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Biosynthesis of the Antibiotic Nematophin and Its Elongated Derivatives in Entomopathogenic Bacteria

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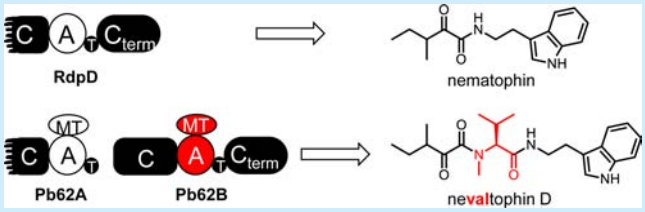
Supporting Information

ABSTRACT: Nematophin, a known antibiotic natural product against *Staphylococcus aureus* for almost 20 years, is produced by all strains of *Xenorhabdus nematophila*. Despite its simple structure, its biosynthesis was unknown. Its biosynthetic pathway is reported using heterologous production in *Escherichia coli*. Additionally, the identification, structure elucidation, and biosynthesis of six extended nematophin derivatives from *Xenorhabdus* PB62.4 carrying an additional valine are reported. Preliminary bioactivity studies suggest a biological role of these compounds in the bacteria−nematode−insect symbiosis.



Xenorhabdus is a well-studied genus of entomopathogenic bacteria that lives in symbiosis with *Steinernema* nematodes.[1,2] The nematode−bacteria complex infects and kills insect larvae so efficiently that they are applied in agriculture.[3] Recent research by us and others[4] has revealed *Xenorhabdus* to be a rich source for natural products with potent biological activities, such as insecticidal, antifungal, antimalarial, or cytotoxic properties.[5−7] Nematophin (**1**)[8] is one of the oldest compounds, described in 1997, and is produced by all strains of *Xenorhabdus nematophila*. It was identified as a potential drug candidate with strong antifungal and antibacterial bioactivities, especially against wild-type and drug-resistant strains of *Staphyloccous aureus* (MIC = 0.7 μg/mL).[8−11] Chemically **1** is a 3-indoleethyl 3′-methyl-2′-oxopentanamide consisting of an N-terminal α-keto acid and a C-terminal tryptamine (TRA), showing some structural similarity to the rhabdopeptide/xenortide peptides (RXPs) reported previously.[12−14] RXPs are non-ribosomally made linear peptides that contain nonpolar amino acids (L-Val, L-Leu, L-Phe), often with *N*-methylation as well as a C-terminal amine. The use of these different building blocks led to the generation of large libraries of RXPs in single strains.[14] Some RXPs display bioactivity against the human parasites causing certain tropical diseases[14] and are also cytotoxic against hemocytes of the model insect *Galleria mellonella*.[12] Because of the structural similarity between **1** and RXPs as well as its potent biological activity, our goal was the elucidation of its biosynthesis.

Here we report that in *X. nematophila* ATCC 19601, a monomodular non-ribosomal peptide synthetase (NRPS) is responsible for the biosynthesis of **1**. This NRPS, named RdpD, is closely related to the rhabdopeptide-producing NRPSs RdpABC but differs from them by the use of an α-keto carboxylic acid as a starting unit. Two related NRPSs encoding biosynthetic gene clusters have been identified in two other *Xenorhabdus* strains, leading to the identification of the nevaltophins, nematophin derivatives with an additional valine moiety and TRA or phenylethylamine (PEA) as the terminal amine.

Bioinformatic analysis revealed that the *X. nematophila* ATCC 19601 strain (GenBank accession code NC_014228.1) encodes two RXP-NRPS biosynthetic gene clusters (BGCs) involved in the production of xenortides[13] and rhabdopeptides[12] that were not responsible for the production of **1** as analyzed by heterologous expression (Figure 1a).[14] Interestingly, there is an additional gene, *rdpD* (XNC1_RS09410), located downstream of *rdpC*, and the cognate proteins share similar features (Figure S1). RdpD is a monomodular NRPS but contains an N-terminally truncated condensation (C) domain that might not be functional as well as an adenylation (A) domain with embedded methyltransferase (MT), a thiolation (T) domain, and a terminal C (C_{term}) domain. In order to investigate the function of RdpD, the corresponding gene *rdpD* was expressed in *Escherichia coli*, resulting in the production of **1** and its derivatives **2**−**4** (Figures 1a and S2 and Table S1) upon addition of the terminal amines

