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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Biochemical investigation of Amm3, an unusual flavin-dependent tryptophan halogenase
involved in the cryptic biosynthesis of the ammosamides

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Marine Biology

by

Kimberly Chang

Committee in charge:

Professor Bradley S. Moore, Chair
Professor William H. Fenical
Professor Amr M. Hamdoun

2017

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Chair

University of California, San Diego

2017

DEDICATION

This thesis is dedicated to my parents who have not only showed me what hard work, perseverance and compassion are, but also encouraged and supported me in all my life pursuits. I would like to dedicate this thesis to my roommate and fellow classmate, Leeann Alferness for being right by my side throughout the ups and downs of this shaping year as a graduate student. Lastly, I would also like to dedicate this thesis to Bimo El Gamal and Imran Rahman for allowing me to be their third musketeer.

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ABSTRACT OF THE THESIS

Biochemical investigation of Amm3, an unusual flavin-dependent tryptophan halogenase involved in the cryptic biosynthesis of the ammosamides

by

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Master of Science in Marine Biology

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Pyrroloquinoline alkaloids have been extensively isolated in the past three decades due to their broad-spectrum bioactivities. Although many total synthesis reports have described the unique chemistry of these tricyclic compounds, the biosynthesis of this well-studied natural product class remains elusive despite their simple structures. In this work, cryptic genes of the previously validated ammosamide biosynthetic gene

cluster were subjects of *in vitro* biochemical investigation. *Amm3*, the putative FADH₂-dependent tryptophan halogenase, was successfully expressed, purified, and screened for optimal crystallization conditions. *Amm3* does not appear to bind flavin, nor does it halogenate a free-tryptophan substrate. Amid these experiments, it was reported by Ortega and colleagues that MibH, the *Microbispora* tryptophan-5-halogenase that is the closest homologue to *Amm3*, set the first precedent of acting on a peptide substrate. In this vein, the *amm6* peptide was found to successfully express under quick and aggressive growth conditions in *Escherichia coli*, and appears to be a promising substrate candidate for *Amm3* halogenation.

Introduction

The pyrroloquinoline alkaloids are a well-studied class of natural products targeted for their diverse structural features and extensive bioactivity. Examples of alkaloids carrying the characteristic tricyclic pyrrolo[4,3,2-*de*]quinolone core have been predominantly isolated from marine sponges since the earliest discoveries of the batzellines, damirones, and makulavones in the late 1980's (Antunes et al. 2005, Figure 1). A notable example is the cytotoxic discorhabdin C, a major pigment of a red-brown sponge, which first demonstrated potent cytotoxicity against L1210 tumor cells at $ED_{50} < 100$ ng/mL, as well as strong antimicrobial activity against *Escherichia coli* and *Bacillus subtilis* (Hu et al. 2011). In addition, the discorhabdins and related alkaloids were also found to exhibit pronounced antiviral, antimalarial, caspase inhibition, and immunomodulatory biological activities (Hu et al. 2011). Because of their broad-spectrum bioactivities and intriguing ring structures, members of the pyrroloquinoline alkaloid group have garnered great interest from synthetic chemists.

Despite years of interest in pyrroloquinoline alkaloid therapeutic potential and chemistry, the biosynthesis of these secondary metabolites has remained unknown due to the genetic difficulty of working with their dominant ecological source, marine sponges. Fortuitously, two marine bacteria derived pyrroloquinoline alkaloids, the lymphostins and ammosamides, have emerged as model members of this natural products class for biosynthetic studies.

Lymphostin, a potent immunosuppressant, was first isolated from *Streptomyces* sp. KY11783 (Hayashi et al. 1982), and displayed both potent lymphocyte kinase (IC_{50}

0.05 μM) and phosphatidylinositol 2-kinase (IC_{50} 0.001 μM) activity. This biological activity, together with a structural cue from lymphostin's pyrroloquinoline skeleton, motivated a biomimetic total synthesis of lymphostin starting from tryptophan. (Tatsuta et al. 2004). In 2011, the Moore Lab confirmed the tryptophan origin of lymphostin's tricyclic core through isotope feeding experiments and identified lymphostin as a product of a unique hybrid nonribosomal peptide synthetase-polyketide synthetase (NRPS-PKS) from three sequenced strains of *Salinispora* (Miyanaga et al. 2011). This work represents the first genetic study of alkaloids bearing a pyrrolo[4,3,2-*de*]quinolone core.

In addition to the lymphostins, only one other example of bacterial pyrroloquinoline alkaloids has been described. The structurally related anti-cancer compounds ammosamide A and B were first isolated from the marine sediment-dwelling actinomycete, *Streptomyces* sp. CNR-698, and subsequently characterized by the Fenical Group in 2009 in a sequence of publications (Hughes, Macmillan, Gaudêncio, Jensen, et al. 2009; Hughes, Macmillan, Gaudêncio, Fenical, et al. 2009). Displaying potent antitumor activity, it was later determined that ammosamides A and B target the cellular cytokinetic protein myosin, a rare occurrence for a natural product.

Since the ammosamides and lymphostins share the same characteristic core structure and are produced by two different bacteria, they provide the opportunity to explore pyrroloquinoline alkaloid biosynthesis through comparative genomics (Jordan and Moore 2016). Jordan and Moore capitalized on this opportunity and showed that the actinomycete-produced lymphostin and ammosamides are derived from a novel biosynthetic machinery that strikingly resembles ribosomally synthesized, post-

translationally modified peptide (RiPP) natural products (Figure 2). RiPP biosynthesis begins with the production of linear ribosomally synthesized precursor peptides that can be modified post-translationally by enzymes, which contributes to the structural diversity and flexible augmentation of this natural product family. Often these modifying enzymes include lantibiotic dehydratases (LDs), four of which are included in the *amm* pathway. Genetic manipulation of these four LDs, as well as other putative RiPP-related open reading frames (ORFs), demonstrated their necessity, further supporting this unusual biosynthetic proposal (Chatterjee et al. 2005).

Bioinformatic studies of the lymphostin and ammosamide gene clusters also point to a conserved short ORF encoding a peptide bearing a C-terminal tryptophan residue that bears a striking resemblance to RiPP precursor peptides (Jordan and Moore 2016). Precursor peptides, which are much larger than final peptide products, consist of two key domains: a leader peptide at the *N*-terminus that carries recognition elements for posttranslational modification, and the core peptide which is post-translationally modified before proteolytic cleavage of the leader peptide (Repka et al. 2016). Jordan's work originally proposed this short ORF, *amm6* to be the starting point of biosynthesis in which the encoded peptide delivers its C-terminal tryptophan residue for posttranslational modification by downstream pathway enzymes (Jordan and Moore 2016). Deletion of *amm6* resulted in the loss of ammosamide production, supporting its requirement in biosynthesis. However, point mutations of the C-terminal tryptophan residue showed that tryptophan residue was not necessary for biosynthesis. In their work, Jordan and Moore propose one possibility for how a highly conserved, but not directly required, tryptophan

residue could relate to a predicted precursor peptide sequence that seems to be integral to ammosamide biosynthesis. They offer that the peptide product of *amm6*, lacking a C-terminal tryptophan residue, could potentially drive product formation of 6-chlorotryptophan by *trans* activation of *amm* pathway enzymes that could then utilize free tryptophan as a substrate.

Although genetic experiments indicate that a peptide-bound tryptophan is not required for ammosamide biosynthesis, other lines of evidence support the role that RiPP genes play in pyrroloquinoline alkaloid biosynthesis. Notably, a gene unique to the *amm* cluster, *amm3* codes for an unusual FADH₂-dependent tryptophan halogenase (Jordan and Moore 2016). Flavin-dependent halogenases are enzymes responsible for the incorporation of chloride and bromide atoms into aromatic and aliphatic compounds (Pée 2012). These halogenases fall into two categories: 1) those that utilize free substrate, i.e. free tryptophan in solution, or 2) those that utilize acyl carrier protein (ACP)-bound substrates (e.g. the dichlorinating PltA in pyoluteorin biosynthesis) (Dorrestein et al. 2005). Instead of yielding a non-halogenated advanced pyrroloquinoline intermediate as hypothesized, Jordan and Moore found that the deletion of *amm3* resulted in abolished ammosamide production. However, unlike other biochemically characterized FADH₂-dependent tryptophan halogenases that act early in biosynthesis, chemical complementation of M512-pCAP01/*ammΔamm3* knockout cultures could not rescue production of compounds when supplemented with 6-chlorotryptophan (Zeng and Zhan 2011; Jordan and Moore 2016). Ammosamide production was restored only after genetic complementation with pKY01/*amm3* when under the control of the strong promoter

ermE (Yamanaka et al. 2012). These gene deletion and complementation studies demonstrated the necessity of *amm3* for ammosamide production.

By amino acid sequence identity, the closest homologue of Amm3 is MibH (61%), a tryptophan-5 halogenase involved in the biosynthesis of microbisporicin (Jordan and Moore 2016). Microbisporicin is a RiPP-class peptide antibiotic from *Microbispora* sp. Intriguingly, phylogenetic evidence shows that Amm3 and MibH clade distinctly from all other characterized flavin-dependent halogenases (Figure 3). In 2010, Foulston and Bibb first indicated that MibH halogenation is presumably post-translational due to limited aminoacyl-tRNA substrates available for ribosomal peptide synthesis (Foulston and Bibb 2010). The hypothesis that that MibH works on a novel substrate type was recently verified by the van der Donk Lab when crystal structures and activity tests showed that MibH specifically halogenates a tryptophan within a peptide, exhibiting highly specific substrate selectivity (Ortega et al. 2017).

