BIOACTIVITY ASSESSMENT OF *VERATRUM CALIFORNICUM* ALKALOIDS

by

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DEDICATION

This thesis is dedicated to my family Jim, Lorna, and Jonah Dirks. Thank you for your encouragement and prayers, as well as your constant love and support. I love you all.

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ABSTRACT

The native Idaho plant *Veratrum californicum* is known to contain steroidal alkaloids that function as inhibitors of hedgehog signaling, a pathway utilized for the growth and differentiation of cells as well as proper tissue development. *Veratrum californicum* was originally noticed when pregnant ewes consumed the plant and later gave birth to lambs with craniofacial mutations such as a cyclopean eyes. These malformations were caused by the plant's steroidal alkaloid cyclopamine blocking the hedgehog signaling pathway. This same pathway is used by more than 20 types of cancer for multiplication of cells. Additional alkaloids have been found in *Veratrum californicum*, which are being studied in order to identify additional hedgehog signaling inhibitors. There are at least seven compounds detected in extracts that are completely uncharacterized, twelve with proposed identities, and the six known alkaloids veratrosine, cycloposine, veratramine, cyclopamine, isorubijervine, and muldamine all within *Veratrum californicum.* In this thesis these molecules were extracted together from the root and rhizome of the plant, and then separated into five fractions using high performance liquid chromatography. Mass spectrometry analysis identified the presence of twenty five alkaloids (nine more than literature precedent), and software processing of the data predicted the molecular formulas for each known, suspected, and unknown alkaloid. The bioactivity of the raw extract and each fraction was assessed by comparison to 0.1 μM cyclopamine, and it was found that three fractions suppressed hedgehog signaling to a greater extent, indicating the presence of bioactive constituents worthy of

further investigation. Five fractions were collected at 2.5 min intervals, beginning at 10.25 min from time of raw extract injection. Fraction 1 was most potent at Hh signal inhibition, and was determined to contain veratrosine, cycloposine, and an isomer of each, followed by fractions 2 and 4, which possessed elevated bioactivity, but to a lesser extent than fraction 1. Fraction 2 was predicted to be comprised of cycloposine, its isomer, an isomer of veratrosine, tetrahydrojervine, veratramine, dihydrojervine, its isomer, etioline, and five unknown compounds. Fraction 4 was composed of cyclopamine, and suspected to contain isomers of veratramine and cyclopamine, isorubijervine, and muldamine, as well as two unknown alkaloids

.

TABLE OF CONTENTS

DEDICATION iv

ACKNOWLEDGMENTS v

ABSTRACT vi

LIST OF TABLES xi

LIST OF FIGURES xii

LIST OF ABBREVIATIONS xiv

CHAPTER ONE: VERATRUM CALIFORNICUM ALKALOIDS 1

Abstract: 1

1. Introduction 2

1.1. *V. album*: subspecies *V. lobelianum*, *V. grandiflorum* and *V. oxysepalum* 6

1.1.1. *V. lobelianum* 7

1.1.2. *V. grandiflorum* 7

1.1.3. *V. oxysepalum* 8

1.2. *V. maackii* 9

1.3. *V. nigrum* 10

1.4. *V. taliense* 10

1.5. *V. viride*: subspecies *V. viride* & *V. eschscholtzii* 11

1.5.1. *V. viride* 11

1.5.2. *V. eschscholtzii* 12

1.6. *V. californicum* 13

2. Alkaloids Identified in *Veratrum californicum* 15

2.1. Cyclopamine (**1**) 16

2.2. Veratramine (**2**) 22

2.3. Isorubijervine (**3**) 24

2.4. Muldamine (**4**) 25

2.5. Cycloposine (**5**) 26

2.6. Veratrosine (**6**) 27

2.7. Verazine (**7**) 28

2.8. Etioline (**8**) 30

2.9. Tetrahydrojervine (**9**) 31

2.10. Dihydrojervine (**10**) 32

2.11. 22-Keto-26-aminocholesterol (**11**) 32

3. Conclusions 35

CHAPTER TWO: EXTRACTION AND SEPARATION OF *VERATRUM CALIFORNICUM* ALKALOIDS 36

Introduction 36

Results 39

Discussion 55

Materials and Methods 57

Plant Material 57

Extraction 57

Separation 58

Identification 59

Chemicals and Solvents 60

CHAPTER THREE: BIOACTIVITY ANALYSIS OF *VERATRUM CALIFORNICUM* EXTRACTED ALKALOID COMBINATIONS 61

Introduction 61

Results 66

Discussion 70

Materials and Methods 72

Cell Culture 72

CHAPTER FOUR: CONCLUSION AND FUTURE DIRECTIONS 74

REFERENCES 77

APPENDIX A 94

APPENDIX B 96

LIST OF TABLES

Table 1.1. Flower and geographic region for *Veratrum* spp. addressed in this review. 3

Table 1.2. Qualitative assessment of *Veratrum californicum* alkaloids. 15

Table 1.3. Methods of extraction, separation, and identification of *Veratrum* alkaloids. 19

Table 1.4. Summary of bioactivity in *Veratrum californicum* alkaloids. 34

Table 2.1 Summary of findings from five fractions of Veratrum californicum extract 52

Table 3.1 Summary of MS data for Fractions 1 (blue), 2 (purple), and 4 (pink) 69

LIST OF FIGURES

Figure 1.1. Structures of 11 known and proposed alkaloids in *Veratrum californicum.* 5

Figure 2.1 Chromatograms of Veratrum californicum raw extract using HPLC-DAD. A. most recent chromatogram of literature extract, B. extract chromatogram from current work, and C. overlay of the two extracts, where the literature extract is in red and the current work is shown in black 40

Figure 2.2 Chromatograms of *Veratrum californicum* raw extract and five fractions using HPLC-CAD 42

Figure 2.3 Chromatogram and extracted ion chromatogram for each alkaloid present in fraction 1, which corresponds to R_t from 10.75 – 13.25 min of crude extract chromatogram. 44

Figure 2.4 Chromatogram and extracted ion chromatogram for each alkaloid present in fraction 2, which corresponds to R_t from 13.25 – 15.75 min of crude extract chromatogram. 45

Figure 2.5 Chromatogram and extracted ion chromatogram for each alkaloid present in fraction 3, which corresponds to Rt from 15.75 – 18.25 min of crude extract chromatogram. 47

Figure 2.6 Chromatogram and extracted ion chromatogram for each alkaloid present in fraction 4, which corresponds to R_t from 18.25 – 20.75 min of crude extract chromatogram. 48

Figure 2.7 Chromatogram and extracted ion chromatogram for each alkaloid present in fraction 5, which corresponds to R_t from 20.75 – 23.25 min of crude extract chromatogram. 50

Figure 2.8 Extracted ion chromatograms previously identified alkaloids present in fractions 2 and 4 of crude alkaloid extract. 53

Figure 2.9 Chromatogram and extracted ion chromatogram for 355 μM veratramine and 390 nM cyclopamine. 54

Figure 3.1 The hedgehog signaling pathway, where the left side of the figure represents when the pathway is off due to PTCH inhibiting SMO, and the right side represents when the pathway is activated by Hh protein binding to PTCH allowing SMO to function.....62

Figure 3.2 Structures of hedgehog signaling pathway inhibitors cyclopamine, modified forms of cyclopamine, and computationally derived, FDA approved chemotherapeutics.....65

Figure 3.3 Results from bioactivity assessment of cyclopamine, raw extract, and five fractions. a. Both high (1:200 dilution for extract and 1:20 dilution for fractions) and low (1:1000 dilution for extract and 1:100 dilution for fractions) concentrations of each treatment. b. Only high concentrations of each treatment, where * signifies $P < 0.05$ and ** signifies $P < 0.01$ as compared to 0.1 μM cyclopamine.....68

Figure 3.4 Relative concentrations of fractions 1, 2, and 4 as compared to 0.1 μM cyclopamine. 71

Figure A.1 Figure S1. Structures of Veratrum spp. steroidal alkaloids, excluding those found in V. californicum[1] 95

Figure B.1 Data on Cyclopamine from SwissADME [126].....97

Figure B.2 Data on Veratramine from SwissADME [126].....98

Figure B.3 Data on Isorubijervine from SwissADME [126].....99

Figure B.4 Data on Muldamine from SwissADME [126].....100

Figure B.5 Data on Verazine from SwissADME [126] 101

Figure B.6 Data on Etioline from SwissADME [126] 102

Figure B.7 Data on Dihydrojervine from SwissADME [126].....103

LIST OF ABBREVIATIONS

°C	Degrees Celsius
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar
µmol	Micromoles
aBCC	Advanced Basal Cell Carcinoma
AML	Acute Myeloid Leukemia
amu	Atomic Mass Units
BCC	Basal Cell Carcinoma
CAD	Charged Aerosol Detector
CC	Column Chromatography
cm	Centimeter
COSY	Correlation Spectroscopy
DAD	Diode Array Detector
DEPT	Distortions Enhancement by Polarization Transfer
Dhh	Desert Hedgehog
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ELSD	Evaporative Light Scattering Detector

ESI	Electrospray Ionization
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
g	Grams
GC	Gas Chromatography
HETCOR	Heteronuclear Correlation
Hh	Hedgehog
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HPV-18	Human Papillomavirus Virus-18
hrs	Hours
hRSV	Human Respiratory Syncytial Virus
HSCCC	High-Speed Counter-Current Chromatography
IC_{50}	Half Maximal Inhibitory Concentration
Ihh	Indian Hedgehog
IR	Infrared Spectroscopy
ITS	Internal Transcribed Spacers
KAAD	3-keto *N*-(aminoethyl-aminocaproyl-dihydrocinnamoyl)
kg	Kilograms
L	Liters
laBCC	Locally Advanced Basal Cell Carcinoma
LC	Liquid Chromatography

LDAC	Low-Dose Cytarabine
LDL	Low-Density Lipoprotein
mBCC	Metastatic Basal Cell Carcinoma
mg	Milligrams
MHz	MegaHertZ
min	Minutes
mL	Milliliter
mm	Millimeter
mp	Melting Point
MPLC	Medium Pressure Liquid Chromatography
MS	Mass Spectrometry
MSQ	Mass Single Quadrupole
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
nM	Nanomolar
NMR	Nuclear Magnetic Resonance Spectroscopy
NOESY	Nuclear Overhauser Effect Spectroscopy
ORR	Overall Response Rate
ppm	Parts Per Million
PPRL	Poisonous Plant Research Laboratory
PTCH	Patched1
QTOF	Quadropole Time of Flight
RRR	Relative Response Ratio
R_t	Retention Time

Shh	Sonic Hedgehog
SLE	Supported Liquid Extraction
Smo	Smoothened
TCI	Tokyo Chemical Industry
TFA	Trifluoroactic Acid
TLC	Thin Layer Chromatography
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
UV	Ultraviolet
v/v	Volume per Volume

CHAPTER ONE: VERATRUM CALIFORNICUM ALKALOIDS

The following chapter was published in the journal *Molecules* in September 2021.[1]

Abstract: *Veratrum* spp. grow throughout the world and are especially prevalent in high mountain meadows of North America. All parts of *Veratrum* plants have been used for the treatment of ailments including injuries, hypertension, and rheumatic pain since as far back as the 1600s. Of the 17–45 *Veratrum* spp., *Veratrum californicum* alkaloids have been proven to possess favorable medicinal properties associated with inhibition of hedgehog (Hh) pathway signaling. Aberrant Hh signaling leads to proliferation of over 20 cancers, including basal cell carcinoma, prostate and colon among others. Six of the most well-studied *V. californicum* alkaloids are cyclopamine (**1**), veratramine (**2**), isorubijervine (**3**), muldamine (**4**), cycloposine (**5**), and veratrosine (**6**). Recent inspection of the ethanolic extract from *V. californicum* root and rhizome via liquid chromatography–mass spectrometry has detected up to five additional alkaloids that are proposed to be verazine (**7**)**,** etioline (**8**), tetrahydrojervine (**9**)**,** dihydrojervine (**10**), 22-keto-26-aminocholesterol (**11**). For each alkaloid identified or proposed in *V. californicum*, this review surveys literature precedents for extraction methods, isolation,

identification, characterization and bioactivity to guide natural product drug discovery associated with this medicinal plant.

1. Introduction

The genus Veratrum consists of 17–45 spp., most of which naturally occur in Asia, and all of them located exclusively in the Northern hemisphere. These perennials can either be classified as part of the Liliaceae or the Melanthiaceae family.[2] Typically, the species of Veratrum have been classified by their gross morphology, but the wide variety within the species has led to an absence of defined taxonomy.[3] In 2003, Zomlefer et al. classified Veratrum spp. by examination of their nuclear ribosomal internal transcribed spacers (ITS).[4] This ITS method was correlated to traditional taxonomic classification by corresponding analysis of flower color and geographical location.[4] **Table 1.1** lists the geographic region, a corresponding flower image, and the alkaloids and other relevant active components for representative Veratrum spp. that are pertinent to the current review. Based on alkaloid composition, the Veratrum spp. that will be discussed in this review are V. lobelianum, V. grandiflorum, V. oxysepalum, V. maackii, V. nigrum, V. taliense, V. viride, V. eschscholtzii and V. californicum (**Table 1.1**). Chemical structures of V. californicum steroidal alkaloids may be found in **Figure 1.1** and are identified by a number in bold font. The structures of additional alkaloids described in this review may be found in Figure S1.

Table 1.1. Flower and geographic region for *Veratrum* spp. addressed in this review.

Species	Flower	Region	Alkaloid(s)	Other Bioactive Components	Reference
V. album var. *lobelianum*		Northern Asia, parts of Europe	Verabenzoamine, veratroilzigadenine, 15-O-(2-Methylbutyroyl)germine, veralosinine, veranigrine, 7, jervine, pseudojervine, rubijervine, veralosine, veralosidine	Et linoleate, β-Sitosterol, resveratrol, oxyresveratrol	[5–8]
V. album var. *grandiflorum*		Asia	2	Resveratrol	[3,4,9–18]
V. album var. *oxysepalum*		Parts of Europe and northeastern Asia	2, veratridine, cevadine		[19–25]
V. maackii		Asia	Verussurine, verabenzoamine, 7, isoverazine, verazinine, 23-methoxycyclopamine 3-O-β-D-glucopyranoside, isoecliptalbine	Stilbenes, flavonoids, phenols, glyceride	[26–28]

V. nigrum		Europe and Asia	7, epiverazine, 2	[29–32]
V. taliense		Europe and Asia	Alkaloids including isorubijervine and rubijervine	[16–18,33,34]
V. viride var. *viride*		North America	2	[2,35,36]
V. viride var. *eschscholtzii*		North America	Isorubijervosine, pseudojervine, 6	[2,37–40]
V. californicum var. *californicum*		North America	1, 2, 3, 4, 5, 6	[2,41–43]

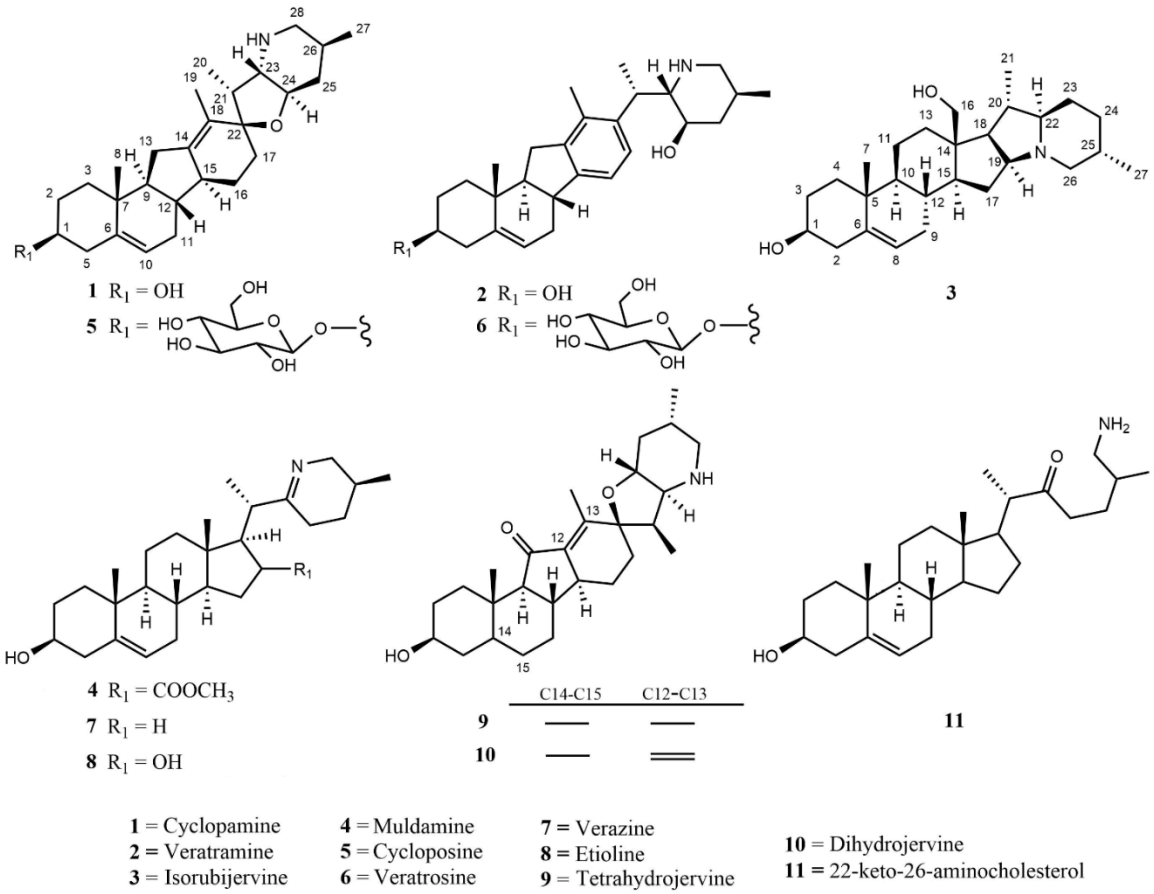

Figure 1.1. Structures of 11 known and proposed alkaloids in *Veratrum californicum.*

Interest in the *Veratrum* genus is attributable to the medicinal and therapeutic properties of steroidal alkaloids produced by the plants. Over 100 alkaloids have been identified, mostly from extraction of the root and rhizome, with several of the alkaloids demonstrating cancer suppression, induction of bradycardia, analgesia, and other effects.[2] Bioactive components from *Veratrum* have been identified within 16 species, but only *V. album*, *V. viride*, and *V. nigrum* have been thoroughly studied.[4] A brief overview of *Veratrum* spp. from which researchers have identified bioactive constituents for potential use as phytotherapeutics is provided. Much of our understanding of *Veratrum* spp.

component activity originates from animal or human poisoning associated with accidental ingestion of plant material. Observations of one-eyed sheep birth defects, low heart rate, vomiting, diarrhea, and other side effects attributable to *Veratrum* poisoning have led to investigations identifying active constituents consisting of alkaloid and non-alkaloid compounds. To provide a background of instances of *Veratrum* poisoning that have led to studies of active components and ultimately alkaloid (and non-alkaloid) identification, several case studies are included in this review.

