The Investigation of Production of Brominated Cladoniamides through Medium Enrichment and Precursor Directed Biosynthesis

by

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Abstract

Cladoniamides are a set of bisindole compounds that contain an indolotryptoline rather than the more common indolocarbazole scaffold. Besides their interesting structures, several of the cladoniamides have been found to be potent cytotoxic agents. We set out to isolate the brominated analogues of known cladoniamides by supplementing the fermentation medium with KBr, which led to the production of 5-bromocladoniamide A. However, the observed production levels were very low. To determine whether the selection against the bromo-substrates is early or late in the cladoniamide biosynthetic pathway, we synthesized 3-chloroarcyriaflavin and 3-bromoarcyriaflavin. These substrates were then fed into *Streptomyces albus + cla (ΔclaC),* which contains the complete cladoniamide biosynthetic pathway, except one crucial gene required for the production of cladoniamides. Through the feeding experiment, we found approximately equal amount of incorporation of the chloro and bromo substrates. The results suggest that the substrate selectivity against bromo precursors is upstream in the pathway from the enzyme encoded by the inactivated gene. Overall, we have observed the production of brominated cladoniamides through the two different methods of modifying the growth conditions and of precursor directed biosynthesis. Furthermore, this work presents a facile way to generate new indolotryptoline molecules through synthetic generation of desired indolocarbazole substrates and then biological conversion using the cladoniamide biosynthetic pathway.

Preface

This dissertation is original, currently in the submission for publication and was completed independently by the author, T. Ding.

The investigation of potassium bromide supplementation was initiated by Chen Chang, who observed production of a brominated cladoniamide through HPLC and mass spectral analysis.

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Abbreviations	Definition
AcOH	Acetic acid
AgOAc	Silver Acetate
$AlBr_3$	Aluminum Bromide
$AlCl_3$	Aluminum Chloride
ATP	Adenosine Triphosphate
$CHCl_3$	Chloroform
CH_3OH	Methanol
COSY	Correlation spectroscopy
CPA	Chromopyrrolic acid
$Cu(OTf)_2$	Copper(II) trifluoromethanesulfonate
DCM	Dichloromethane
DMF	Dimethylformamide
EGFR	Epidermal Growth Factor Receptor
ESI	Electrospray Ionization
EtOH	Ethanol
EtOAc	Ethyl Acetate
$FADH_2$	Reduced flavin adenine dinucleotide
GTP	Guanosine triphosphate
HCl	Hydrochloric Acid
H_2NNH_2	Hydrazine
H_2O	Water
H_2O_2	Hydrogen Peroxide
HOCl	Hydrochlorous Acid
HPLC	High Performance Liquid Chromatography
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
h	Hour
I_2	Iodine
IC_{50}	Half of maximum inhibitory concentration
IPA	Indole pyruvic acid
ISP4	International Streptomyces Project medium # 4
KB cell	Mouth Epidermal Carcinoma Cell
KBr	Potassium Bromide
KCN	Potassium Cyanide
KI	Potassium Iodide
KOH	Potassium Hydroxide
KOtBu	Potassium *tert*-butyloxide
K_3PO_4	Potassium Phosphate
LB	Luria Broth
MAPs	Microtubule-associated Proteins
MeI	Methyl iodide

MeOH	Methanol
Me_2SO_4	Dimethylsulfate
MHz	Megahertz
$MgCl_2$	Magnesium Chloride
MS	Mass Spectrometry/Spectrometer
MS agar	Mannitol/ Soya flour agar
$NaBH_4$	Sodium Borohydride
NaCl	Sodium Chloride
Na_2CO_3	Sodium Carbonate
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
$NaHSO_3$	Sodium Bisulfate
NaOH	Sodium Hydroxide
$NaSO_4$	Sodium Sulfate
NH_2CHO	Formamide
NMR	Nuclear Magnetic Resonance
O_2	Oxygen
O.D.	Optical Density
ORF	Open Reading Frame
OsO_4	Osmium Tetroxide
Pd	Palladium
$Pd_2(dba)_3$	Tris(dibenzylideneacetone)dipalladium
$PdCl_2$	Palladium chloride
$Pd(OAc)_2$	Palladium acetate
pH	Negative log concentration of hydrogen ions
$PhNHNH_2$	Phenylhydrazine
PKC	Protein Kinase C
PPh3	Triphenylphosphine
RT	Room temperature
SCLC	Small Cell Lung Cancer
SEM	[2-(Trimethylsilyl)ethoxy]methyl acetal
$SnCl_2$	Tin Chloride
tBuOH	*tert*-Butyl alcohol
TBAF	Tetrabutylammonium fluoride
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran
TIA	Terpenoid Indole Alkaloids
TLC	Thin Layer Chromatography
TOF	Time of Flight
UV	Ultraviolet

Acknowledgements

Most people would agree on that research is not an easy field. Countless scientists have invested their lives in the goal of advancing the scientific knowledge. I would like to acknowledge the individuals who have assisted me through this intellectually intriguing and challenging journey. There is my principle investigator, Dr. Katherine Ryan. I am grateful for her patience and knowledge. Without her, I would have been completely lost. Furthermore, I would like to thank postdoctoral fellow Dr. Yiling Du. He taught me much about the basic molecular biology techniques and provided me with many tools need for me to complete my project. Lastly, I would like to thank my other fellow colleagues. There were too many and too numerous ways they have assisted me along my way. Once again, thank you everyone. I am grateful.

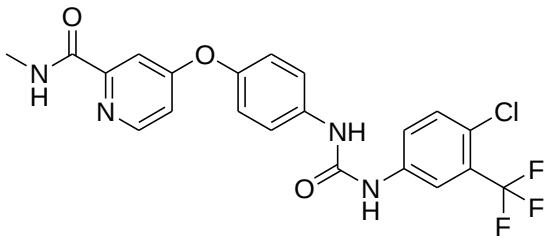
Chapter One: Introduction

1.1 Natural Products

Natural products are molecules obtained from biological sources such as plants, fungi and bacteria. These molecules have historically played important roles in medicine. For example, *Astragalus propinquus* seeds have been applied as traditional Chinese medicines for the past 2000 years as a way to improve the general immune system of ailing individuals.[1] Recent studies into the active compounds have shown that extracts from *A. propinquus,* can activate cellular telomerases within human keratinocytes, fibroblasts, and immune cells.[2]

Natural products can be categorized into two major groups. The first group consists of primary metabolites. Despite the wide diversity of life, the primary metabolites of all living organisms are quite similar. The list includes molecules such as carbohydrates, nucleotides and fatty acids.[3] It is believed that the observed productions are an integral part of the growing process.[3] Fermentation of microorganisms is one of the more economical methods to isolate large quantities of these primary metabolites. One application of these metabolites is in enhancing the taste and nutritional values in food through addition of organic acids and vitamins. Another major field of interest is using renewable primary metabolites as a suitable replacement for the dependence on petroleum.[3]

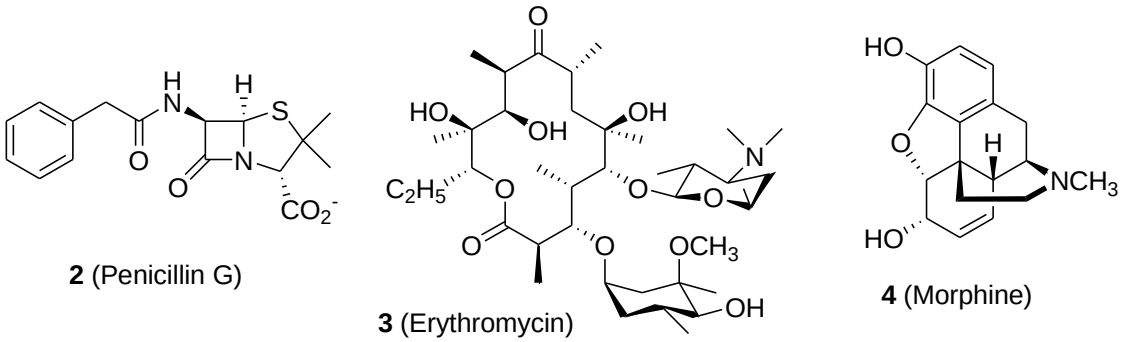
The second group of natural products is made up of secondary metabolites. These molecules are generally molecules produced by a limited group of organisms,[4] and are not absolutely required for survival of the organism. These secondary metabolites are one of the largest sources of valuable pharmaceuticals. For instance, between 1981 and 2010, over 48.6 percent of all new pharmaceutically relevant compounds were either directly from natural sources or were synthetic mimics of a natural product.[5] Comparatively, combinatorial chemistry, another major source of novel compounds, only produced one *de novo* molecule approved for drug use during the same period, the antitumor agent sorafenib (**1**).[5]



1 (sorafenib)

Figure 1 Chemical structure of sorafenib

One particularly important natural product is the β-lactam antibiotic penicillin G, from the fungi *Penicillium notatum*, isolated by Dr. Alexander Fleming.[6] In 1928, Dr. Fleming observed the absence of *Staphylococcus aureus*, a common laboratory contaminant, surrounding a particular mold colony.[6] Deducing that something produced by the mould must be inhibiting the growth of the bacteria, he was able to isolate Penicillin G (**2**).[6] Even 80 years after its initial discovery, penicillin and modified derivatives such as amoxicillin are still used clinically to combat bacterial infections.



2 (Penicillin G)

3 (Erythromycin)

4 (Morphine)

Figure 2 Well-known natural products including penicillin G, erythromycin and morphine

Another prominent example is the isolation of erythromycin (**3**) from the bacteria *Saccharopolyspora erythraea.*[7] The chemical structure of the compound consists of a 14-membered macrolide that is glycosylated at two positions with L-cladinose and D-desosamine. Erythromycin is a broad spectrum antibiotic and was originally administered for individuals who were allergic to penicillin.[7] The initial discovery of its antibiotic activity was by the physician scientist, Abelardo Aguilar. Isolation and full characterization of erythromycin was accomplished by a research group at Eli Lilly, headed by J. M. McGuire in 1949.[7]

Natural opioids are also well known, especially morphine (**4**) which is commonly applied to relieve intense pain. It was initially isolated from seedpods of *Papaver*

somniferum (opium poppy) by Friedrich Sertürner in 1804.[8] The mechanisms of action is believed to involve direct interactions with the central nervous system.[8] Morphine was then commercialized by Merck in 1827.[8] The drug is now on the World Health Organization's List of Essential Medicines required for a healthcare system due to its potency and applicability.

The aforementioned discoveries are only a tiny sample of all the medicinally active compounds that have been isolated from microorganisms. Antimalarial, antiviral and even antihypertensive agents have all been discovered from natural sources.[1,3,4,5,9] Another major field in which secondary natural metabolites are contributing is in chemotherapy and treatment of cancer.

1.2 Chemotherapeutic Applications

One of the most well-known chemotherapeutic drugs is paclitaxel, better known by its commercial name Taxol® (**5)**. Paclitaxel is a secondary metabolite initially isolated from the bark of *Taxus brevifolia* by Monroe Wall and Mansukh Wani.[10] It has been found that paclitaxel can combat cancer growth by preventing mitotic division.[11] Cancer can often be characterized by the uncontrollable rapid replication of malignant cells. For the cells to replicate, they must undergo mitotic replication. In normal mitotic division, there is an equilibrium between the condensed microtubules and free floating tubulin dimers.[12] This equilibrium is naturally regulated to allow for separation of replicated chromosomes. The role that paclitaxel plays in the process is as a microtubule stabilizer.[11] Depending on the stage of the mitotic replication, paclitaxel promotes the condensation of tubulin dimers into microtubules.[10] In turn, cancerous cells cannot replicate and divide, thus growth is controlled. Various studies have been conducted to confirm the inhibitory effects. For instance it was found that even with concentrations as low as 0.05 μmol/L, paclitaxel can promote microtubules formation *in vitro* despite the absence of guanosine-5'-triphosphate (GTP) and microtubule-associated proteins (MAPs).[12]

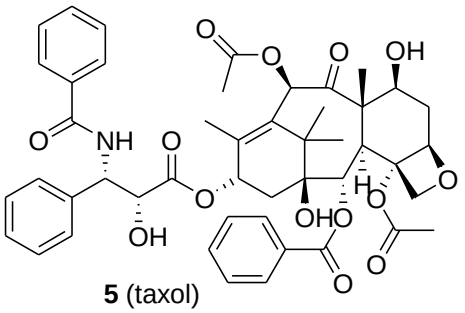


Figure 3 Chemical structure of Taxol

Beside paclitaxel, there are several other promising naturally derived molecules that have gone through clinical trials as potential anticancer drugs. One is staurosporine (**6**) and its derivative 7-hydroxy-staurosporine. Staurosporine was originally isolated by Omura and colleagues in 1977 from the strain *Streptomyces staurosporeus*.[13] Extracts of the culture initially displayed antimicrobial activity against various tested fungi and yeast.[13] It was later found that staurosporine also possessed potent inhibitory effects against protein kinases, in particular protein kinase C (PKC).[14] PKC is a common regulatory protein that can activate other proteins through the process of phosphorylation.[15] In the case of cancer progression, prolonged activation of PKC is believed to increase the risk of spontaneous mutations.[16] The strong affinity of staurosporine towards the PKC complex is believed to inhibit ATP binding and halts the phosphorylation process.[17] It has also been found that 7-hydroxy-staurosporine (UCN-01), a staurosporine analog isolated from *Streptomyces sp*. N-126 has an IC_{50} value of 4.1 nM when tested against PKC.[18] Due to the observed potent cytotoxic effects, UCN-01 has been put through Phase I and Phase II clinical trials against diseases such as melanoma.[19]

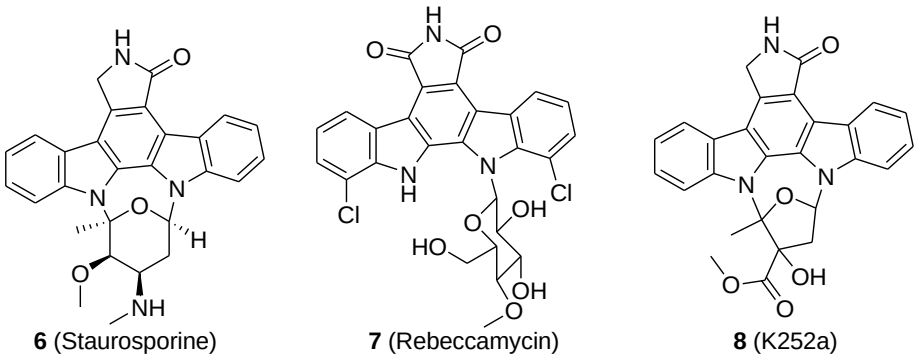


Figure 4 Potential anticancer agents including Staurosporine, Rebeccamycin and K252a.

Another potential anticancer agent is rebeccamycin (**7**) produced by *Lechevalieria aerocolonigenes*, which was isolated from a soil sample collected from Panama.[20] Initial interest into the bacteria culture was due to observed inhibition effects of the organic extracts from the producing strain against KB cell (Mouth Epidermal Carcinoma Cells) culture growth.[20] Through further culturing and isolation, a compound somewhat similar to the aforementioned staurosporine was discovered. The structure of rebeccamycin can be seen in **Figure 4**. It consists of an indolocarbazole backbone with one *N*-glycosylation. Similar indolocarbazole containing compounds had been isolated previously, such as the topoisomerase I inhibitor K252a (**8**).[21] Therefore, rebeccamycin was believed to be a potential anticancer candidate. Topoisomerases are DNA altering enzymes that break and reform the phosphodiester backbone of nucleotide strands.[21] Inhibitors of topoisomerases can impede this process, especially the re-ligation, which results in damaged sections of DNA.[21] The damage sustained can promote cellular apoptosis and leads to control of cancerous growth.[21] In the case of rebeccamycin, the original compound was only found to be weakly inhibitory toward topoisomerase I.[21] However an analogue of rebeccamycin (becatecarin) entered Phase II clinical trial against relapsed small cell lung cancer (SCLC) in 2012.[22]

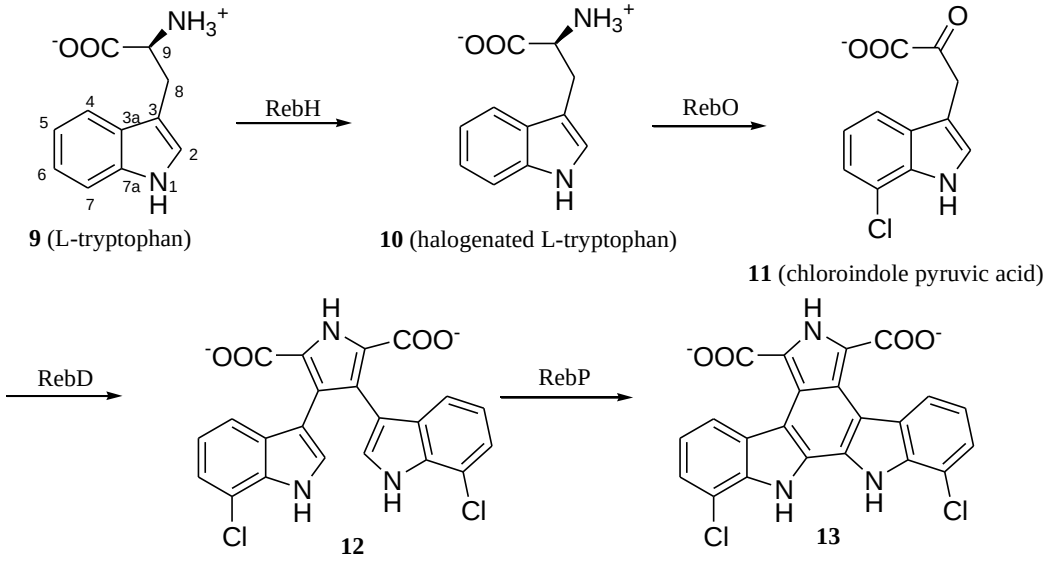
1.2.1 Biosynthesis of Indolocarbazole Rebeccamycin

Due to the interest into the above indolocarbazoles, much has been learned about the biological synthesis of these bisindole alkaloids. One of the first studies involved probing the natural substrates for associated enzymes. In 1988, researchers selectively fed ^{13}C labeled substrates in to the rebeccamycin producer, *Lechevalieria aerocolonigenes* ATCC39243.[23] Of the added substrates, D-[1-^{13}C]glucose and L-[Methyl-^{13}C]methionine both led to increased signal detected during ^{13}C NMR analysis.[23] In the case where D/L-[2-^{13}C]tryptophan were separately supplemented, only the L-[2-^{13}C]tryptophan feeding led to an increase in ^{13}C detected.[23] These results support that indolocarbazoles such as rebeccamycin are indeed derived from L-tryptophans. However, very little was known about the mechanism of indolocarbazole assembly.

One important finding was the discovery of gene *ngt* from *L. aerocolonigenes* ATCC39243, which encodes for an indolocarbazole *N*-glycosyltransferase.[24] It was initially observed that the feeding of an indolocarbazole into the *L. aerocolonigenes* system can lead to the isolation of the N-glycosylated product.[24] The function of *ngt* was then confirmed by cloning and expression.[24] Further along, based on the observation that bacterial genes commonly occur in discrete clusters, it was correctly reasoned that the indolocarbazole genes must be located in proximity to *ngt.*[25] Therefore, the Salas group set out to construct a genomic library of ATCC39243 in *Escherichia coli (E. coli)-Streptomyces* shuttle vector pKC505 and the library was screened for cosmids containing the *ngt* gene.[25] To confirm the function of the genes, the positive cosmids were heterologously expressed in *Streptomyces albus* J1074 and production of rebeccamycin was observed.[25] The sequenced cluster was 25,681 base pairs (bps) long, spanning 18 open reading frames (ORFs).[25] Annotation of the gene cluster suggested that *rebO, D, C, P* genes coded for enzymes that were similar to reported proteins which could participate in the condensation of two tryptophan units into an indolocarbazole.[25] Further insights on the function of these genes were gained by construction of plasmids containing particular inserts from this cluster and then analyzing the product of corresponding heterologous expressions. It was found that the insert containing r*ebO*, *rebD*, *rebC*, *rebP*, and *rebM* led to the production of deschloro-rebeccamycin aglycone (also known as arcyriaflavin A).[25] The insert with *rebG*, *rebO*, *rebD*, *rebC*, *rebP*, *rebM*, *rebR*, *rebF*, and a 3′-truncated *rebU* led to the production of deschloro-rebeccamycin.[25] The results confirmed that the genes, *rebO, D, C,* and *P* may have been crucial for construction of the indolocarbazole backbone. The annotation also allowed for assignment of the modification genes such as *rebM* believed to responsible for methylation of the 4-hydroxyl position of the indolocarbazole and the *rebH* gene, believed to be responsible for chlorination.[25]

Further insight into the indolocarbazole assembly was provided by the Onaka *et al,* where they constructed knock-out mutants of *L. aerocolonigenes* ATCC39243 to analyze the corresponding production of metabolites.[26] The first step is believed to be a halogenation of **9** to generate **10,** catalyzed by the $FADH_2$-dependent tryptophan halogenase RebH. Due to the sequence similarity between RebH and pyrrolnitrin tryptophan halogenase found in *Pseudomonas chlororaphis*, it was reasoned that

rebeccamycin halogenation also occurs at the beginning of the biosynthetic pathway.[26] Confirmation of the function of RebH as a halogenase came from genetics studies that showed that inactivation of the *rebH* gene gave production of deschloro derivative of rebeccamycin.[26] Since the initial discovery, many similar tryptophan halogenases



9 (L-tryptophan) **10** (halogenated L-tryptophan) **11** (chloroindole pyruvic acid) **12** **13**

Figure 5 Proposed functions of RebH, O, D, and P enzymes

have been reported. The site of halogenation depends on the specific halogenase. For example, the ClaH from cladoniamide biosynthesis appear to be selective for C-5 only while RebH targets the C-7 position.[26, 27, 28]

The next step along the biosynthetic cascade is believed to involve RebO, an amino acid oxidase which reacts with chlorotryptophan to give rise to an indole pyruvate (**11**)**.**[26] The initial assignment of its function was based on sequence similarity to known amino oxidases such as StaO from staurosporine biosynthesis.[26] Further confirmation came from the Sherman group where they heterologously expressed the *rebO* gene, purified RebO, and then feed in various tryptophan substrates to isolate out the corresponding indole pyruvic acids.[29] The oxidative process catalyzed by RebO is required to prepare the substrate and allow it to undergo a RebD-catalyzed coupling reaction to give chromopyrrolic acid (CPA, **12**). Inactivation of *rebD* led to abolishment of downstream products.[26] Further insight into the specific substrate requirement for this reaction was obtained by feeding of indole pyruvic acid (IPA) into an *in vitro* system containing StaD, a homolog of RebD.[30] Addition of ammonium ions equilibrates the

substrate between the IPA and imine forms. The results suggest that it is the IPA enamine which is utilized for StaD-catalyzed coupling to ultimately give CPA.[30] The formed CPA is then cyclized by the cytochrome P450 enzyme RebP with the formation of a new C-C bond at the C-2 positions of each tryptophan-indole to give compound **13**.[26] It was observed that inactivation of the *rebP* gene led to the accumulation of CPA.[26]

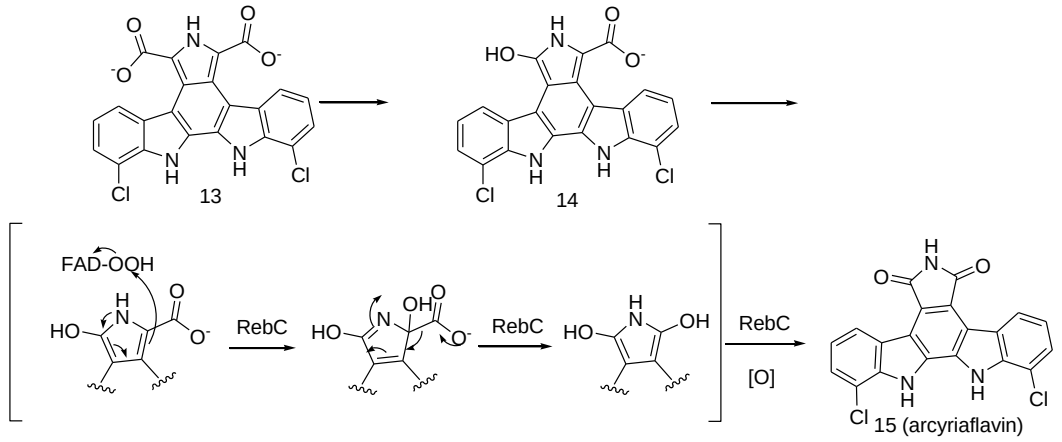


Figure 6 Proposed function of RebC[31,34]

RebC is then believed to be involved in the conversion the cyclized CPA to arcyriaflavin.[26] The exact mechanisms by which RebC accomplishes this reaction is not yet fully understood. It was proposed that one of the two carboxyl groups can readily undergo decarboxylation with the release of CO_2 to generate **14** (**Figure 6**). [31] FAD is then believed to be involved in the addition of a second hydroxyl group which was supported by the reported crystal structure of flavin-bound RebC.[32] Furthermore, the homolog StaC, which does not normally utilize FAD can be reconstituted with the cofactor to increase the activity of the enzyme.[31] Following the FAD assisted hydroxylation, a second decarboxylation is believed to occur before the intermediate is oxidized once more and rearranges to generate an arcyriaflavin derivative (**15**).[33] Interestingly, the product produced by StaC/RebC can be altered by selective mutations.[33] RebC can behave as StaC and produce K252c (staurosporine aglycone) if the active site phenylalanine at 216 is mutated to a valine, while the arginine at 239 is changed to an asparagine.[33] The final product formed through the indolocarbazole genes is an arcyriaflavin derivative, which can be processed into rebeccamycin by further glycosylation with RebG and methylation by RebM.[26]

Further studies into the rebeccamycin cluster include more work performed by the Salas group, in which they co-expressed different combinations of rebeccamycin biosynthetic genes, including staurosporine biosynthetic gene *staC* in order to obtain novel molecules.[35] Using this method, they were able to generate over 30 derivatives.[35] Furthermore, in a later study, they also introduced alternative, independently transformed, glycosylating genes to generate a wide array of glycosylated products.[36] In particular, an L-olivose derivative was found to be a picomolar inhibitor of the human kinase Ikkb.[36]

In recent years, there have been more novel and potentially chemotherapeutic compounds discovered. One particular class of interest are the cladoniamides.

1.3 Cladoniamides

1.3.1 Isolation of Cladoniamides

Cladoniamides (**Figure 7**, **16-23**) are a set of tryptophan-derived bisindole compounds that were initially isolated in 2008 by collaboration between the Andersen group and the Davies group at the University of British Columbia.[28] The producer of these compounds, *Streptomyces uncialis* was isolated from the surface of the lichen *Cladonia uncialis.*[28] The lichen itself was collected in British Columbia at a site near Pitt River.[28] Beside the isolated cladoniamides, this strain of *S. uncialis* was found to also be the producer of a potent antibiotic known as uncialamycin (**23**).[28] For sufficient isolation, *S. uncialis* was grown on solid agar medium of ISP4 (International Streptomyces Project medium # 4, soluble starch 10 g/L, dipotassium phosphate 1g/L, magnesium sulfate 1 g/L, sodium chloride 1 g/L, ammonium sulfate 2 g/L, calcium carbonate 2 g/L, ferrous sulfate 1 g/L, manganous chloride 1 g/L, zinc sulfate 1 g/L, agar 20 g/L) at 30°C.[28] Following cultivation, the cultures were then extracted with ethyl-acetate and purified via open column reverse-phase chromatography, Sephadex LH20, and reverse-phase HPLC to ultimately afford seven related molecules, named the cladoniamides, after the lichen *Cladonia uncialis.*[28]

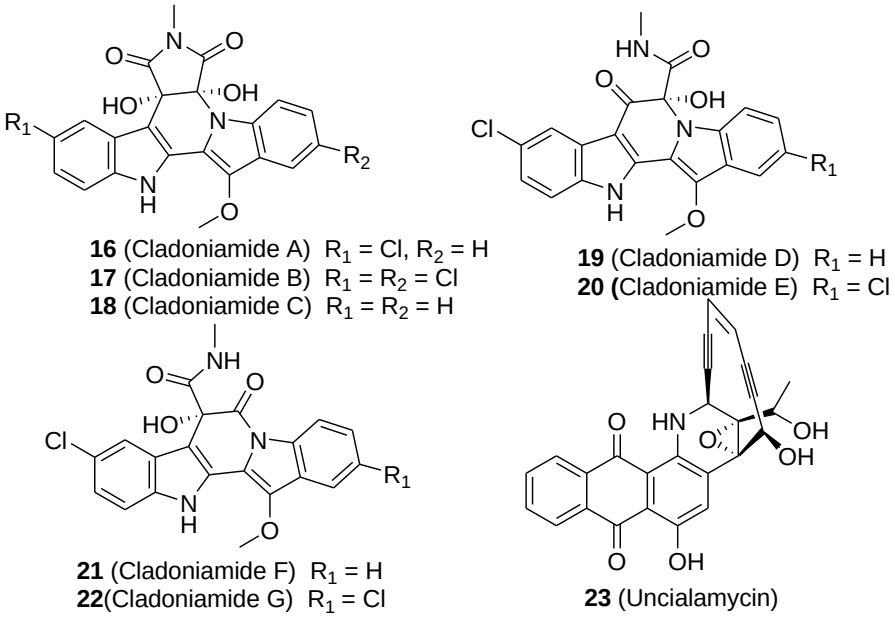


Figure 7 Interesting natural products isolated from *Streptomyces uncialis,* including cladoniamides and uncialamycin.

There are several interesting aspects to the cladoniamides, including their chemical structures, which are related to both staurosporine and rebeccamycin. The previously discussed bisindoles staurosporine (**6**) and rebeccamycin (**7**), both contain an indolocarbazole scaffold (**Figure 8, 24**) in which the two tryptophan-derived indoles are arranged in a mirrored fashion across the vertical midline. By contrast, while cladoniamides appear to also derive from condensation of two units of tryptophans, the symmetry is actually disrupted and one of the tryptophans is "flipped." This novel arrangement is now known as an indolotryptoline scaffold (**25**).[19]

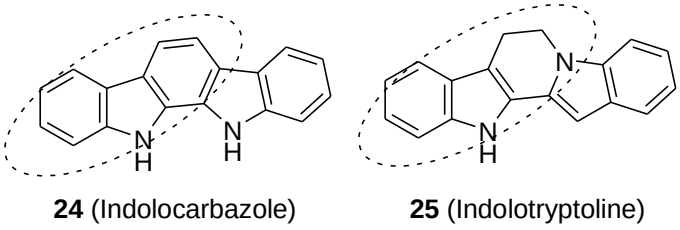


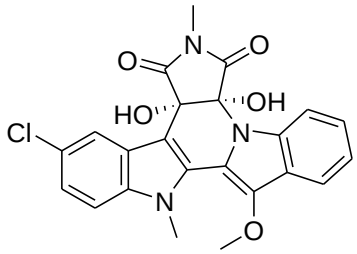
Figure 8 Comparison between indolocarbazole and indolotryptoline scaffolds

1.3.2 Biological Activities of Cladoniamides

The other appealing aspect of the cladoniamides is their biological activities, since several of these compounds have been found to be potent anticancer agents. Cladoniamide G has moderate activity against *in vitro* human breast cancer MCF-7 cells.[28] By contrast, cladoniamide A has been found to display potent activity, with an IC_{50} of 8.8 ng/mL against HCT-116 cells.[27] Cladoniamide B had a similar IC_{50} at 10 ng/mL.[37] The methylated analog of cladoniamide A, BE-54017 (**Figure 9**, **26**), is less active, with an IC_{50} of 79 ng/mL against HCT-116.[27] These anticancer activities may be unsurprising considering the structural similarities between the cladoniamides and the aforementioned rebeccamycin/staurosporine (IC_{50} values against HCT-116: rebeccamycin at 410 ng/mL, and staurosporine at 40 ng/mL).[19,38] One possible mechanism by which cladoniamides can act as an inhibitor is proposed to involve the vacuolar H^+-ATPase, a conserved eukaryotic protein complex responsible for acidifying cellular compartments such as organelles.[39] It is believed that acidification around cancerous tissue may contribute to resistance to chemotherapeutic drugs and tumor progression.[39] Therefore, interference of the process may offer control over the progression of the tumor. A strain of *Schizosaccharomyces pombe*, which shares many similar cellular processes with human cancer cell lines, were grown with cladoniamide A or BE-54017 and resistant mutants were isolated.[39] Whole genome screening was then employed on the resistant mutants, which revealed mutations in the c/c' subunits of the ATPase complex, suggesting that may be the site of action for this group of indolotryptoline compounds.[39]

1.3.3 Biosynthesis of Indolotryptoline Cladoniamide

Biosynthesis of the cladoniamides was originally hypothesized by Andersen and colleagues to start with the synthesis of an indolocarbazole scaffold. This was the predominant view because the observed indolotryptoline scaffold has never been previously reported. And, based on structural analysis, the most similar known class of compounds are the indolocarbazoles.



