

D-Peptidase Activity in a Marine Mollusk Detoxifies a Nonribosomal Cyclic Lipopeptide: An Ecological Model to Study Antibiotic Resistance

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Laurine Darcel, Louis Bornancin, Delphine Raviglione, Isabelle Bonnard, Suzanne Mills, et al.. D-Peptidase Activity in a Marine Mollusk Detoxifies a Nonribosomal Cyclic Lipopeptide: An Ecological Model to Study Antibiotic Resistance. Journal of Medicinal Chemistry, In press, 10.1021/acs.jmedchem.1c00249 . hal-03212973

HAL Id: hal-03212973

https://univ-perp.hal.science/hal-03212973

Submitted on 30 Apr 2021

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D-peptidase activity in a marine mollusk detoxifies a non-ribosomal cyclic lipopeptide: an ecological model to study antibiotic resistance.

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ABSTRACT

In the marine environment, sessile cyanobacteria have developed chemical strategies for protection against grazers. In turn, herbivores have to circumvent these defenses and in certain cases even take advantage of them as shelter from their own predators. This is the case of *Stylocheilus striatus*,

a sea hare that feeds on *Anabaena torulosa*, a cyanobacterium that produces toxic cyclic lipopeptides of the laxaphycin B family. *Stylocheilus striatus* consumes the cyanobacterium without being affected by the toxicity of its compounds, but also uses it as an invisibility cloak against predators. In this article, using different substrates analogous to laxaphycin B, we demonstrate the presence of an enzyme in the digestive gland of the mollusk that is able to biotransform laxaphycin B derivatives. The enzyme belongs to the poorly known family of D-peptidases that are suspected to be involved in antibiotic resistance.

INTRODUCTION

Overcoming and understanding resistance to antibiotics is of major importance for human health worldwide.[1] Bacteria develop resistance to antibiotics through three main mechanisms: preventing the antibiotic from accessing the target, modifying the target or inactivating the antibiotic.[2] For example, D-peptidases are able to reengineer bacterial cell-wall which prevents antibiotics from accessing their molecular target resulting in antibiotic resistance, and β-lactamases are able to hydrolyze carbapenems antibiotics destroying their activity.[3,4] Recently D-peptidases have been found to act on antibiotics biosynthesized by non-ribosomal peptide synthetases (NRPS).[5] D-stereospecific resistance peptidases (DSRP) regulate the concentration of the antibiotic within the producer organism to avoid self-toxicity and are therefore closely associated with NRPS modules. A recent genome mining study reports the presence of DSRP in a large number of bacteria.[5] For example, BogQ and TriF are DSRPs in Firmicutes (mostly Gram positive bacteria), and cleave the linear peptides bogorol and tridecaptin on the C-terminal side of D-amino acids (D-AA), but BogQ also cleaves the antibiotic bacitracin, a cyclic-non-ribosomal lipopeptide. Therefore, BogQ and TriF peptidases provide microbes with a potential of antibiotic resistance by having an enzymatic

site that recognizes a minimal pattern containing D-amino acids. As such, D-peptidases might constitute a threat for the future of non-ribosomal lipopeptides used as antibiotics. However, these enzymes are poorly studied and their cleavage mechanisms are not well known. The aim of this project was to understand the specificities of this type of enzyme using a new model, a marine trophic chain.

A natural model system requires the presence of an organism with the antibiotic properties in question (non-ribosomal peptides), as well as an organism that produces the resistance-bestowing enzyme. One class of natural products with promising antibiotic properties are cyclic lipopeptides (CLPs), which are among the most intriguing peptides produced by fungi and bacteria as they contain a fatty acid linked to either a D-, N-methyl, β-hydroxy- or α,β−dehydro-, unusual amino acids that confer them antiparasitic, antifungal and antibacterial activities.[6,7] Due to these non-ribosomal amino acids and the lipophilic acyl chain, CLPs are expected to show little or no susceptibility to cleavage by animal peptidases and some of these CLPs (colistin and polymyxin B) are therefore considered to be the last resort in the treatment of antibiotic resistant microbes.[8] Among bacteria, cyanobacteria produce a diverse array of secondary metabolites that, during blooms, are often associated with hepatotoxic and neurotoxic poisoning in humans and marine animals.[9,10] CLPs with biological activities on multiple targets account for the majority of secondary metabolites produced by these cyanobacteria.[11] The function of these peptides in the physiology and ecology of cyanobacteria is still debated, but has been ascribed to either protection against grazing and/or allelopathy.[12] The abundance of CLPs in cyanobacteria coupled with the presence of a grazing herbivore would be a novel model system providing the potential to investigate cleavage mechanisms and resistance.

The secondary metabolites of two benthic filamentous bloom-forming cyanobacteria present in French Polynesia, *Lyngbya majuscula* (*Lm*) and *Anabaena torulosa* (*At*), have been largely characterized. In both cases the major compounds are lipopeptides, cyclic hexadepsipeptides from the kulolide family[13] in *L. majuscula*, and cyclic undeca- and dodeca-peptides from the laxaphycin family[14] in *A. torulosa*. The laxaphycin secondary metabolites in *A. torulosa* can be classified into two types, laxaphycin A-type and laxaphycin B-type peptides, the major compounds being laxaphycin A (laxA) **1a**, and laxaphycins B (laxB) **2a** and B3. While laxaphycin A has no cytotoxic activity, laxaphycins B and B3 have cytotoxic effects on different cell lines, which are synergistically enhanced when laxA and laxB are combined.[15] LaxB is made up of 12 amino acids with a stereochemistry alternating between L and D. This peptide contains non-proteinogenic amino acids, (3R)-β-aminodecanoic acid (Ade), (2R,3S)-hydroxyleucine (HyLeu), D-Leu, D-Asn, and N-Methyl-Ile. Laxaphycin A (laxA) is also cyclic and composed of 11 amino acids including D-AA (D-Phe, D-Leu, D-Ile) and a fatty acid (3R)-β-aminooctanoic acid (Aoc) as well as the dehydroamino acid (E-Dhb).[16] Their cyclic structure, combined with the presence of D-amino acids, should make these peptides very resistant to proteolysis.[17]

An herbivorous gastropod, the sea hare *Stylocheilus striatus (Ss)*, has been found living and feeding on both cyanobacteria in French Polynesia.[18,19] *S. striatus*, like many sea hares feeding on chemically-defended seaweeds or cyanobacteria, sequesters the whole set of lipopeptides from *L. majuscula*, for which no cytotoxic activity has been found, at high concentrations in its digestive gland[20], while only sequesters laxaphycin A-type peptides from *A. torulosa*. Interestingly, *S. striatus* is also able to biotransform diet-derived compounds into less repellent molecules.[21] Indeed, *S. striatus* biotransforms laxaphycin B-type peptides from *A. torulosa* into non-toxic acyclic compounds.

