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Original Research Article

Chemo-enzymatic synthesis of Neu5Gc-containing sialylated lactulose

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Abstract

Purpose: *To synthesize novel sialylated lactuloses, namely, Neu5Gc-α2,3-lactulose and Neu5Gc-α2,6-lactulose.*
Methods: *ManNGc was chemically synthesized from commercially available N-acetylmannosaime (ManNAc), which was used as the donor substrate to synthesize α-(2→3) linkage and α-(2→6) linkage sialyllactulose from lactulose via sialyltransferases-catalyzed one-pot multienzyme (OPME) approach. The sialylated products were purified by silica gel flash chromatography column. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) were used to confirm the purity and characterize the structure of the new compounds.*
Results: *Sialyllactulose with α-(2→3) linkage (Neu5Gc-α2,3-lactulose) and α-(2→6) linkage (Neu5Gc-α2,6-lactulose) were efficiently synthesized by an efficient one-pot multienzyme sialylation approach from ManNGc, sodium pyruvate, CTP, and lactulose. The molecular weight of the two products, based on mass spectral data was 648 Da while NMR data indicated the formation of sialylated glycans.*
Conclusion: *Novel sialylated oligosaccharides have been efficiently synthesized from lactulose using highly efficient OPME sialylation approaches. Further investigations are required to ascertain the probiotic activities for possible applications in pharmaceutical and food industries.*

Keywords: *Neu5Gc, Chemo-enzymatic synthesis, Sialylation, Sialyllactulose, Lactulose*

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INTRODUCTION

Sialic acids are nine-carbon sugars and there are over fifty structurally distinct forms of sialic acids found in nature. Sialic acids have three basic forms, including N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminc acid (KDN) [1-3]. Sialylated glycoconjugates play important roles in physiological and pathological processes, such as cellular recognition, bacterial and viral infection, and tumor development, differentiation, etc [3,4]. Currently, the intestinal microbiome is increasingly being recognized as an important determinant of health. Sialylated oligosaccharides are known to be one of the receptors for pathogens and they could inhibit pathogen adhere to the intestinal epithelia [5,6] and block the adhesion of *Helicobacter pylori* to intestinal cells [7].

The particular difficulty of the application of sialylated oligosaccharides is the lack of sufficient material for performing preclinical trials. Enzymatic approaches have approved to be one of the efficient way to produce many novel

synthetic sialylated oligosaccharides which are most likely to have antimicrobial actions.

Lactulose (4-O-β-D-galactopyranosyl-D-fructose) does not exist in nature. It is usually synthesized from lactose by isomerization and bioconversion [8-10]. Lactulose is non-absorbable in the upper gastrointestinal tract but can be metabolized by colonic bacteria [12]. It has been used to treat hepatic encephalopathy in patients with hyperammonemia [13], and also widely used in food industries because of its prebiotic activities [14]. On the other hand, non-human sialic acid *N*-glycolylneuraminic acid (Neu5Gc) is broadly presented in tissues of animals other than human and has also been shown to be expressed on glycolipids and glycoproteins in human melanoma, colon, and breast cancers [15,16]. It is interesting to get knowledge of whether Neu5Gc-containing lactuloses have any increased prebiotic activities.

In this paper, we report here the efficient chemo-enzymatic synthesis of Neu5Gc-containing sialylated lactuloses with α-(2→3) linkage and α-(2→6) linkage, via one-pot multienzyme sialylation approach from chemically synthesized ManNGc as the donor substrate and commercially available lactulose as the acceptor substrate. The outcome of this work should provide useful information in the development of Neu5Gc containing sialylated oligosacchride.

