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Shotgun Cloning and Heterologous Expression of the Patellamide Gene Cluster as a Strategy to Achieving Sustained Metabolite Production**

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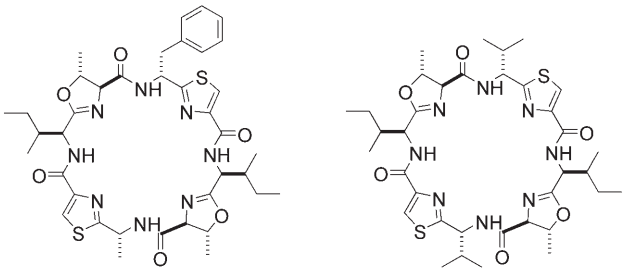
Secondary metabolites of marine invertebrates show exceptional promise as potential pharmaceuticals in many therapeutic areas.[1] The main obstacle to the exploitation of these chemically diverse and often complex bioactive marine metabolites is the economic issue of a sustainable supply. Different strategies have been attempted to overcome this impediment, as long-term harvesting of wild stocks from the environment is generally considered unsound.[2] Whole organism aquaculture, tissue culture, symbiont culture and chemical synthesis are possible, but seldom provide the robust and economic yield needed for a commercially viable global supply. One promising option is to clone the biosynthetic genes that encode the expression of a lead metabolite into a surrogate host suitable for industrial-scale fermentation. Accordingly, we demonstrate the recombinant expression for biosynthesis of a bioactive marine metabolite. The patellamide gene cluster is cloned from *Prochloron* sp., a photosynthetic endosymbiont of the ascidian *Lissoclinum patella* (Urochordata) that is recalcitrant to laboratory culture.[3] *Prochloron* sp. genes encoding the biosynthesis of patellamides are expressed in *Escherichia coli* to yield the cyclic octapeptides, patellamide D (**1**) and ascidiacyclamide (**2**).



1 **2**

Most marine invertebrates harbour microorganisms that include bacteria, cyanobacteria, fungi and eukaryotic algae within host tissues, where they reside as extra- and intracellular symbionts.[4] Numerous products isolated from marine invertebrates are structurally homologous to known metabolites of strict microbial origin; this suggests that commensal microbiota contribute substantially to the biosynthesis of metabolites elicited from the macro-organism host. Several metabolites from marine invertebrates that are in preclinical or clinical trial phases, such as discodermolide and the halichondrins, are alleged to be products derived from their microbiotic consortia.[5] In the case of the bryostatins from *Bugula neritina*, molecular evidence from in situ hybridisation experiments favours a microbial origin of these macrocyclic lactones produced by a characterised, but yet uncultured, γ-protobacterial endobiont.[6] In addition, the same group has characterised a single gene that is proposed to be responsible for the early steps of the bryostatin biosynthesis.[7] Similarly, DNA sequencing suggests that the biosynthetic source of the onnamides and theopederins from the sponge *Theonella swinhoei* is an uncultured symbiotic prokaryote.[8] In contrast, symbiotic actinomycetes from the marine tunicate *Aplidium lenticulum* have been cultured to yield isolate *Streptomyces* sp. JP95, which produces the telomerase inhibitor griseorhodin A, from which the biosynthetic gene cluster has been sequenced.[9] In addition, genes that encode the biosynthesis of several other marine metabolites have been entirely sequenced.[10–13] While production of several

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bioactives such as 6-deoxyerythronolide B,[14] epothilones[15] and yersiniabactin[16] from terrestrial sources has been accomplished by heterologous expression of biosynthetic genes, the challenge remained to clone genomic DNA from the uncultured microbiota of a marine invertebrate for the production of a lead candidate for pharmaceutical development in a heterologous host.

