BRIDGE

Discovery

Project description

Rebecca Buller, Rémy Bruggmann & Vincent Perreten

Microbial Epimerases: A Toolbox for the Synthesis of Novel Peptide-Based Drugs

FNSNF
Swiss National Science Foundation

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Swiss Confederation

Federal Department of Economic Affairs,
Education and Research EAER
Commission for Technology and Innovation CTI
Innovation Promotion Agency

1. Summary

25,000 deaths in the European Union alone are estimated to be caused by drug-resistant bacteria every year. In addition to the increased suffering it causes, antimicrobial resistance has huge economic implications. Multidrug-resistant bacteria have been connected to an economic loss of more than US $ 4 billion each year,[1] some predictions even estimate global costs that range between US$ 2.1 trillion and US$ 124.5 trillion by 2050 in the absence of any progress tackling the challenge.[2] These numbers justify a large financial investment to push innovation in research and development for the discovery of antimicrobial medicines, vaccines and diagnostic tools.

Modified short peptide antibiotics are recognized as promising candidates to address antimicrobial resistance.[3] Antimicrobial peptides are potent, broad-spectrum antibiotics functioning via a combination of different antimicrobial mechanisms.[4] However, a major challenge for peptide therapeutics in general is their fast degradation, usually by proteases, which can be greatly slowed down via introduction of D-amino acids.[5-6] Furthermore, the introduction of D-amino acids offers a means to expand the compound diversity within this class of antimicrobial drugs.

Biosynthesis of D-amino acid-containing natural products has long been thought to be mostly carried out via non-ribosomal peptide synthethases. Recently, the enzyme superfamily of radical SAM epimerases has been recognized to be capable of converting a “normal”, all L-peptide into its D-amino acid containing counterpart.[7] This way, multiple epimerizations can be produced by a single enzyme, avoiding utilization of multiple peptide synthethases.

To find new rSAM epimerases we will screen metagenomic databases and repositories that comprise diverse data from many different sources. From the identified genes we will build an epimerase toolbox by recombinantly expressing and engineering novel members of this superfamily.[8-9] Special priority will be given to the development of *in vitro* assays that allow combining a set of different epimerases with a library of native or synthetic peptide substrates in a combinatorial fashion: In this way we can match the newly identified epimerases with many different natural and synthetic peptides thus multiplying the accessible diversity.

The Institute of Veterinary Bacteriology, University of Bern, will test the antibiotic activity by the measurement of the minimal inhibitory concentration (MIC) of our microbiome-derived peptide library against antibiotic susceptible and multidrug resistant pathogens of the ESKAPE group which represent the most challenging bacteria for therapy in healthcare settings.[10] Once a peptide with improved properties has been identified through our drug discovery platform, it can be synthesized via chemical means for the industrial scale.

Overall, our project paves the way to translate the hidden biocatalytic potential of the microbiome into an innovative platform for drug discovery with the focus on novel antibiotics. It bridges the gap between academic research and industrial development by providing a new drug discovery and synthesis tool for the pharmaceutical industry thus underscoring Switzerland’s importance as an R&D innovation hub and production site.

2. Project description

2.1 Current state of research in the field

Multidrug-resistant bacteria are nowadays challenging therapy and even the latest generations of antibiotics became obsolete. February 2017, the WHO has published a list of antibiotic-resistant priority pathogens for which new antibiotics are urgently needed. This list should promote research and development of new antibiotics.[11] Antimicrobial peptides represent one of the alternatives to classic antibiotic molecules. Several compounds from different origins including insects, arachnids, animals, milk, animals and humans have already been shown to have antimicrobial activities against different types of bacteria .[12-14]

However, natural antimicrobial peptides typically have very short half-lives (*e.g.* in the range of minutes) resulting from extensive proteolysis in blood, kidneys, or liver.[15-16] The high first-pass extraction is caused by enzymatic- and pH-mediated hydrolysis in the gastrointestinal (GI) tract and liver.[17] As a consequence, successful peptidic drugs need to be modified to improve their absorption, distribution, metabolism, and excretion (ADME) properties. One way to achieve improved plasma-half life is the introduction of D-amino acids: In several examples the modification of peptidic drugs by the introduction of D-amino acids has been shown to successfully improve stability against enzymatic degradation.[6, 15, 18-19] However, the synthesis of modified peptides is expensive and drives the need for a more flexible approach to discovery within the pharmaceutical industry such as the establishment of molecular biology approaches.[20] In this way, peptide synthesis and modification can be carried out *in situ* simultaneously allowing for the generation of a diverse set of novel peptide drug leads.

Biosynthesis of D-amino acid-containing natural products has long been assumed to be products of non-ribosomal peptide synthetases (NRPSs), a large multi-domain protein complex that can generate peptides with unnatural residues (including D-amino acids).[21] Recently, an exciting discovery was made that the enzyme superfamily of radical SAM (*S*-adenyl methionine) epimerases is able to convert a “normal” all L-peptide into its D-amino acid containing counterpart.[7, 22-23] With these enzymes, proteins/peptides containing multiple non-canonical D-amino acids can be obtained without the use of the large and complex NRPS machinery.

Notable examples of the epimerases include PoyD from cyanobacterium Oscillatoria sp. PCC6506, a bacterium symbiont of the marine sponge *Theonella swinhoei* and YydG from *Bacillus subtilis*.[7, 23] These enzymes have been shown to catalyze up to 18 epimerization reactions of multiple natural amino acids on single peptide/protein substrates ranging from 2 to 13 kDa in size, exhibiting significant promiscuity while retaining high stereo-selectivity (i.e., exclusively L- to D-conversion). Along with other post-translational modifications (PTMs) including methylation and hydroxylation, epimerization play an essential role in maturation of bioactive natural products.[7]

The overall mechanism of product formation appears to follow 1) ribosomal synthesis of a precursor peptide/protein, 2) PTMs of the precursor peptide/protein and 3) proteolysis to yield mature peptides (Figure 1).[24] First, a precursor protein is produced by the ribosomal machinery, which consists of a core peptide (resulting in the final product) fused to a leader peptide on the N terminus. After translation, specific amino acid residues

on the core peptide are epimerized (along with other PTMs if applicable). This modified core peptide is then cleaved by proteases, usually concomitant with export to the outside of the cell via peptidase-containing ABC transporters.[25] Notably, the proteins responsible for the PMTs, including epimerization, protease cleavage, and transport are encoded within the same operon.

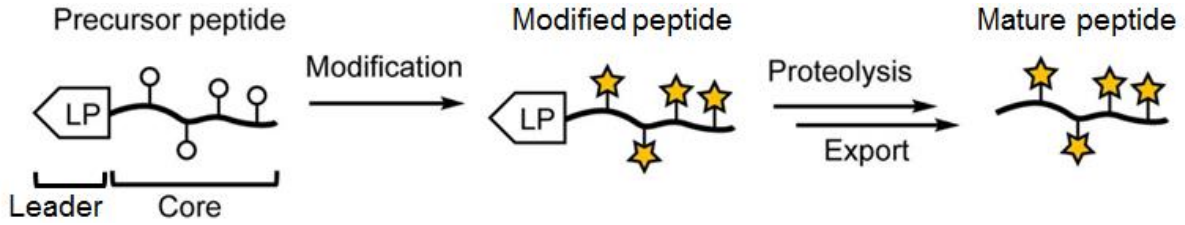

Figure 1. Schematic representation of post-translationally modified peptide (RIPP).

