]

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Using the same 3-azidopropyl lactoside **20** as the acceptor for Pd2,6ST, unnatural sialosides containing a 4,6-bis-*epi*-KDO (**38**) and an *N*-(benzyloxycarboxyamido) glycinylamido-neuraminic acid (NeuGlyCbz) (**39**) were successfully synthesized in high yields (entries **m** and **n**). The successful synthesis of the KDO analogue containing trisaccharide **38** demonstrates that Pd2,6ST can transfer sialic acids with carbon backbones shorter than nine to galactoside with high efficiency. Furthermore, a bulky Cbz group at C-9 of sialic acid residue of the donor does not affect the activity of Pd2,6ST, which further demonstrates the extremely flexible donor substrate specificity of this enzyme.

Using GalβOMe (**21**) as an acceptor for Pd2,6ST, α2,6-linked sialosides containing sialic acid modified with unnatural substitutents (**40**–**44**), such as an azido or an acetylene group, were also synthesized in excellent yields (86%–93%) from their C2- or C6- modified ManNAc or mannose bearing azide or alkyne functional groups **15**–**19**. The resulting sialosides tagged with an azide or alkyne group can be easily linked to other molecules via Staudinger ligation[32] or Click chemistry.[33]

Acceptor specificity of the recombinant Pd2,6ST was explored using an *N*-acetyl β-galactosaminide **22**, an *N*-acetyl α-galactosaminide **23**, an α-galactoside (methyl α-D-galactopyranoside), β-galactosides **20**, **21** and **24**, and an α2,3-linked sialoside **25**. Similar to that reported previously,[8c,23] Pd2,6ST showed a much broader acceptor specificity compared to mammalian sialyltransferases.[24] The recombinant Pd2,6ST could use both α or β-linked GalNAc derivatives. Furthermore, β-galactosides and α2,3-linked sialosides were all excellent acceptors for the sialyltransferase. In contrast to that reported,[23] however, α-galactosides were not acceptors for the enzyme.

In conclusion, we have demonstrated that the recombinant *P. damsela* α2,6-sialyltransferase does not only have relaxed acceptor substrate specificity, but also have extremely flexible donor substrate specificity. We have also proved the practical application of the one-pot three-enzyme chemoenzymatic approach in effective preparative-scale synthesis of diverse sialosides with naturally occurring or unnaturally sialic acid modifications. This system can be easily extended to large-scale synthesis. Sialyltransferases, together with sialic acid aldolases and CMP-sialic acid synthetases, are important tools for simple and efficient preparation of sialoside libraries. These structurally defined synthetic sialosides will be essential standards for developing analytical methods. They are key probes to elucidate the biological significance of nature’s sialic acid diversity, the biosynthetic and degradation pathways of structurally modified sialic acids, and their involvement in the physiological and pathological processes of human and other vertebrates. The synthetic sialosides are also important ligands for identifying specific lectins and producing specific antibodies, which in turn, are vital tools in histochemical studies of organ-and species-specific presentation of modified sialic acids.

