Synthetic Investigation of Natural Products Causing Dopaminergic Neurodegeneration

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University of Mississippi
SYNTHETIC INVESTIGATION OF NATURAL PRODUCTS CAUSING DOPAMINERGIC NEURODEGENERATION

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ABSTRACT

The terrestrial organisms Streptomyces venezuelae and Rhaponticum repens produce toxic secondary metabolites that likely function as chemical deterrents. The polyketide SV-6 from S. venezuelae and the sesquiterpene lactone repin from R. repens both produce dose-dependent and selective degeneration of dopaminergic neurons. These molecules represent two possible tools that can be used to explore chemotoxic induction of Parkinson’s disease. In the case of SV-6, a newly isolated metabolite, total synthesis was undertaken to confirm its structure and biological activity. The natural product was produced from methacrolein and Roche ester starting materials that were elaborated to their respective fragments, a vinyl iodide and a Weinreb amide. These fragments were coupled to form SV-6 and the synthesis allows for many additional opportunities to easily generate stereoisomers and analogs for further study. In the case of repin, previously isolated material was used as a starting material to create a biotin-labeled probe. There were considerable challenges due to the electrophilic nature of the compound, but a Nicolas reaction followed by copper catalyzed cycloaddition produced an impure biotin-repin probe. Repin was also subjected to screening in multiple biological assays which identified proteins potentially responsible for its neurodegenerative activity.
DEDICATION

To my family, who encouraged me to dream in a pragmatic way.
<table>
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<tr>
<th>Symbol</th>
<th>Abbreviation</th>
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<tr>
<td>$^{13}\text{C}$ NMR</td>
<td>Carbon Nuclear Magnetic Resonance</td>
<td></td>
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<tr>
<td>$^{1}\text{H}$ NMR</td>
<td>Hydrogen Nuclear Magnetic Resonance</td>
<td></td>
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<tr>
<td>AcOH</td>
<td>Acetic acid</td>
<td></td>
</tr>
<tr>
<td>BuLi</td>
<td>Butyllithium</td>
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<tr>
<td>DMAP</td>
<td>N,N-4-Dimethyaminopyridine</td>
<td></td>
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<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<tr>
<td>Ph</td>
<td>Phenyl</td>
<td></td>
</tr>
<tr>
<td>PPTS</td>
<td>Pyridinium p-toluenesulfonate</td>
<td></td>
</tr>
<tr>
<td>pTSA</td>
<td>para-Tolylsulfonic acid</td>
<td></td>
</tr>
<tr>
<td>Py</td>
<td>Pyridine</td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
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<tr>
<td>TEMPO</td>
<td>(2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl</td>
<td></td>
</tr>
<tr>
<td>TBAF</td>
<td>Tetra-N-butylammonium fluoride</td>
<td></td>
</tr>
<tr>
<td>TBS</td>
<td>tert-Butyldimethylsilyl</td>
<td></td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
<td></td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
<td></td>
</tr>
<tr>
<td><strong>TLC</strong></td>
<td>Thin layer chromatography</td>
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<tr>
<td><strong>USDA</strong></td>
<td>United States Department of Agriculture</td>
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ACKNOWLEDGEMENTS

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I. BACKGROUND INFORMATION

Dopamine is the neurotransmitter most often regarded as a “reward molecule”, with its release punctuating food consumption and sexual intercourse, as well as the use of psychoactive drugs such as nicotine, amphetamines and cocaine\(^1\). Interestingly, diseases that reduce dopamine production often effect movement and motor control as well as mood and behavior. In 1817, James Parkinson described in “An Essay on the Shaking Palsy” the disease that now bears his name\(^2\). The term “parkinsonism” is a blanket label of any condition consisting of bradykinesia or akinesia \textit{plus} muscle rigidity, tremor, or unstable posture. Increased emphasis has more recently been placed on non-motor components of this Parkinson’s disease (PD). These aspects may include pain, fatigue, constipation, cognitive impairment and psychosis\(^3\). The disease is progressive and may start with a slight tremor on one side of the body. It can diminish facial expression, vocal tone and clarity of speech. It may also cause stiffness or slowed movement \(^4\). Approximately 1.5\% of world’s population above the age of 65 is impacted by the disease and the number of PD sufferers is expected to reach 8.7 million sufferers by 2030\(^5\).

1.1 Parkinson’s Disease

Parkinson’s disease is routinely broken into two groups, \textit{sporadic} and \textit{familial} cases. The disorder was once considered to be entirely sporadic- meaning it has no genetic basis\(^6\).
However, there have been great advances in understanding the genetic origins of the diseases. It is now thought that roughly 10% of patients have monogenic (familial) forms of PD. The disease is a complex manifestation of various environmental factors in combination with some specific genetic susceptibilities. Among the genes consistently implicated in PD development are SNCA, LRRK2, GBA, PRKN, PINK1, PARK7, VPS35, EIF4G1, DNAJC13 and CHCHD2. Some of these genes (SNCA, LRRK2, GBA) are considered key in development of both familial and sporadic forms. The discovery of these genes and their products has led to the development of some useful genetic animal models of the disease. While these models are helpful, the exact role of genetic factors with the environment is lacking and therefore remains a great need for chemotoxic models.

1.2 Mitochondria, Metabolism and Neurodegeneration

Mitochondria are cellular organelles which produce energy via a series of coupled biochemical processes. Through ATP and the electron transport chain, they generate the largest proportion of cellular energy and are common to nearly all eukaryotic cells. Mitochondria form reticular networks in cells and density of this network varies between cell and tissue type relative to oxidative phosphorylation-dependent energy demands. Among those with the highest requirements are cardiac and skeletal muscles as well as neurons. Due to a heritage predating eukaryotic incorporation, mitochondria bear DNA which is distinct from that of their host cells. Human mitochondrial DNA (mtDNA) is roughly 16.6 kilobase pairs in length. This material consists almost entirely of exons and encodes two ribosomal RNA units, 22 transfer RNA units, and 13 proteins. The dependence of these organelles upon the eukaryotic home it resides in for replication, repair, transcription and translation is probably at the root of many disease states.
the aforementioned 13 mtDNA encoded proteins, all are subunits of oxidative phosphorylation system and respiratory chain (OXPHOS). The inner mitochondrial membrane contains the five OXPHOS complexes. There are about 85 protein subunits across all OXPHOS complexes and all complexes are required for sustenance of energetic requirements.

In addition to mutations in mtDNA, the release of and exposure to reactive oxygen species (ROS) are considered significant drivers of the aging process (Figure 1). There are a number of factors for which the brain—and its dopaminergic neurons—are disproportionally affected by these insults. The brain is rich in easily oxidized fatty acids and it also has a high rate of oxygen consumption. This intense ROS generation coupled with a lower amount of antioxidant enzymes relative to other tissues sets the stage for numerous cellular damage scenarios to unfold.

Figure 1: Interplay between genetic and environmental factors in PD
Dopaminergic neurons are especially susceptible to ROS damage via metabolic products of dopamine \(^1\)\(^{12,13}\).

Medical researchers now believe that this fundamental energy metabolism may have far-reaching implications in disease and cellular dysfunction\(^1\)\(^4\). Nuclear or mitochondrial gene mutations can have direct consequences on mitochondrial health. The overlap of genomic susceptibility and environmental exposure may account for the incidence and variety of diseases of mitochondrial origin that seems to be unexplained when looking at each factor individually. Environmental exposure to cyanide and rotenone are known to inhibit mitochondrial components complex IV and complex I \(^1\)\(^{15,15b}\). For example, a group of 50,000 people suffering from folate deficiency responded to cyanide or methanol exposure with symptoms of optical neuropathy that were similar to Leber’s Hereditary Optical Neuropathy\(^1\)\(^6\). Leber’s is a mitochondrial disease of genetic predisposition. Other diseases of mitochondrial origin include Leigh Syndrome, Diabetes Mellitus and potentially many others. Parkinson’s Disease, while typically associated with degeneration of dopaminergic neurons is thought to have some mitochondrial origin\(^1\)\(^7,18\).

1.3 Molecular Factors in Biology of Parkinson’s and Related Diseases

There are some well-known players in this degenerative pathway such as \(\alpha\)-synuclein, Parkin and PINK1. \(\alpha\)-Synuclein is a neuronal protein thought to contribute to Parkinson’s through formation of soluble oligomeric aggregates known as protofibrils. While presynaptic in origin, \(\alpha\)-synuclein can be secreted, leading to disease propagation by seeding further aggregation in neighboring cells\(^1\)\(^9\). While the extent of \(\alpha\)-synuclein’s role in Parkinson’s is not entirely clear, it is thought to participate in other neurodegenerative conditions collectively labeled
synucleinopathies. α-Synuclein which is naturally unfolded, can be phosphorylated at Ser129, which plays some role in aggregation into Lewy bodies. Parkin, a cytosolic ubiquitin ligase, was once implicated in this phosphorylation at Ser129, although it now thought to de-phosphorylate at both Ser87 and Ser129, which reduces cellular damage and death in synucleopatheies\(^20\).

PINK1 (PTEN-induced putative kinase 1) aggregation occurs on the outer membrane of damaged mitochondria and acts as a signal for their destruction. It is believed that PINK1 and Parkin work cooperatively. PINK1 activates and recruits Parkin from the cytosol, which in turn phosphorylates and ubiquitinates the outer membrane, initiating selective autophagy.\(^21\) In this model, both excessive damage to these organelles by toxins (MPTP, paraquat, rotenone and others), and the inability to clear dysfunctional mitochondria may both lead to neuronal cell death and Parkinson’s disease.

1.4 Environmental Toxins and PD: 6-OHDA and MPTP

The two most explored toxin models for PD are 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) \(^1,2\) (Figure 2). They are the oldest models and also share some common mechanistic traits and limitations. 6-OHDA was introduced in the late 1900s making it the first PD toxin model to be developed\(^14\). When 6-OHDA is administered systemically, it causes destruction of sympathetic nerve terminals in the peripheral nervous system\(^22\). Due to the chemical similarity of both 6-OHDA and the active metabolite of MPTP to endogenous neurotransmitters, they actively cross the blood-brain barrier (BBB) via catecholamine transporters\(^23,24\). A possible explanation for the regioselectivity of these toxins is that midbrain neurons have the highest concentration of dopamine transporter (DAT) per cell\(^25\). DAT presence is required for MPTP-induced toxicity, as evidenced by mice mutants with DAT
deletions\textsuperscript{26}. To enhance the selectivity of 6-OHDA, it is sometimes co-administered with desipramine \textsuperscript{1.3}, a selective noradrenaline reuptake inhibitor\textsuperscript{27}.

![Chemical structures of 6-hydroxydopamine, MPTP, desipramine, and levodopa (L-DOPA) in Figure 2]

1.5 Metabolism and Activation of 6-OHDA

The location of 6-OHDA injection into the brain directly relates to its neurodegenerative effect. If injected into the midforebrain (MFB), non-apoptotic morphology is exhibited within 24 hours whereas protracted retrograde degeneration can take place for a window of up to three weeks if injected into the striatum\textsuperscript{28}. Striatal injection produces morphology akin to apoptic degeneration. For this reason 6-OHDA models must be differentiated by injection site. MFB injections lead to rapid degeneration within a few days followed by a gradual decline to near complete lesion in five weeks\textsuperscript{29}. Striatal injection causes more gradual damage in a dose-dependent pattern. For this reason, it is preferred when modeling initial stages of PD\textsuperscript{30}.

