Natural Products

International Edition: DOI: 10.1002/anie.201603312
German Edition: DOI: 10.1002/ange.201603312

Bacterial Alkaloids Prevent Amoebal Predation

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Abstract: *Bacterial defense mechanisms have evolved to protect bacteria against predation by nematodes, predatory bacteria, or amoebae. We identified novel bacterial alkaloids (pyreudiones A–D) that protect the producer, Pseudomonas fluorescens HKI0770, against amoebal predation. Isolation, structure elucidation, total synthesis, and a proposed biosynthetic pathway for these structures are presented. The generation of P. fluorescens gene-deletion mutants unable to produce pyreudiones rendered the bacterium edible to a variety of soil-dwelling amoebae.*

Whilst soil-dwelling bacteria compete with other bacteria for resources, they are also under constant threat from predators, such as amoebae and nematodes. These evolutionary pressures have shaped intricate bacterial defense strategies including biofilm formation, cell-surface alteration, or increase in swimming or swarming speed.[1] Secondary metabolites are selective and efficient weapons that can repel and kill both competitors and predators. Whilst bacterial natural products have received great attention as therapeutically useful antibacterial agents, their ecological roles in predator defense are less well studied.[2] In these microbial predator–prey interactions lies an almost untapped source of novel secondary metabolites with multifarious functions. Protozoan grazing is a major source of bacterial mortality and is thus a significant selection pressure in soil ecosystems.[3–5] Therefore, we investigate bacterial secondary metabolites involved in defense against amoebal predation. As a model organism, we use the highly bacterivorous social amoeba *Dictyostelium discoideum*.[6] Dictyostelids are ubiquitous and commonly encountered soil inhabitants.[7]

We isolated bacteria from forest soil in Germany and subjected them to a *D. discoideum* plaque assay to determine whether the bacteria were eaten by the amoeba. By using a co-cultivation approach, we tested inedible strains for secretion of soluble toxins.[8] In short, the toxic strains were cultured with amoeba whilst separated by a semipermeable membrane to allow the diffusion of metabolites without direct contact between the different organisms. Bacterial strains producing diffusible toxins caused decreased amoebal growth or cell lysis. We identified one strain in particular, HKI0770, that exhibits potent amoebicidal activity. To determine the phylogenetic placement of this strain, we sequenced its 16S-rRNA gene, which revealed that HKI0770 belongs to the *Pseudomonas fluorescencs* group. Bacteria of the genus *Pseudomonas* are extremely ubiquitous and prolific producers of secondary metabolites,[9,10] and *P. fluorescens* strains in particular are common soil inhabitants that are known to engage in antagonistic and mutualistic associations with amoebae.[10–12]

In order to isolate secreted amoebicides, *P. fluorescens* HKI0770 was cultured in liquid medium and the supernatant was extracted with ethyl acetate. The dried extract was fractionated by solid-phase extraction on C18-functionalized silica gel and subfractionated by reversed-phase high-performance liquid chromatography (RP-HPLC). Bioassay-guided fractionation using a cell-based *D. discoideum* growth inhibition assay was performed to identify amoebicidal compounds. The active fractions were purified to homogeneity and the planar structures of the amoebicides were elucidated by nuclear magnetic resonance (NMR) spectroscopy, as well as high-resolution mass spectrometry (HRMS).

We identified a set of pyrrolizidine diones, namely pyreudiones A–D (**1**–**4**, Figure 1), that did not match any previously reported compound. Key correlations of isolated pyreudiones observed by 2D-NMR spectroscopy are shown in Figure 2. Their bicyclic pyrrolizidine alkaloid (PA) core structure is a commonly found structural motif in plant alkaloids, yet rarely found in bacterial metabolites.[13,14] Bacterial PAs such as jenamidines,[15] and structures similar to PAs such as brabantamides,[16] have been reported to show antitumor or antibacterial activity. The pyreudiones additionally belong to the class of tetramic acids, which also often display potent biological activities.[17,18] Members of this structural class occur predominantly in the *Z-exo*-enol form,

