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Total Biosynthesis of Legionaminic Acid, a Bacterial Sialic Acid Analogue

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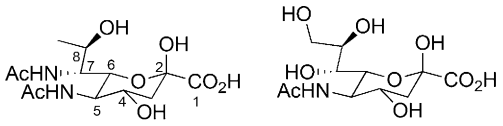
Abstract: *Legionaminic acid, Leg5,7Ac$_2$, a nonulosonic acid like 5-acetamido neuraminic acid (Neu5Ac, sialic acid), is found in cell surface glycoconjugates of bacteria including the pathogens Campylobacter jejuni, Acinetobacter baumanii and Legionella pneumophila. The presence of Leg5,7Ac$_2$ has been correlated with virulence in humans by mechanisms that likely involve subversion of the host's immune system or interactions with host cell surfaces due to its similarity to Neu5Ac. Investigation into its role in bacterial physiology and pathogenicity is limited as there are no effective sources of it. Herein, we construct a de novo Leg5,7Ac$_2$ biosynthetic pathway by combining multiple metabolic modules from three different microbial sources (Saccharomyces cerevisiae, C. jejuni, and L. pneumophila). Over-expression of this de novo pathway in Escherichia coli that has been engineered to lack two native catabolic pathways, enables significant quantities of Leg5,7Ac$_2$ ($\approx$120 mg L^{-1} of culture broth) to be produced. Pure Leg5,7Ac$_2$ could be isolated and converted into CMP-activated sugar for biochemical applications and a phenyl thioglycoside for chemical synthesis applications. This first total biosynthesis provides an essential source of Leg5,7Ac$_2$ enabling study of its role in prokaryotic and eukaryotic glycobiology.*

The nine-carbon alpha keto acid sugar 5-acetamido neuraminic acid (**2**, Neu5Ac, Figure 1) is a member of the nonulosonic acid family of sugars, and plays a major role in prokaryotic and eukaryotic glycobiology. Neu5Ac, along with its approximately 50 derivatives, is commonly referred to as sialic acid. In eukaryotes, Neu5Ac is abundant on cell surface glycoconjugates and is involved in a wide array of processes such as cell-cell interactions and immune response.[1] The amounts and types of sialic acids on eukaryotic cell surfaces are regulated spatially and temporally by biosynthetic and catabolic enzymes that are responsive to intracellular *N*-acetylglucosamine levels and to numerous extracellular sialic acid binding proteins such as the SigLec family of proteins.[2] Many human diseases are associated with alterations in Neu5Ac homeostasis, especially diseases associated with immune dysfunction like cancer.[3a] In addition, many bacteria and viruses rely on binding host cell-surface sialic acids as part of their infectious life cycle. Some viruses, such as influenza A, require neuraminidase to cleave these host cell-surface sialic acids and release the virus. Selective inhibition of influenza neuraminidase by zanamivir or oseltamivir provides one of the few pharmacological treatment options for combating an influenza pandemic.[3b] In addition to Neu5Ac, many bacteria have pathways to produce and use unique nonulosonic acid analogues, notably legionaminic acid (**1**, Leg5,7Ac$_2$,[4] Figure 1) and pseudaminic acid.[5]



1, Legionaminic Acid (Leg5,7Ac$_2$) **2**, 5-acetamido neuraminic acid (Neu5Ac)

Figure 1. Comparison of sialic acid (Neu5Ac) and legionaminic acid (Leg5,7Ac$_2$) structures.

Legionaminic acid was identified in 1994 from the lipopolysaccharide of *Legionella pneumophila*, the bacteria responsible for Legionnaires' disease.[6] While structurally homologous to Neu5Ac, the role of legionaminic acid in bacterial physiology and its impact on host–pathogen interaction is largely unknown.[7] Studies examining the biology of Leg5,7Ac$_2$ have been critically hampered by the lack of availability of this sugar.

