Bioconjugate Chemistry

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Communication

Structure–Activity Relationship of Fluorinated Sialic Acid Inhibitors for Bacterial Sialylation

Sam J. Moons,[¶] Emiel Rossing,[¶] Jurriaan J. A. Heming, Mathilde A. C. H. Janssen, Monique van Scherpenzeel, Dirk J. Lefeber, Marien I. de Jonge, Jeroen D. Langereis,* and Thomas J. Boltje*

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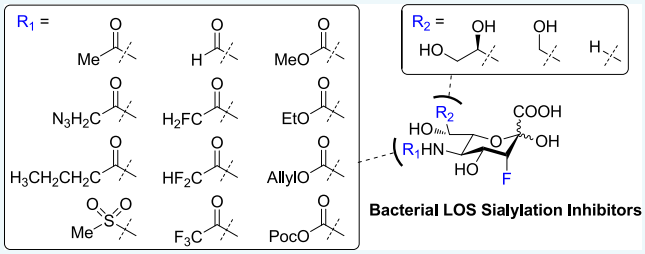
ABSTRACT: Bacterial pathogens such as Nontypeable *Haemophilus influenzae* (NTHi) can evade the immune system by taking up and presenting host-derived sialic acids. Herein, we report a detailed structure–activity relationship of sialic acid-based inhibitors that prevent the transfer of host sialic acids to NTHi. We report the synthesis and biological evaluation of C-5, C-8, and C-9 derivatives of the parent compound 3-fluorosialic acid (SiaNFAc). Small modifications are tolerated at the C-5 and C-9 positions, while the C-8 position does not allow for modification. These structure–activity relationships define the chemical space available to develop selective bacterial sialylation inhibitors.



Gram negative bacterium Nontypeable *Haemophilus influenzae* (NTHi) is a commensal organism in the upper respiratory tract microbiome, which can become an opportunistic pathogen in children and the elderly.[1,2] NTHi has evolved the ability to evade the immune system by expressing host-like molecular structures at its cell surface such as host-derived sialic acids.[3] The switch of NTHi from a symbiotic colonizing bacterium to an opportunistic pathogen is associated with uptake and expression of host sialic acids on its lipooligosaccharide (LOS).[4]

Sialic acids are complex nine-carbon sugars abundantly expressed on human glycoconjugates and can be released by the action of sialidases. These released sialic acids can be taken up by NTHi using a tripartite ATP-independent periplasmic (TRAP) transporter system and utilized for LOS sialylation or as a carbon source.[5,6] NTHi expresses SiaP to take up sialic acid, SiaB to generate cytidine monophosphate (CMP) sialic acid, which is the donor substrate for sialyltransferases LsgB, Lic3A, and Lic3A2 that enable LOS sialylation[4] (Figure 1). Alternatively, sialic acid is converted by aldolase NanA to enable the utilization of sialic acid as a carbon source. A clear link between virulence and sialic acid utilization by NTHi has been established, and LOS sialylation has been shown to confer NTHi resistance to serum killing and increases biofilm formation.[7,8] Conversely, NTHi strains defective in sialic acid utilization are no longer virulent in animal models for otitis media highlighting the therapeutic potential of the NTHi sialic acid utilization pathway.[9]

We have recently reported selective sialic acid-based inhibitors of sialylation in NTHi or host-derived cells.[11] The

