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Mechanisms of Toxicity and the Structure-Activity Relationships of Molinate and Dieldrin

Erin Marie Gagan Allen
University of Iowa

MECHANISMS OF TOXICITY AND THE STRUCTURE-ACTIVITY RELATIONSHIPS OF MOLINATE AND DIELDRIN

by

Erin Marie Gagan Allen

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Pharmacy
(Medicinal and Natural Products Chemistry)
in the Graduate College of
The University of Iowa

July 2011

Thesis Supervisor: Associate Professor Jonathan A. Doorn

ABSTRACT

Pesticides have been used to control various types of pests, including plants and insects, for thousands of years, however the impact of exposure to these toxic chemicals, with respect to environmental and health consequences, is not fully understood. Two pesticides of interest, molinate and dieldrin, have been shown to cause neurotoxicity in humans, but their mechanisms of toxicity are still unknown. In order to better understand how exposure to these chemicals can cause toxicity, the structure-activity relationship (SAR) was defined to determine how specific changes to the structure of each pesticide affects the toxicity profiles of each of these compounds.

Results of this study demonstrated that oxidation of molinate to molinate sulfoxide, and then further to molinate sulfone, a more potent inhibitor of aldehyde dehydrogenase. The sulfone metabolite is capable of covalently modifying the active-site cysteine residue of aldehyde dehydrogenase, accounting for the observed enzyme inhibition. These results indicate that the compound responsible for the toxicity from molinate exposure is not the parent compound, but rather one of the sulfoxidation metabolites.

When the SAR of dieldrin was investigated with respect to a Parkinson's disease model, it was determined that the compounds that were previously found to be the least potent insecticides were the most toxic with respect to dopaminergic cells. Each of the compounds tested was observed to disrupt dopamine metabolism in accordance with their toxicity profiles in dopaminergic cells. In combination, these results implicate important structural features responsible for the toxicity with respect to Parkinson's disease. This information is critical for the development of new pesticides, and will be important to increase the selective toxicity for insects while minimizing adverse/off-target effects. This can lead to the development of safer, more effective pesticides that will be essential for future environmental and human health.

Abstract Approved: _____
Thesis Supervisor

_____
Title and Department

_____
Date

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Iowa City, Iowa

CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

Erin Marie Gagan Allen

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Pharmacy (Medicinal and Natural Products Chemistry)
at the July 2011 graduation.

Thesis Committee: _____
Jonathan A. Doorn, Thesis Supervisor

_____
Michael W. Duffel

_____
Robert J. Kerns

_____
David L. Roman

_____
Stefan Strack

To Ruth H. Bidlack

All stories, even the ones we love, must eventually come to an end and when they do, it is only an opportunity for another story to begin.

Mr. Magorium's Wonder Emporium
Directed by Zach Helm

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Results of this study demonstrated that oxidation of molinate to molinate sulfoxide, and then further to molinate sulfone, a more potent inhibitor of aldehyde dehydrogenase. The sulfone metabolite is capable of covalently modifying the active-site cysteine residue of aldehyde dehydrogenase, accounting for the observed enzyme inhibition. These results indicate that the compound responsible for the toxicity from molinate exposure is not the parent compound, but rather one of the sulfoxidation metabolites.

When the SAR of dieldrin was investigated with respect to a Parkinson's disease model, it was determined that the compounds that were previously found to be the least potent insecticides were the most toxic with respect to dopaminergic cells. Each of the compounds tested was observed to disrupt dopamine metabolism in accordance with their toxicity profiles in dopaminergic cells. In combination, these results implicate important structural features responsible for the toxicity with respect to Parkinson's disease. This information is critical for the development of new pesticides, and will be important to increase the selective toxicity for insects while minimizing adverse/off-target effects. This can lead to the development of safer, more effective pesticides that will be essential for future environmental and human health.

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LIST OF ABBREVIATIONS

1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane ..... DDT

1-methyl-4-phenyl-1,2,3-tetrahydropyridine ..... MPTP

2,4,6-trinitrobenzenesulfonic acid ..... TNBSA

2',7'-dichlorodihydrofluorescein diacetate ..... H_2DCFDA

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide .....MTT

3,4-dihydroxyphenylacetaldehyde .....DOPAL

3,4-dihydroxyphenylacetic acid ..... DOPAC

3,4-dihydroxyphenylethanol .....DOPET

3-chloroperbenzoic acid ..... *m*CPBA

4-hydroxynonenal.....4HNE

5,5'-dithiobis(2-nitrobenzoic acid) .....DTNB

Acetonitrile .....ACN

Aldehyde dehydrogenase .....ALDH

Aldehyde dehydrogenase 2 .....ALDH2

Aldehyde reductase.....ALR

Aldrin ..... Adn

α-synuclein ..... SNCA

Attention deficit and hyperactivity disorder ..... ADHD

Band pass.....BP

Calculated ..... Calcd

Carbon nuclear magnetic resonance ..... ^{13}C NMR

Central nervous system ..... CNS

Cis Aldrin Diol .....CAD

Comprehensive Environmental Response, Compensation, and Liability Act .....CERCLA

Cysteine ..... Cys

Desmethylene Aldrin ..... dAdn or des Aldrin

Desmethylene Dieldrin ..... dDI or des Dieldrin

Dieldrin ..... DI

Dihydroethidium ..... DHE

Dimethylsulfoxide ..... DMSO

Dopamine transporter ..... DAT

Endrin ..... End

Electrospray ionization ..... ESI

γ-aminobutyric acid ..... GABA

Gas Chromatography tandem Mass Spectrometer ..... GC/MS

High Performance Liquid Chromatography ..... HPLC

Hour ..... h

Human recombinant aldehyde dehydrogenase 2 ..... hALDH2

Inhibitor concentration at 50% inhibition ..... IC_{50}

Integrated Pest Management ..... IPM

Isodrin ..... Idn

Lactate dehydrogenase ..... LDH

leucine-rich repeat kinase 2 ..... LRRK2

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

Lysine ..... Lys

Malondialdehyde ..... MDA

Mass to charge ratio ..... *m/z*

Monoamine oxidase ..... MAO

Nerve growth factor ..... NGF

Nicotinamide adenine dinucleotide ..... NAD

Parkin PARK2

Parkinson's disease ..... PD

Plasma membrane Ca^{+2}-ATPases ..... PMCA

Proton nuclear magnetic resonance ..... ^{1}H NMR

PTEN-induced putative kinase 1 ..... PINK1

Reactive oxygen species ..... ROS

Room temperature ..... RT

Serine ..... Ser

Sodium dodecyl sulfate polyacrylamide gel electrophoresis ..... SDS-PAGE

Structure-activity relationship ..... SAR

Superoxide dismutase ..... SOD

Time of flight mass spectrometer ..... TOF MS

Tris buffered saline and Tween20 ..... TBST

Tumor necrosis factor α ..... TNF- α

United States Environmental Protection Agency ..... US EPA

Vesicular monoamine transporter ..... VMAT

CHAPTER ONE. ENVIRONMENTAL CONSEQUENCES OF PESTICIDE USE

> Sprays, dusts, and aerosols are now applied almost universally to farms, gardens, forests, and homes – nonselective chemicals that have the power to kill every insect, the "good" and the "bad," to still the song of birds and the leaping of fish in the streams, to coat the leaves with a deadly film, and to linger on in soil – all this though the intended target may be only a few weeds or insects.
>
> Rachel Carson, *Silent Spring*, p7

We encounter pests every day, whether it is a colony of bees that has chosen your mailbox as the perfect location for a hive, a brilliant yellow dandelion dotting your otherwise green yard, or a brown-furred mouse that has found refuge in your basement. The list of pests, or organisms that cause a nuisance or harm, may include insects, plants, animals, fungi, or microorganisms. Some of these pests can cause significant health hazards, such as *Yersinia pestis*, the bacteria responsible for the Bubonic plague. This is a type of bacteria that can infect the fleas that are typically found on rodents. In areas heavily infested with rats, this bacteria can be transmitted to humans, resulting in a deadly bacterial infection. While modern antibiotics are successful in treating this infection if caught early, *Yersinia pestis* has been responsible for millions of deaths across the world (*1*).

The degree of damage and harm pests can do is highly variable, such as the lethality of *Yersinia pestis* or the mere inconvenience of a dandelion. Regardless of the degree of risk they pose, people have developed various methods for dealing with them, such as through the use of chemical toxins. According to the Environmental Protection

Agency (EPA), a pesticide is an agent that can be used to prevent, destroy, repel, or mitigate any pest; and may include insecticides, herbicides, fungicides, etc. that can be used to control various types of organisms. One of the first reported uses of pesticides was the use of sulfur prior to 1000BC. This was followed by the use of arsenic sulfide in China in the 16th century (*2-4*). By the early 1900s, a majority of pests were controlled with inorganic compounds such as sodium arsenate or by-products of coal gas production, i.e. petroleum oils (*4*). These compounds were not selective pesticides, and led to a shift in the 1940s to the use of synthetic compounds, such as organophosphates and 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane (DDT) (*2*). This compound was first discovered in Germany in 1873, but insecticidal properties of DDT were not determined until 1939, by the Swiss entomologist Paul Muller – a discovery for which he was awarded the Nobel Prize in Medicine in 1948 (*4*).

Throughout the 1940s and 50s the commercial production and application of pesticides expanded in the United States (*2*). Despite their widespread use, there was not much concern regarding the environmental and health consequences of the use of these pesticides until 1960s when Rachel Carson's "Silent Spring" opened the eyes of the public to "A Fable For Tomorrow." This was the opening chapter for her book and described the consequences of pesticide use as a "shadow of death" that would creep across the land. She described the "white granular powder" that had "had fallen like snow upon the roofs and the lawns, the fields and streams" and the aftermath that resulted in "browned and withered vegetation," "deserted by all livings things" (*5*). This was accompanied by an eerie silence of a "spring without voices," with no birds singing

or bees droning – a silent spring (*5*). While the exact scene she described was not representative of an actual city, she stressed that "this imagined tragedy could easily become a stark reality we all shall know" if we do not begin to take the adverse effects of pesticide use seriously (*5*).

Carson's heart-wrenching depiction of the destruction caused by the misuse and mistrust of pesticides inspired the establishment of policies and organizations, such as the Environmental Protection Agency in 1970, aimed to monitor the safety, regulation, and registration of chemicals intended for pesticide use. Currently, the EPA is responsible for evaluating potential uses of new pesticides and reviewing the safety of older pesticides, in addition to enforcing and developing safety policies and regulations, and responding in emergency situations. While the EPA has been instrumental in initiating increased safety measures with respect to the consequences of pesticide exposure, a full understanding of the health and environmental consequences of the use (past, present, and future) of these toxic compounds is yet to be realized. Without an appreciation for the inherent toxicity of these chemicals and a complete understanding for the long-term adverse effects, the misuse of pesticides and unintended toxicity from exposure to these chemicals will continue.

The known adverse effects related to exposure to the toxic chemicals administered to control pests are numerous. In addition to the intended lethality of the target organism, pesticide exposure has been associated with the unintentional death of birds, fish, insects, plants, and mammals (including humans). There have also been substantial reports on exposure to these chemicals increasing the risk for cancer, birth

defects, neurodegenerative disorders, attention deficit and hyperactivity disorder (ADHD), autism, reproductive abnormalities, and autoimmune diseases (*2, 3, 6-19*).

Contributing to these adverse effects are the inherent characteristics of these compounds, their toxicity, the reason they are being used as pesticides; and their persistence in the environment, which allows for fewer applications. Theoretically these pesticides are applied as infrequently as possible, and in the smallest amounts possible in order to treat the problem, and to also minimize the unintentional toxicity. Unfortunately this is not always the case, and pesticides are often used more frequently and in higher concentrations than are necessary for their purpose, which can increase the risk for potential adverse effects.

As Paracelsus, the "Father of Toxicology," emphasized, the dose makes the poison. If we are considering a compound that is already known to be toxic, such as a pesticide, the dose required to treat the target pests is lower than it would be to cause harm to people. However, if a particular compound is highly persistent in the environment, and when repeated applications are taken into account, the concentration of the pesticide could inadvertently reach levels high enough to result in human toxicity. Another concern is the repeated exposure to low levels of these toxic, persistent compounds. While the concentration of the compounds when considering a one-time dose may not cause any observable effect, it is often chronic insult from these pesticides that are thought to be responsible for many of the known adverse effects (*13, 20-23*). This slow, chronic exposure is much more difficult to monitor and the long-term effects and disease implications are not fully understood or realized.

One potential solution to eliminate the adverse effects of pesticide exposure would be to ban their use altogether. While this would eliminate any concerns of further damage to the environment, it would not solve all of the current problems, including the presence of persistent chemicals already in the water, soil, and food chain; it would also create additional issues. By banning the use of current pesticides, without alternative control methods established, the pests they are currently controlling would take over the crops potentially causing massive destruction to the food supply across the United States. Therefore, it would be important to ensure that there are alternative methods for pest control available, which improve upon the safety and effectiveness of current pesticides, prior to an outright ban. Additional factors that contribute to the problems that may arise as a result of pesticide bans are the number of pests that threaten the agricultural industry by destroying crops, as well as the consumer expectation of unblemished produce. These factors pressure farmers to provide high yield of products that are visually appealing, results that are often forced through the overuse of pesticides.

Unfortunately the cost of pesticide abuse is not fully realized, and the public needs to ask “What are we willing to sacrifice to meet these demands for high yields and unblemished products?” While the “cost” may not be reflected in the price of the products, it will more likely effect things like our health, the health of our children and grandchildren, the safety of our pets, and the lifespan of livestock and wild animals. It is important to consider what we are willing to “pay” to get rid of the dandelions in our

yard, to minimize the number of Japanese beetles, or to be able to purchase a “perfect,” red apple from the grocery store.

There are current approaches being implemented, such as the concept of Integrated Pest Management (IPM), that are intended to minimize the use of pesticides not only on crops but also within homes, schools, and on lawns and gardens (*24*). This protocol combines the genetic manipulation of crops to produce pest-resistant strains, with the use of biological pesticides. These biological pesticides take advantage of the checks and balances already established in nature, such as the relationship between predator and prey. An additional component of IPM is the more efficient use of pesticides. This involves a more targeted approach using fewer applications and reduced quantity of chemicals. This is accomplished with an application cycle that is intended to target the pest during the most susceptible period of their life cycle. While this method still employs pesticides, their use is minimized, and therefore also limits the adverse effects on the environment.

In addition to optimizing the Integrated Pest Management method, there are other ways to protect society and the environment from pesticide exposure. This includes the education of the public, consumers, and farmers in regards to the inherent toxicity of these chemicals and the potential long-term effects. Without a full appreciation for the dangers of the use of these pesticides the misuse and mistrust of these compounds will continue. It is also important to develop more specific pesticides that are both more effective and have minimal toxicity to non-target organisms. This

would be accompanied by stricter regulations and testing regimens before the chemicals are introduced into the environment and food supply.

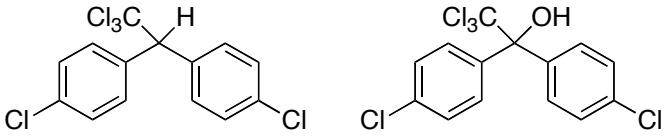
In addition to the better screening of new compounds, additional research is needed to understand the current threat of toxicity from chemicals that are still persistent in the environment. These chemicals, such as DDT and dieldrin, may have been banned for several decades, but due to their long half lives in the environment, still pose a significant health risk. It is important to fully understand the potential of pesticides (past and present) to cause adverse effects and to understand their mechanisms of toxicity, particularly in humans. This research is not only important to help predict long-term effects of their exposure and to potentially develop therapeutics and methods to combat these adverse effects; but these results can also be applied to the design of new, safer pesticides. Regardless of the approach taken to manage and regulate the use of pesticides, it is important that the public demands that "the methods employed [to control pests] must be such that they do not destroy us along with the insects" (*5*).

CHAPTER TWO. STATEMENT OF HYPOTHESIS

Many insecticides and herbicides have been used throughout the world for thousands of years, without much thought on the long-term effects on the environment and human health (*5*). A number of these pesticides have since been associated with the development of diseases such as Parkinson's Disease, Alzheimer's Disease, and many forms of cancer (*3*). These disease links, as well as other possible adverse effects, have prompted the United States Environmental Protection Agency (US EPA) to reevaluate and/or ban the use of a large number of chemicals in an effort to limit human exposure to these toxic substances. Two banned pesticides of interest are molinate, a thiocarbamate herbicide shown to cause neurotoxicity; and dieldrin, an organochlorine pesticide considered one of the twelve most persistent, bioaccumulative, and toxic chemicals (*25, 26*).

Molinate and dieldrin are members of two different classes of synthetic pesticides, carbamates and organochlorines, respectively. These classes, based on structure, can vary significantly with respect to their target species, adverse effects in mammalian systems, and environmental outlook. For example, pyrethroids, another class of synthetic pesticides, have low mammalian toxicity, and minimal persistence in the environment. These compounds are typically highly toxic to insects, allowing them to be used at relatively low doses. In contrast, the organochlorines, such as dieldrin, are highly persistent in the environment, and while acutely, may not pose a significant mammalian toxicity risk, due to their ability to bioaccumulate, they are considered a major hazard to human health (*27*).

Between synthetic classes there can be great diversity, i.e. carbamates and organochlorines, but there can also be variety between compounds within these classes. For example, if we consider the class of organochlorines, most of the compounds, i.e. dieldrin, are highly lipophilic and persist in the environment for a long time. This is true for DDT, and was a major factor contributing to the ban of this pesticide in the US in the 1970s. However, if the hydrogen on the central carbon atom of this compound is switched to a hydroxyl group, as in dicofol (see Figure 2.1), the resulting compound is more water soluble, and not as stable. This causes the pesticide to break down more rapidly than DDT, limiting its ability to bioaccumulate and to cause long-term adverse effects in the environment. This is why dicofol is currently still in use for the treatment of mites on fruit and cotton crops (*3*).



DDT Dicofol

Figure 2.1. Structures of DDT and dicofol

As in the case of DDT and dicofol, the correlation between structure and function can have important implications and insight into the mechanism of pesticide toxicity. It can also help in the identification of specific proteins these compounds may target, as well as to understand the interaction between the compounds and these proteins. The two pesticides investigated in this study, molinate and dieldrin, were chosen based on

their risk to human health and environmental impact. By taking a structure-activity relationship (SAR) approach, defining how specific changes to the structure of each pesticide affects their toxicity profile, important information regarding how these pesticides interact within mammalian model systems can be determined.

Hypothesis

The central hypothesis of this study is that specific structural modifications affect the toxicity profiles of pesticides, i.e. dieldrin and molinate, and can be used to define the reactivity and possible proteins targets of these compounds. This hypothesis was tested using four specific aims.

Specific Aims

Specific Aim 1: Determine the relative inhibitory potency of molinate and its metabolites with ALDH2. Previous studies have implicated ALDH2 as a target of molinate, but identification of which compound, molinate or one of its sulfoxidation metabolites, is responsible for this enzyme inhibition has not been determined. This is an important consideration to better understand the adverse effects from molinate exposure.

Specific Aim 2: Define the protein reactivity of molinate and its metabolites. The results of this aim will provide the mechanism of ALDH2 inhibition by molinate or one of its metabolites.

Specific Aim 3: Examine the localization of dieldrin and the toxicity of dieldrin analogs. Completion of this aim will provide critical information regarding the trafficking

of dieldrin in a dopaminergic cell and will determine which of its structural features are important for its toxicity.

Specific Aim 4: Assess the impact of dieldrin analogs on oxidative insult. There is a clear link between oxidative insult and increased risk for Parkinson's disease. In this aim, the structural features of dieldrin responsible for oxidative insult in a dopaminergic system will be defined.

CHAPTER THREE. MECHANISM OF MOLINATE INHIBITION OF ALDEHYDE DEHYDROGENASE 2

Introduction

Molinate, also known as Ordram, is a thiocarbamate herbicide. It was commonly used as a pre-emergent in rice paddy fields to control broad-leaved and grassy plants. It was used in United States, primarily in California, and only for a six-week long season. Even though it was only used for short durations, the United States Environmental Protection Agency (US EPA) banned the use of this pesticide, in effect at the conclusion of the 2009 growing season, due to toxicity risks (*28*).

The primary exposure risks for molinate include dermal and inhalation for field workers applying this pesticide, as well as oral consumption of contaminated water from field run-off in areas surrounding rice fields (*29*). Numerous adverse effects have been attributed to molinate exposure. These include reproductive toxicity (*30*), as well as neurotoxic consequences, such as peripheral neuropathy, indicating damaged or abnormally functioning neurons (*29*). The toxic effects on the reproductive system include lesions in Leydig cells and inhibition of testicular esterases (*30-32*).

The neurotoxic effects of molinate have been evaluated in a number of different animals, including rats, mice, and dogs. In rats, this herbicide was found to cause neuronal cell necrosis in the pyriform cortex, an area primarily responsible for olfactory sensation, and a dose-response relationship was observed for disruptions in both motor activity and sensory response (tail-flick) (*25, 33*). It should be noted that no peripheral

neuropathy was observed in rats dosed with molinate (*34*). When mice were dosed with molinate, peripheral nerve demyelination was observed in addition to eosinophilic bodies (swollen, degenerated axons) in the medulla and spinal cord. Hindlimb adduction, splayed hindlimbs, and ataxia were also observed in these studies (*25, 35*). In dogs dosed chronically with this herbicide, irreversible motor function deficits were found (*25, 36*). These motor deficits included splayed limbs, reduced motor activity, and tremors. This was accompanied by minor demyelination in the spinal cord in these animals. The irreversibility of the adverse effects observed in these animals is a very important consideration, because even though molinate was used for short durations each year, the neurotoxic effects in humans resulting from this exposure period may also be irreversible.

In addition to motor function and effects on neuronal pathology, the consequences of molinate exposure on the function and activity of particular enzymes has also been evaluated. One previously identified target of molinate is aldehyde dehydrogenase (ALDH), indicated by an *in vivo* decrease in liver ALDH activity in rats treated with molinate (*37-39*). Elevated levels of acetaldehyde in the blood and brain of ethanol-challenged rats dosed with molinate were also found, indicative of ALDH inhibition (*39*).