Toxicity initiates as the dopamine transporter releases 6-OHDA into the cell followed by non-enzymatic auto-oxidation\textsuperscript{31}. This oxidation leads to formation of superoxides, quinones and other radical species and causes downstream damage. This oxidative stress hypothesis is bolstered by the fact that overexpression of glutathione peroxidase and superoxide dismutase protect mice from 6-OHDA-induced damage\textsuperscript{32,33}. \textit{In vitro} analysis of the pathways of MFB and striatally-induced 6-OHDA degeneration has been performed. MFB injection more closely
resembles necrotic cell death than striatal administration and can be rescued by use of a general caspase inhibitor\textsuperscript{33}. Inhibition of the c-jun-N-terminal kinase (JNK) in rats and JNK deletions in mice have both demonstrated protective effects in MFB and striatal 6-OHDA studies, highlighting a common pathway in both models\textsuperscript{34,35}.

1.6 MPTP Discovery, Metabolism and Activation

MPTP \textbf{1.2} was discovered serendipitously at the interface of clandestine drug synthesis and forensic chemistry. A chemistry graduate student was seeking to synthesize meperidine analog (1-methyl-4-phenyl-4-propionipiperidine) \textbf{1.5} for personal use. Despite early successes, he allowed the reaction temperatures elevate, generating an ester that, following metabolism, forms an alcohol that is subject to elimination reaction and accidentally created MPTP (see Figure 3)\textsuperscript{36}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Synthesis of desmethylprodine and MPTP (MP= 1-methylpiperidin-4-one, HPMP= 1-methyl-4-phenylpiperidin-4-ol)}
\end{figure}

Following self-administration of this batch, he rapidly developed bradykinesia that was alleviated with L-dopa \textbf{1.4}. Subsequent forensic analysis of his labware determined these details. Initial injection studies in mice failed to produce such dramatic symptoms. However, later
rediscovery of MPTP across the country several years later by a similar synthetic mistake lead to injection of the material into monkeys. This chapter confirmed the acute toxicity of MPTP in humans while underlining important neurochemical differences between rodents and primates. MPTP was confirmed as a chemical probe capable of producing all of the PD hallmarks of rigidity, tremor and instability among others\textsuperscript{37}.

Although MPTP was overlooked in rodents initially, much effort has gone into developing this model. As it turns out, rats have are uniquely resistant to the effects of MPTP whereas mice are more susceptible. However, certain susceptibilities within mouse subpopulations (strain, age, gender) must be taken into account when selecting a model.

Due to the popularity of the MPTP model, much has been elucidated of the pathways by which it causes neuronal cell death. MPTP acts indirectly as a protoxin that eventually forms the metabolite 1-methyl-4-phenylpyridinium 1.5, or MPP+\textsuperscript{38}. Following systemic administration in primates, the lipophilic character of MPTP allows rapid crossing of the blood-brain barrier. Monoamine oxidase B (MAO-B) performs oxidation to 1-methyl-4-phenyl-2,3,-dihydropyridinium (MPDP+) is thought to take place in the glial or serotonergic cells\textsuperscript{39}. From here, MPDP+ undergoes further oxidation to MPP+, and gains access to dopaminergic neurons via the dopamine transporter (DAT) for which it has high affinity. This role of DAT is supported by genetic deletion and inhibition of DAT by small molecules- both measures prevent MPTP neurotoxicity \textit{in vivo}\textsuperscript{40,41}. Also of note is that DAT concentrations are highest in the midbrain cells which may explain the unique sensitivity of these cells toward MPTP-induced damage\textsuperscript{26}. As MPP+ enters neurons, it begins to accumulate in the synaptic vesicles and in the mitochondrial matrix by passive transport. MPP+ then collects in the mitochondria, it binds to complex I of the electron transport chain. This step of the chain allows for oxidation of NADH.
substrates and inhibits further electron shuttling steps from taking place. This also leads to a decrease in ATP production and increased ROS production. Meanwhile, there is an accompanying spike in vesicular dopamine release induced by MPP+. This dopamine is subject to auto-oxidation that acts as another source of damaging ROS.[42]

Both 6-OHDA and MPTP have been explored thoroughly in animal research models. There are specific, important differences such as animal selected, and which brain region is the target of degeneration. Despite their limitations, they represent the best explored tools available and these limitations highlight the importance of increasing the chemotoxic toolkit for PD research.
II. DISCOVERY AND SYNTHESIS OF BACTERIAL METABOLITE SV-6

2.1 Discovery of a Novel Neurotoxic Metabolite from *S. venezuelae*

A metabolite isolated from *Streptomyces venezuelae* caused age-related and dose-related degeneration of all *Caenorhabditis elegans* neurons, with dopaminergic neurons specifically degenerating more rapidly. Human neuroblastoma cells were also exposed to the metabolite. In this assay, the SH-SY5Y-derived cells in culture showed degeneration in a dose-dependent fashion following exposure. Initial investigations of the metabolite suggest that it causes cell death through decreased ATP production, increased reactive oxygen species (ROS) and modulation of mitochondrial complex I. The metabolite also disrupts protein homeostasis, ubiquitin proteasome activity and glutathione response to α-synuclein toxicity by loss-of-function of *pink1*. These molecular changes were seen in all cells of *C. elegans*, but due to the high energy demands of muscle and neuronal cells, these changes were more obvious.

Our collaborators at the University of Alabama (Department of Biology; Tuscaloosa, AL) have worked for some time on modeling PD in a genetically invariant hermaphroditic *C.elegans* (Nematode) model. The worm has considerable evolutionary distance from humans, but bears many important features that make it a useful for understanding PD neuropathology and also a testing ground for potential therapies. Beginning with work performed by Sydney Brenner in the 1970s to understand neuromuscular development, it has since revolutionized our
understanding of genomics, epigenetics, cell biology and aging\textsuperscript{46}. The worm has only 952 total cells, and almost a third of which are neurons, and represents the most thoroughly mapped neuronal circuitry and cell lineage of any model species\textsuperscript{47}. Its 302 neurons retain many important features of its mammalian counterpart such as receptors, ion channels, neurotransmitters, vesicular transporters and other synaptic components. It is rapidly cultured and transparent, which allows for degenerative assessment of six anterior dopaminergic neurons using an indicator such as green fluorescent protein (GFP)\textsuperscript{48}. \textit{C. elegans} has been exploited for PD research with dopaminergic circuitry consisting of a mere eight anatomically defined neurons. The organism’s genetic loci for PD risk factors are similarly defined and are termed “PARK”, with 21 sites currently identified. Of these, only six are connected to familial/monogenic PD. These are SNCA (\(\alpha\)-synuclein), VPS35, LRRK2, PINK1, PRKN (Parkin), and DJ-1\textsuperscript{49}. \textit{C. elegans} expresses all of these homologs \textit{except} \(\alpha\)-synuclein, which is a considerable exception. However, a variety of \(\alpha\)-synuclein overexpression experiments have accurately modeled the progressive degenerative and motor deficits expected in PD\textsuperscript{50}. Additionally, \(\alpha\)-synuclein misfolding can be traced by use of green-fluorescent fusion proteins which express into the bodywall muscle cells\textsuperscript{51}.

Our collaborators’ work is not the only evidence that microbial communities may contribute to PD. A study by Sampson et al. reported the effect of “gut microbiota on regulation of motor deficits and neuroinflammation” in a mouse PD model\textsuperscript{52}. This study demonstrated a number of interesting patterns connecting gut microbes to PD. In mice overexpressing \(\alpha\)-synuclein, gut microbes are required to produce microglial activation and synuclein pathology. Treatment with antibiotics ameliorated these effects, while microbial recolonization promoted pathophysiological symptoms. Oral administration of microbial metabolites also promoted
neuroinflammation and motor disturbances. Finally, the mice overexpressing α-synuclein were colonized with microbiota from either healthy or PD-affected human donors. Those mice exposed to the microbiota from PD-affected humans displayed deterioration in motor function in agreement with PD symptoms.

2.2 Innovation Toward a Better Chemotoxic Toolkit

Our understanding and treatment of Parkinson’s Disease (and other neurodegenerative diseases of mitochondrial origin) has been limited by the existing animal models used to study the disease. These animal models often require exposure to toxins such as MPTP and rotenone. These compounds are useful, but still leave a gap in our understanding. Increasing research into the chemical induction of PD-like symptoms is a much needed innovation not just Parkinson’s but for other mitochondrial diseases of neurodegeneration. Natural products provide an interesting subset of these chemicals as they may have been preserved in the environment over evolutionary time as a protection mechanism. It is speculated that only 5-15% of the Parkinson’s population stems from hereditary factors, leading researchers to look into environmental factors (toxins, microbiota) as important contributors to disease development. Figure 4 shows some natural products with mitochondrial toxicity are rotenone, iromycin, repin and the recently identified molecule SV-6 2.8 15a, 53.
Figure 4. Mitochondrial toxins
2.3 Discovery and Significance of SV-6

The target molecule SV-6 \textbf{2.8} was isolated from a bioassay-guided fractionation and purification. Spent \textit{S. venezuelae} growth media was extracted, yielding 300 mg of crude material that was subjected to reverse-phase liquid chromatography (RP-HPLC). To test these fractions, they were exposed to \textit{C. elegans} in an established neurodegeneration assay\textsuperscript{45}. Further purification of this active fraction lead to isolation of one milligram of material that displayed significant activity. This material was subjected to high-resolution electrospray ionization mass spectrometry and nuclear magnetic resonance to generate the putative structure.

In a related study performed by our collaborators, field collections of various \textit{Streptomyces} species not limited to \textit{S. venezuelae} were performed across the state of Alabama with the goal of determining how prevalent this toxin might be (Figure 4). Samples were collected and organized by three categories of land-use type and physiography. The first was agricultural soils (AG) used for crops and livestock grazing. The second was soils from undeveloped (UD) soils with multiple tree species and dense undergrowth. The last category was developed or urban (UR) soils near human populations but excluding lawns and landscaped soils\textsuperscript{54}. All three soil types were collected from across the major physiographic provinces of Alabama to represent the topographic and geologic diversity of the state (Figure 4).
Figure 5 Colors represent the physiographic provinces and data points (•) indicate sampling locations. (Map courtesy of the University of Alabama Cartography Lab)
The study showed that *Streptomyces* isolates by soil type varied with “a significant difference found in the number of isolates obtained between agricultural and undeveloped soils” while there was no difference between isolates when compared across physiographic provinces (Figures 5 and 6).

Isolates of *Streptomyces* from each land use were selected and cultured where possible—approximately half the Streptomyces species isolated did not grow in the SYZ media that was selected to aid in production of secondary metabolites. Of those that did grow successfully, supernatants were collected and assessed in the neurodegeneration *C. elegans* model system. From this group, 28.3% the *Streptomyces* spp. isolates (51 of 180 strains) produced dopaminergic neurodegeneration in *C.elegans* versus 0.4% in the control group used in this study.

