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Characterizing non-hydrolyzing *Neisseria meningitidis* serogroup A UDP-N-acetylglucosamine (UDP-GlcNAc) 2-epimerase using UDP-N-acetylmannosamine (UDP-ManNAc) and derivatives

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Abstract

Neisseria meningitidis serogroup A non-hydrolyzing uridine 5′-diphosphate-N-acetylglucosamine (UDP-GlcNAc) 2-epimerase (NmSacA) catalyzes the interconversion between UDP-GlcNAc and uridine 5′-diphosphate-N-acetylmannosamine (UDP-ManNAc). It is a key enzyme involved in the biosynthesis of the capsular polysaccharide [-6ManNAcα1-phosphate-]$_n$ of *N. meningitidis* serogroup A, one of the six serogroups (A, B, C, W-135, X, and Y) that account for most cases of *N. meningitidis-caused* bacterial septicemia and meningitis. *N. meningitidis* serogroup A is responsible for large epidemics in the developing world, especially in Africa. Here we report that UDP-ManNAc could be used as a substrate for C-terminal His$_6$-tagged recombinant NmSacA (NmSacA-His$_6$) in the absence of UDP-GlcNAc. NmSacA-His$_6$ was activated by UDP-GlcNAc and inhibited by 2-acetamidoglucal and UDP. Substrate specificity study showed that NmSacA-His$_6$ could tolerate several chemoenzymatically synthesized UDP-ManNAc derivatives as substrates although its activity was much lower than non-modified UDP-ManNAc. Homology modeling and molecular docking revealed likely structural determinants of NmSacA substrate specificity. This is the first detailed study of *N. meningitidis* serogroup A UDP-GlcNAc 2-epimerase.

Keywords

Epimerization; *Neisseria meningitidis*; UDP-GlcNAc; UDP-ManNAc; UDP-GlcNAc 2-epimerase

1. Introduction

Neisseria meningitidis is a Gram-negative bacterium and a causative agent of bacterial septicemia and meningitis.[1] Among the thirteen known serogroups which are classified

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based on their distinct structures of capsular polysaccharides (CPSs), six (A, B, C, W-135, X, and Y) account for most cases of diseases.[2] Of these six major disease-causing meningococcal serogroups, only serogroups A and X produce capsular polysaccharides (CPSs) that do not have N-acetylneuraminic acid (Neu5Ac, sialic acid is a more general term) residues.[3] Unlike the CPSs of serogroups B and C which are homopolymers of Neu5Ac with α2–8- and α2–9-linkages respectively,[4] or serogroups W-135 and Y CPSs which are heteropolymers of [-6Gal/Glcα1–4Neu5Acα2-]$_n$ with alternating Neu5Ac and Gal/Glc as disaccharide repeating units,[5] the CPS of serogroup A is a homopolymer of [–6ManNAcα1-phosphate-]$_n$.[6–9] Correspondingly, the genetic organization for serogroup A capsule is different from those for serogroups B, C, W-135, and Y.

Like other *N. meningitidis* serotypes, the serogroup A *cps* operon (of a size of 4701 bp) is located between the outer membrane capsule transporter gene (*ctrA*) and the *galE* gene encoding a UDP-glucose-4-epimerase.[10] The *cps* operon has four open reading frames (ORFs 1–4 designated as *sacA–sacD*). The first ORF was predicted to encode a 372-amino acid protein (SacA) that has homology to *Escherichia coli* UDP-N-acetyl-D-glucosamine (UDP-GlcNAc) 2-epimerase WecB (also referred to as RffE) which is responsible for the conversion of UDP-GlcNAc to its C2-epimer UDP-N-acetyl-D-mannosamine (UDP-ManNAc). The second ORF encodes SacB of 545 aa whose C-terminal sequence shares 35% sequence identity and 53% sequence similarity with a hypothetic UDP-glucose-4-epimerase CpsY from *Mycobacterium leprae* and *Mycobacterium tuberculosis* but was proposed to be a polymerase for linking the repeating unit ManNAcα1-phosphate via α1–6-linkage.[10] The third ORF encodes SacC, an O-acetyltransferase of 247 aa that catalyzes the transfer of acetyl group to the ManNAc residues in CPS.[11] The last ORF encodes SacD of 287 aa which shares about 25% sequence identity and 43% sequence similarity with bacterial glycosyltransferases such as AcbVII in *Aeromonas hydrophila*,[12] hypothetic GT2 (GTA type) glycosyltransferase from *Shewanella violacea* DSS12,[13] and a putative glycosyltransferase from *Halomonas elongata*.[14]

N. meningitidis serogroup A is responsible for large epidemics in the developing world, especially in Africa, where incidence rate approaches 1% of the population during epidemics.[15,16] Of these four ORFs involved in *N. meningitidis* serogroup A CPS synthesis, only one gene product, SacC which was demonstrated to catalyze the acetylation of the ManNAc moieties, has been characterized biochemically. SacA was proposed to be involved in the first step in serogroup A CPS biosynthetic pathway and is critical for serogroup A CPS biosynthesis.[10] It shows high degree of homology to several functionally characterized UDP-GlcNAc 2-epimerases [EC:5.1.3.14] which catalyze the interconversion between UDP-GlcNAc and UDP-ManNAc.[17] This epimerization process is independent of nicotinamide adenine dinucleotide oxidized form (NAD^+) and is critical for synthesizing bacterial ManNAc-containing CPSs (in both Gram-positive and Gram-negative bacteria[10] such as *N. meningitidis*[6] and *Streptococcus pneumoniae* types 19F and 19A),[18] cell wall polysaccharides of *Bacillus* species[19–21] and *Paenibacillus polymyxa* (or *Bacillus polymyxa)*,[22] teichoic acid linkage units of Gram-positive bacteria including *Listeria monocytogenes*,[23] *Staphylococcus aureus* H and *Bacillus subtilis*,[20,24–27] and lipopolysaccharides.[28] It is also involved in the biosynthesis of UDP-N-

acetylmannosaminuronic acid (UDP-ManNAcA) for producing ManNAcA-containing CPSs[29,30] and enterobacterial common surface antigen (ECA) in Enterobacteriaceae (Gram-negative) such as *E. coli*.[31–33]

Here we present the cloning, expression, and characterization of NmSacA, an *N. meningitidis* serogroup A non-hydrolyzing UDP-GlcNAc 2-epimerase. The catalytic function of NmSacA is demonstrated using chemoenzymatically synthesized substrates. UDP-ManNAc and several of its derivatives can be used as substrates for C-terminal His_6-tagged recombinant NmSacA (NmSacA-His_6) in the absence of UDP-GlcNAc. NmSacA-His_6 can be activated by UDP-GlcNAc and inhibited by 2-acetamidoglucal and UDP. This is the first detailed study of *N. meningitidis* serogroup A UDP-GlcNAc 2-epimerase.

2. Results and discussion

2.1. Sequence alignment of NmSacA and other bacterial non-hydrolyzing UDP-GlcNAc 2-epimerases

Sequence alignment of NmSacA and other functionally characterized UDP-GlcNAc 2-epimerases (Fig. 1), including *E. coli* RffE, *Streptococcus pneumonia* Cps19fK, *Staphylococcus aureus* Cap5P, and *Salmonella enterica* RfbC, showed that NmSacA shares 58%, 51%, 47%, and 52% identity to RffE, Cps19fK, Cap5P, and RfbC, respectively. These characterized UDP-GlcNAc 2-epimerases play important functional or structural roles in the bacteria. RffE is responsible for the formation of UDP-ManNAc (from UDP-GlcNAc) which is used as an intermediate for the biosynthesis of enterobacterial common antigen (ECA).[34] Cps19fK and RfbC are involved in the biosynthesis of ManNAc-containing *Streptococcus pneumonia* type 19F CPS[35] and *Salmonella enterica* serogroup O:54 polysaccharide,[28] respectively. Cap5P is responsible for the formation of UDP-ManNAc (from UDP-GlcNAc) which is oxidized to UDP-N-acetylmannosaminuronic acid (UDP-ManNAcA) for the synthesis of ManNAcA-containing *Staphylococcus aureus* serotype 5 CPS.[36]

The bacterial non-hydrolyzing UDP-GlcNAc 2-epimerases, including SacA from *N. meningitidis* serogroup A (NmSacA), share some sequence homology to the epimerase domain (the N-terminal domain of 378 amino acids) of mammalian bifunctional hydrolyzing UDP-GlcNAc 2-epimerases/ManNAc kinases and bacterial hydrolyzing UDP-GlcNAc 2-epimerases. For example, NmSacA shares 20% identity with *N. meningitidis* serogroup B SiaA (AAA20475), 25% identity with *E. coli* K1 NeuC (YP_854392) in the N-terminal sequence of 166 amino acids, and 22% identity with the rat (NP_446217) or human (NP_005467) bifunctional UDP-GlcNAc 2-epimease/ManNAc kinase in the N-terminal sequence of 200 amino acids. Apparently, the sequence homologies between NmSacA and these hydrolyzing UDP-GlcNAc 2-epimerases are lower than those of NmSacA and other bacterial non-hydrolyzing UDP-GlcNAc 2-epimerases as shown in Fig. 1.

Nonetheless, bacterial non-hydrolyzing UDP-GlcNAc 2-epimerases as well as mammalian and bacterial hydrolyzing UDP-GlcNAc 2-epimerases all catalyze the epimerization of the C ″-2 of UDP-GlcNAc (the carbon 2 at the GlcNAc structure). However, their biological functions and the products formed are different. Both the epimerase domain of the

mammalian hydrolyzing UDP-GlcNAc 2-epimerases/ManNAc kinases[37,38] and bacterial hydrolyzing UDP-GlcNAc 2-epimerases[39] produce α-ManNAc and UDP (Scheme 1A) and are involved in the biosynthesis of sialic acids. In comparison, bacterial non-hydrolyzing UDP-GlcNAc 2-epimerases catalyze the interconversion of UDP-GlcNAc and UDP-ManNAc (Scheme 1B) and are essential for the biosynthesis of ManNAc- and/or ManNAcA-containing polysaccharides or glycolipids.[10,19,28–32]

2.2. Cloning, expression, and purification of NmSacA-His_6

NmSacA was cloned as a C-His_6-tagged recombinant protein (NmSacA-His_6) in pET22b(+) vector. Expression of NmSacA-His_6 in *E. coli* BL21 (DE3) followed by Ni^{2+}-column purification produced, on average, 83 mg purified protein per liter cell culture. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 2) showed that the purified protein has a molecular weight close to the calculated value of 43 kD.