EXPERIMENTAL

Materials and reagent

Lactulose was purchased from Carbosynth Ltd (Berkshire, UK); CTP was purchased from Hangzhou Meiya Pharmaceutical (Hangzhou, China); Neu5Ac and N-Acetyl-D-mannosamine (ManNAc) were purchased from Ningbo Hongxiang Bio-chem (Ningbo, China); Mannose was purchased from Shanghai Institute of Biotechnology (Shanghai, China); Magnesium chloride ($MgCl_2$), *p*-anisaldehyde, acetoxyacetyl chloride, ethyl acetate (EtOAc), methyl alcohol (MeOH), n-propanol, ammonia, ethyl alcohol (EtOH), acetic acid (HOAc), sodium chloride and sodium hydrogen sulfite were of analytical grade (AR). Pmaldolase (from *Pasteurella multocida*), NmCSS (a CMPsialic acid synthetase from *N. meningitidis*), PmST1_M144D (α2–3-sialyltransferase, a *Pasteurella multocida* multifunctional α-2,3-sialyltransferase 1 M144D mutant), Pd2-6ST (a recombinant *Photobacterium damsela* α-2,6-sialyltransferase) were kindly provided by National Glycoengineering research center (NGRC) in Shandong University.

Chemical synthesis of ManNGc

(1) $ManNH_2 \cdot HCl$

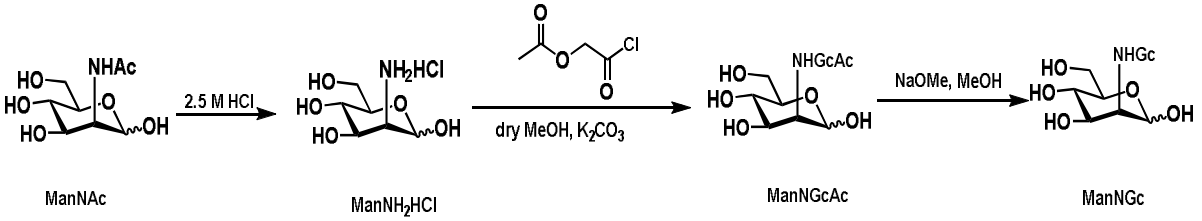
ManNAc (5 g) was dissolved in 20 mL of 2.5 M HCl. The mixture was reacted at 60 °C (water-bath) for 1 to 2 h. After removal of the solvent, the residue was dried under vacuum.

(2) ManNGcAc

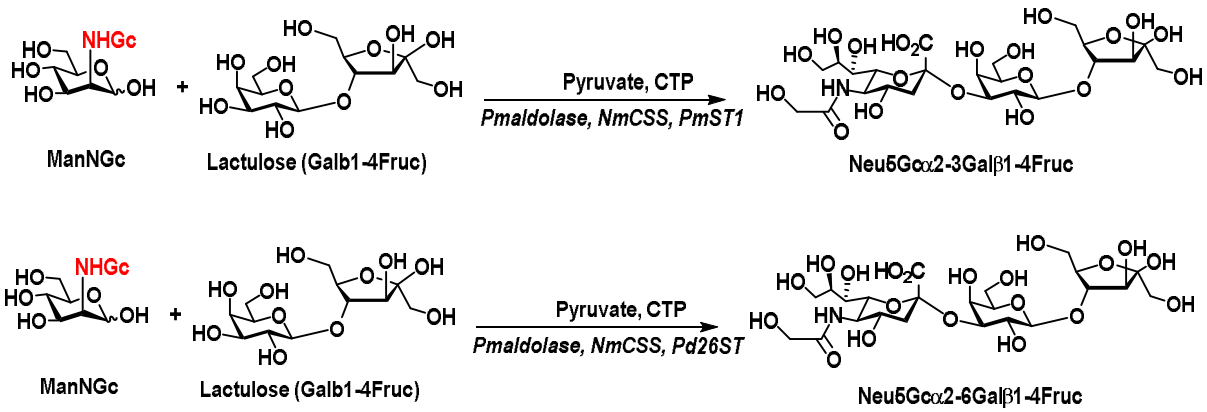
To a stirred mixture of $ManNH_2$-HCl (2.16 g) in dry MeOH (50 mL), solid K_2CO_3 (6.91 g) was added. The mixture was stirred for 30 min and acetoxyacetyl chloride (2.15 mL) was added. When the starting material was almost consumed, the reaction mixture was concentrated and purified by silica flash chromatography (EtOAc:MeOH: H_2O = 5 : 2 : 1) to give the ManNGcAc (80 %).

