Investigation on the diversity and antimicrobial potential of marine predatory bacteria enabled elucidation of the thio-peptide kocurin and aminophenylpyrrole-derived alkaloids biosynthesis

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Luis Jesus Linares-Otoya

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Dedicado a mi abuela Marina, mis padres y hermanos

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Contents

Abbreviations

°C	Degrees Celsius
2D	Two-dimensional
3-HPQ	3H-pyrrolo [2,3-c]quinoline
δ	NMR chemical shift in ppm
µL	microliter
antiSMASH	antibiotic and Secondary Metabolites Analysis Shell
APPA	Aminophenylpyrrole Derived Alkaloids
ATP	Adenosine triphosphate
BALOs	Bdellovibrio and like organisms
BLAST	Basic Local Alignment Search Tool
BGC	Biosynthetic gene cluster
br	broad(NMR data)
calcd	calculated
COSY	Correlated spectroscopy
d	doublet (NMR data)
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
e.g.	"Exempli gratia"
EF-Tu	Elongation Factor Thermo Unstable
EIC	Extracted ion chromatogram
ESI	Electrospray ionization
et al.	"Et alii"
EtOAc	Ethyl acetate

FAD	Flavin Adenine Nucleotide
FMN	Flavin Mononucleotide
GNPS	Global Natural Product Social Molecular Networking
h	hour
HCCA	α-Cyano-4-hydroxycinnamic acid
HMBC	Heteronuclear Multiple-Bond Correlation
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
Hz	Hertz
IC_{50}	Inhibitory concentration (required for 50 % inhibition level)
i.e.	"Id est"
IDO	Indolamine 2,3 dioxygenase
IPTG	Isopropyl-β-D-thiogalactopyranosid
J	Spin-spin coupling constant in Hz
Kbp	kilobase pairs
L	Liter
LB	Luria Bertani medium
m	multiplet (NMR data)
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization – Time Of Flight
MB	Marine Broth
MeOH	Methanol
mg	milligrams
MHz	Megahertz
MIA	Methyl Indolic Acid
MIC	Minimal Inhibitory Concentration
min	minutes

mL	millilitres
mM	millimolar
MM	Minimal Medium
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
m/z	Mass-to-charge ratio
n	number
NMR	Nuclear Magnetic Resonance
NOS	Nosiheptide
NRPS	Non-Ribosomal Peptide Synthase
OD_{600}	Optical density at wavelength of 600nm
ORF	Open Reading Frame
PAS	Per-Anrt-Sim domain
PCR	Polymerase Chain Reaction
PKS	Polyketide synthase
ppm	Parts per million
q	quadruplet (NMR data)
RiPPs	Ribosomally Synthetized and Post-translationally modified Peptides
rpm	revolutions per minute
s	Singlet (NMR data)
SAM	S-Adenosyl Methionine
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
sp.	species
t	Triplet (NMR data)
UPLC	Ultra-High Performance Liquid Chromatography
UV	Ultraviolet

Introduction

Prokaryotes like bacteria have evolved various strategies and impressive metabolic capabilities to colonize harsh habitats, such as marine in which they have to cope with variations in temperature, salinity, and in some case low nutrient concentration (Eom *et al*. 2013). Many bacteria in the marine environment are living in a symbiotic consortium with higher organisms, e.g., sponges or other invertebrates like tunicates and sea slugs; seem to be highly dependent on their microbiome. For instance, several organisms such as sponges acquired and accumulated highly bioactive molecules from their bacterial microbiome that could serve as chemical defense molecules to protect themselves from predators. In the last decades, it was recognized that many compounds described before from sponges are indeed produced by associated bacteria. For example, it was reported that polytheonamides isolated from sponges of the genus *Theonella* have actually a biosynthetic origin in a bacterial symbiont (Hamada *et al*. 2005). This proteusin-type of compound is produced by *Candidatus Entotheonella*, a phylum of uncultured bacteria detected by metagenomics analysis that lives associated with sponges of the genus *Theonella* (Freeman *et al.* 2012).

As certain bacteria like the *Candidatus entotheonella* live in association with larger organisms, others have evolved a totally independent lifestyle (Korp *et al*. 2016). This group of autonomous bacteria in most cases possess larger genomes, which provide them with a larger metabolic repertoire, enabling them: (i) to get access to scarce nutrients, and/or (ii) to produce bioactive specialized metabolites to protect themselves from predators or even to play this role (Korp *et al*. 2016).

In the present study, we focused on the investigation of the antimicrobial potential of marine predatory bacteria. The assumption that these organisms use antimicrobial compounds to weaken or even kill their prey predicts a great potential of these bacteria as bioresource for novel antibiotics (Korp *et al*. 2016).

1.1 Predatory strategies of bacteria

Predatory behavior is widely distributed among bacteria and can be found in several phyla, e.g., in Actinobacteria (Byrd *et al*. 1985), Bacteroidetes (Lewin 1997), Chloroflexi (Mohseni *et al*. 2016), and Proteobacteria (Sockett 2009).

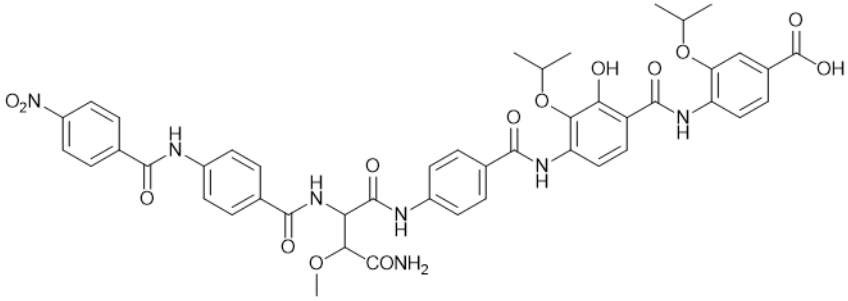
However, the predatory behavior can vary significantly among these mentioned taxa. Epibiotic predation involves attachment to the outer surface of the prey, which is then followed by a degradation of the prey's cell wall and assimilation of cell components through specialized structures. Such an epibiotic predation was reported for *Cytophaga* species (IMAI *et al*. 1993), as well as for the flavobacterial genus *Tenacibaculum (e.g., T. discolor LL*04 11.1.1 *and T. gallaicum* A37.1T*)* , whereby for the latter even an intra-genus prey specificity was reported (Banning *et al*. 2010). Other predatory bacteria are known to use diacytosis (penetration of the prey cell) (Taylor *et al*. 1974), or to invade the periplasm of Gram-negative bacteria (Kandel *et al*. 2015). The corresponding behaviors are referred to as endobiotic and periplasmic predation, respectively. An example of a bacterium using the periplasmatic type of predation are the Proteobacteria *Bdellovibrio* spp. These are obligatory predatory bacteria, which grow epibiotically attached to the cell envelope of their prey. They are dividing in a binary manner by penetrating the prey's periplasm to generate a number of progeny cells (Jurkevitch and Jacquet 2017; Sockett 2009).

Finally, some bacteria apply a so-called 'wolf pack strategy' to prey on cells. This strategy involves a quorum of predatory cells showing gliding behavior, which pool hydrolytic enzymes, proteases or nucleases in order to lyse and feed on nearby prey (Martin 2002). A well-known example are myxobacteria (order of Myxococcales) , e.g., the marine *Enhygromyxa salina* (Schaeberle *et al*. 2010) and *Cystobacter* sp. (Baumann *et al*. 2014), proliferative producers of antibiotically active metabolites (Schaeberle *et al*. 2014). Further examples can be found within the Gammaproteobacteria, e.g., *Lysobacter* spp. (Seccareccia *et al*. 2015), the Bacteroidetes, e.g., *Rapidithrix thailandica (Srisukchayakul et al. 2007)*, *Porifericola rhodea* (Yoon *et al*. 2011), and the Chloroflexi, e.g., *Herpetosiphon* spp. (Mohseni *et al*. 2016).

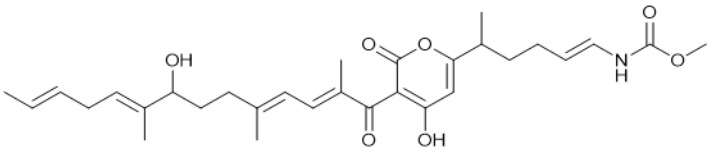
1.2. Antibiotic potential of predatory bacteria

It was speculated that the biosynthesis of antibiotic compounds is linked to the predatory lifestyle of these organisms (Berleman and Kirby 2009). As mentioned before, a characteristic feature of these taxa is their large genome size with a high number of biosynthetic gene clusters (BGCs) involved presumably in the synthesis of specialized metabolites. Among these compounds, antibiotics would play an important role in their predatory lifestyle (Korp *et al*. 2016).

Myxobacteria have shown their ability to biosynthesize several structural classes of specialized metabolites with different biological effects, e.g., antibiotics. Prominent examples of myxobacterial compounds that are currently in preclinical evaluation for the development as antibiotics are the non-ribosomal peptides (NRP) cystobactamids produced by *Cystobacter* sp. (Baumann *et al.* 2014) and the hybrid NRP-polyketide corallopyronin A (Jansen *et al.* 1985) from *Corallococcus coralloides*, both terrestrial myxobacteria (Figure 1).



Cystobactamide 919-2



Corallopyronin A

Figure 1. Chemical structure of cystobactamid 919-2 and corallopyronin A

In 2001 the first antibiotic metabolite of truly marine myxobacteria was isolated from *Haliangium ochraceum.* The active principle was identified as the polyketide haliangicin (Figure 2) (Fudou *et al.* 2001). More than ten years later the first antibiotics from the genus *Enhygromyxa salina* , an obligatory marine myxobacteria were isolated (Felder *et al.* 2013).The terpenoid salimyxin B and the peptide derived enhygrolide A showed inhibitory activity towards *A. crystallopoietes* (MIC value of 8 and 4 μg mL^{-1}, respectively).

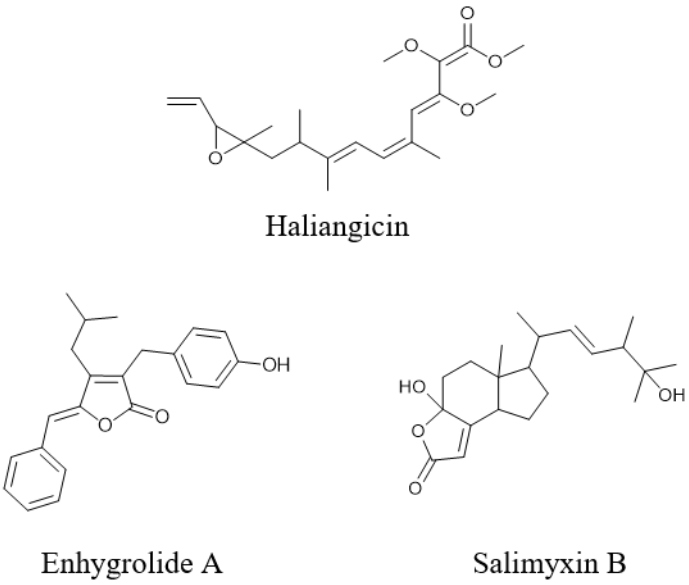


Figure 2. Chemical structure of haliangicin and salimyxin A

Besides myxobacteria, which are in the meanwhile regarded as one of the most promising source for novel specialized metabolites, also members of the *Cytophagales* clade have been proven to produce several antibiotics, and can be regarded as emerging natural product producers beside the classical Actinobacteria, Myxobacteria or Bacilliales.

One example are the ariakemicins (Figure 3), unusual linear hybrid polyketide-nonribosomal peptide antibiotics, isolated from *Rapidithrix* sp., (Ariake Inland Sea, Japan). The mixture of ariakemicins A and B inhibited Gram-positive bacteria (*S. aureus* MIC 0.46 μg/mL) (Oku *et al*. 2008). An intriguing fact is that other predatory bacteria, e.g., *Herpetosiphon* sp. B060 and *Sorangium* sp. (formerly reported as *Polyangium* sp.), produce specialized metabolites, which show structural similarities to the polyketide peptide hybrids

ariakemicins. Siphonazole, produced by *Herpetosiphon* sp. B060 (Nett *et al*. 2006), and phenoxan, produced by *Sorangium* sp. (Kunze *et al*. 1992), have the benzoic acid derivative and the oxazole rings interlinked by unsaturated polyketide-peptide chain(s) in common.

It was speculated that the production of related metabolites by phylogenetically distant taxa may imply that these natural products have the same molecular ancestor, diversified during evolution (Oku *et al*. 2008). However, also more recent horizontal transfer events should be taken into account, since it was suggested that this could be the case for the peptide antibiotic althiomycin that was initially reported in *Streptomyces* and later in myxobacterial strains (Korp *et al*. 2016).

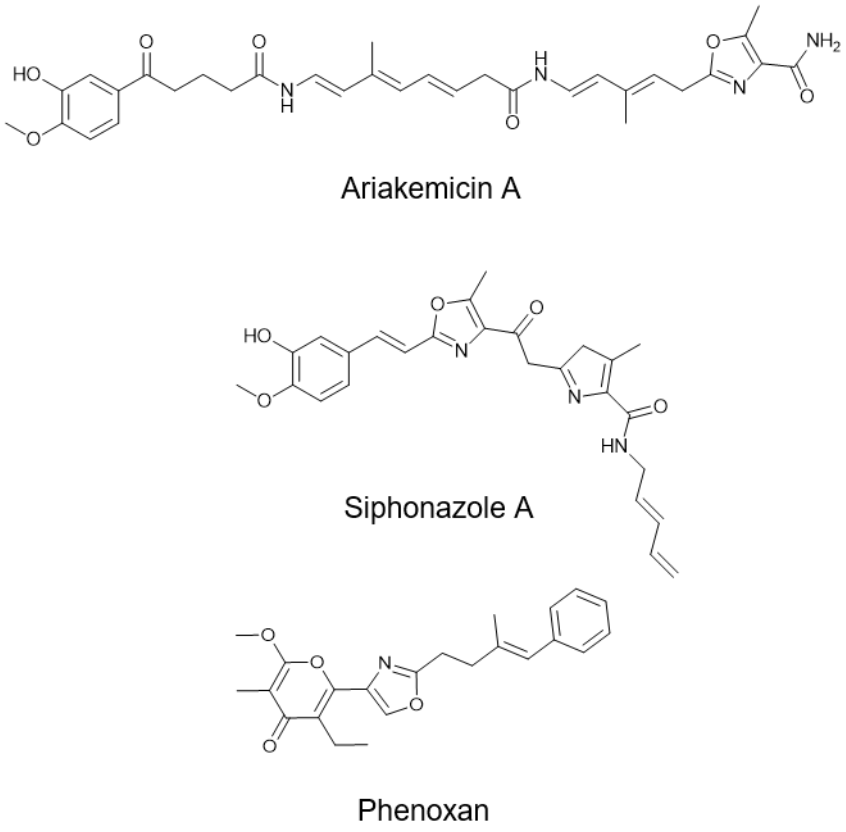


Figure 3. Chemical structure of ariakemicin A, siphonazole A and phenoxan

Another antibiotic produced by *Cytophaga* strains is the antibiotic G-1499-2, showing antibiotic activity against a narrow range of organisms (*Flavobacterium sp.*1980 and of *Staphylococcus aureus* Oxford H strain VI). It was isolated from the culture broth of *Cytophaga johnsonii* ATCC 21123 (Evans *et al*. 1978). The monobactams PB-5266 (A-C) were isolated and purified from the culture broth *of Cytophaga johnsonae* PB-5266 and showed activity against gram positive bacteria (Kato *et al*. 1987). A 22-membered macrolide antibiotic, YM-32890 A (1) was isolated from the culture broth of *Cytophaga* sp. YL-02905S. It showed a potent antimicrobial activity against *Staphylococci* including a macrolide-resistant strain (Kamigiri *et al*. 1997) (Figure 4).

Katanosins (A-B) are potent anti-gram-positive bacteria antibiotics (also known as lysobactins). The were isolated from the fermentation broth of different microorganisms, such as *Cytophaga* (Shoji *et al*. 1988) and proteobacteria, i.e. *Lysobacter* sp. (Bonner *et al*. 1988). Katanosins are cyclic depsipeptides (acylcyclodepsipeptides). Despite the phylogenetic distance between these taxa both are reported as predatory bacteria (Figure 4).

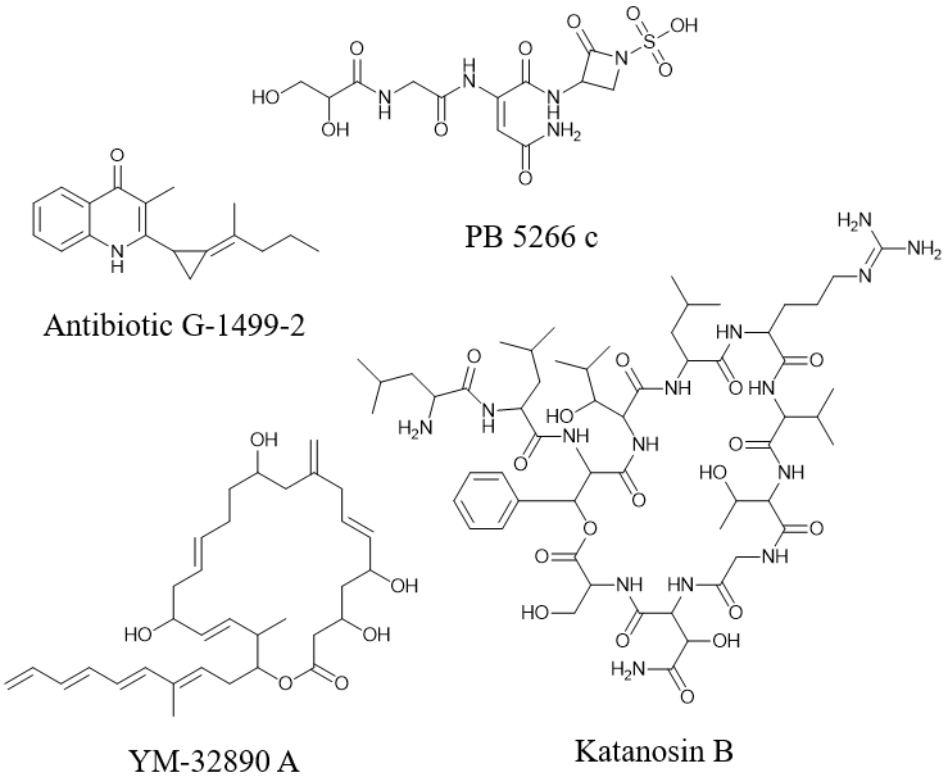


Figure 4. Chemical structure of the antibiotics G-1499-2, PB-5266, YM-32890 A and katanosin B.

1.3. Biosynthesis of antibiotics from predatory bacteria: Thiopeptides and aminophenylpyrrole-derived alkaloids (APPA)

In this project several bacterial species, which possess the potential to biosynthesize antibiotically active natural products were identified. In this sense several known antibiotic could be dereplicated, e.g., ariakemicin, kocurin, naphthyridinomycin, pumilacidins, resistomycin, surfactin and APPAs.

Despite the fact that many of these compounds represent promising lead structures for the development of novel antibiotic, such as the thiopeptide kocurin and the diverse family of APPAs, their biosynthetic basis was still unknown (Schinke *et al*. 2017). Understanding the biosynthetic routes of these compounds by identification of the biosynthetic gene cluster, as well as the elucidation of the biosynthetic pathway, would contribute to the future development of diverse analogs.

In this study, we focused on understanding the biosynthesis of the highly interesting bioactive compounds kocurin and APPAs. Therefore, in the following section it is described what is known about the biosynthesis of thiopeptides and APPAs in general. This formed the basis of the detailed investigations into the biosynthesis of kocurin and APPAs.

1.3.1. Biosynthesis of ribosomally synthesized and post-translationally modified peptides (RiPPs)

RiPPs are peptides that are ribosomally-produced and post-translational modified due to the action of several diverse enzymes (van der Donk 2013). RiPPs are a highly diverse family of natural products, consisting of more than 20 sub-classes, produced by prokaryotes and eukaryotes. These compounds exert a wide range of biological functions, for instance antibiotic, antifungal, morphogenetic, cytotoxic (Arnison *et al*. 2013).

The biosynthetic gene clusters of RiPPs are small in size (usually between 5-20 kb), since the core peptide is encoded in a single ORF called precursor peptide (Figure 5). This fact together with the better predictability of the chemical products make RiPPs an attractive target for genome mining oriented natural product discovery (Bachmann *et al*. 2014). Almost all the compounds produced by RiPPs are initially synthesized as a longer precursor peptide, typically ~20–110 residues in length, which is encoded by a structural gene (Arnison *et al*. 2013). As exemplified in Figure 5, in most RiPPs, a so-called "leader peptide" (also called "leader sequence") is appended to the *N*-terminus of the core peptide. This leader is usually important for recognition by many of the post-translational modification enzymes and for export (Oman and van der Donk 2010). In some more rare examples such as the bottromycins, a leader peptide is not attached at the *N*-terminus, but rather at the *C*-terminus of the core peptide and has been termed a "follower" peptide (Huo *et al*. 2012).

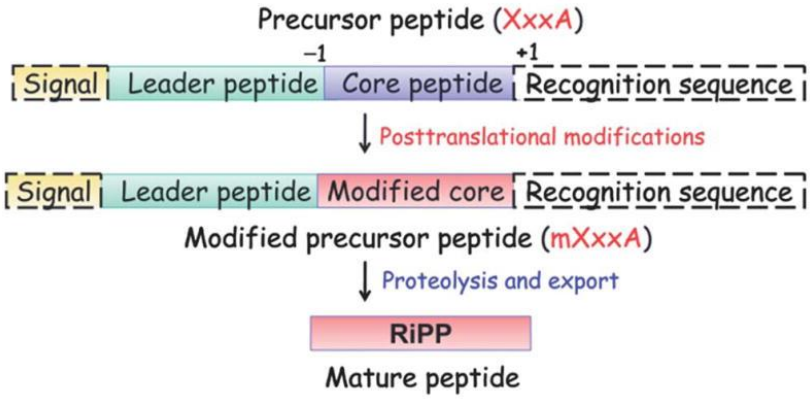


Figure 5. General scheme of RiPPs biosynthesis highlighting the precursor peptide domain organization (Arnison *et al*. 2013).

RiPPs can be divided into subclasses based on the posttranslational modification the propeptides undergo, e.g., lanthipeptides, thiopeptides, lassopeptides, microviridins, proteusins. In the following section the thiopeptides biosynthesis will be described (Arnison *et al*. 2013).

1.3.1.1 Biosynthesis of thiopeptides

Nearly all thiopeptides exhibit potent antibiotic activities, targeting either the elongation factor thermo unstable (EF-Tu) or the 50S ribosomal subunit (Harms *et al*. 2008; Parmeggiani and Nissen 2006). Given their unique mechanisms of action and low toxicities, thiopeptides have the potential to become novel antibiotic leads (Schinke *et al*. 2017). However, unfavorable pharmacokinetics limit their further applications, and therefore, molecular engineering to overcome these limitations appears as attractive alternative (Walsh *et al*. 2010). Thiopeptide antibiotics exhibit one of the most complex structures among all RiPPs. The typical thiopeptide core structure includes five-membered sulfur (or oxygen) heterocycles, dehydroserines (or dehydrothreonines) and six-membered azacycles (Figure 6).

The structural framework of thiopeptides is constructed in a specific chronological order (Figure 7). First, the methyloxazol or thiazole rings are formed from threonine and cysteine residues, respectively. Second, Ser/Thr residues are dehydratated and finally the six-membered azacycle is generated. The disruption of one of these steps abolishes the production of thiopeptides. The further maturation of thiopeptide compounds requires in many cases additional tailoring reactions (e.g., halogenation, methylation, amidation of the *C*- terminus, glycosylation, formylation) and finally from the cytosol to the outside of the cell (Zheng *et al*. 2017) .

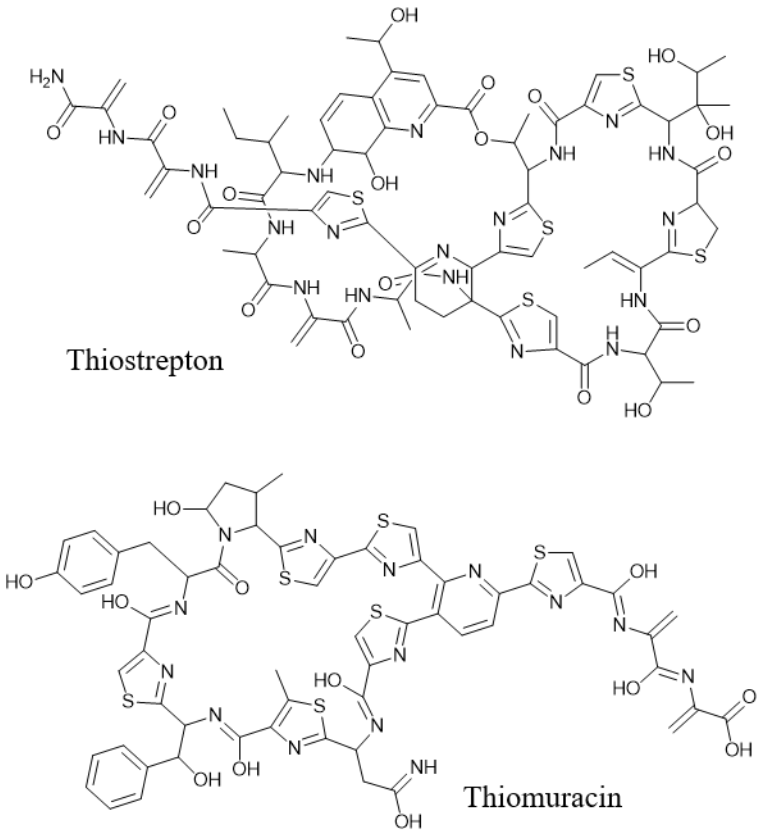


Figure 6. Chemical structure of the thiopeptides thiostrepton and thiomuracin.

1.3.1.1.1 Thiazole-oxazole formation

As mentioned before, thiazoles or oxazoles are formed from cysteines and serine/threonine respectively. This reaction is catalyzed by two enzymes. First an ATP-dependent cyclodehydratase (containing a YcaO-like domain) form the thiazoline/methyloxazoline rings. These intermediate products are later dehydrogenated by the action a FMN-dependent enzyme form the thiazol/oxazole rings. In some cases, an auxiliary protein is serving as docking protein, being the linker between the leader peptide and the enzyme complex (Dunbar *et al*. 2015). For instance, in the thiomuracin biosynthesis, the docking protein TbtF, which is encoded by an alone-standing gene, is indispensable in the formation of thiazoles. Only if TbtF is present, it can recruit the cyclodehydratase TbtG (Hudson *et al*. 2015). Interestingly, the formation of sulfur and oxygen heterocycles in thiopeptides

requires two sets of cyclodehydratase and dehydrogenase. This is most likely due to the different nucleophilicities of the sulfhydryl and hydroxyl groups (Malcolmson *et al*. 2013; Tocchetti *et al*. 2013).

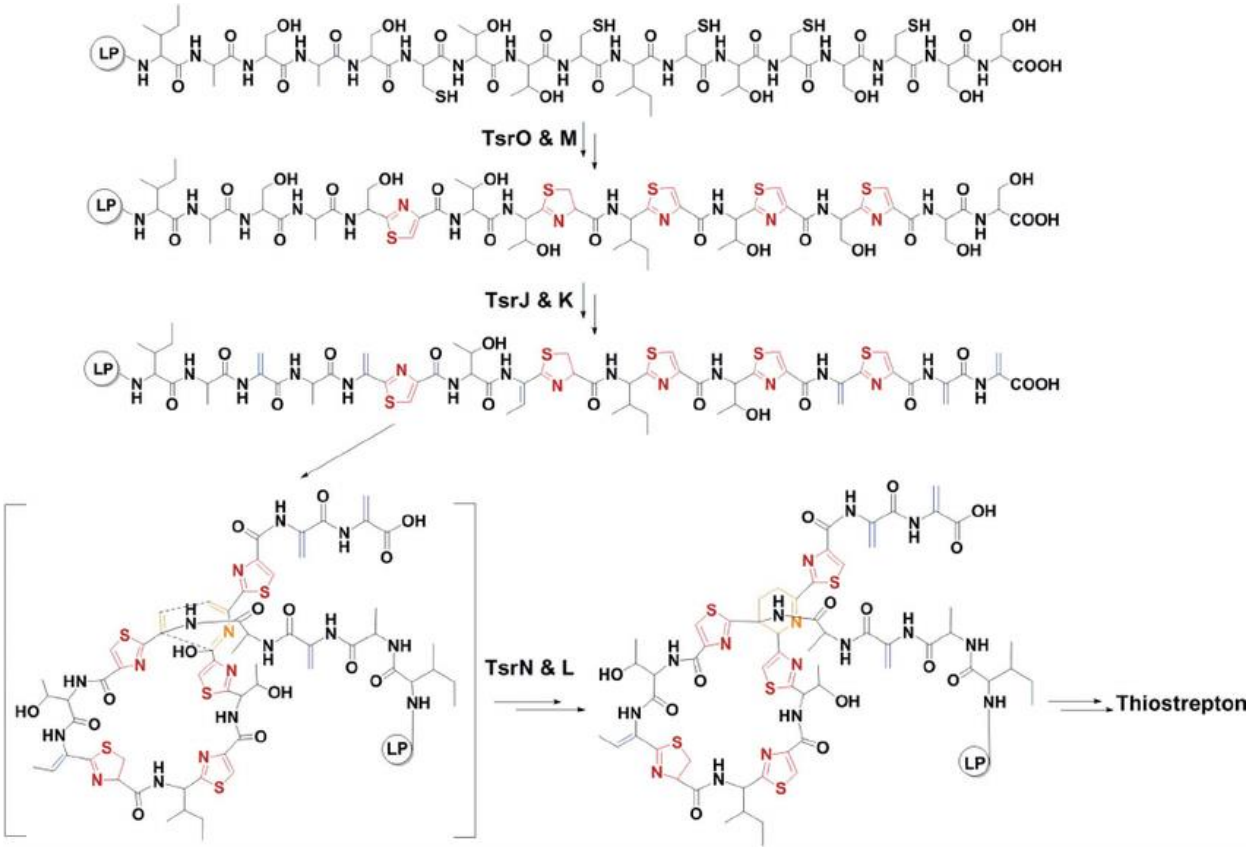


Figure 7. Biosynthetic pathway of thiostrepton (Zheng *et al*. 2017).The thiostrepton biosynthesis starts with the formation of thiazoles and thioazoline rings from Cys catalized by TsrO and TsrM. Later the serine and threonine are dehydrated to dehydroalanine and dehydrobutyrine by TsrJ and TsrK. The next step is the macrocyclization via six-membered ring formation catalized by TsrN and TsrL. Additional tailoring modification including methylation, side-ring formation and cleavage of the leader peptide finally yield thiostrepton.

1.3.1.1.2. Dehydratation

In the dehydratation mechanism of serine and threonine, first, a glutamylation takes place, followed by the loss of one water molecule. The donor of glutamyl is glutamyl-tRNAGlu (Hudson *et al.* 2015). This mechanism is identical to the type I dehydration in lanthipeptides (Repka *et al*. 2017). However, in thiopeptides the LanB dehydratase that is encoded by one open reading frame in lanthipeptide BGCs, is split in two open reading frames (ORFs) (Zheng *et al*. 2017). Each ORF contains either a glutamylation domain, or an elimination domain. In thiomuracin biosynthesis, dehydratation is catalyzed by TbtB and TbtC. Thereby, TbtB catalyzes the transfer of the glutamyl to the hydroxyl of serine from tRNAGlu, and TbtC subsequently catalyzes the elimination of the glutamate from the glutamylated aminoacid, yielding a dehydroamino acid (Hudson *et al*. 2015).

1.3.1.1.3. Pyridine ring formation

In thiopeptides, the generation of a six-membered nitrogen heterocycle is reached by a unique aza-Diels–Alder reaction, which results at the same time in the formation of the macro-ring system. Thereby, the conversion that occurs after the [4 + 2] cycloaddition is different for aromatic and non-aromatic azacycles (Zheng *et al*. 2017). Examples of aromatic azacycle-containing thiopeptides are thiocillin and thiomuracin. In these compounds, a single multifunctional enzyme catalyzes a series of reactions, including isomerization, cycloaddition, dehydration and aromatization that together form the pyridine moiety (Wever *et al*. 2015). Moreover, in some cases, e.g. nocathiacin, pyridine can be transformed into hydroxypyridine via further oxidation reactions (Ding *et al*. 2010). On the other hand, in the biosynthesis of non-aromatic azacycle containing thiopeptides, like thiostrepton, the six-membered ring is constructed in a different manner without aromatization (Zheng *et al*. 2016). After isomerization, cycloaddition and dehydration, the subsequent 1,4-reduction generates a dehydropiperidine. This dehydropiperidine moiety can be further modified by reduction or rearrangement into piperidine (Miyairi *et al*. 1970) or imidazopiperidine (Puar *et al*. 1998).

1.3.1.1.4. Tailoring reactions

Thiopeptides usually lose the free N-terminus and have diverse strategies to protect their C-terminus, including amidation, esterification, cyclization, etc. (Zheng *et al*. 2017). For instance the Cys residue of lactocillin is acylated by an indole-3-carboxylic acid (Donia *et al*. 2014). Formation of side-ring systems is another common feature from thiopeptides, as exemplified in thiostrepton (Weisblum and Demohn 1970). In bimacrocyclic members of the thiopeptide family, such as thiostrepton and nosiheptide (NOS), the mature molecules possess a side-ring system that contains a tryptophan-derived moiety (i.e., quinaldic acid or methyl indole acid (MIA)) (Wang *et al*. 2013). The MIA in the NOS biosynthesis is generated by a radical S-adenosylmethionine (SAM)-dependent rearrangement, mediated by a single enzyme (NosL) (Zhang *et al*. 2011). Further tailoring reactions include halogenations, methylation, formylation, glycosylation and others (Zheng *et al*. 2017).

1.3.2. Bacterial aminophenylpyrrole derived-alkaloids

Pyrrolnitrin was discovered in an antibiotics screening in 1964 from a *Pseudomonas pyrrocinia* strain (Arima *et al*. 1964). The active molecule identified was a phenylpyrrole derivative containing a nitro group and two chlorine atoms and was named pyrrolnitrin. This compound showed mostly antifungal activity but also antimicrobial activity against gram-positive bacteria (Arima *et al*. 1965). In the next decades, this compound was also isolated from a number of different proteobacterial species, such as *Burkholderia (El-Banna and Winkelmann 1998)*, *Myxococcus fulvus, Corallococcus exiguus, Cystobacter ferrugineus* and *Enterobacter agglomerans (Gerth et al. 1982)*. Pyrrolnitrin was later synthetically modified, leading to the more photostable derivatives fenpiclonil and fludioxonil, which are widely used in agriculture as foliar fungicides (Kilani and Fillinger 2016).