2.3. NmSacA-His_6 is an active UDP-GlcNAc 2-epimerase

Previous reports used a coupled enzyme assay with UDP-ManNAc dehydrogenase measuring the formation of nicotinamide adenine dinucleotide reduced form (NADH) to characterize the activity of non-hydrolyzing UDP-GlcNAc 2-epimerases.[18,33] A direct assay using ion-paired reversed-phase high-performance liquid chromatography (HPLC) was also reported.[18] We developed an effective direct capillary electrophoresis approach to allow the separation and detection of UDP-GlcNAc, UDP-ManNAc, 2-acetamidoglucal, and UDP (Figs. S1 and S3). It avoids the complication of the coupled assays and uncertainty about the involvement of cofactor (e.g. NAD^+) in the activity of non-hydrolyzing UDP-GlcNAc 2-epimerase and provides good sensitivity.

Capillary electrophoresis assays using either UDP-GlcNAc or UDP-ManNAc as the starting material confirmed the 2-epimerization activity of NmSacA-His_6 in catalyzing the interconversion between UDP-GlcNAc and UDP-ManNAc. The reactions reached to an equilibrium with a fixed ratio of 11:1 for UDP-GlcNAc:UDP-ManNAc using either starting material. The relatively low percentage of UDP-ManNAc formed (<9%) when UDP-GlcNAc was used as the starting material made it relatively more difficult to characterize the activity of the UDP-GlcNAc 2-epimerase. Therefore, a better way is to use UDP-ManNAc as the starting material to study the UDP-GlcNAc formation which can reach up to 92% yield at equilibrium. UDP-ManNAc was chemoenzymatically synthesized and purified[40] for this purpose.

2.4. pH profile of NmSacA-His_6

Using UDP-ManNAc as the substrate, CE-based pH profile study showed (Fig. 3) that the epimerase activity of NmSacA-His_6 was optimum between pH 8.0 and pH 9.0. No or minimum activity was observed when the pH fell to below 6.0 or rose to 11.0.

2.5. Effects of metal ions, ethylenediaminetetraacetic acid (EDTA), and dithiothreitol (DTT) on the UDP-GlcNAc 2-epimerase activity of NmSacA-His_6

The 2-epimerase activity of NmSacA-His_6 is not dependent on the addition of an external divalent metal ion. As shown in Fig. 4, the enzyme activity was approximately at the same

level in the absence of a metal cation, in the presence of 10 mM EDTA, or in the presence of a monovalent metal ion Li^+. A mild decrease of the activity of NmSacA-His_6 was observed in the presence of 10 mM Mg^{2+}, Cu^{2+}, Ca^{2+}, or Ni^{2+}. The addition of 10 mM Co^{2+} dramatically decreased the epimerase activity, while the presence of 10 mM Mn^{2+} or Zn^{2+} completely depleted the activity of the epimerase and the activity could not be rescued by subsequent addition of EDTA (data not shown). The enzyme was apparently precipitated shortly after being mixed with 10 mM Zn^{2+}. Precipitation was also observed after the enzyme was mixed with Mn^{2+} (10 mM) and incubated at 37 °C for 20 minutes.

NmSacA-His_6 can be a useful reagent for enzymatic production of ManNAc/ManNAcA-containing polysaccharides, including *N. meningitidis* serogroup A CPS, for vaccine development. For this purpose, it is important to be aware of the damaging effect of NmSacA-His_6 by 10 mM Mn^{2+}. Cautions should be taken to avoid the use of Mn^{2+} when NmSacA is applied in multienzyme reactions containing glycosyltransferases and sugar nucleotide biosynthetic enzymes that require a divalent metal ion cofactor.[41] In these cases, Mg^{2+} is a suitable alternative as the presence of 10 mM Mg^{2+} only led to a moderate decrease of the epimerase activity of NmSacA-His_6.

NmSacA-His_6 has two cysteine residues. However, the addition of DTT (1 mM) did not influence the enzyme activity significantly (Fig. 4), indicating that disulfide bond formation is not required for the epimerase activity of NmSacA-His_6.

2.6. Kinetics

As shown in Table 1, when UDP-ManNAc was used as the substrate, the apparent k_{cat} value of NmSacA-His_6 obtained was 52 s^{-1} and the K_M value was 5.2 mM. When UDP-GlcNAc was used as substrate, the apparent k_{cat} value was 31 s^{-1} and the K_M value was 3.6 mM. The efficiencies of the 2-epimerization reactions catalyzed by NmSacA-His_6 were similar when UDP-ManNAc ($kcat/K_M$ = 10 mM^{-1} s^{-1}) or UDP-GlcNAc ($kcat/K_M$ = 8.6 mM^{-1} s^{-1}) was used as the substrate with a slightly higher efficiency for epimerizing UDP-ManNAc. Nevertheless, UDP-GlcNAc seemed to bind to NmSacA-His_6 slightly better than UDP-ManNAc as indicated by a smaller K_M value for UDP-GlcNAc.

Compared to the reported apparent kinetics parameters for *E. coli* RffE using UDP-GlcNAc as the substrate (apparent k_{cat} = 7.1 s^{-1}, K_M = 0.6 mM),[42] the K_M value of UDP-GlcNAc (3.6 mM) for NmSacA-His_6 was about 6-fold higher than that of RffE (0.6 mM), indicating a weaker binding of UDP-GlcNAc to NmSacA-His_6 than to RffE. However, NmSacA-His_6 has a better apparent k_{cat} value (31 s^{-1}) than RffE ($kcat$ = 7.1 s^{-1}).

2.7. UDP-GlcNAc was an activator for NmSacA-His_6

Bacterial non-hydrolyzing UDP-GlcNAc 2-epimerases from *Bacillus cereus*[43] and *E. coli* O14 K7 H[18,33] were shown to be allosteric enzymes and UDP-GlcNAc was absolutely required for their UDP-ManNAc 2-epimerization catalytic activity.[33] In our study, when chemoenzymatically synthesized pure UDP-ManNAc was used as the only starting material, NmSacA-His_6 successfully catalyzed the 2-epimerase reaction for the formation of UDP-GlcNAc. Therefore, UDP-GlcNAc is not an absolute requirement for the 2-epimerase

activity of NmSacA-His_6 despite sharing high sequence homology with known non-hydrolyzing bacterial UDP-GlcNAc 2-epimerases (Fig. 1).

Nevertheless, UDP-GlcNAc was able to activate the epimerization process in a dose-dependent manner. As shown in Fig. 5, in the absence of UDP-GlcNAc (solid line with diamond symbols), epimerization of UDP-ManNAc was quite slow in the initial reaction period ("lag time"). The reaction rate increased significantly when a small amount of UDP-GlcNAc was produced from UDP-ManNAc by NmSacA-His_6-catalyzed reaction. The dose-dependent activation effect of UDP-GlcNAc was obvious when comparing the initial rates of NmSacA-His_6-catalyzed UDP-ManNAc 2-epimerization reactions in the absence of UDP-GlcNAc (solid line), and in the presence of 5% (dashed line), 15% (longer dashed line), or 50% (dotted line) UDP-GlcNAc.

2.8. 2-Acetamidoglucal and UDP were NmSacA-His_6 inhibitors

As shown in Scheme 2, 2-acetamidoglucal **D** was synthesized from N-acetylglucosamine (GlcNAc) **A** with an overall 47% yield by following a reported method.[44] Free GlcNAc was treated with acetyl chloride to prepare peracetylated glycosyl chloride **B** (2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-α-D-glucopyranosyl chloride) in 56% yield,[45] which was used for the formation of peracetylated glucal **C** (85% yield) by treating with isopropenyl acetate[44] in the presence of catalytic amount of *p*-toluenesulfonic acid (*p*-TsOH). Deacetylation of **C** using sodium methoxide in methanol[46] removed the O-acetyl groups and one of the two N-acetyl groups to produce the desired product 2-acetamidoglucal **D** in a 96% yield.

Similar to the reported inhibitory effects of 2-acetamidoglucal and UDP for *E. coli* RffE[33] which were proposed intermediates for its 2-epimerase reaction,[18] both 2-acetamidoglucal and UDP showed competitive inhibitory effects for the UDP-ManNAc 2-epimerization of NmSacA-His_6 (Fig. S2), with K_i values of 14 mM and 0.8 mM respectively.

2.9. 2-Acetamidoglucal and UDP could not be used as starting materials by NmSacA-His_6 for the synthesis ofUDP-GlcNAc or UDP-ManNAc

Consistent with previous observations for the UDP-GlcNAc 2-epimerase from *E. coli*,[18] when either UDP-GlcNAc or UDP-ManNAc was used as the starting material for NmSacA-His_6, the formation of UDP and 2-acetamidoglucal was observed which could be produced quantitatively when the reaction was incubated for a longer period of time (>11 h). In comparison, the UDP-sugars were stable under the experimental conditions in the absence of the enzyme (Fig. S3).

Attempts using 2-acetamidoglucal and UDP as the starting materials for NmSacA-His_6 for the production of UDP-GlcNAc or UDP-ManNAc under different conditions (various pH values, with or without Mg^{2+} or DTT), however, were not successful. Adding a low concentration (1 or 2 mM) of UDP-GlcNAc did not seem to allow the consumption of 2-acetamidoglucal and UDP for the formation of UDP-GlcNAc or UDP-ManNAc.

Strong evidences support the role of 2-acetamidoglucal as an intermediate formed by *anti* elimination of UDP-GlcNAc in both mammalian hydrolyzing UDP-GlcNAc 2-epimerases/ManNAc kinases[37] and bacterial hydrolyzing UDP-GlcNAc 2-epimerase from *N.*

meningitidis MC58 (a serogroup B strain).[39] For example, incubating 2-acetamidoglucal with the enzyme led to the slow formation of ManNAc by *syn*-addition of water.[37,39] 2-Acetamidoglucal was also hypothesized as an intermediate for bacterial hydrolyzing UDP-GlcNAc 2-epimerases.[18,47] We showed here that similar to previous reports,[18,43] incubating NmSacA-His_6 with either UDP-GlcNAc or UDP-ManNAc led to the formation of 2-acetamidoglucal and UDP (Fig. S3). With longer incubation times, UDP-ManNAc and UDP-GlcNAc were completely converted to 2-acetamidoglucal and UDP. However, the formation of UDP-GlcNAc or UDP-ManNAc was not observed by incubating NmSacA-His_6 with 2-acetamidoglucal and UDP. Therefore, it is debatable whether UDP and 2-acetamidoglucal should be considered as intermediates or products.

