Biocatalytic Preparation of *N*-Glycolylneuraminic Acid, Deaminoneuraminic Acid (KDN) and 9-Azido-9-deoxysialic Acid Oligosaccharides

Ola Blixt, James C. Paulson*

The Scripps Research Institute, Department of Molecular Biology, MEM-L71,10550 North Torrey Pines Road, La Jolla, CA 92037, USA
Fax: (+1)-858-784-9690, e-mail: jpaulson@scripps.edu, olablixt@scripps.edu

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Abstract: A facile approach for preparative synthesis of sialyloligosaccharides containing *N*-glycolylneuraminic acid, deaminoneuraminic acid, and two 9-azido derivatives is described. Synthesis is accomplished by use of 6-carbon mannose/mannosamine precursors employing an aldolase, CMP-sialic acid synthetase, and sialyltransferase. Using a combination of five different sialyltransferases and various acceptor substrates, 16 sialosides representing sequences found in glycoproteins and glycolipids were synthesized with typical yields of 60–80%.

Keywords: carbohydrates; enzyme catalysis; neuraminic acid; oligosaccharides; sialic acid; sialyltransferases

Sialic acid-containing carbohydrates comprise a rich diversity of structures on the surface of mammalian cells that mediate host-pathogen interactions, cell to cell adhesion and cell signaling events. Sialic acid itself is a generic designation used for 2-keto-3-deoxynonulosonic acids, with greater than 20 analogues found in nature. The most common derivatives found in animal glycans are derived from *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), while the non-aminated 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (KDN) is frequently found in the lipooligosaccharide (LOS) of various Gram-negative bacteria.[1]

Investigations into the biological roles of sialic acid-containing carbohydrates has stimulated development of chemical and chemo-enzymatic approaches to their synthesis. For preparative synthesis, enzymatic sialylation has been demonstrated to be an efficient route for generating Neu5Ac-containing oligosaccharides. However, the synthesis of oligosaccharides containing other sialic acids has been limited by the availability of the respective monosaccharide and its corresponding nucleotide sugar.[2]

Here we report a facile method for one-pot, two-step preparative enzymatic synthesis of sialyloligosaccharides containing Neu5Gc, KDN and 9-azido-9-deoxy derivatives of Neu5Ac and KDN. The key to this approach is the preparation of the sialic acids themselves and their conversion into the corresponding CMP-sialic acid donor substrates. This is followed by addition of suitable acceptor substrates and sialyltransferase to elaborate the desired sialyloligosaccharide. Preparation of sialyloligosaccharides with the 9-azido-9-deoxy-sialic acids provides a potential route to the synthesis of other 9-substituted derivatives.

General synthetic approach: The general approach used by Wong for enzymatic synthesis of sialosides with *in situ* generation of CMP-NeuAc was adapted for the synthesis of sialosides with other sialic acid derivatives (Figure 1).[3] The desired CMP-sialic acid was first prepared using a reaction half-cycle that involved a one-pot, two-step condensation of mannose or mannose derivative with pyruvic acid using a sialic acid aldolase, and conversion to the corresponding CMP-sialic acid by reaction with cytidine 5′-triphosphate (CTP) using a CMP-sialic acid synthetase. A commercial Neu5Ac-aldolase (NAL-311, Toyobo), was selected since it was previously shown to convert mannose to KDN, and Man2Gc to Neu5Gc with high efficiency.[4] More critical was the choice of the synthetase. Commercially available CMP-sialic acid synthetases derived from *E. coli* will not use Neu5Gc as a substrate (unpublished observations). While mammalian CMP-sialic acid synthetases exhibit acceptable specificity[5] a recombinant source of the enzyme needed for preparative synthesis was not available. The *N. meningitidis* CMP-sialic acid synthetase was used because of its broad specificity and its facile preparation as a recombinant fusion protein.[6,7] A commercial source of the enzyme is also available (Calbiochem). Recently, this synthetase was successfully optimized with other *E. coli* expression con-

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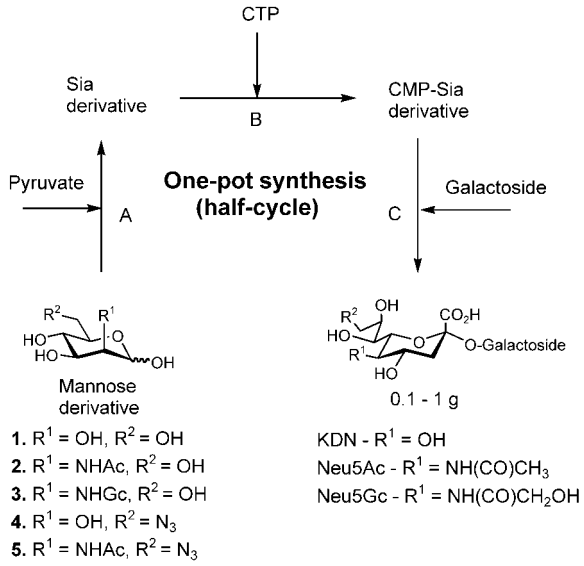

