Supporting Information

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

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Experimental procedures

Bacterial strains and culture conditions

All *Xenorhabdus* and *Escherichia coli* (*E. coli*) DH10BMtaA strains used in this study (Table S4) were cultivated on lysogeny broth (LB, pH 7.0) agar medium at 30 °C and grown in liquid LB with shaking at 200 rpm. When required, antibiotics were added at final concentrations of 34 µg/mL for chloramphenicol, 50 µg/mL for kanamycin, and 50 µg/mL for spectinomycin, respectively. *E. coli* DH10BMtaA was generated by integration of the phosphopantetheinyltransferase gene from the myxothiazol biosynthetic gene cluster (*mtaA*) into the genome of *E. coli* DH10B. This strain was used for direct routine cloning and as a heterologous host for production of nonribosomal peptides.

DNA manipulations

DNA manipulations, such as plasmid isolation from *E. coli* strains, DNA transformations, restriction digestion and DNA gel electrophoresis were performed according to standard protocols.[1] *Xenorhabdus* genomic DNA was isolated according to the instructions described in the QIAGEN kit. PCR amplifications were performed using Q5® High-Fidelity DNA Polymerase (New England Biolabs) or Phire Hot Start II PCR Master Mix (Thermo scientific) according to the manufacturer's protocol. PCR primers used in this study are listed in Table S5.

Construction of *E. coli* strains for heterologous production of nematophin and nevaltophins

The plasmids pCOLA-ara-tacI, and pCDF-ara-tacI were generated in this lab, and are used as expression vectors for cloning of gene clusters under the control of P_{BAD} promoter (Table S6). The plasmids used for production of new nematophin derivatives were constructed by cloning DNA fragments carrying either the *pb62* gene cluster into the above-described expression vectors, using Gibson Assembly Master Mix according

to the manufacturer's instructions. The DNA fragments with 25-30 bp of overlapping regions were obtained by PCR amplification from expression vectors and *Xenorhabdus* genomic DNA, respectively. The assembly reaction was performed by incubation at 50 °C for 60 min, then briefly digested with *Dpn*I for 1 h and quenched at 85 °C for 20 min. Finally, *E. coli* DH10BMtaA was transformed with the assembled DNA plasmid by electroporation. The Genbank accession number for *pb62AB* is KR871223. The Genbank accession number for *the isnAB* (rhabduscin biosynthesis) from *Xenorhabdus* PB62.4 and *X. miraniensis* DSM17902 are KY346861 and KY346862, respectively.

Point mutations on PCP domains of RdpD and Pb62A

Point mutations on PCP domains of RdpD and Pb62A were constructed by replacing the Ser residue on the conserved motif of LGGHSL with Ala.

Heterologous production of compounds in *E. coli*

The *E. coli* strains carrying plasmids containing desired gene or gene clusters were separately inoculated into 5 mL of liquid LB and grown overnight at 30 °C with shaking at 200 rpm. 100 µL of this overnight culture was transferred into 10 mL of fresh LB medium containing appropriate antibiotics, 0.1% arabinose (for induction), 1mM phenylethylamine (PEA) or tryptamine (TRA) and 2% (v/v) of Amberlite XAD-16 resin (Sigma-Aldrich). The culture was kept growing for 1 day at 30 °C and 200 rpm.

Culture extraction and LC-MS analysis of crude extracts from heterologous producers

After 24 h, bacterial cells and the XAD resin were harvested by centrifugation at 4000 rpm for 15 min, followed by resuspending pellets in 10 mL of methanol and rotating for 1 h. The methanol extraction was then collected via a paper filter and evaporated under reduced pressure. The dried residue was dissolved in 2 mL methanol and analyzed (20 µL injection volume) by ESI-HPLC-MS (Dionex UltiMate 3000 system coupled to a Bruker AmaZon X mass spectrometer) using a ACQUITY UPLC™ BEH C18 column (130Å, 2.1 mm × 100 mm, 1.7 µm particle size, Waters GmbH) and an aqueous acetonitrile (containing 0.1 % formic acid, v/v) gradient from 5 to 95% over 16 min at a flow rate of 0.6 mL/min. ESIMS spectra were recorded in a positive ion mode with scanning range of 100-1200 *m*/*z*.

Preliminary structure elucidation of nevaltophins via isotope labelling experiments

Isotope labelling experiments with heterologous producers of nevaltophins were performed as described previously.[2,3] Briefly, the production culture was individually fed with L-methionine-d_3, L-valine-d_8, L-leucine-d_{10} (Sigma-Aldrich) with a final concentration of 1 mM. For inverse feeding experiments[4] were also carried out, the overnight culture (1:100) was inoculated into fresh ISOGRO-^{13}C (Sigma-Aldrich) medium containing 10 mM K_2HPO_4, 10 mM KH_2PO_4, 8 mM $MgSO_4 \cdot 7H_2O$ and 90 µM $CaCl_2 \cdot H_2O$. The ISOGRO-^{13}C (Sigma-Aldrich) production culture was supplemented with 0.1% arabinose, 1 mM PEA or TRA, 2% of adsorbent XAD-16 resin and 1 mM L-valine, 1 mM L-leucine or 1 mM L-isoleucine (Carl-Roth), and grown at 30 °C and 200 rpm for 24 h. Methanol extraction of the XAD resin and subsequent LC-MS analysis were performed as described above.

Isolation of nevaltophin A (5)

To isolate **5**, 20 mL of a *E. coli* DH10B MtaA/pCX4 overnight culture was evenly divided into five 1 L Erlenmeyer flasks containing 400 mL LB medium containing kanamycin (50 µg/mL) and spectinomycin (100 µg/mL), each supplemented with 0.1% arabinose, 2 % (v/v) Amberlite XAD-16 resin (Sigma-Aldrich) and 1 mM PEA. These cultures were grown at 30 °C for 24 h with shaking at 200 rpm. The resulting mixtures of bacteria and XAD resin were harvested by centrifugation, then methanol (5 × 400 mL) was added and the mixtures shaken at room temperature for 1 h at 150 rpm. The mixtures were then filtered and the combined methanol extracts evaporated to dryness under reduced pressure. The crude residue (3.6 g) was dissolved in 15 mL water acidified with 50 µL of a 58% TFA solution (in water), and then extracted with ethyl acetate (3 × 20 mL). The combined organic layers were concentrated to dryness under reduced pressure. The residue (500 mg) was dissolved in MeOH (2 mL) and subjected to reverse phase preparative HPLC-MS (Waters, 515 HPLC 110 Pump, 2545 Binary Gradient Module, 2998 Photodiode Array Detector, SFO System Fluidics Organizer, Selector Value, 2767 Sample Manager coupled to a 3100 mass detector; XBridge C18 column, 5 µm, 4.6 mm × 250 mm) using a gradient of 40-60% aqueous MeOH over 30 min to yield **5** (Rt = 10.8 min; 8 mg/L). Nevaltophin A (**5**) purified from crude extracts as a yellow oil: ^{1}H ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) are identical to those for synthetic **5**; positive-ion ESIMS *m/z* 347; HRESIMS *m/z* 347.2327 $[M + H]^+$ (calcd for $C_{20}H_{30}N_2O_3$, 347.2335).

Activity testing against *Micrococcus luteus*

Crude extracts (0.6 mg) and nematophin (50 µg) dissolved in methanol were separately added to paper filters, then placed on an LB agar plate inoculated with *Micrococcus luteus.* The plate was incubated at 30 °C overnight to observe the development of inhibition zones.

Antiparasite and cytotoxicity assay

Bioactivity assays against *Trypanosoma brucei rhodesiense* (STIB900), *Trypanosoma cruzi* (Tulahuen C4), *Leishmania donovani* (MHOM-ET-67/L82), and *Plasmodium falciparum* (NF54) were conducted as described previously.[5] Cytotoxicity against the rat skeletal myoblasts (L6 cells) was assayed as described previously.[6]

***In vitro* prophenoloxidase (proPO) activation assay**

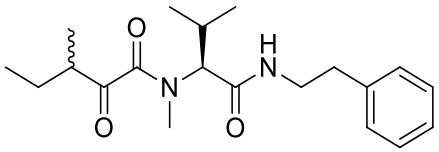
Galleria mellonella larvae were chilled at -20°C for 20 min. The frozen larvae were sterilized with 70% ethanol and thawed on ice, before being cut between the second proleg and tail and bled into a prechilled, sterilized 1.5 mL Eppendorf tube on ice. The collected hemolymph was diluted in a 3:1 ratio (v/v) with 50 mM phosphate buffered saline (PBS, pH 6.5) and centrifuged at 4000 g for 10 min at 4 °C to obtain hemolymph plasma. For each reaction mixture, 20 µL plasma was added to 120 µL PBS containing 5 µL *E. coli* lipopolysaccharide (LPS) (1 mg/mL; Sigma-Aldrich), then the mixture was left to stand at room temperature for 15 min. Finally, 20 µL compounds (5 mg/mL; crude extracts dissolved in PBS) were added, followed by addition of 25 µL 20 mM 4-methyl catechol (Sigma-Aldrich). The absorbance changes in the 96-well plate were measured at 490 nm by a Tecan microplate reader every 1 min for 1 h at room temperature with constant shaking as described previously.[7]

Phylogenetic analysis of C_{term} domains and MT domains

The alignment of protein sequences for terminal condensation (C_{term}) domain of RdpD as well as the C_{term} domains of RXP-NRPSs from seleted *Xenorhabdus* and *Photorhabdus* strains was generated using ClustalW of Geneious 6.1.8 and then applied to MEGA 6.0 to construct the phylogenetic tree using neighbor-joining analysis in a JTT (Jones-Taylor-Thorton) model with 1000 bootstraps, other parameters were set as default values. The same method was applied to construction of phylogenetic tree for MT domains in selected RXP-NRPSs.

General chemical methods

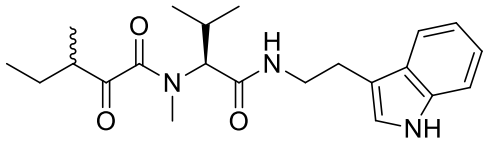
Anhydrous solvents were purchased from commercial suppliers and stored over activated molecular sieves. Reagents were purchased from commercial suppliers and used as received. Reactions were magnetically stirred and monitored by analytical thin layer chromatography (TLC) using aluminium-backed silica gel 60 pre-coated TLC plates, with visualization by $KMnO_4$ staining. Flash column chromatography was performed using silica gel 60. ^1H and ^{13}C NMR spectra were measured on a Bruker Avance 500 instrument (^1H NMR: 500 MHz, ^{13}C NMR: 125 MHz) at 298 K. NMR data are reported in δ (ppm) with $CDCl_3$ used as solvent and internal standard (^1H NMR: 7.26 ppm, ^{13}C NMR: 77.0 ppm). *J* values are reported in Hz with multiplicities given as follows: s = singlet; d = doublet; t = triplet; q = quartet; quin. = quintet; m = multiplet; br = broad. Melting points were acquired on a Krüss M5000 automatic melting point meter.



nevaltophin A (**5**)

Synthesis of nevaltophin A (5): To a stirred solution of *N*-Boc-*N*-methyl-L-valine (1 eq., 115 mg, 0.5 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.1 eq., 85.3 mg, 0.55 mmol) and ethyl (hydroxyimino)cyanoacetate (OxymaPure®, 1 eq., 71 mg, 0.5 mmol) in anhydrous dichloromethane (2.5 m L) at 0˚C under N_2 was added phenethylamine (1.5 eq., 95 µL, 0.75 mmol). After 5 min, the reaction mixture was warmed to room temperature and stirred for a further 20 h. The reaction mixture was then diluted with dichloromethane (5 mL), washed with 1M HCl and saturated solutions of $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. To cleave the Boc protecting group, a solution of HCl (2M) in diethyl ether (1 mL) was added and the reaction mixture stirred at room temperature for 20 h before being concentrated *in vacuo*. Finally, to a stirred solution of the resulting product, 3-methyl-2-oxopentanoic acid (1.1 eq., 41 mg, 0.32 mmol) and 2-bromo-1-ethyl-pyridinium tetrafluoroborate (1.1 eq., 88 mg, 0.32 mmol) in anhydrous dichloromethane (3.2 m L) at –10˚C under N_2 was added *N*,*N*-diisopropylethylamine (3 eq., 151 µL, 0.87 mmol). After 20 min, the reaction mixture was gradually warmed to room temperature and stirred for a further 20 h, before being diluted with dichloromethane (5 mL), washed with 1M HCl and saturated solutions of $NaHCO_3$ and brine, and finally dried over anhydrous Na_2SO_4 and

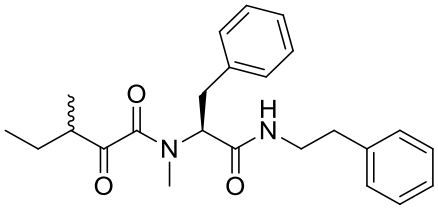
concentrated *in vacuo.* The residue was purified by flash chromatography (2% MeOH in DCM, silica gel 60) to yield **5** as a yellow oil (38.3 mg, 38%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.32-7.13 (m, 11H), 6.06 (br s, 1H), 4.32 (dd, *J* = 11.2, 0.8 Hz, 1H), 3.63 (dqd, *J* = 13.5, 6.8, 3.2 Hz, 1H), 3.56-3.40 (m, 4H), 3.30-3.18 (m, 1H), 3.00-2.92 (m, 1H), 2.90 (s, 3H), 2.82 (d, *J* = 3.1 Hz, 3H), 2.81-2.74 (m, 4H), 2.45-2.29 (m, 2H), 1.84-1.66 (m, 2H), 1.48-1.24 (m, 6H), 1.13 (dd, *J* = 13.6, 6.9 Hz, 3H), 1.10 (dd, *J* = 7.2, 3.0 Hz, 3H), 0.97-0.91 (m, 9H), 0.87-0.82 (m, 6H) and 0.78 (dd, *J* = 6.7, 1.5 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 205.8, 205.4, 203.4 (2C), 168.6 (2C), 168.5, 168.4, 168.0 (2C), 166.7, 166.5, 138.5 (2C), 128.7 (4C), 128.6 (2C), 128.5 (2C), 126.5, 126.4 (2C), 66.1, 65.9, 62.7, 44.4 (2C), 44.0, 43.8, 40.5 (3C), 35.7 (4C), 30.8, 28.2 (2C), 25.6, 25.4 (2C), 24.7, 24.5, 24.3 (2C), 19.7, 19.5 (2C), 18.8, 18.6, 18.4, 15.2, 14.0, 13.9 (2C), 11.7, 11.5 and 11.4 (2C) ppm; positive-ion ESIMS *m/z* 347; HRESIMS *m/z* 347.2331 $[M + H]^+$ (calcd for $C_{20}H_{30}N_2O_3$, 347.2335).



nevaltophin D (**8**)

Synthesis of nevaltophin D (**8**): To a stirred mixture of Boc-*N*-methyl-L-valine (1 eq., 38.5 mg, 0.17 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.1 eq., 35.1 mg, 0.18 mmol) and ethyl (hydroxyimino)cyanoacetate (OxymaPure®, 1 eq., 23.7 mg, 0.17 mmol) in anhydrous dichloromethane (1 m L) at 0˚C under N_2 was added tryptamine (1.5 eq., 40.1 mg, 0.25 mmol). After 5 min, the reaction mixture was warmed to room temperature and stirred overnight. The reaction mixture was then diluted with dichloromethane (5 mL), washed with 1M HCl and saturated solutions of $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. To cleave the Boc protecting group, a solution of HCl (2M) in diethyl ether (1 mL) was added and the reaction mixture stirred at room temperature overnight before being concentrated *in vacuo*. Finally, to a stirred solution of the resulting product, 3-methyl-2-oxopentanoic acid sodium salt (1.1 eq., 16.7 mg, 0.11 mmol) and 2-bromo-1-ethyl-pyridinium tetrafluoroborate (1.1 eq., 29.3 mg, 0.11 mmol) in anhydrous dichloromethane (1.1 mL) at 0˚C under N_2 was added *N*,*N*-diisopropylethylamine (3 eq., 50 µL, 0.29 mmol). After 20 min, the reaction mixture was gradually warmed to room temperature and stirred overnight, before being diluted with dichloromethane (5 mL), washed with 1M HCl and

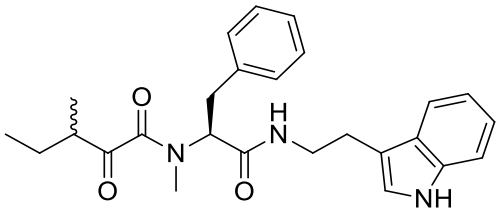
saturated solutions of $NaHCO_3$ and brine, and finally dried over anhydrous Na_2SO_4 and concentrated *in vacuo.* The residue was purified by Agilent HPLC system (30 min gradient from isocratic 50% Acetonitrile in H_2O, Agilent Eclipse XDB-C18 9.4 × 250 mm, 5 µm) to yield **8** as oil (7.6 mg, 32%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.07 (br d, *J* = 25.4 Hz, 2H), 7.61 (t, *J* = 8.6 Hz, 2H), 7.35 (dd, *J* = 10.6, 4.2 Hz, 2H), 7.23-7.15 (m, 2H), 7.15-7.08 (m, 2H), 7.02 (d, *J* = 2.2 Hz, 1H), 6.97 (d, *J* = 1.1 Hz, 1H), 6.07 (s, 1H), 4.33 (d, *J* = 10.9 Hz, 1H), 3.75-3.58 (m, 2H), 3.57-3.43 (m, 7H), 3.26-3.16 (m, 1H), 2.97 (dt, *J* = 9.9, 4.9 Hz, 5H), 2.90 (d, *J* = 2.3 Hz, 4H), 2.83 (d, *J* = 0.9 Hz, 3H), 2.38 (dtt, *J* = 18.4, 12.2, 6.2 Hz, 2H), 1.76 (dddd, *J* = 15.4, 13.4, 7.9, 5.0 Hz, 2H), 1.48-1.24 (m, 3H), 1.15-1.04 (m, 6H), 0.98-0.90 (m, 10H), 0.89-0.83 (m, 6H) and 0.79 (dd, *J* = 6.7, 1.4 Hz, 1H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 205.8, 205.5, 203.9, 168.7, 168.6, 168.2, 167.1, 166.9, 136.6, 127.3, 122.4, 122.4, 122.3, 122.2, 119.6, 119.5, 118.9, 118.8, 112.9, 112.6, 111.4, 111.3, 66.3, 66.2, 62.8, 51.0, 44.7, 44.2, 44.0, 39.7, 39.5, 30.9, 28.4, 28.3, 25.8, 25.5, 24.9, 24.7, 24.4, 19.9, 19.7, 19.6, 19.0, 18.8, 18.5, 15.3, 14.2, 14.0, 11.8 and 11.6 ppm; positive-ion ESIMS *m*/*z* 386; HRESIMS *m/z* 386.2438 $[M + H]^+$ (calcd for $C_{22}H_{32}N_3O_3$, 386.2444).



nevaltophin G (**12**)

