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Bacterial pathogens: threat or treat (a review on bioactive natural products from bacterial pathogens)†

Fleurdeliz Maglangit, *[ab] Yi Yu *[c] and Hai Deng *[b]

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Threat or treat? While pathogenic bacteria pose significant threats, they also represent a huge reservoir of potential pharmaceuticals to treat various diseases. The alarming antimicrobial resistance crisis and the dwindling clinical pipeline urgently call for the discovery and development of new antibiotics. Pathogenic bacteria have an enormous potential for natural products drug discovery, yet they remained untapped and understudied. Herein, we review the specialised metabolites isolated from entomopathogenic, phytopathogenic, and human pathogenic bacteria with antibacterial and antifungal activities, highlighting those currently in pre-clinical trials or with potential for drug development. Selected unusual biosynthetic pathways, the key roles they play (where known) in various ecological niches are described. We also provide an overview of the mode of action (molecular target), activity, and minimum inhibitory concentration (MIC) towards bacteria and fungi. The exploitation of pathogenic bacteria as a rich source of antimicrobials, combined with the recent advances in genomics and natural products research methodology, could pave the way for a new golden age of antibiotic discovery. This review should serve as a compendium to communities of medicinal chemists, organic chemists, natural product chemists, biochemists, clinical researchers, and many others interested in the subject.

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[a]*Department of Biology and Environmental Science, College of Science, University of the Philippines Cebu, Lahug, Cebu City, 6000, Philippines. E-mail: ffmaglangit@up.edu.ph; Tel: +63 32 232 8185*

[b]*Department of Chemistry, University of Aberdeen, Aberdeen AB24 3UE, UK. E-mail: h.deng@abdn.ac.uk; Fax: +44 (0)1224 272291; Tel: +44 (0)1224 272953*

[c]*Key Laboratory of Combinatorial Biosynthesis and Drug Discovery (MOE), Hubei Province Engineering and Technology Research Centre for Fluorinated Pharmaceuticals, School of Pharmaceutical Sciences, Wuhan University, Wuhan 430071, China. E-mail: yu_yi@whu.edu.cn; Tel: +86 27 68752491*

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1. Introduction

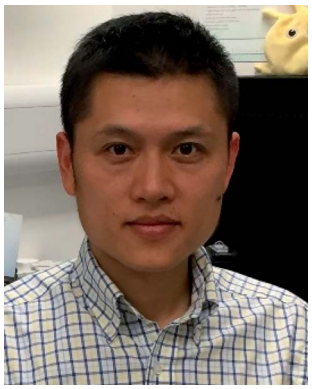
Antimicrobial resistance (AMR) is amongst the major threats to public health and poses a huge economic burden on global health care. The World Health Organization (WHO) has recently published the priority list of drug-resistant bacteria that pose the greatest danger to human health,[1,2] and among these, a majority of Gram-negative bacteria, including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacteriaceae. Resistance has emerged to all clinically used antibiotics including those of "last-resort" such as colistin and polymyxin B, and continues to rise at alarming rates.[3,4]

Despite the severity of the situation, the number of new chemical entities in the antibiotic development pipeline is in substantial decline. Nearly all the classes of antibiotics currently in clinical use were discovered during the 'golden era' (1940s–1960s), with several new drugs that are chemically tailored analogues from existing scaffolds.[5] The problem is compounded by the fact that bacteria are evolving resistance at a faster pace than antibiotic development.[6,7] The last new class of antibiotics that target the Gram-negative bacteria are the synthetic fluoroquinolones which were introduced into the clinic about 50 years ago.[8,9] The high rate of the rediscovery of old known molecules in traditional natural product (NP) screening platforms makes this grim situation even worse. Thus, the research community must find new sources of NPs to cope with the looming antibiotic crisis.

Pathogenic bacteria have shown to be rich sources of novel compounds, yet they remained untapped and understudied.[10–13] Virulence factors involved in their pathogenicity have been the subject of extensive study for many decades.[14–23] In recent years, however, it has become apparent that entomopathogenic, phytopathogenic and human and animal pathogenic bacteria are prolific sources of structurally novel and highly bioactive druggable molecules.[11,12]

Threat or treat? While pathogenic bacteria pose a threat to insects, plants, and humans, they also represent gold mines of potential pharmaceuticals to treat various diseases.[11,12,24,25] The opportunistic human pathogen, *Staphylococcus aureus* is a classic example. Despite being a threat, they produce potent bacteriocins (also known as staphylococcins) and several other compounds active against a wide variety of Gram-positive bacteria.[25]

Dr Fleurdeliz Maglangit finished her Bachelor of Science in Chemistry at the University of the Philippines in the Visayas – Miagao Iloilo. With her continued pursuit for learning, she obtained two Masters degrees, Master in Chemistry and Master of Science in Environmental Studies. She just recently earned her Ph.D. in Chemistry at the University of Aberdeen, Scotland, UK last July 2020. Her PhD work was mainly focused on natural products discovery and biosynthesis. In her research, she was able to isolate novel compounds with excellent antibacterial bioactivities from Streptomyces *bacteria. Currently, she is an Assistant Professor in Chemistry at the University of the Philippines Cebu. She hopes to discover novel bioactive natural products with potential for further drug development using local samples.*



Dr Yi Yu obtained his Bachelor's and Master's degrees in Biotechnology (2002) from Huazhong Agricultural University. He received his Ph.D. degree in Genetics (2007) at the Institute of Microbiology, Chinese Academy of Sciences (CAS). He then undertook postdoctoral research with Professor Wen Liu at Shanghai Institute of Organic Chemistry CAS. In 2010, he joined the School of Pharmaceutical Sciences, Wuhan University, and was promoted to professor in 2016. Now, he is the Director of the Institute of Traditional Chinese Medicine and Natural Products. His research interests centre around deciphering the biochemical logic and molecular machinery of bacterial and plant natural product biosynthesis.

Dr Hai Deng studied chemistry for his Bachelor's and Masters' degrees in China. In 1999, he came to the UK to pursue his Ph.D. in the field of biochemistry and biotransformations at the University of Wales, Swansea. From 2002–2008, he was a postdoctoral researcher of Professor David O'Hagan in University of St Andrews. He was appointed as a lecturer at the Department of Chemistry, University of Aberdeen in 2008, and promoted to senior lecturer in 2014 and reader in 2018. His current research includes discovery of novel bioactive natural products from various sources, tracing biosynthesis pathways, identifying novel enzyme activities and enzyme mechanism. He became the Fellow of Royal Society of Chemistry in 2017.

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Microbial genome-level studies and metabolomic approaches have further revealed the untapped biosynthetic potential of the diverse and underexplored group of pathogenic bacteria. Bacterial genomics has shown that they not only encode for virulence factors but also potential leads for drug development.[11,12] However, it has been estimated that only a very small portion of this gold mine had just been discovered, and that further drug leads or pharmacophores could be mined given the application of suitable and sufficient resources.[11] Thus, this review intends to explore the role that pathogenic bacteria could play in the search for novel compounds and scaffolds. This review should serve as a compendium to communities of medicinal chemists, organic chemists, natural product chemists, biochemists, clinical researchers, and many others interested in the subject.

2. Scope of the review

This review surveys the natural products (NPs) isolated from entomopathogenic, phytopathogenic, human, and animal pathogenic bacteria with antibacterial and/or antifungal activity, highlighting those NPs or NP-modified molecules currently in pre-clinical trials or those with potential for future drug development. These include the polyketides (PKSs), non-ribosomal peptides (NRPs), peptide–polyketide hybrid metabolites, and ribosomally-synthesised and post-translationally modified peptides (RiPPs). Selected unique and interesting pathways involved in their biosynthesis and the key roles they play in pathogenesis (where known) are also summarized.

Entomopathogenic bacteria such as *Photorhabdus* spp., *Xenorhabdus* spp., and *Serratia marcescens* are the focus of the review. The period from 2017 to the second quarter of 2020 saw a huge rise in the number of bioactive NPs from *Photorhabdus* spp. and *Xenorhabdus* spp. that are not covered in previous synopses,[12,26] and thus they are the emphasis in our review. It is worth noting that the honeybee pathogen, *Paenibacillus larvae* also appears as a rich, yet largely understudied source of novel and structurally diverse NPs. The readers are referred to the review by Müller, *et al.* (2015) which details the metabolites identified from *P. larvae*.[27] Although a rich source, no new metabolite has been identified from this bacterium since 2015.

Phytopathogenic bacteria such as *Burkholderia* spp., *Clostridium puniceum*, *Dickeya* spp., *Erwinia amylovora*, *Pseudomonas syringae*, *Streptomyces scabies*, and *Xanthomonas* spp. are among the prolific NP producers, and thus they are the topic of this review. The NPs from the diverse genus *Burkholderia* is summarized in a recent review.[28] Another review provided the genomics perspective of NP biosynthesis in phytopathogenic bacteria *E. amylovora*, *Xanthomonas* spp., *S. scabies*, *P. syringae*, and *Dickeya* spp.[11] Hence, in this review we aim to update and complement previous synopses and cover only those NPs that show the most interesting bioactivities or those that have not been mentioned by Baldeweg, *et al.* (2019)[11] or Kunakom and Eustáquio (2018).[28] Furthermore, we included the phytopathogen *C. puniceum* not mentioned in the above reviews for it produces potent metabolites with antimicrobial activity in nanomolar concentration.

We also explore the human and animal pathogenic bacteria such as *Nocardia* spp., *Staphylococcus* spp., *Streptococcus mutans*, and *Yersinia ruckeri* as sources of antimicrobials with therapeutic potential. These bacteria have been shown to produce structurally diverse NPs with potent bioactivities.[29–33] The antimicrobials from *Nocardia* spp. and bacteriocins from *Staphylococcus* spp. have been summarized in recent reviews,[25,33] and thus those NPs with remarkable activities from these bacteria were highlighted. Finally, we provide a thorough compilation of the antimicrobial NPs from bacterial pathogens, *Burkholderia* spp., *C. puniceum*, *Dickeya* spp., *E. amylovora*, *Nocardia* spp., *Photorhabdus* spp., *P. larvae*, *Pseudomonas* spp., *Staphylococcus* spp., *S. marcescens*, *S. mutans*, *Streptomyces* spp., *Vibrio* spp., *Xanthomonas* spp., *Xenorhabdus* spp., and *Yersinia ruckeri* (see Table S1 in the ESI† of this article listed in alphabetical order). We also provide their mode of action (molecular target), activity, and minimum inhibitory concentration (MIC) towards bacteria and fungi (where known), in the pursuit to demonstrate the exceptional biosynthetic ingenuity of the underexplored source of pathogenic bacteria for the production of novel and druggable chemical entities.

3. Pathogenic bacteria as novel sources of antimicrobial discovery

Pathogenic bacteria are master engineers of highly diverse and biologically active molecules. To thrive and survive in highly competitive and resource-limited microbial communities, pathogenic bacteria have developed an approach to protect themselves by producing a plethora of structurally diverse metabolites that have been fine-tuned by the producing organism to have potent and selective biological activities.[25,34] It is believed that pathogenic bacteria exploit these molecules to regulate virulence and persistence during infections. Additionally, the vast array of antibacterial armamentarium is thought to fight off predators, compete for nutrients, and protect their host. Other roles have also been suggested such as signalling and quorum sensing, gene expression, stress response, cellular growth and iron acquisition.[12,35]

Pathogenic bacteria represent exceptionally prolific sources of potential therapeutics as indicated in their genomes, yet they have been largely ignored.[11,36] Here, we present an overview of the antimicrobial NPs produced by entomopathogenic, phytopathogenic, and human and animal pathogenic bacteria, and highlight a selection of metabolites with antibiotic activity that show promising potential for future development (Fig. 1).

3.1 Entomopathogenic bacteria

Previously regarded as overlooked and neglected sources, the entomopathogenic bacteria have received considerable interest in the last 15 years owing to the novel druggable chemical entities they generate.[13,34] Those that have been described recently as prolific NP producers include *Photorhabdus* spp. and *Xenorhabdus* spp., *S. marcescens*, and *P. larvae*.

Members of the genera *Photorhabdus* and *Xenorhabdus* (*Enterobacteriaceae*) produce a wide array of NPs to support

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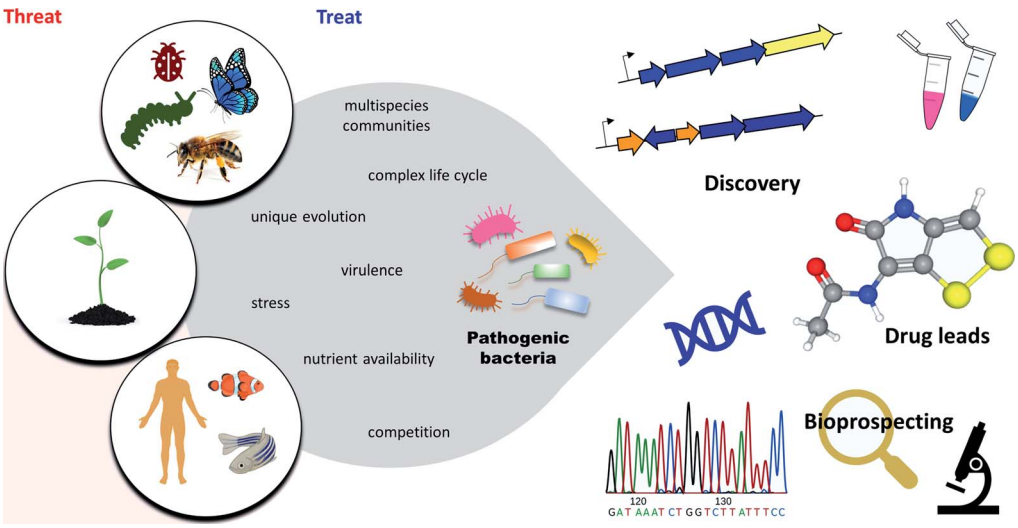

Fig. 1 Overview of pathogenic bacteria. Despite a threat to insects, plants, animals, and humans, pathogenic bacteria represent novel sources of potential pharmaceuticals to treat various diseases.

a complex life cycle involving insect pathogenesis and nematode symbiosis with *Heterorhabditis* spp. and *Steinernema* spp., respectively.[37] The antimicrobial compounds produced by these bacteria are non-toxic to the nematode, but lethal to several insect pathogens and other opportunistic microbes that are direct food competitors.[24] This indicates the production of antimicrobials with favourable toxicity, good pharmacokinetics, and are likely druggable and safe to eukaryotic organisms. *Serratia marcescens* is a Gram-negative, facultatively-anaerobic bacterium (*Enterobacteriaceae*) often associated with insect infection.[38] Several insects are susceptible to *Serratia* species, including crickets, grasshoppers, locusts, cockroach, termites, beetles, butterflies, moths, fruit fly, wasps,[39] and recently has been discovered as being pathogenic to bees.[40] Some members of *S. marcescens* also cause opportunistic nosocomial infections of the respiratory tract, urinary tract, brain, meninges, heart, and wounds.[39,41] Despite a threat, *S. marcescens* has been shown to produce not only the characteristic red pigment prodigiosin but also a huge repertoire of antimicrobial compounds.[41] *Paenibacillus larvae* is a Gram-positive bacterium that causes fatal intestinal infection of honeybee larvae, called American Foulbrood (AFB). This pathogen spreads very rapidly and poses various threats of different severity leading to massive losses of entire bee colonies. *P. larvae* secretes a broad spectrum of antibacterial compounds that are critical virulence factors and also, relevant in the quest for new bioactive compounds for drug development. Readers are referred to the recent review by Müller, *et al.*[27]

It should be mentioned that several other entomopathogenic bacteria such as *Bacillus thuringiensis* and *Pseudomonas entomophila* have the capacity to produce NPs based on their genome sequences but have not been mined further for NP production.[42,43]

3.2 Phytopathogenic bacteria

Plant pathogenic bacteria can have detrimental effects on plant growth, productivity, and yield. They affect a wide range of crops posing a threat to global food production. Hundreds of phytopathogenic bacteria have been identified to date,[44–46] but only a few have been explored for natural product discovery.[11]

Clostridium puniceum, the only known plant pathogenic bacterium from the diverse genus *Clostridium* to date,[44–46] causes potato slimy rot, manifested by the formation of pink pigments by the bacterium.[47] All *Dickeya* species (formerly *Erwinia chrysanthemi*) cause economically important diseases on different plant hosts worldwide.[14,48] *D. zeae* causes soft rot in a variety of plants (*e.g.* potato, chicory, maize, banana, rice). *Erwinia amylovora* is the causative agent of fire blight, a destructive disease of *Rosaceae* plants such as apple and pear trees[49] that is typically accompanied by the development of black necrosis.[50] Historically, *E. amylovora* is the first characterised bacterial plant pathogen.[51] *Pseudomonas* spp. produce a wide spectrum of phytotoxic compounds. *P. syringae* pathovars are the topmost phytotoxic-producing bacteria among all *Pseudomonas*, and all phytopathogens identified to date.[14,52] *Streptomyces* species are particularly renowned for their ability to produce numerous bioactive NPs.[53–58] Several *Streptomyces* strains, however, are phytopathogenic and can cause potato common scab diseases such as *S. caviscabies*, *S. acidiscabies*, *S. turgidiscabies*, and *S. scabies*.[21,59] Among the most notable pathogens of the genus *Xanthomonas* are *X. albilineans*, the causative agent of leaf scald disease on sugar cane[60] and *X. campestris*, the causal agent of black rot of crucifers that affects all cultivated brassicas.[14] Members of the genus *Burkholderia* include strains that can either be beneficial or harmful. Some strains are pathogenic to plants such as *B. glumae*, which causes rice rot, while others cause opportunistic human infections such as the strains of *Burkholderia cepacia* complex (Bcc), which

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include *B. pseudomallei* and *B. mallei*. For detailed information on the diverse *Burkholderia* genus, refer to the recent review.[28]

Virulence-mechanisms of plant pathogenic bacteria have been the subject of several different reviews.[14,21,28,52,59] Despite being a threat to agriculture, phytopathogens *C. puniceum*, *Dickeya* spp., *E. amylovora*, *Pseudomonas* spp., *Streptomyces* spp., *Xanthomonas* spp., *Burkholderia* spp. – some of which belong to the top 10 most important plant pathogenic bacteria[14] – also serve as huge arsenals for potent drug leads. Genome analyses disclosed that their biosynthetic machinery encodes not only for virulence factors but also for antibiotic-like metabolites with no plant disease-associated function.[11] Furthermore, some phytotoxins were found to exhibit potent antimicrobial properties.[11,28,47]

3.3 Human and animal pathogenic bacteria

While the antimicrobials from non-pathogenic strains are studied in-depth, knowledge of the structural and mechanistic diversity of antibiotics particularly from human and animal pathogenic bacteria is limited. Here, we provide an overview of the potential chemistry to be uncovered from the opportunistic pathogens, *Nocardia* spp., *Staphylococci*, *S. mutans*, *Vibrio* spp., and *Y. ruckeri*.

Many different species of *Nocardia* have been identified, and many of these are pathogenic to humans and animals. To date, more than 50 *Nocardia* species are clinically significant.[61] Of these, *N. brasiliensis*, *N. abscessus*, *N. transvalensis*, *N. terpenica*, and *N. pseudobrasiliensis* have been identified to be prolific microbial sources of bioactive novel compounds.[33]

Staphylococci represent the normal flora of the skin and mucous membrane of human and animals.[62] There are more than 40 species, but few are important human pathogens such as *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, and *S. saprophyticus* implicated in various infections, especially in immunocompromised patients.[18] Though they pose a threat, they are also prolific producers of potent bacteriocins (also known as staphylococcins) exhibiting antibacterial activity against closely related species and a wide variety of Gram-positive bacteria.[25,63]

Streptococcus mutans is the major causative agent of human dental caries (tooth decay).[64] In addition to caries, *S. mutans* is also implicated in infective endocarditis, a lethal infection, and inflammation of heart valves.[65] Bacterial sequence analysis of *S. mutans* discloses a small genome (about 2 Mb) yet surprisingly harbours rich and diverse biosynthetic gene clusters (BGC) for the production of PKS, NRPS, hybrid PKS–NRPS, and RiPP metabolites.[66,67] Several bioactive NPs have recently been isolated from *S. mutans*.[30,68–78]

Vibrionaceae includes several species that cause intestinal (diarrhoea, cholera) and extra-intestinal (septicaemia, skin infection) illnesses in both humans and aquatic animals. Among the opportunistic *Vibrio* pathogens, *V. parahaemolyticus* has been shown to produce metabolites with remarkable bioactivity.[79]

Yersinia ruckeri is the etiological agent of yersiniosis or enteric redmouth (ERM) disease in marine and freshwater fish, particularly salmonids.[17] Infections due to *Y. ruckeri* cause high mortalities in fish, contributing to substantial economic losses in the aquaculture industry.[80] *Y. ruckeri* has also been isolated from human wound infection, however, it remains unclear whether *Y. ruckeri* or another bacterium caused the infection.[81] Interestingly, *Y. ruckeri* has been shown to produce the dithiolopyrrolone natural product, holomycin.[31,32]

4. Chemical diversity of antimicrobials produced by pathogens

Pathogenic bacteria produce numerous NPs with highly diverse structures made up of a handful of simple building blocks, usually derived from one or more primary metabolic pathways. These NPs can be classified into five different groups according to their biosynthetic origin: polyketides, nonribosomal peptides, polyketide–nonribosomal peptide hybrid metabolites, ribosomal peptides, and others. Since numerous NPs from pathogenic bacteria are known, only selected compounds with promising therapeutic potential are presented.

4.1 Polyketides

Polyketides, assembled by polyketide synthases (PKS), are among the largest classes of chemically diverse NPs, encompassing molecules such as macrolides, aromatics, and polyenes. The structural diversity exhibited by polyketides is exemplified by the broad spectrum of biological activities they possess, such as antibacterial, antifungal, and anticancer among others (Fig. 2 and Table S1†). PKSs occurring in bacteria are classified into three types (type I, II, and III) depending upon their structure and biochemistry. Type I PKSs are large multifunctional enzymes comprised of multiple functional domains as exemplified by borrelidin **1**, gladiolin **2**, erythromycin **3**, and brasilinolide A **4**. Type II PKSs are formed by discrete catalytic domains and are responsible for the biosynthesis of bacterial aromatic polyketides such as clostrubins **5–6** and nocardicyclin A **7**. Type III PKSs are simpler chalcone synthase-like proteins that catalyse the formation of the product within a single active site. Examples include chalcones, resorcinol, pyrones, and stilbenes (Fig. 2 and Table S1†).

