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Synthesis of Neuraminidase-Resistant Sialyllactose Mimetics from *N*-Acyl Mannosamines using Metabolically Engineered *Escherichia coli*

Paul Rivollier,^[a] Eric Samain,^[a] Sylvie Armand,^[a] Isabelle Jeacomine,^[a] Emeline Richard,^[a] and Sébastien Fort*^[a]

Abstract: Herein, we describe the efficient gram-scale synthesis of α 2,3- and α 2,6-sialyllactose oligosaccharides as well as mimetics from *N*-acyl mannosamines and lactose in metabolically engineered bacterial cells grown at high cell density. We designed new *Escherichia coli* strains co-expressing sialic acid synthase and *N*-acylneuraminate cytidyltransferase from *Campylobacter jejuni* together with the α 2,3-sialyltransferase from *Neisseria meningitidis* or the α 2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224. Using their mannose transporter, these new strains actively internalized *N*-acetylmannosamine (ManNAc) and its *N*-propanoyl (*N*-

Prop), *N*-butanoyl (*N*-But) and *N*-phenylacetyl (*N*-PhAc) analogs and converted them into the corresponding sialylated oligosaccharides, with overall yields between 10% and 39% (200–700 mg.L⁻¹ of culture). The three α 2,6-sialyllactose analogs showed similar binding affinity for *Sambucus nigra* SNA-I lectin as for the natural oligosaccharide. They also proved to be stable competitive inhibitors of *Vibrio cholerae* neuraminidase. These *N*-acyl sialosides therefore hold promise for the development of anti-adhesion therapy against influenza viral infections.

Introduction

N-acetyl-neuraminic acid (Neu5Ac), the main form of sialic acid, is a major carbohydrate in vertebrates. This nine-carbon α -keto acid amino sugar is primarily found at the outermost end of the glycan chains on cell-surface glycoproteins and glycolipids, where it is appended to a galactose or an *N*-acetyl galactosamine residue, through either an α 2,3- or an α 2,6-linkage.^[1]

Given its wide distribution and exposed position, Neu5Ac is an important regulator of cellular recognition in which it plays a dual role. On the one hand, sialylation is essential for physiological events including development processes, immune reactions and reproduction.^[2] On the other hand, it is a hallmark of several cancers and a target for pathogen infection.^[3,4] Highly contagious viruses exploit the wide occurrence of sialylated structures in the human respiratory tract for infection. Influenza and coronaviruses, which represent two of the most important

zoonotic threats, use sialic-acid-binding proteins present on their surface to target host cells. Coronaviruses have received worldwide interest with the global spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2019. Recent studies have indicated that SARS-CoV-2 infection is mediated by specific interactions between its spike protein and α 2,6-sialylated glycans at the surface of human epithelial cells.^[5,6] Similarly, human and avian influenza viruses display two sialic acid binding proteins on their surface.^[7] Hemagglutinins allow adhesion to the host cell to initiate the infectious cycle. Neuraminidases cleave terminal sialic acids on infected cells and possibly on mucins, allowing the circulation and the propagation of the virus in the organism.^[8] The main difference between avian and human viruses lies in their specificity. Human viruses preferentially bind to α 2,6-linked sialic acids, whereas avian viruses are specific to α 2,3-linked sialic acids.^[9] Although seasonal vaccines and antivirals are commonly used to combat influenza infections, their efficiency is being increasingly questioned. During the 2019–2020 flu epidemic in nine European countries, vaccine effectiveness against influenza A and B strains only reached 50% for patients of all ages hospitalized in primary care settings.^[10] Antiviral drugs, in particular the neuraminidase inhibitors Relenza™ and Tamiflu™, are second-line therapeutics used to treat flu. However, their use is scrutinized because, as for antibiotics, these molecules are likely to lead to the emergence of resistant strains.^[11] Although this concern is currently minor, resistance has already been observed in immunocompromised patients infected with influenza A H1N1 pdm09 and H3N2 strains. Overall, human influenza viruses likely cause up to 650 000 deaths annually worldwide.^[12] The development of alternatives to vaccines and

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antivirals thus holds great importance. Inhibition of virus adhesion to the host cell is one way to prevent or mitigate infection. In so-called anti-adhesion therapy, decoy molecules are designed to inhibit the pathogen receptors and prevent their interaction with host cells.^[13,14] This type of strategy against influenza requires the capacity to produce sialylated oligosaccharides on a large scale. In this regard, enzymatic synthesis, which avoids the protection and deprotection steps, is much more efficient than chemical methods.^[15]

In vitro synthesis of α 2,3- or α 2,6-sialosides using bacterial trans-sialidases^[16] or sialyltransferases has been carried out successfully. In particular, one-pot enzymatic cascade reactions using a Neu5Ac aldolase, an *N*-acetylneuraminyltransferase and a sialyltransferase (ST) have been designed to prepare sialyllactose from *N*-acetylmannosamine.^[17–19] In addition, this approach also allows the preparation of oligosaccharides containing C-5-modified sialic acids from *N*-modified mannosamines. These modifications were initially intended for the study of the specificity of sialyltransferases^[20,21] and bacterial neuraminidases,^[22] or the development of ligands targeting siglecs expressed on cancer cells.^[23]

However, in vitro synthesis suffers from limitations such as the requirement to express and purify several proteins, and to devise nucleotide-sugar regeneration steps to prevent the inhibition of the glycosyltransferases. As an alternative, we have developed an in vivo process based on a high-density culture of metabolically engineered bacterial cells.^[24] This approach, which takes advantage of the bacterial metabolism, leads to the synthesis of complex oligosaccharides from readily accessible substrates, through the expression of heterologous glycosyltransferases. The use of live bacteria allows the accumulation of oligosaccharides without the need to isolate enzymes or to add

expensive cofactors. This process produces α 2,3-sialyllactose (3-SL) at 25 g.L⁻¹ using lactose as the unique precursor in an *Escherichia coli* strain engineered to synthesize Neu5Ac from the endogenous pool of UDP-*N*-acetylglucosamine and expressing an α 2,3-sialyltransferase (3-ST) from *Neisseria meningitidis*.^[25]

Despite its efficiency, this method does not provide access to modified sialosides. To do so, Birikaki et al. used an *E. coli* strain able to internalize and accumulate exogenous Neu5Ac.^[26] C-5-modified sialic acids were efficiently transported into the bacteria, activated into the corresponding nucleotide sugars and glycosylated onto lactose to provide analogues of the saccharidic portion of the GM2 and GM3 gangliosides.^[27] The latter, that is, α 2,3-sialyllactose mimetics, were shown to interact with *Maackia amurensis* lectin with the same affinity as the natural ligand, while being resistant to neuraminidases. However, the multi-step synthesis of the *N*-modified sialic acid precursors was a barrier to large-scale development.

In the present work, we report the design of new *E. coli* strains that provide straightforward access to α 2,3- and α 2,6-sialylated oligosaccharides from easily accessible mannosamine precursors. To illustrate the efficiency and versatility of the method, we have synthesized α 2,6-sialyllactose (6-SL) mimetics and assessed their resistance to *Vibrio cholerae* (VC) neuraminidase and binding affinity for *Sambucus nigra* (SNA-I) lectin (Figure 1).

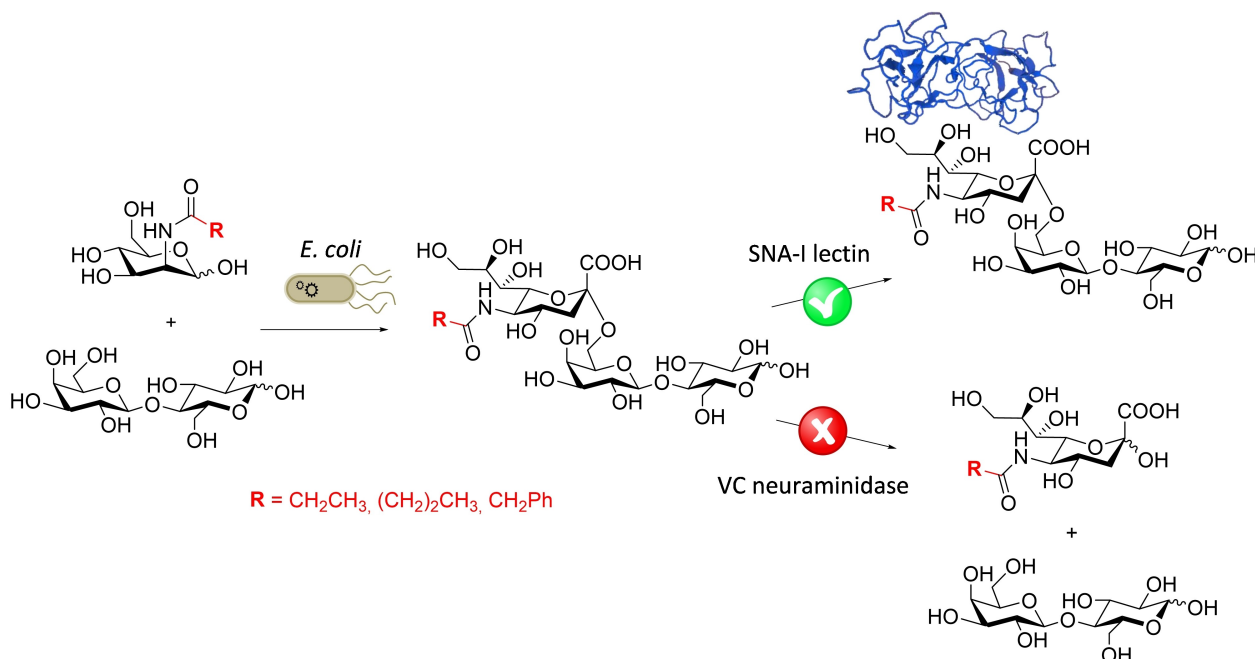


Figure 1. Pathway for the production of 6-SL analogs that interact with SNA-I lectin and are resistant to hydrolysis catalyzed by the VC neuraminidase.

