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Enzymatic synthesis of *N*-formylated sialosides *via* a five-enzyme cascade†

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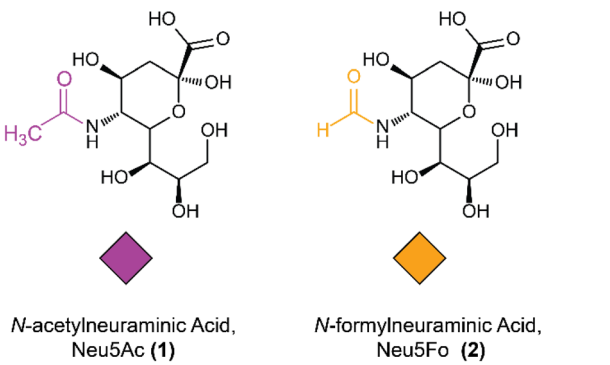
Here we report an enzymatic approach to synthesize *N*-formylneuraminic acid (Neu5Fo) containing sialosides, through a five-enzyme cascade. This method stands as an alternative to traditional chemical syntheses, aiming for precision and efficiency in generating sialosides with a tailored *N*-formyl group generated directly from formic acid. The newly synthesized Neu5Fo was characterized using various NMR techniques revealing a conformational equilibrium at the amide bond of the formyl group in slow exchange on the NMR time scale with a *trans* : *cis* ratio of ~2 : 1. This work not only suggests potential for exploring the biological roles of sialosides but also points to the possibility of developing novel therapeutic agents.

Introduction

Sialic acids are a distinct group of nine-carbon sugars prominently displayed on cell surface glycan chains, playing pivotal roles in cellular interactions, signaling pathways, and immune system modulation.[1–4] *N*-Acetylneuraminic acid (Neu5Ac) is the most common, and in humans the only sialic acid present.[5] The functional diversity of sialic acids is greatly expanded by modifications such as acetylation or sulfation at the C4 or C7–C9 positions (Fig. 1, left panel). These modifications can contribute to the evasion of sialidase activity from pathogens and modulate the recognition by sialic acid-binding proteins. They can markedly reduce the recognition of sialic acids by influenza A virus hemagglutinin, contributing to host resistance.[6] By substituting the *N*-acetyl group for an *N*-glycolyl group at the *N*-acyl position, as seen in *N*-glycolylneuraminic acid (Neu5Gc). This structure cannot be biosynthesized in humans due to a genetic deletion, but it can be metabolically incorporated into human tissues through dietary consumption of red meat and dairy products.[7] These modifications can subtly or significantly alter the biological interactions and stability of these sialosides.[8]

Glycoscience has long been focused on the synthesis of sialic acid derivatives due to their significant role in biological processes. Sialic acids are decorating glycan chains on cell surfaces and play a pivotal role in molecular recognition events that underpin many physiological and pathological processes.[9] Chemical synthesis of sialosides, particularly those with *N*-acyl modifications, has traditionally faced challenges. Typical chemical methods involve multistep processes with protection and deprotection stages, harsh conditions, and often yield suboptimal quantities. These methods underscore the need for more sustainable and efficient synthesis approaches for sialic acid derivatives.[10–12]

In this study, the synthesis of neuraminic acid bearing an *N*-formyl group (Neu5Fo, Fig. 1, right panel)[13] is described using an enzymatic method. *N*-Formylated sugars have been described to be present in Gram-negative bacteria,[14–17] playing crucial roles in the virulence and evasion of antibacterial defences.[18] *N*-Formylated sugars of pathogens like *Brucella*



N-acetylneuraminic Acid, Neu5Ac **(1)**

N-formylneuraminic Acid, Neu5Fo **(2)**

Fig. 1 Structure of Neu5Ac (**1**) and Neu5Fo (**2**). The coloured diamond-shaped icons represent (**1**) and (**2**) throughout the manuscript and are based on the Symbol Nomenclature for Glycans (SNFG).[22]

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abortus and *Campylobacter jejuni* are synthesized by *N*-formyltransferases that utilize 10-formyltetrahydrofolate as activated donor substrate for the modification of amino sugars in lipopolysaccharides.[19–21]

Our method utilizes direct enzymatic formylation of glucosamine using formic acid circumventing the need for an activated formyl donor like 10-formyltetrahydrofolate. It employs a fully biocatalytic synthesis route consisting of a five-enzyme cascade, which features the use of *Cyclobacterium marinum* chitobiose deacetylase (CmCDA), previously reported by our team for its ability to catalyze the *N*-acylation of glucosamine from unactivated carboxylic acids.[23] Later, this technique has been refined to map the *N*-acetylation pattern chito-oligomers, by enzymatically transferring unactivated azidoacetic acid, and then utilizing click chemistry, which exposed specific *N*-acetylation patterns of various bacterial chitobiose deacetylases.[24]

