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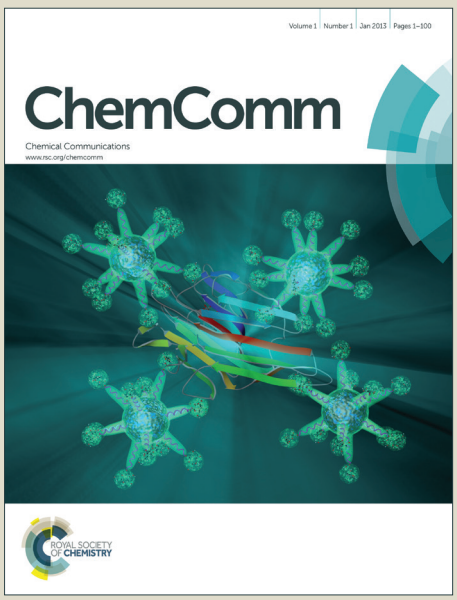

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Sequential one-pot enzymatic synthesis of oligo-*N*-acetyllactosamine and its multi-sialylated extensions

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

A simple and efficient protocol for the preparative-scale synthesis of various lengths of oligo-*N*-acetyllactosamine (oligo-LacNAc) and its multi-sialylated extensions is described. The strategy utilizes one thermophilic bacterial thymidylyltransferase (RmlA) coupled with corresponding sugar-1-phosphate kinases to generate two uridine diphosphate sugars, UDP-galactose and UDP-*N*-acetylglucosamine. By incorporating glycosyltransferases, oligo-LacNAcs and their sialylated analogs were synthesized.

N-Acetyllactosamine oligomers (oligo-LacNAc) are type-2 oligosaccharides composed of repeating units with β(1→3) linked LacNAc (Galβ1→4GalNAc) residues.[1] These oligomers are found on glycoproteins[2] and glycolipids;[3] serve as acceptor substrates for a series of glycosyltransferases (GTs) in the production of various glycoconjugates, such as sialylated, fucosylated, and ABO blood antigens decorated with oligo-LacNAc chains; and constitute glycan ligands involved in specific biological activities.[4] In addition, LacNAc extensions are present on the branched core structures of glycans, sometimes with the linear assembly of several LacNAc units to form so-called poly-LacNAc. It has been well-documented that LacNAc is an essential structural unit recognized by common glycan-binding proteins, such as galectins. Changes in binding specificity associated with the multivalent LacNAc present in branched *N*-glycan or repeated LacNAc chains have also been reported.[5] Moreover, 6'-sialylated compounds with different numbers of LacNAc repeat units show significant differences in binding specificity and affinity to the influenza-hemagglutinin receptor.[6]

The majority of oligo-LacNAc containing carbohydrates cannot be isolated from natural sources. To gain better insight into carbohydrate-mediated biological mechanisms and to tackle such inaccessible complex structures, many advanced chemical synthetic strategies[7] and enzymatic strategies[8] have been developed to synthesize homogenous linear or biantennary oligo-LacNAc extensions and intact glycoproteins bearing *O*- and *N*-glycans.[9] Notably, a chemo-enzymatic strategy was developed to synthesize asymmetrical multi-antennary *N*-glycans.[10] Because enzymatic routes are more efficient for natural and non-natural glycans and glycoconjugates preparation,[11] many efforts are focus on the availability of a variety of sugar-nucleotides for GT-catalyzed oligosaccharide elongation.[12] We herein report a simple and efficient method to produce uridine diphosphate galactose (UDP-Gal) and uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) for the preparative-scale synthesis of various lengths of oligo-LacNAc and its multi-sialylated extensions. During the preparation of this manuscript, Paulson and coworkers demonstrated the feasibility of α-2,6-sialylation at the internal Gal of either linear or branched oligo-LacNAc containing complex saccharides by using α-2,6-sialyltransferase from *Photobacterium damsela* (Pd2,6ST).[13] With the oligo-LacNAc in hand, we further explored the synthesis of multi-sialylated oligo-LacNAc extensions with α-2,3- or α-2,6-linkage by using two bacterial STs, PmST[14] from *Pasteurella multocida* and Pd2,6ST, respectively. We found that different degrees of α-2,6-sialylation on oligo-LacNAc by Pd2,6ST could be achieved under varying concentrations of the sialic acid donor, cytidine monophosphate-sialic acid (CMP-sialic acid).