Results and Discussion

Design of *E. coli* strains utilizing *N*-acyl mannosamine

The metabolic engineering strategy to produce 3- and 6-SL from exogenous *N*-acetylmannosamine (ManNAc) in *E. coli* K12 is described in Figure 2. As a starting point, we used a previously reported pathway for producing 3-SL from lactose and endogenous uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) using a ZLKA-derived strain (DC7) (Figure 2A).^[25] This strain overexpresses the *neuABC* genes from *Campylobacter jejuni* and *3-st* from *N. meningitidis*. UDP-GlcNAc was first converted into ManNAc by the UDP-GlcNAc-2 epimerase NeuC before being transformed into Neu5Ac by the sialic acid synthase NeuB. Then, Neu5Ac was activated by the *N*-acetylneuraminic acid cytidyltransferase NeuA into the corresponding nucleotide sugar. In parallel, lactose was actively internalized in the bacteria via the *lacY* transporter. Finally, 3-ST from *N. meningitidis* catalyzed the glycosylation between the neuraminic acid donor and lactose to produce the target 3-SL. The product accumulated following the inactivation of the *lacZ* and *nankETA* genes responsible for lactose and sialic acid catabolism, respectively. In addition, knocking out the sialic acid transporter NanT prevented the leakage of the monosaccharide into the culture medium.

To synthesize 3- and 6-SL from mannosamine, two new strains (ZW3, ZW4) also derived from ZLKA were engineered (Figure 2B). In contrast to those previously reported by Fierfort et al.,^[25] these strains did not harbor the *neuC* gene, thereby preventing the formation of ManNAc from endogenous UDP-GlcNAc. ZW3 and ZW4 also expressed the genes encoding the 3-ST from *N. meningitidis* and the 6-ST from *Photobacterium* sp.

JT-ISH-224, respectively.^[28] 6-ST, which has been reported to display high activity leading to the formation of oversialylated side products,^[28,29] was expressed under the control of the arabinose-inducible pAraC promoter, whereas the other genes were cloned into isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible plasmids. This should allow a dissociated expression of the 6-ST over the other enzymes.

In *E. coli*, *N*-acetylmannosamine is taken up by the phosphotransferase system ManXYZ.^[30,31] Although this transport is accompanied by sugar phosphorylation at OH-6, a non-negligible part of unmodified ManNAc is also present in the bacterial cytoplasm. One study has reported that, when *E. coli* K92 is grown on ManNAc, both ManNAc and ManNAc-6-phosphate can be found intracellularly at a 1:3 molar ratio.^[32] Thus, we assumed that ManNAc is partly available for sialic acid synthase.

The ZW3 and ZW4 strains were cultivated at high cell density in presence of equimolar quantities of lactose and ManNAc. NeuA, NeuB and 3-ST overexpression was activated by addition of IPTG. Due to the high activity of 6-ST, its gene expression was not induced but relied only on expression leakage.

The fermentation time course was monitored using thin layer chromatography (TLC). Both substrates were totally internalized by the two strains and converted within a few hours into a more polar product localized in the intracellular medium only. Mass spectrometry analysis of the crude cytoplasmic fraction confirmed the presence of the expected sialyllactose with a peak at *m/z* 632 corresponding to a molecular ion $[M-H]^-$ (data not shown). After completion of the cultures, the sialylated oligosaccharides were isolated from the cytoplasmic fractions by centrifugation, cell lysis and then

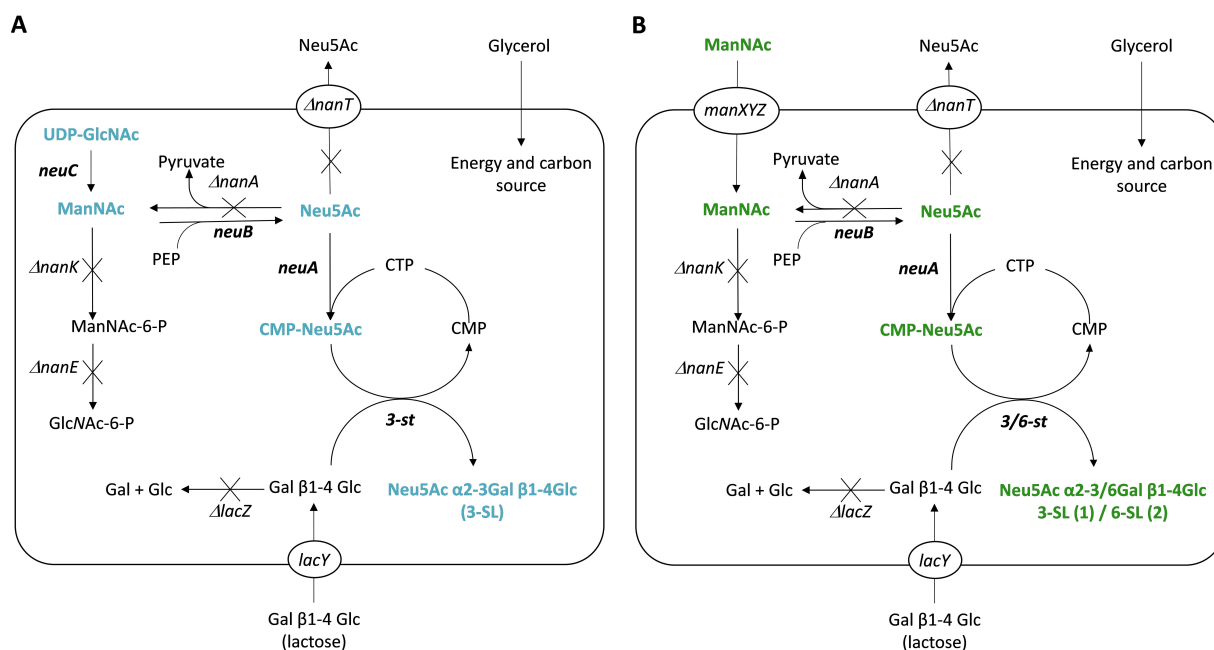


Figure 2. (A) Previously reported engineered metabolic pathway for the production of 3-SL from endogenous UDP-GlcNAc in the *E. coli* DC7 strain.^[25] (B) New engineered metabolic pathway to produce 3-SL 1 and 6-SL 2 from exogenous ManNAc in the *E. coli* ZW3 and ZW4 strains, respectively.

deproteinization with acidification of the medium. After purification using ion-exchange chromatography, pure 3-SL **1** and 6-SL **2** were recovered at a similar yield of 32% (947 mg.L⁻¹). ¹H and ¹³C NMR data of the pure products were in perfect agreement with those reported earlier (Supporting Information S1).^[25,28] The production of 3- and 6-SL from ManNAc with ZW3 and ZW4 strains reached an extremely satisfactory yield, comparable to those reported earlier with strains using Neu5Ac as the exogenous substrate.^[27,33]

Considering that a large part of the ManNAc must have been phosphorylated during its uptake, the production yields were higher than expected. To determine if the monosaccharide can passively diffuse through the cell membrane and thus explain this finding, we designed a new strain (MAC7) differing from ZW3 only by the knockout of the *ManXYZ* transporter gene. TLC analysis of the culture medium showed slower internalization of ManNAc by MAC7 compared with ZW3 under similar fermentation conditions. After 45 h of culture, a more polar product was observed in the bacterial cytoplasm, but a significant amount of ManNAc was still present in the extracellular medium. After purification, 3-SL was isolated with a production yield of 16% (487 mg.L⁻¹), which is half of the production in ZW3. This result suggests that ManNAc passively diffuses through the bacterial cell wall, but at a much lower rate than by active transport with the *ManXYZ* permease. In conclusion, under the culture conditions used, ManNAc was mostly internalized by the bacteria via mannose permease without being phosphorylated, presumably due to the competitive transformation into Neu5Ac.

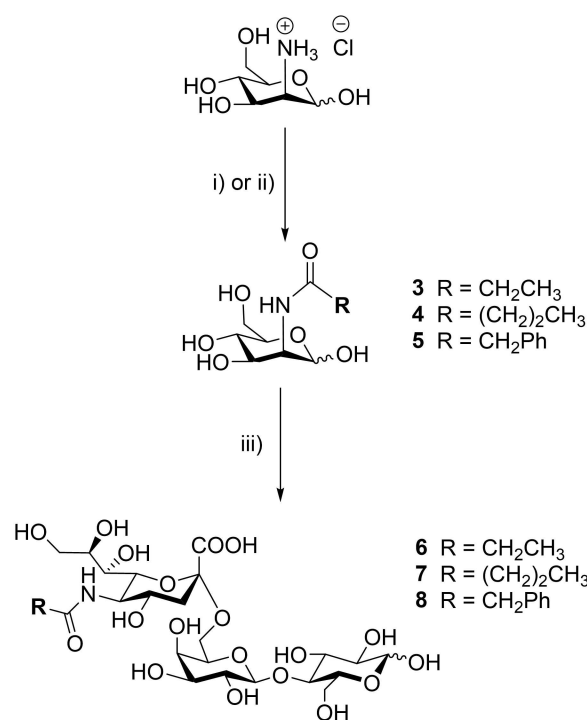
Synthesis of α 2,6-sialyllactose (6-SL) derivatives from *N*-acyl mannosamines

Few studies have investigated the impact of Neu5Ac C-5 modification on both hydrolysis by neuraminidases and interaction with lectins. However, it has been shown that transformation of the *N*-acetyl of sialyl galactosides into *N*-methoxyacetyl or *N*-azidoacetyl groups may confer reduced susceptibility to hydrolysis by bacterial neuraminidases from *Clostridium perfringens* and *Vibrio cholerae* among others^[22] and by neuraminidases from avian and human influenza viruses, respectively.^[34] Heise et al. have reported that cellular sialylated glycans containing a *N*-propargyloxycarbonyl still bind to *M. amurensis* and *S. nigra* lectins, and nonetheless display reduced susceptibility to hydrolysis by *C. perfringens* neuraminidases.^[35] We have previously shown that *N*-propanoyl- and *N*-phenylacetyl-3-SL are resistant to hydrolysis by VC neuraminidase, and interact with *M. amurensis* lectin in a similar way as natural sialyllactose.^[27] The *N*-phenylacetyl analog also proved to be a competitive inhibitor of VC neuraminidase. Therefore, we aimed to produce C-5-modified 6-SL analogs, resistant to hydrolysis and inhibiting neuraminidases, that keep their binding affinity for hemagglutinins. This double effect on both hemagglutinins and neuraminidases can potentially lead to promising anti-adhesive molecules against human influenza infections.

