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Caprazamycins: Biosynthesis and structure activity relationship studies

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ABSTRACT

Cell wall biosynthesis represents a valid target for antibacterial action but only a limited number of chemical structure classes selectively interact with specific enzymes or protein structures like transporters of the cell envelope. The integral membrane protein MraY translocase is essential for peptidoglycan biosynthesis catalysing the transfer of the peptidoglycan precursor phospho-MurNAc-pentapeptide to the lipid carrier undecaprenyl phosphate, thereby generating the cell wall intermediate lipid I. Not present in eukaryotic cells, MraY is a member of the superfamily of yet not well-understood integral membrane enzymes which involve proteins for bacterial lipopolysaccharide and teichoic acid or eukaryotic *N*-linked saccharides biosynthesis. Different natural nucleoside antibiotics as inhibitors of MraY translocase have been discovered comprising a glycosylated heterocyclic pyrimidin base among other potential lipid-, peptidic- or sugar moieties. Caprazamycins are liponucleoside antibiotics isolated from *Streptomyces* sp. MK730-62F2. They possess activity *in vitro* against Gram-positive bacteria, in particular against the genus *Mycobacterium* including *M. intracellulare*, *M. avium* and *M. tuberculosis*. Structural elucidation revealed the (+)-caprazol core skeleton as a unique moiety, the caprazamycins share with other MraY inhibitors such as the liposidomycins, A-90289 and the muraminomicins. They also share structural features such as uridyl-, aminoribosyl- and fatty acyl-moieties with other MraY translocase inhibitors like FR-900493 and the muraymycins. Intensive studies on their biosynthesis during the last decade identified not only common initial biosynthetic steps, but also revealed possible branching points towards individual biosynthesis of the respective compound. Structural diversity of caprazamycins was generated by feeding experiments, genetic engineering of the biosynthetic gene clusters and chemical synthesis for structure activity relationship studies with its target, MraY translocase.

1. Introduction

The first step in the membrane cycle of reactions during peptidoglycan biosynthesis is the transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to undecaprenyl phosphate, catalysed by the integral membrane protein phospho-MurNAc-pentapeptide translocase also referred to as MraY translocase. MraY translocase consists of ten transmembrane α-helices. In 2013, the first X-ray structure from a MraY homologue from the extremophile *Aquifex aeolicus* was reported, which allowed visualization of the overall architecture and localization of the Mg^{2+} ion within the active site, thereby providing a structural basis for studies of catalysis by this class of enzymes (Chung et al., 2013). MraY crystallized as a dimer with the center of the dimer interface as an oval-shaped tunnel. There are three strictly conserved aspartic acid residues, which function as active site nucleophile, and each of them is found on cytoplasmic loops. Replacement of each of the Asp residues by Asn lead to a reduced enzyme activity (Al-Dabbagh et al., 2008; Lloyd et al., 2004; Struve and Neuhaus, 1965). It has been proposed that Asp-117 and Asp-118 may form a binding site for Mg^{2+}, which is required for translocase I activity, suggesting that Asp-265 may represent the active site nucleophile, but other residues have also been found to be important for activity in previous studies (Al-Dabbagh et al., 2008). In 2016, a refined X-ray structure of MraY was published at 2.95 Å, but this time in complex with the known MraY inhibitor muraymycin D2 (Chung et al., 2016). Interestingly, muraymycin D2 does not interact with any of the aforementioned aspartic

Abbreviations: 3-MG, 3-methylglutaryl; ADR, 5-amino-5-deoxyribofuranose; CPZs, caprazamycins; GlcNAc, 2-*N*-acetylglucosamine; GlyU, 6′-*N*-alkyl-5′-*C*-glycyluridine; LPMs, liposidomycins; MRMs, muraymycins; MRSA, methicillin-resistant *Staphylococcus aureus*; MurNAc, 2-*N*-acetylmuramic acid; PAP, 3′-phosphoadenosine-5′-phosphate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PGN, peptidoglycan; PLP, pyridoxalphosphate; SAR, structure activity relationship; UMP, uridine monophosphate; VRE, vancomycin-resistant Enterococci

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acid residues; instead MraY undergoes conformational changes upon ligand binding to form a pocket that binds the uracil base and the 5´-*O*-aminoribosyl moiety of muraymycin D2 (Chung et al., 2016; Hering et al., 2018; Koppermann et al., 2018; Koppermann and Ducho, 2016).