1.1. *V. album*: subspecies *V. lobelianum*, *V. grandiflorum* and *V. oxysepalum*

V. album is a species complex of three subspecies, *V. lobelianum, V. grandiflorum*, and *V. oxysepalum*, sharing the common name white false hellebore.[2] This species is prevalent in northern Eurasia and to a lesser extent may be encountered in localized outcrops in North America, specifically in northern Alaska.[2,3] This plant complex had been used medicinally for centuries for its emetic properties well before the cause of this effect was understood. In 1820 *V. album* became one of the first *Veratrum* species analyzed for the presence of steroidal alkaloids. Eventually it was determined that certain *V. album* alkaloids induced a hypotensive effect by binding to voltage-gated sodium channels, causing the channels to remain open. When a neuronal stimulus occurred, multiple signals were released, because the cell's ability to repolarize was inhibited. This property led to increased use of the plant to treat hypertension. With rising demand, more plants had to be grown, but the supply was unreliable due to slow growth

and germination rates as well as chilling requirements. Phytotherapies incorporating *V. album* alkaloids were also discontinued due to difficulty in achieving the appropriate dosing for patients; the difference between toxic and therapeutic doses was about 30%.[2]

1.1.1. *V. lobelianum*

V. lobelianum grows in most countries in northern Asia, but is also found less frequently in parts of Europe.[5] In Russia it is called "chemeritsa" and has been used as part of Russian traditional medicine for hundreds of years. A tincture made from the plant has been incorporated as an ingredient in ointments that are rubbed onto a patient's skin to treat scabies and neuralgic and rheumatic pain. When the roots and rhizomes are boiled and concentrated, they are applied to the scalp as a treatment for lice. Together, baked roots, rhizomes, and cream have been an applicant for eczemas. Aqua veratri, commonly known as Hellebore water, is a dilution of the *V. lobelianum* tincture used to treat psoriasis by rubbing the ointment into the scalp of the patient. *V. lobelianum* has also been consumed to reduce fever, typhus, and pneumonia. The plant is possibly best known as an antiparasitic, including its use to combat hypodermatosis in cattle and to induce emesis in pigs.[44]

1.1.2. *V. grandiflorum*

V. grandiflorum, also known as white hellebore[4], has a geographical growth region predominantly in Asia.[2,3] Arthritis and gout have been treated using *V. grandiflorum* extract, which contains active components including stilbenoid phenol and

resveratrol, which promote anti-inflammatory and anti-osteoarthritis responses.[9] In the 5th century BCE, it was thought that gout was caused by overeating, and thus Hippocrates recommended consuming large amounts of *V. grandiflorum* in order to induce vomiting.[11] In the 1800s, a controversial treatment for gout was a tincture made from a combination of *V. grandiflorum*, tobacco, foxglove, wild cucumber, and a mix of other plants for flavor and aroma.[12] Resveratrol, the component of red wine that has gained considerable attention in recent years, was first isolated in 1940 from *V. grandiflorum*, a fact that is still evident in its name. Resveratrol has had a long history of clinical trials utilizing different diets and dosages of oral supplements to most effectively employ its anti-oxidative, anti-aging, anti-cancer, and cholesterol lowering properties.[15,45]

1.1.3. *V. oxysepalum*

The final member of the *V. album* complex is *V. oxysepalum,* which primarily grows in parts of Europe and northeastern Asia.[19,20] *V. oxysepalum* is the most common member of the *V. album* complex to be mistaken for an edible plant; when consumed, it induces poisoning due to the presence and abundance of toxic alkaloids. When *V. oxysepalum* is ingested, a person will suffer from vomiting, a drop in blood pressure and heart rate, and a tingling sensation in their mouth.[19] There have been at least seven situations where *V. oxysepalum* was confused for an edible wild plant, and upon ingestion led to accidental poisoning. The first report was associated with gentian wine, which requires the European *Gentiana lutea*, a plant that bears remarkable similarity to *V.*

oxysepalum.[19] In 2004, two men living in Italy consumed a beverage freshly prepared by a friend, thinking it was made from *Gentiana lutea*. Within the hour, they experienced nausea, vomiting, and headaches, and one man had diarrhea. After admittance to the hospital, both patients were treated with activated charcoal and an anti-emetic and discharged within 24 h.[46] A second, similar incident involving confusion of *V. oxysepalum* for *G. lutea* occurred in 2008.[21] A set of four separate cases occurred in the hills near Ljubljana, Slovenia in the months of April and May 2009 where four adults mistook *V. oxysepalum* for the edible *Allium ursinum* (wild garlic).[22] In the final reported incident 11 children at a youth camp poisoned themselves by accidentally consuming small amounts of *V. oxysepalum* while attempting to prepare herbal tea.[23]

1.2. *V. maackii*

V. maackii is native to Asia, commonly encountered in the Henan, Jilin, and Shandong provinces of China. *V. maackii* is used in the Chinese traditional medicine Lilu, which gains its alleged therapeutic benefits from alkaloids, stilbenes, flavonoids, phenols, and glyceride. *V. maackii* has been used for thousands of years to treat symptoms of aphasia caused by jaundice, scabies, apoplexy, and other such ailments. The plant components are teratogens, which is consistent with other *Veratrum* alkaloids, and there are at least nine stilbenes (1. *cis*-mulberroside A, 2. resveratrol-4,3′-*O*-β-D-diglucopyranoside, 3. mulberroside A, 4. gentifolin K, 5. resveratrol-3,5-*O*-β-d-diglucopyranoside, 6. oxyresveratrol-4′-*O*-β-d-glucopyranoside, 7. oxyresveratrol-3-*O*-β-

d-glucopyranoside, 8. oxyresveratrol, and 9. resveratrol) that have been identified as bioactive constituents. Stilbenes have anti-oxidant and anti-radical properties that can lead to prevention of cancer and cardiovascular disease as well as anti-inflammatory and neuroprotective effects. The anti-oxidant properties of *V. maackii* stilbenes have been demonstrated in mice through the reduction of ethanol-associated single-stranded DNA breaks.[26]

1.3. *V. nigrum*

V. nigrum, commonly referred to as black false hellebore, is another plant that grows in Europe and Asia. This particular species of *Veratrum* has been used in both Chinese and Korean medicine. *V. nigrum*, along with a few other species of *Veratrum,* make up the Chinese drug "Yeo-Ro", which has been used for treatment of headache, jaundice, chronic malaria, dysentery, scabies, and hypertension.[29] In Traditional Korean Medicine, *V. nigrum* is fed to patients to induce vomiting as a treatment for dyspnea caused by a stroke or epilepsy.[30] The alkaloids **2**, **7**, and epi-verazine have been extracted and used as melanogenesis inhibitors in mice, but the mechanism of action remains undetermined.[29] *V. nigrum* is used in China to alleviate aphasia in the same way as *V. maackii,* and also to treat symptoms of hypertension.[30,31] Veratramine has a hypotensive effect by blocking sodium ion channels and acting as an agonist for serotonin on presynaptic 5-HT neurons.[31]

1.4. *V. taliense*

Veratrum taliense grows in Europe and Asia, and in the Middle Ages was used in both places to treat high blood pressure, excess of phlegm, epilepsy, and stroke. The alkaloids contained within the plant inhibit voltage gated sodium ion channels. Two examples include **3** and rubijervine, which can block $Na_V1.5$ channels in cardiovascular muscles and induce cardiotoxicity at elevated levels.[33]

The roots and rhizomes of *V. taliense* are a component of the traditional folk medicine "Pimacao" from the Yunnan province of China. The other constituents of Pimacao are *V. grandiflorum*, *V. mengtzeanum*, and *V. stenophyllum*. Pimacao is the main ingredient in "Yunnan Baiyao", a well-known traditional Chinese medicine for the treatment of pain caused by fractures, hemorrhage, epilepsy, and rheumatism. The mechanism of action has not been thoroughly studied because the formula for this medicine has not been disclosed.[16–18,47] Yunnan Baiyao is commonly used for orthopedics, respiratory care, gastroenterology, and gynecology, but some possible adverse drug reactions include abdominal pain, chest tightness, nausea, vomiting, perturbation, and urticaria, among others.[47]

1.5. *V. viride*: subspecies *V. viride* & *V. eschscholtzii*

V. viride is a species complex of V. viride and V. eschscholtzii.

1.5.1. *V. viride*

Growing widely across North America, *V. viride,* also known as green false hellebore, was used by some Native American tribes to treat a variety of infirmities and

ailments. For a cold, one would chew on the root; for a snake bite, crushed rhizome was applied to the wound; and for venereal disease, a tea made from the plant was consumed. The known ability of *V. viride* to induce vomiting was also used to determine a worthy leader in Native American tribes, based on who could resist the emetic properties the longest. As early as 1835, *V. viride* was consumed to treat rheumatism and as an anti-inflammatory.[2] For those with primary hypertension it has also been shown to reduce high blood pressure by consumption of powdered roots and rhizomes. The alkaloids germidine and germitrine are responsible for the hypotensive properties. Germidine is a diester of the known compound germitrine in combination with acetic acid and *l*-α-metylbutyric acid, whereas germitrine is a triester of the same composition, but with the addition of *d*-methylethylglycolic acid. Germitrine, the more effective constituent, was reported to cause a drop in blood pressure in an anesthetized dog with as little as half a microgram per kilogram administered intravenously.[35]

1.5.2. *V. eschscholtzii*

The second member of the *V. viride* subspecies complex is *Veratrum eschscholtzii*.[2] *V. eschscholtzii* is localized in western North America, stretching from California to Alaska. The Native American tribes Bella Coola, Cowlitz, Kwakiutl, Okanagan, Quinault, Salish, Shuswap, and Thompson treated colds and blood, heart, orthopedic, and skin ailments with this plant as well as using it as a poison, analgesic, anti-rheumatic, emetic, and laxative.[37] For example, it was suggested that a poultice of

leaves be applied to the body for treatment of pain, or a decoction of the whole plant be ingested for treatment of rheumatism.[38] Isorubijervosine, the glycoside of **3**, was first isolated from this plant along with the already known alkaloids, pseudojervine and **6.**[39]

1.6. *V. californicum*

V. californicum var. *californicum* is also referred to as California false hellebore. It too grows in the western United States but is often found slightly further south. *V. californicum* was not traditionally used for medicinal purposes, but instead was noticed due to its teratogenic effects. During the 1950s in the high mountain meadows of Idaho, lambs were born with a singular eye in a cyclopean malformation. It was determined that this was caused by the pregnant ewes consuming *V. californicum* before giving birth to offspring. The alkaloids within the plant were determined to be responsible for the interruption of the hedgehog (Hh) signaling pathway retarding the development of the embryo, causing improper tissue formation.[2]

Each of the above listed *Veratrum* spp. has been studied because each contains at least one known or suspected alkaloid found within *V. californicum*. There remain many gaps in the research of *V. californicum* as most of the studies were conducted over fifty years ago; thus, the bioactivity source(s) within each additional *Veratrum* spp. were examined in order to provide more information regarding the possible uses of *V. californicum*. The alkaloids found in *V. californicum* have potential to be developed into cancer therapeutics due to their Hh signaling inhibitory properties.[41] In fact, **1** has been

used as a molecular template to inspire the development of FDA-approved and clinical trial candidate chemotherapeutics, including glasdegib, saridegib (IPI-926), vismodegib, and sonidegib.[48] Ethanolic extracts of below-ground parts of *V. californicum* were examined using high pressure liquid chromatography (HPLC) and mass spectrometry (MS) analysis, revealing the existence of at least 16 unique components (**Table 1.2**). Six of the alkaloids have confirmed identities (green highlight), while five others are proposed (yellow highlight) and another five are completely unknown (red highlight).[42] The remainder of this review will focus on the medicinal relevance, extraction method, characterization, and bioactivity of each of the eleven known and proposed alkaloids.

Table 1.2. Qualitative assessment of *Veratrum californicum* alkaloids.

Identity *	$[M+H]^+$ (*m*/*z*)	Predicted Molecular Formula
Cyclopamine (**1**)	412.326	$C_{27}H_{41}NO_2$
Veratramine (**2**)	410.312	$C_{27}H_{39}NO_2$
Isorubijervine (**3**)	414.343	$C_{27}H_{43}NO_2$
Muldamine (**4**)	458.370	$C_{29}H_{47}NO_3$
Cycloposine (**5**)	574.381	$C_{33}H_{51}NO_7$
Veratrosine (**6**)	572.365	$C_{33}H_{49}NO_7$
Verazine (**7**)	398.347	$C_{27}H_{43}NO$
Etioline (**8**)	414.342	$C_{27}H_{43}NO_2$
Tetrahydrojervine (**9**)	430.337	$C_{27}H_{43}NO_3$
Dihydrojervine (**10**)	428.320	$C_{27}H_{41}NO_3$
22-keto-26-aminocholesterol (**11**)	416.357	$C_{27}H_{45}NO_2$
N/A	576.396	$C_{33}H_{53}NO_7$
N/A	574.381	$C_{33}H_{51}NO_7$
N/A	576.397	$C_{33}H_{53}NO_7$
N/A	410.311	$C_{27}H_{39}NO_2$
N/A	412.326	$C_{27}H_{41}NO_2$

* Color coding of sections in Table 1.2 correspond to alkaloids that have been characterized (green), those that have been proposed, but not confirmed (yellow), and alkaloids that have been detected, but are yet to be identified (red).

2. Alkaloids Identified in *Veratrum californicum*

For each *V. californicum* alkaloid that is known or proposed, the following section details how these components were characterized based on three criteria: (1) method of extraction and isolation, (2) process of identification, and (3) reported bioactivity. The eleven best characterized alkaloids in *V. californicum* (see **Figure 1.1**; green and yellow entries from **Table 1.2**) were first identified from a variety of different plant species, so a description of the original discovery has been detailed. In instances where two or more procedures for extraction from the same species of plant provided the same alkaloid, two methods have been described: the original and the most up to date refined protocol. They are presented in the order of alkaloids with a confirmed presence followed by the

suspected alkaloids. An overview of the methods by which each *V. californicum* alkaloid was extracted and its properties and potential medicinal uses are summarized in **Tables 1.3 and 1.4**.

2.1. Cyclopamine (**1**)

A modern method of **1** extraction from *V. californicum* in 2013 included the use of a Soxhlet apparatus.[2] Biomass from root and rhizome was ground to a fine powder and wetted with 2 mL of 98.3:1.7 Ethanol:Ammonioum Hydroxide (*v*/*v*) solution and then packed into a cellulose extraction thimble. The contents of the thimble were extracted in a Soxhlet apparatus with two 50 mL portions of 98.3:1.7 EtOH:NH_4OH over consecutive 12 h segments. The extract was subjected to rotary evaporation and stored at −20 °C [1]. Beginning with crude alkaloid mixtures, **1** can be separated via HPLC using a C_{18} column.[49,50]

Compound **1** can be quantified through LC chromatograms by first generating a calibration curve from the commercially available standard, then comparing areas under the curve to experimental values. Once isolated, **1** and its derivatives can be characterized by MS.[49,50] A full list of 700 MHz proton and carbon-13 chemical shift assignments for **1** characterization is available in literature.[51]

Compound **1** and its derivatives have been shown to inhibit the Hh signaling pathway. Active during embryonic development, the Hh pathway plays an important role in limb patterning and specification of cells in the nervous system. Typically dormant in

cells after embryonic development, aberrant activation of Hh can result in tumorigenesis.[52],[53] Compound **1** was the first identified molecule to inhibit the Hh signaling pathway.[54] The activation of the Hh pathway requires signal transduction by the transmembrane protein smoothened (Smo).[55] Compound **1** inhibits the Hh signaling pathway by blocking smoothened after it binds to an internal helical fold of the transmembrane portion of the receptor protein.[56] In most adult tissues, the Hh signaling pathway is inactivated.[57] Aberrant Hh activation can lead to oncogenesis, but **1** and its derivatives are promising therapeutic agents that can be used for treatment. Two such derivatives are KAAD-cyclopamine and saridegib.[58,59] Both have higher potency and stability compared to **1**. In addition to targeting Smo and blocking Hh signaling, **1** can also interfere with myriad other harmfully mutated biological processes: it has been shown to inhibit growth of breast cancer and erythroleukemia cells through mechanisms outside of Smo binding [60,61], and has been shown to induce apoptosis in human prostate cancer cells.[62] Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is an extremely useful cancer treatment that kills only cancerous cells without disturbing normal cells. Cancer cells located in the stomach are resistant to TRAIL due to an absence of death receptor 5. Compound **1** causes increased expression of death receptor 5 in TRAIL-resistant gastric cancer cells, making them more susceptible to TRAIL.[63] Outside of cancer cell suppression, **1** blocks transcription of human respiratory syncytial

virus both in vitro and in vivo and also holds some promise in future treatment of psoriasis by promoting healing of psoriatic skin lesions.[64,65]

Table 1.3. Methods of extraction, separation, and identification of *Veratrum* alkaloids.

Alkaloid	Plant(s)	Sample Preparation *	Separation Technique	Identification	References
Cyclopamine (**1**)	*V. californicum*	Soxhlet extraction with ethanol/ammonium hydroxide	HPLC	LC-MS, ^{1}H and ^{13}C NMR	[2,51]
		Ethanol soak			[49,50]
Veratramine (**2**)	*V. viride*	Ethanol and chloroform extraction	Flash chromatography with silica gel	^{1}H and ^{13}C NMR, HPLC-MS, crystallization, melting point, and HPLC-CAD.	[66]
	V. viride	Benzene, ammonia, acetic acid, and NaOH extractions	High-speed counter-current chromatography	HPLC, MS and NMR	[67]
	V. oxysepalum	Diethyl ether and dichloromethane extractions	N/A	HPLC-MS	[68]
	V. nigrum L.	Ethanol and then chloroform extraction	Column chromatography	Crystallization	[69]
	V. grandiflorum	Crystallization and filtration using 2 N-calcium acetate and acetic acid (Unk plant part)	N/A	Crystallization, melting point, and NMR	[70]
	V. californicum	Dried and ground, ethanol then chloroform extraction	HPLC	HPLC, CAD and MS	[71]

Isorubijervine (**3**)	*V. eschscholtzii Gray*	Chloroform extraction (Unknown plant part)	Craig countercurrent distribution	N/A	[72]
	V. taliense	Methanol extraction	Silica gel column chromatography and MPLC	NMR and ESI-MS.	[33]
	V. viride Aiton	Ethanol and chloroform extraction (Unk plant part)	Flash chromatography with silica gel	IR, LC-MS, ^{1}H and ^{13}C NMR	[66]
Muldamine (**4**)	*V. californicum*	Benzene and 5% NH_4OH soak (Unk plant part)	Column chromatography with silica gel/benzene/methanol slurry	HPLC-ELSD, MS *m/z* = 458.37	[73]
Cycloposine (**5**)	*V. californicum*	Dried and ground, ethanol then chloroform extraction	HPLC-ELSD	HPLC-MS spectra confirmation	[71,73,74]
Veratrosine (**6**)	*V. patulum*	Ethanol soak, chloroform extraction, alkaloid residue	Silica gel, recrystallization in acetone	HPLC-MS *m/z* and elution times	[71,73,74]
	V. californicum	Dried and ground, ethanol then chloroform extraction	HPLC-ELSD		
Verazine (**7**)	*Zygadenus sibiricus*	N/A (From aerial plant)	N/A	N/A	[75]

	Solanaceae Solanum hypomalacophyllum	Dried, ground and refluxed with $CHCl_3$	Vacuum liquid chromatography and column chromatography	N/A	[76]
	Asteraceae Eclipta alba	EtOAc and MeOH extraction from leaves	Silica gel columns and preparative TLC	MeOH crystallization and DEPT, HETCOR, HMQC, and HMBC NMR	[77]
	V. nigrum	Ethanol extraction followed by two $CHCl_3$ extractions (Unknown plant part)	Alkali-treated silica gel and TLC	Specific TLC fractions were recrystallized	[78]
	V. nigrum	Three methanol extractions	Silica gel column chromatography twice and HPLC	Recrystallization, ESI-MS, IR, ^{1}H and ^{13}C NMR	[29]
Etioline (**8**)	*Solanum spirale*	Dried and ground, Soxhlet extraction with ethanol, chloroform/methanol extraction	Silica gel	^{13}C NMR and atom probe chromatography	[79]
	Lilium candidum L.	Ethanol extraction from bulbs and then $CHCl_3$ extraction	TLC with chloroform and methanol	TLC with chloroform and methanol	[80]
Tetrahydrojervine (**9**)	N/A	Synthesized only			
Dihydrojervine (**10**)	N/A	Synthesized only			
22-Keto-26-amino cholesterol (**11**)	N/A	Cholesterol metabolite, proposed			[81,82]

2.2. Veratramine (**2**)

Compound **2** has been extracted from many different *Veratrum* spp., including *V. viride* [66,67], *V. oxysepalum* [68], *V. nigrum* L. [69] and *V. grandiflorum* [70]. One method of extraction from *V. viride* involved percolation with 95% ethanol, followed by evaporation, resuspension in 5% tartaric acid, and filtration. Additional percolation of the filtrate was performed with chloroform, where the extract was subjected to pH adjustment and solvent evaporation. Flash chromatography with silica gel was used to separate **2** from other alkaloids.[66] Another method of extraction from *V. viride* involved grinding the roots and rhizomes to a powder, and then performing extractions using benzene and dilute ammonia. Additional extractions were performed with acetic acid, NaOH, and again with benzene until a crude alkaloid mixture was produced. In order to purify **2** from the mixture, ammonium sulfate was added and the resulting mother liquor was diluted with ammonia until the alkaloid could be removed with 50% ethanol.[67] An alkaline solution of powdered *V. oxysepalum* biomass was refluxed with ethanol and filtered. Additional extractions of the filtrate with diethyl ether and dichloromethane were performed, resulting in a crude extract of alkaloids. The isolation of **2** from *V. oxysepalum* was carried out via a two-step use of high-speed counter-current chromatography (HSCCC).[68] In order to remove the alkaloids from *V. nigrum* L., extractions were first performed with 95% ethanol. After concentration, the pH was lowered to 3 using hydrochloric acid, and then the solution was filtered. The pH of the filtrate was raised to 10 using ammonium hydroxide, and additional extractions of crude alkaloids were performed with chloroform. Separation of alkaloids was achieved via column chromatography.[69] The resinous material of *V. grandiflorum* passed through a

series of crystallizations and filtrations over a period of several days. The mother liquor from the crystallization of jervine hydrochloride was dissolved in 0.5 N-acetic acid, and 2 N-sodium sulfate was added, eventually leading to formation of veratramine sulfate and then **2.**[70]

After extraction from the roots and rhizomes of *V. viride*, **2** was characterized in different solvents using ^{1}H and ^{13}C NMR.[83] After **2** was separated from a crude extract of *V. oxysepalum*, the data from HPLC, MS, and NMR were analyzed to confirm its identity.[68] A C_{18} column was used for HPLC-MS isolation of **2** from V. *nigrum* L. This was followed by ESI-MS analysis in a *m*/*z* range of 200–700 amu.[84] The alkaloid quantity from *V. californicum* was determined using HPLC with a charged aerosol detector (CAD) and MS via calibration curves that were determined in triplicate in accordance with a purchased standard (**2**). Identification of the alkaloid was performed with HPLC-MS.[71] Melting point has been used for characterization, as colorless needles of **2** melt at 209.5 to 210.5 °C. The specific rotation values were found to correspond closely to calculated values. The mother liquor of a crude jervine hydrochloride from *V. viride* had excess ammonia added until separation of **2** was achieved. The product was recrystallized and jervine contaminants were removed to achieve the same melting point as above. In order to confirm that **2** had indeed been produced, the product was acetylated and compared to triacetylveratramine, and was found to possess the same properties and melting point. Specific rotation values were also calculated and verified with experimental results.[67]

Compound **2** has been tested for inhibition of prostate cancer by interfering with the Hh signaling pathway, and in conjunction with other alkaloids, has been used for treating high blood pressure, apoplexy, and other ailments.[83,84] Bioactivity of **2** was

measured in various ways. The ability of **2** to inhibit the growth of cancerous cells on a malignant PC-3 cell line was tested, and a wound-healing assay was also performed. Out of nine alkaloids tested in the wound-healing assay, **2** and its derivatives exhibited the highest degree of anti-migratory activity, and at a concentration of 50 μM, **2** displayed the highest degree of prevention of proliferation of cancerous cells.[83] In another study, **2** was introduced to two different groups of rats by gavage at doses of 0.25 or 2.50 μmol/kg for seven days to test its neurotoxicity both in vivo and in vitro using rat liver microsomes. DNA was damaged in the cerebellum and the cerebral cortex at both high and low doses of **2** as detected using the alkaline comet assay.[84]

2.3. Isorubijervine (**3**)

Compound **3** has been found in many different species of *Veratrum*. Isolation of **3** from *V. eschscholtzii* Gray was achieved by crude chloroform extraction. A Craig countercurrent distribution was performed on this extract to separate the alkaloids and identify **3.**[72] In a separate study, the roots and rhizomes of *V. taliense* were dried and ground before being extracted with methanol. After solvent acidification, the extract was filtered, and then base was added before silica gel column chromatography (CC) was utilized for component separation. The second eluted fraction underwent medium pressure liquid chromatography (MPLC) to yield **3.**[33] This alkaloid was also obtained from *V. viride* Aiton through a series of ethanol and chloroform extractions. The identification of specific alkaloids was made possible after flash chromatography was performed using silica gel.[66]

The amount of **3** contained in the extract from *V. californicum* was quantified by creating a calibration curve for HPLC using commercially available **3**. In order to

determine measurement of **3**, the HPLC was attached to a CAD and an MSQ plus MS.[71] Compound **3** was identified from the extract of *V. taliense* using NMR and ESI-MS.[33] The melting point of the colorless needles of isorubijervine was found to be 241–242 °C. IR, LC-MS, and ^{1}H and ^{13}C NMR were used to characterize this compound.[66]

The traditional medicinal benefits of this alkaloid include the treatment of pain and hypertension, but the mechanism remains unknown.[31,33] Compound **3** has been used to treat high blood pressure and pain, but has also been found to cause toxicity to the heart. The LD_{50} of **3** was found to be 1.14 mg/kg in mice. Administration of **3** to rats and macaques resulted in bradycardia and hypotension that lasted for several minutes; the response observed in macaques was dose-dependent. $Na_v1.5$ sodium channels expressed in HEK293t cells, which are critical to cardiovascular function, were treated with **3**. The IC_{50} value was found to be 6.962 ± 0.422 μM, and a 5 μM dose led to a 41% decrease in current. This decrease in flow in the sodium channel may explain the symptoms of bradycardia in the animals.[33]

2.4. Muldamine (**4**)

Compound **4** was extracted by soaking *V. californicum* in a benzene/5% NH_4OH (3:1 *v*/*v*) solution for 24–48 h. This solution was then dried in vacuo to produce a mixture of alkaloids that were recrystallized first in an acetone/water solution and then in a methanol/water solution. The recrystallized alkaloid mixture (300 mg) was dissolved in benzene/methanol (10:1, 3 mL) and loaded onto a chromatography column packed with a slurry of silica gel and benzene/methanol (60:1). Elution was performed using a benzene/methanol solution (60:1, 20:1). Isolation of **4** was performed using HPLC-ELSD in combination with manual fraction collection of individual peaks.[73]

Compound **4** has been characterized by a *m*/*z* of 458.37.[73] The identity of **4** was verified using a commercially available standard.