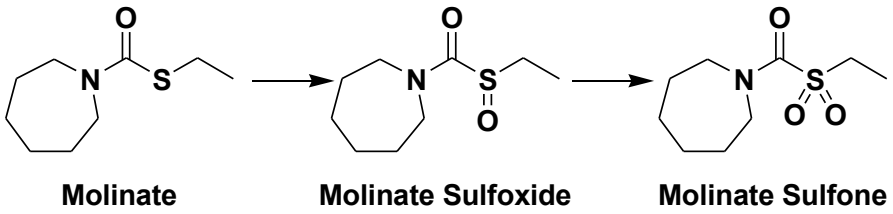
In humans, there are 19 genes attributed to ALDHs (*40, 41*), an enzyme responsible for the metabolism of many toxic aldehydes such as 3,4-dihydroxyphenylacetaldehyde (DOPAL) and 4-hydroxy-2-nonenal (4HNE) (*41*). ALDHs are also responsible for the biotransformation of acetaldehyde, an aldehyde formed as a result of alcohol consumption. DOPAL is the aldehyde metabolite of dopamine, the neurotransmitter important for motor activity, whereas 4HNE is a product of lipid peroxidation. These are both reactive electrophiles, which, at elevated concentrations, have been shown to modify proteins and lead to increased oxidative stress, mitochondrial dysfunction, and toxicity (*41-47*). The accumulation of these neurotoxic

aldehydes results when ALDH is inhibited, and has been implicated in the development of neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease (*41, 47, 48*).

ALDH2 has been found in the locus coeruleus, substantia nigra, and portions of the basal ganglia (*49*). Polymorphisms in this enzyme that render it inactive have been linked to increased risk for late-onset Alzheimer's disease and certain types of cancer (*41, 50-54*). This type of reduction in ALDH2 activity can lead to increased susceptibility to 4HNE and oxidative insult (*44, 46, 55-58*). In a transgenic mouse model with reduced ALDH2 activity, age-dependent neurodegeneration was observed, indicating an increased susceptibility to oxidative stress and neuronal damage (*59*). In addition, the overexpression of this enzyme in primary hippocampal neurons has been shown to be neuroprotective and reduce the neurotoxicity of 4HNE (*60*). Inhibition of ALDH2 has also been shown to cause elevated levels of DOPAL and results in increased sensitivity to levels of 4HNE (*61-69*). Due to the importance of ALDH2 in the detoxification of reactive aldehydes and its role as a neuroprotective enzyme, the effect of molinate and its metabolites on this particular ALDH was investigated.

Previous reports have proposed that it is the metabolites of molinate that are primarily responsible for the toxic effects observed from exposure to this pesticide, not the parent compound (*30, 70, 71*). Molinate can be metabolized by two main pathways, 1.) hydroxylation of the ring or 2.) oxidation of the sulfur of the thiocarbamate first to a sulfoxide then further oxidation to the sulfone (Scheme 3.1) (*71*). Ring hydroxylation of molinate is predominant at lower doses of molinate, and thought to be a detoxification pathway, and was found to be predominant at lower doses of molinate. At higher doses, molinate metabolism is thought to occur via the sulfoxidation route (*71*). Upon sulfoxidation, these metabolites may then undergo glutathione conjugation, followed by excretion of the corresponding mercapturate product. Species differences in the rates and routes of metabolism have been observed (*72, 73*). In humans, it has been shown

that only 1-5% of the dose of molinate is excreted as the mercapturate, and 35-40% is excreted as hydroxymolinate or a comparable conjugate (*72, 74*). Based upon these results, a recent report concluded that at the current recommended exposure limits, human toxicity risk is minimized (*70*). However, the target of the remaining 60% of the initial dose that is not excreted is unknown.



Scheme 3.1. Molinate Sulfoxidation

The role of the sulfoxidation metabolites in the toxicity of molinate has been investigated, particularly with respect to esterase inhibition. It has been shown that in rats and humans, molinate sulfoxide and molinate sulfone are both more potent testicular carboxylesterase inhibitors than molinate, resulting in the carbamylation of an active site Ser residue (*30, 70*). This esterase inhibition is thought to contribute to the reproductive toxicity observed in rats and mice (*32, 70*).

In addition to esterase inhibition, molinate sulfoxide has been shown to be capable of inhibiting liver ALDH (*38*), however, the relative inhibitory potency of both sulfoxidation metabolites of molinate towards ALDH has not been previously addressed. Also, the protein reactivity profile of these three compounds has not been investigated, nor the specific target of protein modification. The goal of this research is to establish the mechanism of ALDH inhibition by molinate and its sulfoxidation metabolites, by comparing their reactivity and potency.

Based upon the relative reactivity of other similar pesticides (*75, 76*) and previously reported studies on molinate (*32, 70, 71*), it is hypothesized that molinate sulfone is a more potent inhibitor of ALDH than molinate or molinate sulfoxide, and that the mechanism of inhibition involves thiol modification. In this report, the relative reactivity of molinate and its metabolites was determined by comparing the inhibition kinetics of these three compounds for human recombinant mitochondrial ALDH (hALDH2). The reactivity of each compound towards various amino acids and hALDH2 was demonstrated, identifying Cys as the target for modification. This work indicates the sulfoxidation of molinate leads to the production of a reactive metabolite, molinate sulfone, capable of inactivating ALDH2.

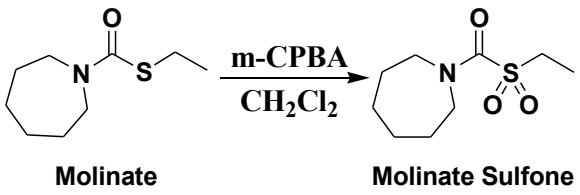
Materials and Methods

Molinate (> 98% purity) was purchased from Chem Service (West Chester, PA), 2,4,6-trinitrobenzenesulfonic acid (TNBSA) was obtained from Pierce (Rockford, IL) and peptides (ANP(1-11) and RKRSRAE) were obtained from American Peptide (Sunnyvale, CA). All other reagents, unless otherwise noted, were purchased from Sigma Aldrich (St. Louis, MO).

Molinate Sulfone (S-Ethyl-hexahydro-1H-azepine-1-carbothioate sulfone)

The synthesis of molinate sulfone, as outlined in Scheme 3.3, was adapted from a literature method (*34*), molinate (54.0 mg, 0.29 mmol) was combined with *m*-chloroperoxybenzoic acid (*m*CPBA, 103 mg, 0.59 mmol) in dichloromethane and stirred in an ice bath overnight. The reaction was filtered and washed with saturated sodium bicarbonate twice. The product was purified using column chromatography on silica gel with a mobile phase consisting of hexanes (A) and ethyl acetate (B) (0 - 17% B). ^{1}H NMR ($CDCl_3$): δ 1.45 (t, 3H, CH_3), 1.64 (m, 4H, ring CH_2), 1.80 (m, 2H, ring CH_2), 1.89 (m, 2H,

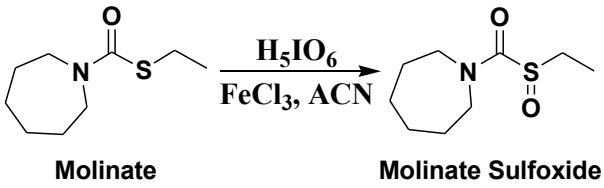
ring CH_2), 3.39 (q, 2H, CH_2), 3.56 (t, 2H, ring CH_2), 3.92 (t, 2H, ring CH_2); TOF MS (ESI) *m/z* calcd for $C_9H_{17}NO_3S$ 219.1, found *m/z* 242.1 $(M + Na)^+$.



Scheme 3.2. Synthesis of Molinate Sulfone

Molinate Sulfoxide (S-Ethyl-hexahydro-1H-azepine-1-carbothioate sulfoxide)

Adapted from a literature method (*77*), molinate (113 mg, 0.600 mmol) was combined with iron trichloride ($FeCl_3$, 3.00 mg, 0.02 mmol) in 600 µL of acetonitrile (ACN) and vigorously vortexed for 5 min. Then, periodic acid (150 mg, 0.66 mmol) was added and let sit for 2 h at room temperature. Next, an excess of sodium thiosulfate was added to quench the reaction, and then allowed to sit at room temperature overnight. The product was extracted four times with dichloromethane (5 mL), and the combined organic layers dried with magnesium sulfate, then filtered. The organic solvent was removed by rotoevaporation and the product purified by column chromatography on silica gel, with a mobile phase consisting of hexanes (A) and ethyl acetate (B) (10% B) to give the racemic product. The reaction is outlined in Scheme 3.2. ^1H NMR ($CDCl_3$): δ 1.38 (t, 3H, CH_3), δ 1.68 (m, 4H, ring CH_2), δ 1.80 (m, 4H, ring CH_2), δ 3.01 (q, 2H, CH_2), δ 3.40 (t, 4H, ring CH_2); TOF MS (ESI) *m/z* calcd for $C_9H_{17}NO_2S$ 203.1, found 226.1 $(M + Na)^+$.



Scheme 3.3. Synthesis of Molinate Sulfoxide

Preparation of hALDH2

Wild-type and C302S hALDH2 were a generous gift from Dr. Thomas Hurley from Indiana University, and prepared as described previously (*46, 78-80*). The proteins were stored in 50% glycerol, therefore, prior to use, the enzymes were dialyzed for 4 h at 4°C with a 4000-fold excess of 50 mM sodium phosphate buffer, pH 7.4, to remove the glycerol. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce) and a Molecular Devices SpectraMax 190 plate reader (Sunnyvale, CA). The specific activity of the wild-type enzyme was measured by incubating hALDH2 (0.3–3.5 µg), propionaldehyde (1.4 µM), and NAD (1.0 mM). The conversion of NAD to NADH was measured as an increase in absorbance at 340 nm (ε = 6220 $M^{-1}cm^{-1}$).

Kinetics of Aldehyde Dehydrogenase Inhibition

One microgram of hALDH2 was incubated and equilibrated with 1 mM NAD, followed by the addition of 0-25 µM of molinate, molinate sulfoxide, or molinate sulfone in 50 mM sodium phosphate buffer, pH 7.4, at 37°C. At time points between 0 and 15 min, propionaldehyde in 50 mM sodium phosphate buffer, pH 7.4, was added for a final concentration of 1.0 mM. Upon addition of substrate, the activity of hALDH2 was measured as an increase in the production of NADH. Percent activity was determined by comparing the initial linear slopes of NADH production in treated wells to the initial slopes from control wells. To determine if this inhibition was reversible, hALDH2 (6 µg)

was incubated with NAD (1.0 mM) and then molinate sulfone (0-50 µM) at 37°C for 0-15 min. This solution was then diluted 1:40 with NAD (1mM) and propionaldehyde (1.0 mM), and absorbance readings taken as before, with initial linear slopes of NADH production monitored.

Determination of k_i Values

The bimolecular rate constants of inhibition (k_i) for each compound (inhibitor) were determined according to literature methods (*81-83*). The inhibitory reaction proceeded with [inhibitor] > 10 [enzyme], such that the [inhibitor] remained relatively constant, and the k_i approximated using pseudo-first order kinetics. Linear regression was used to calculate the slopes of primary kinetic plots of ln (% activity remaining) vs time (t) to obtain apparent first-order rate constants of inhibition (k') at each [inhibitor] and to determine the k_i from the slopes of secondary plots of $-k'$ vs [inhibitor]. All slopes were linear for the duration of the experiment (900s), except for 25 µM molinate sulfone due to complete enzyme inhibition after 600 s. Therefore, for this concentration, the slope (k') was determined only from 0-600 s, whereas all other slopes were determined through 900 s.

Preparation of Striatal Synaptosomes

Striatal synaptosomes (isolated nerve terminals that retain most of the structural and functional characteristics of neurons (*44, 84, 85*)) were prepared from 20-25 g Swiss Webster (CD1 IGS) female mice, as previously described (*44, 86, 87*). Briefly, mice were euthanized with an ip dose of 150 mg/kg sodium pentobarbital, and the brains were quickly removed. The striatal tissue was removed and homogenized in media (0.32 M sucrose, 10.0 mM Tris, and 0.5 mM EDTA, pH 7.5, 4°C) at 10% w/v. The homogenate was then centrifuged at 800 × g for 10 min, and the pellet discarded. The supernatant was mixed (1:1) with 1.2 M sucrose and the solution centrifuged at 18000 × g for 25 min. The pellet was resuspended to the original volume in HEPES-buffered synaptosomal media

(136 mM NaCl, 4.80 mM KCl, 1.20 mM $MgSO_4$, 1.40 mM $CaCl_2$, 10.0 mM glucose, 140 µM EDTA, and 20.0 mM HEPES, pH 7.4). Protein concentration was determined using the BCA assay (Pierce).

Treatment and Analysis of Striatal Synaptosomes

Synaptosomes (0.5 mg/mL) were pre-incubated in 50mM sodium phosphate buffer, pH 7.4, with 0.10 mM dopamine for 15 min at 37°C. Then molinate, molinate sulfoxide, or molinate sulfone were added (50 µM), or sodium phosphate buffer for vehicle controls. Aliquots were removed at various time points (0-60 min) and combined with perchloric acid (5% v/v final concentration), to precipitate proteins and stop the reaction. Solutions were stored at -20°C, thawed, and centrifuged at 10,000 × *g* for 5 min prior to HPLC analysis. Samples were analyzed by an Agilent 1100 Series Capillary HPLC with Phenomenex C18 Luna column (1 x 150 mm) to quantify dopamine metabolites: DOPAL, and 3,4-dihydroxyphenylacetic acid (DOPAC). Separation of these metabolites was achieved using isocratic conditions of 0.1% trifluoroacetic acid in water with 6% ACN (v/v), a flow rate of 50 µL/min, and detection with a photodiode array detector (absorbance at 202 and 280 nm). The peak area was then converted to concentration units through comparison to a standard curve achieved from metabolite standards. The percent activity of ALDH for each sample was determined by comparing the linear slopes (0-60 min) for the time-dependent formation of DOPAC from treated synaptosomes to controls.

Amino Acid Reactivity

Thiol reactivity of molinate, molinate sulfoxide, and molinate sulfone was determined using Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)) and a procedure adapted from the literature (*88*). *N*-acetyl Cys (50 µM) was incubated with each compound (0-100 µM) for 15 min at 37°C. DTNB (0.5 mM) was added, and absorbance monitored at 412 nm using a SpectraMax plate reader to determine free

thiol concentration (ε = 13,600 $M^{-1}cm^{-1}$). In order to determine amine reactivity of each compound, TNBSA was used according to manufacturer specifications (Pierce) and literature (*89*). An amine-containing model peptide (20 µM), RKRSRAE, was incubated with each compound (0-100 µM) for 20min at 37°C in sodium bicarbonate buffer, pH 8.0 in a quartz 96-well plate. TNBSA (100 µM, final concentration) in sodium bicarbonate buffer pH 8.0 was then added and absorbance readings were taken every minute for 2.5h at 335 and 420 nm. The absorbance values were compared to those obtained from a standard curve produced using *N*-acetyl lysine (0-20 µM) and TNBSA (0.003% w/v), in order to determine the primary amine concentration in each sample.

Mass Spectrometry of Modified Peptide

A Cys-containing model peptide, rat ANP(1-11) (SLRRSSCFGGR, 50 µM), from American Peptide, was incubated with either molinate, molinate sulfoxide, or molinate sulfone (500 µM) for 1 h at 37°C in 50 mM sodium phosphate buffer, pH 7.4. These samples (2.5-5 ng/µL) were then analyzed by LC/MS using a Waters Acquity UPLC interfaced with a Q-TOF Premier equipped with an Acquity BEH C18 column (2.1 x 100 mm). The mobile phase consisted of (A) 5% ACN in water with 0.1% formic acid and (B) 0.1% formic acid in ACN with a flow rate of 200 µL/min. The gradient conditions used were 0% B at 1 min to 45% B at 15 min, and held for 10 min. Mass spectrometric detection was conducted using positive ion mode, scanning 100-1500 *m/z*, and analysis was completed using MassLynx 4.0 software. MS/MS data was collected on the samples (2-3 ng/µL) by direct injection using a Harvard Apparatus syringe pump running at 10 µL/min interfaced with a Waters Premier Q-TOF. The collision energy was set to 20 V, scanning 100-1500 *m/z*. Analysis was conducted using MassLynx 4.0 and MaxEnt deconvolution software.

Protein Mass Spectrometry

Wild-type hALDH2 and the C302S mutant of hALDH2 were incubated with molinate and molinate sulfone at molar ratios of 1:2 for 13 h in 10 mM Tris-HCl, pH 7.5, and then analyzed for covalent adducts by mass spectrometry. Samples were introduced into the mass spectrometer using an Agilent 1200 series capillary HPLC with a flow rate of 10 µL/min consisting of 70% H_20 and 30% acetonitrile with 0.1% formic acid. An Agilent 6520 quadrupole-time of flight (Q-TOF) mass spectrometer, operating in positive ion ESI-TOF mode, was interfaced with the HPLC, and the data processed and deconvoluted using Mass Hunter/Bioconfirm software suite.

Statistics

All statistics and linear regressions were calculated using GraphPad Prism version 5.0c (GraphPad Software, San Diego, CA). SEM for the comparison of ALDH activity in mouse striatal synaptosomes was calculated with the standard rules of error propagation for quotients using the SE from linear regression calculations. All other values are reported as the mean ± SEM ($n \geq 3$). One-way ANOVA with Newman-Keuls Multiple Comparison test was conducted to determine significance between all samples for the bimolecular rate constants and percent activity of ALDH (Table 3.1 and Table 3.2). All values are significantly different from control and other treated samples, $p < 0.01$, unless otherwise noted.

Results

Kinetics of Aldehyde Dehydrogenase Inhibition

To compare the inhibitory potency of molinate and its metabolites towards ALDH, the activity of hALDH2 was monitored upon incubation with various concentrations of each of these compounds. The specific activity of this recombinant protein was determined to be 0.375 µmol/min/mg protein using propionaldehyde as

the substrate. This value is comparable to the specific activity previously determined for this recombinant protein (*56*). The natural log transformation of percent activity of hALDH2 treated with each compound (1-25 μM) are presented in Figure 3.1A, C, and E (molinate, molinate sulfoxide, and molinate sulfone, respectively).

The slope (k') from the linear regression of percent activity for each concentration of inhibitor is graphed in Figure 3.1B (molinate), Figure 3.1D (molinate sulfoxide), and Figure 3.1F (molinate sulfone). The linear slope of the secondary plot represents the bimolecular rate constant (k_i ($\mu M^{-1}s^{-1}$)) for each compounds. The bimolecular rate constants for all three compounds are shown in Table 3.1. Based on these values, the relative inhibitory potency was determined to be molinate sulfone > molinate > molinate sulfoxide. The inhibition of hALDH2 was found to be irreversible, because upon dilution, no return of activity was observed (data not shown).

Disruption of Dopamine Metabolism

In addition to using recombinant protein, mouse striatal synaptosomes were also used to monitor ALDH activity. This model system contains multiple carbonyl metabolism enzymes, and is a good model for dopamine metabolism because they retain most of the structural and functional characteristics of neurons, including active MAO (*44, 84, 85*) and multiple ALDHs (*90, 91*). They have also proven to be a good model for assessing the effects of drugs on synaptic processes (*92, 93*). The synaptosomes were pre-incubated with dopamine (0.1 mM) for 15 min, to allow for easily detectable levels of DOPAL to be produced, and then treated with either molinate, molinate sulfoxide, or molinate sulfone.

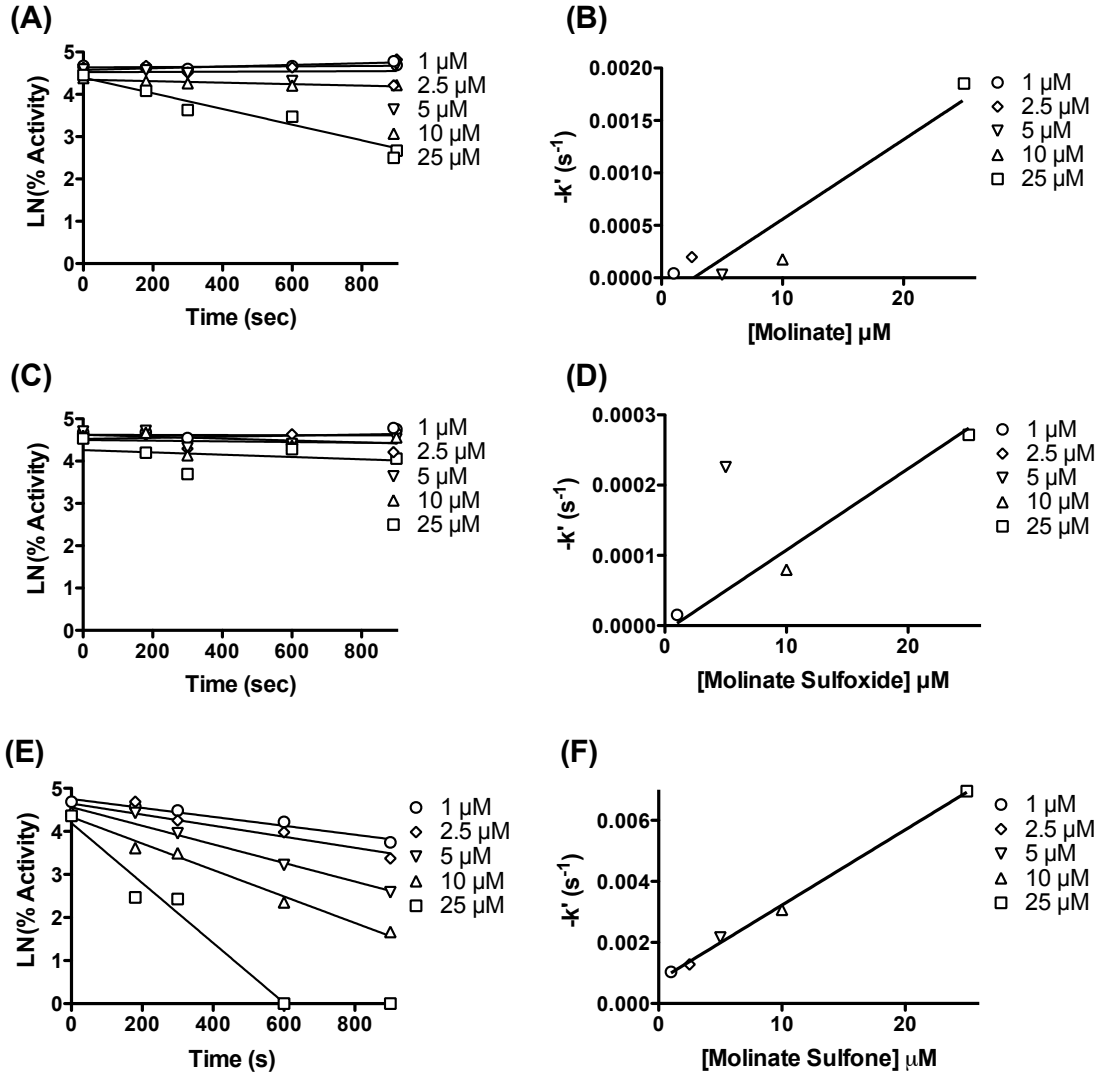


Figure 3.1. Inhibition kinetics of hALDH2 by molinate, molinate sulfoxide, and molinate sulfone, modified from (*94*). (A), (C), (E) Primary plots, representing the natural log transformation of percent activity calculated from treatment of hALDH2 with 1-25 µM molinate, molinate sulfoxide, or molinate sulfone, respectively. The slope of each line gives k' (s^{-1}). (B), (D), (F) Secondary plots, representing the linear correlation between k' (s^{-1}) and [molinate], [molinate sulfoxide], and [molinate sulfone], respectively, with the slope of each line equal to k_i ($\mu M^{-1} s^{-1}$).

