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Review Article

REVIEW ARTICLE

Employing the promiscuity of lantibiotic biosynthetic machineries to produce novel antimicrobials

Manuel Montalbán-López[†], Auke J. van Heel[†] and Oscar P. Kuipers[*]

Department of Molecular Genetics, University of Groningen, Nijenborgh 7, 9747AG Groningen, the Netherlands

[*]**Corresponding author:** Department of Molecular Genetics, University of Groningen, Nijenborgh 7, 9747AG Groningen, the Netherlands. Tel: +31503632093; Fax: +31503632348; E-mail: o.p.kuipers@rug.nl
[†]M. Montalbán-López and Auke J. van Heel contributed equally to this work.

One sentence summary: The ribosomal synthesis and enzymatic modifications of lantibiotics provide excellent opportunities to design and engineer a great variety of novel antimicrobial compounds.

Editor: Alain Filloux

ABSTRACT

As the number of new antibiotics that reach the market is decreasing and the demand for them is rising, alternative sources of novel antimicrobials are needed. Lantibiotics are potent peptide antimicrobials that are ribosomally synthesized and stabilized by post-translationally introduced lanthionine rings. Their ribosomal synthesis and enzymatic modifications provide excellent opportunities to design and engineer a large variety of novel antimicrobial compounds. The research conducted in this area demonstrates that the modularity present in both the peptidic rings as well as in the combination of promiscuous modification enzymes can be exploited to further increase the diversity of lantibiotics. Various approaches, where the modifying enzymes and corresponding leader peptides are decoupled from their natural core peptide and integrated in designed plug-and-play production systems, enable the production of modified peptides that are either derived from vast genomic data or designed using functional parts from a wide diversity of core peptides. These approaches constitute a powerful discovery platform to develop novel antimicrobials with high therapeutic potential.

Keywords: RiPPs: ribosomally produced and post-translationally modified peptides; PTM: post-translational modifications; lantibiotics; antimicrobial

ABBREVIATIONS

Abu: 2-aminobutyric acid
CuAAC: Cu(I)-catalyzed alkyne-azide cycloaddition
Dha: dehydroalanine
Dhb: dehydrobutyrine
ncAA: non-canonical amino acids
PTM: post-translational modifications
RiPPs: ribosomally produced and post-translationally modified peptides

INTRODUCTION

Bacteria that have acquired multiple drug resistances pose a serious problem in the treatment of bacterial infections. Several health organizations and research institutes have emphasized the urgent need for new molecules, particularly with novel mechanisms of action (Boucher *et al.* 2009; Silver 2011; Fraser *et al.* 2013; Kåhrström 2013). The reasons leading to this dramatic situation are a reduced investment in antimicrobial research, abuse of antibiotics during either medical or veterinary

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applications, the high costs for development and clinical trials and a low success rate in the identification of novel compounds by expensive screening and clinical-testing programs (Brötz-Oesterhelt and Sass 2010; Donaldson and WHO 2012).

Various alternative strategies are being investigated, including phage therapy, modulation of the immune system, collateral sensitivity cycling and the use of discarded antibiotics (Bush *et al.* 2011; Bergen *et al.* 2012; Imamovic and Sommer 2013; Riley *et al.* 2013; Ling *et al.* 2015). Importantly, also the quest for novel antimicrobials is ongoing. Although most classic antibiotics are derived from non-ribosomal synthesis in microorganisms, the use of post-translationally modified and ribosomally synthesized bacterial peptides is attracting renewed interest because they also show high antimicrobial activity, are stable with respect to proteolytic degradation and have lower chances of resistance development (Montalbán-López *et al.* 2011; Cotter, Paul Ross and Hill 2012; Draper *et al.* 2015). Here we focus on the possibilities that the plethora of new sequences, biosynthetic machineries and structures of lantibiotics and other modified peptides offer, to develop new antimicrobial molecules (Fig. 1).

LANTIBIOTICS

Biosynthesis

Lanthipeptides (lanthionine containing peptides) form a class of ribosomally synthesized and post-translationally modified peptides (RiPPs) produced by bacteria (Arnison *et al.* 2013). They possess enzymatically created thioether bridges that are derived from a link between a cysteine and a dehydrated threonine or serine residue, yielding beta-methyllanthionine or lanthionine, respectively. This process is strictly leader peptide dependent, since the leader will guide the biosynthetic enzymes to the core peptide to be correctly modified. Typically, lanthionine is present as 2S,6R isomer (DL-lanthionine) and methyllanthionine as 2S,2S,6R (DL-lanthionine). In the last years, the presence of DL-forms together with LL-lanthionine (2R,6R) and LL-methyllanthionine (2R,2R,6R) has been confirmed in certain type II lanthipeptides (Tang *et al.* 2015; Garg *et al.* 2016), highlighting that it is not only the enzyme but also the core peptide sequence that is a determining factor for modification (*vide infra*). The correct stereochemistry of lacticin 481 has been investigated and is crucial for activity (Knerr and van der Donk 2013). In spite of the multiple combinations that might occur between the different cysteines and dehydroamino acids present in a lantibiotic core peptide, the leader peptide-dependent enzymes catalyzing these reactions are regio- and stereoselective, and a single main product is often observed when a post-translational modification (PTM) enzyme modifies its cognate peptide(s).

A subdivision based on the dehydration and cyclization enzymes is broadly accepted. Thus, class I lanthipeptides are dehydrated by a LanB enzyme and cyclized by a LanC enzyme, whereas in classes II, III and IV, both reactions are conducted by a single bifunctional enzyme referred to as LanM, LanKC and LanL, respectively. LanBs dehydrate serine and threonine via glutamylation and elimination using glutamyl-tRNA from the producer cell to do so (Ortega *et al.* 2015, 2016). LanCs cyclases have a conserved zinc ion as activator of the cysteine within the lantibiotic core peptide. This ion is trapped by a typical cysteine-cysteine-histidine triad. LanM and LanL both possess a C-terminal LanC-like cyclase domain but they differ in the dehydration mechanism. Interestingly, a subgroup of highly promiscuous and still regioselective LanM enzymes (e.g. ProcM) coordinate the zinc ion with three cysteines, which has been related to a higher reactivity in the cyclization reaction (Yu, Mukherjee and van der Donk 2015). The fact that mutations in the cysteines binding zinc in this enzyme alter the regioselectivity and completion of the maturation points at the key role of the first lanthionine ring formed in the subsequent completion of the maturation for ProcM-like enzymes, which lack the ability to open lanthionines and reassemble them as other LanM (e.g. HalM2) or NisC can do (Yang and van der Donk 2015a). Whether the ring-opening ability of these enzymes is a sort of proof-control to produce the thermodynamically most favorable compound remains unknown. In LanM enzymes a dehydration domain structurally related to lipid kinases catalyzes the phosphorylation and elimination reactions on Ser and Thr yielding the dehydrated residue (Dong *et al.* 2015). In LanL enzymes, dehydration requires the coordination of a kinase and lyase domains. LanKCs also possess the lyase and kinase domains but differ from LanL in the putative cyclase domain, which lacks the characteristic zinc-binding residues present in LanC and LanC-like cyclases. LanKC enzymes can introduce both lanthionine and labionin, a triamino triacid resulting from the linkage between a lanthionine and a dehydroalanine via a methylene group. An additional particularity of LanKC enzymes is a predominantly C- to N-terminal processivity (Jungmann *et al.* 2014).

