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Peptides

Atropopeptides are a Novel Family of Ribosomally Synthesized and Posttranslationally Modified Peptides with a Complex Molecular Shape

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Abstract: Biomacromolecules are known to feature complex three-dimensional shapes that are essential for their function. Among natural products, ambiguous molecular shapes are a rare phenomenon. The hexapeptide tryptorubin A can adopt one of two unusual atropisomeric configurations. Initially hypothesized to be a non-ribosomal peptide, we show that tryptorubin A is the first characterized member of a new family of ribosomally synthesized and posttranslationally modified peptides (RiPPs) that we named atropopeptides. The sole modifying enzyme encoded in the gene cluster, a cytochrome P450 monooxygenase, is responsible for the atropospecific formation of one carbon-carbon and two carbon-nitrogen bonds. The characterization of two additional atropopeptide biosynthetic pathways revealed a two-step maturation process. Atropopeptides promote pro-angiogenic cell functions as indicated by an increase in endothelial cell proliferation and undirected migration. Our study expands the biochemical space of RiPP-modifying enzymes and paves the way towards the chemoenzymatic utilization of atropopeptide-modifying P450s.

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Molecular shape defines the function of large biomolecules such as proteins, RNA and DNA.[1] In the case of small molecules, structures with ambiguous molecular shapes are rare and most structures can be sufficiently described by considering point chirality, *E/Z* isomerism, and canonical (single axially chiral) atropisomerism.[2] Ambiguous molecular shapes are usually not even taken into consideration during the structure elucidation process.[2] The correct stereochemical assignment of a molecule and the knowledge about possible stereoisomers, however, are of paramount importance as cautionary tales from the past have painfully illustrated. One stereoisomer of thalidomide (Contergan), for instance, can be used as a sleeping pill while the other stereoisomer causes very severe birth defects.[3] One of the few examples of shape-defined natural products is the lassopeptides, a family of ribosomally synthesized and posttranslationally modified peptides (RiPPs).[4] RiPP biosynthetic gene clusters (BGCs) usually encode a precursor peptide, tailoring enzymes, and one or multiple proteases. The precursor peptide consists of a leader and a core peptide. During the RiPP maturation process, the leader peptide serves as a signal and recognition sequence for tailoring enzymes that modify the core peptide. Upon full modification, the core peptide is released from the leader peptide by a protease.[5] Lassopeptide BGCs, for instance, encode a peptidase that is responsible for leader peptide removal and a lasso cyclase (macrolactam synthetase) that forms a macrolactam ring and threads the C-terminal amino acid tail through the macrolactam ring.[4,6] Lassopeptides, like microcin J25 (Figure 1) can theoretically adopt one of two unusual atropisomeric configurations: the natural threaded (**1a**) configuration in which the peptide chain is threaded through the macrolactam ring and held in place by e.g. large bulky amino acid residues to prevent unthreading of the lassopeptides and the non-natural, un-threaded (**1b**) atropisomer.[4,7] Both stereosiomers can only be interconverted by multiple, non-physical bond torsions, a phenomenon referred to as unusual atropisomerism.[2] Unusual atropsiomerism is to be distinguished from classical atropisomerism and topoisomerisms. In the case of canonical atropisomerism, two stereoisomers can be interconverted through hindered bond rotation.[2] Topoisomers, on the other hand, require bond scission and reformation for the interconversion of both isomers.[2,8]

