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Aldolase-Catalyzed Synthesis of β-D-Gal*p*-(1→9)-D-KDN: A Novel Acceptor for Sialyltransferases

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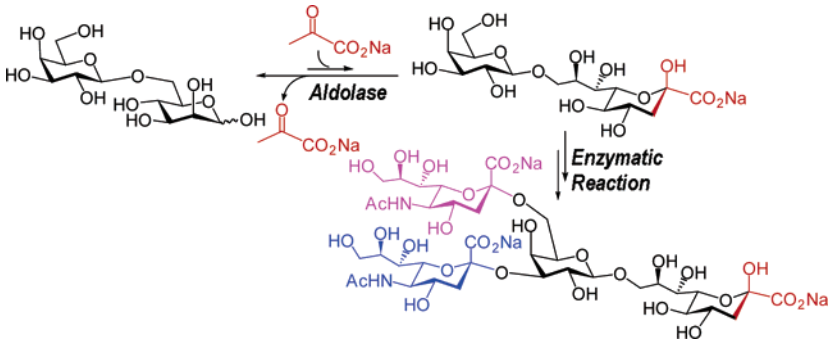
ABSTRACT



β-D-Gal*p*-(1→9)-D-KDN, a disaccharide component of the cell wall of *Streptomyces* sp. MB-8, was synthesized from β-D-Gal*p*-(1→6)-D-Man*p* and pyruvate using a sialic acid aldolase. The obtained KDN-containing compound was a novel acceptor for bacterial sialyltransferases. Unusual α2,3- and α2,6-linked sialyltrisaccharides and a tetrasaccharide were synthesized using a one-pot two-enzyme system containing a *Neisseria meningitidis* CMP-sialic acid synthetase and a *Pasteurella multocida* sialyltransferase or a *Photobacterium damsela* α2,6-sialyltransferase.

Sialic acids are negatively charged nine-carbon monosaccharides that play pivotal roles in many physiologically and pathologically important processes, including cellular recognition and communication, bacterial and viral infection, and tumor metastasis, etc.[1,2] Although having been predominantly found as terminal carbohydrate units on glycoproteins and glycolipids of vertebrates or as components of capsular polysaccharides and lipooligosaccharides of pathogenic bacteria in the forms of polysialic acids or side-chain end units,[1] sialic acids have also been found as internal residues that link to other carbohydrate units in polysaccharide or glycoconjugate forms.

Carbohydrate structures containing nonterminal sialic acid residues have been found mainly in the surface molecules (e.g., cell wall components, capsular polysaccharides or lipopolysaccharides) of some pathogenic bacteria.[3] These

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ionic molecules are believed to be essential for the efficient attachment of the bacteria to host cells[3h] and play important roles in the virulence of the pathogenic organisms.[4] For example, KDN (deoxyneuraminic acid or 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid), one of three basic sialic acid forms, was first found in rainbow trout eggs.[5] It has recently been detected in the cell wall of *Streptomyces* sp. VKM Ac-2090[3i] and strain *Streptomyces* sp. VKM Ac-2124[3h] as β2,4-linked KDN polymers branched with Glcβ1,8-linked side chains. Using the combination of ^{1}H and ^{13}C NMR as well as MALDI-TOF mass spectrometry analyses, KDN-containing oligomers have also been found as cell wall components of *Streptomyces* sp. MB-8. They are tetrasaccharide components with two β-D-Gal*p*-(1→9)-D-KDN disaccharides linked through an α-D-KDN−(2→4)-β-D-KDN linkage, with some of the galactose residues being 3-O-methylated (Figure 1).[3g]

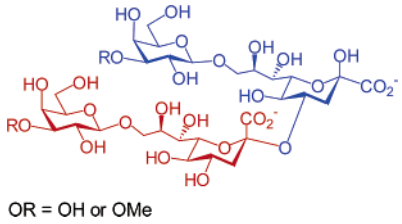
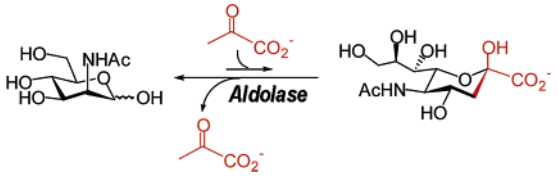