Table 3.1. Bimolecular rate constants, k_i (M^{-1} s^{-1}) of hALDH2 inhibition.

Compound	k_i ($M^{-1}s^{-1}$)
Molinate	76.0 ± 6.94
Molinate Sulfoxide	11.6 ± 15.3[a]
Molinate Sulfone	245 ± 7.38

Note: Rate constants determined for the inhibition of hALDH2 by molinate, molinate sulfoxide, and molinate sulfone (*94*).

[a]Not significantly different than zero ($p > 0.05$).

To assess ALDH activity, the conversion of DOPAL to DOPAC was monitored at various time points using HPLC. A time-dependent increase in DOPAC concentration was observed for the control, as illustrated in Figure 3.2. However, treatment of the synaptosomes with molinate sulfoxide and molinate sulfone yielded significantly decreased DOPAC production, compared to controls, indicating inhibition of ALDH activity in this model system. The percent activity, calculated by comparing the initial linear slopes of the time-dependent DOPAC formation to control, is shown in Table 3.2. Molinate sulfone was found to be the most potent inhibitor of aldehyde metabolism in the synaptosomes, followed by molinate sulfoxide, then molinate.

Amino Acid Reactivity

The reactivity of each compound with two protein nucleophiles, Cys and Lys, was assessed in order to characterize possible sites of protein modification. To determine Cys reactivity, various concentrations of molinate, molinate sulfoxide, or molinate sulfone were incubated with *N*-acetyl Cys, and free thiol concentration was monitored using DTNB. The results are shown in Figure 3.4. (A) HPLC chromatogram of ANP(1-11)

peptide (SLRRSSCFGGR, 50 µM), RT 4.57 min. (B) HPLC chromatogram of ANP(1-11) peptide (50 µM) modified by molinate sulfone (500 µM), RT 7.37min (*94*). and demonstrate that under these conditions, only molinate sulfone is reactive with *N*-acetyl Cys, indicated by a decrease in free thiol concentration. The amine-reactivity of these compounds was determined by incubating an amine-containing peptide (RKRSRAE) with each compound and monitoring the concentration of primary amines using TNBSA. No reactivity towards this peptide was observed with any of the compounds (data not shown).

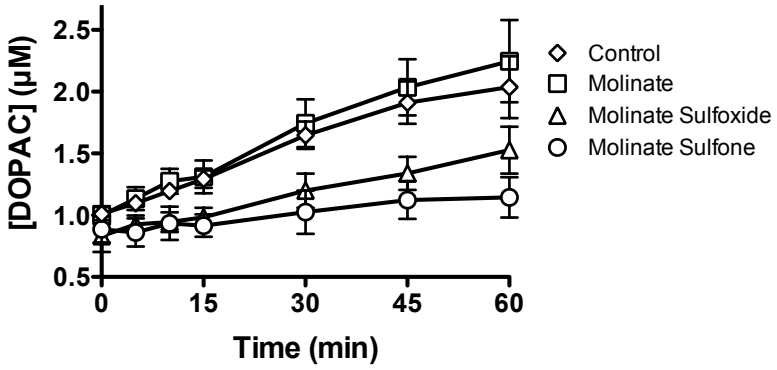


Figure 3.2. Inhibition of aldehyde metabolism (i.e. DOPAL), represented by a decreased production of DOPAC, in mouse striatal synaptosomes (0.5 mg/mL protein) pre-incubated with 0.1 mM dopamine and treated with 50 µM molinate (□), molinate sulfoxide (Δ), molinate sulfone (O), or control (◊). The values shown represent the mean ±SEM (*n* = 3, except for controls where *n* = 6), where peak area was converted to concentration using a standard curve. Adapted from (*94*).

Table 3.2. Percent activity of ALDH in striatal synaptosomes.

Compound	% Activity
Molinate	117 ± 3.67
Molinate Sulfoxide	62.6 ± 2.87
Molinate Sulfone	27.6 ± 2.98

Note: Percent activity determined following treatment with 50µM compound, determined through comparison of the initial linear slopes (0-60 min) of time-dependent DOPAC formation to control (*94*).

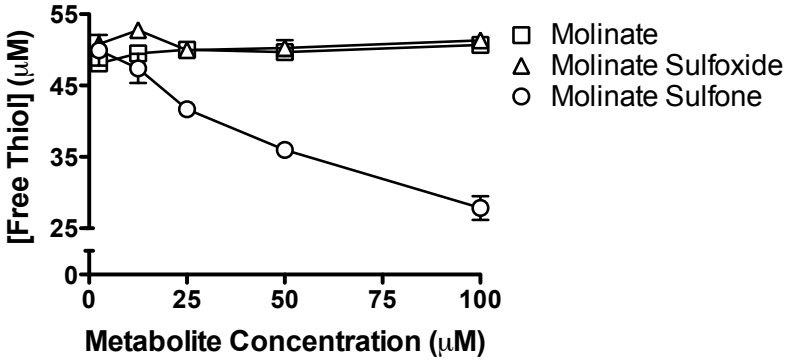


Figure 3.3. Reactivity of molinate (□), molinate sulfoxide (Δ), and molinate sulfone (O), (0-100 µM) with *N*-acetyl Cys (50 µM), monitored using DTNB (500 µM) to quantify free thiol concentration. The values shown represent the mean ±SEM (*n* = 3), and are representative of values reported in (*94*).

Mass Spectrometry of Modified Peptide

In order to further investigate thiol reactivity as well as to assess the reactivity of molinate and its metabolites with Ser residues, a model peptide containing Cys and Ser was used. This peptide (ANP(1-11), SLRRSSCFGGR) was incubated with each compound and then analyzed using LC-MS. The HPLC chromatogram for the unmodified peptide (retention time (RT) 4.57 min) is shown in Figure 3.4A, with the chromatogram for ANP(1-11) incubated with molinate sulfone in Figure 3.4B. The presence of modified peptide (RT 7.37 min) and a small amount of unmodified peptide (RT 5.12 min) can be observed in this HPLC trace (Figure 3.5B). Chromatograms were also obtained for ANP(1-11) incubated with molinate and molinate sulfoxide, however, only a peak corresponding to the unmodified peptide was observed (data not shown).

From the sample eluting at 4.57 min in the HPLC trace, peaks at 613.4 *m/z* (theoretical, 613.2 *m/z*) and 409.2 *m/z* (theoretical, 409.1 *m/z*), corresponding to the doubly and triply charged unmodified peptide, were observed, Figure 3.6A. LC/MS analysis of the sample at RT 7.37 min, revealed *m/z* 676.4, 451.3, and 338.7 (theoretical, 676.3, 451.2, and 338.6 *m/z*), representing the doubly, triply, and quadruply charged ANP(1-11) peptide with a 126 Da thiocarbamate adduct, respectively (Figure 3.6C). In this mass spectrum there is also a peak at 126.1 *m/z*, corresponding to the charged loss of the azepine ring.

In order to verify the exact location of the 126 Da modification of the peptide, MS/MS analysis was conducted on both the control peptide (unmodified) as well as the peptide treated with molinate sulfone (modified) (Figure 3.5). Fragmentation of the triply charged ion corresponding to the unmodified peptide (408.95 *m/z*) (Figure 3.5B), and the following major ions were found: b_2 (201.1), y_2 (232.1), y_3 (289.1), b_3 (357.2), y_4 (436.2), b_4 (513.3), y_5 (539.2), b_5 (600.3), y_6 (626.2), b_6 (687.4), y_7 (713.3), b_7 (790.3), y_8 (869.4), b_9 (994.4), and b_{10} (1051.5). Fragmentation of the triply charged ion corresponding to the modified peptide (450.8 *m/z*) was also analyzed via MS/MS (Figure

3.5D), allowing determination of the target residue (Cys) for molinate sulfone adduction. The following major ions were found: charged loss of adduct (126.1), y_1 (175.1), b_2 (201.1), y_2 (232.1), y_3 (289.2), b_3 (357.2), y_4 (436.2), b_4 (513.3), b_5 (600.3), y_5 (664.3), b_6 (687.4), y_6 (751.3), y_7 (838.3), y_8 (994.4). The y_4, y_5, y_6, y_7, and b_6 ions, along with the charge loss (126.1) corresponding to the carbamate adduct, conclusively map the 126.1 Da, thiocarbamate adduct to the Cys residue of the ANP(1-11) peptide, Figure 3.6. No evidence of primary amine modification (amine terminus of the peptide) or Ser modification was observed. Results further confirm the aforementioned findings conducted using *N*-acetyl Cys and the RKRSRAE peptide, indicating reactivity towards thiols but not primary amines by molinate sulfone.

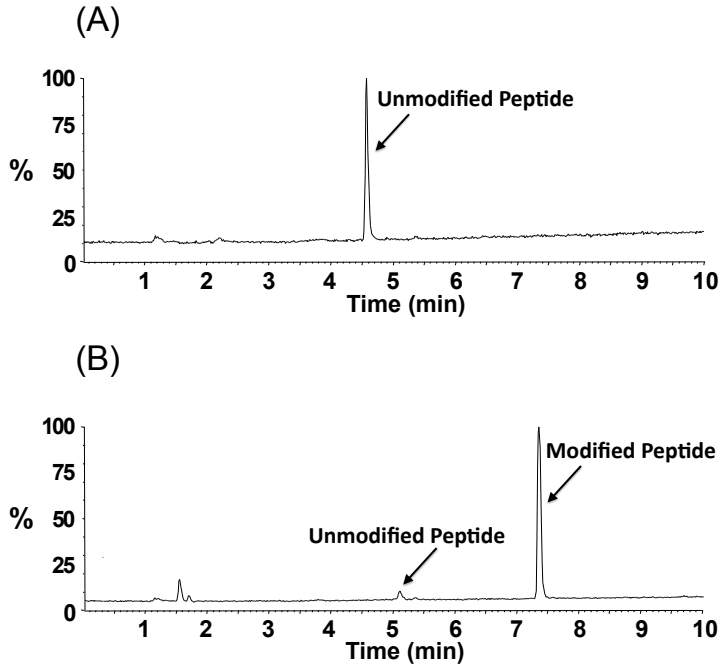


Figure 3.4. (A) HPLC chromatogram of ANP(1-11) peptide (SLRRSSCFGGR, 50 µM), RT 4.57 min. (B) HPLC chromatogram of ANP(1-11) peptide (50 µM) modified by molinate sulfone (500 µM), RT 7.37min (*94*).

Protein Mass Spectrometry

Based on the ability of molinate sulfone to inhibit ALDH and to modify Cys residues, the reactivity of molinate and molinate sulfone with hALDH2 was investigated using mass spectrometry (Table 3.3). When hALDH2 was treated with molinate, no mass shift was observed, indicating no covalent modification occurred. However, when hALDH2 was incubated with molinate sulfone, a mass shift of 125 *m/z* was observed (*m/z* 54445 to *m/z* 54570), indicating the presence of a single carbamate adduct on this protein. Such a finding supports the results obtained using the ANP(1-11) model peptide, demonstrating Cys reactivity with molinate sulfone, but not with molinate. In order to determine if molinate sulfone was reacting with the catalytic Cys302 of hALDH2, a C302S mutant of hALDH2 was also treated with molinate and molinate sulfone. This mutant no longer contains the nucleophilic Cys residue in the active-site, but instead replaces it with a much less reactive Ser residue. The C302S mutation maintains the structure of the wild-type protein, but reduces its catalytic activity. When the C302S hALDH2 was treated with either molinate or molinate sulfone no protein modification was observed, with Δ*m/z* being 0. Since protein reactivity was prevented when Cys302 was changed to a Ser, it may be concluded that molinate sulfone covalently modifies the active-site Cys residue of hALDH2.

Discussion

Exposure to molinate can lead to disruptions in motor activity and other signs of neurotoxicity (*25, 29*). The neurotoxic effects observed may be a consequence of inhibition of ALDH in the brain, resulting in an accumulation of endogenous neurotoxins, such as DOPAL or 4HNE, oxidative stress, or mitochondrial dysfunction (*41-47*). The current study investigates the relative reactivity of molinate and both of its sulfoxidation metabolites, molinate sulfoxide and molinate sulfone, towards hALDH2, as well as

establishes their protein reactivity profiles and identifies the mechanism of ALDH2 inhibition.

Previous work with molinate and its metabolites has indicated that in rat liver mitochondria and microsomes, molinate and molinate sulfoxide both inhibit ALDH (molinate sulfone was not evaluated) (*38*). In rat brain mitochondria the parent molinate was not found to inhibit ALDH, but neither sulfoxidation metabolite was investigated (*48*). There have also been two *in vivo* studies assessing ALDH activity. One study found liver ALDH inhibition in mice dosed ip with molinate (*39*), and the other found inhibition of ALDHs from the brain, liver, and testicle in rats dosed with molinate (*34*). These studies all indicate molinate or a metabolite is capable of inhibiting ALDHs, but the rates of this inhibition and the metabolite responsible for it are not clear. In the current study, molinate and molinate sulfone were both found to inhibit hALDH2, with molinate sulfone (245 $M^{-1}s^{-1}$) being three times more potent than the parent molinate (76 $M^{-1}s^{-1}$). Molinate sulfoxide, however, was not found to be a potent hALDH2 inhibitor (11.6 $M^{-1}s^{-1}$).

In both of the model systems used, hALDH2 and mouse striatal synaptosomes, molinate sulfone was found to be the most potent ALDH inhibitor. Molinate sulfoxide was found to only inhibit ALDH activity in the mouse striatal synaptosomes, which contain multiple ALDHs (*40, 90, 91, 95*) and also have oxygen sources that may be able to oxidize the sulfoxide to the more potent sulfone. However, when this compound was tested against human ALDH2, it was not found to inhibit this enzyme, indicating that in the synaptosome it may be targeting other isoforms of ALDH, or there are species differences affecting its potency (*40, 95*). The parent pesticide, molinate, was found to inhibit hALDH2, but showed no potency towards inhibiting ALDH activity in the synaptosomes. It may be concluded that molinate is a potent inhibitor of ALDH2, particularly in humans, but is not capable of inhibiting the ALDHs found in the striatal

region of the mouse brain, or is less capable than the sulfoxidation metabolites to access these enzymes (*71*).

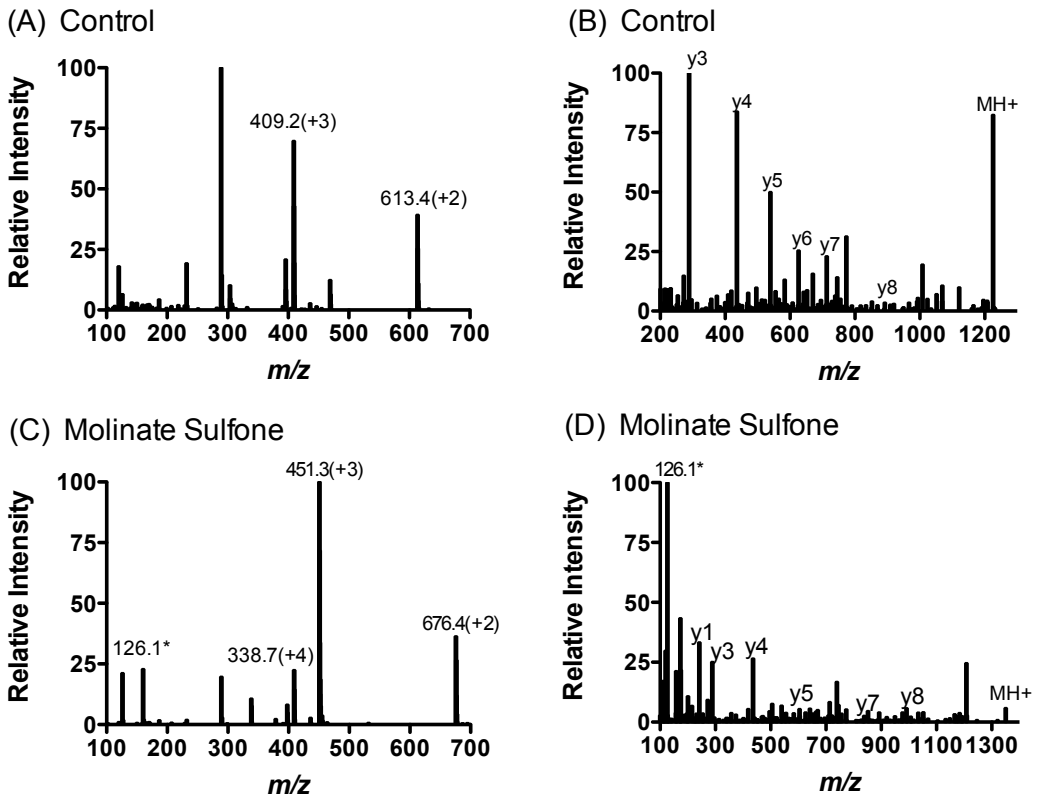


Figure 3.5. LC/MS and MS/MS analysis of the model peptide, ANP(1-11) (MH^+ at *m/z* 1225.4, SLRSSCFGGR) (50 µM), incubated with molinate sulfone (0 µM (panels A, B) or 500µM (panels C, D)). (A) Mass spectrum at 4.57 min with peaks 613.4 and 409.2 *m/z* corresponding to the doubly and triply charged, unmodified peptide. (B) Deconvoluted MS/MS of unmodified peptide (408.95 *m/z)*. (C) Mass spectrum at 7.37min with peaks at 676.4, 451.3, and 338.7 *m/z* corresponding to the doubly, triply, and quadruply charged peptide modified by molinate sulfone. (D) Deconvoluted MS/MS of modified peptide (450.8 *m/z*). *, charged loss of adduct (126.1 *m/z*). Results are representative of those reported in (*94*).

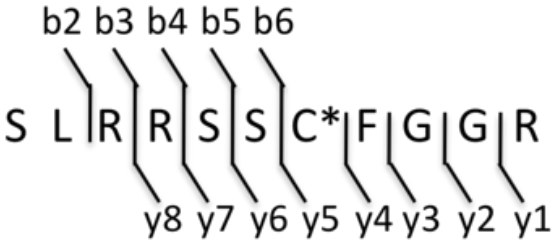


Figure 3.6. Sequence of the thiocarbamate-modified ANP(1-11) peptide with y and b fragment ions identified from MS/MS analysis. C*, modified Cys residue.

Table 3.3. Deconvoluted mass spectrometric analysis of wild-type hALDH2 and C302S hALDH2 treated with molinate or molinate sulfone.

	Wild-type hALDH2		**C302S hALDH2**	
	MH^+ (*m/z*)	**Δ*m/z*[a]**	**MH^+ (*m/z*)**	**Δ*m/z*[a]**
Control	54445	0	54429	0
Molinate	54445	0[b]	54429	0[b]
Molinate Sulfone	54570	125	54429	0[b]

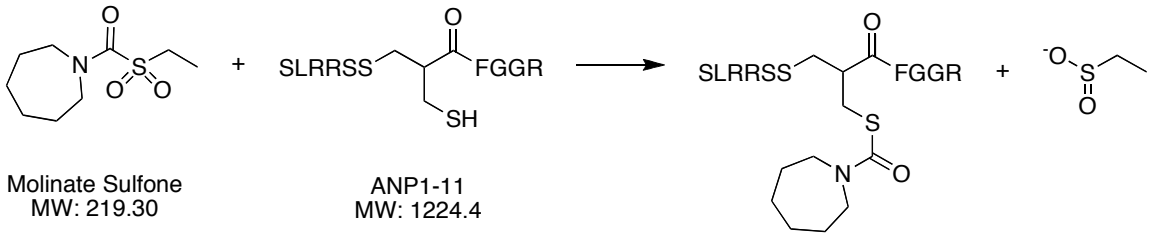
[a] Denotes complete change in mass from control.

[b] No shift in mass from control observed.

Previous studies with molinate have also demonstrated an inconsistency regarding the relative reactivity of molinate vs molinate sulfoxide dependent upon the model system employed (*30, 70*). Extensive work has been previously conducted investigating the inhibition of esterases by molinate and its sulfoxidation metabolites. These studies involved the use of rat liver and testicular microsomes and illustrated molinate sulfoxide was the most potent esterase inhibitor, followed by molinate sulfone, then by molinate (*30*). Another recent report using Leydig cells isolated from rats, found molinate sulfone was the most potent esterase inhibitor followed by molinate sulfoxide and molinate (*70*). Even though both studies addressed rat testicular

carboxylesterase activity, differences in the relative esterase inhibition potency were observed.

The reactivity profile of each of the molinate metabolites with protein nucleophiles (i.e. Cys and Lys) was also established. It was determined that molinate and molinate sulfoxide were not reactive towards *N*-acetyl Cys, ANP(1-11) (containing Cys and Ser residues), or a Lys containing peptide (RKRSRAE). Molinate sulfone was found to modify *N*-acetyl Cys and the Cys residue in the ANP(1-11) peptide, resulting in a mass shift of 126 *m/z*. This confirms the proposed mechanism of action in Scheme 3.4, involving a nucleophilic substitution reaction between Cys and the carbonyl of the thiocarbamate moiety of molinate sulfone. The reactivity of molinate sulfone towards thiols has been utilized in previous studies to form a standard for selected reaction monitoring to compare the total number of carbamate adducts on Cys residues between microsome and mitochondrial fractions from various organs (*34, 96*). From these studies it was concluded that molinate or one of its metabolites was capable of modifying proteins, such as hemoglobin, although specific sites of adduction were not identified, nor were other target proteins. The authors noted that the degree of Cys modification did not necessarily correlate with ALDH inhibition (*34*), but specific modifications on ALDH enzymes were not assessed.