While Leg5,7Ac$_2$ has been produced by total synthesis,[8,9] the synthetic routes are highly demanding and low yielding. A recent total synthesis of Leg5,7Ac$_2$ enabled the immobilization of the 2-thioethyl glycoside onto maleimide coated slides. Probing these slides with human blood sera demonstrated the presence of antibodies that recognize this bacterial specific sugar presumably due to previous bacterial infections.[9] Despite this advance, the low yield and technical challenge of this route limit its utility for studying the glycobiology of Leg5,7Ac$_2$.

In 2009, the biosynthetic pathway for Leg5,7Ac$_2$ in *Campylobacter jejuni*[10] was elucidated. Unexpectedly this pathway used GDP-GlcNAc (**6**, Scheme 1) as the key building block, unlike related nonulosonic acid pathways,

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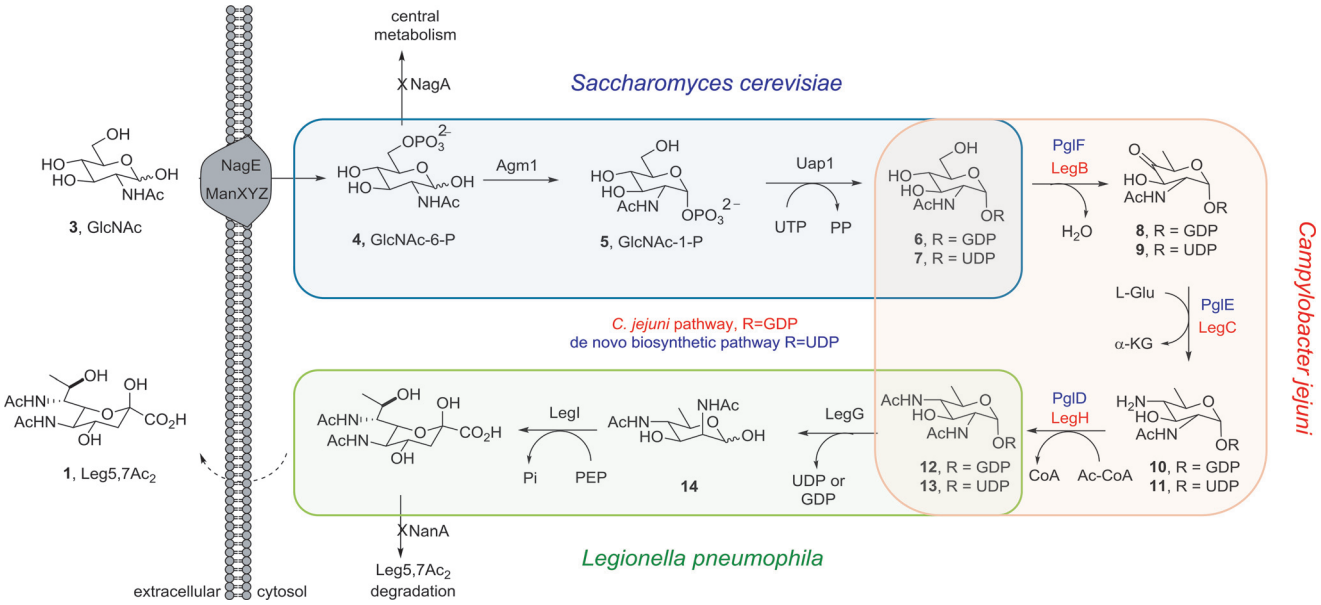


Scheme 1. De novo biosynthetic pathway of Leg5,7Ac$_2$ production in *E. coli*. Enzymes listed in blue are from the engineered UDP-linked pathway and those in red from the native *C. jejuni* GDP-linked biosynthetic pathway.