Figure 1. Tetrasaccharide components found in the cell wall of *Streptomyces* sp. MB-8.[3g]

Sialic acid aldolase (EC 4.1.3.3.) catalyzes a reversible condensation of pyruvate with D-*N*-acetylmannosamine (ManNAc) to form D-*N*-acetylneuraminic acid (Neu5Ac) with the equilibrium favoring the aldol cleavage (Scheme 1).[6]

Scheme 1. Reaction Catalyzed by Sialic Acid Aldolases

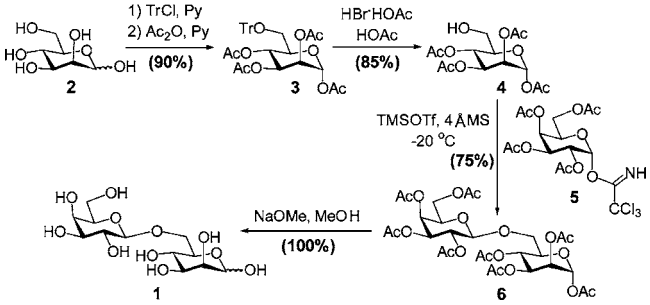


Sialic acid aldolase has flexible substrate specificity and has been widely used in the enzymatic synthesis of naturally occurring and structurally modified sialic acids.[1a,7] We have cloned a full-length sialic acid aldolase (NanA) from *Escherichia coli* K-12 and overexpressed it as a C-terminal His$_6$-tagged fusion protein.[8] When expressed at 37 °C for 3 h with the induction of 0.1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside), the majority of the recombinant aldolase presented in cell lysate as a soluble form, and it can be easily purified by an affinity column packed with Ni^{2+}-NTA-agarose (nickel-nitrilotriacetic acid-agarose) resin. In agreement with previous reports,[6a] the recombinant aldolase can tolerate a wide range of modifications (even bulky groups) at various positions on the ManNAc substrate or mannose and it has been used successfully in one-pot multiple-enzyme systems for the efficient synthesis of CMP-sialic acid derivatives[8] and sialosides.[9]

On the basis of the extremely flexible substrate specificity of the sialic acid aldolase, we hypothesize that the disaccharide structure β-D-Gal*p*-(1→9)-D-KDN observed in the cell wall of *Streptomyces* sp. MB-8[3g] (Figure 1) can be synthesized by an aldolase-catalyzed reaction from pyruvate and a simpler disaccharide β-D-Gal*p*-(1→6)-D-Man*p*, in which the Gal residue can be considered as a substituent replacing the H atom in the 6-*O*-hydroxyl group of the mannose.

To test our hypothesis, disaccharide β-D-Gal*p*-(1→6)-D-Man*p* **1** was synthesized using a conventional synthetic approach (Scheme 2). Direct tritylation followed by acetylation of mannose **2**[10] afforded acetyl 2,3,4-tri-*O*-acetyl-6-*O*-trityl-α-D-mannopyranoside **3** in 90% yield. Although it

Scheme 2. Synthesis of Disaccharide β-D-Gal*p*-(1→6)-D-Man*p* **1**



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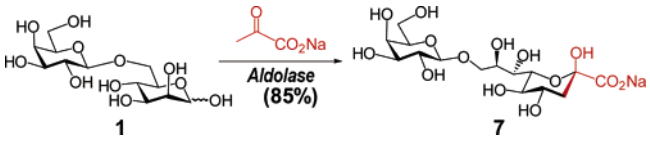
was reported that the trityl ether can be easily removed by acid-catalyzed hydrolysis, removal of the trityl at C6 of **3** under standard conditions (HCl/MeOH, HOAc/H_2O, or HF/CH_2Cl_2) was problematic and produced a mixture containing a byproduct formed by 4→6 migration of the *O*-acetyl at C-4 to newly deprotected C-6. Instead, using hydrogen bromide[10,11] in acetic acid was proven to be a satisfactory alternative approach for selective deprotection of the C-6 hydroxyl group. The desired compound **4** was obtained in 85% yield under this condition. Promoted by trimethylsilyl trifluoromethane sulfonate (TMSOTf) in dichloromethane, Schmidt glycosylation of **4** with donor 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl trichloroacetimidate **5**[12] afforded the peracetylated disaccharide **6** in 75% yield. Deacetylation of **6** by Zémplen reaction in sodium methoxide and methanol produced the desired disaccharide **1** in quantitative yield.

