Review

Roman Šink, Hélène Barreteau, Delphine Patin, Dominique Mengin-Lecreulx, Stanislav Gobec and Didier Blanot*

MurD enzymes: some recent developments

Abstract: The synthesis of the peptide stem of bacterial peptidoglycan involves four enzymes, the Mur ligases (MurC, D, E and F). Among them, MurD is responsible for the ATP-dependent addition of D-glutamic acid to UDP-MurNAc-L-Ala, a reaction which involves acyl-phosphate and tetrahedral intermediates. Like most enzymes of peptidoglycan biosynthesis, MurD constitutes an attractive target for the design and synthesis of new antibacterial agents. *Escherichia coli* MurD has been the first Mur ligase for which the tridimensional (3D) structure was solved. Thereafter, several co-crystal structures with different ligands or inhibitors were released. In the present review, we will deal with work performed on substrate specificity, reaction mechanism and 3D structure of *E. coli* MurD. Then, a part of the review will be devoted to recent work on MurD orthologs from species other than *E. coli* and to cellular organization of Mur ligases and *in vivo* regulation of the MurD activity. Finally, we will review the different classes of MurD inhibitors that have been designed and assayed to date with the hope of obtaining new antibacterial compounds.

Keywords: inhibitors; MurD; Mur ligases; peptidoglycan; three-dimensional structure.

***Corresponding author: Didier Blanot,** Laboratoire des Enveloppes Bactériennes et Antibiotiques, Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR 8619 CNRS, Université Paris-Sud, F-91405 Orsay, France, e-mail: didier.blanot@u-psud.fr
Roman Šink and Stanislav Gobec: Fakulteta za farmacijo, Univerza v Ljubljani, Aškerčeva 7, SI-1000 Ljubljana, Slovenia
Hélène Barreteau, Delphine Patin and Dominique Mengin-Lecreulx: Laboratoire des Enveloppes Bactériennes et Antibiotiques, Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR 8619 CNRS, Université Paris-Sud, F-91405 Orsay, France

Introduction

Peptidoglycan (murein) is an essential component of the bacterial cell wall (1). It is found on the outside of the cytoplasmic membrane of almost all bacteria. It consists of linear glycan chains alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues linked by β-1→4 bonds. The D-lactoyl group of each MurNAc residue is substituted by a peptide stem whose composition is often L-Ala-γ-D-Glu-*meso*-A_2pm (or L-Lys)-D-Ala-D-Ala (A_2pm, 2,6-diaminopimelic acid) in nascent peptidoglycan, the last D-Ala residue being removed in the mature macromolecule. Cross-linking of the glycan strands generally occurs between the carboxyl group of D-Ala at position 4 and the amino group of the diamino acid at position 3, either directly in most Gram-negative bacteria or through a short peptide bridge in most Gram-positive organisms. This results in a rigid tridimensional (3D) network surrounding the entire cell.

The biosynthesis of peptidoglycan is a complex process that involves ca. 20 reactions occurring in the cytoplasm (synthesis of the nucleotide precursors) and on the inner side (synthesis of lipid-linked intermediates) and outer side (polymerization reactions) of the cytoplasmic membrane (2). The cytoplasmic steps lead to the formation of UDP-MurNAc-pentapeptide from UDP-GlcNAc. After the formation of UDP-MurNAc (enzymes MurA and MurB), the successive additions of L-Ala (MurC), D-Glu (MurD), diamino acid (MurE) and D-Ala-D-Ala (MurF) are catalyzed by four enzymes, the Mur ligases (3). Thereafter, the phospho-MurNAc-pentapeptide moiety is transferred (enzyme MraY) to a membrane polyprenol, undecaprenyl phosphate, yielding lipid I. The addition of a GlcNAc residue from UDP-GlcNAc (catalyzed by MurG) leads to the formation of lipid II, which contains the whole monomer motif (4). The disaccharide-pentapeptide motif then crosses the membrane to reach the outer face; recent work has demonstrated that this process is performed by a specific protein, FtsW (5). Finally, the polymerization reactions (transglycosylation and transpeptidation), catalyzed by the penicillin-binding proteins, take place (6).

The main function of peptidoglycan is to preserve cell integrity by withstanding the inner osmotic pressure. Indeed, inhibition of its biosynthesis (mutation, antibiotic) or its specific degradation (e.g., by lysozyme) during bacterial growth will generally result in cell lysis. Owing to the different forms of resistance that pathogenic

bacteria are developing, there is an urgent need to search for new inhibitors (7). In this context, peptidoglycan is an attractive target (8). As a matter of fact, most antibiotics known to interfere with peptidoglycan biosynthesis (e.g., β-lactams, vancomycin, bacitracin) target the polymerization reactions or the synthesis of lipid-linked intermediates. This is the reason why the cytoplasmic steps, and in particular the Mur ligases, which were underexploited in the past, have recently gained more attention.

Among the Mur ligases, MurD from *Escherichia coli* has been the most studied enzyme from biochemical, mechanistic and structural standpoints; perhaps as a consequence of this, many inhibitors have been designed and synthesized. In this paper, we will review the knowledge recently gathered about MurD ligase orthologs from *E. coli* and other species.

Generalities on Mur ligases

As mentioned in the Introduction, four enzymes, MurC, MurD, MurE and MurF, are involved in the *de novo* synthesis of the peptide stem of peptidoglycan (3). A fifth enzyme, Mpl, directly adds tripeptide L-Ala-γ-D-Glu-*meso*-A_2pm to UDP-MurNAc during peptidoglycan recycling in some Gram-negative bacteria (9, 10). The Mur ligases catalyze the formation of an amide or peptide bond with the simultaneous formation of ADP and P_i from ATP. A divalent cation, Mg^{2+} or Mn^{2+}, is necessary. The Mur ligases share the following three characteristics.

i. They have a common reaction mechanism, which consists first in the activation of the carboxyl group of the UDP-containing precursor by ATP to yield an acyl-phosphate and ADP; the acyl-phosphate then undergoes a nucleophilic attack by the amino group of the amino acid (or dipeptide) substrate to form a high-energy tetrahedral intermediate, which eventually breaks down into amide or peptide and P_i [(11) and references therein].
ii. They possess a series of invariant residues in addition to an ATP-binding consensus sequence. This finding led to the definition of the Mur ligases as a new family of enzymes (12–14). The family also includes MurT, an enzyme involved in D-isoglutamine formation in lipid II in *Staphylococcus aureus* (15, 16), and three enzymes not related to peptidoglycan synthesis, folylpoly-γ-L-glutamate synthetase FolC (17), cyanophycin synthetase CphA (18) and poly-γ-glutamate synthetase CapB (19).
iii. They display similar 3D structures in three domains, the N-terminal, central and C-terminal domains, being involved in the binding of the nucleotide precursor, ATP, and the amino acid or dipeptide, respectively. Whereas the topologies of the central and the C-terminal domains are similar among the Mur ligases, that of the N-terminal domain shows differences related to the length of the UDP-precursor substrates, with MurC and MurD on the one hand, and MurE and MurF on the other hand. Moreover, Mur ligases exist as 'open' and 'closed' conformations, the closure being thought to result from ligand binding (20).

Properties of the MurD enzyme from *E. coli*

Substrate specificity

MurD is the enzyme adding the second amino acid on the peptide stem, and in all of the bacteria studied to date, this amino acid turns out to be D-glutamic acid (1, 21). The minor variations that can be encountered in mature peptidoglycan (D-isoglutamine, D-*threo*-3-hydroxyglutamate) are the result of modifications posterior to the action of Mur ligases (15, 16, 22).

The *E. coli* MurD enzyme has been purified and characterized in the 1990s in its native (23–25) and His-tagged (14) forms. Optimal pH value, optimal Mg^{2+} concentration and kinetic parameters can be seen in Table 1. Its substrate specificity has been studied for the addition of various amino acids or derivatives to the radiolabeled precursor UDP-MurNAc-L-[^{14}C]Ala (26). It appeared that (i) an α-amino acid moiety of D configuration, (ii) an anionic group on carbon 4 and (iii) a proper distance between the two acid groups were essential requirements for a substrate. As a matter of fact, apart from D-Glu, very few compounds turned out to be good substrates: DL-homocysteic acid, D-*erythro*-3- and -4-methylglutamic acids, and two cyclic analogs of Glu (five- or six-membered ring). Moreover, L-Glu is absolutely not a substrate of the enzyme (26), even though some *N*-sulfonyl- or *N*-acyl-derivatives of L-Glu were shown to inhibit *E. coli* MurD (27–29).

