(R)-3-AMINOISOBUTYRIC ACID: THE DESIGN OF AN ENZYMATIC ROUTE FOR THE SYNTHESIS OF BETA AMINO ACIDS.

Biocatalysis and green chemistry
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ABSTRACT

One of the most successful applications of bio-catalysis concerns the asymmetric synthesis of β-amino acids. A recent study by Martìnez-Gòmez et al.[1] has paved the way for the synthesis of enantioenriched β-amino acids employing a biomimetic approach that retraces thymine catabolic pathway. Specifically, using a tandem technique, the kinetic resolution of (R)-3-aminoisobutyric acid from dihydrouracils has been achieved. The study proposal presented is aimed to take advantage of these results and extend the retrosynthetic pathway up to glycolysis. The strategy proposed is based on a newly engineered metabolic pathway developed by mutant Saccharomyces cerevisiae strains. Enzyme kinetics experiments will be suggested to investigate if it is possible to improve the resolution step employing O-ureido-serine racemase.

JUSTIFICATION, WITH BACKGROUND INFORMATION

The interest in preparation of beta amino acids has been rekindled in the last decade, mainly because of the intense biological activity ascribed to this family of compounds (Figure 1). Even though less abundant than alfa amino acids, beta peptides could be found in several natural metabolites provided with pharmaceutical properties.[2]

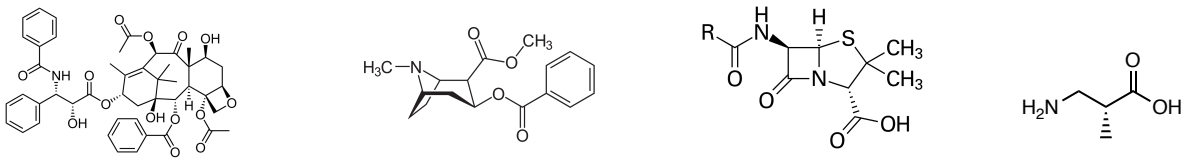

Figure 1: Taxol, Cocaine, Penicillin, R-3-AiBA

Beta amino acids have found a wide range of applications. In fact, the capability of self-assembling into regular secondary structures is employed in peptidomimetics[3], while the encouraging antibiotic properties of these compounds, especially in their cyclized form[4], has become very attractive for designing new drugs.

In this context of valuable chemicals for sciences, (R)-3-aminoisobutyric acid (R-3-AiBA) represents one of the most promising examples. In fact, R-3-AiBA plays an important role in human organism. Produced by the catabolism of thymine[5], R-3-AiBA activates the browning of fat during sustained muscular efforts, it is involved in insulin resistance mechanism and acts as a bone-protective factor preventing osteocyte cell death.[6]
The possibility to design new products for treating eating disorders makes R-3-AiBa and other β-alanine derivatives very attractive from a commercial point of view.[7] [8]
Undoubtedly, the growing interest for β-peptides has encouraged the research for new routes in this field of synthetic chemistry. About this topic, Juaristi and Soloshonok[9] have published an excellent

overview of the most important methods for preparation of beta amino acids. However, the demand for new tools for asymmetric synthesis is still high.[10 11]
To date, the most promising techniques explored consist in the employment of bio-catalytic reactions based on a wide spectrum of enzymes such as aminomutases[12], transaminases[13] or monoxygenases.[14] Nevertheless, there is still a considerable scope of improvement, because even the best exploited kinetic resolutions do not overcome yields of 50 %.[15]
The lack of enzymes capable to catalyze the racemization of β-amino acids represents the main impediment to perform dynamic resolutions.[1]

Up to now, the best results for the resolution of 3-aminoisobutyrric acid has been achieved using a bio-mimetic approach.[1] As mentioned before, R-3-AiBA is naturally produced through the degradation of thymine. In human catabolism, this pathway occurs through hydantoinase enzymes.[16] Recently, some features of these enzymes, enantioselectivity and substrate promiscuity above all, drew the attention of several groups interested in the development of new routes for enantiopure β amino acids.[17]

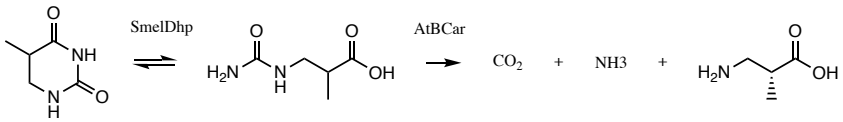

Figure 2: kinetic resolution of R-3-AiBA starting from 5-METDHU using tandem technique

The method proposed by Martìnez-Gòmez et al. uses a racemic mixture of (R, S)-5-METDHU as starting material and is based on a tandem reaction that involves two enzymes: SmelDhp (dihydropyrimidinase) and Atβcar (β-alanine synthase). The kinetic resolution described reaches an enatiomeric excess of 90% at half conversion of the starting material (Figure 3).