Scheme 3.4. Reactivity of Molinate Sulfone with Cys Containing Peptide, ANP(1-11).

Molinate and molinate sulfoxide were not observed to react with Cys, Lys, or Ser residues in either peptide used. It is important to note that *in vivo* molinate or a metabolite has been reported to be able to inhibit carboxylesterases via carbamylation of the active-site Ser, confirmed via MALDI-TOF (*30, 70*). The Ser residues in the active-site of carboxylesterases are highly polarized and activated, rendering them much more reactive than the Ser residues found in other proteins and in ANP(1-11) (*97, 98*).

The last part of this study investigated the covalent modification of hALDH2 by molinate sulfone. Literature reports have indicated that exposure to molinate can cause inhibition of ALDH activity (*34, 38, 39*), but the mechanism of this inhibition, including identifying which metabolite is responsible and the site of covalent modification, has not been previously determined. Using mass spectrometry and site-directed mutagenesis, molinate sulfone was found to be highly reactive towards the active-site Cys302 of hALDH2, resulting in a single thiocarbamate adduct. While it was determined that Cys302 is the primary target of molinate sulfone, other residues (e.g., Cys301 or Cys303) are predicted to be modified by the inhibitor at higher concentrations. It was found that treatment of the Ser302 mutant of hALDH2 with a ten-fold excess of molinate sulfone yielded partial adduction of the enzyme, i.e. peaks at *m/z* 54445 and *m/z* 54570 were observed.

In the present study, molinate sulfone was the only compound observed to inhibit hALDH2 activity and to covalently modify this enzyme. Based upon these results it is concluded that this metabolite is most likely responsible for the inhibition of ALDH, and not molinate or molinate sulfoxide. The production of molinate sulfone is very difficult to monitor *in vivo*, and has not been detected in blood from animals dosed with molinate (*38, 99, 100*). This is most likely due to its high reactivity, but many previous studies have ruled out the role of this metabolite in enzyme inhibition. The results of the current study indicate this metabolite is the most likely contributor to ALDH inhibition

observed *in vivo*. Based on its bimolecular rate constant, only a small amount of molinate sulfone needs to be produced in order for enzyme inhibition to occur.

Conclusions

In summary, molinate sulfone was found to be the most potent inhibitor of ALDH activity, using hALDH2 and mouse striatal synaptosomes. This indicates the sulfoxidation of molinate and then of molinate sulfoxide leads to increased inhibitory potency of this pesticide. The reactivity profile of all three compounds was established against various protein nucleophiles, i.e. Cys and Lys. The only reaction that was observed under the conditions used (pH 7.4 and 37°C) was between Cys and molinate sulfone. It was determined that this reaction involved nucleophilic attack of the thiol to the carbonyl of molinate sulfone, resulting in the formation of a 126 Da carbamate adduct, based upon results from LC/MS and MS/MS. A single 125 Da adduct was also found on Cys302 of hALDH2 upon treatment with molinate sulfone, using mass spectrometry and site-directed mutagenesis. From these results, it may be inferred that the sulfone metabolite of molinate is capable of covalently modifying the active-site Cys residue of ALDH2, resulting in the inhibition of this critical enzyme. The next step in the project is to identify covalently modified proteins *in vivo*, and to investigate the reactivity of molinate and its metabolites towards other isoforms of ALDH, i.e. ALDH1 or ALDH3.

CHAPTER FOUR. ENVIRONMENTAL IMPLICATIONS OF PARKINSON'S DISEASE

Parkinson's Disease (PD) is one of the most common movement disorders in the US, with over 1 million people suffering from this disease and more than 40,000 people diagnosed with PD every year, according to the Parkinson's Disease Foundation. The symptoms of PD include resting tremors, bradykinesia (slow movements), postural instability, and rigidity, all of which contribute to motor dysfunction. The hallmark of this neurodegenerative disorder is the selective loss of dopaminergic neurons in the substantia nigra pars compacta (*101*). Unfortunately, it is not until > 80% of these neurons are lost that the motor deficits become apparent enough to reach a conclusive diagnosis (*101, 102*). This lag time is due, in part, to natural compensatory mechanisms (i.e. increased receptor sensitivity and more rapid dopamine turnover) within the brain that help to maintain "normal" motor function despite loss of dopamine neurons (*103, 104*). Because of the latency between the initial onset of the disease (start of neuronal loss) and the clinical manifestation, little is known about the mechanism underlying the neurodegeneration of these dopaminergic neurons (*105*).

Risk Factors for Parkinson's disease

There are three main risk factors for developing PD; these include aging, genetic predisposition, and environmental exposures (20, 102, 103, 106). It is thought that these factors contribute either on their own, or in conjunction with another risk factor, to cause the selective dopaminergic cell death. This neurodegeneration ultimately results from one (or more) of four main causes: protein aggregation, oxidative stress,

neuroinflammation, or apoptosis (107). The course of events between the initial insult and cell death is still not well understood, but each risk factor (aging, genetics and environmental exposure) affects processes that have been shown to ultimately result in neurodegeneration. This relationship is outlined in Figure 4.1.

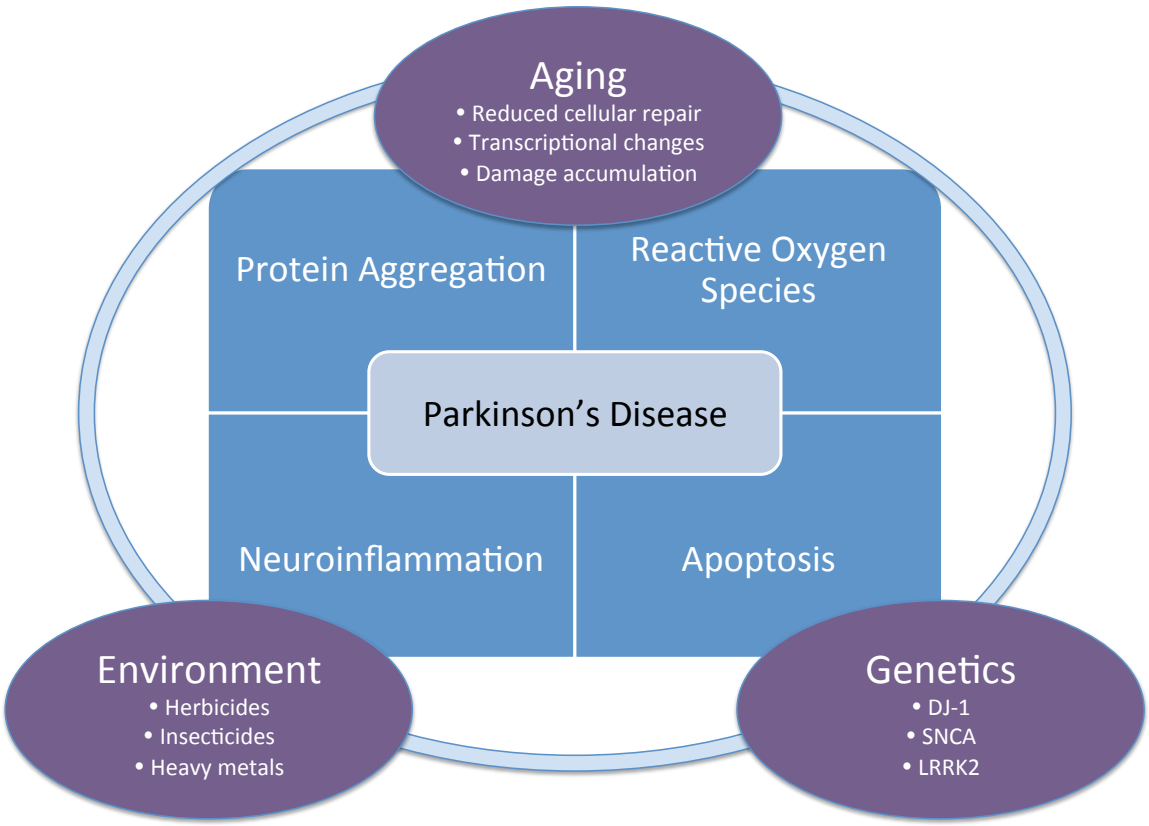


Figure 4.1. Risk factors (aging, environment, and genetics) and the adverse cellular effects they may cause that ultimately lead to neurodegeneration of dopamine neurons and the development of Parkinson's disease. Adapted from (*20, 102, 103*).

Aging and Parkinson's Disease

Age is the largest risk factor for the development of PD, with a majority of cases in patients over the age of 60 (*20, 101-103*). The process of aging is unique for each person, and is dependent mainly upon environmental exposures, with some contribution (< 25%) from genetic predisposition (*103, 108*). As we age, there is an accumulation of unrepaired cellular damage, along with a decrease in the functioning of cellular compensation mechanisms, such as the ability to repair DNA and protein damage (*103, 104, 108*). This is due to a decrease in chaperone efficiency, causing an increase in misfolded proteins; a decrease in proteasome activity, resulting in an accumulation of ubiquinated and damaged proteins that can lead to protein aggregation (*6, 103, 109-111*).

In addition to disruptions in the management of damaged and misfolded proteins, there is also an increase in mitochochondrial dysfunction (*6, 103, 109-111*). This reduction in mitochondrial function results in an accumulation of reactive oxygen species (ROS), which can cause DNA damage, and a decrease in the antioxidant defenses of the cell (*6, 102, 103, 112*). An increase in damaged DNA and proteins is difficult for aging cells to overcome because of the reduced function of their compensatory mechanisms (i.e. proteasome function and DNA repair mechanisms), and often results in the further accumulation of damage to an already injured cell (*103, 112, 113*).

Some cell populations are more susceptible to the insults caused by aging, such as the dopamine neurons in the substantia nigra (101, 107, 114). This susceptibility is influenced by altered levels of expression for many critical proteins that contribute to the antioxidant response, neuronal regulation, and cellular repair (107, 115). For example, the plasma membrane Ca+2-ATPases (PMCA) are pumps responsible for the removal of calcium from the neurons. The catalytic activity and expression of this protein decreases with age (116). This makes it more difficult for the cells to maintain calcium homeostasis. Another enzyme, monoamine oxidase (MAO), is upregulated with

age (*117, 118*). This enzyme is responsible for the oxidative deamination of dopamine to its aldehyde metabolite, 3,4-dihydroxyphenylacetaldehyde (DOPAL). Unfortunately, this metabolite is highly toxic and reactive towards proteins, therefore an upregulation of MAO can result in protein modifications and an increased risk for cell death (*42, 43, 45, 65, 119-121*).

There are many other proteins that have been found to be altered in dopamine neurons that can lead to an increased susceptibility to develop PD, such as the vesicular monoamine transporter and the dopamine transporter (*107, 122*). Altered ratios of these proteins critical for the trafficking of dopamine have been correlated to PD (*22, 122-127*). Changes in the expression of glutathione-related proteins have also been found, coupled with reduced activity of this system, leaving neurons more susceptible to oxidative damage (*107, 128-131*).

In addition to protein changes, there are also alterations to the function of the immune system with age. A mild chronic inflammatory response and altered cytokine activity has been observed with age, i.e. decrease in interleukin 10 (IL-10) and increases in tumor necrosis factor α (TNF-α) (*132-135*). Microglia, the resident macrophages within the central nervous system (CNS), are normally quiescent, and undergo activation when they encounter stimuli such as cytokines or lipopolysaccharide, in order to protect cells within the CNS. Once activated, microglia then release additional cytokines, and nitric oxide and ROS, which can be cytotoxic to neighboring neurons (*132*). With aging, microglia become basally activated and can result in an exaggerated response to stimuli, potentially leading to neuronal damage and/or degeneration (*132, 136, 137*).

Genetic component of Parkinson's disease

In addition to aging, certain genetic mutations have been linked to the development of Parkinson's disease. This correlation between genetics and Parkinson's disease has been estimated to be less than 5%, based on a twin study conducted in 1999

(*138*). However, when considering only early-onset PD cases, where the age of onset is <50, a majority of cases were found to be genetic (*138, 139*). Mutations (single point mutations, deletions, copy number varients, etc) in a number of genes have been associated PD, including *α-synuclein* (*SNCA*), *leucine-rich repeat kinase 2* (*LRRK2*), *parkin* (*PARK2*), *DJ-1* (*PARK7*), and *PTEN-induced putative kinase 1* (PINK1) (*140*).

Mutations in *SNCA* were the first confirmed evidence of a genetic component of Parkinson's disease (*141-143*). The most widely studied mutations of *SNCA* are missense mutations resulting in A53T, A30P, or E46K α-synuclein. The exact function of α-synuclein is still unknown, but is thought to regulate dopamine synthesis, through inhibition of tyrosine hydroxylase; control the synaptic vesicle release of neurotransmitters; and modulate the function of the dopamine transporter (DAT) (*142, 144-151*). When α-synuclein is mutated, as in certain familial forms of PD, this protein is more likely to form oligomers or protofibrils. These protein aggregates have been shown to cause mitochondrial depolarization, the release of cytochrome C, and result in the increased production of reactive oxygen species (*143, 152-159*). These cellular insults have been shown in cellular and animal models to cause apoptosis in dopaminergic cells (*143, 152-159*).

Mutations in LRRK2 have also been correlated to an increased risk to develop PD (*140, 142, 160*). This protein has both GTPase and kinase activity, which can be affected by the presence of mutations, i.e. G2019S that have been implicated in PD (*140, 142, 161*). This particular mutation, G2019S, is the most common LRRK2 mutation (>85% of PD cases with LRRK2 mutation), resulting in increased kinase activity (*161-163*). This augmented enzyme activity has been associated with neuronal toxicity and may help explain the disease pathogenesis (*142, 161, 162, 164*). Despite the research investigating the adverse cellular effects from specific protein mutations, such as in LRRK2 and α-synuclein, the mechanism(s) of neurotoxicity, as it relates to familial forms of PD, is still not completely understood.

Contribution of Environmental Exposures to Parkinson's Disease

In addition to the genetic aspect of PD, there is also a predominant environmental component to disease progression. In fact, a twin study conducted in 1999, addressing the relative roles of genetics and environmental factors in the pathogenesis of PD, found that in PD acquired in patients over the age of 50, which encompasses 80-90% of PD cases, a limited genetic link was found (*138, 139*). This indicates environmental factors are the principal cause of late-onset PD, the predominant form of this neurodegenerative disease (*138, 139*). After exposure to 1-methyl-4-phenyl-1,2,3-tetrahydropyridine (MPTP) was found to cause Parkinson-like symptoms by Langston et al, it prompted a more in-depth investigation into other environment-related compounds that may elicit a similar response (*20, 165-170*).

In order identify particular environmental factors that may lead to an increased risk for developing PD, a number of epidemiological studies have been completed (*20*). These studies have identified rural living, farming, pesticide application, and well-water consumption as environmental risk factors (*8, 139, 171-181*). The correlation between PD and pesticide exposure has been further corroborated by the increased concentrations of certain pesticides in the brains of PD patients (*139, 182-184*). The pesticides found include many organochlorine pesticides such as dieldrin, aldrin, and DDT. In these studies, the substantia nigra of brains of PD patients was analyzed for dieldrin and other pesticides using gas chromatography. They found a significant increase in dieldrin concentration in the brains of PD patients, compared to the brains of patients with other neurodegenerative diseases such as Alzheimer's disease, and control brains (*182*). An increase in dieldrin concentration in the caudate nucleus of PD patients, compared to controls, has also been observed (*183*). Both areas of the brain with high concentrations of dieldrin, the caudate nucleus and substantia nigra, play critical roles in

the nigrostriatal system. Loss of neurons within these two areas are hallmarks of PD neuronal degeneration (*185*).

Conclusion

Parkinson's disease research is not only important to better understand the mechanisms underlying the genetic causes of PD, but also to determine how the disease pathology of familial forms of PD compare to the disease progression observed from sporadic or idiopathic PD due to aging or environmental exposures. Most likely, there is interplay between all three risk factors of PD, genetics, aging, and environmental exposures, and that these risk factors are not mutually exclusive. While there are some cases of PD that are predominantly genetic based, and others, i.e. from MPTP exposure, that can be attributed solely to an environmental cause; a majority of PD cases are likely due to an increased susceptibility from all three risk factors. While this makes it very difficult to determine the precise role of each possible component, by examining the relative mechanisms and disease development from variable causes, it will provide a more complete picture of the cellular events that ultimately result in the selective neuronal loss and motor deficits that define Parkinson's disease. Through this more thorough understanding, earlier detection of PD, the development of more effective therapeutics, and methods for disease prevention may be established.

CHAPTER FIVE. TOXICITY AND CELLULAR LOCALIZATION OF DIELDRIN ANALOGS

Introduction

Parkinson's Disease (PD), a neurodegenerative disease characterized by a selective loss of dopaminergic neurons, affects over 1 million people across the US, according to the Parkinson's Disease Foundation. While the exact mechanism of this neuronal loss is unknown (*105*), a majority of PD cases are thought to be due to environmental exposures, such as insecticides, herbicides, and heavy metals (*138, 139, 182-184*).

The correlation between pesticide exposure and PD has been corroborated by an increased concentration of pesticides found in the brains of PD patients (*139, 182-184*). One pesticide of interest is dieldrin, whose level was elevated in PD patients, a result not found in the brains of patients with other neurodegenerative diseases such as Alzheimer's disease, or in the brains of healthy, control patients (*182*). In addition to finding higher concentrations of dieldrin in the brains of PD patients, this pesticide has also been shown to affect a number of cellular processes associated with PD. The consequences of these effects include increased oxidative stress, disruptions in dopamine metabolism and trafficking, and apoptosis susceptibility (*21, 22, 105, 186-188*). Details of these studies will be discussed in more detail in Chapter 6.

Dieldrin was a common organochlorine pesticide used to control insects on corn and cotton crops. It was a highly effective insecticide due to its high lipophilicity, which allowed it to be absorbed into the cuticle of target insects, and its stability meant that it did not need to be reapplied very often. It was these same characteristics that led the US EPA to ban its use in the 1970s. Currently, dieldrin is still considered one of the twelve most persistent, bioaccumulative, and toxic chemicals by the US EPA, and is

ranked seventeenth on the 2007 Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) Priority List of Hazardous substances (*26*).

The central nervous system is one of the main target organs of dieldrin in humans, and poisoning has been shown to cause headache, nausea, vomiting, convulsions, as well as coma (*189, 190*). The ability of dieldrin to cause these neurotoxic effects is due to the hydrophobic nature of this pesticide, the same characteristics that made it a very effective insecticide. These chemical properties allow dieldrin to cross membranes and accumulate readily in the body, particularly within the central nervous system and adipose tissue.

Due to its lipophilicity and stability, the half-life of this compound in humans is approximately 300 days (*105*), and is significantly longer in the environment, with a half-life around 25 years (*191*). Therefore, even though dieldrin has not been used in the US for over thirty years, it still persists in the environment. It has been estimated that the dietary consumption of dieldrin is 0.03µg/kg/day, a value that is 480 times the recommended benchmark, per the suggested exposure limit calculated by the US EPA (*192*). While dieldrin is currently banned in the US, it is still a major environmental toxicant with a high risk for exposure.

Many of the reported adverse effects, such as mitochondrial dysfunction and apoptosis, resulting from exposure to dieldrin can lead to selective dopaminergic cell dysfunction and/or death (*21, 186*). Such an occurrence *in vivo* is predicted to facilitate the development of PD. Therefore, it is of high importance that the mechanism of the selective dopaminergic toxicity from dieldrin exposure is understood.

In insects, dieldrin is known to suppress the activity of the GABA receptor, through an interaction similar to picrotoxinin (the toxic component of picrotoxin) (*3, 193, 194*). This involves interaction with an allosteric binding site on the GABA receptor-ionophore complex and results in a decrease in Cl^- influx into the postsynaptic neuron

(*193, 195-198*). The human GABA receptor, however, is not as susceptible, and the mechanism of dieldrin toxicity in humans remains unknown (*193, 194, 199-201*).

Studies have been completed assessing how structural changes to dieldrin affect its pesticidal activity in various species of insects (*202-204*). Significant alterations to insect toxicity were found with small changes to dieldrin's structure, such as removal of chlorine atoms or a carbon bridge (*202-204*). This implies that specific structural elements are critical for its insecticidal properties. It is unknown how these same structural features relate to dieldrin's dopaminergic toxicity, and if the compounds with the most potent insecticidal activity are also the most detrimental in a model of dopaminergic cells. This comparison will provide valuable information about the cellular targets of these compounds, and will be used to gain valuable insight into the mechanistic link between dieldrin exposure and the development of Parkinson's disease.

The hypothesis of this study is that dieldrin interacts with proteins noncovalently, due to the hydrophobic nature of this compound. Even though dieldrin contains an epoxide, an electrophilic functional group generally reactive towards nucleophiles, this epoxide is remarkably stable, and predicted to be unreactive to nucleophilic sites on proteins. It is also predicted that the structure activity relationship of dieldrin in a dopaminergic cell is comparable to the one defined in insects. In order to test this hypothesis, the reactivity of dieldrin towards protein nucleophiles was investigated. In addition, the localization of dieldrin in a dopaminergic cell was then monitored using GC/MS to quantify this organochlorine in various parts of the cell. Lastly, the relationship between dieldrin's chemical structure and its toxicity in a cellular dopaminergic model was evaluated. To determine dieldrin's structure-activity relationship, three aspects of its structure were assessed, 1. The relative 3-dimensional orientation and presence of the methano bridge, 2. The identity of polar moiety (olefin,

cis diol, or epoxide) located opposite of the chlorine atoms, and 3. The combined effect of the 3-dimensional orientation and polar group, Figure 5.1.