Synthesis of nevaltophin G (**12**): To a stirred mixture of *N*-Boc-*N*-methyl-L-phenylalanine dicyclohexylamine salt (1 eq., 76.8 mg, 0.17 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.1 eq., 31.2 mg, 0.18 mmol) and ethyl (hydroxyimino)cyanoacetate (OxymaPure®, 1 eq., 23.7 mg, 0.17 mmol) in anhydrous dichloromethane (1 m L) at 0˚C under N_2 was added phenethylamine (1.5 eq., 32 µL, 0.25 mmol). After 5 min, the reaction mixture was warmed to room temperature and stirred overnight. The reaction mixture was then diluted with dichloromethane (5 mL), washed with 1M HCl and saturated solutions of $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. To cleave the Boc protecting group, a solution of HCl (2M) in diethyl ether (1 mL) was added and the reaction mixture stirred at room temperature overnight before being concentrated *in vacuo*. Finally, to a stirred

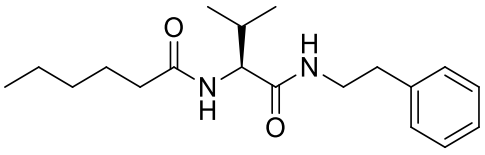
solution of the resulting product, 3-methyl-2-oxopentanoic acid (1.1 eq., 13.7 mg, 0.11 mmol) and 2-bromo-1-ethyl-pyridinium tetrafluoroborate (1.1 eq., 29.3 mg, 0.11 mmol) in anhydrous dichloromethane (1.1 m L) at 0˚C under N_2 was added *N*,*N*-diisopropylethylamine (3 eq., 50 µL, 0.29 mmol). After 20 min, the reaction mixture was gradually warmed to room temperature and stirred overnight, before being diluted with dichloromethane (5 mL), washed with 1M HCl and saturated solutions of $NaHCO_3$ and brine, and finally dried over anhydrous Na_2SO_4 and concentrated *in vacuo.* The residue was purified by Agilent HPLC system (25 min gradient from 55-63% Acetonitrile in H_2O; Agilent Eclipse XDB-C18 9.4 x 250 mm, 5 µm) to yield **12** as a yellow oil (9.5 mg, 22%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.31-7.24 (m, 7H), 7.24-7.18 (m, 7H), 7.15 (d, *J* = 7.8 Hz, 2H), 7.13-7.09 (m, 2H), 7.06 (d, *J* = 7.6 Hz, 2H), 6.00 (br s, 1H), 5.27 (ddd, *J* = 19.6, 9.6, 6.8 Hz, 1H), 4.14 (dd, *J* = 9.8, 4.7 Hz, 1H), 3.66 (tdd, *J* = 13.9, 7.1, 2.2 Hz, 1H), 3.57-3.49 (m, 1H), 3.48-3.41 (m, 1H), 3.35 (dd, *J* = 14.7, 6.7 Hz, 1H), 3.17 (dd, *J* = 14.5, 4.6 Hz, 1H), 3.04-2.93 (m, 2H), 2.88 (d, *J* = 2.8 Hz, 3H), 2.86-2.78 (m, 5H), 2.78-2.70 (m, 3H), 1.66-1.55 (m, 5H), 1.36-1.20 (m, 3H), 1.09-0.98 (m, 1H), 0.95 (dd, *J* = 9.7, 6.8 Hz, 3H) and 0.81 (td, *J* = 7.3, 2.5 Hz, 3H) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz) δ 205.8, 203.7, 203.6, 168.8, 168.5, 167.3, 167.2, 138.6, 137.4, 137.3, 136.6, 136.5, 129.3, 129.2, 129.1, 129.0, 128.9, 128.8, 128.8, 127.2, 127.1, 126.7, 62.4, 62.3, 57.1, 56.9, 44.5, 44.4, 43.9, 43.8, 41.0, 40.9, 35.8, 34.0, 33.5, 28.7, 28.6, 24.8, 24.2, 24.1, 23.9, 13.9, 13.7, 13.5, 11.8, 11.6, and 11.4 ppm; positive-ion ESIMS *m/z* 395; HRESIMS *m/z* 395.2309 $[M + H]^+$ (calcd for $C_{24}H_{31}N_2O_3$, 395.2335).



nevaltophin H (**13**)

Synthesis of nevaltophin H (**13**): To a stirred mixture of *N*-Boc-*N*-methyl-L-phenylalanine dicyclohexylamine salt (1 eq., 76.8 mg, 0.17 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.1 eq., 31.2 mg, 0.18 mmol) and ethyl (hydroxyimino)cyanoacetate (OxymaPure®, 1 eq., 23.7 mg, 0.17 mmol) in anhydrous dichloromethane (1 mL) at 0˚C under N_2 was added tryptamine (1.5 eq., 40.1 mg, 0.25 mmol). After 5 min, the reaction mixture was warmed to room temperature and stirred

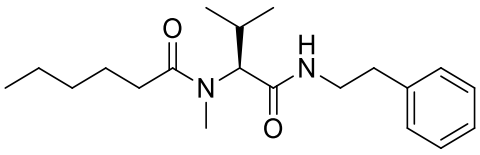
overnight. The reaction mixture was then diluted with dichloromethane (5 mL), washed with 1M HCl and saturated solutions of $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. To cleave the Boc protecting group, a solution of HCl (2M) in diethyl ether (1 mL) was added and the reaction mixture stirred at room temperature overnight before being concentrated *in vacuo*. Finally, to a stirred solution of the resulting product, 3-methyl-2-oxopentanoic acid (1.1 eq., 13.7 mg, 0.11 mmol) and 2-bromo-1-ethyl-pyridinium tetrafluoroborate (1.1 eq., 29.3 mg, 0.11 mmol) in anhydrous dichloromethane (1.1 mL) at 0˚C under N_2 was added *N*,*N*-diisopropylethylamine (3 eq., 50 µL, 0.29 mmol). After 20 min, the reaction mixture was gradually warmed to room temperature and stirred overnight, before being diluted with dichloromethane (5 mL), washed with 1M HCl and saturated solutions of $NaHCO_3$ and brine, and finally dried over anhydrous Na_2SO_4 and concentrated *in vacuo.* The residue was purified by Agilent HPLC system (25 min gradient from 55-63% Acetonitrile in H_2O, Agilent Eclipse XDB-C18 9.4 × 250 mm, 5 µm) to yield **13** as a yellow oil (7.6 mg, 16%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.04 (br d, *J* = 22.6 Hz, 2H), 7.59 (dd, *J* = 14.9, 7.9 Hz, 2H), 7.35 (dd, J = 8.1, 1.8 Hz, 2H), 7.30-7.24 (m, 2H), 7.23-7.17 (m, 6H), 7.12 (t, *J* = 7.5 Hz, 2H), 7.05 (d, *J* = 7.6 Hz, 2H), 6.99-6.92 (m, 2H), 5.99 (d, *J* = 4.5 Hz, 1H), 5.28 (ddd, *J* = 19.6, 9.9, 6.6 Hz, 1H), 4.13 (ddd, *J* = 9.9, 4.6, 1.6 Hz, 1H), 3.73-3.49 (m, 4H), 3.34 (dd, *J* = 14.7, 6.6 Hz, 1H), 3.16 (dd, *J* = 14.5, 3.8 Hz, 1H), 3.05-2.96 (m, 4H), 2.95-2.90 (m, 2H), 2.86 (d, *J* = 4.9 Hz, 3H), 2.78 (d, *J* = 5.9 Hz, 3H), 2.74-2.64 (m, 1H), 1.59 (dtt, *J* = 14.9, 13.6, 7.4 Hz, 2H), 1.33-1.18 (m, 2H), 1.07-0.95 (m, 1H), 0.92 (dd, *J* = 6.8, 3.4 Hz, 3H) and 0.87-0.72 (m, 6H) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz) δ 205.7, 204.0, 203.9, 168.6, 168.5, 168.4, 167.4, 167.3, 137.4, 137.3, 136.6, 136.5, 129.3, 129.1, 129.0, 128.8, 128.7, 127.3, 127.2, 127.0, 122.5, 122.3, 119.6, 118.8, 112.8, 112.7, 112.5, 111.4, 111.3, 62.5, 62.4, 56.9, 56.8, 51.0, 44.6, 44.5, 43.9, 43.89, 40.0, 39.6, 34.0, 33.5, 33.40, 30.9, 30.8, 28.7, 28.6, 25.4, 25.3, 24.8, 24.1, 23.9, 13.8, 13.7, 13.3, 11.8, 11.6, 11.50 and 11.4 ppm; positive-ion ESIMS *m*/*z* 434; HRESIMS *m/z* 434.2419 $[M + H]^+$ (calcd for $C_{26}H_{32}N_3O_3$, 434.2444).



nevaltophin I (**14**)

Synthesis of nevaltophin I (**14**): 182 mg of *N*-Boc-L-valine (1 eq., 0.84 mmol) was dissolved in 4 mL of anhydrous dichloromethane under argon. The solution was cooled

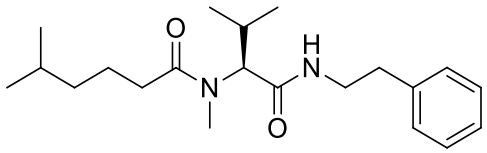
to 0°C then 177 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.1 eq., 0.924 mmol) and 120 mg of ethyl (hydroxyimino)cyanoacetate (OxymaPure®, 1 eq., 0.84 mmol) were added followed by 121 mg of phenylethyl amine (1.5 eq., 1.26 mmol). After 15 min, the mixture was warmed to room temperature and stirred overnight. Water (10 mL) was then added and the organic layer successively extracted with saturated solutions of $NaHCO_3$, brine, $NaHSO_4$ and brine. The dichloromethane was removed under reduced pressure, before addition of 10 mL 2M HCl in diethyl ether. The mixture was then stirred at room temperature overnight, and the resulting precipitate filtered and washed with diethyl ether. 5 mL of anhydrous dichloromethane was then added to the precipitate under argon, followed by 280 µL of diisopropylethylamine (2 eq., 1.68 mmol). The mixture was cooled to 0°C then 124 µL of hexanoyl chloride (1.05 eq., 0.88 mmol) was added slowly and the mixture stirred at room temperature overnight. The mixture was extracted twice with a saturated solution of $NaHCO_3$ and brine, then the solvent was removed under reduced pressure to yield 136 mg (60%) of **14** as a pale yellow solid: m.p. 162°C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.32-7.14 (m, 5H), 6.64 (br t, *J* = 5.3 Hz, 1H), 6.37 (br d, *J* = 8.8 Hz, 1H), 4.22 (dd, *J* = Hz, 1H), 3.57 (m, 1H), 3.43 (m, 1H), 2.81 (t, *J* = 7.2 Hz, 2H), 2.19 (m, 2H), 2.01 (dsextet, *J* = 13.9, 6.9 Hz, 1H), 1.68-1.52 (m, 2H), 1.35-1.23 (m, 4H) and 0.89 (m, 9H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.2, 171.4, 138.6, 128.6 (2C), 128.5 (2C), 126.5, 58.5, 40.6, 36.6, 35.6, 31.4, 31.1, 25.4, 22.3, 19.2, 18.3 and 13.9 ppm; positive-ion ESIMS *m/z* 319; HRESIMS *m/z* 319.2390 $[M + H]^+$ (calcd for $C_{19}H_{31}N_2O_2$, 319.2386).



nevaltophin J (**15**)

Synthesis of nevaltophin J (**15**): 64 mg of *N*-Boc-*N*-methyl-L-valine (1 eq., 0.276 mmol) was dissolved in 1.5 mL of anhydrous dichloromethane under argon. The solution was cooled to 0°C then 58 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.1 eq., 0.303 mmol) and 39 mg of ethyl (hydroxyimino)cyanoacetate (OxymaPure®, 1 eq., 0.276 mmol) were added followed by 121 mg of phenylethyl amine (1.5 eq., 0.404 mmol). After 15 min, the mixture was warmed to room temperature and stirred overnight. Water (5 mL) was then added and the organic layer successively extracted with saturated solutions of $NaHCO_3$, brine, $NaHSO_4$ and brine. The dichloromethane was

removed under reduced pressure, before addition of 4 mL of 2M HCl in diethyl ether. The mixture was stirred overnight at room temperature, and the resulting precipitate filtered and washed with diethyl ether. 2 mL of anhydrous dichloromethane was added to the precipitate under argon, followed by 95 µL of diisopropylethylamine (2 eq., 0.55 mmol). The mixture was cooled to 0°C then 39 µL of hexanoyl chloride (1.05 eq., 0.28 mmol) was added slowly and the mixture stirred at room temperature overnight, before extracted with saturated solutions of $NaHCO_3$ and brine. The solvent was then removed under reduced pressure to yield 66 mg (74%) of **15** as a pale yellow oil: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.32-7.14 (m, 5H), 6.35 (m, 1H), 4.48 (d, *J* = 11.2 Hz, 1H), 3.49 (m, 2H), 2.87 (s, 3H), 2.78 (m, 2H), 2.37-2.15 (m, 3H), 1.60 (m, 2H), 1.39-1.25 (m, 4H), 0.94-0.88 (m, 6H) and 0.80 (d, *J* = 6.7 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 174.4, 170.0, 138.7, 128.6 (2C), 128.3 (2C), 126.3, 62.4, 40.0, 35.6, 33.6, 31.4, 30.6, 25.5, 24.6, 22.4, 19.5, 18.4 and 13.9 ppm; positive-ion ESIMS *m/z* 333; HRESIMS *m/z* 333.2541 $[M + H]^+$ (calcd for $C_{20}H_{33}N_2O_2$, 333.2542).



nevaltophin K (**16**)

Synthesis of nevaltophin K (**16**): To a stirred solution of *N*-Boc-*N*-methyl-L-valine (1 eq., 115 mg, 0.5 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.1 eq., 85.3 mg, 0.55 mmol) and ethyl (hydroxyimino)cyanoacetate (OxymaPure®, 1 eq., 71 mg, 0.5 mmol) in anhydrous dichloromethane (2.5 mL) at 0˚C under N_2 was added phenethylamine (1.5 eq., 95 µL, 0.75 mmol). After 5 min, the reaction mixture was warmed to room temperature and stirred for a further 20 h. The reaction mixture was then diluted with dichloromethane (5 mL) and washed with 1M HCl and saturated solutions of $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. To cleave the Boc protecting group, a solution of HCl (2M) in diethyl ether (1 mL) was added and the reaction mixture stirred at room temperature for 20 h before being concentrated *in vacuo*. To a stirred solution of this product (84.8 mg) in anhydrous dichloromethane (1 mL) at room temperature under N_2 was added 5-methylhexanoyl chloride (1.4 eq., 73 mg, 0.49 mmol) prepared according to the literature in dichloromethane (1.5 mL) and *N*,*N*-diisopropylethylamine (2.7 eq., 170 µL, 0.98 mmol). The reaction mixture was stirred for 16 h before being washed with saturated solutions of

$NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated *in vacuo* to yield **16** as a yellow oil (90.8 mg, 72%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.33-7.13 (m, 13H), 6.14 (br m, 1H), 4.43 (d, *J* = 11.2 Hz, 1H), 3.50 (m, 4H), 2.85 (s, 3H), 2.78 (m, 4H), 2.75 (s, 3H), 2.43 (t, *J* = 7.4 Hz, 2H), 2.34-2.13 (m, 4H), 1.72-1.50 (m, 8H), 1.29-1.15 (m, 6H) and 0.89 (m, 20H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 174.5, 170.1, 169.6, 138.7, 128.7 (2C), 128.5 (2C), 126.4, 64.7, 62.6, 40.2, 40.0, 38.6, 38.0, 35.8, 35.7, 35.5, 33.9, 27.9, 27.7, 25.6, 22.8, 22.5, 22.4, 22.1, 19.6 and 18.5 ppm; positive-ion ESIMS *m/z* 346; HRESIMS *m/z* 347.2694 $[M + H]^+$ (calcd for $C_{21}H_{35}N_2O_2$, 347.2699).

Advanced Marfey's Analysis

Synthetic and authentic **5** (0.5 mg each) were separately hydrolysed by treatment with aqueous HCl (6M, 0.8 mL) in Ace pressure tubes at 110°C for 16 h. The resulting hydrolysates were concentrated to dryness and then resuspended in H_2O (100 μL). To 50 μL aliquots of this solution in black microcentrifuge tubes were added $NaHCO_3$ (1M, 10 μL) and *N*α-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide or -D-leucinamide (L-FDLA or D-FDLA, 100 μL of a 1% solution in acetone), before heating at 40°C for 1 h. The reaction mixtures were then cooled to room temperature, quenched (1M HCl, 10 μL) and concentrated to dryness. The residues were dissolved (MeOH, 400 μL) and analysed by RPHPLC (flow rate of 0.6 mL/min, gradient of aqueous acetonitrile (ACN) containing 0.1% formic acid with the following program: 0-2 min, 5-20% ACN; 2-4 min, 20% ACN; 4-38 min, 20-60% ACN; 38-40 min, 60-95% ACN; 40-45 min, 95% ACN; 45-47 min, 95-5% ACN; 47-50 min, 5% ACN) coupled to positive and negative ion ESI-MS (Figure S16). Authentic standards derived from commercial *N*-Me-L-Val were also prepared using the above-described procedure, with the omission of the hydrolysis step.

Quantification of production titer

Compounds **1** and **8** are the main nematophin and nevaltophin derivatives produced in both wild type strains and heterologous expression strains, which were chosen as standards for the quantification of compounds **1**-**10**. The standard compounds were prepared at different concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0.039 μg/mL) and measured by LC-MS. The peak area for each compound at different concentration was calculated using Bruker Compass Data Analysis program to generate the equations for compounds **1** and **8** with y = 3E+08x (R^2 = 0.9971) and y = 8E+07x (R^2

= 0.9929), respectively. The samples of crude extract from each strain were prepared as described above and analyzed by LC-MS. The peak area of expected compound was obtained and its corresponding production titer was calculated based on the equation generated from standard compound.