Next to the characteristic dehydrated residues and lanthionine rings, some lanthipeptides contain additional post-translational modifications that are usually essential for their activity or increase their resistance against proteolysis (Knerr and van der Donk 2012). These modifications include the reduction of dehydroamino acids to form D-alanine or D-aminobutyrate (LanJ; Cotter *et al.* 2005; Huo and van der Donk 2016), reduction of a C-terminal pyruvic group (ElxO; Ortega *et al.* 2014), oxidation of the thioether bond (GarO; Boakes *et al.* 2009), hydroxylations of proline and aspartate (MibO; Foulston and Bibb 2010 and CinX; Ökesli *et al.* 2011), halogenation of tryptophan (MibH and MibV; Foulston and Bibb 2010), C-terminal decarboxylation (LanD; Kupke *et al.* 1992; Majer *et al.* 2002), covalent linkage of lysine and alanine (Cinorf7; Foulston and Bibb 2010), N-terminal acetylation (PaeN; Huang and Yousef 2015), disulfide bridge formation (Liu *et al.* 2009), and recently the tryptophan N-glycosylation of a lanthipeptide has been reported (Iorio *et al.* 2014).

Lanthipeptides display diverse biological activities, such as antimicrobial, antifungal, antiviral or antinociceptive activity (Férir *et al.* 2013; Iorio *et al.* 2014; Mohr *et al.* 2015), and some are able to generate an immunomodulatory effect (Pablo *et al.* 1999; Kindrachuk *et al.* 2013). Lantibiotics are those lanthipeptides that possess antimicrobial activity and so far all belong to classes I and II. So far, more than 70 lantibiotics and their biosynthetic pathways have been described in literature (Dischinger, Basi Chipalu and Bierbaum 2014). Further investigation of these lantibiotics has resulted in extensive knowledge about their maturation, regulation, biological activity and mechanism of action (Lubelski *et al.* 2008; Knerr and van der Donk 2012) (Fig. 2). Currently, 3D structures of several modification enzymes have been published, i.e. the decarboxylases EpiD and MrsD (Blaesse *et al.* 2000, 2003), the dehydratases NisB and MibB (Ortega *et al.* 2015, 2016), the cyclase NisC (Li *et al.* 2006), the bifunctional dehydratase/cyclase CylM (Dong *et al.* 2015), the reductase ElxO (Ortega *et al.* 2014) and the leader peptidase NisP (Xu *et al.* 2014).

Lantibiotic biosynthesis requires the coordinated expression of a set of genes including the structural gene (*lanA*) and genes encoding the modification enzyme(s) (*lanB* and *lanC*, or *lanM*), a leader peptidase (*lanP*), ATP binding cassette (ABC) transporters (*lanT*), regulatory elements and determinants for

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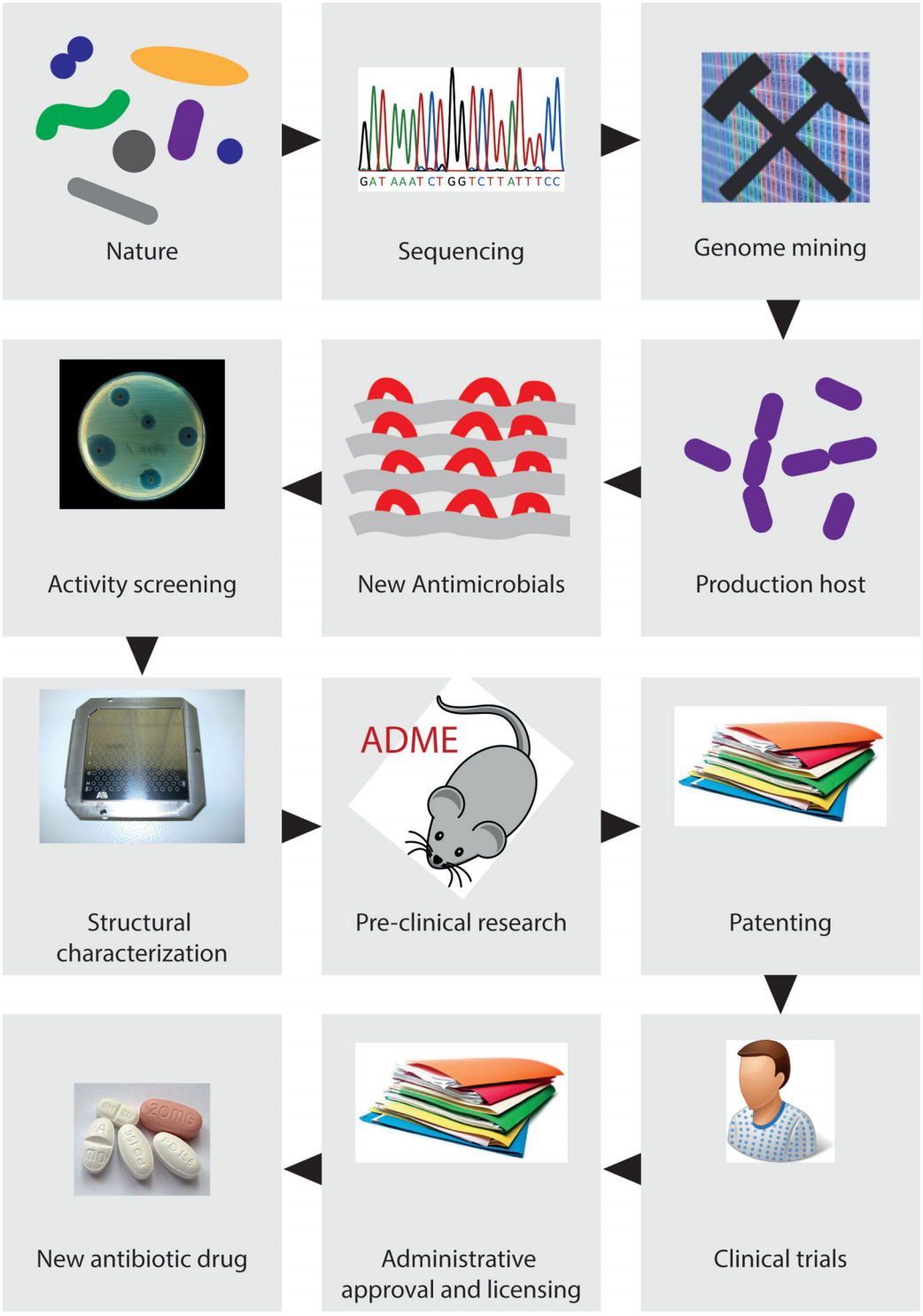

