

Synthetic Disialyl Hexasaccharides Protect Neonatal Rats from Necrotizing Enterocolitis**

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Abstract: Two novel synthetic α 2-6-linked disialyl hexasaccharides, disialyllacto-N-neotetraose (DSLNNt) and α 2-6-linked disialyllacto-N-tetraose (DSLNT), were readily obtained by highly efficient one-pot multienzyme (OPME) reactions. The sequential OPME systems described herein allowed the use of an inexpensive disaccharide and simple monosaccharides to synthesize the desired complex oligosaccharides with high efficiency and selectivity. DSLNNt and DSLNT were shown to protect neonatal rats from necrotizing enterocolitis (NEC) and are good therapeutic candidates for preclinical experiments and clinical application in treating NEC in preterm infants.

Human milk oligosaccharides (HMOs) are a mixture of more than 100 glycans, which constitute the third major component of human milk.^[1] They have been found to contribute significantly to the gut health of breastfed infants. Strong evidence is now available to support the roles of HMOs in promoting the growth of beneficial gut bacteria, inhibiting the binding of pathogenic bacteria, human immunodeficiency virus (HIV), or protozoan parasites to gut epithelial cells, modulating immune responses, and influencing the functions of the gut epithelium.^[2]

Most of the reported HMOS-related studies used HMOS mixtures and thus the key active components are not clear. Among the few individual HMOS compounds with known functions, disialyllacto-*N*-tetraose (DSLNT; Figure 1), but not its nonsialylated or monosialylated analogues, was previously identified as a specific HMOS component that is effective for preventing necrotizing enterocolitis (NEC) in

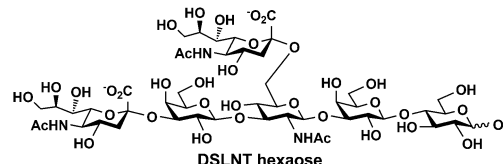


Figure 1. The structure of disialyllacto-*N*-tetraose (DSLNT).

a neonatal rat model.^[3] DSLNT contains two sialic acid residues: one is linked to the terminal galactose (Gal) residue through an α 2-3-sialyl linkage and the other is linked to the internal *N*-acetylglucosamine (GlcNAc) residue through an α 2-6-sialyl linkage (Figure 1). The hexasaccharide is presented at a level of 0.2–0.6 g in a liter of human milk.^[4] However, it is not presented in porcine milk,^[5] and it either is not presented^[6] or exists only in a trace amount in bovine milk.^[4,7] Due to the limited availability of human milk and the absence or low abundance of DSLNT in bovine milk, it is impractical to obtain the compound on a large scale for potential clinical therapeutic applications. Furthermore, despite the identification of the activity of an α 2-6-sialyl-transferase (for example, in the livers of various animals and of humans, as well as in human placenta, bovine mammary glands, human milk, and human mammary tumors, although at a lower level) that catalyzes the transfer of the sialic acid that is α 2-6 linked to the internal GlcNAc residue in DSLNT,^[8] the gene for the enzyme has not been identified. Therefore, it is currently unfeasible to obtain the desired α 2-

