Beilstein-Institut
Beilstein Bozen Symposium on Molecular Engineering and Control
May 14th – 18th, 2012, Prien (Chiemsee), Germany

PLP-dependent Enzymes: a Powerful Tool for Metabolic Synthesis of Non-canonical Amino Acids[1]

Martino L. di Salvo[1]*, Nediljko Budisa[2], and Roberto Contestabile[1]

[1]Dipartimento di Scienze Biochimiche "A. Rossi Fanelli",
Sapienza Università di Roma,
Via degli Apuli 9, 00185 Roma, Italy.

[2]Institut für Biologische Chemie, Biocatalysis Group, Technische Universität Berlin,
Müller-Breslau-Str. 10, 10623 Berlin, Germany.

E-Mail: *martino.disalvo@uniroma1.it

Received: 5th June 2013 / Published: 13th December 2013

An Overview on PLP-dependent Enzymes

Pyridoxal 5'-phosphate (PLP), the biologically active of vitamin B_6, was first identified in the mid-forties as the cofactor for the transamination reaction. Since then, PLP-dependent enzymes have been the focus of extensive biochemical research. The interest aroused by these enzymes is due to their unrivalled catalytic versatility and their widespread involvement in cellular metabolism. As a matter of fact, PLP acts as cofactor in more than 160 different enzymes classified by the Enzyme Commission, representing 4% of all known cellular catalytic activities [1]. PLP-dependent enzymes serve vital roles in all living organisms and catalyze a number of diverse chemical reactions, such as transamination, decarboxylation, racemization, carbon-carbon bond cleavage and formation. PLP-dependent activities are involved in essential biosynthetic pathways including glucose and lipid metabolism,

[1] Non-canonical amino acids (ncAA): amino acids that do not participate to protein translation, i. e. are not genetically encoded. We will use the term ncAA for both naturally and synthetically generated amino acids that are used to expand the scope of protein synthesis

amino acid metabolism, heme and nucleotide synthesis, and neurotransmitter production [2, 3]. As a consequence of their crucial metabolic relevance, a number of these enzymes are widely recognized drug targets [4].

The mechanism of many B_6 enzymes has been studied extensively over the last 50 years, with respect to structure, function, substrate and reaction specificity. Because of their catalytic versatility, in the recent years PLP-dependent enzymes have acquired extraordinary importance in biotechnology, to be exploited in the semi-synthetic production of compounds for medical and industrial use.

PLP chemistry

PLP resembles benzaldehyde in its structure. However, the properties of the carbonyl group are modified by the presence both of an adjacent hydroxyl group and ring nitrogen in the *para* position (Fig. 1), whose protonation state has profound effects on the cofactor's reactivity. These features of PLP were recognized to be of fundamental importance for catalysis in the early model studies carried out on the cofactor alone and on its analogues [5].

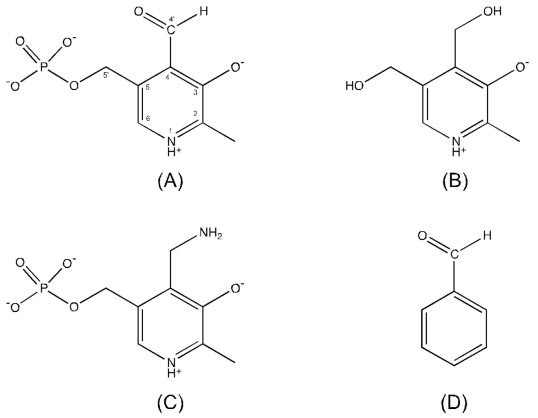

Figure 1. Structure of pyridoxal 5'-phosphate (PLP) and related compounds. **(A)** pyridoxal 5'-phosphate, the active form of vitamin B6 used as enzyme cofactor; **(B)** pyridoxine (PN), the vitamin B6 form that is most commonly given as dietary supplement; **(C)** pyridoxamine 5'-phosphate (PMP), the other natural occurring vitamin B6-derived catalyst; **(D)** benzaldehyde.

Two basic, interdependent chemical properties of PLP are involved in catalysis. First, its ability to form imines with primary amino groups through its aldehyde group, and second its facility to stabilize carbanionic intermediates that develop by heterolytic cleavage of chemical bonds. Typically, the cofactor forms two types of imine. In the free enzyme an *internal aldimine* is formed with a lysine side chain. *External aldimines* are formed with amino acids and closely related compounds (Fig. 2). Both types of imine react reversibly with primary

amines in a transaldimination reaction (step 2 to 5 in Fig. 2), with formation of a *geminal diamine* intermediates, allowing binding of substrates and release of products (forward and reverse of step 3 in Fig. 2).

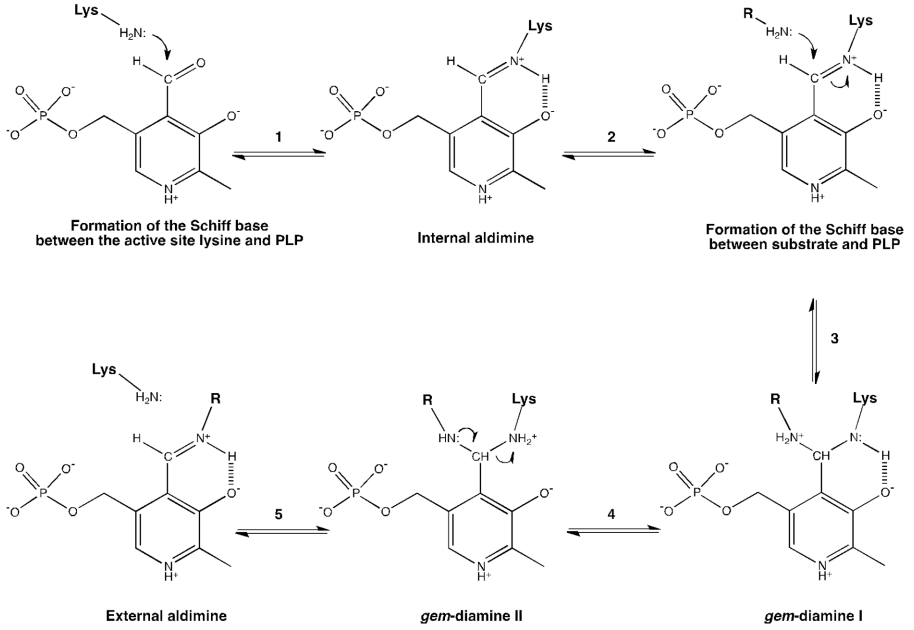

Figure 2. PLP reacts reversibly with primary amines to form imines; **(1)** formation of the *internal aldimine* with the active site lysine residue; **(2 to 5)** transaldimination reaction: formation of the *external aldimine* with the substrate amino acid, with formation of *geminal diamine* intermediates. The transaldimination reaction between the internal and the external aldimines allows substrate binding and product release.

The reactions described so far are characteristic of any carbonyl compound. PLP is unusual because of the high efficacy with which they are accomplished, that relies on the properties of its heteroaromatic pyridine ring. The electrophilicity of C4' is in fact greatly enhanced through the electron withdrawing effect exerted by the protonated pyridinium nitrogen (Fig. 3A). Moreover, the phenoxide anion at C3 stabilizes the protonated state of the imine nitrogen, by means of resonance and hydrogen bonding (Fig. 3B), that further increases the electrophilic character of C4' and easily accommodates an electron lone pair during transaldimination. At the same time, these coulombic (inductive and field) and resonance effects, the former strictly dependent on the latter, are responsible for the second catalytic property of PLP, its action as an ''electron sink''. The protonated N1 of the cofactor in the external aldimine withdraws electrons from Cα of substrates. This electron deficit, further increased if the imine nitrogen is itself protonated, produces a polarization, and therefore a weakening, of σ bonds to Cα.

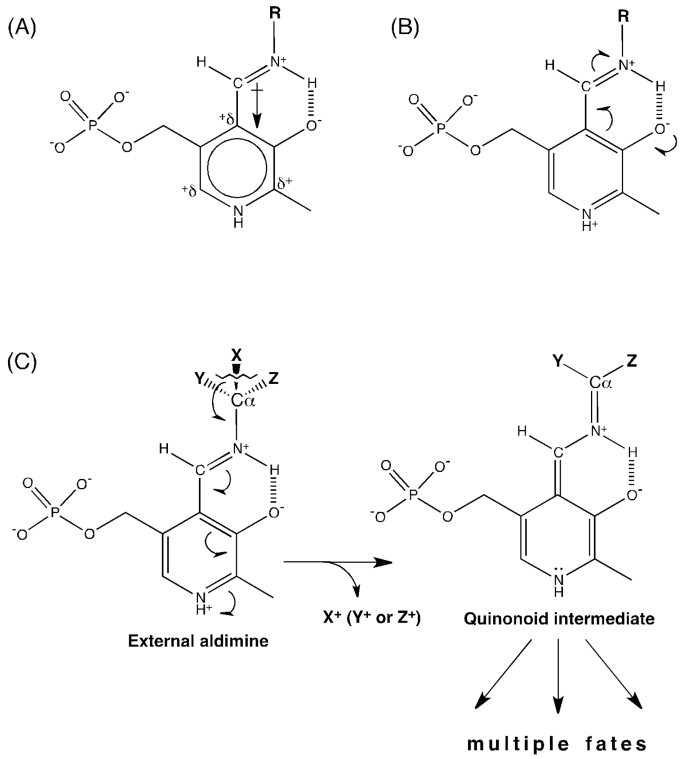

Figure 3. (A) Coulombic and **(B)** resonance effects involved in PLP-dependent catalysis. **(C)** Formation of the *quinonoid intermediate* upon the heterolytic cleavage of one of the bonds around Cα. PLP acts as an electron sink in the formation of the quinonoid intermediate, the common steps in all PLP-catalyzed reactions.

The extensive conjugation of the π-electrons of the ring, which extends to the imine bond and to the oxy substituent at C 3, very efficiently delocalizes the net negative charge arising from the heterolytic cleavage of these bonds. The protonated ring nitrogen plays a major role in stabilizing the net negative charge (Fig. 3C). Besides the gain in delocalization energy, which is a thermodynamic aspect, an additional, kinetic consideration must be taken into account. This focuses on the transition state for bond breaking. An important factor in the activation of σ bonds by a π system is the stereochemical arrangement. Dunathan [6] pointed out that if the gain in delocalization energy is to aid the bond breaking process, the transition state must assume a geometry that approaches that of the coplanar product, i. e. which places the bond to be broken in a plane perpendicular to that of the π system. Figure 4 shows this basic concept and, at the same time, explains how reaction specificity may be controlled.

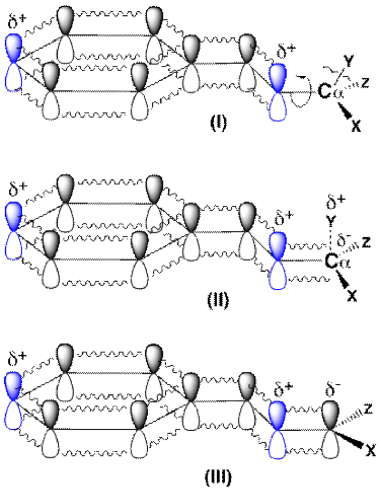

Figure 4. Schematic representation of the π-orbital framework in a PLP-substrate external aldimine. The minimum level of energy of the transition state in the cleavage of a bond to Cα is that in which the π-orbital that is forming is in conjugation with the π-system of the pyridine ring **(II)**, the nitrogen atoms are displayed in blue. This situation takes place when the σ-bond to be cleaved is perpendicular to the pyridine ring, as in **(I)**. This geometry of the transition state, which approaches that of the quinonoid product **(III)**, allows the extension of the electronic delocalisation to the bond that is breaking, stabilising the developing negative charge. As a consequence, the bond to Cα perpendicular to the pyridine ring is the most labile because of its overlap with the π-system. Rotation around the bond between Cα and the imine nitrogen **(I)** determines which of the three bonds to Cα will be cleaved and therefore controls the reaction specificity ('Dunathan's hypothesis' [6]).

The cofactor is able to catalyze multiple reactions in the complete absence of enzymes (although at much slower rates than those characteristic of enzymes) such as transamination of amino acids [5], racemization [7] and, with serine, α,β-elimination [8]. The rates of reactions catalyzed by pyridoxal phosphate alone were found to be accelerated by metal ions, such as Cu^{2+} and Al^{3+} [8]. Since these ions are known to chelate with Schiff's bases of the type formed with PLP, it was concluded that their action is that of providing coplanarity between the imine and the pyrimidine ring. The role played by metal ions in model systems is carried out by the hydrogen bond between O3 and the imine nitrogen in the enzymes. The function of the phosphorylated hydroxymethyl substituent at C 5 is clearly to provide a firm anchor to the coenzyme. PLP-dependent enzymes bind the cofactor phosphate group through a similar set of interactions. This common recognition pattern was named "phosphate-binding cup" [9]. The methyl substituent at C 2 appears not to have a function and may simply arise from metabolic requirements [10].

It is worth of note that the pool of free PLP *in vivo* must be maintained at a very low level, to prevent toxic build-up. In fact, PLP being a very reactive aldehyde, easily combines with amines and thiols in the cell. This characteristic has been related, for example, to the neurotoxic effect of excess consumption of vitamin B_6 [11]. In eukaryotic cells, the concentration of free PLP is maintained as low as 1 μM. There must then be a tight regulation control on PLP homeostasis, that would insure the presence of enough cofactor for all PLP-dependent enzymatic activity in the cell. Failure to maintain the correct tuning between PLP biosynthesis, degradation, and delivering to newly formed PLP-enzymes might end up in poor cell growth or vitamin B_6-associated pathogenesis such as severe neurological disorders (epilepsy, schizophrenia, Alzheimer's and Parkinson's diseases) [12].