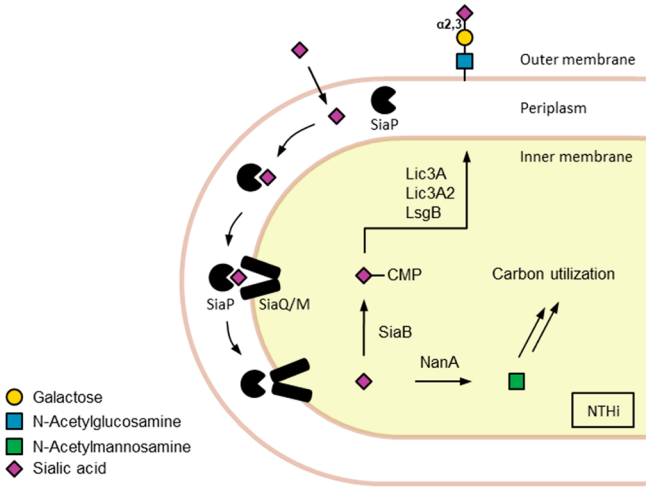


Figure 1. Sialic acid utilization in NTHi. Host-derived sialic acids are taken up by the SiaPQM transporter system. Intracellular sialic acid is either used as a carbon source (via NanA) or used to sialylate the lipooligosaccharide (LOS). To this end, sialic acid is CMP activated by SiaB and incorporated by sialyltransferases Lic3A, Lic3A2, and/or LsgB.[10]

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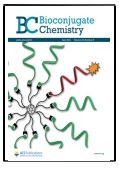

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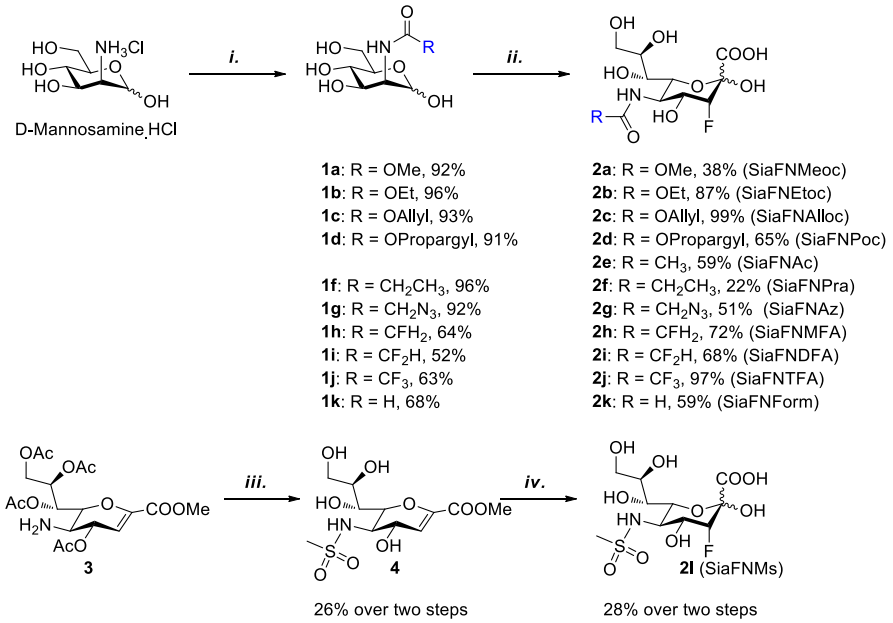
Scheme 1. Synthesis of C-5 Modified SiaFNAc Inhibitors[a]



[a](i) The synthesis of **1a**−**1j** has been described previously;[13−16] **1k**: MeOH, NaOMe, TEA, methyl formate; (ii) Sodium fluoropyruvate, H_2O, Neu_5Ac aldolase, 37 °C; (iii) 1. MsCl, DIPEA, DCM, 0 °C; 2. NaOMe, MeOH; (iv) 1. Selectfluor, DMF, H_2O; 2. NaOH, H_2O.

peracetylated sialic acid inhibitor enabled passive diffusion of the membrane of host cells but not NTHi. Conversely, unprotected sialic acid inhibitors showed active uptake via the TRAP transporter of NTHi (SiaP) but were not taken up by host cells. Selective inhibition of NTHi LOS sialylation using fluorinated sialic acid (SiaFNAc) in NTHi led to a reduced serum resistance confirming that targeting this evasive mechanism in NTHi is a promising therapeutic strategy. In mammalian cells, we have optimized the sialic acid inhibitor potency by modifying the C-5 position of the sialic acid scaffold. We found that C-5 carbamate modified sialic acids showed increased metabolic conversion by *N*-acylneuraminate cytidylyltransferase (CMAS) leading to higher intracellular concentrations of the active CMP-sialic acid derivative.[12] This prompted us to also carry out an extensive structure−activity relationship of fluorinated sialic acids for testing in NTHi as well. Herein, we report the synthesis and biological evaluation of C-5, C-8, and C-9 derivatives of fluorinated sialic acid.

