Design, synthesis and evaluation of bacterial sialic acid uptake inhibitors

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To all companions on this journey.
It has been a tough,
but wonderful ride.

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List of publications

I. **Tiago Bozzola**, Mariafrancesca Scalise, Christer U. Larsson, Michael C. Newton-Vesty, Caterina Rovegno, Ankita Mitra, Jonathan Cramer, Weixiao Yuan Wahlgren, Partha Radhakrishnan Santhakumari, Richard E. Johnsson, Oliver Schwardt, Beat Ernst, Rosmarie Friemann, Renwick C.J. Dobson, Cesare Indiveri, Jenny Schelin, Ulf J. Nilsson, Ulf Ellervik. Sialic acid derivatives target and block bacterial sialic acid uptake by inhibition of the SiaT transporter. *ACS Chemical Biology*, 2022..

Contributions. I performed the synthesis and characterization of the synthetic derivatives, as well as the nanoDSF and ITC experiments. I contributed to the interpretation of data from the other experiments. I wrote the manuscript with contributions from all other authors.

II. **Tiago Bozzola**, Ulf J. Nilsson and Ulf Ellervik. Structure activity relationships of O4, N5 and C9 Neu5Ac derivatives targeting different bacterial sialic acid transporter families *Manuscript*.

Contributions. I conceptualized the work with the other authors. I performed the nanoDSF and ITC experiments. I wrote the manuscript with contributions from the other authors.

III. **Tiago Bozzola**, Richard E. Johnsson, Ulf J. Nilsson and Ulf Ellervik. Sialic acid 4-*N*-piperazine and piperidine derivatives inhibit sialic acid uptake by the *Proteus mirabilis* sodium solute symporter. *Manuscript*.

Contributions. I conceptualized the work with the other authors. I performed the synthesis, characterisation and evaluation via nanoDSF and ITC experiments of all compounds. I wrote the manuscript with contributions from the other authors.

IV. **Tiago Bozzola**, Ulf J. Nilsson and Ulf Ellervik. Direct Sialic Acid 4-OAc substitution by nitrogen, sulfur and carbon nucleophiles with retention of stereochemistry. *RSC Advances*, 2022, **12**, 11992-11995.

Contributions. I conceptualized the work with the other authors and performed all experimental work and compound characterization. I wrote the manuscript with contributions from the other authors.

Popular summary

Antibiotics are one of the major medical advances in human history. Bacterial infections, which until 100 years ago would lead to a patient's death, are now easily treated with antibiotics. This situation could and has, to some extent, changed. Using and misusing antibiotics increases the risk of developing bacteria capable of resisting them. Such process is called "Antibiotic resistance" and is becoming an increasing concern. These resistant bacteria complicate treatments and lead to increased mortality, possibly a step back to pre-antibiotic times. With this thesis, we aim at investigating a potential new class of antibacterial agents with a novel mechanism of action.

Sialic acid is a carbohydrate among many in nature. Its uniqueness is the location on cell surfaces. Cell surfaces are decorated with chains of carbohydrates called glycans. Sialic acids are often found at the terminal position of glycans and therefore are highly important for mechanisms such as recognition and communication between cells. Bacteria generally do not produce sialic acid and therefore their surface glycans do not display it. To avoid our immune system and thus increase their chances of growth and infection, bacteria scavenge sialic acid from humans and display it on their surfaces. Thanks to this mechanism called "molecular mimicry", bacteria make themselves appear like human cells, thus evading our immune system. Bacteria possess dedicated sialic acid transporters. We have worked on developing new molecules based on sialic acid that can target these bacterial transporters to disrupt the bacterial sialic acid supply. We believe that this strategy could lead to the generation of a new class of antibacterial drugs.

In this thesis, we have established new synthetic methodologies to make sialic acid derivatives and developed assays to test them. Based on our results, we successfully managed to target and block the sialic acid transporters from *Proteus mirabilis* and *Staphylococcus aureus*. The work in this thesis opens and paves the way for the development of these compounds as potential antibacterial drugs.

Abbreviations

ABC	ATP binding cassette
ACN	Acetonitrile
ADME	Absorption, distribution, metabolism and excretion
AMR	Antimicrobial resistance
CMAH	Cytidine monophospho-*N*-acetylneuraminic acid hydroxylase
CMP	Cytidine monophosphate
DP	Differential potential
DSF	Differential scanning fluorimetry
EET	Entropy-enthalpy transduction
ITC	Isothermal titration calorimetry
HA-MRSA	Hospital-acquired Methicillin-resistant *Staphylococcus aureus*
LMIC	Low- and medium-income countries
MFS	Major facilitator superfamily
Neu5Ac	5-acetyl neuraminic acid
Neu5Gc	5-glycolyl neuraminic acid
NHH	Non-human hominids
PDB	Protein data bank
PK	Pharmacokinetics
Pyr	Pyridine
RED	Rapid equilibrium dialysis
SAR	Structure-activity relationship
SSS	Sodium solute symporter
TBAI	Tetrabutylammonium iodide
TEA	Triethylamine
TRAP	Tripartite ATP-independent periplasmic

Abbreviations found in the ACS Style Guide are not included

1 Introduction

Antibiotic and antibacterial are not synonyms. An antibiotic is defined as a substance active against bacteria and is produced naturally or derived from a microorganism. Antibacterials enclose antibiotics and are defined as chemical or physical treatments that kill or inhibit the growth of bacteria. Despite the clear definitions, the two terms are used interchangeably in the literature.[1] In this thesis, I will adhere to the traditional definitions, unless the cited literature does otherwise.

1.1 History of antibiotics

Antibiotics are arguably the most significant therapeutic advance in history, having saved countless lives in the last 100 years. Furthermore, the antibiotic advancements are tightly bound to the development of the pharmaceutical research at large.

It was not until 1884 that Robert Koch and others established a causative relation between microbes and diseases.[2] This period is often referred to as the "bacteriological revolution" for the expansion in knowledge regarding bacteria and their roles in diseases.[3] Paul Ehrlich is credited as one of the founding fathers of the "antibiotic era".[4] He was the first to formulate the concept of the *Zauberkugel*, or "magic bullet" in English. This concept affirms that it is possible to selectively target a microbe to cure a disease, without harming the host. Such conviction led him to set up the first drug discovery program as we know it, with synthesis of generations of compounds and systematic testing in model systems. This drug discovery campaign, with the collaboration of bacteriologist Sahachiro Hata, led to the discovery of Salvarsan in 1909, the very first antimicrobial agent active against *Treponema pallidum*, the cause of syphilis. Salvarsan was later replaced by Neosalvarsan, possessing milder side effects. These drugs became the most frequently prescribed ones until the discovery and commercialisation of penicillin in the 1940s.[5]

The following decades witnessed the discovery of a large number of antibacterial drugs, displaying different antibacterial mechanisms of action, thus allowing for efficacy and broad spectrum of use.[6]

1.2 Antibiotic mechanisms and classes

Antibacterial drugs can be classified in different fashions, based on their effects (bacteriostatic or bactericidal), mechanism of action, chemical structure or spectrum of activity. According to the mechanism of action (**Figure 1**), the different classes can be divided based on the inhibition of:

- **Cell wall synthesis**. This class is the most widely used, including the broad group of β-lactams as well as fosfomycin, glyco- and lipopeptides.[7] Disrupting the cell wall biosynthesis leads to a loose membrane, with consequent lysis of the bacterial cells. These drugs are often first line antibiotic treatments.
- **Protein synthesis**. This type of antibiotic effect is achieved by inhibition of different processes.[8] Among those, aminoglycosides and tetracyclines are initiation inhibitors, binding the bacterial ribosome (30S subunit), thus preventing the onset of protein synthesis. Other antibiotics, such as macrolides and chloramphenicol, bind the 50S ribosomal subunit, thus inhibiting the peptidyl transfer. These drugs exploit the differences between prokaryotic and eukaryotic ribosome structure. These drugs are generally second line treatments or used for more specific applications.

- **Cell membrane interference**. Polymyxins are the most prominent member of this class.[9] By binding LPSs, polymyxins disrupt the bacterial membrane of Gram-negative bacteria. These are last resort drugs, due to their neuro- and nephrotoxicity.
- **Folic acid synthesis**. Sulfonamides, as well as trimethoprim, explicit their antibacterial effects by inhibiting bacterial enzymes involved in the folic acid biosynthesis, such as dihydrofolate reductase and dihydropteroate synthetase.[10] Bacteria, unlike mammals, rely solely on biosynthesis for folic acid supply and its disruption leads to bacterial death. Trimethoprim is a first line antibiotic, while sulfonamides are limited to second line and specific uses, due to side effects.
- **DNA replication**. This class of antibiotics acts in different mechanisms. Quinolones inhibit DNA topoisomerases, preventing DNA replication.[11] Nitroimidazoles and nitrofurantoin, similarly, damage bacterial DNA via metabolic activation within the bacteria.[12,13] Quinolones are widely used, while nitroimidazoles and nitrofurantoin are generally employed for specific applications.

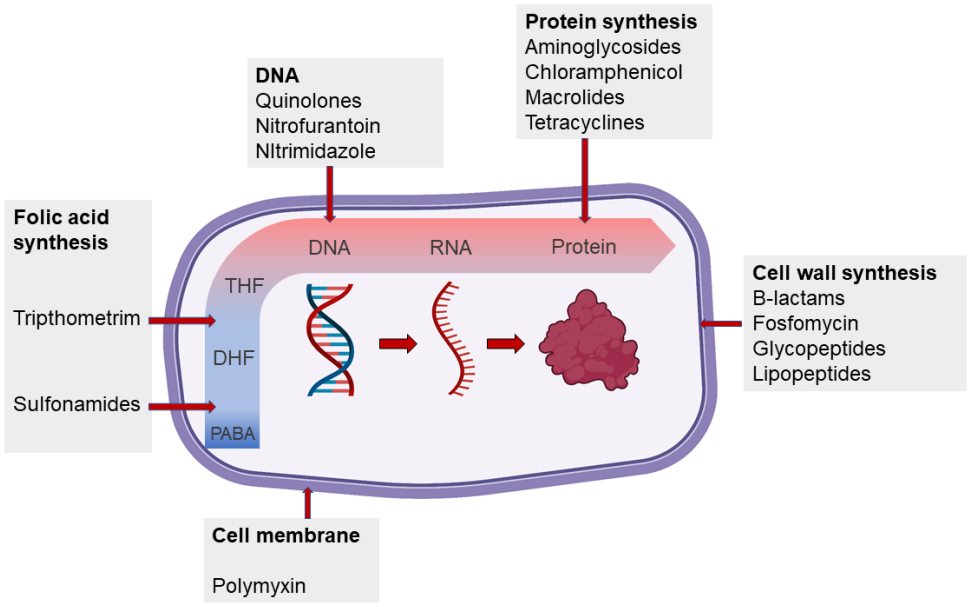


Figure 1. Targets and mechanisms of action of the different antibacterial classes.

1.3 Antibiotic resistance

Antibiotic resistance is a direct result of antibiotic use. Fleming himself, in 1945, warned patients to appropriately use antibiotics to limit the risk of selecting resistant strains.[14] Antibiotics kill or inhibit growth of bacteria, thus placing bacteria under a very high evolutionary pressure to develop resistance mechanisms. For a visual example of such a processes, we suggest the vision of *The Evolution of Bacteria on a "Mega-Plate" Petri Dish* by Kishony Lab on YouTube.[15]

Bacteria can acquire resistance in different ways, such as by gene transfer or mutation of their own DNA. Such acquired drug resistant mechanisms can be described in four main groups: drug target modification, drug uptake reduction, drug inactivation and active efflux. For more information and antibiotic resistance mechanisms, see the reviews by Holmes *et al* or Reygaert.[16,17]

Antibiotic resistance, despite the knowledge of its existence since the 1950s, started to be seen as a threat only recently (**Figure 2**). In the past century, the general attitude towards antibiotic resistance was that new drugs could be discovered and the problem contained.[18] The golden age of antibiotics ended in the 1980s, when the

antibacterial pipeline started to dry up. Ever since, we have not seen any new antibacterial drug class reaching the clinic. This has led to the current situation, where the estimate for global bacterial antimicrobial resistance (AMR) attributed deaths is 1.27 million in 2019.[19] Based on the current situation, the AMR attributable deaths are forecasted to reach 10 million by 2050.[20]

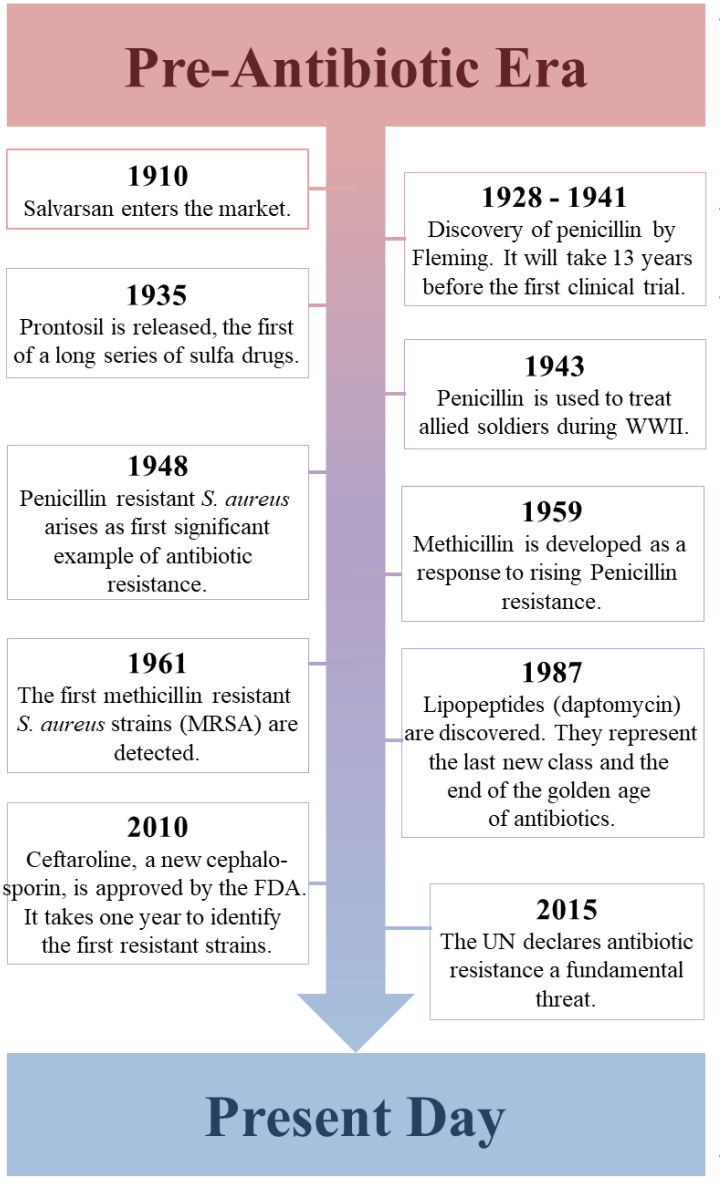


Figure 2. History of antibacterial drugs and antibiotic resistance.

The strategic plans to fight antibiotic resistance generally propose four different groups of actions:

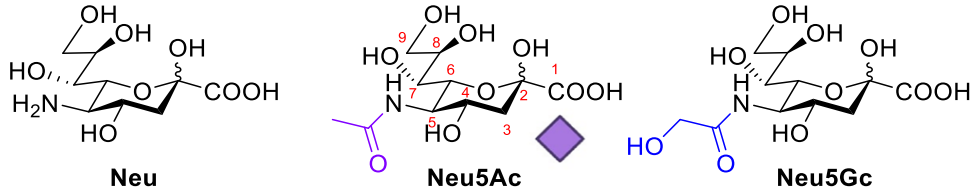
- **Prevention and control**. Most of the AMR infections seem to be acquired in hospital or healthcare settings.[21] Thus, introducing better hygiene and practises could result in a significant reduction of the problem. In low and medium income countries (LMIC) the focus is on water, sanitation and hygiene for community-based programs.[19]
- **Vaccinations**. Vaccinations bypass the need of antibacterial drugs, thus limiting the insurgence of resistance. Currently, a vaccination is available only for *Staphylococcus pneumoniae*, but more are developed.[22]
- **Antibiotic use and misuse**. How the antibiotics have been handled is not sustainable. In the past decades, antibiotics have been misused, with studies showing incorrect prescription in between 30 to 60% of the cases analysed in intense care units.[23] Additionally, adding antibiotics to animal feed is a

common practise required to sustain animal farming. Antibiotic resistant strains can arise in animals, as well as in water and soil. The mutated genes of the antibiotic resistant strains can then reach human pathogens via horizontal gene transfer.[24] Stricter regulations in healthcare as well as agriculture are needed. Additionally, better diagnostic tools to identify the aetiology of a disease need to be implement, to employ antibiotics only in those situations that require them.[16]

- **Development of new antibacterial drugs**. The last 30 years have not witnessed the clinical introduction of new classes of antibiotics. More investments and research need to be directed towards novel antibacterial treatments, to populate the pipeline.

1.4 Sialic acid

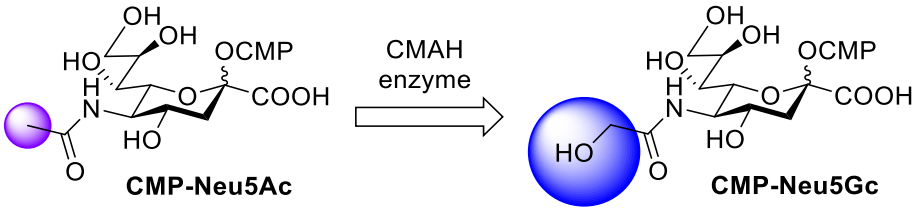
Sialic acid is a general term for a family of acidic nine carbon sugars ubiquitous in vertebrates, with 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid (Neu) as a common core structure.[25] Different animal species display different types of sialic acids, with 5-acetylneuraminic acid (Neu5Ac) and 5-glycolylneuraminic acid (Neu5Gc) being the most common forms (**Scheme 1**). Other sialic acid variations arise from *O*-acetylation, *O*-lactylation, *O*-methylation, and formation of sulphate or phosphate esters at positions 4, 7, 8 and/or 9.[26]



Scheme 1. Sialic acids and numbering of Neu5Ac. The purple diamond is the symbol for Neu5Ac.

Sialic acids play a pivotal role in vertebrate cells. They are found in the terminal position of glycans, in the order of hundreds of millions of chains per cell.[27] Such abundance translates in a multitude of physiological and pathological functions.[28,29]

Humans are among the few mammalian species that do not display Neu5Gc.[30] This condition arose about 2 million years ago, with a point mutation that led to the inactivation of the CMAH gene encoding for the cytidine monophospho-*N*-acetylneuraminic acid hydroxylases (CMAH) enzyme.[31] This enzyme converts CMP-Neu5Ac to CMP-Neu5Gc via hydroxylation of the 5-acetamide (**Scheme 2**).



Scheme 2. Conversion of CMP-Neu5Ac to CMP-Neu5Gc mediated by the CMAH enzyme.

This modification had radical effects on the early human development, especially in respect to its relation with pathogens.[27] CMAH inactivation was not the sole modification. Almost 20% of the human sialic acid related genes show human-specific evolution, when compared to other non-human hominids (NHH).[32] Therefore, sialic acid biology was a "hot spot" in terms of the evolutionary changes that led to branching of humans from NHH.