Given that the *amm* gene cluster lacks any open reading frame resembling an ACP gene, we are left with potentially two different substrates that Amm3 may halogenate, free tryptophan, or a perhaps a MibH-like peptide within the pathway. Previous genetic studies point to a substrate distinct from tryptophan, but *in vitro* investigations of Amm3 have not been pursued. With this framework in place, this work will highlight pointed biochemical investigation of Amm3 and concerted efforts to understand its substrate specificity.

Materials and Methods

1) Cloning of *amm3*, and heterologous expression and purification of Amm3 – an unusual FAD-dependent tryptophan halogenase

To ensure that the correct ORF encoding *amm3* was cloned and consistent with published genetic studies, GeneMark was used to identify and validate the correct predicted gene sequence (Jordan and Moore 2016). *Amm3* was PCR-amplified from pCAP01/*amm* using PrimeStar Max DNA polymerase (Takara) standard protocol (forward primer:CTGGTGCCGCGCGGCAGCCATATGACGCACATGCACGGTACC, reverse primer: TGGTGCTCGAGTGCGGCCGCAAGCTTTCATCGCACGCCCCCG CCC) , and ligated into NdeI/HindIII digested pET28 using T4 DNA Ligase. The resulting pET28/*amm3* vector was then transformed via electroporation into *E.coli* BL21-Gold(DE3) for recombinant protein expression. Four mL LB overnight preculture containing 50 µg/mL kanamycin was inoculated with 100 µL of *E.coli* BL21-Gold(DE3)/pET28*amm3* and grown at 37 °C with shaking. One L TB containing 50 µg/mL kanamycin was then inoculated with overnight preculture and grown at 30 °C until O.D. 0.5. Cultures were cooled to 16 °C for 1 hour before being spiked with 10 mg of riboflavin and induced with 50 µM IPTG. Cultures were incubated for an additional 36 hours at 16°C with shaking at 200 rpm.

Cells were harvested by centrifugation at 8,000 g at 4 °C for 3 min and resuspended in harvest buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl 10% glycerol) before being lysed by sonication. The lysate was centrifuged at 14,000 g for 45 min, after which the supernatant was applied to a 5 mL Ni-NTA column equilibrated with harvest

buffer. The column was washed with 6 column volumes of 20 mM Tris-HCl (pH 8.0), 1 M NaCl and 30 mM imidazole buffer, and protein was eluted in a linear gradient fashion from 0 mM to 250 mM imidazole. After running 10% SDS-PAGE, pure protein fractions were collected, pooled, and dialyzed overnight in 20 mM Tris (pH 8.9) and 50 mM NaCl buffer at 4 °C in the presence of 1 U/mg thrombin protease.

Following dialysis, protein was additionally purified by anion exchange chromatography using a 5 mL Q-FF column. The column was equilibrated with 10 column volumes of 95% 20 mM Tris-HCl pH 8.9 wash buffer before loading protein. The column was washed with 6 column volumes of wash buffer before protein was eluted in a linear gradient from 0 M KCl to 1 M KCl. Ion exchange purified protein fractions were validated by 10% SDS-PAGE, pooled together, and concentrated with a 50 kDA molecular weight cut-off (MWCO) centrifugal filter and desalted in 20 mM Tris-HCl pH 8, 50 mM KCl, 10% glycerol buffer using a PD-10 column and stored at -80 °C.

2) Amm3: Flavin (FAD)-binding experiments

Flavin-binding was first tested by incubating 100 µL of 10 mM FAD and 400 µL of purified Amm3 at room temperature for 15 min before application to a PD-10 column followed by 2 mL of 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 10% glycerol desalting buffer. The sample was then eluted with an additional 3 mL desalting buffer and collected in six 500 µL fractions and scanned on an Agilent Cary 60 UV-Vis spectrophotometer from 800 nm to 200 nm to assess absorbance differences between fractions.

Additionally, flavin-binding was further investigated by titrating Amm3 halogenase into 100 μ L of 0.1 mM FAD. A baseline UV absorbance spectrum of the 0.1 mM FAD solution was first measured on an Agilent Cary 60 UV-Vis spectrophotometer before Amm3 was incrementally added to the 0.1 mM FAD solution in small volumes (\sim 0.5 μ L) starting from a concentration range of 0.02 mM to 0.14 mM. An absorbance spectrum measurement of the FAD solution was made at each addition of Amm3.

3) Amm3: *In vitro* halogenation assay with free tryptophan as substrate

50 μ M L-tryptophan substrate was incubated with 25 μ M purified Amm3 halogenase in 20 mM Tris-HCl pH 8, 50 mM KCl, 10% glycerol reaction buffer containing 50mM of KCl, 2mM NADP⁺, 0.1 mM FAD, 10 mM Na-phosphite, 10 μ M phosphite dehydrogenase (PtdH) and SsuE enzymes at 30 °C for 4 hours. An engineered variant of phosphite dehydrogenase N-His₆-PtdH-17X-A176R was kindly purified and provided by Dr. Abraham El Gamal (Agarwal et al. 2014). The NAD(P)H-dependent flavin reductase, SsuE, derived from the two component *E.coli* alkanesulfonate monooxygenase system was used in the Amm3 halogenation assay (Dorrestein et al. 2005). SsuE uses NAD(P)H to carry out reduction of FAD to FADH₂ required by tryptophan halogenases. After a 4 hour incubation period, reactions (100 μ L) were quenched with 100 μ L of MeOH with 0.2% formic acid to precipitate protein, centrifuges and supernatant was filtered through a 0.22 micron filter. Reaction assays were analyzed by liquid chromatography-mass spectrometry (LC-MS) using with a Phenomenex Kinetex 2.6 μ m XB-C18 100 Å, 150 x 4.6 mm column (0-3 min isocratic 10% MeCN, 3-23 min 10% -100% MeCN; mobile phase buffered with 0.1% formic acid) operating on an Agilent 1260 HPLC in tandem to an Agilent 6530 Accurate Mass Q-TOF mass

spectrometer. In addition to reaction assays, a negative control (with no halogenase), 6-chlorotryptophan, and L-tryptophan standards were also conducted for LC-MS analysis. Mass spectra were acquired in positive ionization mode.

4) Amm3: *In vitro* halogenation assay with *amm3* + pCAP01/*ammΔamm3* cell lysate as substrate

Cultures of *S. coelicolor* mutant strain M512 stock of pCAP01/*ammΔamm3* (kindly provided by Dr. Peter A. Jordan) were started from 20 µL of spores that were germinated at 50 °C for 10 min in 200 µL of 2X YT media (yeast extract, tryptone) and were then added to 3 mL TSB preculture containing 50 µg/mL kanamycin and 30 µg/mL nalidixic acid. The preculture was grown for 5 days at 30 °C after which 1 mL was used to inoculate 50 mL of R5 production medium without antibiotics. Production culture was grown for 5 days at 30 °C prior to centrifugation to pellet M512 cells. Cells were washed twice with 20 mM Tris-HCl (pH 8.0) and 500 mM NaCl 10% glycerol harvest buffer before lysis by sonication. 200 µL of mutant *Streptomyces* pCAP01/*ammΔamm3* lysate was incubated with 25 µM purified Amm3 halogenase in 20 mM Tris-HCl pH 8, 50 mM KCl, 10% glycerol reaction buffer containing 50 mM of KCl, 2 mM NADP⁺, 0.1 mM FAD, 10 mM Na-phosphite, 10 µM PtdH and SsuE enzymes in a 1 mL reaction at 30 °C for 4 hours. A negative control was also prepared with the same reaction components, except for Amm3 halogenase. LC-MS reaction samples were prepared and run in the method described above in method 3.

5) Amm3: Crystallography condition optimization

Initial crystallization conditions for Amm3 were first screened using the following kits from Hampton Research: Index, Index 2, Crystal Screen, Crystal Screen 2, and Natrrix, in addition to the Wizard Classic 3 screen by Rigaku. Amm3 crystals were grown at 6 °C by hanging drop vapor-diffusion by mixing 1 µL of protein sample at 10 mg/mL concentration with an equal volume of reservoir mother liquor. Once a promising preliminary crystallization condition was noted, conditions were further optimized by carrying out a secondary crystal screen testing a close range of buffer concentration, precipitant concentration, or pH of the successful commercial screens. Upon assessment of proper concentrations and pH conditions, a final crystal screen was included with the addition of the following additives to the final concentration of the mother liquor: 5% glycerol, 5% ethylene glycol, 5% methyl pentanediol (MPD), 5% xylose, 5% sucrose, 1 mM tryptophan, and 1 mM FAD.