These biotransformed compounds were characterized as two acyclic laxaphycin B-type peptides coming from the parent cyclic molecule laxB.[22,23] Cyclic lipopeptides are relatively widespread in cyanobacteria, but biotransformation involving the opening of the ring and deletion of amino-acids, has never previously been described. We aimed to identify the mechanism that *S. striatus* set up to detoxify laxaphycin B-type peptides as an adaptation to feed on *A. torulosa* without suffering adverse effects.

This project examines the process of enzymatic detoxification of laxB in *S. striatus*. We report a comprehensive study of the fate of laxB and its synthetic analogues exposed to the molluscan digestive gland in order to describe the enzymatic mechanism performed by an unknown enzyme of the D-peptidase family. Finally, we test the capacity of this new D-peptidase to inactivate four known antibiotics.

RESULTS AND DISCUSSION

The study of the cyanobacterium *Anabaena torulosa* harvested during a bloom in Moorea in French Polynesia, revealed the presence of acyclolaxaphycin B (acyclolaxB) derived from the toxic laxB (Figure 1A). AcyclolaxB is open between (2R,3S)-HyLeu in position 3 and L-alanine in position 4 (Figure 1B).[22] As the starting point of NRPS biosynthesis of laxB is Ade[1] and elongation continues to the C-terminal Thr[12] with the final cyclization mediated by a thioesterase,[24] it is unlikely that the acyclolaxaphycin is the result of a malfunction of the NRPS, but more likely results from a biotransformation process of laxB by external agents. LC-MS analysis of the digestive tract of *S. striatus* showed that acyclolaxB had lost Ala[4] and (2R,3S)-HyLeu[5] confirming a biotransformation process[23] (Figure 1D), while the non-toxic laxA **1a** was bioaccumulated in the same digestive gland (Dg), but not degraded or biotransformed. Subjecting laxB to extracts of the sea hare's digestive gland highlighted that the transformation is a two-steps process beginning with

the opening of the macrocycle accompanied with a mass increase of 18 *m/z*, followed by the deletion of Ala[4] and (2R,3S)-HyLeu[5] and a loss in mass of 200 *m/z*. Hereafter we will refer to the peptides using a number and a letter corresponding to the initial compound and the stage along the cleavage process, respectively. For example, compound **2a** is the cyclic laxB, compound **2b** is the first intermediate after an increase in mass of 18 *m/z* and **2c** is the final stage of the biotransformation process after a loss in mass of 200 *m/z* (Table S1a-b). We hypothesized that the detoxification mechanism involved an enzyme targeting the D-amino acid in positions 3 and 5 of laxB. However, from this analysis it was difficult to determine if the first cleavage occurred at the C-ter of the (2R,3S)-HyLeu in position 3 resulting in the already known acyclolaxB **2d** or in position 5 producing compound **2b** (Figure 1C).

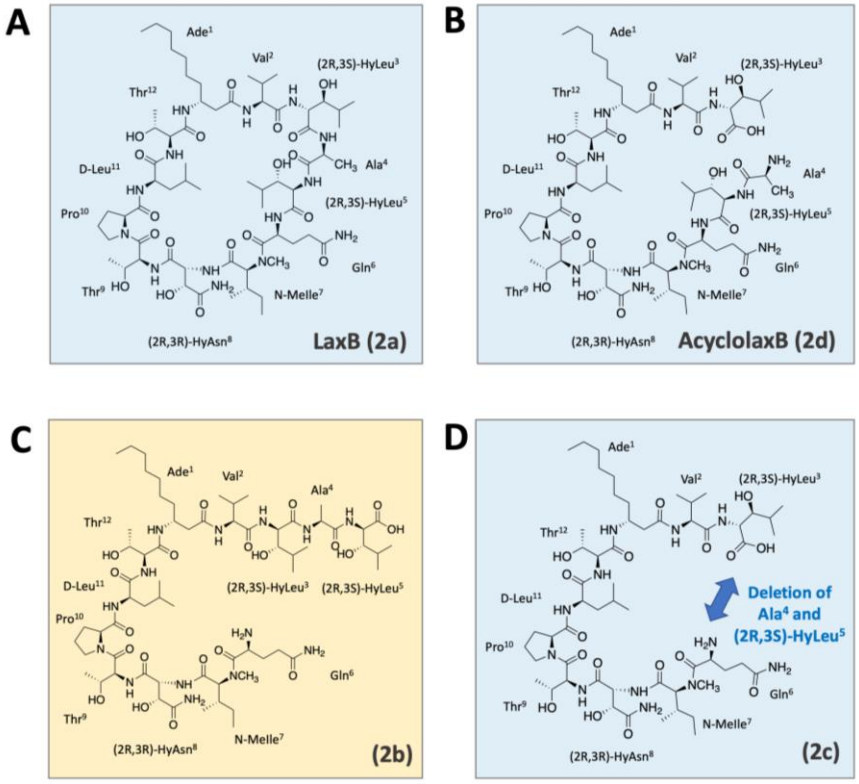


Figure 1. A. Structure of laxB (**2a**) isolated from the cyanobacterium *Anabaena torulosa*. **B.** Structure of acyclolaxB (**2d**) isolated from the cyanobacterium *Anabaena torulosa*. **C.** Hypothetical structure of the intermediate acyclic laxaphycin (**2b**) obtained during the cleavage process of laxB (**2a**). **D.** Structure of acyclic laxaphycin B (**2c**) isolated from the sea hare *Stylocheilus striatus*.

To examine the transformation process of laxB over time we used *S. striatus* collected from a bloom of *L. majuscula* in Moorea. Unlike *A. torulosa*, *L. majuscula* does not produce laxaphycins and thus *S. striatus* will not be contaminated by acyclic laxaphycins, simplifying the monitoring of laxB (**2a**) transformation. In order to ensure that *S. striatus* taken from *L. majuscula* has the capacity to transform **2a**, individual *S. striatus* were collected from, and fed on, *L. majuscula*. All individuals were dissected and their different organs analyzed. As expected, LC-MS analyses detected acyclic laxaphycin B **2c** within the digestive glands of *S. striatus* collected from *A. torulosa* (*Dg-Ss-At*), but not within the digestive glands of *S. striatus* collected from *L. majuscula* (*Dg-Ss-Lm*) (Figure S17). **2a** is thus detoxified within *Dg-Ss-At* to give the non-toxic acyclic laxaphycin B **2c**.[23] Furthermore, adding **2a** to digestive gland extracts of *S. striatus* collected from *L. majuscula* confirms that *S. striatus* does have the capacity to cleave **2a** given that we observed the decrease of **2a** to the benefit of acyclic laxaphycin B **2c** by LC-MS (Figures S16B-S18). These results were reproduced using different batches of digestive glands from different individuals whose proteins levels were kept constant during each experiment via quantification using the Bradford method.[25] To better characterize this enzyme, we hereafter used digestive gland extracts of *S. striatus* fed on *L. majuscula* (*Dg-Ss-Lm*) having confirmed its capacity to cleave **2a**.