1.5 Sialic acid and bacteria

Bacteria are generally not able to biosynthesize sialic acids, with only a few exemptions such as *Escherichia coli K1*, *Neisseria meningitidis* and *Campylobacter jejuni*.[33] Therefore, bacteria mostly rely on the environment for their sialic acid supply.[34]

In the bacterial sialic acid supply, the first step is represented by accessing unbound sialic acid. Some bacteria release sialidases to cleave glycan-bound sialic acid,[35] while others lack such enzymes and therefore rely on the sialidase activity of other bacteria,[36] or of the host.[37]

In Gram-negative bacteria, the second step is crossing the outer membrane. This is done through passive diffusion mediated by porins, by facilitated diffusion, or active transport.[38] Due to the low concentration of unbound sialic acid, passive diffusion is generally not a viable strategy. Therefore, bacteria have developed other ways for the transport of sialic acid in the periplasmic space. These include the sialic acid porin NanC from *E. coli* which has been studied and characterised.[39] More recently, also the NanOU from the Bacteroidetes species has been described.[40]

Lastly, bacteria have developed different mechanisms to transport free sialic acid into the cytosol (**Figure 3**). To date, four different classes of transporters have been identified and characterized:

- ATP binding cassette (ABC) transporter family, which utilises ATP hydrolysis as driving force to enable transport. The first identified member of this class was from *Haemophilus ducreyi*.[41] This ABC transporter is composed of a periplasmic substrate binding protein (SatA), an integral membrane permease domain (SatB) and an ATPase domain (SatD). More examples have been identified and characterized since then.[42,43] Up to date, only the SatA domain from *H. ducreyi* has been crystallized in both its native form and with bound Neu5Ac or Neu5Gc.[44]
- Tripartite ATP-independent periplasmic (TRAP) transporter family. Such transporters take advantage of electrochemical Na^+ gradients and consist of three subunits: a substrate binding protein (SiaP), a large membrane spanning subunit (SiaM) and a small spanning subunit (SiaQ). In the case of *Haemophilus influenzae*, the SiaQ and SiaM are fused in a SiaQM subunit.[45] As for the case of the ABC transporter, also most of the investigative efforts have been placed on the structural elucidations of the substrate binding domain SiaP from *H. influenzae*,[46] as well as *Fusobacterium nucleatum*, *Pasteurella multocida* and *Vibrio cholerae*.[47]
- Major facilitator superfamily (MFS) transporter family, from which the first sialic acid transporter (NanT) was identified from *E. coli*.[48] NanT employs proton gradients for the sialic acid uptake. No crystal structure has been published yet.
- Sodium solute symporter (SSS) transporter family, the most recently discovered transporter.[49] When transporting sialic acid, these transporters are denominated SiaT. Such transporters, alike TRAP ones, depend on sodium gradient for the sialic acid uptake. The crystal structure of SiaT from *Proteus mirabilis* (PmSiaT) has been recently elucidated.[50] Additionally, a homology model of the SiaT from *S. aureus* has been constructed based on the PmSiaT strucuture.[51]

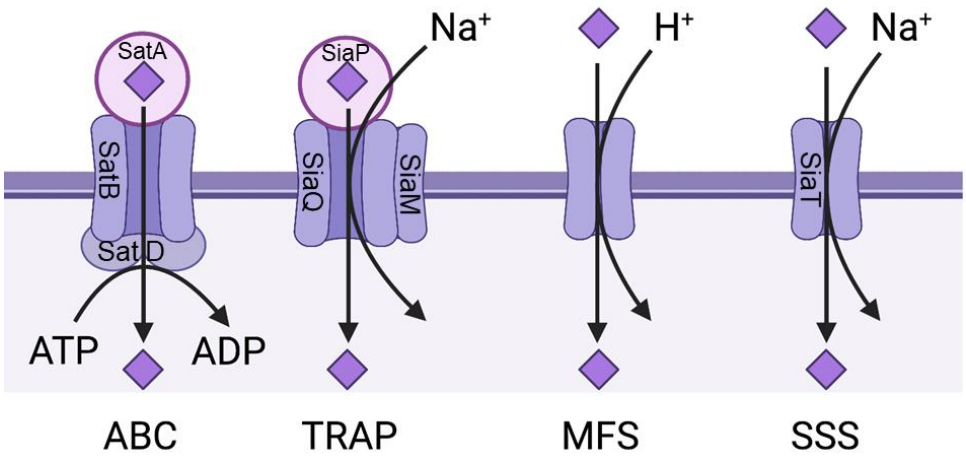


Figure 3. Bacterial sialic acid transporters.

The presence of so many different sialic acid transporters is indicative of the significance of sialic acid for bacteria. Bacteria generally are limited to one type of sialic acid transporter, with a few exceptions predicted such as *Photobacterium profundum*, *S. pneumoniae* and *S. enterica* serovar Typhimurium.[38] The presence for multiple sialic acid transporters is hypothesise to be linked to the increased ability to uptake different sialic acids.

1.6 Bacterial sialic acid utilization

Sialic acids play a paramount role for pathogenic and commensal bacteria.[52] Such bacteria colonise sialic acid-rich niches, such as the respiratory and gastrointestinal tract, and utilise sialic acid in different ways.[53] Once inside the bacteria, sialic acids undergo catabolism or a mechanism called molecular mimicry (**Figure 4**). In the catabolic pathway, sialic acid represents an alternative source of carbon and nitrogen, thus providing an advantage over other bacteria incapable of catabolising sialic acid. Interestingly, Neu5Ac metabolism was found essential for the *E. coli* colonisation of the mouse intestine, when compared to disruption of other sugars metabolism.[54] The sialic acid catabolic pathway is well described.[38]

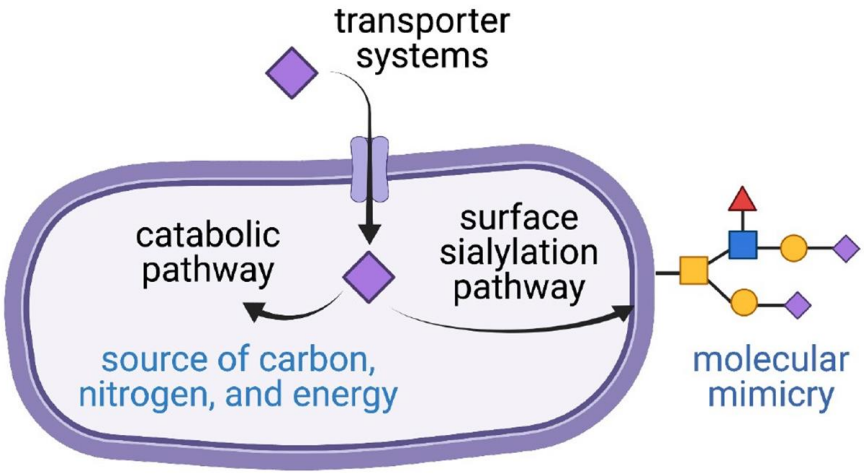


Figure 4. Catabolic pathway and molecular mimicry in bacteria.

Molecular mimicry describes the ability of certain bacteria to mimic the host antigens, thus evading immune recognition.[55] As previously described, bacteria are generally not able to biosynthesise sialic acids, therefore they do not naturally display sialylated glycans. To avoid immunorecognition, bacteria scavenge sialic acids from the host and sialylate their surfaces, thus increasing the chances of colonisation and infection.[56] Such process is mediated by sialyltransferases that attach CMP-Neu5Ac to glycan chains.[57] For a thorough discussion on molecular mimicry, we suggest the recent review by de Jong *et al*.[58]

1.7 Sialic acid uptake inhibition

Sialic acid uptake inhibition represents a novel and attractive mode of action for the development of antibacterial drugs. To begin with, humans synthesise sialic acid, thus we do not rely on the environment for our supply. Only one human sialic acid transporter is known, which transports CMP-Neu5Ac inside the Golgi lumen, where glycans undergo sialylation.[59] This transporter shares little homology with the bacterial sialic acid transporters, especially considering that its substrate is CMP-Neu5Ac, rather than Neu5Ac.[38] For these reasons, bacterial sialic acid uptake inhibition seems promising from the perspective of off-targeting.

Moreover, several studies have demonstrated the potential of bacterial sialic acid supply disruption both *in vitro*, *ex vivo* and *in vivo*. Heise *et al.* inhibited the activity of sialyltransferases in *H. influenzae* with a sialic

acid derivative and demonstrated how this bacterium would lose its sialic acid-induced serum resistance.[60] This effect was caused by the reduced ability of the bacterium to take advantage of molecular mimicry by sialylating its epitopes. In the same study, the authors also presented different methodologies to evaluate bacterial surface sialylation inhibition, serum mediated killing and sialic acid transfer from bronchial cells to *H. influenzae*. Bouchet *et al.* studied sialic acid in *H. influenzae*-associated otitis.[61] The results showed that *H. influenzae* sialic acid-deficient mutants were profoundly impaired in their inflammatory activity. This was a direct effect of the reduction in sialylation of the bacterial epitopes caused by genetic knock-out of the sialyltransferases or CMP-acetylneuraminic acid synthetase genes.

The use of antibiotics alters the intestinal microbiota, which is our first line of defence against microbial infections.[62] Ng *et al.* investigated the role of sialic acid in antibiotic-associated pathogens *S. typhimurium* and *C. difficile* in mice. Sialic acid faecal concentration was found to be much higher after one day of antibiotic treatment, due to concomitant sialidase activity of some residual bacteria and lack of consumption by the commensal microbiota. Free sialic acid played a pivotal role in the bacterial colonisation. Bacteria having their sialic acid transporters genetically knocked-out (nanT for both *S. typhimurium* and *C. difficile*) showed significantly compromised proliferation.

Huang *et al.* demonstrated the role of free sialic acid in the growth of *E. coli* in mouse intestine during colitis.[63] *E. coli* does not release sialidases, therefore relies on the activity of other bacteria, such as *Bacteroides vulgatus*, for the sialic acid supply. *E. coli* growth was associated with aggravated inflammation, while the administration of sialidase inhibitors hindered the *E. coli* growth by 2-3 orders of magnitude and significantly reduced inflammation.

From these studies, bacterial sialic acid transporters represent promising drug targets for the development of small molecules capable of targeting and inhibiting them.

2 Thesis objectives

We embarked on this project with the hypothesis that bacterial sialic acid uptake inhibition may lead to a new class of anti-bacterial drugs. To test this hypothesis, we identified the following objectives:

- Structure-based design of putative inhibitors based on X-ray structures of bacterial sialic acid transporters, starting from PmSiaT.
- Establishment of different biophysical and biochemical methods to test the compounds' affinities and behaviours *in vitro* and *in vivo*.
- Development of compounds with broad-spectrum activity, capable of targeting multiple bacterial sialic acid transporters.
- Characterisation and optimization of affinity, selectivity, PK-ADME properties, toxicity, and cross-reactivities for selected hits.
- Proof-of-concept, demonstrating efficacy of optimized synthetic inhibitors in infectious models with selected pathogens.
- Development of new chemistry to functionalise sialic acids.

In the following chapters, we will describe our efforts in trying to answer these questions.

3 Design, synthesis and evaluation of SiaT inhibitors.

3.1 Design and synthesis

This project began as an attempt to target the SiaT transporters from *Proteus mirabilis* and *Staphylococcus aureus*. The PmSiaT crystal structure was published in 2018 and a homology model based on it allowed for a putative structure of the SaSiaT.[50,51] With the structures at hand, we could pursue a structure-based approach. On this regard, the two structures are similar (~41% sequence identity), therefore we hypothesised that compounds active on one protein could be also active on the other.[51] Additionally, SiaTs are the only sialic acid transporter family whose genes are widely distributed in both Gram-positive and Gram-negative bacteria, thus opening the possibility for broad spectrum activity.[38] Lastly, *P. mirabilis*, but especially *S. aureus*, are problematic bacteria.[64] *S. aureus* is a Gram-positive pathogenic bacterium infamous for the rise of several antibiotic resistance strains, MRSA (methicillin-resistance *S. aureus*). MRSA infections are often difficult to treat and associated with high mortality.[21,65] *P. mirabilis* is a Gram-negative pathogenic bacterium, commonly associated with urinary tract infections (UTI) that is also gaining attention for its antibiotic resistance.[66,67]

The initial efforts aimed at the development of a library of compounds with single modifications at different positions of the natural substrate. The reason was to establish a structure-activity relation (SAR) as starting point for the development of further compound generations. We identified O4, N5 and C9 as sites for the generation of synthetic compound series. As visible from **Figure 5**, the 4-OH points towards the opening of the channel protein and we envisioned the possibility of introducing larger substituents, such as benzyls. The remaining parts of the molecule establish a tight set of interactions with the binding site, with limited room for the installation of large substituents. We therefore planned for a series of compounds bearing small modifications at these positions. The 5-NHAc appears close to Phe243 in PmSiaT and Asn244 in SaSiaT, respectively, and we consequentially planned for substituents capable of interactions with the side chains of such amino acids. The 9-OH is reported to establish a hydrogen bond with Gln82 and Asn83, in PmSiaT and SaSiaT, respectively.[50,51] Accordingly, our choice of substituents were both to probe the importance of this bond and to investigate for alternatives.

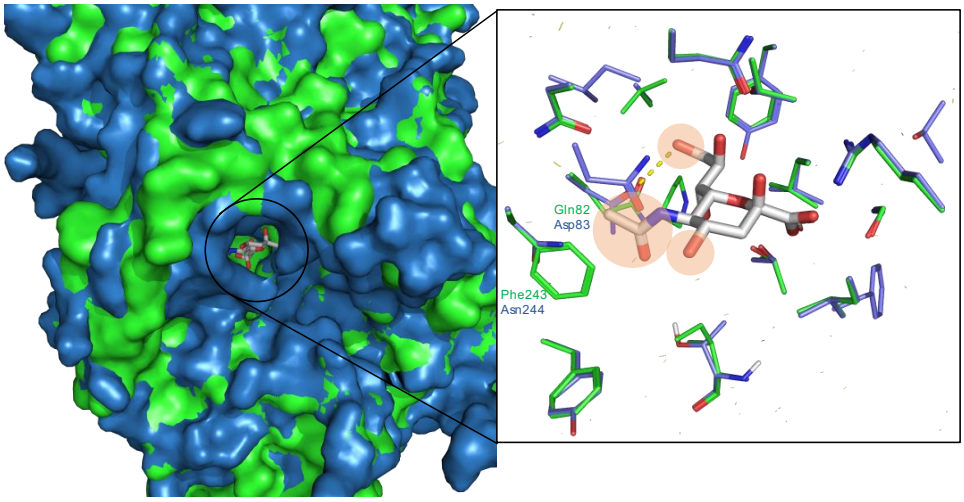
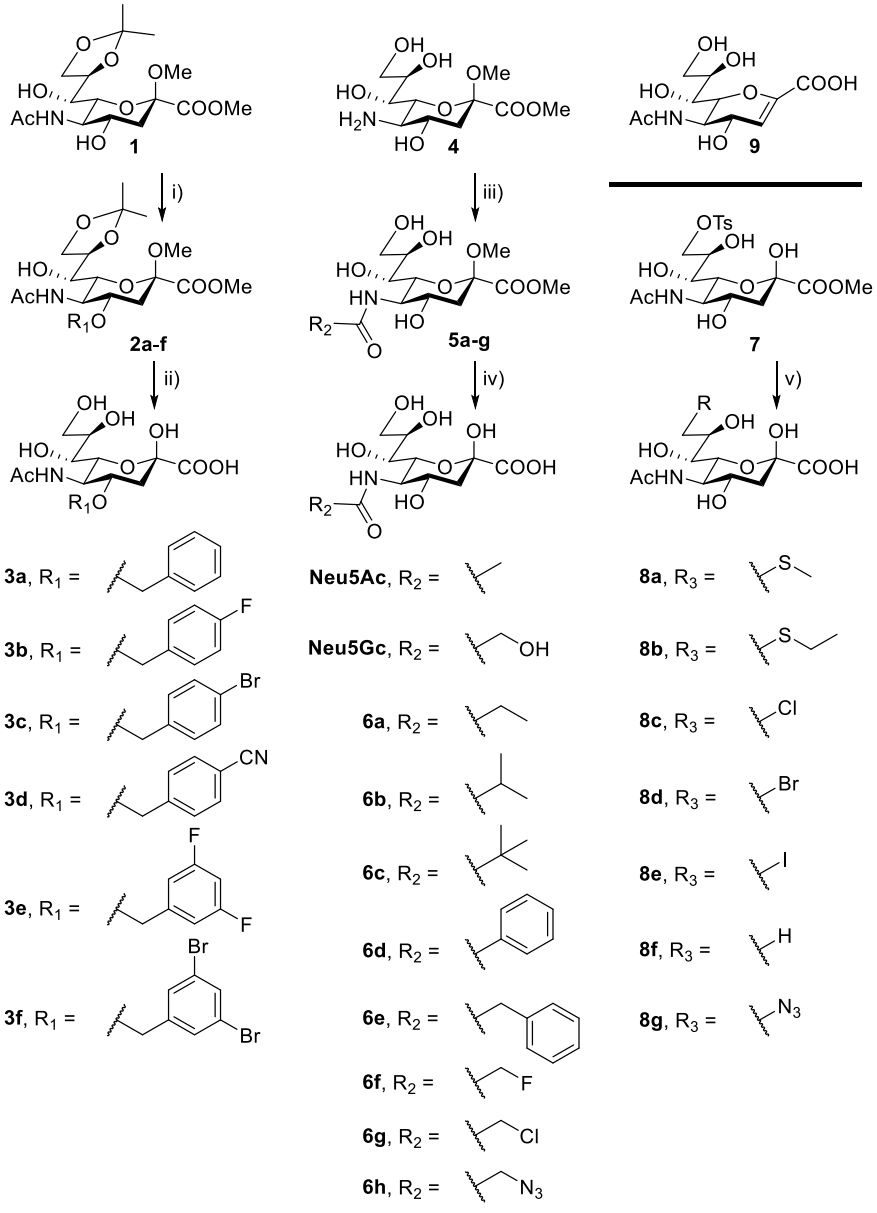


Figure 5. Overlay of the PmSiaT crystal structure (green, PDB ID 5NV9) and SaSiaT homology model (blue) from the outward-facing portion. The binding site is enlarged and the 4-OH, 5-NHAc and 9-OH are highlighted.

From the synthetic standpoint, we focused on reliable and quick derivatisation methods, using common and published intermediates to obtain compounds with a wide array of functional groups and, subsequentially, properties. The synthetic approach is summarised in **Scheme 3**.

To obtain the O4 derivatives, we started from known intermediate **1**[68] and employed Ag_2O-TBAI catalysis to limit the alkylation of the unprotected 7-OH.[69] The approach was successful and we developed a number of compounds with different substitutions on the benzyl functionality, though in moderate yields. For 5N, we chose intermediate **4**[70] and performed selective acylation reactions in the presence of TEA. In hindsight, it would have been advantageous for the acylation to use inorganic bases such as Cs_2CO_3, since TEA was problematic to remove during purification. This possibility, though, was not realized at the time. The 9C derivatives were synthesised via substitution of tosylate **7**[71] with different nucleophiles. The real challenges were found in the deprotections and, especially, purifications of the final compounds. In the case of compounds **2a-f** and **5a-g**, we employed a 2-step method using LiOH to first hydrolyse the ester, and then add Amberlyst® 15 (H^+) until acidic pH. The reactions were then refluxed until disappearance of the methyl sialoside. Such harsh conditions were essential but also detrimental for the compounds, leading to degradation and overall low yields. The presence of intermediates or side products with similar polarities also complicated the final purification via preparative HPLC and a lot of effort was spent there.



Scheme 3. Synthetic approaches in the development of the first sialic acid library. i) $ArCH_2Br$, Ag_2O, TBAI, dry ACN; ii) LiOH at r.t, followed by Amberlyst® 15 H^+ form at 100 °C in ACN-H_2O; iii) RCOCl, TEA, dry MeOH, 0° C; iv) Amberlyst® 15 H^+ form, ACN-H_2O 100 °C; v) Nu in DMF or ACN, followed by either acidic (Amberlyst® 15 H^+ form, H_2O, 95 °C) or basic (NaOH or LiOH) hydrolysis.