A vast array of natural products of both marine and terrestrial origin can be constructed by large multifunctional polyketide synthase (PKS), nonribosomal peptide synthetase (NRPS) and hybrid NRPS-PKS enzymes that contain repeated, coordinated modules, of which each module is responsible for the sequential catalysis of one complete cycle of polyketide or polypeptide chain elongation, together with tailoring enzymes necessary for structural modifications.[17,18] We have elected to express the cyclic octapeptides patellamide D (**1**) and ascidiacyclamide (**2**), isolated from the aplousobranch ascidian (seasquirt) *Lissoclinum patella*,[19,20] which have diverse biological activity, including potent cytotoxicity and reduction of multidrug resistance in certain types of lymphoblasts.[21] This invertebrate offers certain advantages for achieving the production of its bioactive metabolites by heterologous expression of the biosynthetic genes. *L. patella* is endemic to the tropical Indo-Pacific and consistently contains patellamides in relatively high quantities. The ascidian harbours a solitary photosynthetic prokaryotic symbiont, *Prochloron* sp. that is the likely source of the patellamides, since the obligate prochlorophyte is known to contain NRPS genes and their core patellamide structure is typical of a prokaryotic origin,[22] although other studies have shown that these compounds are localised mainly in the host tunicate.[23] Furthermore, *Prochloron* sp. and its surrogate gene host *E. coli* are both prokaryotes and are likely to be biochemically compatible, particularly for sharing post-translational activation of the modular NRPS peptidyl-carrier proteins by homologous 4′-phosphopantetheinyl transferase enzymes.[24] The predicted length for a putative linear NRPS for the patellamides is in the order of 30 kb.[18]

We have used the "shotgun" cloning approach as utilized by others[25–29] to clone large random-sized fragments of genomic DNA from *Prochloron* sp. to obtain heterologous expression of the cloned patellamide D (**1**) and ascidiacyclamide (**2**) biosynthetic pathways in *E. coli*. *Prochloron* sp. was separated from the cloacal cavities of ten specimens of *L. patella*, each showing a matching patellamide composition according to HPLC analysis of the holobiont extracts. Large random-sized fragments of genomic DNA were extracted (Figure 1), shotgun cloned into bacterial *E. coli–Streptomyces* artificial chromosomes (BAC) by using pPAC-S1/2 shuttle vectors[30] and were transformed into *E. coli* DH10B cells. A total of 1433 insert-selected transformants were transferred into 14×96 well microtitre plates for ease of handling in culturing the BAC library. Partial sequence analysis of the 16S rDNA region by using universal bacterial primers confirmed that *Prochloron* sp. was the origin of the genomic DNA used to construct the BAC library. Genomic DNA samples were also utilized as templates to amplify NRPS gene sequences for use as probes in Southern blot hybridisations to detect putative NRPS biosynthetic gene clusters from the genomic libraries. Two pairs of degenerative oligonucleotide primers were used,[22,31] but failed to amplify any products despite a wide range of experimental conditions.

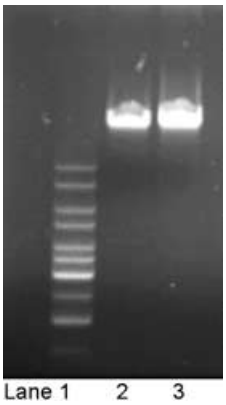

Figure 1. Gel electrophoresis of pooled genomic DNA samples from *Prochloron* sp. (lane 3) isolated from *L. patella* collected at Davies Reef; 1 kb ladder (lane 1); 50 kb λ DNA (lane 2).

Since we were unable to interrogate the BAC library using molecular tools to identify cloned transformants containing expected NRPS sequences, we resorted to direct chemical analysis to screen for the biosynthesis of patellamides. Interference posed by the complex medium (LB broth + 15 μg mL^{-1} kanamycin) used for fermentation required that sample preparation techniques were rigorously optimised for clean metabolite recovery, and the use of sensitive mass-selective detection provided by LC-MS analysis allowed recognition of target metabolites secreted to the broth at very low levels. In this procedure, patellamides were concentrated by solid-phase extraction and eluted with methanol, and the dried residue was cleaned by water/dichloromethane partitioning to remove HPLC interferences. Broth extracts were prepared from 40 mL fermentations prepared by inoculation with the entire 96-well set of clones from each plate. The biosynthetic products obtained from the combined clones of each plate were analysed by HPLC-MS^n in a procedure that proved sensitive for metabolite detection. Primary identification was achieved by comparing the HPLC retention times of the eluting peaks obtained from mass-selected detection with that of authentic patellamide standards obtained from tissue extracts of the ascidian hosts. By LC-MS detection, six of the fourteen-plate BAC library showed detectable levels of patellamides. At this level of examination, only one plate showed clear production of both patellamide D (**1**) and ascidiacylamide (**2**) by mixed-plate fermentation; three other plates showed the presence of **1**; two others showed the presence of **2**, both of which showed tenfold greater levels of **2** than the combined patellamide levels detected in the mixed fermentation broths from all other plates.

