Studies Toward the Biosynthesis of Chimonanthine in *Chimonanthis praecox*

by

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B.Sc. (Spec.), University of Alberta, 2012

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in the
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Abstract

Chimonanthine is the building block of a series of natural products found in terrestrial plants including members of *Psychotria* in the family of *Rubiaceae*. Studies have shown that alkaloids containing the chimonanthine core display interesting analgesic, inhibition of melanogenesis, and anti-cancer activities. The goal of this study is to explore the precursor directed biosynthesis of chimonanthine and the enzymes involved in the biosynthesis of chimoanthine as well as to identify these enzymes for potential use as biocatalysts that can generate libraries of modified natural products. Herein we report the identification of a suitable plant containing these enzymes and demonstrate the feasibility of new assays by showing that feeding of plants with synthetic precursors leads to the production of labelled chimonanthine.

**Keywords:** chimonanthine; precursor-directed biosynthesis; calycantheaceous alkaloid, pyrrolidinoindoline alkaloids;
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<th>Definition</th>
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<td>2,2-DMP</td>
<td>2,2-dimethoxypropane</td>
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<tr>
<td>4’OMT</td>
<td>4'-O-methyltransferase</td>
</tr>
<tr>
<td>6OMT</td>
<td>6-O-methyltransferase</td>
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<tr>
<td>AcOH</td>
<td>Acetic acid</td>
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<td>ACP</td>
<td>Acyl carrier protein</td>
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<td>AT</td>
<td>Acyl transferase</td>
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<tr>
<td>BCl$_3$</td>
<td>Boron trichloride</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CH$_2$Cl$_2$</td>
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<tr>
<td>CH$_2$O</td>
<td>Formaldehyde</td>
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<tr>
<td>CNMT</td>
<td>Coclaurine N-methyltransferase</td>
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<tr>
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<td>Dehydratase</td>
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<td>Diisopropylamine</td>
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<td>DMF</td>
<td>Dimethylformamide</td>
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<td>DMPU</td>
<td>1,3-Dimethyl-3,4,5,6-tetrahydro-2-pyrimidinone</td>
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<tr>
<td>DOPA</td>
<td>Dihydroxyphenylalanine</td>
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<tr>
<td>DRR</td>
<td>1,2-Dehydroreticuline reductase</td>
</tr>
<tr>
<td>DRS</td>
<td>1,2-Dehydroreticuline synthase</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIC</td>
<td>Extracted Ion Chromatogram</td>
</tr>
<tr>
<td>EMCCD</td>
<td>Electron multiplying charge coupled device</td>
</tr>
<tr>
<td>ER</td>
<td>Enoyl reductase</td>
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<td>Et$_2$O</td>
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<td>Triethyl amine</td>
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EtOH  Ethanol
FAD  Flavin adenine dinucleotide
FeCl₃  Iron (III) chloride
G6P  Glucose-6-phosphate
G6PDH  Glucose-6-phosphate dehydrogenase
GC  Gas chromatography
GC-MS  Gas chromatography-mass spectrometry
HMPA  Hexamethylphosphoramidate
HN₃  Hydrazoic acid
HPLC  High performance liquid chromatography
HR  High resolution
I₂  Iodine
INMT  Indoleethylamine N-methyltransferase
JRES  J-resolved spectroscopy
KR  Ketoreductase
KS  Ketosynthase
LC  Liquid chromatography
LC-MS  Liquid chromatography-mass spectrometry
LDA  Lithium diisopropylamide
L-DOPA  L-3,4-dihydroxyphenylalanine
LiAlD₄  Lithium aluminium deuteride
LiAlH₄  Lithium aluminium hydride
LiCl  Lithium chloride
Me₃Al  Trimethyl aluminium
MeMgI  Methyl magnesium iodide
MeOH  Methanol
MES  2-(N-Morpholino)ethanesulfonic acid
MM-NAC  Methylmalonyl N-acetylcysteine
MOPS  3-(N-Morpholino)propanesulfonic acid
MPE  Maximum possible effect
mRNA  Messenger ribonucleic acid
MS  Mass spectrometry
Na  Sodium
NaBH(OAc)₃  Sodium triacetoxyborohydride
NaBH₄  Sodium borohydride
NADH  Nicotinamide adenine dinucleotide
NADP  Nicotinamide adenine dinucleotide phosphate
NaH  Sodium hydride
NaHMDS  Sodium bis(trimethylsilyl)amide
NCS  Norcoclaurine synthase
NH₃  Ammonia
NMR  Nuclear magnetic resonance
PAH  Phenylalanine hydroxylase
Pb(OAc)₄  Lead (IV) acetate
PBS  Phosphate buffered saline
PDB  Precursor-directed biosynthesis
PEG  Polyethyleneglycol
Ph₃P  Triphenylphosphine
PhH  Benzene
PVPP  Poly(vinylpolypyrrolidone)
Red-Al  Sodium bis(2-methoxyethoxy)aluminumhydride
RNA  Ribonucleic acid
SalAT  7-O-Acetyltransferase
SalR  Salutaridine reductase
SDS-PAGE  Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SmI₂  Samarium (II) iodide
T6ODM  Thebaine 6-O-demethylase
TDC  Tryptophandecarboxylase
TE  Thioesterase
TFA  Trifluoroacetic acid
TH-1  Tryptophan hydroxynase 1
THF  Tetrahydrofuran
TLC  Thin layer chromatography
TRP-1  Tyrosinase related protein 1
TRP-2  Tyrosinase related protein 2
TYDC  Tyrosine decarboxylase
Zn      Zinc
Chapter 1.

Introduction

1.1. Enzymes in Nature and in Chemistry

In Nature, living organisms (e.g. mammals, plants) produce useful natural products via numerous biosynthetic processes that rely on enzymes, which possess several unique characteristics such as high substrate specificity and enantioselectivity.\(^1\)\(^-\)\(^4\) A large number of these enzymes have now been identified and structurally characterized. For example, polyketide biosynthesis has been the subject of intense research and is now largely understood to rely on collections of enzymes that form “modules” which are responsible for adding one more acetate or propionate unit to a growing polyketide chain and effect subsequent functional group transformations (e.g., reduction or dehydration). The biosynthesis of polyketides starts with a loading module, and ends with a thioesterase which removes the polyketide natural product from the biosynthetic machinery.\(^5\)\(^-\)\(^7\) Understanding this biosynthetic pathway has allowed scientists to exploit this molecular machinery in the synthesis of many unnatural polyketides. An example of this is shown in Scheme 1.1, where Sherman and coworkers carried out the chemoenzymatic synthesis of 10-deoxymethynolide (3) and acetyl-narbonolide (5). The substrate 2 was prepared in 11 steps from the Roche ester (1) then further elaborated and lactonized by the actions of PikAIII-TE to afford 3 or two modules of PikAIII/PikAIV to afford the related macrolactone 5.\(^8\) This rapid synthesis of these complicated polyketides illustrates the power of using biosynthetic pathways to compliment total syntheses.
Alkaloids are a very large class of natural products that necessarily contain a nitrogen atom and have played an important role historically as a source of natural medicines (e.g. opium poppy from 200 BC). However, the biosynthetic pathways for the production of alkaloids have not been well elucidated compared to pathways for other natural products such as polyketides, terpenoids, and carbohydrates. For example, morphine (6), the most well-known alkaloid, was first isolated in 1805 by Friedrich Sertürner, but elucidation of the enzymes responsible for morphine biosynthesis was only established in the early 2000s.
The difficulties in elucidating pathways involved in alkaloid biosynthesis is in part related to the fact that there exist numerous structurally distinct families of alkaloids whose natural production initiates with a unique starting material and consequently each biosynthetic step requires discrete enzymes. For example, the biosynthesis of morphine (6) is depicted in Scheme 1.2.[11–13] This pathway initiates with decarboxylation of L-DOPA (9) by tyrosine decarboxylase (TYDC) to afford dopamine (10). Norcoclaurine synthase (NCS) utilizes substrates 10 and 4-hydroxyphenylacetaldehyde (11) to yield (S)-norcoclaurine (12), which is then methylated by norcoclaurine 6-O-methyltransferase (6OMT) to obtain (S)-coclaurine (13). Subsequent methylation by coclaurine N-methyltransferase (CNMT) affords (S)-N-methylcoclaurine (14). Then, a selective oxidation of an aromatic ring by N-methylcoclaurine-3-hydroxylase (Cyp80B3) affords (S)-3'-hydroxy-N-methylcoclaurine (15), which undergoes selective methylation on hydroxyl group by 3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase (4'OMT) to obtain (S)-reticuline (16). Iminium ion mediated epimerization of substrate 16 to (R)-reticuline (18) is performed by two enzymes: 1,2-dehydroreticuline synthase (DRS) and 1,2-dehydroreticuline reductase (DRR). Salutaridine synthase (Cyp719B1) cyclizes 18 to yield salutaridine (19). Salutaridine reductase (SalR) reduces ketone function in 19 to produce salutaridinol (20). Salutaridinol 7-O-acetyltransferase (SalAT) specifically acetylates the C7 alcohol function to provide salutaridinol-7-O-acetate (21), which spontaneously cyclizes to thebaine (22).

There are two possible routes from thebaine (22) to morphine (6). The first pathway is from 22 to neopinone 23 via removal of the methyl group at the 6 position by thebaine 6-O-demethylase (T6ODM). Then, 23 spontaneously undergoes rearrangement to codeinone (24). The reduction of the enone to an allylic alcohol by codeine reductase (COR) yields codeine (25). The last step in the first pathway involves
demethylation by codeine \textit{O}-demethylase (CODM) to obtain morphine (6). The second route from 22 to 6 involves demethylation CODM to afford oripavine (26), which undergoes another demethylation by T6ODM to yield mophinone (27). Lastly, 27 undergo reduction by COR to obtain morphine (6).
Scheme 1.2. Biosynthetic route of morphine (42)
Thus, biosynthetic studies on alkaloids are complicated by their unique and complex structures. However, there is no doubt that further studies towards the identification of biosynthetic pathways are useful as they would support the large scale syntheses of these potentially important natural products as well as analogues. In this thesis, the biosynthesis of one member of the family of calycanthaceous alkaloids, chimonanthine, is studied. In the following section, the history, bioactivity, and synthetic and biosynthetic efforts towards chimonanthine will be discussed.

1.2. Calycanthaceous Alkaloids

The study on calycanthaceous alkaloids started with a letter written in November, 1887 by Mr. J. H. H. Boyd. [14]

“Hundreds of cattle and sheep have died here in the past five years from ‘bubby’ (the eccentric name of *Calycanthus glaucus*). The seeds only are poisonous. When a brute gets a sufficient dose, from five to ten well-filled pods, it makes for the nearest water and often falls dead while drinking, or it may live three or four weeks and then die. The symptoms are like those of a man extremely drunk, except that any noise frightens it. Stamp the ground hard close to a brute poisoned with ‘bubby,’ and it will jump and jerk and tremble for several minutes. That is our method of telling when they have taken it. The eyes turn white and glassy, and while lying they throw back the head and look as if dead already. ‘Bubby’ does not seem to hurt a brute so much if it cannot get water. Our best remedy is apple brandy, strong coffee and raw eggs poured down as soon as possible after finding. It is certain that ‘bubby’ is the most poisonous of any shrub or weed in existence here, from the fact that when brutes have once eaten it they will take it every time they can get it. It grows on every hillside, along all branches (creeks), in every fence corner and almost everywhere.”

In 1888, Eccles studied the poisonous “bubby” seeds (*Calycanthus glaucus*) in order to elucidate the toxic components from the extracts, and was the first to report a
new compound, calycanthine (28) which represents the first entry of a new class of alkaloids which have become known as the calycanthaceous alkaloids (Figure 1.2). Interestingly, during this study it was reported that alkaloid 28 was not toxic. The isolated sample of (+)-calycanthine also allowed for the detailed description of several physical properties, including specific bad odour, poor solubility in water and good solubility in diethylether or chloroform. Furthermore, it was noted that the colour of the alkaloids change after coming into contact with several acids. For example, when 28 was treated with concentrated sulphuric acid, the initial mixture turned into a yellow solution. In the presence of concentrated ‘muriatic acid’ (HCl), the solution turned yellow initially and then turned olive green. A combination of concentrated sulphuric acid and sugar made a “lovely” pink red colour. Also, Eccles reported that (+)-calycanthine (28) made up 2% of the total mass of the seed. One year after this initial discovery, Wiley determined that the alkaloid has the molecular formula C_{18}H_{40}N_{2}O_{11}.\[14\]

![Chemical structures](image1.png)

**Figure 1.2 Examples of (bis)indoline calycanthaceous alkaloids.**

Nearly twenty years after these initial studies, Gordin reported that there was no oxygen atom in (+)-calycanthine (28) and determined that the molecular formula was
C\textsubscript{11}H\textsubscript{14}N\textsubscript{2} by measuring the amount of CO\textsubscript{2} and volume of N\textsubscript{2} released from calycanthine (28).\textsuperscript{[16,17]} A further twenty years later, in 1925, the molecular formula was doubled by Späth and Stroh to C\textsubscript{22}H\textsubscript{28}N\textsubscript{4}.\textsuperscript{[18]} In a subsequent study by Barger, the molecular formula of (+)-calycanthine (28) was further revised to C\textsubscript{22}H\textsubscript{28}N\textsubscript{4} in 1939.\textsuperscript{[19]} However, the molecular structure was not elucidated until 1960 when Woodward, Clark and Katz determined the structure of (+)-calycanthine (28) by degradation methods.\textsuperscript{[20]} They also proposed a potential biosynthetic pathway (Scheme 1.3) to (+)-calycanthine (28) beginning with the β,β'-oxidation of N\textsubscript{β}-methyltryptamine (37). The same year, the structure of (+)-calycanthine (28) was confirmed by Hamor via X-ray crystallography of the alkaloid.\textsuperscript{[21]}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Scheme13.png}
\caption{Scheme 1.3. Woodward’s proposed biosynthesis of calycanthine (28)}
\end{figure}

The discovery of a number of new calycanthaceous alkaloids was reported following the isolation of (+)-calycanthine.\textsuperscript{[19,22–26]} (Figure 1.2) Additionally, several homologues of (+)-calycanthine (28) produced by medicinal terrestrial plants (e.g. Psychotria sp.) were identified that contain a central meso-chimonanthine unit as part of their structure (highlighted in blue, Figure 1.3).\textsuperscript{[27–32]}
1.3. Chimonanthine

1.3.1. Background Information

In 1961, (-)-chimonanthine (29), a structural isomer of calycanthine (28), was discovered by Hodson from the leaves of *Chimonanthus fragrans*, Lindl.\[33\] The structure of (-)-chimonanthine (29) was determined in the following year via x-ray crystallography by Hamor.\[34\] Interestingly, the enantiomeric (+)-chimonanthine (30) was later isolated in 1983 by Tokuyama from the skin of colombian poison-dart frog,\[35\] and again in 1999 by Potier from plants of the *Psychotria* species.\[30\] Despite having been synthesized prior to its isolation, *meso*-chimonanthine (31) was also isolated from *Psychotria forsteriana* in 1992.\[36\]
In 2002, Verotta performed a comparative study on the analgesic properties of chimonanthines to morphine (6), and found that the former family of natural products (29-31) exhibit strong binding affinities toward the μ-opioid receptor.[37] In the tail-flick mouse assay (Figure 1.5), Verotta compared (-)- and (+)-chimonanthines (29 and 30) with morphine (6) by measuring the change in the response time after causing pain to the mouse.[38] While morphine (6) displayed 100% maximum possible effect (MPE) at 6 mg/kg, (-)-chimonanthine (29) displayed 40% MPE at 10 mg/kg and (+)-chimonanthine (30) displayed 66% MPE at 5 mg/kg. In the capsaicin induced pain model, Verotta reported that a dosage of 0.25 mg/kg of (-) and (+)-chimonanthines (29, 30), the mice reduced licking at the site where capsaicin was injected, and they observed that inhibition of licking diminished by 47% and 38% when administered with (-)-chimonanthine (29) and (+)-chimonanthine (30), respectively. Furthermore, (-)-chimonanthine (29), (+)-chimonanthine (30) and meso-chimonanthine (31) displayed strong binding affinity ($K_i$) to μ-opioid receptor with 271 ± 85 nM, 652 ± 159 nM and 341 ± 29 nM, respectively. As a comparison, morphine (6) displayed 0.76 ± 0.04 nM binding affinity towards the same receptor. These results suggested that chimonanthines could be good lead candidates as analgesics.