Figure 1. Pipeline for the development of novel RiPP-based antimicrobials. Following the arrows, the process starts with identification of RiPP clusters. Next, the RiPP has to be produced in an appropriate (heterologous) host. Active compounds are further characterized. Finally, patenting and clinical trials of selected compounds will lead to novel antimicrobials for clinical use. The number of steps does not in any way indicate the time and or resources needed during the development.

immunity (*lanFEG* and *lanI*) that protect the producer organism from self-destruction. The structural peptide consists of two parts with separate functions. The first part is the leader peptide that displays three main functions, namely guiding and activating the lanthionine modification enzymes, keeping the core peptide inactive during maturation and allowing efficient transport and leader peptide cleavage (Oman and van der Donk 2010; Plat *et al.* 2013). A structural motif has been identified in NisB and CylM (Dong *et al.* 2015), two model lantibiotic LanB and LanM enzymes, respectively, as well as other RiPP biosynthetic enzymes, even though they do not share high sequence homology (Burkhart *et al.* 2015; Dong *et al.* 2015). This finding points

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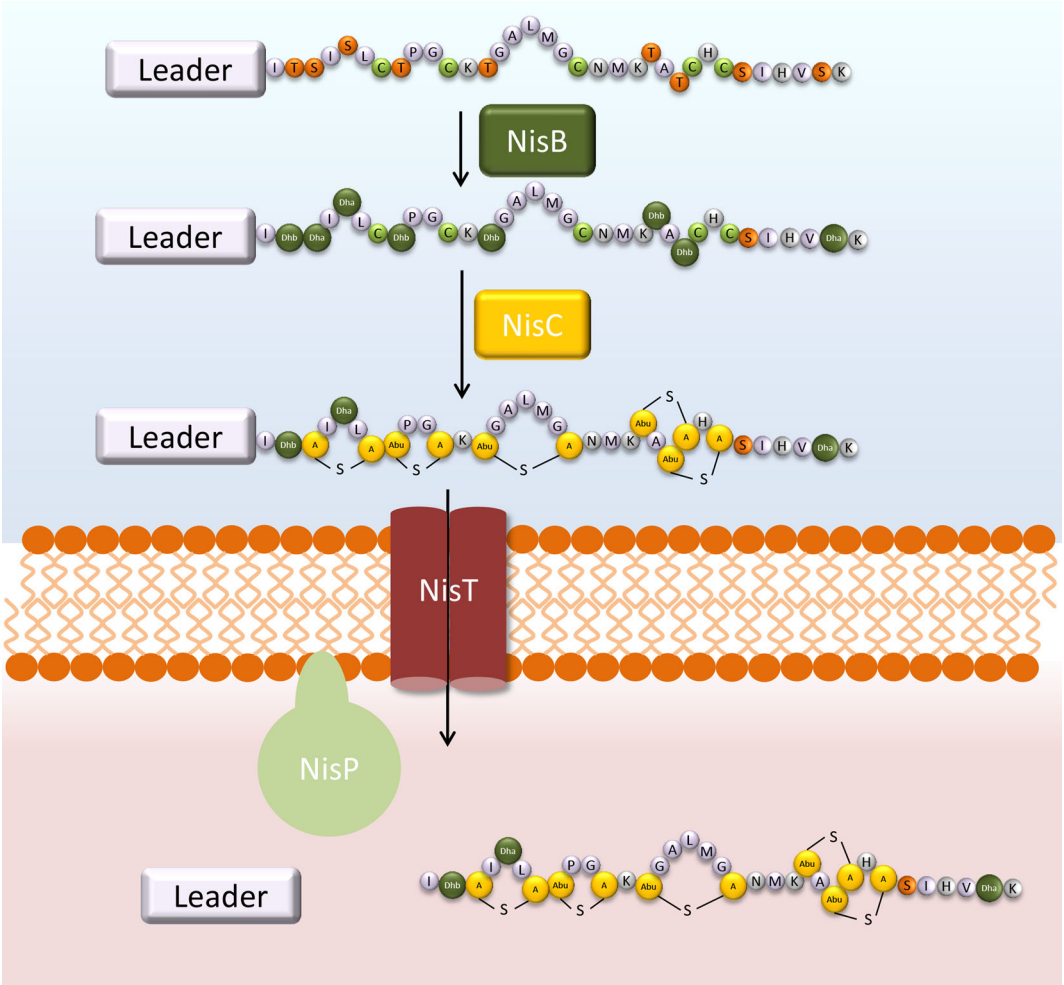

Figure 2. Schematic representation of the maturation of the model lantibiotic nisin. Nisin is produced as a linear peptide consisting of a leader peptide, responsible for the interaction with the PTM enzymes and their activation, and a core peptide that undergoes extensive PTMs. The dehydratase NisB converts serine and threonine (orange) residues into dehydroalanine and dehydrobutyrine (dark green), respectively, and the cyclase NisC catalyzes the addition of a thiol group in cysteine (light green) to an N-terminally located dehydroamino acid. These reactions produce the characteristic lanthionine rings (yellow). The fully modified peptide is translocated across the membrane by a dedicated transporter, NisT. At the outside of the cell, the protease NisP removes the leader peptide and releases mature nisin. The elements in the figure are not to scale.

at a general leader peptide recognition mechanism widespread along PTM enzymes that act before leader peptide removal and can have great impact in future peptide-engineering approaches (Montalbán-López and Kuipers 2016). The second part, the core peptide, is the part that exhibits antimicrobial activity after its maturation. The fully modified peptide with the leader attached needs to undergo proteolytic cleavage to become fully active (Fig. 2). In type I lantibiotics, this is performed usually by a LanP protease, whereas in type II lantibiotics cleavage takes place concomitantly with export with the protease domain of LanT. Even though the type I lantibiotic transporter is also referred to as LanT, it has no proteolytic activity. Exceptionally, some type II lantibiotics (e.g. cytolysin and lichenicidin) are fully activated in a second proteolytic step catalyzed by a LanP-like extracellular protease (Caetano *et al.* 2011).