Polyketides are biosynthesised from two-carbon acetate units derived from activated acetyl-CoA and malonyl-CoA in successive decarboxylative Claisen condensation reactions, in a manner analogous to fatty acid biosynthesis. Typically, this process involves the core domains comprising of the ketosynthase (KS_α and KS_β), malonyl/acyl transferase (AT), and a phosphopantethienylated acyl carrier protein (ACP) which serves as an anchor for the growing PK chain.[82] A series of post-PKS tailoring enzymes such as ketoreductase (KR), methyltransferase (MT), enoyl reductase (ER), and dehydratase (DH) can variously modify the polyketide backbone, either while the intermediates are still bound to the assembly line or after they are released. Installation of different polyketide starter and extender units also represents a significant route to add unusual moieties such as nitrile functionality, carboxylates, and

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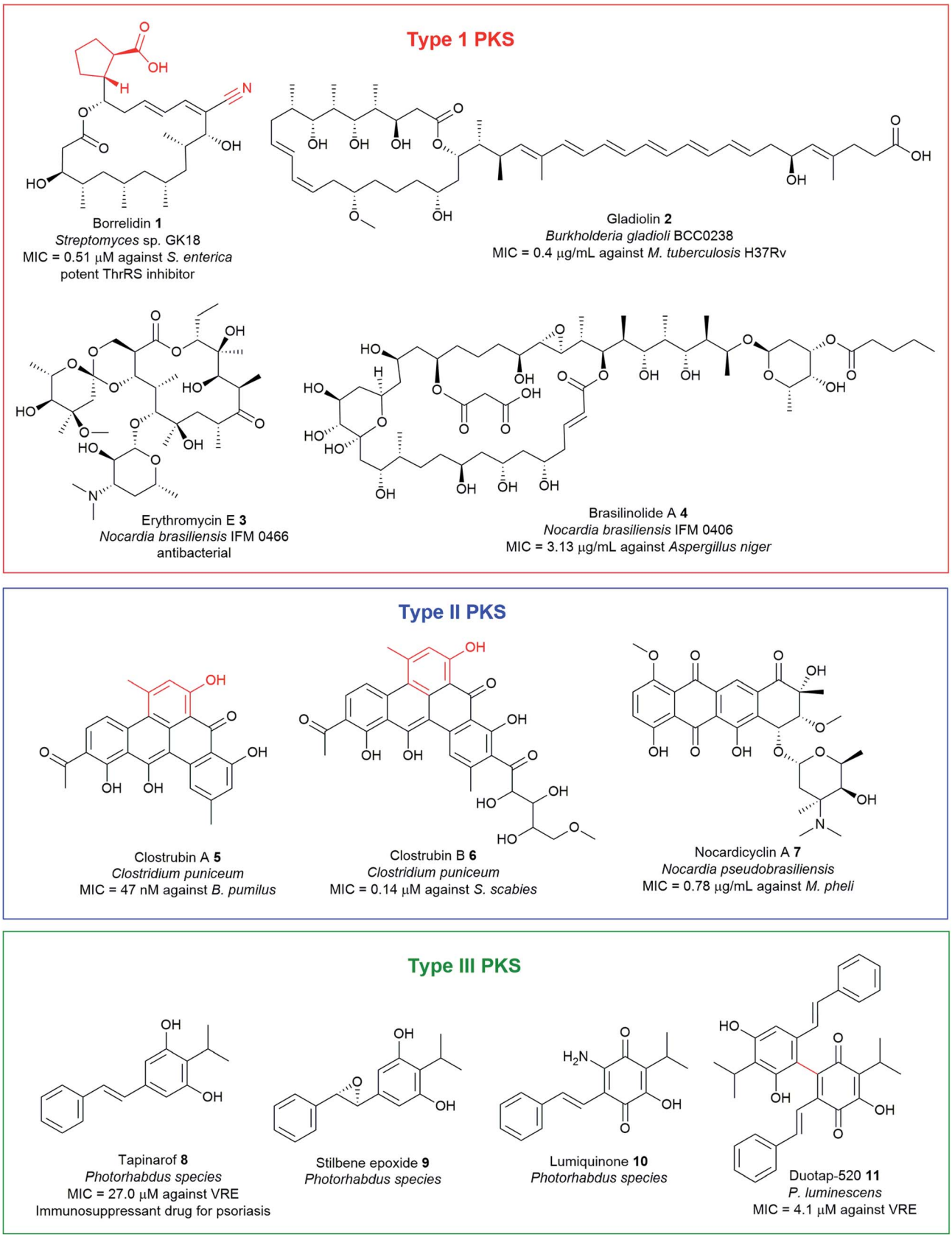

Fig. 2 Examples of antimicrobial polyketide natural products with unusual chemical motifs highlighted in red, isolated from pathogenic bacteria.

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branched-alkyl chains into polyketide scaffolds to generate mature final products with a high degree of chemical complexity and activity. The mechanistic enzymology of diverse polyketide assembly lines has been the subject of comprehensive reviews.[82,83] This section covers some representatives of interesting polyketide antimicrobials containing unusual chemical functionalities from pathogenic bacteria such as PKSI borrelidin **1**, PKSII clostrubins **5** and **6**, and stilbene-containing PKSIII metabolites **8–11**.

4.1.1 Borrelidin. Borrelidin **1** was first isolated from *Streptomyces rochei* in 1949 as an antibiotic exhibiting anti-*Borrelia* activity,[84] and then more recently as a product of the potato pathogen *Streptomyces* GK18 [85] and other *Streptomyces* species[86–90] as well as marine-derived microorganisms (Fig. 2 and Table S1†).[91–94] Borrelidin **1** features an 18-membered macrolide with a nitrile functionality.[95,96] To date, numerous analogues have been discovered including borrelidins B–O,[87,90,91,93,94] acetyl-borrelidin[89] as well as amide containing congeners, borrelidin CR1 and CR2.[92,93,97]

More than 30 nitrile-containing pharmaceuticals are currently marketed for a wide range of medical indications, including vildagliptin for diabetes and anastrozole for breast cancer treatment.[98] The nitrile functionality renders the molecule more water-soluble and less susceptible to oxidative metabolism in the liver.[98] Furthermore, nitrile moiety is rare in natural products, hence the biosynthetic mechanism of borrelidin, particularly the nitrile group has attracted significant interest. The biosynthesis of borrelidin proceeds through the typical pathway known for type 1 PKS to form the macrolide ring except for the unique *trans*-cyclopentane-(1*R*-2*R*)-dicarboxylic acid (CDPA) starter unit (Fig. 3). CDPA is likely derived from tyrosine or 4-hydroxyphenyl acetic acid (4-HPA) catabolism.[86] The nitrile formation in **1** may start from oxidation of the pendant methyl group in pre-borrelidin **1c** to an aldehyde **1e** catalysed by cytochrome P450, BorI, and alcohol dehydrogenase, BorK. This is followed by the conversion of the aldehyde to aminomethyl group (borrelidin B) **1b** catalysed by the putative aminotransferase, BorJ.[94] BorJ is related to CynN1 and CyaN1 aminotransferases in nitrile-containing cyanosporasides that typically act upon carbonyl groups, catalysing conversion to amines.[99] The aminomethyl intermediate **1b** is finally converted to the nitrile catalysed by the putative BorI and BorK enzymes *via* a series of oxidation and dehydration reactions. Mutants obtained by inactivation of either BorI or BorJ failed to generate any borrelidin but led to the production of pre-borrelidin **1c**, suggesting that BorI/J are responsible for nitrile biosynthesis.[86,94] Furthermore, the isolation of borrelidin B **1b** from a marine-derived *Streptomyces* strain supports the plausible mechanism of nitrile formation.[94]

Borrelidin is a potent threonyl-tRNA synthetase inhibitor.[100] Borrelidin **1** is active against a wide range of bacteria, including *Enterococcus faecalis*, *Micrococcus luteus*, *Enterococcus faecium*, *Proteus hauseri*, and *Klebsiella pneumoniae* (MIC = 0.5–65 μM).[90,91,93] Additionally, borrelidin exhibits 3× potent activity against *Salmonella enterica* (MIC = 0.51 μM), the causative agent of foodborne salmonellosis than the antibiotic ampicillin (MIC = 1.4 μM).[91] This remarkable activity has received considerable clinical interest in the search for privileged scaffolds that selectively target *S. enterica*. On the other hand, borrelidin C and D analogues with an additional hydroxy moiety in the cyclopentane ring are inactive against the tested bacteria and show reduced activity in *S. enterica* (MIC = 16–63 μM). SAR investigation of the borrelidin scaffold has

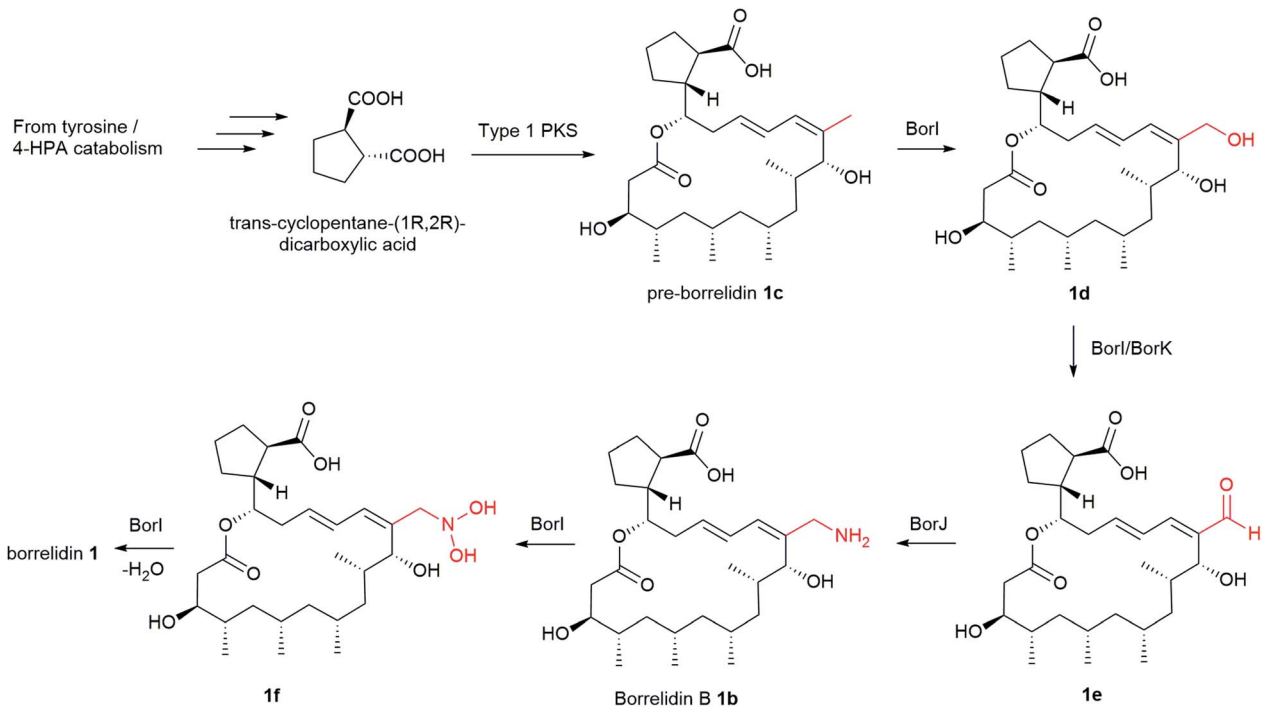

Fig. 3 Proposed nitrile formation in borrelidin biosynthesis.

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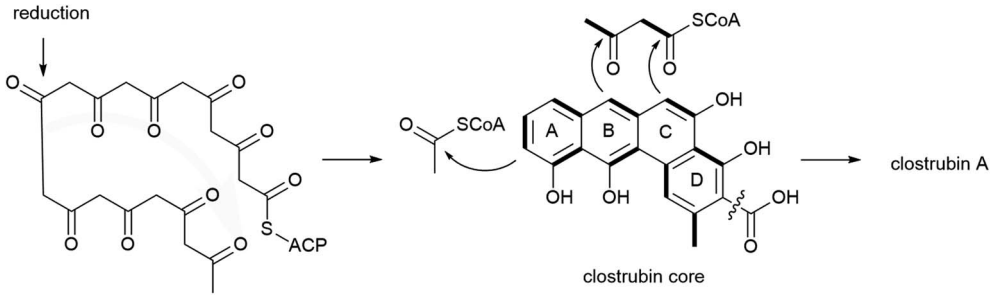

Fig. 4 Noncanonical polyketide cyclisation folding in clostrubin biosynthesis.

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indicated that the vinylic nitrile and the carboxylic acid moieties are essential for the activity.[87,90,93,94,101]

4.1.2 Clostrubins. Clostrubin A **5** was first isolated as a deep purple pigment from the strictly anaerobic bacterium, *Clostridium beijerinckii* (HKI0724) in 2014 (Fig. 2 and Table S1†).[102] A year later, clostrubin A **5** and its related compound clostrubin B **6** were identified from the potato cultures of *C. puniceum*.[47] Both compounds feature a highly unusual pentacyclic polyphenol with an exceptional benzo[*a*]tetraphene scaffold that is rare in anaerobes, and clostrubin B **6** differs from A **5** in the presence of an extra sugar-like linear side chain.[47]

The biosynthesis of clostrubins in the anaerobic *C. puniceum* is proposed to originate from type II PKS (*clr*) with high homology to the pentacyclic resistomycin (*rem*) PKS in aerobic bacteria, *Streptomyces resistomycificus* (Fig. 4).[103] Type II PKSs are very common in actinomycetes; only two examples of type II polyketides have been identified in non-actinomycete bacteria so far. Stable-isotope labelling experiments indicated that the striking perifused ring feature of clostrubin is formed from a noncanonical polyketide folding which delineates from the conserved cyclization patterns of typical angucylic decaketides from aerobic bacteria. Numerous tailoring enzymes catalyse diverse post-modification reactions, such as cyclodehydration steps and decarboxylation leading to a loss of one C1 carbon to afford **5**. Furthermore, labelling experiments suggest that the polycyclic core undergoes acetylation at ring A, and that ring E could be formed by condensation with an activated aceto-acetyl building block.[102] The benzo[*a*]tetraphene scaffold has also recently been identified in borolithochromes from the specimens of the Jurassic putative macroalgae *Solenopora jurassica* that has been preserved for over 150 million years, illustrating the evolutionary significance of clostrubin-type polyketides.[104]

Clostrubin A **5** displayed nanomolar potency against *Bacillus subtilis* (MIC = 75 nm) and superior antibacterial activity against several nosocomial pathogens, methicillin-resistant *S. aureus*, MRSA (MIC = 0.12 μM), vancomycin-resistant *Enterococcus*, VRE (MIC = 0.97 μM), and *Mycobacterium* including *M. smegmatis*, *M. aurum*, *M. vaccae*, and *M. fortuitum* (MIC = 0.12–0.48 μM) than the antibiotic ciprofloxacin.[102] Furthermore, when tested against some common potato disease-causing microbial pathogens like *Clavibacter michiganensis* subsp. sepedonicus (ring rot), *Bacillus pumilus* (soft rot), and *S. scabies* (common scab), clostrubin A **5** displayed nanomolar activity with MIC values of 47 nM, 95 nM, and 95 nM, respectively. Likewise, clostrubin B **6** displayed activity but weaker than clostrubin A **5** against the potato pathogens (MIC = 0.14–0.27 μM).[47]

Clostrubins **5–6** are not virulence factors but rather play dual roles beneficial to the anaerobic bacteria.[47,102] First, being potent antibiotics, they act as chemical arsenals to inhibit other microbial competitors in a resource-limited niche.[47,102] Second, clostrubins promote the survival of the anaerobic *C. puniceum* and *C. beijerinckii* in an oxygen-rich plant environment.[47] Taken together, clostrubins represent promising leads for the development of antibacterial agents for use in fighting off potato infections. Furthermore, the total synthesis of clostrubin was achieved,[105] which may provide insight into structure–activity relationships (SAR) to guide the development of novel antibiotics.

4.1.3 Stilbenes. Stilbenes, a class of polyphenols commonly found in plants, are characterised by the presence of 1,2-diphenylethylene nucleus known to exhibit diverse biological activities such as antioxidant, anticancer, antihyperglycemic, nematicidal, and antimicrobial activities.[106] *Photorhabdus* spp. is the only known bacterial producer of stilbenes, with two major products being 3,5-dihydroxy-4-isopropyl-*trans*-stilbene (also known as tapinarof **8**) and its stilbene epoxide (Fig. 2 and Table S1†).[107] Tapinarof (benvitimod) **8** is a topical non-steroidal anti-inflammatory drug (NSAID) used for the treatment of psoriasis and atopic dermatitis.[108] Its mode of action (MOA) is mediated by activation of the aryl hydrocarbon receptor (AhR) and nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) signaling pathways.[108] The AhR is a conserved ligand-dependent transcription factor involved in the regulation of the metabolism of drugs, xenobiotics, and endogenous small molecules. Nrf2 is involved in the cellular detoxification and defence against reactive oxygen species (ROS) and electrophilic cell stress.[109]

Although the carbon framework of stilbene monomers consists only of 1,2-diphenylethylene units, they demonstrate an enormous structural diversity because they are easily polymerized by oxidative coupling to produce diverse oligomers with intricate structures.[110,111] Since stilbenes possess strong antioxidant/radical scavenging properties,[106] their production in *Photorhabdus* spp. can be induced by supplementation of redox stress that generates reactive oxygen species. Feeding of paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) to *P. luminescens* and *P. asymbiotica* cultures under aerobic

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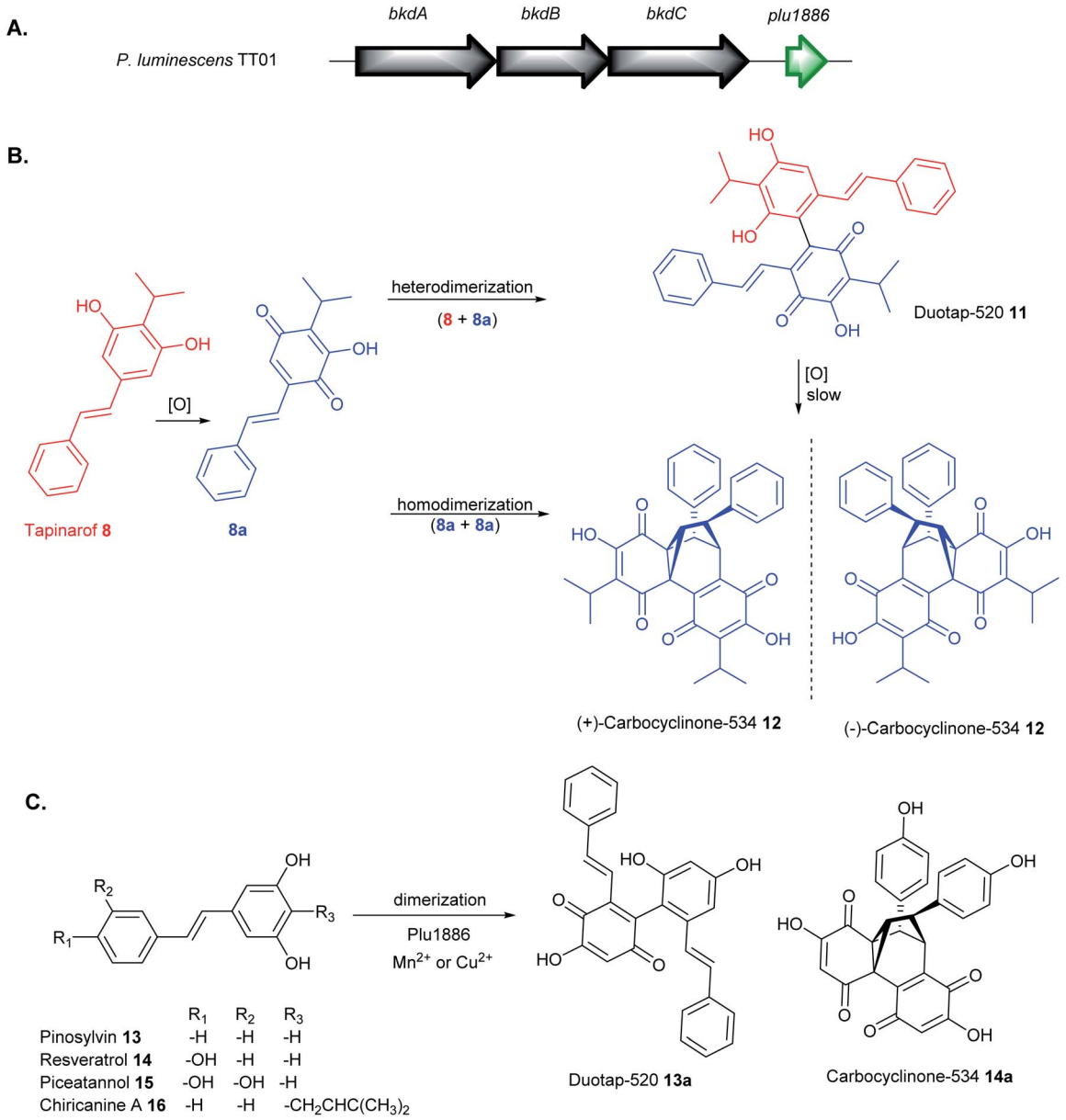

Fig. 5 (A) Annotation of Plu1886, which encodes a cupin enzyme, adjacent to known tapinarof biosynthetic genes in *P. luminescens* TT01 (B) proposed pathway for regioselective oxidative dimerization of tapinarof **8** to duotap-520 **11** and carbocyclinone-534 **12**, and (C) activity of Plu1886 enzyme with plant-derived stilbenes **13–16** in the presence of Mn^{2+} or Cu^{2+}.

conditions produced tapinarof **8** and its stilbene epoxide **9**,[107] lumiquinone **10** [112] and two novel tapinarof dimers, duotap-520 **11** and carbocyclinone-534 **12** (Fig. 5B).[113] Duotap-520 **11** contains a resorcinol–benzoquinone C–C bond linkage whereas carbocyclinone-534 **12** features a novel hexacyclic core with a cyclopropane bridge. The complex structure of **12** was elucidated by nuclear magnetic resonance (NMR) experiments, X-ray crystallographic analysis, and electronic circular dichroism (ECD) spectral measurements and characterised as a racemic mixture of (+)-carbocyclinone-534 and (−)-carbocyclinone-534 **12**.

