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Targeting the forgotten transglycosylases

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ABSTRACT

Forty years ago, moenomycin was reported as a representative of a novel natural product class with strong antibacterial activity against Gram-positive organisms. Moenomycin was developed as an antimicrobial growth promoter in animal feeds. Mechanistically, moenomycin acts via inhibition of the transglycosylation process at the final stage of the peptidoglycan biosynthesis, in particular through binding directly to the transglycosylase enzymes, thereby preventing polymerisation of lipid II into linear peptidoglycan. Despite moenomycin's success, no developments of direct transglycosylase enzyme inhibitors were reported for over 30 years, probably due to the complexities and uncertainties surrounding the transglycosylation process, in particular the number of enzymes involved in the process and their specific roles. The development of better research tools and an improved understanding of the transglycosylation process, together with the increasing threat presented by multidrug-resistant bacteria, have led to a resurfacing of interest in targeting the forgotten transglycosylases. In addition, several new generation glycopeptides in clinical development inhibit the transglycosylation process, adding further value to the approach. In this paper, we summarise some of the developments in the area of transglycosylase inhibitors over the last 10 years.

1. Stage III of the peptidoglycan biosynthesis

The formation of highly cross-linked peptidoglycan, which forms a continuous covalent macromolecular net-like structure in almost all eubacteria, is essential for the preservation of the bacterial cell integrity in the face of high and variable internal osmotic pressure. The biosynthetic process that produces the peptidoglycan takes place in several stages [1–4]. The biosynthetic machinery is conserved across both Gram-negative and Gram-positive bacteria. Transglycosylation occurs in the final stage of the process, once the precursor (lipid II) has been synthesised in the cytoplasm and translocated across the bacterial cell membrane to the cell surface. Transglycosylation and transpeptidation are the final steps in the formation of the macromolecular peptidoglycan net and occur on the outside of this cell surface (Fig. 1).

The transglycosylation step proceeds by the reaction between the disaccharide unit lipid II and the growing peptidoglycan chain, where both lipid II and the growing peptidoglycan chain are attached to the cytoplasmic membrane via a long hydrophobic moiety. There is evidence to suggest, at least in some Gram-positive organisms, that the growing polysaccharide chain acts as the donor and adds to the 4-hydroxy group on the GlcNAc unit of lipid II, which acts as the acceptor [2,5,6]. An alternative mechanism involving lipid II as a donor, is also viable, but is to our knowledge not supported by experimental evidence.

The processes of elongation and septation (division) involve complexes of enzymes attached at the outside surface of the bacterial cell membrane. Transglycosylation is an absolute requirement for these processes to occur. However, the specific role and spatial and temporal distribution of the

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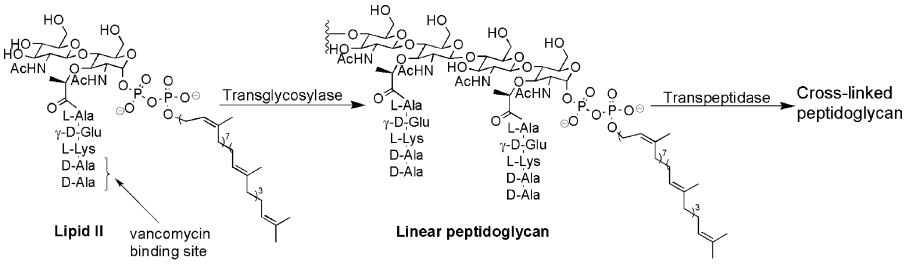


Fig. 1 – Stage III of the peptidoglycan biosynthesis.

transglycosylase (TG) enzymes involved in the process are not well understood. Transglycosylation activity has been shown to be one of the processes found to occur in high molecular-weight bifunctional or multimodular penicillin-binding proteins (PBPs) and has also been identified in lower molecular weight monofunctional transglycosylases (MGTs).

2. Tranglycosylases: enzymatic facts

The multimodular penicillin-binding proteins comprise a large group of proteins that can be divided into class A and class B on the basis of the functional activities of the component domains. Common to all PBPs is a C-terminal domain that encompasses the transpeptidase (TP) or carboxypeptidase activities associated with PBPs. TG activity is found in the N-terminal domain of class A high-molecular-weight penicillin-binding proteins. The majority of class A and class B PBPs have been identified by genome sequence analysis. Goffin and Ghuysen completed a detailed protein sequence analysis of PBPs and demonstrated that class A PBPs had six conserved amino acid motifs in the TG domain [7]. The first extensively characterized PBP having TG and TP activity was PBP1b from *Escherichia coli* [8]. There are more than 30 class A PBPs that have been identified in both Gram-positive and Gram-negative organisms on the basis of genome sequencing [2,7].