**Figure 6** “(A) Exposure to environmental isolates of *Streptomyces* spp. can cause significant neurodegeneration in populations of *C. elegans*. (B) *Streptomyces* spp. isolated from agricultural soils caused significantly more dopaminergic neurodegeneration.”

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16
(E.coli extracts). When activity was tracked back to land use type there were more isolates from agricultural and undeveloped soils with neurodegenerative activity compared to urban soils (Figure 7).

Figure 7 “(A) Comparison of the average number of isolates obtained from dilutions of 0.25 g of agricultural (AG), undeveloped (UD) and urban (UR) soils. (B) n = 60 for each land use. Columns with the same letters are not significantly different from one another (Fisher’s exact test, p < 0.01).”

This led the authors to conclude that there was a neurodegenerative compound(s) being produced in the soil responsible for the Parkinsonian-like symptoms seen in rural populations that were not previously accounted for by other environmental factors such as herbicide, fungicide and pesticide exposure. Furthermore, the authors hypothesize that there is a common mitochondrial toxicant across many of these Streptomyces isolates. The authors believe that SV-6 may be a common toxicant and total synthesis help in understanding its ecological distribution and mechanism of action.
2.4 Structure of SV-6

SV-6 2.8 is a 13-membered linear polyketide that bears structural similarities to other metabolites from *S. venezuelae*. **Figure 8** below highlights the structural similarity of SV-6 with methynolide 2.10 and pikromycin 2.11. Of note is the 10-hydroxy group that is shared across all the structures and may be a product of PikC oxidation instead of polyketide synthase metabolism\(^{55}\). Pik pentakide 2.13 is a common biosynthetic precursor to both methynolide and pikromycin\(^{56}\). It is important to note that the stereochemistry of SV-6 in this figure is only hypothetical and based on the known metabolites from *S. venezuelae*. Only following the total synthesis of SV-6 and comparison to the isolated metabolite can this structure be confirmed.
Figure 8
2.5 Polyketide Synthase and the Biosynthetic Origin of SV-6

While a vast diversity of structures are enabled by strict implementation of polyketide synthase machinery, there are even more possible variations enabled by incorporation of alternate starter or extender units (Figure 9).

![Polyketide Synthase Diagram](image)

**Figure 9** General biosynthetic format of polyketide pathway responsible for aromatics, macrolides and fatty acids\(^57\).

In addition to malonyl and acetyl-CoA units, **Figure 10** shows the PKS can also use propionyl-CoA units which can be converted into methylmalonyl-CoA extender units by addition of CO\(_2\) by propionyl-CoA carboxylase. This allows for methyl groups to be installed between carbonyl-bearing units. Some bacteria can also incorporate iso-fatty acids in the PKS pathway. These branched chain fatty acids are otherwise used to maintain membrane fluidity. They are
generated from branched chain amino acids and serve as alternate PKS starter units. Leucine, isoleucine and valine lead to isovaleryl-CoA, 2-methylbutyryl-CoA and isobutyryl-CoA respectively (Figure 10).

![Diagram of branched chain amino acids and their corresponding CoA derivatives](image)

**Figure 10** Incorporation of branched motifs into PKS pathway products.\(^{57}\)

In the case of SV-6 it seems probable that isobutyryl-CoA was used as a starter unit followed by incorporation of multiple methylmalonyl-CoA units. In addition to the variety of loading and extender units and subsequent reductions and dehydration steps, there is potential for other
tailoring steps following PKS release, such as decarboxylation, Pik oxidation and to form macrocyclic lactone rings. In the case of SV-6, there is a disparity in carbon count and acetate incorporation pattern. It is possible that the parent unit was decarboxylated then subjected to redox transformations to result in the proposed structure for the isolated natural product (see Figure 11).
Figure 11 General biosynthetic hypothesis for PKS assembly of SV-6. Main assembly (top), followed by post-PKS release tailoring steps (bottom).
2.6 Initial SV-6 Synthesis Plan: Andrade and Breit Fragments

To date, neither the structure nor the synthesis of the target molecule SV-6 has been published. Although the synthesis will be new, the plan is to rely on well-established methods for producing polyketides. The final compound could be produced in a convergent fashion by coupling two fragments such as a vinyl iodide and aldehyde (Scheme 1) fragment A 2.13 and fragment B 2.14, followed by oxidation and deprotection (Fig.2). Our confidence in this approach was based on the successful syntheses of similar polyketides tridesmethyl telithromycin, and vittatalactone by the groups of Andrade and Breit, respectively\textsuperscript{58,59,60}.

**Scheme 1** Disconnection between fragments A and B

![Scheme 1 Disconnection between fragments A and B](image)

**Scheme 2** Initially planned fragment A synthesis

![Scheme 2 Initially planned fragment A synthesis](image)

Fragments A and B may be derived from enantioselective alkylations of simple α,β-unsaturated aldehyde starting materials methacrolein 2.15 and crotonaldehyde 2.22 with
diethylzinc. In the case of fragment A, the alcohol 2.16 could be oxidized to the epoxide 2.17 using Sharpless asymmetric epoxidation\(^6\). This is followed by ring opening with pivalic acid to selectively protect the least hindered position\(^5\). At this point, the 1,2 diol 2.18 can be protected with an acetal 2.19. The pivalate group can be removed using methyllithium, and then oxidized to the aldehyde 2.21. Takai-Utimoto olefination will be used to finish fragment A 2.13.

Scheme 3 Initially planned fragment B synthesis

The synthesis of Fragment B 2.14 continues from a distinct allylic alcohol than fragment A. First, a Steglich esterification with ortho-diphenylphosphanylbenzoic acid (o-DPPBA) 2.24 is performed, and a portion of this product 2.25 is retained for a later step (Scheme 3, step g). Stereospecific addition of the branched bromide to the ester in a copper-assisted Grignard reaction (Figure 12) followed by ozonolysis concludes the synthesis of fragment B. This iterative approach using o-DPPB esters has proven effective in longer polyketides by Breit and
co-workers. Cleavage of the double bond with ozonolysis (or alternative method), followed by reduction and Appel reaction leads us to an intermediate 2.26 to be reacted with previously generated o-DPPB ester 2.25.

![Syn-addition reaction](attachment:reaction.png)

**Figure 12** Breit's proposed mechanism of stereoselectivity

Fragment A and fragment B would be connected via a Nozaki-Hiyama-Kishi reaction (Scheme 4). This reaction has previously been proven effective in the synthesis of other polyketides such as telithromycin, palytoxin, and halichondrin B.

**Scheme 4**

![Scheme 4](attachment:scheme.png)

**SV-6 Fragment Coupling and Completion:** (a)CrCl2, cat. NiCl2; (b) DMP; (c) aq. HCl
2.7 Enantioselective Oganozinc Alkylations of Aldehyde Building Blocks

It was envisioned that both starting materials could be derived from enantioselective alkylations of simple enone starting materials methacrolein 2.15 and crotonaldehyde 2.22 with diethylzinc (Scheme 5). This enantioselective counterpart to the Grignard addition reaction is based on the pioneering work of Noyori, which established the utility of β-amino alcohols such as DAIB 2.32 (Scheme 6) to enantioselectively control addition of diorganozincs to aldehydes.65

**Scheme 5**

Diaorganozincs were held to be an ideal alkyl donor due to their sp-hybridized linear geometry. The alkyl-metal bond was relatively nonpolar, but it could be bent to induce reactivity toward carbonyls upon perturbation by external ligands. The formation of stable alkylzinc alkoxide tetramers in hydrocarbon solvents following release of anionic ligands could also be leveraged as a driving force for the reaction (Scheme 6). Addition of diethylzinc addition to aromatic aldehyde substrates fared best in yield, enantiomeric excess and reaction time. Alkyl
aldehydes and longer alkyl groups in the organozinc agents both performed poorly. α,β-unsaturated aldehydes performed somewhere in the middle. Methacrolein and crotonaldehyde were both explored in this initial report, but not optimized.

Scheme 6

Later, Nugent and coworkers at Bristol-Myers Squibb performed parallel synthesis techniques to more thoroughly understand the structure-activity relationships of β-amino alcohol ligands in asymmetric organozinc alkylations. They too focused on benzaldehyde 2.32 as their test substrate. In a follow-up study it was determined that a tertiary amine substructure bearing piperidinyl or morpholinyl rings was optimal in all cases. N-terminal and O-terminal substituents were both deemed critical and simplified amino alcohols performed abysmally. Introduction of a phenyl group to N-terminal position dramatically improved enantioselectivity to 83% while introduction of a cyclohexyl group increased enantioselectivity to 67%. The combination of both features proved yielded a new ligand 2.30 that could be produced in two steps. In this case, catalyst loading of 5% both aromatic and α,β-unsaturated aldehydes (including methacrolein) performed well while alkyl aldehydes still performed poorly.

In a recent update to this method by Myers and co-workers, the conditions for this reaction were optimized. In this case, they needed to produce large quantities of (R)-2-methylpent-1-en-3-ol 2.16 from methacrolein 2.15. In their work, they were able to modify...
addition protocol which resulted in both a reduction in catalyst loading and a reduction in transfers of pyrophoric diethylzinc. They found that by adding diethylzinc to a suspension of catalyst followed by slow addition of methacrolein followed by aqueous-acidic workup they could also recover their ligand in high purity. They also changed the solvent from hexanes-toluene (2:1) to pure hexanes. Using catalyst loading of 1% and reaction temperature of 0°C, they attained product in 76% yield with 99% ee. With these studies from Noyori, Nugent and Myers as a guide, we would attempt to extend this method to crotonaldehyde as a substrate using a tert-butyl morpholine ligand to produce an analogous ethylation product, hex-4-en-3-ol 2.23.

Scheme 7

In regard to SV-6, the work began by synthesizing the ligands to be used (Scheme 7). Next, these were to be applied to the synthesis of fragment B because the starting material, crotonaldehyde was much less expensive than methacrolein ($0.20 versus $6 per milliliter). This
would allow me to test the ligand-directed diethylzinc reaction on a more affordable starting material. Both ligand 2.30 and 2.31 were made in two steps. This was followed by asymmetric ligand-directed alkylation of crotonaldehyde with diethyl zinc and 2.31 based on the work of Noyori and Myers. At small scale this reaction seemed to have some impurities. It was initially performed on a scale too small too effectively perform vacuum distillation on. Initial attempts at purification with column chromatography were challenging due to the small size and nonpolar nature of the compound. Spectra indicated some impurities but there was uncertainty as to if they were oxidation products (unlikely), solvent impurities or residual chromatography solvents. Only after scaling the reaction up was it apparent that the reaction had produced a mixture of products (Scheme 8), with our intended product being the major component. However, this mixture was not separable further by column chromatography or distillation. After realizing this fact, the ligand-directed alkylation was abandoned.

