# DD-Ligases as a Potential Target for Antibiotics: Past, Present and Future

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**Abstract:** DD-ligases catalyze the synthesis of the D-Ala-D-Ala and D-Ala-D-Ser dipeptides or the D-Ala-D-Lac depsipeptide in an early step of peptidoglycan synthesis. Their function is essential for bacterial growth and specific to bacteria, making them attractive targets for the development of novel antibiotics. This review examines the biochemical and structural features of these enzymes and presents the main families of inhibitors described so far. Over the last 20 years, 7 structures of DD-ligases have been solved by X-ray crystallography, giving a detailed view of the general topology of the active site and of the residues in the catalytic pocket that play a central role in substrate recognition. This has paved the way to the rational design of inhibitors, which can be classified as (i) analogues of substrates, (ii) analogues of the product of the reaction, (iii) analogues of the transition state, and (iv) original scaffolds discovered by screening or by rational computer-aided design. The three first strategies have led to molecules that are polar by nature and have therefore poor access to their cytosolic target. The fourth one is potentially most promising as it yields more diverse structures. The most active molecules show affinity constants in the μM range, but microbiological evaluation remains scarce (typical MIC 1-8 mg/L for the tested compounds). These data strongly suggest targeting DD-ligases is a promising approach for discovery of new antibiotics. Future research should, however, aim at finding more potent inhibitors endowed with the appropriate pharmacokinetic properties that ensure access to their intracellular target.

**Keywords:** DD-ligase, antibiotic, peptidoglycan, D-cycloserine, phosphinophosphate.

## INTRODUCTION

Bacterial resistance to current antibiotics is an increasingly worrying issue affecting both the hospital and community settings [1-3], with the emergence of multidrug resistant phenotypes in many bacterial species of clinical importance [4-6], and an upsurge of variety of new resistance mechanisms [7-9]. Searching for molecules acting on still unexploited or ill-exploited targets represents, therefore, an important strategy for maintaining our capacity to effectively fight bacteria and avoiding returning to the pre-antibiotic era [4,10-12].

The cell wall has for long been considered as a privileged target for antibiotics, as it meets the criteria of essentiality for bacterial survival and of specificity to prokaryotic cells. Its main constituent, the peptidoglycan, is already the target of two major therapeutic classes in our current arsenal, namely the β-lactams and the glycopeptides, which inhibit the late stages of its biosynthetic pathway. Acquisition of resistance to these two classes of antibiotics has revealed the high flexibility in this assembly pathway [13], and the need to target other enzymes. Many of them acting on earlier steps of peptidoglycan synthesis are not or only poorly exploited so far [14-17].

Among them, D-alanyl-D-alanine ligases are of particular interest because they use substrates (D-amino acids) that are specific for the bacterial world and are essential for bacterial growth [18]. Closely related enzymes using alternative substrates (D-lactate or D-serine) are found in vancomycin-resistant Gram-positive bacteria [7,19,20]. These originate probably from non-pathogenic bacteria, including glycopeptide producers [21-23]. Resistance to vancomycin was first described in *Enterococcus* species (VRE: Vancomycin Resistant Enterococci) in the 1980's [24]. It rapidly spread and constitutes today a major problem in hospitals of countries having made a large use of glycopeptides such as the US [25,26]. More recently, a few cases of vancomycin-resistant *Staphylococcus aureus* (VRSA) isolates have been described in the US [27,28]. These organisms, which are also resistant to β-lactams, seem to have acquired from enterococci the plasmid bearing the resistance genes to vancomycin. They show high levels of vancomycin resistance [7,29]. This is in contrast to the so-called vancomycin-intermediate *Staphylococcus aureus* (VISA; also referred to as GISA as they tend to also show resistance to teicoplanin, another glycopeptide used in several countries but not in the US) that show low level of resistance due to an increase in the natural target of vancomycin [7,30].

Finding inhibitors of the D-alanyl - D-alanine ligases and related enzymes would therefore be of prime interest. The present paper reviews our current knowledge on these enzymes in terms of mode of action and structural features, and describes the inhibitors that have been designed based on this knowledge.

## DD-LIGASE ROLE IN PEPTIDOGLYCAN SYNTHESIS

The cell wall ensures structural integrity of bacteria by conferring resistance to osmotic pressure and environmental stress, and by maintaining their shape during cell division and elongation. It also plays a role of selective barrier to the access of foreign substances to the bacterial cytoplasmic membrane [31-34]. Its main constituent, the peptidoglycan (also called murein), is a polymer of long glycan chains cross-linked by peptide bridges. Glycan chain is composed

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of alternating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) linked by β1→4 bonds. The carboxyl group of each N-acetyl-muramic acid is substituted by a peptide subunit with the following sequence (in most cases): L-alanine, D-glutamate, *meso*-diaminopimelate, D-alanine and D-alanine. The last D-alanine is lost during the synthesis of the peptidoglycan upon peptidic cross-linkage catalyzed by transpeptidases [35,36]. The chemical structure of the peptidoglycan is very similar in Gram-positive and Gram-negative bacteria, with however some variations in the peptidic chain (for example the replacement of the *meso*-diaminopimelate by a L-lysine in several Gram-positive bacteria [33,37]. The cell wall is much thicker in Gram-positive bacteria due to the apposition of more linked layers.

The synthesis of peptidoglycan can be divided into 3 successive stages consisting each of a series of reactions occurring at different locations in the bacteria (Fig. (**1**)) [38]. The first one takes place in the cytoplasm and leads to the formation of an activated form of a monomeric glycopepentapeptidic unit (UDP-MurNAc-pentapeptide). The second one consists in the attachment of the MurNAc-pentapeptide to a lipid carrier (undecapenoylphosphate) at the inner face of the membrane, the addition of an N-acetylglucosamine, and the translocation of the resulting disaccharide-(peptide)-pyrophosphate undecaprenoyl-carrier to the external face of the membrane. The third stage takes place at the outer side of the cytoplasmic membrane and consists in the integration of new monomer units in the mature peptidoglycan by transpeptidation and transglycosylation reactions. These latter reactions are inhibited by glycopeptides and β-lactams (the latter bind covalently to the corresponding enzymes, hence their name of penicillin-binding proteins [PBPs]).

DD-ligases act at a critical step in the first stage of this whole biosynthetic pathway. They catalyze the formation of a dipeptide of D-amino acids (D-alanyl – D-alanine in most cases) that will occupy the terminal position of the cytosolic UDP-MurNAc-pentapeptide precursor units. Both the substrate and the product of reaction are key molecules at the intersection between the anabolic and catabolic pathways of