To facilitate sample purification, the *N*-formyl sialoside was synthesized using GalX (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) as an acceptor substrate. The enzymatic synthesis of the GalX sialoside was performed in a streamlined fashion using a one-pot cascade that integrates five distinct enzymatic steps (Fig. 2). Initially, the chitobiose deacetylase CmCDA catalyzes the *N*-formylation of glucosamine to yield *N*-formylglucosamine. This is followed by the action of GlcNAc 2 Epimerase, which epimerizes the product to generate the corresponding mannosamine derivative. Subsequently, Sialic Acid Aldolase facilitates an aldol reaction that converts this intermediate into an *N*-formylneuraminic acid derivative. CMP Sialic Acid Synthase is employed for the synthesis of cytidine-5′-monophosphate-*N*-formylneuraminic acid. This activated nucleotide sugar is then used as a donor substrate for a Sialyltransferase-catalyzed sialylation of GalX, resulting in the final Neu5FoGalX sialoside. This method provides a more streamlined and potentially eco-friendlier pathway to sialoside synthesis, circumventing the need for activated intermediates and reducing the number of steps typically required.

The synthesis of Neu5Fo sialosides introduces a valuable entity into the chemoenzymatic toolkit, offering perspectives on probing the functional roles of sialic acids. Given the formyl distinct chemical properties of the formyl group, compared to the common acetyl group, could help to modulate sialosides' interactions with proteins like Siglecs and selectins, and influence pathogen recognition. These aspects carry implications for novel therapeutic agent development, including anti-inflammatory and antimicrobial drugs.[25,26] Our biocatalytic synthesis strategy highlights the potential of enzymatic cascades for crafting sialosides and may spur further research into the myriad functions of sialic acids in biological systems.

Results and discussion

The conventional synthesis of C5-substituted sialosides typically involves chemical routes that are often hampered by low yields, lack of specificity, and the need for protective group strategies.[27–29] These limitations have propelled the search for alternative synthesis approaches, especially those that can replicate the enzymatic pathways found in nature. Moving away from conventional practices that depend on the chemical *N*-acylation of glucosamine or mannosamine,[30–33] our research introduces a completely enzymatic approach to sialoside syn-

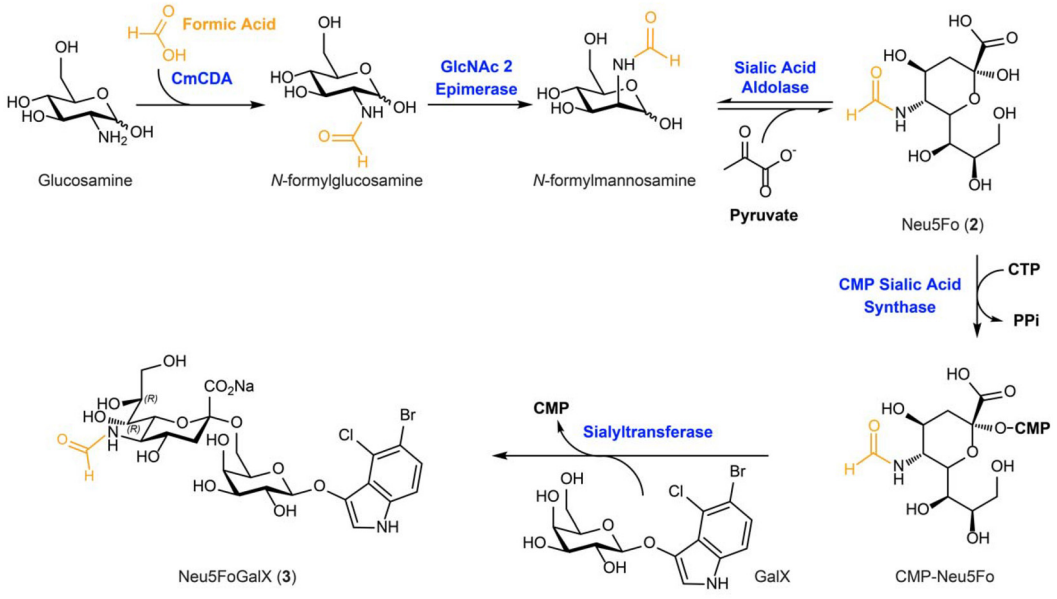

Fig. 2 Enzymatic conversion sequence showing the one-pot synthesis of *N*-formylneuraminic acid (Neu5Fo, **2**) and its subsequent incorporation into Neu5FoGalX sialoside (**3**), *via* a five-step enzymatic cascade. This process starts with CmCDA-catalyzed *N*-formylation of glucosamine, followed by GlcNAc 2-epimerization, aldol reaction mediated Neu5Fo formation, CMP-Neu5Fo synthesis, and culminates with α2,6-sialyltransferase-driven conjugation to GalX.

thesis, utilizing the efficacy of a five-enzyme cascade. This approach circumvents the need for protective groups and harsh chemical conditions, offering a greener and potentially more scalable alternative for the synthesis of sialoside containing non-natural functionalities. The cornerstone of this strategy is the utilization of chitobiose deacetylase, CmCDA, which facilitates the direct *N*-acylation of glucosamine with unprotected carboxylic acids, previously shown for acetic, propionic, butyric, hexanoic, and azidoacetic acids, thereby opening avenues for the synthesis of a diverse array of sialoside analogs.[23] For this work we selected formic acid, the simplest carboxylic acid, as the acyl donor to synthesize a Neu5Fo-bearing sialoside. Eighteen hours of incubating the one-pot reaction system was enough for the synthesis of the Neu5FoGalX sialoside from the starting materials GalX, glucosamine, pyruvate, CTP and formic acid.