A high degree of modularity is present in the biosynthesis gene clusters with the separated modules (i.e. enzymes) being responsible for distinct steps in the biosynthesis. The PTM enzymes have been shown to tolerate a broad range of substrates in a leader-dependent (e.g. the dehydratase NisB or the bifunctional dehydratase-cyclase LctM) or -independent (e.g. GdmD; Kupke *et al.* 1994; van Heel *et al.* 2013b) fashion. Advanced plug-and-play expression systems that make use of this fact have been developed for the incorporation of Dha, Dhb and lanthionine residues into diverse substrates. Systems have been described using LctM from the lacticin 481 biosynthesis (You *et al.* 2009), NisBC from nisin biosynthesis (Rink *et al.* 2005) and NukM from the nukacin ISK-1 biosynthesis (Nagao *et al.* 2005) among others. These studies not only provided valuable mechanistic information but also paved the way for using PTM enzymes in peptide stabilization for therapeutic applications (Moll, Kuipers and Rink 2010).

Mechanism of action and resistance

Lantibiotics display strong inhibitory effects on a wide range of microorganisms, particularly against Gram-positive bacteria, including multidrug-resistant bacteria such as vancomycin-resistant *Enterococcus faecalis* or methicillin-resistant *Staphylococcus aureus* (Mota-Meira *et al.* 2000; Asaduzzaman and Sonomoto 2009; Knerr and van der Donk 2012). The medical potential of lantibiotics is based on their strong antimicrobial effect,

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which is in a similar concentration range as that of antibiotics currently used in medicine, their high stability and their specificity towards prokaryotes (van Heel, Montalban-Lopez and Kuipers 2011).

Several different modes of action have been elucidated for lantibiotics. For example, nisin has a dual mode of action, the first being the sequestering of lipid II from its natural location and thereby blocking cell wall synthesis (Hasper *et al.* 2006). The second involves pore formation in the cytoplasmic membrane, which is stabilized by the pentapeptide (Breukink *et al.* 1999; 't Hart *et al.* 2016). Other modes of action of lantibiotics that have been described include lipid II sequestering without pore formation (for instance mersacidin), inhibition of phospholipases (for instance cinnamycin) or induction of autolysis (for instance Pep5) (Islam *et al.* 2012).

Since the pyrophosphate group of lipid II that binds to the first two rings of nisin is not prone to mutation, the appearance of a significant, stable and genetically transmissible resistance to nisin is not easy to achieve (Draper *et al.* 2015). In addition, this mechanism of action distinguishes from other antimicrobials such as vancomycin or beta-lactams, therefore bacteria resistant to these antibiotics remain sensitive to lantibiotics. Transient resistance to lantibiotics has been demonstrated *in vitro* involving mechanisms such as enzymatic reduction of the negative charge of the cell envelope by alanylation of teichoic acids (Peschel *et al.* 1999; Kramer *et al.* 2006) or lysinylation of phospholipids (Thedieck *et al.* 2006), increasing the thickness/compact structure of the cell wall (Kramer *et al.* 2006), or the lipid composition in the membrane (Verheul *et al.* 1997). Some two-component systems present in Gram-positive bacteria have also been related to increased resistance against lantibiotics. For instance, the *cprABCK* and *cprK* genes in *Clostridium difficile* have been identified to respond to some lantibiotics increasing the expression of the transporter CprABC to reduce toxicity (Suárez, Edwards and McBride 2013). Some other strains such as *Streptococcus agalactiae* produce a protease (the so called nisin resistance protein) specifically targeting the last lanthionine ring of nisin reducing significantly its activity (Khosa *et al.* 2016). Last, resistance due to the presence of specific lantibiotic immunity proteins by cross-resistance between lantibiotic producers applies so far to only closely related lantibiotics. However, the presence of lantibiotic-like immunity-related transporters in non-lantibiotic producers has been discovered and requires a more detailed study. Their transfer and constitutive expression to model laboratory strains increased their resistance to a specific lantibiotic by a phenomenon called immune mimicry, although the specific role in the original bacteria is not yet clear (Draper *et al.* 2009). A more detailed review on lantibiotic resistance has been recently addressed (Draper *et al.* 2015).

Clinical potential

The use of peptide drugs in medicine is expanding with a market increasing not only in turnover but also in the number of approved molecules (Craik *et al.* 2013; Fosgerau and Hoffmann 2015). However, the use of peptides and large molecules in therapeutics remains challenging. The size of lantibiotics excludes them as drugs with good oral absorption for systemic applications and limits their use to local applications or systemic parenteral administration according to Lipinski's rule of five (Lipinski *et al.* 2012). Invasive administration is already in use for other last resort antimicrobials such as vancomycin. Local application is considered a favorable aspect since this is limiting their action towards microorganisms in another restricted biological compartment. Thus, the impact upon administration of these antimicrobials on the natural beneficial flora can be minimized. Oral applications that do not depend on intestinal absorption are feasible, for instance for the treatment of *C. difficile* (Crowther *et al.* 2013) in the gut or *Helicobacter pylori* (Mota-Meira *et al.* 2000) in the stomach. Alternatively, advances made in the pharmaceutical industry to improve oral uptake of high molecular weight drugs (such as insulin and other peptide hormones and heparin) are promising and might in the future provide means to deliver large macromolecules orally for systemic applications (Goldberg and Gomez-Orellana 2003; Pawar *et al.* 2014).

So far, lantibiotics have been successfully tested for the topical treatment of mastitis (Fernández *et al.* 2008), skin infections (Heunis, Smith and Dicks 2013) and oral infection prevention and treatment (Turner, Love and Lyons 2004; Hillman *et al.* 2007; Dobson *et al.* 2011). A particular advantage of lantibiotics is that lanthionine rings and dehydrated residues included in their structure are highly stable at the low pH that is encountered in the gastric juice.

A general problem peptides pose in clinical use is their sensitivity to proteases and the short half-life (Hancock and Sahl 2006; Craik *et al.* 2013). However, this is counteracted by the stabilizing effect of PTMs. Other issues regarding toxicity and stability of lantibiotics have been previously addressed (van Heel, Montalban-Lopez and Kuipers 2011).