26 (BE-54017)

M2 Methyltransferase Type 11
X1 FAD binding monooxygenase
M1 Methyltransferase Type 12
D Chromopyrrolic Acid synthase
X2 FAD binding monooxygenase
M3 O-methyltransferase
H Tryptophan 6-halogenase
T Cation/H+ antiporter
F Flavin reductase
Y Alpha/beta hydrolase
O L-amino acid oxidase
P Cytochrome P450
R Transcription regulator

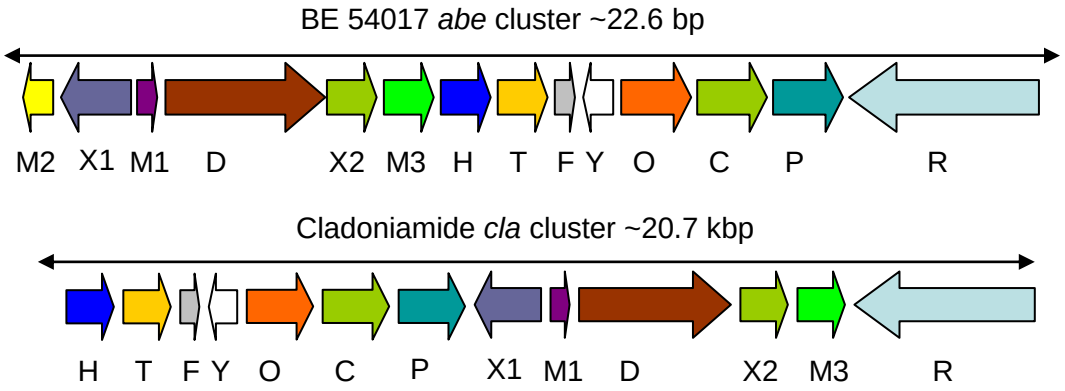


Figure 9 BE-54017 and cladoniamide biosynthetic gene cluster with corresponding annotations

It was hypothesized that the novel indolotryptoline scaffold found in cladoniamide may be derived from the much more prevalent indolocarbazole structure found in rebeccamycin and staurosporine.[28] At some point in the pathway, one of the tryptophan-indole must be rotated to generate the new structure.[28] Further insight in to these initial proposals came from the Brady group, in the search for interesting biosynthetic pathways from environmental DNA.[27] The target of their search was oxy-tryptophan dimerizations genes such as *rebD* and *staD*.[27] Following isolation of eDNA and construction of cosmid libraries, they screened for *staD* homologues using degenerate primers. Each positive cosmid was then sequenced and annotated.[27] From their screening, cosmid AB1650 was found to carry a complete set of conserved indolocarbazole synthetic genes, termed *abeO, D, C,* and *P*.[27] However, unlike the rebeccamycin cluster there were also three methyltransferase genes (*abeM1, M2, M3)* and two monooxygenase genes (*abeX1, X2)*.[27] Since it was unprecedented to have additional monooxygenase genes within indolocarbazole biosynthetic pathways, it was reasoned that this may lead to production of novel compounds.[27] Thus, the AB1650 cosmid was heterologously expressed in *Streptomyces albus,* leading to the isolation of BE-54017 (**26**), a methylated analog of cladoniamide A.[27]

To further confirm the action of each gene, the Brady group then analyzed a series of transposon mutants.[27] It was found that transposon insertion at the *abeO* or *abeD* loci abolished the production of BE-54017, while the expected intermediates of chlorinated tryptophan and chloroindole pyruvic acid were observed.[27] Production of the chromopyrrolic acids was observed if *abeC* and *abeP* were inactivated.[27] The two monooxygenases, *abeX1 and X2* were believed to be crucial for the conversion of the formed indolocarbazole into the indolotropoline.[27] Transposon insertion in either of the genes halted the production of the indolotryptoline final product.[27] The proposed functions of the monooxygenases and methyltransferases will be discussed more in detail in the following section.

The *cla* gene cluster, responsible for cladoniamide biosynthesis, was identified by Ryan and was found to be similar to the BE-54017 gene cluster (*abe*).[40] This is unsurprising due to the close chemical structure of the produced compounds. The complete *cla* cluster is ~20.7 kbps and consists of 13 open reading frames (ORFs).[40] When comparing the homologues between the two clusters, the similarity of the amino acid sequences ranges from 46 to 74%.[40] However, there appears to be spatial rearrangements which might have been caused by transposition of a section of the whole gene cluster somewhere along the evolutionary process.[40] Similar observations have been made by the Koonin group where they compared 25 complete bacterial/archeal genomes and found that rearrangement of gene ordering is a very common event in the biological evolution of bacteria and archaea.[41] In comparison, the conservation of protein sequences is much more consistent.[41]

It is believed that ClaX1/AbeX1 and ClaX2/AbeX2 are crucial for the proposed tryptophan inversion and indolotryptoline forming process. Inactivation of *abeX1* through transposon mutagenesis resulted in the accumulation of chloroarcyriaflavin without further modification (**27**).[19] Inactivating *abeX2* led to the observation of a dihydroxylated derivative of chloroarcyriaflavin (**30**).[19] Based on annotation, both sets of enzymes are believed to be flavin-dependent oxyganeses.[19] Furthermore, they were found to be similar to Class A flavin-dependent monooxygenases, which are capable of epoxidation catalysis.[19,41] Therefore, all the experimental results are in an agreement with the initial mechanism proposed by the Andersen group. First, ClaX1/AbeX1 attaches a single

oxygen across the central double bond to generate an epoxide (**29**).[19] Then the strained epoxide ring is opened to form two hydroxyl groups (**30**).[19] It is still unclear whether the hydrolysis process is enzyme catalyzed or chemically spontaneous. The next step is the methylation of the succinimide nitrogen by ClaM1/AbeM1 to give **31.**[19] This was confirmed by inactivation of the *abeM1* gene, which resulted in the production of BE-54017 lacking the N-methylation.[19] Further experimental evidence for ClaM1 function will be discussed in the following section. Following the formation of the dihydroxylated intermediate, ClaX2/AbeX2 is believed to insert another oxygen across the C2 to C3 double bond located on the dechlorinated indole ring (**32**),[19] resulting in a highly activated region.[24]

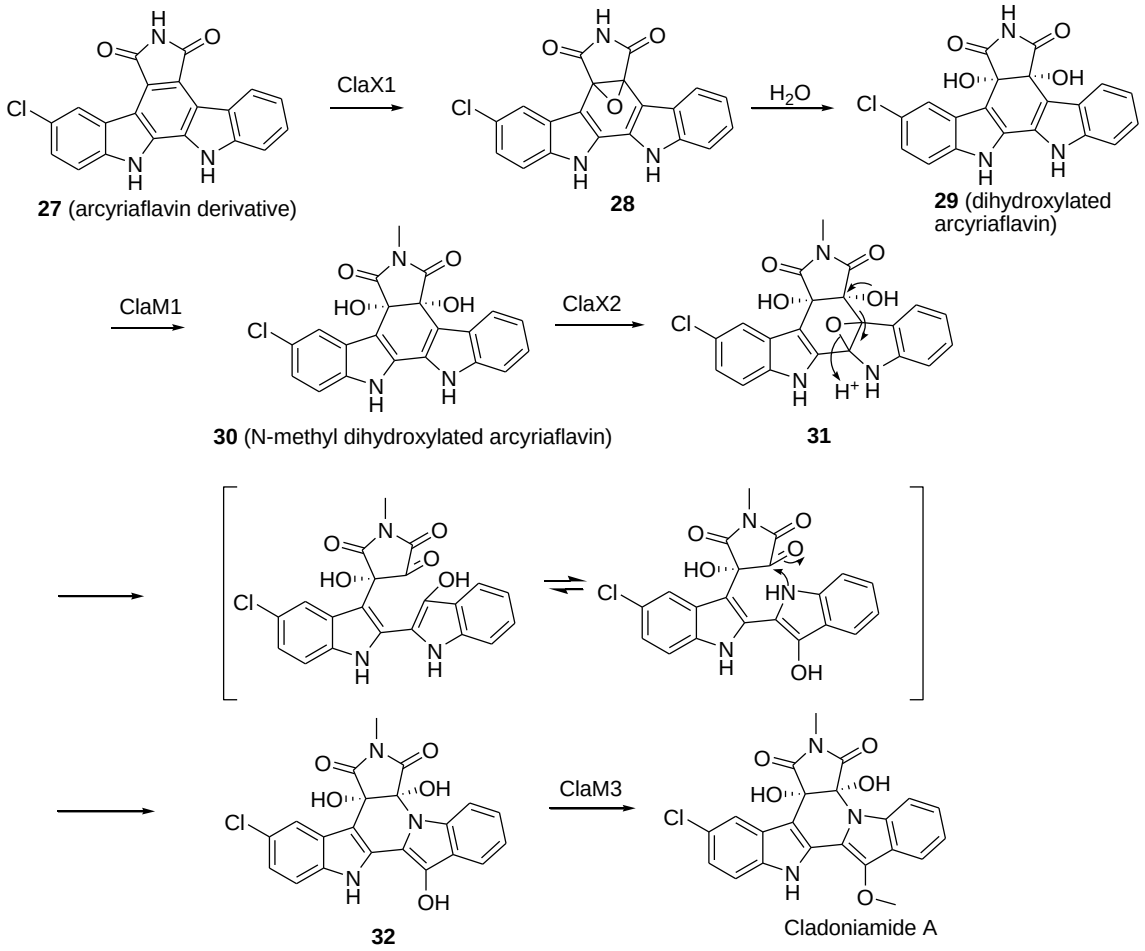


Figure 10 Final steps in cladoniamide synthesis, reactions catalyzed by ClaX1, X2, M1, and M3.

The newly incorporated epoxide then acts as an electron sink to promote cleavage of the central carbon ring, leaving the dechlorinated indole free to rotate around the C-C bond connecting both indoles. Once the nitrogen group from the rotating indole gets close

enough to the formed ketone, it is able to attack the carbonyl and reform the central ring.[19] The initial indolocarbazole scaffold is now replaced with indolotryptoline **32**. Further experimental support for the proposed mechanism has also been obtained. It was found that in an *E. coli* system expressing only *claX1*, it is possible to feed in arcyriaflavin A (**Figure 11, 33**) and isolate out the expected dihydroxylated product **34**.[43] A similar experiment employing *claX2* yielded no products,[43] suggesting the two monooxygenases do not perform identical functions and X1 is responsible for the first epoxidation. The timing of the *claM1* methylation was also confirmed to follow the *claX1* oxidation by showing that it can only accept products following the first epoxidation reaction.[43] To investigate the function of *claX2*, various combinations of *claX1, X2, M1,* and *M3* were expressed in *E. coli* and arcyriaflavin/dichloroarcyriaflavin was fed to the resultant strain.[43] The system with only *X1* and *M1* yielded the expected methylated dihydroxylated product.[43] Only with the four genes of *X1*, *X2*, *M1*, and *M3* co-expressed together was the production of cladoniamide B observed from feeding of dichloroarcyriaflavin A (**Figure 11, 35**) .[43]

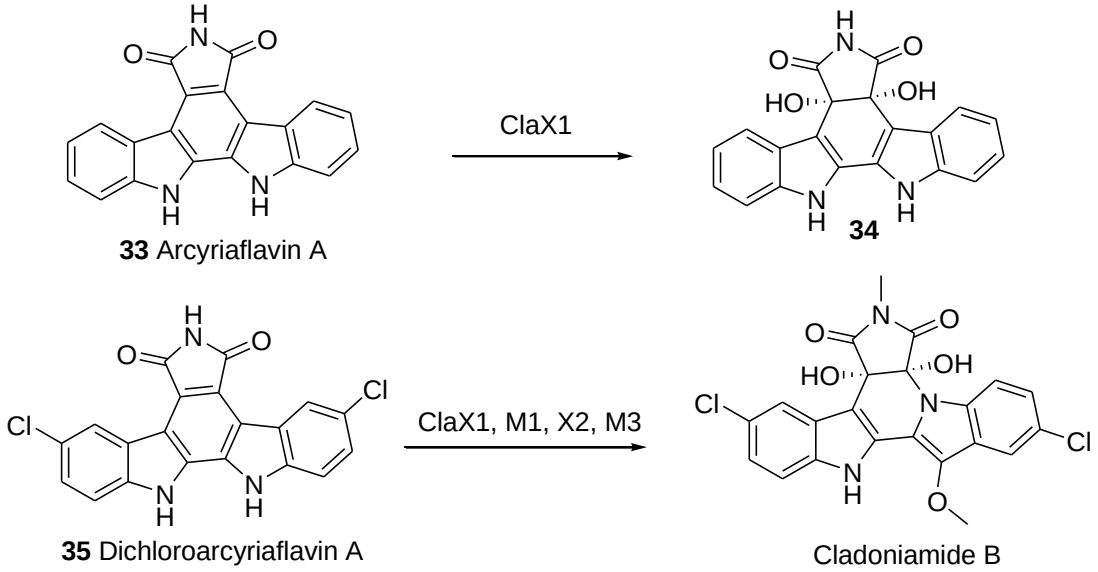
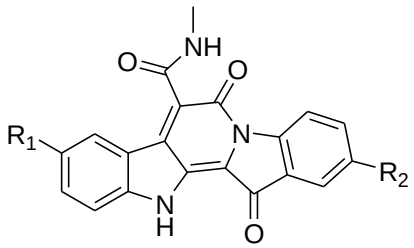


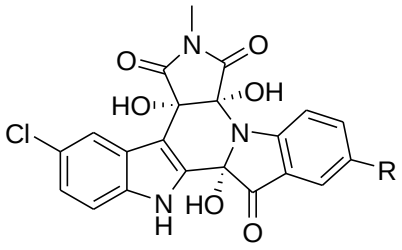
Figure 11 Functions of ClaX1, M1, X2 and M3

The difference between the *abe* cluster and the *cla* cluster is the presence of an extra methyltransferase in the *abe* cluster. There are two found in the *cla* cluster (ClaM1/M3), while BE-54017 contains three (AbeM1/M2/M3).[19,40] ClaM1/AbeM1 has already been mentioned as an N-methyltransferase targeting the succinimide nitrogen.

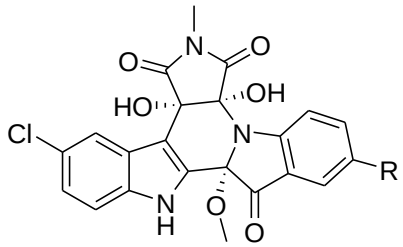
AbeM2, which is only found in BE-54017 gene cluster, is believed to also act as an N-methyltranferase that targets the nitrogen atom on the chlorinated indole.[19] As expected, knockout of *abeM2* accumulated the corresponding product without the specific methyl group.[19] Finally, the last step in the biosynthesis is believed to be carried out by ClaM3/AbeM3 which is an O-methyltransferase that methylates the oxygen attached to C-3 of the right hand indole.[37] A recent study has shown that this O-methylation may be a vital stabilizing mechanism.[37] Without this protection, the synthesized compound may undergo unintended decomposition.[37] Inactivation of the *claM3* gave rise to a series of new indolotryptoline compounds known as xenocladoniamides (**36-43**).[37,44]



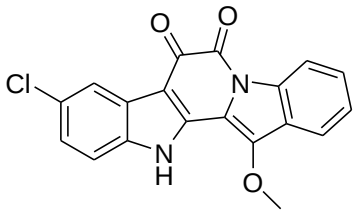
36 (Xenocladoniamide A) R_1= Cl, R_2 = H
37 (Xenocladoniamide B) R_1 = R_2 = Cl
38 (Xenocladoniamide C) R_1 = R_2 = H



39 (Xenocladoniamide D) R = H
40 (Xenocladoniamide E) R = Cl



41 (Methyloxy Xenocladoniamide D) R = H
42 (Mehtyloxy Xenocladoniamide E) R = Cl



43 (Xenocladoniamide F)

Figure 12 Xenocladoniamides isolated by inactivation of *claM3*

Of all the genes found within the cladoniamide and BE 54017 cluster, all of their function in the biosynthetic pathway have been defined expect for one.[37] The lone gene is *claY/abeY*, a putative hydrolase. However, knockout studies on the gene suggested that it was not crucially involved in the cladoniamide biosynthetic pathway.[19,37] Inactivation of it does not prevent the production of the expected products.[19,37] One possible explanation is that the gene is a genetic artifact without major function. It is also interesting to consider the halogenases RebH, ClaH, and AbeH and the effect of halogenation in nature.

Could they also be simple artifacts as well? The production of deschloro-analogs of these compounds has been observed. Therefore, lacking this halogen substituent does not cripple their biosynthesis. However, all the biologically active cladoniamides (BE54017, cladoniamide A, B, and G) are chlorinated. It is conceivable that similar to previously reported active compounds, halogenation plays an important role in the biological activities.[45]

1.3.4 Total synthesis of Arcyriaflavin and Cladoniamides

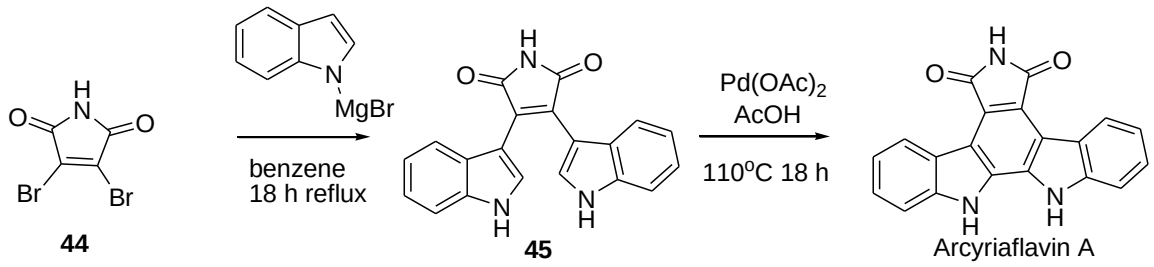


Figure 13 Malsher groups synthesis of arcyriaflavin A

Since the initial isolation of indolocarbazoles, there have been multiple reports on the total chemical synthesis of the bisindole scaffold. One efficient method was reported by the Malsher group which employs dibromomaleimide (**44**) and four equivalents of indolylmagnesium bromide. The mixture is dissolved in benzene and refluxed for 18 h.[46] The end product of the Grignard-like reaction is the bisindolylmaleimide (**45**). Then to cyclize the central ring, the bisindole product is refluxed with palladium acetate in acetic acid for another 18 h.[46] Extraction of the reaction mixture yields arcyriaflavin A as the product.[46] Over the two-step reactions, the calculated yield is about twenty percent.[46] This is a very quick way to generate arcyriaflavin A at a good quantity.

It should be noted that there has been reports of total synthesis of cladoniamides. The total synthesis of BE-54017 has been reported by the Shibasaki group. The initial starting material for the reaction was the stannane (**46**), which can be generated from chlorinated indole.[47] The stannane was then used in a Stille coupling with methoxyacetylchloride to give **47**.[47] The product was then used in a standard Fischer indole synthesis to generate a bisindole (**48**).[47] Due to the unstable nature of the synthesized bisindole, it was directly used in a Michael addition with maleimide to give **49**.[47] Cyclization of the center ring (**50**) was accomplished by Pd black catalysis at

200°C.[47] At this point, the carbon backbone for BE-54017 and cladoniamide A has been formed. The final two steps involved were methylation of the indole nitrogen followed by OsO_4 treatment to oxidize across the central alkene bond to generate two hydroxyl groups.[47]

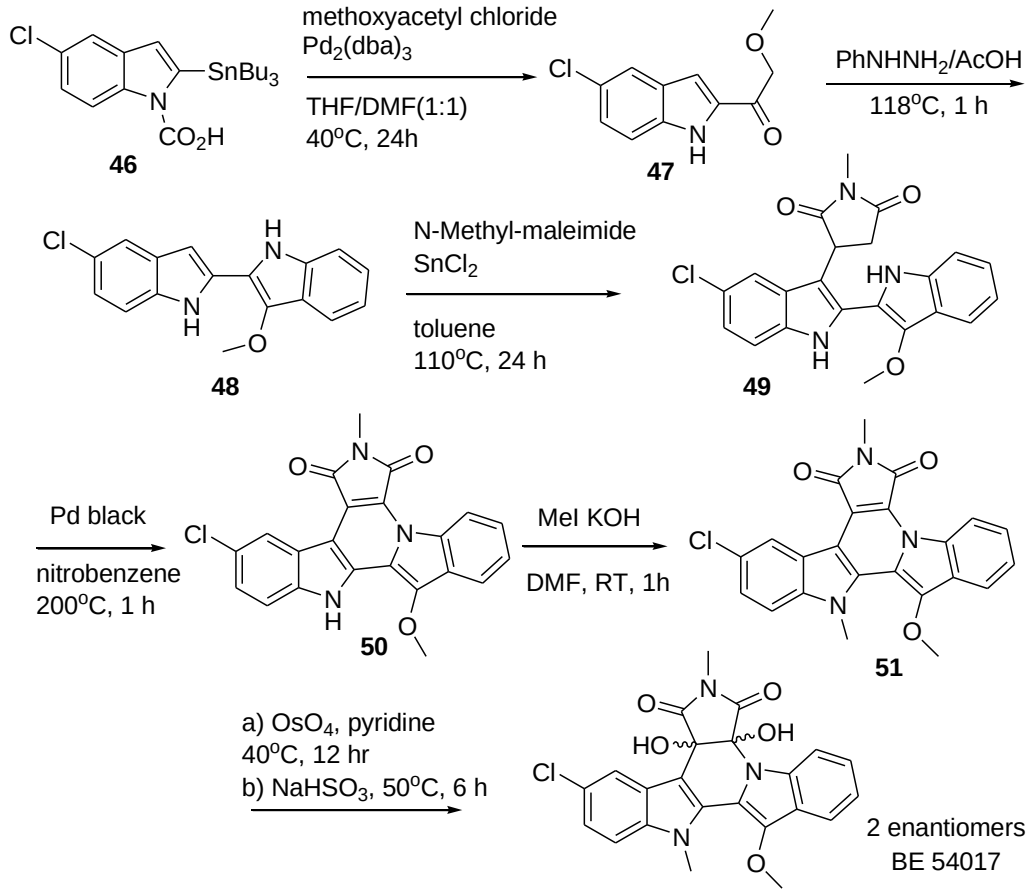


Figure 14 Total synthesis of BE 54017 by the Shibasaki group

The Dake and Andersen groups reported the first synthesis for cladoniamide G. Similar to the BE-54017 synthesis, 5-chloroindole (**52**) was employed as the starting material.[48] Compound **52** was then converted to 3-acetoxy-5-chloroindole (**53**) through an iodate-acetate exchange reaction catalyzed by silver (I).[48] Compound **53** was further condensed with a second unit under basic conditions to afford the dichloroindigo (**54**).[48] Formed dichloroindigo was then reduced using hydrazine and sodium hydroxide, yielding **55**.[48] The final step involved the addition of the reduced form of dichloroindigo with a specially synthesized linchpin reagent (**56**) to generate cladoniamide G.[48]

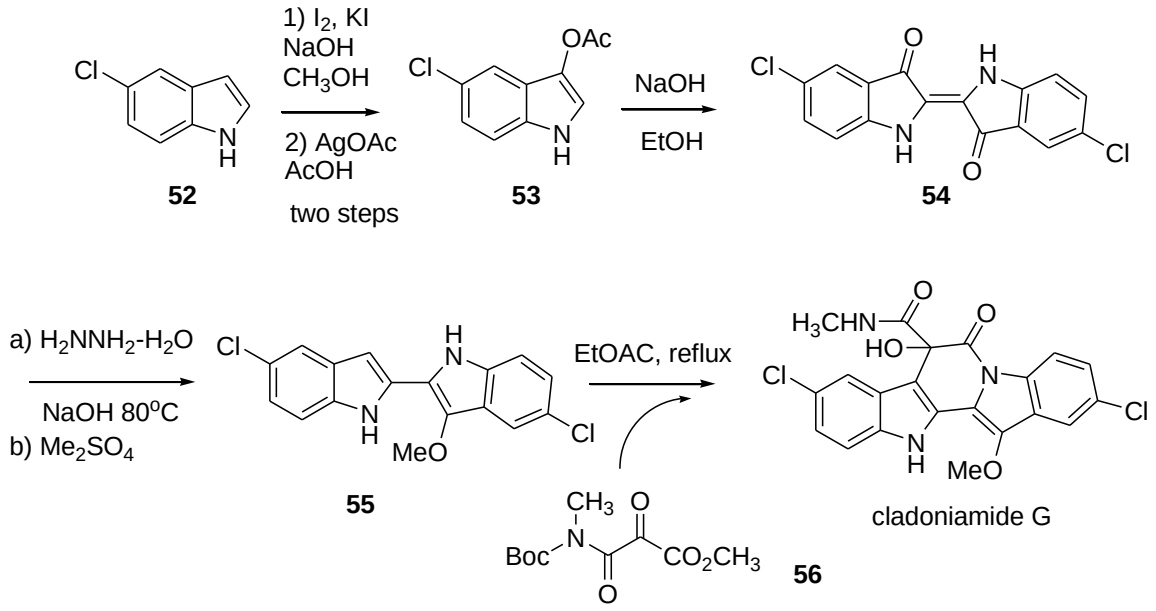


Figure 15 Total synthesis of cladoniamide G by the Dake group

Recently, there have been more reports of the total synthesis of cladoniamide G/F. The Koert group first synthesized the appropriate organobromide (**57**) and the corresponding SEM-protected borate (**58**) and were able to form the bisindole back bone (**59**) seen in cladoniamides through a Suzuki coupling reaction.[49] Subsequent deprotection and then reaction with mesoxalic ester amide (**61**) generated cladoniamide G.[49] They also reported the total synthesis of cladoniamide F, which contains only one chlorine when compared to the G analog. The starting material, compound **62,** was used in a Fischer indole synthesis with phenylhydrazine to give rise to the bisindole intermediate (**63**), which was converted to cladoniamide F by subsequent reaction with **61** under identical conditions as the synthesis of cladoniamide G.[49]

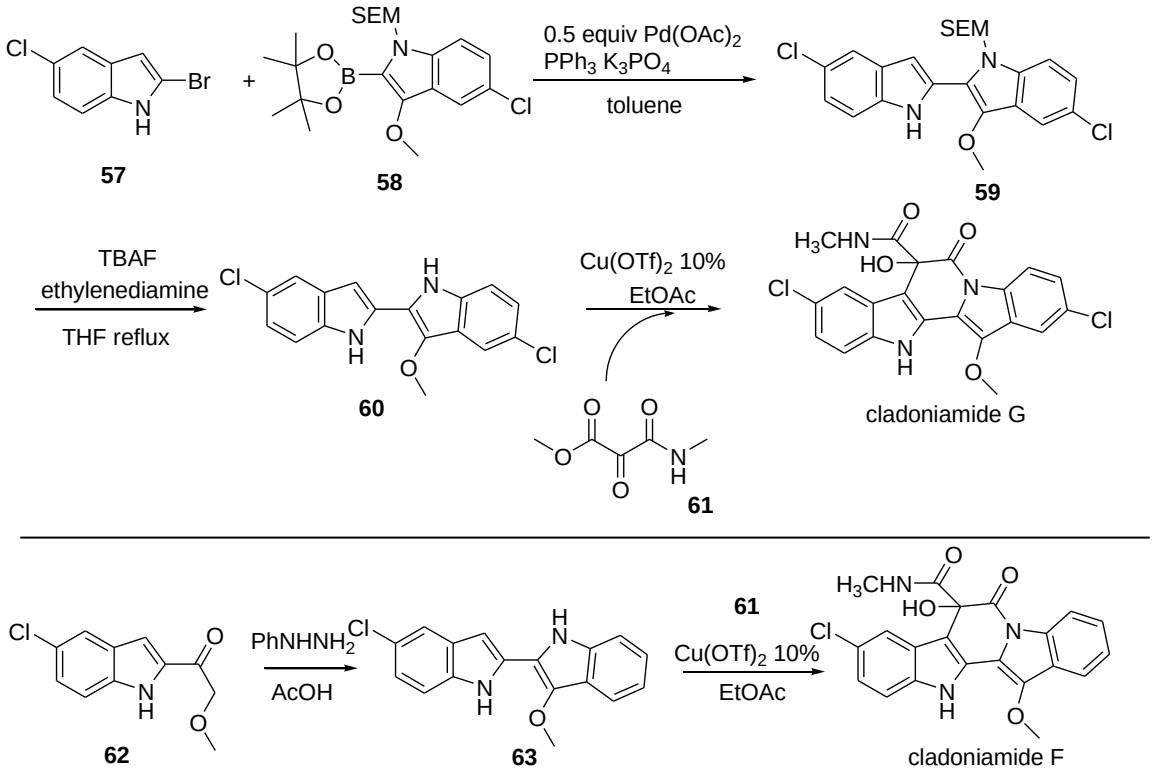


Figure 16 Total synthesis of cladoniamide G/F through Suzuki coupling by the Koert group

A similar method to generate cladoniamide G was also reported by the Kongkathip group.[50] They started by first synthesizing the substrates (**65** & **66**) needed for the Suzuki coupling reaction.[50] The two substrates were then linked together to generate the bisindole intermediate (**67**).[50] Then final step involved the application of the linchpin reagent (**56**) as reported by the Dake group.[50]

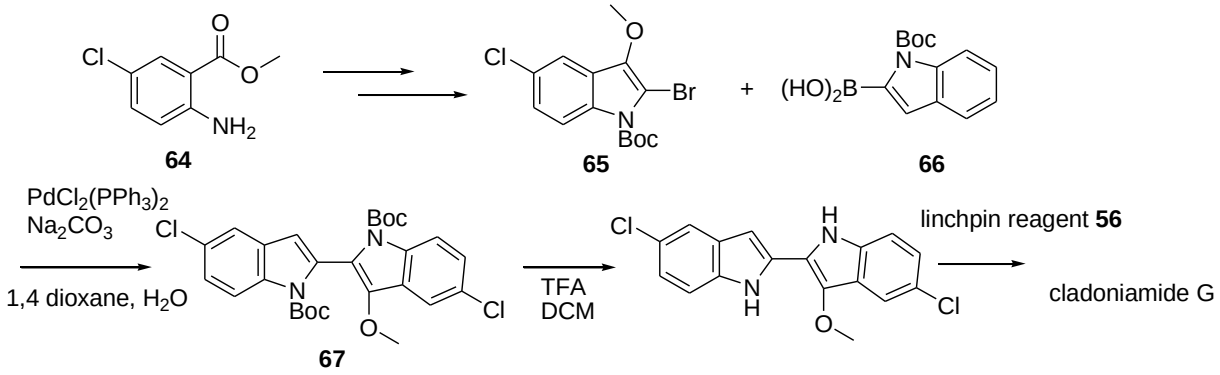


Figure 17 Total synthesis of cladoniamide G reported by the Kongkathip group

In terms of reaction conditions, all the reported synthesis of cladoniamides were somewhat similar and involves the crucial formation of the bisindole complex through condensation, Fischer indole synthesis or transitional metal catalyzed coupling. The

reactions reported by Shibasaki are the only method to generate analogs of cladoniamide A/B/C and BE 54017 through 6 steps and an overall yield of 2.9%.[47] When synthesizing cladoniamide G, the report from the Dake group is the most promising with 35% yield over 5 steps.[48] However, there is a need to also generate the linchpin reagent. In comparison, the steps proposed by the Koert group and Kongkathip group require the synthesis of the appropriate substrates for the coupling reactions which can take five to six reactions to prepare.[49,50]

1.4 Biological Halogenation

There have been a large number of halogenated natural products isolated from various sources, the count is currently over 4000 molecules.[51] Of the 4000 halogenated compounds, they span numerous classes such as enediynes, marcocyclic lactones, and the aforementioned indolocarbazoles.[51] In terms of distribution, the most abundant halogenation is chlorine ion followed by bromine ion.[51] Iodinated and fluorinated compounds are much rarer which is consistent with the relatively lower quantities of these halides in natural sources.[51] It has been confirmed that halogen substituents can often be crucial for desired biological activities. For instance, the well-known antibiotic vancomycin requires the presence of two chlorine groups to appropriately position the molecule for action.[52] When testing the protein binding abilities, having only one chlorine or none at all both greatly decreased the affinity of vancomycin to target peptides.[52] In turn, the antibiotic activity of the deschloro derivative was only half of the original compound.[52] Therefore, halogenation is quite important, and nature has developed various methods of performing this reaction.