3.2 Compound screening by thermal shift assay

With the compounds at hand, we proceeded to establish standard and reliable testing methods. Differential scanning fluorimetry (DSF) came to our attention especially for the many advantages presented in its nanoDSF version.[72] NanoDSF is a methodology in which the melting temperature of the protein is measured based on the intrinsic fluorescence of the native protein itself. The intrinsic fluorescence is based on the fluorescence of the protein's tryptophan and tyrosine residues and directly proportional to the number of those. Upon denaturation, the environment of the fluorescent residues is altered, due to the collapse of the three-dimensional structure of the protein, thus producing a change in the protein's intrinsic fluorescence. In general, compounds binding a protein lead to an increase in the protein's melting temperature.[72] This is due to the compound stabilizing the folded state of the protein, and nanoDSF is therefore a direct measurement of binding of the compound to its target. Moreover, nanoDSF utilises 10 μL capillaries at 1-2 μM of protein and can allocate 48 samples per run, which takes, depending on the settings, generally 90 minutes. For all the previous reasons, nanoDSF represents an excellent screening method for our purposes.

We were delighted to observe how Neu5Ac induces significant thermal stabilisations in the case of both Pm and SaSiaT and we selected 1.25 mM as a screening concentration. At that concentration, we observed a thermal stabilisation with Neu5Ac of +3.7 and +8.6 °C, for PmSiaT and SaSiaT, respectively. With a testing method at hand, we then screened all compounds. It is possible to derive an affinity constant (K_d) from nanoDSF,[73] but we decided to use it as a qualitative method to select the most promising compounds that we would then evaluate with other techniques, such as isothermal titration calorimetry.

The nanoDSF results are shown in **Figure 6**. The results are presented as the difference (ΔT_m) between the compound T_m and the Neu5Ac one. Therefore, compounds with a higher T_m than Neu5Ac will be shown having a positive ΔT_m, while compounds with a lower T_m will display a negative one. Compounds with a thermal stabilisation similar to the protein alone, are to be considered non binders. It is immediately evident how compounds **3a-f**, presenting aromatic moieties on O4, induce higher thermal stabilisations than any other single modification. These results are particularly astounding in the case of **3f** for PmSiaT, where we observe a ΔT_m of +14.4 °C, by far the highest for both proteins. All other benzyl derivatives display ΔT_m between +4.0 and +7.0 °C for PmSiaT, and +2.4 and +5.8 °C for SaSiaT. In the case of SaSiaT, compound **3f** falls in the lower end of the benzyl spectrum with a ΔT_m of +2.6 °C. Modifications at N5 proved useful only when small polar substituents were introduced, as in compounds **6f-h**. Substitutions of the 9-OH did not yield compounds with better binding, compared to Neu5Ac, but the intermediate results of the halogen series (compounds **8c-e**) indicate promising leads. Compound **8f**, the 9-deoxy derivative, showed no thermal stabilisation, indicating the significance of the hydrogen bond for both targets.

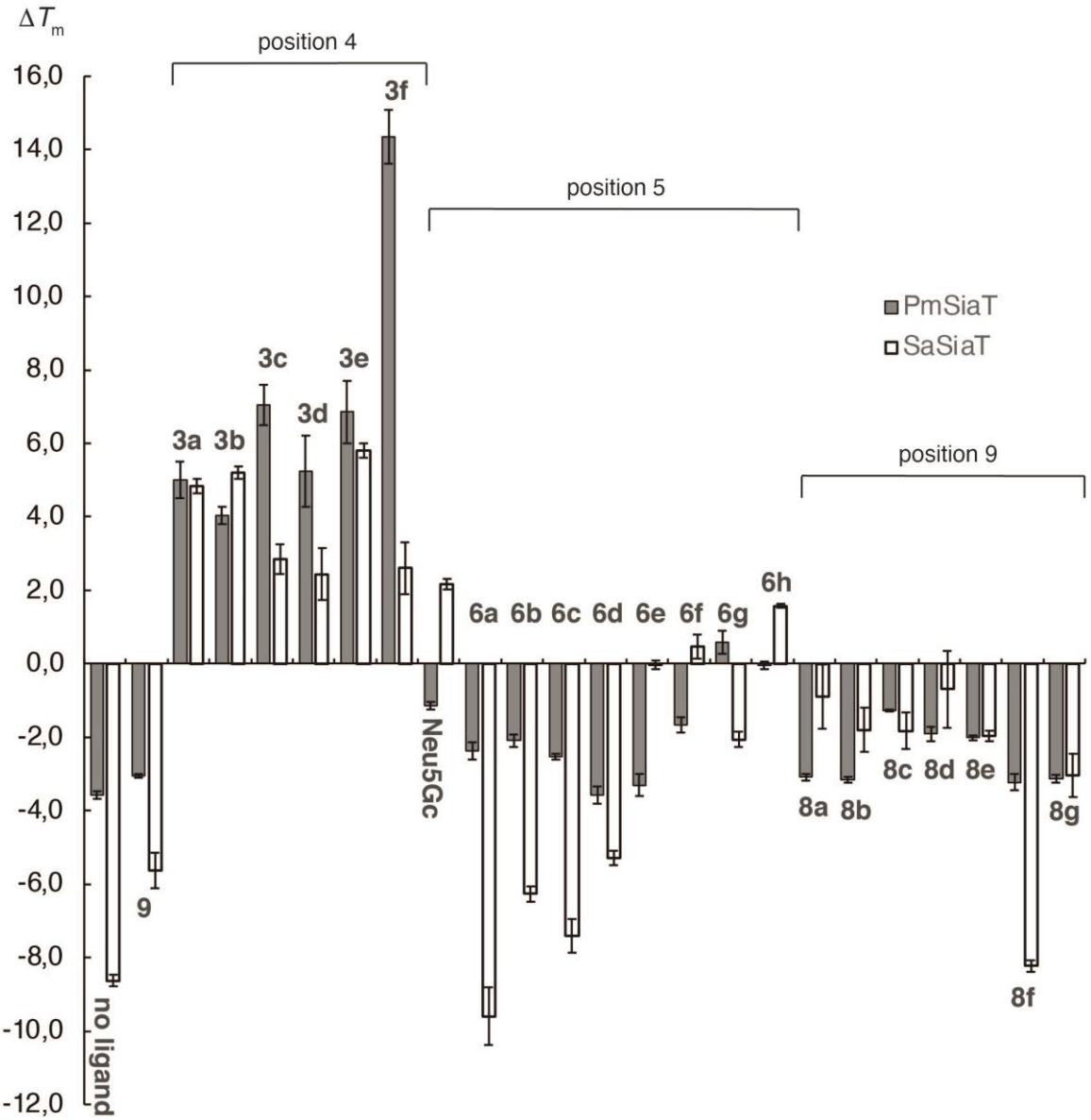


Figure 6. NanoDSF results for PmSiaT (dark) and SaSiaT (white). The ΔT_m reported are given in °C and relative to Neu5Ac, meaning that the ΔT_m of a given compound is the difference between the compound's T_m and the one from Neu5Ac. Accordingly, compounds with a negative ΔT_m have a lower T_m than Neu5Ac, while a positive ΔT_m indicates greater thermal stabilisation. All experiments were performed in triplicates and error bars represent ± s.d.

3.3 Evaluation of binding thermodynamics

To verify the ΔT_m and affinity increases observed with nanoDSF, we investigated the binding thermodynamics with isothermal titration calorimetry (ITC). ITC is a calorimetric methodology capable of measuring the heat released or absorbed during a binding event. In a standard set up, the protein solution is loaded in the sample cell and the ligand solution in the syringe. The compound solution is then titrated into the sample cell and, depending on the nature of the binding, the injections generate (exothermic reaction) or absorb (endothermic reaction) heat. The instrument applies energy (differential power DP) to maintain an equal temperature between the sample cell and a reference cell containing pure water. The resulting thermogram records the time-dependent power usage of this feedback circuit plotted against the mole fraction of reactants. By fitting this data to an appropriate thermodynamic model (i.e., the Wiseman equation),[74] the enthalpy, affinity, and stoichiometry of binding are determined. By applying equations 1 and 2, it is then possible to calculate all other thermodynamic parameters.

$$\Delta G° = -RTlnK_A = RTlnK_D \quad (1)$$

$$\Delta G° = \Delta H° - T\Delta S° \quad (2)$$

We selected three compounds: **3a**, **3e** and **3f**. Compound **3f** and **3e** have the highest ΔT_m for PmSiaT and SaSiaT respectively, while compound **3a** displays intermediate ones for both. Additionally, these compounds were chosen to test the correlation between nanoDSF and ITC results.

Table 1. Affinity data. ITC dissociation (K_d, µM), thermal shift changes from nanoDSF (ΔT_m, °C), and proteoliposome assay inhibitory constants (K_i, µM) of compounds **3a**, **3e** and **3f**.

	PmSiaT			SaSiaT		
Compound	**K_d**	**ΔT_m**	**K_i**	**K_d**	**ΔT_m**	**K_i**
Neu5Ac	50 ± 4[50]	0	-	130 ± 35	0	-
3a	9.0 ± 3.2	4.8 ± 0.5	-	8.8 ± 2.7	4.9 ± 0.2	-
3e	6.2 ± 2.8	6.8 ± 0.9	9.9 ± 2.6	4.1 ± 2.1	5.8 ± 0.2	2.8 ± 0.5
3f	0.27 ± 0.14	14.4 ± 0.7	0.13 ±0.04	15.7 ± 7.3	2.6 ± 0.7	53.6 ± 11.1

Table 1 and **Figure 7** highlight different aspects derived from the same data. In **Table 1** we can observe the affinity data and how it correlates between the different methods, while in **Figure 7** we observe the thermodynamic fingerprints. The ITC data confirms the initial findings from the nanoDSF screen, indicating excellent correlation from these orthogonal methodologies. Hence, compound **3f** displays the highest affinity, in the mid nanomolar range, for PmSiaT with a 185-fold increase compared to Neu5Ac. Compound **3e** proves to be the most potent one for SaSiaT, with a 31-fold affinity increase, while compound **3a** shows intermediate results for the two targets.

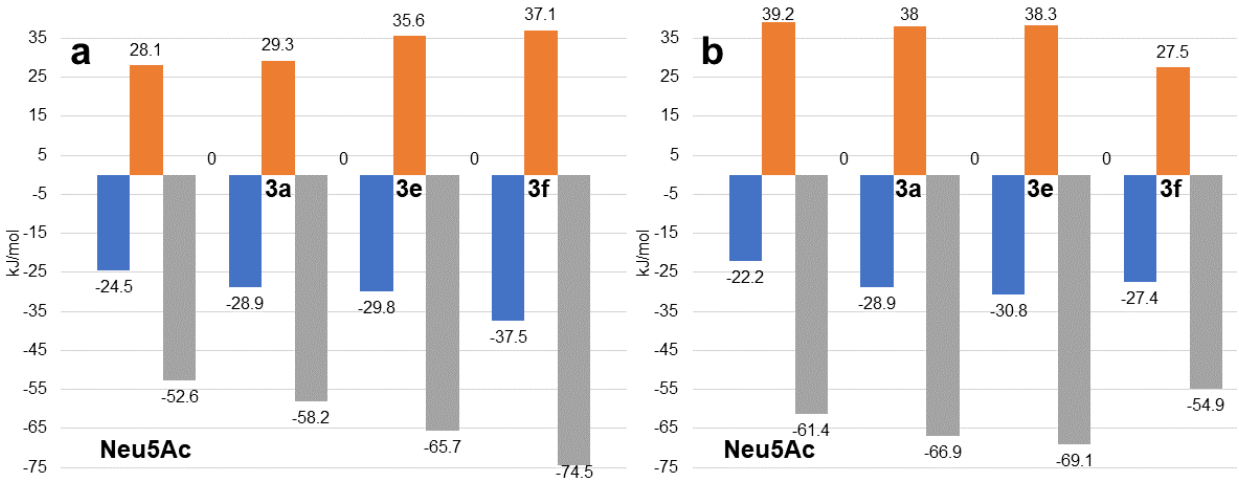


Figure 7. Thermodynamic data in kJ/mol for PmSiaT (**a**) and SaSiaT (**b**). ΔG (blue), ΔH (orange) and -TΔS (gray).

Figure 7 depicts the entropic and enthalpic contributions of compounds **3a**, **3e** and **3f** binding to PmSiaT (**a**) and SaSiaT (**b**). It is immediately evident, that the binding is entropically driven. Entropy is a complex parameter with several contributing factors, such as entropies associated with the ligand, the protein and water molecules. This multitude of contributions makes attributing "responsibilities" complicated. What we can say, is that the entropic component of our ITC experiments is very large and it is likely that changes in the protein conformational landscape contribute to a major extent. We hypothesise that our compounds bind the target and induce an entropy-enthalpy transduction (EET).[75] EET describes those binding events in which a ligand binds its target enthalpically, and induces a change in the conformational dynamics of the protein. Proteins are in equilibria between different conformations and each conformation has different thermodynamic properties. Binding of a ligand can shift the relative equilibrium of the different conformations. In those cases where the equilibrium is directed to a conformation having a higher entropy, the observed overall binding

thermodynamics will present a high entropic contribution. The entropic contribution of the protein overshadows the enthalpic contribution of the ligand, thus resulting in an entropy-driven binding.

The transport mechanism for PmSiaT has been previously modelled and described (Supplementary Movies 3 and 4 from Wahlgren *et al*).[50] From the movies and **Figure 8**, it is possible to see how Neu5Ac binding PmSiaT induces a dramatic change in the protein conformation, which subsequentially leads to uptake. We hypothesise that our compounds bind the target, but the binding event does not allow for the conformational change that would lead to uptake. This hypothesis is also strengthened by the computational and proteoliposome experiments described below.

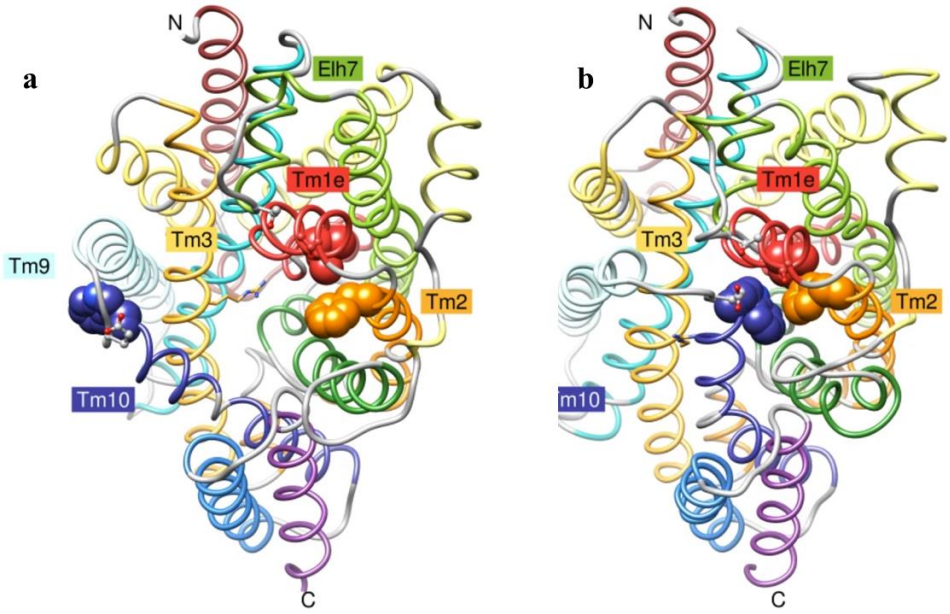


Figure 8. PmSiaT conformational change from the outward open (**a**) to the outward closed (**b**).

3.4 Molecular dynamic simulations

To investigate the affinity enhancement observed experimentally for compounds **3a**, **3e** and **3f,** we then performed molecular dynamics. The PmSiaT crystal structure with Neu5Ac binding is the only structure available to us.[50] Since this transporter has several conformations in which it can equilibrate, we are not sure if building the model from the binding pose of Neu5Ac is descriptive of the actual binding event of our compounds. Our collaborators from Sweden and New Zealand have been trying to cocrystallize compound **3f** with PmSiaT, but the attempts have been so far unsuccesful.

We therefore built starting complexes for molecular dynamics simulations by adding the benzyl functionalities to O4 of Neu5Ac in the PmSiaT crystal structure (PDB ID 5NV9) and in the SaSiaT homology model.[50,51] The simulations were run for 200 ns using OPLS3 force field in Desmond (Schrödinger Release 2020-4). The three simulations with PmSiaT all converged to stable poses with the Neu5Ac portion remaining approximately in the same position, while the ones with SaSiaT did not. We attribute the issue of the SaSiaT simulations to unknown shortcomings in the homology model setup and decided to not investigate these further.

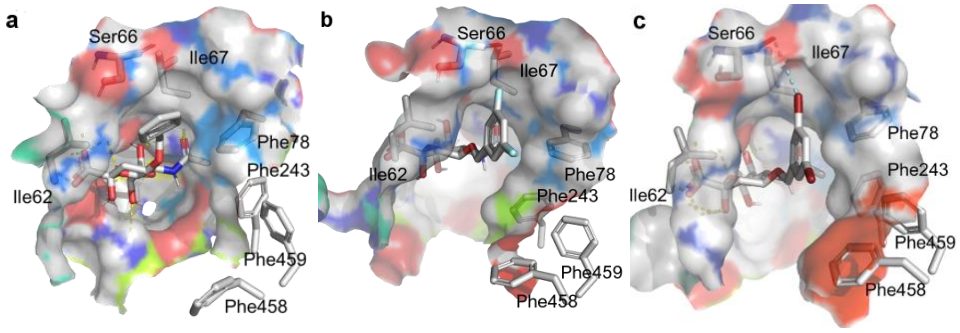


Figure 9. Representative MD snapshots at 195 ns of: (**a**) Compound **3a**; (**b**) Compound **3e**; (**c**) Compound **3f** in complex with PmSiaT (pdb id 5NV9). The bromo to Ser66 backbone carbonyl contact for compound **3f** is indicated with a turquoise dashed line in panel (c).

All three benzyl groups project towards the opening of the channel protein. In this part of the protein, several hydrophobic residues such as Ile62, Ile67, Phe243, Phe458, and Phe459 are present (**Figure 9**). This part of PmSiaT has been previously termed "the hydrophobic gate".[50] Therefore, the benzyl derivatives are capable of establishing different hydrophobic interactions expecially with the phenylalanines.

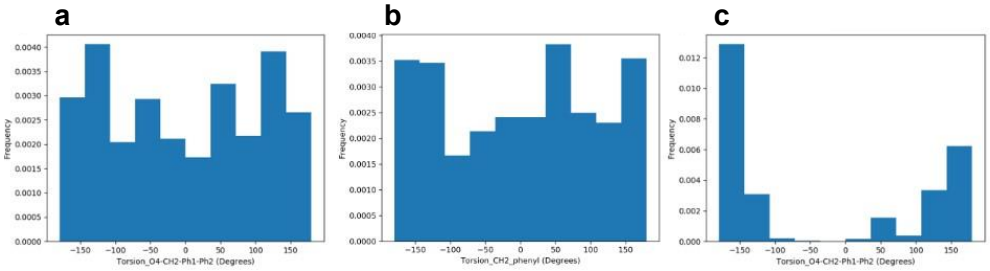


Figure 10. Dihedral angle distribution of the *O*4-CH_2-phenyl bond of (**a**) compound **3a**, (**b**) compound **3e** and (**c**) compound **3f**. These were measured through the 200 ns simulations with PmSiaT.

To investigate the interactions further, we took a closer look at the dihedral angle distribution of the O4-CH_2-phenyl bond of the three compounds (**Figure 10**). What became evident was how compound **3f** sampled significantly less orientations and remained prodominantly settled in one throughout the simulation. Compound **3f** seems to fit the binding pocket with excellent shape complementarity, which could partially explain the reduced movement during the simulation. Additionally, a bromo substituent seems to establish an interaction with the carbonyl of Ser66, thus suggesting the presence of an halogen bond (3-4 Å) which could also contribute to the greater affinity of compound **3f** over the other ones.

