Natural Products from *Nocardia* and Their Role in Pathogenicity

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Abstract

Nocardia spp. are filamentous Actinobacteria of the order Corynebacteriales and mostly known for their ability to cause localized and systemic infections in humans. However, the onset and progression of nocardiosis is only poorly understood, in particular the mechanisms of strain-specific presentations. Recent genome sequencing has revealed an extraordinary capacity for the production of specialized small molecules. Such secondary metabolites are often crucial for the producing microbe to survive the challenges of different environmental conditions. An interesting question thus concerns the role of these natural products in *Nocardia*-associated pathogenicity and immune evasion in a human host. In this review, a summary and discussion of *Nocardia* metabolites is presented, which may play a part in nocardiosis because of their cytotoxic, immunosuppressive and metal-chelating properties or otherwise vitally important functions. This review also contains so far unpublished data concerning the biosynthesis of these molecules that were obtained by detailed bioinformatic analyses.

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Introduction

Infectious diseases caused by bacteria, fungi, parasites or viruses are still a significant cause of death worldwide [Dye, 2014]. Interestingly, the therapeutic agents to combat such infections are also predominantly derived from microorganisms. One of the most important producers of small molecules with pharmaceutical values, such as antibacterial, antiviral, anticancer, antiparasitic, and immunosuppressing activities, are filamentous Actinobacteria [Mann, 2001; Barka et al., 2016; Lee et al., 2020]. The biosynthesis of these natural products is governed by defined sets of genes organized in biosynthetic gene clusters (BGCs), encoding, e.g., polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS) and hybrid NRPS/PKS machineries. Genes that mediate expression control, self-resistance or export are typically clustered together with these biosynthetic genes in the BGCs [Walsh and Fischbach, 2010; Medema et al., 2015]. Such compounds, produced as secondary metabolites, are often crucial elements in bacterial survival strategies, e.g., as “defense weapons” against other microorganisms or within a host organism. Additionally, these molecules can mediate inter-species and intra-species communication or provide an advantage in nutrient acquisition [Netzker et al., 2018].

Nocardia spp. are filamentous Actinobacteria of the order Corynebacteriales. They are closely related to the genera *Corynebacterium* and *Mycobacterium*, but particularly to the genus *Rhodococcus*. They primarily form mycelia, but can also fragment into rod- to coccoid-shaped bacteroid non-motile elements [Dhakal and Sohng, 2015]. A characteristic feature of *Nocardia* spp. is that they contain tuberculostearic acids in their cell wall, similar to members of the genus *Mycobacterium*. However, in contrast to mycobacteria, *Nocardia* spp. produce short-chain (40- to 60-chain) mycolic acids and usually exhibit branching on Gram staining [Brown-Elliott et al., 2006]. *Nocardia* are often found in organic-rich soil as ubiquitous saprophytes. But most prominently, they are recognized as human opportunistic pathogens. Therefore, they have mostly been isolated from human tissue, and comparably few strains were isolated from environmental samples, such as soil and water [McNeil and Brown, 1994]. In general, immunocompromised patients are affected by nocardiosis with a high mortality rate. Nevertheless, cases in immunocompetent patients have also been reported [Wilson, 2012]. Numerous different strains of *Nocardia* have been identified, many of which are described as infectious to humans and animals. To date, more than 50 *Nocardia* species are considered clinically significant [Conville et al., 2018]. The most prevalent human pathogens are *Nocardia farcinica*, *Nocardia asteroides* type VI (*Nocardia cyriacigeorgica*), *Nocardia brasiliensis*, the *Nocardia nova* complex, *Nocardia abscessus* and the *Nocardia transvalensis* complex [Brown-Elliott et al., 2006]. Usually, *Nocardia* cause localized cutaneous or pulmonary infections. From the infection site, the bacteria can disseminate throughout the whole body, leading to systemic forms of nocardiosis, which often includes severe brain infections. *Nocardia* spp., in general, have been studied for a long time, but most of the studies have focused on the characterization and taxonomic classification of new isolates as well as host pathophysiology [Beaman and Beaman, 1994; Brown-Elliott et al., 2006]. In particular because of their clinical importance, many *Nocardia* genomes were sequenced within the last decade. Surprisingly, these efforts showed an extraordinary potential for the production of diverse secondary metabolites for most *Nocardia* strains, rivalling better-known genera such as *Amycolatopsis* [Männle et al., 2020]. Although pathogenic bacteria have been recognized as a source for natural products drug discovery, they remain largely untapped and understudied [Maglangit et al., 2020]. More recently, some *Nocardia* strains have moved into focus as producers of compounds with pharmaceuti-