Moreover, the crystal structure of MraY helped in the identification of a novel inhibition site in MraY translocase based on the already known site of interaction with lysis protein E from bacteriophage ϕX174 (Rodolis et al., 2014b). Lysis protein E has been described as MraY inhibitor in 2001 (Bernhardt et al., 2001), however, the exact interaction has only recently been discovered (Bugg et al., 2016). Construction of a helical wheel model for transmembrane helix 9 of MraY and the transmembrane domain of protein E enabled the identification of an Arg-Trp-x-x-Trp (RWxxW) motif in protein E that might interact with the periplasmic Phe288 of MraY and the neighbouring Glu287 (Bugg et al., 2016). This RWxxW-motif is also found in several cationic antimicrobial peptide sequences like the uridylpeptide antibiotics pacidamycins and muraymycins (Rodolis et al., 2014a). Members of this class of antibiotics showed reduced levels of inhibition to F288 L or E287A mutant MraY enzymes, implying that they interact at this extracellular site as part of the enzyme inhibition mechanism. In contrast, the caprazamycins seem not to interact at this extracellular binding site.

Caprazamycins (CPZs (**1**)) are liponucleoside antibiotics isolated from *Streptomyces* sp. MK730-62F2. They possess activity *in vitro* against Gram-positive bacteria, in particular against the genus *Mycobacterium* including *M. intracellulare*, *M. avium*, *M. tuberculosis* and *M. phlei* (MIC: 1.6 μg mL^{-1}) (Igarashi et al., 2005). Structural elucidation revealed a complex and unique composition of moieties the CPZs share with the liposidomycins (LPMs) (**2**), A-90289 (**3**), muraminomicins (**4**) and muraymycins (**5**) (Fig. 1).

The core skeleton is the (+)-caprazol composed of an *N*-alkylated α-5´-(*β*-1-*O*-aminoribosyl)-uridinyl-glycine which is cyclized to form a rare diazepanone ring. Attached to the 3´´´-OH are *ß*-hydroxy-fatty acids of different chain length and constitution resulting in CPZs A – G (**1**). They differ from the LPMs (**2**) by the presence of a permethylated L-rhamnose glycosidically linked to the 3-methylglutaryl (3-MG) moiety. Structure-activity studies on the liposidomycin family have shown that the nature of the fatty acyl chains strongly influences antimicrobial activity and that the presence of the 3-MG moiety improves activity (Kimura et al., 1998). Aventis groups have reported that LMP-analogues containing the aminoribofuranose monosaccharide attached to the 5´-position of uridine all show good levels of in vitro MraY inhibition (IC_{50} 5–50 μM) (Dini et al., 2000), but variable antimicrobial activity in vivo (Dini et al., 2002).

2. Biosynthesis of caprazamycins

All corresponding biosynthetic gene clusters of known MraY inhibitors have been cloned, sequenced and most of them have in addition been confirmed by heterologous expression in suitable Streptomycetes hosts (Chi et al., 2013; Funabashi et al., 2010; Kaysser et al., 2009, 2010b). The initial enzymatic steps towards the disaccharide core (**11**), a 5-amino-5-deoxyribofuranose (ADR) attached to the 6´-*N*-alkyl-5´-*C*-glycyluridine (GlyU) through *ß*-*O*-glycosidic bond have been elucidated by the Van Lanen group in great detail (Cui et al., 2018a). Notably, homologues of all six genes involved in the initial steps are present in the caprazamycin (**1**), liposidomycin (**2**), muraymycin (**5**), muraminomicin (**4**), sphaerimicin and A-90289 (**3**) biosynthetic gene clusters pointing to a common biosynthesis of the disaccharide core structure in all liponucleoside antibiotics (Cheng et al., 2011; Chi et al., 2013; Funabashi et al., 2010, 2013; Kaysser et al., 2009, 2010b). The biosynthetic mechanisms for ADR and GlyU were originally defined using the enzymes LipK-P from A-90289 (**3**) biosynthesis (Barnard-Britson et al., 2012; Chi et al., 2011; Funabashi et al., 2010; Yang et al., 2011). Recently, the homologue enzymes Mur16-20 and Mur26 in muraymycin (**5**) biosynthesis were also characterized by the Van Lanen group (Cui et al., 2018a). The late biosynthesis steps of e.g., acylation or sulfation however were elucidated using enzymes from caprazamycin (**1**) biosynthesis (Gust et al., 2013). For better understanding, only caprazamycin protein names are mentioned within the following text illustrating the common ground for biosynthesis of ADR and GlyU in all known liponucleoside antibiotics (Fig. 2).

