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Mechanism of Action of Nucleoside Antibacterial Natural Product Antibiotics

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Abstract

This article reviews the structures and biological activities of several classes of uridine-containing nucleoside antibiotics (tunicamycins, mureidomycins/pacidamycins/sansanmycins, liposidomycins/caprazamycins, muraymycins, capuramycins) that target translocase MraY on the peptidoglycan biosynthetic pathway. In particular, recent advances in structure-function studies, and recent X-ray crystal structures of translocase MraY complexed with muraymycin D2 and tunicamycin are described. The inhibition of other phospho-nucleotide transferase enzymes related to MraY by nucleoside antibiotics and analogues is also reviewed.

Dedication. This article is part of a Special Issue commemorating Dr Kiyoshi Isono and his important contributions to the study of nucleoside antibiotics. Dr. Isono led the discovery of the liposidomycin

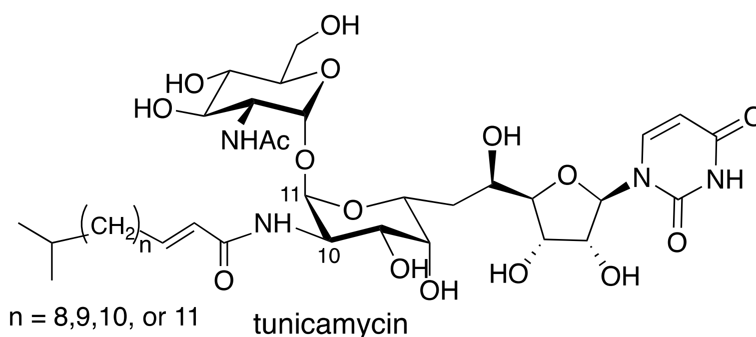
natural products in 1985, one of the first studies in this field, which established that nucleoside antibiotics could be selective antibacterial agents.

The discovery of the liposidomycin nucleoside antibiotics by Dr. Kiyoshi Isono and co-workers in 1985 [1], and the nucleoside antibiotic tunicamycin by Tamura and co-workers [2], has led to the identification and study of a related collection of uridine-containing nucleoside antibiotics with potent antibacterial activity, targetting the enzyme phospho-MurNAc-pentapeptide translocase (MraY) on the peptidoglycan cell wall biosynthetic pathway. The structures of each family have been reviewed in detail in reviews in 2003 [3] and 2010 [4]. This review will discuss recent structure-activity studies on each group of nucleoside antibiotics, and the mechanism of inhibition of translocase MraY, in particular, the recent crystal structures of nucleoside antibiotics bound to MraY.

1. Antibacterial nucleoside antibiotics targetting bacterial peptidoglycan biosynthesis

1.1 The tunicamycin group of GlcNAc-tunicamine nucleoside antibiotics (tunicamycins, streptovirudins, corynetoxins)

The tunicamycin group of nucleoside antibiotics were isolated in 1971 from *Streptomyces lysosuperficus* by Tamara and co-workers [2]. They contain a uracil base attached to a C_{11} tunicamine sugar, glycosylated at C_{11} by a GlcNAc sugar and N-acylated at C_{10} by a C_{12}-C_{15} fatty acid (see Figure 1). They showed antibacterial activity against a range of Gram-positive bacteria, especially those in the *Bacillus* genus (MIC 0.1-20 μg/ml) [2], but also showed toxicity towards eukaryotic cells, due to inhibition of eukaryotic N-linked glycoprotein biosynthesis [5]. The streptovirudins and corynetoxins contain the same uracil-tunicamine skeleton, but are acylated by different fatty acids [3]. The biosynthetic gene cluster for the tunicamycin antibiotics has been identified in *Streptomyces chartreusis* [6], and the biosynthetic pathway has been shown to involve an unusual radical SAM enzyme TunM in the assembly of the tunicamine sugar [7].