Mechanisms of reaction

In spite of all the different reaction carried out by PLP-dependent enzymes, it is possible to envisage a series of common mechanistic features characteristic of PLP-catalyzed reactions. In the early 1950 s, a general mechanism of reaction was postulated based on considerations on the various known PLP-dependent enzymes and model studies on the cofactor alone [5]. All of the many different reactions begin with conversion of the PLP-enzyme internal aldimine to the PLP-substrate external aldimine and the consequent heterolytic cleavage of one of the three bonds to Cα (Fig. 3C and 5). This latter step represents the first point of diversification. The three distinct carbanions formed are called quinonoid intermediates and react further in multiple paths that themselves branch in a series of consecutive steps, invariably ending up in either an aldimine or a ketimine adduct which is transaldiminated or hydrolyzed in order to release the related product. It follows that an exposition of the essential steps in the mechanisms of the varied types of reactions can be approached progressively, starting from the above mentioned common quinonoid intermediates and moving towards increasing levels of diversification (Fig. 5).

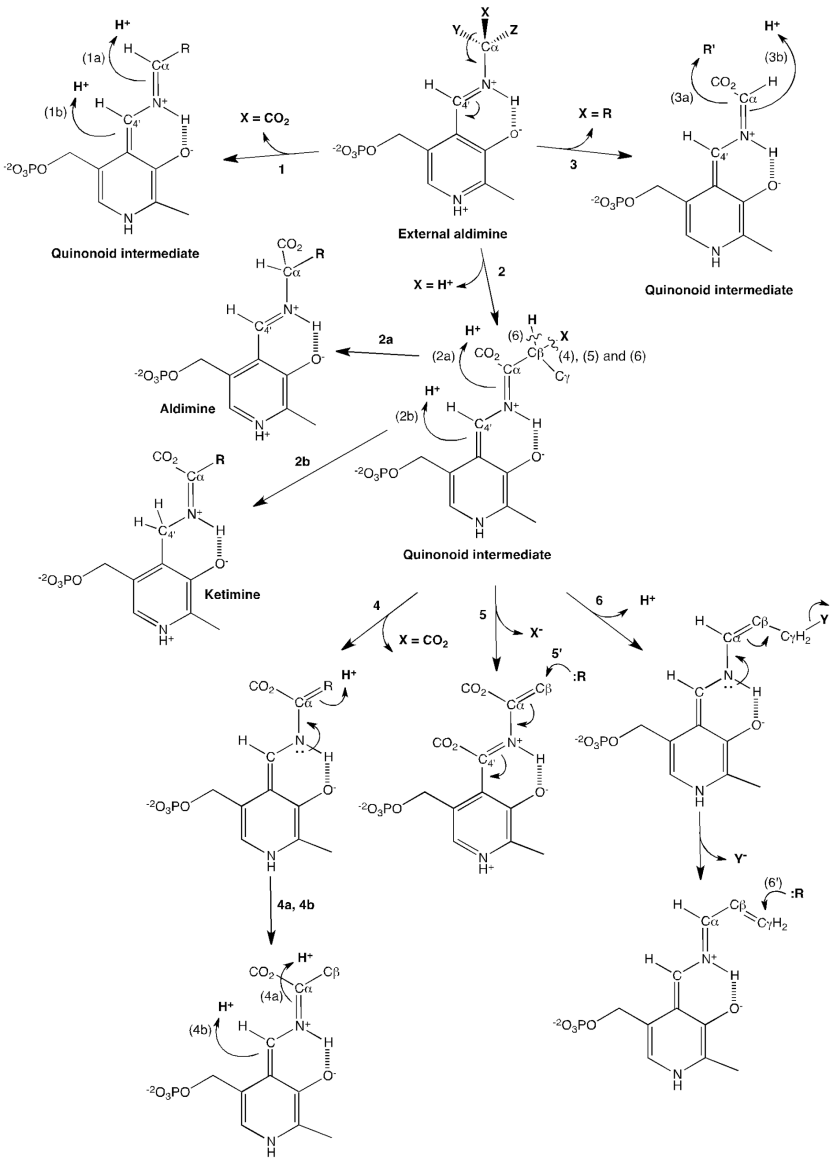

Figure 5. Schematic representation of the various types of reaction catalyzed by PLP-dependent enzymes.

Legend:

(1) Reactions proceeding through elimination of CO_2 from Cα:
  (1a) α-decarboxylation
  (1b) transaminating decarboxylation
(2) Reactions proceeding through deprotonation of Cα:
  (2a) aldimine formation: racemization
  (2b) ketimine formation: transamination

(4) β-decarboxylation
(4a) aldimine formation: β-decarboxylation
(4b) ketimine formation: transaminating β-decarboxylation
(5) β-elimination
(5') β-synthesis
(5'a) aldimine formation: production of a new β-substituted amino acid
(5'b) ketimine formation: production of a new β-substituted keto acid
(6) γ-elimination
(6') γ-synthesis
(3) Reactions proceeding through elimination of the side chain:
(3a) α-synthesis: production of a β-hydroxyamino acid
(3b) retroaldolic cleavage: releasing of glycine and an aldehyde

Pyridoxal phosphate is clearly a tool in the "hands" of proteins. The efficacy of each enzyme relies on its ability to accelerate certain reactions selectively at the expense of others, thereby making its way through the intricate maze of the many possible chemical transformations. The outstanding catalytic diversity of PLP-dependent enzymes arises from modulation and enhancement of the coenzyme intrinsic chemical properties by the surrounding polypeptide matrix.

On the basis of the available structural information, it is generally accepted that PLP-dependent enzymes originated very early in the evolution (before the three biological kingdoms diverged, some 1500 million years ago) from different protein ancestors, which generated at least five independent families, each corresponding to a different fold type [13]. These families have been named from their more representative enzyme. The aspartate aminotransferase family corresponds to fold type I and contains the majority of structurally determined PLP-dependent enzymes. The tryptophan synthase β-subunit family corresponds to the fold type II; the bacterial alanine racemase family corresponds to the fold type III; the D-amino acid aminotransferase family corresponds to the fold type IV; the glycogen phosphorylase corresponds to fold type V. The glycogen phosphorylase family seems to be a totally unrelated example, in which PLP is used in a completely different way. In that case, in fact, the reaction chemistry occurs through a general acid-base mechanism that involves the phosphate moiety of the cofactor. In all other PLP-dependent enzyme families, the phosphate group acts only as an anchor to tightly bind the cofactor to the folded polypeptide, whereas the chemistry is carried out by the pyridine ring moiety.

The observation that PLP-dependent enzymes have multiple evolutionary origins is very interesting: the common mechanistic features of PLP-enzymes are not then accidental historical traits but may reflect evolutionary necessities based on PLP chemistry.In support to this hypothesis is the observation of several examples of convergent evolution, provided by enzymes catalyzing the same reactions but belonging to different fold types, concerning both similarities of cofactor binding sites and catalytic mechanisms [14].

Nowadays, more than a hundred crystal structures of PLP-dependent enzymes, most of them solved in the last 5 years, are accessible from databases.

Catalytic versatility and promiscuity

Enzymes are generally believed to be very specific in their action, i. e. to be endowed with a strict reaction and substrate specificity. However, as a matter of fact, many enzymes are able to catalyze more than one reaction, often using different substrates. These enzymes are called generalist in opposition to specialist enzymes, which evolved to catalyze one reaction on a unique primary substrate. They represent a significant portion of the total enzymes in a living cell and play more than one physiologically important role [15]. Besides this natural eclecticism, many enzymes when acting on their physiological substrate are prone to make ''mistakes'' and catalyze measurable side reactions. Moreover, *in vitro* and when presented with substrates analogues (also unnatural compounds), numerous enzymes show activities that are not part of the organism's physiology. This capability of an enzyme to catalyze alternative reactions (often different types of reactions with different substrates) is referred to as catalytic promiscuity [16, 17]. Catalytic promiscuity is believed to have played a fundamental role in divergent evolution and diversification of catalytic properties. Ancestral enzymes were probably able to catalyze a range of different reactions. Gene duplication and evolutionary pressure may have worked to shape enzymes' active sites so as to confer narrower substrate and reaction specificity.

Catalytic promiscuity offers very important biotechnological opportunities. Many enzymes may be used as such for the enzymatic synthesis of unnatural compounds. Alternatively, they may be engineered so as to catalyze novel reactions. In this context, PLP-dependent enzymes represent a particularly interesting source of promiscuous catalysts. PLP itself is able to catalyze multiple reactions, although at a much slower rate compared to the enzymes that use it as cofactor. This exceptional catalytic versatility is often responsible for a pronounced catalytic promiscuity. For example, the physiological role of aspartate aminotransferase (Asp-AT) is to catalyze the reversible transamination that converts L-aspartate and α-ketoglutarate into oxaloacetate and L-glutamate. Asp-AT catalyzes this reaction almost exclusively with its own natural substrates. L-enantiomers of tyrosine, phenylalanine and alanine undergo transamination 3 – 5 orders of magnitude slower than the dicarboxylic substrates. However, the above-mentioned compounds are also racemized by Asp-AT with a rate of 10^{-5} – 10^{-6} s^{-1} [18, 19].

Moreover, when presented with appropriate, unnatural substrate analogues, Asp-AT efficiently catalyzes β-elimination reactions [20]. This apparent ''imperfection'' in the catalytic machinery of the enzyme is actually due to the presence of a good leaving group at Cβ of such analogues. After deprotonation of the substrate-cofactor aldimine (step 2 in Fig. 5 and step 1 in Fig. 6) Cα and C4' of the quinonoid intermediate have a marked nucleophilic character. When acting on physiological substrates Asp-AT stereospecifically reprotonates

either Cα or C4' to form the related external aldimines (reverse of step 1 and step 2a in Fig. 6). A nucleophilic attack by Cα on Cβ is instead the prevalent reaction if a good leaving group exists at the latter carbon (step 2b in Fig. 6).

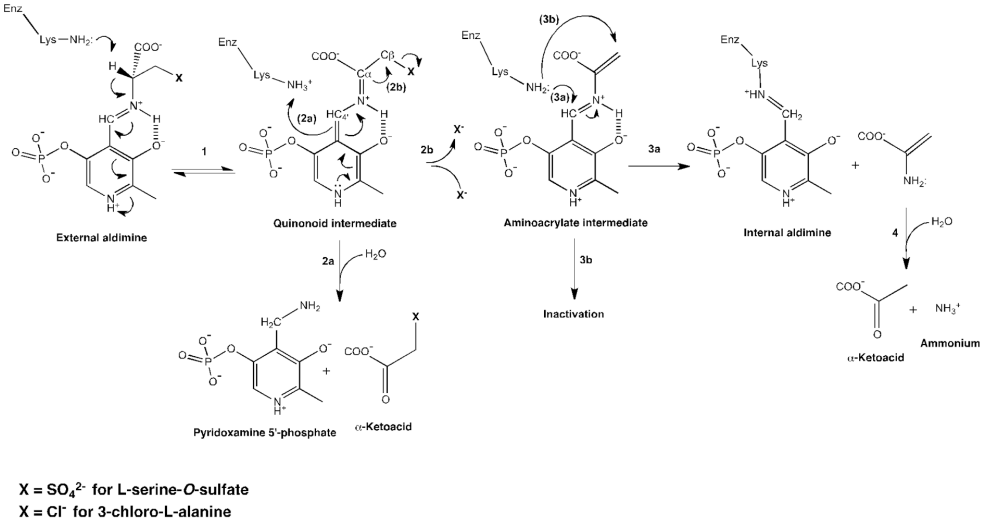

Figure 6. Mechanism of reaction of Asp-AT-catalyzed β-elimination **(2b)** and transamination **(2a).** Details of the mechanism are described in the test.

The subsequent transaldimination then releases the aminoacrylate intermediate, which spontaneously hydrolyses to form ammonium and the related keto acid (step 3a and 4 in Fig. 6). Alternatively, the active site lysine reacts with the aminoacrylate intermediate inactivating the enzyme (step 3b in Fig. 6). As an example, L-serine *O*-sulphate [21] and 3-chloro-L-alanine [22] are both rapidly converted to pyruvate, ammonia and SO_4^{2-} or Cl^- respectively. The ability of Asp-AT to catalyze β-elimination of α-amino acids that have a good leaving group at Cβ has been exploited in the synthesis of unnatural amino acids such as sulfocysteine by the inclusion of appropriate nucleophiles as co-substrates, which attack the aminoacrylate intermediate forming a stable product [23]. Although aminoacrylate is abnormal to the aminotranferase reaction, it is, together with aminocrotonate (same structure of aminoacrylate with a methyl group at Cβ), an intermediate for many PLP-dependent enzymes that catalyze β-elimination reactions. Among them are serine and threonine dehydratases (elimination of OH^-, as H_2O), bacterial tryptophanase (elimination of indole) and alliinase from onions and garlic (elimination of 1-propenylsulphenic acid, the lachrymator formed on crushing these herbs). It is also a natural intermediate in synthetic reactions such as that catalyzed by tryptophan synthase [25]. Some of the above mentioned enzymes have been exploited for the synthesis of non-canonical amino acids (ncAA) based on the reaction of the aminoacrilate intermediate with nucleophilic compounds (see below).

Remarkably, some PLP-dependent enzymes catalyze alternative reactions that correspond to the main reaction catalyzed by evolutionary related enzymes. Serine hydroxymethyltransferase (SHMT), an enzyme with exceptionally broad reaction specificity, is an interesting example of this phenomenon and of catalytic promiscuity. On top of the hydroxymethyltransferase reaction, SHMT is able to catalyze decarboxylation, aldol cleavage, transamination and racemization reactions, using different natural or unnatural substrates. Two other PLP-dependent enzymes, threonine aldolase (TA) and fungal alanine racemase (AlaRac), turned out to be very close to SHMT from the point of view of structural similarities and catalytic properties, being able to catalyze the same reactions, although with different efficiencies [25, 26].

Non-canonical Amino Acids Found in Natural Compounds

As pointed out in the previous chapter, due to their extraordinary versatility and catalytic efficiency PLP-dependent enzymes can be envisaged as powerful tools for the semi-synthetic production of non-canonical amino acids and their related compounds. The following sections review some of the most common non-canonical amino acids found in natural compounds (this section), whose production can be achieved by the use of reactions catalyzed by PLP-dependent enzymes (next section ''Synthesis of non-canonical amino acids by PLP-dependent enzymes'').