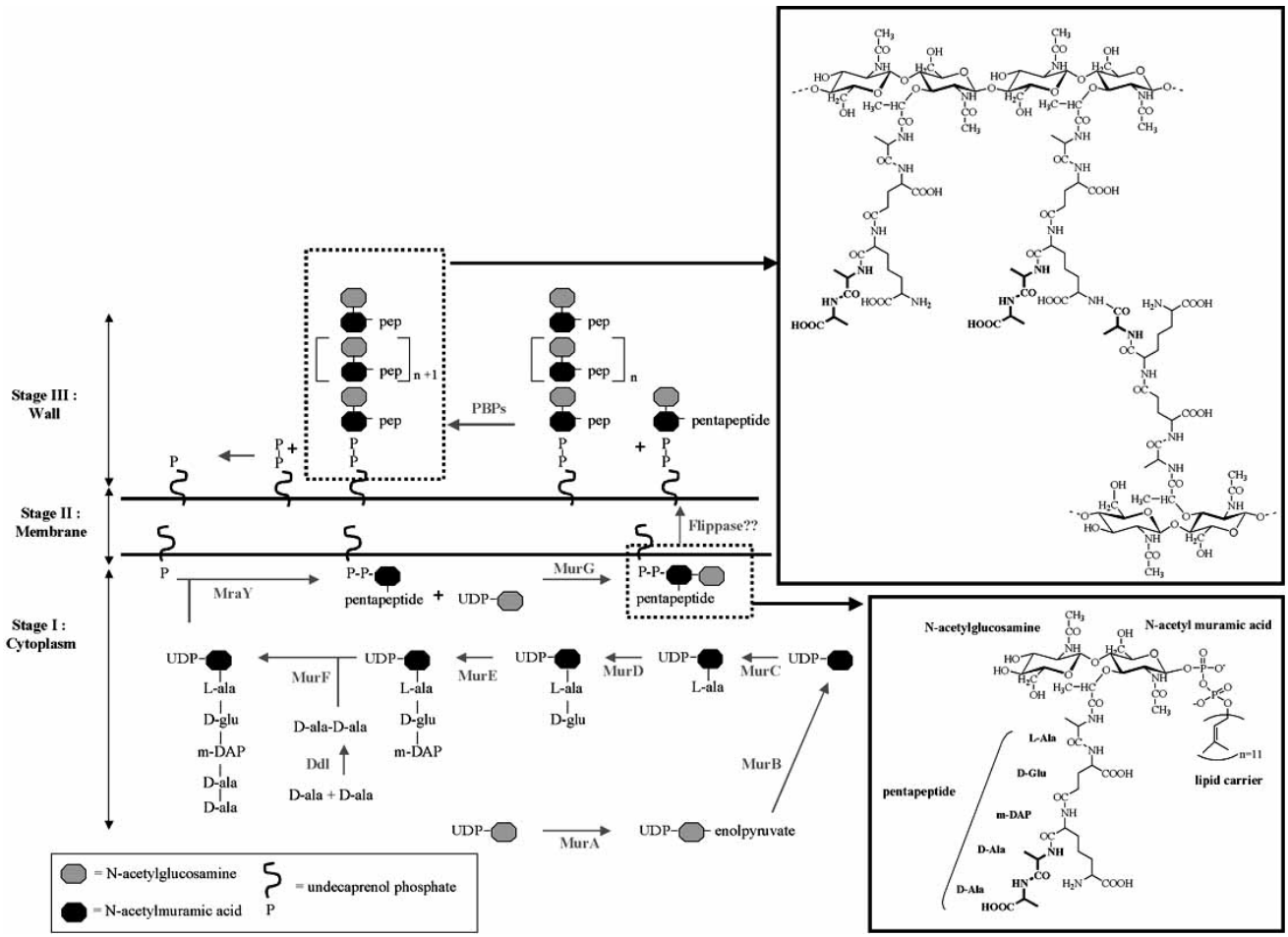



**Fig. (1). General scheme of peptidoglycan biosynthesis.** L-ala: L-alanine; D-glu: D-glutamate; mDAP: meso-diaminopimelate; D-ala: D-alanine; pep: pentapeptide; P: phosphate group; PBPs: Penicillin Binding Proteins; Ddl: DD-ligase. The inserts on the right show the chemical structure of the cytoplasmic precursor (bottom) and of the reticulated peptidoglycan (top), with D-Ala-D-Ala termini highlighted in bold characters. DD-ligases catalyze an early step in the cytoplasmic stage of peptidoglycan synthesis. Being essential for the synthesis of peptidoglycan precursors, they represent an attractive target for new antibiotics.

peptidoglycan. The intracellular pool D-alanyl – D-alanine seems to be finely regulated, as best demonstrated in *Escherichia coli* [39]. In growth phase, D-alanyl – D-alanine is used for the synthesis of monomeric units of peptidoglycan precursors, while D-alanine, released at the external face of the growing cell wall by the activity of transpeptidases during the reticulation step of peptidoglycan precursors, is recycled for the synthesis of new peptidic precursors. In stationary phase, both D-alanine and D-alanyl – D-alanine are liberated at the cell surface by carboxypeptidases, and transported into the cytosol. D-alanyl – D-alanine is then reincorporated in new cell wall precursors or converted back to D-alanine that can be used as energy source in starvation conditions. When present in high concentrations, D-alanyl – D-alanine can also inhibit the D-alanyl-D-alanine ligase activity, regulating thereby its own production [40,41]. Conversion of D-alanyl – D-alanine back to D-alanine is catalyzed by a DD-dipeptidase. This enzyme was first discovered in vancomycin-resistant enterococci (VanX [42,43]) and contribute to resistance by reducing the D-alanyl – D-alanine pool and, thereby, favouring the incorporation of D-Alanine-D-Lactate instead of D-Alanyl - D-Alanine in peptidoglycan precursors (see next section). Homologs of VanX were then identified in vancomycin-producing organisms, where they play, as in resistant enterococci, a role of protection against the antibiotic produced. They are now also described in other bacteria (see for example the DdpX enzyme in *E. coli)*, in which there are thought to play a critical role in the fine regulation of the amount of precursors available for cell wall synthesis [44].

## D-ALANYL – D-ALANINE LIGASES AND ALTERNATIVE LIGASES INVOLVED IN VANCOMYCIN RESISTANCE

DD-ligases (EC 6.3.2.4) belong to the superfamily of enzymes forming Carbon-Nitrogen Bonds, and to the family of Acid-Amino-Acid ligases (Peptide Synthases). The latter consists of enzymes possessing ATP-dependent carboxylate-amine ligase activity, and their catalytic mechanism includes acylphosphate intermediates [45,46].

Most DD-ligases synthesize a D-alanyl - D-alanine dipeptide, but, as seen above, bacteria may also possess enzymes producing a D-alanyl – D-serine dipeptide or a D-alanyl – D-lactate depsipeptide. This simple change in the terminal substituent of the MurNAc-pentapeptide is sufficient to confer vancomycin resistance. In susceptible strains, vancomycin, indeed, acts by binding with a high affinity to the D-alanyl – D-alanine termini of the lipid-PP-disaccharide-pentapeptide on the external face of the cytoplasmic membrane by forming five hydrogen-bonds [47-49]. This prevents the reticulation of peptidoglycan through steric inhibition of the transglycosylase and transpeptidase reactions catalyzed by the PBPs. Replacing the terminal D-alanine by a D-serine or a D-lactate causes a 6 [50] to 1,000 [51,52] fold decrease of vancomycin affinity for the corresponding peptidoglycan termini, due mainly to conformational change with D-alanyl – D-serine and to the loss of one hydrogen bond with D-alanyl- D-lactate [53-55] (see Fig. (**2**) for an illustration). Six types of vancomycin resistance have been differentiated on a phenotypic and genotypic basis. The Table (**1**) summarizes their main features in terms of location and transferability of the operon, regulation of the expression, level of resistance to both vancomycin and teicoplanin, and peptidic precursor produced [19,56]. The name of the resistance type is determined by the ligase produced (see for reviews [19,20]). Thus, VanA- [51], VanB- [57], and VanD- [58] types of resistance result from the preferential incorporation of D-alanyl – D-lactate precursors in peptidoglycan, the main differences between these three phenotypes consisting in their inducibility and in the level of resistance conferred. VanC [59], VanE [60] and VanG [61] types of resistance are due to the production of ligases that form D-alanyl – D-serine [62]. This is associated with a moderate level of resistance to vancomycin only, as the decrease in affinity is less marked than for D-alanyl – D-lactate.

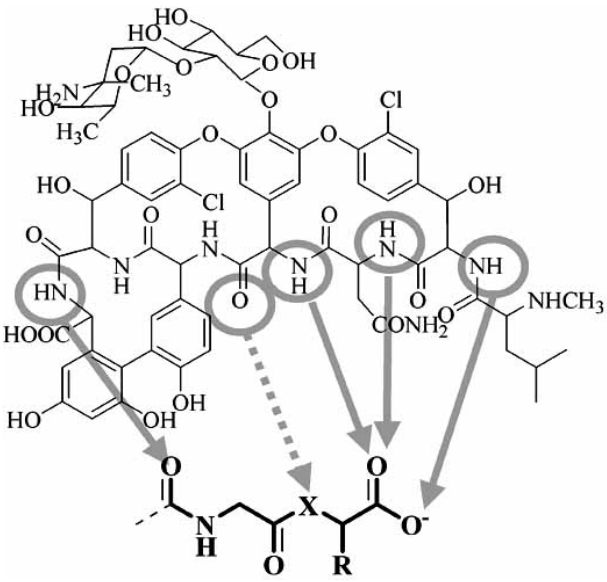


**Fig. (2).** Illustration of the hydrogen bonds established between vancomycin and pentapeptide termini of peptidoglycan precursors. Five hydrogen bonds (in gray) are formed between vancomycin and precursors ending in D-alanyl-D-alanine or D-alanyl-D-serine (but in this case, affinity is 6-fold lower). In strains producing pentapeptides ending in D-alanyl-D-lactate, one hydrogen bond (dotted line) is lost, causing a loss of affinity for the antibiotic of 1 thousand fold.

Of interest, bacterial species producing vancomycin like *Amycolatopsis orientalis* and *Streptomyces toyocaensis* are intrinsically resistant to vancomycin, as they possess a D-alanyl – D-lactate ligase [21,22], which is present on a gene cluster similar to that found in VRE. This suggests that glycopeptide-producing organisms may have been the source of resistance genes in vancomycin-resistant enterococci [63]. Likewise, some lactic bacteria like *Lactobacillus casei*, *Pediococcus pentosaceus*, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* produce precursors ending in D-alanine – D-lactate and are, therefore, intrinsically resistant to vancomycin [53,64].