Both Gram-positive and Gram-negative bacteria also have monofunctional TG enzymes that show a high degree of similarity to the transglycosylase domains of the class A PBPs [9,10]. MGTs are believed to be membrane-associated proteins that play a key role in peptidoglycan biosynthesis.

Table 1 – Putative class A multimodular PBPs and monofunctional TGs in pathogenic organisms

Organism	Class A PBPs	MGT	References
G+			
S. aureus	1 (PBP2)	1	[1,7,10,15]
S. pneumoniae	3 (PBP1a, PBP2a and PBP1b)	1	[1,7,13]
E. faecalis	3 (*ponA*, *pbpF*, *pbpZ*)	0	[16]
B. subtilis	4 (PBP1, PBP2c, PBP2d, PBP4)	0	[14]
G−			
E. coli	3 (PBP1a, PBP1b, PBP1c)	2–3	[7]
H. influenzae	2 (PBP1a, PBP1b)	1	[7]
N. meningitidis	1 (PBP1)	2–4	[7]

As shown in Table 1, a single bacterial species can contain a number of class A PBPs and MGTs. Biochemical tools for the elucidation of the functions of these enzymes have been notoriously difficult to establish [1]. However, recent developments in assay methods, the evolving understanding of the structures of these enzymes [11] and genetic studies continue to provide insights into the various roles these enzymes have. Genetic studies, where various class A PBPs have been deleted, have shown that whilst *E. coli* will tolerate the loss of either PBP1a or PBP1b, if both are deleted the loss is lethal even though PBP1c and an MGT are present [12]. Similar genetic studies in *Streptococcus pneumoniae* have shown that deletion of PBPs 1a and 2a were lethal despite there being PBP1b and an MGT [13]. It has been suggested in *E. coli* the majority of peptidoglycan elongation is carried out by PBP1b [1] (and references there-in).

There appears to be at least one other class of TG-enzymes that bear no homology in the TG domain to the class A PBPs and MGTs. Biochemical characterization of mutant *Bacillus subtilis* cells lacking all four class A PBPs (no MGTs identified) still produce polymerised peptidoglycan [14]. Similarly, in *Enterococcus faecalis* deletion of all three class A PBPs led to a viable organism that demonstrated high resistance to the common TG-inhibitor moenomycin (vide infra).

3. Transglycosylases as viable antibacterial targets

Inhibition of the transglycosylation process is a validated approach for the discovery of new antibacterials. Vancomycin successfully inhibits bacterial growth by binding to the TG-universal substrate lipid II. Targeting the common substrate has obvious benefits in terms of bypassing the complex, diverse and largely unknown TG-enzyme machinery. On the downside, only one substrate needs to be modified to create resistant strains. Vancomycin binds the D-ala-D-ala dipeptide terminus of lipid II through five hydrogen bonds. In vancomycin-resistant *enterococci* (VRE), D-ala-D-ala is replaced with D-ala-D-Lac and one of these hydrogen bonds is lost resulting in a 1000-fold decrease in vancomycin-binding affinity [17–19]. Vancomycin resistance comes in various forms and is of increasing concern, especially in the hospital environment.

A second way to inhibit the TG process is by direct interaction with the TG domain of several (if not all) class A PBPs and MTGs. This is a challenging approach given the need for targeting multiple TG enzymes and the many uncertainties about their role and distribution across many species.

Moenomycin is the only well-studied prototype example that proves this approach can be successful. This natural product isolated from *Streptomyces* species such as *Streptomyces ghanaensis* and *Streptomyces bambergiensis* [20,21] is highly active against a broad range of Gram-positive organisms and binds directly with high affinity to several isolated class A PBPs. Moenomycin was not developed for human use due to poor pharmacokinetic properties [22]. Moenomycin is used as a growth promoter in animal feed under the Hoechst trademark Flavomycin. Despite its extensive use, no prevalence of acquired resistance against moenomycin was detected in a large collection of *E. faecalis* isolates from farm and pet animals [23], nor in a large collection of *Staphylococcus aureus* isolates from chickens [24]. In addition, moenomycin displays a strong 'plasmid-curing' effect of multiresistant *E. coli* in vitro and in vivo under field conditions, leading to a significant decrease in resistance in intestinal *E. coli* in flavomycin-fed animals [25].

4. Inhibitors of the transglycosylation process

4.1. *Glycopeptides and their hydrophobic derivatives*

For some time vancomycin (Fig. 2) and teicoplanin were the only antibiotics available for the treatment of methicillin-resistant *S. aureus* (MRSA). With the emergence of vancomycin-resistant organisms, new glycopeptide derivatives have been generated, with two in late clinical development [26]. These are summarised in Table 2 and structures are shown in Fig. 2.