β-Hydroxy-α-amino acids

β-Hydroxy-α-amino acids (also called 3-Hydroxy-α-Amino Acids, 3-HAA;) constitute a large and widespread class of compounds. They can be found either as naturally occurring building blocks in proteins (threonine, serine and 3-hydroxyproline) and as components of many composite natural products, endowed with a wide range of biological activities such as antibiotics, immunosuppressants and peptide conjugates [27]. The non-canonical amino acids belonging to this class are useful building blocks in synthetic, combinatorial (e. g., for constructing libraries of β-lactams for antibiotic synthesis), and medicinal chemistry. Following is a list of some of the principal β-hydroxyamino acids found in nature (Fig. 7):

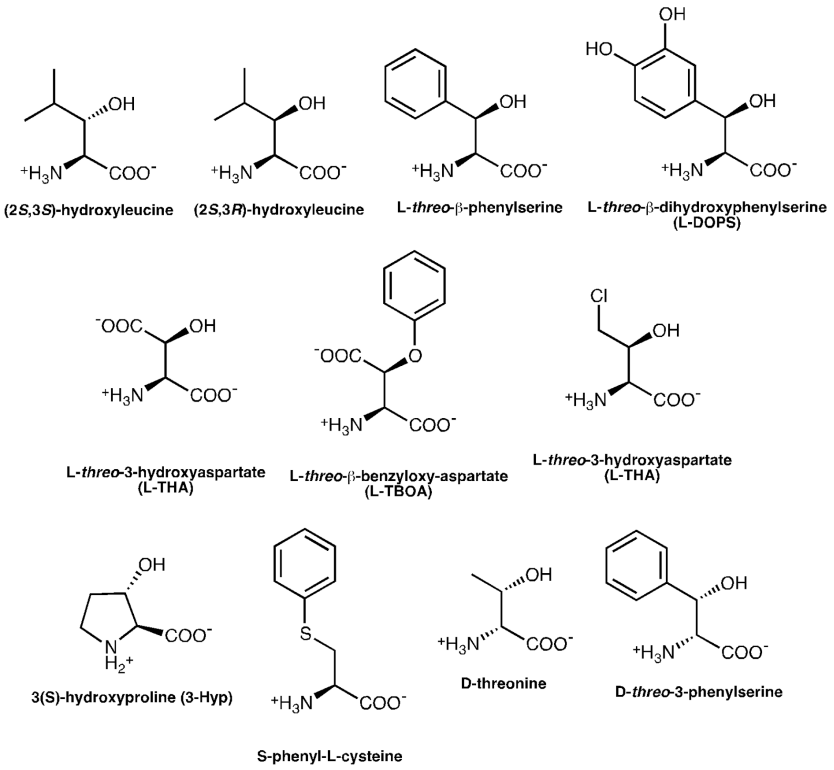

Figure 7. Structures of naturally occurring non-canonical amino acids.

- **3(S)-hydroxy-L-leucine** ((2S,3S)-hydroxyleucine), is a key component of several cyclodepsipeptides, natural peptidic antibiotics isolated from bacteria (*Streptomyces*), fungi and marine sponges, which are often used as lead compounds for pharmacologically more potent and toxicologically safer derivatives. Cyclodepsipeptides containing 3(S)-hydroxy-L-leucine include antibiotics telomycin and variapeptin, the immunosuppressant with anti-inflammatory activity L-156,602, and several potent antitumor agents such as axinothricin, citropeptin, A83586C, and verucopeptin [28].
- **3(R)-hydroxy-L-leucine.** The configuration around the Cβ seems to be important for biological activity. As a matter of fact, the (2S,3R) isomer of hydroxyleucine is also a specific constituent of bioactive compounds, such as lactacystin and omuralide, proteasome inhibitors with neurotrophic properties [29], and lysobactins (or katanosins), a class of macrocyclic peptide lactone (depsipeptide) antibiotics also containing other proteinogenic and nonproteinogenic hydroxylated amino acids (HyPhe, HyAsn, L-*allo*-Thr), together with D-amino acids [30].

- **3-hydroxy-L-lysine**, is a naturally occurring amino acid and a putative intermediate in the synthesis of balanol, a potent protein kinase C inhibitor [31]. Its isomer 4-hydroxylysine is, on the other hand, widely known as a component of collagen.
- Besides being present as building block of antibiotics such as lysobactins [32], **L-*threo*-β-phenylserine** and its ester derivatives possess antiviral activity by themselves. This biological activity is competitively reversed by phenylalanine and abolished by the substitution of the -OH or α-amino group of phenylserine [33]. Moreover, L-*threo*-β-phenylserine was shown to act as an auxin antagonists and to possess antifungal chemotherapeutic activity [34]. An analogue of β-phenylserine, **L-*threo*-dihydroxyphenylserine** (L-DOPS; Droxidopa) is a psychoactive drug and synthetic amino acid precursor, which acts as a prodrug to the neurotransmitters norepinephrine (noradrenaline) and epinephrine (adrenaline) [35]. Unlike norepinephrine and epinephrine themselves, L-DOPS is capable of crossing the blood-brain barrier.
- **3-hydroxy-N^{ε},N^{ε},N^{ε}-trimethyl-L-lysine** is an intermediate of L-carnitine biosynthesis. L-carnitine is an essential molecule for fatty acid entry into the mitochondria and energy metabolism. In humans, L-carnitine is obtained either form the diet or from a four step biosynthetic pathway starting from N^{ε},N^{ε},N^{ε}-trimethyl-L-lysine. The aldolase responsible for the cleavage of the 3-hydroxy-N^{ε},N^{ε},N^{ε}-trimethyl-L-lysine in carnitine biosynthesis has not been yet identified. Cytosolic SHMT, the key metabolic PLP-dependent enzyme that typically catalyzes the interconversion of L-serine to glycine with the formation of 5,10-methylenetetrahydrofolate, was shown to catalyze *in vitro* the above mentioned reaction [36] and might be responsible for the reaction *in vivo* [37]. L-threonine aldolase, the enzyme strictly related to SHMT (see par. 3), might be responsible for the reaction in yeast [38].
- **DL-*threo*-3-hydroxyaspartate** (**DL-THA**) derivatives are important pharmacological agents, as they function as non-transportable inhibitors of the high-affinity Na^{+}-dependent excitatory amino acid transporters (EAATs), that maintain glutamate concentration below the excitotoxic level. L-glutamate is the major excitatory mediator in mammalian central nervous system and its extracellular concentration has to be tightly regulated. In particular, DL-THA derivatives possessing an ethereal bulky substituent (i. e. a benzyl or naphytl group), are able to exert their activity on all EAAT subtypes [39].
- **4-chloro-L-threonine**, is a component of several nonribosomally synthesized phytotoxic and antifungal lipodepsinonapeptides such as syringomycin, syringotoxin, syringostatin, pseudomycin and cormycin produced by many strains of the plant colonizing bacterium *Pseudomonas spp.* [40]. Chlorothreonine is also present in two structural variants of the antitumor antibiotic compound actinomycin Z from *Streptomyces fradiae,* namely actinomycin Z_3 and Z_5. The presence of chlorine contributes to the biological activities of these natural compounds. 4-Chlorothreonine was also isolated as a free amino acid in *Streptomyces* cultures and was reported to inhibit the growth of radish, sorghum and also *Candida albicans* [41]. 4-Chlorothreonine has also been investigated as a potential

antitumor agent, as a result of its ability to inactivate PLP-dependent enzyme serine hydroxymethyltransferase through a mechanism-based (suicide) inhibition. Its fluorinated analogues (such as 4-trifluoro-L-threonine or the corresponding L-*allo*-threonine derivatives) would likewise be expected to react with serine hydroxymethyltransferase and might thus be suitable for chemotherapy [42]. Another non-canonical hydroxylated amino acid, 4-hydroxythreonine, can be found in other actinomycins complexes (i.e. actinomycin Z_1) [43]. Interestingly, the phosphorylated form of this amino acid, 4-phospho hydroxy-L-threonine, is 4-phospho-hydroxy-L-threonine is an obligatory intermediate in PLP coenzyme biosynthesis in the γ-subdivision of eubacteria (such as *Escherichia coli*) [44].

- **3(S)-Hydroxy-L-proline** (**3-Hyp**). First isolated in 1902 by German chemist Hermann E. Fischer from hydrolyzed gelatin, 3-hydroxy-L-proline is a common non-proteinogenic amino acid found in animal tissues. Together with its isomer 4(R)-hydroxy-L-proline, it is one of the essential constituents of collagen and related proteins such as elastin and bacterial surface proteins. The hydroxylation of proline residues highly increases the stability of the collagen triple helix, primarily through stereoelectronic effects [45]. Hydroxyprolines account for roughly 4% of all amino acids in animal tissues. Hyp is found in few proteins other than collagen, for example in hydroxyproline-rich glycoproteins (HRGP) a super-family of extracellular structural proteins found in plant cell walls, with physiological importance in cell signaling and embryogenesis and exploited for protein scaffold design for practical applications in bionanotechnology and medicine [46]. Also, Hyp is found in some snail poisons cyclopeptides, lacking collagen-like sequences [47]. Hydroxyproline is naturally generated as a post-translational modification in a complex reaction requiring oxygen, L-ascorbic acid and α-ketoglutarate and catalyzed by prolylhydroxylase. Other hydroxyprolines (such as 2,3-cis-, 3,4-trans-dihydroxyproline) are also found in nature, as part of repeated sequences in adhesive proteins from mussels, diatom cell walls and fungus poisons [48 – 50]. 3-Hyp analogue **3-hydroxy-5-methylproline** is present as a component of antibiotic actnomycin Z_1 [51].

β-Substituted amino acids

The most important derivatives of canonical sulfur-containing amino acids L-cysteine and L-methionine are their β-substituted seleno-derivatives. Selenium is a trace element essential for normal physiological processes. It is toxic at relatively low levels, and selenium compounds must be carefully administered, due to the narrowness between physiologically required dosage and toxic quantity. Selenium-containing amino acids, such as selenocysteine (Sec), selenocystine and selenomethionine (Sem) can act directly as antioxidants or chelators of redox-active metal ions, or can be incorporated into selenium-dependent antioxidant enzymes (e.g. glutathione peroxidase, thioredoxin reductase, methionine sulphoxide reductase, formate dehydrogenase, glycine reductase, deiodinase and several hydrogenases). Sec is incorporated into proteins at specific positions and participates to enzyme catalysis,

enhancing the kinetic properties. For this reason it has been considered as the 21st proteinogenic amino acid. The biosynthesis of Sec is quite peculiar, in that it happens directly on its tRNA which is then recognized by the ribosome machinery and inserted through a UGA codon-directed cotranslation (mediated by the so-called SElenoCysteine Insertion Sequences (SECIS) elements and involving four specific genes, *selA-D*). The product of *selA*, selenocysteine synthase, is the PLP-dependent enzyme responsible for the last biosynthetic step. Its reaction will be described in par. 1. On the other hand, Sem is randomly incorporated into proteins in place of methionine, because $tRNA^{Met}$ does not discriminate between Met and Sem, and seem to have no effect on protein function. Laboratory-induced incorporation of Sem into proteins allows for the production of protein crystals suitable for three-dimensional structure determination via multiwavelength anomalous dispersion phasing methods. Higher animals are not able to *de novo* synthesizes Sem and must acquire it from the diet. Selenomethionine is then incorporated into proteins and stored in the body, from which is then released by normal metabolic processes.

The unnatural derivative of L-cysteine, **S-phenyl-L-cysteine** (Fig. 7) is a building block of the anti-AIDS drug Nelfinavir (brand name Viracept), an orally bioavailable inhibitor of HIV-1 and HIV-2 proteases [52] and in phase I clinical trials as anti-cancer agent [53].

D-Amino acids

Although not used in ribosomal protein synthesis, D-amino acids are important building molecules for bacterial wall construction (the oligopeptide moiety of peptidoglycan contains D-amino acids in both Gram-positive and Gram-negative bacteria), are found in skin antimicrobial peptides [54] and are used by fungi for the biosynthesis of several antibiotics and ionophores.

D-alanine, present in bacterial peptidoglycan, is produced from the L- enantiomer by alanine racemase, one of the most investigated bacterial PLP-dependent enzyme. A distinct alanine racemase exists in eukaryotic organisms, such as the fungi *Tolypocladium niveum* and *Cochliobolus carbonum*. This enzyme has a completely different protein fold and evolutionary origin with respect to its bacterial homonym. In *T. niveum* it is essential to provide the D-alanine incorporated by non-ribosomal synthesis into the cyclic undecapeptide cyclosporin, a potent immunosuppressant drug. In *C. carbonum*, D-alanine is a component of the cyclic tetrapeptide HC-toxin, an inhibitor of histone deacetylase which is an essential virulence determinant [26].

Gramicidin is a polypeptide with alternating L- and D-amino acids, with the general formula: formyl-L-X/Gly/L-Ala/D-Leu/L-Ala/D-Val/L-Val/D-Val/L-Trp/D-Leu/L-Y/D-Leu/L-Trp/D-Leu/L-Trp-ethanolamine (X and Y being markers of different gramicidin subtypes). The alternating D-/L-amino acid sequence is essential for the formation of the characteristic β-helix, which assembles in the lipid bilayers to form pores and increase bacterial membrane perme-

ability. The presence of D-amino acid is also essential to confer resistance to proteolitic cleavage. Interestingly, all D-enantiomer forms of naturally occurring antibiotics where shown to be completely resistant to enzymatic digestion while retained antibacterial activity [55].

D-amino acids are also widely distributed in vertebrate tissues and body fluids. For example, **D-aspartic acid** is found during the development of brain and peripheral organs on early stages of life but regulates also adult neurogenesis [56]. **D-Serine** is the most biologically active D-amino acid described to date. In the brain, it acts as a potent agonist at the glycine of the NMDA-type glutamate receptor (N-methyl-D-aspartate receptor), dramatically increasing receptor affinity for glutamate; D-serine is then able to produce excitotoxicity, without any change in glutamate concentration *per se* [57]. High concentration of D-amino acids in human serum, such as **D-Ala** (but also D-Ser), are suggested to correlate with damage to renal function [58].