2.10. NmSacA-His_6 could tolerate modified UDP-ManNAc as substrates

The fact that NmSacA-His_6 was active in catalyzing the epimerization of UDP-ManNAc in the absence of externally added UDP-GlcNAc simplified the substrate specificity studies of the enzyme using modified UDP-sugars containing either altered UDP or the sugar components as potential substrates.

An efficient chemoenzymatic approach was used for the synthesis of UDP-ManNAc and its derivatives. This included chemoenzymatic synthesis of UDP-$ManN_3$ using a one-pot multi-enzyme (OPME) system containing a *Bifidobacterium infantis* N-acetylhexosamine 1-kinase (NahK_ATCC15697), a *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP), and a *Pasteurella multocida* inorganic pyrophosphatase (PmPpA).[40] The obtained UDP-$ManN_3$ was chemically reduced followed by chemical N-acylation. The approach allowed the access to a number of UDP-ManNAc derivatives containing different N-acyl groups which were excellent probes to investigate the substrate specificity of NmSacA-His_6. In addition to UDP-GlcNAc and UDP-ManNAc (**1**), UDP-mannose (UDP-Man **2**) and derivatives (**3–4**),[40] UDP-mannosamine (UDP-$ManNH_2$, **5**),[40] as well as a list of UDP-ManNAc derivatives (**6–12**) and two UDP-GlcNAc derivatives (**13–14**)[48] were synthesized and used to investigate the substrate specificity of NmSacA-His_6 (Fig. 6). Among the tested compounds **2–14**, NmSacA-His_6 showed activity for three UDP-ManNAc derivatives (**6**, **7**, and **11**) and a UDP-GlcNAc derivative (**14**). UDP-Man (**2**) and its derivatives (**3–4**) as well as UDP-$ManNH_2$ (**5**) were not suitable substrates for NmSacA-His_6, indicating the essential role of the N-acyl group in the UDP-N-acylhexosamines for enzyme recognition. Among UDP-ManNAc (**6–12**) and UDP-GlcNAc derivatives (**13–14**) tested, only those containing a small N-acyl group, such as an N-acetyl (**11**), an N-hydroxylacetyl (or an N-glycolyl) (**6**), an N-propanoyl (**7**), or an N-trifluoroacetyl (**14**) group, were tolerable substrates for NmSacA-His_6. Those containing a larger N-acyl group, including an N-butanoyl (**8**), N-azidoacetyl (**9**,**12**, and **13**), or an N-phenylacetyl (**10**) group were not accepted by the enzyme as the substrates.

While UDP-GlcNTFA (**14**) with an N-trifluoroacetyl group replacing the N-acetyl group in UDP-GlcNAc was a similarly good substrate as UDP-GlcNAc for NmSacA-His_6 (data not shown), UDP-ManNGc containing an N-glycolyl group (**6**) and UDP-ManNPr containing an N-propanoyl group (**7**) were much poorer substrates than UDP-ManNAc for NmSacA-His_6. As shown in Table 1, the catalytic efficiencies of NmSacA-His_6 for UDP-ManNGc **6**

(k_{cat}/K_M = 0.21 mM^{-1} s^{-1}) and UDP-ManNPr **7** (0.15 mM^{-1} s^{-1}) were much lower than that for UDP-ManNAc **1** (10 mM^{-1} s^{-1}). Both weaker binding (the K_M values of 20 ± 1.9 mM for UDP-ManNGc **6** and 51 ± 5.0 mM for UDP-ManNPr **7** which were about 4- and 10-fold, respectively, higher than the K_m value of 5.2 ± 1.1 mM for UDP-ManNAc **1**) and lower turnover numbers (the k_{cat} values of 4.2±0.2 s^{-1} for UDP-ManNGc **6** and 7.8 ± 0.3 s^{-1} for UDP-ManNPr **7** which were about 12- and 6.7-fold, respectively, lower than the k_{cat} value of 52 ± 5 s^{-1} for UDP-ManNAc **1**) contributed to the lower NmSacA-His_6 catalytic efficiencies for UDP-ManNGc **6** and UDP-ManNPr **7**.

During the synthesis of UDP-ManNAc from chemoenzymatically synthesized UDP-$ManN_3$ **4** by reduction followed by N-acetylation,[40] catalytic hydrogenation of UDP-$ManN_3$ **4** using the Lindlar's catalyst[49] under 5 atm H_2 for 2.5 hours produced a UDP-mannosamine derivative (rUDP-$ManNH_2$), with the C=C double bond in the uracil also being reduced. Although the Lindlar's catalyst is a milder hydrogenation catalyst than palladium on charcoal, the high pressure of H_2 gas used and the relative long reaction time may have caused the reduction of uracil C=C double bond. rUDP-ManNAc **11** and rUDP-ManNAz **12** were synthesized from rUDP-$ManNH_2$ by acylation similar to the procedures reported previously for the synthesis of UDP-GlcNAc derivatives[48] and described in the experimental section for the synthesis of other UDP-ManNAc derivatives. The two UDP-ManNAc derivatives containing a reduced uracil ring provided an opportunity to investigate the effect of the nucleoside base structure on the activity of NmSacA-His_6. It was interesting to find that rUDP-ManNAc, despite its structural modification on the uracil ring, was still a substrate for NmSacA-His_6. The reduction of the C=C bond in the uracil, however, led to a significant (~100-fold) lower catalytic efficiency of NmSacA-His_6 due to a weaker binding (K_M = 63 ± 7.0 mM which was 12-fold higher than the K_M for UDP-ManNAc) and a slower turnover (k_{cat} = 6.7 ± 0.5 s^{-1} which was almost 8-fold lower than the k_{cat} for UDP-ManNAc) (Table 1).

Isotope effect studies showed that for*E. coli* non-hydrolyzing UDP-GlcNAc 2-epimerase, the C—H bond cleavage at the C2″-epimerization site of UDP-GlcNAc occurred during a rate-determining step.[18] In contrast, the C—H bond cleavage at the C2″ of UDP-GlcNAc was not rate limiting during catalysis by either *N. meningitidis* MC58 group B SiaA (a hydrolyzing UDP-GlcNAc 2-epimerase)[39] or a rat recombinant hydrolyzing UDP-GlcNAc 2-epimerase.[37] Interestingly, the positive allosteric effect of UDP-GlcNAc was demonstrated for both hydrolyzing[39] and non-hydrolyzing[3343] bacterial UDP-GlcNAc 2-epimerases but not for the rat enzyme which showed a negative allosteric effect.[50] Therefore, the unique features of bacterial non-hydrolyzing UDP-GlcNAc 2-epimerases and their important role in forming biologically important ManNAc/ManNAcA-containing polymers present a great opportunity for designing specific inhibitors as potential therapeutics against pathogenic bacteria including *N. meningitidis.* Indeed, an inhibitor against bacterial non-hydrolyzing UDP-GlcNAc 2-epimerases by targeting the allosteric binding site was identified as an effective compound in blocking the growth of a broad spectrum of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA).[51]

The substrate specificity study presented here provided additional information that could be used to facilitate the design of inhibitors that are specifically against NmSacA. For example, the presence of the N-acyl group in the sugar component of UDP-sugars seemed to be essential for NmSacA recognition. We were especially interested in testing UDP-ManNAz and UDP-GlcNAz as potential substrates for the enzyme because of broad application of azido-modified sugars in cell surface glycan labeling.[52–54] However, neither UDP-ManNAz (**9**) nor UDP-GlcNAz (**13**) (Fig. 6) containing an N-azidoacetyl group was a tolerable substrate for NmSacA-His_6. This may be due to the bigger size of the azidoacetyl group compared to the acetyl group and the lack of free rotation in the azido group which do not allow proper fitting to the binding pocket of the enzyme.

Despite having low protein sequence identity, *E. coli* RffE[55] shows structural homology to glycosyltransferases, such as glycogen phosphorylase,[56] bacteriophage T4 β-glucosyltransferase,[57] GlcNAc transferase MurG involved in peptidoglycan biosynthesis,[58] glucosyltransferase GtfB in vancomycin biosynthetic process,[59] *Pasteurella multocida* sialyltransferase 1 (PmST1),[60] and other GT-B glycosyltransferase structures.[61] Reports on the crystal structures of *E. coli* (a Gram-negative bacterium) RffE,[55] *Bacillus anthracis* (a Gram-positive bacterium)[62] and *Methanocaldococcus jannaschii* (archaea)[63] non-hydrolyzing UDP-GlcNAc 2-epimerases identified the substrate binding pocket and the critical amino acid residues that are involved in catalysis. The absence of NAD^+ in the crystal structures of these epimerases supported the observation that NAD^+ was not required for the activity of the enzyme. A structural basis for the allosteric effect of UDP-GlcNAc in regulating the activity of these enzymes was also established.[62] Nevertheless, the detailed catalytic mechanism of this important class of enzyme remains to be explored.

The crystal structures of UDP-GlcNAc 2-epimerases from *B. anthracis* [Protein Data Bank (PDB) ID 3BEO], *B. subtilis* (PDB ID 4FKZ), and *M. jannaschii* (PDB ID 4NES) contain UDP as a ligand in the active site and a nearby UDP-GlcNAc ligand proposed to be involved in allosteric activation.[62,63] In order to understand the substrate specificity of NmSacA, homology models were created from the three structures of UDP- and UDP-GlcNAc-bound proteins using SWISS-MODEL.[64] The *B. anthracis* model gave the best QMEAN4 score. Especially, all residues immediately surrounding the substrate are conserved between the *B. anthracis* model and NmSacA. Therefore, the *B. anthracis* model was selected for docking studies using Autodock Vina.[65] UDP-GlcNAc and UDP-ManNAc were docked using the position of UDP and UDP-GlcNAc ligands in the *B. anthracis* structure to define the docking area. The position of the UDP atoms in docked UDP-GlcNAc and UDP-ManNAc was highly consistent with the position of the crystallized UDP ligand. The docking studies reveal that the pocket surrounding the N-acetyl group defines the size of tolerable N-acyl groups in the substrate (Fig. 7) by residues Pro-128, Arg-135, and Glu-311 (not shown) which appears consistent with the observed discrimination against substrates with acyl groups larger than glycoyl and propionyl.