cal value regarding their immunosuppressant, antimicrobial, cytotoxic or antifungal bioactivity, i.e., *N. brasiliensis*, *N. abscessus*, *N. transvalensis*, *Nocardia terpenica* and *Nocardia pseudobrasiliensis* [Shigemori et al., 1998; Mikami, 2007; Luo et al., 2014b; Dhakal et al., 2019]. However, because most *Nocardia* spp. produce secondary metabolites only in trace amounts, it was found difficult to isolate and produce the desired compounds for pre-clinical and clinical characterization and widespread therapeutic use. Metabolic engineering approaches and synthetic biological tools were developed to overcome the bottleneck in terms of cost-effectiveness and scale-up possibilities paving a route towards commercial application and large-scale production. In addition, most *Nocardia* spp. are biosafety level 2 organisms, and genetic manipulation in the native producer can thus be tedious and time consuming. Therefore, heterologous expression of BGCs has emerged as a key strategy to gain access to the promising secondary metabolite gene cluster and to reach titers of industrial levels [Wohlleben et al., 2012; Brown and Dawson, 2015; Baltz, 2016; Dhakal et al., 2016b; Luo et al., 2016; Katz et al., 2018]. As an example, heterologous expression and optimizations of the biosynthetic pathway for brasiliocardins have been employed in *Amycolatopsis*. This resulted in the increased production of BraC and the BraC-aglycon by 27%, a titer that is comparable to production from the native producer *N. terpenica* IFM0406 [Schwarz et al., 2018a; Schwarz et al., 2018b]. Furthermore, nargenicin A1 production has been successfully enhanced up to 24-fold in comparison to the control strain by the application of a combined overexpression and precursor feeding strategy [Dinesh et al., 2012; Dhakal et al., 2015; Dhakal et al., 2016a].

Many *Nocardia* strains combine properties from their sister genus *Rhodococcus* and the slightly more distantly related genus *Mycobacterium*, reflecting their need to survive in different ecological settings. Similar to *Rhodococcus*, *Nocardia* spp. are able to produce diverse hydrolytic enzymes and possess the metabolic versatility to degrade complex organic compounds [Luo et al., 2014a; Luo et al., 2014b]. This allows them to live successfully as highly flexible saprophytes in challenging, e.g., oil- or industrial waste-contaminated environments. On the other hand, *Nocardia* strains can cause pulmonary forms of nocardiosis that resemble tuberculosis in most clinical symptoms and radiological manifestations [Duggal and Chugh, 2020]. *Nocardia* primarily infect the lungs or cutaneous and lymphocutaneous tissues of immunocompromised patients. Such opportunistic behaviour is also known from pathogenic non-tuberculous mycobacteria, includ-

Fig. 1. Natural products from *Nocardia* spp. with immunosuppressant activity.

ing the *Mycobacterium avium* complex, *Mycobacterium kansasii*, *Mycobacterium abscessus* and *Mycobacterium ulcerans*. The common aspects of *Nocardia* and *Mycobacterium* pathogenesis have been attributed to a shared cell wall physiology containing layers of mycolic acid and arabinogalactan as well as more general virulence factors, e.g., catalase and superoxide dismutase enzymes, invasion-like proteins, phospholipase C, haemolysin and the cord factor [Beaman and Beaman, 1994; Vera-Cabrera et al., 2013; Zoropogui et al., 2013; Ji et al., 2020]. However, major parts of *Nocardia* infections remain poorly understood, including strain-specific virulence and progression of nocardiosis. It is noteworthy that a number of molecules, which are considered to be essential for survival and pathogenicity of *Mycobacteria*, are synthesized by biosynthetic machineries, which are usually associated with the secondary metabolism, i.e., PKS and NRPS. The main examples are mycolate-, phthiocerol-, tetrapeptide- and mycoketide-based cell wall glycolipids, various siderophores and the toxin mycolactone [Portevin et al., 2004; Stinear et al., 2004; Chopra et al., 2008; Matsunaga and Sugita, 2011; Yu et al., 2012]. Recently, the analysis of the first *Nocardia* genome sequences showed a high diversity of PKS- and NRPS-containing gene clusters, some of them appear to be conserved in all analysed strains, some of them are unique [Komaki et al., 2014]: the number of secondary metabolite pathways in *Nocardia* species is roughly similar to *Streptomyces* with an average of 36 BGCs per genome [Doroghazi and Metcalf, 2013; Mänle et al., 2020]. How the encoded chemistry exactly contributes to the general and strain-specific pathogenicity of *Nocardia* spp. is a focus of ongoing investigations. In the current review, *Nocardia* metabolites with cytotoxic, im-

munosuppressive, metal-chelating properties or vitally important functions, which may play a part in nocardiosis, will be summarized and discussed. So far unpublished data concerning the biosynthesis of these molecules obtained by detailed bioinformatic analysis will also be included.

Metabolites with Cytotoxic and Immunosuppressive Properties

For a long time, it has been speculated that cytotoxic and immunosuppressive properties of *Nocardia* secondary metabolites may play a role in infection, immune evasion and clinical manifestation of the disease. Mikami and co-workers have been the first to systematically exploit clinical *Nocardia* isolates as a source of pharmaceutically interesting natural products [Mikami, 2007]. Based on this assumption and by similar efforts of other research groups, a number of bioactive molecules could be isolated and characterized.

In the realm of immunosuppressants, *N. terpenica* IFM 0406 (formerly referred to as *N. brasiliensis*) was found to biosynthesize brasilicardins (A–D) as remarkable immunosuppressive entities. This unique molecular family is characterized by featuring a diterpene skeleton decorated with *N*-acetylglucosamine (GlcNAc), *L*-rhamnose, pyruvate and 3-hydroxybenzoate (3-HBA) units [Shigemori et al., 1998]. Outstandingly, brasilicardin A (shown in Fig. 1) selectively revealed antitumour activity with a striking pattern of differential cytotoxicity ($IC_{50} = 0.22 - 100 \mu\text{g/mL}$) against a broad panel of various tumour cell lines [Komaki et al., 1999; Komatsu et al., 2005]. Moreover, via a mouse mixed lymphocyte reaction as a simulating setup for allograft rejection, brasilicardin A