In the last years, more structural variants of APPAs with diverse bioactivities were discovered from bacteria belonging to the order of *Cytophagales* (phylum Bacteroidetes). For instance, 3-(2-aminophenyl)pyrrole and the antibacterial 3-(2-aminophenyl)-5-methoxy-1,5-dihydro2H-pyrrol-2-one were isolated from *Rapidithrix thailandica* TISTR 1749 and TISTR

1741, respectively (Sangnoi *et al*. 2014; Sangnoi *et al*. 2008). Furthermore, marinoquinolines (A− K) and marinoazepinones (A−B) (Figure 8), structurally related compounds, were isolated from the marine bacteria *Ohtaekwangia koreensis* PWU25 and *Mooreia alkaloidigena* CNX-2166 (Okanya *et al*. 2011; Choi *et al*. 2015). For the Cytophagales-derived APPAs, no oxidation of the amino group, nor halogenation, as is the case for pyrrolnitrin, was observed. Instead, the aminophenylpyrrole seems to be fused to several acyl, indole, or aromatic side chains, yielding the 3H-pyrrolo [2,3-c] quinoline (3-HPQ) ring system of the highly diverse marinoquinolines . In addition, N-methylation of the quinolone ring further expands the diversity of these alkaloids (Choi *et al*. 2015).

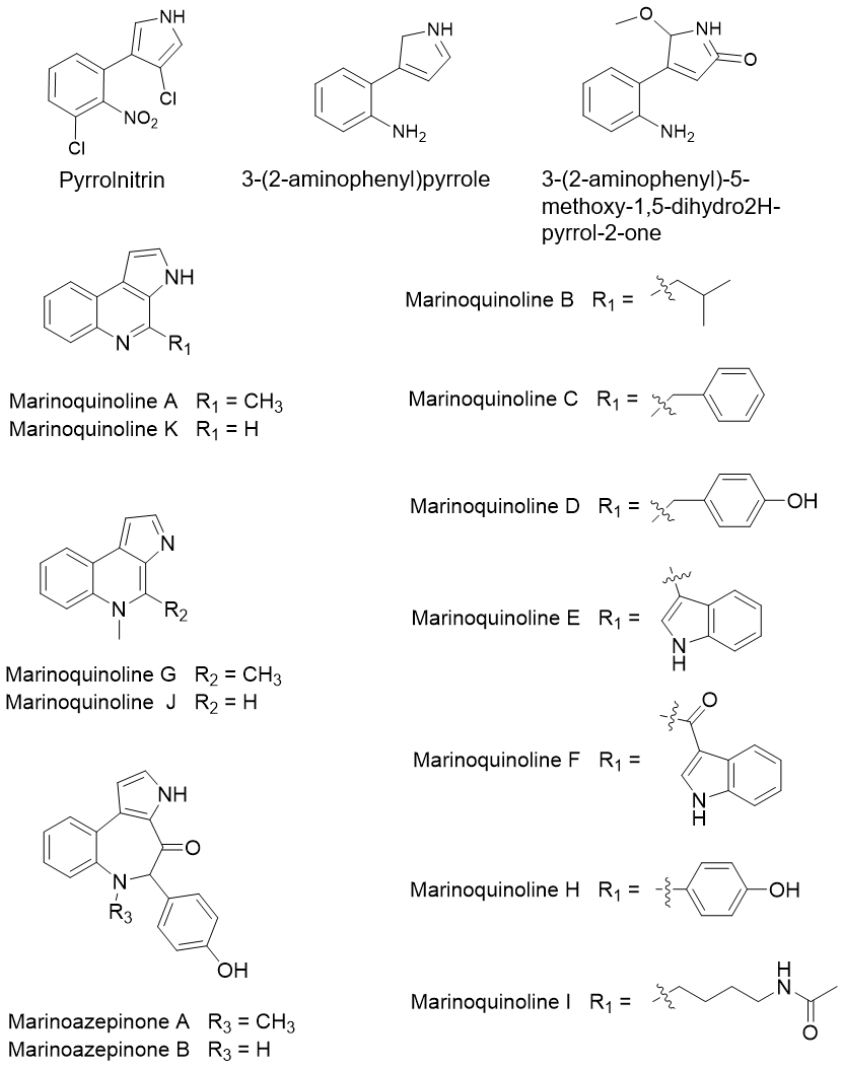


Figure 8. Chemical structure of pyrrolnitrin and of *Cytophagales* produced APPAs.

1.3.2.1. Biosynthetic studies on pyrrolnitrin

In contrast to the recently discovered APPAs from *Cytophagales*, the biosynthesis of pyrrolnitrin was in detail investigated (Kirner *et al*. 1998). However, initial biosynthetic studies were done using feeding experiment. First of all, the authors found that these compounds were tryptophan-derived (Elander *et al*. 1968). Later it was observed that a different halogenation mechanism should be involved in the biosynthesis than the in that time known one catalyzed by haloperoxidases (Wiesner *et al*. 1986). Almost two decades later it was reported that this halogenation reaction is catalyzed by FAD-dependent halogenases (Dong *et al*. 2005).

1.3.2.2. Biosynthetic gene cluster and pathway

After several attempts Hammer *et al.* managed in 1997 to isolate a 32-kb genomic DNA-fragment from *P. fluorescens* BL915 that contained several genes involved in the production of pyrrolnitrin (Hammer *et al*. 1997). Detailed investigations using Tn5 mutagenesis revealed that four genes were required for the production of pyrrolnitrin in *Escherichia coli*. The functions of the individual gene products were elucidated using deletion mutants and feeding experiments (Kirner *et al*. 1998). The initial step is the halogenation of tryptophan to 6-chlorotryptophan, followed by the ring opening and monodechloroaminopyrrolinitrin formation. The latter is further halogenated and finally the amino group is dioxidized to forme a nitro group (Figure 9).

PrnA and PrnC are FAD-dependent enzymes, which were found to be involved in the chlorination of the tryptophan at the 5 position and in the chlorination of the monodechloroaminopyrrolnitrin intermediate (Kirner *et al*. 1998).

A mutant which did not produce PrnB was found to accumulate the L-isomer of chlorinated tryptophan; no D-isomer was detected in this mutant. Therefore, it can be concluded that PrnB catalyzes the ring rearrangement and decarboxylation of tryptophan to monodechloroaminopyrrolnitrin (Laurentis *et al*. 2007).

PrnD was found to have strong homologies to class IA dioxygenases, including a consensus sequence of the [2Fe-2S] Rieske-type binding domain and a region that is highly similar to the proposed mononuclear nonheme Fe(II) binding domain, which is also common to terminal dioxygenases. PrnD is involved in the oxidation of the amino group of aminopyrrolnitrin to the nitro group of pyrrolnitrin (Lee *et al*. 2005).

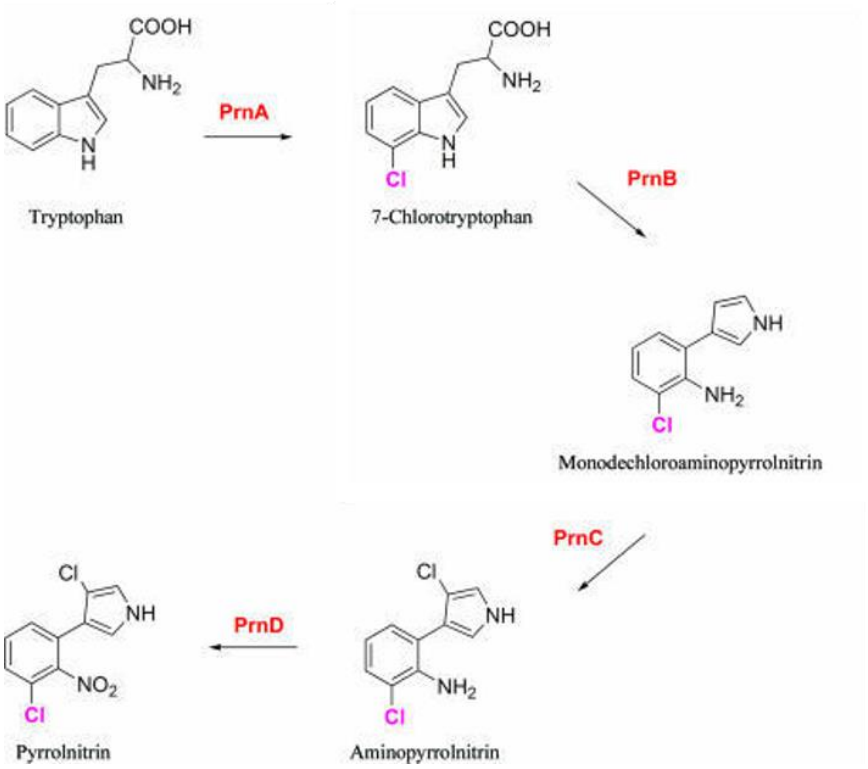


Figure 9. Biosynthesis of pyrrolnitrin by *Pseudomonas* strains (Laurentis *et al*. 2007).

Scope and outline of the dissertation

In the present study, the microbiome from the intertidal zone of Peruvian coastline was investigated in the context to its potential to produce bioactive antibiotic compounds. The focus of this research was on specialized metabolites from predatory bacteria. Given their lifestyle, predatory bacteria are regarded as promising bioresource for antibiotics, since they might use such compounds in order to get access to the prey.

First of all, culture-dependent and -independent studies were performed to get first insights into the bacterial biodiversity and the antimicrobial potential of the bacterial isolates. The results of this preliminary investigation are detailed in chapter 1.

After identifying the basis of the antimicrobial activity for some of the most interesting isolates, we focused on the elucidation of the biosynthesis of the corresponding compounds. In this regard, in chapter 2 the biosynthesis of the potent antibiotic kocurin, biosynthesized by *Kocuria rosea* s17 is described; and in chapter 3 the biosynthesis of the highly diverse aminophenylpyrrole derived alkaloids, which show various bioactivities, was elucidated from the strains *Rapidithrix thailandica* s80 and *Porifericola rhodea* s47.

Chapter I

Diversity and Antimicrobial Potential of Predatory Bacteria from the Peruvian Coastline

Luis Linares-Otoya , Virginia Linares-Otoya , Lizbeth Armas-Mantilla , Cyntia Blanco-Olano , Max Crüsemann , Mayar L. Ganoza-Yupanqui , Julio Campos-Florian , Gabriele M. König and Till F. Schäberle

Mar. Drugs 2017, 15, 308

In this chapter the diversity and antimicrobial potential of the marine microbiome of the intertidal area along the Peruvian coastline was investigated. The Peruvian coastline is regarded as one of the richest in biodiversity of marine higher organisms given that it represents the confluence point of the cold Humboldt coastal current and the warm Caribbean current. It is of interest if this biodiversity is also translated to the bacterial level. Culture-independent and dependent approaches were used to get first insides into the bacterial diversity with a focus on predatory bacteria. The focus on predatory bacteria was chosen based on the assumption that due to their hunting habits, these microorganisms make use of antimicrobial metabolites. The predatory bacteria, which could be isolated and cultured *in vitro* were screened for antimicrobial compounds.

Own contributions: planning of the experiment, sample collection, isolation of bacterial strains, dereplication of antimicrobial specialized metabolites (MS data analysis and GNPS molecular networking), and manuscript writing.

marine drugs

MDPI

Article

Diversity and Antimicrobial Potential of Predatory Bacteria from the Peruvian Coastline

Luis Linares-Otoya [1,2,5], Virginia Linares-Otoya [3,5], Lizbeth Armas-Mantilla [3], Cyntia Blanco-Olano [3], Max Crüsemann [2], Mayar L. Ganoza-Yupanqui [3], Julio Campos-Florian [3], Gabriele M. König [2,4] and Till F. Schäberle [1,2,4,*]

1 Institute for Insect Biotechnology, Justus Liebig University of Giessen, 5392 Giessen, Germany; luis.lioto@hotmail.com
2 Institute for Pharmaceutical Biology, University of Bonn, 3115 Bonn, Germany; mcruesem@uni-bonn.de (M.C.); g.koenig@uni-bonn.de (G.M.K.)
3 Department of Pharmacology, Faculty of Pharmacy and Biochemistry, National University of Trujillo, 13011 Trujillo, Peru; mvlinareso@hotmail.com (V.L.-O.); lizfa_2712@hotmail.com (L.A.-M.); cyntia_0212@outlook.com.pe (C.B.-O.); mganoza@unitru.edu.pe (M.L.G.-Y.); jcamposf@unitru.edu.pe (J.C.-F.)
4 German Centre for Infection Research (DZIF) Partner Site Bonn/Cologne, Bonn 53115, Germany
5 Research Centre for Sustainable Development Uku Pacha, 13011 Uku Pacha, Peru
* Correspondence: till.f.schaeberle@agrar.uni-giessen.de; Tel.: +49-641-99-37140

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Abstract: The microbiome of three different sites at the Peruvian Pacific coast was analyzed, revealing a lower bacterial biodiversity at Isla Foca than at Paracas and Manglares, with 89 bacterial genera identified, as compared to 195 and 173 genera, respectively. Only 47 of the bacterial genera identified were common to all three sites. In order to obtain promising strains for the putative production of novel antimicrobials, predatory bacteria were isolated from these sampling sites, using two different bait organisms. Even though the proportion of predatory bacteria was only around 0.5% in the here investigated environmental microbiomes, by this approach in total 138 bacterial strains were isolated as axenic culture. 25% of strains showed antibacterial activity, thereby nine revealed activity against clinically relevant methicillin resistant *Staphylococcus aureus* (MRSA) and three against enterohemorrhagic *Escherichia coli* (EHEC) strains. Phylogeny and physiological characteristics of the active strains were investigated. First insights into the chemical basis of the antibacterial activity indicated the biosynthetic production of the known compounds ariakemicin, kocurin, naphthyridinomycin, pumilacidins, resistomycin, and surfactin. However, most compounds remained elusive until now. Hence, the obtained results implicate that the microbiome present at the various habitats at the Peruvian coastline is a promising source for heterotrophic bacterial strains showing high potential for the biotechnological production of antibiotics.

Keywords: antibiotics; dereplication; microbiome; natural products; predatory bacteria

1. Introduction

Bacteria have developed diverse strategies and remarkable metabolic capabilities to survive and to colonize harsh environments, such as marine habitats [1]. Certain bacteria apply the strategy to live in association with larger organisms, e.g., sponges or sea slugs, while others have developed a more independent lifestyle [2,3]. The latter group consists of more autonomous bacteria that in most cases possess larger genomes, which provide them with a larger metabolic repertoire enabling them: (i) to get access to scarce nutrients, and/or (ii) to produce bioactive specialized metabolites to protect themselves from predators or even to play this role [4].

Predatory behavior is widely distributed among bacteria and can be found in several phyla, e.g., in Actinobacteria [5], Bacteroidetes [6], Chloroflexi [7], and Proteobacteria [8]. However, the strategies that these microorganisms apply to foster a predatory lifestyle are variable. For a *Cytophaga* species, it was reported that it does epibiotic predation [9]. This means that it attaches to the prey, i.e., phytoplankton, surface, followed by degradation and assimilation of the cell. The Proteobacteria *Bdellovibrio* spp. are obligatory predatory bacteria, which apply a periplasmatic type of predation [8]. They grow epibiotically, whereby the predator cells are attached to the cell envelope of the prey. They are dividing in a binary manner by penetrating the prey's periplasm to generate a number of progeny cells [10]. Another most interesting predatory behavior was denominated as the 'wolfpack strategy' [11]. Here, a predatory attack is performed by using sophisticated communication systems, since it involves a coordinated attack by many bacterial cells, which have the ability to glide on surfaces. A famous example are the Myxobacteria, e.g., the marine *Enhygromyxa salina* [3] and *Cystobacter* sp. [12], well-known proliferative producers of antibiotically active metabolites [13,14]. Further examples come from the Gammaproteobacteria, e.g., *Lysobacter* spp. [15], the Bacteroidetes, e.g., *Rapidithrix thailandica* [16], *Porifericola rhodea* [17], and the Chloroflexi, e.g., *Herpetosiphon* spp. [7]. As mentioned before, a characteristic feature of these taxa is their large genome size with a striking high number of biosynthetic gene clusters (BGCs) putatively coding for specialized metabolites. It is assumed that the biosynthesis of antibiotics plays an important role in their predatory lifestyle [4].

The assumption that these bacteria use antibiotics to weaken or even kill their prey [4] implements the great potential of these organisms as source for novel bioactive compounds. The goal of this project was to identify new bacterial species, which possess the potential to biosynthesize antibiotically active natural products. Therefore, the Peruvian coastline was selected as sampling area, since it represents a biodiverse ecosystem rich in endemic species [18]. It is of interest, if the grade of biodiversity, usually judged by macroorganisms, can also be transferred to microorganisms. Thereby, in this study, the focus is on predatory bacteria. Hence, after obtaining culture-independent insights into the bacterial diversity, a culture-dependent approach using the predatory behavior of the target organisms was applied to isolate promising strains that could potentially produce new antibiotics.

2. Results

2.1. Coastal Microbiome

In order to get an insight into the bacterial diversity present at the three coastal sites, the metagenome of the soil samples was analyzed. The sampling sites represent three marine ecosystems with different seawater temperature (Figure 1).

Paracas is located in the southern coastline of Peru, clearly dominated by the Humboldt Current that brings a cold-water column from southern latitudes. Isla Foca represents the encounter point between the Caribbean and Humboldt Current. Manglares instead, is a mangrove ecosystem, located in the estuary of the Tumbes River, close to the Pacific Ocean. Analysis of the 16S rRNA data obtained by next generation sequencing showed that a less diverse microbiome was found at Isla Foca, IF (213,991 reads) where 89 bacterial genera were found, as compared to 195 genera at Paracas, PA (161,958 reads) and 173 genera at Manglares, MA (163,056 reads). 47 bacterial genera, equivalent to one-half of the IF genera and about one fourth of the PA and MA genera, were found at all of the sites (Figure 2). Approximately half of the genera present in the samples of PA and MA were specific for the respective site, while at the IF site only one sixth of all bacteria could be contributed to specific genera.

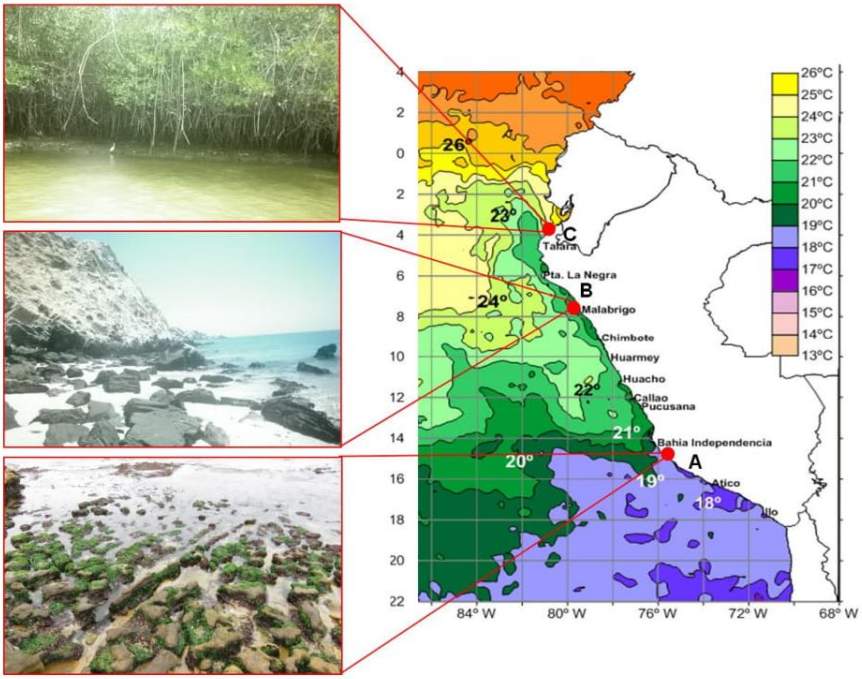

Figure 1. Sample collection sites. The sites are indicated by a red dot at the map. The water temperature (annual average) is given. A: Paracas (18 °C), open beach with presence of halophilic algae and remains of mollusk shells in the collected sediment; B: Isla Foca (22 °C), island located at the confluence of the Humboldt stream and the Caribbean. Presence of coralliferous formations and endemic species in the sampled soil; C: Manglares (26 °C), mangrove ecosystem located at the mouth of the river Tumbes in the Pacific Ocean. Collected samples consisted of mud, located at the base of mangroves.

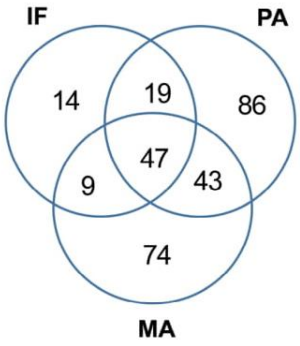

Figure 2. Venn diagram showing site specific and shared bacterial genera. The three sites are Isla Foca (IF), Paracas (PA), and Manglares (MA).

Regarding the phylum level, the sites show a comparable pattern, with Proteobacteria being the most dominant representative (81.45% IF, 63.22% PA, 91.21% MA), followed by Bacteroidetes (10.60% IF, 27.21% PA, 5.69% MA). The remaining portion consists of Firmicutes (7.28% IF, 4.63% PA, 2.50% MA) and in the case of PA 4.94% others (Figure 3).

At the lower taxa level of classes the differences between sites were even more pronounced. Gammaproteobacteria dominated the Proteobacteria clade for PA and MA, while at the IF site the Epsilonproteobacteria were the most abundant class. The latter class was almost completely built by Campylobacterales, which summed up to 98.3 ± 2.33%. In the Gammaproteobacteria phylum the Alteromonadales (IF: 18.81%, PA: 33.18%, MA: 54.55%) and Oceanospirillales (IF: 16.4%, PA: 32.72%,

MA: 18.28%) represented the most abundant orders, with the exception of IF where a high abundance of the Vibrionales (36.97%) and pseudomonads (25.54%) was observed. The most abundant class within the Bacteroidetes phylum was at all of the sites Flavobacteria (78.03 ± 10.36). Therein, the Flavobacteriaceae accounted for 85.69 ± 12.43%. At IF and MA the Bacteroidia class accounted nearly for the complete rest of the bacterial clades in the phylum Bacteroidetes. At PA instead, the Chitinophagia class represented the remaining bacteria. Other orders within the Bacteroidetes phylum were <1%. The Firmicutes split mainly into Clostridiales and Bacillales, other bacteria had a proportion <1%. However, the ratio of Bacillales to Clostridiales was 1:14.9 in PA, 21.8:1 in IF and 1.2:1 in MA. Hence, only in the mangrove coastal environment of Manglares both classes were present in similar proportions.

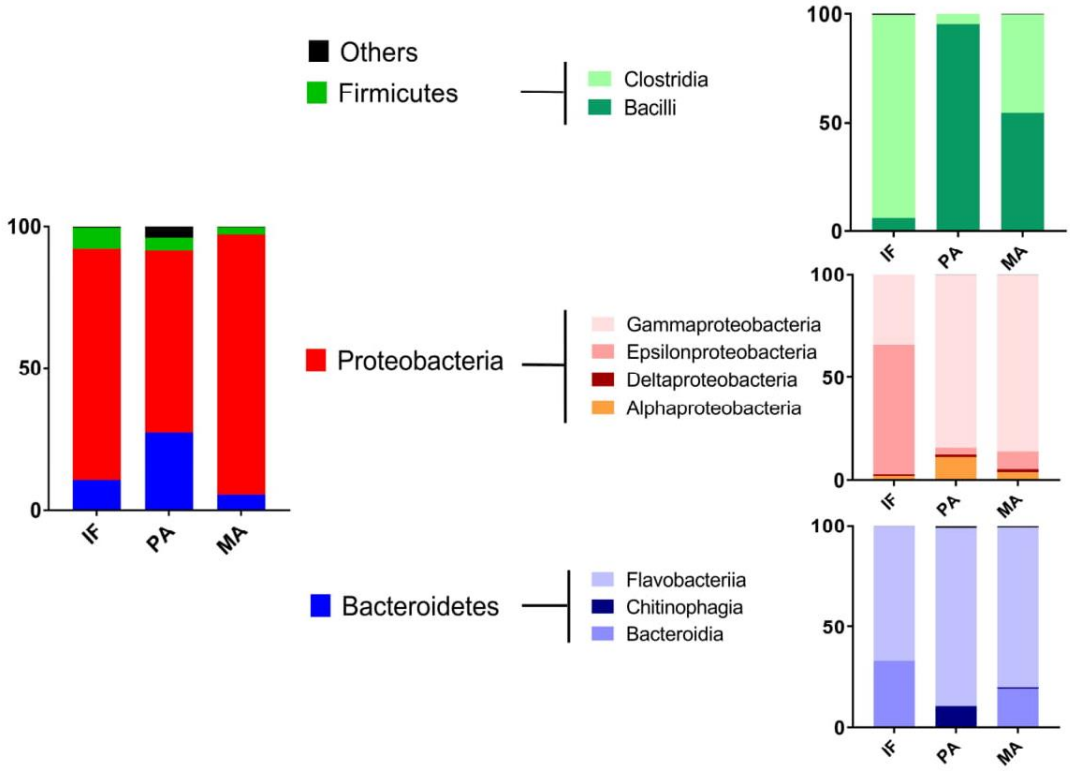

Figure 3. Relative abundance of the bacterial taxa in the metagenomic analysis of the coastal samples.

Beside the general insights into the bacterial diversity present at the Peruvian coastline the potential for detection of antibiotically active metabolites should be investigated. Therefore, it was first planned to target Myxobacteria, as reported proliferative secondary metabolite producers [13]. However, only a neglectable amount of the reads accounted for myxobacterial species, i.e., only 1 read (equivalent to 0.0002%) could be contributed to *Myxococcus xanthus*. However, the rationale to isolate predatory bacteria, since it can be assumed that these organisms produce natural products with antibacterial activity to weaken or even kill their prey [19], could still be followed. A database of predatory bacteria was constructed based on literature (Table S4). Screening the obtained metagenomic data for the presence of reported predatory bacterial taxa, yielded in 0.50 ± 0.44% of the total number of reads (Table 1). At PA and MA, the abundance of the targeted bacteria was 0.67% and 0.85% of total bacteria, respectively. In contrast, IF showed just 19 reads (0.009%).

The most abundant clade of predatory bacteria particularly seen at MA corresponds to *Bdellovibrio*-like organisms (BALOs group), mostly represented by Bdellovibrionales (mainly *Bdellovibrio* and *Bacteriovorax*) (Table 1). The BALOs are characterized by their parasitic lifestyle on other Gram-negative bacteria and represented 86.07% of the predatory bacteria in the

sample, while it summed up to 26.31% and 37.88% at IF and PA, respectively. Most of the predatory bacteria at IF (73.7%) and PA (62.02%) belonged to the taxon Bacteroidetes, while only 13.8% were found at MA. In minute proportions, the myxobacterium *Myxococcus xanthus* (specific for PA) and the cyanobacterium *Vampirovibrio* sp. (specific for MA) were observed.

Table 1. Number of reads of different reported predatory taxa in the metagenomic analysis of 16S rRNA gene sequences.

Taxa	Site of Collection (N° of Reads) [a]		
	Isla Foca (213,991)	Paracas (161,958)	Manglares (163,056)
Bacteroidetes			
Cytophagales	6	203	11
Cellulophaga	2	0	0
Tenacibaculum	4	131	179
Saprospiraceae	2	334	0
Delta-proteobacteria			
Bdellovibrionales-like bacteria	2	81	1184
Bacteriovoracaceae	1	324	2
Bdellovibrionaceae	2	3	0
Myxococcales	0	1	0
Cyanobacteria			
Vampirovibrio	0	0	2

[a] The total number of reads obtained by Illumina sequencing is given.

2.2. Isolation of Antibiotic Producing Predatory Bacteria

In the metagenomic analysis, it was shown that only about 0.5% of the bacteria present in the environmental samples correspond to known predatory bacteria. To get a hand on these underrepresented strains, we used the bait-streak isolation technique (see Material and Methods). Two different bacterial strains were used as bait organisms, i.e., *Escherichia coli* XL1 Blue and the marine Alphaproteobacterium *Pheobacter inhibens* DSM17395. By this approach, 138 bacterial strains were isolated. Thereby, 105 strains were isolated from *E. coli* bait plates and 33 were obtained using *P. inhibens* as bait. It was noticed that the observable growth was significantly slower if *P. inhibens* was used as bait (14–21 days), than if *E. coli* was used (6–10 days). All of the obtained isolates were screened for their antibiotic activity. Liquid cultures were extracted with ethyl acetate and the resulting crude extracts were initially tested against Gram-positive *Arthrobacter psychrolactophilus* and Gram-negative *E. coli* strains. As a result 35 bacterial strains (equivalent to 25%) showed activity against *A. psychrolactophilus* and four strains (3%) against *E. coli* (Figure 4, Table S1).Thereby, ten of the active strains had been isolated using *P. inhibens*, and 25 strains using *E. coli* as prey organism. These strains, showing antibiotic activity in the first screening round, were further tested against clinical isolates, i.e., MRSA and EHEC strains. Now, nine bacterial strains showed activity against MRSA. These bacterial strains were members of the genera *Bacillus* (1), *Euzebyella* (1), *Kocuria* (1), *Labrenzia* (1), *Microbacterium* (1), *Nocardiopsis* (1), *Rapidithrix* (2), and *Streptomyces* (1). Three strains also exhibited antimicrobial activity against EHEC (equivalent to 1.4% of the isolated 138 strains). These strains showed highest homology to *Rapidithrix thailandica* TISTR1768 (99% identity on DNA level), *Streptomyces* sp. NPA1 (99%), and *Euzebyella* sp. B39 (99%).

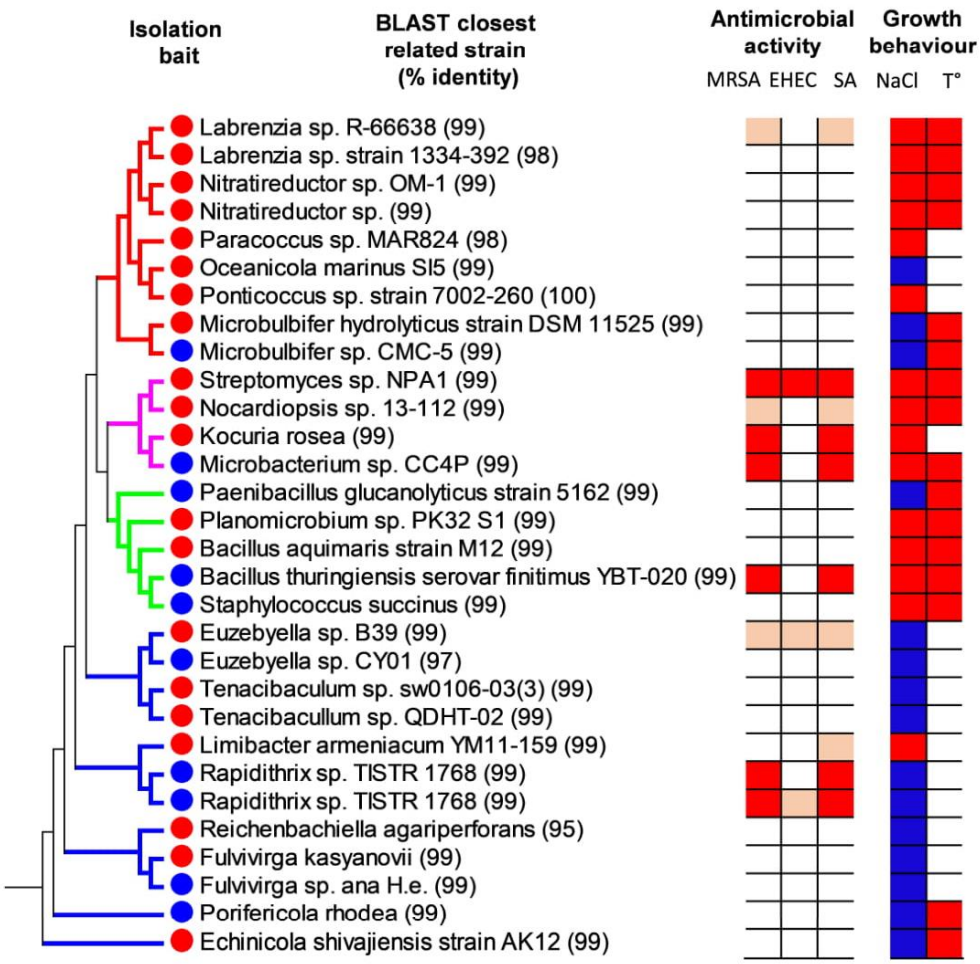

Figure 4. Phylogenetic tree of antimicrobially active isolates. All of the strains indicated here showed activity against *Arthrobacter psychrolactophilus*. The color code of the tree branches indicates Proteobacteria (red), Actinobacteria (green), Firmicutes (purple), and Bacteroidetes (blue). The column "Isolation bait" indicates if the strain was isolated using *E. coli* (red), or *P. inhibens* (blue) as prey organism. Antimicrobial activity is given against methicillin-resistant *Staphylococcus aureus* (MRSA), enterohemorragic *E. coli* (EHEC), and methicillin-sensitive *S. aureus* (SA). Dark and light red indicate strong and moderate antimicrobial activity, respectively; white indicates that no activity was observed. Physiological properties of the strains are indicated on the right side. The NaCl column indicates if the strain is able to grow optimally with low NaCl concentration (<1%, red) or if it needs higher amounts (>1.5%, blue). The T° column indicates which strains were able to grow at an elevated temperature (40 °C). The 16S rRNA sequences of the strains have been deposited at GenBank with the accession numbers MF796603-MF796631 and MF620093.

2.3. Phylogenetic Analysis

From the originally isolated 35 strains showing antimicrobial activity, 30 could be retained in vitro, since five strains stopped growing under our culture conditions. The in vitro culturable strains were identified by 16S rRNA gene sequencing. The NCBI database was used to determine the closest relatives by using BLASTn and a phylogenetic tree was constructed using the minimal evolution method. Most of the active strains (12 strains) belong to the Bacteroidetes phylum; therein especially the Cytophagales family is pronounced (8 strains) (Figure 4). This fact clearly indicates that this clade of predatory bacteria was highly enriched by the isolation method used in this work, since it

represented only 0.1% of the total bacterial counts in the metagenomic analysis. The enrichment of this clade was even more pronounced when *P. inhibens* was used as bait (5 out of 10 strains).

In the Proteobacteria phylum, the Alphaproteobacteria (7 strains) were enriched as compared to the Gammaproteobacteria (2 strains). This differs to the relative abundance observed in the metagenomic data where the latter represent about 80% of the Proteobacteria. The strains isolated from this clade, however, were not described to have a predatory behavior, but could be isolated by the method used. Thereby, Alphaproteobacteria were only isolated by using *E. coli* as bait (7 out of 25 strains as compared to 0 out of 10 strains by using *P. inhibens*).

Apart from the Bacteroidetes and Proteobacteria, the remaining 9 strains were divided between the Firmicutes and Actinobacteria phylum. It was observed that *E. coli* used as a bait during isolation tended to enrich Actinobacteria (3 strains were isolated using *E. coli* and only 1 strain using *P. inhibens*) while *P. inhibens* bait enriched Firmicutes (4 strains were isolated using *P. inhibens* and only 1 strain using *E. coli*).