3. Conclusion

In conclusion, we successfully cloned, expressed, purified, and characterized a non-hydrolyzing UDP-GlcNAc 2-epimerase from *N. meningitidis* serogroup A (NmSacA) which

is involved in the biosynthesis of bacterial CPS. We also developed a capillary electrophoresis (CE)-based assay method which allowed direct separation and detection of compounds in reaction mixtures with a good sensitivity. Different from other bacterial non-hydrolyzing UDP-GlcNAc 2-epimerases reported previously, the presence of UDP-GlcNAc was not an absolute requirement for the epimerization of UDP-ManNAc by NmSacA. Nevertheless, UDP-GlcNAc activated the UDP-ManNAc epimerization activity of NmSacA in a dose-dependent manner. A list of UDP-ManNAc and UDP-GlcNAc analogs obtained by chemoenzymatic synthesis helped to map out the substrate specificity of NmSacA. NmSacA can be used in enzymatic synthesis of *N. meningitidis* serogroup A CPS for vaccine development and is a potential target for drug development.

4. Experimental section

4.1. Materials

E. coli electrocompetent DH5α and chemically competent BL21 (DE3) cells were from Invitrogen (Carlsbad, CA, USA). Vector plasmid pET22b(+) was purchased from Novagen (EMD Biosciences Inc., Madison, WI, USA). QIAprep spin miniprep kit and QIAquick gel extraction kit were from Qiagen (Valencia, CA, USA). Herculase-enhanced DNA polymerase was from Stratagene (La Jolla, CA, USA). T4 DNA ligase and 1 kb DNA ladder were from Promega (Madison, WI, USA). *NdeI* and *Xho*I restriction enzymes were from New England Biolabs (Beverly, MA, USA). Ni^{2+}-NTA agarose (nickel-nitrilotriacetic acid agarose) was from 5 PRIME (Gaithersburg, MD, USA). *N. meningitidis* serogroup A genomic DNA (ATCC 53417D) from strain M1027 was from the American Type Culture Collection (Manassas, VA, USA). Bicinchoninic acid (BCA) protein assay kit was from Pierce Biotechnology Inc. (Rockford, IL, USA). UDP-ManNAc was synthesized chemoenzymatically as reported previously.[40]

4.2. Chemical synthesis of 2-acetamidoglucal

As shown in Scheme 2, GlcNAc **A** (5.0 g, 22.70 mmol) was added to acetyl chloride (15 mL) and the mixture was stirred for overnight. After adding chloroform (50 mL), the resulting solution was poured into an ice-water mixture. The organic phase was washed with saturated aqueous $NaHCO_3$ (50 mL) and concentrated. The resulting residue was purified by silica gel flash chromatography (eluted with EtOAc) to produce glycosyl chloride **B** (4.7 g, 56%). The obtained compound **B** (4.0 g, 10.9 mmol) was dissolved in isopropenyl acetate (70 mL) and *p*-TsOH (70 mg) was added. The mixture was refluxed at 110 °C for 20 h and then was concentrated and purified by silica gel flash chromatography (eluted with EtOAcHexane = 1:1.5 to 1:2) to produce compound **C** (3.45 g, 85%). The obtained compound **C** (1.2 g) was dissolved in dry MeOH (30 mL) and NaOMe was added (pH ~ 8.5). The reaction mixture was stirred for 2 h and then neutralized with Amberlite IR-120 (H^+). After filtration, the solution was concentrated and purified by silica gel flash chromatography (eluted with EtOAc:MeOH:H_2O = 2.5:1.0:0.5) to produce the desired glycal **D** as a white solid (0.63 g, 96%). ^{1}H NMR (600 MHz, D_2O) δ 6.66 (s, 1H, H-1), 4.24 (d, 1H, *J* = 6.6 Hz, H-3), 3.98 (m, 1H, H-5), 3.85 (d, 2H, *J* = 4.2 Hz, H-6a and H-6b), 3.75 (dd, 1H, *J* = 6.6 and 9.0 Hz), 2.03 (s, 3H, CH_3); ^{13}C NMR (125 MHz, D_2O) δ 174.71, 141.89, 113.36, 78.73, 68.97. 68.55, 60.03, 22.04.

4.3. Cloning of NmSacA-His$_6$

Commercially available *N. meningitidis* serogroup A genomic DNA from strain M1027 (ATCC 53417D) was used as a template for polymerase chain reaction (PCR). NmSacA was cloned and expressed as a C-His$_6$-tagged fusion protein. The primers used for cloning the C-His$_6$-tagged protein in pET22b(+) vector were designed based on the gene sequence (GenBank accession number: AL157959, locus tag NMA0199, GenBank accession number for the corresponding putative protein: CAM07513) reported for *N. meningitidis* serogroup A strain Z2491. They were: forward primer 5′-GATCCATATG AAAGTCTTAACCGTCTTTGG-3′ (*Nde*I restriction site is underlined) and reverse primer 5′-AAGCTCGAGTCTATTCTTTAATAAAGTTTCTAC-3′ (*XhoI* restriction site is underlined). PCRs for amplifying the target gene were performed in a 50 μL reaction mixture containing genomic DNA (10 ng), forward and reverse primers (0.2 μM each), 1× Herculase buffer, dNTP mixture (0.2 mM), and 5 U (1 μL) of Herculase-enhanced DNA polymerase. The reaction mixture was subjected to 30 cycles of amplification at an annealing temperature of 55 °C. The resulting PCR product was purified and double digested with *Nde*I and *Xho*I restriction enzymes. The purified and digested PCR product was ligated with the predigested pET22b(+) vector and transformed into electrocompetent *E. coli* DH5α cells. Selected clones were grown for minipreps and characterized by restriction mapping. DNA sequencing was performed by the Davis Sequencing Facility in the University of California-Davis.

4.4. Overexpression of NmSacA-His$_6$

Positive plasmids were selected and transformed into *E. coli* BL21 (DE3) chemical competent cells. The plasmid-bearing *E. coli* strains were cultured in LB-rich medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10gL^{-1} NaCl) containing ampicillin (100 μg mL^{-1}). Overexpression of the target protein was achieved by inducing the *E. coli* culture with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) when the OD_{600} of the culture reached 0.8 followed by incubating at 20 °C for 20 h with vigorous shaking at 250 rpm in a C25KC incubator shaker (New Brunswick Scientific, Edison, NJ).

4.5. Enzyme purification of NmSacA-His$_6$

His$_6$-tagged proteins were purified from cell lysate. To obtain cell lysate, cell pellet harvested by centrifugation at 4000 rpm for 2 h was resuspended in lysis buffer (pH 8.0,100 mMTris-HCl containing 0.1% Triton X-100, 20 mL was used for cells collected from each liter of cell culture). Lysozyme (50 μg mL^{-1}) and DNaseI (3 μg mL^{-1}) were then added and the mixture was incubated at 37 °C for 60 min with vigorous shaking. Cell lysate was obtained by centrifugation at 12,000 rpm for 30 min as the supernatant. Purification of His$_6$-tagged proteins from the lysate was achieved using a Ni^{2+}-resin column. The column was pre-equilibrated with 10 column volumes of binding buffer (5 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5) before the lysate was loaded. After washing with 8 column volumes of binding buffer and 10 column volumes of washing buffer (40 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5), the protein was eluted with an elute buffer (200 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5). The fractions containing the purified enzymes were collected, dialyzed and then stored at 4 °C.

4.6. Quantification of purified NmSacA-His_6

The concentration of purified enzyme was obtained in a 96-well plate using a Bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as a protein standard. The absorbance of samples was measured at 562 nm by a BioTek Synergy™ HT Multi-Mode Microplate Reader.

4.7. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed in a 12% Tris-glycine gel using a Bio-Rad Mini-protein III cell gel electrophoresis unit (Bio-Rad) at DC = 150 V. Bio-Rad Precision Plus Protein Standards (10–250 kDa) were used as molecular weight standards. Gels were stained with Coomassie Blue.

4.8. pH profile by capillary electrophoresis

Assays were performed in a total volume of 10 μL in a buffer (100 mM) with pH varying from 6.0 to 11.0 containing UDP-ManNAc (2 mM) and the recombinant enzyme (33 ng). Reactions were allowed to proceed for 10 min at 37 °C before being quenched by adding pre-chilled ethanol (10 μL) to make 2-fold dilutions. The samples were then kept on ice, centrifuged, and aliquots of 8 μL were withdrawn and analyzed by a Beckman P/ACE MDQ capillary electrophoresis system (60 cm × 75 μm i.d.) with a UV detector (254 nm). All assays were carried out in duplicate. Yields were calculated based on the integrals of peaks in the electropherogram.

4.9. Effects of metal ions, ethylenediaminetetraacetic acid (EDTA), and dithiothreitol (DTT)

EDTA (10 mM), different metal ions with Cl^- as the counter ion (10 mM), and DTT (1 mM) were used in Tris-HCl buffer (pH 9.0, 100 mM) containing 2 mM of UDP-ManNAc in a total volume of 10 μL to analyze their effects on UDP-ManNAc 2-epimerization activity of the epimerase (33 ng). Reactions without EDTA, DTT, or metal ions were used as a negative control. Reactions were allowed to proceed for 10 min at 37 °C before being quenched by adding pre-chilled ethanol (10 μL) to make 2-fold dilutions.

4.10. Kinetics by CE assay

The assays were carried out in a total volume of 10 μL in Tris buffer (100 mM, pH 8.5) containing UDP-ManNAc or UDP-GlcNAc and the epimerase (33 ng). Reactions were allowed to proceed for 5 min at 37 °C. Apparent kinetic parameters were obtained by varying the UDP-ManNAc or UDP-GlcNAc concentrations from 0.5–25.0 mM (0.5, 1.0, 2.0, 3.0, 5.0, 10.0, and 25.0 mM). Apparent kinetic parameters were obtained by fitting the data (the average values of duplicate assay results) into the Michaelis-Menten equation using Grafit 5.0.