We previously described the synthesis in *E. coli* of *N*-propionyl and *N*-phenylacetyl derivatives of 3-SL from modified sialic acid precursors.^[27] This synthesis was possible using the wide substrate promiscuity of bacterial CMP-Neu5Ac synthetases and sialyltransferases.^[19] To synthesize α 2,6-sialylated oligosaccharides with *N*-acyl modifications, mannosamine derivatives must also be internalized by the transporter and processed by the sialic acid synthase NeuB. The broad substrate specificity of *ManXYZ* should allow the internalization of *N*-acyl mannosamines,^[30] and *C. jejuni* NeuB has been shown to transform mannosamine derivatives into the corresponding sialic acids.^[36]

ManNProp **3**, ManNBut **4** and ManNPhAc **5** were synthesized on a multigram scale from mannosamine hydrochloride and the corresponding acid anhydrides (Scheme 1). After purification using silica gel flash chromatography, we obtained **3** and **4** at a yield of 84% and only 22% for **5**. NMR data were in agreement with those previously reported (Supporting Information S1).^[37] The preparation of **5** was further improved by in situ activation of phenyl acetic acid with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM). Under these conditions, **5** was ultimately isolated with a yield of 56%. The production of *N*-acyl-6-SL from *N*-acyl mannosamines **3–5** was carried out in a high cell density culture of the ZW4 strain.

TLC analysis of the culture showed that ManNProp **3** and ManNBut **4** were completely internalized and converted into more polar products within a few hours. ManNPhAc **5** was



Scheme 1. Synthesis of *N*-acyl-6-SL from mannosamine hydrochloride. i) MeONa/MeOH, acid anhydride, rt, overnight, 84% for **3** and **4**. ii) phenylacetic acid, *N*-methylmorpholine, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride, MeOH, rt, overnight, 56% for **5**. iii) Lactose, engineered *E. coli* ZW4, 24 h, 33% (611 mg.L⁻¹) for **6**, 39% (713 mg.L⁻¹) for **7**, 10% (196 mg.L⁻¹) for **8**.

internalized much more slowly, but seemed to be efficiently converted by the sialic acid synthase. After 22 h of culture, **5** was still present in the extracellular medium, but was absent in the intracellular medium, and a more polar product accumulated in the intracellular fraction. After isolation and purification by ion-exchange chromatography as previously described for 3-SL, the products were still contaminated with 10% of α 2,6-(2-keto-3-deoxy-manno-octonyl)lactose (α 2,6-KDO-lactose) as determined by ^1H NMR. The formation of α 2,6-KDO-lactose by 6-ST has indeed previously been observed in some occasions.^{[28][38]} A further purification step using low-pressure size-exclusion chromatography finally afforded pure *N*-Acyl-6-SL **6–8** with 33% (613 mg.L⁻¹), 39% (733 mg.L⁻¹) and 10% (196 mg.L⁻¹) yields, respectively. Obtention of expected products was confirmed using ^1H and ^{13}C NMR (Supporting Information S1), and mass spectrometry with peaks related to $[\text{M}-\text{H}]^-$ molecular ions at m/z 646, 660 and 708, respectively. The good production yields of *N*-Prop 6-SL **6** and *N*-But-6-SL **7** suggest that small aliphatic chains have a minor impact on the internalization of the precursors via ManXYZ and their transformation by the subsequent enzymatic cascade. However, the bulkiness of the phenylacetyl group on mannosamine clearly impairs its internalization, thereby lowering the yield of *N*-PhAc-6-SL **8**.

Neuraminidase resistance and affinity for a sialic acid-binding lectin of modified sialosides

We assessed the ability of modified *N*-acyl-6-SL **6–8** to interact with a specific sialic-acid-binding lectin and to resist to a neuraminidase. A solid-phase competitive enzyme-linked lectin assay (ELLA) was used to evaluate the interaction between **6–8** and SNA-I, a common protein with high specificity for α 2,6-linked sialic acids (Figure 3).^[38–40] Briefly, in a microtiter plate, the binding of horseradish peroxidase-labeled SNA-I with

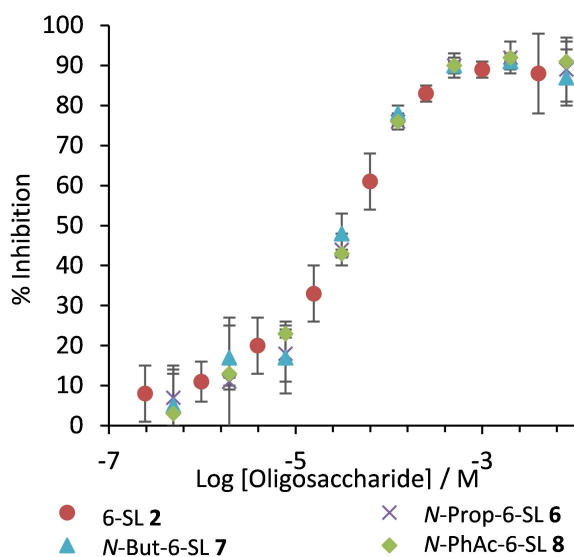


Figure 3. Inhibition of bovine fetuin-SNA-I binding assessed using an enzyme-linked lectin assay (ELLA).

immobilized fetuin was quantified using a colorimetric reaction with *O*-phenylenediamine dihydrochloride in absence and in presence of increasing concentrations of competitive oligosaccharides **6–8**.

Figure 3 shows that all the sialosides displayed similar affinity for SNA-I with a median inhibition concentration (IC_{50}) of 36 μM for 6-SL **2**, and IC_{50} values of 38 μM , 33 μM and 36 μM for **6**, **7** and **8**, respectively. These results are similar to those observed for the interaction of 3-SL derivatives with *M. amurensis* agglutinin confirming a modest impact of the C-5 modification of sialic acid on lectin binding.^[27]

Next, the resistance of **6–8** against VC neuraminidase was assessed using TLC (Supporting Information S2). The *N*-acyl 6-SL derivatives **6–8** remained intact after 24 h in presence of the enzyme, but their *N*-Ac homologue was totally hydrolyzed under similar reaction conditions. This result is consistent with previous observations in the α 2,3-sialylated series.^[27] To confirm the ability of the *N*-acyl 6-SL derivatives to act as decoys, inhibition assays were performed with the fluorogenic substrate 4-methylumbelliferyl- α -Neu5Ac (4-MU-Neu5Ac). As expected, oligosaccharides **6–8** displayed competitive inhibition of the neuraminidase, as shown in the Lineweaver–Burk plot for *N*-Prop-6-SL **6** in Figure 4A (other compounds are presented in Supporting Information S3). Although *N*-But-6-SL **7** appeared to be a slightly weaker inhibitor than *N*-Prop-6-SL **6** and *N*-PhAc-6-SL **8**, the inhibition constants of all compounds were in the low

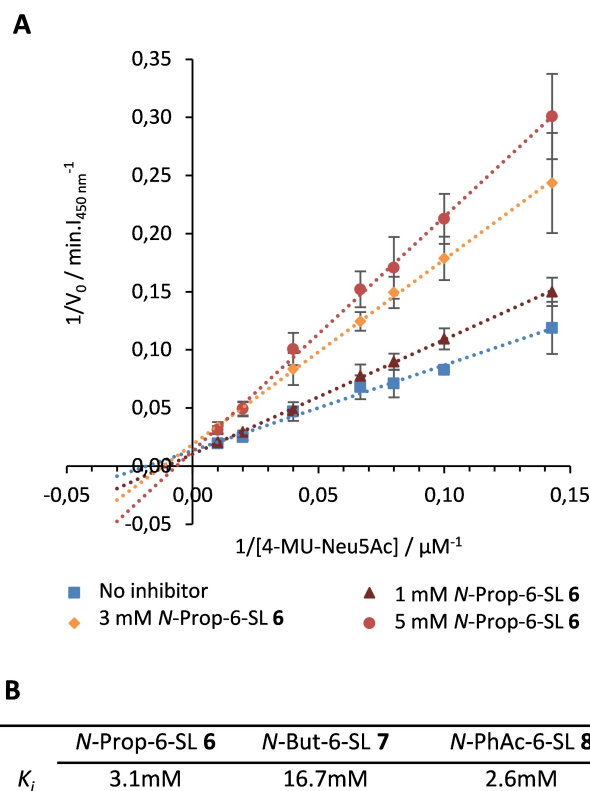


Figure 4. A) Lineweaver–Burk plot of initial-rate kinetics of 4-MU-Neu5Ac hydrolysis by VC neuraminidase in absence or presence of *N*-Prop-6-SL **6**. B) Inhibition constants of *N*-acyl-6SL inhibitors **6–8** towards hydrolysis of 4-MU-Neu5Ac by VC neuraminidase.