The first step is the conversion of UMP to uridine-5´-aldehyde catalysed by the non-heme, Fe(II)-dependent dioxygenase Cpz15 (Goswami et al., 2017; Yang et al., 2011). Cpz15 represents an α-ketoglutarate-dependent dioxygenase catalysing an oxidative dephosphorylation via stereospecific 5´-hydroxylation of UMP to yield uridine-5´-aldehyde (**6**). Uridine-5´-aldehyde serves as precursor for both, ADR and GlyU. Regarding GlyU biosynthesis, uridine-5´-aldehyde subsequently undergoes an aldol-type reaction with glycine to generate the nonproteinogenic *ß*-hydroxy amino acid 5´*S*,6´*S*-GlyU (**7**) by the PLP-dependent aldolase Cpz14 (Barnard-Britson et al., 2012). Biosynthesis of the amino-ribosyl moiety ADR is initiated by action of the aminotransferase Cpz18. Cpz18 represents a distinct PLP-dependent enzyme catalysing the transamination of uridine-5´-aldehyde to 5´-amino-5´-deoxyuridine (**8**) using L-methionine as the amino group donor (Chi et al., 2011). The next step involves the nucleoside phosphorylase Cpz19 to generate uracil and 5-amino-5-deoxy-α-D-ribose-1-phosphate (**9**) which is further activated to UDP-5-amino-5-deoxyribose

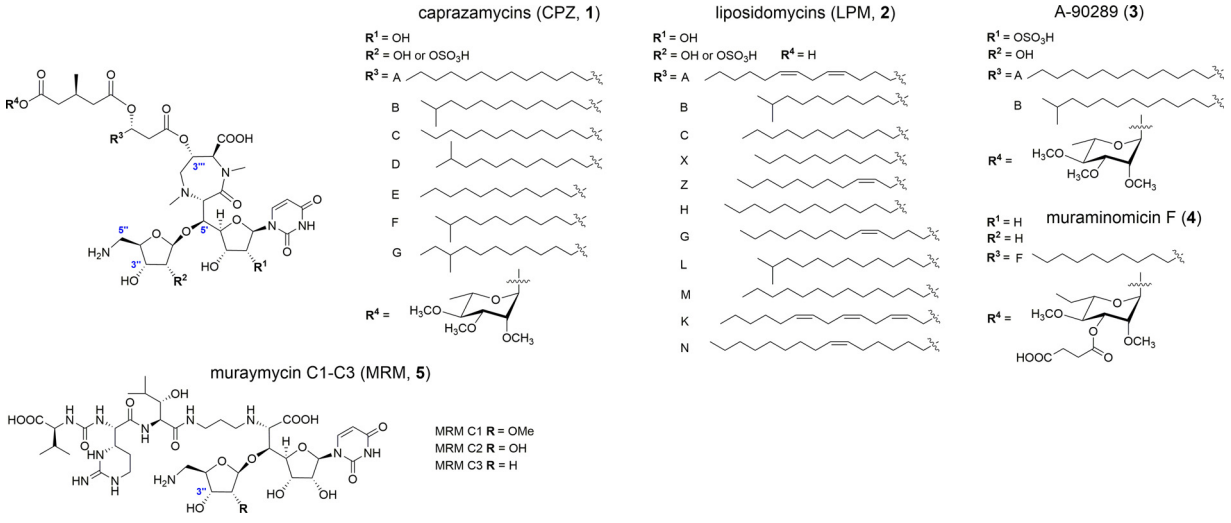

Fig. 1. Chemical structures of liponucleoside MraY inhibitors (1–5).

