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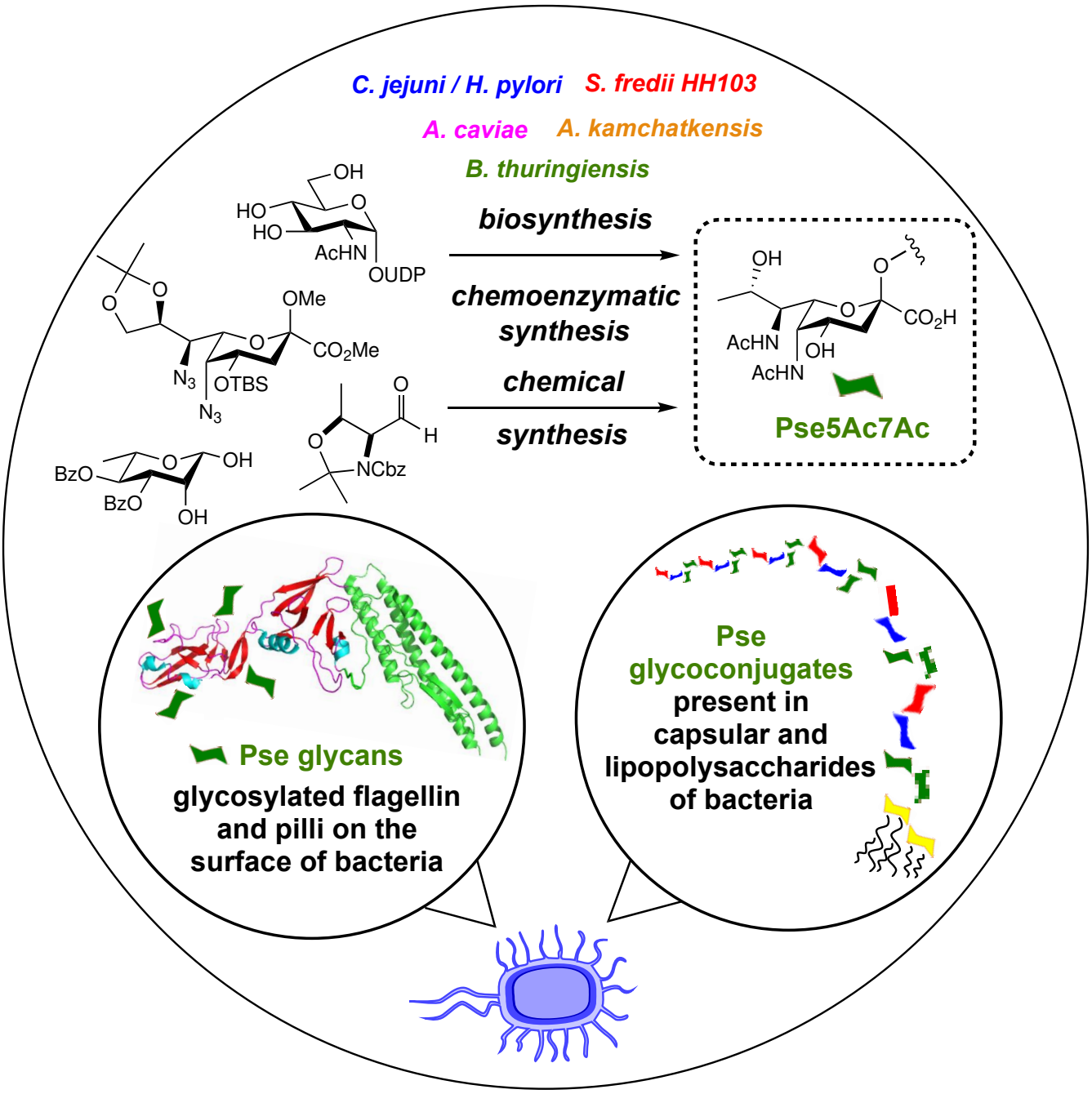
Synthetic approaches for accessing pseudaminic acid (Pse) bacterial glycans

Emily K. P. Flack[a]†, Harriet S. Chidwick[a]†, Matthew Best[a], Gavin H. Thomas[b] and Martin A. Fascione*[a]



[a] Dr. E. K. P. Flack, Dr. H. S. Chidwick, Dr. M. A. Fascione
Department of Chemistry
University of York
York, YO10 5DD, UK
E-mail: martin.fascione@york.ac.uk
[b] Prof G. H. Thomas
Department of Biology
University of York
York, YO10 5DD, UK
† These authors contributed equally.

Abstract: Pseudaminic acids (Pse) are a group of non-mammalian nonulosonic acids (NulOs) that have been shown as an important virulence factor for a number of pathogenic bacteria, including emerging multidrug resistant ESKAPE pathogens. Despite their discovery over 30 years ago, relatively little is known about the biological significance of Pse glycans compared to their sialic acid analogues, primarily due to a lack of access to the synthetically challenging Pse architecture. Recently however the Pse backbone has been subjected to increasing synthetic exploration by carbohydrate (bio)chemists, and the total synthesis of complex Pse glycans achieved using inspiration from the biosynthesis and subsequent detailed study of the chemical glycosylation using Pse donors. In this minireview we provide context for these efforts by summarising the recent synthetic approaches pioneered for accessing Pse glycans, which are set to open up this underexplored area of glycoscience to the wider scientific community.

1. Introduction

At the beginning of the millennium, sugars were defined as the "Cinderella" molecules of biology; the neglected, over-looked sister of the nucleic acids and proteins.[1] Recently however the ubiquity of glycosylation and glycans has become apparent and it is now accepted that sugars play essential roles in biology beyond that of an energy source. [2] Nevertheless, glycan structure and function still remains relatively unexplored in many forms of life including bacteria, archaea, fungi and algae, and until recently many classes of glycosylation were believed to be reserved as a eukaryotic post-translational modification. The discovery of the Pgl pathway within *Campylobacter jejuni* was the first example of a general *N*-linked glycosylation pathway in prokaryotes,[3] and a significant breakthrough in the field of bacterial glycobiology. The types of monosaccharide found in bacteria show greater diversity than eukaryotic monosaccharides and often undergo an extensive range of chemical modifications (including alkylation, acylation, aminoacylation and phosphorylation).[4]

Key cell-surface glycoconjugates in Gram-negative bacteria include peptidoglycan, lipopolysaccharide (LPS), lipooligosaccharide (LOS), extracellular polysaccharide (EPS), capsular polysaccharide (CPS) and glycoproteins, whilst Gram-positive bacteria do not contain LPS or LOS but do have glycosylated lipoteichoic acids and wall teichoic acids.[4] Perhaps one of the most intriguing biological roles of bacterial sugars is in "molecular mimicry", wherein microbial pathogens cover their surfaces with glycans that are similar to the hosts, with the aim of evading host immune responses or increasing host tolerance towards the pathogen.[2a] This is observed with the nonulosonic acids (nulOs),[5] a sub-group of nine carbon α-keto acid sugars, some of which are found as terminal residues in human glycoconjugates and often in bacterial glycoconjugates.[6] A well-studied sub-class of these sugars are the sialic acids, of which over 50 naturally occurring derivatives have been identified.[7] Sialic acids can be found in all three domains of life, and include ubiquitous 2-keto-3-deoxy-5-acetamido-D-glycero-D-galacto-nonulosonic acid (*N*-acetyl-neuraminic acid, Neu5Ac **1**, Figure 1a).[8]