Stilbene monomers such as resveratrol, isorhapontigenin, and piceatannol can undergo spontaneous oxidation and dimerization into an assortment of oxidized oligomers.[110,111] Likewise, it has been shown that the formation of tapinarof-derived products, duotap **11**, and carbocyclinone **12** involved similar oxidation, Diels–Alder cyclization, and dimerization mechanism (Fig. 5B). Under aerobic conditions, duotap **11** was shown to undergo slow spontaneous conversion into **12**. Furthermore, an orphan cupin-type protein, Plu1886 adjacent to tapinarof *bkd* BGC in *P. luminescens* TT01 was identified to enhance the transformation of tapinarof **8** to **11** or **8** to **12** *in vitro* (Fig. 5A).[113] Cupin superfamily of enzymes are widespread in plants and are known to catalyse numerous diverse oxidation reactions, often requiring metal cofactors (*e.g.* Ni^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}) for the activity.[114,115] *In vitro*

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enzymatic tapinarof conversion to carbocyclinone-534 **12** is highest in the presence of Mn^{2+} and to **11** in Cu^{2+}. Microaerobic cultures of Δ*plu1886* mutant showed a substantial decrease in carbocylinone **12** production relative to the WT, supporting its role to enhance tapinarof dimerization reactions.[113]

The bacterial Plu1886 enzyme shows substrate promiscuity towards plant-derived stilbenes such as pinosylvin **13**, resveratrol **14** (Fig. 5C). The cupin catalysed the robust conversion of pinosylvin **13** to the novel duotap **13a** and resveratrol **14** into its new carbocyclinone **14a** scaffold in the presence of Mn^{2+} or Cu^{2+}. The no-enzyme controls only showed a trace amount of dimer **14a** and an undetectable level of **13a**. The new enzyme-derived products **13a** and **14a** were purified and structurally confirmed by 2D NMR experiments. No duotap production from **14** or carbocyclinone production from **13** was observed and no derivatives corresponding to dimerization of piceatannol **15** or chiricanine **16**.[113]

Stilbenes are prolific sources of lead molecules in the search for new drugs and medicines. Even slight structural modifications of monomeric stilbenes dramatically alter their chemical complexity and improve their overall pharmacokinetic properties.[106] Duotap-520 **11** exhibited much higher potency against MRSA (MIC = 6.5 μM) and VRE (MIC = 4.1 μM) compared to tapinarof **8** with MIC values of 50.5 μM and 27.0 μM in MRSA

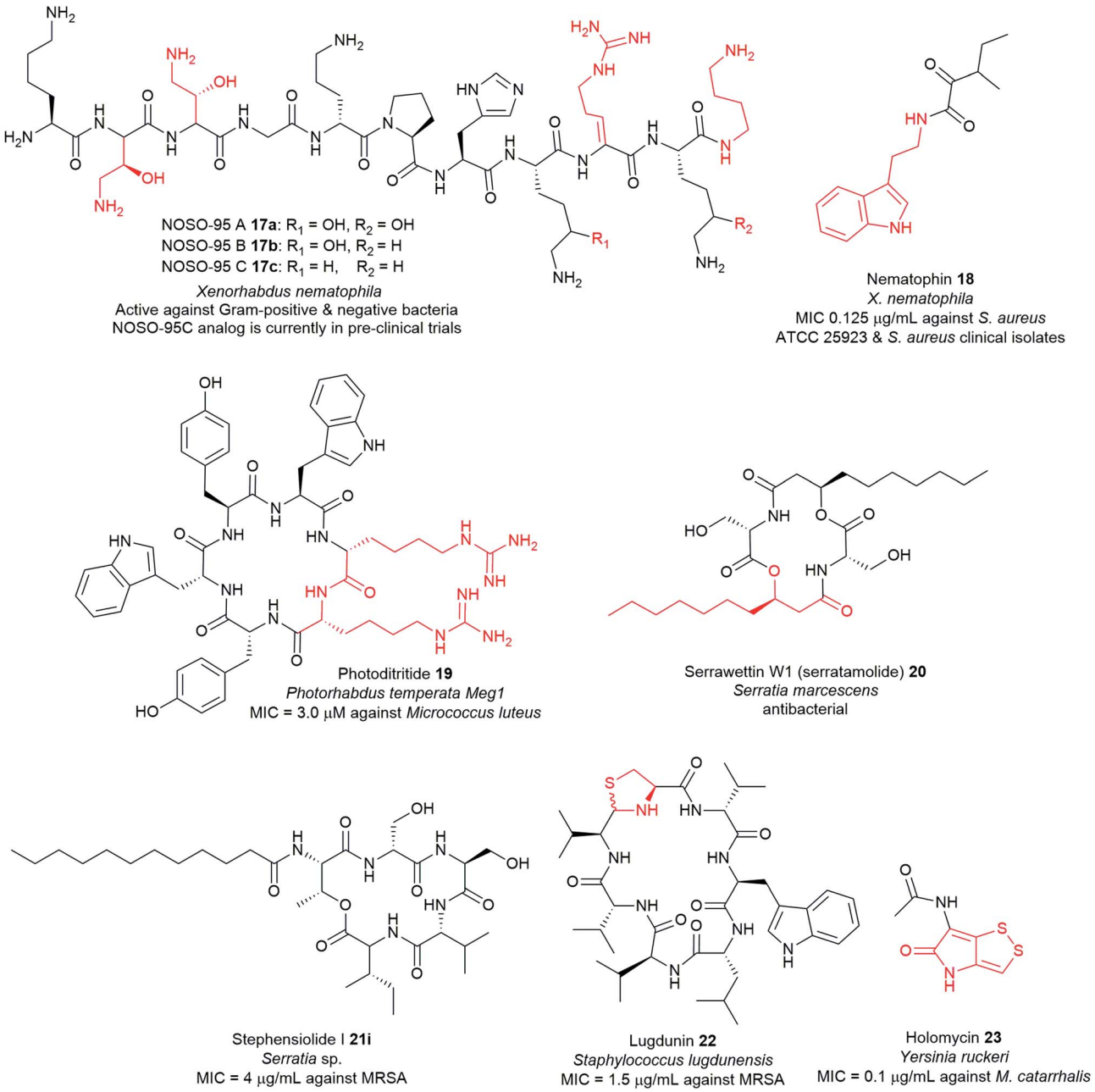

Fig. 6 Examples of antimicrobial nonribosomal peptides with unusual motifs highlighted in red, isolated from pathogenic bacteria.

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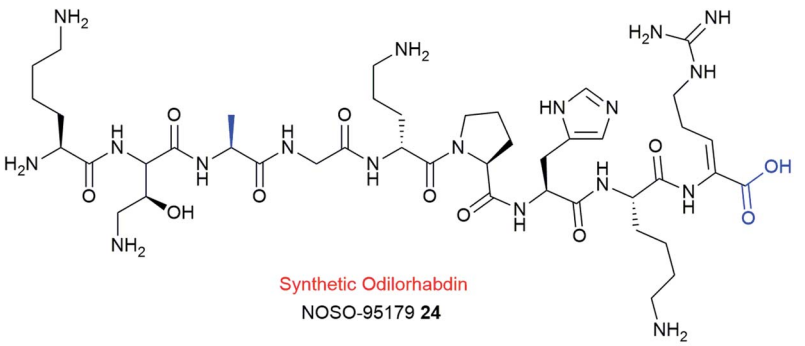
and VRE, respectively. Carbocyclinone-534 **12** did not show any significant antimicrobial activity but exhibited anti-mycobacterial activity against *M. smegmatis*.[113] Duotap **11** showed stronger activity than tapinarof **8** in its ability to regulate the Nrf2 antioxidant reporter gene. Furthermore, dimers **11** and **12** showed little to no efficacy in a colitis mouse model, whereas the monomer reduces disease symptoms. Although **8**, **11** and **12** were only produced in the pathogenic P-form of *Photorhabdus* spp., their varying bioactivity data suggest that the bacterium employs a regulatory mechanism to attain its desired functional outcomes required for symbiosis and pathogenesis.[113] The much weaker antimicrobial activity of tapinarof relative to duotap-520 is probably a means of cellular detoxification by the bacteria to support its symbiosis with the nematode, whereas the more potent duotap-520 presumably support its pathogenic lifestyle.[107,110,113] The promiscuity of Plu1886 biosynthetic enzyme *in vitro* represents a significant cornerstone towards the development of an efficient system to generate novel stilbene dimers with specific activity.

4.2 Nonribosomal peptides

Non-ribosomal peptide synthetases (NRPSs) are multi-modular enzymes that catalyse the synthesis of numerous peptide and peptide-like natural products that have wide applications in medicine, agriculture, and biotechnology among other fields (Fig. 6 and Table S1†). These mega enzyme complexes are not limited to the 22 proteinogenic amino acids; a large breadth of substrates is now known to be integrated and modified by post-synthesis action. NRPSs can incorporate a wide variety of non-proteinogenic amino acids, such as D-isomers, α-hydroxy/keto acids, carboxylic acids, and *N*-methylated residues, as well as several other building blocks such heterocyclic rings and fatty acids. Other common post-synthetic modifications associated with the NRPS machinery include glycosylation and oxidative cross-linking giving rise to diverse molecules with precise functionality for a particular molecular target.[116]

Typical NRPS modules feature an adenylation (A) domain that selects and activates an amino acid monomer (and sometimes other carboxylic acids) as an adenylate followed by acyl transfer to a peptidyl carrier protein (PCP; also known as thiolation domain, T). This thiolation domain loads the activated amino acid on a 4′-phosphopantetheine (4′-Ppant) arm and covalently tethers it to form a peptide bond with an amino acid on the succeeding module, a reaction catalysed by the condensation (C) domain. Together, these three core domains (C, A, T) comprise a minimal NRPS module. In addition to these essential domains, each module may contain an epimerase (E) for the conversion of an L to D-configuration of amino acid, methyltransferase (MT) for *N*-methylation of the amide nitrogen, oxidase (Ox) for the conversion of a thiazoline to a thiazole or for α-hydroxylation of the incorporated amino acid, and reductase (R) for reductive release of an aldehyde product. The C domain replaced by the cyclization (Cy) domain catalyses both condensation and the intramolecular heterocyclisation of Ser, Cys, or Thr to afford thiazoline or oxazaline heterocycles. The release of the final peptide product from the NRPS is catalysed by a C-terminal reductase (R), thioesterase (TE), or a cyclizing C domain to yield linear, cyclic, or branched peptide chain topologies. The structural biology and enzymology of NRPSs have been the subject of several reviews.[83,117,118] This section covers some of the interesting linear and cyclic



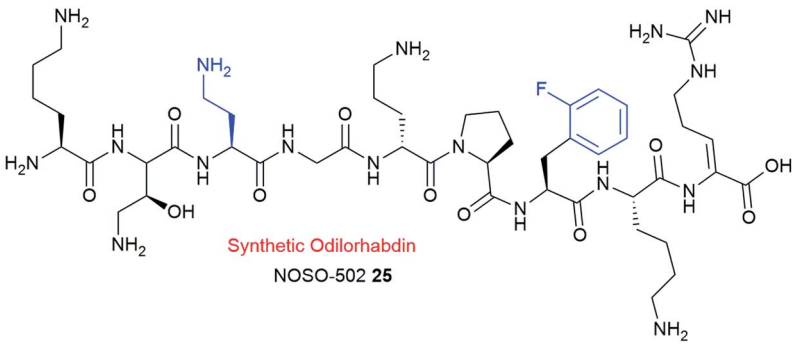


Fig. 7 Chemical structures of synthetic analogues NOSO-95179 **24** and NOSO-502 **25**.

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nonribosomal peptide antimicrobials from pathogenic bacteria such as odilorhabdins **17a–c**, nematophin **18**, photoditritide **19**, serrawettins **20**, stephensiolides **21**, lugdunin **22**, and holomycin **23** (Fig. 6 and Table S1†).

4.2.1 Odilorhabdins. Odilorhabdins (ODLs) are a new class of ribosome-targeting antibiotics produced by the NRPS gene cluster in *Xenorhabdus nematophila* strain K102 (CNCM I-4530) (Fig. 6 and Table S1†).[119] Three ODLs were isolated, NOSO-95A **17a** (1296 Da), NOSO-95B **17b** (1280 Da), and NOSO-95C **17c** (1264 Da). Compounds **17a–c** are 10-mer linear peptides containing four types of non-proteinogenic amino-acid residues: α,γ-diamino-β-hydroxybutyric acid (Dab(βOH)) at positions 2 and 3, δ-hydroxylysine (Dhl) at positions 8 and 10, α,β-dehydro arginine at position 9, and a putrescine moiety at the C-terminal position.[119]

Lead optimization strategies identified a synthetic analogue, NOSO-95179 **24** (Fig. 7)[120] with improved antibacterial properties over the natural compound NOSO-95C **17c**.[120,121] NOSO-95179 **24** differs from NOSO-95C **17c** by the replacement of $Dab(\beta OH)_3$ by alanine and the removal of the lateral lysine10 and putrescine at the C-terminus. Further structural modification at Ala3 and His7 positions of **24** led to the selection of NOSO-502 **25** as the first odilorhabdin clinical candidate (Fig. 7).[9,122,123] NOSO-502 **25** exhibits potent activity to all classes (Ambler A, B, C, and D classification) of carbapenem-resistant *Enterobacteriaceae* (CRE) strains (MIC = 0.5–4 μg mL^{-1}). Furthermore, **25** shows excellent *in vivo* efficacy in several CRE murine infection models, exhibits good *in vitro* safety profile, and has a low potential for resistance development.[119,120,122,123] Notably, **25** exhibits good stability in plasma, microsomes, and hepatocytes.[123] Taken together, NOSO-502 **25** represents a promising drug candidate.

Antimicrobial peptides that interfere with bacterial ribosomes are rare.[124,125] Nine classes of ribosome-targeting antibiotics are known, five of which, including odilorhabdins target the 30S subunit.[125] However, the specific binding site of ODLs on the ribosome and its bactericidal mechanism is distinct from the other four classes.[119] ODLs bind to the decoding centre of the 30S small ribosomal subunit[119] that has never been exploited by any other known ribosome targeting antibiotics such as negamycin, tetracycline, streptomycin and paromomycin.[125–128] ODLs display concentration-dependent bactericidal activity similar to the mechanism described for aminoglycosides and negamycin antibiotics.[126,127,129] At lower concentrations, ODLs induce miscoding of the genetic code, likely by increasing the affinity of aminoacyl-tRNAs to the ribosome,[128] whereas at higher concentrations they inhibit translocation.[119]

4.2.2 Nematophin. Nematophin **18a**, first described in 1997,[130] is produced by all strains of *X. nematophila* (Fig. 6 and Table S1†). Chemically **18a**, 3-indole-ethyl-(3′-methyl-2′-oxo)-pentanamide, contains an N-terminal α-keto group and a C-terminal tryptamine residue, showing structural resemblance to the Rhabdopeptide–Xenortide Peptides (RXPs).[130] Recently, new nematophin analogues **18b–d** and nematophins with valine building blocks, nevaltophins **26a–f** were identified in *Xenorhabdus* strains (Fig. 8).[131]

The biosynthesis of nematophin is proposed to originate from the monomodular NRPS, RdpD, which is closely related to the RXP-producing NRPS, RdpABC but differs in the incorporation of α-keto carboxylic acid as the starting unit.[131] Heterologous expression of the *rdpD* gene from *X. nematophila* ATCC 19601 strain in *Escherichia coli* fed with either phenylethylamine (PEA) or tryptamine (TRA), resulted in the production of new nematophin congeners, **18b–d** (Fig. 8A). In contrast, the wild type (WT) *X. nematophila* strain only produced nematophin **18a** even when fed with PEA or TRA and the presence of the amine compounds did not enhance its production level.[131]

Very few non-ribosomal peptides containing α-keto acid building blocks have been described to date.[131,132] The α-keto acid precursors in nonribosomal cereulide from *Bacillus cereus* and valinomycin from *Streptomyces* spp. occur *via* deamination of α-amino acids such as valine, isoleucine or alanine.[132–134] A similar deamination mechanism to the corresponding acids is proposed in RdpD biosynthesis which is activated by the A domain and subsequently loaded onto the adjacent T domain. Nucleophilic attack by the free amine *via* the C_{term} generates nematophin **18a** and analogues (**18b–d**). The C_{term} domains in RXP-NRPS and RdpD-NRPS indicate that various amines such as TRA and PEA commonly found in *Xenorhabdus* strains can be used as substrates to access the production of TRA- (**18b**) and PEA-containing nematophin derivatives (**18c–d**). The PEA analogues are produced in minor amounts, implying that the substrate preference of the C_{term} domain in RdpD is likely tryptamine over phenylethylamine.[131]

A similar BGC was identified in *Xenorhabdus* PB62.4 containing two monomodular NRPS, Pb62A resembling RdpD with a broken $C_{starter}$ domain, and Pb62B like the RXP RdpC terminal module with a complete C domain. Heterologous expression of the *pb62* gene cluster in *E. coli* fed with either PEA or TRA has permitted to unlock the production of new elongated nematophin derivatives containing an additional valine motif in the structure which was assigned the name nevaltophins **26a–f**. The structures of **26a–f** suggest a biosynthetic pathway very similar to that of **18a–d** but with the incorporation of a valine subunit with α-keto acid building blocks (Fig. 8B).[131] The production of **26a–f** was abolished in the $Ser_{1303}Ala$ mutation on the conserved Ser of the PCP domain in PB62A and led to the accumulation of **26g**, further supporting the proposed biosynthesis (Fig. 8C). Furthermore, when Pb62A was used as a starting module in XndB involved in xenortide biosynthesis,[135] nevaltophins with phenylalanine motif **26h–i** were produced.[131] The results provided a platform for engineered biosynthesis further expanding the nematophin chemical space.

While the crude extracts containing nematophins displayed zone of inhibition against the Gram-positive bacteria *M. luteus*, the nevaltophins containing-extracts did not exhibit activity.[131] The authors, however, only tested the antibacterial activity of nevaltophins against *M. luteus*;[131] and the results may not provide conclusive evidence that the valine unit incorporation in the nematophin core structure may enhance or decrease its bioactivity. In stark contrast, another study indicated that nematophin **18a** has no activity against *M. luteus* at the highest concentration tested (100 μg mL^{-1}). Nematophin, however,

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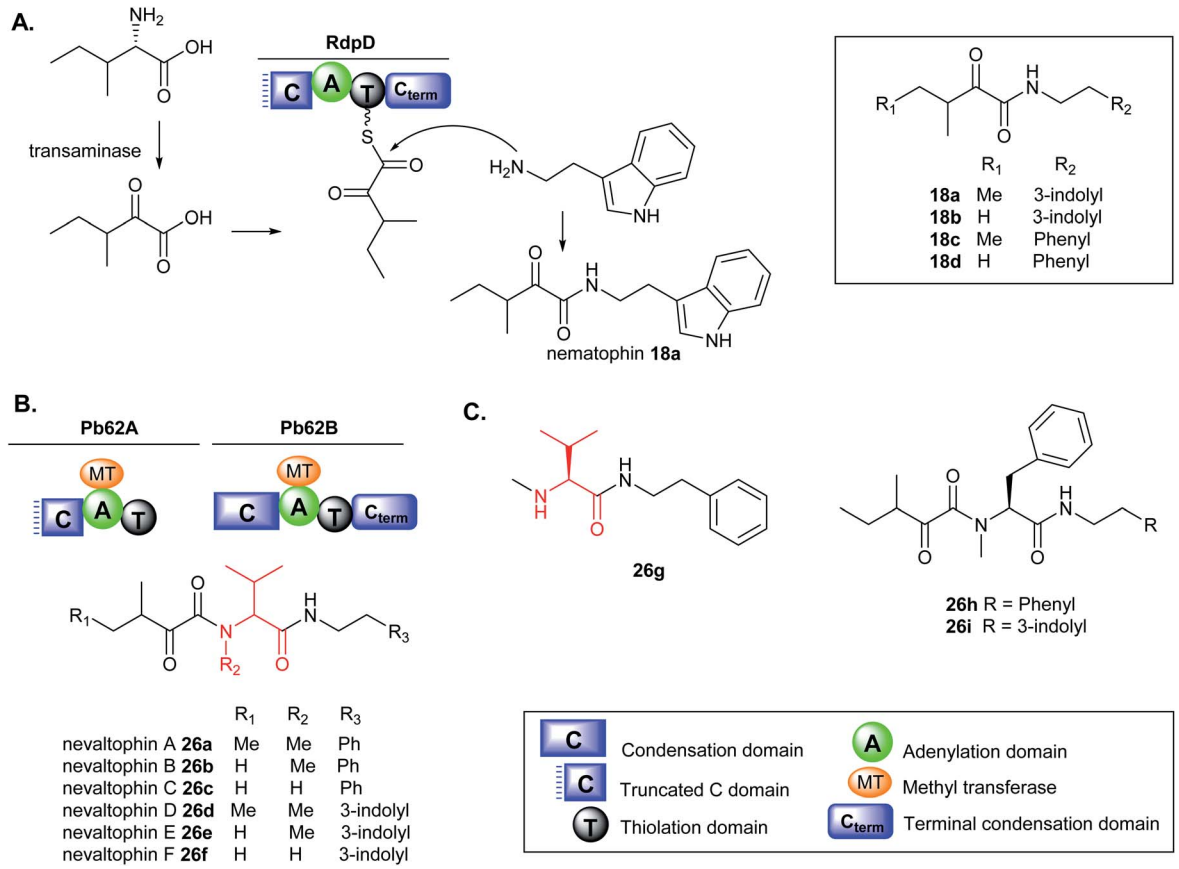

Fig. 8 (A) Nematophin **18a** and analogues **18b–d** from heterologous expression of the *rdpD* gene in *E. coli* and proposed biosynthesis, (B) nevaltophin and analogues **26a–f** from heterologous expression of the *pb62* gene cluster in *E. coli*, and (C) chemical structures of **26g–i**.

showed potent activity against other Gram-positive bacteria such as *S. aureus* (MIC = 0.125 μg mL^{-1}),[130,136] MRSA (MIC = 1.5 μg mL^{-1}) and fungal pathogen, *Botrytis cinerea* (MIC = 12 μg mL^{-1}).[130] Furthermore, the δ-keto amide functionality in nematophin is essential for its anti-staphylococcal activity;[136] and the activity is substantially enhanced by *N*-substitution of the indole ring with an alkyl or a phenyl group.[131,136,137] The synthetic *N*-methyl substituted nematophin analogue displayed nanomolar activity towards several strains of *S. aureus* (15 ng mL^{-1}), *Staphylococcus hyicus* (60 ng mL^{-1}), and *Staphylococcus intermedius* 9503 (50 ng mL^{-1})[136] including MRSA ATCC 43300 (31 ng mL^{-1}) and methicillin-susceptible *S. aureus*, MSSA ATCC 29213 (125 ng mL^{-1}).[137] Conversely, incorporation of azaindole moieties in the nematophin scaffold significantly reduced the antibiotic activity (MIC = 16–128 μg mL^{-1}).[137] Nematophin **18a** and nevaltophin **26a** showed weak activity against parasites, *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani*, and *Plasmodium falciparum*.[131] Phenylethylamide-containing compounds such as nematophin were found to specifically inhibit an insect serotonin receptor facilitating its role in insect pathogenesis.[138]