6) Cloning of *amm6*, and test expression (low induction conditions) of Amm6- a short ORF peptide essential for biosynthesis

Amm6 was PCR-amplified from pCAP01/*amm* PrimeStar HS DNA polymerase (Takara) standard protocol (forward primer: ATCGTCACATATGAGTGAGACCCAAGTGACCGAGA, reverse primer: ACGCCAGAAGCTTTCACCATGAACGCGATTTCCTTGAGG), and ligated into NdeI/HindIII digested pET28 using T4 DNA Ligase. The resulting pET28/*amm3* vector was then transformed via electroporation into *E.coli* Rosetta(DE3) for recombinant protein expression. Four mL LB overnight preculture containing 50 µg/mL kanamycin and 25 µg/mL chloramphenicol were inoculated with 100 µL of Rosetta (DE3)/pET28/*amm6* and grown at 37 °C with shaking. One L TB

containing 50 µg/mL kanamycin and 25 µg/mL chloramphenicol was inoculated with overnight preculture and grown at 30 °C until 0.D. 0.5. Cultures were cooled to 18 °C for 1 hour before being test-induced with 0 µM, 50 µM, 100 µM, and 400 µM IPTG and incubated for an additional 36 hours at 18 °C with shaking at 200 rpm.

Cells were harvested by centrifugation at 8,000 g at 4 °C for 3 min and resuspended in 20 mM Tris-HCl (pH 8.0) and 500 mM NaCl 10% glycerol harvest buffer and treated with 0.1 mM final concentration phenylmethylsulfonyl fluoride (PMSF) protease inhibitor before being lysed by sonication. Pellet and lysate samples at each induction concentration (0 µM IPTG, 50 µM IPTG, 100 µM IPTG, and 400 µM IPTG) were analyzed on a tricine-SDS-PAGE gel (Schägger 2006).

7) Heterologous expression and purification of Amm6 short ORF peptide (“burst” induction)

A 4 mL LB preculture containing 50 µg/mL kanamycin and 25 µg/mL chloramphenicol was inoculated with 100 µL of Rosetta (DE3)/pET28/*amm6* (described in method 6) and grown at 37 °C overnight with shaking. One L TB containing 50 µg/mL kanamycin and 25 µg/mL chloramphenicol was inoculated with overnight preculture and grown at 37 °C until 0.D. 1.5. Cultures were induced with 1 mM IPTG and incubated for an additional hour at 37 °C with shaking at 200 rpm before immediate cell harvest.

Cells were harvested by centrifugation at 8,000 g at 4 °C for 3 min and resuspended in 20 mM Tris-HCl (pH 8.0) and 500 mM NaCl 10% glycerol harvest buffer and treated with 0.1 mM final concentration PMSF protease inhibitor before being lysed by sonication. The lysate was centrifuged at 14,000 g for 45 min, in which the

supernatant was applied to a 5 mL Ni-NTA column equilibrated with harvest buffer. The column was washed with 6 column volumes of 20 mM Tris-HCl (pH 8.0), 1 M NaCl and 30 mM imidazole buffer, and protein was eluted in a linear gradient fashion until 250 mM imidazole. Purified protein fractions were analyzed on a Tris-Tricine SDS-PAGE gel, pooled and concentrated with a 10 kDa MWCO centrifugal filter.

After concentration, an equal volume of MeCN + 0.1% formic acid was added to the concentrated Ni-purified Amm6 sample to precipitate large proteins. The resulting supernatant was then lyophilized and resuspended in 200 μ L of 20 mM Tris-HCl (pH 8.0) buffer and further purified by preparatory HPLC with solvents: water + 0.1% TFA (A) and acetonitrile + 0.1% TFA (B). The HPLC method performed using a reverse phase C₁₈ column (Phenomenex, 250 mm) was a linear gradient of 10% to 95% A for 20 min, held at 95% B for 5 min, and then decreased to 10% B for 5 min. Protein peaks absorbed at 280 nm on the HPLC chromatogram were collected and lyophilized. To confirm peptide isolation, LC-MS characterization was performed on a 10 μ L injection of 25 μ M purified Amm6 in positive ionization mode on a on a Higgins Analytical PROTO 300 C4 5 μ m, 250 x 4.6 mm column (1-10 min isocratic 10% MeCN, 10-15 min 10-30% MeCN, 15-55 min 30-70% MeCN, 55-60 min 70-100%, 60-63 min isocratic 100% MeCN; mobile phase buffered with 0.1% formic acid operating on an Agilent 1260 HPLC in tandem to an Agilent 6530 Accurate Mass Q-TOF mass spectrometer.

8) Amm3: *In vitro* halogenation assay with Amm6 purified peptide as substrate

50 μ M Amm6 substrate purified as described in method 7, was incubated with 50 μ M purified Amm3 halogenase in 20 mM sodium phosphate (pH 7.4) reaction buffer

containing 10 mM of NaCl, 2mM NADP⁺, 5 μ M FAD, 10 mM Na-phosphite, 10 μ M PtdH and SsuE enzymes at 30°C for 4 hours. 100 μ L of reaction was precipitated with 100 μ L of MeOH with 0.2% formic acid, and then spin filtered by centrifugation. Reaction were analyzed by LC-MS as described in method 3. In addition to reaction assays, a negative control (with no halogenase), 6-chlorotryptophan, and L-tryptophan standards were also conducted for LC-MS analysis. Mass spectra were acquired in positive ionization mode.

Results and Discussion

1) *Amm3* prolifically expresses in *E.coli*, but halogenase does not appear to bind flavin

100 mg/L of soluble non-yellow halogenase was obtained following protein expression and purification by Nickel chromatography (Figure 4), overnight thrombin cleavage, and ion exchange chromatography (Figure 5). The 75 kDa *Amm3* protein was diluted to 10 mg/mL with 20 mM Tris-HCl pH 8, 50 mM KCl, 10% glycerol (Figure 6) for subsequent activity assays and crystallization screening.

Amm3 expressed abundantly in *E.coli*, following lower growth temperature (16°C for 36 hours) and induction conditions (50 μ M IPTG). Interestingly, *mibH*, the closest phylogenetic homologue to *amm3*, was reported to yield soluble protein only after coexpression with *E.coli* chaperones *groES/EL* (Ortega et al. 2017). Despite supplementing media with riboflavin, purified *Amm3* did not have the characteristic yellow color indicative of flavin binding. A preliminary flavin binding test involving the addition of FAD to *Amm3* followed by elution via a desalting column demonstrated that the protein did not elute with FAD. A subsequent ultra violet (UV) binding assay, in which FAD solution was titrated up to more than equimolar concentration of *Amm3*, showed no evidence of an absorbance shift characteristic of flavin binding (Figure 7). These findings suggest that *Amm3* does not readily bind FAD. Similar reports were also made of *MibH*, stating that flavin was not present in downstream purification, suggesting that the enzyme has a weak binding affinity toward its cofactor (Ortega et al. 2017). The fact that *Amm3* and *MibH* do not bind FAD is not uncommon, purified *PrnA* from pyrrolnitrin biosynthesis as well as other examples of investigated purified tryptophan

halogenases have also lacked observable binding behavior (Pée 2012). Furthermore, it has also been claimed that all studied flavin-dependent halogenases thus far are strictly dependent on FADH₂, and do not accept reduced FMN or riboflavin.

2) Amm3 does not appear to halogenate free tryptophan substrate

FADH₂ dependent halogenases have rather high substrate specificity and have classically acted *in vitro* on either a free tryptophan substrate or a substrate bound to a carrier protein (Pée 2012). Because the *amm* biosynthetic gene cluster lacks an ORF resembling an ACP gene, purified Amm3 was first tested for activity against a free-tryptophan substrate, in which no transformation of tryptophan substrate to chlorinated product was observed by LC-MS (Figure 8). Interestingly, it was also reported that regardless of conditions tested, halogenation activity by MibH on free tryptophan was not observed (Ortega et al. 2017).

Due to the limited understanding of chemistry at hand, there is a possibility that specific components required for Amm3 activity may be missing. As there are many hypothetical proteins in the *amm* cluster, it may be likely that protein-protein interactions are necessary for successful biosynthesis. To explore whether purified Amm3 was missing necessary components for halogenation activity, a cell-free lysate was prepared from *S. coelicolor* M512 pCAP01/*amm*Δ*amm3*, to complement the Amm3 assay system. M512 pCAP01/*amm*Δ*amm3* carries the entire *amm* cluster, except for the *amm3* gene. This mutant was previously described to have been unable to produce the ammosamides, however upon genetic complementation with *amm3*, *in vivo* ammosamide production was restored (Jordan and Moore 2016). Using this knowledge, it was reasoned whether

Amm3, upon complementation with cell free lysate, would yield halogenation.

Nonetheless, no halogenation activity by Amm3 was seen on free tryptophan substrate upon complementation with cell lysate (Figure 9).

3) Amm3: Successful crystallization growth conditions of apoprotein

Given the high levels of Amm3 protein expression, it became of interest to pursue X-ray crystallography to add content to biochemical characterization. A three-dimensional structure of Amm3 could provide information about potential flavin and substrate-binding domains of the halogenase, especially in reference to the structures available in the protein data bank. Initial crystallization conditions for Amm3 were identified and optimized to 0.15 M sodium citrate, 22% (w/v) PEG8000, 5% sucrose when screened at 6°C (Figure 10). However, further crystallography pursuits were paused until an Amm3 holoprotein structure could be achieved.