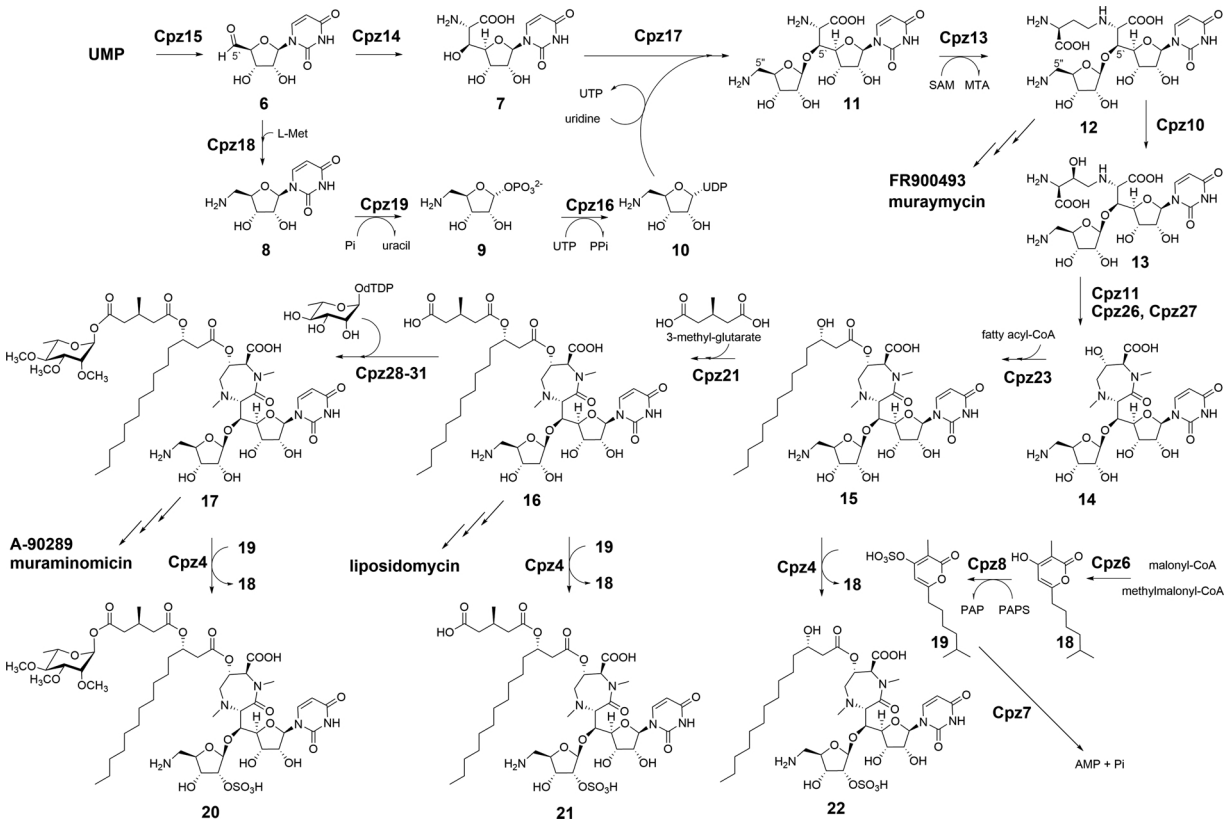

Fig. 2. Caprazamycin biosynthesis. The early biosynthetic steps towards ADR-GlyU (**11**) were elucidated with enzymes from A-90289 (**3**) and muraymycin (**5**) biosynthesis: LipL, Mur16 (orthologue Cpz15); LipK, Mur17 (orthologue Cpz14); LipN, Mur19 (orthologue Cpz17); LipO, Mur20 (orthologue Cpz18); LipP, Mur26 (orthologue Cpz19); LipM, Mur18 (orthologue Cpz16); L-Met = L-methionine; Pi = phosphate; PPi = pyrophosphate; UTP = uridine triphosphate; UDP = uridine diphosphate; CoA = coenzyme A; SAM = S-adenosyl-methionine; MTA = methylthioadenosine; dTDP = deoxy-thymidine-5'-diphosphate.