4.2.3 Nonribosomal peptides *via* promoter exchange. Several known and cryptic nonribosomal peptides were identified in *Photorhabdus* and *Xenorhabdus via* the promoter exchange strategy, including GameXPeptides, xenoamicins, mevalagmapeptides, xenorhabdin, indigoidine,[139,140] and the pentadecapeptide, kolossin.[141] Recently, photoditritide **19** was identified after the photoditritide synthetase (*pdtS*) gene was activated in *Photorhabdus temperata* Meg1 *via* substitution of the native promoter with a transcriptionally active arabinose-inducible promoter, P_{BAD} (Fig. 6 and Table S1†).[142] Overexpression of the *pdtS* gene was achieved with arabinose (induced strain), resulting in the production of a hexapeptide that is not previously detected in the wild type (WT) strain. Photoditritide **19** consists of two homoarginines (Har), two tyrosines (Tyr), and two tryptophans (Trp).[139] Although non-proteinogenic amino acid Har-containing peptides have been reported in various marine organisms such as a sponge (cupolamide A),[143] cyanobacteria (nodularin-Har)[144,145] and marine-derived actinomycetes (lucentamycins A–D),[146] photoditritide is the first peptide from entomopathogenic bacteria that contains the rare homoarginine residue.[142] Photoditritide **19** displayed antimicrobial activity against *M. luteus* (MIC = 3.0 μM) and *E. coli* (MIC = 24 μM) and weak antiprotozoal activity against *T. cruzi* (IC_{50} = 71 μM), *P. falciparum* (IC_{50} = 27 μM) and *T. brucei rhodesiense* (IC_{50} = 13 μM). No cytotoxic activity against mammalian L6 cells was observed.[142] Nonribosomal peptides **27–31** were produced *via* promoter exchange in *Δhfq* mutants of *Photorhabdus* and *Xenorhabdus* strains (Fig. 9 and Table S1†). The global post-transcriptional regulator, Hfq, is widespread in

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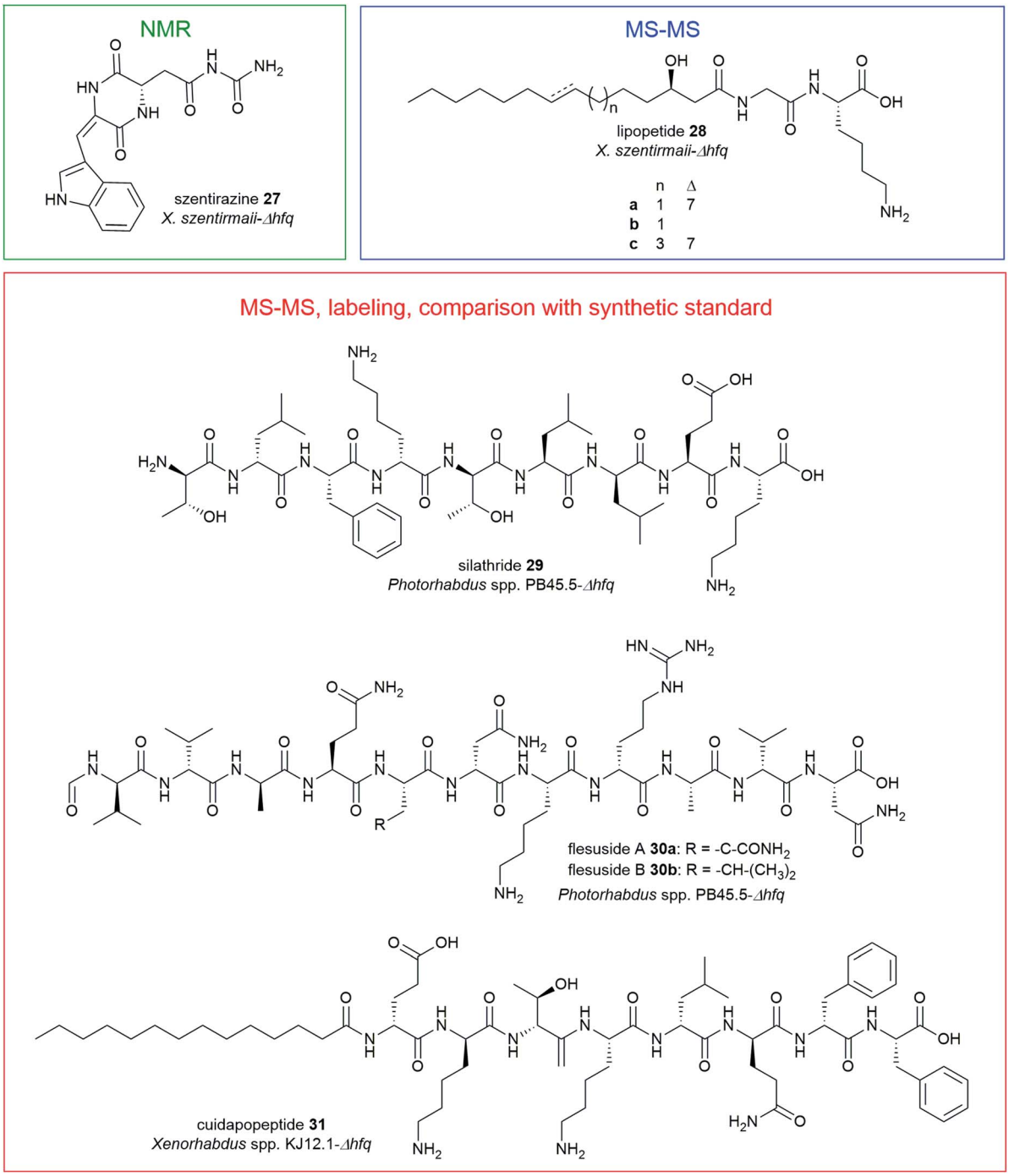

Fig. 9 Structures of nonribosomal peptides identified from *Δhfq* mutants of *X. szentirmaii* (szentirazine **27**, lipopeptides **28a–c**), *Photorhabdus* PB45.5 (silathride **29**, flesusides A and B **30a–b**), *Xenorhabdus* KJ12.1 (cuidadopeptide **31**) *via* promoter exchange.

bacteria and performs diverse functions, one of which is the modulation of BGC expression through mediating interactions between the small regulatory RNAs (sRNAs) and their target mRNAs.[147–149] An *hfq* deletion mutant in *P. luminescens* abolished the production of all known NPs.[150] Exchanging the native promoter of a BGC of interest with a constitutively strong active P_{BAD} promoter in *Δhfq* mutants resulted in the production of numerous known and new compounds **27–31.** While promoter exchange in the wild type strains produces several background peaks,[139–142] promoter substitution in *Δhfq* mutants leads to culture supernatants containing only the compounds of interest, thereby enabling direct bioactivity testing, requiring no laborious and time-consuming isolation and purification steps.[151]

The promoter exchange strategy resulted in overproducing mutants with significantly higher production titres relative to

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the WT strains.[151] In *X. szentirmaii-Δhfq*, two silent BGCs were activated that encode for the known depsipeptides, xenobactin[152] and szentiamide.[153] Additionally, a new oxidized diketopiperazine (DKP), szentirazine **27**, and three new shortened PAX-peptides (**28a–c**) were produced. The new compounds **27–28** were exclusively produced by the induced *Δhfq* mutant. The structures of the lipopeptides (**28a–c**) were elucidated by detailed MS-MS analysis while szentirazine **27** was isolated from a large-scale culture, and its structure was characterized by NMR spectroscopy.[151] Furthermore, new peptides silathride **29** and flesusides A **30a** and B **30b** were identified from *Photorhabdus* PB45.5-*Δhfq* and the new lipopeptide cuidadopeptide **28** from *Xenorhabdus* KJ12.1-*Δhfq via* a similar approach. The structures of **29–31** were elucidated by detailed MS/MS fragmentation analysis, labeling experiments and by comparison with synthetic compounds.[151] All new NPs **27–31** showed weak to moderate antimicrobial activity against several Gram-positive and Gram-negative bacteria, and fungi.[151]

4.2.4 Serrawettins. Serrawettins are non-ionic biosurfactants produced exclusively by the genus *Serratia*. They exhibit diverse activities such as emulsification, surface, antifouling, antitumor, and antimicrobial.[154] Three molecular species have been reported from *S. marcescens*, serrawettin W1 (also known as serratamolide A **20a**), serrawettin W2 **32a**, and serrawettin W3. Serratamolide A, a symmetrical dilactone molecule, was discovered by Wasserman, *et al.* in 1961 (Fig. 10 and Table S1†).[155,156] It is composed of two L-serine amino acids linked to two β-hydroxy serratamic acids (D-3-hydroxydecanoyl-L-serine).[157] It differs from depsipeptides valinomycin,[158] cereulide,[158] and enniatins[159] in the presence of β-hydroxy acids, rather than α-hydroxy acid residues. Several serrawettin W1 **20a** congeners have also been identified from *Serratia* sp., serratamolides B–G (**20b–g**), which varies in the length of the fatty acid chain and the presence of a methoxy unit (**20g**) and a double bond in the alkyl chain (Fig. 10).[160,161]

The general chemical structure of serrawettin W2 consists of five amino acid residues (D-Leu–L-Ser–L-Thr–D-Phe–L-Ile) attached to a β-hydroxy fatty acid moiety (Fig. 10 and Table S1†).[162,163] Four analogues of serrawettin W2 **32b–d** were recently isolated from *Serratia* sp. which differs based on the amino acids present (Ile or Val, Phe or Tyr) or the length of the fatty acid chain (C5 or C7).[162] Further putative analogues (W7–W8)

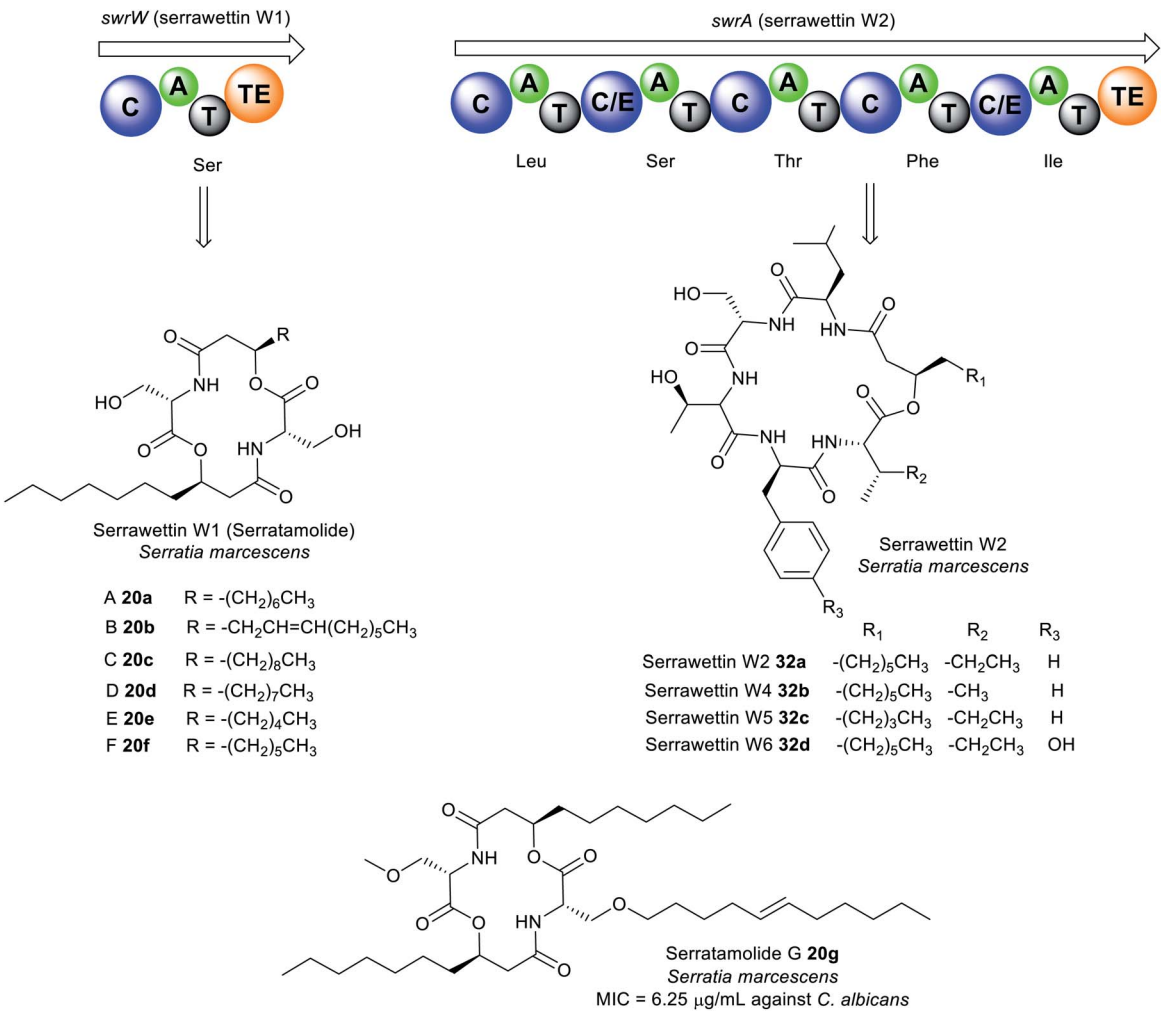

Fig. 10 Analogues of serratamolide (serrawettin W1) A–G (**20a–g**) and serrawettin W2 **32a–d** identified in *Serratia* sp.

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were tentatively identified in *Serratia surfactantfaciens* sp. nov. YD25 by MS/MS fragmentation analysis.[164] The structure of serrawettin W3 described in 1986 is still yet to be determined.[165] It is partially characterised and is composed of five amino acid residues (Thr, Ser, Val, Leu, Ile) and one dodecanoic fatty acid.[163]

The dilactone serrawettin W1 is believed to be formed solely by the action of the monomodular NRPS, SwrW encoding for aminolipid synthetase (Fig. 10). Initially, the biosynthesis of **20a** was thought to occur *via* condensation of two serratamic acid molecules. However, mutational studies indicate the absence of the presumed precursors, suggesting the involvement of NRPS machinery in **16a** production. Consequently, the presence of SwrW was identified in *S. marcescens* 274 by transposon mutagenesis. SwrW exhibits a C–A–T–TE domain architecture specific for only L-serine, and is presumed to be the simplest enzyme in the NRPS family. This simple NRPS system features an unusual dimerization, most likely *via* two following transesterification steps to assemble the symmetric and cyclic product, serrawettin W1 with no peptide bonds.[166] Biosynthesis of serrawettin W1 presumably starts with the adenylation of the L-serine, after which the activated L-serine binds as a thioester to the thiolation domain which has been phosphopantetheinylated through the action of the PPTase, PswP.[167] The amino group of the L-serine bound to the thiolation domain forms a bond with the 3-D-hydroxydecanoyl fatty acid which is speculated to come from a yet unknown ACP domain to form the first serratamic acid intermediate, and then subsequently transferred to the TE active site.[166] Thereafter, biosynthesis of the second serratamic acid occurs and follows similar dimerization and cyclization processes to the ones catalysed by the multi-modular synthetase in the biosynthesis of the symmetric decapeptide gramicidin S from *Brevibacillus brevis*.[168]

Biosynthesis of serrawettin W2 in *S. surfactantfaciens* sp. YD25^T is proposed to be catalysed by the NRPS peptide synthetase, SwrA consisting of five modules (Fig. 10). The unusual feature of SwrA (like SwrW) stems from the assembly of the starter unit. Typical NRPS contains A domains at the initiation site, but the SwrA NRPS harbours a C domain at its N-terminus suggesting that the initiation of peptide synthesis may form from the condensation of a fatty acid rather than an amino acid. It is presumed that a fatty acid adenylate, acyl-ACP, or acyl-CoA is likely the substrate for this C domain, catalysing the *N*-acylation of leucine. The fatty acid precursor in serrawettin W2 is speculated to be synthesised by the putative PKS SwrEFG gene cluster and other unknown enzymes. Chain elongation then occurs *via* the action of the other domains by successive incorporation of serine, threonine, phenylalanine, and isoleucine. Finally, cyclisation and chain release of the oligopeptide is catalysed by the TE domain to yield serrawettin W2.[164]

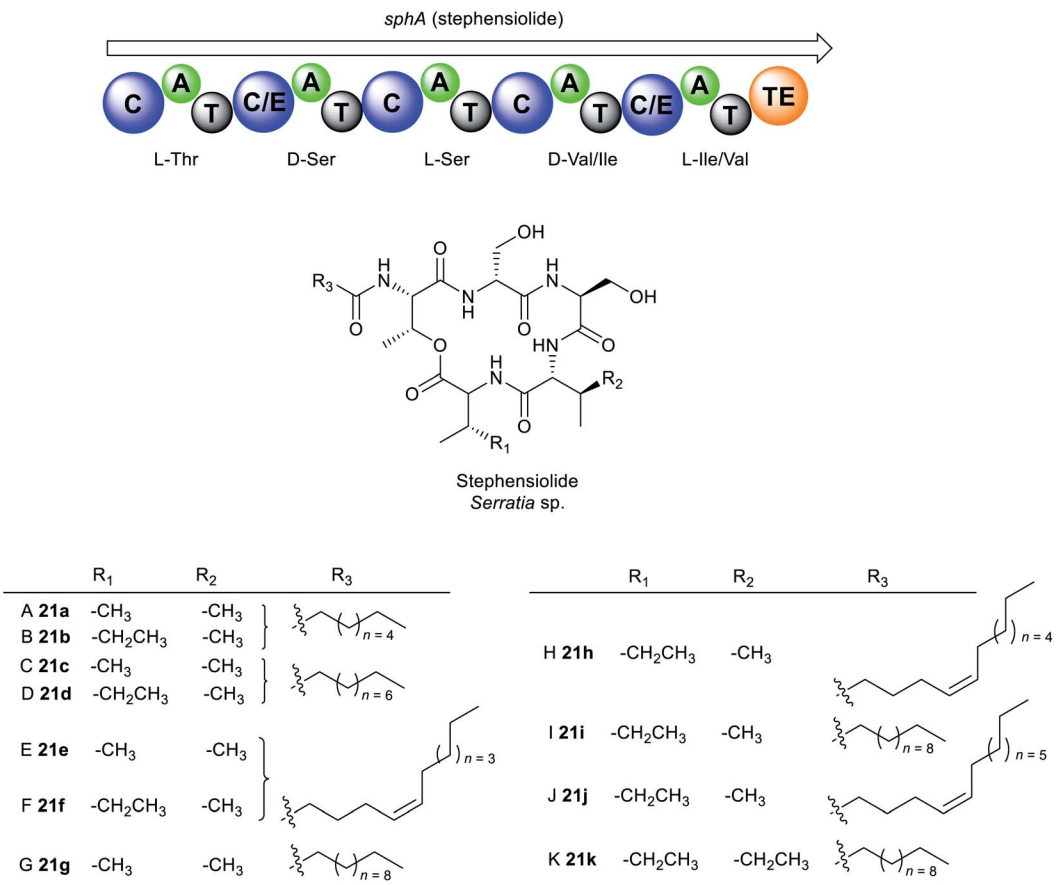

Fig. 11 Structures of stephensiolides A–K (**21a–k**) from *Serratia* sp.

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Serrawettin W1 **20a** exhibits antimycobacterial activity against *M. tuberculosis*, *M. diernhoferi*, and *M. avium* (MIC = 25 μg mL^{-1}),[155,161] and antibacterial and antifungal activities towards *S. aureus*, *B. subtilis*, *M. luteus*, *Trichophyton* spp., and MRSA (MIC = 6.25–50 μg mL^{-1}).[155,169,170] Likewise, serrawettin W2 **32a** is active against Gram-positive (*e.g. S. aureus*, *Rhodococcus* sp. and *Micrococcus* spp.) and Gram-negative bacteria (*e.g. Pseudomonas* spp., *Shigella* spp.) including drug-resistant *S. aureus* clinical isolates.[164] Serrawettin W2 **32a** is a potent biofilm inhibitor of *Candida albicans* (IC_{50} = 7.7 μM), while the W2 analogues **32b–f** are moderately active (IC_{50} = 13.4–60.0 μM).[162] Furthermore, **32a** is cytotoxic towards Hela (IC_{50} = 20.9 μM) and Caco2 (IC_{50} = 54.1 μM) cell lines.

