

Drug Discovery Targeting Amino Acid Racemases

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1. INTRODUCTION

Amino acids are among the most important molecules in nature since they play central roles both as building blocks of proteins and as intermediates in metabolism. The amino acid sequence dictates protein folding, the native three-dimensional structure, and protein stability. Furthermore, the chemical properties of the amino acid residues forming the active site determine the peculiar environment that allows for protein function and regulation.

All amino acids found in proteins, except glycine, possess a stereogenic center at the α -carbon atom. Millions of years of evolution have resulted in the virtually complete homochirality of such a stereogenic center, that is, the L-enantiomer, in mammals.¹ This selection of the L-amino acids by nature is generally considered to be a result of chance.²

Since the cornerstone of the protein–ligand recognition is the multipoint attachment theory, it turns out that the configuration of the α -carbon atom of amino acids strongly affects the protein–ligand interaction. Nevertheless, during the last half of the twentieth century, various studies evidenced the presence of D-amino acids in some plants and bacteria.^{3,4} These compounds were either found in a free state or in peptides and proteins. Most bacteria produce significant amounts of D-alanine (D-Ala) and D-glutamate (D-Glu), which are incorporated into peptidoglycan.⁵ Peptidoglycan is a strong and elastic polymer of the bacterial wall, which is capable to counteract the osmotic pressure of the cell, maintaining cell shape and anchoring components of the cell envelope.⁶ The number of D-amino acids present in the structure of peptidoglycan seems to constitute a measure of protection against peptidase and protease attacks. So far, no peptidase capable of hydrolyzing a peptide bond characterized by the sequence D–D or D–L amino acids has been isolated in mammals. In addition, several antibiotics produced by prokaryotes (e.g., bacitracin, actinomycin D) contain D-amino acids (Figure 1). It has been recently demonstrated that bacteria synthesize a pool of different D-amino

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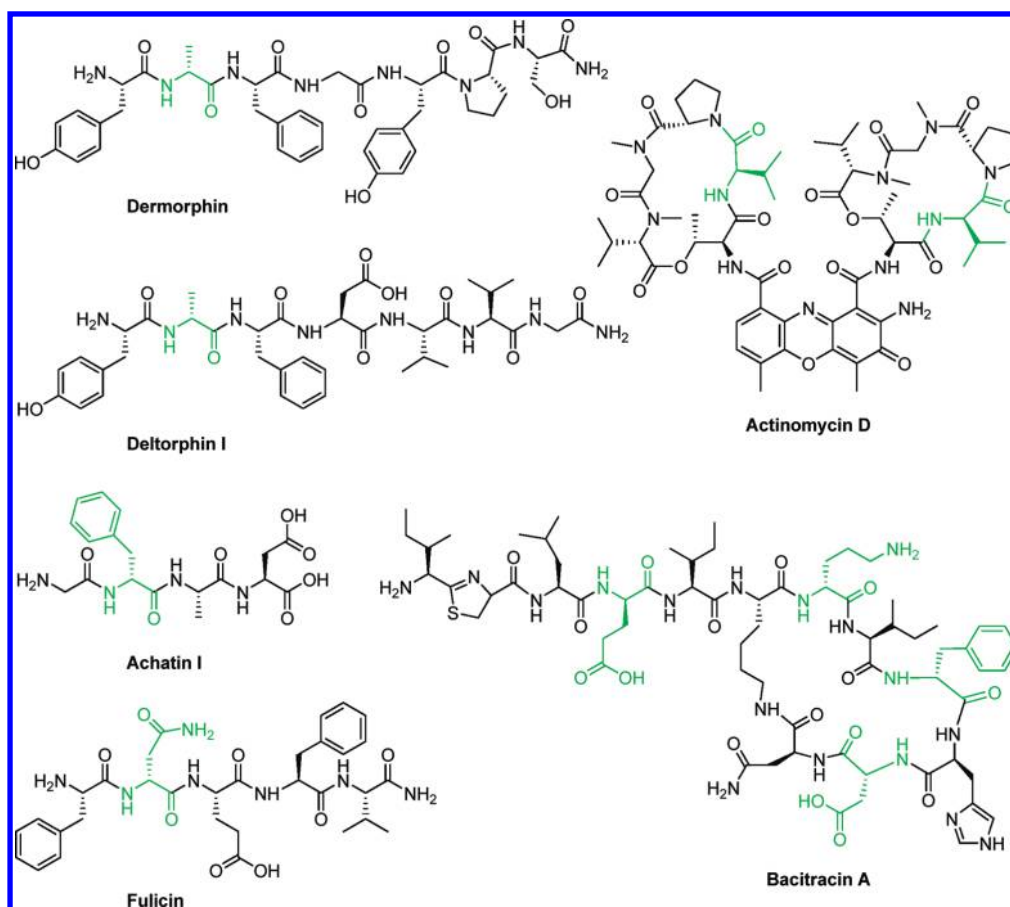


Figure 1

acids, including D-methionine (D-Met) and D-leucine (D-Leu) in *Vibrio cholerae* and D-tyrosine (D-Tyr) and D-phenylalanine (D-Phe) in *Bacillus subtilis*. By selectively incorporating them in the peptidoglycan cell wall, bacteria cope with different environmental stresses.⁷

The first reports on D-amino acids in animal tissues were restricted to amphibians and invertebrates. As a matter of fact, dermorphin (Figure 1) was the first D-amino acid-containing peptide isolated from the skin of a frog; it is provided with an analgesic activity a thousand times greater than that of morphine.⁸ Worth noting, the replacement of D-Ala by its mirror image causes a sharp drop in activity indicating that D-Ala, located at position 2 of the amino acid sequence, is essential for its activity. Subsequently, D-amino acids have been detected in a variety of peptides synthesized by animal cells,⁹ including opioids (deltorphins) (Figure 1), neuropeptides (achatin and fulicin) (Figure 1) and a family of antimicrobial peptides named bombinins H. For example, a tetrapeptide, termed achatin I (Figure 1), containing a D-Phe at position 2, was isolated from the ganglia and atrium of the African giant snail *Achatina fulica*.¹⁰ Also in this case the excitatory activity on heart or other muscles is lost by replacing D-Phe with L-phenylalanine (L-Phe).

Recent developments of highly sensitive chromatographic techniques allowed the detection of low concentrations of free D-amino acids in mammals, including humans. The principal free D-amino acids found in significant amount in animal tissues are D-Ala, D-serine (D-Ser), and D-aspartate (D-Asp). In particular, D-Ser was detected predominantly in the mammalian brain. The specific biological function of these D-amino acids remained

unknown for long time, in spite of the fact that in 1935 Krebs discovered the presence of a flavin-containing enzyme in mammals, the D-amino acid oxidase (DAAO), which catalyzes the oxidative deamination of D-amino acids to the corresponding α -keto acids with high enantiospecificity.¹¹ DAAO is an ubiquitous enzyme in eukaryotes, with a selective distribution that, in humans, includes kidney and cerebellum. The biological role of DAAO remained uncertain for a number of years. Since the presence of D-amino acids in mammalian tissues was initially attributed to the action of endogenous microbial flora, to ingestion with the diet or to spontaneous racemization of L-amino acids incorporated in polypeptides during aging,¹² it was suggested that such an enzyme acts as a detoxifying agent to remove D-amino acids. The subsequent detection of substantial amounts of D-amino acids in various human tissues and in the brain suggested the involvement of DAAO in modulating the level of D-amino acids. Moreover, it has been shown that the human brain contains endogenous D-Ser and D-Asp, which are likely to act as modulators of neurotransmission.^{13,14} Consequently, it has been hypothesized that DAAO may play a role in controlling the level of these neurotransmitters.

At present, the physiological significance of D-amino acids, that is, D-Ser and D-Asp, is well-established. As a consequence, it is conceivable to admit the presence in the human body of a pathway, which involves specific enzymes, for the synthesis of such D-amino acids. These enzymes have been discovered, structurally characterized and termed *racemases*. The different racemases, which have been discovered in mammals and in bacteria, may differ

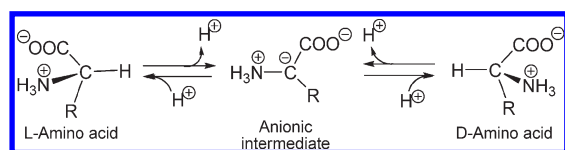
Table 1. PLP-Dependent Amino Acid Racemases

	localization/function	substrates
alanine racemase	bacteria (peptidoglycan synthesis); eukaryotes (biosynthesis of D-aa containing peptides)	Ala
serine racemase	bacteria (vancomycin resistance); mammalian (CNS/biosynthesis of the neuromodulator D-Ser)	Ser
arginine racemase	bacteria (catabolism of D-Arg and D-Lys)	Arg, Lys, ornithine, other basic amino acids
aspartate racemase	Ark shell (storage of D-Asp as an energy source under anaerobic condition); mammalian (CNS, biosynthesis of the neurotransmitter D-Asp)	Asp

Table 2. PLP-Independent Amino Acid Racemases

	localization/function	substrates
proline racemase	bacteria (proline and arginine metabolism); eukaryotes (<i>T. cruzi</i> / <i>T. vivax</i> , proline metabolism and catabolism, B-cell mitogen, parasite evasion mechanisms)	Pro
glutamate racemase	bacteria (peptidoglycan synthesis)	Glu
aspartate racemase	bacteria (peptidoglycan synthesis)	Asp
diaminopimelate epimerase	bacteria and plants (biosynthesis of L-Lys)	DAP

Scheme 1. Racemization of an Amino Acid



considerably with respect to substrate specificity. For example, arginine racemase (ArgR) is mostly active with lysine, arginine and ornithine, and merely weakly active with alanine, whereas alanine racemase (AlaR) acts on alanine only.

In the following chapters we will discuss the classification of racemases, their diffusion, function, localization, structural features and the state of the art in the discovery of novel drugs targeting such enzymes for the treatment of different pathologies.

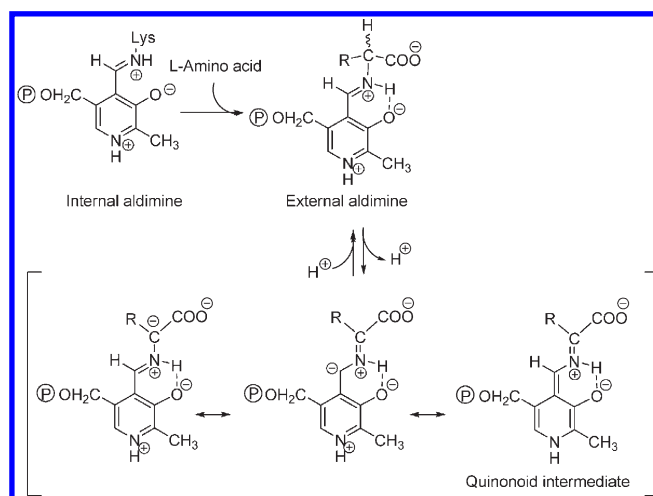
2. CLASSIFICATION AND CATALYTIC MECHANISMS OF RACEMASES

The array of racemases can be divided in two classes on the basis of their mode of action: (1) pyridoxal 5'-phosphate-dependent amino acid racemases and (2) pyridoxal 5'-phosphate-independent racemases.

The first group of racemases depends on pyridoxal 5'-phosphate (PLP), the coenzyme representing the biologically active form of vitamin B6. Its members are exemplified by eukaryotic enzymes, such as serine racemase (SerR), AlaR, and aspartate racemase (DR), and by bacterial enzymes, such as AlaR, SerR, and ArgR (Table 1). The second group of racemases, which are cofactor-independent, includes aspartate racemase (AspR), glutamate racemase (GluR), proline racemase (ProR) as well as diaminopimelate epimerase (DAPE) (Table 2). These enzymes occur in bacteria and archaea, except for ProR that was found in eukaryotic *Trypanosoma spp.*

In both cases, the racemases catalyze the abstraction of the C α -hydrogen of an amino acid as a proton to form a planar carboanionic transition state, followed by the return of the proton to the same carbon from either the same or the opposite side (Scheme 1). The stereochemical inversion of an amino acid works in both directions, that is, L \rightarrow D and D \rightarrow L.

Scheme 2. Role of PLP in the Racemization of Amino Acids



2.1. PLP-Dependent Amino Acid Racemases

Since the pK_a value of the amino acid C α -hydrogen usually spans the range of 21–34,¹⁵ several enzymes evolved to employ the PLP coenzyme in order to increase the acidity of such a hydrogen.^{16–19} All known PLP-dependent enzymes exist in their native state as an internal aldimine (Schiff base) (Scheme 2) with the ϵ -amino group of a lysine residue present in the catalytic site. The amino moiety of the amino acid substrate displaces the lysine from the internal aldimine to form a new aldimine, termed external aldimine, which is characterized by a sizable acidity of the C α -hydrogen of the substrate (Scheme 2). Typically, the formation of such an external aldimine brings about a sharp drop in the pK_a value of the substrate from 21–34 to 6–17.^{20,21} This huge reduction in the pK_a value is due to the delocalization of the negative charge of the anionic intermediate by resonance through the conjugated π system extending on the PLP ring. The scissile bond of the external aldimine must be oriented perpendicularly to the coenzyme planar π -bonding system to maximize the σ – π orbital overlap in the transition state,²² as recognized by crystallographic studies carried out on

Scheme 3. Two-Base Mechanism for PLP-Independent Racemases



PLP-dependent enzymes.^{23–25} The process of racemization takes place because the α -hydrogen of the external aldimine, due to its substantial acidity, can be easily abstracted as a proton to form an anionic planar intermediate which, subsequently, undergoes a reprotonation on either the *re*- or *si*-faces of the substrate-cofactor complex. The mechanism of racemization of the PLP-dependent enzymes as well as the three most significant resonance forms of the anionic intermediate are depicted in Scheme 2. The quinonoid resonance structure is commonly considered the major species responsible for the catalytic power of PLP, in which the electrons from C α are neutralized by the positively charged pyridine nitrogen.

The PLP-dependent enzymes have been classified, on the basis of their tertiary structures, into five groups, namely, fold type I–V^{26,27} and they probably evolved from at least three different ancestral proteins.

The fold type I is the most common and best known structure, and is found in a variety of aminotransferases and decarboxylases, as well as in enzymes that catalyze an α -, β -, or γ -elimination reaction. The prototype of the fold type I enzyme is aspartate aminotransferase. This family includes enzymes that are catalytically active as homodimers, but some of them assemble into larger complexes. Each subunit folds into two domains: a large domain, in which the central feature is a seven stranded β sheet, and a small domain, which folds into a three- or four-stranded β sheet covered with helices on one side. The active site is located in a cleft between the two domains, at the interface between the two subunits of the dimer. All enzymes belonging to this fold type show an aspartate interacting with the pyridine nitrogen. In turn, the enzymes belonging to the fold type I can be divided into eight subtypes.²⁸

The fold type II is found mainly in enzymes that catalyze β -replacement and β -elimination reactions. The prototype of the fold type II enzyme is tryptophan synthase. SerR^{29–31} belongs to this fold type. Each subunit consists of two domains: an N-terminal domain containing a four-stranded sheet surrounded by helices, and a C-terminal domain built up by a six-stranded sheet with flanking helices. The active site is located in a cleft between the C-terminal ends of the two β sheets. At variance with fold type I enzymes, a serine residue interacts with the pyridine nitrogen, rather than an aspartate.

The fold type III family includes AlaR, ArgR, and a subset of amino acid decarboxylases. The subunit is characterized by an eight-stranded α/β barrel and a domain mainly comprising β strands. The side chain of an arginine residue forms a hydrogen bond with the pyridine nitrogen, indicating that protonation of the pyridine nitrogen is not crucial for the catalytic activity.

The fold-type IV subset comprises D-Ala aminotransferase and a few other enzymes. The fold consists of a two-domain structure with the active site located at the domain interface. Two identical subunits form a catalytic dimer. However, branched chain aminotransferase further assembles into a hexamer. The smaller N-terminal domain contains a six-stranded antiparallel β sheet with two α helices on one side. In the larger C-terminal domain, two four-stranded β sheets form a pseudo- β -barrel that is surrounded by a few helices.

Finally, the fold-type V group includes glycogen and starch phosphorylases. The C-terminal domain has a dinucleotide-binding fold and binds the cofactor PLP. In these enzymes, the catalytic role of PLP is via its phosphate moiety, thus diverging from the coenzyme action in the enzymes belonging to the other four types.

In all the five types, a covalent Schiff-base linkage with lysine, present in the active site, is used to anchor the cofactor to the enzyme in the resting state and the phosphate group is bound to the N terminus of an α helix. In four of the five folds, the catalytic lysine residue is located in the connection between a β strand and the following α helix. In contrast to the classical binding mode of, for example, NAD(P)H or thiamine diphosphate to open α/β structures, crossover connections to adjacent parallel strands in the sheet are, with the exception of fold type II, not utilized for PLP binding.

2.2. PLP-Independent Amino Acid Racemases

The members of this second class of enzymes, which includes proline, aspartate, and glutamate racemases, as well as DAPE operate through a “two-base” mechanism in a cofactor-independent manner, as first proposed by pioneering work on ProR from *Abeles’* group^{32,33} (Scheme 3).

All these enzymes are members of the class of proteins that contain both α and β secondary structural elements. In particular, AspR and GluR belong to the aspartate transcarbamylase (ATC)-like fold, while ProR and DAPE belong to the diaminiopimelate epimerase-like fold. The former fold consists of two similar domains related by a pseudodyad axis, whereas the latter fold consists of mixed beta-sheet folds into a barrel around the central helix.

ProR has been considered the prototype of PLP-independent racemases, as it interchanges L and D enantiomers without the aid of any cofactor. A partially purified preparation of ProR from the Gram-positive bacterium *Clostridium sticklandii* was first described by Stadtman and Elliot in 1957.³⁴ The authors also established the sulfhydryl nature of the enzyme and they speculated that PLP was not involved in the racemization of Pro. It was only ten years later that Cardinale and Abeles published, for the first time, the isolation of ProR in a highly purified form and described a conceivable mechanism of catalysis.³² The reported studies on isotope incorporation and enzyme kinetics in D₂O demonstrated a “two-base” mechanism in which one base of the enzyme removes the substrate α -hydrogen as a proton, while the conjugate acid of the second base delivers a proton to the opposite side of the α -carbon. The Authors also observed that deuterium can not be exchanged while the active site is occupied, indicating that the two bases of the catalytic site are monoprotic. In 1975, the same group hypothesized that the active site of ProR is located at the interface of two identical, or nearly identical, subunits.³³ Each of these two subunits supplies one base, which were then identified as the thiol group of two cysteines. Moreover, at saturating substrate levels, the initial rate of the enzyme-catalyzed release of ³H from L-[2-³H]-proline into the solvent is independent of the initial L-proline (L-Pro) concentration, whereas the initial rate of the enzyme-catalyzed release of ³H from (±)-[2-³H]-proline decreases by increasing the (±)-Pro concentration. On the basis of

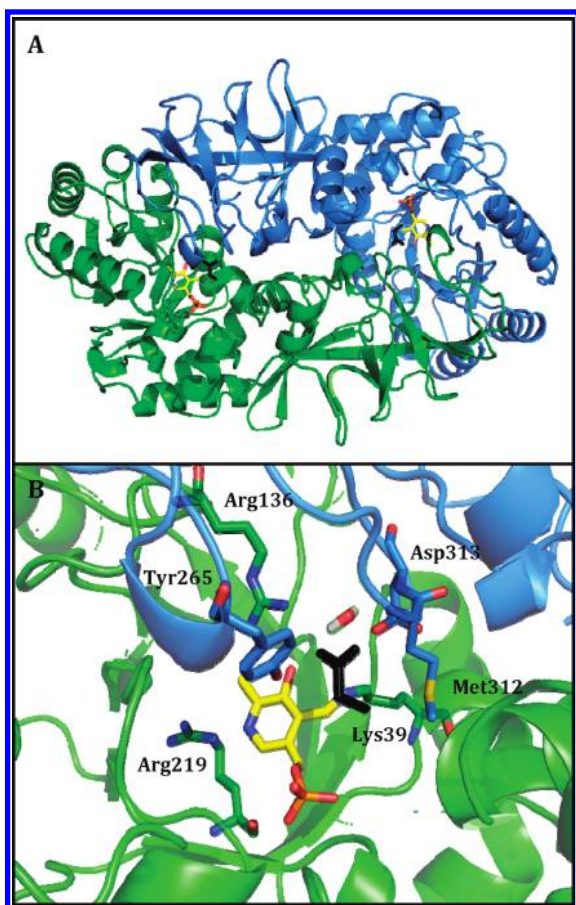


Figure 2. A) Three-dimensional structure of the dimeric AlaR from *B. stearothermophilus* complexed with propionate (PDB code 2sfp⁵⁷). B) Close up of the binding pocket. The PLP is colored in yellow and the propionate in black. The residues involved in PLP and ligand binding, and participating to the catalytic processes are represented in capped sticks. The water molecule located in the binding pocket and likely involved in the stabilization of the complex is also highlighted.

these observations, the Authors speculated that there are two forms of the enzyme, one which is able to bind L-Pro, while the other recognizes solely the D-enantiomer.

Fundamental studies by Knowles and co-workers on ProR³⁵ further clarified the mode of action of this enzyme deepening its energetics and mechanism. Subsequently, the same authors extended their work to GluR,³⁶ confirming the same “two-base” catalytic mechanism of racemization, in which one cysteine residue behaves as a base abstracting the C- α proton to generate a carbanion intermediate, which is then protonated by the second Cys residue, behaving as a conjugated acid. Such a catalytic mechanism is also shared by AspR³⁷ and DAPE.³⁸ Although they all operate through a similar acid/base mechanism, involving two Cys residues, it is possible to separate the PLP-independent racemases into two subgroups on the basis of sequence and structural similarity: GluR and AspR constitute one homologous family of enzymes, whereas ProR and DAPE form the other one.

3. AMINO ACID RACEMASES AS DRUG TARGETS

3.1. PLP-Dependent Racemases

3.1.1. Alanine Racemase. **3.1.1.1. Localization, Structure, and Function.** AlaR (EC 5.1.1.1), one of the PLP-dependent

racemases, is ubiquitous in bacteria and only a few similar enzymes have been found in eukaryotes, such as aquatic animals,³⁹ fungi,^{40,41} fission yeast⁴² and plants.⁴³ The enzyme produced by pathogenic fungi, such as *Tolypocladium niveum* and *Cochliobolus carbonum*, is responsible for the synthesis of D-Ala in cyclosporin A, an immunosuppressant drug widely used in postallogeic organ transplant, as well as in HC-toxin, a cyclic peptide isolated from varieties of maize (*Zea mays* L.) infected by *C. carbonum*.

