# INVESTIGATIONS OF SALVINORIN A: SYNTHETIC ISOLATION, QUANTIFICATION, AND IN VIVO CHARACTERISTICS

BY

MICHAEL J. CASPERS

Submitted to the graduate program in Medicinal Chemistry and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

_____
Chairperson: Dr. Thomas E Prisinzano

_____
Dr. Barbara N. Timmermann

_____
Dr. Todd D. Williams

Date Defended: 10-8-2013

The Thesis Committee for Michael J. Caspers
certifies that this is the approved version of the following thesis:

INVESTIGATIONS OF SALVINORIN A: SYNTHETIC ISOLATION, QUANTIFICATION, AND IN VIVO CHARACTERISTICS

_____

Chairperson: Thomas E Prisinzano

Date approved: 10-8-2013

## ABSTRACT

While many ligands are known to interact with the opioid receptor system, most can be traced back to the common morphine scaffold. In several cases ligands have been developed that are selective for either the mu, kappa, or delta opioid receptors. Selective kappa opioid receptor agonists and antagonists have shown potential to be used to decrease cocaine self-administration and be utilized in the treatment of relapse. The emergence of salvinorin A as a novel scaffold, that selectively interacts with the kappa opioid receptor, retains therapeutic potential with a reduced side effect profile to the current treatments. Traditional mu opioid ligands, such as morphine, have life threatening side effects such as respiratory depression and constipation among other drawbacks like tolerance and dependence.

To fully investigate the potential of salvinorin A as a viable alternative for therapeutic treatment it must be isolated in high purity for pharmacological evaluation. In efforts to isolate high purity salvinorin A, a reactive handle was generated that was shown to undergo cycloaddition to the furan ring of salvinorin A. This handle was developed using the reactivity limitations that were also investigated for the Diels-Alder cycloaddition of electron deficient alkenes and alkynes with the salvinorin A furan ring.

In order to understand the unique mode of interaction salvinorin A imparts on the opioid receptor system, investigations into the pharmacological profile of salvinorin A were explored. Analytical methods were developed for the identification and quantification of salvinorin A from non-human primate cerebrospinal fluid and human plasma. This method was then exploited to develop a time-course graph for the measurement of *in vivo* concentrations of salvinorin A in various biological fluids which could be correlated to subjective and biological observations.

## ACKNOWLEDGEMENTS

Though too numerous to name all of them, there are many people that have supported me and my work during my graduate studies. I would first like to thank all of my lab mates past and present, you were the reason I could crawl out of bed and come to work every day. Anthony, thanks for all the life conversations and the occasional cigar and glass of scotch , Tammi, thanks for the weekend Wal-Mart trips and showing me how to use English in ways I never knew possible, Kim, thanks for clearly defining the word "crazy" for me, Andrew, thanks for teaching me how to write proper rap lyrics, Karrie, thanks for solving all my computer problems even when you didn't want to, Rachel, thanks for all the delicious baked goods, and Mark, thanks for keeping me fully supplied with entertainment. To the post-docs I have worked with, Chris and Chad, you always provided sound chemical and biological advice whenever I had questions.

To all the faculty and staff in the medicinal chemistry department here at the University of Kansas, thanks for welcoming me into your community and helping me develop as a scientist, all of your support and advice was very much appreciated. I would like to thank Dr. Todd Williams for teaching me how to be independent on a mass spectrometer and for the numerous discussions on theory and analytical calculations I constantly pestered him with. Thanks to Dr. Justin Douglas for his extensive help in NMR structure elucidation and for taking the time to try some very unique NMR experiments when he didn't have to. I would like to thank my committee members Drs Thomas Prisinzano, Barbara Timmermann, and Todd Williams for taking time out of their busy schedules to read my thesis and helping me take the next step in my life. A special thanks is in order for my advisor Dr. Thomas Prisinzano, thank you for all your support, patience, encouragement, and understanding, thanks for keeping me well-fed on lab outings and allowing me to get to know your wonderful family, I will always be thankful for everything you did for me.

I must thank all the friends I have made during my graduate studies, you helped me remember to stay human and I will never forget the memories we made. To all the friends I left behind since moving to Kansas, reliving old times over the phone was often what I needed and I hope we build new memories soon. I of course cannot forget my family who has always been there for me. To my father who passed just as my time here began, I think about you every day, thanks for always believing in me and for teaching me how to shoot strait. To my mother, thanks for always being supportive in everything I have done and being patient when I got too busy to call home. To my grandma, thanks for always praying for me, someone had to do that and it undoubtedly helped me. To my brother and uncles, the late night dice games in the fish house over Christmas where more of a relief than you could ever imagine, regardless if any fish where biting.

## TABLE OF CONTENTS

## LIST OF ABBREVIATIONS

5-HT ..... Serotonin

ACSF ..... Artificial cerebrospinal fluid

APCI ..... Atmospheric pressure chemical ionization

APZ-OAV ..... Altered states of consciousness questionaire

AUC ..... Area under the curve

cAMP ..... Cyclic adenosine monophosphate

CHO ..... Chineese hamster ovary

CID ..... Collision induced dissociation

CNS ..... Central nervous system

CNS ..... Central nervous system

COSY ..... Correlation spectroscopy

CSF ..... Cerebrospinal fluid

DMSO ..... Dimethylsulfoxide

DMT ..... Dimethyltryptamine

DOB ..... 2,5-dimethoxy-4-bromoamphetamine

DOP ..... Delta opioid

EEG ..... Electroencephalography

FLAG-hKOP ..... FLAG tagged human kappa opioid

GC x GC-ToFMS ..... Two dimensional gas chromatography time of flight mass spectrometry

GNTI ..... 5’-guanidinonaltrindole

GPCR ..... G-protein coupled receptor

HPLC ..... High performance liquid chromatography

KOP ..... Kappa opioid

LC-MS ..... Liquid chromatography tandem mass spectrometry

LOD ..... Limit of detection

LOQ ..... Limit of quantification

LSD ..... Lysergic acid diethylamide

MOP ..... Mu opioid

MRM ..... Multiple reaction monitoring

HMBC ..... Heteronuclear multiple bond coherence

HSQC ..... Heteronuclear single quantum correlation

NIMH ..... National institute of mental health

NMDA ..... *N*-methyl-D-aspartate

NMR ..... Nuclear magnetic resonance

PANAS ..... Positive and negative affect scale

PANSS ..... Positive and negative syndrome scale

PEG ..... Polyethylene glycol

PET ..... Positron emission tomography

Pgp ..... Permeability glycoprotein

ROMP ..... Ring opening metathesis polymerization

SAR ..... Structure activity relationship

SDEQ ..... Subjective drug effect questionnaire

SIM ..... Selected ion monitoring

SPE ..... Solid phase extraction

$t_r$ ..... Retention time

$t_{1/2}$ ..... Half-life

THF ..... Tetrahydrofuran

TLC ..... Thin layer chromatography

TLC-GC/MS .....Thin layer chromatography gas chromatography tandem mass spectrometry

VAS.....Visual analogue scale

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF SCHEMES

# CHAPTER I

## Natural Product History

Natural products, single chemical entities produced by a living organism, have been around longer than mankind itself. Having the advantage of time, natural products have had hundreds of millions of years to evolve before intelligent life began recognizing their use as medicinal agents.[1] Since early prehistoric times natural products and their extracts have been used in medicine and often as the only known means of treatment.[2-4] After thousands of years of using natural products, their utility and traditional uses have been passed along first by word of mouth between communal members and later becoming systematically written into books for the whole world to share, making once locally known remedies readily available. For instance, it is now common knowledge that extracts or teas made from chamomile can help relax and remove tension, ginger can be used to ease nausea, peppermint will soothe indigestion, and that bacopa will reduce stress and symptoms of depression. The use of natural products for medical purposes in fact includes a wide variety of sources including ginger, lemon balm, catnip, rosemary, aloe, green tea, and sage.[5, 6]

As civilization advanced, we looked first to natural products as treatments, then began eventually isolating their individual active components for more effective treatments.[7] From these isolated compounds, a large percentage of commonly used ancient treatments were found to contain basic nitrogen atoms, becoming known as plant alkaloids.[3] These alkaloids consisted of common compounds such as atropine, nicotine, cocaine, ephedrine, tubocurarine, quinine, and

morphine (**Figure 1**). In today's scientific community many of these compounds have been investigated not only for their therapeutic drug potential, but also to investigate the physiological mechanisms by which they operate. Even with today's scientific advances, over 75% of the world population utilizes natural or traditional treatments for medicinal therapeutics, with a strong popularity remaining even in industrialized areas of the world.[8]

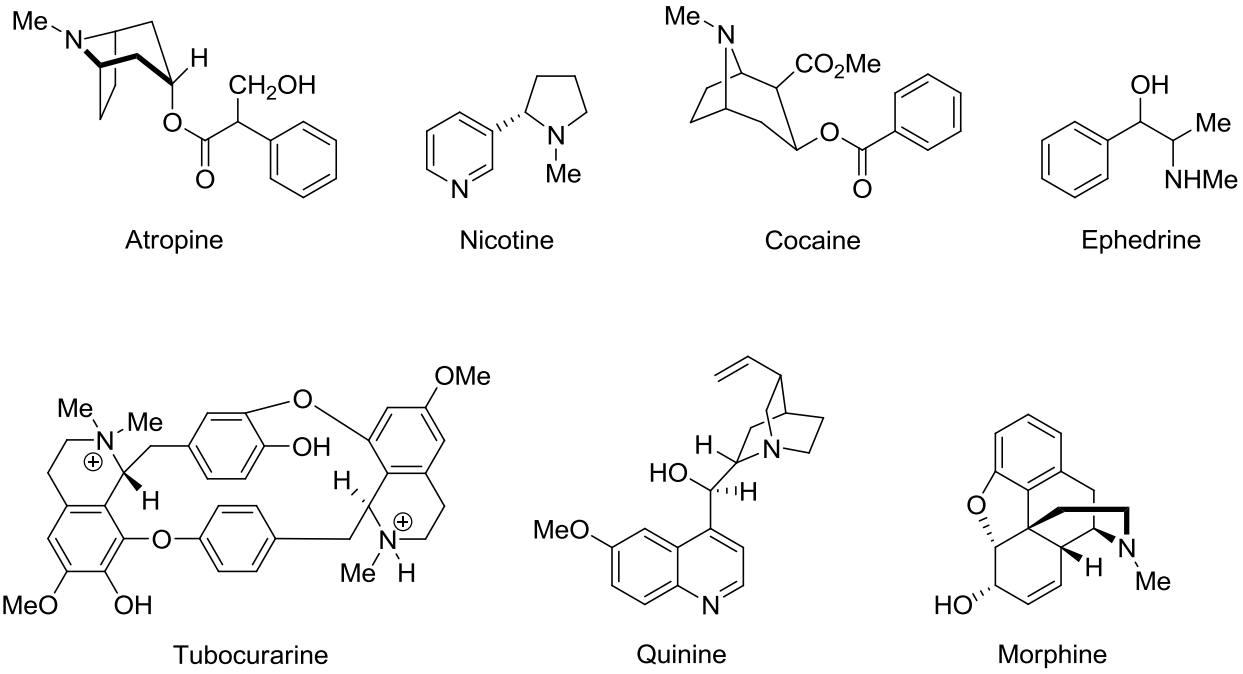



**Figure 1:** Chemical strutures of aklaloid natural products

With their widespread use in traditional medicines and remedies, natural products have vastly influenced the modern pharmaceutical industry, helping shape it into what we know today. The influence that natural products have on the modern pharmaceutical industry has resulted in over half of all modern pharmaceuticals being derived from or based on a natural product.[9, 10] From 1981 to 2010 approximately 70% of the top selling drugs were natural products or inspired by natural products, having a place in nearly every therapeutic area.[9-11] While nature is undisputedly

a great source in the search for biologically active agents, the complexity of natural products can often lead to many problems with their development into usable agents. These very hurdles that encompass natural products on a whole are often times the same reasons which attract scientists to them. Organic chemists have struggled in synthesizing even milligrams of a complex natural product which nature makes in large quantities with ease, while pharmacologists discover unique pathways after a natural product illicits a biological response.[12-14] This study of natural products has lead to developments in the field of organic chemistry by pushing past the limits of known chemical syntheses as well as in pharmacology by providing biochemical probes for the elucidation of pathways of disease.[9, 15] The overall advancement of science through the study of natural products has helped mold modern medicine by providing an assorted set of novel molecules with complex diversity.[7, 14, 16]

## Limitations of Natural Products

Historically natural products have been very successful as sources for leads and drug candidates in pharmaceutical industry.[7, 15] Even though a large percentage of new drugs today are either derived from or are natural products, many of the natural product-based research programs within large pharmaceutical industry are being closed.[16] Traditionally, the sets of compounds tested against drug targets were largely composed of natural products or compounds derived from natural products. However, these libraries were composed mainly of compounds closely related to one another, such as thousands of steroids or β-lactams. As the pharmaceutical industry began expanding its search for lead compounds and drug targets, it also began looking for increased structural diversity within the library of compounds it tested during screening. As the demands of industry increased for a more diverse set of scaffolds for screening, natural products, while still useful, represented only a small portion of the diversity possible and began

taking a back-seat to large synthetic libraries. It was much easier to synthesize structural diversity in-house than to comb through the growing political tensions regarding intellectual property rights with local governments of the foreign countries where many natural products are collected.[17, 18] Limited collection regions are also extended into the oceans where it can be difficult to collect or even explore for diversity beyond the upper ocean.[9] Even if an agreement is reached, the biomass required to isolate practical amounts of a single usable compound can often times be enormous and taxing on the sustainability of the species being harvested for large scale medicinal purposes (ex. Taxol).[19] Additionally, many companies terminated their natural product research programs due to limited financial returns on their investments and instead began searching for "blockbuster" drugs to recoup the investments required to push leads through development and into the clinic.[20]

Many other inherent problems also arise during progression of natural products into clinical trials. Once isolated and identified as leads, some natural products are unable to be reisolated for further evaluation. Occasionally the organism producing the compound of interest may not continue producing it due to environmental stresses, location of collection, or disappearance of the species. Natural products are often limited by structural complexity and can be difficult to be reduced or expanded into useful pharmaceutical agents. While natural products can often display impressive selectivity and potency for a given target, they can also exhibit an unimpressive pharmacokinetic profile in the areas of bioavailability, solubility, metabolic stability, and toxicity.[19, 21] When combined, all these factors determine whether or not a given compound can be used or developed into an agent with successful therapeutic utility. Previous examples have proven that semisynthetic modifications to complex natural products can improve their pharmacological properties.[21] However, with the highly complex structures often including

multiple chiral centers and the highly functionalized scaffolds many natural products possess, it can be very difficult and time consuming to perform even the most basic of chemical transformations during the lead development phase.

As previously mentioned, there are many challenges to natural product chemistry, however, it still remains an excellent source of both new structural diversity as well as leads for potential pharmaceutical agents.[18] Recent trends show pharmaceutical companies terminating their natural product programs while the number of new drugs reaching market that are natural product related remains relatively constant.[15] Despite the rise in combinatorial chemistry techniques, natural products as a whole still have a chance for a rebirth within large pharmaceutical companies as advances in scientific methodology catches up to the limitations of the natural product scaffolds.[14] While more sources of natural products are being explored the vast majority remain undiscovered and available for future applications in medical settings.[9, 16, 17] With the past success of natural products and their numerous unexplored sources, natural products surely have a place in the future of pharmaceutical research.

## Natural Products in Current Medicine

Today as many as 80% of all antibacterial and anti-cancer agents are either natural products or derived from natural product sources.[15, 22] These agents were mostly isolated from either plant sources or microbial life and have been key tools to improving the quality of life and increasing lifespan to anyone in need of treatment. While almost all areas of modern medicine have been influenced by natural products, the areas of antibacterial and anti-cancer agents have been influenced most due to their evolutionary role.[10] Thanks to natural products, many different drug classes for the treatment of cancer and bacterial infections have emerged. Some of these drug

classes include penicillins, tetracyclines, alkylating agents, cephalosporins, aminoglycosides, topoisomerase inhibitors, microtubulin stabilizers, and glycopeptides **(Figure 2)**.[9, 15, 23] The study of these drugs has not only improved treatment options and broadened our understanding of various disease states, but they have also helped identify new drug targets and pathways for further investigation of enhanced therapeutics. The improvement of cancer therapeutics has resulted in an increase in the five-year survival rate by 20% between the years of 1975 and 2004.

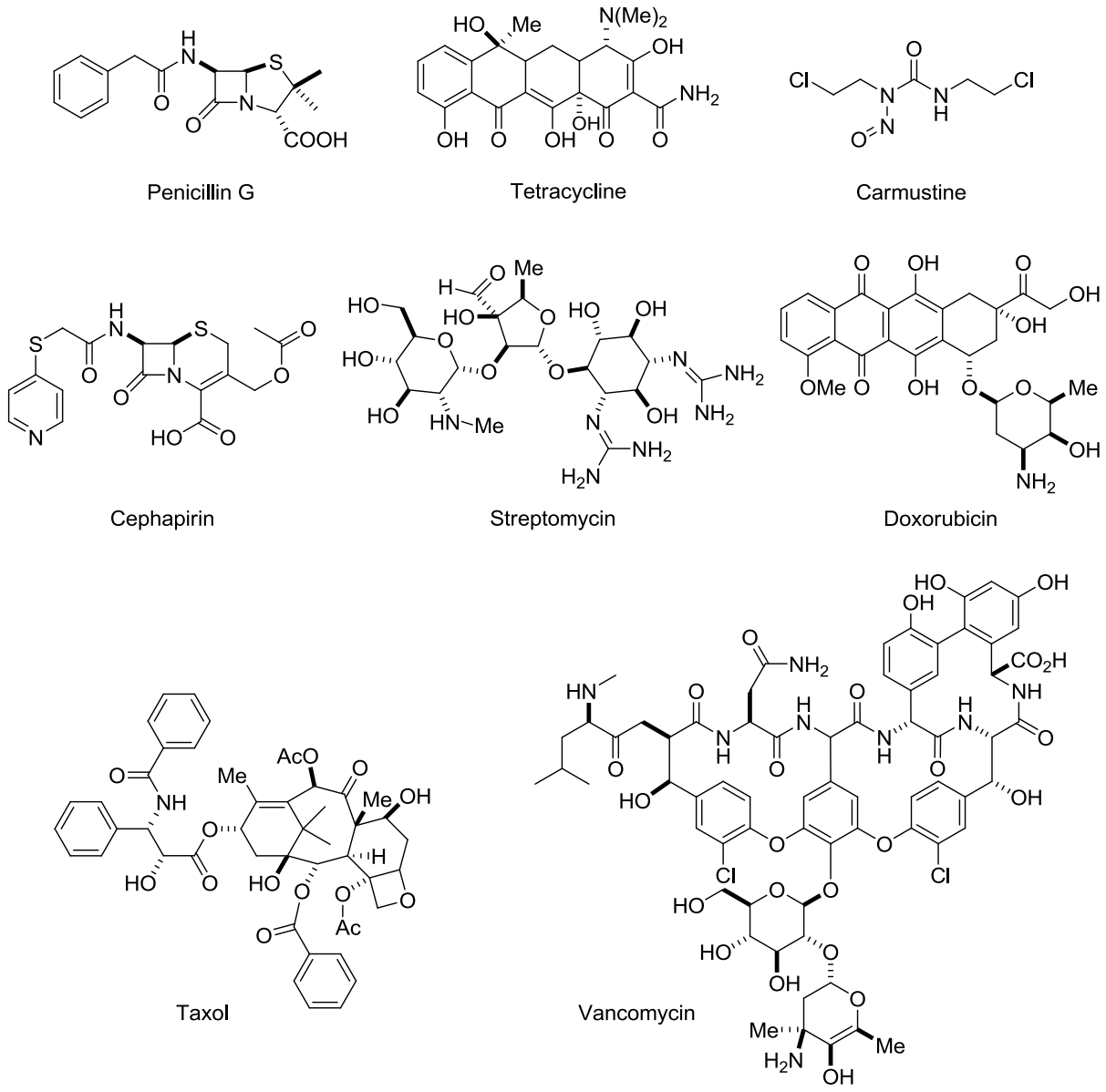



**Figure 2:** Examples of a penicillin (penicillin G), a tetracycline (tetracycline), an alkylating agent (carmustine), a cephalosporin (cephapirin), an aminoglycoside (streptomycin), a topoisomerase inhibitor (doxorubicin), a microtubulin stabilizer (taxol), and a glycopeptide (vancomycin)

## Opioids

One natural product that has drastically changed the pharmaceutical industry is the alkaloid morphine. This natural product was isolated from the latex of the opium poppy *Papaver*

*somniferum* L. (Papaveraceae) by Friedrich Serturner in 1805.[24, 25] Opium in general has been used for centuries and can be traced back to the Sumerians who cultivated and harvested it in the lower Mesopotamia region.[26] Extracts of the opium poppy were used to treat pain and induce sleep. However, once morphine was extracted and isolated from the crude extracts it was sold in pure form around 1827 by Merck. It wasn't until nearly 100 years later that the structure of morphine was proposed by Sir Robert Robinson in 1923.[27, 28] Even after a structure for morphine had been proposed its complexity was not confirmed by total synthesis for an additional 30 years in 1952 by Gates and Tschudi.[29] Much of the subsequent research and thousands of morphine analogues that were synthesized in the following years helped to mold the field of receptor pharmacology as we know it today. Some currently used over-the-counter and prescribed drugs were directly influenced from the early and late work involving morphine. Several of these drugs include codeine, dextromethorphan, hydrocodone, nalbuphine, levorphanol, benzomorphans, fentanyl, and buprenorphine **(Figure 3)**.

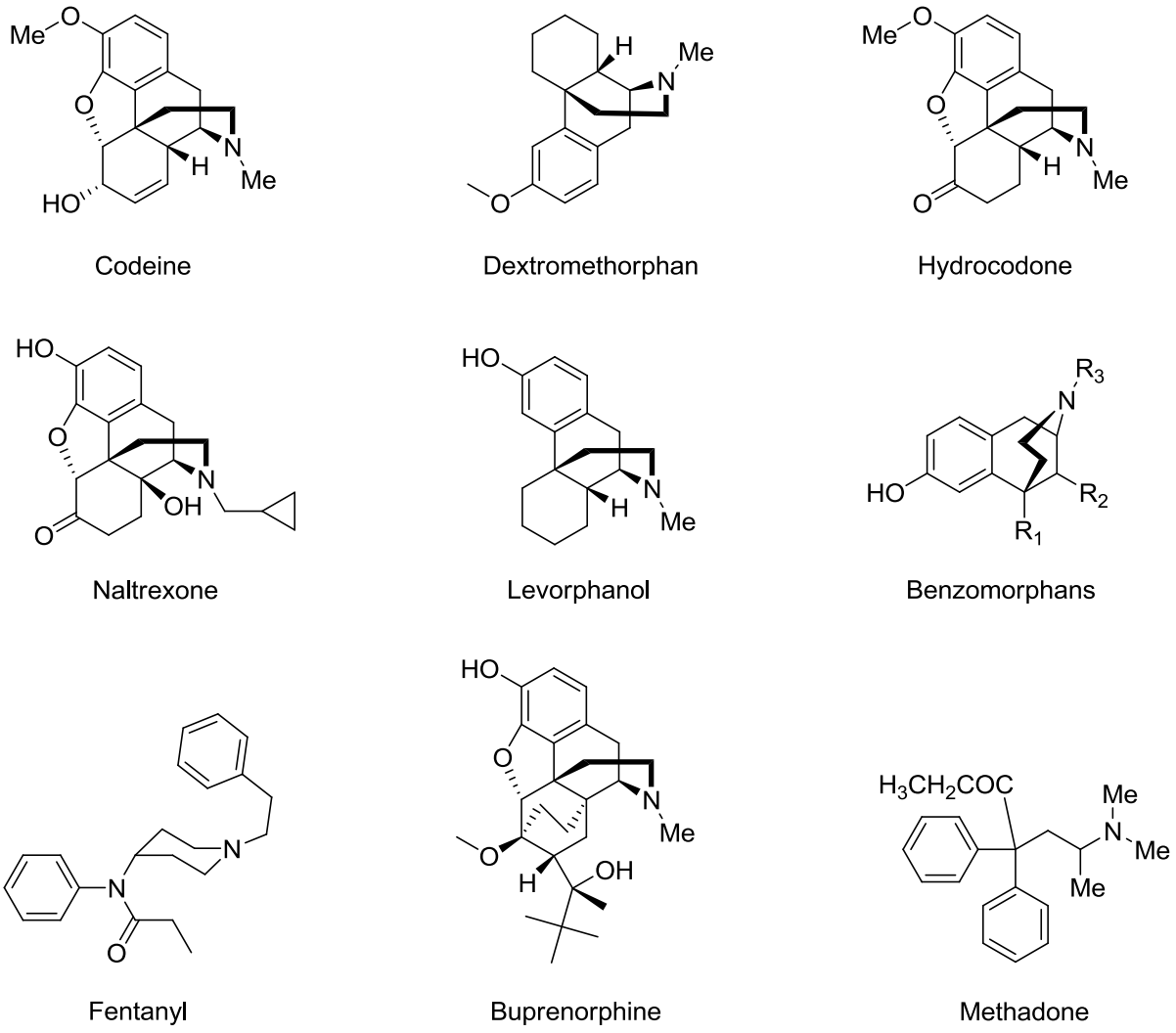



**Figure 3:** Examples of drugs influenced from research involving morphine

## Drawbacks of Opioid Use

Opioids have been the most therapeutically valuable compounds for the treatment of pain for the past 2000 years.[30] These compounds can potentially act at three opioid receptor subtypes, the μ opioid (MOP) receptor, the δ opioid (DOP) receptor, or the κ opioid (KOP) receptor. However, the use of these agents is not without their shortcomings. Use of narcotic analgesics gives rise to adverse side effects such as respiratory depression, constipation, tolerance, and physical

dependence, all of which are mediated by the MOP receptors.[30, 31] Prolonged use of opioids will decrease their therapeutic potential resulting in larger doses to reach the same level of efficacy. Upon building tolerance the body can adapt to the drug and become physically dependent on it to avoid withdrawal symptoms. Overdosing to reach a desired effect will most commonly result in death via respiratory depression.[32] However, in a controlled setting such as the clinic, constipation is a more common side effect to MOP receptor agonists.[32] Common to other marketed drugs, less consequential side effects observed with the use of some opioids also include nausea, vomiting, pruritus, and drug-drug interactions.[30] Regardless of which symptoms are being exhibited, these negative side effects impose limitations to the utility in their therapeutic use.