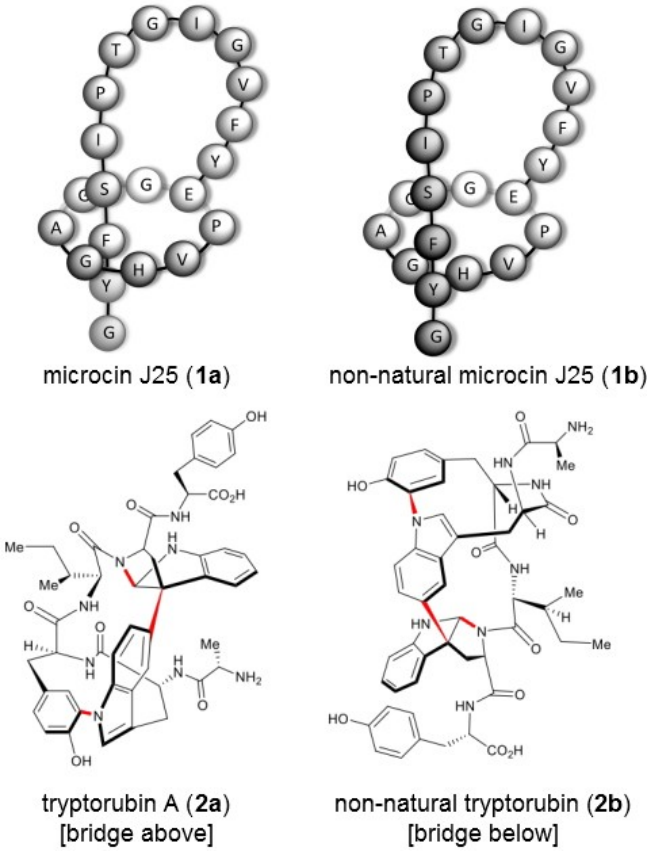

Figure 1. Example of peptide natural products with non-trivial molecular shapes that can adopt one of two unusual atropisomeric configurations.

Tryptorubin A (**2a**) is, to our knowledge, the smallest natural product where molecular shape has to be considered.[2] It was isolated from *Streptomyces* sp. CLI2509, a bacterial symbiont of the bracket fungus *Hymenochaete rubiginosa*.[9] The unusual hexapeptide is characterized by a carbon-carbon and a carbon-nitrogen bond between the side chains of its aromatic amino acids and an additional carbon-nitrogen bond between the tryptophan side chain and the peptide backbone, respectively, resulting in an extremely rigid 3D structure (**2a**) (Figure 1).[9] Synthetic studies by the Baran lab have recently identified that tryptorubin A can adopt two possible unusual atropisomeric configurations, which we described as the bridge above (**2a**) and the bridge below (**2b**) configurations (Figure 1).[2] The Baran lab has developed an atropo-specific total synthetic route towards both unusual atropisomers, yet only the bridge above isomer (**2a**) was isolated from the natural producer, suggesting that the biosynthetic enzymes tightly control the atropo-specific formation of **2a**.[2]

Initially, tryptorubin A was hypothesized to be the product of a non-ribosomal peptide synthetase (NRPS).[9] Genome mining studies pointed to a NRPS biosynthetic gene cluster (BGC) since the putative tryptorubin NRPS BGC was conserved in the original producer and the closely related producer of **2a**, *Streptomyces* sp. Tü6071.[9] Moreover, the putative tryptorubin NRPS harbored the number of modules that would be required for hexapeptide biosynthesis.[9] In addition, similar biaryl-bridges and C–N bridges between aromatic amino acid side chains and side chains and the peptide backbone have been reported for several NRPS-derived peptides including vancomycin-like glycopeptides and diketopiperazines (e.g., aszonalenin), respectively.[10] The predicted adenylation domain substrate specificities, however, were not in good agreement with the tryptorubin sequence. Moreover, the presence of additional genes in the BGC that are likely not involved in tryptorubin A biosynthesis, prompted us to re-evaluate its biosynthesis.

Here, we show that tryptorubin A is a ribosomally synthesized and posttranslationally modified hexapeptide. Only two genes, a precursor, and a cytochrome P450 monooxygenase gene are sufficient for the atropo-specific biosynthesis of tryptorubin A. We expand the family of atropopeptides, as we named peptides that are biosynthesized in a tryptorubin-like fashion and that are characterized by a complex three-dimensional shape. We show that atropopeptide pathways yield two products, a hexa, and a pentapeptide through a two-step maturation process. Both products act as signaling metabolites and stimulate endothelial cell proliferation and migration.

The initially proposed NRPS BGC was the only candidate tryptorubin (*trp*) BGC that was identified by state-of-the-art genome mining pipelines to be potentially involved in hexapeptide biosynthesis.[9] Due to several inconsistencies between the BGC composition and the structure of tryptorubin A, we evaluated an alternative biosynthetic origin of tryptorubin A. Since all amino acids incorporated into tryptorubin A are L-configured,[2] we explored if **2a** might be biosynthesized by a RiPP BGC that has been overlooked by state-of-the-art genome mining pipelines. Manual mining of the six-frame translated genome sequences of both reported tryptorubin A producers for the core amino acid sequence (AWYIWY) resulted in the identification of a putative precursor peptide sequence.[2] The precursor gene was not identified as a gene by any genome annotation algorithm or genome mining pipeline, most likely as it does not show any homology to known RiPP precursor genes. Only a single putative natural product tailoring enzyme, a cytochrome P450 monooxygenase, is encoded next to the putative tryptorubin precursor gene. The two genes are the only genes that are conserved in a variety of other putative producers of tryptorubin-like peptides as revealed by BLAST analysis[11] of the putative tryptorubin-modifying P450 and manual analysis of the surrounding genomic area (Figure S1). These studies suggested that the cytochrome P450 is the only other gene product involved in tryptorubin A maturation. Manual inspection of the genomic neighborhood of the tryptorubin-like P450s identified by BLAST analysis[11] resulted in the identification of several putative tryptorubin-like BGCs[2] that either encode precursor peptides with altered core peptide sequence and/or differ in the presence of additional cytochrome P450 and other tailoring genes.