Bacterial AlaR is one of the best-studied amino acid racemases. In fact, D-Ala, produced by the action of AlaR on L-Alanine (L-Ala), is important to both Gram-positive and Gram-negative bacteria, since it is required for the synthesis of the peptidoglycan in the cell wall. Because peptidoglycan is ubiquitously distributed in bacteria, but not in mammals, AlaR can be considered a target for new antimicrobial drugs. Several bacteria, such as *Salmonella typhimurium*, *Escherichia coli* and *Bacillus subtilis* contain two independent AlaR genes, DadB and Alr,⁴⁴ belonging to the fold-type III. The expression of DadB is induced when cells are grown in high concentration of L- or D-Ala and is probably responsible for the catabolism of D-Ala.⁴⁵ By contrast, Alr is constitutively expressed and is probably involved in the synthesis of D-Ala. Moreover, recent studies have indicated that AlaR, which is accessible in the exosporium, plays a key role in the inhibition of germination in *Bacillus* spores.⁴⁶ As a matter of fact, while L-Ala is an effective germination-promoting compound, its mirror image (D-Ala) is a potent inhibitor of L-Ala-induced germination.⁴⁷ Therefore, a compound capable to inhibit AlaR activity could trigger a premature germination leading to the death of *Bacillus* spores in suboptimal environments.^{48,49} *Bacillus anthracis*, a spore-forming Gram-positive bacterium, is the causative agent of the zoonotic disease anthrax. Although the disease is most common in wild and domestic mammals, it can also occur in humans when exposed to infected animals or living spores.⁵⁰ Inhalation of *B. anthracis* spores can lead to the most severe form of the disease, historically associated with a very high mortality rate.^{51,52} Triggering the premature germination of *B. anthracis* spores by spraying a solution of an AlaR inhibitor on affected areas might be a strategy to speed decontamination efforts and reduce the risk of infection in humans. The crystal structure of AlaR from *Bacillus stearothermophilus* and the complexes between AlaR and two inhibitors, namely propionate and 1-aminoethylphosphonic acid, have been reported (Figure 2).^{53–57} The crystal structure revealed that the enzyme is a homodimer of 388 residues.⁵⁶ Each monomer is composed of two folded domains: (i) a N-terminal domain formed by the portion 1–240 and (ii) a C-terminal domain which involves the remaining portion of the monomer (241–388). The N-terminal domain is made up of an eight-stranded α/β -barrel, while the C-terminal domain contains mainly β -strands. A water molecule was identified in both active sites of the AlaR homodimer.⁵⁷ This water molecule plays an important role and should be taken into account for the design of specific AlaR inhibitors either by utilizing it as a bridging group or by displacing it by a suitable moiety appended to the inhibitor.

An interesting feature of AlaR is that the pyridine ring of PLP is not protonated because of the contiguity with Arg219,⁵⁸ which possesses a pKa value in water of about 13. The X-ray crystal structure evidenced this structural feature where Arg219 interacts with the PLP pyridine nitrogen through a hydrogen bond, preventing the formation of the cationic pyridinium ion (Figure 2B).⁵⁶ Therefore, the main catalytic effect of PLP in

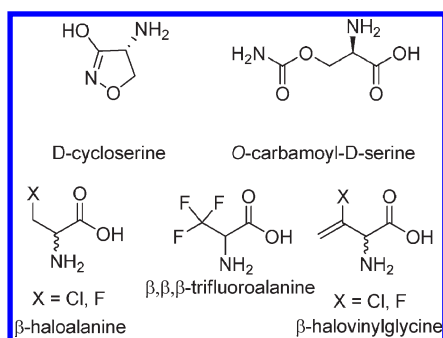


Figure 3

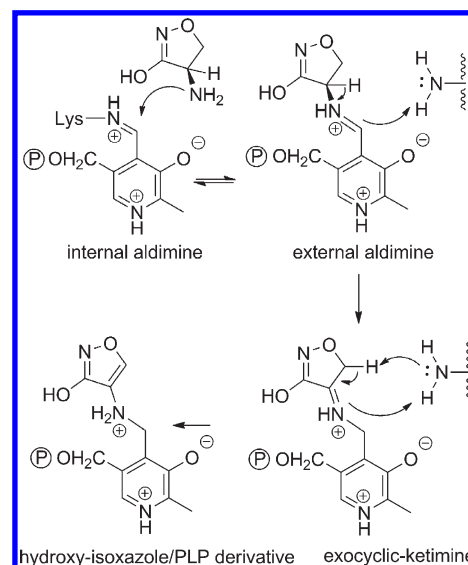
AlaR may be ascribed to solvation.^{59–61} It has been assumed that in such an enzyme the nonprotonated form generates an intermediate with a limited lifetime, which aids the enzyme in avoiding side reactions.⁶² Quantum mechanical and molecular dynamics simulations were recently carried out on the wild type and Arg219Glu mutant.^{59,63} Results suggest that in the wild type the stabilization of the quinonoid intermediate is due to solvent effects whereas in the mutant also Glu219 contributes to catalysis.

An alternative mechanism of action involving two bases, that is, lysine (Lys39) and tyrosine (Tyr265'), has also been proposed. The lysine residue, connected to the PLP cofactor, acts as a base for the conversion of D-Ala into L-Ala, while a nearby tyrosine localized on the other monomer performs as a base to abstract the C α -hydrogen from L-Ala.^{64–66} The role of Tyr265' was supported by the theoretical model published by Ondrechen et al.⁶⁷ Because of the low pK_a value, the phenolic group of Tyr265' is predicted to be in the anionic form in the pH range 7–9 where the racemase is most active. It was also reported that a small fraction of Lys39 is uncharged in the same pH range. Therefore, Lys39 can be available as a neutral amine and can behave as the catalytic base for the conversion of D-Ala into L-Ala. Moreover, AlaR has been shown to be capable to catalyze a transamination reaction.^{26,68} The enzyme catalyzes one or the other transformation at different pH values. While the racemization process has its optimum pH in the range 7–10, the rate of the transamination reaches its peak value around pH 6.5.⁶⁸

3.1.1.2. Inhibitors and Drug Perspectives. Two well-known natural inhibitors of AlaR are D-cycloserine and O-carbamoyl-D-serine (Figure 3).⁶⁹ These compounds inhibit AlaR of many bacteria, e.g., *S. aureus*,⁷⁰ *E. coli*,⁷¹ and *B. subtilis*,⁷² forming a covalent adduct with the PLP cofactor of the protein.⁷³ Both L- and D- enantiomers act as irreversible suicide inhibitors of AlaR^{71,74} and, thus, can be used as antimicrobial agents. Moreover, cycloserine has been shown to inhibit D-Ala–D-Ala ligase in vitro.⁷⁵

D-Cycloserine is an effective second-line drug against *Mycobacterium avium* and *Mycobacterium tuberculosis* and has been commercialized as Seromycin, which is indicated in the treatment of active pulmonary and extrapulmonary tuberculosis, when the treatment with the primary medications (streptomycin, isoniazid, rifampin, and ethambutol) proved to be inadequate. Moreover, the drug is used for treating acute urinary tract infections caused by susceptible strains of Gram-positive and Gram-negative bacteria, especially *Enterobacter spp.* and *E. coli*. Unfortunately, cycloserine can provoke toxic side effects, in particular on central nervous system (CNS), such as seizures, psychosis, confusion, tremor, vertigo, hyperreflexia or dysarthria.⁷⁶

Scheme 4

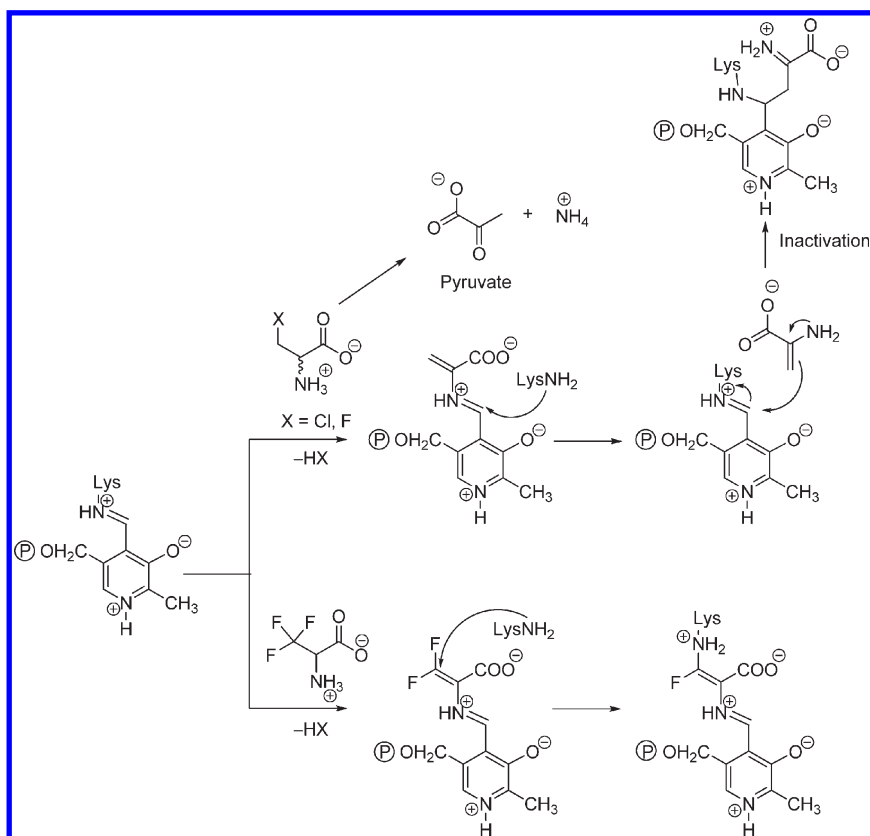


The toxicity of Seromycin is closely related to excessive blood levels (above 30 $\mu\text{g/mL}$) due to a high dosage or to an inadequate renal clearance. The cause of these neuronal disorders is probably connected to the activity of D-cycloserine as partial agonist of the glycine site of N-methyl-D-aspartate (NMDA) receptors⁷⁷ and as an inhibitor of PLP-dependent enzymes involved in the biosynthesis and metabolism of the neurotransmitter γ -aminobutyric acid (GABA).⁷⁸ Moreover, resistance has been observed in many clinical situations.⁷⁹ Since cycloserine is not a specific inhibitor of AlaR but can inhibit several PLP-dependent enzymes,⁸⁰ more than one mechanism leading to cycloserine resistance is likely to occur. Cycloserine may enter the bacterial cell by either diffusion or uptake via the D-Ala/glycine transport system.⁷³ Mutations in the transporter gene, which cause a reduced cycloserine binding or elimination of the transporter from the cell surface, affect the drug uptake and increase the resistance. Analogously, a mutation in a gene coding for an efflux pump may lead to a higher affinity for cycloserine increasing the efficiency of drug expulsion. Alternatively, it is possible that a mutation increases the affinity or the levels of a drug-detoxifying enzyme, which would derivatize or hydrolyze cycloserine. The overproduction of a protein target could lead to increased resistance because of the sequestration or removal of the drug by the excess target protein. To this end, it was shown that a natural mechanism of resistance to the drug is the overproduction of AlaR because of a promoter-up mutation.⁸¹

NMR and IR spectra indicate that cycloserine exists in solution primarily in the lactim form. Recent studies suggest a mechanism of inactivation that results in aromatization of cycloserine (Scheme 4).⁸² This mechanism requires an initial transamination step to form an external aldimine which subsequently undergoes a proton shift to yield an exocyclic ketimine. Such an exocyclic ketimine, which is no more conjugated with the pyridine ring, proceeds through a second prototropic shift to form the stable covalent adduct hydroxy-isoxazole/PLP.⁷³

Cycloserine can be considered a model compound for the development of similar derivatives capable to inactivate PLP-dependent enzymes. The goal is focused on the discovery of drug candidates characterized by a high selectivity for AlaR.

Scheme 5



The most successful approach to design inhibitors of PLP-dependent enzymes consists in incorporating olefinic, acetylenic, or halogen functional groups in the substrates of the target enzymes. In fact, many D-Ala analogues exhibit a remarkable inhibitory activity if they bear a halogen in a position suitable for a nucleophilic attack, i.e. β -chloroalanine, β -fluoroalanine,^{83,84} β,β,β -trifluoroalanine,⁸⁵ β -halovinylglycine (Figure 3).⁸⁶ The inactivation of AlaR by β -haloalanines and trifluoroalanine proceeds via two different mechanisms.⁸⁵ In both cases, the first step is invariably the formation of the cross-conjugated enamino-PLP species through the nucleophilic attack of the nascent aminoacrylate on the PLP-lysine aldimine (Scheme 5).⁸⁷ Such an intermediate can undergo a bifurcated route: (i) if the β -carbon atom bears a single halogen (β -haloalanines), the electrophilicity at the olefinic terminus is insufficient to trap the nearby nucleophile, that is, the ϵ -NH₂ of the active-site Lys38, and the nucleophilic attack occurs at the aldimine carbon atom or (ii) if the β -carbon atom bears more halogens, that is, trifluoroalanine, the amino group of Lys38 acts as a nucleophile and attacks the highly electron deficient difluoroenamino moiety. In both cases the enzyme is irreversibly inhibited but, whereas in the first route the inactivation is caused by the alkylation of the PLP-cofactor, in the second route the inhibition of the enzyme is due to the formation of a covalent bond between the ϵ -NH₂ of Lys38 and the β position of the fluoro-aminoacrylate. An important indicator of potency of irreversible inhibitors is the number of turnovers per inactivation event, termed *partition ratio*. This parameter measures the frequency (for turnover to inactivation) with which an alkylation of the active site occurs causing the inactivation of the enzyme before the electrophilic species uncovered in turnover can

be deactivated chemically or physically (e.g., product release) at the active site. With a lower partition ratio, the amount of drug required to fully inhibit a given amount of enzyme is reduced. Consequently, the release of activated metabolites, which could interact with other cellular constituents, is diminished with an amelioration of the pharmacological profile. In the case of β -haloalanines, a side reaction that leads to the formation of pyruvate and ammonia without inactivation of the enzyme can take place (Scheme 5).⁸⁸

Racemic β -fluoroalanine was the first drug candidate prepared at Merck by Kollonitsch and colleagues; the D isomer was successfully validated as a potent, wide-spectrum, and orally active antibiotic (partition ratio ~ 800).^{83,89,90} Unfortunately, safety issues unrelated to its mechanism of action have hindered its development for clinical applications.

Halovinylglycines represent a class of potent AlaR inhibitors. At variance with haloalanines, in which the loss of HX leads to the formation of a nucleophile (aminoacrylate), the removal of the halide from halovinylglycines generates 2-amino-2,3-dienoic acid **1**, an allene characterized by a highly electrophilic reactivity (Scheme 6). Allene **1** can undergo the following three different transformations: (i) 57% of the reactive intermediate **1** is hydrolyzed to vinylglyoxylate **2**, a reactive Michael acceptor; (ii) 12% of the same intermediate reacts with the amino group of a lysine to yield paraquinoid **3** or its tautomer **4**, then the lysine-enamine linkage is hydrolyzed to α -aminoacetone **5** restoring the active form of the enzyme; (iii) the remaining 31% of allene **1** reacts with the same lysine residue to yield heterodiene **6**, which subsequently O-alkylates a tyrosine residue to give **7** and inactivating the enzyme in an irreversible manner.⁹¹ Fluorovinylglycines are much

Scheme 6

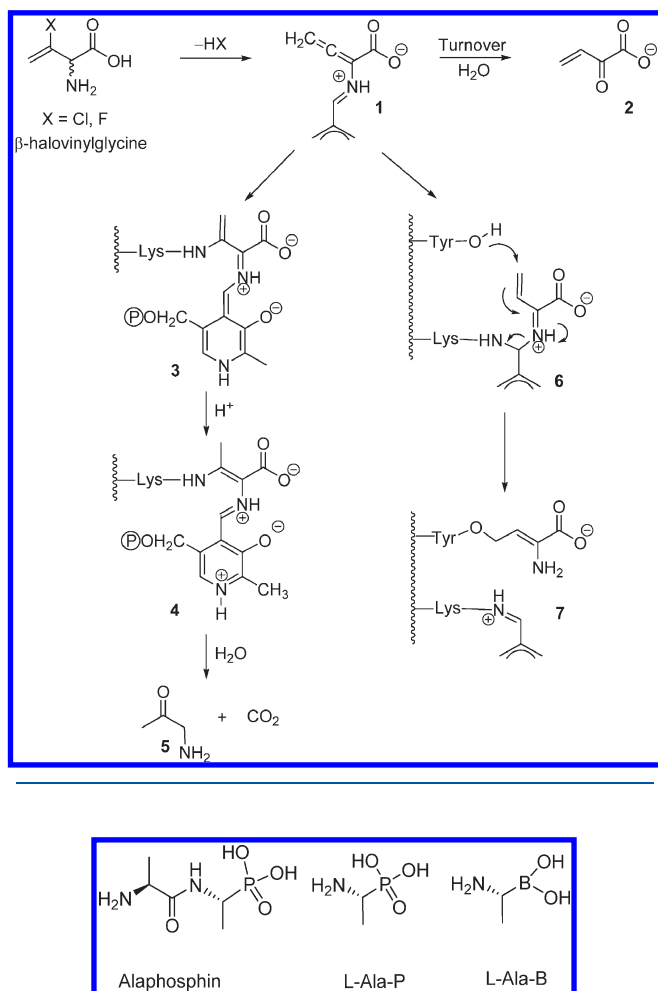


Figure 4

less reactive than chlorovinylglycines due to the higher stability of the C—F bond in comparison to the corresponding C—Cl bond. Furthermore, chlorovinylglycine is much more reactive than D-fluoroalanine, with a partition ratio of 2.2 ± 0.2 , a value 450-fold lower than that of the reference compound (partition ratio ≈ 800).

Unfortunately, their potency as antibiotics *in vivo* is not commensurate to their efficiency as inactivators of AlaR *in vitro*, probably because these inhibitors are not well recognized by the amino acid transport systems of the bacteria.

An interesting compound that selectively inhibits the biosynthesis of the bacterial cell wall is Alaphosphin (L-alanyl-L-1-aminoethylphosphonic acid, Figure 4). Alaphosphin is representative of a series of antibacterial phosphonopeptides which were designed to mimic the terminal dipeptide moiety (D-Ala-D-Ala) of the bacterial cell wall peptidoglycan. Alaphosphin efficiently crosses the bacterial cell wall and is hydrolyzed to L-Ala and (L)-(1-aminoethyl)phosphonic acid (L-Ala-P) by intracellular aminopeptidases. The (L,L) stereochemistry of Alaphosphin is necessary for its recognition by the transporters and to cross the cell wall. Its bactericidal activity arises from the inhibition activity of AlaR brought about by L-Ala-P. It is well documented that L-Ala-P is a competitive inhibitor of AlaR present in crude extracts of Gram-negative bacteria (*E. coli* and *S. typhimurium*).^{71,92-94} It is also a time-dependent and irreversible

inhibitor of AlaR isolated from Gram-positive bacterial sources (*Streptomyces aureus* and *Streptococcus faecalis*).⁹² L-Ala-P is characterized by a slow binding and a slow release process,^{95,96} with an extremely slow off rate ($t_{1/2} \approx 25$ days). The dianionic form of the phosphonate moiety may mimic a potential transition-state intermediate of the reaction and the two negative charges are crucial for the observed inhibitory activity of L-Ala-P. In 1998, Stamper et al. published the crystal structure of the complex of L-Ala-P with AlaR; this was the first report on the X-ray structure of an external aldimine involving this enzyme.⁵⁴

The replacement of the carboxylic group of L-Ala with the boronic moiety leads to (L)-(1-aminoethyl)boronic acid (L-Ala-B, Figure 4), which is a powerful slow binding and time-dependent inhibitor of both AlaR from *B. stearothermophilus* and D-Ala-D-Ala ligase from *S. typhimurium*.⁹⁷ L-Ala-B is less potent than L-Ala-P in terms of both K_i and lifetime of the enzyme-inhibitor complex, the two bioisosteres probably differently interacting with the enzyme active site.

In a perspective of developing more specific inhibitors for AlaR via a structure-based drug design, crystallographic structures from three species (*B. stearothermophilus*, *Pseudomonas aeruginosa*, and *M. tuberculosis*) have been solved and compared in detail.⁹⁸ The identity levels of the amino acid sequence between *M. tuberculosis* AlaR (*MtbAlaR*) and the two other published AlaR structures, that is, *P. aeruginosa* (*PaoAlaR*)⁵³ and *B. stearothermophilus* (*BstAlaR*),⁵⁶ are 32 and 34%, respectively. The secondary structure and overall fold of *MtbAlaR* are very similar to those observed in *PaoAlaR* and *BstAlaR*, confirming a strong architectural resemblance between prokaryotic AlaRs. The β -structure of the C-terminal domain and the geometry of the active site of *MtbAlaR* closely match that of *PaoAlaR* and *BstAlaR* (Figure 5). At the N-terminal domain, structural differences between *MtbAlaR*, *PaoAlaR*, and *BstAlaR* occur near the N-terminus and in some helices and loops located in the periphery of the α/β -barrel (Figure 5). Concerning the active site, the levels of sequence identity between the residues of *MtbAlaR* and those of *PaoAlaR* and *BstAlaR* are 44 and 50%, respectively. From the active site cavity containing the PLP moiety it is possible to access a second cavity on the opposite side. In *BstAlaR*, this accessory cavity contains four water molecules, whereas in *PaoAlaR*, it contains three solvent molecules. In *MtbAlaR*, the presence in this cavity of tryptophan (Trp88) reduces the free space, and only two water molecules can be accommodated. Moreover, it was shown the presence of a constriction site, created by two amino acid residues (Tyr271' and Tyr364), at the entryway corridor nearby the PLP. This corridor is roughly conical with its base oriented toward the outside of the enzyme. The corridor to the active site of AlaR can be ideally divided into three layers: outer, middle, and inner, with the inner layer terminating at the PLP moiety. In the inner and middle layers, all four residues of *MtbAlaR*, *PaoAlaR* and *BstAlaR* are identical and they are located similarly around the corridor. Residues in the outer layer near the surface of the enzyme are not conserved. Consequently, the design of novel inhibitors selective for one of the above-mentioned enzymes should be based on an extended pharmacophore model which takes into account interactions with amino acid residues located in the outer layer of the corridor.