Deconvolution of two of the original six patellamide-expressing plates was accomplished by LC-MS^n analysis to select and authenticate individual metabolite-producing transformants. In this procedure, fermentation broths were analysed for patellamide biosynthesis from mixed cultures sampled from the collective column wells of each test plate followed by the culture

of individual transformants from each row of a metabolite-positive column. The BAC library plate from which both **1** and **2** were originally detected gave just one transformant (1-1-F), which produced both patellamide and ascidiacyclamide metabolites. The other plate examined gave one transformant (3-4-G) that produced both metabolites plus two other BAC clones (3-4-D and 3-6-B) that produced only ascidiacyclamide. Metabolites **1** and **2** have been fully characterised from the broth of clone 3-4-G, and their structures are consistent with the products from the other three patellamide-producing clones. Further work is being carried out to identify the fermentation products from all of the library clones containing patellamide metabolites. HPLC-MS^n analysis showed that **1** and **2** produced by clone 3-4-G had identical HPLC retention times (Figure 2), molecular $[M+H]^+$ and $[M+Na]^+$ ions, and characteristic MS^2 fragmentation patterns (Figures 3 and 4) that proved identical to the fragmentation spectra of authentic samples[19] obtained from *L. patella* extraction. Accurate mass measurements obtained for **1** and **2** produced by clone 3-4-G were within acceptable levels of deviation from the exact molecular mass calculated for each metabolite. Recovered metabolite yields without genetic improvement of the expression system were low (transformant 3-4-G = 77 ng mL^{-1} patellamide D + 94 ng mL^{-1} ascidiacylamide) but are of the same magnitude reported for epothilone yields (50–100 ng mL^{-1}) obtained from expression of the epothilone gene cluster in *Streptomyces coelicolor.*[15]

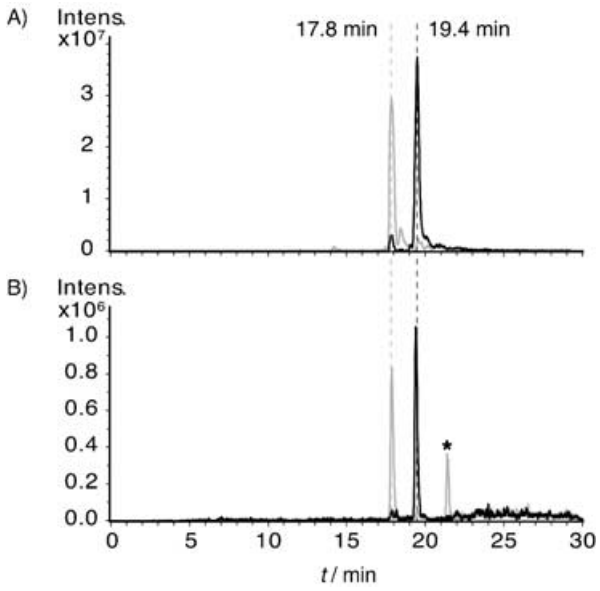

Figure 2. Extracted ion chromatograms for *Lissoclinum patella* and BAC transformant 3-4-G monitored at combined $m/z = 777$ $[M+H]^+$ + 799 $[M+Na]^+$ (grey trace) and $m/z = 757$ $[M+H]^+$ + 779 $[M+Na]^+$ (black trace). A) Methanolic extract of *L. patella* and B) the fermentation extract of clone 3-4-G. The peak in the grey trace at $t_R = 17.8$ min is **1**, and the peak in the black trace at $t_R = 19.4$ min is **2**. The minor peak marked with an asterisk co-elutes with a known contaminant.