As a prerequisite of this study, we verified that laxaphycins **1a** and **2a** do not show any intrinsic susceptibility to cleave when exposed to protease or rat serum even though their cyclic structures

and the presence of non-ribosomal amino acids should prevent their cleavage. Both peptides remained stable in Tris buffer at pH 8 when exposed to rat serum at a protein concentration of 10 mg/mL for 24 hours (Figure S19). To complete this study, compound **2a** was tested against four enzymes with broad substrate specificities: Pepsin, Elastase, Thermolysin (TLN), and Subtilisin (Table S3). Only TLN at pH 8 and 75°C resulted in the linearization of laxB **2a**, but differed from that observed in *S. striatus*, as cleavage occurred at the C-terminus of proline 10 and the hydrolysis kinetics were very different. While laxB was almost completely linearized after 30 minutes in the presence of digestive gland extracts, only 40% of laxB was consumed after 24 hours by TLN (Figure S18 and Figures S21-22). Further inspection of the enzyme highlighted that the biotransformation of **2a** was inactivated if the digestive gland extracts from *S. striatus* (*Dg-Ss-Lm*) were denatured at 90°C for 5 minutes. This result reveals that cleavage involves a heat-sensitive protein similar to the enzymes (Figure S23). It is noteworthy that a 70 μM solution of **2a** in buffer at pH 8 does not degrade at 90°C. Moreover, we showed that linearization of **2a** occurred with cleavage at the C-terminal position of the two HyLeu and was repeatable over 21 batches of digestive gland extracts obtained from 11 *S. striatus*. As previously reported, **2a** is the archetype of a compound family that includes dozens of analogues slightly differing at the sequence level.[22] In order to define if the observed cleavage is restricted to **2a**, we used isolated natural laxB2[22] **3a**, synthesized trichormamide C **4a** and its analogues **5a-6a** to explore the importance of the nature of the amino acids at the cleavage positions 3 and 5, and also to examine the role of (2R,3R)-HyAsn in position 8, as this amino acid is replaced by a D-Asn in **4a-6a** (Figure 2 and Figures S25-28).[19]

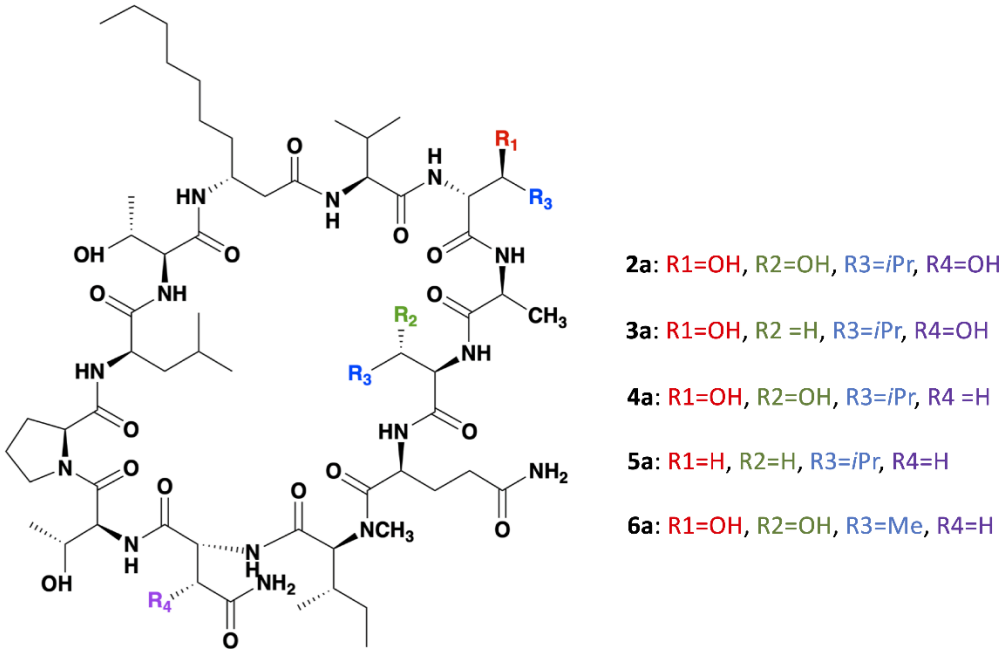


Figure 2. Structure of **2a** and its cyclic analogues **3a**, **4a**, **5a** and **6a**.

The structure of **3a** only differs from **2a** by one amino acid, a D-Leu instead of the (2R,3S)-HyLeu in position 5. **4a** possesses a D-Asn in position 8 instead of the (2R,3R)-HyAsn. These slight differences did not affect the outcome of the cleavage of **3a** and **4a** with *Dg-Ss-Lm* extracts and LC-MS analyses confirmed that the linearized compounds **3c** and **4c** were obtained by a cleavage at the C-ter of amino acids in position 3 and 5 (Figure 3, Figures S25-26). Following this observation, we used compounds **5a** and **6a** that might be considered as **3a** and **4a** hybrids. These fully synthetic compounds **5a** and **6a** have two D-Leu or two D-Thr respectively, instead of the two (2R,3S)-HyLeu present in **2a** and **4a**. For both compounds we observed similar cleavage at the C-ter of the D-Leu or at the D-Thr to give **5c** and **6c**, respectively (Figure 3, Figures S27-28). Thus, **5a** and **6a** are cleaved at the same location as **2a** and **4a** despite the modification of the amino acids at the cleavage site. Furthermore, we observed that the cleavage rate of **2a**, **3a**, **4a** and **5a** was similar during the first few hours and 90% or more of the substrate was consumed within four

hours (Figure 3). For **6a** the macrocycle opening was slightly slower than the others with as much as 25% of **6a** remaining 8 hours after the beginning of the reaction, while a comparable amount of cleavage occurred for the other compounds in less than three hours (Figure 3).