The active site of the epimerases consists of highly conserved (at least one) [4Fe-4S] cluster and S-adenosyl-methionine (SAM) (Figure 2). The epimerase reaction proceeds via electron transfer from the iron-sulfur cluster to SAM (Figure 2), followed by generation of a 5'-deoxyadenosyl radical (5'-dA•) which is then able to abstract the α-hydrogen from an amino acid on the peptide substrate. Subsequent H atom abstraction from the opposite side of the enzyme active site (often a cys residue) yields the amino acid residue with inverted stereochemistry at the α-position. Besides epimerization reaction, recent years have witnessed the emergence of novel radical-based chemical transformations catalyzed by radical SAM enzyme superfamily, most of which are unprecedented in biology.

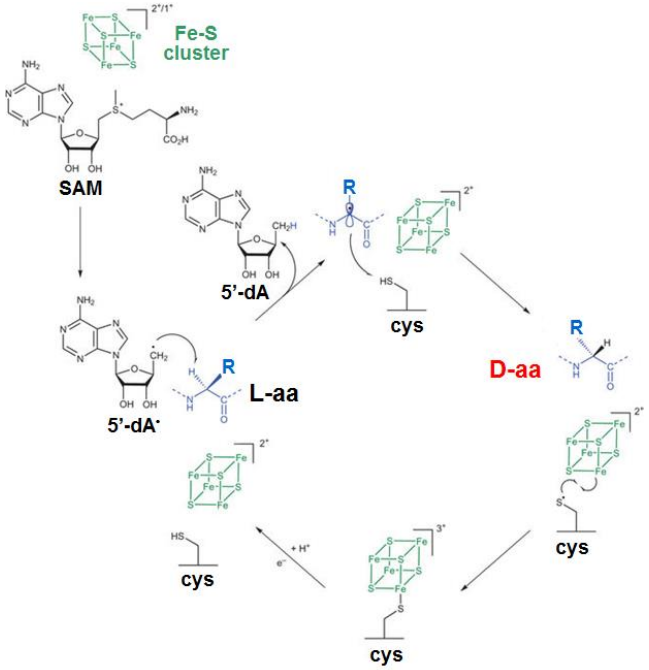

Figure 2. Active site and proposed reaction mechanism of rSAM epimerase.

Several epimerases from distinct organisms have been successfully cloned and heterologously expressed in *E. coli*. Importantly, these epimerases have been shown to be active *in vivo*; co-expression of the epimerases and their natural substrate proteins in *E. coli* resulted in quantitative transformation of the substrates to the products with desired stereoconfiguration.[26] Furthermore, the N-terminus leader peptide sequence has been suggested to affect the overall yield as well as stereoconfiguration of peptide product. In contrast to the *in vivo* epimerization, examples of *in vitro* epimerization are scarce. Most recently, Berteau *et al.* demonstrated that recombinant, anaerobically reconstituted epimerases YydG and PoyD are able to catalyze epimerization of its natural peptide substrates into the desired product with high yield *in vitro*.[23, 27] Significantly, these enzyme

can efficiently modify both full-length and truncated (a core peptide alone) substrates to produce the desired bioactive peptide product, demonstrating a greater degree of flexibility in substrate structure.

The amount of data generated in the different fields of biology has increased enormously in the last decade and presently, no end is foreseeable.[28] Most of these data is attributed to sequence data produced by next generation sequencing (NGS) and the amount generated in 2025 is estimated to one zetta-base/year (10E21 bases). Already now, the sequence data collected by researchers around the globe is very large and data sets are often not analyzed in depth but rather partially with only a particular research question in mind. This leaves a wealth of publicly available data which has yet not been comprehensively analyzed. Consequently, the computational mining of genomes has become a powerful tool in the discovery of novel natural products as well as biosynthetic genes.[29] Due to the relatively conserved genomic arrangement of many of the genes involved in RIPPs, novel class of epimerases can be systematically identified. The literature-known epimerases[22] are only the tip of the iceberg and many more are expected to be present in Nature. The above described revolution of next generation sequencing methods together with a variety of genome mining methods and tools will thus guide our discovery and characterization of new rSAM epimerases with unique catalytic properties (i.e. substrate specificity, regioselectivity).

2.2 Own achievements in the field

"leader less" epimerization of native and unnatural peptides (ZHAW)

In our proof-of-concept study, we focused on an rSAM epimerase OspD from cyanobacterium Oscillatoria sp. PCC6506. Consistent with the previous report by Piel *et al.*[22] co-expression of OspD and the known substrate OspA in *E coli* resulted in conversion of OspA substrate to the desired epimerized product.

Implementation of *in vitro* epimerization protocol is prerequisite for establishment of our epimerase library platform. However, there are only a few precedents of *in vitro* epimerization by rSAM epimerases in literature to date.[8, 27] In an attempt to carry out *in vitro* epimerization reactions, we expressed OspA and OspD separately. The anaerobically reconstituted OspD showed the formation of an iron-sulfer cluster as evidenced by the visible absorbance at 410 nm. To examine the activity of OspD, we assayed the reconstituted enzyme with purified peptide substrate OspA. The enzyme fully converted the substrate to its epimerized product in 3h (Figure 3a). Importantly, the MS/MS analysis of the peptide epimerized in a D_2O buffer showed incorporation of deuterium atoms on the amino acid residues which were previously identified as epimerization points in *in vivo* epimerization experiment (Figure 3b).[22]

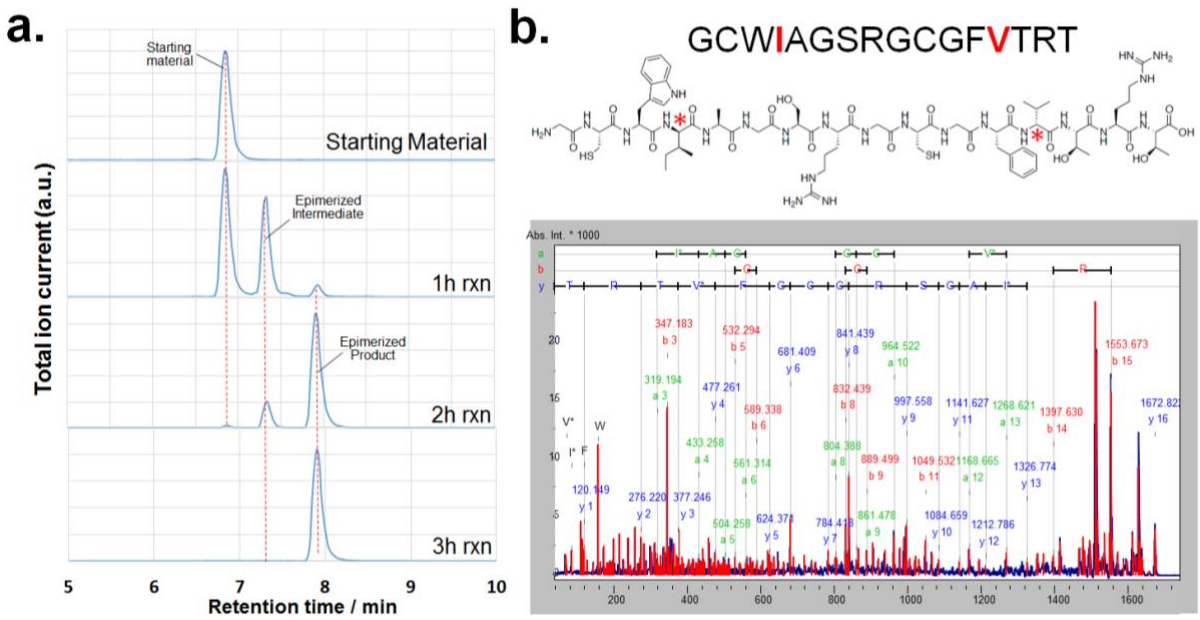

Figure 3. *in vitro* epimerization of OspA with OspD epimerase. a) time course of epimerization analysed by LC-MS, b) sequence and structure of OspA core with epimerized amino acids highlighted in red, and MS/MS analysis of epimerized OspA core.