3.5 Inhibition assay using proteoliposoms.

To further investigate the inhibition, our collaborators at the Università della Calabria performed functional assays using proteoliposomes. In this experimental model, the purified protein is inserted in an artificial membrane (liposome) with the same orientation as in the native membrane. This tool provides the great advantage of studying the activity of a single protein without any interference deriving from other membrane proteins or intracellular enzymes and effectors.[76] Therefore, in a proteoliposome assay, we can study uptake, uptake inhibition and derive the kinetic parameters in a cell-like system.[77]

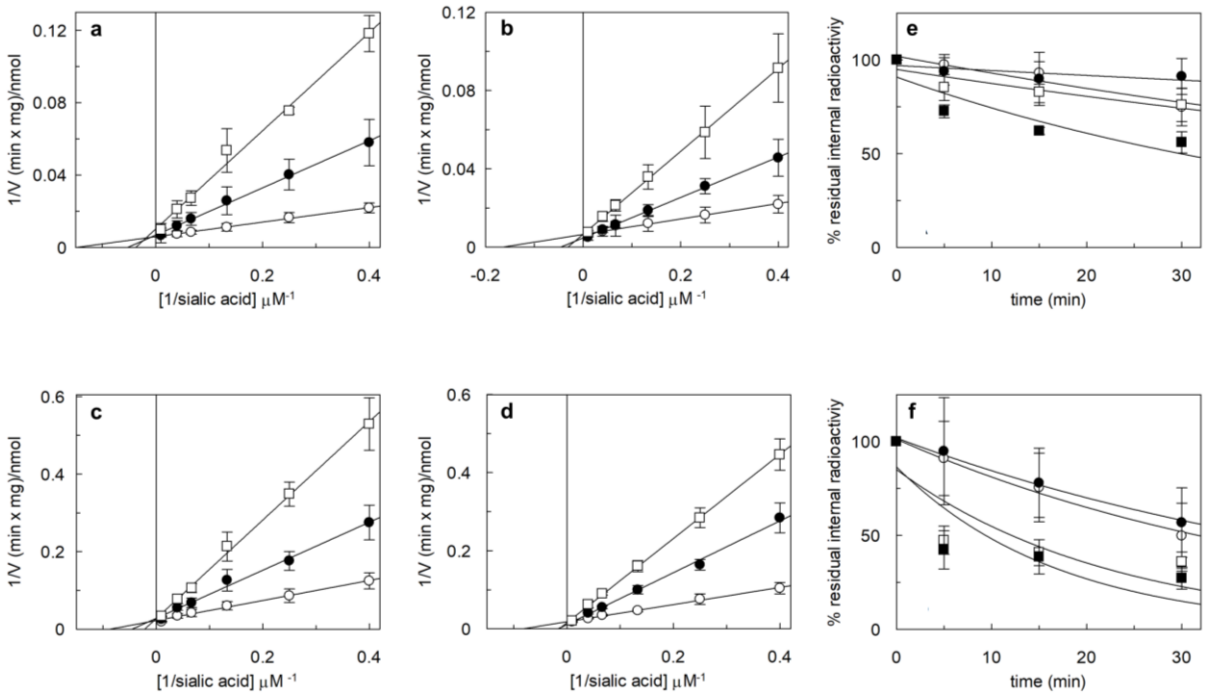


Figure 11. Kinetic analysis of the inhibition of recombinant SiaT from *P. mirabilis* (**a-b**) and *S. aureus* (**c-d**) reconstituted in proteoliposomes and [^{3}H]Neu5Ac efflux in proteoliposomes (**e-f**). **a-d**, Data were plotted according to Lineweaver–Burk as reciprocal transport rate vs reciprocal Neu5Ac concentration. Transport rate was measured, in 5 min, by adding [^{3}H] Neu5Ac at the indicated concentrations to proteoliposomes containing 20 mM K^+-gluconate in the presence of valinomycin as described in the Supporting Information. (**a-b**, *P.mirabilis* SiaT); (**a**) Compound **3f** 0.25 µM (●) or 0.75 µM (□) was added in comparison to samples without inhibitor (○). (**b**) Compound **3e** 12 µM (●) or 40 µM (□) was added in comparison to samples without inhibitor (○). (**c-d**, *S.aureus* SiaT); (**c**) Compound **3f** 80 µM (●) or 200 µM (□) was added in comparison to samples without inhibitor (○). (**d**) Compound **3e** 5 µM (●) or 12 µM (□) was added in comparison to samples without inhibitor (○). Results are mean ± s.d. from four independent experiments (n = 4). In (**e**), the efflux of [^{3}H]Neu5Ac was measured from proteoliposomes harbouring PmSiaT in the absence of external substrate (o), or in the presence of 0.1 mM of external Neu5Ac (■) or 0.1 mM compound **3e** (□) or 0.1 mM compound **3f** (•) at the indicated times. In (**f**), the efflux of [^{3}H]Neu5Ac was measured from proteoliposomes harbouring SaSiaT in the absence of external substrate (o), or in the presence of 0.1 mM of external Neu5Ac (■) or 0.1 mM compound **3e** (□) or 0.1 mM compound **3f** (•) at the indicated times. Data are calculated as the percent of residual activity with respect to the control sample (efflux time zero). Results are means ± SD of three independent experiments.

PmSiaT and SaSiaT were reconstituted in proteoliposomes and the transport activity was measured as uptake of radiolabelled [^{3}H]-Neu5Ac. The kinetic inhibition was studied using two different concentrations of compounds **3e** and **3f** and the results were analysed by Lineweaver-Burk plots (**Figure 11 a-d**).

The kinetic analysis allowed us to measure a K_m of 7.5 ± 1.6 µM and a V_{max} of 180 ± 56 nmol/mg protein/min for Neu5Ac for PmSiaT, while for SaSiaT the respective values were 12.2 ± 2.2 µM and 51 ± 9 nmol/mg protein/min. These values seem to indicate a slightly better transport capacity of PmSiaT over SaSiaT. The inhibitory constants of compounds **3e** and **3f** are shown in **Table 1** and are in line with the ITC and nanoDSF results, indicating robustness of the results over different orthogonal methods. Additionally, the Lineweaver-Burk plot reveals that compounds **3e** and **3f** act as competitive inhibitors, hence binding to the same binding site as Neu5Ac.

To investigate whether these compounds behave as substrates or uptake inhibitors, our collaborators performed efflux studies using proteoliposomes. After uptake of [^{3}H]-Neu5Ac, the proteoliposomes were added to 0.1 mM of Neu5Ac, compound **3e** or **3f** (**Figure 11 e-f**). With Neu5Ac present, a measurable leaking of [^{3}H]-Neu5Ac was observed for both PmSiaT and SaSiaT, indicating efflux of [^{3}H]-Neu5Ac and uptake of Neu5Ac. With the inhibitors present, no counterflow was measured for PmSiaT, indicating that compound **3e** and **3f** block the transporter and therefore do not act as substrates. In the case of SaSiaT, compound **3e** did induce a counterflow similar to Neu5Ac (**Figure 11f)**, suggesting that it might act as a substrate. Such behaviour was not observed for compound **3f**.

3.6 Bacterial growth

To determine the efficacy of our compounds at blocking bacterial sialic acid uptake *in vivo*, we designed bacterial growth assays together with our collaborators at Lund University. To assess the reduction in sialic acid uptake, we grew the bacteria in minimal media with Neu5Ac as additional carbon source. By adding our compounds, an alteration of the bacterial growth would be indicative of a disruption of the sialic acid uptake. We performed the assays with *P. mirabilis* strain HI4320 and a clinical HA-MRSA isolate *S. aureus* strain COL. The results are shown in **Figure 12**. We did not observe any significant effect in the case of *P. mirabilis*, while compounds **3e** and **3f** induced a growth delay of 4.4 and 9.4 hours, respectively in *S. aureus*. These results were surprising since the previous results consistently indicated how compound **3f** had the most pronounced affinity and uptake inhibition on PmSiaT.

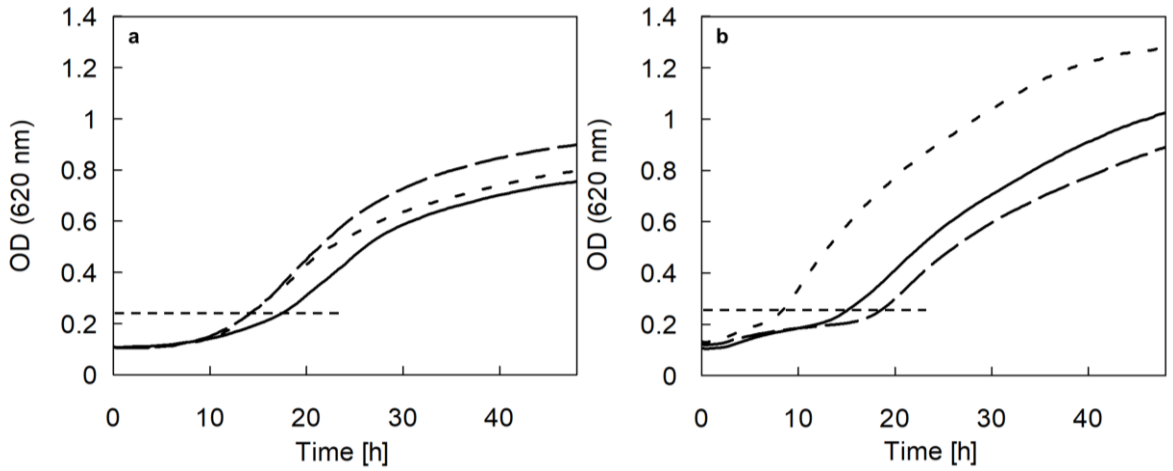


Figure 12. (**a**) *P. mirabilis* HI4320 grown in minimal media supplemented with Neu5Ac (3.2 mM) and no inhibitor (dotted line), compound **3e** (0.5 mM, solid line) and compound **3f** (0.5 mM, dashed line). (**b**) *S. aureus* COL grown in minimal media supplemented with Neu5Ac (3.2 mM) and no inhibitor (dotted line), compound **3e** (0.5 mM, solid line) and compound **3f** (0.5 mM, dashed line). Average values of five independent biological replicates (n = 5) including in total 13 technical replicates are presented. Horizontal dashed line indicates end of lag phase corresponding to the time for the initial population density to increase twofold.

Thus, we performed similar bacterial growth assays using an *E. coli* JW3193 ΔNanT, a mutant with the nanT gene (encoding the endogenous sialic acid transporter) deleted. Such mutant had been previously reported unable to grow on Neu5Ac as sole carbon source.[51] Its ability to grow on Neu5Ac was restored when genes for PmSiaT and SaSiaT were introduced. Our collaborators at University of Canterbury, New Zealand performed the experiments, but again we observed no bacterial growth inhibition caused by compounds **3e** and **3f**.

3.7 Characterisation of physiochemical properties and metabolic stability

The importance of physiochemical and ADME properties is commonly overlooked and such negligence often leads to failure of a drug discovery program.[78] Hence, physiochemical and metabolic stability investigations early in the research have to be taken into account in the choice of lead compounds to bring forth in later stages of the research. Therefore we assessed such properties for compounds **3a**, **3e** and **3f**. These investigations were conducted by our collaborators at Red Glead Discovery AB.

First, the physiochemical properties were analysed and showed how these compounds are, maybe unsurprisingly, polar (logD at 7.4 < 0) and soluble (solid solubility > 1630 μM). These results were expected since compound **3a**, **3e** and **3f** are sialic acid derivatives. We were concerned about the metabolic stability of the benzyl moieties, since it is known that they can be oxidatively removed by cytochromes.[79]

The metabolic stability was tested in human, rat and mouse microsomes and showed intrinsic clearance Cl_{int} <10 mL/min/mg protein. The plasma stability was assessed in five different species (human, dog, mini pig, rat, mouse) and compounds **3a** and **3e** displayed full recovery after 23 hours. Compound **3f** was the least stable, with 91%, 81% and 39% recovery in human, dog and mini pig respectively, while still full recovery in rat and mouse. The last ADME property assessed was plasma protein binding by RED (rapid equilibrium dialysis) in

three different species (human, rat and mouse). Compound **3f** is the least apolar and showed a lower free fraction (21-27%), probably due to this aspect, while compounds **3a** and **3e** had higher ones (63-85%).

Overall, the tested properties were found interesting and suitable for developing these class of molecules further.

3.8 Conclusions

We started this work by asking the question whether it would be possible to inhibit bacterial sialic uptake by targeting the SiaT transporters. We thus designed and synthesised a library of compounds with modifications at O4, N5 and C9 and evaluated their affinity with a thermal shift assay. From this initial screen, we identified the 4-*O*-benzyl series as the most promising lead and selected three compounds for further evaluations. We then investigated the binding thermodynamics of compounds **3a**, **3e** and **3f** with both PmSiaT and SaSiaT and recorded significant affinity increases for both targets. The affinity increase seemed to be largely entropically driven and we hypothesised that it could be explained by EET. Molecular dynamics helped us in understanding the reasons for the affinity increases for PmSiaT. The compounds establish additional interactions with a portion of the binding site rich in aromatic and hydrophobic residues, with compound **3f** displaying excellent shape complementarity and a plausible hydrogen bond to a backbone carbonyl.

Subsequently, we examined the Neu5Ac uptake inhibition with proteoliposomes and found that compounds **3e** and **3f** behave as competitive inhibitors and do not mostly act as substrates. Only compound **3e**, in the case of SaSiaT, indicated a possible substrate behaviour. The compound efficacy was evaluated *in vivo* via bacterial growth assays and compound **3e** and, especially, **3f** caused a pronounced growth delay on a HA-MRSA strain. The compounds did not seem effective in the case of Gram-negative bacteria, such as *P. mirabilis* and *E. coli*. The reasons might be the compounds' inability to enter the outer membrane or metabolic instability in the periplasmic space. Lastly, we investigated the physiochemical properties and metabolic stability of compounds **3a**, **3e** and **3f** and found them to be well suited for further development of this class of compounds.

Overall, these results indicate that it is possible to target bacterial sialic acid uptake by inhibiting the SiaT transporters.

3.9 Key findings

- Introduction of aromatic residues on O4 of Neu5Ac leads to significant affinity increases for the SiaT transporters.
- The affinity increase is largely entropically driven, but this might be explained with entropy-enthalpy transduction (EET). The enthalpic binding of the compounds may induce a conformational change in the protein that leads to a more entropically favoured protein state, resulting in an entropically-looking binding.
- The affinity increase is enabled by additional interactions with hydrophobic residues located in the outer hydrophobic gate.
- Compounds **3e** and **3f** inhibit Neu5Ac uptake in proteoliposome by competitive inhibition. Compound **3f** might be a substrate for SaSiaT, while that is not the case for the other tested combinations.
- Compounds **3e** and **3f** delay bacterial growth of HA-MRSA strain COL when Neu5Ac is the predominant carbon source. That is not true for the tested strains of *P. mirabilis* and *E. coli*.
- Compounds **3a**, **3e** and **3f** possess promising physiochemical and ADME properties.

4 Targeting multiple sialic acid transporter families

In our quest towards broad-spectrum sialic acid uptake inhibitors, we selected two additional protein targets to focus our efforts on. The two proteins are both substrate binding proteins (SBP), but from two different transporter families. We selected FnSiaP, the SBP from the TRAP transporter of *F. nucleautum*, and HdSatA, the SBP from the ABC transporter of *H. ducreyi*. We chose these targets for several reasons. Firstly, FnSiaP and HdSatA are easy to produce, especially when compared to membrane proteins such as the SiaTs. Secondly, their crystal structures have been elucidated both in the liganded and unliganded forms, with the binding affinities and binding thermodynamic investigated by ITC experiments.[44,47] The binding event of Neu5Ac with the two SBPs is quite different when compared to the SiaTs. Neu5Ac binding FnSiaP and HdSatA is reported to induce dramatic conformational changes described as Venus flytrap or Pac-Man models.[44,80,81] These models describe how the substrate induces a shift in the proteins internal interactions, leading to the protein folding and enclosing the substrate. These mechanisms create a tight and defined binding site with distinct interactions (**Figure 13**), which consequently translate in high affinities. Accordingly, FnSiaP and HdSatA have high affinities for sialic acids, both in the low nanomolar range **Table 2**.

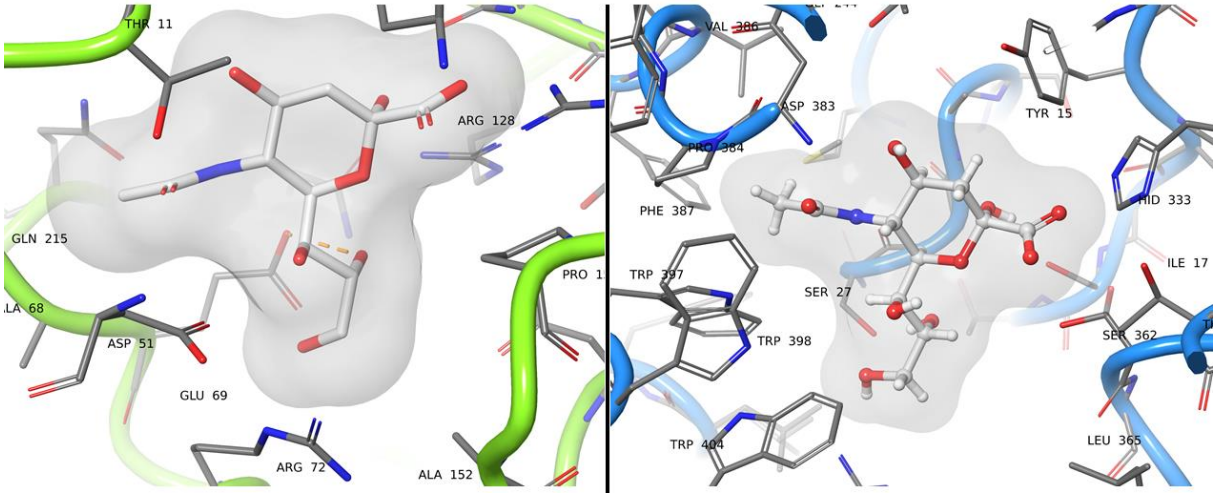


Figure 13. Crystal structures of Neu5Ac binding FnSiaP (green, pdb ID 4mnp) and HdSatA (blue, pdb ID 5z99). The images were genereted using Maestro (Schrödinger Release 2022-1).

4.1 Thermal shift assay

Based on our initial work on the SiaTs, we started by examining whether nanoDSF could be used as a screening method for the new targets. We therefore conducted a Neu5Ac dilution series to identify concentrations capable of significant ΔT_m in FnSiaP and HdSatA (**Figure 14**).

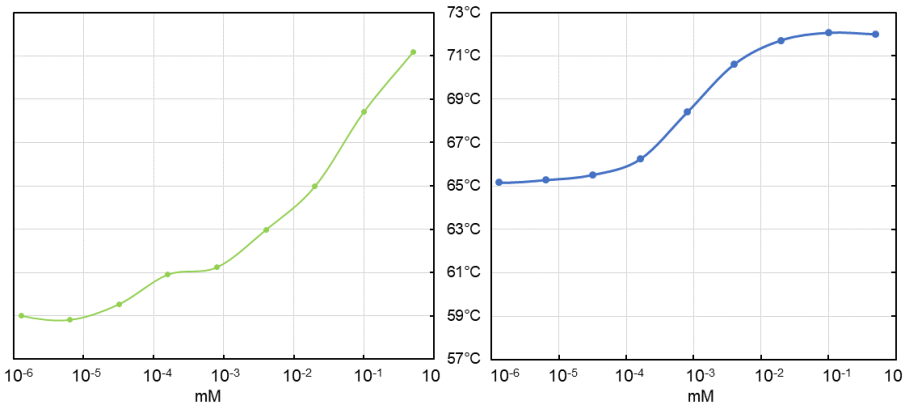


Figure 14. Neu5Ac dilution series with FnSiaP (green) and HdSatA (blue). On the x axis, the Neu5Ac concentration in mM is in logaritmic scale.