2.4. Physiological Properties of the Isolated Bacteria

All of the strains grew optimally when the NaCl concentration in the medium was around 2%, and nearly all of the strains were able to grow in the NaCl range from 0.5 to 3.89% (Table S2). However, four strains from the Bacteroidetes (*Echinicola* sp. and *Tenacibaculum* spp.) and Proteobacteria (*Oceanicola* sp.) clades seemed to be obligatory marine, since they stopped growing when the NaCl concentration in the media was decreased below 1%. The *Oceanicola* sp. even showed the best growth rate around 4% and was unable to grow under 2% NaCl. Most of the bacterial strains grew optimally to around 30 °C (Table S3). However, several strains were able to grow at higher temperatures of 45 °C. These were mainly the Actinobacteria (with the exception of *Kocuria* sp.) and Firmicutes. In addition, Proteobacterial strains, e.g., *Labrenzia* and *Nitratireductor*, and few Bacteroidetes, e.g., *Porifericola* and *Echinicola*, showed a tolerance to higher temperatures.

2.5. Identification of Known Compounds—Dereplication

In order to get a better picture of the metabolome of the isolated bacteria and to identify known antibiotic compounds produced, a Global Natural Product Social Molecular Networking (GNPS) analysis was performed [20]. In addition, a literature database search was done, in order to dereplicate additional compounds.

The ethyl acetate crude extracts were analyzed by HPLC-MS/MS and the GNPS server enabled us to identify several reported compounds with antimicrobial activity. This analysis showed that many compounds are exclusively biosynthesized by a particular strain. When comparing the two isolated *Rapidithrix* strains, which share 99% identity of their 16S rRNA gene, revealed that only one strain, i.e., *Rapidithrix thailandica* s80 produced the hybrid peptide-polyketides ariakemicin A and B. The other strain, i.e., *Rapidithrix thailandica* s68, instead, showed different metabolites that were not found in the former one. Further, we were able to identify several already described antibiotics using mass-based analysis: Kocurin was identified in extracts from *Kocuria rosea* s17 [21]; naphthyridinomycin and resistomycin in *Streptomyces* sp. s120; and, the pumilacidins C and E as well as surfactin in *Paenibacillus glucanolyticus* s102b (see Supplementary Information).

A first draft of the genome sequence of *Streptomyces* sp. s120 (unpublished) was analyzed using antiSMASH [22] and biosynthetic gene clusters showing similarities to reported naphthyridinomycin and resistomycin clusters could be detected (Table S5). However, for most of the antibiotically active extracts the responsible molecule(s) could not be identified yet.

3. Discussion

Predatory bacteria play an important role in marine ecosystems. It was demonstrated that members of the BALOs, i.e., *Bdellovibrio bacteriovorus* and *Bacteriovorax stolpii*, as well as *Micavibrio aeruginosavorus*-like predators are key players in controlling the abundance of several bacteria. All are

epibiotic bacteria, whereby the *M. aeruginosavorus*-like species do not invade the periplasmatic space of their prey. Together they are important modulators of *Vibrio* populations in seawater and oysters [23]. In the here reported sample set, the BALOs represented about 70% of the total predatory bacteria present. Notably, this group was more abundant at the MA and PA sites. It is however difficult to link this to the abundance of a particular prey taxa, since the host specificity from these strains can be variable and differs from one strain to another [24]. However, it can be assumed that their prey was not so abundant at the IF site, at least at the time the samples were taken, since for these predatory taxa only five reads were detected. Instead, Epsilonproteobacteria and Clostridiales were more dominant at IF, indicating a more anaerobic and/or sulfur rich environment, which does not represent a favorable habitat for the before mentioned strictly aerobic taxa [25]. The overall percentage of $0.50 \pm 0.44\%$ of predatory bacteria in the environmental samples corresponds to reported values. In marine aquaculture systems the observed range was 0.13–1.4% [26].

These previously mentioned taxa that require live prey as their growth substrate represented the majority of predatory bacteria found in the investigated sediment samples. However, in regard of proliferative producers of biologically active metabolites, the facultative predators represent the more promising species, e.g., Myxobacteria, Cytophagales and certain Flavobacteria. In order to get nutrients by predation, these bacteria make use of their gliding motility and the biosynthesis of a whole repertoire of bioactive specialized metabolites. These metabolites can be used for intra- and interspecies communication, as well as for chemical warfare [4]. Remarkably, such biologically active metabolites are of high interest for the pharmaceutical industry, due to their potential utility in modern medicine and biomedical research [27]. As here exemplified by two phylogenetically distinct predatory bacteria, i.e., *Myxococcus* species (Myxobacteria) and the *Herpetosiphon* species (Chloroflexi) [4]. Both possess large genomes with an overrepresented number of BGCs, putatively involved in the biosynthesis of specialized metabolites. Corresponding molecules, showing promising activities, were already isolated, e.g., the antibiotics myxovirescins and gulmirecins from *Myxococcus*, as well as siphonazole and auriculamide from *Herpetosiphon* [4].

Even though the share of predatory species in the environmental microbiome is low, they can be isolated and mostly adapted to grow under laboratory conditions. The isolation based on the phenotype is an important selection criterion, since also closely related strains might be non-predatory. Hence, starting with culture-independent screening approaches is difficult. It was reported that the predatory bacterium, *Ensifer adhaerens*, is very closely related to a non-predatory strain, which rendered 16S rRNA gene-based phylogenetic probes incapable of distinguishing predatory from non-predatory organisms [28]. In addition, predatory lifestyle can be encountered in various taxa with different phylogeny and physiology. Using two different Proteobacterial strains as bait, i.e., the Gammaproteobacterium *E. coli* and the Alphaproteobacterium *P. inhibens* resulted in the isolation of targeted bacteria that initially were not even identified by the metagenomic analysis. In that way, two *Rapidithrix* species and one *Porifericola* species were isolated, described as predatory bacteria before [16,17]. Hence, the power of the isolation method is demonstrated, although it became clear that the selection of the prey organism has a strong influence onto the bacterial strains isolated. Particular taxa were isolated using a given prey organisms, e.g., there was a complete lack of Alphaproteobacterial isolates when *P. inhibens* was used as bait. This result is in accordance with previous work performed to assess the prey specificity of several *Tenacibaculum* strains [29]. They reported clear differences in prey preferences even at the intra-genus level. This might also explain why during this work several strains were initially isolated, but after several transfers, they stopped growing in vitro, i.e., *Brumimicrobium*, *Fulvivirga*, and *Saprospira* species. For future bioprospecting projects, the use of several taxa as bait organisms should allow to diversify and/or enrich certain bacteria, thereby increasing the chance for the isolation of novel strains. In addition, the methods to isolate such low abundant species like the predatory bacteria should be developed further. No myxobacterial species could be isolated, even though a *M. xanthus* read was detected by metagenomics. It is assumed that Myxobacteria, which are detected using culture-independent

methods, but cannot be cultivated from an environmental sample, are present as a vegetative cell form and not as dry-resistant myxospores [30]. If diffusion chambers like the iChip, which was successfully applied in the isolation of the teixobactin-producing bacterium *Eleftheria terrae* [31], can be successfully applied to isolate social bacteria, which hunt as wolf packs, must be evaluated in the future.

Interestingly, by the approach applied in the project strains belonging to the Actinobacteria and Bacillales taxa were isolated, which had not been reported before as predatory bacteria. However, strains of these taxa are well known as proliferative producers of specialized metabolites. This means that they developed different features to outcompete other bacteria in the highly competitive environment of the ocean to access nutrients. In fact, about 80% of natural products that are used in human medicine are derived from one genus, i.e., *Streptomyces* [32]. There are some possible explanations why such strains were isolated by our approach. It might be that they produce metabolites lysing the prey cells, and subsequently these metabolites can be used for growth, or they metabolize the agar present in the plates. Further, it has to be considered that in the environment and therewith in the samples used for cultivation, consortia of bacteria are present. Hence, predatory bacteria could initially release the nutrients from the prey, making them accessible for other strains, whereby we isolated the latter as axenic culture.

Even though Actinobacteria, especially Streptomycetes, are well investigated, and this group is still far from being completely exploited. Fostered by the progress in sequencing technologies, many genomes became available and using genome mining tools it can be clearly seen that many more BGCs coding for the synthesis of so far unknown metabolites are encoded in the genomes than compounds are identified. Therefore, such bacteria are still in the focus of natural product research. In this work, four Actinobacteria were isolated, one each from the genera *Kocuria*, *Microbacterium*, *Nocardiopsis* and *Streptomyces*, respectively. By the dereplication approach used, the thiopeptide kocurin was identified in the extract of *Kocuria rosea* s17. This potent anti MRSA antibiotic is produced by several members of the *Micrococcaceae* family. The activity profile of kocurin is comparable to linezolid, a drug in clinical use. In a in vivo mouse model infections with MRSA and VRE could be cured [33]. Coming back to the imbalance between BGCs identified in silico and known metabolites, kocurin is a special case, since it was the other way round. The compound was already known, but the biosynthesis remained unknown. Based on the identification of the compound in this project, we recently were able to identify and clone the corresponding BGC [21]. Moreover, by dereplication the polyketides resistomycin and naphthyridinomycin were identified in the crude extract of *Streptomyces* sp. S120.

Among the bacteria isolated in this project, the Cytophagales were highly enriched. For this phylogenetic clade the gliding behavior and in less degree epibiotic predation seem to be common features. Moreover, the strains belonging to this group seemed to be truly marine, since their growth was significantly reduced, once the NaCl level decreased below 1.5–2%. Three different *Fulvivirga* strains were initially isolated, however, only two could be maintained under the laboratory conditions used. From this genus, only one genome sequence is available in the NCBI database, i.e., *Fulvivirga imtechensis* (NZ_AMZN00000000.1). The genome has a size of approximately 7 mbps and a bioinformatic analysis using antiSMASH [22] revealed the presence of several BGCs that putatively code for specialized metabolites. This suggests *Fulvivirga* strains to be a promising target to continue research on the chemistry of this poorly investigated genus.

Other Bacteroidetes that could potentially offer novel antimicrobially active molecules are the Flavobacteria. In this work, two *Euzebyella* strains, showing 96% identity to each other, were isolated and showed activity against MRSA and *E. coli*. To the best of our knowledge, there is no previous report of antimicrobial compounds produced by this recently discovered genus [34]. Further research is planned to elucidate the chemical basis of the observed activities. The other predatory Flavobacterium isolated belonged to the genus *Tenacibaculum*. This taxa exhibits an epibiotic predation type [29]. Hence, this does not imply the production of specialized metabolites in the magnitude of the previously mentioned strains. This fact is also reflected in the much smaller genome sizes (3–3.5 mbps) of this genus.

However, there have been strains isolated belonging to the Cytophagales group for which antibiotic production was proven. Marine strains of the genus *Rapidithrix* or *Porifericola* were isolated with similar isolation techniques as the ones used here [16,17]. These strains showed complex gliding behavior and exhibited interesting antimicrobial activity. For *Rapidithrix* sp. HC35 the production of the antibiotic ariakemicin was reported [35], as well as several antibiotically active pyrrole derivative molecules [36,37]. In this work we isolated two antibiotically active *Rapidithrix* strains. For one of them the antimicrobial activity was attributed to ariakemicin production, while the other strain should produce different antibiotic compounds. Interestingly, the LC-MS/MS analysis revealed that the latter produced several compounds, which were not observed in the former. An intriguing fact is that other predatory bacteria, e.g., *Herpetosiphon* sp. B060 and *Sorangium* sp. (formerly reported as *Polyangium* sp.), produce specialized metabolites, which show structural similarities to the polyketide peptide hybrids ariakemicins [7,38]. Siphonazole, produced by *Herpetosiphon* sp. B060, and phenoxan, produced by *Sorangium* sp., have the benzoic acid derivative and the oxazole rings interlinked by unsaturated polyketide-peptide chain(s) in common. It was speculated that the production of related metabolites by phylogenetically distant taxa may imply that these natural products have the same molecular ancestor, diversified during evolution [35]. However, also more recent horizontal transfer events should be taken into account, since it was suggested that this could be the case for the peptide antibiotic althiomycin that was initially reported in *Streptomyces* and later in myxobacterial strains [4].

In summary, predatory bacteria play an important role in marine ecosystems and the physiological requirements for their lifestyle renders them a most promising source of bioactive compounds. Bioprospecting projects aiming to discover new producer strains of biologically active natural products have regained interest in the last years, given the notable development of other areas, such as improved analytical methods as well as the advances in sequencing technologies and in bioinformatics. These technologies complement the traditional screening efforts and provide us a more accurate look on the metabolic potential of these bacteria [20]. These new approaches for antibiotic discovery are particularly important given the current scenario, where the number of effective antibiotics is diminishing in an alarming rate and the panorama is not optimistic since, with a few exceptions, no major breakthrough discoveries similar to penicillin and aminoglycosides have been reported in the last 20 years [39]. Here, it could be shown that many strains possess antibacterial activities, whereby the chemical basis of this effect is unknown. Hence, these unexplored specialized metabolites possess likely novel chemical features. This in turn could contribute to the refilling of the antibiotics development pipeline. Further, it suggests using a higher number of prey organisms in future bioprospecting project, since this should diversify, or enrich particular taxa of bacteria to be isolated, thereby increasing the chance to isolate novel strains and compounds.

4. Materials and Methods

4.1. Collection of Samples and Isolation of Bacterial Strains

Intertidal soil samples containing organic material (0 to 0.5 cm deep) were collected from three different locations of the Peruvian coastline. The coordinates were: A: Paracas, S 13°51′44.8″ W 76°16′11.7″; B: Isla Foca, S 5°12′31.3″ W 81°12′17.3″; C: Manglares, S 3°25′33.8″ W 80°16′28.6″. These sampling areas were selected by taking the differences in seawater temperature into account, since variations in biodiversity can be expected in these habitats: Manglares (26 °C, northern coast), Isla Foca (22 °C, nor-central coast), and Paracas (18 °C, southern coast). The location of these areas is shown in Figure 1. The samples were transported in sterile falcon tubes to the Laboratory of Pharmacology of the National University of Trujillo. Bacteria isolation was based on the bacteriolytic properties of the expected strains. Therefore, *E. coli* XL1 Blue cells were incubated overnight in 50 mL LB (Luria Broth) medium at 37 °C, and *Pheobacter inhibens* DSM17395 cells were incubated in Marine Broth 2216 (Difco, Sparks, MD, USA) medium at 30 °C, respectively. Then, cells were collected by centrifugation (4000 g, 5 min) and were subsequently washed with sterile Artificial Sea Water

(ASW, composition per liter: NaCl 23.926 g, Na_2SO_4 4.008 g, KC1 0.677 g, $NaHCO_3$ 0.196 g, KBr 0.098 g, H_3BO_3 0.026 g and NaF 0.003 g). After autoclaving, cyanocobalamin (0.5 mg/L) and cycloheximide (25 mg/L) were added. Cells were resuspended in 4 mL of ASW and 50 µL of this solution were transferred as circular patches onto ASW agar plates, allowing them to dry for 2 h. A small amount of environmental sample was placed on the middle of each *E. coli* or *P. inhibens* patch. The following incubation was performed at 30 °C for 3 weeks. Once, lysis of *E. coli* or *P. inhibens* cells was observed, the agar piece of the lytic zone was cut out and transferred onto a new ASW agar plate. The same procedure was repeated, until an axenic culture was obtained.

4.2. Metagenomic Analysis of 16S rRNA Gene Sequence

For metagenomic analysis, 30 subsamples were collected at each sample site (100 m^2 diameter). From each location the respective sample (1 g wet weight) was mixed with 5 mL deionized water and homogenized using a Potter homogenizator. The resulting mixture was filtered using a 5 µm pore size filter (Sartorius, Göttingen, Germany) to enrich bacterial cells. The cells were collected by centrifugation at 10,000 *g* for 15 min. The metagenomics DNA was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma, Darmstadt, Germany) and the 16S rRNA gene was sequenced by CEMET GMBH (Tübingen, Germany) using an Illumina sequencer and F515/R806 primers. The obtained data was analyzed using Megan6 [40].

4.3. Antimicrobial Activity

To determine the antimicrobial activity of the isolated strains, a small-scale cultivation (50 mL) was carried out in marine broth M2216 (Difco, Sparks, MD, USA) for 4 days at 30 °C. Then, the cultures were extracted 1:1 with ethyl acetate and the organic phase was evaporated to dryness in a rotary evaporator. The resulting crude extract was resuspended in methanol to a final concentration of 10 mg/mL. 50 µL of these extracts were added to sterile filter disks and were placed onto LB agar plates on which the test organisms (*Arthrobacter psychrolactophilus* as Gram-positive and *Escherichia coli* XL1 Blue as Gram-negative) were previously streaked out. The plates were incubated overnight at 30° and 37 °C, respectively. The inhibition of the test strains was quantified by the diameter of the resulting halo (in mm). In the first screening round, 35 strains showed activity and were used for a further antimicrobial activity test against relevant pathogenic strains, i.e., methicillin resistant *Staphylococcus aureus* (MRSA) and enterohemorrhagic *E. coli* (EHEC).

4.4. Growth Characteristics

For the strains that showed antimicrobial activity, the temperature and salinity tolerance was determined. The growth response was investigated by testing several temperatures, ranging from 25 to 45 °C in marine broth agar M2216 (Difco, Sparks, MD, USA). The salinity tolerance was assessed by modifying the NaCl level of the medium from 0.5 to 10%.

4.5. Phylogenetic Analysis of Isolated Strains

The strains that showed antimicrobial activity against any of the test strains were selected for 16S rRNA identification. The genomic DNA of the respective strain was isolated using the DNA purification kit according to Promega manufacturer's instructions. PCR amplification of the 16S rRNA gene was achieved using the primer pair pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCCCA-3′). The resulting DNA fragments were gel purified and then sequenced (GATC, Konstanz, Germany) using the same primers. The data resulting from both sides of the end sequencing were merged and analyzed using BLASTn. The phylogenetic tree was built using the minimum evolution method of the software MEGA 7.

4.6. HPLC-MS/MS Measurements

The previously obtained crude extracts (see Section 2.3) were diluted in methanol to a final concentration of 1 mg/mL. Mass spectra were recorded on a micrOTOF-Q mass spectrometer (Bruker, Billerica, MA, USA) with ESI-source coupled with a HPLC Dionex Ultimate 3000 (Thermo Scientific, Darmstadt, Germany) using an EC10/2 Nucleoshell C18 2.7 µm column (Macherey-Nagel, Düren, Germany). The column temperature was 25 °C. MS data were acquired over a range from 100 to 3000 m/z in positive mode. Auto MS/MS fragmentation was achieved with rising collision energy (35–50 keV over a gradient from 500 to 2000 m/z) with a frequency of 4 Hz for all of the ions over a threshold of 100. HPLC begins with 90% H_2O containing 0.1% acetic acid. The gradient starts after 1 min to 100% acetonitrile (0.1% acetic acid) in 20 min. 5 µL of a 1 mg/mL sample solution was injected; and, flow rate was set to 0.3 mL/min.

Supplementary Materials: The following are available online at www.mdpi.com/1660-3397/15/10/308/s1, Table S1: Isolated strains that showed antibiotic activity, Table S2: Growth behavior at different NaCl concentrations, Table S3: Growth behavior at different temperatures, Table S4: Selected predatory bacteria reported in the literature. The 16S rRNA sequences of the strains have been deposited at GenBank with the accession numbers MF796603-MF796631 and MF620093, Table S4: Antismash bioinformatic of secondary metabolites BGC present in the genome of *Streptomyces* sp. s120 showing the presence of the naphthyridinomycin and resistomycin BGC.

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Chapter I Supplementary

Supplementary Material

Diversity and Antimicrobial Potential of Predatory Bacteria from the Peruvian Coastline

Luis Linares-Otoya,[1,2,5] Virginia Linares-Otoya,[3,5] Lizbeth Armas-Mantilla,[3] Cyntia Blanco-Olano,[3] Max Crüsemann,[2] Mayar L. Ganoza-Yupanqui,[3] Julio Campos-Florian,[3] Gabriele M. König,[2,4] Till F. Schäberle[1,2,4,*]

[1] Institute for Insect Biotechnology, Justus Liebig University of Giessen, Giessen, Germany
[2] Institute for Pharmaceutical Biology, University of Bonn, Bonn, Germany
[3] Department of Pharmacology, Faculty of Pharmacy and Biochemistry, National University of Trujillo, Trujillo, Peru
[4] German Centre for Infection Research (DZIF) Partner Site Bonn/Cologne
[5] Research Centre for Sustainable Development Uku Pacha, Peru
* Correspondence till.f.schaeberle@agrar.uni-giessen.de; Tel.: +49-641-9937140

Supplementary Table S1. Isolated strains that showed antibiotic activity

Strain identifier	Strain name as deposited in NCBI database	Closest 16S rRNA gene sequence hit in BLAST	Identity %	Isolation bait	Isolation site
2	Tenacibacullum sp. s2	Tenacibacullum sp. QDHT-02	99	*E. coli*	Paracas
16	Tenacibaculum sp. S16	Tenacibaculum sp. sw0106-03(3)	99	*E. coli*	Paracas
17	Kocuria rosea s17	Kocuria rosea	99	*E. coli*	Paracas
21	Reichenbachiella sp. S21	Reichenbachiella agariperforans	95	*E. coli*	Paracas
23	Oceanicola marinus s23	Oceanicola marinus Sl5	99	*E. coli*	Paracas
37	Limibacter armeniacum s37	Limibacter armeniacum YM11-159	99	*E. coli*	Paracas
42	Fulvivirga sp. s42	Fulvivirga sp. ana H.e.	99	*P. inhibens*	Isla Foca
47	Porifericola rodea s47	Porifericola rhodea	99	*P. inhibens*	Paracas
48	Fulvivirga kasyanovii s48	Fulvivirga kasyanovii	99	*P. inhibens*	Isla Foca
68	Rapidithrix sp. s68	Rapidithrix sp. TISTR 1768	99	*P. inhibens*	Paracas
80	Rapidithrix sp s80	Rapidithrix sp. TISTR 1768	99	*P. inhibens*	Paracas
82	Labrenzia sp. strain s82	Labrenzia sp. strain 1334-392	98	*E. coli*	Paracas
83	Microbulbifer hydrolyticus s83	Microbulbifer hydrolyticus strain DSM 11525	99	*E. coli*	Manglares
90	Bacillus sp. s90	Bacillus thuringiensis serovar finitimus YBT-020	99	*P. inhibens*	Paracas
93	Labrenzia sp. s93	Labrenzia sp. R-66638	99	*E. coli*	Isla Foca
100	Nitratireductor sp. s100	Nitratireductor sp.	99	*E. coli*	Manglares
106	Staphylococcus succinus s106	Staphylococcus succinus	99	*P. inhibens*	Paracas
107	Microbulbifer sp. s107	Microbulbifer sp. CMC	99	*P. inhibens*	Manglares
108	Euzebyella sp. s108	Euzebyella sp. CY01	97	*P. inhibens*	Manglares
120	Streptomyces sp. s120	Streptomyces sp. NPA1	99	*E. coli*	Manglares
122	Nocardiopsis sp. s122	Nocardiopsis sp. 13-112	99	*E. coli*	Manglares
123	Nitratireductor sp. s123	Nitratireductor sp. OM-1	99	*E. coli*	Isla Foca
102[a]	Microbacterium sp. s102a	Microbacterium sp. CC4P	99	*P. inhibens*	Paracas
102b	Paenibacillus sp. s102b	Paenibacillus glucanolyticus strain 5162	99	*P. inhibens*	Paracas
118b	Echinicola shivajiensis s118b	Echinicola shivajiensis strain AK12	99	*E. coli*	Manglares
17_1c	Planomicrobium sp. s171c	Planomicrobium sp. PK32_S1	99	*E. coli*	Paracas
17_2c	Paracoccus sp. s172c	Paracoccus sp. MAR824	98	*E. coli*	Paracas
30b1	Ponticoccus sp. s30b1	Ponticoccus sp. strain 7002-260	100	*E. coli*	Paracas
49b1	Bacillus aquimaris s49b1	Bacillus aquimaris strain M12	99	*E. coli*	Paracas
49b2	Euzebyella sp. s49b2	Euzebyella sp. B39	99	*E. coli*	Paracas

Supplementary Table S2. Growth behavior at different NaCl concentrations

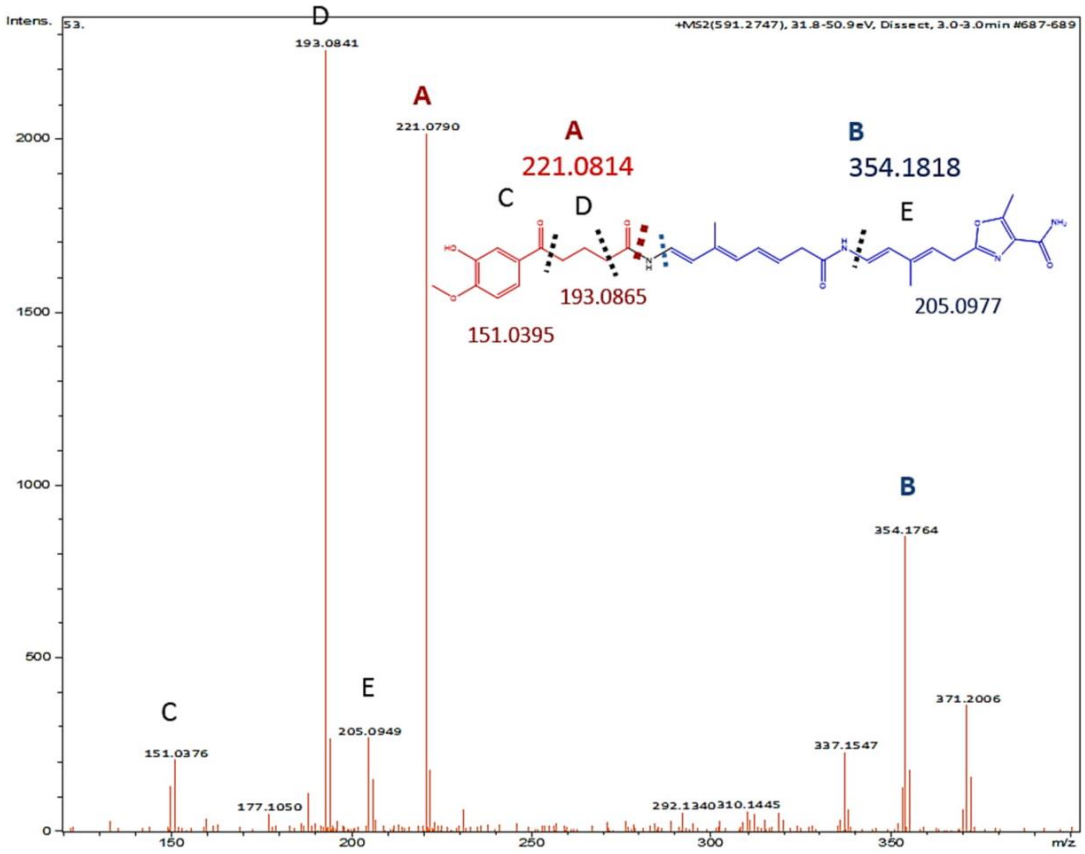
Strain identifier	% NaCl							
	0.5%	1%	1.95%	2.14%	2.23%	3.89%	5%	10%
2	--	++	+++	+++	++	++	--	--
16	--	+	++	+++	++	+	+	--
17	++	++	++	++	++	++	+	--
17_1c	++	++	++	++	++	++	+	--
17_2c	++	++	++	++	++	++	+	--
21	+	+	++	+++	++	++	+	+
23	--	--	--	+	++	+++	++	--
30b1	++	+++	+++	++++	+++	+++	+++	++
37	++	++	++	++	++	++	--	--
42	+	++	+++	+++	++	+	+	+
47	+	++	+++	++	++	++	--	--
48	+	++	++	++	+	--	--	--
49b1	+++	+++	+++	+++	+++	++	+	--
49b2	+	+	++	++	++	++	+	--
68	+	++	+++	+++	++	+	--	--
80	+	++	+++	+++	++	+	--	--
82	++	++	+++	+++	++	++	++	--
83	+	++	+++	+++	++	++	++	+
90	+++	+++	+++	+++	+++	+++	++	--
93	+	++	++	++	+	+	--	--
100	++	++	++	++	++	++	++	-
102a	+	++	++	++	++	+	+	+
102b	+	++	+++	+++	++	+	+	+
106	++	+++	++++	+++	+++	+++	++	++
107	+	++	+++	+++	++	+	+	+
108	+	+	++	+++	++	+	+	--
118b	--	+	++	++	+	+	+	--
120	++	++	++	++	++	++	--	--
122	+	++	++	++	++	+	+	+
123	++	+++	+++	++	++	++	+	+

Supplementary Table S3. Growth behavior at different temperatures

Strain identifier	10ºC		27 ºC		30ºC		45ºC	
	24 H	48 H	24 H	48 H	24 H	48 H	24 H	48 H
2	--	--	++	+++	++	+++	--	--
16	--	--	++	+++	++	+++	--	--
17	--	--	++	++	++	++	--	--
17_1c	--	--	++	++	++	++	+	+
17_2c	--	--	++	++	++	++	--	--
21	--	--	++	+++	++	+++	--	--
23	--	--	++	+++	++	+++	--	--
30b1	--	--	+	++	++	+++	--	--
37	--	--	++	++	++	+++	--	--
42	--	--	--	+	+	++	--	--
47	--	--	--	+	++	+++	+	+
48	--	--	--	+	+	++	--	--
49b1	--	--	+	++	+	++	+	+
49b2	--	--	--	++	++	+++	--	--
68	--	--	--	++	+	++	--	--
80	--	--	+++	+++	+++	+++	--	--
80	--	--	--	++	+	++	--	--
82	--	--	++	+++	++	+++	+	+
83	--	--	+	++	++	+++	--	+
90	--	--	++	+++	++	+++	++	++
93	--	--	+	++	+	++	+	+
100	--	--	++	++	++	++	--	--
102a	--	--	+	++	+	++	+	+
102b	--	--	+	++	+	++	+	+
106	--	--	++	++	++	+++	+++	+++
107	--	--	+	++	++	+++	+	+
108	--	--	++	+++	+++	+++	--	--
118b	--	--	--	+	+	++	+	+
120	--	--	++	+++	+++	+++	++	+++
122	--	--	+	++	+	++	+	+
123	--	--	+	++	+	++	+	+

Supplementary Table S4. Selected predatory bacteria reported in the literature

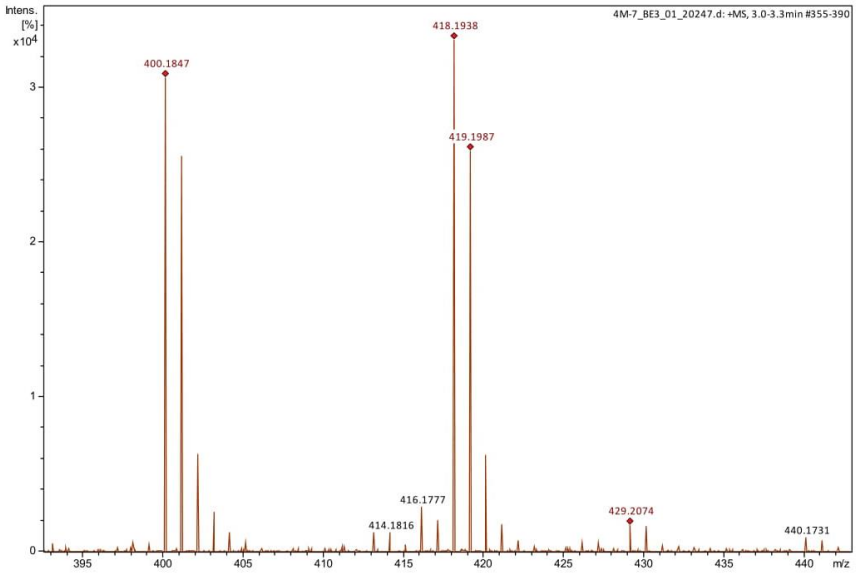
Genus and or species	Reference
Agromyces ramosus	[1]
Herpetosiphon	[2]
Bdellovibrio bacteriovorus	[3]
Halobacteriovorax	[4]
Myxococcus xanthus	[2]
Vampirococcus	[5]
Ensifer	[6]
Cupriavidus necator	[7]
Lysobacter	[8]
Saprospira grandis	[9]
Tenacibaculum	[10]
Olleya	[10]
Cytophaga	[11]
Rapidithrix	[12]
Porifericola rhodea	[13]
Vampirovibrio chlorellavorus	[2]
Micavibrio	[14]



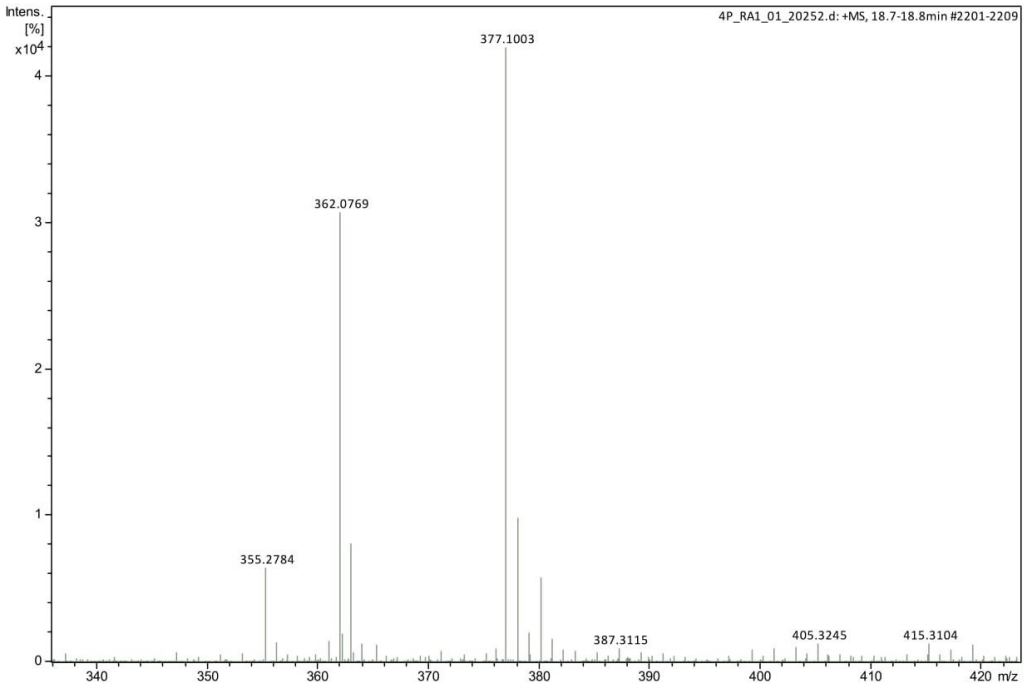
Supplementary Figure S1. MS/MS spectrum of *Rapidithrix thailandica* s80 crude extract showing the fragmentation pattern expected for ariakemicins isolated before from *Rapidithrix* sp [15].