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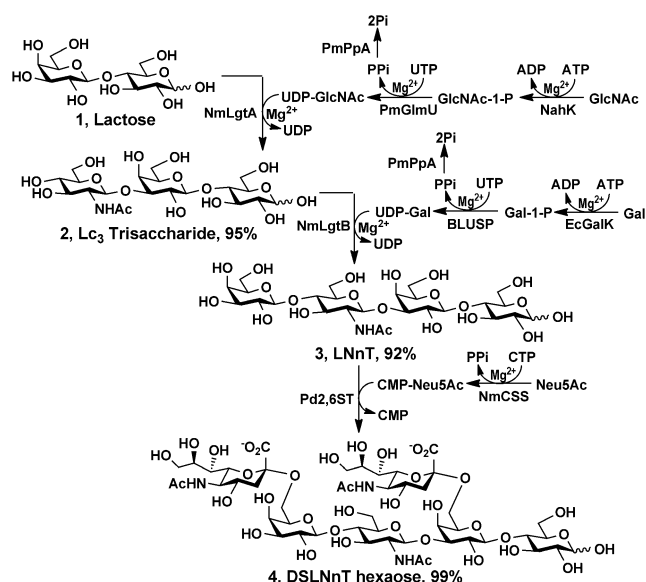
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6-sialyltransferase in large amounts to allow large-scale enzymatic synthesis of DSLNT. On the other hand, sialosides remain challenging targets for large-scale chemical synthesis due to the intrinsic structural features of sialic acids (namely, a sterically hindered anomeric carbon atom with a connecting electron-withdrawing carboxyl group in the sialic acid, which lowers the glycosylation reactivity and efficiency, and the lack of a neighboring participating group that disallows precise control of the sialylation stereospecificity).^[9] Recent advances in the development of chemical methods for synthesizing sialosides overcame some of the challenges and significantly improved the synthetic yields and stereoselectivity. Nevertheless, despite the synthesis of a more complex DSLNT-containing glycosyl ceramide (35 mg) reported by the Kiso group,^[10] the chemical synthesis of DSLNT in a free oligosaccharide form has not been reported.

One strategy to overcome the challenges in obtaining DSLNT in a large enough amount for NEC studies and potential therapies is to identify compounds that have similar or better NEC-preventing effects than DSLNT and that can be easily obtained synthetically. Herein, we report the synthesis of novel synthetic disialyl hexasaccharides, including α 2–6-linked disialyllacto-*N*-neotetraose (DSLNNt), obtained by sequential one-pot multienzyme (OPME) reactions, and α 2–6-linked disialyllacto-*N*-tetraose (DSLNT), obtained by one-pot sialylation of lacto-*N*-tetraose (LNT). We also report that these two disialyl hexasaccharides have potent NEC-preventing effects. DSLNNt can be produced in a large amount from simple starting materials for potential therapeutic applications.

In nature, the key enzymes that catalyze glycosidic bond formation are glycosyltransferases (GlyT). The GlyT-catalyzed transfer of monosaccharides other than sialic acids can be achieved most efficiently through a three-enzyme process: activation of a monosaccharide by a glycosyltransferase (GlyK) to form a sugar-1-phosphate (monosaccharide-1-P), which can be used by a nucleotidyltransferase (NucT) for the synthesis of a nucleoside diphosphate monosaccharide that acts as the sugar nucleotide donor substrate of a suitable glycosyltransferase for the formation of the desired glycosidic bond in the product.^[11] An inorganic pyrophosphatase (PpA) can also be added to push the reaction toward completion in the direction of product formation.^[12] These enzymes can be used in one-pot (a so-called OPME reaction) for the efficient synthesis of glycans. Each OPME reaction is usually used to add one monosaccharide to a glycosyltransferase acceptor. Utilization of the OPME reactions sequentially allows the formation of complex carbohydrates and glycoconjugates. The stereo- and regiospecificities of the glycosidic bond formed, the nucleotide triphosphate required, and the selection of the related sugar nucleotide biosynthetic enzymes are defined by the glycosyltransferases chosen based on the structures of the desired carbohydrate products. OPME approaches are limited by the availability, expression level, solubility, stability for storage, and substrate specificity of the enzymes involved, so the identification of suitable glycosyltransferases and the corresponding sugar nucleotide biosynthetic enzymes is critical for developing efficient OPME systems.