Figure 1. One-pot preparative synthesis of sialyl-oligosaccharides using mannose derivatives (**1** – **5**). A: Neu5Ac aldolase; B: ST3-CMP-Neu5Ac Synthase fusion protein; C: Sialyltransferase.

structs.[7] In our reactions the formation of sialic acid and CMP-sialic acid could easily be monitored by thin layer chromatography.[8] Because the synthetase fusion protein also contains the *N. meningitidis* α(2–3) sialyltransferase, proteins were removed from the crude sugar nucleotide mixture before the sialyltransferase and acceptor saccharide were introduced to complete the synthesis of the desired sialoside.

Five recombinant sialyltransferases were evaluated for their ability to transfer the various sialic acids to their acceptor substrates including *N. meningitidis* ST3Gal,[6,9] porcine ST3Gal I,[10] rat ST3Gal III,[11] humanST6Gal I,[12] chicken ST6GalNAc I.[13] Acceptor substrates comprised common core structures found in glycoprotein and glycolipid carbohydrates groups including Galβ(1–4)Glc-spacer (lactose), Galβ(1–4)GlcNAc-spacer (LacNAc; Type 2), Galβ(1–3)GlcNAc-spacer (Type 1), Galβ(1–3)GalNAcαThr-spacer (T-antigen) and GalNAcαThr-spacer (Tn-antigen). As summarized in Figure 2, a total of 16 sialosides were synthesized at a 50–500 mg scale, and yields were typically 60–80% (See Supporting Information).

Synthesis of KDN-oligosaccharides: Several sialyltransferases have previously been demonstrated to transfer KDN from CMP-KDN to oligosaccharide acceptors, including rST6Gal-I,[14] pST3Gal-I[15] and rST3Gal-III[16]. We have evaluated the five different sialyltransferases mentioned above in the half-cycle for their ability to generate preparative amounts of KDN-oligosaccharides (Figure 2). Recombinant rST3Gal-III readily transferred KDN to lacto-*N*-biose **6**[17] and LacNAc derivatives **7**[12] to give **13** (65%) and **15** (75%), respectively. ST3Gal (*N. m.*) was also tested but no transfer of KDN could be detected, as observed previously for the ST3Gal from *N. gonorrheae*[18]. Recombinant hST6Gal-I[12] was successfully used to sialylate LacNAc derivative **9**[19] for synthesis of the novel oligosaccharide **20** (85%) not yet described in nature. The enzymes pST3Gal-I and chST6GalNAc-I, efficiently converted the T- (**11**) and Tn-antigens (**12**)[20] to the corresponding KDN products **25** (96%) and **28** (17%), respectively. The lower yield of **28** is due to slower and incomplete catalytic transfer by the chST6GalNAc-I enzyme.

Synthesis of Neu5Gc-oligosaccharides: While the syntheses of several *N*-glycolyl-sialosides have been reported,[6,12,21] no commercial sources of such structures are currently available. As summarized in Figure 2, several α2–3-*N*-glycolylsialosides were prepared using rST3Gal-III, ST3Gal(*N. m.*) and pST3Gal-I starting from the acceptor substrates **6**, **7**, **10**, and **11**, to generate Neu5Gcα(2–3)Galβ(1–3)GlcNAcβOsp (**14**, 88%), Neu5Gcα(2–3)Galβ(1–4)Glc-ßOsp (**18**, 53%), Neu5Gcα(2–3)Galβ(1–4)GlcNAc-βOsp (**19**, 44%) and Neu5Gcα(2–3)Galβ(1–3)GalNAcα(1–1)Thr-(NHAc)OMe (**26**, not isolated). α2,6-Sialosides Neu5Gcα(2–6)Galβ(1–4)GlcNAcβOR (**21**, 75%), Neu5Gcα(2–6)Galβ(1–4)GlcNAcβOR (**23**, 75%) and NeuGcα(2–6)GalNAcα(1–1)OThr(NHAc)OMe (**21**, 87%) were also synthesized using hST6Gal-I and chST6GalNAc-I enzymes.[13] Compound **23** was synthesized from *N*-acetoxy-acetylmannosamine *via* the acetoxy derivative (**22**) which after Zemplén deacetylation gave **23** (95%).