While MurD is very specific with respect to its amino acid substrate, it differs from the other Mur ligases by its less strict specificity towards the nucleotide precursor. Indeed, it has been shown that the removal of the UMP moiety leads to a substrate (1-phospho-MurNAc-L-Ala) having a fairly good affinity for the enzyme (K_m=0.2 mmol/l) (30).

Table 1 Optimal pH, optimal Mg^{2+} concentration, and kinetic parameters of MurD orthologs.

Species	Peptide extension	Optimal pH	Optimal $[Mg]^{2+}$ (mmol/l)	K_m ATP (mmol/l)	K_m UMA[a] (mmol/l)	K_m D-Glu (mmol/l)	V_{max} (μmol/[min mg])	References
E. coli	No	8.9–9.2	5	0.138	0.0075	0.055	8.4	(23, 24)
	No	8[b]	5[c]	0.135±0.033	0.007±0.003	0.042±0.011	4.78±0.42	(25)
	His-tag	9.4[b]	5	0.057	0.005	0.064	8.4	(14)
S. aureus	No	8[b]	20–25	0.084±0.010	0.29±0.034	0.534±0.025	32.1±3.7	(25)
	His-tag	8[b]	5[c]	n.d.[d]	n.d.	n.d.	5[e]	(105)
	His-tag	8.4–9.6	15	5.4±1.9	0.041±0.020[f]	0.13±0.044[f]	35±6	(51)
S. pneumoniae	His-tag	7.7	20	n.d.	0.03	0.105	n.d.	(106)
	His-tag	8.2–9.6	5	~2[g]	0.096±0.039[h]	0.19±0.026[h]	15±3	(52)
P. aeruginosa	His-tag	8[b]	5[c]	n.d.	n.d.	n.d.	n.d.	(107)
	His-tag	n.s.[i]	5[c]	n.d.	n.d.	n.d.	3.12[j]	(108)
E. faecalis	No	8[b]	10–25	0.047±0.004	0.036±0.007	0.118±0.014	29.8±5.9	(25)
H. influenzae	No	8[b]	5 or 10[c]	0.102±0.006	0.008±0.004	0.169±0.020	13.1±2.2	(25)
B. burgdorferi	His-tag	8.2–9.0	5	0.053±0.012	0.0063±0.0030	0.11±0.029	1.6±0.3	(52)
M. tuberculosis	His-tag	8.8–9.8	10	0.71±0.29	0.34±0.01	0.70±0.18	0.49±0.11	(52)
	Yes[k]	8.5	5	0.106±0.0001	0.053±0.0001	0.085±0.0002	0.8±0.1	(63)

Unless noted otherwise, K_m values were determined at saturating concentrations of the other substrates. Detailed conditions can be found in the references. Partially purified enzymes, or purified enzymes whose activity was not checked, were not considered.

[a]UMA, UDP-MurNAc-L-Ala.

[b]pH of the assay, no optimal value specified.

[c]Concentration used in the assay, no optimal value specified.

[d]n.d., not determined.

[e]Specific activity at 1 mmol/l ATP, 0.12 mmol/l UMA and 1 mmol/l D-Glu.

[f]Determined at subsaturating ATP concentration (2 mmol/l) owing to high K_m ATP value.

[g]Marked inhibition by excess ATP over 1.5 mmol/l.

[h]Determined at subsaturating ATP concentration (1.5 mmol/l) owing to inhibition by excess ATP.

[i]n.s., not specified.

[j]Specific activity at 1 mmol/l ATP, 0.1 mmol/l UMA and 0.5 mmol/l D-Glu.

[k]21-amino acid N-terminal extension.

Reaction mechanism and order of binding

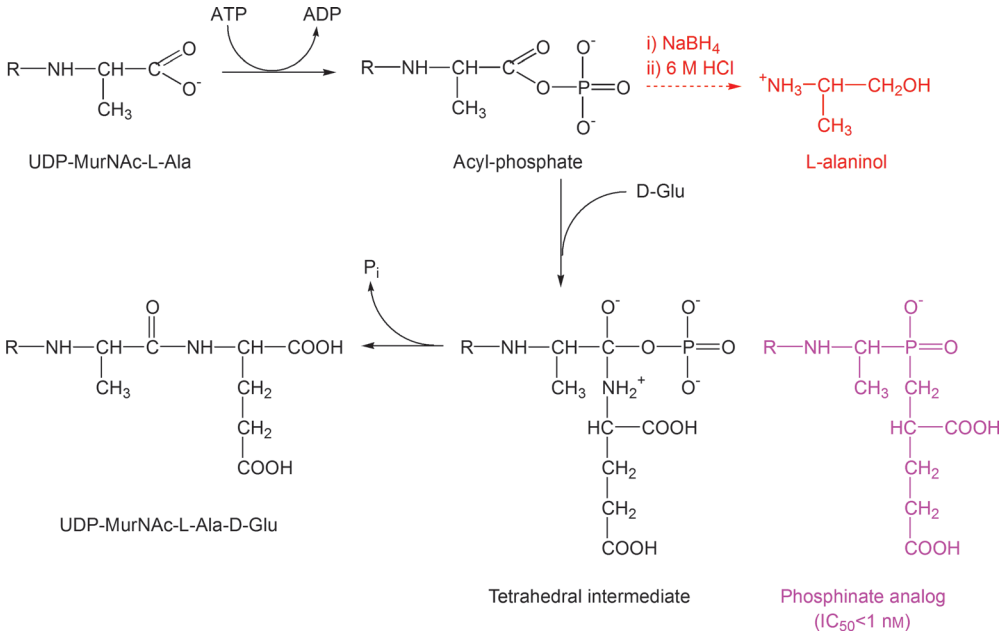
As mentioned above, the Mur ligases share the same reaction mechanism involving two intermediates, an acyl-phosphate and a high-energy tetrahedral intermediate (Scheme 1). The formation of acyl-phosphate was first suggested indirectly, mainly with MurC [see references in (11)]. It was then firmly established for MurC and MurD through the use of the borohydride reduction method, introduced by Degani and Boyer (31) and Todhunter and Purich (32). This method allowed to chemically trap the acyl-phosphate intermediates into stable derivatives (2-amino-2-deoxy-3-*O*-[(*S*)-(1-hydroxymethyl)ethyl]-D-glucose for MurC, L-alaninol for MurD) that were clearly identified (Scheme 1) (11, 33). The existence of the tetrahedral intermediate was suggested by the strong inhibition of the MurD activity with phosphinate analogues (Scheme 1) (34–36).

A particular reaction was observed with MurD: the synthesis of adenosine tetraphosphate (33). This compound was formed upon incubation of the enzyme with ATP and UDP-MurNAc-L-Ala (without D-Glu). This synthesis was explained by a reaction of ATP with the acyl-phosphate intermediate. No adenosine tetraphosphate formation could be detected with MurC (33), but this molecule was present in the crystal structure of FolC (37).

The kinetic mechanism was established for MurC (38) and MurF (39). Data were unambiguously consistent with a sequential ordered kinetic mechanism, with ATP binding first, followed by the nucleotide precursor and, finally, amino acid (or dipeptide). Although such experiments were never performed with MurD, it is reasonable to assume that it follows the same type of mechanism. However, the fact that the molecular isotope exchange reaction was not strictly ADP-dependent (35), contrary to MurC (40), suggested some randomness in the kinetic mechanism.