Scheme 1. Sequential one-pot multienzyme (OPME) synthesis of lacto-*N*-neotetraose (LNnT) and DSLNNt. Enzymes: NahK: *N*-acetylhexosamine-1-kinase; PmGlmU: *Pasteurella multocida* *N*-acetylglucosamine uridylyltransferase; PmPpA: a *Pasteurella multocida* inorganic pyrophosphatase; NmLgtA: β 1–3-*N*-acetylglucosaminyltransferase; EcGalK: *Escherichia coli* galactokinase; BLUSP: *Bifidobacterium longum* UDP-sugar pyrophosphorylase; NmLgtB: *Neisseria meningitidis* β 1–4-galactosyltransferase; NmCSS: *Neisseria meningitidis* CMP-sialic acid synthetase; Pd2,6ST: *Photobacterium damsela* α 2–6-sialyltransferase.

To obtain lacto-*N*-neotetraose (LNnT, **3**), a common human milk tetrasaccharide, the Lc₃ trisaccharide (GlcNAc β 1–3Gal β 1–4Glc, **2**; Scheme 1) was synthesized from the inexpensive disaccharide lactose and the monosaccharide GlcNAc by using a one-pot four-enzyme GlcNAc activation and transfer system containing *Bifidobacterium longum* (strain ATCC 55813) *N*-acetylglucosamine-1-kinase (NahK),^[13] *Pasteurella multocida* *N*-acetylglucosamine uridylyltransferase (PmGlmU),^[14] *Pasteurella multocida* inorganic pyrophosphatase (PmPpA),^[12] and *Neisseria meningitidis* β 1–3-*N*-acetylglucosaminyltransferase (NmLgtA).^[15] In this system, adenosine 5'-triphosphate (ATP) and GlcNAc were used in an NahK-catalyzed reaction to form *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P), which was used with uridine 5'-triphosphate (UTP) by PmGlmU to form uridine 5'-diphosphate (UDP)-GlcNAc, the sugar nucleotide donor for NmLgtA for the production of Lc₃ from lactose. All four enzymes were quite active in tris(hydroxymethyl)aminomethane (Tris)-HCl buffer at pH 8.0, and Lc₃ trisaccharide (1.36 g) was obtained in an excellent yield (95%) by incubation at 37 °C for 2 days.

By taking advantage of a promiscuous *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP),^[16] which can produce uridine 5'-diphosphate galactose (UDP-Gal) directly from UTP and galactose-1-phosphate (Gal-1-P), LNnT (Gal β 1–4GlcNAc β 1–3Gal β 1–4Glc, **3**; 1.19 g) was synthesized from Lc₃ (**2**) and simple galactose in an excellent yield (92%). This synthesis used a one-pot four-enzyme galactosylation system^[17] containing *Escherichia coli* galacto-

kinase (EcGalK),^[18] BLUSP,^[16] PmPpA,^[12] and *Neisseria meningitidis* β 1–4-galactosyltransferase (NmLgtB).^[12] This is a more effective system than our previously reported OPME β 1–4-galactosylation process, which involved the formation of UDP-glucose (UDP-Glc) from glucose-1-phosphate (Glc-1-P) and subsequent C4 epimerization to produce UDP-Gal indirectly.^[12]