RESULTS AND DISCUSSION

Design, Synthesis, and Evaluation of C-5 Modified 3-Fluorosialic Acid Derivatives. Since the C-5 carbamate derivatives of 3-fluorosialic acid showed increased metabolism to the corresponding CMP derivative in mammalian cells, we set out to prepare a set of C-5 modified 3-fluorosialic acids to evaluate the effect on the use in NTHi.[12] This requires derivatives devoid of protecting groups as sialic acid is actively taken up by the NTHi TRAP system. Hence, we opted to use a chemoenzymatic strategy starting from D-mannosamine·HCl (Scheme 1). Acylation reactions proceeded with moderate to excellent yields (52−96%) to afford a set of carbamate (**1a**−**1d**),[13] amide (**1e**−**1j**),[13−16] and formyl (**1k**) modified mannosamines. A subsequent reaction with fluoropyruvate catalyzed by neuraminic acid lyase (NAL) afforded 3-fluorosialic acids **2a**−**2k** in poor to good yield (22−99%) and with the desired axial substitution in line with earlier reports.[17] To evaluate an entirely different chemotype, we also prepared methanesulfonyl derivative **2l**. In this case, NAL did not accept the methanesulfonyl mannosamine as a substrate, so a chemical procedure starting from sialic acid glycal **3** was used instead. Introduction of the mesyl group and subsequent electrophilic fluorination with SelectFluor, followed by ester hydrolysis, afforded derivative **2l**.

To evaluate the efficacy of sialic acid-based inhibitors on LOS sialylation, we used an assay to measure LOS sialylation in NTHi. Neuraminic acid incorporation into NTHi LOS was measured using a metabolic sialic acid glycoengineering approach with azidoacetyl modified sialic acid (SiaNAz). NTHi bacteria were grown in sialic acid-deficient medium supplemented with SiaNAz, and incorporation into LOS was detected by reacting them with biotin-alkyne using copper-catalyzed alkyne−azide cycloaddition (CuAAC) followed by staining with fluorescent streptavidin. Evaluation of the ability of inhibitors **2a**−**2l** to block the metabolic incorporation of SiaNAz was done by growing NTHi in defined media containing 100 μM SiaNAz and increasing concentrations of **2a**−**2l** (0.1−1000 μM).[18] It is important to note that the inhibitors were used to outcompete an unnatural sialic acid (SiaNAz) and therefore likely differ from their ability to outcompete the natural substrate, SiaNAc. Nevertheless, it is expected that the relative ability of **2a**−**2l** to outcompete SiaNAz is consistent when translated to their ability to inhibit the incorporation of SiaNAc.

The derivative most closely related to the natural substrate SiaNAc is SiaFNAc, and it also proved to be the best inhibitor with an EC50 of 0.085 μM (Figure 2). Previously, we reported an IC50 of 0.7 μM.[11] The difference in inhibitory capacity is likely caused by the change in growth conditions (shaking versus static growth), resulting in a more efficient inhibition of SiaNAz incorporation. Modification of the C-5 amide to various carbamates reduced their inhibitory potency compared to SiaFNAc. The methyl and allyl carbamates **2a** and **2c** did show inhibition in contrast to ethyl and propargyl carbamates **2b** and **2d**. The activity of the carbamate series is very different