2. Occurrence and biological significance of Pseudaminic acid glycans

A less common group of nulOs which are of particular interest are pseudaminic acids (Pse). These "sialic acid-like" sugars are not present in mammalian cells, but have been found in many bacterial species.[5, 9] The general term pseudaminic acid arose from the initial discovery of α-5,7-diacetamido-3,5,7,9-tetradeoxy-L-glycero-L-manno-non-2-ulosonic acid (Pse5Ac7Ac **2**, Figure 1a), within the LPS O-antigen of *Pseudomonas aeruginosa O7* and *O9*.[10] Pse5Ac7Ac **2** varies from Neu5Ac **1** in stereochemistry at carbons 5, 7 and 8 and functionality at 7 and 9. Derivatives of Pse5Ac7Ac **2** have since been characterised in which *N*-linked substituents at C5 and C7 can show wide diversity (Figure 1b),[5] and the Bacterial Carbohydrate Structure Database currently lists 149 bacterial glycan structures in which Pse5Ac7Ac **2** or a derivative is present (http://csdb.glycoscience.ru/bacterial/) (May 2019). Pse glycoconjugates have also been identified in human gut archaea *Methanobrevibacter smithii*, where Pse5Ac7Ac **2** is present in the CPS.[11] Although putative nulO biosynthesis genes have also been found in several other species of archaea, complete pathways within these genomes are yet to be assigned.[11b] Unlike Neu5Ac **1** which is most commonly a terminal glycan residue, Pse is predominantly found as an internal component of glycoconjugates, although there are some examples of terminal Pse residues.[12]

Pse glycoconjugates are notably present in the LPS and CPS of many pathogenic bacteria, including nosocomial pathogens *P. aeruginosa* and *Acinetobacter baumannii*.[9a, 9c, 9e, 10, 12-13] Furthermore serine and threonine residues of flagella and pili may be *O*-glycosylated with Pse.[10, 12, 14] For example, in the gastric pathogen *Helicobacter pylori*, flagella are exclusively glycosylated with Pse5Ac7Ac **2** and experimental evidence has shown that disruption of flagellin glycosylation resulted in a loss of bacterial motility due to a lack of detectable flagella.[15] Similarly in *C. jejuni*, the leading cause of gastroenteritis, the FlaA1 flagellin protein is also glycosylated with Pse at multiple residues,[14a] and mutation of these residues resulted in truncated flagella and reduced motility.[16] It has been

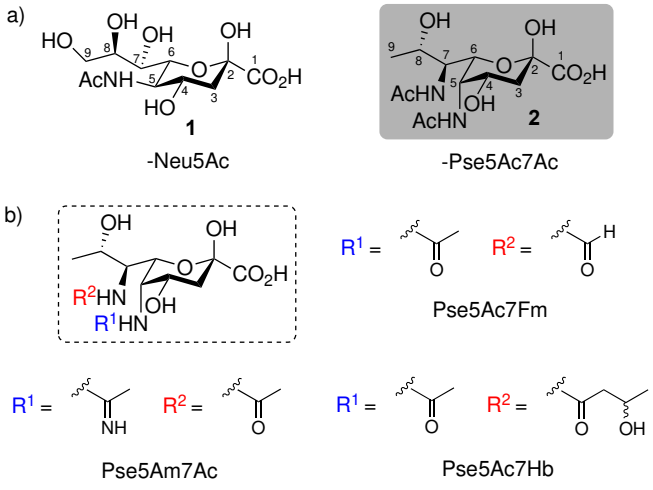


Figure 1. a. Ubiquitous sialic acid Neu5Ac **1**, and nonulosonic acid Pse5Ac7Ac **2**. **b.** Naturally occuring Pse derivatives, modified at *N*5 and *N*7.

demonstrated that host immune responses to *C. jejuni* may be dampened by the presence of the cell surface Pse modified flagella, which were shown to bind to the human immune-modulatory receptor siglec-10, resulting in increased murine dendritic cell interleukin-10 expression.[6] As both motility and a capacity to evade host immune response are established key virulence factors in the ability of pathogenic bacteria to colonize the host,[17] novel therapeutics with the potential to prevent bacterial presentation of Pse are therefore clearly of critical significance. However, further exploration of this area of glycoscience has been severely hampered by limited access to Pse based glycans and probes. In this review, we summarise recent ground breaking work in the field of Pse glycan synthesis, which is set to open up the study of Pse glycobiology to the wider scientific community.

3. Biosynthesis of pseudaminic acids

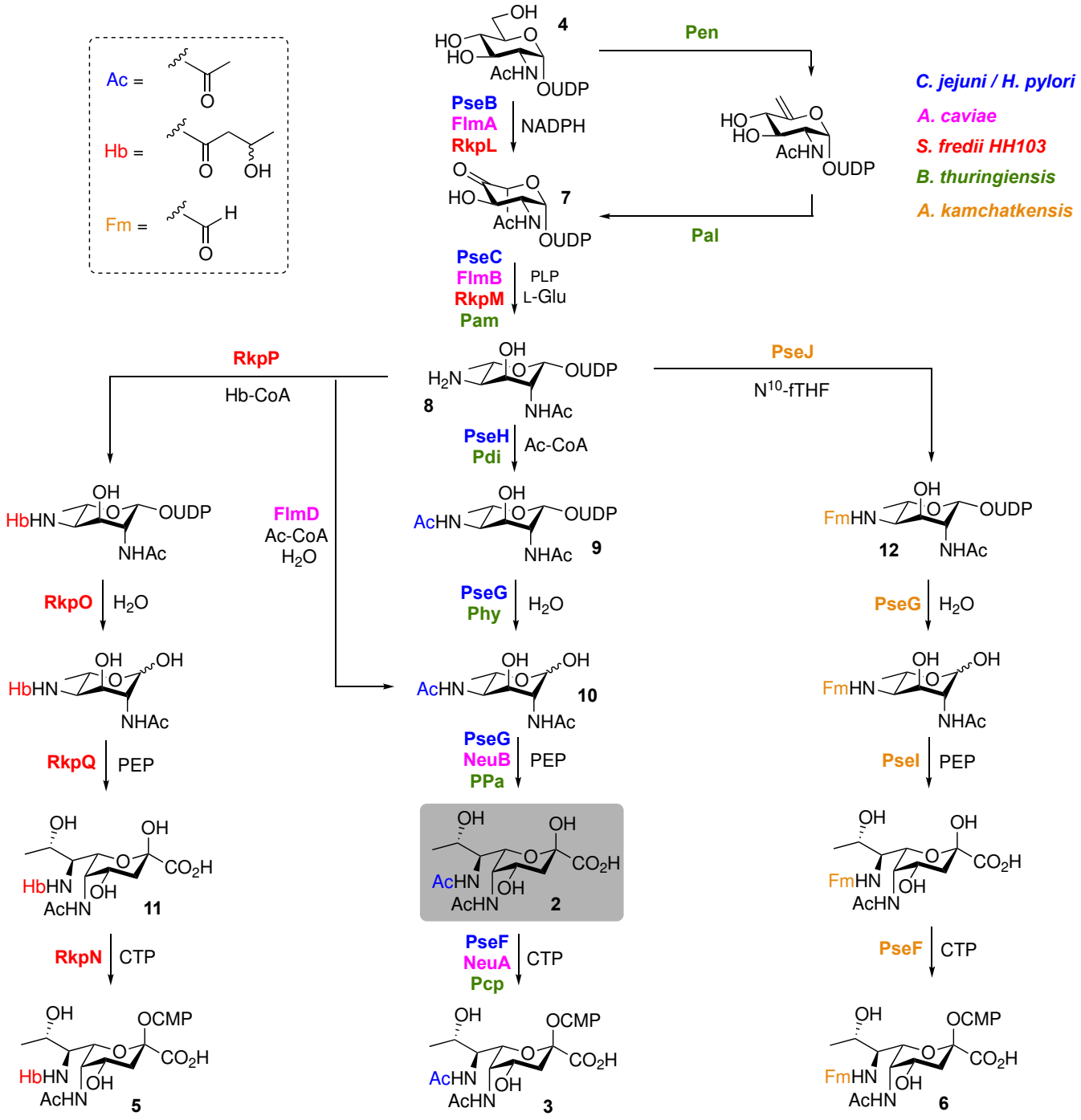
The biosynthetic pathways of nucleotide-activated α-CMP-Pse5Ac7Ac **3** and its derivatives have been proposed in multiple organisms including multidrug resistant pathogens (all pathways described in this section are depicted in Scheme 1). These organisms include the aforementioned *C. jejuni* and *H. pylori*, where the α-CMP-Pse5Ac7Ac **3** biosynthetic pathways have been well characterised *in vitro*. [9b, 18] The biosynthesis of CMP-Pse has a number of features common to the biosynthesis of all CMP-nulOs, and have proved inspiring for novel chemical approaches, *vide infra*. In all CMP-nulO biosynthesis pathways an activated hexose is initially utilised, in Pse biosynthesis this is UDP-GlcNAc **4**. Varying numbers of steps including hydrolysis of the nucleotide are required to convert the activated hexose into the desired hexose, before condensation with a three-carbon substrate, phosphoenol pyruvate (PEP), yields a nulO. Finally, all nulOs are activated at the anomeric position to form reactive glycosyl donors. *In vivo* this is often through installation of a cytidine monophosphate group (CMP), and this CMP-donor then acts as a substrate for glycosyltransferase enzyme (GTs) which incorporate the NulO into glycoconjugates.[5]