Several enzymes acting on a variety of **3-hydroxy-D-amino acids** have been described and characterized from different microorganisms. These enzymes are highly specific for the D-amino acid forms. One example is **D-*threo*-3-phenylserine/D-threonine** (Fig. 7) dehydrogenase from *Pseudomonas cruciviae*, that catalyzes the oxidation of the 3-hydroxyl group of to yield D-2-amino-3-ketobutyrate, which is spontaneously decarboxylated into aminoacetone [59]. D-Threonine and D-*threo*-3-phenylserine are synthetic compounds and have not been found in the free form in nature, although the toxic peptide phalloidine contains D-threonine [60]. Another example is D-3-hydroxyaspartate aldolase from *Paracoccus denitrificans*, a PLP-dependent enzyme which catalyzes the conversion of **D-3-hydroxyaspartate** to glyoxylate and glycine. This enzyme is strictly specific for the D-amino acid form, but does not distinguish between the *threo*/*erythro* for at β-carbon. It also acts on D-threonine, D-3-phenylserine and on norepinephrine analogues D-3 – 3,4-dihydroxyphenylserine and D-3 – 3,4-methylenedioxyphenylserine [61]. D-hydroxyaspartate is found in phallacidin, the major *Amanita* mushroom toxin [62]. The physiological role of these D-hydroxyamino acids (and of the enzymes acting on them), is not clear, but could be related to the fungus *vs.* bacteria host-pathogen interaction.

Biogenic amines

Biogenic amines are important compounds found in microbial, vegetable and animal cells. They are mainly produced via amino acid decarboxylation although some of the aliphatic amines can be formed *in vivo* by amination or transamination of the corresponding aldehydes and ketones. Besides their role as nitrogen source for the biosynthesis of hormones, alkaloids, nucleic acids and proteins, biogenic amines are precursors of food aroma components and can be related to food spoilage and fermentation processes. In addition, they can be precursors of carcinogenic *N*-nitroso compounds. The biological function of biogenic

amines in humans is of paramount importance in the central nervous system. A list of common amino acid-derived amines acting as neurotransmitter and hormones is shown in Table 1 (and Fig. 8).

Table 1. Biogenic amines derived from amino acids and their biological effects.

BIOGENIC AMINE	PRECURSOR AMINO ACID	BIOLOGICAL ACTIVITY	
Histamine	Histidine	Neurotransmitter Pro-inflammatory signal from mast cells Mediator	Mediates arousal/attention Allergic reactions/tissue damage Stimulation of HC l secretion in the stomach. Lowers blood pressure
Serotonin	Tryptophan	CNS neurotransmitter	Regulates mood, appetite, sleep
Norepinephrine (noradrenaline) **Epinephrine** (adrenaline) **Dopamine**	Tyrosine	Neurotransmitter Hormone Neurotransmitter	Involved in wakefulness and sleep Regulates heart rate, blood vessel and air passage diameters. Fight-or-flight response, stress hormone Important roles in behavior and cognition, voluntary movement, motivation, punishment and reward, sleep, mood, attention, working memory, learning.
Tyramine/ Phenylethylamine	Tyrosine	Mediator	Rise of blood pressure. Catecholamine releasing agent.
3-Iodothyronamine	Thyroid hormones	Hormone	Decreased body temperature and cardiac output
Tryptamine and its derivatives	Tryptophan	Neuromodulators	Modulator of serotonin effects on CNS. Functions by itself in central neurotransmission still debated

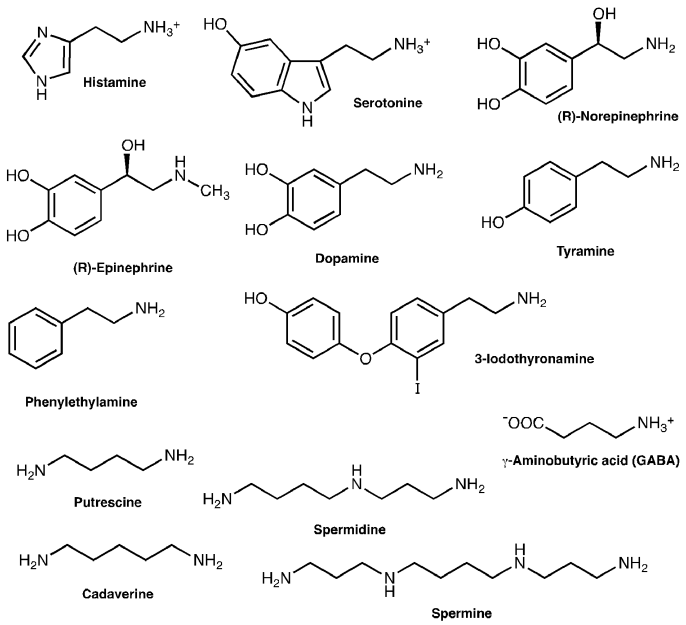

Figure 8. Structure of physiologically relevant biogenic amines.

Polyamines such as cadaverine, putrescine, spermidine, and spermine (Fig. 8) (derived from amino acids arginine, lysine and ornithine) are linear cationic molecules that interact with DNA and regulate nucleic acid function. They have also been shown to promote programmed ribosomal frame shifting during translation [63] and to modulate membrane permeability acting on a variety of ion channels. Although every cell is able to synthesize polyamines, the body relies on a continuous dietary supply of these compounds, stored in different organs and then release in a strictly regulated manner.

Many PLP-dependent decarboxylases are involved in the production of biologically important amines. DOPA decarboxylase catalyzes the conversion of L-3,4-diidroxyphenylalanine (L-DOPA) into dopamine, a neurotransmitter found in the nervous system and peripheral tissues of both vertebrates and invertebrates and also in plants where it is implicated in the biosynthesis of benzylisoquinoline alkaloids. It also catalyzes the decarboxylation of 5-hydroxy-L-tryptophan to give 5-hydroxytryptamine (serotonin). Ornithine decarboxylase catalyzes the rate-limiting step in the biosynthesis of polyamines, i. e. the decarboxylation of the urea cycle intermediate L-ornithine to give putrescine. Glutamate decarboxylase is involved in the formation of γ-aminobutyric acid (GABA; Fig. 8), the major inhibitory neurotransmitter in the central nervous system.

Synthesis of Non-canonical Amino Acids by PLP-dependent Enzymes

Synthesis of β-hydroxy-α-amino acids

The synthesis of β-hydroxy-α-amino acids (3-HAAs) has been attracting a lot of attention because many of these compounds are biologically active or constitute intermediates or building blocks of drugs. The industrial production of 3-HAAs has been mainly restricted to chemical synthesis, which yields all four stereoisomers (D- and L- *threo* and *erythro* forms) and therefore requires further steps of purification. However, in the recent past many communications concerning the use of enzymes for the synthesis of 3-HAAs have appeared in the literature. Two types of fold type I PLP-dependent aldolases have been used for synthetic purposes, both catalyzing the reversible aldol reaction of glycine with an aldehyde: **serine hydroxymethyltransferase** (SHMT; systematic name *5,10-methylenetetrahydrofolate:glycine hydroxymethyltransferase*, EC 2.1.2.1) and **threonine aldolase** (TA; systematic name *threonine acetaldehyde-lyase*, EC 4.1.2.x). 3-HAAs contain two chiral centers, one at Cα, which determines the L- or D- configuration, and the second at Cβ, responsible for the *erytro* or *threo* configuration of L- and D-3-HAAs. Although SHMT is selective for the l-3-HAA, it lacks specificity for the configuration of Cβ (SHMT from corn and rabbit liver yielded 3-HAAs with prevailing *erythro* configurations; pig liver SHMT has been used in large preparative reactions, obtaining mixtures of *erythro* and *threo* compounds [64]. For this reason, the synthesis of 3-HAAs catalyzed by SHMT has not been pursued with great passion.

On the other hand, many TAs with different stereospecificity have been isolated from a number of bacteria and fungi and characterized [65]. According to their stereospecificity at Cα, TAs are classified into L- and D- type enzymes. The former ones are further divided into low-specificity TAs (EC 4.1.2.48), L-threonine aldolases (EC 4.1.2.5) and L-*allo*-threonine aldolase (EC 4.1.2.49), depending on their preference for the configuration at Cβ. Only low specific D-TAs (EC 4.1.2.42) are known so far.

TA have been used for the synthesis of 3-HAAs following two strategies: the enzymatic resolution of racemic mixtures obtained via chemical synthesis or the direct enzymatic asymmetric synthesis.

The enzymatic resolution strategy has been extensively employed [64, 66] and, significantly, has been reported in multi-step procedures for the synthesis of the antibiotic thiamphenicol [67] and the anti-Parkinson's disease drug L-*threo*-DOPS [68]. It consists in the enantioselective cleavage of one enantiomer of a diastereomerically highly enriched racemate, previously obtained through chemical synthesis. The drawback of this strategy is that its maximum yield is 50% and therefore its attractiveness relies on the economical chemical synthesis of the racemate.

The TA-based enzymatic asymmetric synthesis of 3-HAAs is a very attractive synthetic route, which has been followed by many researchers. Several are the successful achievements in this field, although the low to medium diastereoselectivity of the biotransformations obtained still needs to be improved. Detailed research on the synthetic capabilities [69, 70] and stereospecificity [71] of TAs has been carried out. Attention has also been focused on the production of the anti-Parkinson's disease drug L-*threo*-DOPS [72] and 3-HAAs to be used in the synthesis of complex organic compounds, such as peptide mimetics [73].

The selectivity in the synthesis of either *threo* or *erythro* 3-HAAs depends on both the stereospecificity of the enzymes and on the thermodynamic equilibrium of the aldol reaction. A high *erythro* over *threo* selectivity could be obtained in the TA-catalyzed condensation of γ-benzyloxybutanal and glycine under kinetically controlled conditions [74]. The *erythro* product was used in the synthesis of mycestericin D, a potent immunosuppressant. On the other hand, mutant forms of TA were produced by error-prone PCR followed by a high-throughput screening, which displayed an increased diastereoselectivity for L-*threo*-DOPS synthesis [75, 76]. Recently, a novel genetic selection system has been devised in the attempt to expand the substrate scope and enhance the selectivity of TAs [77].

The catalytic capabilities of TAs have been also exploited using substrates other than glycine and simple aldehydes. A two-step, one-pot bioenzymatic reaction, involving TA and a PLP-dependent decarboxylase, has been devised to produce β-amino alcohols [71, 78]. L-TA from *Escherichia coli* was shown to accept aldehydes bearing a carboxylic acid, forming ω-carboxy-β-hydroxy-L-α-amino acids [39]. Moreover, the enantio- and diastereoselective

synthesis of α,α-disubstituted β-hydroxy-L-α-amino acids was obtained using L-*allo*-threonine aldolase from *A. jandeii* and D-threonine aldolase from *Pseudomonas* sp. as catalysts and D-amino acids (alanine, serine and cysteine) as substrates [79]. Interestingly, an engineered D-alanine racemase was employed as an aldolase with D-alanine as substrate [80–83]. This latter achievement supports the hypothesized strict structural and functional relationship between PLP-dependent racemases and aldolases [14].

Finally, the use of whole cells for the threonine aldolase-based synthesis of L-*threo*-DOPS by TA has been recently reported [75, 84].

β-Substitution reactions

This kind of reactions, catalyzed by several PLP-dependent enzymes, allow for the addition of different nucleophiles to an aminoacrylate intermediate previously formed from the starting substrate upon a β-elimination reaction. Subsequently, the product of condensation (the amino acid analogue) is released from the cofactor. These reactions can accomplish carbon-carbon, carbon-nitrogen or carbon-sulfur (or selenium) bond formation, depending on the nucleophile used as second substrate (indoles, phenols, cyanides for C-C bond formation; pyrroles, azides for C-N bond; thiols/selenols for C-S or C-Se bond formation). The most used PLP-dependent enzymes for β-substitution reactions and production of amino acid analogues are herein described.

L-Tyrosine phenol-lyase (TPL; EC 4.1.99.2)**.** The synthesis of fluorinated analogues of tyrosine by use of bacterial tyrosine phenol-lyase has been described many years ago [85]. The physiological reaction catalyzed by TPL (a fold type I PLP-dependent enzyme) is the hydrolytic cleavage of L-tyrosine to yield phenol and ammonium pyruvate. In addition to this reaction, participating in tyrosine metabolism, TPL also efficiently catalyzes the β-elimination of a number of β-substituted amino acids with good leaving groups, such as S-methyl-L-cysteine, β-chloroalanine, and S-(*O*-nitrophenyl)-L-cysteine. TPL has also been shown to catalyze the racemization of alanine, but at a much slower rate [86]. The reaction of TPL is readily reversible at high concentration of ammonia and pyruvate. Under these conditions the enzyme binds ammonia first, followed by pyruvate and then phenol. The reverse reaction catalyzed by TPL can be used then as a biosynthetic tool for the production of tyrosine and tyrosine analogues. When an appropriate phenol derivative is substituted for phenol, for example, the corresponding tyrosine analogues can be synthesized. Several differently substituted fluoro-, chloro-, bromo-, iodo-, methyl-, methoxy-L-tyrosines have been produced by this method. If the phenol ring is substitute by a catechol, 3,4-dihydroxyphenil-L-alanine (L-DOPA) is produced. L-DOPA is used in the treatment of Parkinson's disease and more than 250 tons of it is made every year for pharmaceutical application. Most of it is indeed produced by an industrialized process that use engineered microorganisms overexpressing TPL activity [87].

Substantially homologous to TPL in sequence, three-dimensional structure and reaction mechanism is **tryptophanase** (Tnase or Trpase; systematic name *L-tryptophan indole-lyase*, EC 4.1.99.1) a bacterial PLP-dependent lyase that catalyzes in vivo degradation of L-tryptophan to yield indole, pyruvate and ammonia [88]. Also in this case, the enzyme can act in the reverse direction, to synthesize L-tryptophan in conditions of excess pyruvate, ammonia and a supply of indole. The enzyme also catalyzes α,β-elimination and β-replacement reactions on several other β-substituted L-amino acids. To our knowledge, this enzyme has not been exploited for biotechnology applications.