The ^{1}H and ^{13}C NMR chemical shifts of Neu5FoGalX (Table 1) were assigned by 1D and 2D NMR experiments,[34,35] which for the indolyl aglycone were aided by noting that in benzene $^{2}J_{CH}$ = 1.0 Hz whereas $^{3}J_{CH}$ = 7.4 Hz, *i.e.*, in a ^{1}H,^{13}C-HMBC NMR spectrum three-bond heteronuclear correlations should readily be observed in the aglycone but two-bond correlations should be absent or of low intensity. Interestingly, the population at the amide bond of the *N*-formyl group in Neu5FoGalX showed an equilibrium between *trans* and *cis* conformations of ~2 : 1, evident from the ^{13}C NMR spectrum (Fig. 3), similar to conformational equilibria observed for other *N*-formylated sugar residues.[36–40] Subsequently the ^{1}H NMR spectral refinement was performed by spin-simulation.

By replacing formic acid with acetic acid, we also synthesized the sialoside Neu5AcGalX using the same enzymatic strategy. The products were analyzed using High-Performance Liquid Chromatography coupled with Electrospray Ionization Mass Spectrometry (HPLC-ESI-MS, ESI Fig. S3 and 4†) and Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-ToF-MS, Fig. 4). The elemental composition of Neu5FoGalX was further verified through high-resolution (ESI-MS) in negative ion mode, demonstrating the anticipated isotope distribution pattern for the bromide and chloride atoms within the GalX moiety (ESI Table S1†).

Importantly, the enzyme mixture was able to fully sialylate GalX to Neu5FoGalX, whereas the yield of Neu5AcGalX was around 60%. These findings underscore the efficiency of the enzymatic cascade when formic acid is employed as the acyl donor, suggesting that the steric and electronic properties of the formyl group are particularly conducive to this synthetic pathway.

The successful synthesis of Neu5FoGalX in high yields paves the way for the exploration of its biological roles. The *N*-formyl group is less commonly found in nature compared to the *N*-acetyl group, and its introduction into sialic acid could influence a myriad of biological interactions, including cell–cell communication, immune response modulation, and pathogen–host interactions. For instance, the altered *N*-acyl group could affect the binding affinity of sialic acids to sialic acid-binding immunoglobulin-type lectins, a family of receptors that are key regulators in the immune system. The *N*-formyl modification may also impact the recognition and internalization of sialosides by sialidases and sialyltransferases, enzymes that are crucial in sialoglycoconjugate metabolism and thus in cellular signaling and communication.

Table 1 ^{1}H and ^{13}C NMR chemical shifts of Neu5FoGalX at 309 K in D_2O referenced to TSP (δ_H 0.00) and dioxane in D_2O (δ_C 67.40). ^{1}H NMR chemical shifts were refined by NMR spin-simulation (Cosmic Truth). The two conformations of the formyl (Fo) group are referred to by *trans* (64%) and *cis* (36%) with respect to the torsion angle H–N–C=O of the amide bond. See ESI for information on atom numbering†

Residue	Atom #	^{1}H (*trans*)	^{13}C (*trans*)	^{1}H (*cis*)	^{13}C (*cis*)
α-Neu5Fo-(2 → 6)	**1**		174.25		174.20
	2		101.17		101.14
	3a	1.67	40.93	1.67	40.82
	3b	2.77		2.78	
	4	3.74	68.95	3.69	68.61
	5	3.92	51.42	3.41	55.96
	6	3.77	73.10	3.80	72.79
	7	3.61	68.89	3.67	68.67
	8	3.88	72.55	3.89	72.70
	9a	3.62	63.96	3.57	63.27
	9b	3.82		3.65	
	10	8.21	165.81	8.00	168.60
→ 6)-β-D-Gal-(1 → 3)	**1**	4.83	104.51	4.84	104.53
	2	3.86	71.57	3.85	71.54
	3	3.76	73.48	3.76	73.48
	4	4.02	69.33	4.01	69.33
	5	3.86	74.64	3.86	74.64
	6a	3.70	63.87	3.72	63.87
	6b	4.00		4.01	
5-Br-4-Cl-3-indolyl (X)	**2**	7.33	115.12	7.31	114.76
	3		136.21		136.36
	4		123.87		123.89
	5		113.23		113.23
	6	7.44	127.12	7.44	127.15
	7	7.30	113.00	7.30	113.00
	8		134.10		134.10
	9		118.23		118.20