One of the better-studied methods of biological halogenation is the use of reduced flavin adenine dinucleotide ($FADH_2$)-dependent halogenases. This class of halogenase includes enzymes such as the aforementioned RebH/ClaH/AbeH. It also includes PrnA from the biosynthesis of pyrrolnitrin and ThaI, responsible for the halogenation of thienodolin[35, 38].

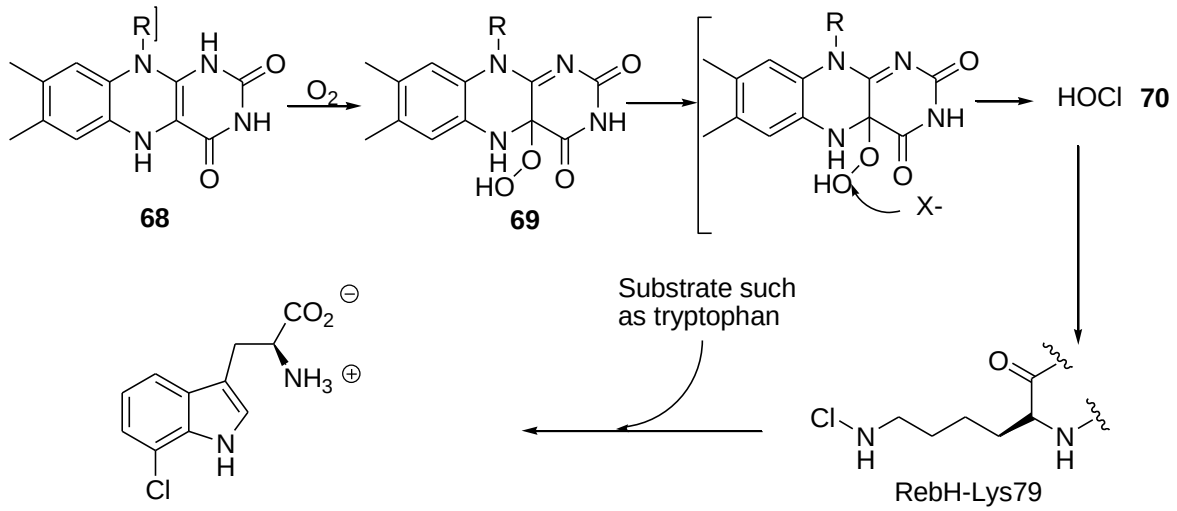


Figure 18 Proposed mechanism of $FADH_2$ dependent halogenases

Much of the insight into the mechanistic action of these halogenases came from detailed investigation of PrnA and RebH. The FAD cofactor is believed to bind onto the "GxGxxG" amino acid motif near the N-terminal of the enzyme.[53] In order to proceed with the reaction, FAD must be in the reduced form. To satisfy this criteria, many biosynthetic gene clusters include a dedicated reductase, such as RebF/ClaF/AbeF, which uses NAD(P)H to regenerate $FADH_2$.[45] However, cellular reductases can also be recruited, as is the case with pyrrolnitrin.[45] With $FADH_2$ (**68**), molecular oxygen can then be incorporated in a fashion that is believed to be similar to previously known flavin monooxygenases to form flavin hydroperoxide (**69**).[45] The formation of a flavin hydroperoxide intermediate has been detected during RebH catalysis.[54] Then halides such as chloride can attack the distal oxygen of the peroxide to liberate halide species such as hydrochlorous acid (HOCl, **70**).[45] However, while HOCl is known to be capable of halogenation, it is not selective and will react with various functional groups within proteins.[55] In the case of RebH, an active site Lys79 residue is believed to react rapidly with HOCl to generate a more stable chloroamine.[55] The chloroamine in turn has reduced reactivity but is able to selectively proceed with the halogenation.[55] Aligned with the theory, it was observed that with ^{36}Cl labeling, the radiolabel sequentially moved from free floating chloride to protein and finally to the product.[55]

Although the exact regioselective mechanisms of $FADH_2$-dependent halogenases are still not fully understood, they have been shown to be highly selective especially when employing tryptophan as a substrate. For instance, PrnA and RebH are tryptophan

7-halogenases.[54,56,57] Tryptophan 5-halogenases include enzymes such as PyrH in the biosynthesis of pyrroindomycin and of course ClaH/AbeH are likely to act as tryptophan 5-halogenases in the biosynthesis of the cladoniamides.[58] There have also been reports on the halogenases targeting the C-6 position which include STTH found within *Streptomyces toxytricini* NRRL 15443 and Thal which was isolated from *Streptomyces albogriseolus* MJ286-76F7.[53,59] Interestingly, *in vitro* analysis of STTH enzyme activity, found it was capable of catalyzing both installation of bromine and chlorine at the C-6 position of D and L-tryptophan.[53] It is note-worthy that there have been no reports of selective addition of halogen onto the C-4 position.

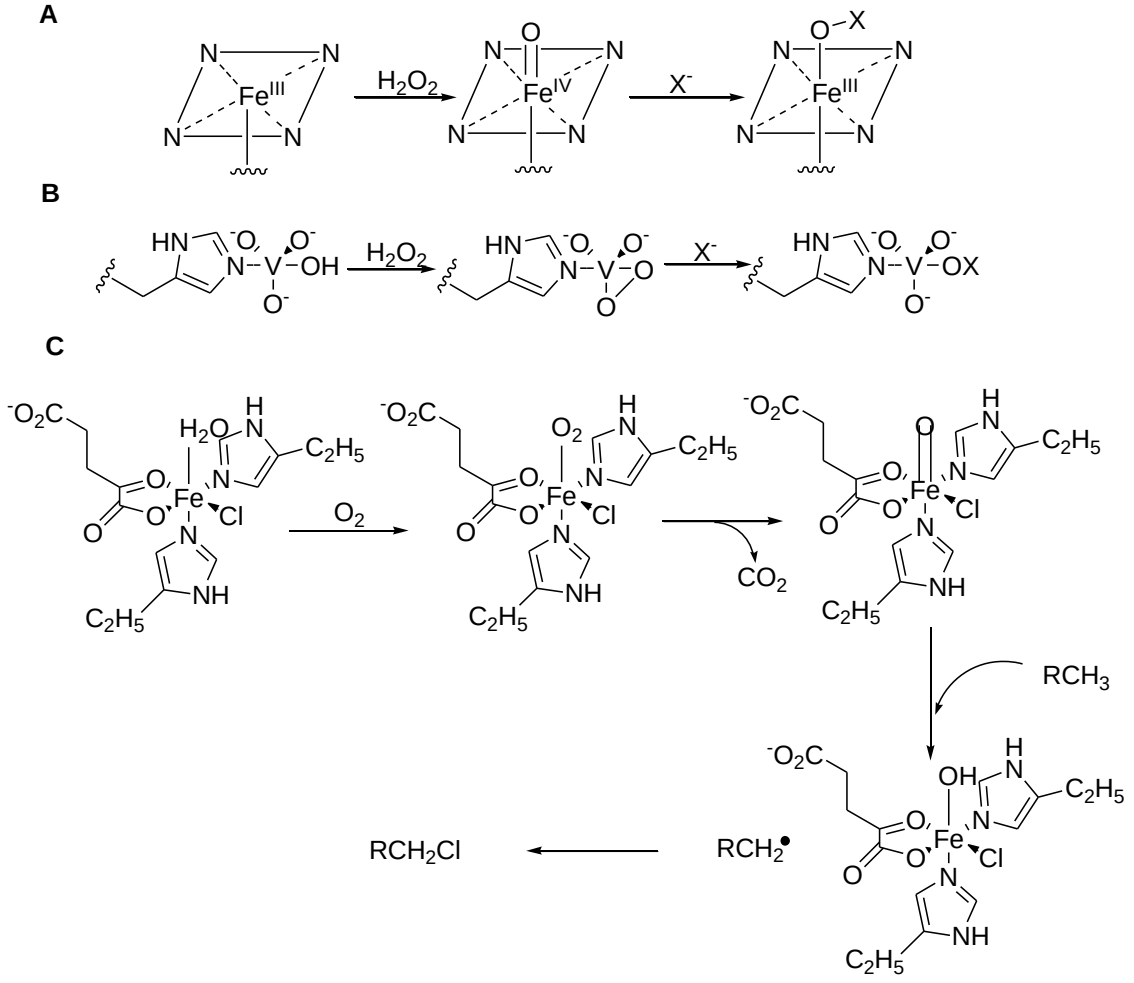


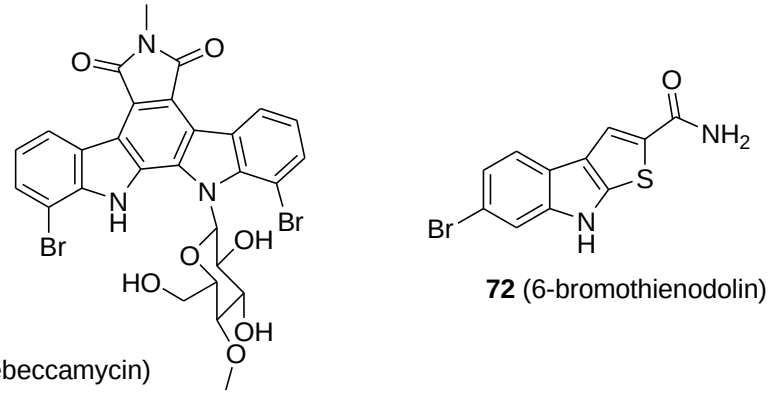
Figure 19 Other well studied biological halogenation mechanisms: a)heme dependent, b) vanadium dependent, and c) non-heme halogenase

Beside the $FADH_2$-dependent halogenases, there are several other common biological methods as well. First, there is the heme-iron dependent halogenases that employ hydrogen peroxide as the oxidizing reagent (**Figure 19A**).[45] The active species is

believed to be a halide ion coordinated onto an oxygen atom which is bond onto the iron center.[45] In turn this complex behaves similar to a hypohalite. The second type uses a vanadium center and functions similarly to the heme-iron halogenase (**Figure 19B**).[45] Another variation is the oxygen dependent non-heme iron halogenase in the biosynthesis of compounds such as armentomycin.[45] The reaction requires α-ketoglutarate, oxygen and halide as cofactors (**Figure 19C**).[45] Halogenation in is believed to involve the formation of radicals that can then quickly form carbon-halide bonds.[45]

1.4.1 Inducing Brominated Compound Production

When investigating the $FADH_2$-dependent halogenases, it has been reported that several of the enzymes are capable of using both bromide and choride. Considering the similar chemical properties of the two ions, this is not surprising. There have been several publications detailing the practice of supplementing growth mediums with bromine sources such as potassium bromide (KBr) in order to promote the formation of brominated analogs. For instance, it was reported that brominated rebeccamycin (**71**) can be isolated by simply adding 0.05% w/v KBr to a culture of *L. aerocolonigenes ATCC 392431*.[60] What was even more interesting was the observed suppression of the original rebeccamycin production.[60] Attempts have also been made to promote the biological production of the fluorinated and iodinated analogues as well, but these have not been observed, possibly due to toxic effects.[61]



71 (Bromorebeccamycin)

72 (6-bromothienodolin)

Figure 20 Isolation of brominated analog of rebeccamycin and thienodolin

Similarly, the brominated analogs of thienodolin have also been observed. Culturing of *S. albogriseolus* MJ286-76F7 in previously reported medium yielded the

production of the expected thienodolin with a C-6 chlorine.[59] However, culturing the strain with added bromine source led to the observation of a new peak in the EtOAc extract.[59] By HPLC/MS, the peak was found to have the characteristic twin bromine peaks of the bromothienodolin (**72**).[59]

Another KBr supplementing experiment reported the production of bromobalhimycin, a vancomycin-class antibiotic.[62] The experiment involved the replacement of all chlorine sources in the growth medium for other halides including fluoride, iodide and bromide. It was found that both fluoride and iodide supplements were toxic to the producer, *Amycolatopsis mediterranei*, and inhibited normal growth.[62] However, a biologically active analog was isolated with bromide supplementation, and it was characterized as the bromobalhimycin.[62] Interestingly, when equal portions of chloride and bromide were added to the medium, the observed products included the production of a chlorobromobalhimycin.[62]

1.5 Aim: Isolation of Bromocladoniamide

Similar to the already reported brominated analogs of the above compounds, it would also be interesting to isolate the bromocladoniamides. Considering the already potent effects of compounds such as cladoniamide A and B, modification of the halogenation may lead to further improve activities and selectivity. One possible course of action is to try to promote the biological synthesis by supplementing the bacterial growth medium with bromine sources. Another route to generate the derivatives is through precursor-directed biosynthesis. Depending on the efficiency of the natural bromine incorporation into cladoniamides, feeding in specific substrates may prove to be more efficient. Precursor-directed synthesis has been well reported for different biosynthetic pathways, one such case is the feeding of unsaturated triketides into the erythromycin PKS system.[63] Analogously, we wanted to interrogate if precursor directed synthesis could also be used in cladoniamides synthesis, specifically to produce 5-bromocladoniamide A. The substrates we decided to employ for supplementation were 3-chloroarcyriaflavin and the 3-bromoarcyriaflavin.

1.5.1 Synthesis of Halogenated Arcyriaflavin

There are several potential methods to synthesize the desired arcyriaflavins. The most direct method would be to purchase arcyriaflavin A and then treat it under electrophilic aromatic substitution conditions with a halogen source such as $AlBr_3$ or $AlCl_3$. However, due to the large number of possible sites of addition it would be challenging to direct the production to a single intended product. In turn, a whole series of differently halogenated compounds can lead to poor yield and difficult purification. Furthermore, the cost of arcyriaflavin A is high.

It is also possible to employ the previously mentioned method by the Malsher group. However, their method may not practical in terms producing what is needed for this project. The main issue lies with the indolylmagnesium bromide. In order to generate halogenated final products, the appropriate halogenated analogs of the substrates must be added. Considering that the intended arcyriaflavin should only have one halogen attached, using this method would require the addition of both the halogenated and non-halogenated indolylmagnesium bromide. Then the three possible reactions crude can include the non-halogenated, mono-halogenated, and dihalogenated products, which would prove to be difficult to further purify.

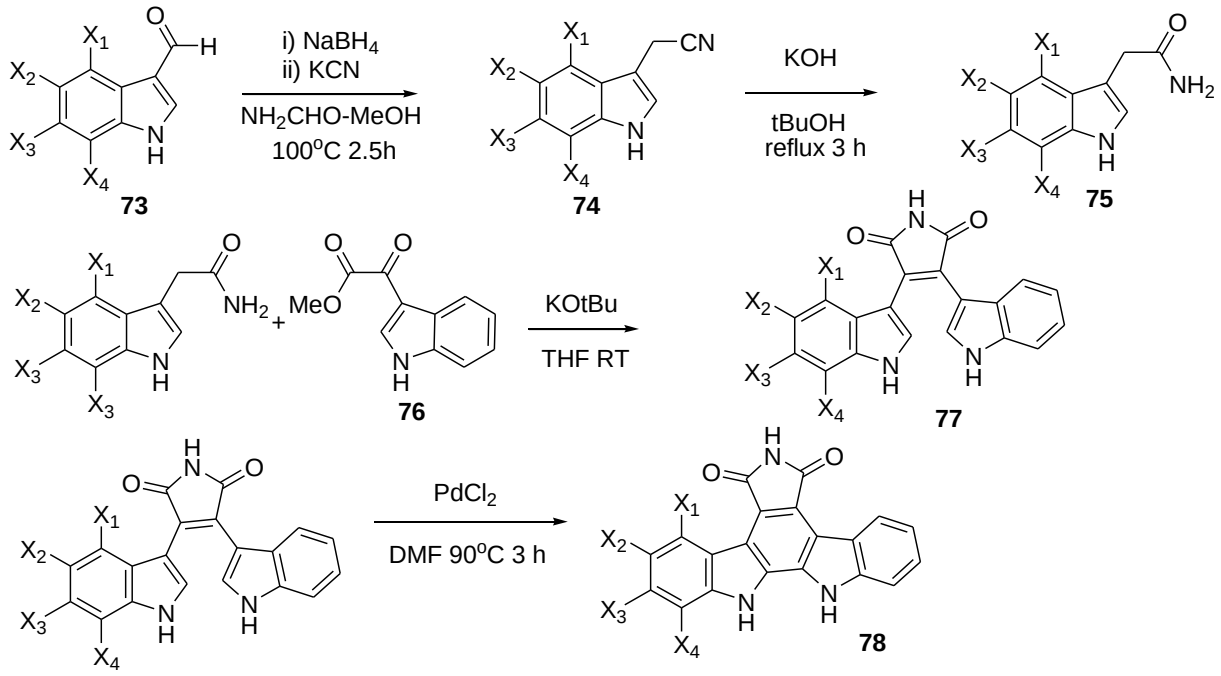


Figure 21 Planned pathway for the synthesis of halogenated arcyriaflavin substrates

In the interest of time and flexibility, the synthetic pathway that was proposed during this research involves a total of four steps, starting with halogenated indolecarbaldehydes (**73**). Various indolecarbaldehydes are commercially available with alterations on locations from C5 to C7. This strategy then allows for the potential to generate a library of bis-indole compounds. The indolecarbaldehyde is first treated with sodium borohydride to selectively reduce the aldehyde into an alcohol.[64] Following the reduction, potassium cyanide is then added to displace the hydroxyl group and add a nitrile group to the indole back bone (**74**).[64]. The formed acetonitrile is then converted into an amide through oxidation of the newly added carbon atom with *tert*-butoxide (**75**).[64] The next step involves a Perkin condensation of the synthesized acetamide with purchased glyoxylate (**76**) to give the pyrrole-2,5-dione (**77**).[65] The final step in the synthetic pathway is to cyclize the C2 positions of the two coupled indoles through a palladium (II) catalyzed oxidation (**78**).[65] The end product of the pathway is the halogenated arcyriaflavin. The location and type of halogen installed on the arcyriaflavin depends entirely on the initial choice of indolecarbaldehyde used.

Chapter Two: Experimental

General Procedures

All the chemical reactions and bacterial cultures were conducted in EMD Extran 300 detergent-washed glassware that was then air-dried. To minimize the amount of moisture in the chemical reactions, the air-dried round-bottom flasks were also flame dried and then fitted with rubber septa when appropriate. Solvents were used directly from tightly sealed bottles without further purification. Analytical thin layer chromatography was performed with TLC Silica gel $60F_{254}$ on aluminum plates with visualization with 254 nm UV-light. For normal phase flash chromatography, SilicaFlash F60 230-400 mesh was used. The C-18 reverse phase purification was carried out using Water Sep-Pak Vac 20cc C18 Cartridges.

Materials

Reagents and solvents for chemical synthesis were purchased from Sigma-Aldrich. $NaBH_4$ and bacterial growth media were purchased from Fisher Scientific. Yeast extract was obtained from Becton Dickinson. Water for HPLC was obtained from Millipore filtration through a Quantum® Ex Ultrapure Organex cartridge, while HPLC-grade acetonitrile was from Sigma-Aldrich.

Instrumentation

A Bruker AV-600 spectrometer with a 5 mm CPTCI cryoprobe was employed for analysis of purified metabolites by ^{1}H, ^{13}C, and 2D NMR. Chemical shifts were recorded in parts per million with reference to residual solvent signals ($CHCl_3$ at δ 7.26 ppm, MeOH at δ 3.31/4.78ppm, and DMSO at δ 2.49 ppm). Data were reported as the following: chemical shift [multiplicity (s = singlet, d = doublet, t =triplet, q = quartet, m = multiplet, br = broad), coupling constant(s) in Hertz, integration]. For ^{13}C, the reference was DMSO at δ 39.5 ppm. Low-resolution ESI-MS data was collected using a Waters ZQ

LC-MS spectrometer. High-resolution ESI-MS data were recorded with a Micromass LCT time-of-flight (TOF) mass spectrometer equipped with electrospray ionization. High-performance liquid chromatography (HPLC) was conducted on an Agilent 1260 with a diode array UV detector. HPLC sample analysis was ran on an Agilent Poroshell 120 EC-C18 2.7 μm column with 0.05% (v/v) TFA containing Milli-Q water and ACN. The HPLC protocol used was 90% Milli-Q water and 10 % ACN at 0 min, reaching 100% ACN at 6 min, which then return to 90% Milli-Q water/10% ACN at 7 min. Bacteria were cultivated in an INFORS HT Thermotron shaking incubators under appropriate conditions. A Buchi Rotavapor R-210 was used to remove excess low-boiling solvents. A Savant SpeedVac SC110A was used to remove small quantities of residual water from samples. For refluxing the chemical reactions, a Thermo Scientific Cimarec Digital Stirring Hotplate was employed. A Denver Instrument SI-114 analytical balance was used for weighing of reagents. For pH measurements, A-980 Alkaacid Test Paper and Φ 350pH/Temp/mV meter were used.

Synthesis of (5-Choro-1H-3-indolyl) Acetonitrile (79) and (5-Bromo-1H-3- indolyl) Acetonitrile (83)

The synthesis of 2-(5-bromo-1*H*-3-indolyl) acetonitrile and 2-(5-chloro-1*H*-3-indolyl) acetonitrile were accomplished as described previously[64] and carried out as follows. 5-bromo-1*H*-indole-3-carbaldehyde (2.000 g, 8.927 mmol, 1 equiv) was first dissolved in 8 mL of formamide/methanol (1:1). Sodium borohydride (0.6764 g, 17.88 mmol, 2 equiv) was slowly added into the solution with stirring. Gas formed following the addition of $NaBH_4$, which was believed to be hydrogen gas. The white cloudy mixture was then stirred at RT for 1 h to allow for complete reduction of the aldehyde into the corresponding primary alcohol, as monitored by TLC comparison with starting material. To the reaction mixture, potassium cyanide (5.000 g, 76.78 mmol, 8.6 equiv), dissolved in 5 mL formamide/methanol (1:1), was then slowly added to the reaction mixture which was then refluxed at 85°C for 3 h. After the reflux, the reaction mixture was cooled to RT before being quenched with 20 mL of saturated NaCl solution. The aqueous solution was transferred into a 50 mL separation funnel and extracted with

chloroform ($CHCl_3$, 3 x 20 mL). These organic extracts were then combined and then dried with anhydrous sodium sulfate for 10 min. Drying reagent were then filtered off through fluted filter paper. The resulting solution was further dried under vacuum to yield a yellow-brownish solid. The crude reaction mixture was then purified with normal phase silica flash column (EtOAc: Petroleum Ether (1:3)). The purified product appeared as a white solid. It should be noted that due the highly toxic nature of KCN, special precautions were taken, including extra personal protective equipment, treatment of glassware, and the separation of waste. Yield: 0.8331 g, 40 %. ^{1}H NMR (300 MHz $CDCl_3$): δ 3.85 (s, 2H), 7.34-7.42 (m, 3H), 7.77 (s, 1H), 8.27 (br, 1H). MS (ESI): m/z 257.3/259.3 $[M+Na]^+$, 233.2/235.2 $[M-H]^-$ (**Figure 36**). The procedure for synthesis of the chlorinated acetonitrile used 5-chloro analog as starting material. Yield: 0.8916 g, 42 %. ^{1}H NMR (300 MHz $CDCl_3$): δ 3.85 (s, 2H), 7.25-7.38 (m, 3H), 7.60 (s, 1H), 8.30 (br, 1H). MS (ESI): m/z 213.3/215.3 $[M+Na]^+$ (**Figure 37**).

Synthesis of (5-Chloro-1H-3- indolyl) acetamide (80) and (5-Bromo-1H-3-indolyl) acetamide (84)

The synthesis of 2-(5-bromo-1*H*-3-indolyl) acetamide and 2-(5-chloro-1*H*-3-indolyl) acetamide were accomplished as described previously and carried out as follows.[64] Compound **83** (0.8331 g, 3.518 mmol, 1 equiv), was first dissolved in 5 mL tert-Butyl alcohol. Potassium hydroxide (1.740 g, 31.07 mmol, 8.8 equiv), dissolved in 5 mL tert-Butyl alcohol, was then added to the acetonitrile solution. The resulting mixture was refluxed at 80°C for 3 h. Following reflux, the reaction mixture was cooled to RT. 6 M concentrated HCl was slowly added to the stirring mixture until a neutral pH of 7 was achieved. The resulting mixture was then transferred into a separation flask containing 20 mL H_2O. Then EtOAc (3 x 30 mL) was used to extract out the crude product. In order to minimize water content, the organic extract was washed with equal volume of saturated NaCl brine solution before briefly dried over anhydrous sodium sulfate. Drying reagent was removed via filtering over fluted filter paper. Finally the EtOAc solvent was removed while drying under vacuum to yield a brown crystalline-like solid. The crude product was purified using normal phase silica flash column with DCM/MeOH (95:5) as

the solvent system. The purified product appeared as brown crystals. Yield: 0.3313 g, 37%. ^{1}H NMR (300 MHz DMSO-d_6): δ 3.45 (s, 2H), 6.85 (br, 1H), 7.18 (dd, J_1=1.9Hz, J_2=8.6 Hz, 1H), 7.26 (d, J= 2.2 Hz , 1H), 7.32 (d, J=8.6Hz, 1H), 7.39 (br, 1H), 7.74 (d, J=1.7Hz, 1H), 11.08 (s, 1H). MS (ESI): m/z 251.3/253.3 $[M-H]^-$ (**Figure 38**). Similar steps were taken for the chlorinated analog. Yield: 0.3904 g, 40 %. ^{1}H NMR (600 MHz DMSO-d_6): δ 3.45 (s, 2H), 6.85 (br, 1H), 7.07 (dd, $J1$=1.9Hz, J_2=8.6 Hz, 1H), 7.27 (d, J=1.9Hz, 1H). 7.35 (s, 1H), 7.38 (s, 1H), 7.60 (d, J=1.7Hz, 1H), 11.07 (s, 1H). MS (ESI): m/z 231.4/233.4 $[M+Na]^+$, 207.3/209.3 $[M-H]^-$ (**Figure 39**).

Synthesis of 3-(5-Chloro-3-indolyl)-4-(3-indolyl)-1H-pyrrole-2,5-dione (81) and 3-(5-Bromo-3-indolyl)-4-(3-indolyl)-1H-pyrrole-2,5-dione (85)

The synthesis of 3-(5-bromo-1*H*-3-indolyl)-4-(1*H*-3-indolyl)-1*H*-pyrrole-2,5-dione and 3-(5-chloro-1*H*-3-indolyl)-4-(1*H*-3-indolyl)-1H-pyrrole-2,5-dione were accomplished as described previously and carried out as follows.[65] Compound **84** (0.2604 g, 1.031 mmol, 1 equiv), and methyl indolyl-3-glyoxylate (0.3142 g, 1.547 mmol, 1.5 equiv), were first dissolved in 2 mL THF. Potassium *tert*-butoxide (0.3473 g, 3.095 mmol, 3 equiv), was dissolve in 1 mL THF and then quickly transferred to the reaction mixture. While sealed with rubber septum, the reaction mixture was stirred for 1 h at 0°C and then further stirred at RT for 3 h. The reaction was then quenched with 6M concentrated HCl until a pH of 7 and extracted with EtOAc (3x 30 mL). The organic extract was washed with equal volume of saturated NaCl brine solution and dried over $NaSO_4$. The drying reagent was removed via filtering over fluted filter paper. The resulting mixture was then dried under vacuum to afford a dark red crude product. Purification involved normal phase silica flash chromatography with 100% DCM to remove the more polar impurities. The less polar fraction was added onto a reverse phase Waters Sep-Pak Vac 20cc column, using H_2O/MeOH (1:1) as the solvent. The desired fraction appeared bright orange when in solution. Following evaporation of the solvents, an orange/red solid product remained. Yield: 0.0405 g, 10%. ^{1}H NMR (600 MHz DMSO-d_6): δ 6.62 (t, J_1=7.2, 1H), 6.68 (d, J_1=8.0 Hz, 1H), 6.95 (s, 1H), 6.96 (t, J_1=7.2 Hz 1H), 7.08 (d, J_1=8.6 Hz , 1H), 7.32 (d, J_1=8.4 Hz, 1H), 7.40 (d, J_1=8.0 Hz 1H), 7.74 (s, 1H). 7.79 (s, 1H), 10.93, (br, 1H), 11.75

(br, 1H), 11.81 (br, 1H). ^{13}C NMR (600 MHz DMSO-d_6): δ 112.1, 114.6, 119.6, 121.6, 122.6, 123.4, 123.5, 123.8, 124.7, 130.4, 134.6, 136.1, 172.9. MS (ESI): m/z 428.3/430.3 $[M+Na]^+$, 404.2/406.2 $[M-H]^-$ (**Figure 40**). The procedure for synthesis of the chlorinated analog was the same except **81** was used in place of **84**. Yield: 0.0677 g, 10%. ^{1}H NMR (600 MHz DMSO-d_6): δ 6.62 (td, J_1=0.7Hz, J_2=8.7Hz, 1H), 6.68 (t, J=8.5Hz, 1H), 6.81 (d, J=1.9Hz, 1H), 6.96 (dd, J_1=2.0Hz, J_2= 8.4Hz, 1H), 6.98 (td, J_1=1.0Hz, J_2= 8.0Hz, 1H), 7.38 (d, J=8.4Hz, 1H), 7.39 (d, J=7.1Hz, 1H), 7.76 (s, 1H), 7.80 (s, 1H), 10.94, (br, 1H), 11.74 (br, 1H), 11.81 (br, 1H). ^{13}C NMR (600 MHz DMSO-d_6): δ 105.3, 105.4, 111.9, 113.2, 119.5, 120.3, 120.7, 121.7, 123.9, 125.2, 126.6, 126.9, 128.2, 129.3, 130.5, 134.4, 136.1, 172.9. MS (ESI): m/z 384.3/386.4 $[M+Na]^+$, 360.2.2/362.2 $[M-H]^-$ (**Figure 41**).