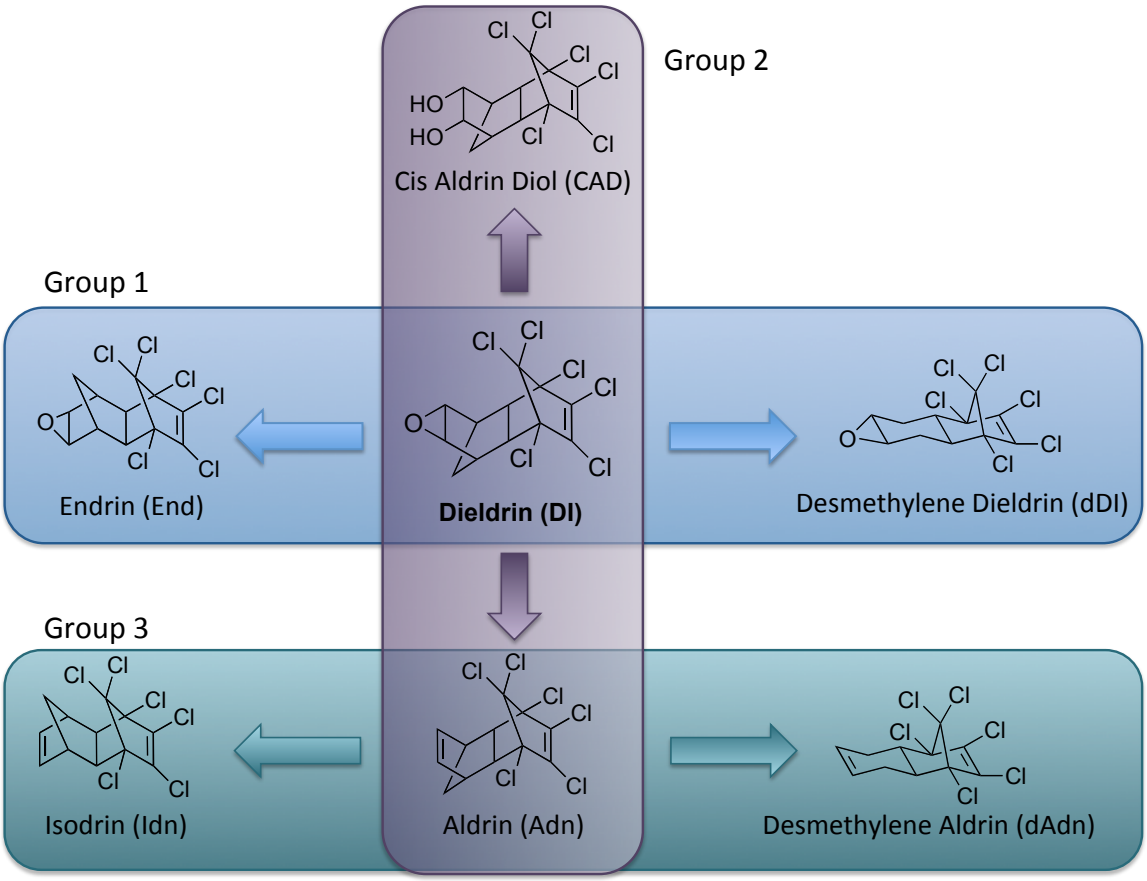


Figure 5.1. Structures and groupings of dieldrin analogs

A total of six analogs were compared to dieldrin, two for each category, as depicted in Figure 5.1. The effects of each compound on dopaminergic cells (PC6-3) were then assessed, and the corresponding structure-activity relationship defined. Despite the research looking at the structural effects of dieldrin on insect toxicity (*202-204*), the structure-activity relationship of dieldrin is not well-defined (*205*), particularly for mammalian systems. The completion of this study will provide information critical

for understanding the interaction of dieldrin with proteins within a dopaminergic neuron, as it relates to Parkinson's disease.

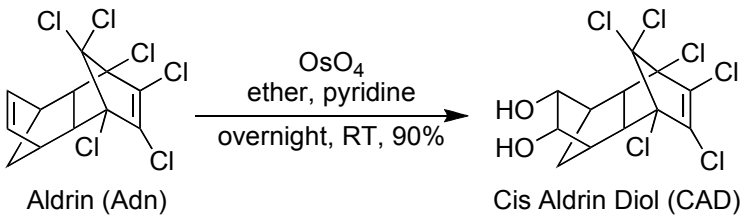
Experimental Procedures

Chemicals

Dieldrin, aldrin, endrin, and isodrin were purchased from Chem Service (West Chester, PA). All other reagents, unless otherwise noted, were purchased from Sigma Aldrich (St. Louis, MO) without further purification. Organochlorine analogs were named based on the von Baeyer/IUPAC system for polycyclic compounds and as discussed previously (*206*).

Cis Aldrin Diol (1,8,9,10,11,11-hexachloro-4,5-(*exo*)*cis*-dihydroxy-2,3-7,6-*endo*-2,1-7,8-*exo*-tetracyclo[6.2.1.$1^{3,6}$.$0^{2,7}$]dodec-9-ene)

Cis aldrin diol (CAD) was synthesized using a method adapted from literature (*207, 208*). Briefly, aldrin (86 mg, 0.24 mmol) was combined with dry ether (6.4 mL), pyridine (0.15 mL), and osmium tetroxide (50 mg, 0.20 mmol). The reaction was stirred for 2h at room temperature, and then let stand overnight in the dark. The ether was evaporated with nitrogen, giving a brown residue that was then dissolved in dichloromethane (10 mL) and stirred vigorously overnight with a solution of water (6.5 mL), potassium hydroxide (71 mg, 1.3 mmol), and *D*-mannitol (630 mg, 3.46 mmol). The dichloromethane layer was removed, washed with water, and dried with sodium sulfate, giving the product in 90% yield. ^{1}H NMR (DMSO-d6): δ 1.12 (d, 1H, CH_2), 1.60 (d, 1H, CH_2), 2.06 (m, 2H, CH), 2.60 (m, 2H, CH), 3.57 (m, 2H, CH), 4.93 (m, 2H, OH); ^{13}C NMR (DMSO-d6): δ 28.3, 43.0, 51.7, 73.6, 81.0, 104.7, 130.8; MS (EI) *m/z* calcd for $C_{12}H_{10}Cl_6O_2$ 395.9, found *m/z* 396.1.



Scheme 5.1. Synthesis of cis aldrin diol

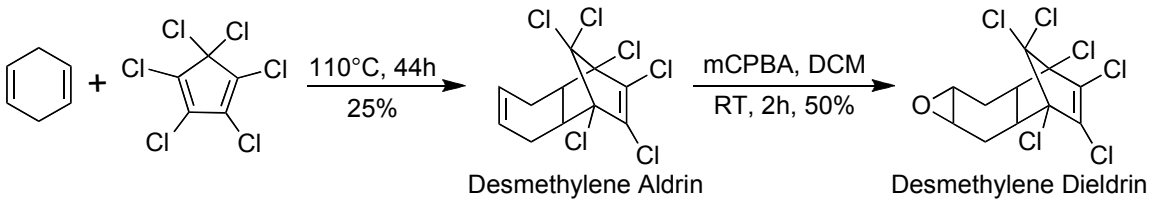
Desmethylene Aldrin (1,8,9,10,11,11-hexachloro-2,3-7,6-*endo*-tricyclo[6.2.1.0^{2,7}]undeca-4,9-diene)

Adapted from literature methods (*202, 208-210*), hexachlorocyclopentadiene (1.0 g, 0.004 mol, 0.6 mL) and 1,4-cyclohexadiene (1.3 g, 0.016 mol, 1.51 mL) were placed under argon and stirred at 110°C for 44 h. Product was purified by column chromatography on silica gel with hexanes as mobile phase, resulting in a 25% yield. ^{1}H NMR ($CDCl_3$): δ 1.90-1.99 and 2.35-2.44 (m, 4H, CH_2), 2.98-3.09 (m, 2H, CH), 5.86 (m, 2H, HC=CH); ^{13}C NMR ($CDCl_3$): δ 21.9, 45.8, 82.5, 103.2, 126.7, 131.6; MS (EI) *m/z* calcd for $C_{11}H_8Cl_6$ 349.9, found *m/z* 349.9.

Desmethylene Dieldrin (1,8,9,10,11,11-hexachloro-4,5-epoxy-2,3-7,6-*endo*-tricyclo[6.2.1.0^{2,7}]undec-9-ene)

Adapted from literature methods (*202, 208, 210, 211*), 3-chloroperbenzoic acid (*m*CPBA) (0.08 g, 0.5 mmol) in 2.0 mL of dichloromethane was added dropwise to a desmethylene aldrin (0.1 g, 0.3 mmol) in 2.0 mL dichloromethane, and stirred at room temperature for 2 h. Reaction mixture was extracted twice with water. The organic layer was then dried with magnesium sulfate and the product purified by column chromatography on silica gel with a mobile phase of 4:1 hexanes to acetone, resulting in a 50% yield. ^{1}H NMR ($CDCl_3$): δ 1.59 - 1.67 and 2.40 - 2.48 (m, 4H, CH_2), 2.91-2.99 (m, 2H,

CH), 3.20 - 3.24 (m, 2H, CH-O); ^{13}C NMR ($CDCl_3$): δ 22.2, 42.2, 49.1, 82.3, 102.8, 132.0; MS (EI) *m/z* calcd for $C_{11}H_8Cl_6O$ 365.9, found *m/z* 365.9.



Scheme 5.2. Synthesis of desmethylene analogs

Thiol Reactivity

Thiol reactivity of dieldrin was determined using Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)) and a procedure adapted from the literature (*88, 94*). N-acetyl Cys (100 µM) was incubated with each compound (0-2.5 mM) overnight at 37°C in DMSO. DTNB (0.5 mM) in DMSO was added along with 50mM sodium phosphate buffer, pH 7.4 (25% v/v), and absorbance monitored at 412 nm using a Molecular Devices SpectraMax plate reader. The free thiol concentration in each well was determined by comparing the absorbance to a standard curve using *N*-acetyl Cys, Figure 5.2A.

Primary amine reactivity with HPLC analysis

The reactivity of dieldrin with primary amines was assessed using *N*-acetyl Lys. Dieldrin (0-100 µM in DMSO) was incubated with *N*-acetyl Lys (100 µM in DMSO) at 37°C for 24h. The concentration of dieldrin was monitored via an HPLC method adapted from Mowafy et al. (*212*). Briefly, the samples were analyzed on an Agilent 1100 Series Capillary HPLC with a Phenomenex C18 Jupiter column (5 µ, 300 Å, 150 x 1.0 mm) using isocratic conditions of water (25%) and methanol (75%), a flow rate of 50 µL/min, and

the absorbance measured via photodiode array at 214 and 234nm. Differences in peak area between dieldrin incubated with and without *N*-acetyl Lys would indicate reactivity between these two compounds, Figure 5.2B.

Cell Culture

PC6-3 cells, cultured in RPMI1640 medium (GIBCO, Invitrogen, Carlsbad, CA) supplemented with heat-inactivated horse serum (10%) (HyClone, Thermo Scientific, Rockford, IL), fetal bovine serum (5%) (GIBCO), penicillin (10 IU/mL), and streptomycin (10 mg/mL), were grown in 100 mm^2 tissue culture dishes at 37°C with 5% CO_2. Cells (3 x 10^4 cells/well) were seeded into six-well plates for four days at 37°C with 5% CO_2. The cells were then differentiated with 2.5s nerve growth factor (NGF) (BD Biosciences, Bedford, MA) at 50 ng/mL for 4 days. Images of undifferentiated and differentiated PC6-3 cells are shown in Figure 3A and B.

Treatment of Cells with Dieldrin and analogs

Media was removed from cells and replaced with HEPES-buffered media containing 115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, 1.0 mM NaH_2PO_4, and 15 mM HEPES (pH 7.4). The cells were treated with compound (dieldrin, aldrin, endrin, isodrin, cis-aldrin diol, desmethylene aldrin, or desmethylene dieldrin) at 0 – 900 μM in dimethylsulfoxide (DMSO, 0.6%), for 4 h.

Cell fractionation and GC/MS Analysis

Upon 4h treatment of the PC6-3 cells with dieldrin (0-300 μM), the HEPES-buffered media used for treatment was collected (extracellular fraction) and then lysis buffer (10 mM K_2PO_4 with 0.1% Triton X-100 (v/v)) was added to each well. The cell lysate was collected and sonicated for 5 min using a sonicator bath. The membrane/lipid fraction was separated from the cytosol and lysed organelles (intracellular fraction) using centrifugation at 1000 x *g* for 10 min at 4°C. The pellet (membrane/lipid fraction)

was resuspended in phosphate-buffered saline and all samples were stored at -70°C pending analysis.

Prior to extraction, each sample was spiked with an internal standard (endrin, 1 μg/mL). The organochlorines were extracted three times using hexanes with a volume equal to the sample volume. If an emulsion formed, one part ethanol was added to the sample. The solvent was removed from the organic layer using nitrogen gas, and then dissolved in ethyl acetate. Dieldrin and endrin were separated using a Thermo Voyager coupled with a single quadrapole mass spectrometer. Flow of the gas carrier was 20mL/min and the temperature was 50°C for 2 min, increased by 10°/min to 280°C, and then held for 10 min. The mass spectrometer monitored ionization at 79 *m/z* and 263 *m/z*, and the samples were run by Lynn Teesch at the High Resolution Mass Spectrometry Facility at the University of Iowa. Representative chromatograms (total ion chromatograms and extracted ion chromatograms) are shown in Figures 5.4 and 5.5. Peak area quantification was completed using Xcaliber 2.0. Dieldrin peak area at 263 *m/z* was first normalized to the internal standard (endrin at 263 *m/z*) and then compared to a standard curve. The amount of dieldrin (μg) in each of the fractions is reported as the mean ±SD, n = 3, in Figure 5.6.

Mitochondrial Viability

The effect of each compound on mitochondrial viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. This involves the conversion of the yellow, MTT tetrazolium to a purple formazan salt by mitochondrial reductases (e.g. succinate dehydrogenase). After a 4 h treatment of the cells with each compound, the cells were incubated for 1.25 h at 37°C with MTT (0.5 mg/mL). The HEPES-buffered media was then removed from each well and centrifuged at 10,000 x *g* for 5 min. This pellet and the formazan salt remaining in each well were dissolved in DMSO and combined. The absorbance was then measured at 570 nm and

650 nm using a Molecular Devices Spectra-Max plate reader. To determine the percent control, the absorbance at 650 nm was subtracted from the absorbance at 570 nm, this value was then divided by the normalized value obtained from the control well and multiplied by one hundred, Figures 5.7 and 5.8.

IC_{50} Value Calculations

In order to calculate the IC_{50} values for each compound based on their inhibition of mitochondrial function, the dose-response data was normalized between 0 and 100%, and graphed against the log[inhibitor], Figure 5.9. A nonlinear curve fitting was applied based on log[inhibitor] vs normalized response with variable slope. The concentration at which 50% normalized activity was observed is reported in Table 5.1 as the IC_{50} value ± SE.

Cytotoxicity Assay

The cytotoxicity of each compound was determined using the Cytotoxicity Detection kit^{plus} (LDH) (Roche Applied Science, Mannheim, Germany), according to the manufacturer's specifications. Briefly, for each experiment, a high control and a low control were used for reference with each treatment set. The high control consisted of cells incubated with 0.6% DMSO, and then for the last 15 min of the experiment, lysis buffer, as provided by the manufacturer. The low control was a well incubated with 0.6% DMSO, no lysis buffer. At 4h, an aliquot of media was removed from each well and incubated with the reaction mixture provided for 20 min in the dark at RT. The reaction was then stopped and the absorbance at 492 and 690 nm measured using a Molecular Devices Spectra-Max plate reader. The value for each well was determined by subtracting the 690nm absorbance from the absorbance at 492 nm. In order to determine the percent cytotoxicity, the value obtained for the low control was subtracted from each treated well, then divided by the difference between the high

control and the low control, Figures 5.10 and 5.11. The compounds did not interfere with the absorbance readings for this assay.

Lactate Dehydrogenase Inhibition

The ability of each compound to inhibit lactate dehydrogenase was evaluated by incubating rabbit L-LDH (0.5 U/mL) with various concentrations of each compound (0-900 µM, 0.6% DMSO) for 30min in HEPES-buffered media. At this point the reaction mixture containing the necessary enzyme substrates and cofactors were added and incubated in the dark at room temperature for 5 h. Absorbance at 492 and 690 nm were then measured and the normalized absorbance determined by subtracting the 690 nm absorbance from the absorbance at 492 nm, Figure 5.12.

Statistics

All statistics and curve fittings were calculated using GraphPad Prism version 5.0c (GraphPad Software, San Diego, CA). For the MTT and LDH results, the difference between each pair of treated groups was analyzed using one-way ANOVA with Newman-Keuls post test. Significance from control for the thiol and amine reactivity was determined using a two-tailed *t* test ($p < 0.05$).

Results

Amino acid reactivity of dieldrin

In an effort to better understand the interaction of dieldrin with target protein(s), dieldrin's ability to covalently modify nucleophilic amino acids (Cys and Lys) was investigated. The thiol reactivity of dieldrin was assessed using N-acetyl cysteine and the concentration of free thiols monitored using DTNB. An observed decrease in free thiol concentration would indicate covalent modification of Cys by dieldrin. No such decrease was observed. It may be concluded that dieldrin does not react with free thiols under the conditions tested, Figure 5.2A.

The ability of dieldrin to react with primary amines was also investigated. In this case, dieldrin was incubated with and without N-acetyl Lys with the concentration of dieldrin measured using HPLC. Again, no significant decrease in dieldrin concentration was observed upon addition of N-acetyl Lys, indicating there was no reaction under these conditions (Figure 5.2B). These results indicate dieldrin does not covalently modify target proteins, and that any observed protein interactions are most likely non-covalent.

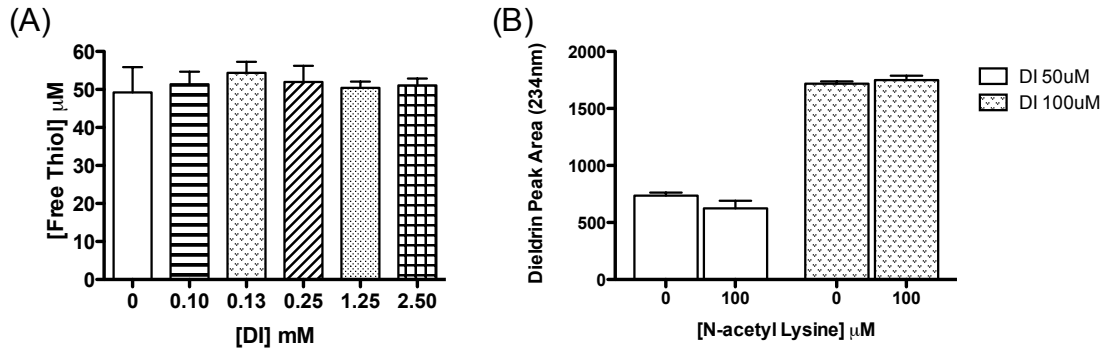
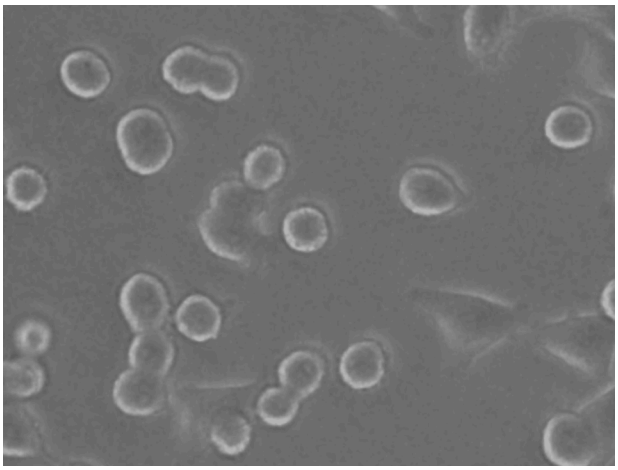


Figure 5.2. Amino acid reactivity of dieldrin. (A) Reactivity of dieldrin (0-2.5 mM) with *N*-acetyl Cys (50 μM), monitored using DTNB (500 μM) ([free thiol] μM ±SD (*n* = 3). (B) Reactivity of dieldrin (0-100 μM) with *N*-acetyl Lys (0-100 μM). [Dieldrin] monitored by HPLC (Peak Area ± SD, n = 3). No significant change from control ($p > 0.1$) was observed in A or B, using a two-tailed t test.

Localization of Dieldrin in Dopaminergic PC6-3 Cells

In order to investigate the movement of dieldrin in a system relevant to Parkinson's disease and compare the toxicity of each dieldrin analog, experiments were conducted in differentiated PC6-3 cells. These cells are a subline of the established cellular model for dopaminergic neurons, PC12 cells. PC6-3 cells, upon differentiation with nerve-growth factor (NGF), assume a neuronal phenotype (*213*) and have important cellular processes relevant to dopaminergic neurons (*214-216*). Images of PC6-3 cells before and after differentiation are shown in Figure 5.3.

(A)



(B)

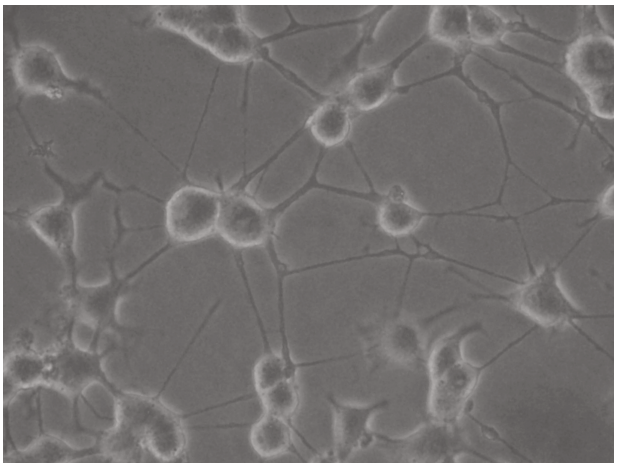


Figure 5.3. (A) undifferentiated PC6-3 cells, (B) PC6-3 cells after differentiation with nerve growth factor (NGF).

Upon treatment with dieldrin (0 - 300 μM), the cells were fractionated giving extracellular, membrane/lipid, and intracellular fractions. Each sample was then spiked with an internal standard (endrin) and extracted prior to quantification with GC/MS. Representative chromatograms of the extract of a cellular fraction are shown in Figures 5.4 and 5.5. The peak area of dieldrin in each sample was normalized to the internal

standard and then compared to a standard curve in order to determine the amount of dieldrin in each sample.