Given the key role of AlaR in the formation of the bacterial wall, it is not surprising that activities aimed at developing efficient enzyme inhibitors are covering more than two decades. In spite of many unsuccessful strategies, new avenues are opened by the recent determinations of the crystal structures of AlaR

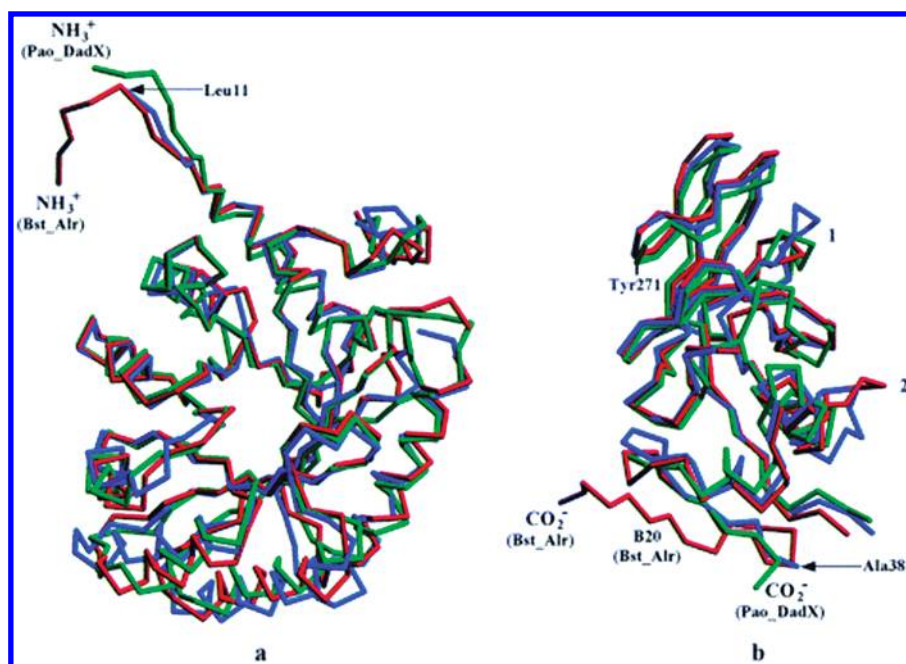


Figure 5. Least-squares superposition, reported by LeMagueres et al.⁹⁸ of *Mtb*AlaR (blue), *Pao*AlaR (green), and *Bst*AlaR (red) of (a) the N-terminal domain and (b) the C-terminal domain.

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from *Bacillus anthracis* in the presence and absence of L-Ala-P,⁹⁹ AlaR from *Streptococcus pneumoniae*,¹⁰⁰ AlaR from *Enterococcus faecalis*,¹⁰¹ AlaR from *Escherichia coli* in the absence and in the presence of cycloserine and four mutants of the active site residues Pro219 and Glu221,¹⁰² as well as the characterization and preliminary crystallographic studies on AlaR from *Bacillus pseudofirmus* OF4.^{103,104} These recent structural studies indicate that the entryway of the active site is very narrow, conserved residues are present in the active site as well as a string of water molecules, and dimer formation is a critical step for AlaR activity. Thus, it has been proposed¹⁰⁰ that strategies for AlaR inhibitors should be aimed at a high throughput screening (HTS) and structure-based methods for the identification of compounds that (i) occupy and block the entryway of the active site, (ii) interfere with active site assembly by preventing dimer formation,¹⁰⁵ (iii) enter in the active site with a specific conformation and, by interacting with active site residues, change conformation, eventually displacing water molecules.

Recently, modified forms of AlaR have been used as catalysts for chemical transformation. In 2003 Hilvert and co-workers showed that AlaR from *Geobacillus stearothermophilus* can be converted into a retro-aldolase enzyme by a single point mutation.¹⁰⁶ As a matter of fact, replacement of Tyr265, one of the catalytic bases, with Ala (alrY265A) decreased the original epimerase activity by more than 3 orders of magnitude.¹⁰⁷ In a following paper from the same group,¹⁰⁸ the alrY265A enzyme was used for the semipreparative production of β -hydroxy- α -amino acids. In fact, the mutant racemase promotes the PLP-dependent aldol condensation of glycine with a range of aromatic aldehydes, working similarly to natural D-threonine aldolases when substrate specificity and stereoselectivity are taken into account.

3.1.2. Serine Racemase. **3.1.2.1. Localization, Structure, and Function.** SerR (EC 5.1.1.18), another PLP-dependent racemase, was first discovered in 1998 in pupae of the silkworm *Bombyx mori*,¹⁰⁹ while the first purification of brain mammalian

SerR was performed by Wolosker and co-workers.^{110,111} SerR is the enzyme involved both in the reversible conversion of L- to D-Ser and serine catabolism by α,β -elimination of water, thereby regulating D-Ser levels.

In their pioneering work, Wolosker et al.¹¹⁰ showed that SerR is abundant in the glial cells of young rats. Recent immunohistochemical and in situ hybridization studies revealed the localization of the enzyme¹¹² and its mRNA¹¹³ in neurons and glia. Expression of SerR has also been shown in the peripheral tissues including retinal ganglion cells,¹¹⁴ Schwann cells,¹¹⁵ and chondrocytes.¹¹⁶

The existence of free D-Ser in the mammalian brain of both rats and humans was first reported by two Japanese laboratories.^{117–119} Sizable amounts of D-Ser are released by astrocytes, a type of glia cells, where SerR was later detected.¹¹⁰ D-Ser behaves as a “neuromodulator” acting as an agonist at the glycine site of NMDA receptors in the mammalian nervous system.¹²⁰ L-glutamate (L-Glu) is the main excitatory neurotransmitter in the CNS, where it is involved in the modulation of many physiological processes, such as learning, memory, and synaptic plasticity.¹²¹ However, a massive influx of L-Glu into the synapses can lead to acute and chronic neurodegenerative diseases (for example, cerebral ischemia, traumatic brain injury, stroke, spinal injury, epilepsy, and Parkinson’s, Alzheimer’s, and Huntington’s diseases).¹²² Memantine, a non competitive antagonist of the NMDA receptors, is the only glutamatergic drug currently approved for the treatment of moderate to severe Alzheimer’s disease. Because of its low affinity for the NMDA receptors and capability to block the channel in the open state, memantine does not substantially accumulate in the channel to interfere with the normal synaptic transmission.¹²³ An alternative approach to reduce glutamatergic hyperactivity is the use of blockers of the glycine site of the NMDA receptors. Unfortunately, the development of drugs with such a mode of action was discontinued since clinical trials evidenced the appearance of heavy adverse

effects, such as hallucinations.¹²⁴ These side effects were attributed to an excessive blockage of the NMDA receptors and alteration of the normal neurotransmission. In this context, inhibition of SerR may provide an alternative therapeutic approach,¹²⁵ since inhibitors of SerR could offer a more gentle and indirect way to decrease NMDA receptor function, with less undesired side effects.

A few years ago, Sasabe et al.¹²⁶ demonstrated that elevated levels of D-Ser in the glia may enhance Glu toxicity in Amyotrophic Lateral Sclerosis (ALS). Although the discovery of mutations in the gene encoding superoxide dismutase 1 (SOD 1) resulted in a considerable number of studies, the reason of the selective motoneuronal death is still unclear. An inefficient GluA2 RNA editing has been proposed as a cause of the death of motoneurons in sporadic ALS patients.¹²⁷ In addition, based on the experimental evidence that elevated level of Glu remained unchanged during the progression of the disease at variance with D-Ser whose level increases progressively along with the course of the pathology, Sasabe et al. speculated that the amount of D-Ser can be used to test the progress of ALS.¹²⁶ Consequently, the reduction of the concentration of D-Ser could be set forth to slow down or to halt the progression of ALS.

The increased glial production of D-Ser seems to be caused by pro-inflammatory stimuli that induce release of D-Ser from microglia by elevating the transcription of SerR.¹²⁸ At the same time, the excessive amounts of L-Glu in the spinal cord of ALS patients can improve the enzymatic activity of SerR in astrocytes, determining an increased level of D-Ser. A further cause of increased levels of D-Ser in ALS patients could be a failure in the D-Ser metabolic pathway, caused by the inactivation of DAAO, the degradation enzyme, and a decrease in the glial uptake. Thus, decreasing the D-Ser levels by inhibiting the enzymatic activity of SerR or by stimulating the activity of DAAO may represent a new therapeutic strategy to treat patients affected by ALS.¹²⁶

An altered level of D-Ser seems to be involved also in schizophrenia.¹²⁹ In this severely debilitating psychiatric disorder, reduced levels of D-Ser have been observed in the cerebrospinal fluid and serum, with a corresponding increase in its precursor, L-Ser.^{130,131} This finding points out the need to deepen the investigation on the involvement of D-Ser modulation in schizophrenia. Some recent studies indicated the malfunctioning of enzymes such as SerR, DAAO and G72, a putative activator of DAAO, as risk factors for the appearance of schizophrenia.^{132,133} SerR knockout mice have been used for determining the biological role of SerR and D-Ser. A recent review has summarized the phenotypes of the presently available three SerR-KO mice and discusses the role of SerR and D-Ser in vivo.¹³⁴

The initial isolation of SerR from mammalian brains¹¹⁰ also allowed the establishment of its molecular weight (37 kDa), optimum operational pH and K_m value. Subsequent studies from the same group¹³ led to the identification of Lys56 as the residue involved in the formation of the internal Schiff base with the PLP moiety. The crystal structure of a homologue of mammalian SerR from *Schizosaccharomyces pombe* was determined in the absence and presence of serine and in the absence and presence of an adenosine-5'-triphosphate (ATP) analogue.¹³⁵ More recently, the first crystal structure of mammalian SerR, both in the presence and absence of the inhibitor malonate, was determined (Figure 6).¹³⁶ A conformational change affecting the small domain and the active site was detected upon ligand binding

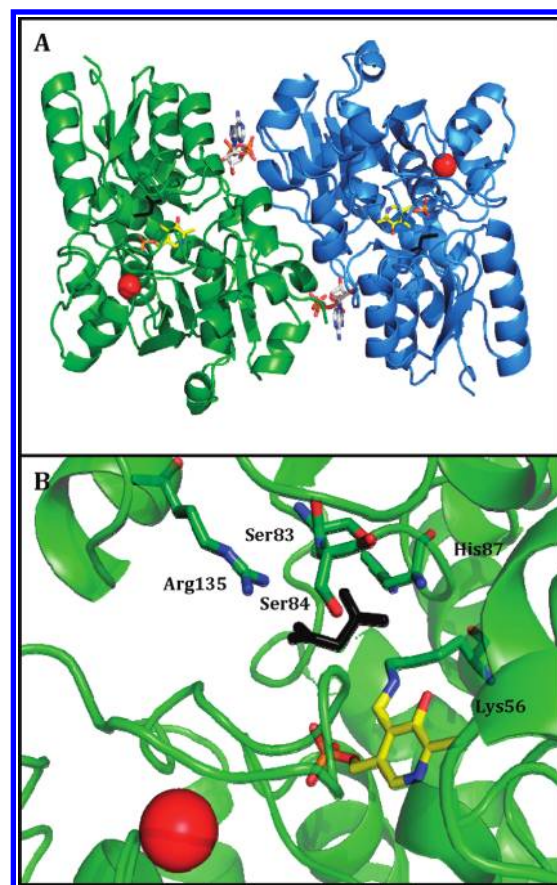


Figure 6. (A) Three-dimensional structure of human SerR complexed with malonate (PDB code 3l6b¹³⁶). (B) Close up view of the binding pocket. The PLP is colored in yellow, the malonate in black, and the manganese ion is represented as a red sphere. The nucleotide analogue phosphomethylphosphonic acid adenylate ester (AMP-PCP) molecules located at the dimer interface were extracted from the crystallographic structure of mammalian SerR from *Schizosaccharomyces pombe* (PDB code 1wtc¹³⁵) and superposed to the human structure.¹³⁶ The residues involved in the ligand binding and in the catalytic processes are highlighted in capped sticks.

with the volume of the active site significantly decreasing in the transition from the open to the closed state. Moreover, Ser84 was identified as the residue that plays a critical role in the catalytic racemization of serine, providing the C- α proton (Figure 6B). The X-ray data show that human and rat SerRs are 90% identical in sequence and are structurally almost indistinguishable, confirming the dimeric structure of the enzyme.

SerR, like AlaR, is a bifunctional enzyme since, in addition to the racemization of serine, it is able to catalyze the dehydration of serine to produce pyruvate and ammonia. The physiological relevance of this dual activity has been investigated.¹³⁷ By generating a SerR mutant (Q155D) with impaired elimination activity, it was demonstrated that the levels of D-Ser were several fold higher both in vitro and in vivo. This finding suggests that the irreversible elimination reaction, coupled to the reversible racemization controls the physiological level of D-Ser especially in the brain tissues lacking DAAO. Random mutagenesis studies on human SerR provided further evidence on the role of Ser84 in the enzyme activity, and on the role of Cys217 and Lys221 in Mg²⁺ binding and protein stability.¹³⁸

A peculiar feature of SerR is the multiple catalytic regulatory mechanisms, involving the interaction with (i) divalent cations, (ii) nucleotides, (iii) specific proteins, and (iv) NO.

- (i) Divalent cations, that is, calcium, magnesium and manganese, were shown to bind to the enzyme at a cation-binding site (Figure 6) via a hexavalent coordination composed by two carboxylate anions of amino acid residues, a carbonyl oxygen of the main-chain and three well-ordered water molecules.¹³⁹ In human SerR, the residues directly involved in the coordination of calcium are Glu210, Asp216, and Ala214. Divalent cations at physiological concentrations activate 5 to 10-fold the racemization and elimination reactions,¹⁴⁰ with EDTA favoring racemization over elimination. Since the cation binding site is relatively far from the active site, the cation effect is likely associated to a conformational change more than a direct involvement in the catalytic reaction. This behavior resembles the scenario previously observed in tryptophan synthase where monovalent cations bind to a site distinct from the active site affecting catalytic rates and intermediate equilibrium distribution.^{141,142}
- (ii) Nucleotides, such as ATP, adenosine diphosphate (ADP), and guanosine diphosphate (GDP), bind at a site localized at the subunit interface, although with different affinities (Figure 6A), increasing 5 to 10-fold the racemase and elimination activity. Since ATP is not hydrolyzed during the catalytic cycle, ATP acts as an allosteric effector.
- (iii) By means of the yeast two-hybrid approach, three proteins have been shown to interact with brain SerR: glutamate receptor interacting protein (GRIP),^{143,144} protein interacting with C kinase 1 (PICK1)¹⁴⁵ and Golga3.¹⁴⁶ Both GRIP and PICK1 bind to the four amino acids of SerR C-terminal (-Ser-Val-Ser-Val-COOH in the human enzyme and -Thr-Val-Ser-Val-COOH in the mouse enzyme), whereas Golga3 binds to the N-terminal 66 amino acids of brain SerR generating a conformational change of the enzyme which gives rise to an increased synthesis of D-Ser. PICK1 is known to direct protein kinase C (PKC) to its targets in cells. It has been recently found that PKC phosphorylating SerR at serine residues decreases SerR activity, thus, regulating D-Ser availability in the brain.¹⁴⁷ The level of SerR is dynamically regulated by glutamate. In fact, activation of metabotropic glutamate receptors leads to the cleavage of phosphatidylinositol (4,5)-bisphosphate (PIP2) by phospholipase C (PLC); the reduced concentration of PIP2 diminishes its inhibition effect on SerR.¹⁴⁸ On the other hand, GRIP, which is normally bound to 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) receptors, dissociates from them upon neuronal depolarization to induce SerR activation as a consequence of its association with the enzyme.¹⁴⁹ The activation of NMDA receptors by L-Glu (the agonist) and D-Ser (the coagonist) leads to the formation of nitric oxide which, by binding to SerR, inactivates the enzyme; this mechanism is a feedback homeostatic regulation of SerR.¹⁴⁹
- (iv) SerR is selectively nitrosylated at Cys113,¹⁴⁹ that is localized near the ATP binding site. The Cys-133 nitrosylation inhibits the enzyme activity likely by decreasing the stimulatory effect brought about by ATP. It was found that

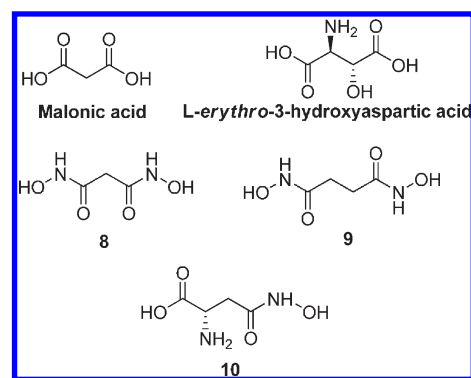


Figure 7

such a nitrosylation interferes with ATP binding causing a 40-fold increase in K_m for ATP without affecting V_{max} .

The S-nitrosylation does not affect K_m for L-Ser but causes a 2-fold decrease in V_{max} .

In bacteria, SerR has been implicated in vancomycin resistance.¹⁵⁰ Vancomycin is a glycopeptide antibiotic that inhibits peptidoglycan synthesis by formation of a complex with the D-Ala—D-Ala residue of the peptidoglycan precursors. Resistance to vancomycin is acquired by modification of the D-Ala—D-Ala residue. Depending on the phenotype, the second D-Ala residue can be mutated in D-lactate or in D-Ser, in turn produced by the action of SerR (VanT).¹⁵⁰

3.1.2.2. Inhibitors and Drug Perspectives. Very few potent and specific inhibitors of SerR have been identified to date; the two most potent competitive inhibitors, malonic acid and L-erythro-3-hydroxyaspartic acid,¹⁵¹ (Figure 7) possess inhibitory constants in the micromolar range ($K_i = 77 \mu\text{M}$ and $49 \mu\text{M}$, respectively). Recently, a series of hydroxamic and dihydroxamic acids (Figure 7) with potent inhibitory activity was identified by Hoffman et al.¹⁵² In this series, succinodihydroxamic acid **9** appears to be the most potent competitive SerR inhibitor identified to date ($K_i = 3.6 \pm 0.6 \mu\text{M}$). Unfortunately, although some dihydroxamic acids are effective SerR inhibitors (e.g. compounds **8** and **9**), their lack of specificity renders them unattractive candidates for further drug development. Their potent inhibition activity was initially attributed to their capacity to chelate Mg^{2+} , a potent SerR activator present in the screening assay. Subsequently, this hypothesis was discarded by the experimental evidence that the hydroxamic acid derivatives maintained their activity even in the presence of a large molar excess of Mg^{2+} , indicating that the capacity of the substrate to chelate Mg^{2+} plays a marginal role in SerR inhibition. Probably, the inhibition is due to a modification of the cofactor, which forms a catalytically inactive aldoxime species. Among the hydroxamic acid inhibitors, compound **10** (Figure 7) exhibits a moderate selectivity for mouse and human SerR. Compound **10** ($K_i = 98 \mu\text{M}$) is an L-aspartic acid (L-Asp) analogue and is 20-fold more potent as an inhibitor of mouse SerR than L-Asp ($K_i = 1900 \mu\text{M}$). For these reasons, compound **10** could serve as a lead compound for the development of the next generation of SerR inhibitors.

The very recent determination of the structure of SerR in the absence and presence of ligands has triggered a few studies aimed at the development of SerR inhibitors. In this quest, an added difficulty is represented by the need for such compounds to cross the blood-brain barrier.

The comparison of the structures of SerR in the absence and presence of malonate^{135,136} indicates that the enzyme undergoes

an open to closed transition upon inhibitor binding. A similar conformational change is likely to occur upon L-Ser binding. Ligand-induced open-closed transitions are common in PLP-dependent enzymes and usually involve the reorientation of the small and large domains that form a subunit. As a result the volume of the active site is significantly reduced and active site residues are relocated. This molecular event might hamper or make more difficult the identification of high affinity ligands. In fact, if a virtual screening is carried using the open conformation, less specific compounds might be identified, whereas if the screening is carried out using the closed conformation, the reduced space may lead to the identification of ligands with reduced size and affinity. In an effort to overcome these limitations and to include SerR conformational flexibility in virtual screening investigations, targeted molecular dynamics of SerR was combined with conformational sampling and docking studies.¹⁵³ Results suggest that a virtual screenings of SerR carried out sampling a defined number of protein conformations along the open-to-closed state pathway might lead to the identification of selective and high affinity enzyme inhibitors.

An ongoing investigation is carrying out an *in silico* screening exploiting the open structure of human SerR and the searching engine FLAP.¹⁵⁴ Selected hits were docked in the active site using GOLD¹⁵⁵ and the free energy of binding was evaluated by HINT.^{156–158} Compounds that exhibit dissociation constants in the low micromolar range were identified.¹⁵⁹ A similar approach is applied to the identification of PLP-dependent enzyme inhibitors of O-acetylserine sulphydrylase.^{159,160}

Finally, SerR activation by ATP and inhibition by Cys133 nitrosylation suggest an alternative route for the modulation of the D-Ser level in the brain. Compounds that target the ATP allosteric site displacing ATP or causing selective nitrosylation of Cys 133 are predicted to inhibit SerR activity.

3.1.3. Arginine Racemase. **3.1.3.1. Localization, Structure, and Function.** ArgR (EC 5.1.1.9) is an example of a PLP-dependent racemase with broad substrate specificity because it catalyzes the racemization of arginine, lysine, ornithine and various other amino acids, including ethionine and citrulline. However, ArgR is unable to recognize and transform hydrophobic, acidic, or aromatic amino acids. The enzyme was first isolated from *Pseudomonas graveolens* as early as 1971.¹⁶¹ It was shown that four moles of the cofactor are bound to one mole of ArgR. In 2009, Matsui et al.¹⁶² established that in both *Pseudomonas taetrolens* and *E. coli*, ArgR resided almost exclusively in the periplasm, a feature so far unknown for any other amino acid racemase. The experiments performed with *P. taetrolens* on the utilization of D- and L- amino acids as a carbon source demonstrated that ArgR is a catabolic enzyme necessary for the efficient utilization of the two basic amino acids D-Lys and D-arginine (D-Arg). ArgR from *P. taetrolens* was found to contain a disulfide bridge. However, enzyme activity and stability were unaffected by either removal of the disulfide bond through a reduction process or by mutating the involved cysteines.¹⁶³

The existence of an ArgR in *Pseudomonas aeruginosa*, an opportunistic human pathogen with an enormous catabolic capability, was suggested by Jann et al in 1988.¹⁶⁴ Generalized inflammation and sepsis are the symptoms of infections by *P. aeruginosa*. If colonization occurs in organs such as lungs, urinary tract, and kidneys, the result can be fatal. *P. aeruginosa* is capable of growing on D-Arg as the sole source of carbon and nitrogen.¹⁶⁵

3.1.3.2. Inhibitors and Drug Perspectives. The observed broad substrate specificity of ArgR suggests the presence in the

active site of only a few well-defined anchor points. This feature and the lack of three-dimensional structure of ArgR has so far prevented studies aimed at the identification of enzyme inhibitors. Toward this goal, protein expression, careful biochemical investigations and crystallization trials are the prerequisites. A possible short-cut might be a homology modeling study using as a parent structure the PLP-dependent enzyme that more closely matches ArgR sequence.

3.1.4. Aspartate Racemase. **3.1.4.1. Localization, Structure, and Function.** D-Asp has been detected in the brain and neuroendocrine tissues.^{163,165–168} This finding triggered a genomic sequence analysis that led to the identification and cloning of a PLP-dependent enzyme.¹⁴ The recombinant enzyme exhibits a K_m value for L-Asp of 3.1 mM and a V_{max} of 0.46 mmol/mg/min at the optimum pH and temperature of 7.5 and 37 °C, respectively. On the basis of *in vivo* experiments in which the enzyme was depleted, it was proposed that D-Asp may function as a modulator of adult neurogenesis.¹⁴ Furthermore, biochemical investigations, coupled to tissue immunostaining, suggest that D-Asp is a novel endogenous neurotransmitter,¹⁶⁹ thus indicating that DR is a potential target for neuropathological disorders.