Figure 1.5. Illustration of tail-flick model with mouse

Further investigations into the biological activity of the chimonanthines revealed that (-)-chimonanthine (29) inhibits melanogenesis, in vitro (see Scheme 1.4). This pathway is responsible for the generation of melanin in the body. The study reported that (-)-chimonanthine (29) showed inhibition of melanin production in B16 melanoma 4A5 cells ($IC_{50} = 1.4 \, \mu M$), and compared favourably to the commercially available tyrosinase inhibitor arbutin ($IC_{50} = 174 \, \mu M$). However, the inhibition mechanism associated with chimonanthine has not been reported yet.[39-41]
Scheme 1.4. Melanin biosynthetic route
The blue arrows in chemical structures of eumelanin and pheomelanin indicate where elongation occurs, and the COOH groups in parenthesis can be substituted with H.

1.3.2. Early Syntheses of Chimonanthine

The interesting biological activity and intriguing structural characteristics of chimonanthine has inspired the development of several synthetic routes to these
compounds. The major challenge in these syntheses relates to the controlled introduction of the two adjacent quaternary all-carbon stereocentres, for which several new methods were devised. In 1962, Hendrickson reported the first total synthesis of rac-chimonanthine (rac-29) and meso-chimonanthine (31) (Scheme 1.5).[^42[^43]] This biomimetic total synthesis of chimonanthine relied on an oxidative dimerization of 55 to afford the key C-C bond. This biomimetic sequence supported the previously proposed biosynthesis of calycanthine by Woodward (see Scheme 1.3).

![Scheme 1.5. Hendrickson's total synthesis of chimonanthine](image)

Two years later, Scott reported the second total synthesis of chimonanthine via oxidative dimerization of N₆-methyltryptamine (37) (Scheme 1.6).[^44] This synthesis utilized a common biological building block 37 and was achieved in a single step with an improved yield compared to that reported by Hendrickson. The proposed mechanism for this key transformation is depicted in Scheme 1.6. Thus, deprotonation of the indole nitrogen by methylmagnesium iodide affords the resonance-stabilised anion 58. The anion then undergoes a single electron oxidation with iron (III) chloride to afford a C3 radical which undergoes radical recombination with an equivalent coupling partner to provide the key C3-C3’ bond to yield chimonanthine (rac-29, meso-31).
1.3.3. First Enantioselective Syntheses of Chimonanthine

While the racemic syntheses of the chimonanthines were achieved with few synthetic steps, their enantioselective synthesis presented a major challenge. Overman and coworkers completed the first total synthesis of meso, (+)- and (-)-chimonanthine through a series of enantioselective syntheses that proved to be landmarks in asymmetric synthesis. For meso-chimonanthine, the readily available isoindigo 59 was converted to 60 via samarium mediated reductive dialkylation (Scheme 1.7).[45] Reduction by Red-Al afforded hexacyclic intermediate 61, which was dihydroxylated and cleaved to afford diol 62. Subsequent Mitsunobu reaction, azide reduction and exposure to trimethylaluminum provided bis(pyrroloindoline) 63. Finally, methylation and deprotection afforded desired product meso-31.
Scheme 1.7. Overman’s total synthesis of meso-chimonanthine

In 1999, Overman reported an enantioselective total synthesis of (-)-chimonanthine (29, Scheme 1.8). The key reaction in the total synthesis of 29 is an intramolecular double Heck reaction cascade of 69 to 70. In this single step, the vicinal quaternary all-carbon stereocentres were introduced in high yield. Following synthetic steps that included cleavage of the cyclohexene ring to provide diol 71. Further reduction and a Mitsunobu reaction afforded 72, the heating of which in methanol and subsequent bis-methylation yielded bispyrroloindoline 73. Lastly, removal of benzyl group from 73 provided the desired product (-)-chimonanthine (29).
Scheme 1.8. Overman's total synthesis of (-)-chimonanthine

Following his successful synthesis of (-)-chimonanthine (43), Overman reported a modified synthesis of (+)-chimonanthine (30, Scheme 1.9) in 2000.[47] An interesting highlight in Overman’s synthesis of 30 involves dialkylation of dihydroisoindigo 74 to afford bisoxindole 75. In a single step, the desired all-carbon quaternary stereocentres
were introduced in excellent yield. The remaining synthetic steps to (+)-30 were identical to those employed in the total synthesis of (-)-30.

Scheme 1.9. Overman’s total synthesis of (+)-chimonanthine

1.3.4. Additional Total Syntheses of Chimonanthine.

Following Overman’s elegant enantioselective syntheses of meso, (-) and (+)-chimonanthines (31, 29, 30), numerous total syntheses of chimonanthine have been reported. In 2002, Takayama achieved a two-step synthesis of rac-chimonanthine (rac-29) and meso-chimonanthine (31).\[48]\ In 2007, Movassaghi reported the total synthesis of (+)-chimonanthine (30) via the Co-mediated homodimerization of tryptophan after benzylic bromination.\[49]\ In 2012, Matunaga developed an enantioselective synthesis of (+)-chimonanthine (30) in six steps through the use of a Schiff base.\[50]\ In 2013, Ma
reported a highly enantioselective synthesis of (-)-chimonanthine (29) in three steps via bromocyclization of tryptamine.\[^{[51]}\] Due in part to these efforts, there have been significant advances in achieving the C3-C3’ dimerization of indole derivatives leading to the synthesis of several chimonanthine analogues.\[^{[52–61]}\]

1.3.5. **Biosynthetic Studies of Chimonanthine**

While there have been multiple syntheses of chimonanthine and related analogues reported, only one study relating to the biosynthesis of this unique alkaloid has been reported.\[^{[62,63]}\]

![Figure 1.6](image)

**Figure 1.6.** *Kirby’s Radiolabelled Precursors and Radiolabelled Chimonanthine products from Chimonanthus praecox*

The radiolabelled precursors were fed to *Chimonanthus praecox*, and the percentage of incorporation rate was determined by the ratio of radioactivity of isolated chimonanthine (counts per minute) over weight of isolated chimonanthine.

In this single study, Kirby synthesized radiolabelled tryptamine derivatives (80-82, Figure 1.6) and then fed solutions of these compounds to the leaves of *Chimonanthus fragrans*, a terrestrial plant from southern China. After seven days, the alkaloids were isolated by extraction, and the incorporation of radiolabelled precursors
into chimonanthine was evaluated. From the radioactivity data, it was determined that the radiolabelled tryptophan (80), tryptamine (81) and N<sub>b</sub>-methyltryptamine (82) were incorporated to the isolated sample of chimonanthine at a rate of 3.6%, 11.1% and 0.1%, respectively. These results indicated that tryptamine in particular was a biological starting point for the biosynthesis of chimonanthine. The low incorporation rate for the radiolabelled N<sub>b</sub>-methyltryptamine (82) compared to that of tryptamine (81) was postulated to result from the low solubility of this compound in water.

Unfortunately, since Kirby’s study 45 years ago, there have been no further studies on the biosynthesis of chimonanthine.

1.4. Proposed Biosynthesis of Chimonanthine

The biosynthetic pathway proposed by Kirby involves the steps tryptophan (85) → tryptamine (86) → N<sub>b</sub>-methyltryptamine (37) → chimonanthine. (Scheme 1.10) It is reasonable that tryptophan decarboxylase (TDC) is involved in the removal of carboxylate group from tryptophan (85) to yield tryptamine (86).<sup>[64–66]</sup> Then, indolethylamine N-methyltransferase (INMT),<sup>[67–69]</sup> a well-characterized enzyme, could effect methylation of the primary amine to afford N<sub>b</sub>-methyltryptamine (37). The final dimerization step of N<sub>b</sub>-methyltryptamine (37) into chimonanthine involves unidentified enzyme(s).

Scheme 1.10. Proposed biosynthetic pathway of chimonanthine from tryptophan.

The unknown dimerase that carries out the conversion of 37 into chimonanthine was proposed to be responsible for the oxidation of 37 required for recombination of the resultant radicals to form the two adjacent quaternary all-carbon stereocentres (Scheme 1.11).
1.5. Main Goals of This Study

Since the discovery of (-)-chimonanthine (29) approximately 55 years ago, the chemical syntheses of chimonanthines and their derivatives have attracted considerable interest from the synthetic community owing to their interesting molecular structures and potentially useful biological activities. However, the biosynthesis of chimonanthine is not well established. More specifically, the key enzyme responsible for the dimerization of $N$-methyltryptamine (37) to provide chimonanthine is unknown. Based on Kirby’s proposal in 1969, the protein is postulated to have two important functions: i) oxidation of a reactive indole intermediate to form a radical, and ii) promoting an enantioselective carbon-carbon bond formation reaction with an equivalent radical intermediate. Importantly, identification of this dimerase could allow for the chemoenzymatic synthesis of chimonanthines and chimonanthine analogues using cheap and commercially available starting materials.

The goal of the thesis was to develop methods to investigate the unknown enzyme responsible for the oxidative dimerization of $N$-methyltryptamine (37). The following chapter of this thesis includes a discussion of *in planta* experiments. Similar to Kirby’s study, halogenated or isotope labelled tryptamine derivatives were prepared (e.g.
fluorinated tryptamine derivative, deuterated tryptamine derivative), and these precursors were fed into a chimonanthine-producing plant, *Chimonanthus praecox*. Once suitable biosynthetic precursors were identified, *in vitro* experiments were initiated. Then, the results from protein extractions, cell component preparation (protoplast and cell wall) are presented, and examination reaction buffers with an ultimate goal of producing chimonanthine analogues using cell extracts.
Chapter 2.

Studies towards the Biosynthesis of (-)- and meso-Chimonanthine

2.1. Background Information

Precursor-directed biosynthesis (PDB) is a method used to exploit existing biosynthetic pathways to natural products by feeding unnatural precursors that can be transformed through these pathways into structurally related unnatural products.\textsuperscript{[70,71]} As illustrated in Figure 2.1, PDB relies on the administration of unnatural precursor of the natural biosynthetic precursor into a target tissue or organism. In Nature, only natural precursors are used to synthesize a natural product. However, when a synthetic unnatural precursor analogue is administered to a particular organism, this unnatural substrate can, in some cases, be assimilated into the biosynthetic pathways of the natural product, leading to the formation of an unnatural product that is structurally related to the natural product. The biosynthetic machinery (e.g., enzymes) is then exploited to produce analogues of the targeted natural products.\textsuperscript{[72–74]}

![Figure 2.1. Illustration of unnatural product synthesis by PDB\textsuperscript{[70,71]}](image-url)
The application of PDB begins with the careful selection of suitable precursor analogues. Early stages of these experiments focus specifically on understanding how the administered unnatural precursors are tolerated by the biosynthetic machinery of a particular organism. The selected unnatural precursors must be able to reach the sites where the organism maintains the enzymes required to produce the natural product of interest. The production of unnatural products can then be tracked by analytical techniques such as HPLC, TLC, LC-MS, GC-MS or NMR. After confirming their production, anticipated unnatural products are then isolated and their structure characterized using modern spectroscopic methods. In some cases, at the same time, unnatural biosynthetic intermediates can also be isolated from this process such that one can track molecules produced during the biosynthetic cycle en route to the target natural product.[70,71,75] For example, in Figure 2.2, nostocarboline (88) is biosynthesized from tryptophan (87) in a strain of cyanobacterium Nostoc 78-12A, and one of the key steps in the biosynthesis of nostocarboline is chlorination of the C5 position. By feeding halogenated or methylated precursor analogues (89-93) into this strain, these precursors bypass the halogenation step and enter into the biosynthetic pathway to yield various nostocarboline analogues (94-98).[76]

Efforts toward the PDB of chimonanthine are discussed in this Chapter. The species Chimonanthus praecox, notably the identical one used in Kirby’s studies described earlier (Section 1.3.5), was cultivated in the SFU greenhouse facility. We initiated our study with the chemical synthesis of potentially useful unnatural precursors that would be used in subsequent in planta feeding experiments (Figure 2.2. (a)). These feeding experiments were performed by delivering the unnatural precursors into Chimonanthus praecox plant tissues, including leaves and roots by placing the plant tissues in series of unnatural precursor solutions. Then allowing the plant tissue several days to incorporate the precursors forms chimonanthine analogues. After a sufficient incubation period, the alkaloids were extracted from the leaves and roots, then the crude samples were analyzed by LC-MS to monitor production of specific m/z of target chimonanthine analogues (Table 2.1). The main goals of these initial experiments were to screen for precursors that could be incorporated in the biosynthetic pathway of (-)- and meso-chimonanthine (29, 31), as well as to then isolate and characterize potential unnatural chimonanthines.
Figure 2.2. Precursor directed biosynthesis of nostocarboline analogues
Figure 2.3. Illustration of experimental scheme. (a) *in planta* assay (b) *in vitro* assay.

(a) *in planta* experiment: unnatural precursors screening and isolation of unnatural products. (b) *in vitro* experiment: studies toward the identification of a dimerase catalyzing the production of chimonanthine. Analytical tools such as NMR and/or LC-MS allow detection of targeted unnatural product(s).