Typically, within Gram-negative bacteria, biosynthesis of α-CMP-Pse5Ac7Ac **3** and its derivatives **5** and **6** is performed in five, six or seven enzymatic steps.[18a] In *C. jejuni* and *H. pylori*, the UDP-GlcNAc **4** starting material is dehydrated and epimerised by PseB to yield ketone **7**, the substrate for L-glutamine dependent aminotransferase PseC, which subsequently affords 4-amino-AltNAc sugar **8**.[19] *N*-acetyltransferase, PseH, then uses amine **8** and acetyl-coenzyme A (Ac-CoA) to afford acetylated **9**, which then undergoes hydrolysis of UDP, facilitated by nucleotidase PseG, yielding the key biosynthetic intermediate 6-deoxy-AltdiNAc **10**. A pseudaminic acid synthase, PseI, then utilises PEP to convert **10** to the nulO Pse5Ac7Ac **2** in an aldol condensation, a strategy often mirrored in chemical syntheses of nine carbon α-keto acid sugars. The sixth enzyme in this biosynthetic pathway, CMP-synthetase PseF, finally installs the CMP-moiety at the anomeric hydroxyl, using CTP to afford the CMP-Pse5Ac7Ac **3** glycosyl donor. This biosynthetic route differs slightly in the human pathogen *Aeromonas caviae*, where only five enzymes are required due to the dual-functionality of FlmD which performs the acetyl-transferase and UDP-hydrolase roles which are carried out by PseH and PseG within *C. jejuni* and *H. pylori*.[20] Alternatively, in the Gram-positive bacterium *Bacillus thuringiensis*, the biosynthesis of α-CMP-Pse5Ac7Ac **3** is carried out by seven enzymatic reactions, where two enzymes "Pen" and "Pal" are required to perform the PseB UDP-GlcNAc 5-epimerase and 4,6-dehydratase roles.[21] This yields the PseC or Pam substrate **7**, from which point the remainder of the pathway is analogous to that in *C. jejuni* and *H. pylori*. The biosynthesis of Pse derivatives have also been characterised including α-CMP-5-acetamido-7-*N*-(3-hydroxybutanoyl)-3,5,7,9-tetradeoxy-L-glycero-L-manno-non-2-ulosonic acid (CMP-Pse5Ac7Hb **5**) found within *Sinorhizobium fredii HH103*, where Pse5Ac7Hb **11** makes up a homopolysaccharide K-antigen.[22] The biosynthesis in this plant symbiont begins with two enzymes,[14b] homologous to that of *C. jejuni* PseB and PseC. The pathways diverge at the third enzyme, where in *S. fredii* the primary amine of C7 in **8** is derivatised with a 3-hydoxybutyryl (Hb) group by the acyl transferase RkpP, in place of an *N*-acetylation that occurs in Pse5Ac7Ac **2** biosynthesis. The remainder of the CMP-Pse5Ac7Hb **5** pathway, comprises three subsequent enzymes homologous to those of the CMP-Pse5Ac7Ac pathway.

Most recently the α-CMP-5-acetamido-7-N-formyl-3,5,7,9-tetradeoxy-L-glycero-L-manno-non-2-ulosonic acid (CMP-Pse5Ac7Fm **6**) biosynthetic pathway from Gram-positive *Anoxybacillus kamchatkensis* has also been partially elucidated.[23] This pathway requires six-enzymes, demonstrating that a six-enzyme pathway is not an exclusive feature of Gram-negative species. The activities of the first three enzymes, PseB, PseC and PseJ were confirmed *in vitro*. PseB and PseC were shown to function similarly to their Gram-negative homologues to produce amine **8**. In a one-pot three enzyme synthesis, the formyl donor *N*10-formyltetrahydrofolate (*N*10-fTHF) was then used to formylate the C4 amine of **8** to produce UDP-4,6-dideoxy-4-formamido-L-AltNAc **12**. Three downstream enzymes have been putatively assigned as those required to produce CMP-Pse5Ac7Fm **6** from **12** in three steps analogous to those used in CMP-Pse5Ac7Ac **3** biosynthesis. There are currently no other examples of *in vitro* characterisation of enzymes involved in biosynthesis of Pse derivatives.

In addition to Pse5Ac7Ac **2**, the derivative 5-acetamido-7-acetamidino-3,5,7,9-tetradeoxy-L-glycero-L-manno-non-2-ulosonic acid (Pse5Ac7Am), is found on the flagella of *C. jejuni*.[24] While the 3-hydroxybutyryl and formyl substituents present in **5** and **6** are installed early in the biosynthetic pathway, it is currently unclear at which point in the pathway the acetamido group is converted into to an acetamidino group, but it has been suggested that Pse5Ac7Ac **2** or CMP-Pse5Ac7Ac **3** may be a late stage substrate for the candidate enzyme, PseA.[24] It is notable that both early and late stage functionalization approaches have been explored in the chemical derivatisation of Pse glycans *vide infra*.

The elucidation of the CMP-Pse5Ac7Ac **3** biosynthetic pathway has been complemented by detailed biochemical and structural characterisation of the individual enzymes from *H. pylori* and *C. jejuni*,[9b] and this has enabled the one-pot multi-enzyme synthesis of Pse5Ac7Ac **2** and CMP-Pse5Ac7Ac **3**.[18a] To date however there is very limited knowledge of glycosyltransferases (GTs) which utilise CMP-Pse donors (pseudaminyltransferases, PseTs), with only a handful of genes putatively assigned as PseTs based upon sequence homology to sialyltransferases (SiaTs) or transferases that process



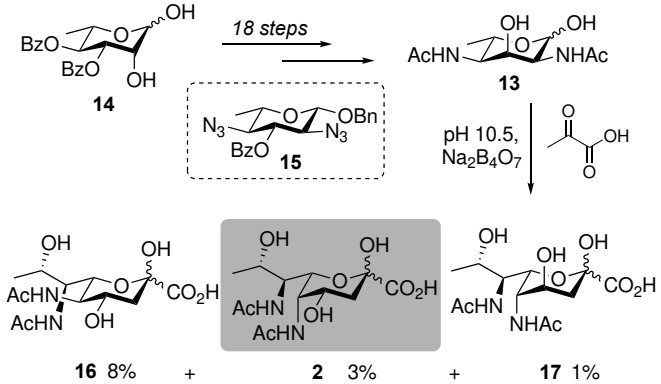
Scheme 1. Biosynthetic pathways for CMP-Pse5Ac7Hb **5**, CMP-Pse5Ac7Ac **3** and CMP-Pse5Ac7Fm **6.** *C. jejuni* and *H. pylori* enzymes in blue, *S. fredii HH103* enzymes in red, *B. thuringiensis* enzymes in green, *A. caviae* enzymes in pink and *A. kamchatkensis* enzymes in orange.

structurally related α-keto sugars.[9g, 25] Furthermore, none of the proteins encoded by the putative PseTs have been functionally characterised *in vitro*, precluding the use of enzymes for accessing Pse-glycosides, which are an essential requirement for biological studies into the significance of Pse. Thus chemical synthesis of these glycans has been adopted as an alternative approach.