Although still relatively scarce, the pharmacokinetic studies on lantibiotics unambiguously show their therapeutic potential (van Heel, Montalban-Lopez and Kuipers 2011). Some lantibiotics are already under development by diverse companies: NVB302 and NVB333 by Novacta Biosystems Limited; NAI-107 by NAICONS; and Mu1140-S by Oragenics (Dischinger, Basi Chipalu and Bierbaum 2014). Mu1140 and NVB333 show strong binding to human and rat plasma, respectively (Ghobrial, Derendorf and Hillman 2010). However, their activities in these models are still remarkable against different infection models. A yet unexplored effect of serum on the minimal inhibitory concentration (MIC) value of Mu1140 has been reported. Thus, the MIC against *S. pneumoniae* was increased 2–4-fold, whereas it was reduced 8-fold against *Staph. aureus* (Ghobrial, Derendorf and Hillman 2010). In the few pharmacokinetic studies available, the elimination rate and half-life of lantibiotics are dose independent, whereas the concentration in plasma and area under the curve (AUC) are dose dependent (Ghobrial, Derendorf and Hillman 2009; Lepak *et al.* 2015; Boakes *et al.* 2016). Collectively, these studies indicated that the relation AUC/MIC is a good indicative of the pharmacodynamic index.

NVB333 shows in the different models tested an activity comparable or even better than conventional antimicrobials such as vancomycin or daptomycin. Notably, the activity *in vivo* of NVB333, a chemical derivative of actagardine, and mersacidin is better than could be expected from the *in vitro* tests (Chatterjee *et al.* 1992; Boakes *et al.* 2016) and highlights the interest of chemical semisynthesis to improve the pharmacological properties of lantibiotics. Interestingly, the resistance to vancomycin or methicillin does not have a strong influence on the efficacy of lantibiotics *in vivo* due to different mechanisms of action (Lepak *et al.* 2015; Boakes *et al.* 2016).

GENOME MINING

In the post-genomic era, the number of (publicly) available genome sequences is exploding. This has enabled the possibility of high-throughput genome mining, either using simple

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homology based approaches (e.g. BLAST), or using more advanced dedicated tools developed for this purpose, such as BAGEL 3 and antiSMASH 2 (Blin *et al.* 2013; van Heel *et al.* 2013a). These tools automate the mining process and make all information accessible to non-expert users. By looking at the presence of multiple defined conserved elements (e.g. sequences of modification enzymes, dedicated transporters, processing enzymes, immunity proteins and regulatory proteins) in parallel, the software identifies sequences of interest in the genome. Next, these sequences are analyzed in detail to find the structural peptide, which, due to the sequence variability and small size, is commonly overlooked in a homology-based search. These tools can easily be extended to predict additional classes of RiPPs as they are discovered assuming that these classes possess discriminating features that are conserved within the class. The rule for a new class could for example be that a DNA sequence of 20 kb should encode at least three out of four defined protein domains (often associated with modification enzymes) and must encode a core peptide that has a maximum size and must contain certain signature amino acids. Using lantibiotics as an example, the genome mining process for class I lantibiotics can be based on the search for a lantibiotic dehydratase C-terminus (PF04738 or PF14028) and LanC-like (PF05147) motifs and a small open reading frame (ORF) containing a typical N-terminal FN/DLD motif and serine, threonine and cysteine in the C-terminal moiety. Multiple classes of RiPPs can be mined for in parallel provided that rules for the identification of these classes have been defined. Although the principles these tools are based on are not very complex, they can greatly improve the annotation of genomes.

The recent identification of a common leader recognition structural element in several RiPP PTM enzymes (Burkhart *et al.* 2015) is an additional feature that can be mined for. The advantage of including this structural motif in genome mining is the possibility to find not only described RiPP gene clusters but also novel classes in which one or more enzymes contain the conserved element. Recent efforts to create a systematic deposition of data about a RiPPs cluster facilitate the definition of search parameters to identify novel compounds and RiPP classes (Medema *et al.* 2015). Once a putative structural gene has been identified *in silico*, automatically finding the correct processing site between the leader and the core peptides remains a challenging problem in genome mining due to the diversity of processing sites found in several RiPP classes. Typically, in class I lantibiotics two subfamilies of LanPs recognize a F/A-A/G-X-X-R\I or E/D-L/V-X-X-Q\S/T motif (X denotes any amino acid,\indicates the cleavage site) (Ortega *et al.* 2014), whereas type II LanT recognizes a double glycine processing site (Knerr and van der Donk 2012). The fact that these typical motifs are absent in lantibiotics such as cytolysin (Booth *et al.* 1996) or that the double glycine motif can also tolerate glycine to alanine mutations can complicate the prediction of the correct processing site.

Mining for PTMs resulted in the identification of several new lantibiotic gene clusters such as lichenicidin and Bsa, which were identified by searching for LanM analogs (Begley *et al.* 2009; Daly *et al.* 2010). Also the subclass IV of lanthipeptides, those that are modified by LanL, was identified by genome mining (Goto *et al.* 2010). Natural product screening efforts can benefit from automated *in silico* predictions of novel RiPPs. The predictions contain information that can be used to simplify the detection and purification of the peptide of interest based on existing methods for similar peptides. Also the prediction of a RiPP class provides information about the expected activity and an indication of its antimicrobial spectrum, facilitating further testing.

PRODUCTION SYSTEMS FOR NOVEL LANTIBIOTICS

The traditional screening methods depending on the isolation of a producer bacterium still constitute a valuable source of novel compounds. For instance, the lantibiotic pinensin, produced by a Gram-negative bacterium, was detected in such a screening campaign and turned out to be a potent antifungal (Mohr *et al.* 2015). However, the production of novel lantibiotics no longer exclusively depends on the host in which it was originally identified. Achieving the expression of detected lantibiotic clusters in its natural host can be hampered by culturing conditions (e.g. the microorganism is not culturable or the production is not detected in the normal culture conditions). In addition to the *in vitro* modification methodologies exposed below, there are various possibilities to achieve the production of lantibiotics identified *in silico* using *in vivo* approaches.