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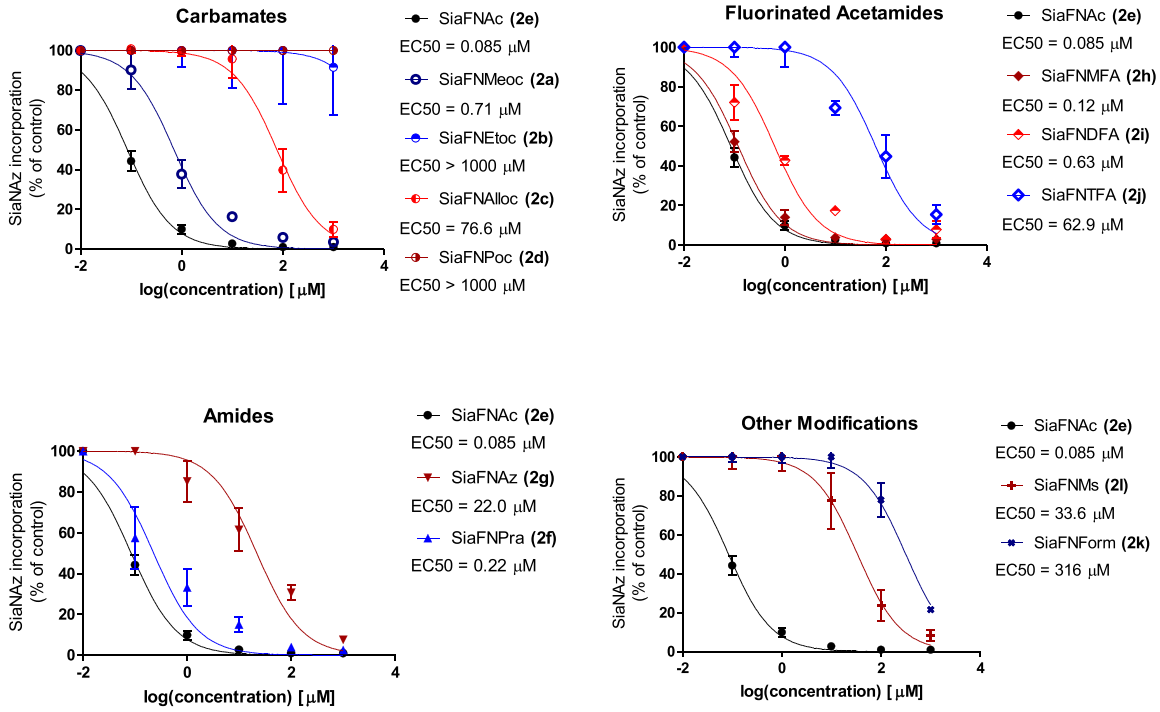

Figure 2. Inhibitory potency and EC_{50} curves of C-5 modified SiaFNAc inhibitors on the incorporation of 100 μM SiaNAz by NTHi. EC_{50} values were calculated based on the fitted S-curves, see the Supporting Information for more details.

from the mammalian setting where the C-5 ethyl and propargyl carbamates proved to be much better inhibitors than the corresponding natural C-5 acetamido counterpart. Interestingly, in NTHi, it is clear that the amide series performs better with the acetamido proving to be the most potent inhibitor. Hence, a set of amides derivatives varying the chain length of the amide was evaluated next (**2e**−**2g**). Increasing the chain length led to a decrease of inhibitory potency with the inhibitory potency order of acetyl > propionyl > azidoacetyl. The electronics of the amide were also varied by introducing a mono-, di-, or trifluoroacetamido group at C-5 (**2h**−**2j**). A clear trend was found indicating that increasing the degree of fluorination leads to a corresponding decrease in inhibitory potency. Hence, increasingly bulky and electron poor amides lead to a loss of inhibitory potency. Finally, the formyl and mesyl derivatives **2k** and **2l** were tested. Even though these groups differ substantially from the parent acetamido compound, they did still show inhibition albeit with much decreased potency. These results demonstrate that the C-5 position of SiaFNAc is amenable to modification, yet all modifications lead to loss of inhibitory potency compared to the SiaFNAc parent structure and are in line with earlier research, which shows preferential utilization of SiaNAc compared to the C-5 modified SiaNGc by NTHi.[19]