millimolar range (Figure 4B). This result is consistent with the fact that these substrate analogs should display an affinity for the enzyme similar to that of sialyllactose, whose K_M for VC neuraminidase is 1.2 mM.^[41]

Conclusions

In this study, we designed a straightforward chemo-biotechnological pathway to produce sialylated oligosaccharides from ManNAc. α 2,3- and α 2,6-sialyllactose were produced at a gram scale in metabolically engineered *E. coli* bacteria. The use of *N*-acyl mannosamine derivatives then allowed the synthesis α 2,6-sialyllactose containing C5-modifications. We showed for the first time that *N*-acyl mannosamine derivatives are actively internalized in *E. coli* via the ManXYZ transporter, albeit at different rates, and converted into the corresponding sialic acids without undergoing significant phosphorylation. ELLA and inhibition assays with SNA-I lectin and VC neuraminidase, respectively, confirmed that these glycomimetics are stable competitive inhibitors of sialidase, and serve as lectin ligands with an affinity similar to that of the natural oligosaccharide. One obvious application of this work is the synthesis of multivalent glycoconjugates as potential anti-adhesion therapy against influenza viruses.^[42] The proposed methodology might also be applied to the production of therapeutic glycoproteins with improved pharmacokinetic properties^[43] and carbohydrate-based antitumor vaccines.^[44]

Experimental Section

General Methods: ^1H and ^{13}C NMR were recorded in D_2O at 298 K using a Bruker Avance III 400 MHz spectrometer (Bruker, Wissembourg, France). For ^1H NMR the solvent residual peaks of D_2O were used as internal standard at 4.79 ppm. Chemical shifts are expressed as parts per million (ppm, δ) and the coupling constant J in Hz. Multiplicities are denoted as s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet), dd (doublet of doublet). Full ^1H and ^{13}C attribution of compounds 1–8 has been done using combination of COSY, HMQC, and HMBC experiments. NMR spectra are presented in Supporting Information S1.

Electrospray ionization (ESI) low resolution mass spectra were recorded on a Bruker amaZone speed spectrometer (Bruker, Wissembourg, France). High-resolution mass spectra (HRMS) were recorded on a Thermo Scientific LTQ Orbitrap XL spectrometer (Thermo Scientific, Villebon-sur-Yvette, France).

Reactions were monitored by TLC (Merck Silica gel 60 F254, Darmstadt, Germany) and visualized under a UV lamp ($\lambda = 254$ nm) and/or by staining with a 30% ammonium bisulfate solution in H_2O or an orcinol (1 g orcinol, 30 mL H_2SO_4 96%, 225 mL H_2O , 725 mL EtOH) solution, followed by charring with a heat gun.

Flash column chromatography purifications were performed using Merk silica gel 60 (0.040–0.063 mm). Purifications by ion-exchange resins were performed using Amberlite IR-120 (H^+ form, Fluka), Amberlite IRA-400 (OH^- form, Sigma), Dowex 66 (free base form, Sigma), Dowex 1 (HCO_3^- form, Sigma) and AG501-X8 mixed bed resin (Bio-Rad, Marnes-la-Coquette, France). Preparative size exclusion chromatography was performed with three Hiload Superdex 30 columns (26x600 mm, GE Healthcare, Uppsala, Sweden) in series

using refractive index detection. Elution was performed with 0.1 M carbonate ammonium at a flow rate of 1.2 mL \cdot min $^{-1}$. Solution conductivity was measured using a PC 52+ DHS conductometer (Dostmann electronic GmbH, Wertheim-Reicholzheim, Germany).

96-Well plates UV-vis absorbance measurements were realized using a TECAN Spark 10 M (Tecan, Grödig, Austria), and fluorometric measurements were realized with a BMG LabTech ClarioStar (BMG Labtech, Ortenberg, Germany). HRP conjugated SNA-I lectin (*Sambucus nigra*, SNA-I, buffered in 0.01 M phosphate - 0.15 M NaCl, labeled with horseradish peroxidase (HRP)) was provided by EY Laboratories (Biovalley, Marne-la-Vallée, France), fetuin from fetal bovine serum and HRP detection kit (SigmaFast OPD) by Sigma Aldrich (Saint-Quentin-Fallavier, France). IC_{50} were calculated using Quest Graph™ IC50 Calculator by AAT Bioquest (Quest Graph™ IC50 Calculator. AAT Bioquest, Inc., 17 Aug. 2022, <http://www.aatbio.com/tools/ic50-calculator>). Neuraminidase from *Vibrio cholerae* was provided by Sigma Aldrich (N7885, Type III, buffered aqueous solution, sterile-filtered, 6.11 units per mL of solution). Other enzymes were obtained from Thermo Fisher Scientific and recombinant DNAs were isolated using miniprep columns (Qiagen Inc.). DNA sequencings were performed by MWG Eurofins Genomics. All other chemicals were provided by Sigma Aldrich (Saint Quentin Fallavier, France).

Chemical synthesis

***N*-Acyl derivatives of D-mannosamine (3-4):** To a solution of D-mannosamineHCl (5 g; 23.0 mmol, 1 equiv.) in 1 M MeONa/MeOH (35 mL) cooled at 0°C was added acid anhydride (34.5 mmol, 1.5 equiv.). The reaction mixture was then stirred overnight at room temperature. Silica gel was added into the flask and the solution was concentrated. After purification by silica gel column chromatography (dry loading, elution with acetonitrile/water 90:10 to 75:25), *N*-acyl-mannosamine was solubilized in water and desalted using mixed bed resin (Biorad AG501-X8) until the medium conductivity dropped below 20 $\mu\text{S}\cdot\text{cm}^{-1}$. The solution was filtered and lyophilized.

***N*-Propionyl-D-mannosamine (ManNProp 3):**^[37] 4.60 g (84%), ^1H NMR (400 MHz, D_2O): (α -isomer) δ 5.12 (d, 1H, $J = 1.7$ Hz, H-1), 4.33 (dd, 1H, $J = 4.7$ Hz, H-2), 4.06 (dd, 1H, $J = 9.8$ Hz, H-3), 3.76–3.93 (m, 3H, H-5,6,6'), 3.63 (t, 1H, $J = 9.5$ Hz, H-4), 2.32 (q, 2H, $J = 7.7$ Hz, COCH_2CH_3), 1.12 (t, 3H, $J = 7.1$ Hz, COCH_2CH_3); (β -isomer) δ 5.03 (d, 1H, $J = 1.7$ Hz, H-1), 4.46 (dd, 1H, $J = 4.5$ Hz, H-2), 3.76–3.93 (m, 3H, H-3,6,6'), 3.52 (t, 1H, $J = 9.8$ Hz, H-4), 3.42 (m, 1H, H-5), 2.38 (q, 2H, $J = 7.7$ Hz, COCH_2CH_3), 1.12 (t, 3H, $J = 7.7$ Hz, COCH_2CH_3); ^{13}C NMR (101 MHz, D_2O): (α isomer) δ 178.7 (CO), 93.2 (C-1), 72.0 (C-5), 68.8 (C-3), 66.7 (C-4), 60.4 (C-6), 53.0 (C-2), 28.9 (COCH_2CH_3), 9.4 (COCH_2CH_3); (β -isomer) δ 179.6 (CO), 93.0 (C-1), 76.3 (C-5), 72.0 (C-3), 66.5 (C-4), 60.4 (C-6), 53.9 (C-2), 29.0 (COCH_2CH_3), 9.4 (COCH_2CH_3); MS (ESI $^+$): m/z 236.03 [$\text{M} + \text{H}$] $^+$.

***N*-Butanoyl-D-mannosamine (ManNBut 4):**^[37] 4.86 g (84%), ^1H NMR (400 MHz, D_2O): (α isomer) δ 5.12 (d, 1H, $J = 1.6$ Hz, H-1), 4.34 (dd, 1H, $J = 4.7$ Hz, H-2), 4.06 (dd, 1H, $J = 9.5$ Hz, H-3), 3.77–3.95 (m, 3H, H-5,6,6'), 3.63 (t, 1H, $J = 9.5$ Hz, H-4), 2.30 (t, 2H, $J = 7.3$ Hz, $\text{COCH}_2\text{CH}_2\text{CH}_3$), 1.63 (m, 2H, $\text{COCH}_2\text{CH}_2\text{CH}_3$), 0.91 (t, 3H, $J = 7.3$ Hz, $\text{COCH}_2\text{CH}_2\text{CH}_3$); (β -isomer) δ 5.03 (d, 1H, $J = 1.7$ Hz, H-1), 4.46 (dd, 1H, $J = 4.5$, 1.7 Hz, H-2), 3.77–3.95 (m, 3H,

H-3,6,6'), 3.53 (t, 1H, $J=9.8$ Hz, H-4), 3.42 (m, 1H, H-5), 2.34 (t, 2H, $J=7.4$ Hz, $\text{COCH}_2\text{CH}_2\text{CH}_3$), 1.63 (m, 2H, $\text{COCH}_2\text{CH}_2\text{CH}_3$), 0.94 (t, 3H, $J=7.7$ Hz, $\text{COCH}_2\text{CH}_2\text{CH}_3$); ^{13}C NMR (101 MHz, D_2O): (α -isomer) δ 177.9 (CO), 93.2 (C-1), 72.0 (C-5), 68.7 (C-3), 66.7 (C-4), 60.4 (C-6), 53.1 (C-2), 37.5 ($\text{COCH}_2\text{CH}_2\text{CH}_3$), 19.0 ($\text{COCH}_2\text{CH}_2\text{CH}_3$), 12.7 ($\text{COCH}_2\text{CH}_2\text{CH}_3$); (β -isomer) δ 178.7 (CO), 92.9 (C-1), 76.3 (C-5), 72.0 (C-3), 66.5 (C-4), 60.4 (C-6), 53.9 (C-2), 37.7 ($\text{COCH}_2\text{CH}_2\text{CH}_3$), 19.0 ($\text{COCH}_2\text{CH}_2\text{CH}_3$), 12.9 ($\text{COCH}_2\text{CH}_2\text{CH}_3$); MS (ESI^+): m/z 250.05 $[\text{M}+\text{H}]^+$.