Supplementary Table S5. Antismash bioinformatic of secondary metabolites BGC present in the genome of *Streptomyces sp*. s120 showing the presence of the naphthyridinomycin and resistomycin BGC [16].

Type	From	To	Most similar known cluster	MIBiG BGC-ID
Otherks-T1pks-Nrps	1	58334	Naphthyridinomycin_biosynthetic_gene_cluster (60% of genes show similarity)	BGC0000394_c1
Other	1	16023	Naphthyridinomycin_biosynthetic_gene_cluster (39% of genes show similarity)	BGC0000394_c1
T2pks	422	42943	Resistomycin_biosynthetic_gene_cluster (88% of genes show similarity)	BGC0000264_c1



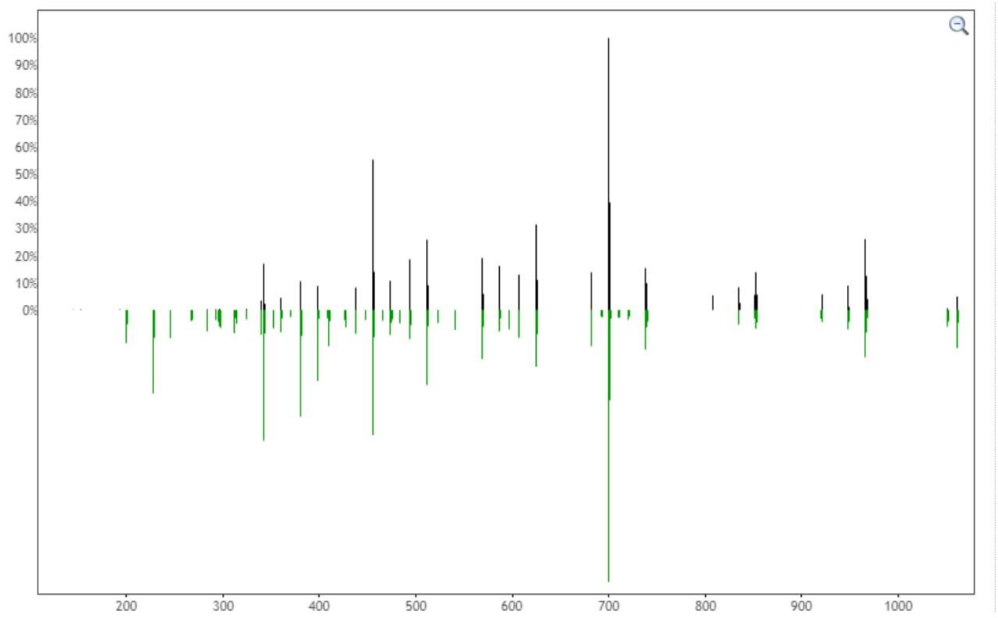
Supplementary Figure S2. MS spectrum of *Streptomyces sp*. s120 extract showing the presence of naphthyridinomycin (observed *m/z* 418.1938, expected 418.1978) [17].



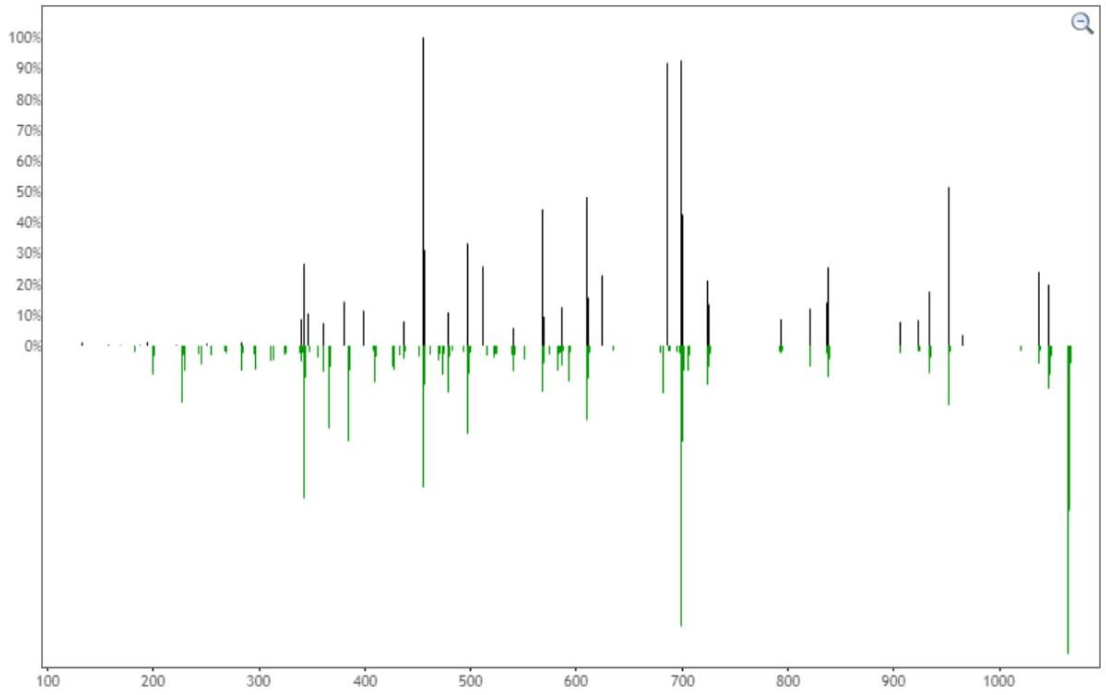
Supplementary Figure S3. MS spectrum of *Streptomyces sp*. s120 extract showing the presence of resistomycin (observed *m/z* 377.1003, expected 377.1025) [18].

Supplementary Table S6. GNPS dereplication analysis of crude extract of *Paenibacillus sp*. s102b showing the presence of pumilacidin C and E as well as surfactin [19].

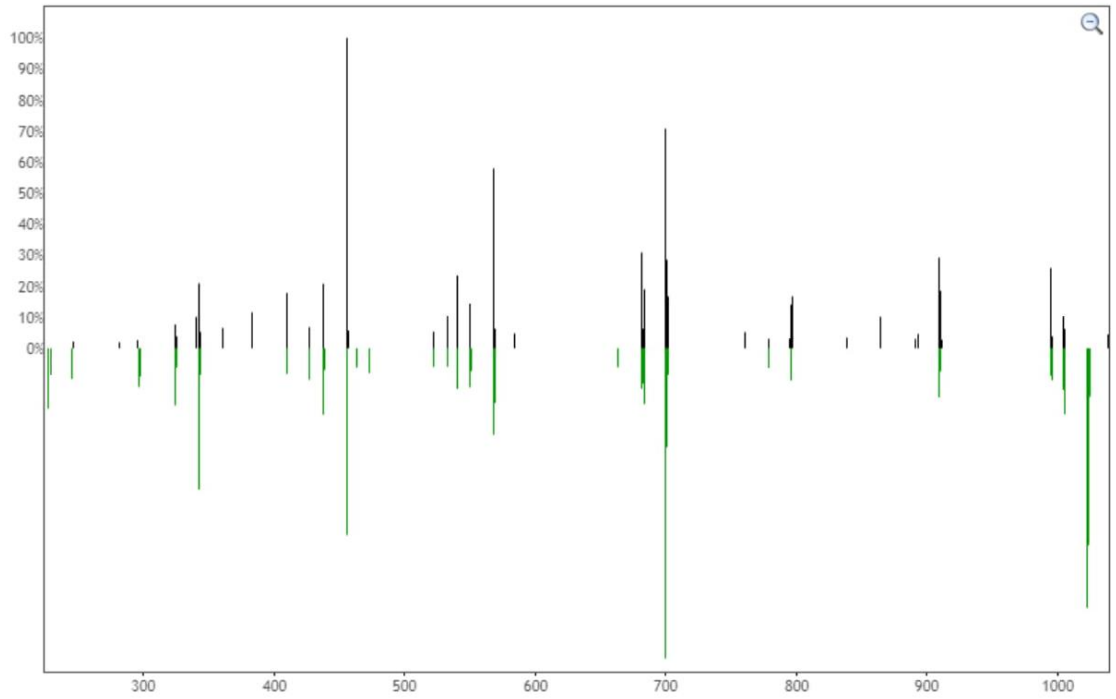
Compound_Name	Library Class	Cosine	Shared Peaks	TIC	RT	Mass Diff	SpecMZ	LibMZ	PI
pumilacidin_C_14344_dereplictor_pv_2.93242e-26	Bronze	0.82	36	6691.19	419.89	0.01	1078.75	1078.74	Pevzner
pumilacidin_E_14342_dereplictor_pv_1.13518e-25	Bronze	0.65	25	4486.22	402.57	0.02	1064.73	1064.72	Pevzner
[Ile2_4_7]Surfactin_43293_dereplictor_pv_2.3158e-16	Bronze	0.73	20	1797.96	1152.71	0.01	1022.68	1022.67	Pevzner



Supplementary Figure S4. MS/MS mirror plot of crude extract of *Paenibacillus sp*. s102b against GNPS database showing the presence of pumilacidin C [19]. Source: Green: Pumilacidin C MS/MS spectrum from GNPS database, black: Crude extract of *Paenibacillus sp*. s102b.



Supplementary Figure S5. MS/MS mirror plot of crude extract of *Paenibacillus sp*. s102b against GNPS database showing the presence of pumilacidin E [19]. Source: Green: Pumilacidin E MS/MS spectrum from GNPS database Black: Crude extract of *Paenibacillus sp*. s102b.



Supplementary Figure S6. MS/MS mirror plot of crude extract of *Paenibacillus sp*. s102b against GNPS database showing the presence of surfactin [19]. Source: Green: Surfactin MS/MS spectrum from GNPS database, black: Crude extract of *Paenibacillus sp*. s102b.

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Summary Chapter I

This chapter provides an overview of the under-investigated microbial biodiversity of the Peruvian coastline with a special focus on predatory bacteria. The microbiome of different sites at the Peruvian coast was analyzed, showing clear differences among the sampling sites. In total 138 bacterial strains were retrieved and screened for production of antimicrobial compounds. The obtained results implicate that the microbiomes present at the various habitats (Isla Foca, Manglares and Bahia Paracas) at the Peruvian coastline are a promising source for heterotrophic bacterial strains, showing high potential for the biotechnological production of antibiotics. First insights into the chemical basis of the antibacterial activities observed indicated the biosynthetic production of the known compounds ariakemicin, kocurin, naphthyridinomycin, pumilacidins, resistomycin, and surfactin. However, most active compounds remained elusive until now.

Despite several highly interesting compounds that were identified in this project had been previously isolated by other authors, the biosynthetic basis of several of them was not elucidated. In the next chapters, we focused on elucidating the biosynthesis of the thiopeptide antibiotic kocurin and of the APPAs.

Chapter II

Identification and heterologous expression of the kocurin biosynthetic gene cluster

Luis Linares-Otoya, Virginia Linares-Otoya, Lizbeth Armas-Mantilla, Cyntia Blanco-Olano, Max Crüsemann, Mayar L. Ganoza-Yupanqui, Julio Campos-Florian, Gabriele M. König, Till F. Schäberle*

Linares-Otoya *et al.*, *Microbiology* 2017;163:1409–1414

By the characterization of the antimicrobial potential of the marine microbiome of the Peruvian Coastline, the biosynthesis of several promising antimicrobial compound was shown. Among them the potent antibiotic kocurin produced by *Kocuria rosea* s17. Despite its potential to become developed into an anti-gram-positive antibiotic for use in the clinic, the biosynthetic basis was still unknown. The chapter deals with the *in silico* identification of the kocurin biosynthetic gene cluster, its cloning and heterologous expression.

Own contributions: planning of the research project, identification of the biosynthetic gene cluster, cloning and heterologous expression, writing the manuscript.

MICROBIOLOGY

SHORT COMMUNICATION

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Identification and heterologous expression of the kocurin biosynthetic gene cluster

Luis Linares-Otoya,[1,2,3] Virginia Linares-Otoya,[3,4] Lizbeth Armas-Mantilla,[4] Cyntia Blanco-Olano,[4] Max Crüsemann,[2] Mayar L. Ganoza-Yupanqui,[4] Julio Campos-Florian,[4] Gabriele M. König[2,5] and Till F. Schäberle[1,2,5,*]

Abstract

The antibiotically bioactive thiopeptide compound kocurin was identified in extracts from a newly isolated *Kocuria rosea* strain. The axenic strain was retrieved from a soil sample of the intertidal area at the Paracas National Park, Peru. The genetic basis of this promising natural product with activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strains was revealed by comparative genome analysis of this new isolate and other reported thiopeptide producer strains. The functionality of the predicted gene locus was experimentally proven by heterologous expression in *Streptomyces coelicolor* M1146. Expression of the gene cluster under the control of a constitutive promoter enabled the transgenic strain to produce kocurin in selected media. The kocurin biosynthetic gene cluster comprises nine open reading frames and spans around 12 kbp of the genome.

The increasing antibiotic resistance of pathogenic bacteria has become a serious threat towards human health [1]. Hence, new treatment options, e.g. novel antibiotics, are urgently needed [2]. To refill the antibiotic development pipeline, bioprospecting projects have been revived, especially in the academic environment, all around the globe. Natural products have always been the most rewarding resource for novel compounds with intriguing bioactivities. Even though the low-hanging fruits seem to have been harvested, since previously known compounds have been re-isolated in activity-based approaches, nature still has an untapped reservoir of natural products harbouring interesting activities from a pharmaceutical point of view [3].

In the present report, the search for new antibiotically active compounds from marine bacteria, isolated from the Peruvian coastline, led to the isolation of a strain whose ethyl acetate crude extract showed strong antibiotic activity against Gram-positive test strains, i.e. *Arthrobacter cristallopoietes*, *Bacillus megaterium* and MRSA. 16S rDNA identification revealed the isolate to be 99 % identical to *Kocuria rosea* DSM 20447 (accession number: NR_044871.1). Analysing the fractionated crude extract by subsequent HPLC-MS/MS resulted in the identification of the known thiopeptide compound kocurin as the molecule responsible for the antibiotic activity (Fig. S1, Table S1, available in the online Supplementary Material).

Kocurin is regarded as a compound with promising properties, which could potentially lead to the development of a novel antibiotic [3]. The substance was first described as compound PM181104, and a patent for the treatment of *Clostridium difficile*-associated infections was claimed (WO2014102570 A1). In 2013 the group of Genilloud and Reyes published an alternative structure to the one shown in the patent and named the compound kocurin. They investigated marine actinomycetes of the family *Micrococcaceae*, whereby the strains had been isolated from sponges. Structure elucidation was achieved using a combination of spectroscopic and chemical methods, which included extensive NMR analysis, HRMS and MS/MS fragmentation, as well as chemical degradation and Marfey's analysis of the resulting amino acid residues [4]. Thiazolyl peptide antibiotics are a class of natural products showing *in vitro* activity against Gram-positive bacteria. Meanwhile, about 100 molecules of this type were reported [5]. Based on the promising *in vitro* activity of

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Author affiliations: [1]Institute for Insect Biotechnology, Justus Liebig University of Giessen, Giessen, Germany; [2]Institute for Pharmaceutical Biology, University of Bonn, Bonn, Germany; [3]Research Centre for Sustainable Development Uku Pacha, Peru; [4]Department of Pharmacology, Faculty of Pharmacy and Biochemistry, National University of Trujillo, Trujillo, Peru; [5]German Centre for Infection Research (DZIF) Partner Site Bonn, Cologne, Germany.

***Correspondence:** Till F. Schäberle, till.f.schaeberle@agrar.uni-giessen.de

Keywords: antibiotic; thiopeptide; heterologous expression; *Kocuria*; *Streptomyces*.

Abbreviations: BGC, biosynthetic gene cluster; bp, base pairs; mbp, mega base pairs; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase; RiPP, ribosomally biosynthesized and posttranslational modified peptide.

Accession number of new sequence data: MF620092 and MF620093.

Three supplementary tables and one supplementary figure are available with the online Supplementary Material.

kocurin, *in vivo* tests were performed. Using a BALB/c mouse septicaemia model, kocurin showed promising activity. It displayed 100 % effective dose (ED_{100}) values of 2.5 and 5.0 mg kg^{-1} body weight against MRSA and of 10.0 mg kg^{-1} against vancomycin-resistant enterococci [6].

In this study, it was projected to identify the genetic basis of kocurin biosynthesis, i.e. to identify the corresponding biosynthetic gene cluster (BGC). Genomic sequence data from several *Kocuria* strains were analysed using antiSMASH [7]. In general, members of the family *Micrococcaceae* possess a small genome (2.7–4 Mbp) compared with other families of actinobacteria such as *Streptomycetaceae* [8]. The overall number of BGCs observed after analysis of the eight available genomes of *Kocuria* species ranged between 2 and 9. Most of the predicted BGCs were type III polyketide synthases (PKSs), bacteriocins, siderophores and thiopeptides, with only a very small number of modular PKSs and non-ribosomal peptide synthetases (NRPSs).

Formerly it was speculated that the biosynthesis of kocurin is encoded by a NRPS/PKS system [3]. However, this was proven wrong following investigation of the biosynthesis of thiomuracins in *Nonomuraea* species [9], and a closer look at the predicted BGCs in our strain revealed that no hybrid NRPS/PKS BGC seems to be associated with kocurin production. Instead, a predicted ribosomally biosynthesized and post-translational modified peptide (RiPP) gene cluster harboured a small gene that might encode the precursor peptide of kocurin. Hence, this BGC was attributed to kocurin production.

In silico analysis revealed two putative kocurin BGCs, in *Kocuria* UCD-OTCP (accession number: NZ_AOSQ01000025) and in *Kocuria flava* HO-9041 (accession number: NZ_CP013254), that could be related to thiopeptide biosynthesis. The biosynthesis of the thiopeptide antibiotics GE2270 (from *Planobispora rosea*) and GE37468 (from *Streptomyces* ATCC55365) was recently characterized [10, 11]. Both molecules show structural similarity, as well as a related mode of action to kocurin. Both groups reported the ribosomal origin of the investigated peptide. The RiPPs are initially synthesized from a larger propeptide, which is post-translationally modified by heterocyclization, serine dehydratation, amidation and macrocyclization to the final product. The resulting molecules normally have a mass in the range of 1100–1600 Da.

Screening the sequence data for a putative kocurin propeptide (amino acid sequence: STNCFCYPCCSCSAPSS) revealed suitable genes in both predicted BGCs. Based on this strong indication for the *in silico* identification of the kocurin BGC, the presence of a homologous BGC in our producer strain was attempted. The primer pair *Koc_scre_f* and *Koc_scre_r* was thus designed, which targets the propeptide gene region and a fragment of the adjacent dehydratase gene (Supplementary information). PCR using the genomic DNA of *K. rosea* s17 as template resulted in the amplification of a fragment with an expected size of 1.1 kbp. Sequencing revealed 100 % identity at the nucleotide level with *Kocuria* sp. UCD-OTCP and 91 % with *K. flava* HO-9041. As a next step, *K. rosea* s17 was genome sequenced using Illumina sequencing and assembled using Spades. Performing antiSMASH analysis on the assembled sequence data, a contig was identified harbouring the complete gene locus linked to kocurin production. The overall identity at DNA level to sequences available in databases is 99.6 % and the organization of the cluster is identical.

To prove the predicted link between the BGC and the metabolite experimentally, heterologous expression was envisaged. Therefore, the boundaries of the cluster were first defined. A closer examination of the predicted function of the proteins encoded by the open reading frames neighbouring the propeptide sequence enabled the identification of eight genes downstream in the same orientation that have a predicted role in kocurin biosynthesis (Fig. 1). This region spans 12.1 kbp and the genes were named *kocA-I* (from kocurin). Upstream of the propeptide encoding *kocA*, a gene coding for a HEAT repeat containing protein was identified in the opposite orientation, but its function was considered not to be involved in kocurin biosynthesis. Downstream of the ABC transporter gene *kocI*, a group of genes involved in general stress response was detected, forming the other border of the BGC (Fig. 1).

Two different *Streptomyces* strains were chosen for heterologous expression. One strain was the well-characterized *Streptomyces coelicolor* M1146 that is optimized for the expression of specialized metabolites due to the deletion of host-specific BGCs [12]. The thiopeptide GE2270 was expressed successfully in this strain [10]. The second strain was *Streptomyces* sp. s120, a strain that was isolated in the same bioprospecting project as *K. rosea* s17. This strain was chosen due to its fast growth rate and genetic tractability.

To clone the kocurin BGC to express it in a heterologous host, it was amplified by PCR in three parts using the reverse and forward primer pairs *koc1-3* (Table S2). The internal primers created a 35 bp homologous region between the DNA fragments generated by PCR and the external primers added 35 bp with homology to both flanks of the *Hin*dIII-linearized, integrative expression vector pSET152*ermE** [13]. The three PCR products were joined with the linearized vector using Gibson assembly (1 h at 50 °C). Subsequently, 5 µl of the reaction mixture were added to 200 µl CaCl-competent *E. coli* XL1blue cells. Transformation was achieved by 20 min on ice, 90 s heat shock at 42 °C, followed by addition of 1 ml Luria Bertani (LB) medium and 90 min incubation at 37 °C for regeneration. Subsequently, aliquots were plated onto apramycin-containing (50 µg ml^{-1}) LB agar plates. After overnight incubation few colonies were observed. These were tested by colony PCR for the presence of the BGC (Supporting information).

From positive clones, the plasmid pSET152-Koc was isolated (Pureyield Promega plasmid isolation Kit, following the manufacture's protocol) and restricted with *Sac*I. Eight out of ten

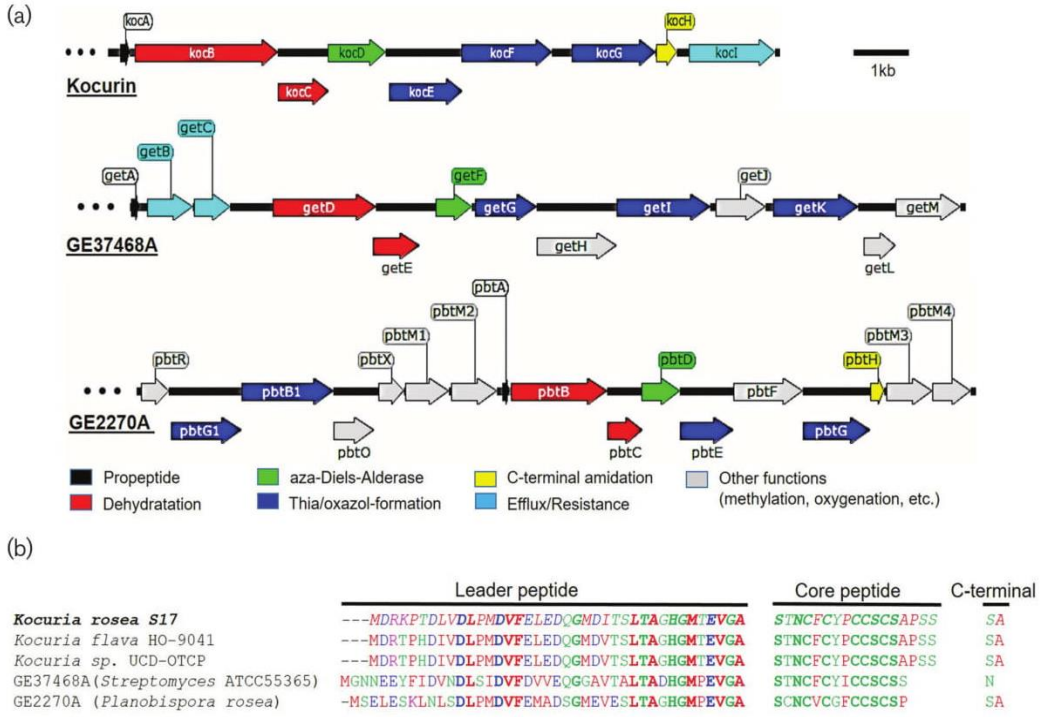

Fig. 1. Biosynthetic gene cluster (BGC) of kocurin and homologues. (a) Gene cluster organization of the related thiopeptide molecules kocurin GE2270 and GE37468. The colour coding indicates the predicted function of the encoded proteins. The names of the respective genes are indicated [10, 11]. Genes are drawn to scale; size bar is on the top line. (b) Amino acid alignment of selected thiopeptide propeptides. Three kocurin producers (*K. rosea* s17, *K. flava* HO9041 and *K.* UCD-OTCP), as well as the producers of GE37468 (*S.* ATCC 55365) and GE2270 (*P. rosea*), are shown. Black lines indicate the three parts of the propeptide chain; both the leader peptide and the C-terminal residues are cleaved off to form the mature peptide.

plasmids showed the expected restriction pattern and were sequenced. The validated plasmid pSET152-Koc was transferred into *E. coli* ET12567, a methylation-deficient strain [14]. Then, triparental conjugation was performed using additionally *E. coli* ET12567 cells carrying the pUB307 helper plasmid and the targeted *Streptomyces* host strain. In brief, the *E. coli* strains were cultured overnight in chloramphenicol (25 µg ml^{-1}) containing LB broth plus kanamycin (50 µg ml^{-1}) in the case of *E. coli* ET12567-pUB307, and apramycin (50 µg ml^{-1}) for *E. coli* ET12567-pSET152-Koc. A volume of 200 µl of this preculture was used to inoculate 20 ml LB broth containing the same antibiotics, and incubation was performed at 37 °C. When the cultures reached an OD_{600} of 0.5, cells were recovered by centrifugation (3500 g, 5 min) and washed twice with 20 ml LB medium. The resulting pellet was resuspended in 500–1000 µl LB medium. A volume of 100 µl of each was mixed together with 100 µl of a *Streptomyces* spore solution, which was previously activated (10 min, 50 °C in 2YT medium). The mixture was directly plated on SFM agar plates supplemented with 10 mM $MgCl_2$. After 16 h at 30 °C, the plates were covered with 1 ml H_2O containing 400 µg nalidixic acid and 1 mg apramycin. Conjugants were observed after 24 and 48 h for *Streptomyces* sp. S120 and *S. coelicolor* M1146, respectively. Colonies were picked and streaked onto new apramycin/nalidixic acid containing SFM agar plates. After 72 h single colonies were picked and tested by colony PCR for kocurin BGC integration. Subsequent sequencing verified the integration of the kocurin BGC for both strains.

Heterologous expression of thiopeptide gene clusters is most challenging. Hence, the projected expression of the thiopeptide TP1161 in *Streptomyces coelicolor* M512 remained unsuccessful [15], and the compound GE2270 was only expressed in 1 out of 35 different media tested [10]. Using the two transgenic strains generated here, five different production media were used, i.e. SM13 and medium C used previously for GE2270 expression in *Streptomyces* and *Planobispora* [10, 16], as well as AF, SFM and ISP2 medium [17]. A preculture was inoculated by using spores of the transgenic strains in ISP2 medium and incubated 48 h at 30 °C. Then, 2 ml were used to inoculate 100 ml of the various media; fermentation was performed for 4 d at 30 °C in 300 ml baffled flasks. Subsequently, the cultures were extracted using 2×300 ml of ethyl acetate for liquid–liquid separation. The organic layer was collected, combined and dried by evaporation. The resulting extract was dissolved in methanol (2 mg ml^{-1}). Samples were analysed by HPLC/MS-MS [gradient: 10 to 100 % acetonitrile over 20 min, EC10/2 Nucleoshell C18 2.7 µm column (Macherey-Nagel)].

An extract of the native producer *K. rosea* s17 served as positive control, and *S. coelicolor* M1146 carrying the empty pSET152 vector and the respective cell-free production medium as negative control. A peak matching the expected kocurin retention time (12.2–12.4 min) was only detected in *S. coelicolor* M1146-Koc grown in ISP2 medium. The observed mass was 1515.3745 $m\ z^{-1}$ [M+H$^+$] (calculated: 1515.3733 $m\ z^{-1}$). In AF medium, only traces were observed, while no kocurin production was observed for *Streptomyces* sp s120 in any of the culture media used (Fig. 2).

The observed production level is very low under the conditions used. This is in accordance with previous heterologous expression attempts for thiopeptides in *Streptomyces*, whereby the media used seem to have a strong influence on expression [10]. In future, further optimization will be necessary to improve the kocurin titre. It might be speculated that the toxicity of the compound can affect the growth and metabolism of the heterologous host at early stages of the life cycle, since visible biomass production started 3 days post-inoculation. Hence, using inducible promoters could be a feasible option to overcome this. Another strategy would be the overexpression of the resistance gene. It was shown before that this resulted in improved GE2270 production yields [10]. However, a reported self-resistance gene for an alternative copy of the elongation factor EF-Tu was not found in the entire genome. Therefore, it can be assumed that the ABC transporter KocI fulfils the immunity function, as suggested for GE37468 [11]. Furthermore, the EF-Tu of the original producer can be resistant towards the thiopeptide as is the case for the GE2270 producer *Planobispora rosea* [10]. This might explain why growth of the heterologous host used in this study was retarded, since no additional resistant version of an EF-Tu gene was cloned with the BGC. Another option is to use *Nonomuraea* species as the heterologous host, since GE2270 production levels could be increased up to 250 mg l^{-1} [18].

A putative kocurin biosynthesis is proposed based on comparative analysis with the BGCs of GE2270 and GE37468 and considering the recent work performed by the van der Donk and Mitchell groups [19] (Fig. 3 and Table S3). The propeptide-coding gene *kocA* is located at the upstream end of the BGC and codes for 58 amino acids, divided into a leader peptide (39 amino acids), a core peptide (17 amino acids) and a C-terminal Ser-Ala residue that is cleaved off during maturation. Also in GE2270 and GE37468, either terminal Ser-Ala or Asp is excised during maturation (Fig. 1). The genes coding for the post-translational modifying enzymes are situated downstream of *kocA*. First, the oxazole and thiazol(ine) rings are formed, catalysed by KocE-G (Fig. 3). These proteins resemble McbC-type dehydrogenases (KocE, G) and a YcaO-like cyclodehydratase (KocF) [20].

The serine residues present in the core peptide should be dehydrated through the action of KocB and KocC, both lantibiotic-type dehydratases. However, the exact timing of the reactions is still unknown. The C-terminus is shortened by

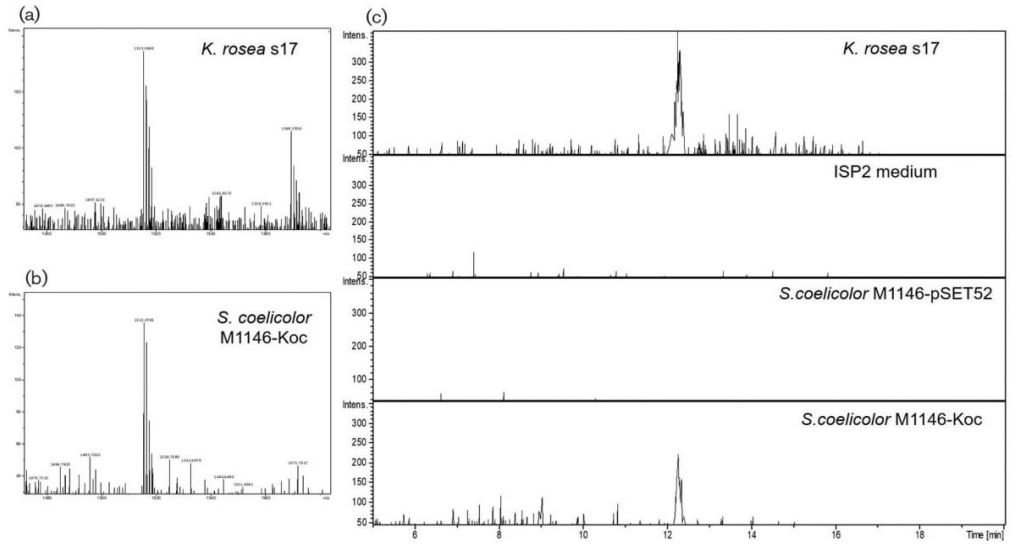

Fig. 2. HPLC/MS-MS analysis of crude extracts of selected *S. coelicolor* M1146-Koc fermentations and controls. MS spectrum of the kocurin peak observed in the native producer *K. rosea* s17 and (b) of *S. coelicolor* M1146-Koc. (c) Extracted-ion chromatogram ($m\ z^{-1}$: 1515.37±0.01) of *K. rosea* s17 extract [fermented in Marine broth (Difco) medium], ISP2 medium and *S. coelicolor* M1146-pSET152 (both negative controls), and the heterologous producer strain *S. coelicolor* M1146-Koc (fermented in ISP2 medium). The peak corresponding to kocurin at 12.2 min is highlighted; the scale is the same in all chromatograms.

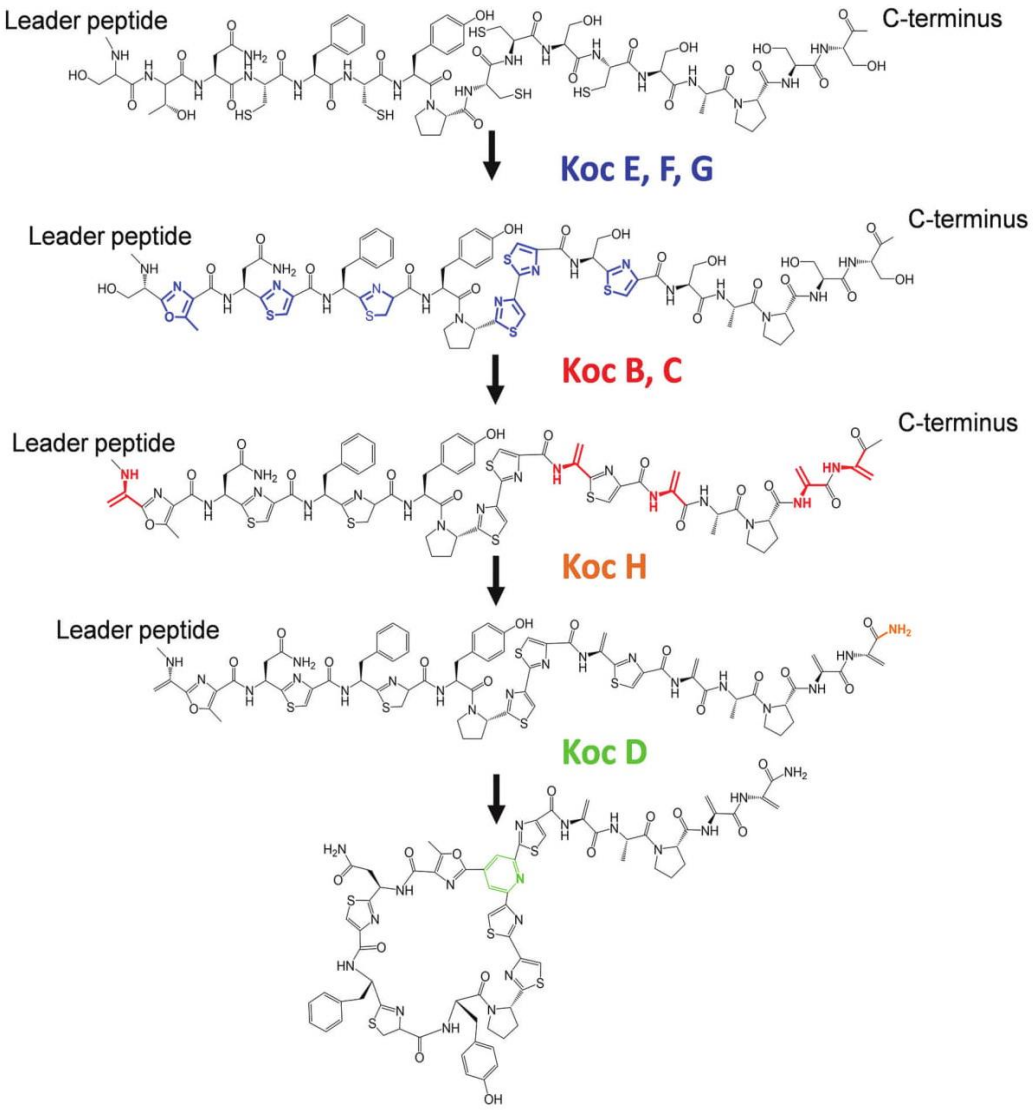

Fig. 3. Proposed pathway for kocurin biosynthesis in *Kocuria rosea* s17.

the excision of the last two amino acid residues and amidated by the action of KocH. For the thiopeptides berninamycin and micrococcin C, it was shown that this step is essential for the final cyclization reaction [21, 22]. Finally, the last step is the +1 to +11 cyclization, yielding a 29-membered macrocycle, by forming the central pyridine ring through an aza–Diels–Alder reaction catalysed by KocD. In *koc*-BGC, *kocI* is additionally found at the downstream end encoding for an ABC transporter.

