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Biosynthesis of uridine diphosphate *N*-Acetylglucosamine: An underexploited pathway in the search for novel antibiotics?

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Abstract

Although the prevalence of antibiotic resistance is increasing at an alarming rate, there are a dwindling number of effective antibiotics available. Thus, the development of novel antibacterial agents should be of utmost importance. Peptidoglycan biosynthesis has been and is still an attractive source for antibiotic targets; however, there are several components that remain underexploited. In this review, we examine the enzymes involved in the biosynthesis of one such component, UDP-*N*-acetylglucosamine, an essential building block and precursor of bacterial peptidoglycan. Furthermore, given the presence of a similar biosynthesis pathway in eukaryotes, we discuss the current knowledge on the differences and similarities between the bacterial and eukaryotic enzymes. Finally, this review also summarises the recent advances made in the development of inhibitors targeting the bacterial enzymes.

KEYWORDS

antibiotic resistance, bifunctional GlmU, glucosamine-6-phosphate synthase, peptidoglycan biosynthesis, phosphoglucosamine mutase, UDP-*N*-acetylglucosamine biosynthesis

1 | INTRODUCTION

Modern medicine has long relied on the effectiveness of antibiotics to treat common bacterial infections.[1] However, this dependence has led to their widespread inappropriate use and a rise in resistance to available drug classes.[2] Currently, antibiotic resistance results in 700,000 deaths annually and, without intervention, this number is expected to rise to over 10 million per year by 2050.[3] In response, priority pathogen reports have been released by the Centers for Disease Control and Prevention and the World Health Organization, which highlight the urgent

Abbreviations: AcCoA, acetyl-coenzyme A; ADGP, 2-amino-2-deoxy-D-glucitol-6-phosphate; ASO, antisense oligonucleotide; DAP, L-2,3-diaminopropanoic acid; DON, 6-diazo-5-oxo-L-norleucine; Fru-6-P, fructose-6-phosphate; GlcN-1-P, glucosamine-1-phosphate; GlcN-1,6-diP, glucosamine-1,6-diphosphate; GlcN-6-P, glucosamine-6-phosphate; GlcNAc-1-P, *N*-acetylglucosamine-1-phosphate; GlcNAc-6-P, *N*-acetylglucosamine-6-phosphate; LβH, left handed β-helix; PDB, protein data base; PPi, pyrophosphate; sRNA, small noncoding RNA; UDP, uridine diphosphate; UDP-GlcNAc, UDP-*N*-acetylglucosamine; UTP, uridine triphosphate; UTR, untranslated region.

need for the development of novel antibiotics against drug-resistant bacteria.[2,4] These reports include the previously identified ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* sp.), which contribute significantly to patient morbidity and mortality.[5] Thus, there is an urgent need to develop novel antibacterial agents of new classes that are not subject to existing resistance mechanisms.[1,6]

Peptidoglycan biosynthesis is the target for several antibiotics currently on the market.[7] These include β-lactam antibiotics that interact with penicillin-binding proteins, glycopeptide antibiotics that bind to the *D*-Ala-*D*-Ala dipeptide moiety of a peptidoglycan precursor and fosfomycin, which inhibits enolpyruvyltransferase, an enzyme that catalyses the first committed step of peptidoglycan biosynthesis.[7] Despite the clinical success of these antibiotic classes, only a fraction of the components in peptidoglycan biosynthesis have been exploited as antibacterial targets.[8] Targets that remain underexploited include the enzymes involved in the biosynthesis of UDP-*N*-acetylglucosamine (UDP-GlcNAc), an essential building block and precursor for bacterial peptidoglycan. UDP-GlcNAc feeds into the first committed stage of peptidoglycan biosynthesis, but it is also crucial for other bacterial pathways, including the biosynthesis of neomycin, kanamycin, gentamycin, lipopolysaccharides, and teichoic acid. In bacteria, UDP-GlcNAc is synthesised from fructose-6-phosphate (Fru-6-P) through four successive enzyme-catalysed reactions (Figure 1).[9] The first reaction is catalysed by glucosamine-6-phosphate (GlcN-6-P) synthase (GlmS) and involves the conversion of Fru-6-P to GlcN-6-P.[10] Phosphoglucosamine mutase (GlmM) then catalyses the interconversion of GlcN-6-P to glucosamine-1-phosphate (GlcN-1-P).[11] This product undergoes acetylation to *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) by GlcN-1-P acetyltransferase and finally uridylation to UDP-GlcNAc by GlcNAc-1-P uridyltransferase.[12–14] The latter two activities are carried out by the bifunctional GlcN-1-P acetyltransferase/GlcNAc-1-P uridyltransferase (GlmU) enzyme that possesses both acetyltransfer and uridyltransfer activity.[13,14]

Whilst UDP-GlcNAc is essential for bacterial peptidoglycan, it is also an essential metabolite in eukaryotes. UDP-GlcNAc is an important building block for major biomolecules such as chitin and glycoproteins, and is involved in several pathways and conditions including glycosylphosphatidylinositol-anchor biosynthesis, insulin resistance, and diabetic cardiomyopathy.[15] Although the product is the same, the eukaryotic biosynthesis pathway for UDP-GlcNAc differs to the prokaryotic pathway, including differences in the equivalent enzymes' amino acid and structural homology (Figure 1).[16] While the first reaction in eukaryotes also involves the interconversion of Fru-6-P and GlcN-6-P, unlike the prokaryotic pathway, it is catalysed by glutamine: fructose-6-phosphate amidotransferase (GFAT) in mammals and glucosamine-6-phosphate synthase (GFA1) in yeasts.[16,17]

In comparison to the biosynthetic pathway in prokaryotes, the sequence of enzymatic transformations occurs in a different order in eukaryotes. Unlike the prokaryotic pathway, the amine acetylation reaction in eukaryotes occurs directly on GlcN-6-P, a reaction catalysed by glucosamine-6-phosphate *N*-acetyltransferase (GNA1).[16,17] The subsequent phosphate interconversion, catalysed by phosphoacetylglucosamine mutase (GNPNAT in mammals and AGM1 in yeasts), then occurs on *N*-acetylglucosamine-6-phosphate (GlcNAc-6-P), yielding GlcN-1-P. This species undergoes uridylation by UDP-GlcNAc pyrophosphorylase (AGX1/AGX2 in mammals and UAP1 in yeasts) to produce UDP-GlcNAc.[16,17] In eukaryotes, the acetyltransferase and uridyltransferase reactions are carried out by two distinct monofunctional enzymes. Given the differences in the biosynthesis of UDP-GlcNAc, the bacterial enzymes are still considered to be significant pharmacological targets for antibiotic development.

Here, we provide a comprehensive review of the current literature around the enzymes involved in the bacterial biosynthesis of UDP-GlcNAc, including their structural and functional features. Given that UDP-GlcNAc is also produced in eukaryotes, we compare the bacterial enzymes with the equivalent eukaryotic enzymes and highlight the structural and functional differences. Furthermore, as the bacterial enzymes are promising targets in the search for novel antibiotics, we review the recent advances towards the development of specific inhibitors.

2 | GLUCOSAMINE-6-PHOSPHATE SYNTHASE (GlmS)

2.1 | Catalytic activity

In bacteria, the initial and rate limiting reaction of UDP-GlcNAc biosynthesis involves the conversion of Fruc-6-P to GlcN-6-P, catalysed by GlmS (EC 2.6.1.16). GlmS is a member of the glutamine amidotransferase enzyme family that catalyses the transfer of the amido nitrogen of glutamine to different nitrogen acceptors such as amino acids, nucleotides, antibiotics, and coenzymes.[18,19] Furthermore, GlmS belongs to the N-terminal nucleophile class of amidotransferases, which are characterised by a conserved N-terminal catalytic cysteine residue (Cys2, *Escherichia coli* numbering). The glutamine hydrolysis

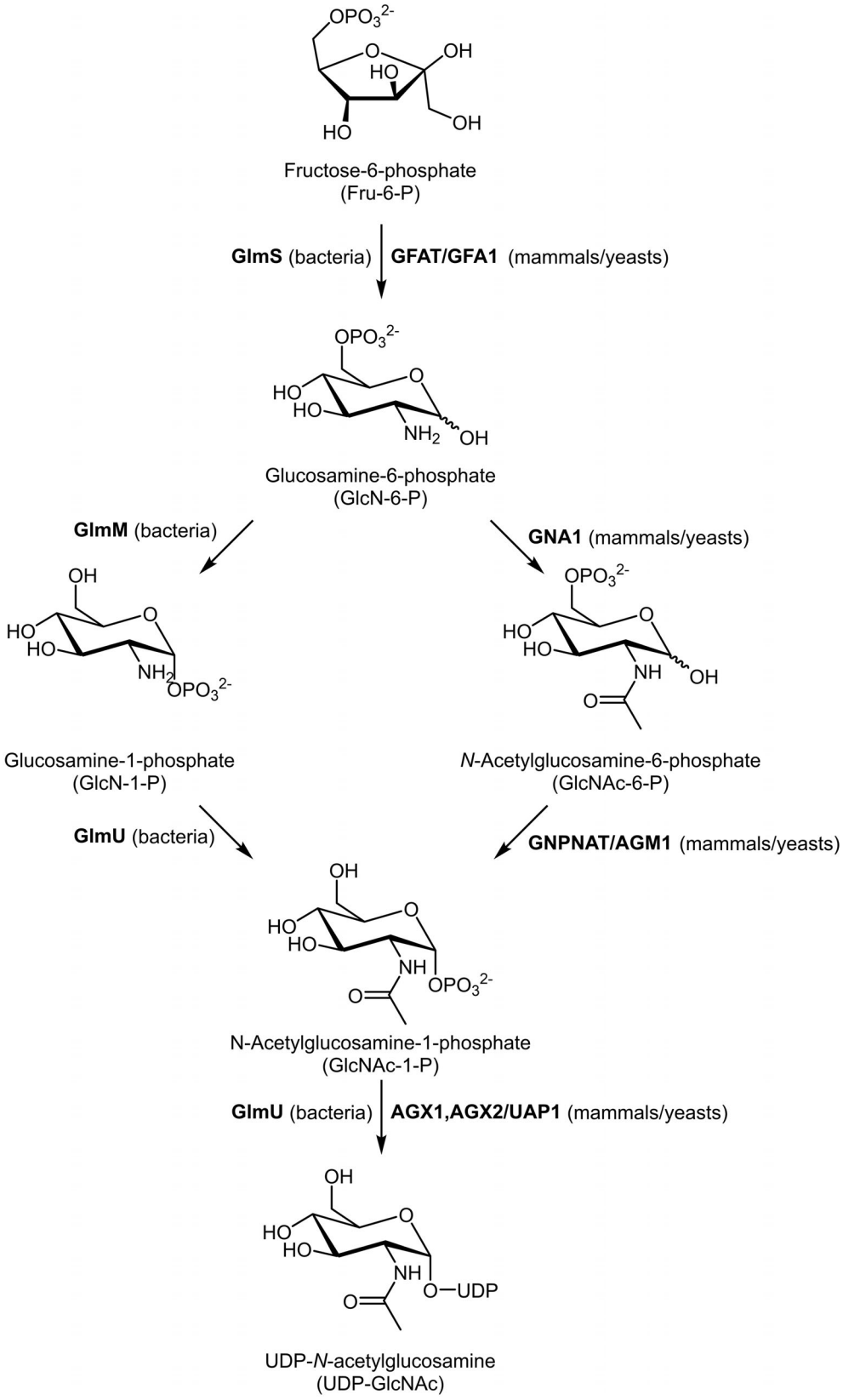


FIGURE 1 Biosynthesis pathway of UDP-*N*-acetylglucosamine in prokaryotes (left) and eukaryotes (right)