Initially given the name alkaloid Q, **4** is produced by *V. californicum* and is known to be a Hh signaling pathway antagonist as well as a non-depolarizing action potential blocker in the giant axon of squid and crayfish.[2,74,81] Compound **4** is not teratogenic in sheep, but did demonstrate marginal activity as a teratogen in hamsters.[82] The bioactivity of **4** was tested in combination with **2** and **3** by measuring Gli protein activity in Shh-Light II cells.[74] The Gli family members are transcription factors that can be monitored in order to determine whether the Hh pathway is activated or not.[85,86] The results demonstrated that the combination of alkaloids did have an inhibitory effect on Hh signaling.[74]

2.5. Cycloposine (**5**)

In a 2019 study by Turner et al., **5** was obtained by first cutting the rhizome/root of *V. californicum* into 1–2 cm cubes and lyophilizing it to dryness over 24–48 h. The dried biomass was then submerged in liquid nitrogen and ground to a fine powder using a mortar and pestle. Powdered biomass (2.0 g) was added to 95% ethanol (100 mL) and sonicated for 30 min, then mixed on a stir plate for 24 hrs. Plant material was removed via vacuum filtration and discarded. Ethanol was separated from the resulting solution by rotary evaporation, yielding brown oil. The crude alkaloid product was dissolved in 95% ethanol (10 mL), warmed to 40 °C, and sonicated to ensure all alkaloid product was dissolved. An aqueous ammonia solution (35% *v*/*v*) was added to obtain an alkaline solution (pH ≥ 10). This solution was eluted through a supported liquid extraction column with chloroform (3 × 10 mL) using a vacuum manifold (2 mbar). The fractions were

combined, filtered and dried. The dried alkaloids were then dissolved in 100% ethanol.[71] HPLC-ELSD was used to separate individual alkaloids from the mixture.[73]

Compound **5** was identified by HPLC-MS by comparison to a commercially available standard.[71,74]

Compound **5** is a teratogenic alkaloid produced exclusively in *V. californicum.*[2] This alkaloid is relevant to medicine through its use in the development of modern cancer treatments.[2] Shh-Light II cell models demonstrated that **5** did not contribute to Hh signaling inhibition in this method of bioactivity analysis; however, deglycosylation from hydrolysis during digestion may contribute to teratogenicity.[74]

2.6. Veratrosine (**6**)

In 1998, researchers conducted an extraction of *V. patulum* by cutting the roots into small pieces and soaking them in ethanol (4 × 7 L). The four ethanol solutions were concentrated in vacuo to form a residue that was dissolved in 5% aqueous tartaric acid solution (2.5 L); insoluble materials were removed by filtration. The solution was defatted with ether (4 × 3 L) and made alkaline with 20% Na_2CO_3 to pH 10. Extraction was performed with $CHCl_3$ (4 × 500 mL), where all chloroform extractions were combined and dried to form an alkaloid residue.[87] The alkaloid components in the *V. patulum* extract were separated by column chromatography using alkali-treated silica gel (400 g) and a mobile phase of MeOH-$CHCl_3$ (2:98, 6:94, 10:90, and 15:85). Thin layer chromatography (TLC) was used to monitor eluates with a total of 26 fractions collected. Fraction 23 was recrystallized with acetone to yield **6** (mp 242–244 °C).[87]

In 2013, **6** was identified from a *V. californicum* ethanol extract using HPLC-ELSD.[73] Most recently, the identification of **6** has been accomplished using HPLC-MS and verified by comparing the elution time of a commercially available standard.[71,74]

When ingested, **6** is an antagonist of epinephrine and norepinephrine.[88] In Shh-Light II cell models it was shown that **6** did not contribute to Hh signaling inhibition; however, hydrolysis of the glycosidic linkage during digestion may contribute to teratogenic effects.[74]

2.7. Verazine (**7**)

Compound **7** has been extracted from different plant families including *Lilaceae* in both species *Veratrum* (*nigrum)* and *Zygadenus* (*sibiricus*), *Solanaceae Solanum (hypomalacophyllum*), and *Eclipta* (*alba*).[29,76–78] The roots of *Solanum hypomalacophyllum* were dried and ground to a powder before 10% NH_4OH was added. The basic mixture was refluxed with $CHCl_3$ and dried. Fractions were collected after using vacuum liquid chromatography and various forms of column chromatography with $CHCl_3$-MeOH and H_2O-MeOH gradients, resulting in both epimers of **7** (20S and 20R).[76] Dried leaves from *Asteraceae Eclipta alba* were extracted using both EtOAc and MeOH. A silica gel column was used with Me_2CO in $CHCl_3$ to elute fractions, which were purified using a second silica gel column and preparative TLC. Compound **7** was also crystallized from MeOH.[77] Extraction from the roots and rhizomes of *V. nigrum* L. var. *ussuriense* was done using ethanol after the plant matter had been dried and portioned into small pieces. The extract was concentrated in vacuo before the addition of a 5% aqueous tartaric acid solution. After the sample was filtered, ether was added, and then another extraction was performed using $CHCl_3$. A 20% aqueous Na_2CO_3 solution was

added to the aqueous portion until a pH of 6 was achieved. Another $CHCl_3$ extraction was performed, and the product was dried before separation by chromatography using alkali-treated silica gel with different concentrations of mobile phase MeOH-$CHCl_3$. TLC separated the fractions, and fractions 5–7 were combined and recrystallized from acetone yielding **7.**[78] Another extraction from the crushed roots of *V. nigrum* was performed using methanol, repeated three times. The extract was diluted with water before being separated into different layers using n-hexane, chloroform, and butanol. The butanol layer underwent silica gel column chromatography twice, first using acetonitrile-methanol, and then using n-hexane-acetone-methanol. Finally, three different compounds were isolated using HPLC.[29]

Compound **7** is usually in the form of colorless needles, and the melting point is between 175 and 177 °C. High resolution ESI-MS was used in order to identify the molecular weight as 397.65 amu and the molecular formula as $C_{27}H_{43}NO$.[29] Compound **7** is typically characterized via NMR and sometimes IR. The spectra are well established, so the presence of **7** can be confirmed by spectral comparison.[89] Important IR, ^{13}C and ^{1}H NMR chemical shifts are those of the alcohol, imine, specific methyl groups, and singular hydrogen and carbon atoms, which have all been described.[29] Assignments of chemical shifts have also been obtained using a variety of NMR experiments including DEPT, HETCOR, HMQC, and HMBC.[77] Deuterochloroform was used as the solvent for the 1D ^{1}H and ^{13}C and 2D double quantum filter COSY and NOESY NMR spectra of **7.**[90]

The medicinal properties of **7** have been examined for use as an antifungal agent or a potential melanogenesis inhibitor, but the mechanism of action is still being explored.[29,77,82,91] Most **7** bioactivity appears to be antifungal. In one study, steroidal

alkaloids were used as inhibitors of *Candida albicans* and *Trichophyton* spp.; **7** was a successful inhibitor with a minimum inhibitory concentration of 6.2 µg/mL for *C. albicans* and 3.1 µg/mL for *T. rubrum.*[91] Yeast assays using the Sc7 yeast strain were inhibited by **7**, but when examining cytotoxicity, the results were not as favorable; IC_{50} values were greater than 10 µg/mL. Due to this cytotoxicity, antifungal studies were aborted.[77] Compound **7** was also studied for potential use as a melanogenesis inhibitor. The IC_{50} was less than 1 µg/mL for inhibiting melanin biosynthesis in B16 FI mouse melanoma cells.[29]

2.8. Etioline (**8**)

Compound **8** was extracted from the root of *Solanum spirale*. The roots were heated, dried, and ground to a powder prior to undergoing a Soxhlet extraction with ethanol. The ethanol was evaporated under vacuum and partitioned evenly between 10% C_6H_6-Et_2O and HOAc. Ammonia was added, and a second extraction was performed with $CHCl_3$-EtOH. The solvents were again evaporated, and silica gel was used to obtain an elution of **8** with $CHCl_3$-MeOH.[79] Compound **8** has also been extracted from *Lilium candidum* L. bulbs using ethanol. This extract was combined with HCl for three days before the pH was raised using ammonia, and the resulting aqueous portions were extracted with $CHCl_3$.[80]

To characterize the *Solanum spirale* extracted alkaloids, analysis by NMR spectroscopy was pursued. The ^{13}C NMR spectrum of the alkaloid suspected to be **8** was compared to solafloridine, 20-isosolafloridine and 20,25-bisisoetioline, and supported by atom probe chromatography measurements.[79] The *Lilium candidum* L. extract was

examined via TLC with $CHCl_3$:MeOH, resulting in the identification of jatropham and 22,26-epiminocholestane-type steroidal alkaloid, which is consistent with **8.**[80]

Compound **8** has been tested for the treatment of Hepatitis B, and has proven to be effective in specific contexts.[92] PLC/PRF/5 cells were prepared from human hepatoma, which were constantly excreting hepatitis B surface antigen, while KB cells were of the HeLa cell line and believed to possess human papillomavirus-18 (HPV-18).[92,93] Compound **8** was applied to these human PLC/PRF/5 and KB cells in vitro, and it was found that it only significantly inhibited the human PLC/PRF/5 cells, showing inhibition of Hepatitis B virus, but not of HPV-18.[92]

2.9. Tetrahydrojervine (**9**)

Compound **9** results from the reduction of the C5-C6 and C12-C13 double bonds in jervine and has been hypothesized to occur in *V. californicum* due to the presence of jervine.[41,94] In past experiments, **9** was artificially synthesized through the reduction of jervine using PtO_2 in acetic acid.[95]

A physical property that assists identification of **9** is its melting point of 221 °C (decomposition).[95] Characterization of **9** has involved specific rotation measurements and use of the Zerewitinoff determination to identify active hydrogen atoms for the purpose of aiding structure determination.[96,97] Possible methods of characterizing this alkaloid such as NMR, HPLC, and GC-MS have not been reported in the literature.

Compound **9** has not been used as a medicine, but has been used as a tool to examine the effects of *Veratrum* alkaloids on embryonic development.[41] Studies involving **9** investigated the relative teratogenic potency of jervines and how saturation of the C5-C6 bond led to less severe Hh pathway inhibition.[95] Compound **9** was tested on

explants of the intermediate neural plate region of Stage 9–10 chick embryos. Explants were treated with **9** in 48 nM and 240 nM concentrations and tested for induction markers of floor plate (HNF-3β) and motor neuron (Isl1/2) differentiation with the goal of inhibiting their growth. Compound **9** produced only 43% inhibition of HNF-3β at 240 nM.[98] In another study, it was determined that **9** was significantly less teratogenic than jervine, **10**, and **1**. Pregnant Syrian hamsters that were dosed with jervine produced fetuses where 92% possessed malformations, whereas only 14% of fetuses had malformations when exposed to **9** during gestation.[95]

2.10. Dihydrojervine (**10**)

Compound **10** is synthetically obtained by reducing the C12-C13 double bond of jervine using PtO_2 or $LiAlH_4$. The latter yielded a mixture of oils and crystalline products which included **10** as the major one.[99]

The successful formation of **10** is confirmed by ^{13}C NMR through loss of the double bond signals at 137.2 ppm and 146.4 ppm and a shift of the methyl carbon C21 of jervine found at 12.4 ppm to 10.5 ppm in the spectrum.[100]

Compound **10** was tested on the human metastatic prostate cancer PC-3 cell line with wound-healing assays to monitor migration and with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to test proliferation. At a 50 μM concentration, **10** did inhibit cancer growth, but it was ineffective in the wound-healing assay.[83]

2.11. 22-Keto-26-aminocholesterol (**11**)

Compound **11** has not yet been definitively identified from *V. californicum*, but is suspected based on mass spectrum data and a compound library screen.[81,82] Compound **11** is not typically a stable compound and thus will undergo ring-closing to yield **7**; it has

not been isolated previously. 22-Hydroxy-26-aminocholesterol can be oxidized by 22-hydroxy-26-aminocholesterol 22-oxidase to form **11.**[81,82] It is a potential intermediate in the biosynthesis of **7** and **1.**[82]

A summary of bioactive *V. californicum* alkaloids can be found in **Table 1.4**.

Table 1.4. Summary of bioactivity in *Veratrum californicum* alkaloids.

Alkaloid	Method of Testing	Efficacy	Reference
Cyclopamine (**1**)	Inhibition of growth of estrogen receptor positive cell line MCF7	Significant effects at both 10 and 20 μM	[60]
	Inhibition of proliferation of HEL and TF1a cells	Strong effect at 40 μM	[61]
	Induced apoptosis in human erythroleukemia cells	40 μM	[61]
	Inhibition of growth of LNCaP C4-2B cells	Significant inhibition at 100 nmol/L and IC_{50} of 11 μmol/L	[62]
	Decreased cell viability	<75% viability at 20 μM with 50 ng/mL TRAIL in TRAIL-resistant AGS cells	[63]
	Inhibition of hRSV infection in vitro	IC_{50} of 36 nM	[101]
	Reduction of lung hRSV titers	Reduction by 1.5 logs at 100 mg/kg	[101]
Veratramine (**2**)	Inhibition of progression of human prostate metastatic cancer cell line PC-3	<40% proliferation at 50 μM dose	[83]
	Inhibition of progression of human prostate metastatic cancer cell line PC-3	<20% migration at 50 μM dose	[83]
	Number of DNA-strand breaks in the cerebellum and cerebral cortex of mice	In both cerebellum and cerebral cortex: >0.5 μm tail moment with 0.25 μmol/kg dose, >1.0 μm tail moment with 2.5 μmol/kg	[84]
Isorubijervine (**3**)	Inhibition of rNaV1.3	IC_{50} of 12.14 ± 0.77 μM	[33]
	Inhibition of rNaV1.4	IC_{50} of 9.82 ± 0.84 μM	[33]
	Inhibition of rNaV1.5	IC_{50} of 6.962 ± 0.422 μM	[33]
	Inhibition of rNaV1.5	5 μM dose led to 41% decrease in current	[33]
	Lethal dose in mice	LD_{50} of 1.14 mg/kg	[33]
Muldamine (**4**)	Blocks action potential in squid and crayfish giant axons	Little or no depolarization at 1×10^{-4} M	[102]
Verazine (**7**)	Antifungal	Minimum inhibitory concentration of 6.2 μg/mL for *C. albicans*	[91]
	Antifungal	Minimum inhibitory concentration of 3.1 μg/mL for *T. rubrum*	[91]

	Inhibition of melanogenesis in B16 FI mouse melanoma cells	IC_{50} < 1 μg/mL	[29]
Etioline (**8**)	Inhibition of hepatitis B in PLC/PRF/5 cells	EC_{50} of 2.67 μg/mL	[92]
Dihydrojervine (**10**)	Inhibition of progression of human prostate metastatic cancer cell line PC-3	<40% proliferation at 50 μM dose	[83]

3. Conclusions

V. californicum contains a multitude of bioactive alkaloids with potential to be developed into therapeutic drugs. Compound **1** is an alkaloid from V. californicum that has inspired novel Hh pathway inhibiting cancer treatments. There remain many alkaloids in V. californicum that have yet to be fully characterized. Advances in separation and identification methods, materials, and instrumentation sensitivity have permitted detection of alkaloids beyond those that have been characterized prior. An assessment of the strategies for extraction, isolation, and characterization of known alkaloids has been presented in an effort to extend existing knowledge of these compounds to alkaloids that have been detected, but not yet characterized (**Figure 1.1**, **Table 1.2**). Utilization of the methods summarized in **Table 1.3** may permit the identification of additional alkaloids beyond those that are known, so that the identity of all 16 detected components can be accomplished and sufficient quantities of purified alkaloids can be assessed for mechanisms of activity. Veratrum plants are a well-known source of medicinal components and the discovery of new compounds in Veratrum californicum with favorable therapeutic properties serves as a compelling pursuit.

CHAPTER TWO: EXTRACTION AND SEPARATION OF *VERATRUM CALIFORNICUM* ALKALOIDS

Introduction

In the 1950's in the high mountain meadows of central and southern Idaho, sheepherders found that 1%-25% of their lambs were born with a craniofacial mutation.[86,103] These "monkey-faced" sheep had malformations affecting their eyes, jaws, skulls, and sometimes brains, with the most obvious mutation being a single, large, cyclopean eye located centrally on their face.[103] The Poisonous Plant Research Laboratory (PPRL) in Logan, Utah was tasked to determine the cause of these deformities in 1954.[86] It was originally believed the cause of the deformities was due to a recessive genetic trait, but in 1957 a breeding experiment provided evidence that negated this theory.[103] Field and feeding studies were conducted on the sheep population to identify causative agents.

Veratrum californicum wasn't considered a possible source of developmental deformities in the lambs' until 1958 when a sheepherder mentioned it can cause illness in the sheep when they consume it. Soon PPRL scientists initiated feeding trials where their results showed the sheep had a range of symptoms including excessive salivation and frothing at the mouth, vomiting, walking unusually, irregular heartbeat, vomiting, dyspnea, convulsions, coma, and death. In 1959 the first lamb with a cyclopic malformation was born during a feeding trial, and six years later enough studies had been conducted to definitively correlate deformities in lambs to the day of gestation the

pregnant ewes grazed on *V. californicum.* It was determined that a ewe consuming the plant on day 14 of gestation gave birth to a lamb having a proboscis-like nose above a cyclopic eye.[2,86]

In 1968 the steroidal alkaloid cyclopamine was isolated from the extract of *V. californicum,* and by 1969 the structure was published.[104,105] Upon analysis of the scientific literature, it was discovered that cyclopamine had originally been isolated from *Veratrum grandiflorum* and had been named 11-deoxojervine by a Japanese research group in 1965.[86] Cyclopamine in combination with cycloposine and jervine were determined to cause cyclopia in lambs. This was the first time that *Veratrum* alkaloids had been considered teratogenic, whereas previously cevanine-type alkaloids had been focused on due to their hypotensive properties.[2]

The extraction and isolation of alkaloids from *V. californicum* continued into the 1970's, followed by several decades of inactivity in the research outside of cyclopamine bioactivity exploration. By 2008, access to modern instrumentation with more sensitive detectors, and analytical techniques like solid phase extraction with increased analyte recovery have greatly improved the extraction, separation, isolation, characterization, and bioactivity assessment of steroidal alkaloids like those prevalent in *V. californicum* extract. In our lab we have been able to detect and identify less abundant alkaloids that were not previously detected from *V. californicum*.[73] Evaluation of eight literature protocols for the extraction of cyclopamine from *V. californicum*, dating back to the 1950's, led to determination that ethanol soak yielded the greatest magnitude of total alkaloids with the highest level of bioactivity.[49] An investigation was performed quantitatively to establish the composition of cyclopamine, veratramine, muldamine, and

isorubijervine contained within the leaf, stem, and root/rhizome of the plant.[74] This study was developed further by uncovering quantitative variation in the amount of the four previously listed alkaloids by plant part, harvest location, and growth stage. During this process at least six new, uncharacterized alkaloids were discovered.[71] These uncharacterized alkaloids exhibited Hedgehog pathway suppression activity, which inspired the current work. First, Turner's ethanol extraction method of 2 g plant material in 100 mL of 95% ethanol was attempted, but the resulting HPLC chromatograms had very little detection. [71] The concentration of starting material was modified until the chromatograms permitted correlation of retention times and predicted alkaloid identification (25 g plant material in 50.0 mL of 95%). A separation method consisting of a 30 min run duration was divided into five segments by time. Collection began for fraction 1 starting at 10.75 min and ending at 13.25 min. Fraction 2 was started immediately after for another collection period of 2.5 min, and this pattern continued until all five fractions were gathered. Each fraction contained between three and ten peaks of interest. Chromatographic analysis and mass spectrometry were used to identify the alkaloids.