In conclusion, the results presented here provide the experimental proof for kocurin BGC and enable future studies on this antibiotic molecule. On the one hand, a heterologous expression system using well-investigated actinobacterial strains is the method of choice for up-scaling experiments. Hence, in addition to biosynthetic derivatization it might become feasible to produce sufficient quantities in a sustainable approach to perform chemical modifications on the mother molecule. On the other hand, the generation of derivatives, e.g. molecules with exchanged amino acids, is facilitated by this system.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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Chapter II Supplementary

Supplementary information

Identification and heterologous expression of the kocurin biosynthetic gene cluster

Luis Linares-Otoya,[1,2,5],Virginia Linares-Otoya,[3,5] Lizbeth Armas-Mantilla,[3] Cyntia Blanco-Olano,[3] Max Crüsemann,[2] Mayar L. Ganoza-Yupanqui,[3] Julio Campos-Florian,[3] Gabriele M. König,[2,4] Till F. Schäberle[1,2,4,*]

[1]Institute for Insect Biotechnology, Justus Liebig University of Giessen, Giessen, Germany

[2]Institute for Pharmaceutical Biology, University of Bonn, Bonn, Germany

[3]Department of Pharmacology, Faculty of Pharmacy and Biochemistry, National University of Trujillo, Trujillo, Peru

[4]German Centre for Infection Research (DZIF) Partner Site Bonn/Cologne

[5]Research Centre for Sustainable Development Uku Pacha, Peru

*Correspondence: Till F. Schäberle, till.f.schaeberle@agrar.uni-giessen.de

Keywords: antibiotic, thiopeptide, heterologous expression, *Kocuria*, *Streptomyces*

Abbreviations: BGC, biosynthetic gene cluster; bp, base pairs; mbp, mega base pairs; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase; RiPP, ribosomally biosynthesized and posttranslational modified peptide

Table of Content

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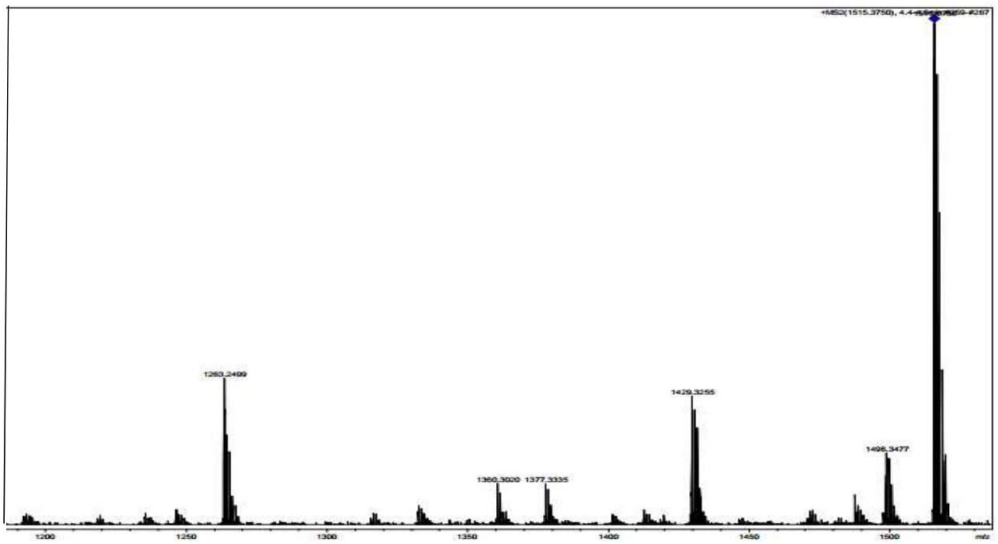
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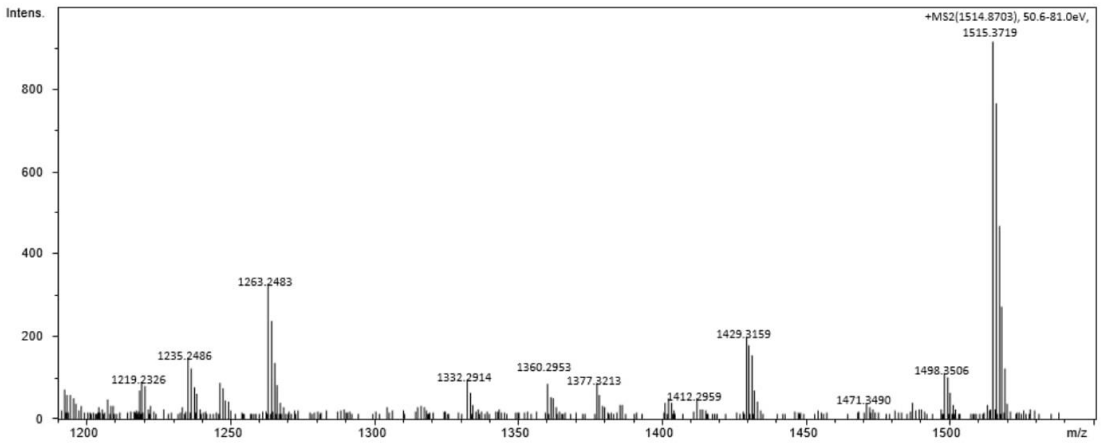
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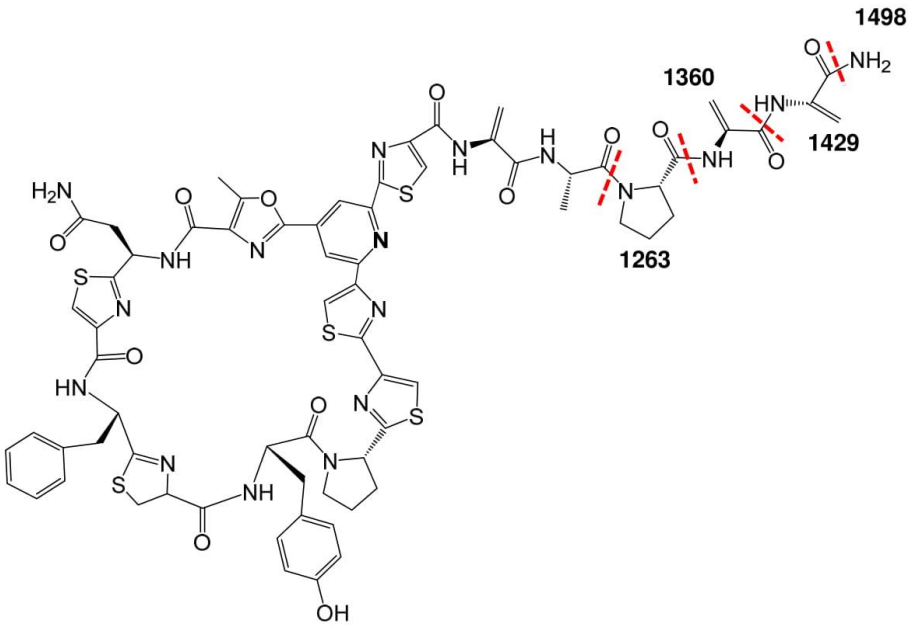

Figure S1. MS/MS data of Kocurin. A: Kocurin from *Kocuria palustris* (Martin *et al.*, 2013) B: Kocurin from *Kocuria rosea* s17 (present work). The region between 1190 and 1550 *m/z* is depicted. C: Structure of kocurin indicating the fragments observed in A and B.

Table S1. MS/MS data comparison between the reported Kocurin from *Kocuria palustris* (Martin *et al.*, 2013) and from *Kocuria rosea* s17 (present work)

Kocuria palustris[a] (Martin *et al.*, 2013)	*Kocuria rosea* s17[a] (present work)	Calculated values[a]
1498.3477	1498.3506	1498.35
1429.3255	1429.3159	1429.33
1360.3020	1360.2953	1360.30
1263.4990	1263.2483	1263.25

[a]The values represent m/z

Table S2. Primer list

Name	Amplified fragment	Sequence
Koc_scre_f	*kocAB*(partial)	gcg ctc ggg mag cag acg cag c
Koc_scre_r	~1.1kb	gcc cgc cac gac agg aga rat ca
Koc_test1_f	*kocI*(partial)	cag gcc tac tac tac gtc gcc c
Koc_test1_r	~0.8kb	ggg tcy tcc acr agg tag ac
Koc_test2_f	*kocG*(partial)	ctg ccc cac ccc ttc ccy tga
Koc_test2_r	~0.8kb	gac atg atg ggy tgs acc cag g
koc1_f	*kocA-kocC*	atc gtg ccg gtt ggt agg atc gac ggt atc gat agg agg tca gcc atg gac cgt aag ccc act ga
koc1_r	~3.9kb	ccc acc acg ggt ccg tgg gcc atg tca tga gct gat acc tgc cc
koc2_f	*kocD-kocG*	ggg cag gta tca gct cat gac atg gcc cac gga ccc gtg gtg gg
koc2_r	~4.5kb	gct acg acg agc gcg gct ccg ggg cgc aat agg act gtg att tc
koc3_f	*kocH-kocI*	gaa atc aca gtc cta ttg cgc ccc gga gcc gcg ctc gtc gta gc
koc3_r	~3.8kb	tag tgg atc ccc cgg gct gca gga att cga tat cat cag acc agc aat tga tgg g

Table S3. Amino acid sequence comparison and predicted gene function between the reported Kocurin BGC (*Kocuria rosea* s17), GE2270 (*Planobispora rosea*) and GE37468 *(Streptomyces ATCC55365)*

Kocuria rosea s17	*Planobispora rosea*[a]	*Streptomyces ATCC55365*[a]	Predicted gene function
KocA	PbtA (66%)	GetA(68%)	Propeptide
KocB	PbtB (29%)	GetD(28%)	Lantibiotic dehydratase
KocC	PbtC (32%)	GetE(32%)	Lantibiotic dehydratase
KocD	PbtD (27%)	-	Aza Diels alderase
KocE	PbtE (42%)	GetG (40%)	Mcb-like dehydrogenase
KocF	-	-	YcaO like protein
KocG	PbtG/G1(46%)	GetI(47%)	Mcb-like dehydrogenase
KocH	PbtH(37%)		Putative peptidase
KocI	-	-	ABC transporter

[a]The identity of the amino acids between *K. rosea* s17 and the other strain is given in brackets

Colony PCR to validate the presence of the BGC

Single *Streptomyces* colonies were picked using a plastic tip and suspended in 20 µL of sterile H_2O. Then the tubes were heated in the microwave for 5 minutes. Subsequently, the PCR reaction with a final volume of 10 µl was set up: 2 µL of the cell suspension, 5 pmol of each primer (*Koc_test1_f/ Koc_test1_r* and *Koc_test2_f/Koc_test2_r*), 0.5 U GoTaq DNA polymerase (Promega), PCR buffer, 1µL $MgCl_2$ (equivalent to a final concentration of 5 mM), 4% DMSO, deoxynucleoside triphosphates. PCR program: Initial denaturation step of 10 min at 98°C, followed by 30 cycles denaturation 35 s at 98°C, annealing 30 s at 60°C, and extension 50 s at 72°C. The expected fragment sizes using these primer pairs were 0.8 kbp.

Summary Chapter II

Following the identification by LC/MS-based analysis of kocurin, an antibiotically bioactive thiopeptide in Chapter I, the genetic basis of this promising natural product with activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strains was investigated.

By comparative genome analysis of a newly isolated and sequenced *Kocuria rosea* s17 strain and other reported thiopeptide producer strains, the putative biosynthetic gene cluster was identified. The kocurin biosynthetic gene cluster comprises nine open reading frames and spans around 12 kbp of the genome.

To prove the functionality of the predicted biosynthetic gene cluster, a heterologous expression *in Streptomyces coelicolor* M1146 was carried out. Expression of the biosynthetic gene cluster under the control of a constitutive promoter enabled the transgenic strain to produce kocurin in selected media.

Chapter III
Biosynthetic basis of structural diversity of aminophenyl-derived alkaloids (APPA)

Linares-Otoya, L.; Liu Y.; Linares-Otoya, V.; Armas-Mantilla, L.; Crüsemann, M.; Ganoza-Yupanqui, M.L.; Campos-Florian, J.; König, G.M.; Schäberle, T.F.2019.

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Aminophenylpyrrole derived alkaloids represent high value lead compounds for the pharmaceutical and agricultural industry. Recently, highly diverse APPAs produced by strains belonging to the order *Cytophagales* were reported. Their structure differed from the pyrrolnitrin, whose production was described from proteobacteria, since no halogenation nor oxidation of the amino group was observed. Instead, the aminophenylpyrrole core seems to be fused to different acyl, aryl or aromatic side chains to form the highly bioactive marinoquinolines

In this chapter the APPAs 3- (2′-aminophenyl)-pyrrole and marinoquinolines were identified in the crude extract of the Cytophagales strains *Rapidithrix thailandica* S80 *and Rapidithrix thailandica* s68 whose isolation was described in chapter 1.

Despite their interesting bioactivities, including antibiotic, antimalarial and inhibition of acetylcholinesterase the biosynthetic basis of these metabolites was not known. A bioinformatics and phylogenetic approach enabled the elucidation of the biosynthesis of APPAs in *Cytophagales* bacteria and their chemical diversification strategy.

Own contributions: planning the project, LC-MS analysis, identification of BGC, heterologous expression of BGC and individual enzymes, *in vitro* assays, writing the manuscript.

Biosynthetic basis for structural diversity of aminophenylpyrrole-derived alkaloids

Luis Linares-Otoya[a,b], Yang Liu[a,b], Virginia Linares-Otoya[c,d], Lizbeth Armas-Mantilla[c,d], Max Crüsemann[e], Mayar L. Ganoza-Yupanqui[d], Julio Campos-Florian[d], Gabriele M. König[e], Till F. Schäberle*[a,b]

a) Institute for Insect Biotechnology, Justus-Liebig-University of Giessen, Giessen, Germany.

b) Department of Bioresources of the Fraunhofer Institute for Molecular Biology and Applied Ecology, Giessen, Germany.

c) Research Centre for Sustainable Development Uku Pacha, Trujillo, Peru.

d) Department of Pharmacology, Faculty of Pharmacy and Biochemistry, National University of Trujillo, Trujillo, Peru.

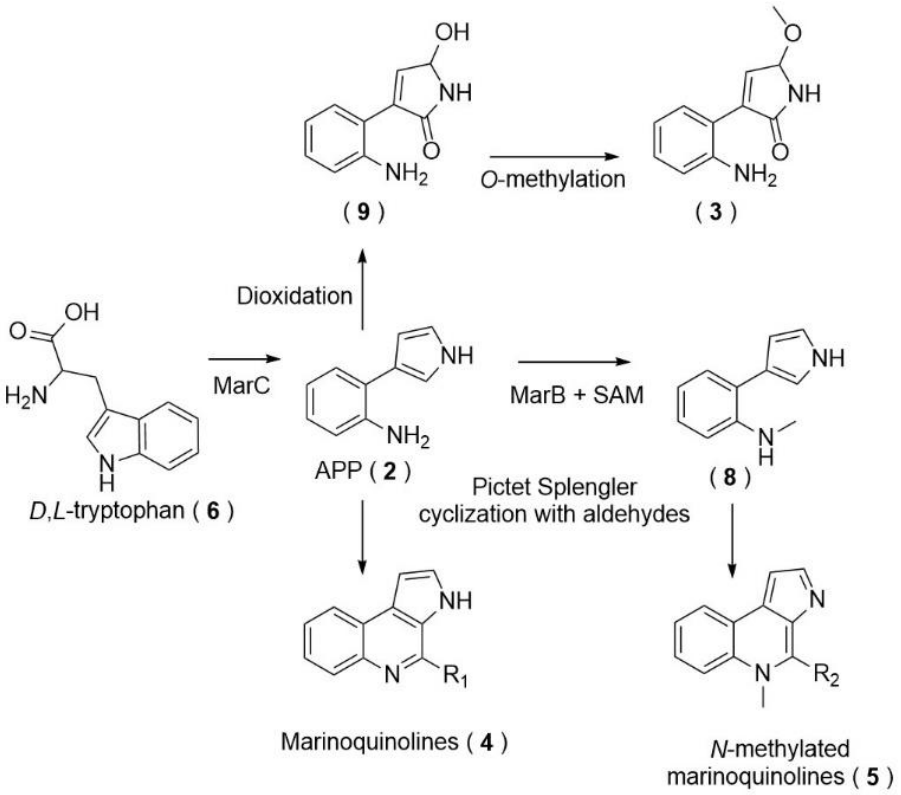
e) Institute for Pharmaceutical Biology, University of Bonn, Germany.

Abstract

Bacterial aminophenylpyrrole-derived alkaloids (APPAs) represent high value lead compounds. Pyrrolnitrin, which was developed into globally important fungicides, is the only reported APPA produced by *Proteobacteria.* Recently, various APPAs showing diverse bioactivities were discovered from *Bacteroidetes*. Here, a bioinformatics and phylogenetic approach enabled the elucidation of the biosynthesis of the highly diverse APPAs in *Cytophagales* bacteria and their chemical diversification strategy. The biosynthetic gene clusters were identified in producer strains and the biosynthesis was experimentally validated by heterologous expression experiments in *E. coli*. First, one enzyme-dependent biosynthetic step yields the tryptophan-derived precursor 3-(2'-aminophenyl)-pyrrole. Second, a spontaneous Pictet-Spengler-like coupling reaction enables the bacterial producer strains to create a library of tricyclic alkaloids, since several aldehydes can be applied as substrates. The diversity of this natural products class is further enlarged by the catalytic action of a methyltransferase, which adds one or more methyl groups to the aminophenyl intermediate.

The antifungal pyrrolnitrin (**1**) is an aminophenylpyrrole-derived alkaloid (APPA), initially isolated from *Pseudomonas pyrrocinia* and later from several other proteobacterial strains[1,2]. The natural product **1** was discovered in 1964 as a broad-spectrum antifungal compound and later synthetically modified, leading to the more photo-stable derivatives fenpiclonil and fludioxonil, which are widely used in agriculture as foliar fungicides[3]. The biosynthetic gene cluster and the function of the gene products corresponding to biosynthesis of **1** were reported[1,2]. In the last decade, more structural variants of APPAs with diverse bioactivities were discovered from bacteria belonging to the order of *Cytophagales* (phylum *Bacteroidetes*). For instance, 3-(2-Aminophenyl)pyrrole (**2**) and the antibacterial 3-(2-Aminophenyl)-5-methoxy-1,5-dihydro-*2H*-pyrrol-2-one (**3**) were isolated from *Rapidithrix thailandica* TISTR 1749 and TISTR 1741, respectively[4,5]. Additionally, structurally related compounds, such as marinoquinolines (A–K) (**4**) and marinoazepinones (A–B) were isolated from the marine bacteria *Ohtaekwangia koreensis* PWU25 and *Mooreia alkaloidigena* CNX-216[6,7] (Figure 1 and S1).

For the *Cytophagales*-derived APPAs no halogenation, nor oxidation of the amino group, as it is the case for **1**, was observed. Instead, the aminophenylpyrrole (**2**) seems to be fused to several acyl, indole or aromatic side chains, yielding the 3H-pyrrolo [2,3-c]quinoline (3-HPQ) ring system of the highly diverse marinoquinolines (**4**). In addition, *N*-methylation of the quinolone ring further expands the diversity of these alkaloids (**5**)[6,7]. In the present work, we report a bioinformatics and phylogenetic approach, which enabled the elucidation of the genetic basis as well as the non-enzymatic processes involved in the biosynthesis of the highly diverse APPAs. Furthermore, the discovery of a new aminophenylpyrrolidone suggests a degradation route of these aminophenylpyrroles.



Scheme 1. Proposed biosynthetic and degradation route of APPAs in *Cytophagales* bacteria. Possible derivatives (R_1 and R_2) of compounds **4** and **5** are described in Figure S1.

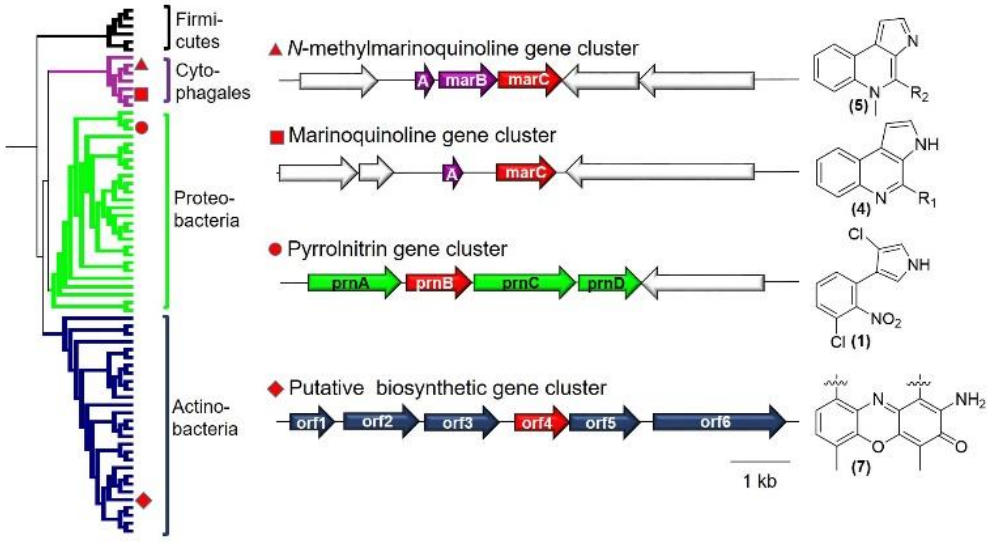


Figure 1. Phylogeny and genomic context of *prn*B-like genes in bacteria. The symbols indicate from which branch of the tree the gene cluster is derived. *prnB* homologs are colored in red. The arrows indicate the genes corresponding to their size. Genes colored in white are not conserved within the clade. The strains that constitute this tree are detailed in Figure S5. Known derivatives for R_1 and R_2 of compounds **4** and **5** are shown in Figure S1. Phylogenetic tree branches: Black: *Firmicutes*, Purple: *Cytophagales,* Green: *Proteobacteria,* Blue: *Actinobacteria*.

In a previous bioprospecting study, several predatory bacterial strains (n=10) belonging to the order *Cytophagales* were isolated and identified [8]. This gave us the opportunity to investigate the biosynthesis of the APPAs (*i.e*., marinoquinolines), which were reported uniquely from this bacterial taxon. First, extraction of the fermentation broths and analysis by LC-HRMS of the isolated *Cytophagales* strains revealed two *Rapidithrix* strains as potential APPA producers: *Rapidithrix thailandica* s80 and *Rapidithrix thailandica* s68 (accession numbers: MF796623.1 and MF796622.1, respectively). Subsequently, a large-scale cultivation (20 L) of *R. thailandica* s80, led to the isolation of **2** (320 mg) and **3** (0.9 mg) (Figure S2 and S3). In addition, metabolomic analysis using mass spectral Global Natural Products Social Molecular Networking (GNPS)[9] revealed the presence of the marinoquinolines C, E, F and H (Figure S4 and Table S1). These compounds, which share the 3-HPQ ring structure, were identified based on the MS/MS fragmentation pattern that showed the corresponding signature ions *m/z* of 167 and 185.

In order to elucidate the biosynthesis of the identified APPAs, we sequenced the *R. thailandica* s80 genome. We considered compound **2**, previously reported as a dead-end product in pyrrolnitrin biosynthesis[10], as a common APPA precursor candidate. In biosynthesis of **1**, a chlorinated version of **2** (monodechloropyrrolnitrin) is formed via oxidative conversion of chlorotryptophan, catalyzed by the enzyme PrnB (ABW69175.1)[11], which belongs to the family of indolamine 2,3 dioxygenases (IDO)[1,2]. Enzymes of this family catalyze O_2-dependent oxidation of *L*-tryptophan (**6**) to *N*-formylkynurenine or, in the case of PrnB, aminophenylpyrrole. The *in silico* search for PrnB homologues allowed the identification of a predicted protein with 40% identity in the *R. thailandica* genome. To form the tricyclic 3-HPQ structure of **4**, a Pictet-Spengler (PS)-like coupling reaction should follow[12,13]. In the same way, the formation of the pyrrolidone moiety in **3** would involve a dioxidation reaction. However, no genes corresponding to these expected functionalities were found adjacent to the *prnB* homologue. Indeed, no clear operon structure as usually expected for a natural product biosynthetic gene cluster (BGC) was observed. The next open reading frame (*orf*), *marA*, following upstream after a 500 bp gap, was annotated as a hypothetical protein with low similarity to a Per-Arnt-Sim (PAS) domain containing protein.

In a next step, additional accessible bacterial genomes possessing a *prnB*-like gene were analyzed to find evidence of operon conservation. Interestingly, it was observed that

the genomic context differs between the phyla. In *Proteobacteria*, the *prnB* homologue is part of the pyrrolnitrin BGC, while in Streptomycetes it was found clustering with oxidoreductases, homologous to the ones involved in actinomycin biosynthesis. These enzymes could be involved in the phenoxazine ring formation of several compounds (**7**)[14]. In *Cytophagales*, two types of BGCs were identified. Thereby, the *prnB* homologue *marC* either clusters with one gene, *marA*, separated by the gap as described before, or with two genes, whereby in the second case a predicted SAM-dependent methyltransferase (MT), *marB*, is present (Figure 1). The latter set-up is represented by strains of the *Catalinimonadaceae* clade.

To verify that the putative BGC identified in *R. thailandica* s80 is encoding for the enzymes necessary for marinoquinoline biosynthesis, we expressed the mar_{Rt} gene cluster in *E. coli* (homologous genes have the same name throughout the manuscript, and we refer to the original strain by the index used). Indeed, the same marinoquinoline derivatives (**4**, marinoquinoline H: R_1=4-hydroxy-benzol (**4a**), marinoquinoline E: R_1= indole (**4b**), Figure 2A) identified in *R. thailandica* s80 were identified in the fermentation broth of the heterologous *E. coli* mar_{Rt} host. However, heterologous expression of either $marA_{Rt}$ or $marC_{Rt}$ alone clearly indicated that the protein $MarC_{Rt}$ is sufficient for the formation of all the mentioned compounds (Figure 2A). Furthermore, the *in vivo* experiments prove that $MarC_{Rt}$ is catalyzing the conversion of the substrate tryptophan to compound **2** (Figure 2). Hence, it might be that MarC exhibits a different substrate specificity than PrnB. In pyrrolnitrin biosynthesis, PrnB uses preferentially 7-chlorotryptophan, yielding the compound monodechloropyrrolnitrin, while only traces of **2** were found[15]. In fermentations of *R. thailandica* s80 instead, **2** is the major compound produced. This shift in specificities among IDO enzymes was also reported for the biosynthesis of the mating pheromone gamone 2 in the ciliate *Blepharisma stoltei*[16].

Our experiments further revealed that compound **2** represents the intermediate towards the 3-HPQ ring of marinoquinolines, which are formed via non-enzymatic PS reactions. Hence, aldehydes can be attached, thereby resembling the chemical synthesis approach towards marinoquinolines[17]. The non-enzymatic conversion of **2** to **4** was supported by an *in vitro* assay, in which no PS activity could be attributed neither to the activity of MarA or MarC, since no influence of these enzymes on the reaction efficiency was detected (Figure S6). The results indicated a fast non-enzymatic, spontaneous reaction. Under physiological conditions (30 °C in aqueous solution), **4** was detected as soon

as **2** was faced to a suitable aldehyde (Figure 2B). Upscaling of the *in vitro* assay, using **2** (isolated before from the original producer *R. thailandica* s80) and 4-hydroxybenzaldehyde (4-HBA) under physiological conditions yielded marinoquinoline H (structure unambiguously confirmed by NMR measurements, Figure S7).

In a natural environment, various aldehydes, which can subsequently be linked with **2**, should be present in the cell from primary metabolism. Furthermore, by cultivating *E. coli mar*AC$_{Rt}$ in minimal medium supplemented with tryptophan, only marinoquinoline E (**4b**, R_1=Indole) was detected (Figure S8). In this derivative, the side chain is derived from tryptophan as well, thereby indicating that the abundance of a precursor directs the biosynthesis.

MarA$_{Rt}$ was obsolete in our *in vivo* and *in vitro* assays. As mentioned before, this gene is distantly related to genes coding for PAS domain containing proteins. The PAS domain is found embedded in several proteins involved in sensing external or intracellular stimuli[18], including visible light[19], redox potential[20], dissolved gasses[21] and others. Therefore, a regulatory role can be proposed for this protein in the *mar* BGC.

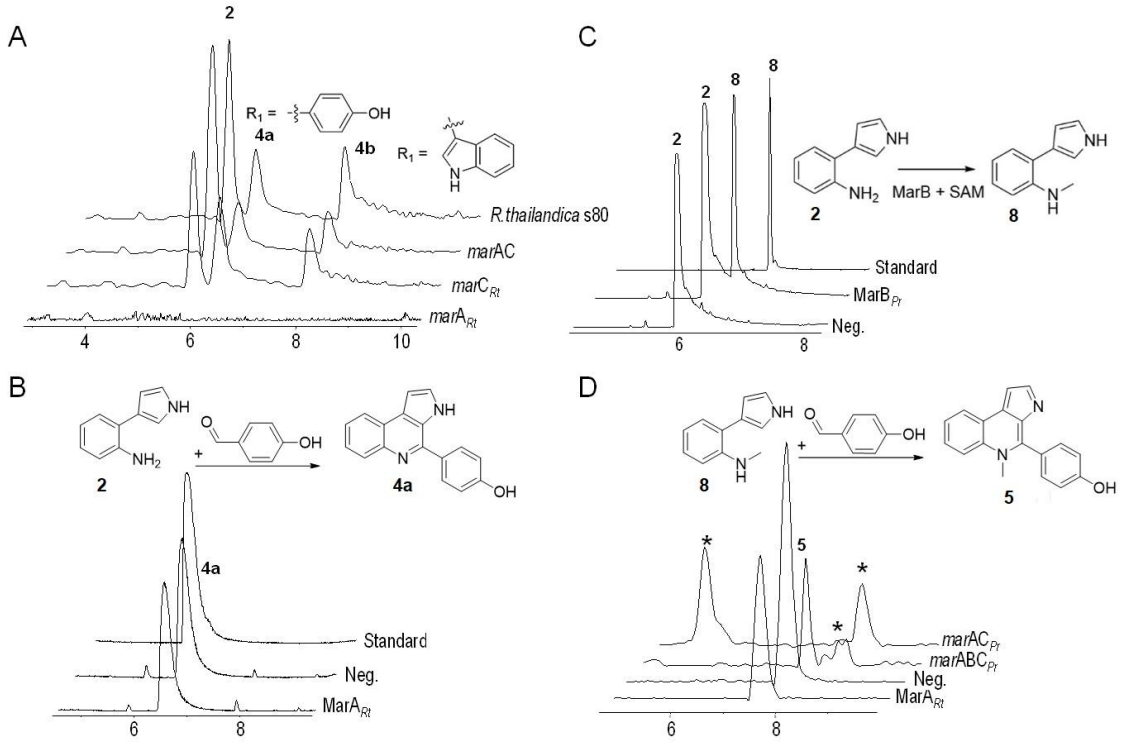


Figure 2. Heterologous expression of the marinoquinoline BGC, and *in vitro* characterization of enzymatic and non-enzymatic processes involved in marinoquinoline formation. **a)** Heterologous expression of the marinoquinoline BGC (*marAC$_{Rt}$*) and of individual genes (*marA$_{Rt}$, marC$_{Rt}$)* in *E. coli.* Extracted Ion Chromatogram (EIC) of **2** (*m/z*: 159.1 $[M+H]^+$) and marinoquinolines H (*m/z*: 261.1 $[M+H]^+$) and E (*m/z*: 284.1 $[M+H]^+$). **b)** Spontaneous formation of marinoquinoline H (EIC *m/z*: 261.1 $[M+H]^+$) from educts **2**

and 4-HBA in *in vitro* assays with or without $MarA_{Rt}$. **c)** *In vitro* methylation activity of $MarC_{Pr}$ (EIC of **2** (*m/z*: 159.1 $[M+H]^+$) and **8** (*m/z*: 173.1 $[M+H]^+$). **d)** Spontaneous formation of *N*-methylmarinoquinoline H (EIC 275.1 m/z $[M+H]^+$) from **8** and 4-HBA. *In vitro* (with or without $MarA_{Rt}$) and *in vivo* (*$marABC_{Pr}$ and $marAC_{Pr}$*) assays. Mar.: marinoquinoline; Neg.: negative control, without any enzyme in the *in vitro* assay. Asterisk (*): unknown compound with a similar *m/z* value as **5**, but not related to marinoquinolines (only present in the *in vivo* assays). Axes X: Time (min) Y: Intensity.

To get further insights into the different types of the APPA BGCs and into the variety of biosynthesized compounds, a *mar* BGC harboring the additional gene *marB*, a putative SAM-dependent MT, was investigated. By *in silico* analysis, we could identify the *marABC* BGC in a recently published *Catalinimonas alkaloidigena* genome. Therefore, we genome sequenced a strain of our collection, which was isolated from the same soil sample and belongs to the *Catalinimonadaceae* clade[22]. Indeed, the sequence data of strain *Porifericola rhodea* s47 (MF796606) from our collection[8] revealed a *mar* BGC, which includes a MT, thereby indicating the potential for biosynthesis of methylated marinoquinoline derivatives. The encoded MarB protein is closely related to the C20 type family of SAM-dependent MTs reported in the BGCs of the tryptophan-derived alkaloid reductasporin (*redM*, MIBiG ID. AKG47110.1)[23] and in several type II polyketides[24,25,26].