which use UDP-GlcNAc (**7**). The Leg5,7Ac$_2$ pathway starts with the NAD$^+$-dependent dehydratase LegB that eliminates water from C4,6 of GDP-GlcNAc (**6**) generating the 4-keto intermediate, GDP-2-acetamido-2,6-dideoxy-α-D-*xylo*-hexos-4-ulose (**8**). LegC, a PLP-dependent aminotransferase, catalyzes the transfer of the amino group from L-glutamate to the 4-keto intermediate producing the amino sugar GDP-4-amino-4,6-dideoxy-α-D-GlcNAc (**10**).[11] Acetylation by the acetyltransferase LegH produces GDP-2,4-diacetamido-2,4,6-trideoxy-α-D-glucopyranose (**12**, GDP-diNAcBac),[12] which is converted into 2,4-diacetamido-2,4,6-trideoxy-D-mannopyranose (**14**) by LegG, a hydrolyzing 2-epimerase. Finally, the synthase LegI condenses this sugar with pyruvate to produce Leg5,7Ac$_2$ (**1**). While this native pathway provides a route to produce Leg5,7Ac$_2$, the rare GDP-linked intermediates suggested that it may be challenging to overproduce significant quantities of **1**.

Previously we had produced multiple grams per litre of the biosynthetically less complex Neu5Ac from UDP-GlcNAc in *Escherichia coli*.[13] We thus hypothesized it would be possible to produce significant quantities of Leg5,7Ac$_2$ in *E. coli* by creating a de novo pathway relying on UDP-GlcNAc rather than GDP-GlcNAc. This required accessing UDP-2,4-diacetamido-2,4,6-trideoxy-α-D-glucopyranose (UDP-diNAcBac, **13**), a known metabolite from the N-linked protein glycosylation pathway (Pgl) in *C. jejuni*.[14] This pathway converts UDP-GlcNAc (**7**) to UDP-diNAcBac (**13**) which is converted to membrane-bound undecaprenyl diphosphate-diNAcBac and serves as the core for subsequent glycan assembly and transfer to Asn residues on proteins.[15]

The design of our de novo biosynthetic pathway thus relied on the dehydratase PglF, the aminotransferase PglE and the acetyltransferase PglD from the *C. jejuni* Pgl pathway to convert UDP-GlcNAc (**7**) into UDP-diNAcBac (**13**, Scheme 1). At this point **13** must intercept the Leg5,7Ac$_2$ pathway and be converted by LegG and LegI into the final product. While in vitro data with purified LegI and LegG from *L. pneumophila* suggested this was possible, the reported efficiencies were very low.[10,16] Herein we show that our de novo pathway is highly effective, producing Leg5,7Ac$_2$ with a titer of circa 120 mg L^{-1} of *E. coli* culture broth. This is the first in vivo production system for accessing this key carbohydrate and demonstrates the power of de novo biosynthetic pathway design.

To construct this system, the individual genes *pglFED* and *legGI* were codon optimized for expression in *E. coli* and cloned into pKH22, a low copy expression vector.[13] This installed a C-terminal or N-terminal hexa-His tag into each gene product and enabled all five genes to be combined into a single operon under the control of an inducible T7 promoter. This polycistronic plasmid, pMIH37 was introduced into the robust protein expression strain *E. coli* BL21 (λDE3). Western blot analysis of the cell lysate 24 h post induction with an anti-hexa-His antibody showed all five proteins were produced (Figure S1 in the Supporting Information). Surprisingly, no Leg5,7Ac$_2$ was detected in the culture broth (Figure 2). We hypothesized that this result was due to degradation of Leg5,7Ac$_2$ as it was being produced.