4. Chemical approaches to the synthesis of pseudaminic acids and glycosides

Chemical synthetic routes to complex molecules are often inspired by biosynthesis pathways and Pse glycans are no different. Whilst Pse5Ac7Ac **2** is fairly similar to commercially available Neu5Ac **1**, its synthesis has proved more challenging. Many of the complications in the synthesis of Pse can be attributed to the epimeric stereochemistry at C5, C7 and C8 and deoxygenation at C9. To date, all literature approaches to chemical synthesis can be classified as one of three strategies: i) synthetic conversion from an analogous nulO acid, which requires inversion of several stereocentres; ii) *de novo* synthesis from a four carbon chiral precursor; or iii) a biomimetic approach targeting synthesis of the biosynthetic intermediate 6-deoxy-AltdiNAc **10**, or analogues, followed by an aldol-type condensation.

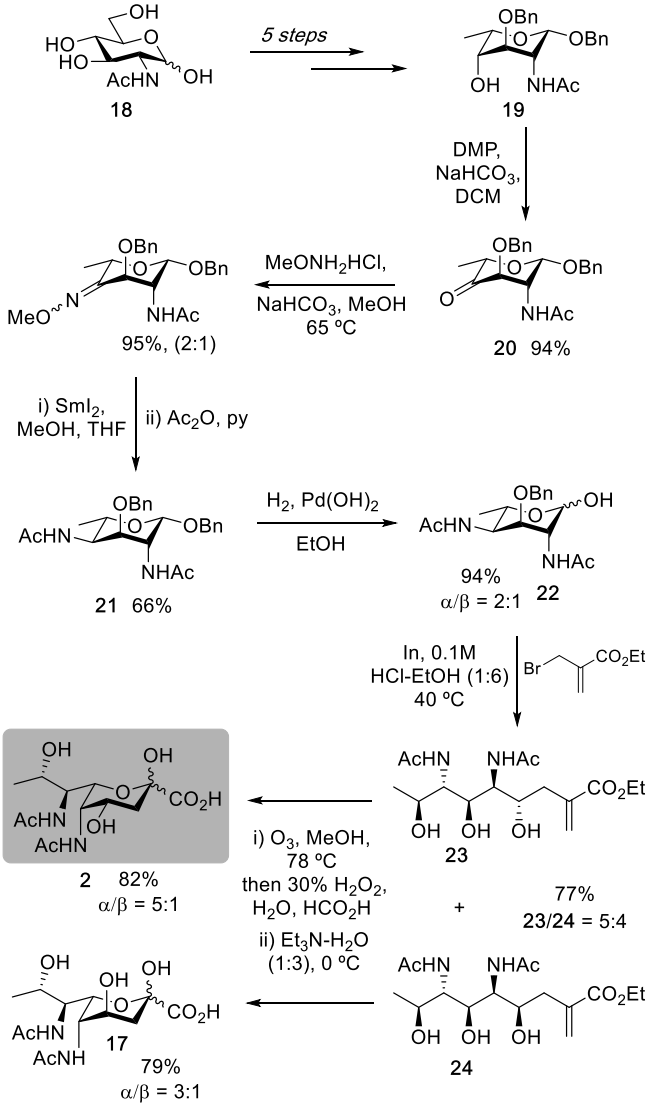
The first publication reporting the synthesis of Pse5Ac7Ac **2** came from Tsvetkov and co-workers in 2001,[26] 17 years after the discovery of Pse5Ac7Ac **2** by Knirel,[10] and falls into the latter category. The authors used an approach that was analogous to the strategies previously deployed in the synthesis of sialic acids, where a six-carbon scaffold is subjected to an aldol-type condensation reaction with oxaloacetic acid, followed by decarboxylation, to yield the nine-carbon product.[27] In this synthesis 2,4-diacetamido-2,4,6-trideoxy-L-allose **13** (Scheme 2) was as used the six-carbon intermediate, an epimer of naturally occurring biosynthetic intermediate **10**. A multi-step synthesis



Scheme 2. Tsvetkov and co-workers approach to Pse5Ac7Ac **2**.

beginning from 3,4-di-O-benzoyl-β-L-rhamnopyranoside **14** was employed in the synthesis of diacetamido sugar **13**, and proceeded through the key diequatorial azide **15**. The condensation of reducing sugar **13** with oxaloacetic acid produced three nonulosonic acids, **16**, Pse5Ac7Ac **2** and **17**, in 8%, 3% and 1% yields respectively. The authors remarked that the stereoselectivity of the condensation reaction was unexpected, and the harsh conditions of the reaction presumably resulted in initial epimerisation at C2 in **13**. Although a landmark as the first chemical synthesis of Pse5Ac7Ac **2**, the low yield of the desired product, left clear room for improvement.

Ito and co-workers reported the next chemical synthesis of Pse5Ac7Ac **2** (Scheme 3)[28] *via* biosynthetic intermediate 6-deoxy-L-AltdiNAc **10** from inexpensive starting material *N*-acetyl-glucosamine **18** (GlcNAc). GlcNAc **18** was manipulated into L-idosamine **19**, a precursor of the biomimetic intermediate **21**. The initial strategy proposed proceeding through azide inversion at C4 in **19**, but was unsuccessful. Therefore an alternative strategy was employed using a Dess-Martin periodane (DMP) oxidation of 19 to afford ketone **20**, followed by oxime formation. Reduction with samarium iodide, and immediate acetylation of the resultant free *N*5 amine afforded diacetamido **21** in 66% yield. The benzyl protected biosynthetic precursor **22** was then accessed through hydrogenolysis, and subjected to the key synthetic step, an indium mediated allylation using bromomethylacrylate ester, which yielded a mixture of both anti **23** and syn **24** (5:4) chain elongated products in 77% yield. Subsequent cyclisation to afford the ring closed 9-carbon Pse5Ac7Ac **2** and 4-epi-Pse5Ac7Ac **17**, by ozonolysis and saponification proceeded in excellent yield for both isomers. The

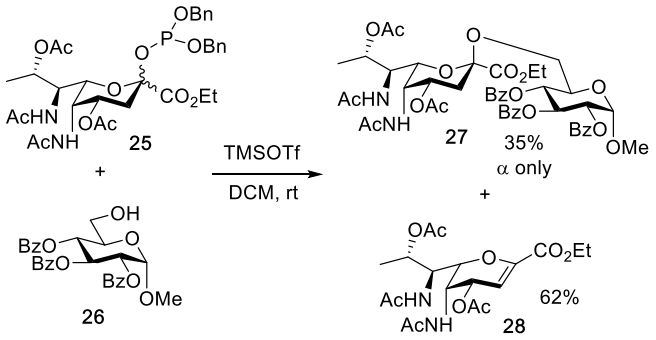


Scheme 3. Synthesis of Pse5Ac7Ac **2** and 4-epi-Pse5Ac7Ac **17** via **21** as reported by Ito and coworkers.