4) Amm6 requires quick, high temperature, high induction (“burst”) expression conditions

It was recently reported that MibH is the first characterized halogenase exhibiting specificity to tryptophan within an unprecedented polypeptide substrate (Ortega et al. 2017). As MibH is the closest homologue to Amm3, it became of timely interest to explore the possibility of an analogous peptide substrate encoded within the *amm* gene cluster, specifically the 56-amino acid peptide product of *amm6* that carries a conserved C-terminal tryptophan residue analogous to the short peptide in the *lym* pathway. *Amm6* carries features resembling that of RiPP precursor peptides; *amm6* and its *lym* counterpart both share a conserved “FDLD” amino acid motif, pointing to leader peptide recognition

elements common to class I lantibiotics (Jordan and Moore 2016; Chatterjee et al. 2005; Figure 2).

Previous attempts to express and purify *amm6* showed C-terminal proteolysis, in which the residues Ser-Arg-Ser-Trp were cleaved prematurely (Figure 11). For this reason, when first revisiting expression of *amm6* for this project, the mild temperature (16 °C) and induction concentration (50 µM IPTG) growth conditions used for *amm3* expression were employed. However, test expressions resulted in inconclusive protein production between varying induction concentrations in both soluble lysate and insoluble pellet samples (Figure 12).

To further investigate *amm6* expression conditions, an aggressive approach was pursued in which *E.coli* cultures were induced at high optical density (O.D. 1.5) with a high isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration (1 mM) and temperature (37°C) and subsequently harvested just one hour after induction. This burst induction strategy resulted in specific production of a predominant peptide product (< 15 kDa) and a minor high molecular weight product (>100 kDa) following FPLC purification (Figure 13). It is possible that the high molecular weight band is a result of the harsh growth conditions when overexpressing *E.coli*. To remove this impurity, but retain the small peptide product, the concentrated Ni-purified protein fractions were first precipitated with acetonitrile, and then subsequently purified by reverse phase high-performance liquid chromatography (HPLC). The peak eluting at 9.6 minutes was collected and lyophilized to yield 4 mg/L white peptide powder (Figure 14).

To confirm if Amm6 was indeed isolated and expressed as the complete peptide, the purified sample was subjected to LC-MS analysis, giving an experimental m/z of 8191.52 (Figure 16), which matched that of the ExPASy theoretical monoisotopic weight (8191.83) (Figure 15).

The successful expression and purification of the Amm6 peptide suggests that the *E.coli* heterologous host benefits from growing to high optical density prior to induction. Furthermore, expression conditions may have benefitted from inducing the protein at a high concentration of IPTG for immediate production before proteolytic processes could begin.

5) Amm6 N-terminal His-tag is cleaved under halogenation conditions

Amm6 was tested as a substrate candidate for Amm3 halogenation activity using reaction components and concentrations as closely to those used in the confirmation of deschloro-microbisporicin as the substrate of MibH halogenation (Ortega et al. 2017). LC-MS results indicate that instead of observing a chlorinated or unchlorinated peptide product, we identify a truncated mass that corresponds to the mass of Amm6 when cleaved at the thrombin cut site in the peptide sequence (6442.2 m/z) (Figure 17). It is suspected that perhaps thrombin may have inadvertently been introduced from other reaction elements. Currently, the reexpression of *amm6* to preemptively cleave the peptide at the thrombin cleavage site is ongoing.

It should be noted in attempting to replicate halogenation reaction conditions reported by Ortega *et.al.*, MibH was accompanied by the flavin reductase encoded in the

microbisporicin pathway, MibS. FADH₂-dependent halogenases require a flavin reductase for catalysis (Keller et al. 2000). After brainstorming more optimal halogenation conditions, it was questioned whether SsuE, a flavin reductase from an *E.coli* heterologous system, was an adequate reductase to complement with *amm3*. Due to this, a future target that remains to be explored is *amm17*, a pathway gene encoding a native flavin reductase that could be cloned and included in Amm3 halogenation assay conditions. In examining the relationship between MibH and MibS more closely, the *mibS* gene is located directly downstream of *mibH*. Because these two genes work closely together in halogenation, we suspect that perhaps *amm3* is closely associated with the neighboring *amm4* F420-dependent oxidase as a downstream partner. We hope to better understand the potential substrate target of Amm3 by ensuring that it has the necessary components to drive activity.

Conclusion

Pyrroloquinoline alkaloids have been extensively isolated in the past three decades due to their broad-spectrum bioactivities. Major total synthesis reports have been published describing the unique chemistry that these tricyclic core-containing compounds confer upon substitution. However, because these natural products have been predominantly isolated from marine sponges, which have been traditionally intractable to lab cultivation and genetic studies, it has been notoriously difficult to understand how pyrroloquinoline alkaloids are biosynthesized by their natural producers. Fortunately, lymphostin and the ammosamides are the first two reported examples of bacterially-produced pyrroloquinoline alkaloids, which can together serve as a microbial model systems to further understand this class of secondary metabolites.

The high sequence and structural homology between the *amm* and *lym* pathway has provided genomic insight on pyrroloquinoline alkaloid biosynthesis, including the notable resemblance to RiPP gene clusters. Bioinformatic analysis of the *amm* gene cluster led to the identification of a unique FADH₂-dependent tryptophan halogenase gene, *amm3*. The major goal of this work was to biochemically characterize the halogenation activity and substrate specificity of Amm3. Upon further investigation, it became increasingly promising that Amm3 could halogenate a peptide substrate.

Amm3 was found to produce abundantly from an *E.coli* heterologous host and was of high enough purity to be able to identify optimal crystallization conditions. Once purified Amm3 was in hand, potential substrates were pursued. FADH₂-dependent halogenases typically utilize one of two substrates: free tryptophan or an ACP-bound

moiety. Amm3 did not show any halogenating activity against free tryptophan in solution. Additionally, the *amm* gene cluster lacked any ORF resembling that of a potential ACP gene.

The similarities between Amm3 and MibH, a recently characterized FAD-dependent halogenase that acts on an unprecedented peptide substrate provided an interesting context to further understand the activity and substrate specificity of Amm3. It was recently verified that MibH has a high specificity towards its substrate, deschloromicrobisporicin, and failed to act upon many structurally similar analogs. This is the first example of a halogenase working on a tryptophan within a peptide backbone.

To determine if Amm3 could also potentially halogenate a similar peptide substrate, the peptide product of *amm6* was identified, expressed and purified. Successful purification of the intact Amm6 was conferred after subjecting cultures to high and quick induction conditions. However, when assessing Amm6 as a substrate of Amm3 in halogenation conditions like that of MibH, we identified a cleaved peptide mass corresponding to cleavage at the thrombin cut site. The introduction of thrombin contamination into the assay reaction is unknown, but may potentially be attributable to other reaction elements where His-tag removal was a step involved in protein purification. Currently, the reexpression of *amm6* to preemptively cleave the peptide at the thrombin cleavage site is ongoing.

Given that MibH demonstrated high specificity to its sole substrate, it is not surprising that a peptide substrate for Amm3 to not have been identified. Among the greater than 20 genes associated with the *amm* pathway, are a variety of peptidases,

oxidoreductases and lantibiotic dehydratase, any of which may modify a yet unknown peptide precursor prior halogenation. It should also be noted that the attempted halogenation conditions may lack the necessary components needed to drive the addition of chlorine. For example, the microbisporicin gene cluster encodes the *mibS* gene, a flavin reductase used to regenerate required FADH₂. Perhaps specific interactions between Amm3 and a partner reductase are not met by SsuE. Future efforts may benefit from exploring the respective roles of *amm4* and *amm17*, a neighboring F420-dependent oxidase and flavin reductase, in supporting Amm3 activity.

The bacterially produced *lym* and *amm* pathways are unique from any biosynthetic gene cluster thus far described and currently represent the only two examples of genetically studied pathways that connect to the pyrroloquinoline alkaloid class. As these alkaloids are dominantly produced by marine sponges, it is likely that there will continue to be abundant opportunities to further understand pyrroloquinoline biosynthesis from this prolific source. However, like the age-old case with many sponge-derived natural products, pyrroloquinolines may be products of associated symbiotic bacteria, in which biosynthetic pursuits can be gained through increasingly expanding metagenomic sequencing (Jordan and Moore 2016). Thus, the study of pyrroloquinoline biosynthesis from a bacterial context not only enhances our basic knowledge of unique chemical routes, but also sets the stage for generalizable assembly of the prominent pyrroloquinoline alkaloid family.