Encouraged by those results, we then carried out "leader-free" epimerization in which enzymes directly epimerize the substrate peptide without the need of leader peptides/proteins (Figure 4a). Toward this end, we obtained 16-amino-acid-long OspA core peptide and incubated it with the reconstituted OspD epimerase in the presence of the reductants and SAM cofactor. Significantly, the OspA core peptide was transformed into the epimerized products even in the absence of the leader peptide (Figure 4b). Furthermore, when this reaction was performed in D_2O-enriched buffer solution, incorporation of deuterium atoms to the substrate peptide was observed, consistent with the proposed mechanisms for this class of enzymes. Our MS/MS analysis of the peptide identified the deuterated amino acids, indicating that those amino acids were epimerized (converted to L → D configuration).

With the epimerized peptide in hand, we subsequently perfromed a protease resistance assay using a conventional protease chymotrypsin. The native (all-L) and epimerized OspA core peptides were incubated in the presence of 2 wt% of chymotrypsin from bovine pancreas at 37°C. A 370-fold increase in protease resistance was observe for the epimerized OspA peptide (Figure 4c), highlighting the stability of D-amino acid containing peptide against proteases.

Having demonstrated that the epimerized peptide exhibits significant protease resistance, we were encouraged to investigate the substrate scope of OspD. We have tested several antimicrobial peptides for epimerization by the OspD epimerase. Interestingly, an antimicrobial peptide lactoferricin, which has a completely different structure compared to that of OspA core, was epimerized by OspD as evidenced by the retention time shift observed in the LC-MS analysis (Figure 5). We are currently in the process of characterizing the property of the peptides (i.e., epimerization point(s), protease resistance, antimicrobial activity).

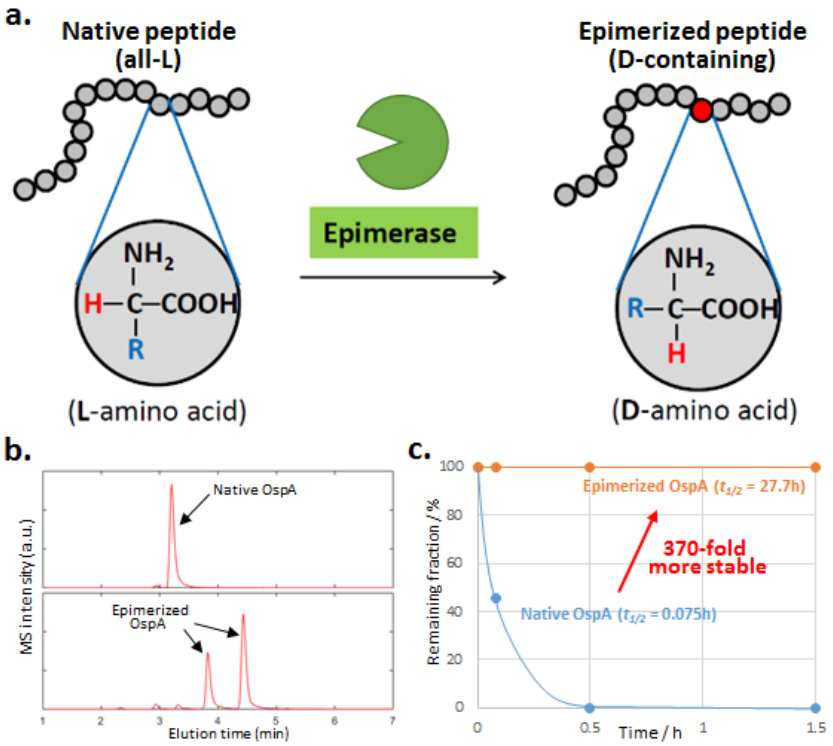

Figure 4. "Leader less" epimerization of native and non-native(antimicrobial) peptide substrates by OspD erimerase. a) Schematic representation of "leader-free" epimerization, b) direct epimerization of OspA core, and c) protease digestion of native and epimerized OspA core.

Taken together, our preliminary results show that the reconstituted OspD can act on free peptide substrates (both native and non-native peptide substrates), demonstrating the great potential of this class of enzymes for the discovery and synthesis of novel peptide-based drugs. Importantly, we have implemented all the nessesary infrastructures for peptide synthesis, epimerization, thorough characterization (structural characterization and protease assay) in-house as demonstrated above.

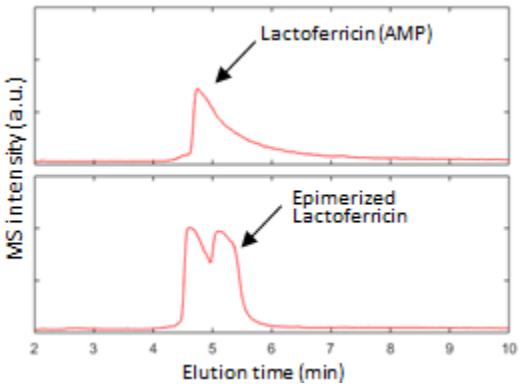

Figure 5. Epimerization of an antimicrobial peptide Lactoferricin by OspD.

Bioinformatic sourcing of the enzyme and substrate diversity in microbiomes (ZHAW and Uni Bern)

Since the discovery of the first rSAM epimerase, PoyD,[7] several of its orthologs have been identified using the power of bioinformatics. More than 10 enzymes have been described as potential rSAM epimerases, and several of these enzymes were biochemically characterized.[22] Despite the success in identifying and characterizing orthologs within the PoyD family, orthologs of YydG have not been characterized.[23] The two

families of epimerases (i.e., PoyD family and YydG family) appear to share no sequence homology, except for the CXXXCXXC motif responsible for the formation of the Fe-S cluster.

In our proof-of-concept study, we searched for YydG orthologs using NCBI Blast. A phylogenetic tree of the identified enzymes was generated and three enzymes from different organisms were chosen for further analysis of the surrounding region. Subsequently, sequences within ~20-kb upsteam and downstream from the identified enzyme were extracted from the respective genome and aligned with the previously described YydG enzyme (Figure 6).

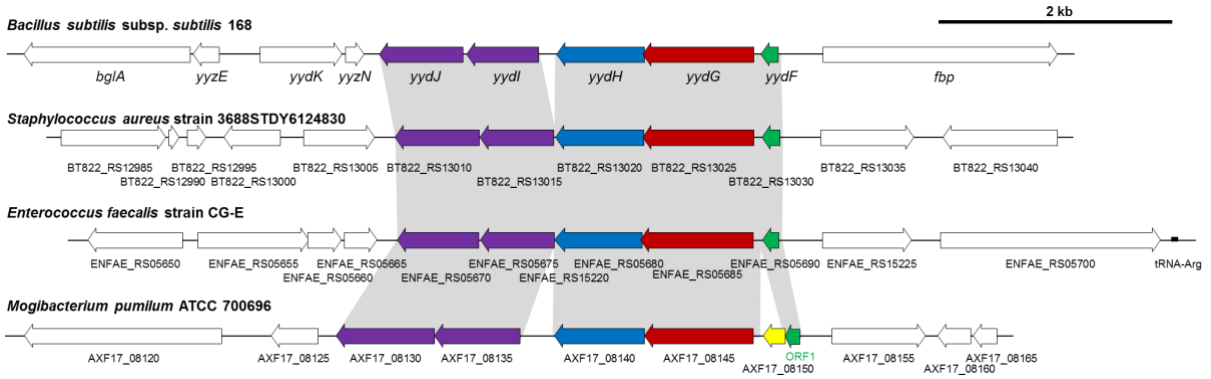

Figure 6: Region maps around the *yydG* gene in *B. subtilis* and orthologous regions in *S. aureus, E. faecalis,* and *M. pumilum*. The same color means similar function. Red: epimerase, green: prepeptide, blue: peptidase, purple: ABC transporter units.