Tridimensional structure

MurD from *E. coli* was the first Mur ligase for which a crystal structure, in complex with substrate UDP-MurNAc-L-Ala,



Scheme 1 Reaction mechanism of MurD showing the acyl-phosphate and tetrahedral intermediates (black). The acyl-phosphate intermediate can be trapped by reduction with sodium borohydride followed by acid hydrolysis, yielding L-alaninol (red). The existence of the high-energy tetrahedral intermediate is suggested by the strong inhibition of the MurD activity by a phosphinate analog of tetrahedral geometry (purple). Stereochemistry was omitted for the sake of simplicity. R, UDP-MurNAc.

was solved (41). Thereafter, other structures containing ADP, Mg^{2+} or Mn^{2+}, the product UDP-MurNAc-L-Ala-D-Glu, or inhibitors, were reported (27, 42), as well as 'open' conformations (43). The 3D structure of MurD from *E. coli* consists of three domains (Figure 1A). The N-terminal domain is responsible for the binding of UDP-MurNAc-L-Ala, the central domain binds ATP and the C-terminal domain binds D-glutamic acid. The most important residues for UDP-MurNAc-L-Ala binding are Leu15, Thr16, Asp35, Thr36, Arg37, Gly73, Asn138 and His183, the last one via Mg^{2+} (Figure 1B) (41–43). ADP is held in place thanks to the interactions with Gly114, Lys115, Ser116, Thr117, Asn271, Arg302 and Asp317 (Figure 1C) (27, 42), while D-glutamic acid interacts with Thr321, Lys348, Ser415, Leu416 and Phe422 (Figure 1D) (42). Interestingly, the topologies of the central and C-terminal domains of *E. coli* MurD were found to be similar to those of FolC (17, 43).

Tridimensional structures were also determined for MurD enzymes from *Streptococcus agalactiae* (44) and *Thermotoga maritima* (45). The domain arrangement is similar to that of the *E. coli* ortholog. An important feature that was observed in *E. coli* MurD was the rigid body C-terminal domain rotation. The position of the C-terminal domain dictates the conformation of MurD: the closed conformation (C-terminal domain rotated towards the central and N-terminal domains) is considered as the active conformation, while open conformations (C-terminal domain rotated away from the N-terminal and central domains) are considered as inactive (43, 46). Binding of substrates (UDP-MurNAc-L-Ala-D-Glu and ATP, or only ATP) provokes C-terminal domain rotation to the closed conformation, while binding of UDP-MurNAc-L-Ala-D-Glu provokes its rotation either to the closed conformation or to the distinct open conformation (27, 41–43). Computational simulations revealed that a small energy barrier exists between the open conformation without ligands and the closed conformation (47). Different C-terminal domain conformations are presented in Figure 1E. C-terminal domain rotation was also observed in *T. maritima* MurD (45).

A catalytic mechanism for MurD at the molecular level was proposed by analyzing the biochemical data together with the 3D structural data of MurD complexes with ligands. The two ADP-bound crystal structures of MurD revealed the ATP-binding mode and two important divalent cation (Mg^{2+} or Mn^{2+}) binding sites (site 1 and site 2), which seem to be very important for the phosphorylation of UDP-MurNAc-L-Ala in the presence of ATP (Scheme 2). Although the electron density for the γ-phosphate of ATP was never visible, it was presumed that one of the γ-phosphoryl oxygen atoms interacts with Mg^{2+} in site 1. This approximation suggests that the

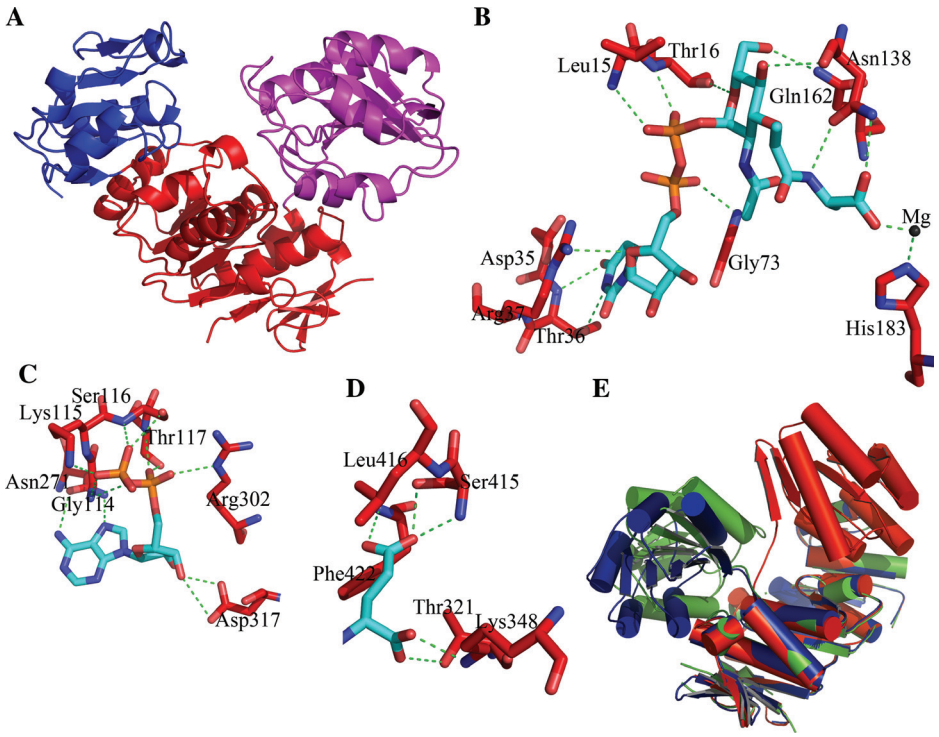


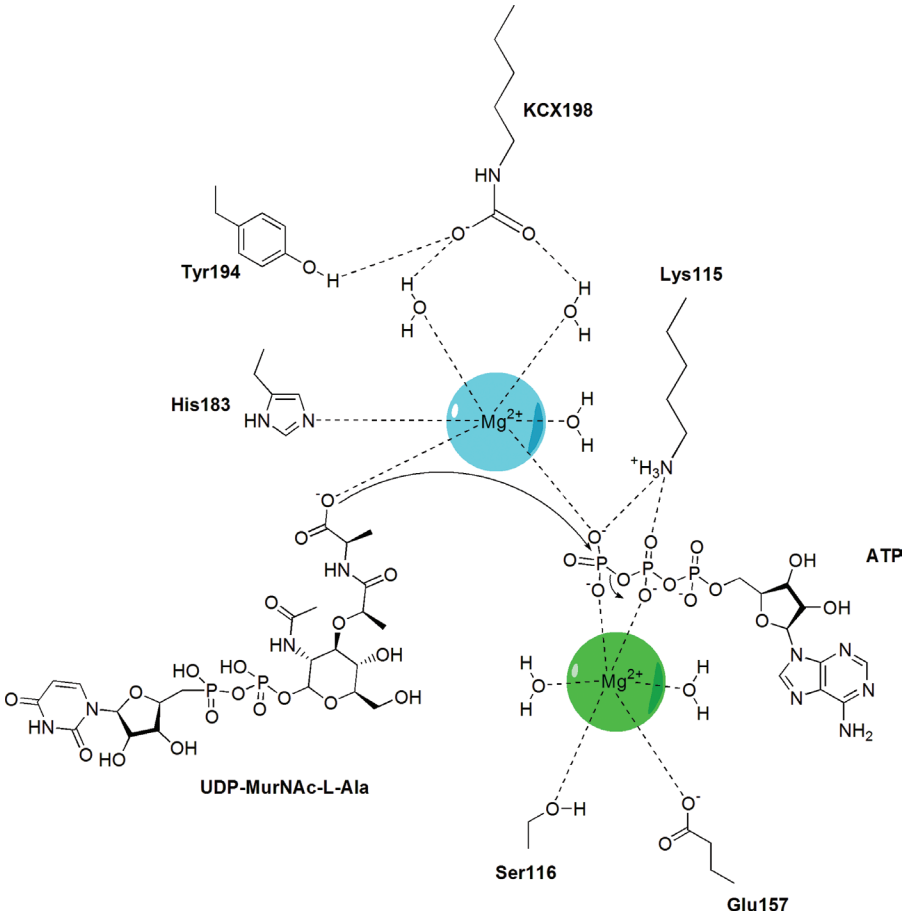
Figure 1 Tridimensional structure of, and interactions of ligands with, *E. coli* MurD.
(A) Tridimensional structure of *E. coli* MurD (PDB entry: 4UAG). Blue: N-terminal domain; red: central domain; magenta: C-terminal domain. (B) Interactions of UDP-MurNAc-L-Ala with MurD. (C) Interactions of ADP with MurD. (D) Interactions of D-glutamic acid with MurD. (E) Superposition of closed conformation (green, PDB entry: 2UAG) with two open conformations (blue, PDB entry: 1E0D; red, PDB entry: 1EEH) of *E. coli* MurD. N-terminal and central domains were aligned.