A strategy successfully used is the expression of the whole gene cluster that was previously identified *in silico* in a well-characterized chassis (i.e. a heterologous host). The production of cinnamycin was first achieved transferring the whole cluster from *Streptomyces cinnamoneus* to *Strep. lividans* (Widdick *et al.* 2003). This approach is hampered by the dependence on the original host and for the availability of the source DNA, but alternatively the DNA can be synthesized. An additional key factor is the selection of an appropriate heterologous host. For instance, planosporicin was identified in *Planomonospora alba* and the initial attempts to express it in *Streptomyces* failed, while later *Nonomuraea* was found to be a more suitable host (Sherwood, Hesketh and Bibb 2013) (Fig. 3, example A). Although the exact topology of the heterologously expressed planosporicin was not determined, the activity assays strongly suggest the correct rings being formed. A feasible explanation for the failed expression in *Streptomyces* is the lack of recognition of the promoter regions and regulators or the preference of the LanB dehydratase for a cognate $tRNA^{Glu}$ (Ortega *et al.* 2015, 2016). The supplementation of a specific *Microbispora* $tRNA^{Glu}$ in *Escherichia coli* increased the dehydration extent of microbisporicin, whereas the usual $tRNA^{Glu}$ naturally present in the host were not a suitable substrate for the dehydratase MibB (Ortega *et al.* 2016). So far, this is the only case where $tRNA^{Glu}$ supplementation has been used to reconstitute the heterologous expression of class I lanthipeptides, but it may be a suitable strategy for their (increased) production in species phylogenetically distant from the wild-type producer organism or when unnatural substrates are expressed. In order to gain control of the production of heterologously expressed lantibiotics, one can replace the original promoters with inducible promoters suitable for the heterologous host as was done for the controlled expression in *E. coli* of geobacillin (Garg *et al.* 2012), several prochlorosins (Tang and van der Donk 2012), lichenicidin (Caetano *et al.* 2011; Kuthning, Mösker and Süssmuth 2015) (Fig. 3, example B), nisin, haloduracin and others (Shi *et al.* 2011). Surprisingly, lichenicidin expression in *E. coli* including the specific lantibiotic transporter achieved the extracellular production of the active compound via an additional TolC transporter. Although the structure of the heterologously expressed peptides was not determined, the activity indicates correct ring formation (Caetano *et al.* 2011; Kuthning, Mösker and Süssmuth 2015). An additional advantage of these systems (and the ones presented below) is that the

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Figure 3. Examples of lantibiotics produced and modified using different approaches. The top panel shows the origin of the sequences and the design. The bottom panel shows the production setup and outcome (it should be noted that not for all examples structures of the resulting lantibiotics have been fully determined). (A) Heterologous expression of the whole planosporicin biosynthesis gene cluster in *Nonomuraea* sp. (Sherwood, Hesketh and Bibb 2013). (B) Heterologous expression of lichenicidin A1 (shown here) and A2 in *E. coli* under control of inducible promoters (Caetano *et al.* 2011). (C) Heterologous expression of flavucin linked to the nisin leader using the nisin modification machinery in *L. lactis* (van Heel *et al.* 2016). (D) Expression of a combined lantibiotic (nisin and subtilin) in *B. subtilis* (Chakicherla and Hansen 1995). (E) Expression of a combined lantibiotic (nisin and gallidermin) and a combination of two modification machineries (NisBTC and GdmD) (van Heel *et al.* 2013b). (F) Nisin extended with C-terminal part of the antimicrobial peptide apidaecin 1b, to improve its activity against Gram-negative pathogens (Zhou *et al.* 2016a). Violet boxes indicate LanBC or LanM enzymes, green boxes indicate leader peptidases and purple boxes denote additional post-translational modification enzymes.

expression of the structural gene and the modification enzyme(s) allows separating the modification of the core peptide from the leader peptidase thereby maintaining the fully modified peptide inactive. This can on one hand increase the production yield and on the other hand avoid the necessity for immunity determinants since the fully modified prelantibiotic is inactive (Kuipers *et al.* 1993; Valsesia *et al.* 2007; Oman and van der Donk 2010; Kuthning, Mösker and Süssmuth 2015). This poses the challenge of selecting the right protease for activation. LanP enzymes and the LanT-protease domain have been successfully expressed and used on unnatural substrates (Geissler, Götz and Kupke 1996; Furgerson Ihnken, Chatterjee and van der Donk 2008; Lin *et al.* 2011; Ortega *et al.* 2014; van Heel *et al.* 2016) (although with low *in vitro* activity) and might be suitable for this purpose once their specificity if fully characterized. The selection of an appropriate protease can have a strong influence on the overall yield of this strategy, since the production level might be dramatically reduced due to changes in the leader

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peptide cleavage sequence (Plat *et al.* 2011; van Heel *et al.* 2016). At present, core peptide activation using trypsin or endoprotease Glu-C is often used due to the partial protection that lanthionine rings constitute.

A powerful alternative is the use of a modular production system that decouples the modification machinery from its native substrate. Thus, a putative novel lantibiotic sequence is expressed together with well-characterized modification enzymes of a different lantibiotic cluster (Fig. 3, example C). Therefore, the lantibiotic leader peptide, which is recognized by its specific lantibiotic synthetases, is combined with the predicted core peptide in order to get it modified. Previously, it has also been shown that related leader peptides can guide the synthesis of a lantibiotic as exemplified by a subtilin leader guiding the synthesis of nisin in *Lactococcus lactis* (Kuipers *et al.* 1993). For example, the nisin modification machinery has already been exploited to produce novel lantibiotics, which were detected *in silico* using Bagel, by fusing the predicted core peptides to the nisin leader peptide and coexpressing it together with the NisBTC enzymes (Majchrzykiewicz *et al.* 2010). The regio- and stereoselectivity of the heterologous machinery acting on non-cognate core peptides remain uncertain, although the fact that they display activity is an indication of a correct ring pattern due to the importance of the correct positioning of the rings and their steric arrangement (Knerr and van der Donk 2012). In a more extensive study, five novel active lantibiotics were characterized two of which, namely flavucin and bagelicin, displayed a potent activity against a multidrug resistant *Enterococcus faecium* strain (van Heel *et al.* 2016). The characterization of these peptides by tandem mass spectrometry showed that at least the first three rings, similar to those of nisin, are most likely formed (Fig. 3, example C). In such studies, the lack of production in their wild-type producer strains in the conditions tested has limited a fair comparison between the final products and those produced in the heterologous expression system. This approach is of particular interest in cases in which either the sequence has been identified in metagenomic data, or when the natural producer microorganism is not culturable. Apart from the nisin biosynthesis machinery the prochlorosin and lacticin 481 machineries have been decoupled from their natural substrates and can be used for this purpose (Zhang *et al.* 2012). The prochlorosins are a good example of this decoupling in nature since one modification enzyme (ProcM) is able to modify 27 different core peptides, yet with high regioselectivity, spread in the genome of the producer organism and producing linear or globular lantibiotics (Tang and van der Donk 2012; Zhang *et al.* 2014). ProcM can also successfully modify other lantibiotics from diverse bacteria fused to one of its cognate leader peptides. Also a *Streptococcus pneumoniae* strain uses this strategy to produce four different lantibiotics with a single LanM enzyme and outcompetes other streptococci in animal models (Maricic *et al.* 2016). The success of this method depends on the promiscuity of the modification enzymes used and the correct prediction of the leader cleavage site. For instance, the promiscuity of different LanM enzymes can greatly vary (Thibodeaux, Ha and van der Donk 2014) (as was shown for HalM2 and ProcM, highly selective and promiscuous, respectively) and limit the applicability of this approach. Different reports point at a coevolution of PTM modification enzymes and substrates (Zhang *et al.* 2012, 2014; Tang, Thibodeaux and van der Donk 2016). Therefore, a possibility to further increase the success rate of this approach might be using a well-characterized enzyme that phylogenetically resembles the wild-type PTM enzyme(s) of the core peptide that has been identified. The success of this method also depends on the correct prediction of the leader cleavage site when the fusions between the characterized leader peptide with the newly identified core peptide are designed and the selection of an appropriate leader peptidase (see above).