In this study we demonstrate that secondary metabolites from the uncultured symbiont of a marine invertebrate are amenable to surrogate expression of the biosynthetic genes in a heterologous host. In our attempt, we obtained patellamide metabolites from *Prochloron* sp. DNA expressed in *E. coli* without needing to shuttle cloned DNA fragments into a *Streptomyces* host to achieve translational activation of the NRPS domain. By extending these methods to the development of a "universal" expression system for genetic recombination, we intend to produce other high-value marine products, particularly those having therapeutic potential for which clinical development has stalled due to a lack of a renewable supply. Furthermore, gene manipulation offers the prospect to produce pharmacophore analogues with more desirable therapeutic properties such as potency, specificity or bioavailability. Alternatively, new "unnatural" combinatorial libraries can be created by iterative exchange of key biosynthetic gene modules[32,33] or by precursor-directed biosynthesis, as recently shown for epothilone.[34]

Our failure to amplify *Prochloron* sp. NRPS gene sequences from cyanobacterial degenerative primers[22] and other primers specific for conserved NRPS sequences[31] suggests that the biosynthetic pathway leading to the formation of the patellamides is a previously unrecognised pathway. Similarly, as part of the genome-sequencing project for *Prochloron didemni*, the biosynthetic pathway for patelamides A and C has also very recently been cloned and expressed in an *E. coli* surrogate host.[35] Gene-sequence analysis conducted in this study indeed reveals that the patellamides might in fact be synthesised by a ribosomal mechanism. The approach reported here did not require a priori knowledge of the producing organism's genome, but rather utilised a universal expression system in tandem with direct chemical analysis that might serve as a more suitable strategy for general application to obtain a sustainable supply of a target marine natural product for preclinical and clinical investigation. As a final note, we conclude, in agreement with Schmidt et al.,[35] that *Prochloron* sp. is the ultimate biosynthetic source of the patellamides isolated from the marine ascidian, *Lissoclinum patella.*

Experimental Section

Sample collection and DNA extraction: Ten specimens of *Lissoclinum patella* taken from Davies Reef in the central region of the Great Barrier Reef, Australia, (147°38′ E:18°51′ S) were processed immediately aboard the R.V. Lady Basten (Australian Institute of Marine Science), and sub-samples were stored at −20 °C for future use. *Prochloron* sp. could easily be separated from fresh *L. patella* with minimal contamination by pressing the animal to release photobionts held in the cloacal cavities of the animal. The cells were subsequently collected by gentle centrifugation and washed with a 5× volume of Tris-EDTA Buffer (10 mM Tris-HCl + 10 mM EDTA; pH 8.0). Isolated *Prochloron* sp. cells (ca. 0.2 g wet weight) were resuspended in Tris-EDTA Buffer (1.5 mL), treated with lysozyme reagent (40 μL, 100 mg mL^{-1} in Tris-EDTA Buffer) and were incubated for 1 h at 37 °C with gentle shaking. The cells were then lysed by the addition of EDTA (3.5 mL, 10 mM, pH 8.0) containing sodium dodecyl sulfate (10% *v/v*), Triton-X (0.5% *v/v*) and proteinase K reagent (40 μL, Qiagen Pty Ltd, VIC, Australia). The lysis solution was incubated at 50 °C for 1 h with gentle shaking. Genomic DNA was obtained from standard procedures[36] by phenol/chloroform (equil-