Scheme 8

![Scheme 8](image)

*Reaction Conditions: (a) diethyl zinc, (S)-3,3-dimethyl-1-morpholinobutan-2-ol, hexanes, 0°C, 3 h*

2.8 Overhauled SV-6 Synthesis Route

Due possibility for rearrangements and associated loss of optical purity, a new route was adopted with some advantages. It would also be safer- no longer requiring large-scale
diethylzinc and ozonolysis reactions. Existing procedures for many intermediates were detailed\textsuperscript{58,56}. This route would also simplify access to isomers and analogs of SV-6 (\textbf{Figure 13}). The commercially available Roche ester starting materials contain a stereogenic center that will appear in the final product. The only downsides were is that this route did not have as many opportunities to explore reaction development and the Roche ester starting material is more expensive.

\textbf{Figure 13}: Modular access to various stereoisomers and analogs

2.9 Fragment A Synthesis: Cossy Fragment

Following the mixed results of the zinc alkylation, it was decided to use a route that was based on the best documented chemistry possible (\textbf{Scheme 9}). There was a related route taken by Cossy et al. that also ended with haloolefination to yield the final product\textsuperscript{58}. It began with a reaction of methacrolein with an ethyl Grignard reagent. This formed the racemic mixture of (E)-hex-4-en-3-ol enantiomers. These were effectively resolved using Sharpless conditions\textsuperscript{61,71}. Silyl protection of the allylic alcohol followed by dihydroxylation formed a mixture of
diastereomers that were chromatographically inseparable. These were carried forward though oxidation to the aldehyde, haloolefination and deprotection. Following deprotection of the silyl ether, the mixture became easily separable on silica gel. Treatment of the diol with dimethoxypropane and pyridinium p-toluenesulfonate yielded the protected vinyl iodide that would be used to couple with fragment B.

Scheme 9

2.10 Fragment B Synthesis: Roche Ester and Pseudoephedrine Chiral Auxiliary

Synthesis of pseudoephedrine propionamide 2.53 and TBS-protected iodide 2.57 proceeded smoothly before encountering problems (Scheme 10). First, the chiral Roche ester was purchased from Sigma-Aldrich, protected with a tert-butyldimethylsilyl protecting group. The protected ester was reduced with DIBAL to the alcohol which was displaced to the iodide under Appel conditions.
By far the most troublesome reaction of the entire SV-6 synthesis was the alkylation of (R,R)-pseudoephedrine propionamide \(2.53\) with the TBS-protected iodide \(2.57\). The related reactions were reported in the literature with yields up to 95% so this difficulty was unforeseen\(^{72,73,56}\). Moisture sensitivity was expected for this reaction but even with careful application of anhydrous techniques the yield plateaued at 15%. The purified product matched data published for the compound but attempts to increase yield were met with failure. Different batches pre-prepared LDA and LiHMDS were used to no success. Diisopropylamine was repeatedly distilled over activated molecular sieves and n-butyllithium sources where repeatedly titrated. The reaction duration and temperatures were varied as were equivalents of reagents used in the reactions. Both pseudoephedrine propionamide \(2.53\) and protected iodide \(2.57\) starting materials were resynthesized numerous times to no avail. Test reactions using benzyl bromide instead of the protected iodide \(2.57\) were also performed to narrow down the cause of the problem.
This well-supported transformation presented a surprising challenge, and was temporarily placed on hiatus while I synthesized fragment A. After returning to this reaction, it was rerun with a previously opened bottle of LDA which surprisingly yielded 80%. The reaction was immediately repeated at larger scale using the same batches of every reagent, resulting in a yield of 75% (Scheme 11). Although this reaction worked well enough to move forward, there was never a scientifically satisfying conclusion as to why it did not work initially.

The first plan was to use alkyllithium reagents to displace the chiral auxiliary and then reduce the carbonyl. This exact reaction is unreported, but its closest analogs use t-butyllithium, phenyllithium and n-butyllithium, with maximum reported yields of 71%, 98% and 95% respectively \textsuperscript{74,75,76}. At this point, the Clemmensen approach was reappraised with an eye to possible issues such as epimerization (Scheme 12).
Other routes were all considered and the step-count and reaction yields from similar systems in the literature were all compared before finding a suitable replacement. In the selected approach (Scheme 13), I opted to first reduce the amide auxiliary to the primary alcohol 2.61. It could then be transformed to a tosylate 2.62, which would be displaced with a copper-assisted alkyllithium. The ammonia-borane reduction worked well but the tosylation had yields of 30-40%. The solvent was changed from dichloromethane to pyridine, improving conversion to 99%. The resulting product 2.60 was deprotected with tetrabutylammonium fluoride and oxidized with nickel (II) chloride and commercial bleach (sodium hypochlorite). 

Scheme 13

Unfortunately, the yield for the nickel (II) chloride oxidation was less than 15%. It was a very complex TLC and the main polar product was isolated and matched expected molecular weight when analyzed with ESI mass spectrometry. It was not characterized further. Instead of optimizing a reaction with limited literature support, the reaction was abandoned. I attempted an alternative oxidation using TEMPO, sodium hypochlorite and sodium chlorite. This reaction went smoothly and after achieving a yield of 77% (Scheme 14), the reaction scale was increased and the resulting material was taken forward to the next step.
Water soluble carbodiimide (EDC) in DMF with standard amide coupling conditions was used to form the Weinreb amide product 2.65 (Scheme 15). The reaction yielded 45% but without any more acid starting material, this was all that would be used for the remaining steps of the synthesis.

The second to last step was a coupling reaction between the fragments 2.13 and 2.14 (Scheme 16). A vinyl lithium species from vinyl iodide 2.13 and t-butyllithium is prepared in situ to attack the Weinreb amide fragment 2.14. This was performed on ten-milligram scale with a yield of 46%.
Six milligrams of the protected enone product \textbf{2.29} was subjected to acidic deprotection conditions (Scheme 17) using a mixture of tetrahydrofuran and aqueous hydrochloric acid at room temperature. This last step proceeded slowly, but it was ultimately successful with 95% yield.

2.11 Future Work

The construction of the proposed SV-6 structure has been completed to supply six milligrams for spectroscopic analysis and biological assay. This material was transferred to the original investigators and will be compared to the spectra of the natural product. The initial synthesis can be repeated to generate more material if needed for biological analysis. In the event that the structures do not match, computational studies in combination with synthesis of isomers may be used to ascertain the configuration of the natural product\textsuperscript{79}. The potential for
diversity is an advantage of the second route that cannot be understated (Figure 14). By using the same experimental procedures outlined herein, it is possible to generate countless isomers and unnatural analogs for the study of structure-activity relationships. The figure below shows just a few examples of compounds that can be generated by following these procedures but only substituting commercially available reagents. Different enantiomers of Roche ester, pseudoephedrine diisopropyl tartrate (DIPT) can be used to influence the stereochemistry of the branched alkyl units (C-4 and C-6) and hydroxyl group (C-10 and C-11). Different groups can be substituted at the C-4 position by use of different pseudoephedrine amides. In this case, any number of anhydrides or acyl chlorides may be used to generate chiral amides in a single chromatography-free step. Substitution of different groups can be added to C-3 position but replacing the isopropyllithium used for SV-6 with other organolithiums or Grignard reagents.

Figure 14

Increasing Divergence from SV-6
III. CHEMICAL MODIFICATION OF REPIN

3.1 *Rhaponticum repens* and Toxic Parkinsonism in Horses

Russian knapweed or *Rhaponticum repens* (L.) Hidalgo of the family Asteraceae is the most recognized toxic plant contributor to equine nigropallidal encephalomalacia (also called toxic equine parkinsonism or “chewing disease”)\(^8\). It has been previously referred to as *Centaurea repens* or *Acroptilon repens*. It is an invasive perennial plant species that is native to Eurasia but was brought to the United States in the early 1900’s. It bears broad, oblong lower leaves but toothed upper leaves. The plant has and thin, stiff, pithy stems and stands erect, with the potential to grow up to one meter in height. It bears urn shaped flowers that vary from pink to purple in color. Once past maturity, these flowers fade into a straw color. The seeds are also a straw or ivory color, oval in shape and 2 mm wide, 4 mm long and 1 mm thick (Figure 15). Both the seeds and the stems are covered with fine hairs. The plant has invasive character, leading to infestations in fields. In the case where they do not entirely dominate an area to the point of monoculture, they can suppress the growth, size and number of plants it shares location with. According to Watson as of 1980 they were capable of reducing grain density by 28-75%. Plant extracts of *R. repens* have demonstrated allelopathic inhibitory effect of crop plants. It seems to only require tillable soil, with some preference for saline or alkaline soils. Russian knapweed is robust in its ability to survive without regard for other species of plant it is surrounded by (or lack thereof). It is this co-habitation with crop plants that is thought
to be responsible for its spread south and east, most likely from sale and distribution of hay contaminated with Russian knapweed seeds\textsuperscript{82}. The majority of these \textit{R. repens} traits are common to \textit{Centaurea solistialis}, with some difference in flower morphology. For instance, \textit{C. solistialis} has yellow flowers with sharp thorns extending from the base of the buds.

\textbf{Figure 15} Images of \textit{Rhaponticum repens} (A) full plant (B) seeds (C) flowering tops in hand for scale (D) single flower\textsuperscript{83}. 
Figure 16 Distribution of Russian knapweed in the United States

3.2 Toxic Effects of *R. repens*

Cordy first reported that horses in the northern and central region of California were beset by a neurological disorder linked to the consumption of Russian knapweed and yellow star thistle. It had been previously identified by local veterinarians as “chewing disease” or “yellow star thistle poisoning”\(^6\)\(^5\). According to this initial report, there was always a sudden onset to the symptoms which included impairment of eating and drinking. Some animals displayed complete inability to eat or drink while others might be “dropping feed from the mouth, [partially unable] to move the material back to the molars, or some difficulty swallowing”\(^6\)\(^5\). Eating was typically affected more than drinking, hence the name “chewing disease”. Also common was a fixed expression and tongue protruding from between incisor teeth which indicated paralysis of the
facial musculature. Of note were changes in the behavior of horses, they often stood motionless or with a drowsy pose. This was accompanied by aimless, abnormally slow or awkward walking which deteriorated to a wobbling or shuffling gait in later stages. Initially a parasitic or viral cause of these symptoms was considered, but further anatomical investigation of horse tissues were ruled inconsistent with either hypothesis. Eventually, this constellation of movement impairments combined with the presence of neuronal legions was termed “equine nigropallidal encephalomalacia” (ENE)\textsuperscript{84}. Despite the sudden onset of ENE, animals must consume 59-71\% of their body weight before these symptoms manifest.

3.3 Structure of SQLs from \textit{R. repens}

Due to the allelopathic and toxic activity of the \textit{R. repens} plant, it has been the target of thorough phytochemical examinations. The focus of this work is on terpenes although alkaloids, flavonoids, tannins, polyacetylenes and glycosides have all been isolated from the plant. Studies of volatile components by Buttery and Binder demonstrated that SQLs comprise between 50\% and 90\% of the plant material by weight. Specifically, there were 58\% of aggregate volatiles in buds, 56\% in blooms, and over 89\% in leaves and stems\textsuperscript{85,86}. In a series of studies led by Stevens for the USDA, a number of SQLs were identified from \textit{Centaurea solstitialis}, particularly noteworthy among these structures are the presence of many chlorohydrins (3.3, 3.7, 3.8, 3.11) products from the opening of epoxides and picrolide 3.2, which bears \textit{p}-hydroxybenzoyl methylene moiety on 4-position of the A ring (\textbf{Figure 17})\textsuperscript{87}. 