4.11. Activation effect of UDP-GlcNAc

The activation effect of UDP-GlcNAc was assayed by fixing the sum of the concentrations of UDP-ManNAc and UDP-GlcNAc to 2 mM and varying the percentage (0, 5%, 15%, and 50%) of UDP-GlcNAc.

4.12. Activity assays using 2-acetamidoglucal and UDP as starting materials

The reactions were carried out in a total volume of 10 μL containing 2-acetylamidoglucal (10 mM), UDP (10 mM), and the epimerase (18 μg) in MES (pH 5.0, 100 mM) or Tris-HCl (pH8.5, 100 mM) buffer in the presence or the absence of Mg^{2+} (20 mM) or DTT (2 mM). Furthermore, assays were also carried out in a total volume of 10 μL in Hepes buffer (pH 7.0, 100 mM) containing 2-acetylamidoglucal (10 mM), UDP (10 mM), Mg^{2+} (20 mM), and the epimerase (18 μg) with or without a low concentration of UDP-GlcNAc (1 mM or 2 mM).

4.13. Inhibition effect of 2-acetamidoglucal and UDP

The inhibition curves and K_i values of 2-acetamidoglucal and UDP for NmSacA were obtained by varying the concentration of the substrate UDP-ManNAc (1.0, 1.5, 2.5, 4.0, 10.0 mM) at different concentrations of 2-acetamidoglucal (0, 2, 10 mM) or UDP (0, 0.5, 1.5 mM).

4.14. Substrate specificity assay of UDP-ManNAc derivatives

The assays were carried out in a total volume of 20 μL in Tris-HCl buffer (100 mM, pH 8.5) containing UDP-ManNAc derivatives (2 mM) and the epimerase (3.3 μg). Reactions were allowed to proceed for 30 min at 37 °C. Aliquots of 10 μL were withdrawn and quenched by adding pre-chilled ethanol (10 μL) to make 2-fold dilutions. The remaining reactions were allowed to continue to a total reaction time of 4 h at 37 °C before being quenched by adding pre-chilled ethanol (10 μL) to make 2-fold dilutions. The samples were then kept on ice until aliquots of 8 μL were withdrawn and analyzed by a Beckman P/ACE MDQ capillary electrophoresis system (60 cm × 75 μm i.d.) with a UV detector (254 nm for rUDP-ManNAc and 215 nm for rUDP-ManNAz).

4.15. Docking studies

Homology modeling was performed with SWISS-MODEL.[64] Models were constructed from template PDB structures 3BEO, 4FKZ, and 4NES. Mol2 files for UDP-GlcNAc and UDP-ManNAc were obtained from the ZINC database.[66] Using AutoDockTools,[67] the docking grid was set based on the position of 3BEO UDP and UDP-GlcNAc ligands. Autodock Vina was used to dock UDP-GlcNAc and UDP-ManNAc into the homology model from 3BEO.[5] The docked structures were analyzed using PyMOL.

4.16. Synthesis of UDP-ManNAc derivatives 6–12

4.16.1. General methods—Chemicals were purchased and used without further purification. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded on a Varian Mercury 600 MHz spectrometer or a Bruker 800 MHz spectrometer. High resolution electrospray ionization (ESI) mass spectra were obtained in negative mode using Thermo Electron LTQ-Orbitrap mass spectrometer. Silica gel 60 Å (Sorbent Technologies) was used for flash column chromatography. Thin-layer chromatography (TLC) was performed on silica gel plates 60 GF254 (Sorbent Technologies) using anisaldehyde sugar stain for detection. Gel filtration chromatography was performed using a column (100 cm × 2.5 cm) packed with BioGel P-2 Fine resins (Bio-Rad). UDP-ManNAc (**1**), UDP-Man (**2**), UDP-

ManF (**3**), UDP-$ManN_3$ (**4**), and UDP-$ManNH_2$ (**5**)[40] as well as UDP-GlcNAz (**13**) and UDP-GlcNTFA (**14**)[48] were previously synthesized. NahK_ATCC15697, BLUSP, and PmPpA were overexpressed as reported previously.[40,48]

4.16.2. Uridine 5′-diphospho-2-hydroxyacetamido-2-deoxy-α-D-mannopyranoside (UDP-ManNGc, 6)—To a solution of UDP-$ManNH_2$ **5** (30 mg, 0.049 mmol) in CH_3CN-H_2O (10 mL, 1:1 v/v) in the presence of $NaHCO_3$ (40 mg, 0.49 mmol), acetoxyacetyl chloride (6.9 μL, 0.098 mmol) in CH_3CN (1 mL) was added. The reaction mixture was stirred for 4 h at 0 °C and was neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The crude product was dissolved in dry methanol (10 mL) containing catalytic amount of sodium methoxide. The resulting mixture was stirred at r.t. for overnight. The reaction mixture was then neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The residue was purified by silica gel column flash chromatography (EtOAc:MeOH:H_2O = 3:2:1, by volume) to produce UDP-ManNGc in 62% yield (20.6 mg). ^{1}H NMR (600 MHz, D_2O) δ 7.98 (d, *J* = 8.4 Hz, 1H, H-6), 6.01−5.92 (m, 2H, H-1′, H-5), 5.51 (dd, *J* = 4.8 and 0.8 Hz, 1H, H-1″), 4.52 (dd, *J* = *4.8* and 0.8 Hz, 1H, H-2″), 4.46 (s, 2H, $COC\underline{H_2}OH$), 4.40−4.12 (m, 6H, H-2′, H-3′, H-4′, H-5a′, H-5b′, H-3″), 3.96−3.65 (m, 4H, H-5″, H-6a″, H-6b″, H-4″). ^{13}C NMR (150 MHz, D_2O) δ 175.01, 173.84, 154.76, 141.53, 102.60, 95.15, 86.88, 82.50, 73.72, 73.33, 70.02, 68.63, 66.30, 63.29, 60.84, 60.10, 52.58. HRMS (ESI) *m/z* calcd for $C_{17}H_{27}N_3O_{18}P_2$ (M – H) 622.0687, found 622.0687.

4.16.3. Uridine 5′-diphospho-2-propionamido-2-deoxy-α-D-mannopyranoside (UDP-ManNPr, 7)—To a solution of UDP-$ManNH_2$ **5** (31.0 mg, 0.051 mmol) in CH_3CN-H_2O (10 mL, 1:1 v/v) in the presence of $NaHCO_3$ (41.6 mg, 0.49 mmol), propionyl chloride (8.9 μL, 0.102 mmol) in CH_3CN (1 mL) was added. The reaction mixture was stirred for 4 h at 0 °C and was neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The residue was purified by silica gel column flash chromatography (EtOAc:MeOH:H_2O = 5:2:1, by volume) to produce UDP-ManNPr in 47% yield (15.9 mg). ^{1}H NMR (800 MHz, D_2O) δ 7.92 (d, *J* = 8.0 Hz, 1H, H-6), 5.93−5.91 (m, 2H, H-5, H-1′), 5.40 (dd, *J* = 4.8 and 0.8 Hz, 1H, H-1″), 4.40 (dd, *J* = 4.8 and 0.8 Hz, 1H, H-2″), 4.33−4.29 (m, 2H, H-2′, H-3′), 4.25−4.04 (m, 4H, H-4′, H-5a′, H-5b′, H-3″), 3.86−3.80 (m, 3H, H-5″, H-6a″, H-6b″), 3.58 (t, *J* = 8.8 Hz, 1H, H-4″), 2.23 (m, 2H, $NHCO\underline{CH_2}CH_3$), 1.05 (t, *J* = *7.2* Hz, 3H, $NHCOCH_2\underline{CH_3}$). ^{13}C NMR (200 MHz, D_2O) δ 178.48, 166.34, 151.86, 141.46, 102.52, 95.33, 88.17, 83.09 (d, *J* = 9.4 Hz), 73.67, 73.15, 69.52, 68.60, 66.18, 64.76 (d, *J* = 5.2 Hz), 59.99, 52.71, 28.77, 9.28. HRMS (ESI) *m/z* calcd for $C_{18}H_{29}N_3O_{17}P_2$ (M – H) 620.0894, found 620.0872.

4.16.4. Uridine 5′-diphospho-2-butyramido-2-deoxy-α-D-mannopyranoside (UDP-ManNBt, 8)—To a solution of UDP-$ManNH_2$ **5** (30.0 mg, 0.049 mmol) in CH_3CN-H_2O (10 mL, 1:1 v/v) in the presence of $NaHCO_3$ (40 mg, 0.49 mmol), butyryl chloride (10.1 μL, 0.098 mmol) in CH_3CN (1 mL) was added. The reaction mixture was stirred for 4 h at 0 °C and was neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The residue was purified by silica gel column flash chromatography (EtOAc:MeOH:H_2O = 5:2:1, by volume) to produce UDP-ManNBt in 43% yield (14.7 mg). ^{1}H NMR (800 MHz,

D_2O) δ 7.93 (d, *J* = 8.0 Hz, 1H, H-6), 5.94 (d, *J* = 4.8 Hz, 1H, H-1′), 5.93 (d, *J* = 8.0 Hz, 1H, H-5), 5.40 (dd, *J* = 7.2 and 0.8 Hz, 1H, H-1″), 4.41 (dd, *J* = 4.8 and 0.8 Hz, 1H, H-2″), 4.33−4.31 (m, 2H, H-2′, H-3′), 4.24 (m, 1H, H-4′), 4.21−4.13 (m, 2H, H-5a′, H-5b′), 4.09 (dd, *J* = 10.4 and 4.8 Hz, 1H, H-3″), 3.87−3.81 (m, 3H, H-5″, H-6a″, H-6b″), 3.60 (t, *J* = 10.4 Hz, 1H, H-4″), 2.23 (t, *J* = 8.0 Hz, 2H, $NHCO\underline{CH_2}CH_2CH_3$), 1.55 (m, 2H, $NHCOCH_2\underline{CH_2}CH_3$), 0.86 (t, *J* = 7.2 Hz, 3H, $NHCOCH_2CH_2\underline{CH_3}$). ^{13}C NMR (200 MHz, D_2O) δ 177.63, 166.37, 151.88, 141.45, 102.53, 95.38, 88.16, 83.09 (d, *J* = 9.0 Hz), 73.67, 73.15, 69.52, 68.54, 66.16, 64.76 (d, *J* = 5.2 Hz), 59.99, 52.80, 37.36, 18.86, 12.67. HRMS (ESI) *m/z* calcd for $C_{19}H_{31}N_3O_{17}P_2$ (M – H) 634.1050, found 634.1028.