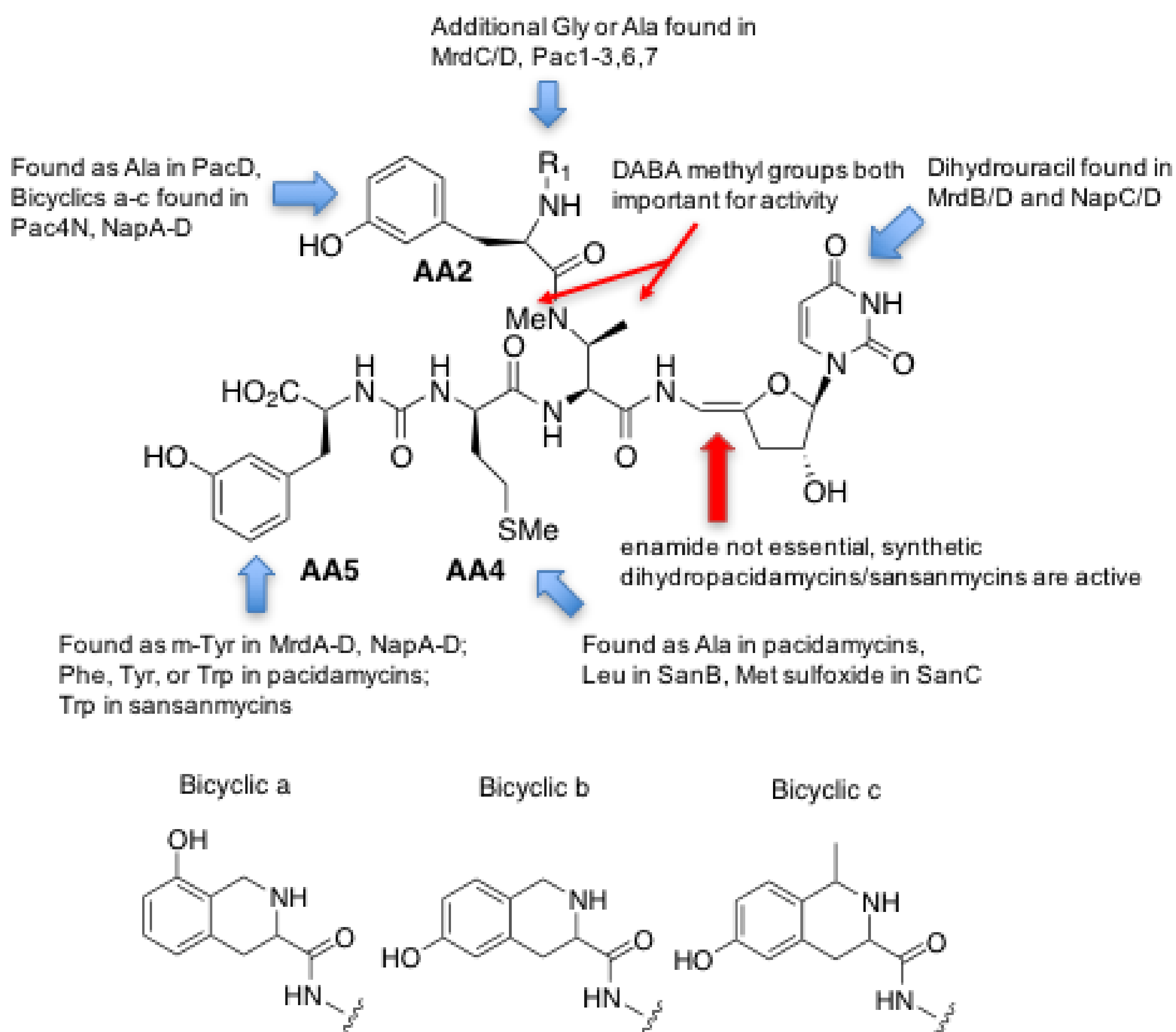


1 Figure 1. Structures of tunicamycins
2
3 A total synthesis of tunicamycin V was reported in 2017 by Ichikawa and co-workers [8],
4 which has enabled the synthesis of tunicamycin analogues for structure-activity study [9]. A lipid-
5 truncated analogue and an analogue lacking the GlcNAc sugar both lost 1000-fold in MraY
6 inhibition activity but retained some enzyme inhibition, while an analogue lacking the nucleoside
7 base was completely inactive [9]. The preseence of the uracil base has been shown to be required in
8 other nucleoside antibiotic families [3,4], which can be rationalised by the MraY structural studies
9 described in Section 2.2.
10

11 **1.2 The mureidomycin group of ureidyl-peptide nucleoside antibiotics (mureidomycins,**
12 **pacidamycins, napsamycins, sansanmycins)**

13 Mureidomycins A-D were isolated from *Streptomyces flavidoviridens* SANK 60486, and
14 first reported in 1989 [10]. They showed potent antimicrobial activity against a range of
15 *Pseudomonas* strains (MIC 0.1 – 3 μg/mL), and protected mice against infection by *Pseudomonas*
16 *aeruginosa* (ED_{50} 50 mg/kg for MrdC) [11,12]. Phospho-MurNAc-pentapeptide translocase (MraY)
17 on the bacterial peptidoglycan biosynthetic pathway was identified as the molecular target of these
18 compounds [11]. A closely related series of pacidamycins 1–7, isolated from *Streptomyces*
19 *coeruleorubidus* strain AB 1183F-64, were also reported in 1989 [13-15]. The pacidamycins also
20 showed antimicrobial activity against *Pseudomonas* strains (MIC's 8 – 64 μg / mL), but they were
21 found not to protect mice against infection by *Pseudomonas aeruginosa* [15].
22 The structures of both families contain a 3'-deoxyuridine sugar attached via an 4',5'-
23 enamide linkage to the carboxyl group of an N-methyl 2,3-diaminobutyric acid (DABA) residue, to
24 which amino acids are attached on both nitrogen substituents (see Figure 2). To the α-amino group
25 of DABA is attached either Met (mureidomycins) or Ala (pacidamycins), which is in turn attached
26 via a urea linkage to a C-terminal aromatic amino acid, either *meta*-tyrosine (mureidomycins), or
27 Trp or Phe (pacidamycins). To the β-amino group of the DABA residue is attached in most cases a
28 *meta*-tyrosine residue, except in pacidamycin D, which contains Ala. Two further mureidomycins E
29 and F were later reported, containing a bicylic derivative of *meta*-tyrosine at the amino-terminal
30 position [16], also found in the closely related napsamycins, which were reported in 1994 [17]. The
31 sansanmycins were reported in 2007, and contain the same structural sekeleton as the
32 mureidomycins, but contain Trp at the C-terminal position, and contain either Met, Leu, or
33 methionine sulfoxide at position 4 [18,19]. The sansanmycins showed antipseudomonal activity,
34 but also showed activity against *Mycobacterium tuberculosis* (MIC 8-20 μg/ml) [19]. The structures
35 of these classes of uridyl peptide antibiotics are shown in Figure 2.

1 A series of synthetic dihydropacidamycin analogues in which the 4'-5' enamide was absent
2 were prepared by Microcide Inc. The parent 4*R*-dihydropacidamycin retained antipseudomonal
3 activity, but with somewhat reduced MIC (64 µg/ml) compared to pacidamycin D [20]. Synthetic
4 analogues containing Phe or Leu at position 4, and Trp or Tyr at position 5, showed best
5 antipseudomonal activity (MIC 4-16 µg/ml) [20]. An analogue containing 4-fluorophenylalanine in
6 place of Met at position 4 showed antimicrobial activity against clinical *E. coli* strains (MIC 4-8
7 µg/ml), as well as *Mycobacterium tuberculosis* (MIC 4-10 µg/ml) [21]. In 2011, Okamoto *et al*
8 published a total synthesis of pacidamycin D [22], which they have used to synthesise further
9 analogues varying the N-terminal dipeptide chain [23]. They have reported that meta-tyrosine in the
10 amino-terminal position is considerably more active than L-Tyr, and that the stereochemistry of the
11 2,3-diaminobutyric acid is important for both MraY inhibition and antimicrobial activity [23].



12

13 Figure 2. Structural features of mureidomycin, pacidamycin, napsamycin, and sansanmycin families
14 of ureidyl peptide antibiotics. Structural variation show by blue arrows, structure-activity
15 observations shown by red arrows.
16

The anti-TB activity of the sansanmycin series has been developed signifcantly by Payne and co-workers, via chemical synthesis of a set of dihydrosansanmycin analogues [24]. They found that dihydrosansamycin B had significantly improved anti-TB activity (MIC_{50} 0.3 μM) compared with sansanmycin B (MIC_{50} 9.5 μM). Structure-activity studies revealed that analogues containing glycine at the N-terminal amino acid showed comparable activity (MIC_{50} 0.63 μM), and that a further modification of a cyclohexyl group at position 4 led to an analogue with MIC_{50} 80 nM that was a potent MraY inhibitor (IC_{50} 30 nM), as shown in Figure 3 [24].