Figure 17
3.4 Collection of Plant Material and Isolation of Repin

The plant material (*R. repens*) was collected by Dr. Gunatilaka (September 2002) and Dr. Rimoldi (July-August 2004 and May 2007) in Riverside, California. It was collected in a somewhat developed semiurban area (near a golf course). This location was selected based on previous positive identification and collections on the same plot of land. Mature flowering plants were collected and dried and voucher specimens were deposited at the University of California at Riverside herbarium.

A variety of methods have been used for isolation of SQLs from *Centaurea* species. These approaches involve the use of many different combinations of solvents and stationary phases or treatment of extracts with lead (II) acetate to facilitate removal of chlorophyll. Rimoldi et al. have used various methods.

**Method A**

The first method begins with hexane extraction for 24 h, then two acetone extractions for 2 h each. These acetone extracts were combined, evaporated and subjected to vacuum liquid chromatography (VLC) using a ethyl acetate/hexane gradient (30% ethyl acetate increasing to 100%). Following analysis and combination of subfractions, repin was isolated after another round of column chromatography.

**Method B**

Aerial plant material was macerated in hexanes for 24 h followed by two 48 h acetone extractions. This extract was evaporated then reconstituted in anhydrous ethanol. The ethanolic solution was slowly treated with a 5% aqueous solution of lead (II) acetate which formed a
precipitate that was stirred for 24 h before vacuum filtration. This filtrate was evaporated and re-extracted into anhydrous dichloromethane. Extracts were dried over sodium sulfate, evaporated and subjected to VLC using a ethyl acetate/hexanes gradient (50% ethyl acetate increasing to 100% followed by 60:40 isopropanol/ethyl acetate). Subfractions from the 75:25 EtOAc-hexanes were rich in repin and were subjected to recrystallization from ethyl acetate.
3.5 Biosynthesis of Sequiterpenes

There is a vast array of terpenoids present across the plant kingdom whose structures range from simple to incredibly complex. All these structures can be traced back to only a couple of simple biosynthetic intermediates- either mevalonic acid (MVA) and methylerthritol phosphate (MEP)\(^5\).
The conventional names for these structures fall into terms determined by the size of their carbon skeletons. Some discrepancy between named category and final carbon count may be attributed to loss of carbons by decomposition of a larger terpene structures or late-stage addition of small units. The overall structure is represented by \((C_5)_n\). With this system, the family of terpenes is subclassified as hemiterpenes \((C_5)\), monoterpenes \((C_{10})\), sesquiterpenes \((C_{15})\), diterpenes \((C_{20})\), sesterterpenes \((C_{25})\), triterpenes \((C_{30})\), and tetraterpenes \((C_{40})\), followed larger isoprene polymers such as rubbers (Figure 18). These units are rarely seen as simple linear polymers and instead often undergo a number of oxidations, rearrangements and combinations with intermediates of acetate, peptide and shikimate pathways to form their isolated products\(^{57}\).

MVA is formed by the fusion of three molecules of acetyl-coenzyme A. The eventual decarboxylation and dehydration of mevalonic acid diphosphate (MVAPP) yields isopentyl phosphate (IPP). Figure 19 displays enzyme-mediated isomerization of IPP to DMAPP creates the system where SN1-mediated loss of diphosphate anion creates stabilized cation that will serve as the electrophilic partner to the nucleophilic terminal alkene of IPP. These two initially combine to form geranyl diphosphate (GPP), which can now act as an electrophile to another IPP unit\(^{57}\).
This iterative process continues for another cycle and the result is farnesyl diphosphate (FPP). It is important to note that stereospecific proton loss is responsible for E-stereochemistry of the double-bond. This stereochemical outcome is an important factor in the subsequent creation of intramolecular carbon-carbon bonds on the E,E-farnesyl scaffold. FPP is a key intermediate in the formation of multicyclic terpenes, including the sesquiterpene lactones. Loss of diphosphate from farnesyl-PP generates a cation facilitating E1 elimination to form (+)-germacrene A. This is followed by a series of enzymatic oxidations using NADPH, to generate parthenolide (Figure 20).
Figure 20: Biosynthesis of medicinally valuable parthenolide

Parthenolide is a secondary metabolite with well-known cytotoxic activity⁹⁰. But parthenolide represents only one example of the potential products produced from the germacryl cation intermediate. Figure 21 below shows the diversity of structures generated from three products of the germacryl cation intermediate. Fermacrene, costunolide and parthenolide serve as the biosynthetic precursor to many biologically active sesquiterpenes including repin, the subject of our group’s research. What we understand of the biosynthesis of terpenes is the product of many years of study using isotopic labeling and biochemical experimentation⁵⁷. However, the exact path of repin’s synthesis in Centaurea solstitialis remains unclear.
Figure 21 Structural diversity generated from germacrene, parthenolide and costunolide
3.6 Modification to Repin

There are numerous cytotoxic compounds in *R. repens*, but the exact toxin and mode of action are not entirely understood. Repin 3.15 is prevalent in the plant and a definite contributor to neurotoxicity but outward indicators of degeneration are not completely consistent with the established models of Parkinson’s disease. The primary aim of this work has been to create derivatives of repin to act as molecular probes to determine its protein target.

Preliminary work in the Rimoldi lab has identified the portions of the molecule that seem to contribute to the neurotoxic activity and those that do not ([Figure 22](#))\(^{89}\). The next stage of our investigation is to create a biotin-linked conjugate which will be used to identify the molecular target(s) of repin.

![Figure 22](#)

The early work focused on using esterification reactions to directly bind the secondary alcohol of repin to the carboxylic acid of biotin. Unfortunately due to the multiple sensitive electrophoric groups of repin, these attempts at esterification were met with failure. The primary issue with all these approaches was that nucleophilic reagents can attack repin directly at these
sites. Non-nucleophilic bases can catalyze intramolecular attack while acidic conditions facilitate carbocationic rearrangements.

<table>
<thead>
<tr>
<th>X</th>
<th>Conditions</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>DIC, HOBT, DMAP, DMF*</td>
<td>0%</td>
</tr>
<tr>
<td>H</td>
<td>Ceric ammonium nitrate, DMF*</td>
<td>0%</td>
</tr>
<tr>
<td>H</td>
<td>Ph₃P, DIAD, DMF*</td>
<td>0%</td>
</tr>
<tr>
<td>C₆F₅</td>
<td>TEA, DMF*</td>
<td>0%</td>
</tr>
<tr>
<td>C₆H₅NO₂</td>
<td>DMAP, DMF*</td>
<td>0%</td>
</tr>
</tbody>
</table>

*Varying temperatures, times and equivalents of base were used.

Table 1

However, esterification using the smaller acrylic acid 3.18 was successful. This now provided a handle for olefin cross-coupling chemistry (Scheme 18). A complementary biotin allyl 3.19 amide was produced successfully, but the Grubbs cross-coupling attempts did not work.
The Huisgen 1,3-dipolar cycloaddition (Scheme 19) is known to be extremely chemoselective and performs well in complex mixtures including biological systems. The regiochemistry can be controlled depending on whether copper or ruthenium is used to catalyze the reaction. The system we planned to use is Cu(I) catalyzed alkyne-azide cycloaddition (CuAAC) as it creates the stable triazole with the linker and repin scaffold oriented trans to from each another.
3.7 Alternate Route: Nicholas and Click Cycloaddition

Recently, a modification of the Nicholas reaction has been reported by the groups of Harki and Brummond in which the nucleophile is the limiting reagent, reducing the capacity for nucleophilic attack onto reactive scaffolds. This method has shown utility in attaching alkyne ligation handles onto serine alcohols, cysteine thiols, tyrosine phenols, pyrrolidine nitrogens of proline, as well as amino and carboxy groups of amino acids. Sesquiterpene lactones bearing similar functionalities such as Melampomagnolide B and a guaianolide analog (Figure 23) have also been successfully modified using this method. One foreseeable problem with this approach is the possible steric interference of adjacent epoxide.
3.8 Nicholas Reaction: Success and Failure

The Nicholas reaction was attempted on repin as a substrate and after many failed attempts, we produced the propargyl ether product 3.21 of interest. Over two steps, the yield was approximately 15% (10mg), and was never fully purified away from a co-eluting impurity (Scheme 20). The reaction was repeated at larger scale in order isolate enough material for the CuAAC reaction with biotin-azide and subsequent biological studies. In these subsequent reactions, it never fared better in terms of yield or purity. The addition of the propargyl group was evident on NMR and high-resolution mass spectral data matched that of our intended product.
3.9 Copper-catalyzed Alkyne-Azide Cycloaddition

As the supply of isolated repin dwindled, the limited amount of the propargylated material that remained was taken forward for CuAAC reactions (Scheme 21). These cycloadditions proved more challenging than expected (Table 2). The stability of the repin scaffold under acidic CuAAC conditions was first tested using repin instead of the propargylated material. Following this, the reaction was attempted under various conditions (~1mg scale) with varying success before scaling up.
The reaction yields were not quantified, only monitored by TLC and ESI-MS for consumption of starting materials and formation of product 3.28. When using CuI as a copper (I) source, transition of solution from clear or yellow to bright blue corresponded with the consumption of starting material. Despite this useful indicator of reaction progress, this reaction did not behave in the same manner when scaled up. This is likely due to inaccuracy of measuring reagents in those test reactions. After moving to CuSO₄/ sodium ascorbate conditions, the reaction worked well and scale up was more successful.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Eq. Alkyne</th>
<th>Eq. Azide</th>
<th>Cu Source (Eq)</th>
<th>Acid (Eq.)</th>
<th>(Base Eq.)</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1 05</td>
<td>CuI (0.02)</td>
<td>acetic (0.04)</td>
<td>DIPEA (0.04)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1 05</td>
<td>CuI (0.04)</td>
<td>acetic (0.05)</td>
<td>DIPEA (0.05)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>2</td>
<td>CuI (0.04)</td>
<td>acetic (0.05)</td>
<td>DIPEA (0.05)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1 02</td>
<td>CuSO₄ (0.44)</td>
<td></td>
<td></td>
<td>sodium ascorbate</td>
</tr>
</tbody>
</table>

Table 2

It was immediately obvious that the product of interest had almost the same retention factor on silica as the biotin-PEG3-azide starting material 3.27. The material was subjected to preparative thin layer chromatography, but the material produced was not pure. This material was subsequently subjected to HPLC, but the two peaks were not separable, possibly due to the weak UV absorbance of the components of the mixture in the range of the UV detector. The same material was subjected to LC-MS analysis, where product was clearly identified. However, the purity was approximated to be 30%-50%, with uncertainty due to variable levels of ionizability between components of the mixture. NMR analysis of the product showed presence
of biotin, polyethylene glycol and repin sections of the molecule but also with areas of unidentifiable impurities.