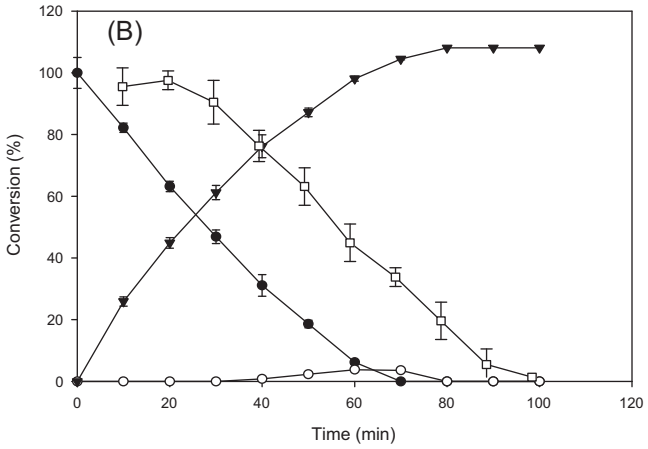

Figure 3: Progression curve showing the conversion of 5-METDHU into 3-AiBA (Black dots represent substrate; triangles are used for product formation while squares depict ee %)[1].

Another essential tool developed for the synthesis of β-amino acids is metabolic engineering. Since beta amino acids are produced by living organisms, the metabolic pathways can be used as a primary source of building blocks, or even to synthetize fine chemicals. Taking advantage of metabolic routes is extremely advantageous since fermentation processes allow to cut the costs of production related to enzymes purification, reduce the number of steps and generally require raw starting materials from renewable feedstock.[18]

Back to the synthesis of beta amino acids, it is known that β-alanine is a crossing intermediate of several metabolic pathways.[19] For this reason, β-alanine is an optimal precursor for the synthesis of its derivatives.

Saccharomyces cerevisiae, the bread yeast, is an excellent example of versatile organism that has been massively employed in metabolic engineering.[20] Recently, Borodina et al.[21] have proved that it is possible to open a new metabolic route expressing aspartate-1-decarboxylase in the yeast. Moreover, the decarboxylation of aspartate induced in the modified strains is the end line of a

metabolic pathway that starts with pyruvate. Consequently, the modified strains of S. cerevisiae can be cultivated on glucose as the only carbon source.
Although this synthetic pathway for *de novo* biosynthesis of β-alanine has been engineered to its final conversion into 3-hydroxypropionic acid (3HP), the process could be easily modified for our purposes.

GOAL OF THE PROJECT:

The goal of this study proposal is to take advantage of the recent results achieved by enzymatic catalysis and use these techniques to synthetize (R)-3-aminoisobutyric acid extending the retro-synthetic pathway up to glycolysis. Pyruvate has been fixed as minimum target, although tracing the route back to glucose is the final objective. The second goal of this project is to optimize the last enzymatic step, specifically evaluating the employment of new enzymes that may allow a dynamic resolution of R-3-AiBA. Lastly, the sustainability of the whole process designed is considered the third major objective of this work.

APPROACH THAT WILL BE FOLLOWED:

At this juncture, it is necessary to clarify the choice of pyruvate as starting material. Generally, industrial scale productions that rely on glucose or its derivatives as a renewable feedstock tend to compete with the food supply chain. Nevertheless, the target molecule of this bio-enzymatic route is a drug-market-oriented product, therefore, the pyruvate's consumption will never reach the proportion of a food-fuel conflict.

The review of literature has shown that the tools needed to design a new enzymatic synthesis are available.