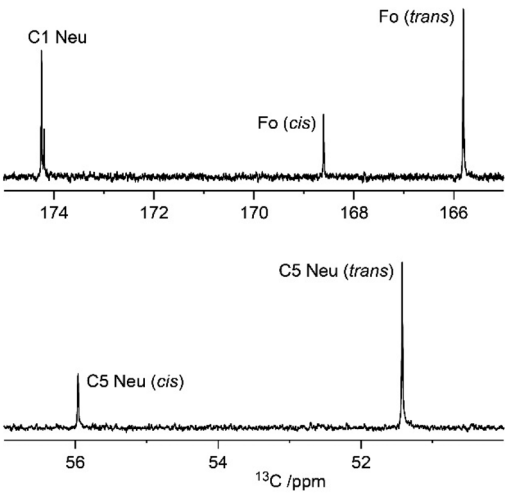

Fig. 3 Selected ^{13}C NMR spectral regions of Neu5FoGalX (**3**).

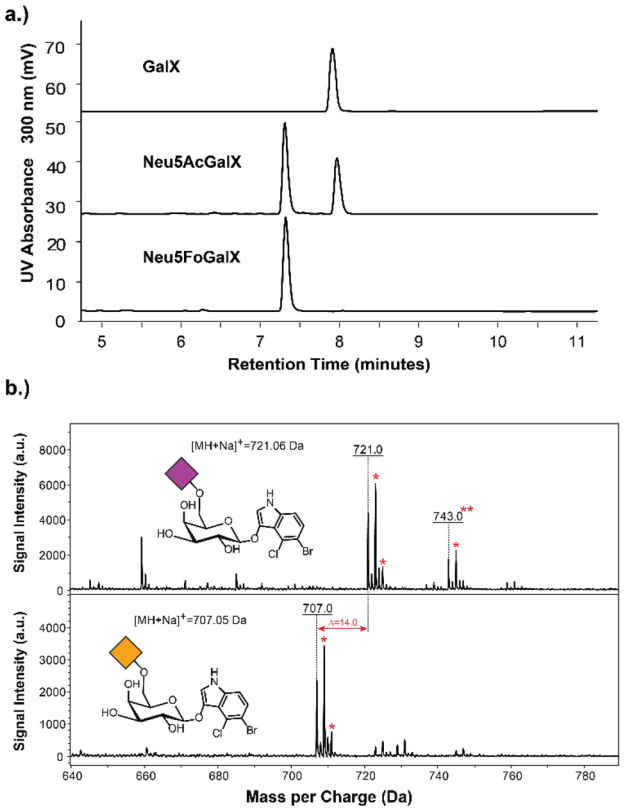

Fig. 4 HPLC chromatograms and MALDI-ToF-MS spectra of Neu5AcGalX and Neu5FoGalX sialosides. The UV absorbance was monitored at 300 nm.

We also assessed the stability of GalX sialosides in acidic environments. Comparing the controlled hydrolysis rates of Neu5Fo and Neu5Ac sialosides at various concentrations (0.05 mM and 0.5 mM) is beneficial when using these compounds as internal standards for precise quantification—an approach that has been previously demonstrated with different types of meats.[31] Notably, within 150 minutes, all GalX sialosides were thoroughly broken down, independent of their concentrations (Fig. 5).

To showcase the practical applicability of Neu5FoGalX as an internal standard, we examined the Neu5Ac content in poultry

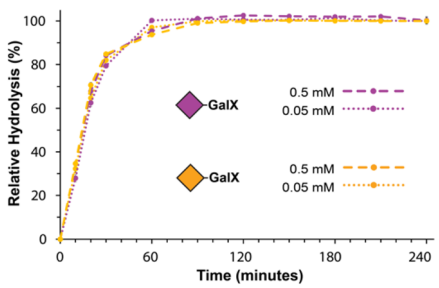

Fig. 5 Controlled hydrolysis time course of Neu5AcGalX and Neu5FoGalX sialosides at various sample concentrations. Standard errors (<2%) was calculated from sample triplicates.

Table 2 Neu5Ac content in egg white and egg yolk powder of different species. Standard errors were calculated from quadruplicates

Poultry type	Neu5Ac in egg white powder (mg g^{-1})	Neu5Ac in egg yolk powder (mg g^{-1})
Chicken	2.8 ± 0.2	3.3 ± 0.3
Quail	2.2 ± 0.7	2.7 ± 0.4
Duck	1.3 ± 0.1	2.1 ± 0.1

egg powders (Table 2). Specifically, chicken egg whites and yolks exhibited significantly higher Neu5Ac levels (2.8 mg g^{-1} and 3.3 mg g^{-1}, respectively) when compared to those from quail and duck eggs. These observations are consistent with previously reported Neu5Ac contents in these egg types.[41] This utilization of Neu5FoGalX not only underscores its value in biochemical analyses but also highlights the natural abundance and dietary relevance of sialic acids.