3.10 Additional Target Screens for Repin

The narrow goal of this project was to create a biotin tagged version of repin. The overarching goal of this project has been to better understand the molecular mechanisms at work behind repin’s unique toxicity. The failure to produce analytically pure biotinylated repin is only one aspect of a project in which there were some noteworthy revelations. Large scale screening of protein targets has become increasingly practical and multiple lines of investigation were pursued. Repin was submitted multiple different screens including a broad, multiplexed serine protease assay (ENPLEX) and a panel of kinases (KinomeScan)\textsuperscript{93,94}. Despite the possibility that a compound bearing multiple electrophilic sites may not show selectivity against enzymes, this was not the case.

Repin was screened against a total of 158 enzyme targets and only showed activity at two different targets (\textbf{APPENDIX I}). Of the 158 targets, 119 were serine hydrolases and 39 were kinases. Repin showed no interaction with the set of serine proteases that included HTRA2, which is correlated with essential tremor and PD\textsuperscript{95}. Of the 39 kinases selected based on literature support correlating them to dopaminergic signaling, mitophagy, PD, or neurodegeneration, only two targets were to be inhibited: GRK3 and LKB1. LKB1 regulates the ability of presynaptic mitochondria to clear calcium by regulating the density of a Ca\textsuperscript{2+}-selective ion channel on mitochondria’s inner membrane. This LKB1-regulated calcium clearing also controls neurotransmitter release from excitatory synapses on cortical axons\textsuperscript{96}. LKB1 demonstrates a role...
in autophagy, a pathway that is the only route to degrade protein aggregates that cannot be processed by the proteasome. This autophagy pathway is of particular importance in autosomal dominant PD (α-synuclein and LRRK2 associated)\(^97\). The other hit, GRK3, is a kinase that phosphorylates dopamine G protein-coupled receptors following activation by a ligand. This activation creates a high-affinity interaction between the G proteins and arrestin- a feedback loop that leads to deactivation and receptor degradation. The interplay of GPCRs, G proteins, arrestin and GRKs regulates striatal dopamine signaling and can lead to dyskinesia that is typical of PD\(^98\). While much remains to be understood, there are now two previously unreported protein targets for repin to guide further studies. Almost as important are the negative results from the 119 proteases targets, highlighting the incredible selectivity that is possible with sesquiterpene Michael acceptors such as repin\(^99\).

3.11 Future Work

Studying the molecular mechanism of repin’s toxicity has been an object of our attention for some time. Despite not purifying a pure biotinylated product, this work represents a way forward. Another path considered but never attempted was a modification of repin to the azide. This seemed much more risky due to the nucleophilicity of the azide anion involved, but it may be facilitated by activation with Schwartz’s reagent/ zirconocene hydrochloride. Hydrozirconation can be followed with displacement with a nucleophile, such as halogen or azide (Figure 24). This may be further complicated by iodination prior to azide substitution.
The modification of repin is all predicated on the amount of natural product available to attempt said modifications. Further synthetic studies would all require more repin to be isolated from Knapweed extracts or plant material. Purification of the biotin-repin from its mixture may be enabled by semi-preparative LC-MS.

Biological studies have failed to produce a complete picture of how repin manifests its toxicity (APPENDIX I). A streptavidin pulldown assay may still provide a missing piece of evidence to tie some of these activities together. It is possible that such an assay using the crude biotinylated material could yield this information if HRMS-based proteomic analysis could differentiate between various covalent adducts.
EXPERIMENTAL

General

All reagents were purchased from Sigma-Aldrich, Acros Organics or Alfa Aesar. Chromatography solvents were purchased as certified A.C.S. grade from Fisher Scientific. Acros extra dry solvents over molecular sieves were used for all reactions. Thin layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} aluminum sheets. To visualize TLCs, both UV light and staining reagents (phosphomolybdic acid, ceric ammonium molybdate, and potassium permanganate) were used. Flash chromatography was performed using standard grade silica gel particle size of 32-62 μm from Sorbent Technologies. \textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectra were recorded using Bruker 400 or 500 MHz instruments. Low resolution mass spectral data was acquired using Waters ZQ with electrospray ionization (ESI) conditions. Optical and specific rotation were measured using a Rudolph Autopol IV polarimeter.
This method was adapted from a procedure by Myers et al. A 3000ml flame dried three neck flask with magnetic stir bar was removed from oven and flame dried under vacuum and filled with argon three times. After cooling, (S)-3,3-dimethyl-2-morpholinobutan-1-ol ligand (1.54 g, 8.24 mmol, 0.02 eq.) was added followed by 300 mL of anhydrous hexane. After ligand was fully dissolved in hexanes, the flask was cooled to 0°C. At this point, a solution of Et₂Zn in hexanes (800 mL, 1.0 M, 800 mmol, 1.94 eq.) was added via cannula, immediately causing a dense white vapor to form the receiving vessel. This solution was stirred for 30 min at 0°C, then crotonaldehyde (33.6 mL, 412 mmol, 1 eq., freshly distilled) was added over 90 minutes by syringe pump.

After 3 h, magnetic stir bar was carefully removed and stirring was changed to an overhead, mechanical apparatus. Ice-chilled HCl (850 mL, 3 M) was added dropwise via pressure equalizing addition funnel. A thick white precipitate formed during the addition, but eventually dissolved near the end of the quenching step. The entire biphasic mixture was added to a 4000 mL separating funnel and layers were separated. The aqueous layer was extracted (2 x 500 mL), then combined and washed with brine (500 mL). The washed solution was dried over Na₂SO₄ and filtered. This bulk volume of the filtrate was removed by rotary evaporator at a pressure of ~40 Torr and temperature of 10°C. The clear oil concentrate was transferred to a 250 mL round-bottom flask using diethyl ether to quantitate transfer. Remaining solvent was removed at atmospheric pressure using a short-path distillation head and heating at 90°C (oil bath). This bath was then removed; distillation flask was cooled to 23°C. Pressure was then lowered again to ~40 Torr and receiving flask was placed in a ice-water bath. Distillation resumed as the oil
bath reached 90°C and product distilled at a temperature of °C at ~40 Torr. The resulting clear liquid weighed 31 g (yield 75%). Enantiomeric excess was not determined as the NMR data showed presence of major impurities. Side product produced was consistent with literature values for the constitutional isomer hex-3-en-2-ol\textsuperscript{100}.

\textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) δ 5.68 – 5.53 (m, 2H), 5.50 – 5.36 (m, 1H), 4.20 (p, J = 6.4 Hz, 1H), 3.89 (q, J = 6.6 Hz, 1H), 2.07 (s, 1H), 1.99 (p, J = 7.4 Hz, 1H), 1.65 (dd, J = 6.4, 1.5 Hz, 3H), 1.48 (ddt, J = 16.9, 13.9, 7.2 Hz, 2H), 1.19 (d, J = 6.3 Hz, 2H), 0.94 (t, J = 7.5 Hz, 2H), 0.84 (d, J = 14.9 Hz, 3H).

\textsuperscript{13}C NMR (101 MHz, CDCl\textsubscript{3}) δ 134.07, *133.16, *132.33, 126.62, 74.33, *68.73, 30.03, *25.03, *23.32, 17.56, *13.34, 9.69. (asterisks denote impurity)
Acetal-protected vinyl iodide (“Fragment A”) was produced wholly in accord with the published synthesis by Cossy\textsuperscript{58}. Spectra were found to be in agreement.

(R,R)-Pseudoephedrine propionamide was produced wholly in accord with the published synthesis by Myers\textsuperscript{73}. Spectra were found to be in agreement.

(1R,2S)-1-cyclohexyl-2-morpholino-2-phenylethan-1-ol (“Ligand 1”) was produced wholly in accord with the published synthesis by Nugent\textsuperscript{67}. Spectra were found to be in agreement.

(S)-3,3-dimethyl-1-morpholinobutan-2-ol (“Ligand 2”) was produced wholly in accord with the published synthesis by Hayashi\textsuperscript{101}. Spectra were found to be in agreement.
Using an adapted method from Sherman, starting material iodide was dried azeotropically with benzene twice before use\(^56\). To an oven-dried 300 mL round bottom flask was added lithium chloride (6.69 g, 157.73 mmol, 12 eq.). This vessel was then iteratively flame dried under vacuum and back-filled with argon gas for five cycles. While cooling, an oven-dried 50ml pear-shaped flask was charged with pseudoephedrine propionyl amide (4.65 g, 21.03 mmol, 1.6 eq.). After careful heating under vacuum and backfilling with argon for three cycles followed by cooling, dry THF (40ml) was added via cannula.

50mL was then added to the 300mL flask containing lithium chloride and the resulting slurry was allowed to stir for 30 minutes. This flask was cooled -78°C. Lithium diisopropylamine solution (21ml, 42.06 mmol, 3.2 eq. of ACROS, 1.7M in THF/n-heptane/ethylbenzene) was added and maintained at -78°C. At this time, the contents of the 50mL flask were transferred via cannula to the 300mL flask. The resulting solution was stirred for 1 h at -78°C, 30 min at 0°C, and 10 min at RT before it was cooled to 0 °C. Protected alkyl iodide 2 was dissolved in 15mL THF and added dropwise followed by an 8mL THF wash. Solution was allowed to warm to room temp and stirred for 12 h. The reaction was quenched with saturated NH\(_4\)Cl (60 mL), and poured into a 250mL separatory funnel containing H\(_2\)O (100 mL), and the aqueous layer was extracted 3x with CH\(_2\)Cl\(_2\). The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was then rinsed 2x with CH\(_2\)Cl\(_2\). Concentration and flash chromatography: EtOAc/Hexanes (3:7) afforded a white, waxy solid (4 g, 9.81 mmol, 75% yield). Spectral data matched that of previously published synthesis\(^72\).
To a 100mL flame-dried round bottom flask under argon was added 20ml dry THF. The flask was cooled to 0°C. Lithium diisopropylamine solution was added (8.1 mL, 16.2 mmol, 4.4 eq. of ACROS, 1.7M in THF/n-heptane/ethylbenzene) and allowed to stir 3 min. Borane-ammonia complex (468 mg, 14.7 mmol, 4 eq., 97% Sigma-Millipore) was added quickly in one portion. Solution was stirred 15 min at 0°C, then stirred 15 min at RT, then returned to 0°C. The amide (1.5g, 3.7 mmol, 1 eq.) dissolved in 10 mL THF and added followed by a 10 mL THF wash.

Solution was warmed to RT with a water bath, stirred 4.5 h then cooled to 0°C and cautiously quenched with 40 mL (chilled) 1N HCl then stirred at RT for 30 min. Reaction mixture was added to separatory funnel and extracted 4x 50mL Et₂O. Organic phase was washed with 50mL 1N HCl, 50mL 1M NaOH, and 100mL brine. Organic phase was dried over MgSO₄ and solvent was removed under reduced pressure (rotovap bath temperature >10°C.) Flash chromatography: Et₂O/Hexanes (4:6) afforded a clear liquid (780 mg, 3.16 mmol, 86% yield). Product material matched previously published spectroscopic data⁵⁵.
To a solution of alcohol (628 mg, 2.55 mmol, 1 eq.) in pyridine (2.5 mL) was added para-toluenesulfonyl chloride (971 mg, 5.1 mmol, 2 eq.) and DMAP (47 mg, 0.382 mmol, 0.15 eq.) at 0°C.