In the case of Neu5Ac production in *E. coli*, genes encoding the inner membrane sialic acid transporter NanT and the degrading sialic acid aldolase NanA needed to be inactivated to prevent Neu5Ac catabolism.[13] While there is no known Leg5,7Ac$_2$ specific transporter and degradation pathway in *E. coli*, we hypothesized that due to the homology between Neu5Ac and Leg5,7Ac$_2$, the Neu5Ac catabolism pathway could also act on Leg5,7Ac$_2$.[17] To evaluate this hypothesis, we investigated if NanA was capable of degrading Leg5,7Ac$_2$. Treatment of Leg5,7Ac$_2$ with recombinant purified NanA (Figure S2) showed consumption of Leg5,7Ac$_2$. Quantification of the pyruvate produced from the aldolase

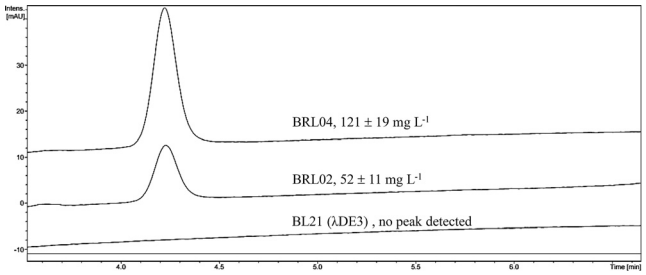


Figure 2. Leg5,7Ac$_2$ production in *E. coli* strains. Broth from BRL04, BRL02 and BL21(λDE3) cultures was derivatized with 1,2-diamino-4,5-dimethoxybenzene and analyzed by HPLC. The peak for derivatized Leg5,7Ac$_2$ was observed eluting at 4.20 minutes.

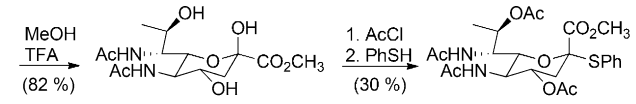
activity enabled determination of the kinetic parameters of NanA ($k_{cat} = 0.52 \pm 0.1$ min^{-1}, $K_M = 1.80 \pm 0.5$ mM, $k_{cat}/K_M = 4.8 \pm 2.2$ M^{-1}s^{-1}, Figure S3). To test if deletion of the Neu5Ac catabolism genes impacted Leg5,7Ac$_2$ production, *E. coli* BRL02, which is NanT$^-$ and NanA^{-13} was transformed with the synthetic *pglFED-LegGI* operon and cultivated in a shake flask. Consistent with our hypothesis, Leg5,7Ac$_2$ was detected at 52 ± 11 mg L^{-1} when this strain was fed 0.3% GlcNAc and 0.3% glycerol (Figure 2).

To further improve Leg5,7Ac$_2$ production, we focused on maximizing intracellular UDP-GlcNAc pools. Its feedstock, GlcNAc, is transported by the phosphotransferase system NagE and concomitantly phosphorylated to generate intracellular GlcNAc-6-phosphate (GlcNAc-6-P, **4**).[18] GlcNAc-6-P binds the transcriptional repressor, NagC, relieving repression of the *nag* operon and leads to conversion of GlcNAc-6-P into fructose-6-phosphate.[19] The first step in this pathway is NagA catalyzed deacetylation of the GlcNAc-6-P 2-acetamido group forming GlcN-6-P. Deletion of *nagA* was thus expected to eliminate catabolism of GlcNAc-6-P.[13d] To ensure robust conversion of intracellular GlcNAc-6-P into UDP-GlcNAc, **7**, the *Saccharomyces cerevisiae* enzymes Agm1, a GlcNAc-6-P to GlcNAc-1-P mutase, and Uap1, a GlcNAc-1-P uridyltransferase, were added to our de novo pathway.[20] These enzymes were particularly advantageous since they circumvented the highly regulated GlcN-6-P metabolic node that connects central metabolism to cell wall biosynthesis in *E. coli* and limits UDP-GlcNAc pools.[13d,21]