Materials and methods

Chemicals and reagents

5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (GalX), D-glucosamine hydrochloride, and cytidine-5′-triphosphate disodium salt (CTP) were purchased from Aladdin (Shanghai, China). *N*-Acetylneuraminic acid (Neu5Ac) was obtained from Shanghai DEMO Medical Technology Co., Ltd. *Escherichia coli* β-galactosidase was sourced from Qlyco Ltd (Nanjing, China). Formic acid, acetic acid, propionic acid, sodium pyruvate, isopropyl 1-thio-β-D-galactopyranoside (IPTG), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (Shanghai, China). Ni-NTA agarose was supplied by Bio-Rad Laboratories, Inc. (Shanghai, China). All other chemicals and reagents were acquired from local commercial suppliers and were of the highest purity available.

Expression and purification of enzymes

Five distinct enzymes were recombinantly expressed in *E. coli*. These included *Cyclobacterium marinum* chitobiose deacetylase (CmCDA, WP_014022743.1), *Pedobacter heparinus* GlcNAc 2-Epimerase (WP_015809055.1), *Dyadobacter fermentans* Sialic Acid Aldolase (WP_015812487.1), CMP Sialic Acid Synthase from *Neisseria meningitidis* (AAB60780.1), and *Photobacterium damsela* α2,6-Sialyltransferase (BAA25316). *E. coli* BL21 (DE3) strains harboring plasmids pET30a-CmCDA, pET30a-PhGn2E, pET30a-DfAldolase, pET30a-NmCTT, and pET28a-PdST6 were cultured in 400 mL LB medium at 37 °C with agitation at 180 rpm. At an optical density (OD600) of 0.5, protein expression was induced by adding IPTG to a final concentration of 0.1 mM, followed by incubation at 18 °C for 24 hours. Cells were harvested by centrifugation at 4000*g* for 20 minutes, resuspended in 10 mL of cell lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% v/v Triton X-100, and 1 mM PMSF) to prevent degradation by proteases, and lysed by sonication on ice for 20 minutes. The cell lysate was then centrifuged at 12 000*g* for 20 minutes, and the supernatant was collected and applied to a

Ni-NTA column at room temperature. The column was washed with wash buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) and eluted with elution buffer (500 mM imidazole, 50 mM Tris-HCl, 100 mM NaCl, pH 8.0). Fractions containing the target enzyme were monitored by UV absorbance at 280 nm, pooled, and stored at 4 °C for subsequent use. The purified enzymes were further concentrated to approximately 5 mg ml^{-1} using Sartorius Vivaspin 6 Centrifugal Concentrators with a molecular weight cut-off (MWCO) of 10 000 Da.

Synthesis of GalX sialosides

Neu5FoGalX and Neu5AcGalX were synthesized on a 50 mL scale. The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 36 mM GlcN, 20 mM pyruvate, 2 mM $MgCl_2$, 0.48 mM GalX, 9.6 mM CTP, and 216 mM formic acid or acetic acid (for the synthesis of Neu5FoGalX or Neu5AcGalX, respectively), along with 5 mU each of CmCDA, GlcNAc 2-Epimerase, Sialic Acid Aldolase, CMP Sialic Acid Synthase, and Sialyltransferase. The mixtures were incubated at 37 °C with shaking at 180 rpm for 18 hours. To monitor the production of GalX sialosides, 20 μL aliquots were sampled periodically and analyzed by HPLC. In the case of the Neu5AcGalX synthesis reaction, *E. coli* β-galactosidase (10 mU) was added to remove residual GalX by incubation at 37 °C for 30 minutes. The reaction was halted by heating at 95 °C for 30 minutes. Proteins were removed by centrifugation at 12 000*g* for 20 minutes, and the supernatant was freeze-dried for purification. The dried sample was re-dissolved in distilled water and purified using preparative HPLC equipped with a reversed-phase column (Cosmosil® 5C18-MS-II, 20 mm ID × 250 mm). The mobile phase consisted of 50 mM ammonium formate (pH 4.5) in water (solvent A) and acetonitrile (solvent B). The flow rate was set at 5 mL min^{-1}, and a linear gradient from 10% to 30% solvent B was applied over 40 minutes, followed by an increase to 90% solvent B over 1 minute, which was then maintained for 9 minutes (ESI Table S2†). The mobile phase was re-adjusted to obtain 10% of solvent B over 5 minutes and held for 20 minutes to re-equilibrate the column. Fractions containing Neu5FoGalX were pooled and lyophilized for further analysis.