Despite their drawbacks, opioid analgesics derived from morphine are still the most widely prescribed medication for pain.[30] The chemical scaffold possessed by the natural product morphine has been extensively explored in efforts to tune out the associated negative side effects. Through these structure-activity relationships (SAR) a vast amount of knowledge has been gained into the understanding of the opioid receptor system.

## Opioid Receptors

Although morphine was isolated in 1806, it was not until 1954 that Beckett and co-workers proposed the idea of opioid receptors and then not until 1973 when opioid receptors were identified in mammalian brain tissue by Pert and co-workers.[33, 34] Later in 1976 Martin and co-workers used morphine, *N*-allylnormetazocine, and ketocyclazocine to classify opioid receptors into three different subtypes **(Figure 4)**.[35, 36] Upon noticing these three structures resulted in

unique pharmacological effects, opioid receptors were classified into MOP, DOP, and KOP receptors.[35, 36]

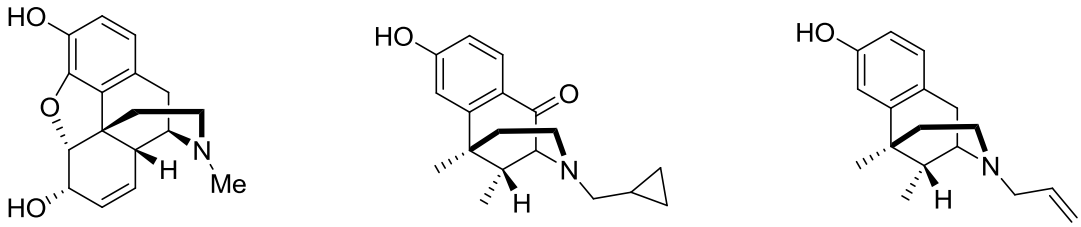



**Figure 4:** Opioids used by Martin for classification of the MOP, DOP, and KOP receptors

Each of the MOP, DOP, and KOP receptor subtypes belong to the G-protein coupled receptor (GPCR) superfamily and commonly associate with the $G_{i/o}$ proteins.[30] The coupling to the $G_{i/o}$ proteins decreases both intracellular calcium and cAMP production, which results in an inhibition of adenylate cyclase and many other downstream signaling effects used in cell communication.[37, 38] Despite their similarities, each opioid receptor differs in receptor distribution, density, and the endogenous ligand used to activate it.[39]

## Expanding Opioid Receptor Research

While the vast majority of opioid research originally focused on morphine and the structure activity relationships of the morphine scaffold at MOP receptors, the therapeutic utility of these ligands are limited due to the negative side effects associated with them.[30] Due to these negative characteristics, the need for the development of novel ligands free of harmful side effects still remains. Expanding research to explore the DOP and KOP receptors has shown promise in both pharmacological profile as well as a reduced side effect profile.[40, 41] As nature has provided many bioactive molecular scaffolds such as morphine, the ideal ligand may remain to be discovered from a different structural class of molecules found in nature. In particular, scaffolds

that have shown promise for such activity are peptides, alkaloids (beyond the morphine scaffold), flavonoids, and terpenes. These scaffolds provide an excellent starting point for the investigation of the DOP and KOP receptors. Terpenes represent an enormous amount of diversity encompassing more than 55,000 unique molecules found in nature.[42, 43] However, when compared to alkaloids such as morphine, terpenes remain an underexplored area in opioid research despite their almost ubiquitous presence in nature.

Much of the diversity found between terpenes and alkaloids can be explained from their biosynthetic origins in nature. Alkaloids contain one or more nitrogen atoms, which usually imparts a varying degree of basicity. These nitrogen atoms originate from amino acids and often contain the carbon skeleton from the precursor amino acid that is being incorporated into the alkaloid structure. While over 27,000 different alkaloids are known, only a few amino acids are utilized in alkaloid biosynthesis, namely ornithine, tyrosine, tryptophan, lysine, histidine, nicotinic acid, and anthranilic acid. Terpenes however represent a larger set of known molecules and originate from only two building blocks, dimethylallyl pyrophosphate and 3-isopentenyl pyrophosphate, each of these being a biochemically activated isoprene unit. Connection of these activated isoprene units in a head-to-tail fashion force all terpenes to have carbon skeletons that can be represented by $(C_5)_n$ with any further modifications happening in conjunction with another pathway such as the acetate or shikimate pathways.

One terpene that has recently gained attention due to its interesting pharmacological profile is salvinorin A. The neoclerodane diterpene salvinorin A does not resemble traditional opioid ligands like the morphine scaffold, including the absence of a basic nitrogen, yet it possess both affinity and efficacy for opioid receptors.[44] Several studies have shown that modification to the salvinorin A scaffold can modulate activity at the opioid receptors and even alter the selectivity

between individual subtypes of opioid receptors.[45-51] Interestingly, the KOP receptor has shown therapeutic potential over the MOP receptor in the treatment of peripheral pain management, drug abuse, anxiety, and depression.[30, 52-55] These results suggest salvinorin A may be an excellent starting point for the investigation into the KOP receptor as a drugable target with a reduced side effect profile.

## Salvinorin A Background

The hallucinogenic sage plant *Salvia divinorum* Epling & Játiva from the mint family Lamiaceae has been used for centuries by the indigenous people of Oaxaca, Mexico.[56-58] The locals of Oaxaca have used *S. divinorum* for ethnomedical purposes, as well as during traditional divination ceremonies. The hallucinogenic nature of *S. divinorum* is favored during divination ceremonies for its abilities to produce visions, while local shaman have used it as a treatment for headaches, rheumatism and the semimagical disease called *panzón de barrego*, which roughly translates to swollen belly.[59, 60] Despite being widely used within the culture of the Mazatecs of Oaxaca for many years, *S. divinorum* did not emerge in industrialized nations until the late 1930's.[61] Traditional modes of use include chewing the leaves or making an infusion and then ingesting.[56-58] In western culture, the dried leaves of *S. divinorum* are typically smoked or less often volatilized.[62, 63] Originally isolated by Ortega in 1982, the main active component of *S. divinorum* was found to be the neoclerodane diterpene salvinorin A **(Figure 5)**.[44, 56, 59, 64] Upon isolation and testing, salvinorin A was determined to be a potent hallucinogen that is a selective and highly efficacious κ-opioid receptor agonist.[44, 65, 66] Furthermore it was shown that salvinorin A does not illicit its action through activation of the serotonin 5-$HT_{2A}$ receptor as do many of the known classical hallucinogens such as psilocybin, mescaline, dimethyltryptamine (DMT), 2,5-dimethoxy-4-bromoamphetamine (DOB) and lysergic acid diethylamide (LSD) **(Figure 5)**.[44, 67]

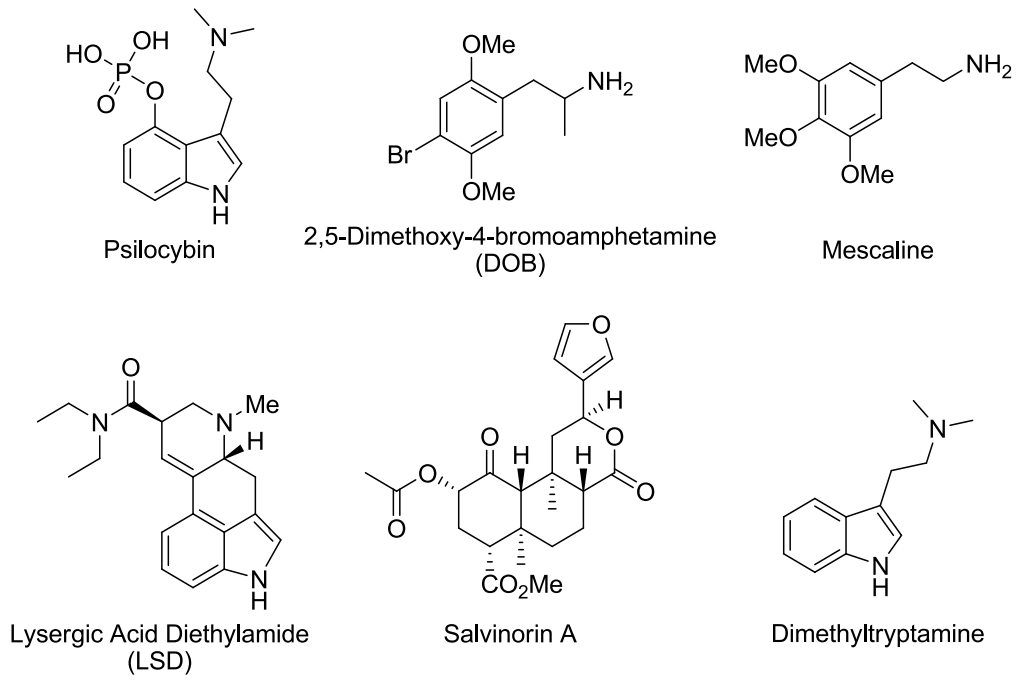



**Figure 5:** Structures of selected known hallucinogens

Interestingly enough, salvinorin A rivals the potency of classical hallucinogens such as LSD and DOB having an active dose in humans ranging from 6 to 21 μg/kg.[68] These findings are made even more intriguing to the science community based on the structure of salvinorin A, which bears little structural resemblance to any of the other known hallucinogens previously mentioned, in particular with respect to its lack of a basic nitrogen, which for years had been considered a necessity for binding and efficacy at opioid receptors.[69-72]

## Current Salvinorin A Use

With the ease of obtaining *S. divinorum* or salvinorin A through local "head shops" and internet sales, salvia use has become popular within certain cultures around the globe. In the United States *S. divinorum* plants, leaf extracts, and dried leaves with enhanced salvinorin A concentrations are readily available for use as a recreational or legal alternative to other

hallucinogens.[62, 73] The use of such products has been increasing since the mid-1990's, and as of 2006 in the United States at least 1.8 million people aged 12 or older have used *S. divinorum* or salvinorin A containing products, while in 2008 4.4% of college students had reported using salvia at least once.[57, 58, 73, 74] Use, however, has recently seen a stabilization at non-trivial levels, with lifetime use among youth remaining roughly constant around 6% between the years (2009-2011).[75] As the popularity of legal hallucinogens such as salvinorin A increases amongst the general public of the United States, Europe, and the rest of the world, its own popularity brings with regulations on salvinorin A and *S. divinorum* as a controlled substance.[76] Regulation of salvinorin A in Florida has changed the views of some users about its use in terms of safety, frequency of use, and the ease of obtaining the drug.[77] The resulting ban on its possession or use in certain areas by law has left many in the scientific community curious to the mechanism of action and pharmacological profile of such an agent, which has sparked much investigation into the pharmacology of salvinorin A.

## Salvinorin A *in vitro* Pharmacology

Salvinorin A was identified in 2002 by Roth and coworkers as the first non-nitrogenous, naturally occurring opioid receptor agonist.[44] Furthermore, salvinorin A was identified as a potent and selective ligand which targeted the KOP receptor. This was determined through the NIMH Psychoactive Drug Screening Program by screening against 51 molecular targets which included ligand-gated ion channels, molecular transporters, and human GPCR's. In this screening panel salvinorin A did not bind to MOP or DOP receptors; instead salvinorin A selectively inhibited bremazocine-labeled KOP receptors, effectively identifying an opioid receptor subtype specific ligand.[44] Later in 2004 a detailed report of salvinorin A's functional properties were evaluated by Chavkin and coworkers.[65] Salvinorin A was compared to the

known KOP receptor agonist U69,593 and again found to be a potent agonist in all assays performed based on its ability to inhibit forskolin stimulated cAMP activity, potassium conductance, and intracellular calcium mobilization. Further KOP receptor subtype selectivity has been suggested by Ansonoff and coworkers by assessing salvinorin A's ability to bind either $KOP_1$ or $KOP_2$ employing ligand binding as evidence.[78] Ansonoff and coworkers showed that salvinorin A imparted selective affinity towards the $KOP_1$ receptor over the $KOP_2$ receptor ($K_i$ = 18.7 nM vs. $K_i$ = 10,000 nM).[78] These results are of course only suggestive, as KOP receptor subtypes have not been identified through gene coding but are only hypothesized through ligand binding.[79-81]

Salvinorin A was shown to have similar affinity and potency as compared to U50,488 as well as being shown to promote KOP receptor internalization by Wang and coworkers.[66] Salvinorin A, in CHO cells, had reported affinity and activity values of $K_i$ = 7.9 nM and $EC_{50}$ = 4.6 nM while U50,488 had reported values of $K_i$ = 11.0 nM and $EC_{50}$ = 2.2 nM. U50,488 and U69,593, both KOP receptor agonists, have previously been shown to down regulate and internalize KOP receptors.[82-84] This internalization is often attributed to the development and mechanism of action of tolerance.[85-87] After incubation of salvinorin A with cells expressing FLAG-hKOP receptors, a reduction of cell surface fluorescence was noted in a dose dependent manner. The reduction of cell surface fluorescence largely indicates the internalization of the FLAG-hKOP receptors. As compared to U50,488 salvinorin A was about half as effective at internalizing KOP receptors as U50,488 (15% vs 30 - 40% respectively).[66]

## Salvinorin A in Non-human Primates

The pharmacokinetic profile of salvinorin A has been investigated in non-human primates and in rare instances during controlled human studies. In 2004 salvinorin A was administered to non-human primates by Butelman and coworkers and was shown to produce κ-opioid agonist-like discriminative effects in rhesus monkeys.[88] Using rhesus monkeys trained to discriminate for U69,593 against vehicle, salvinorin A was shown to fully substitute for U69,593 in a dose dependent manner. Upon pretreatment with the general opioid antagonist quadazocine, at the pharmacologically active dose of 0.32 mg/kg, the discriminative effects of salvinorin A and U69,593 were fully blocked in all test subjects. Pretreatment with the longer acting KOP selective antagonist 5'-Guanidinonaltrindole (GNTI) blocked the effects of salvinorin A in two of the three subjects. However the NMDA antagonist ketamine, known to produce psychotomimetic effects in humans, was not generalized by the U69,593 discriminante rhesus monkeys.

Pharmacokinetic profiling of salvinorin A was described in non-human primates by Schmidt and coworkers in 2005.[89] In this study a single bolus dose of salvinorin A (0.032 mg/kg) was administered intravenously to 2 male and 2 female rhesus monkeys. Across the four subjects the overall elimination $t_{1/2}$ was 56.6 ± 24.8 min; however, gender differences were noticed for both the distribution and elimination of salvinorin A. In males, the distribution $t_{1/2}$ was rapid, the elimination $t_{1/2}$ was 37.9 ± 5.6 min, and the area under the curve (AUC) was 572 ± 133 ng min/ml. In females, the distribution $t_{1/2}$ was slower than males at 0.95 ± 0.20 min, the elimination $t_{1/2}$ was higher than males at 80.0 ± 13.1 min, and the AUC was larger than males at 1087 ± 46 ng min/ml. These results are consistent with other observations involving KOP receptors and non-human primates.[90, 91]

Later, in 2007, Butelman and coworkers used gonadally intact male and female rhesus monkeys to investigate the effects of salvinorin A in a neuroendocrine biomarker assay of prolactin.[92] Upon administration of salvinorin A and U69,593 (0.0032-0.056 mg/kg i.v.) a dose-dependent and time-dependent increase in prolactin was observed. In male subjects, salvinorin A showed increased prolactin levels 5 minutes after injection. However, U69,593 had increased prolactin levels and a longer duration of action lasting out to 120 minute post administration as compared to salvinorin A which at 90 minutes was not significantly different than vehicle prolactin levels. Switching the route of salvinorin A administration to subcutaneous injection produced a slower onset and lower prolactin levels with peak effects not occurring until the 60 minute time point. Additionally using nalmefene, which antagonizes MOP receptors at low concentrations and antagonizes both MOP and KOP receptors at high concentrations, Butelman was able to show that nalmefene (0.01 mg/kg, s.c.) did not antagonize the prolactin effects of salvinorin A, but at an elevated dose of 0.1 mg/kg the prolactin effects of salvinorin A were antagonized. Ketanserin, a 5-$HT_2$ antagonist, when administered i.m. at an active dose of 0.1 mg/kg, did not antagonize the neuroendocrine effects of salvinorin A. This further implicates that the effects of salvinorin A act through modulation of the KOP receptor.

The pharmacokinetic parameters of salvinorin A were measured in female baboons by Hooker and coworkers in 2008.[93] Using positron emission tomography (PET) with an $^{11}$C-acetyl derivative of salvinorin A, Hooker was able to show that 3.3% of the total salvinorin A administration reached a peak concentration in only 40 seconds with a half-life of 8 minutes. The highest concentrations within the brain were reached in the cerebellum with a notable amount being found in the visual cortex, which is hypothesized to account for, in part, the hallucinogenic effects observed when smoking salvinorin A. Administration of the opioid receptor antagonist

naloxone did not reduce or alter the distribution of salvinorin A administration. Peripheral organ kinetics suggested salvinorin A is metabolized through either the renal or biliary systems. Based on the maximum brain concentrations observed, it was presumed that less than 10 mg of salvinorin A within the brain is required to cause hallucinations. These findings are consistent with the observed and anecdotal effects described of humans when smoking salvinorin A.

In 2009, Butelman and coworkers investigated the entry of salvinorin A into the central nervous system (CNS) and its unconditioned behavioral effects in non-human primates.[94] Sedation, postural relaxation, ptosis, and facial relaxation were all measured as a means to quantify the effects of centrally penetrating KOP receptor agonists. Upon administration of salvinorin A (0.032 and 0.1 mg/kg i.v.) dose dependent increases in sedation were observed in both chaired and home-cage subjects, as well as dose dependent increases in postural effects in home cage subjects out to 30 minutes. Salvinorin A was also shown to cause dose-dependent and time-dependent effects on facial relaxation and ptosis following intravenous administration. Compared to the known KOP receptor agonist U69,593, a shorter duration of action and more pronounced time-dependent behavioral effects were observed when administering salvinorin A. Upon pretreatment with the opioid antagonist nalmefene (0.1 mg/kg), the effects of salvinorin A were prevented and after administration of nalmefene 11 minutes post salvinorin A administration. Additionally, nalmefene rapidly reversed salvinorin A induced ptosis with facial relaxation being observed. These behavioral effects were further linked to KOP receptor modulation when pretreatment of $CB_1$ receptor antagonist rimonabant and 5-$HT_2$ antagonist ketanserin were both ineffective at preventing salvinorin A induced behavioral effects.[94]

Continued research by Butelman and coworkers in 2010 investigated the discriminative effects of salvinorin A in non-human primates.[95] Butelman and coworkers showed rhesus

monkeys trained to discriminate salvinorin A generalized the centrally acting KOP receptor agonists bremazocine, U69,593 and U50488. However, only one of the three subjects generalized the peripherally restricted KOP receptor agonist ICI204,448. These results link the discriminative effects of salvinorin A to centrally acting KOP receptor agonists. Other MOP and DOP receptor agonists, fentanyl and SNC80 respectively, did not exhibit generalization with salvinorin A. Additionally, the serotonergic 5-$HT_2$ hallucinogen psilocybin and the NMDA antagonist ketamine were not generalized. Quadazocine (0.32 mg/kg) blocked the discriminative effects of salvinorin A, while the 5-$HT_2$ antagonist ketanserin (0.1 mg/kg) did not. This further supports the discriminative cue produced by salvinorin A is mediated by activation of the KOP receptor and a distinct mechanism from other hallucinogens.

## Salvinorin A in Humans

The effects of salvinorin A in humans are relatively unexplored based on volume of experiments being conducted in a controlled laboratory setting. Most of the original hypotheses to the pharmacokinetic parameters were anecdotal, being based upon first hand witnesses and user experiences with salvia or salvinorin A. Many user experiences can be seen over the internet on popular video sharing websites such as YouTube.com which Lange and coworkers have used in an attempt to quantify the observable effects of salvinorin A after watching several of these videos.[96] Many of these videos show symptoms commonly associated with salvinorin A such as speech and coordination impairment, hallucinogenic experiences, and a dysphoric "loss of self" feeling. KOP receptors were first implicated to be associated with psychotomimesis by Pfeiffer and coworkers in 1986.[97] Exactly how KOP receptors mediate their hallucinogenic effects remains unknown, however since the work of Pfeiffer, dysphoria has been the best surrogate marker of KOP receptor agonism.

The first reports of controlled studies involving salvinorin A in humans have only appeared recently in the past few years. In early 2011, two reports were published only four days apart from two separate groups involving administration of salvinorin A to humans. One group, led by Mendelson and coworkers, investigated the sublingual effects of salvinorin A, while the other group led by Johnson and coworkers investigated the smoked effects of salvinorin A.[68, 98] Upon sublingual administration of salvinorin A in a DMSO/PEG-400 (25:75) solution Mendelson and coworkers did not observe any physiological or subjective effects from salvinorin A greater than placebo.[98] Even doses up to 4 mg did not affect pulse, blood pressure, $O_2$ saturation, or core temperature. Subjects were questioned after dosing using the Subjective Drug Effects Questionnaire (SDEQ), Altered States of Conciousness Questionnaire (APZ-OAV), and Positive and Negative Affect Schedule (PANAS) but lacked to produce results significantly different from placebo. Attempts were also made at quantification of salvinorin A in the blood and urine but in all accounts was found to be below their limit of quantification. While previous claims have been made to the sublingual effects of salvinorin A these results indicate the bioavailability of salvinorin A to be low at best.[58] However, when Johnson and coworkers administered salvinorin A via inhalation, definite subjective effects were observed and recorded out to 20 minutes after inhalation.[68] In agreement with Mendelson's work, Johnson and coworkers did not see a significant increase in blood pressure or heart rate. After administration of salvinorin A ranging from 0.375 μg/kg to 21 μg/kg dose-related increases were observed in drug strength on a mystical-type experience questionnaire (Mysticism Scale) and a subjective effects of serotonergic hallucinogens questionnaire (Hallucinogen Rating Scale). Salvinorin A exhibited a unique profile of subjective effects with similarities to classical hallucinogens, including mystical effects. Time dependent drug strength ratings were assessed every two minutes for one

hour with peak ratings at the two minute time point. Participant narratives indicated the user experienced intense and unusual experiences that were characterized by feelings of energy, changes in spatial orientation, pressure on body parts, and strange and sometimes recurring themes between individual dosing sessions such as revisiting childhood memories, contact with entities, and cartoon-like imagery. The participants were largely behaviorally inactive which is in contrast to the chaotic effects observed in the online videos by Lange and coworkers.[96]

A larger population of participants was used by Addy in 2012 to assess the acute and post-acute behavioral and psychological effects of salvinorin A in humans.[99] Consistent with previous reports, vital signs were recorded before and after inhalation and were found to be unaffected by salvinorin A. Participants were dosed with either 1017 or 100 μg of purified salvinorin A dissolved onto 25mg of dried leaf material two weeks apart in a counterbalanced order. The participants' behavior during each session was recorded by the researcher followed by a subjective effects questionnaire (Hallucinogen Rating Scale) immediately following each session. While on active doses (1017 μg) participants moved, laughed, and talked much more often than on inactive doses (100 μg), this is contrary to the findings of Johnson and coworkers.[68] Participants also rated the subjective effects on the hallucinogen rating scale significantly higher on active doses, indicating hallucinogenic experiences. These results indicate similarities and differences between salvinorin A and other more classical hallucinogens.

Ranganathan and coworkers, in 2012, explored the neuroendrocrine effects, psychotomimetic effects, perceptual alterations, and quantitative electroencephalographic (EEG) effects in humans after inhalation of biologically active doses of salvinorin A.[100] Over three days, 10 healthy individuals who had previously used salvinorin A were administered either 0 mg, 8 mg, or 12 mg of salvinorin A in a randomized order. On the visual analog scale (VAS) salvinorin A produced

less drowsiness when administered at both low and high doses, common to anecdotal reports of a heightened sense of awareness. However, the VAS feelings of "high", "calm", "sad", "irritable", or "anxious" were unaffected by salvinorin A administration. Salvinorin A administration resulted in transient psychotomimetic effects as measured by increases on the positive and negative syndrome scale (PANSS), as well as the psychotomimetic states inventory (PSI). Salvinorin A administration also produced transient increases in perceptual alterations for both intensity and somaesthesia as measured by the HRS. Elevated levels of plasma cortisol and prolactin were observed, making this the first study to demonstrate the endocrine effects of salvinorin A in humans. As prolactin is well known as a biomarker of KOP receptor agonism in non-human primates, this provides compelling evidence of the centrally mediated effects of salvinorin A.[92, 101, 102] The elevation in cortisol levels are additionally consistent with the elevating effects of other KOP receptor specific agonists observed in animals and humans.[103, 104] These data and observations may suggest that salvinorin A lowers dopamine levels within the tuberoinfundibular pathway to elevate prolactin levels while stimulating the hypothalamic-pituitary-axis to increase cortisol levels.