Appendix

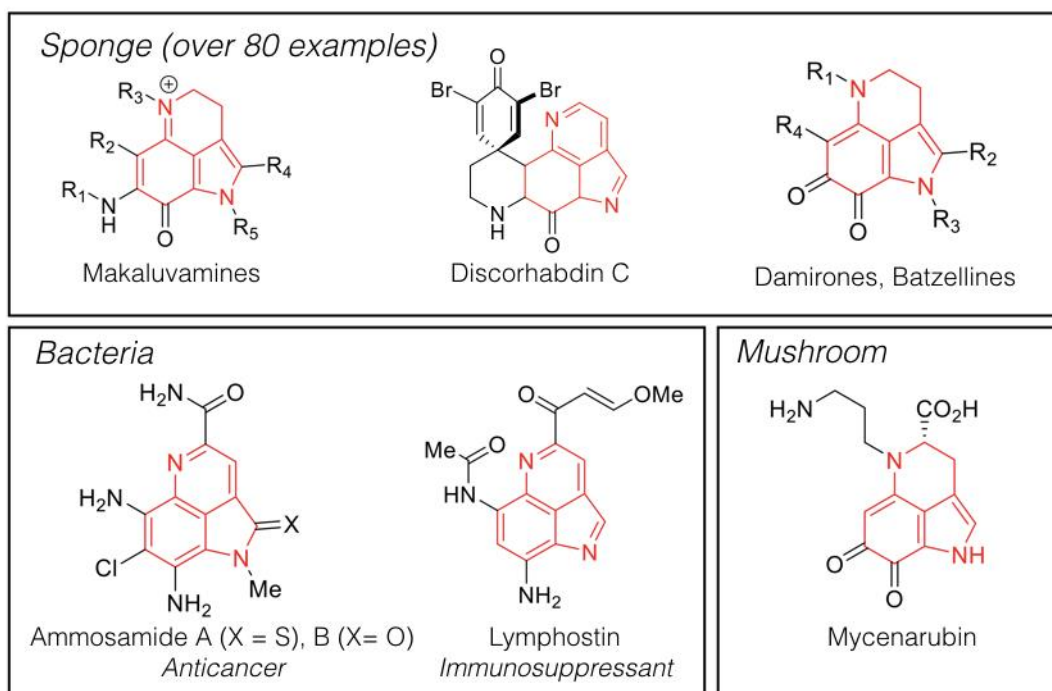


Figure 1: Pyrroloquinoline alkaloids are diverse and potent natural products that are derived from a variety of sources.

The characteristic pyrrolo[4,3,2,-de]quinolone core is indicated in red.

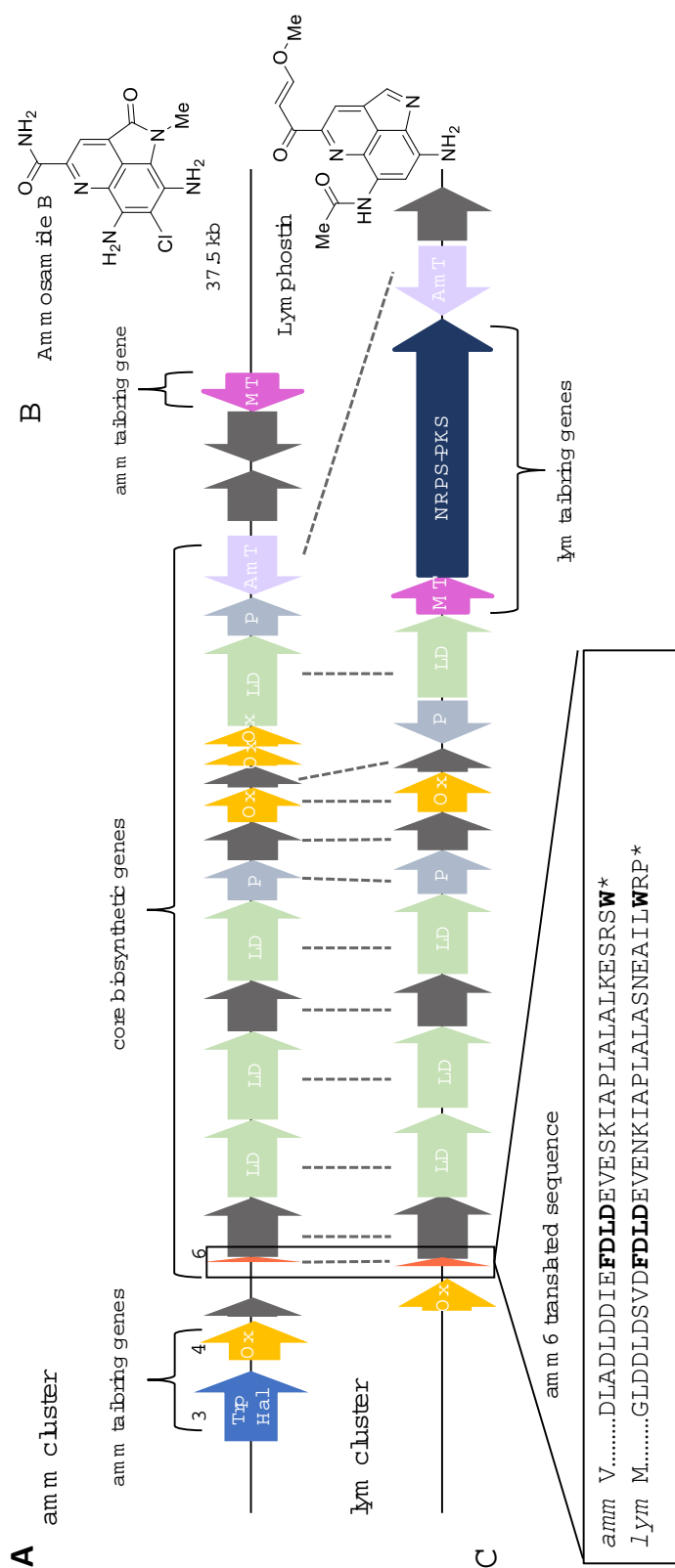


Figure 2: The ammosamide and lymphostin biosynthetic gene clusters show high gene sequence homology.

(A) Sequence alignment of ammosamide (*amm*) and lymphostin (*lym*) gene cluster. Brackets are used to indicate conserved biosynthetic genes and tailoring genes, respectively.

(B) Structural features derived from tailoring enzymes are highlighted for ammosamide B and lymphostin.

(C) Aligned translated sequences of the short ORFs in *amm* and *lym* pathways.

Trp Hal, tryptophan halogenase; LD; lantibiotic dehydratase; P, peptidase, Ox, oxidoreductase; Amt, amidotransferase; MT, methyltransferase. Unmarked genes in gray encode for hypothetical proteins.

Halogenation sites of corresponding metabolites are indicated in red. Red branches indicate halogenation of an ACP-bound substrate. Green branches indicate halogenation of a free substrate. Blue branches direct to Amm3, described in this work, and MibH, a halogenase that has been reported to modify a tryptophan residue within a peptide substrate. Figure courtesy of Dr. Peter A. Jordan.

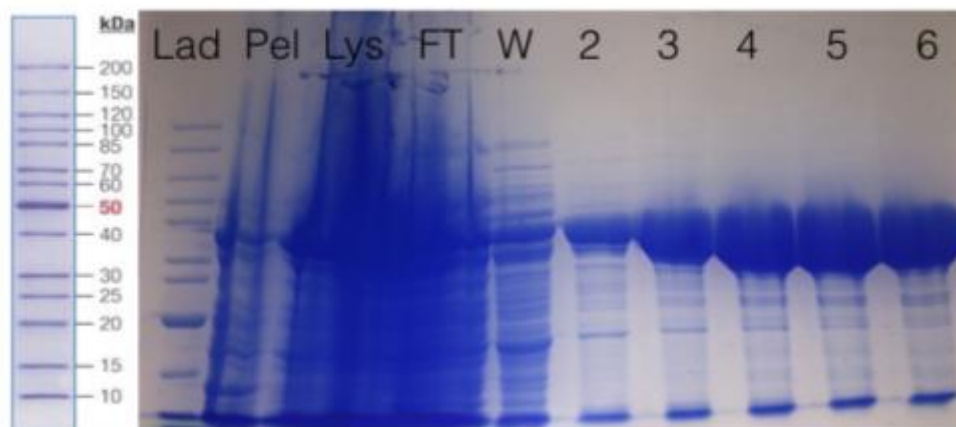


Figure 4: 10% SDS-PAGE gel of Ni-purified Amm3 tryptophan halogenase.

From left to right: ladder (Lad), pellet (Pel), lysate (Lys), flow through (FT), wash (W), fraction 2, fraction 3, fraction 4, fraction 5, fraction 6.

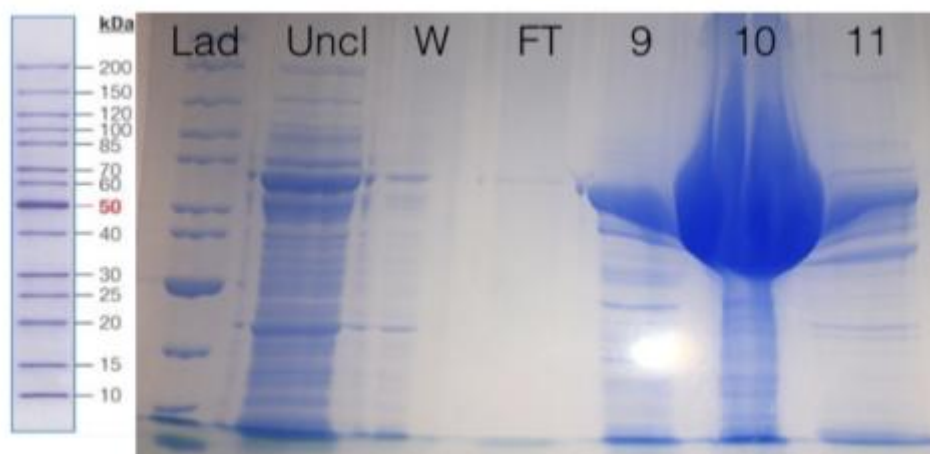


Figure 5: 10% SDS-PAGE gel of Ion-exchange-purified Amm3 tryptophan halogenase following Nickel purification and overnight thrombin proteolysis/dialysis.

From left to right: ladder (Lad), uncleaved with His-tag (Uncl), wash (W), flow through (FT), fraction 9, fraction 10, fraction 11.