The arrangement of the gene cluster appears to be similar among the four different organisms. Importantly, the potential peptide substrates were identified. Based on the sequence alignment and secondary structure analysis of these substrate peptides, the identified epimerases are predicted to possess slightly different substrate specificity. Biochemical characterization of these enzyems is currently underway in Buller group at ZHAW.

Competence Center for Biocatalysis (CCBIO), ZHAW

The CCBIO has a proven track record of expressing and characterizing newly identified enzymes as highlighted by the recently developed ene reductase library consisting of > 20 recombinantly expressed proteins. Additionally, CCBIO can build on its extensive knowledge in protein engineering to tackle the challenge of optimizing the selected rSAM epimerase systems.[9, 30-33] Over the last few years, the necessary laboratory infrastructure dedicated to the proposed project has been set up, including an anaerobic workstation (Bactron Shellab) and LC-MS (Agilent LC/MSD) system for the identification of epimerized peptides. Furthermore, we have full access to in-house state-of-the-art infrastructures, including MALDI-TOF-TOF MS/MS and LC-MS/MS systems to fully characterize the physico-chemical property of the resulting epimerized peptides (i.e., identification of epimerization points).

Rebecca Buller (née *Blomberg*), the head of the Competence Center for Biocatalysis and the project manager of the here-proposed research project, has over ten years of experience in biocatalysis and protein engineering [9,

[30-33] of which she worked five years in the Flavour & Fragrance industry as laboratory head (biocatalysis) and project manager.[34] Additionally, a well experienced scientist in the field of biocatalysis, Dr. Takahiro Hayashi [35-36], will also be working on the project. Dr. Hayashi has extensive working knowledge of anaerobic handling of metalloproteins including iron-sulfur proteins and non-heme iron enzymes.[37-38]

Interfaculty Bioinformatics Group, University of Bern

Rémy Bruggmann is the head of the Interfaculty Bioinformatics Unit and a group leader of the Swiss Institute of Bioinformatics. He has been working with next generation sequencing (NGS) data since a decade and has analyzed data from all NGS as well as 3rd generation technologies. He has a track record in NGS based publications and in particular in genome sequencing as well as genome annotation (three Nature publications and one Nature genetics publication), metagenomics of viruses in cattle and bacterial genomics. He and his team developed a state-of-the-art bioinformatics pipeline to detect novel viruses in animals with viral infections of unknown etiology. Another focus of his research is the sequencing and assembly of genomes as well as metagenomes. For instance, more than 600 bacterial genomes were sequenced and analyzed in his group. To cope with these vast data, he has established a unique high performance compute infrastructure at the University of Bern.

Institute of Veterinary Bacteriology, University of Bern

Vincent Perreten is Professor and Head of Bacterial Molecular Epidemiology and Infectiology at the Institute of Veterinary Bacteriology, Vetsuisse Faculty of the University of Bern, Switzerland. He is expert in the field of antibiotic resistance studying transmission of zoonotic bacteria and their genetic elements between animal and humans. He has expertise in antibiotic resistance determination and molecular characterization of antibiotic resistance mechanisms, and in the application of novel techniques in bacteriology, such as antimicrobial testing of fastidious microorganisms, microarrays and whole genome sequencing. He has recently contributed to a study evaluating the antimicrobial activities of peptides.[14] He has published more than 100 publications in the field of antibiotic resistance.

2.3 Scientific Contents

One of the world's most pressing problems is the lack of new potent antibiotics.[39-41] In order to address this challenge, we plan to transfer the untapped information in Nature's microbiome into the production of pharmaceutically active compounds. We will focus on the enzyme family of radical SAM epimerases which has been recently discovered in the marine microbiome but has by now also been identified in the human microbiome and several bacterial species.[8, 42] As described above, rSAM epimerases can post-translationally modify peptides by epimerizing individual amino acids from the L to the D configuration and can thus aid in the discovery of innovative peptide-based drugs.

The collaboration between the Competence Center of Biocatalysis (ZHAW), the Bioinformatics Center (University of Bern) and Institute of Veterinary Bacteriology (University of Bern) gives us the tools to harness the

catalytic power of epimerases for practical applications: We will build an epimerase toolbox by recombinantly expressing and engineering bioinformatically identified members of this superfamily. Special priority will be given to the development of *in vitro* assays that allow combining a set of different epimerases with a library of native or synthetic peptide substrates in a combinatorial fashion. The resulting library of modified peptides will be tested by investigating the resistance against protease degradation as well as minimal inhibitory concentration (MIC) of our microbiome-derived peptide library against pathogens of the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) using both, antibiotic susceptible and multidrug resistant strains.

Bioinformatic sourcing of the enzyme and substrate diversity in microbiomes

To expand our epimerase search bexond the taxonomic ID2 (Bacteria) (see section 2.2), we will screen publicly available metagenomics databases among others various repositories such as TARA Oceans (http://ocean-microbiome.embl.de), EBI Metagenomics (https://www.ebi.ac.uk/metagenomics/), NIH Human Microbiome project (http://hmpdacc.org), Sorcerer II (http://www.jcvi.org/cms/research/projects/gos/overview/) and SCOPE/C-MORE (http://cmore.soest.hawaii.edu/datasearch/data.php). These databanks comprise diverse data from many different sources like ocean sampling expeditions, human microbiomes, soil, grassland, freshwater and others. We will use data from whole genome sequencing, whole transcriptome sequencing and assembled genome data for our purpose.

Initially, we will focus on the metagenome assembly data sets and screen for rSAM epimerases both on nucleotide (DNA, RNA) and protein level (translated and annotated). The advantage of using assembled data (e.g. TARA Oceans) is that after identification of an rSAM epimerase, the gene(s) of the natural peptide substrate can potentially also be found as they are typically on the same operon/contig.[24] Subsequently, we will also screen raw unassembled data for novel rSAM epimerases and target peptides. Because the raw sequences are relatively short (Illumina data: 76-300bp and 454 data up to 500/600bp) we will extend the initial sequences of the partial epimerase using a De Bruijn/k-mer local assembly approach.[43-44] For the local reassembly, we will limit the sequence data to the smallest unit available which ideally is a single sampling point reducing the assembly complexity enormously and hence the necessary compute resources. We will optimize and automatize these steps to efficiently screen the metagenomics data repositories available at the time of the project start.

We have already retrieved a large part of the required data and stored it on our high performance compute infrastructure which consists of 1 Petabyte of fast storage and a cluster of 600 CPU-cores. In case these compute resources are not sufficient, we have full access to the compute cluster of the University of Bern which has ~4500 CPU-cores and to the clusters of Vital-IT (https://www.vital-it.ch/) which have a total of ~4000 CPU cores.

All novel epimerases will be classified according to sequence similarities (amino acid and nucleotide level) and assign them to the sampling source and – if possible – to the bacteria species. In addition, an in depth

in silico analysis of the potential epimerase target proteins will be performed to unravel possible common features of these proteins. Preliminary results already revealed a potential new motif CXXGXXXNXXXXXXXXXP downstream of the CXXXCXXC (Fe-S Cluster of active site) which will be used to more specifically screen novel epimerases. Both, the classification of the epimerases and the analysis of the targets, will be used to prioritize candidates for the *in vitro* epimerization experiments and allow us to better understand the enzymes' role in Nature.