N-Phenylacetyl-D-mannosamine (ManNPhAc 5).^[37] 1.52 g (22%), ^1H NMR (400 MHz, D_2O): (α -isomer) δ 7.31–7.46 (m, 5H, Ph), 5.11 (d, 1H, $J=1.6$ Hz, H-1), 4.34 (dd, 1H, $J=4.7$ Hz, H-2), 4.08 (dd, 1H, $J=9.7$ Hz, H-3), 3.82–3.92 (m, 3H, H-5,6,6'), 3.68 (m, 2H, COCH_2Ph), 3.62 (d, 1H, $J=9.7$ Hz, H-4); (β -isomer) δ 7.31–7.46 (m, 5H, Ph), 5.04 (d, 1H, $J=1.6$ Hz, H-1), 4.47 (dd, 1H, $J=4.4$ Hz, H-2), 3.79–3.92 (m, 2H, H-3,6), 3.76–3.79 (m, 1H, H-6'), 3.75 (s, 2H, COCH_2Ph), 3.51 (t, 1H, $J=9.8$ Hz, H-4), 3.40 (m, 1H, H-5); ^{13}C NMR (101 MHz, D_2O): (α -isomer) δ 175.2 (CO), 135.0 (C^{Ph}), 129.3–127.2 (C^{Ph}), 93.0 (C-1), 72.0 (C-5), 68.8 (C-3), 66.7 (C-4), 60.3 (C-6), 53.3 (C-2), 42.0 (COCH_2Ph); (β -isomer) δ 176.1 (CO), 135.0 (C^{Ph}), 129.3–127.2 (C^{Ph}), 92.9 (C-1), 76.3 (C-5), 72.0 (C-3), 66.5 (C-4), 60.3 (C-6), 54.2 (C-2), 42.1 (COCH_2Ph); MS (ESI^+): m/z 320.04 $[\text{M}+\text{H}]^+$.

Alternatively, phenylacetic acid (5.7 mmol; 1.2 equiv.) and 4-methylmorpholine (7.5 mmol; 1.6 equiv.) were added at room temperature to a stirred suspension of D-mannosamine·HCl (1 g; 4.8 mmol; 1 equiv.) in MeOH (25 mL). Then DMTMM (5.7 mmol; 1.2 equiv.) was added. After stirring overnight at room temperature, the solution was concentrated and purified by silica gel column chromatography (dry loading, elution acetonitrile/water 10:0 to 9:1). The product was solubilized in water and desalted using a mixed bed resin (AG501-X8). The solution was filtered and lyophilized to provide pure **5** at a yield of 56% (774 mg).

Strains, plasmids, and fermentation - general methods: A summary of plasmids and bacterial strains used in this study can be found in Table 1.

The strain MAC was constructed from the strain ZLKA (Table 1).^[25] To knockout the ManXYZ transporter, a 1.219 kb segment located between nucleotides 332 of *manX* and 522 of *manY* was deleted as follows: two DNA segments flanking the deleted sequence were amplified by PCR using *E. coli* K12 DNA

as template. The upstream 0.915 kb segment was amplified with primers 5' AAGTCGACACTCGAGAATGGCGATGAAGACCAG 3' and 5' CCAAGTCCACCAGTTCATCAAGCTTAA 3' and the downstream 0.902 kb segment with primers 5' CAAGCTTCGATTCCGGAAGTGGTGAC 3' and 5' AGGATCCATGCCCCATGACAAACAG 3'. The two amplified fragments were ligated at their terminal *HindIII* restriction site, and cloned together into the *BamHI* and *Sall* sites of the suicide vector pKO3. The deletion was then carried out according to the pKO3 gene replacement protocol.^[45]

To construct the plasmid pBS-*neuBA*, a 1.07 kb DNA segment containing the *neuB* gene for sialic acid synthase was amplified by PCR using pBBR3-SS plasmid as template.²⁵ The *KpnI* and *BamHI* sites were added using the primers 5' GGTACCTAAGGAGGAAAATAAATGAAAGAAATAAAAATACAA 3' and 5' TGGATCCTCAGCAAAATCCTCATAGCTTAAC 3'. Similarly, a 0.84 kb DNA segment containing the *neuA* gene for CMP-Neu5Ac synthetase was amplified by PCR using the same template. The *BamHI* and *XhoI* sites were added using the primers 5' AGGATCCAGGAGGATATAAATGAGCTTAGCAA-TAATCC 3' and 5' CTCGAGTTAAGTCTCTAATCGATTGTTTC-CAATG 3'. The two amplified fragments were cloned into pCR4Blunt-TOPO vector to form pCR4-*neuB* and pCR4-*neuA*. Finally, the two genes *neuB* and *neuA* were excised from pCR4-*neuB* and pCR4-*neuA* by a *KpnI/BamHI* and a *BamHI/XhoI* digestion respectively, and were simultaneously cloned into the *KpnI* and *XhoI* sites of the pBlueScript KS+ vector to form the plasmid pBS-*neuBA*.

The plasmid pBAD-6st containing the α -2,6-sialyltransferase from *Photobacterium sp. JT-ISH-224* was constructed by subcloning a 1.548 kb DNA fragment obtained by *Acc651* and *XbaI* digestion of pBBR3-6ST plasmid into the *Acc651* and *XbaI* sites of pBAD33.^[28]

The plasmid pSU-3st containing the *N. meningitidis* α -2,3-sialyltransferase gene was constructed by subcloning the 2.15 kb DNA fragment obtained by *SacI* and *KpnI* digestion of plasmid NST-01 into the *SacI* and *KpnI* sites of pSU2718.^[24]

Cultures were carried out in 3 L reactors containing 1.5 L of a mineral culture medium composed of: $\text{NH}_4\text{H}_2\text{PO}_4$ (5 g.L⁻¹), KH_2PO_4 (5 g.L⁻¹), trace mineral solution (7.5 mL.L⁻¹), citric acid (0.5 g.L⁻¹), KOH (1.65 g.L⁻¹), NaOH (0.65 g.L⁻¹), glycerol

Table 1. Genes, plasmids and *E. coli* strains used in this study.

Gene	Description	Reference or source
neuA	CMP-Neu5Ac synthetase from <i>C. jejuni</i>	AF400048
neuB	Sialic acid synthase from <i>C. jejuni</i>	AF400048
6-st	α 2,6-sialyltransferase from <i>Photobacterium sp. JT-ISH-224</i>	AB293985
3-st	α 2,3-sialyltransferase from <i>N. meningitidis</i>	U60660
Plasmids		
pBS-NeuBA	Amp ^R , pLac promoter, <i>colE1</i> ori, high copy, carrying <i>neuBA</i> between <i>KpnI</i> and <i>XhoI</i>	This study
pBAD-6-st	Cm ^R , pAraC promoter, P15 A ori, low copy, carrying 6-ST between <i>Acc651</i> and <i>XbaI</i>	This study
pSU-3-st	Cm ^R , pLac promoter, P15 A ori, low copy, carrying 3-ST between <i>SacI</i> and <i>KpnI</i>	This study
Strains		
ZLKA	DH1, Δ LacZ, Δ LacA, Δ nanKETA	Fierfort et al. (2008) ^[25]
MAC	ZLKA, Δ manXYZ	This study
ZW4	ZLKA, pBS- <i>neuBA</i> , pBAD-6-st	This study
ZW3	ZLKA, pBS- <i>neuBA</i> , pSU-3-st	This study
MAC7	MAC, pBS- <i>neuBA</i> , pSU-3-st	This study

(2.4 g.L⁻¹), MgSO₄·7H₂O (1 g.L⁻¹), glucose (20 g.L⁻¹), thiamine HCl (4.5 mg.L⁻¹). MgSO₄ and glucose were added from concentrated solutions that were autoclaved separately. Thiamine was sterilized by filtration. The trace mineral stock solution contained: nitrilotriacetate (70 mM, pH 6.5), ferric citrate (7.5 g.L⁻¹), MnCl₂·4H₂O (1.3 g.L⁻¹), CoCl₂·6H₂O (0.21 g.L⁻¹), CuCl₂·2H₂O (0.13 g.L⁻¹), H₃BO₃ (0.25 g.L⁻¹), ZnSO₄·7H₂O (1.2 g.L⁻¹), Na₂MoO₄·2H₂O (0.15 g.L⁻¹). Antibiotics were used to ensure the maintenance of the different plasmids. Their final concentrations were 50 mg.L⁻¹ of ampicillin and 25 mg.L⁻¹ of chloramphenicol. The feeding solution contained glycerol (750 g.L⁻¹), MgSO₄·7H₂O (12 g.L⁻¹), trace mineral solution (25 mL.L⁻¹). Throughout the culture, the dissolved oxygen was maintained at 30% of air saturation by automatically adjusting the stirrer speed. The pH of the culture medium was regulated at 6.8 with automatic addition of 30% NH₄OH.

The high cell density culture consisted of two phases: (1) an exponential-growth phase, which started with the inoculation of the bioreactor at 2% (v/v) with a culture grown in LB medium and lasted until exhaustion of the initially added glucose and glycerol (15 h). The temperature was maintained at 33 °C. (2) A fed-batch phase of approximately 24 h with glycerol feeding (4.5 g.L⁻¹.h⁻¹) and a temperature maintained at 28 °C.