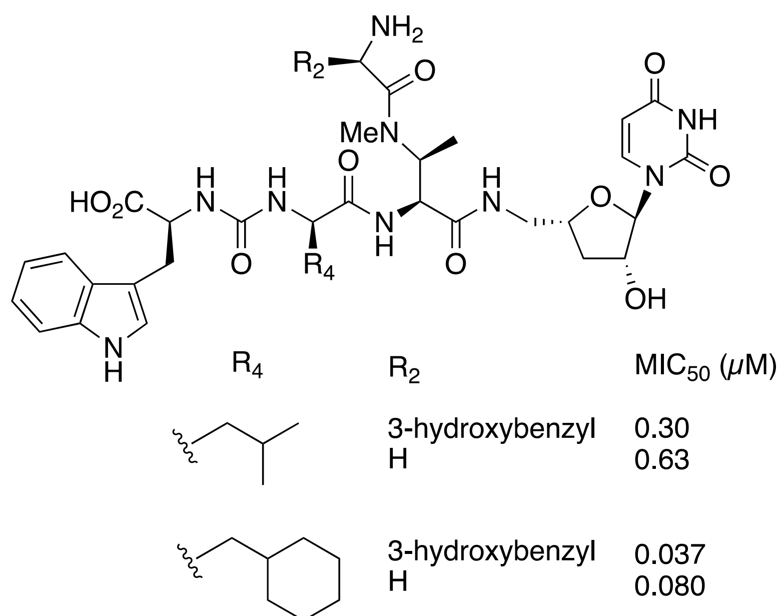


Figure 3. Antimicrobial activity of synthetic dihydrosansanmycin analogues modified at positions 2 and 4 against *Mycobacterium tuberculosis* H37Rv.

The biosynthetic gene cluster for the pacidamycin antibiotics, containing a number of non-ribosomal peptide synthetase genes, was identified in *Streptomyces coeruleorubidus* in 2010 by the groups of Goss [25] and Walsh [26]. The unusual ureidopeptide moiety at the carboxyl terminus of the peptide chain is assembled via carboxyl activation of Ala by PacN, followed by carboxylation, and then peptide bond formation catalysed by ligase PacL [27]. The diamino acid DABA is biosynthesised from L-threonine by a pyridoxal 5'-phosphate-dependent β-replacement reaction, also observed for mureidomycin biosynthesis in *Streptomyces flavidovirens* [28], using L-aspartate as a nucleophile, followed by a β-elimination reaction [26]. The modified uridine nucleoside is formed via oxidation of uridine to the 5'-aldehyde, followed by transamination to 5'-amino-uridine, followed dehydration of the 4'-hydroxyl group [29]. The crystal structure of the novel dehydratase enzyme Pac13 has been determined, implicating His-42 in the catalytic mechanism [30]. The additional N-terminal Ala found in some pacidamycins and mureidomycins is added by ligase PacB, that uses Ala-tRNA as an amino acid donor [31]. The unusual amino acid meta-tyrosine is

biosynthesised from L-Phe by a novel non-heme iron- and tetrahydrobiopterin-dependent hydroxylase [32]. Mutasynthesis has been used to generate novel chlorinated pacidamycin derivatives [33], and modified sansanmycins [34]. The modified sansanmycins were reported to retain antimicrobial activity, in some cases with reduced activity, but MX-6 containing 4-fluorophenylalanine at the C-terminus showed enhanced antimicrobial activity against *B. subtilis* and *M. tuberculosis* [34].

1.3 The liposidomycin group of liponucleoside antibiotics (liposidomycins, caprazamycins)

The liposidomycins are liponucleoside natural products containing an aminoglycoside sugar, which were reported by K. Isono and coworkers in 1985 [1], and their molecular structures reported in 1988 [35]. They show antimicrobial activity against *Mycobacterium* strains (MIC 1.6 μg/ml) [1]. The caprazamycins were reported in 2003: they share the same structural skeleton as the liposidomycins, as shown in Figure 4, but the 3-methylglutaryl substituent is glycosylated by an additional L-rhamnose sugar [36,37].

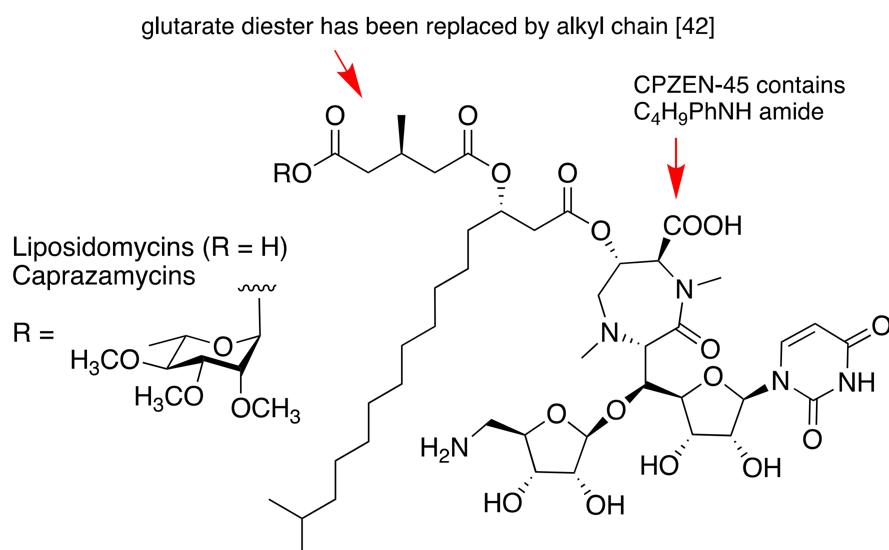


Figure 4. Structures of the liposidomycins and caprazamycins, and synthetic analogues

Synthetic uridine-based analogues of the liposidomycins containing the aminoribofuranoside sugar retain MraY inhibition activity (IC_{50} 0.14-50 μM), but show weaker antimicrobial activity, demonstrating the importance of the lipophilic substituent, probably needed for cellular uptake [38-40]. Fer et al have published a further series of uridine-based analogues containing a lipophilic group linked via a triazole heterocycle, which show antimicrobial activity against *Staphylococcus aureus*, and inhibit MraY with IC_{50} values in the range 100-1000 μM [41]. Matsuda and coworkers have synthesised analogues of caprazamycin containing an alkyl chain in place of the glutarate diester sidechain, which retain antimicrobial activity but show enhanced stability [42]. A semisynthetic caprazamycin derivative CPZEN-45 is active in animal models for

protection against tuberculosis infection, and Ichizaki et al published in 2013 that CPZEN-45 inhibits transferase WecA in *M. tuberculosis*, involved in lipoloysaccharide biosynthesis, rather than MraY [43].