Oritavancin, telavancin and dalbavancin are all glycopeptide analogues containing an additional hydrophobic moiety. These hydrophobic *N*-alkylated glycopeptide derivatives [26–32] have potent antibacterial activity against many Gram-positive vancomycin-susceptible and -resistant strains. Oritavancin, the most potent agent against all vancomycin-resistant Gram-positive phenotypes, was in phase III clinical trials, but development of this product has halted recently due to problems encountered in the production process. Dalbavancin is currently in pre-registration for skin and soft tissue infections, but is not as effective against vancomycin-resistant *S. aureus* and Van A-type *enterococci*. Telavancin has been granted fast track approval by the FDA and is in Phase III trials for hospital-acquired pneumonia and complicated skin and skin structure infections.

The fact that these compounds only differ in an additional hydrophobic moiety and yet, at least in the case of oritavancin and televancin, are so successful against vancomycin-resistant bacteria has raised the question of whether these compounds have a different mechanism of action. One suggestion is that oritavancin and dalbavancin have a greater ability to dimerize and that this results in improved binding at the dipeptide termini of lipid II [32]. The hydrophobic chain could also act as a membrane anchor bringing the molecule into closer contact with lipid II. It is proposed that these effects overcome the problem of the D-ala-D-Lac binding resulting in greatly improved activity.

Kahne [22,38–40], and later others [41], put forward the theory that these vancomycin analogues have a second mechanism of action involving direct binding of the compounds to the proteins involved in transglycosylation. Subsequently, these derivatives have been demonstrated to interact directly with *E. coli* PBP1b [42] and to inhibit the tranglycosylation reaction for *S. aureus* PBP2 [43]. After removing an N-terminal leucine from these glycopeptides the 'damaged' glycopeptides lost all ability to bind to lipid II, but nevertheless maintained good antibacterial activity and were shown to directly inhibit the *S. aureus* PBP2 transglycosylation reaction [43].

Telavancin has at least two modes of action, one vancomycin-like, and one involving depolarisation of the bacterial cell membrane [44].

4.2. *Ramoplanin*

Ramoplanin (Fig. 2) is a lipoglycodepsipeptide that is highly active against a variety of Gram-positive bacteria including MRSA and VRE, and is in phase II clinical trials for the treatment of clostridium difficile-associated diarrhea (CDAD). Many articles have been published on ramoplanin [33,34,45] and its mechanism of action has been a topic of discussion for some time. The focus has moved away from the proposed binding of lipid I and inhibition of MurG to the binding of lipid II resulting in inhibition of transglycosylation [33]. Analysis of the lipid II-ramoplanin complex has been difficult since it tends to form insoluble fibrils. Proposals have been put forward suggesting an octapeptide unit is involved in complexation [34,46,47]. Ramoplanin has also been reported to bind to lipid II as a dimer [48] and Walker has shown the stoichiometry of the complex to be 2:1 (ramoplanin:lipid II) [49].

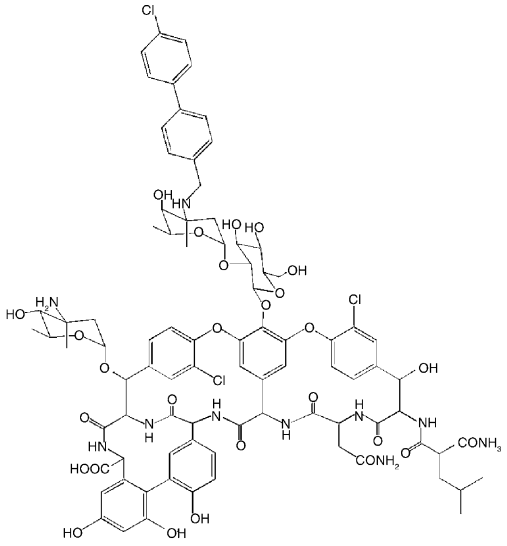
4.3. *Lantibiotics*

The lantibiotics are peptide antibiotics containing the thioether amino acids lanthionine or methyllanthionine. The type A lantibiotics (of which the most intensely studied is Nisin) are elongated, flexible and amphipathic molecules.