As for the SiaTs, Neu5Ac induces significant thermal stabilisations in both FnSiaP and HdSatA. Pleasantly, the high affinity of both proteins for Neu5Ac was reflected in the nanoDSF screen, with large thermal shifts observable already at low micromolar concentrations. We identified 0.2 and 0.04 mM as screening concentrations for FnSiaP and HdSatA, respectively.

Previously, we have developed a compound library with single modifications of Neu5Ac at O4, N5 and C9 (**Scheme 3**). We decided to employ this compound library to test which portions of Neu5Ac could tolerate derivatisations, probe for additional interactions and try to observe overall trends to guide the design of broad-spectrum activity compounds. Thus, we performed nanoDSF experiments on both FnSiaP and HdSatA. The results are presented in **Figure 15**. The results from the PmSiaT screen, previously reported in **Figure 6**, are included to observe trends and draw general conclusions.

It is immediately evident that no compound elicits the same degree of thermal stabilisation as the 4-*O*-benzyl series **3a-f** on PmSiaT.

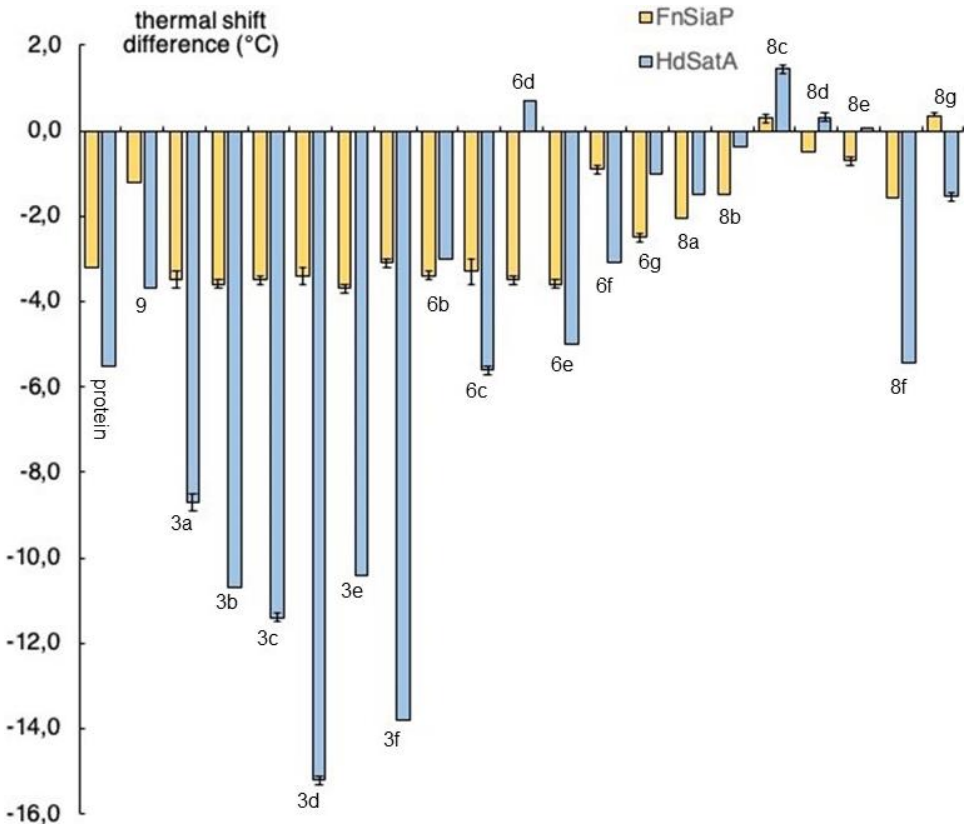


Figure 15. nanoDSF screens of compounds **3a-f**, **6a-g**, **8a-g** and **9** on PmSiaT (brown), FnSiaP (yellow) and HdSatA (blue). The ΔT_m reported are given in °C and relative to Neu5Ac, meaning that the ΔT_m of a given compound is the difference between the compound's T_m and the one from Neu5Ac. Accordingly, compounds with a negative ΔT_m have a lower T_m than Neu5Ac, while a positive ΔT_m indicates greater thermal stabilisation.

For FnSiaP, no significant stabilisation is observed, with modifications only tolerated at C9. Compounds 9-chloro **8c** and 9-azido **8g** display the best thermal stabilisations, in line with Neu5Ac. The tightness of the FnSiaP binding site is also exemplified by the decreasing ΔT_m trend observed for the 9-halogen series **8c-e**, with the 9-chloro **8c** showing the highest ΔT_m, opposed to the lowest one of the 9-iodo **8e**. The only permitted modification at N5 is the fluoroacetyl of compound **6f**, likely due to the small size, similar to the 5-glycolyl of Neu5Gc.

For HdSatA, the results indicate a higher degree of freedom for derivatisations, in a still tight binding site. The 9-chloro derivative **8c** again shows the highest ΔT_m, with good to intermediate results for the C9 derivatives **8a-e** and **g**. Dissimilar to FnSiaP, the 5-benzoyl functionality of **6d** retains affinity for the target. Compound **6d** seems to represent the upper limit size at N5, since the phenylacetyl group of **6e** displays no thermal stabilisation. A similar trend is also observed for compounds **6b** and **6c**, where the addition of one methyl (from 5-isobutyryl to 5-trimethylacetyl) causes complete loss of affinity.

4.1.1 Thermal destabilisations

The 4-*O*-benzyl series **3a-f** induce significant thermal destabilisation for HdSatA. Such observations are not uncommon for DSF experiments. Negative thermal shifts are often regarded as the compound binding the unfolded state of the protein,[72] while others claim binding to the native state or other protein conformations with lower melting temperatures.[82,83] We tried to investigate these destabilisations via ITC and microscale thermophoresis (MST). Unfortunately, our attempts have so far been unsuccessful. Up to date, we believe that these thermal destabilisations indicate binding to a different protein state. However, more work is needed, to fully understand the nature of such protein state.

4.2 Evaluation of binding thermodynamics

To investigate the affinities and the forces driving the binding event, we selected compounds **8c** and **8g** for FnSiaP, and **6d** and **8c** for HdSatA and performed ITC experiments. The results are presented in **Table 2**.

Table 2. K_d values and thermodynamic parameters of compounds **6d**, **8c** and **8g** for FnSiaP and HdSAtA. The thermodynamic values are expressed in kJ/mol.

	FnSiaP				HdSatA			
Compound	**K_d (nM)**	**Δ*G*°**	**Δ*H*°**	**-TΔ*S*°**	**K_d (nM)**	**Δ*G*°**	**Δ*H*°**	**-TΔ*S*°**
Neu5Ac	45.5[47]	-41.9	-38.4	-3.6	133[44]	-39.3	-9.3	-30.0
6d	-	-	-	-	223[a]	-38.0	5.2	-43.2
8c	578 ± 550	-35.6 ± 1.2	-7.9 ± 1.5	-27.7 ± 2.5	469[a]	-36.2	3.9	-40.1
8g	159 ± 60	-38.8 ± 0.8	-14.6 ± 0.8	-24.2 ± 1.6	-	-	-	-

[a] single replicate.

For FnSiaP, the nanoDSF and ITC data do not seemingly correlate. Despite very similar thermal shifts from nanoDSF, we recorded a decrease in affinity of 13- and 3-fold for compounds **8c** and **8g,** respectively. This is not surprising when the differences of the two methods are taken in account. In a nanoDSF experiment, FnSiaP denaturates between 58 and 62 °C. That is 33-37 °C higher than the temperature in which an ITC experiment is performed. Dissociation constants, as shown in **Eq. 1**, are temperature-depended. Moreover, the specific binding thermodynamics of Neu5Ac and compounds **8c** and **8g** need to be taken into account to comprehend the divergence between the two methods. From **Table 2** and the literature,[47] it is evident that Neu5Ac binds FnSiaP enthalpically, with formation of salt bridges and H-bonds. Compounds **8c** and **8g** display different behaviours, with a predominantly entropy-driven binding. Entropy, as in **Eq. 2**, is also temperature dependent. Consequentially, entropic binders will artificially increase their affinity for the target as the temperature increases, while the opposite is true for enthalpic binders.[72] In the light of this knowledge, the nanoDSF and ITC data give complementary information, which results in good agreement.

For HdSatA, the change in the binding thermodynamics is even more pronounced. Neu5Ac binds HdSatA with a predominant entropic component (**Table 2**). Both compounds **6d** and **8c** display positive $\Delta H°$, accentuating how the entropy dominates the binding event.

4.3 Conclusions

We investigated which sites are most suited for the development of Neu5Ac derivatives capable of targeting different bacterial sialic acid transporter families, namely PmSiaT, FnSiaP and HdSatA. To do so, we established testing methods to evaluate the affinities of the compounds using nanoDSF and ITC. To study initial SARs for the three targets, we employed the previously described compound library (**Scheme 3**).

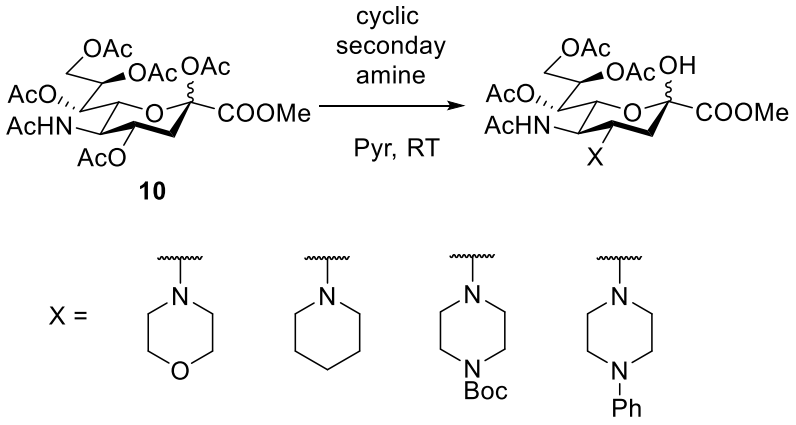
Our results indicate challenges in such a pursuit. The challenges reside in the differences associated with the diverse binding sites. 4-*O*-benzyl derivatives **3a-f** show promising results for PmSiaT, but negative and unaccountable ones for FnSiaP and HdSatA, respectively. Derivatisations at N5 are largely not tolerated by all targets, with the 5-benzoyl derivative **6d** as a lonely exception for HdSatA. C9 of Neu5Ac appears as the most promising site of derivatisation, since all targets retain affinity for compounds **8a-e** and **8g**, although to different degrees.

4.4 Key findings

- FnSiaP and HdSatA possess tight binding sites that allow only for the introduction of small substituents.
- Modifications of the Neu5Ac structure led to reduction of the enthalpic thermodynamic component, compensated by entropic gains.
- C9 of Neu5Ac appears as the most promising site for derivatisation to develop compounds capable of targeting the three proteins.
- The 4-*O*-benzyls **3a-f** induce thermal destabilisations for HdSatA which will require further investigations.

5 Development of alternative scaffolds at C4

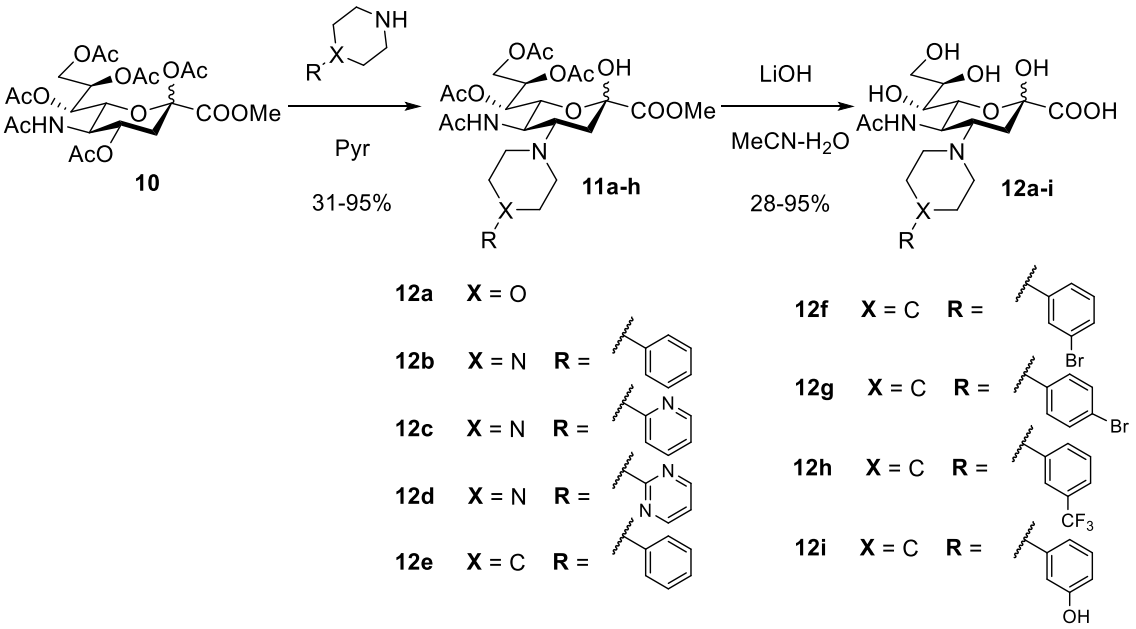
Based on the results from Paper I, the introduction of aromatic substituents at O4 is an effective strategy to develop Neu5Ac derivatives with significantly higher affinities for PmSiaT. We therefore envisioned introducing different scaffolds between C4 and the aromatic functionality, both to sample different portions of the "hydrophobic gate" and to achieve additional interactions. The procedure described by Ye *et al* sparked our interest for the possibility of introducing cyclic secondary amines at C4, in a single step, with retention of the configuration, from the commonly employed intermediate **10** (**Scheme 4**).[84]



Scheme 4. Reaction as reported by Ye *et al* with a selection of the cyclic secondary amines employed.[84]

The procedure allows for the introduction, among others, of piperidine and piperazine scaffolds which could function as linkers between C4 and the aromatic group. In the view of their cyclic structure, piperidines and piperazines could restrain the position of the aromatic moiety, thus facilitating interactions with the hydrophobic residues in the protein. Moreover, the nitrogen atoms could engage in additional interactions such as H-bonds, cation-π or salt bridges. Piperidines and piperazines are privileged scaffolds in drug discovery programs and are found in several marketed drugs.[85,86]

We therefore selected morpholine, 4-phenylpiperazine and 4-phenylpiperidine (compounds **11a**, **b** and **e**) as an initial screen to test the synthetic feasibility and probe the novel structural motives on PmSiaT (**Scheme 5**). The synthesis was unproblematic, and we swiftly obtained final compounds **12a**, **b** and **e** after basic hydrolysis. Given the rapid results, we also synthesised compounds **12c** and **d**.



Scheme 5. Synthesis of compounds **12a-i**.

Compounds **12b-e** are similar, with single point differences. Their selection and evaluation were conceived to identify the most promising scaffold for further functionalisation. We evaluated the affinity of compounds **12a-e** with our screening method of choice, nanoDSF (**Figure 16**).

The initial nanoDSF screen was fruitful and gave us several insights. Compounds **12b-e**, *i.e.* the ones bearing aromatic substituents, exhibited favourable thermal shifts. The best result came from the 4-phenylpiperidine **12e**, with a ΔT_m of 7.4 °C, the second highest one in absolute terms, after the 3,5-dibromobenzyl **3f**. Accordingly, we selected the 4-phenylpiperidine as starting point for further derivatisations. To better understand in which direction to carry on, we selected functionalisations at the *ortho* and *para* positions of the phenyl group, with electron donating and withdrawing substituents (compounds **12f-i**).

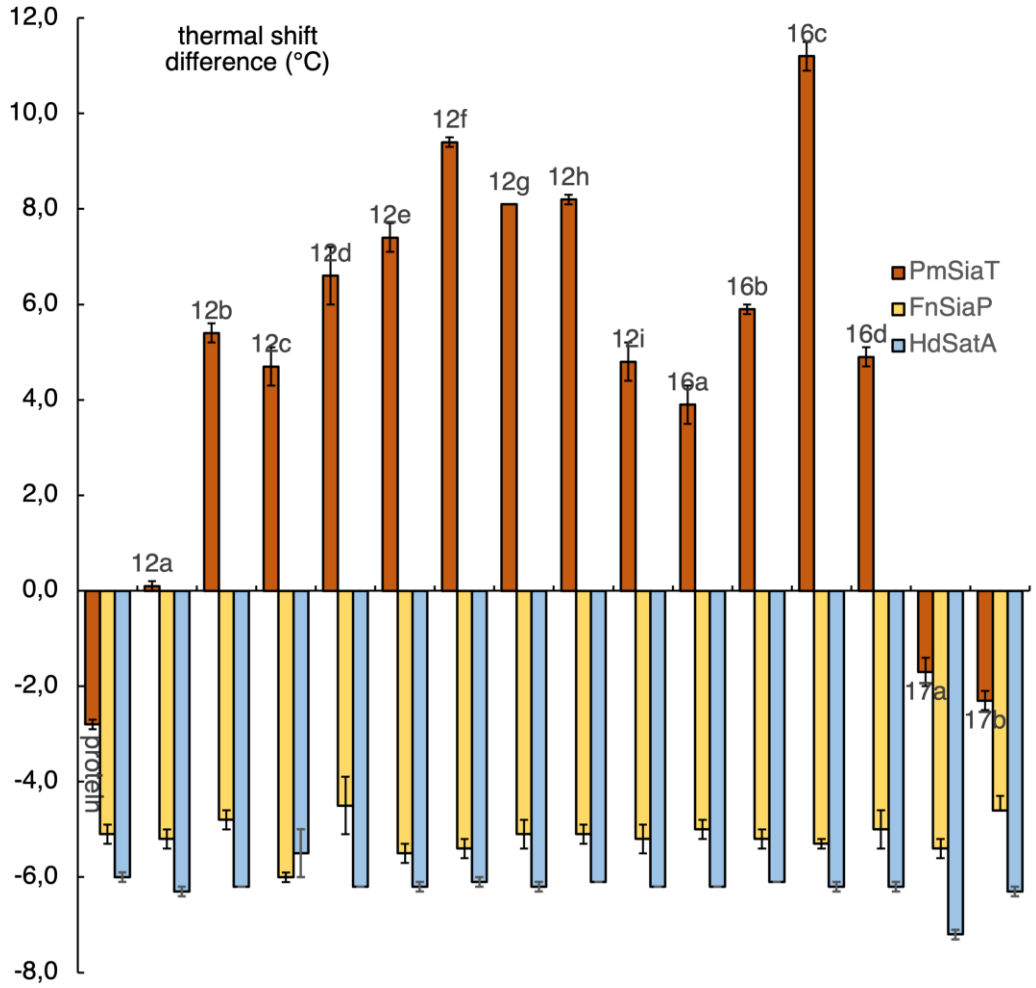
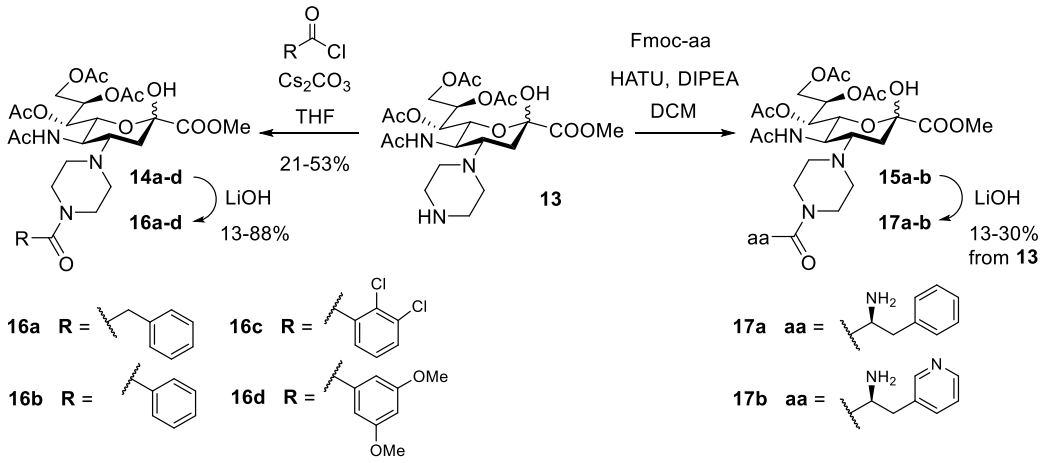


Figure 16. nanoDSF results for compounds **12a-i, 16a-d** and **17a-b** The ΔT_m reported are given in °C and relative to Neu5Ac, meaning that the ΔT_m of a given compound is the difference between the compound's T_m and the one from Neu5Ac. Accordingly, compounds with a negative ΔT_m have a lower T_m than Neu5Ac, while a positive ΔT_m indicates greater thermal stabilisation.