(3) ManNGc

ManNGcAc (1 g) was dissolved in dry MeOH (20 mL), and NaOMe (200 mg) was then added to give pH about 9. The reaction mixture was stirred for overnight at rt. Resin (H^+) was added to the reaction mixture to neutralize the NaOMe. After filtration and concentration, the desired ManNGc was obtained (Yield: around 50 %).



Scheme 1: Chemical synthesis of ManNGc from ManNAc



Scheme 2: Enzymatic sialylation of lactulose via OPME sialylation system

Enzymatically synthesis of Neu5Gcα2-3Lactulose and Neu5Gcα2-6Lactulose

(1) Small-scale reaction assays

The enzymatic assays were carried out in a total volume of 10 µL in Tris–HCl buffer (100 mM, pH 8.5) containing Lactulose (10 mM), ManNGc (15 mM), sodium pyruvate (60 mM), CTP (15 mM), $MgCl_2$ (20 mM), Pmaldolse (2 µg), and PmST1_M144D (5 µg) or Pd26ST (5 µg). Reactions were allowed to proceed for 4.5 h at 37 °C. The reaction process was monitored by thin layer chromatography (TLC) and mass spectra.

(2) Preparation-scale reactions

ManNGc (100 mg), sodium pyruvate (278 mg), lactulose (119 mg), and CTP (285 mg) were dissolved in Tris-HCl buffer (10 mL, 100 mM, pH 8.5) containing $MgCl_2$ (20 mM) and Pmaldolase (3 mg), NmCSS (3 mg), PmST1_M144D (5 mg, for synthesis of Neu5Gcα2-3Lactulose) or Pd26ST (5 mg, for synthesis of Neu5Gcα2-6Lactulose). The reactions were incubated in an incubator shaker at 37 °C for overnight with agitation at 110 rpm. The reactions were monitored by TLC (n-propanol:H_2O:NH_4·OH = 5: 2:1, by volume). When an optimal yields were achieved, the reactions were stopped by adding the same volume of cold EtOH and kept at 4 °C for 30 min, then centrifuged (7000 rpm, 30 min). The supernatant was concentrated and the residue was purified by silica gel column (eluting solvent: EtOAc : MeOH : H_2O = 6 : 2 : 1 and EtOAc:MeOH:H_2O = 5:2:1).

Mass spectrometry

Purified samples (0.5 mg) were dissolved in 100 µL distilled water at room temperature. Mass Spectrum analyses were carried out on a TQD LC/MS system (Waters, USA), equipped with quaternary pump, vacuum degasser, column compartment, auto-injector, diode-array detector (DAD), and ion-trap mass spectrometer with electrospray ionization (ESI) interface. Scan ranges were 150 - 800 m/z.

NMR

Purified sample (15 mg) were dissolved in 0.5 mL D_2O at room temperature. NMR spectra were recorded on a Bruker Avance III HD 600 spectrometer (Bruker BioSpin, Billerica, MA, USA) at 600 MHz for ^1H and ^{13}C.

RESULTS

ManNGc

As shown in Figure 1, ManNAc, $ManNH_2$, ManNGc, and ManNGcAc were separated by the developing solvent (EtOAc:MeOH:H_2O = 6:2:1) in thin layer chromatography (TLC). Silica gel flash chromatography column was used to purify the products in series of gradient eluting solvent.