HPLC-ESI-MS profiling of Neu5FoGalX

The analysis of the products was performed using a Shimadzu LCMS-8040 system equipped with an LC-30AD pump, a SIL-30AC autosampler, an SPD-20A UV detector, and an electrospray ionization (ESI) mass spectrometer. A 10 μL sample was separated using a reversed-phase C18 column (Phenomenex HyperClone® 5 μm C18, 250 × 4.60 mm). The mobile phase consisted of 50 mM ammonium formate (pH 4.5) in water (solvent A) and acetonitrile (solvent B). The flow rate was 0.8 mL min^{-1}, and a linear gradient from 10% to 60% solvent B was applied over 5 minutes. Solvent B was then increased to 90% over 1 minute and held for 1 minute, followed by a decrease to 10% over 1 minute and held for 6 minutes to re-equilibrate the column. Mass spectrometric analysis was conducted in both positive and negative ion modes with a scan range from 400 to 1000 Da. The data were processed using the LabSolutions software package (Shimadzu, Kyoto, Japan).

For analysis of the Neu5Ac contents of poultry egg powder samples, the whites and yolks of fresh chicken, duck and quail eggs were separated, then freeze-dried, ground into powder, and stored until analysis. For each assay, 10 mg of egg white powder or 5 mg of egg yolk powder was dissolved in 1 mL of 2 M acetic acid solution, followed by the addition of 10 μL of 0.05 mM Neu5FoGalX as the internal standard. The mixture was vortexed to ensure maximum dissolution.

MALDI-ToF and HR-ESI mass spectrometric analysis

The reaction mixture was diluted 50-fold with distilled water. A 1 μL aliquot of the diluted sample was subjected to MALDI-TOF-MS (matrix-assisted laser desorption ionization-time-of-flight MS) analysis. Spectra were acquired using a Bruker Autoflex Speed MALDI-ToF mass spectrometer with 2,5-dihydroxybenzoic acid as the matrix. The data were analyzed using Bruker FlexAnalysis software version 3.3. High-resolution ESI-MS was recorded on Neu5FoGalX in the negative mode on a MicrOTOF mass spectrometer (Bruker Daltonics) using a mixture of isopropanol and water.

NMR spectroscopy

The oligosaccharide Neu5FoGalXs was deuterium-exchanged by freeze-drying from D_2O and examined by NMR spectroscopy as a solution in 99.96% D_2O (0.55 mL) prepared in an NMR tube with an outer diameter of 5 mm. The NMR experiments were performed at 309 K on a Bruker Avance III 700 MHz spectrometer with a 5 mm TCI ($^1H/^{13}C/^{15}N$) Z-Gradient (53.0 G cm^{-1}) CryoProbe. Chemical shifts are reported in ppm using internal sodium 3-(trimethylsilyl)(2,2,3,3-d_4)propanoate (TSP, δ_H 0.00) for ^1H NMR and external 1,4-dioxane 10% in D_2O (δ_C 67.40) for ^{13}C NMR as references. 1D and 2D NMR experiments suitable for assignment of ^1H and ^{13}C resonances in carbohydrate compounds were used.[34,35] Processing of the acquired data was carried out using TopSpin 4.1.4 (Bruker). ^1H NMR spectral refinement was performed by spin-simulation[42,43] using a pre-release version of Cosmic Truth (CT; ct.nmrsolutions.io), a web-based, client/server software for automated and semi-automated spectrum analysis from NMR Solutions Ltd (Kuopio, Finland).

Acid hydrolysis of GalX Sialosides and HPLC analysis after fluorescence derivatization

Lyophilized GalX sialosides were dissolved in distilled water to a final concentration of 0.05 mM or 0.5 mM. A 10 μL aliquot of each sample was mixed with 1 mL of 2 M acetic acid and incubated at 80 °C for varying times up to 4 hours. The mixtures were then cooled to room temperature and centrifuged at 12 000*g* for 10 minutes. The top 500 μL of the supernatant was dried by vacuum centrifugation and re-dissolved in 100 μL of 0.1 M NaCl solution. The pH was adjusted to 7.0 using 1 M NaOH, followed by centrifugation at 12 000*g* for 10 minutes. A 50 μL aliquot of the supernatant was mixed with 10 μL of an *o*-phenylenediamine (OPD) solution (10 mg mL^{-1} OPD in

200 mM $NaHSO_3$) and derivatized at 80 °C for 40 minutes in the dark. After filtration, 35 μL of the sample was injected into a Shimadzu HPLC system equipped with an RF-20Axs fluorescence detector (Ex/Em = 373/448 nm) and separated using a reversed-phase column (Phenomenex HyperClone® 5 μm C18, 250 × 4.60 mm). The flow rate was set at 1 mL min^{-1}, with water as solvent A, acetonitrile as solvent B, and methanol as solvent C. A linear gradient of 5–12% solvents B and C was applied over the first ten minutes, followed by an increase to 40% solvents B and C over the next 2 minutes. Solvent B was then decreased to 5% over 1 minute and held for 5 minutes to re-equilibrate the column. Data were processed using the LabSolutions software package.