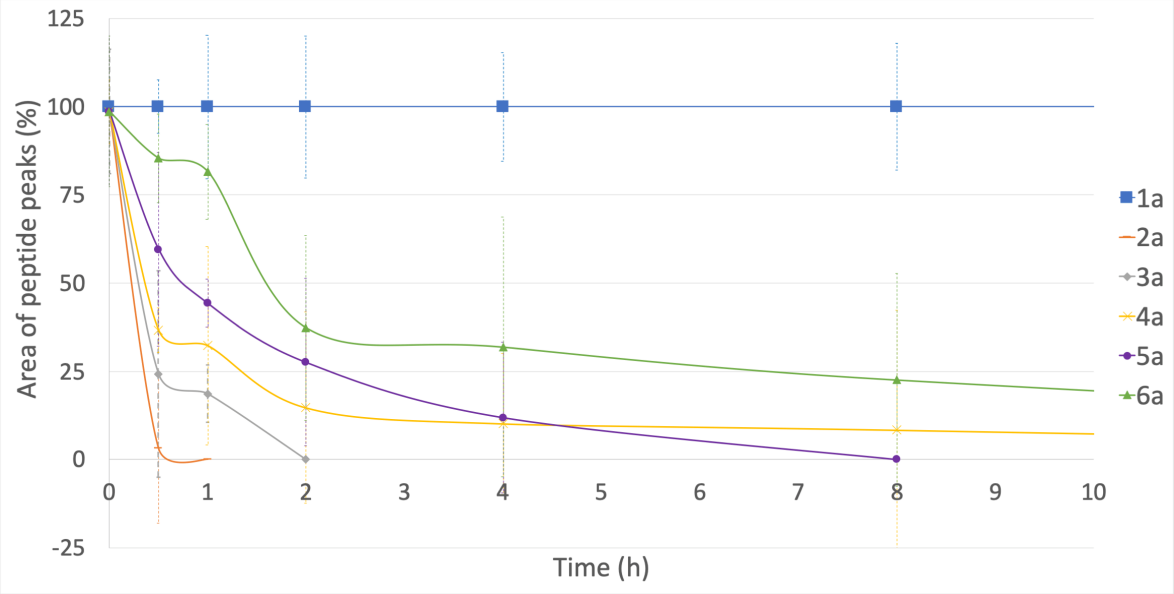


Figure 3. Cleavage rate of laxaphycin-B type peptides **2a-6a**, compared to that of laxaphycin A **1a** (averaged rate), as a function of the incubation time of the peptide with *Dg-Ss-Lm*. Error bars represent standard deviation.

The peptides **4a-6a** confirmed that the enzymatic cleavage is a two-step process that deletes the amino acids in positions 4 and 5 of the macrocycle, but defining the location of the first cleavage point using MS-MS was not straightforward due to the observed dehydration of Thr or (2R,3S)-HyLeu that complicated the interpretation of the mass spectra as already reported[26] (Figures S36-S40). To determine the order of the two successive cleavages we focused on compound **5a** to avoid synthesizing hydroxyleucines and hydroxyasparagine contained in **2a**, as these amino acids are not commercially available. However, stereochemistry was respected by using D-leucines and D-

asparagine. From **5a**, we produced two linear peptides corresponding to the two possible cleavage points (steps 1' and 2' in path A or steps 1 and 2 in path B; Figure 4). Compound **5b** is a linear analogue of **5a** that is disconnected at the C-ter of D-Leu^{5}. Compound **5d** refers to the open compound **5a** at the C-ter of D-Leu^{3}. These two candidate peptides allowed us to determine the first cleavage site and subsequently the two-step enzymatic reaction pathway that produces compound **5c**. It is worth noting that when both candidates **5b** and **5d** were exposed to digestive gland extracts, they were cleaved and produced compound **5c** after deletion of compound **5e** (Figures S29-30). Optimization of HPLC conditions enabled us to differentiate between peptides **5b** and **5d** through their retention times of 13.0 and 13.3 min respectively which correlated to their peak mass of *m/z* 1365.6 (Figure 4B). Subsequently, separation of both peaks remained when co-injected (Figure 4B). Based on these observations, a solution containing compound **5a** was exposed to digestive gland extracts and LC-MS revealed that only the peak corresponding to **5b** along with the final product **5c** were observed (Figure 4C, solution B). Mixing solution B with solution A containing compounds **5b** and **5d** in equivalent amounts confirmed that only compound **5b** was formed during the enzymatic process (Figure 4C). Thus, our test of the enzymatic processing of compound **5a** provided evidence supporting path B that the ring is first cleaved at the C-ter of the amino acid in position 5 delivering compound **5b** (Figure 4A, step 1 in red) and then cleaved at the C-ter of the amino acid in position 3 to form **5c** (Figure 4A, step 2 in red).

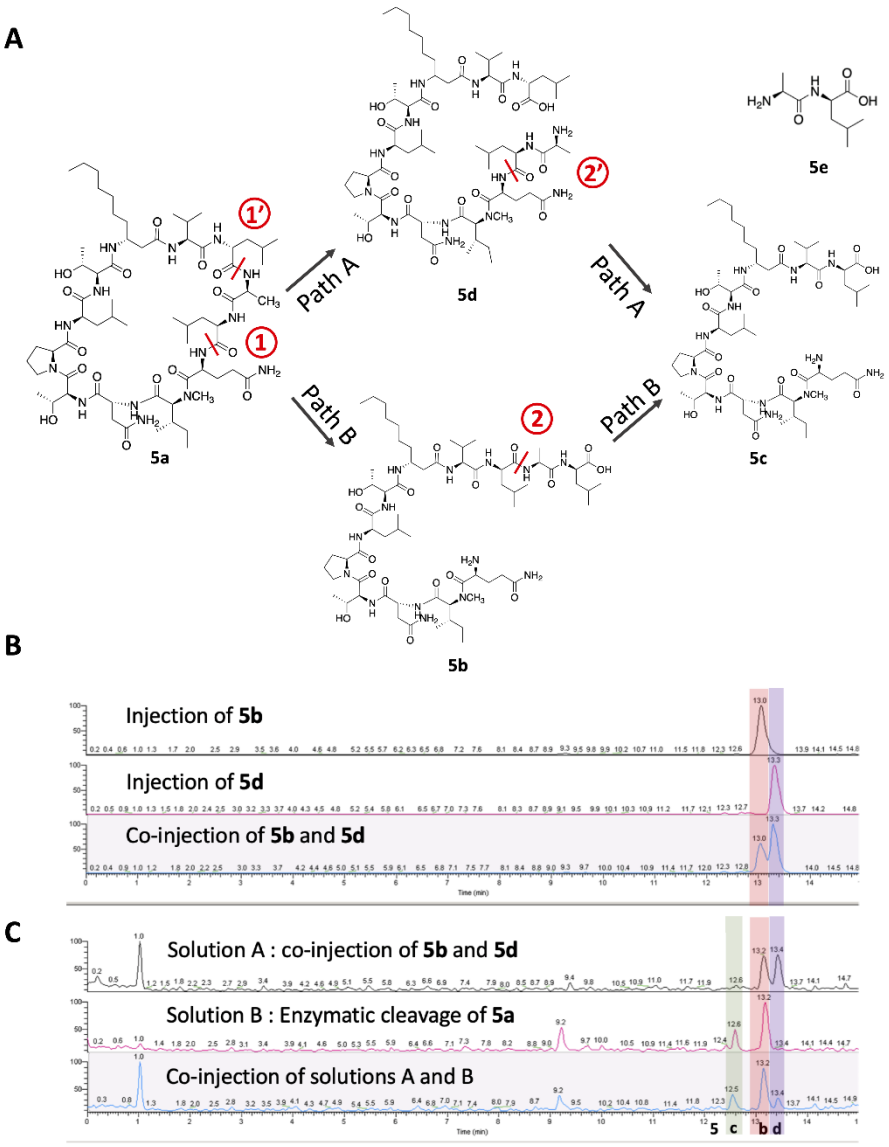


Figure 4. Study of the two possible enzymatic cleavage paths of compound **5a** which leads to the formation of compounds **5c** and **5e** consecutively from the intermediates **5b** or **5d. A.** Potential intermediates produced by Path A or B. **B.** LC-MS chromatograms of postulated cleavage intermediates **5b** (pink strip) and **5d** (purple strip) and their co-injection. **C.** LC-MS chromatogram of **5a** exposed to enzymatic degradation producing compound **5b** as confirmed by co-injection with synthetic **5b** and **5d** compounds. Compound **5c** resulting from the second cleavage is also observed (green strip).