The isopropyl-β-D-thiogalactopyranoside inducer was added at a final concentration of 0.2 mM at the beginning of the second phase. At the same time, lactose and ManNAc derivatives were added in equimolar concentration. To monitor the fermentation time-course, culture aliquots (1 mL) were taken at different time points throughout fermentation. Samples were centrifuged and the culture supernatants (then referred to as extracellular fractions) were separated from the pellets by centrifugation. Then the pellets were suspended in water and boiled for 10 min at 100 °C to lyse the cells. After centrifugation, the new supernatants containing the intracellular compounds were referred to as intracellular fractions. Intra- and extracellular fractions were analyzed by TLC (elution with butanol/acetic acid/water 2:1:1, revelation with orcinol solution followed by heating). At the end of the culture, bacterial cells were recovered by centrifugation (7,000 x g for 20 min) and the pellet was resuspended in 1.5 L of distilled water. The cells were permeabilized by heating at 100 °C for 45 min and after cooling, the mixture was grinded and centrifuged again (7,000 x g for 20 min). The pH of the supernatant, corresponding to the intracellular fraction, was lowered to 3.0 by the addition of a strong cation exchanger resin (Amberlite IR-120 H⁺ form). This resulted in the precipitation of proteins, which were removed by centrifugation (7,000 x g for 20 min). The pH of the clear supernatant was then adjusted to 6.0 by the addition of a weak anion exchanger (Dowex 66 free base form), and the supernatant was then loaded on a Dowex 1 (HCO₃⁻ form) column (5 cm x 30 cm). Sialoside was adsorbed on Dowex 1 resin and, after washing with distilled water, eluted with continuous gradient of (NH₄)₂CO₃ (2 L, 0–500 mM, 10 mL.min⁻¹). Fractions containing the sialoside were pooled, concentrated under reduced pressure, and lyophilized. Sialyllactose derivatives (7–9) were additionally purified by low-pressure size-exclusion chromatography

on Superdex 30 resin (26x600 mm) by elution with 0.1 M (NH₄)₂CO₃ at 1.2 mL.min⁻¹. Fractions containing the sialoside were pooled, concentrated under reduced pressure, and lyophilized.

(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranose (1): Strains ZW3 and MAC7 were cultured in presence of ManNAc (1.5 g, 4.5 mM) and lactose (2.31 g, 4.5 mM) to give 3-SL 1 (1.41 g, 32% with ZW3 ; 730 mg, 16% with MAC7). ¹H NMR (400 MHz, D₂O): δ 5.24 (d, J = 3.8 Hz, 1H, H-1^{α-Glc}), 4.68 (d, J = 8.0 Hz, 1H, H-1^{β-Glc}), 4.55 (d, J = 7.9 Hz, 1H, H-1^{Gal}), 4.13 (ddd, J = 9.9, 3.2, 1.5 Hz, 1H, H-3^{Gal}), 3.99 (m, 1H, H-6^{Gal}), 3.98 (m, 1H, H-6^{β-Glc}), 3.97 (m, 1H, H-4^{Gal}), 3.96 (m, 1H, H-6^{α-Glc}), 3.96 (m, 1H, H-5^{α-Glc}), 3.90 (m, 1H, H-6^{α-Glc}), 3.90 (m, 1H, H-6^{Sia}), 3.88 (m, 1H, H-9^{Neu}), 3.86 (m, 1H, H-5^{Neu}), 3.84 (m, 1H, H-6^{β-Glc}), 3.83 (m, 1H, H-3^{α-Glc}), 3.76 (m, 2H, H-6,6^{Gal}), 3.73 (m, 1H, H-5^{Gal}), 3.71 (m, 1H, H-4^{Neu}), 3.69 (m, 1H, H-4^{α-Glc}), 3.69 (m, 1H, H-4^{β-Glc}), 3.66 (m, 1H, H-9^{Neu}), 3.65 (m, 1H, H-8^{Neu}), 3.65 (m, 1H, H-3^{β-Glc}), 3.62 (m, 1H, H-5^{β-Glc}), 3.60 (m, 1H, H-7^{Neu}), 3.59 (m, 1H, H-2^{α-Glc}), 3.59 (m, 1H, H-2^{Gal}), 3.30 (t, J = 8.9 Hz, 1H, H-2^{β-Glc}), 2.77 (dd, J = 12.4, 4.6 Hz, 1H, H-3^{Neu}), 2.05 (s, 3H, COCH₃^{Neu}), 1.81 (t, J = 12.1 Hz, 1H, H-3^{Neu}); ¹³C NMR (101 MHz, D₂O): δ 175.0 (NCO^{Neu}), 173.9 (COONa^{Neu}), 102.6 (C-1^{Gal}), 99.8 (C-2^{Neu}), 95.8 (C-1^{β-Glc}), 91.8 (C-1^{α-Glc}), 78.3 (C-4^{α-Glc}), 78.1 (C-4^{β-Glc}), 75.5 (C-3^{Gal}), 75.1 (C-5^{Gal}), 74.8 (C-5^{β-Glc}), 74.3 (C-3^{β-Glc}), 73.8 (C-2^{β-Glc}), 72.8 (C-8^{Neu}), 71.7 (C-6^{Neu}), 71.4 (C-3^{α-Glc}), 71.1 (C-2^{α-Glc}), 70.1 (C-5^{α-Glc}), 68.1 (C-7^{Neu}), 69.3 (C-2^{Gal}), 68.3 (C-4^{Neu}), 67.4 (C-4^{Gal}), 62.5 (C-9^{Neu}), 61.0 (C-6^{Gal}), 60.0 (C-6^{β-Glc}), 59.9 (C-6^{α-Glc}), 51.7 (C-5^{Neu}), 39.6 (C-3^{Neu}), 22.0 (COCH₃^{Neu}); MS (ESI⁻): m/z 632.2 [M-H]⁻.

(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→6)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranose (2): Strain ZW4 was cultured in presence of ManNAc (1.5 g, 4.5 mM) and lactose (2.31 g.L⁻¹, 4.5 mM) to give 6-SL 2 (1.42 g, 32%); ¹H NMR (400 MHz, D₂O): δ 5.24 (d, 1H, J = 3.8 Hz, H-1^{α-Glc}), 4.68 (d, 1H, J = 8.0 Hz, H-1^{β-Glc}), 4.44 (d, 1H, J = 7.8 Hz, H-1^{Gal}), 3.99 (m, 1H, H-6^{Gal}), 3.95 (m, 1H, H-6^{Neu}), 3.95 (m, 1H, H-5^{α-Glc}), 3.95 (m, 1H, H-6^{β-Glc}), 3.90 (m, 1H, H-8^{Neu}), 3.88 (m, 1H, H-9^{Neu}), 3.88 (m, 1H, H-6^{α-Glc}), 3.86 (m, 1H, H-5^{Neu}), 3.84 (m, 1H, H-3^{α-Glc}), 3.83 (m, 1H, H-5^{Gal}), 3.82 (m, 1H, H-6^{α-Glc}), 3.79 (m, 1H, H-6^{β-Glc}), 3.74 (m, 1H, H-4^{Gal}), 3.68 (m, 1H, H-3^{Gal}), 3.67 (m, 1H, H-4^{Neu}), 3.65 (m, 1H, H-9^{Neu}), 3.65 (m, 1H, H-3^{β-Glc}), 3.65 (m, 1H, H-5^{β-Glc}), 3.64 (m, 1H, H-4^{α-Glc}), 3.64 (m, 1H, H-4^{β-Glc}), 3.61 (m, 1H, H-6^{Gal}), 3.61 (m, 1H, H-2^{α-Glc}), 3.57 (m, 1H, H-7^{Neu}), 3.55 (m, 1H, H-2^{Gal}), 3.31 (d, 1H, J = 9.0 Hz, H-2^{β-Glc}), 2.73 (dd, 1H, J = 12.4, 4.7 Hz, H-3^{Neu}), 2.04 (s, 3H, NCH₃^{Neu}), 1.76 (t, 1H, J = 12.2 Hz, H-3^{Neu}); ¹³C NMR (101 MHz, D₂O): δ 174.9 (NCO^{Neu}), 173.5 (COONa^{Neu}), 103.2 (C-1^{Gal}), 100.3 (C-2^{Neu}), 95.6 (C-1^{β-Glc}), 91.8 (C-1^{α-Glc}), 79.7 (C-4^{α-Glc}), 79.6 (C-4^{β-Glc}), 74.6 (C-5^{β-Glc}), 74.6 (C-3^{β-Glc}), 73.7 (C-2^{β-Glc}), 73.7 (C-5^{Gal}), 72.5 (C-4^{Gal}), 72.3 (C-3^{Gal}), 71.8 (C-8^{Neu}), 71.6 (C-3^{α-Glc}), 71.0 (C-2^{α-Glc}), 70.8 (C-2^{Gal}), 69.9 (C-5^{α-Glc}), 68.5 (C-6^{Neu}), 68.4 (C-7^{Neu}), 68.4 (C-4^{Neu}), 63.5 (C-6^{Gal}), 62.6 (C-9^{Neu}), 60.2 (C-6^{β-Glc}), 60.1 (C-6^{α-Glc}), 51.8 (C-5^{Neu}), 40.1 (C-3^{Neu}), 22.0 (CH₃^{Neu}); MS (ESI⁻): m/z 632.2 [M-H]⁻.