obvious limitation of this route was the low levels of

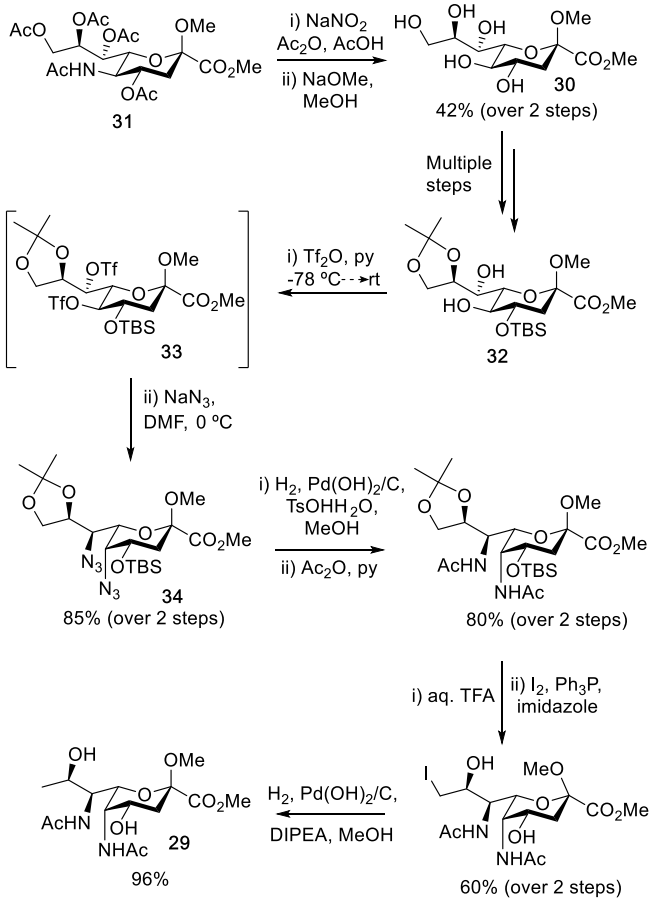
stereocontrol achieved in the allylation, which could not be improved.

However, enough Pse5Ac7Ac **2** was accessed to enable the first chemical glycosylation of a Pse glycosyl donor to be performed (Scheme 4). The authors subjected dibenzyl phosphate donor **25** to a TMSOTf mediated glycosylation in dichloromethane (DCM) using methyl glycoside acceptor **26**, and isolated the α-glycoside **27** in 35% yield, however there was also significant formation of the glycal byproduct **28**.



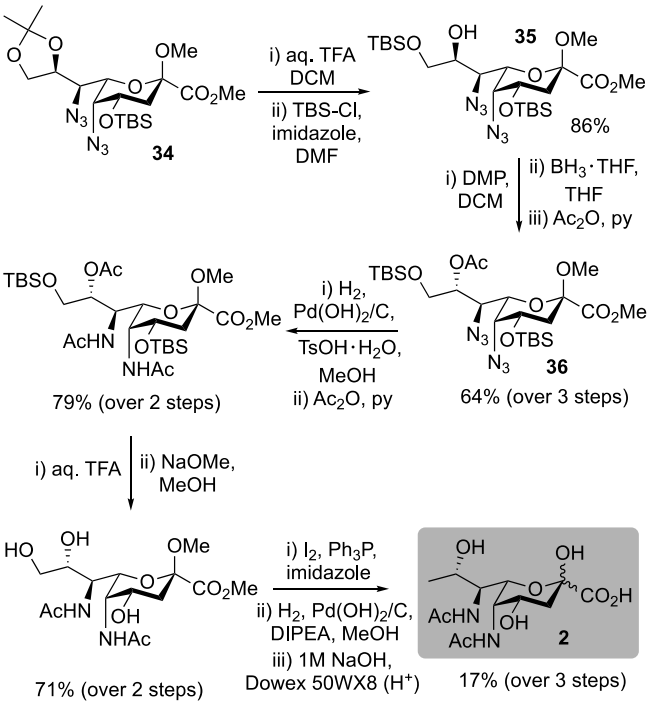
Scheme 4. Glycosylation of dibenzyl phosphite Pse glycosyl donor **25**.

In 2014 Kiefel and co-workers reported the synthesis of a Pse5Ac7Ac analogue, 8-epi-Pse5Ac7Ac methyl ester **29**, over 13 steps.[29] Their initial retrosynthetic analysis highlighted sialic acid 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (KDN) as a potential precursor that would furnish the correct stereochemistry required at C5 and C7 through initial azide inversion. However, due to difficulties in obtaining KDN from commercial sources, the authors first had to synthesise the methyl ester protected KDN **30** from protected Neu5Ac **31** *via* a Zbiral deamination[30] (Scheme 5) of the *N*5 equatorial acetamido group and Zemplen deprotection. Following a number of protecting group manipulations, key diol **32** was produced. The diol was then subjected to a parallel inversion, proceeding through the bis-triflate intermediate **33**, before treatment with sodium azide yielded the diazido derivative **34**, bearing the correct Pse stereochemistry at C5 and C7. Following optimisation of conditions the diazido compound **34** was reduced and bis-acetylated before concomitant acetal and TBS protection and then deoxygenation of the primary alcohol, via iodination and $Pd(OH)_2$ catalysed reduction to afford the 8-epi-Pse5Ac7Ac methyl ester **29** (Scheme 5).



Scheme 5. Synthesis of 8-epi-Pse5Ac7Ac methyl ester **29** as reported by Kiefel and co-workers.

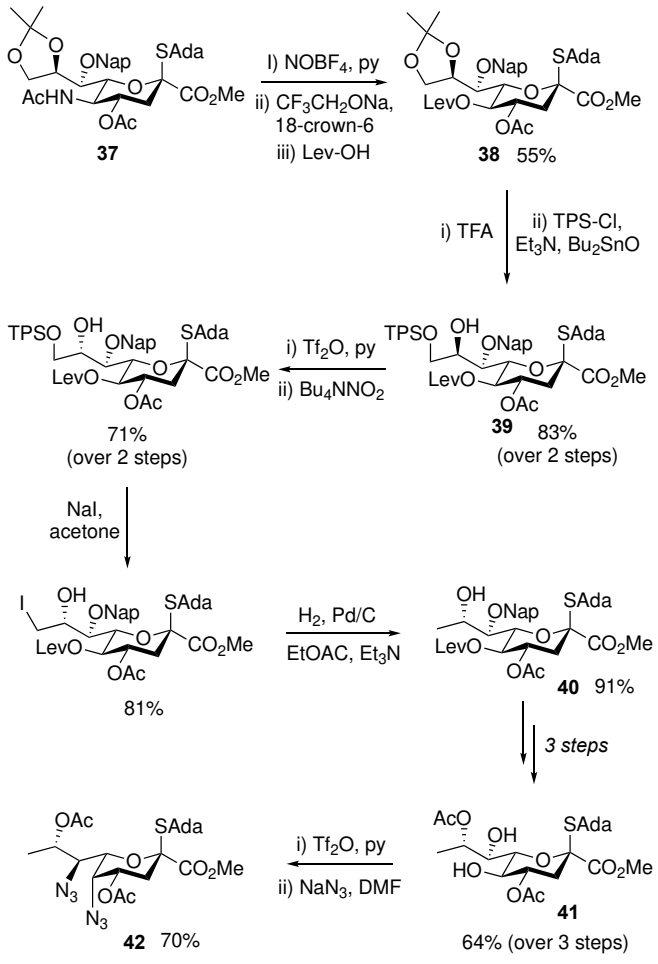
Two years on from reporting the synthesis of 8-epi-Pse5Ac7Ac **29**, Kiefel, Payne and co-workers disclosed the synthesis of Pse5Ac7Ac **2** in an overall 17-step sythesis from Neu5Ac **1** (Scheme 6).[31] This strategy also required the synthesis of diazido acetonide **34**, established by Kiefel,[29] which was then subjected to acetal clevage and selective TBS reprotection of the primary alcohol at *O*9. The free *O*7 hydroxy of **35** was then available for DMP oxidation, followed by stereoselective reduction with BH_3·THF, and protection to deliver the C8 epimerised acetyl **36**. From therein the endgame involved similar protecting group manipulations, and an iodination-deoxygenation strategy as employed in the 8-epi-Pse5Ac7Ac methyl ester **29** synthesis. Culminating in hydrolysis and saponification to yield the parent Pse5Ac7Ac **2**.