γ-phosphate is transferred to UDP-MurNAc-L-Ala via a bimolecular nucleophilic substitution type mechanism (S_n2). After the transfer of the γ-phosphate, the acyl-phosphate intermediate is stabilized with interactions with Lys115 and the two Mg^{2+} ions. Then, the properly oriented incoming D-Glu is added to the acyl-phosphate intermediate, resulting in the formation of the tetrahedral intermediate. A catalytic base is required to withdraw a proton from the amino group of D-Glu. In the disruption of the tetrahedral intermediate, another catalytic base (probably His183) is essential for the withdrawal of the second proton (42) and thus the formation of UDP-MurNAc-L-Ala-D-Glu. Enzymatic reaction pathways were recently studied using a hybrid quantum mechanical/molecular mechanical replica path method and found to be in line with the proposed sequential mechanism (48). However, it was discovered that proton transfer from D-Glu to either the γ-phosphate of ATP or the nitrogen of His183 was energetically too demanding to fit in the normal energy scope of enzymatic reactions. This suggested that D-Glu most likely enters the enzyme reaction in its deprotonated form obtained by an as-yet unidentified mechanism (48).

An important chemically modified residue in the proximity of the active site is carbamylated lysine 198 (KCX198), which interacts via a water bridge with the Mg^{2+} ion in site 2, thus stabilizing its position (Scheme 2) (42). KCX198 was only observed in the ligand-bound closed *E. coli* MurD crystal structures (27, 41, 42), while in open structures (43) Lys198 was not carbamylated. From an enzymatic point of view, the importance of the carbamyl group, which is also present in MurE and MurF, was investigated by chemical rescue experiments (49). It was shown that mutant proteins MurD Lys198Ala/Phe/Glu/Cys, which displayed low enzymatic activity, were rescued upon incubation with short-chain carboxylic acids, but not amines. These observations are in favor of a functional role for the carbamate in MurD.

MurD enzymes from other bacteria

After MurD from *E. coli*, several orthologs of the MurD enzyme from various bacteria were isolated and characterized. A list, along with optimal pH values, optimal Mg^{2+} concentrations and kinetic parameters, is presented in



Scheme 2 Schematic representation of the γ-phosphate transfer from ATP to UDP-MurNAc-L-Ala, and important residues that are involved in the reaction. Mg^{2+} ions in sites 1 and 2 are depicted as green and cyan spheres, respectively.

Table 1. In almost all cases, K_m values are in the micromoles per liter range. A few discrepancies between data from different laboratories were encountered (e.g., for *S. aureus* or *Mycobacterium tuberculosis*), presumably owing to different assay conditions. For two MurD orthologs (*S. aureus* and *Streptococcus pneumoniae*), high K_m values for ATP (≥2 mmol/l) were observed. Such unusual values have also been found for MurC from *S. aureus* and MurE from *T. maritima* (50, 51). Maximum velocities of enzymes from Gram-positive bacteria are higher than those from Gram-negative bacteria. In two cases (*Borrelia burgdorferi* and *M. tuberculosis*), V_{max} values are quite low. This might be related to the slow-growth properties of the bacterial species in question (52).

It is interesting to mention that D-isoglutamine was not a substrate for the enzymes originating from *S. aureus*, *S. pneumoniae* and *M. tuberculosis* [(51); H. Barreteau and D. Blanot, unpublished results], species in which this amino acid is found at position 2 of the peptide stem of mature peptidoglycan. This is in keeping with amidation occurring at a later stage of peptidoglycan synthesis, namely, lipid II (15, 16, 53).

Genetic aspects, cellular organization and regulation

Conditional-lethal (thermosensitive) mutants of *E. coli* altered at different levels of the peptidoglycan pathway were isolated in the early 1970s, and the mutations were mapped in several chromosomal regions (54–56). One region of particular interest, located at 2 min of the *E. coli* map, was named *mra* (murein region A) or *dcw* (division cell wall) because it contained a large cluster of genes, from *mraZ* to *envA*, that coded for proteins involved in peptidoglycan biosynthesis and cell division. The *murD* gene encoding the D-glutamic acid-adding enzyme was

identified within this cluster (57, 58), together with the *murC*, *murE* and *murF* genes encoding the other three Mur ligases, and the *mraY* and *murG* genes encoding lipids I and II synthesizing activities. These different genes, tightly packed and overlapping in some cases, were transcribed in the same direction, and their expression was mainly dependent on a promoter, P_{mra}, identified upstream of the cluster (59). Repression of P_{mra} resulted in a dramatic depletion of MurD ligase *in vivo* and was followed by the arrest of peptidoglycan synthesis and cell lysis (60). Demonstration that the *E. coli murD* gene is essential for growth was recently provided by the construction of a conditional mutant strain carrying the *murD* gene on a plasmid bearing a thermosensitive replicon (51). A similar cluster organization with interspersed *mur* (murein synthesis) and *fts* (division) genes was found in many other bacterial species. As discussed by Mingorance et al. (61), such a 'genomic channeling' could favor the formation of Mur and Fts protein complexes which then contribute together to the channeling of peptidoglycan precursors from the cytoplasm to the cell wall-synthesizing machinery operating at the division site. However, this is not a general rule as Mur ligase-encoding genes of many species, for instance, *murC* in *Bacillus subtilis* or *murE* in *Thermus thermophilus*, appear variably dispersed in other chromosomal locations. No evidence that Mur ligases are assembled into functional complexes *in vivo* has been provided to date, but interactions between these enzymes and other proteins involved in peptidoglycan synthesis or cell division were reported in *Caulobacter crescentus* (62), *M. tuberculosis* (63) and *T. maritima* (45). It has been speculated, but not demonstrated, that a concerted action of Mur ligases in this pathway, achieved through the formation of a multi-enzyme complex and allowing the channeling of intermediates, may explain that the lack of efficiency of some potent Mur ligase inhibitors observed *in vivo* is due to a restricted accessibility of these compounds to the active sites of these enzymes (64, 65).

It was earlier shown that *E. coli* Mur ligases were expressed constitutively at a relatively high level as compared to the cell requirements for peptidoglycan synthesis (66), suggesting that there was no rate-limiting step in this part of the pathway. This was consistent with the low pool levels observed for these different UDP-MurNAc-peptide intermediates in growing cells. The unrestricted accumulation of some of these intermediates that is observed in wild-type cells treated with antibiotics [UDP-MurNAc-tripeptide following treatment with D-cycloserine (67), a D-Ala:D-Ala ligase inhibitor] or in conditional mutants grown in restrictive conditions [UDP-MurNAc-L-Ala in a D-glutamate-requiring mutant starved for this amino acid (68)] further suggested that these pools were not tightly regulated and that the intermediates can be released from the enzyme active sites and escape a putative channeling process. However, this does not exclude that the activity of Mur ligases could be regulated in some way. Feedback inhibition of the MurD activity by its reaction product, UDP-MurNAc-L-Ala-D-Glu, as well as by the final nucleotide precursor of the pathway, UDP-MurNAc-pentapeptide, has been reported earlier, which could contribute to the regulation of this enzyme activity (68, 69). Moreover, the fact that MurD enzymes from *E. coli* and *Haemophilus influenzae* are (i) inhibited by moderate (15–30 μmol/l) concentrations of the substrate UDP-MurNAc-L-Ala and (ii) require monocations such as NH_4^+ and/or K^+ for optimal activity, contrary to *S. aureus* and *Enterococcus faecalis* orthologs, has led Walsh et al. (25) to postulate a key role for MurD in the regulation of peptidoglycan synthesis in Gram-negative bacteria. More recently, Mur ligases of some species were identified as targets of serine-threonine protein kinases (STPKs) and shown to be phosphorylated by these activities both *in vitro* and *in vivo*. This was in particular reported for MurC from *Corynebacterium glutamicum* (70) and *S. pneumoniae* (71), and for MurD from *M. tuberculosis* (72). The phosphorylation of *C. glutamicum* MurC protein by the PknA kinase, which occurs on six different threonine residues, was shown to negatively modulate its ligase activity *in vitro* (70). Protein phosphorylation was also demonstrated for other enzymes catalyzing earlier cytoplasmic steps or the final polymerization steps of this pathway: GlmM (73), GlmU (74) and penicillin-binding proteins (75). Deletion of *S. aureus* STP phosphatase and *pknB* serine/threonine kinase genes brought about alterations of the pool levels of peptidoglycan nucleotide precursors that also suggested a regulation of Mur ligase activities by phosphorylation in this bacterial species; according to the quantitative changes in the relevant metabolites, the effect was stronger for MurC/F than for MurD/E (76).