Interest in the *V. californicum* alkaloids originates from their activity to inhibit the Sonic hedgehog (Shh) signaling pathway.[106] This pathway is responsible for proper cell polarization, limb formation, central nervous system development, and most epithelial tissue differentiation in animals leading to bilateral symmetry.[71,107–110] Over twenty cancers correlate to aberrant Hedgehog signaling (Hh) for propagation. When the Hh pathway is blocked during normal embryonic development, the consequences are severe, but this same mechanism of action can be used to treat cancer.[2,71]

Results

Raw extract was collected from the root and rhizome of *V. californicum* and was compared to the literature extract reported by Turner et al., using high performance liquid chromatography (HPLC) with a diode array detector (DAD) as seen in **Figure 2.1 a-c.** Buffer A was 0.1% Trifluoroacetic acid in water, and Buffer B was HPLC grade acetonitrile. A linear gradient was used starting at 15% Buffer B and increasing to 60% Buffer B over a period of 25 min. From 25.1 to 30.0 min Buffer B was set to 15%. A semi-preparatory Agilent Zorbax SB-C_{18} column (9.4 x 250 mm, 5 µm) was used with a flow rate of 3.0 mL/min. Five fractions were collected by time using this same method, with the collection periods described below. **Figure 2.1 a.** shows Turner's literature extract, **Figure 2.1 b.** shows the raw alkaloid extract for the current work, and **Figure 2.1 c.** shows the two overlaid.

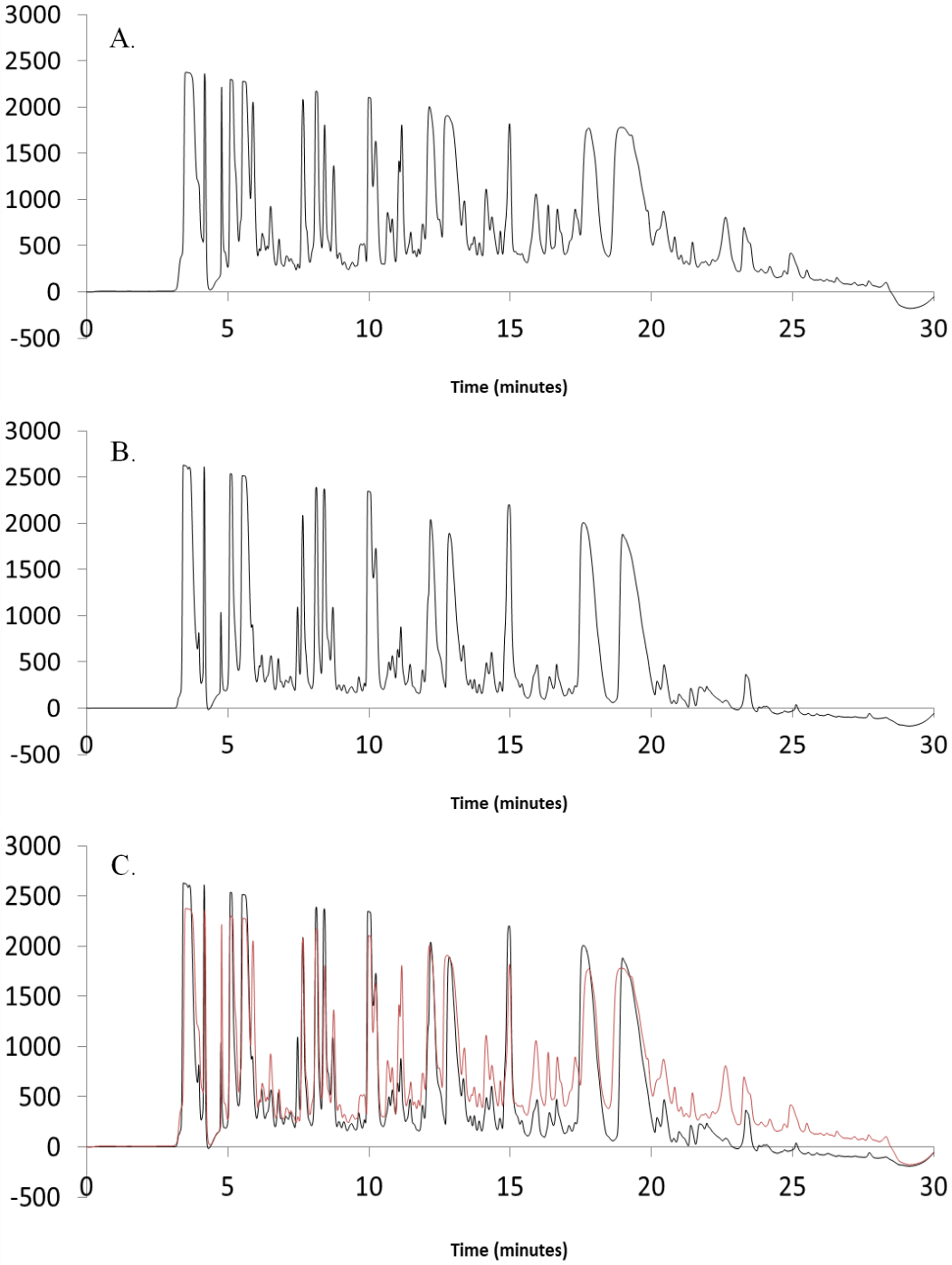

Figure 2.1 Chromatograms of Veratrum californicum raw extract using HPLC-DAD. A. most recent chromatogram of literature extract, B. extract chromatogram from current work, and C. overlay of the two extracts, where the literature extract is in red and the current work is shown in black

The extract was also analyzed using a charged aerosol detector (CAD) as in **Figure 2.2 a**. The solvents and gradient were the same for this method as the one described above, but the flow rate was lowered to 0.3 mL/min for a Thermo Acclaim 120 C_{18} column (2.1 x 150 mm, 3 μm). The chromatograms of the fractions as captured by the CAD can be seen in **Figure 2.2 b-f.,** where **Figure 2.2 b.** shows fraction 1 (collection time 10.75 to 13.25 min), **Figure 2.2 c.** shows fraction 2 (collection time 13.25 to 15.75 min), **Figure 2.2 d.** shows fraction 3 (collection time 15.75 to 18.25 min), **Figure 2.2 e.** shows fraction 4 (collection time 18.25 to 20.75 min), **Figure 2.2 f.** shows fraction 5 (collection time 20.75 to 23.25 min), and **Figure 2.2 g** shows as an overlaid view of each fraction.

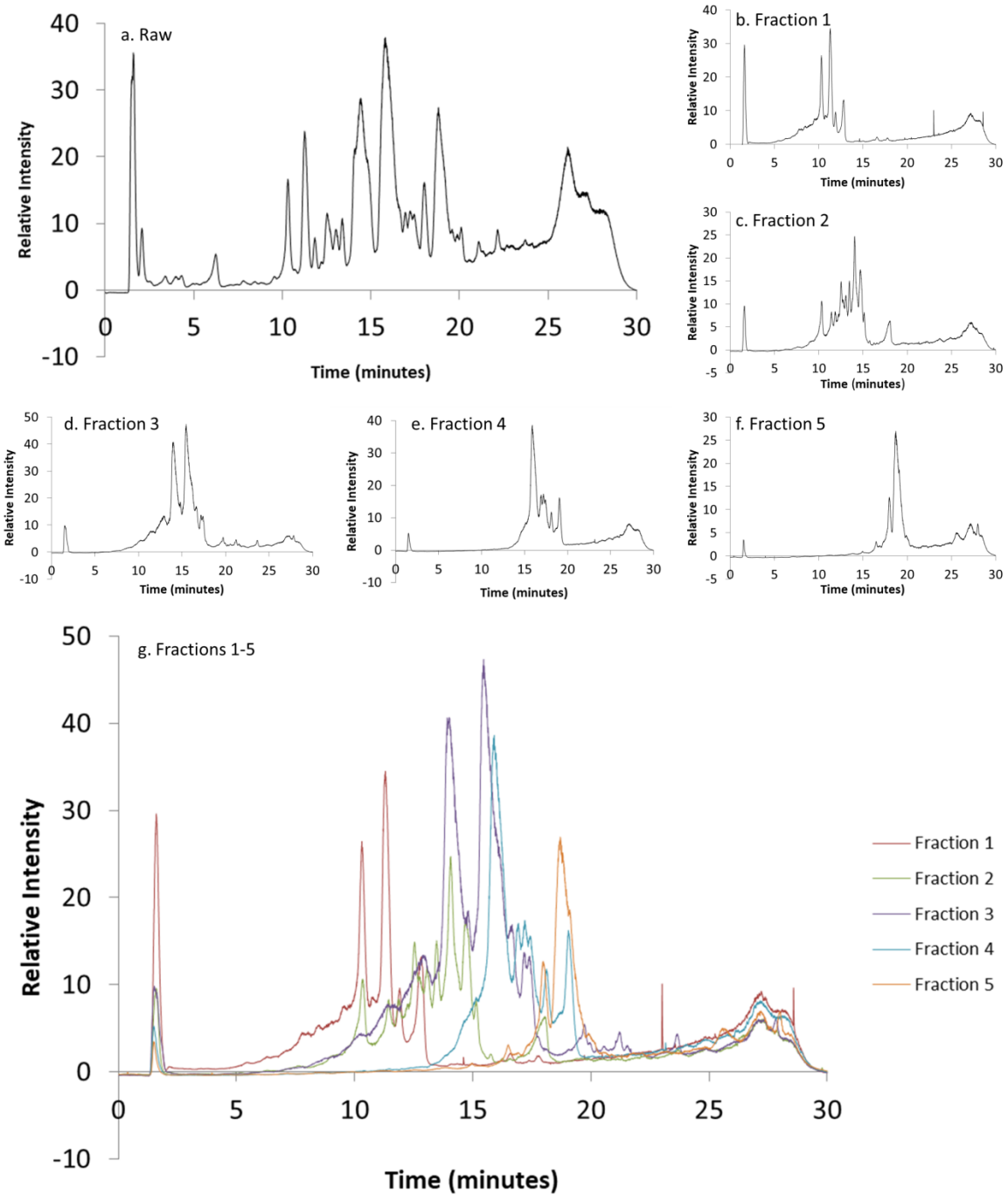

Figure 2.2 Chromatograms of *Veratrum californicum* raw extract and five fractions using HPLC-CAD

Each fraction as well as the raw extract was analyzed by high resolution mass spectrometry (MS) in order to determine molecular weight (m/z) and predicted molecular formula for alkaloid identification. An XTerra MS C_{18} column, 3.5 µm, 2.1 x 150 mm

(Waters, Milford, MA, USA) was utilized with a flow rate of 200 μL/min. The solvent system was 5% acetonitrile and 0.1% formic acid in water (Buffer A) and acetonitrile and 0.1% formic acid (Buffer B). A linear gradient was used starting at 5% Buffer B and increasing to 70% Buffer B over a period of 25 min. The mass spectra and extracted ion chromatograms of prominent peaks corresponding to abundant alkaloids in each of the five fractions collected from the extract are shown in **Figures 2.3-2.7**.

The LC-MS examination of fraction 1 revealed the presence of four alkaloids peaks, which can be seen with their corresponding extracted ion chromatograms in **Figure 2.3.**

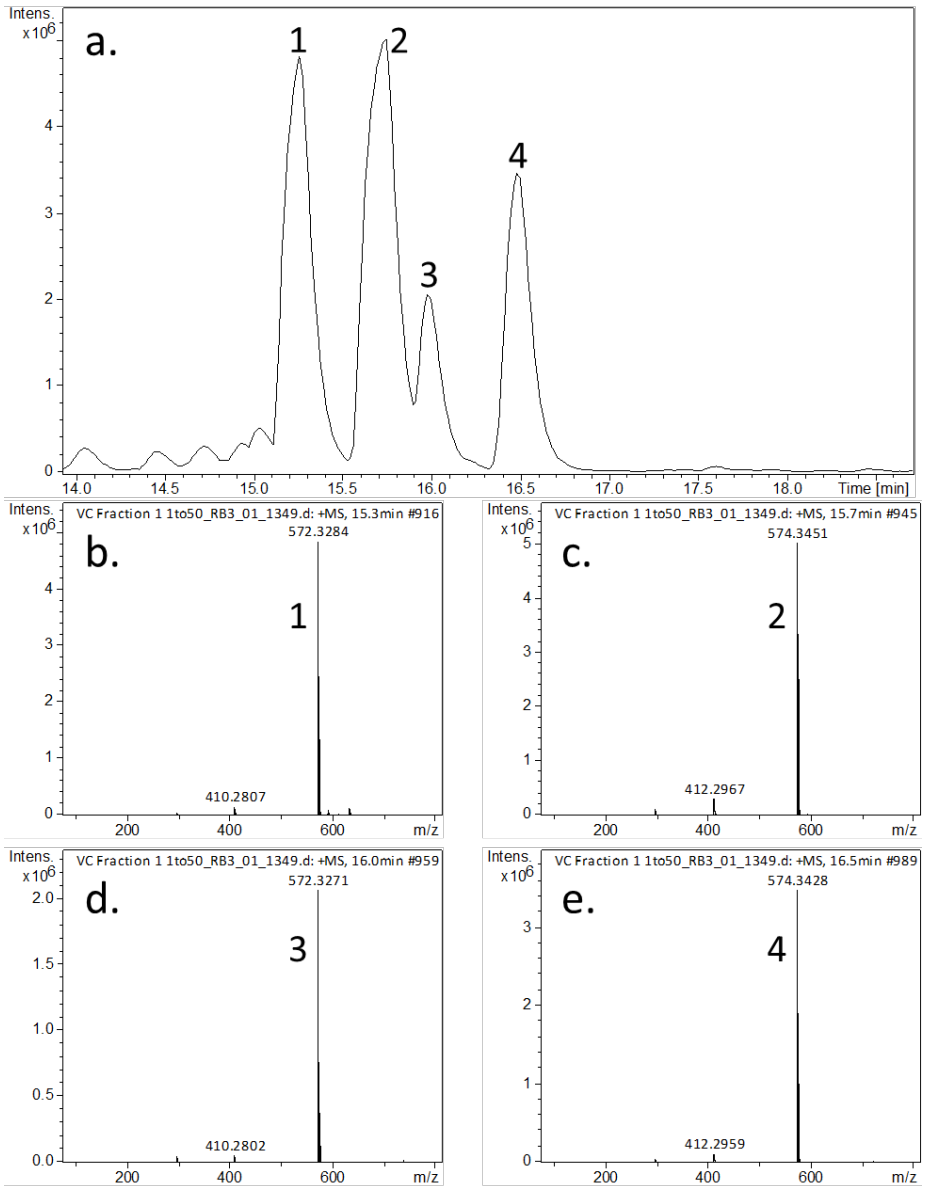

Figure 2.3 Chromatogram and extracted ion chromatogram for each alkaloid present in fraction 1, which corresponds to R_t from 10.75 – 13.25 min of crude extract chromatogram.

Fraction 1 contains four alkaloids as shown in **Figure 2.3 a.** The peaks appeared to be paired with 1 and 3 sharing nearly identical m/z ratios of 572.3284 and 572.3271 (**Figure 2.3 b.** and **d.**) and peaks 2 and 4 following the same pattern with m/z ratios of 574.3451 and 574.3428 **(Figure 2.3 c.** and **e.**) respectively. The predicted chemical

formulas are $C_{33}H_{49}NO_7$ for peaks 1 and 3 and $C_{33}H_{51}NO_7$ for the peaks 2 and 4. The reasonable identity of these alkaloids is veratrosine, cycloposine, and isomers of each.[71]

Fraction 2 had more alkaloid peaks than any other fraction. The mass spectrometry data aligned with the HPLC chromatogram for these ten compounds can be found in **Figure 2.4.**

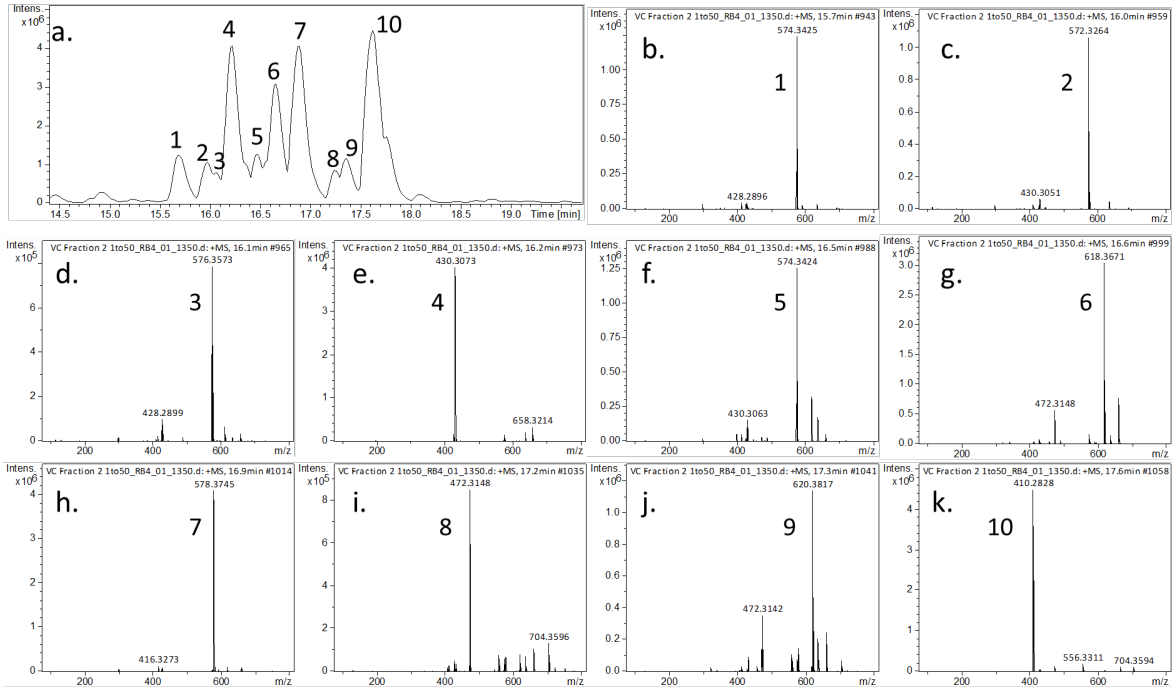

Figure 2.4 Chromatogram and extracted ion chromatogram for each alkaloid present in fraction 2, which corresponds to R_t from 13.25 – 15.75 min of crude extract chromatogram.

Fraction 2 demonstrates at least 10 distinct peaks as shown within the chromatogram found in **Figure 2.4 a.** There is some overlap in peak retention times between fractions 1 and 2. **Figure 2.4 b.** shows peak 1 with an m/z of 574.3425 and an elution time of 15.7 minutes, which corresponds to peak 2 of fraction 1 (**Figure 2.3 c.**). Peak 2 in **Figure 2.4 c.** has an m/z of 572.3264 and elution time of 16.0 min, which is the same as peak 3 from fraction 1 (**Figure 2.3 d.**), which was predicted to be an isomer of

veratrosine. Peak 3 from fraction 2 (**Figure 2.4 d.**) with an m/z of 576.3573, an elution time of 16.1 min, and a predicted molecular formula of $C_{33}H_{49}NO_7$ (identity unknown) does not have a peak corresponding to fraction 1. Neither does peak 4 (**Figure 2.4 e.**) with an m/z of 430.3073, elution time 16.2 min, and predicted molecular formula of $C_{27}H_{43}NO_3$. This molecular formula is consistent with tetrahydrojervine.[71] **Figure 2.4 f.** contains peak 5 with an m/z of 574.3424 and an elution time of 16.5 min, which matches that of peak 4 from fraction 1 (**Figure 2.3 e.**). Thus, this peak is also predicted to be an isomer of cycloposine. Peak 6 (**Figure 2.4 g.**) had an m/z of 618.3671, an elution time of 16.6 min, and a predicted molecular formula of $C_{35}H_{55}NO_8$ (identity unknown). **Figure 2.4 h.** shows peak 7 with an m/z of 578.3745, elution time of 16.9 min, and predicted molecular formula of $C_{33}H_{55}NO_7$ (identity unknown). **Figure 2.4 i.** displays peak 8, which had an m/z of 472.3148, elution time of 17.2 min, and predicted molecular formula of $C_{29}H_{45}NO_4$ (identity unknown). Peak 9 (**Figure 2.4 j.**) had an m/z of 620.3871, an elution time of 17.3 min, and a predicted molecular formula of $C_{35}H_{57}NO_8$ (identity unknown). Finally, peak 10 (**Figure 2.4 k.**) had an m/z of 410.2828, an elution time of 17.6 min, and a predicted molecular formula of $C_{27}H_{39}NO_2$, which was confirmed to be veratramine.

Fraction 3 had the fewest number of alkaloids of any fraction. The mass spectra for each of the three peaks identified on the HPLC can be seen in **Figure 2.5**.

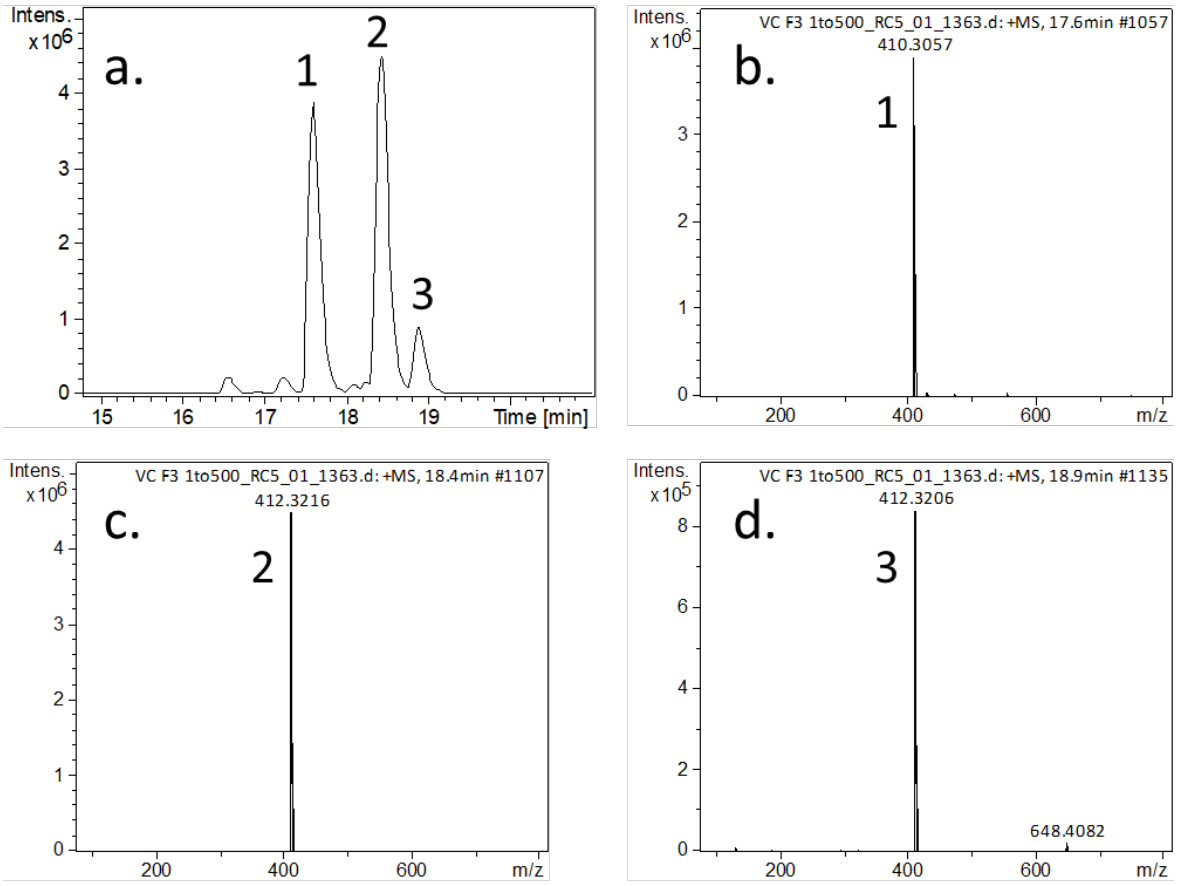

Figure 2.5 Chromatogram and extracted ion chromatogram for each alkaloid present in fraction 3, which corresponds to Rt from 15.75 – 18.25 min of crude extract chromatogram.