Initial sialylation of LNNt by using *N*-acetylneuraminic acid (Neu5Ac) in a one-pot two-enzyme sialylation system^[19] containing *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS)^[19a] and *Photobacterium damselae* α 2–6-sialyltransferase (Pd2,6ST)^[20] with a Neu5Ac to LNNt ratio of 1.5 to 1 produced an unexpected mixture of monosialylated and disialylated LNNt (DSLNNt), which was difficult to separate. An increase in the Neu5Ac to LNNt ratio to 2.4 to 1 led to the formation of DSLNNt hexasaccharide (Neu5Ac α 2–6Gal β 1–4GlcNAc β 1–3(Neu5Ac α 2–6)Gal β 1–4Glc, **4**; 236 mg) in an excellent yield (99%). NMR spectroscopy data confirmed that Pd2,6ST not only adds a Neu5Ac that is α 2–6 linked to the terminal Gal, but it also adds an α 2–6-linked Neu5Ac to the internal Gal residue in LNNt, which is consistent with the observation in a recent report.^[21] As shown in Table 1 by using the β anomers (the major forms in D₂O solution) of the glycans for comparison, the attachment of Neu5Ac to the C6 atom of the internal Gal (Gal^{II}) and the terminal Gal (Gal^{IV}) of LNNt results in significant downfield shifts of the substituted carbon atoms (a downfield shift of δ = 2.39 ppm for the C6 atom of Gal^{II} and a downfield shift of δ = 2.52 ppm for the C6 atom of Gal^{IV}) in DSLNNt. There are obvious interactions of the Neu5Ac residues with GlcNAc^{III} and Glc^I, which result in a significant downfield shift of δ = 2.58 ppm for the C4 atom of GlcNAc^{III} and a downfield shift of δ = 1.55 ppm for the C4 atom of Glc^I. These unusual chemical shift changes seen in Neu5Ac α 2–6Gal sialosides are in accordance with those observed for glycans with the same or similar structural elements.^[22]

DSLNT hexaose (Neu5Ac α 2–6Gal β 1–3GlcNAc β 1–3(Neu5Ac α 2–6)Gal β 1–4Glc, **5**; 268 mg; Figure 2) containing two sialic acid residues that are α 2–6 linked to the terminal and internal Gal residues of LNT, respectively, was also synthesized in an excellent yield (98%) by using the same one-pot two-enzyme sialylation system containing NmCSS and Pd2,6ST with a Neu5Ac to LNT ratio of 2.6 to 1.

Two other disialyl glycans (Figure 2), GD3 tetrasaccharide (Neu5Ac α 2–8Neu5Ac α 2–3Gal β 1–4Glc, **6**; 239 mg) and disialyllactose (DSLac, Neu5Ac α 2–3(Neu5Ac α 2–6)Gal β 1–4Glc, **7**; 112 mg), were also synthesized from Neu5Ac α 2–3-Lac^[23] by using a one-pot two-enzyme sialylation system containing NmCSS and *Campylobacter jejuni* α 2–3/8-sialyltransferase (CjCstII; for GD3)^[24] or NmCSS and Pd2,6ST (for DSLac)^[20] (see the Supporting Information for details).

As a control, a monosialyl pentasaccharide 3'''-sialyl LNNt (3'''-sLNNt, **8**; 138 mg; Figure 2) was synthesized from LNNt (**3**) by using a one-pot two-enzyme sialylation system with NmCSS and a single-site mutant of *Pasteurella multocida* multifunctional α 2–3-sialyltransferase 1 (PmST1 M144D).^[25] Unlike the Pd2,6ST-catalyzed sialylation reaction, which could add either one or two α 2–6-linked sialic acid residues to LNNt, the PmST1M144D-catalyzed sialylation

Table 1: ¹³C NMR chemical shifts for compounds Gal β 1–4Glc (Lac), GlcNAc β 1–3Gal β 1–4Glc (Lc₃ glycan), Gal β 1–4GlcNAc β 1–3Gal β 1–4Glc (LNNt), and Neu5Ac α 2–6Gal β 1–4GlcNAc β 1–3(Neu5Ac α 2–6)Gal β 1–4Glc (DSLNNt). Significant chemical shift changes after sialylation for the formation of DSLNNt from LNNt are highlighted in bold.