exhibits comparable IC₅₀ immunosuppressive readings to cyclosporine A, slightly lower than the ones of tacrolimus, thereby employing a possible new strategy of immunosuppression that modulates the amino acid transporter system L of T-lymphocytes [Usui et al., 2006]. In contrast, the immunosuppressive evaluation of brasilicardins B and C dropped approximately 50 times compared to brasilicardin A, while brasilicardin D did not afford such readings, pinpointing the necessity of the methoxy group at C-16, GlcNAc and 3-HBA for immunosuppressive activity. Due to the extraordinary potency of brasilicardins as promising immunosuppressants and the urgency to glean additional biological and pharmacological studies, many total synthetic trials were recalled to successfully de novo construct this molecular family to overcome their limited availability from natural sources [Anada et al., 2017]. Biosynthetically, the implementation of D-[1-¹³C] glucose in an incremental manner at C2, C6, C11, C15, C19, C21, C22 and C23 in the perhydrophenanthrene skeleton validated that geranylgeraniol was the precursor of the diterpene. Moreover, the amino acid moiety was found to possibly be derived from [3-¹³C] pyruvate via the non-mevalonate pathway. Cloning and inactivating a geranylgeranyl diphosphate synthase enabled the identification of the responsible gene cluster [Shigemori et al., 1999; Hayashi et al., 2008]. In a follow-up investigation, Schwarz et al. [2018a] filled the gaps of the putative roles of some genes located within the cluster. Bra0 as a putative dioxygenase in cooperation with Bra11 is envisioned to be responsible for methoxylation at C16 of the brasilicardins backbone. Furthermore, Bra12 was found to act as a vital positive regulator for the brasilicardin production, pinpointing an essential consideration for any future biotechnological setup of producing such entities.

The retrieval of brasilinolides (A–C) as multifaceted bioactive macrolides from *N. terpenica* (formerly *N. brasiliensis*) IFM 0406 provided further proof of the fascinating molecular chemotypes that *Nocardia* can assemble. Structurally, these architectures frame a glycosylated 32-membered polyhydroxy macrolactone with a tetrahydropyran hemiketal ring entrenched within the macrocycle and 2-deoxy-L-fucose (2dF) as a deoxysugar decoration. Biologically, this molecular family was found to address bio-versatile purposes as immunosuppressive, antitumour and antifungal bioactivities [Hideyuki et al., 1996]. Interestingly, brasilinolide A (shown in Fig. 1), besides being a potent immunosuppressant, displayed inherited chemo- and bio-tractability via its favourable physicochemical properties accompanied with a notable low cytotoxicity index. Differently, brasilinolide B did not

disclose any immunosuppressive effectiveness but antifungal and antibacterial activities, which has been speculatively referred to the structural variations in the unusual deoxysugar moiety [Tanaka et al., 1997b; Mikami et al., 2000]. Lacking both the 2dF and macrolide (C23-OH) motifs, brasilinolide C, in contrast to brasilinolide A, showed anticancer activity towards a murine lymphoma cell line. However, the suppressive effect on mouse mixed lymphocytes was lost, which was structurally attributed to the 23-O-malonyl and 3'-O-pentanoyl groups' significance in the immunosuppression [Komatsu et al., 2004]. In light of the scaffold biosynthesis of brasilinolides and their various impressive biological properties, the possible engineering of their type I modular PKS and decorating enzymes (C37-O-glycosylation, C23-O-[alkyl]malonyl acid esterification, C28–29 epoxidation, C16-hydroxylation and O-acylation/methylation of the sugar moiety) can be harnessed as a rationale for leveraging such bioactive chemical space and other related macrolides to be appended with different structural elements like tunable glycosyl patterns [Chiu et al., 2016].

Nargenicin (shown in Fig. 1) is a highly unusual polyketide macrolactone containing a rare oxa-bridged decalin core as its key structural feature [Celmer et al., 1980]. It has been isolated from a number of different *Nocardia* species, e.g., *Nocardia* sp. CS682, *N. argentesis* and *N. arthritidis*, but the genetic basis for the production of nargenicin has remained unknown until recently. Pidot et al. [2019] have identified the BGC and the iron- and α -ketoglutarate-dependent dioxygenase responsible for the formation of the ether bridge. Nargenicin targets the bacterial replicative DNA polymerase DnaE, but also inhibits cell proliferation and induces cell differentiation in acute myeloid leukemia cell lines, if co-administered with 1,25-dihydroxyvitamin D₃ or all-*trans*-retinoic acid [Kim et al., 2009]. Additionally, it has been reported to attenuate lipopolysaccharide-induced inflammatory responses and shown to protect cells from tacrolimus-induced DNA damage and apoptosis [Yoo et al., 2009]. The latter activity was at least partially due to scavenging of reactive oxygen species and, hence, suppression of the mitochondrion-dependent apoptotic pathway [Park et al., 2019]. Very recently, a nargenicin A1 derivative was identified as a potent anticancer agent [Dhakal et al., 2020]. It was shown to inhibit angiogenesis by downregulating endothelial VEGF/VEGFR2 signalling and the tumoural HIF-1 α /VEGF pathway [Han et al., 2020].