Inhibitors acting on the constitutive DD-ligase, but also on those conferring resistance to vancomycin (and related glycopeptides), would thus represent a significant advance in

**Table 1. Types of Resistance to Vancomycin in Enterococci[a], in Relation with Alternative Peptidoglycan Precursors Produced by Alternative DD-Ligases**

| Type | MIC (mg/L) | | Bacterial species | Location of genes | Expression | Conjugation | Alternative dimer |
|---|---|---|---|---|---|---|---|
| | Vancomycin | Teicoplanin | | | | | |
| VanA | 64-1000 | 16-512 | *Enterococci* (*faecium, faecalis, avium, durans, raffinosus, gallinarum, casseliflavus*) ; *S. aureus* | Transposon on plasmid or chromosome (Tn*1546*) | Inducible | Yes | D-ala – D-lac |
| VanB | 4-1000 | 0.5-1 | *Enterococci* (*faecium, faecalis, bovis*) | transposon on plasmid or chromosome (Tn*1547* or Tn*1549*) | Inducible | Yes | D-ala – D-lac |
| VanC | 2-32 | 0.5-1 | *Enterococci* (*gallinarum, casseliflavus, flavescens*) | Chromosome | Constitutive | No | D-ala – D-ser |
| VanD | 64-128 | 4-64 | *E. faecium* | Chromosome | Constitutive or Inducible | No | D-ala – D-lac |
| VanE | 8-32 | 0.5 | *E. faecalis* | Chromosome | Inducible | No | D-ala – D-ser |
| VanG | 16 | 0.5 | *E. faecalis* | Chromosome | Inducible | Yes | D-ala – D-ser |

[a]Glycopeptide resistance is mediated by the acquisition of a gene operon, located on a mobile genetic element, which codes for the concerted production of enzymes involved in the synthesis of low-affinity precursors termini ending in D-lactate or D-serine, and in the elimination of high-affinity precursors ending in D-alanine, as well as for a regulatory system allowing induction by glycopeptides [116].

the current antibiotic arsenal, as alternatives are scarce to act upon these strains. More broadly speaking, inhibitors of DD-ligases would also be of interest for use against clinically-important pathogens with resistance mechanisms towards major antibiotic classes, such as methicillin-resistant *Staphylococci* or multi-resistant pneumococci. In this context, the knowledge of the mode of action and of the tridimensional structure of these different types of ligases is of prime interest to help in the rational design of inhibitors able to act on enzymes with different substrate specificities.

## DD-LIGASES: GENERAL STRUCTURE AND MECHANISM OF ACTION

Several atomic resolution structures of D-alanyl – D-alanine and D-alanyl – D-lactate ligases of different species have been reported (Table (**2**)) in absence or presence of different inhibitors. All these structures can be divided in three domains: N-terminal, central and C-terminal domains, each consisting of a β-sheet surrounded by helices and loops characteristic of the superfamily of ATP-dependent carboxylate-amine/thiol ligases [65,66]. Alignment of the sequences (Fig. (**3**)) shows that identity percentages varying from 99.1 % for the D-alanyl – D-alanine ligases *of Thermus caldophilus* and *Thermus thermophilus* to 24.2 % for the D-alanyl – D-alanine ligases of *E. coli* (DdlB) and *Helicobacter pylori*.

The catalytic mechanism of DD-ligases was first elucidated for one of the D-alanyl – D-alanine ligase of *E. coli* (DdlB) based on the crystal structure obtained in the presence of ADP and methyl-phosphinophosphate (a good mime of the transition state intermediate [65]). The reaction consists in a two-step process, with the transfer of the γ-phosphate of ATP to the first D-alanine (D-$Ala_1$) leading to an acetylphosphate product (D-alanyl-phosphate), followed by the nucleophilic attack on the D-alanylphosphate by the amino group of the second substrate (D-$Ala_2$) producing a tetrahedral intermediate. The latter is then dissociated to generate the product of the reaction (D-alanyl – D-alanine) and a phosphate group (Fig. (**4**)). Kinetics study of the *E. coli* ligase (DdlB) showed a $k_{cat}$ value for D-alanyl – D-alanine synthesis of 1870 $min^{-1}$ at pH 7.8, with no detectable D-alanyl – D-lactate activity. The affinity of the enzyme for D-$Ala_1$ ($K_m$ ~ 1 μM) is about 1000-fold higher than for D-$ala_2$ ($K_m$ ~ 1.1 mM) [67].

### Structural Comparison of the Active Sites

Most of the structures of the complexed enzymes were obtained with a phosphonate or phosphinate ligand. ADP appears sandwiched between two β-sheets from the central and C-terminal domains and the inhibitor is accommodated between the central and N-terminal domains (Fig. (**4**)). Three loops cover the ligand binding site, which is less than 2 % surface-accessible to the solvent. In *E. coli* D-alanyl – D-alanine ligase, these loops are connected by a triad of hydrogen bonds Glu15-Ser150-Tyr216, which are thought to break and re-form to allow entry and exit of ligands. One of these loops, called ω-loop (205-220 in *E. coli*), is suggested to close over ADP and the phosphinophosphate ligand upon binding, defining a well-circumscribed active site. The potential motion of this ω-loop is further supported by recent solved structures, which provide insight into the ligand- induced conformational changes [68-71]). Among them are the crystal structure of the apo form of *T. caldophilus* D-alanyl – D-alanine enzyme [69] in which the ω-loop has an open conformation and extends away from the active site, as well as the crystal structure of the *S. aureus* D-alanyl – D-alanine enzyme which was solved with a non-competitive inhibitor and features a highly disordered ω-loop [70].

D-alanyl – D-Lactate ligases (from *Leuconostoc mesenteroides* and *Enterococcus faecium)* possess a five-residue insertion in the ω-loop (residues 248-252 and 238-242

**Table 2. Amino acids in the active site of the D-alanyl – D-alanine or D-alanyl – D-lactate ligases interacting with ADP, $Mg^{2+}$ or the phosphinophosphate analog (PP) or Adenylyl Imidodiphosphate (AMPPNP), as observed in the crystal structures of the enzymes. The equivalent residues in the other two structures featuring no ligand in their active sites are also listed after superposition of on the *E. coli* structure**

| | | **Ligase and ligand in active site** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **D-alanyl – D-alanine ligase** | | **D-alanyl – D-lactate ligase** | | **D-alanyl – D-alanine ligase** | | |
| **Interactions with** | | **(ADP + PP analogue)** | **(AMPPNP)** | **(ADP + PP analogue)** | | **(none)** | | |
| **Ligand** | **Sub-ligand** | ***Escherichia coli* (1DLN)** | ***Thermus thermophilus* (2YZN)** | ***Leuconostoc mesenteroides* (1EHI)** | ***Enterococcus faecium* (1E4E)** | ***Staphylococcus aureus* (2I80)** | ***Thermus caldophilus* (2FB9)** | ***Helicobacter pylori* (2PVP)** |
| ADP | Adenine | I142 | F151 | F178 | F169 | F175 | F154 | I169 |
| | | K144 | K153 | K180 | K171 | K177 | K156 | K171 |
| | | M154 | I163 | I190 | V181 | I187 | I166 | V181 |
| | | E180 | E189 | E216 | E207 | E213 | E192 | E207 |
| | | W182 | A191 | A218 | A209 | G215 | A194 | F209 |
| | | L183 | L192 | V219 | V210 | V216 | L195 | I210 |
| | | F209 | F222* | W254 | I240 | F245* | F225* | F240* |
| | | M259 | F272 | L305 | F294 | F295 | F275 | F290 |
| | | L269 | N281 | G315 | N304 | N305 | N284 | N299 |
| | Ribose | M154 | I163 | I190 | V181 | I187 | I166 | V181 |
| | | E187 | E197 | E224 | E214 | E220 | E200 | E215 |
| | | Y210 | Y223* | Y255 | F241 | Y246* | Y226* | -- |
| | | K215 | K228* | K260 | -- | K251* | K231* | K246 |
| | α-Phosphate | K97 | K116 | K136 | K133 | K130 | K119 | K130 |
| | | K144 | K153 | K180 | K171 | K177 | K156 | K171 |
| | β-Phosphate | K97 | K116 | K136 | K133 | K130 | K119 | K130 |
| | | S151 | S160 | S187 | S178 | S183 | S163 | S178 |
| | | K215 | K228* | K260 | -- | K251* | K231* | K246* |
| | $Mg^{2+}$ | E270 | E282 | E316 | E305 | E306 | E285 | E300 |
| | | N272 | N284 | N318 | N307 | N308 | N287 | N302 |
| | $Mg^{2+}$ | D257 | D270 | D303 | D292 | D293 | D273 | D288 |
| PP analogue | $NH_3^+$ | E15 | E13 | E16 | E16 | E16 | E16 | E13 |
| | | E68 | E87 | E107 | E104 | D102 | E90 | E101 |
| | | S150 | S159 | S186 | S177 | S183 | S162 | S177 |
| | $PO_4^-$ | K215 | K228* | K260 | -- | K251* | K231* | K246* |
| | | R255 | R268 | R301 | R290 | R291 | R271 | R286 |
| | $CH_3$(1) | V18 | V16 | V19 | V19 | V19 | V19 | I16 |
| | | H63 | H82 | H102 | H99 | H96* | H85 | H96 |
| | C1' * | Y216 | Y229* | F261 | H244 | Y252* | Y232* | Y247 |
| | $CH_3$(2) | Y210 | Y223* | Y255 | F241 | Y246* | Y226* | -- |
| | | K215 | K228* | K260 | -- | K251* | K231* | K246* |
| | | L282 | M294 | L328 | R317 | M318 | M297 | -- |
| | P=O | R255 | R268 | R301 | R290 | R291 | R271 | R286 |
| | | G276 | G288 | G322 | G311 | G312 | G291 | G306 |
| | $COO^-$ | S281 | S293 | S327 | S316 | S317 | S296 | Y311 |
| | | L282 | M294 | L328 | R317 | M318 | M297 | -- |