Experimental Section

Preparative scale (50–150 mg) synthesis: Reactions were typically carried out in a 50 mL centrifuge tube in 10 mL of Tris-HCl buffer (100 mM; pH 8.5 or pH 7.5) containing an acceptor (lactose, GalNAc, or galactose derivatives, 50–100 mg), ManNAc or mannose derivatives (1.5 equiv.), sodium pyruvate (7.5 equiv.), CTP (1.5 equiv.), $MgCl_2$ (20 mM), aldolase, NmCSS, and Pd2,6ST. The reaction mixture was incubated at 25 °C for 12 h with shaking (120 rpm). After adding the same volume of ice-cold methanol to stop the reaction, insoluble material was removed by centrifugation. The supernatant was concentrated by rotor evaporation and purified by Bio-Gel P2 gel filtration chromatography. Lyophilized sialoside products were characterized by NMR and high resolution mass spectrometry (HRMS).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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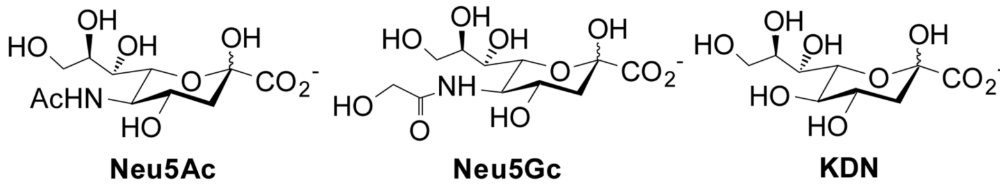
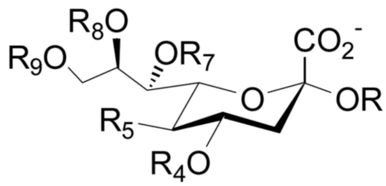
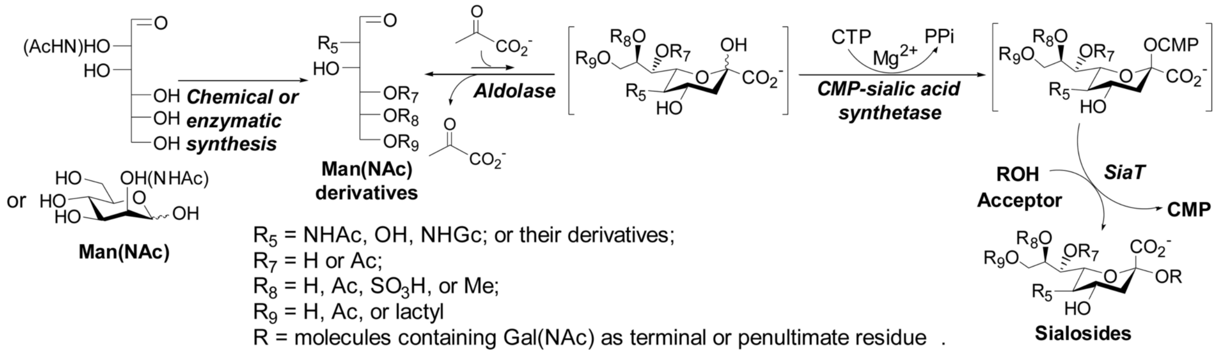


Scheme 1.
Three basic forms of naturally occurring sialic acids.



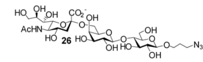
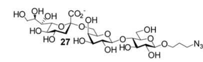
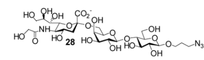
R_4 = H or acetyl
R_5 = NHAc, OH, NHGc; or their derivatives
R_7 = H or acetyl
R_8 = H, acetyl, sulfate, or methyl
R_9 = H, acetyl, lactyl, or phosphate

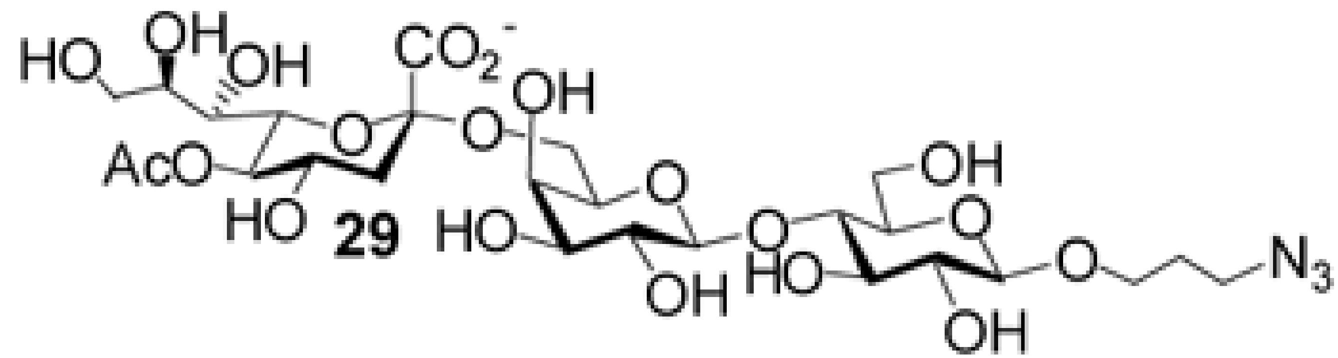
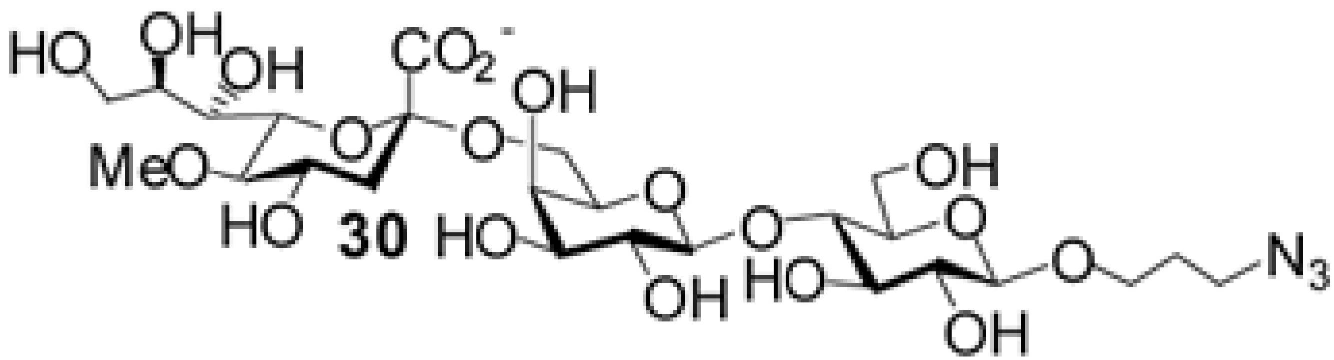
Scheme 2.
Naturally occurring sialic acid modifications.