Most recently, MacLean and coworkers again investigated the dissociative, hallucinogenic, and memory effects of salvinorin A in individuals with histories of hallucinogen use.[105] Consistent with previous findings, the dose-related effects peaked at 2 minutes and rapidly dissipated, not having any effect on heart rate or blood pressure. Doses of 0.375 μg/kg to 21 μg/kg were administered to a total of 8 healthy participants previously experienced with hallucinogens. Two participants reached a voluntarily stopping point where they absolutely refused to receive a dose equal to or higher than the 19.5 μg/kg (second highest dose) previously received. Additionally half of the participants provided a maximal drug rating of 10 (the

strongest effect imaginable for salvinorin A) at one or more time points, either verbally or due to a completely unresponsive nature when prompted. Participants stated that salvinorin A effects were unique and very intense as compared to other hallucinogens they had experienced. While the average ratings of similarity to other hallucinogens were relatively low on the quantitative pharmacological class questionnaire, the ratings of overall similarity to classical hallucinogens increased linearly as a dose function, indicating participants judged salvinorin A to be only somewhat similar to classic hallucinogens. All except one participant exhibited a dose-related impairment in word recall and recognition for an auditory memory task. Additionally, ratings on "liking" and "good" effects increased across doses while feelings of "disliking" and "bad" effects were not significantly affected by dose, indicating a possible abuse liability.

## Conclusions

While natural products have been exploited for many years in the pursuit of remedies to treat medical ailments, there still remains a large area for investigation and improvement of their current uses. As natural products have been used in nearly all areas of modern medicine and are still being used as a large portion of the present treatments available and being developed, their utility is invaluable to medical practitioners. Specifically, the field of opioid pharmacology could greatly benefit from the investigation of novel scaffolds and receptor selective ligands. Many natural products have limitations to their use, while opioids have severely limiting drawback, yet the use of morphine and its derivatives remains the gold standard for treatment of pain today. Since the discovery of salvinorin A as a novel scaffold for opioid receptors, it has garnered much attention for its potential to modulate opioid receptors with a reduced side-effect profile. Therefore the continued investigation of salvinorin A and its place in opioid pharmacology remains to be fully investigated, including its isolation and pharmacokinetic profiling.

# CHAPTER II

## Rationale and Specific Aims

Salvinorin A is a structurally unique KOP receptor agonist that has shown potential for therapeutic use. While structural optimization is still needed to eliminate the drawbacks of its use in humans, salvinorin A provides an excellent scaffold with altered pharmacology for the investigation of opioid receptors. With opioid receptors linked to therapeutically relevant physiological functions such as pain, mood disorders, and gastrointestinal problems, the need for a novel treatment beyond morphine is a large void yet to be filled by researchers. It is known that salvinorin A imparts a hallucinogenic effect on its user, however it does not impart this effect through a typical 5-$HT_{2A}$ mechanism that most classical hallucinogens operate by. As much effort has gone into the modification of the salvinorin A scaffold in attempts to alter the pharmacology and reduce its side effect profile, there still remains much to explore with the absorption, distribution, metabolism, excretion, and toxicity (ADMET) associated with salvinorin A.

### Specific Aim #1: Attempt to isolate salvinorin A in a fast and selective manner

As salvinorin A has shown potential to be a therapeutically useful agent, the isolation of high purity salvinorin A from its source would be a great aid to the investigation to the utility of salvinorin A. Generation of a reactive handle for a polymeric resin could lead to an efficient process for the selective removal of specific compounds from a complex mixture. This method could potentially reduce the need for large solvent volumes, the time required for isolation, and the labor required for a process. Previous work has shown solid phase chemistry to be successful

and amendable to natural products. To further explore the utility of solid phase extraction, a reactive solid phase handle was designed and shown to be chemically reactive towards the salvinorin A scaffold.

**Specific Aim #2: Investigate the pharmacokinetics of salvinorin A in non-human primates and humans**

Before salvinorin A can be fully developed into a useful agent with altered pharmacological properties, the pharmacokinetic profile of salvinorin A itself must first be understood. Without knowledge of the basic pharmacokinetic properties such as bioavailability, distribution, and metabolic stability, accurate predictions for the modification to the salvinorin A scaffold cannot be made to enhance a desired parameter. While much work has been done to investigate salvinorin A in lower order animals, little has been done with the investigation of salvinorin A in higher order animals such as non-human primates and humans. To expand the understanding of the pharmacokinetic parameters of salvinorin A, time course graphs were generated to relate in vivo concentrations of salvinorin A in non-human primate cerebrospinal fluid and in human plasma, using a reliable and reproducible LC-MS/MS quantification method.

# CHAPTER III

## Solid Support Reagents in Natural Product Chemistry

The use of immobilized reagents and scavengers provides an exciting and practical means for working with and efficient preparation of novel compounds from natural product extracts. Whether isolating or derivatizing a compound, solid supports within the field of natural products provide an excellent opportunity for the future exploration, design, and synthesis programs in chemistry. Natural products, whose novel structures and molecular complexity are unlikely to come from a combinatorial library, are more likely to arise from the development of an efficient method for isolating novel natural products. These newly identified compounds could then be used for testing within biological assays or against the biological macromolecules associated with many complex disease states. Much of the more current research has been focused on anti-cancer agents, while the search for natural products possessing utility for the CNS or neurosciences has taken a back seat, being underdeveloped.[106] While natural products could provide many novel scaffolds and selective agents to better characterize receptor types and or biological pathways associated with CNS disorders such as anxiety, chronic pain, depression, and schizophrenia, they may be under investigated for multiple reasons. Historically the isolation and purification of natural products has not been easily adapted to a high-throughput format since it most often requires large amounts of time and labor. Additionally, even after isolation and purification are complete there remains the issue of structure elucidation, which can often be assigned incorrectly.[107] These troubles have de-emphasized the exploration for novel biological probes from natural sources. At present there remains a lack of a general and efficient method for

the rapid isolation of natural products, which is a vital problem because until fully explored, the vast amount of structural and chemical diversity in nature will not be available for use as biological probes for investigating complex disease states.

The use of polymer-supported reagents is one approach for the development of a general and efficient method for the isolation of natural products. Polymer-supported reagents and scavengers are able to simplify both synthetic and isolation procedures, and as such they have been widely employed though organic chemistry for this purpose.[108] When compared to traditional solution phase chemistry, solid supported reagents have a major advantage in the work-up and isolation steps being significantly simplified to a simple filtration.[109] Additionally, almost all organic transformation performed in homogenous solution phase can also be performed with the appropriate polymer-supported reagent.[110] The commercialization of nearly every type of functionally supported resin makes solid phase synthesis a very useful and versatile resource for organic chemists.[109] The versatility of solid-supported reagents is exemplified in the reported polymer-supported synthesis of the natural products (±)-epibatidine, (±)-oxomaritidine, (±)-plicamine, and carpanone **(Figure 6)**.[111-116] The successful syntheses of these natural products highlight the value, versatility, and applications of using immobilized reagents in natural product chemistry.

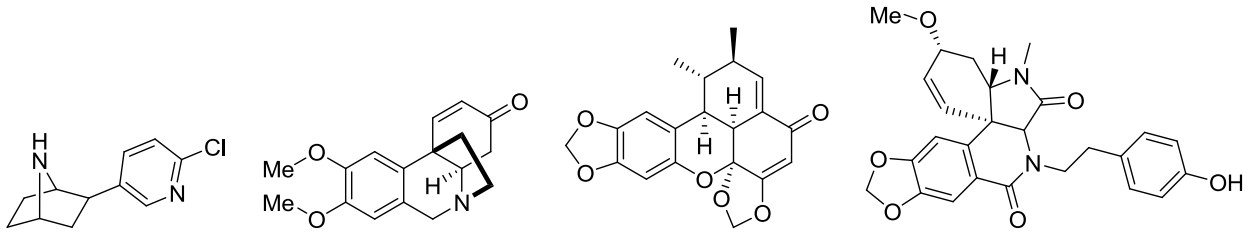



**Figure 6:** Chemical strutures synthesized on solid support resins

There are many advantages provided when using solid supported reagents instead of conventional solution phase synthesis.[110] Most notably, excess reagents can be used to force reactions to completion and the remaining excess simply washed away during filtration without causing messy or extensive workup procedures. Chemical reagents or their by-products that are normally toxic, noxious, or in general hazardous to work with can also be immobilized onto a solid support thereby reducing the risk of inhalation or direct physical contact. Solid support systems are also easily adapted to automated synthesis, such as the split and pool method. Many of the standard analytical methods such as nuclear magnetic resonance (NMR), liquid chromatography-mass spectrometry (LC-MS), thin layer chromatrography (TLC), and high performance liquid chromatography (HPLC) can be applied to solid-supported systems as well since the chemistry is carried out in solution. Additionally, reagents immobilized on solid support permits the simultaneous use of multiple reagents that would not be compatible if used together in standard homogeneous solution phase chemistry. For instance it has been shown that using acidic and basic solid support reagents in "tea bags" can be used together in a single reaction vessel to perform chemistry.[117]

While immobilized reagents are an excellent tool for the rapid cleanup of reaction mixtures facilitated by sequestration of by-products, used reagents, and remaining excess reagents, the functionality of the solid support may be limiting its usefulness as a specific "trap" for a target compound. Using an appropriately functionalized solid support that could selectively capture a natural product based on its inherent functionality such that the contaminating impurities could be washed away would be a very powerful tool in natural products research. Additionally, employing a clean and effective method of releasing the polymer bound molecule thus releasing the desired product in a pure form would speed and simplify the often extensive chromatographic

procedures used to isolate single compounds from natural product extracts. This could be accomplished using any number of reversible physical or chemical methods. Both ionic and covalent bonding interactions have previously been exploited to successfully purify products from complex reaction mixtures.[118-123] Many solid phase extraction (SPE) kits are commercially available for analytical sample preparation that utilize these basic principles to isolate a desired analyte under a wide variety of conditions. Following these principles, a solid supported reagent could be generated to select for a specific functionality contained by a molecule such that a single entity is selectively removed from a mixture.

One process uses a reversible and selective interaction between a resin bound metal which is linked to an organic metal chelating tag which in turn is then linked to a specific natural product.[124] This approach combines the principles of conventional homogenous phase chemistry with both tagged reagent and catch-and-release methodologies. In this case the link used as the tag can be tailored to a specific need and subsequently immobilized onto the resin bound metal rendering it reactive yet insoluble. After use, a simple filtration can then isolate the resin and any unbound impurities or reactants will be washed away. The desired product can then be cleaved from the resin and the tag recaptured onto the resin, regenerating it for additional use. Many metals bound to resin and organic metal chelating molecules can be used for this style of procedure.[125-128] However, linking a organic metal chelating molecule to an unknown natural product is difficult. Instead, isolating a natural product based on its inherent functionality and chemical properties would generalize the procedure rather than attempting to link the compound to a suitable pendant group.

Another functionality, that is more specific than acid/base properties, is the presence of a furan ring. Furan rings are known to undergo Diels-Alder reactions with acetylenedicarboxylic

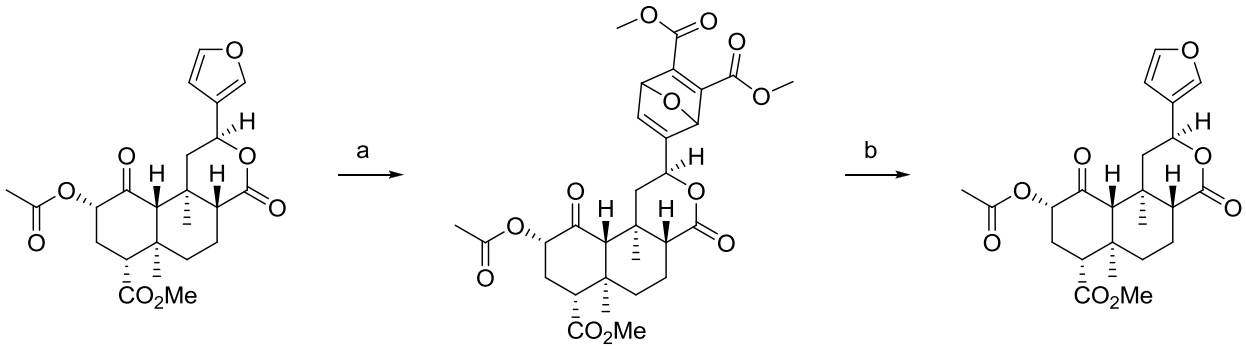
acid derivatives.[129-131] Additionally, it is known that by heating a Diels-Alder cycloadduct a retro Diels-Alder reaction will occur, which is driven by rearomatization. This inherent chemical property can then be used as a new catch-and-release method if bound to a solid support. This would allow for the capture and release of furan containing natural products via immobilization onto a solid support and then released after washing away the remaining unbound impurities. While Diels-Alder chemistry has previously been employed on solid support, this method has not been applied to natural product isolation.[132, 133] Furthermore, this methodology has been amendable to solid support via use of a stabilized iminonitroso Diels-Alder reaction to derivatize complex diene containing natural products.[134-137]

With the previously mentioned concepts in mind, efforts were then begun to develop a reactive resin, or resin appendage, that could react with salvinorin A. The selective reaction of a polymer with salvinorin A could potentially be used to molecularly sequester salvinorin A in an efficient manner. Taking known salvinorin A reactivity into account, the selective reaction with salvinorin A would need to be reversible, high yielding, and proceed under mild conditions to retain the salvinorin A scaffold intact. Investigations therefore proceeded into the reactivity and reversibility of Diels-Alder cycloadditions with the salvinorin A furan ring.

## Results and Discussion

Screening of a diverse set of dienophiles was conducted to understand the reactivity of the salvinorin A furan ring to participate in Diels-Alder cycloadditions. Initial reaction of dimethyl acetylenedicarboxylate with salvinorin A yielded a cycloaddition product that could be isolated and reheated in solution to perform the retro reaction and regenerate starting material **(Scheme 1)**. The cycloaddition of various electron deficient alkynes with the furan ring of salvinorin A and subsequent retro Diels-Alder to reform salvinorin A was not unexpected. There have been many reports of retro Diels-Alder reactions previously reported and used during chemical synthesis.[138-140] These cycloaddition products were primarily being explored in efforts to modulate activity at the opioid receptors using salvinorin A as a chemical scaffold.

**Scheme 1:** Diels-Alder cycloaddition with salvinorin A and subsequent retro reaction regenerating salvinorin A

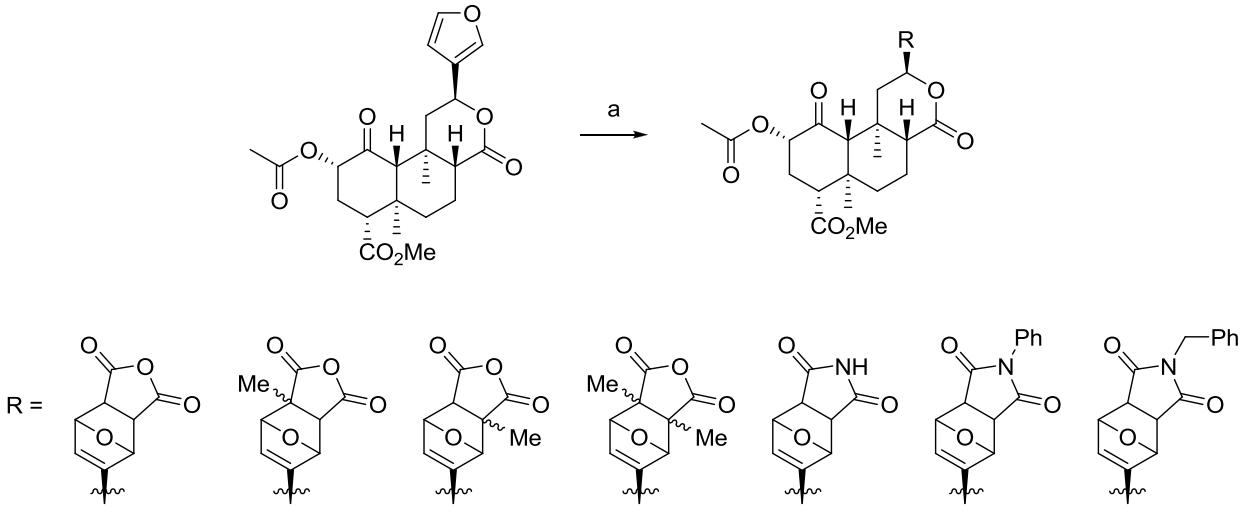



a) dimethyl acetylenedicarboxylate, toluene, heat b) toluene, heat

This transformation and regeneration procedure is reaction specific between the dienes and dienophile partners. Therefore the utility of this reaction specificity was explored in an attempt to selectively isolate salvinorin A based on its inherent reactivity. Going beyond the use of alkynes to form cycloadducts, alkene reactivity with the furan ring of salvinorin A was also investigated. Attempts with maleic anhydride and maleimide to form cycloadducts with salvinorin A under

similar reaction conditions observed with dimethyl acetylenedicarboxylate yielded no reaction. In efforts to force the reaction towards the cycloaddition products, many variations in reaction conditions were performed that can affect Diels-Alder type reactions. After manipulation of time, temperature, solvent, addition of water, lewis acid catalysis, and microwave conditions, no new products were formed, as seen on TLC. However it was noticed that varying the concentration of staring materials in solution did begin to develop a new product as the starting materials became more concentrated in solution. Workable amounts of products were found after the concentration was near 100 mg of salvinorin A in 1 ml of chloroform. Using this information, the reactions were then performed as concentrated as possible by dissolving salvinorin A into liquid maleic anhydride. Carrying out these reactions neat resulted in near complete consumption of the salvinorin A starting material. While many of the alkene derivatives used were solids at room temperature, they needed to be heated to their melting points before the reaction could be run neat. Exploration of reaction scope with alkene dienophiles can be seen in **(Scheme 2)**.

**Scheme 2:** Various maleic anhydride and maleimide cycloadducts formed with salvinorin A

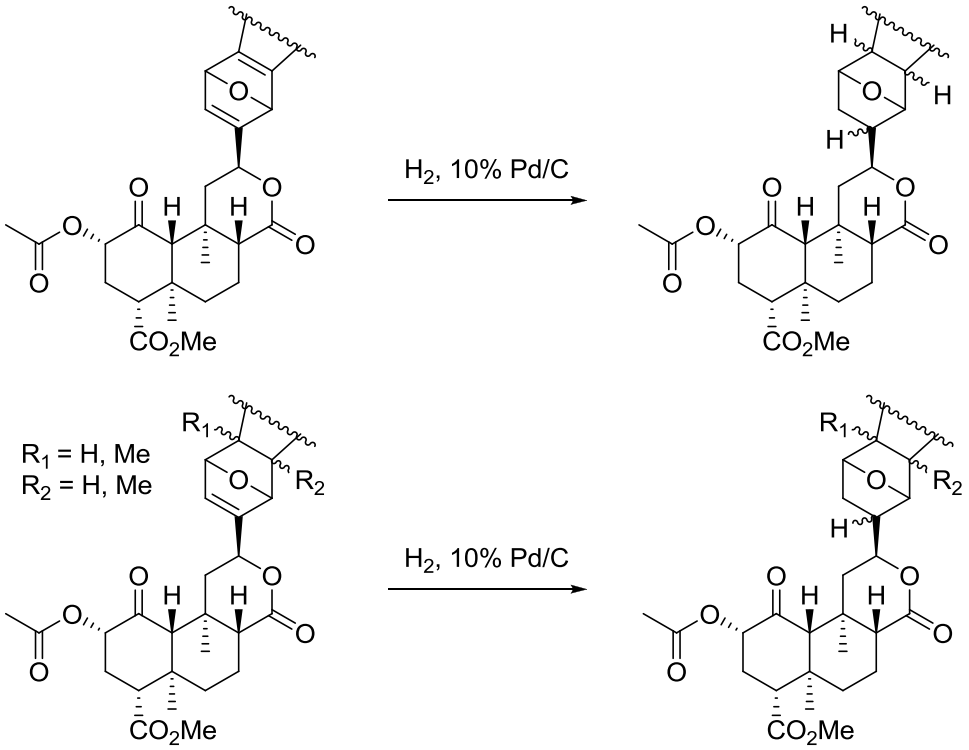



a) Respective diene, heat, neat

Upon isolation of the cycloaddition products derived from the reaction of salvinorin A with alkene dienophiles, the products were found to immediately degrade back to the respective starting materials. Degradation was noted via spotting an NMR sample onto a TLC plate and observing the reemergence of several different chemical entities. Clean NMR and HPLC spectra were unable to be obtained due to the regeneration of starting materials which introduced impurities into the samples. Even after HPLC purification, reinjection of the purified sample would begin to show additional peaks enter into the spectra, indicating that the cycloaddition products were not chemically stable rather than an initial purification problem. This dissociation of cycloadducts into respective starting materials may be due to the nature of the substituent and substitution pattern of the furan diene. Alkyl substituted furans have, when reacted with maleimide dienophiles, been shown to have an increased dynamic reversibility.[141] Additionally 3-substituted furans exhibit a decreased transition state free energy barrier making interconversion between cycloadduct and respective diene and dienophile easier.[141]

While the information gained about the reactivity of the salvinorin A furan ring was valuable for development of a reactive resin, it was also disappointing to produce multiple salvinorin analogues that were unable to be evaluated at opioid receptors due to instability. In an effort to gain some information from these analogues the cycloadducts were attempted to be "trapped" via reduction of the oxanorbornene and oxanorbornadiene systems that results from the Diels-Alder reaction of furan with an alkene **(Scheme 3)**.

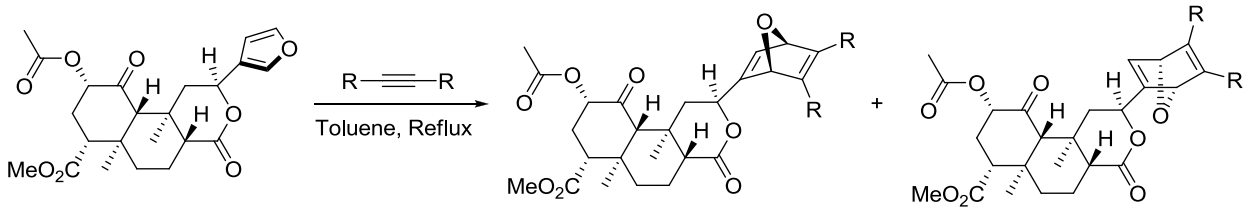
**Scheme 3:** Reduction of the oxanorbornadiene and oxanorbornene systems results in multiple isomers depending on the face hydrogen is delivered to




Although this did prove to be successful the resulting reduced systems proved to be very complex during structure elucidation. There are potentially four diastereomers that can be produced during the reduction, all of which would produce nearly identical spectroscopic data. These compounds were ultimately too hard to structurally verify even after attempts at inducing solid state NMR properties to gain residual dipolar coupling constants between unidentified protons.[142-144]

Additional complications can arise from the cycloaddition with unsymmetrical alkynes. Facial attack of asymmetric alkyne dienophiles leads to additional isomers during the formation of the oxanorbornadiene system **(Scheme 4)**.

**Scheme 4:** Cycloaddition to the top or bottom face of the salvinorin A furan ring forces the oxygen up or down resulting in two isomers



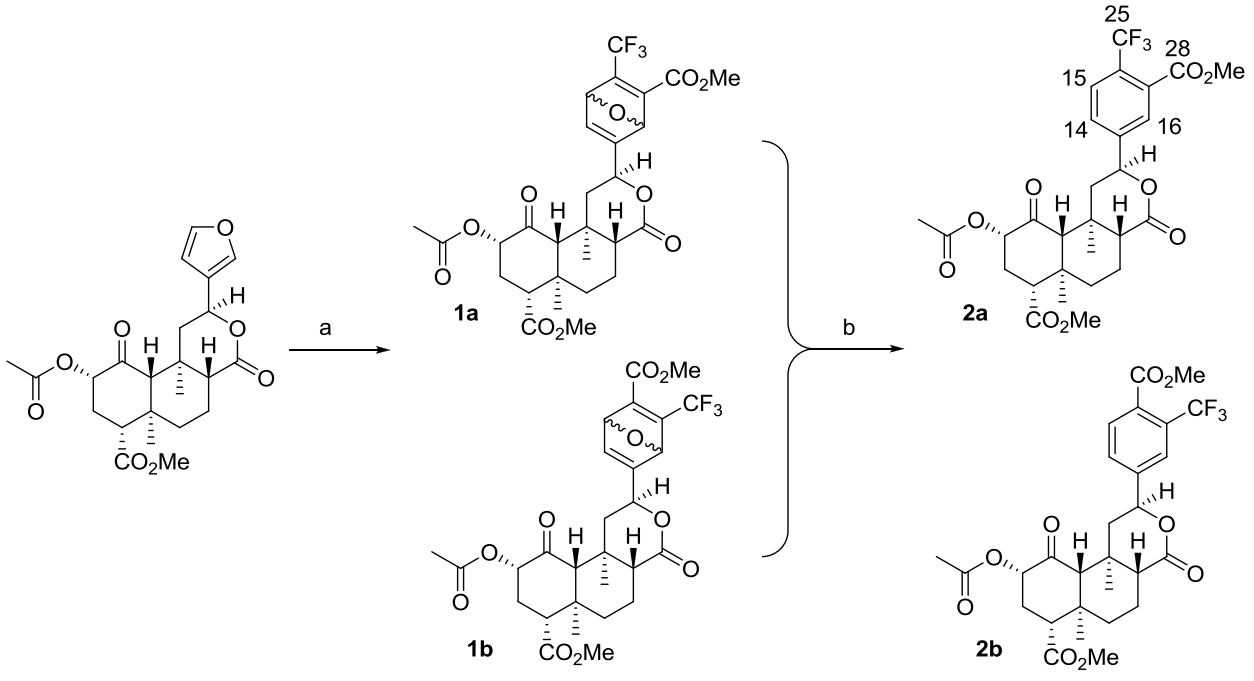

Asymmetric alkyne adducts were still unable to be separated similar to previously mentioned alkene adducts. The regiochemistry remained scrambled and required additional synthetic procedures before isolation was successful **(Scheme 5)**.