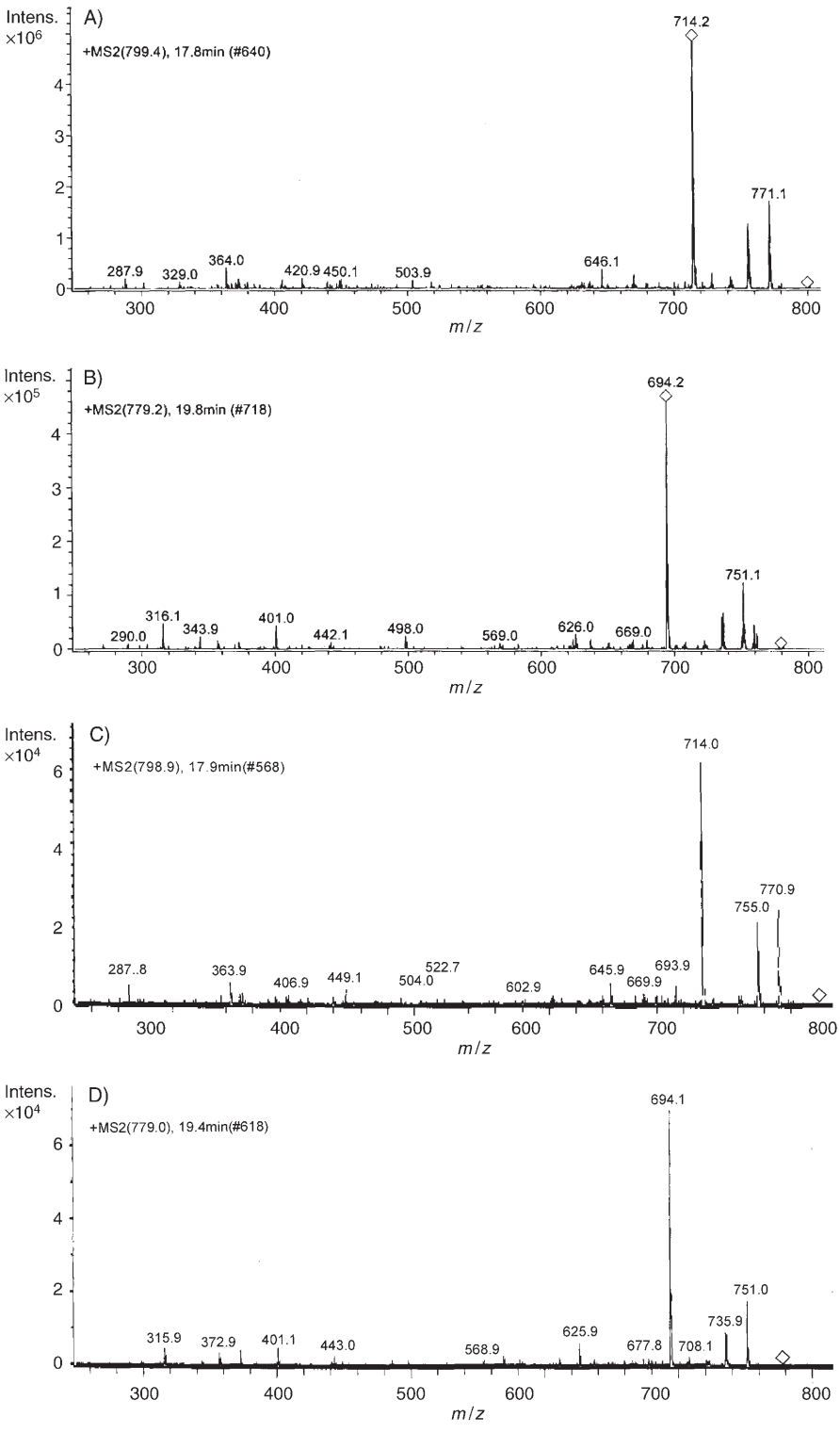

Figure 3. MS^2 spectra for $[M+Na]^+$ obtained online during a HPLC elution of **1** and **2** from *Lissoclinum patella* (A and B, respectively) and BAC transformant 3-4-G (C and D, respectively).

ibrated) extraction and ethanol precipitation. The DNA was then treated with RNase A reagent (100 μL, 100 μM mL^{-1}; Qiagen Pty Ltd), re-extracted with phenol/chloroform (equilibrated), precipitated with ethanol and finally resuspended in Tris-EDTA buffer. The yield of DNA was determined by absorbance at 260 nm (Genequant, Amersham Biosciences UK Ltd), and the DNA was diluted to a final concentration of about 100 ng mL^{-1}. In the absence of pulsed-field gel electrophoresis facilities, the sizes of genomic fragments were estimated by comparison with the 50 kb genome of bacteriophage Lambda following agarose gel electrophoresis (Figure 1).