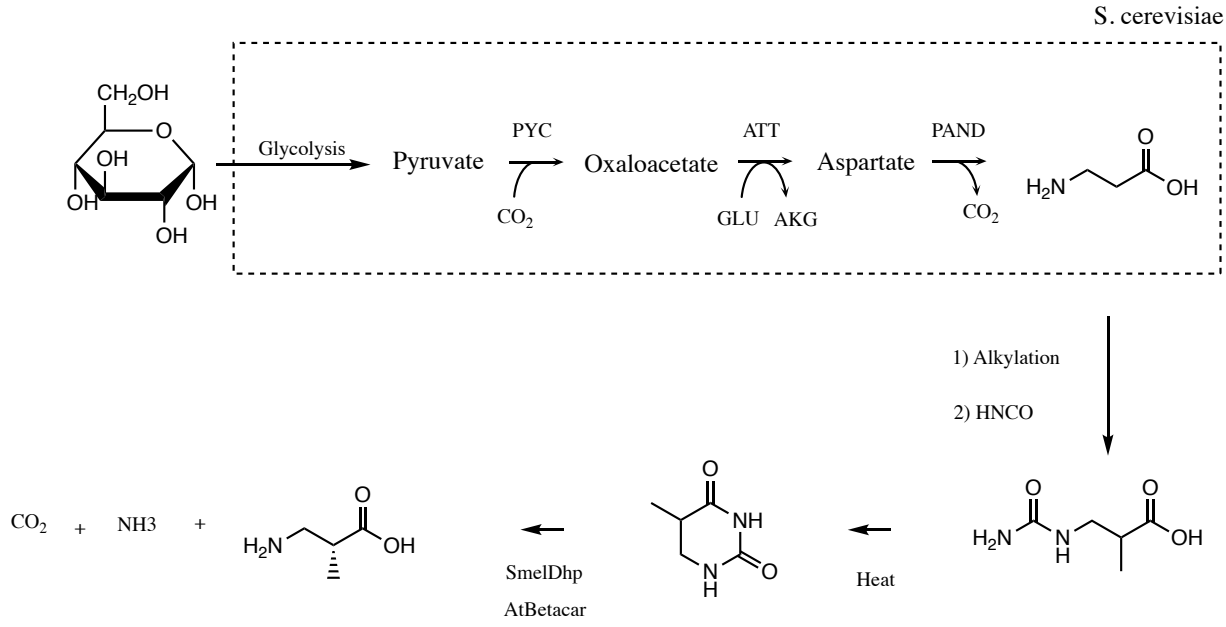

Figure 4: complete bio-enzymatic route from glucose to R-3-AiBA, dotted line circumscribes the metabolic step performed by S. cerevisiae strains.

However, during the first phase of the project, the consistency of the route must be proved. Specifically, the bio-conversion of glucose into β-alanine is the only step that does not have a direct reference in literature. For this reason, the first stage consists in adjusting the procedure described by Borodina et al. to accumulate β-alanine in the yeast strains.

As shown by White et al.[22], S. cerevisiae is not able to convert glucose into β-alanine, because lacking in aspartate-1-decarboxylase. The enzyme production can be induced in the yeast metabolism overexpressing TcPAND from T. castaneum or C. glutamicum. The new organism converts L-Aspartate into β-alanine by drawing the resources directly from the glycolysis pathway. In addition, the newly introduced metabolic pathway can be promoted by increasing the expression of other genes encoding pyruvate carboxylases (PYC1, PYC2) and aspartate aminotransferase (AAT2).
The mutant strains will be obtained using Easy-Clone plasmids as described by Jensen et al.[23], while the details for strain cultivation on mineral medium are provided by Borodina et al. The batch (dL scale) will be fed in time with dextrose in aerobic conditions and at the end of the process the concentration of metabolites will be measured using HPLC.

Before channeling efforts on the second part of mutation experiments, it would be useful to study the metabolic model through computational analysis using COBRA Toolbox (SBML format).[24]

Despite the outstanding results obtained by Borodina et al., the productivity of the newly engineered metabolic pathway (0.17 ± 0.0 $gL^{-1}h^{-1}$ yield of 3HP on glucose) can still be improved by far. An analysis of β-alanine metabolism, reveals that a relevant consumption of this metabolite is caused by the pantothenate and CoA metabolism.[25] It has been shown by Olzhausen et al.[26] that the enzyme that regulates the consumption of β-alanine downstream is pantothenate synthase, encoded in the gene YIL145C (PAN6). However, a pantothenic acid uptake deficiency will lead to a CoA limitation that will affect the yeast's growth, therefore, it seems counter-productive to silence this gene. On the other hand, wild type cerevisiae can overcome the path closure relying on exogenous pantothenate (Figure 5).[26] Thus, using a deletion mutant strain (JWH2 Δpan6*::*His3) supplied with pantothenic acid would maximize the accumulation of β-alanine without affecting the yeast growth in the fed-batch reactor. It is possible to silence the gene YIL145C using plasmid construction and site-directed mutagenesis, as described in detail by Olzhausen et al.