Following the identification of suitable unnatural precursors, this study focused on the extraction of protein and components of cells from *Chimonanthus praecox* followed by *in vitro* assays on the biosynthesis of chimonanthine (Figure 2.2. (b)). Specifically, the extracted protein or cell components are incubated with unnatural precursors shown to be competent in the PDB studies, then the presence of chimonanthine analogues is assessed using LC-MS analyses. Following these studies, the proteins or cells are further separated by either protein size or cell fractionation, respectively. These different types of fractions can then be analyzed for their ability to produce the unnatural products. The ultimate goal of this study is to isolate the unknown enzyme(s) that is(are)
responsible for the C-C bond formation/dimerization between two \( N_b \)-methyl-tryptamines (37) that yields chimonanthine.

### 2.2. Preparation of Precursor Analogues

As described in Section 1.3.5., Kirby’s study led to the proposal for a biosynthetic pathway involving the conversion of tryptophan (85) to tryptamine (86), which is then converted to \( N_b \)-methyl-tryptamine (37) and finally dimerized to provide chimonanthine (e.g. 29) (Scheme 1.10). In order to select suitable unnatural precursor candidates for our feeding experiments, we began our investigation by evaluating commercially available and synthetically accessible analogues of tryptamine and \( N_b \)-methyl-tryptamine.

**Scheme 2.1. Synthesis of fluorinated unnatural precursor derivatives**

In Scheme 2.1, 5-fluoroindole (100) undergoes alkylation to afford nitroalkene 101. Then, without purification of 101, the nitro group is reduced using NaBH₄ to yield 5-fluorotryptamine (102). Addition of chloroethylformate to 102 yields carbamate 103, which after reduction using LiAlH₄ or LiAlD₄ furnishes the desired unnatural precursors 104 or 105. The same chemistry was applied to prepare compounds 37, 110, 112, and 115 (Figure 2.4, see experimental for details). The other precursor analogs used in this study were purchased from AK Scientifics and/or Alfa Aesar.
The unnatural precursors have unique molecular weight distinguishable from natural precursor, and also have unique characteristics, for example, the fluorinated precursors 108, 109, 113, and 114 can be tracked by $^{19}$F-NMR, and brominated precursors (111 and 115) display specific isotope patterns in MS analysis (Figure 2.4).

![Chemical structures of precursors](image)

Figure 2.4. Precursor analogues to study PDB of chimonanthine

### 2.3. *In Planta* Experiment (Feeding Experiment)

#### 2.3.1. Screening Precursor Analogues

The precursor analogues were dissolved independently in weakly acidic water (pH = 4) and fed to the leaves of *Chimonanthus praecox* by standing the stems of leaves in the water solution as described by Kirby. This process required a five-day incubation period in a greenhouse and, after this time, the leaves were collected and ground using a mortar and pestle with freezing in liquid nitrogen. The powdered green leaves were suspended in methanol and stirred overnight. The solvent was collected by filtration to remove insoluble plant debris and then concentrated *in vacuo*. The crude alkaloid samples were then obtained by acid/base and chloroform extractions (see Experimental 2.7.6). The resulting crude mixtures were submitted for LC-MS analyses to detect the presence of potential unnatural products.
Figure 2.5. Feeding precursor analogues into leaves of *chimonanthus praecox*

In our studies, we used the extracted ion chromatogram (EIC) function for our high resolution-liquid chromatography-mass spectrometry experiments. Specifically, EIC allows the detection of the specific m/z of interest from the entire data acquired in chromatographic run. The use of this technique is critical for the detection of small amounts of unnatural products in the presence of larger amounts of the natural products and other small molecules. In Table 2.1, the expected unnatural products from feeding experiments involving either one or two unnatural precursors are presented.

From the results of LC-MS analyses the formation of unnatural products was preliminarily established by monitoring their unique m/z. Administration of a single precursor or two precursors simultaneously were performed. The relative level of incorporation was estimated by calculating by the ratio of areas of m/z of unnatural product over areas of m/z of (-)-chimonanthine (29) observed in the LC chromatograms using the MS as a detector in EIC mode. The LC-MS analysis in EIC mode indicated that precursor analogues 107, 108, 109, 112, 113, and 114 were incorporated into the expected unnatural chimonanthine analogues (117, 118, 119, 122, and 123). Furthermore, 6-methyltryptamine 106, 5-chlorotryptamine (110), 5-bromotryptamine (111), and 5-bromo-Nβ-methyltryptamine (115) did not provide the m/z expected for the potential chimonanthine analogues (116, 120, and 121). Interestingly, after feeding with 7-methyltryptamine (107), LC-MS analysis detected the expected m/z of the corresponding chimonanthine analogue (117). As shown in Table 2.1, two other precursor analogues also gave rise to the anticipated m/z of their corresponding chimonanthine analogues. Based on these preliminary results suggesting that C5 and
C6 substituted products were not formed, we postulate that somewhat larger substituents, such as Cl, Br, and CH₃, at these positions may not be tolerated by the enzymes in the biosynthetic pathway leading to chimonanthine.

Table 2.1. Results after feeding experiment

<table>
<thead>
<tr>
<th>Entry</th>
<th>Precursors</th>
<th>Expected chimonanthine analogue</th>
<th>Exact m/z of chimonanthine analogue</th>
<th>Detection of the m/z of chimonanthine analogue</th>
<th>Incorporation (%)</th>
</tr>
</thead>
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<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td>375.2543</td>
<td>No</td>
<td>NI</td>
</tr>
<tr>
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<td><img src="image4.png" alt="Image" /></td>
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<td>Yes, 373.2545</td>
<td>1.97</td>
</tr>
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<td>17.1</td>
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<td><img src="image10.png" alt="Image" /></td>
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<td>No</td>
<td>NI</td>
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<td></td>
<td>Structure 1</td>
<td>Structure 2</td>
<td>Mass 1</td>
<td>Mass 2</td>
<td>Yes/No</td>
</tr>
<tr>
<td>---</td>
<td>-------------</td>
<td>-------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
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<td><img src="image12.png" alt="Structure 12" /></td>
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<td>0.54</td>
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<td><img src="image12.png" alt="Structure 12" /></td>
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<td>0.54</td>
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<tr>
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</tr>
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<td><img src="image121.png" alt="Structure 121" /></td>
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<td>0.54</td>
</tr>
<tr>
<td>11</td>
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<td>Yes, 378.2325</td>
<td>0.79</td>
</tr>
</tbody>
</table>
12  
\[
\text{113} 
\text{+} 
\text{112} 
\text{N-CD₃} 
\text{125} 
\text{F} 
\text{368.2324} 
\text{Yes, 368.2325} 
\text{1.12} 
\]

13  
\[
\text{112} 
\text{+} 
\text{108} 
\text{NH₂} 
\text{125} 
\text{F} 
\text{368.2324} 
\text{Yes, 368.2319} 
\text{1.06} 
\]

14  
\[
\text{112} 
\text{+} 
\text{114} 
\text{N-CD₃} 
\text{126} 
\text{D₃C₂} 
\text{371.2512} 
\text{Yes, 371.2508} 
\text{0.79} 
\]

15  
\[
\text{37} 
\text{+} 
\text{108} 
\text{NH₂} 
\text{127} 
\text{F} 
\text{365.2136} 
\text{Yes, 365.2137} 
\text{1.30} 
\]

16  
\[
\text{37} 
\text{+} 
\text{127} 
\text{F} 
\text{365.2136} 
\text{Yes, 365.2144} 
\text{1.11} 
\]
2.3.2. Isolation of Unnatural Products, (-)-5,5'-Difluoro-chimonanthine and meso-5,5'-Difluoro-chimonanthine

After identifying several putative precursor analogues, we decided to utilize 5-fluorotryptamine (108) as our model precursor analogue for our in planta experiments. This choice was largely driven by the generation of a strong m/z signal associated with that of the target unnatural product by LC-MS analyses (entry 3, Table 2.1) but also by the ability to monitor formation of this product by $^{19}$F NMR spectroscopy. The feeding experiments were therefore scaled-up in order to isolate sufficient quantities of the unnatural products 128 and 129 (Figure 2.6) that would enable proper characterization to confirm results from LC-MS analyses.

Figure 2.6. Target unnatural chimonanthine analogues after feeding Chimonanthus praecox with 108: (-)-5,5'-fluorochimonanthine (128), and meso-5,5'-fluorochimonanthine (129)

The extracted crude alkaloids (40 mg) obtained from plant tissues after the feeding experiments were solubilized in methanol prior to purification by preparative HPLC (Figure 2.7).
Figure 2.7.  HPLC separation of alkaloid extracts after 5-fluorotryptamine (108) was fed to *Chimonanthus praecox*.

Table 2.2.  Isolation of chimonanthines and unnatural chimonanthines by HPLC

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Isolated product</th>
<th>Amount isolated (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.62</td>
<td>5-fluorotryptamine</td>
<td>6</td>
</tr>
<tr>
<td>7.6</td>
<td>(-)-5,5'-difluoro-chimonanthine</td>
<td>~0.7</td>
</tr>
<tr>
<td>7.8</td>
<td>(-)-chimonanthine</td>
<td>10</td>
</tr>
<tr>
<td>8.4</td>
<td>meso-5,5'-difluoro-chimonanthine</td>
<td>~0.05</td>
</tr>
</tbody>
</table>
Only the characterized products are reported in the table. The other fractions were not fully characterized.

Table 2.2 summarizes the characterization of the isolated alkaloids. Among the 40 mg of the crude alkaloid preparation, 6 mg (15%) was the administered compound. The amounts of the known natural products, (−)-chimonanthine (29) and meso-chimonanthine (31) were 10 mg (25%), and 6 mg (15%), respectively. The desired chimonanthine analogues, 128, and 129 were collected, and the amounts were approximately 0.7 mg (1.75%) and 0.05 mg (0.1%), respectively. Some remaining material may be present in the additional peaks observed in the chromatogram but these have not been characterized.

After separation, each fraction was submitted for LC-MS analysis. The fractions with the expected m/z were combined and characterized by 1H-NMR and 19F-NMR spectroscopy. (−)-5,5′-Difluorochimonanthine (128, Figure 2.6) showed the desired m/z when analyzed by LC-MS and eluted at a retention time of 1.5 minutes. As expected, the 1H-NMR spectrum displayed three characteristic aromatic proton resonances (δ 6.4 to 6.9 ppm) and a single fluorine resonance (δ 127.1 ppm) in the 19F-NMR spectrum. This provided good evidence for the incorporation of fluorine into the unnatural product 128.

While the characterization of (−)-5,5′-difluorochimonanthine (128) was not particularly challenging, the characterization of meso-5,5′-difluorochimonanthine (129) presented several interesting challenges. Based on the LC-MS analyses of meso-5,5′-difluorochimonanthine (129), we observed the desired m/z signal corresponding to the unnatural product at a retention time of 4.2 minutes. As depicted in Figure 2.8, 1H-NMR and 19F-NMR analyses performed at room temperature were not enough to allow proper characterization. The resonances from the 1H-NMR spectra were very broad and J-couplings could not be determined. The 19F-NMR spectrum included two fluorine
resonances at room temperature (δ 129.2 and 129.4 ppm), which did not match with the expected single fluorine resonance.

We speculated that the broad signals may stem from the presence of slowly interconverting atropisomers. Accordingly, we were able to resolve this issue by increasing the temperature of the sample being analyzed by NMR spectroscopy up to 80 °C. As indicated in Figure 2.6, an increase in temperature during acquisition of ¹H-NMR spectra resulted in spectra with much sharper resonances, presumably due to the more rapid interconversion of two atropisomers. Additionally, the corresponding ¹⁹F-NMR spectra recorded at higher temperatures also indicated coalescence of two separate fluorine resonances into a single resonance.

![Figure 2.8. ¹H and ¹⁹F NMR spectra of isolated meso-5,5'-difluorochimonanthine (129) acquired at various temperatures](image)

The NMR experiments were performed at four different temperatures, 25, 40, 60 and 80 °C).

Based on these NMR spectroscopic experiments, we were able to characterize meso-5,5'-difluorochimonanthine (129). It indicated meso-129 is not formally meso at room temperature, but rather is a mixture of two atropisomers. Also, at higher temperature, the atropisomers rapidly interconvert such that the atropisomers display a spectrum characteristic of a meso compound.[77–82]. It is expected that an energy barrier must be overcome in order to rotate the C3-C3' bond; thus, at room temperature there are two possible conformations of meso-5,5'-difluorochimonanthine (129).
2.4. In Vitro Assay

2.4.1. Background Information

It was previously proposed\textsuperscript{[20,62]} that an unknown dimerase is responsible for catalyzing the dimerization of \( N_b \)-methyltryptamine (37) to provide chimonanthine (29, see Scheme 1.10). While our initial work has shown that \textit{Chimonanthus praecox} can incorporate \( N_b \)-methyltryptamine (37) into the synthesis of chimonanthine, it was necessary to extract proteins from \textit{C. praecox} to further investigate and ultimately isolate the enzyme responsible for the transformation of interest. An important aspect of our study also aimed at addressing the location of this enzyme within the plant.

![Figure 2.9. Feeding D\textsubscript{3}-N\textsubscript{b}-methyltryptamine into different plant parts. Order from left to right. A) One whole leaf. B) Small pieces of a leaf. C) Stem. D) Branch. E) Root. F) Control. The different parts of \textit{Chimonanthus praecox} were incubated with precursors at room temperature, and stirred for three days.](image)

In order to pinpoint the location of the enzyme catalyzing the dimerization reaction en route to chimonanthine (29), we first selected specific components of \textit{Chimonanthus praecox} including leaves, stems, branches and roots (Figure 2.9). These individual plant parts were placed in an aqueous solution of D\textsubscript{3}-\( N_b \)-methyltryptamine (112) and 5-fluorotryptamine (108) and incubated for three days (Figure 2.9). The plant components were then taken out of solutions, and were suspended individually in methanol and the samples were sonicated for one hour. The methanol extracts were then filtered, and aliquots of the filtrate were subjected to LC-MS analyses. As indicated in Table 2.3, the biosynthesis of chimonanthine may occur in either the leaves or the roots of the plant.
### Table 2.3  Localization of Biosynthesis of Chimonanthine

<table>
<thead>
<tr>
<th>Entry</th>
<th>Plant Part</th>
<th>Detection of (118) m/z 383.2042</th>
<th>Detection of (122) m/z 353.2607</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leaf</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>Stem</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>Branch</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>Root</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>Control (No plant part)</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

$m/z$ of 118: 383.2042, of 122: 353.2607

#### 2.4.2. Cytoplasmic Protein Extraction from Leaf

The initial attempts to extract the proteins involved in this transformation were performed using commercially available plant protein extraction kits in combination with mechanical extraction methods. For our experiments, we opted to use the P-PER™ Plant Protein Extraction Kit, which has the advantage of being fast and providing pure protein that can be used directly for enzymatic reactions. Specifically, this kit is designed for the protein extraction up to 80 mg of plant sample but can only extract cytoplasmic proteins.