In complement to the intriguing immunosuppressive entities that *Nocardia* can offer, a wide chemical space with cytotoxic properties was validated as well. Examples

Fig. 2. Structures of representative cytotoxically active compounds.

of *Nocardia*-specific anthracycline antibiotics are nothramycin, SO-75R1 and the nocardicyclins [Mikami et al., 1992; Tanaka et al., 1997a; Momose et al., 1998]. In vitro antitumour activity of nocardicyclin A against the P388 leukemia cell line and its multi-drug-resistant derivative P388/ADR was shown [Tanaka et al., 1998]. DNA-damaging agents, such as anthracyclines, are promising candidate drug leads for cancer therapy, but their use is limited by low production titer, dose-related toxicities (e.g., cardiotoxicity), as well as development of drug resistance. These problems are still essential to overcome to provide novel therapeutic options [Bauermeister et al., 2016]. Further cytotoxic compounds from *Nocardia* spp. include the benz[a]anthraquinone antibiotics brasiliquinones A–D (shown in Fig. 2), produced by *N. brasiliensis* IFM 0089. Brasiliquinones were active against L1210 and KB tumour cells in vitro with IC_{50} values ranging from 0.55 to 3.4 $\mu\text{g/mL}$ [Nemoto et al., 1997; Tsuda et al., 1999]. Moreover, brasiliquinone C showed strong inhibitory activity against the epidermal growth factor (EGF) receptor kinase and some activity against *c-erbB-2* kinase, involved in cell proliferation. Brasilidine A (shown in Fig. 2) is an isonitrile-containing indole alkaloid which was obtained from the same *N. brasiliensis* strain. The molecule showed cytotoxicity against several tumour cell lines, e.g., the multidrug-resistant P388/ADM (IC_{50} , 0.56 $\mu\text{g/mL}$) and CHO/MDR (IC_{50} , 3.43 $\mu\text{g/mL}$) [Kobayashi et al., 1997]. *N. brasiliensis* IFM 0667 produces the geranylated benzenoid metabolites nocarasins A–C (shown in Fig. 2), which showed considerably cytotoxicity against human leukemia cell lines in vitro, with IC_{50} values of 0.91, 0.51 and

0.60 $\mu\text{g/mL}$, respectively [Tsuda et al., 1999]. These compounds exhibit promising bioactivity in vitro, but whether they represent good drug candidates for further clinical development remains to be explored.

Driven by the pathogenicity of several members of the genus *Nocardia*, *Nocardia tenerifensis* IFM 10554^T was cocultured with the mouse macrophage-like cell line J774.1, in an elegant attempt to replicate the initial infection state to induce the secondary metabolism. Interestingly, nocarjamide (shown in Fig. 3), a cyclic nonapeptide with Wnt signal-alleviating activities, was exclusively expressed under these conditions, exemplifying the need of non-classical coculture setups to unlock the silent biosynthetic machineries of *Nocardia* [Hara et al., 2018]. The identification of such Wnt signal-modulating secondary metabolites might contribute to untangling *Nocardia*'s pathogenicity, counting on the involvement of these signalling cascades in numerous crucial processes, such as the differentiation/proliferation of eukaryotic cells and various disease-underlying mechanisms like osteoporosis-pseudoglioma syndrome and Parkinson's ailment [Hara et al., 2018]. Although the encoding BGC for nocarjamide was not identified, it could be speculated that such chemistry derives from an NRPS assembly line. We have recently reported on a bioinformatic survey of the biosynthetic capacity of *Nocardia* spp. with a focus on secondary metabolites [Männle et al., 2020]. A revisit of our original data while preparing the current article revealed a putative biosynthetic pathway for the production of a nocarjamide-like compound in *N. tenerifensis* DSM 44704 (syn. IFM 10554). An overview of the gene cluster analysis is shown in Figure 4. The respective gene cluster encodes an NRPS machinery of nine modules featuring epimerase domains in modules 6 and 7 and methyltransferase domains in modules 3, 4 and 9. The A-domain specificities for the first and last domains are predicted to be for threonine and valine, respectively. Such properties would perfectly match the required functionalities to assemble the L-Thr-L-Val-L-NmetLeu-L-NmetPhe-L-Leu-D-Phe-D-Val-L-Ala-L-NmetVal nocarjamide peptide chain. Intriguingly, similar BGCs were found in several other *Nocardia* strains, e.g., *N. brasiliensis* ATCC 700358, *Nocardia vulneris* W9851, *Nocardia* sp. CS682 and *Nocardia suis* massiliense S-137. This finding, together with the specific conditions under which nocarjamide is produced, suggests a potential role in *Nocardia* pathogenesis. Still, further experiments are required to firmly establish the presumed genes-to-molecule link.

Another approach to unlock the hidden *Nocardia* chemistry was followed by the Khosla group. They were

Fig. 3. Recently identified bioactive molecules isolated from *Nocardia* spp., NOCAP (NOCardiosis-Associated Polyketide).

able to completely reconstitute and deorphanize a 3-MDa *trans*-AT PKS system that is predominantly found in clinical *Nocardia* isolates. The PKS was hence termed nocardiosis-associated polyketide synthase (NOCAP) [Kuo et al., 2016]. They were the first to describe the full in vitro reconstitution of an assembly line PKS that mainly contains *trans*-AT modules. The thereby synthesized compound consists of a substituted resorcylaldehyde moiety linked to a 15-carbon chain with 2 conjugated all-*trans* trienes separated by a stereogenic hydroxyl group. This molecule likely represents the aglycon of a yet to be discovered natural product which would be decorated with a 6-deoxy sugar (shown in Fig. 3). Further investigations of its biological role are underway [Yuet et al., 2020].