We also employed the piperazine scaffold as a tool to further distance the aromatic moiety via the formation of amide bonds at the distal nitrogen (**Scheme 6**). In such fashion, we planned and synthesised compound **16a**, with an *N*-benzoyl group and **16b**, with an *N*-phenylacetyl. Additionally, peptide coupling was utilised to increase the linker length and examine the introduction of an extra amino group. Thus, we achieved the synthesis of compounds **17a-b** bearing an L-phenylalanine and the unnatural L-pyridylalanine, respectively. Lastly, we developed two derivatives of **16b** with electron-withdrawing and donating groups (compounds **16c-d**). The overall nanoDSF results are presented in **Figure 16**.



Scheme 6. Synthesis of compounds **16a-d** and **17a-b**.

The nanoDSF indicated some clear trends, with electron-withdrawing groups enhancing affinities for both the 4-phenylpiperidine and 4-benzoylpiperazine series (compounds **12f-h** and **16c**). To the contrary, electron-donating groups such as the 3-hydroxy and 3,5-dimethoxy led to loss of affinity compared to the parent structure (compounds **12e** > **12i** and **16b** > **16d**). The two observations might be explained by the increased hydrophobicity of the halogenated derivatives, as well as the possible establishment of hydrophobic interactions and halogen bonds. Moreover, the halogenated derivatives should possess reduced desolvation penalties, when compared to compounds **12i** and **16d**, which would contribute to a gain in entropy.

From preliminary molecular dynamics experiments, the aromatic group of **16c** extends towards the hydrophobic gate, but to a different portion compared to the one sampled by the 4-*O*-benzyl derivatives (**Figure 17**). Compound **16c** forces a change in the orientation of Phe243, Phe458 and Phe459 to an induced fit. Experimental structural studies are required to investigate the ligand-binding interactions and verify the simulated results.

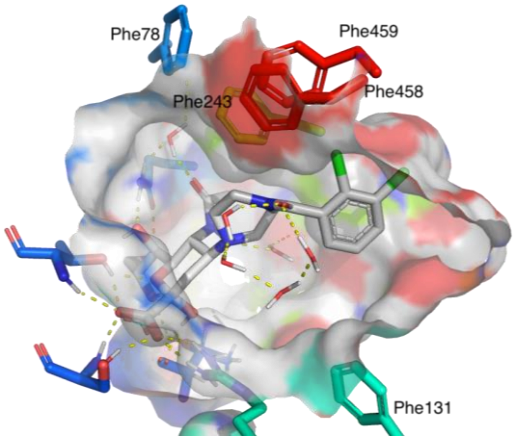


Figure 17. Representative MD snapshot at 90 ns from a 100ns molecular dynamic simulation of **16c** in complex with PmSiaT. The complex for the simulations was constructed by placing **16c** with its Neu5Ac part identically positioned as Neu5Ac in the x-ray structure of Neu5Ac in complex with the outward open PmSiaT (pdb id 5NV9).

5.1 Conclusions

We have developed a varied compound library, using both 4-*N*-piperidines and piperazines as linkers. The goal was to distance and limit the degrees of movement of the aromatic moiety, when compared to the 4-*O*-benzyls. An initial set of derivatives was used to identify the best scaffolds to be further derivatised. The 4-phenylpiperidine of compound **12e** induced the highest ΔT_m for PmSiaT and was thus selected for further derivatisation. The 4-piperazine was also further functionalised with amide bond formations. In both

compound series, we observed strong correlations with the effects of electron-withdrawing and donating groups, with the former significantly increasing affinity. FnSiaP and HdSatA did not show any thermal stabilisation induced by the new compound library. This is partially expected by the tightness of the binding sites. Contrary to the 4-*O*-benzyl series, we did not observe any thermal destabilisation for HdSatA.

Further work is required and planned to identify the affinity-enhancing interactions that led to the very high thermal shifts of compounds **12f** and **16g**.

5.2 Key findings

- The Ye *et al* procedure tolerates a wide array of cyclic amines.
- It is possible to utilise the 4-*N*-piperazine **13** as a handle for amide bond formations and to attach amino acids.
- Introduction of aromatic moieties on the 4 position of 4-*N*-piperidines and piperazines lead to high affinity compounds for PmSiaT.
- Functionalising the aromatic moieties with electron-withdrawing substituents significantly increases the affinity for PmSiaT, while the opposite is true for electron-donating compounds.
- FnSiaP and HdSatA do not tolerate the introduction of these scaffolds.

-

6 Nucleophilic substitution of the 4-OAc

We initially selected a few interesting 5- and 6-membered cyclic amines to investigate the 4-amination method published by Ye *et al.*[84] The chosen cyclic amines were morpholine, 4-phenylpiperidine, 4-phenylpiperazine and what we thought was isoindoline. The first three reactions were completed within 3 days, while the fourth initially showed no conversion. However, the reaction slowly proceeded and took about a month to reach completion. When we finally characterized the compound, we realized that, by mistake, we had used indoline (compound **20e**, **Scheme 7**), rather than isoindoline. The nitrogen on the indoline ring is aromatic, thus having a very different reactivity compared to all the previously tested reagents by Ye *et al*. The long reaction time also reflected the reduced reactivity of indoline, when compared to the cyclic secondary amines. We hypothesized that the aminations could be performed with a much wider variety of amines, than originally proposed by Ye, and that the outcome of the reactions were dependent on the nucleophilicity of the amine. Cyclic secondary amines are more nucleophilic compared to primary, or acyclic secondary ones.[87] To test the hypothesis, we selected benzylamine, *N*-methylbenzylamine, aniline and *N*-methylaniline and performed the reactions using the published conditions, i.e. room temperature and 10 equivalents of the amine.

The reaction with benzylamine was completed within a day and we isolated a compound that corresponded to the partially deacetylated glycal **18** (**Chart 1**). To prevent the deacetylated side products and the glycal formation, the temperature was lowered to -5 °C and the equivalents of benzylamine lowered to three. From thin-layer chromatography (TLC), the desired 4-benzylamino product is the first to form, but it then easily undergoes glycal formation (compound **19**). The purification was challenging because of multiple side products and unreacted starting material present. Nonetheless, the 4-benzylamino compound **20a** was isolated after 3 days, though in moderate yield (25%).

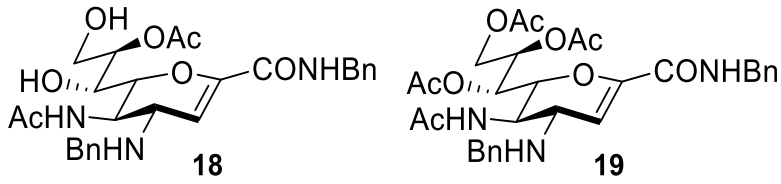
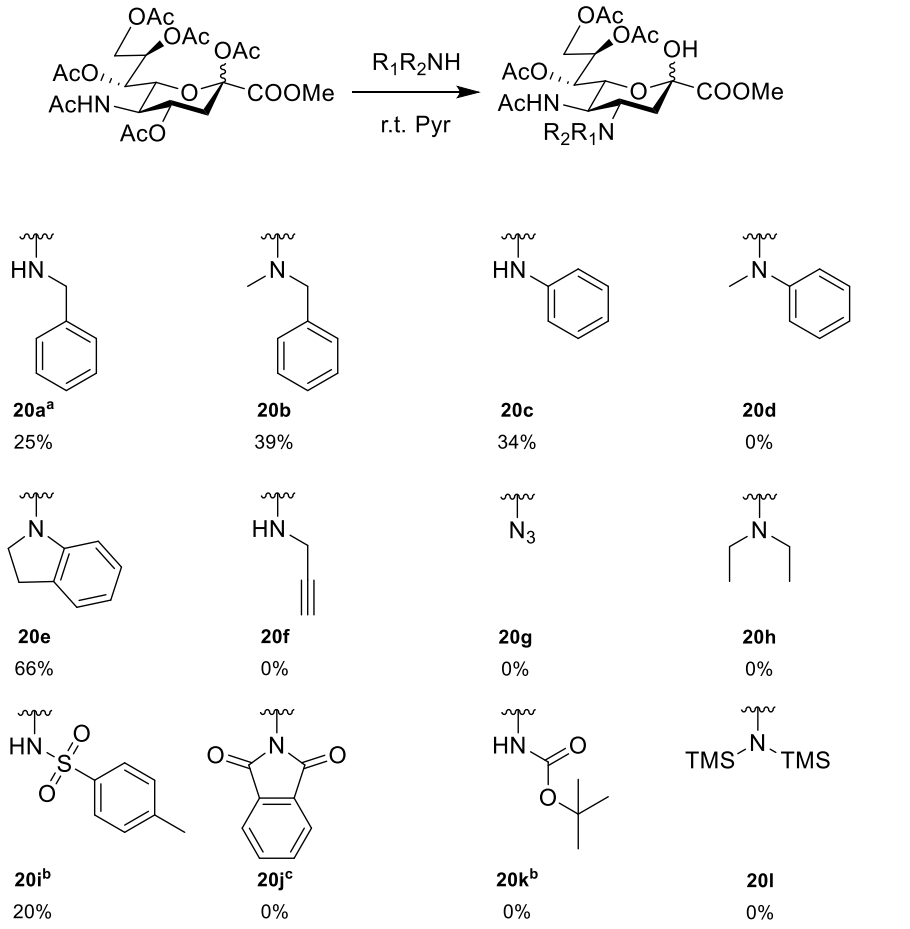


Chart 1. Side products isolated from 4-amination using benzylamine.

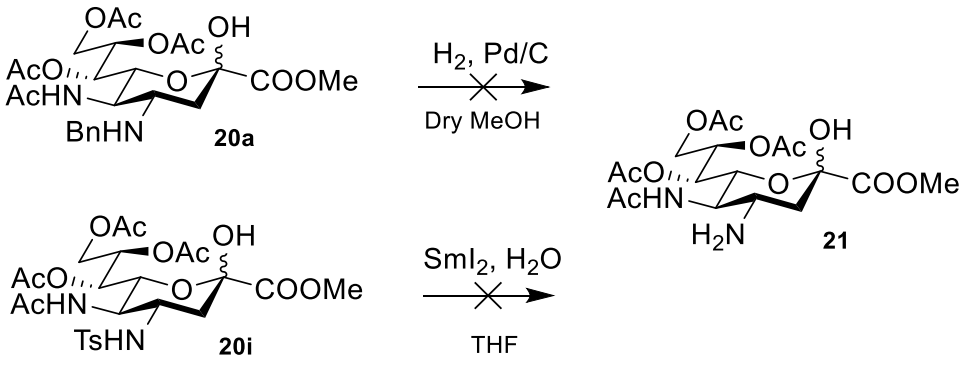
The reaction with *N*-methylbenzylamine did not require any optimization and we could easily isolate compound **20b** in 7 days in 39% yield. The reaction with aniline **20c** did not require any adjustment, but we observed prolonged reaction time as in the case of indoline. This was also not surprising due to aniline's reduced nucleophilicity. Lastly, *N*-methylaniline did not show conversion neither through prolonged reaction times (>30 days), nor increased temperature.



Scheme 7. 4-Aminations starting from **10**. General conditions, 3-10 eq of the nitrogen nucleophile in pyridine at room temperature. [a] The temperature was lowered to -5°C. [b] 3 eq of *t*-BuOK present. [c] Potassium phtalimide was used.

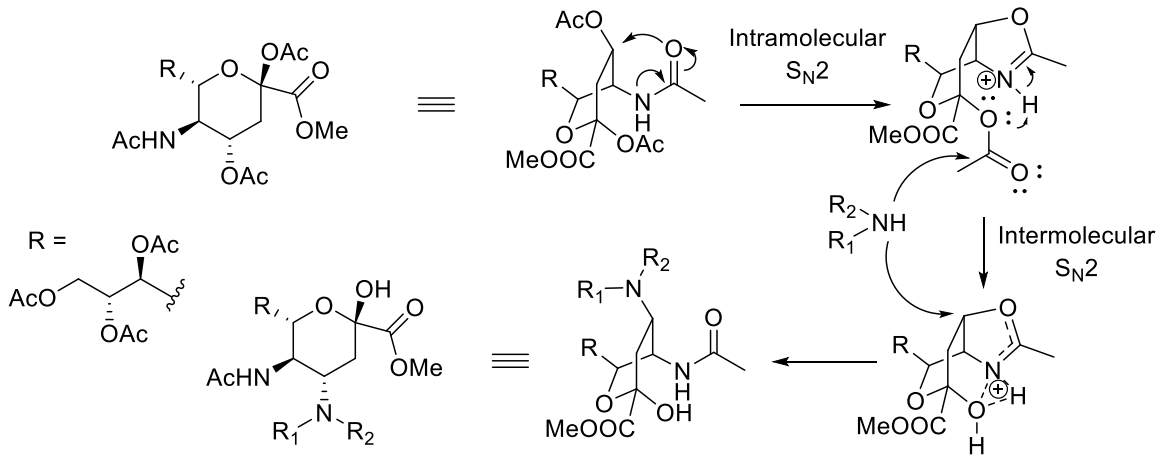
With these results, it became clear that we could further expand the scope of the reaction. We also realized how we might be able to introduce an amino or azide functionality at C4 of sialic acid. This prospective was particularly exciting for the extensive research on neuraminidase inhibitors bearing modifications in this position.[88,89]

We selected a series of amines, amides, carbamates and sulfonamides as well as azide to both test the scope of the reaction and obtain the 4-amino functionality (**Scheme 7**). Unfortunately, most of the attempts were unsuccessful. We then tried to deprotect benzylamino **20a** and tosyl **20i** (**Scheme 7**), but again our efforts failed to unmask the 4-amino. The hydrogenolysis of **20a** led to reduction of the α-ketoester, as previously described,[90] while no conversion was observed with SmI_2 in the attempt of deprotection of the tosyl group **20i**.[91]



Scheme 8. Attempts at deprotecting compound **20a** and **20i**.

One of the results that surprised us (**Scheme 7**) was the lack of product formation when using sodium azide, which is an excellent nucleophile.[92] We therefore took a closer look at the mechanism as proposed by Ye *et al* (**Scheme 9**).



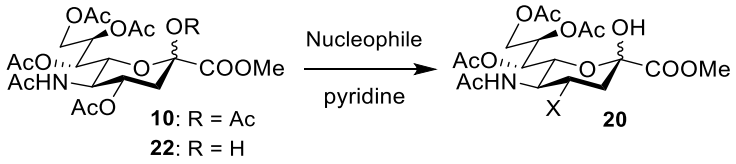
Scheme 9. 4-Amination mechanism as proposed by Ye *et al*.

In the proposed mechanism, the first step involves an intramolecular S_N2 to form the oxazolinium ring. The resulting positive charge is stabilized through space by the anomeric oxygen, which in turn is activated and undergoes deacetylation by a first equivalent of the amine. The reaction is then completed by a second equivalent of the amine that attacks C4, resulting in substitution with retention of the initial stereochemistry. To test this proposed mechanism, Ye *et al* also run the 4-amination with hemiacetal **22**, and the 2-deoxy derivative **23** (**Chart 2**). Interestingly, the reaction with **22** was reported as faster, but the results were not followed up with further experiments. Based on these results, we hypothesized that the rate limiting step is the deacetylation of the anomeric position.

We therefore synthesised compound **22**[93] and rerun the reaction with sodium azide. To our delight, the reaction the reaction led to 45% isolated yield after 7 days. We then decided to repeat some of the previous reactions performed with **10**, but with **22** as the electrophile. The results are summarised in **Table 3**.

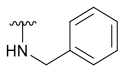
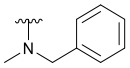
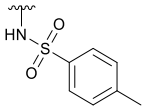
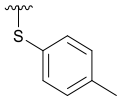
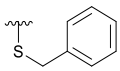
These results support our hypothesis that the rate limiting step of the reaction is the anomeric deacetylation. Only compounds able to perform the aminolysis of the 2-acetate were capable of substituting C4, starting from **10**. On the other hand, such requirement was not necessary when starting from **22**, enabling more reactions to succeed. Additionally, all reactions became significantly faster. We then also tested the reaction with potassium cyanide, thiocresol and benzyl mercaptan. All three reactions worked in good to excellent yields (**Table 3**), indicating how the scope could be expanded to carbon and sulphur nucleophiles.

Table 3. 4-Substitutions reactions starting from compounds **10** and **22**. The results from compound **10** are reported again to allow for comparisons.



10: R = Ac
22: R = H

20

Compound	X	R	Time	Temperature	Yield
20a		Ac	3 days	-5°C	25%
		H	20 h	-10°C	34%
20b		Ac	7 days	RT	39%
		H	-	-	Not tested
20c		Ac	30 days	RT	34%
		H	17 days	RT	39%
20e		Ac	30 days	RT	66%
		H	-	-	Not tested
20g		Ac	>30 days	RT	0%
		H	7 days	RT	39%
20h		Ac	>30 days	RT	0%
		H	4 h	RT	41%
20i		Ac	3 days[a]	RT	20%
		H	2 h[b]	RT	25%
20m		Ac	days	RT	21%
		H	2 days	RT	38%
20n		Ac	-	-	Not tested
		H	4 h	RT	89%
20o		Ac	-	-	Not tested
		H	4 h	RT	63%

Reaction conditions: 3-10 eq of nucleophile in pyridine at room temperature until disappearance of starting material or appearance of side products. [a] 3 eq of *t*-BuOK are additionally present. [b] 3 eq of Cs_2CO_3 are additionally present.

In the light of the new observations, we re-evaluated the mechanism proposed by Ye *et al*. When building a model of **22**, we noticed that the distance between O2 and C4 corresponds to 2.87 Å (**Figure 18**). This distance matches previously published distances suitable for through-space interactions, as reported by Miljkovic *et al*.[94]

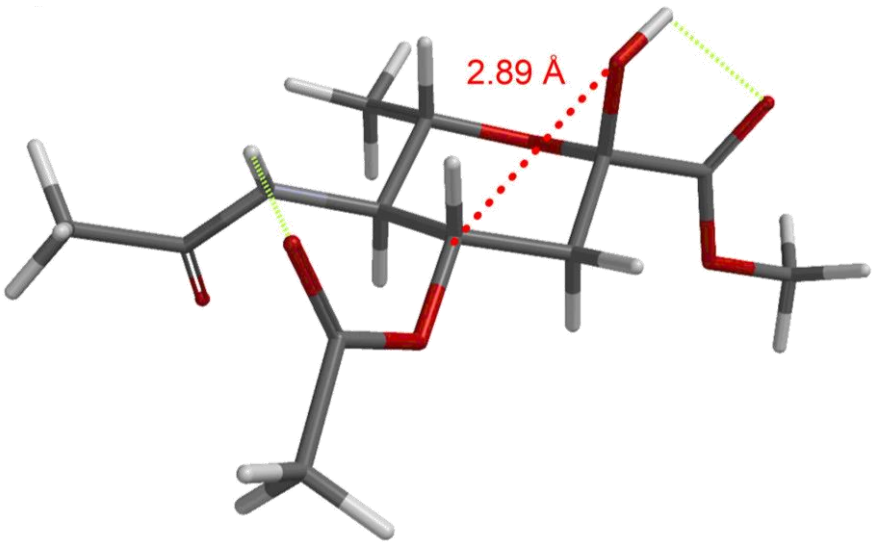


Figure 18. Stick model of **22** highliting the distances between O2 and C4 (red) and the hydrogen bond between the proton of the 2-OH and the C1 carbonyl (green).