Reaction of salvinorin A with ethyl 4,4,4-trifluoro-2-butynoate in toluene at 95°C afforded **1a** and **1b** as a mixture of isomers in 48% yield. The isolated mixture was subsequently treated with $Fe_2(CO)_9$ in refluxing toluene to aromatize the oxanorbornadiene scaffold by deoxygenation. This process gave a mixture of isomers **2a** and **2b** which were separated by flash column chromatography using a gradient of 10 to 30% ethyl acetate in hexanes to afford **2a** in 3.6% yield and **2b** in 62% yield.

Determination of the regiochemistry relied heavily on NMR using the HMBC, HSQC, and COSY correlation of protons coupled to the splitting pattern observed in the fluorine coupled $^{13}C$ NMR. Structures **1a – 2b** were elucidated in a similar fashion to one another using the previously mentioned techniques. Specifically, in the case of structure **2a,** the three aromatic protons at δ 7.72, 7.69, and 7.50, which all correlate to aromatic carbons in the HSQC were identified first. Next the two protons at δ 7.72 and 7.50 were found to correlate to each other in the COSY spectrum, which would represent C-14 and C-15 **(Scheme 5)**. Further analysis of the COSY spectrum showed that the proton signal at δ 7.69 did not correlate to any other protons, indicating that it was attached to the C-16 position. Examination of the HMBC spectrum then showed that

the proton signal at δ 7.72 was correlated to C-25, C-27, and C-13, while the proton signal at δ 7.69 was correlated to C-28, C-26, and C-12. This would suggest that the COSY uncoupled proton signal from δ 7.69 is attached to C-16 and the COSY coupled proton signal from δ 7.72 is attached to C-15.

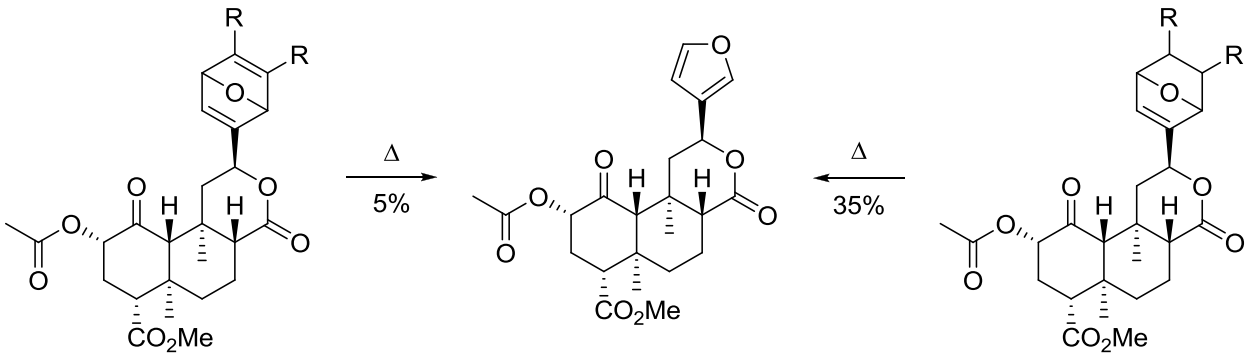
**Scheme 5:** Asymmetric alkynes lead to multiple isomers of difficult to separate mixtures




Reagents and conditions: a) ethyl 4,4,4-trifluoro-2-butynoate, tolene, 95°C b) $Fe_2(CO)_9$ , toluene, reflux

Applying the information gained through probing the Diels-Alder reactivity of the salvinorin A furan ring towards the development of a reactive polymer, it was decided to proceed with an alkyne linked to a solid supported reagent. While the retro Diels-Alder reaction proceeded in lower yields for cycloadducts generated from alkynes than from alkenes **(Scheme 6)**, an alkene dienophile could not be appended to the solid support due to the concentrations needed for the reaction to proceed.

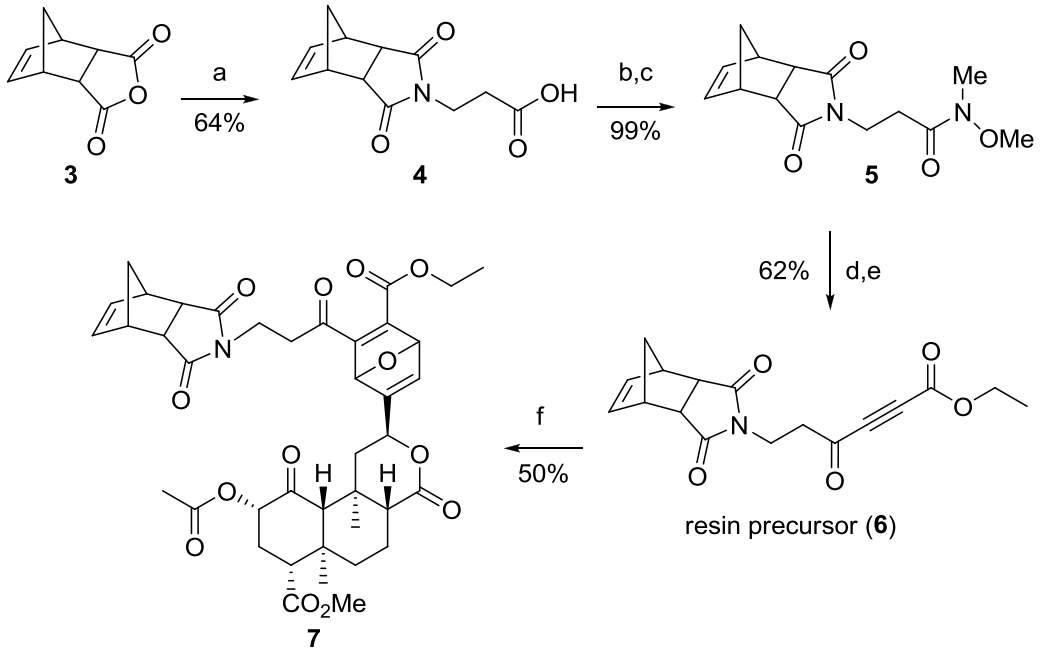
**Scheme 6:** Heating cycloadducts after isolation will regenerate salvinorin A




A high reaction concentration could not be used since a natural product extract is often very thick and would mechanically prevent the reaction rather than if it was chemically reactive or not. Additionally, to aid in the chemical reaction of the Diels-Alder cycloaddition it would be beneficial to have a polymer precursor that could be reacted with salvinorin A and then polymerized. Efforts were then focus on ring opening metathesis polymerization (ROMP) for its ability to perform a late-stage polymerization. A late-stage polymerization would allow for harsh reaction conditions, if needed, while generating a tailor made reactive "arm" that could be used to sequester salvinorin A.

One type of system that will undergo ROMP is the norbornene scaffold. The norbornene system had been previously shown to be useful in applications involving scavange-ROMP-filter and capture-ROMP-release techniques.[145, 146] With the idea of capture-ROMP-release or ROMP-catpture-release in mind for the molecular sequestration of salvinorin A from a plant extract via cycloaddition to the furan ring, this system was used as a building block for the generation of a reactive polymeric resin. Using the reactive properties of the salvinorin A scaffold towards alkyne dienophiles, a tether was modified for attachment to the norbornene system which contained an alkyne very similar to those known to react with salvinorin A **(Scheme 7)**.[50]

**Scheme 7:** Method for the synthesis of a selectively reactive resin shown to react with salvinorin A

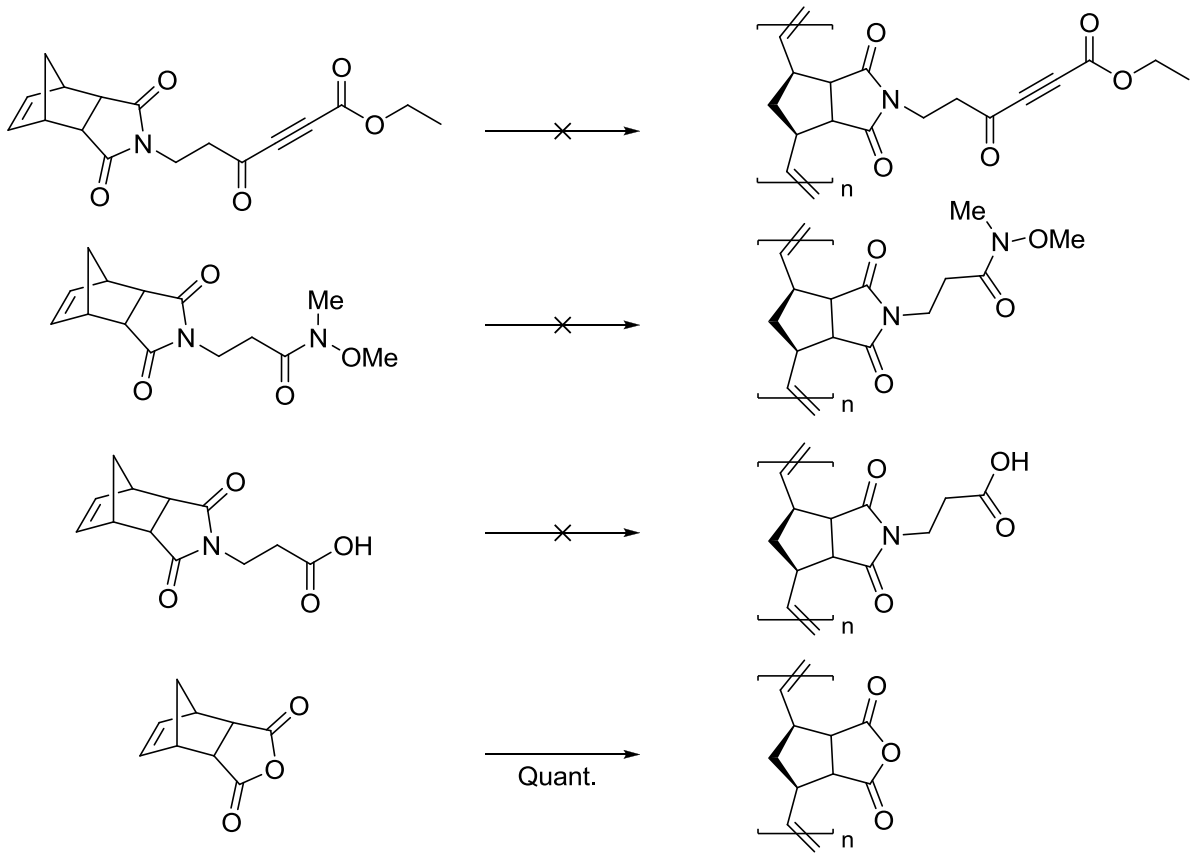



Reagents and Conditions: a) ß-alanine, toluene b) CDI, THF, 30min c) (MeO)MeNH$_2$Cl d) *n*-BuLi, THF, Ethyl Propiolate -78°C e) 5% HCl(aq) f) Salvinorin A, toluene, Δ

Slow addition of β-alanine to a solution of *cis*-5-norbornene-*endo*-2,3-dicarboxylic anhydride (**3**) in refluxing toluene yielded **4** in 65% yield, which could be used without purification. Compound **4** was then converted to the Weinreb amide via a 1,1'-carbonyldiimidazole mediated coupling to *N*,*O*-dimethylhydroxylamine to yield **5** in 99% yield, which could then be used without further purification. **5** was then converted to the resin precursor **6** by addition of a lithiate generated from ethyl propiolate and *n*-butyl lithium at -78°C. Resin precursor **6** was then used to test its reactivity towards Diels-Alder cycloaddition with the furan ring of salvinorin A as proof of concept that the polymerized resin would be adequate to link with salvinorin A. Compound **6** was reacted with salvinorin A under standard cycloaddition conditions using toluene and heat to produce cycloadduct **7** in 50% yield.

Starting from **3** allowed for an easy point of divergence by simply adding in any amine necessary. The resulting products could then be easily purified by extraction, which would eliminate the need for large-scale chromatograph. β-Alanine was selected since a two carbon linker between the functionality of the resin and polymeric backbone was believed to be enough space to allow for cycloaddition with salvinorin A as seen with the formation of **7**. With proof of concept in hand, the reactive resin precursor was subjected to ROMP conditions in hopes of generating a polymeric resin. However, after several attempts at polymerization, a polymeric resin was unable to be generated. Only at the initial stage of resin synthesis with carbic anhydride **1** was polymerization successful **(Scheme 8)**.

**Scheme 8:** Failed stages of ring opening metathesis polymerization during resin development



The failed attempts at ROMP were detrimental to project success. Without being able to start from the resin precursor there would be no way of knowing the loading capacity of the resin. While many solid phase reactions are monitored via disappearance of starting materials in infra red (IR) spectroscopy there would be no guarantee that disappearance of starting materials resulted in their addition to a polymerized resin. The subsequent chemical reactions would then be performed on faith hoping that the previous reaction had worked. Additionally any of the reactions during conversion of **4** to **6** that had not gone to completion would reduce yield, having been carried though the entire synthesis lowering the overall potential for loading capacity of salvinorin A onto the resin. Ideally the capture of salvinorin A onto solid support would occur with a resin of known loading capacity such that a percent recovery could be calculated when extracting salvinorin A from a plant extract. Minor attempts were conducted starting from a polymeric TentaGel resin of known loading capacity in which the polymeric backbone was insoluble, yet the chemically reactive "arms" were soluble in solution. However these arms, although soluble in solution, were unable to be seen on NMR without the use of a technique known as magic angle spinning. Therefore the use of a premade polymeric resin presented the same problems as the chemical synthesis of a custom resin.

## Conclusions and Future Directions

The chemical reactivity of the salvinorin A furan ring has been probed in efforts to understand its amenability towards reversible Diels-Alder cycloadditions. Reaction of salvinorin A with electron deficient alkynes formed more stable adducts than when reacted with electron deficient alkenes. However with asymmetric cycloadducts structure elucidation becomes very time consuming and product isolation required additional synthetic procedures before isolation was able to be achieved. Being unstable, alkene cycloadducts that were formed immediately

degraded but were also 30% more likely to perform a retro Diels-Alder reaction under heated conditions. Taking these results into account a reactive handle was designed which contained an electron deficient alkyne similar to those known to form cycloadducts with the salvinorin A furan ring.

The design of a reactive handle included the possibility that it could be polymerized into a functional resin. Upon generation of a functional resin precursor, it was reacted with salvinorin A and shown to form a stable cycloadduct. With the proof of concept in hand, the resin precursor was attempted to be polymerized. However, attempts at polymerization resulted in no reaction at any step along the synthetic pathway other than at the first step.

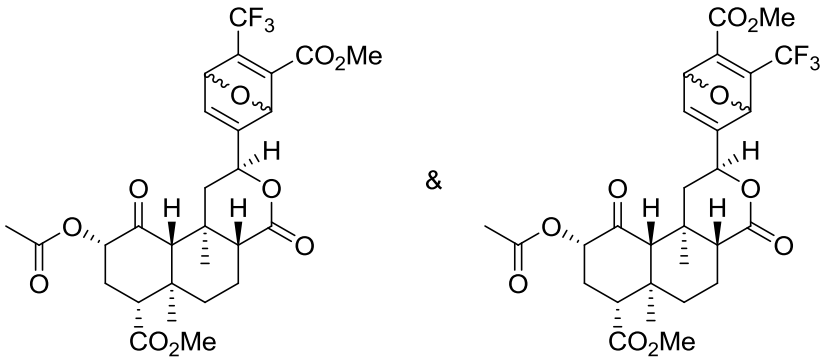
While attempts at polymerization were unsuccessful, future work should focus on utilizing a premade polymer that can be functionalized directly in a single step to include an electron deficient alkyne for selective reaction with the salvinorin A furan ring. While this approach will encounter many of the same problems for product identification, a single step addition to a premade polymer would be amendable to using a large excess of reagent in order to drive the reaction to completion. However, a large excess approach in a multi-step synthesis is still subject to miscalculation of the resin loading capacity due to small amounts of unreacted material carried through the procedure. Using a premade polymer also has the advantage to be totally contained within a "teabag" for sequestering of the resin. This would be useful because the oxanorbornadiene systems that were being explored can potentially be soluble in some solvents. In this case, if the resin dissolved out of the "teabag" it would be lost to the plant extract, unable to be recovered in a selective fashion.

## Experimentals

All reagents purchased from chemical suppliers were used without further purification and reactions monitored using Thin-layer chromatography (TLC) on 0.25 mm Analtech GHLF silica gel plates using EtOAc/*n*-hexanes and visualized at 254 nm. Column chromatography was performed on silica gel (40 – 63 μm particle size, 230-400 mesh) from Sorbent Technologies (Atlanta, GA). NMR spectra were recorded on either a Bruker DRX-400 with a H/C/P/F QNP gradient probe or a Bruker Avance AV-III 500 with a dual carbon/proton cryoprobe using δ values in ppm as standardized from tetramethylsilane (TMS) and *J* (Hz) assignments for $^{1}H$ resonance coupling and $^{13}C$ fluorine coupling. High resolution mass spectrometry data were collected on a LCT Premier (Waters Corp.) time-of-flight mass spectrometer. Analytical HPLC was performed on an Agilent 1100 Series Capillary HPLC system with diode array detection at 254.8 nm on an Agilent Eclipse XDB-C18 column (4.6 × 150 mm, 5 mm) with isocratic elution in 60% $CH_3CN$/40% $H_2O$ at a flow rate of 5.0 mL/min unless otherwise noted.

**General procedure for the cycloaddition of salvinorin A with alkynes:**

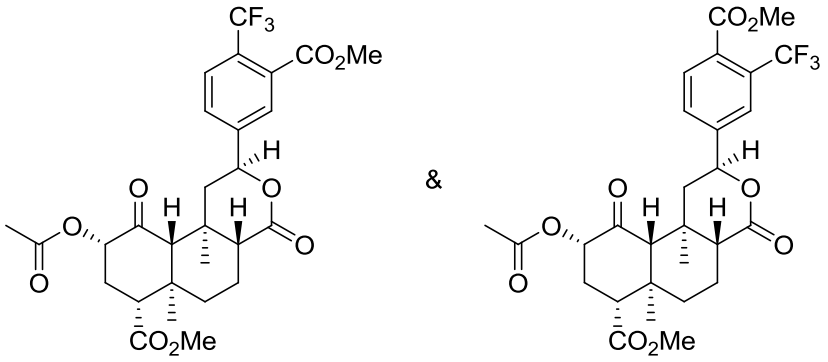
A solution of salvinorin A (0.462 mmol), appropriate alkyne (0.554 mmol), and toluene was allowed to stir at room temperature until homogeneous. The solution was then slowly heated to reflux over the course of an hour and allowed to stir overnight at reflux. The solvent was removed under reduced pressure and the resulting residue was subjected to flash column chromatography using a mixture of EtOAc/hexanes.



**(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-methyl 9-acetoxy-2-(5-(ethoxycarbonyl)-6-(trifluoromethyl)-7-oxabicyclo[2.2.1]hepta-2,5-dien-2-yl)-6a,10b-dimethyl-4,10-dioxododecahydro-1*H*-benzo[*f*]isochromene-7-carboxylate (1a) and (2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-methyl 9-acetoxy-2-(6-(ethoxycarbonyl)-5-(trifluoromethyl)-7-oxabicyclo[2.2.1]hepta-2,5-dien-2-yl)-6a,10b-dimethyl-4,10-dioxododecahydro-1*H*-benzo[*f*]isochromene-7-carboxylate (1b)**.

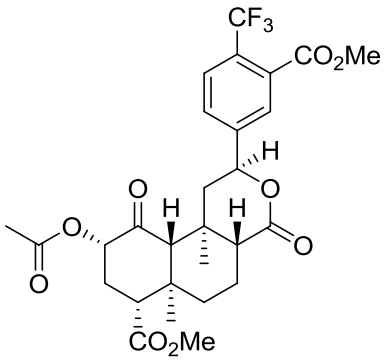
Asymmetric cycloadducts **1a** and **1b** were synthesized as described by the general procedure using ethyl 4,4,4-trifluoro-2-butynoate to afford 402 mg of the combined mixture (48%) as a white powder, mp 119 – 121 °C; $^{1}$H NMR (500 MHz, $CDCl_3$) δ 6.91 (dd, *J* = 1.9, 3.9, 1H), 5.76 (s, 1H), 5.64 (dt, *J* = 1.8, 5.5, 1H), 5.61 (s, 1H), 5.39 (dd, *J* = 4.7, 11.0, 1H), 5.33 – 5.27 (m, 1H), 5.15 (dd, *J* = 11.0, 19.9, 1H), 4.35 – 4.23 (m, 3H), 3.73 (s, 4H), 2.79 – 2.72 (m, 1H), 2.43 (td, *J* = 5.4, 13.3, 1H), 2.31 (dd, *J* = 4.3, 10.6, 2H), 2.17 (d, *J* = 2.5, 5H), 2.13 (s, 1H), 2.09 (d, *J* = 8.7, 1H), 2.03 (d, *J* = 12.3, 1H), 1.78 (d, *J* = 10.1, 1H), 1.61 (dd, *J* = 12.4, 21.2, 5H), 1.54 – 1.46 (m, 2H), 1.41 (s, 4H), 1.37 – 1.29 (m, 4H), 1.11 (s, 4H); $^{13}$C NMR (126 MHz, $CDCl_3$) δ 202.0, 171.54, 171.53, 170.7, 170.4, 170.0, 169.9, 162.0, 161.5, 158.5, 157.9, 151.3 (q, $^{3}J_{CF}$ = 4.66), 151.0 (q, $^{2}J_{CF}$ = 35.57), 150.8 (q, $^{3}J_{CF}$ = 4.87), 137.4, 135.8, 121.5 (q, $^{1}J_{CF}$ = 269.57), 121.4 (q, $^{1}J_{CF}$ = 269.63), 85.7 (d, $^{3}J_{CF}$ = 2.67), 84.5 (d, $^{3}J_{CF}$ = 2.52), 75.0, 74.9, 73.9, 72.7, 64.0, 64.0, 62.2, 62.1, 53.50, 53.47, 52.0, 51.2, 51.0, 42.04, 41.99, 41.1, 40.4, 38.02, 37.96, 35.5, 35.3, 30.8, 30.7,

20.61, 20.58, 18.12, 18.08, 16.4, 15.3, 15.1, 13.9. HRESIMS (*m*/*z*): [M+Na] calculated for $C_{29}H_{33}F_3O_{10}Na$, 621.1924; found, 621.1986. HPLC **1a** $t_R$ = 12.858 min; purity = 91.33%; **1b** $t_R$ = 11.759 min; purity = 8.67%.

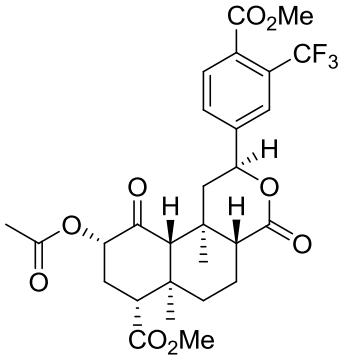



**(2S,4aR,6aR,7R,9S,10aS,10bR)-methyl 9-acetoxy-2-(3-(ethoxycarbonyl)-4-(trifluoromethyl)-phenyl)-6a,10b-dimethyl-4,10-dioxododecahydro-1H-benzo[f]isochromene-7-carboxylate (2a) and (2S,4aR,6aR,7R,9S,10aS,10bR)-methyl 9-acetoxy-2-(4-(ethoxycarbonyl)-3-(trifluoromethyl)-phenyl)-6a,10b-dimethyl-4,10-dioxododecahydro-1H-benzo[f]isochromene-7-carboxylate (2b).**

A solution of **1a** and **1b** (200 mg, 0.33 mmol), $Fe_2(CO)_9$ (150 mg, 0.41 mmol), and toluene (15 mL) was allowed to stir at 60 °C for 20 minutes. Once the solution turned black, it was gradually heated to reflux and allowed to stir for 2 hours. The solution was filtered through a pad of celite and solvent was removed under reduced pressure. The residue was purified by column chromatography (gradient eluent: 10% ethyl acetate/90% *n*-hexanes - 30% ethyl acetate/70% *n*-hexanes) to afford 121 mg of **2a** (62%) and 8.2 mg of **2b** (4.2%).