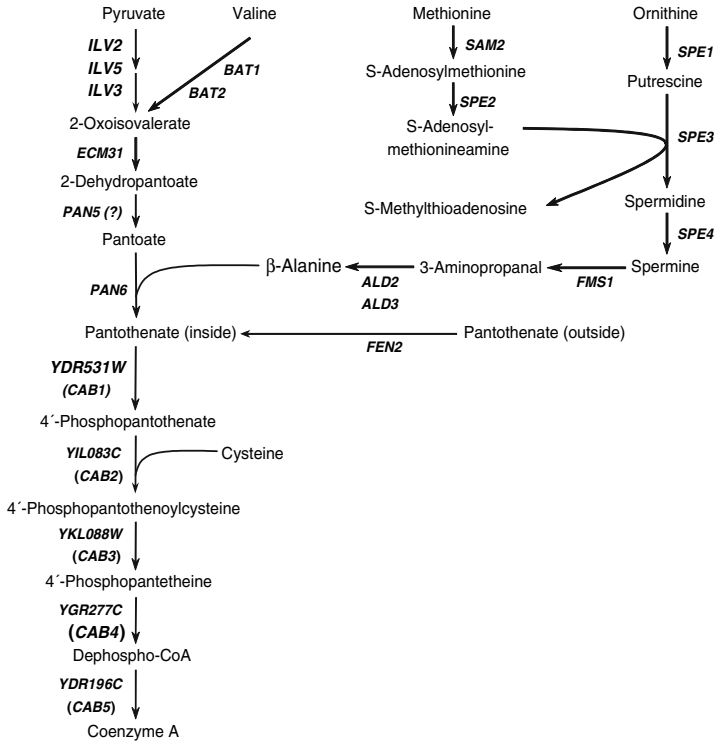

Figure 5: detail of S. cerevisiae metabolism, intersection between the pathways of β-alanine and Coenzyme A.[26]