| ^{VI} Neu5Ac α 2–6Gal β 1–4GlcNAc β 1–3(^V Neu5Ac α 2–6)Gal β 1–4Glc | | | | | |
|--|-----------------|--------|-----------------|--------------|--------------|
| Sugar Unit | C atoms | Lac | Lc ₃ | LNNt | DSLNNt |
| β -D-Glc ^I | 1 | 95.64 | 95.66 | 95.61 | 95.70 |
| | 2 | 73.70 | 73.71 | 73.65 | 73.37 |
| | 3 | 74.26 | 74.20 | 74.22 | 74.36 |
| | 4 | 78.19 | 78.21 | 78.21 | 79.76 |
| | 5 | 74.69 | 74.71 | 74.76 | 74.76 |
| | 6 | 59.78 | 60.01 | 60.34 | 60.23 |
| β -D-Gal ^{II} (1–4) | 1 | 102.79 | 102.84 | 102.76 | 103.35 |
| | 2 | 70.86 | 70.03 | 69.88 | 69.76 |
| | 3 | 72.42 | 81.87 | 81.82 | 82.29 |
| | 4 | 68.46 | 68.26 | 68.22 | 68.32 |
| | 5 | 75.25 | 74.80 | 75.52 | 73.77 |
| | 6 | 60.94 | 60.88 | 60.84 | 63.23 |
| β -D-GlcNAc ^{III} (1–3) | 1 | | 102.75 | 102.73 | 102.74 |
| | 2 | | 56.58 | 56.52 | 54.83 |
| | 3 | | 73.49 | 73.42 | 72.50 |
| | 4 | | 69.92 | 78.11 | 80.69 |
| | 5 | | 75.57 | 74.66 | 74.72 |
| | 6 | | 60.41 | 59.93 | 60.05 |
| β -D-Gal ^{IV} (1–4) | C=O | | 174.87 | 174.83 | 175.01 |
| | CH ₃ | | 22.09 | 22.03 | 22.38 |
| | 1 | | | 102.79 | 103.65 |
| | 2 | | | 70.99 | 70.83 |
| | 3 | | | 73.42 | 72.62 |
| | 4 | | | 68.24 | 68.47 |
| α -D-Neu5Ac ^V (2–6) | 5 | | | 75.52 | 73.80 |
| | 6 | | | 60.85 | 63.37 |
| | 1 | | | | 173.59 |
| | 2 | | | | 100.38 |
| | 3 | | | | 40.17 |
| | 4 | | | | 68.47 |
| α -D-Neu5Ac ^{VI} (2–6) | 5 | | | | 51.69 |
| | 6 | | | | 72.64 |
| | 7 | | | | 68.50 |
| | 8 | | | | 71.86 |
| | 9 | | | | 62.53 |
| | C=O | | | | 175.01 |
| α -D-Neu5Ac ^{VI} (2–6) | CH ₃ | | | | 22.12 |
| | 1 | | | | 173.66 |
| | 2 | | | | 100.23 |
| | 3 | | | | 40.17 |
| | 4 | | | | 68.47 |
| | 5 | | | | 51.79 |
| α -D-Neu5Ac ^{VI} (2–6) | 6 | | | | 72.64 |
| | 7 | | | | 68.50 |
| | 8 | | | | 71.81 |
| | 9 | | | | 62.53 |
| | C=O | | | | 175.01 |
| | CH ₃ | | | | 22.15 |

reaction only added one α 2–3-linked sialic acid residue to the terminal Gal in LNNt. The use of the PmST1 M144D mutant^[25] instead of the wild-type PmST1^[23] avoided product hydrolysis caused by the α 2–3-sialidase activity of the wild-type enzyme and thus improved the yield of the one-pot two-

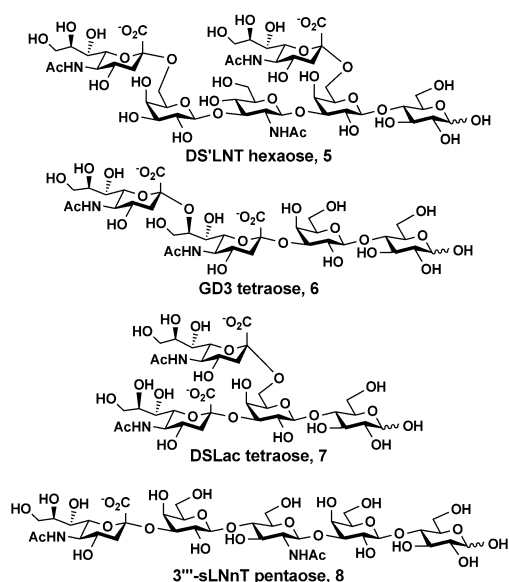
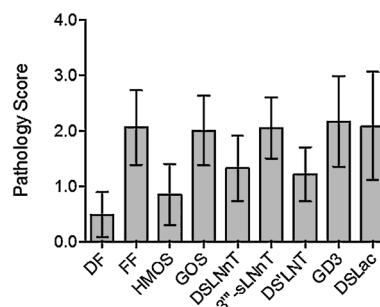