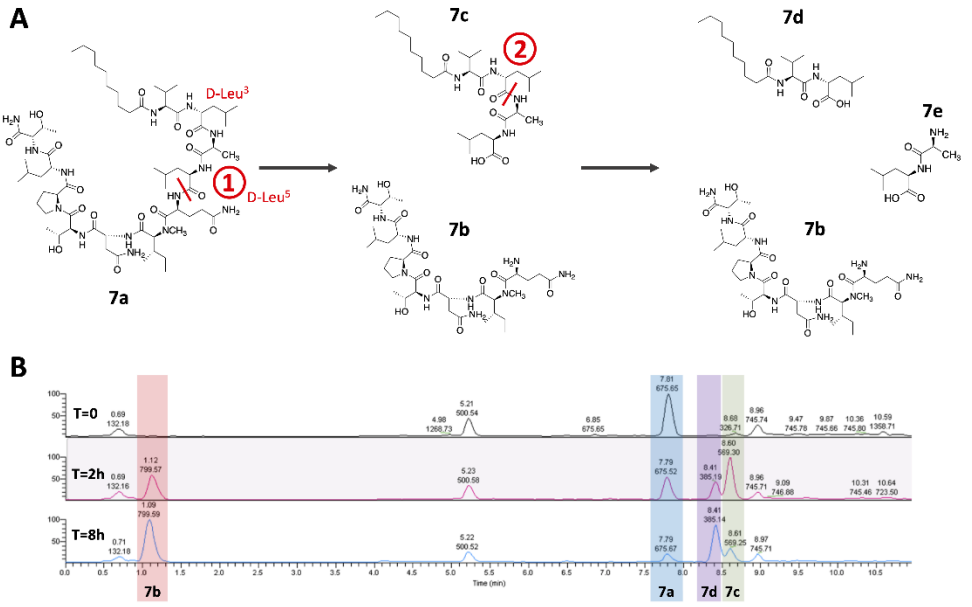


Figure 5. Study of the cleavage path of **7a**. **A.** Successive cleavage observed by LC-MS for compound **7a**. **B.** Chromatograms of **7a** successive cleavage over time.

Intrigued by the fact that linear compounds **5b** and **5d** were substrates of the enzyme, we synthesized compound **7a** that corresponds to a linear analogue of **5a** in which the Ade has been replaced by a decanoic acid at the N-terminus. Despite a linear structure, the peptide was still cleaved at the same location as its cyclic analogue (Figure S31) indicating that **7a** manages to structure itself in order to be recognized by the enzyme as its cyclic analogue. Furthermore, compound **7a** enabled us to differentiate between the fragments by mass spectrometry and to confirm the order of the two-step cleavage process (Figure 5 and Figure S41). The two cleavage studies of **5a** and **7a** demonstrate that **2a** and its cyclic analogues (**3a**, **4a** and **6a)** are cleaved in the same way, according to path B (Figure 4A). The discovery of acyclolaxB **2d** in *Anabaena torulosa* (Figure 1B) suggests that cleavage of **2a** would produce the intermediates **2d** then **2c**, however, they are in fact **2b** then **2c** (Figure 6). The origin of acyclolaxaphycin B **2d** is still unknown to date.

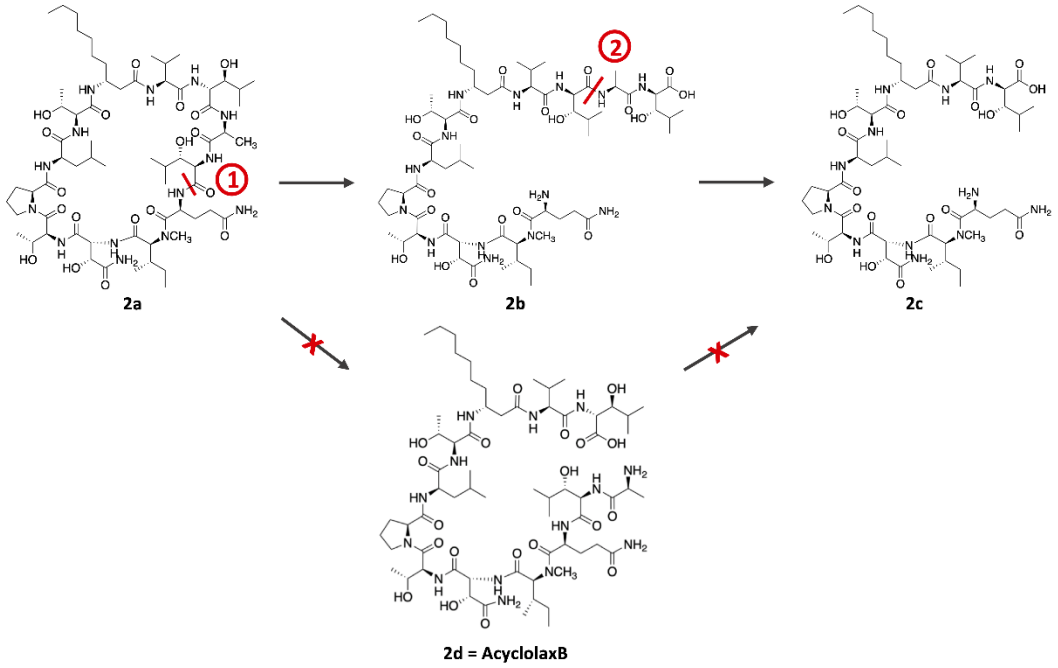


Figure 6. Cleavage path of **2a** determined following the studies on **5a** and **7a**.

Given the length of Ade[1]'s carbon chain, this amino acid could play a crucial role in the enzyme's ability to recognize the substrate. We therefore replaced Ade[1] with β-Alanine and synthesized compound **8a**. This peptide was not cleaved by digestive gland extracts (*Dg-Ss-Lm*), demonstrating that the enzyme requires Ade[1]'s long carbon chain for insertion of the peptide into the enzyme active site (Figure S32). Stereochemistry can also impact the enzyme's ability to recognize the substrate. **2a**'s structure is particular with an alternation of L- and D-amino acids and is cleaved at the C-terminal end of D-amino acids (D-Thr, D-Leu and (2R,3S)-HyLeu). We therefore synthesized compound **9a**, an analogue of **5a**, which contained two L-Leu instead of the two D-Leu at the cleavage site (Figure 7). This peptide was not cleaved by digestive gland extracts (*Dg-Ss-Lm*), emphasizing the importance of amino acid stereochemistry at the enzyme's recognition site (Figure S33).