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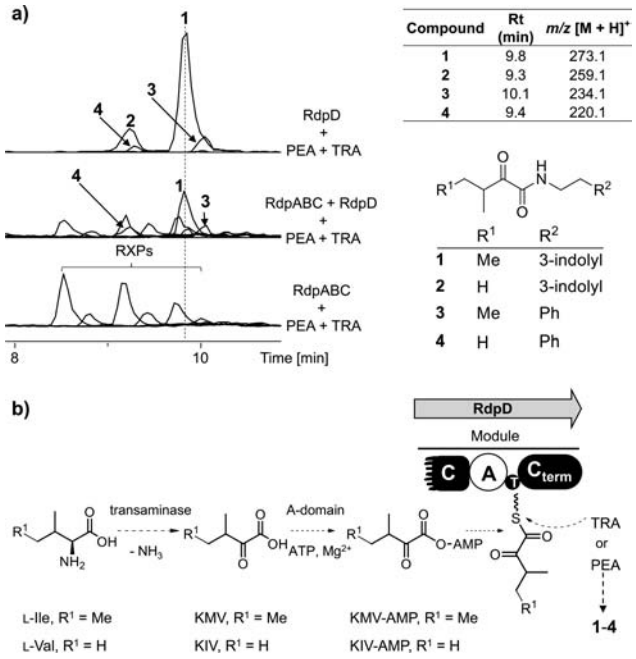

Figure 1. Heterologous production of nematophin (**1**) in *E. coli* and its proposed biosynthesis. (a) LC−MS analysis of LB culture for expression of RdpABC and RdpD from *X. nematophila*, together or alone in *E. coli* fed with TRA and PEA, produced **1** and its related compounds **2**−**4** and RXPs as shown in extracted ion chromatograms (EICs) (for details on rhabdopeptides, see Figure S4). (b) Proposed biosynthesis of **1** and its related compounds **2**−**4**. RdpD encodes a single NRPS module consisting of a truncated condensation (C) domain, an adenylation (A) domain, a thiolation (T) domain, and a C-terminal C (C_{term}) domain.

PEA and TRA at 0.25 mM. In *X. nematophila*, **1** is the only product (Figure S1) even when PEA or TRA is fed, and it is produced at a similar level (0.9−1.6 mg/L) as in *X. nematophila* (0.6 mg/L) (Figure S3a). Since crosstalk between the xenortide and rhabdopeptide RXP-NRPSs was described previously,[14] potential crosstalk between RdpABC (producing rhabdopeptides; Figure S4) and RdpD was studied, but no new products were detected (Figure 1a). Despite the close proximity of *rdpC* and *rdpD* in the genome, suggesting a recent duplication event, a phylogenetic analysis of their C_{term} domains revealed that they are not closely related and might belong to different subclades (Figure S5). The involvement of RdpD in the production of **1** was confirmed by analysis of an RdpD Ser911Ala mutation, which led to a loss of the attachment of the phosphopantetheinyl arm at the peptidyl carrier protein domain (Figures 1b and S1). Most likely **1**−**4** are composed of isoleucine- or valine-derived α-keto carboxylic acids, L-α-keto-β-methylvaleric acid (KMV) or L-α-ketoisovalerate (KIV), and TRA or PEA: the α-keto-carboxylic acids are activated by the A domain and are subsequently loaded onto the T domain, where nucleophilic attack by the amine catalyzed by C_{term} releases the nematophin derivatives (Figure 1b). Recent work on the substrate flexibility of the C_{term} domains in RXP-NRPSs[14] suggested that the C_{term} domain in RdpD can incorporate different amines that occur naturally in *Xenorhabdus* strains, including TRA and PEA, resulting also in PEA-containing derivatives (**3** and **4**) in minor amounts (Figure 1a). Thus, TRA might be the preferred substrate for RdpD.