reaction has been extensively studied and involves a nucleophilic attack on the δ-carbonyl group of L-glutamine by the thiol group of the conserved cysteine.[20–22] The intermediate, γ-glutamyl thioester, is subsequently hydrolysed, resulting in the release of free ammonia and glutamate (Figure 2a).[20–22] The newly formed ammonia is then transferred to the ring-opened form of Fruc-6-P, forming a fructosamine intermediate, which undergoes

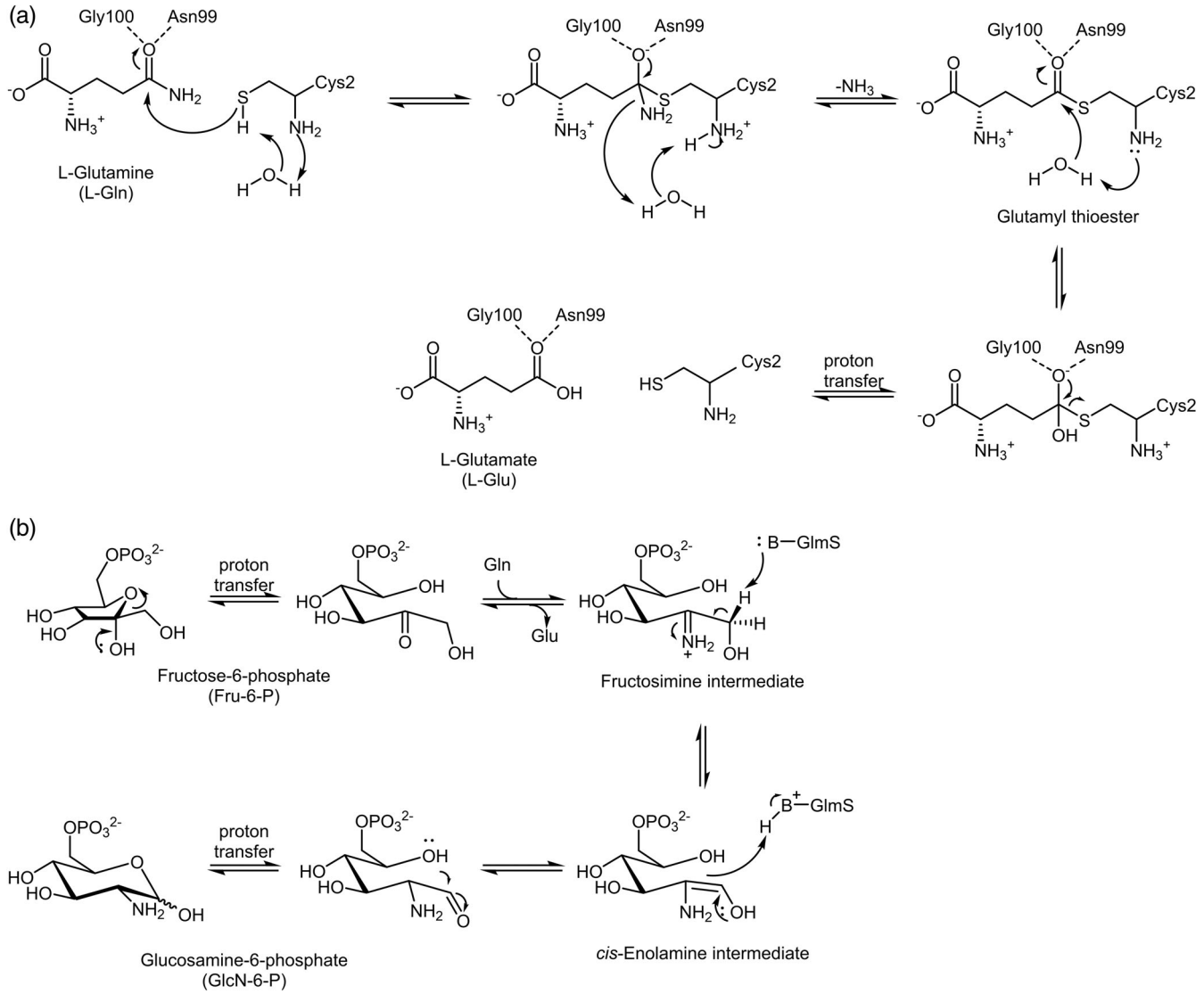


FIGURE 2 Reaction mechanisms of the (a) glutamine hydrolysis and (b) conversion of fructose-6-phosphate to glucosamine-6-phosphate catalysed by GlmS

isomerisation to yield GlcN-6-P (Figure 2b).[23] The GlmS-catalysed reaction is irreversible and obeys an ordered bi-bi mechanism in which Fruc-6-P binds prior to the binding and release of glutamate, followed by the release of GlcN-6-P.[24]

The mechanism underpinning the regulation of GlmS differs depending on the bacterium. One mechanism observed primarily in Gram-positive bacteria involves the controlled expression of *glmS* by a metabolite-binding "riboswitch," which responds to GlcN-6-P bioavailability.[25,26] Riboswitches are not found in mammals and are highly conserved elements located in the 5′-untranslated region (UTR) of messenger RNA (mRNA).[26] Once sufficient levels of GlcN-6-P have accumulated, the *glmS* ribozyme is activated and gene expression is controlled through the self-cleavage of mRNA.[26] The other mechanism observed solely in Gram-negative bacteria is the post-transcriptional regulation of *glmS*, which is controlled by small noncoding RNAs (sRNAs), GlmY and GlmZ, in response to altered levels of GlcN-6-P and is also believed to contribute to antibiotic resistance.[27]

2.2 | Structural features

The structural features of GlmS are well understood as numerous crystal structures of the full enzyme as well as the individual domains have been published. The monomeric enzyme consists of two structural domains, the N-terminal glutaminase domain (Met1-Gln240) and the C-terminal isomerase domain (Arg250-Glu609) (Figure 3a).[28] Structural analysis of *E. coli* GlmS has