Following the extraction protocol on leaves as described by the manufacturer, the extracted sample was incubated with $N_b$-methyltryptamine derivatives 112 and 113 overnight under several conditions. Table 2.4 summarizes the experimental data. The samples were then filtered through Amicon® 3K centrifuge tubes and aliquots from each sample were subjected to LC-MS analyses. Unfortunately, the products having desired $m/z$ were not detected in any of the samples. The lack of success from these experiments may be due to the use of an inappropriate buffer during the *in vitro* enzymatic reaction or protein extraction.
Table 2.4  In vitro assay with protein extracts by P-PER™ Plant Protein Extraction Kit

<table>
<thead>
<tr>
<th>Entry</th>
<th>Precursor</th>
<th>Buffer</th>
<th>Cofactor</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Detection of m/z of 353.2607</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>N/A</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>N/A</td>
<td>7.4</td>
<td>4</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>N/A</td>
<td>7.4</td>
<td>30</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>N/A</td>
<td>4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>N/A</td>
<td>9</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>Vitamin C</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>Mg</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>FeCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>NADH</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>FAD</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>NAD</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>B</td>
<td>N/A</td>
<td>7.8</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>B</td>
<td>N/A</td>
<td>7.8</td>
<td>4</td>
<td>No</td>
</tr>
<tr>
<td>14</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>B</td>
<td>N/A</td>
<td>7.8</td>
<td>30</td>
<td>No</td>
</tr>
<tr>
<td>15</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>B</td>
<td>MgBr&lt;sub&gt;2&lt;/sub&gt;</td>
<td>7.8</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>16</td>
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<td>B</td>
<td>FeCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>7.8</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>17</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>B</td>
<td>FeSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>7.8</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>18</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>B</td>
<td>NADH</td>
<td>7.8</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>19</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>B</td>
<td>FAD</td>
<td>7.8</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>20</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>B</td>
<td>NAD</td>
<td>7.8</td>
<td>rt</td>
<td>No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Entry</th>
<th>Precursor</th>
<th>Buffer</th>
<th>Cofactor</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Detection of m/z of 353.2607</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>N/A</td>
<td>4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>22</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>N/A</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>23</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>N/A</td>
<td>9</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>24</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>N/A</td>
<td>7.4</td>
<td>4</td>
<td>No</td>
</tr>
<tr>
<td>25</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>N/A</td>
<td>7.4</td>
<td>30</td>
<td>No</td>
</tr>
<tr>
<td>26</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>Vitamin C</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>27</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>MgBr&lt;sub&gt;2&lt;/sub&gt;</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>28</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>FeCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
</tbody>
</table>
The second attempt to obtain protein extracts used homogenized extracts of leaves. In a cold room (4 °C), 20 g of freshly removed leaves of *Chimonanthus praecox* were placed in a blender with 150 ml of various cold buffers (Table 2.5). The leaves were homogenized in a blender for 4 × 10 seconds pulses. The plant debris were filtered over cheesecloth and the filtrate was centrifuged at 14,000 rpm for 30 min at 4 °C. The resulting supernatants were collected and concentrated by using Amicon® centrifuge tubes. The protein samples were then incubated with precursor analogues 103 and 104 in various conditions (Table 2.5), but the enzymatic reactions were not conclusive.

**Table 2.5. In vitro assay with protein extracts from homogenized plant leaf samples after blending**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Precursor</th>
<th>Extraction Buffer</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5-F-(N_0)-methyltryptamine</td>
<td>A</td>
<td>7.4</td>
<td>4, rt, 37</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>5-F-(N_0)-methyltryptamine</td>
<td>B</td>
<td>7.8</td>
<td>4, rt, 37</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>5-F-(N_0)-methyltryptamine</td>
<td>C</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>Entry</td>
<td>Precursor</td>
<td>Extraction Buffer</td>
<td>pH</td>
<td>Temperature (°C)</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-----------</td>
<td>--------------------</td>
<td>----</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>D</td>
<td>7.1</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>E</td>
<td>7.0</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>5-F-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>F</td>
<td>4.3, 5.3, 6.4, 7.3, 8.3, 9.1,10</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>D&lt;sub&gt;3&lt;/sub&gt;-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>A</td>
<td>4, 7.4, 9</td>
<td>4, rt, 37</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>D&lt;sub&gt;3&lt;/sub&gt;-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>B</td>
<td>7.8</td>
<td>4, rt, 37</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>D&lt;sub&gt;3&lt;/sub&gt;-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>C</td>
<td>7.4</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>D&lt;sub&gt;3&lt;/sub&gt;-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>D</td>
<td>7.1</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>D&lt;sub&gt;3&lt;/sub&gt;-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>E</td>
<td>7.0</td>
<td>rt</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>D&lt;sub&gt;3&lt;/sub&gt;-N&lt;sub&gt;6&lt;/sub&gt;-methyltryptamine</td>
<td>F</td>
<td>4.3, 5.3, 6.4, 7.3, 8.3, 9.1,10</td>
<td>4,rt,37</td>
<td>No</td>
</tr>
</tbody>
</table>

**Buffer A:** PBS. **Buffer B:** 0.2M Tris-HCl pH of 7.8. **Buffer C:** PBS, 1 mM PEG, 5% (w/v) PVPP, 0.01% Triton X-100. **Buffer D:** 25mM Hepes-Na, 0.5 mM EDTA, 8 mM MgCl₂, 8 mM DTT. **Buffer E:** 0.2M MOPS, 5% w/v PVPP, 1% Triton X-100 (v/v), 10% glycerol, 2 mM DTT. **Buffer F:** 0.45 M Mannitol, 50 mM sodium phosphate, 2 mM EDTA. rt: room temperature.

## 2.4.3. Cytoplasmic Protein Extraction from Root

Following the failure to isolate the enzymes responsible for the biosynthetic pathway from the leaves of *C. praecox*, the next target was the root of the plant (Figure 2.10). The roots of *C. praecox* were collected and subjected to the same extraction procedure as that previously described for the leaves, with the notable exception that a pH 4.5 sodium acetate buffer was used because we assumed that in lower pH the
precursors have better solubility in water.[83] Further purification and separation of root proteins was performed by ammonium sulfate precipitations at concentrations of 0-20%, 20-40%, 40-60%, 60-80% and 80-95%, and the proteins from each precipitation were collected after dialysis in pH 4.5 sodium acetate buffer.

Figure 2.10. Image of roots of *Chimonanthus praecox* washed with distilled water.

The crude protein samples were concentrated by Amicon® centrifugation tubes followed by ammonium sulfate precipitation. The crude root protein samples were incubated with D$_3$-N$_b$-methyltryptamine (112) or 5-fluoro-N$_b$-methyltryptamine (113) (Scheme 2.2).

Scheme 2.2. Expected incorporation of 113 to 118 *in vitro* assay with root protein extract

Unfortunately, these experiments were unsuccessful (Table 2.6). In light of the failures encountered in the protein extract-based experiments, we suspended further investigation into isolating the key enzyme from the roots, primarily due to the detrimental effects on the plant as a result of removing its roots for our studies.
Table 2.6. In vitro assay with 118 and protein extracts from homogenized plant root samples after blending and ammonium sulfate precipitation

<table>
<thead>
<tr>
<th>Entry</th>
<th>Cofactor(s)</th>
<th>Ammonium sulfate (%)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N/A</td>
<td>crude</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>FAD</td>
<td>crude</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>NADP⁺, NADPH</td>
<td>crude</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>NADP⁺, NADPH, FeCl₃, FeSO₄</td>
<td>crude</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>N/A</td>
<td>0-20</td>
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<td>6</td>
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<td>No</td>
</tr>
<tr>
<td>7</td>
<td>NADP⁺, NADPH</td>
<td>0-20</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>NADP⁺, NADPH, FeCl₃, FeSO₄</td>
<td>0-20</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>N/A</td>
<td>20-40</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>FAD</td>
<td>20-40</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>NADP⁺, NADPH</td>
<td>20-40</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>NADP⁺, NADPH, FeCl₃, FeSO₄</td>
<td>20-40</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td>N/A</td>
<td>40-60</td>
<td>No</td>
</tr>
<tr>
<td>14</td>
<td>FAD</td>
<td>40-60</td>
<td>No</td>
</tr>
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<td>NADP⁺, NADPH</td>
<td>40-60</td>
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</tr>
<tr>
<td>16</td>
<td>NADP⁺, NADPH, FeCl₃, FeSO₄</td>
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<td>17</td>
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<tr>
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<td>NADP⁺, NADPH</td>
<td>60-80</td>
<td>No</td>
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<tr>
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<td>NADP⁺, NADPH, FeCl₃, FeSO₄</td>
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</tr>
<tr>
<td>24</td>
<td>NADP⁺, NADPH, FeCl₃, FeSO₄</td>
<td>80-95</td>
<td>No</td>
</tr>
</tbody>
</table>
Buffer: 50 mM sodium acetate, pH 4.5, reaction at room temperature overnight. \( m/z \) of difluorinated chimonanthine is 383.2042

2.4.4. \textit{In vitro} Assay with Protoplast

The cell wall that surrounds the plasma membrane is a distinct cellular component of plant cells. Our lack of success in the preceding experiments led us to believe that the cell wall may hinder the homogenization of plant protein samples during blending. Thus, we next removed the cell wall to obtain the protoplasts, which are plant cells without a cell wall. The protoplast preparation was adapted from Sheen’s methods\cite{84} (for detailed procedures, see the experimental section 2.7.11). The cell wall was digested enzymatically by incubation of leaves of \textit{Chimonanthus praecox} with two enzymes: cellulase and pectinase (Figure 2.11). After preparation of protoplasts, 5-fluorotryptamine (108) was added to the protoplast samples and incubated overnight at room temperature. The samples were filtered through Amicon® ultra centrifugal tubes. Unfortunately, these experiments did not produce the desired difluorochimonanthine.

![Fluorescent microscopy images of plant cells. Native plant cells (left) and a protoplast after enzymatic digestion (Right).](image)

The images were taken with WaveFX spinning disc confocal microscopy with 40x objective and Hamamatsu 9100 EMCCD camera. The native plant cells (left) are in shape in the presence of cell wall, but the protoplast (right) after enzymatic digestion of cell wall in native cells becomes rounded. The scale bars indicate 10 \( \mu \text{m} \)
2.4.5. Proteins from Cell Walls

The cell wall was then considered as a possible site for this elusive enzymatic reaction, since no chimonanthine analogues were produced during our cytoplasmic protein extracts and protoplasts feeding experiments. The plant cell wall is known to protect cells from insects, and provide rigidity to maintain the shape of the cell as well as to help it endure internal osmotic pressure. Also, it is mainly composed of carbohydrates including cellulose, hemicellulose, and pectin. Other compositions of cell walls are lignin, suberin, waxes and proteins. However, the protein and enzyme content of cell walls is not well known.[85–87]

In 1997, however, the Lewis group at Washington State University reported a new kind of protein involved in the synthesis of (+)-pinoresinol called ‘dirigent protein’ found in the cell wall of Forsythia suspensa.[88–93] The meaning of dirigent is ‘to align’, and the main function of the dirigent protein is to provide only the desired stereoselective product by avoiding undesired C-C bond formation by aligning two substrates with specific orientation. The enzyme was also proposed to stabilize one of the radical intermediates involved in the reaction. Their study examined the biosynthesis of (+)-pinoresinol (135, Scheme 2.3), which is a dimer of (E)-coniferyl alcohol (130). Lewis and coworkers postulated that an enzyme is responsible for coupling two (E)-coniferyl (130) alcohols selectively to form (+)-pinoresinol. They reported that pure (E)-coniferyl alcohol (130) spontaneously led to several different isomers (132-134). However, the ‘dirigent protein’ offered region-and stereospecific control in the production of (+)-pinoresinol in the presence of an oxidant. Several years later, another dirigent protein which afforded (−)-pinoresinol (136) from (E)-coniferyl alcohol (130) was discovered in 2010 (Scheme 2.3).[94,95]

The evidence for dirigent proteins in the cell wall of F. suspensa provided an important precedent for the investigation of enzymes embedded in the cell wall. With this in mind, we decided to investigate the cell wall of C. praecox to determine if it contained the elusive dimerase we were seeking. Several important biosynthetic similarities exist in regards to the formation of the downstream metabolites present in F. suspense and Chimonanthus praecox. For example, the dimeric nature of pinoresinol is similar to that observed in chimonanthine, albeit synthesized from different monomers. Furthermore,
the biosynthesis of pinoresinol occurs via an oxidative dimerization reaction, which is also postulated to occur during the biosynthesis of chimonanthine. Based on these topical similarities, we set out to isolate the cell wall proteins from *C. praecox* with the rationale being that the enzyme involved in the formation of chimonanthine might be present in this cellular component.

Scheme 2.3. Comparison between uncontrolled and controlled oxidative dimerization to afford natural product
The procedure used for the cell wall preparation was that described by Lewis (see experimental section 2.7.13 for details) (Figure 2.12). Following the preparation of cell wall material, 0 mg, 100 mg, 200 mg, 300 mg and 400 mg of this cell wall preparation was incubated with an aqueous solution of 1 mL of 50 mM potassium phosphate buffer (pH 7), 5 mM 5-fluoro-N-methyltryptamine (113), and 2.5 µM ammonium persulfate overnight at 30 °C. The samples were then filtered through 3K Amicon® centrifugal tubes, to remove proteins and insoluble materials, and then submitted for analyses by LC-MS.

![Figure 2.12](image)

**Figure 2.12.** Images of ground leaves in liquid nitrogen in a mortar (a), cell wall powders after removing cellular components (b) and cell wall proteins on SDS-PAGE (c)

A green power was obtained after grinding leaves of *C. praecox* in liquid nitrogen using a mortar and pestle (a). Mild detergent and acetone works to remove cytoplasmic components by opening up the cell membrane to afford pale-yellow cell wall materials (b). Proteins were then extracted from the crude cell wall materials and separated by SDS-PAGE (c).

We were delighted to find that the crude cell wall preparation appeared to promote the dimerization of our fluorinated precursors as demonstrated by the presence of m/z signal of 383.2042 ± 0.002 corresponding to difluorinated chimonanthine analogue (128) (Figure 2.13). This observation provides good evidence for the presence of the dimerase within the cell walls of *Chimonanthus praecox*.