*indicates residues in the ω-loop which are conserved in the sequence alignment (Fig. (**3**)) but which do not occupy similar spatial positions as their w-loop adopts an open conformation due to the absence of inhibitor in the active site (see text).

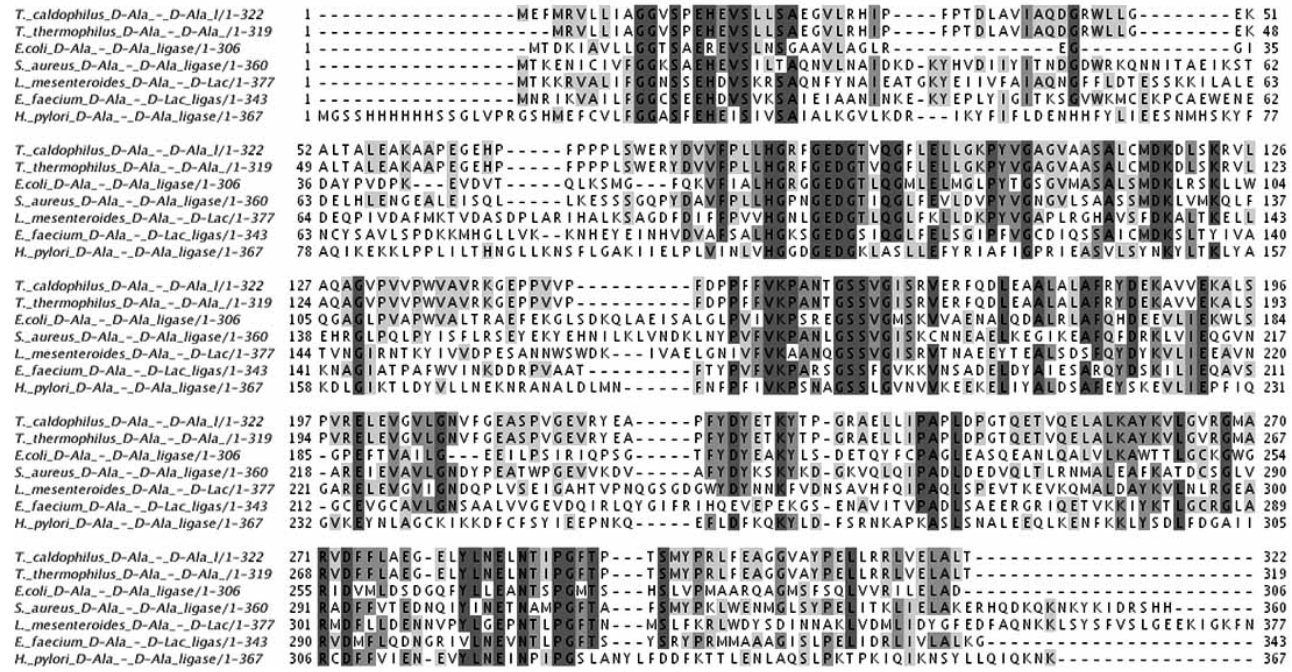



**Fig. (3). Multiple sequence alignment.** Stars above the sequence point to residues interacting with the phosphinophosphate inhibitor ; the thick black line above the sequence highlights the ω- loop. Dark gray: highly conserved residues ; light gray: residues conserved in most of the sequences. The alignment was generated by Clustalw.

respectively; Fig. (**3**)). Their crystal structures in complex with phosphinophosphate analogues [72,73] show that this ω-loop adopts a closed conformation as well. Here also, the importance of the ω-loop lid motion is confirmed by the finding that one of the monomers in the structure of the *L. mesenteroides* D-alanyl – D-lactate ligase shows a disordered ω-loop and accommodates no ligand, while in the other monomer complexed to a ligand, the ω-loop adopts a structured conformation containing a two-turn helix similar to that observed in *E. coli* D-alanine – D-alanine ligase [72]. In *E. faecium* D-alanyl – D-lactate ligase, the ω-loop, which also closes the active site, shows however a more extended conformation compared to *E. coli* and *L. mesenteroides* structures [73].

Tyr216 (*E. coli*) is critical not only for activity but also for substrate specificity, as its replacement by Phe converts the dipeptide ligase to an enzyme that has now gained substantial depsipeptide ligase activity [74]. This residue establishes a triad of H bonds with the Glu15 and Ser150 (in the D-alanyl – D-alanine ligase) fixing the ω-loop to lock the substrates and the intermediates in the active site. Glu15 also interacts with the amino group of D-$Ala_1$ to orient this electrophilic substrate in the appropriate direction. Despite its important role in substrate positioning, Tyr216 does not seem to be required for loop motion and function. First, the crystal structure of a Tyr to Phe mutant in *E. coli* [74] showed that the OH group of the phenol moiety in Tyr216 is not essential for loop closure and that other residues can form H bonds with the other 2 loops, namely the carbonyl main chain group of Tyr210 (in *E. coli*) with the neighboring Ser151. Second, in D-alanyl – D-lactate ligases that naturally lack this Tyr residue (position 261 in *L. mesenteroides* and 251 in *E. faecium* [75,76]), an equivalent hydrogen bond is formed either between Tyr277 main chain and Ser187 or between Phe241 main chain and Ser178.

The region of the active site is well-defined in the complexed enzyme structures, making it possible to identify residues establishing strong interactions with ADP or the methylphosphinate inhibitor. Site-directed mutagenesis experiments have confirmed the role of most residues proposed to take part in the reaction process [67]. Table (**2**) lists the residues in the different complexed structures that are in contact with ADP and the methylphosphinophosphate inhibitor (**21,** Fig. (**5**)) through their main and/or side chain. These residues in the *E. coli* structure are shown in Fig. (**4**). The equivalent residues in the apo form of the *T. caldophilus* and *H. pylori* enzymes and in the *S. aureus* enzyme complexed with a non-competitive inhibitor are also listed. The main observation is that a large body of residues is conserved between the active sites of the different enzymes. The adenine ring lies in a pocket mainly composed of hydrophobic and aromatic residues. Two charged residues are located in the neighborhood of adenine to form H-bonds with the hydrogen bond donor and acceptors of the base. The ribose, α- and β-phosphate groups as well as the two $Mg^{2+}$ ions interact with almost identical residues in all complexed enzymes. With the notable exception of *E. faecium* D-alanyl – D-lactate ligase, each ligase possesses three lysines (two for *E. faecium*), which form electrostatic links with the phosphate groups of ADP. The two $Mg^{2+}$ ions, which are essential for phosphate transfer and bridge ADP and the inhibitor phosphate group, interact with conserved Glu, Asn and Asp residues. Thus clusters of negatively- and positively-charged residues provide the electrostatic environment to counterbalance the two $Mg^{2+}$