The *mar_{Pr}* BGC was subsequently cloned and heterologously expressed in *E. coli*. Fermentation of the heterologous host *E. coli mar*ABC_{Pr} resulted in the detection of novel masses compared with the empty host strain. MS/MS analysis attributed these masses to mono-, di- and trimethylated aminophenylpyrroles (Figure S9 and Table S2). The expected mono- and dimethylated pyrrolidone degradation products were as well identified. In addition, traces of putatively methylated marinoquinoline H were detected by LC-HRMS analysis as well (Figure 2D). In a next step, the heterologous *E. coli* strain *$marAC_{Pr}$* (without the MT gene *$marB_{Pr}$*) was generated. No methylated marinoquinolines were detected in fermentations of the latter (Figure 2D).

To verify the results, *$marB_{Pr}$* was expressed in *E. coli* and purified to perform *in vitro* assays. These assays should give an indication about the time point of methylation. In both *in vitro* as well as *in vivo* assays, neither methylated tryptophan nor methylated marinoquinolines were detected. Instead, the methylated intermediate was identified (**8**) (Figure 2C). To unambiguously identify the methylated compound, the reaction was up-scaled, leading to isolation of 0.2 mg of **8**. ^{1}H-NMR spectra, as well as COSY correlation, showed that the methylation occurred at the amino group of **2** (Figure S10 and

Table S3). Interestingly, an ion indicating double methylation, as it was observed in the *in vivo* assays, could be detected *in vitro*, however, in minor amounts. Double methylation was also observed in the biosynthesis of reductasporin, where the MarB orthologue RedM catalyzes the double *N*-methylation of a dimeric tryptophan structure[23].

From the obtained results it can be concluded that in a first step **6** is converted into **2** by the catalytic activity of MarC and subsequently MarB catalyzes the addition of a SAM-derived methyl group to the latter molecule, yielding **8** (Figure 2C). To verify that **8** represents the intermediate product towards *N*-methylated marinoquinolines, it was mixed in equimolar concentration with 4-HBA. Indeed, methylated marinoquinoline H could be detected by LC MS/MS analysis in accordance to the *in vivo* assays (Figure 2D). However, the compound indeed showed a clear peak in the ESI-MS analysis, indicating that it is ionizing well, but the produced amounts were very low and we could not isolate it preparatively.
From the data, it can be concluded that **2** and **8** are intermediates in the biosynthesis of **4** and **5**, respectively. The proposed mechanism of the 3HPQ formation including a PS-like reaction followed by a spontaneous oxidation is depicted in the Figure S11.

Finally, the pyrrolidone **3** was found in the native strains and in the heterologous hosts. Since there is no gene encoding for the expected dioxygenase within the BGC, it is likely that the conversion of **2** to **3** occurs either by unspecific enzymes, *e.g.* oxygenases, or enzyme-independent. Previous work observed analogs of **3** formed after exposition of pyrrolnitrin to intense UV light[27] or to rat liver oxidases[28]. Fermentation and extraction of both, *R. thailandica* and *E. coli* mar_{Rt}, was conducted by applying special care of exposition to light, which might explain the low concentration of **3**, due to reduced photo-oxidation. Alternatively, it was proposed that the formation of **3** from compound **2** proceeds by spontaneous or enzymatic oxidation during fermentation[29]. This might proceed via a [4+2] cycloaddition of the diene group of the pyrrole ring with oxygen, followed by rearrangement of a pyrrolinone intermediate and subsequent methylation of the hydroxyl group[29]. The LC-HRMS-based search for the putative intermediate pyrrolinone allowed the identification of a compound matching the expected mass. After purification, its structure was elucidated by NMR measurements confirming the identity of the unreported compound **9** as [3-(2-amino-phenyl)-5-hydroxy-1,5-dihydro-*2H*-

pyrrol-2-one] (Figure S12 and Table S4). This result supports the proposed degradation mechanism of **2** via oxygenation and methylation towards **3** (Scheme 1).

3-HPQ alkaloids are particularly rare in nature. In prokaryotes, to the best of our knowledge, these compounds have only been found in members of the order *Cytophagales*. In spite of producing similar APP-intermediates during the biosynthesis, pyrrolnitrin is the only APPA produced by *Proteobacteria*. Instead, *Cytophagales* bacteria direct the production of APPA towards chemical diversification. This difference can be explained by the fact that only the latter pathway favors the non-enzymatic reaction, which forms 3-HPQ and thereby diversifies this family of metabolites. In pyrrolnitrin biosynthesis the formation of APP is followed by oxidation of the amino group, preventing in this way the PS coupling reaction to occur[1,2]. In nature, there are several examples of bacteria making use of non-enzymatic reactions for the formation of structurally intriguing heterocycles that are essential for the bioactivity of the compounds; exemplified by the rubrolone pyridine ring assembly of the endophytic strain *Streptomyces* sp. KIB-H033[30], or the formation of discoipyrrole from 4-hydroxysattabacin, anthranilic acid and 4-HBA in *Bacillus hunanensis* SNA-048[31]. The same principle seems to be applied in marinoquinoline biosynthesis.

In the present work the antimicrobial activity of compounds **2**, **3**, **4a**, **8** and **9** were assessed against *E. coli* and *Bacillus megaterium*, whereby only for **4a** activity against the Gram-positive test strain was observed (Table S5).
In marinoquinoline biosynthesis the methylation of the amino group does not prevent 3-HPQ formation, instead it increases the molecular diversity; thereby allowing the formation of both, methylated and non-methylated marinoquinoline congeners. This can be observed for the marinoquinolines G and A, J and K, as well as for the marinoazepinones A and B, where both versions were isolated from the same organism *Mooreia alkaloidigena* CNX-216 in equal amounts[7]. It is highly remarkable that the relatively small 3-HPQ containing molecules show a very diverse range of bioactivities, *e.g.*, antibiotic, acetylcholinesterase inhibition, cytotoxic, as well as promising activities against chloroquine-resistant *Plasmodium falciparum* K1[10]. The antimalarial aplidiopsamine A that consists of a 3-HPQ core plus an adenine side chain was isolated from the marine tunicate *Aplidiopsis confluata*[10]. It is likely that this compound plays a role in the symbiotic relationship between bacterium and metazoan organism[10]. Further research on the regulation of the corresponding BGCs, *e.g.* by elucidating the role of Ma-

rA, would contribute to the understanding of the diversification strategy developed by this bacterial taxon. The synthesis of a reactive molecule, which can react with a wide range of aldehydes, can be regarded as a combinatorial synthesis strategy of the bacteria. Therefore, it is hard to name the final product of the biosynthesis, since the reactive compound **2** represents the major metabolite; but, as well as the degradation products, has no observed bioactivities. Furthermore, compound **2** might play a role in detoxification by capturing aldehydes. However, the very diverse range of bioactivities[6,7,10] of the relatively small 3-HPQ containing molecules supports the idea that they represent the final step of this pathway.

Methods

General Experimental Procedures. A complete description of methods, strains, cloning, protein expression, and enzymatic activity assays is given in the Supporting Information.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Associated Content

Supporting Information: Experimental details and supplementary figures (*e.g.*, marinoquinolines and marinoazepinones structures, LC-HRMS and NMR data of the compounds, extended phylogenetic tree, and assay results). This material is available free of charge via the internet at http://pubs.acs.org.

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Chapter III Supplementary

Biosynthetic basis for structural diversity of aminophenylpyrrole-derived alkaloids

Luis Linares-Otoya[a,b], Yang Liu[a,b], Virginia Linares-Otoya[c,d], Lizbeth Armas-Mantilla[c,d], Max Crüsemann[e], Mayar L. Ganoza-Yupanqui[d], Julio Campos-Florian[d], Gabriele M. König[e], Till F. Schäberle*[a,b]

Experimental Section

Small scale cultivation of *Cytophagales* strains

For screening, a small scale cultivation of 10 strains belonging to the genera *Echinicola* (1 strain), *Fulvivirga* (3), *Limibacter* (1), *Porifericola* (1), *Rapidithrix* (2) *and Reichenbachiella* (2) was carried out. A 20 µL cryoculture was inoculated in 50 mL Erlenmeyer flasks containing 10 mL Difco™ Marine Broth2216 (MB) and cultured for 72 h at 30 °C at 100 rpm. (Table S6). 200 µL of the preculture was used to inoculate 30 mL MB medium, incubated at 30 °C in an orbital shaker (100 rpm) for 96 h. The cells were disrupted by sonication followed by a liquid-liquid extraction using ethyl acetate (1:1). The organic phase was dried in a rotary evaporator and re-suspended in MeOH to a concentration of 1 mg mL^{-1} for the LC-HRMS analyses.

Large scale cultivation of *R. thailandica* s80 and *P. rhodea* s47

An agar piece containing a single colony was excised from the MB agar plate and used to inoculated 1 L flasks containing 300 mL of MB medium, followed by cultivation at 30 °C. After 48 h, 20 L of MB medium were inoculated using 10 mL of pre-culture per liter, incubation was done for 72 h at 30 °C on an orbital shaker (110 rpm).

Crude extract generation and LC-HRMS analysis

The bacterial cultures were extracted by liquid-liquid separation (1:1) using ethyl acetate. The organic phase was transferred to a spherical flask and completely dried by rotary evaporator. The extracts were weighed and resuspended in MeOH to a concentration of 1 mg mL^{-1} for LC-HRMS measurements. Mass spectra were recorded on a micrOTOF-Q mass spectrometer (Bruker) with ESI-source coupled with a HPLC Dionex Ultimate 3000 (Thermo Scientific) using an EC10/2 Nucleoshell C18 2.7 µm column (Macherey-Nagel). The column temperature was 25 °C. MS data were acquired over a range from 100 to 3000 *m*/*z* in positive mode. Auto MS/MS fragmentation was achieved with rising collision energy (35–50 keV over a gradient from 500 to 2000 *m*/*z*) with a frequency of 4 Hz for all of the ions over a threshold of 100. HPLC begins with 90 % H_2O containing 0.1 % acetic acid. The gradient starts after 1 min to 100% acetonitrile (0.1 % acetic acid) in 20 min. 5 µL of a 1 mg mL^{-1} sample solution was injected; and, flow rate was set to 0.3 mL min^{-1}

Isolation and characterization of compounds 2, 3 and 9 from *Rapidithrix thailandica* s80

The cells and the medium of *R. thailandica* were separated by centrifugation. The medium was extract with ethyl acetate (see above). The resulting ethyl acetate fraction (1.3 g) was separated by medium pressure LC (Puriflash) on C18 silica gel, using H_2O and MeOH in a gradient of increasing polarity from 5 % MeOH to 100 % MeOH, to generate eight fractions. Fraction 4 (~30 % MeOH and 70 % H_2O) containing compounds **3** and **9**, and fraction 6 (~50 % MeOH and 50 % H_2O) containing compound **2** were submitted to a Sephadex LH-20 column (eluent MeOH) for further purification based on the molecular weight. TLC plates with silica gel F_{254}

(Merck) were used to monitor resulting fractions; detection was by spraying the plates with vanillin-concentrated sulfuric acid reagent followed by heating at 110 °C. Final purifications were carried out by semi-preparative HPLC. Column chromatography included Sephadex LH-20 and Merck MN Silica gel 60 M (0.04–0.063 mm). The analysis column (EU250/4.6) and the semi-preparative column (VP250/10) were Nucleodur C18 Gravity SB 5 µm (MACHEREY-NAGEL), and the analysis gradient used was (MeOH, 0.1 % HCOOH in H_2O): 0 min (10 % MeOH); 5 min (10 % MeOH); 35 min (100 % MeOH); 45 min (100 % MeOH). ^{1}H, ^{13}C, and 2D NMR spectra were recorded at 25 °C in DMSO-d_6 and MeOH-d_4 on Bruker AVANCE DMX 400 and 600 NMR spectrometers. Chemical shifts were referenced to the solvent residual peaks, δ_H 2.50 (DMSO-d_6) and 3.31 (MeOH-d_4) ppm for ^{1}H, and δc 39.51 (DMSO-d_6) and 49.15 (MeOH-d_4) ppm for ^{13}C. Optical rotations were determined with a Perkin-Elmer-241 MC polarimeter. By above mentioned analysis program, compound **2** (320 mg) was eluted at 70 % MeOH and 30% H_2O, retention time 30 min; compound **3** (0.9 mg) eluted at 46 % MeOH and 54 % H_2O, retention time 22 min; compound **9** (1.3 mg) eluted at 58 % MeOH and 42% H_2O, retention time 26 min.

Antibacterial activity assays

The antimicrobial activities against *Bacillus megaterium and Escherichia coli* were tested using the agar disc-diffusion bioassay method. Therefore, the test strains were cultured overnight in LB medium, and then the cultures were diluted to an OD_{600} of 0.1 and spread onto LB agar plates. Compounds were resuspended in methanol (final concentration of 1 mg/mL), and 25 µl of this solution were transferred to a paper disc. Negative control discs (solvent only) and positive control discs (5 µg of chloramphenicol) were also placed on the test plates.

Strains and plasmids

The plasmids used in this study were constructed using PCR products obtained from genomic DNA as template and primer pairs are described in the Table S7**.** PCR products were assembled using Gibson assembly into pRSFduet or pET28a expression vectors for *in vivo* assays or protein purification, respectively. 3 µL of the reaction were used for transforming chemically competent *E. coli* Rosetta cells. For this purpose the competent cells were thawed on ice for 5 min and the Gibson assembly reaction buffer prepared by us was added, mixed and left on ice for 20 min. Then, a heat shock (42 °C) was applied to the mixture for 90 sec and 800 µL of Luria-Bertani medium (LB) (Table S6) were added to the mixture, followed by incubation for additional 60 min in a shaking heat block. Finally, the culture was centrifuged for 3 min at 4000 rpm and the supernatant was discarded, leaving 100 µL in the vial to resuspend the pellet. The solution was spread on 60 µg mL^{-1} kanamycin containing LB agar plates for clone selection.

Heterologous expression of the marinoquinoline BGC

For *in vivo* assays, the transgenic *E. coli* strains, carrying the respective Mar_{Rt} or Mar_{Pr} BGCs, and the deletion genotypes Δ$marA_{Rt}$, Δ$marC_{Rt}$ and Δ$marB_{Pr}$, were grown overnight in LB medium containing 60 µg mL^{-1} kanamycin. 0.3 mL of these cultures was used to inoculate 30 mL of LB or Minimal Medium (kanamycin added for selection) (Table S6). IPTG (final concentration 0.1 mM) was added to the medium to induce the protein expression when the OD_{600} reached 0.6 and the flasks were place on an orbitory shaker, 200 rpm at 30 °C for 12 h. At the moment of induction the medium was also supplemented with 10 mM *DL*-tryptophan. As negative controls, the *E. coli* Rosetta containing empty pET28a or pRSF-duet vectors, were used and as a positive control served the 3 days cultured *Rapidithrix thailandica* s80 strain (MB medium, 30 °C).

Heterologous expression and purification of MarA, MarB and MarC

The transgenic *E. coli* strains $marA_{Rt}$, $marC_{Rt}$ and $marB_{Pr}$, carrying pET28a with an N-terminal His tag, were grown overnight in LB medium containing 60 µg mL^{-1} kanamycin at 37 °C. 10 mL of these cultures was used to inoculate 1 L of LB containing 60 µg mL^{-1} kanamycin. IPTG (0.1 mM) was added to the medium to induce the protein expression when the OD_{600} reached

0.6 and the flasks were placed on an orbital shaker, 200 rpm at 30 °C for 12 h. Then, cells were collected by centrifugation at 4 °C and resuspended in ice cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole; pH 8.0). The resulting suspensions were sonicated and the supernatant, containing the soluble protein, was loaded onto pre-equilibrated 1 mL Qiagen® Ni-NTA columns. The protein purification was carried out at 4 °C. After washing to remove unspecifically bound protein (using 10 and 20 mM imidazole lysis buffer), the His-tagged proteins were eluted from the column using lysis buffer with increasing imidazole concentrations from 50 to 200 mM (Figure S13). The proteins were rebuffered into an imidazole-free buffer (50 mM NaH_2PO_4, 300 mM NaCl; pH 8.0) and concentrated using the Amicon® Ultra-15 centrifugation membrane columns.

Evaluation of marinoquinoline H formation and isolation

10 mM of compound **2** and of 4-hydroxy-benzaldehyde were mixed in the protein buffer in the presence or absence of 20 µL MarA or MarB in 200µL reaction volume. The reaction was incubated at 30 °C and stopped by freeze drying after 5, 15, 30, 60, 120 and 420 min each. The samples resuspended in 200 µL of 90 % MeOH, 0.05 % formic acid were measured by MALDI-TOF (Bruker Autoflex III MALDI-TOF/TOF) using α-Cyano-4-hydroxycinnamic acid (HCCA) matrix. The corresponding software (Autoflex III Smartbeam, Bruker / FlexAnalysis, Bruker) was used for data evaluation. To isolate marinoquinoline H, the same reaction was up-scaled 100-fold. Then, the samples were freeze dried and extracted with MeOH. The isolation of marinoquinoline H (16 mg) was achieved by HPLC with 61 % MeOH and 39 % H_2O, retention time 27 min.

MarB methylation assay and isolation of 8

15 mM *S*-Adenosyl methionine 97 % (Sigma) and 10 mM of compound **2** were mixed in imidazole-free protein buffer and the reaction was started by adding 100 µM MarB. Total volume was 200 µL and reaction was performed at 30 °C for 24 h. Reactions were freeze-dried and extracted with MeOH for LC-HRMS analysis. For the isolation of compound **8**, the reaction was up-scaled 20-fold. After freeze-drying the samples were extracted with MeOH, compound **8** was purified by HPLC. The analysis column (EU250/4.6) and the analysis gradient used before were used. The peak corresponding to **8** (0.2 mg) eluted at 75 % MeOH and 25 % H_2O, retention time 34.8 min.

Accession Codes

Rapidithrix thailandica s80 and *Rapidithrix thailandica* s68 (accession numbers: MF796623.1 and MF796622.1;

Porifericola rhodea s47 (MF796606)

Marinoquinoline BGC: MH566999
N-methylmarinoquinoline BGC: MH567000

Supplementary Figures

Supplementary Figure 1: Reported naturally produced marinoquinolines and marinoazepinones.

Supplementary Figure 2: LC-HRMS and NMR spectra of compound **2**.

Supplementary Figure 3: LC-HRMS and NMR spectra of compound **3**.

Supplementary Figure 4: LC/MSMS-based molecular mass networking of the *Rapidithrix thailandica* s80 and *E. coli* mar_{Rt} crude extracts showing the network of marinoquinolines derivatives.

Supplementary Figure 5: Phylogeny of *prnB*-like genes in bacteria.

Supplementary Figure 6: Spontaneous, non-enzymatic formation of Marinoquinoline H.

Supplementary Figure 7: LC-HRMS and NMR spectra of compound **4**.

Supplementary Figure 8: *In vivo* marinoquinoline formation by *E. coli* $marAC_{Rt}$ in LB and minimal medium.

Supplementary Figure 9: LC/MS chromatogram of the *in vivo* assay using the heterologous strain *E. coli* $marABC_{Pr}$.

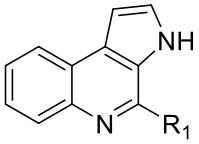
Supplementary Figure 10: LC-HRMS and NMR spectra of compound **8**.

Supplementary Figure 11: LC-HRMS and NMR spectra of compound **9**.

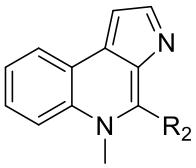
Supplementary Figure 12. Proposed mechanism of the 3HPQ formation including a Pictet-Spengler-like reaction, followed by spontaneous oxidation.

Supplementary Figure 13: SDS-PAGE gel showing the purification of His-tagged MarA, MarB and MarC from *E. coli* ROSETTA (washing and elution steps).

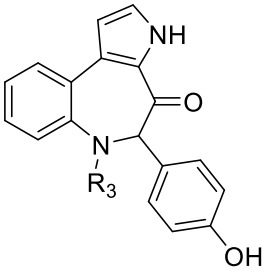
Supplementary Figure 1. Reported naturally produced marinoquinolines and marinoazepinones[1,2].



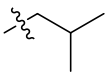
Marinoquinoline A $R_1 = CH_3$
Marinoquinoline K $R_1 = H$

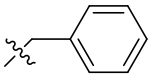


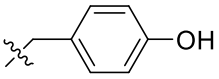
Marinoquinoline G $R_2 = CH_3$
Marinoquinoline J $R_2 = H$

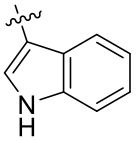


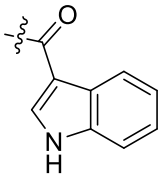
Marinoazepinone A $R_3 = CH_3$
Marinoazepinone B $R_3 = H$

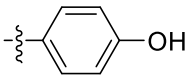
Marinoquinoline B $R_1 =$ 

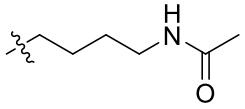
Marinoquinoline C $R_1 =$ 

Marinoquinoline D $R_1 =$ 

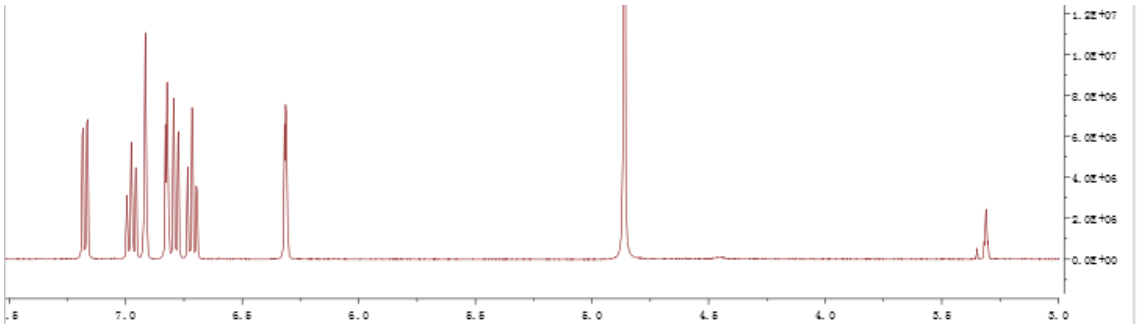
Marinoquinoline E $R_1 =$ 

Marinoquinoline F $R_1 =$ 

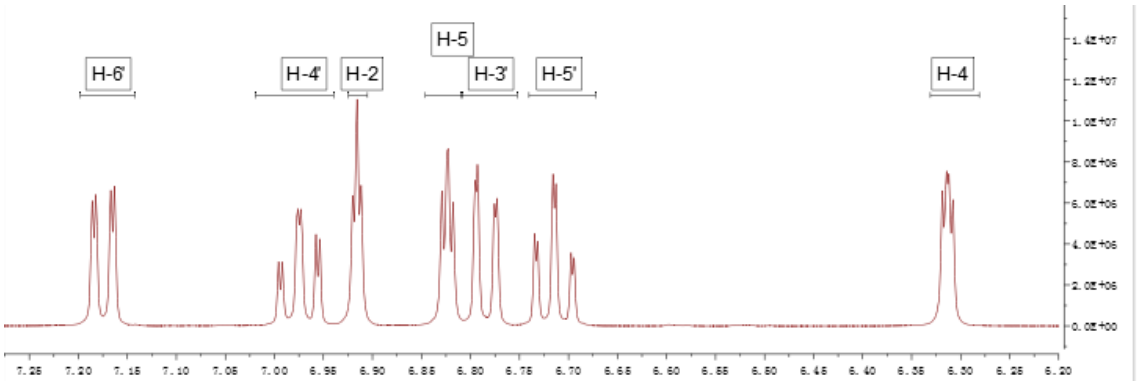
Marinoquinoline H $R_1 =$ 

Marinoquinoline I $R_1 =$ 

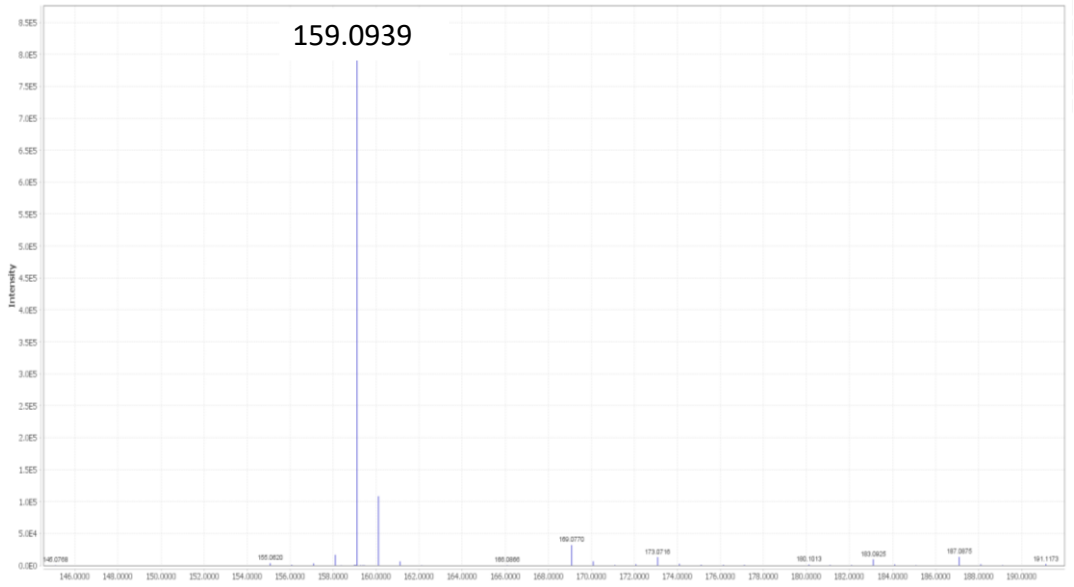
Supplementary Figure 2. LC-HRMS and NMR spectra of compound **2**.
2A. ^{1}H NMR (400 MHz, CH_3OH-d_4,) spectrum of the compound **2**



2B. Expansion ^{1}H NMR (400 MHz, MeOD-d_4,) spectrum of compound **2**



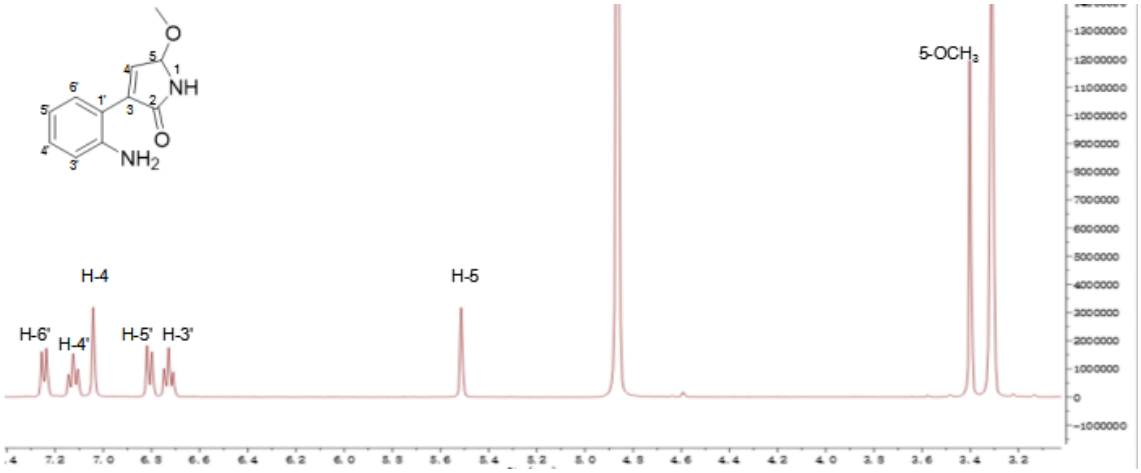
2C. LC-HRMS spectrum of compound **2**



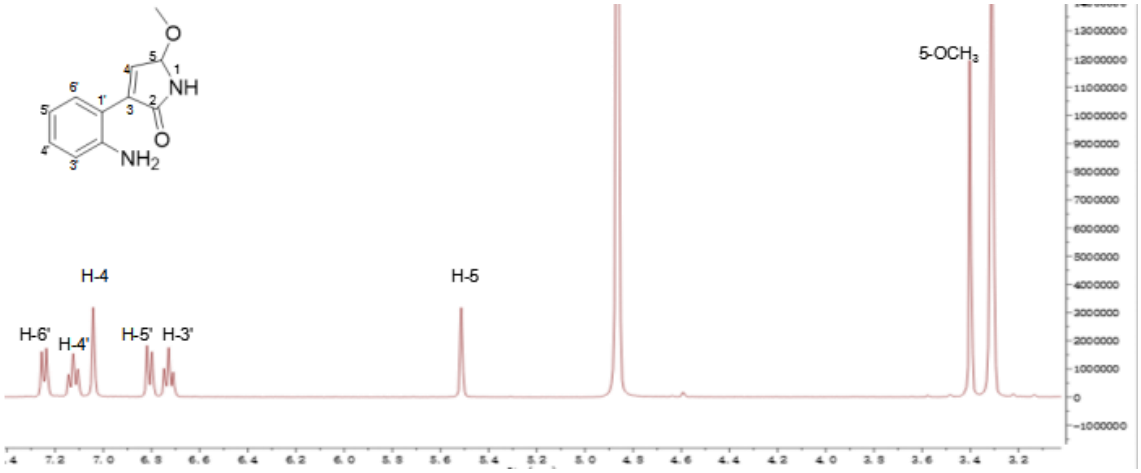
Expected *m/z* $[M+H]^+$ for $C_{10}H_{10}N_2$: 159.0922

Supplementary Figure 3. LC-HRMS and NMR spectra of compound **3**.