Figure 2.13 displays the EIC for the m/z signal of 383.2042 ± 0.002. Notably the intensity of the signals at a retention time of 4.7 min becomes stronger as the amount of cell wall materials used in the assays is increased. This data indicates that more product
was formed in the reaction mixture containing the cell wall components. One reservation, however, is that the retention time observed is not the same as that observed for the isolated difluorochimonanthines. Furthermore, we did not perform a control experiment in which no precursor was added to test for the presence of molecules having the expected mass within the cell wall fraction. Nevertheless, this preliminary observation provided some impetus to investigate further these cell wall materials as a potential source of the putative dimerase.

Figure 2.13. Extracted ion chromatograms of m/z of 383.2042 ± 0.002 in the time interval of 4.4 min to 5.2 min after incubation of precursor with different amount of cell walls

To further test for the presence of the putative dimerase enzyme in cell walls, the cell wall proteins were extracted with 1M NaCl from the pale yellow crude cell walls (Figure 2.12. (b)). After concentrating the extracted protein samples by 3K Amicon® centrifugal tubes, 200 µL of the resulting crude protein sample was obtained having a protein concentration of 1.8 mg/mL as determined by using DC Protein Assay (Bio-Rad). Incubation of the resulting extracted proteins (0 µL, 15 µL, 30 µL, 45 µL and 60 µL of 1.8 µg/µL protein solution) were added to the same reaction buffer that was previously developed (ammonium persulfate in potassium phosphate buffer) and the 5-fluoro-Nα-
methyltryptamine precursor was added. The mixtures were incubated overnight at 30 °C and the samples were then filtered through 3k Amicon® centrifugal tubes. As shown in Figure 2.14a, EIC analysis indicated the presence of a compound having an m/z of 383.2042 ± 0.002, which corresponds to the predicted m/z for 128 (m/z = 383.2042). We were able to further confirm the identity of this species as 5,5'-difluorochimonanthine by overlaying the chromatograms of (-)-5,5'-difluorochimonanthine (Figure 2.14a) and meso-5,5'-difluorochimonanthine (Figure 2.14b) with that obtained from our incubation experiments. In the case of (-)-5,5'-difluorochimonanthine (128, t_R = 1.4-1.5 min) and meso-5,5'-difluorochimonanthine (129, t_R = 4.2-4.4 min), the peak area and peak height also increased with larger amounts of the extracted cell wall proteins. These results provide good support for our hypothesis that an enzyme associated with the cell wall is responsible for the dimerization of 5-fluoro-Nb-methyltryptamine (113).
Notably, these signals did not appear in the absence of an oxidant or cell wall preparation. This observation suggests that both an oxidant and cell wall proteins are required for the biosynthesis of chimonanthine.
2.5. Summary

Although the identification and characterization of the unknown chimonanthine dimerase has not yet been achieved, the experiments described in this thesis provide an advanced starting point for solving this problem. First, we identified that *Chimonanthis praecox* is able to use several precursor analogues for the biosynthesis of the corresponding unnatural chimonanthines. Also, feeding experiments followed by LC-MS analyses indicated that larger substituents on the C5 and C6 position of tryptamine hindered the incorporation of precursor analogues into the active site during the biosynthesis of unnatural chimonanthines. Then, through incubating different plant parts with a suitable precursor analogue (5-fluoro-\(N_b\)-methyltryptamine), we identified that the biosynthesis of chimonanthine should take place either in the leaves or the roots of *Chimonanthis praecox*. During our attempts to purify proteins from different components of plant cells, we were delighted to find good data supporting the presence of protein(s) in the cell wall that catalyze the biosynthesis of chimonanthine in the presence of an oxidant such as ammonium persulfate.

![Scheme 2.4. Possible route of dirigent protein mediated biosynthesis of chimonanthine](image)

We propose that the biosynthesis of chimonanthine in *C. praecox* involves a dirigent protein. This may occur by the oxidation of starting compound 37 to radical species 38, upon which an unknown dirigent protein stabilizes the radical on C3, and aligns two \(N_b\)-methyltryptamine together to afford (-)-chimonanthine, but not (+)-chimonanthine. There exist, however, alternative possible mechanisms that may be addressed through detailed studied of the enzyme once purified.
2.6. Future Direction

The short-term goal of future work should be the investigation of the cell wall fractions in more detail in order to isolate and characterize the putative dimerase. Even though we were able to demonstrate the production of unnatural products through incubation with cell wall protein extracts, we encountered difficulties in subsequent reproduction of the experiments. An improvement of the extraction or reaction protocols through buffer screening would represent the next logical step. After the development of a robust protocol, our next objective would require fractionating the extracts by separation techniques including ion exchange, size exclusion or precursor attached-affinity chromatography,\[^{96-99}\] native gel running,\[^{100-102}\] ammonium sulfate precipitation\[^{103}\] or Rotofor, a preparatory isoelectric focusing apparatus\[^{104,105}\] to search a fraction where the target protein is present. We expect to be able to purify and isolate the target protein through well-established protein separation techniques. Finally, we propose using tryptic digestion of the protein in tandem with MS to help identify protein sequence.\[^{106-108}\]

Another possible approach is via mutasynthesis,\[^{70,71,75}\] which would first involve a viable leaf cell culture of *Chimonanthus praecox*\[^{109}\] followed by silencing tryptamine biosynthesis in the cell.\[^{110}\] Through such a method, the producing organism cannot use tryptamine as a feedstock for chimonanthine biosynthesis, which means unnatural chimonanthine analogues would be the major product as only unnatural precursors would be available to the plant cells. The advantage of this technique is that the separation of unnatural products and chimonanthine analogues would be more facile in the absence of large quantities of the natural product, chimonanthine. This technique has previously been successfully implemented by O’Connor in producing fluorinated unnatural products (138-141) from *Catharanthus roseus* hairy root culture (Figure 2.15). In this study, she successfully silenced the tryptophan decarboxylase in hairy root of *C. roseus* which resulted in the absence of tryptamine biosynthesis and the production of unnatural products.\[^{110}\]
Figure 2.15. O’Connor’s mutasynthesis in Catharanthus roseus.

The administration of unnatural precursor, 5-fluorotryptamine, into the hairy root of C. roseus causes production of fluorinated alkaloids, fluorinated-ajmalicine (138), fluorinated-serpentine (139), fluorinated-catharanthine (140), and fluorinated-tabersonine (141) in hairy root culture.

The long-term goal of this project will be the recombinant expression of the dimerase enzyme in vitro. Specifically, the identification of complete protein sequence of the key enzyme would allow for the direct synthesis of the corresponding mRNA. More likely, some protein sequence would allow identification of the gene by genome sequencing or through traditional cloning methods. The resulting gene sequence would be used to recombinantly express the target protein in microbiological systems or convenient plant systems to facilitate scale-up production of the enzyme. Once enough enzyme is obtained, chemoenzymatic reactions could be performed to prepare various chimonanthine analogues in one step from N6-methyltryptamine and other alkaloid precursors. Additionally, the chemoenzymatic activity tolerance will be tested in reactions designed to generate quaternary carbon centre(s) in the final downstream
products, which would potentially allow rapid access to structurally complex and diverse natural products.

2.7. Experimental

2.7.1. General Considerations

All reactions described were performed under an atmosphere of dry nitrogen using oven dried glassware unless otherwise specified. Flash chromatography was carried out with 230-400 mesh silica gel (SiliCycle, SiliaFlash® P60). Concentration and removal of trace solvents was done via a Büchi rotary evaporator using dry ice/acetone condenser, and vacuum applied from an aspirator or Büchi V-500 pump.

All reagents and starting materials were purchased from Sigma Aldrich, Alfa Aesar, TCI America, and/or Strem, and were used without further purification. All solvents were purchased from Sigma Aldrich, AK Scientific, EMD, Anachemia, Caledon, Fisher, or ACP and used without further purification, unless otherwise specified. Diisopropylamine (DIPA) and CH₂Cl₂ were freshly distilled over calcium hydride. Tetrahydrofuran (THF) was freshly distilled over Na metal/benzophenone.

Cold temperatures were maintained by use of the following conditions: 5 °C, fridge (True Manufacturing, TS-49G); 0 °C, ice-water bath; −40 °C, acetonitrile-dry ice bath; −78 °C, acetone-dry ice bath; temperatures between −78 °C and 0 °C required for longer reaction times were maintained with a Neslab Cryocool Immersion Cooler (CC-100 II) in a EtOH/2-propanol bath.

Nuclear magnetic resonance (NMR) spectra were recorded using chloroform-d₃ (CDCl₃) or methanol-d₄ (CD₃OD) or dimethyl sulfoxide-d₆ (CD₃SOCD₃) as solvents. Signal positions (δ) are given in parts per million from tetramethylsilane (δ 0) and were measured relative to the signal of the solvent (¹H NMR: CDCl₃: δ 7.26, CD₃OD: δ 3.31, CD₃SOCD₃: δ 2.50; ¹³C NMR: CDCl₃: δ 77.16, CD₃OD: δ 49.15, CD₃SOCD₃: δ 39.51).
Coupling constants (J values) are given in Hertz (Hz) and are reported to the nearest 0.1 Hz. 1H NMR spectral data are tabulated in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet), coupling constants, experimental integration providing the number of protons. NMR spectra were recorded on a Bruker Avance 600 equipped with a QNP or TCI cryoprobe (600 MHz), Bruker 500 (500 MHz), or Bruker 400 (400 MHz). Assignments of 1H and 13C NMR spectra are based on analysis of 1H- 1H COSY, HSQC, HMBC spectra, where applicable.

Optical rotations were measured using a PerkinElmer 341 polarimeter at a wavelength of 589 nm.

High resolution mass spectra were performed using a Bruker MaXis Impact TOF LC/MS. The HRMS was calibrated with internal standard sodium formate.

High performance liquid chromatography (HPLC) was performed using an Agilent 1200 Series equipped with a variable wavelength UV-Vis detector (λ = 254 nm) and XBridge™ PREP C18 5µm 10x150mm column.

The plant, Chimonanthus praecox, was purchased from Flora Exootica, QC, Canada, and then was cultivated in SFU greenhouse facility all the time. They have been watered twice a week to maintain the plant.

In planta feeding experiments were performed in a temperature-controlled greenhouse (SFU greenhouse facility). Plant protein samples were concentrated by using 10 kDa Amicon® centrifugation tubes (15 mL, 0.5 mL). They were centrifuged at 4,000 g (15 mL) or 14,000 g (0.5 mL) for 60-minute at 4 °C. The filtrates are discarded and the concentrated samples are all collected, and repeat this procedure again to prepare concentrated protein sample.

Fluorescence microscopy was performed using a WaveFX spinning disc confocal system equipped with a Yogogawa CSU-10 confocal head, lasers with λ = 441, 491, 561, 647 nm, and a Hammamatsu 9100 EMCCD camera. An objective lens of 40x magnification was used.
2.7.2. Experimental Information for Tryptamine Analogues

Preparation of 5-fluorotryptamine (108)

To a heterogeneous mixture of 5-fluoroindole (100, 1.5g, 11.1 mmol) and 1-dimethylamino-2-nitroethylene (1.4 g, 12.2 mmol) was added trifluoroacetic acid (20 ml, 0.55 M) and the reaction mixture was stirred for two hours. After this time, the reaction mixture turned black and was then diluted with EtOAc (50 mL) and 10 % aqueous Na₂CO₃ (150 mL). The phases were separated and the aqueous phase was washed with EtOAc (4 x 50 mL). The combined organic phases were then washed with brine (150 mL), dried over MgSO₄ and the solvent was removed in vacuo. The crude mixture was then suspended in hot Et₂O, filtered through Celite® and the solvent was removed in vacuo to give the crude product (2.1 g) as a yellow solid.

To a stirred flask containing THF (75 mL) at 0 °C was added sodium borohydride (1.26 g, 33.4 mmol). BF₃•OEt₂ (6.3 g, 44.5 mmol, 4 equiv) was then added slowly to the reaction mixture and stirred for 15 minutes at room temperature. The crude product from the first step was then added dropwise as a solution in THF (20 mL). The reaction mixture was heated at reflux for two hours, then cooled to room temperature and acidified to pH 1 by the addition of 1N HCl solution (~25 mL). The reaction mixture was refluxed for an additional two hours, then cooled to room temperature and washed with Et₂O (100 ml x 4). The combined organic phases were then washed with MgSO₄ and filtered, then the solvent was removed in vacuo. Purification of the crude material by flash chromatography (10% ammonia solution (7N in MeOH), 90% CH₂Cl₂) to afford 5-fluorotryptamine (108, 1.05 g, 51 %) as a brown solid.

¹H NMR (400 MHz, CD₃OD) δ: 7.28 (dd, J= 4.4, 8.6 Hz, 1H), 7.20 (dd, J= 2.5, 10.0 Hz, 1H), 7.14 (s, 1H), 6.85 (dt, J = 2.5, 9.1 Hz, 1H), 2.93 (dt, J = 2.4, 6.6 Hz, 2H), 2.85 (dt, J = 2.4, 6.6 Hz, 2H)
$^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$: 158.9 (d, $J = 231.9$ Hz), 134.81, 129.01 (d, $J = 9.6$ Hz), 125.6, 113.5 (d, $J = 4.7$ Hz), 113.0 (d, $J = 9.7$ Hz), 110.4 (d, $J = 26.5$ Hz), 103.8 (d, $J = 23.4$ Hz), 42.9, 29.15

HRMS: $m/z$ calculated for C$_{10}$H$_{11}$FN$_2$: 179.0979 (M+H); Found: 179.0975 (M+H)

**Preparation of 5-chlorotryptamine (110)**

![Chemical Structure](image)

To a heterogeneous mixture of 5-chloroindole (500 mg, 3.397 mmol) and 1-dimethylamino-2-nitroethylene (435 mg, 3.74 mmol) was added trifluoroacetic acid (7 ml, 0.48 M), and the reaction mixture was stirred for two hours. After this time, the reaction mixture turned black and was then diluted with EtOAc (50 mL) and 10 % aqueous Na$_2$CO$_3$ (150 mL). The phases were separated and the aqueous phase was washed with EtOAc (4 x 50 mL). The combined organic phases were then washed with brine (150 mL), dried over MgSO$_4$ and the solvent was removed *in vacuo*. The crude mixture was then suspended in hot Et$_2$O, filtered through Celite® and the solvent was removed *in vacuo* to give the crude product (0.7 g) as an orange solid.