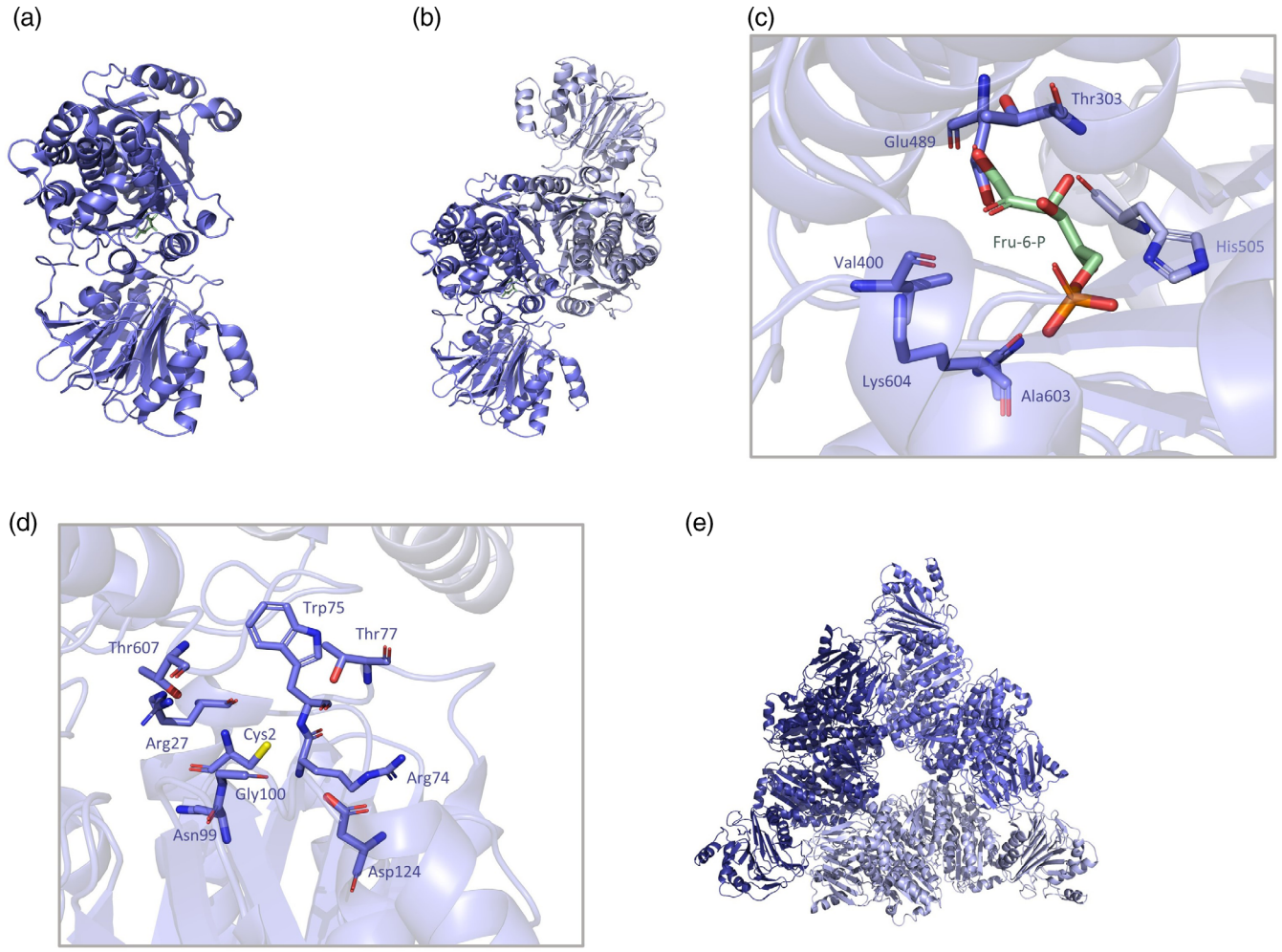


FIGURE 3 Structure of *Escherichia coli* (Ec) GlmS enzyme. (a) Cartoon structure of EcGlmS (PDB: 4AMV) monomeric unit bound to fructose-6-phosphate (Fru-6-P) (green). (b) Cartoon structure of bound EcGlmS demonstrating the dimer conformation. (c) Cartoon structure of EcGlmS isomerase active site bound with Fru-6-P, with key residues shown as sticks. (d) Cartoon structure of EcGlmS glutaminase active site, with key residues shown as sticks. (e) Cartoon structure of EcGlmS (PDB: 3OOJ) in the unliganded form demonstrating the inactive hexamer conformation. Residues are coloured by nitrogen (blue), oxygen (red), and sulfur (yellow). Images were generated using PyMOL v 2.2 (Schrödinger)

revealed that the functional enzyme adopts a dimeric conformation consisting of two identical monomers (Figure 3b).[23] The monomers interact at the C-terminal domain, with the isomerase active site composed of residues from the two monomers (Figure 3c).[23] A 10 peptide (Gln240-Arg250) linker connects the two domains; however, it does not form any secondary structure and is not closely related to either domain, indicating the potential for flexibility of the domains.[23,29] Furthermore, crystallisation of the intact protein proposed that an 18 Å hydrophobic channel forms between the glutaminase and isomerase domains and is responsible for the transfer of ammonia.[23] Comparisons between the apo- and substrate bound-GlmS structures have revealed conformational changes that occur during the catalytic cycle.[29] Binding of Fru-6-P leads to the activation of the GlmS enzyme, increasing glutaminase activity by 100-fold.[20] Moreover, activation of the enzyme leads to the ordering of the C-terminal peptide, C-tail (Asn601-Glu609), resulting in the closure of the isomerase site.[29] Upon glutamine binding, the glutaminase domain is activated and the ammonia channel is formed (Figure 3D).[30] Furthermore, structural studies of GlmS have revealed that the inactive enzyme adopts a hexameric conformation that exists in equilibrium with the active dimeric conformation of GlmS, the formation of which is potentially regulated through an allosteric site (Figure 3e).[31]

2.3 | Homology

Unlike the prokaryotic enzyme, the eukaryotic enzymes GFA1 (in fungi) and GFAT (in mammals) have not been as extensively studied. Despite this, a number of

structural differences between the prokaryotic and eukaryotic enzymes have been identified. Structural studies of the fungal GFA1 enzyme have demonstrated a tetrameric conformation both in solution and in crystalline structures.[32,33] Comparatively, the mammalian GFAT enzyme adopts different conformations, with a tetramer present in solution and a dimer formed in crystalline structures.[34,35] It is suggested that the dimeric conformation is the biologically-relevant state due to the similarities in structure to the prokaryotic enzyme.[36] In fact, the active site residues of the prokaryotic and eukaryotic enzymes are highly conserved and structurally similar, which is expected as the enzymes both use Fru-6-P and produce GlcN-6-P.[34] The eukaryotic enzyme is also feedback regulated by UDP-GlcNAc through a conserved binding site.[37] Studies of the mammalian enzyme have revealed that it does not follow an ordered bi-bi mechanism like the bacterial GlmS enzyme.[35] Moreover, humans have three isoforms of GFAT, which differ in structure, catalytic efficiency, and allosteric regulation.[35]

2.4 | Inhibitors

Due to the promise of GlmS as an antibacterial target, there have been several attempts at identifying inhibitors of the enzyme (Figure 4). Glutamine analogues have been studied as affinity label inhibitors, possessing electrophilic functionality at the γ-position of glutamate, which can react irreversibly with the N-terminal cysteine residue located in the glutaminase domain (Figure 4).[18] These inhibitors include azaserine and albizzin (Compounds **1–2**), which were initially identified as inhibitors of the mammalian enzyme.[38] However, they have since been shown to have activity against *Salmonella typhimurium* GlmS at high concentrations (azaserine, $K_i = 0.77$ mM; albizzin, $K_i = 1.45$ mM).[39] Anticapsin (Compound **3**), another natural analogue of glutamine, is a potent inhibitor of *E. coli* and *S. typhimurium* ($K_i = 0.28$ μM), albeit it exhibits poor antibacterial activity.[39,40] Synthetic glutamine inhibitors have also been explored including N^3-fumaroyl-L-2,3-diaminopropanoic acid and 6-diazo-5-oxo-L-norleucine (DON) (Compounds **4–5**), which exhibit activity against the bacterial *S. typhimurium* GlmS enzyme ($K_i = 8.0$ μM and 7.5 μM, respectively).[39] Furthermore, analogues of L-2,-3-diaminopropanoic acid (DAP) have been explored, including haloketone ($IC_{50} = 0.4–62$ μM) (Compounds **6–8**), maleimide ($IC_{50} = 175$ μM) (Compound **9**), and epoxide ($k_{inact}/K_{irr} = 5.2\ M^{-1}\ s^{-1}$) (Compound **10**) derivatives.[41–43] While these derivatives showed mixed potency against bacterial GlmS, DAP derivatives have also been explored as inhibitors of fungal GFA1, indicating that specificity for the bacterial enzymes may be difficult to achieve.[41–46] Moreover, a series of electrophilic glutamine analogues based on DON have been investigated with two derivatives, a bromomethyl ketone analogue and a dimethylsulfonium salt (Compounds **11–12**), demonstrating enhanced activity against *E. coli* GlmS ($K_i = 2.7$ μM and 0.37 μM, respectively).[47]

Other inhibitors of GlmS include synthetic *cis*-enolamine transition state intermediate analogues. Indeed, 2-amino-2-deoxy-D-glucitol-6-phosphate (ADGP) (Compound **13**) inhibits *E. coli* GlmS ($K_i = 25$ μM); however, it possesses poor antibacterial activity due to low uptake by microbial cells (Figure 4).[48] Analogues of ADGP have also been examined (Compounds **14–19**) against the *E. coli* enzyme, although potency greatly varies among these compounds, and they lack whole cell activity against both Gram-positive and Gram-negative bacteria.[49,50] Another class of analogue inhibitors include those of the reaction product, GlcN-6-P. While two of these analogues (Compounds **20–21**) possess only low millimolar potency ($IC_{50} = 0.21 - >5$ mM) and poor whole cell activity, one inhibitor, an α-iodoketone derivative (Compound **22**), has low micromolar potency against *E. coli* GlmS ($K_i = 0.22$ μM) and is predicted to interact with the glutamine binding site.[49,51] Furthermore, the potential of a mechanism-based inhibitor of GlmS has been explored resulting in a prodrug (Compound **23**) that undergoes enzyme-catalysed hydrolysis to produce a 4-thioquinone inhibitor with millimolar potency ($K_{irr} = 35.8$ mM).[52] Following the detailed structural characterisation of GlmS, in silico screening approaches have been undertaken to identify potential inhibitors. Three compounds (Compounds **24–26**) were found to inhibit GlmS in vitro ($IC_{50} = 70$ μM) and predicted to bind at the interface between the two GlmS monomers (Figure 4).[53] However, these compounds have poor water solubility, which has prevented their further development as potential antibiotics.[53]

Furthermore, the availability of GlmS crystal structures have allowed for molecular docking studies to identify the targets of inhibitors that have previously demonstrated antibacterial activity (Figure 4). This includes catechin derivatives (Compounds **27–30**), a metabolite abundant in teas known to possess antibacterial potential, and phenothiazine and phenoxazine derivatives (Compounds **31–39**) that have demonstrated whole cell activity against Gram-positive and Gram-negative bacteria (MICs = 45.7–200 μM).[54,55]

More recently, exogenous activation of the *glmS* riboswitch is being investigated as a potential bacterial growth inhibitor. High throughput screening assays identified a carba-analogue of GlcN-6-P, which has the ability to activate the *glmS* riboswitch with the same potency as GlcN-