Figure 2. Structures of DS'LNT hexaose, GD3 tetraose, DSLac tetraose, and 3'''-sLNnT pentaose.

enzyme α 2–3-sialylation reaction. Indeed, an excellent yield (98%) was achieved without the need for close monitoring and prompt stopping of the reaction process.

The NEC-preventing effects of the disialyl compounds DSLNnT (**4**), DS'LNT (**5**), GD3 (**6**), and DSLac (**7**), as well as the monosialyl compound 3'''-sLNnT (**8**), were tested in the same neonatal rat model that was used previously.^[3] A mixture of HMOS isolated from pooled human milk was used as a positive intervention control, and a galactooligosaccharide (GOS) sample that was shown to be ineffective in preventing NEC^[3] was used as a negative intervention control. As shown in Figure 3, dam-fed (DF) animals hardly developed any signs of NEC (mean pathology score of 0.48 ± 0.41). The pathology scores were significantly higher in animals that were orally gavaged with rodent formula (FF) without the addition of glycans (2.06 ± 0.67 , $p < 0.0001$ relative to DF). The addition of HMOS to the formula led to significantly lower pathology scores (0.85 ± 0.55 , $p < 0.0001$ relative to FF), which were not significantly different from the DF control ($p = 0.106$). The addition of GOS had no effect of lowering the pathology scores (2.00 ± 0.63 , $p = 0.790$ relative to FF). All of these results are in accordance with the previously reported data.^[3] The addition of the synthesized DSLNnT to the formula led to significantly lower pathology scores (1.32 ± 0.59 , $p < 0.001$ relative to FF); this result was not significantly different from the effect seen in animals that received HMOS ($p = 0.062$) but was still different from the DF control ($p < 0.001$). The addition of the synthesized 3'''-sLNnT to the formula did not lower the pathology scores (2.05 ± 0.55 , $p = 0.987$ relative to FF). The addition of the synthesized DS'LNT to the formula significantly reduced the pathology scores (1.21 ± 0.49 , $p < 0.001$ compared to FF); this result, again, was not significantly different from the effect seen in animals receiving HMOS ($p = 0.120$) but was still different from the DF control ($p < 0.001$). Neither GD3 nor



| | DF | FF | HMOS | GOS | DSLNnT | 3'''-sLNnT | DS'LNT | GD3 |
|------------|---------|---------|---------|-------|--------|------------|--------|-----|
| FF | <0.0001 | | | | | | | |
| HMOS | <0.01 | <0.0001 | | | | | | |
| GOS | <0.0001 | ns | <0.0001 | | | | | |
| DSLNnT | <0.001 | <0.001 | <0.01 | <0.01 | | | | |
| 3'''-sLNnT | <0.0001 | ns | <0.0001 | ns | <0.001 | | | |
| DS'LNT | <0.001 | <0.001 | ns | <0.01 | ns | <0.01 | | |
| GD3 | <0.001 | ns | <0.001 | ns | <0.01 | ns | <0.01 | |
| DSLac | <0.001 | ns | <0.001 | ns | <0.01 | ns | <0.01 | ns |