Scheme 6. Synthesis of Pse5Ac7Ac **2** from Neu5Ac **1**, proceeding through key diazido intermediate **34**.

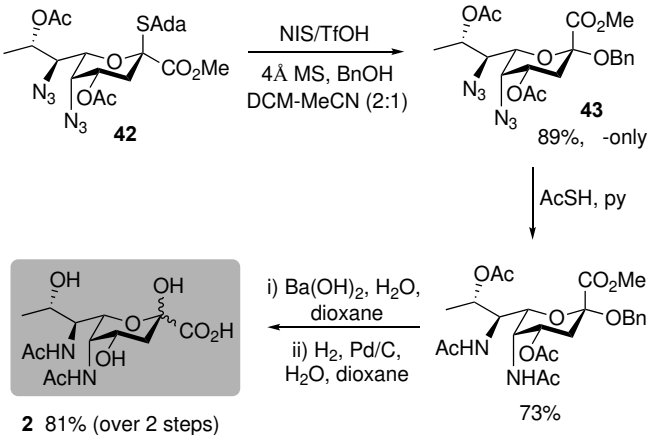
Dhakal and Crich also developed a synthesis of Pse sugars using Neu5Ac 1 as a starting material.[32] A Zbrial deamination by nitrosylation was similarly employed, on this occasion using a thioadamantyl (Ada) donor **37** as the substrate (Scheme 7). Levulinic acid was employed as the nucleophile and advantageously furnished a protected hydroxyl **38** in 55% overall yield. In exploring this reaction the authors noted that the

deamination reaction was more stereoselective when the C9 position was still functionalised with an oxygen. They therefore opted to deaminate **37** before performing protecting group manipulations followed by inversion of the hydroxyl stereochemistry at C8 of **39** by triflation and nucleophilic attack. Deoxygenation of C9 was then achieved through iodination and subsequent hydrogenation of iodide **40** in 91% yield. Three further protecting group manipulations yielded the diol **41**, which was then subjected to double parallel inversion through triflation and attack with sodium azide, affording the diazido SAda donor **42** bearing the correct Pse stereochemistry at positions 5 and 7 (Scheme 7). Glycosylation of this donor under NIS/TfOH activation (Scheme 8) with benzyl alcohol as an acceptor afforded the benzyl glycoside **43** in an excellent 89% yield as only the β-anomer. The benzyl glycoside **43** was then reduced and acetylated at N5 and N7 in a single step, before deprotection by ester hydrolysis and hydrogenation in two steps to afford Pse5Ac7Ac **2** in 81% yield. The authors noted that the β-selectivity achieved in a dichloromethane-acetonitrile (2:1) solvent mix at −78 °C using benzyl alcohol as an acceptor was also observed with more hindered glycosyl acceptors, and



Scheme 7. Dhakal and Crich's Zbiral deamination of Pse thioglycoside **37** with a levunoic acid nucleophile enabled expedient synthesis of diazido donor **42**.

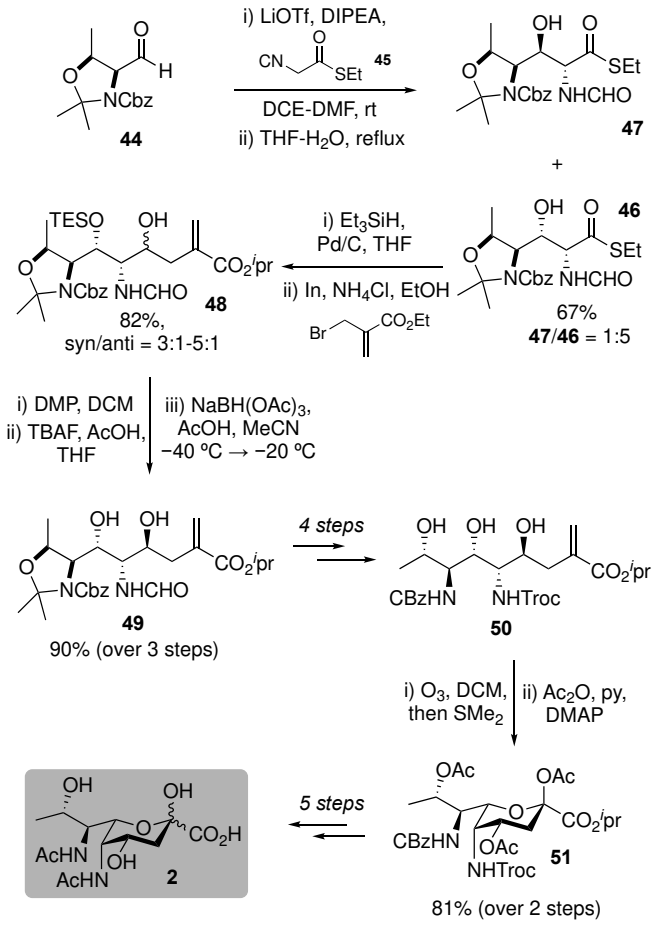
attributed the exquisite equatorial selectivity to the preference for the side chain in Pse glycans to adopt a *trans,gauche* conformation. Finally the authors also demonstrated that regioselective deprotection of the 5-azido and 7-azido positions could be achieved under orthogonal conditions, enabling the late



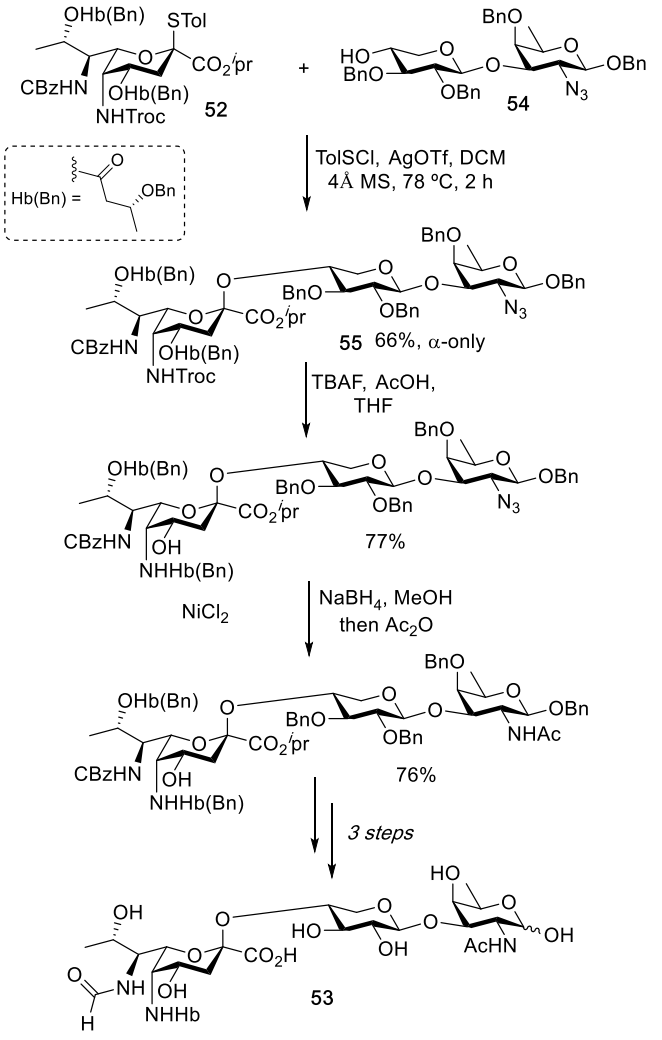
Scheme 8. Glycosylation of diazido Pse donor **42** with benzyl alcohol *en route* to the synthesis of Pse5Ac7Ac **2**.

stage selective functionalisation and differentiation of the two positions, as is sometimes observed in the biosynthesis of Pse *in vivo*.