Transfer of rSAM epimerases from microbiomes to the laboratory

Cloning and functional expression of the novel epimerases and their target peptides will constitute the base for the enzyme and substrate libraries used as a tool for drug discovery and synthesis. Putative epimerases genes and their peptide substrates discovered via the newly developed bioinformatic algorithms are amplified or chemically synthesized, depending on the availability of genomic DNA. Activity data generated from the biocatalytic characterization of the novel epimerases will be fed back to the bioinformatics team. This way, phylogenetic studies to discover relatives of the most-suited epimerases can be undertaken which will subsequently be incorporated in the discovery platform (Figure 7).

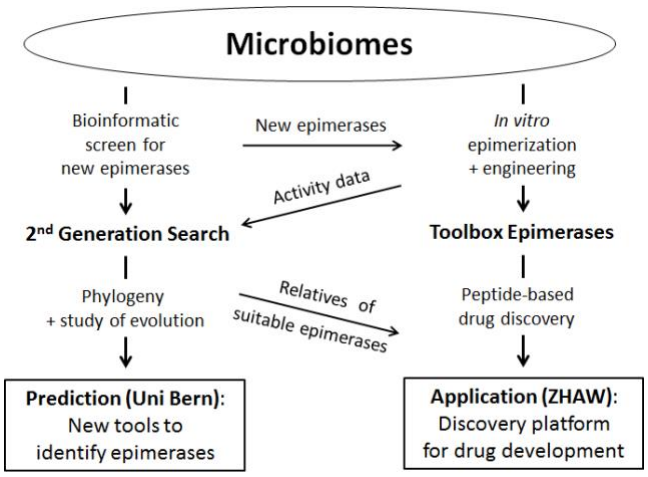

Figure 7. Transfer of rSAM epimerases from microbiomes to the laboratory.

***In vitro* epimerization and characterization of rSAM epimerases**

In parallel to the bioinformatics-driven research, we will optimize the already discovered peptide/epimerase pairs, i.e., OspA/OspD and YydF/YydG system[22] to meet the properties required for application. These requirements include the engineering of substrate and enzyme toward better applicability (see "Enzyme and substrate engineering for enhanced applicability").

Epimerizations were typically achieved via the co-expression of the epimerase with the full-length peptide substrate.[22] As a consequence, any biocatalytic approach requires that genes are introduced into the same host organism and epimerized products are harvested via cell lysis and purification. In contrast, *in vitro* epimerization enables to separate expression of epimerases and peptide substrates, followed by biocatalysis in

a subsequent step. Via the separation of expression and biocatalysis, multiple different epimerases can act on a library of both biologically and chemically synthesized peptide substrates in a combinatorial fashion thus multiplying the number of accessible peptide structures (Figure 8).

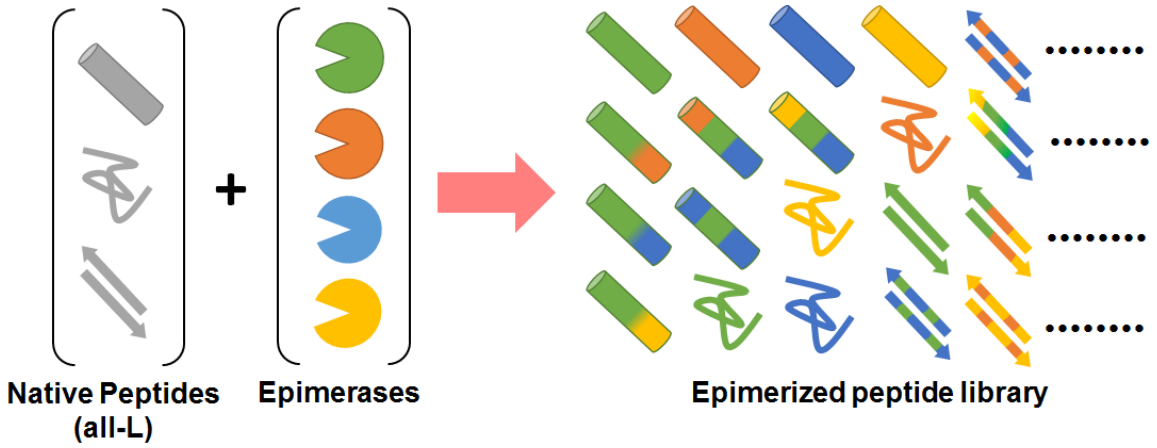

Figure 8. Combinatorial epimerization of peptide substrate (antimicrobial peptide) by epimerases, generating a large diversity of peptide library.

Over the last year, a few *in vitro* epimerizations via an rSAM epimerase were reported: *B.subtilis* YydG and Oscillatoria sp. PoyD enzymes were overexpressed in *E.coli* and anaerobically reconstituted with Fe-S clusters. [8, 27] As demonstrated in "Own achievement", we recombinantly expressed rSAM epimerase OspD for *in vitro* epimerizations. The reconstituted OspD can epimerize free peptide substrates (both native and non-native peptide substrates) *in vitro*. This demonstrates the great potential of this class of enzymes for the discovery of novel peptide-based drugs.

In vitro biocatalysis with purified epimerases requires SAM as a cofactor in stoichiometric amounts. Once general assay conditions are established, they can be further refined by introducing a re-cycling system for the expensive molecule. Methods for regenerating SAM from methionine and ATP are available and will be combined with the epimerization system.[45]

With respect to the mechanistic understanding of epimerases, *in vitro* epimerizations allow to monitor the progress of the reaction (for example enzyme kinetics, saturation concentrations, total turnover number, etc) as well as testing reaction conditions that are not suitable for the host organism but common in an industrial context (non-physiological pH, organic solvent, temperature). Investigations with purified epimerases allow for ultimate control of the biocatalytic reaction.

Enzyme engineering for enhanced applicability

Although epimerization of unnatural peptide substrates was demonstrated, transformations of unnatural substrates seem substantially less efficient than their natural counterparts, requiring optimization through a combination of *in silico* enzyme design and laboratory evolution.[9] State-of-the-art protein

engineering methodologies[9] will be complemented by computer-driven approaches, including the use of algorithms for homology modeling[46] and protein stabilization.[47] To this end, we will utilize the award-winning Rosetta-based software of newly founded start-up company Cyrus Biotechnology in whose beta–testing we are involved. In laboratory evolution, efficiencies rivaling those of natural enzymes can be achieved by recursive cycles of mutagenesis and screening (i.e., directed evolution).[48-49] The ideal strategy for gene diversification will probably involve a combination of randomization methods. Structure guided mutagenesis of residues that line the epimerase active site may be most effective for achieving substantial jumps in activity. Due to the nature of the reaction and the substrate/product pair, screening very large libraries of enzymes is unrealistic. We will therefore employ reduced library design by constructing restricted libraries (e.g. mixture of NDT, VHG and TGG which encode 22 codons for all 20 amino acids in equal frequency and NDT codon which encodes 12 amino acids of different physicochemical properties without encoding stop codons) to minimize the number of variants to be screened while maintaining the diversity of the constructed libraries. Identified beneficial mutations will be combined and used for a starting point for next round of evolution cycle.[50]

In a parallel enzyme engineering approach, we will optimize protein characteristics such as protein expression, solvent stability and thermostability for the best performing epimerase from our biocatalyst toolbox and improve its catalytic properties for a model antimicrobial peptide. Enhanced stability of enzymes will be achieved via multiple means: a) “conventional” addition of protein tags,[51] b) computationally derived mutations.[47] The results of the combined engineering approaches will allow us to elucidate general engineering principles for rSAM epimerases and thus accelerate the development of our microbiome based drug discovery platform. Additionally, our engineering insights will likely be transferable to other enzyme classes involved in the synthesis of RIPPs and thus boost innovation in this emerging field.