Supplementary Tables

Table S1. HR-Masses and chemical formula of compounds in this study

Compound	Calculated mass $[M+H]^+$	Detected mass $[M+H]^+$	Chemical formula	RDB	Delta ppm
1	273.1598	273.1595	$C_{16}H_{21}O_2N_2$	7.5	0.9
2	259.1441	259.1438	$C_{15}H_{19}O_2N_2$	7.5	1.1
3	234.1494	234.1495	$C_{14}H_{20}O_2N$	5.5	0.8
4	220.1696	220.1694	$C_{14}H_{22}ON$	4.5	1.1
5	347.2323	347.2329	$C_{20}H_{31}O_3N_2$	6.5	1.8
6	333.2173	333.2169	$C_{19}H_{29}O_3N_2$	6.5	1.1
7	319.2016	319.2012	$C_{18}H_{27}O_3N_2$	6.5	1.3
8	386.2435	386.2438	$C_{22}H_{32}O_3N_3$	8.5	0.7
9	372.2282	372.2277	$C_{21}H_{30}O_3N_3$	8.5	1.2
10	358.2486	358.2489	$C_{21}H_{32}O_2N_3$	7.5	0.8
11	235.1805	235.1806	$C_{14}H_{23}ON_2$	4.5	-0.4
12	395.2329	395.2326	$C_{24}H_{31}O_3N_2$	10.5	0.8
13	434.2438	434.2432	$C_{26}H_{32}O_3N_3$	12.5	1.4
14	319.2380	319.2384	$C_{19}H_{31}O_2N_2$	5.5	-1.2
15	333.2537	333.2528	$C_{20}H_{33}O_2N_2$	5.5	2.6
16	347.2693	347.2688	$C_{21}H_{35}O_2N_2$	5.5	1.4

Table S2. Bioactivity assay of compounds against selected tropical pathogens

Pathogen	Disease	IC_{50} (mg/mL) of compounds against different parasites					
		1	**5**	**14**	**15**	**16**	**Positive control**
Trypanosoma brucei rhodesiense	Sleeping sickness	34.5	14.5	1.3	5.2	3.7	0.003 (melarsoprol)
Trypanosoma cruzi	Chagas	47.3	35.9	28.7	16.7	14.3	0.665 (benznidazole)
Leishmania donovani	Cutaneous leishmaniasis	17.1	12.7	9.4	6.5	4.5	0.056 (milterfosine)
Plasmodium falciparum	Malaria	27.1	13.1	11.5	7.4	4.4	0.002 (chloroquine)
Cytotoxicity		49.3	49	30.3	18.1	14.1	0.009 (podophyllotoxin)

Table S3. A domain specificity-conferring codes

A domain specificity-conferring codes											
Variability	3%	16%	16%	39%	52%	13%	26%	23%	26%	0%	**Specificity**
Position	235	236	239	278	299	301	322	330	331	517	
Pb62A-A	V	A	I	M	L	A	A	S	M	K	α-ketoisovaleryl
RdpD-A	V	A	I	M	L	G	A	S	M	K	α-ketoisovaleryl
BarE-A	V	G	I	L	V	G	G	T	M	K	α-ketoisocapryl
CesA-A1	V	G	V	W	V	G	T	S	G	K	α-ketoisocapryl
CesB-A1	V	G	F	W	V	A	V	S	D	K	α-ketoisovaleryl
Vlm1-A1	A	A	L	W	I	A	V	S	G	K	α-ketoisovaleryl
Vlm2-A1	V	V	I	W	I	A	E	N	M	K	pyruvate
GreA	V	A	E	F	S	G	G	A	C	K	4-hydroxyphenylpyruvic acid
Pb62B-A	D	A	L	V	L	A	V	S	I	K	Val
BacA	D	G	F	F	L	G	V	V	Y	K	Ile
FenB	D	A	F	F	Y	G	I	T	F	K	Ile

Table S4. Bacterial strains used in this study

Strain	**Relevant Genotype**	**Reference/Strain No.**
E.coli		
DH10BMtaA	F– *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, *end*A1, *ara*D139, Δ(*ara leu*)7697, *gal*U, *gal*K, *rps*L, *nup*G, λ– , *entD::mtaA*	This lab
Xenorhabdus		
X. nematophila ATCC 19601	Wild type	This lab
RdpD-PCP_{Ala}	Point mutation on RdpD-PCP domain in wild type	This study
PB62.4	Wild type	This lab

Table S5. Primers used in this study

Primer name	Sequence (5'-3')	Targeting DNA fragment
XC65-Fw	ATGAAAAATGCAATTCAAATTATTAATG	*rdpD* gene from *X. nematophila* ATCC 19601
XC65-Rv	TTAGTCATAAAAATGATGGATGCTCTC	
XC66-Fw	AAGAGAGCATCCATCATTTTTATGACTAACAATTAATCATCG GCTCGTATAATG	pCDF-ara-tacI vector backbone for *rdpD* (pCX47)
XC66-Rv	ATTAATAATTTGAATTGCATTTTTCATGGAATTCCTCCTGTTA GCCCAAAAAAAC	
XC7-Fw	ATGAAAAATGCAGCACAAATGTTC	*pb62* gene cluster from *Xenorhabdus* PB62.4
XC7-Rv	TCAGTGATAAAAATGACTAATAC	
XC8-Fw	AAAGAAAGTATTAGTCATTTTTATCACTGACAATTAATCATC GGCTCGTATAATG	pCOLA-ara-tacI vector backbone for pCX4
XC8-Rv	AAAGAAAGTATTAGTCATTTTTATCACTGACAATTAATCATC GGCTCGTATAATG	
XC7-Fw	ATGAAAAATGCAGCACAAATGTTC	*pb62A* in *pb62* gene cluster from *Xenorhabdus* PB62.4
XC50-Rv	TCATATATCACCTTCCAATAGTTCC	
XC50-Fw	ATGCTGGAACTATTGGAAGGTGATATATGACAATTAATCATC GGCTCGTATAATGTGTG	pCOLA-ara-tacI vector backbone for pCX34
XC8-Rv	AAAGAAAGTATTAGTCATTTTTATCACTGACAATTAATCATC GGCTCGTATAATG	
XC51-Fw	ATGAGAAATGCAGCACAAATTATTAATG	*pb62B* in *pb62* gene cluster from *Xenorhabdus* PB62.4
XC7-Rv	TCAGTGATAAAAATGACTAATAC	
XC8-Fw	AAAGAAAGTATTAGTCATTTTTATCACTGACAATTAATCATC GGCTCGTATAATG	pCDF-ara-tacI vector backbone for pCX35
XC51-Rv	ATTAATAATTTGTGCTGCATTTCTCATGGAATTCCTCCTGTT AGCCCAAAAAAACG	
XC7-Fw	ATGAAAAATGCAGCACAAATGTTC	Ser in the conserved motif of LGGHSL from PCP domain of Pb62A was replaced by Ala, the encoding sequence was assembled into pCOLA-ara-tacI for pCX68
XC89-Rv	AGGCGTGTGGCTTGTAATGCATGCCCGCCCAG	
XC89-Fw	ACGCTGGGCGGGCATGCATTACAAGCCACACG	
XC50-Rv	TCATATATCACCTTCCAATAGTTCC	
XC50-Fw	ATGCTGGAACTATTGGAAGGTGATATATGACAATTAATCATC GGCTCGTATAATGTGTG	
XC8-Rv	AAAGAAAGTATTAGTCATTTTTATCACTGACAATTAATCATC GGCTCGTATAATG	

Table S6. Plasmids used in this study

Plasmid	Description	References
pCOLA-ara-tacI	3,345 bp, modified from pCOLA_tacI/I that contains arabinose-inducible promotor and kanamycin resistance gene (Km^R)	This lab
pCDF-ara-tacI	3,404 bp, modified from pCDF_tacI/I that contains arabinose-inducible promotor and spectinomycin resistance gene (Sm^R)	This lab
pCX41	18,643 bp, *rdpABC* gene cluster from *X. nematophila* ATCC 19601 genomic DNA assembled into pCOLA-ara-tacI, Km^R	[3]
pCX47	7,577 bp, *rdpD* from *X. nematophila* ATCC 19601 genome assembled into pCDF-ara-tacI, Sm^R	This study
pCX4	16,048 bp, *Pb62AB* from *Xenorhabdus* PB62.4 genome assembled into pCOLA-ara-tacI, Km^R	This study
pCX34	7,464 bp, *pb62A* from *Xenorhabdus* PB62.4 genome assembled into pCOLA-ara-tacI, Km^R	This study
pCX35	9,424 bp, *pb62B* from *Xenorhabdus* PB62.4 genome assembled into pCDF-ara-tacI, Sm^R	This study
pCX55	9,368 bp, *xndB* from *xnd* gene cluster, assembled into pCDF-ara-tacI, Sm^R	[3]
pCX68	7,407 bp,Ser in the conserved motif of LGGHSL from PCP domain of Pb62A was replaced by Ala, the encoding sequence was assembled into pCOLA-ara-tacI, Km^R	This study

Km^R : kanamycin resistance; Sm^R: spectinomycin resistance

Supplementary Figures

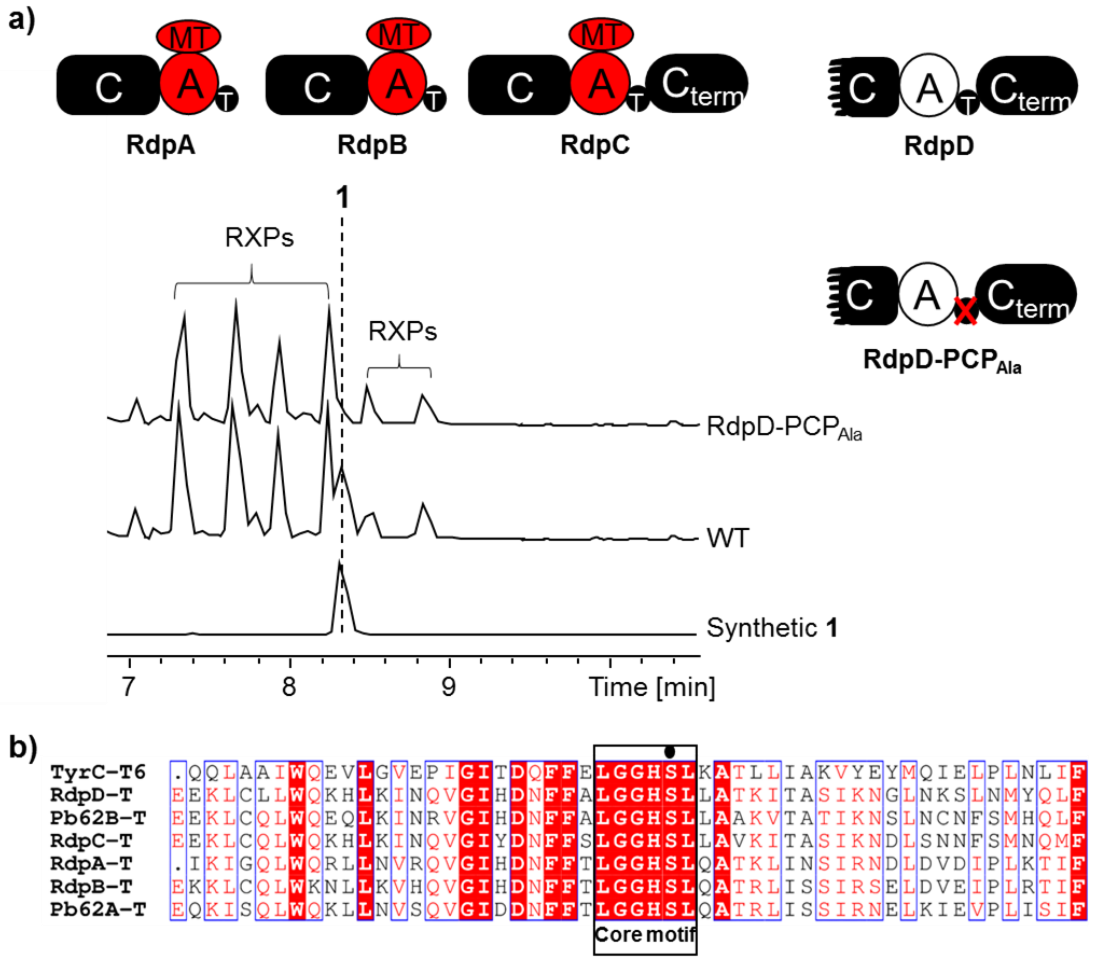


Figure S1. Construction of RdpABCD and analysis of RdpD-PCP mutation. a) General compositions of RdpABC, RdpD and RdpD-PCP mutation as well as comparison of BPCs from LC-MS analysis of *X. nematophila* HGB081 (WT), RdpD-PCP mutant and synthetic nematophin (**1**). b) Alignment of PCP domains from selective NRPSs, the conserved motif of PCP domain was shown, Ser was replaced by Ala to generate RdpD-PCP mutation.

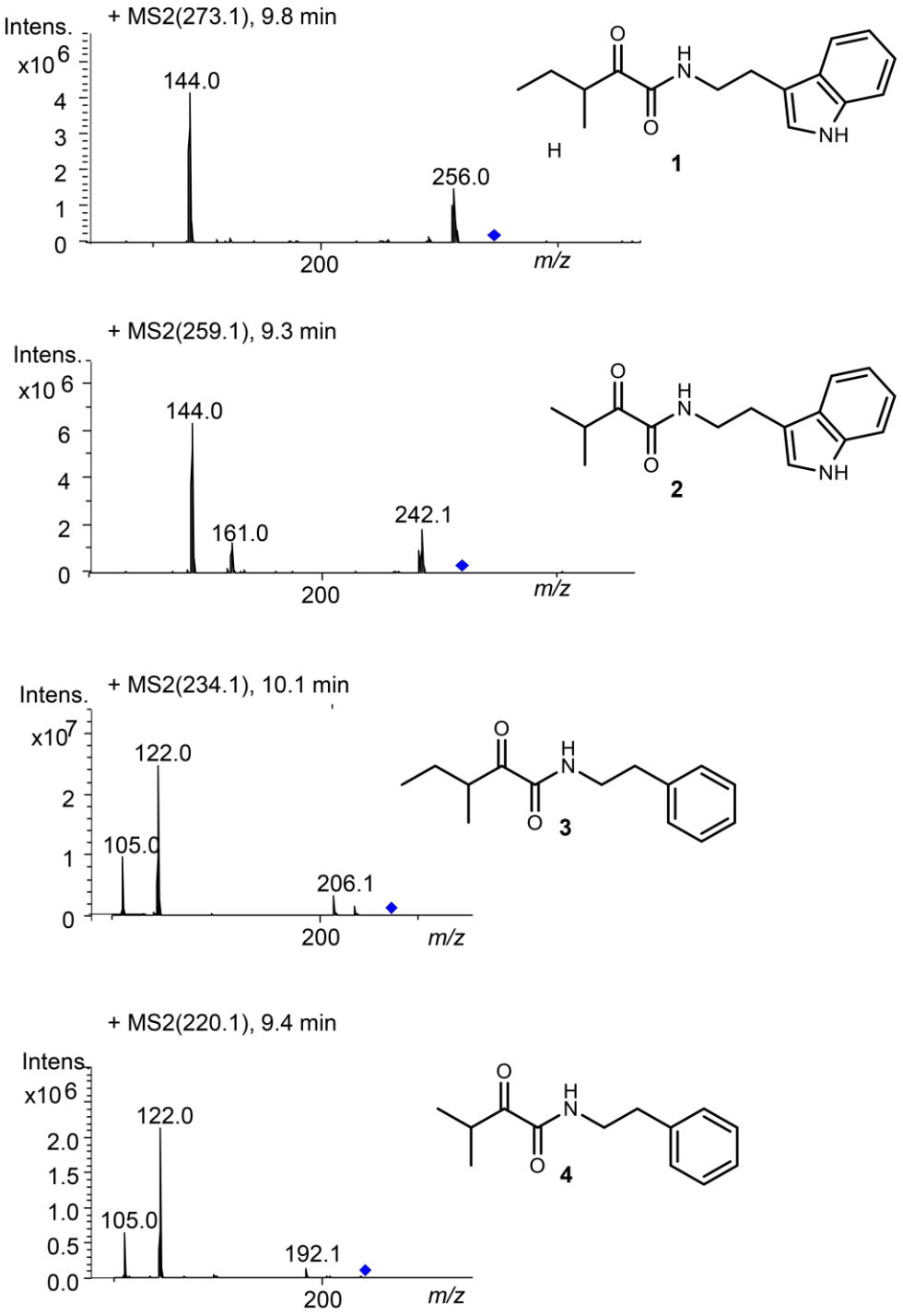


Figure S2. LCMS-MS fragmentations of compounds **1**-**4**.

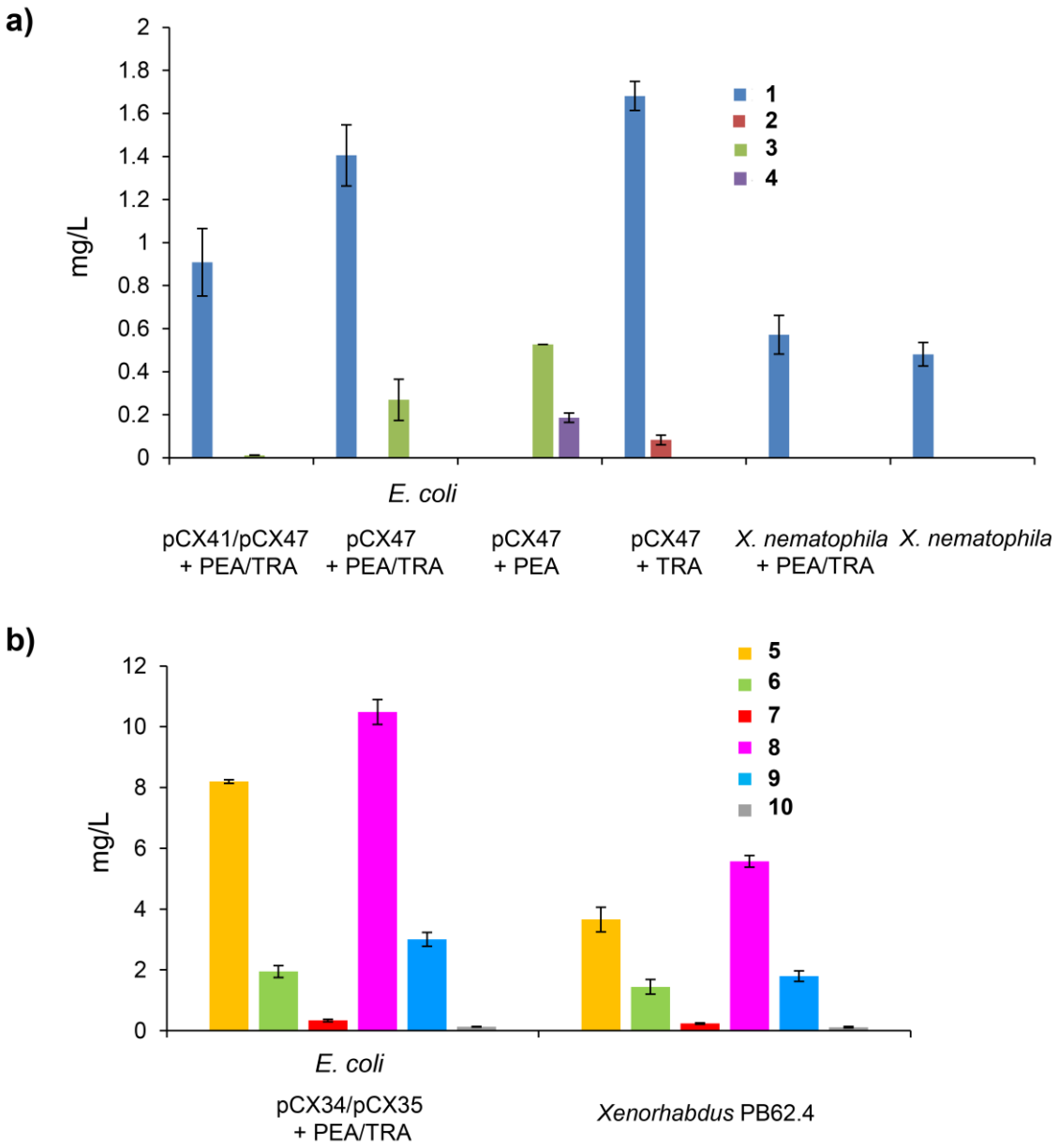


Figure S3. Comparision of production titer of compounds **1**-**10** in original *Xenorhabdus* producers and heterologous expression strains (*E. coli*). a), Quantification of production level for compounds **1**-**4** produced in both heterologous *E. coli* MtaA strains and *X. nematophila* HGB081 (against **1** as standard); b), Quantification of production level for compounds **5**-**10** produced in both heterologous *E. coli* MtaA strain and *Xenorhabdus* PB62.4 (WT) (against **8** as standard).