The biosynthetic gene cluster for production of the caprazamycins in *Streptomyces* sp. MK730-62F2 was identified in 2009 by Kaysser et al [44]. The biosynthetic pathway involves the formation of uridine 5'-aldehyde, followed by a pyridoxal-5'-phosphate dependent reaction with glycine to form a uridine-amino acid adduct, followed by an *S*-adenosylmethionine-dependent reaction transferring a 3-amino-3-carboxypropyl group [44]. A gene deletion strain, in which *cpz21* encoding an acyltransferase enzyme acting late in the biosynthetic pathway had been deleted, was found to accumulate the caprazamycin aglycone [44]. The genes responsible for addition of the L-rhamnose sugar found in caprazamycins have been identified, allowing the heterologous gene expression of intact caprazamycins [45]. The biosynthetic pathway for the aminoribosyl sugar moiety found in the caprazamycin, muraymycin and other nucleoside natural products has also been shown to proceed via uridine 5'-aldehyde, and was reported by van Lanen and co-workers [46-48].

1.4 The muraymycin group of lipo-ureidylpeptide nucleoside antibiotics

The muraymycins were reported in 2002 by McDonald et al, isolated from a *Streptomyces sp*. strain [49]. Their structure contains an aminoribofuranoside monosaccharide attached to the 5'-position of a uridine-amino acid, similar to that found in the liposidomycins & caprazamycins, as shown in Figure 5, and a ureidopeptide structure linked via a 3-aminopropyl moiety [49]. The muraymycins also target translocase MraY (IC_{50} 0.027 μg/ml), show antimicrobial activity against strains of *Staphylococcus aureus* (MIC 2-16 μg/ml) and *Enterococcus* (MIC 16-64 μg/ml), and were reported to protect mice against *S. aureus* infection (ED_{50} 1.1 mg/kg) [49].

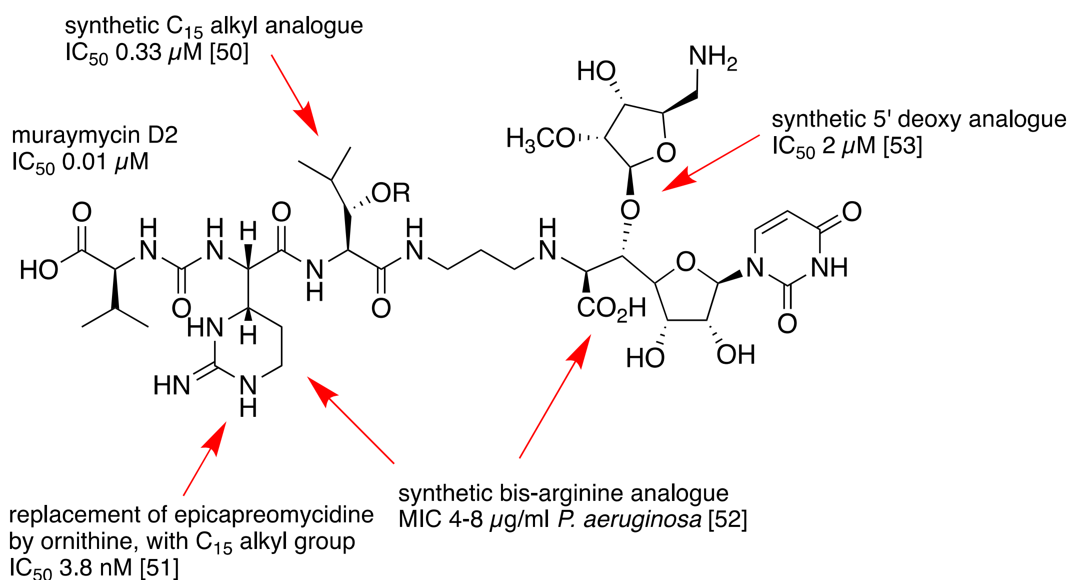


Figure 5. Structures of muraymycin antibiotics and synthetic analogues

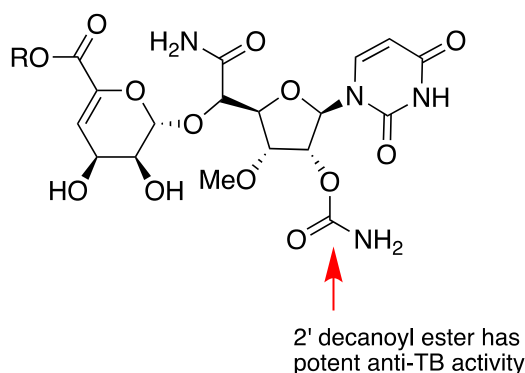
Several bioactive muraymycin analogues have been generated via total chemical synthesis. Tanino *et al* have synthesised analogues in which the hydroxyleucine residue is replaced by an alkyl sidechain, which show MraY inhibition activity (IC_{50} 0.33 μM), and retain antimicrobial activity [50]. The same group have reported that the epicapreomycidine amino acid (a cyclic analogue of arginine) can be replaced by arginine, lysine or ornithine residues, and that these synthetic analogues retain antimicrobial activity [51]. Takeoka et al. have prepared further analogues with L-arginine in place of epicapreomycidine, in which the C-terminal amino acid is removed, which retain full MraY inhibition activity, and show enhanced antimicrobial activity against *Pseudomonas* strains [52]. Spork *et al* have synthesised an analogue of muraymycin lacking the aminoribose sugar which retains activity for MraY inhibition (IC_{50} 2 μM) [53]. The ω-guanylated fatty acid which is found in the most active muraymycins has been shown to assist localisation of the antibiotic into the cell membrane [54]. The total synthesis of muraymycin D1 was reported in 2016 by Mitachi et al [55], enabling the synthesis of further analogues. The biosynthetic gene cluster for the biosynthesis of muraymycin in *Streptomyces* sp. NRRL 30471 was reported in 2011 [56].

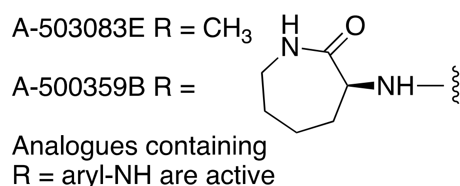
1.5 The capuramycin group of caprolactam nucleoside antibiotics (capuramycin, A-500359A)

Capuramycin, a nucleoside antibiotic produced by *Streptomyces griseus*, containing a uronic acid monosaccharide attached to the 5' position of a modified uridine nucleoside, to which is attached a 7-membered caprolactam ring, as shown in Figure 6, was first reported in 1986 [57,58]. Capuramycin and a methylated derivative A-500359A which shows antimicrobial activity against *Mycobacterium smegmatis* (MIC 2-16 μg/ml) and potent MraY inhibition (IC_{50} 0.017 μg/ml) was then reported in 2003 [59,60]. A-500359E, which lacks the aminocaprolactam ring, shows potent inhibition of MraY (IC_{50} 0.027 μM), but lacks antimicrobial activity [61]. Semi-synthetic derivatives of A-500359E have been reported, in which the aminocaprolactam is replaced by synthetic arylamines, which show potent MraY inhibition (IC_{50} 10-40 ng/ml), and antimicrobial activity against *Mycobacterium* strains (MIC 0.5-2 μg/ml) [62]. Acylation of capuramycin on the 2' hydroxyl group gave a further series of bioactive derivatives, including a decanoyl derivative which shows very potent activity against *M. tuberculosis* (MIC 0.06 μg/ml) [63].