Patellamide analysis: Subsamples (ca. 1 g wet weight) of *L. patella* were extracted with MeOH (3× 2 mL), and the extract was passed through a C-18 solid-phase extraction cartridge (Sep-Pak; Waters/Millipore) prior to HPLC analysis (Phenomenex Phenosphere ODS-2, 5 μm, 80 Å, 250×4.6 mm column with isocratic MeOH/H_2O (80:20) elution at 1 mL min^{-1} and detection at 234 nm) to determine the composition of the patellamides present in the ascidian samples from which *Prochloron* sp. DNA was extracted.

Molecular methods: Large random-sized fragments of genomic DNA pooled from ten freshly isolated *Prochloron* sp. samples were end repaired by using Klenow fragments and blunt-end cloned into *Bam*HI cut vectors that had also been end repaired and dephosphorylated for transformation into chemically competent *E. coli* DH10B cells by using the pPAC-S1 and pPAC-S2 bacterial *E. coli-Streptomyces* artificial chromosome (BAC) shuttle vectors[37] according to standard methods.[36] The shuttle vectors are relatively large at 22.3 kb but easy to handle after cloning large DNA fragments ($\geq$ 100 kb). The vectors are reputed to be more stable than other cosmid and BAC systems and contain the necessary origins for replication and resistance markers to be shuttled amongst *E coli* and *Streptomyces*.[37] The BACs contain the *sacB* gene and promoter at the polylinker site, such that DNA insertion inactivates the *sacB* gene for positive selection of cloned transformants.[30] Transformants were selected for resistance to kanamycin (15 μg mL^{-1}) and sucrose (5% *w/v*) supplemented Luria–Bertani (LB) agar for insert selection. A library of 1433 insert selected clones was generated and stored in 8×12 well (96) well plates for ease of handling.

Deconvolution of the genomic library: Combined clones from each of the 96-well plates were fermented in LB broth (40 mL) containing of kanamycin (15 μg mL^{-1}). After removal of cells by centri-