The identified cytochrome P450 belongs to a new subfamily of cytochrome P450s that is exclusively associated

with homologs of *trp*-like pathways and does not group with other cytochrome P450s encoded in other RiPP BGCs (e.g., biarylitide and citillin BGCs) (Figure S2 and Table S1).[12] While biarylitide precursor peptides are significantly shorter, atropopeptide and cittilin precursor peptides have the same length but do not show sequence conservation. BGCs of biarylitides and cittilins lack a pathway-encoded protease.[12] Since no protease is encoded in the putative tryptorubin BGC either, we speculated that an ubiquitous protease might be involved in proteolytic cleavage to release **2a**.[2] It is tempting to speculate that all tryptorubin-like peptides are processed by a member of the same protease family irrespective of the taxonomic grouping of the producer organism. To identify the likely protease, a selection of genomes of putative tryptorubin producers were mined for gene products that might be involved in proteolytic cleavage (Figure S3). In addition to the genomes of putative tryptorubin producers, genomes of frequently used bacterial heterologous host strains were likewise mined for all genes annotated as proteases or peptidases to identify suitable host strains for subsequent heterologous expression studies of the putative minimal *trp* BGC. Phylogenetic analyses revealed that putative producers of tryptorubin-like peptides encode members of four conserved protease families, three of which (M41 family of unassigned peptidases,[13] S16 family of unassigned peptidases[14] and AA223 family of peptidases[13]) belong to the superfamily of AAA+ proteases that are intracellular, membrane-associated enzymes that catalyze the degradation of a wide range of proteins. Moreover, members of the family of M24B unassigned extracellular metallo-peptidases[15] were identified in all putative tryptorubin producers and all commonly used *Streptomyces* host strains (Figure S3).

To verify our biosynthetic model, we synthesized the putative *trp* BGC, cloned it into different *Streptomyces* vectors (integrative and non-integrative) and heterologously expressed it in a range of *Streptomyces* host strains that harbor copies of all identified protease/peptidase genes putatively involved in tryptorubin maturation using different media and culture conditions. Extracts were screened for the presence of tryptorubin A. The highest yields of **2a** were obtained from the heterologous expression of the *trp* BGC in the replicative plasmid pUWL201-oriTaac(3)IV in *Streptomyces albus* J1074 (Figure S4–S5). To our surprise, tryptorubin A was only produced in the presence of adsorber resin, indicating that **2a** might have a negative effect on its biosynthesis. To verify whether the heterologously produced tryptorubin A is the bridge-above atropisomer that has been reported from the natural producer, we compared the retention time of the heterologously produced tryptorubin A with authentic standards of both unusual atropisomers that were either obtained through total synthesis[2] or isolated from the native producer.[9] These studies verified that only the bridge-above atropisomer is produced and that the minimal *trp* BGC is sufficient for tryptorubin biosynthesis (Figure S6). The identification of multiple tryptorubin-like BGCs suggests that tryptorubin A is the first characterized member of a new family of RiPPs. We suggest the name atropopeptides for the novel RiPP family to account for their complex molecular shape.