(5-Propionamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→6)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranose (6): Strain ZW4 was cultured in presence of ManNProp 3 (0.99 g, 2.8 mM) and lactose (1.44 g, 2.8 mM) to

give *N*-Prop-6-SL **6** (0.92 g, 33%); $^1\text{H NMR}$ (400 MHz, D_2O): δ 5.26 (d, $J = 3.8$ Hz, 1H, H-1 $^{\alpha\text{-Glc}}$), 4.69 (d, $J = 8.0$ Hz, 1H, H-1 $^{\beta\text{-Glc}}$), 4.45 (d, $J = 7.9$, 1H, H-1 $^{\text{Gal}}$), 4.00 (t, $J = 2.1$ Hz, 1H, H-6 $^{\text{Gal}}$), 3.98 (m, H-6 $^{\beta\text{-Glc}}$), 3.98 (m, 1H, H-5 $^{\alpha\text{-Glc}}$), 3.98 (m, 1H, H-5 $^{\text{Neu}}$), 3.95 (m, 1H, H-7 $^{\text{Neu}}$), 3.92 (m, 1H, H-4 $^{\text{Gal}}$), 3.90 (m, 2H, H-6,6 $^{\alpha\text{-Glc}}$), 3.90 (m, 1H, H-9 $^{\text{Neu}}$), 3.88 (m, 1H, H-3 $^{\alpha\text{-Glc}}$), 3.83 (m, 1H, H-5 $^{\text{Gal}}$), 3.82 (m, H-6 $^{\beta\text{-Glc}}$), 3.76 (m, 1H, H-6 $^{\text{Neu}}$), 3.73 (m, 1H, H-4 $^{\text{Neu}}$), 3.72 (m, 1H, H-3 $^{\text{Gal}}$), 3.69 (m, 1H, H-4 $^{\text{Neu}}$), 3.66 (m, H-3 $^{\beta\text{-Glc}}$), 3.66 (m, H-4 $^{\beta\text{-Glc}}$), 3.66 (m, 1H, H-4 $^{\alpha\text{-Glc}}$), 3.67 (m, 1H, H-9 $^{\text{Neu}}$), 3.63 (m, 1H, H-6 $^{\text{Gal}}$), 3.63 (m, H-5 $^{\beta\text{-Glc}}$), 3.62 (m, 1H, H-2 $^{\alpha\text{-Glc}}$), 3.58 (m, 1H, H-8 $^{\text{Neu}}$), 3.56 (m, 1H, H-2 $^{\text{Gal}}$), 3.33 (t, 1H, $J = 8.6$ Hz, H-2 $^{\beta\text{-Glc}}$), 2.74 (dd, $J = 12.4$, 4.7 Hz, 1H, H-3 $^{\text{Neu}}$), 2.31 (q, $J = 7.6$ Hz, 2H, $\text{NCOCH}_2\text{CH}_3$), 1.77 (t, $J = 12.2$ Hz, 1H, H-3 $^{\text{Neu}}$), 1.14 (t, $J = 7.6$ Hz, 3H, $\text{NCOCH}_2\text{CH}_3$); $^{13}\text{C NMR}$ (101 MHz, D_2O): δ 178.9 (NCO^{Neu}), 173.5 ($\text{COONa}^{\text{Neu}}$), 103.3 (C-1 $^{\text{Gal}}$), 100.3 (C-2 $^{\text{Neu}}$), 95.7 (C-1 $^{\beta\text{Glc}}$), 91.9 (C-1 $^{\alpha\text{-Glc}}$), 79.8 (C-4 $^{\alpha\text{-Glc}}$), 79.7 (C-4 $^{\beta\text{-Glc}}$), 74.7 (C-5 $^{\beta\text{-Glc}}$), 74.7 (C-3 $^{\beta\text{-Glc}}$), 73.8 (C-2 $^{\beta\text{-Glc}}$), 73.7 (C-5 $^{\text{Gal}}$), 72.6 (C-6 $^{\text{Neu}}$), 72.4 (C-3 $^{\text{Gal}}$), 71.8 (C-4 $^{\text{Gal}}$), 71.7 (C-3 $^{\alpha\text{-Glc}}$), 71.1 (C-2 $^{\alpha\text{-Glc}}$), 70.8 (C-2 $^{\text{Gal}}$), 70.0 (C-5 $^{\alpha\text{-Glc}}$), 68.5 (C-7 $^{\text{Neu}}$), 68.5 (C-8 $^{\text{Neu}}$), 68.3 (C-4 $^{\text{Neu}}$), 63.6 (C-6 $^{\text{Gal}}$), 62.7 (C-9 $^{\text{Neu}}$), 60.3 (C-6 $^{\beta\text{-Glc}}$), 60.1 (C-6 $^{\alpha\text{-Glc}}$), 51.7 (C-5 $^{\text{Neu}}$), 40.2 (C-3 $^{\text{Neu}}$), 29.3 ($\text{NCOCH}_2\text{CH}_3$), 9.6 ($\text{NCOCH}_2\text{CH}_3$); HRMS (ESI-LTQ Orbitrap) m/z calcd for $\text{C}_{24}\text{H}_{40}\text{NO}_{19}$ ($[\text{M}-\text{H}]^-$), 646.22000; found: 646.21872.

(5-Butanamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylonic acid)-(2 \rightarrow 6)- β -D-galactopyranosyl-(1 \rightarrow 4)- β -D-glucopyranose (7**):** Strain ZW4 was cultured in presence of ManNBut 4 (1.0 g, 2.7 mM) and lactose (1.47 g, 2.7 mM) to give *N*-But-6-SL **7** (1.1 g, 39%); $^1\text{H NMR}$ (400 MHz, D_2O): δ 5.24 (d, $J = 3.7$ Hz, 1H, H-1 $^{\alpha\text{-Glc}}$), 4.68 (d, $J = 8.1$ Hz, 1H, H-1 $^{\beta\text{-Glc}}$), 4.45 (d, $J = 7.8$, 1H, H-1 $^{\text{Gal}}$), 3.98 (m, 1H, H-6 $^{\text{Gal}}$), 3.95 (m, H-6 $^{\beta\text{-Glc}}$), 3.95 (m, 1H, H-5 $^{\alpha\text{-Glc}}$), 3.95 (m, 1H, H-7 $^{\text{Neu}}$), 3.90 (m, 1H, H-4 $^{\text{Gal}}$), 3.88 (m, 1H, H-9 $^{\text{Neu}}$), 3.87 (m, 1H, H-5 $^{\text{Neu}}$), 3.87 (m, 2H, H-6,6 $^{\alpha\text{-Glc}}$), 3.85 (m, 1H, H-3 $^{\alpha\text{-Glc}}$), 3.82 (m, 1H, H-5 $^{\text{Gal}}$), 3.81 (m, H-6 $^{\beta\text{-Glc}}$), 3.73 (m, 1H, H-6 $^{\text{Neu}}$), 3.73 (m, 1H, H-4 $^{\text{Neu}}$), 3.67 (m, 1H, H-3 $^{\text{Gal}}$), 3.67 (m, 1H, H-4 $^{\text{Neu}}$), 3.65 (m, 1H, H-9 $^{\text{Neu}}$), 3.65 (m, H-5 $^{\beta\text{-Glc}}$), 3.64 (m, H-3 $^{\beta\text{-Glc}}$), 3.64 (m, 1H, H-4 $^{\alpha\text{-Glc}}$), 3.63 (m, H-4 $^{\beta\text{-Glc}}$), 3.60 (m, 1H, H-6 $^{\alpha\text{-Glc}}$), 3.60 (m, 1H, H-2 $^{\alpha\text{-Glc}}$), 3.56 (m, 1H, H-8 $^{\text{Neu}}$), 3.56 (m, 1H, H-2 $^{\text{Gal}}$), 3.34 (t, 1H, $J = 8.4$ Hz, H-2 $^{\beta\text{-Glc}}$), 2.71 (dd, $J = 12.4$, 4.6 Hz, 1H, H-3 $^{\text{Neu}}$), 2.27 (t, $J = 7.4$ Hz, 2H, $\text{NCOCH}_2\text{CH}_2\text{CH}_3$), 1.75 (t, $J = 12.2$ Hz, 1H, H-3 $^{\text{Neu}}$), 1.61 (m, 2H, $\text{NCOCH}_2\text{CH}_2\text{CH}_3$), 0.91 (t, $J = 7.5$ Hz, 3H, $\text{NCOCH}_2\text{CH}_2\text{CH}_3$); $^{13}\text{C NMR}$ (101 MHz, D_2O): δ 178.0 (NCO^{Neu}), 173.5 ($\text{COONa}^{\text{Neu}}$), 103.2 (C-1 $^{\text{Gal}}$), 100.3 (C-2 $^{\text{Neu}}$), 95.6 (C-1 $^{\beta\text{Glc}}$), 91.8 (C-1 $^{\alpha\text{-Glc}}$), 79.7 (C-4 $^{\alpha\text{-Glc}}$), 79.6 (C-4 $^{\beta\text{-Glc}}$), 74.6 (C-5 $^{\beta\text{-Glc}}$), 74.6 (C-3 $^{\beta\text{-Glc}}$), 73.7 (C-2 $^{\beta\text{-Glc}}$), 73.7 (C-5 $^{\text{Gal}}$), 72.5 (C-6 $^{\text{Neu}}$), 72.3 (C-3 $^{\text{Gal}}$), 71.8 (C-4 $^{\text{Gal}}$), 71.6 (C-3 $^{\alpha\text{-Glc}}$), 71.0 (C-2 $^{\alpha\text{-Glc}}$), 70.8 (C-2 $^{\text{Gal}}$), 69.9 (C-5 $^{\alpha\text{-Glc}}$), 68.5 (C-7 $^{\text{Neu}}$), 68.5 (C-8 $^{\text{Neu}}$), 68.2 (C-4 $^{\text{Neu}}$), 63.5 (C-6 $^{\text{Gal}}$), 62.6 (C-9 $^{\text{Neu}}$), 60.2 (C-6 $^{\beta\text{-Glc}}$), 60.1 (C-6 $^{\alpha\text{-Glc}}$), 51.7 (C-5 $^{\text{Neu}}$), 40.2 (C-3 $^{\text{Neu}}$), 37.9 ($\text{NCOCH}_2\text{CH}_2\text{CH}_3$), 19.0 ($\text{NCOCH}_2\text{CH}_2\text{CH}_3$), 12.8 ($\text{NCOCH}_2\text{CH}_2\text{CH}_3$); HRMS (ESI-LTQ Orbitrap) m/z calcd for $\text{C}_{25}\text{H}_{42}\text{NO}_{19}$ ($[\text{M}-\text{H}]^-$), 660.23565; found: 660.23453.