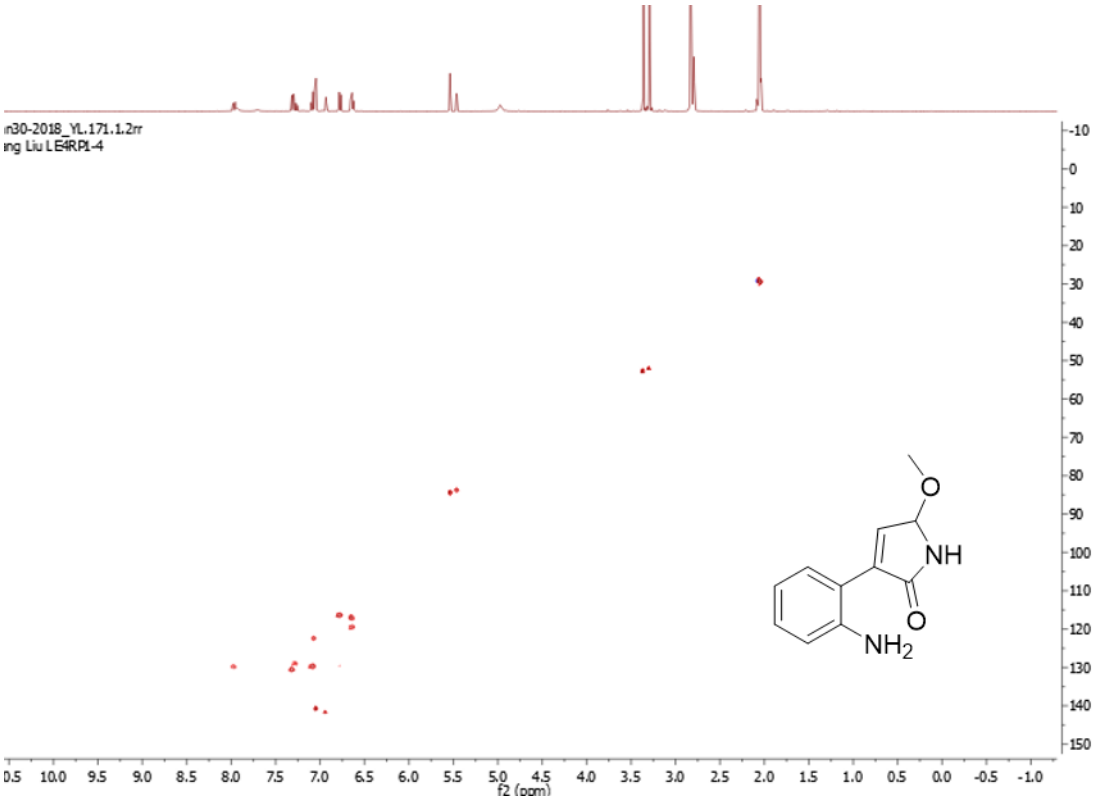
3A. ^{1}H NMR (400 MHz, CH_3OH-d_4,) spectrum of the compound **3**



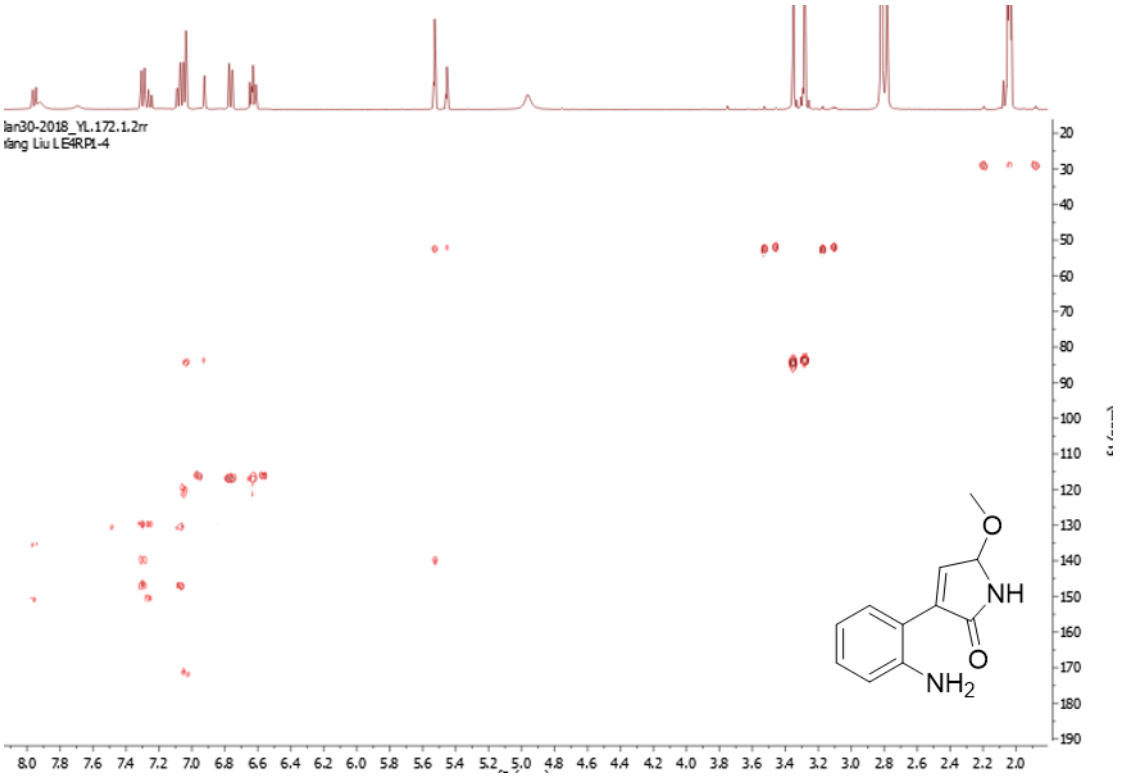
3B. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of the compound **3**



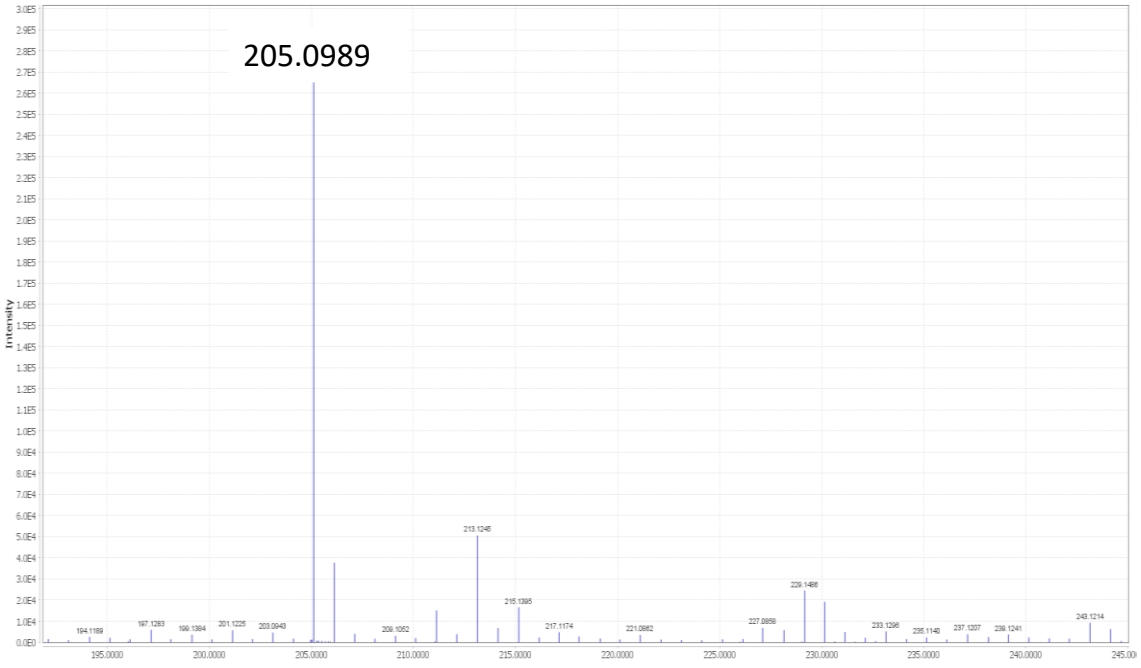
3C. HSQC spectrum of the compound **3**



3D. HMBC spectrum of the compound **3**

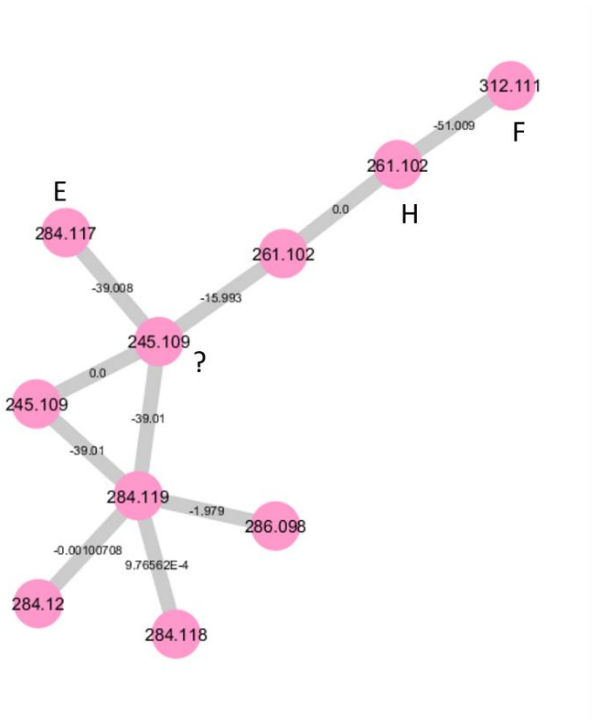


3E. LC-HRMS spectrum of compound **3**



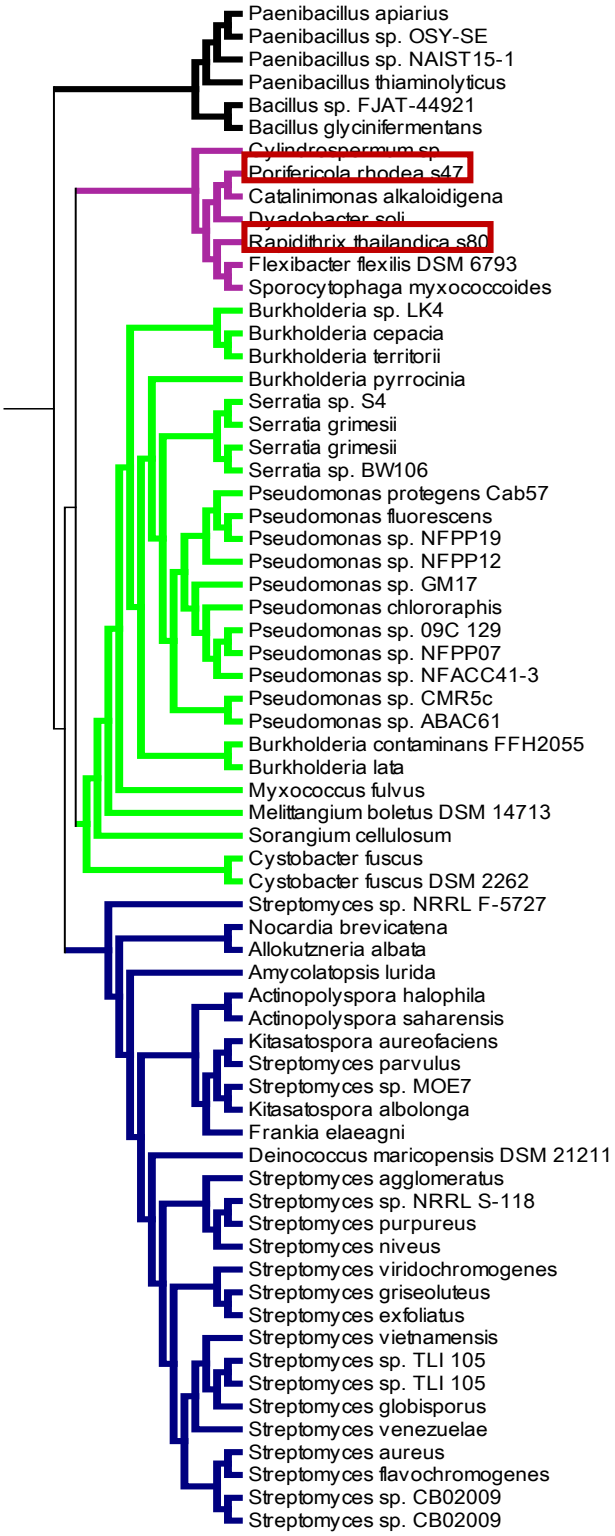
Expected *m/z* $[M+H]^+$ for $C_{11}H_{12}O_2N_2$: 205.0977

Supplementary figure 4. LC/MSMS-based molecular mass networking of the *Rapidithrix thailandica* s80 and *E. coli* mar_{Rt} crude extracts. The network of marinoquinolines derivatives is shown.



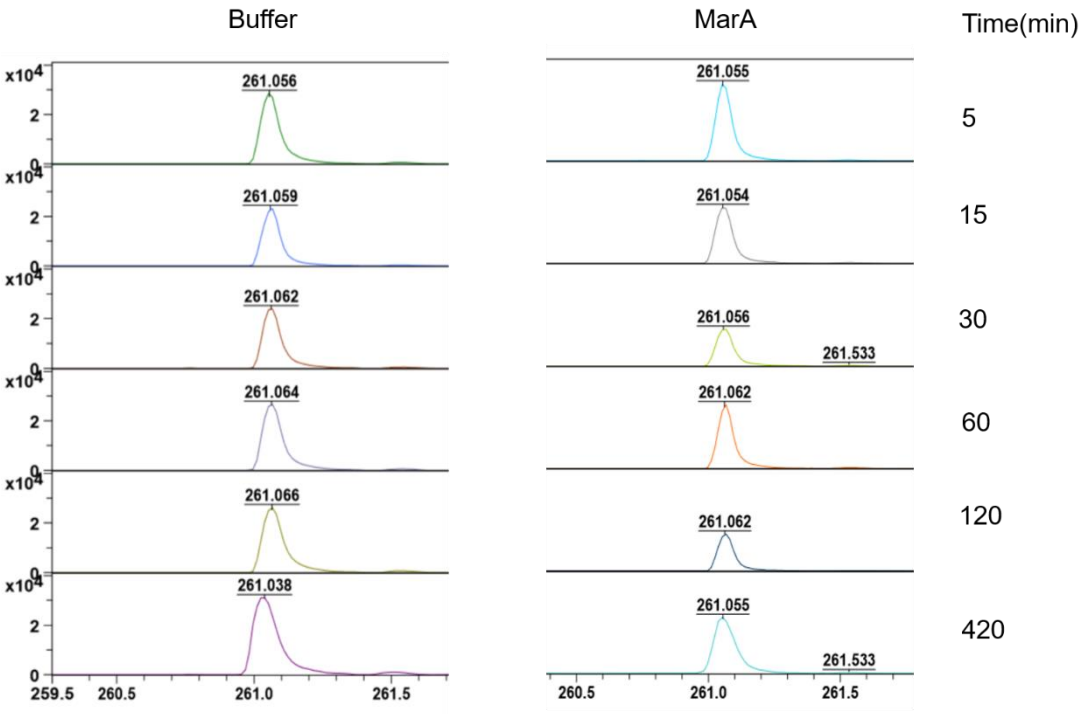
This part of the molecular mass network of the LC/MS analysis of *E. coli* mar_{Rt} crude extracts shows the presence of Marinoquinoline E, H and F. Marinoquinoline C was found separated as a single node. The *m/z* of 245.1090 corresponds putatively to an unknown marinoquinoline derivative.

Supplementary figure 5. Phylogeny of *prnB*-like genes in bacteria



Branches are colored according to their phylum. Black: *Firmicutes*; Purple: *Bacteroidetes*; Green: *Proteobacteria*; Blue: *Actinobacteria*. The strains used in this studies are highlighted.

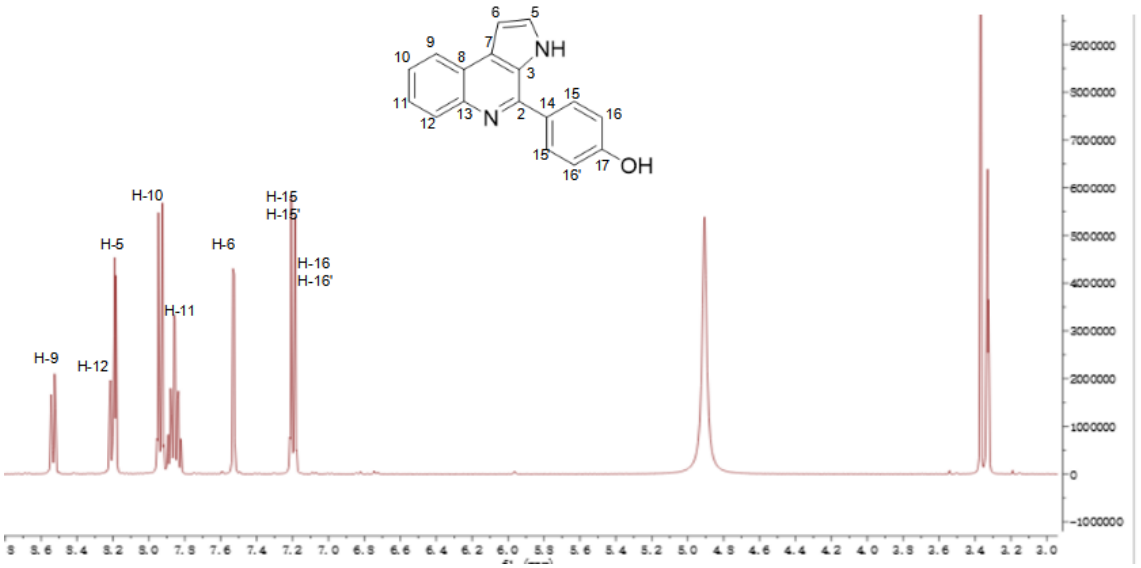
Supplementary Figure 6. Spontaneous, non-enzymatic formation of Marinoquinoline H. The measurement was performed by MALDI-TOF analysis.



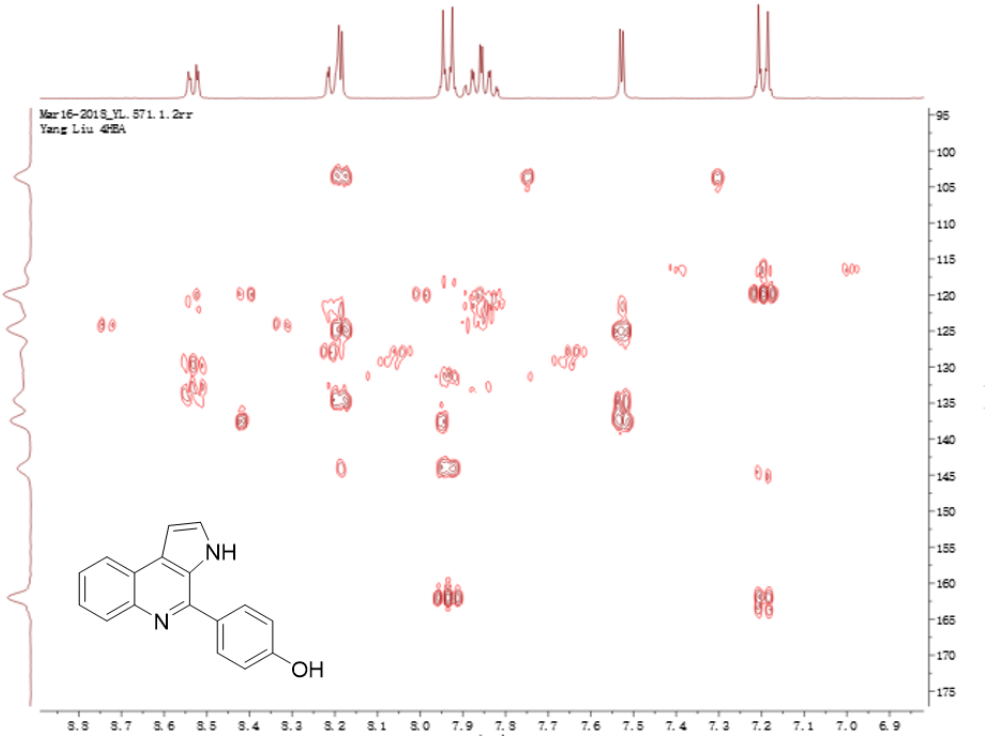
No effect of the enzyme was observed on the marinoquinoline H formation. This compound can be detected from 5 min at the same intensity than in other time point measurements.

Supplementary Figure 7. LC-HRMS and NMR spectra of compound **4**.

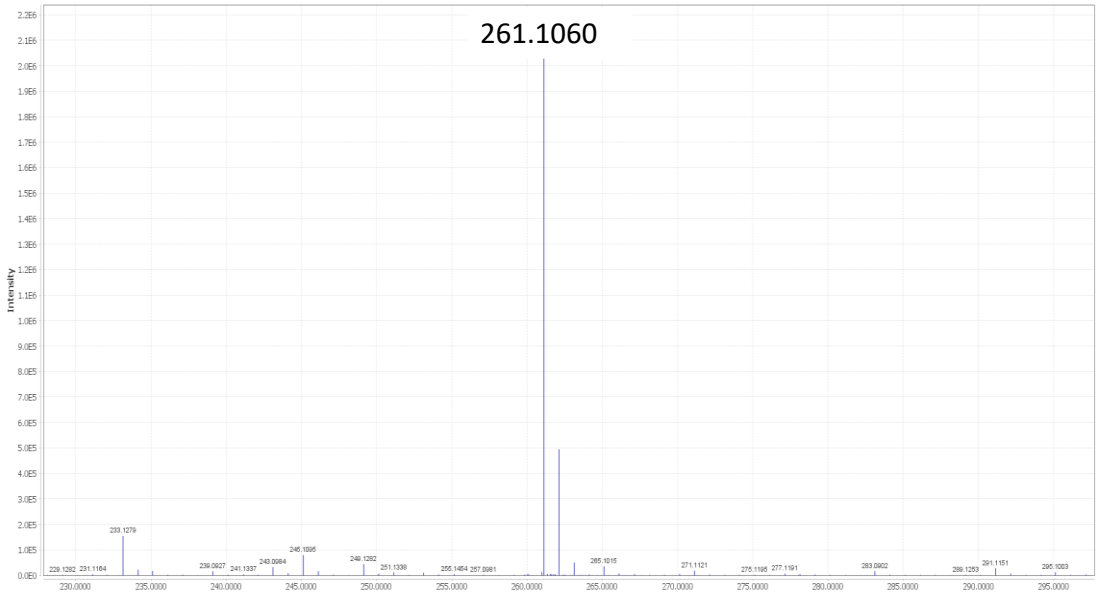
7A. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of the compound **4**



7B. HMBC spectrum of the compound **4**

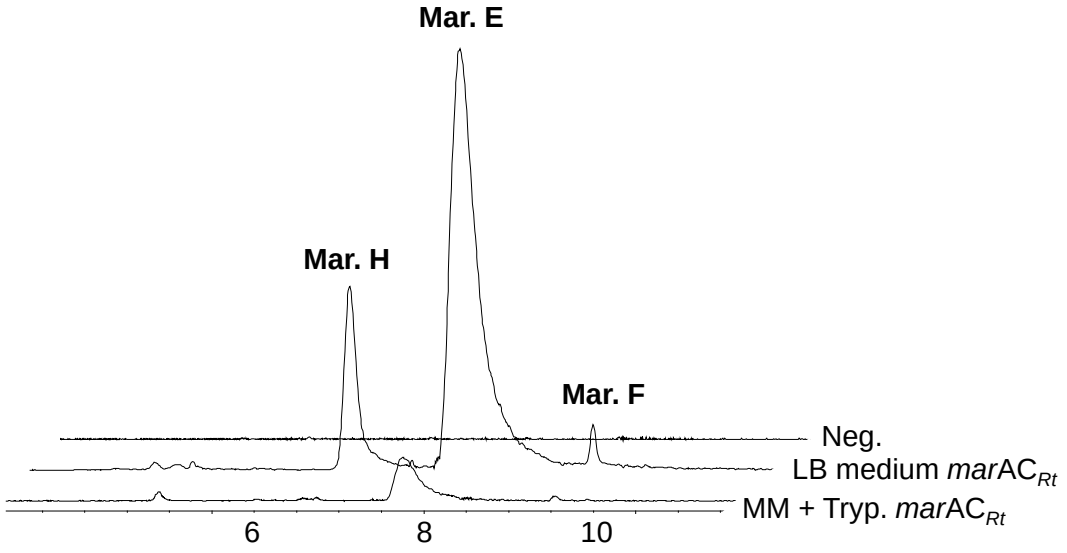


7C. LC-HRMS spectrum of compound **4** (marinoquinoline H)



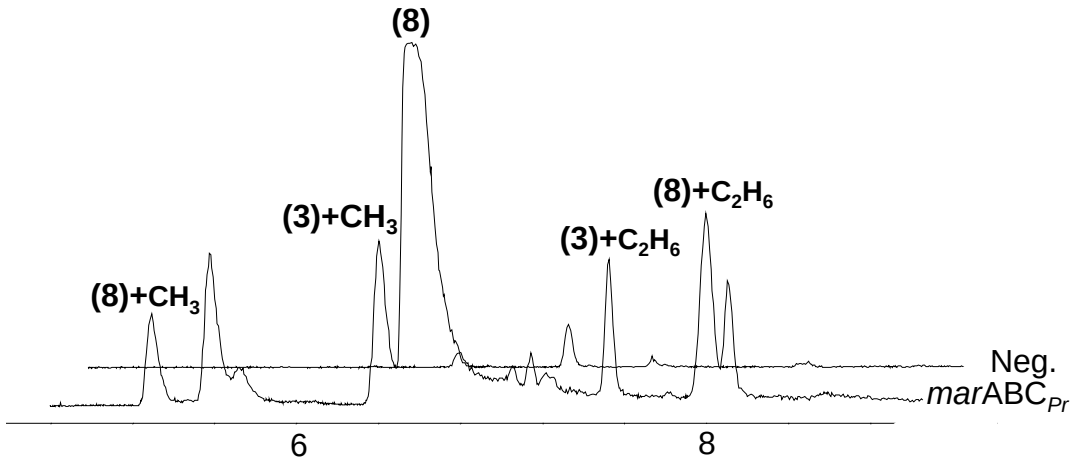
Expected *m/z* $[M+H]^+$ for $C_{17}H_{13}N_2O$: 261.1022

Supplementary Figure 8. *In vivo* marinoquinoline formation by *E. coli marAC*$_{Rt}$ in LB and minimal medium



Extracted Ion Chromatogram (EIC) of Marinoquinoline H (*m/z*: 261.1 $[M+H]^+$), E (*m/z*: 284.1 $[M+H]^+$) and F (*m/z*: 312.1 $[M+H]^{+)}$. Mar.: marinoquinoline, MM.: Minimal medium, Neg.: negative control. Axes X: Time (min) Y: Intensity.

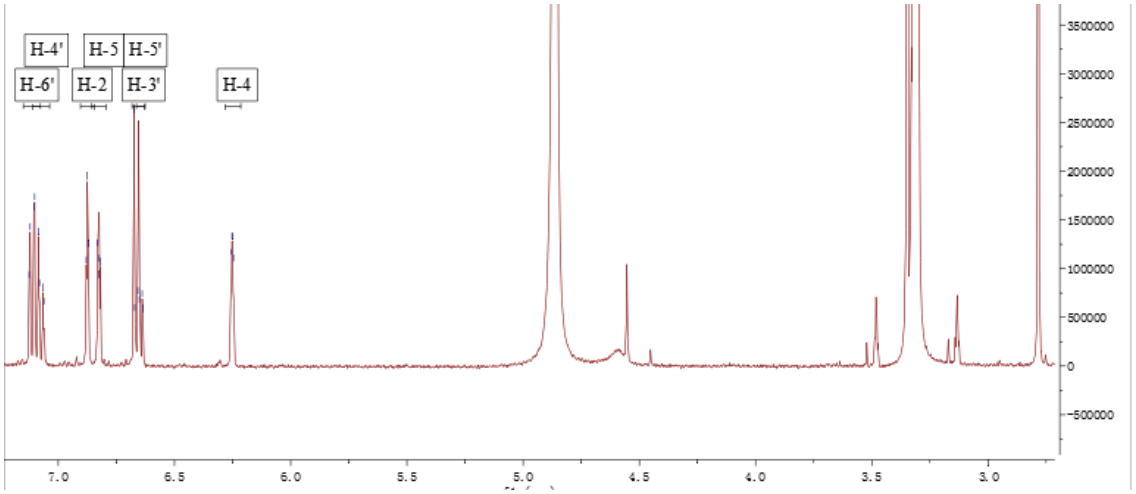
Supplementary Figure 9. LC/MS chromatogram of the *in vivo* assay using the heterologous strain *E. coli marABC*$_{Pr}$.



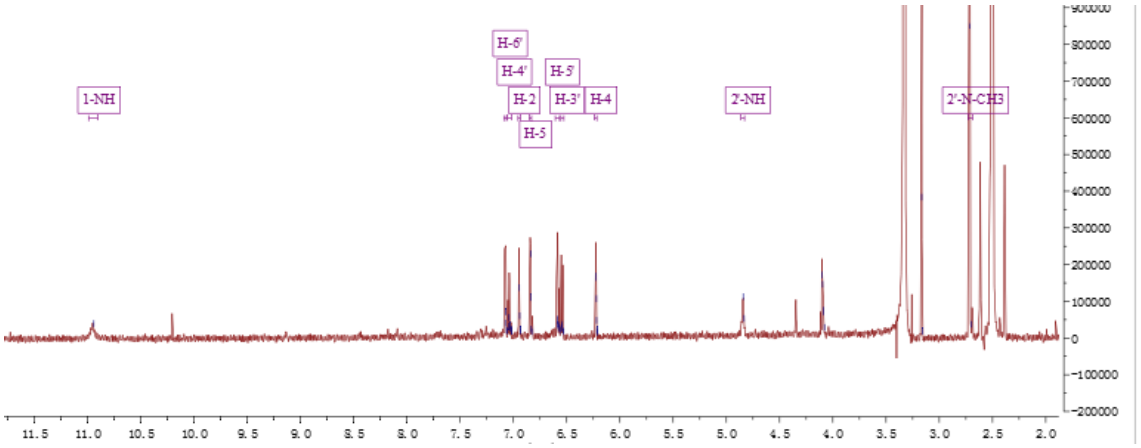
Extracted Ion Chromatogram (EIC) of compound **8** (*m/z*: 173.1 $[M+H]^+$), compound **8**+CH_2 (*m/z*: 187.1 $[M+H]^+$) Compound **8**+C_2H_4 (*m/z*: 201.1 $[M+H]^+$), Compound **3**+CH_2 (*m/z*: 219.1 $[M+H]^+$), Compound **3**+C_2H_4 (*m/z*: 233.1.1 $[M+H]^+$). Neg.: negative control. X-axes: time (min) y-axes: intensity.

Supplementary Figure 10. LC-HRMS and NMR spectra of compound **8**.

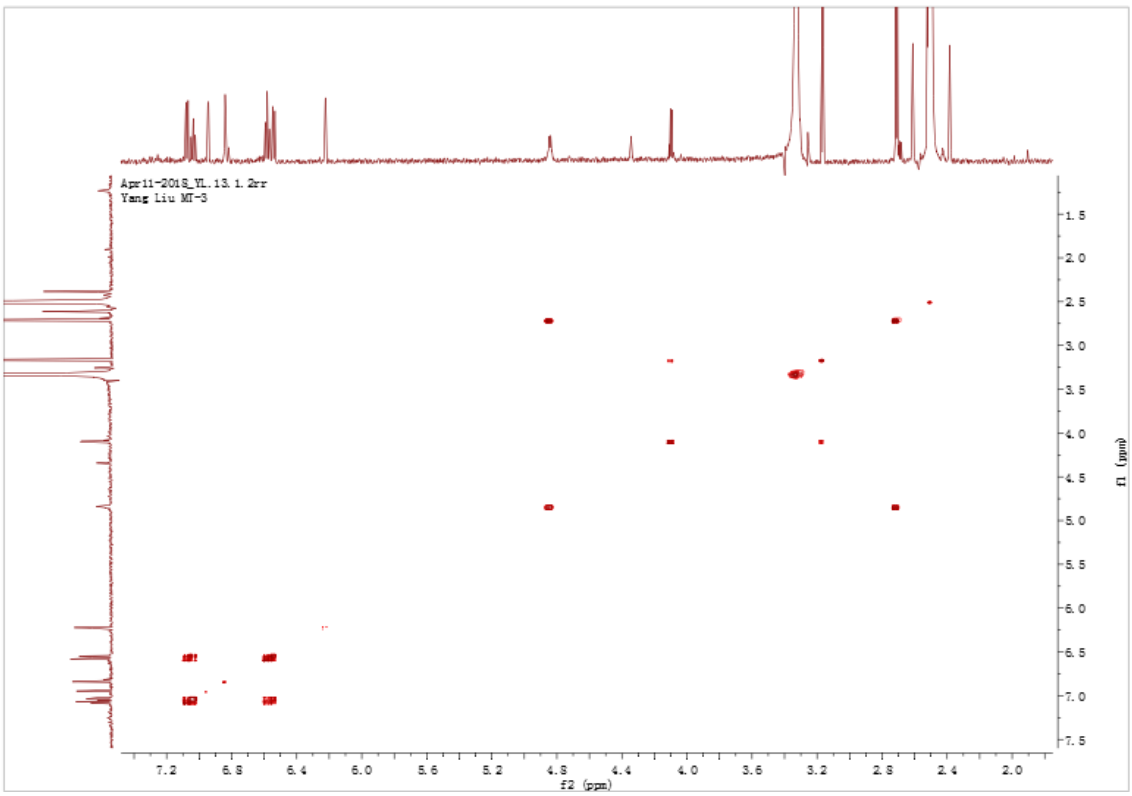
10A. ^{1}H NMR (400 MHz, MeOH-d_4) spectrum of the compound **8**



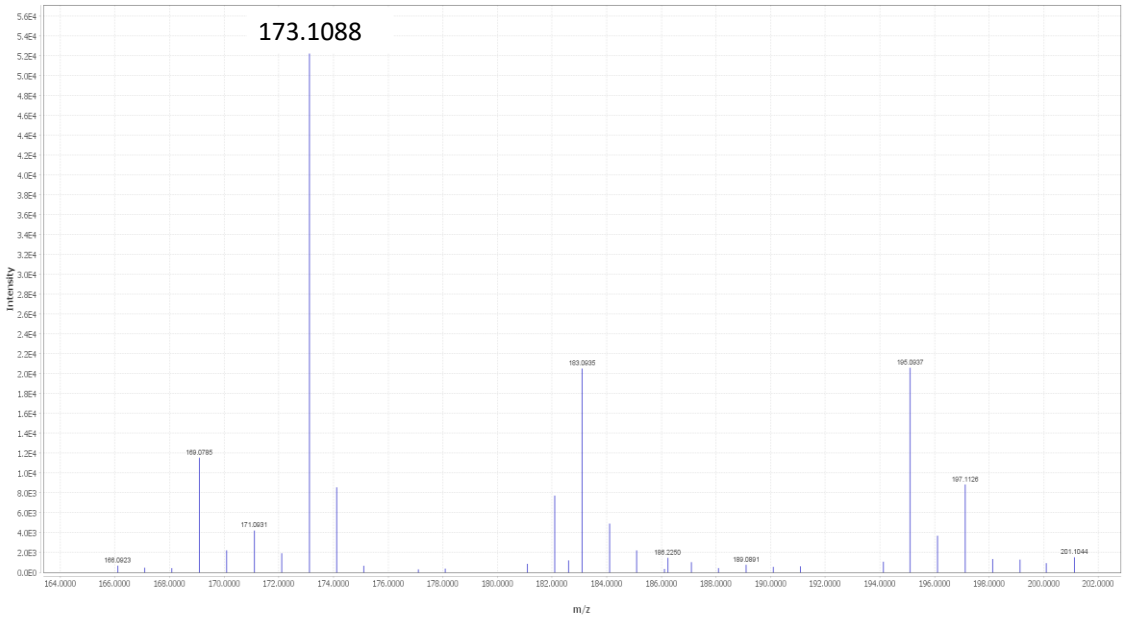
10B. ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of the compound **8**



10C. COSY spectrum of the compound **8**

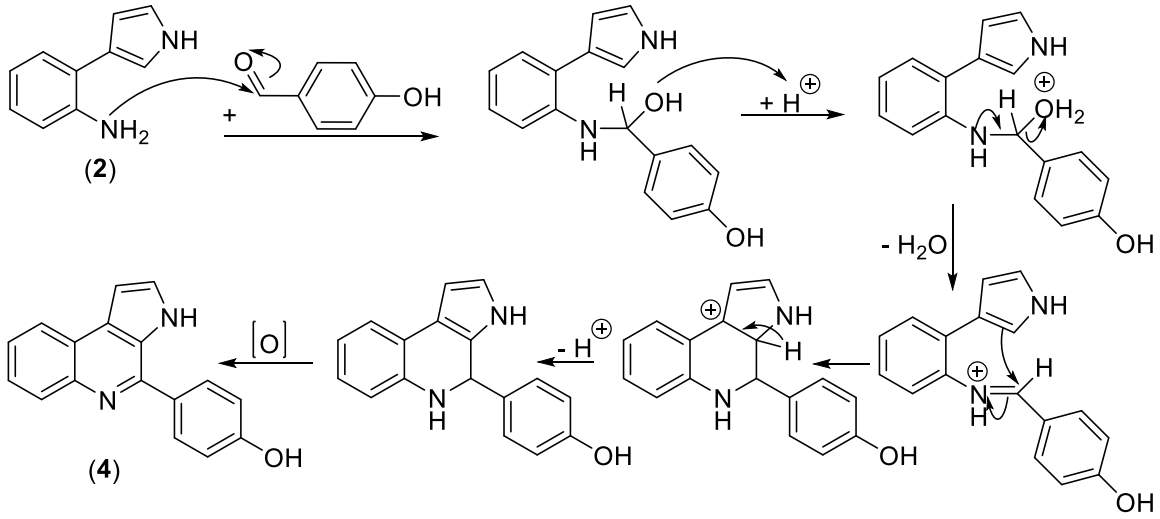


10D. LC-HRMS spectrum of compound **8**



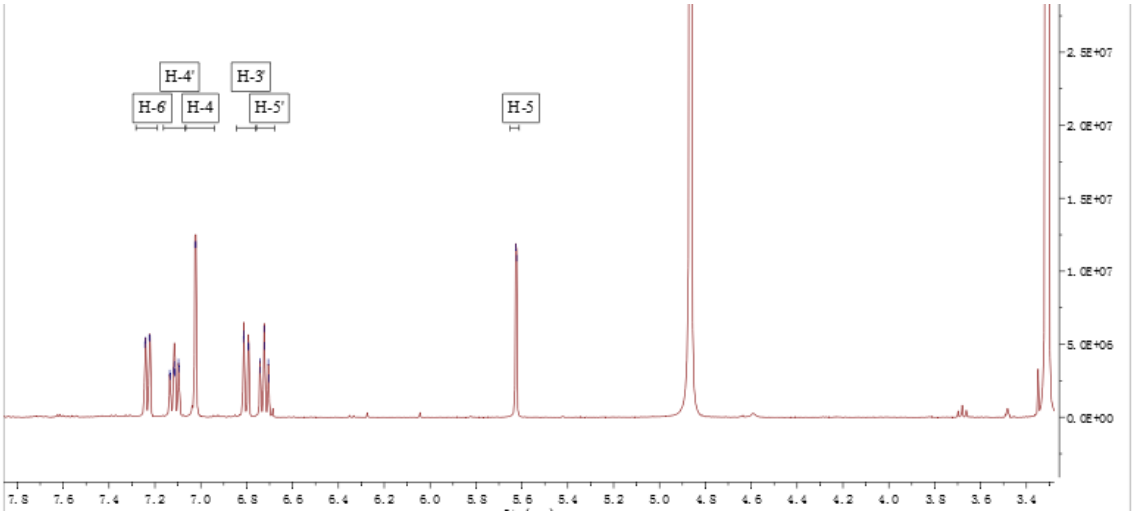
Expected *m/z* $[M+H]^+$ for $C_{11}H_{12}N_2$: 173.1078

Supplementary Figure 11. Proposed mechanism of the 3HPQ formation including a Pictet-Spengler-like reaction, followed by spontaneous oxidation.