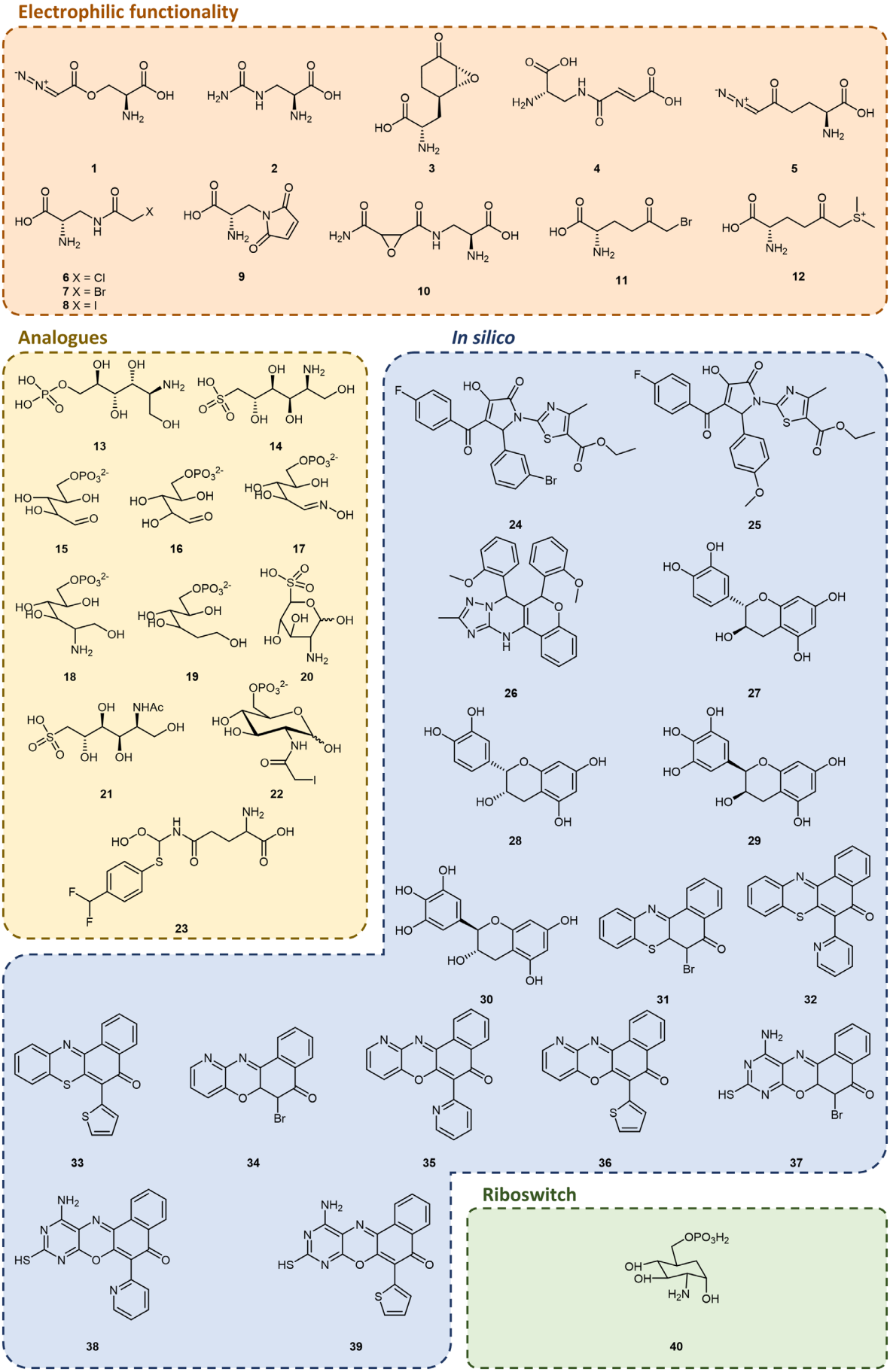


FIGURE 4 Inhibitors of the bacterial GlmS enzyme

6-P (Figure 4).[56] In fact, carba-α-D-glucosamine-6-phosphate (Compound **40**) has been tested in vivo and demonstrates inhibition of Gram-positive bacterial growth (MICs = 150–625 μM).[57] Complementary antisense oligonucleotides (ASOs), which target the UTR region of the *glmS* riboswitch, have also been explored as GlmS inhibitors, demonstrating significant antibacterial activity against Gram-positive pathogens (MIC_{80} = 700 nM).[58] Following the binding of an ASO to the metabolite-binding domain, the *glmS* riboswitch is activated, leading to degradation of *glmS* mRNA and the inhibition of gene expression.[58] The alternative regulatory mechanism of GlmS has also been postulated to be a potential antibiotic target. Due to the contribution of sRNAs to antibiotic resistance, it has been suggested that interfering with GlmY and GlmZ may increase bacterial susceptibility to GlmS inhibitors; however, there are currently no inhibitors that target this regulatory mechanism.[59]

3 | PHOSPHOGLUCOSAMINE MUTASE (GlmM)

3.1 | Catalytic activity

The second enzyme in the pathway, GlmM (EC 5.4.2.10), catalyses the interconversion of GlcN-6-P and GlcN-1-P.[11] GlmM was first characterised in *E. coli* and belongs to the α-D-phosphohexomutase enzyme superfamily.[11] Like other members of this enzyme family, GlmM is only active when it is phosphorylated at Ser100 (*Bacillus anthracis* numbering), although the dephosphorylated form also exists in vitro and can be separated by HPLC.[11,60] In vitro studies have demonstrated that the dephosphorylated form can be phosphorylated by the reaction intermediate glucosamine-1,6-diphosphate (GlcN-1,6-diP), as well as ATP and serine/threonine kinases.[61–63] The conversion of GlcN-6-P to GlcN-1-P follows a ping-pong bi-bi mechanism, whereby the reaction of GlcN-6-P with the phosphorylated GlmM enzyme results in the first phosphoryl transfer, generating the diphosphate intermediate. This intermediate then undergoes a reorientation process, which allows for a second phosphoryl transfer. This results in the GlcN-1-P product, as well as the regeneration of the active form of the enzyme (Figure 5).[11,60] Substrate specificity studies have demonstrated that GlmM from *E. coli* and *Bacillus subtilis* can catalyse the interconversion of the 1-phosphate and 6-phosphate isomers of glucose, although at reduced rate constants.[60,64,65] Furthermore, GlmM is considered a moonlighting protein in Gram-positive bacteria due to its involvement in the regulation of c-di-AMP production, a second messenger molecule essential for bacterial growth.[66–69]

3.2 | Structural features

There are a limited number of crystal structures of GlmM published in the Protein Data Bank (PDB). In fact, the first structure of GlmM was not published until 2010 from *Francisella tularensis* (PDB: 3I3W). There are now structures published from *B. anthracis*, *B. subtilis*, and *S. aureus*, with the majority of structural studies focusing on *B. anthracis* GlmM.[69–71] Overall, GlmM is structurally similar to other enzymes in the α-D-phosphohexomutase superfamily. The monomeric unit, a 47 kDa protein, is composed of four structural domains arranged in a heart shape (Figure 6a).[70] Domains 1–3 share a fold consisting of a mixed α/β fold, while domain 4 is topologically distinct with a β-sheet flanked by 2 α-helices.[70] Dissimilar to most members of the α-D-phosphohexomutase superfamily that appear to be monomeric, GlmM forms a stable dimer in solution, which could offer potential functional advantages including the opportunity for allosteric inhibition of activity (Figure 6b).[69,70] The dimer interface of GlmM is largely hydrophobic, primarily involving residues from domain 1 (Figure 6c).[70] Although there are a number of conserved residues in the interface, there is no distinct dimerisation sequence motif.[70] The active site is located at the centre of the molecule in a large, open cleft composed of highly conserved catalytic residues in the superfamily.[70] The site is highly hydrophilic with an overall positive electrostatic potential, consistent with the negatively charged substrate and product.[70] There are four key regions in the GlmM active site involving residues from all four structural domains: (a) the phosphoserine residue involved in the phosphoryl transfer reaction (Ser100); (b) the metal-binding site (Asp240, Asp242 and Asp244); (c) the sugar-binding loop that interacts with the sugar moiety of the substrate and product (Glu325 and Ser327); and (d) the phosphate-binding site that interacts with the phosphate group of the substrate and product (Arg410, Ser412 and Arg419)[70] (Figure 6d). These regions are unable to function independently and must be correctly positioned relative to each other for activity.[70] To allow for this positioning, domain 4 rotates to form a lid over the active site following the phosphorylation of Ser100.[70,72]

3.3 | Homology

Structural alignments have revealed a high degree of similarity between GlmM and the eukaryotic GNPNAT/AGM1 enzymes, which are also members of the α-D-phosphohexomutase enzyme superfamily.[73] One key difference, however, is that the eukaryotic enzymes are monomeric like most phosphohexomutase enzymes, compared to

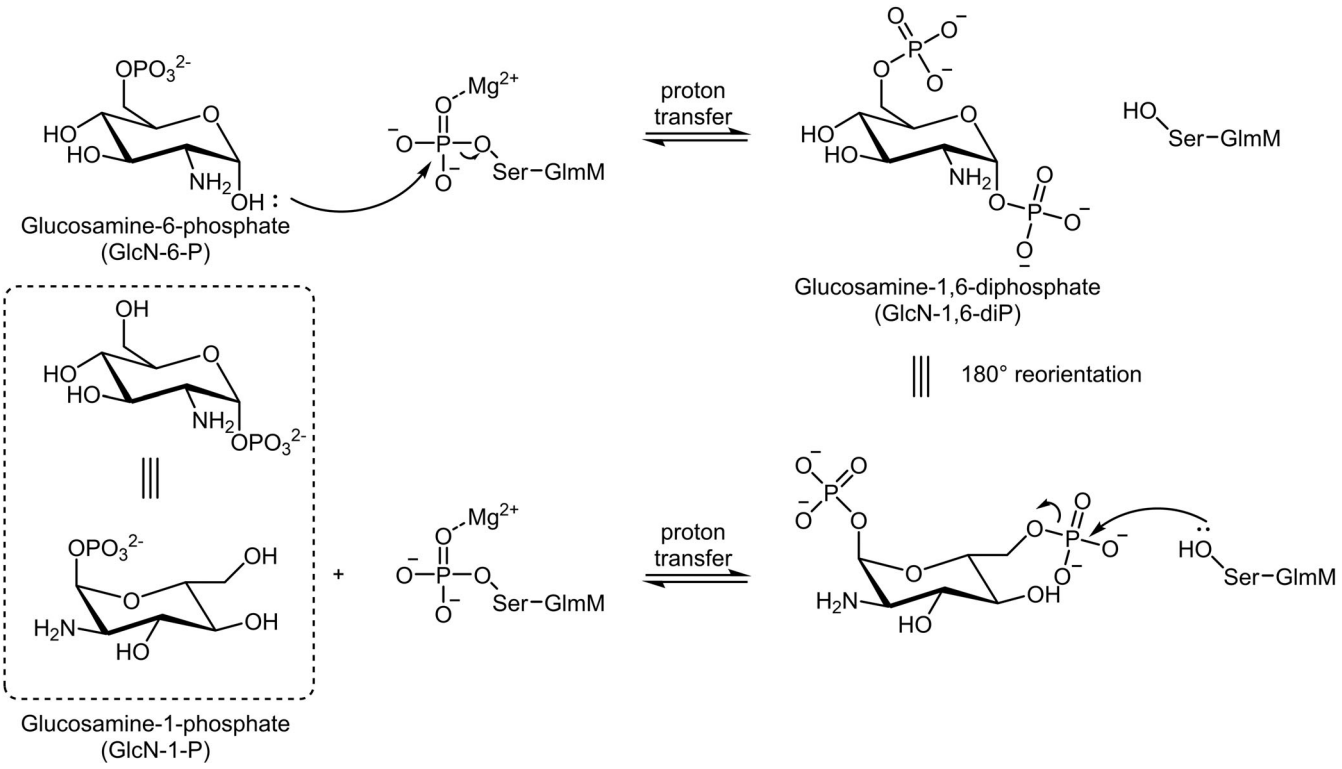


FIGURE 5 Reaction mechanism of the interconversion of glucosamine-6-phosphate and glucosamine-1-phosphate catalysed by GlmM