Synthesis of 3-Chloro-arcryiaflavin (82) and 3-Bromo-arcyriaflavin (86)

The synthesis of 3-bromo-arcyriaflavin and 3-chloro-arcryiaflavin were accomplished as described previously and carried out as follows.[65] Compound **85** (0.0406 g, 0.0988 mmol, 1 equiv), was first dissolve in 2 mL of DMF. Palladium (II) chloride (0.0320 g, 0.1805 mmol, 2 equiv) was then added to the mixture. The reaction mixture was refluxed at 90°C for 4 h. Following reflux, the mixture was cooled to RT before being quenched with saturated NaCl solution. The aqueous mixture was transferred into a separation flask where it was extracted with EtOAc (30 mL). The aqueous phase from the separation was discarded while the organic phase was washed with saturated NaCl (3x 10 mL) to minimize the amount of DMF leftover. Finally, the organic phased was briefly dried with $NaSO_4$ and then removed the drying reagent by filtering over fluted filter paper. The collected solution was then evaporated to dryness under vacuum. The product was purified by Waters Sep-Pak Vac 20cc reverse phase column with H_2O/MeOH (1:1) as the solvent. A yellow band corresponding to halogenated arcyriaflavin was observed. Following evaporation of the solvents, a yellow/lightly orange solid product remained. For the brominated product the yield was 0.0100 g, 25%. ^{1}H NMR (600 MHz DMSO-d_6): 7.37 (td, J_1=2.2 Hz, J_2=8.1 Hz, 1H), 7.58 (m, 1H), 7.59 (m, 1H), 7.83 (d, J=8.1Hz, 1H), 7.87 (d, J=8.6 Hz,1H), 8.98 (br, 1H), 8.99 (d, J= 2.2Hz, 1H) 11.08 (br, 1H), 11.90 (br, 1H), 11.94 (br, 1H). ^{13}C NMR (600 MHz DMSO-d_6): δ 112.3, 113.9, 114.5, 115.9, 119.9,

120.1, 120.5, 121.5, 122.7, 123.2, 124.4, 126.6, 126.9, 129.1, 129.7, 138.8, 140.3, 171.2, 171.3. MS (ESI): 402.2/404.2 $[M-H]^-$ (**Figure 42**). The procedure for synthesis of the chlorinated analog was the same except **81** was used in place of **85**. Yield: 0.0170 g, 25%. ^{1}H NMR (600 MHz DMSO-d_6): δ 7.37 (t, J_1=7.2 Hz, 1H), 7.57 (td, J1=1.1Hz, J2= 7.0Hz, 1H), 7.58 (dd, J_1=2.3 Hz, J_2=8.5 Hz, 1H), 7.82 (d, J_1=8.6 H, 1H), 7.83 (d, J_1=8.1 Hz, 1H), 8.98 (d, J= 7.9Hz, 1H), 8.99 (d, J=1.9Hz, 1H), 11.07 (s, 1H), 11.96 (s, 1H), 12.05 (s, 1H). ^{13}C NMR (600 MHz DMSO-d_6): δ 112.1, 113.9, 114.5, 115.9 119.9, 120.5, 121.5, 122.5, 123.2, 124.3, 126.6, 126.9, 129.1, 129.3, 139.1, 140.4, 171.2, 171.4. MS (ESI): 358.2.2/360.2 $[M-H]^-$ (**Figure 43**).

Construction of the heterologous expression systems

Cladoniamides were initially isolated from *Streptomyces uncialis.* However, due to the limited production of cladoniamides in the natural system, a new host *Streptomyces albus* J1074 + *cla*(*S. albus* J1074 + *cla*) was employed.[37] To promote the highest production of the desired compounds, the cosmid containing the *cla* cluster was freshly conjugated into *S. albus* J1074. For feeding experiments, the cosmid containing the *cla* (Δ*claC*) was conjugated into *S. albus* J1074. The two gene clusters used were both constructed by Dr. Yiling Du as described previously.[37,43]

***S. albus* J1074 spore preparation**

The first component required for conjugation with Streptomyces strains is high quality bacterial spores. To isolate fresh spores, 100 uL of *S. albus* J1074 glycerol stock was added and spread over a 25 mL MS agar plate (20 g/L of each of soya flour, mannitol and agar) under sterile conditions. The cell culture was the incubated at 30°C for 7 days until a uniform white spread of bacterial colonies/mycelium could be seen. Initial cell growth was observable after the first two days. These colonies turn snow white following the formation of mycelium. After 7 d the spores were then harvested by addition of 10 mL of sterile water into the agar plate. A sterile loop was then used to scrape the bacterial colonies off the agar plate. Then 10 mL of water containing

colonies/spores was transferred to a 15 mL Falcon tube and vortexed at maximum velocity for 1 min. The resulting solution was then filtered through wool to remove the mycelium while retaining the desired spores (black colored). Finally the filtered solution was centrifuged at 7000 rpm for 10 minutes to pellet the spores. The supernatant was removed and 500 uL of 50% glycerol was added to re-dissolve the spore pellet.

E.coli ET12567/pUZ8002 preparation

The second component needed for conjugation is competent *E. coli* containing the intended gene cluster and a conjugation promoting plasmid. Initially 5 mLs of Luria-Bertani (LB, tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) were prepared with apramycin (50 μg/ml), chloramphenicol (25 μg/ml) and kanamycin (50 μg/ml). The prepared mediums were then inoculated with the E. *coli* ET12567/pUZ8002 containing either of the vectors pYD1 (complete *cla* genes) or pYD84 (*cla* genes with knockout of *claC*) and grown overnight at 37°C. Addition of the antibiotics was used to maintain selective pressure (kanamycin for pUZ8002, chloramphenicol for the non-methylating *E. coli* ET12567, and apramycin was selecting for pYD1 or pYD84) while decreasing the possibility of contaminations. Of the 5 mL overnight culture, 500 uL was used to inoculate a new 50 mL LB culture containing the corresponding antibiotics. The new 50 mL culture was then grown at 37°C for 5 hr until an optical density ($O.D._{600}$) between 0.4 to 0.6 was reached. As soon as the desired values were seen, the culture was cooled in ice to slow down the cell growth. While on ice, the culture was transferred into a 50 mL Falcon tube and centrifuged at 4000 rpm for 15 minutes. The supernatant was removed and the pellet was re-suspended in 20 mL of ice cold 10% glycerol. The culture was then centrifuged once more and re-suspended in 10 mL of ice-cold 10% glycerol. One final centrifugation and re-suspension in 1 mL 10% glycerol generated the required competent *E. coli*.

Conjugation Procedure

Of the prepared *S. albus* spore stock, 10 uL was added to 500 uL of 2 X YT medium (16 g/L Bacto tryptone, 10 g/L yeast extract, 5 g/L NaCl, then adjusted to pH of 7.0 with NaOH) and then heat shocked at 50°C for 10 minutes. Once the heated cells had cooled to RT, 500 uL of the competent *E. coli* stock was added and well mixed. The mixture was then centrifuged at 6000 rpm for 5 minutes. The supernatant was removed and the pellet was re-suspended in the residual liquid. On two MS agar plates containing 10 mM $MgCl_2$, the mixture of *E. coli* and S. *albus* was then carefully spread out. The plates were incubated overnight at 30°C. Following incubation, the MS agar plates were overlaid with 0.5 mg nalidixic acid and 1.25 mg apramycin in 200 uL of water. The plates were allowed to dry before sealing the plates once more for incubation at 30°C. Colonies that appeared over the next few days were then replicated on a new MS agar plate containing apramycin and kanamycin to minimize the possibility of contaminations. To further rule out false positives, the replicated colonies were then cultured under appropriate conditions to confirm the expected biological productions.

Feeding Experiment

Chlorinated arcyriaflavin was first used to optimize the feeding conditions. A total of 60 mL of dechlorinated R5 medium K_2SO_4 0.25 g/L, $MgSO_4 \cdot 7H_2O$ 12.27 g/L, glucose 10 g/L, Casamino acids 0.1 g/L, Bacto yeast extract 5 g/L, $CaCO_3$ 2 g/L, chlorine containing trace elements 2 mL/L ($ZnSO_4$ 84.4 mg/L, $FeSO_4 \cdot 7H_2O$ 205.7mg/L, $CuSO_4 \cdot 5H_2O$ 14.65 mg/L, $MnSO_4 \cdot H_2O$ 8.54 mg/L, $Na_2B_4O_3 \cdot 10H_2O$ 10 mg/L and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 10 mg/L), pH 6.7-7.0) was prepared. Then 300 uL of 1 g/L of chlorinated arcyriaflavin in MeOH was added to the growth medium. *S. albus* expressing the *cla* cluster but with a *ΔclaC* knockout was then used to inoculate each of the 12 x 5 mL medium. The prepared cultures where then cultivated at 30°C with 220 rpm for a length of time ranging from 0 to 10 days. One sample for each day was extracted with EtOAc (3 x 5 mL). The organic phase was concentrated in vacuum and re-dissolved in 1 mL MeOH for HPLC analysis under the protocol discussed in the Instrumentation section.

The aqueous phase leftover was tested with pH probe and indicator paper to determine the relative pH.

An alternative method involved similar steps as above however the feeding substrates were not added until large quantities of bacterial colonies were seen (48-72 hr following inoculation).

To further confirm the best time for bacterial culture extraction, 50 mLs of modified R5 mediums were inoculated with the *S. albus* strain and then cultivated for 48 to 72 h before the addition of 500 uL of 1 g/L of prepared 5-bromoarcyriaflavin in MeOH. These cultures were then grown from 12 days. On each day, 2 mL was taken out from each culture and extracted with EtOAc (2x 2 mL). The organic extracts then removed via speedvac. The crude extracts were then redissolved in 40 uL of MeOH for HPLC analysis.

Comparative Feeding

The previously reported *S.albus + cla (Δ claC)* strain was used to inoculate 50 mL cultures of modified R5 medium. Then, following observation of abundant mycelium growth, 500 uL of 1 g/L halogenated arcyriaflavins dissolved in MeOH were added. The culture was incubated for another 48 h. At the end of the incubation period, each 50 mL culture was extracted with 2 x volume equivalents of EtOAc. The organic phase was first suction filtered and then concentrated in vacuum. Isolated samples were re-dissolved in MeOH for further analysis.

Isolation of Brominated Cladoniamide A

Stock samples of *S. albus + cla* (containing the complete cladoniamide gene cluster) were used to inoculate volumes of dechlorinated R5 medium with 0.05% KBr. Prepared cultures were then incubated for 10 to 12 d at 30°C with 220 rpm. Following cultivation, cultures were transferred into separation funnels and extracted with 2x volume equivalents of EtOAc. The isolated organic extracts were then combined suctioned filtered to remove left over culture medium and cell colonies. The filtered EtOAc was then dried under vacuum to afford a dark yellow crude mass. The crude

product was then redissolved in minimal amount of MeOH and loaded onto a normal phase flash column using 100% DCM as the solvent system for initial partition. Each of the isolation fractions was then analyzed by Agilent 1100 high-pressure liquid chromatography (HPLC) with diode array UV detector to determine the location of the cladoniamides. Fractions of interest were combined and loaded on to a Waters Sep-Pak Vac 20cc reverse phase column with H_2O/MeOH (1:1). Finally, pure brominated cladoniamide A was isolated using Phenomenex Luna c18 250 x 10.00 mm 5 µm column with a constant gradient of acetonitrile and water (60:40, both containing 0.05% (v/v) trifluoroacetic acid). ^{1}H NMR (600 MHz MeOH-d_4): 2.96 (s, 3H), 4.13 (s, 3H), 7.16-19(m, 2H), 7.21 (dd, J1=8.6 Hz, J2=1.9 Hz, 1H), 7.46 (d, J=8.6 Hz, 1H), 7.66 (d, J=1.6 Hz, 1H), 8.01 (d, J= 1.8Hz, 1H), 8.16 (d, J=8.9 Hz, 1H). HRESI MS: 480.0191 and 482.0192 *m/z* $[M-H]^-$ (**Figure 59**).

Chapter Three: Results and Discussion

3.1 Bromocladoniamide production by KBr supplementation

Our initial attempts to isolate the bromocladoniamides involved the straightforward addition of 0.05% w/v KBr to an already established cladoniamide production system.[31] A similar procedure had been used previously to produce bromorebeccamycin.[60] In this study, we utilized *Streptomyces albus* J1074 + *cla*, a cladoniamide production system, grown in the previously reported modified R5 medium with KBr supplementation.[37] Unfortunately, following routine cultivation and extraction, no brominated analogs were observed. The only cladoniamides observed (identified by HPLC comparison to authentic standards) were A, B and C (**Figure 22**), even after repeated attempts. A potential problem was that chloride ions, which are much more

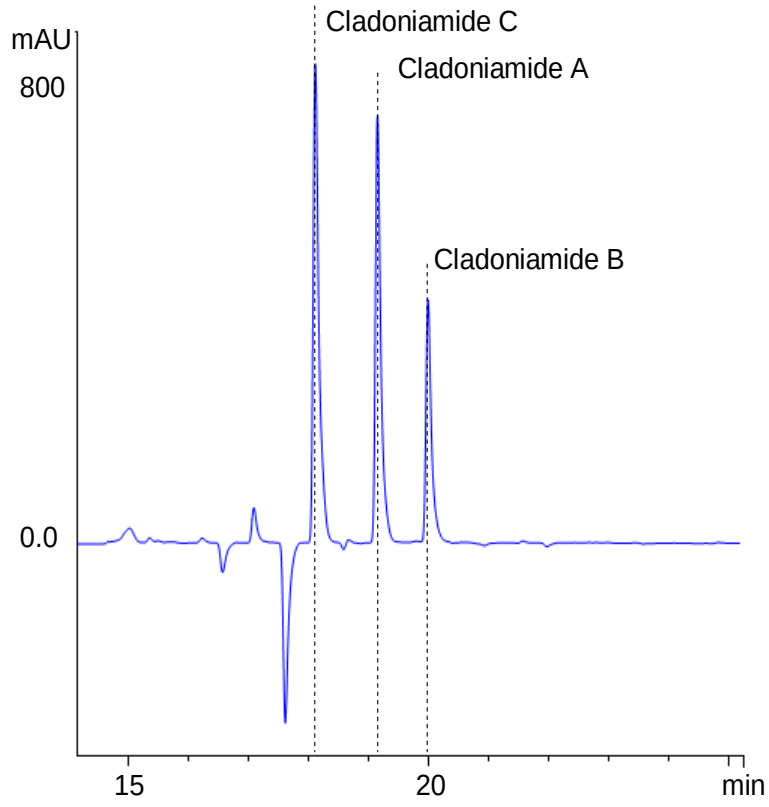


Figure 22 Observed production of previously reported cladoniamide A-C in R5 medium with 0.05% (w/v) KBr supplement.

abundant in the medium, might have been preferentially incorporated into the biosynthetic pathway, thus overshadowing bromide uptake. To address this possibility, the modified R5 medium was "dechlorinated". In particular, all apparent sources of chloride were replaced with suitable alternatives, including replacement of all chloride-containing metal salts. Attempts to culture *S. albus* J1074+*cla* in the modified medium yielded a new peak at the 348 nm detection wavelength that exhibited absorbance spectra highly similar to those of cladoniamide A/B (**Figure 23**). The collected product corresponding to the new peak had *m/z* twin peaks of equal intensity at values of 504.1/506.1 on low a resolution ESI mass spectrometer (**Figure 34**).

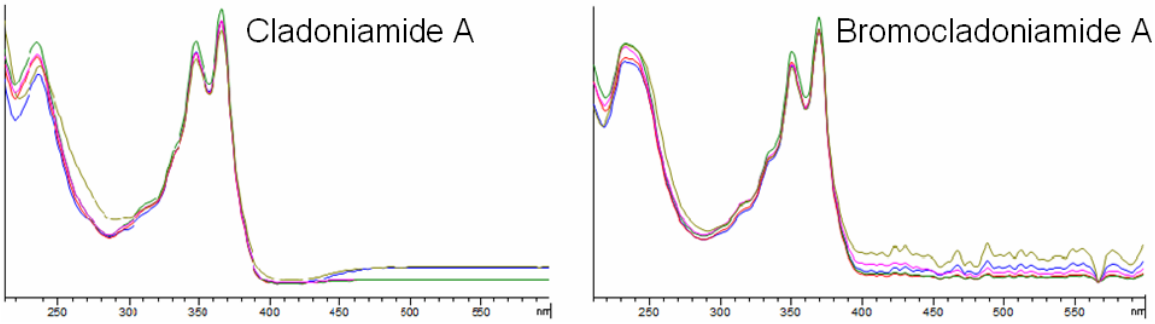


Figure 23 UV-Vis absorbance for cladoniamide A (left) and bromocladoniamide A (right)

The observed mass values were consistent with bromocladoniamide A plus a sodium ion. However, a peak believed to be the chlorinated cladoniamide A still persisted despite removal of all the major chlorine sources within the growth medium (**Figure 24**, cladoniamide A RT =3.7 min, bromocladoniamide A RT=3.8 min). The fact that the cladoniamides A analogues has a similar retention time as cladoniamide A is reasonable considering the similar chemical properties of the two halides.

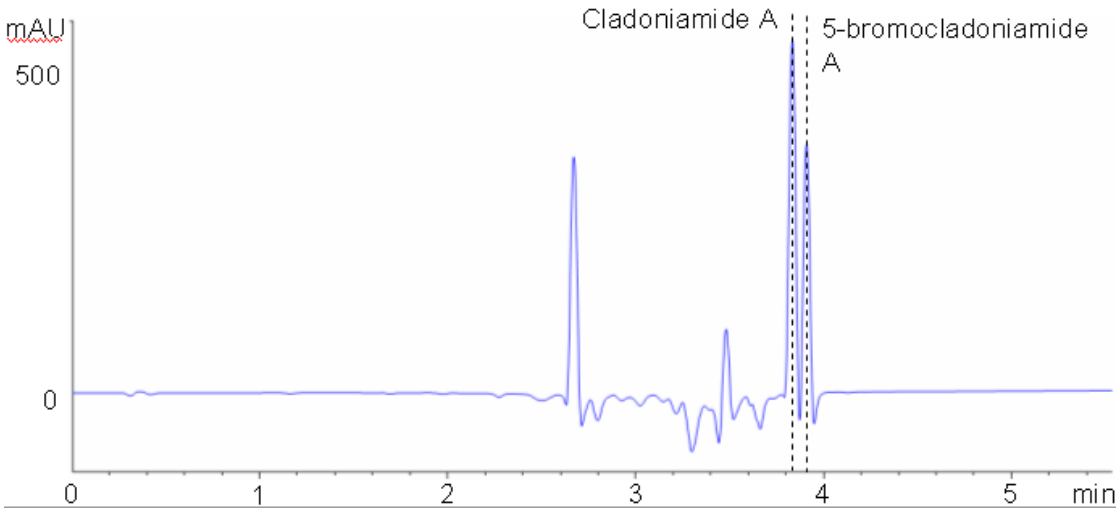


Figure 24 HPLC analysis of purified organic extract from dechlorinated R5 medium with 0.05% KBr supplement. The twin peaks at 3.7 to 3.8 minute are believed to be cladoniamide A and the bromo analog

The observed production of the original cladoniamides, despite removal of the major chloride sources, suggests that the putative 5-tryptophan halogenase, ClaH, may have greater preference for chloride than for bromide. Another possibility is that tryptophan substrates were becoming brominated, but enzymes downstream were hindering the complete production due to poor interaction between substrate/active sites. With the original growth in modified R5 medium, approximately 9 mg/L of cladoniamide A can be isolated[31]. With the "dechlorinated" medium, only meager quantities of the putative bromocladoniamide A can be isolated. As a result of the low yield, over twenty-four liters of the KBr supplemented culture were extracted EtOAc before isolating a small quantity of the putative bromocladoniamide A. The purified product was then characterized. First, the recorded ^{1}H NMR data are consistent with the expected signals corresponding to 5-bromocladoniamide A. ^{1}H NMR (600 MHz MeOH-d_4): 2.96 (s, 3H), 4.13 (s, 3H), 7.16-19 (m, 2H), 7.21 (dd, J1=8.6 Hz, J2=1.9 Hz, 1H), 7.46 (d, J=8.6 Hz, 1H), 7.66 (d, J=1.6 Hz, 1H), 8.01 (d, J= 1.8Hz, 1H), 8.16 (d, J=8.9 Hz, 1H). The singlets at δ 2.96 and 4.13 ppm were believed to correspond to the N-methyl and O-methyl groups with three protons each. The aromatic region between δ 7.16 to 8.16 ppm integrated to approximately seven protons, which is expected based on the chemical structure of cladoniamide A. Furthermore, the signal at 8.01 ppm appeared as a doublet with a coupling constant of 1.8 Hz. This was reasoned to be the isolated proton next to the halide, which in turn can only couple to the proton that is over four bonds away. However, the isolation of the bromocladoniamide A was further confirmed by HRESI-

MS analysis. The observed twin bromine $[M-H]^-$ *m/z:* were 480.0191 and 482.0192 (**Figure 59**), corresponding to the formula of $C_{22}H_{15}N_3O_5Br$ (calcd for $C_{22}H_{15}N_3O_5Br$, 480.0195). Unfortunately, the low quantity of pure sample prevented more in depth NMR analysis.

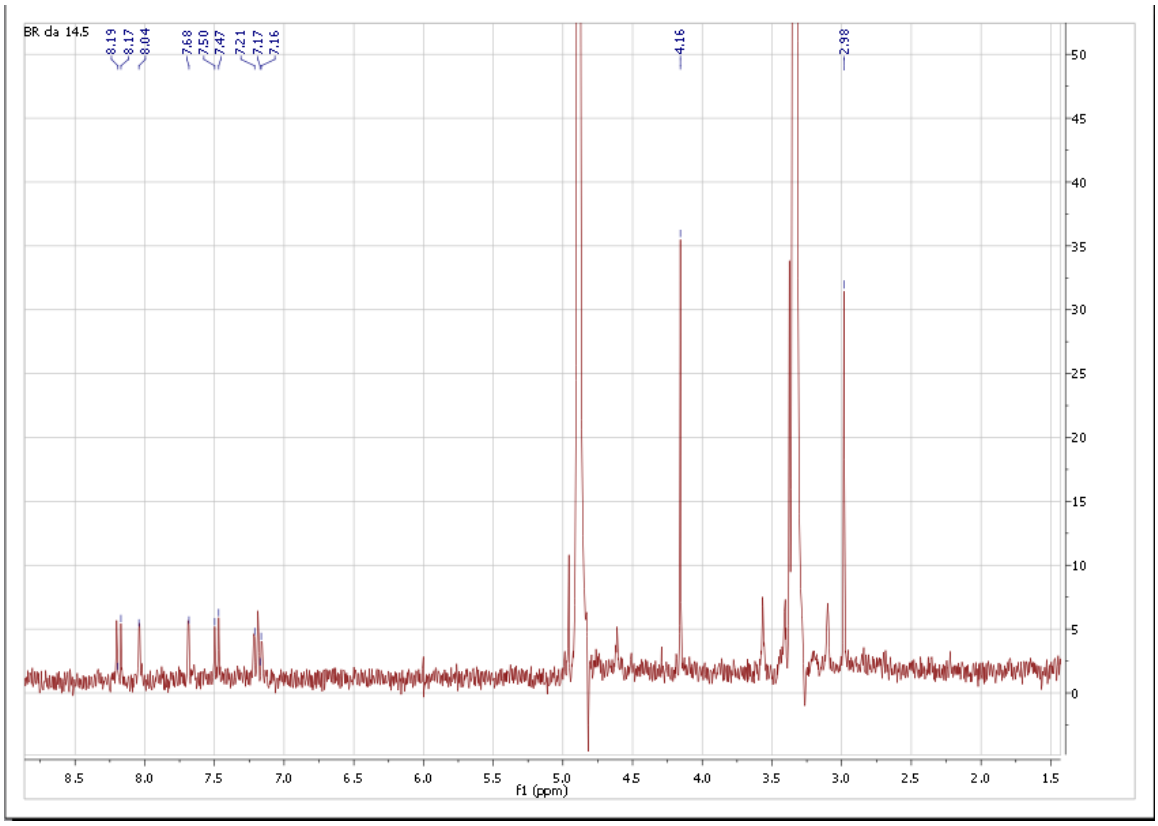


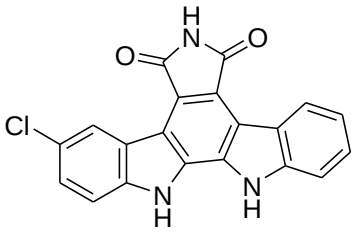
Figure 25 ^{1}H NMR of 5-bromocladoniamide A in MeOH d_4

Therefore, it can be concluded that the production of bromocladoniamide A can be induced by KBr supplement into the growth medium. However, the production level is quite low. A reasonable explanation could be poor enzyme/substrate interaction somewhere along the biosynthetic pathway. As mentioned, previous indolocarbazole biosynthesis studies has shown the tryptophan halogenase substrate specificity allows for addition of both bromide and chloride.[59,60,64] However, there appears to be some preference for chloride. For example, both the chlorinated and brominated analogs of thienodolin can be isolated by modifying the culturing medium.[59] Yet, when comparing the cultured extract of a mutant in which the halogenase was inactivated, and specific halogenated tryptophans were added to reestablish the system, there was clearly more thienodolin produced than the 6-bromo analog.[59] It was suggested that the production of

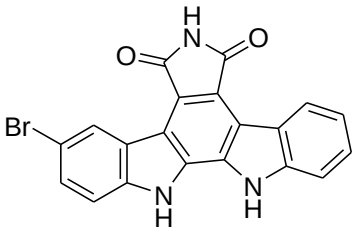
the chloro analog was more abundant due to the preference for the chlorine substituent by downstream enzymes. Another possibility is that the bottle-neck for bromocladoniamide may also lie downstream from the tryptophan halogenase. ClaO is a reasonable candidate. Studies by the Sherman group into the biological activities of the homologous RebO an amino acid oxidase, have shown that there may be selective preference on both the type of halogenation and location.[29] They were able to overexpress and purify the protein from an *E. coli* vector. Then substrates including L-Tryptophan, 1-Methyl-L-Tryptophan, 5-Fluoro-L-Tryptophan, and 7-Chloro-L-Tryptophan were compared.[29] The rates of turnover were analyzed by measuring the rate of catalytic H_2O_2 production.[29] Of those substrates, 7-Chloro-L-Tryptophan displayed the highest k_{cat} value of 39.82 min^{-1}, with a k_{cat}/K_m value of 453 $mM^{-1}min^{-1}$.[29] In comparison, the corresponding k_{cat}/K_m values of the other three substrates were 7.94 mM^{-1} $min^{-1}$for L-Tryptophan, 1.07 $mM^{-1}min^{-1}$ for 1-Methyl-L-Tryptophan and 7.80 $mM^{-1}min^{-1}$ for 5-Fluoro-L-Tryptophan.[29] Interestingly, L-tryptophan also showed decreased conversion compared to the 7-chloro analog, suggesting that the halide positioning may be important for catalysis.[29] Based on these observations, it is reasonable to believe that ClaO, a homologue of RebO, may have selectivity toward 5-chloro-L-tryptophan.

There are also other possible sites of strict selectivity along the biosynthetic pathway beside ClaO. Despite the insights into the biosynthesis of cladoniamides, the relative specificity of many downstream enzymes for the 5-chloro versus 5-bromo precursors remains unknown. Indeed, a study on the last four enzymes in the pathway (X1, M1, X2, and M3) revealed that non-chlorinated metabolites could progress through the complete pathway, and the accumulation of cladoniamide C in both wild-type and heterologous expression systems suggests that the downstream enzymes might not be specific for the type of halogenation at C-5.[43] These possibilities raised the intriguing idea that feeding of synthetic intermediates to the latter part of the pathway could enable production of new derivatives. To probe this process, we decided to construct a feeding experiment where both chlorinated and brominated indolocarbazole substrates could be fed into the latter part of the cladoniamide pathway. The system which we decided to employ was a knockout mutant of the *cla* gene cluster in which *claC* has been inactivated. The encoded enzyme, ClaC, is highly similar to RebC/StaC, which control the oxidative

outcome following cyclization of CPA (**12**).[37] Furthermore, the in-frame deletion of *claC* had a polar effect on *claP* which is the cytochrome P450 enzyme responsible for cyclization of CPA (**12**) to generate **13**. Thus, the only observed product following the inactivation of *claC* was CPA.[37] Without ClaP/C, chlorochromopyrrolic acid (**13**) cannot be converted into chloroarcyriaflavin (**27**).[37] Specific halogenated substrates could be fed into this system to re-establish the complete pathway. Selectivity of these enzymes can then be inferred based on the relative conversion of the different substrates. The substrates of choice were 3-bromoarcyriaflavin and 3-chloroarcyriaflavin.



83 3-chloroarcyriaflavin A



87 3-bromoarcyriaflavin A

Figure 26 Desired substrates for feeding experiment

3.2 Synthesis of 3-chloroarcyriaflavin A (82) and 3-bromoarcyriaflavin A (86)

Due to the potential to generate a wider variety of cladoniamides, we decided to undertake a synthetic plan that allowed for incorporation of numerous modifications simply based on the starting material. The initial work involved the preparation of the two major indole subunits which would then be coupled together. First, we needed to generate an indole acetamide. While there are various ways to obtain the acetamide, we found that there is a large source of commercial halogenated indole carboaldehydes which appeared as a good starting point. The formed acetamide would then be coupled to indolylglyoxylate to generate the desired carbon backbone. Another way to introduce more derivatives would be to employ indolylglyoxylates with different substituents.

3.2.1 Synthesis of 3-chloroarcyriaflavin (82)

As described in the previous sections, we decided to start with halogenated indolecarbaldehydes that were purchased from Sigma-Aldrich. The first reaction used was to convert the indolecarbaldehyde into the corresponding nitrile with $NaBH_4$ as reported by the Casey group.[64] There should only be one possible site of reduction due to the limited number of functional groups. The reaction was monitored by TLC. Following reduction, the original spot of the starting material was no longer seen while a new and more polar compound could be seen under UV analysis. Due to the lone pair on the indole ring, the newly formed alcohol is able to undergo an E1 elimination to generate an alkene intermediate. After complete reduction, KCN was then slowly added to the reaction mixture. The cyanide nucleophile is believed to attack the formed reactive alkene intermediate to form the nitrile product. TLC of the second step of the reaction showed the formation of a new UV active spot that was less polar than the alcohol intermediate.