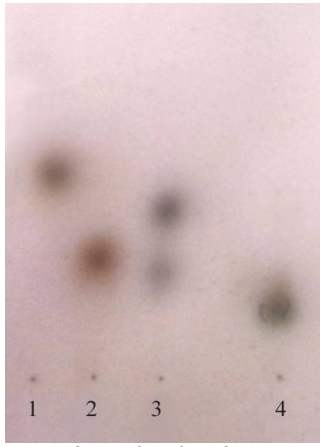


Figure 1: TLC of synthesis of ManNGc. ***Key*:** 1 - ManNAc; 2 - $ManNH_2$; 3 - ManNGcAc; 4 - ManNGc

Formation of Neu5Gcα2-3Lactulose and Neu5Gcα2-6Lactulose

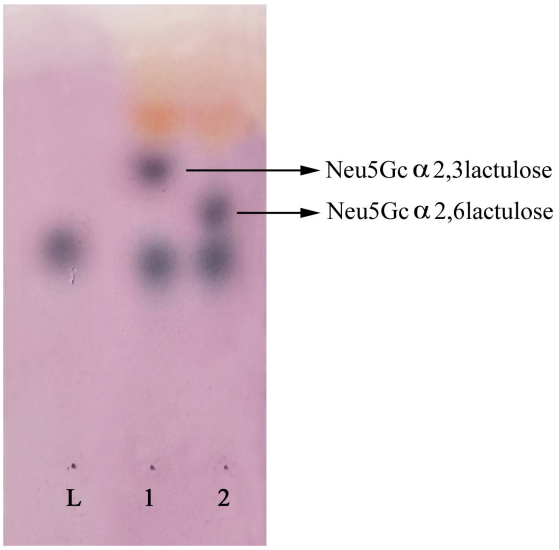


Figure 2: TLC of small reactions of Neu5Gcα2-3Lactulose and Neu5Gcα2-6Lactulose. L, Lactulose; 1, reaction of Neu5Gcα2-3lactulose; 2, reaction of Neu5Gcα2-6lactulose)

As shown in Figure 2, using lactulose as the sialyltransferase acceptor, α2-3-sialylated and α2-6-sialylated trisaccharides were readily obtained using an efficient OPME approach.

Mass spectra

Figure 3: TLC of purified Neu5Gcα2-3Lactulose and Neu5Gcα2-6Lactulose. L, Lactulose; 1, Neu5Gcα2-3Lactulose; 2, Neu5Gcα2-6Lactulose