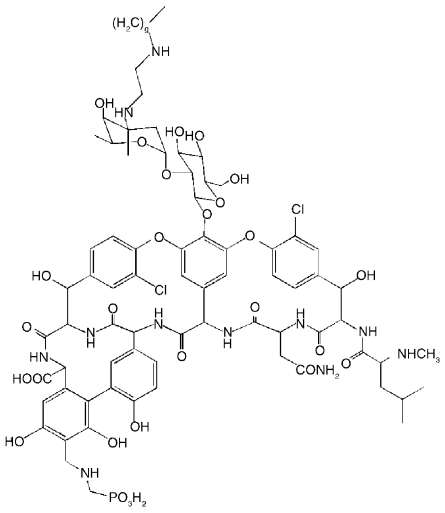
Nisin forms transient pores in model membrane vesicles at micromolar concentrations but causes rapid bacterial cell death at nanomolar MIC's against many strains [50]. Nisin has been shown to use lipid II as a docking molecule in its mode of action. Various experiments [51–53] have led to a much clearer picture of this binding and the ensuing pore formation [54]. The headgroup of lipid II is recognized by the N-terminal portion of nisin. The flexible 'hinge region' of nisin allows a conformational change so that nisin goes from being parallel to perpendicular with respect to the membrane surface. The C-terminus is thus buried in the membrane and the nisin and lipid II continue to associate to form relatively stable pores. It has been proposed that the pore consists of eight nisin and four lipid II molecules [54].

Type B lantibiotics (e.g. Mersacidin, Actagardine) have a more rigid, globular shape than type A and exert their antibiotic effect by inhibiting cell wall biosynthesis. This occurs by the compounds binding to lipid II and so blocking the substrate from binding to the TG enzyme site. Mersacidin (Fig. 2) has been shown to bind lipid II but in a different manner to vancomycin [36,55,56]. This lessens the likelihood of cross-resistance developing.

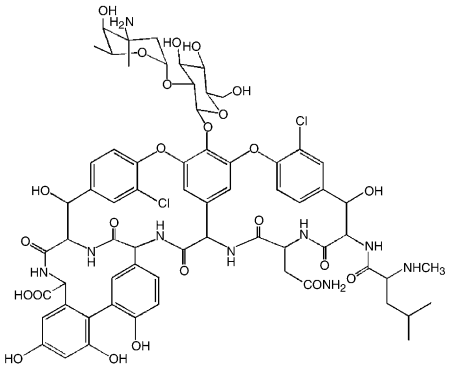
In recent years, a few unusual two-peptide lantibiotics have been discovered such as Lacticin 3147 [57], demonstrat-



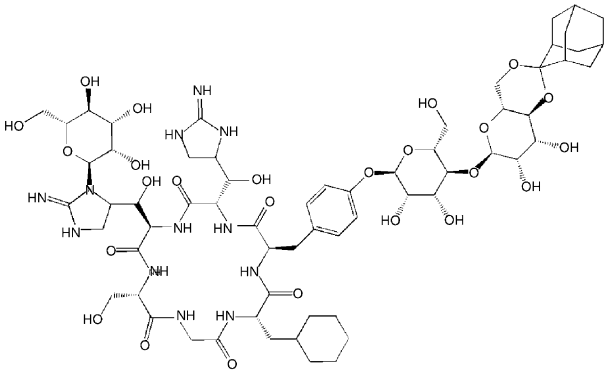
Oritavancin



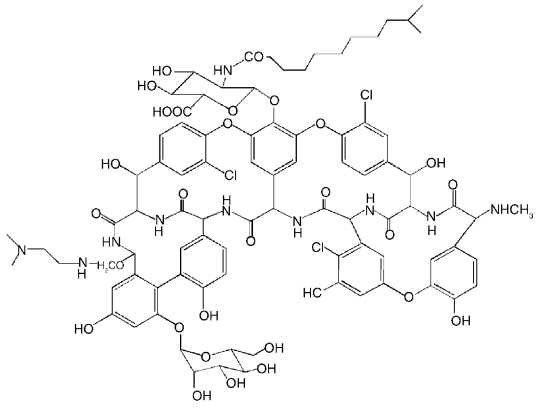
Telavancin



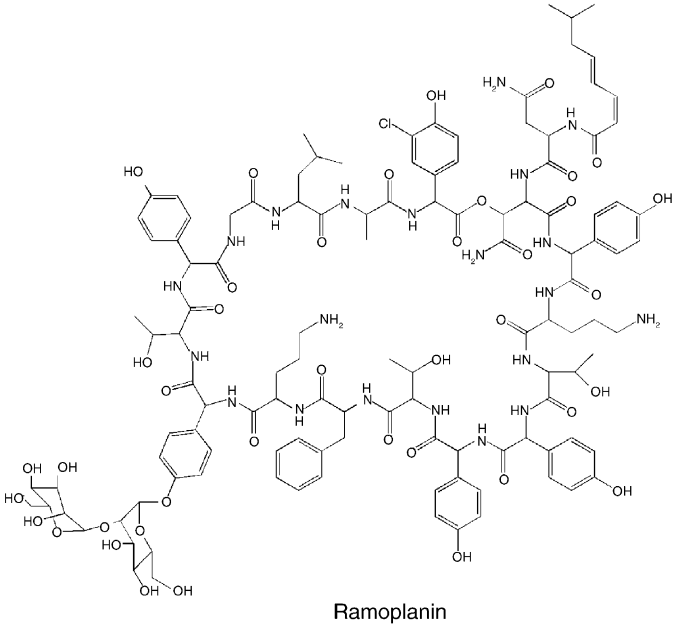
Vancomycin



AC98-6446



(a) Dalbavancin



Ramoplanin

Fig. 2 – Structures for compounds contained in Table 2. Shaded circles represent amino acids conserved between Mersacidin and Actagardine and so are postulated to be responsible for activity.