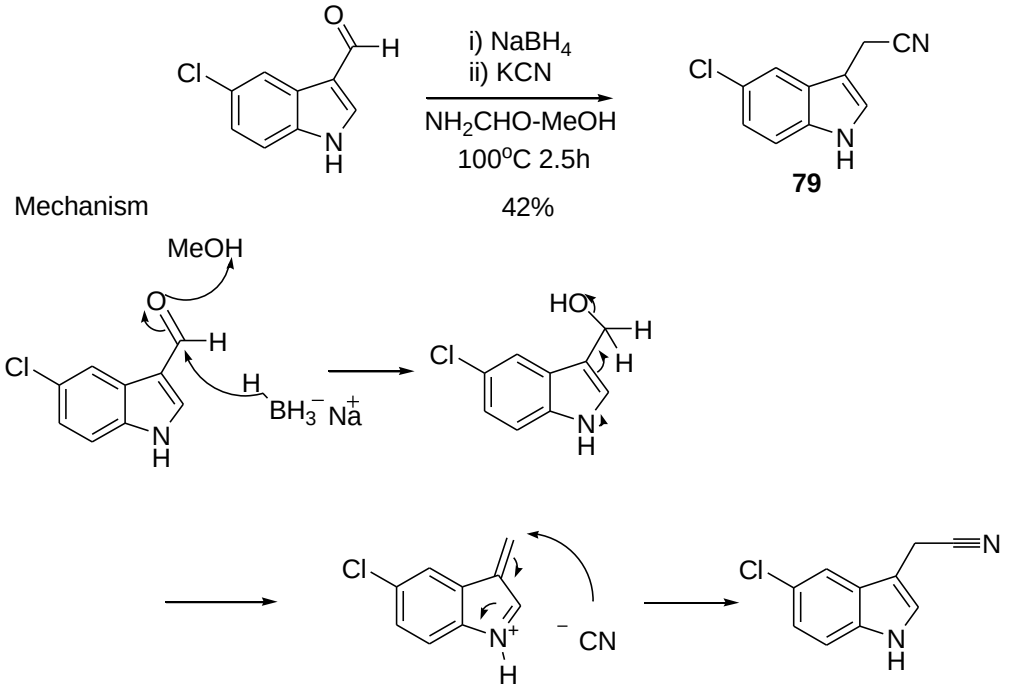


Figure 27 Mechanism of the reduction of indolecarbaldehydes with $NaBH_4$, followed by addition of potassium cyanide to form the indole-acetonitrile **80**

The synthesis of the halogenated indole-acetonitrile was confirmed by ESI-MS and ^{1}H NMR. For (5-chloro-1*H*-3-indolyl) acetonitrile (**79**), the ion $[M+Na]^+$ at *m/z* 213.3/215.3 (**Figure 36**) was seen. For ^{1}H NMR analysis in $CDCl_3$, there were a total of seven hydrogens detected. Four of the peaks were within the aromatic region, corresponding to the C-H carbons around the indole ring. A broad peak at 8.3 ppm is believed to the N-H signal. The final two protons can be seen at 3.8 ppm as a large singlet which corresponds to the CH_2 group in between the indole and nitrile functional groups. The observed values were identical to the published values, suggesting that the correct compounds were formed.[64]

The second reaction in the pathway was conversion of the formed halogenated indole-acetonitriles into the corresponding acetamide. This reaction was carried out as reported by the Casey group.[64] Compound **79** was refluxed in *tert*-butyl alcohol with KOH for 3 h. Due to the partial positive charge on the nitrile carbon, the nitrile carbon is thought to be an electrophile, reacting with the abundant hydroxide. The formed imine intermediate would then quickly rearrange to form the stable amide. Completion of the reaction was monitored by TLC by loss of starting material. The resulting basic reaction mixture was neutralized with concentrated HCl during workup to fully protonate the formed amide. The formed product was (5-Chloro-1H-3- indolyl) acetamide (**80**).

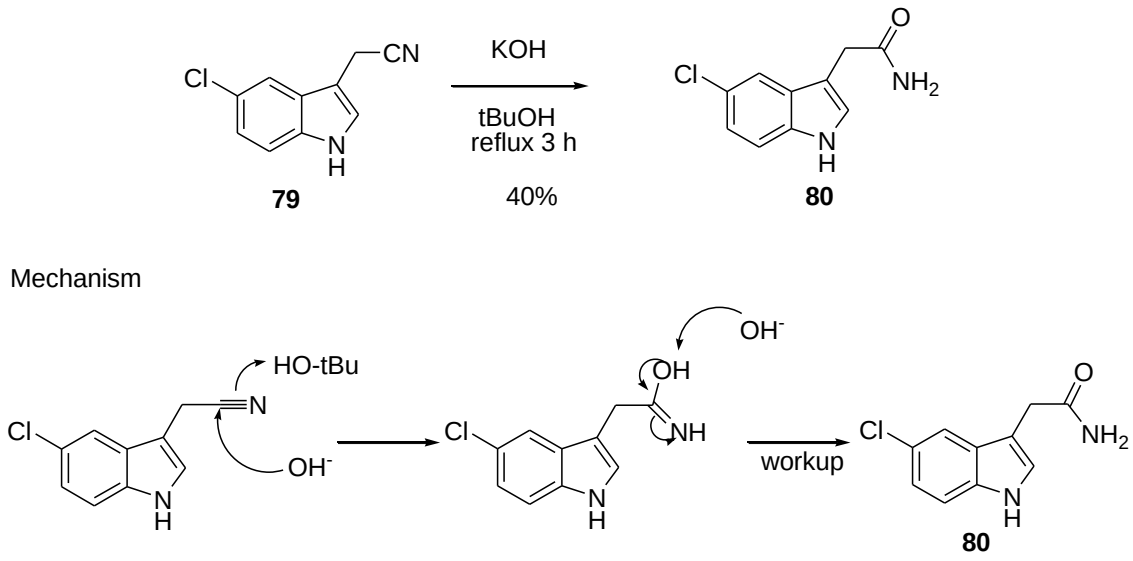


Figure 28 Conversion of the indole acetonitrile (**80**) into the corresponding acetamide (**81**) under a basic condition

Characterization of the compound was through ESI-MS and ^{1}H NMR. For (5-chloro-1*H*-3-indolyl) acetamide (**80**), $[M-H]^-$ at 207.3/209.3 *m/z* and $[M+Na]^+$ at 231.4/233.4 *m/z* were observed. ^{1}H NMR, which was conducted in DMSO-d_6, showed the α-carbon protons can be seen at 3.45 ppm as a singlet that integrates to two protons. The N-H proton from the indole ring can be seen at 11.07 ppm. The two amidic protons can be separately seen as broad peaks at 6.85 and 7.39 ppm. The remaining peaks integrate to exactly four protons and are believed to be the protons around the indole ring. Based on the coupling constants, the proton at 7.32 ppm is vinylically coupled to the proton at 7.18 ppm which is in turn is four-bond coupled to the proton at 7.74 ppm. These signals should account for the benzene hydrogens. Finally, the proton at C2 of the indole can be seen at 7.26 ppm as a doublet with the coupling constant of 2.3 Hz which suggests distant coupling from the N-H proton at 11.07 ppm.

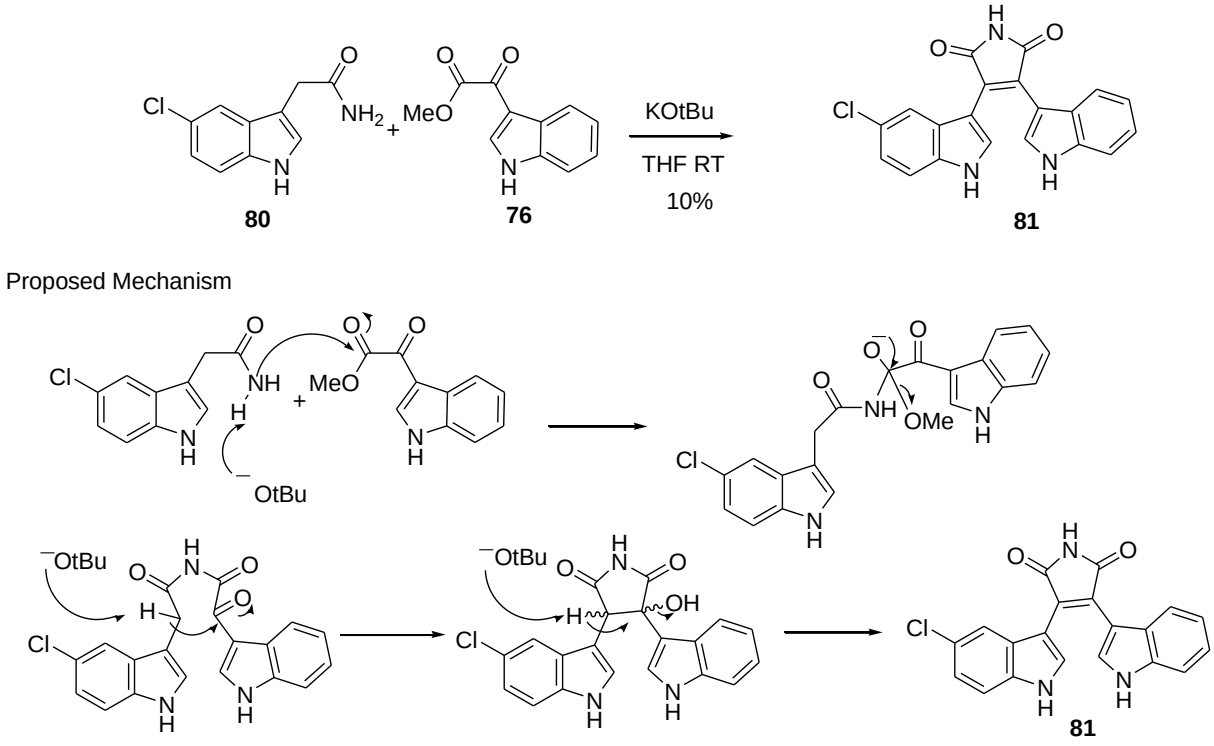


Figure 29 Mechanism of Perkin condensation of synthesized indole-acetamide (**81**) with methyl indolyl-3-glyoxylate into compound (**82**).

Following the formation of the halogenated indole-acetamide (**80**), the next step was to couple it with methyl indolyl-3-glyoxylate through a Perkin condensation to generate the desired bisindole (**81**) as described by Yang *et al.* [65] The formed acetamide, indolylglyoxylate and excess potassium *tert*-butoxide were dissolved in THF. While

cooled at 0°C the mixture was stirred for 1 h. Following the first hour, the mixture was warmed to RT with further stirring for 3 h. The starting materials were believed to undergo a Perkin condensation where the amide first nucleophilically attacks the ester functional group on the indolylglyoxylate, with elimination of MeOH generating the imide. Then, due to the relatively basic conditions of the mixture, the α-hydrogen of the acetamide can be deprotonated and the resulting nucleophilic carbon anion in turn add to the ketone functional group on the glyoxylate side. The formed intermediate would be a bisindole succinimide that can further undergo an E1cB (Elimination Unimolecular conjugate Base) dehydration to afford 3-(5-chloro-3-indolyl)-4-(3-indolyl)-1*H*-pyrrole-2,5-dione (**81**). As reported previously by Yang *et al*, the isolated product was red/orange colored solids. Initial confirmation of desired products was by ESI-MS. For the chlorinated dione $[M-H]^-$ at 360.2/362.2 *m/z* and $[M+Na]^+$ at 384.3/386.3 *m/z* were observed (**Figure 40)**. NMR data also supports the expected product.

When looking at the ^{1}H NMR of the chlorinated analog in DMSO-$_{d6}$, a total of twelve hydrogens were detected. Nine of the signals were between 6.5 to 8.0 ppm which should be the C-H hydrogens along the two indole rings. Of the remaining three proton signals, the broad singlet at 10.94 ppm is believed to be the succinimide proton as it was seen correlated to carbon signals of 126.9/128.2 and 172.9 ppm in the HMBC. The two H NMR signals at 11.74 and 11.81 were thought to be the N-H hydrogens from the two indole rings. In HMBC analysis, they can be individually seen correlated to two similar sets of four different carbon signals (105.3, 125.2, 129.2, and 136.5 for 11.74/105.4, 126.6, 130.5, 134.4 for 11.81). Of these carbon signals only 129.2 and 130.5 can be seen directly bonded to hydrogens at 7.80 and 7.76 ppm based on the HSQC. The proton signals at 7.76 and 7.80 ppm appeared as singlets which suggest that they were

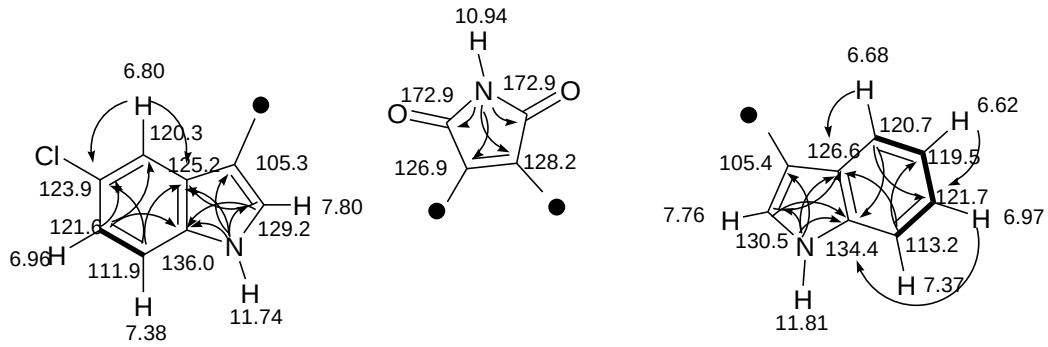


Figure 30 Assignment of proton and carbon NMR signals for compound **81**

the enamine hydrogens at C-2 and C-2'. This then left a total of eight carbon and seven proton signals unaccounted for. Of the eight carbons, only the one at 123.9 ppm was not directly connected to hydrogen based on HSQC data, which in turn suggested that it was the one connected to the halide at C-5. Then three separate protons can be seen correlated to the carbon at 123.9 ppm (6.80, 6.96, and 7.38 ppm). Of those three proton signals, 7.38 ppm appeared as a doublet that was vinylically coupled to the proton at 6.96 ppm. The peak at 6.96 ppm appeared as a doublet of doublets where it was further split by the proton at 6.80 ppm. However, the coupling constant between 6.80 ppm and 6.96 ppm was only 1 Hz, suggested that it was a distant coupling where 6.80 ppm is the proton connected to C-4. Furthermore, these protons were seen correlated to the aforementioned carbon signals of 125.2 ppm at C-3a and 136.0 ppm at C-7a. This established the indole ring containing the halide. The four hydrogen signals left were 6.62, 6.68, 6.98, 7.37 ppm which were reasoned to be the four benzene protons located on the second indole ring (C-4' to C-7'). The COSY analysis was also in an agreement where observed correlations included the signals between 6.68 and 6.62 ppm, 6.62 and 6.98 ppm, 6.98 ppm was further correlated to 7.37 ppm. The observed HMBC correlation between these four protons and nearby 126.6 ppm of C-3a' and 134.4 ppm of C-7a' further confirms the assignments. Therefore, it was believed that the correct product was formed from synthesis.

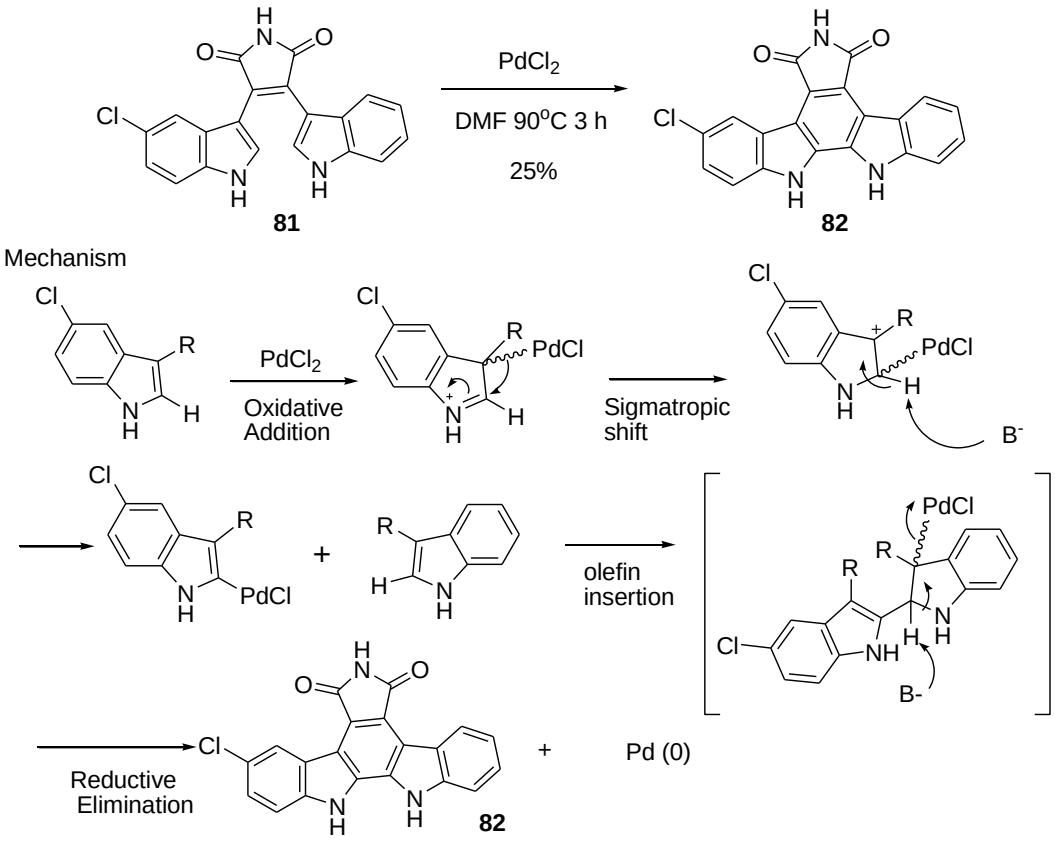


Figure 31 Fujiwara-Moritani oxidative Heck reaction cyclization of **81** to **82**

The last reaction involved the cyclization of the central ring to generate the halogenated arcyriaflavin (**82**) as reported by Yang *et al*.[65] In order to do so, the synthesized dione (**81**) was subjected to Fujiwara-Moritani oxidative Heck conditions. The starting materials were dissolved in DMF with greater than 2 equiv of $PdCl_2$ and then refluxed for 3 h. Following refluxing, a new spot could be seen by TLC that was very closely located to the starting material. However, while the starting material was red/orange in color, the new spot formed was yellow. ESI-MS data on the purified yellow products are in an agreement with the desired products. The *m/z* of 358.2/360.2 were seen for the 3-chloroarcyriaflavin and matches $[M-H]^-$ (**Figure 42).** Further confirmation on the chemical structure came from ^{1}NMR data in DMSO-d_6. The proton signals observed were quite similar to the ones seen for the compound **81**. However, based on integration of the peaks, only ten protons can be seen instead of the original twelve, as expected from the cyclization process that removes the two hydrogens at C-2 and C-2'. Similar to the 2D NMR data of the above diones, the succinimide proton was observed at' 11.07 ppm for the chloro analog (11.08 ppm for the bromo analog). The 11.07 ppm proton was then

seen correlated to the carbonyl carbon at 171.2/171.3 ppm and then alkene carbon at 121.5 ppm. The N-H protons from the indole rings were observed at 11.84 and 11.90 ppm. These protons were also seen correlated to two different sets of four carbon signals (114.5, 122.7, 129.3, and 139.1 ppm correlated to 12.05 ppm; 115.9, 121.5, 129.1, and 140.3 ppm correlated to 11.96 ppm). However, unlike the dione NMR data, the proton signals at C-2 and C-2' can no longer be seen from any of the spectrums. This then left once again eight carbon signals with the one without a HSQC signal at 119.9 ppm reasoned to be the carbon connected to the halide. The rest of the signals were assigned in a similar fashion to the dione compounds. To further confirm the production of the desired compound, the UV absorbance of the starting dione and the formed halogenated arcyriaflavins were compared and found to be distinct. By contrast, the synthesized halogenated arcyriaflavins were compared to a non-halogenated arcyriaflavin A standard,[37] the UV absorbance were identical (**Figure 33**). Major peaks can be seen at ~240/~320 nm, and a small bump can been seen at ~420 nm. Therefore it can be confidently concluded that the desired substrates of 3-chloroarcyriaflavin have been synthesized.

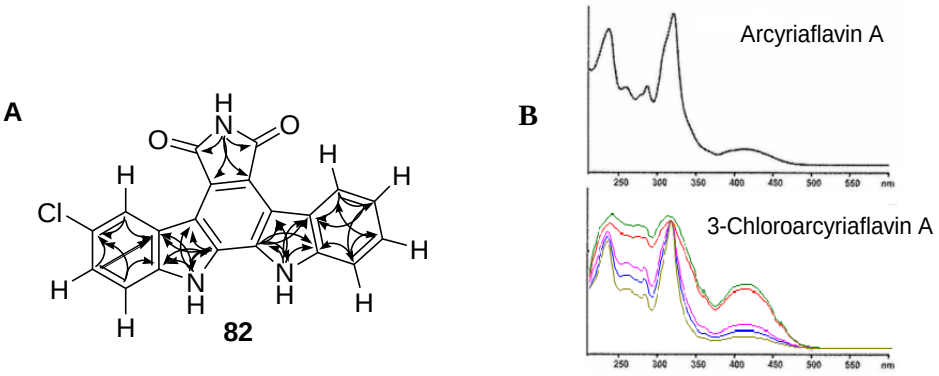


Figure 32 A) HMBC correlations seen in the synthesized 3-chloroarcyriaflavin (**83**). B) Comparison between the UV absorbance of synthesized 3-chloroarcyriaflavin with arcyriaflavin A standard.

3.2.2 Synthesis of 3-bromoarcyriaflavin (86)

The steps taken to synthesize the 3-bromoarcyriaflavin were identical to the 3-chloroarcyriaflavin except 5-bromo-indolecarbaldehyde was used as the starting material. Confirmation of the synthesis of 5-bromo-1H-3-indolyl acetonitrile (**83**) was by ESI-MS

and ^{1}H NMR. The *m/z* values of 257.3/259.3 $[M+Na]^+$ (**Figure 37**) were seen along with *m/z* value of 233.2/235.2 $[M-H]^-$. The detected protons signals in $CDCl_3$ were also in an agreement with published values.[64] For the production of the bromo acetonitrile, the yield was slightly lower than the chloro analog, only 40%. Compound **83** was then converted to 5-bromo-1H-3-indolyl acetamide (**84**). The identity of the product was confirmed by ESI-MS and ^{1}H NMR. The *m/z* values of 251.2/253.2 $[M-H]^-$ were observed (**Figure 39**). The observed proton signals were also consistent with published values.[64] Yield of this reaction was found to be 37%, which was also slightly lower when compared to the conversion of the chloro analog. Compound **84** was then coupled with indolyl-3-glyoxylate (**77**) to generate **85**. The formation of the dione was first confirmed by ESI-MS where *m/z* values of 404.2/406.2 $[M-H]^-$ and 428.3/430.3 $[M+Na]^+$ were observed (**Figure 41**). 2D NMR analysis was then used to confirm the structure. The yield for the synthesis of **85** was only 10%, identical to what was observed with the chloro analog. Compound **85** was then treated with $PdCl_2$ to generate 3-bromoarcyriaflavin (**86**). The expected *m/z* values of 402.2/404.2 $[M-H]^-$ were observed (**Figure 43**). Signals observed in NMR analysis were also very similar to those seen in the 3-chloroarcyriaflavin. Yield of cyclization of **85** to generate **86** was identical to the values observed for the chloro substrate, at 25%.

3.3 Feeding Experiment

With the establishment of the viable feeding system and synthesis of the required in chemical substrates, the next step involved selectively supplementing the growth of the modified *S. albus* strain. There has only been one brief mentioning of feeding arcyriaflavin into *S. albus* therefore there was a need to optimize the feeding conditions.[35]

To optimize for favorable feeding protocol, the first attempt involved direct supplement of the initial growth medium with the synthesized chlorinated arcyriaflavin. This substrate was used for optimization because a larger quantity was available. Furthermore, the chemical properties of the desired product cladoniamide A are known and it is likely a better substrate for the enzymes in the pathway. 300 μL of **82** (1mg/mL in MeOH) was added to a 60 mL volume of modified R5 medium. Of the 60 mL, 12x 5

mL samples were prepared and inoculated with Δ*claC S. albus* which was then grown at 30°C. Over the next 10 days, one sample was analyzed each day to monitor the production of the converted product and the relative change in pH of the medium.

From this three interesting observations were made. First, no conversion of the arcyriaflavin into cladoniamide was seen even after 10 d. Second, the pH was observed to increase over time. Initial pH was at 6.5 which then reached 8.2 by 10 d. Third, the arcyriaflavin may have been modified in an unintended fashion. Over time, a new peak nearby to the chlorinated analog could be seen in the HPLC trace with the identical UV absorbance to arcyriaflavin. This was even observed in the blank culture where no bacteria were added. One possible explanation was that the chlorinated arcyriaflavin was somehow modified. These modifications in turn could have interfered with the conversion of it into cladoniamide. A separate 50 mL culture supplemented with the same concentration of substrate was also analyzed to confirm that low conversion was not the issue. However, similar results were seen. Therefore, it appeared that direct supplementation of the substrate during the preparation of the medium was not effective.

Based on the hypothesis that arcyriaflavin can be modified if exposed to medium conditions for prolonged periods, the next attempt involved cultivating the cell culture first. As previously, 50 mL cultures of modified R5 medium were inoculated. However, chlorinated arcyriaflavin was only supplemented following observation of an abundance of bacterial mycelium (48 to 72 h after inoculation). After another 48 h of incubation at 30°C, cultures were extracted and analyzed with HPLC. A new peak was observed under at 348 nm detection wavelength. It had the exact retention time and expected UV absorbance as the chlorinated cladoniamide A. This process was then replicated for the brominated substrate and the corresponding product peak for brominated cladoniamide A was observed. The retention times were identical to those seen during the KBr supplemented growth of *S. albus* expressing the complete *cla* cluster. Despite a relatively low quantity of purified product, the low resolution mass spectrum matched exactly to those seen before. Therefore, the previously isolated compound believed to be the 5-bromocladoniamide A was likely correctly assigned. Furthermore, the constructed feeding system appeared to be functional.

The next goal was to compare the relative conversion of the two halogenated substrates. In order to do so, an estimated equal molar quantities of each substrate was added to the same 50 mL culture which was then cultivated under the aforementioned conditions. To ensure that any observations were repeatable, the cultures were prepared in triplicate, where the medium was prepared as a whole and then accurately divided. To further ensure that relatively equal quantities of each substrate were added, a 50 mL volume of the prepared medium was set aside and then supplemented along with the grown bacterial cultures. This 50 mL volume was then immediately extracted using EtOAc, and following HPLC analysis, displayed nearly identical detection level for the two substrates. Therefore, the triplicate bacterial cultures should all have started with nearly identical quantities of each substrate. Following the above cultivation protocol, each of the 50 mL cultures were extracted with a total of 90 mL of EtOAc. The organic extracts were then individually evaporated to dryness and the resulting crude product was re-dissolved in 1 mL MeOH. From 1 mL solutions, 10 uL of each were subjected to HPLC analysis.

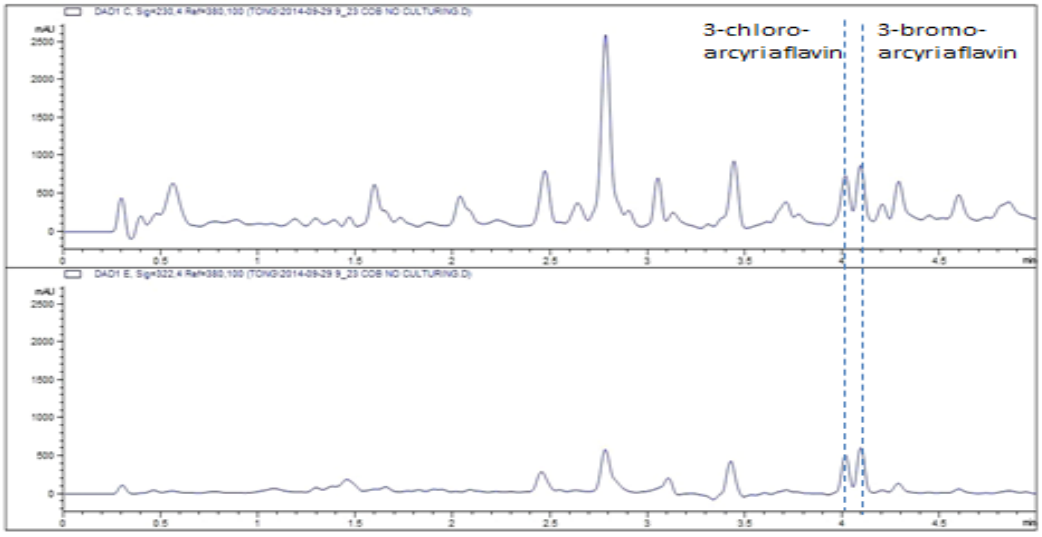


Figure 33 EtOAc extracts of medium supplemented with equal amounts of 3-chloro arcyriaflavin and 3-bromo arcyriaflavin without cultivation. The added substrates can be seen between 4.0 to 4.2 minute. Scans at two different wavelength, 230 (top) and 322 nm (bottom).

An HPLC run of the blank cultures where no substrates were supplemented displayed the expected result of no conversion products. Then, with the 50 mL cultures that were supplemented with equal molar of each substrate, twin peaks can be seen between 3.7 to 3.9 minutes at 348 nm wavelength of detection. All of the replicates of the combined feeding exhibited nearly identical results of relatively equal quantities of

conversion. For example, when looking at one of the replicates, the measurement of the HPLC peak areas of the produced cladoniamide A had a value of 1523.9, while 5-bromocladoniamide A had a similar value of 1582.2. When considering that about equal amounts of substrates were added and equal amounts of converted products were seen following bacterial growth, it appeared that the selectivity leading to limited bromocladoniamide production with KBr supplement perhaps lies upstream from ClaC. The observation also suggests that ClaX1/X2 do not have a specific preference in terms of chlorine and bromine at the C-5 position of tryptophan indole.

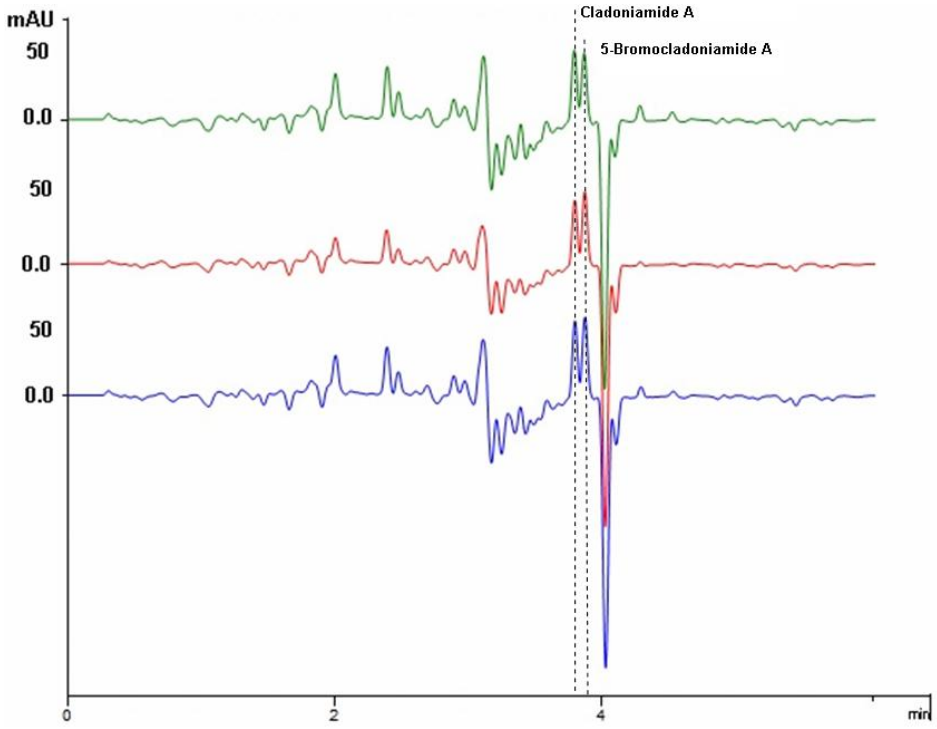


Figure 34 Triplcates of 50 mL cultures of the *S. albus* strain with both feeding substrates added which were then extracted following 48 h of cultivation. Expected twin peaks between 3.7 and 3.8 minute can be seen under 348 nm.