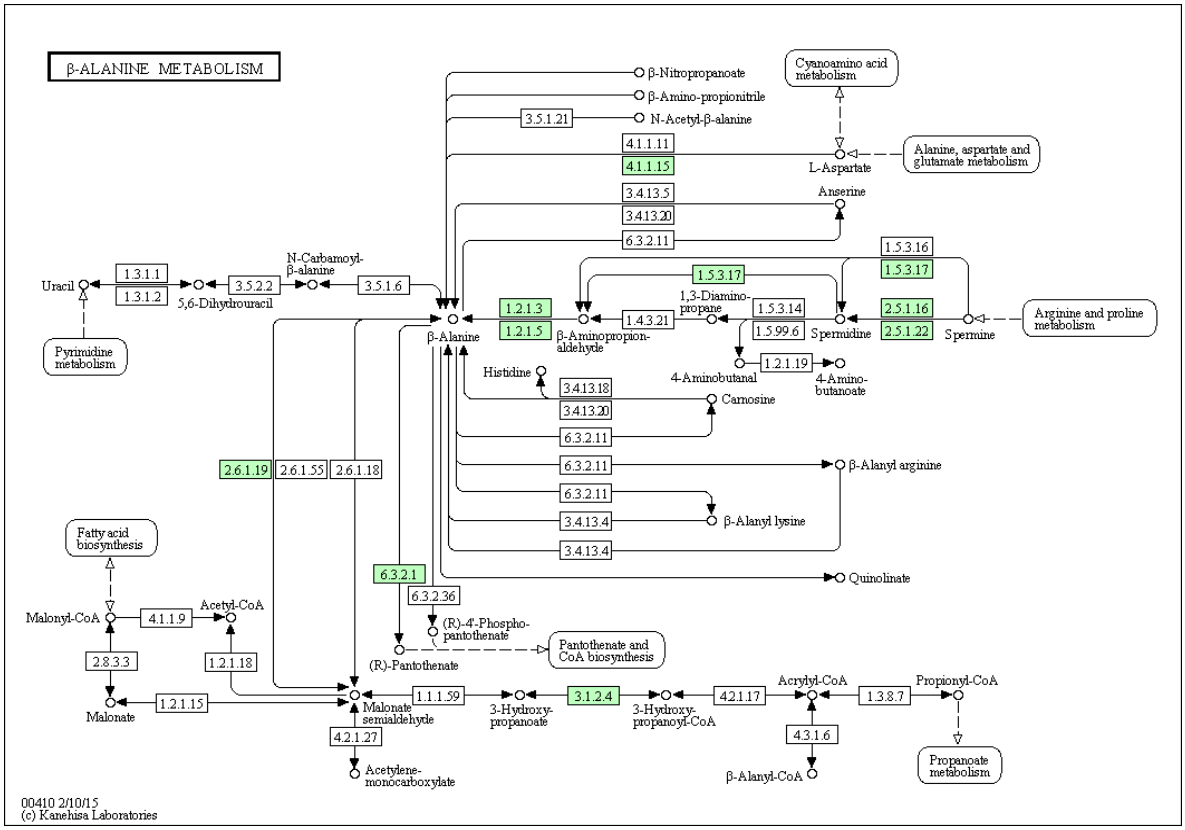

Figure 6: overview of β-alanine metabolism in Saccharomyces cerevisiae.[25]

Additional improvements to the metabolic route can be suggested on the basis of the diagram represented in Figure 6.[25] In fact, the metabolic map depicts two other main routes involved in the production of β-alanine. Modifying the catabolism of uracil seems a risky strategy, because of the side effects on cellular growth that may arise from a misbalanced metabolism of pyrimidines. By contrast, β-alanine accumulation could be encouraged by promoting arginine and proline metabolism via spermine. According to White et al., this route can be favored by overexpressing the gene FMS1 that encodes an amine oxidase, identified as the rate-limiting enzyme in this pathway. Again, the mutation can be induced using an overexpression vector as reported by White et al.

The next phase of the project will focus on the conversion of β-alanine into 3-AiBA and the subsequent resolution of the racemic mixture.

The first reaction of the chemical step is the alkylation of β-alanine. Unfortunately, this reaction cannot be performed in water as well as in other protic solvents, since generating the nucleophilic enolate requires the use of a strong base.[27] Therefore, the approach that will be followed consists in employing 2eq of LDA in 2-meTHF, a suitable green solvent for alkylations.[28] In addition, lithium chloride will be used to stabilize the enolate, while methyl iodide will be employed as alkylating agent. Furthermore, to avoid the competition with the N-metallation reaction[29], benzyl chloroformate will be employed to protect the amine function.

Subsequently, the condensation of 3-AiBA with isocyanic acid will produce β-ureidoisobutyric acid.[30] Cyclization of this acid can occur under various conditions: treatment with acetyl chloride, digestion with acetic anhydride or acqueous hydrocloridric acid or simply by heating.[31]

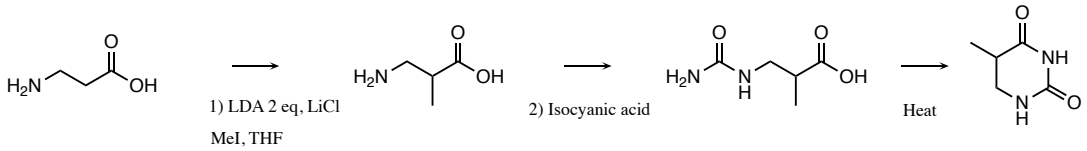

Figure 7: two step conversion of β-alanine into 5-METDHU.

A second issue that must be pointed out is the possibility of following a different retrosynthetic pathway to lead back to pyruvate (Figure 8). β-ureidoisobutyric acid can be obtained through Michael addition of urea on methacrylic acid.[1] Despite the lower yields reported for these reactions[30], α,β addiction introduces a considerable shortcut. Indeed, the α,β unsaturated acid can be directly synthetized from pyruvate by promoting the elimination of the alpha hydroxyl alcohol, produced after the nucleophilic addition of methyl lithium on the carbonyl.

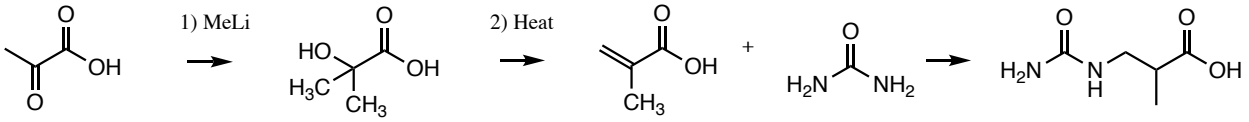

Figure 8: alternative route from pyruvate to β-ureidoisobutyric intermidiate via methacrylic acid.