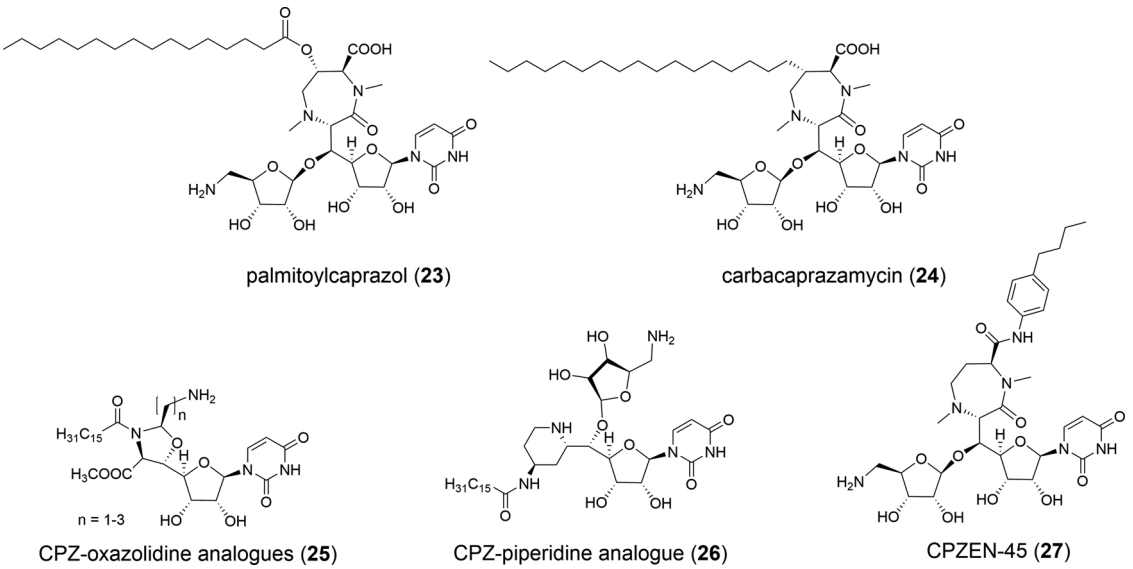

Fig. 3. Structure of caprazamycin analogues obtained by chemical synthesis.

(**10**) catalysed by the nucleotidyl-transferase Cpz16. The final transfer of ADR onto GlyU to form the disaccharide core structure of liponucleoside antibiotics **11** has not been characterized so far biochemically. Van Lanen and colleagues have demonstrated that the Cpz17 homologue LipN from A-90289 (**3**) biosynthesis, encoding for a glycosyltransferase, is capable to form a *ß-O*-glycosidic bond of an unnatural disaccharide using uridine as a surrogate sugar acceptor (Chi et al., 2011). Recently, a six-enzyme reaction (Mur16-20 + Mur26) was published showing the successful formation of the ADR-GlyU disaccharide, the common precursor of all known liponucleoside antibiotics (Cui et al., 2018a).

For caprazamycins (**1**), liposidomycins (**2**), A-90289 s (**3**), muraymycins (**5**) and muraminomicins (**4**), biosynthesis would continue with a transfer of a 3-amino-3-carboxypropyl group from S-adenosylmethionine to the 5′-amino group of the disaccharide ADR-GlyU by the aminotransferase Cpz13 resulting in **12**. Although this step is

speculative at this time, a similar reaction is known to occur in nocardicin biosynthesis (Gunsior et al., 2004). Hydroxylation of the 3-amino-3-carboxypropyl group could be catalysed by the hydroxylase Cpz10 resulting in **13**. Subsequent steps, including cyclisation, N-methylation of the diazepanone ring and attachment of the fatty acid are speculative at present. N-methylation by Cpz11 and/or Cpz26 and subsequent cyclisation by amide bond formation between the carboxyl group and the secondary amino group would immediately result in the characteristic (+)-caprazol (**14**). The putative kinase Cpz27 may be involved in activation of the carboxyl group for this reaction. Cpz23 is a possible candidate for the attachment of the *ß*-hydroxy fatty acids, resulting in the formation of the *ß*-hydroxyacylcaprazols (**15**). Attachment of the 3-methylglutaryl moiety is catalysed by the carboxyesterase Cpz21 to generate the caprazamycin aglycones (**16**). Heterologous expression of a Δ*cpz21* containing caprazamycin gene cluster in *Streptomyces coelicolor* M1154 resulted in the accumulation of *ß*-hydroxyacylcaprazols (**15**). Interestingly, these simplified caprazamycin structures still maintained their biological activity against *Mycobacterium phlei* (Kaysser et al., 2009). Since caprazamycins contain a permethylated L-rhamnose moiety, genes for the formation of L-rhamnose are required in the caprazamycin producer strain *Streptomyces* sp. MK730-62F2. Formation of L-rhamnose involves four genes, a dTDP-glucose synthase, a dTDP-glucose dehydratase, an 4-ketoreductase and a 3,5-epimerase, respectively, encoded by *cpzDII*, *cpzDIII*, *cpzDVI* and *cpzDIV* outside of the gene cluster (Kaysser et al., 2010c). Gene deletion of *cpzDIII* in the native producer confirmed the involvement of the L-rhamnose subcluster in caprazamycin biosynthesis. The cluster encoded rhamnosyltransferase Cpz31 is responsible for the attachment of L-rhamnose to the caprazamycin aglycone (**16**) (Kaysser et al., 2010c) and three putative *O*-methyltransferases, Cpz28-30, are candidates for sequential methylation towards caprazamycins (**17**).