Determination of Neu5Ac content in poultry egg powder

For analyzing the Neu5Ac contents of poultry egg powder samples, the whites and yolks of fresh chicken, duck and quail eggs were separated, then freeze-dried, ground into powder, and stored until analysis. In preparation for each assay, 10 mg of egg white powder or 5 mg of egg yolk powder was reconstituted in 1 mL of 2 M acetic acid solution. To this solution, 10 μL of 0.05 mM Neu5FoGalX as added as the internal standard. The samples were thoroughly vortexed to ensure optimum dissolution of the powders within the acidic medium, and incubated at 80 °C for 2 hours. After cooling the mixtures to room temperature and centrifugation (12 000*g* for 10 minutes), the sample supernatants (top 500 μL) were subject to the OPD derivatization procedure and HPLC analysis as described in the preceding section.

Conclusions

In conclusion, this study has successfully demonstrated a novel, sustainable, and effective biocatalytic synthesis of *N*-formyl sialoside, namely Neu5FoGalX. By leveraging the specificity and mild conditions of enzyme-catalyzed processes, our research offers an alternative approach to traditional chemical synthesis methods for sialic acid derivatives. Importantly, the *N*-formyl group present in this newly synthesized sialoside introduces distinctive chemical properties that may also influence sialoside interaction with biologically significant proteins and potentially alter pathogen–host recognition. These results will therefore contribute to expanding the chemoenzymatic toolbox available for the exploration of the functional roles of sialic acids and underscore the potential of enzymatic synthesis in generating novel glycoconjugates for biotechnological and analytical applications.

Author contributions

Conceptualization: J. Voglmeir, L. Liu, G. Widmalm; funding acquisition: J. Voglmeir, L. Liu, G. Widmalm; investigation: Z. X. Hu, S. R. Li, Q. J. Xia, G. Widmalm; methodology: Z. X. Hu, S. R. Li, Q. J. Xia, J. Voglmeir, L. Liu, G. Widmalm; supervision: J. Voglmeir, L. Liu; visualization: J. Voglmeir; writing – original draft: T. Wang, J. Voglmeir, G. Widmalm; writing – review & editing: J. Voglmeir, L. Liu, G. Widmalm.

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

There are no conflicts to declare.

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References

1 C. Büll, T. J. Boltje, N. Balneger, S. M. Weischer, M. Wassink, J. J. van Gemst, V. R. Bloemendal, L. Boon, J. van der Vlag, T. Heise, M. H. den Brok and G. J. Adema, *Cancer Res.*, 2018, **78**, 3574–3588.
2 A. L. Lewis, X. Chen, R. L. Schnaar and A. Varki, Sialic Acids and Other Nonulosonic Acids, in *Essentials of Glycobiology*, ed. A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, D. Mohnen, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar and P. H. Seeberger, Cold Spring Harbor, NY, 4th edn, 2022, ch. 15.
3 A. E. Awofiranye, C. Dhar, P. He, A. Varki, M. A. G. Koffas and R. J. Linhardt, *Glycobiology*, 2022, **32**, 921–932.
4 A. Gonzalez-Gil, T. A. Li, J. Kim and R. L. Schnaar, *Mol. Aspects Med.*, 2023, **90**, 101110.
5 A. Varki, *J. Autoimmun.*, 2017, **83**, 134–142.
6 R. Schauer, *Curr. Opin. Struct. Biol.*, 2009, **19**, 507–514.
7 A. N. Samraj, O. M. T. Pearce, H. Läubli, A. N. Crittenden, A. K. Bergfeld, K. Banda, C. J. Gregg, A. E. Bingman, P. Secrest, S. L. Diaz, N. M. Varki and A. Varki, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 542–547.
8 S. A. Springer and P. Gagneux, *J. Biol. Chem.*, 2013, **288**, 6904–6911.
9 S. Ghosh, *Sialic Acids Sialoglycoconjugates in the Biology of Life, Health and Disease*, Elsevier Science, 1st edn, 2020.
10 Y. Norimura, D. Yamamoto and K. Makino, *Org. Biomol. Chem.*, 2017, **15**, 640–648.
11 X. Zhu and R. R. Schmidt, *Angew. Chem., Int. Ed.*, 2009, **48**, 1900–1934.
12 E. Rossing, J. F. A. Pijnenborg and T. J. Boltje, *Chem. Commun.*, 2022, **58**, 12139–12150.
13 R. Brossmer and E. Nebelin, *FEBS Lett.*, 1969, **4**, 335–336.