D-Asp is an agonist of the NMDA receptors, equipotent with L-Glu and NMDA itself.^{120,170,171} It has been demonstrated that in human brain very high levels of D-Asp occur transiently during the last stage of embryonic life or in the early postnatal life.¹¹⁷ In human fetal cortex, the concentration of D-Asp exceeds that of L-Asp, but diminishes rapidly to trace levels after birth. D-Asp is crucial for neurotransmission and neurosecretion in the CNS, as well as for the biosynthesis or secretion of hormones in endocrine glands.^{172–174} D-Asp is present in some neuronal populations of the brain and of neuroendocrine tissues, such as the catecholaminergic cells of the adrenal medulla, the anterior and posterior lobes of pituitary gland, the pineal gland, and the testes.^{120,167} D-Asp is highly concentrated in the supraoptic and paraventricular hypothalamic nuclei, whose axons terminate in the posterior pituitary.¹⁷⁵ D-Asp occurs also in a high concentration in the pineal gland,¹⁷⁶ where it modulates melatonin synthesis in rat pinealocytes.¹⁷⁷ Its localization in different areas of the hypothalamic–pituitary axis suggested that D-Asp might have a role in neuroendocrine modulation. In fact, D-Asp has been shown to increase serum growth hormone (GH), luteinizing hormone (LH), and prolactin levels, through modulation of the release of some of the neuropeptides and neurotransmitters involved in their regulation, such as luteinizing hormone-releasing hormone (LHRH), α -melanocyte-stimulating hormone (α -MSH), GABA, and dopamine.^{170,178} The high concentrations of D-Asp in the cortical plate, subventricular zone, and discrete portions of the hippocampal formation, during early neonatal stages, imply an important role during the developmental phase.¹⁷⁹ In adult hippocampus, D-Asp persists in dentate gyrus where new neurons are generated^{180,181} and integrated into existing neural circuitries which are involved in process of learning and memory formation. If D-Asp is physiologically formed by DR, the enzyme should have similar localizations.^{14,182,183} It was demonstrated that DR plays an important role in neuronal development, consistent with the high levels of D-Asp in early neonatal stages.¹⁴

Although many studies on the physiological relevance of D-Asp have been published, at present, there are no reports dealing with the connection of altered levels of D-Asp with any pathological state. Yamada and his co-workers also reported the occurrence of a DR in an ark shell, *Scapharca broughtonii*.¹⁸⁴ A peculiar

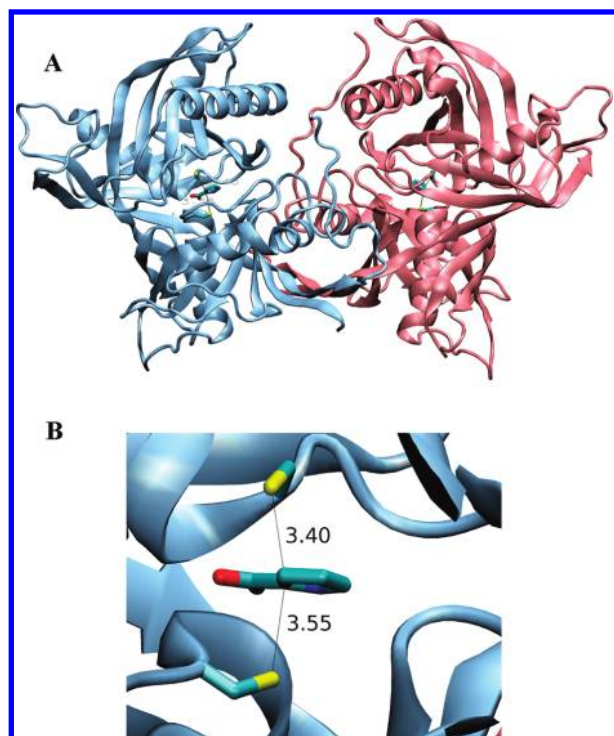


Figure 8. Ribbon diagram of the ProR (PDB: 1W61) in complex with two PYC molecules, displayed as sticks. (a) a global view of the enzyme, (b) a close view of the catalytic site. The catalytic Cys 300 and Cys 130, are displayed below and above, respectively. A broken line shows the distances (in Å) between the two catalytic sulfur atoms and the C2 atom of PYC.

characteristic of DR from *S. broughtonii*, is that it is markedly affected by AMP, which maximally enhances its activity up to 7-fold. By contrast, ATP lowers the activity to less than 7%.¹⁸⁵

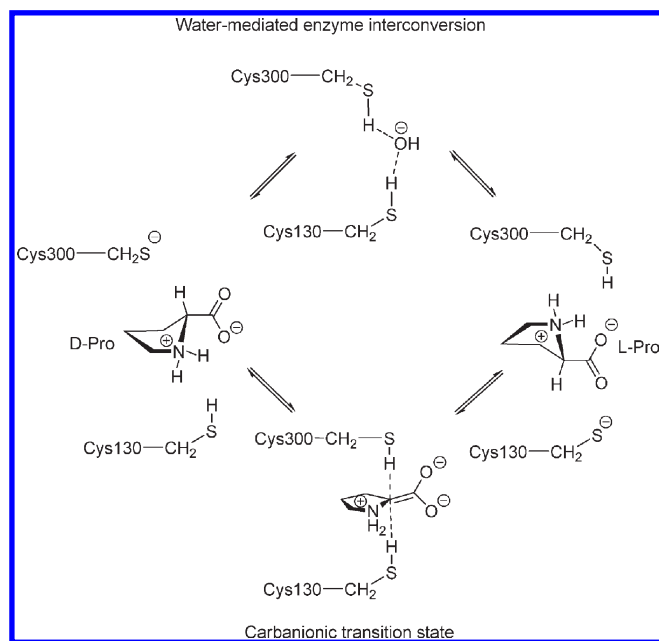
3.1.4.2. Inhibitors and Drug Perspectives. The interest in DR as a potential drug target for neurological disorders is very recent.¹⁴ So far, no high yield expression system for DR has been reported, thus preventing detailed enzyme characterization, biochemical assays and extensive crystallization campaign for the determination of enzyme structure. Given the DR role in modulating NMDA-associated signals, intensive activities should be first focused on a deep understanding of enzyme structure–function relationships.

The considerations addressed to DR, the PLP-dependent aspartate racemase, as a potential drug target hold also true for AspR, its PLP-independent variant (see Section 3.2.3).

3.2. PLP-Independent Racemases

3.2.1. Proline Racemase. **3.2.1.1. Localization, Structure, and Function.** A partially purified preparation of ProR from the Gram-positive bacterium *Clostridium sticklandii* (Cs) was first described by Stadtman and Elliot in 1957.³⁴ The first eukaryotic ProR was identified in 2000 by Minoprio et al. in the human parasite *Trypanosoma cruzi* (Tc),¹⁸⁶ the etiological agent of Chagas disease.¹⁸⁷ Investigating the in vitro-induced differentiated metacyclic trypomastigotes of Tc, Reina San Martin et al.¹⁸⁸ were able to isolate a 45 kDa mitogen (Tc45) which is involved in the nonspecific polyclonal activation of B lymphocytes. Subsequently, it was hypothesized the existence of two homologous of the Tc45 genes (Tc45-A and Tc45-B),¹⁸⁶ where the Tc45-A gene copy shares a high sequence homology with

Scheme 7. Mechanism of Action of ProR



CsProR, the only ProR then described in the literature. Worth noting, since the active site of CsProR^{33,186} is fully conserved in the Tc45 protein, both proteins catalyze the racemization of L- and D-proline (D-Pro) without the aid of any cofactor while leaving unaffected the stereochemistry of the other amino acids. Furthermore, specific or nonspecific inhibitors, such as pyrrole-2-carboxylate (PYC), iodoacetamide, and iodoacetate, abolish or severely compromise both the mitogenic activity of TcProR45 and its enzymatic activity.¹⁸⁶ Through a site-directed mutagenesis investigation, the Cys330 residue was identified as the key amino acid, since its replacement completely abolished the ProR activity;¹⁸⁹ the catalytic mechanism was further deepened in 2006.¹⁹⁰ The crystal structure of the TcProR-A was obtained with its competitive inhibitor PYC and showed that the enzyme is in fact a homodimer, with each monomer folded in two equally sized α/β domains separated by a deep crevice. The refined model revealed the presence of two molecules of the inhibitor, each one occupying the central hydrophobic cavity of the monomer (see Figure 8). Each of the active site cavities found in the interdomain crevices, includes two cysteines (Cys130 and Cys300 in TcProR-B; Cys160 and Cys330 in TcProR-A), which are suitably located to perform the acid–base catalysis of L/D-Pro (Scheme 7), according to the general mechanism proposed for PLP-independent racemases.

Since the pK_a value of the Cys sulphydryl group in aqueous solution is 8.4, it should be almost completely protonated at physiological pH and therefore unable to remove the α -proton of Pro. However, it has been suggested that the electrostatic field inside the enzyme could highly perturb its aqueous pK_a value, generating the anionic species needed for the catalytic process.

In the crystal structure of the complex of ProR with PYC, an inhibitor representing a transition state analogue, the C2 atom of PYC is in close contact with the two sulphydryl groups of residues Cys130 and Cys300 from equivalent α -helices in each structural domain (Figure 9).¹⁹⁰ This experimental evidence strongly supports the “two-base” mechanism. PYC is firmly fixed in place through H-bonding interactions of its carboxylate group with

five amino acidic residues of the enzyme; additionally, the Pro quaternary amine group is involved in hydrogen bonding interactions with His132 and Asp296. The pK_a of proline inside the ProR active site may be estimated to be about 23.4, reflecting a reduction of more than 7 pK_a units when compared to the calculated value of 30.9 in aqueous solution. This relatively high pK_a of Pro in the active site raises the question of why C α -deprotonation occurs and not deprotonation of the ammonium group of the zwitterionic Pro (pK_a \approx 9.6). Probably the answer is the tight interactions with His132 and Asp296, which prevent Pro from reorienting in a position enabling ammonium deprotonation.^{61,190}

As in other PLP independent amino acid racemases, the two domains of each monomer in the dimeric *TcProR-A* are structurally equivalent. The symmetric properties correlating both subunit domains stand on a dyad axis crossing the reaction center granting to the enzyme the ability to react with both enantiomeric forms of proline. A complete thermodynamic characterization of *TcProR-A* association with its inhibitor was obtained by isothermal titration calorimetry (ITC) using temperatures between 15 and 35 °C. The binding isotherm was best fitted to a model with two interacting sites with the enzyme expressed in terms of protein-dimer concentration. This data confirmed that *TcProR-A* binds with high affinity ($K_1 = 4.6 \times 10^6$ M⁻¹, $K_2 = 3.0 \times 10^5$ M⁻¹) two molecules of PYC as a dimer in solution. The ITC also supported the negative cooperativity and a differential packing constraint among the subunits. Thus, the binding of the first ligand molecule triggers conformational changes of one of the monomers resulting in a \sim 15 times lower affinity to bind the second inhibitor molecule. This property may explain the initial conclusions of Rudnick and Abeles on one active site per dimer by quantifying the binding stoichiometry of the inhibitor at equilibrium.³³

Recently, the corresponding *TvProR* gene and functional enzyme have been found also in *Trypanosoma vivax* (*Tv*), a livestock trypanosome cyclically and mechanically transmitted between domestic and wild ruminants by *tsetse* flies, tabanids and other varieties of biting flies.¹⁹¹

The human parasite *Tc*, as well as the livestock-infective *Tv*, take advantage of the presence of ProR in their genome as a way to escape the host immune responses. It has been hypothesized that *TcProR* is also involved in the machinery of the parasite differentiation by regulating intracellular proline metabolic pathways through the conversion of free L-Pro, internalized from the vector digestive tract, into D-Pro, that would be integrated into expressed peptides or stocked in reservosomes.^{191,192} The replacement of the L-Pro moiety with its mirror image, that is, D-Pro, into a peptide sequence increases its half-life, stability and resistance to host proteases. Furthermore, the presence of D-Pro would make the peptide less immunogenic than the corresponding one made up exclusively by L-amino acids.^{193,194}

In addition to the above-mentioned enzymatic properties, ProR of both *Tc* and *Tv* possesses a T-cell-independent B-cell mitogenic activity.

Attempts to better explore the direct or indirect association between *TcProR* mitogenic and enzymatic activities have demonstrated that they are dissociated.^{186,189} It is believed that the structure of *TcProR* active site may expose protein conformational motifs that bind to B-cell expressed molecules, thus triggering lymphocyte activation. Indeed, modification of key catalytic site residues of the enzyme aiming at abolishing catalysis without altering *TcProR* conformational structure, produced *TcProR* mutants in which the mitogenic activity was preserved. On the other hand, B cell proliferation assays showed that *TcProR* inactivated by PYC was unable to trigger B-cell proliferative activity.

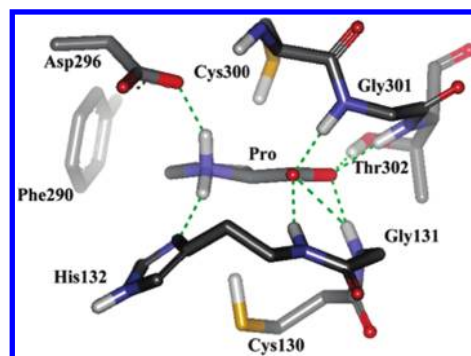


Figure 9. Active-site interactions of ProR with proline in the transition state (see Scheme 7).

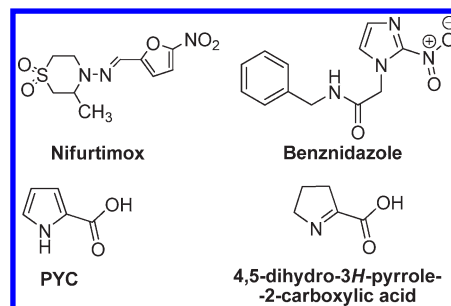


Figure 10

These data confirmed that conformational changes of *TcProR* take place upon inhibitor binding preventing its direct interaction with B-cell expressed ligands and that polyclonal B-cell activation observed with *TcProR* is unambiguously dependent on conformational epitopes displayed by the active protein.

3.2.1.2. Inhibitors and Drug Perspectives. In the previous section we have discussed how the first eukaryotic ProR was identified in *Tc*. This intracellular protozoan causes Chagas disease, one of the most neglected diseases. Endemic in several regions of Latin America, this disease persists as the major infectious heart disease in the world.^{195,196} It is estimated that 13 million people are currently infected in Central and South America and that the global incidence of the disease is 300 000 new cases per year.¹⁹⁷ Natural transmission of the disease occurs through faeces of the vector (a hematophagous bug belonging to the subfamily *Triatominae*, family *Reduviidae*) deposited near a skin lesion or mucosa (80–90%) or via organ transplantation/blood transfusion (5–20%) or congenital transmission (0.5–8%). The majority of infections occur during early childhood, and around 30% infected people develop chronic cardiac involvement, usually after decades of asymptomatic infection.

The available therapeutic options for Chagas disease are very limited. At the moment, there are only two drugs, nifurtimox and benznidazole (Figure 10), which may be helpful in the acute stages of the infection. Both of these drugs cure around 80% of the acute form of the disease and 20% of the chronic one. Patients require 60 days of treatment, with 2–3 doses per day. The drugs are of difficult access for patients in non endemic areas, and the distribution of benznidazole is restricted to specialized clinics that require medical monitoring during the course of treatment. Moreover, both medications have serious side effects limiting their use. Benznidazole is contraindicated to treat pregnant women

and, more generally triggers liver and kidney failure. Nifurtimox has been gradually discontinued and particularly contraindicated to patients previously diagnosed with psychiatric and neurological disorders. Furthermore, some strains of *Tc* are resistant to the treatment.¹⁹⁸ Therefore, it is clear that it is of utmost importance to find out new therapeutic targets and novel drugs for the treatment of Chagas disease, particularly for late stages of the disease.

ProR is an essential enzyme in the parasite metabolism and in the interactions of the parasite with the host immune defenses. Chamond and colleagues constructed transgenic parasites for *Tc*ProR genes and analyzed the ability of mutants to pursue differentiation into infective forms and to interact with host cells.¹⁹² The data determined that overexpression, but not reduction, of *Tc*ProR in noninfective forms leads to an increase in parasite differentiation to metacyclic infective forms and enhance parasite infectivity to host cells. The existence of proline transport systems in *Tc* has added importance to the role of *Tc*ProR to parasite bioenergetics,¹⁹⁹ since L-Pro is known as the most important carbon source in trypanosomatids.²⁰⁰ Consequently, inhibitors of ProR could represent a new therapeutic approach for the treatment of Chagas disease. Unfortunately, at the moment only few molecules capable to inhibit ProR have been reported.

ProR of *Clostridium sticklandii* and of *Tc* is inhibited by PYC (Figure 10). Modifications of the heterocyclic ring lead to a reduction of the inhibitory activity. In fact, the thiophene analogue is less effective while the furan analogue is almost inactive. The high affinity for the enzyme of PYC could be due to the planar arrangement of its key substituents, which mimic the transition state of the proline racemization process represented by 4,5-dihydro-3H-pyrrole-2-carboxylic acid, which turned out to be a good inhibitor of ProR.

Crystallographic data have shown that incubation of *Tc*ProR in the presence of PYC promotes considerable alteration of the enzyme structure, thus interfering with its binding to B-cell expressed ligands.¹⁹⁰ Furthermore, studies of *Tc*ProR localization showed that the enzyme localizes in the cytoplasm of intracellular dividing parasites (tissue amastigotes) and nearby the flagellar pocket, the principal site of excretion/secretion of trypanosomes. Interestingly, the addition of PYC to *Tc*-infected cultures results in a dose-dependent decrease of the conversion of amastigotes to circulating trypomastigotes, suggesting that the enzyme, which is up-regulated in actively multiplying parasites, may be related to the parasite differentiation triggering steps.²⁰¹ The addition of PYC during the initial steps of the parasite-host cell interaction led to a significant initial reduction of *Tc* invasion in vitro. The inhibition of the enzyme has a direct or an indirect influence on the parasite metabolism, differentiation and virulence. Unfortunately, PYC has a very poor solubility in water and this problem limits its further development as a drug. Minoprio's and Blondel's groups and collaborators had dedicated lots of efforts to produce, by medicinal chemistry, more soluble PYC variants and to design pharmacophoric models based on the catalytic site of *Tc*ProR to be further used in virtual screening of compound libraries. However, at present, those attempts have not yet yielded better inhibitors with a higher affinity for the enzyme than PYC.²⁰² The active site of *Tc*ProR in its complexed form defines a small pocket of 100 Å³ presenting a very specific set of oriented functional groups. Hence, the design of active inhibitors for this enzyme by the classical strategies of drug design represents a particular challenge. Therefore, a more aggressive and less traditional approach has been conceived. It takes advantage of

the conformational changes observed in the *Tc*ProR upon ligand binding. In effect, ongoing innovative approaches and modeling technologies making use of molecular dynamics and transition path calculations²⁰³ can be used to build new docking models for enzymes, either in the catalytic site or by identifying previously unknown allosteric sites. This approach, based on a new powerful transition Path Optimization and Exploration (POE) method, has proved to allow a drastic expansion of virtual screening strategies and, thus, of the chemical space accessible through computational aided drug design, as successfully validated in other systems.²⁰⁴

A different approach was first reported by Minoprio's group, which tried to use *Tc*ProR45 as a "vaccine".²⁰⁵ The aim was to induce specific responses against *Tc*ProR45 and neutralize its mitogenic activity. They developed two protocols. In the first one, intramuscular DNA 'vaccination' containing the *Tc*ProR45 gene was used. It turned out that 'immunoprotection' is possible, since an 85% decrease in the levels of parasitaemia, after a challenge with infective forms of the parasite, was obtained. Moreover, in the second protocol, an *i.p.* injection of submitogenic doses of recombinant protein (r*Tc*ProR45) was used. The outcome was a decrease up to 95% of the mice parasitaemia, after challenge with a lethal inoculum of *Tc*. Therefore, both protocols of immunization were able to trigger specific B cells and consequently high levels of antibodies anti-r*Tc*ProR45 were detected in sera.

In 2010, Bryan et al. showed that *Tc*ProR may contribute to delay host immune effector mechanisms by nonspecifically activating B cells, especially marginal zone B cells, causing hypergammaglobulinemia and inducing production of interleukin-10, a cytokine known to increase host susceptibility to *Tc*.²⁰⁶ While *Tc*ProR45 is apparently nonantigenic during experimental *T. cruzi* infection, it can be delivered as a potent antigen via genetic immunization. Genetic immunization with *Tc*ProR did not lead to detectable systemic B-cell expansion and did not interfere with the immune response to a different test of immunogenicity. Moreover, genetic vaccination with *Tc*ProR induces the triggering of memory B cells, underlying the usefulness of *Tc*ProR as a target to the development of new therapies against trypanosomiasis.

Finally, it is also possible to suppose that the parasite uses the racemization process to synthesize and/or express new peptides containing D-Pro to avoid or reduce the host-induced proteolysis. The identities of the parasite proteins bearing D-Pro should be discovered and investigated since they may become attractive targets involved in parasite evasion.¹⁹³ These last studies offer the basis for further development of novel strategies to treat Chagas and other infectious diseases.

3.2.2. Glutamate Racemase. **3.2.2.1. Localization, Structure, and Function.** GluR (EC 5.1.1.3) is another PLP-independent racemase that occurs in prokaryotes. The first evidence of GluR from *Lactobacillus arabinosus* dates back to 1952, but its biochemical function was not clarified.^{207,208} In early nineties, it was demonstrated that, like ProR, GluR uses a "two-base" mechanism in which the sulfhydryl groups of two cysteines serve as the general base/acid catalysts.³⁶ The involvement of the two Cys residues was demonstrated with the enzyme from *Lactobacillus fermenti*, since their replacement, one at a time, with an alanine completely abolished the racemase activity.²⁰⁹ Similar results were obtained by replacing the Cys residues with Thr/Ala in the enzyme from *E. coli*.^{210,211} It was also suggested that Cys73 is responsible for the deprotonation of D-Glu, while Cys184 is responsible for the deprotonation of L-Glu. Moreover, it was

shown that neighboring residues, i.e. Asp10 for the conversion of D into L and His186 for the reverse conversion, assist the catalytic thiol groups in acting as bases.²¹²

The structure determination of GluR from *Aquifex pyrophilus* in the absence and presence of D-glutamine, a weak inhibitor, provided the first structural elucidation of the architecture of the active site.²¹³ However, no strong interactions between D-glutamine and the amino acid residues present in the active site were noticed. In 2005, it was reported the crystal structure of the GluR from *L. fermenti*,²¹⁴ which shares approximately 32% sequence identity with *A. pyrophilus* GluR. It represents the first three-dimensional structure of a cofactor-independent racemase from a Gram-positive bacterium.

NMR studies carried out on *B. anthracis* GluR²¹⁵ and on monomeric *Helicobacter pylori* GluR,²¹⁶ allowed the establishment that the enzyme is quite flexible, adopting multiple conformations. The enzyme active site is rather small, therefore substrate and product can access it exploiting the enzyme flexibility.