Fraction 3 contains three alkaloids (**Figure 2.5 a.**), of which only peak 10 from fraction 2 (veratramine), corresponds to peak 1 in Fraction 3 (**Figure 2.5 b.)** with m/z ratios of 410.2828 and 410.3057 respectively. The elution time for this alkaloid is consistent between fractions at 17.6 min, lending further support for the identification. **Figure 2.5 c.** shows peak 2 with an m/z of 412.3216, elution time of 18.4 min, and predicted molecular formula $C_{27}H_{41}NO_2$, which was confirmed to be cyclopamine. **Figure 2.5 d.** displays peak 3 with an m/z of 412.3206, elution time of 18.9 min, and a predicted molecular formula of $C_{27}H_{41}NO_2$ (predicted to be cyclopamine's isomer[42]).

Figure 2.6 displays the six detected alkaloids found in fraction 4.

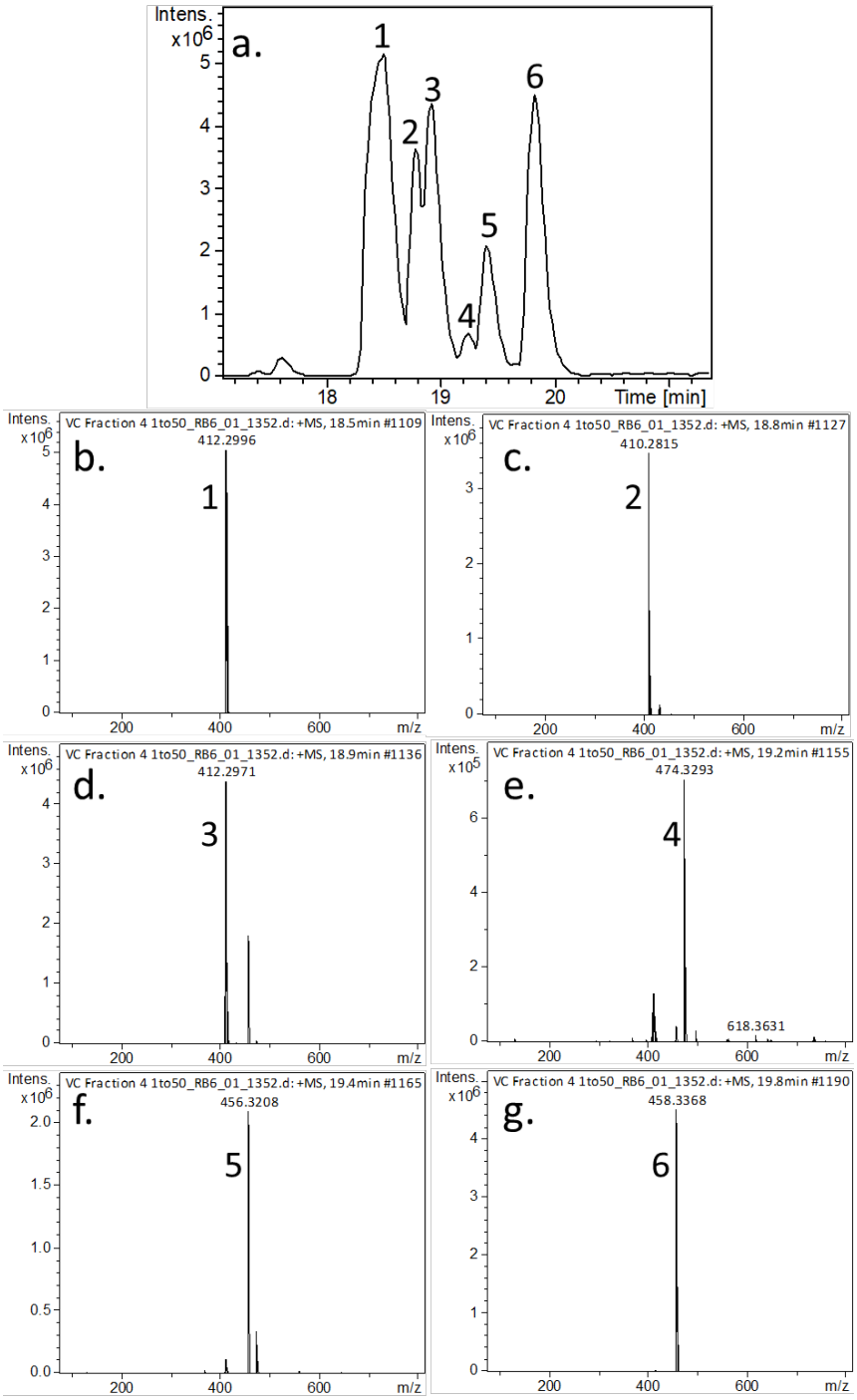

Figure 2.6 Chromatogram and extracted ion chromatogram for each alkaloid present in fraction 4, which corresponds to R_t from 18.25 – 20.75 min of crude extract chromatogram.

In **Figure 2.6 a.** there are six alkaloids present. **Figure 2.6 b.** shows peak 1 with m/z 412.2996, elution time 18.4 min, and predicted molecular formula $C_{27}H_{41}NO_2$

consistent with cyclopamine. **Figure 2.6 c.** displays peak 2 with an m/z of 410.2815, elution time of 18.8 minutes, and predicted molecular formula of $C_{27}H_{39}NO_2$. This may be an isomer of veratramine. Peak 3 as seen in **Figure 2.6 d.** has an m/z of 412.2971, an elution time of 18.9, and a predicted molecular formula of $C_{27}H_{41}NO_2$. This is potentially an isomer of cyclopamine.[42] **Figure 2.6 e.** contains peak 4 which has an m/z of 474.3293, elution time of 19.2 min, and predicted molecular formula of $C_{29}H_{47}NO_4$ (identity unknown). Peak 5 (**Figure 2.6 f**) has an m/z of 456.3208, an elution time of 19.4 min, and a predicted molecular formula of $C_{29}H_{45}NO_3$ (identity unknown). Lastly, peak 6 (**Figure 2.6 g**) has an m/z of 458.3368, an elution time of 19.8 min, and a predicted molecular formula of $C_{29}H_{47}NO_3$, which is consistent with muldamine[71].

Within the final fraction eight different compounds were noted. **Figure 2.7** shows the HPLC chromatogram for fraction 5 as well as the corresponding mass spectrum for each alkaloid peak.

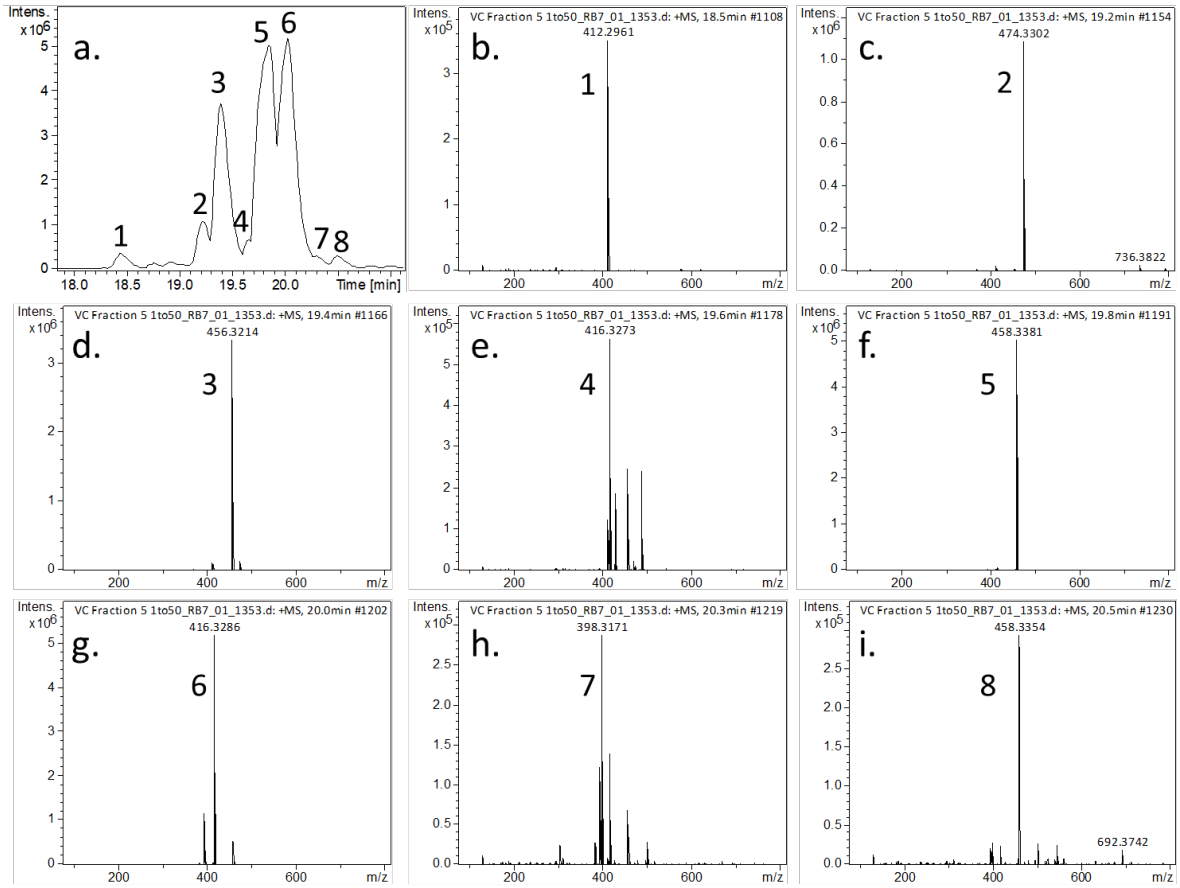

Figure 2.7 Chromatogram and extracted ion chromatogram for each alkaloid present in fraction 5, which corresponds to R_t from 20.75 – 23.25 min of crude extract chromatogram.

Figure 2.7 a. shows the eight alkaloids present in the chromatogram for fraction 5. Peak 1 (**Fig. 2.7 b.**) has an m/z of 412.2931, an elution time of 18.5 min, and a predicted molecular formula of $C_{27}H_{41}NO_2$, again corresponding to cyclopamine. **Figure 2.7 c.** displays peak 2 with an m/z of 474.3302, an elution time of 19.2 min, and a predicted molecular formula of $C_{29}H_{47}NO_4$. This is consistent with peak 4 from fraction 4 (**Figure 2.6 e.**), which has an unknown identity. Peak 3 (**Figure 2.7 d.**) has an m/z of 456.3214, an elution time of 19.4 min, and a predicted molecular formula of $C_{29}H_{45}NO_3$, which matches peak 5 from fraction 4 (**Figure 2.6 f.**), but has an unknown identity. Peak 4 (**Figure 2.7 e.**) has an m/z of 416.3273, an elution time of 19.6 min, and a predicted

molecular formula of $C_{27}H_{45}NO_2$, which has a suspected identity of 22-keto-26-aminocholesterol[71]. **Figure 2.7 f.** contains peak 5 with an m/z of 458.3381, an elution time of 19.8 min, and a predicted molecular formula of $C_{29}H_{47}NO_3$. This peak matches peak 6 from fraction 4 (**Figure 2.6 g.**), which was predicted to be muldamine. Peak 6 (**Figure 2.7 g.**) has an m/z of 416.3286, an elution time of 20.0 min, and a predicted molecular formula of $C_{27}H_{45}NO_2$. This predicted molecular formula is the same as that of peak 4 (**Figure 2.7 e.**) which is consistent with that of 22-keto-26-aminocholesterol[71]. **Figure 2.7 h.** has peak 7 with an m/z of 398.3171, an elution time of 20.3 min, and a predicted molecular formula of $C_{27}H_{43}NO$, which is consistent with verazine[71]. The final peak, peak 8 (**Figure 2.7 i.**), has an m/z of 458.3354, an elution time of 20.5 min, and a predicted molecular formula of $C_{29}H_{47}NO_3$, which is representative of an isomer of muldamine.

Twenty-five unique compounds were detected across the five fractions with nine compounds repeated across more than one fraction and sixteen compounds found within only a single fraction. The presence of veratramine and cyclopamine was confirmed using the elution times of standards (**Figure 2.9**), and the identities of sixteen alkaloids were suspected, leaving seven unknown. **Table 2.1** demonstrates these findings.

Table 2.1 Summary of findings from five fractions of Veratrum californicum extract

R_t	m/z	Molecular Formula	Identity	Fraction
15.3	572.3	$C_{33}H_{49}NO_7$	Veratrosine	1
15.7	574.3	$C_{33}H_{51}NO_7$	Cycloposine	1,2
16	572.3	$C_{33}H_{49}NO_7$	Isomer of veratrosine*	1,2
16.1	576.4	$C_{33}H_{53}NO_7$	?	2
16.2	430.3	$C_{27}H_{43}NO_3$	Tetrahydrojervine*	2
16.5	574.3	$C_{33}H_{51}NO_7$	Isomer of cycloposine*	1,2
16.6	618.4	$C_{35}H_{55}NO_8$	?	2
16.9	578.4	$C_{33}H_{55}NO_7$	?	2
17.1	428.3	$C_{27}H_{41}NO_3$	Dihydrojervine* **	2
17.2	472.3	$C_{29}H_{45}NO_4$	?	2
17.3	620.4	$C_{35}H_{57}NO_8$	?	2
17.4	414.3	$C_{27}H_{43}NO_2$	Etioline* **	2
17.5	428.3	$C_{27}H_{41}NO_3$	Isomer of dihydrojervine* **	2
17.6	410.3	$C_{27}H_{39}NO_2$	Veratramine	2,3
18.4/18.5	412.3	$C_{27}H_{41}NO_2$	Cyclopamine	3,4,5
18.8	410.3	$C_{27}H_{39}NO_2$	Isomer of veratramine*	4
18.9	412.3	$C_{27}H_{41}NO_2$	Isomer of cyclopamine*	3,4
19.2	474.3	$C_{29}H_{47}NO_4$	?	4,5
19.4	456.3	$C_{29}H_{45}NO_3$	?	4,5
19.6	416.3	$C_{27}H_{45}NO_2$	22-keto-26-aminocholesterol*	5
19.5	414.3	$C_{27}H_{43}NO_2$	Isorubijervine**	4
19.8	458.3	$C_{29}H_{47}NO_3$	Muldamine	4,5
20	416.3	$C_{27}H_{45}NO_2$	Isomer of 22-keto-26-aminocholesterol*	5
20.3	398.3	$C_{27}H_{43}NO$	Verazine*	5
20.5	458.3	$C_{29}H_{47}NO_3$	Isomer of muldamine*	5

*Signifies a prediction based on the m/z and molecular formula, but it has not been confirmed in **Table 2.1**
See **Figure 2.8 for data

There were several alkaloids previously identified in *V. californicum* that did not have a corresponding peak in any of the UV chromatograms including etioline, dihydrojervine, and isorubijervine. These compounds were identified by retention time and m/z ratios corresponding to data from prior reports.[71] The results are shown in **Figure 2.8.**

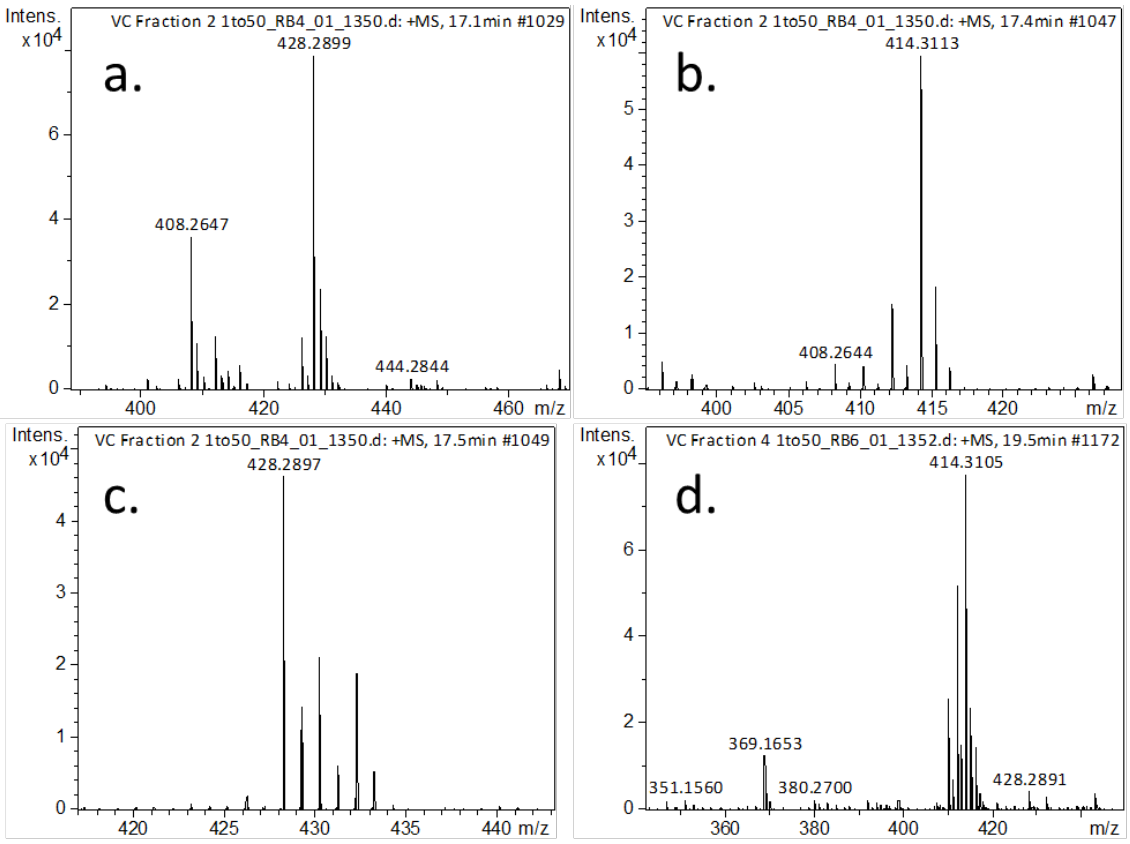

Figure 2.8 Extracted ion chromatograms previously identified alkaloids present in fractions 2 and 4 of crude alkaloid extract.

Figure 2.8 contains the alkaloids missing from the *V. californicum* extract as described in the literature.[71] The peak seen in **Figure 2.8 a.** has an m/z of 428.2899, an elution time of 17.1 min (fraction 2), and a predicted molecular formula of $C_{27}H_{41}NO_3$, representing dihydrojervine. **Figure 2.8 b.** shows a peak with an m/z of 414.3113, an elution time of 17.4 min (fraction 2), and a predicted molecular formula of $C_{27}H_{43}NO_2$ corresponding to etioline. The peak seen in **Figure 2.8 c.** has an m/z of 428.2897, an elution time of 17.5 min (fraction 2), and a predicted molecular formula of $C_{27}H_{41}NO_3$, which is consistent with dihydrojervine, and is thus assumed to be an isomer. The final peak in **Figure 2.8 d.** has an m/z of 414.3105, an elution time of 19.5 min (fraction 4), and a predicted molecular formula of $C_{27}H_{43}NO_2$, which is isorubijervine.

Figure 2.9 shows two peaks from the standard made of 355 µM veratramine and 390 nM cyclopamine.

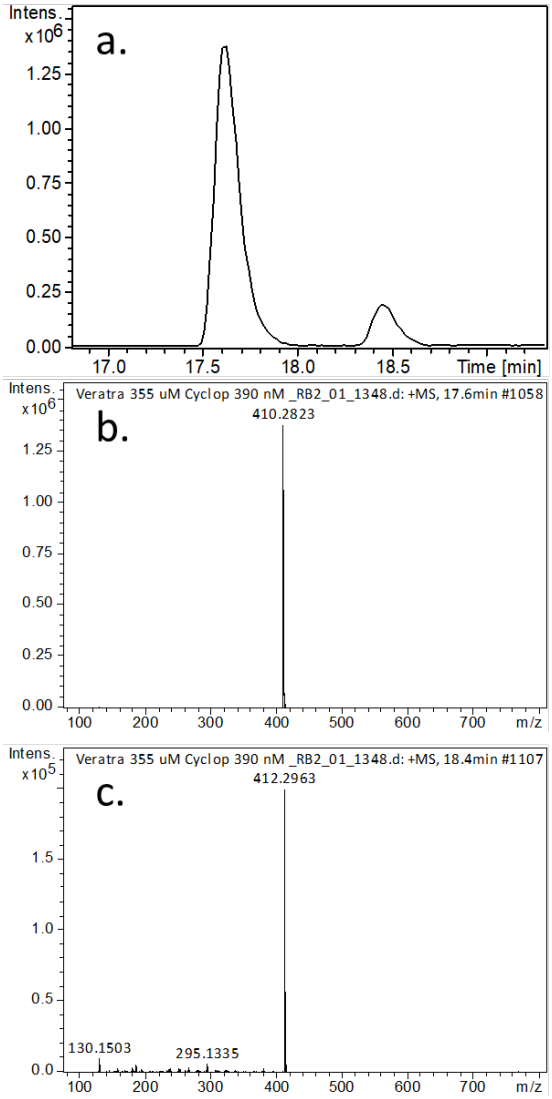

Figure 2.9 Chromatogram and extracted ion chromatogram for 355 µM veratramine and 390 nM cyclopamine.