As a part of our ongoing investigation of RXP-producing strains, an identical BGC (*pb62* and *mir*) was identified in the genomes of two strains of our in-house strain collection of entomopathogenic bacteria, *Xenorhabdus* PB62.4 (GenBank accession code KR871223) and *Xenorhabdus mirianensis* DSM17902 (GenBank accession code KR871228), that both also carry a broken $C_{starter}$ domain, as confirmed by antiSMASH[15] analysis. When the *pb62* gene cluster was studied in detail, it showed two monomodular NRPSs: Pb62A being similar to RdpD with a truncated N-terminal C domain, an A domain, an MT domain, and a T domain, and Pb62B, resembling a terminal module like RdpC with a complete C domain (Figure 2b). To gain insight into the biosynthesis of the RXPs derived from this cluster, a plasmid (pCX4) covering the *pb62* gene cluster was constructed and expressed in *E. coli*. LC−ESI-MS/MS analysis of the crude culture extracts of the resulting *E. coli* strain fed with either PEA or TRA revealed six new compounds **5**−**10** with *m*/*z* values between 319 and 386 $[M + H]^+$ whose sum formulas were

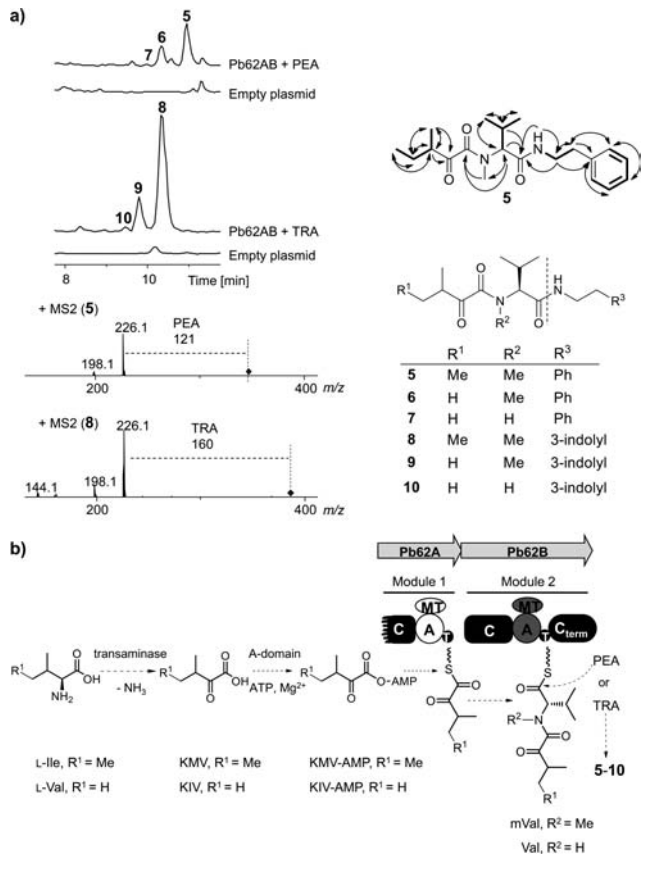

Figure 2. Heterologous production of new nevaltophins in *E. coli* and their biosynthesis. (a) LC−MS analysis of LB culture for expression of Pb62A and Pb62B from *Xenorhabdus* PB62.4 in *E. coli* fed with PEA and TRA, respectively. BPCs for nevaltophins A−F (**5**−**10**) and MS/MS data for the main compounds **5** and **8** (left) as well as their structures (right) are shown. COSY (bold) and HMBC (H → C) correlations observed in the NMR spectra are shown for **5** ($CDCl_3$, 500 MHz). (b) Proposed biosynthetic pathway for nevaltophins. Pb62A encodes an NRPS module consisting of a truncated C domain, an A domain with an embedded methyltransferase (MT) domain, and a T domain, while Pb62B encodes one NRPS module containing a C domain, an A−MT domain, a T domain, and a C_{term} domain. The first A domain (shown in white) activates KIV and KMV derived from L-Val and L-Ile, respectively, while the second A domain (gray) activates L-Val. The terminal C_{term} domain can incorporate PEA and TRA, respectively.