To verify whether atropopeptide BGCs that harbor precursor genes that encode the tryptorubin A core peptide sequence all produce the bridge-above stereoisomer or whether tryptorubin-like cytochrome P450s generate different patterns of carbon-carbon and carbon-nitrogen bonds, we characterized the atropopeptide BGC encoded in *Streptomyces misionensis* DSM 40306. To our surprise, we observed the production of two atropopeptides with a mass difference of 71 Da that is indicative of a loss of the N-terminal alanine residue. We extracted both peptides from a 6 L culture and purified them to homogeneity using a combination of flash chromatography and semi-preparative high-performance liquid chromatography (HPLC). High resolution mass spectrometry (HR-MS) and nuclear magnetic resonance (NMR) data of the hexapeptide (Figure S8–S16, Table S3) in combination with the comparison with authentic standards of **2a** and **2b** (Figure S7) revealed that the hexapeptide is virtually identical to tryptorubin A. The structure of the pentapeptide was determined by 1- and 2-dimensional NMR experiments that showed that the pentapeptide (**3**) lacks the N-terminal alanine residue but retains the 3-dimensional shape described for **2a** as revealed by characteristic NOE correlations (Figure S17–S26, Table S4). The perfect agreement with the bridge-above structure of the penta and hexapeptide with the reported structure of tryptorubin A points to a highly atropo-specific biosynthesis despite the only 72 % homology of the cytochrome P450 when compared to the cytochrome P450 encoded in the *trp* BGC. The observation of a tryptorubin A analog that lacks the N-terminal alanine residue (further referred to as tryptorubin B (**3**) (Figure 2)), indicates that a) the protease that releases the mature tryptorubin A might not be specific and gives rise to both products, b) that tryptorubin B is the bioactive molecule that results from the N-terminal removal of alanine in a pro-drug like mechanism or c) tryptorubin B is the N-terminal degradation product of the bioactive tryptorubin A. To verify which of our hypotheses is correct, we reinvestigated the natural producer of **2a** and observed the production of both tryptorubin A and B after prolonged culturing times. We next set up a time-course experiment to verify if tryptorubin A is converted into tryptorubin B over time (Figure S27–S28). These experiments indeed revealed that over the course of the first two days, tryptorubin A is almost exclusively produced. Upon longer culturing times, more and more tryptorubin B (**3**) is biosynthesized and the relative amount of tryptorubin A (**2a**) is decreasing. This observation suggests a conversion of tryptorubin A into tryptorubin B (Figure 2). As members of the M24B peptidases, one of the four peptidase families present in all putative atropopeptide producers, have been reported to remove N-terminal amino acids,[15] we speculate that homologs of the peptidase are responsible for the N-terminal alanine removal. Since no bioactivity had been reported for tryptorubin A, we subjected both tryptorubin A and B to a large panel of bioactivity assays. Both compounds were tested for their i) antibiotic activity against a wide range of bacteria across the bacterial branch of life

including all ESKAPE pathogens, ii) antifungal activity against a selection of fungi, including fungal pathogens such as *Candida albicans*, iii) cytotoxic activity against several cancer cell lines, and iv) anti-inflammatory activity. Since **2a** and **3** did not show any activity in physiologically relevant concentrations in any of the bioactivity assays, we evaluated alternative bioactivities. A potential influence on the proliferation of the human microvascular endothelial cell line (HMEC-1) was assessed. All atropopeptides showed strong proliferative effects in HMEC cultures indicating a pro-angiogenic activity (Figure 3a). Moreover, a migration assay was performed to assess undirected endothelial cell migration. All tested atropopeptides promoted cell migration to close the artificially introduced cell-free gap (Figure 3b and Figure S29), suggesting pro-angiogenic properties. We next verified if **2a** and **3** might have similar effects on bacterial growth and studied the effect of both compounds on the complex life cycle of *Streptomyces*. The presence of the atropopeptide BGC significantly accelerates *Streptomyces* differentiation as indicated by extensive aerial mycelia formation in comparison to strains harboring the empty vector control or the wild-type strain, respectively (Figure S30).

We next set out to explore the effect of a different core peptide sequence on the three-dimensional shape, study whether amino acid alterations result in different biaryl and carbon-nitrogen bridge patterns and obtain first insights into the structure–activity relationship of **2a**. We therefore generated a multiple sequence alignment of 30 putative atropopeptide precursor peptides that are encoded in the neighborhood of putative atropopeptide-modifying P450 genes (Figure S31). The relative degree of conservation of the amino acids at each point was analyzed (Figure S31). The precursor peptides are 23–27 amino acids in length. The multiple sequence alignment revealed that the leader peptide harbors two relatively conserved motifs that are separated by a variable region. While positions 1–3 and 5 in the core peptide sequence of the identified atropopeptides are conserved, positions 4 and 6 vary and frequently harbor aromatic amino acids (Figure S31). These additional aromatic amino acid bearing atropopeptides are prime targets for subsequent studies to identify putative novel atropopeptide bridge patterns. We therefore selected the atropopeptide BGC (further referred to as amyxirubin or *amy* BGC) in the genome of *Amycolatopsis xylanica* with the putative core peptide sequence AWYIWW. Two atropopeptides that we named amyxirubin A (**4**) and B (**5**) (Figure 2b) that differ by the presence or absence of the N-terminal alanine residue were extracted from 6 L cultures and purified to homogeneity. The structure of the purified hexapeptide amyxirubin A was determined by 1- and 2-dimensional NMR experiments (Figure 2b, Figure S33–S41 and Table S5) revealing that while the core amino acid sequence differs, the regioselectivity of biaryl and carbon-nitrogen bridges are identical to those in tryptorubin A. In addition, key NOE correlations showed that the isolated peptides adopt the same atropisomeric configuration as reported for tryptorubin A indicating that the atropopeptide-modifying P450s show a high degree of conserved stereo- and regioselectivity