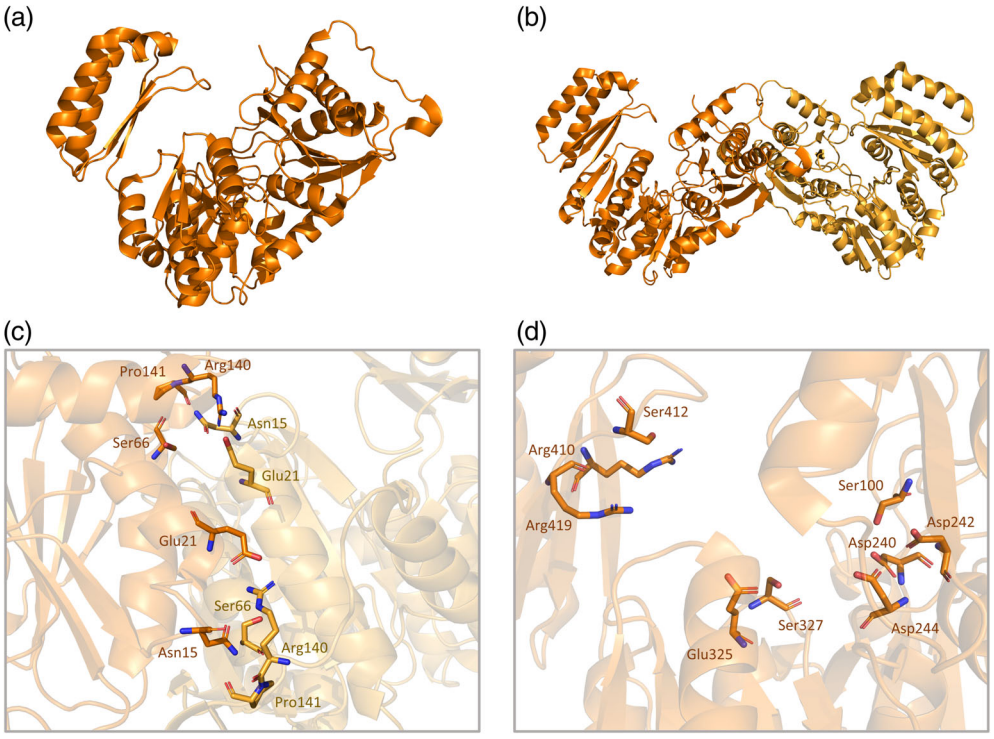
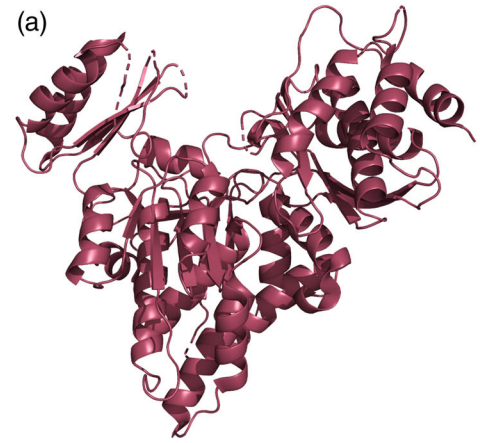


FIGURE 6 Structure of *Bacillus anthracis* (Ba) GlmM enzyme. (a) Cartoon structure of BaGlmM (PDB: 3PDK) monomeric unit. (b) Cartoon structure of BaGlmM in the unliganded form demonstrating the dimer conformation. (c) Cartoon structure of BaGlmM dimerisation interface, with key residues shown as sticks. (d) Cartoon structure of BaGlmM, with residues within the active site shown as sticks. Residues are coloured by nitrogen (blue) and oxygen (red). Images were generated using PyMOL v 2.2 (Schrödinger)

the dimer conformation of the bacterial enzyme (Figure 7a).[74] Sequence alignments have identified a shared common sequence from domain 1, Ser/Thr-X-Ser-His-Asn-Pro, that is conserved among phosphoglucomutases, phosphoglucosamine mutases, and phosphoacetylglucosamine mutases, including the eukaryotic phosphoacetylglucosamine mutase AGM1 (Figure 7b).[60,73] The serine at the third position in this motif corresponds to the



(b)

```
B. anthracis     95  VMISASHNPVQD  106
H. sapiens       59  VMVTASHNPEED   70
C. albicans      61  VMITASHNPPED   72
                     **::***** :*
```

(c) Metal-binding site:

```
B. anthracis    237  LAFDGDGDRLI  247
H. sapiens      273  CSFDGDADRIV  283
C. albicans     287  ASFDGDADRLI  297
                      :****.**::
```

Sugar-binding loop:

```
B. anthracis    322  LGGEQ------SGHI  330
H. sapiens      394  LEDKKRKAAKMLENI  408
C. albicans     410  NNDNEAKAIKVLQNF  424
                       .::        ::
```