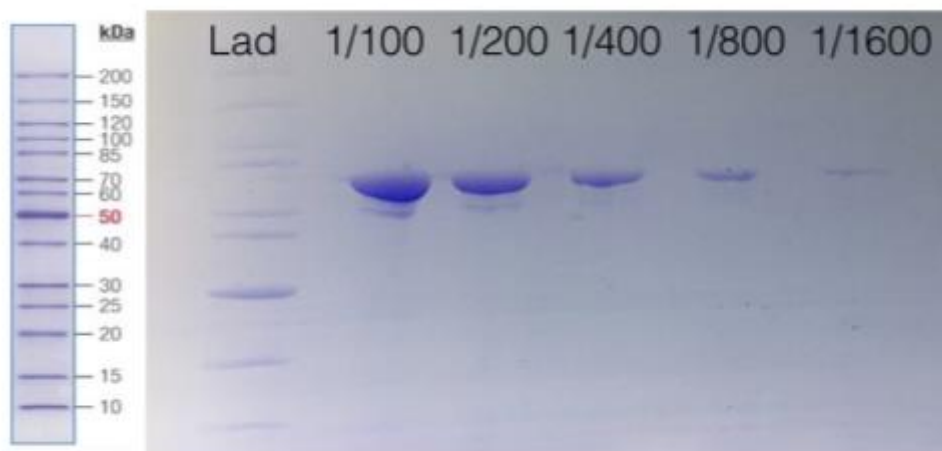


Figure 6: 10% SDS-PAGE gel of serially-diluted purified Amm3.

Concentrated, purified 32.5 mg/mL Amm3 was diluted to the following concentrations for better gel visualization: 1/100, 1/200, 1/400, 1/800, and 1/1600.

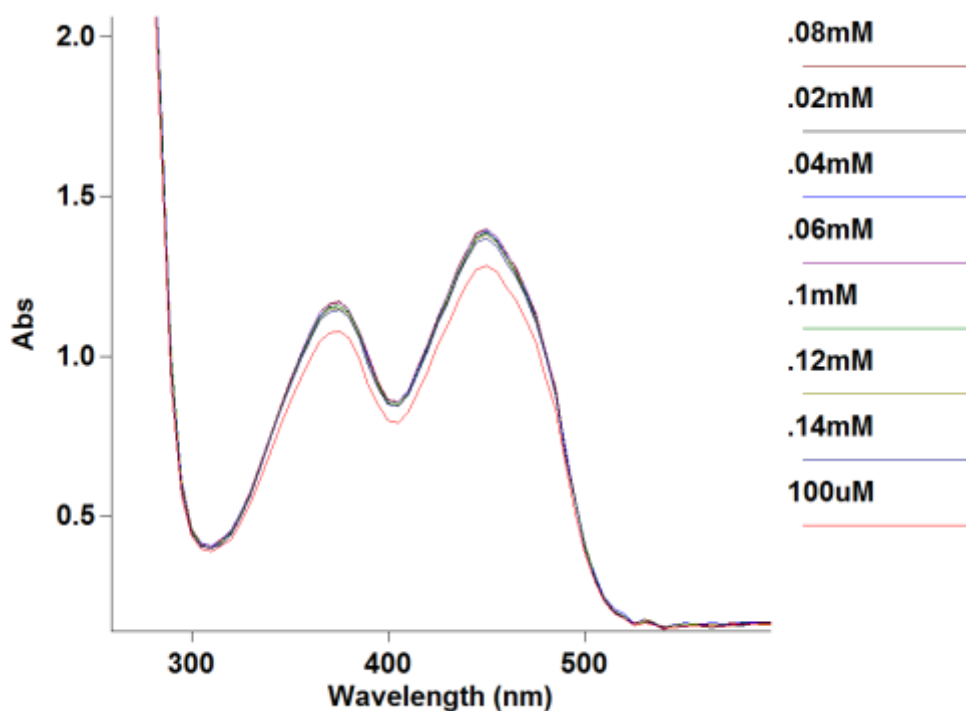


Figure 7: UV absorbance of 100 μ M FAD solution with increasing concentration of Amm3.

Absorbance spectrum does not indicate a UV shift to correspond to Amm3 flavin binding. 100 μ M FAD solution is in red, traces of FAD solution titrated with increasing millimolar concentrations of Amm3 are indicated in respective colors.

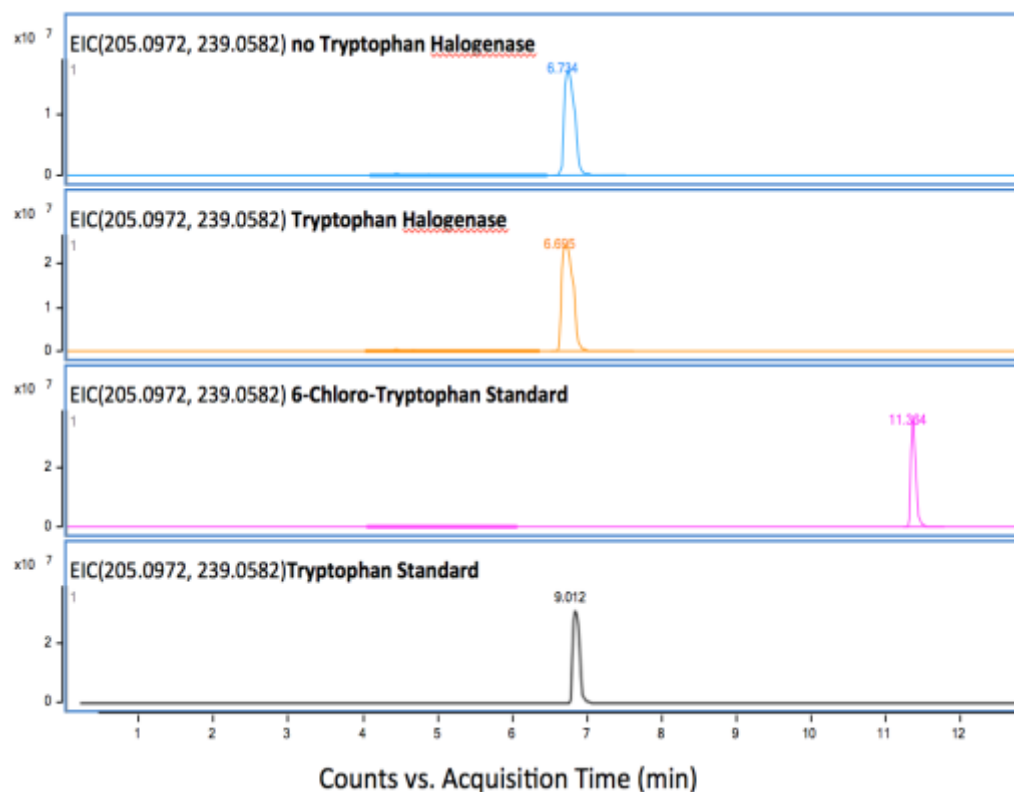


Figure 8: LC-MS analysis of halogenation assay with free tryptophan substrate.

6-Chloro-tryptophan and tryptophan standards were run as standards on the LC-MS. The experimental condition with containing enzyme “Tryptophan Halogenase” showed no difference from the negative control “no Tryptophan Halogenase.” Thus, no halogenation activity was seen upon free tryptophan substrate.

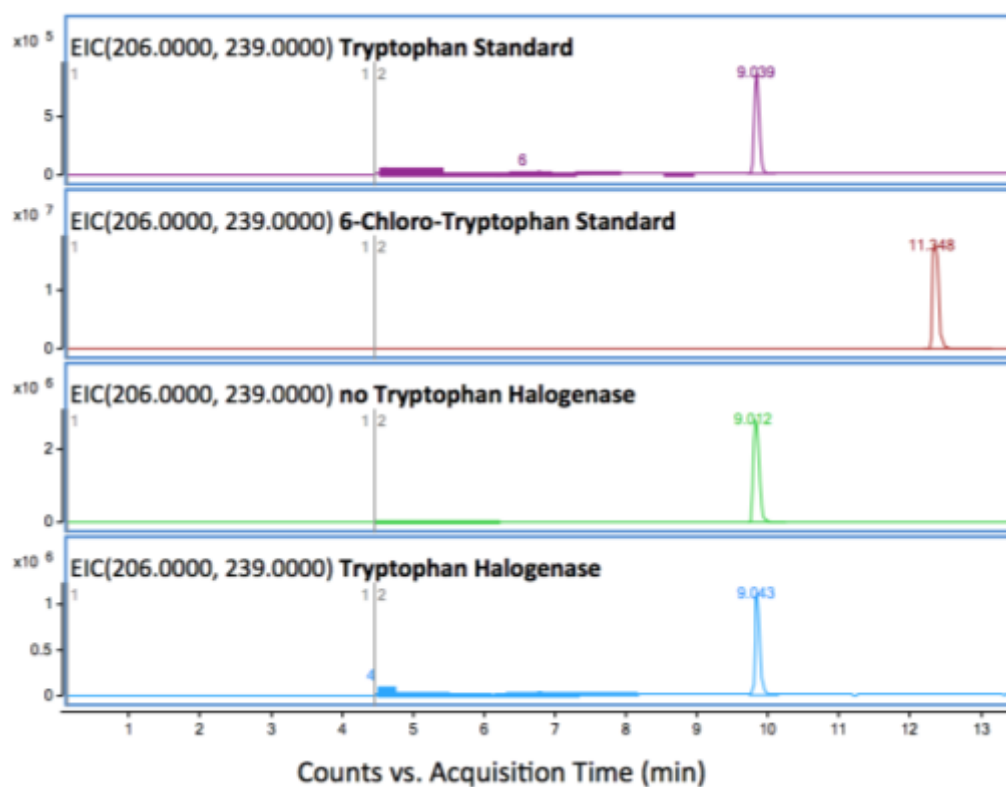


Figure 9: LC-MS analysis of halogenation assay with Amm3 complemented with pCAP01/*amm* Δ *amm3* cell lysate

6-chloro-tryptophan and tryptophan standards were run as standards on the LC-MS. The experimental condition with containing enzyme “Tryptophan Halogenase” showed no difference from the negative control “no Tryptophan Halogenase.” Thus, no halogenation activity was seen upon addition of *S. coelicolor* M512 pCAP01/*amm* Δ *amm3* cell lysate in attempt to complement *E.coli* expressed *amm3* with the *in vivo* reaction conditions the native host, *Streptomyces* sp. CNR-698.