Our hypothesis is that the 2-OH of **22**, in contrast to the 2-OAc of **10**, can donate its electrons through space to the antibonding orbital (σ*) of the C4-O4 σ bond. This interaction is enough to weaken the σ bond, thus allowing for the oxazoline formation. The 2-OAc of **22** is not electron-dense enough to establish such interaction. To reproduce the reactivity trend observed by Miljkovic *et al*, we synthesized compound **24** and subjected it to the reaction with KCN. The choice of KCN was for its clear differences between **22** and **10** (**Table 3**). Against our expectations, **24** was found unreactive. Our hypothesis is that the 2-OH of **22** is additionally activated by an internal hydrogen bond between the proton of the 2-OH and the carbonyl at C1 (**Figure 18**). This increases the electron-density on the O2, results in the observed reactivity.

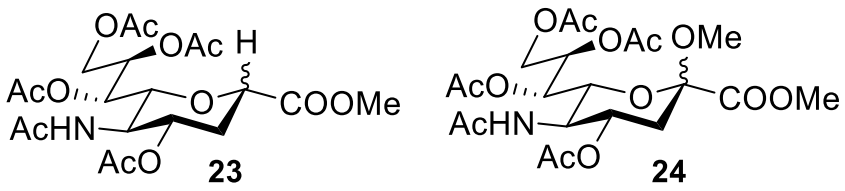


Chart 2. 2-Deoxy derivative **23** and the methyl sialoside **24**.

6.1 Conclusions

To date, the challenges associated with introducing modifications at C4 of sialic acid hampered the development of 4-derivatised sialic acid derivatives.[95,96] As described in the introduction, sialic acids mediate a number of physiological and pathological processes. Therefore, developing synthetic tools to explore unnatural sialic acid derivatives is of paramount importance.

With our methodology, we enable the introduction of a wide variety of functional groups. These functional groups, such as the azido, cyano and sulfide groups, can be further modified and used for the generation of collections of compounds. Our methodology allows for 4-substitution in 3 steps from starting material Neu5Ac, with retention of the configuration. We also proposed a new mechanism on how the 4-amination works, identifying the 2-OH as a crucial feature in weakening the σ C4-O4 bond by donating electrons through-space to the σ* antibonding orbital.

6.2 Key findings

- Substitutions at C4 of acetyl protected Neu5Ac are possible not just with cyclic secondary amines as previously reported, but with a wide array of nucleophiles.
- The rate limiting step of the reaction is the 2-deacetylation. Therefore, starting from hemiacetal **22** allows for faster reactions and a broader scope.
- The 2-OH seems to be involved in a through-space interaction with the σ* antibonding orbital of the C4-O4 bond. By donating electrons, the 2-OH weakens the C4-O4 bond, thus allowing for the formation of the oxazolinium ring.
- The reported reactions greatly increase the scope of 4-substitutions and allow for further derivatisations thanks to the introduced groups such the azide, cyano and sulfides.

7 Conclusions and future prospects

Our starting point was the idea that bacterial sialic acid uptake inhibitors could have an antibacterial effect. Initially, we did not have any sialic acid derivative capable of binding the target proteins. Additionally, we did not have any standard methodology to evaluate the synthesised compounds. The research has consequentially proceeded to establish synthetic, computational, biophysical and biochemical methodologies to reliably get results to later build upon.

After four years, we can address the initial objectives:

- With our structure-based approach, we succeeded in developing compounds capable of targeting and inhibiting the SiaTs. Introduction of electron-poor aromatic moieties on C4 of Neu5Ac proved to be a successful strategy, with compounds displaying nanomolar affinities.
- Several methodologies have been employed for evaluating the synthesised compounds, such as nanoDSF, ITC, MD, physiochemical and ADME properties, and proteoliposome and bacterial growth assays. These evaluations have become standard for us and will be valuable for the future research endeavours.
- It could be possible to develop compounds capable of inhibiting multiple bacterial sialic acid transporter families with derivatisation at C9 and, potentially, O4 of Neu5Ac.
- Thanks to our new methodology, it is now possible to functionalise C4 of sialic acid with a varied range of nucleophiles, in a simple, single step reaction.

This work represents the first steps towards the development of this class of compounds as antibacterial drugs, but there is a lot more work to be done. We did not manage to investigate the toxicity and cross reactivity of our compounds. It is true that we humans do not have sialic acid transporters, but we do have a lot of sialic acid-binding proteins.

Even more importantly, we are still lacking a proof of concept. Demonstrating an antibacterial effect is complicated with this mechanism of action, since these compounds are not bactericidal *per se*. The antibacterial effect would be mediated by the bacterium's inability to reach a carbon source, but especially by suppression of molecular mimicry. Our results from the bacterial growth assays are promising on *S. aureus*, which is not known to sialylate its epitopes. Future research will need to address the inefficacy of our compounds towards Gram-negative pathogens and try to solve it, since Gram-negative bacteria are more frequently found to sialylate their surfaces.[58]

In the future, the research will need to focus on obtaining compounds with broad spectrum activity, capable of targeting multiple transporter families. To sustain and drive the drug-design, more structural information is needed, with crystal structures in complex with the most promising compounds, to critically establish beneficial and detrimental interactions. With more structural information, it will be possible to implement and strengthen the computational approach to guide the drug design.

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9 References

(1) Bhattacharjee, M. K. *Chemistry of Antibiotics and Related Drugs*; Springer International Publishing AG Switzerland, 2016.

(2) Cohen, J.; Powderly, W. G.; Opal, S. M. The Evolution of Koch's Postulates. In *Infectious Diseases*; Elsevier, 2017; pp 1–3.

(3) Fredricks, D. N.; Relman, D. A. Sequence-Based Identification of Microbial Pathogens: A Reconsideration of Koch's Postulates. *Clin. Microbiol. Rev.* **1996**, *9* (1), 18–33.

(4) Aminov, R. I. A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. *Front. Microbiol.* **2010**, *1*, 134.

(5) Mahoney, J. F.; Arnold, R. C.; Harris, A. Penicillin Treatment of Early Syphilis—A Preliminary Report. *Am. J. Public Heal. Nations Heal.* **1943**, *33* (12), 1387–1391.

(6) Hutchings, M. I.; Truman, A. W.; Wilkinson, B. Antibiotics : Past, Present and Future. *Curr. Opin. Microbiol.* **2019**, *51*, 72–80.

(7) Schneider, T.; Sahl, H. G. An Oldie but a Goodie – Cell Wall Biosynthesis as Antibiotic Target Pathway. *Int. J. Med. Microbiol.* **2010**, *300* (2), 161–169.

(8) Arenz, S.; Wilson, D. N. Bacterial Protein Synthesis as a Target for Antibiotic Inhibition. *Cold Spring Harb. Perspect. Med.* **2016**, *6* (9).

(9) Vaara, M. Polymyxins and Their Potential next Generation as Therapeutic Antibiotics. *Front. Microbiol.* **2019**, *10*, 1686–1689.

(10) Stringer, J. L. Folate Antagonists. In *Basic Concepts in Pharmacology: What You Need to Know for Each Drug Class, 5e*; McGraw-Hill Education: New York, NY, 2017.

(11) Blondeau, J. M. Fluoroquinolones: Mechanism of Action, Classification, and Development of Resistance. *Surv. Ophthalmol.* **2004**, *49* (2, Supplement 2), S73–S78.

(12) Ang, C. W.; Jarrad, A. M.; Cooper, M. A.; Blaskovich, M. A. T. Nitroimidazoles: Molecular Fireworks That Combat a Broad Spectrum of Infectious Diseases. *J. Med. Chem.* **2017**, *60* (18), 7636–7657.

(13) Huttner, A.; Verhaegh, E. M.; Harbarth, S.; Muller, A. E.; Theuretzbacher, U.; Mouton, J. W. Nitrofurantoin Revisited: A Systematic Review and Meta-Analysis of Controlled Trials. *J. Antimicrob. Chemother.* **2015**, *70* (9), 2456–2464.

(14) Fleming, A. Penicillin's Finder Assays Its Future. *New York Times* **1945**, No. June 26, 21.

(15) KishonyLab. The Evolution of Bacteria on a "Mega-Plate" Petri Dish https://www.youtube.com/watch?v=plVk4NVIUh8.

(16) Holmes, A. H.; Moore, L. S. P.; Sundsfjord, A.; Steinbakk, M.; Regmi, S.; Karkey, A.; Guerin, P. J.; Piddock, L. J. V. Understanding the Mechanisms and Drivers of Antimicrobial Resistance. *Lancet* **2016**, *387* (10014), 176–187.

(17) Reygaert, W. C. An Overview of the Antimicrobial Resistance Mechanisms of Bacteria. *AIMS Microbiol.* **2018**, *4* (3), 482–501.

(18) Spellberg, B.; Gilbert, D. N. The Future of Antibiotics and Resistance: A Tribute to a Career of Leadership by John Bartlett. *Clin. Infect. Dis.* **2014**, *59 Suppl 2* (Suppl 2), S71–S75.

(19) Collaborators, A. R. Global Burden of Bacterial Antimicrobial Resistance in 2019 : A Systematic Analysis. *Lancet* **2022**, *399*, 629–655.

(20) O'Neill, J. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. London: Review on Antimicrobial Resistance, 2016.

(21) Cassini, A.; Högberg, L. D.; Plachouras, D.; Quattrocchi, A.; Hoxha, A.; Simonsen, G. S.; Colomb-Cotinat, M.; Kretzschmar, M. E.; Devleesschauwer, B.; Cecchini, M.; Ouakrim, D. A.; Oliveira, T. C.; Struelens, M. J.; Suetens, C.; Monnet, D. L.; Strauss, R.; Mertens, K.; Struyf, T.; Catry, B.; Latour, K.; Ivanov, I. N.; Dobreva, E. G.; Tambic Andraševic, A.; Soprek, S.; Budimir, A.; Paphitou, N.; Žemlicková, H.; Schytte Olsen, S.; Wolff Sönksen, U.; Märtin, P.; Ivanova, M.; Lyytikäinen, O.; Jalava, J.; Coignard, B.; Eckmanns, T.; Abu Sin, M.; Haller, S.; Daikos, G. L.; Gikas, A.; Tsiodras, S.; Kontopidou, F.; Tóth, Á.; Hajdu, Á.; Guólaugsson, Ó.; Kristinsson, K. G.; Murchan, S.; Burns, K.; Pezzotti, P.; Gagliotti, C.; Dumpis, U.; Liuimiene, A.; Perrin, M.; Borg, M. A.; de Greeff, S. C.; Monen, J. C.; Koek, M. B.; Elstrøm, P.; Zabicka, D.; Deptula, A.; Hryniewicz, W.; Caniça, M.; Nogueira, P. J.; Fernandes, P. A.; Manageiro, V.; Popescu, G. A.; Serban, R. I.; Schréterová, E.; Litvová, S.; Štefkovicová, M.; Kolman, J.; Klavs, I.; Korošec, A.; Aracil, B.; Asensio, A.; Pérez-Vázquez, M.; Billström, H.; Larsson, S.; Reilly, J. S.; Johnson, A.; Hopkins, S. Attributable Deaths and Disability-Adjusted Life-Years Caused by Infections with Antibiotic-Resistant Bacteria in the EU and the European Economic Area in 2015: A Population-Level Modelling Analysis. *Lancet Infect. Dis.* **2019**, *19* (1), 56–66.

(22) Jansen, K. U.; Knirsch, C.; Anderson, A. S. The Role of Vaccines in Preventing Bacterial Antimicrobial Resistance. *Nat. Med.* **2018**, *24* (1), 10–19.

(23) Luyt, C.-E.; Bréchot, N.; Trouillet, J.-L.; Chastre, J. Antibiotic Stewardship in the Intensive Care Unit. *Crit. Care* **2014**, *18* (5), 480.

(24) Allen, H. K.; Donato, J.; Wang, H. H.; Cloud-hansen, K. A.; Davies, J.; Handelsman, J. Call of the Wild: Antibiotic Resistance Genes in Natural Environments. *Nat. Rev. Microbiol.* **2010**, *8*, 251–259.

(25) Schauer, R. Sialic Acids as Regulators of Molecular and Cellular Interactions. *Curr. Opin. Struct. Biol.* **2009**, *19* (5), 507–514.

(26) Chen, X. .; Varki, A. Advances in the Biology and Chemistry of Sialic Acids. *ACS Chem. Biol.* **2010**, *5* (2), 163–176.

(27) Ajit, V. Uniquely Human Evolution of Sialic Acid Genetics and Biology. *Proc. Natl. Acad. Sci.* **2010**, *107* (supplement_2), 8939–8946.

(28) Varki, N. M.; Varki, A. Diversity in Cell Surface Sialic Acid Presentations: Implications for Biology and Disease. *Lab. Investig.* **2007**, *87* (9), 851–857.

(29) Bauer, J.; Osborn, H. M. Sialic Acids in Biological and Therapeutic Processes: Opportunities and Challenges. *Future Med. Chem.* **2015**, *7* (16), 2131–2141.

(30) Altman, M. O.; Gagneux, P. Absence of Neu5Gc and Presence of Anti-Neu5Gc Antibodies in Humans-An Evolutionary Perspective. *Front. Immunol.* **2019**, *10* (April), 789.

(31) Hsun-Hua, C.; Hiromu, T.; Sandra, D.; Jane, I.; Elizabeth, N.; L., W. K.; A., M. E.; L., N. D.; T., W. S.; Ajit, V. A Mutation in Human CMP-Sialic Acid Hydroxylase Occurred after the Homo-Pan Divergence. *Proc. Natl. Acad. Sci.* **1998**, *95* (20), 11751–11756.

(32) Altheide, T. K.; Hayakawa, T.; Mikkelsen, T. S.; Diaz, S.; Varki, N.; Varki, A. System-Wide Genomic and Biochemical Comparisons of Sialic Acid Biology among Primates and Rodents: Evidence for Two Modes of Rapid Evolution. *J. Biol. Chem.* **2006**, *281* (35), 25689–25702.

(33) Vimr, E. R.; Kalivoda, K. A.; Deszo, E. L.; Steenbergen, S. M. Diversity of Microbial Sialic Acid Metabolism. *Microbiol. Mol. Biol. Rev.* **2004**, *68* (1), 132–153.

(34) Severi, E. .; Hood, D. W. .; Thomas, G. H. Sialic Acid Utilization by Bacterial Pathogens. *Microbiology* **2007**, *153* (9), 2817–2822.

(35) Corfield, T. Bacterial Sialidases--Roles in Pathogenicity and Nutrition. *Glycobiology* **1992**, *2* (6), 509–521.

(36) Shakhnovich, E. A.; King, S. J.; Weiser, J. N. Neuraminidase Expressed by Streptococcus Pneumoniae

Desialylates the Lipopolysaccharide of Neisseria Meningitidis and Haemophilus Influenzae: A Paradigm for Interbacterial Competition among Pathogens of the Human Respiratory Tract. *Infect. Immun.* **2002**, *70* (12), 7161–7164.

(37) Sohanpal, B. K.; Friar, S.; Roobol, J.; Plumbridge, J. A.; Blomfield, I. C. Multiple Co-Regulatory Elements and IHF Are Necessary for the Control of FimB Expression in Response to Sialic Acid and N-Acetylglucosamine in Escherichia Coli K-12. *Mol. Microbiol.* **2007**, *63* (4), 1223–1236.

(38) North, R. A.; Horne, C. R.; Davies, J. S.; Remus, D. M.; Muscroft-Taylor, A. C.; Goyal, P.; Wahlgren, W. Y.; Ramaswamy, S.; Friemann, R.; Dobson, R. C. J. "Just a Spoonful of Sugar..": Import of Sialic Acid across Bacterial Cell Membranes. *Biophys. Rev.* **2018**, *10* (2), 219–227.

(39) Wirth, C.; Condemine, G.; Boiteux, C.; Bernèche, S.; Schirmer, T.; Peneff, C. M. NanC Crystal Structure, a Model for Outer-Membrane Channels of the Acidic Sugar-Specific KdgM Porin Family. *J. Mol. Biol.* **2009**, *394* (4), 718–731.

(40) Phansopa, C.; Roy, S.; Rafferty, J. B.; Douglas, C. W. I.; Pandhal, J.; Wright, P. C.; Kelly, D. J.; Stafford, G. P. Structural and Functional Characterization of NanU, a Novel High-Affinity Sialic Acid-Inducible Binding Protein of Oral and Gut-Dwelling Bacteroidetes Species. *Biochem. J.* **2014**, *458* (3), 499–511.

(41) Post, D. M. B.; Mungur, R.; Gibson, B. W.; Munson, R. S. J. Identification of a Novel Sialic Acid Transporter in Haemophilus Ducreyi. *Infect. Immun.* **2005**, *73* (10), 6727–6735.

(42) Holder, J. W.; Ulrich, J. C.; DeBono, A. C.; Godfrey, P. A.; Desjardins, C. A.; Zucker, J.; Zeng, Q.; Leach, A. L. B.; Ghiviriga, I.; Dancel, C.; Abeel, T.; Gevers, D.; Kodira, C. D.; Desany, B.; Affourtit, J. P.; Birren, B. W.; Sinskey, A. J. Comparative and Functional Genomics of Rhodococcus Opacus PD630 for Biofuels Development. *PLOS Genet.* **2011**, *7* (9), e1002219.

(43) Gruteser, N.; Marin, K.; Krämer, R.; Thomas, G. H. Sialic Acid Utilization by the Soil Bacterium Corynebacterium Glutamicum. *FEMS Microbiol. Lett.* **2012**, *336* (2), 131–138.

(44) Setty, T. G.; Mowers, J. C.; Hobbs, A. G.; Maiya, S. P.; Syed, S.; Munson, R. S.; Apicella, M. A.; Subramanian, X. R. Molecular Characterization of the Interaction of Sialic Acid with the Periplasmic Binding Protein from Haemophilus Ducreyi. *J. Biol. Chem.* **2018**, *293* (52), 20073–20084.

(45) Mulligan, C.; Geertsma, E. R.; Severi, E.; Kelly, D. J.; Poolman, B.; Thomas, G. H. The Substrate-Binding Protein Imposes Directionality on an Electrochemical Sodium Gradient-Driven TRAP Transporter. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (13), 4957–4958.

(46) Müller, A.; Severi, E.; Mulligan, C.; Watts, A. G.; Kelly, D. J.; Wilson, K. S.; Wilkinson, A. J.; Thomas, G. H. Conservation of Structure and Mechanism in Primary and Secondary Transporters Exemplified by SiaP, a Sialic Acid Binding Virulence Factor from Haemophilus Influenzae. *J. Biol. Chem.* **2006**, *281* (31), 22212–22222.

(47) Setty, T. G.; Cho, C.; Govindappa, S.; Apicella, M.; Ramaswamy, S. Bacterial Periplasmic Sialic Acid-Binding Proteins Exhibit a Conserved Binding Site. *Acta Crystallogr D Struct Biol* **2014**, *70*, 1801–1811.

(48) Vimr, E. R.; Troy, F. A. Identification of an Inducible Catabolic System for Sialic Acids (Nan) in Escherichia Coli. *J. Bacteriol.* **1985**, *164* (2), 845–853.

(49) Severi, E.; Hosie, A. H. F.; Hawkhead, J. A.; Thomas, G. H. Characterization of a Novel Sialic Acid Transporter of the Sodium Solute Symporter (SSS) Family and in Vivo Comparison with Known Bacterial Sialic Acid Transporters. *FEMS Microbiol. Lett.* **2010**, *304* (1), 47–54.