Phosphate-binding site:

```
B. anthracis    407  ILVRPSGTEPLIRVMA  422
H. sapiens      493  AFVRPSGTEDVVRVYA  508
C. albicans     509  SFVRASGTEDAVRVYA  524
                      :** **** :** *
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FIGURE 7 Comparison of prokaryotic GlmM and eukaryotic GNPNAT/AGM1 enzymes. (a) Cartoon structure of *Candida albicans* AGM1 (PDB: 2DKA) in the unliganded form illustrating monomeric conformation. Image was generated using PyMOL v 2.2 (Schrödinger). (b) Sequence alignment of *B. anthracis* GlmM, *Homo sapiens* GNPNAT and *C. albicans* AGM1 demonstrating conservation of a common motif (bold) among phosphoglucomutases, phosphoglucosamine mutases, and phosphoacetylglucosamine mutases. (c) Sequence alignment of the active site residues of *B. anthracis* GlmM, *H. sapiens* GNPNAT and *C. albicans* AGM1 demonstrating conservation of the metal-binding and phosphate-binding sites (bold) and difference in sugar-binding loop (bold) between the prokaryotic and eukaryotic enzymes

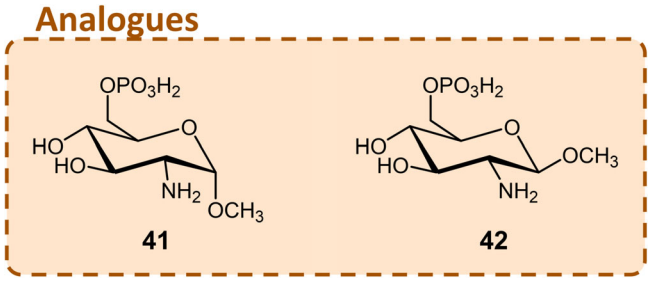

FIGURE 8 Glucosamine-6-phosphate analogues as inhibitors of GlmM

phosphorylation site, Ser100, while the threonine or serine at the first position contributes to substrate specificity.[60] Furthermore, the active site residues of GlmM are highly conserved in AGM1, indicating that these residues are crucial for the activity of phosphohexomutase enzymes (Figure 7c).[73]

3.4 | Inhibitors

There has only been one study focused on the development of inhibitors of bacterial GlmM. Two analogues (Compounds **41–42**) of GlcN-6-P have been characterised against *Mycobacterium tuberculosis* GlmM; however, they did not result in inhibition of the enzyme (Figure 8).[75]

4 | GLUCOSAMINE-1-PHOSPHATE ACETYLTRANSFERASE/*N*-ACETYLGLUCOSAMINE-1-PHOSPHATE URIDYLTRANSFERASE (BIFUNCTIONAL GlmU)

4.1 | Catalytic activity

The final two steps of UDP-GlcNAc biosynthesis (acetylation and uridylation) are catalysed by the bifunctional GlmU enzyme (EC 2.7.7.23).[13] When it was first identified in *B. subtilis*, the GlmU protein was initially thought to be an GlcNAc-1-P uridyltransferase.[12,76] However, later studies of the *E. coli* GlmU protein demonstrated its ability to catalyse both the acetyltransfer and uridyltransfer of GlcN-1-P, thereby identifying GlmU as a bifunctional enzyme.[13] Studies on N- and C-terminal truncated forms of GlmU have revealed the enzyme encompasses two domains with two separate functional sites that are individually active.[14,77] GlmU first catalyses the *N*-

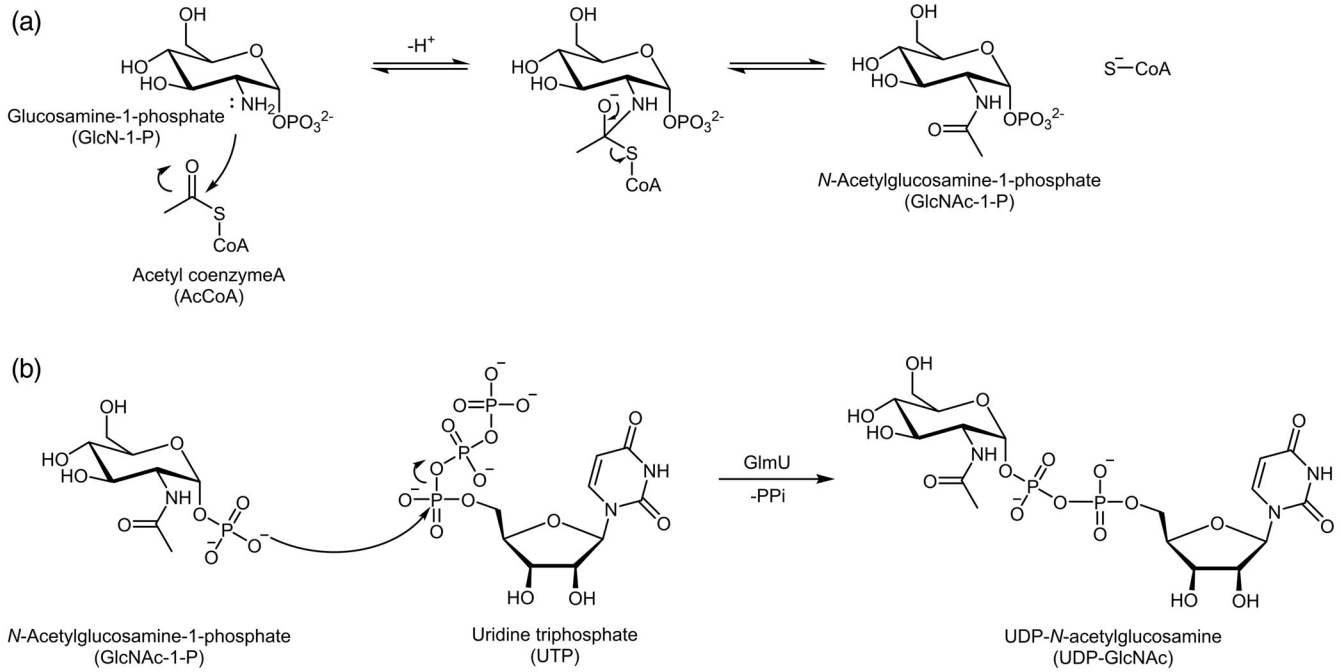

FIGURE 9 Reaction mechanism of the (a) acetylation and (b) uridylation of GlcN-1-P catalysed by the bifunctional GlmU enzyme

acetylation of GlcN-1-P with acetyl-coenzyme A (AcCoA) to produce GlcNAc-1-P in the C-terminal domain (Figure 9a), followed by a uridyltransfer reaction with uridine triphosphate (UTP) at the N-terminal domain, resulting in the formation of UDP-GlcNAc and pyrophosphate (PPi) (Figure 9b).[13,14,78,79] Furthermore, steady-state kinetic experiments with truncated forms of GlmU showed that the order of the chemical reactions is not random but imposed by the enzyme, with the acetyltransfer occurring before the uridyltransfer.[14] However, under certain physiological conditions, GlmU can catalyse the uridyltransfer prior to the acetyltransfer, albeit under greatly reduced catalytic parameters.[77] Steady-state kinetic experiments with full length *E. coli* GlmU have demonstrated that the acetyltransferase reaction proceeds four times faster than the uridyltransferase reaction, with approximate turnover rates of 80 and 20 s^{-1}, respectively.[14] Substrate specificity studies have revealed that *E. coli* GlmU can utilise galactosamine-1-phosphate and *N*-acetylgalactosamine-1-phosphate as substrates, though with lower efficiency.[14]

4.2 | Structural features

To date, there have been 39 crystal structures of GlmU deposited in the PDB for various bacterial species. These include truncated and full forms of the enzyme, both in apo and in complex with ligands.[80–82] Overall, the 3D structure of the GlmU enzymes are similar, with the two domains linked by a long α-helical arm (Leu230-Ala250, *E. coli* numbering) (Figure 10a).[80,81] The C-terminal acetyltransferase domain (Gly251-Ala437) shares sequence similarities with other acetyltransferases and is characterised by an imperfect, tandem hexapeptide repeat sequence motif, [LIV]-[GAED]-X_2-[STAV]-X, which folds into a left-handed β-helix (LβH) (Figure 10c).[80,81,83,84] The N-terminal uridyltransferase domain, on the other hand, shares sequence homology with various nucleotidyltransferases (or nucleotide diphosphate sugar pyrophosphorylases) over residues Met1-Ala120, with strict conservation of the pyrophosphorylase fingerprint sequence L-$(X)_2$-G-X-G-T-X-M-$(X)_4$-P-K motif.[80,81,85] The uridine binding site is a large open pocket bound by two lobes (Figure 10d).[80,81] The first lobe interacts with the nucleotide (Asn2-Val111 and His216-Asn227), while the second lobe interacts with the sugar moiety (Glu112-Val215).[80,81] In the absence of UDP-GlcNAc, the uridyltransferase domain adopts an open conformation.[85] Upon the binding of UDP-GlcNAc in the active site, two regions within lobe 2 move towards each other to adopt a closed conformation.[85,86] Structural analysis has revealed that GlmU forms a trimeric arrangement, with the LβH domains tightly packed in parallel, the long α-helical arm seated on top of the arrangement and the N-terminal domains projected away from the trimer-axis (Figure 10b).[77,80,86] Moreover, this trimeric organisation is common among enzymes with LβH domains and is essential for acetyltransferase activity.[85] In fact, the acetyltransfer catalytic site is formed by complimentary

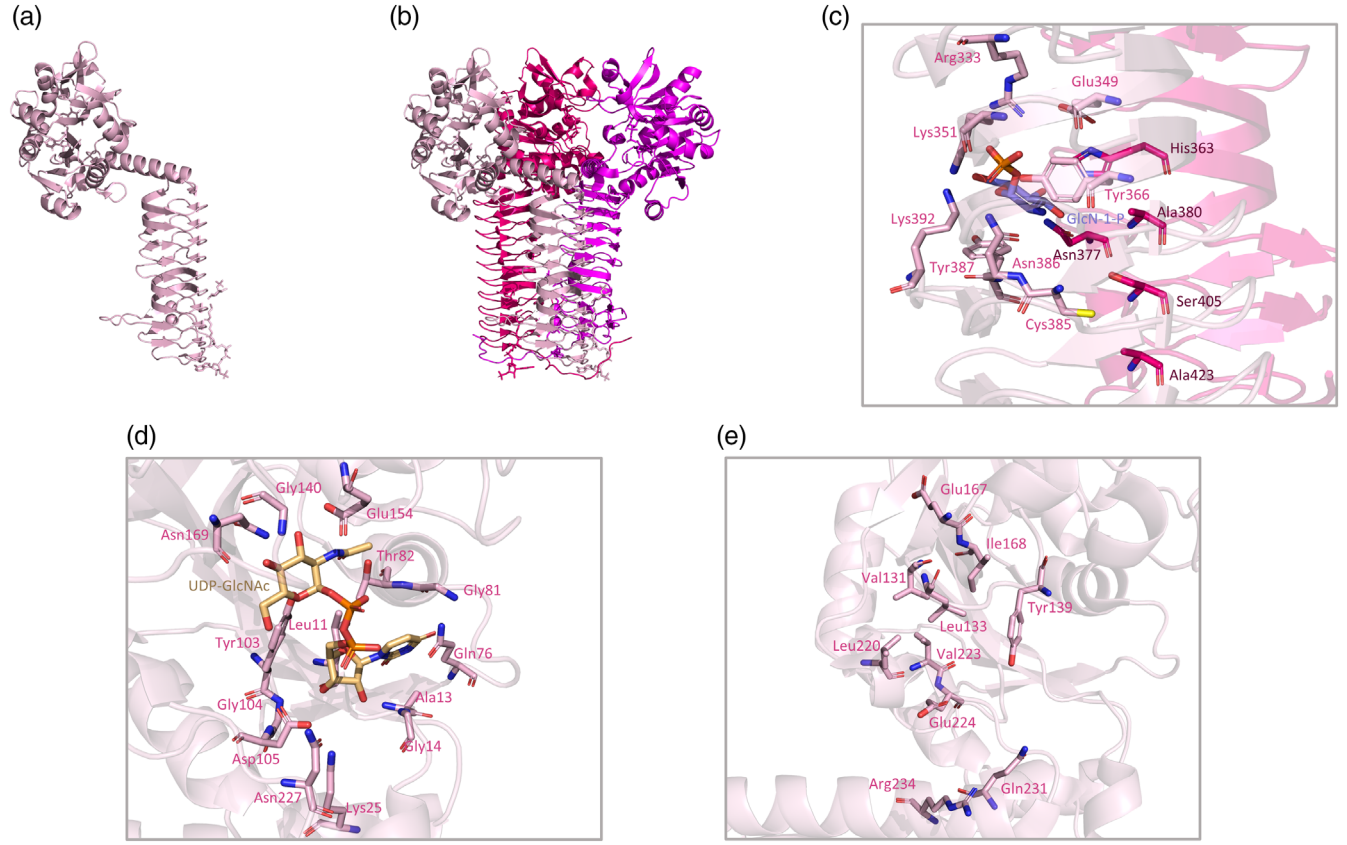

FIGURE 10 Structure of *E. coli* (Ec) GlmU enzyme. (a) Cartoon structure of EcGlmU (PDB: 2OI6) monomeric unit. (b) Cartoon structure of EcGlmU in complex with UDP-GlcNAc, CoA and GlcN-1-P illustrating the trimeric conformation. (c) Cartoon structure of EcGlmU acetyltransferase site bound with the substrate glucosamine-1-phospahet (GlcN-1-P), with key active site residues shown as sticks. (d) Cartoon structure of EcGlmU uridyltransferase site bound with the reaction product UDP-*N*-acetylglucosamine (UDP-GlcNAc) (PDB: 2OI7), with key active site residues shown as sticks. (e) Cartoon structure of EcGlmU allosteric site (PDB: 2OI6), with key residues involved shown as sticks. Residues are coloured by nitrogen (blue), oxygen (red), and sulfur (yellow). Images were generated using PyMOL v 2.2 (Schrödinger)