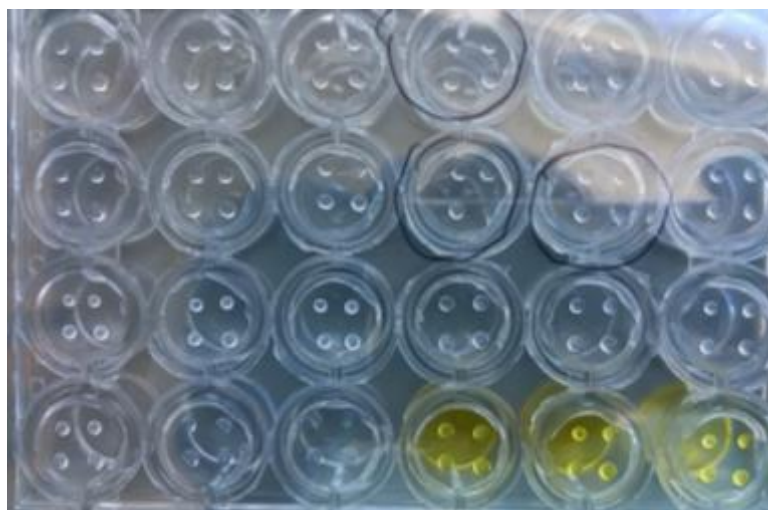


Figure 10: Protein crystallization tray of Amm3.

Initial crystallization conditions for Amm3 were tested using the following crystal screen kits from Hampton Research: Index, Index 2, Crystal Screen, Crystal Screen 2, and Natrrix, in addition to the Wizard Classic 3 screen by Rigaku

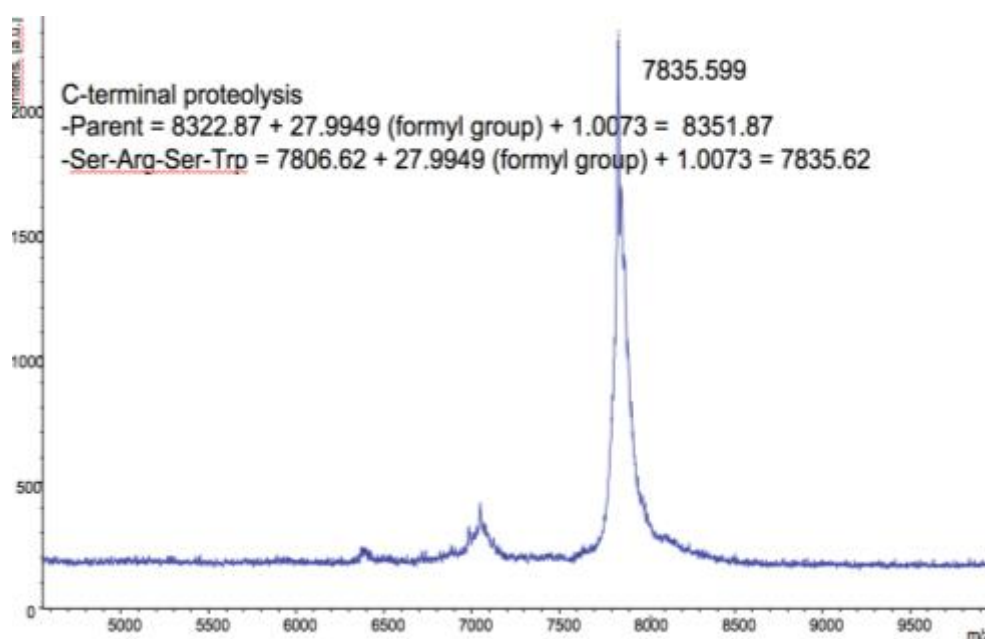


Figure 11: MALDI MS analysis of purified Amm6

Previous attempt to purify Amm6 resulted in a cleaved peptide product in which the last four C-terminal residues (Ser, Arg, Ser, Trp) were cleaved. Chromatogram courtesy of Dr. Peter A. Jordan.

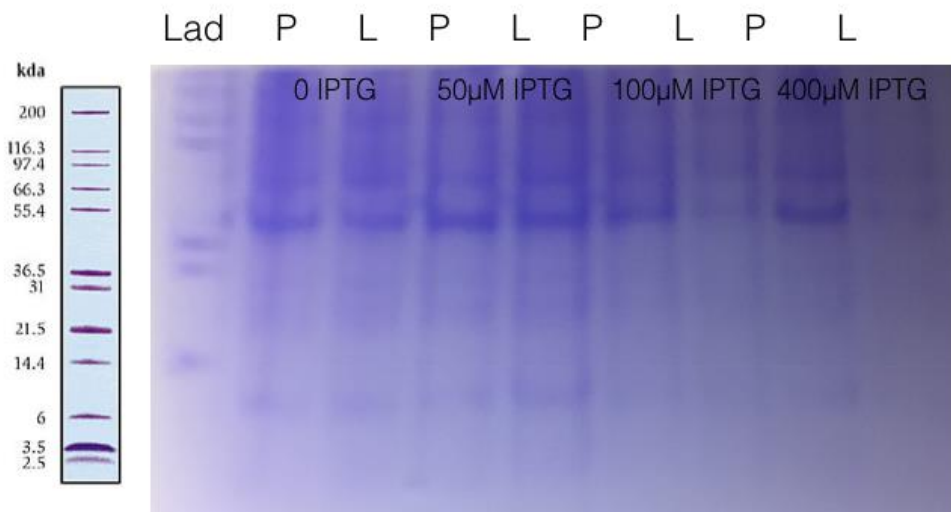


Figure 12: Tris-tricine gel of Ni-purified Amm6 shows unspecific protein production.

From left to right: Mark12 ladder (Lad), 0 μ M IPTG pellet (P), 0 μ M IPTG lysate (L), 50 μ M IPTG pellet (P), 50 μ M IPTG lysate (L), 100 μ M IPTG pellet (P), 100 μ M IPTG lysate (L), 400 μ M IPTG pellet (P), 400 μ M IPTG lysate (L).

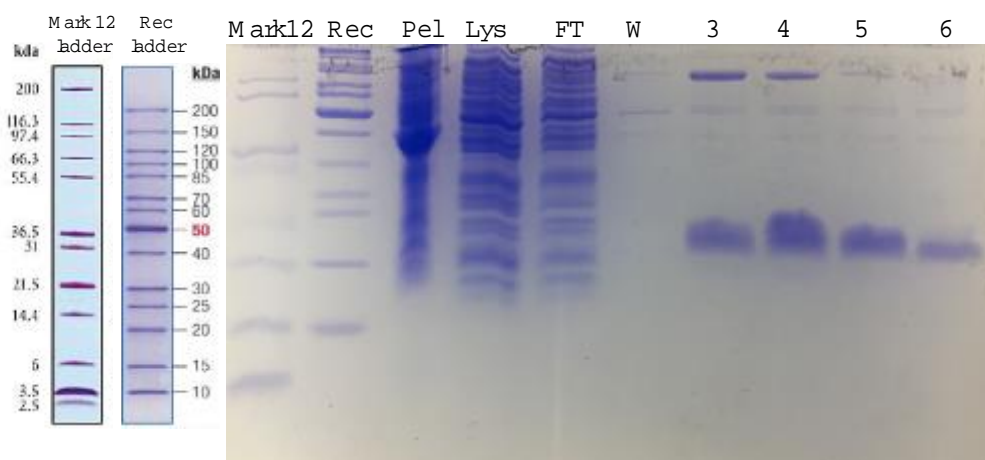


Figure 13: Tris-tricine gel of Ni-purified Amm6 peptide after “burst” short and high O.D. induction.

From left to right: Mark12 ladder, Rec ladder (Rec), pellet (Pel), lysate (Lys), flow through (FT), wash (W), fraction 3, fraction 4, fraction 5, and fraction 6.