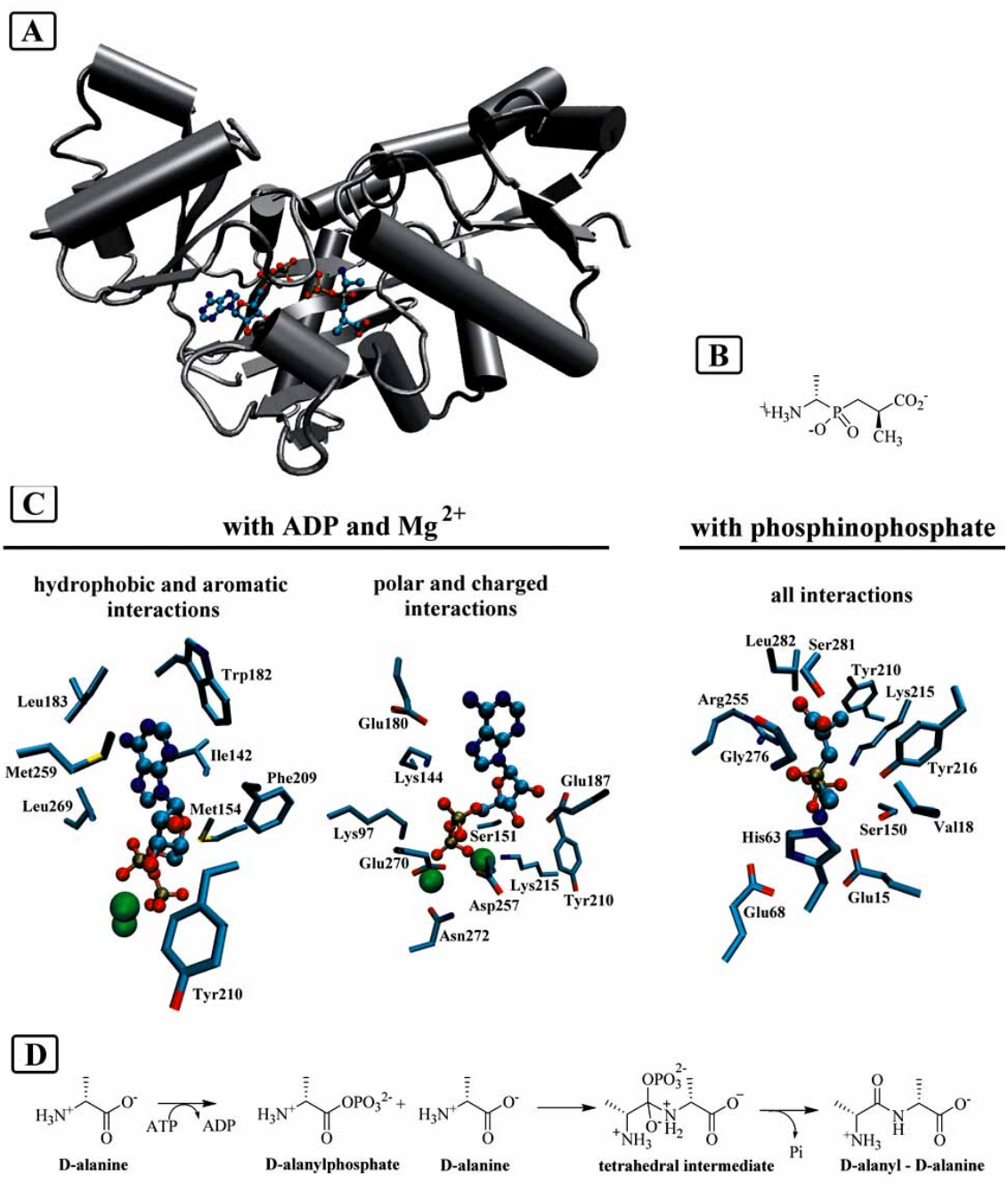
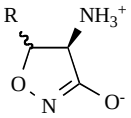


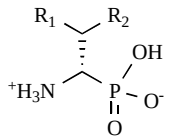

**Fig. (4). Structure and mechanism of action of DD-ligase**. **A:** Cartoon diagram of the *E. coli* D-alanyl – D-alanine ligase (DdlB) structure [65]. ADP and methylphosphinophosphate analogue inhibitor are depicted as ball-and-stick and colored according to their chemical type. **B**: Structure of the phosphinophosphate inhibitor (see also (**21**) in Fig. (**5**)). **C:** Residues interacting with ADP and the 2 magnesium ions: hydrophobic and aromatic residues are shown on the left panel; polar and charged residues are depicted on the middle panel. Residues interacting with the methylphosphinophosphate inhibitor are shown on the right panel. **D:** Mechanism of the reaction catalyzed by this enzyme, as deduced from structural data.

and the phosphoryl groups, respectively. In all complexed structures, the ammonium group of the inhibitor interacts with two glutamic acids (the equivalent of one of the Glu's is Asp in *S. aureus*) and one serine. In all structures but that of *H. pylori*, the carboxylic group is maintained at the other end of the inhibitor by one hydrogen bond to a serine side chain (281 in *E. coli*) and one hydrogen bond to a residue main chain (282 in *E. coli*). The *H. pylori* D-alanine – D-alanine ligase displays a low affinity for D-alanine. This could be partly related to the presence of a unique helix of seven residues (residues 306-312) in the vicinity of the D-$Ala_2$ binding site since the other ligases possess an α-helix of eight residues in this region [71]. The hydroxyl group of Tyr311 located in the $3_{10}$ helix of *H. pylori* structure could potentially

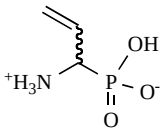
## ANALOGUES OF THE SUBSTRATE D-ALA



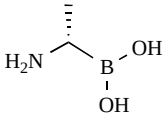
(**1**) R = H D-cycloserine (0.25 mM)
(**2**) R = *cis*-$CH_3$ *cis*-D-cyclothreonine (0.12 mM)
(**3**) R = *trans*-$CH_3$ *trans*-D-cyclothreonine (0.54 mM)



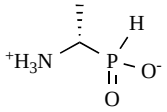
(**4**) $R_1$ = H $R_2$ = H D-Ala(P) (0.5 mM)
(**5**) $R_1$ = H $R_2$ = Cl Cl-Ala(P) (15 mM)
(**6**) $R_1$ = Cl $R_2$ = Cl $Cl_2$-Ala(P) (24 mM)



(**7**) VGP (5 mM)

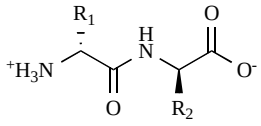


(**8**) Ala-B (35 μM)



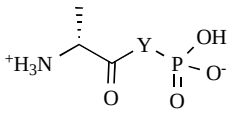
(**9**) D-(1-aminoethyl)phosphinic acid (0.4 mM)

## ANALOGUES OF D-ALA-D-ALA DIPEPTIDE

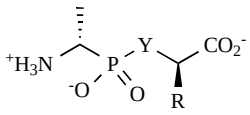


(**10**) $R_1$ = $CH_3$ $R_2$ = $CH_3$ D-Ala-D-Ala (1.2 mM)
(**11**) $R_2$ = $CH_2CH_3$ D-Ala-D-butyr (0.6 mM)
(**12**) $R_2$ = $(CH_2)_2CH_3$ D-Ala-D-norval (0.55 mM)
(**13**) $R_2$ = $CH(CH_3)_2$ D-Ala-D-Val (2.61 mM)
(**14**) $R_2$ = $CH_2OH$ D-Ala-D-Ser (0.91 mM)
(**15**) $R_2$ = $CH(CH_3)OH$ D-Ala-D-Thr (3.3 mM)
(**16**) $R_1$ = $CH_2CH_3$ $R_2$ = $CH_3$ D-butyr-D-Ala (6 mM)

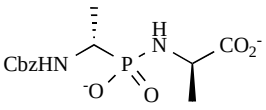
## ANALOGUES OF ENZYMATIC TRANSITION STATE



(**17**) Y = $CH_2$ (510 μM)
(**18**) Y = NH (50 μM)
(**19**) Y = *E*-CH=CH (1 μM)



(**21**) Y = $CH_2$, R = $CH_3$ (4 μM)
(**22**) Y = $CH_2$, R = $SCH_3$ (1.2 μM)
(**23**) Y = $CH_2$, R = *n*-Heptyl
(**24**) Y = $CH_2$, R = $CH_2CH(CH_3)_2$ (4 μM)
(**25**) Y = $CH_2$, R = $CH_2Ph$ (3 μM)
(**26**) Y = O, R = $CH_3$
(**27**) Y = O, R = $CH_2CH_3$



(**20**)