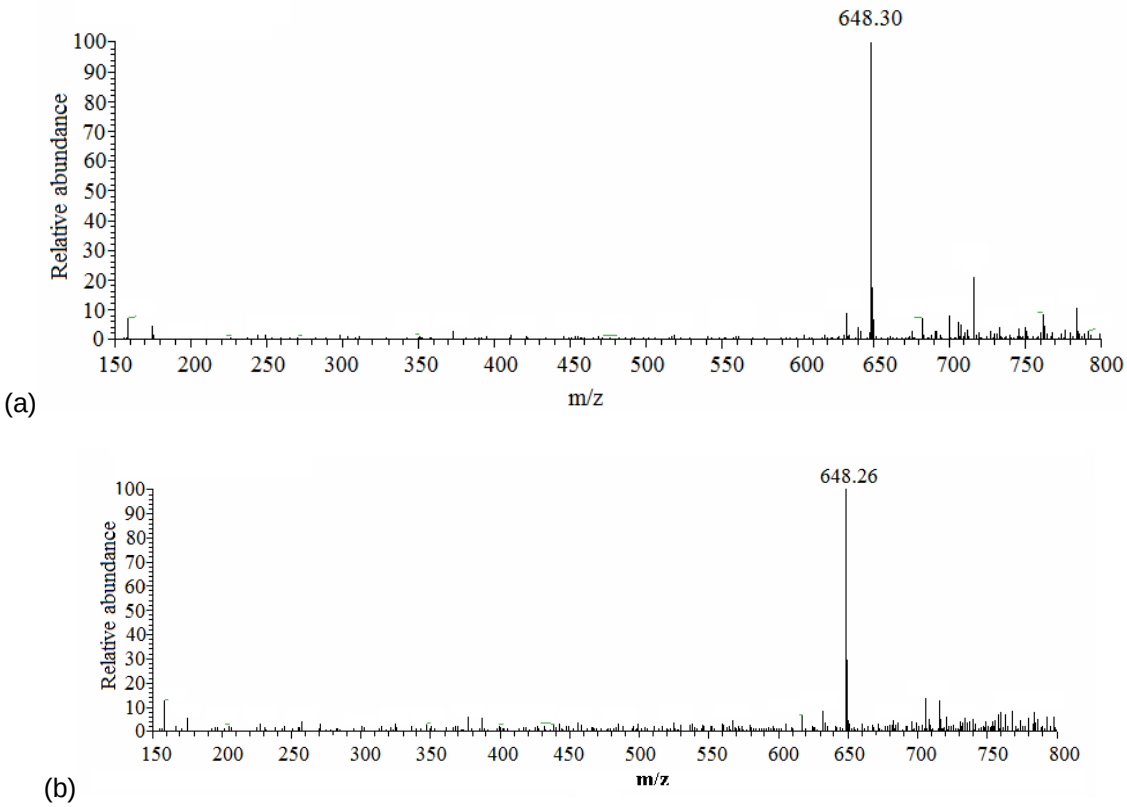


Figure 4: Mass spectrum of purified Neu5Gcα2-3Lactulose and Neu5Gcα2-6Lactulose. ***Note:*** a = Neu5Gcα2-3Lactulose; b = Neu5Gcα2-6Lactulose.

The purified products were checked by TLC and Mass spectrum. Figure 3 showed the compounds were pure. From Figure 4, the molecular weight of Neu5Gcα2-3lactulose and Neu5Gcα2-

6Lactulose based on MS data both showed about 648 Da (the mass of lactulose is 342 Da), these results confirmed that the obtained products are the desired sialylated lactuloses.

NMR data

As shown in Figure 5, the structures of sialyllactuloses were confirmed by ^{1}H and ^{13}C NMR spectra. The NMR data are as follows:

Neu5Gcα2-3Lactulose: Yield, 85 %; white solid. ^{13}C NMR (151 MHz, D_2O, Figure 5a) δ 175.73, 173.83, 100.32, 99.77, 98.02, 77.24, 75.61, 75.12, 72.53, 71.81, 69.19, 68.05, 67.96, 67.48, 66.55, 66.03, 63.87, 62.91, 62.48, 61.09, 60.94, 60.47, 51.35, 39.71. ^{1}H NMR (600 MHz, D_2O, Figure 5b) δ 4.55 (d, *J* = 7.8 Hz, 0.7 H), 4.45 (d, *J* = 7.8 Hz, 0.3 H), 4.20– 3.43 (m, 23 H), 2.67 (dd, *J* = 12.0 and 4.8 Hz, 1 H), 1.71 (t, *J* = 12.0 Hz, 1 H).

Neu5Gcα2-6Lactulose: Yield, 80 %; white solid. ^{13}C NMR (151 MHz, D_2O, Figure 6a) δ 175.69, 173.47, 100.30, 98.09, 96.60, 78.18, 74.32, 73.61, 72.42, 72.23, 71.75, 70.64, 68.61, 68.24, 68.00, 67.05, 66.26, 63.85, 62.97, 62.54, 60.94, 51.45, 40.12. ^{1}H NMR (600 MHz, D_2O, Figure 6b) δ 4.44 (d, *J* = 7.8 Hz, 0.6 H), 4.35 (d, *J* = 7.8 Hz, 0.4 H), 4.24– 3.46 (m, 23 H), 2.62 (dd, *J* = 12.0 and 4.8 Hz, 1 H), 1.64 (t, *J* = 12.0 Hz, 1 H).