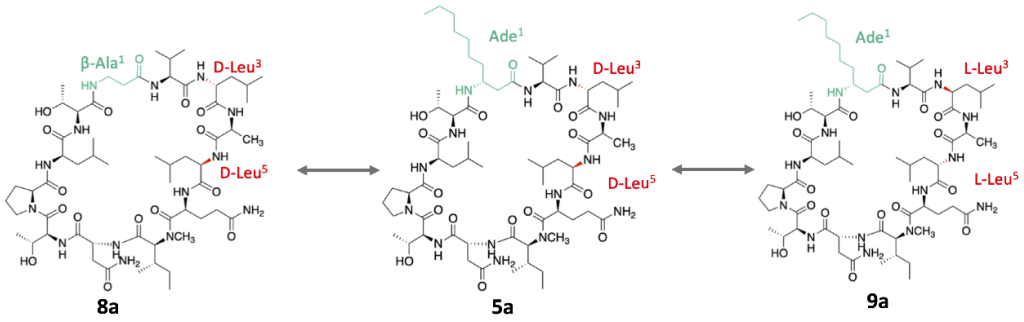


Figure 7. Structures of non-cleaved compounds **8a** and **9a** compared to **5a**.

To reinforce our findings, we replaced only one D-Leu with a L-Leu. Knowing that **7a** is recognized by the enzyme although it is a linear peptide, and that it is cleaved at the C-ter of the two D-Leu starting with D-Leu^5 then D-Leu^3, we synthesized the same peptide sequence by replacing D-Leu in position 3 by L-Leu, while keeping D-Leu in position 5, resulting in compound **10a**. LC-MS analysis showed that the peptide was cleaved at the C-ter of D-Leu^5 but no longer in position 3, where L-Leu was present (Figure 8 and Figure S34).

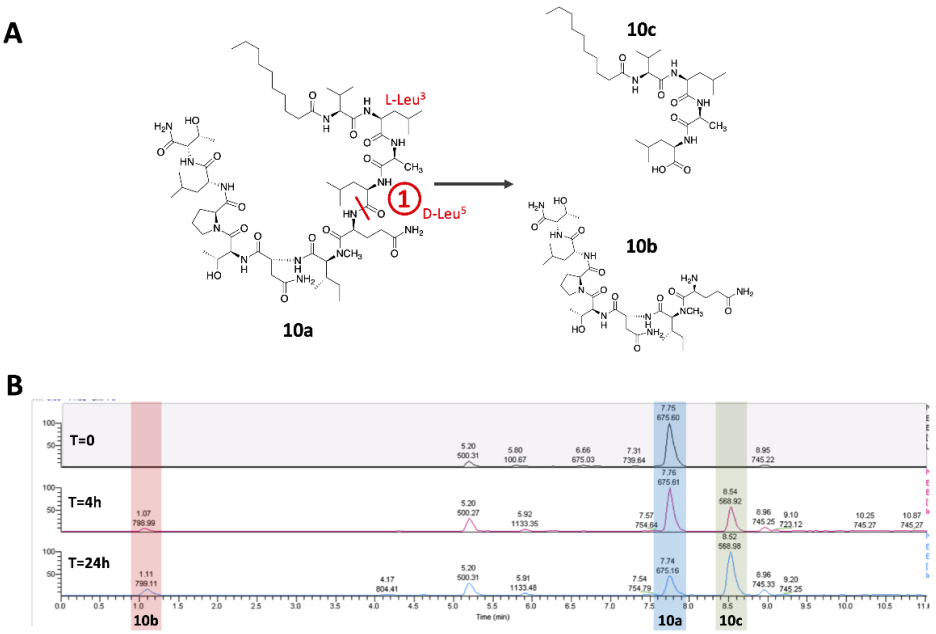


Figure 8. Study of the cleavage path of **10a**. **A.** Cleavage observed by LC-MS for compound **10a**. **B.** Chromatograms of **10a** cleavage over time. Compounds **10d** and **10e** were not observed.

Taken together, our results confirm that a D-peptidase within the digestive gland of *S. striatus* acts on cyclic lipopeptides in the laxaphycin B-type peptide family, but also on synthetic linear analogues provided that they contain D-leucine like amino acids with suitable surroundings. Indeed, the D-leucine in position 11 does not constitute a preferred cleavage site. D-peptidases are attracting increasing interest as they may be involved in the resistance of bacteria to antibiotics if transferred to pathogenic bacteria.[5] With regard to this, we tested the stability of four antibiotics, the vancomycin glycopeptide, the CLPS daptomycin, bacitracin and colistin all containing D-amino acids as well as a cyclic structure like laxaphycins, against these same digestive gland extracts from *S. striatus*. None of these four peptides were cleaved in contact with the digestive gland extracts of the mollusk (Figure S35). This result shows that the specificity of the investigated D-peptidase is restricted to the laxB family and that the D, L alternating stereochemistry observed may be an important prerequisite for the substrate-enzyme association.

CONCLUSION

The stability of laxaphycin B was demonstrated in buffers at pH 3, pH 5 and pH 8, as well as at temperatures between 30°C and 90°C. LaxA (**1a**) was bioaccumulated in the digestive gland of *S. striatus,* while laxB (**2a**) was recovered as an acyclic laxB despite the fact that **2a** is stable in a complex medium such as rat serum, as well as against known isolated enzymes. As the biotransformation of **2a** in the digestive tract of *S. striatus* was the same regardless of whether *S. striatus* had been reared on *At* or *Lm*, we can confirm the hypothesis that the enzyme is either produced within the mollusk itself or recovered by the latter from its environment. **2a** is cleaved in two steps, by a first C-terminal opening of a HyLeu and then by the loss of 2 amino acids with a C-terminal cleavage of the second HyLeu. The structure-activity relationship (SAR) study with different cyclic analogues of **2a** showed that the side chain size of amino acids 3 or 5 impact