To a stirred flask containing THF (55 mL) at 0 °C was added sodium borohydride (400 mg, 10.5 mmol). BF$_3$•OEt$_2$ (1.9 g, 13.6 mmol) was then added slowly to the reaction mixture and stirred for 15 minutes at room temperature. The crude product from the first step was then added dropwise as a solution in THF (10 mL). The reaction mixture was heated at reflux for two hours, then cooled to room temperature and acidified to pH 1 by the addition of 1N HCl solution (~15 mL). The reaction mixture was refluxed for an additional two hours, then cooled to room temperature and washed with Et$_2$O (100 ml x 4). The combined organic phases were dried over MgSO$_4$ and filtered, then the solvent was removed *in vacuo*. Purification of the crude material by flash
chromatography (10% ammonia solution (7N in MeOH), 90% CH\textsubscript{2}Cl\textsubscript{2}) to afford 5-chlorotryptamine (110, 0.21 g, 32%) as a brown solid.

\(^1\)H NMR (600 MHz, CD\textsubscript{3}OD) \(\delta\): 7.52 (dd, \(J = 0.5, 2.0\) Hz, 1H), 7.30 (dd, \(J = 0.5, 8.6\) Hz, 1H), 7.12 (s, 1H), 7.05 (dd, \(J = 2.0, 8.6\) Hz, 1H), 2.92 (dt, \(J = 1.6, 7.1\) Hz, 2H), 2.85 (dt, \(J = 1.6, 7.1\) Hz, 2H)

\(^13\)C NMR (150 MHz, CD\textsubscript{3}OD) \(\delta\): 136.6, 129.8, 125.4, 125.3, 122.5, 118.7, 113.5, 113.2, 42.9, 29.0

HRMS: \(m/z\) calculated for C\textsubscript{10}H\textsubscript{11}ClN\textsubscript{2}: 195.0684 (M+H); Found: 195.0682 (M+H)

**Preparation of 5-bromotryptamine (111)**

5-bromotryptamine hydrochloride salt (1.0 g, 3.6 mmol) was dissolved in water (30 mL) and basified with 15% NaOH in water (7 mL). The aqueous phase was extracted with dichloromethane (20 ml x 3). The combined organic phases were dried over MgSO\textsubscript{4} and filtered, then the solvent was removed \textit{in vacuo} to afford 5-bromotryptamine (111) as a brown solid (0.77 g, 3.2 mmol, 89%) 

\(^1\)H NMR (600 MHz, CD\textsubscript{3}OD) \(\delta\): 7.68 (dd, \(J = 1.7\) Hz, 1H), 7.25 (dd, \(J = 8.6\) Hz, 1H), 7.17 (dd, \(J = 1.8, 8.6\) Hz, 1H), 7.10 (s, 1H), 2.91 (t, \(J = 6.6\) Hz, 2H), 2.85 (t, \(J = 6.6\) Hz, 2H)

\(^13\)C NMR (150 MHz, CD\textsubscript{3}OD) \(\delta\): 136.8, 130.6, 125.1, 125.0, 121.9, 113.9, 113.2, 112.8, 43.0, 29.2

HRMS: \(m/z\) calculated for C\textsubscript{10}H\textsubscript{11}BrN\textsubscript{2}: 239.0178, 241.0158 (M+H); Found: 239.0182, 241.0163 (M+H)
**Preparation of carbamate 142**

To a stirred solution of tryptamine (88, 1.0 g, 6.2 mmol) in CH$_2$Cl$_2$ (16 mL) was added triethylamine (0.87 mL, 12 mmol) then cooled to 0 °C. Ethyl chloroformate (0.62 mL, 6.5 mmol) was then added dropwise then the reaction mixture was warmed to room temperature then diluted with H$_2$O (25 mL) and the phases were separated. The organic phase was washed with 1N HCl (15 mL), 5% aqueous NaHCO$_3$ (15 mL), H$_2$O (15 mL) and brine (15 mL) then dried (MgSO$_4$) and filtered and the solvent removed *in vacuo*. Purification of the crude material by flash chromatography (EtOAc-hexanes 30:70) provided carbamate 142. (1.35 g, 5.8 mmol, 93%) as a yellow oil.

$^1$H NMR (400 MHz, CD$_3$OD) δ: 7.56 (d, $J = 7.9$ Hz, 1H), 7.32 (dt, $J = 0.8$, 8.1 Hz, 1H), 7.08 (dt, $J = 1.0$, 7.1 Hz, 1H), 7.04 (s, 1H), 6.99 (dt, $J = 1.0$, 7.1 Hz, 1H), 4.05 (q, $J = 7.1$ Hz, 2H), 3.38 (m, 2H), 2.91 (t, $J = 7.5$ Hz, 2H), 1.21 (t, $J = 7.1$ Hz, 3H)

$^{13}$C NMR (150 MHz, CD$_3$OD) δ: 159.2, 138.1, 128.8, 123.3, 122.2, 119.5, 119.3, 113.3, 112.2, 61.6, 42.8, 26.8, 15.0

HRMS: $m/z$ calculated for C$_{13}$H$_{16}$N$_2$O$_2$: 255.1104 (M+Na); Found: 255.1103 (M+Na)

**Preparation of 5-fluorocarbamate 103**
To a stirred solution of 5-fluorotryptamine (108, 180 mg, 1.0 mmol) in CH₂Cl₂ (10 mL) was added triethylamine (0.15 mL, 1.1 mmol) then cooled to 0 °C. Ethyl chloroformate (0.10 mL, 1.1 mmol) was then added dropwise then the reaction mixture was warmed to room temperature then diluted with H₂O (15 mL) and the phases were separated. The organic phase was washed with 1N HCl (15 mL), 5% aqueous NaHCO₃ (15 mL), H₂O (15 mL) and brine (15 mL) then dried (MgSO₄) and filtered and the solvent removed in vacuo. Purification of the crude material by flash chromatography (EtOAc-hexanes 30:70) provided 5-fluorocarbamate 103. (0.24 g, 0.97 mmol, 97%) as a yellow oil.

¹H NMR (500 MHz, CD₃OD) δ: 7.56 (d, J = 7.9 Hz, 1H), 7.32 (dt, J = 0.8, 8.1 Hz, 1H), 7.08 (dt, J = 1.0, 7.1 Hz, 1H), 7.04 (s, 1H), 6.99 (dt, J = 1.0, 7.1 Hz, 1H), 4.05 (q, J = 7.1 Hz, 2H), 3.38 (m, 2H), 2.91 (t, J = 7.5 Hz, 2H), 1.21 (t, J = 7.1 Hz, 3H)

¹³C NMR (125 MHz, CD₃OD) δ: 159.3, 158.8 (d, J = 231.9 Hz), 134.6, 129.1 (d, J = 9.4 Hz), 125.4, 113.6 (d, J = 5.0 Hz), 112.9 (d, J = 9.7 Hz), 110.3 (d, J = 26.5 Hz), 103.9 (d, J = 23.4 Hz), 61.6, 42.8, 26.8, 15.0

HRMS: m/z calculated for C₁₃H₁₅N₂FO₂: 273.1010 (M+Na); Found: 273.1009 (M+Na)

Preparation of 5-bromocarbamate 143
To a stirred solution of 5-bromotryptamine (111, 630 mg, 2.6 mmol) in CH₂Cl₂ (25 mL) was added triethylamine (0.37 mL, 5.2 mmol) then cooled to 0 °C. Ethyl chloroformate (0.47 mL, 5.2 mmol) was then added dropwise then the reaction mixture was warmed to room temperature then diluted with H₂O (35 mL) and the phases were separated. The organic phase was washed with 1N HCl (35 mL), 5% aqueous NaHCO₃ (35 mL), H₂O (35 mL) and brine (35 mL) then dried (MgSO₄) and filtered and the solvent removed in vacuo. Purification of the crude material by flash chromatography (EtOAc-hexanes 30:70) provided 5-bromocarbamate 143. (0.78 g, 2.5 mmol, 96%) as a brown oil.

¹H NMR (400 MHz, CD₃OD) δ: 7.67 (s, 1H), 7.21 (d, J = 8.6 Hz, 1H), 7.13 (d, J = 8.6 Hz, 1H), 7.05 (s, 1H), 4.02 (q, J = 6.7 Hz, 2H), 3.1 (t, J = 7.4 Hz, 2H), 2.84 (t, J = 7.4 Hz, 2H), 1.18 (t, J = 6.7 Hz, 3H)

¹³C NMR (100 MHz, CD₃OD) δ: 159.2, 136.7, 130.7, 125.0, 121.9.0, 113.8, 113.2, 112.8, 61.6, 42.7, 26.6, 15.0

HRMS: m/z calculated for C₁₃H₁₅BrN₂O₂: 333.0209, 335.0189 (M+Na); Found: 333.0206, 335.0185(M+Na)

2.7.3. Experimental Information for Nᵦ-methyltryptamine analogues

Preparation of Nᵦ-methyltryptamine (37)

To a cold (0 °C), stirred solution of carbamate 42 (2.52 g, 10.8 mmol) in THF (100 mL) was added LiAlH₄ (1.2 g, 31.6 mmol) and the reaction was heated to reflux for 90 minutes. Following this, the reaction was cooled to 0 °C, diluted with Et₂O (50 mL) and treated by the dropwise addition of H₂O (3 mL) followed by 15% aqueous NaOH (3
mL) and a further addition of H₂O (9 mL). MgSO₄ was then added and the reaction mixture was stirred for 15 minutes then filtered and the solvent was removed in vacuo to afford N₆-methyltryptamine (7) as a yellow solid (1.58 g, 9.1 mmol, 84%)

¹H NMR (400 MHz, CD₃OD) δ: 7.53 (td, J = 0.9, 8.0 Hz, 1H), 7.32 (td, J =0.5, 8.1 Hz, 1H), 7.08 (ddd, J = 1.1, 7.1, 8.1 Hz, 1H), 7.03 (s, 1H), 6.99 (ddd, J = 1.1, 7.1, 8.1 Hz, 1H), 2.91 (m, 2H), 2.81 (m, 2H), 2.33 (s, 3H)

¹³C NMR (150 MHz, CD₃OD) δ: 138.2, 128.6, 123.4, 122.4, 119.6, 119.2, 113.4, 112.3, 53.0, 35.9, 26.0

HRMS: m/z calculated for C₁₁H₁₄N₂: 175.1230 (M+H); Found: 175.1236 (M+H)

Preparation of D₃-N₆-methyltryptamine (112)

To a cold (0 ºC), stirred solution of carbamate 42 (0.65 g, 2.8 mmol) in THF (50 mL) was added LiAlD₄ (0.46 g, 2.8 mmol) and the reaction was heated to reflux for 16 hours. Following this, the reaction was cooled to 0 ºC, diluted with Et₂O (50 mL) and treated by the dropwise addition of H₂O (0.5 mL) followed by 15% aqueous NaOH (0.5 mL) and a further addition of H₂O (1.5 mL). MgSO₄ was then added and the reaction mixture was stirred for 15 minutes then filtered and the solvent was removed in vacuo. Purification of the crude material by flash chromatography (10% ammonia solution (7N in MeOH), 90% CH₂Cl₂) to afford D₃-N₆-methyltryptamine (112) as a yellow solid (0.22 g, 1.2 mmol, 44%).

¹H NMR (400 MHz, CD₃OD) δ: 7.55 (td, J = 0.9, 8.0 Hz, 1H), 7.33 (td, J =0.5, 8.1 Hz, 1H), 7.09 (ddd, J = 1.1, 7.1, 8.1 Hz, 1H), 7.08 (s, 1H), 7.00 (ddd, J = 1.1, 7.1, 8.1 Hz, 1H), 2.98 (m, 2H), 2.92 (m, 2H)
\(^{13}\)C NMR (150 MHz, CD\(_3\)OD) \(\delta\): 138.3, 128.6, 123.5, 122.4, 119.7, 119.2, 112.9, 112.3, 52.6, 34.8 (m), 25.6

HRMS: \(m/z\) calculated for C\(_{11}\)H\(_{11}\)D\(_3\)N\(_2\): 178.1418 (M+H); Found: 178.1420 (M+H)

**Preparation of 5-fluoro-\(N\_p\)-methyltryptamine (113)**

\[
\begin{array}{c}
\begin{tikzpicture}
  \node at (0,0) {\text{F}};
  \node at (1,0) {\text{N}};
  \node at (2,0) {\text{H}};
  \node at (3,0) {\text{H}};
  \node at (3.5,0) {\text{H}};
\end{tikzpicture}
\end{array}
\]

To a cold (0 °C), stirred solution of 5-fluorocarbamate 103 (0.10 g, 0.40 mmol) in THF (10 mL) was added LiAlH\(_4\) (46 mg, 1.2 mmol) and the reaction was heated to reflux for two hours. Following this, the reaction was cooled to 0 °C, diluted with Et\(_2\)O (25 mL) and treated by the dropwise addition of H\(_2\)O (50 \(\mu\)L) followed by 15% aqueous NaOH (50 \(\mu\)L) and a further addition of H\(_2\)O (150 \(\mu\)L). MgSO\(_4\) was then added and the reaction mixture was stirred for 15 minutes then filtered and the solvent was removed \textit{in vacuo} to afford 5-fluoro-\(N\_p\)-methyltryptamine (113) as a brown oil (81.9 mg, 9.1 mmol, 84%)

\(^1\)H NMR (500 MHz, CD\(_3\)OD) \(\delta\): 7.28 (dd, \(J=4.4, 8.7\) Hz, 1H), 7.21 (dd, \(J=2.4, 9.9\) Hz, 1H), 7.12 (s, 1H), 6.85 (dt, \(J=2.6, 9.2\) Hz, 1H), 2.90 (m, 2H), 2.85 (m, 2H), 2.4 (s, 3H)

\(^{13}\)C NMR (125 MHz, CD\(_3\)OD) \(\delta\): 158.8 (d, \(J=231.9\) Hz), 134.8, 128.9 (d, \(J=9.5\) Hz), 125.5, 113.4 (d, \(J=4.6\) Hz), 113.0 (d, \(J=9.6\) Hz), 110.4 (d, \(J=26.4\) Hz), 103.8 (d, \(J=23.5\) Hz), 52.7, 35.8, 25.7

HRMS: \(m/z\) calculated for C\(_{11}\)H\(_{14}\)FN\(_2\): 193.1136 (M+H); Found: 193.1136 (M+H)

**Preparation of 5-fluoro-D\(_3\)-\(N\_p\)-methyltryptamine (114)**
To a cold (0 °C), stirred solution of 5-fluorocarbamate 103 (0.10 g, 0.40 mmol) in THF (10 mL) was added LiAlD₄ (0.10 g, 2.4 mmol) and the reaction was heated to reflux for 16 hours. Following this, the reaction was cooled to 0 °C, diluted with Et₂O (25 mL) and treated by the dropwise addition of H₂O (100 μL) followed by 15% aqueous NaOH (100 μL) and a further addition of H₂O (300 μL). MgSO₄ was then added and the reaction mixture was stirred for 15 minutes then filtered and the solvent was removed in vacuo. Purification of the crude material by flash chromatography (10% ammonia solution (7N in MeOH), 90% CH₂Cl₂) to afford 5-fluoro-D₃-N₆-methyltryptamine (114) as a brown solid (0.22 g, 1.2 mmol, 44%).