4.16.5. Uridine 5′-diphospho-2-azidoacetamido-2-deoxy-α-D-mannopyranoside (UDP-ManNAz, 9)—To a solution of UDP-$ManNH_2$ **5** (16.1 mg, 0.026 mmol) in CH_3CN-H_2O (10 mL, 1:1 v/v) in the presence of $NaHCO_3$ (21 mg, 0.26 mmol), azidoacetic acid N-hydroxysuccinimide (NHS) ester (25.8 mg, 0.13 mmol) in CH_3CN (1 mL) was added. The reaction mixture was stirred for 4 h at 0 °C and was neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The residue was purified by silica gel column flash chromatography (EtOAc:MeOH:H_2O = 5:2:1, by volume) to produce UDP-ManNAz in 50% yield (9.2 mg). ^{1}H NMR (600 MHz, D_2O) δ 7.97 (d, *J* = 7.8 Hz, 1H, H-6), 5.99 (d, *J* = 4.2 Hz, 1H, H-1′), 5.97 (d, *J* = 8.4 Hz, 1H, H-5), 5.49 (dd, *J* = 7.8 and 1.8 Hz, 1H, H-1″), 4.50 (dd, *J* = 4.8, 1.8 Hz, 1H, H-2″), 4.39−4.36 (m, 2H, H-2′, H-3′), 4.31−4.17 (m, 3H, H-4′, H-5a′, H-5b′), 4.15 (dd, *J* = 10.2 and 4.8 Hz, 1H, H-3″), 4.07 (s, 2H, $\underline{CH_2}N_3$), 3.94−3.85 (m, 3H, H-5″, H-6a″, H-6b″), 3.63 (t, *J* = 10.2 Hz, 1H, H-4″). ^{13}C NMR (150 MHz, D_2O) δ 173.92, 170.84, 154.65, 141.52, 102.58, 95.11, 86.85, 82.49 (d, *J* = 9.0 Hz), 73.73, 73.24, 70.48, 70.01, 68.64, 66.26, 60.07, 53.08 (d, *J* = 9.3 Hz), 51.53. HRMS (ESI) *m/z* calcd for $C_{17}H_{26}N_6O_{17}P_2$ (M – H) 647.0751, found 647.0722.

4.16.6. Uridine 5′-diphospho-2-phenylacetamido-2-deoxy-α-D-mannopyranoside (UDP-ManPhAc, 10)—2-Phenylacetyl acid (67 mg, 0.49 mmol) was dissolved in 10 mL of dry CH_2Cl_2 and two drops of DMF were added. The mixture was cooled to 0 °C. Oxalyl chloride (83 μL, 0.98 mmol) was slowly added over 15 min using a syringe. The reaction was allowed to warm up to r.t. for overnight. The solvent was then removed under reduced pressure to produce 2-phenylacetyl chloride as a light pink solid. To a solution of UDP-$ManNH_2$ **5** (31.4 mg, 0.051 mmol) in CH_3CN-H_2O (10 mL, 1:1 v/v) in the presence of $NaHCO_3$ (42 mg, 0.51 mmol), 2-phenylacetyl chloride in CH_3CN (1 mL) was added. The reaction mixture was stirred for 4 h at 0 °C and was neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The residue was purified by silica gel column flash chromatography (EtOAc:MeOH:H_2O = 5:2:1, by volume) to produce UDP-ManPhAc in 63% yield (24.1 mg). ^{1}H NMR (800 MHz, D_2O) δ 7.87 (d, *J* = 8.8 Hz, 1H, H-6), 7.37−7.28 (m, 5H, Phenyl), 5.91 (d, *J* = 4.0 Hz, 1H, H-1′), 5.89 (d, *J* = 8.0 Hz, 1H, H-5), 5.43 (dd, *J* = 7.2 and 1.6 Hz, 1H, H-1″), 4.42 (dd, *J* = 4.8 and 1.6 Hz, 1H, H-2″), 4.30−4.10 (m, 6H, H-2′, H-3′, H-4′, H-5a′, H-5b′, H-3″), 3.84−3.80 (m, 3H, H-5″, H-6a″, H-6b″), 3.68−3.56 (m, 3H, H-4″, $\underline{CH_2}$Ph). ^{13}CNMR(200 MHz, D_2O) δ 174.97, 166.35, 151.82, 141.39, 134.82, 129.04, 128.72, 127.08, 102.46, 95.20, 88.23, 82.99, 73.67, 73.22,

69.45, 68.54, 66.17, 64.74, 60.06, 53.01, 41.85. HRMS (ESI) *m/z* calcd for $C_{23}H_{31}N_3O_{17}P_2$ (M – H) 682.1050, found 682.1024.

4.16.7. Dihydropyrimidine-2,4(1H, 3H)-dione-α-D-ribofuranosyl-5′-diphospho-2-acetamido-2-deoxy-α-D-mannopyranoside (rUDP-ManNAc, 11)— UDP-$ManN_3$ **4** (167 mg, 0.28 mmol) was dissolved in MeOH-H_2O (20 mL, 1:1 v/v) in the presence of the Lindlar's catalyst (200 mg). Catalytic hydrogenation under 5 atm hydrogen gas for 2.5 h produced the product quantitatively with the reduction of both the double bond on the uridine base ring and the azido group (reduced UDP-$ManNH_2$ or rUDP-$ManNH_2$). To a solution of rUDP-$ManNH_2$ (45.0 mg, 0.080 mmol) in CH_3CN-H_2O (10 mL, 1:1 v/v) in the presence of $NaHCO_3$ (66 mg, 0.80 mmol), acetyl chloride (11.4 μL, 0.16 mmol) in CH_3CN (1 mL) was added. The reaction mixture was stirred for 4 h at 0 °C and was neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The residue was purified by silica gel column flash chromatography (EtOAc:MeOH:H_2O = 5:2:1, by volume) to produce rUDP-ManNAc in 84% yield (40.8 mg). ^{1}H NMR (600 MHz, D_2O) δ 5.92 (d, *J* = 7.2 Hz, 1H, H-1′), 5.46 (dd, *J* = 7.8 and 1.8 Hz, 1H, H-1″), 4.46 (dd, *J* = 4.8 and 1.8 Hz, 1H, H-2″), 4.37 (dd, *J* = 7.2 and 5.4 Hz, 1H, H-2′), 4.32 (dd, *J* = 5.4 and 2.4 Hz, 1H, H-3′), 4.19 (m, 1H, H-4′), 4.14 (dd, *J* = 10.2 and 4.8 Hz, 1H, H-3″), 4.11 (dd, *J* = 5.4 and 3.0 Hz, 2H, H-5a′, H-5b′), 3.94−3.91 (m, 1H, H-5″), 3.88−3.87 (m, 2H, H-6a″, H-6b″), 3.70−3.58 (m, 3H, H-4″, H-6), 2.78 (t, *J* = 7.2 Hz, 2H, H-5), 2.06 (s, 3H, NHCO$\underline{CH_3}$). ^{13}C NMR (150 MHz, D_2O) δ 174.63, 173.90, 154.65, 104.99, 95.32, 86.86, 73.24, 70.49, 70.01, 68.73, 66.34, 65.55, 60.11, 52.99, 35.98, 30.12, 21.89. HRMS (ESI) *m/z* calcd for $C_{17}H_{29}N_3O_{17}P_2$ (M – H) 608.0894, found 608.0867.