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determined by HR-ESI-MS (Table S1 and Figure 2a). Isotopic labeling experiments allowed the identification of their biosynthetic building blocks (Figures S6 and S7). In order to confirm the proposed structures, the main derivative **5** was isolated. The 1D (^{1}H, ^{13}C) and 2D (COSY, HSQC, and HMBC) NMR spectra of **5** were consistent with the presence of an α-keto amide as well as *N*-methylvaline and PEA units (Figures S8–S12). However, both the ^{1}H and ^{13}C spectra of **5** were complicated by overlapping signals, which suggested the presence of multiple diastereomers. Therefore, chemical synthesis of **5** was performed to facilitate its NMR assignment and to provide further material for bioactivity testing. Compounds **5** and **8** (Figures S13–S16) were synthesized by standard methods using racemic 3-methyl-2-oxopentanoic acid, providing a mixture of diastereomers that displayed spectroscopic properties identical to those of natural **5**. The complex NMR spectra of natural and synthetic **5** arise from the presence of multiple diastereomers due to keto–enol tautomerization and thus racemization of the 3-methyl-2-oxopentanoyl moiety.[16] Furthermore, slowly interconverting conformers at the *N*-methyl amide, consistent with those reported for the related peptides xenortides A and B from *X. nematophila*,[17] add to the complex NMR spectra of **5**. Finally, the absolute configuration of the *N*-methyl-L-valine residue was determined via the "advanced Marfey's method" (Figure S17), confirming the structure as shown in Figure 2a.

Compounds **5**, **6**, **8**, and **9** were also identified in the original producer PB62.4 (Figure S18), and they were named nevaltophins because of their formal insertion of Val into the nematophin structure. The production titer of **8** in *E. coli* (10.5 mg/L) was almost twice that in strain PB62.4 (5.8 mg/L) (Figure S3b).The structures of **5**–**10** suggest a biosynthesis pathway (Figure 2b) very similar to that of **1** but with the additional "insertion" of a valine moiety. The MT domain of Pb62A shows only one deviation from the conserved binding motif of LLEIGCGSLLL, and thus, it is not clear whether it is fully functional (Figure S19). However, since the substrate for the first A domain is not an amino acid, the MT function is not required anymore. A Ser1303Ala mutation in PB62A on the conserved Ser of the PCP domain resulted in no production of **5**–**10** but accumulation of **11** (Figures 3 and S20), confirming the proposed biosynthesis pathway. Very few non-ribosomal peptides containing α-keto acid building blocks have been reported to date. Examples are cereulide from *Bacillus cereus*,[18] valinomycin from *Streptomyces levoris* A-9,[19] barbamide from the marine cyanobacterium *Lyngbya majuscula*,[20] and quinone/furanone from the fungus *Suillus grevillei*.[21] A comparison of A domains that incorporate standard amino acids and those that incorporate α-keto acids revealed a specific replacement of the first amino acid residue, Asp235, with nonpolar residues in the binding pockets (Table S3). The presence of a nonpolar residue might increase the size of the binding pocket and might ensure that only α-keto acids and not amino acids are incorporated by the A domain.

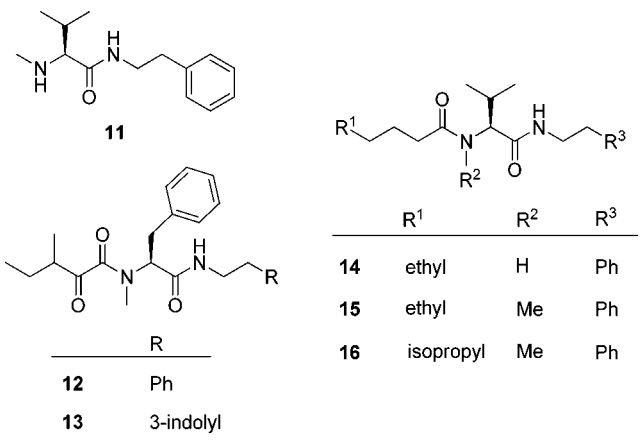

Figure 3. Chemical structures of compounds **11**–**16**.

Besides natural crosstalk between different RXP-producing NRPSs, artificial crosstalk has also been observed between different proteins, resulting in a variety of non-natural RXPs.[14] We therefore investigated whether Pb62A can act as a starting module for XndB involved in xenortide biosynthesis, and indeed, the formation of Phe-containing nevaltophins (**12** and **13**) was observed (Figures 3 and S21), whose structures were confirmed by chemical synthesis (Figures S22–S25).