The reaction mixture was allowed to warm and was stirred for 3 h at RT before it was quenched with HCl (1.0 N, 7 mL) and extracted with ethyl acetate (2×10 mL).

The combined organic extracts were washed with saturated Na$_2$CO$_3$ solution (2×7 mL) and brine (1×7 mL), dried with anhydrous Na$_2$SO$_4$ and filtered. Concentration and flash chromatography: (pentane/ethyl acetate, 9:1) afforded 1.01 g of tosylate as clear oil (99%). Spectral data matched that of previously published synthesis$^{102}$. 

\[
\text{\includegraphics[width=0.5\textwidth]{image.png}}
\]
To a flame-dried, argon filled flask was added CuI (1.62 g, 8.51 mmol, 3.3 eq.), followed by two vacuum-argon cycles. Flask was cooled to 0 °C, 15mL of dry Et₂O was added, followed by 25mL a 0.7 M solution of isopropyllithium (ACROS in pentanes; 17.27 mmol, 6.7 eq) via cannula.

After 45 min, the mixture was cooled to -78°C and treated with tosylate (1.03 g, 2.57 mmol, 1 eq.) in 3 mL of dry Et₂O, followed by 3ml Et₂O wash. After 3 hr, the reaction mixture was quenched with 50mL of a 4:1 mixture of NH₄Cl (sat.)/NH₄OH (29%) at 0 °C, extracted with Et₂O, washed with brine and dried with MgSO₄. Concentration and flash chromatography: (100% hexanes) afforded 525 mg of alkylated product as clear oil (75%).

1H NMR (CDCl₃, 400 MHz) δ 3.45 (dd, J = 9.7, 5.1 Hz, 1H), 3.33 (dd, J = 9.7, 6.7 Hz, 1H), 1.72 – 1.51 (m, 3H), 1.34 – 1.20 (m, 2H), 1.14 – 1.05 (m, 1H), 1.00 – 0.81 (m, 22H), 0.04 (s, 6H).

13C NMR (CDCl₃, 101 MHz) δ 68.46, 46.91, 41.83, 33.27, 29.91, 27.91, 26.13, 26.13, 26.13, 25.38, 23.86, 22.2, 20.70, 17.82, -5.1, -5.20.

HRMS: Calculated [M+Cs]+ calculated for C16H36OSiCs: 405.1590 m/z; found 405.1793 m/z.
To a stirred solution of protected alcohol in 3.5 mL THF at 0°C was added tetrabutylammonium fluoride solution (ACROS 1M in THF; 2.5 mL, 2.5 mmol, 1.9 eq.). The cooling bath was removed and the reaction was stirred at RT overnight. Concentration under reduced pressure and flash chromatography (100% hexanes to 100% CH₂Cl₂; Rf 0.36 in 100% CH₂Cl₂) afforded 188mg of product (90%). Spectral data matched that of previously published synthesis.⁶⁰
To a solution of alcohol in CH$_3$CN (8 mL) and phosphate buffer (8 mL, pH 7.0, 100 mM) were subsequently added NaClO$_2$ (310 mg, 3.42 mmol, 6.3 eq.), NaClO (1.55 mL; store-bought Clorox brand, 8.5% chlorine content) and TEMPO (7 mg, 0.046 mmol, 0.09 eq.) at room temperature. After being stirred for 1 h, the pH value of the reaction mixture was adjusted to 3 with 2M HCl.

Volatile were removed in vacuo; the aqueous residue was extracted with diethyl ether (2 x 30 mL). The combined organic layers were washed with brine (30 mL), dried over anhydrous Na$_2$SO$_4$, concentrated in vacuo. Flash chromatography: 15% ethyl acetate/hexanes (Rf: 0.34, 20% ethyl acetate/ hexanes) afforded a clear, liquid product with a minty aroma (72 mg, 77% yield).

$^1$H NMR (CDCl$_3$, 400 MHz): δ 10.17 (brs, 1H), 2.67 – 2.62 (m, 1H), 1.79 – 1.69 (m, 2H), 1.61 – 1.56 (m, 1H), 1.24 (d, J = 6.5 Hz, 3H), 1.21 – 1.01 (m, 4H), 0.98 – 0.84 (m, 11H).

$^{13}$C NMR (CDCl$_3$, 101 MHz) δ 182.79, 45.49, 40.30, 36.37, 27.09, 23.91, 22.19, 21.21, 18.72, 16.81.

HRMS: Calculated [M-H]+ calculated for C$_{10}$H$_{20}$O$_2$: 171.1385 m/z; found 171.1359 m/z.
To an ice-cooled and stirred solution of trimethylheptanotic acid (57 mg, 0.33 mmol, 1 eq.), was added N,O-dimethylhydroxylamine hydrochloride (34 mg, 0.35 mmol, 1.05 eq), and triethylamine (0.04 mL, 0.35 mmol, 1.05 eq) in DMF (6mL) HOBt (51 mg, 0.38 mmol, 1.15 eq) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (73 mg, .38 mmol, 1.15 eq) were added. The mixture was stirred at room temperature for 15 h.

At this point, reaction was diluted with toluene, washed with 1N aq. HCl followed by saturated NaHCO₃, and brine, dried over Na₂SO₄, and concentrated under reduced pressure. Flash chromatography: 1:9 ethyl acetate/hexanes to 1:4 ethyl acetate/hexane yielded 32 mg of amide (45% yield).

¹H NMR (CDCl₃, 400 MHz): δ 3.69 (s, 3H), 3.17 (s, 3H), 1.78 – 1.59 (m, 2H), 1.50 – 1.37 (m, 1H), 1.15 – 0.89 (m, 7H), 0.83 (m, 9H).


HRMS: Calculated [M-H]+ calculated for C₁₂H₂₅NO₂: 214.1807 m/z; found 214.1966 m/z.
To a stirring solution of vinyl iodide (10 mg, 0.046 mmol, 1 eq.) in dry Et$_2$O (0.75mL) at -78°C was added t-BuLi (ACROS 1.7M in pentane) dropwise. After stirring 45 minutes at -78°C, a solution of amide (30 mg, 0.102 mmol, 2.2 eq.) in 0.5mL Et$_2$O was added quickly followed by a 0.5mL rinse. Reaction mixture was then warmed to -42°C (acetonitrile and dry ice bath) and stirred 1hr.

After this time, the reaction mixture was diluted with Et$_2$O and quenched by addition of saturated aqueous NH$_4$Cl solution at 0°C. The solution was extracted with Et$_2$O; the organic layers were combined, washed with brine, dried over MgSO$_4$ and concentrated under reduced pressure. Flash chromatography: 1:9 diethyl ether/pentanes afforded a clear liquid product (7 mg, 46%).

*Note: Some solvent signals and dibutylhydroxytoluene (from diethylether) were present in NMR spectra. Due to the unknown volatility of this compound, it was only exposed to high-vacuum for a brief period.*

$^1$H NMR (CDCl$_3$, 500 MHz): $\delta$ 6.78 (d, J = 15.5 Hz, 1H), 6.43 (d, J = 15.5 Hz, 1H), 3.82 (t, J = 6.6 Hz, 1H), 2.81 (dt, J = 13.6, 6.8 Hz, 1H), 1.71 – 1.60 (m, 3H), 1.53 – 1.49 (m, 5H), 1.45 – 1.37 (m, 11H), 1.25 (s, 3H), 1.13 – 0.92 (m, 11H), 0.88 – 0.79 (m, 11H).

$^{13}$C NMR (CDCl$_3$, 126 MHz) $\delta$ 203.82, 146.93, 127.26, 86.24, 77.16, 46.88, 42.79, 41.40, 28.47 (2C), 28.28, 26.62, 25.26 (2C), 24.83, 23.59, 23.49, 22.25, 17.32, 11.40.

HRMS: Calculated [M+Cs]$^+$ calculated for C$_{20}$H$_{36}$O$_3$Cs: 457.1719 m/z; found 457.1695 m/z.
To a 20 mL scintillation vial was added 5 ml of 1:1 THF/1N HCl and stirred at RT for 18 h. After 24 h, 60 µL 3N HCl was added and reaction was monitored. After 24 additional hours, 40 µL 3N HCl was added and the reaction was monitored for disappearance of starting material. After 24 additional hours, the reaction was quenched with NaHCO$_3$. This mixture was extracted with ethyl acetate (6 x 5 mL) and washed with brine. Flash chromatography: CH$_2$Cl$_2$ to 1:6 ethyl acetate/CH$_2$Cl$_2$ yielded 5 mg of product (95% of theoretical yield).

$^1$H NMR (CDCl$_3$, 400 MHz): δ 6.86 (d, J = 15.6 Hz, 1H), 6.48 (d, J = 15.6 Hz, 1H), 3.4 (dd, 8, 4 Hz, 2H), 2.81 (dt, J = 13.6, 6.8 Hz, 1H), 2.56 (s, 1H), 2.02 (s, 1H), 1.75 – 1.50 (m, 6H), 1.50 – 1.39 (m, 2H), 1.34 (s, 3H), 1.27 (d, J = 14.4 Hz, 1H), 1.08 (d, J = 6.9 Hz, 3H), 1.00 (t, J = 7.4 Hz, 3H), 0.88 – 0.78 (m, 10H).

$^{13}$C NMR (CDCl$_3$, 101 MHz) δ 204.10, 148.02, 126.90, 79.72, 75.71, 46.83, 42.87, 41.17, 28.44, 25.27, 25.16, 24.92, 23.57, 22.28, 20.21, 17.37, 11.14.

HRMS: Calculated [M+Cs]$^+$ calculated for C17H32O3Cs: 417.1406 m/z; found 417.1575 m/z.

$[\alpha]_D^{21} = +13.5$ (c 0.67, CHCl$_3$)

IR (neat) 3373, 2958, 2929, 1675
Prepare silica column for flash chromatography before or while starting reaction procedure. Remove round-bottom flask from the oven, fill with argon, cover and cool to room temperature on bench. Dissolve 16μL propargyl alcohol in 1.5mL of anyhydrous CH₂Cl₂. Weigh and transfer cobalt carbonyl into a separate flask. Add propargyl alcohol/CH₂Cl₂ solution to cobalt carbonyl containing flask and stir at RT for 1.5 hours. Dissolve 50mg of repin into 2.3mL anhydrous CH₂Cl₂. Cool reaction flask to 0°C. Add repin solution followed by dropwise addition of BF₃-etherate. Stir only 10 minutes, add 1ml saturated NaHCO₃. Stir one minute before addition of (4ml) water.