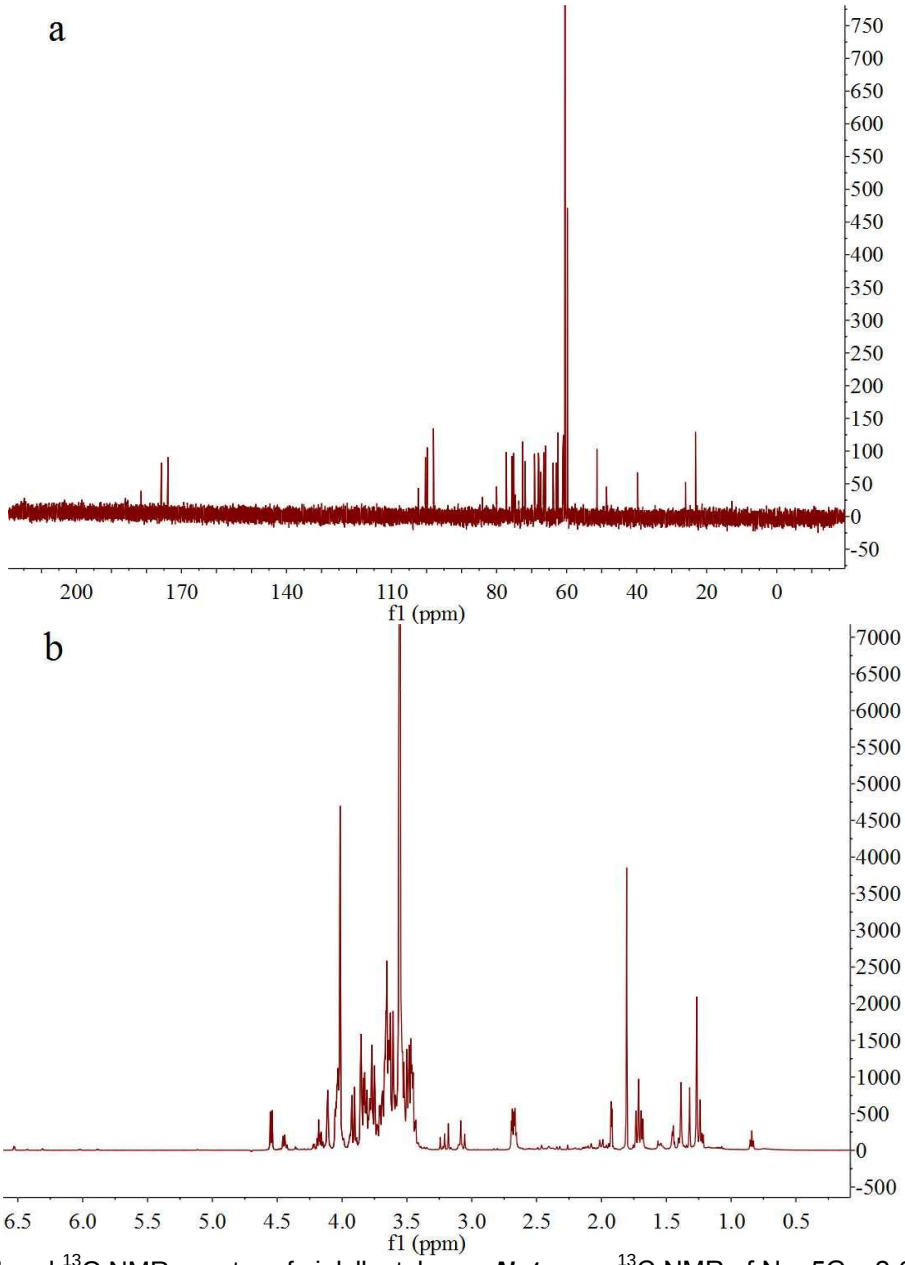


Figure 5: ^{1}H and ^{13}C NMR spectra of sialyllactuloses. ***Note:*** a = ^{13}C NMR of Neu5Gcα2-3Lactulose; b = ^{1}H NMR of Neu5Gcα2-3lactulose