¹H NMR (600 MHz, CD₃OD) δ: 7.28 (dd, J = 4.4, 8.7 Hz, 1H), 7.21 (dd, J = 2.4, 9.9 Hz, 1H), 7.11 (s, 1H), 6.85 (dt, J = 2.5, 9.1 Hz, 1H), 2.90 (m, 2H), 2.83 (m, 2H)

¹³C NMR (150 MHz, CD₃OD) δ: 158.8 (d, J = 231.9 Hz), 134.8, 128.9 (d, J = 9.5 Hz), 125.5, 113.5 (d, J = 4.7 Hz), 113.0 (d, J = 9.6 Hz), 110.4 (d, J = 26.5 Hz), 103.8 (d, J = 23.4 Hz), 52.7, 35 (hept, J = 20.5 Hz), 25.8

HRMS: m/z calculated for C₁₁H₁₄D₃FN₂: 196.1324 (M+H); Found: 196.1326 (M+H)

**Preparation of 5-bromo-N₆-methyltryptamine (115)**

To a cold (0 °C), stirred solution of 5-bromocarbamate 143 (0.81 g, 2.6 mmol) in THF (25 mL) was added LiAlH₄ (380 mg, 10 mmol) and the reaction was heated to reflux
for two hours. Following this, the reaction was cooled to 0 °C, diluted with Et₂O (30 mL) and treated by the dropwise addition of H₂O (0.4 mL) followed by 15% aqueous NaOH (0.4 mL) and a further addition of H₂O (1.2 mL). MgSO₄ was then added and the reaction mixture was stirred for 15 minutes then filtered and the solvent was removed in vacuo to afford 5-bromo-Ν₉-methyltryptamine (115) as a brown oil (650 mg, 2.5, 99%)

^1^H NMR (400 MHz, CD₃OD) δ: 7.68 (d, J = 1.7 Hz, 1H), 7.25 (d, J = 8.6 Hz, 1H), 7.16 (dd, J = 1.7, 8.6 Hz, 1H), 7.09 (s, 1H), 2.90 (m, 2H), 2.83 (m, 2H), 2.34 (s, 3H)

^1^3^C NMR (100 MHz, CD₃OD) δ: 136.8, 130.5, 125.1, 125.0, 121.8, 113.9 113.2, 112.8, 52.9, 35.9, 25.7

HRMS: m/z calculated for C₁₁H₁₄BrN₂: 253.0335, 255.0315 (M+H); Found: 253.0338, 255.0318 (M+H)

2.7.4. Synthetic Standards of D₆-chimonanthines

Preparation of D₆-chimonanthine 144 and 145

This procedure was adapted from Takayama’s chimonanthine synthesis.

To a cold (-30 °C), stirred solution of carbamate 142 (1.5 g, 6.35 mmol) in trifluoroethanol (8 mL) was added [bis(trifluoroacetoxy)iodo]benzene (1.8 g, 4.1 mmol) was added over 3 hours by the addition of 0.3 g portions every 30 minutes, and the reaction was stirred at -30 °C for two hours. Following this, the reaction was warmed to room temperature, and the solvent was removed in vacuo. The crude mixture was dissolved in freshly distilled THF (60 mL), and the reaction mixture was cooled to 0 °C. To a cold (0 °C), stirred solution was added LiAlD₄ (0.80 g, 19 mmol) and the reaction was heated to reflux for 16 hours. Following this the reaction was cooled to 0 °C, diluted with Et₂O (100 mL) and treated by the dropwise addition of H₂O (0.8 mL) followed by 15% aqueous NaOH (0.8 mL) and a further addition of H₂O (2.4 mL). MgSO₄ was then added and the reaction mixture was stirred for 15 minutes then filtered and the solvent was removed in vacuo. Purification of the crude material by flash chromatography (10%
ammonia solution (7N in MeOH), 90% CH₂Cl₂) to afford D₆-rac-144 (5 mg, 1%) and D₆-meso-155 (trace)

![rac-144 diagram]

¹H NMR (600 MHz, CD₃OD) δ: 7.23 (broad s, 2H), 6.98 (broad s, 2H), 6.63 (broad s, 2H), 6.54 (broad s, 2H), 2.70-2.09 (m, 10H)

²H NMR (600 MHz, CH₂Cl₂) δ: 2.39 (s)

HRMS: m/z calculated for C₂₂H₂₀D₆N₄: 353.2607 (M+H); Found: 353.2606 (M+H)

![meso-155 diagram]

¹H NMR (600 MHz, CD₃Cl) δ: 7.04 (m, 4H), 6.49 (d, J = 7.9 Hz, 4H), 6.63 (broad s, 2H), 2.60-2.35 (m, 6H), 2.06 (m, 2H)

HRMS: m/z calculated for C₂₂H₂₀D₆N₄: 353.2607 (M+H); Found: 353.2604 (M+H)
2.7.5. Feeding Precursors \textit{in planta} and Screening Precursors by LC-MS

Fresh stems with several leaves were collected from \textit{Chimonanthus praecox}, then the tips of stems were placed to the solutions of each synthetic precursor or two-precursor mixed (106-115, 5 mM, H$_2$O-DMSO 90:10, 10 mL, pH 4-5, see Table 2.1) and incubated in greenhouse overnight. Then, a leaf from each solution were collected, and the leaf was cut into small pieces individually. The small pieces were placed in MeOH (0.5 mL) in 1.5 mL Eppendorf® tubes, then, the samples were sonicated for one hour. The green methanol solutions was filtered and submitted to LC-MS.

LC-MS was run over 10 minutes with the eluent system (Table 2.7). MS was calibrated with internal standard, sodium formate. Then, the LC-MS chromatograms performed EIC analysis at \textit{m/z} of target unnatural products.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent: A (%)</th>
<th>Solvent: B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10.1</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Solvent A: H$_2$O with 0.1 % formic acid. Solvent B: acetonitrile with 0.1 % formic acid. Column temperature: 30 °C. Initial flow rate: 0.3 mL/min, ramp to 0.5 mL/min in 8min and Hold till 12 min. Total run time: 12 min

2.7.6. Isolation of Fluorinated Unnatural Products

The scale-up experiments commenced with the feeding of 50 mg of 5-fluorotryptamine (109) to 20 g of fresh leaves of \textit{Chimonanthus praecox} over 5 days. After 5 days, the leaves were collected and cut into small pieces. The small pieces of leaves were suspended in MeOH (250 mL) and stirred overnight to extract alkaloids, then filtered. The solvent was removed \textit{in vacuo}, and the plant extracts were dissolved in chloroform (75 mL). The mixture was acidified with 1M HCl (75 mL), and the phases were separated. The organic phase was washed with 15% NaOH (75 mL), then the
phases were separated again. The combined organic phases were then washed with brine (100 mL), dried over MgSO₄ and the solvent was removed *in vacuo* to afford brown plant extracts (40 mg).

The plant extracts were dissolved in methanol (1 mL) and placed in fridge overnight, then filtered to remove solid impurities. The filtered sample was submitted to HPLC to purify the alkaloids. After HPLC isolation, each fraction was submitted to LC-MS to monitor if the desired *m/z* of 383.2042 was present or not. The fractions that contained the desired *m/z* of 383.2042 were collected and concentrated under vacuum to afford 128 (~0.7 mg) and 129 (~0.05 mg).

**Table 2.8. HPLC eluent system to isolate difluorochimonanthines**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent: A (%)</th>
<th>Solvent: B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>8.5</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>30</td>
</tr>
</tbody>
</table>

Solvent A: H₂O with 0.1 % formic acid, Solvent B: methanol with 0.1 % formic acid. Injection volume 200 µL.

**Characterization of isolated alkaloid (-)-128**

![128](image)

¹H NMR (600 MHz, CD₃Cl) δ: 6.86 (broad s, 2H), 6.66 (broad s, 2H), 6.33 (broad s, 2H), 2.64-2.44 (m, 8H), 2.34 (s, 6H), 2.07 (broad s, 2H)

¹³C NMR (150 MHz, CD₃Cl) δ: 157.1 (d, *J* = 239.7 Hz), 146.7, 135.1 (d, *J* = 7.2 Hz), 129.9, 114.5 (d, *J* = 22 Hz), 110.0, 86.1, 53.6, 52.6, 37.3, 35.8
HRMS: $m/z$ calculated for C$_{22}$H$_{24}$F$_2$N$_4$: 383.2042 (M+H); Found: 383.2049 (M+H)

$[\alpha]_D^{20}$: -35 (c: 0.06, CHCl$_3$)

**Characterization of isolated alkaloid meso-129**

![Chemical Structure](image)

$^1$H NMR (600 MHz, (CD$_3$)$_2$SO) $\delta$: 6.70 (m, 2H), 6.34 (d, $J = 0.5$, 8.5 Hz, 2H), 6.33 (d, $J = 8.5$ Hz, 2H), 2.28 (m, 10H), 2.11 (s, 6H)

HRMS: $m/z$ calculated for C$_{22}$H$_{24}$F$_2$N$_4$: 383.2042 (M+H); Found: 383.2038 (M+H)

### 2.7.7. Plant Protein Extraction with P-PER™ Plant Protein Extraction Kit and *in vitro* Assay

A small leaf (~80 mg) was collected from *Chimonanthus praecox*, and protein extraction procedures were followed as described in P-PER™ Plant Protein Extraction Kit. 100 $\mu$L of protein samples were used in the *in vitro* assay in combination with precursor analogues in the following reaction buffer system (1mL, 1 mM precursor, 0.1 mM cofactor as indicated in Table 2.4).

**Reaction Buffer**

Buffer A: PBS

Buffer B: 0.2 M Tris-HCl
After overnight incubation at the specified temperatures, the samples were filtered in 3K Amicon® centrifugation tube, and the filtrate was submitted for LC-MS analysis.

2.7.8. **Plant Protein Extraction from Leaves Using Blender and *in vitro* Assay**

Fresh leaves (~20-50 g) were collected from *Chimonanthus praecox*. These leaves were frozen and then ground into a powder using a mortar and pestle cooled in liquid nitrogen. The powdered leaves were then placed in a blender (Oster®) and cold (4 °C) extraction buffer (200 mL) and protease inhibitor (Roche® cOmplete, Mini, one tablet) were added in a cold (4 °C) room. The blender was run for 15 seconds and this blending was repeated five times. After the blending, the homogenized samples were filtered through 4-layers of cheese cloth, then centrifuged at 14,000 rpm for 30 minutes at 4 °C to remove cellular debris and large cellular components. The supernatant was concentrated by using Amicon® centrifugal tubes as described above and the concentrated supernatant sample was then transferred into pre-cooled 2 mL Eppendorf tubes.

The collected protein samples were then added into the reaction buffers specified below and precursor analogues 112 or 113 (1 mM). After overnight incubation at the specified temperatures, the samples were filtered through 3K Amicon® centrifugal tubes, and finally subjected to LC-MS analysis.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Extraction Buffer</th>
<th>Reaction Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>2</td>
<td>0.2 M Tris-HCl</td>
<td>0.2 M Tris-HCl</td>
</tr>
<tr>
<td>3</td>
<td>PBS, 1 mM PEG, 5% (w/v) PVPP, 0.01% Triton X-100</td>
<td>PBS</td>
</tr>
<tr>
<td>4</td>
<td>25 mM Heps-Na, 0.5 mM EDTA, DTT, 8 mM MgCl₂</td>
<td>25 mM Heps-Na, 8 mM MgCl₂</td>
</tr>
<tr>
<td>5</td>
<td>50 mM Tris-HCl, 10 mM DTT, 0.5 M sucrose.</td>
<td>50 mM Tris-HCl, 0.5 M sucrose.</td>
</tr>
<tr>
<td>6</td>
<td>0.45 M Mannitol, 50 mM sodium phosphate, 2 mM EDTA</td>
<td>1 M Mannitol, 0.2 М sodium phosphate</td>
</tr>
</tbody>
</table>
2.7.9. **Plant Protein Extraction From Roots Using Blender and *in vitro* Assay** \(^{[83]}\)

Fresh roots (~100 g) were collected from *Chimonanthus praecox*. The roots were washed thoroughly with distilled water (1 L X 5) to remove any residual soil. These roots were ground to provide a powder using a mortar and pestle cooled with liquid nitrogen. The powdered roots were placed in a blender (Oster®) and cooled (4 °C) extraction buffer (500 mL) and protease inhibitor (Roche® cOmplete, Mini, 1 tablet) were added to the sample in a cold (4 °C) room. The blender was run for 15 seconds intervals and repeated 5 times. After blending, the homogenized samples were filtered through 4-layers of cheesecloth, then centrifuged at 14,000 rpm for 30 minutes at 4 °C to remove cellular debris and large cellular components. The supernatants were then collected and subjected to ammonium sulfate precipitation to separate and concentrate protein samples.

2.7.10. **Root protein separation by Ammonium Sulfate Precipitation**

To a beaker of plant protein supernatant sample, in a cold (4 °C) room, was slowly added ammonium sulfate to obtain a 20% solution, which was then stirred for 12 hours. After this time the sample was centrifuged at 14,000 rpm for one hour at 4 °C and the solids were collected. More ammonium sulfate was then added to the filtrate to increase the concentration to 40% and the resulting mixture was stirred for a further 12 hours and then centrifuged at 14,000 rpm for one hour at 4 °C and the solids were collected. This procedure was repeated with ammonium sulfate concentrations of 60%, 80%, and 95% to afford the ammonium sulfate plant protein fractions.

Each solid fraction was later dissolved in sodium acetate buffer (10 mL, 50 mM, pH 4.5), and the ammonium sulfate was removed by dialysis against this same buffer over 16 hours. The protein solution was then centrifuged at 15,000 for 30 minutes to remove residual solids and afford a solution of the protein extracts.
2.7.11. Preparation of Protoplasts \[^{[84]}\]  

Fresh leaves (~3-5 g) were collected from *Chimonanthis praecox*, and they were cut into 0.5-1 mm strips using a razor blade. The short strips were placed in digestive enzyme solution (20 mM MES (pH 5.7) containing 1.5% (w/v) cellulose R10, 0.4% (wt/vol) macrzyme R10, 0.4 M mannitol and 20 mM KCl) in a 50 mL Erlenmeyer flask. The solution was kept in the dark by wrapping the flask with aluminium foil and the solution was gently shaken (100 rpm) for 4 hours at room temperature. The solution was then transferred into a 15 mL Falcon® centrifuge tube and centrifuged at 2,000 g. The solids were collected and washed with washing buffer (0.5 M mannitol, 4 mM MES-KOH, pH 5.5, 20 mM KCl, and 3 x 20 mL). The washed protoplasts were directly used for assays and microscopy.