14 M. Zaretsky, E. Roine and J. Eichler, *Front. Microbiol.*, 2018, **9**, 2133.
15 L. Kandiba, O. Aitio, J. Helin, Z. Guan, P. Permi, D. H. Bamford, J. Eichler and E. Roine, *Mol. Microbiol.*, 2012, **84**, 578–593.
16 P. Castric, F. J. Cassels and R. W. Carlson, *J. Biol. Chem.*, 2001, **276**, 26479–26485.
17 M. Caroff, D. R. Bundle and M. B. Perry, *Eur. J. Biochem.*, 1984, **139**, 195–200.
18 B. Liu, Y. A. Knirel, L. Feng, A. V. Perepelov, S. N. Senchenkova, Q. Wang, P. R. Reeves and L. Wang, *FEMS Microbiol. Rev.*, 2008, **32**, 627–653.
19 J. B. Thoden, M.-F. Goneau, M. Gilbert and H. M. Holden, *Biochemistry*, 2013, **52**, 6114–6126.
20 R. A. C. Hughes and D. R. Cornblath, *Lancet*, 2005, **366**, 1653–1666.
21 S. D. Breazeale, A. A. Ribeiro, A. L. McClerren and C. R. H. Raetz, *J. Biol. Chem.*, 2005, **280**, 14154–14167.
22 S. Neelamegham, K. Aoki-Kinoshita, E. Bolton, M. Frank, F. Lisacek, T. Lütteke, N. O'Boyle, N. H. Packer, P. Stanley, P. Toukach, A. Varki and R. J. Woods, *Glycobiology*, 2019, **29**, 620–624.
23 P. Laborda, Y. M. Lyu, F. Parmeggiani, A. M. Lu, W. J. Wang, Y. Y. Huang, K. Huang, J. Guo, L. Liu, S. L. Flitsch and J. Voglmeir, *Angew. Chem., Int. Ed.*, 2020, **59**, 5308–5311.
24 M. Linhorst, J. Wattjes and B. M. Moerschbacher, *ACS Catal.*, 2021, **11**, 14456–14466.
25 B. A. H. Smith and C. R. Bertozzi, *Nat. Rev. Drug Discovery*, 2021, **20**, 217–243.
26 W. Li, J. B. McArthur and X. Chen, *Carbohydr. Res.*, 2019, **472**, 86–97.
27 N. M. Podvalnyy, N. N. Malysheva, M. V. Panova, A. I. Zinin, A. O. Chizhov, A. V. Orlova and L. O. Kononov, *Carbohydr. Res.*, 2017, **451**, 12–28.
28 M. Panza, S. G. Pistorio, K. J. Stine and A. V. Demchenko, *Chem. Rev.*, 2018, **118**, 8105–8150.
29 G. J. Boons and A. V. Demchenko, *Chem. Rev.*, 2000, **100**, 4539–4566.
30 H. Yu, H. A. Chokhawala, S. Huang and X. Chen, *Nat. Protoc.*, 2006, **1**, 2485–2492.
31 H. L. Yao, L. P. Conway, M. M. Wang, K. Huang, L. Liu and J. Voglmeir, *Glycoconjugate J.*, 2016, **33**, 219–226.
32 Z.-P. Cai, L. P. Conway, Y. Y. Huang, W. J. Wang, P. Laborda, T. Wang, A. M. Lu, H. L. Yao, K. Huang, S. L. Flitsch, L. Liu and J. Voglmeir, *Molecules*, 2019, **24**, 1368.
33 S.-Y. Wang, P. Laborda, A.-M. Lu, X.-C. Duan, H.-Y. Ma, L. Liu and J. Voglmeir, *Catalysts*, 2016, **6**, 212.
34 G. Widmalm, in *Comprehensive Glycoscience*, ed. J. Barchi Jr., Elsevier, Oxford, 2nd edn, 2021, pp. 340–373.
35 C. Fontana and G. Widmalm, *Chem. Rev.*, 2023, **123**, 1040–1102.
36 E. Martínez-Gómez, J. Ståhle, Y. Gil-Ramírez, A. Zúñiga-Ripa, M. Zaccheus, I. Moriyón, M. Iriarte, G. Widmalm and R. Conde-Álvarez, *Front. Microbiol.*, 2018, **9**, 1092.
37 O. Engström, H. Mobarak, J. Ståhle and G. Widmalm, *J. Phys. Chem. B*, 2017, **121**, 9487–9497.
38 M. V. Zaccheus, T. Ali, A. Cloeckaert, M. S. Zygmunt, A. Weintraub, M. Iriarte, I. Moriyón and G. Widmalm, *PLoS One*, 2013, **8**, e53941.
39 X. Hu, W. Zhang, I. Carmichael and A. S. Serianni, *J. Am. Chem. Soc.*, 2010, **132**, 4641–4652.
40 L. Kenne, P. Unger and T. Wehler, *J. Chem. Soc., Perkin Trans. 1*, 1988, 1183–1186.
41 M. M. Wang, Y. Y. Zhang, H. L. Yao, L. Liu and J. Voglmeir, *Sci. Technol. Food Ind.*, 2021, **42**, 28–41.
42 R. S. Phansalkar, C. Simmler, J. Bisson, S.-N. Chen, D. C. Lankin, J. B. McAlpine, M. Niemitz and G. F. Pauli, *J. Nat. Prod.*, 2017, **80**, 634–647.
43 G. F. Pauli, S.-N. Chen, D. C. Lankin, J. Bisson, R. J. Case, L. R. Chadwick, T. Gödecke, T. Inui, A. Krunic, B. U. Jaki, J. B. McAlpine, S. Mo, J. G. Napolitano, J. Orjala, J. Lehtivarjo, S.-P. Korhonen and M. Niemitz, *J. Nat. Prod.*, 2014, **77**, 1473–1487.