To investigate the nevaltophin bioactivity and potential structure–activity relationships, we synthesized three further analogues, nevaltophin I–K (**14**–**16**), containing acyl chains instead of α-keto acids (Figures S26–S31). While crude extracts containing **1** showed the expected good antibiotic activity against Gram-positive *Micrococcus luteus*, **5**–**10** showed no antibacterial activity (Figure S32). Compounds **5** and synthetic **14**–**16** were also evaluated against the human parasites *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donnovani*, and *Plasmodium falciparum*, responsible for the tropical diseases sleeping sickness, Chagas disease, tropical sore, and malaria, respectively.

Their cytotoxicity was analyzed against rat skeletal myoblasts (L6 cells). As shown in Table S2, synthetic **14**–**16** having an N-terminal fatty acid chain displayed the best activity against *T. brucei*. Since a recent study suggested that RXPs act as inhibitors involved in the insect immune response, we performed a prophenoloxidase (proPO) assay as described previously.[4] PO is the main insect defense system leading to melanin deposition at any foreign organism in the insect hemolymph. Unexpectedly, neither natural **5** nor synthetic **14**–**16** acted as proPO inhibitors as described for the natural product rhabduscin but instead as proPO activators (Figure 4). This might suggest a decoy and countermeasure strategy similar to aircraft protection systems using decoy flares, where in *Xenorhabdus* the secretion of proPO inducers leads to melanization still far away from the toxin-producing bacterial cell, giving it a growth advantage. Alternatively, excessive melanization can also result in the accumulation of toxic byproducts and ultimately insect death,[22] while the bacteria themselves are protected by the potent PO inhibitor rhabduscin,[4] whose biosynthesis genes have been also found in strain PB62.4 (Figure S33).

Taken together, these results demonstrate for the first time the biosynthetic pathway of the antibiotic nematophin (**1**) in *X. nematophila* and close derivatives. In addition, we have also identified elongated nematophin derivatives (compounds **5**–**10**) and their corresponding gene cluster, *pb62AB*, from *Xenorhabdus* PB62.4. Heterologous production of these metabolites in *E. coli* allowed their isolation and structure elucidation via MS, isotopic labeling experiments, 1D and 2D NMR spectroscopy, and chemical synthesis. This study adds a new structural family to the arsenal of antimicrobial and anti-insect compounds employed by entomopathogenic *Xenorhabdus* bacteria.

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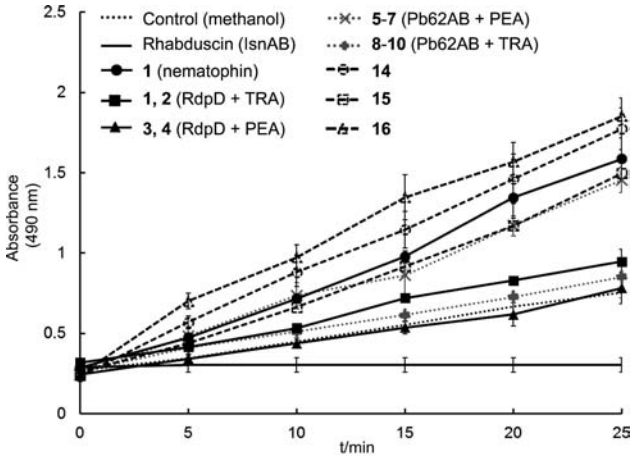

Figure 4. *In vitro* prophenoloxidase (proPO) activity assay. Methanol (negative control); IsnAB (heterologous expression of rhabduscin gene cluster, as a strong inhibitor of proPO[4]); RdpD + TRA/PEA (crude extracts from *E. coli* strains carrying *rdpD* fed with TRA or PEA, producing compounds **1**−**2**/**3**−**4**); Pb62AB + PEA/TRA (crude extracts from *E. coli* strains carrying *pb62AB* fed with PEA or TRA, producing compounds **5**−**7**/**8**−**10**); synthetic nematophin derivatives **14**−**16**. Data are shown as mean ± SD for $n = 3$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03796.

Experimental procedures and chemical synthesis of all compounds (PDF)

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Notes

The authors declare no competing financial interest.

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