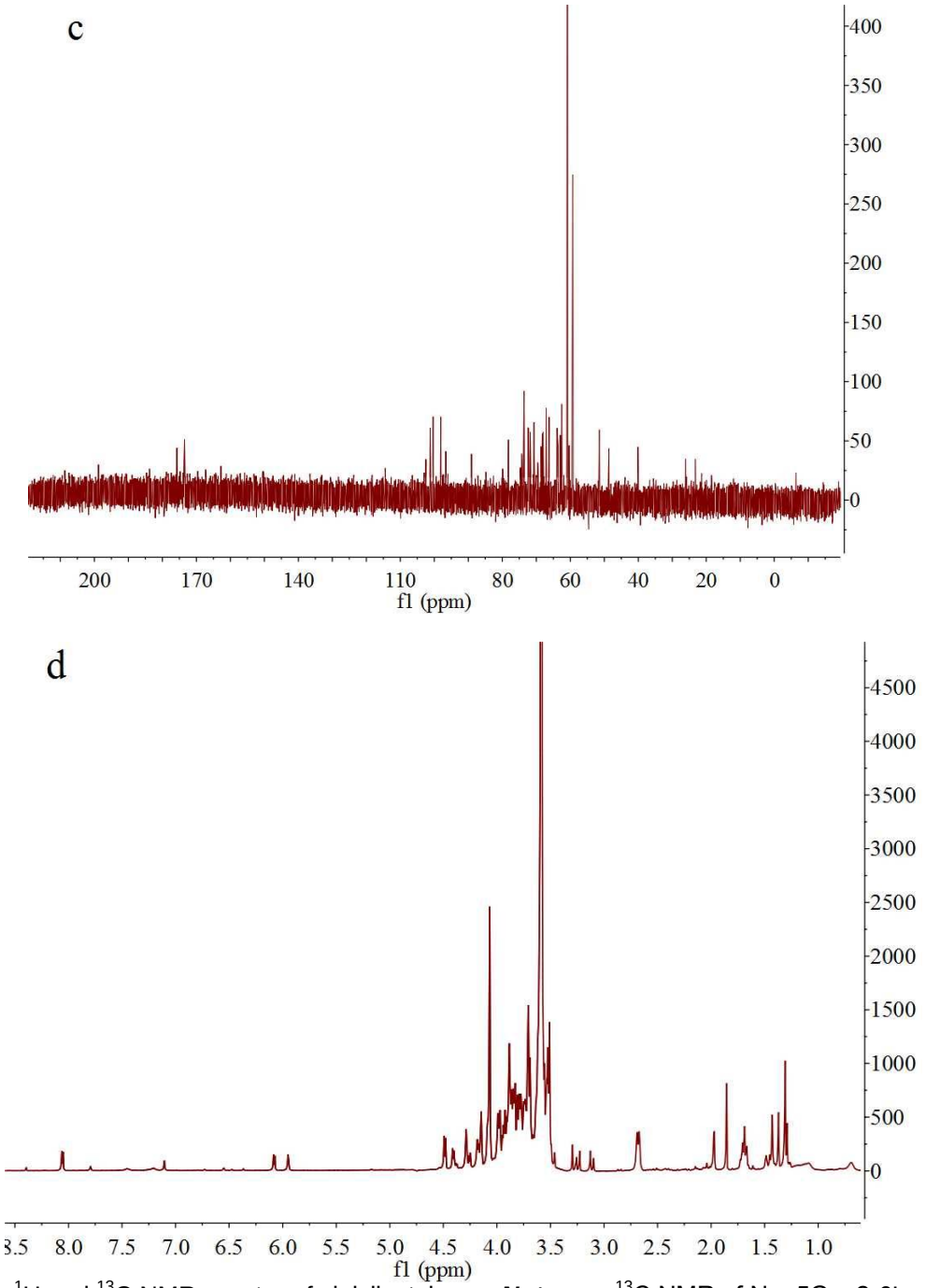
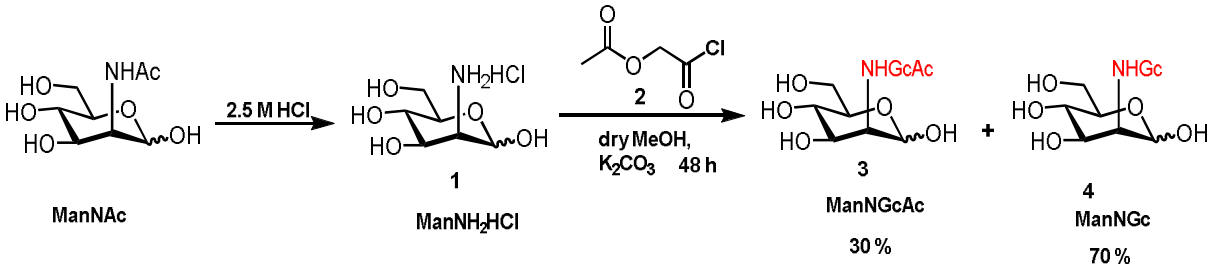