MurD inhibitors

Most of the efforts in designing MurD inhibitors were concentrated on MurD from *E. coli*, for which several classes and generations of inhibitors have been developed. MurD inhibitors can be divided into several distinct structural classes as presented in Figure 2.

The most fundamental distinction of the classification is between peptide and non-peptide inhibitors. Among the former, two cyclic nonapeptides are worth mentioning,

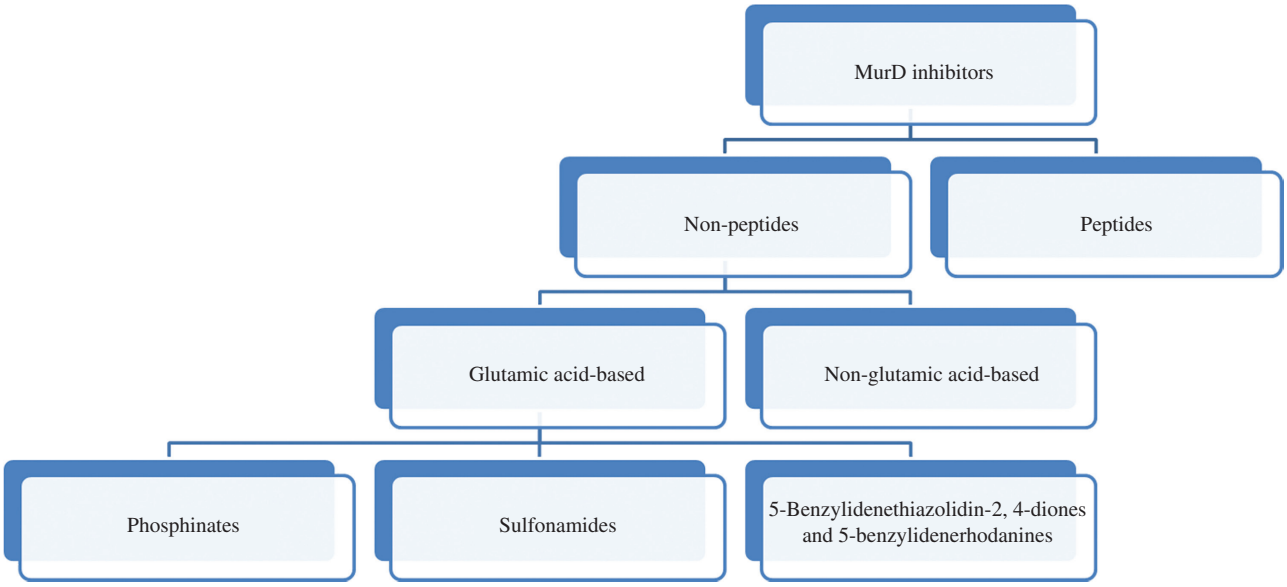
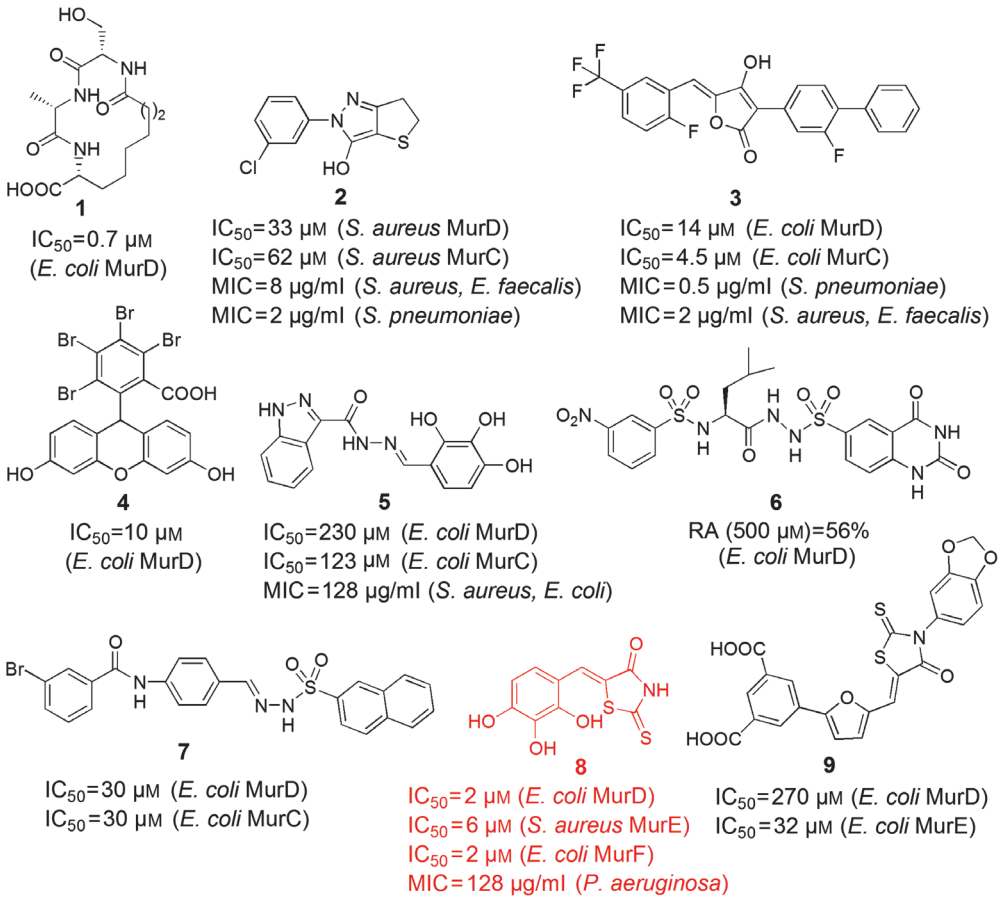


Figure 2 Different classes of MurD inhibitors.

with amino acid sequences Cys-Pro-Ala-His-Trp-Pro-His-Pro-Cys and Cys-Ser-Ala-Trp-Ser-Asn-Lys-Phe-Cys. They inhibit *E. coli* MurD with IC_{50} values of 1.5 and 0.62 mmol/l, respectively (77). Linear peptide Arg-Pro-Thr-His-Ser-Pro-Ile, which inhibits *Pseudomonas aeruginosa* MurD in the low micromoles per liter range (IC_{50}, 4 µmol/l), was also discovered (78).