Comparing the two strategies, the metabolic pathway appears more challenging, but at the same time more promising in terms of costs efficiency, sustainability and production capacity. Therefore, this study proposal will focus on the first strategy. However, the use of traditional chemical steps for producing β-ureidoisobutyric acid will not be completely discarded. In fact, if the metabolic route will prove to be unworkable, this method will step in as a back-up plan. Moreover, this second method represents a good term of reference to estimate the competitiveness of the metabolic route designed.

The last synthetic step consists in hydrolyzing 5-METDHU with SmelDhp and Atβcar using the tandem technique described by Martìnez-Gòmez. From previous studies[32], it is known that Atβcar preferably hydrolyzes the R enantiomer of N-carbamoylated-β^2-alanine derivatives, even though the enzyme is not sufficiently selective to run a kinetic resolution on its own. By contrast, the role of SmelDhp in the kinetic resolution has not been entirely clarified. In fact, the authors claimed that it was not possible to determine the enantioselectivity toward 5-METDHU by chiral HPLC. Therefore, different spectroscopic techniques will be applied in this project to follow the SmelDhp kinetics. Specifically, DNMR spectroscopy in combination with chiral co-solvents for aliquots quenching could be able to unravel the enzymatic mechanism and provide the kinetic parameters.

Once again, in order to produce the required enzymes, it will be necessary to resort to the expression of specific genes in E. Coli (DH5α and BL21 Gold) bacteria's cultures as described in detail by Martìnez-Gòmez et al.[1] The ideal conditions for running the hydrolysation, are reported by the authors.

In the last phase of the project, it will be tested whether it is possible to convert the kinetic resolution of 5-METDHU into a dynamic resolution of β-ureidoisobutyric acid using Atβcar and DcsC (Figure 9). Because of the enzyme specificity of DcsC (O-ureido-L-serine racemase), this step is considered a risky strategy. However, the advantage of employing a racemase is twofold: the enantioselective hydrolysis would maintain a higher ee allowing the full conversion into product; furthermore, the condensation step of β-ureidoisobutyric acid into 5-METDHU would be avoided.

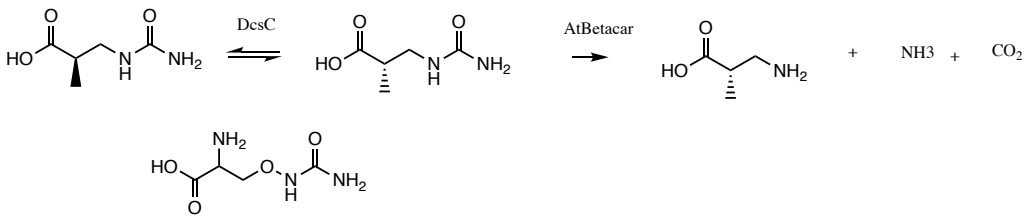

Figure 9: Dynamic resolution scheme, DcsC and Atβcarin used in tandem for the hydrolization of β-ureidoisobutyric acid. On the bottom, O-ureido-L-serine racemase, the natural substrate of DcsC.

However, it is difficult to find a suitable enzyme able to perform this task, because most of the racemases have a PLP-based active site that turned to be highly selective upon the natural substrate. This cofactor primarily interacts with the amine function located on the chiral center of alpha amino acids: therefore, its application on β-ureidoisobutyric is inconceivable.

Despite the complexity of the problem, it is still possible to identify proteins that satisfy these conditions. DcsC (O-ureido-L-serine racemase) is a PLP-independent enzyme that belongs to the cluster of proteins responsible for the biosynthesis of D-cycloserine.[33] This racemase has a thiolate-thiol pair in the active site capable to remove the acidic proton and subsequently re-protonate the alpha carbon, inverting the configuration of the substrate (Figure 10). In addition, the substantial structural similarity between O-ureido-L-serine and β-ureidoisobutyric acid (comparable chain length and steric features, the presence of carboxilate and ureido functions) might allow the recognition of the intermediate in the active site. Specifically, the carboxylate displaced charge should form a hydrogen bond with the protein amide backbone. This would provide the main binding, while the π system of the carbanion formed should hold the substrate in a rigid conformation through stereoelectronic interactions.[34]

Approaching the practical aspect, the procedure to isolate DcsC is described in detail by Dietrich et al. and consists in cloning mutant E. Coli obtained by plasmids introduction. After cell lysis, purification occurs through chromatographic separation and dialysis.