Two peaks are seen in **Figure 2.9 a.** The peak in **Figure 2.9 b.** has an m/z of 410.2823, an elution time of 17.6 min, and a predicted molecular formula of $C_{27}H_{39}NO_2$, confirming its identity of veratramine. **Figure 2.9 c.** has an m/z of 412.2963, an elution

time of 18.4 min, and a predicted molecular formula of $C_{27}H_{41}NO_2$, verifying the presence of cyclopamine.

Discussion

The chromatogram representing alkaloids extracted from *V. californicum* root and rhizome that served as the basis for the present thesis is shown in **Figure 2.1**. There is good correlation between these results and those most recently appearing in literature.[71] This validated that the modified extraction method, using 25 g of plant material in 50.0 mL of 95% ethanol instead of 2 g in 100 mL, was effective at removing similar alkaloid content from the roots and rhizomes of *V. californicum* as the work previously performed.[42] The fraction collection at intervals of 150 seconds, beginning at 10.25 min, was intended to assess the alkaloids with the most desirable bioactivity from within the crude extract (**Figure 2.1**). The region of the chromatogram based on the published HPLC protocol, was selected based on the presence of alkaloids with suspected Hh signal suppression activity, leading to the selection of fraction collection across the range of 10.25-23.75 min.[74] Chromatograms for the five fractions collected over the 10.25-23.75 min crude extract (**Figure 2.2** b-f) confirmed the presence of alkaloids separated into smaller buckets that could be pursued for bioactivity characteristics.

Examination of chromatograms corresponding to fractions 1-5 (**Figures 2.3-2.8**), show that fraction 1 contained four alkaloids, fraction 2 contained thirteen (including those from **Figure 2.8**), fraction 3 contained three, fraction 4 contained seven (including one from **Figure 2.8**), and fraction 5 contained eight. Veratrosine, cycloposine and their isomers were identified in fraction 1 (**Figure 2.3**). Fraction 2 was made up of cyloposine, its isomer, veratrosine's isomer, tetrahydrojervine, dihydrojervine, its isomer, etioline,

and veratramine, and five unknown alkaloids (**Figures 2.4** and **2.8**). Fraction 3 did not have any compounds in it that were not found in other fractions. The presence of veratramine and cyclopamine were confirmed by elution times of veratramine and cyclopamine standards, and an isomer of cyclopamine was detected as well (**Figures 2.5** and **2.9**). Fraction 4's only unique compounds were isorubijervine and an isomer of veratramine, and it too included cyclopamine and its isomer, as well as muldamine, and two unknown compounds (**Figures 2.6** and **2.8**). Lastly, Fraction 5 was made up of cyclopamine, muldamine and its isomer, 22-keto-26-aminocholesterol and its isomer, verazine, and two unknown compounds (**Figure 2.7**).[71] This data is found summarized in **Table 2.1**.

When examining the m/z ratios of the standards for veratramine and cyclopamine, they were found to be 410.2823 and 412.2963 respectively (**Figure 2.9**), but the expected m/z ratios were about 55 parts per million (ppm) lower than those values. This was likely due to the sample concentration exceeding the limit of detection, and thus all the molecular formulas were generated with a margin of error within the range of 54.5-61.8 ppm. Fraction 3 was an exception, as it was diluted by an additional order of magnitude, resulting in an error of less than one ppm for veratramine and less than 2 ppm for cyclopamine.

Table 2.1 confirms a lack in knowledge of the alkaloid content present in *V. californicum.* Further work should be done to identify the detected alkaloids and pursue the compounds with notable bioactivity. The plant roots and rhizomes used in this experiment were gathered from the Shindig Trail (N43 45.719" W 116 05.327") on July, 3, 2014. The chromatograms fitting this same description from Turner et al. 2019, did not

detect the presence of etioline or dihydrojervine, and only a small peak for isorubijervine.[71] The missing alkaloids of etioline, dihydrojervine, and isorubijervine (**Figure 2.8**) may not be detected sufficiently well by UV-Vis and may require detection by CAD or MS.

Materials and Methods

Plant Material

Plants were harvested from the high mountain meadows of the Boise National Forest, Idaho by the Shindig Trail at 6901 feet elevation (N43 45.719" W 116 05.327") on July, 3, 2014. The roots/rhizomes were separated from the above ground plant parts and transported to the laboratory on ice. The roots/rhizomes were cut into 2 cm pieces and freeze dried for 14 hrs with a LabConco Freezone 4.5 freeze drying unit (Labconco Corporation, Kansas City, MO, USA). The pieces were then stored in sealable plastic bags at -20°C until use.

Extraction

Root/rhizome pieces were removed from the freezer, thawed, and cut to about 2 cm in size. They were frozen in liquid nitrogen followed by freeze drying a second time for 24-48 hours using a LabConco Freezone 4.5 freeze drying unit (Labconco Corporation, Kansas City, MO, USA). A coffee grinder (Mr. Coffee, IDS77) was used to create a fine powder, which was either used immediately or stored in a vacuum-sealed bag in the freezer. A 50.0 mL volume of 95% ethanol was added to 25 g of powdered plant material and the solution sonicated for 30 min. All solvents were purchased from Fisher Scientific unless otherwise specified. This mixture was stirred overnight on a stir plate before vacuum filtration (Whatman filter paper, 0.45 μm). The solid plant material

was discarded, and the ethanol was removed from the filtrate by rotary evaporation at reduced pressure. The remaining solid was resuspended in 10.0 mL 95% ethanol, warmed to 40°C, and sonicated for five minutes. Ammonium hydroxide was added until the pH of the solution exceeded 10. This solution was then added to a supported liquid extraction (SLE) column (Chem Elut, Agilent, Santa Clara, CA, USA or HyperSep SLE, ThermoFisher Scientific, Pittsburgh, PA, USA) and allowed to absorb for 10 min. Alkaloids were eluted with chloroform (3 x 10 mL) using a vacuum manifold. The combined chloroform fractions were dried using rotary evaporation, and the alkaloids were dissolved in 1.0 mL of 100% ethanol.

Separation

For the preliminary examination of the extract and fraction collection a Dionex UltiMate® 3000 uHPLC system coupled to a DAD and an automated fraction collector was used. Separation was performed using a semi-preparative Agilent Zorbax SB-C_{18} column (9.4 x 250 mm, 5 µm) with mobile phases of 0.1% trifluoroacetic acid (TFA) in water (Buffer A) and HPLC grade acetonitrile (Buffer B). The flow rate was set to 3.0 mL/min starting with 15% Buffer B with a linear gradient up to 60% Buffer B for 25 minutes. From 25.1 minutes to 30 minutes Buffer B was set to 15%. Five fractions were collected starting at 10.75 minutes in 150 sec intervals. Fractions of the same retention times were pooled, evaporated to dryness by rotary evaporation, and resuspended in 2.0 mL of 100% ethanol. Fractions 1-5 were stored at -20 °C until they could be analyzed by LC-MS.

Identification

The raw extract and fractions were examined using a Thermo Scientific UltiMate 3000 HPLC paired with a Corona Veo RS CAD. A Thermo Acclaim 120 C_{18} column (2.1 x 150 mm, 3 µm) with mobile phases of 0.1% (TFA) in water (Buffer A) and acetonitrile (Buffer B) was used. The flow rate was set to 0.3 mL/min starting with 15% Buffer B with a linear gradient up to 60% Buffer B for 25 minutes. From 25.1 minutes to 30 minutes 15% Buffer B was used. Standards for cyclopamine (10.1 mM) and veratramine (10.1 mM) were run using the same method in order to compare elution times.

To more precisely identify the alkaloids present, LC-MS analysis was performed using an ultra-high resolution Quadrupole Time of Flight (QTOF) instrument (Bruker maXis). The electrospray ionization (ESI) source was operated under the following conditions: positive ion mode, 1.2 bar nebulizer pressure, 8 L/min flow of N_2 drying gas heated to a temperature of 200 °C, 3000 V to -500 V voltage between HV capillary and HV end-plate offset, mass range set from 80 to 800 m/z, and the quadrupole ion energy at 4.0 eV. Sodium formate was used to calibrate the system in the mass range of 80 to 800 m/z. HPLC separation was achieved using an XTerra MS C_{18} column, 3.5 µm, 2.1 x 150 mm (Waters, Milford, MA, USA). The flow rate was set to 200 µL/min. The mobile phases were 5% acetonitrile and 0.1% formic acid in water (Buffer A) and acetonitrile and 0.1% formic acid (Buffer B). The linear gradient method was used to separate analytes starting at 5% Buffer B and increasing to 70% Buffer B over 25 min. A 1 µL sample injection was used. The data was analyzed with the Compass Data Analysis software package (Bruker Corporation, Billerica, MA, USA).

Chemicals and Solvents

The cyclopamine standard (>99% purity) was purchased from Alfa Aesar (Ward Hill, MA, USA), and the veratramine standard (>98.0% purity) was from Tokyo Chemical Industry (TCI) (Tokyo, Japan). The extraction solvents 95% ethanol, ammonium hydroxide, and chloroform were purchased from Fisher Scientific (Pittsburgh, PA, USA), and the 100% ethanol was from Decon Labs (King of Prussia, PA, USA). The HPLC mobile phases TFA and acetonitrile (>99% purity) were also obtained from Fisher Scientific.

CHAPTER THREE: BIOACTIVITY ANALYSIS OF *VERATRUM CALIFORNICUM* EXTRACTED ALKALOID COMBINATIONS

Introduction

The Hedgehog (Hh) signaling pathway is one of the most important signaling pathways, and it is one of a small number that is active for intercellular communication during development.[52,85] It plays a crucial role for proper formation of vertebrate embryos in regards to bilateral symmetry, the central nervous system, skeleton, limbs, teeth, eyes, and several vital organs.[52,86] When there is loss of proper function of this pathway the results can range from under-developed facial features to cyclopia to nervous system disorders.[86]

The Hh gene was first discovered in the Nobel prize-awarded work in 1978 during the gene knockout trials in *Drosophila* by Christiane Nusslein-Volhard and Eric Wieschaus. The fruit fly embryo resembled a hedgehog with a short body and spine-like projections when the Hh gene was silenced. [86,111] The vertebrate Hh pathway is dependent upon three signaling molecules: Desert hedgehog (Dhh), Indian hedgehog (Ihh), and Sonic hedgehog (Shh), which get their names from two hedgehog breeds and a fictional character.[86] In humans, when Shh protein binds *Patched 1* (PTCH), a 12-span transmembrane protein, a seven-span transmembrane protein, Smoothened (Smo), initiates a signaling cascade. The levels of zinc-finger transcription factors from the Ci (cubitus interruptus)/Gli family determines if the Hh signal is transmitted. Gli 1 and Gli 2 function as activators while Gli 3 is a repressor. Gli 1 is directly connected to Hh

pathway activation that its function is monitored to determine the activation state of the signaling pathway. The teratogenic alkaloid cyclopamine was the first discovered Hh pathway suppressor. Instead of PTCH binding to Smo as an inhibitor, cyclopamine takes PTCH's place, stopping signal transduction.[85,86] Chris Chandler designed and created a cell signaling image that can be seen in **Figure 3.1**.

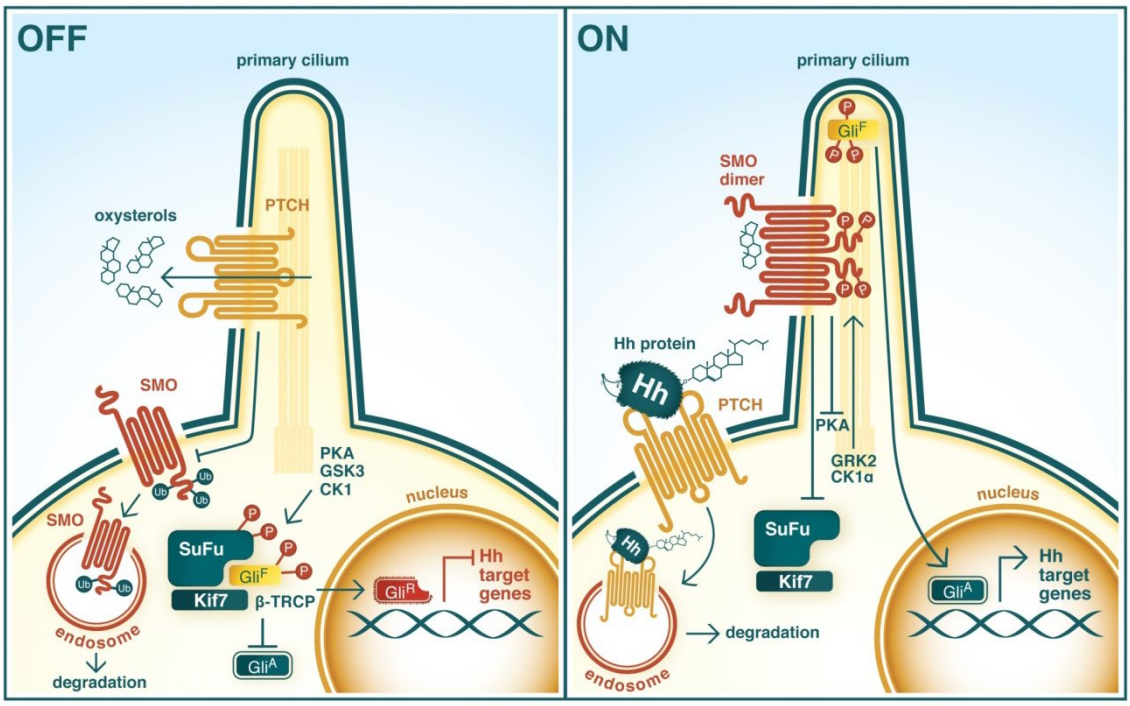

Figure 3.1 The hedgehog signaling pathway, where the left side of the figure represents when the pathway is off due to PTCH inhibiting SMO, and the right side represents when the pathway is activated by Hh protein binding to PTCH allowing SMO to function

Once most cells have matured, the Hh signaling pathway is no longer in use, but its aberrant activation is associated with the development of over twenty different types of cancer.[2,86] A promising realm of treatment of these cancers includes finding inhibitors to block the pathway's succession without the typical side effects of traditional methods, which is usually done by targeting Smo and more recently Gli.[48,86] In mouse models cyclopamine has suppressed tumor growth for eight different types of cancer, and in

humans it has been used in the form of a topical ointment applied directly to tumors, leading to shrinkage of cancerous tissue without adverse side effects.[86]

There are some drawbacks with using cyclopamine as a chemotherapeutic: it has little solubility in physiological conditions or water, it breaks down under acidic conditions (such as in the presence of human stomach acid), and as some specific cell types do still utilize the Hh pathway into maturity, it can have some off-target side effects.[48,71,86] The cyclopamine molecular scaffold has been used as a model for drug development in therapeutics targeting the blockage of Hh pathway signaling. Initial drug development efforts at Johns Hopkins University began by adding a 3-keto *N*-(aminoethyl-aminocaproyl-dihydrocinnamoyl) moiety to cyclopamine (KAAD-cyclopamine). In mouse model studies, KAAD-cyclopamine exhibited inhibition levels that exceeded cyclopamine by 10-20 fold.[86] Improving solubility of cyclopamine does not eliminate deleterious side effects. Another effort to develop a therapeutic resulted in IPI-926, a compound based on alteration of native cyclopamine, making the natural product more stable and potent. Phase 2 clinical trials with IPI-926 were aborted due to the median survival rate for patients being less than that of the placebo group.[86] Yet another drug discovery pathway inspired by cyclopamine was pursued, but this time it was a computational predictive approach to small molecule creation. One such high throughput virtual screening workflow surveyed a high volume of small molecules for Smo binding.[112] In January of 2012 Vismodegib (GDC-0449 or Erivedge) was approved by the FDA for treatment of metastatic basal cell carcinoma (mBCC) or locally advanced basal cell carcinoma (laBCC) in adults that are ineligible for radiation therapy or surgery.[112,113] Created by Genentech, Inc., in phase II clinical trials Vismodegib had an

overall response rate (ORR) of 60% for patients with laBCC and 46% for patients with mBCC. 43% of patients with laBCC demonstrated significant tumor shrinkage or healed lesions, and 30% of patients with mBCC noticed tumor shrinkage. The most common side effects were: decreased appetite, diarrhea, dysgeusia, fatigue, hair loss, muscle spasms, and nausea.[112] Sonidegib (LDE225, erismodegib, or ODOMZO), also identified in a small-molecule in vitro screening study, was designed by Sun Pharma Global and approved by the FDA in 2015 for treatment of recurrent advanced basal cell carcinoma (aBCC) or patients not suitable for radiation of surgery.[48,114,115]In Phase II clinical trials, over 50% of patients taking 200 mg daily saw objective responses. 18 out of 94 patients with laBCC either died or had their condition progress, but more than 50% had a response for longer than 6 months. 80% of patients with mBCC maintained an objective response. The most common to least common side effects when taking Sonidegib were: muscle spasms, alopecia, dysgeusia, nausea, increased creatinine kinase, fatigue, decreased weight, diarrhea, decreased appetite, myalgia, and vomiting.[114] It has been found that over time patients' bodies have developed methods of resisting the inhibitory properties of these two drugs. Sometimes Smo mutates, or an alternative Hh pathway initiates.[116] The most recent FDA-approved Hh signaling pathway inhibitor is Glasdegib (PF-0449913 or DAURISMO™), which is an oral drug developed by Pfizer and approved in 2018 for use in combination with low-dose cytarabine (LDAC) to treat acute myeloid leukemia (AML) for patients ages 75 and older or those who have co-morbidities which do not allow the use of intensive induction chemotherapy.[48,117] The BRIGHT AML 1003 study showed a 54% reduction in mortality with glasdegib in combination with LDAC compared to LDAC alone. Pneumonia, fatigue, dyspnea, hyponatremia, sepsis, and

syncope were the most common side effects of using glasdegib with LDAC.[117] **Figure 3.2** shows the structures for each of the above mentioned modified alkaloids (KAAD-cyclopamine and IPI-926) and chemotherapeutics (vismodegib, sonidegib and glasdegib).

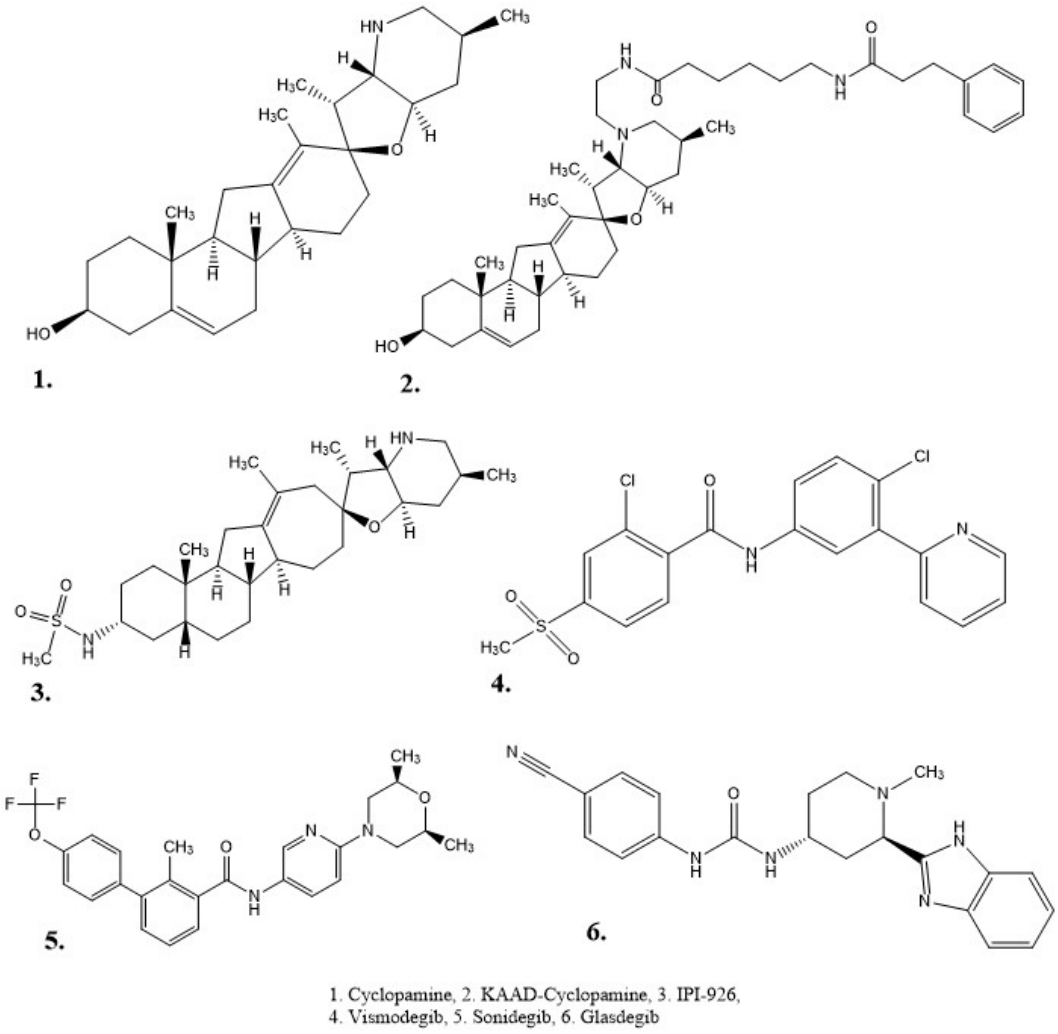

Figure 3.2 Structures of hedgehog signaling pathway inhibitors cyclopamine, modified forms of cyclopamine, and computationally derived, FDA approved chemotherapeutics

Cyclopamine was the first discovered inhibitor of the Hh pathway, and many derivatives of it have improved function to suppress Hh signaling, so it is logical to return to the plant source in search of additional Hh signaling suppressors that may have been missed at time of the original work in the 1950's. It took until 1968, for cyclopamine to

be extracted from the native Idaho plant *Veratrum californicum* and finally characterized.[86] The plant was initially noticed when it was determined to be the cause of cyclopic malformations in newborn lambs in central and southern Idaho.[86,103] Pregnant ewes fed on the plant, which contained toxic alkaloids with the ability to block the Hh pathway, leading to malformed embyos.[2,86]

Preliminary work has been done in our lab demonstrating that a raw alkaloid extract has more potent inhibitory effects on the Hh signaling pathway than cyclopamine alone at commensurate concentration.[42,71] This preliminary study result motivated the current investigation. Five fractions of alkaloids were collected from the raw extract chromatogram using HPLC-DAD and an automated fraction collector based on elution time. A cyclopamine standard, the raw extract, and the five fractions were then tested for bioactivity using a Shh-Light II cell line obtained from Johns Hopkins University, in order to measure Gli activity. It was found that three of the fractions had a statistically significant enhanced potency to suppress Hh signaling as compared to cyclopamine standard. Further separation and isolation of *V. californicum* alkaloids contained within fractions 1, 2 and 4 constitutes a worthy pursuit for discovery of cancer therapeutics.