A biosynthetic gene cluster for a closely related capuramycin antibiotic A-503083B in *Streptomyces* sp. SANK 62799 was reported in 2010 [64]. The biosynthetic steps for attachment of a caprolactam moiety were elucidated, via carboxy methyltransferase CapS and transferase CapW [64]. The biosynthetic gene cluster for capuramycin A-102395 has also been reported, involving the incorporation of L-threonine into uridine 5'-carboxamide [65]. Transferase CapW has been used to

1 prepare of a set of 43 semi-synthetic bioactive capuramycin derivatives [66]. Several of these
2 analogues retained similar antimicrobial activity to the parent compound, with three analogues
3 showing enhanced activity against *M. smegmatis* and *M. tuberculosis* [66].
4





5
6 Figure 6. Capuramycin natural products and synthetic analogues
7

8 **1.6 Comparison of antimicrobial activities of nucleoside natural products.**

9 The nucleoside natural product antibiotics show very interesting and varied antimicrobial
10 activities. The mureidomycins show particularly potent antimicrobial activity against *Pseudomonas*
11 *aeruginosa* (MIC 0.1 – 3 μg/mL), a bacterium responsible for antibiotic-resistant infections around
12 the world, and can protect mice against infection by *Pseudomonas aeruginosa* [11,12]. The
13 pacidamycins and napsamycins also show anti-pseudomonal activity, but synthetic
14 dihydropacidamycins containing modifications at position 4 (see Figure 2) showed new
15 antimicrobial spectrum against *Escherichia coli* (MIC 4-8 μg/mL) and *Citrobacter freundii* (1.0
16 μg/mL) [20,21]. Given that the MraY sequences from these organisms are quite closely related, it
17 seems likely that these changes in antibacterial spectrum are caused by changes in uptake.

18 The liposidomycin and caprazamycin liponucleosides ahow activity against strains of
19 *Mycobacterium* (MIC 1.6 μg/ml) [2]. The synthetic caprazamycin derivative CPZEN-45 has shown
20 efficacy against both drug-sensitive and extremely drug-resistant (XDR) Mtb in a mouse model of
21 acute tuberculosis, and is in clinical trials against TB infection [43]. Capuramycins also show
22 potent activity against *Mycobacterium smegmatis* (MIC 2-16 μg/ml) [59,60], and semi-synthetic
23 derivatives show enhanced anti-Mtb activity [62,63]. The activity of the sansanmycins against
24 *Mycobacterium tuberculosis* has been greatly enhanced in synthetic dihydrosansanmycins
25 containing modifications at position 4 (MIC_{50} 0.04-0.6 μM) [24].

The muraymycin antibiotics show antimicrobial activity against *Staphylococcus aureus* (MIC 2-16 µg/ml) and *Enterococcus* (MIC 16-64 µg/ml), and can protect mice against *S. aureus* infection [49]. Synthetic analogues containing two L-arginine residues show modified antimicrobial spectrum, notably against *Pseudomonas* strains (MIC 4-8 µg/mL) [52].

2. Mechanism of inhibition of translocase MraY by nucleoside antibiotics

2.1 Kinetic mechanism of inhibition of translocase MraY

Translocase MraY catalyses the first step of the lipid cycle of bacterial peptidoglycan biosynthesis, namely the reaction of UDPMurNAc-L-Ala-γ-D-Glu-m-DAP-D-Ala-D-Ala (UDPMurNAc-pentapeptide) with lipid carrier undecaprenyl phosphate, to form lipid intermediate 1 (undecaprenyl-diphospho-MurNAc-pentapeptide), releasing uridine 5'-monophosphate (UMP) [67]. Translocase MraY is an integral membrane protein, shown to contain ten transmembrane helices [68]. The MraY-catalysed reaction is a phosphotransfer reaction, shown in Figure 7, whose catalytic mechanism could either proceed via a single step phosphotransfer, or a two-step mechanism involving an active site nucleophile [67]. Three aspartic acid residues in *E. coli* MraY (Asp-115, Asp-116, Asp-267), found on cytoplasmic loops, were shown to be essential for activity, and it has been proposed that two Asp residues bind the active site Mg^{2+} cofactor, while the third may be a catalytic nucleophile [69].