The cyclic lipodepsipeptides, stephensiolides A–K **21a–k** were produced by a *Serratia* strain that was isolated from the midgut and salivary glands of *Anopheles stephensi* mosquitoes (Fig. 11).[171] Stephensiolides were also isolated from the fungal endophyte, *Lecanicillium* sp. (*Hypocreales*) obtained from the latex of *Sandwithia guyanensis* plant.[172] Stephensiolides **21a–k** mimic the core structure of serrawettin W2 **32a** as both are cyclic pentapeptides[162,163] but differ in the sequence of the amino acid constituents.[171] The peptide sequence in stephensiolides is Thr–Ser–Ser–Val/Ile–Ile/Val while serrawettin W2 is Leu–Ser–Thr–Phe–Ile. Furthermore, the lactone in stephensiolides is cyclized through the hydroxy group of the threonine, whereas serrawettin W2 is cyclized *via* a 3-hydroxy group of the fatty acid chain. Stephensiolide congeners (A to K) **21a–k** vary in the length of the alkyl chain, amino acid residues (Ile or Val) or the presence of a double bond in the lipid side chain.[171]

Like serrawettin W1 **20a** and serrawettin W2 **32a**, stephensiolides are biosynthesised by a similar NRPS machinery (Fig. 11). Bioinformatics analysis identified the penta-modular NRPS, *sphA* which is presumed to be responsible for the incorporation of five amino acids, threonine, serine, serine, valine/isoleucine, and isoleucine/valine.[171] SphA contains a unique initial C domain that is homologous to the lipopeptide-loading C module of EndA in the enduracidin biosynthesis,[173] which is probably responsible for the incorporation of the fatty acid in **21a–k** from an ACP.[171]

Antimicrobial testing of the stephensiolide mixture (A to K) revealed activity against *B. subtilis* 3610 (IC_{50} = 15 μg mL^{-1}), *P. falciparum* Dd2 (IC_{50} = 14 μg mL^{-1}), and the human hepatocytes, HepG2 (IC_{50} = 21 μg mL^{-1}).[171] Stephensiolides also demonstrated antibacterial activity against MRSA with stephensiolide I **21i** as the most active (MIC = 4 μg mL^{-1}).[172] Like serrawettins, stephensiolides facilitate bacterial surface motility as biosurfactants.[171] The primary role of swarming motility within mosquitoes is not fully understood, however, it is speculated that an enhanced swarming ability enables the bacteria to colonize and migrate in the different tissues within the insect host. A close relative to *S. marcescens*, *Serratia* strain AS1 colonizes diverse anopheline species and infect multiple different tissues within mosquitoes, including the midgut, female ovaries, and male accessory glands.[174]

4.2.5 Lugdunin. Lugdunin **22** is a macrocyclic peptide antibiotic isolated from the nasal and skin commensal bacterium, *S. lugdunensis* (Fig. 12 and Table S1†). Structural features of lugdunin comprise an unusual thiazolidine heterocycle and five amino acids (Val, Trp, Leu, Val, and Val) in alternating D- and L-configuration.[29] This five-membered thiazolidine resembles a clasp that "adorns" the peptide backbone, hence the term fibupeptides was coined for this new class of compounds (Latin *fibula*, meaning clasp).[175]

The biosynthetic mechanism for lugdunin production features several unusual aspects of the domains and their overall organization (Fig. 12). Four NRPS genes, *lugA, B, C, and D*, are proposed to direct the biosynthesis of lugdunin. Interestingly for a heptapeptide, the gene cluster encodes adenylation domains for only five amino acids. Biosynthesis presumably starts at the characteristic initiation module of LugD specific for L-cysteine, followed by sequential addition of D-valine and L-tryptophan by LugA, and D-leucine by LugB. The modules encoded in LugC exhibit a very peculiar organization, featuring a single valine-incorporating A domain but two downstream condensation and three PCP domains for peptide bond formation and amino acid transfer, respectively.[29] This suggests an iterative biosynthetic logic similar to that of koranimine[176] and yersiniabactin,[177] where the single LugC adenylation domain activates three successive valine residues for subsequent installation in alternating L- and D-configurations. Chain release of the thioester-bound heptapeptide is catalysed by the terminal reductase of LugC, followed by subsequent cyclisation. Finally, the nucleophilic attack of the cysteine thiol group at either the *re* or *si* face of the imine yields two thiazolidine-containing structural diastereomers (depicted with wavy bond). The thiazolidine heterocycle is present in some linear NRPS compounds, such as watasemycins[178] and yersiniabactin,[177] but is yet unreported in macrocyclic peptides. Lugdunin is the first thiazolidine-containing macrocyclic peptide. Interestingly, production of lugdunin in ample amounts for chemical characterisation and biological profiling was only obtained *via* substitution of the native *tetR*-like regulatory gene, *lugR*, with a xylose-based expression approach.[29]

Lugdunin **22** exhibits potent bactericidal activities against a wide range of Gram-positive bacteria, including *B. subtilis*, *Listeria monocytogenes*, *S. aureus*, *Streptococcus pneumoniae*, and opportunistic pathogens MRSA, VRE, and glycopeptide-intermediate resistant *S. aureus* (GISA) (MIC = 1.5–12 μg mL^{-1}).[29] In contrast to rifampicin, *S. aureus* did not show any resistance to lugdunin even under prolonged exposure to sub-optimal doses of the compound for over 30 days. Furthermore, it shows no toxicity in primary human erythrocytes, neutrophils, or human monocytic cell line HL60, and demonstrates good *in vivo* efficacy in the mouse model of *S. aureus* skin infection. *In vivo* tests show significant reduction and even total eradication of viable *S. aureus* on the skin surface and in the mouse tissue indicating that the compound can penetrate the deeper layers of the skin.[29] This inhibitory mechanism is achieved by the bactericidal activity of lugdunin as well as by the increased innate defence of epithelial cells resulting in efficient protection against *S. aureus* skin colonization. Lugdunin offers the host three layers of protection. Firstly, it can directly inhibit and kill *S. aureus*. Secondly, it can work synergistically with the

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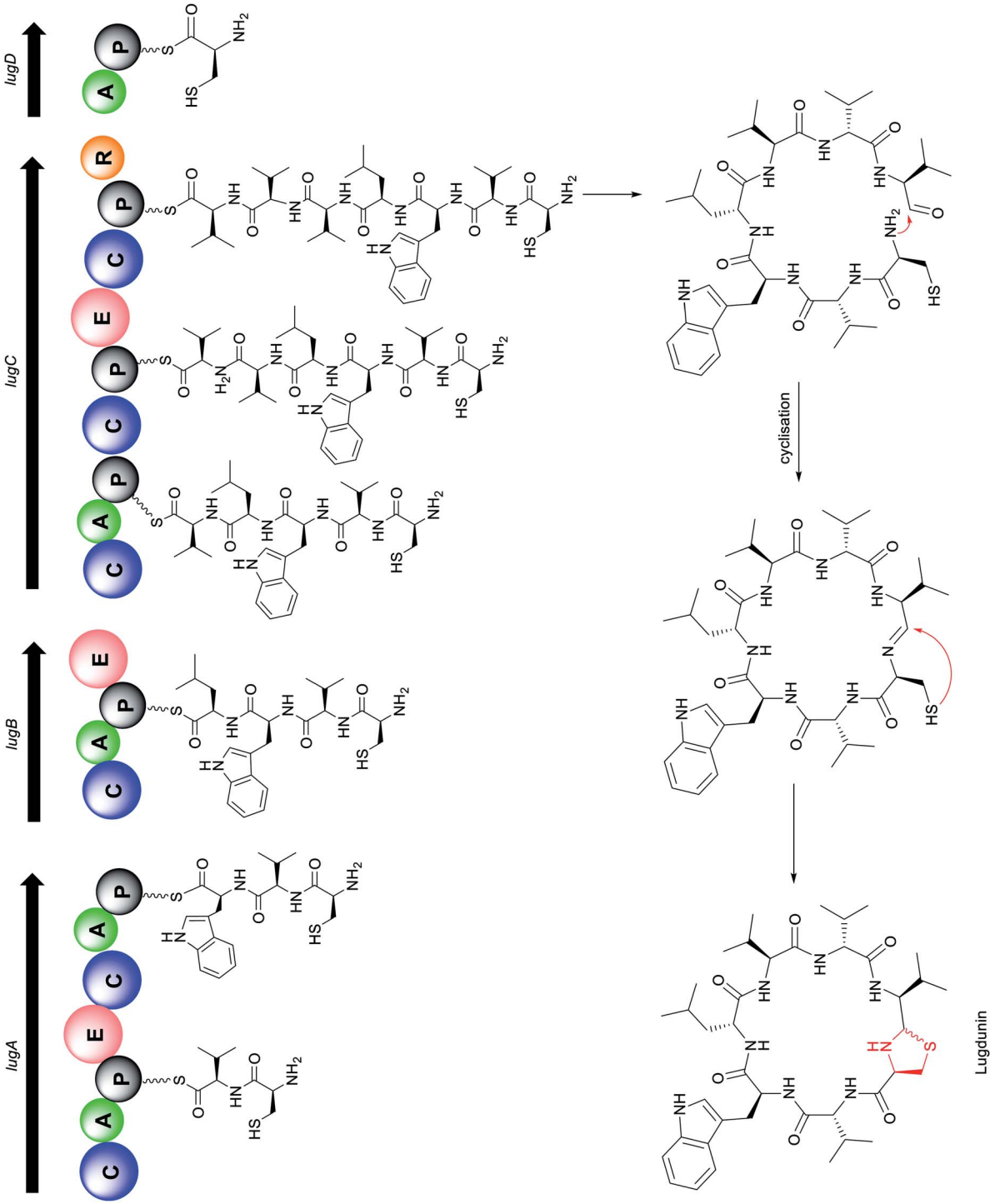

Fig. 12 Proposed lugdunin biosynthesis.

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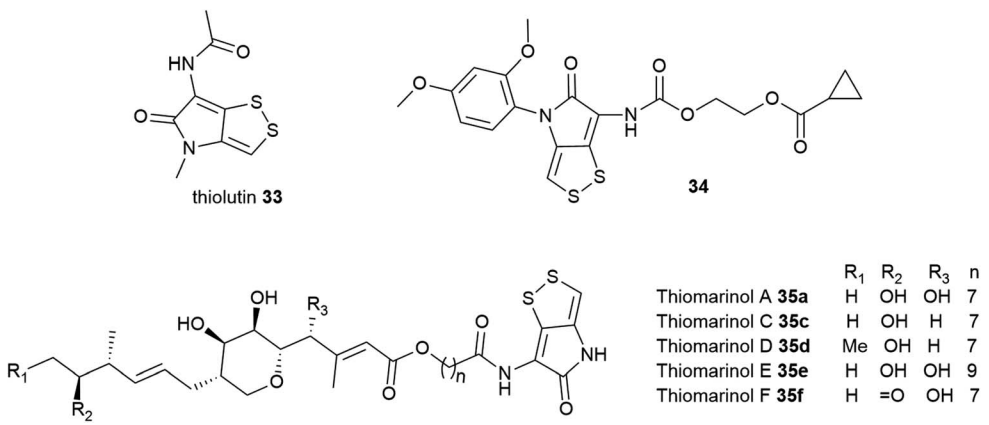
antimicrobial peptides produced naturally by the host as part of the immune response (for example, hCAP18/LL-37 and the dermcidin-derived peptides DCD-1L), enhancing their ability to kill *S. aureus*. Finally, it can induce an immune response within the skin, thus enabling it to recruit phagocytic immune cells to aid with the clearing of the competing pathogen. Other factors derived from the skin commensal *S. epidermidis* may serve to amplify this response, increasing efficacy.[179]

SAR studies indicate that the cyclic structure of the peptide, the *N*-unsubstituted thiazolidine "clasp", two amino acids tryptophan and leucine, and an alternating D- and L-amino acid backbone are integral to the activity.[175] The nonpolar tryptophan and leucine residues interact with the hydrophobic regions of the bacterial cell membranes similar to the activity of poly-(Trp–Leu)-octapeptides.[180] Fibupeptides like lugdunin carry electronically charged particles across the membrane and consequently disintegrate the membrane potential, thereby killing the bacteria. Incorporation of an additional tryptophan motif in the peptide backbone intensifies this membrane interaction and further strengthens the antibacterial effect, exhibiting two-fold increased activity over the parent compound.[175] Lugdunin or analogues thereof are promising candidates for the treatment of multi-drug resistant Gram-positive infections. However, it may be challenging to develop these into systemic therapeutics considering that they are membrane-targeting antibiotics. Such compounds also tend to perturb mammalian plasma membranes.[181]

4.2.6 Holomycin. Holomycin **23** was first discovered in *Streptomyces griseus* in 1959 [182] and later was reported to be produced by several other *Streptomyces* species[183–188] and other bacteria, including the marine Gram-negative bacterium *Photobacterium halotolerans*[79] and the fish pathogen *Y. ruckeri* (Fig. 6 and Table S1†).[31,32] Structurally, holomycin belongs to a class of dithiolopyrrolone (DTP) natural products[189] which contains a unique heterobicyclic core with a disulfide bridge and a variety of *N*-alkyl and *N*-acyl substituents.[31,79,182–184]

Dithiolopyrrolones possess broad-spectrum inhibitory activity against bacteria, fungi, and cancer cell lines.[189–191] Holomycin **23** is potent against several Gram-positive and Gram-negative bacteria including *E. coli* (MIC = 0.2–2 μg mL^{-1}), *S. aureus* (MIC = 2–4 μg mL^{-1}), *S. epidermidis* (MIC = 1 μg mL^{-1}), *S. pneumoniae* (MIC = 0.1–0.3 μg mL^{-1}), *Haemophilus influenzae* (MIC = 0.3 μg mL^{-1}), and *Moraxella catarrhalis* (MIC = 0.1–0.3 μg mL^{-1}),[192] as well as rifampicin-resistant *S. aureus* (RRSA) mutants containing modified RNA polymerase β-subunit (MIC = 4–8 μg mL^{-1}).[193] Despite this attractive biological activity, holomycin is toxic, so it may need to be modified for possible future antibiotic use. Chemical synthesis of DTP analogues with modifications at the *N*-positions has attracted significant interest by several groups.[194–199] *N*-Aryl DTP analogues have been shown antitumor activity[198] and antileukopenia activity.[194,197] *N*-Aryl DTP with 2,4-dimethoxyphenyl moiety displayed potent antibacterial activity against clinical isolates of MRSA, RRSA, vancomycin-resistant *S. aureus* (VRSA), and moderately penicillin-resistant *S. pneumoniae* (MPRSP) with MIC values in the range of 0.125–2 μg mL^{-1} comparable to the antibiotic rifampin.[195] Previous works also showed that the biosynthetic pathway of DTPs is susceptible to be manipulated by feeding different organic acids or fatty acids to the cultures to modify the lateral acyl chain.[200–202] Another approach involved the generation of hybrid-type antimicrobials by incorporating the holomycin antibiotic into the myxopyronin core.[203] The holomycin nucleus has also been more recently identified in the marine hybrid antibiotic thiomarinol **35**, in which it is joined to a pseudomonic acid motif, an analogue of the FDA-approved topical antibiotic mupirocin (Bactroban®) (Fig. 13).[190,204] The biosynthetic hybridity of thiomarinol may have advantageous effects; when one antibiotic fragment is modified by inactivating enzymes, the other constituent might remain functionally active.[205,206] Attempts to stimulate holomycin production have also received considerable interest. Holomycin-high producing variants of *S. clavuligerus* were obtained *via* competition-based adaptive evolution against MRSA N315 (ref. 207) as well as manipulation of the regulatory gene, *argR* which regulates the expression of arginine biosynthesis.[208]

Owing to the promising antimicrobial activity of DTPs, several studies into their mode of action (MOA) have been conducted using some of the more well-studied group



	R_1	R_2	R_3	n
Thiomarinol A **35a**	H	OH	OH	7
Thiomarinol C **35c**	H	OH	H	7
Thiomarinol D **35d**	Me	OH	H	7
Thiomarinol E **35e**	H	OH	OH	9
Thiomarinol F **35f**	H	=O	OH	7

Fig. 13 Structures of thiolutin **33**, thiomarinols **35**, and synthetic holomycin analogue **31** that exhibits notable bacterial RNA polymerase inhibition.

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members. Two opposing plausible mechanisms of action have been proposed. The first one identifies DTPs as inhibitors of bacterial RNA polymerase (RNAP). Thiolutin **33**, a holomycin variant, has been shown to reversibly inhibit RNA and protein synthesis of *Saccharomyces cerevisiae* at a concentration of 2–4 μg mL^{-1} in the whole-cell and spheroplasts assays and inactivates yeast RNA transcription *in vitro*.[191,209–211] However, subsequent studies of holomycin or thiolutin in *E. coli* RNA synthesis inhibition have indicated that although both exhibit activity *in vivo*, they show weak (or no activity) *in vitro*. Furthermore, it was also not clear which step of RNA synthesis thiolutin inhibits. Induction of β-galactosidase in *E. coli* has suggested both RNA transcription initiation and chain elongation as possible targets of thiolutin. These opposing results cast doubt as to whether RNAP is the main target of the antibiotic in *E. coli*.[186,192,212–214] To uncover the intriguing aspects of DTP mechanisms, Tan and co-workers synthesised various *N*-aryl DTP analogues and investigated their *in vitro* inhibitory against *E. coli* RNAP. Among all the tested compounds, synthetic **34** inhibited the most potent RNAP activity *in vitro* and is also the least cytotoxic. Additionally, molecular docking studies (Fig. 13) of **34** revealed interaction and high binding affinity with the amino acid residues in the switch region of the *E. coli* RNAP in the same manner as myxopyronin A, indicating that DTP and analogues are bacterial RNA inhibitors.[195,199]

The second alternative mechanism is proposed by Li and co-workers in which holomycin **23** is considered as an intracellular metal-chelating antibiotic that sequesters free metal ions and selectively targets *E. coli* metalloenzymes, and not RNA polymerase *in vitro*.[215] The proposed model suggested holomycin acts as a prodrug[192,216] whose activation involves the conversion of the ene-disulfide in the cytoplasm to the active ene-dithiol, reduced holomycin (red-holomycin) with high affinity for zinc ions.[215,216] The mechanism by which the cyclic disulfide **23** is reduced in the cells is as yet unknown. After entering the cells, the red-holomycin **23a** is proposed to exert its metallophoric activity *via* two different routes (Fig. 14): (1) red-holomycin **23a** sequesters essential metals, especially zinc, thereby limiting zinc availability in the bacterial cell, and (2) red-holomycin **23a** removes zinc from a subset of zinc-dependent metalloproteins (*i.e. E. coli* class II fructose bisphosphate aldolase, FbaA), thereby disrupting the cell's metal homeostasis and potentially interfering the essential metabolic processes such as glucose utilization, RNA synthesis, and respiration. Although both routes contribute to the inhibitory effect of holomycin, route two may play a more prominent role in the MOA, consistent with the findings that an increased zinc concentration renders no enhanced effect on the *E. coli* growth inhibition. Disruption of the zinc import machinery involved in the maintenance of metal homeostasis, such as ZnuABC restricts zinc uptake and further sensitizes *E. coli* to holomycin.[215] This MOA is unique amongst antibiotics and may be further explored to understand the specificity of holomycin and other DTPs against metalloenzymes for the development of novel potent chelators.

4.3 Hybrid polyketide–nonribosomal peptide natural products

Owing to the structural and catalytic resemblances between PKS and NRPS, they have evolved the ability to communicate with each other and combine modules to form hybrid assembly lines. During the transfer of the growing peptide or polyketide intermediate across NRPS/PKS interfaces, ketosynthase (KS) and condensation (C) domains facilitate chain elongation by accepting upstream PCP-bound peptidyl thioesters and ACP-bound polyketide thioesters, respectively, thereby switching efficiently between C–C bond and C–N bond formation. Together, the biosynthetic versatility of PKS machinery and the substrate flexibility of NRPS modules that can incorporate almost 500 different proteinogenic and nonproteinogenic amino acids coalesce to yield hybrid natural products with astounding structural and biological diversity (Fig. 15 and Table S1†). This biosynthetic machinery has been described extensively elsewhere.[83,217] Examples of antimicrobial hybrid polyketide–peptide metabolites produced by pathogenic bacteria include the red-pigment prodigiosin, the broad-spectrum antibiotic althiomycin, the DNA-gyrase inhibitor albicidin, and the

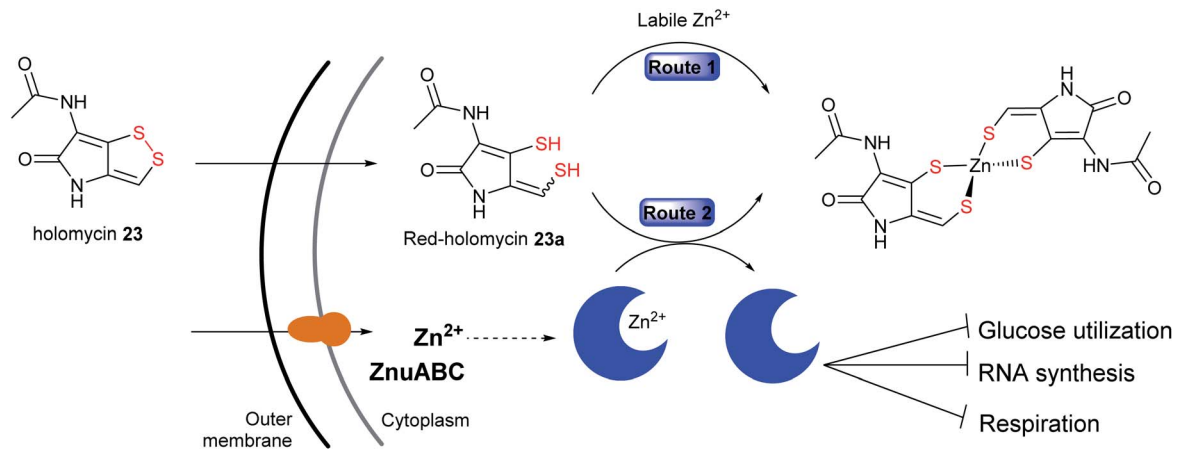

Fig. 14 A model for the mechanism of action of holomycin in which it acts as a prodrug which undergoes intracellular reduction to the active red-holomycin that sequester free metal ions, particularly zinc (route 1) or removes zinc from metalloproteins (route 2).

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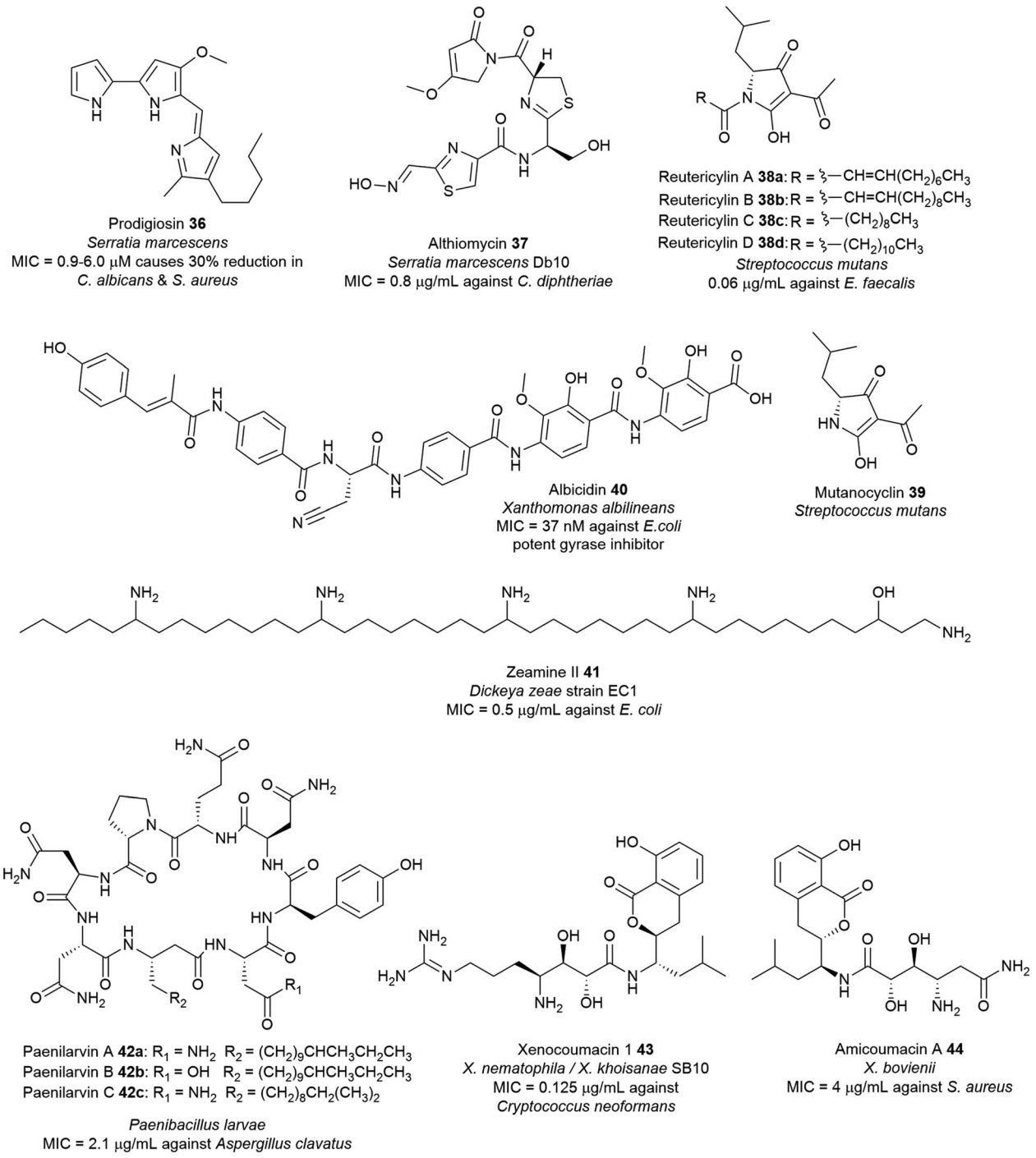

Fig. 15 Examples of hybrid polyketide–nonribosomal peptide natural products with antimicrobial activity from pathogenic bacteria.

antibacterial metabolite associated with dental caries reutericyclin.