Tryptophan synthase (TS; systematic name L-*serine hydro-lyase*, EC 4.2.1.20.) can be used for the synthesis of tryptophan analogues. Tryptophan synthase catalyzes, in bacteria fungi and plants, the final two steps in tryptophan biosynthesis. The enzyme is a $\alpha_2\beta_2$ tetramer. The physiological reaction of TS is the combination of the reactions occurring at the α and β-sites, which are tightly coupled through allosteric interactions. The reaction catalyzed by the α-subunit is the reversible retroaldol cleavage of indole-3-glycerol phosphate to give indole and D-glyceraldehyde-3-phosphate. The indole is not released into the solvent, but is directly transferred to the β-subunit (a fold type II PLP-enzyme) where it is condensed to the aminoacrylate intermediate formed by the elimination of water from the L-serine-PLP adduct. The protonation of the α-carbon of the following quinonoid intermediate produces the external aldimine with L-tryptophan ([24, 89] and references within).

The α-subunit reaction is catalyzed by general acid-base catalysis; although it is readily reversible and thus could be used for the synthesis of indole-3-glycerol phosphate and its derivatives (through indole and aldehydes analogues), there have been very few investigations about its synthetic applications. On the other hand, the β-reaction of TS has been used for the preparation of a large variety of non-canonical α-L-amino acids. In fact, a wide range of indole analogues are recognized by the β-subunit active site and give rise to the corresponding L-tryptophan derivatives. Substituted methyl-, fluoro-, chloro-, hydroxy-, methoxy-, dipluoromrthyl-, azido-indoles in various positions have been proved to be accepted as substrates by tryptophan synthases from several organisms (*Neurospora crassa, E. coli, S. typhimurium*) although with catalytic constants different from unsubstituted indole. For example, the much lower nucleophilicity of azaindoles requires the reaction for the formation of azatryptophans to be carried out for longer times (even days or weeks). TS is also able to accommodate and condense heterocyclic double 5-membered rings such as thienopyrroles and selenopyrroles with the formation of thiatryptophans and selenatryptophans, whose preparation by synthetic chemistry would prove difficult due to their extreme acid sensitivity; these compounds, incorporated into protein, may be useful as spectroscopic probes and heavy atom derivatives in protein crystallography [90 – 92]. Notably, reaction with all indole derivatives take place with high stereospecificity, since the reprotonation of Cα only happens on the *re*-face of the quinonoid intermediate.

***O*-acetylserine sulfhydrylase** (OASS, cysteine synthase; systematic name *O3-acetyl-L-serine:hydrogen-sulfide 2-amino-2-carboxyethyltransferase*, former EC 4.2.99.8, now 2.5.1.47) the enzyme responsible of the final step of cysteine biosynthetic pathway in bacteria, fungi, plants and protozoan parasites can be used for the production of a variety of β-substituted amino acids. This enzyme catalyzes a β-replacement reaction in which the β-acetoxy group of *O*-acetyl-L-serine (OAS) is replaced by bisulfide to give L-cysteine and acetate. The substrate *O*-acetyl-L-serine is synthetized by serine acetyltransferase (SAT), from L-serine and acetyl-CoA. Interestingly, SAT modulates OASS activity by directly interacting with it. The reaction follows a ping-pong bi-bi mechanism with a stable α-amino-acrylate intermediate. The enzyme is a homodimer belonging to fold type II PLP-dependent enzymes, the active site residues being contributed by a single subunit [93]. Given the key biological role played by *O*-acetylserine sulfhydrylase in bacteria, inhibitors with potential antibiotic activity have been developed [94]. Bacteria such as *E. coli*, *S. typhimurium* and *Haemophilus influenzae* possess two OASS isoforms (namely OASS-A and OASS-B, encoded by *cysK* and *cysM* genes, respectively). The two enzymes share a 43% sequence identity, have almost superimposable structure, and similar activities. Whereas OASS-A is highly expressed at basal level, the physiological role of OASS-B is still controversial. Both enzymes have relaxed substrate specificity, with OASS-B being more promiscuous and able to accept bigger substrates. A recent study on both isoforms of *E. coli* OASS showed that many different nucleophiles can replace bisulfide in the reaction catalyzed by OASS, such as several types of thiols (methane-, ethane-, propylene-, phenyl-thiol) and selenols, phenol, azide, cyanide, 5-memberd rings containing at least two adjacent nitrogens (i.e. pyrazole, but not imidazole), giving rise to a variety of non-canonical β-substituted-L-α-amino acids [95, 96]. OASS-B isoform was able to synthetize, starting from *O*-acetyl-L-serine and 1,2,4-oxadiazolidine-3,5-dione quisqualic acid, an amino acid extracted from plants with neuro-stimolatory effects by acting as an agonist of AMPA and metabotropic glutamate receptors. Interestingly, its decarboxylated counterpart, quisqualamine, shows central depressant and neuroprotective properties and appears to act predominantly as an agonist of the $GABA_A$ (γ-aminobutyric acid, the decarboxylated form of glutamate) receptor.

Similar to OASS is ***O*-phosphoserine sulfhydrylase** (OPSS; systematic name *O-phospho-L-serine:hydrogen-sulfide 2-amino-2-carboxyethyltransferase*, EC 2.5.1.65), an enzyme recently discovered in hypothermophilic aerobic archea which catalyzes the sulfhydrylation of *O*-phospho-L-serine to form L-cysteine. *O*-phosphoserine is much more stable than *O*-acetylserine and might be the precursor involved in cysteine biosynthesis in extremophiles. As for OASS, OPSS is able to catalyze synthetic reactions using a variety of nucleophiles, giving rise to unnatural amino acids. This enzyme was actually also found in organisms such as the human parasite *Trichomonas vaginalis* and pathogens *Mycobacterium tuberculosis*, revealing a new cysteine biosynthetic pathway, with interesting implication for the design of innovative drugs.

Another enzyme closely related to OASS is ***O*-acetyl-L-homoserine sulfhydrylase** (**OAHS**; homocysteine synthase, *O*-acetylhomoserine(thiol)-lyase; EC 4.2.99.10). This is a sulfide-utilizing enzyme involved in the complex and diversified L-cysteine and L-methionine biosynthetic pathways of various bacteria, filamentous fungi and yeast [97]. OAHS is known to catalyze the conversion of *O*-acetyl-L-homoserine to L-homocysteine using bisulfide as direct sulphur donor. L-Homocysteine is then converted to L-methionine through tetrahydrofolate dependent methylation catalyzed by methionine synthase. OAHS is essential in certain microorganisms, whereas others use alternative methionine biosynthetic pathways. Remarkably, OAHS is structurally related to cystathionine α-, β-, γ-lyases from bacteria, plants, yeast and animals, all enzymes involved in methionine biosynthesis and requiring PLP as cofactor. The reaction catalyzed by OAHS is a γ-substitution reaction. The first half reaction requires two deprotonation steps, followed by γ-elimination of the acetyl group, leaving an aminoacrylate intermediate. Bisulfide then makes a nucleophilic attack to form homocysteine. As for OASS, different nucleophiles can perform this second step of the reaction, including Na_2Se_2 [98] cyanide, sodium azide, methanethiol (directly generating methionine), ethanethiol and thiophenol [99]. Recent studies indicated that in *Wolinella succinogenes*, a close relative of *Helicobacter pylori*, the source of sulphur in methionine biosynthesis is a protein thiocarboxylate. The substrate of OAHS, in this case, would then not be a small nucleophile, but an entire sulphur-carrier low molecular weight protein (with ubiquitin-like fold) [100].

Finally, to conclude this general view on PLP-dependent enzymes catalysing β-substitution reactions, it is worth mentioning the enzymes that naturally produce seleno-amino acids. The reaction mechanism of **selenocysteine synthase** (systematic name *selenophosphate:O-phospho-L-seryl-tRNASec selenium transferase*, EC 2.9.1.2), the enzyme responsible of the last step of selenocysteine biosynthesis in eukaryotes and Archea is very similar to the one catalyzed by OPSS. Interestingly, in this case the substrate is not free *O*-phospho-L-serine, but *O*-phosphoseryl-$tRNA^{[Ser]Sec}$, which is generated by enzymatic phosphorylation of seryl-$tRNA^{[Ser]Sec}$. First, the enzyme removes the phosphate group from *O*-phosphoseryl moiety to yield the aminoacrylate intermediate, and then accepts activated monoseleniumphosphate as condensing nucleophile. The bacterial (*E. coli*) counterpart, **L-Seryl-tRNASec selenium transferase** (systematic name *Selenophosphate:L-seryl-tRNASec selenium transferase*, EC 2.9.1.1), catalyzes the same reaction using seryl-$tRNA^{[Ser]Sec}$, generating the aminoacrylate through the elimination of the hydroxyl group from the seryl moiety.

Non-canonical Amino Acids for Genetic Code Expansion

What is genetic code expansion?

The number of amino acids in ribosomal protein synthesis is restricted to the 20 canonical amino acids. Additional functionalities into protein structures can be introduced by expanding the repertoire of amino acids that make up the basic structure of the resulting protein

molecule by re-assigning one (or several) of the codons that are normally used to encode conventional amino acids (or to terminate protein synthesis) into another, non-canonical amino acid (ncAA). By incorporating an alternative amino acid into proteins, the available chemical options to achieve additional functionalities is substantially enlarged. This approach relies on

1. the availability of a tRNAs/amino acyl-tRNA synthetase pairs that allow to "mischarge" the tRNA of the re-assigned codon with the ncAA and
2. a mechanism that prevents the re-assigned codon to fulfill its original function with a canonical amino acid or a termination function (STOP codon).

The whole system is defined to consist of four elements: a re-assigned codon, a cognate mischarged tRNA, a cognate mischarging amino acyl-tRNA synthetase, and the charged ncAA. Through this approach, the biological, chemical, or physical properties of new amino acids are precisely defined by the chemist at the bench and, due to the genetic encoding of these ncAAs, their incorporation into proteins should occur with exquisite fidelity and efficiency (for review see: [101]).

In this way, microorganisms with an engineered genetic code are capable of delivering the biological, chemical, or physical properties of many unnatural or synthetic non-canonical amino acids, into resulting polypeptide sequences. For example, ncAAs leading to changes in the protein backbones, such as α-hyroxy acids, can be site-specifically installed into proteins to define specific sites for chemical hydrolysis [102]. Other examples are special ncAAs building blocks such as fluorinated analogues that have highly attractive unique features for pharmaceutical industry, since fluorination improves bioavailability (i.e. membrane interaction and passage activity) of drugs and their metabolic stability [103]. In addition, ncAAs can be used to site-specifically derivatize proteins with PEG molecules, sugars, oligonucleotides, fluorophores, peptides, and other unique synthetic moieties. Important bioorthogonal[2] chemical functionalities such as azides, olefins, carbonyl compounds (ketones/aldehydes), strained- and unstrained-alkynes, halogens, boronic esters/acids, oximes/hydrazones can be used for specific coupling with these moieties.

Bioorthogonal click-reactions for site-specific chemoselective ligations include copper-click, photo-click, metathesis, and catalyzed oxime/hydrazone chemistries. Remarkably, these chemistries are often mutually orthogonal: e.g., photoclick chemistry is bioorthogonal to both oxime/hydrazone and copper-click chemistry, and should enable a variety of different coupling reactions to the same protein in a single expression experiment (for review see [104]).

[2] The term bioorthogonal chemistry refers to any chemical reaction that can occur inside a living system without interfering with native biochemical processes.

While *in vitro* incorporation of ncAAs has been known for decades, *in vivo* approaches that are currently available for the incorporation of desirable ncAAs in a controlled manner have only been recently developed and can be divided into two methodologies. In the first approach, residue-specific incorporation of different ncAAs into target proteins occurs via sense codon reassignment using auxotrophic host strain that exploits wide substrate tolerance in activating similar amino acids (analogs, surrogates) [105]; the second approach includes suppression methodologies and use orthogonal pairs to reassign termination or non-triplet coding units for site-specific addition of ncAAs to the existing amino acid repertoire during translation. In this case, aminoacyl-tRNA synthetases needed to activate the desired ncAAs are selected using standard positive/negative selection methods [106, 107].

Code expansion and metabolic engineering: crucial role of PLP-dependent enzymes

One of the most straightforward methods, using expanded genetic code to generate unnatural polypeptides with non-canonical amino acids usually require the addition of the starting building blocks to the growth medium and their subsequent uptake by the cellular transport machinery. While this is perfectly acceptable for small-scale experiments, there is still no solution for mass production. Here, the ncAAs should be produced from medium nutrients, just as all other cell components, to prevent complicated feeding schemes and additional costly substrates. To tackle this general problem it is necessary to perform intracellular synthetic pathway engineering. Thereby, PLP-dependent enzymes could be of prime importance, as they are well known to be catalytically promiscuous and to contain members from thermophilic group of bacteria, which provide a rather stable scaffold for bioengineering purposes [108].

Certainly, one of the best documented semisynthetic approach of non-canonical amino acids synthesis through metabolic engineering using PLP-dependent enzymes is the engineering of cysteine biosynthetic pathway as reported by Maier [95]. In particular, the final step in this biosynthetic pathway is catalyzed by *O*-acetylserine sulfhydrylase, a PLP-dependent enzyme which catalyzes β-substitution reaction on *O*-acetylserine. The catalytic promiscuity of this enzyme is reflected in its broad substrate specificity in a similar manner as tryptophan synthase (see above). Combined with this feature, the intracellular deregulation of cysteine pathway enables the biosynthesis of amino acid derivatives characterized by diverse side chains with interesting chemical functionalities. For example, the fermentation media supplied with toxic substances such as azide, cyanide or triazole allows their biotransformation into amino acids such as azidoalanine, cyanoalanine and triazole-1-yl-alanine (Fig. 9). Using this approach, high yield production of non-canonical amino acids are reported as opening a fairly good perspective to make a further step and couple such reengineered metabolic processes with reprogrammed protein translation apparatus for tailor-made protein production on industrial scale.