(50) Wahlgren, W. Y.; Dunevall, E.; North, R. A.; Paz, A.; Scalise, M.; Bisignano, P.; Bengtsson-palme, J.; Goyal, P.; Claesson, E.; Caing-carlsson, R.; Andersson, R.; Beis, K.; Nilsson, U. J.; Farewell, A.; Pochini, L.; Indiveri, C.; Grabe, M.; Dobson, R. C. J.; Abramson, J. Substrate-Bound Outward-Open Structure of a Na+-Coupled Sialic Acid Symporter Reveals a New Na+ Site. *Nat. Commun.* **2018**, 1–14.

(51) North, R. A.; Wahlgren, W. Y.; Remus, D. M.; Scalise, M.; Kessans, S. A.; Dunevall, E.; Claesson, E.; Indiveri, C.; Friemann, R.; Dobson, R. C. J. The Sodium Sialic Acid Symporter from Staphylococcus

Aureus Has Altered Substrate Specificity. *Front. Chem.* **2018**, *6*, 1–11.

(52) Almagro-Moreno, S.; Boyd, E. F. Insights into the Evolution of Sialic Acid Catabolism among Bacteria. *BMC Evol. Biol.* **2009**, *9* (1), 118.

(53) Lewis, A. L.; Lewis, W. G. Host Sialoglycans and Bacterial Sialidases: A Mucosal Perspective. *Cell. Microbiol.* **2012**, *14* (8), 1174–1182.

(54) Fabich, A. J.; Jones, S. A.; Chowdhury, F. Z.; Cernosek, A.; Anderson, A.; Smalley, D.; McHargue, J. W.; Hightower, G. A.; Smith, J. T.; Autieri, S. M.; Leatham, M. P.; Lins, J. J.; Allen, R. L.; Laux, D. C.; Cohen, P. S.; Conway, T. Comparison of Carbon Nutrition for Pathogenic and Commensal Escherichia Coli Strains in the Mouse Intestine. *Infect. Immun.* **2008**, *76* (3), 1143–1152.

(55) Damian, R. T. Molecular Mimicry : Antigen Sharing by Parasite and Host and Its Consequences. *Am. Nat.* **1964**, *98* (900), 129–149.

(56) Poole, J.; Day, C. J.; von Itzstein, M.; Paton, J. C.; Jennings, M. P. Glycointeractions in Bacterial Pathogenesis. *Nat. Rev. Microbiol.* **2018**, *16* (7), 440–452.

(57) Vimr, E.; Lichtensteiger, C. To Sialylate, or Not to Sialylate: That Is the Question. *Trends Microbiol.* **2002**, *10* (6), 254–257.

(58) de Jong, H.; Wösten, M. M. S. M.; Wennekes, T. Sweet Impersonators: Molecular Mimicry of Host Glycans by Bacteria. *Glycobiology* **2022**, *32* (1), 11–22.

(59) Nji, E.; Gulati, A.; Qureshi, A. A.; Coincon, M.; Drew, D. Structural Basis for the Delivery of Activated Sialic Acid into Golgi for Sialyation. *Nat. Struct. Mol. Biol.* **2019**, *26* (6), 415–423.

(60) Heise, T.; Langereis, J. D.; Rossing, E.; De Jonge, M. I.; Adema, G. J.; Bull, C.; Boltje, T. J. Selective Inhibition of Sialic Acid-Based Molecular Mimicry in Haemophilus Influenzae Abrogates Serum Brief Communication Selective Inhibition of Sialic Acid-Based Molecular Mimicry in Haemophilus Influenzae Abrogates Serum Resistance. *Cell Chem. Biol.* **2018**, *25*, 1279–1285.

(61) Bouchet, V.; Hood, D. W.; Li, J.; Brisson, J.; Randle, G. A.; Martin, A.; Li, Z.; Goldstein, R.; Schweda, E. K. H.; Pelton, S. I.; Richards, J. C.; Moxon, E. R. Host-Derived Sialic Acid Is Incorporated into Haemophilus Influenzae Lipopolysaccharide and Is a Major Virulence Factor in Experimental Otitis Media. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100* (15), 8898–8903.

(62) Stecher, B.; Chaffron, S.; Käppeli, R.; Hapfelmeier, S.; Freedrich, S.; Weber, T. C.; Kirundi, J.; Suar, M.; McCoy, K. D.; von Mering, C.; Macpherson, A. J.; Hardt, W.-D. Like Will to Like: Abundances of Closely Related Species Can Predict Susceptibility to Intestinal Colonization by Pathogenic and Commensal Bacteria. *PLOS Pathog.* **2010**, *6* (1), e1000711.

(63) Huang, Y. L.; Chassard, C.; Hausmann, M.; Von Itzstein, M.; Hennet, T. Sialic Acid Catabolism Drives Intestinal Inflammation and Microbial Dysbiosis in Mice. *Nat. Commun.* **2015**, *6* (8141).

(64) WHO. Antimicrobial Resistance. Global Report on Surveillance. *Bull. World Health Organ.* **2014**, *61* (3), 383–394.

(65) (U.S.), C. for D. C. and P. *Antibiotic Resistance Threats in the United States*; 2019.

(66) Schaffer, J. N.; Pearson, M. M. Proteus Mirabilis and Urinary Tract Infections. *Microbiol. Spectr.* **2016**, 383–433.

(67) Girlich, D.; Bonnin, R. A.; Dortet, L.; Naas, T. Genetics of Acquired Antibiotic Resistance Genes in Proteus Spp. *Front. Microbiol.* **2020**, *11*, 256.

(68) Buda, S.; Crich, D. Oxidative Deamination of N-Acetyl Neuraminic Acid: Substituent Effects and Mechanism. *J. Am. Chem. Soc.* **2016**, *138* (3), 1084–1092.

(69) Ren, B. .; Wang, M. .; Liu, J. .; Ge, J. .; Dong, H. Enhanced Basicity of Ag2O by Coordination to Soft Anions. *ChemCatChem* **2015**, *7* (5), 761–765.

(70) Yu, C. S.; Niikura, K.; Lin, C. C.; Wong, C. H. The Thioglycoside and Glycosyl Phosphite of 5-Azido Sialic Acid: Excellent Donors for the α-Glycosylation of Primary Hydroxy Groups. *Angew. Chemie - Int. Ed.* **2001**, *40* (15), 2900–2903.

(71) Kiefel, M. J. .; Wilson, J. C. .; Bennett, S. .; Gredley, M. .; von Itzstein, M. Synthesis and Evaluation of C-9 Modifed N-Acetylneuraminic Acid Derivatives as Substrates for N-Acetylneuraminic Acid Aldolase. *Bioorg. Med. Chem.* **2000**, *8*, 657–664.

(72) Scott, D. E.; Spry, C.; Abell, C. Differential Scanning Fluorimetry as Part of a Biophysical Screening Cascade. In *Fragment-based Drug Discovery Lessons and Outlook*; 2016; pp 139–172.

(73) Bai, N.; Roder, H.; Dickson, A.; Karanicolas, J. Isothermal Analysis of ThermoFluor Data Can Readily Provide Quantitative Binding Affinities. *Sci. Rep.* **2019**, *9* (1), 1–15.

(74) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L.-N. Rapid Measurement of Binding Constants and Heats of Binding Using a New Titration Calorimeter. *Anal. Biochem.* **1989**, *179* (1), 131–137.

(75) Fenley, A. T.; Muddana, H. S.; Gilson, M. K. Entropy-Enthalpy Transduction Caused by Conformational Shifts Can Obscure the Forces Driving Protein-Ligand Binding. *Proc. Natl. Acad. Sci.* **2012**, *109* (49), 20006–20011.

(76) Scalise, M.; Pochini, L.; Giangregorio, N.; Tonazzi, A.; Indiveri, C. Proteoliposomes as Tool for Assaying Membrane Transporter Functions and Interactions with Xenobiotics. *Pharmaceutics* . 2013.

(77) Ciancaglini, P.; Simão, A. M. S.; Bolean, M.; Millán, J. L.; Rigos, C. F.; Yoneda, J. S.; Colhone, M. C.; Stabeli, R. G. Proteoliposomes in Nanobiotechnology. *Biophys. Rev.* **2012**, *4* (1), 67–81.

(78) Vrbanac, J.; Slauter, R. A Comprehensive Guide to Toxicology in Preclinical Drug Development; Faqi, A. S., Ed.; Academic Press, 2013; pp 3–30.

(79) Bissada, J. E.; Truong, V.; Abouda, A. A.; Wines, K. J.; Crouch, R. D.; Jackson, K. D. Inter-Individual Variation in CYP3A Activity Influences Lapatinib Bioactivation. *Drug Metab. Dispos.* **2019**, dmd.119.088823.

(80) Felder, C. B.; Graul, R. C.; Lee, A. Y.; Merkle, H. P.; Sadee, W. The Venus Flytrap of Periplasmic Binding Proteins: An Ancient Protein Module Present in Multiple Drug Receptors. *AAPS PharmSci* **1999**, *1* (2), E2.

(81) Oh, B. H.; Pandit, J.; Kang, C. H.; Nikaido, K.; Gokcen, S.; Ames, G. F.; Kim, S. H. Three-Dimensional Structures of the Periplasmic Lysine/Arginine/Ornithine-Binding Protein with and without a Ligand. *J. Biol. Chem.* **1993**, *268* (15), 11348–11355.

(82) Askin, S.; Bond, T. E. H.; Sorenson, A. E.; Moreau, M. J. J.; Antony, H.; Davis, R. A.; Schaeffer, P. M. Selective Protein Unfolding: A Universal Mechanism of Action for the Development of Irreversible Inhibitors. *Chem. Commun.* **2018**, *54* (14), 1738–1741.

(83) Cimmperman, P.; Baranauskienė, L.; Jachimovičiūtė, S.; Jachno, J.; Torresan, J.; Michailovienė, V.; Matulienė, J.; Sereikaitė, J.; Bumelis, V.; Matulis, D. A Quantitative Model of Thermal Stabilization and Destabilization of Proteins by Ligands. *Biophys. J.* **2008**, *95* (7), 3222–3231.

(84) Ye, D.; Li, J.; Zhang, J.; Jiang, H.; Neuac, R. N. Simultaneous Stereoselective 4-Amination with Cyclic Secondary Amines and 2-O-Deacetylation of Peracetylated Sialic Acid Derivatives OH. *Tetrahedron Lett.* **2007**, *48*, 4023–4027.

(85) Vardanyan, R. *Piperidine-Based Drug Discovery*, 1st Editio.; Elsevier, 2017.

(86) Durand, C.; Szostak, M. Recent Advances in the Synthesis of Piperazines: Focus on C–H Functionalization. *Organics* . 2021.

(87) Brotzel, F.; Ying, C. C.; Mayr, H. Nucleophilicities of Primary and Secondary Amines in Water. *J. Org. Chem.* **2007**, *72* (10), 3679–3688.

(88) Kelm, S.; Madge, P.; Islam, T.; Bennett, R.; Koliwer-Brandl, H.; Waespy, M.; von Itzstein, M.; Haselhorst, T. C-4 Modified Sialosides Enhance Binding to Siglec-2 (CD22): Towards Potent Siglec Inhibitors for Immunoglycotherapy. *Angew. Chemie Int. Ed.* **2013**, *52* (13), 3616–3620.

(89) Bourguet, E.; Figurska, S.; Frączek, M. M. Human Neuraminidases: Structures and Stereoselective Inhibitors. *J. Med. Chem.* **2022**, *65* (4), 3002–3025.

(90) Martin, R.; Witte, K. L.; Wong, C. H. The Synthesis and Enzymatic Incorporation of Sialic Acid

Derivatives for Use as Tools to Study the Structure, Activity, and Inhibition of Glycoproteins and Other Glycoconjugates. *Bioorg Med Chem* **1998**, *6* (8), 1283–1292.

(91) Kumar, V.; Ramesh, N. G. Iodine Catalyzed One-Pot Diamination of Glycals with Chloramine-T: A New Approach to 2-Amino-β-Glycosylamines for Applications in N-Glycopeptide Synthesis. *Chem. Commun.* **2006**, No. 47, 4952–4954.

(92) Phan, T. B.; Mayr, H. Nucleophilic Reactivity of the Azide Ion in Various Solvents. *J. Phys. Org. Chem.* **2006**, *19* (11), 706–713.

(93) Kartha, K. P. R.; Field, R. A. Iodine : A Versatile Reagent in Carbohydrate Chemistry IV. Per-O-Acetylation, Regioseleetive Acylation and Acetolysis. *Tetrahedron* **1997**, *53* (34), 11753–11766.

(94) Miljković, M.; Yeagley, D.; Deslongchamps, P.; Dory, Y. L. Experimental and Theoretical Evidence of Through-Space Electrostatic Stabilization of the Incipient Oxocarbenium Ion by an Axially Oriented Electronegative Substituent During Glycopyranoside Acetolysis. *J. Org. Chem.* **1997**, *62* (22), 7597–7604.

(95) Bandgar, B. P.; Patil, S. V; Zbiral, E. Synthesis of Methyl 4-Acetamido-N-Acetyl-4-Deoxy-α- and β-4-Epi-Neuraminic Acids. *Carbohydr. Res.* **1995**, *276* (2), 337–345.

(96) Ciccotosto, S.; von Itzstein, M. Synthesis of Methyl 5-Acetamido-3,4,5-Trideoxy-4-Guanidinyl-d-Glycero-d-Galacto-2-Nonulopyranosidonic Acid (4-Deoxy-4-Guanidino-Neu5Acα2Me). *Tetrahedron Lett.* **1995**, *36* (30), 5405–5408.

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EXPERIENCE

03/2018 - 06/2022 **European Joint Doctoral Studies in Medicinal Chemistry**
Department of Chemistry, Lund University, Lund, Sweden.
Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland.
Supervisors: Prof. Ulf Ellervik (ulf.ellervik@chem.lu.se), Prof. Ulf Nilsson (ulf.nilsson@chem.lu.se), Oliver Schwardt PhD (oliver.schwardt@unibas.ch).
Title of the dissertation: Design, synthesis, and evaluation of bacterial sialic acid uptake inhibitors.
Responsible for:

- Design, synthesis and characterisation of glycomimetics.
- Development and optimisation of biophysical assays for the compounds' evaluations.
- Computational investigations via molecular dynamics.
- Project leading and coordinating with the partners involved in the biochemical and microbiological aspects of the project from Sweden, Switzerland, Italy, India, and New Zealand.

06/2021 - 07/2022 **Industrial Secondment**
Red Glead Discovery AB, Lund, Sweden.
Supervisor: Richard E. Johnsson PhD (richard.johnsson@redglead.com).

02/2017 - 09/2017 **Master Thesis in Medicinal Chemistry**
Department of Chemistry, Lund University, Lund, Sweden.
Supervisor: Prof. Ulf Nilsson.
Title of the dissertation: Synthesis of sialic acid 9-C and 3-OH derivatives: the challenge of developing new antibacterial drugs by inhibition of sialic acid uptake.

11/2016 - 01/2017 **Internship in Medicinal Chemistry**
Department of Drug Sciences, Università degli Studi di Torino, Torino, Italy.
Supervisor: Prof Marco Lucio Lolli (marco.lolli@unito.it).
Aim of the project: Synthesis of human DHODH inhibitors.

08/2016 - 11/2016
09/2017 - 12/2017 **Internship in Community Pharmacy**
Farmacia Croce, Chivasso (TO), Italy.
Supervisor: Dott. Ernesto Demarchis.

09/2011 - 01/2017 **Volunteering in Intercultura Onlus (AFS)**
National office: Via Gracco del Secco, 100, 53034 Colle di Val d'Elsa (SI) Italy
Experience in several roles, both locally and nationally:

- Part of the 12 members national committee in 2016 in charge of designing a strategic development plan for the whole organization for the following triennium (2017-18-19).
- Responsible person for the organisation visibility, selection of candidates and training of the students in the local chapter (Ivrea and Canavese) from 2014 to 2017.
- National responsible for training students going to India in 2014 and 2015.
- Logistic team member in national and international events (*Il Corpo e La Rete*, Florence 2013, *Saper vivere insieme,* Trento 2015, *Volunteer Summer Summit*, Venice 2016.

EDUCATION

09/2012 - 04/2018 **MSc in Medicinal Chemistry and Pharmaceutical Technology**
Department of Drug Sciences, Università degli Studi di Torino, Torino, Italy.
Final grade: 110/110 cum laude.
Main subjects: Organic chemistry, Medicinal chemistry, pharmacology, biochemistry, molecular biology, toxicology, pharmaceutical technology, regulatory affairs, quality assurance.

07/2010 - 05/2011 **Exchange student at Kumararani Meena Muthiah Matriculation Higher Secondary School**
Chennai, Tamil Nadu, India,

09/2007 - 07/2012 **Student at Liceo scientifico statale "Isaac Newton"**
Final grade 80/100, Chivasso, Italy.

CONFERENCES AND TRAINING EVENTS

12/03/2021 **Determination of pharmacokinetic properties of drugs**
Training event by PhD4Glycodrugs, https://www.phd4glycodrug.eu/events-2/
Basel, Sweden.

22-25/10/2019 **Biotech Entrepreneurship and Life-science Start-ups**
Training event by PhD4Glycodrugs, https://www.phd4glycodrug.eu/events-2/
Lund, Sweden.

3-4/05/2019 **Træf for Organisk Kemi-Studerende (TOKS) XVII**
Conference on *Synthesis and Application of Biomolecules* at School of Pharmaceutical Sciences, University of Copenhagen, Denmark. http://toks.dk/.
Selected for oral presentation.

15-18/04/2019 **Computer-Aided Drug Design**
Training event by PhD4Glycodrugs, https://www.phd4glycodrug.eu/events-2/
Ljubljana, Slovenia.

7-9/11/2018 **Multivalency in GlycoDrug Design**
Training event by PhD4Glycodrugs, https://www.phd4glycodrug.eu/events-2/
Utrecht, Netherlands.

19-21/12/2018 **CARBO XXXIII, Sweet'18–Glycochemistry, Biology and Technology (SGBT'18)**
Conference at IISER Kolkata, India.
Poster contribution awarded.

2-4/07/2018 **Structural Glycosciences Summer School**
Summer school, https://glycoalps.univ-grenoble-alpes.fr/glyco-club/glyco-club-s-activities/structural-glycoscience-summer-school-2020-827229.htm
Grenoble, France.

10-16/07/2018 **International Summer School on Organic Synthesis "A. Corbella"**
Summer school, https://corbellasummerschool.unimi.it/
Gargnano (BS), Italy.

PUBBLICATIONS

1. **T. Bozzola**, M. Scalise, C.U. Larsson, M.C. Newton-Vesty, C. Rovegno, A. Mitra, J. Cramer, W.Y. Wahlgren, P. Radhakrishnan Santhakumari, R.E. Johnsson, O. Schwardt, B. Ernst, R. Friemann, R.C.J. Dobson, C. Indiveri, J. Schelin, U.J. Nilsson, U. Ellervik. Sialic acid derivatives inhibit SiaT transporters and delay bacterial growth. *ACS Chemical Biology,* 2022.
DOI https://doi.org/10.1021/acschembio.2c00321.

2. **T. Bozzola**, U.J. Nilsson, U. Ellervik. Direct Sialic Acid 4-OAc substitution by nitrogen, sulfur and carbon nucleophiles with retention of stereochemistry. *RSC Advances, 2022, **12**, 11992-11995.*

3. **T. Bozzola**, U.J. Nilsson, U. Ellervik. Structure activity relationships of sialic acid derivatives targeting three different bacterial sialic acid transporters. *In preparation,* 2022.

4. **T. Bozzola**, R. E. Johnsson, U. J. Nilsson, and U. Ellervik. Sialic acid 4-*N*-piperazine and piperidine

derivatives target sialic acid uptake by the *P. mirabilis* sodium solute symporter. *Submitted*, 2022.

LANGUAGES

Italian Native
English Fluent
Spanish Intermediate