**2a:** white powder, mp 110-112 °C, $^{1}$H NMR (500 MHz, $CDCl_3$) δ 7.72 (d, *J* = 8.2, 1H), 7.69 (s, 1H), 7.50 (d, *J* = 8.0, 1H), 5.62 (dd, *J* = 5.0, 12.0, 1H), 5.13 – 5.06 (m, 1H), 4.40 (q, *J* = 6.9, 2H), 3.73 (s, 3H), 2.75 – 2.71 (m, 1H), 2.56 (dd, *J* = 5.1, 13.6, 1H), 2.30 (dd, *J* = 7.8, 13.5, 2H), 2.24 – 2.18 (m, 1H), 2.16 (s, 3H), 2.13 (d, *J* = 8.3, 2H), 1.82 (d, *J* = 13.2, 1H), 1.66 (dd, *J* = 14.2, 26.0, 2H), 1.52 (s, 3H), 1.49 – 1.43 (m, 1H), 1.39 (t, *J* = 7.2, 3H), 1.13 (s, 3H). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 202.0, 171.5, 170.8, 169.9, 166.5, 144.4, 132.2 (q, $^{3}J_{CF}$ = 1.80), 128.4 (q, $^{2}J_{CF}$ = 32.99), 127.8, 127.2 (q, $^{3}J_{CF}$ = 5.20), 127.0, 123.2 (q, $^{1}J_{CF}$ = 273.41), 77.6, 75.0, 63.8, 62.3, 53.6, 52.0, 51.6, 45.1, 42.1, 38.1, 35.8, 30.7, 20.6, 18.1, 16.4, 15.2, 13.9. HRESIMS (m/z): [M+Na] calculated for $C_{29}H_{33}F_3O_9$, 605.1974; found 605.1959. HPLC $t_R$ = 16.767 min; purity = 99.93%.



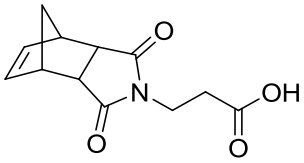

**2b:** white powder, mp 100-102 °C, $^{1}$H NMR (500 MHz, $CDCl_3$) δ 7.78 (d, *J* = 8.0, 1H), 7.66 (s, 1H), 7.51 (d, *J* = 8.0, 1H), 5.62 (dd, *J* = 5.0, 12.0, 1H), 5.12 – 5.07 (m, 1H), 4.39 (q, *J* = 7.1, 2H), 3.73 (s, 3H), 2.78 – 2.71 (m, 1H), 2.55 (dd, *J* = 5.1, 13.6, 1H), 2.30 (dd, *J* = 7.7, 13.5, 2H),

2.21 (d, $J$ = 13.9, 1H), 2.17 (d, $J$ = 5.8, 3H), 1.82 (d, $J$ = 13.2, 1H), 1.69 (d, $J$ = 14.4, 1H), 1.60 (s, 3H), 1.52 (s, 3H), 1.46 (t, $J$ = 12.8, 1H), 1.38 (t, $J$ = 7.1, 3H), 1.14 (s, 3H). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 202.0, 171.5, 170.8, 169.9, 166.4, 143.7, 131.3 (q, $^3J_{CF}$ = 1.83), 130.8, 129.4 (q, $^2J_{CF}$ = 32.66), 128.6, 123.7 (q, $^3J_{CF}$ = 5.38), 123.1 (q, $^1J_{CF}$ = 273.83), 77.7, 75.0, 63.8, 62.2, 53.6, 52.1, 51.7, 45.1, 42.1, 38.1, 35.9, 30.7, 20.6, 18.1, 16.5, 15.2, 13.9 HRESIMS (m/z): [M+Na] calculated for $C_{29}H_{33}F_3O_9$, 605.1974; found 605.1979. HPLC $t_R$ = 16.705 min; purity = 89.59%.

**General procedure for the cycloaddition of salvinorin A with alkenes:**

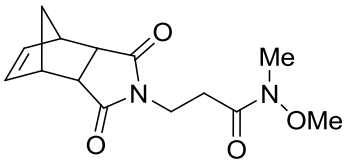
Salvinorin A (0.231 mmol) and an excess of appropriate alkene were mixed heterogeneously in a 2 ml conical vial and slowly heated in an oil bath until the mixture became homogeneous, additional alkene was added as necessary until the salvinorin A was completely dissolved. The resulting mixture was stirred warm for 2 hours at which time the solution was diluted with THF (5 ml), 10% Pd/C (20 mg) added, and charged with $H_2$. The resulting solution was allowed to stir overnight at room temperature. The solution was then filtered through a pad of celite and the resulting filtrate concentrated under reduced pressure to a solid residue. The residue was then purified by flash column chromatography using a mixture of EtOAc/hexanes.

**Procedure for the synthesis of resin precursor:**



**(4)**

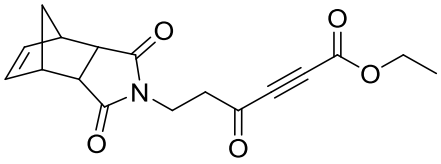
To a solution of *cis*-5-Norbornene-*endo*-2,3-dicarboxylic anhydride (7.39 g, 45 mmol) in refluxing toluene was added β-alanine (8.02 g, 90 mmol) over the course of 15 minutes. The solution was then allowed to cool just below reflux and the reaction vessel fitted with a dean stark apparatus at which time the solution was brought back to reflux and allowed to stir overnight. To the solution was added 1 M HCl solution until the flask was free of solid particulates. The remaining toluene was then removed under reduced pressure and the resulting solution was extracted with $CH_2Cl_2$ and the combined organics dried over $Na_2SO_4$. $Na_2SO_4$ was removed via filtration and the filtrate concentrated under reduced pressure to yield 4 as a white solid (6.82 g, 64.4 %) which was used without further purification. Spectral data matches previously reported results.[147]



**(5)**

To a stirring solution of **(4)** (6.64 g, 28.23 mmol) in THF (70 ml) was added 1,1'-Carbonyldiimidazole (5.03 g, 31.05 mmol) and the resulting solution was allowed to stir at room temperature. After 1 hour, *N*,*O*-Dimethylhydroxylamine (3.17 g, 32.46 mmol) was added and allowed to stir overnight. Solvent was removed under reduced pressure and the resulting solid

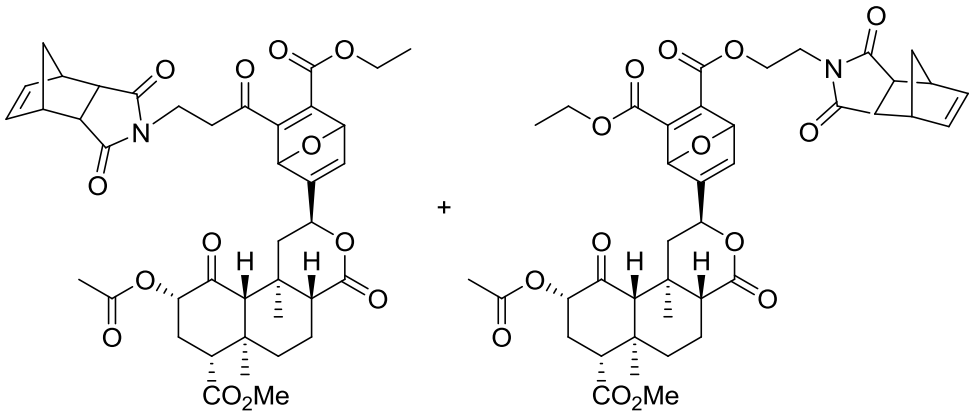
was flushed with excess water and extracted with EtOAc. The combined organics were dried ($Na_2SO_4$) and the solvent was removed under reduced pressure to yield **5** as a white solid (7.767 g, 98.8 %) which was used without further purification. $^1$H NMR (500 MHz, Chloroform-*d*) δ 6.10 (s, 2H), 3.70 – 3.62 (m, 5H), 3.38 (d, *J* = 1.5 Hz, 2H), 3.26 (dd, *J* = 2.8, 1.5 Hz, 2H), 3.15 (s, 3H), 2.60 (t, *J* = 6.9 Hz, 2H), 1.73 (dd, *J* = 8.8, 1.6 Hz, 1H), 1.53 (d, *J* = 8.8 Hz, 1H) $^{13}$C NMR (126 MHz, $CDCl_3$) δ 177.49, 171.47, 171.45, 171.43, 134.41, 61.27, 52.17, 45.77, 44.91, 34.08, 32.18, 32.15, 29.98 m.p. (88-89 °C)




**(6)**

To an anhydrous solution of ethyl propiolate (0.546 g, 5.388 mmol) stirring in THF (30 ml) at -78 °C was added *n*-BuLi (3.367 ml, 5.388 mmol) dropwise and allowed to stir. After 15 minutes an anhydrous solution of **(5)** (0.500 g, 1.796 mmol) in THF (6 ml) was added dropwise and the combined solutions allowed to stir at -78 °C for 1.5 hours. The reaction was then quenched with 15 ml of 1 M HCl, allowed to warm to room temperature, and extracted with EtOAc. The combined organics were dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure to yield an orange oil. The oil was then subjected to flash column chromatography using a mixture of EtOAc/Hexanes (4:6) to yield **6** as a yellow oil (349 mg, 61.6%). $^1$H NMR (500 MHz, Chloroform-*d*) δ 6.10 (d, *J* = 1.7 Hz, 2H), 4.31 (q, *J* = 7.1 Hz, 2H), 3.69 (t, *J* = 7.1 Hz, 2H), 3.39 (s, 2H), 3.30 – 3.22 (m, 2H), 2.84 (t, *J* = 7.1 Hz, 2H), 1.74 (d, *J* = 8.8 Hz, 1H), 1.54 (d, *J* = 8.8 Hz, 1H), 1.34 (t, *J* = 7.1 Hz, 3H) $^{13}$C NMR (126 MHz, $CDCl_3$) δ 182.47, 177.22, 152.00, 134.50, 79.68, 78.85, 63.12, 52.24, 45.73, 45.00, 42.55, 32.53, 13.92

**Cycloaddition of Salvinorin A with Resin Precursor:**



**(7)**

A solution of salvinorin A (200 mg, 0.462 mmol), resin precursor **(6)** (200 mg, 0.634 mmol), and toluene was allowed to stir at room temperature until homogeneous. The solution was then slowly heated to reflux over the course of an hour and allowed to stir overnight at reflux. The solvent was removed under reduced pressure and the resulting residue was subjected to flash column chromatography using a mixture of EtOAc/hexanes (3:1) to yield **7** as a white solid (173 mg, 50.1%). $^{1}$H NMR (500 MHz, Chloroform-*d*) δ 6.98 – 6.76 (m, 1H), 6.10 (ddt, *J* = 6.6, 5.0, 2.1 Hz, 2H), 5.82 – 5.51 (m, 2H), 5.51 – 5.03 (m, 2H), 4.38 – 4.18 (m, 2H), 3.73 (d, *J* = 3.2 Hz, 3H), 3.70 – 3.62 (m, 2H), 3.38 (dq, *J* = 3.8, 1.8 Hz, 2H), 3.25 (ddd, *J* = 9.9, 3.0, 1.6 Hz, 2H), 3.20 – 3.00 (m, 2H), 2.79 (ddd, *J* = 56.8, 11.9, 4.7 Hz, 1H), 2.36 – 2.25 (m, 3H), 2.25 – 2.19 (m, 1H), 2.19 – 2.14 (m, 4H), 1.81 – 1.70 (m, 2H), 1.66 – 1.51 (m, 5H), 1.42 – 1.24 (m, 8H), 1.10 (d, *J* = 3.3 Hz, 3H), 0.98 – 0.82 (m, 1H). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 202.15, 201.86, 196.91, 177.37, 177.35, 177.30, 177.27, 171.62, 171.41, 170.96, 169.80, 161.95, 159.76, 157.76, 150.76, 135.30, 134.36, 134.34, 86.13, 85.89, 85.35, 74.84, 74.81, 74.07, 63.55, 61.97, 61.75, 53.37, 53.06, 52.03, 51.88, 51.83, 50.49, 45.68, 45.65, 45.62, 44.85, 44.83, 44.80, 41.90, 41.81, 41.24,

40.65, 37.83, 35.34, 35.31, 33.29, 30.67, 20.53, 20.48, 18.00, 16.24, 16.22, 15.12, 14.86, 14.02, 13.97.

# CHAPTER IV

## Quantification of Salvinorin A

In order to proceed with the investigations of the biological, psychological, and pharmacokinetic effects salvinorin A imparts, a need for detection of salvinorin A in human and non-human primate biological fluids has arisen. Several methods have been developed for the detection of salvinorin A in human biological fluids.[148-153] Other methods have focused on the quantification of salvinorin A in non-human fluids, herbal products related to *S. divinorum*, or directly from the extract or leaves of *S. divinorum*.[61, 65, 150, 153-158]

One study quantified salvinorin A from a screen of commercially available leaves and leaf extracts of *S. divinorum*.[61] In this study, Wolowich and coworkers found that samples from commercial sources contained significantly lower amounts of salvinorin A than advertised, ranging from 1 to 16% of the claimed amount. Additionally three of the five samples obtained contained contaminates of caffeine or vitamin E not normally found in *S. divinorum* or its extracts. HPLC-UV/Vis was used to analytically quantify salvinorin A at 208 nm while the caffeine and vitamin E were identified by TLC-GC/MS. This analysis method was first reported by Gruber and coworkers in 1999.[157] This method however did not establish a limit of quantification, but was able to accurately measure amounts from 0.255 to 1.53 μg, as seen from linear plots of the average peak height that achieved a correlation coefficient of 0.9997.

Similar studies have been performed to investigate salvinorin A concentrations from leaves and extracts of *S. divinorum* even where salvinorin A and closely related neoclerodane diterpenes are produced in *S. divinorum* plants. Siebert in 2004 published a method for analyzing

chloroform extracts of fresh leaves by using TLC.[158] It was concluded that salvinorin A and its closely related compounds are secreted as components of a complex resin that accumulates in the peltate glandular trichomes, specifically in the subcuticular space between the trichome head cells and the enclosing cuticle. Another study by Tsujikawa and coworkers used LC/MS in product ion scan mode to detect salvinorin A and salvinorin B and quantify them by HPLC-UV/Vis from extract products circulating in Japan.[154] After analyzing nine dried leaf and extract products salvinorin A and salvinorin B were found in be in the range of 3.2 to 5.0 / 0.10 to 0.17 μg/ml respectively for dried leaf products and 4.1 to 38.9 / 0.26 to 2.42 μg/ml respectively in the extract products. Later in 2006 Medana and coworkers switched to a liquid chromatography method coupled to electrospray ionization multistage ion trap mass spectrometry (LC/ESI-IT-MS) to identify salvinorins and divinatorins in *S. divinorum* leaves.[153] The plant leaves were extracted with a mixture of acetonitrile/water (1:1) microfiltered and then subjected to LC/MS. The limit of detection (LOD) varied for each of the salvinorin and divinatorin compounds identified, however the reported LOD for salvinorin was found to be 3 ng/ml.

Other types of analysis have also been performed for the quantification of salvinorin A including two-dimensional gas chromatography–time of flight mass spectrometry (GC x GC-ToFMS) analysis of salvinorin A in plants, water, and urine.[150] This study produced a LOD similar to LC-MS methods at 5 ng/ml with a linear range from 8 to 500 ng/ml. The benefit of using GC x GC was seen in the narrow time range of peak elutions, which allowed for effective separation of peaks of interest from matrix and chromatographic interferants. Additionally, this method only provides information on human samples that were spiked *ex vivo* with salvinorin A rather than collection of urine after administration of salvinorin A. Other studies have been conducted using the urine of participants who were believed to have consumed *S. divinorum*

after observation of eventually intoxicated patients.[148] Using microextraction by packed sorbent (MEPS) and GC-MS/MS Morano and coworkers also achieved LOD and linear ranges comparable to previously reported results. However by using MEPS instead of the more common extraction technique of solid phase extraction (SPE), they were able to reuse a single packed syringe up to 80 times.

There have been four attempts at the quantification of salvinorin A out of human blood or blood plasma. One study by McDonough and coworkers validated their method for blood by using certified drug-free urine instead of certified drug-free blood in hopes to prevent blood esterases converting salvinorin A into salvinorin B on the basis that salvinorin A has a short half life.[149] The group used LC-MS as their analytical method and had similar LOD and linear ranges as previously reported results. They later claim to investigate a unique case of salvinorin A testing where only blood was available for analysis, but do not provide any of the results. Another method was developed for detection of salvinorin A in plasma, urine, saliva, and sweat using GC-MS after extraction from their respective matrices.[159] In this study Pichini and coworkers administered, via smoking, 75 mg of dried *S. divinorum* leaves containing 0.58 mg of salvinorin A to two participants. Unfortunately, both individuals refused to have blood samples drawn due to a vivid hallucination stage after smoking the dried leaves. However, salvinorin A was detected in saliva and urine but not in sweat after administration, indicating the mode of excretion is either via metabolism into salvinorin B or direct elimination in saliva and urine. Most recently Margalho and coworkers validated a procedure for the detection and quantification of salvinorin A in pericaridial fluid, vitreous humor, whole blood and plasma using GC-MS.[160] They report linear ranges from 5.0 to 100 ng/mL with determination coefficients higher than 0.99 in each matrix with a LOD and LLOQ value of 5.0 ng/mL. Beyond new matrices (pericardial

fluid, vitreous humor, and whole blood) for detection and quantification of salvinorin A, this method didn't improve on the previously known LLOQ or techniques and instrumentation for analysis. Perhaps the most successful method was conducted by Schmidt and coworkers in 2005.[151] Blood and cerebrospinal fluid (CSF) samples were used from a male rhesus monkey never exposed to salvinorin A or *S. divinorum*. The only major drawback to this study is that samples were artificially spiked with salvinorin A after collection. In this fashion a metabolism study was done by incubating salvinorin A in blood and periodically sampling for concentrations of salvinorin A and salvinorin B. After 30 minutes the concentration of salvinorin B exceeded the concentration of salvinorin A, suggesting blood esterases rapidly perform this transformation. A linear range was reported between 2 and 100 ng/ml with a limit of quantification (LOQ) of 2 ng/ml according to FDA guidelines. This method was then applied to the quantification of salvinorin A in the CSF of rhesus monkeys.[89]

As these methods had used artificially spiked samples of human plasma and non-human primate CSF, efforts were then begun to develop a reliable and reproducible LC-MS/MS method for the quantification of salvinorin A from unspiked biological fluids. With a reliable method for quantification, investigations into the pharmacokinetic profile of salvinorin A in humans and non-human primates could then be explored. Beginning attempts for elucidation of the salvinorin A pharmacokinetic profile would be focused on its duration of action within a biological system by creation of time-course graphs to show the *in vivo* concentrations of salvinorin A after dosing. As salvinorin A is also CNS active, the anecdotal short duration of action would also be investigated by testing whether or not salvinorin A is a Pgp substrate, which could explain its rapid entry and exit from the CNS.

## Results and Discussion

The behavioral effects of salvinorin A in rhesus monkeys, the central nervous system (CNS) levels of salvinorin A and its sensitivity to modulation by the P-glycoprotein (Pgp) transporter in the blood brain barrier were investigated.[161] Previous *in vitro* work with salvinorin A had suggested it may be a substrate of Pgp, being effectively effluxed from the CNS, thus giving rise to its short duration of action.[162] To investigate whether salvinorin A was a Pgp substrate in an *in vivo* system, the competing Pgp substrate loperamide (0.032-0.32 mg/kg) and the selective Pgp inhibitor tariquidar (0.32-3.2 mg/kg) were administered independently to examine their effects on the unconditioned behavioral effects salvinorin A imparts. Control experiments with single doses of salvinorin A (0.0032 – 0.01 mg/kg) yielded non-zero scores for both ptosis and facial relaxation with a fast onset of action. In another control experiment, U69,593 known to be a very poor Pgp substrate and potent KOP receptor agonist was not affected upon pretreatment with loperamide or tariquidar. Upon pretreatment with either loperamide or tariquidar, a dose-dependent enhancement of salvinorin A-induced ptosis, but not facial relaxation was observed **(Figures 7 & 8)**.

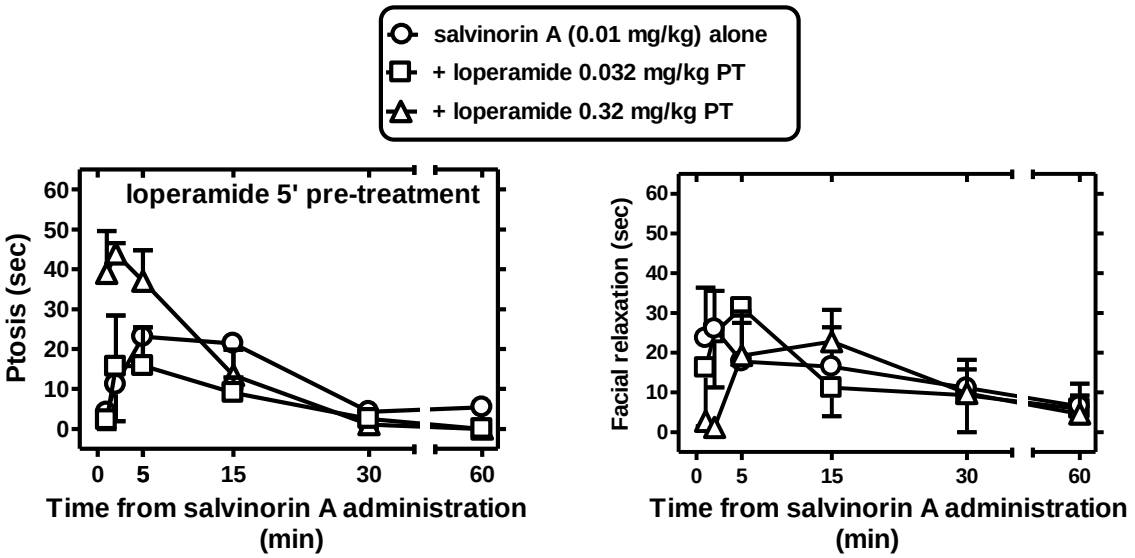



**Figure 7:** 5-minute Pretreatment with loperamide (0.032 – 0.32 mg/kg) effects salvinorin A (0.01 mg/kg) induced ptosis, but not facial relaxation.[161]

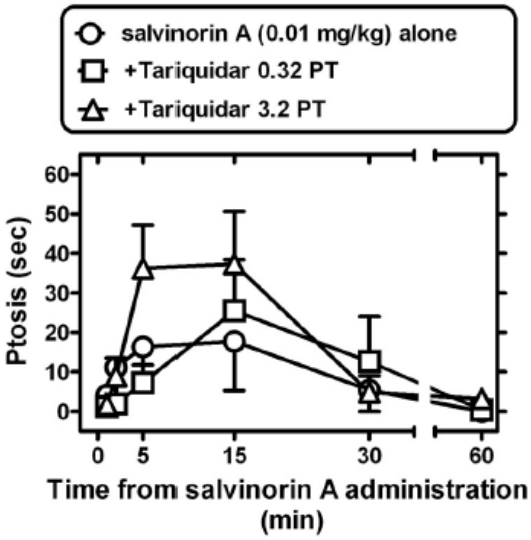



**Figure 8:** 30-minute Pretreatment with tariquidar (0.32 – 3.2 mg/kg) effects salvinorin A (0.01 mg/kg) induced ptosis, facial relaxation data not shown.[161]

At the largest doses of loperamide (0.32 mg/kg) and tariquidar (3.2 mg/kg) alone, there was no effect on ptosis or facial relaxation. However upon pretreatment with these agents for the proper time period they elicited a dose-dependent increase in salvinorin A induced ptosis but not facial relaxation. Cerebrospinal fluid (CSF) samples were also analyzed via LC/MS/MS and the amount of salvinorin A present was quantified in experiments with and without pretreatment with the Pgp inhibitor tariquidar. It was shown that upon pretreatment with tariquidar (3.2 mg/kg i.v.) enhanced peak levels of salvinorin A in CSF were observed, making this the first *in vivo* model to show the sensitivity of salvinorin A effects by modulation of Pgp **(Figure 9)**.

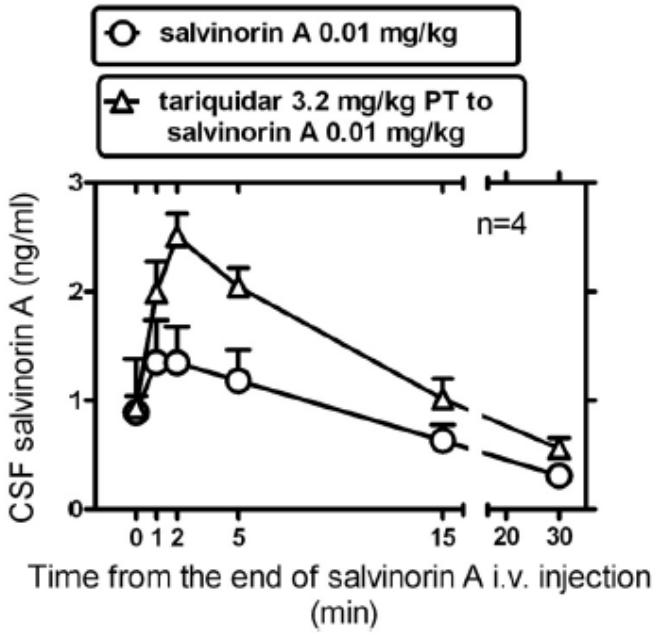



**Figure 9:** Pretreatment with tariquidar (3.2 mg/kg) increases peak salvinorin A (0.01 mg/kg) levels within the CSF

Salvinorin A (0.01 mg/kg) exhibited a profile of consistent CSF levels after injection of salvinorin A in each subject, even immediately after intravenous injection, likely due to the time taken to administer salvinorin A intravenously. Peak salvinorin A levels were recorded at 1 and 2

minutes after administration and declined to <25% of peak values by 30 minutes post administration. Upon pretreatment with tariquidar a similar time course profile was observed but with higher recorded CSF levels. Peak levels of salvinorin A post tariquidar pretreatment were noted as being an approximately 90% increase over levels recorded for salvinorin A alone.