4.3.1 Prodigiosin. Prodigiosin **36** was first characterized in *S. marcescens*[218,219] and was later identified in several other bacterial genera including *Streptomyces*,[220] *Vibrio*,[221] *Zoogloea*,[219] *Hahella*,[222] and *Pseudoalteromonas* (Fig. 15 and Table S1†).[219] Chemically, prodigiosin **36** is 2-methyl-3-pentyl-6-methoxyprodiginine consisting of three pyrroles in the structure. Prodigiosin production yields are greatly influenced by various nutritional and environmental factors, such as carbon, phosphate, and nitrogen sources, inorganic salts, media composition, oxygen availability, temperature, pH, and incubation time.[41,223,224] The biosynthesis of **36** in the genus *Serratia* is dependent on the *pig* gene cluster consisting of *pigA–N* or *pigA–O*.[225,226] The regulation and biosynthesis of prodiginines in *Serratia* spp., *Streptomyces* spp. and *Pseudoalteromonas* spp. have been reviewed recently.[227,228]

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Prodigiosin **36** has numerous potential beneficial properties such as antibacterial,[229] antifungal,[223] antimalarial,[230] antiprotozoal,[231] anticancer,[232] immunosuppressant,[233] and as natural colourants for the dyeing of silk and wool.[227,234] It is active against a wide range of Gram-positive bacteria including *S. aureus* and *B. subtilis*,[221] and Gram-negative *E. coli*, *Erwinia carotovora*, *S. enterica*, as well as drug-resistant strains such as MRSA and oxacillin-resistant *S. aureus* (ORSA).[223] Prodigiosin targets the bacterial plasma membrane and causes disruption and loss of vital intracellular substances (K^+ ions, sugars, amino acids, proteins) *via* a chaotropicity-mediated mode-of-action.[235] Bacterial prodigiosin and related analogues exhibit *in vitro* antiproliferative activity against over 60 human cancer cell lines with an average inhibitory concentration of 2 μM. Furthermore, they are also potent inhibitors of T lymphocyte proliferation.[223] Findings associated with anticancer and immunosuppressive properties of prodiginines and their possible modes of action have been subject to several reviews.[236–238] Prodigiosin has also been used as inspiration to develop potent analogues such as obatoclax mesylate (GX15-070) which is currently in clinical trials for the treatment of various types of cancer including lymphoma, myelofibrosis, leukaemia, and mastocytosis.[236,239–241]

The physiological and ecological function of prodigiosin remains elusive. Its ubiquitous nature suggests that it may be ecologically beneficial to the producer organism. However, the precise role of the pigment remains elusive due to the diversity of prodiginine producers.[228] In *S. marcescens*, prodigiosin **36** is not an essential virulence factor.[218] Some reports have suggested potential roles of the pigment which is likely a mode of defence against microbial competitors in a continuously dynamic environment or as a response to natural stressors.[223,228] Apart from its protective function against predators, prodigiosin may also serve as a metabolic sink (energy overflow) through the consumption of the excess NAD(P)H or proline from primary metabolism.[242] *S. marcescens* colonizes and propagates in the environment *via* swarming, swimming, and air dispersal. It is speculated that prodigiosin contributes to *Serratia's* cell surface hydrophobicity and consequently its improved motility facilitates bacterial dispersion through the air.[223,243]

4.3.2 Althiomycin. The broad spectrum-antibiotic, althiomycin **37** ($C_{18}H_{17}N_5O_6S_2$, 439 Da) was first isolated from *Streptomyces althioticus* in 1957 (Fig. 15 and Table S1†).[244] Its structure consisting of two glycines, two cysteines, and one serine was elucidated in 1974 by X-ray crystallography.[245,246] Althiomycin **37**, which is also produced by myxobacteria of the genera *Cystobacter*[247] and *Myxococcus*[248] and other *Streptomyces* species,[246] was only identified from the entomopathogen *S. marcescens* Db10 in 2012.[249] The biosynthesis of althiomycin in *S. marcescens* is proposed to involve six genes (*alb1–6*) that encode a hybrid of NRPS and PKS systems closely related to *Myxococcus xanthus* DK897.[248]

Althiomycin **36** displays wide spectrum antibiotic activity against several Gram-positive bacteria including strains of *S. aureus* (MIC = 16–25 μg mL^{-1}),[250,251] *E. faecalis* (MIC = 16 μg mL^{-1}),[251] *Corynebacterium diphtheriae* (MIC = 0.8 μg mL^{-1})[250] and Gram-negative bacteria including *E. coli* (MIC = 1 μg mL^{-1}),[251] *K. pneumoniae* (MIC = 6.3 μg mL^{-1})[250] and *Shigella flexneri* (MIC = 25 μg mL^{-1})[250] but exhibits no such effects in mammalian cells.[252] Althiomycin **36** blocks the action of the peptidyl transferase by binding to the 50S ribosomal subunit, thus inhibiting prokaryotic protein synthesis.[244,252] Althiomycin and derivatives have been chemically synthesised (albeit with low efficiency).[251,253] The synthetic de(hydroxymethyl) althiomycin analogue showed comparable antibiotic activity to that of the parent compound. SAR studies indicated that the 4-methoxy-3-pyrrolin-2-one moiety, and the configuration of the oxime group and thiazoline ring are relevant to its bioactivity.[250] This methoxypyrrolinone pharmacophoric feature in althiomycin is also present in other bioactive natural products such as malyngamide A,[254] sintokamide A,[255] and mirabimide E.[256] To date, the difficulties encountered in chemical synthesis have hampered further investigations into the potential of althiomycin-based compounds as antibacterial drugs.[249]

4.3.3 Albicidin. The antibiotic albicidin **40** was first characterized in 1985 from the chlorosis-inducing cultures of *X. albilineans* isolated from diseased sugarcane (*Saccharum officinarum* L.) (Fig. 15 and Table S1†).[257–259] It took 30 years before the structure of **40** was fully elucidated owing to its extremely low yields in *X. albilineans* cultures.[257,260] The development of a viable heterologous expression system in a fast-growing bacterium, *Xanthomonas axonopodis* pv. vesicatoria optimized albicidin production[261] (albeit with low efficiency ~1 mg per 100 L cell culture), which enabled unambiguous structural elucidation of **40**.[260] Albicidin **40** is a rather extraordinary linear polyaromatic oligopeptide composed of a cinnamoyl residue at the N-terminus, an unusual β-cyano-L-alanine (Cya), two *para*-aminobenzoic acids and a dipeptidic moiety at the C-terminus (4-amino-2-hydroxy-3-methoxybenzoic acids).[260]

The structure determination of **40** paved the way for chemical synthesis providing multigram quantities of albicidin and enabling SAR studies of the albicidin scaffold.[262] Albicidin **40** targets the GyrA subunit of the DNA gyrase (topoisomerase II),[263] an essential enzyme that catalyses and modulates the extent of supercoiling of double-stranded DNA.[264] Albicidin inhibits this supercoiling activity of *E. coli* DNA gyrase with half-maximal inhibitory concentrations (~40 nM) lower than those of most coumarins and quinolones.[263] Albicidin is bactericidal against a wide range of Gram-positive and Gram-negative bacteria with nanomolar potency particularly against fluoroquinolone-resistant strains of *E. coli* (MIC = 0.031–0.5 μg mL^{-1}), *Salmonella enteritidis* (MIC = 0.5 μg mL^{-1}), and *P. aeruginosa* DSM 117 (MIC = 1.0 μg mL^{-1}).[265] Structural modifications of **40** such as the substitution of the central amino acid β-cyanoalanine with polar threonine residue[266] or azahistidine leads to analogues with increased bioactivity over the natural albicidin.[267] Replacement of the N-terminal methylcoumaric acid moiety with benzoyl or acyl residues leads to inactivity towards the *E. coli* gyrase[268,269] whereas carbamoylation of the N-terminus motif, which is most likely a post-NRPS reaction gives rise to a more potent bacterial gyrase inhibitor (IC_{50} ~ 8 nM).[270] Synthetic azahistidine–albicidin variants with ethoxy group substitution on the C-terminal dipeptide motif exhibits increased potency against Gram-positive *B. subtilis*, *Mycobacterium phlei* and ciprofloxacin-sensitive (MIC = 0.031 μg mL^{-1}) and -resistant *S. aureus* (MIC = 0.063 μg mL^{-1}).[267] Variation

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in the molecule's stereocenter has minimal effect on the activity as indicated by *ent*-albicidin containing the D-Cya exhibiting comparable gyrase activity (IC_{50} ~ 40 nM) with the natural product albicidin.[265] Furthermore, replacing the central amide bond with a triazole moiety leads to a novel albicidin analogue that can overcome the serine endopeptidase AlbD resistance while preserving biological activity.[267,271]

4.3.4 Reutericyclin. Reutericyclin **38a**, *N*-acyl tetramic acid, was initially isolated from the cultures of lactic acid bacteria *Lactobacillus reuteri* LTH2584 originating from an industrial sourdough isolate (Fig. 15 and Table S1†).[71,272,273] Its chemical structure was confirmed by chemical synthesis.[272,274] More recently, reutericyclin (renamed reutericyclin A **38a**) and analogues reutericyclin B **38b** and C **38c** including the unacylated tetramic acid mutanocylin **39** were produced from the *muc* gene cluster in *S. mutans* B04Sm5 isolated from the mouth of a child with severe dental caries.[30] Mutanocylin **39** was also reported to be produced after the unidentified BGC1 in *S. mutans* 35 was activated *via* a new heterologous expression system.[275] The chemical structures of reutericylins A–B **38a–b** differ from C–D **38c–d** in the presence of the *N*-substituted α,β-unsaturated fatty acid whereas the latter have saturated acyl chains.[30] In solution, tetramic acids undergo keto–enol tautomerism, and the preferred tautomeric form of reutericyclin is the pyrrolidine-2,4-dione which differs from all other naturally-occurring 3-acyl-tetramic acids.[272,276]

The reutericyclin BGC in *S. mutans* comprises 9 genes (*mucA–I*) that encode a hybrid modular PKS–NRPS assembly line, as well as enzymes involved in transport and regulation. Reutericyclin **38a–c** are proposed to be assembled from C10 or C11 fatty acids as starter units through elongation with leucine, which is subsequently extended *via* a malonyl-CoA unit (Fig. 16). The reutericyclin genomic island does not code for enzymes related to fatty acid metabolism,[277] thus the C10 or C11 lipid chain in **38a–c** may come from the general metabolism through the action of hydroxyacyl-ACP dehydratases to generate *trans*-2-decenoyl-ACP, decanoyl-ACP, and *trans*-2-dodecenoyl-ACP.[30]

Another interesting feature of the *muc* assembly line is the lack of an epimerase (E) domain or dual functioning C/E domains required in the conversion of L-to D-leucine residue. The A domain in MucD appears to incorporate the D-leucine building block in **38a–c**.[30] Most Gram-positive bacteria have the ability to synthesise D-alanine and D-glutamic acid as components of the peptidoglycan cell wall, however, the synthesis of other D-amino acids is less common.[278] Feeding of [$^{13}C_1$] L- and D-leucine to fermentation cultures of *S. mutans* and *L. reuteri* revealed incorporation of only [$^{13}C_1$] L-leucine.[30] Conversely, an isoleucine 2-epimerase with leucine epimerase activity has been characterised in lactobacilli,[279] and *L. reuteri* strains have been reported to produce D-leucine.[277] Presumably, *S. mutans* may also contain isoleucine 2-epimerase homologues responsible for D-leucine synthesis. Additionally, the *muc* TE domain may also act as epimerase as exemplified by the NocTE domain in nocardicin biosynthesis.[280] However, MucTE exhibits very low homology to the dual functioning NocTE domain.[30] It is currently unclear which enzyme is responsible for the epimerization reaction in reutericyclin biosynthesis. The first three genes, *mucABC* are homologous to the phloroglucinol biosynthetic proteins PhlABC, and are believed to catalyse the acetylation of the pyrrolidine ring in **38a–c**. Expression of the MucA–E in *E. coli* BAP1 strain resulted in the production of **38a–c** and a new analogue reutericyclin D **38d** containing an *N*-dodecanoyl substituent, indicating that genes *mucA–E* indeed compose the minimal BGC for **38a–c** production. Furthermore, heterologous expression and deletion experiments characterised MucF as a new deacylase responsible for converting reutericyclin **38a–c** to the tetramic acid **38d** lacking the lipid chain.

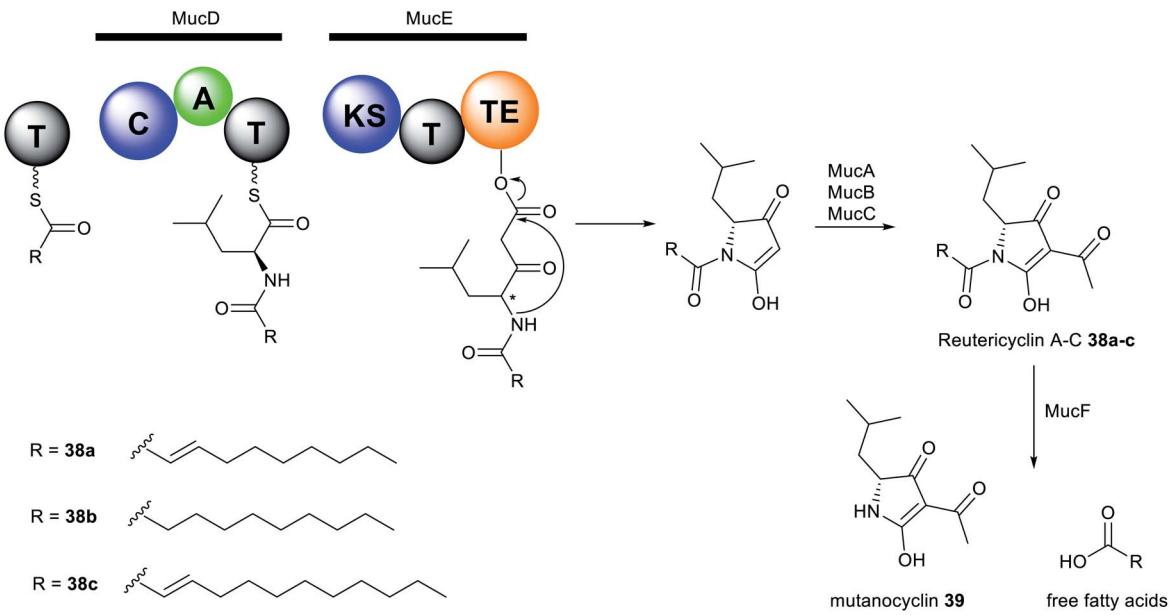

Fig. 16 Proposed reutericyclin and mutanocyclin biosynthesis.

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Reutericyclin exhibits potent activity against a broad range of Gram-positive bacteria, including *B. cereus*, *B. subtilis*, *E. faecalis*, *S. aureus*, *Lactobacillus* spp., *Weissella confusa* and clinical isolates of *E. faecium* (MIC = 0.06–6.5 μg mL^{-1})[71] as well as pathogens associated with topical infections such as mupirocin-resistant MRSA (MIC = 0.8–3.12 μg mL^{-1}),[72] macrolide-resistant *Streptococcus pyogenes* (MIC = 0.012–0.4 μg mL^{-1})[72] and *Clostridium difficile* (MIC = 0.09–0.38 μg mL^{-1}).[71,281] Gram-negative bacteria, yeast, and fungi are resistant to reutericyclin.[71] The natural reutericyclin exhibits slightly higher antibacterial activity compared to the synthetic reutericyclin racemate, indicating that the stereochemistry is vital to the compound's bioactivity.[282] Reutericyclin is an amphiphilic molecule consisting of a hydrophilic negatively charged group and two hydrophobic side chains. Thus, it acts as a proton ionophore and targets the cytoplasmic membrane causing dissipation of the transmembrane proton potential (ΔpH) in sensitive cells.[72,73,283] SAR revealed that substitution of these hydrophobic groups with polar or charged substituents diminishes the antibacterial activity. The loss of activity in polar-substituted reutericyclins is probably due to the decreased interaction with the hydrophobic regions of the bacterial membrane.[283] Although the *in vitro* profile of reutericyclin **38a** is comparable to the antibiotic mupirocin, it's *in vivo* activity in *S. aureus* murine infection model is 5-fold weaker compared to the antibiotic. The primary factor that may decrease the efficacy of **38a** *in vivo* is likely the slow partitioning of the aqueous dermis by the highly lipophilic reutericyclin molecules.[72] Reutericyclin is cytotoxic towards Vero epithelial cells and causes hemolysis in mammalian cells.[283] Conversely, modifications of the substituents in the *N*-substituted position has shown to modulate the cytopathic effects of this class of compounds.[284] Mutanocyclin **39** consisting mainly of the tetramic acid core lacks antibacterial activity, demonstrating that the presence of the appropriate ring moieties plays a critical role in the bioactivity.[30,275,276,284] Taken together, reutericyclins appear to be potent candidates for controlling recalcitrant skin infections caused by Gram-positive pathogens. Further medicinal chemistry optimization efforts are necessary to discover reutericyclin-based chemotypes with reduced toxicity whilst retaining or increasing antibacterial activity.

The production of reutericyclin **38a** in sourdough is thought to inhibit other competing Gram-positive competitor *L. sanfranciscensis* while enabling the stable persistence of the producing organism *L. reuteri*. A wide variety of food-related spoilage pathogens is inhibited by reutericyclin. Hence, reutericyclin-producing strains may find application in food preservation and fermentations.[68]

In *S. mutans*, the tetramic acids reutericyclins **38a–d** and mutanocyclin **39** are found to inhibit the growth of healthy oral microbes, suggesting that the pathogen likely use these molecules to remove the bacteria that block its growth to further cause severe dental caries.[30] The findings lay a foundation for the continued exploration of antibiotic-producing strains within the complex competing microbial niche of the human microbiota.

4.4 Ribosomally synthesized and post-translationally modified peptides (RiPPs)

Ribosomally synthesised and post-translationally modified peptides (RiPPs) are a large class of structurally diverse natural products (Fig. 17 and Table S1†). RiPPs are produced from a short precursor peptide comprising of a leader peptide and a core peptide. Biosynthesis begins with the synthesis of a precursor peptide by the ribosome. Then, the core peptide is subject to post-translational modifications (PTMs) beyond the 20 canonical amino acids; many of which are guided by leader peptides and recognition sequences to install a wide variety of unusual structural features onto the peptide backbone. Such PTMs can often render significant advantages over unmodified linear peptides, including enhanced target affinity and stability, as well as resistance to proteolytic degradation. Following modifications, the leader peptide and recognition sequences are cleaved by proteolysis, sometimes concomitant with cyclisation of the polypeptide chain, to produce the mature active product. In some cases, additional post-translational modifications occur after cleavage of the flanking sequences. For further information and perspectives on RiPP biosynthesis, we direct the readers to several recent reviews.[285,286] Numerous ribosomally-synthesised bacteriocins have been isolated from pathogenic bacteria, and they have been the subject of several different reviews.[25,287,288] In this section, we highlight those interesting antibiotic RiPPs with unusual PTMs from bacterial pathogens such as darobactin **45**, bottromycin **46**, and nocardithiocin **47** (Fig. 17 and Table S1†).

4.4.1 Darobactin. Darobactin **45** is the first member of a new class of antibiotics that selectively kills Gram-negative bacteria produced by *Photorhabdus khanii* (Fig. 17 and Table S1†).[289] Darobactin **45** is a 7-mer modified peptide with an amino acid sequence of Trp1–Asn2–Trp3–Ser4–Lys5–Ser6–Phe7. The unprecedented chemical architecture of darobactin features a novel scaffold with two fused macrocycles, an aromatic-aliphatic ether linkage between two tryptophans and a unique tryptophan–lysine bond between two inactivated carbons.[289] This unusual lysine–tryptophan crosslink was also previously identified in the peptide pheromone, streptide from *Streptococcus thermophilus*. Genetic and biochemical studies implicated a radical *S*-adenosylmethionine (rSAM) enzyme, StrB containing two [4Fe–4S] clusters likely responsible for the installation of the unique lysine-to-tryptophan crosslink in streptide.[290] Enzymes of the rSAM class catalyse free radical based reactions to incorporate a wide variety of unique and difficult modifications during RiPP biosynthesis, including α-, β-, and γ-thioether bridge, tyramine excision, epimerization, methylation, aliphatic-ether crosslink, and carbon–carbon bond formation.[291,292]

The putative BGC involved in darobactin **45** biosynthesis consists of a propeptide DarA, transporters DarB and DarD, membrane fusion protein DarC, and a RaS enzyme DarE. Deletion of the *dar* operon in *P. khanii* DSM3369 by double crossover abrogated production of **45**. Notably, heterologous expression of the *dar* BGC into *E. coli* produced the peptide suggesting that the *dar* is sufficient for darobactin production.