4.16.8. Dihydropyrimidine-2, 4(1H, 3H)-dione-α-D-ribofuranosyl-5′-diphospho-2-azidoxyacetamido-2-deoxy-α-D-mannopyranoside (rUDP-ManNAz, 12)—To a solution of reduced UDP-$ManNH_2$ (10.0 mg, 0.017 mmol) in CH_3CN-H_2O (10 mL, 1:1 v/v) in the presence of $NaHCO_3$ (14 mg, 0.17 mmol), azidoacetic acid NHS ester (16.9 mg, 0.085 mmol) in CH_3CN (1 mL) was added. The reaction mixture was stirred for 4 h at 0 °C and was neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The residue was purified by silica gel column flash chromatography (EtOAc:MeOH:H_2O = 5:2:1, by volume) to produce rUDP-ManNAz in 87% yield (9.3 mg). ^{1}H NMR (800 MHz, D_2O) δ 5.87 (d, *J* = 7.2 Hz, 1H, H-1′), 5.44 (d, *J* = 7.2 and 1.6 Hz, 1H, H-1″), 4.48 (dd, *J* = 4.8 and 1.6 Hz, 1H, H-2″), 4.34 (dd, *J* = 6.4 and 5.6 Hz, 1H, H-2′), 4.28 (dd, *J* = 5.6 and 2.4 Hz, 1H, H-3′), 4.15 (m, 1H, H-4′), 4.13 (dd, *J* = 10.4 and 4.8 Hz, 1H, H-3″), 4.07−3.83 (m, 7H, H-5a′, H-5b′, $\underline{CH_2}N_3$, H-5″, H-6a″, H-6b″), 3.66−3.53 (m, 3H, H-4″, H-6), 2.74 (*t, J* = 7.2 Hz, 2H, H-5). ^{13}C NMR (150 MHz, D_2O) δ 173.87, 170.80, 154.61, 95.06, 86.75, 82.48, 74.09, 73.18, 70.44, 69.93, 68.58, 66.17, 65.48, 59.98, 51.45, 35.89, 32.34. HRMS (ESI) *m/z* calcd for $C_{17}H_{28}N_6O_{17}P_2$ (M – H) 649.0908, found 649.0878.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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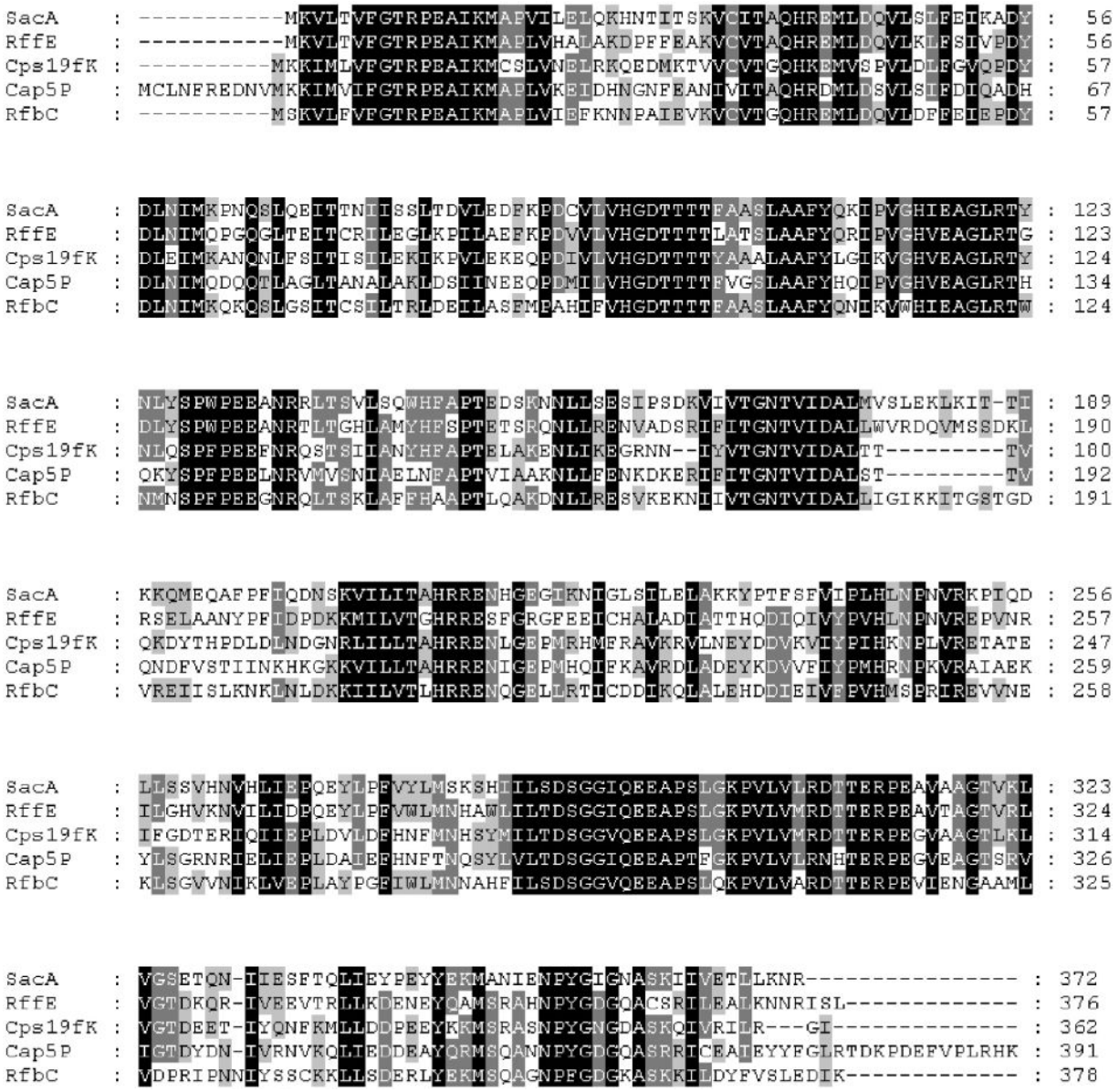


Fig. 1.

Sequence alignment of *Neisseria meningitidis* serogroup A SacA (NmSacA) (GenBank accession number CAM07513) and other non-hydrolyzing bacterial UDP-GlcNAc 2-epimerases including *Escherichia coli* RffE (GenBank accession number AAT48211), *Streptococcus pneumonia* Cps19fK (GenBank accession number AAC44968), *Staphylococcus aureus* Cap5P (GenBank accession number AAC46099), and *Salmonella enterica* RfbC (GenBank accession number AAC98403).

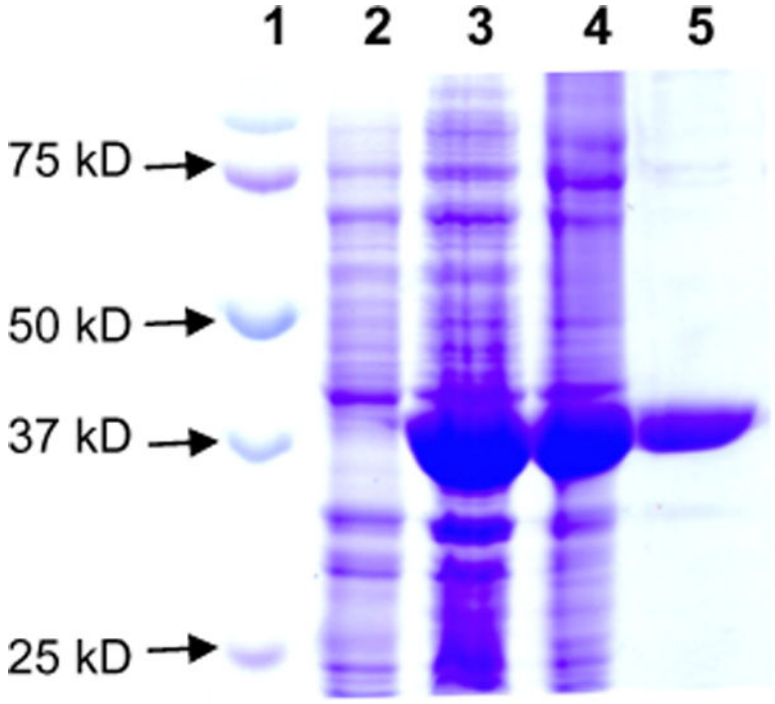


Fig. 2.

SDS–PAGE analysis of NmSacA-His_6 expression and purification. Lanes: 1, protein standards; 2, whole cell extraction before induction; 3, whole cell extraction after induction; 4, cell lysate after induction; 5, Ni^{2+}-column purified protein.

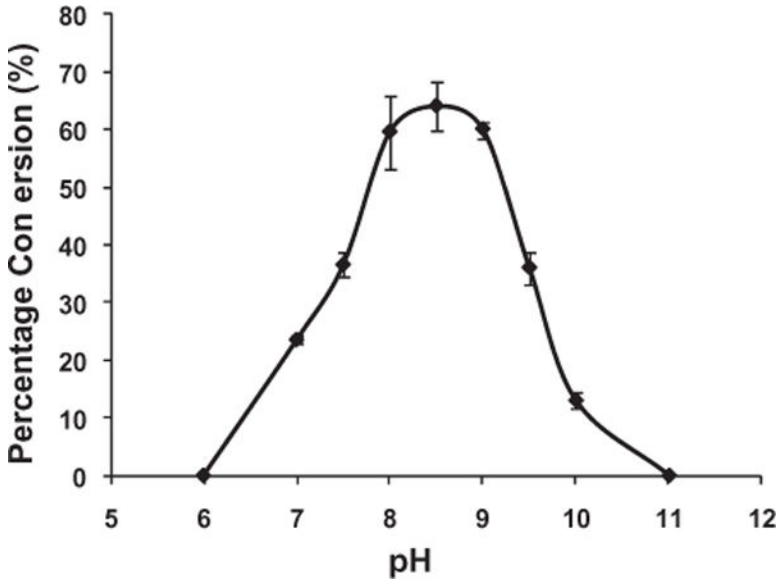


Fig. 3.

The pH profile of NmSacA-His_6 when UDP-ManNAc was used as the substrate. Buffers used: MES (pH 6.0), Tris–HCl (pH 7.0–9.0), CAPS (pH 9.5–11.0).

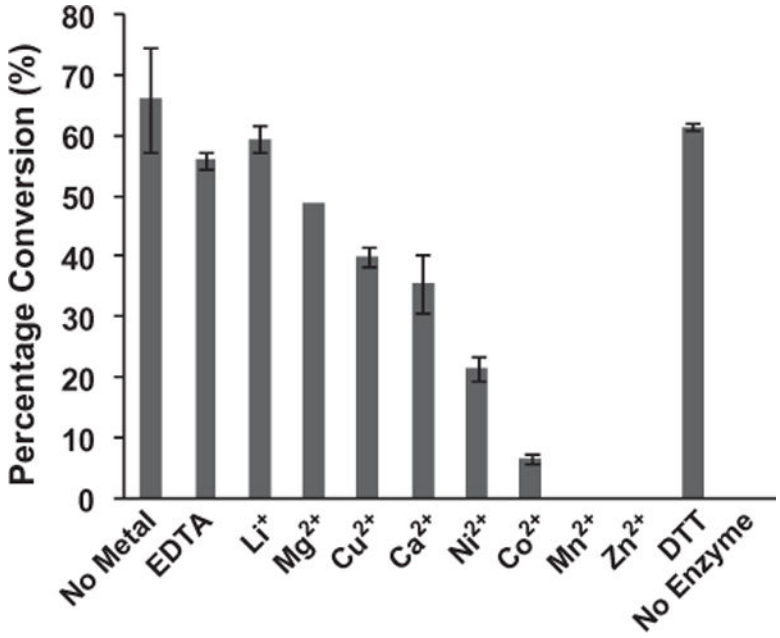


Fig. 4.

Effects of metal ions (10 mM), EDTA (10 mM), and DTT (1 mM)on theUDP-ManNAc 2-epimerase activity of NmSacA.

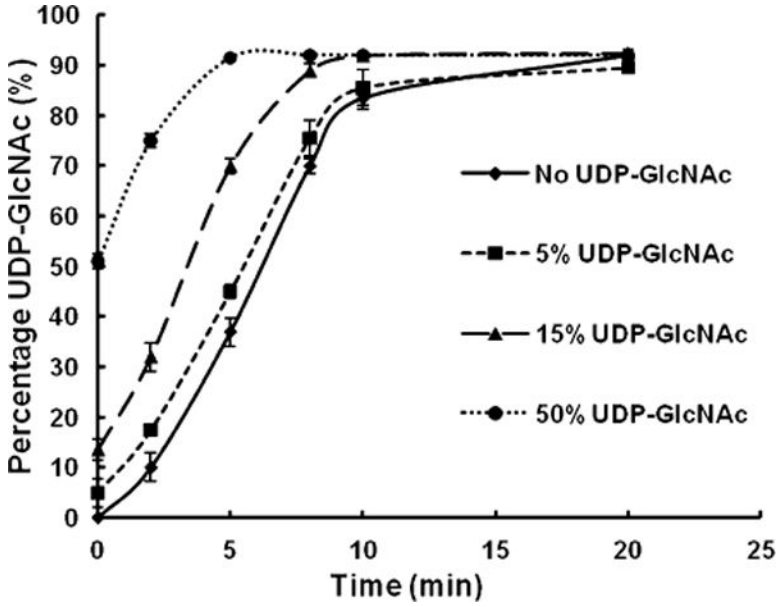


Fig. 5.