To efficiently and quickly produce oligo-LacNAc, we used two recombinant bacterial GTs, β-1,3-*N*-acetyl-glucosaminyltransferase from *Helicobacter pylori* (HpGnT)[15] and β-1,4-galactosyltransferase from *Neisseria meningitidis* (NmGalT), overexpressed in *E. coli*.[16] Defined lengths of oligo-LacNAc were synthesized using these enzymes in the presence of the sugar donors UDP-Gal and UDP-GlcNAc. To reduce cost and enable the simple production of the UDP-sugars used in oligo-LacNAc synthesis, an enzymatic one-pot synthesis of two UDP-sugars (UDP-Gal and UDP-GlcNAc) using one thermophilic enzyme, RmlA (glucose-1-phosphate thymidylyltransferase, EC 2.7.7.24, from *Aneurinibacillus thermoaerophilus* DSM10155),[17] was exploited. RmlA, which was

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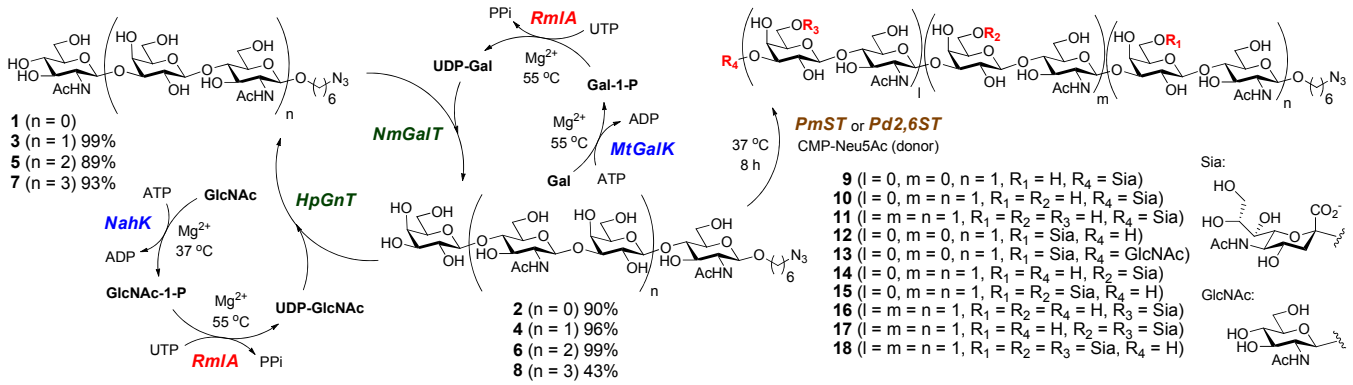

Scheme 1. Sequential synthesis of oligo-LacNAcs and Sialylation of oligo-LacNAcs

found to synthesize thymidine diphosphate glucose (dTDP-Glc) in nature, has been reported to possess broad substrate tolerance toward sugar-1-phosphates (sugar-1-P) in the preparation of NDP-sugars.[12a] Additionally, we explored its potential to produce UDP-Gal in a preparative scale, which was further used in the multi-hundred milligram scale synthesis of LacNAc.[17] Importantly, compare to other enzymes which can produce UDP-sugars directly from the condensation reaction of uridine triphosphate (UTP) and sugar-1-P,[12b-d] the capability of producing both UDP-Gal and UDP-GlcNAc makes RmlA an ideal enzyme for the enzymatic oligo-LacNAc synthesis. The corresponding sugar-1-P required for RmlA, galactose-1-phosphate (Gal-1-P) and *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P), were produced by using recombinant galactokinase (MtGalK, from *Meiothermus taiwanensis* sp. nov. WR-220)[18] and *N*-acetylhexosamine kinase (NahK, from *Bifidobacterium longum*)[19], respectively. In addition to RmlA, MtGalK is also a thermophilic enzyme and was coupled with RmlA in the one-pot synthesis of UDP-Gal in our group.[18] Combining RmlA with the corresponding kinases and GTs, the GlcNAc analog (**1**) was smoothly converted to defined lengths of oligo-LacNAc (**2-8**) by a sequential one-pot enzymatic synthesis starting from cheap materials (GlcNAc and Gal), as shown in Scheme 1.