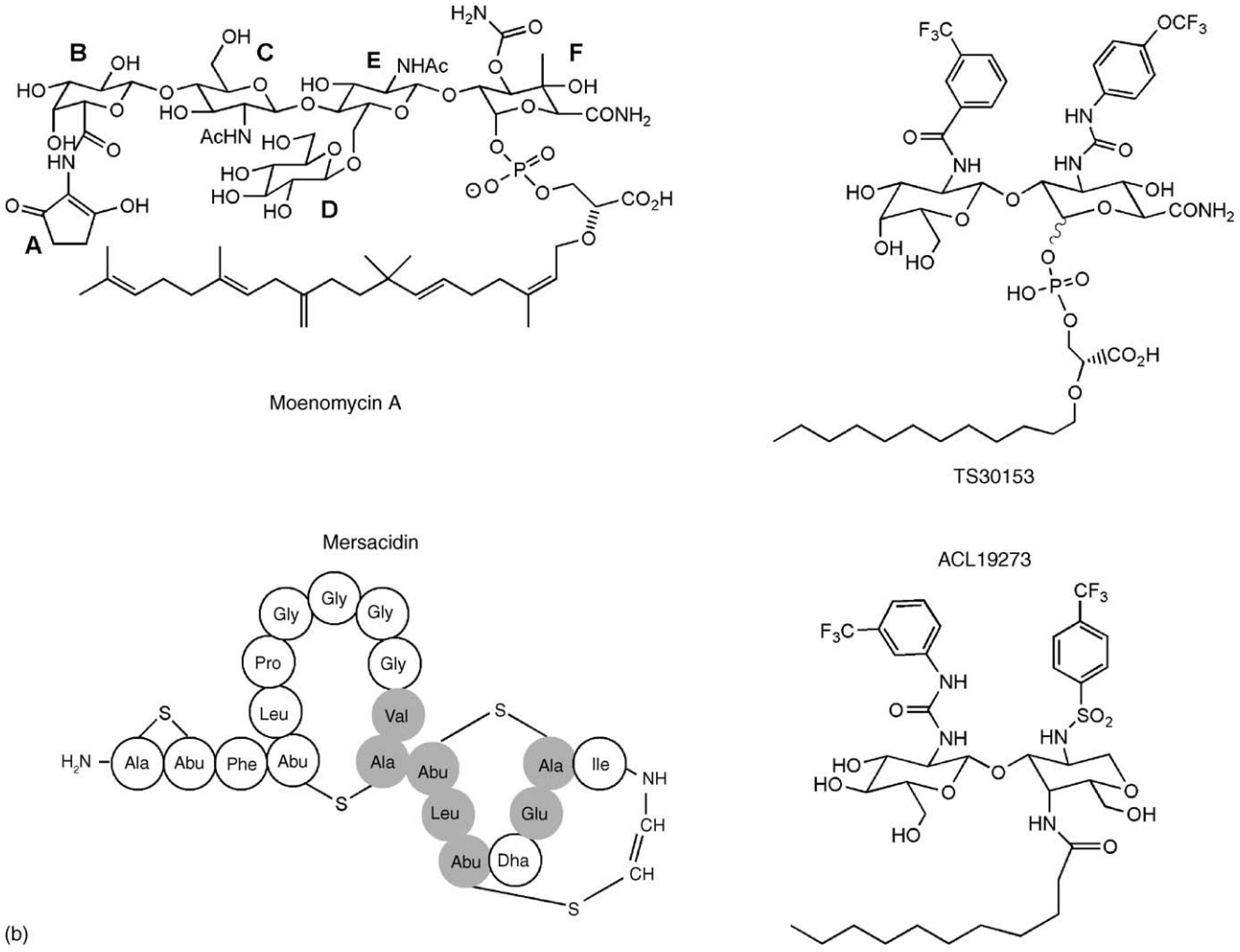


Fig. 2. (Continued).

ing high activity against MRSA and VRE, with a similar dual mode of action to nisin.