Application of epimerases to modify existing peptide drug leads

Antimicrobial peptides (AMPs) are potent, broad-spectrum antibiotics functioning via a combination of different antimicrobial mechanisms.[4] However, a major challenge for peptide therapeutics in general is their fast degradation, usually by proteases, which can be greatly slowed down via introduction of D-amino acids.[5-6, 18] Thus, to enhance their potency and pharmacokinetic properties, we will submit several model AMPs to epimerization. With their length of ca. 12-50 amino acids they are ideal targets for our drug discovery platform and are likely to be accepted by the promiscuous epimerases.

In addition to our initial target peptides (see “Own achievement in the field”), we will begin our investigations with other antimicrobial peptides with clinical applications. Our target peptides include Indolicidin, a 13-amino acid cationic extended peptide which has successfully been used as a template for many analogs that exhibit improved biological properties tested in phase III clinical trials.[4] Many peptides are commercial available. Further candidates to be epimerized are the α-helical Magainin-2 and Melittin, β-sheet fold Bactenecin and human β-defensins, cyclic polymixin B, and epinecidin-1, Parasin and Alloferon 2 exhibiting random coil

structures. Generally, model antimicrobial peptides are chosen based on their classification in their antimicrobial potency, 3D structure (different structural peptide classes), and number of hydrophobic/charged.

In addition to established antimicrobial peptides, lead structures that failed due to their short plasma life time may be re-investigated after introduction of D-amino-acids, as these modified variants are expected to be less prone to degradation. We will use the industrial (e.g. Swiss Industrial Biocatalysis Consortium (SIBC)) and academic connections (antibiotic platform, biotechnet Switzerland) of the Competence Center of Biocatalysis at the ZHAW to identify these alternative drug targets.

Application of environmentally-derived peptides for drug discovery

Despite the computational power available, prediction of peptide antimicrobial properties is still not straightforward.[4] Hence, the search for new peptide-derived antibiotics is based on extensive empirical testing requiring a large pool of diverse lead structures. To expand the pool of screenable compounds, we will access the natural target peptides of rSAM epimerases derived from microbiomes by bioinformatics identification and functional expression. In addition to producing the peptides in their naturally epimerized form, we will generate peptides with altered D-amino acid incorporation pattern by combinatorically matching available peptides and epimerases, thus multiplying the number of potential lead structures for drug discovery (Figure 7).

The antibiotic activity of our microbiome-inspired peptide library will be tested at the Institute of Veterinary Bacteriology, University of Bern. The minimal inhibitory concentration (MIC) of the peptides will be determined for a collection of bacteria of the ESKAPE group by microbroth dilution testing using serial two-fold dilution in microtiter plates. MIC will be determined following the same guidelines which are recommended for susceptibility testing of antibiotics[52] and as previously established.[14]

2.4 Innovative potential

According to the World Health Organization[39], antibiotic resistance is one of the biggest threats to global health today and development of novel antibiotics is urgently needed. Owing to the discovery gap for novel antibiotic therapies during the last decades, public health is running out of treatment options for multi-drug resistant gram negative bacteria such as *Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa*.[41]

To address the rising threat of antibiotic resistance we will harness the microbiome's diversity in a dual fashion: A biocatalytic toolbox consisting of novel rSAM Epimerases will allow the introduction of unprecedented diversity into existing peptide-based antibiotics and thus modulate and improve the peptides' characteristics, for example their mode-of-action and pharmacokinetics.[5] The Buller group has succefully implemented the *in vitro* epimerization of leader-less and non-natural peptide substrates and showed the beneficial effect of D-amino acid incorporation on protease degradation (Figure 4) paving the way for the utilization of epimerases for drug discovery. At the same time, the identification of the epimerases' native target peptides within the microbiome by the Bruggmann group will yield more template peptides with potential antimicrobial activity. Owing to the

impressive variety of biologically active molecules in nature which have been optimized by selective pressure, the isolation of natural products has proven a successful strategy to identify new potential therapeutics.[53]

The innovative cornerstone of our novel approach builds on the combinatorial use of rSAM Epimerases: By submitting known antimicrobials and newly identified peptides to multiple rounds of diversification, we will maximize structural diversity to be tested for antimicrobial properties by the Perreten group. In addition, the elucidation of an antimicrobial fingerprint motif for each tested peptide will facilitate the construction of a structure-function roadmap that may further guide antimicrobial peptide research.

A successful project outcome will serve as a lighthouse example for the successful exploitation of the microbiomes' enzymatic diversity in the discovery of new drugs and our study will supply the Swiss pharmaceutical industry with valuable tools for further development efforts in the emerging field of RIPP application.

The unprecedented use of rSAM epimerases for the introduction of structural diversity into synthetic and natural peptides will make accessible an innovative platform for peptide drug discovery. Building on the recent findings in the emerging field of RIPP synthesis, we will exploit newly developed concepts and methods to engineer rSAM epimerases according to industrial needs. This transfer is only made possible by building on key innovations from multiple research fields such as the utilization of big data or of computationally aided enzyme design.

2.5 Project plan

2.5.1 Methods & Milestones

The project targets the utilization of untapped information stored in Nature's microbiome for the production of pharmaceutically active compounds with the focus on novel antibiotics. To reach this goal, a consortium of three complementary research groups has been formed (Figure 9), which in accordance to the project plan (Table 1) will work on the four main objectives that have been set and linked to the expected results and deliverables (see below). In addition to the scientific milestones listed within the Gantt chart (Table 1), the overall deliverables - including economic indicators of success - proposed to measure the success of the project are:

I. Bioinformatic sourcing of the microbiome's diversity

D1.1: Expansion and improvement of bioinformatic algorithms for the identification of new rSAM epimerases and peptides.

D1.2: Identification of > 25 new rSAM epimerases and their target peptides.

II. Set-up of a biocatalytic toolbox of rSAM Epimerase in an industrially useful format

D2.1: Toolbox "rSAM Epimerases" is expanded by 5-10 newly identified members from the microbiome and their activity is verified *in vitro.*

D2.2: A mutant library of the best performing microbiome epimerase is optimized toward the epimerization of non-native antimicrobial peptides. Best clones generate mg quantities of epimerized product.

D2.3: An *in vitro* protocol for the combinatorial use of rSAM epimerases is established.

D2.4: General engineering principles for the leader-peptide free epimerization and altered substrate specificity are elucidated.

III. Evaluation of antimicrobial properties of the generated novel peptides

D3.1: A library of 20 - 30 novel peptide based drugs is established.

D3.2: Native and non-native epimerization products are tested for their antimicrobial activity.

IV. Industrial implementation & Knowledge Dissemination

D4.1 A patent will be filed to secure IP and promote technology transfer of the D-amino acid containing novel antimicrobial peptides from bench to the bedside.

D4.2 Two scientific publication reporting on the bioinformatic sourcing and the set-up and engineering of the rSAM epimerases will be provided, including information on sourcing algorithm and elucidated engineering principles for rSAM Epimerases.

D4.3 Relationships with a minimum of three potential partners in a future CTI project will be established/intensified and the transition of succesful antimicrobial peptides toward the clinic will established.