Scheme 3.

An efficient chemoenzymatic approach for the synthesis of sialosides containing sialic acid modifications.

Product	Yield (%)
	**98**
	**97**
	**95**

Product	Yield (%)
 29	75
 30	76

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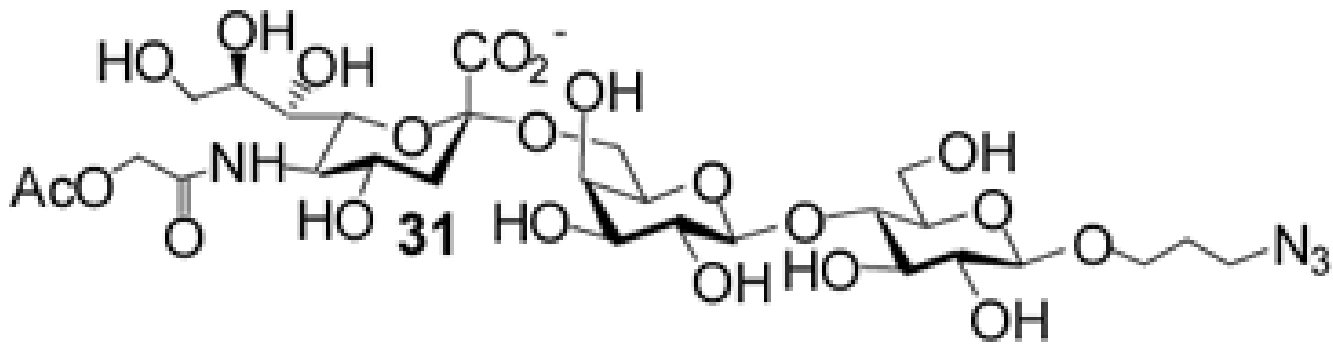