Putative Components of the Cell Envelope

Nocardia spp. encode a remarkable number of comparably simple pathways driven by ketosynthase biochemistry. One such example is the gene cluster for the biosynthesis of nocardiolactones (shown in Fig. 3) which has been found in 23 isolates [Mikami et al., 1999; Robinson et al., 2020]. An NltA/NltB heterodimer of ketoacyl-ACP synthase III (KASIII; FabH) homologs catalyzes the “head-to-head” Claisen condensation of two long-chain acyl-CoAs. Subsequent reduction by NltD generates a di-alkyl β -hydroxy acid which is converted by the adenylate-forming enzyme NltC to a di-alkyl β -lactone product. Such β -lactone synthetases were associated with the formation of various small molecules that act as inhibitors of hydrolytic enzymes [Wolf et al., 2017; Robinson et al., 2019]. A similar set of enzymes, for instance, is encoded in the BGC for lipstatin: LstA-D [Hochuli et al., 1987; Bai

et al., 2014]. Whereas lipstatin is an acylated nocardiolactone derivative with potent activity against the pancreatic lipase, other β -lactone-containing compounds are inhibitors of proteases, e.g., the proteasome [Kaysser, 2019]. Given their lipophilic nature, it is likely that nocardiolactones are components of the bacterial cell envelope and might thus contribute to the interaction with the host during *Nocardia* infections. Notably, the respective pathway has been found to cumulate in clinically relevant *Nocardia* strains.

In addition, *Nocardia* genomes are rich in monomolecular type I PKS. The clinically important pathogen *N. cyriacigeorgica* GUH-2, for example, harbours 5 BGCs with this organization (out of 19 predicted BGCs in total) [Zoropogui et al., 2013; Komaki et al., 2014]. Similar pathways in *Mycobacterium tuberculosis* were determined to participate in the synthesis of diverse surface-exposed glycolipids including the assembly of mycolic acids, mycocerosic acids and pthiocerols [Matsunaga and Sugita, 2011]. The most abundant of such glycolipids are trehalose-6,6'-dimyco-late (TDM), as well as the pthiocerol containing phenolic glycolipid (PGL) (shown in Fig. 5). Mycolic acid-containing glycolipids are known to affect the host immune system, e.g. by stimulation of cellular and humoral immunity, granuloma formation and anti-tumour activity. TDM, also named the cord factor, has been linked to intracellular survival of virulent *Nocardia* strains, thereby constituting potential virulence mechanisms. This may include favoured uptake into macrophages by activating the alternative complement pathway without triggering respiratory burst. And it was shown in in vitro systems that TDM potentially inhibits

N. tenerifensis DSM 44704

N. brasiliensis ATCC 70035

N. vulneris W9851

N. suismassiliense S-137

Nocardia sp. CS682

N. tenerifensis IFM10554
transvalencin A

N. farcinica W6977

N. carnea NBRC 14403

N. nova SH22a

N. puris DSM 44599

S. coelicolor A3(2)

Fig. 4. Biosynthetic gene cluster analysis for the production of nocarjamide-like compound (**a**) and transvalencin A (**b**).

Fig. 5. Glycolipids TDM (trehalose-6,6'-DiMyco-late) and PGL (phenolic glycolipid).

membrane fusion between the phagosome containing the bacterial cell and lysosomes of the host. Furthermore, TDM stimulates important effector cells in innate, early adaptive and both humoral and cellular adaptive immunity [Beaman, 1994; Ryll et al., 2001]. Indeed, the PKS cluster 1.1 of *N. cyriacigeorgica* GUH-2 is analogous to the mycolic acid BGC from *M. tuberculosis* and conserved in all *Nocardia* genomes [Männle et al., 2020; Blin et al., 2021]. Moreover, the type I PKS enzymes from clusters 1.10 and 1.13 show significant homology (48%) to pthiocerol and pthioceranic acid synthases PpsC and Pks2, respectively [Blin et al., 2021]. This suggests that these BGCs could be involved in glycolipid biosynthesis, too. Remarkably, most sequenced *Nocardia* strains contain at least 1 such monomolecular type I PKS cluster in addition to the mycolic-acid PKS. The PKS cluster 1.10 of *N. cyriacigeorgica* GUH-2 (and other *Nocardia*) also encodes a putative acyl-CoA dehydrogenase (ACAD) with homology to EpxF and MatG from the epoxomycin and matlystatin biosynthetic pathways, respectively [Schorn et al., 2014; Zettler et al., 2016; Leipoldt et al., 2017]. These enzymes are known to generate electrophilic groups for the production of potent small-molecule protease and proteasome inhibitors [Kaysser, 2019]. It could thus be speculated that the ACAD in cluster 1.10 might modify a CoA- or ACP-activated fatty acyl product of the encoded PKS, accordingly. Notably, *N. cyriacigeorgica* GUH-2 has been shown to inhibit proteasome activity and induce apoptosis in cells by a yet unidentified secreted small molecule [Loeffler et al., 2004; Barry and Beaman, 2007]. However, whether this activity correlates with product from cluster 1.10 has yet to be determined.