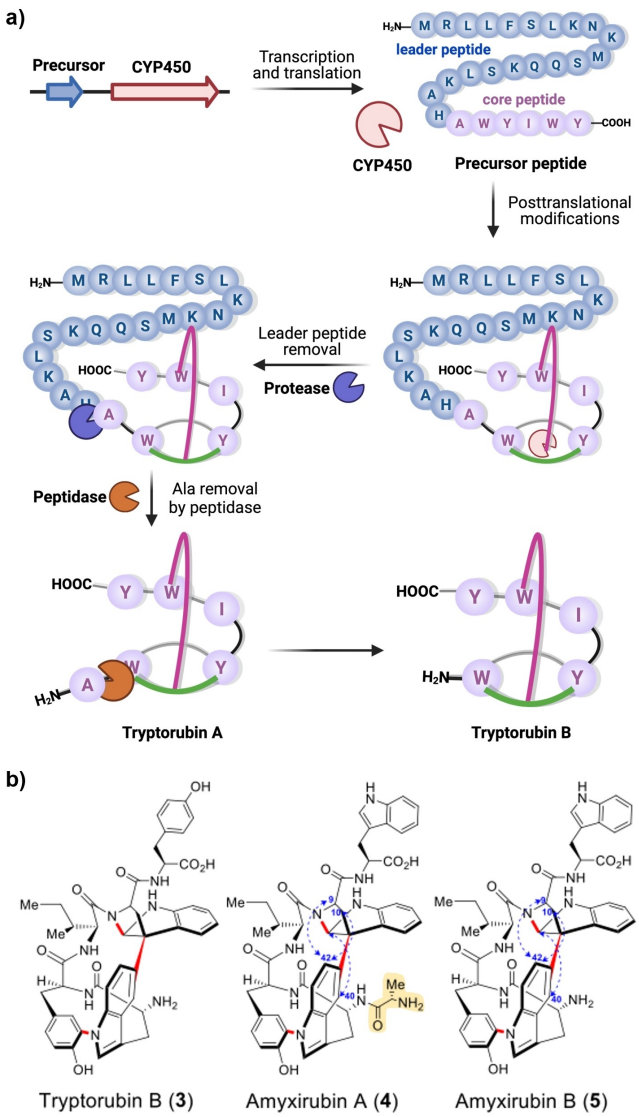

Figure 2. Proposed model for atropopeptide biosynthesis and structures of atropopeptides characterized in this study. a) Model for the biosynthesis of tryptorubin A and B. b) Hexa- and pentapeptide structures characterized in this study. Key NOE correlations that are indicative of the bridge above atropisomer are highlighted in blue. Characteristic C–C and C–N bonds are highlighted in red.

(Figure S42–S49 and Table S6). Moreover, **4** and **5** showed similar proliferative and pro-migratory properties in endothelial cell cultures indicating that the amino acid substitution does not affect bioactivity (Figure 3a and Figure S29).

Tryptorubin A and B are the first members of a new family of RiPPs that we named atropopeptides to account for their unusual complex three-dimensional shape. Our studies show that the single modifying enzyme, a member of a new subfamily of cytochrome P450 enzymes that is not related to characterized RiPP modifying-cytochrome P450s,[16] is sufficient for the atropo-specific formation of one biaryl link and two carbon-nitrogen bridges. All characterized atropopeptides feature the conserved bridge-above configuration suggesting an important function of the