**Fig. (5). Condt…..**

**OTHER INHIBITORS**

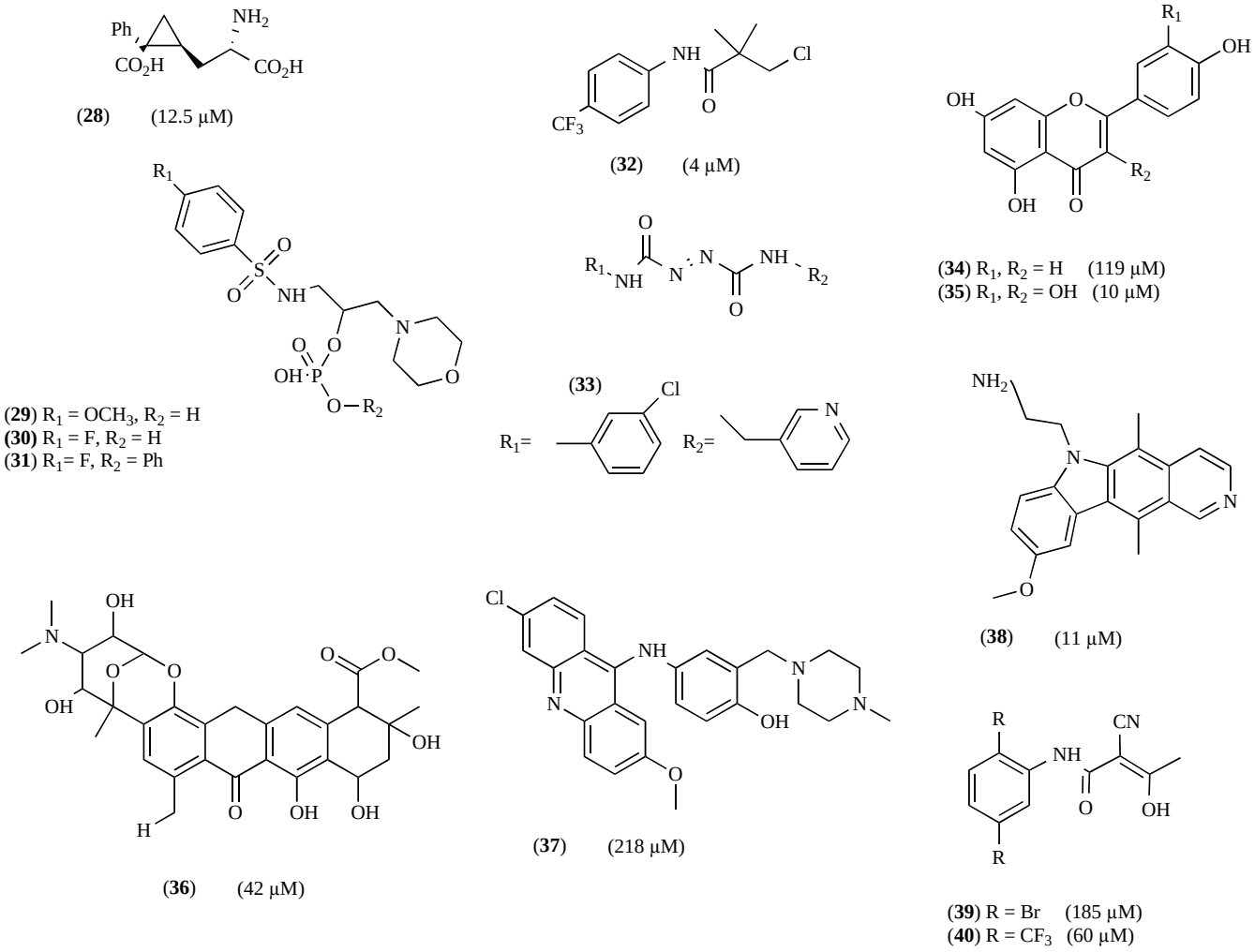



**Fig. (5). Chemical structure of current D-alanyl – D-alanine ligase inhibitors.** Affinities constants ($K_i$ for DdlB from *E. coli* or the corresponding enzyme in *Salmonella typhimurium*) are shown between parentheses when available. Note that most of the studies report a $K_i$ value for D-cycloserine of about $10^{-4}$ M, but lower values ~ 1 μM have also been published [110].

establish a hydrogen bond with the carboxylate group of the phosphinate, substituting for the serine present in the other ligases. However, no counterpart to the main chain hydrogen bond, formed for instance by Leu282 in *E. coli* with the carboxylate group, is found in *H. pylori* structure, providing a possible explanation for its much weaker affinity for the substrate D-Ala. Yet, the picture could be more complex as previous modeling studies on inactive *Enterococcus faecalis* and *E. faecium* mutants suggested that several residues not directly facing the active site may nevertheless be important for enzymatic activity by preserving the structural integrity of the binding pocket [77,78].

In the center of the inhibitor, the P=O bond interacts with identical residues (Arg, Gly) in all complexed structures. The phosphate group of the phosphorylated inhibitor interacts with a lysine residue. This lysine (Lys215 in *E. coli*) is conserved in all enzyme structures except in *E. faecium* in which it is replaced by a network of four water molecules [73]. The two side chains of the inhibitor make contacts with hydrophobic groups that are conserved in all DD-ligases examined. The $CH_2$ group in the inhibitor that corresponds to the NH group of the second D-alanine or the OH group of the lactate substrate interacts in most structures with an aromatic residue (Tyr, Phe or His).

## Specificity for the Second Ligand

The *L. mesenteroides* and *E. faecium* D-alanyl – D-lactate ligases have substantially higher ratios of alanyl-lactate versus alanyl-alanine activity than the *E. coli* Tyr216Phe mutant for instance (3000:1 and 30,000:1 respectively, as compared to 40:1 [72]). These two ligases must thus possess structural characteristics at the second substrate site that allow them to discriminate between the zwitterionic D-alanine and the anionic D-lactate. In the *L. mesenteroides* structure, the carboxylic acid group of the phosphinophosphate is not as strongly bound to the OH group of the conserved Ser327, and no hydrogen bond is formed with the

conserved Leu328 main chain [72], suggesting that the second D-alanine substrate should be less strongly bound than in the *E. coli* structure. In the *E. faecium* structure, the higher selectivity for D-lactate has been ascribed to the presence of His244 [73], which occupies the same spatial position as Tyr216 in *E. coli* or Phe261 in *L. mesenteroides*. Noticeably, this correspondence is not seen in sequence alignments (see Fig. (**3**)). The fact that this histidine is not present in *L. mesenteroides* D-alanyl – D-lactate ligase might explain the difference in ratio of D-alanyl – D-lactate versus D-alanyl – D-alanine activity with the *E. faecium* enzyme.

## INHIBITORS OF D-ALANYL – D-ALANINE LIGASE

Over the last 50 years, 4 main categories of inhibitors of the D-alanyl – D-alanine ligase have been described, the structures of which are presented in Fig. (**5**). A complete evaluation of the potential interest of these molecules should include not only a demonstration of their capacity of inhibiting the enzyme *in vitro*, but also of preventing bacterial growth. In the absence of a transport system, this second property implies that the molecules are sufficiently amphiphilic to cross the bacterial membrane and reach the DD-ligase in the cytosol. Yet, they must remain capable of interacting with an enzyme characterized by a highly hydrophilic catalytic cavity. Some bacteria (like *E. coli*) also produce two ligases (DdlA and DdlB), which may not be equally susceptible to a given inhibitor. This point would need to be more systematically evaluated, as it questions the universality of the ligase inhibitors and, therefore, their potential interest as broad-spectrum antimicrobial agents.

### Analogues of the Substrate

The first inhibitor of DD-ligase to be described in the literature is the D-4-amino-3-isoxazolidone [D-cycloserine (**1**)], a structural analogue of the natural substrate D-alanine which acts as competitive inhibitor of D-alanyl – D-alanine ligase from *S. aureus* [79]. Further studies with the *E. faecalis* enzyme indicate that both D-alanine binding sites of DD-ligase are sensitive to D-cycloserine (**1**), with the donor site showing a higher 'affinity' ($K_I = 2.2 \times 10^{-5}$ M) for D-cycloserine (**1**) than the acceptor site ($K_{AI} = 1.4 \times 10^{-4}$ M) and that the inhibition is competitive with respect to D-alanine, instantaneous and completely reversible ($K_i = 2.5 \times 10^{-4}$ M) [80]. D-cycloserine access to the bacterial cytosol is mediated by the D-Alanine-glycine transport system [81].