regions of contact between the three adjacent monomers, as confirmed by structural studies of both the truncated and full length GlmU enzymes.[80,81] Comparatively, the uridyltransferase activity of GlmU does not require trimerisation, though some interactions between domains participate in the folding and stability of the N-terminal domain.[77]

4.3 | Homology

Unlike the prokaryotic pathway, the acetyltransferase and uridyltransferase reactions are carried out by two separate enzymes in the eukaryotic pathway. Although GNA1 catalyses the equivalent acetyltransfer reaction, there is limited sequence similarity with the C-terminal acetyltransferase domain of GlmU.[87] The eukaryotic uridyltransferase enzymes, AGX1/AGX2/UAP1, on the other hand, share numerous structural and sequential homologies with the N-terminal domain of GlmU.[88,89] The start of the C-terminal domain of AGX1/AGX2 is characterised by a long α-helix that corresponds to the α-helical arm that connects the two domains of GlmU (Figure 11a).[81,88] The conservation of this structure between the two domains suggests a common ancestor and indicates that this structure is important for enzymatic activity.[88] As a nucleotidyltransferase enzyme, AGX1/AGX2 contains the pyrophosphorylase fingerprint sequence motif (Figure 11b). Furthermore, the mode of binding of the nucleotide and sugar moieties is conserved between AGX1/AGX2 and GlmU. In fact, there is strict conservation of 7 of the hydrophobic and hydrogen bonds between the proteins and the amino sugar.[88] However, AGX1/AGX2 diverges from GlmU by the presence of two extra domains, one of which is unique to the eukaryotic enzyme and is suggested to play a role in the regulation of AGX1/AGX2.[88] Moreover, AGX1/AGX2 forms a dimeric arrangement, in contrast to the trimeric

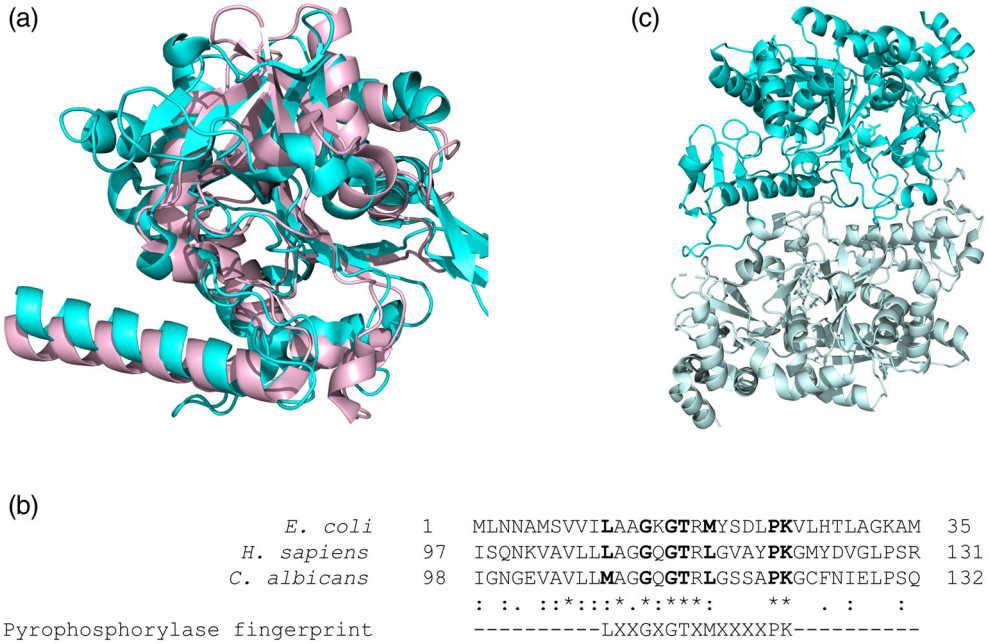

FIGURE 11 Comparison of the prokaryotic GlmU enzyme and the eukaryotic UDP-GlcNAc pyrophosphorylase enzymes. (a) Cartoon structure of the uridyltransferase domain of *E. coli* GlmU (pink) (PDB: 2OI6) superimposed on the cartoon structure of *H. sapiens* AGX2 (cyan) (PDB: 1JVD). (b) Sequence alignment of pyrophosphorylase fingerprint from *E. coli* GlmU, *H. sapiens* AGX1 and *C. albicans* UAP1, demonstrating conservation of the motif (bold) across UDP-GlcNAc pyrophosphorylase enzymes from different domains. (c) Cartoon structure of *H. sapiens* AGX2 (PDB: 1JVD) in complex with UDP-GlcNAc illustrating dimeric conformation. Cartoon structure images were generated using PyMOL v 2.2 (Schrödinger)

arrangement of GlmU, although it is not known if dimerisation is required for activity (Figure 11c).[88]

4.4 | Acetyltransferase inhibitors

Due to the acetyltransferase activity of GlmU being unique to bacteria, the acetyltransferase active site has been a focus for inhibitor development (Figure 12). Analogues of the substrate GlcN-1-P (Compounds **43**–**44**) have been explored; however, only one of the two compounds (Compound **43**) showed promising activity, inhibiting *M. tuberculosis* GlmU at millimolar concentration (IC_{50} = 12.8 mM).[75] Furthermore, the acetyltransferase site of GlmU is sensitive to thiol-specific reagents, including iodoacetamide and *N*-substituted maleimides (Compounds **45**–**49**), which demonstrate antibiofilm activity against Gram-negative and Gram-positive bacteria.[90–92] In vitro high throughput screening has led to the identification of sulfonamide inhibitors (Compounds **50**–**60**) that possess low micromolar potency (IC_{50} = 0.001–103 μM) but lack significant whole cell activity, likely due to poor penetration of the compounds into the bacterial cell.[93–96]

Screening has also been performed in silico to identify novel leads, resulting in the identification of *M. tuberculosis* GlmU and *E. coli* GlmM inhibitors (Figure 12). Not only did the three most active *M. tuberculosis* GlmU inhibitors (Compounds **61**–**63**) demonstrate low micromolar potency (IC_{50} = 5.3–65.2 μM), but they also exhibited significant whole cell activity (MICs = 2–25 μg/mL).[97,98] The EcGlmU inhibitors (Compounds **64**–**68**), on the other hand, possessed low micromolar potency (IC_{50} = 4.1–24.9 μM) in vitro with four inhibitors (Compounds **64**–**67**) showing low μg/mL (MIC = 2–8 μg/mL) activity against *E. coli* and *A. baumannii*.[99]

It is common for antibacterial compounds to be derived from naturally occurring products, including those produced in plants and bacteria (Figure 12). Dicumarol (Compound **69**), a naturally occurring anticoagulant drug, has recently been identified to have antibacterial activity, inhibiting *M. tuberculosis* GlmU at low micromolar concentrations (IC_{50} = 13.7 μM) and increasing the sensitivity of *M. tuberculosis* to other anti-tuberculosis drugs.[100] A phenolic acid derivative (Compound **70**), isolated from the parasitic plant *Balanophore involucrate*, has demonstrated inhibition of the acetyltransfer activity of GlmU at low micromolar concentration (IC_{50} = 18.2 μM).[101] Moreover, a secondary metabolite from *Aspergillus terreus*, terreic acid (Compound **71**), possesses mid-micromolar potency against EcGlmU (IC_{50} = 44.2 μM) and *Haemophilus influenzae* GlmU (IC_{50} = 95.6 μM), and whole cell activity against a number of Gram-negative bacteria (MIC = 23–184 μg/

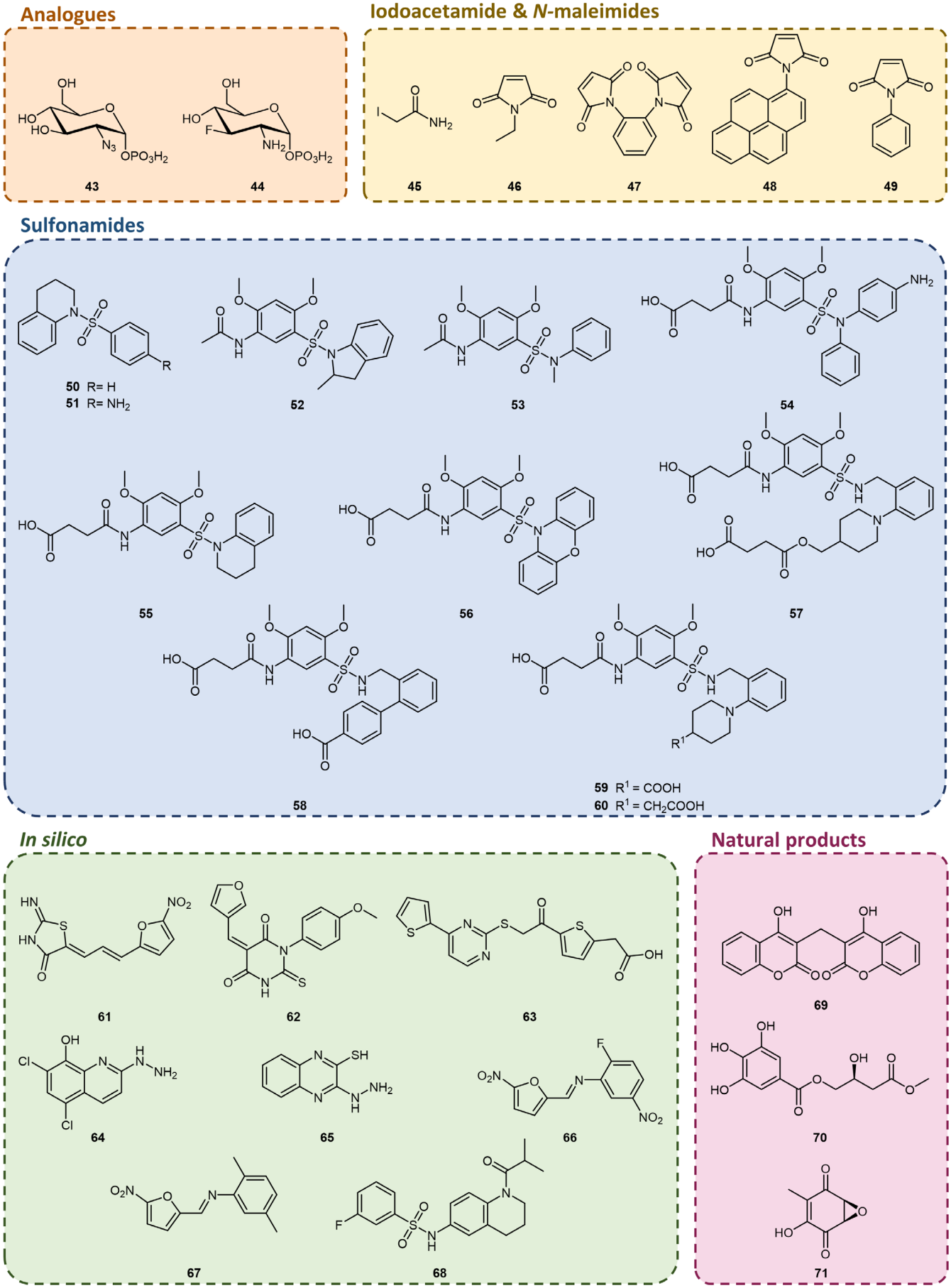

FIGURE 12 Inhibitors of the acetyltransferase activity of the bifunctional GlmU enzyme

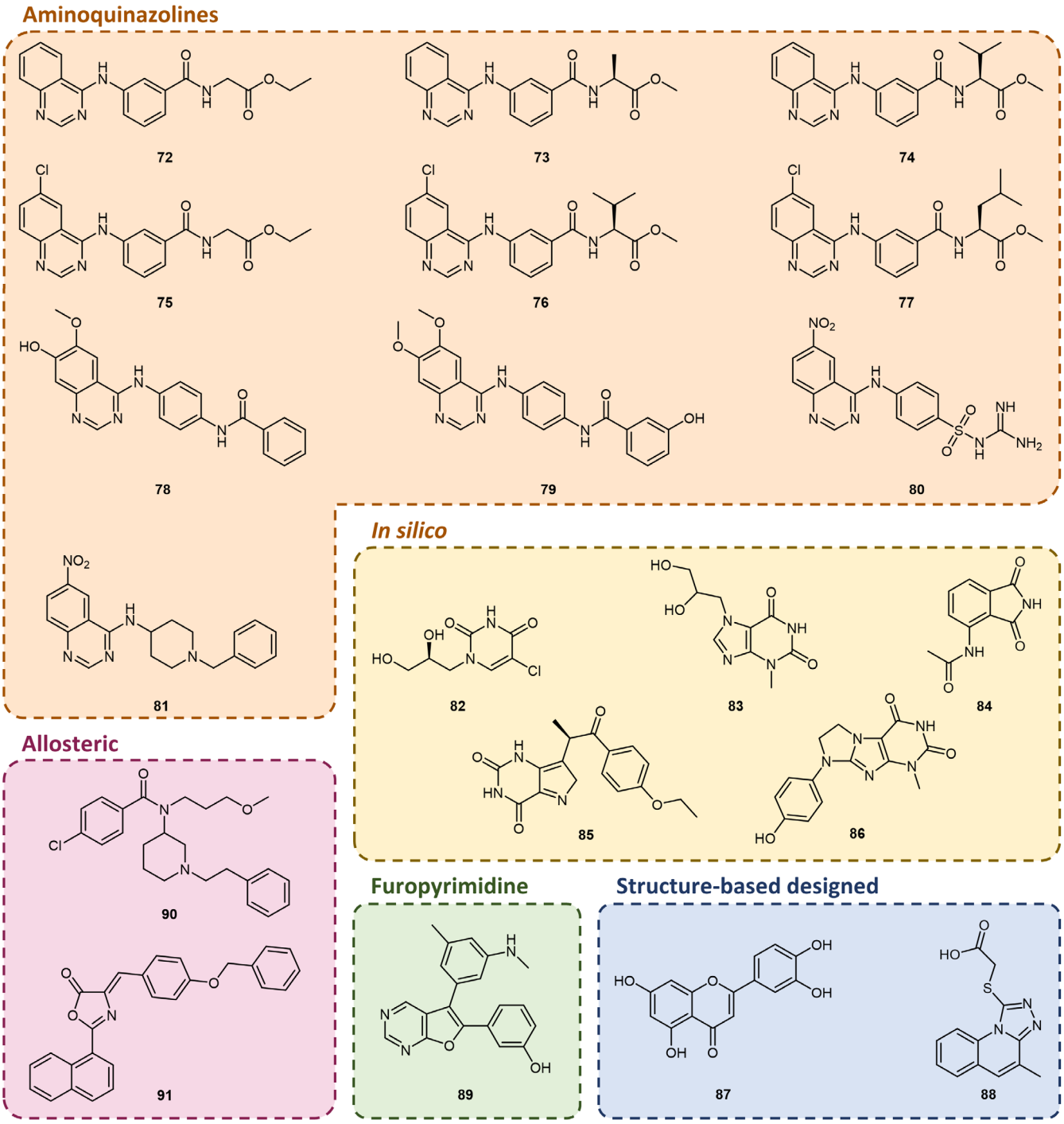

FIGURE 13 Inhibitors of the uridyltransferase activity of the bifunctional GlmU enzyme

mL).[102] Furthermore, *Streptococcus pneumoniae* GlmU has recently been shown to be sensitive to zinc inhibition, resulting in a significant impairment of activity.[103]

4.5 | Uridyltransferase inhibitors

While the uridyltransferase domain of GlmU shares homology with AGX1/AGX2, it has still been explored as a novel antibiotic target (Figure 13). High throughput screening has identified multiple series of inhibitors based on aminoquinazoline cores. Compounds **72–77** demonstrated varied whole cell activity against Gram-positive bacteria (MIC = 0.51–264 μM), while compounds **78–81** possessed micromolar potency (IC_{50} = 1.3–74 μM).[79,104–106] These series of inhibitors are predicted to bind within the hydrophobic pocket, occupying part of the UTP-binding site and locking GlmU in an apo-