While CSF samples are an acceptable biological fluid to use when working with rhesus monkeys, CSF is not an ideal source for quantification of salvinorin A in humans. The standard biological fluid to use when investigating pharmacokinetics in humans is blood plasma. The ease of acquiring, quantity, and versatile working conditions make blood plasma an ideal medium for the investigation of salvinorin A action *in vivo* with human participants.

In a trial of seven human participants who smoked salvinorin A, plasma samples were collected at multiple times and various intervals (t = -2, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 60, 90 min) in attempts to link the salvinorin A drug profile to the amount present in the blood plasma. Upon quantification and plotting salvinorin A can be found in blood plasma with a similar rapid increase in concentration and sharp decline in concentration as found in the rhesus monkey CSF. The peak salvinorin A concentrations found in blood plasma were also similar in profile (1 to 3 minutes after smoking) which is consistent with the observed pharmacokinetic profile of salvinorin A **(Figure 10)**.

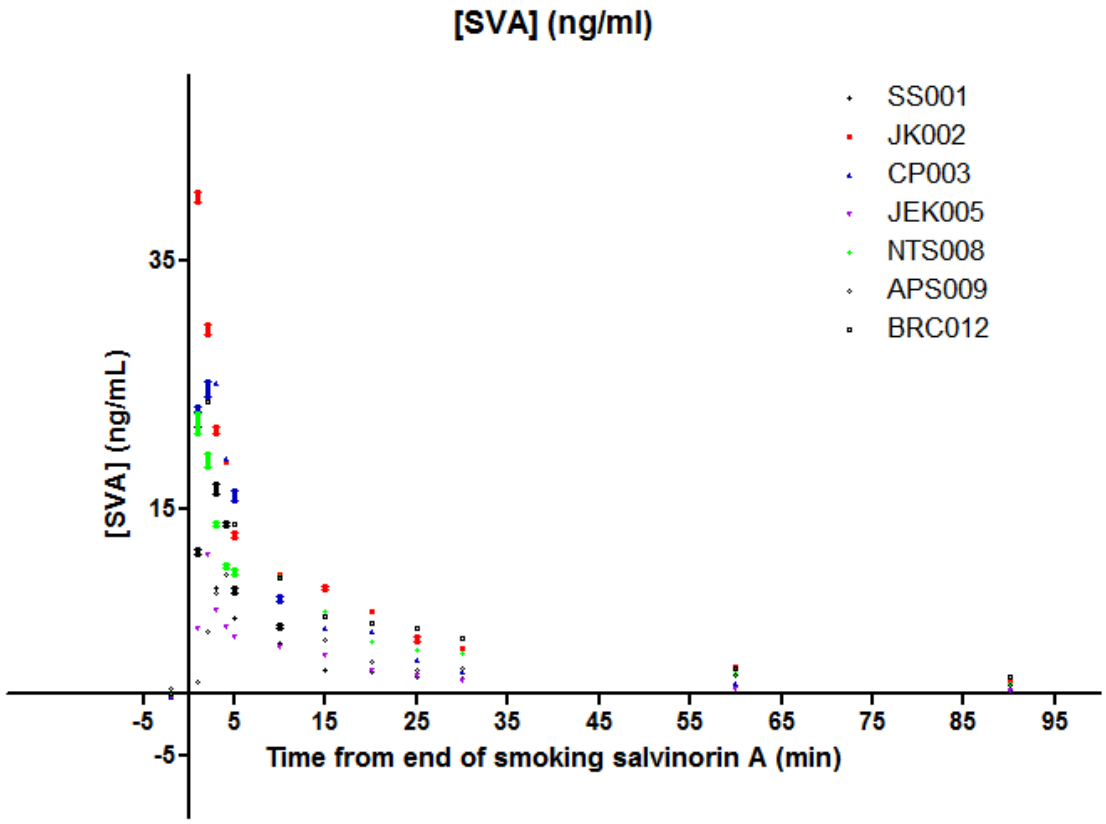



**Figure 10:** Salvinorin A blood plasma concentrations for seven human participants over a 90 minute time frame after smoking 18.0 μg / kg (2 participants) or 21.0 μg / kg (5 participants)

While a previous method has reported the detection of salvinorin A in CSF following i.v. administration, and in blood plasma samples spiked with salvinorin A, they employed lengthy SPE procedures and were limited by the use of MS1 scanning.[151] With the preparation of CSF samples, lengthy SPE procedures were able to be removed which greatly reduced the sample preparation time to about one minute per sample. However with blood samples, SPE cartridges were repeatedly clogged such that it was impossible to pass solvent through. Early in the development it was apparent there was a broad variability in the propensity to clog an SPE column with plasma across participants, individual participant samples, and the commercially

processed pooled plasma used for standard curves. Therefore, the potential clogging of an SPE column was of real concern since a data point may be lost due to a limited supply of blood plasma being used up on a SPE column that clogs. Changing to a higher loading capacity SPE cartridge did slightly remedy the issue but did not completely stop the columns from becoming plugged. **(Table 1)**. Due to the large injection volumes used on the instrumentation, it was not possible to reduce the amount of plasma loaded onto the SPE column and using a SPE column beyond 100 mg / mL would require a large elution volume making the reconstitution step less consistent and precise. With these restrictions in mind the Strata C18-E columns were used in all subsequent SPE work.

**Table 1:** SPE columns used for salvinorin A extraction from human plasma

| SPE Column | Loading Capacity | SPE Attempts | Attempts Plugged | Plasma Volume |
|---|---|---|---|---|
| Strata C8 | 100 mg $mL^{-1}$ | 5 | 2 | 400 μL |
| Strata C18-E | 100 mg $mL^{-1}$ | 5 | 2 | 400 μL |
| Oasis HLB | 30 mg $mL^{-1}$ | 5 | 5 | 200 μL |
| Strata-X | 30 mg $mL^{-1}$ | 5 | 5 | 200 μL |
| Strata-X-C | 30 mg $mL^{-1}$ | 5 | 5 | 200 μL |
| Strata-X-CW | 30 mg $mL^{-1}$ | 5 | 5 | 200 μL |

Even after pretreatment of plasma samples with cold ethanol or cold acetonitrile, the SPE columns were still clogged at approximately the same rate as previously noted. It was then assumed that while the SPE columns were still being overloaded that they might be mechanically blocked by particulates within the samples themselves. Upon centrifugation at 21,000 rpm for 4 minutes the plasma was split into a pellet, clear plasma, and a fatty lipid-like layer. After loading the clear plasma, middle layer, onto an SPE cartridge it easily passed through and allowed for free flow of solvent through the column with minimal vacuum pressure.

Extra workload was placed on the HPLC guard column due to the removal of SPE workup from the CSF samples and residual impurities in the plasma samples. A slow clog in the HPLC guard column raised the pressure within the instrument over a period of injection cycles, which required changing the guard column after the pressure had increased to over 4600 psi, but never until all injections had been completed from a single participant series. Injections ranged from 70 to 400, depending on sample quality, before the pressure dictated repacking the guard column.

Once the sample preparation was reproducible for both CSF and plasma the appropriate internal standard was selected. Previously a +3 mass analogue had been used as an internal standard with an APCI source and SIM in MS1 successfully.[151] However when applied to MS/MS the increased sensitivity reveals a slight impurity of salvinorin A within the +3 internal standard, observed as cross-talk between mrm channels with pure internal standard injections. Presumably the small impurity is derived from deuteron-proton exchange with the glassware during synthesis. Following the published procedure in three separate incidences by two separate chemists yielded 2-3% of (433u)-salvinorin A mixed into the (435u)-salvinorin A in each case. To avoid mathematical manipulation during data processing a unique analogue was used as internal standard while a feasible synthesis could be worked out for a (438u)-salvinorin A analogue **(Figure 11)**.

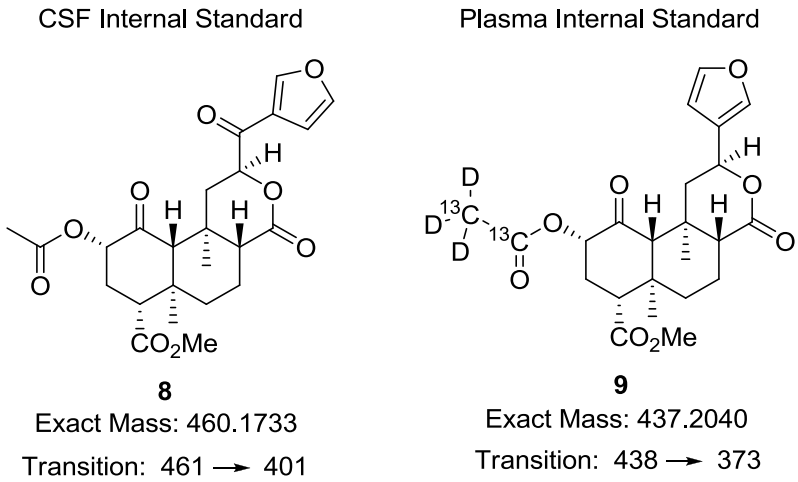



**Figure 11:** Internal standards used for processing CSF and plasma samples

Internal standard **8** was used for CSF samples because of its structural similarity, column retention, ionization similarity, and +28 mass units in relation to salvinorin A. In blank rhesus monkey CSF, collected in the absence of salvinorin A, no interference was detected on the chemical transitions monitored for the internal standard **8** and salvinorin A. Preparation of standard curve samples were done in artificial cerebrospinal fluid (ACSF) due to the limited supply of natural CSF. Upon alteration of previous synthetic methodology it was found that with extensive washing of glassware with $D_2O$ and performing the synthesis in a "perdeuterium" environment the cross-talk between the internal standard (**9**) and salvinorin A channels was eliminated by >99.99% as observed in the mass spectrometer. Since a separate internal standard had already been used in the processing of most CSF samples the isotopically pure (438u)-salvinorin A was only used during the processing of human plasma samples.

Percent recovery was determined using the ratio of integrated peak areas of salvinorin A undergoing SPE procedures versus salvinorin A injected from pure organic solvent. Precision was defined as the average standard deviation for each sample for three injections. Accuracy was defined as the deviation of the calculated concentration from the theoretical standard

concentration. Statistical data for CSF and plasma samples is summarized in **Table 2 and Table 3**. The limit of quantification was defined as the point where signal to noise remained greater than 5:1 **(Figure 12)**. Analytes remained stable for at least two weeks as that was the longest timeframe between injections of prepared samples. Matrix effects were not encountered in either CSF or plasma samples.

**Table 2:** Salvinorin A statistical data from rhesus monkey CSF

| Parameter | Value |
|---|---|
| Linearity[1] | 0.0125 - 50 ng $mL^{-1}$ |
| ($R^2$) | ≥.998 |
| Limit of Quantification | 0.0125 ng $mL^{-1}$ (5.8 % , 10.96 %) |
| Precision (Interday, Intraday)[2] | CV ( < 1.7 % , < 1.3 %) |
| Accuracy (Interday, Intraday)[2] | (9.42 % , 4.94 %) |

[1] Nine-point calibration curve ran in triplicate

[2] Calculated from 3 sets of 27 injections each

**Table 3:** Salvinorin A statistical data from human plasma samples

| Parameter | Value |
|---|---|
| Linearity[1] | 0.25 - 50 ng $mL^{-1}$ |
| ($R^2$)[2] | 0.997 - .999 |
| Limit of Quantification | 0.05 ng $mL^{-1}$ (4.7 % , 7.47 %) |
| Precision (Interday, Intraday)[3] | CV ( < 3.47 % , < 2.85 %) |
| Accuracy (Interday, Intraday)[3] | (12.37 % , 7.08 %) |
| Percent Recovery | 93% – 114% |

[1] Eight-point calibration curve ran in triplicate

[2] Range of correlation coefficients over seven trials

[3] Calculated from 7 sets of 24 injections each

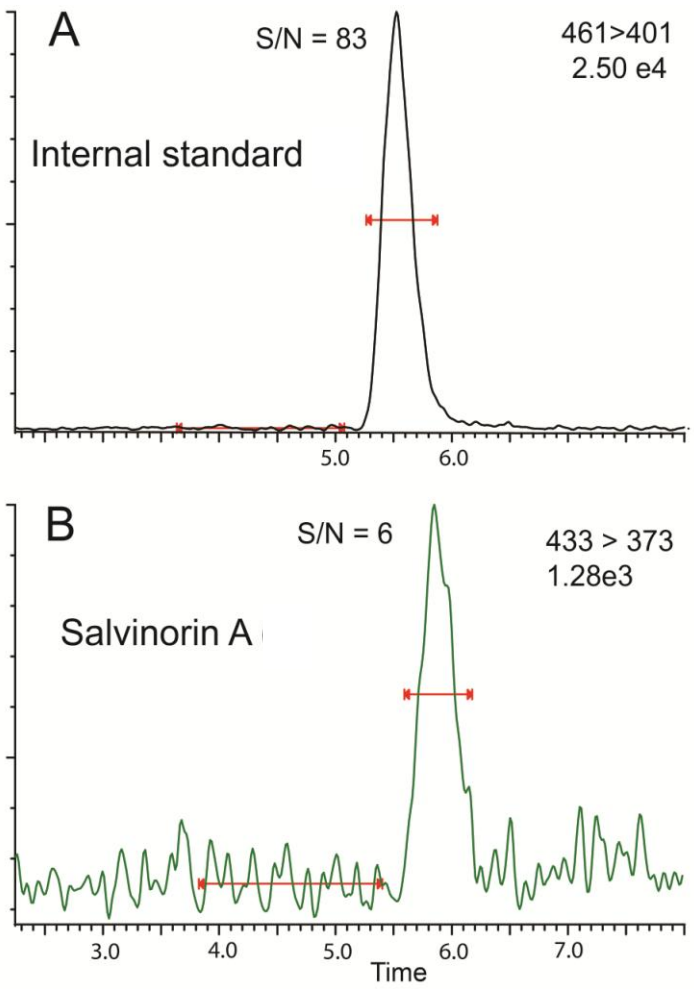



**Figure 12:** (Panel A): LC/MS/MS chromatogram with a signal to noise measurement for the internal standard at a concentration of 2.86 ng $mL^{-1}$. (Panel B): LC/MS/MS chromatogram and signal to noise measurement for salvinorin A at a concentration of 0.0125 ng $mL^{-1}$

## Conclusions and Future Directions

The method developed allows for the quantification of salvinorin A at and below behaviorally active doses from both non-human primate CSF and human plasma. The plasma samples showed an order of magnitude increase in sensitivity from previously reported results, while the CSF samples showed two orders of magnitude increase in sensitivity while eliminating

the SPE procedures. Major increases in sensitivity were derived from the increased peak concentration that resulted from analyte focusing large injection volumes on a small bore column. Additionally MS/MS transitions reduced chemical noise in the spectral output. However, backpressure on the LC and guard column does need to be monitored as reduction in sample work-up (CSF samples) and large injection volumes onto the small bore column slowly obstructed the guard column and increased the backpressure. The backpressure was relieved as needed by repacking guard columns upon reaching the pressure maximum (4600 psi) depending on sample cleanliness. The synthesis and use of new internal standards prevented cross-talk between mrm channels while eliminating the need for mathematical manipulation of the data due to impurities generated from deuteron-proton exchange during chemical synthesis. The method developed was then successfully applied to processing of human plasma samples, as well as non-human primate CSF samples.

Time course graphs generated from the processing of CSF and plasma samples indicate a rapid absorption and distribution of salvinorin A into the body and rapid clearance. Consistent with previous results and anecdotal reports, salvinorin A was found to reach a peak concentration in non-human primate CSF at 1 and 2 minutes post administration and 1 to 3 minutes post inhalation in human plasma. Additionally, salvinorin A was found to be a Pgp substrate in non-human primates as indicated by the increase in peak concentration after administration pretreatment with the Pgp inhibitor tariquidar.

These studies focused on the in vivo concentrations of salvinorin A and the behavioral effects it imparts, future work should focus on other basic pharmacokinetic parameters such as the metabolic stability and excretion of salvinorin A. With knowledge about the concentration required to impart a biological effect and the short duration of action observed, to be a useful

agent other basic pharmacokinetic parameters need to be investigated. As a theory of metabolism, the deacetylation of salvinorin A by blood esterases renders it inactive. By identifying the metabolites of salvinorin A, the metabolic pathway could be interrupted, extending its biological duration of action. Modification of the salvinorin A scaffold to increase its water solubility and its bioavailability would provide a preferred method of administration beyond inhalation or injection. While much more information about salvinorin A scaffold is needed before it can be properly developed, future studies need to focus on obtaining the basic pharmacokinetic profile of salvinorin A before an accurate conclusion can be made about its usefulness for future pharmaceutical use.

## Experimentals

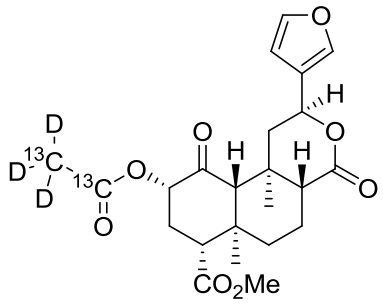
### Experimental Subjects

Experimental studies in non-human primates were approved by the Rockefeller University Animal Care and Use Committee.[161] Experimental studies conducted in humans were approved by the Institutional Review Board at the Johns Hopkins University School of Medicine. Human participants had given their informed consent before participation in any experimental trials.

### Chemicals and Solutions

Salvinorin A was extracted from the dried leaves of *S. divinorum* following previously described methods.[163, 164] All solvents used were purchased from the Sigma-Aldrich chemical company and were of HPLC quality. Preparation of the internal standard, (**9**), was conducted starting from salvinorin A by following procedures developed by Lovell and coworkers.[165] All stock solutions were prepared at a starting concentration of 1.0 mg $mL^{-1}$ in acetonitrile and cooled to 2 °C in a refrigerator for storage. All stock solutions were made again from fresh materials after a two week period to prevent changes in concentration or degradation of the analyte. SPE column washing solution contained 90 % Millipore $H_2O$ and 10 % methanol (v/v). SPE column elution solution consisted of 75 % dichloromethane and 25 % acetonitrile (v/v). Reconstitution solution was made of 64 % Millipore $H_2O$, 35 % acetonitrile, and 1 % formic acid (v/v/v).

**Synthesis of Plasma Internal Standard**



(Internal standard, 438-Salvinorin A)

**[22,22,22-$^{2}$H],[22,23-$^{13}$C]-(2S,4aR,6aR,7R,9S,10aS,10bR)-methyl 9-acetoxy-2-(furan-3-yl)-6a,10b-dimethyl-4,10-dioxododecahydro-1H-benzo[f]isochromene-7-carboxylate**

A 10 mL round bottom flask was first rinsed with $D_2O$ and then washed with soap/$D_2O$ and rinsed again with $D_2O$ then placed in an oven for drying. Once dry the flask was capped inside the oven with a septum and injected with 5.0 ml of $CDCl_3$, before cooling the hot glass, then flushed with argon. After letting the glassware cool, 4-(dimethylamino)pyridine (6 mg, 0.05 mmol) and pure salvinorin B (50 mg, 0.128 mmol) were added to the flask, after which the reaction vessel was again flushed with argon. Once dissolved, acetic anhydride – $^{13}C_4$, $d_6$ 99 % $^{13}C$, 97 % D (55 μL, 0.64 mmol) was added to the solution and allowed to stir at room temperature. While monitoring the reaction by TLC it appeared that the reaction was complete after only 3 hours, however it was allowed to stir for 7 hours to assure complete conversion of salvinorin B to the deuterium labeled 438-salvinorin A. Deuterated methanol (2 mL) was then added to the stirring solution and concentrated under reduced pressure. To the residue was added $CDCl_3$ (6 mL) and washed with DCl (35 wt. %) in $D_2O$ (4 x 4mL). The organic layer was then concentrated under reduced pressure to produce a white solid in 95.3% yield (52.8 mg, 0.122 mmol), which did not need further purification. Upon HPLC analysis it was found to be at least 97.44% pure with no traces of salvinorin B in the chromatogram, $t_r$=5.739 min. After analysis by

LC/MS/MS the isotopic purity of 438-salvinorin A to 433-salvinorin A was determined to be >99.99%. $^{1}$H NMR (500 MHz, Chloroform-*d*) δ 7.41 (dt, *J* = 1.7, 0.9 Hz, 1H), 7.39 (t, *J* = 1.7 Hz, 1H), 6.38 (dd, *J* = 1.9, 0.9 Hz, 1H), 5.53 (ddd, *J* = 11.8, 5.2, 0.9 Hz, 1H), 5.15 (ddd, *J* = 10.7, 8.8, 0.9 Hz, 1H), 3.73 (s, 3H), 2.80 – 2.71 (m, 1H), 2.51 (dd, *J* = 13.4, 5.2 Hz, 1H), 2.34 – 2.27 (m, 2H), 2.15 (q, *J* = 2.6, 1.9 Hz, 1H), 2.08 (dd, *J* = 11.7, 3.0 Hz, 1H), 1.83 – 1.76 (m, 1H), 1.68 – 1.56 (m, 4H), 1.45 (s, 3H), 1.12 (s, 3H $^{13}$C NMR (126 MHz, $CDCl_3$) δ 200.27, 169.79, 169.38, 168.22, 141.96, 137.66, 123.43, 106.62, 73.27, 70.29, 62.30, 51.81, 50.24, 49.63, 41.61, 40.34, 36.39, 33.69, 29.00, 18.84, 16.38, 14.66, 13.45

## Preparation of Plasma Blanks

Calibration standards were prepared by serial diluting stock solutions of salvinorin A and **8**, these dilutions were then added to pooled human plasma containing the anticoagulant acid-citrate-dextrose. Specific preparation was done by adding 350 μL of plasma to a 1.5 mL microcentrifuge tube, to which was then added 100 μL from a 10 ng $mL^{-1}$ solution of **8**, and 100 μL from an individual dilution of **1** from the full serial dilution set. Each sample was vortexed for approximately 3 or 4 seconds before submitting to solid phase extraction. Final standards contained a volume of 350 μL of reconstitution solution containing salvinorin A at concentrations of 0.050, 0.10, 0.25, 0.50, 1.0, 2.5, 5.0, 10, 25, 50 ng $mL^{-1}$, with **9** at a concentration of 2.86 ng $mL^{-1}$ for each sample.

## Cerebrospinal Fluid Blanks

Calibration standards were prepared by serial diluting stock solutions of salvinorin A and **8** which was added to sufficient artificial cerebrospinal fluid (ACSF). ACSF contained 126 mM NaCl, 2.5 mM KCl, 20 mM HEPES, 1.2 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 2.4 mM $CaCl_2$, and 1.2 mM MgCl solution at pH 7.4. Final standard samples were composed in 1.0 mL volumes of ACSF containing salvinorin A at 0.0125, 0.125, 0.25, 0.50, 1.0, 2.5, 5.0, 10, 25, 50 ng $mL^{-1}$, internal standard (**8**) at 2.86 ng $mL^{-1}$, 10 % (v/v) acetonitrile, and 1.0 % formic acid (v/v).

## Preparation of Human Plasma Samples

Vaporized doses of salvinorin A, either 18.0 (2 participants) or 21 μg $kg^{-1}$ (5 participants) were inhaled by seven human participants. 12 blood samples from each participant were collected up to 90 minutes post inhalation followed by cold centrifuged to obtain blood plasma. Plasma samples were thawed on ice from storage at -80 °C until free of any frozen particulates. Plasma samples were then made by adding 400 μL of plasma to a 1.5 mL microcentrifuge tube and centrifuged at 21000 × g for 4 minutes. Upon centrifugation the plasma sample was split into three distinct layers, particulates on bottom, clear plasma in the middle, and a fatty layer on top, with the majority of the volume composing the middle layer. From these layers 350 μL of the middle layer was cautiously pipetted out leaving behind the top and bottom layers. This 350 μL was then transferred to a new 1.5 mL microcentrifuge tube to which was added 100 μL of a 10 ng $mL^{-1}$ solution of **9** in reconstitution solution. The resulting solution was then mixed by vortexing for approximately 3 or 4 seconds and subjected to solid phase extraction.

## Preparation of Rhesus Monkey CSF Samples

CSF samples containing salvinorin A were stored at – 80 °C until processing when samples were thawed on ice until they were free flowing and free of any ice crystals. Upon thawing 300 μL of CSF sample was transferred via pipette to a high recovery sample vial and diluted to a total volume of 350 μL. Final solutions contained 10 % acetonitrile, 1.0 % formic acid, and 1.0 ng of internal standard (**8**). Samples were then analyzed using multiple reaction monitoring (mrm) on a LC-MS/MS system.