Specificity studies with analogues of D-cycloserine (**1**) have evidenced the features of the molecule contributing to the inhibitory activity and some of the modifications that can be made in the molecule while maintaining activity. For example, *cis*-5-methyl-D-4-amino-3-isoxazolidone [*cis*-D-cyclothreonine (**2**)] affects the binding of the antibiotic at the donor site ($K_I = 1.2 \times 10^{-4}$ M) but has little effect at the acceptor site ($K_{AI} = 1.9 \times 10^{-4}$ M). In contrast, *trans*-5-methyl-D-4-amino-3-isoxazolidone, [*trans*-D-cyclothreonine (**3**)] hinders binding at both the donor [$K_I = 5.4 \times 10^{-4}$ M] and acceptor sites [$K_{AI} = 5.6 \times 10^{-4}$ M] of DD-ligase [80].

D-cycloserine (**1**) has been used as an antibiotic for many years, mainly in combination with other antibiotics for the treatment of tuberculosis. Its interest is limited by high MIC values (~ 50 mg/L against *Mycobacterium tuberculosis* [82]), a bacteriostatic activity only, the frequent development of resistance [83], and a propensity to induce neurotoxicity [84]. It has been almost completely abandoned after the introduction of more active and less toxic molecules from other pharmacological classes.

D-(1-Aminoethyl)phosphonic acid (**4**) named also D-Ala(P) [85], exhibits a strong inhibition against the D-alanyl – D-alanine ligase of *E. faecalis* ($IC_{50} = 9.3 \times 10^{-4}$ M) [86-88] and a rapidly dissociating inhibitor of D-alanyl – D-alanine ligase from *Salmonella* [$K_i = 0.5$ mM] [89].

D-alanyl – D-alanine ligase of *E. faecalis* enzyme is also competitively inhibited by the mono- and dichloro derivatives of D-Ala-P (**4**), namely β-chloro- (**5**) and (β-β-dichloro-α-aminoethyl)-phosphonic acid (**6**), with $K_i$ values of 15 and 24 mM respectively [90]. D-(1-amino-2-propenyl)phosphonic acid (**7**) (VPG), the phosphonic analogue of D-vinylglycine in which the carboxyl group is replaced by a phosphonic one, inhibits the same enzyme with $K_i$ values of 5 mM [91]. These compounds show poor or no antibacterial activity, most probably because they are unable to gain access to their cytoplasmic target due to their highly polar character.

A different analogue of alanine (1-aminoethyl)boronic acid [Ala-B, (**8**)] in which the carboxyl group is replaced by a boronic acid substituent, proved also able to inhibit D-alanyl – D-alanine ligase from *Salmonella typhymurium* [89,92]. Kinetic analysis suggests that Ala-B (**8**) [$K_i = 35$ μM] can compete with D-alanine at either of the two D-alanine binding sites, according to a mixed inhibition (more than one mode of interaction between enzyme and inhibitor), but no D-alanyl – D-alanine-B dipeptide was detected. The ability of Ala-B (**8**) to generate ATP-dependent, slow binding inhibition may rely on the electrophilic nature of the boron atom in the $-B(OH)_2$ group, which has a propensity to generate a tetrahedral boronate anion at the active site by addition of an $H_2O$ molecule.

The amino acid isoster 1-(aminoethyl)phosphinic acid (**9**) [$K_i = 0.4$ mM], a phosphinic analogue of Ala(P) (**4**), was synthesized to analyze the effect of the oxidation state of the phosphorus atom on the inhibition [93,94]. In the phosphinate, the C-P bond found in the phosphonate is present as well, but one of the P-O bonds is replaced by a P-H bond. As compared to the phosphonate group $PO_3^{2-}$, this $PHO_2^-$ group has a behavior closer to that of a $COO^-$ group in enzymatic studies at pH 7.4. Phosphinic acid (**9**) behaves as a simple competitive inhibitor ($K_i = 0.4$ mM) of D-alanyl – D-alanine ligase from *S. typhimurium* [89]. This compound was shown to inhibit the bacterial growth of *Klebsiella pneumoniae* at a concentration of 0.3 mM (32 mg/L) and under aerobic conditions, but this was interpreted as resulting from the inhibition of pyruvate dehydrogenase [95].

### Analogues of the Product

In *E. faecalis*, D-alanyl – D-alanine ligase has a high specificity for D-amino acids in the N-terminal site and a low specificity for D-amino acids in the C-terminal site [96,97], explaining why the enzyme is able to synthesize a

wide variety of mixed dipeptides. Furthermore, dipeptides can produce inhibition, and several of these mixed peptides are slightly more effective inhibitors than D-alanyl – D-alanine itself. For this reason, a series of D-alanyl – D-alanine analogues were tested as potential inhibitors. Specific inhibition was observed only for dipeptides with the DD configuration. The N-terminal site has a high specificity for D-amino acids (D-alanine, D-α-amino-*n*-butyric acid), while the C-terminal site has a low specificity for D-amino acids (D-alanine, D-valine, D-α-amino-*n*-butyric acid, D-serine, D-threonine, and D-norvaline) [97]. The addition of substituents to the N-terminal residue usually decreases the effectiveness of the dipeptide as inhibitor, but certain additions to the C-terminal residue enhance the effectiveness of the dipeptide as an inhibitor. For example, D-Ala – D-butyr (**11**) ($K_i = 0.60 \times 10^{-3}$ M) and D-Ala – D-norval (**12**) ($K_i = 0.55 \times 10^{-3}$ M) are more effective than D-Ala – D-Ala (**10**) ($K_i = 1.2 \times 10^{-3}$ M and $IC_{50} = 6.6 \times 10^{-4}$ M) [88]. In contrast, D-norvaline and D-α-amino-*n*-butyric acid are less effective than D-alanine as substrates on the acceptor site [97]. Incubation of D-alanine and D-valine with the ligase and ATP does not result in the synthesis of D-Ala – D-Val (**13**). In contrast, D-Ala – D-Val (**13**) will inhibit the ligase as effectively as D-butyr – D-Ala (**16**) [98]. From Lineweaver-Burk plots, it has been established that D-Ala – D-Ala (**10**), D-Ala – D-butyr (**11**), D-Ala – D-Val (**13**) and D-butyr – D-Ala (**16**) are competitive inhibitors. In contrast, D-Ala – D-Ser (**14**) and D-Ala – D-Thr (**15**) are non-competitive inhibitors [41,98].

## Analogues of the Transition State

Another class of D-alanyl – D-alanine ligase inhibitors is represented by synthetic dipeptide mimetics of D-alanine – D-alanine (**10**), (3-amino-2-oxoalkyl)phosphonic acids [**17-19**]) [99,100] which mimic the presumed transition state of the enzyme catalytic reaction [101-103]. They serve as false substrates in which the C-terminal residue was replaced by an α-amino acid with a phosphoryl acidic function substituting for the carboxyl group. [3-(R)-amino-2-oxobutyl]phosphonic acid (**17**) ($K_i = 5.1 \times 10^{-4}$ M) and the corresponding aza analogue (**18**) ($K_i = 5.0 \times 10^{-5}$ M) were effective ligase inhibitors although they had no significant antibacterial activity [100]. Varying the $COCH_2PO_3H_2$ part of the structure of (**17**) generally reduces the activity. The carbonyl group is required, and several substitutions on the methylene-phosphonate group decrease the inhibitory potency. The aza analogue (**18**) is a better inhibitor than the methylene analogue (**17**) since the polarity and bond length of the NH-P group better mimics the P-O bond of the activated complex D-alanyl phosphate formed in the acceptor site. Compound (**18**) is a more potent inhibitor of the enzyme than is D-cycloserine (**1**) [100]. *E*-3-aminobutenylphosphonic acid (**19**) is a good competitive inhibitor of D-alanyl – D-alanine ligase, with a $K_i$ value close to $10^{-6}$ M, which is two orders of magnitude smaller than the $K_m$ [104]. These highly polar compounds again do not show significant antibacterial activity [100]. *N*-Carboxybenzyl-aminophosphonamidic acid (**20**) [$K_i$ = 1 μM] is also a competitive inhibitor of DD-ligase [104].

D-alanyl – D-alanine ligases are also inhibited by a series of phosphinic [89,105] and phosphonic acids [100,106]. Their inhibitory properties were established with the help of kinetic [41,107] and NMR spectroscopy studies [108].