Among the non-peptide inhibitors, the non-glutamic acid-based inhibitors constitute a distinct class (Scheme 3). Macrocyclic inhibitors of MurD from *E. coli* were discovered using computer-based molecular design. The most potent inhibitor **1** had an IC_{50} value of 0.7 µmol/l (79). 2-Phenyl-5,6-dihydro-2*H*-thieno[3,2-*c*]pyrazol-3-ol derivatives were found to inhibit MurD from *S. aureus* (but also MurB and MurC) in the micromoles per liter range. The most potent compound **2** inhibited MurD with an IC_{50} value of 33 µmol/l (80). An important property of these compounds is their antibacterial activity: compound **2** inhibited the growth of some Gram-positive strains, while the antibacterial activity against Gram-negative strains was much lower (80). Pulvinones inhibited the MurA-D enzymes from *E. coli* and *S. aureus*. Representative inhibitor **3** also expressed antibacterial activity against some Gram-positive strains (81). Structurally diverse 9*H*-xanthene derivatives and polycyclic inhibitors were discovered with structure-based virtual screening. The most potent representative from this campaign (compound **4**) inhibited *E. coli* MurD with an IC_{50} value of 10 µmol/l (82). *N*-Acyl-hydrazones were discovered by screening an in-house bank of compounds on MurD (83). One promising inhibitor (compound **5**) can be highlighted in the synthesized series. It inhibited *E. coli* MurD and MurC with IC_{50} values of 230 and 123 µmol/l, respectively. Another feature of this compound is a weak antibacterial activity of 128 µg/ml against *S. aureus, E. coli* and the efflux pump AcrAB-deficient *E. coli* strain (83). Sulfonohydrazides were designed and synthesized as analogs that mimic a diphosphate part of the MurD substrate UDP-MurNAc-L-Ala. In the presence of a 500 µmol/l concentration of the most potent inhibitor (compound **6**), *E. coli* MurD still retained 56% residual activity (84). The sulfonohydrazone moiety was incorporated into previously known MurD inhibitors, thus forming a new class. The most potent compound of this series (compound **7**) inhibited *E. coli* MurC and MurD (IC_{50}, 30 µmol/l for both enzymes) (85). 5-Benzylidenethiazolidin-4-ones were discovered by combining rhodanine, which had previously been employed as a diphosphate surrogate or a phosphate mimetic, with the 2,3,4-trihydroxyphenyl group, which had been incorporated in earlier MurD inhibitors. The best compound (**8**) inhibited *E. coli* MurD, *S. aureus* MurE and *E. coli* MurF in the low micromoles per liter range, thus appearing as a multi-target inhibitor. Unfortunately, it displayed only weak antibacterial activity against *P. aeruginosa* (MIC, 128 µg/ml) (86). Benzene-1,3-dicarboxylic acid derivatives were discovered in a virtual screening campaign. The most potent compound (**9**) had an IC_{50} value of 270 µmol/l with *E. coli* MurD. It was also a fairly good MurE inhibitor (IC_{50}, 32 µmol/l) (87).

The largest class of *E. coli* MurD inhibitors consists of glutamic acid-based inhibitors. As the reaction catalyzed

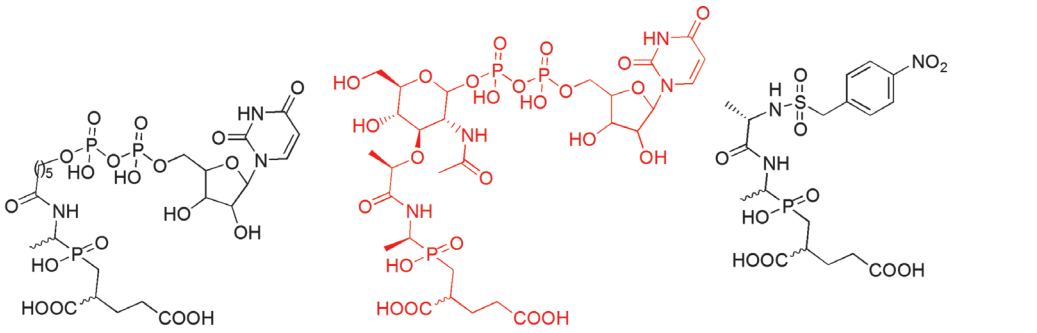


Scheme 3 Representative non-glutamic acid-based inhibitors of MurD. Multi-target inhibitor **8** is depicted in red.

by MurD proceeds via a tetrahedral intermediate, the phosphinate transition-state analogs were the first potent inhibitors of MurD that were described in the literature. Compound **10** (Scheme 4), in which the MurNAc moiety was replaced by a hydrophobic linker of appropriate length, inhibited MurD from *E. coli* with an IC_{50} value of 680 nmol/l (34). The potency of phosphinate inhibitors was improved with the inclusion of the MurNAc moiety, which led to the best inhibitor reported to date (compound **11**, IC_{50} <1 nmol/l, Scheme 4) (36). The next generation of phosphinate transition-state analogs featured simplified compounds with the key phosphinodipeptide Ala-Ψ(PO_2-CH_2)-Glu structural motif. The inhibitory activities of such compounds (i.e., **12**) were in the micromoles per liter range (88), and some of them also inhibited MurE (89).

Several 5-benzylidenethiazolidin-2,4-dione and 5-benzylidenerhodanine inhibitors that contained D-(or L-)glutamic acid in their structures were developed. Starting with virtual screening and taking into consideration the fact that rhodanine-containing compounds possess *in vitro* antimicrobial activity, a first generation was developed. An interesting finding of this research was the observation that the most potent MurD inhibitor did not lose significantly its activity when D-glutamic acid (compound **13**, Scheme 5) was replaced with L-glutamic acid (compound **14**) (28).

In the next generation of 5-benzylidenethiazolidin-2,4-dione- and 5-benzylidenerhodanine-substituted glutamic acid, the substitution of the aromatic rings was altered, which resulted in *E. coli* MurD inhibitors (compounds **15** and **16**, Scheme 6) with IC_{50} values of 45 and 85 μmol/l, respectively (90). These compounds were further improved with minor alterations of the ring substituents and variations in the linker regions between the two phenyl rings. Such efforts produced inhibitor **17**, which was the best of the series (IC_{50}, 3 μmol/l). Another interesting inhibitor, compound **18**, contained a sulfonamide function instead of the carboxamide one (IC_{50}, 45 μmol/l) (91, 92). The next step in the development of this class of inhibitors featured a shorter linker between the glutamic acid part and the 4-thiazolidinone part of the molecule (29). Compound **19** inhibited *E. coli* MurD with an IC_{50} value of 10 μmol/l (Scheme 6). An interesting fact here is that the compound containing L-glutamic acid was the most

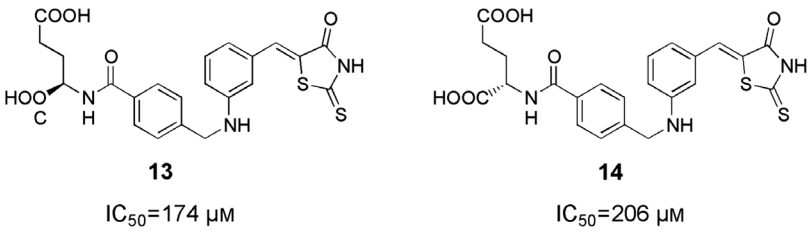


Scheme 4 Representative phosphinate inhibitors of MurD. The most potent inhibitor reported to date (**11**) is depicted in red.

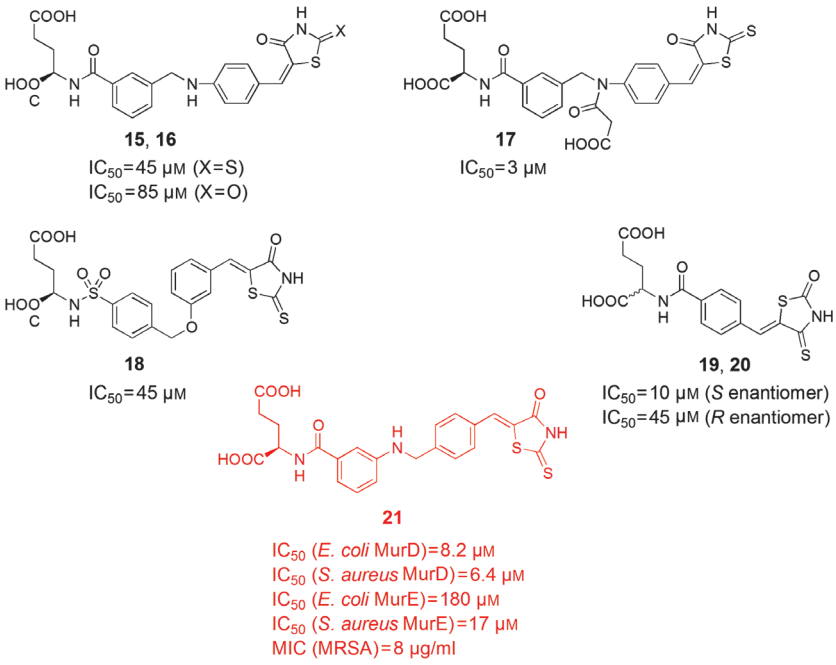
potent one; the D-Glu-containing derivative **20** displayed a lower potency (IC_{50}, 45 μmol/l) (29). Finally, an important progress in this series was the design and synthesis of compound **21**, in which the NH-CH_2 group connecting the two phenyl rings had been reversed (Scheme 6). This compound turned out to be a dual MurD and MurE inhibitor. It was endowed with antibacterial activity against *S. aureus* and its methicillin-resistant strain (MRSA) (MIC, 8 μg/ml) (93).