2.7.12. *In Vitro* Assay with Protoplasts

The collected protoplasts (~0.5 g) were suspended in PBS (1.5 mL) in a 2 mL Eppendorf® tube. Then 5-fluorotryptamine (108, 20 μL, 0.2 M) was added and the mixture was incubated at 30 °C overnight. MeOH (0.5 mL) was added after which the sample was sonicated for one hour. The sample was filtered using a 3K Amicon® centrifugal tube and then subjected to analysis by LC-MS.

2.7.13. Preparation of Cell Wall\[^{[88]}\]

Leaves of *Chimonanthis praecox* were collected (15 g), frozen in liquid nitrogen and ground into powder using a cooled mortar and pestle. The resulting green powder was transferred into a 500 mL-beaker containing potassium phosphate buffer (250 mL, 50 mM, and pH 7.0) with 1% (v/v) of Triton X-100 and stirred for 4 hours at 4 °C. The mixture was then filtered through one layer of cheesecloth, the insoluble materials were then washed with cold distilled water (1 L) and squeezed in the cheesecloth to remove any residual water. The insoluble materials were washed overnight with 0.5 M of NaCl (250 mL) and filtered through one layer of cheesecloth. The insoluble materials were washed with cold distilled water (1 L) and squeezed in cheesecloth to remove residual water. 4 g of the cell wall materials were collected in this manner.
2.7.14. Preparation of Cell Wall proteins[89]

25 g of leaves were collected and frozen in liquid nitrogen. They were then ground using a cooled mortar and pestle into a fine green powder. The green powder was homogenized in 0.1 M potassium phosphate buffer (pH 7.0, 300 mL) containing 5 mM DTT and then filtered through four-layers of cheese cloth. The insoluble materials were collected, and shaken sequentially with pre-chilled (-20 °C) acetone (3 x 300 mL, 30 min each), solution A (0.1 M potassium phosphate buffer (pH 6.5) with 0.1 % beta-mercaptoethanol, 4 °C 300 mL, 30 min), solution A containing 1% (v/v) Triton X-100 (4 °C, 300 mL, 4 hours), and finally with solution A to remove remaining detergent (4 °C, 300 mL, 16 hours). Between each procedure, the homogenate was filtered through 4 layers of cheesecloth and the insoluble materials were collected.

The collected insoluble materials (pale yellow) were placed into a cooled (4 °C) solution of 200 mL of 1 M NaCl solution (4 hours) and stirred with a magnetic stir bar to extract the proteins from the insoluble residues. The homogenate was decanted and filtered through 4-layers of cheesecloth and the yellow solution (protein sample) was concentrated using 15 mL 10K Amicon® centrifugation tubes (4000 g, 45 mins) and then concentrated again using 0.5 mL 10K Amicon® centrifugation tubes (14,000 g, 45 mins). The protein concentration of the resulting samples was determined by Bradford assay (BioRad), using BSA as a protein standard, to afford 200 µL of yellow protein sample (1.8 mg / mL).

The protein extracts (0, 15, 30, 45, and 60 µL) were assayed in a solution of 0.1 M potassium phosphate (pH 7) containing 5 mM of 5-fluoro-\(N\)\(_{6}\)-methyltryptamine and 2.5 µL of ammonium persulfate in 250 µL overnight at 30 °C. After the incubation the samples were filtered through 3K Amicon® centrifugation tubes and the filtrates were submitted for analysis by LC-MS.
References


Appendix A.

Total Synthesis of Ascospiroketal A Through a Ag\(^1\)-Promoted Cyclization Cascade

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Natural Product Synthesis

Total Synthesis of Ascospiroketal A Through a Ag⁺-Promoted Cyclization Cascade**

Stanley Chang, Soo Hur, and Robert Britton*

Abstract: The total synthesis of four candidate stereocore structures for the marine octaketide ascospiroketal A have been achieved. These concise and highly stereodecontrolled syntheses feature a unique Ag⁺-promoted cyclization cascade involving an oxetanyl ketochlorohydrin to access the entire tricyclic core of the natural product in one step. These syntheses also establish the full stereochemistry for the ascospiroketal natural products.

In 2007, König reported the isolation of ascospiroketal A (1) and B (2) (Figure 1) as single spiroacetals epimers from the marine-derived fungus Ascochyta sulcicosti. Following extensive analysis of 1D and 2D NMR spectra, it was proposed that 1 and 2 possess a rare octaketide tricyclic core, in which the terminal ring is cyclized through an ether or ester linkage and includes a quaternary stereogenic center at C2 installed through geminal SAM methylation. The relative stereochemistry within the tricyclic core of the ascospiroketal was established from analysis of NOESY experiments and confirmed that these compounds are anomic spiroacetals. Unfortunately, no information could be obtained regarding their absolute stereochemistry, the configurational relationship between the tricyclic core and side chain, or within the side chain itself. Thus, the relative stereochemistry of the three remote stereocenters remained undefined. Structurally, these compounds represent the most complex members of a small family of tricyclic 5,5-spiroacetals that includes the cephalosporolides, penicilrolides, and opalinolides, for which several potentially useful biological activities have been ascribed.

Herein we describe the first total synthesis and full stereochemical assignment of ascospiroketal A (1) through a highly efficient Ag⁺-promoted cyclization cascade that assembles the entire tricyclic core in one step from a bicyclic precursor.

We have recently reported that aldo adducts of chloroaldehydes 3 (Scheme 1) undergo Ag⁺-promoted cyclization to afford various spiroacetals 5 including 5,5-spiroacetals such as those embedded in the tricyclic core of the ascospiroketal. This unique spirocyclization strategy involves intramolecular alkylation of a hemiacetal by a Ag⁺-activated chloromethylene (e.g., 4). In contemplating a total synthesis of the ascospiroketal, we envisaged that ultimately the side chain 6 (Scheme 1) would be appended to the fully functionalized core 7 through a Sonogashira coupling. Importantly, this late-stage coupling would allow for the rapid production of configurational isomers at the undefined stereocenters C15, C2′, and C3′. In turn, a Ag⁺-promoted spirocyclization of the ketochlorohydrin 9 would provide the spiroacetal 8 and the terminal ring would be accessed through...
rerrangement of the hydroxy oxetane function (i.e., 8 → 7). Molecular models suggested that Lewis acid activation of the oxetane function in 8 could involve a secondary coordination to the central ring oxygen (Scheme 1). Importantly, this bidentate chelate structure would enforce the desired sense of diastereoselectivity on this critical rearrangement and secure the correct configuration at the all-carbon quaternary center C2. While without precedent, the potential for these two distinct cyclization reactions to be promoted in tandem by a Ag⁺ salt was a particularly appealing aspect of this route. Building on our experiences in aldol reactions of α-chloroaldehydes,[6] we expected the ketochlorohydrin 9 to be readily available from the union of suitably functionalized aldol coupling partners 10 and 11.

In an attempt to address stereochemical uncertainties regarding the ascospiroket A side chain,[13] we first targeted the four potential diastereomeric truncated side chains 16–19 (Scheme 2). Thus, readily available TBS-protected (2S,3R)-3-hydroxy-2-methylbutyric acid 14[11] was coupled with the homopropargyl alcohol 13 derived from TMS-acetylene addition to (−)-propylene oxide (12). Deprotection of the resulting silyloxy ester 15 gave the hydroxy ester 16. Repeating this sequence of reactions separately with (+)-propylene oxide and/or (2S,3S)-3-hydroxy-2-methylbutyric acid[12] (see the Supporting Information (SI) for full details) afforded the corresponding esters 17–19. With these materials in hand, comparison of their 1H and 13C NMR spectral data (Scheme 2 and SI) with that reported for the equivalent portion of ascospiroket A[13] suggested that the natural products possess a (2S,3R,3'R) relative configuration as depicted for esters 16 and 19. Unfortunately, we were not able to confidently assign the relative configuration at C15 using these model compounds. Considering this uncertainty, the complete stereochemical assignment of ascospiroket A would ultimately require the synthesis of four candidate stereoisomers using the side-chain precursors 16, 19, and ent-16, ent-19, and comparison of their spectral data to that reported for the natural product.

Synthesis of the tricyclic core of ascospiroket A was initiated with the addition of vinyl lithium reagent 21 to the known aldehyde 20 (Scheme 3).[13] Sharpless asymmetric epoxidation[14] of 22 afforded the corresponding epoxide (not shown) along with recovered alcohol (+)-22 in high enantiomeric purity (98% ee) at 60% conversion. Removal of the acetal protecting group and protection of the secondary alcohol function in (+)-22 then yielded the methyl ketone 23. The preparation of α-chloroaldehyde 10 involved one-carbon homologation of commercially available acetaldehyde 24 by displacement of the corresponding mesylate with cyanide and subsequent reduction. The organocatalytic asymmetric α-chlorination[15] was explored using the conditions reported by MacMillan,[16] Jørgensen,[17] and Christmann.[18] After some experimentation with this unusual substrate, we found that a combination of MacMillan's catalyst 26[19] and NCS[20] gave α-chloroaldehyde 10 in optimal enantiomeric purity (85% ee). Finally, coupling of the lithium enolate derived from methyl ketone 23 with the α-chloroaldehyde 10 provided the aldo adduct 9 in good yield and excellent diastereoselectivity (d.r. = 12:1).[20]

Having established a concise, 6-step synthesis of ketochlorohydrin 9 we next explored the key spirocyclization reaction. Using our optimized conditions[21] for the formation of simple spiroacetals, we were delighted to find that overnight reaction of 9 with AgOTf and AgOAc proceeded smoothly to afford the anomic spiroacetals 27 in good combined yield. Pleasingly, the only by-products produced in
any appreciable quantity were the tricycles 29 and 30. Further optimization of the formation of these tricycles involved a brief screen of \( \text{Ag}^+ \) salts, whereupon a combination of \( \text{AgBF}_4 \) and \( \text{Ag}_2\text{O} \) was identified as optimal, delivering 29 and 30 in a combined isolated yield of 82%. As highlighted in Scheme 4, the complete diastereoselectivity observed in the opening of the prochiral oxetane may be attributed to chelation of the central ring oxygen and that of the oxetane to the \( \text{Ag}^+ \) salt (see 27). This bidentate chelation\(^{19}\) is not possible in the pro-3 transition structure 28. It is notable that the \( \text{Ag}^+ \)-promoted cyclization cascade effects the formation of three heterocyclic rings and a spiroacetal center, and secures the relative stereochemistry at the all-carbon quaternary center required for ascosporiketal A. To improve the overall efficiency of this reaction, the undesired spiroacetal 29 was readily epimerized using Dudley's conditions (\( \text{ZnCl}_2, \text{MgO} \))\(^{34,35}\) to provide a mixture of the anomic spiroacetics 29 and 30 and a means to recycle the former material.

Completion of the total synthesis of the candidate stereocenters for ascosporiketal A is depicted in Scheme 5. A two-step oxidation\(^{21,22}\) of the primary alcohol function in tricycle 30 provided the carboxylic acid 32 without epimerization of the spiroacetal center. At this point, suitable crystals were obtained for X-ray crystallographic analysis (see ORTEP, Scheme 5), which confirmed our stereochemical assignment of the tricyclic core. Moreover, the spectral data (\( ^1\text{H} \) and \( ^13\text{C} \) NMR) derived from 32 were in close agreement with that reported for the same region of ascosporiketal A, supporting the assigned stereochemistry for the natural product. Following silicon to iodine exchange (32-33)\(^{23}\), Sonogashira coupling\(^{24}\) of the side chain 16 with vinyl iodide 33 provided the full carbon skeleton of ascosporiketal A. Lindlar reduction\(^{25}\) of the resulting enyne completed the total synthesis of candidate stereocstructure 34, which required only 12 steps from the readily available aldehyde 20. Repetition of the final two reactions using the stereochemically unique side chains 19, ent-16, and ent-19 gave the candidate stereocstructures 35, 36, and 37, respectively (Figure 2). Whereas the \( ^1\text{H} \) NMR spectra derived from these synthetic materials were similar, the resonances for the diastereotopic protons at C14 proved to be diagnostic for the C15-(S) and C15-(R) series. Notably, only the spectra derived from the C15-(S) diastereomers 34 and 37 contained H14a/H14b resonances characteristic of ascosporiketal A (see truncated spectra, Figure 2).\(^{21}\) Furthermore, whereas the \( ^1\text{H} \) NMR spectrum of 37 acquired at 500 MHz closely matched that of the natural product, several subtle differences in the chemical shift (\( \Delta \delta > 0.01 \text{ ppm} \)) and/or shape of resonances for H10, H13, H14, and H13 were observed between the spectra of 34 and ascosporiketal A.\(^{21}\) Thus, based on the distinct \( ^1\text{H} \) NMR spectra of the candidate stereocstructures 34-37, the relative configuration for ascosporiketal A was unambiguously assigned as that depicted for 37. The specific rotation for 37 (\( [\alpha]_{{20}^{2}D}^{20} = +5 \left( c \ 0.20 \text{ in MeOH} \right) \)) was also consistent with that reported for the natural product (\( [\alpha]_{{20}^{2}D}^{20} = +20 \left( c \ 0.45 \text{ in MeOH} \right) \)) confirming the absolute stereochemistry of ascosporiketal A as shown for 37. Interestingly, the structurally related octaketide cephalosporin C,\(^{26}\) also isolated from a marine-derived fungus, possesses the same absolute stereochemistry within its tricyclic spiroacetal core.

In summary, exploiting a \( \text{Ag}^+ \)-promoted cyclization cascade, concise (14-step) syntheses of four candidate stereoc\( \text{str} \)uctures of the naturally occurring polyketide ascosporiketal A were realized. Comparison of their spectral data with that reported for 34 allowed for the confident assignment of the relative and absolute stereochemistry for the natural product as (2R,3R,4R,6R,9S,15S,2R,3S). Considering the similarities in structure between the ascosporiktals A (1) and
Figure 2. Comparison of the $^1$H NMR spectra (D$_2$-acetone) of candidate stereostereocchemistry 34-37 to 1 and enlargement of the H14 resonances from the $^1$H NMR spectra of 31$^0$ and 34-37.

B (2), the relative and absolute stereochemistry of acascopiol B should also be revised accordingly.

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[20] Treating compound 29 or a structurally related model system with the following Lewis or Brønsted acids also resulted in the production of $S_1,1$ mixtures of spirooctal: tetradinoesterate, BF$_4$-OEt$_2$, camphorsulfonic acid, Sn(OTf)$_2$, InCl$_3$.