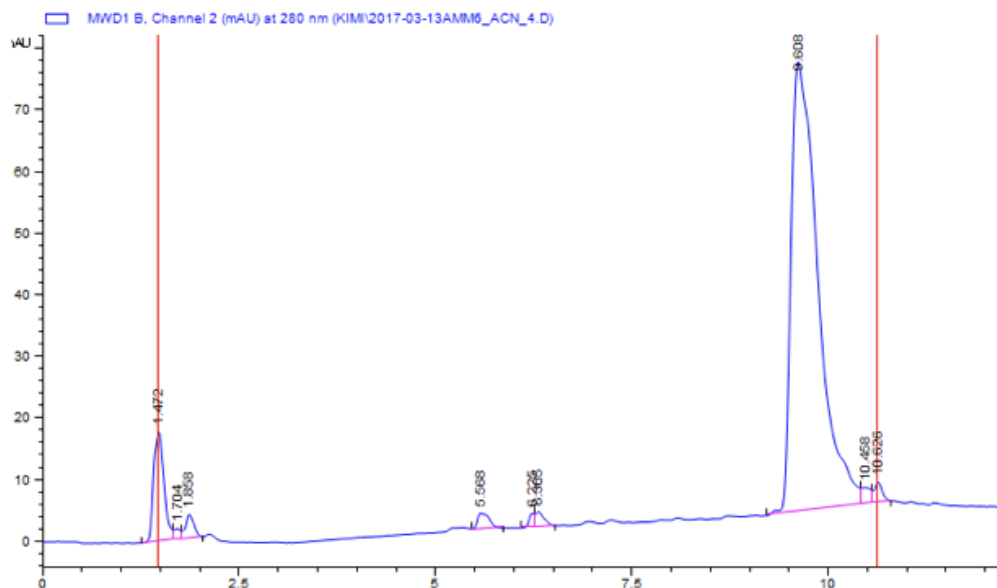


Figure 14: Preparative HPLC chromatogram of Ni-purified Amm6 following “burst” high and quick induction conditions.

The peak eluting at ~9 minutes was collected and lyophilized prior to characterization.

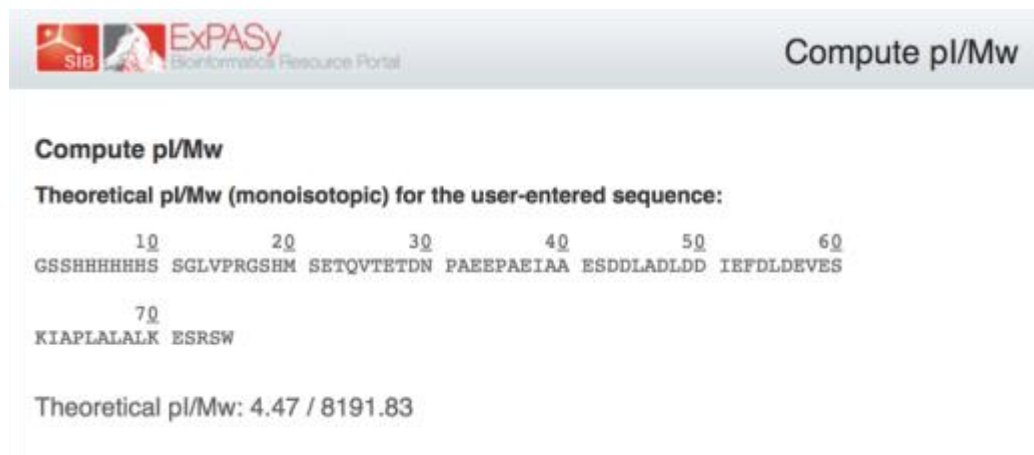


Figure 15: ExPASy theoretical molecular weight of Amm6 peptide

To confirm that purified Amm6 was the correct mass, the theoretical monoisotopic pI/Mw was calculated on ExPASy by inputting the peptide sequence.

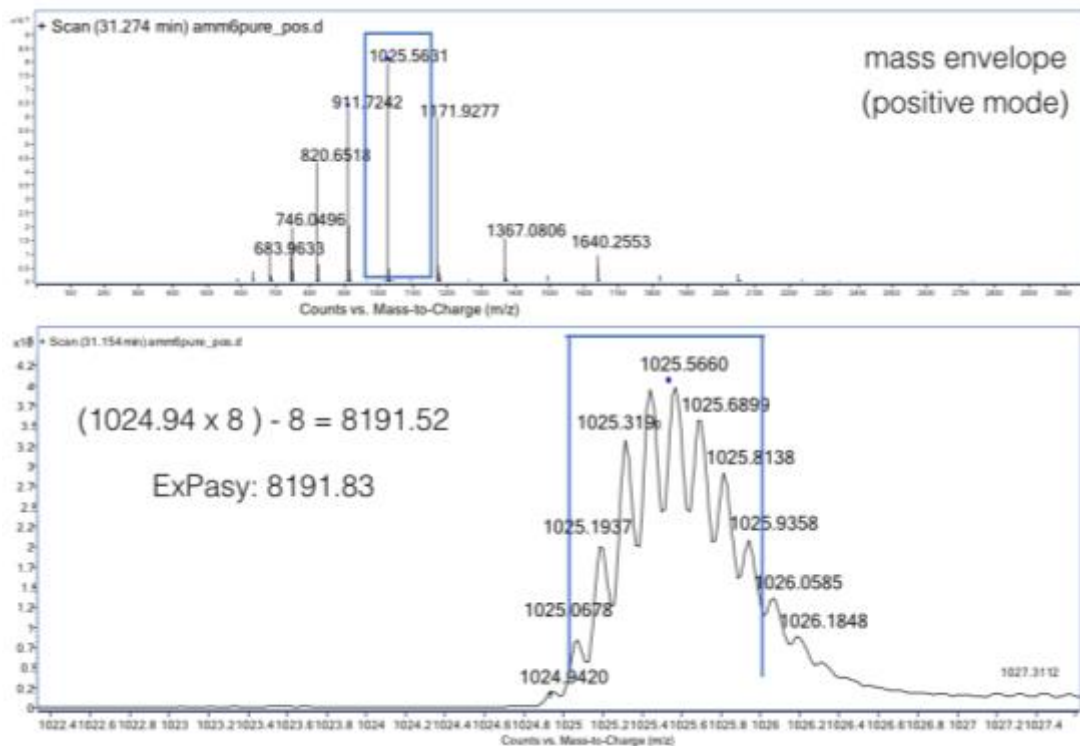


Figure 16: LC-MS purified Amm6 is the correct mass

LC-MS results confirm that purified Amm6 mass (8191.52 Mw) closely matches that predicted by ExPASy software (8191.83 Mw).

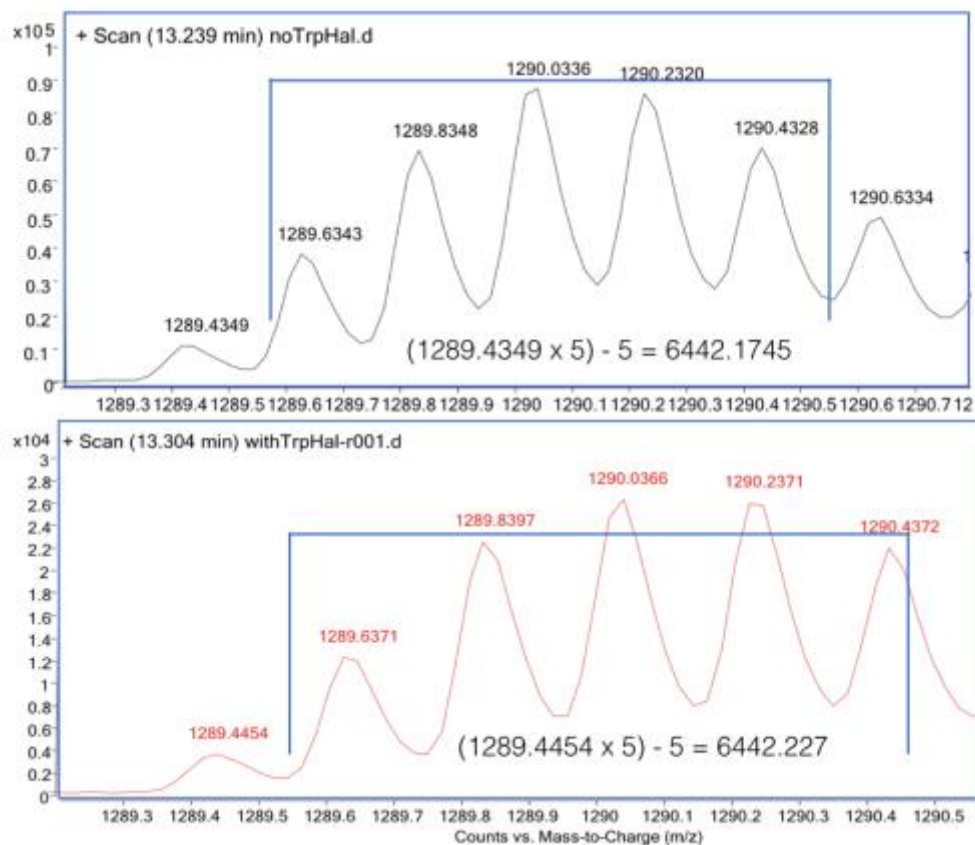


Figure 17: LC-MS analysis indicates that Amm6 cleaves during assay conditions. Halogenation assay results using Amm6 purified peptide as a substrate for Amm3 shows no evidence of halogenation activity, but instead a cleaved peptide product in both conditions that corresponds to a cut at the thrombin cleavage site.

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