Siderophores

Since iron is an indispensable nutrient factor for most living systems, *Nocardia* spp. have developed their molecular entities to sequester such a valuable element from their diverse nutrient-limited niches [Dhakal et al., 2019]. Siderophores as Fe(III) scavengers are the most abundantly represented secondary metabolites from *Nocardia* with many biological properties. We conducted a first rough analysis within the antiSMASH BGC database [Blin et al., 2021] of relevant *Nocardia* strains, estimating that in general *Nocardia* genomes harbour on average at least two gene clusters for the production of siderophores. Iron is an essential element for pathogenic bacteria to survive and grow inside their human hosts. Therefore, the human body has invented a range of defence mechanisms that limit the availability of free iron to the invading pathogens. Most of the iron is tightly bound to specific proteins, such as haemoglobin, ferritin, or transferrin, thus severely restricting iron acquisition by the bacteria [Miethke and Marahiel, 2007]. To be able to access the iron pool inside a living system, microorganisms produce small molecules as high-affinity ferric iron chelators. Such siderophores form extracellular Fe(III) complexes with stabilities up to 300-times higher than, e.g., transferrin. Siderophores are thus considered important virulence factors for diverse pathogens including *Mycobacterium* spp. [Sritharan, 2000].

Over the last 25 years, suites of mycobactin-like siderophores, named nocobactins, have been disclosed from different pathogenic and non-pathogenic *Nocardia* spp. encompassing structurally variable subfamilies, e.g., the nocardimicins, amamistatins, BE-32030s, formobactin,

brasilibactin, or terpenibactins [Dhakal et al., 2019; Chen et al., 2020; Männle et al., 2020]. Their architectures commonly frame two units of lysine, a C-terminal cyclic and a central acyclic one, usually decorated with N^{ϵ} -OH and N^{ϵ} -acyl functional groups. In addition, the 2-OH-phenyl-(5/6)-methyl-oxazol(in)e moiety defines a further scaffold backbone unit. The variation in the substitution patterns of the oxazol(in)e, the phenyl residues and N^{ϵ} of both lysine units in tandem with the fatty acid nature affords a broad panel of molecular congeners supporting their cognate producers to cope with their environments [Hoshino et al., 2011; Dhakal et al., 2019]. Biosynthetically, a non-linear assembly mechanism is hired to generate such entities recruiting three NRPS modules and one PKS module. Exemplified by the nocobactin NA split gene cluster, NbtF in tandem with a stand-alone A-domain NbtT are assumed to condensate salicylic acid with threonine which in turn is heterocyclized into an oxazole unit. NbtD in synergy with NbtH and NbtG incorporates the decorated N^{ϵ} -lysine into the backbone. In parallel, NbtB coupled with NbtC is envisioned to biosynthesize the 3-hydroxy fatty acid fragment which is subsequently esterified with the last tethered lysine, mediated by NbtD. Finally, NbtE propagates the skeleton with another morphed N^{ϵ} -hydroxylysine unit which undergoes ϵ -caprolactamization [Hoshino et al., 2011]. It could be shown that the diverse subfamilies of the nocobactin-like siderophores originate from one gene cluster family which is highly conserved in almost all *Nocardia* strains. The structural differences derive from subtle changes and additions in the genetic and thus biosynthetic repertoire of the respective pathways [Männle et al., 2020].

In addition to their biological potencies which were assessed in cytotoxicity, antitumour and antimuscarinic contexts, nocobactin-like siderophores are assumed to be correlated with *Nocardia*'s pathogenicity during the progression of nocardiosis. Such an assumption was gleaned from their structural resemblance to the known virulence factor mycobactin from *M. tuberculosis*. Indeed, an $\Delta nbtE$ -deficient mutant of *N. farcinica* IFM 10152 was attenuated cytotoxically towards murine macrophage-like J774A.1 cells, proposing an association between nocobactin NA (shown in Fig. 5) production and the observed virulence [Hoshino et al., 2011]. Recently, nocobactin NA was identified in a screening campaign, searching for small-molecule immune suppressors secreted by *Nocardia* species. Arai et al. [2020] found that this compound inhibits Notch signalling in a dose-dependent manner with IC_{50} values of up to 12.4 μ M and decreased the expression of hairy and enhancer of split 1 (Hes1) protein.

Notch is an important regulator of immune cell development and function, and interference by the pathogen may serve as an immune evasion mechanism.

Actinomycetes strain Acta 3026, which was found to belong to the genus *Nocardia*, produces two cytotoxic metabolites, nocardichelins A and B. Both compounds show inhibition of several human tumour cell lines with GI_{50} values in a low micromolar to nanomolar range. The nocardichelins represent a new group of siderophores by combining structural elements of mycobactin/nocobactin-type siderophores from mycobacteria or *Nocardia* spp. and hydroxamate-type siderophores. Nocardichelins may thus facilitate similar virulence mechanisms [Schneider et al., 2007].