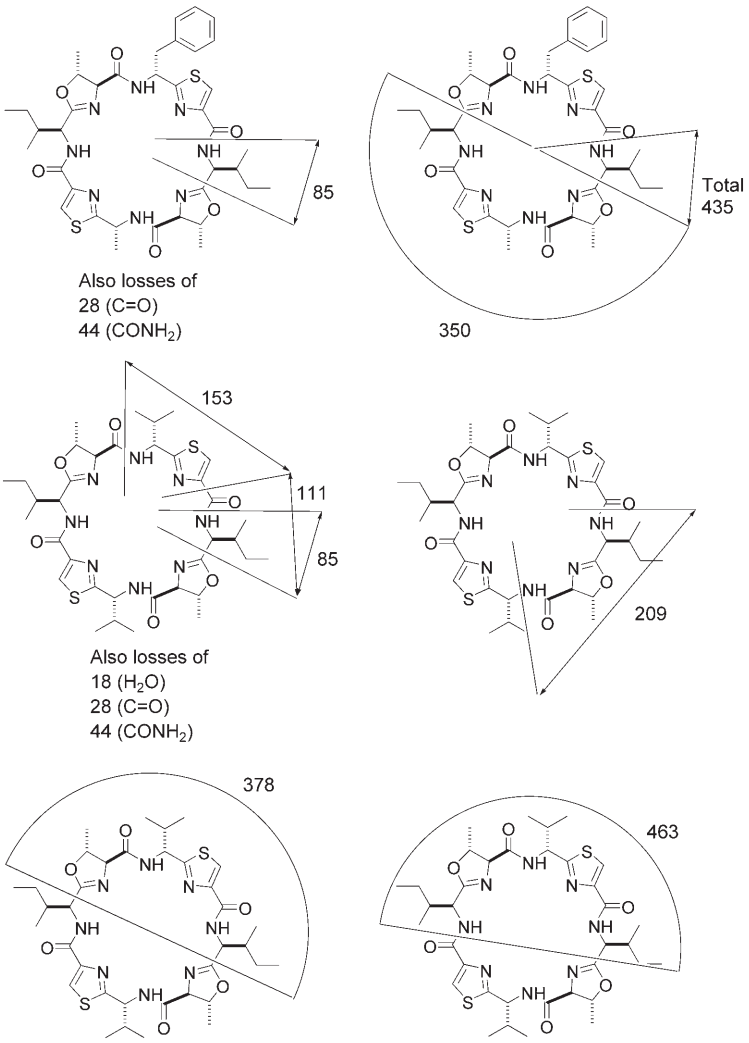

Figure 4. Interpretation of the MS^2 fragmentation patterns obtained online during a HPLC elution of **1** and **2** obtained from BAC transformant 3-4-G. The fragmentation patterns are identical to those obtained for authentic **1** and **2** from *Lissoclinum patella*.

fugation, lipophilic components of the broth were absorbed onto a C-18 solid-phase support (60 mL, 10 g, Alltech, Deerfield, IL, USA), washed with H_2O (40 mL) and eluted with methanol (2×40 mL). After evaporation of the methanol under reduced pressure, the residue was partitioned between water (40 mL) and dichloromethane (2×40 mL). The organic phases were combined and reduced under vacuum, the residue was redissolved in MeOH (400 μl), and the sample extract was subsequently analysed by HPLC-MS (Bruker Daltonics Esquire 3000 Plus coupled with an Agilent 1100 HPLC) by using linear solvent gradients (Phenomenex Synergi Fusion RP 4 μm, 80 Å, 250×4.6 mm column, $t=0$ min, MeOH/H_2O (50:50); $t=$ 20 min, 100% MeOH; $t=25$ min, 100% MeOH; $t=30$ min, MeOH/H_2O (50:50); $t=35$ min MeOH/H_2O (50:50) at 1 mL min^{-1}; $T=30\,^{\circ}C$). Extracted ion currents ($[M+H]^+$ and $[M+Na]^+$) for both patellamides, shown to be present in collected specimens of *L. patella*, were monitored. Once plates containing **1** and/or **2** were located, combined clones from each column were cultured and analysed by LC-MS, and individual clones from each column containing the desired metabolites were fermented separately for patellamide clone selection. Authentic **1** and **2** obtained from *L. patella* were used as reference standards.[19] Analytical data obtained for the patellamide products isolated from the fermentation broth of BAC transformant 3-4-G are given below.

*Patellamide D (**1**)* eluted at 17.8 min and gave characteristic ions by MS analysis at $m/z=777$ $[M+H]^+$ and 799 $[M+Na]^+$ and characteristic fragment ions by MS-MS analysis at $m/z=771$, 755, 714, 449, 364, 329. Accurate mass measurement of $m/z=799$ $[M+Na]^+$ yielded 799.3036; $[C_{38}H_{48}N_8O_6S_2Na]^+$ requires 799.3030 ($\Delta=+0.8$ ppm). Patellamide D was recovered at 77 ng mL^{-1} from the fermentation broth (18 h at 37 °C) obtained from transformant 3-4-G.

*Ascidiacyclamide (**2**)* eluted at 19.4 min and gave characteristic ions by MS analysis at $m/z=757$ $[M+H]^+$ and 779 $[M+Na]^+$ and characteristic fragment ions by MS-MS analysis at $m/z=751$, 735, 694, 668, 626, 570, 401, 316. Accurate mass measurement of $m/z=779$ $[M+Na]^+$ yielded 779.3314; $[C_{36}H_{52}N_8O_6S_2Na]^+$ requires 779.3343 ($\Delta=-3.7$ ppm). Ascidiacyclamide was recovered at 94 ng mL^{-1} from the fermentation broth (18 h at 37 °C) of transformant 3-4-G.

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Keywords: peptides · *Escherichia coli* · heterologous expression · HPLC-MS · natural products · patellamides

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