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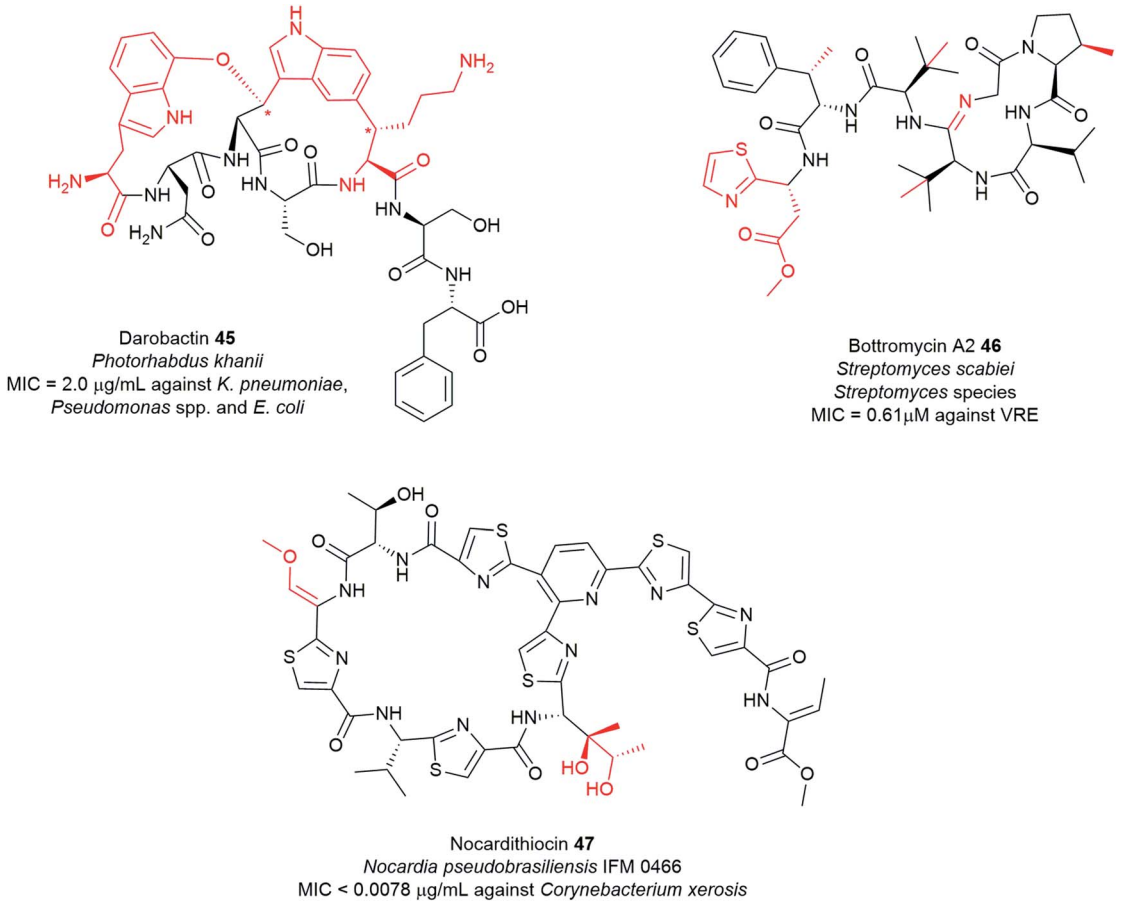

Fig. 17 Examples of antimicrobial ribosomal peptides with unusual motifs highlighted in red from pathogenic bacteria. The stereogenic centres of **45** labelled in star (*) were deduced from DFT calculations and ROESY correlations.

DarE showed little homology to StrB, nonetheless, DarE harbours the rSAM and SPASM/Twitch domains that are characteristic of this diverse protein superfamily. It is speculated that DarE catalyses the versatile formation of Lys-to-Trp macrocyclic crosslink in darobactin. Recently, a novel rSAM enzyme TqqB has been shown to install a C–O–C Thr–Gln ether cross-link.[291] The *dar* operon does not encode a separate putative enzyme that incorporates the C–O–C Trp–Trp ether bond. It was speculated that DarE may not only catalyse the linkage of the Trp–Lys C–C bond but also the formation of the aromatic–aliphatic ether linkage in darobactin. RiPP operons often encode a protease that cleaves out the mature peptide; however, this is not present in the *dar* operon. Generic proteolysis is presumed to be involved in the maturation of the propeptide.[289] It is anticipated that structural and biochemical investigations of the novel darobactin enzymatic system will further expand the repertoire of rSAM enzymes and will aid future engineering efforts of RiPP natural products.

Darobactin **45** is effective against multiple Gram-negative bacteria *in vitro*, including drug-resistant human pathogens such as polymyxin-resistant *P. aeruginosa* and extended-spectrum β-lactam-resistant *K. pneumoniae* and *E. coli* and carbapenem-resistant clinical isolates (MIC = 2–64 μg mL^{-1}). It exhibits better efficacy in several mouse septicaemia infection models than the antibiotic gentamicin. Darobactin, however, showed little to no activity on Gram-positive bacteria, gut commensals including *Bacteroides* and human cell lines (HepG2, FaDu, HEK293) up to 128 μg mL^{-1} concentration.[289]

Gram-negative bacteria are difficult to treat due to their double-membrane cell wall, which forms a protective barrier from antibiotics.[293] The outer membrane contains a layer of negatively charged lipopolysaccharides in addition to proteins and phospholipids that blocks the entry of large and hydrophobic molecules.[9] The cut-off size for compounds that can penetrate the membrane is about 600 Da. Given the size of darobactin (966 Da), it cannot breach this permeability barrier but instead acts on the surface of the cell. Darobactin binds to the β-barrel assembly machine (BAM) A protein and induces the closed-gate conformation, thereby preventing the normal protein folding and membrane insertion necessary for bacterial survival.[289] The discovery of darobactin **45** offers a promising lead in the dwindling pipeline of antibiotics that selectively target the Gram-negatives. Currently, darobactin **45** is in pre-clinical stage.[9]

The production of darobactin **45** in large amounts remains a challenge. Heterologous expression in different *Photorhabdus* species yielded highest in *P. khanii* DSM 3369 strain (3 mg L^{-1}),[289] yet the production titre is still low. The complexity

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of the structure and the stereochemistry make it difficult to be obtained by chemical synthesis. The poor yield complicates both drug development and further biosynthetic studies of the molecule. Nonetheless, bacterial genome sequences identified further tentative analogues, darobactins B–E from *Yersinia*, and *Photorhabdus* species.[289] Expression of these putative darobactins may provide insight into the structure–activity relationships (SAR) and determine the pharmacophoric regions of the molecule. The identification of the biosynthetic route of **45** should facilitate the generation of a library of darobactin-like antibiotics that selectively targets the Gram-negatives.

4.4.2 Bottromycin. Bottromycin **46** was first isolated from the fermentation cultures of *Streptomyces bottropensis* in 1957 (ref. 294) and was later characterised in several other *Streptomyces* species[295] including the plant pathogen *S. scabies* (Fig. 17 and Table S1†).[296,297] The structure elucidation process involved several repeated revisions since its first isolation[298–301] which ultimately led to the assignment of bottromycin **46** and was later confirmed by chemical synthesis.[302] The structure features an unprecedented macrolactamidine ring, rare β-methylated amino acid residues, and a terminal methyl ester and a thiazole heterocycle.[294,296,298] Through untargeted metabolomics and mass spectral networking analysis, the biosynthetic pathway of bottromycins in *S. scabies* was determined which involves a series of complex and unprecedented modifications from the precursor peptide (BtmD) catalysed by the enzymes encoded in the *btm* gene cluster (Fig. 18).[303] The regulation of bottromycin biosynthesis in *S. scabies* was recently elucidated. The only regulatory gene *btmL* encoded in the cluster was identified to be a positive modulator of *btmD* and not a master regulator that controls bottromycin expression.[304] The mechanism by which BtmL modulates BtmD transcription in *S. scabies* remains elusive. An understanding of the regulation of bottromycin biosynthesis may shed light into further engineering and overproduction of medicinally promising bottromycin-based compounds as well as expression of other RiPP pathways.

Total synthesis of bottromycins and analogues enabled evaluation of their antibacterial activity.[302,305–307] Bottromycin inhibits the growth of a wide range of microorganisms by blocking the binding of aminoacyl tRNAs to the A-site on the 50S ribosome, ultimately leading to inhibition of bacterial protein synthesis.[308,309] The bottromycins, particularly bottromycin A2 **46**, display potent antibacterial activity against Gram-positive bacteria including clinically-isolated MRSA and VRE strains (MIC = 0.5–2.0 μg mL^{-1})[305,306,310] and mycoplasma.[306] A natural de-methyl analogue of **46**, bottromycin B2, exhibits slightly reduced antibacterial activity (MIC = 4 μg mL^{-1}).[305] The three-dimensional structure of bottromycin is

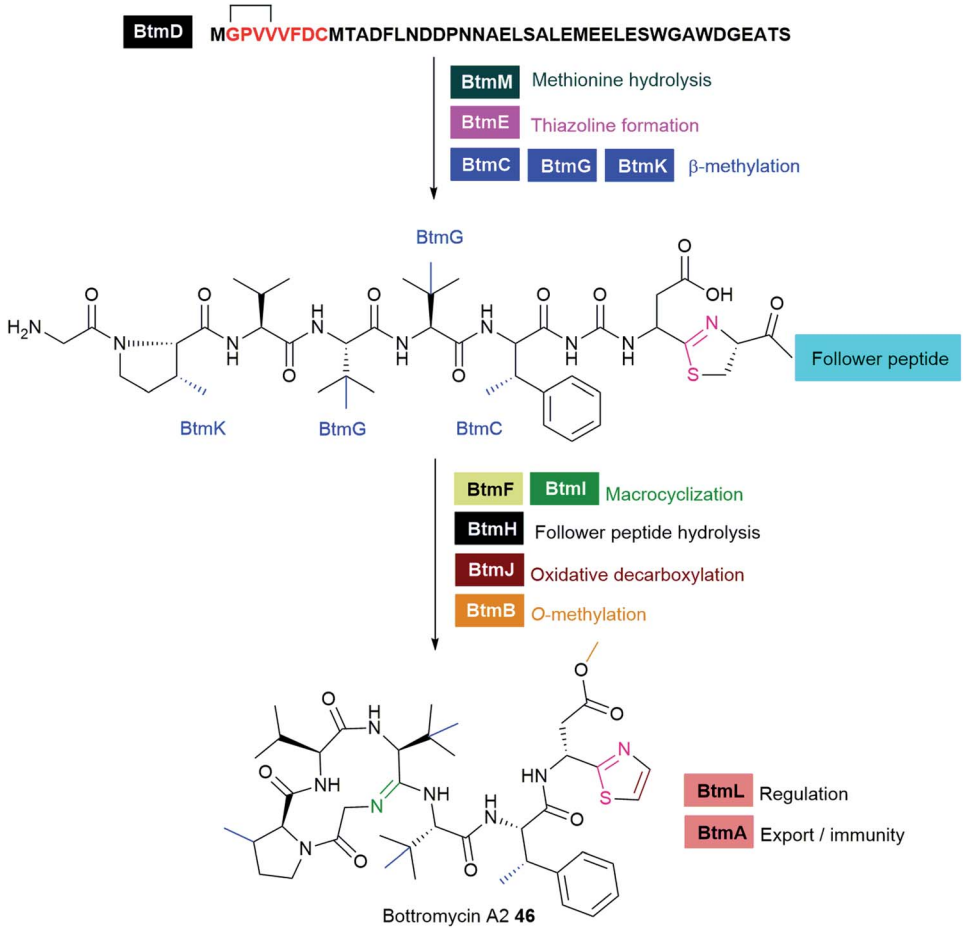

Fig. 18 Biosynthesis of bottromycin A2 in *S. scabies*.

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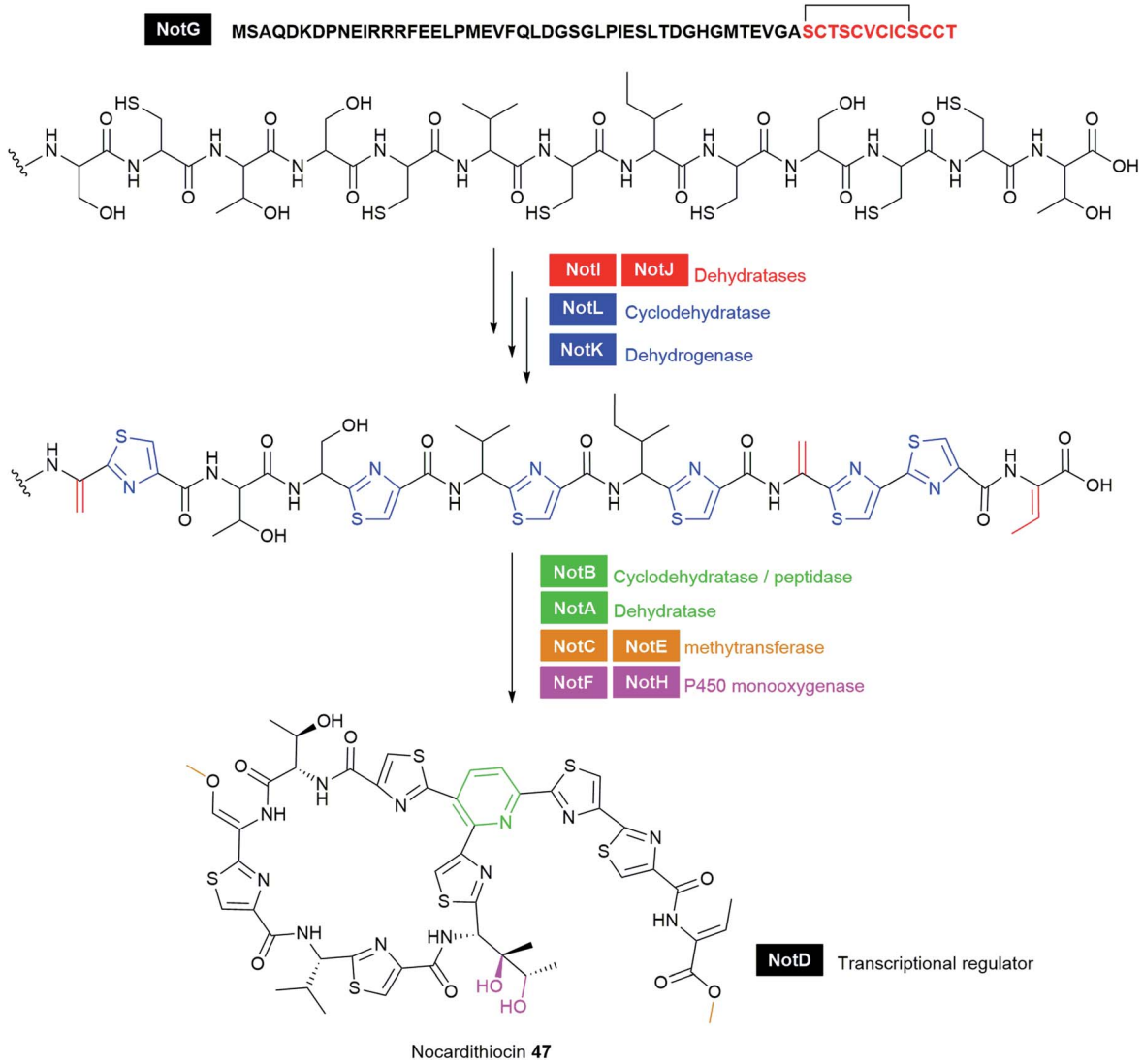
essential for the antibacterial activity while the thiazole and methyl ester moieties are not required.[305]

The development of bottromycin as an antibacterial drug is impeded by the reduced *in vivo* efficacy in MRSA-infected mice. This reduced efficacy is mainly due to the instability of the terminal methyl ester moiety, which undergoes rapid hydrolysis to carboxylic acid in blood plasma rendering it inactive. Notably, the substitution of this ester with a ketone functionality resulted in potent and stable analogues with improved pharmacological properties and superior *in vivo* efficacy in the mouse infection model than **46**.[306] Therefore, further structural optimization of bottromycin-based compounds *via* engineering of the biosynthetic pathway or chemical synthesis offers promising leads for the development of new bottromycin-based anti-infectives. Recently, yeast-mediated pathway engineering of the bottromycin BGC through an inducible, theophylline-controlled riboswitch system led to an overall 120-fold increase in pathway productivity in a heterologous *Streptomycete* host.[311] Another approach involved promoter exchange that resulted in 5–50 fold higher productivity of a suite of new bottromycin-related compounds compared to the wild type strain.[312] Application of these strategies to turn-up or upregulate biosynthetic pathways that are involved in controlling metabolic yields will undoubtedly facilitate the discovery of known NPs and new bioactive NPs in Actinobacteria.

4.4.3 Nocardithiocin. Nocardithiocin **47** is a thiocillin-like thiopeptide produced by the opportunistic pathogen *N. pseudobrasiliensis* strain IFM 0757 obtained from a clinical sample (Fig. 17 and Table S1†).[313] Thiopeptides (or thiazolyl peptides) are a family of highly modified sulfur-rich peptides, characterised by a macrocycle bearing a nitrogen-containing six-membered ring core, numerous thiazole rings, and several dehydrated amino acid residues.[314] Nocardithiocin contains the characteristic 2,3,6-trisubstituted pyridine core, and hence it is classified into series d.[313]

Biosynthesis of nocardithiocin **47** is proposed to be directed by a 12 gene-cluster (*notA–L*), with NotG as the precursor peptide (Fig. 19).[315] The characteristic pyridine core in **47** is likely



Nocardithiocin **47**

Fig. 19 Proposed biosynthetic pathway of nocardithiocin **47**.

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formed by macrocyclization of the precursor peptide at sites Ser1 and Ser10. The structure of **47** features isoleucine (Ile8) bearing two hydroxy moieties resembling PTMs observed in thiostrepton that is probably catalysed by a cytochrome P450.[316] A second P450 is predicted to hydroxylate a dehydroalanine (Dha4), similar to those observed in berninamycin compounds,[317] which is subsequently methylated by a putative methyltransferase (NotC or NotE). Another methyltransferase (NotC or NotE) likely installs a methyl group at the C-terminus.[315]

Nocardithiocin **47** exhibits potent bacteriostatic activity against a variety of bacteria including *Corynebacterium xerosis* (MIC < 0.0078 μg mL^{-1}), *M. smegmatis* (MIC = 0.062 μg mL^{-1}), *Nocardia asteroides* (MIC = 0.062 μg mL^{-1}), and *Gordonia bronchialis* (MIC = 0.03 μg mL^{-1}). It is also highly active against rifampicin-resistant bacteria as well as -sensitive *M. tuberculosis* strains, and most of the resistant strains were inhibited at concentrations ranging from 0.025 to 1.56 μg mL^{-1}.[313] Despite the impressive antibiotic activity of **47**, its clinical use is hampered by poor aqueous solubility and light instability.[313,314] The identification of the nocardithiocin BGC expands the possibility for further structural modifications to generate stable analogues with improved pharmacokinetic properties. Genetic modification of the nocardithiocin scaffold *via* substitution of Val6 of the core peptide by ten mostly hydrophobic amino acids yielded nocardithiocin analogues, two of which showed improved MIC against a panel of Gram-positive bacteria. Furthermore, nocardithiocin and all analogues were stable to light. However, they remained poorly water-soluble.[318] Introduction of polar groups at the

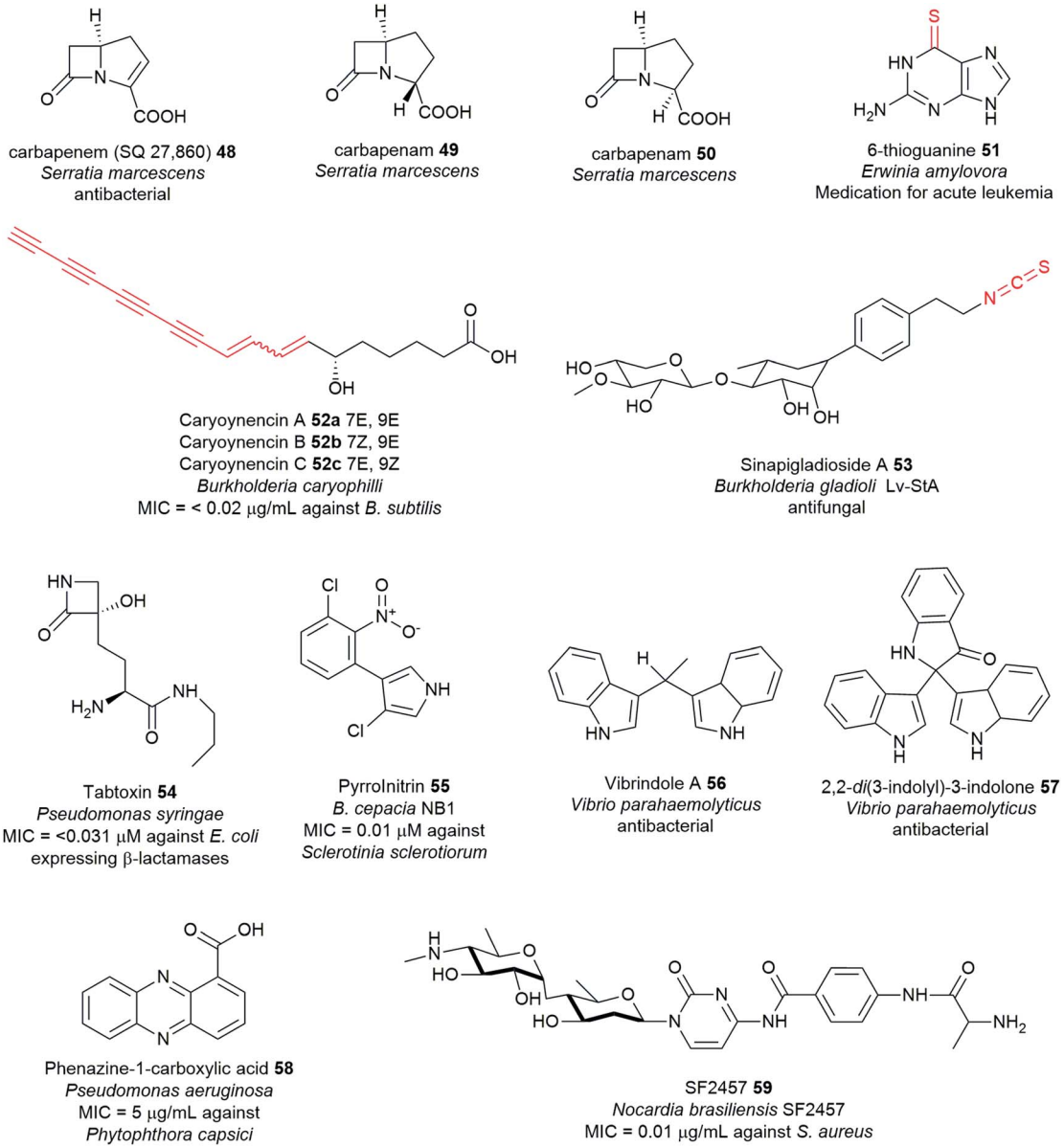

Fig. 20 Other categories of metabolites discovered from pathogens.