4.4. *Mannopeptimycins*

The mannopeptimycins are a novel class of cyclic glycopeptide antibiotics produced by *Streptomyces hygroscopicus* [58]. Their mechanism of action appears to involve binding to lipid II at a site other than the D-ala-D-ala bound by vancomycin [59,60]. The form this complex takes is still unclear [61] but as a result of this substrate binding the TG reaction is inhibited. The original mannopeptimycins displayed reasonable activity against Gram-positive bacteria, including MRSA. A variety of derivatives including esters, carbonates [62], ethers, halogenated compounds [63,64], acetals and ketals [65] have since been synthesised and evaluated for their antibacterial activity. A recent semisynthetic mannopeptimycin, AC98-6446 (Fig. 2), has shown good activity both in vitro [35] and in vivo [66].

4.5. *Moenomycin*

Moenomycin is highly active against a broad range of Gram-positive organisms, but is largely inactive against Gram-negative organisms, probably due to its inability to penetrate the outer bacterial membrane. Moenomycin directly inhibits the TG activity of PBP1b and 1c from *E. coli*, and PBP1a [67], 1b [68] and 2a of *S. pneumoniae* [69]. Moenomycin also inhibits an MTG from *S. aureus* [10].

From genetic modification studies in *E. faecalis*, it appears that deletion of all PBP class A enzymes still leads to viable organisms, which as a result of the deletion have become entirely resistant to moenomycin. This indicates that moenomycin, upon binding to PBP in *E. faecalis*, causes a toxicity signal [16], which, as the authors suggest, may result from poisoning of the polymerisation complexes containing class A PBPs rather than simply inhibiting the TG activity.

4.5.1. *Binding site on the TG-domain*

In 1987, it was proposed, based on structural similarities, that the moenomycins inhibit the transglycosylation reaction by competing with lipid II at the binding site of the TG enzymes [70,71]. Over the last two decades a lot of work has been done on evaluating the structure–activity relationships of the moenomycins both in degradation studies and by synthesis of analogues [70,72–87]. Both trisaccharide (C-E-F) and disaccharide (E-F) degradation products of moenomycin A (Fig. 3) inhibit the in vitro transglycosylation process in *E. coli*, but only the trisaccharide demonstrates significant antibacterial activ-

Table 2 – Summary of inhibitors of the transglycosylation process

Compound	Class	Proposed mode of action	*S. aureus*			*E. faecium*		*E. faecalis*		*S. pneum*	Reference
			MSSA	MRSA	VISA	VSE	VRE	VSE	VRE	PR	
Vancomycin	Glycopeptide	Binds to lipid II	0.13–1.0	0.5–4.0	8.0	0.25–4.0	>128[a]	0.25–4.0	>128	0.25–2.0	[31]
Oritavancin	Semi-synthetic glycopeptide	Dimerization, hydrophobic anchoring and direct TG binding	0.13–1.0	0.13–4.0	1.0–8.0	0.06–0.25	0.06–1.0[a]	0.06–0.25	0.06–1.0	0.002–0.06	[31]
Dalbavancin	Semi-synthetic glycopeptide	Dimerization, hydrophobic anchoring and direct TG binding	0.03–0.5	0.06–1.0	2.0	0.06–0.13	0.5–128[a]	0.06–0.13	0.5–128[a]	0.008–0.13	[31]
Telavancin	Semi-synthetic glycopeptide	Multiple. Lipid II binding and membrane depolarisation	0.5	0.5–1.0	2.0	0.5	4–8[a]	0.5	4–8[a]	–	[29]
Ramoplanin	Glycolipodepsi-peptide	Binds lipids I and II. Fibril formation and TG inhibition	0.5–1.56	<2.0	–	0.1	<1.0	0.1	<1.0	<2.0	[33,34]
AC98-6446	Cyclic glycopeptide	Binds lipid II but not at vancomycin region	0.03–0.06	0.015–0.06	0.015–0.06	0.06–0.25	0.06–0.12	0.06–0.25	0.06–0.12	0.008	[35]
Mersacidin	Lantibiotic (type B)	Binds to lipid II	12.5	12.5	–	25	25	–	–	–	[36]
Moenomycin A	Natural product glycolipid	Direct TG enzyme binding	0.05	0.062	–	>200	0.39–1.56	0.078	0.062	–	[37,38]
TS3O153 (23)	Disaccharide	Direct TG enzyme binding	6.25	6.25	–	6.25	6.25–12.5	6.25	6.25–25.0	–	[22,37]
ACL 19273	Disaccharide	Direct TG enzyme binding	1.0	4.0[b]	–	4.0[b]	4.0[b]	4.0[b]	4.0[b]	–	–

MIC's are in μg/mL. MSSA/MRSA are methicillin-susceptible and methicillin-resistant *S. aureus*, respectively, and VISA is vancomycin intermediate-resistant *S. aureus*. *S. pneum* PR is penicillin-resistant *S. pneumoniae*. VSE/VRE are vancomycin-susceptible/resistant *enterococci*. Telavancin figures are MIC_{90} all others MIC range.