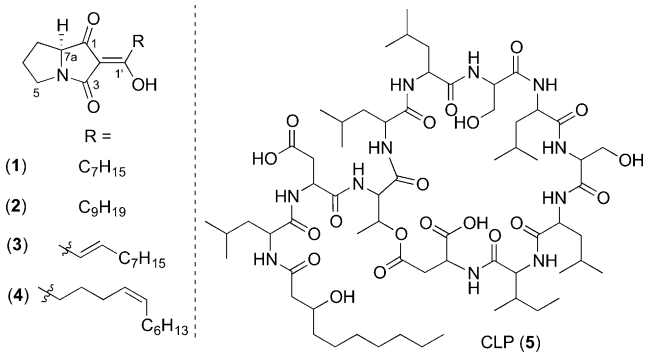

Figure 1. Structures of pyreudiones A–D (**1**–**4**) and the cyclic lipopeptide **5** isolated from cultures of *P. fluorescens* HKI0770.

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Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/anie.201603312.

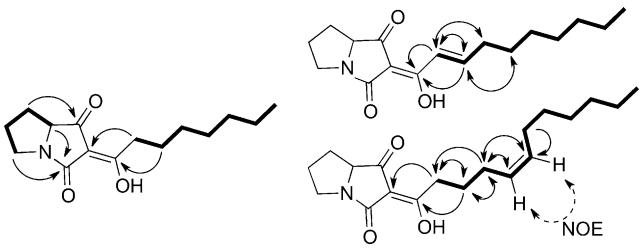
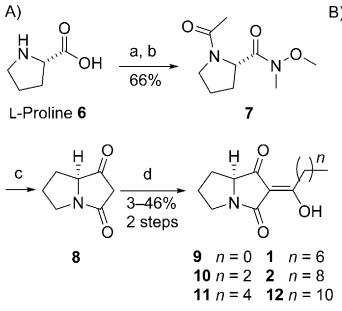


Figure 2. Selected correlations observed by 2D NMR spectroscopy. Bold lines: ^{1}H-^{1}H COSY correlations; solid arrows: HMBC correlations; dashed arrow: nuclear Overhauser effect (NOE) correlation.



B)

n =	IC_{50} (μg mL^{-1}) *D. discoideum*
0 (**9**)	>100
2 (**10**)	>100
4 (**11**)	>100
6* (**1**)	4
8* (**2**)	1
10 (**12**)	2

Scheme 1. A) Synthesis of pyreudiones A and B (**1** and **2**) and analogues **9**–**12**. Reagents and conditions: a. Ac_2O, EtOAc, RT, ultrasound; b. DIPEA, EDCI, *N*,*O*- dimethyl hydroxylamine, CH_2Cl_2, RT; c. LHMDS, THF, −78 °C; d. EDCI, CH_2Cl_2, $CH_3(CH_2)_nCO_2H$ acid, DMAP, RT. Overall yields for pyrrolizidine alkaloids over 4 steps from L-proline: **9:** 9%; **10**: 30%; **11**: 2%; (*S*)-**1**: 7%; (*R*)-**1**: 7%; **2**: 9%; **12**: 6%. B) Amoebicidal activities of pyreudione analogues against *D. discoideum*. The asterisk indicates that these compounds were identified in *P. fluorescens* HKI0770 cultures. DIPEA = diisopropylethylamine; EDCI = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; LHMDS = lithium bis(trimethylsilyl)amide, DMAP = 4-dimethylaminopyridine.

with the hydroxy group hydrogen bonded to the lactam carbonyl group.[19] The ratio between the *Z-exo*-enol and *E-exo*-enol form of the pyreudiones was approximately 7:2.