(5-Phenylacetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylonic acid)-(2 \rightarrow 6)- β -D-galactopyranosyl-(1 \rightarrow 4)- β -D-glucopyranose (8**):** Strain ZW4 was cultured in presence of ManNPhAc 5 (1.2 g, 2.7 mM) and lactose (1.47 g, 2.7 mM) to give *N*-PhAc-6-SL **8** (290 mg, 10%); $^1\text{H NMR}$ (400 MHz, D_2O): δ 7.42 (m, 1H, H_o^{Ph}), 7.36 (m, 1H, H_p^{Ph}), 7.35 (m, 1H, H_m^{Ph}), 5.23 (d, $J = 3.8$ Hz, 1H, H-1 $^{\alpha\text{-Glc}}$), 4.67 (d, $J = 8.0$ Hz, 1H, H-1 $^{\beta\text{-Glc}}$), 4.43 (d, $J = 7.9$, 1H, H-1 $^{\text{Gal}}$), 3.97 (m, 1H, H-6 $^{\text{Gal}}$), 3.96 (m,

H-6 $^{\beta\text{-Glc}}$), 3.95 (m, 1H, H-5 $^{\alpha\text{-Glc}}$), 3.94 (m, 1H, H-6 $^{\text{Neu}}$), 3.88 (m, 2H, H-6,6 $^{\alpha\text{-Glc}}$), 3.86 (m, 1H, H-5 $^{\text{Neu}}$), 3.85 (m, 1H, H-8 $^{\text{Neu}}$), 3.85 (m, 1H, H-7 $^{\text{Neu}}$), 3.83 (m, 1H, H-3 $^{\alpha\text{-Glc}}$), 3.80 (m, 1H, H-5 $^{\text{Gal}}$), 3.80 (m, 1H, H-9 $^{\text{Neu}}$), 3.80 (m, H-6 $^{\beta\text{-Glc}}$), 3.73 (m, 1H, H-4 $^{\text{Gal}}$), 3.70 (m, 1H, H-4 $^{\text{Neu}}$), 3.70 (m, 1H, H-4 $^{\text{Neu}}$), 3.66 (m, 1H, H-3 $^{\text{Gal}}$), 3.64 (m, 2H, COCH_2Ph), 3.64 (m, H-3 $^{\beta\text{-Glc}}$), 3.63 (m, H-5 $^{\beta\text{-Glc}}$), 3.63 (m, H-4 $^{\beta\text{-Glc}}$), 3.63 (m, 1H, H-4 $^{\alpha\text{-Glc}}$), 3.61 (m, 1H, H-6 $^{\alpha\text{-Glc}}$), 3.60 (m, 1H, H-2 $^{\alpha\text{-Glc}}$), 3.55 (m, 1H, H-2 $^{\text{Gal}}$), 3.54 (m, 1H, H-9 $^{\text{Neu}}$), 3.30 (t, 1H, $J = 8.6$ Hz, H-2 $^{\beta\text{-Glc}}$), 2.73 (dd, $J = 12.6$, 4.7 Hz, 1H, H-3 $^{\text{Neu}}$), 1.75 (t, $J = 12.2$ Hz, 1H, H-3 $^{\text{Neu}}$); $^{13}\text{C NMR}$ (101 MHz, D_2O): δ 175.5 (NCO^{Neu}), 173.4 ($\text{COONa}^{\text{Neu}}$), 135.0 (C^{Ph}), 129.1 (C- m^{Ph}), 128.9 (C- o^{Ph}), 127.3 (C- p^{Ph}), 103.2 (C-1 $^{\text{Gal}}$), 100.3 (C-2 $^{\text{Neu}}$), 95.6 (C-1 $^{\beta\text{Glc}}$), 91.8 (C-1 $^{\alpha\text{-Glc}}$), 79.7 (C-4 $^{\alpha\text{-Glc}}$), 79.6 (C-4 $^{\beta\text{-Glc}}$), 74.6 (C-5 $^{\beta\text{-Glc}}$), 74.6 (C-3 $^{\beta\text{-Glc}}$), 73.7 (C-2 $^{\beta\text{-Glc}}$), 73.7 (C-5 $^{\text{Gal}}$), 72.5 (C-4 $^{\text{Gal}}$), 72.3 (C-3 $^{\text{Gal}}$), 71.8 (C-7 $^{\text{Neu}}$), 71.6 (C-3 $^{\alpha\text{-Glc}}$), 71.0 (C-2 $^{\alpha\text{-Glc}}$), 70.8 (C-2 $^{\text{Gal}}$), 70.9 (C-5 $^{\alpha\text{-Glc}}$), 68.5 (C-6 $^{\text{Neu}}$), 68.4 (C-8 $^{\text{Neu}}$), 68.1 (C-4 $^{\text{Neu}}$), 63.5 (C-6 $^{\text{Gal}}$), 62.7 (C-9 $^{\text{Neu}}$), 60.2 (C-6 $^{\beta\text{-Glc}}$), 60.1 (C-6 $^{\alpha\text{-Glc}}$), 51.8 (C-5 $^{\text{Neu}}$), 42.6 (COCH_2Ph), 40.2 (C-3 $^{\text{Neu}}$); HRMS (ESI-LTQ Orbitrap) m/z calcd for $\text{C}_{29}\text{H}_{42}\text{NO}_{19}$ ($[\text{M}-\text{H}]^-$), 708.23565; found: 708.23478.

Biological assays

Enzyme-linked lectin assay with SNA-I lectin: ELLA experiments were conducted in 96-well microtiter plates coated with fetuin ($20 \mu\text{g}\cdot\text{mL}^{-1}$, $100 \mu\text{L}$), in PBS buffer (10 mM, pH 7.4), overnight at 4°C . After washing three times with $100 \mu\text{L}$ of 1 mM PBS containing 0.05% Tween 20, hereafter named PBST, the wells were incubated for 2 h at 37°C with a blocking solution of BSA (1% BSA in PBST, $200 \mu\text{L}$ per well). The plates were washed again three times with PBST ($100 \mu\text{L}$). Solutions containing sialylated oligosaccharides at different concentrations (0.5 μM –8 mM) and SNA-I-HPR conjugate ($15 \mu\text{g}\cdot\text{mL}^{-1}$) in PBST were pre-incubated 1 h at 37°C . The wells were filled with $50 \mu\text{L}$ of these solutions and incubated 2 h at 37°C . After washing three times with PBST ($100 \mu\text{L}$), the color was developed by reaction with the HRP substrate *O*-phenylenediamine dihydrochloride (SigmaFast OPD, $200 \mu\text{L}$) for 30 min at room temperature following instructions from the manufacturer. Absorbance was measured at 450 nm. % Inhibition was calculated according to formula:

$$\% \text{ Inhibition} = \frac{A_{\text{no inhibitor}} - A_{\text{sample}}}{A_{\text{no inhibitor}}} \times 100$$
 where A is the absorbance.

VC Neuraminidase Inhibition Fluorescent Assay: VC neuraminidase activity was determined using a fluorometric assay based on the measurement of 4-methylumbelliferone (MU) released upon cleavage of 4-methylumbelliferyl- α -Neu5Ac (4-MU-Neu5Ac). Enzyme ($1 \text{ mU}\cdot\text{mL}^{-1}$) was incubated at 39°C with different substrate concentrations (7 μM –100 μM) in $200 \mu\text{L}$ of 50 mM sodium acetate buffer containing 10 mM sodium chloride, 50 mM calcium chloride (pH 5.5), in absence or presence of sialyllactose inhibitor at different concentrations (0.5 mM–5 mM). Samples were excited at 365 nm and emission was read at 450 nm at fixed gain during 5 min after mixing the enzyme with the substrate. Data analysis was done on values taken between 1 min and 5 min.

Supporting Information

TLC analysis of *N*-acyl-sialyllactose hydrolysis by *V. Cholerae* neuraminidase, *V. Cholerae* neuraminidase inhibition florescent assays, NMR spectra of the products.

CRedit author statement

Paul Rivollier: Investigation, Writing - original draft. **Eric Samain:** Conceptualization, Investigation. **Sylvie Armand:** Supervision. **Isabelle Jeacomine:** Investigation. **Emeline Richard:** Conceptualization, Investigation, Supervision, Writing - original draft. **Sébastien Fort:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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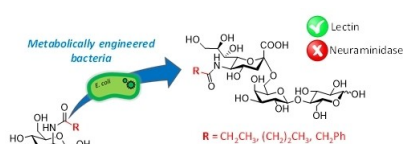
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RESEARCH ARTICLE

Sialyllactose mimetics showing lectin affinity but resistant to neuraminidase hydrolysis were produced on a gram scale from *N*-acyl-mannosamines using metabolically engineered *E. coli* cells.



*P. Rivollier, E. Samain, S. Armand, I. Jeacomine, E. Richard, S. Fort**

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Synthesis of Neuraminidase-Resistant Sialyllactose Mimetics from *N*-Acyl Mannosamines using Metabolically Engineered *Escherichia coli*