Approximately 20% of caprazamycins are sulfated analogues (Tang et al., 2013). Investigations on Cpz4, Cpz6, Cpz7 and Cpz8 demonstrated a novel mechanism to be responsible for caprazamycin sulfation (Tang et al., 2013). The type-III polyketide synthase Cpz6 is employed in the biosynthesis of a group of novel triketide α-pyrones (presulficidins) (**18**) that are subsequently sulfated by the 3′-phosphoadenosine-5′-phosphosulfate (PAPS)-dependent sulfotransferase Cpz8. The resulting sulficidins (**19**) then serve as sulfate donors for the PAPS-independent arylsulfate sulfotransferase Cpz4 to generate sulfated caprazamycins (**20**), sulfated caprazamycin aglycones (**21**) and sulfated hydroxyacylcaprazols (**22**). Cpz4 therefore seems to accept a variety of different substrates, however, biosynthetic intermediates lacking either the uridyl-, aminoribosyl- or fatty acyl-moieties were not accepted (Kaysser et al., 2010a). Since PAP has been demonstrated to inhibit PAPS-dependent sulfotransferases, Cpz7 acts as a 3′-phosphoadenosine-5′-phosphatase, converting PAP to AMP and thereby modulating the intracellular concentration of PAP (Tang et al., 2013).

3. Caprazamycin regulation, transport and resistance

Cpz9, a putative regulator of the AraC family is the only regulator found in the caprazamycin (**1**) biosynthetic gene cluster. Members of this family are most commonly positive transcriptional activators, also known from sugar degradation and other pathways. For the muraymycins (**5**), a novel negative regulator, Mur34 was investigated in more detail (Xu et al., 2013). *mur34* encodes for a protein of 158 amino acids which shows significant homologies to LivI (74% identities), RacA (76% identities) and KanI (74% identities) involved in the biosynthesis or regulation of lividomycin, ribostamycin and kanamycin, respectively. However, their exact function remains unclear. Mur34 belongs to an all-alpha protein class and λ repressor-like DNA-binding domains superfamily. Deletion of *mur34* resulted in a ca. 30-fold higher expression of some randomly chosen biosynthetic genes and because of this, in an approximately 10-fold increased muraymycin (**5**) production. Interestingly, homologues of Mur34 can be found in other gene clusters of MraY inhibitors comprising uridylpeptide antibiotics such as napsamycins, pacidamycins and sansanmycins. The predicted gene product of *cpz22* shows homology to ABC-transporters. Similar proteins can be found in many antibiotic gene clusters and are usually involved in self-resistance. Another self-resistance mechanism could involve Cpz12 and Cpz27, two putative aminoglycoside phosphotransferases similar to the tunicamycin resistance proteins, e.g. TmrD from *Deinococcus radiodurans*, which structure has been reported (Kapp et al., 2008). The 2′-, 3′- and 5′-hydroxy groups of the uridine have been suggested as potential targets for phosphorylation by TmrD resulting in the inactivation of tunicamycin. For a similar protein, CapP from the capuramycin-related gene cluster encoding for the biosynthesis of A-503083 s, a region-specific transfer of the gamma-phosphate to the 3′′-hydroxyl of the unsaturated hexuronic acid moiety of A-503083 B was demonstrated (Yang et al., 2010). Recently, a self-resistance mechanism has also been described for the muraymycins (**5**) (Cui et al., 2018b) involving the phosphotransferase Mur28 and the nucleotidyltransferase Mur29. Biochemical characterization surprisingly revealed that both enzymes preferred the same 3′′-hydroxy substituent of the aminoribose. While Mur28 preferentially phosphorylates this site, Mur29 adenylates instead. Both modifications lead to a drastic reduced bioactivity of muraymycins indicating that both self-resistance mechanisms may work complementary with a distinct temporal frame or during muraymycin biosynthesis.