[a] VanA-resistant species.

[b] Based on complete inhibition of bacterial growth for a broad range of Gram-positive organisms at single concentration on agar plate.

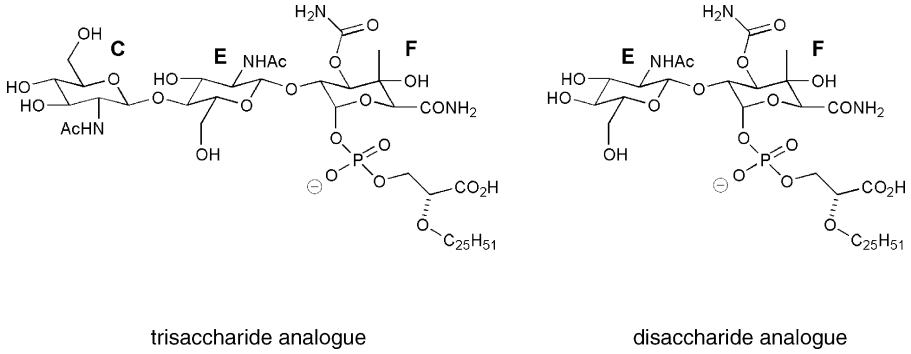


Fig. 3 – Degradation products of moenomycin A.

ity against *S. aureus* [70,72,78]. More recently, it has been observed that moenomycin A does not compete with lipid II for binding to the TG PBP1b in a competitive binding assay [40]. Using simplified models it was found that moenomycin A overlaps well with four sugar units of the growing peptidoglycan [79]. These results indicate that moenomycin A competes with the growing peptidoglycan chain for a larger binding site rather than with the disaccharide unit lipid II.

Welzel et al. have proposed that the different position of the lipid group relative to the second sugar unit in moenomycin A (i.e. 1-lipid, 2-sugar) as compared to the growing peptidoglycan chain (i.e. 1-lipid, 4-sugar), is responsible for the inhibition of the TG enzymes [5]. Structure–activity relationship studies have shown that the minimum requirements for antibacterial activity in vivo are the three rings, C, E and F, which has led to the proposal that these three sugar rings in moenomycin A are involved in binding to the donor-binding site [88] (Fig. 4). In a recent study on *S. pneumoniae* PBP1b [68], moenomycin prevented binding of lipid II to the PBP, suggesting at least that it can interfere with binding of lipid II to the enzyme.

4.5.2. *Moecinol*

The chemical structure of the lipid moiety (moecinol) itself is very important for antibacterial activity [70,73,74,80,81]. Hydrogenation of the lipid is tolerated but any introduction of polar groups into the lipid moiety leads to complete loss of antibacterial activity. It is thought that the lipid moiety of moenomycin A is required for the non-specific binding to the cytoplasmic membrane but recent enzyme-binding studies of delipidated moenomycin A with PBP1b have shown that it is over two orders of magnitude less potent as an inhibitor than moenomycin A [40]. This result indicates that the lipid moiety is also substantially involved in the binding to the enzyme. Decreasing the length of the lipid chain leads to a gradual loss of antibacterial activity until the lipid chain becomes too short and all activity is lost [78]. Esterification of the carboxylic acid group in the lipid or cleavage of the acid group from the lipid chain leads to complete loss of activity [70,80]. It has been proposed that the acid group mimics the second phosphate group of the growing peptidoglycan and binds in the cytoplasmic membrane [80].

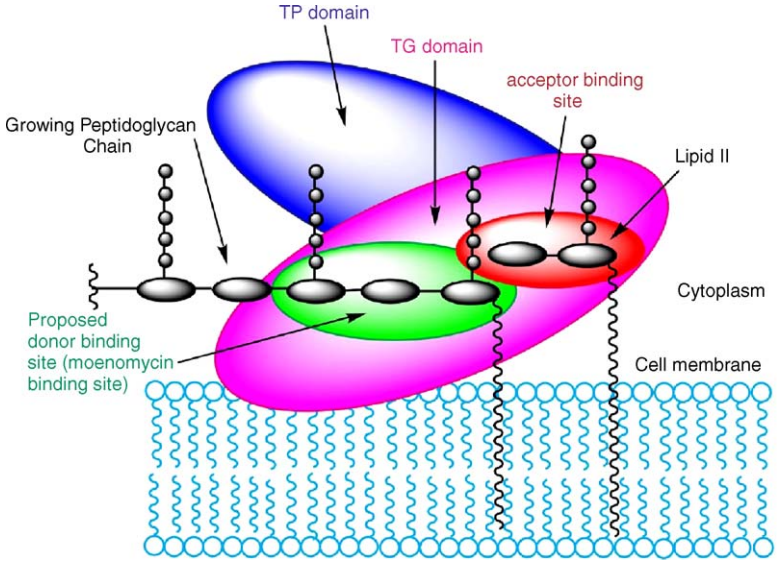


Fig. 4 – Pictorial representation of a class A PBP and one proposed mechanism for the transglycosylation process, in this case involving the growing peptidoglycan chain at the donor site and lipid II at the acceptor site.