The studies were commenced from the preparation of LacNAc by using a 6-azidohexyl-GlcNAc acceptor (**1**, azido-GlcNAc);[20] the azido group could be employed for further modification. The following β-1,4-galactosidic linkage was constructed by NmGalT in the presence of UDP-Gal. Both MtGalK and RmlA are thermally stable and show good activity at 55 °C under similar reaction conditions; thus, the one-pot synthesis of UDP-Gal is feasible using these two enzymes. As shown in Scheme 1, in the one-pot synthesis of UDP-Gal, Gal (20 mM), adenosine triphosphate (ATP, 20 mM), and uridine triphosphate (UTP, 20 mM) were first dissolved in 100 mM Tris-HCl (pH 7.5) in the presence of 40 mM $MgCl_2$ and of MtGalK (10 μg mL^{-1}) and RmlA (1 mg mL^{-1}) at 55 °C for 2 h. The formation of UDP-Gal was monitored by RP-HPLC. However, due to the use of high reaction temperatures, longer incubation times resulted in the production of hydrolysis products of UDP-Gal, UMP, and 1,2-cyclic phosphate Gal. To circumvent this problem and avoid the purification of UDP-Gal from the mixture with ADP and UDP, two equivalents (to the sugar acceptor) of Gal, ATP, and UTP were used. When the yield of UDP-Gal reached 50% within 90 min, at which point the UDP-Gal amount was sufficient to supply the glycoside transfer reaction in the synthesis of LacNAc, the reaction temperature was lowered to 25 °C. Thus, the remaining starting materials, ATP, UTP, and Gal, continued to be utilized to produce UDP-Gal while the hydrolysis of UDP-Gal was slowed. NmGalT is a thermo-unstable enzyme but retains good catalytic activity below physiological temperatures, such as at 25 °C. The addition of 0.2 mM (final concentration) dithiothreitol (DTT) and 1 mg mL^{-1} bovine serum albumin (BSA) can stabilize NmGalT and maintain its activity.[17] Furthermore, to prevent the hydrolysis of UDP-Gal and the decomposition of NmGalT at 55 °C, the above reaction mixture was cooled to 25 °C, and NmGalT was then added. After incubation for 4 h, the desired LacNAc (**2**) was obtained in 90% yield (46 mg). Thus, in this sequential addition one-pot synthesis, only the purification of the final desired saccharide product is needed, which is more efficient and simpler in comparison with the purification of Gal-1-P and UDP-Gal.

The procedure for the assembly of GlcNAc-LacNAc trisaccharide **3** is similar to that described for the synthesis of **2**. The transfer of a GlcNAc moiety to LacNAc was catalyzed by HpGnT and required UDP-GlcNAc as a donor substrate (Scheme 1). We found that RmlA could also be used to prepare UDP-GlcNAc from the condensation reaction of UTP and GlcNAc-1-P, and the latter was known to be obtained by enzymatic reaction using NahK.[19] The optimal reaction condition for NahK is at 37 °C, and prolonged reaction times at elevated temperatures, such as at 55 °C, degrade the enzyme activity. Thus, the one-pot UDP-GlcNAc synthesis procedure was slightly modified, and the sequential addition of NahK and RmlA was employed (Scheme 1). GlcNAc (20 mM) and ATP (20 mM) were first dissolved in 100 mM Tris-HCl (pH 7.5) in the presence of 40 mM $MgCl_2$ and NahK (1 mg mL^{-1}) at 37 °C. The progress of GlcNAc-1-P formation was monitored by RP-HPLC, as indicated by the formation of ADP and the consumption of ATP. The yield of GlcNAc-1-P reached 90% within 4 h. Then, the temperature of the above reaction mixture was increased to 55 °C, followed by the addition of UTP (20 mM) and RmlA (1 mg mL^{-1}). When the yield of UDP-GlcNAc reached 50% in 90 min as monitored by the formation of UDP-GlcNAc using RP-HPLC, the above reaction mixture was cooled to room temperature (25 °C), followed by the addition of HpGnT (40 μg mL^{-1}). After incubation for 4 h, the desired trisaccharide **3** was obtained in 99% yield (70 mg).