In addition, we identified a previously reported cyclic lipopeptide (CLP **5**, Figure 1).[20,21] Bacterially produced CLPs constitute a very diverse group of secondary metabolites with equally diverse, yet often poorly understood ecological functions.[22]

Next, we determined the amoebicidal activity of the isolated bacterial metabolites. While CLP **5** was modestly active, with a half maximal inhibitory concentration (IC_{50}) of 94 μg mL^{-1}, pyreudiones A–D (**1**–**4**) were responsible for most of the amoebicidal activity, with IC_{50} values in the range of 1–11 μg mL^{-1}. Furthermore, IC_{50} values from combinations[23] of pyreudione A and CLP **5** showed additive effects, with no observable synergism in the *D. discoideum* growth inhibition assay (see the Supporting Information). The main metabolites isolated from a liquid culture of *P. fluorescens* HKI0770 were, in descending order, CLP **5** (31 mg L^{-1}), pyreudione A (15 mg L^{-1}), and pyreudione B (4 mg L^{-1}). Pyreudiones C and D were produced in small concentrations of approximately 1 mg L^{-1}. Since we could not identify any congeners with either shorter chain length ($n<6$; Scheme 1) or longer chain length ($n>10$), we sought to investigate the influence of the alkyl chain length on amoebicidal activity. From this, we expected to gain insight into why **1** and **2** constitute the major isolated metabolites.

Enantioselective synthesis of the pyreudiones also allowed structural verification and determination of the absolute stereochemistry of the isolated natural products. Both (*S*)- and (*R*)-enantiomers of the main metabolite **1** were prepared, as well as the chain-length analogues **9**–**12** (Scheme 1).

Starting from L-proline, *N*-acetylation was performed with acetic anhydride in ethyl acetate,[24] and the corresponding Weinreb amide **7** was formed with *N*,*O*-dimethyl hydroxylamine and EDCI as coupling reagent. LHMDS-mediated cyclization yielded pyrrolizidine-1,3-dione core **8**. Subsequent acylation using various linear-chain fatty acids furnished the desired pyrrolizidine alkaloids **1**, **2**, and **9**–**12**.

The ^{1}H, ^{13}C NMR, and HRMS spectra of synthetic pyreudione A (**1**) matched those of isolated **1**, as did their HPLC retention times. Importantly, the specific optical rotation values were in good agreement (see the Supporting Information). Synthetic enantiomer (*R*)-**1** displayed a specific optical rotation with same magnitude yet opposite sign. This confirms an (*S*)-configuration for the pyreudiones, thus indicating a putative biosynthesis from L-proline.

We tested the chain-length analogues of **1** for amoebicidal activity and found that the short-chain-length analogues **9**–**11** showed no detectable activity up to a concentration of 100 μg mL^{-1} (see Scheme 1). The compounds with longer chain lengths (**1**, **2**, and **12**) showed considerable activity, with IC_{50} values of 4, 1, and 2 μg mL^{-1}, respectively. Chain-length analogues with $n>8$ did not display a strong increase in activity when compared to pyreudione (**2**; $n=8$).

Bioassay-guided fractionation enabled us to identify the most potent amoebicidal secondary metabolites, yet we did not know whether these metabolites actually prevent amoebal predation. In order to address this question, mutant strains with impaired CLP and pyreudione syntheses were required, and thus, we set out to identify the respective biosynthetic genes. We sequenced the genomic DNA of *P. fluorescens* HKI0770 using Illumina next-generation sequencing.[35] Sequence analysis of a variety of genes confirmed the genus and species of *P. fluorescens* HKI0770. We used AntiSmash[25] to identify a gene locus corresponding to a multimodular nonribosomal peptide synthetase (NRPS).[26,27] Bioinformatic prediction of the amino acid sequence of the respective metabolite was in excellent agreement with the amino acid sequence of CLP **5** (see the Supporting Information).