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tail end of the nocathiacin thiopeptide enhanced its water-solubility while retaining its potent *in vitro* and *in vivo* antibacterial activity.[319] Similar tail modifications could also improve the solubility of nocardithiocin without diminishing antibacterial potency.

4.5 Other categories of metabolites from pathogens

Most of the PKS and peptidyl compounds discussed in this review can be classified based on their biosynthetic class. Other antimicrobial metabolites, including nucleosides, indoles, guanine, β-lactams, and carbapenems will be covered in this section, highlighting those with remarkable activity.

4.5.1 Carbapenems. *Serratia* strains generate antibiotics of the carbapenem group (Fig. 20 and Table S1†).[320] Carbapenems belong to a diverse group of β-lactam antibiotics, which are now the most widely used class of clinical antibiotics to date.[321] Their biosynthesis involves a biochemical route unique from the other four known classes of β-lactams, such as penicillins,[322] cephalosporins,[322–324] monobactams,[325] and clavams.[326] The biosynthesis and regulation of carbapenems have been extensively studied in *Serratia* and involve a nine-gene cluster, *carRABCDEFGH*.[327,328]

Carbapenems have broad-spectrum activity against important Gram-positive and Gram-negative pathogens, particularly nosocomial multidrug-resistant bacteria.[321,328] Furthermore, they exhibit potent antibacterial and β-lactamase-inhibitory activity.[328,329] *S. marcescens* makes the simplest known β-lactam antibiotic containing only the bicyclic nucleus, 1-carbapen-2-em-3-carboxylic acid (SQ 27860) **48** and two saturated diastereomers, (3*R*,5*R*)- and (3*S*,5*R*)-carbapenam-3-carboxylic acids **49–50** (Fig. 20 and Table S1†).[320,330–332] Gram-negative enteric *Erwinia* strains also produce carbapenem **48** and carbapenams **49–50**, and later **48** was identified as a metabolite of the entomopathogen, *P. luminescens*.[330–332] Unlike **48**, these carbapenams **49–50** lack antibacterial activity but are resistant to β-lactamases I and II from *B. cereus*.[332] Carbapenem **48** is a potent antibacterial, but it is highly unstable and requires initial derivatization to the *p*-nitrobenzyl ester for isolation. Carbapenem **48** is active against several strains of *S. aureus*, *E. coli*, and *Enterobacter cloacae*.[331]

4.5.2 Thioguanine. Thioguanine **51** (also known as tioguanine or 6-thioguanine, 6-TG or 2-aminopurine-6-thiol), introduced in the early 1950s for antimetabolite therapy,[333] is now in clinical use for the treatment of various diseases including psoriasis, inflammatory bowel disease and acute and chronic myelogenous leukaemia (Fig. 20 and Table S1†).[334–337] Thioguanine **51** is a sulfur-containing guanine analogue that works by disrupting DNA and RNA. Originally known as a synthetic compound,[333] thioguanine was first isolated from the cultures of *Pseudomonas* sp. GH[338] and later identified as a critical virulence factor of the plant pathogen, *E. amylovora*.[50,339]

The biosynthesis of thioguanine **51** has been recently elucidated in *E. amylovora* and is encoded by the *ycf* gene cluster (Fig. 21A). The rare thioamide moiety in thioguanine is likely derived from the action of two key enzymes YcfA and YcfC which constitutes a bipartite enzyme system, unique from those previously described thionation in RNA systems (Fig. 21B).[337,339] The ATP-dependent YcfA enzyme catalyses the transfer of sulfur onto the guanine backbone[339] and uses a pyridoxal phosphate

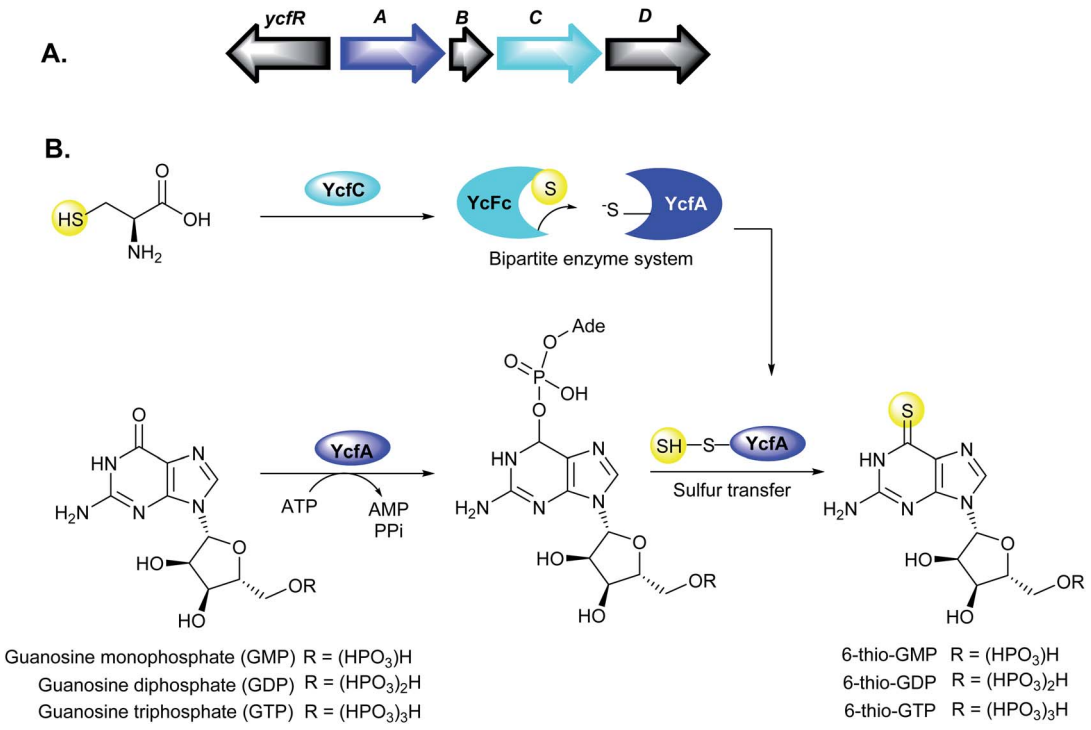

Fig. 21 (A) Organization of the *ycf* gene cluster in *Erwinia amylovora*, and (B) model for the bipartite enzyme system, YcfA and YcfC for the oxygen-to-sulfur substitution in thioamide formation.

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(PLP)-dependent specialised sulfur shuttle enzyme, YcfC that functions independently from the general sulfur mobilization pathways.[337] While the sulfur source in universal RNA-systems often originates from L-cysteine through the action of cysteine desulfurases (IscS),[340] the cysteine-derived sulfur nucleophile in thioguanine biosynthesis is provided by YcfC and then, transferred and bound onto one of the cysteine residues (Cys113) of the YcfA active site.[337,339] Meanwhile, no thionated products were detected using the IscS homologue (Ea-IscS)-catalysed reaction in *E. amylovora*.[337] YcfA initially activates the guanine backbone by adenylating the carbonyl oxygen prior to thionation.[339] Subsequent YcfA-mediated sulfur transfer to the activated substrate generates the thioamides with concomitant release of adenosine monophosphate (AMP).[337,339]

In addition to its anticancer properties,[334] thioguanine **51** is bacteriostatic towards *E. coli*[338] and strains of *Salmonella typhimurium* and *Pantoea agglomerans*,[341] with the resumption of cell growth after prolonged incubation. At 0.25 μM concentration, thioguanine completely inhibited the growth of *E. coli* strains B and K12.[341] This inhibition did not occur when either adenine or guanine was present in the assay medium.[338] Growth inhibition was also reported for *B. subtilis*,[342,343] and the abolishment of flagella formation for *B. cereus*.[344] Other organisms like *Streptococcusfaecium* strain, *S. cerevisiae*, *Rahnella aquatilis*, and *Gibbsiella* sp. (strain BK1) were insensitive to thioguanine.[50,341,345]

4.5.3 Caryoynencin. Caryoynencin A, B, and C **52a–c** were isolated from the cultures of the plant carnation pathogen *Burkholderia caryophylli* through bioactivity guided screening. The structure features an exceedingly rare polyacetylene functionality, and remarkably, caryoynencin is the only known bacterial polyyne with four conjugated triple bonds. However, caryoynencin is extremely unstable. Isolation of **52** from the active extract was carried out by applying argon at 0 °C to obtain a concentrated mixture of caryoynencins A, B, and C. By contrast, concentration on a rotary evaporator (20 °C) or storage at −20 °C led to a complete loss of bioactivity. Furthermore, the individual component was not achievable since they equilibrate rapidly.[346] Recently, a derivatization strategy that selectively targets terminal alkynes was used to isolate and characterise these extremely unstable compounds directly from the culture extracts (Fig. 22).[347] The so-called CuAAC (copper-catalysed azide–alkyne cycloaddition) "click reaction"[348] was carried out by treatment of the *B. caryophilli* extracts with benzyl azide and copper(I) catalyst to obtain the triazoles **60a–b** and **61b**, the structures of which were fully elucidated by NMR.[347]

Transposon mutagenesis and genome sequencing of *B. caryophilli* (DSM50341) have provided the first insight into the unusual polyyne biosynthesis in bacteria which involves novel desaturases and a cytochrome P450 monooxygenase (Fig. 23A). Disruption of the transposon site points to a Δ^9 desaturase-like gene, orthologs of which were identified in the genomes of the plant pathogens *Burkholderia gladioli* BSR3 and *B. gladioli* pv. cocovenenans. Comparative genomics analyses further revealed that the caryoynencin (*cay*) locus is conserved in several *Burkholderia* strains, and homologous gene clusters were also identified in various other bacteria.[347] Metabolomic analyses also revealed that strain BSR3 and *B. gladioli* Lv-StA are capable of caryoynencin production.[347,349] The characterisation of the *cay* BGC will thus facilitate the discovery of numerous polyyne-bearing NPs from bacteria and lead to the expansion of the polyyne biosynthetic machinery capable of producing polyacetylenes.

Caryoynencin is likely derived from fatty acid-ACP, followed by desaturation to yield the alkyne motifs, **62** (Fig. 23B). Three putative desaturase genes (*cayB*, *cayC*, *cayE*) were implicated to be responsible for the incorporation of the triple bonds. Phylogenetic analyses have revealed that the CayBCE desaturases are unique and have probably evolved independently from those found in fungi, plants, and insects.[347] Notably, CayB and CayC form a separate clade with the closest desaturase homologue JamB,[350] which has been suggested to introduce the terminal alkyne functionality in the jamaicamide pathway of *Moorea producens*. Subsequent hydroxylation and elimination reactions of **62** catalysed by the putative cytochrome P450

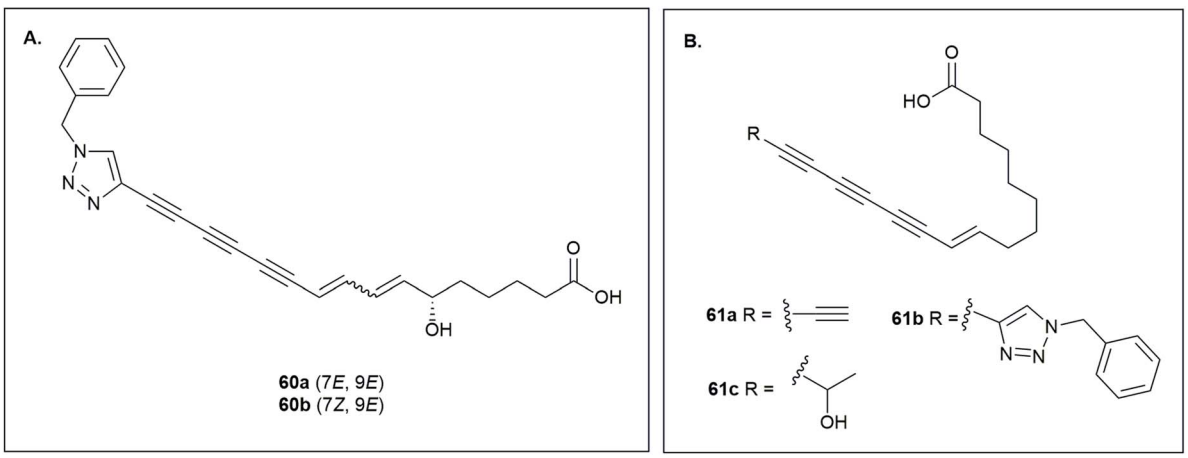

Fig. 22 Chemical trapping of the tetraynes by an *in situ* copper(I)-catalysed azide–alkyne cycloaddition (CuAAC) click reaction. (A) Structures of triazoles **60a–b** produced from *B. caryophylli* wild type after click reaction, and (B) structures of **61a** from *B. caryophylli* ΔcayG and **61b** after click reaction, and **61c** shunt product.

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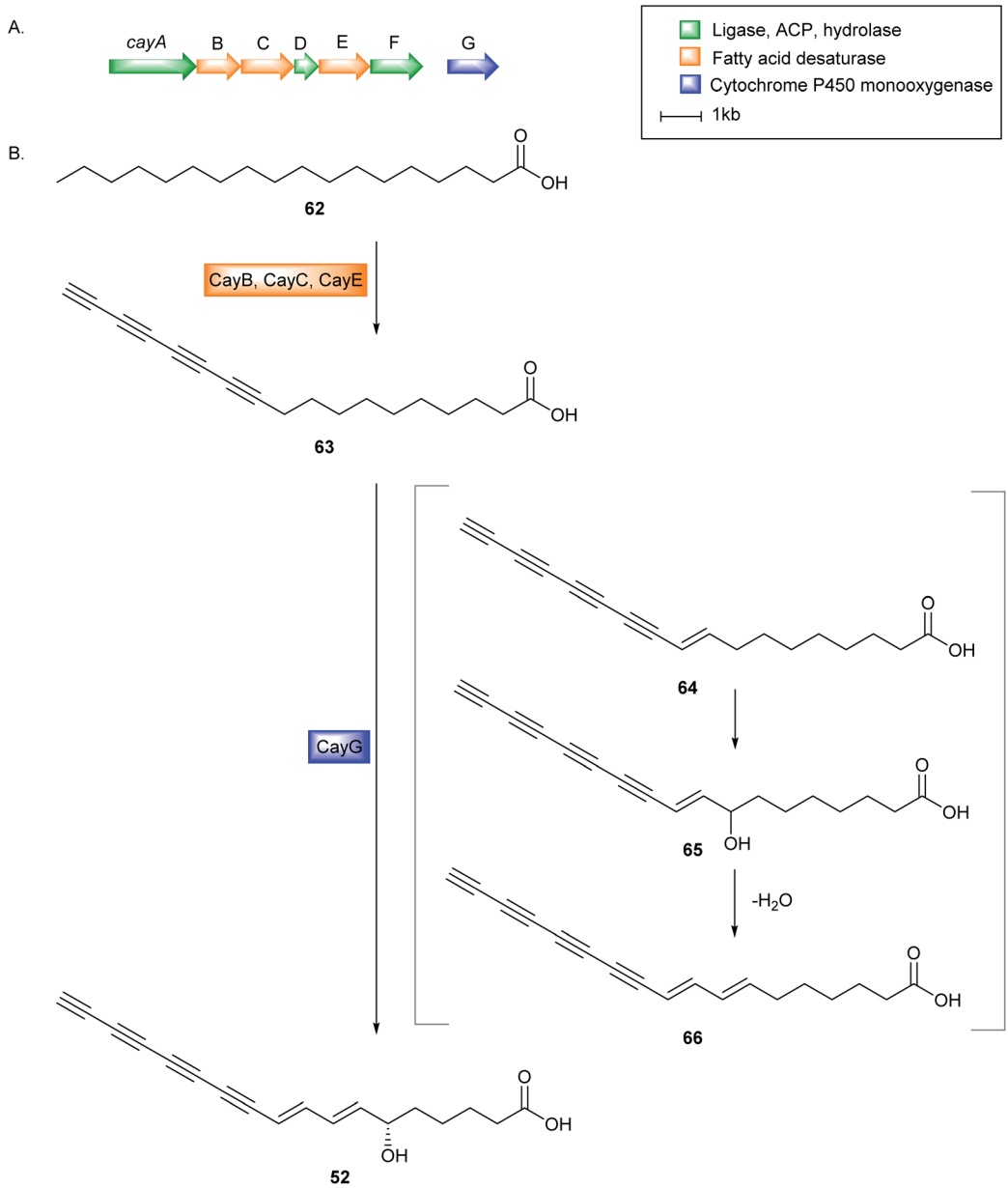

Fig. 23 (A) Biosynthetic gene cluster of caryoynencin (*cay*) in *B. caryophilli*, and (B) Proposed biosynthetic pathway of caryoynencin.

(CayG) generates the allylic alcohol moiety in caryoynencin **52**. Formation of the triazole **61b** lacking a hydroxyl group in Δ*cayG* mutant after an *in situ* click reaction supports the plausible function of CayG. Compound **61c** with an alcohol moiety instead of a triple bond is likely a shunt product of terminal alkyne formation.[347]

Caryoynencin has been shown to possess outstanding activity than the antibiotic kanamycin A. It is active against a wide range of bacteria and fungi including *E. coli* (MIC = 0.63 μg mL^{-1}), *K. pneumoniae* (MIC = 0.04 μg mL^{-1}), *S. aureus* (MIC = 0.02 μg mL^{-1}), *B. subtilis* (MIC = <0.02 μg mL^{-1}), *C. albicans* (MIC = 0.05 μg mL^{-1}), and several *Trichophyton* species (MIC = 0.02–0.05 μg mL^{-1}).[346,351] Owing to their high instability, synthetic approaches have been developed to gain more insight into their structure and function.[352,353] The terminal alkyne and the hydroxy group were crucial for the antibacterial activity while the diene motif and the butanoic acid were not essential.[351,353] The triazole **60b** is active against *B. subtilis* (MIC = 3.12 μg mL^{-1}) and MRSA (12.5 μg mL^{-1}),[346] consistent with earlier studies that the tetrayne molecules were more potent than the corresponding triyne or diyne analogues.[351] Remarkably, the introduction of a trimethylsilyl motif generates stable polyacetylene derivatives with potent antibacterial activity. Hydrophilicity also plays an important role in the bioactivity, and thus conjugates of polyynes and sugars, amino acids, and nucleic acids are attractive molecules.[353]

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5. Conclusions and future perspective

Pathogenic bacteria have an enormous yet unexploited potential for natural product drug discovery. Entomopathogenic, phytopathogenic, and human and animal pathogenic bacteria produce a repertoire of novel potential therapeutics, with an assortment of unprecedented structures, activities, and modes of action. Some of them are in pre-clinical trials (darobactin, NOSO-95C analogue) or have huge potential for drug development. Darobactin, odilorhabdin, and albicidin are promising candidates in the dwindling pipeline of antibiotics that selectively target the Gram-negatives.

Although darobactin is a potent drug lead for Gram-negative bacteria, the greater bottleneck is to produce it in large amounts for pre-clinical and clinical development. Moreover, the complexity and stereochemistry of darobactin make it difficult to be obtained by chemical synthesis, and thus SAR studies to determine the key bioactive moieties remain a challenge. Nevertheless, modifications of the complex vancomycin antibiotic have been achieved by several groups to produce potent analogues with less propensity to antibiotic resistance.[354] Lead optimisation of the odilorhabdin scaffold was also achieved and identified NOSO-502 as a clinical candidate for carbapenem-resistant Enterobacteriaceae.[128] Several other metabolites that possess potent *in vitro* activity can be chemically-modified to increase *in vivo* efficacy and further enhance pharmacokinetic properties without diminishing activity such as bottromycin, althiomycin, caryoynencin, nocardithiocin, lugdunin, nematophin, holomycin, and reutericyclin. The NPs covered in this review could be clinical leads or could provide structural templates for further medicinal chemistry optimisation efforts.

The ecological functions of currently known NPs in pathogenic bacteria remain to be deciphered. Understanding this role might be key to determining their potential use. For example, clostrubins which serve dual functions – kills potential microbial competitors and permits survival of the pathogenic anaerobe in an oxygen-rich potato niche – represent promising leads for the design and development of antibacterial therapeutics and plant protection agents.[47] Impairing clostrubin production in *C. puniceum* could also help prevent potatoes from "soft rot".

Given that some pathogenic bacteria are a threat to humans, how can one prioritize natural products discovery from these huge untapped resources? Developments in culture-independent meta-omics approaches have provided greater access to underinvestigated taxa that contain unique metabolic profiles that probably encode novel chemistry. The exploitation of these metagenomic data has proven to be beneficial in the characterisation and isolation of cryptic metabolites from the complex human microbiota.[355–357] For example, the thiopeptide antibiotic lactocillin was discovered *via* a sequence-based metagenomic analysis.[358] Such techniques can be exploited to identify potential genetic markers of disease in NP producing bacterial pathogens, thereby circumventing the threat of opportunistic infections caused by pathogenic bacteria. Metagenomic approaches also provide a means to access novel bioactive molecules with diverse structures. Integrating other emerging techniques in these NP discovery efforts, such as elicitation of cryptic biosynthetic pathways and refactoring of silent BGCs should help illuminate the chemical "dark matter" in bacterial pathogens. The substitution of native promoters with strong constitutive promoters in cryptic gene clusters has been one such productive strategy in activating biosynthesis and improving antibiotic expression. For example, promoter exchange in *Photorhabdus* spp. and *Xenorhabdus* spp. led to the expression of several cryptic nonribosomal peptides. Additionally, promoter exchange in Δ*hfq* mutants resulted in the production of desired metabolites for further bioactivity testing.[151] The development of high-throughput next-generation sequencing methods, together with the development of new bioinformatics tools that can assemble nearly complete genomes, will continue to revolutionize microbial "dark matter" exploration. Furthermore, improvements in analytical platforms (mass spectrometry, NMR) coupled with recent advancements in metabolomics enable the detection and identification of compounds in minute quantities from complex biological samples.[359–361] Application of recent machine learning tools for structure recognition, bioactivity prediction, drug–target interactions[362] such as the NMR-based Small Molecule Accurate Recognition Technology (SMART 2.0)[363] further accelerates the drug discovery process.

Taken together, access to the immense repertoire of novel cryptic metabolites encoded in pathogenic bacteria is only achievable through improvements in, and integration of, various approaches and available methods from multiple disciplines. Further exploitation of the untapped chemical diversity of pathogenic bacteria will undoubtedly yield many more novel bioactive molecules and might reboot the antibiotic pipeline. Soon, we can predict a second "Golden Era of Antibiotics" discovery.

6. Conflicts of interest

The authors declare no conflicts of interest.

7. Acknowledgements

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