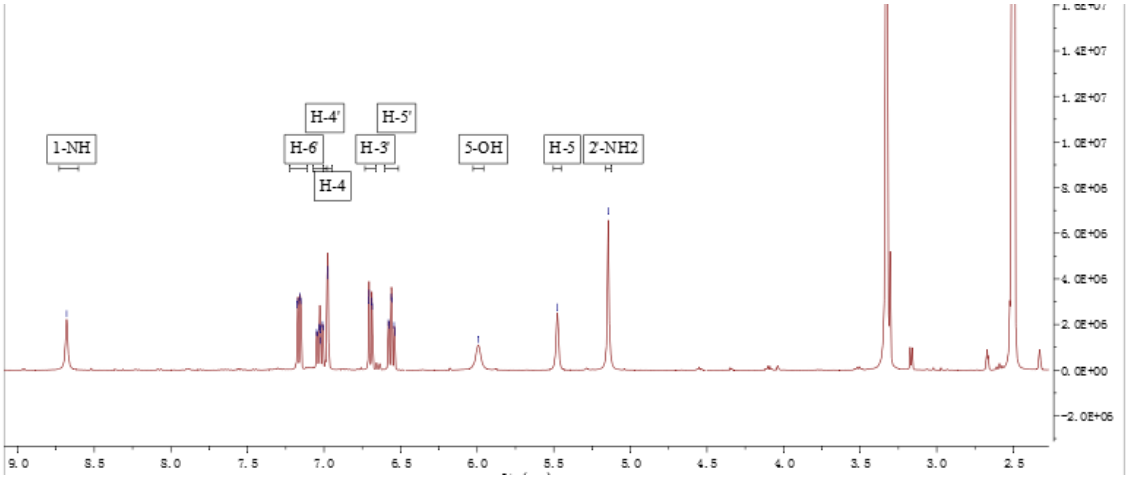


Supplementary Figure 12. LC-HRMS and NMR spectra of compound **9**.

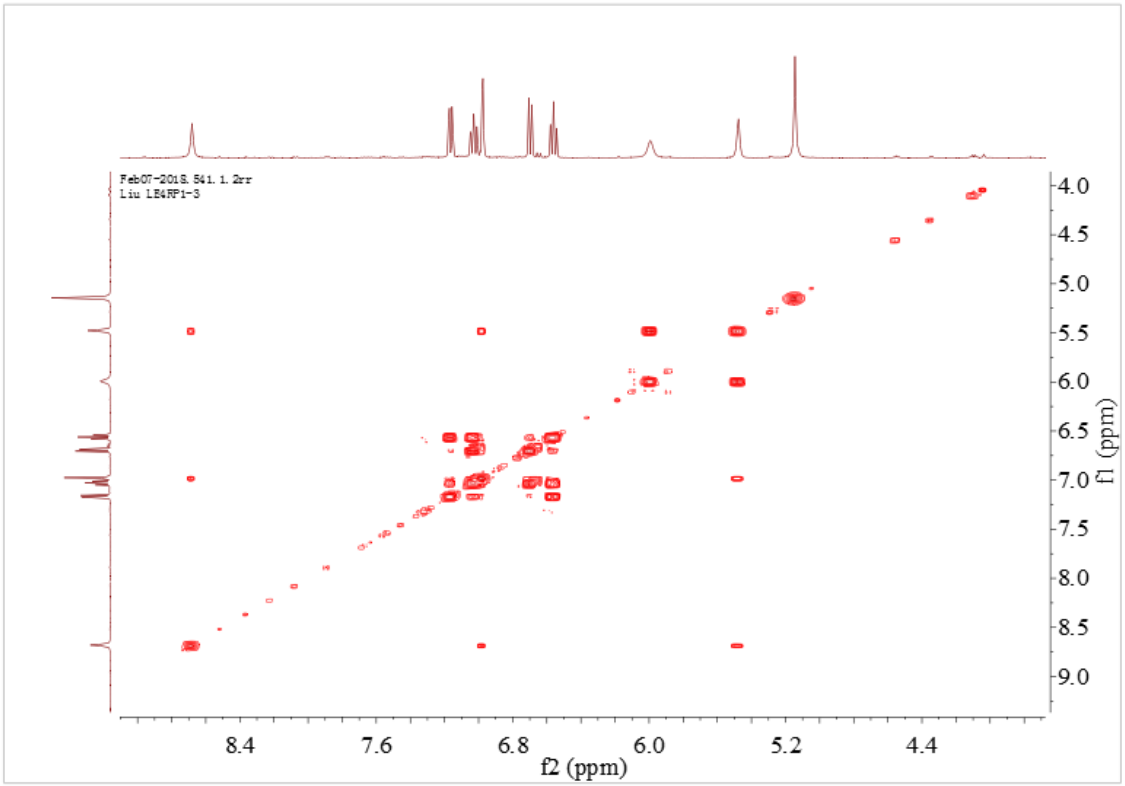
12A. ^{1}H NMR (400 MHz, MeOH-d_4,) spectrum of the compound **9**



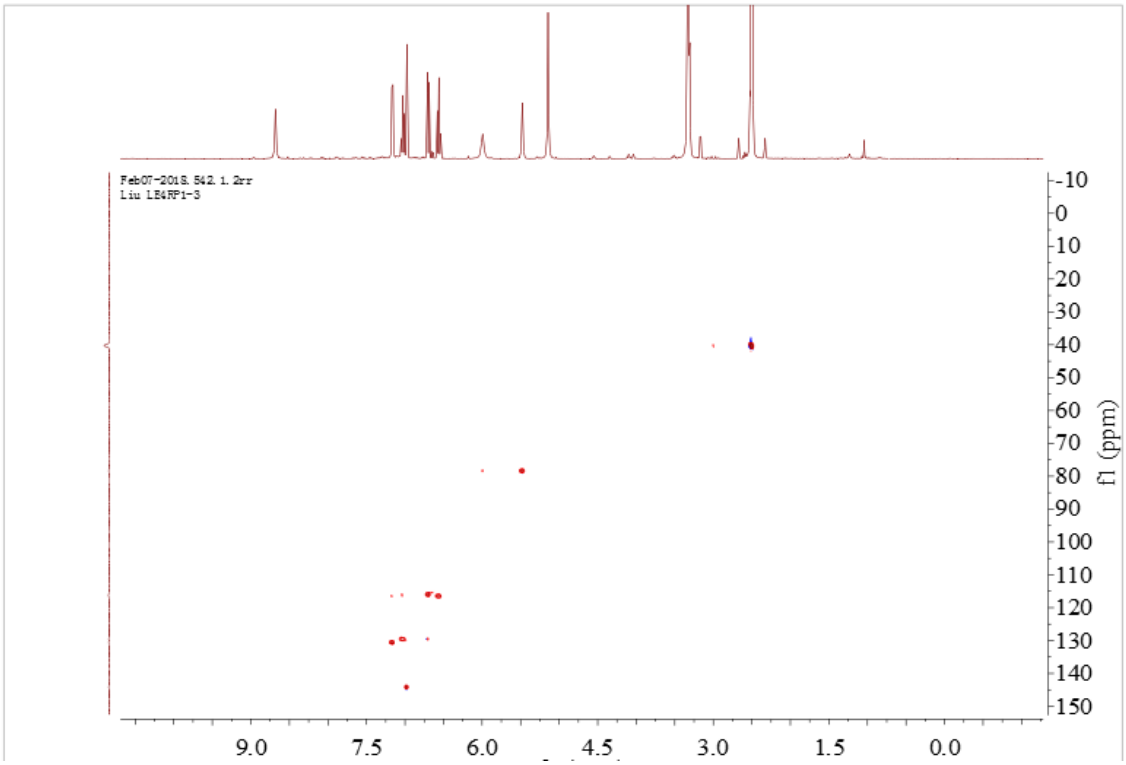
12B. ^{1}H NMR (400 MHz, DMSO-d_6,) spectrum of the compound **9**



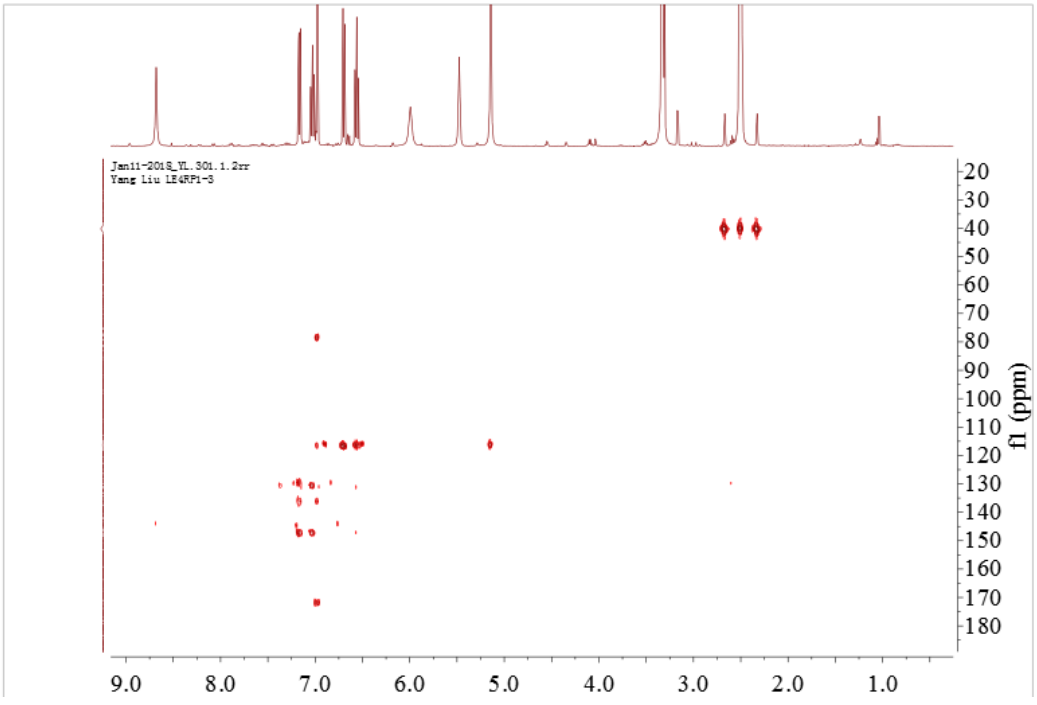
12C. COSY spectrum of the compound **9**



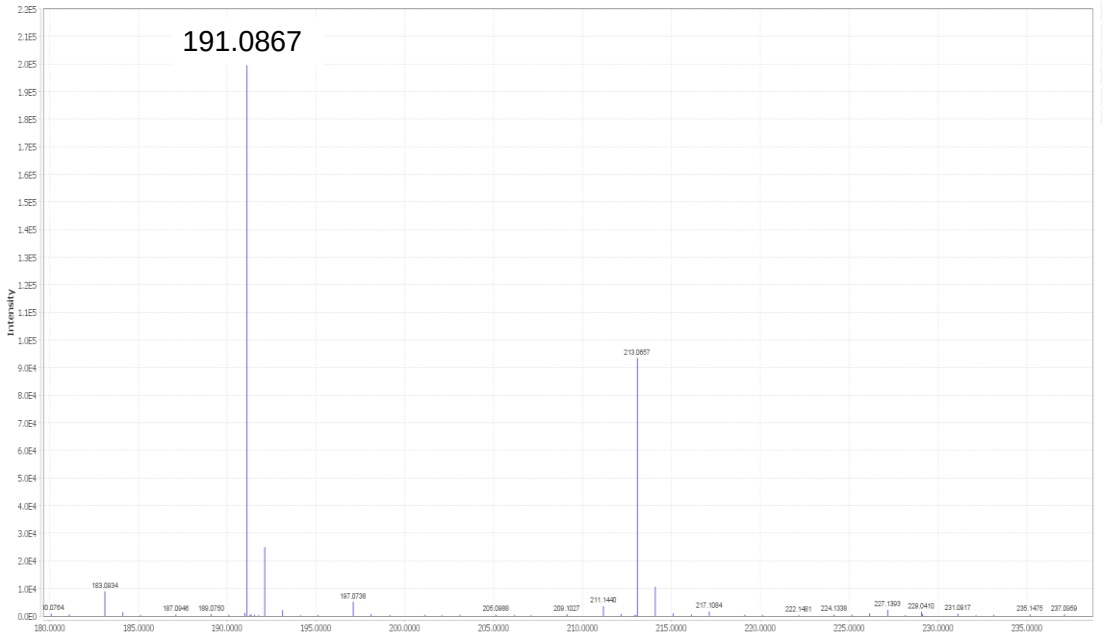
12D. HSQC spectrum of the compound **9**



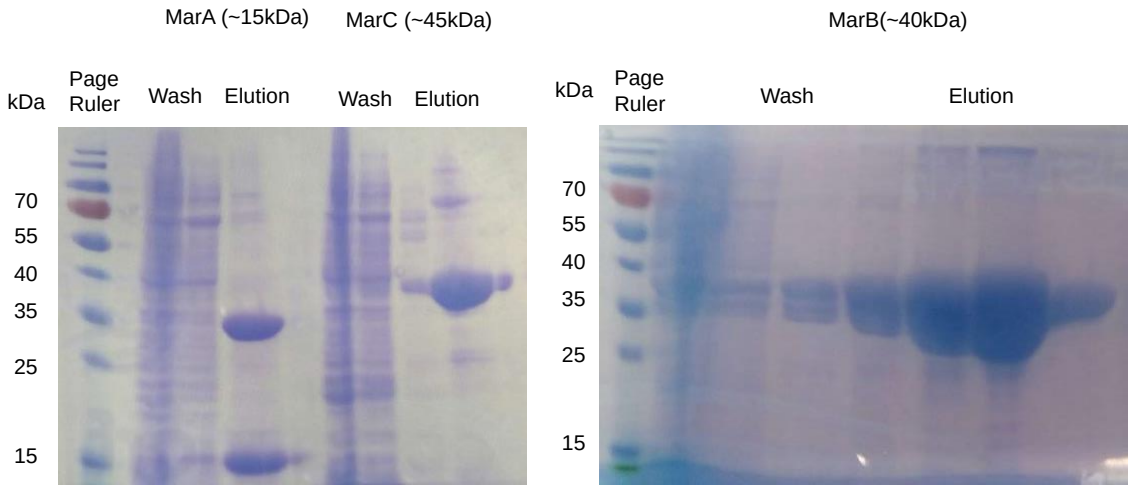
12E. HMBC spectrum of the compound **9**



12F. LC-HRMS spectrum of compound **9**



Supplementary Figure 13. SDS-PAGE gel showing the purification of His-tagged MarA, MarB and MarC from *E. coli* ROSETTA (washing and elution steps).



For MarA, the monomer (~15 kDa), as well as the dimer can be seen.

Supplementary Tables

Supplementary Table 1. Marinoquinoline derivatives detected from *E. coli* $mar_{Rt.}$

Compound	Retention time(min)	Detected mass	Expected mass
Marinoquinoline H	6.16-6.30	261.1025	261.1022
Marinoquinoline C	7.26-7.49	259.1217	259.1230
Marinoquinoline E	7.87-9.32	284.1193	284.1182
Marinoquinoline F	13.46-13.48	312.1107	312.1131

Supplementary Table 2. APPA derivatives detected from *E. coli* $mar_{Pr.}$

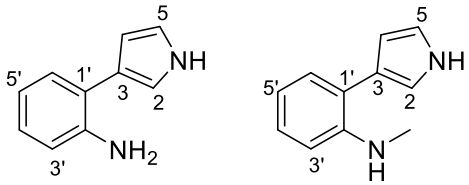
Compound	Retention time(min)	Detected mass	Expected mass
Compound 8	7.50-8.10	173.1080	173.1078
Compound 8+ CH_2	4.06-4.36	187.1244	187.1235
Compound 8+ C_2H_4	7.87-9.32	201.1193	201.1391
Compound 3+ CH_2	6.80-7.00	312.1107	219.1133
Compound 3+ C_2H_4	7.20-7.50	233.1287	233.1290

Supplementary Table 3. Coupling constant and COSY correlation of the novel compound **8**.

	Compound **2**	Compound **8**	
	MeOH-d_4	MeOH-d_4	DMSO-d_6
1-NH			10.95, br s
2	6.92, t (1.77)	6.87, t (1.73)	6.95, td (1.73, 1.94)
4	6.31, dd (2.71, 1.65)	6.25, dd (2.65, 1.64)	6.23, td (2.54, 1.73)
5	6.82, dd (2.71, 1.94)	6.83, dd (2.65, 1.94)	6.85, dd (2.54, 1.94)
2'-NH			4.85, q (5.15)
2'-N-CH_3		2.78, s	2.71, d (5.15)
3'	6.78, dd (7.94, 1.25)	6.66, d (7.74)	6.55, dd (8.07, 1.20)
4'	6.97, ddd (7.94, 7.29, 1.58)	7.07, dd (7.64, 1.70)	7.04, td (7.68, 1.64)
5'	6.71, td, (7.42, 1.25)	6.65, dd (7.44, 1.11)	6.59, td (7.39, 1.20)
6'	7.17, dd, (7.58, 1.58)	7.11, dd (7.90, 1.57)	7.08, dd (7.41, 1.64)

^{1}H NMR spectroscopic data of **2** and **8** (MeOH-d_4 recorded at 400MHz, and DMSO-d_6 recorded at 600MHz, δ in ppm, J in Hz).

Methylation of **2** due to the $MarB_{Pr}$ activity occurred at the amino group of the phenyl moiety.



Compound **2** compound **8**

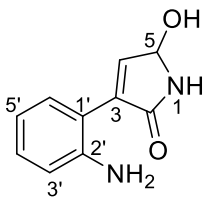
The position of methyl group of compound **8** was proved by coupling constant and COSY correlation of δ 4.85 and δ 2.71.

Supplementary Table 4. NMR spectroscopic data of the novel compound **9**

position	δ_H, multiplicity (*J*)	δc	HMBC	COSY
1-NH	8.68, s			H-5
2		171.7		
3		136.2		
4	6.98, d (1.73)	144.1	C-1';C-2; C-3; C-5	H-5
5	5.48, br s	78.5		1-NH; H-4; 5-OH
5-OH	5.99, br s			H-5
1'		116.6		
2'-NH_2	5.14	147.5	C-1';C-3'	
3'	6.70, dd (8.14, 1.13)	115.9	C-1'; C-5'	H-4'
4'	7.03, ddd (8.08, 7.26, 1.65)	129.5	C-2'; C-6'	H-3'; H-5'
5'	6.56, dd (7.46, 1.22)	116.4	C-1'; C-3'	H-4'; H-6'
6'	7.16, dd (7.69, 1.60)	130.6	C-2'; C-3'; C-3	H-5'
6'-NH_2	5.14, s			

NMR spectroscopic data of **9** recorded in DMSO-d_6 (δ in ppm, *J* in Hz; ^{1}H at 400MHz, ^{13}C at 100 MHz; quaternary carbons observed from HMBC spectroscopic data).

Light yellowish, amorphous solid; [α]22 D = -17.5 (*c* 0.25, MeOH); ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR, see Table . The molecular formular of compound **9** was established as C10H10N2O2 based on the ion peak observed at m/z = 191.0867 $[M+H]^+$ and m/z=213.0672 $[M+Na]^+$ in the LC-HRMS spectrum. The ^{1}H and ^{13}C NMR data of **9** was similar to the known compound **3**. In ^{1}H NMR spectrum, the one additional hydroxy proton of compound **9** resonated at δ_H 5.99 in stead of the methoxy group of compound **3** revealed the difference of the two co-isolated compounds. The COSY correaltions of 5-OH to 5-H, and of 5-H to 1-NH and 4-H demonstrated the position of hydroxy group, which also can be proved by HMBC correlations from 4-H to C-1', C-2, C-3, and C-5. So compound **9** was isolated as a new amino phenyl pyrrolidone derivative.



Supplementary Table 5. Antibiotic and Antifungal activity of APPA derivatives

Compound	Anti Gram+[a]	Anti Gram-[a]	Fungicidal	Reference[b]
Marinoquinoline A	+	+	-	[1,2]
Marinoquinoline B	-	-	-	[1]
Marinoquinoline C	+	-	+	[1,2]
Marinoquinoline D	+	-	-	[1,2]
Marinoquinoline E	+	-	+	[1]
Marinoquinoline F	-	-	+	[1]
Marinoquinoline G	-	-	-	[2]
Marinoquinoline H	+	-	-	This study
Marinoquinoline I	+	-	-	[2]
Marinoquinoline J	-	-	-	[2]
Marinoquinoline K	-	-	-	[2]
Marinoazepinone A	-	-	-	[2]
Marinoazepinone B	+	-	-	[2]
Compound 2	-	-	-	This study
Compound 3	-	-	-	This study
Compound 9	-	-	-	This study
Compound 8	-	-	-	This study

[a]: + indicates that antibacterial activity was observed and – indicates the lack of it. In this study, *Escherichia coli* and *Bacillus megaterium* were used as Gram-negative and Gram-positive test strains, respectively. Details are given above in the experimental section.

[b]: In [1] the MIC tests were conducted in 96-well microtiter well plates in EBS medium for *Staphylococcus aureus*, *N. flava*, and *M. luteus* and the Gram-negative bacteria *E. coli* and *Chromobacterium violaceum*. In addition, the fungi *M. hiemalis*, the yeasts *Candida albicans*, *R. glutinis*, and *Pichia anomala* as well as the fission yeast *S. pombe* were tested.In [2] the activities were measured in an agar diffusion test against *Pontibacillus* sp. (strain CNJ-912) and *V. shiloi* (strain CUA-364).

Supplementary Table 6. Media composition (per liter)

Name	**Composition**
LB medium	Tryptone 10g
	Yeast extract 5g
	NaCl 10g
Marine Broth 2216	Peptone 5.00 g
	Yeast extract 1.00 g
	Fe(III) citrate 0.10 g
	NaCl 19.45 g
	$MgCl_2$ (anhydrous) 5.90 g
	Na_2SO_4 3.24 g
	$CaCl_2$ 1.80 g
	KCl 0.55 g
	$NaHCO_3$ 0.16 g
	KBr 0.08 g
	$SrCl_2$ 34.00 mg
	H_3BO_3 22.00 mg
	Na-silicate 4.00 mg
	NaF 2.40 mg
	$(NH_4)NO_3$ 1.60 mg
	Na_2HPO_4 8.00 mg
	Distilled water 1000.00 ml
Minimal Medium	Glucose 5g
	NaCl 5g
	$FeSO4*7H_2O$ 0.0011g
	$MgSO4*7H_2O$ 0.25g
	$(NH_4)SO_4$ 1.98g
	KOH 4.23g
	KH_2PO_4 13.63g

Supplementary Table 7. Primers, DNA templates, PCR products and constructs used during this study.

Primer name	Sequence	Construct	DNA template	PCR product amplified
marRT1_f	Aataattttgtttaactttaataaggagatataccatgagtgagttag-taacatc		*R. thailandica*	
marRT1_r	gtgatgatggtgatggctgcttactcattcaaccaatcaa		s80	$MarA_{Rt}$
marRT2_f	ttgattggttgaatgagtaagcagccatcaccatcatcac		pRSF	T7 prom.
marRT2_r	attgacttaacaacttccatatgtatatctccttcttata			
marRT3_f	tataagaaggagatatacatatggaagttgttaagtcaat			
marRT3_r	ggccggccgatatccaattgagatctgccattataactccactgattgata ctttt	pRSF-Mar_{Rt}	*R. thailandica* s80	$MarC_{Rt}$
marPR1_f	aataattttgtttaactttaataaggagatataccatgattactacaagtaaa gc			$MarA_{Pr}$
marPR1_r	tgggtttgtgatgttatcatatgtatatctccttcctactcgtttaagtactcaa			
marPR2_f	ttgagtacttaaacgagtaggaaggagatatacatatgataacatcacaa accca		*Porifericola rhodea* s47	$MarB_{Pr}$
marPR2_r	gtgatgatggtgatggctgcttatttgtaagctatacctg			
marPR3_f	caggtatagcttacaaataagcagccatcaccatcatcac		pRSF	T7 prom.
marPR3_r	tcaaaagctaatgttttcatatgtatatctccttcttata			
marPR4_f	tataagaaggagatatacatatgaaaacattagcttttga			
marPR4_r	cgcgtggccggccgatatccaattgagatctgccattatctgttttcgtaac ttt	pRSF-Mar_{Pr}	*Porifericola rhodea* s47	$MarC_{Pr}$
marPR_DMT_f	ttgagtacttaaacgagtagaaggagatatacatgaaaacattagcttttg a	pRSF-$Mar_{Pr}\Delta$MarB	*Porifericola rhodea* s47	ΔMarB in pRSF-Mar_{Pr} used instead marPR1_r marPR4_f
mrPR_DMT_r	tcaaaagctaatgttttcatgtatatctccttctactcgtttaagtactcaa			
marA_pET28_f	catgactggtggacagcaaatgggtcgcggatccggcatgagtgagtta gtaaca			
marA_pET28_r	tagcagccggatctcagtggtggtggtggtggtgcttactcattcaacca atcaa	pET28 marA	*R thailandica* s80	$MarA_{Rt}$
marB_pET28_f	gactggtggacagcaaatgggtcgcggatccggcatgataacatcaca aacccaa			
marB_pET28_r	tagcagccggatctcagtggtggtggtggtggtgcttatttgtaagctata cctg	pET28 marB	*Porifericola rhodea* s47	$MarB_{Pr}$
marC_pET28_f	catgactggtggacagcaaatgggtcgcggatccggcatggaagttgtt aagtca			
marC_pET28_r	tagcagccggatctcagtggtggtggtggtggtgcttataactccactgat tgat	pET28 marC	*R. thailandica* s80	$MarC_{Rt}$

PCR reaction conditions (Q5® High-Fidelity DNA Polymerase) Initial denaturation 30s at 98°C; 30 Cycles : 10 s(98°C), 15 s(52°C) , 72°C (20s per kb) and final extensión 2 min at 72°C.

References

[1] P. W. Okanya, K. I. Mohr, K. Gerth, R. Jansen, R. Mueller, *Journal of natural products* **2011**, *74*, 603.

[2] E. J. Choi, S.-J. Nam, L. Paul, D. Beatty, C. A. Kauffman, P. R. Jensen, W. Fenical, *Chemistry & Biology* **2015**, *22*, 1270.

Summary Chapter III

The last chapter summarizes the results on the biosynthetic investigation of APPAs in bacteria belonging to the *Cytophagales* order. A bioinformatics and phylogenetic analysis allowed the identification of the biosynthetic gene cluster in *Rapidithrix thailandica* s80 (corresponding to marinoquinolines production) and *Porifericola rhodea* s47 (corresponding to *N*-methylmarinoquinolines production).

The biosynthesis of these alkaloids, including enzymatic and spontaneous reactions, was elucidated by different *in vivo* and *in vitro* experiments. This experiments include the heterologous expression of the BGCs and construction of deletion mutants as well as the expression and purification of single enzyme for further *in vitro* assays. In addition , the discovery of a new aminophenylpyrrolidone suggests a degradation route of these aminophenylpyrroles.

The experiments revealed that the in a first step tryptophan is converted into aminophenylpyrrole in a reaction catalized by MarC(homologue to PrnB from pyrrolnitrin biosynthesis). This enzymatic product is a common precursor for the diverse class of APPA. From this point on, a spontaneous Pictet−Spengler-like coupling reaction enables the bacterial producer strains to create a library of tricyclic marinoquinolines, since several aldehydes can be applied as substrates. In an alternative route a SAM dependent methyltransferase MarB adds one or more methyl groups to the aminophenyl intermediate that later reacts with aldehydes to form the *N*-methylated marinoquinolines.

Conclusions and Outlook

The microbiomes of the intertidal area of different sites at the Peruvian coast vary considerably most likely in response to the seawater temperature. From the total microbiome, the known predatory bacterial taxa represented only 0.5%. However, by applying the bait streak isolation method using *E. coli* and *Pheobacter inhibens* as prey, several predatory strains were highly enriched and isolated.

The culturable predatory bacteria showed a high potential for the production of antibiotics. From the isolates, several antibacterial compounds were identified, including the known compounds ariakemicin, kocurin, naphthyridinomycin, pumilacidins, resistomycin, surfactin and APPAs. However, most compounds remained elusive until now.

Kocurin, a compound with antibiotic activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strains was identified in the broth of *Kocuria rosea* s17. The biosynthetic gene cluster was identified by comparative genome analysis with other reported thiopeptide producers. It comprises nine open reading frames and spans around 12 kbp of the genome. Heterologous expression was used to prove the functionality of the predicted kocurin biosynthetic gene cluster.

The highly diverse APPAs were isolated from the predatory bacterial strains *Rapidithrix thailandica* s68, *Rapidithrix thailandica* s80 and *Porifericola rhodea* s47. Bioinformatics and phylogenetic analyses allowed the identification of the biosynthetic gene cluster, which seems to be only present in the *Cytophagales* order. The biosynthesis of the *Cytophagales* APP alkaloids, their chemical diversification strategy, as well as the putative degradation pathway were elucidated making use of different *in vivo* and *in vitro* experiments.

The first step in the biosynthesis consists in a one enzyme dependent reaction that yields 3- (2′-aminophenyl)-pyrrole from tryptophan. Second, a spontaneous Pictet−Spengler-like coupling reaction followed by oxidation enables the bacterial producer strains to create a library of tricyclic marinoquinolines alkaloids, since several alde-

hydes can be applied as substrates. The diversity of this natural products class is further enlarged by the catalytic action of a methyltransferase, which adds one or more methyl groups to the aminophenyl intermediate. Furthermore, the discovery of a new aminophenylpyrrolidone suggests a degradation route of these aminophenylpyrroles via dioxidation and methylation.

The elucidation of the promising antibiotic kocurin and APPAs might facilitate the further development of such compound classes. In the case of kocurin, synthetic biology approaches to obtained analogs with exchanged aminoacids appears as an interesting option to improve the physicochemical limitations of this compounds.

With the elucidation of the APPA biosynthesis, the idea to use a chemoenzymatic approach to build a library of natural and unnatural marinoquinolines derivatives with diverse bioactivies appears feasible. In this sense experiments using the methyltransferase MarB will be carried out for this approach.

Summary

Bacteria are a remarkable source of bioactive compounds, of which many served as lead compounds for the development of medicinal drugs currently used for diverse applications in clinic. Despite the immense bacterial diversity, only a few taxa commonly isolated in bioprospecting projects are known as proliferative producers of these compounds (e.g., 70% of antibiotics are derived from *Streptomyces* species). In recent years, the re-isolation of known compounds became a recurrent problem, making the search for novel talented taxa a priority.

Predatory bacteria possess large genomes, which provide them with a large metabolic repertoire. The assumption that these bacteria use antibiotics to weaken or even kill their prey implements the great potential of these organisms as source for novel bioactive compounds. In the present work, the isolation of predatory bacterial strains, characterization of their produced specialized metabolites and elucidation of the biosynthesis of these metabolites was pursued. The Peruvian coastline was selected as sampling area, since it represents a biodiverse ecosystem, rich in endemic species. It is of interest, if the grade of biodiversity, usually judged by macroorganisms, can also be transferred to microorganisms. Therefore, a culture-independent metagenomics study was carried out to obtain first insights into the bacterial diversity. The microbiome of three different sites at the Peruvian Pacific coast was analyzed, revealing a lower bacterial biodiversity at Isla Foca than at Paracas and Manglares, with 89 bacterial genera identified, as compared to 195 and 173 genera, respectively. Only 47 of the bacterial genera identified were common to all three sites.

Then, in order to obtain promising strains for the production of novel antimicrobials, predatory bacteria were isolated from these sampling sites, using two different bait organisms. Even though the proportion of predatory bacteria was only around 0.5% in the here investigated environmental microbiomes, by this approach in total 138 bacterial strains were isolated as axenic culture. 25% of these strains showed antibacterial activity, thereby nine revealed activity against clinically relevant methicillin resistant *Staphylococcus aureus* (MRSA) and three against enterohemorrhagic *Escherichia coli* (EHEC) strains. Phylogeny and physiological characteristics of the active strains were

investigated. Metabolomic analyses enabled first insights into the chemical basis of the antibacterial activity and indicated production of the known compounds ariakemicin, kocurin, naphthyridinomycin, pumilacidins, resistomycin, surfactin and aminophenylderived alkaloids. However, most antibacterial compounds remained elusive until now.

Among the reported compounds, it was aimed to understand the biosynthesis of the promising antibiotic kocurin. Kocurin is a ribosomally synthetized and posttranslational modified (RIPP) thiopeptide produced by *Kocuria rosea* s17. The genetic basis of this natural product with activity against MRSA was revealed by comparative genome analysis of this new isolate and other reported thiopeptide producer strains. The functionality of the predicted gene locus was experimentally proven by heterologous expression in *Streptomyces coelicolor* M1146. Expression of the gene cluster under the control of a constitutive promoter enabled the transgenic strain to produce kocurin in selected media. The kocurin biosynthetic gene cluster (BGC) comprises 9 open reading frames and spans around 12 kbp of the genome.

Another class of highly interesting natural products, showing various bioactivities, for which the biosynthesis was investigated in detail, were the highly diverse aminophenyl-derived alkaloids (APPAs). APPAs, like the antifungal pyrrolnitrin, which was already discovered in the 60ies, represent high value lead compounds that were developed into globally important fungicides. More recently, diverse APPAs were discovered from bacteria belonging to the Cytophagales order (phylum Bacteroidetes). In the present work, a bioinformatics and phylogenetic approach enabled the elucidation of the genetic basis, as well as the non-enzymatic processes involved in the biosynthesis of these natural products. A bioinformatic analysis of the producer genomes (*Rapidithrix thailandica* s80 and *Porifericola rhodea* s47) allowed the identification of the putative APPA BGC. The biosynthesis was experimentally validated by heterologous expression of the BGCs in *E. coli* and revealed that first, one enzyme-dependent biosynthetic step yields the tryptophan-derived precursor 3-(2'-aminophenyl)-pyrrole. Second, a spontaneous Pictet-Spengler-like coupling reaction enables the producer strains to create a library of tricyclic alkaloids. Which alkaloids are formed is highly dependent on the chemical environment, since several aldehydes can be applied as substrates. The diversity of these natural products class is further enlarged by the catalytic action of a methyltransferase (MT), which adds a methyl group to the aminophenyl intermediate. This

N-MT is encoded in some of the bacterial BGCs, and was investigated in detail, using *in vivo* and *in vitro* experiments.

In summary, predatory bacteria represent an important bioresource for the discovery of novel metabolites. In the present study, the combination of bioinformatics and metabolomics analyses enabled the elucidation of the BGCs for kocurin and APPAs. The results obtained might facilitate the further development of such compound classes in the future and might contribute to enlarge the toolbox for synthetic biology approaches.

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