## Soild Phase Extraction

Strata C18-E 55 micron, 70 Å SPE cartridges (Phenomenex, Torrance, CA) were used for preparation of authentic human samples as well as standard curve samples. A Phenomenex 12-port SPE vacuum manifold was used at vacuum pressure between 1 and 10 inches of Hg, depending on the viscosity of plasma, to maintain a flow rate of approximately 1 mL $min^{-1}$. SPE columns were conditioned by washing sequentially with 3 mL of methanol and 3 mL of Millipore $H_2O$. The columns were then loaded with plasma samples and rinsed with 3 mL of washing solution before removing solvent under gentle vacuum for approximately 5 minutes. Salvinorin A was then extracted from the columns using 2 mL of eluting solution, collected in 2 dram vials, and solvent evaporated to dryness under a gentle stream of nitrogen gas at room temperature. Vials containing the dried extracts were then reconstituted into 350 μL of mobile phase and transferred to a total recovery autosample vial.

## Instrumentation

Samples were injected and chromatographed using a Waters Acquity UPLC (Waters corp Milford, MA) which eluted into a Micromass Quattro Ultima (Micromass LTD Manchester, UK) triple quadrupole mass spectrometer operating with a positive electrospray source. Salvinorin A was separated from internal standard and any impurities using a Micro-Tech Scientific 1 mm ID × 5 cm Zorbax C18 300 Å 3.5 micron column preceded by a 2.0 cm × 1 mm ID guard column. Table 4 outlines the HPLC solvent gradient used for elution of analyte and internal standard. Data was collected and processed using MassLynx version 4.1 (Waters corp.), and all instrumental parameters used are summarized in Table 5. Repacking of guard columns was done manually 1 mm "kits" (upchurch / idex P/N C-128).

**Table 4**: Solvent gradient program used for elution during LC-MS/MS analysis[161]

| Time (min) | Flow Rate | % Solvent A† | % Solvent B‡ |
|---|---|---|---|
| Initial | 0.135 | 99.0 | 1.0 |
| 1.0 | 0.135 | 99.0 | 1.0 |
| 2.0 | 0.135 | 71.0 | 29.0 |
| 10.0 | 0.135 | 67.0 | 33.0 |
| 10.1 | 0.145 | 5.0 | 95.0 |
| 11.0 | 0.145 | 5.0 | 95.0 |
| 12.0 | 0.130 | 99.0 | 1.0 |

† Solvent A consisted of 99.0 % $H_2O$ and 1.0 % acetonitrile

‡ Solvent B consisted of 99.92 % acetonitrile and 0.08 % formic acid

**Table 5:** Set parameters of instrumentation

| Parameter | Set Value |
|---|---|
| Ionization Mode | ES+ |
| Injection Volume | 50.00 µL |
| Capillary | 2.81 kV |
| Source Temperature | 100 °C |
| Desolvation Temperature | 250 °C |
| Q1 & Q3 Resolution | 0.8 amu, $w_h$ |
| Collision Cell Pressure | $1.55e^{-3}$ mbar |

# CHAPTER V

## Conclusions

This work sought to isolate salvinorin A in a fast and selective manner, as well as investigate the pharmacokinetics and behavioral effects of salvinorin A in non-human primates and humans. These goals would allow for the investigation of salvinorin A and its utility to be a useful pharmacological agent. The selective and efficient removal of salvinorin A from a complex reaction mixture could also potentially be amended to other natural products or unknown reaction mixtures in search of a specific functional group. The pure salvinorin A could then be used to begin development of a pharmacokinetic profile in higher order species.

In order to understand the retro Diels-Alder reaction of cycloadducts formed with the salvinorin A furan ring, several dienophiles were used to probe its chemical reactivity. It was found that the cycloadducts formed from reaction with electron deficient alkenes were unstable and performed a retro Diels-Alder reaction in a 35% yield. With electron deficient alkynes, cycloadducts were found to be stable for isolation, but only underwent a retro reaction in 5% yield. However asymmetrical alkynes complicate the reactivity by forming multiple inseparable isomers. While multiple isomers do not necessarily affect a retro reaction from occurring, they do complicate structure elucidation for applications such as SAR studies at opioid receptors. From these results, a handle for a functional resin was designed similar to alkynes known to react with salvinorin A.

Using a oxanorbornadiene scaffold for the potential of polymerization, a reactive resin precursor was synthesized in a fast and efficient manner. This reactive resin precursor was

shown to form a stable cycloadduct with salvinorin A, but was not able to be polymerized once attached. Furthermore the oxanorbornadiene scaffold was unable to be polymerized at any stage of the synthetic procedure other than in the first step where it was unmodified. With these results, there appears to be far more disadvantages and obstacles than benefits for this method of extracting salvinorin A. In theory, the selective removal of a specific compound based on its inherent reactivity would seem like a novel technique for natural product exploration. However, with salvinorin A specifically the retro reaction proceeded in such low yields it would require an extremely large amount of both polymerized resin and solvent. This inevitably is characteristic of the traits we are trying to avoid and is counterproductive to the project. Instead future work should remained focused on the reactivity of the salvinorin A furan ring and its SAR for the opioid receptors. By manipulating the chemical properties of salvinorin A, investigations into developing improved pharmacokinetic and pharmacodynamic properties could be explored. Current isolation and purification techniques for salvinorin A are well investigated and already work on large scale, therefore focusing efforts instead to improving the water solubility and metabolic stability of salvinorin A would likely fill a much larger gap in the literature.

The method for quantification of salvinorin A allows for behaviorally active doses to be detected in cerebrospinal fluid after i.v. injection in non-human primates and from plasma after inhalation in humans. Compared to previous methods, plasma samples showed an order of magnitude increase in sensitivity, while CSF samples showed two orders of magnitude increase in sensitivity while bypassing SPE sample preparation procedures. The major increases in sensitivity were derived from larger injection volumes with analyte focusing on a small inner diameter column thus increasing peak concentration. Additionally MS/MS transitions reduced chemical noise as expected. However, reducing sample preparation and large injection volumes

onto a small bore column slowly obstructed the guard column and increased back pressure due to slow clogging during repeated sample injections. Therefore these techniques do require the monitoring of back pressure on the LC and guard column which can be remedied by repacking the guard column as needed depending on sample cleanliness. New internal standards were synthesized and used to prevent cross-talk between mrm channels which eliminated the need for mathematical manipulation during data processing from impurities in internal standard.

Time course graphs for the salvinorin A concentration in non-human primate CSF and in human plasma were produced. Peak concentrations were found at 1 and 2 minutes post injection in CSF and 1 to 3 minutes post inhalation in human plasma, with salvinorin A concentrations quickly declined over the next 15 to 30 minutes. The rapid absorbance and clearance were consistent with previously reported observations and anecdotal reports. One possible explanation for the fast off-set of salvinorin A is that it is a Pgp substrate as it was also shown that pretreatment with the Pgp inhibitor tariquidar increased peak salvinorin A concentrations within non-human primate CSF by around 90%.

This work has completed the development of a method for the rapid quantification of salvinorin A from non-human primate CSF and from human plasma. This method then allowed for the generation of time-course graphs measuring the *in vivo* concentrations of salvinorin A during the short time it is biologically active after administration. These results, for the first time, where then quantitatively coupled to behavioral effects that salvinorin A imparts. Future work should focus on identification of other basic pharmacokinetic parameters such as the metabolic stability and excretion pathway of salvinorin A. Identification of the salvinorin A metabolites could lead to novel biomarkers for the future studies involving salvinorin A quantification *in vivo* and their correlation to behavioral effects.

## REFERENCES


1. Des Marais, D. J. When Did Photosynthesis Emerge on Earth? *Science* **2000,** 289, 1703-1705.

2. Borchardt, J. K. The Beginnings of Drug Therapy: Ancient Mesopotamian Medicine. *Drug News and Perspectives* **2002,** 15, 187 - 192.

3. Borchardt, J. K. Beginnings of drug therapy : drug therapy in ancient India. *Drug News and Perspectives* **2003,** 16, 403 - 408.

4. Borchardt, J. K. Arabic Pharmacy During the Age of the Celiphis. *Drug News and Perspectives* **2002,** 15, 383 -388.

5. Walls, D. Herbs and Natural Therapies for Pregnancy, Birth and Breastfeeding. *International Journal of Childbirth Education* **2009,** 24, 29-37.

6. Sharma, R. Recommendations on Herbs and Herbal Formula in Cancer Prevention. *The Open Nutraceuticals Journal* **2010,** 3, 129 - 140.

7. Li, J. W.-H.; Vederas, J. C. Drug Discovery and Natural Products: End of an Era or an Endless Frontier? *Science* **2009,** 325, 161-165.

8. Borchardt, J. K. Myrrh: an analgesic with a 4,000-year history. *Drug News and Perspectives* **1996,** 9, 554 - 557.

9. Baker, D. D.; Chu, M.; Oza, U.; Rajgarhia, V. The value of natural products to future pharmaceutical discovery. *Natural Product Reports* **2007,** 24, 1225 - 1244.

10. Newman, D. J.; Cragg, G. M. Natural Products As Sources of New Drugs over the 30 Years from 1981 to 2010. *Journal of Natural Products* **2012,** 75, 311-335.

11. Strohl, W. R. The role of natural products in a modern drug discovery program. *Drug Discovery Today* **2000,** 5, 39-41.

12. Hagiwara, H.; Suka, Y.; Nojima, T.; Hoshi, T.; Suzuki, T. Second-generation synthesis of salvinorin A. *Tetrahedron* **2009,** 65, 4820-4825.

13. Scheerer, J. R.; Lawrence, J. F.; Wang, G. C.; Evans, D. A. Asymmetric Synthesis of Salvinorin A, A Potent k Opioid Receptor Agonist. *Journal of the American Chemical Society* **2007,** 129, 8968-8969.

14. Lam, K. S. New aspects of natural products in drug discovery. *Trends in Microbiology* **2007,** 15, 279-289.

15. Cragg, G. M.; Grothaus, P. G.; Newman, D. J. Impact of Natural Products on Developing New Anti-Cancer Agents. *Chemical Reviews* **2009,** 109, 3012-3043.

16. Harvey, A. L. Medicines from nature: are natural products still relevant to drug discovery? *Trends in Pharmacological Sciences* **1999,** 20, 196-198.

17. Harvey, A. L. Natural products in drug discovery. *Drug Discovery Today* **2008,** 13, 894-901.

18. Galm, U.; Shen, B. Natural Product Drug Discovery: The Times Have Never Been Better. *Chemistry & Biology* **2007,** 14, 1098-1104.

19. Ginsburg, H.; Deharo, E. A call for using natural compounds in the development of new antimalarial treatments - an introduction. *Malaria Journal* **2011,** 10, S1.

20. Adnani, N.; Michel, C. R.; Bugni, T. S. Universal Quantification of Structurally Diverse Natural Products Using an Evaporative Light Scattering Detector. *Journal of Natural Products* **2012,** 75, 802-806.

21. Kennedy, J. Mutasynthesis, chemobiosynthesis, and back to semi-synthesis: combining synthetic chemistry and biosynthetic engineering for diversifying natural products. *Natural Product Reports* **2008,** 25, 25-34.

22. Demain, A. L. Antibiotics: Natural products essential to human health. *Medicinal Research Reviews* **2009,** 29, 821-842.

23. Newman, D. J.; Cragg, G. M. Natural products as drugs and leads to drugs: the historical perspective. *Natural Product Chemistry For Drug Discovery* **2010**, 3 - 27.

24. Serturner, F. W. Uber die Entdeckung des Morphiums. *J Pharm (Leipzig)* **1805,** 13, 29 - 32.

25. Sandkuhler, J.; Kress, H. G. Opioids for chronic nonmalignant and neuropathic pain. *European Journal of Pain* **2005,** 9, 99-100.

26. Booth, M. The Discovery of Dreams. In *Opium: A History*, Simon & Schuster, Ltd.: London, 1996.

27. Gulland, J. M.; Robinson, R. CXII.-The morphine group. Part I. A discussion of the constitutional problem. *Journal of the Chemical Society, Transactions* **1923,** 123, 980-998.

28. Gulland, J. M.; Robinson, R. CXIII.-The morphine group. Part II. Thebainone, thebainol, and dihydrothebainone. *Journal of the Chemical Society, Transactions* **1923,** 123, 998-1011.

29. Gates, M.; Tschudi, G. THE SYNTHESIS OF MORPHINE. *Journal of the American Chemical Society* **1952,** 74, 1109-1110.

30. McCurdy, C. R.; Prisinzano, T. Opioid Receptor Ligands. In *Burger's Medicinal Chemistry and Drug Discovery*, John Wiley & Sons, Inc.: 2010; pp 1 - 168.

31. McNicol, E.; Horowicz-Mehler, N.; Fisk, R. A.; Bennett, K.; Gialeli-Goudas, M.; Chew, P. W.; Lau, J.; Carr, D. Management of opioid side effects in cancer-related and chronic noncancer pain: a systematic review. *The Journal of Pain* **2003,** 4, 231-256.

32. Gutstein, H. B.; Akin, H. Opioid Analgesics. In *Goodman & Gilman's the pharmacological basis of therapeutics*, Goodman, L.; Hardman, J.; Limbird, L.; Gilman, A., Eds. McGraw-Hill: New York, 2001; pp 569 - 619.

33. Beckett, A. H.; Casy, A. F. SYNTHETIC ANALGESICS: STEREOCHEMICAL CONSIDERATIONS. *Journal of Pharmacy and Pharmacology* **1954,** 6, 986-1001.

34. Pert, C. B.; Snyder, S. H. Opiate Receptor: Demonstration in Nervous Tissue. *Science* **1973,** 179, 1011-1014.

35. Martin, W. R.; Eades, C. G.; Thompson, J. A.; Huppler, R. E.; Gilbert, P. E. The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. *Journal of Pharmacology and Experimental Therapeutics* **1976,** 197, 517-532.

36. Gilbert, P. E.; Martin, W. R. The effects of morphine and nalorphine-like drugs in the nondependent, morphine-dependent and cyclazocine-dependent chronic spinal dog. *Journal of Pharmacology and Experimental Therapeutics* **1976,** 198, 66-82.

37. Kieffer, B. L.; Evans, C. J. Opioid Tolerance-In Search of the Holy Grail. *Cell* **2002,** 108, 587-590.

38. Maudsley, S.; Martin, B.; Luttrell, L. M. The Origins of Diversity and Specificity in G Protein-Coupled Receptor Signaling. *Journal of Pharmacology and Experimental Therapeutics* **2005,** 314, 485-494.

39. Ko, M. C. H.; Lee, H.; Harrison, C.; Clark, M. J.; Song, H. F.; Naughton, N. N.; Woods, J. H.; Traynor, J. R. Studies of m-, k-, and d-Opioid Receptor Density and G Protein Activation

in the Cortex and Thalamus of Monkeys. *Journal of Pharmacology and Experimental Therapeutics* **2003,** 306, 179-186.

40. Lovell, K. M.; Simpson, D. S.; Cunningham, C. W.; Prisinzano, T. E. Utilizing nature as a source of new probes for opioid pharmacology. *Future Medicinal Chemistry* **2009,** 1, 285-301.

41. Prisinzano, T. E.; Rothman, R. B. Salvinorin A Analogs as Probes in Opioid Pharmacology. *Chemical Reviews* **2008,** 108, 1732-1743.

42. Christianson, D. W. Roots of Biosynthetic Diversity. *Science* **2007,** 316, 60-61.

43. Christianson, D. W. Unearthing the roots of the terpenome. *Current Opinion in Chemical Biology* **2008,** 12, 141-150.

44. Roth, B. L.; Baner, K.; Westkaemper, R.; Siebert, D.; Rice, K. C.; Steinberg, S.; Ernsberger, P.; Rothman, R. B. Salvinorin A: A potent naturally occurring nonnitrogenous k opioid selective agonist. *Proceedings of the National Academy of Sciences* **2002,** 99, 11934-11939.

45. Simpson, D. S.; Lovell, K. M.; Lozama, A.; Han, N.; Day, V. W.; Dersch, C. M.; Rothman, R. B.; Prisinzano, T. E. Synthetic studies of neoclerodane diterpenes from Salvia divinorum: role of the furan in affinity for opioid receptors. *Organic & Biomolecular Chemistry* **2009,** 7, 3748-3756.

46. Beguin, C.; Duncan, K. K.; Munro, T. A.; Ho, D. M.; Xu, W.; Liu-Chen, L.-Y.; Carlezon Jr, W. A.; Cohen, B. M. Modification of the furan ring of salvinorin A: Identification of a selective partial agonist at the kappa opioid receptor. *Bioorganic & Medicinal Chemistry* **2009,** 17, 1370-1380.

47. Beguin, C.; Potter, D. N.; DiNieri, J. A.; Munro, T. A.; Richards, M. R.; Paine, T. A.; Berry, L.; Zhao, Z.; Roth, B. L.; Xu, W.; Liu-Chen, L.-Y.; Carlezon, W. A.; Cohen, B. M. N-

Methylacetamide Analog of Salvinorin A: A Highly Potent and Selective k-Opioid Receptor Agonist with Oral Efficacy. *Journal of Pharmacology and Experimental Therapeutics* **2008,** 324, 188-195.

48. Harding, W. W.; Tidgewell, K.; Byrd, N.; Cobb, H.; Dersch, C. M.; Butelman, E. R.; Rothman, R. B.; Prisinzano, T. E. Neoclerodane Diterpenes as a Novel Scaffold for m Opioid Receptor Ligands. *Journal of Medicinal Chemistry* **2005,** 48, 4765-4771.

49. Tidgewell, K.; Groer, C. E.; Harding, W. W.; Lozama, A.; Schmidt, M.; Marquam, A.; Hiemstra, J.; Partilla, J. S.; Dersch, C. M.; Rothman, R. B.; Bohn, L. M.; Prisinzano, T. E. Herkinorin Analogues with Differential b-Arrestin-2 Interactions. *Journal of Medicinal Chemistry* **2008,** 51, 2421-2431.

50. Lozama, A.; Cunningham, C. W.; Caspers, M. J.; Douglas, J. T.; Dersch, C. M.; Rothman, R. B.; Prisinzano, T. E. Opioid Receptor Probes Derived from Cycloaddition of the Hallucinogen Natural Product Salvinorin A. *Journal of Natural Products* **2011,** 74, 718-726.

51. Lee, D. Y. W.; Karnati, V. V. R.; He, M.; Liu-Chen, L.-Y.; Kondaveti, L.; Ma, Z.; Wang, Y.; Chen, Y.; Beguin, C.; Carlezon Jr, W. A.; Cohen, B. Synthesis and in vitro pharmacological studies of new C(2) modified salvinorin A analogues. *Bioorganic & Medicinal Chemistry Letters* **2005,** 15, 3744-3747.

52. Carlezon, W. A.; Beguin, C.; DiNieri, J. A.; Baumann, M. H.; Richards, M. R.; Todtenkopf, M. S.; Rothman, R. B.; Ma, Z.; Lee, D. Y.-W.; Cohen, B. M. Depressive-Like Effects of the k-Opioid Receptor Agonist Salvinorin A on Behavior and Neurochemistry in Rats. *Journal of Pharmacology and Experimental Therapeutics* **2006,** 316, 440-447.

53. Shippenberg, T. S.; Zapata, A.; Chefer, V. I. Dynorphin and the pathophysiology of drug addiction. *Pharmacology & Therapeutics* **2007,** 116, 306-321.

54. Kivell, B.; Prisinzano, T. Kappa opioids and the modulation of pain. *Psychopharmacology* **2010,** 210, 109-119.

55. White, K. L.; Roth, B. L. Psychotomimetic Effects of Kappa Opioid Receptor Agonists. *Biological Psychiatry* **2012,** 72, 797-798.

56. Valdes III, L. J. Salvia divinorum and the unique diterpene hallucinogen, Salvinorin (divinorin) A. *Journal of Psychoactive Drugs* **1994,** 26, 277-283.

57. Ott, J. Ethnopharmacognosy and human pharmacology of *Salvia divinorum* and salvinorin A. *Curare* **1995,** 18, 103-129.

58. Siebert, D. J. Salvia divinorum and salvinorin A: new pharmacologic findings. *Journal of Ethnopharmacology* **1994,** 43, 53-56.

59. Valdes III, L. J.; Butler, W. M.; Hatfield, G. M.; Paul, A. G.; Koreeda, M. Divinorin A, a psychotropic terpenoid, and divinorin B from the hallucinogenic Mexican mint, Salvia divinorum. *The Journal of Organic Chemistry* **1984,** 49, 4716-4720.

60. Valdes III, L. J.; Diaz, J.; Paul, A. G. Ethnopharmacology of ska Maria Pastora (Salvia divinorum, Epling and Jativa-M.). *Journal of Ethnopharmacology* **1983,** 7, 287-312.

61. Wolowich, W. R.; Perkins, A. M.; Cienki, J. J. Analysis of the Psychoactive Terpenoid Salvinorin A Content in Five Salvia divinorum Herbal Products. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* **2006,** 26, 1268-1272.

62. Gonzalez, D.; Riba, J.; Bouso, J. C.; Gomez-Jarabo, G.; Barbanoj, M. J. Pattern of use and subjective effects of Salvia divinorum among recreational users. *Drug and Alcohol Dependence* **2006,** 85, 157-162.

63. Baggott, M. J.; Erowid, E.; Erowid, F.; Galloway, G. P.; Mendelson, J. Use patterns and self-reported effects of Salvia divinorum: An internet-based survey. *Drug and Alcohol Dependence* **2010,** 111, 250-256.

64. Ortega, A.; Blount, J. F.; Manchand, P. S. Salvinorin, a new trans-neoclerodane diterpene from Salvia divinorum(Labiatae). *Journal of the Chemical Society, Perkin Transactions 1* **1982**, 2505-2508.

65. Chavkin, C.; Sud, S.; Jin, W.; Stewart, J.; Zjawiony, J. K.; Siebert, D. J.; Toth, B. A.; Hufeisen, S. J.; Roth, B. L. Salvinorin A, an Active Component of the Hallucinogenic Sage Salvia divinorum Is a Highly Efficacious k-Opioid Receptor Agonist: Structural and Functional Considerations. *Journal of Pharmacology and Experimental Therapeutics* **2004,** 308, 1197-1203.

66. Wang, Y.; Tang, K.; Inan, S.; Siebert, D.; Holzgrabe, U.; Lee, D. Y. W.; Huang, P.; Li, J.-G.; Cowan, A.; Liu-Chen, L.-Y. Comparison of Pharmacological Activities of Three Distinct k Ligands (Salvinorin A, TRK-820 and 3FLB) on k Opioid Receptors in Vitro and Their Antipruritic and Antinociceptive Activities in Vivo. *Journal of Pharmacology and Experimental Therapeutics* **2005,** 312, 220-230.

67. Prisinzano, T. E. Psychopharmacology of the hallucinogenic sage Salvia divinorum. *Life Sciences* **2005,** 78, 527-531.

68. Johnson, M. W.; MacLean, K. A.; Reissig, C. J.; Prisinzano, T. E.; Griffiths, R. R. Human psychopharmacology and dose-effects of salvinorin A, a kappa opioid agonist hallucinogen present in the plant Salvia divinorum. *Drug and Alcohol Dependence* **2011,** 115, 150-155.

69. Cunningham, C. W.; Rothman, R. B.; Prisinzano, T. E. Neuropharmacology of the Naturally Occurring k-Opioid Hallucinogen Salvinorin A. *Pharmacological Reviews* **2011,** 63, 316-347.

70. Kaczor, A.; Matosiuk, D. Non-peptide opioid receptor ligands - recent advances. Part I - Agonists. *Current Medicinal Chemistry* **2002,** 9, 1567 - 1589.

71. Kaczor, A.; Matosiuk, D. Non-peptide opioid receptor ligands - recent advances. Part II.Antagonists. *Current Medicinal Chemistry* **2002,** 9, 1591 - 1603.

72. Rees, D. C.; Hunter, J. C. Opioid Receptors. In *Comprehensive Medicinal Chemistry*, Emmet, J. C., Ed. Pergamon Press: New York, 1990; pp 805 - 846.

73. Lange, J. E.; Reed, M. B.; Croff, J. M. K.; Clapp, J. D. College student use of Salvia divinorum. *Drug and Alcohol Dependence* **2008,** 94, 263-266.

74. Office of Applied Studies. In *Results from the 2007 National Survey on Drug Use and Health: National Findings*, SAMHSA (Substance Abuse and Mental Health Services Administration): Rockville, MD, 2008.

75. Johnston, L. D., O'Malley, P. M., Bachman, J. G., & Schulenberg, J. E. Monitoring the Future national results on adolescent drug use: Overview of key findings, 2011. In *Institute for Social Research, The University of Michigan.*, Ann Arbor, 2012.

76. Siebert, D. J. The legal status of *Salvia divinorum*. *The Salvia divinorum Research and Information Center*, *http://www.sagewisdom.org/legalstatus.html* **2012**.

77. Stogner, J.; Khey, D. N.; Griffin Iii, O. H.; Miller, B. L.; Boman Iv, J. H. Regulating a novel drug: An evaluation of changes in use of Salvia divinorum in the first year of Florida's ban. *International Journal of Drug Policy* **2012,** 23, 512-521.

78. Ansonoff, M. A.; Zhang, J.; Czyzyk, T.; Rothman, R. B.; Stewart, J.; Xu, H.; Zjwiony, J.; Siebert, D. J.; Yang, F.; Roth, B. L.; Pintar, J. E. Antinociceptive and Hypothermic Effects of Salvinorin A Are Abolished in a Novel Strain of k-Opioid Receptor-1 Knockout Mice. *Journal of Pharmacology and Experimental Therapeutics* **2006,** 318, 641-648.