enzyme-like conformation.[79,104–106] Two of the inhibitors (Compounds **80–81**) demonstrate significant whole cell activity against *M. tuberculosis* (MICs = 6.25–25 μM) and a lack of toxicity in mammalian Vero cell lines, indicating that aminoquinazoline inhibitors hold potential as antibiotic candidates.[106]

In silico high throughput screening has been used to identify five structures (Compounds **82–86**) that are predicted to be selective for the bacterial enzyme with binding to the eukaryotic enzymes expected to be hindered by pocket residues in AGX1/AGX2; however, these leads have yet to be examined for in vitro activity (Figure 13).[107] In silico screening has also been used to develop inhibitors of the uridyltransferase site through structure-based design, leading to the identification of a *Xanthomonas oryzae* GlmU inhibitor, luteolin (Compound **87**), with low micromolar potency (IC_{50} = 0.81 μM), and a *M. tuberculosis* GlmU inhibitor (Compound **88**) with mid-micromolar potency (IC_{50} = 42.1 μM).[108,109]

Additionally, inhibitors of GlmU have been identified through the screening of bacterial products, for example, a furopyrimidine product (Compound **89**) isolated from *Actinomadura* sp. (Figure 13).[110] This furopyrimidine product inhibited four targets in bacteria with activity against Gram-positive and Gram-negative bacteria; however, GlmU was found to be the least favourable docking site of the furopyrimidine.[110] Analysis of the *H. influenzae* GlmU crystal structure has led to the identification of a lipophilic pocket that has not been previously characterised. This pocket is adjacent to the GlcNAc binding site and undergoes conformational changes following the binding of UDP-GlcNAc (Figure 10e).[111] There have been two inhibitors identified that interact with the allosteric sites of *H. influenzae* GlmU and *M. tuberculosis* GlmU (Compounds **90–91**, respectively) with low micromolar potency (IC_{50} = 9.96–18 μM).[112,113] Binding of these inhibitors obstructs the conformational change required for phosphotransfer between the substrates, resulting in diminished GlmU activity.[112,113] The *M. tuberculosis* GlmU inhibitor, Oxa33 (Compound **91**), has demonstrated whole cell activity against *M. tuberculosis* (MIC = 30 μg/mL) indicating that the allosteric site of GlmU could serve as a potential antibacterial target.[113]

5 | THE FUTURE OF UDP-GlcNac IN ANTIBIOTIC DEVELOPMENT

Peptidoglycan biosynthesis is a rich source of antibiotic targets and yet there are several components that remain underexploited. There has been a significant focus on inhibiting the Mur class of enzymes over the last three decades; however, this has only yielded one clinically available antibiotic, fosfomycin.[7] The UDP-GlcNAc biosynthesis enzymes, on the other hand, have only gained traction as antibiotic targets in the last decade. The availability of enzyme functional and structural characterisation data, including enzyme kinetics, site-directed mutagenesis, and X-ray crystal structures, has allowed us to determine reaction mechanisms, substrate specificity, and conformational changes that occur upon ligand binding. Although we now have a strong understanding of the functional and structural features of GlmS, GlmM and GlmU, we still have a lot to learn about the enzymes in the UDP-GlcNAc biosynthesis pathway. Bacterial GlmM and GlmU form different quaternary structures to their eukaryotic homologues.[69,70,77,80,86,88] This potentially indicates that different residues are involved in the quaternary structure formation in bacteria and, therefore, targeting the dimerisation/trimerisation interfaces of these bacterial enzymes could be a viable approach to inhibitor development. In fact, this approach has already been exploited in GlmS, even though it possesses the same quaternary structure as the eukaryotic enzyme, with three inhibitors (Compounds **24–26**) demonstrating micromolar potency. However, the specificity of these inhibitors for the bacterial enzymes has yet to be explored.[53] This could be particularly successful for the bifunctional GlmU enzyme given that trimerisation has been shown to be essential for acetyltransferase activity.[77]

As mentioned earlier, antibacterial compounds are commonly derived from naturally occurring products, including those produced in plants and bacteria. There are currently six antibiotic classes that originated from naturally occurring compounds, two of which target bacterial cell wall synthesis.[114] Although there are a handful of compounds that have demonstrated activity against the bifunctional GlmU enzyme, natural compounds have yet to be explored as potential inhibitors of GlmS and GlmM and could be a promising approach to inhibitor discovery.[100–102] Moreover, a common problem encountered across the inhibitors for GlmS, GlmM, and GlmU is a lack of activity against whole organisms. Whilst some inhibitors demonstrate micromolar potency, they are unable to penetrate bacterial cells to exert their activity.[49,93,95,96] The challenge is, therefore, to develop compounds that, while retaining their affinities for their respective targets, can cross the cytoplasmic membrane. This could be achieved through combining an inhibitor with a membrane permeabilising agent, avoiding the potential loss of target affinity that comes with redesigning inhibitors. One of the biggest hurdles in targeting UDP-GlcNAc biosynthesis is the presence of a similar

pathway in eukaryotes. However, the bacterial enzymes possess significant differences to their eukaryotic enzymes in both structure and function as highlighted here, which could allow for specific targeting of the bacterial enzymes. Nevertheless, the assessment of potential off-target effects of bacterial enzyme inhibitors within eukaryotic cells remains crucial in the antibiotic development process. Despite this and the available knowledge of the bacterial UDP-GlcNAc biosynthesis, the pathway remains underexploited in the search for novel antibiotics. Future research should focus on developing inhibitors of the bacterial enzymes that show specificity and activity against not only the bacterial enzymes but the whole organism.

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