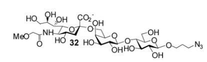
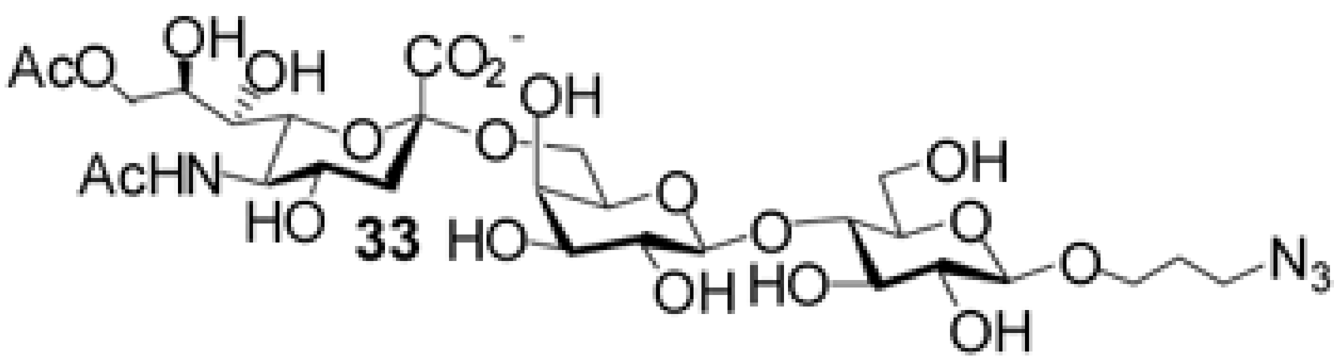
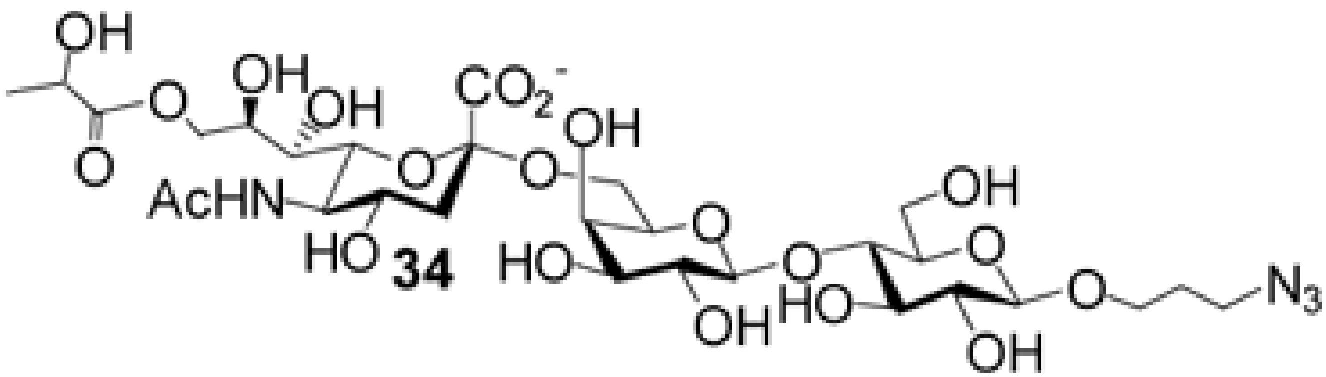
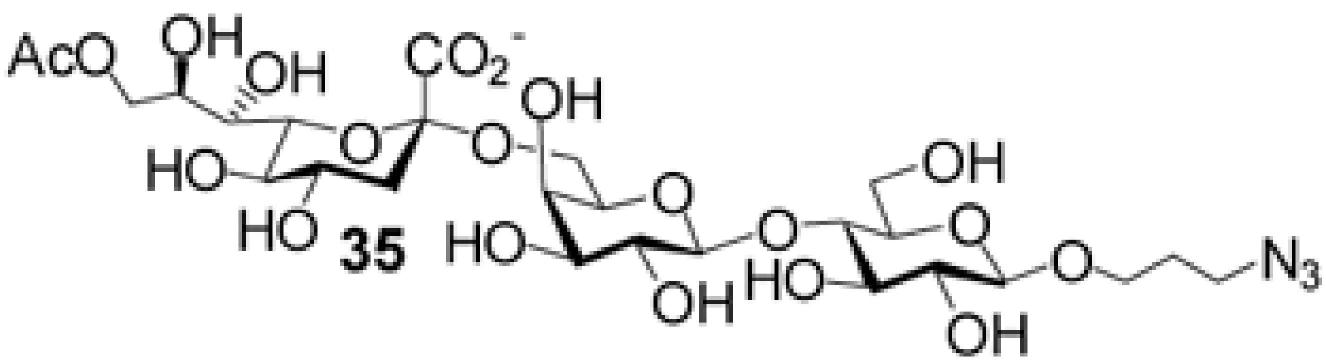
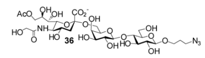
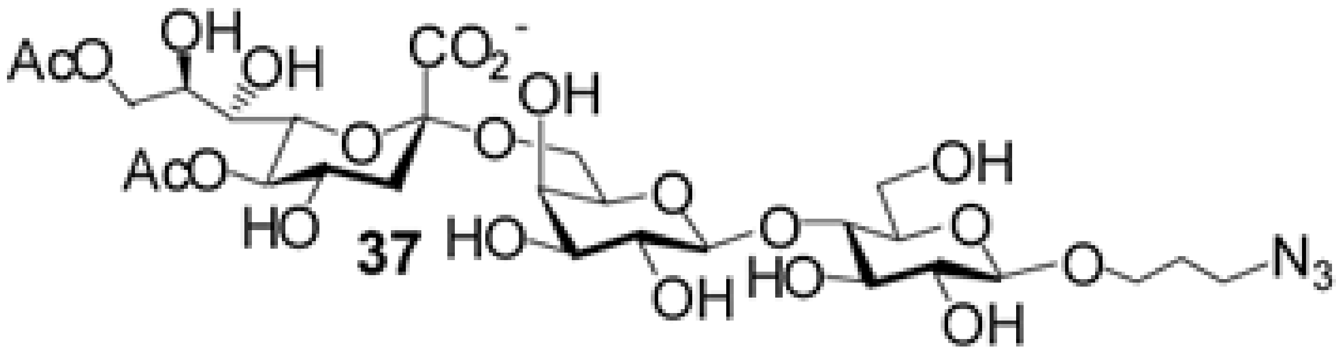