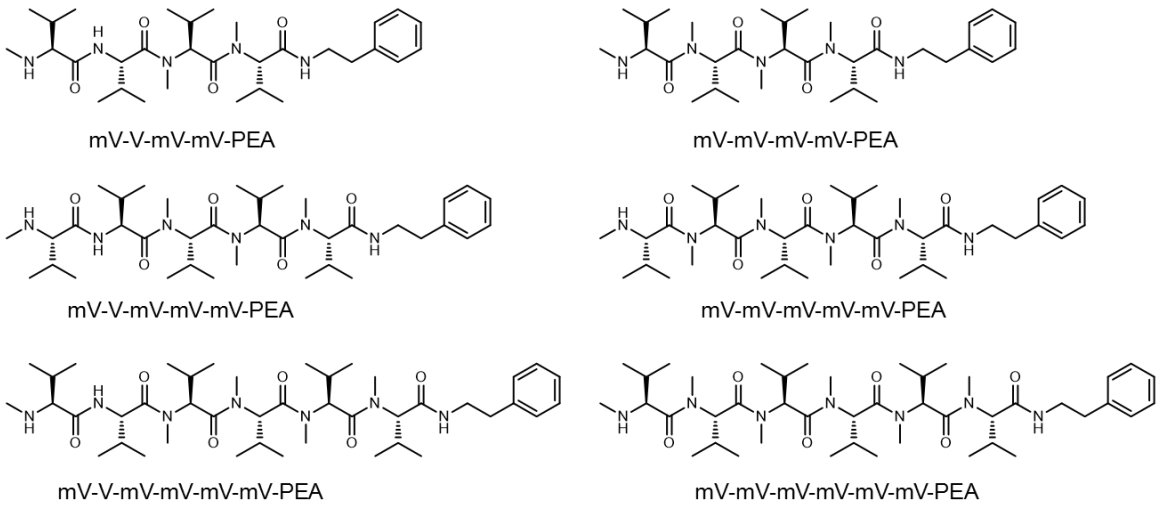


Figure S4. Chemical structures of selected rhabdopeptides from *X. nematophila*.[3]

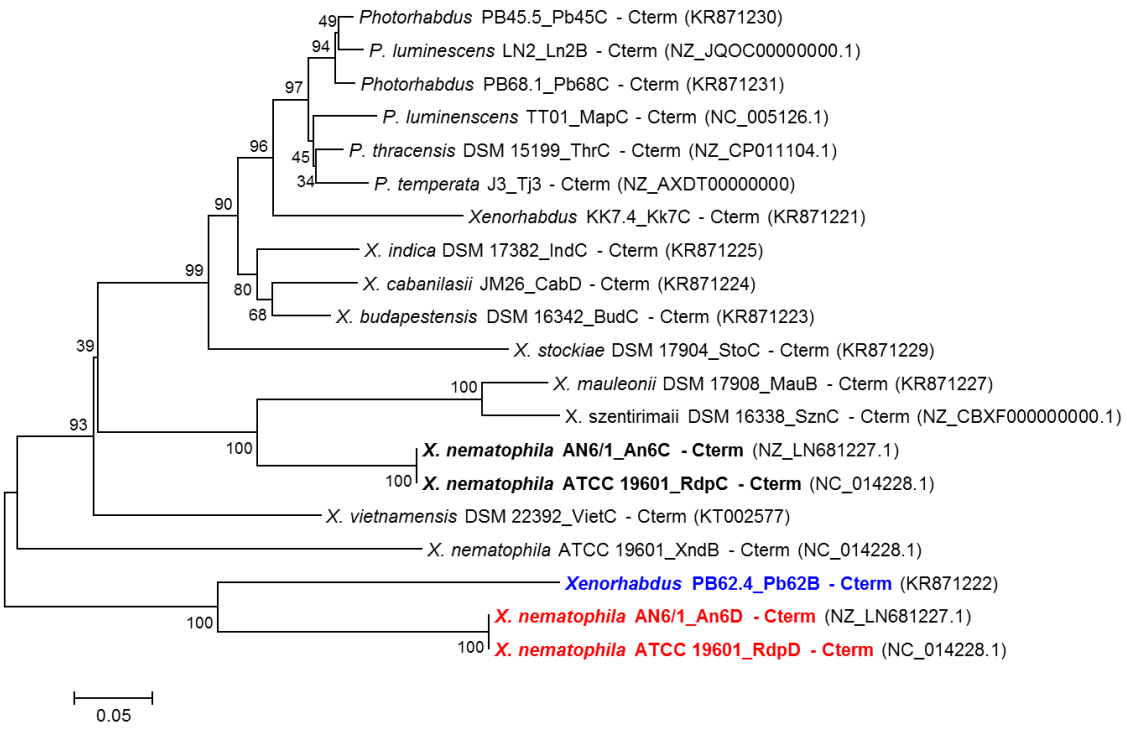


Figure S5. RdpD-C_{term} represents a distinct subclade in the phylogenetic tree of C_{term} domains from selected RXP-NRPSs in different *Xenorhabdus* and *Photorhabdus* strains.[3]

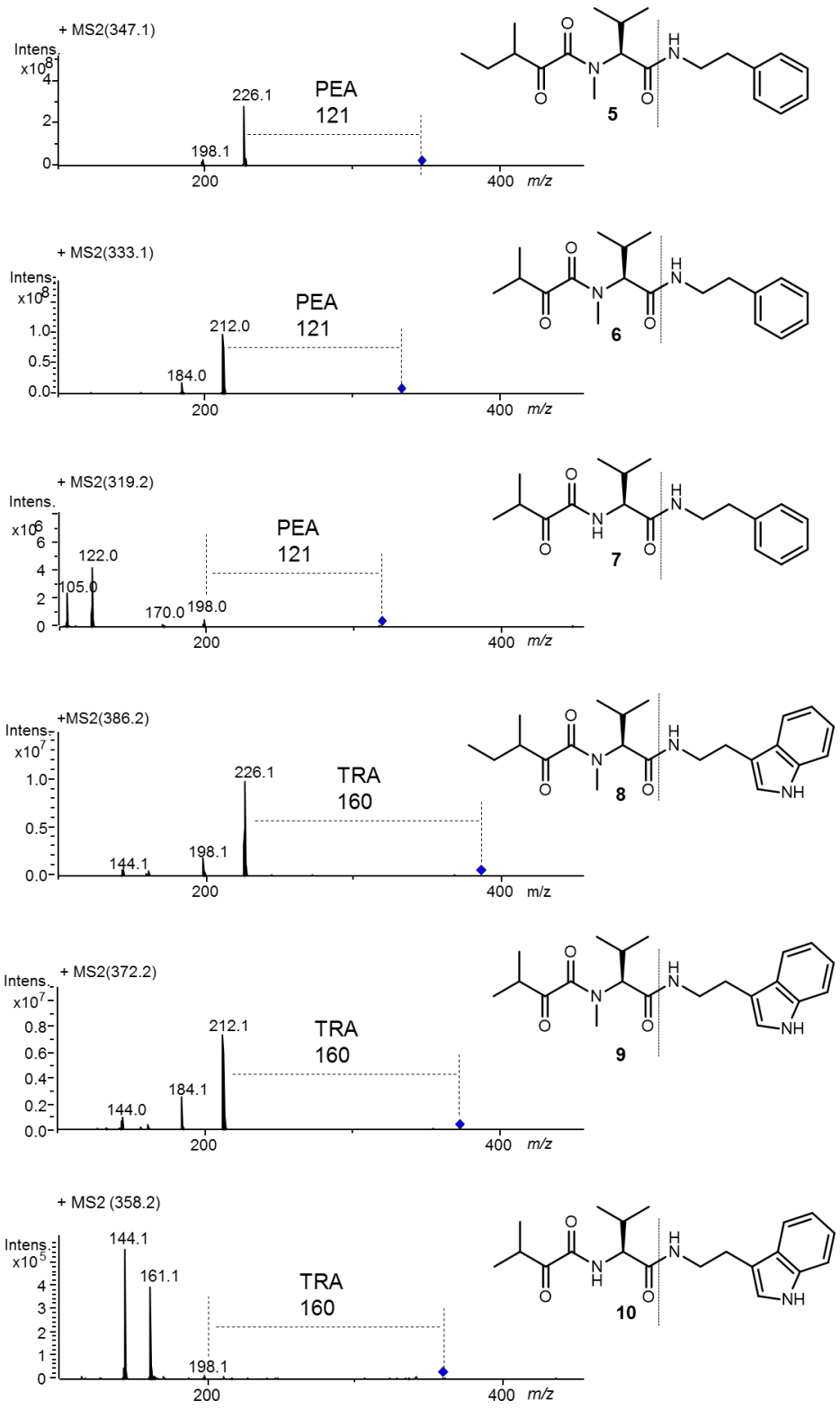
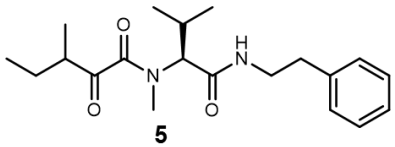
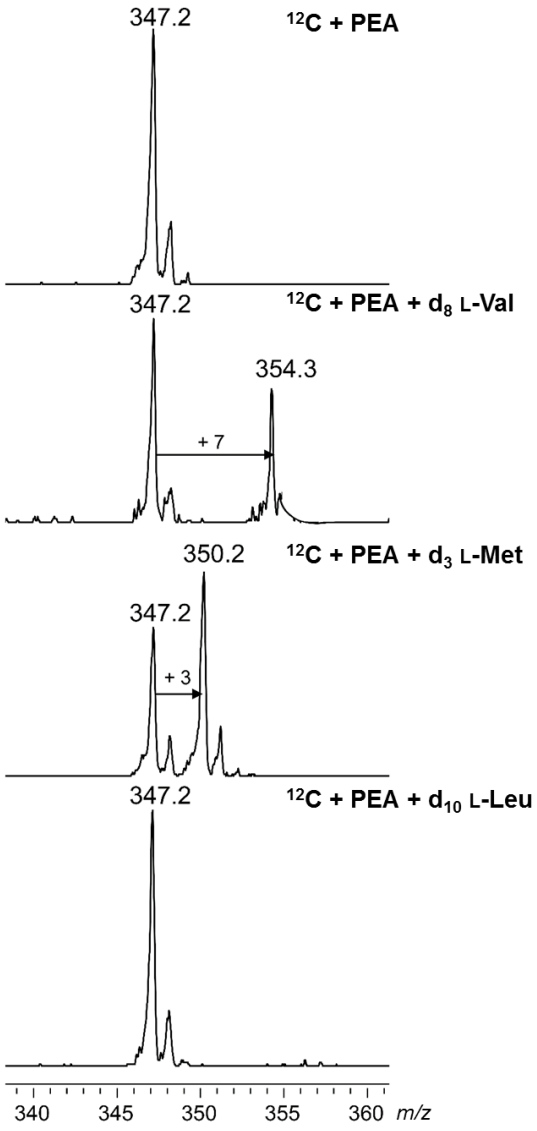


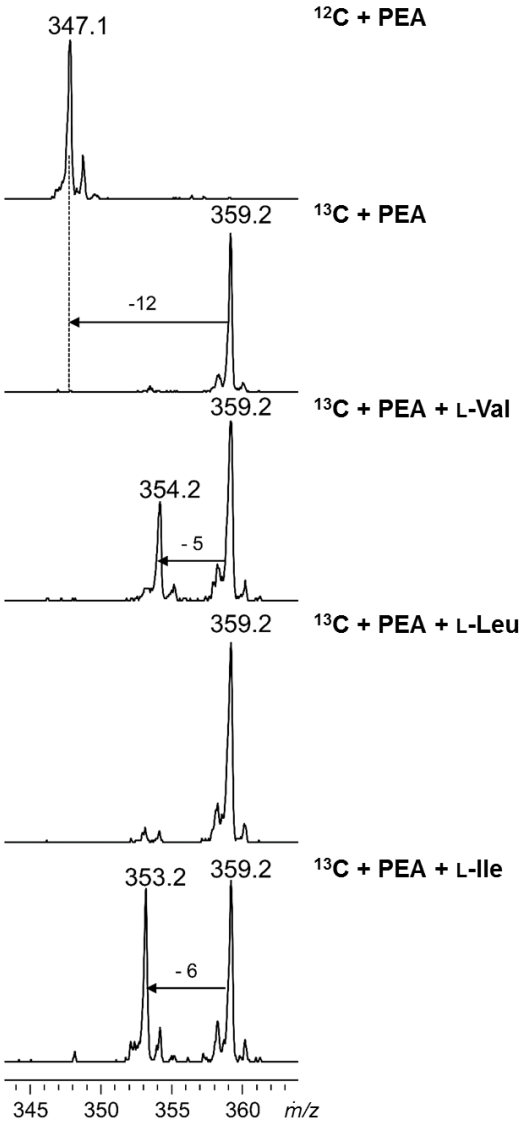
Figure S6. LCMS-MS fragmentation of nevaltophins A-F (**5**-**10**).

a) ***m/z*** **[M+H$^+$]: 347.2**

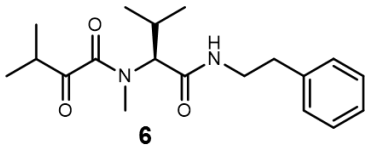


5

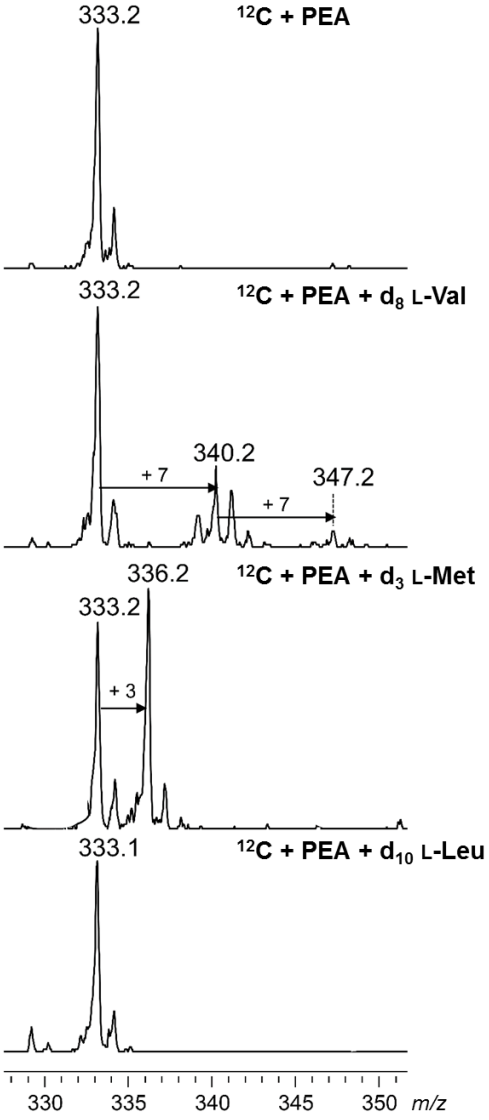

347.2
12C + PEA
347.2
12C + PEA + d8 L-Val
354.3
+ 7
350.2
12C + PEA + d3 L-Met
347.2
+ 3
347.2
12C + PEA + d10 L-Leu
340
345
350
355
360
m/z

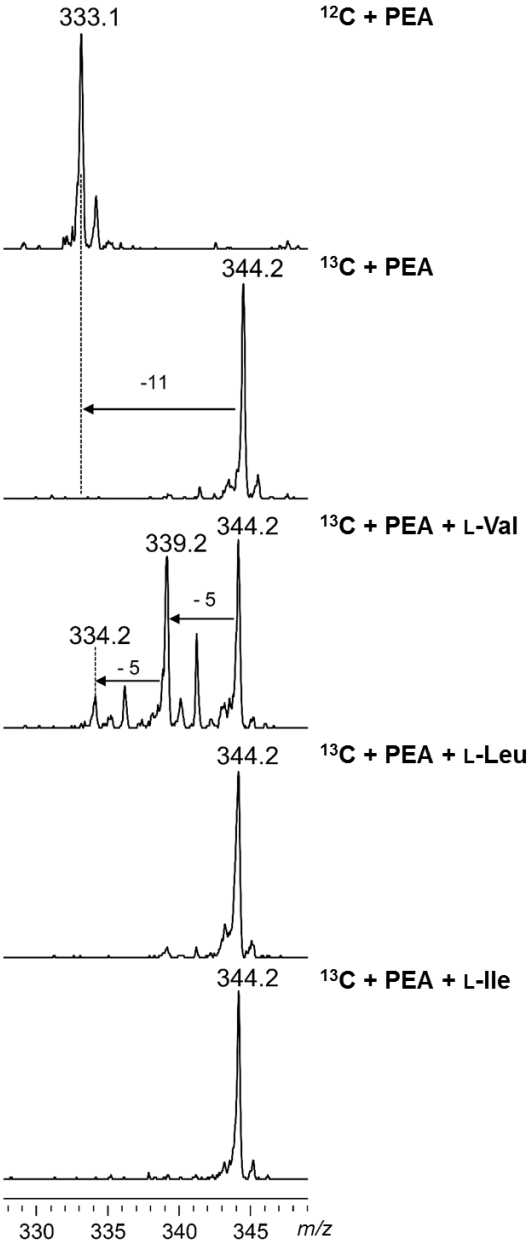

347.1
12C + PEA
359.2
13C + PEA
-12
359.2
13C + PEA + L-Val
354.2
- 5
359.2
13C + PEA + L-Leu
353.2
359.2
- 6
13C + PEA + L-Ile
345
350
355
360
m/z

b) ***m/z*** **[M+H⁺]: 333.2**



6


333.2
12C + PEA
333.2
12C + PEA + d8 L-Val
340.2
+ 7
347.2
+ 7
336.2
12C + PEA + d3 L-Met
333.2
+ 3
333.1
12C + PEA + d10 L-Leu
330
335
340
345
350
m/z


333.1
12C + PEA
344.2
13C + PEA
-11
344.2
13C + PEA + L-Val
339.2
- 5
334.2
- 5
344.2
13C + PEA + L-Leu
344.2
13C + PEA + L-Ile
330
335
340
345
m/z