Synthesis of 9-azido-9-deoxy-sialyloligosaccharides: Sialyltransferases are often permissive to substitutions at the 9-O-position allowing the synthesis of unnatural, but useful, functionalized sialic acids.[22] The synthesis of 9′-azido-9′-deoxysialic acid analogues from 6-azido-6-deoxymannose derivatives **4** and **5**[23] was accomplished using the half-cycle and the recombinant sialyltransferases rST3Gal-III, hST6Gal-I and pST3Gal-I. rST3Gal-III transferred 9-azido-9-deoxy-Neu5Ac and 9-azido-9-deoxy-KDN to acceptor **8** forming compounds **16** (98%) and **17** (85%). Of the α(2–6)sialyltransferases tested, hST6Gal-I utilized the CMP-9-azido-9-deoxy-Neu5Ac donor substrate to generate **24** (85%). chST6GalNAc-I, however, was unable to utilize 9-azido-9-deoxy-modified sialic acid donor substrates.

Experimental Section

General Procedure for One-Pot, Two-Step Sialoside Synthesis

The appropriate mannose derivative (1–5, 1 equiv.), sodium pyruvate (3 equiv.) and CTP (1.5 equiv.) were dissolved in Tris-HCl (100 mM, 40 mL/mmol CTP), pH 9.5 containing $MgCl_2$ (20 mM) and pH adjusted to 8.6 with NaOH (1 M). Neu5Ac-

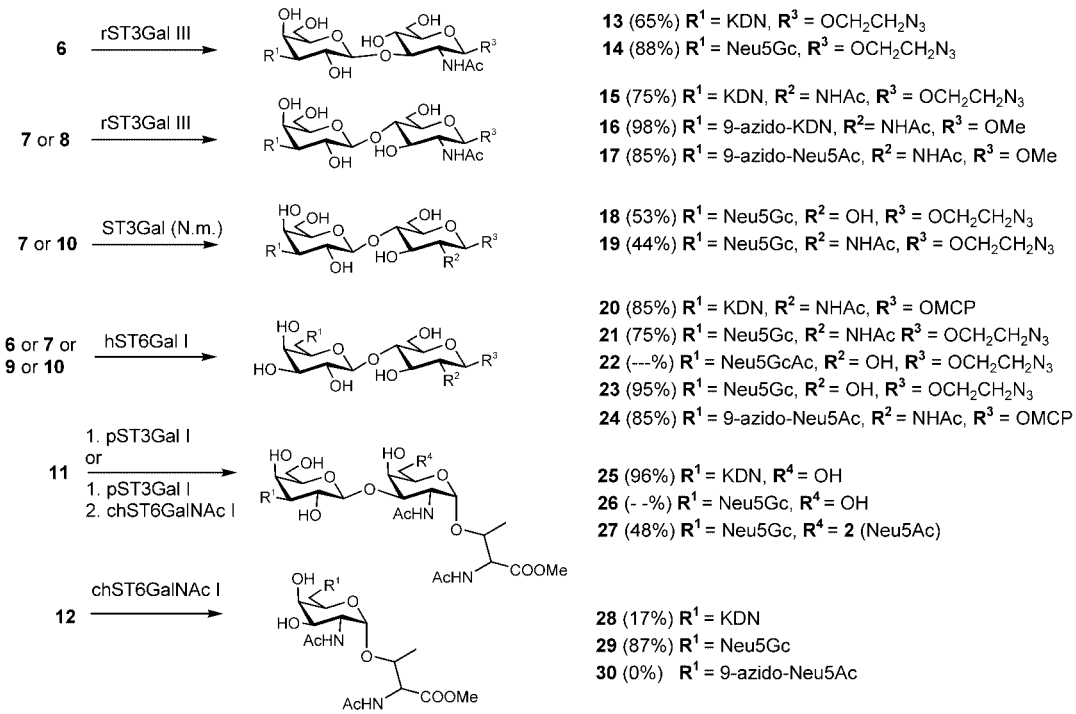

Figure 2. Synthesized structures from mannose derivatives and acceptor substrates using the one-pot, two-step half cycle (see Figure 1). MCP = 5-(methylcarboxy)pentyl, (– – –) = not isolated.

aldolase (Nal-311 (Toyobo), 500 U/mmol mannose derivative) and *N. meningitidis* ST3Gal-CMPNeu5Ac synthetase fusion protein (20 U/mmol CTP) were added and the reaction mixture was kept at 37 °C. The pH was constantly monitored and adjusted with 1 M NaOH to pH 8.3 – 9.0 as needed. After 14 h the reaction was terminated by passing the mixture through a Prep/Scale-TFF Cartridge cellulose membrane filter MWCO 10 kDa (Millipore, Bedford, MA.). To the filtrate, oligosaccharide acceptor (0.5 equiv.), $MnCl_2$ (20 mM) and sialyltransferase (5 U/mmol acceptor) were added and the pH was adjusted to 7.0 with HCl (1 M). After 14 h the product was purified by size exclusion chromatography (Sephadex G15) and compounds were isolated in various yields ranging from 17 – 98% (typically 60 – 80%) with a purity > 90% as judged by TLC and NMR (See Supporting Information).

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