INCREASING THE EXTENT OF MODIFICATION

The high structural diversity found in lantibiotics originates from the PTMs that they undergo. Next to lanthionine incorporation, about 15 different additional modifications have been described and for 9 of these modifications the responsible enzymes have been identified. By defining these enzymes as separate modules to extend the library of PTMs, one can envision an endless number of different lantibiotic production systems. For example, it was shown that two enzymes originating from lantibiotic biosynthesis, i.e. a hydrogenase converting Dha into D-alanine and a C-terminal decarboxylase producing aminovinylcysteine, could be implemented in *Lactococcus lactis* to work in conjunction with an engineered lanthionine introduction system (van Heel *et al.* 2013b). In this case, novel modifications were introduced into another lantibiotic (i.e. nisin) that naturally does not possess these modifications. A similar approach was also recently used for the production of D-alanine-containing lantibiotics in *Escherichia coli* and *in vitro* using a different LanJ enzyme (Yang and van der Donk 2015b) coupled to non-cognate substrates and PTM enzymes. In this report, the assessment of the chirality in the reduced Dha was thoroughly studied and a clear D-alanine was identified.

Such systems can also be used for the production of lantibiotics fused to a heterologous leader peptide in a plug-and-play system that requires the additional PTMs to become fully active. As a proof of principle, van Heel *et al.* (2013b) achieved the production of gallidermin fused to the nisin leader peptide combining the nisin modification machinery (NisBTC) and the decarboxylase GdmD. The three main challenges for this approach are: (i) understanding the substrate requirements for the PTM enzyme, (ii) the availability of sufficient active enzyme, (iii) adequate production levels of the desired peptide. Hence, the success of this strategy heavily depends on fundamental knowledge of RiPPs and their PTM enzymes. The definition of the sequence that these enzymes recognize or the necessity for a leader peptide will facilitate their application on non-cognate peptides. Several studies probed the substrate tolerance of the C-terminal decarboxylase EpiD from the epidermin gene cluster (Kupke *et al.* 1995), the lactate dehydrogenase ElxO from the epilancin 15X cluster (Ortega *et al.* 2014) or the reductases LtnJ from the lacticin 3147 cluster and NpnJ from *Nostoc punctiforme* (Zhang *et al.* 2014; Mu *et al.* 2015; Yang and van der Donk 2015b). Studies gaining insights on minimal leader peptide requirements (Cheung, Pan and Link 2010; Plat *et al.* 2011) and mechanistic information on wild-type or improved engineered enzymes (Oman *et al.* 2012; Dunbar and Mitchell 2013; Sardar *et al.* 2015) are also providing the required information, not only in the lantibiotic field but also in other RiPP classes.

In addition, varying the culture conditions can also affect the PTM enzymes and lead to improvements in the lantibiotic biosynthesis. The replacement of chloride ions in the growth medium of microbisporicin by other halogens led to the isolation of brominated microbisporicin. This variant shows an increased potency compared to the chlorinated one (Cruz *et al.* 2015).

A different approach to achieve a larger chemical diversity in lantibiotics is the incorporation of non-canonical amino acids (ncAAs) *in vivo* (see below for ncAAs incorporation *in vitro* in

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lantibiotics). Remarkably, this method is compatible with the insertion of additional PTMs into lanthionine-containing antimicrobials. The insertion of ncAAs into lantibiotics *in vivo* has been achieved in *E. coli* using the lichenicidin biosynthesis machinery in an auxotrophic strain (Oldach *et al.* 2012) and by codon reprogramming in the production of prochlorosins (Shi *et al.* 2011) and lacticin 481 (Bindman *et al.* 2015). In the last case, the inserted ncAA was a hydroxyacid that facilitates leader peptide removal by alkalyne hydrolysis of the ester bond without interfering with the PTM enzyme. Recently, Zhou *et al.* (2016b) achieved the incorporation of Trp-analogs in nisin by using a cross-feeding system in an auxotrophic *L. lactis*, paving the way for many more amino acid analog incorporations in this multi-auxotrophic strain.

COMBINATORIAL APPROACHES TO GENERATE NOVEL STRUCTURAL PEPTIDES

Lantibiotic structural peptides consist of several parts with distinct functions. For example, nisin can be dissected into four parts, i.e. the modification enzyme interaction and leader cleavage site in the leader peptide, and the lipid II binding- and membrane insertion domains in the core peptide (Rink *et al.* 2007). In class II lantibiotics, the structural motif responsible for lipid II binding is also described (TxS/TxD/EC motif) and so is the interaction between the two-component lantibiotic lacticin 3147 with lipid II (Wiedemann *et al.* 2006), their pore-forming moiety and the sequences responsible for the interaction with each other. It is possible to combine various structural modules of different lantibiotics and get active hybrids as in the case of nisin and subtilin produced in *Bacillus subtilis*, that was successfully processed into the expected active structure (Chakicherla and Hansen 1995) (Fig. 3, example D). Additional modules might need additional PTMs to be fully active as is the case of a nisin-gallidermin hybrid that was produced expressing the nisin biosynthesis machinery together with GdmD to obtain full activity (van Heel *et al.* 2013b) (Fig. 3, example E). The activity profile and mass suggest that the modifications were correctly installed. The rational design of lantibiotic core peptides to improve their activity has also been proven for nisin by modifying the length of the hinge region and, therefore, playing with the ability to insert in the membrane of species with different membrane thicknesses. This approach has rendered variants with improved activity against certain pathogens including *Enterococcus faecalis*, *Listeria monocytogenes* or *B. cereus* (Zhou, van Heel and Kuipers 2015).