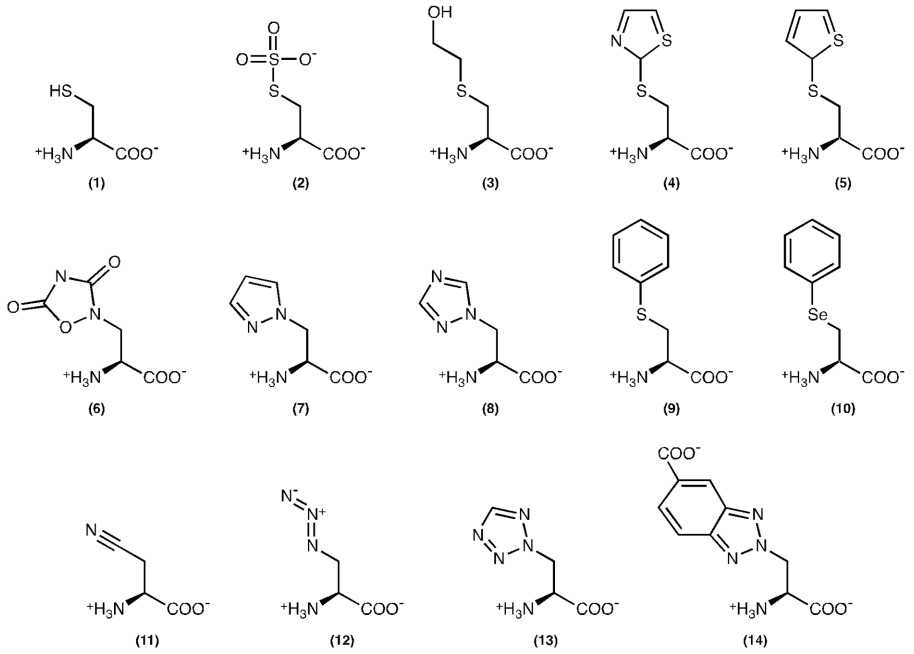

Figure 9. Structures of amino acids generated by metabolic engineering by means of the catalytic promiscuity of *O*-acetylserine sulfhydrylase, the last enzyme in cysteine biosynthetic pathway. The canonical amino acid cysteine **(1)** is the natural product of the intracellular catalytic activity of this enzyme. Non-canonical amino acids such as azidoalanine **(12)** are useful for protein surface diversification by Staudinger ligation or for 'click' chemistry of proteins; cyanoalanine **(11)** might serve as biophysical marker (IR probe); *S*-phenylcysteine **(9)** is an important building block in the design of inhibitors for AIDS therapy. Common names of the other non-canonical amino acids shown in the figure are: *S*-sulfocysteine **(2)**, *S*-hydroxyethylcysteine **(3)**, *S*-thiazole-2-yl-cysteine **(4)**, *S*-thien-2-yl-cysteine **(5)**, 1,2,4-oxadiazolidinedionyl-alanine **(6)**, pyrazole-1-yl-alanine **(7)**, triazole-1-yl-alanine **(8)**, tetrazole-2-yl-alanine **(13)**, S-phenyl-cysteine **(9)**, phenyl-selenocysteine **(10)** and 5-carboxybenzotriazole-2-yl-alanine **(14)**. (Adapted from [95])

The manipulation of the tryptophan biosynthetic pathway in combination with incorporation of non-canonical amino acids into proteins is another very instructive example of biosystems engineering by coupling expanded genetic code and semisynthetic/synthetic metabolism mediated by PLP-dependent enzymes. In particular, last steps in tryptophan biosynthesis include indole production and its condensation with L-serine to make L-tryptophan. This two combined reactions are performed by tryptophan synthase, through the well characterized reaction mechanism described before, which includes direct transfer of the indole intermediate between α and β subunits through a tunnel in the enzyme complex (the so-called 'channeling effect'). PLP-dependent β subunit of tryptophan synthase, which catalyzes β-substitution reaction on indole, is one of the best-studied enzymatic systems in biochemistry. One remarkable property of this enzyme, essential for amino acid derivative

production, is its broad substrate specificity; even amino acids not structurally and chemically related to tryptophan can be synthesized by using this enzyme [109] (but, significantly, these related amino acids are not substrates for endogenous tryptophanyl-tRNA synthetase). Thus, in a fermentation medium provided with a variety of (natural or synthetic) indole analogues/surrogates and controlled expression system, related amino acids analogues can be synthesized intracellularly and subsequently incorporated into target proteins as recently demonstrated [110].

From a biotechnological point of view, the great advantage of an expanded genetic code efficiently coupled with engineered PLP-dependent enzymes would be the possibility to achieve high chemical diversity at low genetic cost and to avoid supply of expensive precursors. In this way, a pathway for the biosynthesis of desired non-canonical amino acids can be engineered, imported and integrated into cellular metabolism enabling microbial hosts to generate desired amino acid from simple precursors or carbon sources.

Outlook

Chemical synthesis of desired non-canonical amino acids is usually expensive. Moreover, a main drawback of chemical syntheses of ncAAs as substrates for ribosomal polypeptides synthesis, with only a few exceptions, is that the substance of interest has to be added to the growth medium and subsequently taken up by the cellular uptake machinery. This can be suitable for small laboratory-scale experiments; however, it is highly unsuitable for large-scale fed-batch production. Therefore, it is highly desirable that synthetic amino acids of interest are produced by the host from medium nutrients, i. e. from simple and economically-favorable substrates without any complicated feeding procedures. Thus, in addition to expanding the scope of protein synthesis, one of the biggest challenge in the field will be to engineer metabolic pathways so as to provide cells with target synthetic amino acids produced intracellularly from simple carbon sources or precursors (Fig. 10). In this respect, enzymatic pathways mediated by PLP-dependent enzymes are most promising targets.

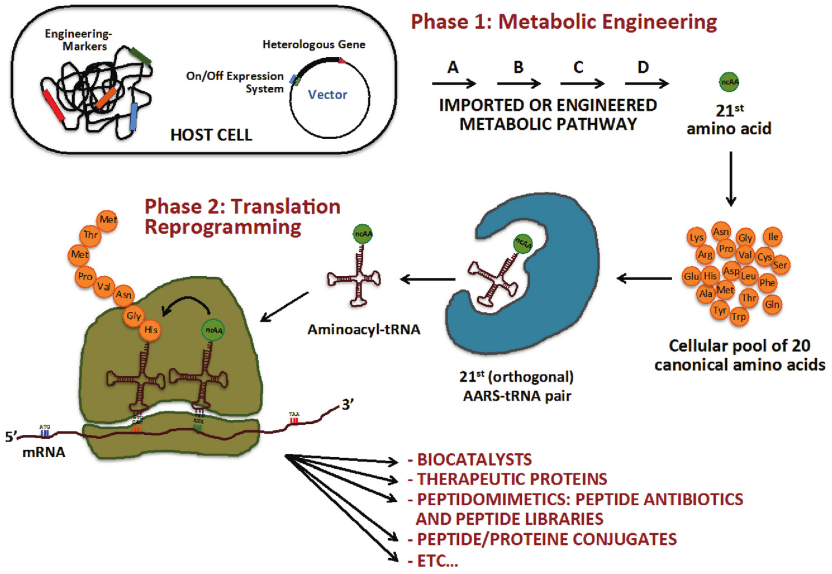

Figure 10. Coupling metabolic engineering with re-programmed protein translation. Combined together, natural products chemistry and genetic code engineering would lead to a larger scale screening for suitable amino acids as candidates for entry into the genetic code from intermediary metabolism of various species. The natural biosynthetic pathways can be imported and integrated into the metabolism of host cells in order to generate intracellular production of desired non-canonical amino acids (which should be exclusive substrates for AARS, aminoacyl-tRNA synthatase). These pathways can be attached to the existing ones in host cells, further modified, reduced, extended or optimized for balanced synthesis of desired substrates. The ultimate goal is the *in-vivo* evolution of novel synthetic pathways capable to generate substrate diversity to an extent far beyond natural one (adapted form [111]).

References

[1] Percudani, R., and Peracchi, A. (2003) A genomic overview of pyridoxal-phosphate-dependent enzymes. *EMBO reports* **4**:850 – 854. http://dx.doi.org/10.1038/sj.embor.embor914.

[2] John, R.A. (1995) Pyridoxal phosphate-dependent enzymes. *Biochim. Biophys. Acta* **1248**:81 – 96. http://dx.doi.org/10.1016/0167-4838(95)00025-P.

[3] Eliot, A.C., and Kirsch, J.F. (2004) Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. *Annual review of biochemistry* **73**:383 – 415.
http://dx.doi.org/10.1146/annurev.biochem.73.011303.074021.

[4] Amadasi, A., Bertoldi, M., Contestabile, R., Bettati, S., Cellini, B., di Salvo, M.L., Borri-Voltattorni, C., Bossa, F., and Mozzarelli, A. (2007) Pyridoxal 5'-phosphate enzymes as targets for therapeutic agents. *Current medicinal chemistry* **14**:1291 – 1324.
http://dx.doi.org/10.2174/092986707780597899.

[5] Metzler, D.E., Ikawa, M., Snell, E.E. (1954) A General Mechanism for Vitamin B6-catalyzed Reactions. *J. Am. Chem. Soc.* **76**(3):648 – 652.
http://dx.doi.org/10.1021/ja01632a004.

[6] Dunathan, H. C. (1966) Conformation and reaction specificity in pyridoxal phosphate enzymes. *Proceedings of the National Academy of Sciences of the U.S.A.* **55**:712 – 716.
http://dx.doi.org/10.1073/pnas.55.4.712.

[7] Olivard, J., Metzler, D.E., and Snell, E.E. (1952) Catalytic racemization of amino acids by pyridoxal and metal salts *J. Biol. Chem.* **199**:669 – 674.

[8] Metzler, D.E., and Snell, E.E. (1952) Deamination of serine. I. Catalytic deamination of serine and cysteine by pyridoxal and metal salts. *J. Biol. Chem*. **198**:353 – 361.

[9] Denesyuk, A.I., Denessiouk, K.A., Korpela, T., and Johnson, M.S. (2002) Functional attributes of the phosphate group binding cup of pyridoxal phosphate-dependent enzymes. *Journal of molecular biology* **316**:155 – 172.
http://dx.doi.org/10.1006/jmbi.2001.5310.

[10] Spenser, I.D., and Hill, R.E. (1995) The biosynthesis of pyridoxine. *Natural product reports* **12**:555 – 565.
http://dx.doi.org/10.1039/np9951200555.

[11] Surtees, R., Mills, P. and Clayton, P. (2006) Inborn errors affecting vitamin B6 metabolism, *Future neurol.* 1F(5):615 – 620.
http://dx.doi.org/10.2217/14796708.1.5.615.

[12] di Salvo, M.L., Safo, M.K., and Contestabile, R. (2012) Biomedical aspects of pyridoxal 5'-phosphate availability. *Front. Biosci. (Elite Ed)* **4**:897 – 913.

[13] Mehta, P.K., and Christen, P. (2000) The molecular evolution of pyridoxal-5'-phosphate-dependent enzymes. *Advances in enzymology and related areas of molecular biology* **74**:129 – 184.

[14] Paiardini, A., Contestabile, R., D'Aguanno, S., Pascarella, S., and Bossa, F. (2003) Threonine aldolase and alanine racemase: novel examples of convergent evolution in the superfamily of vitamin B6-dependent enzymes. *Biochim. Biophysi. Acta* **1647**:214 – 219.
http://dx.doi.org/10.1016/S1570-9639(03)00050-5.

[15] Nam, H., Lewis, N.E., Lerman, J.A., Lee, D.H., Chang, R.L., Kim, D., and Palsson, B.O. (2012) Network context and selection in the evolution to enzyme specificity. *Science* **337**:1101 – 1104.
http://dx.doi.org/10.1126/science.1216861.

[16] O'Brien, P.J., and Herschlag, D. (1999) Catalytic promiscuity and the evolution of new enzymatic activities. *Chemistry & biology* **6**:R91-R105.
http://dx.doi.org/10.1016/S1074-5521(99)80033-7.

[17] Khersonsky, O., and Tawfik, D.S. (2010) Enzyme promiscuity: a mechanistic and evolutionary perspective. *Annual reviews of biochemistry* **79**:471 – 505.
http://dx.doi.org/10.1146/annurev-biochem-030409-143718.

[18] Kochhar, S., and Christen, P. (1992) Mechanism of racemization of amino acids by aspartate aminotransferase. *Eur. J. Biochem.* **203**:563 – 569.
http://dx.doi.org/10.1111/j.1432-1033.1992.tb16584.x.

[19] Kochhar, S., and Christen, P. (1988) The enantiomeric error frequency of aspartate aminotransferase. *Eur. J. Biochem.* **175**:433 – 438.
http://dx.doi.org/10.1111/j.1432-1033.1988.tb14213.x.

[20] Soper, T.S., Ueno, H., and Manning, J.M. (1985) Substrate-induced changes in sulfhydryl reactivity of bacterial D-amino acid transaminase. *Archives of Biochemistry and Biophysics* **240**:1 – 8.
http://dx.doi.org/10.1016/0003-9861(85)90001-3.

[21] John, R.A., and Fasella, P. (1969) The reaction of L-serine O-sulfate with aspartate aminotransferase. *Biochemistry* **8**:4477 – 4482.
http://dx.doi.org/10.1021/bi00839a038.

[22] John, R.A., and Tudball, N. (1972) Evidence for induced fit of a pseudo-substrate of aspartate aminotransferase. *Eur. J. Biochem.* **31**:135 – 138.
http://dx.doi.org/10.1111/j.1432-1033.1972.tb02510.x.

[23] Contestabile, R., and John, R.A. (1996) The mechanism of high-yielding chiral syntheses catalysed by wild-type and mutant forms of aspartate aminotransferase. *Eur. J. Biochem.* **240**:150 – 155.
http://dx.doi.org/10.1111/j.1432-1033.1996.0150h.x.

[24] Miles, E.W. (1991) Structural basis for catalysis by tryptophan synthase. *Advances in enzymology and related areas of molecular biology* **64**:93 – 172.