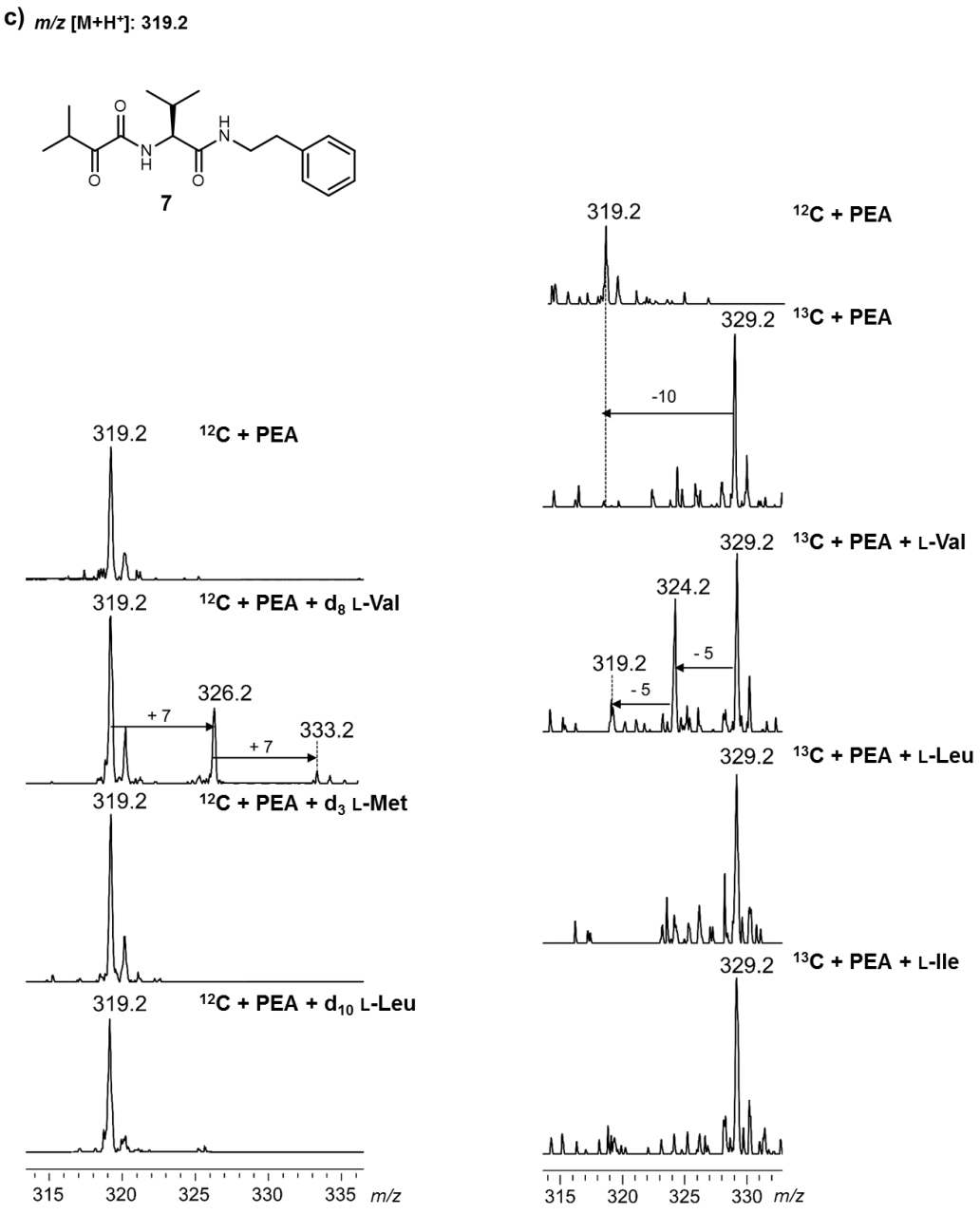


Figure S7. EICs of LC-MS analysis of crude extracts from isotope labeling experiments for determination of compounds **5**-**7**.

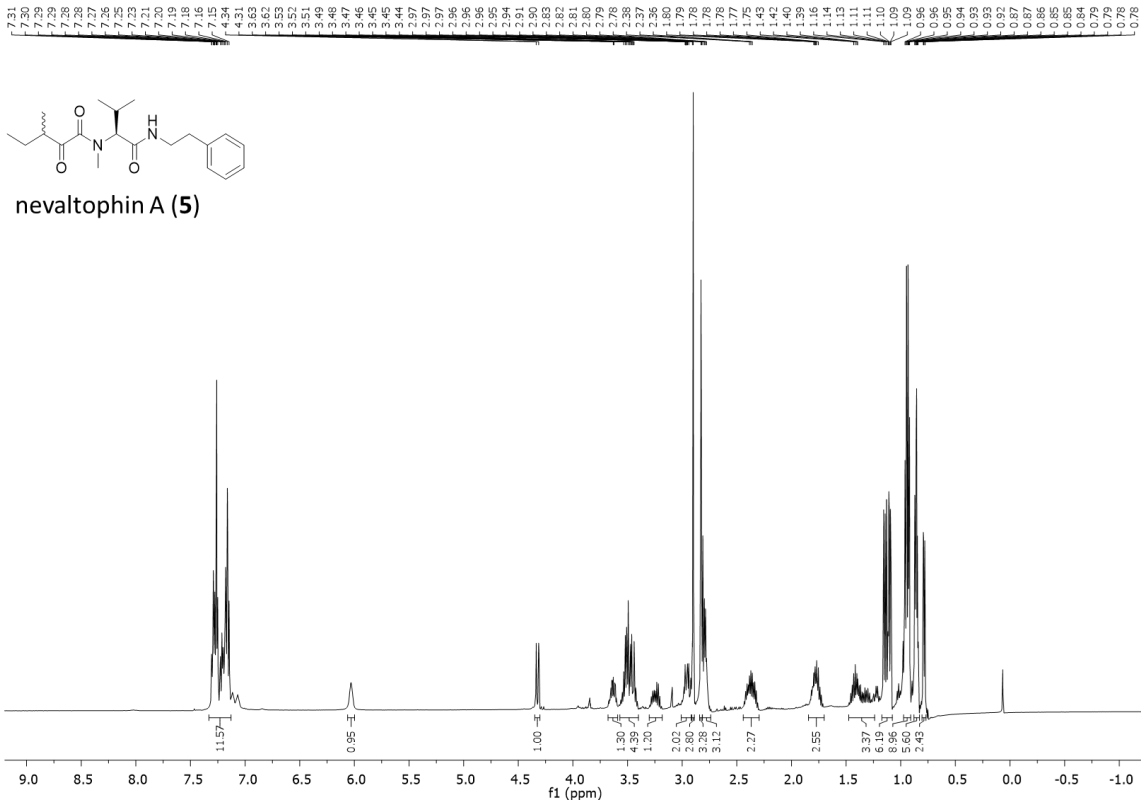


Figure S8. ^{1}H NMR spectrum of **5** (CDCl3, 500 MHz).

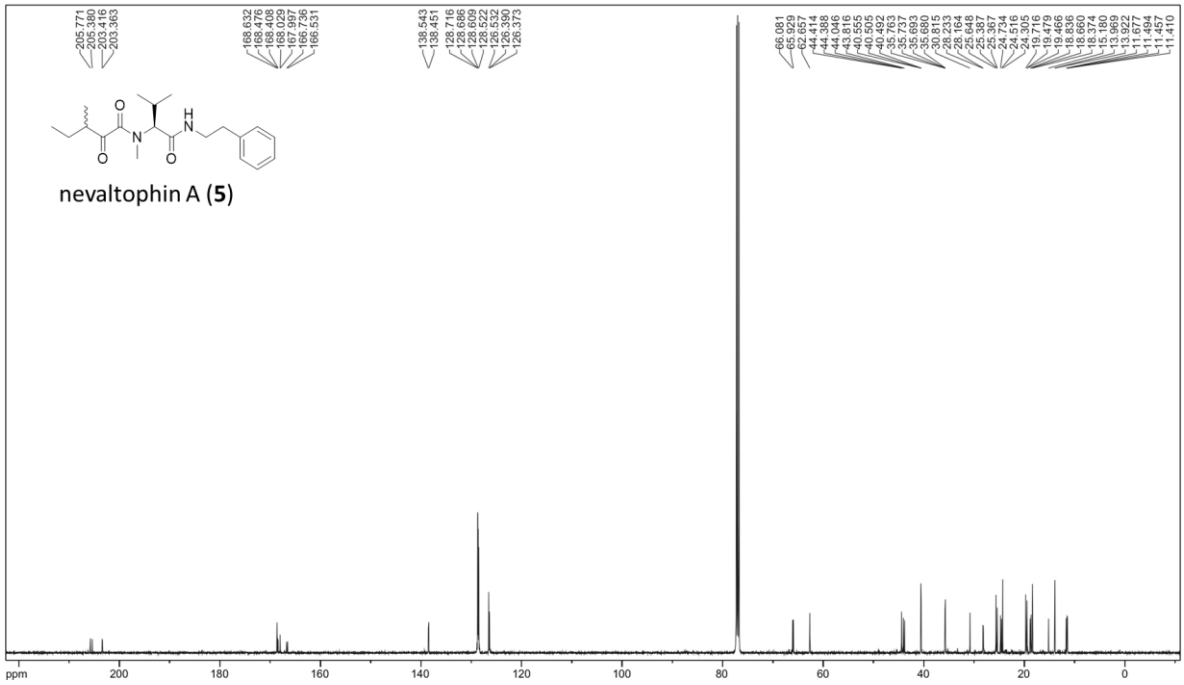


Figure S9. ^{13}C NMR spectrum of **5** ($CDCl_3$, 125 MHz).

c

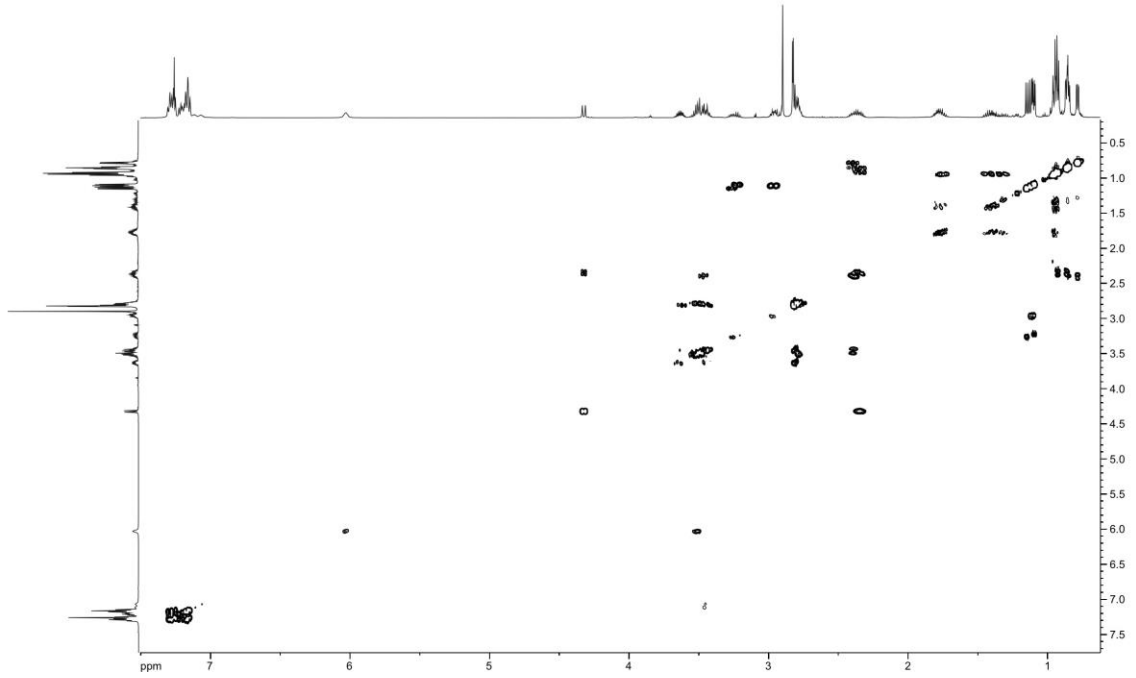


Figure S10. COSY spectrum of **5** ($CDCl_3$, 500 MHz).

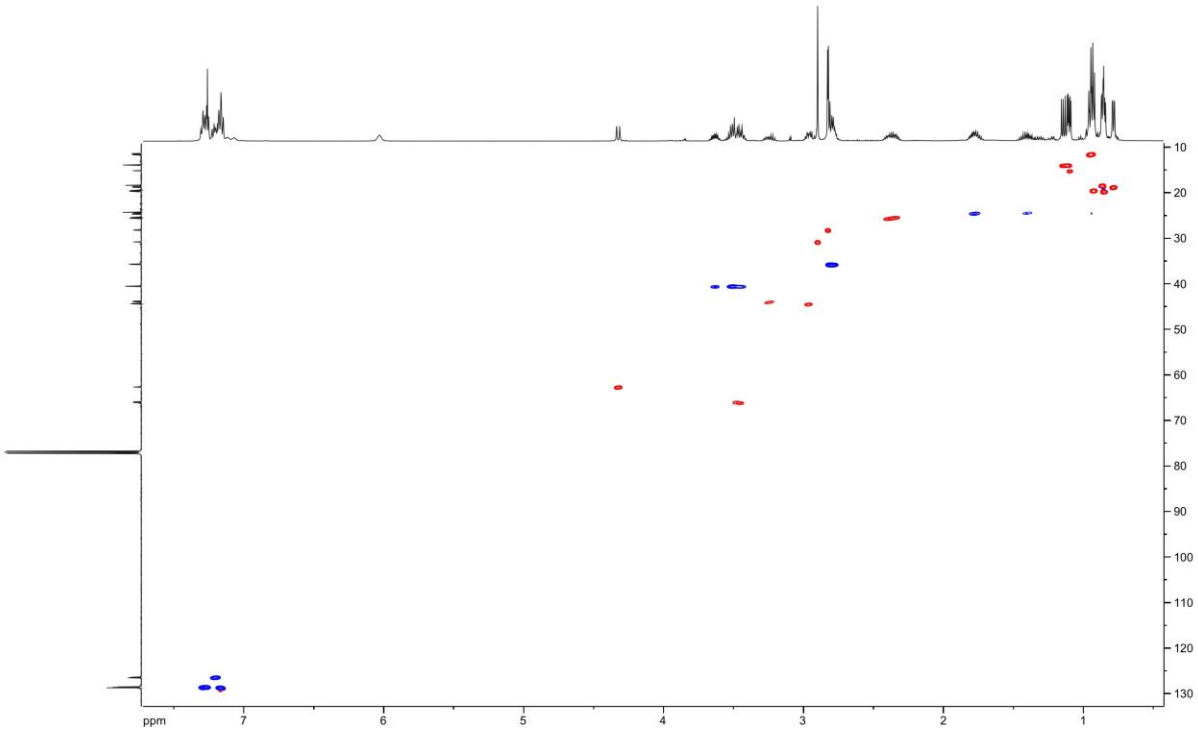


Figure S11. HSQC spectrum of **5** ($CDCl_3$, 500 MHz).

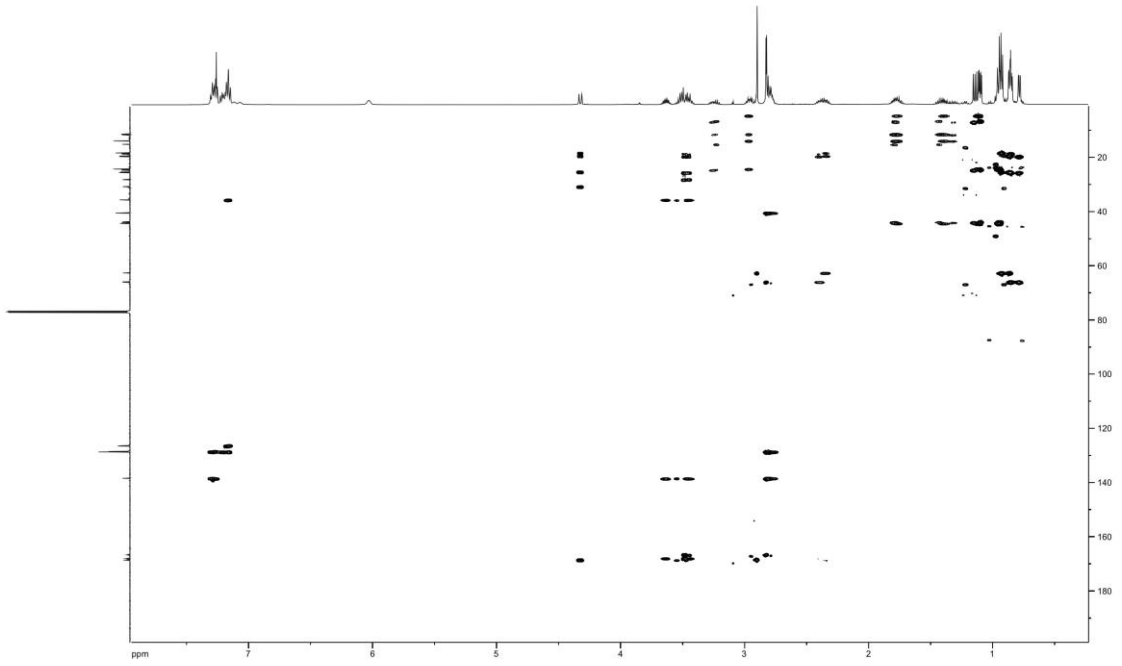


Figure S12. HMBC spectrum of **5** ($CDCl_3$, 500 MHz).

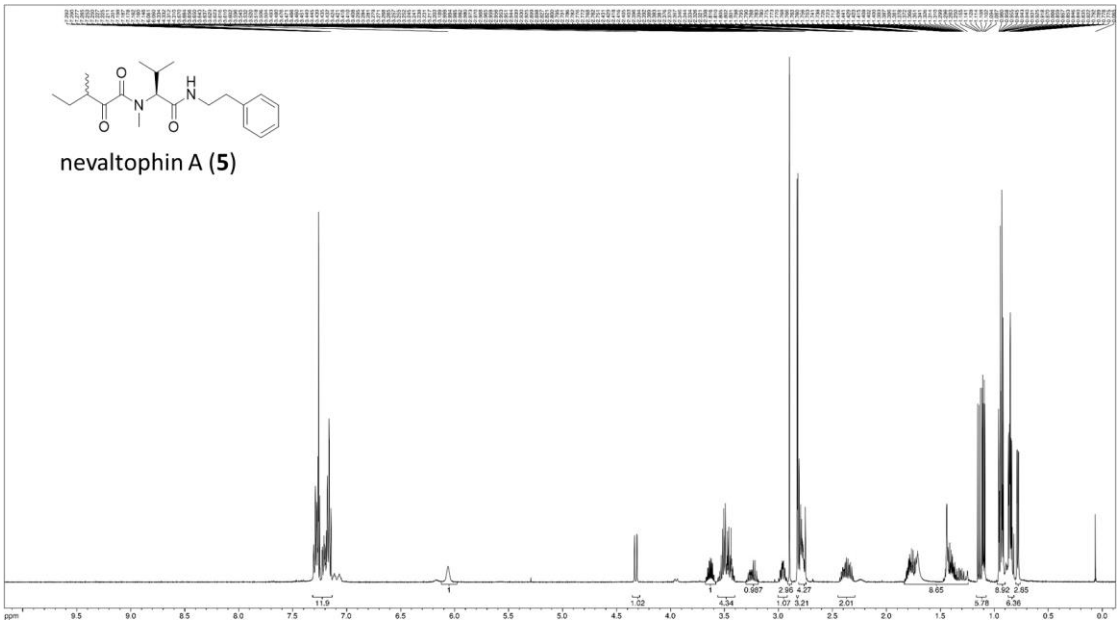


Figure S13. ^{1}H NMR spectrum of synthetic **5** ($CDCl_3$, 500 MHz).