The presence of this essential lipid moiety is the likely cause of significantly reduced activity of moenomycin in the presence of serum [89].

4.5.3. *Other moenomycin SAR results*

A 380-fold decrease in antibacterial activity is observed when the carbamoyl group (C3 of ring F) is hydrolysed to give the free hydroxy group [72,75]. For the trisaccharide series of analogues (Fig. 4), it has been shown that the *N*-acetyl group at C2 on the C ring is essential for antibacterial activity [76,78,79].

The following modifications on the disaccharide degradation product have led to a complete loss of TG-activity in *E. coli* [70,77,78]:

- Replacement of the amide ($CONH_2$) at C5 of ring F with a methyl ester (CO_2Me).
- Replacement of the hydroxy group (OH) at C4 of ring F with a hydrogen (H).
- Replacement of the carbamoyl group ($OCONH_2$) at C3 of ring F with a hydroxy group (OH).
- Replacement of the *N*-acetyl group (NHAc) at C2 of ring E with either a free amine (NH_2) or a hydroxy group (OH).

The methyl group (Me) at C4 of ring F was not required for biological activity.

4.6. *Moenomycin-like inhibitors*

Since it was demonstrated that a moenomycin trisaccharide degradation product maintained the full antibacterial activity profile of the parent compound, and that a disaccharide degradation product could still inhibit TG activity in vitro, the challenge has been to synthesise simple analogues of moenomycin with antibacterial activity [70,76,84,87].

Sofia et al. used a solid phase combinatorial approach to synthesise a library of disaccharides based on the moenomycin A disaccharide degradation product [37]. A 1300-member library was prepared and tested for antibacterial activity. Certain criteria such as a substituted urea at the C3 position were shown to be important for activity. Several compounds were discovered with activity against a selection of Gram-positive bacteria in the 3–25 μg/mL range (Table 2 and Fig. 2, TS30153). Importantly, these compounds were shown to have activity against the moenomycin-resistant *Enterococcus faecium* strain. This clearly demonstrated that synthetically simpler analogues of moenomycin could be expected to show activity comparable to that of vancomycin against clinically important bacterial strains. One of these disaccharide products was employed by Kahne and co-workers [90] to link to the vancomycin aglycone, resulting in a 'hybrid glycopeptide' with good activity. A similar approach involved fusing ampicillin onto moenomycin, giving modest TG binding [91].

Attention has also been focused on the synthesis of analogues of the moenomycin trisaccharide degradation product (the smallest fragment that maintains full activity). This has resulted in a body of useful synthetic and SAR information being built up [5,79,92,93]. Interestingly, some modest activity has also been reported on some simple monosaccharides [94] in contrast to earlier attempts [95,96]. This may lead to further investigations into whether or not substituted monosaccharides have sufficient molecular complexity to fulfil the requirements for TG inhibition.

A library of compounds produced by Alchemia was built on a disaccharide scaffold (unpublished results) with each molecule containing essential binding elements from moenomycin and Sofia's active disaccharide. The essential lipid in these molecules is not linked through a phosphate group but rather through an amide functionality. The scaffold and the relative positioning of the substituents on the scaffold were designed to mimic the positioning of equivalent substituents on other ligands; however, the final molecule does not contain labile elements at its reducing end, resulting in excellent metabolic stability. Several hits of this type were identified, with MIC values ranging between 1 and 4 μg/mL (Table 2 and Fig. 2, ACL19273) and they were found to be active against a broad panel of Gram-positive organisms including many clinical isolates of VRE and MRSA. An example molecule of this class demonstrated specific inhibition of the transglycosylation process.

5. Conclusion

It is becoming increasingly evident that new glycopeptides that demonstrate excellent activity against vancomycin-resistant organisms have novel mechanisms of action. Direct binding to the TG enzymes is an effective way of overcoming vancomycin resistance, but produces many challenges for the rational drug designer. Those challenges include lack of understanding of the separate function of the various class A PBPs and MTGs, and their spatial and temporal distribution across species, the apparent need for membrane-binding lipids in the drug, and the lack of structural information. With the development of more accessible lipid II substrate analogues [43,97,98], and high-throughput in vitro screening of TG inhibition [99], more tools are at hand to investigate inhibition profiles of new and existing TG inhibitors. This will provide more practical entry routes for the discovery and development of novel TG inhibitors.

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