GluR is essential to the bacterial cell wall biosynthesis pathway, since the incorporation of D-Glu as the second residue of the pentapeptide is strictly conserved across the bacterial kingdom.⁶ GluR has been implicated in the production and maintenance of sufficient D-Glu pool levels required for growth.

Peptidoglycan biosynthesis is classified into three distinct phases based on the cellular location of the synthetic pathway. Most of the active drugs act through inhibition of the phase III

segment, which involves the extracellular cross-linking and final maturation of the cellular envelope. Even if the discovery of improved agents that target this phase remains a dominant area of research and development, the onset of resistance limits the therapeutic utility of this class of compounds. Moreover, the emergence of multidrug-resistant (MDR) bacteria, such as methicillin-resistant *Staphylococcus aureus*, MDR-*Pseudomonas aeruginosa* and MDR-*Acinetobacter baumannii*, has resulted in significantly increased mortality rates with limited or no options for therapeutic intervention.²¹⁷ This crisis has resulted in a call for the discovery of drugs that have a novel mode of action.²¹⁸ Therefore, targeting GluR, which is involved in phase I of peptidoglycan biosynthesis, represents a novel strategy in the search for new antibacterial agents.

3.2.2.2. Inhibitors and Drug Perspectives. Despite the officially recognized importance of GluR as a target for antibacterial drugs, until 1997 only two GluR inhibitors were reported, L-serine-O-sulfate (L-SOS)²¹⁹ and aziridino glutamate (Figure 11).²²⁰ Both drugs are irreversible inhibitors and, in addition, suffer either from inefficiency of the process or instability.

In the subsequent years, an increased number of GluR inhibitors have been published. Most of the first generation inhibitors are characterized by the presence of an amino acidic chain in their structure. D-N-hydroxyglutamate (Figure 11) belongs to this class; it is a good competitive inhibitor ($K_i = 56 \mu\text{M}$) of GluR from *L. fermenti*.²²¹ N-hydroxyglutamate undergoes an enzyme-catalyzed elimination reaction, that leads to the formation of an imine intermediate (Scheme 8) which is responsible for the strong inhibition. Such an imine could mimic the normal carbanionic intermediate and thus be strongly bound by the racemase. Moreover, hydration of the imine and subsequent elimination of ammonia produces α -ketoglutarate as depicted in Scheme 8.

In 2002, the discovery of a new class of (2R,4S)-4-substituted D-Glu analogues (Figure 11, general structure 11) was reported by Lilly researchers;²²² this class of compounds showed a promising activity against *S. pneumoniae*, one of the most frequent cause of bacterial respiratory infection and meningitis. One of the most potent compound of this series is the 2-naphthyl derivative (Figure 11, compound 12), with a K_i of 16 nM. A further increasing in activity was achieved by replacing the naphthyl moiety with a heteroaromatic bicyclic groups, that is, 2-benzothienyl, 2-(3-Cl-benzothienyl) and 3-(2'-furyl) groups; these new derivatives exhibited inhibition of bacterial infection in an in vivo murine model.

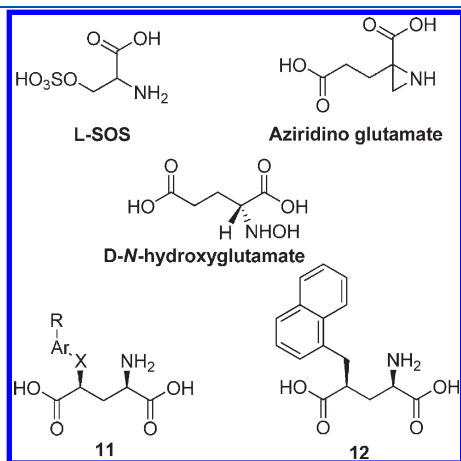
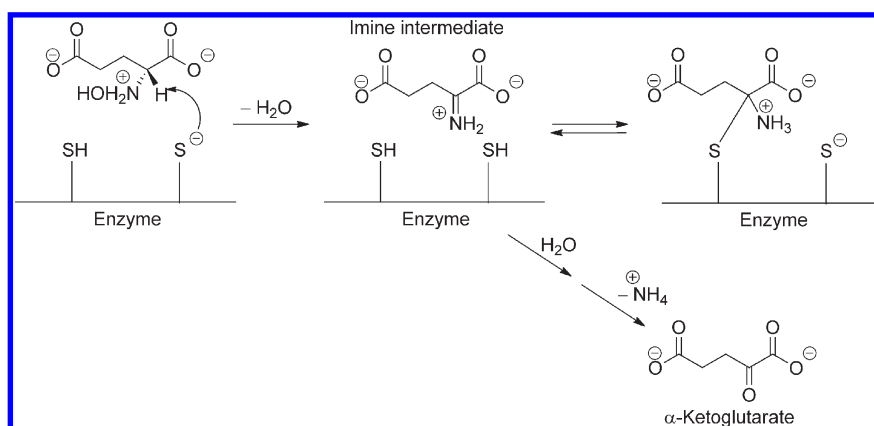


Figure 11

Scheme 8



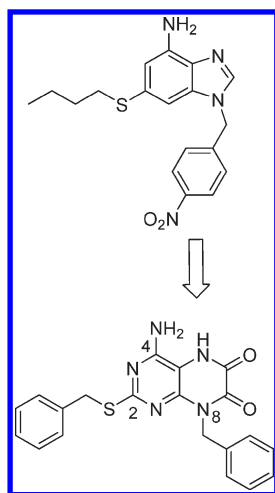


Figure 12

In the past few years, the research was focused mostly on non amino acidic scaffolds. In this contest, high-throughput screening of the AstraZeneca collection compounds identified a number of hits including 9-benzyl purines that showed good activity against *Enterococcus faecalis* and *Enterococcus faecium* GluR. Despite a number of efforts to improve the pharmacological profile of the lead compound through strategies based on an iterative structure-based drug design, only modest improvements in potency was observed for the *Enterococcus* spp. and no activity was detected against the *S. aureus* enzyme. Furthermore, many members of the series exhibited relatively poor solubility and only moderate antibacterial activity. In 2008, researchers from the same company reported a successful ‘scaffold-hopping’ endeavor from the 9-benzyl purines to the 8-benzyl pteridinediones and a hit-to-lead work on this series (Figure 12). Among the tested compounds, 2-benzylthio 8-benzyl pteridinedione showed activity against the *S. aureus* enzyme as well as *E. faecalis* and *E. faecium*.²²³ Moreover, by decorating the scaffold with different substituents at the 2-, 4- and 8-positions emerged some derivatives provided with a relevant inhibition potency and improved physical properties. The broad spectrum of action exhibited by some derivatives of this series represents a breakthrough in the development of small molecule inhibitors. Unfortunately, this series of compounds generally exhibits a poor solubility. Attempts to improve their physical properties by incorporating polar substituents gave a moderate success.

Recently, a very interesting research plan focused on the design and synthesis of selective *H. pylori* GluR inhibitors has been published.²²⁴ *H. pylori* erodes the stomach mucosa and causes gastritis and peptic ulcers.²²⁵ Moreover, a relationship between infection by such a bacterium and an increased incidence of a gastric cancer has been proposed.^{226,227} Current therapies, based on the combined administration of two antibiotics with a proton pump inhibitor (Triple Therapy), give rise to numerous drawbacks, such as stomach upset, diarrhea, headache, a metallic taste and sensitivity to the sun, that consequently involve a poor patient compliance.²²⁸ Additionally, the appearance of *H. pylori* strains resistant to the drugs diminished the effectiveness of the multiple therapies. In this contest, researchers at AstraZeneca focused their attention on the design of new *H. pylori* GluR inhibitors. An initial high-throughput screening of the AstraZeneca compound collection and a subsequent lead

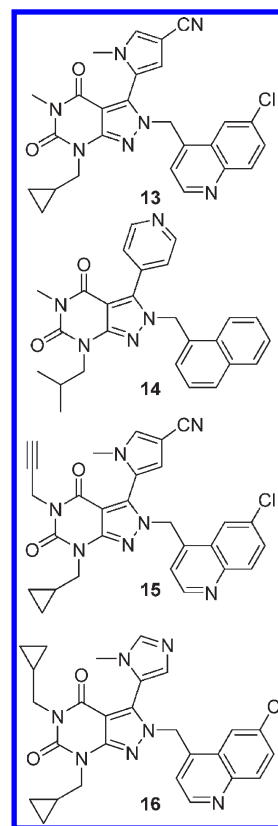


Figure 13

optimization process allowed the identification of pyrazolopyrimidinedione **13** (Figure 13). Such a compound possesses a low nanomolar inhibitory activity at GluR ($IC_{50} = 26$ nM) but, due to its low aqueous solubility and bioavailability, it is not suitable for oral administration. With the aim to improve its bioavailability, a series of analogues bearing polar functionalities onto the fused pyrimidinedione scaffold were prepared and tested.

The structures of derivatives characterized by a high inhibitory activity against *H. pylori* GluR are reported in Figure 13. Remarkably, studies of kinetic inhibition on compound **14** indicated that it does not compete with glutamate and, quite interestingly, glutamate binding was required for inhibition. *H. pylori* GluR was co-crystallized with both a substrate (D-Glu) (Figure 14A, B) and an inhibitor (compound **14**) (Figure 14C) and the structure of the complexes were solved at high-resolution.²²⁹ The major feature of the crystalline structure is the presence of a cryptic pocket, formed by a dislocation of the C-terminal helix, capable to accommodate the inhibitor and located ~ 9 Å away from the catalytic center. The inhibitor binding pocket is formed by displacement and rotation of the Trp252 side-chain with the pyrazolopyrimidinedione core of derivative **14**, as well as the other members of the cluster, forming a π -stacking interaction which involves its naphthyl moiety and the indole ring of Trp252 (Figure 14C). Worth of note, the structure of the active site did not change on binding of the inhibitor.

The pyrazolopyrimidinedione series of compounds represents the first example of noncompetitive inhibitors of a single-substrate enzyme, which fit a cryptic allosteric site. This outcome further highlights the power of a high-throughput screening to search enzyme inhibitors with a novel mode of action. Once again, physicochemical properties need to be optimized to achieve

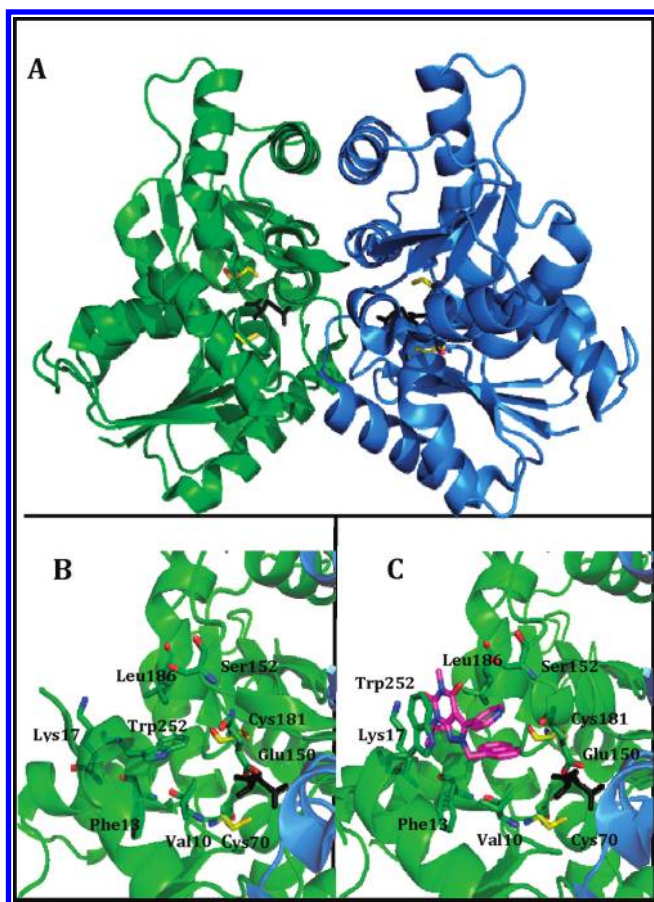


Figure 14. (A) Three-dimensional structure of GluR from *H. pylori* complexed with D-Glu (PDB code 2jfy²²⁹). (B) Close up view of the enzyme active site in the presence of the substrate and (C) in the presence of the substrate and an inhibitor (PDB code 2jtz). The catalytic cysteines are colored in yellow, D-Glu in black, and the pyrazolopyrimidinedione-based inhibitor in magenta. The residues lining the binding pocket are represented in capped sticks. The pocket vacated by the indole ring movement of Trp252 is filled with the naphthyl moiety of the inhibitor and further stabilized by interactions with Val10, Gly11 (not shown), His183, Leu186, and Trp244 (not shown). Additional interactions are formed between the isobutyl moiety and the Phe13, Ser14 (not shown), Lys17, Leu253 (not shown) residues, while the pyridyl ring substituent makes contacts with the main-chain atoms of residues Glu150 and Ser152.

values of solubility and membrane permeability suitable for oral administration.²²⁴ Potent analogues, characterized by good in vivo clearance, oral bioavailability, and low susceptibility to bacterial efflux pump transport, that is, derivatives **15**, **16**, were identified; unfortunately, an improved microbial potency is paralleled by an increase in lipophilicity which enhances the protein binding and lowers the solubility (Figure 13). As a result, the lack of efficacy of the above-reported optimized compounds in a murine mouse model of *H. pylori* colonization was attributed to an insufficient plasma concentration of the free drug. Anyway, the high degree of selectivity for *H. pylori* GluR showed by these compounds makes them attractive candidates for a novel *H. pylori*-selective therapy.

A second series of allosteric inhibitors targeting the *H. pylori* GluR enzyme has been reported.²³⁰ In this series, a novel scaffold represented by the benzodiazepine amine **17** (Figure 15) was discovered by a high-throughput campaign of the former Zeneca

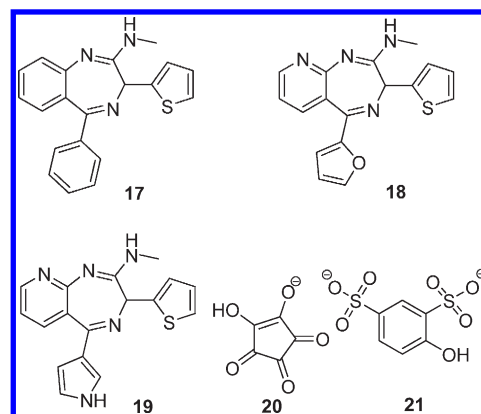


Figure 15

compound collection. Also these compounds show selectivity for the *H. pylori* GluR. X-ray crystallographic studies demonstrated that these inhibitors bind at a site that is clearly distinct from the one that accommodates the pyrazolopyrimidinedione derivatives. This binding site is located along the symmetry axis of the dimer interface. In contrast to the pyrazolopyrimidinedione series, the optimized benzodiazepine analogues generally exhibited improved physico-chemical properties. Physico-chemical parameters such as protein binding, solubility, lipophilicity and molecular weight suitable for oral administration were the hallmarks of the series (e.g., compounds **18** and **19**). Despite such an excellent profile, attempts to demonstrate efficacy in a murine *H. pylori* colonization model turned out to be unsuccessful; the lack of activity was attributed to insufficient concentration levels of the free drug in the plasma of mice caused by a high intrinsic clearance.²³¹

A recent paper by Whalen et al.²³² reports a virtual screening using a transition-state conformation of the *B. subtilis* GluR enzyme. This study resulted in the discovery of several competitive inhibitors, that is, **20** and **21** (Figure 15), provided with IC₅₀ values in the micromolar range ($K_i = 42 \mu\text{M}$ for **20** and $59 \mu\text{M}$ for **21**). Since the structures of these novel inhibitors are dissimilar from those of current amino acid like inhibitors, they could represent novel scaffolds to be used in the drug discovery process.

Besides these classes of small molecules that inhibit GluR, two reports on large-molecule inhibitors (molecular weight >650) have been published. The first report identified hemein as a moderately potent inhibitor of the *P. pentosaceus* GluR enzyme.²³³ Haemin was found to be a noncompetitive inhibitor ($K_i = 3.7 \mu\text{M}$), hypothesized to bind either to the orthosteric site, or to a distal site inducing a conformational change. The second report, published in 2000, showed a series of peptides²³⁴ provided with a weak inhibitory potency (IC₅₀ = $160 \mu\text{M}$) for the *E. coli* GluR enzyme.

3.2.3. Aspartate Racemase. **3.2.3.1. Localization, Structure, and Function.** The PLP-independent AspR (EC 5.1.1.13) shares with the other members of the AspR group the same “two-base” mode of action. AspR was discovered in *S. faecalis* and partially purified in 1972.²³⁵ In 1991, Yohda et al. found that AspR occurs exclusively in lactic acid bacteria, such as *Streptococcus species* and *Lactobacillus species*, and succeeded to purify the enzyme to homogeneity from *Streptococcus thermophilus*.²³⁶ Lactic acid bacteria are characterized by the presence of a D-Asp residue in the peptidoglycan crossbridge, which is produced from L-Asp by the action of AspR. The same Authors were able to clone the AspR gene of *S. thermophilus* and to determine the nucleotide sequence of the gene.²³⁷

The crystal structure of the *Pyrococcus horikoshii* OT3 AspR (PhAspR), disclosed in 2002, exhibits a dimeric assembly (Figure 16A).²³⁸ As expected for a PLP-independent racemase, it showed two strictly conserved Cys residues (Cys82 and Cys194) in its catalytic site (Figure 16B). These two cysteine residues, as well as other surrounding amino acid residues, that is, Asn83, Thr84, Gly193, and Thr195, are highly conserved among all AspR isoforms and some other PLP-independent racemases (Figure 16B).

Furthermore, the active site of all known AspRs contains two basic amino acids, that is, Arg48 and Lys164 (Figure 16B), which are essential to the functionality of the enzyme since they seem to play an important role in the substrate recognition and binding.²³⁹ In particular, the X-ray structure of an inactive mutant of PhAspR complexed with citric acid suggested that Arg48 is responsible for recognizing the carboxylate moieties of the substrate and to stabilize the reaction intermediate, while Lys164 is responsible for stabilizing the closed state structure.²⁴⁰

The AspR shows approximately 26% sequence identity with the GluR from *A. pyrophilus*. Worth noting, the two enzymes show major differences in the structure of their catalytic site despite the marginal difference among the structure of their natural substrates, namely, Asp and Glu, and the similar sequence homology in the core center.²³⁸ This reflects into a restricted substrate specificity. In fact, GluR exclusively acts on D- and L-Glu,²⁴¹ while both enantiomers of Asp are inactive as substrates and do not affect Glu racemization. In analogy, AspR is highly specific for Asp. In the crystal structure of both the enzymes, the position of the two regions Cys82-Asn83-Thr84 and Gly193-Cys194-Thr195, as well as of two other threonine residues, are highly conserved. These features allow the interaction between two moieties of the enzyme (the sites of Cys, Thr and Asn) with the amino acidic portion of both enantiomers of the substrate. As mentioned above, also the residues Arg48 and Lys164 are strictly conserved in all AspR. Their positively charged guanidinium or ammonium group can form an ion–ion interaction with the negatively charged L-/D-Asp carboxylate moiety. In addition, Thr84/Thr124 can interact with the amino or the positively charged ammonium group of L-/D-Asp through a hydrogen bonding.

The Arg and Lys residues of the active site are not conserved in the GluR of *A. pyrophilus*, which means that the interaction between these residues and the side-chain carboxylate group of glutamate enantiomers may be pointless for their recognition by GluR. The distance between the γ -sulfur atoms of the two Cys residues present in the active-site of *A. pyrophilus* GluR is 7.6 Å, a value definitely lower than that (about 9.6 Å) found in PhAspR.²³⁹

3.2.4. Diaminopimelate Epimerase. 3.2.4.1. *Localization, Structure, and Function.* DAPE (EC 5.1.1.7) is a member of the PLP-independent amino-acid racemases. It catalyzes the stereoinversion of (L,L)-2,6-diaminopimelate to meso-2,6-diaminopimelate in the lysine-biosynthetic pathway in plants and bacteria (Scheme 9). Since lysine biosynthesis does not occur in animals, the members of such a pathway, such as DAPes, are attractive targets for the rational design of antibiotics.^{242,243}

DAPE is a unique member of this family of racemases, because its substrates [(L,L)-DAP and (D,L)-DAP] contain two stereocenters but the enzyme recognizes the sole two stereoisomers with the L configuration at the distal site. The DAP epimerase was detected over 50 years ago in *E. coli*,²⁴⁴ but its purification and

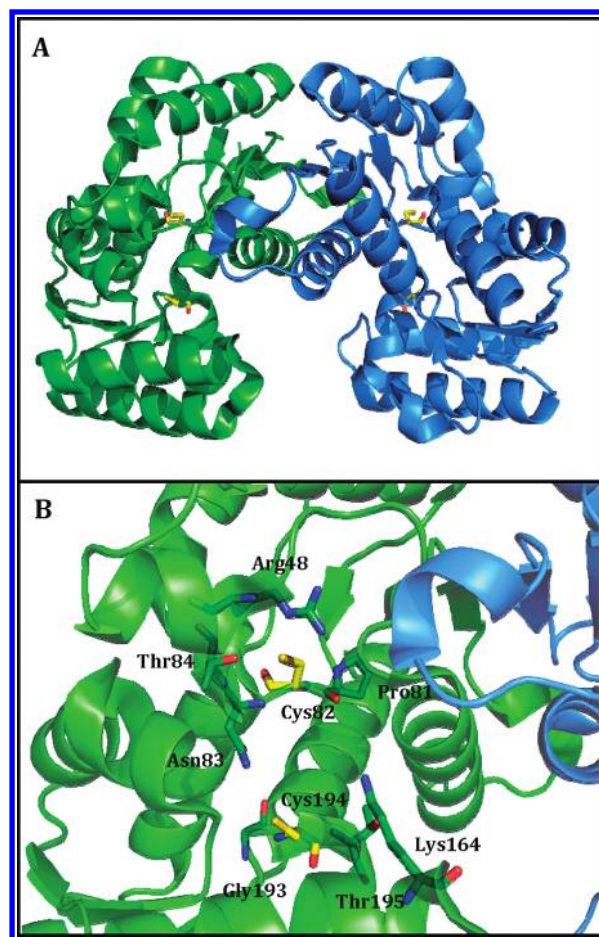
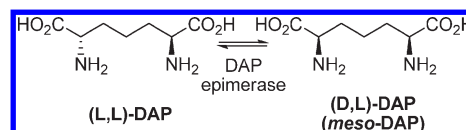


Figure 16. (A) Three-dimensional structure of AspR from *P. horikoshii* OT3 (PDB code 1jfl²³⁸). (B) Close up view of the binding pocket. The catalytic cysteines are colored in yellow, the residues lining the binding pocket are represented in capped sticks.