A highlight in the development of 4-thiazolidinone inhibitors was the determination of their binding mode by solving several X-ray structures of MurD/inhibitor complexes (90–93). The binding modes of these compounds are very similar, as can be seen from the structures of complexes with typical inhibitors **16** and **21** (Figure 3). A superposition of the crystal structures of inhibitor **16** [Protein Data Bank (PDB) entry: 2X5O] (90) and UDP-MurNAc-dipeptide (PDB entry: 4UAG) (42) within *E. coli* MurD reveals that the inhibitor occupies the binding site of the product (Figure 3A). The thiazolidin-2,4-dione ring occupies the uracil-binding site with interactions with Asp35, Thr36 and Arg37. The carboxylic groups of the D-glutamic acid part of the inhibitor occupy exactly the same position as those of UDP-MurNAc-dipeptide. The γ-carboxyl group interacts with Ser415, Leu416 and Phe422, while the α-carboxyl group is held in position through interactions with Thr321 and Lys348 directly. In the MurD/**21** complex (PDB entry: 2Y1O), the α-carboxyl group interacts with Lys115 and Lys348 via water molecules (Figure 3B), which is a unique property of the binding mode of inhibitor **21** (93). No polar contacts between the protein and the central linker part of the inhibitors exist. Here, only π-π stacking of the aminophenyl ring with Phe161 and hydrophobic interactions of the benzylidene ring with Gly73 are worth mentioning.

Sulfonamides represent the last class of important MurD inhibitors reported to date. The sulfonamide function was, similarly to the phosphinate one, incorporated into the target compounds in order to mimic the MurD tetrahedral intermediate (27, 94, 95). The first generation of naphthalene sulfonamides was N-substituted glutamic acid derivatives (27, 95) The inhibitory properties of 2-naphthalene sulfonamides **22** and **23** (Scheme 7), which differ only in the configuration of the glutamic acid residue, revealed that their mechanism of inhibition was competitive towards D-glutamic acid and non-competitive towards UDP-MurNAc-L-Ala or ATP. However, the potency of the L-enantiomer was significantly lower than that of the D-enantiomer (27). An important achievement of the thorough investigation of the inhibitory mechanism was



Scheme 5 5-Benzylidenerhodanines containing D-glutamic acid (**13**) and L-glutamic acid (**14**). IC_{50} values were determined with *E. coli* MurD.



Scheme 6 5-Benzylidenethiazolidin-2,4-dione and 5-benzylidenerhodanine inhibitors. Unless stated otherwise, IC_{50} values were determined with *E. coli* MurD. Dual MurD and MurE inhibitor **21** is depicted in red.

the solution of the X-ray co-crystal structures of both enantiomers in the active site of MurD. Crystal structures revealed almost exactly the same binding mode of the two enantiomers, as both the L- and D-glutamic acid parts of the inhibitors occupy the same position as the glutamic acid part of UDP-MurNAc-L-Ala-D-Glu, while the naphthalene-sulfonamido groups are displaced by approximately 1.5 Å (27). Naphthalene sulfonamides were later further modified and biochemically and structurally evaluated (95). The outcome was a series of new inhibitors with the most potent compound (**24**), which inhibits MurD with an IC_{50} value of 85 μmol/l. The binding energies of the naphthalene sulfonamides were calculated using the linear interaction energy method; the calculated energies correlated very well with the experimentally obtained free energies (96). Further analysis of the interactions between naphthalene sulfonamides and MurD was performed by nuclear magnetic resonance followed by molecular dynamics, which takes into account the ligand flexibility and its effect on particular ligand-enzyme contact, thus offering potential explanations for moderate inhibitory activities (97).

Armed with a considerable knowledge about the binding modes of naphthalene sulfonamides, a second generation of sulfonamide inhibitors was developed. Instead of the D-glutamic acid moiety, they possessed cyclic, rigidified mimetics, as for example in compounds **25** and **26** (Scheme 7) (98). Incorporation of rigid mimetics

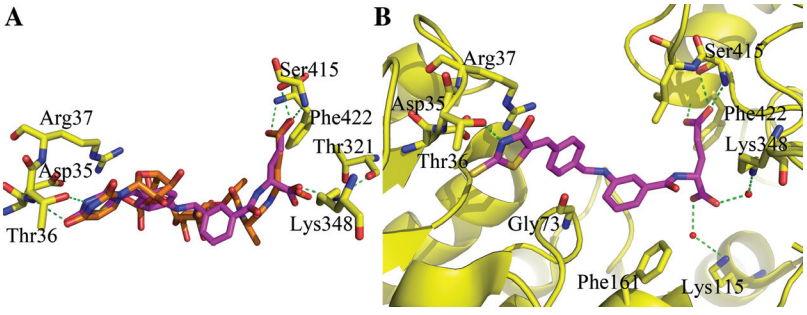
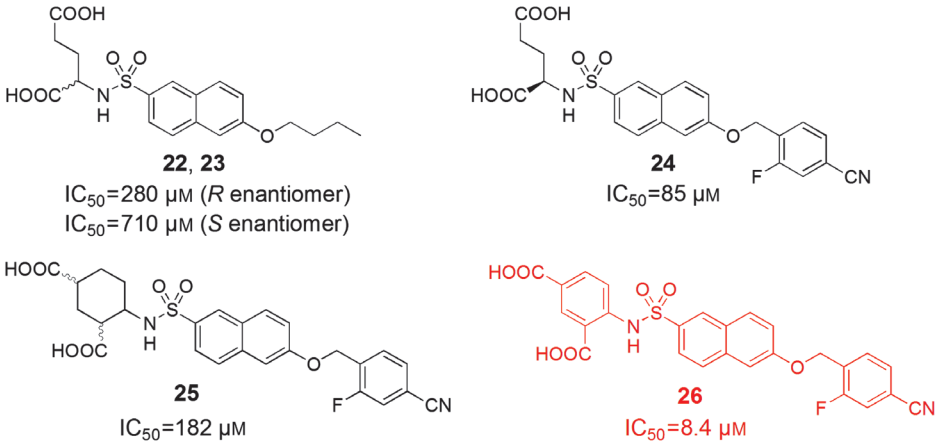


Figure 3 Binding modes of 5-benzylidenethiazolidin-2,4-dione and 5-benzylidenerhodanine inhibitors.
(A) Binding mode of MurD inhibitor **16** (magenta; PDB entry: 2X5O) superimposed to UDP-MurNAc-L-Ala-D-Glu (orange; PDB entry: 4UAG).
(B) Binding mode of MurD inhibitor **21** (PDB entry: 2Y1O).



Scheme 7 Representative naphthalene sulfonamides containing glutamic acid or rigid mimetics thereof. IC_{50} values were determined with *E. coli* MurD. The best inhibitor **26** is depicted in red.

at the position of D-glutamic acid improved the inhibitory activities against MurD when compared to the parent compounds, thereby confirming the advantage of conformational restriction. The co-crystal structure of MurD with rigidified inhibitor **25** revealed that both carboxyl groups occupied exactly the same binding sites as the carboxyl groups of D-glutamic acid (Figure 4) (98). They formed typical interactions with Ser415, Leu416 and Phe422 on one hand, and Lys348 and His183 on the other hand. The 2-cyano-4-fluoro-phenyl ring occupied the uracil-binding pocket of the nucleotide substrate with a hydrogen bond between the cyano group and Thr36. The central naphthalene moiety did not interact with the protein at all, and this represented a major opportunity for the improvement in the potency of future generations of sulfonamide inhibitors, which should interact with MurD also in this region.