enzyme recognition by affecting some of the kinetics of the cleavage. However, suppression of the hydroxy function on these amino acids does not significantly affect hydrolysis rate. On the other hand, the Ade in position 1 is crucial in CLP recognition by the enzyme as its absence precludes association with the enzyme. More importantly, the enzyme involved in the cleavage of the compounds described in this study is part of the little studied D-stereospecific peptidase for which only few examples have been previously reported. However, recently Li et al. (2018) identified 403 putative D-stereospecific resistance peptidases (DSRP) in the Bacillales order and found a strong association between these peptidases and the non-ribosomal peptide biosynthetic gene cluster.[5] These DSRPs cleave peptides at the C-terminal side of D-cationic amino acids, but in the present case cleavage occurs at the C-ter of D-HyLeu (**2a**, **3a**, **4a**), D-Leu (**3a**, **5a**, **7a**, **10a**) and D-Thr (**6a**) thus highlighting a different and yet unreported specificity. We thus provide evidence of a new D-peptidase acting on CLPs that extends our knowledge of these little studied enzymes. D-peptidases have only been reported in the Bacillales order to date, but they raise the question of their potential horizontal gene transfer to other pathogenic bacterial strains that might impact the development of CLP antibiotics. This study showed that this enzyme had no impact on three CLP known antibiotics. Furthermore, as all known D-peptidases are of bacterial origin we hypothesize that the soluble enzyme found in the digestive gland of *S. striatus* originates from bacteria associated with the intestinal microbiome of *S. striatus*. This study revealed that *S. striatus* contains, and potentially also concentrates, bacteria producing enzymes with unexpected specificity against laxB, transforming it into a less toxic linearized compound. Further examination of the microbiome associated with the molluscan digestive gland, as well as the chemistry of cyanobacteria surfaces, could provide new information that will be examined in the near future. Simplified analogues of laxB provided us with more information concerning the specificity of this

enzyme and will serve as a basis for the creation of new peptides which, by photo-crosslinking, will allow us to recover the enzyme more efficiently.

EXPERIMENTAL SECTION

Peptide synthesis

Linear peptides were synthesized with an automated microwave peptide synthesizer (CEM Liberty One). The synthesis was performed at 50 °C under microwaves (25 W) and at 0.1 mmol scale with PyOxim (4.9 equiv)/DIEA (10 equiv) as coupling reagents. 20% piperidine in DMF were used for deprotection steps. Depending on the peptide to be synthesized, either Rink Amide resin (0.67 mmol/g) or 2-chlorotrityl chloride resin (1.6 mmol/g-Cl) preloaded with the first amino acid was used. For each amino acid the standard coupling cycle was as follows: after Fmoc deprotection of the amino acid supported by the resin, 2.5 mL of the requested amino acid at a concentration of 0.2 M in DMF was added to the resin, followed by 1 mL of PyOxim in DMF at 0.49 M and lastly by 0.5 mL 2M DIEA in NMP. The coupling of all amino acids was performed for 20 minutes except for difficult couplings which were carried out 2 or 3 times such as Fmoc-L-Gln(Trt)-OH that was coupled three times for 20 minutes, followed by capping with a solution of 100 mL of DMF, 5 mL of anhydride acetic (0.5 M, 1 equiv), 2 mL of DIEA (0.21 equiv) and 135 mg of HOBt (0.019 equiv) for 3 minutes. The Fmoc-group was deprotected by adding 20% piperidine solution in DMF for 3 minutes. At the end of the synthesis, the resin was washed three times with DMF and three times with DCM and then was dried under *vacuum*.

In the case of the linear peptides **5b**, **5d**, **7a** and **10a** a total cleavage was done using a solution of TFA/TIS/H_2O 95:2.5:2.5 (v/v) under agitation over 2 hours. The resin was then filtered and the filtrate was concentrated *in vacuo* with cyclohexane as a co-solvent. Peptides were precipitated in ice-cold diethyl ether and freeze-dried. Peptides were then purified by semi-preparative HPLC.

In the case of cyclic peptides **8a** and **9a**, a cleavage of the protected peptide from the resin was carried out using a solution of 20% TFE in DCM under agitation for 2 hours. The resin was then filtered and the filtrate was concentrated *in vacuo* with cyclohexane as a co-solvent. The peptide was then freeze-dried but not purified. The linear peptide (45 μmol) was dissolved in DMF (4.5 mL) into a syringe. EDC (3 equiv) was dissolved in the same amount of DMF (4.5 mL) into another syringe. OxymaPure (3 equiv) was dissolved in 4.5 mL of DMF into a three-neck round-bottom flask purged under nitrogen atmosphere. The two syringes were put on both sides of the flask and both solutions were added under a nitrogen atmosphere at a rate of 0.01 mL/min. Once the addition was complete, the mixture was stirred for 48 hours under a nitrogen atmosphere. DMF was evaporated using Genevac (T=36°C, Medium Boiling Point) over 3 hours and the resulting sediments were dissolved with 10 mL of ethyl acetate. The organic layer was washed three times with citric acid (1M), three times with $NaHCO_3$ (1M) and three times with brine in order to separate the cyclic peptide from any potential remaining linear ones. The organic layer was dried over magnesium sulfate and then concentrated *in vacuo*. To allow side-chain deprotection, the resulting solid was mixed with a solution of TFA/TIS/H_2O 95:2.5:2.5 (v/v) over 2 hours. TFA was evaporated under *vacuum* and the peptide was precipitated using ice-cold diethyl ether. The peptide was then purified by semi-preparative HPLC.

Syntheses of laxaphycin B, trichormamide C (compound **4a**) and its two cyclic analogs **5a** and **6a** have been previously published.[19,27] For LC-MS profiles, as well as the exact masses of peptides **5b**, **5d**, **7a**, **8a**, **9a** and **10a**, see Figures S4 to S9.

As the peptides do not have chromophores to visualize them by HPLC coupled with UV detection, their purity were estimated by LC-MS analysis. As purity was estimated from the detected peaks of the mass spectra, we cannot exclude that purity was overestimated due to the

presence of trace compounds associated with the main peak. Thus the reported purities (Table S2) should be considered to be qualitative rather than quantitative. Moreover, if impurities are present, they do not impact the biotransformation process which was the main aim of the study. Indeed, it is noteworthy that no associated impurities were detected by NMR. Thus analyses of the peptides by LC-MS and NMR confirmed that the purity of the peptides was higher than 95%.

Peptide stability in buffer solutions

Two buffer solutions were prepared and sterilized by autoclave: a pH 8 buffer (50 mM Tris, pH adjusted with HCl 1M) and a pH 3 buffer (citric acid 0.1 M, sodium citrate 0.1 M, pH adjusted with NaOH 1M). Stock solutions of peptides were prepared at 10 mg/mL in DMSO. These solutions were diluted 10 times in buffer pH 3 and pH 8. Peptide solutions at 1 mg/mL (10% DMSO in buffer) were diluted 10 times with the same buffer used for previous dilutions resulting in peptides at 0.1 mg/mL (1% DMSO in buffer) in both buffers. Solutions were incubated under agitation (different temperatures were tested to fit with enzyme specificities, see supporting information). For each kinetic point, the same procedure was applied: solutions were centrifuged for a few seconds, then part of the solution was removed to be quenched by 90% MeOH, resulting in 0.01 mg/mL peptide solutions (DMSO/buffer/MeOH, 0.1:9.9:90, v/v). The quenched solutions were centrifuged at 21,952 x *g* for 20 minutes at 4°C. The maximum amount of supernatant was recovered in a LC-MS vial for analysis. All tests were performed in triplicate.