4. Structure activity relationship studies of caprazamycins with MraY translocase

Liponucleosides of type **1**-**5** have been shown to inhibit the biosynthesis of the bacterial cell wall by targeting the formation of lipid I catalysed by MraY translocase. Earlier investigations indicated that the 3′′-OH group (Fig. 1), the amino group of the ADR-GlyU and an intact uracil moiety are essential for the inhibition of the *Escherichia coli* MraY translocase (Kimura et al., 1998). In 2005, total synthesis of the (+)-caprazol was first accomplished by Ichikawa and colleagues (Hirano et al., 2005, 2007), however, this compound only showed weak antibacterial activity. In contrast, the acylated compounds exhibit strong growth inhibition of mycobacteria, suggesting a potential role of the fatty acid side chain in penetration of the bacterial cell (Hirano et al., 2008b). To define the pharmacophore of caprazamycins (**1**), simplified analogues were synthesized, e.g. palmitoylcaprazol (**23**) and carbacaprazamycin (**24**) (Fig. 3).

Both compounds, **23** and **24** exhibited antibacterial activity against *Mycobacterium smegmatis* ATCC607 (MIC 6.25 μg mL^{-1}) with a potency like that of natural CPZs (**1**) (Hirano et al., 2008c; Ichikawa et al., 2015). Therefore, simplification of the fatty acyl side chain lacking substituents was tolerated. Further truncated analogues lacking either the aminoribose, the uridine or both moieties were inactive demonstrating that the diazepanone ring itself might not be essential for biological activity, however, it plays an important role presumably as scaffold to spatial orientate the attached aminoribosyluridine and fatty acyl side chains for efficient interaction with MraY translocase (Ichikawa, 2016). In order to replace the diazepanone ring with structures easier to synthesize, oxazolidine (**25**), isoxazolidine and lactam-fused isoxazolidine-containing uridine derivatives were generated (Ii et al., 2010; Yamaguchi et al., 2015). These compounds showed moderate biological activities (MIC 8–16 μg mL^{-1}) not reaching the same level of MraY inhibition as natural CPZs (**1**). A similar goal was pursued by using a piperidine as a scaffold linking the crucial structural units of caprazamycins (Hirano et al., 2008a; Nakaya et al., 2015). Among these compounds, only one analogue (**26**) exhibited good MraY inhibition and bioactivity against Gram-positive bacteria such as MRSA and VRE.

Another interesting compound is CPZEN-45 (**27**). Acidic treatment of caprazamycins (**1**) results in the deacylation and therefore accumulation of the bio-inactive (+)-caprazol (**14**) in high yields. The carboxyl

group of (+)-caprazol was targeted to introduce different side chains to generate alkylamide-type and anilide-type compounds. Ester derivatives were obtained in addition by condensing (+)-caprazol with various alcohols. The anilide derivative CPZEN-45 (**27**) showed best activity against *M. tuberculosis* H37Rv (MIC 1.56 μg mL^{-1}) while maintaining minimal hemolytic effects (Hanif et al., 2014; Nakamura et al., 2016). The biological activity was even superior to that the natural caprazamycin B and it showed excellent therapeutic efficacy in a murine tuberculosis model infected with an extensively drug-resistant *M. tuberculosis* strain (Salomon et al., 2013). Furthermore, the mode of action has been demonstrated to be different from the one of caprazamycins (**1**). Unlike caprazamycins, CPZEN-45 strongly inhibits caprazamycin-resistant strains including those overexpressing MraY. Analysis of a spontaneous CPZEN-45 resistant *Bacillus subtilis* strain identified a mutation in the gene *tagO*, suggesting TagO is the primary target of CPZEN-45. The undecaprenyl-phosphate-GlcNAc-1-phosphate transferase TagO is involved in teichoic acid biosynthesis. CPZEN-45 also inhibits the TagO orthologue WecA of *M. tuberculosis* involved in the biosynthesis of the mucolylarabinogalactan of the cell wall (Ishizaki et al., 2013). Hitherto, no insights into the molecular binding sites of CPZs to TagO exist. In the future, detailed structural knowledge of the mode of action of liponucleosides to different protein targets can uncover the high innovative potential of the particular spatial arrangement of nucleobase-, sugar- and lipid moieties attached to heterocycles - namely deduced from the diazepanone in CPZs - towards novel antibacterial structural classes for anti-infective drug development. The caprazamycin core skeleton therefore represents a formidable precursor to design novel anti-infectives for the treatment of mycobacterial diseases.

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