The second generation of naphthalene sulfonamide inhibitors of MurD was investigated by NMR using $^{1}H/^{13}C$ heteronuclear single quantum correlation. Conformational and dynamic properties of the bound ligands and their binding interactions were examined using the transferred nuclear Overhauser effect and saturation transfer difference, while the binding mode was examined using unrestrained molecular dynamics simulations. The discoveries in this research shed light on the dynamic behavior of MurD/ligand complexes and its influence on contacts between both partners (99).

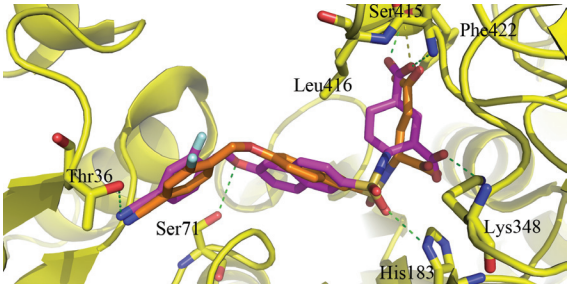


Figure 4 Binding mode of naphthalene sulfonamide **25** containing a rigid glutamic acid mimetic (magenta; PDB entry: 2XPC) superimposed to its D-glutamic acid-containing counterpart **24** (orange; PDB entry: 2VTD).

Amino acid sequence alignment of MurD enzymes from different bacterial species revealed that they do share conserved residues that are essential for the catalytic activity. However, the overall similarity is small. Considering this, it was not quite surprising that compounds that had been designed as inhibitors of MurD from *E. coli* turned out to be weaker inhibitors of other MurD orthologs (52). For example, compounds **15, 16, 20** (Scheme 6) and **22**, **24**, **25**, **26** (Scheme 7) either inhibited MurD enzymes from *S. aureus*, *S. pneumoniae*, *B. burgdorferi* and *M. tuberculosis* in the 0.1–1 mmol/l range or were not inhibitors at all. This divergent result could be explained by differences in amino acid sequences and topologies of the active sites of the MurD ligases in question (52).

Recently, computational efforts were devoted to finding new MurD inhibitors (100–103), which resulted in some new and potentially interesting scaffolds (104).

Expert opinion

In part because of its propensity to yield high-quality crystal structures, MurD from *E. coli* is the Mur ligase for which a great deal of mechanistic and structural data has been gathered. For the same reason, many MurD inhibitors belonging to different classes have been described, some being

endowed with antibacterial activity. However, the fact that MurD orthologs from other pathogenic bacteria display less sensitivity to certain classes of inhibitors suggests that differences in active site topologies may be crucial for inhibitor recognition. Moreover, it remains to be demonstrated that the antibacterial activities already observed are not due to off-target activities. Therefore, we think that efforts should be devoted to the structural studies of MurD orthologs in order to find more powerful, panactive MurD inhibitors that could be developed into broad-spectrum antibacterial agents. Moreover, the best inhibitors should now be assayed for MurD inhibition *in vivo* to demonstrate that they indeed provoked an arrest of peptidoglycan synthesis in the susceptible species, which resulted from the depletion of the intracellular pool of UDP-MurNAc-pentapeptide. From cellular and physiological standpoints, the studies of protein complexes in which Mur ligases participate as well as the search for regulation mechanisms, which are still in their infancy, should be developed.

Outlook

The last two decades have seen major advances in the discovery of mechanistic and structural properties of MurD from *E. coli*. The study of MurD orthologs from important pathogenic organisms has started and should be pursued. Several classes of MurD inhibitors have been reported. Among the enzymes of peptidoglycan synthesis, the large amount of data now available makes MurD one of the best candidates as a target for new antibacterial agents. One of the most significant problems in the development of MurD inhibitors has been the lack of antibacterial activity of the majority of compounds synthesized so far. We believe that this is due to their poor penetration through the bacterial cell wall and/or to the rapid extrusion from the cell interior to the external environment via efflux pumps. A possible solution to this problem could be the design and synthesis of conjugates with siderophores, which would improve the penetration of inhibitors and enable them to accumulate within bacteria. The involvement of MurD (and other Mur ligases) in protein complexes and the deciphering of mechanisms of regulation are more fundamental topics that will undoubtedly be further developed.

Highlights

- MurD catalyzes the ATP-dependent addition of D-glutamic acid to UDP-MurNAc-L-Ala, a reaction which involves acyl-phosphate and tetrahedral intermediates.
- Several co-crystal structures of *E. coli* MurD with different ligands or inhibitors are available.
- Several MurD orthologs from pathogenic bacteria have been purified and characterized.
- Different classes of MurD inhibitors have been reported, some being endowed with antibacterial activity.
- The search for panactive MurD inhibitors, for which the mode of action is clearly demonstrated, has to be performed now.
- The study of cellular organization of Mur ligases and *in vivo* regulation of the MurD activity should be further developed.

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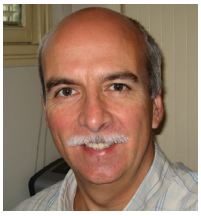
Roman Šink is a post-doctoral researcher at the Faculty of Pharmacy of the University of Ljubljana. He was trained in organic and medicinal chemistry, enzymology and X-ray crystallography. He obtained his PhD degree in pharmaceutical sciences from the University of Ljubljana in 2010. His main research topics have been the design, synthesis, and study of the binding modes of peptidoglycan biosynthetic pathway inhibitors. Currently his research activity focuses on the design and synthesis of new antituberculotics.

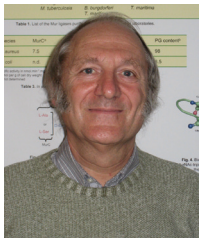
Hélène Barreteau is assistant professor at the University Paris-Sud, Orsay, and a member of the Laboratory of Bacterial Envelopes and Antibiotics. She was trained in biochemistry and microbiology, and received her PhD degree in biochemistry from the University Picardie Jules-Verne, Amiens, in 2004. She is especially interested in the search for antimicrobial compounds and has been working on peptidoglycan as an antibacterial target for 8 years.

Delphine Patin was trained in genetics and molecular biology at the University Paris Diderot, Paris. She received a Diplôme d'Etudes Approfondies in human genetics from the University Paris Diderot in 1999. She joined the Laboratory of Bacterial Envelopes and Antibiotics as an engineer in 2007. Her main research interests have been the study of Mur ligases from different bacterial species and the search for inhibitors of these enzymes.



Dominique Mengin-Lecreulx was trained in biochemistry, enzymology, molecular biology and organic chemistry and obtained his PhD degree at the University Paris-Sud in 1987. He is director of research at CNRS and, since 2001, is the head of the Laboratory of Bacterial Envelopes and Antibiotics which, for more than 30 years, has been studying the biochemistry, genetics and physiology of the bacterial cell-wall peptidoglycan biosynthesis pathway as a whole. His main research interests concern the identification of genes and enzymes involved in this pathway, the regulation of this metabolism, the mode of action of antibiotics and bacteriocins interfering with this pathway and the corresponding resistance mechanisms, as well as the molecular bases for the recognition of bacterial cell-wall peptidoglycan by host innate immunity mechanisms.

Stanislav Gobec is professor of medicinal chemistry at the Faculty of Pharmacy, University of Ljubljana. He graduated in pharmacy and received his PhD degree in medicinal chemistry from the University of Ljubljana in 1999. His main research interests are the structure-based design, synthesis and evaluation of small-molecule enzyme inhibitors and receptor ligands. For more than 15 years he has been involved in discovery of inhibitors of bacterial cell-wall biosynthesis as potential antibacterial agents.



Didier Blanot is director of research at CNRS and a member of the Laboratory of Bacterial Envelopes and Antibiotics, University Paris-Sud, Orsay. He was trained in organic chemistry, biochemistry and enzymology, and received his PhD degree in natural sciences from the University Paris-Sud in 1979. For more than 30 years, his main research interests have been the functional and structural study of the enzymes of peptidoglycan biosynthesis, the determination of the structure of peptidoglycan from certain bacterial species, and the search for peptidoglycan biosynthesis inhibitors that could act as antibacterial agents.