After the successful and stepwise assembly of Gal and GlcNAc on azido-GlcNAc **1**, we extended our sequential addition one-pot procedure to synthesize oligo-LacNAc, as shown in Scheme 1. Defined lengths of oligo-LacNAc were synthesized by alternative addition of pre-synthesized UDP-Gal and NmGalT and pre-synthesized UDP-GlcNAc and HpGnT. The progress of each enzymatic elongation step was monitored by TLC, as indicated by the complete conversion of the corresponding acceptor substrates. As shown in Scheme 1, the glycosylation yields of Gal on the non-reducing end GlcNAc (**3** and **5**) acceptors by NmGalT were almost quantitative, yielding $(LacNAc)_2$ **4** and $(LacNAc)_3$ **6** in quantities of 84 mg (96%) and 62 mg (99%), respectively. Likewise, the assembly of GlcNAc by HpGnT to the non-reducing end Gal acceptor (**4** and **6**) also gave very good yields of the desired products. The yields of GlcNAc-$(LacNAc)_2$ **5** and GlcNAc-$(LacNAc)_3$ **7** were 89% (49 mg) and 93% (34 mg), respectively. Notably, the synthetic yield of

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$(LacNAc)_4$, **8** was significantly lower (43%, 15 mg). This low yield may be due to the poor substrate acceptance of NmGalT to heptasaccharide acceptor **7** and to the low water solubility of the octasaccharide **8** as the chain length increased. An attempt to use buffer-DMSO co-solvent for the reaction was unsuccessful due to the poor solubility of **8** in DMSO. Interestingly, the water solubility of GlcNAc terminated oligo-LacNAcs was observed to be better than that of the Gal terminated oligo-saccharides.

We also investigated the potential one-pot synthesis of oligo-LacNAc by using MtGalK, NahK, and RmlA to prepare sugar donors at 55 °C followed by cooling the reaction temperature to 25 °C and adding GlcNAc acceptor **1**, NmGalT, and HpGnT simultaneously. However, the synthetic efficiency of UDP-GlcNAc was slightly higher than that of UDP-Gal. To precisely control the formation of each UDP-sugar, UDP-Gal and UDP-GlcNAc were prepared separately in two vessels. When the formation of 90% of UDP-Gal and UDP-GlcNAc was achieved, as monitored by RP-HPLC, they were poured together into one vessel that contained GlcNAc **1**. After cooling the temperature of the vessel to 25 °C, NmGalT and HpGnT were added, and the resulting mixture was stirred for 24 h. After the removal of the rest of the UDP-sugars and of the buffer salt by BioRad P-2 gel, the resulting oligo-LacNAc products were analyzed by MALDI-MS. The results revealed that compounds **2**, **3**, **4** and **5** were obtained, and no major product dominated (see ESI, Figure S1). Furthermore, the amounts (based on the intensities of peaks in the MS spectrum) of the GlcNAc terminated oligo-saccharides (**3** and **5**) were higher than those of the Gal terminated oligo-saccharides (**2** and **4**), suggesting that HpGnT may be more efficient for the conversion of corresponding acceptor substrates than NmGalT in this reaction system.

The sialic acids on the LacNAc chains of glycoproteins are typically linked α-2,3 or α-2,6 to the terminal (non-reducing end) Gal. These sialylated oligo-LacNAc were well-documented to interact with glycan-binding proteins such as selectins, galectins, and siglecs (sialic acid-binding immunoglobulin-type lectins) as well as with viral hemagglutinins.[21] To prepare a small library of sialylated glycans, two bacterial STs, α-2,3-ST and α-2,6-ST, were used to assemble a sialic acid moiety onto oligo-LacNAc at the C-3 position of the terminal Gal and at the C-6 positions of the internal Gals, respectively. Thus, recombinant PmST (an α-2,3-ST) and Pd2,6ST (an α-2,6-ST) were overexpressed in *E. coli* using an intein expression system,[12] and the donor, CMP-sialic acid (CMP-Neu5Ac), was prepared by CMP sialic acid synthetase.[22] PmST was used to perform α-2,3-sialylation of the terminal Gal of **2**, **4**, and **6**, yielding sialylated products **9** (82%), **10** (80%) and **11** (73%), respectively (Table 1). Notably, all the acceptors provide similar reaction rate in the PmST catalyzed sialylation.