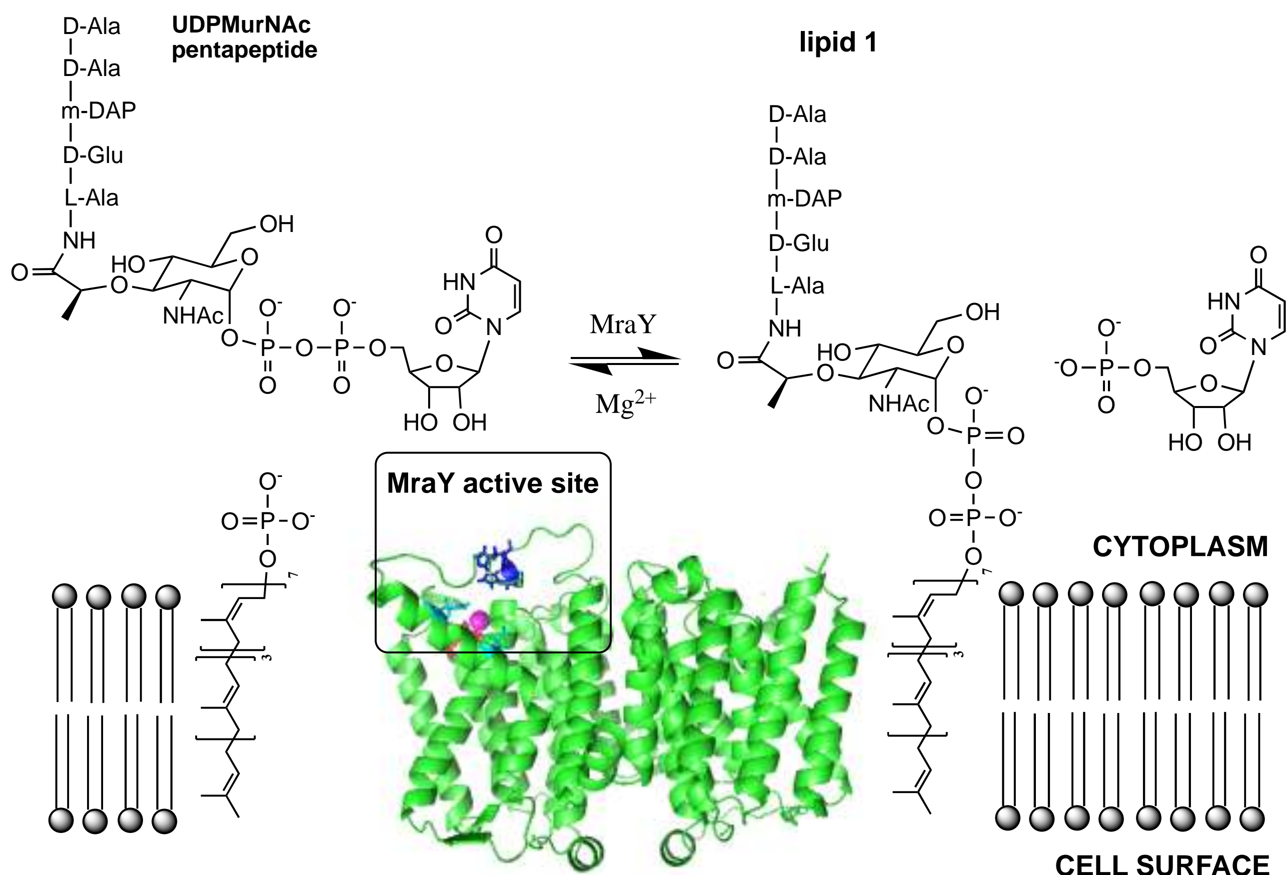


Figure 7. Reaction catalysed by translocase MraY, showing MraY dimer structure and location of MraY active site.

Mureidomycin A has been found to act as a slow-binding inhibitor (K_i 35 nM, K_i* 2 nM) for solubilised *E. coli* MraY, using a continuous fluorescnce enhancement assay, showing competitive enzyme inhibition towards both UDPMurNAc-pentapeptide and polyprenyl phosphate substrates [70]. Liposidomycin B also acts as a slow-binding inhibitor of *E. coli* MraY (K_i* 90 nM), showing non-competitive enzyme inhibition towards UDPMurNAc-pentapeptide, but competitive inhibition towards dodecaprenyl phosphate [71]. By contrast, tunicamycin is a reversible inhibitor of *E. coli* MraY (K_i 0.6 μM) showing competitive enzyme inhibition towards UDPMurNAc-pentapeptide, but non-competitive inhibition towards dodecaprenyl phosphate [71]. Muraymycin D2 and synthetic analogues thereof were found by Tanino et al to show competitive inhibition towards UDPMurNAc-pentapeptide (K_i 7.6 nM), but non-competitive inhibition versus undecaprenyl phosphate, against *B. subtilis* MraY using a radiochemical assay [51]. Hence there are some differences in the kinetic mechanism of MraY inhibition shown by the different classes of nucleoside antibiotics.

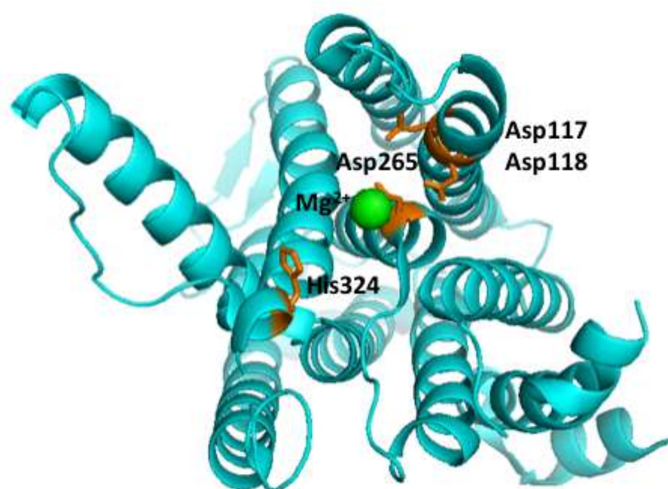
The possibility that the mureidomycins might be mechanism-based inhibitors, reacting via the enamide functional group, which might be expected to be chemically reactive, has been found not to be the case, from studies on model enamide-containing analogues [72], and since synthetic

dihydropacidamycin and dihydrosansanmycin analogues retain MraY inhibition [20,21,24]. The observed slow-binding inhibition is therefore probably due to a conformational change in the protein structure, discussed in Section 2.2. Studies on analogues of mureidomycins have found that the amino terminus of the peptide chain and the N-methyl amide group of DABA are both important for MraY inhibition [73,74], leading to a proposal that the amino terminus might bind in place of the Mg^{2+} cofactor, positioned via a *cis*-amide rotamer in the peptide chain [74]. The amino group of the aminoribofuranose monosaccharide of liposidomycins and caprazamycins is also known to be important for activity [39].

2.2 Structure of *Aquifex aeolicus* MraY and its complexes with nucleoside antibiotics

In 2013 the crystal structure of the *Aquifex aeolicus* MraY was determined, confirming the arrangement of ten transmembrane α-helices [75]. The protein was found to crystallise as a dimer, with transmembrane helix 9 strongly bent and protruding into the membrane. The active site contained the three catalytic Asp residues, close to the Mg^{2+} cofactor, with Asp-265 positioned closest to the Mg^{2+} ion [75]. There is a triad of three histidine residues (His-324, His-325, His-326; HHH motif) conserved in bacterial sequences of the polyprenyl-phosphate N-acetylhexosamine 1-phosphate transferase (PNPT) superfamily, which are positioned on loop E on the opposite side of the active site, 10-13 Å from the three catalytic Asp residues, shown in Figure 8.

The structure of a complex of *A. aeolicus* MraY with muraymycin D2 was published in 2016 [76]. Upon binding of muraymycin D2, transmembrane helix 9b (TM9b) moves away from the active site and the HHH motif (conserved across the PNPT family) in loop E extends, which widens and reshapes the active site, hence there is a significant conformational change upon ligand binding [76], which may be important for the catalytic cycle of MraY, and potentially could explain the slow-binding inhibition of MraY observed for some nucleoside natural product inhibitors [70,71]. Binding of muraymycin D2 to the MraY active site does not involve the catalytic Asp residues and does not require Mg^{2+}, but several other binding interactions were elucidated [76]. The uracil base is bound via π–π stacking interactions to Phe-262 (see Figure 9A), as well as hydrogen-bonding interactions to the uracil carbonyl and NH groups. The amino group of the aminoribose moiety is bound by Asp-193, whose mutation greatly reduces affinity for muraymycin D2, and Asn-190. The strong binding of the amino group of the aminoribose helps to rationalise why this group is important for activity in several nucleoside antibiotics [39,73]. The carboxyl terminus of the peptide chain is bound by Gln-305, a residue that is conserved in bacterial MraY homologues, and the *epi*-capreomycidine amino acid is bound by His-324 and His-325 [76].