An attempt was also made to isolate 5-bromocladoniamide A. First two liters of modified R5 were supplemented with an estimate of 1 mg of 3-bromoarcyriaflavin each. Following two days of growth, the 2 liters were extracted with 2 volumes of EtOAc. Unfortunately, only a small quantity of the 5-bromocladoniamide A was isolated and then analyzed with mass spectrometry to give the aforementioned *m/z* values. To further probe the limited production seen, 50 mL cultures were prepared and supplemented with 500

uL of 5-bromoarcyriaflavin (1 g/L). The cultures were then grown for a total of 12 day. Each day, 2 mL of the culture were extracted and analyzed by HPLC to relatively determine the conversion of the substrate. It was seen that conversion occurred most around 2 and 3 d. Then, over time, the formed bromocladoniamides appeared to decrease. At the 12th day, only a very small signal was seen. This observation was in an agreement with a previous report on the degradation of cladoniamides even under mildly basic conditions[32]. However, this suggested that the extraction around 2 days should have yielded the largest quantities of the desired product. In turn, it appeared that while the precursor directed biosynthesis is functional, the rate of conversion in this case was very low.

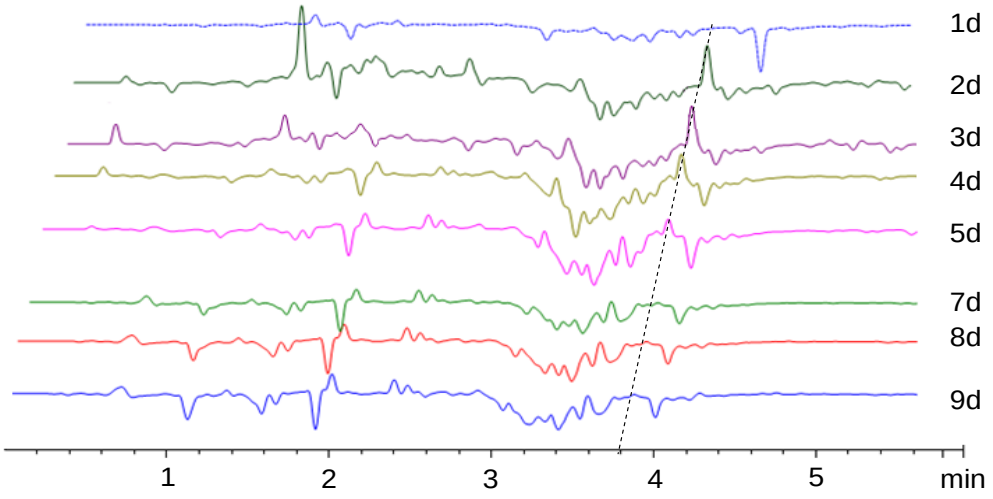


Figure 35 Time course production of 5-bromoarcyriaflavin through precursor directed biosynthesis. The highest product peaks were seen around 2 and 3 d. Then the formed cladoniamide appeared to decompose over time.

3.5 Future Directions

It can be concluded that the brominated analog of cladoniamide A can be produced by direct KBr supplement into the growth medium. Furthermore, precursor directed biosynthesis through feeding of arcyriaflavin analogous to a *claC* mutant also appeared to be a suitable method. However, one persistent issue was the low quantities of the brominated analog isolated. Despite attempted efforts through both of the production methods, very small quantities of the purified product were obtained. The

characterization of the brominated cladoniamide A would be greatly bolstered if ^{13}C and 2D NMR data could be obtained.

In order to address the low conversion of substrate seen, one possible experiment would be to pre-treat the bacterial cells with certain conditions such as electroporation or DMSO washes to weaken the bacterial cell wall. Similar techniques have been employed to transport genetic material into the cell, it may also allow for more efficient uptake of the added substrates. Another interesting aspect would be to further probe the substrate specificity of ClaH and ClaO. It is possible that they have selective preference for 5-chloro tryptophan substrates. Then, by analyzing the enzyme active sites, it is conceivable to alter the specificity by selective replacements of key amino acid residues.

In terms of further applying the synthetic/biosynthetic production system that has been established in this study as a whole, a very wide array of molecules can potentially be applied. As previously mentioned, the synthetic scheme was designed to allow for incorporation of different indolecarbaldehydes as the initial starting material. Luckily, the commercially available indolecarbaldehydes come with all the possible halogens (other than astatine which is radioactive) located along the indole tryptophan ring. Another point where varieties can be introduced is the addition of the methyl-(1-methylindolyl)-3-glyoxylate. A wide array of different glyoxylate derivatives are also readily available which then allow for modifications of the second indole ring. Then similar steps can be taken to synthesize the desired arcyriaflavin substrate. Feeding the synthesized substrate into the cladoniamide biosynthetic system could produce an array of cladoniamide analogous. Considering that attempts to induce the biological production of exotic halide compounds can be challenging due to the toxic nature of free floating ions, this reported system could be a very efficient method to generate fluorinated and even iodinated cladoniamides.

Chapter Four: Conclusion

Natural products still remain one of the major sources of pharmaceutically interesting drugs. Cladoniamides are a recently discovered group of potential anticancer natural products. Rather than having the indolocarbazole scaffold often seen with bisindole molecules, the cladoniamides have an indolotryptoline core structure. Of the known indolotryptoline compounds, some have even been found to be nanomolar inhibitors of cancerous cells. Here we report the isolation of the brominated analog of cladoniamide A. We were able to promote the production of 5-bromocladoniamide A through two different methods. One method was by removing the chlorine sources within the growth medium and then supplementing in KBr as the bromine source. Interestingly, despite the active effort to minimize chlorine concentration, the production of cladoniamide A was still observed along with limited production of the bromo analog. Another method employed was precursor directed biosynthesis where synthesized halogenated substrates were fed into the cladoniamide biosynthetic machinery, leading to the production of the expected chloro and bromo analogs. An alternative facet of the feeding study was to probe for possible halogen selectivity along the pathway which resulted in the observed lower production of the bromo cladoniamide. Through first disrupting the pathway by the inactivation of the arcyriaflavin biosynthesis and then supplementing in 3-chloro and 3-bromo analogs, we were able to observe relatively equal conversion of the substrates. This implies that, collectively, the downstream enzymes ClaX1, M1, X2, and M3 were not affected by the halogen substituent. Therefore, the possible substrate selectivity may be upstream at either ClaH or ClaO. Considering the number of possible arcyriaflavin derivatives that can be chemically synthesized, this system offers a way to generate a wide array of cladoniamide derivatives.

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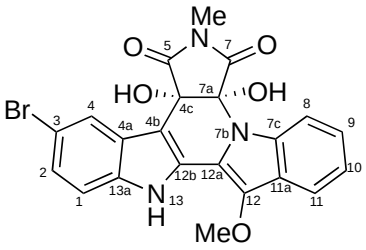
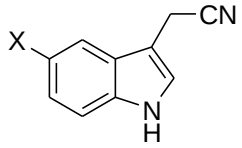
Appendix

Table 1 ^{1}H NMR of bromocladoniamide A at 600 MHz in MeOH-d_4, compared to the previously reported cladoniamide A[21].



Position	5-Bromocladoniamide A	Cladoniamide A reference
1	7.46 (d, J=8.6 Hz, 1H)	7.56 (d, J=8.6 Hz, 1H)
2	7.16 (dd, J_1 =8.6 Hz, J_2 = 1.9 Hz, 1H)	7.20 (dd, J1= 8.6 Hz, J2=2.0 Hz, 1H)
3		
4	8.01 (d, J=1.8 Hz, 1H)	7.94 (d, J=2.0 Hz, 1H)
4a		
4b		
4c		
5		
6		
7		
7a		
7b		
7c		
8	8.16 (d, J=8.9Hz, 1H)	8.15 (d, J=7.9 Hz, 1H)
9	7.16-19 (m, 2H)	7.23 (t, J=7.9 Hz, 1H)
10		7.12 (t, J=7.9 Hz, 1H)
11	7.66 (d, J=1.6Hz, 1H)	7.71 (d, J=7.9 Hz, 1H)
11a		
12		
12a		
12b		
13	Not observed	11.62 (s, 1H)
13a		
NMe	2.96 (s, 3H)	2.88 (s, 3H)
OH (C4c)	Not observed	7.21 (s, 1H)
OH (C7a)	Not observed	8.29 (s, 1H)
OMe	4.13 (s, 3H)	4.11 (s, 3H)

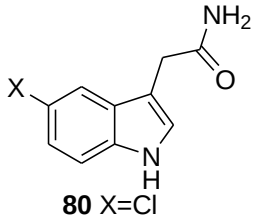
Table 2. ^{1}H NMR data of compounds (**79** and **83**) recorded at 300 MHz in $CDCl_3$.and compared to reported reference values [44] .



79 X= Cl
83 X= Br

2-(5-Bromo-1*H*-indol-3-yl) Acetonitrile Reference	**79**	**83**
3.73 (s, 2H)	3.85 (s, 2H)	3.85 (s, 2H)
7.19-7.33 (m, 3H)	7.25-7.38 (m, 3H)	7.34-7.42 (m, 3H)
7.69 (s, 1H)	7.60 (s, 1H)	7.77(s, 1H)
	8.30 (br, 1H)	8.27 (br, 1H)

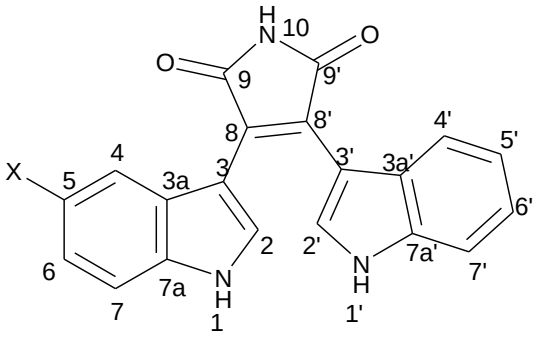
Table 3 ^{1}H NMR data of compounds (**80** and **84**) recorded at 600 MHz in DMSO-d_6, compared to reported values for **84** [44] .

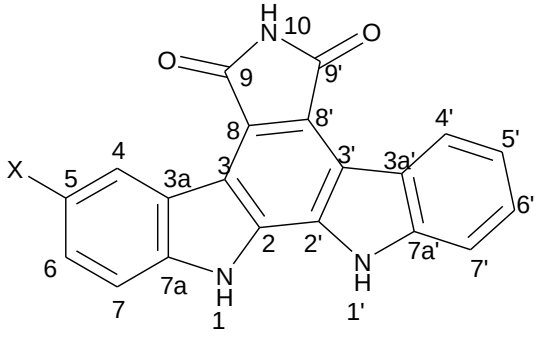


80 X=Cl
84 X=Br

2-(5-Bromo-1*H*-indol-3-yl) Acetamide Reference	**80**	**84**
3.44 (s, 2H)	3.45 (s, 2H)	3.45 (s, 2H)
7.15-7.38 (m, 4H)	6.85 (br, 1H)	6.85 (br, 1H)
	7.07 (dd,J1=1.9Hz, J2=8.6Hz, 1H)	7.18 (dd, J1=1.9Hz, J2=8.6Hz, 1H)
	7.27 (d, J=1.9Hz, 1H)	7.26 (d, J=2.2Hz, 1H)
	7.35 (s, 1H)	7.32 (d, J=8.6Hz, 1H)
	7.38 (s, 1H)	7.39 (br, 1H)
7.73 (s, 1H)	7.60 (d, J=1.7Hz, 1H)	7.74 (d, J=1.7Hz, 1H)
11.07 (s, 1H)	11.07 (s, 1H)	11.08 (s, 1H)

Table 4. ^{1}H NMR data of compounds (**81-82** and **85-86**) recorded at 600 MHz in DMSO-d_6[71]





81 X = Cl
85 X = Br

82 X = Cl
86 X = Br

	81	**85**	**82**	**86**
1	11.74 (br, 1H)	11.81 (br, 1H)	12.05 (br, 1H)	11.94 (br, 1H)
2	7.80 (d, J= 2.6Hz, 1H)	7.74 (s, 1H)		
3				
3a				
4	6.81 (d, J=1.9Hz, 1H)	6.95 (s, 1H)	8.99 (d, J1=1.9Hz, 1H)	8.99 (d, J=2.2Hz, 1H)
5				
6	6.96 (dd, J1= 2.0Hz J2=8.4Hz, 1H)	7.08 (d, J=8.5Hz, 1H)	7.58 (dd, J1=2.0Hz, J2=8.6Hz, 1H)	7.59 (m, 1H)
7	7.38 (d, J=8.4Hz, 1H)	7.32 (d, J=8.5Hz, 1H)	7.82 (d, J=8.6Hz, 1H)	7.87 (d, J=8.6Hz, 1H)
7a				
8				
9				
10	10.94 (br, 1H)	10.93 (br, 1H)	11.07 (br, 1H)	11.08 (br, 1H)
1'	11.81 (br, 1H)	11.75 (br, 1H)	11.96 (br, 1H)	11.90 (br, 1H)
2'	7.76(s, 1H)	7.79 (s, 1H)		
3'				
3a'				
4'	6.68 (t, J=8.5Hz, 1H)	6.68 (t, J=7.2Hz, 1H)	8.98 (d, J=7.9Hz, 1H)	8.98 (br, 1H)
5'	6.62 (td, J1= 0.7Hz, J2=8.7Hz, 1H)	6.62 (t, J=7.1Hz, 1H)	7.37 (td, J1=0.7Hz, J2=7.8Hz, 1H)	7.37 (td,J1=2.2Hz, J2=8.1Hz, 1H)
6'	6.98 (td, J1=1.0Hz, J2=8.0Hz, 1H)	6.96 (d, J=7.9Hz, 1H)	7.57 (td, J1=1.1Hz, J2=7.0Hz, 1H)	7.58 (m, 1H)
7'	7.39, (d, J=7.1Hz, 1H)	7.40 (d, J=8.0Hz, 1H)	7.83 (d, J=8.1Hz, 1H)	7.83 (d, J=8.1Hz, 1H)
7a'				
8'				
9'				

Table 5. ^{13}C NMR data of compounds (**81-82** and **85-86**) recorded at 600 MHz in DMSO-d_6. (NS is when the signals were difficult to confidently assign)[71]

	81	**85**	**82**	**86**
1				
2	129.2	130.4	129.3	129.7
3	105.3	NS	114.5	115.9
3a	125.2	NS	122.5	121.5
4	120.3	123.8	123.2	123.2
5	123.9	123.5	119.9	126.9
6	121.7	124.7	126.6	126.6
7	111.9	114.6	112.1	113.9
7a	136.0	134.6	139.1	138.8
8	126.9	NS	121.5	120.1
9	172.9	172.9	171.4	171.3
10				
1'				
2'	130.5	130.4	129.1	129.1
3'	105.4	NS	115.9	114.5
3a'	126.6	NS	121.5	122.7
4'	120.7	122.6	124.3	124.4
5'	119.5	119.6	120.5	120.5
6'	121.7	121.6	126.9	126.6
7'	113.2	112.1	113.9	112.3
7a'	134.4	136.1	140.4	140.3
8'	128.2	NS	121.5	119.9
9'	172.9	172.9	171.2	171.2

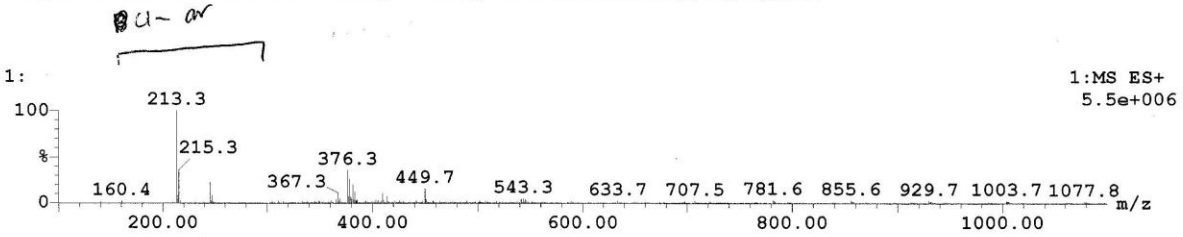
Mass Spec Report: Page 1

File:Ryan114-1 Sample: 1 Date:08-Jul-2014
Time:16:21:54 Group Name:Ryan Method:C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp
User Sample ID: OAMS#:Ryan114-1 Vial:1:36

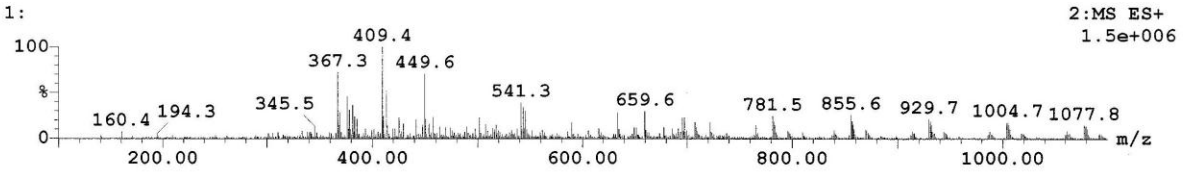
Printed: Tue Jul 08 16:26:19 2014

Sample Report:

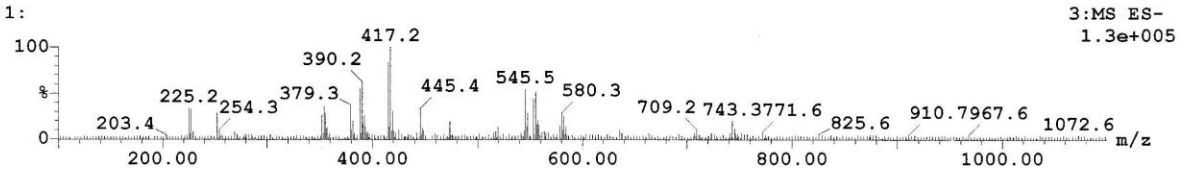
Sample 1 Instrument Method C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp



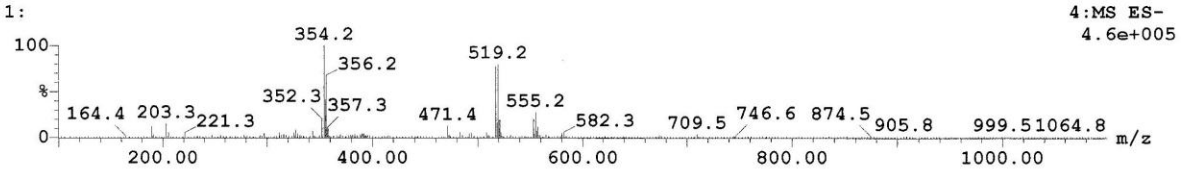
No elements were found.



No elements were found.



No elements were found.



No elements were found.

Figure 36 Low RES ESI MS analysis of compound **79**

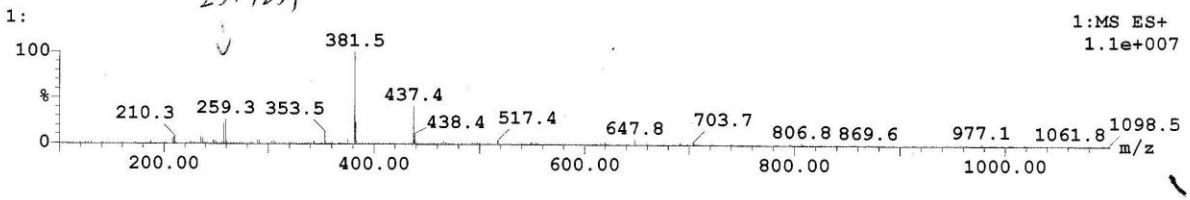
Mass Spec Report:

File:Ryan113-2 Sample: 2 Date:02-Jul-2014
Time:14:57:40 Group Name:Ryan Method:C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp
User Sample ID:br cn OAMS#:Ryan113-2 Vial:1:36

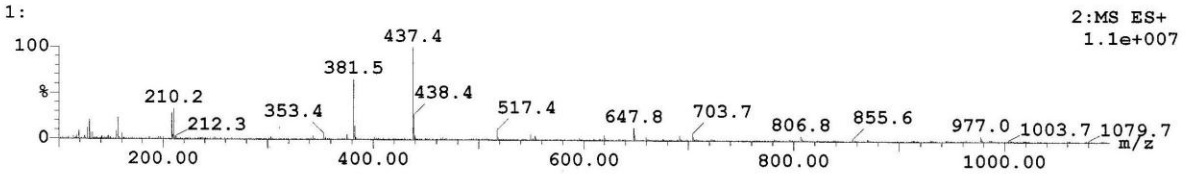
Printed: Wed Jul 02 15:01:38 2014

Sample Report (continued):

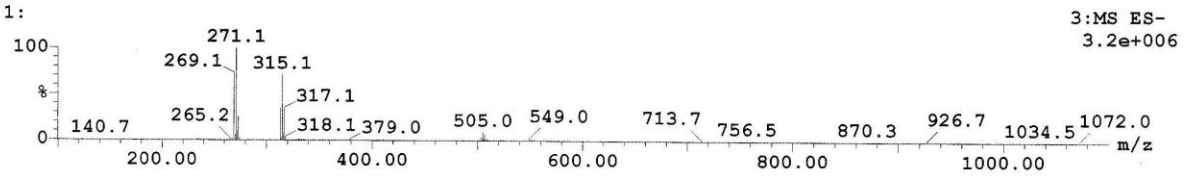
257 /259



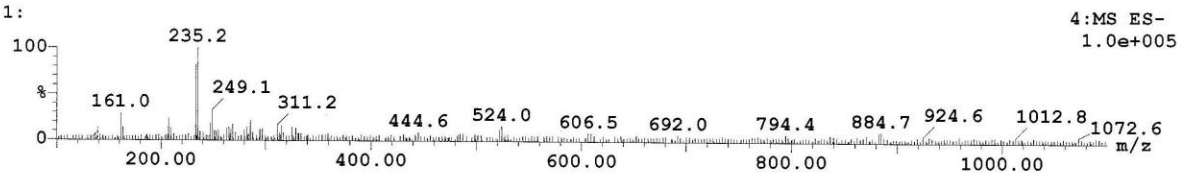
No elements were found.



No elements were found.



No elements were found.



No elements were found.

233/235

3A 213

3 20

Figure 37 Low RES ESI MS analysis of compound **83**

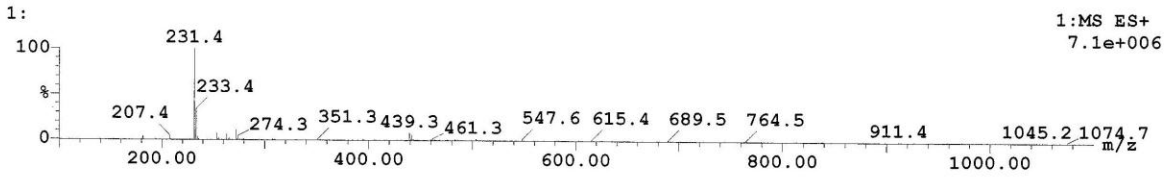
Mass Spec Report:

File:Ryan117-1 Sample: 1 Date:15-Jul-2014
Time:12:12:24 Group Name:Ryan Method:C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp
User Sample ID:1 OAMS#:Ryan117-1 Vial:1:65

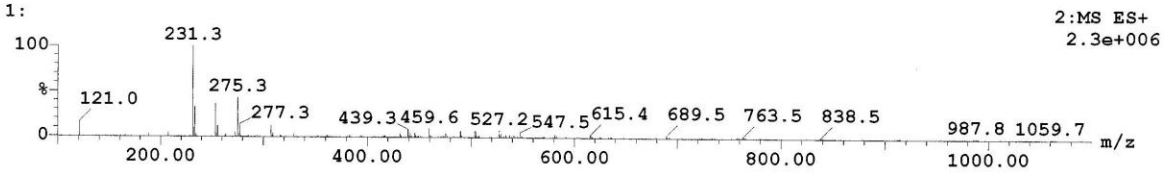
Printed: Tue Jul 15 12:28:33 2014 Cl- NH_2

Sample Report:

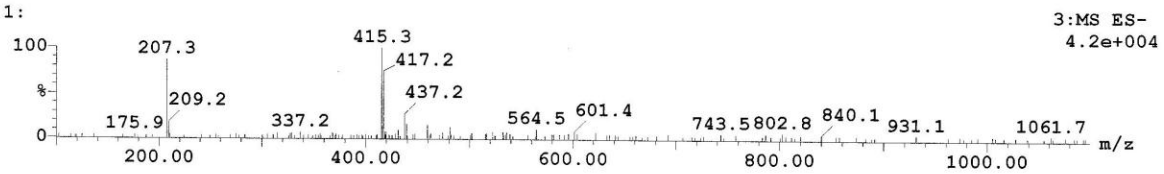
Sample 1 Instrument Method C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp



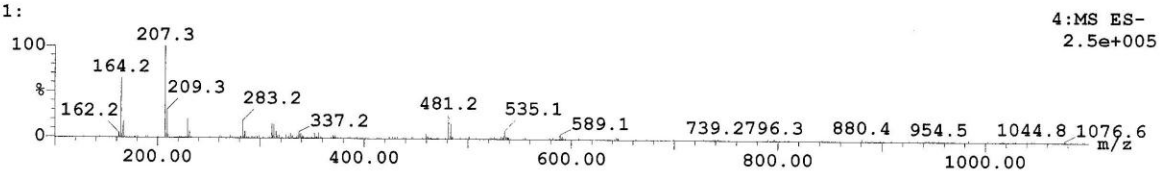
No elements were found.



No elements were found.



No elements were found.



No elements were found.

Sample 2 Instrument Method C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp

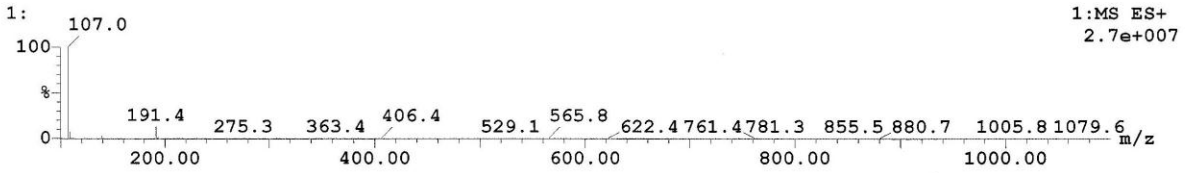
Figure 38 Low RES ESI MS analysis of compound **80**

Mass Spec Report: Page 3

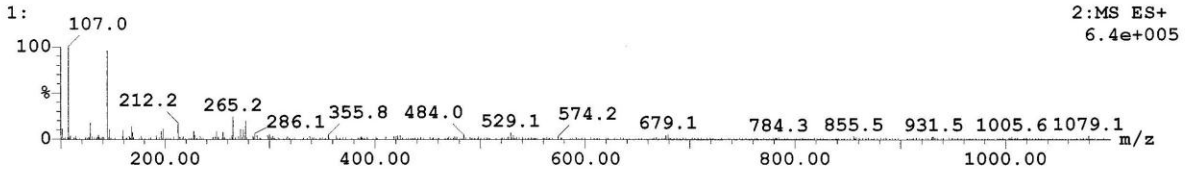
File:Ryan118-3 Sample: 3 Date:17-Jul-2014
Time:11:21:50 Group Name:Ryan Method:C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp
User Sample ID:br nh2 pure OAMS#:Ryan118-3 Vial:1:41

Printed: Thu Jul 17 11:26:00 2014

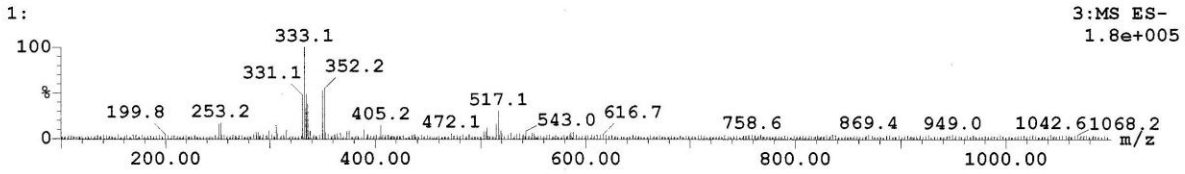
Sample Report (continued):



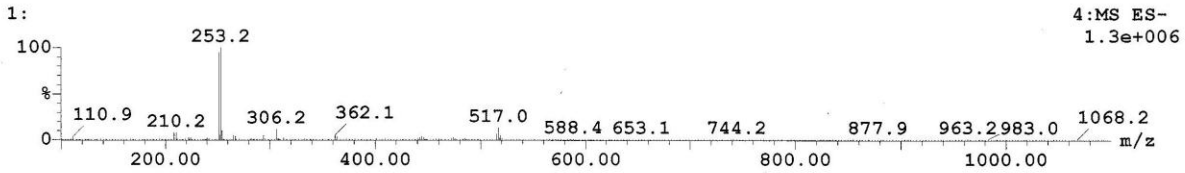
No elements were found.



No elements were found.



No elements were found.



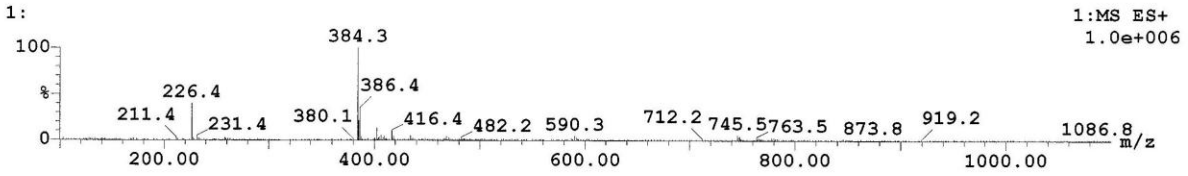
No elements were found.

Figure 39 Low RES ESI MS analysis of compound **84**

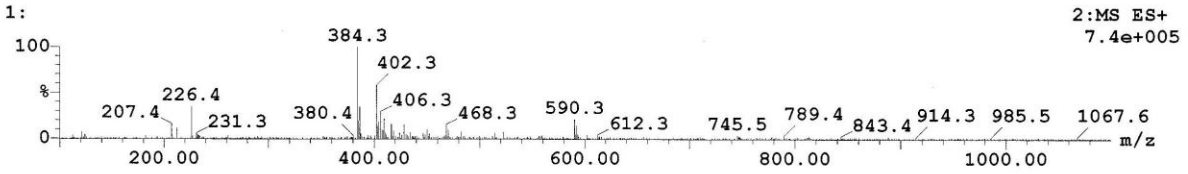
File:Ryan130-2 Sample: 2 Date:01-Aug-2014
Time:16:07:49 Group Name:Ryan Method:C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp
User Sample ID:cl link OAMS#:Ryan130-2 Vial:1:76

Printed: Fri Aug 01 16:12:15 2014

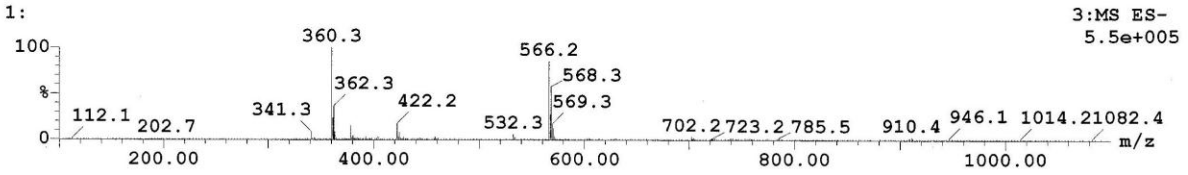
Sample Report (continued):



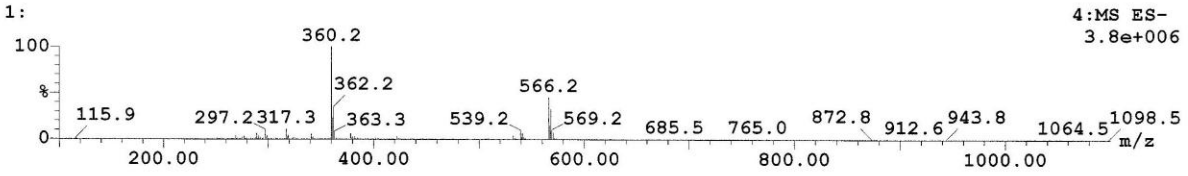
No elements were found.