Besides nocobactins, further metal-chelating compounds have been isolated from *Nocardia* over the last decades. One example is JBIR-16 (shown in Fig. 6) from cultures of *N. tenerifensis* NBRC 101015 with structural resemblance to heterobactin from *Rhodococcus erythropolis* PR4 [Carrano et al., 2001; Mukai et al., 2009b; Bosello et al., 2013]. Notably, the genome of *N. tenerifensis* DSM 44704 (syn. NBRC 101015) encodes a heterobactin-like gene cluster with a trimodular NRPS system and a stand-alone adenylation (A) domain. The predicted product would be a X-Gly-Orn tripeptide decorated with dihydroxybenzoic (Dhb) acid moieties, perfectly fitting the JBIR-16 hOrn-Gly-Orn skeleton. The putative JBIR-16 BGC can be found in other *Nocardia*, e.g., *N. brasiliensis* ATCC 700358 and *N. vulneris* W9851. In the course of our genomic survey for secondary metabolite pathways in *Nocardia*, another gene cluster family was identified and also recognized as heterobactin-like, which is present in about one-third of the strains. Here, the putative pathways comprise a bimodular NRPS system with various and/or unknown specificities, a Dhb-specific A domain, oxidoreductases and a number of siderophore transporters. A corresponding compound has so far not been isolated, but given the widespread occurrence of the BGC, an important physiological function could be assumed. Another gene cluster family from *Nocardia* exhibits similarity to the pathway of the predicted metal-chelating molecule coelibactin from *Streptomyces coelicolor* A3 (2) [Bentley et al., 2002; Männle et al., 2020]. The proposed coelibactin structure consists of a phenoxazoline and two thiazoline moieties derived from the incorporation of salicylic acid (Sal), threonine or serine and two molecules of cysteine. The coelibactin-like BGCs from *Nocardia* spp. contains an additional Cys-specific NRPS module, another methyltransferase and, in some cases, a putative halogenase. Intriguingly, a compound that would match the

Fig. 6. Metal chelating compounds nocobactin NA and JBIR-16.

predicted product from this gene cluster family has been isolated previously: the thiazolidine zinc complex antibiotic transvalencin A produced by *N. transvalensis* IFM 10065 [Hoshino et al., 2004]. We would speculate that cluster 1.1 in *N. farcinica* W6977 and 1.10 in *N. carnea* NBRC 14403 may direct the formation of transvalencin A or at least a highly similar molecule (see Fig. 4) [Blin et al., 2021]. In contrast to the coelibactin BGC, the identified clusters in *Nocardia* do not feature a specific A domain for the activation of salicylic acid. However, all *Nocardia* strains that produce nocobactins contain such an enzyme to provide salicylic acid for nocobactin biosynthesis [Männle et al., 2020]. In fact, the respective gene, *nbtF*, is not co-located with the majority of the nocobactin BGC but can be found elsewhere on the genome in a small discrete BGC, also termed cluster II [Hoshino et al., 2011]. It might thus be possible that NbtF interacts with both the nocobactin and the putative transvalencin A pathway to channel the salicylic acid precursor and to control the siderophore profile in the respective *Nocardia* strains.

Antibiotics

Nocardia strains have been found to produce a variety of different antimicrobial compounds mainly as ribosomally synthesized and post-translationally modified peptides (RiPPs) or NRPS products. As such molecules are likely more important to establish the producing organism in its natural habitat in the environment than of clinical significance, we will only briefly summarize a few major examples. However, it has recently been shown by

Peschel and coworkers that antibiotic natural products may well play a role in disease-relevant competition processes in human microbiota [Zipperer et al., 2016].

Thiopeptides are macrocyclic RiPPs with several thiazole rings and multiple occurrences of dehydrated amino acid residues. The prominent class-defining feature of such a molecular family is the 6-membered nitrogenous ring that can exist in several oxidation states as a piperidine, dehydropiperidine or pyridine [Burkhart et al., 2017; Montalban-Lopez et al., 2020]. Nocardithiicin (shown in Fig. 7) is a thiocillin-similar thiopeptide produced by *N. pseudobrasiliensis* strain IFM 0757, structurally tailored with six thiazoles, two dehydrated amino acid residues besides a central heterocyclic frame. The highly bioactive peptide showed potent minimal inhibitory concentration (MIC) values at 0.025–6.25 µg/mL against clinical pathogens, such as rifampicin-resistant along with -sensitive *M. tuberculosis* strains [Mukai et al., 2009a; Sakai et al., 2015]. From *Nocardia* sp. ATCC 202099, further substantially altered thiopeptides, the nocardthiicins (shown in Fig. 7), were isolated with structural similarities to a nosiheptide indolic scaffold with distinctive functionalization, e.g., glycosylation with a rare sugar unit which improves their water solubility [Li et al., 2003; Bai et al., 2020]. Interestingly, nocardthiicins (I–III) displayed an impressive in vitro nanomolar potency against a broad spectrum of gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), multi-drug resistant *Enterococcus faecium* (MREF), and penicillin-resistant *Streptococcus pneumoniae* (PRSP). More importantly, in a systemic *S. aureus* infection mice model, nocardthiicins showed equivalent efficacy to vancomycin [Leet et al., 2003]. However, nocardthiicins have not yet proceeded to clinical application, largely due to solubility issues. A wide range of chemical modifications were made on different moieties to increase the solubility. The thereby generated derivatives maintained good activity at least in vitro, but in vivo activities were largely disappointing [Brown and Dawson, 2015]. Nevertheless, the most promising compound LFF571 [LaMarche et al., 2012] has successfully completed a phase II clinical trial for treatment of *Clostridium difficile* infections [Mullane et al., 2015]. So far, no phase III trial has been reported.