To our delight, the obtained disaccharide *β*-D-Gal*p*-(1→6)-D-Man*p* **1** was a good substrate for the recombinant sialic acid aldolase. In fact, disaccharide *β*-D-Gal*p*-(1→9)-D-KDN **7** was obtained in an excellent (85%) yield from **1** and 5 equiv of pyruvate in Tris−HCl buffer (100 mM, pH 7.5) by incubating with the aldolase at 37 °C for 24 h followed by a Bio-gel P-2 gel filtration column purification step (Scheme 3). The structure of the product was confirmed by NMR and

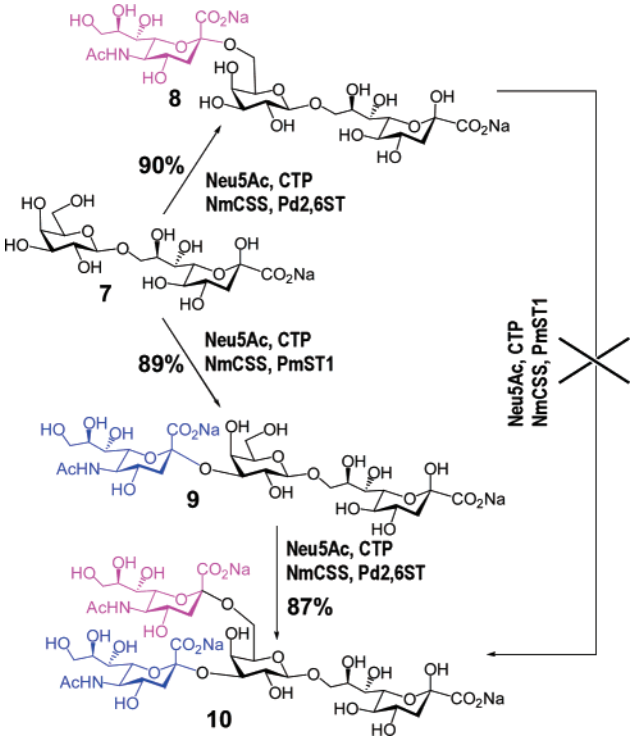
Scheme 3. Aldolase-Catalyzed Synthesis of Disaccharide *β*-D-Gal*p*-(1→9)-D-KDN



high-resolution mass spectrometry. To our knowledge, the synthesis of this type of oligosaccharides which contain a sialic acid residue at the reducing end has only been achieved enzymatically by using the trans-glycosylation activity of a *Bacillus circulans* *β*-galactosidase with lactose as the galactosyl donor.[13] An earlier attempt to convert *β*-D-Glc*p*-(1→6)-D-Man*p*NAc to *β*-D-Glc*p*-(1→6)-D-Neu5Ac using aldolase[14] was not successful. The enzymatic synthesis we report here, thus, is the very first example to show that the reducing-terminal mannose in a disaccharide can be converted to KDN by a sialic acid aldolse.

Examining the structure of **7** identified a terminal galactose at the nonreducing end of the disaccharide. The galactose residue in **7**, thus, would be a potential acceptor candidate for sialyltransferases. A one-pot multiple-enzyme system established in our lab[9] was used to test this hypothesis. As a potential acceptor for sialyltransferases, disaccharide **7** was incubated with Neu5Ac and CTP, as well as two enzymes including the *N. meningitidis* CMP-sialic acid synthetase (NmCSS) and a sialyltransferase (an α2,3- or α2,6-sialyltransferase) in a Tris−HCl buffer (pH = 8.5) containing 20 mM Mg^{2+}. In this system, Neu5Ac was activated by NmCSS to form CMP−Neu5Ac, an activated sugar nucleotide donor for sialyltransferases, from which the Neu5Ac moiety can be transferred to the galactose residue in **7** (a Gal-terminated acceptor structure) to form an α2,3- or α2,6-linked sialoside depending on the sialyltransferase used. We found that disaccharide **7** indeed was a good acceptor for both the *Photobacterium damsela* α2,6-sialyltransferase (Pd2,6ST) and the *Pasteurella multocida* multifunctional sialyltransferase (PmST1).[9,15] Using the one-pot two-enzyme approach,[16] we obtained novel trisaccharides α-D-Neu5Ac−(2→6)-*β*-D-Gal*p*-(1→6)-D-KDN **8** and α-D-Neu5Ac−(2→3)-*β*-D-Gal*p*-(1→6)-D-KDN **9** in 90% and 89% yields, respectively (Scheme 4).