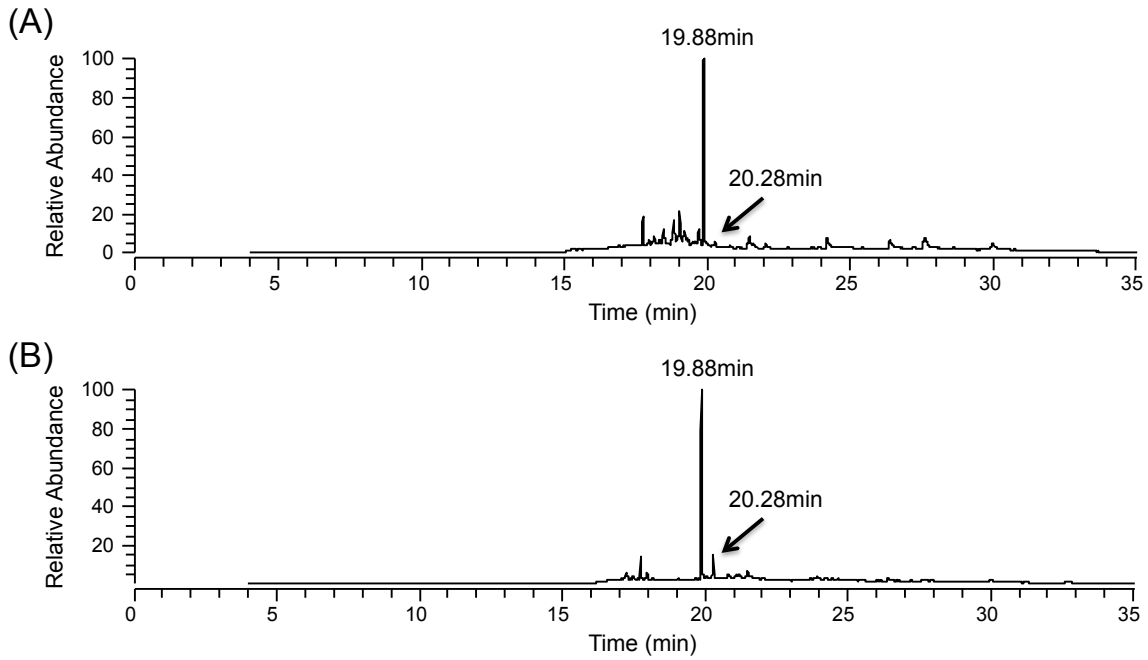


Figure 5.4. Representative chromatograms depicting dieldrin (19.88 min) and endrin (20.28 min) (A) total ion chromatogram and (B) extracted ion chromatogram at 263 *m/z*.

As depicted in Figure 5.6A, the amount of dieldrin in the extracellular fraction increases with the initial dose of dieldrin. A similar trend is also observed in the membrane/lipid fraction, Figure 5.6B. When the amount of dieldrin in the intracellular fraction was quantified, Figure 5.6C, the amount of dieldrin increased through 50 µM treatment, but did not increase to the same extent as observed in the other fractions, even with 300 µM dieldrin. This indicates there may be a limiting factor contributing to the leveling out effect observed in the intracellular fraction. These results indicate that dieldrin is capable of entering the PC6-3 cells, but most of the dieldrin is found in the membrane/lipid portion of the cells, Figure 5.6C.

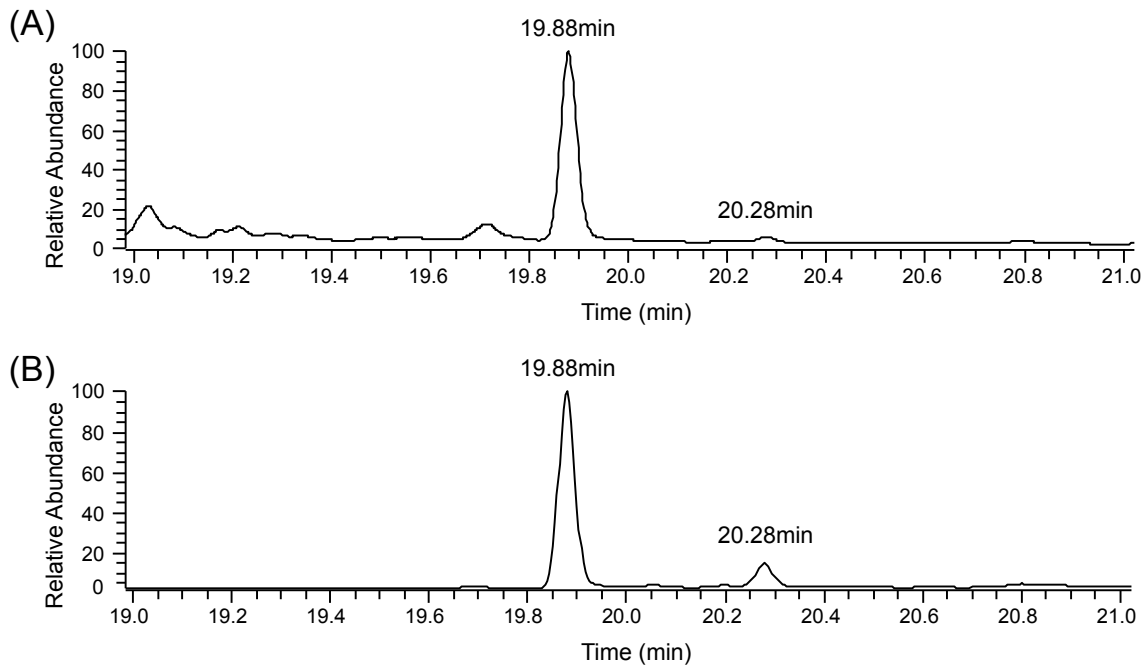


Figure 5.5. Representative chromatograms (19-21 min) depicting dieldrin (19.88min) and endrin (20.28 min) (A) total ion chromatogram and (B) extracted ion chromatogram at 263 *m/z*.

Dieldrin Analogs

Six dieldrin analogs were used to assess the structure-activity relationship of dieldrin as it relates its toxicity in dopaminergic cells. The analogs were chosen based on their structure, allowing for the assessment of three chemical characteristics, 1. The importance of the 3-dimensional orientation and presence of the methano bridge (endrin and desmethylene dieldrin), 2. The effect of the polar group opposite of the chlorine atoms (aldrin and cis aldrin diol), and 3. The interplay between both the polar moiety and the 3-dimensional orientation (isodrin and desmethylene aldrin).

The first group of dieldrin analogs addressed the 3-dimensional orientation of dieldrin by comparing it to the effects of its isomer (endrin) and desmethylene dieldrin. Endrin is identical to dieldrin, except that the relative orientation of the methano bridge and chlorinated bridge is opposite. This compound was also used as a pesticide, with its

insect toxicity only slightly less than that of dieldrin (*202*). Endrin is listed as #41 on the CERCLA priority list of hazardous substances (*26*). The desmethylene analog was used to assess the importance of this bridge and the rigidity of the parent compound. This compound completely lacks the unsubstituted, methano bridge, rendering it much more flexible than dieldrin. In insects, this compound showed very minimal toxicity, and was never used as a pesticide (*202, 203*).

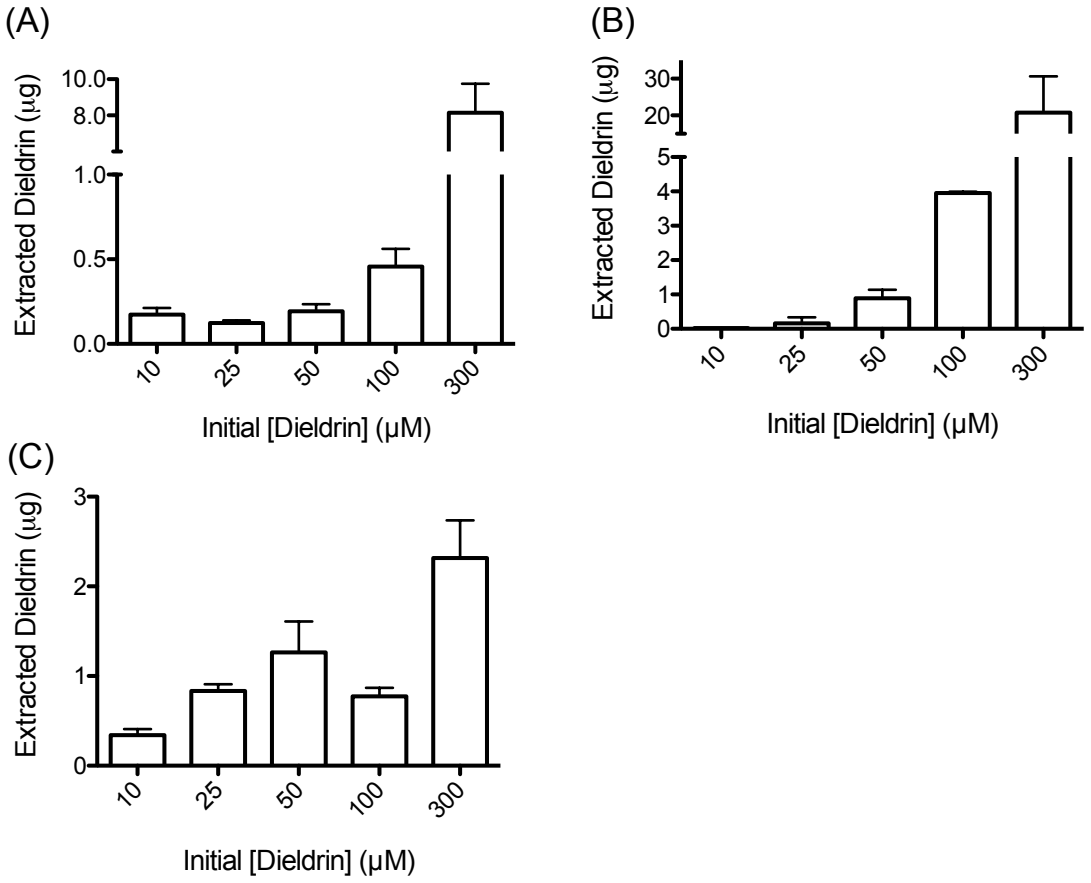


Figure 5.6. Amount of dieldrin (µg), normalized to internal standard and standard curve, from hexane extraction of cellular fractions from cells treated with 0 - 300 µM dieldrin. Each bar represents the concentration of dieldrin (µg ± SD, *n* = 3). (A) extracellular fractions, (B) membrane/lipid fractions, (C) extracellular fractions.

Dieldrin contains an epoxide opposite of the chlorine atoms, whereas the compounds in Group Two contain either an olefin (aldrin) or a cis diol (cis aldrin diol). Aldrin was widely used as a pesticide around the same time as dieldrin, and is slightly less toxic than dieldrin in various species of insects (*202, 217*). Aldrin's half-life is not as long as dieldrin's, mostly due to increased volatility and rate of metabolism in the environment and in mammals, as compared to dieldrin (*218*). Despite these characteristics limiting the adverse effects of aldrin exposure, it is still listed as #24 on the CERCLA priority list of hazardous substances (*26*). Cis aldrin diol is a metabolite of dieldrin that can then be epimerized to the trans diol in mammals (*219*). It was found not be as toxic in insects as dieldrin (*220*). Due to the environmental and metabolic relevancy, aldrin and cis aldrin diol were chosen for this study. The structural differences in these compounds from dieldrin were also useful to assess the importance of hydrogen bonding and polarity of the epoxide in dieldrin toxicity.

The third group aimed to assess the combined effects of altering the polar moiety (epoxide to olefin) along with the 3-dimensional structure. The two compounds in this group are based on aldrin, with isodrin being its isomer, and desmethylene aldrin lacking the methano bridge found in aldrin and dieldrin. Isodrin was never used commercially as a pesticide, but in *Musca domestica* it is only slightly less toxic than endrin (*202*). The toxicity profile of desmethylene aldrin has not yet been investigated.

Mitochondrial Viability

To assess the structure-activity relationship of dieldrin, the viability of the mitochondria in the PC6-3 cells was monitored after 4 h treatment with various concentrations of each dieldrin analog, Figures 5.7 and 5.8. The concentrations used in this study (10-900 μM) are slightly higher than the concentrations detected in the brains of Parkinson's patients (2.4 μM, based on reported values of μg of dieldrin per g of lipid in the striatum) (*139, 182-184*), but were chosen to represent the full range of

mitochondrial dysfunction in this model system (< 20-100% mitochondrial function). This range was required to calculate the IC_{50} values, which were used for accurate comparisons between compounds, and were also comparable to the concentrations used in similar studies (*186, 188, 221-225*).

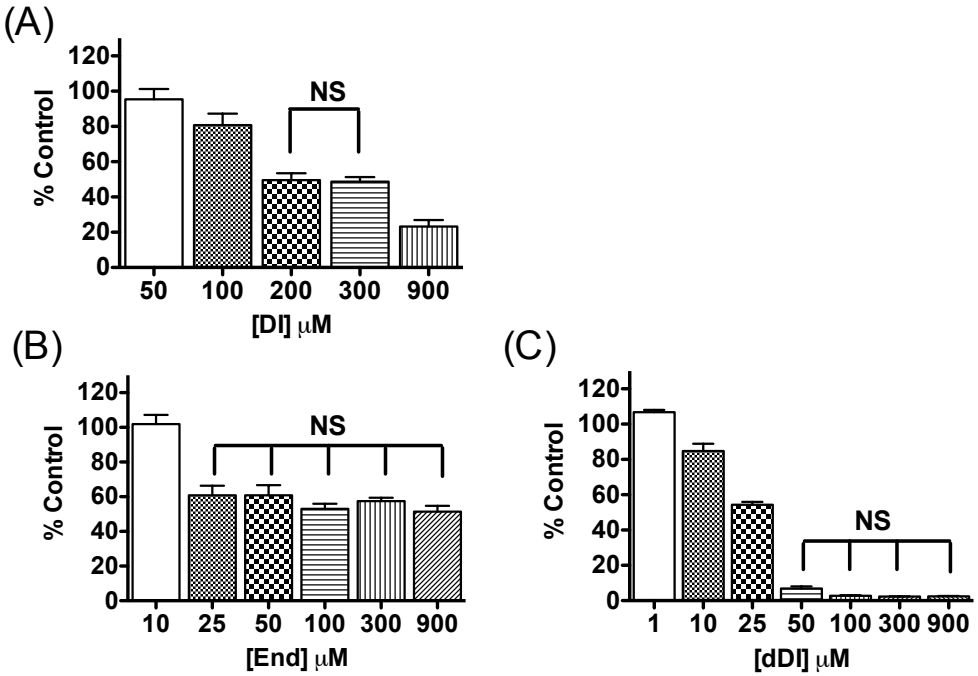


Figure 5.7. Mitochondrial activity in PC6-3 cells treated with dieldrin or group 1 compounds (%control ± SD, n = 4-6), obtained from MTT assay after 4h incubation with (A) dieldrin, (B) endrin, (C) desmethylene dieldrin. The difference between each pair of treated groups is significant ($p < 0.05$) using one-way ANOVA with Newman-Keuls post test unless otherwise noted (NS).

Mitochondrial function was quantified using a colorimetric agent dependent upon the activity of cellular dehydrogenases such as succinate dehydrogenase, a critical mitochondrial enzyme. A decrease in percent control (values < 100%) indicates impairment of mitochondrial function. Results are depicted for dieldrin and group 1 analogs in Figure 5.7, with groups 2 and 3 shown in Figure 5.8. All of the compounds,

except for endrin, displayed a dose-dependent decrease in mitochondria function in the PC6-3 cells after 4 h. Based on these results, IC_{50} values were calculated in order to easily compare the potency of each compound, Table 5.1.

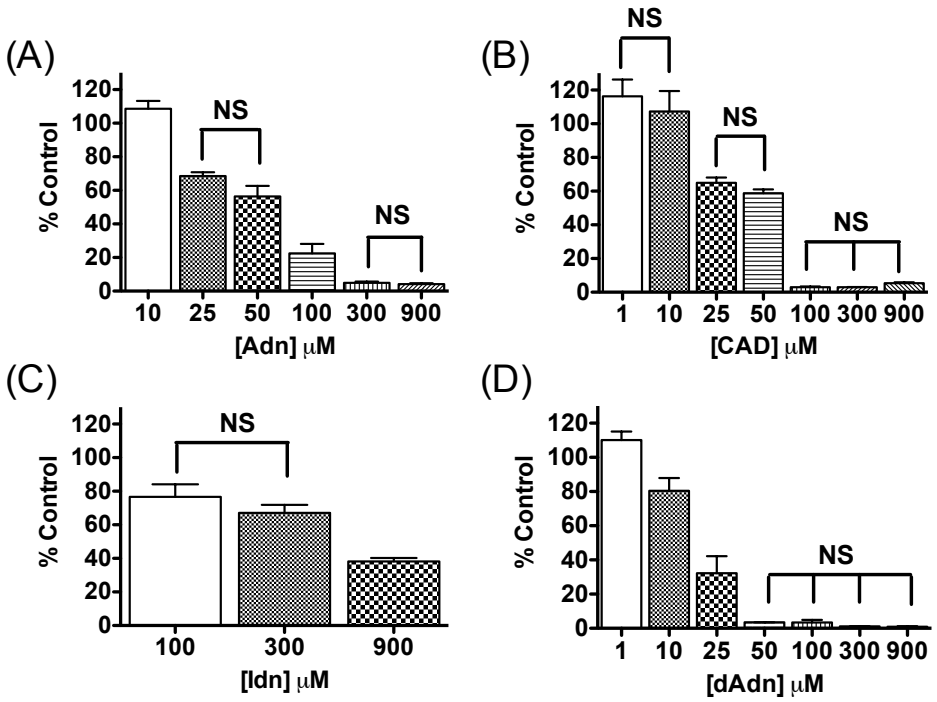


Figure 5.8. Mitochondrial activity in PC6-3 cells treated with compounds in groups 2 and 3 (%control ± SD, n = 4-6), obtained from MTT assay after 4 h incubation with (A) aldrin, (B) cis aldrin diol, (C) isodrin, and (D) desmethylene aldrin. The difference between each pair of treated groups is significant ($p < 0.05$) using one-way ANOVA with Newman-Keuls post test unless otherwise noted (NS).

For comparison with each analog, the parent compound dieldrin showed a dose-dependent increase in mitochondrial dysfunction with an IC_{50} value of 293 μM. When the 3-dimensional orientation was assessed using the group 1 analogs, it was found that the isomer, endrin did not exhibit a dose-dependent increase in toxicity, therefore no IC_{50} value could be calculated. This implies that there may be a disruption in the

localization and processing of this analog, and it requires further investigation. When the methano bridge was removed (desmethylene dieldrin), yielding a more flexible structure, the compound was substantially more toxic than dieldrin with an IC_{50} value of 22 μM.

When dieldrin was compared to group 2, compounds with changes to the polar moiety, it was found that the substitution of the epoxide for an olefin or a cis diol both resulted in decreased mitochondria viability (IC_{50} values of 46 and 36 μM respectively), as compared to dieldrin. Such a result implies that a moiety capable of being a hydrogen bond donor (i.e. cis diol) enhances the toxicity, as does the presence of an olefin.

Table 5.1. IC_{50} values (μM ± SE) for each compound.

Compound	**IC_{50} (μM) ± SE**
Dieldrin	293 ± 1.08
Endrin	NA
Desmethylene Dieldrin	22.3 ± 1.05
Aldrin	46.4 ± 1.08
Cis Aldrin Diol	36.9 ± 1.11
Isodrin	578 ± 1.22
Desmethylene Aldrin	15.8 ± 1.05

Note: IC_{50} values are based on the concentration of each inhibitor at 50% of the normalized mitochondrial viability.

The last group of compounds takes into account the presence of the olefin, already shown to enhance toxicity, as well as the 3-dimensional orientation of the compound. Looking at the isomer of aldrin (isodrin), there was very little toxicity observed (IC_{50} value was almost 600 μM). When the desmethylene analog of aldrin was

assessed, it resulted in the most substantial mitochondria dysfunction of all of the analogs with an IC_{50} value of 16 μM. These results imply that the presence of the olefin augments the toxicity of dieldrin, and when this modification is coupled with the removal of the methano bridge, the toxicity is enhanced even more.

Release of Lactate Dehydrogenase

The MTT assay is used to monitor mitochondrial function, and because mitochondria are responsible for many critical cellular processes, i.e. energy production, if this organelle is rendered inactive it can indicate cell death. However, for compounds that are known to target the mitochondria, i.e. dieldrin, a decrease in mitochondrial viability is not always a good predictor for cell death. In this case, an alternative assessment is needed to verify that the loss of mitochondrial function is also indicative of cell death. In order to accomplish this, the release of lactate dehydrogenase, a cytosolic enzyme, was monitored spectrophotometrically, Figures 5.10 and 5.11. This assay relies upon the increased membrane permeability (corresponding to release of cytosolic components including lactate dehydrogenase) as an indicator of cell death.

Just as in the MTT assay, PC6-3 cells were treated for 4h with various concentrations of each dieldrin analog. The release of lactate dehydrogenase was then determined by quantifying the enzyme activity in the extracellular media from treated wells. These values were compared to a vehicle control (baseline level of lactate dehydrogenase release) as well as a high control (chemically lysed cells, and maximum possible amount of lactate dehydrogenase release). Based on these results the percent cytotoxicity was calculated and then graphed in Figures 5.10 (dieldrin and group 1 analogs) and in 5.11 (groups 2 and 3). In this experiment, the larger the bar, the more lactate dehydrogenase released, indicating cytotoxicity. For each of the compounds a dose-dependent increase in %cytotoxicity was observed, except for endrin, which showed no cytotoxicity, even at high concentrations (900 μM).