Results

The bioactivity of the Shh-Light II cell line was measured using the Dual-Luciferase® Reporter Assay System.[118] Shh protein was added in order to activate the pathway and trigger an increase in the presence of Gli transcription factors, which was detected as luminescence by a plate reader.[85,86] Each 96-well plate contained three wells with each of the following treatments: negative controls (media only), positive controls (media and Shh protein), and treatments consisting of 0.1 μM cyclopamine, 0.01 or 0.05

μM cyclopamine, 1:200 dilutions of raw alkaloid extract, 1:1000 dilutions of raw alkaloid extract, 1:20 dilutions of each fraction, and 1:100 dilutions of each fraction in media with added Shh protein. The results of this assessment for four plates (a total of 12 wells for each treatment), is shown in **Figure 3.3 a-b.** along with a detailed view of all high concentration treatments.

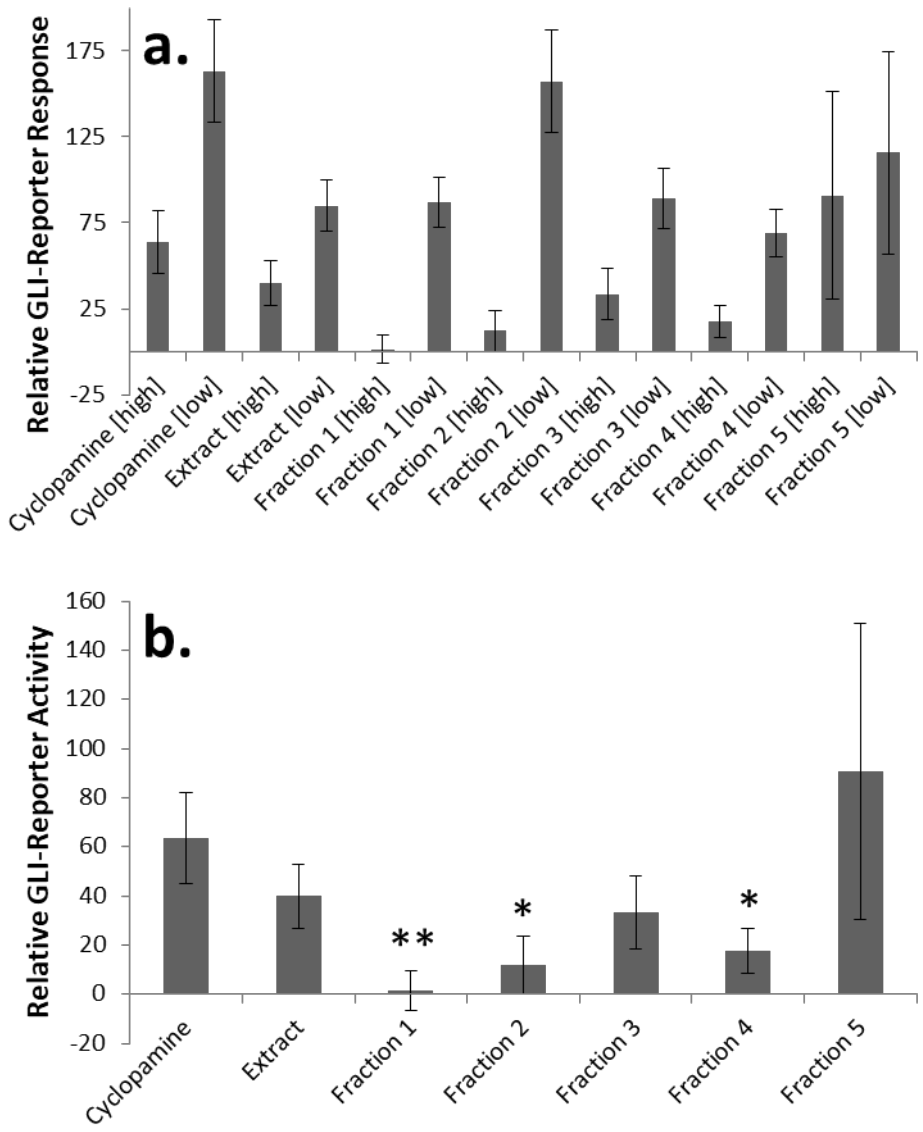

Figure 3.3 Results from bioactivity assessment of cyclopamine, raw extract, and five fractions. a. Both high (1:200 dilution for extract and 1:20 dilution for fractions) and low (1:1000 dilution for extract and 1:100 dilution for fractions) concentrations of each treatment. b. Only high concentrations of each treatment, where * signifies P < 0.05 and ** signifies P < 0.01 as compared to 0.1 µM cyclopamine.

Figure 3.3 a shows that for every individual treatment, the higher concentration always showed greater Gli-reporter inhibition than the lower concentration. In addition, none of the low concentration treatments were more potent than the high concentration of cyclopamine standard. For **Figure 3.3 b** cyclopamine (final concentration of 0.1 µM) had

relative Gli-reporter activity of 63.56 ± 18.40, while fraction 1 was 1.376 ± 8.231, fraction 2 was 12.04 ± 11.66, and fraction 4 was 17.73 ± 9.10. Fraction 1 was significantly more suppressive of the Hh signaling pathway than cyclopamine ($P<0.01$) as were fractions 2 and 4 ($P<0.05$).

Table 3.1 Summary of MS data for Fractions 1 (blue), 2 (purple), and 4 (pink)

	Elution Time	m/z	Formula	Identity
Fraction 1	15.3	572.3	$C_{33}H_{49}NO_7$	Veratrosine?
	15.7	574.3	$C_{33}H_{51}NO_7$	Cycloposine?
	16.0	572.3	$C_{33}H_{49}NO_7$	Isomer of veratrosine?
	16.5	574.3	$C_{33}H_{51}NO_7$	Isomer of cycloposine?
Fraction 2	15.7	574.3	$C_{33}H_{51}NO_7$	Cycloposine?
	16.0	572.3	$C_{33}H_{49}NO_7$	Isomer of veratrosine?
	16.1	576.4	$C_{33}H_{53}NO_7$	?
	16.2	430.3	$C_{27}H_{43}NO_3$	Tetrahydrojervine?
	16.5	574.3	$C_{33}H_{51}NO_7$	Isomer of cycloposine?
	16.6	618.4	$C_{35}H_{55}NO_8$	?
	16.9	578.4	$C_{33}H_{55}NO_7$	?
	17.1	428.3	$C_{27}H_{41}NO_3$	Dihydrojervine?
	17.2	472.3	$C_{29}H_{45}NO_4$	?
	17.3	620.4	$C_{35}H_{57}NO_8$	?
	17.4	414.3	$C_{27}H_{43}NO_2$	Etioline?
	17.5	428.3	$C_{27}H_{41}NO_3$	Isomer of dihydrojervine?
	17.6	410.3	$C_{27}H_{39}NO_2$	Veratramine
Fraction 4	18.5	412.3	$C_{27}H_{41}NO_2$	Cyclopamine
	18.8	410.3	$C_{27}H_{39}NO_2$	Isomer of veratramine?
	18.9	412.3	$C_{27}H_{41}NO_2$	Isomer of cyclopamine?
	19.2	474.3	$C_{29}H_{47}NO_4$	?
	19.4	456.3	$C_{29}H_{45}NO_3$	?
	19.5	414.3	$C_{27}H_{43}NO_2$	Isorubijervine?
	19.8	458.3	$C_{29}H_{47}NO_3$	Muldamine?

Table 3.1 shows a summary of the MS data presented in Chapter 2 for fractions 1, 2, and 4. Fraction 1 contained four alkaloids hypothesized to be veratrosine, cycloposine,

and an isomer of each. Fraction 2 had 10 identified compounds: cycloposine, its isomer, the isomer of veratrosine, and tetrahydrojervine are all suspected to be present, veratramine was confirmed, and five molecules remain unknown. Cyclopamine has a verified presence within fraction 4, and its isomer, the isomer of veratramine, and muldamine are all suspected to be present, along with two unknown compounds.

Discussion

The data tabulated in **Table 3.1** identifies the compounds present in each alkaloid fraction that demonstrate better activity at Gli-reporter inhibition than cyclopamine. Fraction 1 demonstrated the best Gli-reporter suppression, with only four alkaloids, cycloposine, veratrosine, and their isomers. While the activity of cycloposine and veratrosine has been determined to be lower than cyclopamine, little is known about the activity of their isomers.[74] With regard to fraction 2, there were at least thirteen different alkaloids, including veratramine, a known Hh signaling inhibitor, and it was not nearly as potent. Fraction 2 also contained every alkaloid present within fraction 1 except for veratrosine. **Figure 3.4** shows the relative concentrations of fractions 1, 2, and 4 compared to 0.1 μM cyclopamine as derived from the areas of the relevant alkaloid peaks in the total ion chromatograms. Fraction 4 contained an abundance of cyclopamine, but despite that, it too was not as effective as fraction 1. Cyclopamine was found to be present across three of the five fractions collected from the extract, so the cyclopamine concentration in any give fraction may have been low. Fraction 4 contained six alkaloids in addition to cyclopamine, including muldamine, which does not have a well-defined Hh signaling effect.

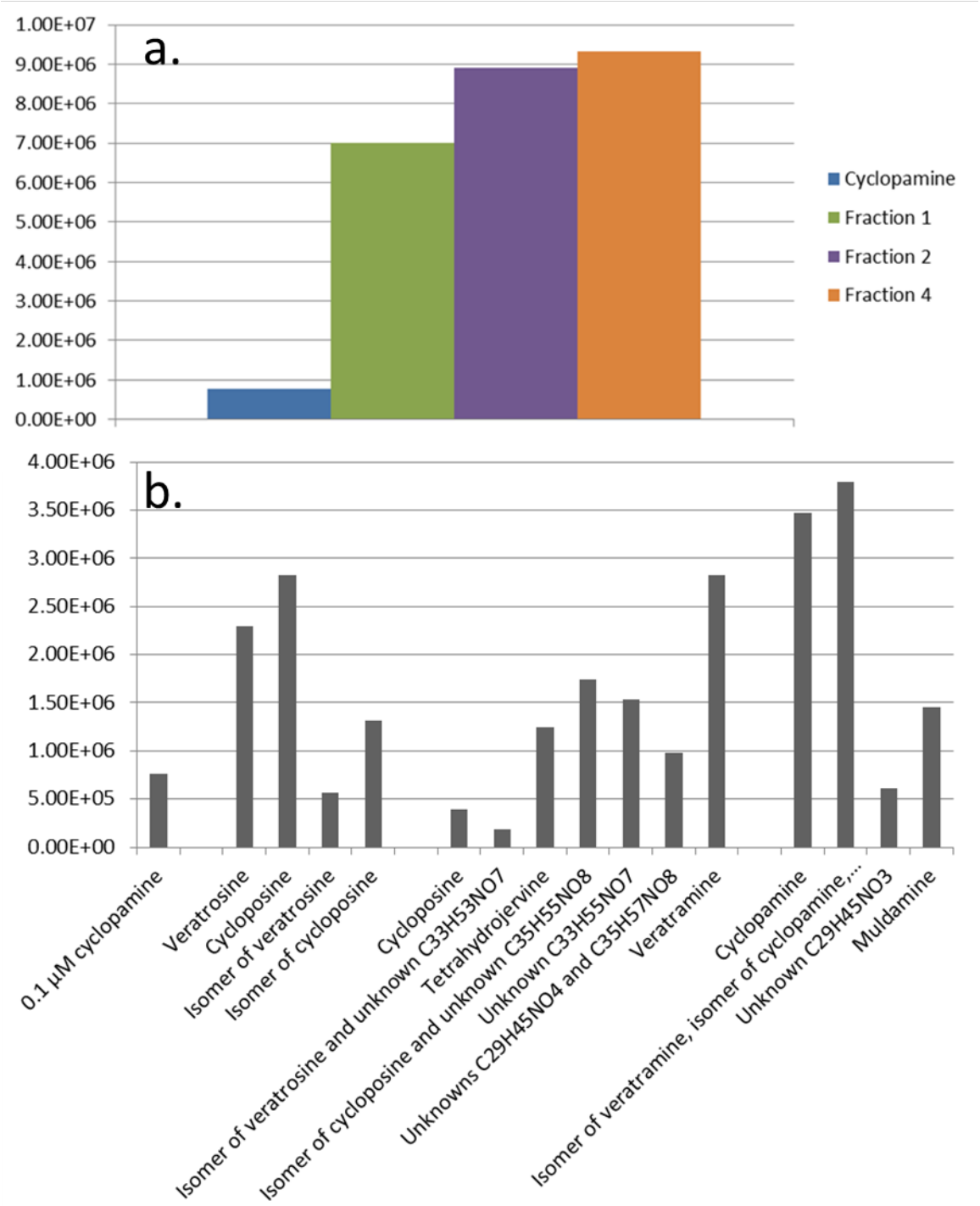

Figure 3.4 Relative concentrations of fractions 1, 2, and 4 as compared to 0.1 µM cyclopamine.

Figure 3.4 a shows that the sum of the relevant alkaloid content within individual fractions 1, 2, and 4 exceeds that of 0.1 µM cyclopamine. Cyclopamine has already been

established as having the greatest Hh pathway suppression of any alkaloid within *V. californicum.*[74] In fraction 1, veratrosine and cycloposine are ineffective in this regard, and thus their isomers at must be contributing to the Hh signaling inhibition.[74] **Figure 3.4 b** shows the isomers' relative content is 5.65 x 10^5 for the isomer of veratrosine and 1.31 x 10^6 for the isomer of cycloposine. The isomer of veratrosine had an m/z of 572.3, an elution time of 16.0 minutes, and a predicted molecular formula of $C_{33}H_{49}NO_7$. The isomer of cycloposine had an m/z of 574.3, an elution time of 16.5 min, and a predicted molecular formula of $C_{33}H_{51}NO_7$. Based upon their low, relative concentration within fraction 1, and fraction 1 being the most potent, these two alkaloids are most worthy of pursuit for further characterization.

Materials and Methods

See Materials and Methods section of Chapter 2.

Cell Culture

Shh-Light II cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with added geneticin (0.4 mg/mL), Zeocin™ (0.15 mg/mL from Invitrogen), 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. The conditions for cell growth were as follows: 37 °C, atmosphere of 5% CO_2, and 100% relative humidity. The Dual-Luciferase® Reporter Assay System was used on four different plates. The cells for first three plates were all generated from the same stock, where the first was passaged five times, the second eight times, and the third eleven times, and the fourth plate's cells were generated from a different cell stock that had been passaged twice. 10,000 cells in 100 µL growth media were added to each well of the 96-well plate and allowed to grow to at least 80% confluency before the media was swapped

for DMEM with added 0.5% FBS, 0.5% penicillin-streptomycin, and 2.5-5.0 µg N-terminal mouse recombinant Shh (R&D Systems, Minneapolis, MN, USA). Each control and treated well contained a final concentration of 1% ethanol. Forty eight hours after treatment, Gli activity was assayed using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) by measuring the luminescence from the cells with a BioTek Synergy H1m Microplate reader. In order to induce luminescence the cells were first lysed, then 100 µL of Luciferase Assay Substrate in Luciferase Assay Buffer II (LAR II) was added to each well, and the firefly luciferase luminescence measurement was recorded. Then 100 µL of Stop & Glo® Substrate in Stop & Glo® Buffer (Stop & Glo® Reagent) was dispensed into each well and the *Renilla* luciferase luminescence was measured. The relative response ratio (RRR) was calculated as described in the Dual-Luciferase® Reporter Assay System manual in order to display the results. Each experiment was performed in triplicate across four plates for a total of 12 trials.

CHAPTER FOUR: CONCLUSION AND FUTURE DIRECTIONS

In the pursuit of more effective and on-target cancer therapeutics, the alkaloids contained within *V. californicum* are worthy of natural product drug discovery investigation. Currently, there are six alkaloids that have been identified and characterized, twelve suspected, and at least seven alkaloids that have been detected, but remain to be identified from the root and rhizome extract of *V. californicum*. Based off of the literature some additional predictions can be made concerning the identities of the seven detected alkaloids marked with question marks in **Table 2.1**.

The alkaloid detected at 16.1 minutes in fraction 2 with a predicted molecular formula of $C_{33}H_{53}NO_7$ may be isorubijervosine. It has been previously extracted from *V. eschscholtzii*.[39] Isorubijervosine is the glycosylated version of isorubijervine, one of the six known alkaloids in *V. californicum*.[71] The glycosylated versions of cyclopamine and veratramine (cycloposine and veratrosine) also have a confirmed presence within *V. californicum*, lending further support to this prediction.[71] No hedgehog signaling bioactivity assessments of this molecule have been reported in the literature.

At 16.6 minutes, also within fraction 2, an alkaloid with the predicted molecular formula $C_{35}H_{55}NO_8$ was detected. Isoveralosine matches the molecular formula and has been previously identified within *V. grandiflorum*[119] and *V. lobelianum*[120]. It has been tested for hedgehog signaling activity using the same Shh-Light II cell line and Dual-Luciferase® Reporter Assay, but was found to be inactive.[119]

A molecule was detected at 16.9 minutes in fraction 2 with a predicted molecular formula of $C_{33}H_{55}NO_7$. This is consistent with the molecular formula for Stenophylline B 3-O-β-D-glucopyranoside, which has been previously extracted from *V. taliense*.[121,122] It has demonstrated antifungal activity, but no work has been done concerning the hedgehog signaling pathway.[122]

At 17.2 minutes within fraction 2, an alkaloid was detected with the predicted molecular formula $C_{29}H_{45}NO_4$. This is thought to be (20R,25R)-12b-O-acetyl-20β-hydroxyisoverazine, which has been previously extracted from *V. grandiflorum*.[119] Using the same Shh-Light II cell line and Dual- Luciferase® Reporter Assay as described previously, it showed suppression of hedgehog signaling.[119]

The last unknown alkaloid found within fraction 2 was reported at 17.3 minutes and has a predicted molecular formula of $C_{35}H_{57}NO_8$. (3β,5α,16α,20S)-16-(acetyloxy)-20-[(5S)-3,4,5,6-tetrahydro-5-methyl-2-pyridinyl]-β-D-glucopyranoside has the same molecular formula, but it has not been previously identified within the *Veratrum* genus. It has been extracted from *Solanum pseudoquina*, and it has unknown activity in relation to the hedgehog signaling pathway.[123]

The alkaloid detected at 19.2 minutes within fraction 4 has the predicted molecular formula $C_{29}H_{47}NO_4$. The molecule baikeidine has the same formula and has been identified within *V. grandiflorum*. The bioactivity has not been tested for this alkaloid.[124]

Lastly, at 19.4 minutes in fraction 4 a molecule was extracted with the predicted molecular formula $C_{29}H_{45}NO_3$. Veralosinine has the same molecular formula and has

been identified in *V. lobelianum*. It has demonstrated favorable inhibition of multidrug resistance, but has not been tested for hedgehog signaling inhibition.[125]

The crude extract of *V. californicum*, when separated by time into five fractions, provides bioactivity efficacy superior to 0.1 μM cyclopamine alone for fractions 1, 2, and 4. The alkaloid composition of these fractions consists of between four and thirteen components. Bioactivity testing of isolated alkaloids from fractions 1, 2, and 4 constitute the logical next step to measure performance with the Shh-Light II cell line, followed by subsequent testing of the most potent Hh suppressors in BCC cells. The most effective inhibitors would need to be isolated in sufficient quantity to be characterized by nuclear resonance spectroscopy in order to determine their molecular structure. Any newly characterized alkaloids have the potential to be vetted as chemotherapeutics or precursors to chemotherapeutics for cancers involving Hh signaling.