Both *agm1* and *uap1* were cloned onto an inducible T7-controlled expression vector (pBRL178). Co-transformation of the *nagA* deletion strain (BRL04)[13c] with pBRL178 and pMIH37 generated clones capable of producing high titers of Leg5,7Ac$_2$ (121 ± 19 mg L^{-1}, Figure 2). Leg5,7Ac$_2$ could be readily isolated from strains grown in 500 mL minimal media, induced with IPTG, and supplemented with antibiotics, 0.3% glycerol (*w*/*v*) and 0.3% GlcNAc (*w*/*v*) at 0 h, 18 h, 36 h and 54 h post induction. The culture broth was harvested at 168 h, lyophilized, triturated with methanol, and processed by ion exchange chromatography. Silica gel column chromatography then afforded nearly 50 mg of pure Leg5,7Ac$_2$ (as judged by ^{1}H NMR,[22] see SI) from 500 mL of production broth.

Many cell surface glycoconjugates containing Neu5Ac have been synthesized chemoenzymatically or chemically.[23] These compounds have been essential for understanding sialobiology. The analogous synthesis of Leg5,7Ac$_2$ containing glycoconjugates is thus a necessity for understanding its glycobiology.[24] To evaluate whether CMP-activated Leg5,7Ac$_2$, a substrate for glycosyltransferase (GT) mediated glycoconjugate synthesis, could be produced from our purified Leg5,7Ac$_2$, we treated our product with recombinant purified CMP-legionaminic acid synthetase, LegF, and CTP.[10] The expected CMP-Leg5,7Ac$_2$ was quantitatively produced (Figure S4), isolated, and characterized by 1D and 2D NMR (see SI for NMR). As some known sialyltransferases[25] tolerate CMP-Leg5,7Ac$_2$ as a donor (Table S1), this nucleotide activated sugar can be used for glycoconjugate synthesis.[26] In addition it will prove indispensible in efforts to identify the first legionaminic acid specific GTs, such as the candidate GTs from the lipopolysaccharide (LPS) pathway in *Acinetobacter baumannii* and flagellar glycosylation in *C. jejuni*.[27] Furthermore, CMP-Leg5,7Ac$_2$ has proven to be a promising antibiotic for the treatment of *Neisseria gonorrhoeae* infections in a mouse model.[28] Presumably Leg5,7Ac$_2$ can replace native Neu5Ac during *N. gonorrhoeae* LPS biosynthesis, leading to the bacteria becoming sensitive to serum factors and being eliminated from the host.

To obtain activated Leg5,7Ac$_2$ for chemical glycosylation, we generated the thioglycoside, **16** (Scheme 2). Leg5,7Ac$_2$ (**1**) was converted into its methyl ester **15** and purified by silica gel chromatography. Like Neu5Ac, **15** is predominantly its β-anomer in solution.[29] The protected, activated α-phenylthioglycoside **16** was generated via the β-chloride. Thus, our system enables the synthesis of a key intermediate, which will be essential for the development of efficient chemical glycosylation protocols.[30]



Scheme 2. Synthesis of thiophenol derivative **16** of Leg5,7Ac$_2$.

In summary, we have developed the first total biosynthesis of Leg5,7Ac$_2$. Our *E. coli* system relies on a de novo biosynthetic pathway created from three different metabolic modules from three different organisms. We show that manipulations of *E. coli* catabolic pathways capable of degrading Leg5,7Ac$_2$ and its biosynthetic precursor GlcNAc-6-P are essential for increasing titers of Leg5,7Ac$_2$. Our system now provides for the first time access to sufficient purified Leg5,7Ac$_2$ to begin elucidating its biological role. We have shown that Leg5,7Ac$_2$ can be used to enzymatically synthesize CMP-Leg5,7Ac$_2$, which is being tested as a novel antibiotic against *N. gonorrhoeae* and affords a route to numerous biologically relevant glycoconjugates. Leg5,7Ac$_2$ can also be chemically activated as the thioglycoside for further synthetic work. The presence of antibodies to Leg5,7Ac$_2$ in human serum[9] underscores its biological relevance in human health and disease and our study provides the necessary tools to begin understanding its function.

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