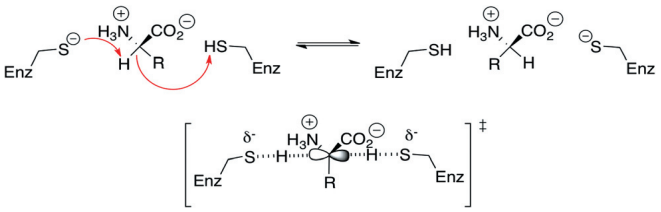

Figure 10: Mechanism of thiolate-thiol pair active site in O-ureido-L-serine racemase.[33]

Subsequently, kinetic studies will be carried out in this project to verify if the enzyme shows activity on β-ureidoisobutyric acid. The preliminary experiments will consist in comparing the progression curves of the kinetic resolutions. Therefore, a racemic mixture of β-ureidoisobutyric acid, previously synthetized, will be hydrolyzed by Atβcat in the presence of DcsC. Since Km values of DcsC have shown a substantially different affinity for D and L enantiomers of O-ureidoserine[33] ($Km_{(D\text{-}>L)}$ 17 mM, $Km_{(L\text{-}>D)}$ 110mM $L \rightarrow D$), the experiment will be repeated (mL scale) in this range of substrate concentration.
In case of success, the kinetic parameters will be determined to optimize the dynamic resolution. This could be done using dynamic NMR or circular dichroism spectroscopy or alternatively by chiral HPLC. Lastly, to identify the critical mechanistic steps for further implementations, pre-steady state analysis can be used to determine the extent of substate binding and to follow possible structural rearrangements.

The last part of the project will consist in determining the overall yield of the enzymatic route. The competitiveness of the process will be evaluated by comparing the metabolic and the traditional chemical pathway. The results will be justified on the base of cost analysis.

WORK PLAN:

1. First Year

- Engineering the expression vectors for s. cerevisiae
- Cloning and cultivating the mutant strains
- Computational analysis of metabolic pathways

2. Second Year

- Engineering the second set of expression vectors for s. cerevisiae mutant strains
- Cloning and cultivating the mutant strains optimized for metabolite overproduction
- Converting of β-alanine into 5-METDHU
- Testing the alternative route from pyruvate (via methacrylic acid)

3. Third Year

- Isolating and purifying the enzymes for kinetic resolutions (SmelDhp, Atβcar, DcsC)
- Performing kinetic resolution of R-3-AiBA
- Defining kinetic parameters of SmelDhp
- Testing dynamic resolution using DcsC
- Evaluation of the overall process based on costs analysis

ORGANIZATION:

The first phase of the project requires a specific equipment. The metabolic engineering experiments demand a feed batch reactor, easy clone plasmids kits and gene sequences described in detail in Figure 11. Furthermore, commercially available strains of S. cerevisiae and E. coli are requested.

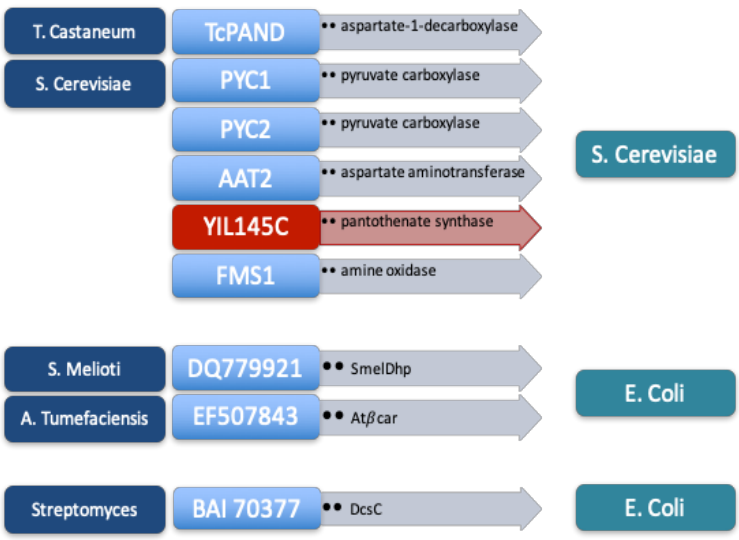

Figure 11: resume of the gene sequences required for the project. From left to right: organism of origin, gene sequence accession number (overexpressed in blue, silenced in red), enzyme encoded, hosting organism.