Peptide stability in the presence of *Dg-Ss-Lm* or rat serum

A pH 8 buffer was prepared and sterilized by autoclave (50 mM Tris, pH adjusted with HCl 1M). Stock solutions of peptides were prepared at 10 mg/mL in DMSO. These solutions were diluted 10 times in buffer pH 8 to give peptide solutions at 1 mg/mL (10% DMSO in buffer). These solutions (1 mg/mL) were mixed with *Dg-Ss-Lm* extracts (10 mg/mL) or rat serum (between 60

and 80 mg/mL) and completed with the same buffer resulting in peptides at 0.1 mg/mL (1% DMSO in buffer) and extracts at 1 mg/mL (concentration varying between 0.4 and 1 mg/mL depending on the extracts) or rat serum around 10 mg/mL. Solutions were incubated at 30°C under agitation. For each kinetic point, the same procedure was applied: solutions were centrifuged for a few seconds, then part of the solution was removed to be quenched by 90% MeOH, obtaining peptide solutions at 0.01 mg/mL (DMSO/buffer/MeOH, 0.1:9.9:90, v/v). The quenched solutions were centrifuged at 21,952 x *g* for 20 minutes at 4°C. The maximum amount of supernatant was recovered in a LC-MS vial for analysis. All the tests were carried out in triplicate on the same extract and in several replicates with other extracts.

Mollusk harvesting, dissection and solid-liquid extraction

Adult specimens of *Stylocheilus striatus* were collected from either the lagoon of Moorea Island (Society Archipelago, French Polynesia) or from populations residing in flow-through aquaria at the University of California Gump Field Research Station situated in Moorea. Specimens obtained from the lagoon were collected at depths of 1-4 m within large cyanobacterial blooms of either *Lyngbya majuscula* or *Anabaena torulosa* on sandy substrates. Animals were transferred in cool boxes within 3 hours of collection into outdoor, flow-through aquaria (40 L) supplied with oxygenated running seawater (0.5 L/min) at CRIOBE. Sea hares were starved for 48 hours prior to dissection to ensure that all food items had been digested and excreted.

Opisthobranches were dissected following cold anesthesia at 4 °C for 20 minutes. A shallow incision was made through the mantle, ventrally from the head to tail. The mantle was removed to expose all internal organs (Figure S1A). The internal organs were then dissected to expose the mouth and buccal mass, gizzard, digestive gland, ovo-testis, hermaphroditic duct, and mucus and albumin gland (Figure S1B). Each body segment was placed individually into labeled 1.5 mL

Eppendorfs and immediately frozen and stored at −20 °C. Ethical approval for the study was granted from The Animal Ethics Committee, Centre National de la Recherche Scientifique (permit number 006725).

Dissected digestive glands were stored in Eppendorf tubes and immersed in liquid nitrogen. Eppendorf tubes were then placed in a Silamat S6 mill (from Ivoclar vivadent) with 8 glass beads. 4 minimum cycles of alternation between liquid nitrogen and grinding were chained. After several cycles of 8 seconds, the whole sample was completely ground. The buffer (50 mM Tris, pH 8) was added and then solutions were mixed by vortexing. Mixtures were centrifuged at 4°C for 20 minutes at 21,952 x *g*. The supernatants of the centrifuged solutions were recovered and placed in aliquots. Solutions were stored at –25°C.

Instruments used

Purifications were carried out using a HPLC from Waters, Waters 1525 chromatography system fitted with a Waters 2487 tunable absorbance detector with detection at 214 nm and 254 nm. The eluents used for purification were ACN (0.1% formic acid) and H_2O (0.1% formic acid). The column used was a GRACE Vydac C-18 column (250x10 mm, 5 µm) at a flow rate of 3 mL/min.

LC-MS analyses were carried out with a Thermo Fisher Scientific LC-MS device, Accela HPLC coupled to a LCQ Fleet fitted with an electrospray ionization source and a 3D ion-trap analyzer. The column used was a Phenomenex BioZen™ 2.6 µm Peptide XB-C18 (LC Column 50 x 2.1 mm) and the gradient ran from 20% to 90% of ACN (0.1 % formic acid) in H_2O (0.1 % formic acid) during 6 min at a flow rate of 0.5 mL/min for a 10 µL injection. The LC-MS data were acquired on the XCalibur software from ThermoFisher. Once the chromatograms were obtained, ion extractions were carried out for each peptide or fragment. Areas were recovered on the peaks of these ion extractions.

High resolution mass spectra were recorded through direct infusion on a ThermoFisher Q Exactive Plus spectrometer.

NMR spectra were acquired on a JEOL ECZ 500 spectrometer and were treated on Delta software from JEOL.

ASSOCIATED CONTENT

Supporting Information

The following files are available free of charge.

- Molecular formula strings "SMILES"
- Structures of all the compounds mentioned in this manuscript, experimental procedures for enzymatic cleavage of peptides and mollusk dissection, LC-MS parameters, LC-HRMS (Figures S4-S9) and NMR (1H, 13C, DEPT, TOCSY, ROESY, HMBC, HSQC, HSQC-TOCSY) characterizations of all peptides studied (Figures S10-S15 and Tables S10-S15), and additional graphs of kinetics of enzymatic reactions.

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ACKNOWLEDGMENTS

We thank Anne Haguenauer for laboratory help and the University of California Gump Field Research Station for their help in providing specimens.

This study is set within the framework of the “Laboratoires d’Excellences (LABEX)” TULIP (ANR-10-LABX-41) and of the "Ecole Universitaire de Recherche (EUR)" TULP-GS (ANR-18-EURE-00019)

All analyses were performed using the facilities of the “Biodiversité et Biotechnologies Marines” platform at the University of Perpignan (Bio2Mar, http://bio2mar.obs-banyuls.fr).

Picture credit for the photograph used in the TOC: Suzanne C. Mills for *Anabaena torulosa* and *Stylocheilus striatus*.

ABBREVIATIONS

ACN, acetonitrile; Acyclolax, acyclolaxaphycin; Ade, (3R)-β-aminodecanoic acid; Aoc, (3R)-β-aminooctanoic acid; *At*, *Anabaena torulosa*; CLPs, cyclic lipopeptides; D-AA, D-amino acid; Dg, digestive gland; *Dg-Ss-At*, digestive gland extracts of *Stylocheilus striatus* fed on *Anabaena torulosa*; *Dg-Ss-Lm*, digestive gland extracts of *Stylocheilus striatus* fed on *Lyngbya majuscula*; Dhb, dehydrobutyrine; DIEA, N,N-diisopropylethylamine; DSRP, D-stereospecific resistance

peptidases; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HCl, hydrochloric acid; HOBt, hydroxybenzotriazole; HyAsn, hydroxyasparagine; HyLeu, hydroxyleucine; lax, laxaphycin; *Lm*, *Lyngbya majuscula*; MeOH, methanol; $NaHCO_3$, sodium bicarbonate; NRPS, Non-ribosomal peptide synthetases; *Ss*, *Stylocheilus striatus*; TFE, trifluoroethanol; TIS, triisopropylsilane; TLN, Thermolysin; W, watt

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