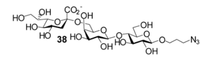
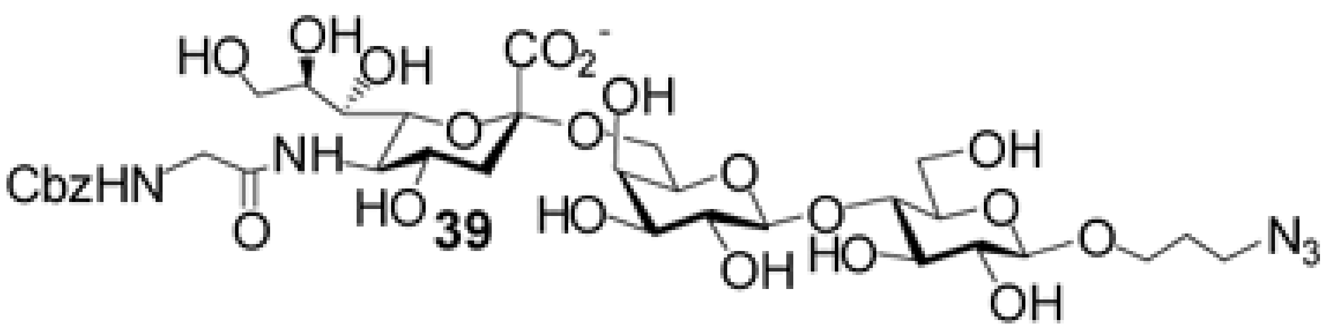
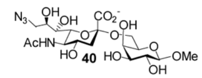
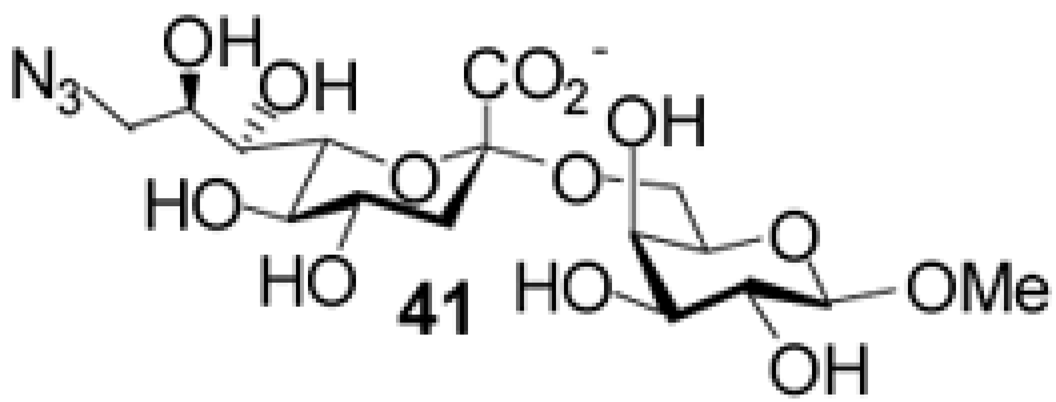
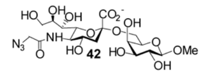
 31	**87**
 32	**99**
 33	**84**