[25] Contestabile, R., Paiardini, A., Pascarella, S., di Salvo, M.L., D'Aguanno, S., and Bossa, F. (2001) L-Threonine aldolase, serine hydroxymethyltransferase and fungal alanine racemase. A subgroup of strictly related enzymes specialized for different functions. *Eur. J. Biochem./FEBS J* **268**:6508 – 6525.

[26] Di Salvo, M.L., Florio, R., Paiardini, A., Vivoli, M., DàAguanno, S. and Contestabile, R. (2012) Alanine Racemase from Tolypocladium Inflatum: a Key PLP-dependent Enzyme in Cyclosporin Biosynthesis and a Model of Catalytic Promiscuity. *Arch. Biochem. Biophys. in press*.

[27] Kimura, T., Vassilev, V.P., Shen, G-J., Wong C-H. (1997) Enzymatic Synthesis of β-Hydroxy-α-amino Acids Based on Recombinant D- and L-Threonine Aldolases. *J. Am. Chem. Soc.* **119**(49):11734 – 11742.
http://dx.doi.org/10.1021/ja9720422.

[28] Karl, J., Hale, K.J., Manaviazar, S., and Delisser, V.M. (1994) A practical new asymmetric synthesis of (2S,3S)- and (2R,3R)-3-hydroxyleucine. *Tetrahedron* **50**(30):9181 – 9188.
http://dx.doi.org/10.1016/S0040-4020(01)85384-9.

[29] Gu, W., and Silverman, R.B. (2011) Stereospecific total syntheses of proteasome inhibitors omuralide and lactacystin. *The Journal of Organic Chemistry* **76**:8287 – 8293.
http://dx.doi.org/10.1021/jo201453x.

[30] Guzman-Martinez, A., Lamer, R., and Van Nieuwenhze, M.S. (2007) Total synthesis of lysobactin. *J. Am. Chem. Soc.* **129**:6017 – 6021.
http://dx.doi.org/10.1021/ja067648h.

[31] Morrison, R. B., and Scott, A. (1966) Swarming of Proteus – a solution to an old problem? *Nature* **211**:255 – 257.
http://dx.doi.org/10.1038/211255a0.

[32] Maki, H., Miura, K., and Yamano, Y. (2001) Katanosin B and plusbacin A(3), inhibitors of peptidoglycan synthesis in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial agents and chemotherapy* **45**:1823 – 1827.
http://dx.doi.org/10.1128/AAC.45.6.1823-1827.2001.

[33] Dickinson, L., and Thompson, M.J. (1957) [The antiviral action of threo-beta-phenylserine]. *British journal of pharmacology and chemotherapy* **12**:66 – 73.
http://dx.doi.org/10.1111/j.1476-5381.1957.tb01364.x.

[34] van Andel, O.M. (1966) Mode of Action of L-Threo-β-phenylserine as a Chemotherapeutant of *Cucumber Scab*. *Nature* **211**:326 – 327.
http://dx.doi.org/10.1038/211326a0.

[35] Goldstein, D.S. (2006) L-Dihydroxyphenylserine (L-DOPS): a norepinephrine prodrug. *Cardiovascular drug reviews* **24**:189 – 203.
http://dx.doi.org/10.1111/j.1527-3466.2006.00189.x.

[36] Schirch, L., and Peterson, D. (1980) Purification and properties of mitochondrial serine hydroxymethyltransferase. *J. Biol. Chem.* **255**:7801 – 7806.

[37] Shapiro, A.L., Scharff, M.D., Maizel, J.V., and Uhr, J.W. (1966) Synthesis of excess light chains of gamma globulin by rabbit lymph node cells. *Nature* **211**:243 – 245.
http://dx.doi.org/10.1038/211243a0.

[38] Brenner, S., and Milstein, C. (1966) Origin of antibody variation. *Nature* **211**:242 – 243.
http://dx.doi.org/10.1038/211242a0.

[39] Sagui, F., Conti, P., Roda, G., Contestabile, R., Riva, S. (2008) Enzymatic synthesis of ω-carboxy-β-hydroxy-(L)-α-amino acids. *Tetrahedron* **64**(22):5079 – 5084.
http://dx.doi.org/10.1016/j.tet.2008.03.070.

[40] Fullone, M.R., Paiardini, A., Miele, R., Marsango, S., Gross, D.C., Omura, S., Ros-Herrera, E., Bonaccorsi di Patti, M.C., Lagana, A., Pascarella, S., and Grgurina, I. (2012) Insight into the structure-function relationship of the nonheme iron halogenases involved in the biosynthesis of 4-chlorothreonine – Thr3 from *Streptomyces* sp. OH-5093 and SyrB2 from Pseudomonas syringae pv. syringae B301DR. *FEBS J*. **279**(23):4269 – 4282.
http://dx.doi.org/10.1111/febs.12017.

[41] Yoshida, H., Arai, N., Sugoh, M., Iwabuchi, J., Shiomi, K., Shinose, M., Tanaka, Y., and Omura, S. (1994) 4-chlorothreonine, a herbicidal antimetabolite produced by *Streptomyces* sp. OH-5093. *The Journal of Antibiotics* **47**:1165 – 1166.
http://dx.doi.org/10.7164/antibiotics.47.1165.

[42] Webb, H.K., and Matthews, R.G. (1995) 4-Chlorothreonine is substrate, mechanistic probe, and mechanism-based inactivator of serine hydroxymethyltransferase. *J. Biol. Chem.* **270**:17204 – 17209.
http://dx.doi.org/10.1074/jbc.270.29.17204.

[43] Lackner, H., Bahner, I., Shigematsu, N., Pannell, L.K., and Mauger, A.B. (2000) Structures of five components of the actinomycin Z complex from *Streptomyces fradiae*, two of which contain 4-chlorothreonine. *Journal of Natural Products* **63**:352 – 356.
http://dx.doi.org/10.1021/np990416u.

[44] di Salvo, M.L., Contestabile, R., and Safo, M.K. (2011) Vitamin B(6) salvage enzymes: mechanism, structure and regulation. *Biochim. Biophys. Acta* **1814**:1597 – 1608.
http://dx.doi.org/10.1016/j.bbapap.2010.12.006.

[45] Kotch, F.W., Guzei, I.A., and Raines, R.T. (2008) Stabilization of the collagen triple helix by O-methylation of hydroxyproline residues. *J. Am. Chem. Soc.* **130**:2952 – 2953.
http://dx.doi.org/10.1021/ja800225k.

[46] Wegenhart, B., Li Tan, L., Held, M., Kieliszewski, M., and Chen, L. (2006) Aggregate structure of hydroxyproline-rich glycoprotein (HRGP) and HRGP assisted dispersion of carbon nanotubes. *Nanoscale Research Letters* **1**:154 – 159.
http://dx.doi.org/10.1007/s11671-006-9006-8.

[47] Buczek, O., Bulaj, G., and Olivera, B.M. (2005) Conotoxins and the posttranslational modification of secreted gene products. *Cellular and Molecular Life Sciences* **62**:3067 – 3079.
http://dx.doi.org/10.1007/s00018-005-5283-0.

[48] Taylor, S.W., Waite, J.H., Ross, M.M., Shabanowitz, J., Hunta, D.F. (1994) trans-2,3-cis-3,4-Dihydroxyproline, a New Naturally Occurring Amino Acid, Is the Sixth Residue in the Tandemly Repeated Consensus Decapeptides of an Adhesive Protein from *Mytilus edulis*. *J. Am. Chem. Soc.* **116**:10803 – 10804.
http://dx.doi.org/10.1021/ja00102a063.

[49] Nakajima, T., and Volcani, B.E. (1969) 3,4-dihydroxyproline: a new amino acid in diatom cell walls. *Science* **164**:1400 – 1401.
http://dx.doi.org/10.1126/science.164.3886.1400.

[50] Buku, A., Faulstich, H., Wieland, T., and Dabrowski, J. (1980) 2,3-trans-3,4-trans-3,4-Dihydroxy-L-proline: An amino acid in toxic peptides of Amanita virosa mushrooms. *Proceedings of the National Academy of Sciences of the U.S.A.* **77**:2370 – 2371.
http://dx.doi.org/10.1073/pnas.77.5.2370.

[51] Katz, E., Mason, K.T., and Mauger, A.B. (1975) 3-hydroxy-5-methylproline, a new amino acid identified as a component of actinomycin Z1. *Biochem. Biophys. Res. Commun.* **63**:502 – 508.
http://dx.doi.org/10.1016/0006-291X(75)90716-0.

[52] Kaldor, S.W., Kalish, V.J., Davies, J.F., 2nd, Shetty, B.V., Fritz, J.E., Appelt, K., Burgess, J.A., Campanale, K.M., Chirgadze, N.Y., Clawson, D.K., Dressman, B.A., Hatch, S.D., Khalil, D.A., Kosa, M.B., Lubbehusen, P.P., Muesing, M.A., Patick, A.K., Reich, S.H., Su, K.S., and Tatlock, J.H. (1997) Viracept (nelfinavir mesylate,

AG1343): a potent, orally bioavailable inhibitor of HIV-1 protease. *J. Med. Chem.* **40**:3979 – 3985.
http://dx.doi.org/10.1021/jm9704098.

[53] Chow, W.A., Jiang, C., and Guan, M. (2009) Anti-HIV drugs for cancer therapeutics: back to the future? *The Lancet Oncology* **10**:61 – 71.
http://dx.doi.org/10.1016/S1470-2045(08)70334-6.

[54] Simmaco, M., Kreil, G., and Barra, D. (2009) Bombinins, antimicrobial peptides from Bombina species. *Biochim. Biophys. Acta* **1788**:1551 – 1555.
http://dx.doi.org/10.1016/j.bbamem.2009.01.004.

[55] Wade, D., Boman, A., Wahlin, B., Drain, C.M., Andreu, D., Boman, H.G., and Merrifield, R. B. (1990) All-D amino acid-containing channel-forming antibiotic peptides. *Proceedings of the National Academy of Sciences of the U.S.A.* **87**:4761 – 4765.
http://dx.doi.org/10.1073/pnas.87.12.4761.

[56] Kim, P.M., Duan, X., Huang, A.S., Liu, C.Y., Ming, G.L., Song, H., and Snyder, S.H. (2010) Aspartate racemase, generating neuronal D-aspartate, regulates adult neurogenesis. *Proceedings of the National Academy of Sciences of the U.S.A.* **107**:3175 – 3179.
http://dx.doi.org/10.1073/pnas.0914706107.

[57] Crow, J.P., Marecki, J.C., and Thompson, M. (2012) D-Serine Production, Degradation, and Transport in ALS: Critical Role of Methodology. *Neurology Research International,* 625245.

[58] Imai, K., Fukushima, T., Santa, T., Homma, H., Huang, Y., Sakai, K., and Kato, M. (1997) Distribution of free D-amino acids in tissues and body fluids of vertebrates. *Enantiomer* **2**:143 – 145.

[59] Misono, H., Kato, I., Packdibamrung, K., Nagata, S., and Nagasaki, S. (1993) NADP(+)-dependent D-threonine dehydrogenase from *Pseudomonas cruciviae* IFO 12047. *Applied and Environmental Microbiology* **59**:2963 – 2968.

[60] Lengsfeld, A.M., Low, I., Wieland, T., Dancker, P., and Hasselbach, W. (1974) Interaction of phalloidin with actin. *Proceedings of the National Academy of Sciences of the U.S.A.* **71**:2803 – 2807.
http://dx.doi.org/10.1073/pnas.71.7.2803.

[61] Liu, J. Q., Dairi, T., Itoh, N., Kataoka, M., and Shimizu, S. (2003) A novel enzyme, D-3-hydroxyaspartate aldolase from *Paracoccus denitrificans* IFO 13301: purification, characterization, and gene cloning. *Applied Microbiology and Biotechnology* **62**:53 – 60.
http://dx.doi.org/10.1007/s00253-003-1238-2.

[62] Hallen, H.E., Luo, H., Scott-Craig, J.S., and Walton, J.D. (2007) Gene family encoding the major toxins of lethal Amanita mushrooms. *Proceedings of the National Academy of Sciences of the U.S.A.* **104**:19097 – 19101. http://dx.doi.org/10.1073/pnas.0707340104.

[63] Rato, C., Amirova, S.R., Bates, D.G., Stansfield, I., and Wallace, H.M. (2011) Translational recoding as a feedback controller: systems approaches reveal polyamine-specific effects on the antizyme ribosomal frameshift. *Nucleic Acids Research* **39**:4587 – 4597. http://dx.doi.org/10.1093/nar/gkq1349.

[64] Gijsen, H.J., Qiao, L., Fitz, W., and Wong, C.H. (1996) Recent Advances in the Chemoenzymatic Synthesis of Carbohydrates and Carbohydrate Mimetics. *Chemical Reviews* 96:443 – 474. http://dx.doi.org/10.1021/cr950031q.

[65] Liu, J.Q., Dairi, T., Kataoka, M., Shimizu, S., and Yama, H. (2000) Diversity of microbial threonine aldolases and their application. *Mol. Catal. B. Enzym.* **10**:107 – 115. http://dx.doi.org/10.1016/S1381-1177(00)00118-1.

[66] Duckers, N., Baer, K., Simon, S., Groger, H., and Hummel, W. (2010) Threonine aldolases-screening, properties and applications in the synthesis of non-proteinogenic beta-hydroxy-alpha-amino acids. *Applied Microbiology and Biotechnology* **88**:409 – 424. http://dx.doi.org/10.1007/s00253-010-2751-8.

[67] Liu, J.Q., Odani, M., Dairi, T., Itoh, N., Shimizu, S., and Yamada, H. (1999) A new route to L-threo-3-[4-(methylthio)phenylserine], a key intermediate for the synthesis of antibiotics: recombinant low-specificity D-threonine aldolase-catalyzed stereospecific resolution. *Applied Microbiology and Biotechnology* **51**:586 – 591. http://dx.doi.org/10.1007/s002530051436.