Identification of the biosynthetic gene(s) corresponding to the pyreudiones proved more complex since both an NRPS and alternative enzymatic machineries were conceivable. We were able to locate a monomodular NRPS gene of 3.9 kb containing putative condensation (C), adenylation (A), thiolation (T), and thioesterase (TE) domains (Figure 3, box). The A domain was predicted to be specific for activating a hydrophobic aliphatic amino acid (using NRPSpredictor2).[28] This gene thus seemed likely to code for a pyreudione synthetase. We inactivated both the putative pyreudione (*pys*)

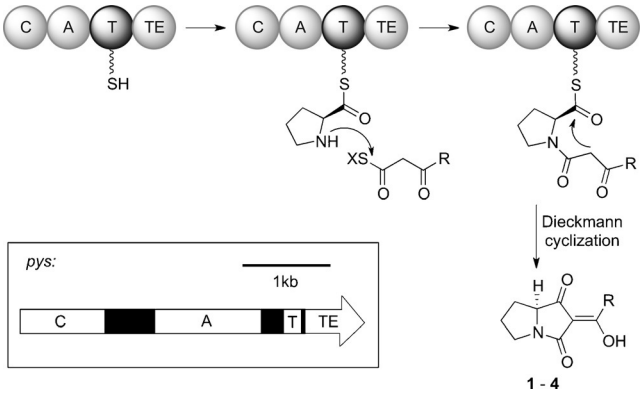


Figure 3. Proposed biosynthesis of pyreudiones **1**–**4** and architecture of the NRPS gene *pys* (box). C: condensation domain; A: adenylation domain; T: thiolation domain; TE: thioesterase domain. See the Supporting Information for details.

and CLP (*clp*) NRPS genes through homologous recombination based allelic replacement.[29,30] Chromosomal deletion of the A + T domains of the putative pyreudione synthetase *pys* gave *P. fluorescens* HKI0770Δ*pys*, and deletion of the A_{Thr} domain of the cyclic lipopeptide synthetase gave *P. fluorescens* HKI0770Δ*clp*. The corresponding metabolite profiles were analyzed by HPLC–MS. Both gene deletions led to complete loss of production of pyreudiones **1**–**4** (Δ*pys*) and CLP **5** (Δ*clp*). The double deletion mutant Δ*clp*Δ*pys* produced none of the metabolites **1**–**5** (Figure 4 A).

These results led us to propose an NRPS-based biosynthesis for the pyreudiones (Figure 3).[31] The A domain selects L-proline and loads the adenylated amino acid onto the T domain. Amide bond formation occurs in a subsequent step between the secondary amine of proline and a β-ketoacid, presumably catalyzed by the C domain. This domain would have to show enough substrate promiscuity to accept a variety of β-ketoacids in order to produce the different alkyl chain congeners. Whether the fatty acids stem from primary metabolism or dedicated polyketide synthases requires further investigations. The TE domain likely catalyzes a Dieckmann-type cyclization, releasing the tetramic acids. This mode of action has previously been reported for other thioesterases.[32] Interestingly, this biosynthesis bears some resemblance to that of the fungal metabolite tenuazonic acid.[33]

Further phenotypic analysis showed that the mutant Δ*clp* lost its swarming capabilities (see Figure S2 in the Supporting Information). While the role of CLPs in bacterial swarming is well known,[22] this finding is significant in this context since swarming is considered to be a predator-defense strategy.

We also tested the different mutants with respect to amoebal predation. Neither the wild type (WT) nor the Δ*clp* strains showed any amoebal plaques, which indicates that CLP **5** does not cause inedibility (Figure 4). Interestingly, amoebal plaques were clearly visible for Δ*pys*, the strain with impaired pyreudione biosynthesis. The double mutant Δ*clp*Δ*pys* showed even larger plaques when compared to the single mutant Δ*pys*. The same phenotypes were observed with other amoebae, namely *D. mucoroides* and *Polysphondylium pallidum*, two strains that are particularly common in Germany (Figure 4).[34]

In order to quantify the amoebicidal effect of the bacterial secondary metabolites, *D. discoideum* was co-cultured with the *P. fluorescens* mutant strains (Figure 5). As previously observed, the WT strain inhibited amoebal growth and caused complete lysis within 50 h. However, the Δ*clp* strain showed virtually no decrease in amoebicidal activity compared to the WT. However, the Δ*pys* strain showed greatly reduced amoebicidal activity, comparable to that of the Δ*clp*Δ*pys* strain. These experiments demonstrate that bacterial production of pyreudiones A–D is necessary and sufficient for preventing amoebal predation in *P. fluorescens* HKI0770.