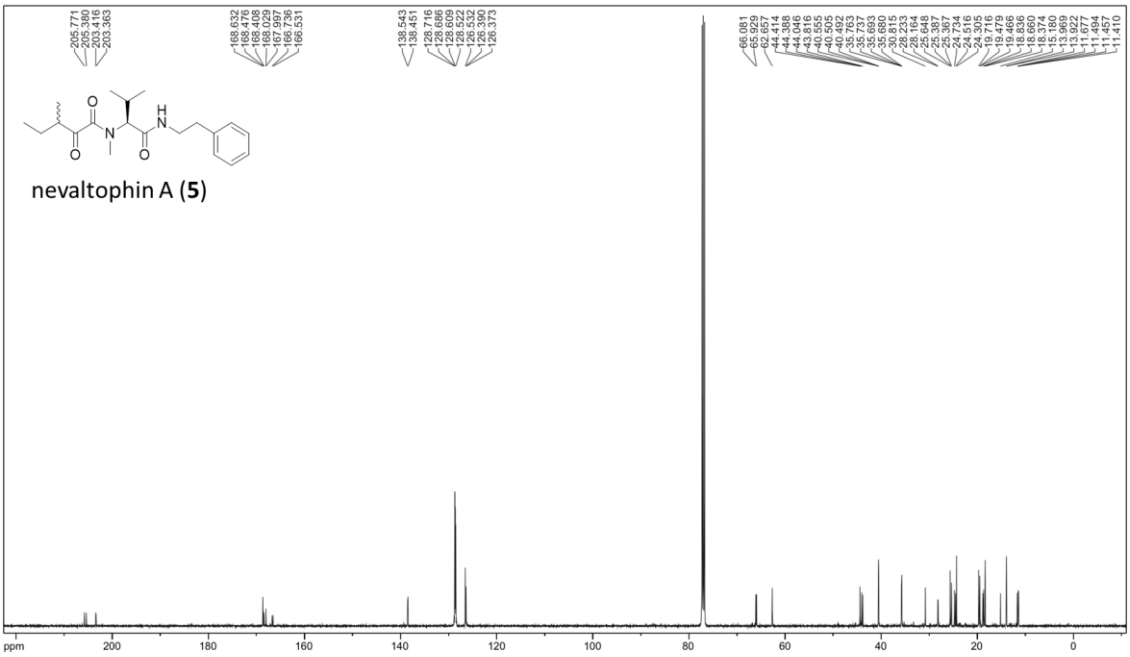


Figure S14. ^{13}C NMR spectrum of synthetic **5** ($CDCl_3$, 125 MHz).

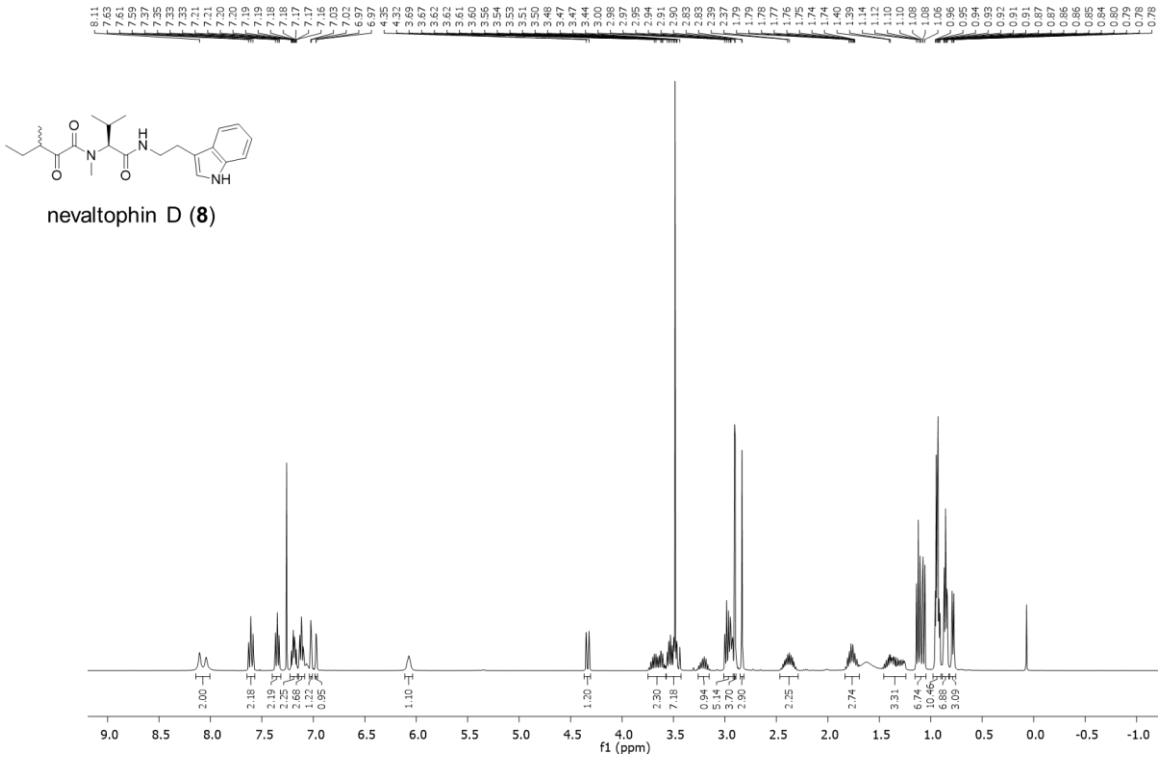


Figure S15. ^{1}H NMR spectrum of synthetic **8** ($CDCl_3$, 400 MHz).

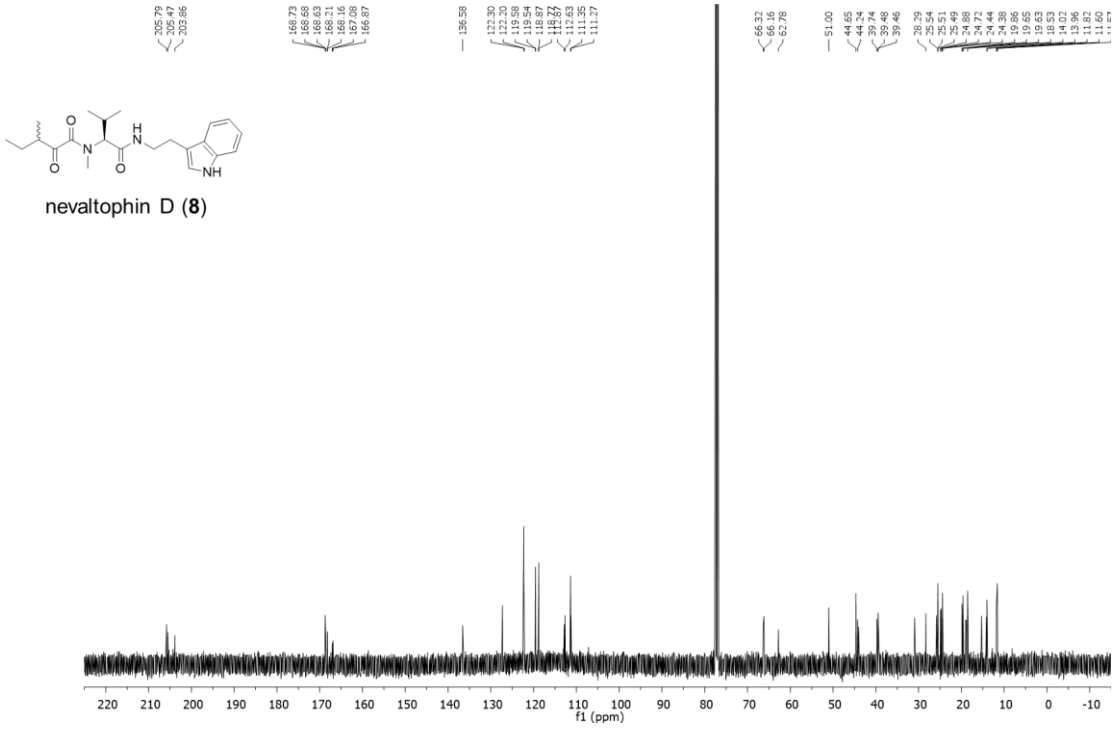


Figure S16. ^{13}C NMR spectrum of synthetic **8** ($CDCl_3$, 125 MHz).

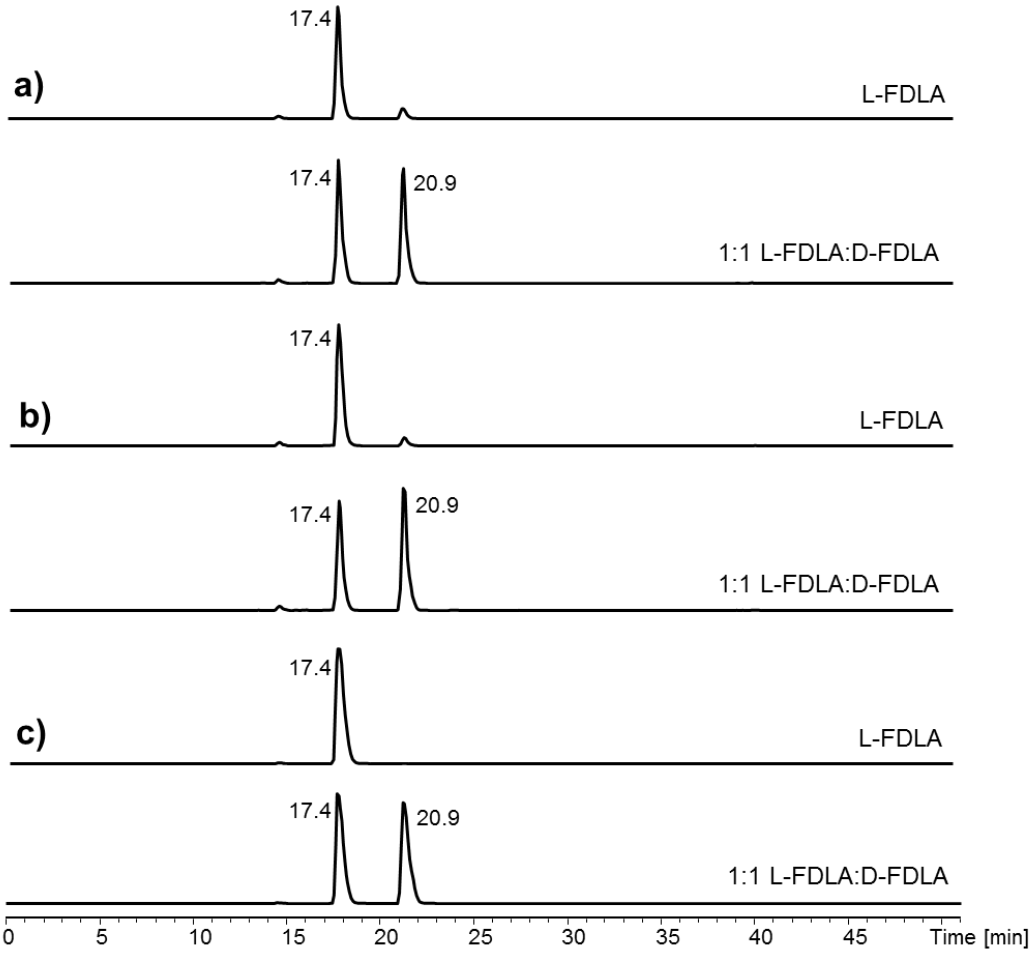


Figure S17. Determination of amino acid absolute configuration via the advanced Marfey's method.[8] Extracted ion chromatograms (*m/z* 426, $[M + H]^+$) are shown for hydrolysed and derivatised synthetic **5** (a), natural **5** (b), and authentic *N*-Me-L-Val (c). Retention times are shown in min. L-FDLA: *N*α-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide. D-FDLA: *N*α-(5-fluoro-2,4-dinitrophenyl)-D-leucinamide.

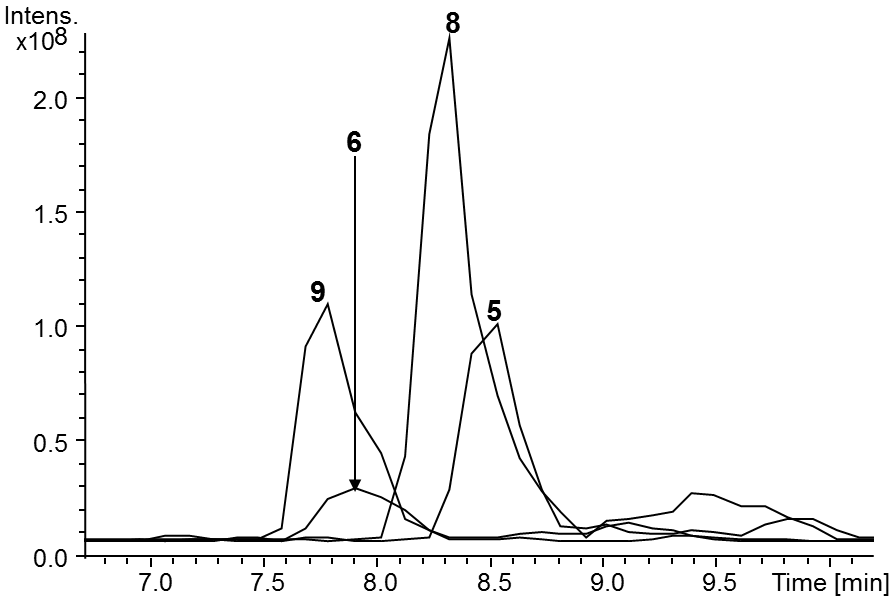


Figure S18. EICs of compounds **5**, **6**, **8** and **9** produced in wild type strain *Xenorhabdus* PB62.4.

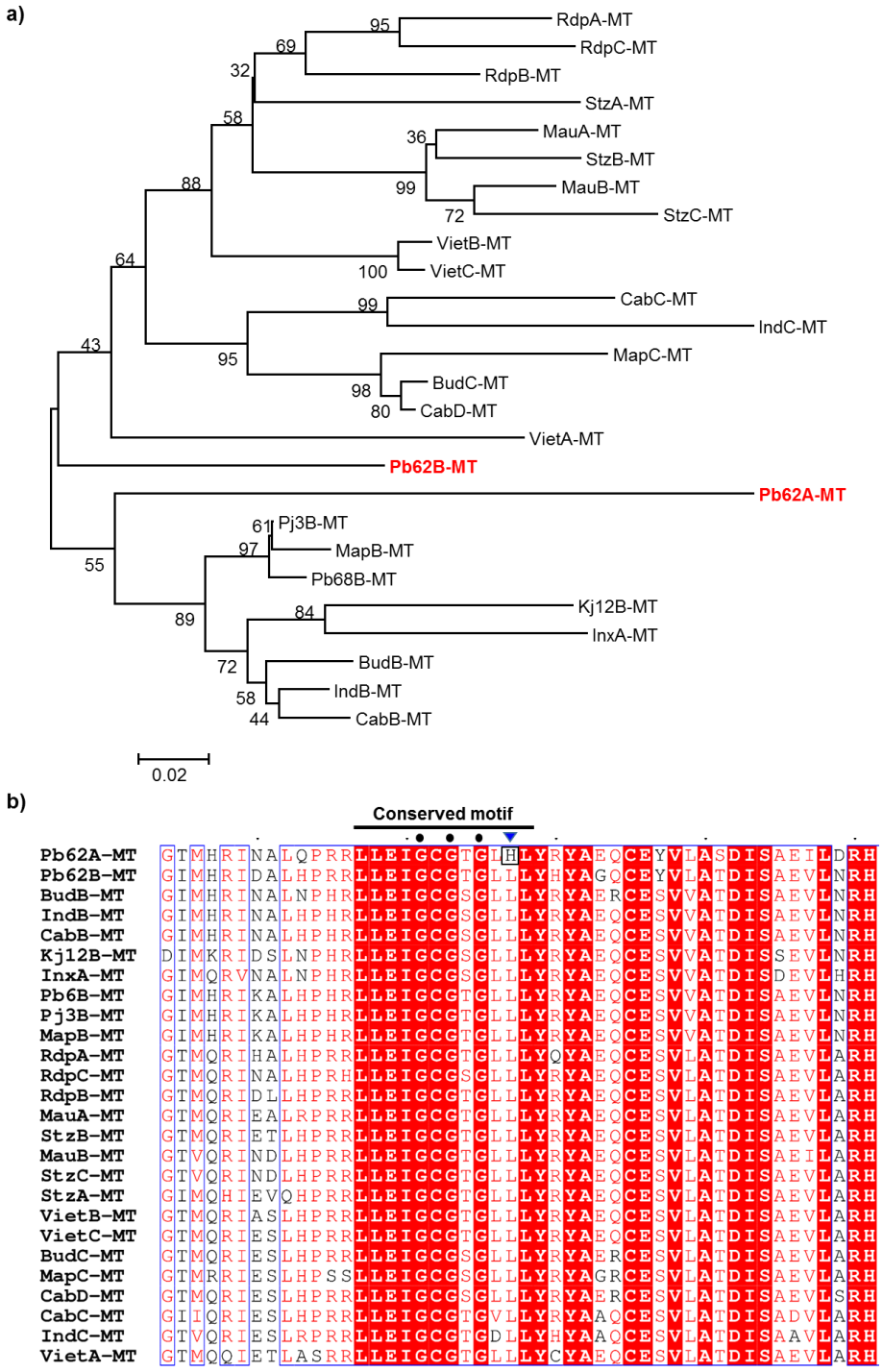


Figure S19. Comparative analysis of Pb62A-MT domain to the other RXP-NRPS-MT domains. a) Phylogenetic tree of MT domains from selected RXP-NRPSs. Pb62A-MT domain represents a distinct subclade. b) Alignment of MT domains from selected RXP-NRPSs, the conserved motif of LLEIGCT/SGLL was shown. In the core motif of Pb62A-MT, one L is replaced by H.