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APPENDIX A

Figure S1: Structures of *Veratrum* spp. steroidal alkaloids, excluding those found in *V. californicum*

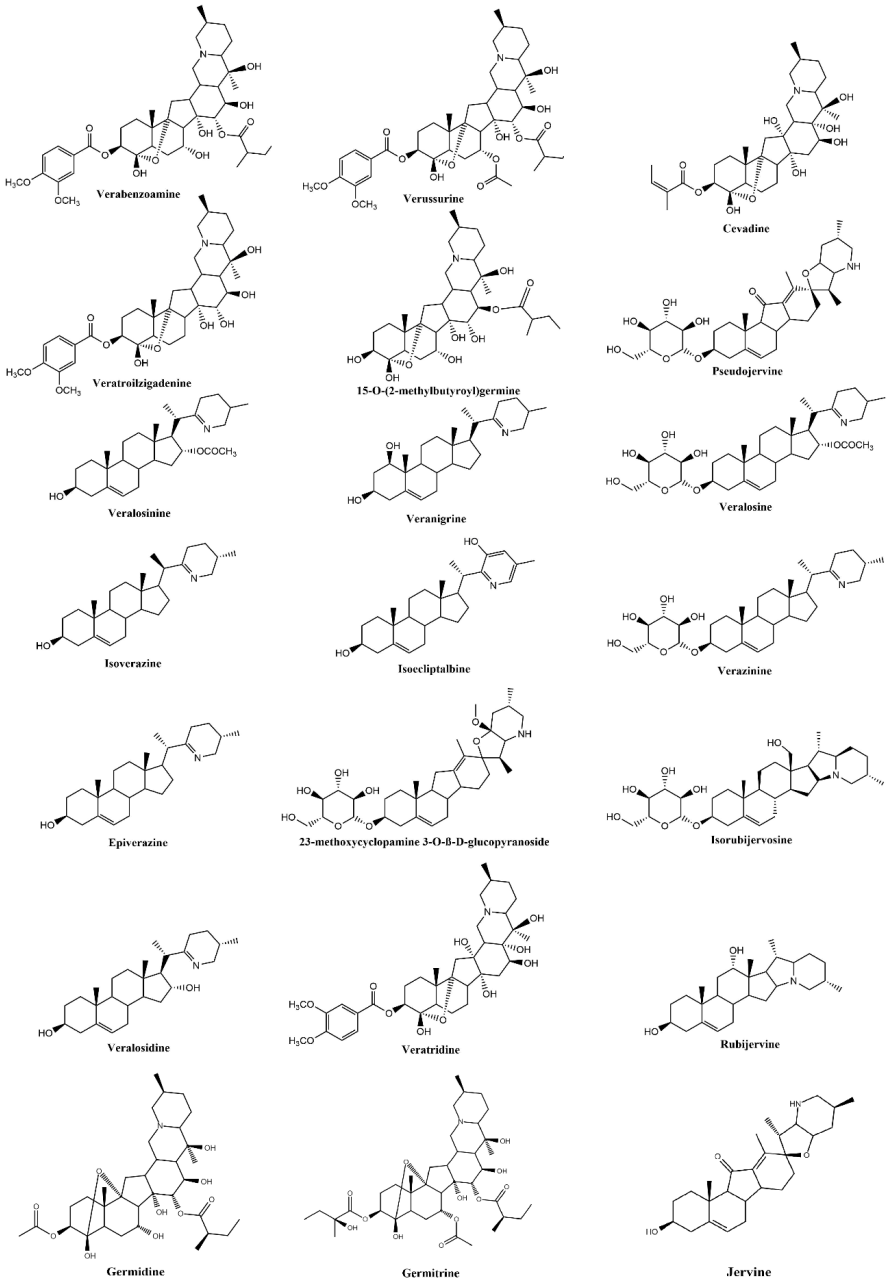
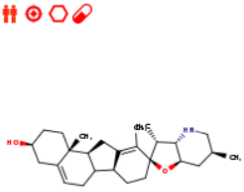


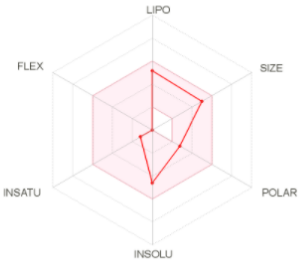
Figure A.1 Figure S1. Structures of Veratrum spp. steroidal alkaloids, excluding those found in V. californicum[1]

APPENDIX B

Further Assessment of Bioactive Alkaloids from *Veratrum californicum* found in Table 1.4

Molecule 1





SMILES O[C@H]1CC[C@]2(C(=CCC3[C@@H]2CC2=C(C)[C@]4(CC[C@@H]32)O[C@H]2[C@H]([C@H]4C)NC[C@H](C2)C)C1)C

Physicochemical Properties	
Formula	C27H41NO2
Molecular weight	411.62 g/mol
Num. heavy atoms	30
Num. arom. heavy atoms	0
Fraction Csp3	0.85
Num. rotatable bonds	0
Num. H-bond acceptors	3
Num. H-bond donors	2
Molar Refractivity	127.01
TPSA	41.49 Å²

Lipophilicity	
Log $P_{o/w}$ (iLOGP)	4.25
Log $P_{o/w}$ (XLOGP3)	3.52
Log $P_{o/w}$ (WLOGP)	4.62
Log $P_{o/w}$ (MLOGP)	4.42
Log $P_{o/w}$ (SILICOS-IT)	3.96
Consensus Log $P_{o/w}$	4.16

Water Solubility	
Log *S* (ESOL)	-4.61
Solubility	1.01e-02 mg/ml ; 2.46e-05 mol/l
Class	Moderately soluble
Log *S* (Ali)	-4.08
Solubility	3.46e-02 mg/ml ; 8.41e-05 mol/l
Class	Moderately soluble
Log *S* (SILICOS-IT)	-4.79
Solubility	6.72e-03 mg/ml ; 1.63e-05 mol/l
Class	Moderately soluble

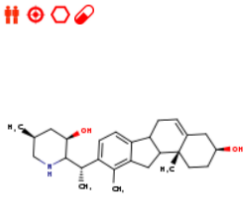
Pharmacokinetics	
GI absorption	High
BBB permeant	Yes
P-gp substrate	Yes
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeation)	-6.31 cm/s

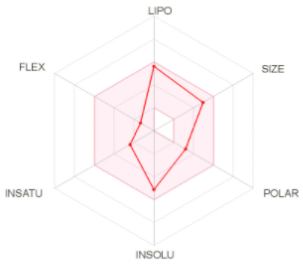
Druglikeness	
Lipinski	Yes; 1 violation: MLOGP>4.15
Ghose	No; 1 violation: #atoms>70
Veber	Yes
Egan	Yes
Muegge	Yes
Bioavailability Score	0.55

Medicinal Chemistry	
PAINS	0 alert
Brenk	1 alert: isolated_alkene
Leadlikeness	No; 2 violations: MW>350, XLOGP3>3.5
Synthetic accessibility	6.30

Figure B.1 Data on Cyclopamine from SwissADME [126]

Molecule 2





SMILES C[C@@H]1CNC([C@@H](C1)O)[C@H](c1ccc2c(c1C)CC1C2CC=C2[C@]1(C)CC[C@@H](C2)O)C

Physicochemical Properties	
Formula	C27H39NO2
Molecular weight	409.60 g/mol
Num. heavy atoms	30
Num. arom. heavy atoms	6
Fraction Csp3	0.70
Num. rotatable bonds	2
Num. H-bond acceptors	3
Num. H-bond donors	3
Molar Refractivity	127.88
TPSA	52.49 Å²

Lipophilicity	
Log $P_{o/w}$ (iLOGP)	4.12
Log $P_{o/w}$ (XLOGP3)	4.34
Log $P_{o/w}$ (WLOGP)	4.21
Log $P_{o/w}$ (MLOGP)	4.24
Log $P_{o/w}$ (SILICOS-IT)	4.76
Consensus Log $P_{o/w}$	4.33

Water Solubility	
Log *S* (ESOL)	-5.13
Solubility	3.04e-03 mg/ml ; 7.42e-06 mol/l
Class	Moderately soluble
Log *S* (Ali)	-5.16
Solubility	2.85e-03 mg/ml ; 6.96e-06 mol/l
Class	Moderately soluble
Log *S* (SILICOS-IT)	-5.60
Solubility	1.02e-03 mg/ml ; 2.50e-06 mol/l
Class	Moderately soluble

Pharmacokinetics	
GI absorption	High
BBB permeant	Yes
P-gp substrate	Yes
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeation)	-5.72 cm/s

Druglikeness	
Lipinski	Yes; 1 violation: MLOGP>4.15
Ghose	Yes
Veber	Yes
Egan	Yes
Muegge	Yes
Bioavailability Score	0.55

Medicinal Chemistry	
PAINS	0 alert
Brenk	1 alert: isolated_alkene
Leadlikeness	No; 2 violations: MW>350, XLOGP3>3.5
Synthetic accessibility	5.19

Figure B.2 Data on Veratramine from SwissADME [126]

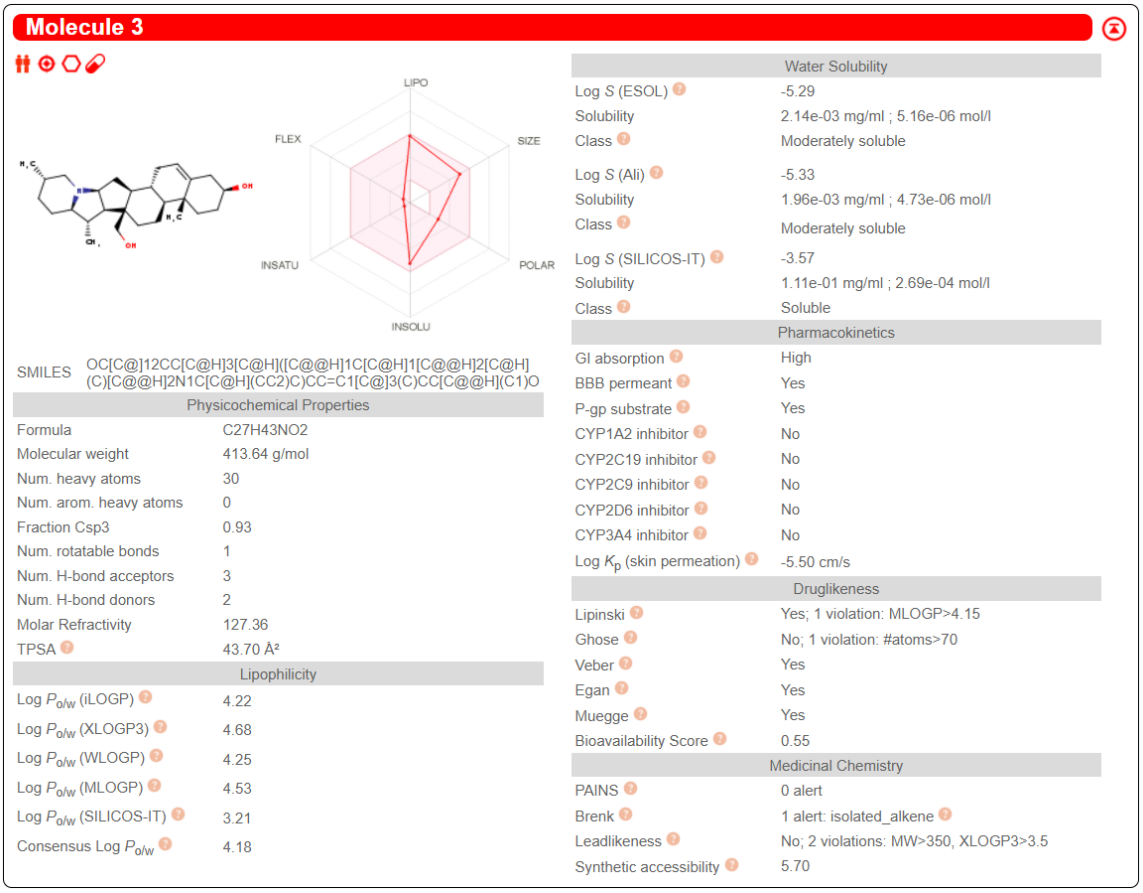


Figure B.3 Data on Isorubijervine from SwissADME [126]

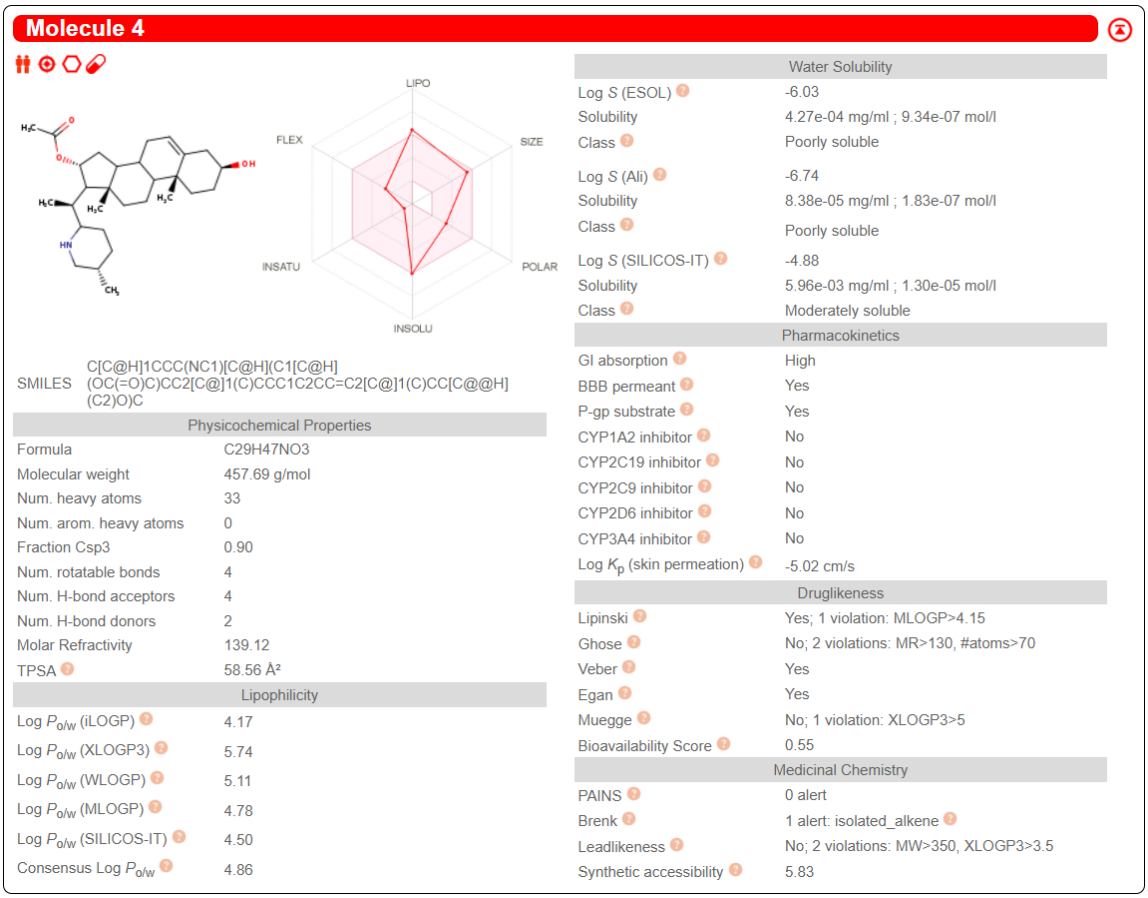


Figure B.4 Data on Muldamine from SwissADME [126]

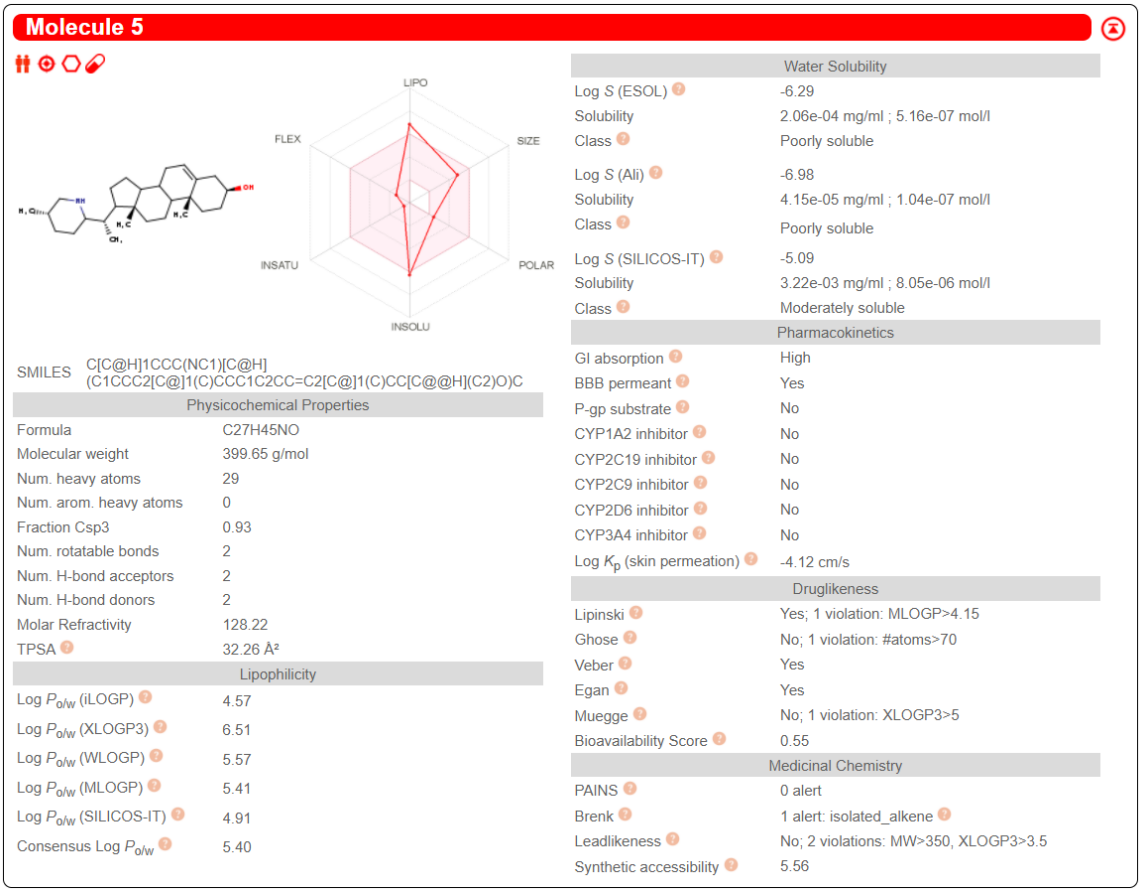
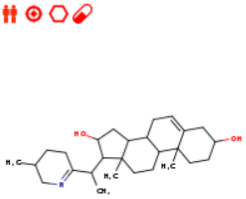
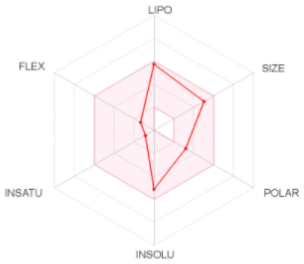


Figure B.5 Data on Verazine from SwissADME [126]

Molecule 6





SMILES CC1CCC(=NC1)C(C1C(O)CC2C1(C)CCC1C2CC=C2C1(C)CCC(C2)O)C

Physicochemical Properties	
Formula	C27H43NO2
Molecular weight	413.64 g/mol
Num. heavy atoms	30
Num. arom. heavy atoms	0
Fraction Csp3	0.89
Num. rotatable bonds	2
Num. H-bond acceptors	3
Num. H-bond donors	2
Molar Refractivity	129.67
TPSA	52.82 Å²

Lipophilicity	
Log $P_{o/w}$ (iLOGP)	4.13
Log $P_{o/w}$ (XLOGP3)	4.54
Log $P_{o/w}$ (WLOGP)	5.02
Log $P_{o/w}$ (MLOGP)	4.42
Log $P_{o/w}$ (SILICOS-IT)	5.08
Consensus Log $P_{o/w}$	4.64

Water Solubility	
Log *S* (ESOL)	-5.13
Solubility	3.05e-03 mg/ml ; 7.37e-06 mol/l
Class	Moderately soluble
Log *S* (Ali)	-5.37
Solubility	1.76e-03 mg/ml ; 4.25e-06 mol/l
Class	Moderately soluble
Log *S* (SILICOS-IT)	-4.49
Solubility	1.34e-02 mg/ml ; 3.25e-05 mol/l
Class	Moderately soluble

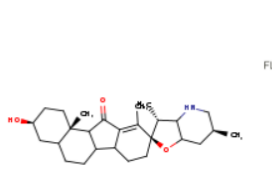
Pharmacokinetics	
GI absorption	High
BBB permeant	Yes
P-gp substrate	Yes
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeation)	-5.60 cm/s

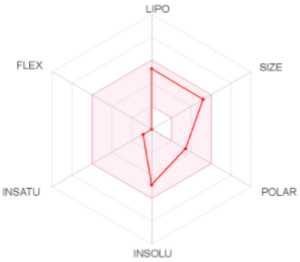
Druglikeness	
Lipinski	Yes; 1 violation: MLOGP>4.15
Ghose	No; 1 violation: #atoms>70
Veber	Yes
Egan	Yes
Muegge	Yes
Bioavailability Score	0.55

Medicinal Chemistry	
PAINS	0 alert
Brenk	1 alert: isolated_alkene
Leadlikeness	No; 2 violations: MW>350, XLOGP3>3.5
Synthetic accessibility	6.26

Figure B.6 Data on Etioline from SwissADME [126]

Molecule 7





SMILES O[C@H]1CC[C@]2(C(C1)CCC1C2C(=O)C2=C(C)[C@]3(CCC12)OC1C([C@H]3C)NC[C@H](C1)C)C

Physicochemical Properties	
Formula	C27H41NO3
Molecular weight	427.62 g/mol
Num. heavy atoms	31
Num. arom. heavy atoms	0
Fraction Csp3	0.89
Num. rotatable bonds	0
Num. H-bond acceptors	4
Num. H-bond donors	2
Molar Refractivity	127.69
TPSA	58.56 Å²

Lipophilicity	
Log $P_{o/w}$ (iLOGP)	3.85
Log $P_{o/w}$ (XLOGP3)	3.73
Log $P_{o/w}$ (WLOGP)	3.88
Log $P_{o/w}$ (MLOGP)	3.57
Log $P_{o/w}$ (SILICOS-IT)	3.66
Consensus Log $P_{o/w}$	3.74

Water Solubility	
Log *S* (ESOL)	-4.84
Solubility	6.16e-03 mg/ml ; 1.44e-05 mol/l
Class	Moderately soluble
Log *S* (Ali)	-4.65
Solubility	9.54e-03 mg/ml ; 2.23e-05 mol/l
Class	Moderately soluble
Log *S* (SILICOS-IT)	-4.68
Solubility	8.93e-03 mg/ml ; 2.09e-05 mol/l
Class	Moderately soluble

Pharmacokinetics	
GI absorption	High
BBB permeant	Yes
P-gp substrate	Yes
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeation)	-6.26 cm/s

Druglikeness	
Lipinski	Yes; 0 violation
Ghose	No; 1 violation: #atoms>70
Veber	Yes
Egan	Yes
Muegge	Yes
Bioavailability Score	0.55

Medicinal Chemistry	
PAINS	0 alert
Brenk	0 alert
Leadlikeness	No; 2 violations: MW>350, XLOGP3>3.5
Synthetic accessibility	6.34

Figure B.7 Data on Dihydrojervine from SwissADME [126]