[68] Liu, J.Q., Odani, M., Yasuoka, T., Dairi, T., Itoh, N., Kataoka, M., Shimizu, S., and Yamada, H. (2000) Gene cloning and overproduction of low-specificity D-threonine aldolase from *Alcaligenes xylosoxidans* and its application for production of a key intermediate for parkinsonism drug. *Applied Microbiology and Biotechnology* **54**:44 – 51. http://dx.doi.org/10.1007/s002539900301.

[69] Vassilev, V.P., Uchiyama, T., Kajimoto, T., and Wong, C.H. (1995) L-threonine aldolase in organic synthesis-preaparation of novel beta-hydroxy-alpha-amino acids. *Tetrahedron Lett.* **36**:4081 – 4084. http://dx.doi.org/10.1016/0040-4039(95)00720-W.

[70] Kimura, T., Vassilev, V.P., Shen, G.J., and Wong, C.H. (1997) Enzymatic synthesis of beta-hydroxy-alpha-amino acods based on recombinant D- and L-threonine aldolases. *Journal of the American Chemical Society* **119**:11734 – 11742. http://dx.doi.org/10.1021/ja9720422.

[71] Steinreiber, J., Schurmann, M., Wolberg, M., van Assema, F., Reisinger, C., Fesko, K., Mink, D., and Griengl, H. (2007) Overcoming thermodynamic and kinetic limitations of aldolase-catalyzed reactions by applying multienzymatic dynamic kinetic asymmetric transformations. *Angew. Chem. Int. Ed. Engl.* **46**:1624 – 1626.

[72] Liu, J.Q., Ito, A., Dairi, T., Itoh, A., Shimizu, S., and Yamada, H. (1998) Low-specificity L-threonine aldolase of *Pseudomonas sp.* NCIMB 10558: purification, characterization and its application to beta-hydroxy-alpha-amino acids synthesis. *Applied Microbiology and Biotechnology* **49.**

[73] Tanaka, T., Tsuda, C., Miura, T., Inazu, T., Tsuji, S., Nishihara, S., Hisamatsu, M., and Kajimoto, T. (2004) Design and synthesis of peptide mimetics of GDP-fucose: targeting inhibitors of fucosyltransferases. *Synlett* **2.**

[74] Shibata, K., Shingu, K., Vassilev, V. P., Nishide, K., Fujita, T., Node, M., Kajimoto, T., and Wong, C.H. (1996) Kinetic and thermodynamic control of L-threonine aldolase catalyzed reaction and its application to the synthesis of mycestericin D. *Tetrahedron Letters* **37**:2791 – 2794. http://dx.doi.org/10.1016/0040-4039(96)00430-3.

[75] Gwon, H.J., Yoshioka, H., Song, N.E., Kim, J.H., Song, Y.R., Jeong, D.Y., and Baik, S.H. (2012) Optimal production of L-threo-2,3-dihydroxyphenylserine (L-threo-DOPS) on a large scale by diastereoselectivity-enhanced variant of L-threonine aldolase expressed in *Escherichia coli. Preparative Biochemistry & Biotechnology* **42**:143 – 154. http://dx.doi.org/10.1080/10826068.2011.583975.

[76] Gwon, H.J., and Baik, S.H. (2010) Diastereoselective synthesis of L-threo-3,4-dihydroxyphenylserine by low-specific L-threonine aldolase mutants. *Biotechnology Letters* **32**:143 – 149. http://dx.doi.org/10.1007/s10529-009-0125-z.

[77] Giger, L., Toscano, M.D., Madeleine, B., Marlierre, P., and Hilvert, D. (2012) A novel genetic selection system for PLP-dependent threonine aldolases. *Tetrahedron* **68**:7549 – 7557. http://dx.doi.org/10.1016/j.tet.2012.05.097.

[78] Steinreiber, J., Schurmann, M., van Assema, F., Wolberg, M., Fesko, K., Reisinger, C., Mink, D., and Griengl, H. (2007) Synthesis of aromatic L-2-amino alcohols using a bienzymatic dynamic kinetic asymmetric transformation. *Adv. Synth. Catal.* **349**:1379 – 1386.
http://dx.doi.org/10.1002/adsc.200700051.

[79] Fesko, K., Uhl, M., Steinreiber, J., Gruber, K., and Griengl, H. (2010) Biocatalytic access to alpha,alpha-dialkyl-alpha-amino acids by a mechanism-based approach. *Angew. Chem. Int. Ed. Engl.* **49**:121 – 124.
http://dx.doi.org/10.1002/anie.200904395.

[80] Fesko, K., Giger, L., and Hilvert, D. (2008) Synthesis of beta-hydroxy-alpha-amino acids with a reengineered alanine racemase. *Bioorganic & Medicinal Chemistry Letters* **18**:5987 – 5990.
http://dx.doi.org/10.1016/j.bmcl.2008.08.031.

[81] Toscano, M.D., Muller, M.M., and Hilvert, D. (2007) Enhancing activity and controlling stereoselectivity in a designed PLP-dependent aldolase. *Angew. Chem. Int. Ed. Engl.* **46**:4468 – 4470.
http://dx.doi.org/10.1002/anie.200700710.

[82] Seebeck, F.P., Guainazzi, A., Amoreira, C., Baldridge, K.K., and Hilvert, D. (2006) Stereoselectivity and expanded substrate scope of an engineered PLP-dependent aldolase. *Angew. Chem. Int. Ed. Engl.* **45**:6824 – 6826.
http://dx.doi.org/10.1002/anie.200602529.

[83] Seebeck, F.., and Hilvert, D. (2003) Conversion of a PLP-dependent racemase into an aldolase by a single active site mutation. *Journal of the American Chemical Society* **125**:10158 – 10159.
http://dx.doi.org/10.1021/ja036707d.

[84] Baik, S.H., and Yoshioka, H. (2009) Enhanced synthesis of L-threo-3,4-dihydroxyphenylserine by high-density whole-cell biocatalyst of recombinant L-threonine aldolase from *Streptomyces avelmitilis. Biotechnology Letters* **31**:443 – 448.
http://dx.doi.org/10.1007/s10529-008-9885-0.

[85] Nagasawa, T., Utagawa, T., Goto, J., Kim, C. J., Tani, Y., Kumagai, H., and Yamada, H. (1981) Syntheses of L-tyrosine-related amino acids by tyrosine phenol-lyase of *Citrobacter intermedius. Eur. J. Biochem.* **117**:33 – 40.
http://dx.doi.org/10.1111/j.1432-1033.1981.tb06299.x.

[86] Phillips, R.S., Chen, H.Y., and Faleev, N.G. (2006) Aminoacrylate intermediates in the reaction of *Citrobacter freundii* tyrosine phenol-lyase. *Biochemistry* **45**:9575 – 9583.
http://dx.doi.org/10.1021/bi060561o.

[87] Koyanagi, T., Katayama, T., Suzuki, H., Onishi, A., Yokozeki, K., and Kumagai, H. (2009) Hyperproduction of 3,4-dihydroxyphenyl-L-alanine (L-Dopa) using *Erwinia herbicola* cells carrying a mutant transcriptional regulator TyrR. *Bioscience, Biotechnology, and Biochemistry* **73**:1221 – 1223.
http://dx.doi.org/10.1271/bbb.90019.

[88] Phillips, R.S., Demidkina, T.V., and Faleev, N.G. (2003) Structure and mechanism of tryptophan indole-lyase and tyrosine phenol-lyase. *Biochim. Biophys. Acta* **1647**:167 – 172.
http://dx.doi.org/10.1016/S1570-9639(03)00089-X.

[89] Miles, E.W. (2013) The tryptophan synthase alpha2beta2 complex: a model for substrate channeling, allosteric communication, and pyridoxal phosphate catalysis. *J. Biol. Chem.* **288**:10084 – 10091.
http://dx.doi.org/10.1074/jbc.X113.463331.

[90] Phillips, R.S., Cohen, L.A., Annby, U., Wensbo, D., Gronowitz, S. (1995) Enzymatic synthesis of Thia-L-tryptophans. *Bioorganic & Medicinal Chemistry Letters* **5**(11):1103 – 1206.
http://dx.doi.org/10.1016/0960-894X(95)00181-R.

[91] Milton, J.S., Phillips, R.S. (1992) Enzymatic synthesis of aza-l-tryptophans: The preparation of 5- and 6-Aza-L-tryptophan. *Bioorganic & Medicinal Chemistry Letters* **2**:1053 – 1056.
http://dx.doi.org/10.1016/S0960-894X(00)80617-4.

[92] Lee, M. *et al.* (1992) Enzymatic synthesis of chloro-L-tryptophans. *Bioorganic & Medicinal Chemistry Letters* **2**:1563 – 1564.

[93] Rabeh, W.M., and Cook, P.F. (2004) Structure and mechanism of O-acetylserine sulfhydrylase. *J. Biol. Chem*. **279**:26803 – 26806.
http://dx.doi.org/10.1074/jbc.R400001200.

[94] Mozzarelli, A., Bettati, S., Campanini, B., Salsi, E., Raboni, S., Singh, R., Spyrakis, F., Kumar, V.P., and Cook, P.F. (2011) The multifaceted pyridoxal 5'-phosphate-dependent O-acetylserine sulfhydrylase. *Biochim. Biophys. Acta* **1814**:1497 – 1510.
http://dx.doi.org/10.1016/j.bbapap.2011.04.011.

[95] Maier, T.H. (2003) Semisynthetic production of unnatural L-alpha-amino acids by metabolic engineering of the cysteine-biosynthetic pathway. *Nature Biotechnology* **21**:422 – 427.
http://dx.doi.org/10.1038/nbt807.

[96] Omura, H., Kuroda, M., Kobayashi, M., Shimizu, S., Yoshida, T., and Nagasawa, T. (2003) Purification, characterization and gene cloning of thermostable O-acetyl-L-serine sulfhydrylase forming beta-cyano-L-alanine. *Journal of Bioscience and Bioengineering* **95**:470 – 475.

[97] Yamagata, S. (1989) Roles of O-acetyl-L-homoserine sulfhydrylases in micro-organisms. *Biochimie* **71**:1125 – 1143.
http://dx.doi.org/10.1016/0300-9084(89)90016-3.

[98] Chocat, P., Esaki, N., Tanaka, H. and Soda, K. (1985) Synthesis of Selenocystine and Selenohomocystine with O-Acetylhomoserine Sulfhydrylase. *Agric. Biol. Chem.* **49**(4):1143 – 1150.
http://dx.doi.org/10.1271/bbb1961.49.1143.

[99] Omura, H., Ikemoto, M., Kobayashi, M., Shimizu, S., Yoshida, T., and Nagasawa, T. (2003) Purification, characterization and gene cloning of thermostable O-acetyl-L-homoserine sulfhydrylase forming gamma-cyano-alpha-aminobutyric acid. *Journal of Bioscience and Bioengineering* **96**:53 – 58.

[100] Tran, T.H., Krishnamoorthy, K., Begley, T.P., and Ealick, S.E. (2011) A novel mechanism of sulfur transfer catalyzed by O-acetylhomoserine sulfhydrylase in the methionine-biosynthetic pathway of *Wolinella succinogenes*. *Acta Crystallogr. D Biol. Crystallogr.* **67**:831 – 838.
http://dx.doi.org/10.1107/S0907444911028010.

[101] Budisa, N. (2004) Prolegomena to future experimental efforts on genetic code engineering by expanding its amino acid repertoire. *Angew. Chem. Int. Ed. Engl.* **43**:6426 – 6463.
http://dx.doi.org/10.1002/anie.200300646.

[102] Kobayashi, T., Yanagisawa, T., Sakamoto, K., and Yokoyama, S. (2009) Recognition of non-alpha-amino substrates by pyrrolysyl-tRNA synthetase. *Journal of Molecular Biology* **385**:1352 – 1360.
http://dx.doi.org/10.1016/j.jmb.2008.11.059.

[103] Budisa, N., Wenger, W., and Wiltschi, B. (2010) Residue-specific global fluorination of Candida antarctica lipase B in *Pichia pastoris*. *Molecular bioSystems* **6**:1630 – 1639.
http://dx.doi.org/10.1039/c002256j.

[104] Young, T.S., and Schultz, P.G. (2010) Beyond the canonical 20 amino acids: expanding the genetic lexicon. *J. Biol. Chem.* **285**:11039 – 11044.
http://dx.doi.org/10.1074/jbc.R109.091306.

[105] Budisa, N., Minks, C., Alefelder, S., Wenger, W., Dong, F., Moroder, L., and Huber, R. (1999) Toward the experimental codon reassignment *in vivo*: protein building with an expanded amino acid repertoire. *FASEB J.* **13**:41 – 51.

[106] Furter, R. (1998) Expansion of the genetic code: site-directed p-fluoro-phenylalanine incorporation in *Escherichia coli. Protein Science* **7**:419 – 426. http://dx.doi.org/10.1002/pro.5560070223.

[107] Wang, L., and Schultz, P. G. (2001) A general approach for the generation of orthogonal tRNAs. *Chemistry & Biology* **8**:883 – 890. http://dx.doi.org/10.1016/S1074-5521(01)00063-1.

[108] Angelaccio, S. (2013) Extremophilic SHMTs: from structure to biotecnology. *Bio.Med. Research International, in press*.

[109] Welch, M., and Phillips, R. S. (1999) Enzymatic syntheses of 6-(4 H-selenolo[3,2-b] pyrrolyl)-L-alanine, 4-(6 H-selenolo[2,3-b]pyrrolyl)-L-alanine, and 6-(4 H-furo[3,2-b] pyrrolyl-L-alanine. *Bioorg. Med. Chem. Lett.* **9**:637 – 640. http://dx.doi.org/10.1016/S0960-894X(99)00067-0.

[110] Lepthien, S., Hoesl, M.G., Merkel, L., and Budisa, N. (2008) Azatryptophans endow proteins with intrinsic blue fluorescence. *Proceedings of the National Academy of Sciences of the U.S.A.* **105**:16095 – 16100. http://dx.doi.org/10.1073/pnas.0802804105.

[111] Budisa, N. (2006) *Engineering the Genetic Code: Expanding the Amino Acid Repertoire for the Design of Novel Proteins*, Wiley-VCH Verlag GmbH, Weinheim.