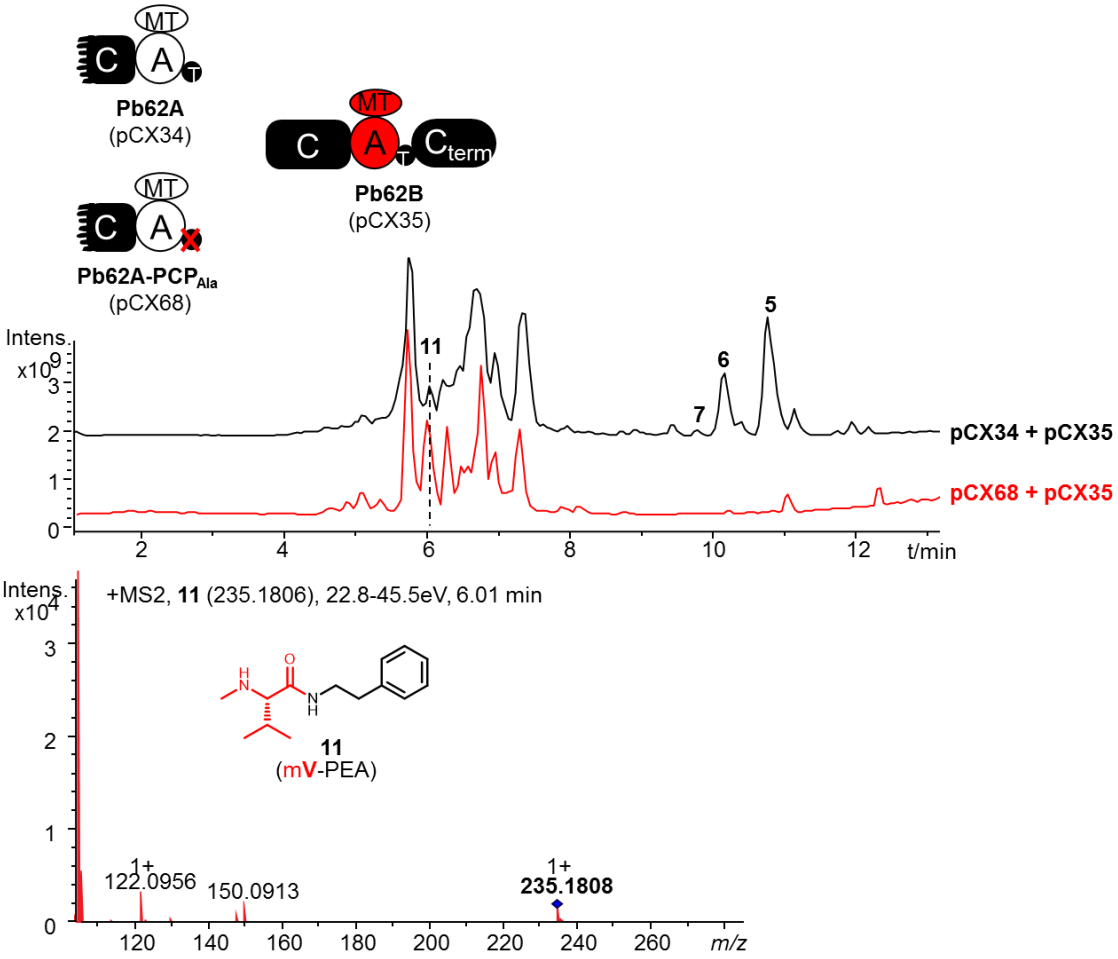


Figure S20. Comparison of BPCs from HR-EIS-HPLC-MS analysis for coexpressions of pCX34 (Pb62A) and pCX35 (Pb62B) (black) as well as pCX68 (Pb62A with point mutation on PCP domain) and pCX35 (red). The point mutation on T domain in Pb62A led to no production of nematophin derivatives but accumulation of compound **11**.

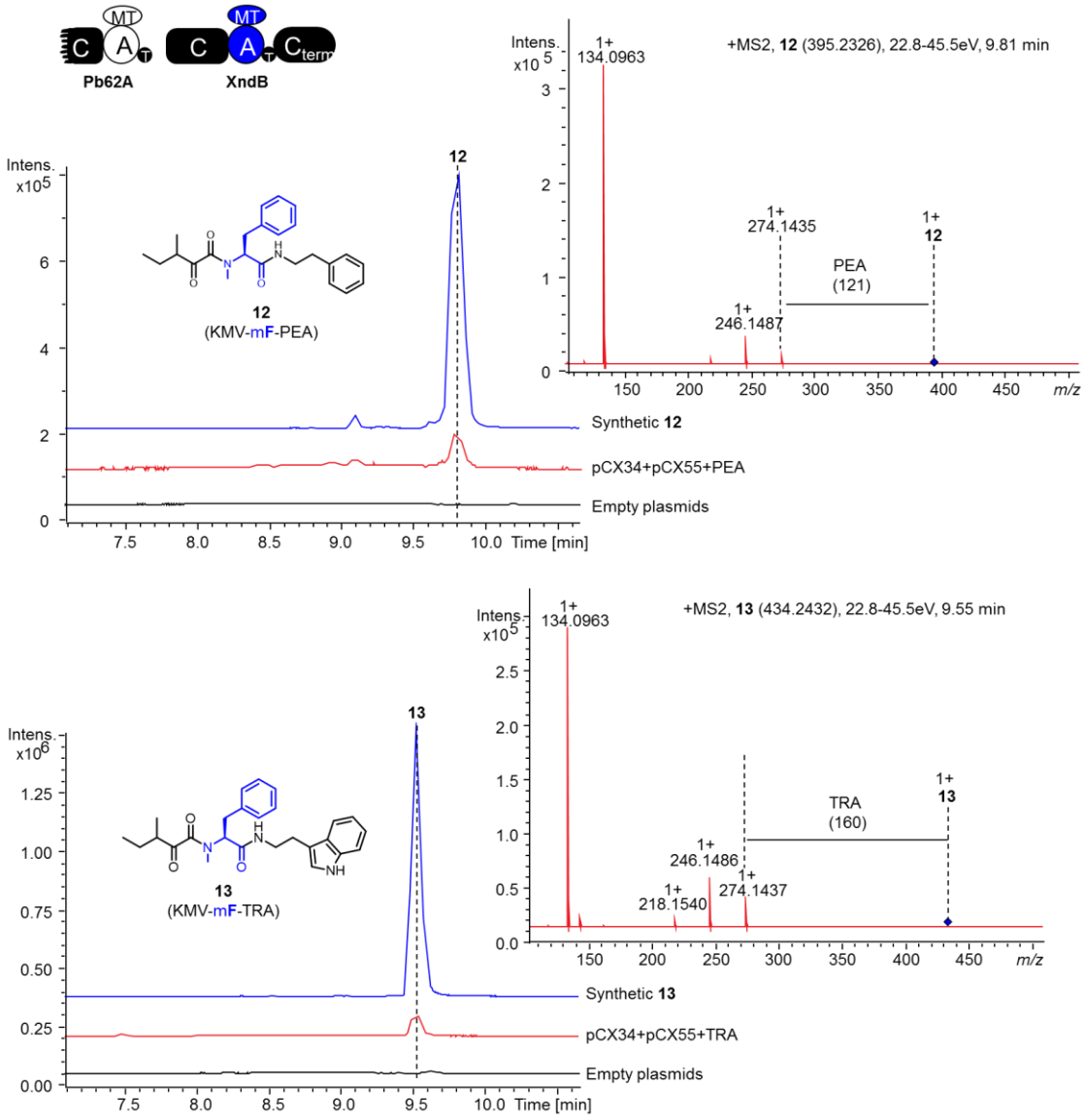


Figure S21. HR-EIS-HPLC-MS analysis for coexpression of Pb62A and XndB after feeding with PEA and TRA, respectively as well as expression of empty plasmids as a negative control. Comparisons of EICs and MS2 fragmentations for compounds **12** and **13** were shown. Synthetic compounds **12** and **13** were used as positive controls.

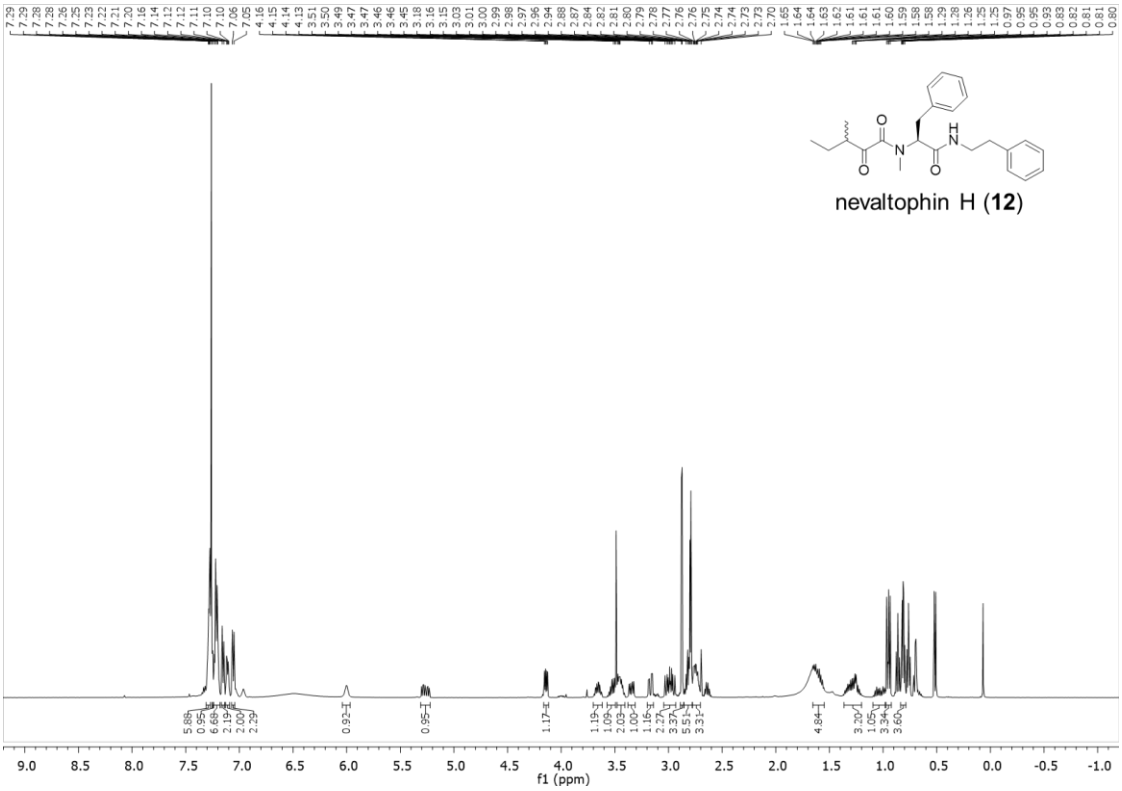


Figure S22. ^{1}H NMR spectrum of synthetic **12** ($CDCl_3$, 500 MHz).

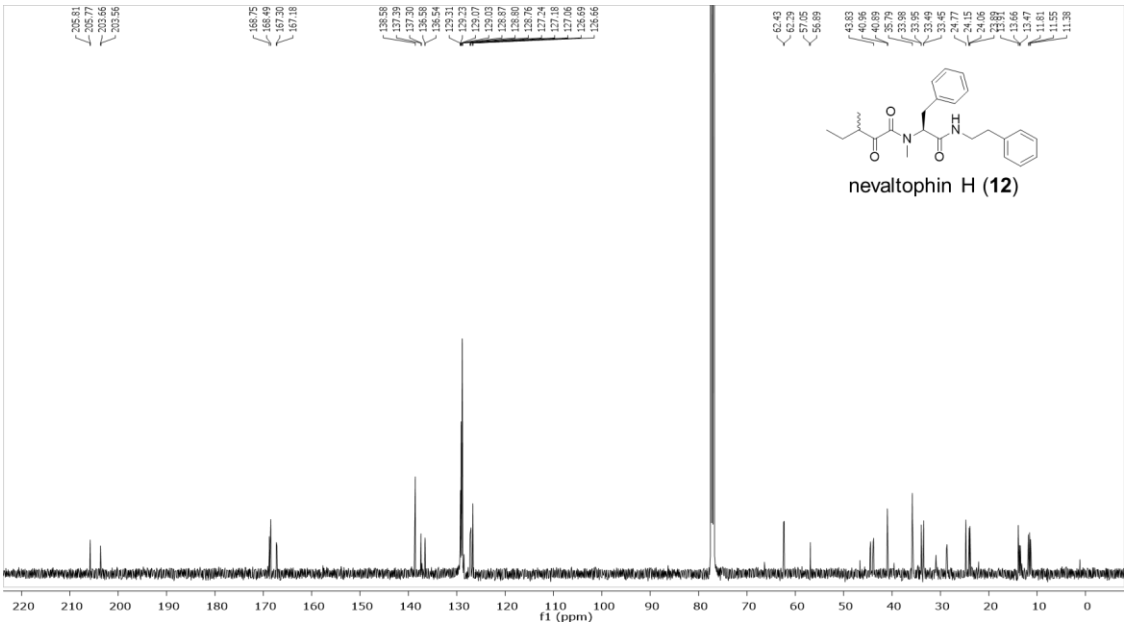


Figure S23. ^{13}C NMR spectrum of synthetic **12** ($CDCl_3$, 125 MHz).

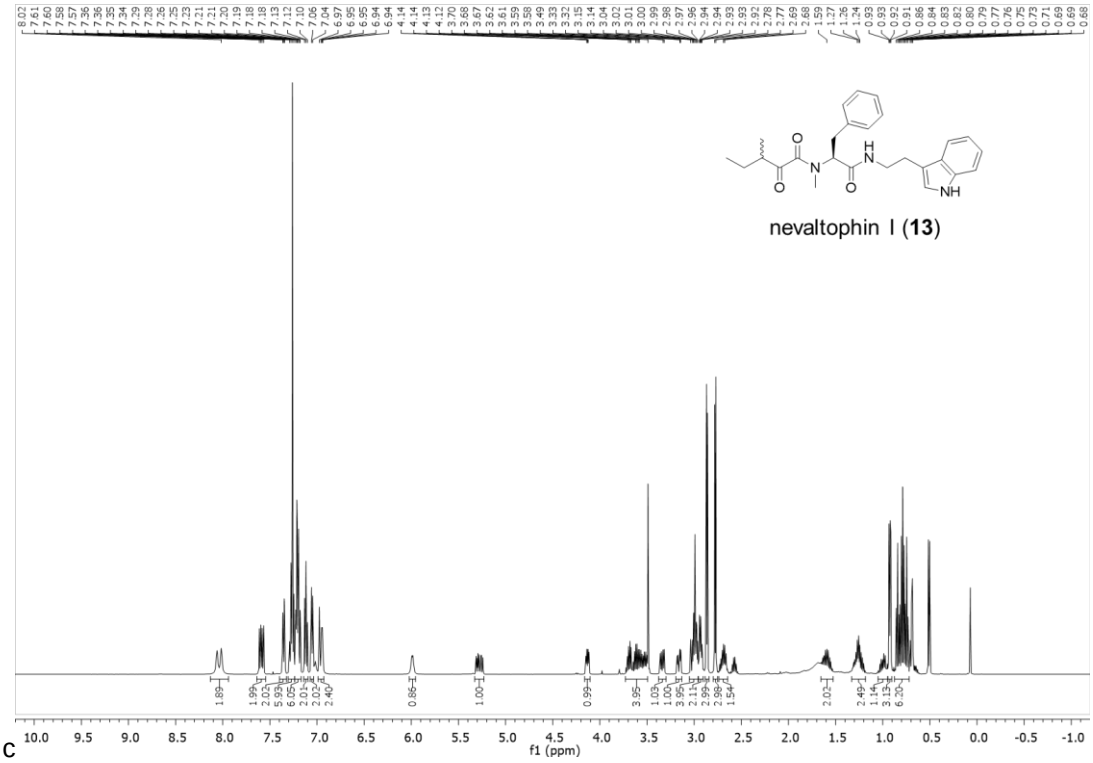


Figure S24. ^{1}H NMR spectrum of synthetic **13** ($CDCl_3$, 500 MHz).

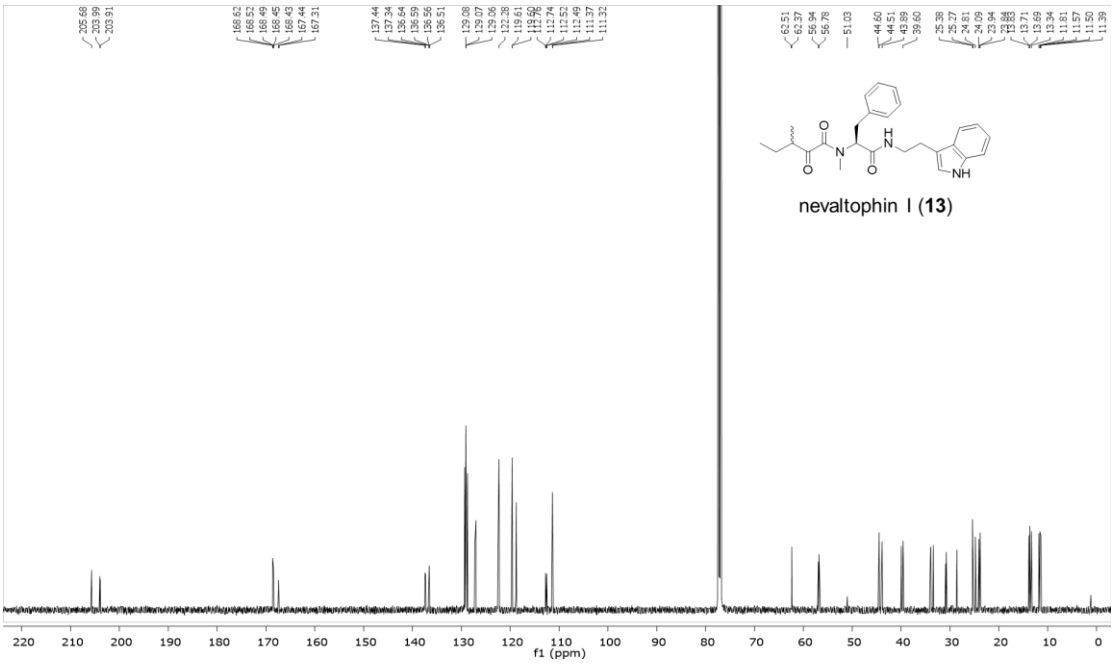


Figure S25. ^{13}C NMR spectrum of synthetic **13** ($CDCl_3$, 125 MHz).