A hybrid lipidated lanthipeptide, lipolanthine, was recently isolated from *N. terpenica* IFM 0406 by a bioactivity-guided strategy in synergy with genome mining efforts. Nocavionin (shown in Fig. 7) represents the first example of a lipidated ribosomally synthesized peptide featuring an unusual decarboxylated motif termed as avionin. Even though so far no antibacterial evaluations for

Fig. 7. Structures of recently identified antibioticly active molecules.

nocavionin have been reported, its structural variant microvionin exhibited strong antibacterial effects with MICs <0.46 and $0.15 \mu\text{g/mL}$ against MRSA and *S. pneumoniae*, respectively [Wiebach et al., 2018].

Nocardicins (A–B) from *N. uniformis* subsp. *tsuyamanensis* are the smallest representatives of NRPS-based compounds containing a monocyclic β -lactam ring, alongside an oxime and double *p*-hydroxyphenylglycine units. They exhibit moderate potency against a broad variety of gram-negative bacteria and some β -lactamase-resistant species. Unexpectedly, the *in vivo* readings of nocardicin A were higher than *in vitro* upon its administration to mice infected with gram-negative bacilli [Aoki et al., 1976; Hashimoto et al., 1976; Mine et al., 1977; Nishida et al., 1977]. Townsend and co-workers conducted several studies on nocardicin biosynthesis, leading to a detailed understanding of the respective biochemical mechanisms [Hosoda et al., 1977; Townsend and Brown, 1982; Townsend and Brown, 1983; Townsend et al., 1983; Townsend and Salituro, 1984]. Genome sequencing revealed the nocardicin A BGC, in which the open reading frames to biosynthesize the nonproteogenic *p*-hydroxyphenylglycine precursor are arranged alongside the NRPS for the assembly [Gunsior et al., 2004; Davidsen et al., 2013].

Peptidolipin NA is a cyclic lipopeptide from the terrestrial *N. asteroides* in which cyclization is executed via an ester to an unusually long lipophilic tail. It was found to exert an antibiotic effect by interacting with the membrane lipid bilayers, generating ion-conducting pores. Recently, peptidolipins (B–F) were reported from a marine *Nocardia* strain WMMB215 with moderate bacteriostatic potency against MRSA [Maget-Dana et al., 1985; Wyche et al., 2012].

Furthermore, during our genomic and chemical exploration of *Nocardia* spp., we and others [Navarro-Muñoz et al., 2020] became aware that the genome of *N. terpenica* IFM 0406 contains the BGC coding for the outstanding anti-antibiotic detoxin (Fig. 8). Subsequent chemical analyses of the crude extract of IFM 0406, using high-resolution LC/MS and MS/MS experiments (Fig. 8a–f), confirmed that IFM 0406 is actually able to produce detoxin D1 and D3. The detoxin complex was originally isolated from *Streptomyces* spp. and is a selective antagonist of the antibiotic blastidicin S [Yonehara et al., 1968; Shimazu et al., 1981]. Thus, a subset of *Nocardia* species do not only produce antibiotics, they are also capable to antagonize the activity of antibiotics from other organisms.

Fig. 8. Biosynthetic gene cluster of detoxin in *S. mobarensis* NRRL B-3729 and *N. terpenica* IFM 0406 and proof of production of detoxins by *N. terpenica* IFM 0406. LC/MS-extracted-ion chromatograms for the pseudomolecular masses of detoxin D1 (**a**) and detoxin D3 (**b**), respectively. From accurate mass measurements, the sum formulae $C_{28}H_{42}N_3O_8$ (measured m/z 548.2964 $[M + H]^+$, D 0.5 ppm) and $C_{26}H_{40}N_3O_6$ (measured m/z 490.2905 $[M + H]^+$, D

0.7 ppm) could be delineated, which were in perfect agreement with those of detoxin D1 and D3, respectively. Further tandem MS experiments (**c**, **d**) and their interpretation (**e**, **f**) corroborated the chemical structure of detoxin D1 (**e**) and detoxin D3 (**f**). The bolded green line (**e**) highlights the structural difference between detoxin D1 and D3.

Conclusion

Natural products from *Nocardia* spp. are an important and continuously growing source for the discovery of diverse and effective bioactive molecules. The majority of the so far obtained secondary metabolites from *Nocardia* spp. are unique and exhibit a high degree of structural novelty; only occasionally the production of typical *Streptomyces*-derived compounds can be detected. To access the encoded chemistry, it is necessary to understand their natural triggers and to expand the genetic toolbox for *Nocardia*. The function of small molecules in bacterial pathogenesis has been established for entomopathogenic and phytopathogenic bacteria, including diverse *Photorhabdus*, *Xenorhabdus*, *Burkholderia*, *Pseudomonas* and *Streptomyces* species. In many human pathogens, siderophores and cell wall components are also well described as virulence factors. An increasing number of examples now show that other specifically produced bioactive compounds can also have considerable impact on the progression and severity of bacterial infectious diseases [Maglangit et al., 2020]. Although *Nocardia* spp. are known as clinically relevant opportunistic pathogens, the role of their prolific secondary metabolism in nocardiosis is not understood. As we have summarized in this review, the chemical arsenal of *Nocardia* exhibits diverse cytotoxic, immunosuppressive and -modulatory bioactivities which could be relevant for their survival and dissemination in the human body. However, if such a link actually exists, it has to be experimentally corroborated. With in-

creasing technical capabilities and interest in the field of chemical ecology and microbiology, the role of specialized molecules in *Nocardia* pathogenesis makes a fascinating subject for future investigations.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.E., H.S., and L.K. generated and analyzed the data. A.E., H.S., L.K., and H.G. wrote the manuscript.

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