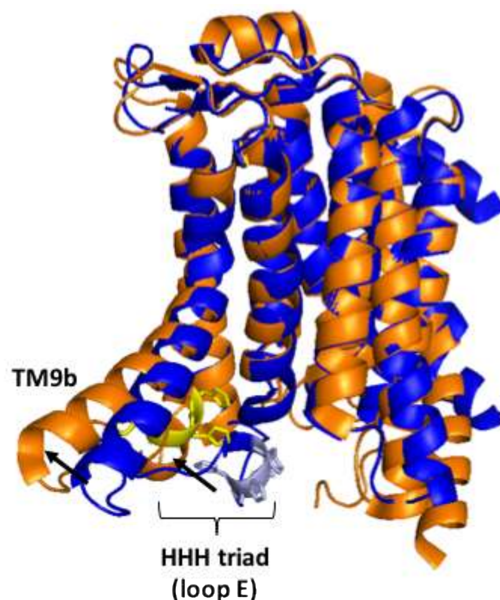
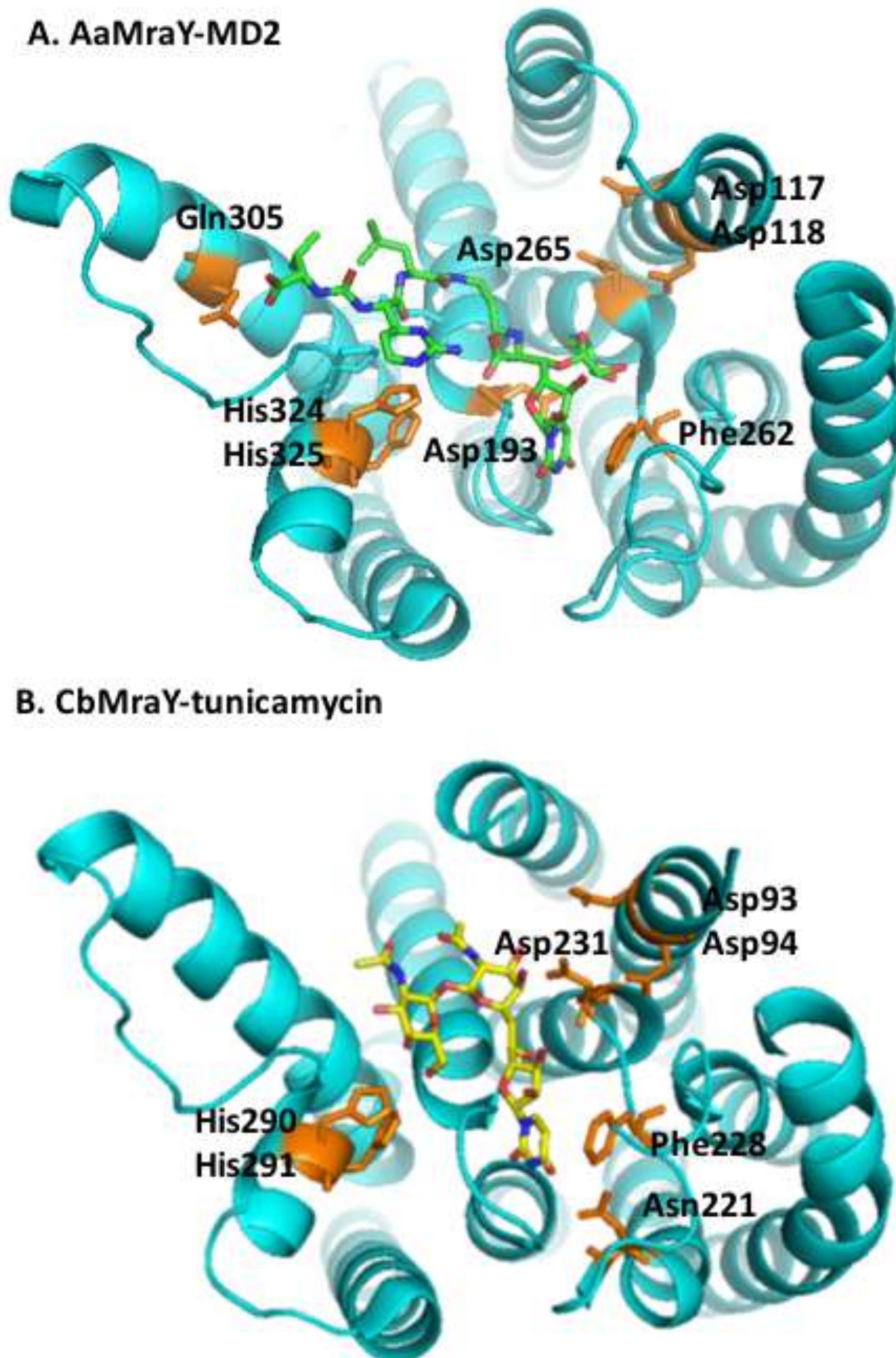


Figure 8. Structures of *A. aeolicus* MraY (A) containing no ligand, (B) complexed with muraymycin D2, showing active site residues (A) and conformational change upon muraymycin binding (B).

In 2017 the structure of a complex of *Clostridium boltae* MraY with tunicamycin was published [77]. The overall conformation of the protein was similar to the MraY-muraymycin complex, but some similarities and differences in the enzyme-ligand binding interactions were observed [77,78]. As in the MraY-muraymycin complex, the uracil base was bound in a small cavity, interacting via $\pi-\pi$ stacking interactions to Phe-228 (see Figure 9B), and the 4-carbonyl group binding to Asn-221. The HHH motif (His-290, His-291) are also involved in ligand binding, in this case to the GlcNAc 4'- and 6'-hydroxyl groups. However, unlike the MraY-muraymycin complex, tunicamycin was found to interact with the catalytic Asp residues, with the tunicamine 9'-hydroxyl group interacting with Asp-231, but in the absence of Mg^{2+} [77]. The structure of tunicamycin bound to its eukaryotic target enzyme GlcNAc-1-phosphate transferase involved in N-linked glycoprotein biosynthesis was also reported in 2018, showing some differences in active site binding, compared to MraY [79].

Hence both nucleosides bind to an MraY structure that has undergone a conformational change, both show specific binding for the uracil base and some involvement of the HHH motif in ligand binding, but muraymycin and tunicamycin show different polar contacts in the MraY active site [78].



1

2 Figure 9. Structures of MraY-ligand complexes, showing the location of selected binding
3 interactions. A. *A. aeolicus* MraY complexed with muraymycin D2. B. *C. boltae* MraY complexed
4 with tunicamycin.