Figure 3. DSLNnT and DS'LNT protect neonatal rats from necrotizing enterocolitis. Ileum pathology scores (0: healthy; 4: complete destruction) are plotted for each animal in the different intervention groups. DF: dam fed (number of rats $n = 33$); FF: fed formula without additional glycans ($n = 27$); HMOS: FF contains oligosaccharides isolated from pooled human milk (2 mg mL^{-1} , $n = 23$); GOS: FF contains galactooligosaccharides (2 mg mL^{-1} , $n = 15$); DSLNnT: FF contains DSLNnT ($300 \text{ } \mu\text{g mL}^{-1}$, $n = 20$); 3'''-sLNnT: FF contains 3'''-sLNnT ($300 \text{ } \mu\text{g mL}^{-1}$, $n = 19$); DS'LNT: FF contains DS'LNT ($300 \text{ } \mu\text{g mL}^{-1}$, $n = 14$); GD3: FF contains GD3 ($300 \text{ } \mu\text{g mL}^{-1}$, $n = 12$); DSLac: FF contains DSLac ($300 \text{ } \mu\text{g mL}^{-1}$, $n = 11$). Bars represent mean \pm standard deviation. p values are listed in the table below the figure. ns: not significant.

DSLac had a significant effect on the pathology scores relative to those in animals that received formula alone.

These results show that, similar to DSLNT, DSLNnT and DS'LNT reduce the pathology scores in an NEC neonatal rat model. All three compounds are disialyl hexasaccharides but with noticeable structural differences. Firstly, both DSLNT and DS'LNT are disialyl type I glycans, the core tetrasaccharide (LNT) of which has a Gal residue β 1–3-linked to Lc_3 trisaccharide, whereas DSLNnT is a disialyl type II glycan, in which the core tetrasaccharide (LNnT) has a Gal residue β 1–4-linked to the Lc_3 trisaccharide. Secondly, all three compounds have a Neu5Ac that is α 2–6 linked to an internal monosaccharide; the internal monosaccharide is GlcNAc in DSLNT, whereas it is Gal in DSLNnT and DS'LNT. Thirdly, the outermost Neu5Ac is linked to the penultimate Gal by an α 2–3 linkage in DSLNT but an α 2–6 linkage in DSLNnT and DS'LNT. These structural differences between DSLNT, DSLNnT, and DS'LNT and their similarity in protecting neonatal rats from NEC indicate that the negatively charged disialyl component is important for the NEC-preventing effect, whereas the tetrasaccharide scaffold (type I or II) does not seem to be important. The importance of the disialyl component is further supported by the lack of NEC-preventing effect in monosialyl pentasaccharides, shown previously for LSTb^[3] and shown herein for 3'''-sLNnT. However, the presence of the disialyl component alone is not sufficient to explain the beneficial effects because GD3 and DSLac showed no effect.

In conclusion, we have shown herein that novel synthetic disialyl hexasaccharides, including disialyllacto-*N*-neotetraose (DSLNT) and α 2-6-linked disialyllacto-*N*-tetraose (DSLNT), can protect neonatal rats from NEC. Unlike the NEC-preventing DSLNT previously identified from human milk, which is not easily obtainable by either purification or synthesis, the newly identified DSLNT and DSLNT are readily available by enzymatic synthesis. The sequential OPME systems described herein allow the use of an inexpensive disaccharide and simple monosaccharides to synthesize desired complex oligosaccharides, such as DSLNT, with high efficiency and selectivity. The readily available DSLNT and DSLNT are good therapeutic candidates for preclinical experiments and clinical application in treating NEC in preterm infants.

Experimental Section

Oligosaccharides 2–8 were prepared by using one-pot multienzyme (OPME) reactions. Animal studies were reviewed and approved by the Institutional Animal Care Use Committee at the University of California, San Diego (AAALAC accreditation number 000503). Detailed synthetic procedures, NMR spectroscopy and HRMS characterization of the products including NMR spectra, and procedures for rat studies are available in the Supporting Information.

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