No elements were found.



No elements were found.



No elements were found.

Figure 40 Low RES ESI MS analysis of compound **81**

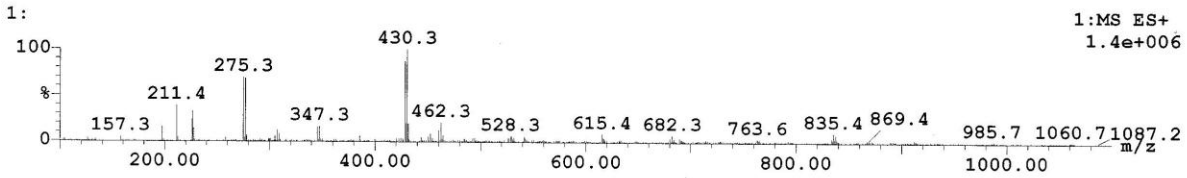
Mass Spec Report:

File:Ryan128-1 Sample: 1 Date:29-Jul-2014
Time:10:31:15 Group Name:Ryan Method:C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp
User Sample ID: OAMS#:Ryan128-1 Vial:1:119

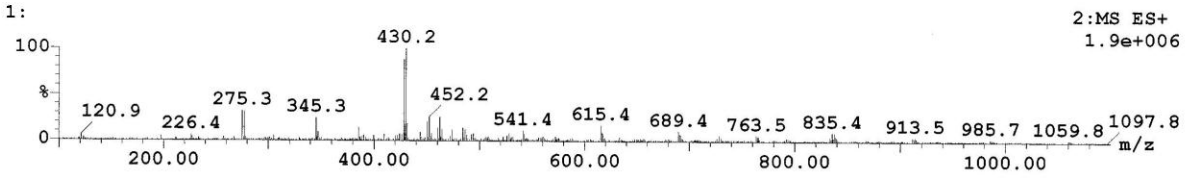
Printed: Tue Jul 29 10:35:25 2014

Sample Report:

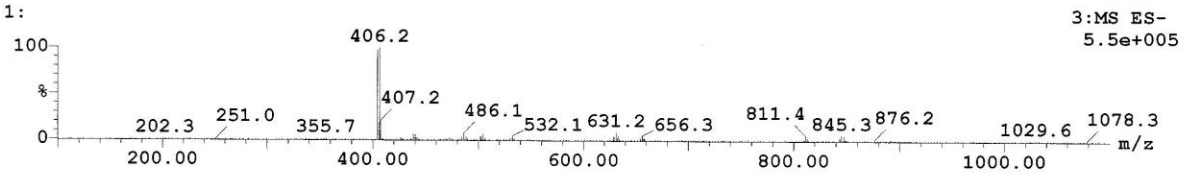
Sample 1 Instrument Method C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp



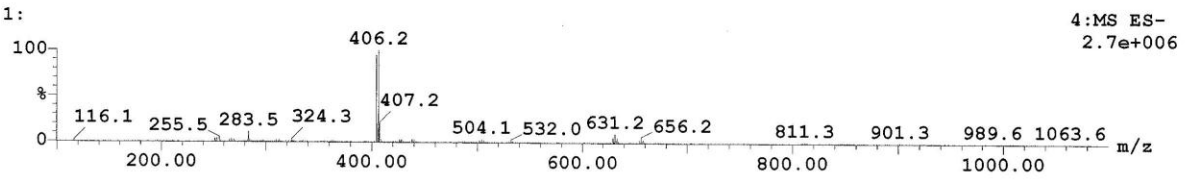
No elements were found.



No elements were found.



No elements were found.



No elements were found.

Figure 41 Low RES ESI MS analysis of compound **85**

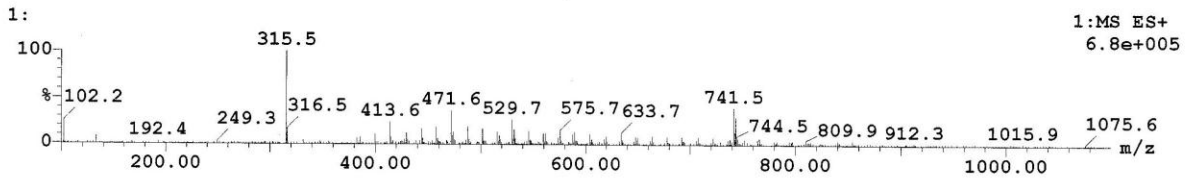
Mass Spec Report:

File:Ryan134-1 Sample: 1 Date:06-Aug-2014
Time:14:23:42 Group Name:Ryan Method:C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp
User Sample ID:cl cy OAMS#:Ryan134-1 Vial:1:98

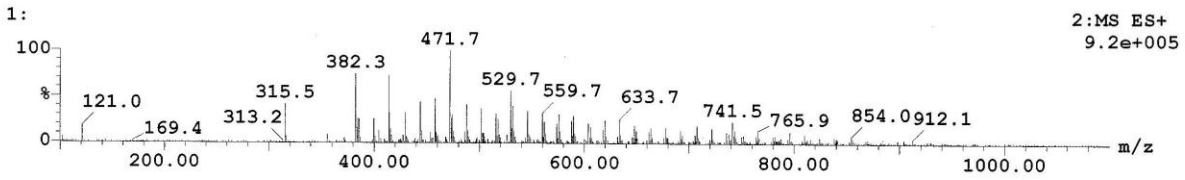
Printed: Wed Aug 06 14:27:53 2014

Sample Report:

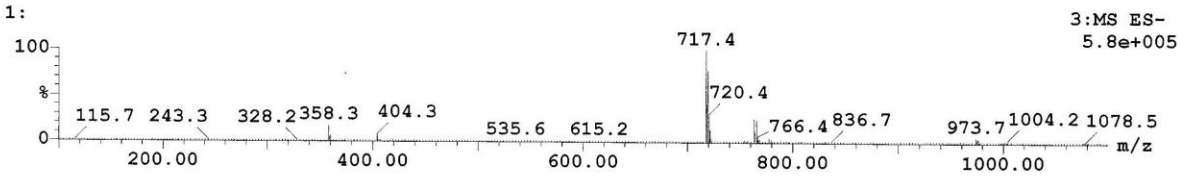
Sample 1 Instrument Method C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp



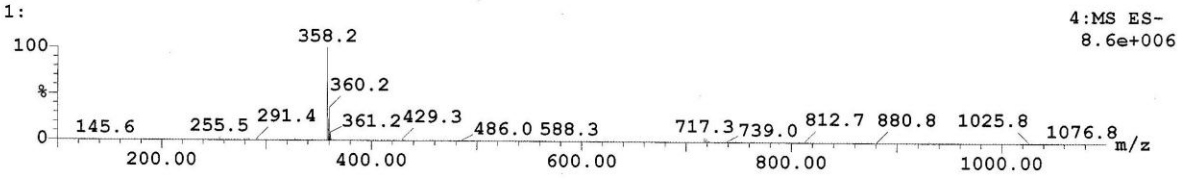
No elements were found.



No elements were found.



No elements were found.



No elements were found.

Figure 42 Low RES ESI MS analysis of compound **82**

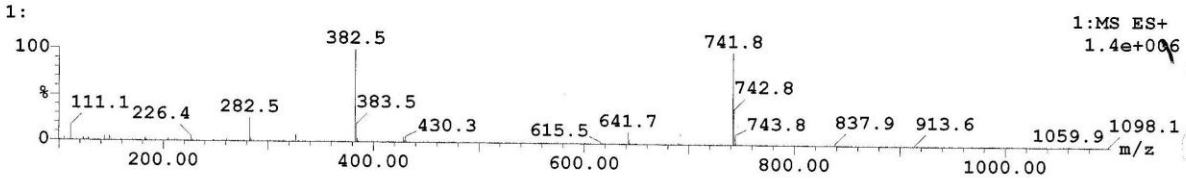
Mass Spec Report:

File:Ryan129-1 Sample: 1 Date:31-Jul-2014
Time:15:36:33 Group Name:Ryan Method:C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp
User Sample ID:br cy OAMS#:Ryan129-1 Vial:1:23

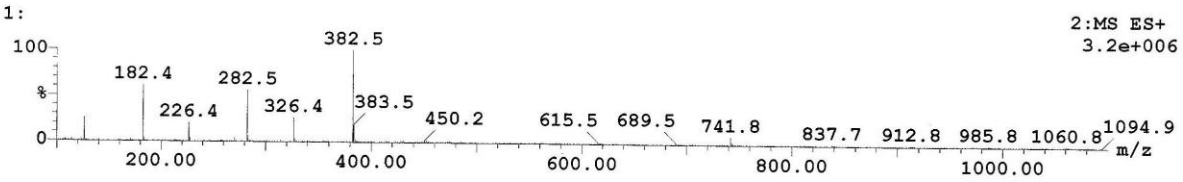
Printed: Thu Jul 31 15:45:07 2014

Sample Report:

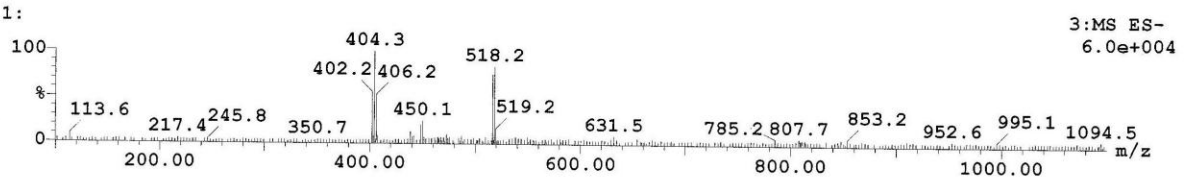
Sample 1 Instrument Method C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp



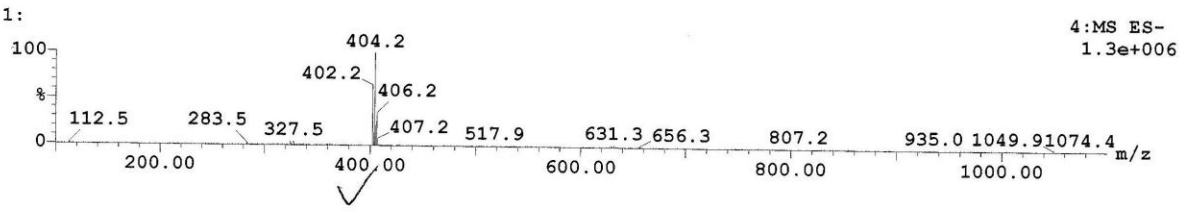
No elements were found.



No elements were found.



No elements were found.



No elements were found.

Sample 2 Instrument Method C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp

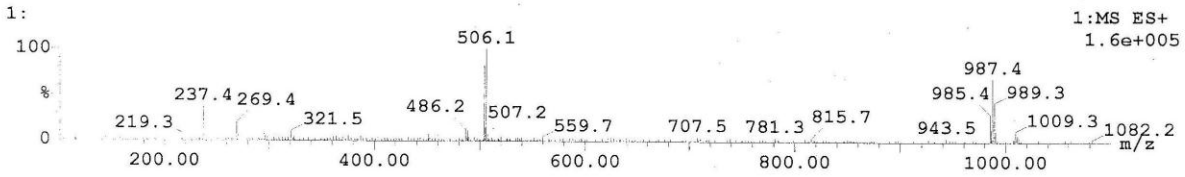
Figure 43 Low RES ESI MS analysis of compound **86**

Mass Spec Report:

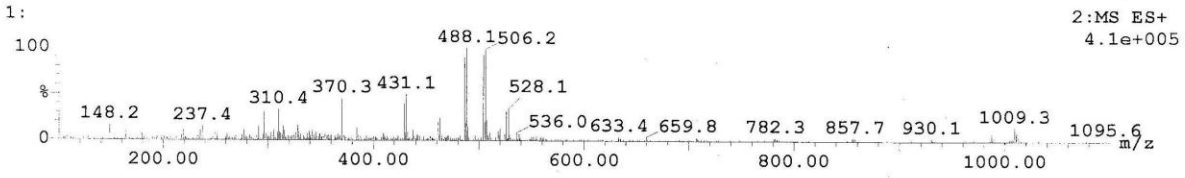
File:Ryan108-3 Sample: 3 Date:20-Jun-2014
Time:13:24:12 Group Name:Ryan Method:C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp
User Sample ID:13 OAMS#:Ryan108-3 Vial:1:81

Printed: Fri Jun 20 13:40:27 2014

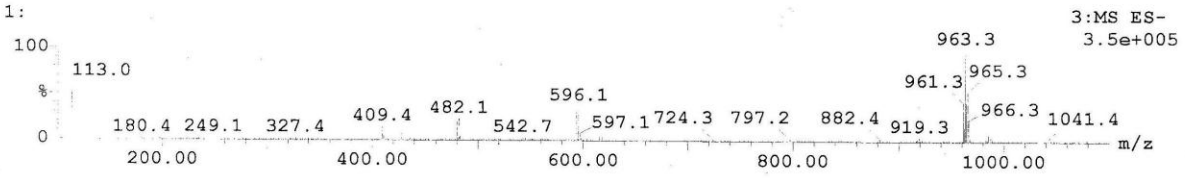
Sample Report (continued):



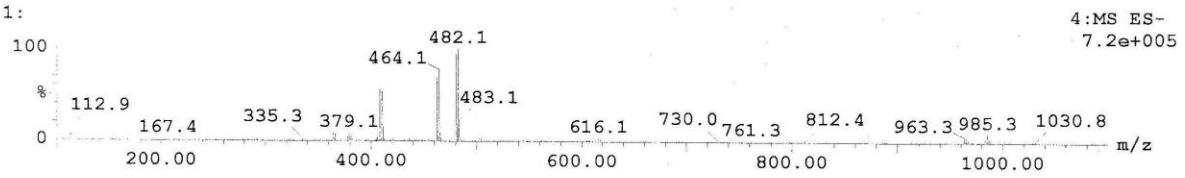
No elements were found.



No elements were found.



No elements were found.



No elements were found.

Sample 4 Instrument Method C:\MassLynx\OALogin\OAMethods\MEOH_FIA_ES_1.olp

Figure 44 Low Res-ESI mass spectral analysis of bromocladoniamide A

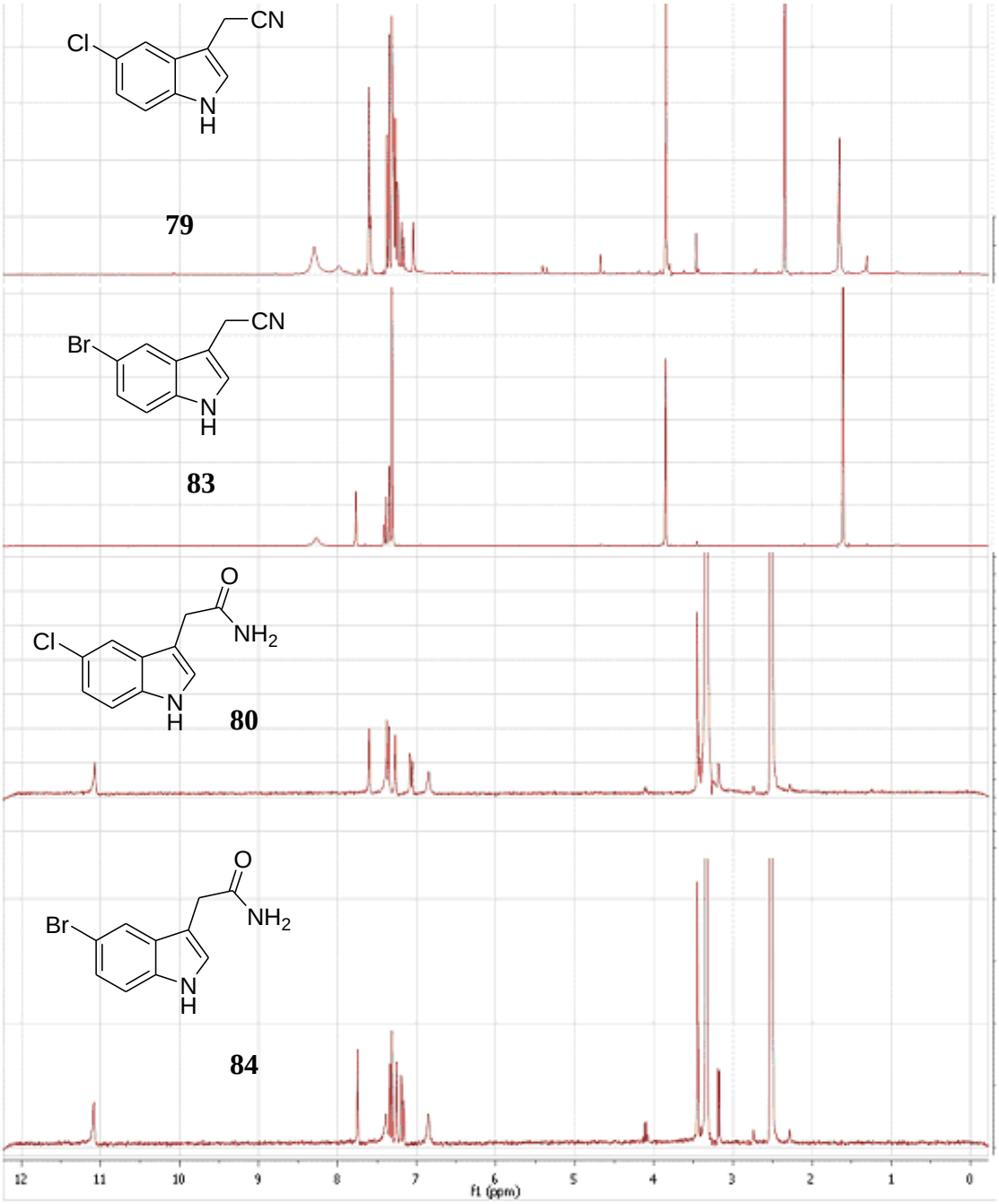


Figure 45 ^{1}H NMR spectra of compounds **79-80** and **83-84** at 300 MHz in $CDCl_3$ and compounds **80** and **84** at 600 MHz in DMSO-d_6

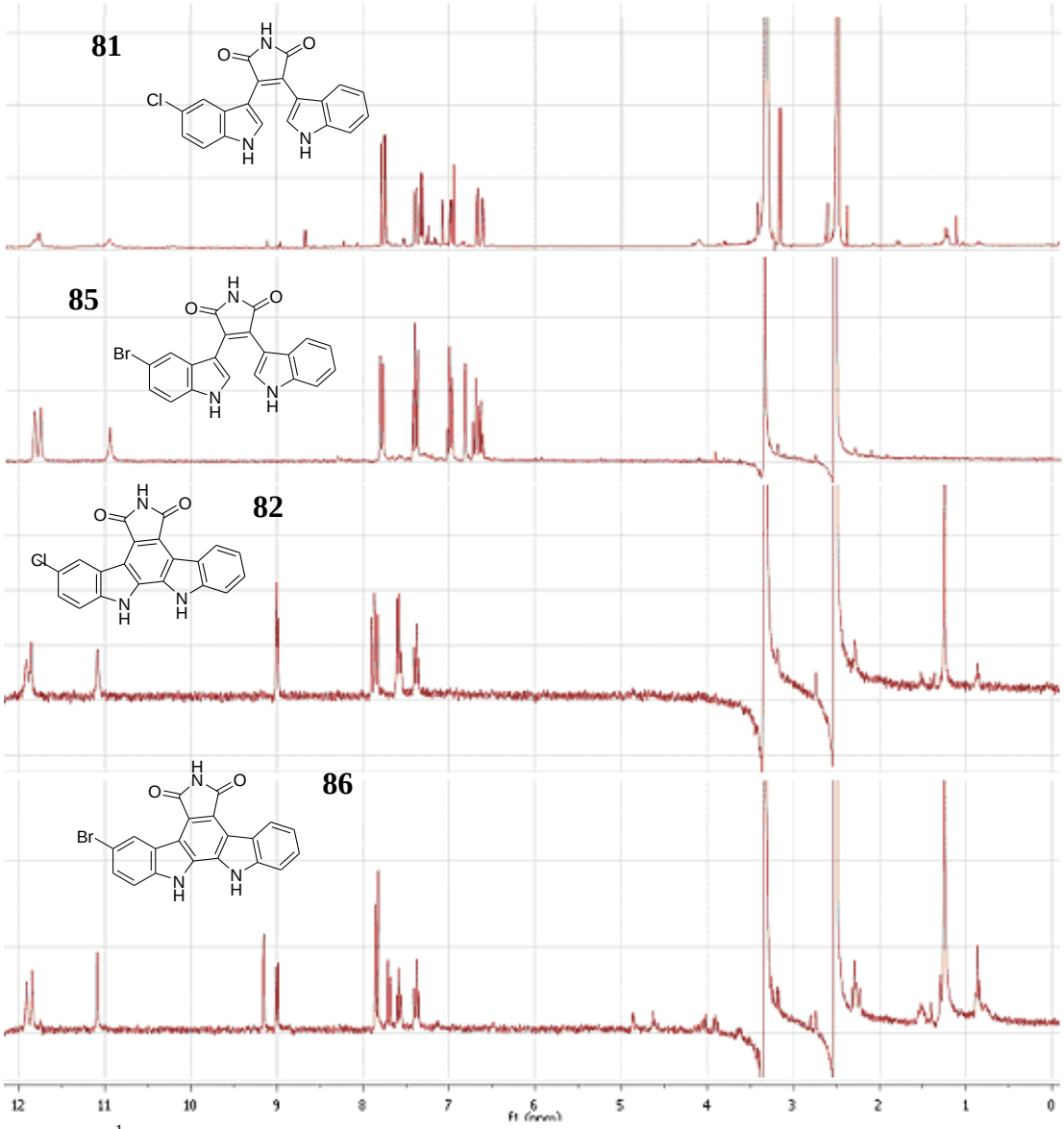


Figure 46 ^{1}H NMR spectra of compounds **81-82** and **85-86** at 600 MHz in DMSO-d_6

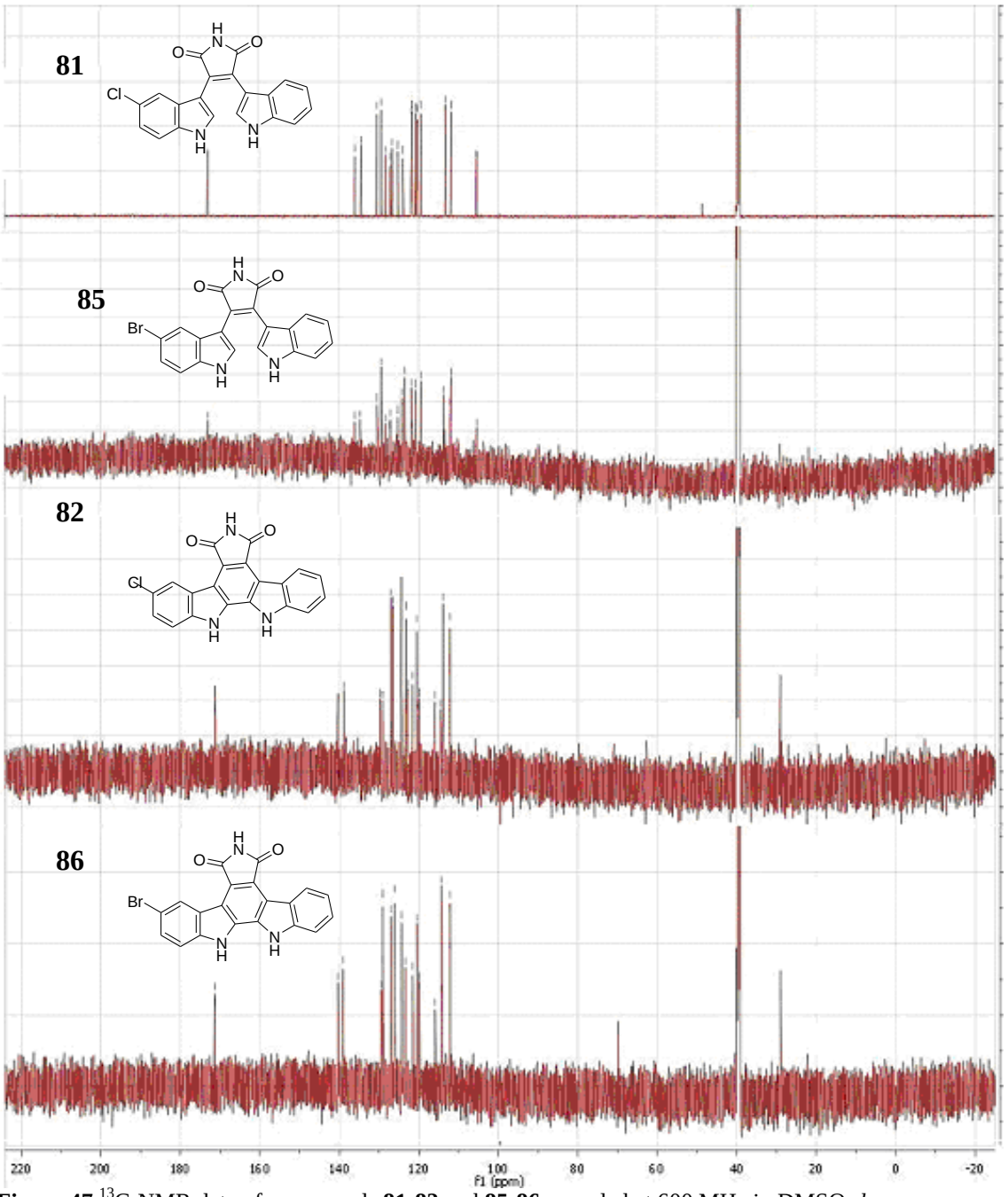


Figure 47 ^{13}C-NMR data of compounds **81-82** and **85-86** recorded at 600 MHz in DMSO-d_6.

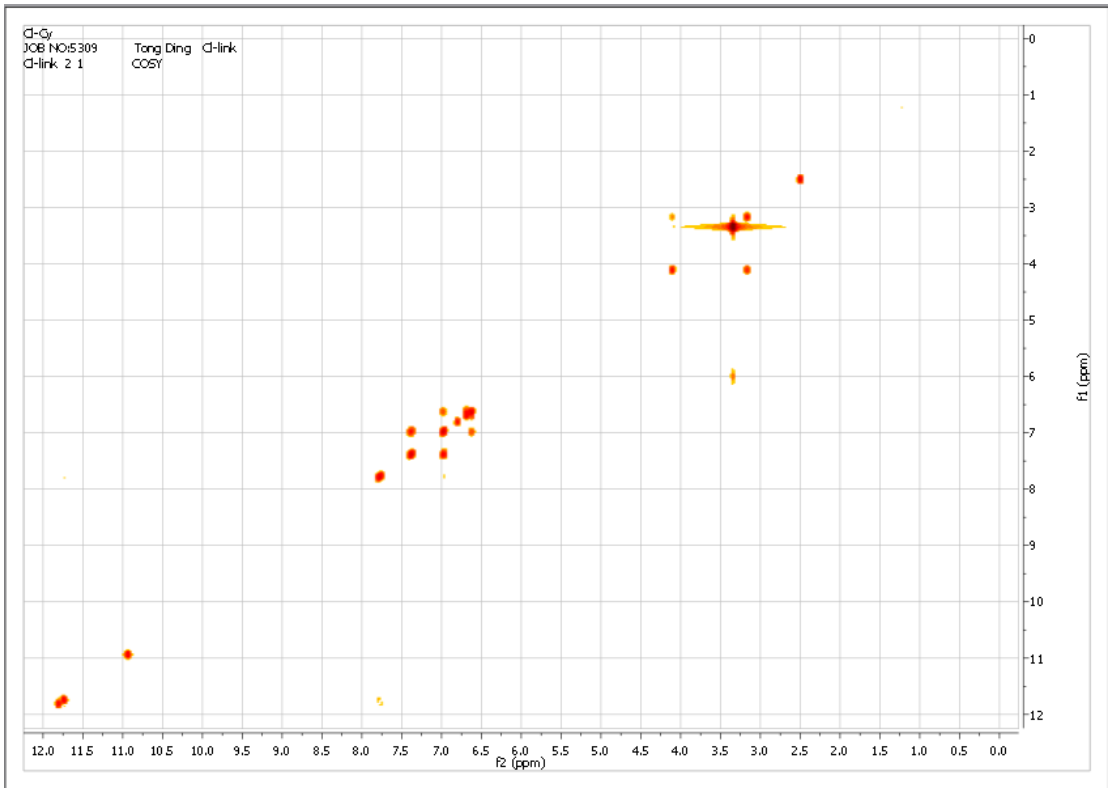


Figure 48 COSY spectra of compound **81** at 600 MHz in DMSO-d_6.

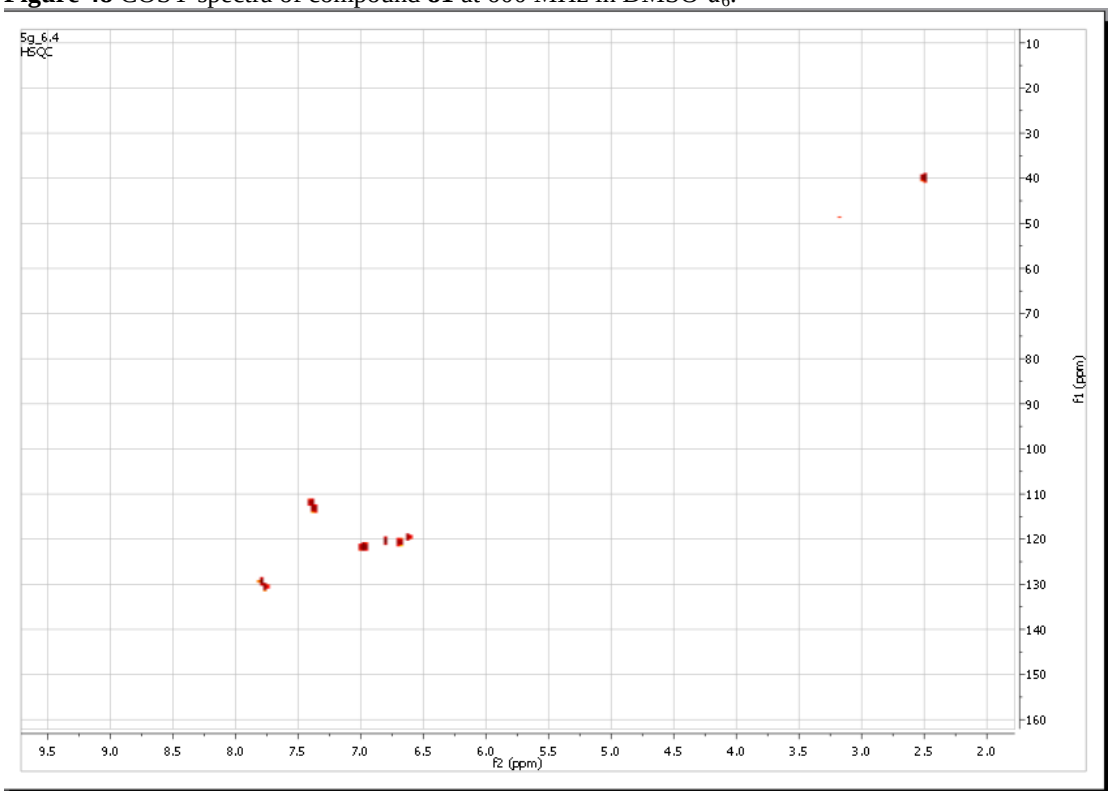


Figure 49 HSQC spectra of compound **81** at 600 MHz in DMSO-d_6.