Scheme 9



characterization was made roughly 30 years ago.²⁴⁵ Similarly to the other PLP-independent racemases, the catalytic mechanism of DAPE involves two cysteine residues, in a “two-base” mechanism.³⁸ This mechanism was later elucidated by Stenta et al. through QM/MM studies.²⁴⁶ The structure of DAPE isoforms from many bacteria^{247–249} and from a plant have been determined recently.²⁵⁰ Particular attention has to be paid to the crystal structure of DAPE from *M. tuberculosis*, since it represents a new potential target to overcome the problem of drug resistance in the treatment of tuberculosis.²⁴⁸ Differently from Asp and Glu racemases, in which the cysteine residues are assisted in the racemization by a histidine (His132) and an aspartate (Asp296), it was proposed that the active site of DAPE exists as a thiol–thiolate pair at neutral pH and does not use any neighboring active-site base to assist the deprotonation process.²⁵⁰

DAPE catalyzes the interconversion of (*L,L*)-2,6-diaminopimelic acid (DAP) into meso-DAP. The meso-DAP, a precursor of *L*-Lys, is a key component of the pentapeptide linker in bacterial peptidoglycan.²⁵¹ Interestingly, mycobacteria incorporate two distinct interpeptide linkages, the common D-Ala/meso-DAP linkage during exponential growth and a meso-DAP/meso-DAP linkage, a penicillin-resistant mode of ligation, upon entering the stationary phase.²⁵² This unique structural feature could be the cause of the β -lactam resistance of *M. tuberculosis*.²⁵³ Tuberculosis is a common and often deadly infectious disease among the world's poor and latent infections are estimated to affect one-third of the world population.²⁵⁴

3.2.4.2. Inhibitors and Drug Perspectives. In 1986, Girodeau et al. published the synthesis and the biological evaluation of dihydro-DAP analogues as potential inhibitors of DAPE (Figure 17).²⁵⁵ Only one compound of the series, γ -methylen-DAP **22** (Figure 17), in all its isomeric forms, showed good antibacterial properties against a wide number of bacteria strains.

In 1988, it was reported that 3-chloro-DAP (Scheme 10), as a mixture of four possible isomers, is a potent inhibitor of *E. coli* DAPE in vitro.²⁵⁶ In analogy with inhibitors of ProR, which are transition state analogues and bear a sp^2 hybrid at C-2, it was proposed that the active form of 3-Cl-DAP is the corresponding enamine **25**, resulting from the elimination of HCl. Moreover, it was speculated that compound **25** binds tightly to DAPE and is slowly released from the enzyme; in this respect, 3-Cl-DAP is a mechanism-based inactivator. It is an innocuous compound by itself but is transformed at the active site of DAPE, becoming a potent competitive inhibitor of the enzyme. After release from the enzyme, **25** could tautomerize to the corresponding imine, which then undergoes a cyclization process to yield tetrahydro-dipicolinate (**26**). It can not be excluded that the predominant epimerase-bound intermediate is the imine tautomer since it has the same planar geometry as enamine **25**.

The 3-fluoro-DAP analogues **23** and **24** present the same mechanism of activation, namely the formation of planar enamine

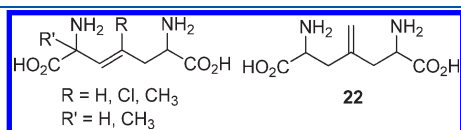
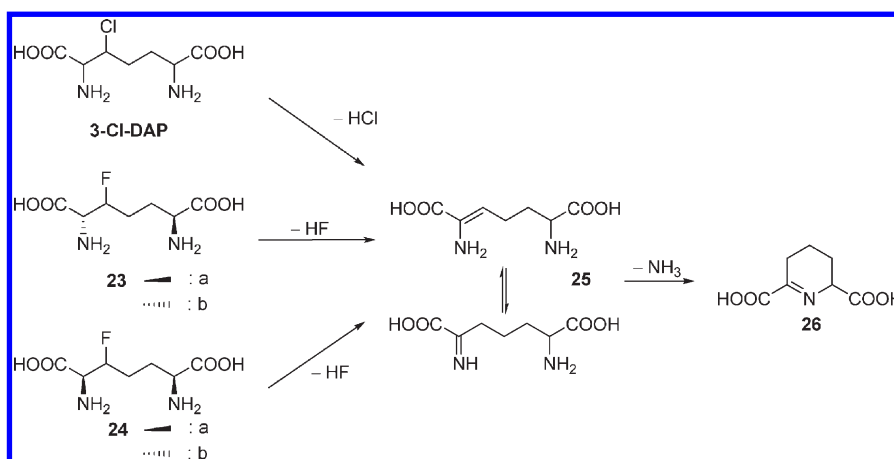


Figure 17

Scheme 10



25 by elimination of HF (Scheme 10). All the four stereoisomers are good DAPE inhibitors, with K_i values in the range 4–25 μ M.²⁵⁷ On the contrary, the 3-hydroxy-DAP analogues lack any inhibitory activity since they do not undergo the enzyme-catalyzed elimination of water.

In the same years, Lam et al. reported a series of DAP analogues that showed inhibitory activity on DAPE isolated from *E. coli*.²⁵⁸ To this group of compounds belong the meso-lanthionine **27a,b** (Figure 18) and its (*L,L*)-isomer **27c**, which are mixed-competitive (allosteric) and competitive inhibitors, respectively ($K_i = 0.18$ and 0.42 mM, respectively), while the (*D,D*)-isomer **27d** is about 20 times less effective, in accord with the stereochemical requirements of the enzyme for its natural substrates. Oxidation of the sulfur of compounds **27** to the corresponding sulfoxide or sulfone derivatives drastically lowers the affinity for the epimerase active site.

The most active compound of this series is the *N*-hydroxy analogue **28** ($K_i = 5.6 \mu$ M). The reason could be the possibility to eliminate water from stereoisomers **28a** or **28c** (Figure 18) to generate the corresponding α -imine as a planar transition state analogue (for details see Scheme 8). This reactive species could be attacked by a nucleophile, such as the Cys thiol group present in the active site, at the α -carbon in a reversible fashion. Interestingly, the *N*-amino analogue **29** is about 500 times less effective than compound **28** as a competitive inhibitor. This may reflect the inability of the hydrazino group to release ammonia through elimination.

In 1990, the irreversible inactivation of DAPE was reported^{259,260} using a crude mixture of all possible diastereomers of 2-(4-amino-4-carboxybutyl)aziridine-2-carboxylic acid (azi-DAP, Figure 19). Enzymatic digestion studies established that Cys73 is alkylated by the aziridino moiety, thereby demonstrating its presence in the active site. The K_i values could not be determined because of the extremely fast conversion of the enzyme–inhibitor complex to the inactivated enzyme. Further studies determined that (*L,L*)-azi-DAP was bound exclusively to Cys73, while (*D,L*)-azi-DAP was linked solely to Cys217;²⁶¹ this result was confirmed by the crystal structure reported by the same group in 2009.²⁵⁰

It was also reported that unlike azi-DAP, which rapidly and irreversibly inactivates DAPE, aziridine derivative **30** (Figure 19) is a weak reversible inhibitor of DAPE, with an IC_{50} value of 2.88 mM.²⁶¹ This different behavior could be explained by taking

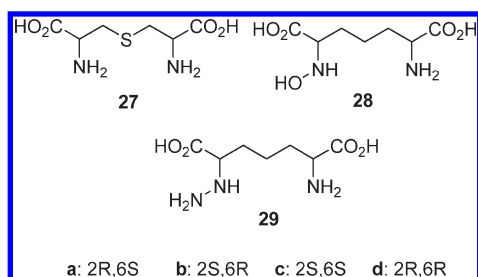


Figure 18

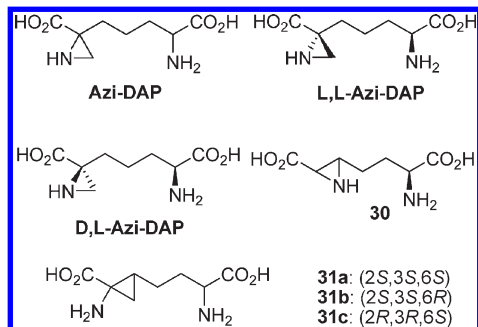


Figure 19

into account the different arrangement of the aziridino moiety in the two structures. While azi-DAP can undergo a nucleophilic attack by the thiol group of a cysteine at its unhindered methylene position of the aziridine ring, the same reaction occurs on derivative **30** with the involvement of a tertiary carbon atom which, by definition, is more crowded.

Another class of compounds that have been designed as inhibitors of DAP is represented by 2,3-methano-2,6-diaminopimelic acids (**31a–c**, Figure 19). These compounds did not exhibit any antibacterial properties; probably they mimic DAP and act as substrates for the enzyme; as a matter of fact, they are incorporated into the cell wall.²⁶²

4. CONCLUSIONS AND FUTURE PERSPECTIVES

L-amino acids are the common building blocks of proteins and are also deeply involved in metabolism to produce energy. They are currently termed “natural amino acids” in contrast to their mirror images, that is, D-amino acids, which could be tagged as “unnatural amino acids” and, according to a long-standing belief, have been considered inactive substances. Along the years there have been compelling evidence of the presence of D-amino acids in bacterial cell wall, in antibiotics, as well as in peptides produced by molluscs and amphibians. These peptides are provided with an array of biological properties spanning from antimicrobial to analgesic activity. Worth noting, the biological activity of the peptides is strictly dependent, not only on the presence of D-amino acids, but also upon the position of such building blocks along the amino acid sequence. Furthermore, free D-amino acids have been detected in mammals, including humans, where they play physiological roles of utmost importance. For example, substantial amounts of D-Asp and D-Ser have been found in the brain of newborn rats and humans, where they perform as neurotransmitter. Their concentration decreases gradually with the development.

The present review deals with the enzymes, termed *amino acid racemases*, involved in the production of D-amino acids through a racemization process of the L counterparts. We took into account solely the amino acid racemases which are specific for a single amino acid or recognize as a substrate a selected number of amino acids. These enzymes have been classified into two groups according to the mechanism used to interconvert the stereo-center of the amino acids: (i) PLP-dependent racemases and (ii) PLP-independent racemases.

Among the PLP-dependent racemases, bacterial AlaR is one of the best-studied amino acid racemases. The crystal structure of AlaR from *B. stearothermophilus*, as well as its complexes with two inhibitors, have been reported and allowed the definition of the active site features. This information is of utmost importance to design new antimicrobial drugs since AlaR, ubiquitously distributed in bacteria and not in mammals, produces D-Ala, which is an essential component of peptidoglycan. D-Cycloserine, an irreversible suicide inhibitor of AlaR, is clinically used as a second-line drug against *M. avium* and *M. tuberculosis*. The major drawback associated to the administration of this drug is related to its capacity to cross the blood brain barrier causing side effects, probably as a result of an interaction with the NMDA receptors as well as to its low specificity for AlaR. D-Cycloserine is also able to inhibit several PLP-dependent enzymes. Novel structural information has opened new avenues for the design of specific and potent AlaR inhibitors. In the future, we expect antimicrobial drugs characterized by a higher selectivity for AlaR and with reduced side effects. In this set of enzymes, SerR and DR will be the targets of novel drugs capable to treat neuropathological disorders. Sizable amount of D-Ser and D-Asp have been detected in the mammalian brain of both rats and humans where they perform as neuromodulators of the NMDA receptors.

Within the set of PLP-independent racemases in depth investigations have been carried out both in academia and in industry with the goal to find out new antiparasitic, antibiotic, and antibacterial agents. Most of the antibacterial drugs present in the market act through inhibition of the phase III segment of the peptidoglycan biosynthetic route. Even if this phase remains the dominant area of research and drug development, the onset of resistance limits the therapeutic utility of this class of compounds. Therefore, targeting GluR, which is involved in phase I, represents a novel strategy in the search for new antibacterial agents. The major problem associated to the development of drugs targeting the cofactor-independent family is the need to find out potent inhibitors selective for a single species of racemases. In the field of GluR, selective inhibitors of the *H. pylori* GluR have been discovered through a high-throughput screening of a large collection of compounds. Quite interestingly, the drug candidates are noncompetitive inhibitors, since they fit a cryptic allosteric site. This outcome further highlights the power of a high-throughput screening to search enzyme inhibitors with a novel mode of action. In addition, investigations carried out with the aim to uncover allosteric inhibitors of racemases could be a fruitful way to obtain compounds provided with a selective profile.

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BIOGRAPHIES



Paola Conti is an Associate Professor of Medicinal Chemistry at the Faculty of Pharmacy, University of Milan. She graduated in Medicinal & Pharmaceutical Chemistry at the University of Milan and in 1998 she received her PhD in Medicinal Chemistry from the same University. She was a postdoctoral fellow of Alan P. Kozikowski at Georgetown University Medical Center (Washington, D.C., U.S.A.), where she was part of the “Drug Discovery Program”. Her main research activity is focused on the design and synthesis of biologically active unnatural amino acids. In particular she is interested in new ligands acting at glutamate receptors and transporters, as potential neuroprotective agents, and in inhibitors of glutamine-dependent enzymes that play a key role in the biosynthesis of amino acids and nucleotides, as potential anticancer agents and for the chemotherapy of parasitic diseases.



Lucia Tamborini graduated in 2004 in Medicinal & Pharmaceutical Chemistry at the University of Milan, and in 2007 received her PhD in Medicinal Chemistry from the same University. During the PhD course, she developed part of the research project (2006–2007) in the group directed by Prof. Steven V. Ley, at the Chemistry Department of Cambridge University (U.K.), where she was affiliated to the Innovative Technology Centre, under the supervision of Dr Ian R. Baxendale. She is currently a Post-Doc at the University of Milan working in Prof. De Micheli's group. Her research activity mainly concerns the design and synthesis of new conformationally constrained analogues of endogenous amino acids, in order to obtain ligands able to interact selectively with Glu or GABA receptors and transporters. Moreover, her current research is focused on the

development of innovative synthetic methodologies based on the use of flow reactors aimed to the multistep synthesis of biologically active compounds.



Andrea Pinto, graduated in Medicinal & Pharmaceutical Chemistry at the University of Milan and is at present Assistant Professor at the DISFARM, Faculty of Pharmacy, University of Milan. In 2006, he received the PhD in Medicinal Chemistry from the University of Milan, within the International Doctoral School in Medicinal Chemistry, Pharmacy and Pharmacology, University of Milan, Semmelweis University of Budapest, where he developed part of his research activity under the supervision of Prof. P. Matyus. He did his postdoctoral work in the research group headed by Prof. S. V. Ley (2007), Department of Organic Chemistry, University of Cambridge, U.K., and in Prof. De Micheli's laboratories (2007–2010). The main research interests of Dr. Pinto are the design and synthesis of new potent and selective ligands active at the glutamatergic system and the development of synthetic methodologies for the preparation of bioactive natural compounds.

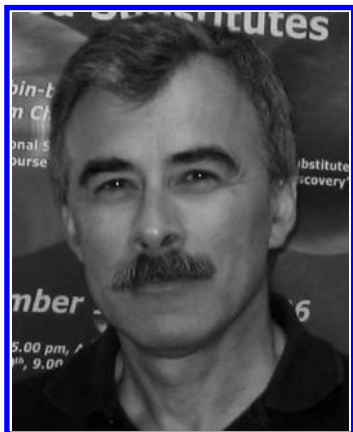


Arnaud Blondel received a diploma of Engineering Science from the Ecole Polytechnique, Paris, France, and trained for his PhD in Biochemistry in Pasteur Institut and University of Paris, France. He was a postdoctoral fellow of Martin Karplus in Harvard University, Cambridge, MA, U.S.A. and University of Strasbourg, France. Since 1997, he is a research scientist at the Institut Pasteur France. He developed protein design and physical-chemistry approaches to unravel the determinants of protein ligand affinity, conceived and developed free energy calculation theory and methods. More recently, he developed new methods to calculate complex transition paths and showed

how such paths can be used to develop innovative drug design strategies

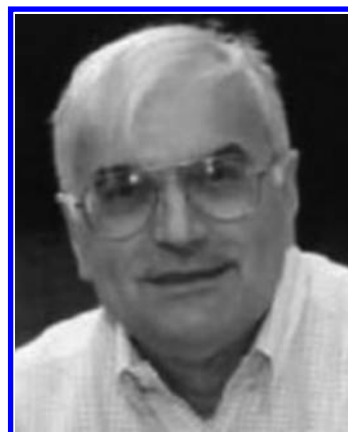


Paola Minoprio received her MSc in Parasitology from University of Sao Paulo, Brazil, and her Ph.D. in Immunology from University of Paris 6, France. She was a postdoctoral fellow under H. Eisen in Fred Hutchinson Cancer Research Center, Seattle, WA, and under A. O' Garra in DNAX Research Center for Molecular and Cellular immunology, Palo Alto, CA, U.S.A. Since 1984, as a research scientist at Institut Pasteur, Paris, France, she has been now leading the laboratory *Trypanosoma* Infectious Processes. Her scientific approaches are focused on mechanisms of parasite escape and persistence in the host. She pursues multidisciplinary strategies to identify lead compounds for new therapies against trypanosomiasis using mitogenic moieties as targets.



Andrea Mozzarelli is Full Professor in Biochemistry, Faculty of Pharmacy, University of Parma, Parma, Italy. Member of the Editorial Board of *Current Medicinal Chemistry*, *Biochimica et Biophysica Acta: Proteins and Proteomics*, *Burger's Medicinal Chemistry VI* and *VII* Edition. The scientific activities are aimed at (i) the elucidation of structure-dynamics-function relationships of hemoglobins, pyridoxal phosphate-dependent enzymes and green fluorescent proteins; (ii) the development of haemoglobin-based oxygen carriers and enzyme inhibitors with potential antibiotic activities; (iii) protein immobilization via either silica gel entrapment or crystallization to select distinct protein conformations, analyzed by spectroscopic and microspectroscopic methods; (iv) the exploitation of computational methods to predict ligand-protein interactions for the design of novel protein ligands; (v) the application of proteomic approaches to

the analysis of food and biological fluids. He has published 132 peer-reviewed papers, several contributions to books and is Editor with Dr. S. Bettati of a forthcoming book on *Chemistry and Biochemistry of Oxygen Therapeutics: from Transfusion to Artificial Blood*, Wiley.



Carlo De Micheli, graduated in Chemistry and in Pharmacy at the University of Pavia, is at present Full Professor of Medicinal Chemistry at the Faculty of Pharmacy of the University of Milan. He has been associated with S.U.N.Y at Buffalo (1981) and University of Rochester (N.Y., 1986) as visiting professor. In 1990, he was promoted to Full Professor at the University of Trieste where he spent four years (1990–1994). He is a member of the International Advisory Board of GluTarget, a research project within the Programme of Excellence at the University of Copenhagen. He is also Coordinator of the Ph.D. programme in medicinal chemistry at the University of Milan and a member of the scientific committee of the "European School of Medicinal Chemistry" which annually takes place in Urbino. His research interest is oriented to the study of the relationships between structure and activity of ligands acting at muscarinic, beta-adrenergic and glutamatergic receptors. He designed and accomplished the synthesis of a number of glutamic acid analogues characterized by a locked conformation. Some of the new derivatives are provided with a pharmacological profile suitable for the development as neuroprotective drugs. Furthermore, he discovered blockers of the glutamate transporters with a pharmacological profile significantly different from that of derivatives reported in the literature.

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ACRONYMS

D-Asp	D-aspartate
L-Asp	L-aspartate
D-Ala	D-alanine
L-Ala	L-alanine
D-Glu	D-glutamate
L-Glu	L-glutamate
D-Leu	D-leucine
L-Pro	L-proline

D-Pro	D-proline
D-Ser	D-serine
L-Ser	L-serine
D-Phe	D-phenylalanine
L-Phe	L-phenylalanine
D-Arg	D-arginine
D-Met	D-methionine
D-Tyr	D-tyrosine
DR	PLP-dependent aspartate racemase
AspR	PLP-independent aspartate racemase
PhAspR	<i>Pyrococcus horikoshii</i> OT3 AspR
AlaR	alanine racemase
MtbAlaR	<i>M. tuberculosis</i> AlaR
PaoAlaR	<i>P. aeruginosa</i> AlaR
BstAlaR	<i>B. stearothermophilus</i> AlaR
GluR	glutamate racemase
ProR	proline racemase
SerR	serine racemase
ArgR	arginine racemase
DAPE	diaminopimelate epimerase
ATC	aspartate transcarbamylase
DAAO	D-amino acid oxidase
SOD 1	Superoxide Dismutase 1
PKC	protein kinase C
PLC	phospholipase C
PICK1	protein interacting with C kinase 1
GRIP	glutamate receptor interacting protein
GH	growth hormone
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
α-MSH	α-melanocyte-stimulating hormone
L-Ala-P	(L)-(1-aminoethyl)phosphonic acid
L-Ala-B	(L)-(1-aminoethyl)boronic acid
L-SOS	L-serine-O-sulfate
azi-DAP	2-(4-amino-4-carboxybutyl)aziridine-2-carboxylic acid
DAP	(L,L)-2,6-diaminopimelic acid
PYC	pyrrole-2-carboxylic acid
NMDA	N-methyl-D-aspartate
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid
GABA	γ-aminobutyric acid
PLP	pyridoxal-5'-phosphate
PIP2	phosphatidylinositol-(4,5)-bisphosphate
ATP	adenosine-5'-triphosphate
ADP	adenosine diphosphate
GDP	guanosine diphosphate
ITC	isothermal titration calorimetry
CNS	central nervous system
HTC	high throughput screening
MDR	multi-drug-resistant
POE	Path Optimization and Exploration
Tc	<i>Trypanosoma cruzi</i>
Tv	<i>Trypanosoma vivax</i>
Cs	<i>Clostridium sticklandii</i>
ALS	Amyotrophic Lateral Sclerosis

REFERENCES

- (1) Lamzin, V. S.; Dauter, Z.; Wisoln, K. S. *Curr. Opin. Struct. Biol.* **1995**, *5*, 830.
- (2) Prelog, V. *Science* **1976**, *193*, 17.