5

2.3 Interaction with protein-protein interaction site for bacteriophage lysis protein E

E. coli MraY is also targetted by an antibacterial lysis protein E from bacteriophage ϕX174, which interacts with Phe-288 and Glu-287 of MraY, on the exterior face of transmembrane 9 of MraY, via an Arg-Trp-x-x-Trp sequence motif near the N-terminus of the E protein [80]. The presence of a guanidine-containing amino acid *epi*-capreomycidine in muraymycin, and two aromatic residues (Trp or m-Tyr and a second m-Tyr) in the mureidomycin/pacidamycin structures, is reminiscent of this Arg-Trp-x-x-Trp motif. Rodolis et al have shown that pacidamycin 1 and a synthetic muraymycin analogue showed significantly reduced activity against site-directed F288L and E287A MraY mutant enzymes [81], suggesting that parts of the antibiotic structure somehow aid the targetting of MraY *in vivo*, perhaps aiding uptake into the cell via a hydrophobic channel present in the structure of MraY [75], as shown in Figure 10. This hypothesis might explain how these agents of molecular weight 600-1200 Da are able to access the MraY active site on the inner face of the cytoplasmic membrane, and also why it has proved difficult to design small analogues of these nucleoside antibiotics that retain both MraY inhibition and antimicrobial activity.

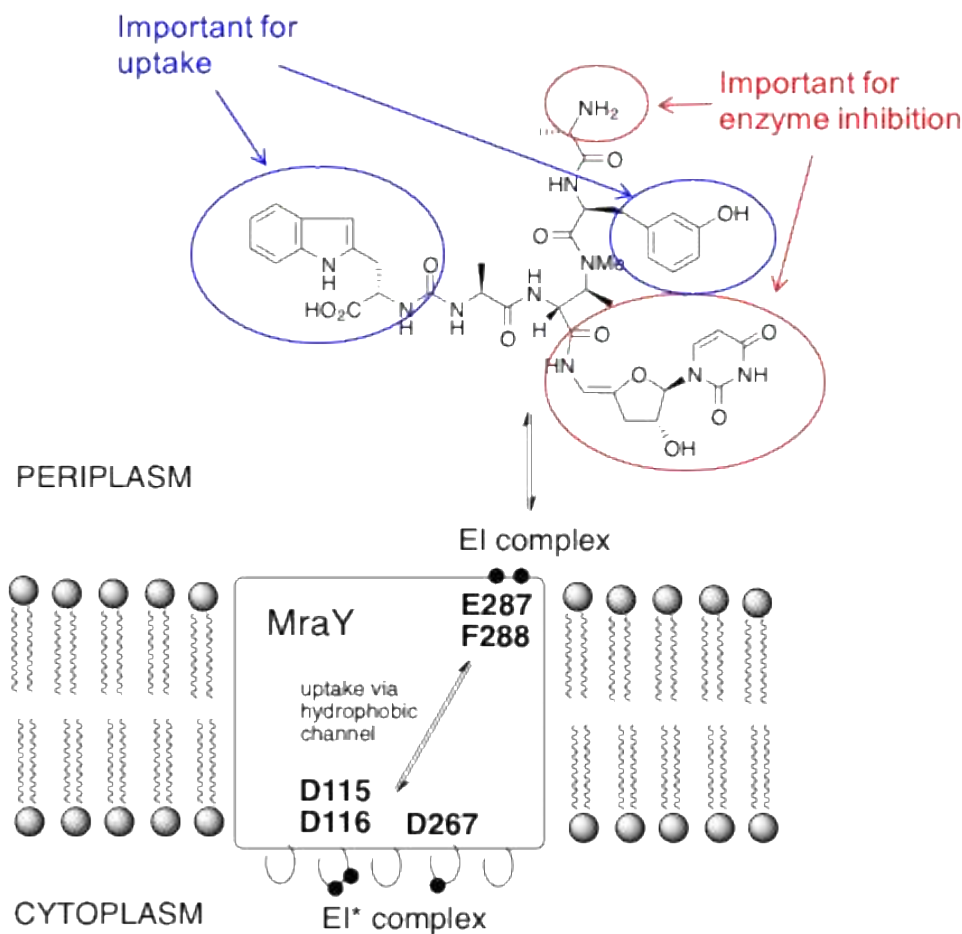


Figure 10. Interaction with the E protein binding site in MraY (Phe-288 and Glu-287) by nucleoside antibiotics, and hypothesis for uptake to the MraY active site.

3. Inhibition of other bacterial phospho-nucleotide transferase enzymes by nucleoside natural product analogues.

There are homologues of MraY involved in lipid-linked cycles responsible for the biosynthesis of enterobacterial common antigen and O-antigen lipopolysaccharide (LPS) in Gram-negative bacteria (WecA) [82], and teichoic acid in Gram-positive bacteria (TagO), both of which are integral membrane proteins that utilise UDP-GlcNAc and undecaprenyl phosphate as substrates [83]. CPZEN-45, a semisynthetic caprazamycin derivative undergoing clinical trials for treatment of tuberculosis, was found to inhibit transferase WecA in *M. tuberculosis* >20 fold more strongly than MraY, and CPZEN-45 also inhibits *B. subtilis* TagO 8-fold more tightly than *B. subtilis* MraY [43]. Selective synthetic inhibitors for TagO have also been published, based on the existing drug ticlopidine [84]. In contrast, the muraymycin analogues prepared by Tanino *et al* were highly selective for MraY inhibition over *E. coli* WecA [51], as were the dihydrosansanmycin analogues of Tan et al [24].

A homologue of WecA (GacO) has been found to catalyse the formation of undecaprenyl-diphospho-GlcNAc in the biosynthesis of the Lancefield group A carbohydrate in *Streptococcus pyogenes* [85]. A further group of small 20-25 kDa phospho-sugar transferase enzymes utilising a UDP-di-*N*-acetyl-bacillosamine substrate are involved in *N*-glycoconjugate biosynthesis in *Campylobacter jejuni* [86]. Synthetic peptidyl-uridine inhibitors have been synthesised as inhibitors of *C. jejuni* PglC, with IC_{50} values in the range 40-250 μM [87].

In conclusion, Nature produces several classes of uridine-based nucleoside antibiotics that target translocase MraY in the bacterial peptidoglycan biosynthetic pathway. Total synthesis of modified nucleoside analogues, and exploitation of the biosynthetic machinery for these natural products, offer considerable promise for the development of highly active antimicrobial agents to treat antibiotic-resistant infections. Understanding of the MraY structure will aid the development of selective MraY inhibitors, and the discovery of MraY homologues such as WecA and TagO has identified further interesting targets for antibacterial drug discovery by nucleoside analogues.

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