To investigate the mechanism of sialylation inhibition by SiaFNAc, we performed a nucleotide sugar analysis on NTHi grown in minimal media with DMSO or 100 μM SiaFNAc. In the samples treated with SiaFNAc, CMP-SiaFNAc was clearly detected and represented the major portion of the nucleotide sugar pool (Figure S1). This is consistent with a mechanism where SiaFNAc is transported by SiaPQM and converted by SiaB into CMP-SiaFNAc which subsequently blocks the activity of the NTHi sialyltransferases. This mechanism is analogous to the mechanism observed for the mammalian sialylation pathway.[12,20,21] However, in the mammalian setting, we have observed that C-5 carbamate derivatives of SiaFNAc lead to higher intracellular concentrations of their corresponding CMP derivatives and a more potent inhibition of sialylation.[12] This is attributed to their more efficient conversion by CMAS. Possibly, SiaB in NTHi is less tolerant of C-5 modified sialic acids leading to a lower metabolic conversion to CMP analogues.

Design, Synthesis, and Evaluation of 3-Fluorosialic Acid Derivatives Modified at the Glycerol Side Chain. The glycerol side chain of sialic acid is one of the features that makes sialic acid stand out from other mammalian monosaccharides and known to undergo modification such as acylation, sulfation, and methylation.[22,23] We were interested in probing to what extent the glycerol side chain contributes to the recognition and activity of SiaFNAc. Hence, we investigated the impact of modifications at the glycerol side chain of SiaFNAc by preparing truncated derivatives **8** and **9** and 9-azido derivative **11** (Scheme 2). Sialic acid glycal **5** was converted using Malaprade oxidation[24] followed by a sodium borohydride reduction to afford truncated derivatives **6** and **7**. Subsequent electrophilic fluorination using SelectFluor[25] followed by ester hydrolysis afforded octulosonic acid **8** and heptulosonic acid **9**. 9-Azido derivative **11** was also obtained from sialic acid glycal **5** by C-9 tosylation, azidation, and subsequent fluorination and hydrolysis.

Testing of derivatives **8**, **9**, and **11** was carried out using the aforementioned protocol. The inhibitory potency of **8** and **9** demonstrates that truncation of the glycerol sides chain was tolerated to some extent (Figure 3). Introduction of a C-9 azide onto the SiaFNAc scaffold led to a very notable reduction of the inhibitory potency (**11**). In contrast, truncation of the carbon skeleton by one carbon (octulosonic acid **8**) led to a less dramatic loss of activity. However, heptulosonic acid **9** did not show any inhibitory activity.

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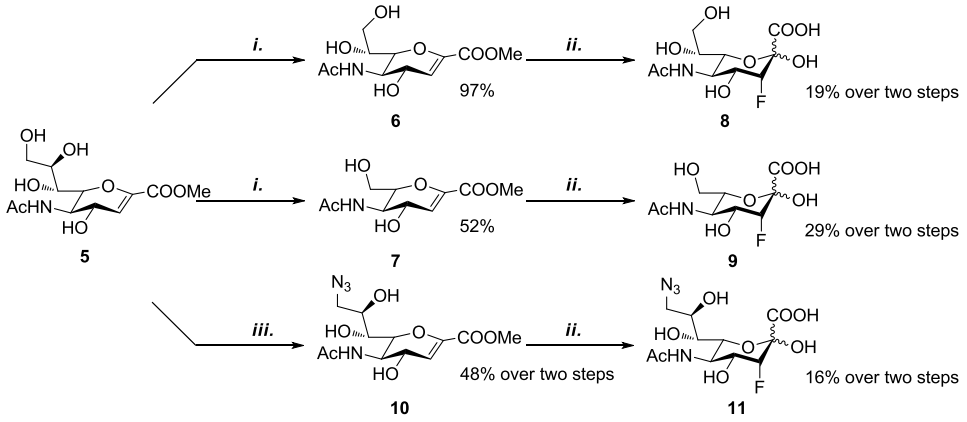
Scheme 2. Synthesis of C-8 and C-9 Modified SiaFNAc Inhibitors[a]



[a](i) 1. $NaIO_4$, MeOH; 2. $NaBH_4$, MeOH; (ii) 1. Selectfluor, DMF, H_2O; 2. NaOH, MeOH; (iii) 1. TsCl, pyridine; 2. NaN_3, acetone, H_2O.