Figure 6: ^{1}H and ^{13}C NMR spectra of sialyllactuloses. ***Note:*** a = ^{13}C NMR of Neu5Gcα2-6Lactulose; b = ^{1}H NMR of Neu5Gcα2-6-lactulose

DISCUSSION

The sialic acid modification on cell surface glycoproteins and glycolipids plays a crucial role in many biological processes, including cell adhesion, antigen recognition and signal transduction [17]. Studies have shown that sialylated glycans have a good prospect in food, especially the developing nervous system in infants [18]. Chemical synthesis of sialylated glycans is very difficult, and the enzymatic synthesis provides an effective method for generating sialyl linkages [19].

In this paper, in order to obtain Neu5Gc-containing sialosides via enzymatic approaches, firstly ManNGc was synthezied from ManNAc. We found that most ManNGcAc (around 70 %) was deacetylated to ManNGc because of alkaline condition when the reaction of formation of ManNGcAc prolonged to 48 h. The two products were separated by silica gel flash



Scheme 3: Synthesis of ManNGc

Chromatography. Therefore, Scheme 1 can be presented as following Scheme 3:

Then ManNGc was used as the donor substrate in the OPME sialylation system to produce the corresponding sialosides. Therein, sialic acid aldolase (Pmaldolase, for synthesis of Neu5Gc), CMP-sialic acid synthetase (NmCSS, for synthesis of CMP-Neu5Gc), and sialyltransferase (PmST1_M144D, for synthesis of α2-3-linked sialoside; Pd26ST, for α2-6-linked sialoside) without the isolation of intermediates. In small-scale reactions assays, around 55% lactulose in the reaction system could not reacted completely, probably due to non-sufficient enzymes or decomposition of CTP. The yield can be solved by adding enough enzyme or CTP during the progress of the enzymatic reactions. Indeed, during large-scale reactions additional amounts of enzyme and CTP were added when TLC analysis showed the reactions not going further. The sialylation yield was up to around 85 % for Neu5Gcα2-3Lactulose and 80 % for Neu5Gcα2-6Lactulose.

CONCLUSION

Two novel Neu5Gc-containing sialyllactulose with α-(2→3) linkage and α-(2→6) linkage have been efficiently synthesized by OPME sialylation from ManNGc, sodium pyruvate, CTP and lactulose. As it's important to find new sialylated oligosaccharides with stronger antibacterial and/or probiotic activities, the two obtained sialylated lactuloses would be very useful substrates for determining their abilities of inhibiting pathogenic bacterial binding to intestinal cells.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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