Extract 3x with CH₂Cl₂. Dry on MgSO₄, filter and evaporate onto Celite under reduced pressure. Separation on silica (0% to 30% gradient of ethyl acetate/hexanes). Main spot elutes at 30% ethyl acetate, and solvent is immediately removed before carrying Co₂(CO)₆-alkyne intermediate rapidly to the next step. To 15mg cobalt complex is added 1.5 mL acetone is added 60mg of ceric ammonium nitrate. Reaction is allowed to stir 10 minutes (or until consumption of starting material is complete). The reaction was diluted with distilled water and diethyl ether. The mixture was transferred to a separatory funnel. The layers were separated and the aqueous layer was extracted with diethyl ether (3x). The combined organics were dried over magnesium sulfate, filtered, and concentrated under reduced pressure rotary evaporation. Yields over two steps ranged between 5%-15%.
$^1$H NMR (CDCl$_3$, 400 MHz) δ 6.21 (d, $J = 3.5$ Hz, 1H), 5.54 (d, $J = 3.1$ Hz, 1H), 5.21 – 5.09 (m, 3H), 4.94 (s, 1H), 4.18 (d, $J = 2.4$ Hz, 2H), 3.34 (d, $J = 4.3$ Hz, 1H), 3.17 (dd, $J = 14.4$, 5.0 Hz, 3H), 2.81 (d, $J = 5.9$ Hz, 2H), 2.45 (d, $J = 2.4$ Hz, 2H), 2.03 (s, 1H), 1.60 (s, 4H), 1.24 (s, 3H).

$^{13}$C NMR (CDCl$_3$, 101 MHz) δ 169.99, 168.80, 141.13, 137.36, 122.29, 118.99, 82.58, 77.16, 76.53, 75.48, 75.11, 65.87, 56.76, 53.88, 52.93, 49.51, 47.79, 45.89, 36.24, 36.00, 17.61, 17.49.

HRMS:

[M+NH$_4$]+ calculated for C$_{22}$H$_{28}$O$_7$N: 418.1866 m/z; found 418.1808 m/z.

[M+Na]+ calculated for C$_{22}$H$_{24}$O$_7$Na: 423.1866 m/z; found 423.1845 m/z.

[2M+Na]+ calculated for C$_{44}$H$_{48}$O$_{14}$Na 823.2942 m/z; found 823.2908 m/z.
The procedure was adapted from biotin labeling of sterols\textsuperscript{103}. To a solution of propargyl repin (7mg, 1 eq) and biotin-PEG3-azide (8mg, 1.03 eq) in tetrahydrofuran (0.08 mL), t-butanol (0.04 mL) and water (0.02) was added a solution of copper(II) sulfate pentahydrate (.62mg, 0.22 eq in 0.01 mL of water) and a solution of sodium ascorbate (1.5 mg, 0.44 eq in water (0.01 mL), respectively. After stirring at 30\textdegree C for 36 h, the solvent was removed by evaporation and residue was extracted by ethyl acetate (2 x 5 mL). Combined organic extracts were washed with water (3 x 5 mL), saturated aqueous NaCl (2 x 10 mL) and dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}. The residue was then purified by preparative thin layer chromatography (15:8:4:1 ethyl acetate/chloroform/methanol/water). This gave impure biotin labeled repin (4mg, 27\%) as a white solid.

HRMS: [M+Na]\textsuperscript{+} calculated for C\textsubscript{40}H\textsubscript{56}N\textsubscript{6}NaO\textsubscript{12}S: 867.3575 m/z; found 867.3570 m/z.


91. Hein, J. E.; Fokin, V. V., Copper-catalyzed azide–alkyne cycloaddition (CuAAC) and beyond: new reactivity of copper(i) acetylides†. Chemical Society reviews 2010, 39 (4), 1302-15.


LIST OF APPENDICES
APPENDIX I: REPIN BIOLOGICAL ASSAY RESULTS
General Description of Repin Kinase Assay

The assay used for repin (KINOMEScan™ by DiscoverX), does not require ATP and allow reporting of thermodynamic interaction affinities instead of IC50 values which are often used and can be influenced by ATP concentration. The kinase may bind the ligand in either an orthosteric (in active site) or allosteric fashion (indirectly). Both cases may inhibit enzymatic activity and potentially downstream signaling associated with the kinase. In the figure below (A), target kinases (squiggly, blue) are allowed to interact with immobilized ligand (orange) in the absence of test compound. Test compounds (gray) either interfere with kinase binding to immobilized ligands (B) or do not interfere (C). The compounds and unbound kinase is washed away and the amount captured is compared to control samples and quantitatively measured using qPCR of the associated DNA label for each target protein.94
Experimental details of Kinome Scan Assay

For the majority of the assays, kinase-tagged T7 phage strains were grown in 24-well blocks in an E. coli host (derived from the BL21 strain). After being grown to log-phase, the E.coli was infected with T7 phage (multiplicity of infection = 0.4) and incubated with shaking at 32°C until lysis (90-150 minutes). The lysates were and filtered (0.2µm) to remove cell debris. The remaining kinases were produced in HEK-293 cells and subsequently tagged with DNA for qPCR detection.

Affinity resins for kinase assays were generated by treating streptavidin-coated magnetic beads with biotinylated small molecule ligands for 30 minutes at room temperature.

These beads were then blocked with excess biotin and washed with blocking buffer (SeaBlock by Pierce Biotechnology), 1 % BSA, 0.05 % Tween 20, 1 mM DTT) to wash unbound ligand and to reduce non-specific phage binding. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compounds in 1x binding buffer (20 % SeaBlock, 0.17x PBS, 0.05 % Tween 20, 6 mM DTT). Test compounds were prepared in DMSO as 40x stocks and directly diluted into the assay. All reactions were performed in 384-well plates in a final volume of 0.02 ml. The assay plates were incubated at room temperature for 1 hour with shaking. At this point, the affinity beads were washed buffer consisting of 1x PBS, 0.05 % Tween 20. The beads were then re-suspended in elution buffer (1x PBS, 0.05 % Tween 20, 0.5 µM non-biotinylated affinity ligand) and incubated at room temperature with shaking for 30 minutes. The kinase concentration in the eluates was measured by qPCR.

The compounds were screened at 1 micromolar concentration. The results were reported as “percent of control” (% Ctrl). To calculate percent of control, the following formula was used:
Where:

**test compound** = compound submitted by our lab (repin)

**negative control** = DMSO (100% control)

**positive control** = control compound (0% control)
Results of KinomeScan assay (DiscoverX - Eurofins)

Table 3

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EnPlex Assay Results and Description (Bachovchin Lab)

“Purified enzymes are coupled to Luminex microspheres, with a different bead color for each enzyme. Multiplexed bead complexes are incubated with a compound before being treated with a biotinylated activity-based probe and a streptavidin R–phycoerythrin conjugate (SASAPE). The mixtures are scanned on a Luminex flow cytometer, where one laser detects the bead color (enzyme identity) and a second laser detects the R-phycoerythrin signal (enzyme activity). Changes in absorbance are due to interruption of SAPE interaction by an inhibitor$^93$.”
**Figure 25:** EnPlex Assay Results: Repin showed no activity pattern across any protease. Positive DPP4 hit (ValboroPro, left lane) compared to repin (listed as MJC-1-21, right lane).
APPENDIX II: SPECTRAL DATA
VITA

EDUCATION

Ph.D., Medicinal Chemistry  
University of Mississippi, University, MS  
May 2018

M.S., Pharmacognosy  
University of Mississippi, University, MS  
Dec. 2013

B.S., Biochemistry & Molecular Biology  
Mississippi State University, Mississippi State, MS  
May 2007

RESEARCH POSITIONS

Graduate Research Assistant (John Rimoldi)  
The University of Mississippi, Department of Medicinal Chemistry, University, MS  
Aug. 2013 – Present
- Total synthesis of a polyketide implicated in dopaminergic neurodegeneration
- Derivatization of sesquiterpene lactone implicated in dopaminergic neurodegeneration
- Synthesis and design synthetic cannabinoid receptor ligands
- Scale-up synthesis of Sigma receptor ligand for in vivo
- Synthesis and design of prolylcarboxypeptidase enzyme inhibitors

Graduate Research Assistant (Ikhlas Khan/ Amar Chittiboyina)  
The University of Mississippi, Department of Pharmacognosy, University, MS  
- Synthesis, isolation and structure elucidation of intermediates en route to psychoactive natural products mesembrine and mesembrenone
- Extraction and isolation of natural products using chromatography
- Structure elucidation of terpenes, alkaloids and sterols from Eurycoma longifolia Jack using IR, MS, 1D and 2D NMR
- In silico screening of natural products for phosphodiesterase 5 activity

Graduate Research Assistant (Jennifer Irvin, Steve Whitten)  
Texas State University, San Marcos, TX  
May 2009 – July 2010
- Synthesis, isolation and structure elucidation of monomers for use in construction of electroactive polymeric materials
- Bacterial culture, expression and isolation of p53 protein
- Thermodynamic ensemble modeling of p53 protein using Fyrestar (RedStorm) software

Undergraduate Research Assistant (Nara Gavini, Lisa Wallace)  
Mississippi State University, Mississippi State, MS  
- Used microbial culture and molecular methods (PCR, blotting) studying nitrogenase fusion proteins, effects of gold nanoparticle exposure to Azotobacter vinelandii
- Extracted, isolated genetic material from Platanthera (Orchidaceae) species using molecular methods
- Analyzed DNA sequence information using Sequencher and Mr.Bayes software to evaluate phylogenetic classifications of orchids
TEACHING POSITIONS

University of Mississippi, University, MS

Department of Medicinal Chemistry 2014
− Pharmacogenomics Teaching Assistant
− Tutor for pharmacy students for medicinal, organic and biochemistry coursework

Texas State University, San Marcos, TX

Department of Chemistry and Biochemistry 2009-2010
− Instructor for general (II), analytical and organic (I) chemistry lab sections
− Chemistry tutor for undergraduate general and organic chemistry

PUBLICATIONS

Design and Synthesis of Icariin Analogues as PDE-5 inhibitors (Oxford ICSB 2011 Poster)

In silico-screening of natural products for potential phosphodiesterase (PDE) 5A1 inhibitors (Oxford ICSB 2014 Poster)

‘Use of broad molecular screening assay to determine selectivity of prolylcarboxypeptidase inhibitors’ (MALTO 2016 Podium Presentation)

‘Synthesis and evaluation of dual-acting opioid and neuropeptide FF Ligands’ (AAPS 2017 Poster)

‘Sceletorine A and B, two unprecedented dimeric alkaloids from Sceletium tortuosum’ (Organic Letters, Submitted)

‘Progress towards the enantioselective total synthesis of a novel metabolite’ from Streptomyces venezuelae” (Poster, 255th ACS National Meeting 2018)

‘Total synthesis and toxicological evaluation of Streptomyces venezuelae Metabolite SV6’ (In Preparation)

‘Identification and characterization of the protein targets of neurotoxic sesquiterpene lactone repin” (In Preparation)

LEADERSHIP POSITIONS

President of Medicinal Chemistry Journal Club 2012 - 2016
Director of Graduate Affairs, Graduate Student Council 2012 - 2013

OTHER POSITIONS, AWARDS AND DISTINCTIONS

Member, Rho Chi (Pharmaceutical Sciences Honors Society) 2017
Instrumentation Assistantship, Elemental Analysis (CHN) 2016
Division of Organic Chemistry Travel Award (ACS National Meeting, San Diego) 2016
Graduate Assistant to Vice Chancellor of Research (Dr. Alice Clark) 2013 - 2015
Graduate Representative, Chancellor’s Standing Committee on Libraries 2012 - 2013
Pharmacognosy Senator, Graduate Student Council 2010 - 2012
PTCB Certified to work as Pharmacy Technician (Certified in Texas) 2009