The extension of lantibiotic activities with other functionalities could be achieved. Previously, the two antimicrobial peptides enterocin CRL35 and microcin V, belonging to the class II bacteriocins, have been linked by a flexible linker which resulted in an extended antimicrobial spectrum (Acuña *et al.* 2012). Similarly, the combination of part of nisin with peptide sequences that are known to traverse the outer membrane of Gram-negative bacteria yielded a nisin-apidaecin hybrid with improved MIC values against *Escherichia coli* despite the reduced activity against *Lactococcus lactis* (Fig. 3, example F) (Zhou *et al.* 2016a). Research to link lantibiotics (or parts thereof) *in vivo* to other classes of antimicrobial compounds is currently ongoing.

IN VITRO APPROACHES FOR THE PRODUCTION OF NOVEL LANTIBIOTICS

The methodology for synthesis of (modified) peptides has progressed substantially in the last years. Still, the PTMs present in lantibiotics are a challenge for chemical synthesis, especially obtaining the correct bridging pattern with the original regio- and stereoselectivity, which is essential for the antimicrobial activity and determined by both the enzyme and the substrate (Tang *et al.* 2015). Full synthesis of nisin and other lantibiotics has been achieved using several different methods being solid phase synthesis the most promising one (Tabor 2011). These new methodologies enabled the replacement of lanthionine bridges by structural analogs which often reduced or abolished the antimicrobial activity (Tabor 2011, 2014). However, chemical synthesis remains a costly process compared to biological production (Ongey and Neubauer 2016).

Another *in vitro* strategy, developed by the van der Donk group, is to use purified PTM enzymes to modify linear (synthetic) precursor peptides that do or do not contain ncAAs (Levengood *et al.* 2009). The *in vitro* synthesis using lantibiotic synthetases has been achieved with several class II synthetases and with the class I cyclase NisC (Li *et al.* 2006) and recently with the dehydratase NisB (Garg, Salazar-Ocampo and van der Donk 2013). Other PTM enzymes have also been tested for this purpose, such as NpnJ (Yang and van der Donk 2015b). Similarly, other lanthipeptide biosynthesis enzymes could potentially be used to produce labionine or lanthionine rings with different directionality and stereospecificity (Krawczyk *et al.* 2012; Tang and van der Donk 2013). The insertion of ncAAs can improve the stability or potency (Knerr *et al.* 2012) of the modified lantibiotic, can facilitate lantibiotic activation by removal of the leader peptide (e.g. using a photocleavable linkage (Bindman *et al.* 2010)) and can serve as a starting point for click-chemistry or other reactions to attach a variety of moieties (Hoesl and Budisa 2011; Budisa 2013). Another starting point for chemical additions are (enzymatically) modified proteinogenic amino acids, such as lysine or N-terminal dehydrated amino acids as was demonstrated earlier (Yoganathan, Sit and Vederas 2011; Bindman and van der Donk 2013). Chemically derivatized nisin can be linked by copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) to different groups such as fluorophores or another nisin molecule by intramolecular bridges (Slootweg *et al.* 2013). Using a similar method, the lipid II-binding motif of nisin was covalently linked to vancomycin increasing the activity of the conjugate against vancomycin resistant *Enterococcus faecalis* (*Arnusch et al.* 2008). The chemical ligation of rings AB of nisin (able to bind lipid II but inactive by themselves) to lipidic moieties, by either CuAAC or direct linkage of a lipid amine to the C-terminus, rendered molecules with high resistance to proteases and potent activity. It is noteworthy that the linkage of terphenyl yielded molecules with an activity similar to that of wild-type nisin (Koopmans *et al.* 2015).

Noteworthy, the chemical modification of lantibiotics by semisynthesis can drastically improve the pharmacokinetic and pharmacodynamic properties. Following this approach, the amidation of carboxylic groups of actagardine has yielded compounds with improved pharmacological applications to feed the preclinical pipeline (Malabarba *et al.* 1990; Boakes *et al.* 2016).

CONCLUSION

Lantibiotics have been repeatedly reported as efficient antimicrobials targeting (multidrug resistant) pathogens *in vitro*. The available data regarding their effect *in vivo* are promising and highlight their potential. Developing novel lantibiotics that are active against Gram-negative bacteria, or specific pathogens, will increase their applicability. Additionally, improving their

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physicochemical properties in terms of stability or bioavailability depending on the envisioned use is desirable.

The application of synthetic biology principles to lantibiotic research enables the production of a large variety of compounds with great chemical diversity. The natural modular design of lantibiotic peptides and the modularity of their PTMs can be exploited to produce novel antimicrobial compounds (Fig. 3, example C). One can envision that modification enzymes from other classes of RiPPs can be used to increase the structural diversity of hybrid compounds further. For this, efficient decoupling of substrates and their PTMs is required, underlining the need for obtaining further mechanistic insight into PTMs. Studies gaining insights on minimal leader peptide requirements (Cheung, Pan and Link 2010; Plat *et al.* 2011) and mechanistic information on wild-type or improved engineered enzymes (Oman *et al.* 2012; Dunbar and Mitchell 2013; Burkhart *et al.* 2015; Sardar *et al.* 2015) are providing the required information. The identification of the leader recognition motif in PTM enzymes can lead to the rational design of combined leader peptide parts that allow modification by PTM enzymes from different RiPP classes (Burkhart *et al.* 2015). Additionally, for the production of these compounds efficient systems that are compatible with the desired PTMs are needed.

Thus, a large library of potential substrates can be created based on genome mining, combinatorial synthesis, mutagenesis, click-chemistry or a combination thereof. Combining these substrates with diverse (enzymatic) PTMs will result in the production of new molecules with desired properties. As it is not predictable which combinations of modules will render the desired molecules, success greatly depends on the number of combinations that can be tested. Therefore, efficient screening methods are required to select for compounds with the desired properties (Lewis 2013). Recently, several screening methods suitable for antibiotic screening have been published based on particle sorting with cells inside (Eun *et al.* 2011). The approaches discussed above show that it is now feasible to create novel (additionally modified) lantibiotics from synthetic or genomic origin that can help satisfy the need for novel antimicrobials.

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