To analyze the metabolites, isolate enzymes and perform kinetics experiments, HPLC with UV detector is needed, possibly equipped with Aminex HPX-87H ion exclusion columns and Luna C_{18}, adjustable for Ni^{+} and Cobalt affinity chromatography. Pre-steady state kinetics require stopped-flow equipment.

The purification of SmelDhp and Atβcar requires FPLC (fast performance liquid chromatography) and Cobalt affinity chromatography.

INNOVATIVE ELEMENTS

1. Coupling metabolic engineering with enzymatic resolution is one of the merits of this project. Indeed, the retrosynthetic scheme presented has been newly designed. In case of success it will turn the biomimetic approach from a valuable tool into a complete synthetic process for the synthesis of beta amino acids.

2. The metabolic engineered pathway proposed has been specifically modelled for this project. In fact, despite the techniques have already been proved to be workable methods, their synergic combination described is an innovative strategy.

3. Lastly, to the best of my knowledge, the dynamic resolutions of a beta amino acids employing DcsC racemase has never been reported. This ambitious element has been introduced with the support of a set of kinetic experiments, such as the pre-steady-state analysis, purposely outlined to evaluate the enzyme compatibility.

EXPECTED RESULTS AND IMPACT:

The final goal of this work is to develop a process that has the necessary features to start a large-scale production. Successful fine tuning of the metabolic route would lead to a suitable process for an industrial application. Furthermore, positive results, especially regarding the dynamic resolution of β-ureidoisobutyric acid, would deliver a new valuable tool for asymmetric synthesis of β-alanine derivatives.

PITFALLS & BOTTLENECKS:

One of the major advantages of employing a metabolic route is that fermentation is carried out in milder conditions. However, this feature of the process is partially neutralized by the following alkylation step. Replacing this step using more sustainable chemistry would be an important achievement.

The second critical point regards the proposal of employing O-ureido-serine racemase for a dynamic resolution of β-ureidoisobutyric acid. The fact that the protonated alpha amine function eases the most thermodynamically unfavorable step in the natural substrate is a concern. Specifically, the deprotonation of the β-aminoacid could be impeded by the absence of this EWD group on the alpha carbon.

FURTHER WORK TOWARDS IMPLEMENTATION:

It has been already underlined that due to the enzyme specificity, it must be taken in account that the chances of employing DcsC without any kind of protein manipulation are, in the best hypothesis,

very optimistic. However, if this experiment will lead to a no go, it would be interesting to face the problem trough protein engineering studies.

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MIRMRTPSTL PFTKMHGAGN DFVVLDLRDG PDPSPELCRA LADRHKGVGC DLVLGIREPR
SARAVAAFDI WTADGSRSAQ CGNGARCVAA WAVRAGLARG PRFALDSPSG THEVDVLDAD
TFRVALAVPR FAPESIPLFG HDGEQDLYEA DLGDGTRVRF AAVSMGNPHA VIEVDDTATA
PVARVGRAVQ ASGLFLPTVN VGFARVESRD RVHLRVHEYG AGETLACGSG ACAAAAVLMR
RGRVDRNVSV VLPGGELRIS WPDDAADVLM TGPAAFVYEG TFLHASV
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Figure 12: Protein sequence of DcsC o-ureido-serine racemase. (The residues that play a role in the active site have been underlined: thiolate-thiol pair 227,81; substrate binding 20,167, 200; hydrophobic pocket used for modulate pka of cysteine residues 169, 218; residues responsible for dimerization 278).[35]

The protein sequence of O-ureido-serine racemase is known, and so are the residues that play a role in the active site tuning the basicity of the thiolate (Figure 12).[35] Modifying the residues in thiolate's environment may balance the lack of the electron withdrawing group in the substrate.

Another strategy that may be taken in account is derivatization. In this case, it will be challenging to find a removable substituent able to enhance the acidity of the alpha-proton, without compromising the substrate recognition.

Lastly, it has been reported[33] that upon the substrate binding a structural reorganization provides the correct orientation, locking the molecule in the active site. Monitoring the structural changes induced by the mutant studies would be a useful tool to understand how to improve the enzyme promiscuity.

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