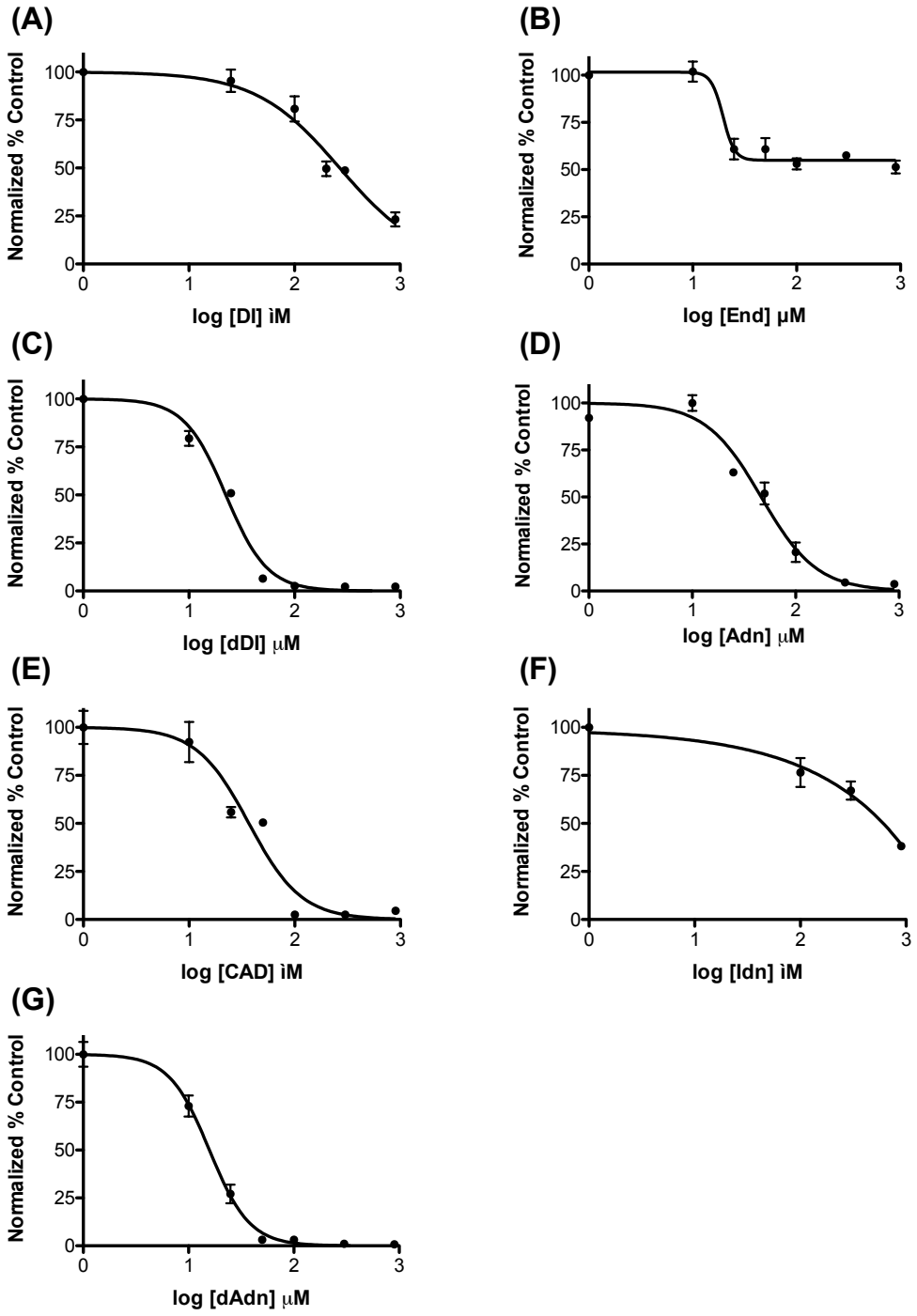


Figure 5.9 Normalized mitochondrial activity (0-100%) obtained from MTT assay with (A) dieldrin, (B) endrin, (C) desmethylene dieldrin, (D) aldrin, (E) cis aldrin diol, (F) isodrin, and (G) desmethylene aldrin. A nonlinear curve fitting was applied based on log[inhibitor] vs normalized response with variable slope.

The results from the lactate dehydrogenase experiment correlate well to those observed using MTT to monitor mitochondrial function and in a previous study (*186*). The relative order of potency between each analog was also comparable, with the desmethylene analogs being the most potent. This was then followed by both compounds in group 2 (aldrin and cis aldrin), then dieldrin, and isodrin and endrin were both the least potent. This indicates that the affect on the mitochondria was indicative of cell death, and was not specific to mitochondrial impairment.

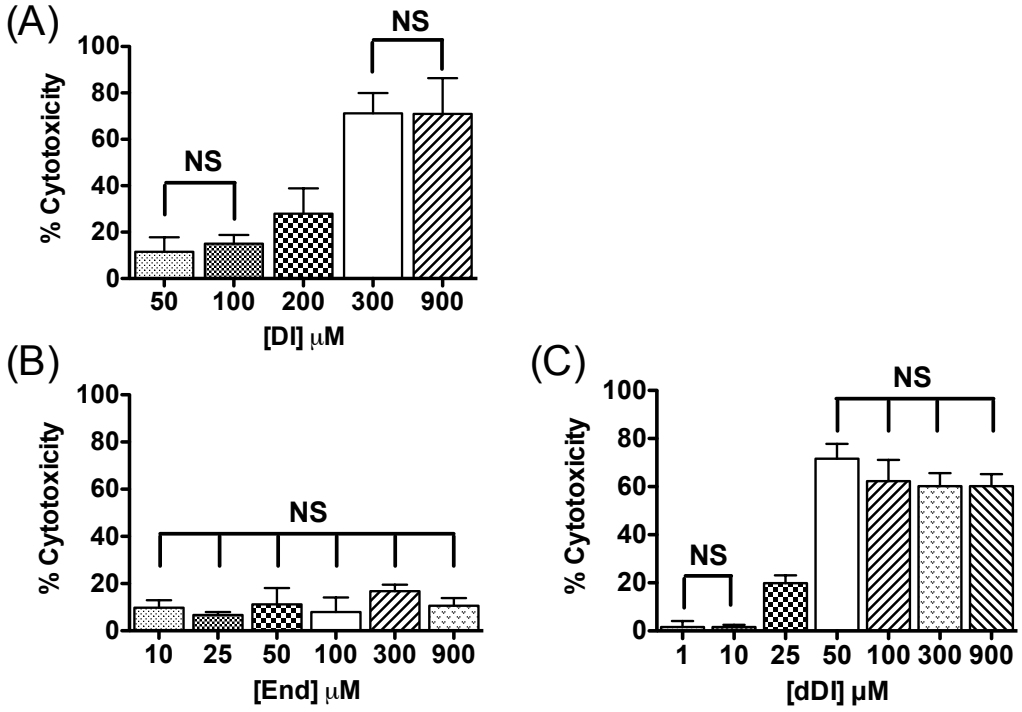


Figure 5.10. Lactate dehydrogenase release from PC6-3 cells after 4 h incubation with dieldrin or group 1 compounds (%cytotoxicity ± SD, n = 4-6). (A) dieldrin, (B) endrin, (C) desmethylene dieldrin. The difference between each pair of treated groups is significant ($p < 0.05$) using one-way ANOVA with Newman-Keuls post test unless otherwise noted (NS).

In the lactate dehydrogenase results, a deviation from the dose-dependent trend was observed at higher concentrations, particularly with the more toxic compounds. Each compound showed increased release of lactate dehydrogenase until the concentration at which roughly 65% cytotoxicity was achieved. At concentrations that would be expected to be greater than 65% cytotoxicity, the values either remained at the same level (desmethylene dieldrin), or started to decrease (cis aldrin diol). This deviation from the dose-dependent trajectory was not seen with the MTT results. A number of experiments were completed to understand the cause of this effect. It was determined that the compounds did not absorb at the wavelengths monitored or react with the reagents used in this experiment. Since this experiment was based on enzyme activity, the compounds were then tested for inhibition of the enzyme, lactate dehydrogenase, Figure 5.12. It was determined that each compound, particularly at higher concentrations, caused inhibition of this enzyme.

When each compound was incubated with rabbit L-lactate dehydrogenase, at higher concentrations, a decrease in enzyme activity was observed. It can be inferred that when the cells were treated with higher concentrations of the dieldrin analogs, the outer membrane of the cells was disrupted, causing a release of lactate dehydrogenase. However, due to the substantial amount of the dieldrin analog still remaining in the extracellular media or that was released from the cell during apoptosis, the lactate dehydrogenase was inhibited by the compound. This resulted in the decreased enzyme activity corresponding to the observed deviation from the dose-dependent increase in cytotoxicity for each analog at higher concentrations (Figures 5.10 and 5.11). Therefore, while it may appear that compounds such as desmethylene aldrin and cis aldrin diol lost potency at higher concentrations, this was actually a result of enzyme inhibition interfering with the assay.

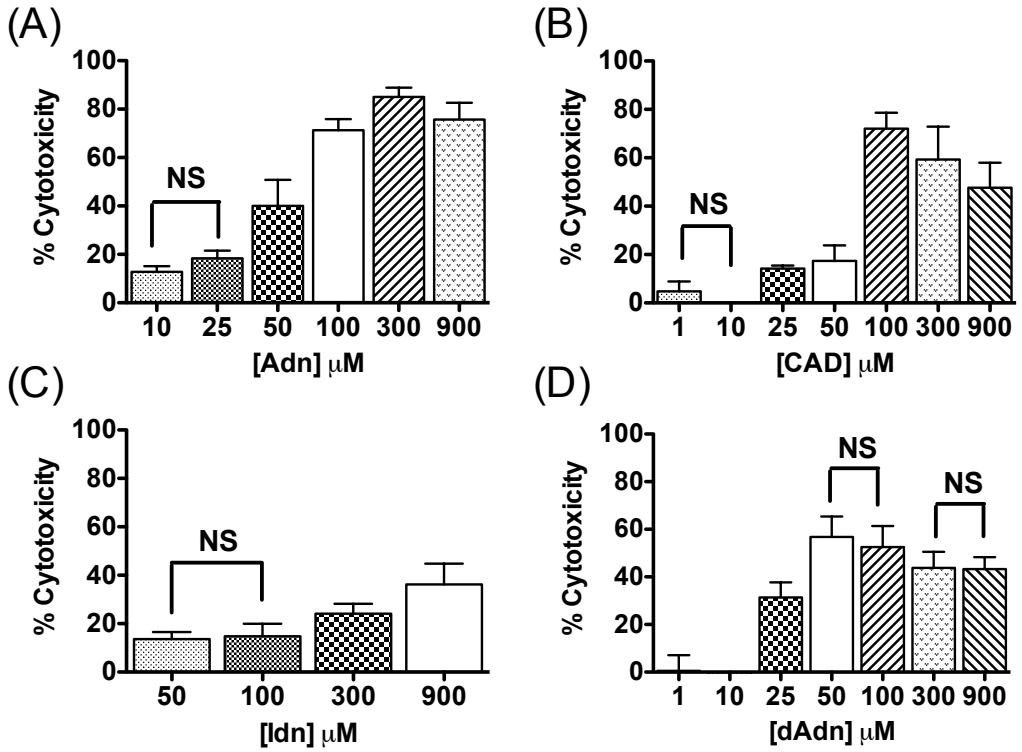


Figure 5.11. Lactate dehydrogenase release from PC6-3 cells after 4h treatment with groups 2 and 3 compounds (%cytotoxicity ± SD, n = 4-6). (A) aldrin, (B) cis aldrin diol, (C) isodrin, and (D) desmethylene aldrin. The difference between each pair of treated groups is significant ($p < 0.05$) using one-way ANOVA with Newman-Keuls post test unless otherwise noted (NS).

Discussion

The goal of this study was to analyze the protein reactivity, localization, and structure-activity relationship of dieldrin in a Parkinson's disease model. This information is important for determining the mechanism of toxicity as it relates to the development of Parkinson's disease. The model system, differentiated PC6-3 cells, was chosen because of its relevancy to this neurodegenerative disease that selectively affects dopaminergic neurons. The correlation between Parkinson's disease and dieldrin has been well documented (*21, 22, 105, 139, 182-184, 186-188*), however the cellular

interactions and structural features of dieldrin contributing to this pathogenesis are unknown.

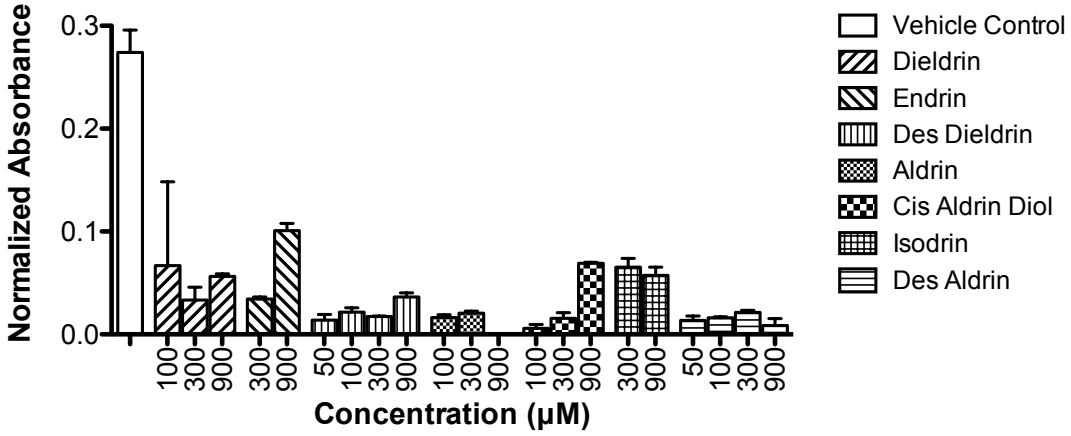


Figure 5.12. Direct inhibition of lactate dehydrogenase upon incubation with each organochlorine. (normalized absorbance (492 nm – 690 nm) ± SD, n = 3).

The first portion of this research addressed the interaction of dieldrin with protein nucleophiles. Dieldrin contains an epoxide, a moiety that is typically electrophilic and highly reactive towards nucleophiles (*226*). It is conceivable that the nucleophilic sites on proteins (i.e. Cys or Lys residues) would be able to react with the epoxide, causing the opening of this three-membered ring and protein modification (*226*). Previous studies have shown that while dieldrin "binds" to proteins, such as those found in spinal cord homogenate from rats and cockroaches, and to a lesser extent, albumin; these interactions are most likely not covalent (*227, 228*). While isolated amino acids cannot completely model the unique environment of these amino acids in each possible protein target, they can provide a valuable reactivity profile. In the current study, it was determined that dieldrin was not capable of covalently modifying Lys or Cys residues,

supporting the previous assumptions of noncovalent interactions (Figures 5.2A and 5.2B). Even though dieldrin contains an epoxide, which is typically very reactive, this particular epoxide is stabilized due to the rigid polycyclic structure, thereby limiting the likelihood of nucleophilic attack by amino acids such as Lys and Cys.

The ability of dieldrin to enter the PC6-3 cells was investigated using cell fractionation and GC/MS. Based on the results, Figure 5.6, dieldrin was found to accumulate in the cell, particularly in the membrane and lipid fraction, Figure 5.6B. The amount of dieldrin in the intracellular fraction increased with increasing initial concentration through 50 μM. At higher concentrations, the amount of dieldrin in the intracellular fraction began to level out indicating decreased diffusion or saturation of a transporter. From the results in Figure 5.6, it may be concluded that dieldrin is capable of entering the PC6-3 cells, and accumulates readily in the membrane and lipid portion. This trend is most likely due to the lipophilicity of this organochlorine.

The effect of dieldrin on various aspects of mitochondrial activity has been previously investigated (*186, 188, 229, 230*). Bergen et al (*229*) demonstrated dieldrin's ability to disrupt respiration in rat liver mitochondria and inhibit electron transport. In a dopaminergic system similar to the PC6-3 cells (PC12 cells), dieldrin has been shown to cause a decrease in mitochondrial membrane potential and a reduction in the activity of mitochondrial dehydrogenase enzymes, using an MTT assay (*186*). They also assessed dieldrin's effect on cell viability using a Trypan blue experiment. In this previous report, dieldrin was shown to cause a 50% decrease in cell viability at 143 μM at 1 h in PC12 cells (*186*). Even with variations in cell line, cell preparation, and timeframe, this value is very comparable with the IC_{50} value of 293 μM obtained using the MTT assay in the current study.

The order of potency for each group of compounds on mitochondrial activity and the release of lactate dehydrogenase were very similar, with dieldrin and Group Two showing moderate toxicity, the isomers (endrin and isodrin) having little to no toxicity,

and the desmethylene compounds being the most toxic in the PC6-3 cells. These results demonstrate the importance of the three-dimensional structure in the potency of the cyclodiene pesticides in a dopaminergic model.

This structure-activity relationship differs significantly from the structural features found to be important for insect toxicity, in opposition to the initial hypothesis. The toxicities of many organochlorines have been assessed in various species of insects, including *Musca domestica* (house fly), *Anopheles stephensi* (mosquito), *Glossina austeni* (tsetse fly), and *Periplaneta americana* (American cockroach) *(202-204, 217, 220)*. When considering only the compounds investigated in the current study, dieldrin was the most toxic, usually followed by aldrin, endrin, and then isodrin. Cis aldrin diol, desmethylene aldrin, and desmethylene dieldrin were all found to have minimal toxicity in the insect species investigated. There were many other compounds also investigated in these studies (> 51 compounds in total), and some species differences in terms of the relative potency of each compound (*202-204*).

The observed species differences were mainly attributed to variances in metabolism and the absorption/distribution of each compound in the insects. The most potent insect toxicants were found to be the dechlorinated dieldrin analogs (*202, 204*) (not included in this investigation). Overall, the structural features critical for insect toxicity seem to be the presence of two electronegative/polar centers, one being the chlorine atoms (> 2), the other centered around either an epoxide or olefin. While changes to the portion of the molecule opposite the chlorines can be made and still retain toxicity (no clear structural relationship observed), the removal of the chlorine atoms, either from the bridge or from the olefin, resulted in the most substantial increase in toxicity (*202*).

While the general characteristics of the structural features that are important for toxicity in both insects and dopaminergic cells are similar, i.e. importance of the 3-dimensional structure and identity of electronegative/polar moiety opposite of the

chlorine atoms (i.e. epoxide or cis diol), the relative potency of these compounds is in direct opposition. There are significant differences in the systems used between these studies, whole insect vs. immortalized cell line, that affect the metabolism, localization, and absorption of these compounds. However, it may still be concluded that there are significant differences in the target proteins that warrant further investigation. It is these differences that will be useful in developing new, potent insecticides that do not also target the dopaminergic system of humans.

Conclusions

It was determined that dieldrin does not covalently modify proteins (via nucleophilic attack by Cys or Lys residues). This conclusion supports previous assertions that while dieldrin "binds" to proteins, these interactions are noncovalent (*227, 228*). It was determined that dieldrin is capable of entering the PC6-3 cells, and after 4h, accumulates readily in the membrane and lipid fraction of the cell.

The structure-activity relationship of dieldrin was investigated using compounds targeting three different structural aspects of this organochlorine. It was determined that the desmethylene compounds were much more toxic in a dopaminergic cell line. The desmethylene compounds were the most toxic to the PC6-3 cells, based on the results of the MTT assay. These two compounds have increased flexibility on the side of the molecule containing the polar moiety (epoxide or olefin). There is also less steric bulk without the additional methano bridge. If it is assumed that all of the compounds interact with the same protein(s) and in a similar manner, it may be concluded that the unchlorinated methano bridge is not a binding contact since its absence increases potency. Also, when this bridge is oriented in the same direction as the chlorinated methano bridge, as in endrin and isodrin, decreased toxicity is observed. This implies that this orientation of the steric bulk (methano bridge) may block the interaction of these compounds with the target protein(s). When considering dieldrin, aldrin, and cis

aldrin diol, the unsubstituted methano bridge is oriented in the opposite direction of the chlorinated bridge, and moderate toxicity is observed. This indicates that while the methano bridge may not be an important binding contact, in this orientation it does not interfere with the interaction of the compounds with the protein target(s).

The identity of the polar group (cis diol, olefin, or epoxide) was found to also be important for toxicity. When the orientation of the methano bridge is the same, the cis diol was found to be more potent than the olefin and the epoxide. Compounds with an olefin (aldrin and desmethylene aldrin) were found to be more potent than their structural analogs (dieldrin and desmethylene dieldrin, respectively). Indicating that the polar group is important for protein interactions, and by changing the epoxide to an olefin or cis diol increases the toxicity in PC6-3 cells.

Interestingly, while these same structural features play an important role in their potency as insecticides, the order of potency in various insects varied from the PC6-3 cells significantly, with endrin and isodrin being more toxic than cis aldrin diol and desmethylene dieldrin, and dieldrin being more toxic than aldrin (*202*). This indicates that the mechanism of toxicity between insects and dopaminergic cells is quite different and also implies variances with the processing (metabolism and distribution) of these compounds.

This information regarding the reactivity and localization of dieldrin is important to better understand the interaction of this pesticide with proteins within dopaminergic cells. Since dieldrin is able to localize within these cells, target proteins may not only be receptors, such as in insects with respect to the GABA receptors. Additional information regarding the structural features that are important for toxicity was also determined. These results implicated the 3-dimensional structure of dieldrin as the most important structural characteristic. This is very useful information to better understand how this compound interacts with proteins within a dopaminergic neuron, and the type of hydrophobic pockets it is likely to interact with. Further investigation is necessary to

identify specific protein targets, which will be useful in determining how exposure to this insecticide can lead to an increased risk for developing Parkinson's disease.

Future work will include analysis of the metabolism and stability of dieldrin and each of the analogs in a dopaminergic system, i.e. PC6-3 cells. It is already known that dieldrin accumulates in the brain, but information regarding stability and metabolism, as it relates it to neurodegenerative diseases, has not been investigated. This information can then be compared to the previous results obtained in rat liver microsomes (*219, 231, 232*) to investigate differences between how this pesticide may be processed in the central nervous system as compared to hepatic metabolism pathways.

An investigation between the structure-activity relationship observed in this dopaminergic system will also be compared to their potency as GABA antagonists, with the inclusion of the dechlorinated analogs of dieldrin. The GABA receptor is the known target in insects, however these pesticides do not seem to affect this receptor in humans. Through a better understanding of the structural features important for toxicity in each of these model systems, more effective insecticides may be developed that also limit neurotoxicity. The development of safer, more effective pesticides will be critical for future human and environmental health.

CHAPTER SIX. THE STRUCTURE-ACTIVITY RELATIONSHIP OF DIELDRIN – IMPACT ON OXIDATIVE INSULT

Introduction

Parkinson's Disease (PD), one of the most common neurodegenerative disorders in the United States, is characterized by the selective loss of dopaminergic neurons in the substantia nigra pars compacta. The mechanism of this neuronal loss is still unknown (*105*), but it is thought that greater than 90% of PD cases are due to environmental causes (*138, 139*). These environmental exposures may include insecticides, herbicides, and heavy metals. One pesticide of interest is dieldrin, whose concentration was elevated specifically in PD patients (*139, 182-184*).

In addition to finding an increased concentration of dieldrin in the brains of PD patients, this pesticide has also been shown to affect a number of cellular processes known to lead to the development of PD. These include increases in oxidative stress, disruptions in dopamine metabolism, and apoptosis (*21, 22, 105, 186-188*), as outlined in Figure 6.1. A study conducted by Kitazawa *et al* examined neurochemical changes in dopaminergic cells (PC12) resulting from dieldrin exposure (*186*). They found an increase in oxidative stress in this model system upon dieldrin treatment, supported by an intracellular increase in superoxide anions, a reactive oxygen species (ROS). These researchers also found dieldrin exposure caused mitochondrial dysfunction, based upon decreased absorbance using an MTT assay (3-(4,5-dimethylthiazol 3,yl)-2,5-diphenyl tetrazolium bromide) (*186*). This result is supported by the data presented in Chapter 5, Figures 5.5 and 5.6.