In summary, we have identified a group of bacterial alkaloids that protect the producing strain from being eaten by a variety of common soil amoebae. Mutational analysis suggests that a monomodular NRPS catalyzes the formation of the pyreudiones. Monomodular NRPSs are an interesting

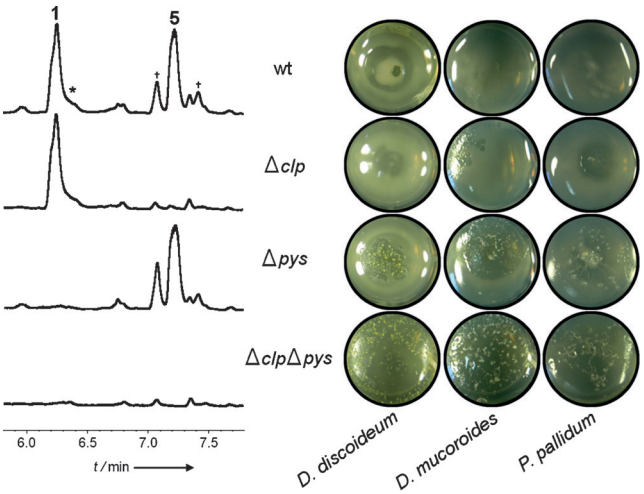


Figure 4. Phenotypic analysis of *P. fluorescens* HKI0770 WT, Δ*pys*, Δ*clp*, and Δ*clp*Δ*pys*. HPLC profiles (left; 190 nm) with pyreudione **1** (congeners **2**–**4** indicated by *) and cyclic lipopeptide **5** (CLP-associated metabolites indicated by †) are shown. All profiles are displayed at the same scale. Amoebal plaque assays (right) show no plaque formation for any of the three amoebae on the WT and Δ*clp* strains, whilst plaques and fruiting bodies are visible on the Δ*pys* and Δ*clp*Δ*pys* strains. Fruiting bodies are macroscopic objects that contain the spores of *D. discoideum*. They occur once the amoeba has depleted its bacterial food source.

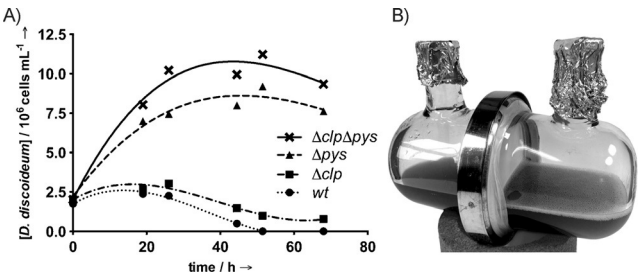


Figure 5. A) Concentration of *D. discoideum* cells during co-cultivation with *P. fluorescens* strains. Data were fitted with a third-order polynomial for better visualization by using GraphPadPrism software. B) The corresponding co-culture setup with amoebal culture (right chamber) and bacterial culture (left chamber).

class of biosynthetic genes that have been identified in many bacterial genomes, yet very few secondary metabolites have so far been ascribed to them. Currently, we are investigating the regulation and biosynthetic details of these potent antifeedants.

Acknowledgements

We thank A. Perner, and H. Heinecke (Hans Knöll Institute, Jena) for MS and NMR measurements. H. Brangsch is thanked for providing *E. coli* ET12567 strain. We are grateful for financial support from the Leibniz Association, for a postdoctoral fellowship from the Daimler and Benz foundation, and for an Aventis Foundation Ph.D. fellowship (M.K.). This work was also supported by the DFG-funded graduate school of excellence Jena School for Microbial Communication (R.B.).

Keywords: alkaloids · bacterial defense · biosynthesis · natural products · pyrrolizidine alkaloids

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 8944–8947
Angew. Chem. **2016**, *128*, 9090–9093

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Received: April 5, 2016
Published online: June 13, 2016