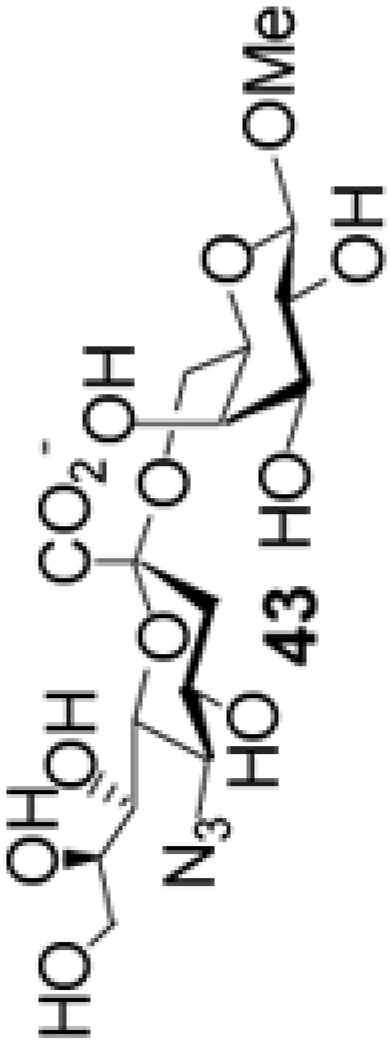
Product	Yield (%)
 34	72
 35	75

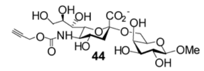
Product	Yield (%)
	78
	65

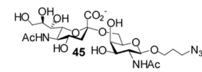
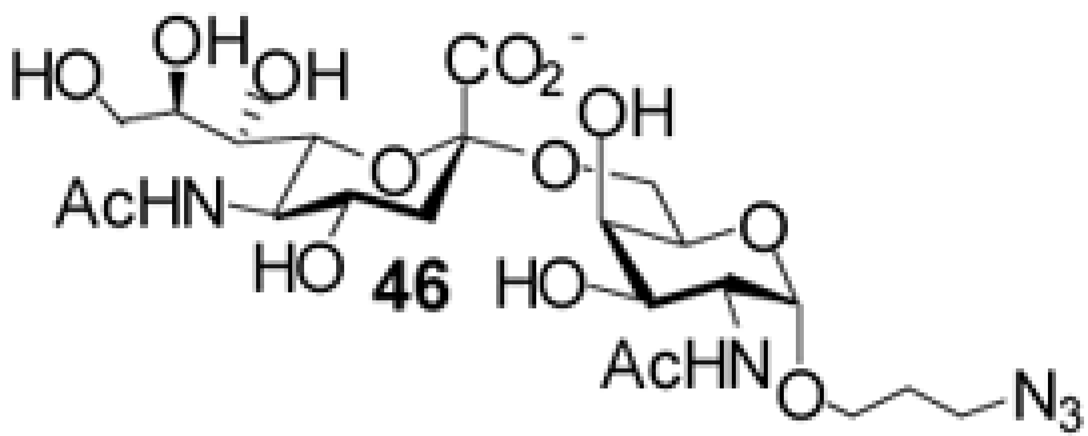
Product	Yield (%)
 38	92
 39	99

Product	Yield (%)
	**93**
	**91**

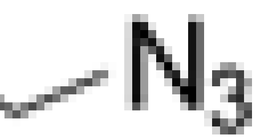
Product	Yield (%)
 **42**	**90**

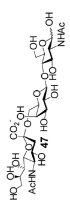
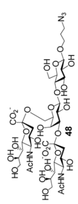
Product	Yield (%)
 **43**	**86**

Product	Yield (%)
 **44**	**87**

Product	Yield (%)
	**87**
	**61**





Product	Yield (%)
 47	92
 48	91