Scheme 4. One-Pot Two-Enzyme Synthesis of Novel α2,3- and α2,6-Linked Sialyltrisaccharides and an Unusual Tetrasaccharide Containing Three Sialic Acid Residues[16]



These results further confirmed the flexible acceptor specificity of both Pd2,6ST and PmST1[9] as reported earlier. Using the similar one-pot two-enzyme approach, we can obtain unusual tetrasaccharide **10** containing three sialic acid residues from **9** in 87% yield using the NmCSS and the Pd2,6ST.[17] An attempt to synthesize **10** from **8** using the one-pot two-enzyme system containing PmST1, however,

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was not successful. This indicates that a Neu5Ac residue linked to the C-3 of the galactose residue in **7** does not block the C-6 site for sialylation by Pd2,6ST. The Neu5Ac residue on C-6 of the galactose residue in **7**, however, blocks the C-3 site for PmST1-catalyzed sialylation.

(16) General experimental procedures for the preparation of sialosides using a one-pot two-enzyme system: Reactions were typically carried out in a 50 mL centrifuge tube in 10 mL of Tris−HCl buffer (100 mM, pH 8.5) containing an acceptor substrate (disaccharide **7** or trisaccharide **9**) (50−100 mg), Neu5Ac (1.5 equiv), CTP (1.5 equiv), $MgCl_2$ (20 mM), NmCSS (0.8 mg), PmST1 (0.04 mg for preparing α2,3-linked sialosides), or Pd2,6ST (0.2 mg for preparing α2,6-linked sialosides). The reaction mixture was incubated at 37 °C for 12 h with shaking (120 rpm). After the addition of 10 mL of ice-cold methanol to stop the reaction, precipitates were removed by centrifugation. The supernatant was concentrated by rotor evaporation and purified by Bio-gel P-2 gel filtration chromatography. Lyophilized sialoside products were characterized by NMR and high-resolution mass spectrometry (HRMS). Notice that under the reaction conditions used (pH 8.5), the α2,6-sialyltransferase activity of the PmST1 was not observed. The PmST1, thus, was used as an α2,3-sialyltransferase here.

(17) The sialosides **8**−**10** could also be prepared from disaccharide **7** or trisaccharide **9** in a one-pot three-enzyme system containing the *E. coli* sialic acid aldolase, NmCSS, and a sialyltransferase (PmST1 or Pd2,6ST). In this system, Neu5Ac was generated in situ from ManNAc and pyruvate by the aldolase, activated to form CMP−Neu5Ac by NmCSS, and transferred to acceptors by a sialyltransferase. As the aldolase-catalyzed reactions are reversible, a large excess of pyruvate (10 equiv) has to be used in the one-pot three-enzyme system to prevent the KDN residue at the reducing end in **7** and **9** from converting back to mannose.

In conclusion, we report herein the very first example of an aldolase-catalyzed synthesis of an unusual disaccharide *β*-D-Gal*p*-(1→9)-D-KDN which is a component of the cell wall of *Streptomyces* sp. MB-8. Using the disaccharide obtained from the aldolase reaction as a novel sialyltransferase acceptor, we have also synthesized two novel sialyltrisaccharides and an unusual sialyltetrasaccharide. We have demonstrated here that the recombinant *E. coli* sialic acid aldolase has extremely flexible substrate flexibility. Together with the one-pot multiple-enzyme system established in our lab, it is a powerful catalyst in the efficient synthesis of sialic acid-containing structures.

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Supporting Information Available: Experimental procedures and NMR spectra for compounds **1** and **4**−**10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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