- (3) Corrigan, J. J. *Science* **1969**, *164*, 142.
- (4) Meister, A. *Biochemistry of the Amino Acids*, 2nd ed; Meister, A., Ed.; Academic Press: New York, 1965; Vol 1, pp 113–139.
- (5) Holtje, J. V. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 181.
- (6) van Heijenoort, J. *Nat. Prod. Rep.* **2001**, *18*, 503.
- (7) Lam, H.; Oh, D. C.; Cava, F.; Takacs, C. N.; Clardy, J.; de Pedro, M. A.; Waldor, M. K. *Science* **2009**, *325*, 1552.
- (8) Broccardo, M.; Erspamer, V.; Falconieri Erspamer, G.; Improta, G.; Linari, G.; Melchiorri, P.; Montecucchi, P. C. *Br. J. Pharmacol.* **1981**, *73*, 625.
- (9) Kreil, G. *Annu. Rev. Biochem.* **1997**, *66*, 337.
- (10) Kamatani, Y.; Minakata, H.; Kenny, P. T.; Iwashita, T.; Watanabe, K.; Funase, K.; Sun, X. P.; Yongsiri, A.; Kim, K. H.; Novales-Li, P.; Novales, E. T.; Kanapi, C. G.; Takeuchi, H.; Nomoto, K. *Biochem. Biophys. Res. Commun.* **1989**, *160*, 1015.
- (11) Krebs, H. A. *Biochem. J.* **1935**, *29*, 1620.
- (12) Helfman, P. M.; Bada, J. L.; Shou, M. Y. *Gerontology* **1977**, *23*, 419.
- (13) Wolosker, H.; Blackshaw, S.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 13409.
- (14) Kim, P. M.; Duan, X.; Huang, A. S.; Liu, C. Y.; Ming, G.-l.; Song, H.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 3175.
- (15) Tanner, M. E. *Acc. Chem. Res.* **2002**, *35*, 237.
- (16) John, R. A. *Biochim. Biophys. Acta* **1995**, *1248*, 81.
- (17) Eliot, A. C.; Kirsch, J. F. *Annu. Rev. Biochem.* **2004**, *73*, 383.
- (18) Toney, M. D. *Arch. Biochem. Biophys.* **2005**, *433*, 279.
- (19) Amadasi, A.; Bertoldi, M.; Contestabile, R.; Bettati, S.; Cellini, B.; di Salvo, M. L.; Borri-Voltattorni, C.; Bossa, F.; Mozzarelli, A. *Curr. Med. Chem.* **2007**, *14*, 1291.
- (20) Dixon, J. E.; Bruice, T. C. *Biochemistry* **1973**, *12*, 4762.
- (21) Toth, K.; Richard, J. P. *J. Am. Chem. Soc.* **2007**, *129*, 3013.
- (22) Dunathan, H. C. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1971**, *35*, 79.
- (23) Kirsch, J. F.; Eichele, G.; Ford, G. C.; Vincent, M. G.; Jansonius, J. N.; Gehring, H.; Christen, P. *J. Mol. Biol.* **1984**, *174*, 497.
- (24) Momany, C.; Ernst, S.; Ghosh, R.; Chang, N. L.; Hackert, M. L. *Protein Sci.* **1995**, *4*, 84.
- (25) Toney, M. D.; Hohenester, S. W.; Cowan, S. W.; Jansonius, J. N. *Science* **1993**, *261*, 756.
- (26) Grishin, N. V.; Phillips, M. A.; Goldsmith, E. J. *Protein Sci.* **1995**, *4*, 1291.
- (27) Soda, K.; Yoshimura, T.; Esaki, N. *Chem. Rec.* **2001**, *1*, 373.
- (28) Schneider, G.; Käck, H.; Lindqvist, Y. *Structure* **2000**, *8*, R1.
- (29) Hyde, C. C.; Ahmed, S. A.; Padlan, E. A.; Miles, E. W.; Davies, D. R. *J. Biol. Chem.* **1988**, *263*, 17857.
- (30) Gallagher, D. T.; Gilliland, G. L.; Xiao, G.; Zondlo, J.; Fisher, K. E.; Chinchilla, D.; Eisenstein, E. *Structure* **1998**, *6*, 465.
- (31) Burkhard, P.; Rao, G. S. J.; Hohenester, E.; Schnackerz, K. D.; Cook, P. F.; Jansonius, J. N. *J. Mol. Biol.* **1998**, *283*, 121.
- (32) Cardinale, G. J.; Abeles, R. H. *Biochemistry* **1968**, *7*, 3970.
- (33) Rudnick, G.; Abeles, R. H. *Biochemistry* **1975**, *14*, 4515.
- (34) Stadtman, T. C.; Elliot, P. J. *Biol. Chem.* **1957**, *228*, 983.
- (35) Albery, W. J.; Knowles, J. R. *Biochemistry* **1986**, *25*, 2572.
- (36) Gallo, K. A.; Tanner, M. E.; Knowles, J. R. *Biochemistry* **1993**, *32*, 3991.
- (37) Yamauchi, T.; Choi, S. Y.; Okada, H.; Yohda, M.; Kumagai, H.; Esaki, N.; Soda, K. *J. Biol. Chem.* **1992**, *267*, 18361.
- (38) Pillai, B.; Cherney, M. M.; Diaper, C. M.; Sutherland, A.; Blanchard, J. S.; Vederas, J. C.; James, M. N. G. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 8668.
- (39) Abe, H.; Yoshikawa, N.; Sarower, M. G.; Okada, S. *Biol. Pharm. Bull.* **2005**, *28*, 1571.
- (40) Hoffmann, K.; Schneider-Scherzer, E.; Kleinkauf, H.; Zocher, R. *J. Biol. Chem.* **1994**, *269*, 12710.
- (41) Cheng, Y. Q.; Walton, J. D. *J. Biol. Chem.* **2000**, *275*, 4906.
- (42) Uo, T.; Yoshimura, T.; Tanaka, N.; Takegawa, K.; Esaki, N. *J. Bacteriol.* **2001**, *183*, 2226.

- (43) Ono, K.; Yanagida, K.; Oikawa, T.; Ogawa, T.; Soda, K. *Phytochemistry* **2006**, *67*, 856.
- (44) Walsh, C. T. *J. Biol. Chem.* **1989**, *64*, 2393.
- (45) McFall, E.; Newman, E. B. *Escherichia coli and Salmonella: Cellular and Molecular biology*; Neidhart, F. C./Am. Soc. Microbiol.: Washington, D.C., 1996; pp 358–379.
- (46) Yan, X.; Gai, Y.; Liang, L.; Liu, G.; Tan, H. *Arch. Microbiol.* **2007**, *187*, 371.
- (47) Preston, R. A.; Douthit, H. A. *J. Gen. Microbiol.* **1988**, *134*, 3001.
- (48) Huang, C. M.; Elmetts, C. A.; Tang, D. C.; Li, F.; Yusuf, N. *Genomics, Proteomics Bioinf.* **2004**, *2*, 143.
- (49) Chesnokova, O. N.; McPherson, S. A.; Steichen, C. T.; Turnbough, C. L., Jr. *J. Bacteriol.* **2009**, *191*, 1303.
- (50) Dixon, T. C.; Meselson, M.; Guillemin, J.; Hanna, P. C. *N. Engl. J. Med.* **1999**, *341*, 815.
- (51) Dahlgren, C. M.; Buchanan, L. M.; Decker, H. M.; Freed, S. W.; Phillips, C. R.; Brachman, P. S. *Am. J. Hyg.* **1960**, *72*, 24.
- (52) Meselson, M.; Guillemin, J.; Hugh-Jones, M.; Langmuir, A.; Popova, L.; Shelokov, A.; Yampolskaya, O. *Science* **1994**, *266*, 1202.
- (53) Morollo, A. A.; Petsko, G. A.; Ringe, D. *Biochemistry* **1999**, *38*, 3293.
- (54) Stamper, C. G. F.; Morollo, A. A.; Ringe, D. *Biochemistry* **1998**, *37*, 10438.
- (55) Watanabe, A.; Yoshimura, T.; Mikami, B.; Hayashi, H.; Kagamiyama, H.; Esaki, N. *J. Biol. Chem.* **2002**, *277*, 19166.
- (56) Shaw, J. P.; Petsko, G. A.; Ringe, D. *Biochemistry* **1997**, *36*, 1329.
- (57) Iurcu Mustata, G.; Soares, T. A.; Briggs, J. M. *Biopolymers* **2003**, *70*, 186.
- (58) Spies, M. A.; Woodward, J. J.; Watnik, M. R.; Toney, M. D. *J. Am. Chem. Soc.* **2004**, *126*, 7464.
- (59) Rubinstein, A.; Major, D. T. *Biochemistry* **2010**, *49*, 3957.
- (60) Major, D. T.; Gao, J. J. *Am. Chem. Soc.* **2006**, *128*, 16345.
- (61) Rubinstein, A.; Major, D. T. *J. Am. Chem. Soc.* **2009**, *131*, 8513.
- (62) Toney, M. D. *Arch. Biochem. Biophys.* **2005**, *433*, 279.
- (63) Lin, Y. L.; Gao, J.; Rubinstein, A.; Major, D. T. *Biochim. Biophys. Acta* **2011**; doi: 10.1016/j.bbapap.2011.05.002.
- (64) Strych, U.; Davlieva, M.; Longtin, J. P.; Murphy, E. L.; Im, H.; Benedik, M. J.; Krause, K. L. *BMC Microbiol.* **2007**, *7*, 40.
- (65) Watanabe, A.; Yoshimura, T.; Mikami, B.; Esaki, N. *J. Biochem.* **1999**, *125*, 987.
- (66) Yoshimura, T.; Goto, M. *FEBS Journal* **2008**, *275*, 3527.
- (67) Ondrechen, M. J.; Briggs, J. M.; McCammon, J. A. *J. Am. Chem. Soc.* **2001**, *123*, 2830.
- (68) Kurokawa, Y.; Watanabe, A.; Yoshimura, T.; Esaki, N.; Soda, K. *J. Biochem.* **1998**, *124*, 1163.
- (69) Neuhaus, F. C. *Antimicrob. Agents Chemother.* **1967**, *7*, 304.
- (70) Roze, U.; Strominger, J. L. *Mol. Pharmacol.* **1966**, *2*, 92.
- (71) Lambert, M. P.; Neuhaus, F. C. *J. Bacteriol.* **1972**, *110*, 978.
- (72) Johnston, R. B.; Scholz, J. J.; Diven, W. F.; Shepard, S.; Pyridoxal Catalysis: Enzymes and Model Systems; Snell, E. E., Braunstein, A. E., Severin, E. S., Torchinsky, Y. M., Eds.; Wiley-Interscience: New York, 1968; p 537.
- (73) Wargel, R. J.; Shadur, C. A.; Neuhaus, F. C. *J. Bacteriol.* **1970**, *103*, 778.
- (74) Fenn, T. D.; Stamper, G. F.; Morollo, A. A.; Ringe, D. *Biochemistry* **2003**, *42*, 5775.
- (75) David, H. L.; Takayama, K.; Goldman, D. S. *Am. Rev. Respir. Dis.* **1969**, *100*, 579.
- (76) Lee, H. K. *Chest* **1960**, *37*, 378.
- (77) Pitkanen, M.; Sirvio, J.; MacDonald, E.; Ekonsalo, T.; Riekinen, P., Sr. *J. Neural Transm.* **1995**, *9*, 133.
- (78) Wood, J. D.; Peesker, S. J.; Gorecki, D. K. J.; Tsui, D. *Can. J. Physiol. Pharmacol.* **1978**, *52*, 62.
- (79) Reitz, R. H.; Slade, H. D.; Neuhaus, F. C. *Biochemistry* **1967**, *6*, 2561.
- (80) Neuhaus, F. C.: *Antibiotics, Vol. 1. Mechanisms of Action*; Gottlieb, D., Shaw, P. L., Ed.; Springer-Verlag: Heidelberg, Germany, 1967; p 40.
- (81) Cáceres, N. E.; Harris, N. B.; Wellehan, J. F.; Feng, Z.; Kapur, V.; Barletta, R. G. *J. Bacteriol.* **1997**, *179*, 5046.
- (82) Peisach, D.; Chipman, D. M.; Van Ophem, P. W.; Manning, J. M.; Ringe, D. *J. Am. Chem. Soc.* **1998**, *120*, 2268.
- (83) Kollonitsch, J.; Barash, L.; Kahan, F. H.; Kropp, H. *Nature* **1973**, *243*, 346.
- (84) Badet, B.; Roise, D.; Walsh, C. T. *Biochemistry* **1984**, *23*, 5188.
- (85) Faraci, W. S.; Walsh, C. T. *Biochemistry* **1989**, *28*, 431.
- (86) Thornberry, N. A.; Bull, H. G.; Taub, D.; Greenlee, W. J.; Patchett, A. A.; Cordes, E. H. *J. Am. Chem. Soc.* **1987**, *109*, 1543.
- (87) Wang, E.; Walsh, C. T. *Biochemistry* **1981**, *20*, 7539.
- (88) Kaczorowski, G.; Shaw, L.; Laura, R.; Walsh, C. T. *J. Biol. Chem.* **1975**, *250*, 8921.
- (89) Wang, E.; Walsh, C. *Biochemistry* **1978**, *17*, 1313.
- (90) Kollonitsch, J.; Barash, L. *J. Am. Chem. Soc.* **1976**, *98*, 5591.
- (91) Thornberry, N. A.; Bull, H. G.; Taub, D.; Wilson, K. E.; Giménez-Gallego, G.; Rosegay, A.; Soderman, D. D.; Patchett, A. A. *J. Biol. Chem.* **1991**, *266*, 21657.
- (92) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Lambert, R. W.; Lloyd, W. J.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 696.
- (93) Adams, E.; Mukherjee, K. L.; Dunathan, H. C. *Arch. Biochem. Biophys.* **1974**, *165*, 126.
- (94) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Lambert, R. W.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 677.
- (95) Badet, B.; Inagaki, K.; Soda, D.; Walsh, C. T. *Biochemistry* **1986**, *25*, 3275.
- (96) Copie, V.; Faraci, W. S.; Walsh, C. T.; Griffin, R. G. *Biochemistry* **1988**, *27*, 4966.
- (97) Duncan, K.; Faraci, S.; Matteson, D. S.; Walsh, C. T. *Biochemistry* **1989**, *28*, 3541.
- (98) LeMagueres, P.; Im, H.; Ebalunode, J.; Strych, U.; Benedik, M. J.; Briggs, J. M.; Kohn, H.; Krause, K. L. *Biochemistry* **2005**, *44*, 1471.
- (99) Au, K.; Ren, J.; Walter, T. S.; Harlos, K.; Nettleship, J. E.; Owens, R. J.; Stuart, D. I.; Esnouf, R. M. *Acta Crystallogr., Sect. F* **2008**, *64*, 327.
- (100) Im, H.; Sharpe, M. L.; Strych, U.; Davlieva, M.; Krause, K. L. *BMC Microbiol.* **2011**, *11*, 116.
- (101) Priyadarshi, A.; Lee, E. H.; Sung, M. W.; Nam, K. H.; Lee, W. H.; Kim, E. E.; Hwang, K. Y. *Biochim. Biophys. Acta* **2009**, *1794*, 1030.
- (102) Wu, D.; Hu, T.; Zhang, L.; Chen, J.; Du, J.; Ding, J.; Jiang, H.; Shen, X. *Protein Sci.* **2008**, *17*, 1066.
- (103) Ju, J.; Qi, J.; Xu, S.; Ohnishi, K.; Benedik, M. J.; Xue, Y.; Ma, Y. *Acta Crystallogr., Sect. F* **2009**, *65*, 166.
- (104) Ju, J.; Xu, S.; Wen, J.; Li, G.; Ohnishi, K.; Xue, Y.; Ma, Y. *J. Biosci. Bioeng.* **2009**, *107*, 225.
- (105) Ju, J.; Xu, S.; Furukawa, Y.; Zhang, Y.; Misono, H.; Minamino, T.; Namba, K.; Zhao, B.; Ohnishi, K. *J. Biochem.* **2011**, *149*, 83.
- (106) Seebeck, F. P.; Hilvert, D. *J. Am. Chem. Soc.* **2003**, *125*, 10158.
- (107) Watanabe, A.; Yoshimura, T.; Mikami, B.; Esaki, N. *J. Biochem.* **1999**, *126*, 781.
- (108) Fesko, K.; Giger, L.; Hilvert, D. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5987.
- (109) Uo, T.; Yoshimura, T.; Shimizu, S.; Esaki, N. *Biochem. Biophys. Res. Commun.* **1998**, *246*, 31.
- (110) Wolosker, H.; Sheth, K. N.; Takahashi, M.; Mothet, J. P.; Brady, R. O., Jr; Ferris, C. D.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 721.
- (111) Wolosker, H. *Biochim. Biophys. Acta* **2011**; doi: 10.1016/j.bbapap.2011.01.001.
- (112) Kartvelishvili, E.; Shleper, M.; Balan, L.; Dumin, E.; Wolosker, H. *J. Biol. Chem.* **2006**, *281*, 14151.
- (113) Yoshikawa, M.; Takayasu, N.; Hashimoto, A.; Sato, Y.; Tamaki, R.; Tsukamoto, H.; Kobayashi, H.; Noda, S. *Arch. Histol. Cytol.* **2007**, *70*, 127.
- (114) Stevens, E. R.; Esguerra, M.; Kim, P. M.; Newman, E. A.; Snyder, S. H.; Zahs, K. R.; Miller, R. F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6789.
- (115) Wu, S.; Barger, S. W.; Sims, T. J. *Brain Res.* **2004**, *1020*, 161.

- (116) Takarada, T.; Takahata, Y.; Iemata, M.; Hinoi, E.; Uno, K.; Hirai, T.; Yamamoto, T.; Yoneda, Y. *J. Cell. Physiol.* **2009**, *220*, 756.
- (117) Hashimoto, A.; Kumashiro, S.; Nishikawa, T.; Oka, T.; Takahashi, K.; Mito, T.; Takashima, S.; Doi, N.; Mizutani, Y.; Yamazaki, T.; Kaneko, T.; Ootomo, E. *J. Neurochem.* **1993**, *1*, 348.
- (118) Hashimoto, A.; Oka, T.; Nishikawa, T. *Eur. J. Neurosci.* **1995**, *7*, 1657.
- (119) Nagata, Y.; Horiike, K.; Maeda, T. *Brain Res.* **1994**, *634*, 291.
- (120) Dunlop, D. S.; Neidle, A.; McHale, D.; Dunlop, D. M.; Lajtha, A. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 27.
- (121) *Excitatory Amino Acids and Synaptic Transmission*; Wheal, H. V., Thomson, A. M., Ed.; Academic Press: London, 1995.
- (122) Bräuner-Osborne, H.; Egebjerg, J.; Nielsen, E. Ø.; Madsen, U.; Krogsgaard-Larsen, P. *J. Med. Chem.* **2000**, *43*, 2609.
- (123) Lipton, S. A. *J. Alzheimer's Dis.* **2004**, *6*, S61.
- (124) Kemp, J. A.; McKernan, R. M. *Nat. Neurosci.* **2002**, *5*, 1039.
- (125) Hashimoto, K.; Fukushima, T.; Shimizu, E.; Okada, S.; Komatsu, N.; Okamura, N.; Koike, K.; Koizumi, H.; Kumakiri, C.; Imai, K.; Iyo, M. *Biol. Psychiatry* **2004**, *28*, 385.
- (126) Sasabe, J.; Chiba, T.; Yamada, M.; Okamoto, K.; Nishimoto, I.; Matsuoka, M.; Aiso, S. *EMBO J.* **2007**, *26*, 4149.
- (127) Kwak, S.; Hideyama, T.; Yamashita, T.; Aizawa, H. *Neuropathology* **2010**, *30*, 182.
- (128) Wu, S.; Barger, S. W. *Ann. N.Y. Acad. Sci.* **2004**, *1035*, 133.
- (129) Labrie, V.; Fukumura, R.; Rastogi, A.; Fick, L. J.; Wang, W.; Boutros, P. C.; Kennedy, J. L.; Semeralul, M. O.; Lee, F. H.; Baker, G. B.; Belsham, D. D.; Barger, S. W.; Gondo, Y.; Wong, A. H. C.; Roder, J. C. *Hum. Mol. Genet.* **2009**, *18*, 3227.
- (130) Bendikov, I.; Nadri, C.; Amar, S.; Panizzutti, R.; De Miranda, J.; Wolosker, H.; Agam, G. *Schizophr. Res.* **2007**, *90*, 41.
- (131) Hashimoto, K.; Fukushima, T.; Shimizu, E.; Komatsu, N.; Watanabe, H.; Shinoda, N.; Nakazato, M.; Kumakiri, C.; Okada, S.; Hasegawa, H.; Kazuhiro, I.; Masaomi, I. *Arch. Gen. Psychiatry* **2003**, *60*, 572.
- (132) Morita, Y.; Ujike, H.; Tanaka, Y.; Otani, K.; Kishimoto, M.; Morio, A.; Kotaka, T.; Okahisa, Y.; Matsushita, M.; Morikawa, A.; Hamase, K.; Zaitu, K.; Kuroda, S. *Biol. Psychiatry* **2007**, *61*, 1200.
- (133) Chumakov, I.; Blumenfeld, M.; Guerassimenko, O.; Cavarec, L.; Palicio, M.; Abderrahim, H.; Bougueleret, L.; Barry, C.; Tanaka, H.; La Rosa, P.; Puech, A.; Tahri, N.; Cohen-Akenine, A.; Delabrosse, S.; Lissarrague, S.; Picard, F.-P.; Maurice, K.; Essioux, L.; Millasseau, P.; Grel, P.; Debailleul, V.; Simon, A.-M.; Caterina, D.; Dufaure, I.; Malekzadeh, K.; Belova, M.; Luan, J.-J.; Bouillot, M.; Sambucy, J.-L.; Primas, G.; Saumier, M.; Boubkiri, N.; Martin-Saumier, S.; Nasroune, M.; Peixoto, H.; Delaye, A.; Pinchot, V.; Bastucci, M.; Guillou, S.; Chevillon, M.; Sainz-Fuertes, R.; Meguenni, S.; Aurich-Costa, J.; Cherif, D.; Gimalac, A.; Van Duijn, C.; Gauvreau, D.; Ouelette, G.; Fortier, I.; Realson, J.; Sherbatich, T.; Riazanskaia, N.; Rogaev, E.; Raeymaekers, P.; Aerssens, J.; Konings, F.; Luyten, W.; Maciardi, F.; Sham, P. C.; Straub, R. E.; Weinberger, D. R.; Cohen, N.; Cohen, D. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 13675.
- (134) Mori, H.; Inoue, R. *Chem. Biodiversity* **2010**, *7*, 1573.
- (135) Goto, M.; Yamauchi, T.; Kamiya, N.; Miyahara, I.; Yoshimura, T.; Mihara, H.; Kurihara, T.; Hirotsu, K.; Esaki, N. *J. Biol. Chem.* **2009**, *284*, 25944.
- (136) Smith, M. A.; Mack, V.; Ebner, A.; Moraes, I.; Felicetti, B.; Wood, M.; Schonfeld, D.; Mather, O.; Cesura, A.; Barker, J. *J. Biol. Chem.* **2010**, *285*, 12873.
- (137) Foltyn, V. N.; Bendikov, I.; De Miranda, J.; Panizzutti, R.; Dumin, E.; Shleper, M.; Li, P.; Toney, M. D.; Kartvelishvili, E.; Wolosker, H. *J. Biol. Chem.* **2005**, *280*, 1754.
- (138) Hoffman, H. E.; Jiraskova, J.; Zvebil, M.; Konvalinka, J. *Collect. Czech. Chem. Commun.* **2010**, *75*, 59.
- (139) Baumgart, F.; Mancheno, J. M.; Rodriguez-Crespo, I. *FEBS J.* **2007**, *274*, 4561.
- (140) De Miranda, J.; Panizzutti, R.; Foltyn, V. N.; Wolosker, H. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14542.
- (141) Peracchi, A.; Mozzarelli, A.; Rossi, G. L. *Biochemistry* **1995**, *34*, 9459.
- (142) Raboni, S.; Bettati, S.; Mozzarelli, A. *Cell. Mol. Life Sci.* **2009**, *66*, 2391.
- (143) Boehning, D.; Snyder, S. H. *Annu. Rev. Neurosci.* **2003**, *26*, 105.
- (144) Kim, P. M.; Aizawa, H.; Kim, P. S.; Huang, A. S.; Wickramasinghe, S. R.; Kashani, A. H.; Barrow, R. K.; Haganir, R. L.; Ghosh, A.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 2105.
- (145) Fujii, K.; Maeda, K.; Hikida, T.; Mustafa, A. K.; Balkissoon, R.; Xia, J.; Yamada, T.; Ozeki, Y.; Kawahara, R.; Okawa, M.; Haganir, R. L.; Ujike, H.; Snyder, S. H.; Sawa, A. *Mol. Psychiatry* **2006**, *11*, 150.
- (146) Dumin, E.; Bendikov, I.; Foltyn, V. N.; Misumi, Y.; Ikehara, Y.; Kartvelishvili, E.; Wolosker, H. *J. Biol. Chem.* **2006**, *281*, 20291.
- (147) Vargas-Lopes, C.; Madeira, C.; Kahn, S. A.; Albino do Couto, I.; Bado, P.; Houzel, J. C.; De Miranda, J.; de Freitas, M. S.; Ferreira, S. T.; Panizzutti, R. *J. Neurochem.* **2011**, *116*, 281.
- (148) Mustafa, A. K.; van Rossum, D. B.; Patterson, R. L.; Maag, D.; Ehmsen, J. T.; Gazi, S. K.; Chakraborty, A.; Barrow, R. K.; Amzel, L. M.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 2921.
- (149) Mustafa, A. K.; Kumar, M.; Selvakumar, B.; Ho, G. P.; Ehmsen, J. T.; Barrow, R. K.; Amzel, L. M.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 2950.
- (150) Arias, C. A.; Martin-Martinez, M.; Blundell, T. L.; Arthur, M.; Courvalin, P.; Reynolds, P. E. *Mol. Microbiol.* **1999**, *31*, 1653.
- (151) Strisovsky, K.; Jiraskova, J.; Mikulova, A.; Rulisek, L.; Konvalinka, J. *Biochem. J.* **2005**, *44*, 13091.
- (152) Hoffman, H. E.; Jirasková, J.; Cígler, P.; Šanda, M.; Schraml, J.; Konvalinka, J. *J. Med. Chem.* **2009**, *52*, 6032.
- (153) Bruno, A.; Amori, L.; Costantino, G. *Mol. Inf.* **2011**, *30*, 317.
- (154) Baroni, M.; Cruciani, G.; Sciabola, S.; Perruccio, F.; Mason, J. S. *J. Chem. Inf. Model* **2007**, *47*, 279.
- (155) Jones, G.; Willett, P.; Glen, R. C. *J. Mol. Biol.* **1995**, *245*, 43.
- (156) Kellogg, E. G.; Abraham, D. J. *Eur. J. Med. Chem.* **2000**, *35*, 651.
- (157) Cozzini, P.; Fornabaio, M.; Marabotti, A.; Abraham, D. J.; Kellogg, G. E.; Mozzarelli, A. *J. Med. Chem.* **2002**, *45*, 2469.
- (158) Spyrikis, F.; Amadasi, A.; Fornabaio, M.; Abraham, D. J.; Mozzarelli, A.; Kellogg, G. E.; Cozzini, P. *Eur. J. Med. Chem.* **2007**, *42*, 921.
- (159) Spyrikis, F.; Dellafiora, L.; Cozzini, P.; Singh, R.; Salsi, E.; Benedetti, P.; Cruciani, G.; Carosati, E.; Campanini, B.; Cellini, B.; Mozzarelli, A. Presented at the 2nd Workshop in New Trends in Computational Chemistry for Industry Applications; Barcelona, 2011.
- (160) Salsi, E.; Bayden, A.; Spyrikis, F.; Amadasi, A.; Campanini, B.; Bettati, S.; Cozzini, P.; Kellogg, G. E.; Cook, P. F.; Dodatko, T.; Roderick, S. L.; Mozzarelli, A. *J. Med. Chem.* **2010**, *53*, 345.
- (161) Yorifuji, T.; Misono, H.; Soda, K. *J. Biol. Chem.* **1971**, *246*, 5093.
- (162) Matsui, D.; Oikawa, T.; Arakawa, N.; Osumi, S.; Lausberg, F.; Stäbler, N.; Freudl, R.; Eggeling, L. *Appl. Microbiol. Biotechnol.* **2009**, *83*, 1045.
- (163) Matsui, D.; Oikawa, T. *Chem. Biodiversity* **2010**, *7*, 1591.
- (164) Jann, A.; Matsumoto, H.; Haas, D. *J. Gen. Microbiol.* **1988**, *134*, 1043.
- (165) Haas, D.; Matsumoto, H.; Moretti, P.; Stalon, V.; Mercenier, A. *Mol. Gen. Genet.* **1984**, *193*, 437.
- (166) Dunlop, D. S.; Neidle, A.; McHale, D.; Dunlop, D. M.; Lajtha, A. *Biochem. Biophys. Res. Commun.* **1996**, *141*, 27.
- (167) Hashimoto, A.; Nishikawa, T.; Konno, R.; Niwa, A.; Yasumura, Y.; Oka, T.; Takahashi, K. *Neurosci. Lett.* **1993**, *152*, 33.
- (168) D'Aniello, A.; Di Cosmo, A.; Di Cristo, C.; Annunziato, L.; Petrucci, L.; Fisher, G.; D'Aniello, A. *Life Sci.* **1996**, *59*, 97.
- (169) D'Aniello, S.; Somorjai, I.; Garcia-Fernández, J.; Topo, E.; D'Aniello, A. *FASEB J.* **2011**, *25*, 1014.
- (170) Erreger, K.; Geballe, M. T.; Kristensen, A.; Chen, P. E.; Hansen, K. B.; Lee, C. J.; Yuan, H.; Le, P.; Lyuboslavsky, P. N.; Micale,