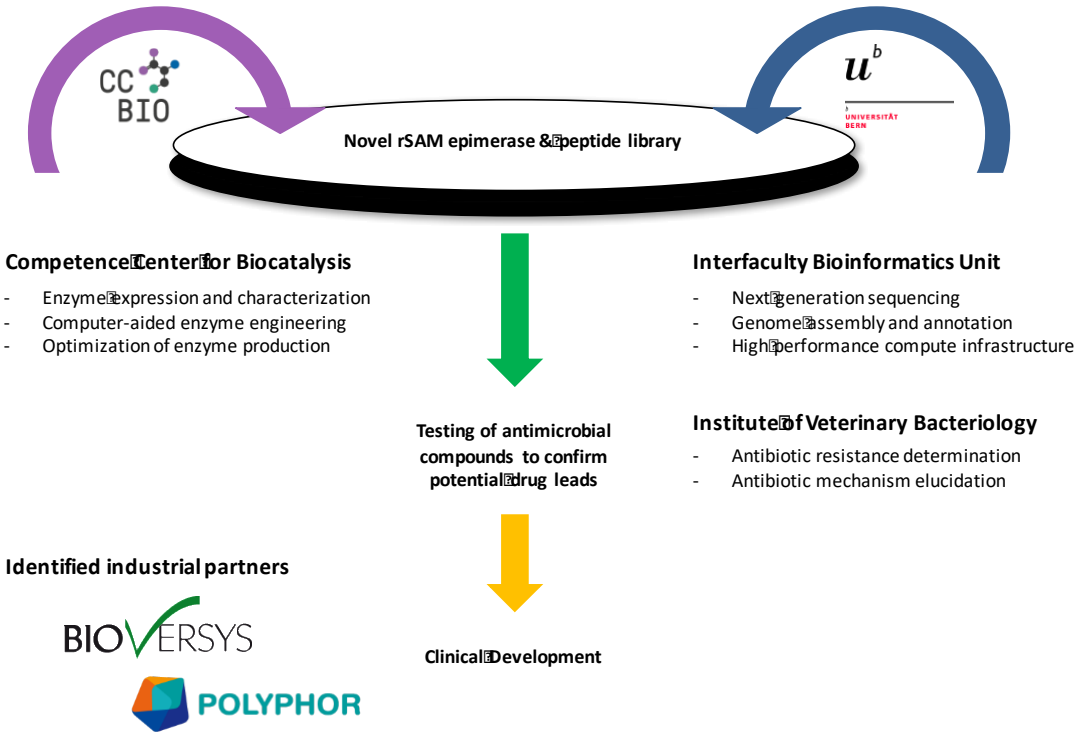

Figure 9: Project structure and orgnanisation - The complementary expertise of the consortium members will allow the transit of untapped information stored in Nature's microbiome to the clinical development stage.

	Workpackages	Responsibilty
WP1	**Bioinformatic Sourcing**	**Rémy / Uni Bern**
T1.1	Elaborate bioinformatic algorithms for the identification of new rSAM epimerases	
T1.2	Screening of databanks for new rSAM epimerases and their target peptides	
M1.1	List of newly identified rSAM epimerases and peptides	
T1.3	Ranking of hits and algorithms based on experimental feedback	
T1.4	Improved, validated second generation algorithm for the identification of epimerases	
M1.2	Expanded list of newly identified rSAM epimerases and peptides	
WP2	**Set-up of Biocatalytic Toolbox of rSAM Epimerases**	**Rebecca / ZHAW**
T2.1	Clone/synthesize 5 -10 newly identified rSAM epimerases	
T2.2	Expand toolbox "rSAM Epimerases" by newly identified members (functional expression)	
T2.3	Set-up and optimize *in vitro* epimerization conditions rSAM Epimerase toolbox	
M2.1	Feedback of epimerase activity data to bioinformatics team	
T2.4	Preparation of peptide material for antimicrobial testing	
WP3	**Enzyme Engineering**	**Rebecca / ZHAW**
T3.1	Explore epimerase system (OspA/OspD) for epimerization of non-native substrates	
T3.2	Transfer of leader peptide free epimerization method to newly identified epimerases	
T3.3	Screening of conversion of model antimicrobial peptides by epimerase library	
T3.4	Optimization of best performing epimerase for model antimicrobial peptides	
T3.5	Characterization of native and engineered epimerases	
M3.1	Functional rSAM Epimerase library established	
M3.2	Establishment of general engineering principles for rSAM Epimerases	
WP4	**Evaluation of antimicrobial properties of the novel peptide library**	**Vincent / Uni Bern**
T4.1	Establishment of library of novel peptide based drugs	
T4.2	Native and non-native epimerization products are tested for their antimicrobial activity	
M4.1	Activity roadmap for novel antimicrobial peptides	
WP5	**Know-How transfer and industrial implementation**	**all**
T5.1	Knowledge dissemination (via CCBIO, biotechnet): Workshop with industry experts	
T5.2	Submit manuscripts about results "toolbox epimerases" and "bioinformatics"	
T5.3	Set-up of CTI project(s) for the clinical development of D-antimicrobial peptides	
WP6	**Project Management and Reporting**	**all**
T6.1	Project coordination (meetings every 3 months)	
T6.2	Project controlling (reporting 1-3)	
T6.3	Final poject meeting	
T6.4	Project closure and final report	
M6.1	Annual reports and final report to BRIDGE	

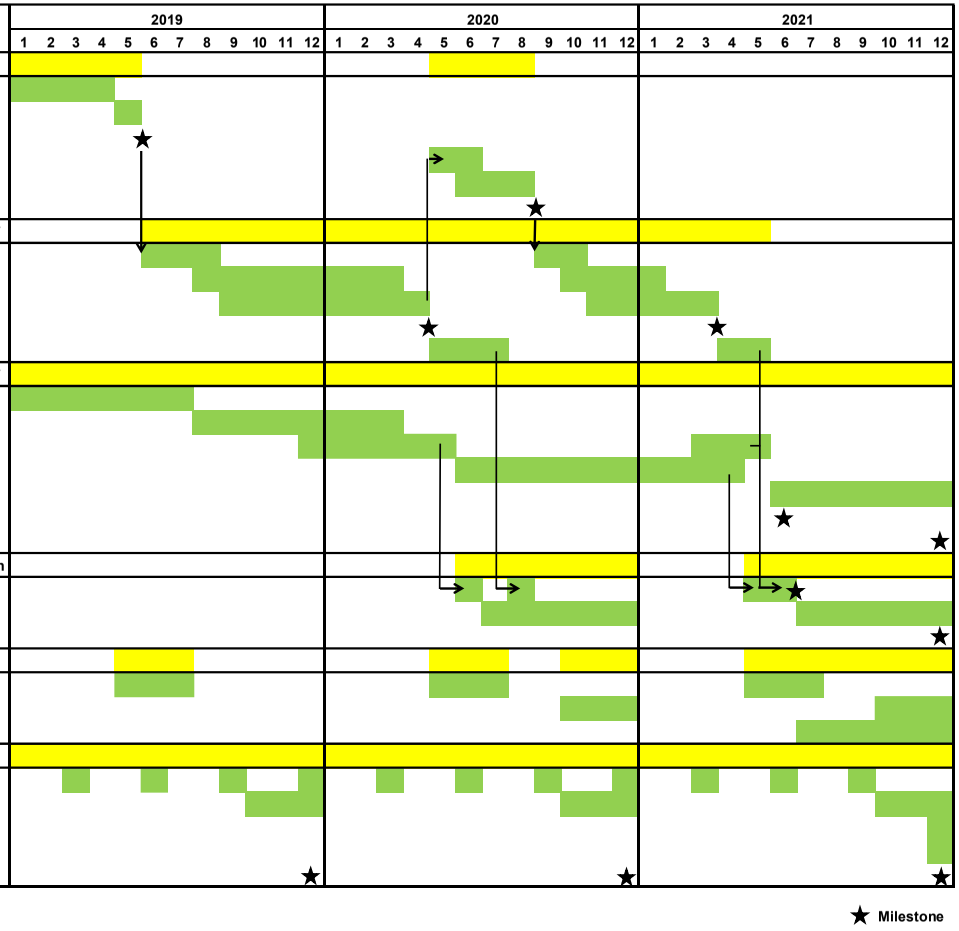

Table 1: Gantt chart of the Microbbial Epimerase Toolbox listing the six workpackages (WP), tasks (T) and milestones (M).