The most potent inhibitor of D-alanyl – D-alanine ligase from *E. faecalis* is 1(*S*)-aminoethyl-(2(*R*)-carboxy-1-*n*-propyl)]phosphinic acid [R = $CH_3$ (**21**), $K_i$ = 4 μM], which is a tight-binding inhibitor of the enzyme, acting according to a mechanism involving an ATP-dependent formation of phosphorylated inhibitor within the enzyme active site [105]. Reacting with ATP and the slow-binding phosphinate (**21**), the D-alanyl – D-alanine ligase from *E. coli* produces ADP and a phosphorylated, mixed anhydride, transition state intermediate in the active site. Its stability is sufficient to allow crystallographic analysis, allowing to propose the catalytic mechanism for the formation of the dipeptide described here above [45]. Larger groups in R position enhance the activity, the most active of which are the thiomethylanalogue D-3-[(1-aminoethyl)phosphinyl]-2-thiomethyl acid (**22**) ($IC_{50}$ = 4 μM) and the *n*-heptyl analogue D-3-[(1-aminoethyl)phosphinyl]-2-heptylpropionic acid (**23**) ($IC_{50}$ = 4 μM), with a preferred stereochemistry corresponding to that of a D-amino acid. These phosphinodipeptides are also more potent inhibitors than the enzyme product D-alanyl – D-alanine ($IC_{50}$ = 1000 μM), as seen in comparison with compound (**21**) ($IC_{50}$ = 35 μM). The potency of compound (**22**) was also tested on *Salmonella* ligase, revealing a $K_i$ = 1.2 μM [89]. The universality of the inhibition mechanism [65] has, however, been questioned, since no kinetic role for ATP in inhibition of the *E. coli* D-alanyl – D-alanine ligase by the phosphonate analogue (**23**) was found [109]. Despite their potent activity against isolated enzymes, these compounds show an antibacterial activity only marginally superior to that of D-cycloserine, probably related to poor transport inside the bacteria [105]. Of interest however, (**21**) shows a broad spectrum activity (with an MIC range of 2-8 mg/L) when combined with the racemase inhibitor fluoro-D-alanine [105].

Other phosphinates, corresponding to D-Ala – D-Leu (**24**) [$K_i$ = 4 μM] and D-Ala – D-Phe (**25**) [$K_i$ = 3 μM] and phosphonates, corresponding to D-Ala – D-lactate (**26**) and D-Ala – D-α-hydroxybutyrate (**27**) were synthesized. The latter compounds are analogues in which the group in the amino position is changed from methylene to oxygen. (**21, 24-27**) were evaluated as inhibitors of 3 ligase enzymes, the two isoforms wild-type (DdlA and DdlB) D-alanyl – D-alanine ligase from *E. coli* and the Y216F mutant that rather shows a D-alanine-D-lactate ligase activity [74,106]. DdlA is potently inhibited by phosphinates (**21, 24** and **25**) but not by phosphonate (**26** and **27**), while DdlB and the mutant enzyme show little discrimination, illustrating that when a single bacterium does express two ligases, they can differ in their susceptibility to inhibitors. Both series of compounds inhibit DdlB strongly and the mutant weakly, the latter showing reduced affinity for all the ligands studied [106].

## Other Inhibitors

Over the last 3 years, several newer inhibitor scaffolds have been published that show no structural similarity with the substrate, the product or an intermediate of the reaction, but proved potent inhibitors of D-alanyl – D-alanine ligase activity. These scaffolds were evidenced either by screening

or by modeling, indicative of the currently more promising routes for drug discovery.

First, *de novo* structure-based molecular design using the SPROUT program allowed to identify two new classes of inhibitors of DdlB. A cyclopropyl-based amino acid tested as a diastereomeric mixture (**28**) has an apparent $K_i$ = 12.5 μM for DdlB, but no activity against the D-alanyl – D-lactate ligase VanA [110]. Hydroxyethylamines (**29-31**) inhibit 75 to 83 % of the activity of both *E. coli* DdlB and VanA D-alanyl-D-lactate ligases at 500 μM [111]. The presence of a phosphate group on the hydroxyl moiety of the hydroxyethylamine is required for activity, since (**31**), which harbors a phenylphosphate, is inactive.

Second, experimental screening of libraries of chemical substances or of natural products and virtual screening of chemical databases in the modeled enzymes has allowed to identify different types on inhibitors. This has led to several families of compounds.

A high-throughput screening against *S. aureus* D-alanyl – D-alanine ligase (StaDdl) has allowed to identify 3-chloro-2,2-dimethyl-*N*-[4-(trifluoromethyl)phenyl] propanamide (**32**) as a potent inhibitor, with a $K_i$ = 4 μM acting through an allosteric mechanism [70]. This inhibitor is non-competitive because it does not interfere with the binding of D-alanine to either site or that of ATP, but instead leads to the formation of an unproductive enzyme-substrate-inhibitor complex. This mode of inhibition is thus significantly different from that of existing inhibitors such as phosphinates, phosphonates, and D-cycloserine, paving the way to more original approaches to inhibit D-alanyl – D-alanine ligase activity.

The screening of a bank of compounds suggested an inhibitory activity for a family of diazenecarboxamides [112]. Further structure-activity relationships demonstrated that the more efficient compounds in the series possessed 2-chloroethyl or cyclohexyl moiety as $R_1$, or phenyl as $R_1$ and 2-,3-,or 4- picolyl substituent in $R_2$. In contrast, a flexible substituant in $R_2$ (N,N-diethylamino-2-ethyl residue, e.g.) makes the molecule inactive. The most active molecule in the series (**33**) has an $IC_{50}$ towards the DdlB of *E. coli* 20-fold lower than that of D-cycloserine, and MIC of 64 mg/L and 256 mg/L against *E. coli* and *S. aureus*, respectively. These inhibitors are structurally distinct from both ADP and D-alanine, suggesting that they inhibit D-alanyl – D-alanine ligase by a novel binding mode.

A library of 3000 natural compounds was used screen potential inhibitors of the Ddl enzyme from *H. pylori.* Two flavoinoids were identified, namely apigenin (**34**) and quercetin (**35**), with $IC_{50}$ of 132 (**34**) and 48.5 (**35**) μM, respectively [113]. $K_i$ values of 85.1 (**34**) and 28.1 (**35**) and of 119.5 (**34**), and 10.1 (**35**) μM were measured towards the *H. pylori* and *E. coli* DdlB enzymes, respectively. The compounds, however, were poor inhibitors of the growth of *H. pylori*, with MICs of 25 (**34**) and 200 (**35**) mg/L. These molecules were described previously as acting on other targets in bacteria (DNA gyrase, fatty acid biosynthesis, membrane [113]), making it difficult to attribute their activity to the inhibition of cell wall synthesis only.

A virtual screening of the 1990 molecules of the NCI (National Cancer Institute) database using AutoDock 4.0 and the DdlB model allowed select for *in vitro* assay the 130 top-ranked compounds. Among them, 3 showed significant inhibitory on the Ddl ligase, with $K_i$ of 42 (**36**), 218 (**37**) and 11 (**38**) μM. Moreover, (**37**) had an MIC of 32 mg/L against a reference strain of *S. aureus* but lower activity on *E. coli,* while (**38**) had MICs of 8-32 mg/L for both species [114]. These compounds, however, are likely to be toxic, as there were submitted to the NCI for potential activity as anticancer agents.

Third, structural alignments of the ATP binding site of D-alanyl – D-alanine ligase and different classes of kinases highlighted a common topology of the co-factor binding site and a same orientation of the adenine part of ATP. This prompted the evaluation of a series of ATP competitive kinase inhibitors and allowed identifying the tyrosine kinase inhibitor LFM-A13 (**39**) as a potent ATP competitive inhibitor of D-alanyl – D-alanine ligase activity, with a $K_i$ of 185 μM [115]. Replacing bromines by trifluoromethyl groups (**40**) further increases the activity ($K_i$ 60 μM).

Search of inhibitors has thus led over the years to the discovery of more potent molecules. Among classical approaches using analogues of substrates, reaction intermediates or products of enzymatic reaction, analogues of the transition state appear the most efficient. Development of potentially useful compounds in this series would, however, require further developments aimed at improving the penetration of molecules that are highly polar by nature inside the bacteria.

More original molecules, obtained either from molecular modeling approaches or by screening, proved efficient against the isolated enzyme. This underlines the interest of such strategies for discovery of pharmacologically active compounds, and justifies further studies of the structure of the target enzymes. Because these inhibitors can be quite different from those made based on templates of the reaction intermediate, it may be easier to modulate their scaffold to obtain adequate bioavailability. Unfortunately, however, many of these molecules have not yet been examined for antibacterial activity, which makes difficult to evaluate whether they could constitute promising leads for development of new classes of antibacterial agents.

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