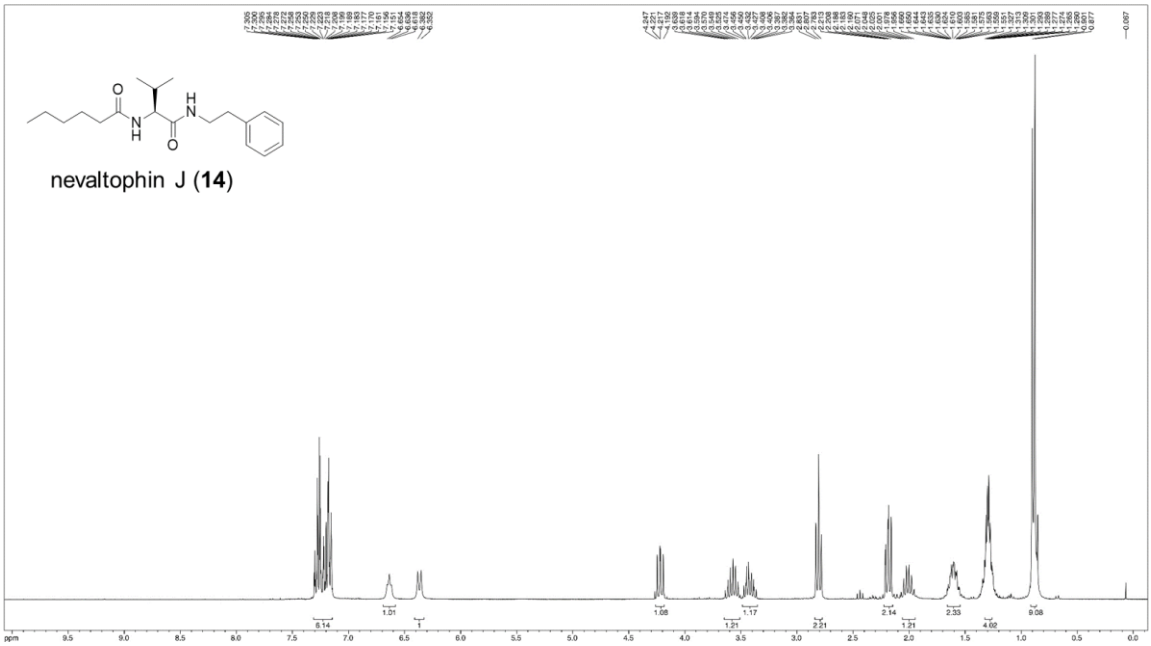


Figure S26. ^{1}H NMR spectrum of synthetic **14** ($CDCl_3$, 300 MHz).

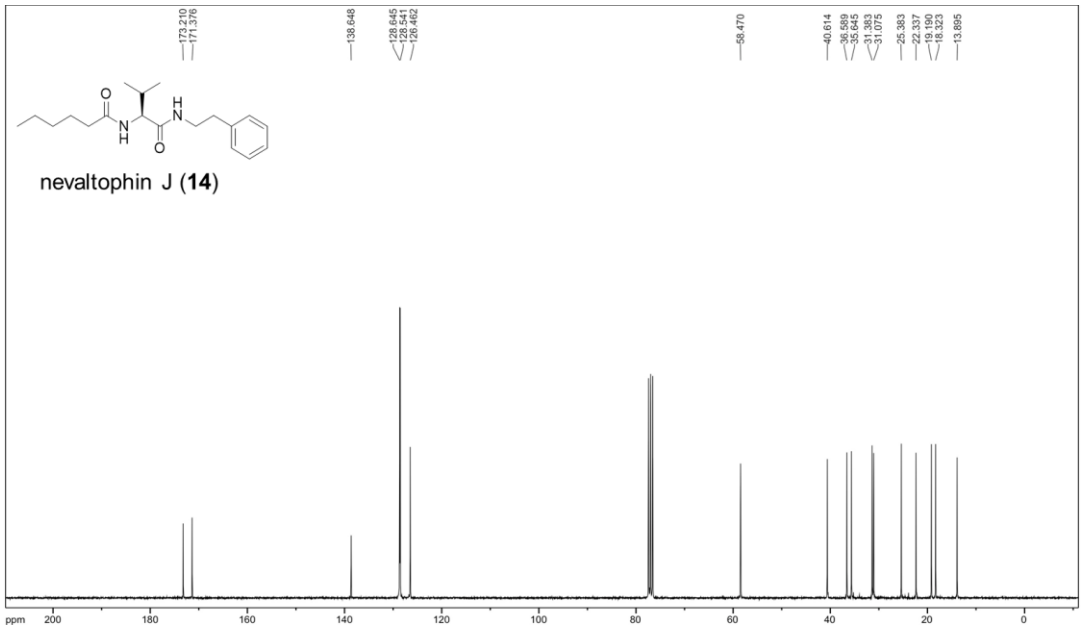


Figure S27. ^{13}C NMR spectrum of synthetic **14** ($CDCl_3$, 75 MHz).

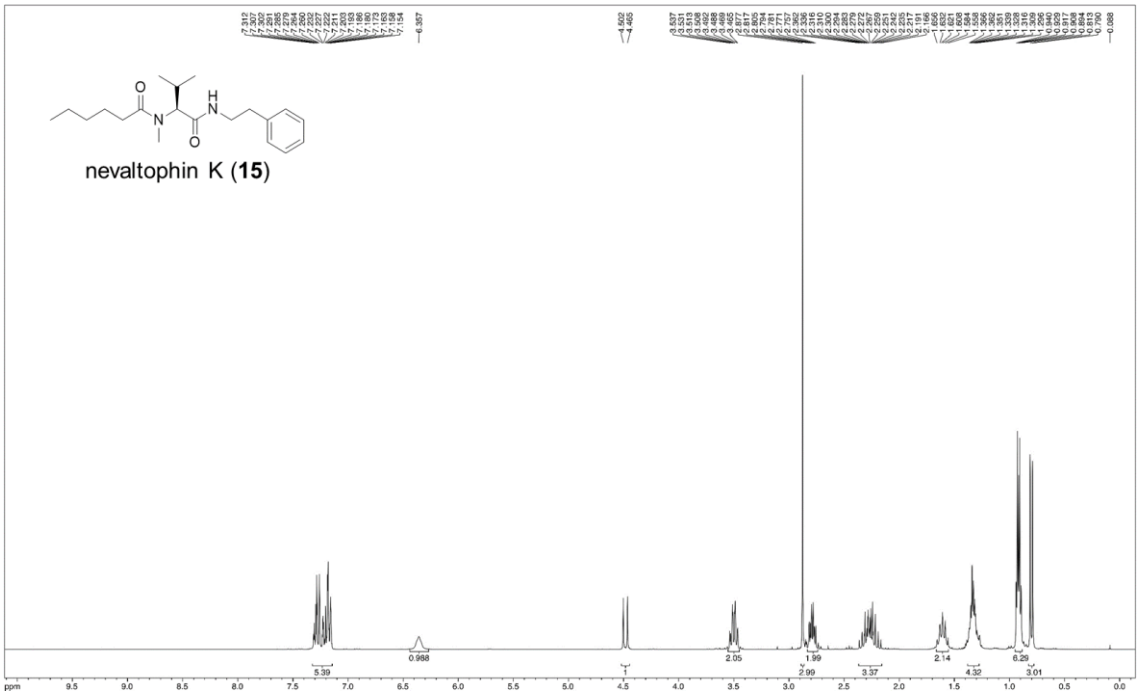


Figure S28. ^{1}H NMR spectrum of synthetic **15** ($CDCl_3$, 300 MHz).

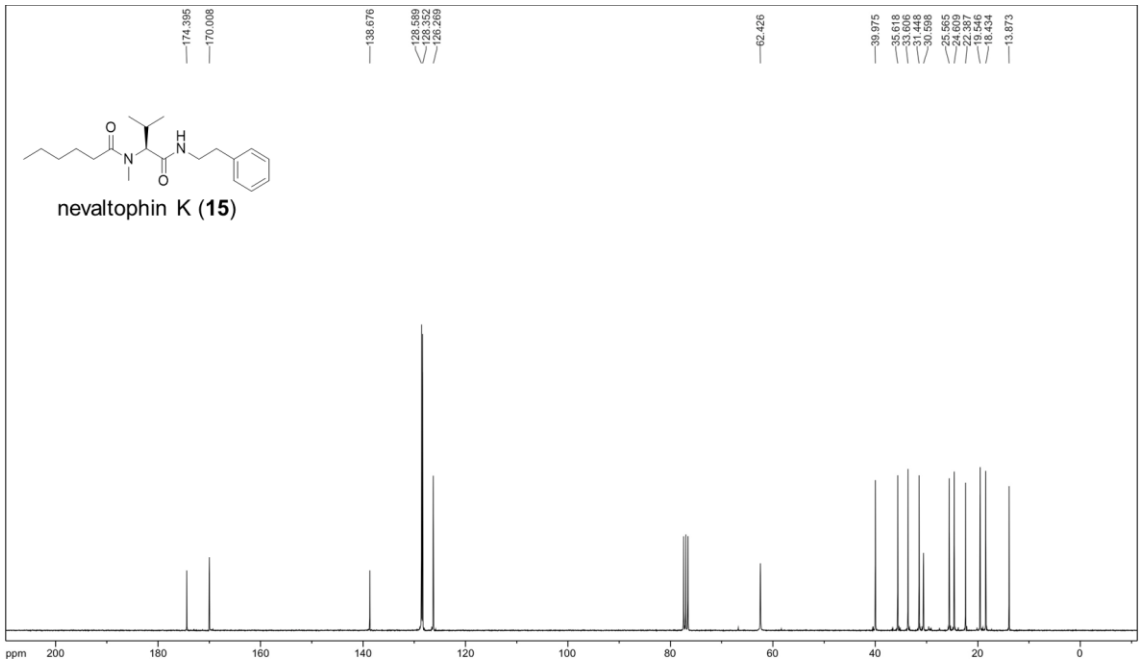


Figure S29. ^{13}C NMR spectrum of synthetic **15** ($CDCl_3$, 75 MHz).

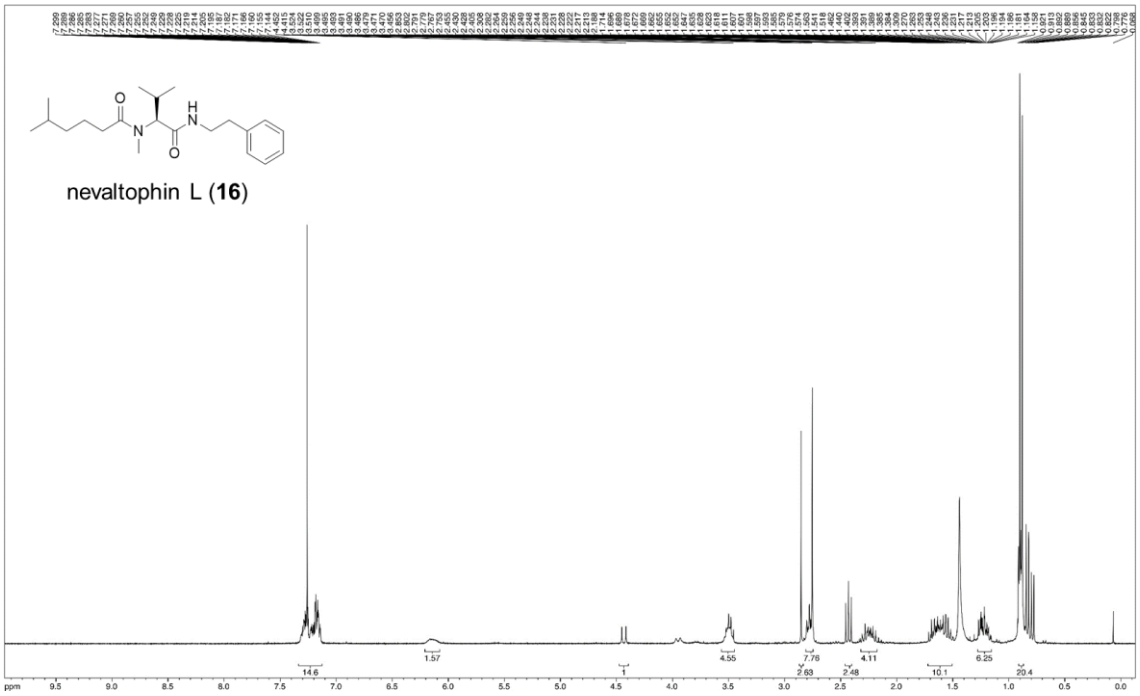


Figure S30. ^{1}H NMR spectrum of synthetic **16** ($CDCl_3$, 300 MHz).

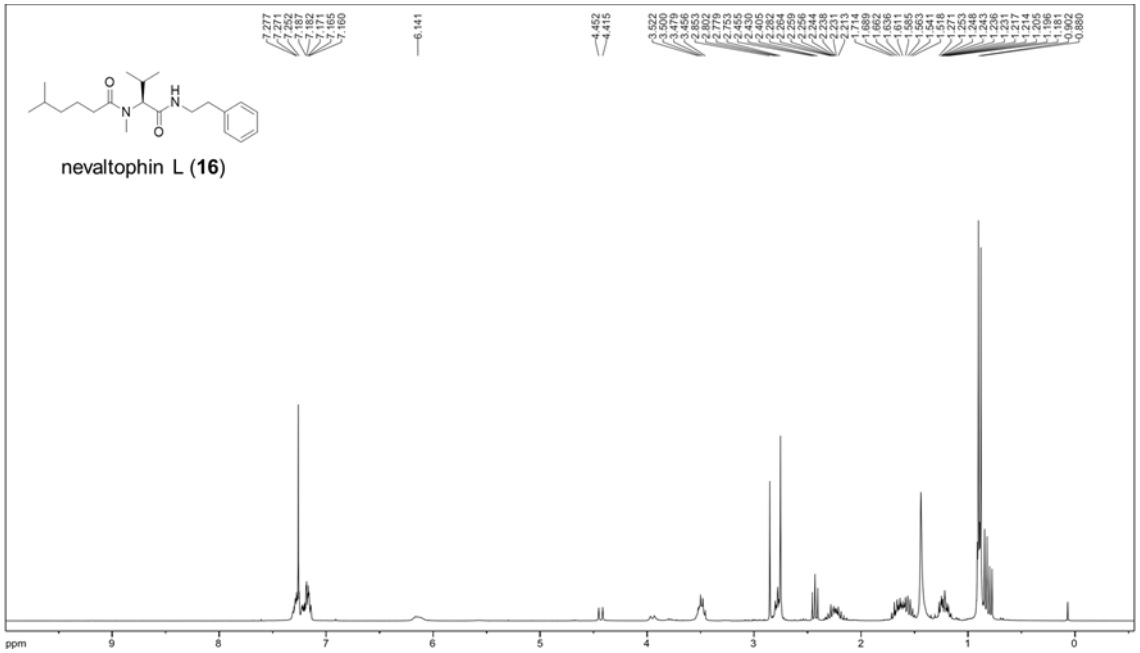


Figure S31. ^{13}C NMR spectrum of synthetic **16** ($CDCl_3$, 75 MHz).

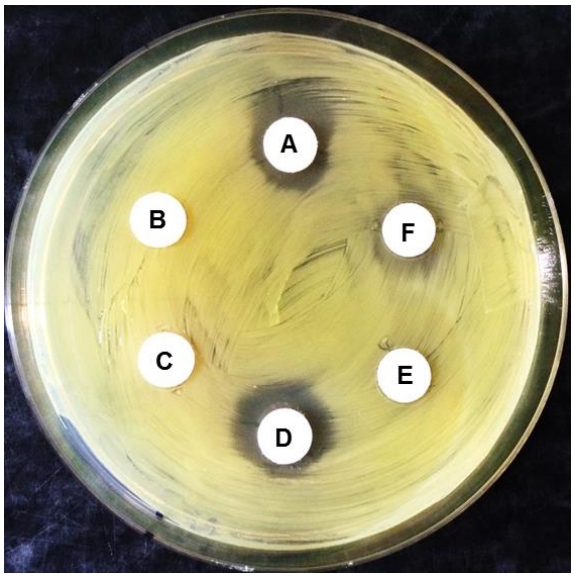


Figure S32. Bioactivity assay of nematophin and nevaltophins against Gram-positive *Micrococcus luteus*. **A**, synthetic nematophin (**1**); **B**, nevaltophins A-C (**5**-**7**) (crude extracts from LB culture of *E. coli* strains carrying *pb62* gene cluster fed with PEA); **C**, nevaltophins D-F (**8**-**10**) (crude extracts from LB culture of *E. coli* strains carrying *pb62* gene cluster fed with TRA); **D**, compounds **1** and **2** (crude extracts from LB culture of *E. coli* carrying *rdpD* fed with TRA); **E**, compounds **3** and **4** (crude extracts from LB culture of *E. coli* carrying *rdpD* fed with PEA); **F**, RDPs and compounds **3**-**4** RdpABC + RdpD +TRA (crude extracts from LB culture of *E. coli* carrying *rdpABC* gene cluster and *rdpD* fed with TRA). Synthetic nematophin (**1**) was applied as a pure compound (50 µg), while **B**-**F** were used as crude culture extracts (0.6 mg).

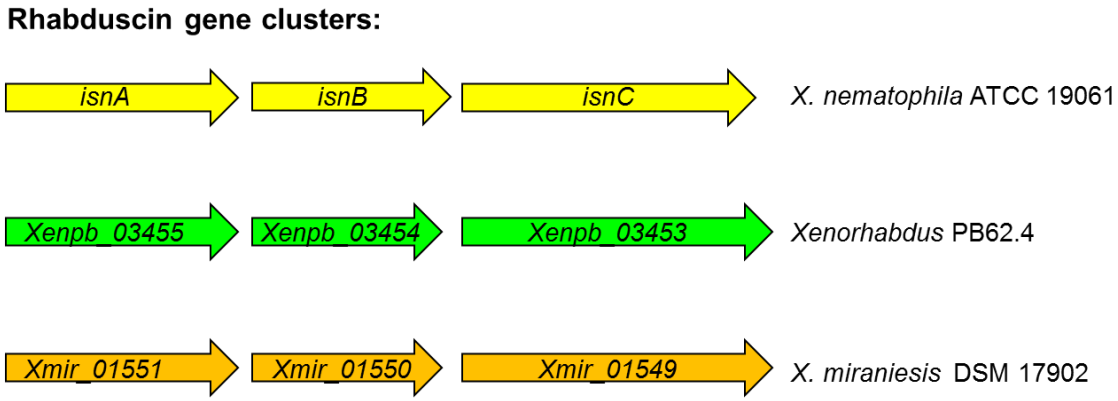


Figure S33. Rhabduscin biosynthetic gene clusters found in *Xenorhabdus* PB62.4 and *X. miraniensis* DSM 17902 strains.

References

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(3) Cai, X.; Nowak, S.; Wesche, F; Bischoff, I.; Kaiser, M.; Fürst, R.; Bode, H. B. *Nat. Chem.* **2016**. doi:10.1038/nchem.2671.
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(5) Orhan, I.; Sener, B.; Kaiser, M.; Brun, R.; Tasdemir, D. *Mar. Drugs* **2010**, *8*, 47-58.
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(7) Crawford, J. M.; Portmann, C.; Zhang, X.; Roeffaers, M. B. J.; Clardy, J. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 10821-10826.
(8) Bhushan, R.; Bruckner, H. *J. Chromatogr.* **2011**, *B879*, 3148-3161.