- N.; Jorgensen, L.; Clausen, R. P.; Wyllie, D. J. A.; Snyder, J. P.; Traynelis, S. F. *Mol. Pharmacol.* **2007**, *72*, 907.
- (171) Skerritt, J. H.; Johnston, G. A. *J. Neurochem.* **1981**, *36*, 881.
- (172) D'Aniello, A.; Di Fiore, M. M.; Fisher, G. H.; Milone, A.; Seleni, A.; D'Aniello, S.; Perna, A. F.; Ingrosso, D. *FASEB J.* **2000**, *14*, 699.
- (173) Wang, H.; Wolosker, H.; Pevsner, J.; Snyder, S. H.; Selkoe, D. J. *J. Endocrinol.* **2000**, *167*, 247.
- (174) Topo, E.; Soricelli, A.; D'Aniello, A.; Ronsini, S.; D'Aniello, G. *Reprod. Biol. End.* **2009**, *7*, 120.
- (175) Schell, M. J.; Cooper, O. B.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2013.
- (176) Imai, K.; Fukushima, T.; Hagiwara, K.; Santa, T. *Biomed. Chromatogr.* **1995**, *9*, 106.
- (177) Ishio, S.; Yamada, H.; Hayashi, M.; Yatsushiro, S.; Noumi, T.; Yamaguchi, A.; Moriyama, Y. *Neurosci. Lett.* **1998**, *249*, 143.
- (178) Pampillo, M.; Scimonelli, T.; Bottin, M. C.; Duvilanski, B. H.; Rettori, V.; Seilicovich, A.; Lasaga, M. *Neuroreport* **2002**, *13*, 2341.
- (179) Wolosker, H.; D'Aniello, A.; Snyder, S. H. *Neurosci.* **2000**, *100*, 183.
- (180) Ming, G. L.; Song, H. *Annu. Rev. Neurosci.* **2005**, *28*, 223.
- (181) Zhao, C.; Deng, W.; Gage, F. H. *Cell* **2008**, *132*, 645.
- (182) Lee, J. A.; Homma, H.; Tashiro, K.; Iwatsubo, T.; Imai, K. *Brain Res.* **1999**, *838*, 193.
- (183) Sakai, K.; Homma, H.; Lee, J. A.; Fukushima, T.; Santa, T.; Tashiro, K.; Iwatsubo, T.; Imai, K. *Arch. Biochem. Biophys.* **1998**, *351*, 96.
- (184) Shibata, K.; Watanabe, T.; Yoshikawa, H.; Abe, K.; Takahashi, S.; Kera, Y.; Yamada, R.-h. *Comp. Biochem. Physiol.* **2003**, *B134*, 307.
- (185) Shibata, K.; Watanabe, T.; Yoshikawa, H.; Abe, K.; Takahashi, S.; Kera, Y.; Yamada, R.-h. *Comp. Biochem. Physiol.* **2003**, *B134*, 713.
- (186) Reina-San-Martin, B.; Degraeve, W.; Rougeot, C.; Cosson, A.; Chamond, N.; Cordeiro-Da-Silva, A.; Arala-Chaves, M.; Coutinho, A.; Minoprio, P. *Nat. Med.* **2000**, *6*, 890.
- (187) Minoprio, P.; Itoharu, S.; Heusser, C.; Tonegawa, S.; Coutinho, A. *Immunol. Rev.* **1989**, *112*, 183.
- (188) Reina-San-Martin, B.; Cosson, A.; Minoprio, P. *Parasitol. Today* **2000**, *16*, 62.
- (189) Chamond, N.; Grégoire, C.; Coatnoan, N.; Rougeot, C.; Holanda Freitas-Junior, L.; Franco da Silveira, J.; Degraeve, W. M.; Minoprio, P. *J. Biol. Chem.* **2003**, *278*, 15484.
- (190) Buschiazzo, A.; Goytia, M.; Schaeffer, F.; Degraeve, W.; Shepard, W.; Grégoire, C.; Chamond, N.; Cosson, A.; Berneman, A.; Coatnoan, N.; Alzari, P. M.; Minoprio, P. *Proc. Nat. Acad. Sci. U.S.A.* **2006**, *103*, 1705.
- (191) Chamond, N.; Cosson, A.; Coatnoan, N.; Minoprio, P. *Mol. Biochem. Parasitol.* **2009**, *165*, 170.
- (192) Chamond, N.; Goytia, M.; Coatnoan, N.; Barale, J.-C.; Cosson, A.; Degraeve, W. M.; Minoprio, P. *Mol. Microbiol.* **2005**, *58*, 46.
- (193) Coatnoan, N.; Berneman, A.; Chamond, N.; Minoprio, P. *Mem. Inst. Oswaldo Cruz, Rio de Janeiro* **2009**, *104*, 295.
- (194) Sela, M.; Zisman, E. *FASEB J.* **1997**, *11*, 449.
- (195) *Working to Overcome the Global Impact of Neglected Tropical Diseases*. First WHO Report on Neglected Tropical Diseases; Crompton, D. W. T., Ed.; World Health Organization: Geneva, 2010, p 75.
- (196) Kirchhoff, L. V.; Weiss, L. M.; Wittner, M.; Tanowitz, H. B. *Front. Biosci.* **2004**, *9*, 706.
- (197) Gutierrez, F. R. S.; Guedes, P. M. M.; Gazzinelli, R. T.; Silva, J. S. *Parasite Immunol.* **2009**, *31*, 673.
- (198) Coura, J. R.; Pinto Dias, J. C. *Mem. Inst. Oswaldo Cruz, Rio de Janeiro* **2009**, *104*, 31.
- (199) Silber, A. M.; Tonelli, R. R.; Martinelli, M.; Colli, W.; Alves, M. J. *J. Eukaryot. Microbiol.* **2002**, *49*, 441.
- (200) Sylvester, D.; Krassner, S. M. *Comp. Biochem. Physiol.* **1976**, *55*, 443.
- (201) Coutinho, L.; Alves Ferreira, M.; Cosson, A.; Meuser Batista, M.; da Gama Jaén Batista, D.; Minoprio, P.; Degraeve, W. M.; Berneman, A.; de Nazaré Correia Soeiro, M. *Mem. Inst. Oswaldo Cruz, Rio de Janeiro* **2009**, *104*, 1055.
- (202) Berneman, A.; Alves-Ferreira, M.; Coatnoan, N.; Chamond, N.; Minoprio, P. *J. Microbiol. Biochem. Technol.* **2010**, *2*, 139.
- (203) Blondel, A.; Renaud, J. P.; Fischer, S.; Moras, D.; Karplus, M. *J. Mol. Biol.* **1999**, *291*, 101.
- (204) Laine, E.; Goncalves, C.; Karst, J. C.; Lesnard, A.; Rault, S.; Tang, W. J.; Malliavin, T. E.; Ladant, D.; Blondel, A. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 11277.
- (205) Chamond, N.; Coatnoan, N.; Minoprio, P. *Curr. Drug Targets Immune Endocr. Metabol. Disord.* **2002**, *2*, 247.
- (206) Bryan, M. A.; Norris, K. A. *Infect. Imm.* **2010**, *78*, 810.
- (207) Ayengar, P.; Roberts, E. *J. Biol. Chem.* **1952**, *197*, 453.
- (208) Narrod, S. A.; Wood, W. A. *Arch. Biochem. Biophys.* **1952**, *35*, 462.
- (209) Tanner, M. E.; Gallo, K. A.; Knowles, J. R. *Biochemistry* **1993**, *32*, 3998.
- (210) Doublet, P.; van Heijenoort, J.; Mengin-Lecreux, D. *Microb. Drug Resist.* **1996**, *2*, 43.
- (211) Ho, H.-T.; Falk, P. J.; Ervin, K. M.; Krishnan, B. S.; Discotto, L. F.; Dougherty, T. J.; Pucci, M. J. *Biochem.* **1995**, *34*, 2464.
- (212) Glavas, S.; Tanner, M. E. *Biochemistry* **2001**, *40*, 6199.
- (213) Hwang, K. Y.; Cho, C. S.; Kim, S. S.; Sung, H. C.; Yu, Y. G.; Cho, Y. *Nat. Struct. Biol.* **1999**, *6*, 422.
- (214) Lee, K.-S.; Park, S.-M.; Yeon Hwang, K.; Chi, Y.-M. *Acta Crystallogr., Sect. F* **2005**, *61*, 199.
- (215) Mehboob, S.; Guo, L.; Fu, W.; Mittal, A.; Yau, T.; Truong, K.; Johlfs, M.; Long, F.; Fung, L. W.-M.; Johnson, M. E. *Biochemistry* **2009**, *48*, 7045.
- (216) Fisher, S. L.; Kern, G.; Newton, T.; Lundqvist, T.; Folmer, R.; Xue, Y. *45th Annual ICAAC*, American Society for Microbiology: Washington, DC, 2005.
- (217) Klevens, R. M.; Morrison, M. A.; Nadle, J.; Petit, S.; Gershman, K.; Ray, S.; Harrison, L. H.; Lynfield, R.; Dumyati, G.; Townes, J. M.; Craig, A. S.; Zell, E. R.; Fosheim, G. E.; McDougal, L. K.; Carey, R. B.; Fridkin, S. K. *J. Am. Med. Assoc.* **2007**, *98*, 1763.
- (218) Spellberg, B.; Powers, J. H.; Brass, E. P.; Miller, L. G.; Edwards, J. E., Jr. *Clin. Infect. Dis.* **2004**, *38*, 1279.
- (219) Ashiuchi, M.; Yoshimura, T.; Esaki, N.; Ueno, H.; Soda, K. *Biosci. Biotech. Biochem.* **1993**, *57*, 1978.
- (220) Tanner, M. E.; Miao, S. *Tetrahedron Lett.* **1994**, *35*, 4073.
- (221) Glavas, S.; Tanner, M. E. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2265.
- (222) de Dios, A.; Prieto, L.; Martín, J. A.; Rubio, A.; Ezquerro, J.; Tebbe, M.; López de Uralde, B.; Martín, J.; Sánchez, A.; LeTourneau, D. L.; McGee, J. E.; Boylan, C.; Parr, T. R., Jr.; Smith, M. C. *J. Med. Chem.* **2002**, *45*, 4559.
- (223) Breault, G. A.; Comita-Prevoir, J.; Eyermann, C. J.; Geng, B.; Petrichko, R.; Doig, P.; Gorseth, E.; Noonan, B. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6100.
- (224) Basarab, G. S.; Hill, P. J.; Rastagar, A.; Webborn, P. J. H. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4716.
- (225) Megraud, F. *Cell* **2005**, *123*, 975.
- (226) Peek, R. M., Jr. *Springer Semin. Immunopathol.* **2005**, *2*, 197.
- (227) Nomura, A. M. Y.; Lee, J.; Stemmermann, G. N.; Nomura, Y.; Perez-Perez, G. I.; Blaser, M. J. *J. Infect. Dis.* **2002**, *186*, 1138.
- (228) Suerbaum, S.; Michetti, P. *New Engl. J. Med.* **2002**, *347*, 1175.
- (229) Lundqvist, T.; Fisher, S. L.; Kern, G.; Folmer, R. H. A.; Xue, Y.; Newton, D. T.; Keating, T. A.; Alm, R. A.; de Jonge, B. L. M. *Nature* **2007**, *447*, 817.
- (230) Geng, B.; Basarab, G.; Comita-Prevoir, J.; Gowravaram, M.; Hill, P.; Kiely, A.; Loch, J.; MacPherson, L.; Morningstar, M.; Mullen, G.; Osimboni, E.; Satz, A.; Eyermann, C.; Lundqvist, T. *Med. Chem. Lett.* **2009**, *19*, 930.

- (231) Newman, J. V.; Rooney, M. T.; Cederberg, C. Presented at the 46th ICAAC Meeting, San Francisco, CA, U.S.A., 2006, Abstract A-1106.
- (232) Whalen, K. L.; Pankow, K. L.; Blanke, S. R.; Spies, M. A. *ACS Med. Chem. Lett.* **2010**, *1*, 9.
- (233) Choi, S.-Y.; Esaki, N.; Ashiuchi, M.; Yoshimura, T.; Soda, K. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 10144.
- (234) Kim, W.-C.; Rhee, H.-I.; Park, B.-K.; Suk, K.-H.; Cha, S.-H. *J. Biomol. Screening* **2000**, *5*, 435.
- (235) Lamont, H. C.; Staudenbauer, W. L.; Strominger, J. L. *J. Biol. Chem.* **1972**, *247*, 5103.
- (236) Okada, H.; Yohda, M.; Giga-Haama, Y.; Ueno, Y.; Ohdo, S.; Kumagai, H. *Biochim. Biophys. Acta* **1991**, *1078*, 377.
- (237) Yohda, M.; Okada, H.; Kumagai, H. *Biochem. Biophys. Acta* **1991**, *1089*, 234.
- (238) Liu, L.; Iwata, K.; Kita, A.; Kawarabayasi, Y.; Yohda, M.; Miki, K. *J. Mol. Biol.* **2002**, *319*, 479.
- (239) Yoshida, T.; Seko, T.; Okada, O.; Iwata, K.; Liu, L.; Miki, K.; Yohda, M. *Proteins Struct., Funct. Bioinf.* **2006**, *64*, 502.
- (240) Ohtaki, A.; Nakano, Y.; Iizuka, R.; Arakawa, T.; Yamada, K.; Odaka, M.; Yohda, M. *Proteins Struct., Funct. Bioinf.* **2008**, *70*, 1167.
- (241) Ashiuchi, M.; Tani, K.; Soda, K.; Risono, H. *J. Biochem.* **1998**, *123*, 1156.
- (242) Hutton, C. A.; Perugini, M. A.; Gerrard, J. A. *Mol. Biosyst.* **2007**, *3*, 458.
- (243) Hutton, C. A.; Southwood, T. J.; Turner, J. J. *Mini-Rev. Med. Chem.* **2003**, *3*, 115.
- (244) Antia, M.; Hoare, D. S.; Work, E. *J. Biochem.* **1957**, *65*, 448.
- (245) Wiseman, J. S.; Nichols, J. S. *J. Biol. Chem.* **1984**, *259*, 8907.
- (246) Stenta, M.; Calvaresi, M.; Altoè, P.; Spinelli, D.; Garavelli, M.; Galeazzi, R.; Bottoni, A. *J. Chem. Theory Comput.* **2009**, *5*, 1915.
- (247) Cirilli, M.; Zheng, R.; Scapin, G.; Blanchard, J. S. *Biochemistry* **1998**, *37*, 16452.
- (248) Usha, V.; Dover, L. G.; Roper, D. I.; Fütterer, K.; Besra, G. S. *Acta Crystallogr.* **2009**, *D65*, 383.
- (249) Hor, L.; Dobson, R. C. J.; Dogovski, C.; Hutton, C. A.; Perugini, M. A. *Acta Crystallogr.* **2010**, *F66*, 37.
- (250) Pillai, B.; Moorthie, V. A.; van Belkum, M. J.; Marcus, S. L.; Cherney, M. M.; Diaper, C. M.; Vederas, J. C.; James, M. N. G. *J. Mol. Biol.* **2009**, *385*, 580.
- (251) Schleifer, K. H.; Kandler, O. *Bacteriol. Rev.* **1972**, *36*, 407.
- (252) Wietzerbin, J.; Das, B. C.; Petit, J. F.; Lederer, E.; Leyh-Bouille, M.; Ghuysen, J. M. *Biochemistry* **1974**, *13*, 3471.
- (253) Goffin, C.; Ghuysen, J. M. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 702.
- (254) Dye, C.; Scheele, S.; Dolin, P.; Pathania, V.; Raviglione, M. C. *J. Am. Med. Assoc.* **1999**, *282*, 677.
- (255) Girodeau, J.-M.; Agouridas, C.; Masson, M.; Pineau, R.; Le Goffict, F. *J. Med. Chem.* **1986**, *29*, 1023.
- (256) Baumann, R. J.; Bohme, E. H.; Wiseman, J. S.; Vaal, M.; Nichols, J. S. *Antimicrob. Agents Chemother.* **1988**, *32*, 1119.
- (257) Gelb, M. H.; Lin, Y.; Pickard, M. A.; Song, Y.; Vederas, J. C. *J. Am. Chem. Soc.* **1990**, *112*, 4932.
- (258) Lam, L. K. P.; Arnold, L. D.; Kalantar, T. H.; Kelland, J. G.; Lane-Bell, P. M.; Palcic, M. M.; Pickard, M. A.; Vederas, J. C. *J. Biol. Chem.* **1988**, *263*, 11814.
- (259) Gerhart, F.; Higgins, W.; Tardif, C.; Ducep, J. B. *J. Med. Chem.* **1990**, *33*, 2157.
- (260) Higgins, W.; Tardif, C.; Richaud, C.; Krivanek, M.; Cardin, A. *Eur. J. Biochem.* **1989**, *186*, 137.
- (261) Diaper, C. M.; Sutherland, A.; Pillai, B.; James, M. N. G.; Semchuk, P.; Blanchard, J. S.; Vederas, J. C. *Org. Biomol. Chem.* **2005**, *3*, 4402.
- (262) Williams, R. M.; Fegley, G. J.; Gallegos, R.; Schaefer, F.; Pruess, D. L. *Tetrahedron* **1996**, *52*, 1149.