Time course comparison for the UDP-ManNAc 2-epimerization activity of NmSacA-His_6 in the absence and the presence of different concentrations of UDP-GlcNAc. For each reaction, data points are connected with a smoothed line for illustrative purpose only.

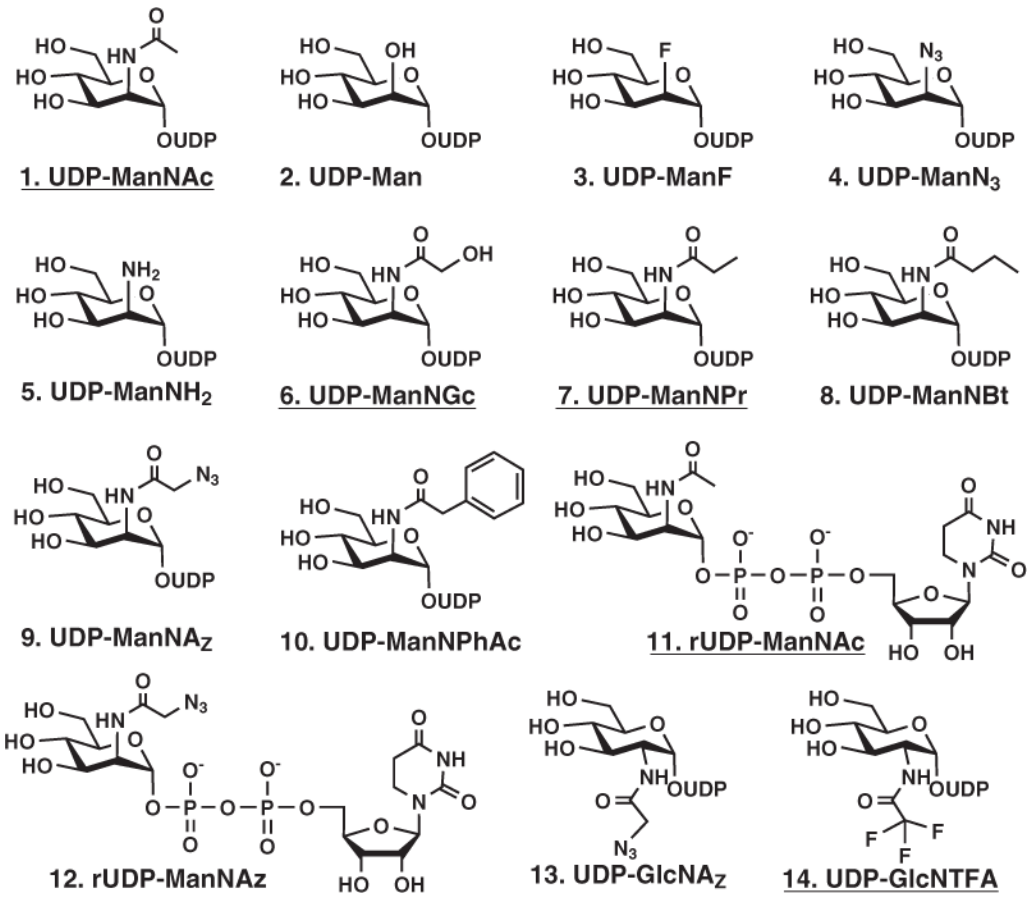


Fig. 6.

Structures of the compounds tested for the substrate specificity of NmSacA-His_6. The structures that can be tolerated by the enzyme are underlined.

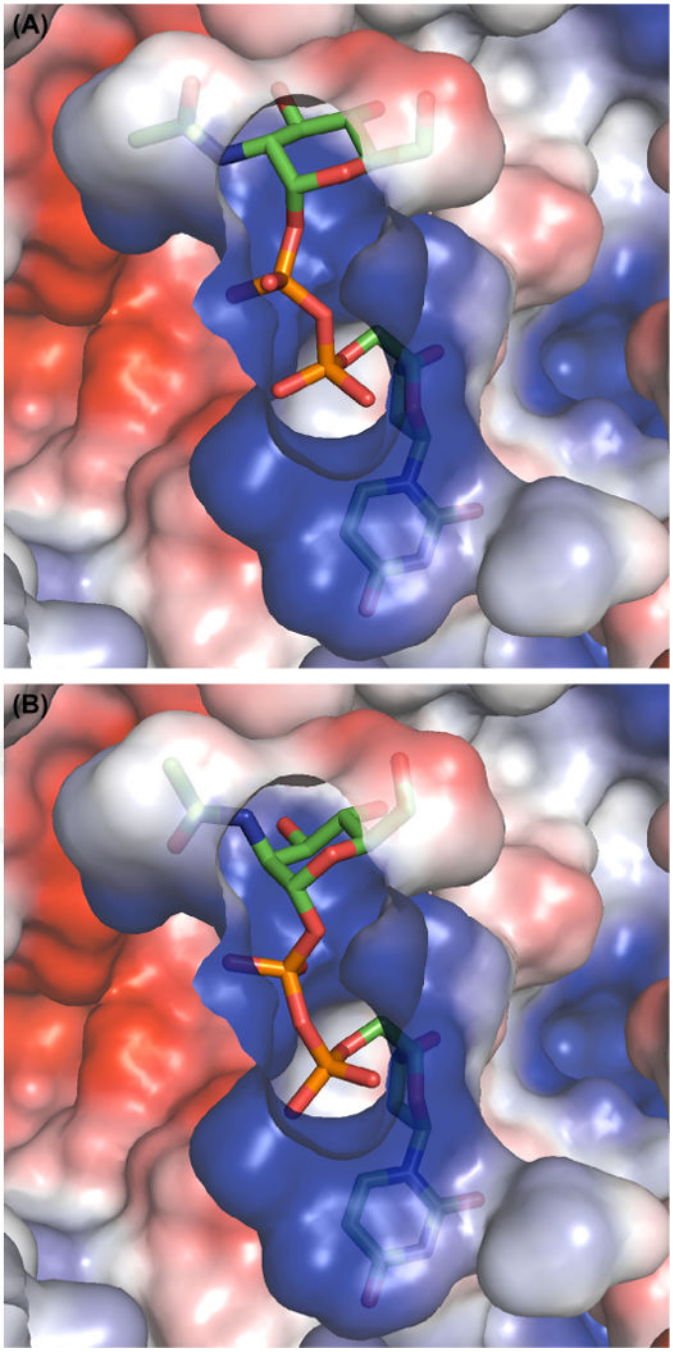
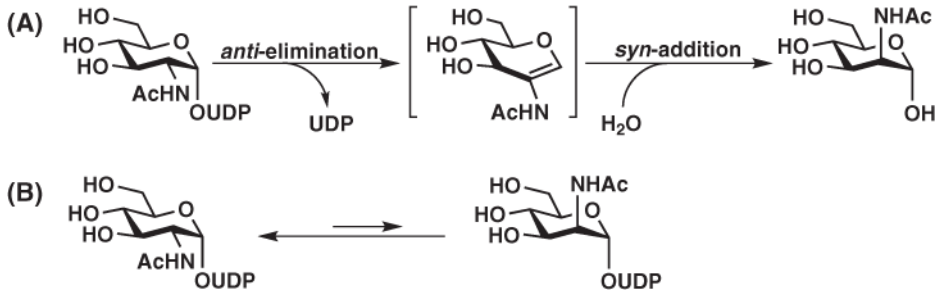


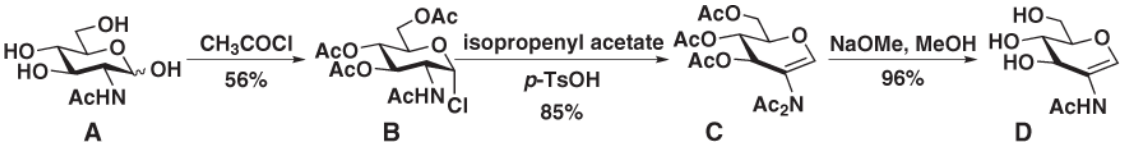
Fig. 7.

Modeled NmSacA active site with docked UDP-GlcNAc (A) or UDP-ManNAc (B) explaining the limited tolerance of NmSacA toward N-acyl group substitution in the substrate. The protein surface is colored according to the electrostatic potential. Positive potentials are shown in blue, negative potentials are in red, and neutral are in white. The sugar nucleotide substrates are shown in a stick model (carbon, green; oxygen, red; phosphorus, orange; nitrogen, blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



Scheme 1.

Comparisons of the reactions catalyzed by the epimerase domain of the mammalian hydrolyzing UDP-GlcNAc 2-epimerases/ManNAc kinases or bacterial hydrolyzing UDP-GlcNAc 2-epimerases (A) and bacterial non-hydrolyzing UDP-GlcNAc 2-epimerases (B).



Scheme 2.

Synthesis of 2-acetamidoglucal from GlcNAc.

Table 1

Apparent kinetics parameters of NmSacA-His_6 in comparison to *Escherichia coli* RffE

	Substrates	k_{cat} (s^{-1})	K_M (mM)[3]	k_{cat}/K_M (s^{-1} mM^{-1})
NmSacA-His_6	UDP-GlcNAc	31 ± 2	3.6 ± 0.5	8.6
	UDP-ManNAc 1	52 ± 5	5.2 ± 1.1	10
	UDP-ManNGc 6	4.2 ± 0.2	20 ± 1.9	0.21
	UDP-ManNPr 7	7.8 ± 0.3	51 ± 5.0	0.15
	rUDP-ManNAc 11	6.7 ± 0.5	63 ± 7.0	0.11
RffE	UDP-GlcNAc[42]	7.1 ± 0.3	0.6 ± 0.1	14.2