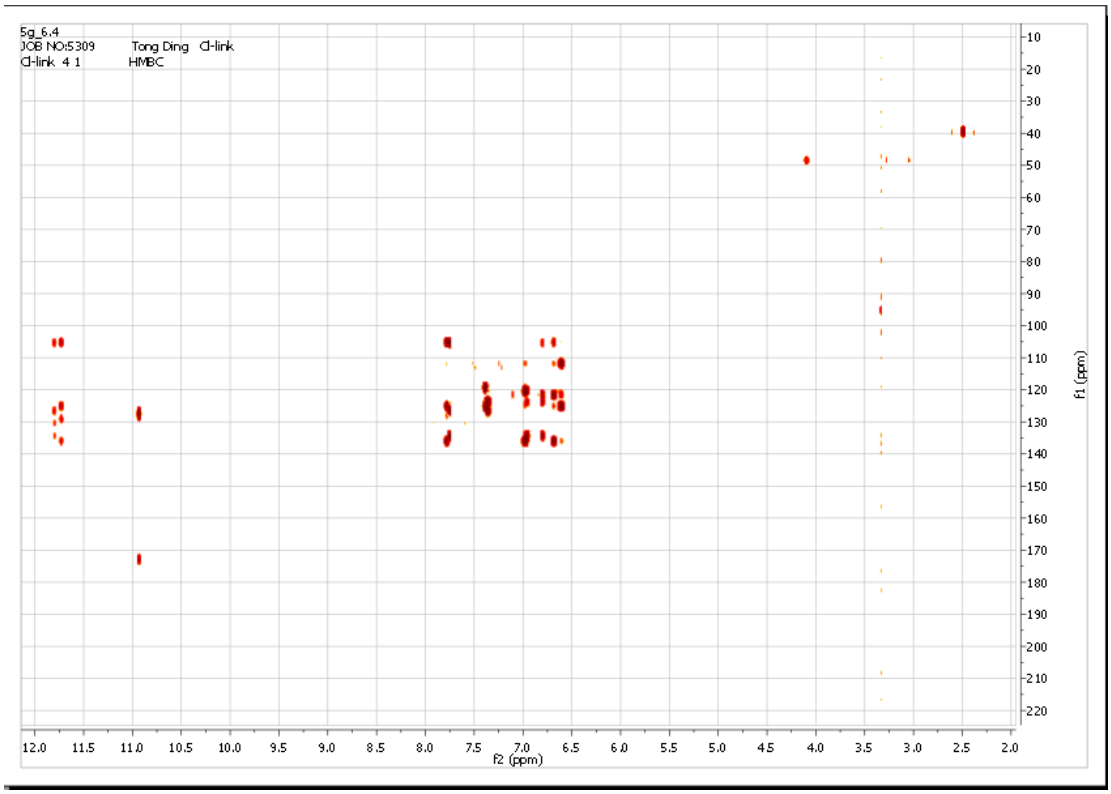


Figure 50 HMBC spectra of compound **81** at 600 MHz in DMSO-d_6.

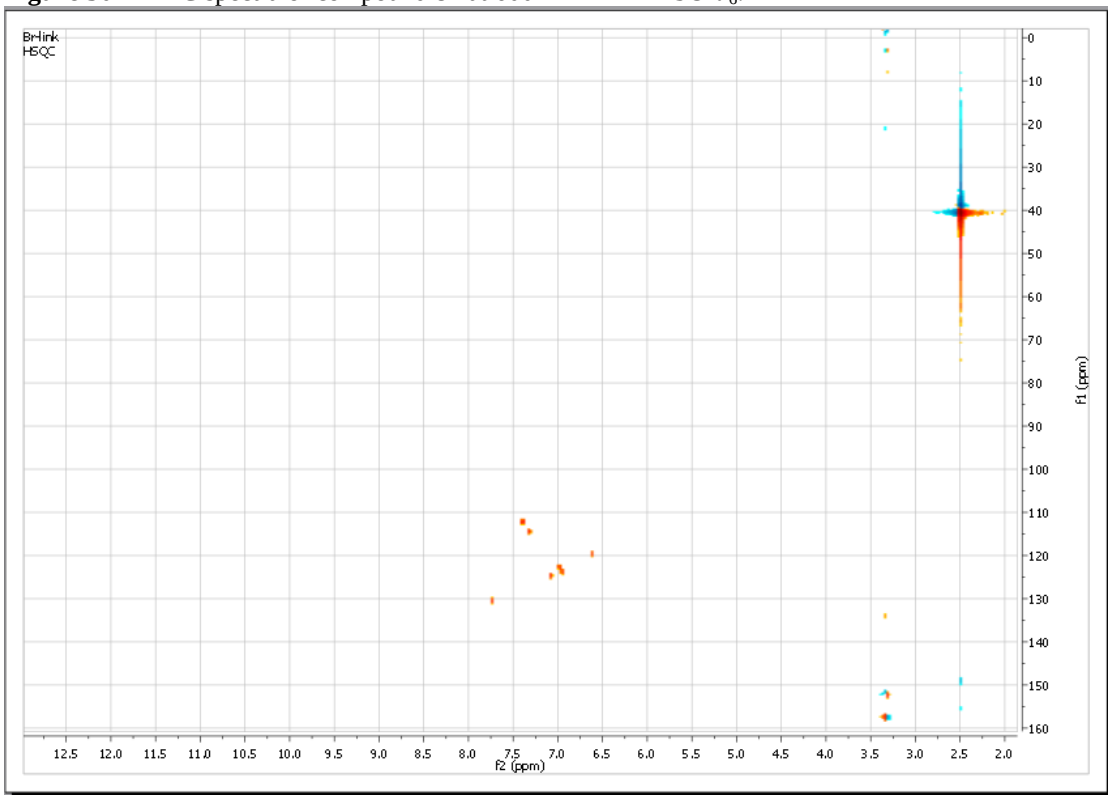


Figure 51 HSQC spectra of compound **85** at 600 MHz in DMSO-d_6

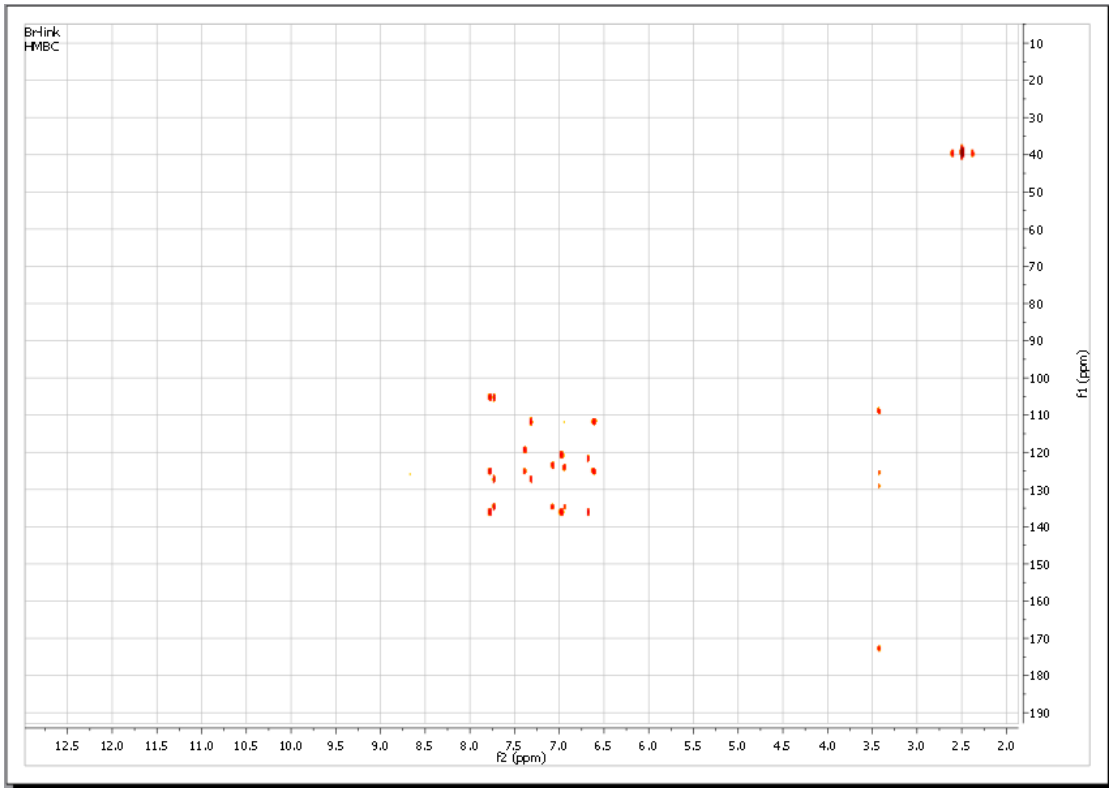


Figure 52 HMBC spectra of compound **85** at 600 MHz in DMSO-d_6

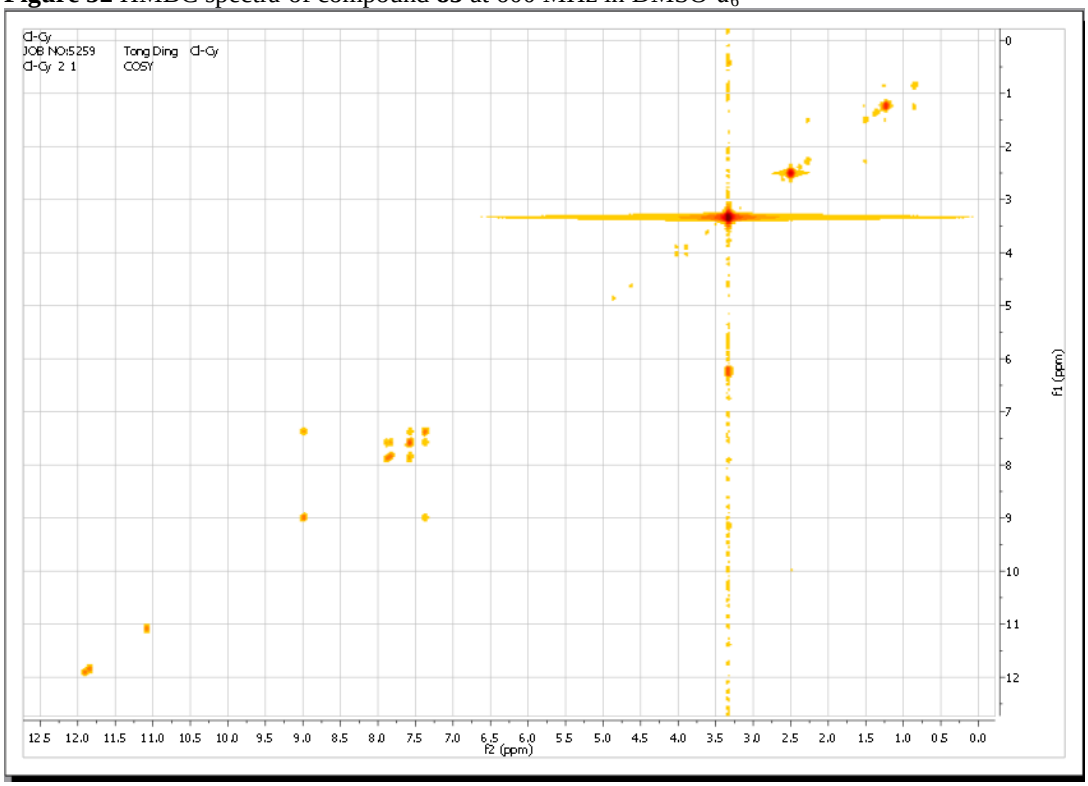


Figure 53 COSY spectra of compound **82** at 600 MHz in DMSO-d_6.

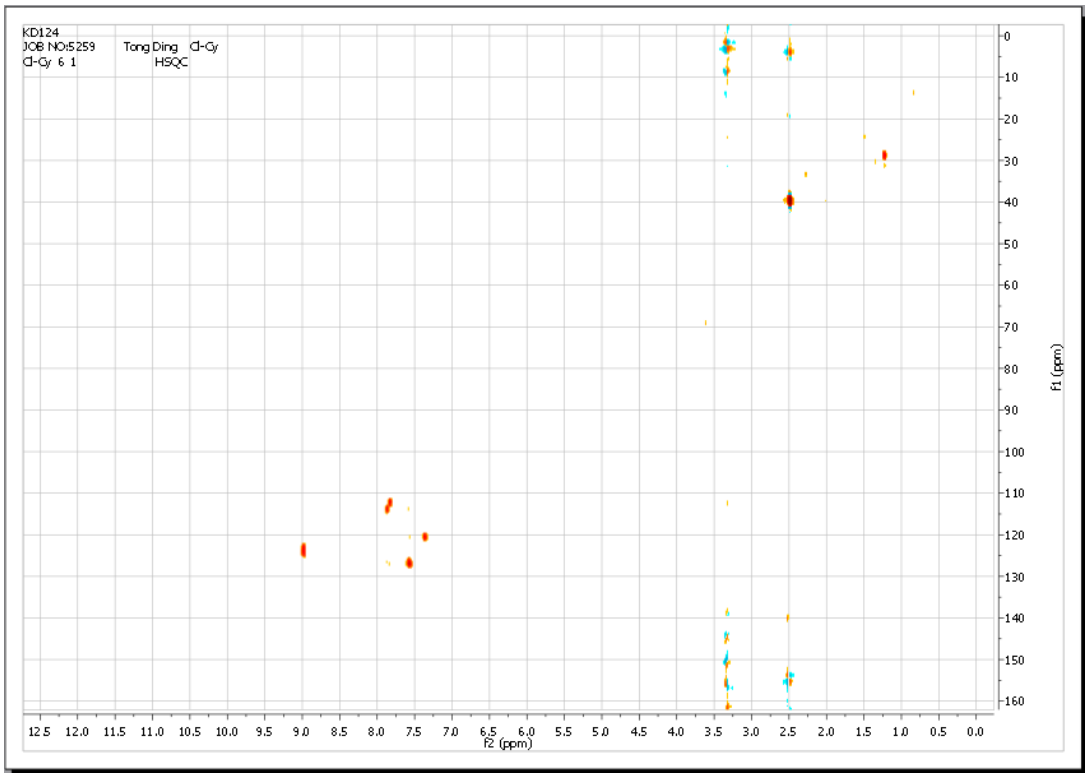


Figure 54 HSQC spectra of compound **82** at 600 MHz in DMSO-d_6.

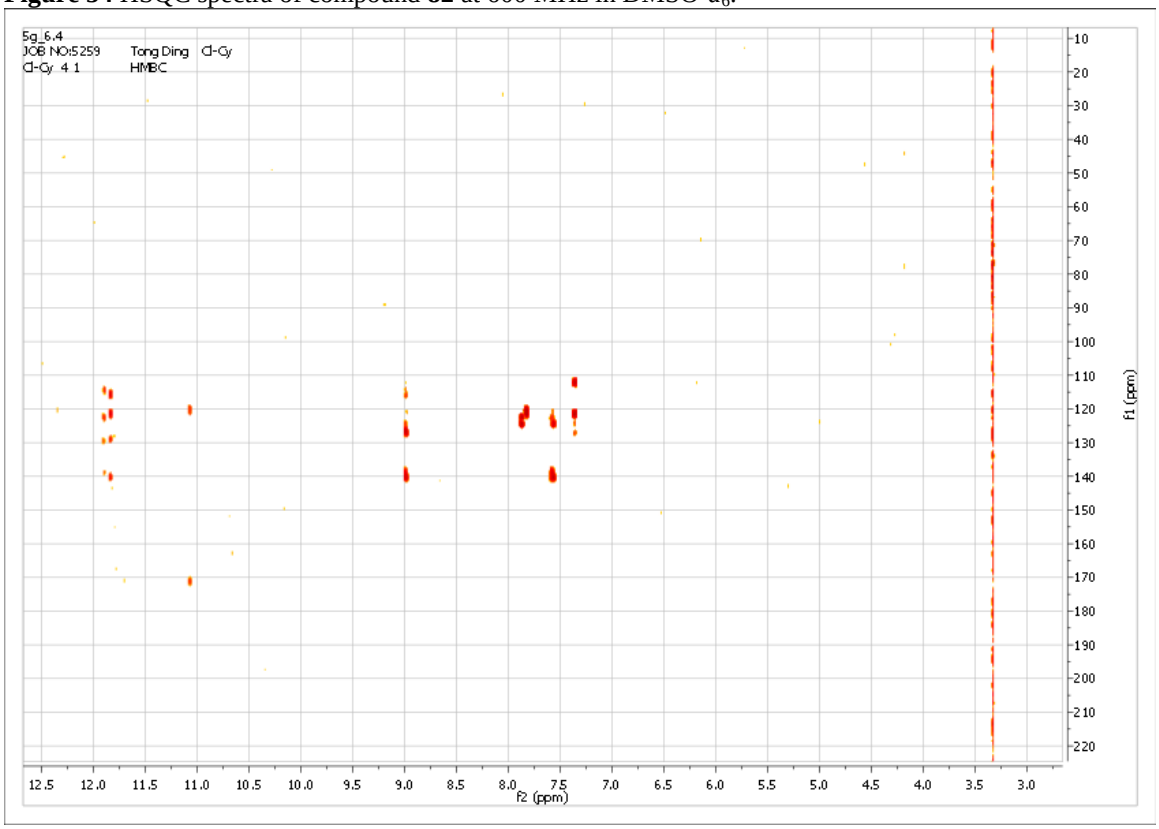


Figure 55 HMBC spectra of compound **82** at 600 MHz in DMSO-d_6.

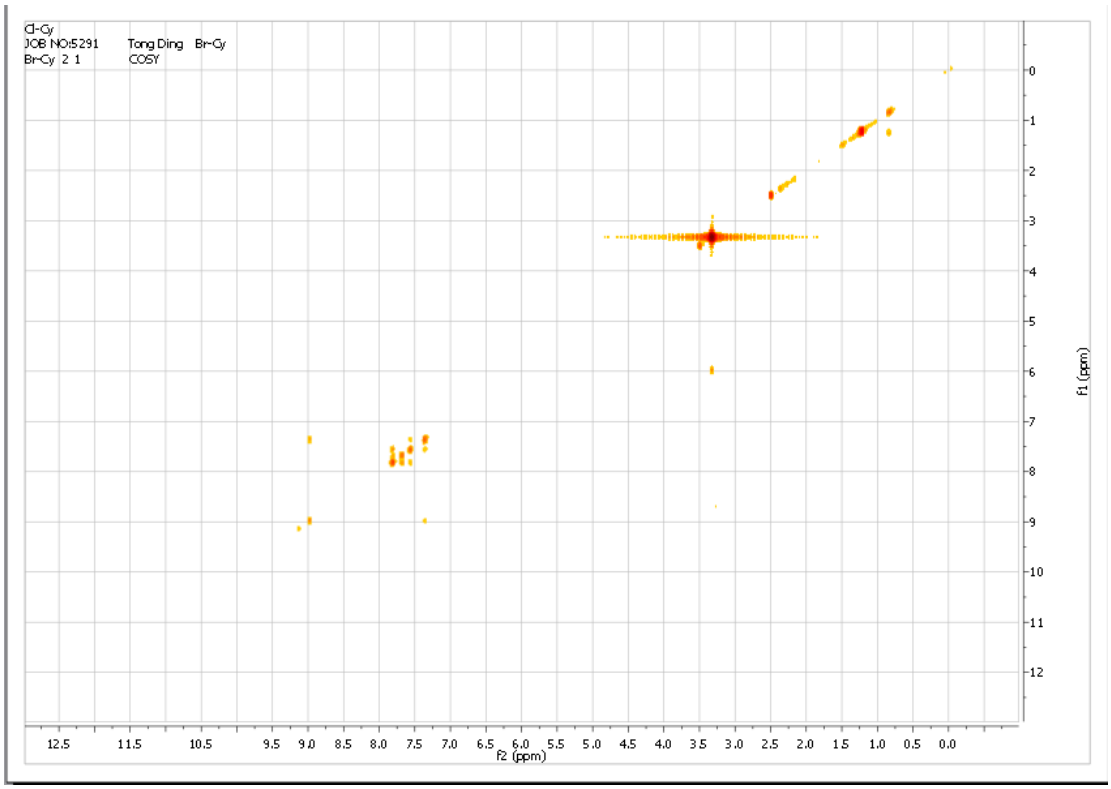


Figure 56 COSY spectra of compound **86** at 600 MHz in DMSO-d_6.

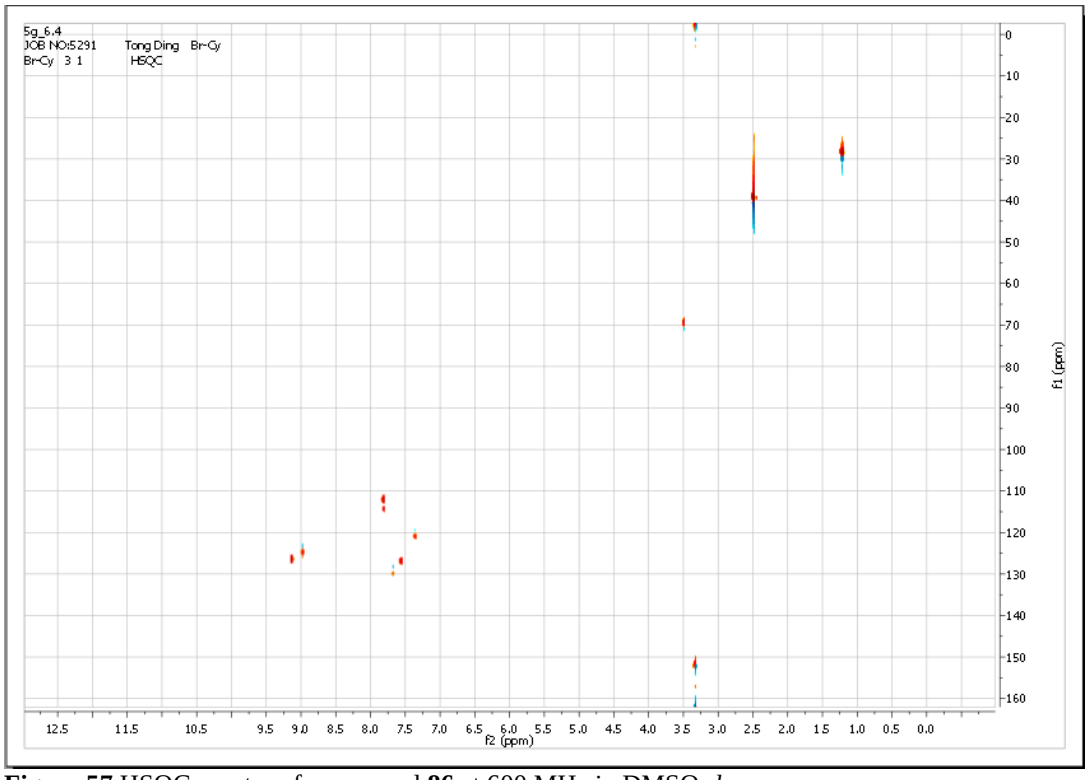


Figure 57 HSQC spectra of compound **86** at 600 MHz in DMSO-d_6.

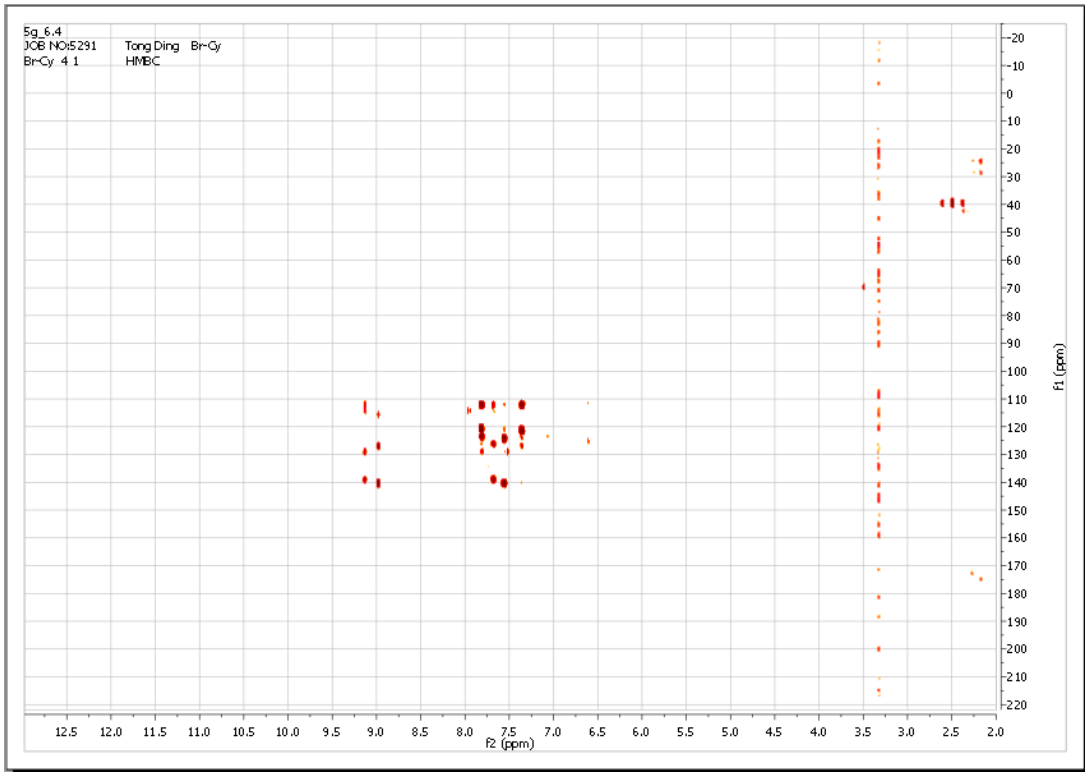


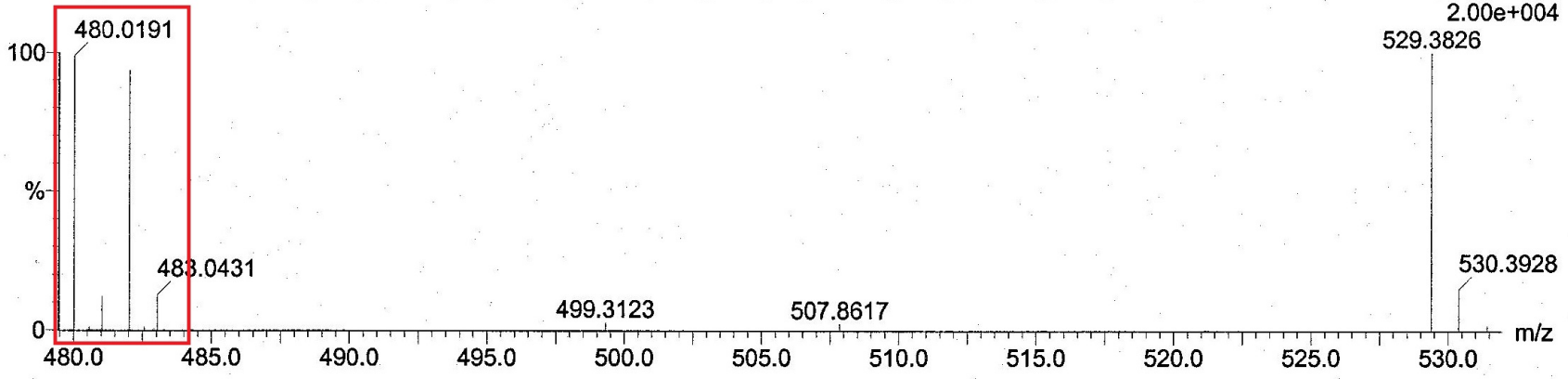
Figure 58 HMBC spectra of compound **86** at 600 MHz in DMSO-d_6.

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -3.0, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
2339 formula(e) evaluated with 12 results within limits (up to 40 closest results for each mass)
Elements Used:
C: 0-80 H: 0-100 N: 0-10 O: 0-10 Na: 0-1 Br: 0-1

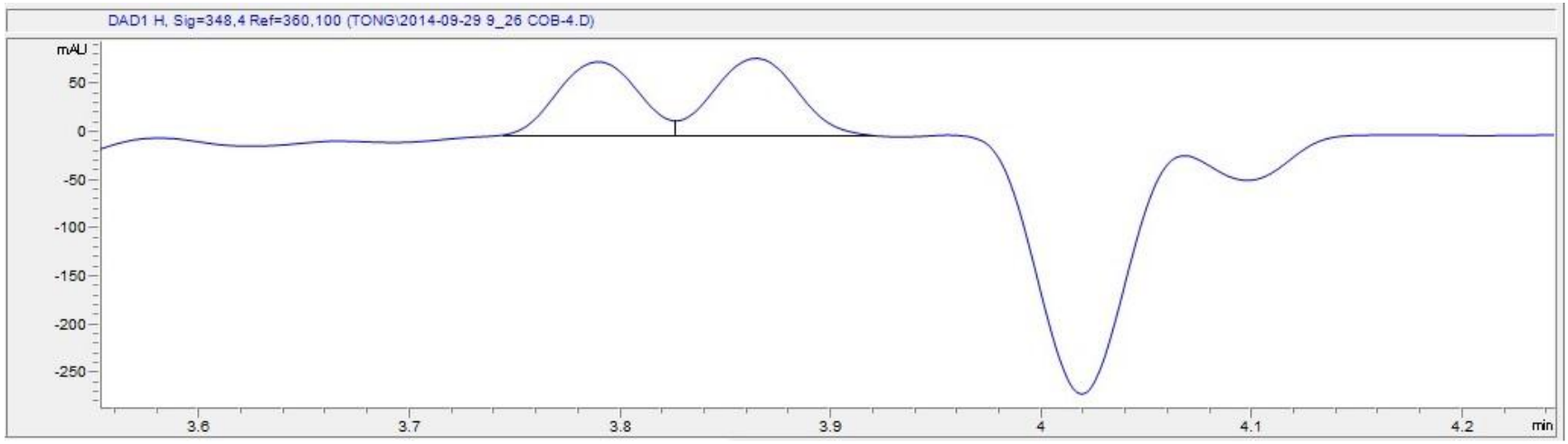
Br-ClaA
EH8585_2 692 (12.694) AM (Cen,4, 50.00, Ar,5000.0,529.38,1.00); Sm (Mn, 1x1.00); Sb (1,40.00); Cm (687:693)

03-Oct-2014
TOF MS ES-
2.00e+004



Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
Minimum:				-3.0		
Maximum:		5.0	5.0	50.0		
480.0191	480.0192	-0.1	-0.2	20.5	9544.8	C21 H7 N5 O8 Na
	480.0195	-0.4	-0.8	16.5	1071.0	C22 H15 N3 O5 Br
	480.0198	-0.7	-1.5	36.5	11121.2	C35 H2 N3 O
	480.0184	0.7	1.5	18.5	1106.7	C21 H12 N7 O Na Br

Figure 59 HRESI-MS analysis of the isolated bromocladoniamide A.



#	Time	Area	Height	Width	Area%	Symmetry
28	3.382	85.2	31.3	0.0418	0.573	1.32
29	3.456	71.9	26.9	0.0392	0.483	0.666
30	3.515	139.7	51.6	0.0396	0.939	1.718
31	3.581	479.6	94.5	0.0712	3.224	0.895
32	3.666	479.7	124.1	0.0551	3.224	1.527
33	3.789	1523.9	253.9	0.084	10.243	2.203
34	3.864	1582.2	286.3	0.0765	10.634	0.539
35	3.954	899.8	241.1	0.0595	6.047	0.496

Figure 60 Measurement of peak areas of produced cladoniamide A at 3.789 min and 5-bromocladoniamide A at 3.864