2.5.2 Innovation Roadmap

The market for protein- / peptide-based drugs is currently estimated at >$40 billion per year which corresponds to 10% of the ethical pharmaceutical market. This market share is growing much faster than that of other pharmaceuticals, and success rates for bringing biologics to market are now about twice that of small molecule drugs.[20, 53] Generally, peptide drugs have played a notable role in medical practice since the advent of insulin therapy in the 1920s. Today, over 60 peptide drugs are approved in the United States and other major markets and more than 150 peptides are in active development.[54] Advantages of peptide therapeutics compared over small molecule drugs are among others their higher potency at peptidic receptors, their reduced risk of CYP inhibition (dimished drug-drug intercations) and reduced risk for side effects caused by off-target binding.[54]

Peptides have been investigated across the therapeutic spectrum, reflecting their potential utilty across a wide range of indication. Going beyond the manufacture of antimicrobial peptides, our rSAM Epimerase discovery platform will allow us to generate lead structures for the treatment of metabolic and cardiovascular disease as well as oncology, all of which are areas of high interest to the pharmaceutical industry.

However, similar to many other fields of science, technical challenges in the peptide-based drug market are tackled keeping in mind financial considerations. One trend to prime the pharmaceutical company pipelines has been to partner discovery programs to academic institutions. Due to this demand, the Competence Center for Biocatalysis (CCBIO) has been founded at the ZHAW in January 2016. Thanks to the expert know-how of its members, it can cover wide areas of the biocatalytic value chain to help industry develop new industrial processes and products.

Following the filing of a patent for all relevant IP, promising peptide candidates will be further explored with the help of our industrial partners BioVersys and Polyphor which have already expressed their interest in our drug discovery platfrom (see attached letters of support). At BioVersys, for example, recent clinical isolates are available allowing to further verify the efficacy of drug candidates on relevant antibiotic resistant strains. Analysis of the mode-of-action can be further supported by BioVersys' extensive collaboration network.

We will keep our industry partners up-to-date about the progress of our project thus facilitating the transformation of the basic applied research that we propose here into industrially sponsored development projects and finally, into marketable drugs. The product development phase, which may be either CTI sponsored or directly financed by industrial partners, can be supported by CCBIO with its expertise in areas ranging from microbiology to bioprocess technology and industrialization (www.zhaw.ch/ccbio).

Overall, we expect two ways in which we can further develop and implement our scientific achievements:

1) ***Industry sponsored development projects***: Further pharmaceutical development of the D-amino acid containing antimicrobial drugs in collaboration with SMEs, namely Polyphor and BioVersys.
2) As a ***service provider****:* The use of our rSAM epimerase library for screening purposes for the conversion of proprietary pharmaceutical peptide leads (antimicrobials and other) into their D-amino acid containing counterparts to improve e.g. ADME properties in the frame of a future spin-off company.

2.5.3 Risk Management

Risk	Probability	Impact on Project	Risk Mitigation
Experimental strategy: delays or failures			
Datamining does not identify novel rSAM epimerases	low	high	1) Our own and other's studies have revealed rSAM epimerases in a wide set organisms, thus we estimate the likelihood of identifying new members of this superfamily as high. 2) Learnings of the experimental work (e.g. activity of identified enzymes) will be fed back to the bioinformatics team to generate improved algorithms with better hit rates. 3) Additionally, we can functionally express the set of already discovered rSAM epimerases as the basis for our drug discovery platform.
Novel rSAM epimerases cannot be recombinantly expressed in *E.coli*	low	medium	1) Alternative host strains such as Rosetta strains and yeasts (*S. cerevisiae, P. pastoris*) will be employed for expression tests and optimized via Design of Experiment (MODDE Software, Umetrics). 2) Functional expression will also be attempted via cell free protein expression (CFPS) using commercial kits (e.g. PURE expression kit), the use of protein tags (e.g. STREP and GST tags) and the introduction of computationally derived stabilizing mutations (e.g. software suite CyrusBench).
Novel rSAM epimerases do not exhibit actitvity *in vitro*	medium	medium	Preliminary studies in our laboratory show that anaerobically reconstituted epimerases are active. However, should that not be the case for all newly identified epimerases, *in vivo* epimerization will be carried out. In this case, non-natural peptide substrates (e.g. existing antimicrobial peptides) will be encoded on plasmids and co-expressed in the rSAM epimerase *E.coli* host.

rSAM epimerases do not accept non-natural peptide substrates (e.g. existing antimicrobial peptides)	medium	medium	1) If novel rSAM epimerases do not accept non-natural peptide substrates, an extensive protein engineering approach will be used. Best qualified epimerases (stability, expression level) will be submitted to computer-aided directed evolution to evolve activity toward a candidate antimicrobial peptide. Learnings will be transferred to other members of the epimerase superfamily. 2) The natural peptidic substrate repertoire of the epimerases will serve as an additional starting point to build a library of potential antimicrobial drug leads.
D-amino acid containing peptides do not exhibit antimicrobial activity.	low	high	Our project will focus on two types of substrates: The natural rSAM epimerase substrates with unknown antimicrobial activity as well as non-natural peptide substrates that possess antimicrobial activity. By combining these two approaches we expect to generate a diverse peptide library with a high likelihood of containing structures with antimicrobial properties.
Implementation strategy & partners: delays and failures			
Consortium failure	low	high	The members of the *Epimerase Toolbox* team already successfuly collaborate on the sourcing of Epimerases. The high industry interest has further cemented the relationship and the team expects that it will be able to sustain the productive and constructive collaboration into the future.
Transfer to clinic: Selected start-up fails	low	high	1) Our closest industrial partner, BioVersys, has been spun out off the ETH in 2008 and since then has a successful track-record of raising money. A failure within the next five years is consequently unlikely. 2) However, as a back-up we have secured support from Polyphor and the SIBC (including several big pharma companies), allowing us develop our product using alternative avenues.

			3) Within the project time frame, we will secure our IP, allowing us to found a spin-off company after accomplishing this BRIDGE grant. Located at the same campus as the ZHAW in Wädenswil, the organization GROW (http://www.grow-waedenswil.ch) facilitates the founding of new start-ups and we plan to employ their expertise.
Existing market prohibits market access	low	medium	An analysis focussed on the Swiss biotech landscape[55] revealed that only a few SMEs / start-ups focus on the development of new antibiotics (e.g. BioVersys, Polyphor, Basilea Pharmaceutica) or are active as service providers for enzyme platforms and screenings. Two of the aforementioned companies will be involved in the co-development of the novel antimicrobial drugs generated in this project. Additionally, multiple global enterprises with headquarters or major sites located in Switzerland (among others Novartis, Roche, Johnson & Johnson) are potential future customers for successful clinical trial phase 1 and 2 leads.

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