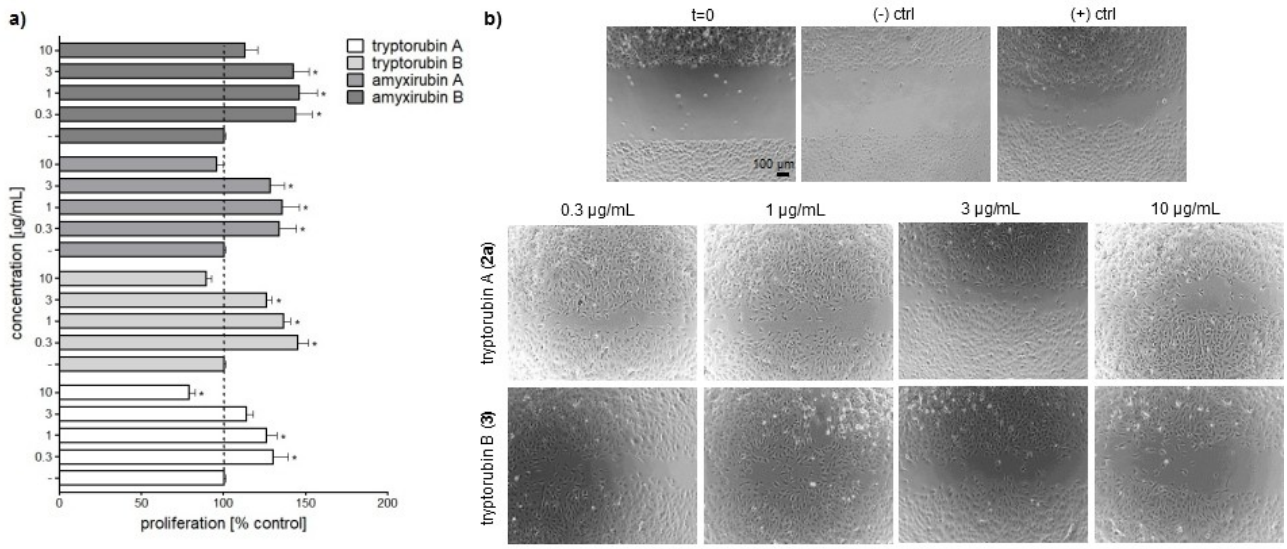

Figure 3. Bioactivity studies. a) Proliferation assay. HMECs were seeded in low-density and treated with atropopeptides for 72 h. Cells were stained with crystal violet solution. The amount of DNA-bound crystal violet was detected by absorbance measurements. b) Undirected migration assay. A scratch was inflicted on a confluent HMEC monolayer. The cells were incubated in growth medium or growth medium containing the indicated concentrations of atropopeptides and incubated for 4 h followed by fixation and microscopical analysis. All data are expressed as mean ± standard error of the mean (SEM); (A) $n=4$; (B) $n=3$; $p \leq 0.05$ vs. ctrl was considered as statistically significant.

three-dimensional shape of the signaling metabolites. BLAST analysis[11] revealed that atropopeptides are encoded in a wide range of bacterial genera. The frequently encountered substitutions at position 4 and 6 in the core structure of tryptorubin A with aromatic amino acids might lead to the identification of atropopeptides with different bridge patterns. Since BLAST analysis[11] excels in the identification of related sequences, more sophisticated bioinformatic approaches might expand the atropopeptide biosynthetic space beyond the variability reported here. Our study indicates that atropopeptides are first modified by the characteristic cytochrome P450 before they undergo a two-step maturation process to yield the bioactive pentapeptide (Figure 2) by proteases that are not encoded in the minimal atropopeptide BGCs. The proteolytic cleavage of RiPPs by ubiquitous proteases is rare since most RiPP families are processed by BGC-encoded peptidases.[5] The strong proliferative properties of **2a** and **3** and their function as bacterial signaling metabolites points to a conserved mode of action. The proliferative and pro-migratory effects of atropopeptides suggest pro-angiogenic properties. Our study on tryptorubin A adds to the increasing number of complex peptide natural products that were initially believed to be biosynthesized by NRPS and that, upon manual inspection of the genome sequence and functional validation of the putative BGC, have been identified as RiPPs.[17]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Information on the biosynthetic gene clusters characterized in this study have been deposited to the MIBiG database (BGC0002750 (*trp*), BGC0002751 (*amy*)), and information about the corresponding natural products will be deposited to the Natural Product Atlas. Links to access the phylogenetic trees displayed in the Supporting Information data file are provided in the manuscript. All remaining data will be made available upon request.

Keywords: Atropisomerism · Cytochrome P450 Monooxygenase · Genome Mining · RiPPs · Stereoisomerism

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