Li and co-workers developed an alternative *de novo* strategy to access Pse5Ac7Ac **2** and Pse glycosides (Scheme 9), beginning from the readily avaliable chiral precursor L-threonine,[33] which was epimerised to L-allo threonine, and transformed into aldehyde **44**. In an analogous strategy to that employed for the synthesis of an epimeric nonulosonic acid-legionaminic acid,[34] the authors opted to deploy the aldehyde in a diastereoselective aldol-type condensation to build the 6-deoxy AltdiNAc biosynthetic backbone, which could then be subjected to a chain elongation by the indium mediated allylation, pioneered in the approach used by Ito and co-workers.[28] The key step in this synthesis was the condensation of the aldehyde **44** and glycine thioester isonitrile **45**, which required significant optimisation to achieve diastereocontrol to deliver the desired diastereomer **46** over the undesired **47** in a 5:1 ratio, with the use of lithium triflate as a Lewis acid and a dicholoroethane (DCE)-dichloromethane solvent mix proving essential. Following triethylsilane reduction of the thioester **46** to an aldehyde, and unexpected but concomitant TES protection of a hydroxyl, poorer levels of the required anti-selectivity were then achieved in the indium mediated allylation (syn/anti = 3:1-5:1) than those reported by Ito using an unprotected substrate (syn/anti = 4:5, Scheme 3). Therefore, the authors opted to oxidise the newly formed alcohol **48** to a ketone using DMP, remove the TES protecting group from the adjacent position and then reduce the ketone with sodium triacetoxy borohydride to selectively afford only the desired anti-stereoisomer **49** in 90% yield over 3 steps. Following a number of protecting group manipulations, the *N*-Troc protected alkene **50** was then subjected to ozonolysis and ring closing, followed by acetylation to yield protected Pse5Ac7Ac **51**. The isopropyl ester **51** could then be deprotected over multiple steps to afford the parent Pse5Ac7Ac sugar **2**, but the protected precursor was also a useful intermediate *en route* to Pse thioglycoside donors for chemical glycosylation studies.



Scheme 9. Li and co-worker's *de novo* synthesis of Pse5Ac7Ac from an L-allo threonine starting material.

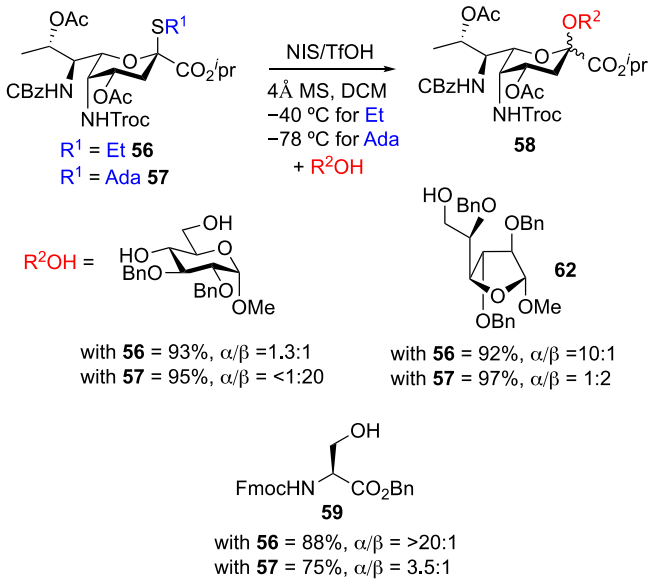


Scheme 10. Total synthesis of Pse containing pili trisaccharide **53** from *P. aeruginosa 1255*.

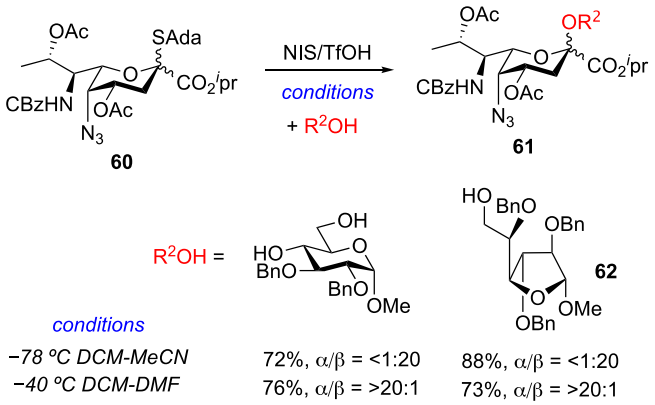
Following significant exploration of Pse glycosylation protocols, the authors set out to perform the first total synthesis of a complex Pse glycan and synthesised STol donor **52**, from **51**, bearing a benzyl protected protected 3-hydroxyl-butyryl group (Scheme 10). This pseudo protecting group was chosen for glycosylations because it could potentially undergo *O*-*N* acyl transfer to afford the *N*5 substituent of the known trisaccharide **53**, which is present on the pili of *P. aeruginosa* strain 1244.[33] When this donor was incubated with protected xylose-FucNAc disaccharide acceptor **54** and activated with *p*-TolSCl/AgOTf the desired α-linked trisaccharide **55** was formed with complete stereoselectivity in 66% yield. Subsequent TBAF mediated Troc deprotection at *N*5 on the Pse sugar triggered the anticipated transfer of the (*R*)-3-benzyloxybutyryl group from *O*4 to *N*5, as required in the target glycan. Reduction and acetylation of the azido group on the FucNAc moiety set the stage for the culmination of this *tour de force* synthesis by removal of all protecting groups and formylation of *N*7 in three steps to yield the final trisaccharide **53**.

In a subsequent study Li and co-workers noted that the stereocontrol observed in glycosylation using the Pse STol donor bearing the *N*5-Troc/*N*7-Cbz protecting group combination (as in **52**) was dependant on the acceptor,[35] with reactive acceptors such as benzyl alcohol alternatively affording the β-anomer with complete stereocontrol. Therefore they explored whether tuning the reactivity of the thioglycoside aglycon could affect stereocontrol, focussing specifically on the SEt donor **56**, and less reactive SAda donor **57**, in NIS/TfOH mediated glycosylations (Scheme 11). All glycosylations proceeded in excellent yield but the authors noted that the SEt donor **56** gave moderate α–selectivity in the synthesis of Pse glycosides **58**, while the β-anomer was generally favoured by the SAda donor **57**. However the overall selectivity was seemingly still acceptor d ependent as demonstrated by glycosylation of serine acceptor **59** which favoured α-glycoside formation with both donors. Taking inspiration from Dhakal and Crich's use of an azido substituted Pse donor,[32] the authors next screened the modifed *N*5-azido SAda donor **60**, which lacked the potential participating Troc group, in an attempt to maximise β-stereoselectivity (Scheme 12). On this occasion NIS/TfOH activation in dichloromethane-acetonitrile solvent mix afforded disaccharides **61** with complete stereoselectivity. Furthermore, the same donor showed bimodal reactivity and afforded disaccharides **61** with complete α-stereoselectivity when subjected to glycosylation under similar activating conditions but in a dichloromethane-*N,N*-dimethylformamide (DMF) solvent mix. The authors proposed that DMF forms a transient glycosyl imidate intermediate, which is converted to the reactive β-imidate prior to S_N2-like glycosylation from the α-face, in a Curtin-Hammett kinetic scenario. Notably the glycosylations of *N*5-Troc SAda donor **57** were also completely α-stereoselective if DMF was used as an additive. The power of the β-selective strategy was showcased by elaborating the β-disaccharide product of glycosylation with

acceptor **62**, into a trisaccharide which mimics the skeletal framework of a repeating LPS unit from a Pse presenting bacterium.[35]