Pd2,6ST also displayed good catalytic efficiency toward oligo-LacNAc. It was recently reported that Pd2,6ST cannot only transfer sialic acid to the terminal Gal but can also perform α-2,6-sialylation on internal Gals.[11] In our approach, we controlled the amount of CMP-Neu5Ac to produce different degrees of sialyated oligo-LacNAc, as shown in Table 1. Pd2,6ST was first applied to convert **2** and **3** to sialylated product **12** (99%) and **13** (57%) in the presence of 1.5 equivalents of CMP-Neu5Ac. When the same equivalent of CMP-Neu5Ac was used in the Pd2,6ST catalyzed sialylation of $(LacNAc)_2$ **4**, two products, mono-sialyl $(LacNAc)_2$ **14** and di-sialyl $(LacNAc)_2$ **15**, were obtained with yields of 72% and 26%, respectively. Increasing the CMP-Neu5Ac amount to 2.5 eq gave a 99% yield of di-sialyl $(LacNAc)_2$ **15**. Although increasing the oligo-LacNAc length decreases its water solubility, the addition of sialic acid to oligo-LacNAc significantly enhanced its water solubility. When CMP-Neu5Ac and $(LacNAc)_3$ **6** (2 to 1 equivalents) were used, a 56% yield of mono-sialyl $(LacNAc)_3$ **16** and a 32% yield of di-sialyl $(LacNAc)_3$ **17** were obtained. Notably, no tri-sialylated product was obtained. The position of sialic acid on sialyl-$(LacNAc)_3$ **16** was determined on the terminal Gal by MS/MS fragmentation analysis, while **17** had two sialic acids, including one attached to the terminal Gal and the other attached to an internal Gal (see ESI, Figure S2). However, when 5.0 eq of CMP-Neu5Ac was used, an 85% yield of tri-sialyl **18** and a 12% yield of di-sialyl **17** were obtained, while no mono-sialylated product was obtained. The results revealed that the internal Gal can be successfully α-2,6-sialylated and that the number of sialic acids attached to the oligo-LacNAc chains can be controlled by using varying concentrations of CMP-Neu5Ac.

Table 1. Sialylation of oligo-LacNAc by sialyltransferases

	entry	acceptor	donor (eq)	product	yield (%)
PmST	1[a]	**2**	1.5	**9**	82
	2[a]	**4**	1.5	**10**	80
	3[a]	**6**	1.5	**11**	73
Pd2,6ST	4	**2**	1.5	**12**	99
	5	**3**	1.5	**13**	57
	6	**4**	1.5	**14**	72
				15	26
	7	**4**	2.5	**15**	99
	8	**6**	2	**16**	32
				17	56
	9	**6**	5	**17**	12
				18	85

[a] reaction for 4 h.

In this study, we developed an economical and straightforward strategy to prepare UDP-Gal and UDP-GlcNAc at a preparative scale by coupling the corresponding kinases with RmlA. In combination with Leloir GTs, NmGalT and HpGnT, oligo-LacNAcs were synthesized. By using our newly developed enzymatic system, defined lengths of oligo-LacNAcs were synthesized in a sequential addition, one-pot fashion. A panel of α-2,3- and α-2,6-sialyated oligo-LacNAcs were further synthesized by PmST and Pd2,6ST. In addition, different degrees of α-2,6-sialylation on oligo-LacNAcs were achieved by controlling the amount of CMP-Neu5Ac in the enzymatic reaction. Thus, our approach efficiently provides diverse structures of sialylated LacNAcs for studying LacNAc associated biology.

This work was financially supported by National Tsing Hua University, Academia Sinica, and Ministry of Science and Technology, Taiwan.

Notes and references

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Electronic Supplementary Information (ESI) available: Cloning and overexpression of enzymes and NMR spectral characterization of the synthesized oligo-LacNAcs. See DOI: 10.1039/c000000x/

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