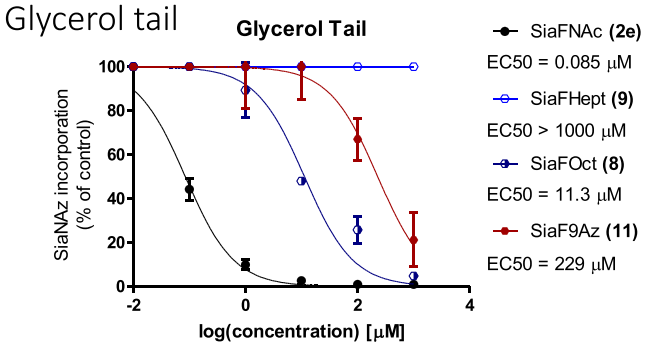


Figure 3. Inhibitory potency of SiaFNAc inhibitors modified at the glycerol side chain.

CONCLUSION

We have prepared and tested a number of C-5, C-8, and C-9 derivatives of 3-fluorosialic acid. In contrast to sialylation in mammalian cells, C-5 carbamate derivatives are much less potent inhibitors than the corresponding C-5 amides in NTHi. This indicates that the enzymes involved in the NTHi LOS sialylation pathway differ substantially from those in the mammalian biosynthesis and are less tolerant for modification of the sialic acid scaffold. Alteration to the glycerol side chain was tolerated to some extent. These results provide clear guidelines for future inhibitor development and may also be the basis for the incorporation of unnatural sialic acids lacking the 3-fluoride.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.1c00194.

General procedures, experimental procedure for synthesis and biological evaluation, and NMR data (PDF)

AUTHOR INFORMATION

Corresponding Authors

Jeroen D. Langereis − *Laboratory of Medical Immunology, Radboud Center for Infectious Diseases, Radboud Institute for Molecular Sciences, Radboud University Medical Center, Nijmegen 6525 GA, The Netherlands*; Email: Jeroen.Langereis@radboudumc.nl

Thomas J. Boltje − *Cluster of Molecular Chemistry, Institute for Molecules and Materials, Radboud University Nijmegen, Nijmegen 6525 AJ, The Netherlands*; orcid.org/0000-0001-9141-8784; Email: t.boltje@ru.nl

Authors

Sam J. Moons − *Cluster of Molecular Chemistry, Institute for Molecules and Materials, Radboud University Nijmegen, Nijmegen 6525 AJ, The Netherlands*

Emiel Rossing − *Cluster of Molecular Chemistry, Institute for Molecules and Materials, Radboud University Nijmegen, Nijmegen 6525 AJ, The Netherlands*

Jurriaan J. A. Heming − *Cluster of Molecular Chemistry, Institute for Molecules and Materials, Radboud University Nijmegen, Nijmegen 6525 AJ, The Netherlands*

Mathilde A. C. H. Janssen − *Cluster of Molecular Chemistry, Institute for Molecules and Materials, Radboud University Nijmegen, Nijmegen 6525 AJ, The Netherlands*

Monique van Scherpenzeel − *Translational Metabolic Laboratory, Department of Neurology, Donders Center for Brain Cognition and Behavior, Radboud University Medical Center, Nijmegen 6525 GA, The Netherlands*

Dirk J. Lefeber − *Translational Metabolic Laboratory, Department of Neurology, Donders Center for Brain Cognition and Behavior, Radboud University Medical Center, Nijmegen 6525 GA, The Netherlands*

Marien I. de Jonge − *Laboratory of Medical Immunology, Radboud Center for Infectious Diseases, Radboud Institute for Molecular Sciences, Radboud University Medical Center, Nijmegen 6525 GA, The Netherlands*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.bioconjchem.1c00194

Author Contributions

¶S.J.M. and E.R. contributed equally.

Notes

The authors declare no competing financial interest.

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