79. Zukin, R. S.; Eghbali, M.; Olive, D.; Unterwald, E. M.; Tempel, A. Characterization and visualization of rat and guinea pig brain kappa opioid receptors: evidence for kappa 1 and kappa 2 opioid receptors. *Proceedings of the National Academy of Sciences* **1988,** 85, 4061-4065.

80. Iyengar, S.; Kim, H. S.; Wood, P. L. Effects of kappa opiate agonists on neurochemical and neuroendocrine indices: Evidence for kappa receptor subtypes. *Life Sciences* **1986,** 39, 637-644.

81. Rothman, R. B.; Bykov, V.; de Costa, B. R.; Jacobson, A. E.; Rice, K. C.; Brady, L. S. Interaction of endogenous opioid peptides and other drugs with four kappa opioid binding sites in guinea pig brain. *Peptides* **1990,** 11, 311-331.

82. Li, J.-G.; Benovic, J. L.; Liu-Chen, L.-Y. Mechanisms of Agonist-Induced Down-Regulation of the Human k-Opioid Receptor: Internalization Is Required for Down-Regulation. *Molecular Pharmacology* **2000,** 58, 795-801.

83. Li, J.-G.; Zhang, F.; Jin, X.-L.; Liu-Chen, L.-Y. Differential Regulation of the Human k Opioid Receptor by Agonists: Etorphine and Levorphanol Reduced Dynorphin A- and U50,488H-Induced Internalization and Phosphorylation. *Journal of Pharmacology and Experimental Therapeutics* **2003,** 305, 531-540.

84. Li, J.-G.; Luo, L.-Y.; Krupnick, J. G.; Benovic, J. L.; Liu-Chen, L.-Y. U50,488H-induced Internalization of the Human k Opioid Receptor Involves a b-Arrestin- and Dynamin-dependent Mechanism: k RECEPTOR INTERNALIZATION IS NOT REQUIRED FOR MITOGEN-

ACTIVATED PROTEIN KINASE ACTIVATION. *Journal of Biological Chemistry* **1999,** 274, 12087-12094.

85. Bohn, L. M.; Dykstra, L. A.; Lefkowitz, R. J.; Caron, M. G.; Barak, L. S. Relative Opioid Efficacy Is Determined by the Complements of the G Protein-Coupled Receptor Desensitization Machinery. *Molecular Pharmacology* **2004,** 66, 106-112.

86. He, L.; Fong, J.; von Zastrow, M.; Whistler, J. L. Regulation of Opioid Receptor Trafficking and Morphine Tolerance by Receptor Oligomerization. *Cell* **2002,** 108, 271-282.

87. Gabra, B. H.; Bailey, C. P.; Kelly, E.; Sanders, A. V.; Henderson, G.; Smith, F. L.; Dewey, W. L. Evidence for an important role of protein phosphatases in the mechanism of morphine tolerance. *Brain Research* **2007,** 1159, 86-93.

88. Butelman, E. R.; Harris, T. J.; Kreek, M. J. The plant-derived hallucinogen, salvinorin A, produces k-opioid agonist-like discriminative effects in rhesus monkeys. *Psychopharmacology* **2004,** 172, 220-224.

89. Schmidt, M. D.; Schmidt, M. S.; Butelman, E. R.; Harding, W. W.; Tidgewell, K.; Murry, D. J.; Kreek, M. J.; Prisinzano, T. E. Pharmacokinetics of the plant-derived κ-opioid hallucinogen salvinorin A in nonhuman primates. *Synapse* **2005,** 58, 208-210.

90. Negus, S. S.; Mello, N. K. Opioid Antinociception in Ovariectomized Monkeys: Comparison with Antinociception in Males and Effects of Estradiol Replacement. *Journal of Pharmacology and Experimental Therapeutics* **1999,** 290, 1132-1140.

91. Negus, S. S.; Zuzga, D. S.; Mello, N. K. Sex differences in opioid antinociception in rhesus monkeys: Antagonism of fentanyl and U50,488 by quadazocine. *The journal of pain : official journal of the American Pain Society* **2002,** 3, 218-226.

92. Butelman, E. R.; Mandau, M.; Tidgewell, K.; Prisinzano, T. E.; Yuferov, V.; Kreek, M. J. Effects of Salvinorin A, a k-Opioid Hallucinogen, on a Neuroendocrine Biomarker Assay in Nonhuman Primates with High k-Receptor Homology to Humans. *Journal of Pharmacology and Experimental Therapeutics* **2007,** 320, 300-306.

93. Hooker, J. M.; Xu, Y.; Schiffer, W.; Shea, C.; Carter, P.; Fowler, J. S. Pharmacokinetics of the potent hallucinogen, salvinorin A in primates parallels the rapid onset and short duration of effects in humans. *NeuroImage* **2008,** 41, 1044-1050.

94. Butelman, E. R.; Prisinzano, T. E.; Deng, H.; Rus, S.; Kreek, M. J. Unconditioned Behavioral Effects of the Powerful k-Opioid Hallucinogen Salvinorin A in Nonhuman Primates: Fast Onset and Entry into Cerebrospinal Fluid. *Journal of Pharmacology and Experimental Therapeutics* **2009,** 328, 588-597.

95. Butelman, E.; Rus, S.; Prisinzano, T.; Kreek, M. The discriminative effects of the k-opioid hallucinogen salvinorin A in nonhuman primates: dissociation from classic hallucinogen effects. *Psychopharmacology* **2010,** 210, 253-262.

96. Lange, J. E.; Daniel, J.; Homer, K.; Reed, M. B.; Clapp, J. D. Salvia divinorum: Effects and use among YouTube users. *Drug and Alcohol Dependence* **2010,** 108, 138-140.

97. Pfeiffer, A.; Brantl, V.; Herz, A.; Emrich, H. Psychotomimesis mediated by kappa opiate receptors. *Science* **1986,** 233, 774-776.

98. Mendelson, J.; Coyle, J.; Lopez, J.; Baggott, M.; Flower, K.; Everhart, E. T.; Munro, T.; Galloway, G.; Cohen, B. Lack of effect of sublingual salvinorin A, a naturally occurring kappa opioid, in humans: a placebo-controlled trial. *Psychopharmacology* **2011,** 214, 933-939.

99. Addy, P. Acute and post-acute behavioral and psychological effects of salvinorin A in humans. *Psychopharmacology* **2012,** 220, 195-204.

100. Ranganathan, M.; Schnakenberg, A.; Skosnik, P. D.; Cohen, B. M.; Pittman, B.; Sewell, R. A.; D'Souza, D. C. Dose-Related Behavioral, Subjective, Endocrine, and Psychophysiological Effects of the k Opioid Agonist Salvinorin A in Humans. *Biological Psychiatry* **2012,** 72, 871-879.

101. Butelman, E. R.; Harris, T. J.; Kreek, M.-J. Apparent efficacy of k-opioid receptor ligands on serum prolactin levels in rhesus monkeys. *European Journal of Pharmacology* **1999,** 383, 305-309.

102. Butelman, E. R.; Kreek, M.-J. k-Opioid receptor agonist-induced prolactin release in primates is blocked by dopamine D2-like receptor agonists. *European Journal of Pharmacology* **2001,** 423, 243-249.

103. Pascoe, J. E.; Williams, K. L.; Mukhopadhyay, P.; Rice, K. C.; Woods, J. H.; Ko, M.-C. Effects of mu, kappa, and delta opioid receptor agonists on the function of hypothalamic-pituitary-adrenal axis in monkeys. *Psychoneuroendocrinology* **2008,** 33, 478-486.

104. Ur, E.; Wright, D. M.; Bouloux, P. M. G.; Grossman, A. The effects of spiradoline (U-62066E), a κ-opioid receptor agonist, on neuroendocrine function in man. *British Journal of Pharmacology* **1997,** 120, 781-784.

105. MacLean, K.; Johnson, M.; Reissig, C.; Prisinzano, T.; Griffiths, R. Dose-related effects of salvinorin A in humans: dissociative, hallucinogenic, and memory effects. *Psychopharmacology* **2013,** 226, 381-392.

106. Clement, J. A.; Yoder, B. J.; Kingston, D. G. I. Natural Products as a Source of CNS-Active Agents. *Mini-Reviews in Organic Chemistry* **2004,** 1, 183 - 208.

107. Nicolaou, K. C.; Snyder, S. A. Chasing Molecules That Were Never There: Misassigned Natural Products and the Role of Chemical Synthesis in Modern Structure Elucidation. *Angewandte Chemie International Edition* **2005,** 44, 1012-1044.

108. Bhattacharyya, S. New developments in polymer-supported reagents, scavengers and cataylsis for organic synthesis. *Current opinion in drug discovery & development* **2004,** 7, 752 - 764.

109. Colombo, M.; Peretto, I. Chemistry strategies in early drug discovery: an overview of recent trends. *Drug Discovery Today* **2008,** 13, 677-684.

110. Ley, S. V.; Baxendale, I. R.; Bream, R. N.; Jackson, P. S.; Leach, A. G.; Longbottom, D. A.; Nesi, M.; Scott, J. S.; Storer, R. I.; Taylor, S. J. Multi-step organic synthesis using solid-supported reagents and scavengers: a new paradigm in chemical library generation. *Journal of the Chemical Society, Perkin Transactions 1* **2000,** 0, 3815-4195.

111. Habermann, J.; V. Ley, S.; S. Scott, J. Synthesis of the potent analgesic compound (+/-)-epibatidine using an orchestrated multi-step sequence of polymer supported reagents. *Journal of the Chemical Society, Perkin Transactions 1* **1999**, 1253-1256.

112. Baxendale, I. R.; Lee, A.-L.; Ley, S. V. A Concise Synthesis of the Natural Product Carpanone Using Solid-Supported Reagents and Scavengers. *Synlett* **2001,** 2001, 1482-1484.

113. Baxendale, I. R.; Lee, A.-L.; Ley, S. V. A concise synthesis of carpanone using solid-supported reagents and scavengers. *Journal of the Chemical Society, Perkin Transactions 1* **2002,** 0, 1850-1857.

114. Baxendale, I. R.; Ley, S. V.; Nessi, M.; Piutti, C. Total synthesis of the amaryllidaceae alkaloid (+)-plicamine using solid-supported reagents. *Tetrahedron* **2002,** 58, 6285-6304.

115. Baxendale, I. R.; Ley, S. V.; Piutti, C. Total Synthesis of the Amaryllidaceae Alkaloid (+)-Plicamine and Its Unnatural Enantiomer by Using Solid-Supported Reagents and Scavengers in a Multistep Sequence of Reactions. *Angewandte Chemie International Edition* **2002,** 41, 2194-2197.

116. Ley, S. V.; Schucht, O.; Thomas, A. W.; Murray, J. P. Synthesis of the alkaloids (+/-)-oxomaritidine and (+/-)-epimaritidine using an orchestrated multi-step sequence of polymer supported reagents. *Journal of the Chemical Society, Perkin Transactions 1* **1999**, 1251-1252.

117. Araya, J. J.; Montenegro, G.; Mitscher, L. A.; Timmermann, B. N. Application of Phase-Trafficking Methods to Natural Products Research. *Journal of Natural Products* **2010,** 73, 1568-1572.

118. Kaldor, S. W.; Fritz, J. E.; Tang, J.; McKinney, E. R. Discovery of antirhinoviral leads by screening a combinatorial library of ureas prepared using covalent scavengers. *Bioorganic & Medicinal Chemistry Letters* **1996,** 6, 3041-3044.

119. Kaldor, S. W.; Siegel, M. G.; Fritz, J. E.; Dressman, B. A.; Hahn, P. J. Use of solid supported nucleophiles and electrophiles for the purification of non-peptide small molecule libraries. *Tetrahedron Letters* **1996,** 37, 7193-7196.

120. Booth, R. J.; Hodges, J. C. Polymer-Supported Quenching Reagents for Parallel Purification. *Journal of the American Chemical Society* **1997,** 119, 4882-4886.

121. Flynn, D. L.; Crich, J. Z.; Devraj, R. V.; Hockerman, S. L.; Parlow, J. J.; South, M. S.; Woodard, S. Chemical Library Purification Strategies Based on Principles of Complementary Molecular Reactivity and Molecular Recognition. *Journal of the American Chemical Society* **1997,** 119, 4874-4881.

122. Flynn, D. L. Recent advances in polymer-assisted solution-phase chemical library synthesis and purification. *Current opinion in drug discovery & development* **1998,** 1, 41 - 50.

123. Parlow, J. J.; Mischke, D. A.; Woodard, S. S. Utility of Complementary Molecular Reactivity and Molecular Recognition (CMR/R) Technology and Polymer-Supported Reagents in the Solution-Phase Synthesis of Heterocyclic Carboxamides. *The Journal of Organic Chemistry* **1997,** 62, 5908-5919.

124. Flynn, D. L. Phase-trafficking reagents and phase-switching strategies for parallel synthesis. *Medicinal Research Reviews* **1999,** 19, 408-431.

125. Han, H.; Wolfe, M. M.; Brenner, S.; Janda, K. D. Liquid-phase combinatorial synthesis. *Proceedings of the National Academy of Sciences* **1995,** 92, 6419-6423.

126. Gravert, D. J.; Datta, A.; Wentworth, P.; Janda, K. D. Soluble Supports Tailored for Organic Synthesis: Parallel Polymer Synthesis via Sequential Normal/Living Free Radical Processes. *Journal of the American Chemical Society* **1998,** 120, 9481-9495.

127. Toy, P. H.; Janda, K. D. Soluble Polymer-Supported Organic Synthesis. *Accounts of Chemical Research* **2000,** 33, 546-554.

128. Ley, S. V.; Massi, A.; Rodríguez, F.; Horwell, D. C.; Lewthwaite, R. A.; Pritchard, M. C.; Reid, A. M. A New Phase-Switch Method for Application in Organic Synthesis Programs Employing Immobilization through Metal-Chelated Tagging. *Angewandte Chemie International Edition* **2001,** 40, 1053-1055.

129. Xing, Y. D.; Huang, N. Z. Deoxygenation of 7-oxabicyclo[2.2.1]hepta-2,5-diene systems to substituted benzenes by titanium tetrachloride-lithium aluminum hydride. *The Journal of Organic Chemistry* **1982,** 47, 140-142.

130. Duval, R. A.; Poupon, E.; Romero, V.; Peris, E.; Lewin, G.; Cortes, D.; Brandt, U.; Hocquemiller, R. Analogues of cytotoxic squamocin using reliable reactions: new insights into the reactivity and role of the a,b-unsaturated lactone of the annonaceous acetogenins. *Tetrahedron* **2006,** 62, 6248-6257.

131. van Berkel, S. S.; Dirks, A. J.; Debets, M. F.; van Delft, F. L.; Cornelissen, J. J. L. M.; Nolte, R. J. M.; Rutjes, F. P. J. T. Metal-Free Triazole Formation as a Tool for Bioconjugation. *ChemBioChem* **2007,** 8, 1504-1508.

132. Brase, S.; Gil, C.; Knepper, K. The recent impact of solid-phase synthesis on medicinally relevant benzoannelated nitrogen heterocycles. *Bioorganic & Medicinal Chemistry* **2002,** 10, 2415-2437.

133. Lei, X.; Porco, J. A. Synthesis of a Polymer-Supported Anthracene and Its Application as a Dienophile Scavenger. *Organic Letters* **2004,** 6, 795-798.

134. Li, F.; Yang, B.; Miller, M. J.; Zajicek, J.; Noll, B. C.; Mollmann, U.; Dahse, H.-M.; Miller, P. A. Iminonitroso Diels-Alder Reactions for Efficient Derivatization and Functionalization of Complex Diene-Containing Natural Products. *Organic Letters* **2007,** 9, 2923-2926.

135. Krchnak, V.; Moellmann, U.; Dahse, H.-M.; Miller, M. J. Solid-Supported Nitroso Hetero-Diels-Alder Reactions. 3. Acid-Mediated Transformation of Cycloadducts by Scission of the Oxazine C-O Bonds. *Journal of Combinatorial Chemistry* **2007,** 10, 112-117.

136. Krchnak, V.; Moellmann, U.; Dahse, H.-M.; Miller, M. J. Solid-Supported Nitroso Hetero Diels-Alder Reactions. 1. Acylnitroso Dienophiles: Scope and Limitations. *Journal of Combinatorial Chemistry* **2007,** 10, 94-103.

137. Krchnak, V.; Moellmann, U.; Dahse, H.-M.; Miller, M. J. Solid-Supported Nitroso Hetero Diels-Alder Reactions. 2. Arylnitroso Dienophiles: Scope and Limitations. *Journal of Combinatorial Chemistry* **2007,** 10, 104-111.

138. Wijnen, J. W.; Engberts, J. B. F. N. Retro-Diels-Alder Reaction in Aqueous Solution: Toward a Better Understanding of Organic Reactivity in Water. *The Journal of Organic Chemistry* **1997,** 62, 2039-2044.

139. Gousse, C.; Gandini, A.; Hodge, P. Application of the Diels-Alder Reaction to Polymers Bearing Furan Moieties. 2. Diels-Alder and Retro-Diels-Alder Reactions Involving Furan Rings in Some Styrene Copolymers. *Macromolecules* **1998,** 31, 314-321.

140. Grieco, P. A.; Abood, N. Cycloalkenone synthesis via Lewis acid-catalyzed retro Diels-Alder reactions of norbornene derivatives: synthesis of 12-oxophytodienoic acid (12-oxoPDA). *The Journal of Organic Chemistry* **1989,** 54, 6008-6010.

141. Boutelle, R. C.; Northrop, B. H. Substituent Effects on the Reversibility of Furan-Maleimide Cycloadditions. *The Journal of Organic Chemistry* **2011,** 76, 7994-8002.

142. Gil, R. R.; Gayathri, C.; Tsarevsky, N. V.; Matyjaszewski, K. Stretched Poly(methyl methacrylate) Gel Aligns Small Organic Molecules in Chloroform. Stereochemical Analysis and Diastereotopic Proton NMR Assignment in Ludartin Using Residual Dipolar Couplings and 3J Coupling Constant Analysis. *The Journal of Organic Chemistry* **2008,** 73, 840-848.

143. Gayathri, C.; de la Fuente, M. C.; Luy, B.; Gil, R. R.; Navarro-Vazquez, A. Probing heterocycle conformation with residual dipolar couplings. *Chemical Communications* **2010,** 46, 5879-5881.

144. Gayathri, C.; Tsarevsky, N. V.; Gil, R. R. Residual Dipolar Couplings (RDCs) Analysis of Small Molecules Made Easy: Fast and Tuneable Alignment by Reversible

Compression/Relaxation of Reusable PMMA Gels. *Chemistry – A European Journal* **2010,** 16, 3622-3626.

145. Harned, A. M.; Hanson, P. R. Capture-ROMP-Release: Application for the Synthesis of O-Alkylhydroxylamines. *Organic Letters* **2002,** 4, 1007-1010.

146. Moore, J. D.; Harned, A. M.; Henle, J.; Flynn, D. L.; Hanson, P. R. Scavenge-ROMP-Filter: A Facile Strategy for Soluble Scavenging via Norbornenyl Tagging of Electrophilic Reagents. *Organic Letters* **2002,** 4, 1847-1849.

147. Shang, M.; Warrener, R.; Butler, D.; Murata, Y.; Margetic, D. Synthesis of bis-peptides attached on poly[n]norbornene molecular scaffolds with well-defined relative positions and distances. *Molecular Diversity* **2011,** 15, 541-560.

148. Moreno, I.; da Fonseca, B.; Oppolzer, D.; Martinho, A.; Barroso, M.; Cruz, A.; Queiroz, J. A.; Gallardo, E. Analysis of Salvinorin A in urine using microextraction in packed syringe and GC-MS/MS. *Bioanalysis* **2013,** 5, 661-668.

149. McDonough, P. C.; Holler, J. M.; Vorce, S. P.; Bosy, T. Z.; Magluilo Jr, J.; Past, M. R. The Detection and Quantitative Analysis of the Psychoactive Component of Salvia divinorum, Salvinorin A, in Human Biological Fluids Using Liquid Chromatography Mass Spectrometry. *Journal of Analytical Toxicology* **2008,** 32, 417-421.

150. Barnes, B. B.; Snow, N. H. Analysis of Salvinorin A in plants, water, and urine using solid-phase microextraction-comprehensive two-dimensional gas chromatography-time of flight mass spectrometry. *Journal of Chromatography A* **2012,** 1226, 110-115.

151. Schmidt, M. S.; Prisinzano, T. E.; Tidgewell, K.; Harding, W.; Butelman, E. R.; Kreek, M. J.; Murry, D. J. Determination of Salvinorin A in body fluids by high performance liquid

chromatography-atmospheric pressure chemical ionization. *Journal of Chromatography B* **2005,** 818, 221-225.

152. Grundmann, O.; Phipps, S. M.; Zadezensky, I.; Butterweck, V. Salvia divinorum and Salvinorin A: An Update on Pharmacology and Analytical Methodology. *Planta Med* **2007,** 73, 1039-1046.

153. Medana, C.; Massolino, C.; Pazzi, M.; Baiocchi, C. Determination of salvinorins and divinatorins in Salvia divinorum leaves by liquid chromatography/multistage mass spectrometry. *Rapid Communications in Mass Spectrometry* **2006,** 20, 131-136.

154. Tsujikawa, K.; Kuwayama, K.; Miyaguchi, H.; Kanamori, T.; Iwata, Y. T.; Yoshida, T.; Inoue, H. Determination of salvinorin A and salvinorin B in Salvia divinorum-related products circulated in Japan. *Forensic Science International* **2008,** 180, 105-109.

155. Tsujikawa, K.; Kuwayama, K.; Miyaguchi, H.; Kanamori, T.; Iwata, Y. T.; Inoue, H. In vitro stability and metabolism of salvinorin A in rat plasma. *Xenobiotica* **2009,** 39, 391-398.

156. Barnes, S.; Prasain, J. K.; Wang, C.-C.; Moore Ii, D. R. Applications of LC-MS in the study of the uptake, distribution, metabolism and excretion of bioactive polyphenols from dietary supplements. *Life Sciences* **2006,** 78, 2054-2059.

157. Gruber, J. W.; Siebert, D. J.; Marderosian, A. H. D.; Hock, R. S. High performance liquid chromatographic quantification of salvinorin A from tissues of salvia divinorum epling & játiva-m. *Phytochemical Analysis* **1999,** 10, 22-25.

158. Siebert, D. J. Localization of Salvinorin A and Related Compounds in Glandular Trichomes of the Psychoactive Sage, Salvia divinorum. *Annals of Botany* **2004,** 93, 763-771.

159. Pichini, S.; Abanades, S.; Farré, M.; Pellegrini, M.; Marchei, E.; Pacifici, R.; Torre, R. d. l.; Zuccaro, P. Quantification of the plant-derived hallucinogen Salvinorin A in conventional and

non-conventional biological fluids by gas chromatography/mass spectrometry after Salvia divinorum smoking. *Rapid Communications in Mass Spectrometry* **2005,** 19, 1649-1656.

160. Margalho, C. u.; Gallardo, E.; Castanheira, A.; Vieira, D. N.; LÃ³pez-Rivadulla, M.; Real, F. C. A validated procedure for detection and quantitation of salvinorin a in pericardial fluid, vitreous humor, whole blood and plasma using solid phase extraction and gas chromatography-mass spectrometry. *Journal of Chromatography A* **2013,** 1304, 203-210.

161. Butelman, E. R.; Caspers, M.; Lovell, K. M.; Kreek, M. J.; Prisinzano, T. E. Behavioral Effects and Central Nervous System Levels of the Broadly Available k-Agonist Hallucinogen Salvinorin A Are Affected by P-Glycoprotein Modulation In Vivo. *Journal of Pharmacology and Experimental Therapeutics* **2012,** 341, 802-808.

162. Teksin, Z. S.; Lee, I. J.; Nemieboka, N. N.; Othman, A. A.; Upreti, V. V.; Hassan, H. E.; Syed, S. S.; Prisinzano, T. E.; Eddington, N. D. Evaluation of the transport, in vitro metabolism and pharmacokinetics of Salvinorin A, a potent hallucinogen. *European Journal of Pharmaceutics and Biopharmaceutics* **2009,** 72, 471-477.

163. Munro, T. A.; Rizzacasa, M. A. Salvinorins D-F, New Neoclerodane Diterpenoids from Salvia divinorum, and an Improved Method for the Isolation of Salvinorin A. *Journal of Natural Products* **2003,** 66, 703-705.

164. Tidgewell, K.; Harding, W. W.; Schmidt, M.; Holden, K. G.; Murry, D. J.; E. Prisinzano, T. A facile method for the preparation of deuterium labeled salvinorin A: synthesis of [2,2,2-2H3]-salvinorin A. *Bioorganic & Medicinal Chemistry Letters* **2004,** 14, 5099-5102.

165. Lovell, K. M.; Vasiljevik, T.; Araya, J. J.; Lozama, A.; Prevatt-Smith, K. M.; Day, V. W.; Dersch, C. M.; Rothman, R. B.; Butelman, E. R.; Kreek, M. J.; Prisinzano, T. E.

Semisynthetic Neoclerodanes as Kappa Opioid Receptor Probes. *Bioorganic & Medicinal Chemistry* **2012,** 20, 3100-3110.