Scheme 11. Pse glycosylations of SEt donor **56** and SAda donor **57**

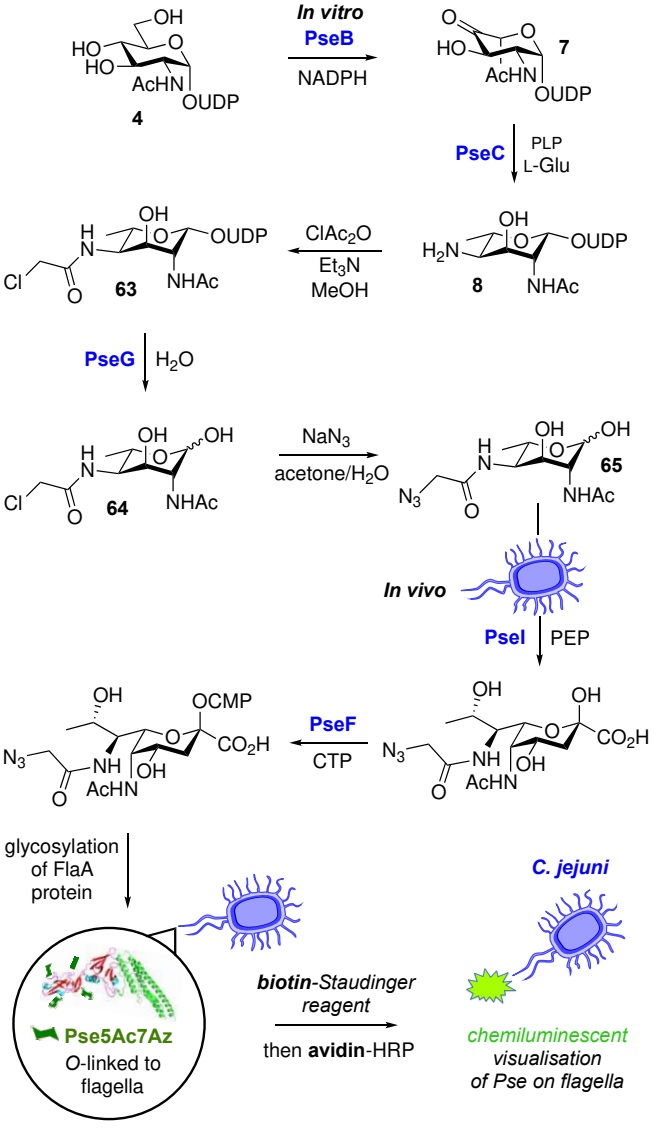


Scheme 12. Comparative stereoselective glycosylations of bimodal Pse donor **60** under different activating conditions.

5. Chemoenzymatic approaches to the synthesis of pseudaminic acid

Notably, several of the total syntheses of Pse glycans reported required installation of azido groups. This functional group may also be used as bioorthogonal handles when present on Pse derivatives, as demonstrated by the work of Tanner and colleagues who used a chemoenzymatic approach to azido-modify *C. jejuni* flagella (Scheme 13).[36] Firstly enzymes PseB and PseC were employed *in vitro* to afford amine **8** from the UDP-GlcNAc **4** biosynthetic starting material. Following chemically acetylation with chloroacetic anhydride, the PseG substrate analogue **63** was produced in a chemoenzymatic process. The UDP group of **63** could still then be hydolysed by PseG yielding reducing sugar **64** which was then treated with sodium azide to yield the altrose diamino functionalised sugar **65**, which is an azido analogue of key biosynthetic intermediate 6-deoxy-AltDiNAc **10** (Scheme 1). The subsequent Pse biosynthesis transformations proved promiscuous enough to utilise this azido analogue when it was fed to *C. jejuni* cells in growth media. Following *in vivo* biosynthetic transformation by PseI and PseF, and presumably as yet uncharacterised glycosyl transferases, the azido group incorporated into the Pse backbone could then be visualised on *C. jejuni* flagella by a Staudinger ligation using a biotin-linked reagent and western blot using an avidin-horseradishperoxidase (HRP) chemiluminescent reporter system.

Li and co-workers built upon this approach and also the methodology used in their total synthesis of Pse5Ac7Ac **2**, by using L-allo-threonine as the starting material for synthesis of 6-deoxy-AltDiNAc analogues bearing azido groups at the *N*5 or *N*7 positions.[37] Supplementing rich growth media for *P. aeruginosa*, *A. baumannii* and *Vibrio vulnificus* with both these Pse biosynthetic precursors, enabled Cu-free click fluorescent visualisation of any resulting cell-surface azido-modified Pse. Studies confirmed that the LPS had been labelled within some of the *P. aeruginosa* strains screened, indicating that azido labelled



Scheme 13. *In vitro* chemoenzymatic synthesis of bioorthogonal biosynthetic precursor 6-deoxy-AltNAc4Az **65**, enables metabolic incorporation and biotinylation of azides on Pse5Ac7Az modified flagella.

Pse was present in the *O*-antigen. Further examination confirmed that only the *N*7-azido precursor allowed labelling in all organisms tested, which suggested that within the strains and serotypes tested, an enzyme downstream of PseG was unable to utilise *N*5-azido sugar. This study demonstrated that whilst chemoenzymatic synthesis of Pse glycans by metabolic engineering can provide valuable information in the biological relevance of Pse, the strategy is not infallible.

6. Summary and Outlook

In conclusion we have summarised the burgeoning work in the field of Pse synthesis. This non-mammalian sugar is of increasing biological importance as an essential component in the cell surface glycoconjugates of a number of pathogenic bacteria, including the multidrug resistant *P. aeruginosa*, and *A. baumannii*, which are emerging causes of outbreaks of hospital acquired infections. Pioneering studies into the biosynthesis of Pse5Ac7Ac **2** in a number of bacteria have provided inspiration to synthetic carbohydrate chemists, who have recently reported the chemical synthesis of Pse5Ac7Ac and derivatives. These studies have also provided test-bed substrates to explore the nuances of Pse chemical glycosylation for the synthesis of more complex Pse glycosides. Such is the arduous challenge presented by Pse total chemical synthesis however, the use of enzymes in tandem with chemical synthesis has also proved increasingly attractive. Chemoenzymatic strategies demonstrate the benefits of utilising enzymes in synthesis due to their inherent regio- and stereoselectivity, but also demonstrate that a degree of enzyme promiscuity may be exploited. This potentially allows access to products ranging from naturally occurring Pse5Ac7Ac derivatives, to Pse bioorthogonal chemical probes, which will undoubtedly be used to study the biological relevance of Pse in the future. However the outstanding challenge in this field still remaining is to combine synthesis of Pse monosaccharides with enzymatic glycosylations, which will provide more routine access to complex glycans containing Pse sugars for in depth glycobiology studies. In order to achieve this goal the unexplored power of pseudaminyltransferase enzymes and other Pse processing enzymes must first be harnessed.

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Keywords: Pse5Ac7Ac • chemical synthesis • carbohydrate chemistry • chemoenzymatic • pseudaminic acid

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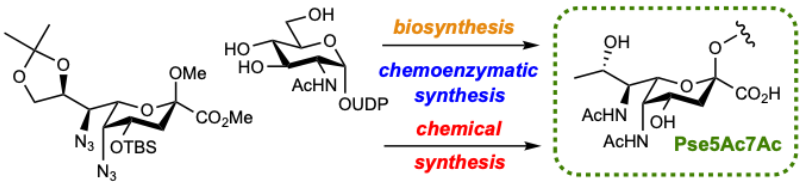

Pse sugars to order: In this minireview we summarise work in the field of pseudaminic acid (Pse) synthesis. This non-mammalian sugar is of increasing biological importance as an essential component in cell surface glycoconjugates of a number of pathogenic bacteria. Pioneering studies into Pse5Ac7Ac biosynthesis have provided inspiration to carbohydrate chemists, who have recently reported the chemical and chemoenzymatic synthesis of Pse glycans.