Additional studies analyzing the adverse effects of dieldrin have shown a disruption in the metabolism of dopamine, a monoamine neurotransmitter important for the function of dopaminergic neurons. As outlined in Scheme 6.1, in a normal dopaminergic neuron, dopamine is first metabolized by monoamine oxidase (MAO) to

form 3,4-dihydroxyphenylacetaldehyde (DOPAL). This neurotoxic aldehyde metabolite may be further metabolized by either aldehyde dehydrogenase (ALDH) to form 3,4-dihydroxyphenylacetic acid (DOPAC) or by aldehyde reductase (ALR) to form the alcohol metabolite (3,4-dihydroxyphenylethanol, DOPET). Disruptions in the metabolism and processing of dopamine have been observed from exposure to dieldrin in a number of studies and in various model systems (*21, 22, 186, 233-236*). In ring doves and mallards, a depletion of dopamine was observed (*233, 234*); in mice a selective release of striatal dopamine was caused by treatment with dieldrin (*236*).

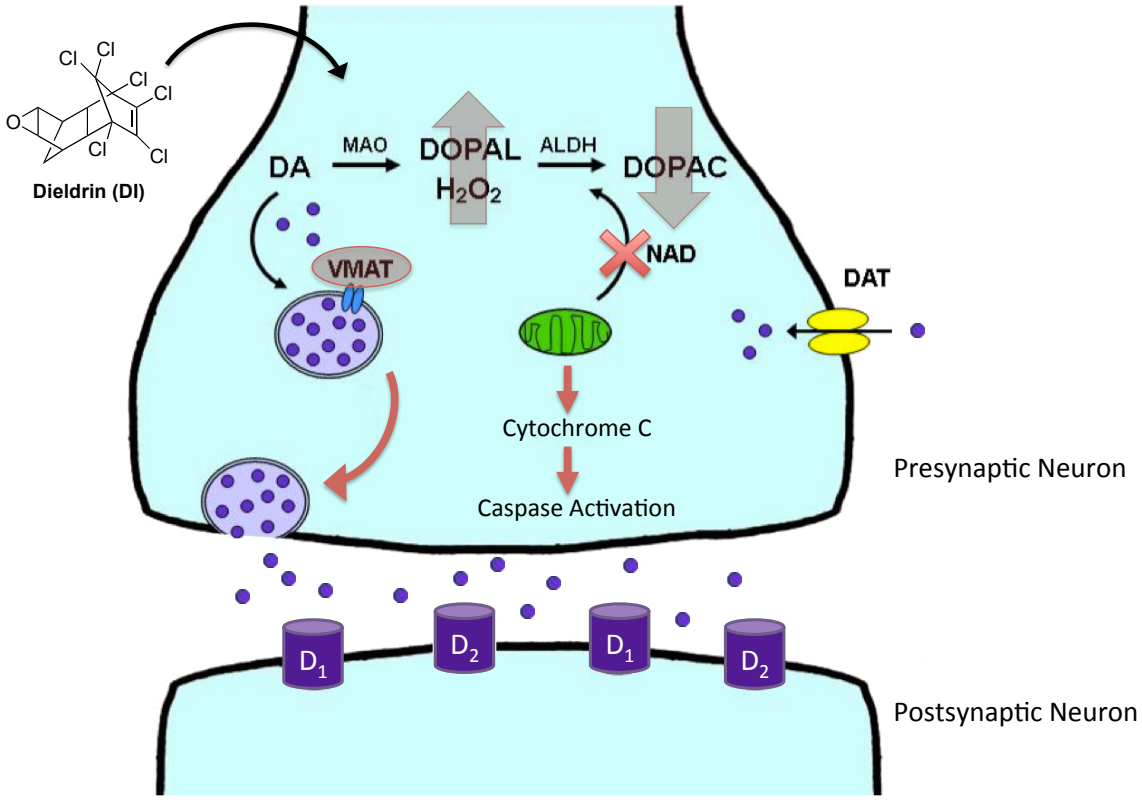
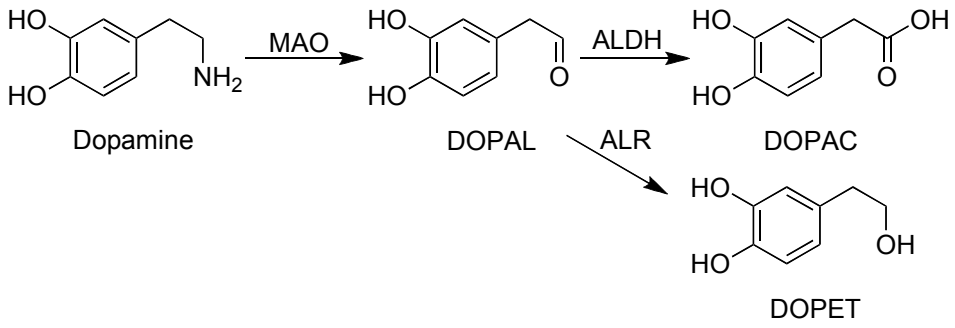


Figure 6.1. Cellular consequences (highlighted in red) of dieldrin exposure in a dopamine synapse, including reduction in NAD production resulting in ALDH inhibition, disruption in dopamine trafficking, and release of cytochrome C leading to caspase activation and apoptosis.

Many of the known adverse effects of dieldrin exposure, such as mitochondrial dysfunction and the disruption of dopamine metabolism, have been correlated to the selective death of dopaminergic cells (*21, 22, 105, 186-188*), and to the development of Parkinson's disease (*42, 43, 114, 120, 121, 143, 237-240*). Therefore, it is of high importance that the mechanism of the selective toxicity towards dopaminergic neurons exposed to dieldrin is understood. The research discussed in Chapter 5 demonstrated a correlation between specific structural features of dieldrin and its toxicity profile in a dopaminergic system. This structure-activity relationship is inconsistent with the one attributed to insect toxicity (*202-204, 217, 220*), implying that there are differences in the protein targets, metabolism, distribution, and processing of these dieldrin analogs between model systems (dopaminergic cell line vs insect). More understanding of these differences is important for determining how dieldrin causes oxidative stress in a dopaminergic model, as it relates to Parkinson's disease susceptibility.



Scheme 6.1. Dopamine Metabolism

Due to the various adverse effects dieldrin has been correlated to, including production of reactive oxygen species and altering the metabolism of dopamine, it is possible there may be multiple protein targets within a dopaminergic system. By examining the effect of each dieldrin analog (as described in Chapter 5), a better

understanding of how the established structure-activity relationship applies to some of the other known endpoints of dieldrin exposure will be determined.

The hypothesis of this study is that the toxicity profile established in Chapter 5, also applies to the ability of these compounds to disrupt the oxidative balance within dopaminergic cells. In order to test this hypothesis, three specific aspects of dieldrin's structure were assessed, 1. The relative 3-dimensional orientation of the bridgehead carbons, 2. The polar moiety opposite of the chlorine atoms, and 3. The effect of the polar moiety coupled with the 3-dimensional orientation. A total of six analogs were compared to dieldrin, two analogs for each of the aforementioned categories. The effects of each compound on dopaminergic neurons with respect to dopamine metabolism, production of reactive oxygen species, and protein modification by lipid peroxidation products were monitored.

Experimental Procedures

Chemicals

Dieldrin, aldrin, endrin, and isodrin were purchased from Chem Service (West Chester, PA). All other reagents, unless otherwise noted, were purchased from Sigma Aldrich (St. Louis, MO) without further purification. Organochlorine analogs were named based on the von Baeyer/IUPAC system for naming polycyclic compounds as discussed previously (*206*).

Cis Aldrin Diol (1,8,9,10,11,11-hexachloro-4,5-(*exo*)*cis*-dihydroxy-2,3-7,6-*endo*-2,1-7,8-*exo*-tetracyclo[6.2.1.$1^{3,6}$.$0^{2,7}$]dodec-9-ene)

Cis aldrin diol (CAD) was synthesized using a method adapted from literature (*207, 208*). Briefly, aldrin (86 mg, 0.24 mmol) was combined with dry ether (6.4 mL), pyridine (0.15 mL), and osmium tetroxide (50 mg, 0.2 mmol). The reaction was stirred

for 2 h at room temperature, and then let stand overnight in the dark. The ether was evaporated with nitrogen, giving a brown residue that was then dissolved in dichloromethane (10 mL) and stirred vigorously overnight with a solution of water (6.5 mL), potassium hydroxide (71 mg, 1.3 mmol), and *D*-mannitol (630 mg, 3.5 mmol). The dichloromethane layer was removed, washed with water, and dried with sodium sulfate, giving the product in 90% yield. ^{1}H NMR (DMSO-d6): δ 1.12 (d, 1H, CH_2), 1.59 (d, 1H, CH_2), 2.06 (m, 2H, CH), 2.60 (m, 2H, CH), 3.57 (m, 2H, CH), 4.93 (m, 2H, OH); ^{13}C NMR (DMSO-d6): δ 28.3, 43.0, 51.7, 73.6, 81.0, 104.7, 130.8; MS (EI) *m/z* calcd for $C_{12}H_{10}Cl_6O_2$ 395.9, found *m/z* 396.1.

Desmethylene Aldrin (1,8,9,10,11,11-hexachloro-2,3-7,6-*endo*-tricyclo[6.2.1.0^{2,7}]undeca-4,9-diene)

Adapted from literature methods (*202, 208-210*), hexachlorocyclopentadiene (1.0 g, 0.004 mol, 0.6 mL) and 1,4-cyclohexadiene (1.3 g, 0.016 mol, 1.5 mL) were placed under argon and stirred at 110°C for 44h. Product was purified by column chromatography on silica gel with hexanes as mobile phase, resulting in a 25% yield. ^{1}H NMR ($CDCl_3$): δ 1.90-1.99 and 2.35-2.44 (m, 4H, CH_2), 2.98-3.09 (m, 2H, CH), 5.86 (m, 2H, HC=CH); ^{13}C NMR ($CDCl_3$): δ 21.9, 45.8, 82.5, 103.2, 126.7, 131.6; MS (EI) *m/z* calcd for $C_{11}H_8Cl_6$ 349.9, found *m/z* 349.9.

Desmethylene Dieldrin (1,8,9,10,11,11-hexachloro-4,5-epoxy-2,3-7,6-*endo*-tricyclo[6.2.1.0^{2,7}]undec-9-ene)

Adapted from literature methods (*202, 208, 210, 211*), 3-chloroperbenzoic acid (*m*CPBA) (0.08 g, 0.5 mmol) in 2 mL of dichloromethane was added dropwise to a desmethylene aldrin (0.1 g, 0.3 mmol) in 2 mL dichloromethane, and stirred at RT for 2 h. Reaction mixture was extracted twice with water. The organic layer was then dried with magnesium sulfate and the product purified by column chromatography on silica gel with a mobile phase of 4:1 hexanes to acetone, resulting in a 50% yield. 1H NMR

($CDCl_3$): δ 1.59-1.67 and 2.40-2.48 (m, 4H, CH_2), 2.91-2.99 (m, 2H, CH), 3.20-3.24 (m, 2H, CH-O); ^{13}C NMR ($CDCl_3$): δ 22.2, 42.2, 49.1, 82.3, 102.8, 132.0; MS (EI) *m/z* calcd for $C_{11}H_8Cl_6O$ 365.9, found *m/z* 365.9.

Cell Culture

PC6-3 cells, cultured in RPMI1640 medium (GIBCO, Invitrogen, Carlsbad, CA) supplemented with heat-inactivated horse serum (10%) (HyClone, Thermo Scientific, Rockford, IL), fetal bovine serum (5%) (GIBCO), penicillin (10 IU/mL), and streptomycin (10 mg/mL), were grown in 100 mm^2 tissue culture dishes at 37°C with 5% CO_2. Cells (3 x 10^4 cells/well) were seeded into six-well plates for four days at 37°C with 5% CO_2. The cells were then differentiated with 2.5s nerve growth factor (NGF) (BD Biosciences, Bedford, MA) at 50 ng/mL for 4 days.

Treatment of Cells with Dieldrin and analogs

Media was removed from cells and replaced with HEPES-buffered media containing 115 mM NaCl, 5.40 mM KCl, 1.80 mM $CaCl_2$, 0.80 mM $MgSO_4$, 5.50 mM glucose, 1.00 mM NaH_2PO_4, and 15.0 mM HEPES (pH 7.4). The cells were treated with compound (dieldrin, aldrin, endrin, isodrin, cis-aldrin diol, desmethylene aldrin, or desmethylene dieldrin) at 0 – 300 μM in dimethylsulfoxide (DMSO, 0.6%).

Dopamine Metabolism

The effect of each compound on the extracellular concentration of dopamine metabolites was monitored. An aliquot of the extracellular media was removed at 0, 1, and 4 h and then combined with perchloric acid (5% v/v) in order to precipitate the proteins and terminate the reaction. The samples were stored at -70°C, thawed, and centrifuged at 10,000 x *g* for 5 min prior to HPLC analysis. Samples were analyzed by an Agilent 1200 Series Capillary HPLC with a Phenomenex C18 Luna column (1 x 150 mm). The dopamine metabolites DOPAL, 3,4-dihydroxyphenylacetic acid (DOPAC), and 3,4-

dihydroxyphenylethanol (DOPET) were separated using isocratic conditions of 0.1% trifluoroacetic acid in water with 6% ACN (v/v), a flow rate of 50 µL/min, and detection with a photodiode array detector (absorbance at 202 and 280 nm). The peak area was then converted to concentration by comparing the area under the curve to a standard curve achieved from metabolite standards. These standards were prepared as previously described (*44, 45, 56, 57*).

Flow Cytometry

In order to monitor the production of reactive oxygen species, PC6-3 cells were pretreated with dihydroethidium (DHE, 10 µM, 0.2% DMSO) and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, 50 µM, 0.2% DMSO) for 20 min at 37°C in HEPES-buffered saline. Cells were then washed and treated with each dieldrin analog at a non-toxic dose (< 20% cell death according to MTT assay) for 1h. The doses of each organochlorine used were dieldrin (25 µM), aldrin (25 µM), cis aldrin diol (25 µM), desmethylene dieldrin (10 µM), isodrin (100 µM), and desmethylene aldrin (10 µM). Cells were then removed from the wells, washed with phosphate-buffered saline, and resuspended in HEPES-buffered saline prior to filtration (70 µM). Hoechst 33258 (4 µg/mL) was added, and the absorbance within the cells measured using a Becton Dickinson LSR II flow cytometer with UV with 440/40 band pass (BP) (Hoechst 33258), 530/30 BP (H_2DCFDA), and 610/20 BP (DHE). Data was processed using CellQuest (BD Bioscience), and results reported as the geometric mean ± SE.

Western Blot Analysis for 4HNE Modified Proteins

PC6-3 cells were pre-treated with 100 µM of dopamine for 15 min, and then incubated with each compound (300 µM) for 4 h. At this time the HEPES-buffered media was removed and lysis buffer was added (10 mM K_2PO_4 with 0.1% triton x-100, pH 7.4). The lysed cells were then collected and sonicated with 10, 1 s pulses. This lysate was stored at -70°C until separation via sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel. The molecular weight marker used was the Amersham ECL DualVue Western Blotting Marker. The proteins were then transferred to a nitrocellulose membrane and blocked using 5% BSA. The membranes were incubated with primary antibody (goat anti-4HNE primary, 1:2500 dilution, Abcam), and then secondary antibodies (bovine anti-goat IgG HRP, 1:10000, Santa Cruz Biotechnology; and S-protein-HRP, 1:10000, Amersham). Bands were detected using Amersham ECL Plus Western Blotting Reagents (GE Healthcare, Piscataway, NJ) and visualized using High Performance Chemiluminescence Film (GE Healthcare). To ensure equal protein load, membranes were stripped using Abcam's mild stripping buffer (0.2 M glycine, 0.2 M SDS, 1% Tween20 (v/v), pH 2.2) at 60°C for 30 min, rinsed under running deionized water for 10 min, and then with TBST for 10 min prior to staining with Ponceau S. Relative band density from the Western blot films was determined using ImageJ 1.44o, and are shown in Table 6.2.

Statistics

All statistics were calculated using GraphPad Prism version 5.0c (GraphPad Software, San Diego, CA). Significance from control for the dopamine metabolism and flow cytometry results was determined using a two-tailed *t* test.

Results

In order to test the hypothesis of this study, that the toxicity of dieldrin analogs is indicative of their effect on oxidative insult in dopaminergic cells, the six analogs of dieldrin utilized in Chapter 5 were tested, Figure 5.1. The analogs were chosen based on their structure, allowing for the assessment of three chemical characteristics, 1. The importance of the methano bridge, 2. The effect of the polar moiety opposite of the chlorine atoms (cis diol or olefin), and 3. The interplay between both the polar moiety and the 3-dimensional orientation. Each of the dieldrin analogs were tested in three assays in differentiated PC6-3 cells and the results compared to their established toxicity

profiles, Chapter 5. The assays monitored dopamine metabolism, ROS production, and protein modification by lipid peroxidation products. PC6-3 cells were chosen as the model system for this study because they are an excellent model for dopaminergic neurons and contain many of the key components and proteins that are affected in PD (*214-216*). They have also been previously used to establish the effect of dieldrin in a dopaminergic system (Chapter 5).

Extracellular dopamine metabolites

The first set of experiments quantified the extracellular release of dopamine metabolites, DOPAL, DOPAC, and DOPET by HPLC, presented in Table 6.1, and Figures 6.2, 6.3, and 6.4. In a normal, functioning dopaminergic neuron, dopamine is packaged into vesicles, affording cytoprotection, as unregulated, cytosolic dopamine is neurotoxic (*241*). Dopamine can be released from vesicles via synaptic transmission, passive diffusion, and/or toxicant-mediated vesicle disruption. Cytosolic dopamine may also be managed through oxidative deamination to the aldehyde metabolite (DOPAL), and then further oxidized to the carboxylic acid (DOPAC), Scheme 6.1. A disruption at any of these levels of control may cause an imbalance of the dopamine metabolites, which can be observed extracellularly (*186*). Observed changes in the concentrations of these metabolites are indicative of disruptions in dopamine metabolism and/or trafficking (*186*).

The concentration of extracellular DOPAL is shown for vehicle treated, dieldrin, and Group one compounds (endrin and desmethylene dieldrin) in Figure 6.2. The vehicle treated cells showed a slight increase in DOPAL over time, as well as an increase in DOPAC. When the cells were treated with dieldrin, a time and dose-dependent increase in [DOPAL] was observed, as compared to control. No significant change in DOPAC was observed, Figure 6.4 and Table 6.1. When the isomer of dieldrin was analyzed (endrin), a slight decrease in DOPAL and an increase in DOPAC was observed. For desmethylene

dieldrin (300µM), the analog of dieldrin lacking the methano bridge, the [DOPAL] increased ten-fold. In addition, the concentration of DOPAC was significantly reduced from control with treatment with this analog, Figure 6.4. This implies a disruption in dopamine metabolism and the processing of these metabolites.

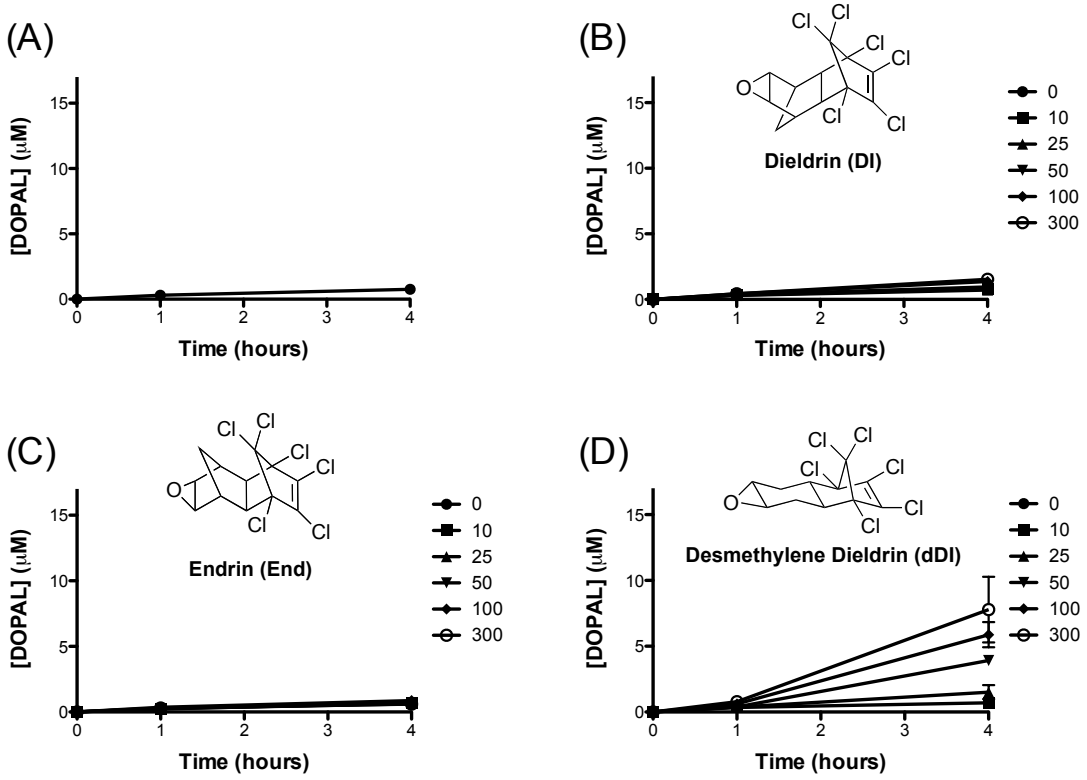


Figure 6.2. Quantification of the extracellular concentration of DOPAL from PC6-3 cells pre-incubated with dopamine (100 µM) and then incubated for 0-4 h with (A) vehicle control (0.6% DMSO), (B) dieldrin, or group 1 compounds (C) endrin, or (D) desmethylene dieldrin. The values shown represent the mean ±SD (*n* = 3, except for controls where *n* = 21), where peak area was converted to concentration using a standard curve.

Group two, with changes to the polar group opposite of the chlorine atoms, also showed a profound effect on the extracellular concentration of DOPAL in comparison to dieldrin, with both aldrin and cis aldrin diol resulting in significant and substantial

increases in DOPAL levels, as compared to control, Figure 6.3. This observation was accompanied by a marked decrease in the production of DOPAC, Table 6.1. For the third group of compounds, isodrin and desmethylene aldrin, results similar to endrin and desmethylene dieldrin were observed, respectively. Isodrin, showed only a small change from control for both DOPAL (slight increase) and DOPAC (slight decrease), whereas desmethylene aldrin showed marked increase in DOPAL concentration and a corresponding decrease in DOPAC.

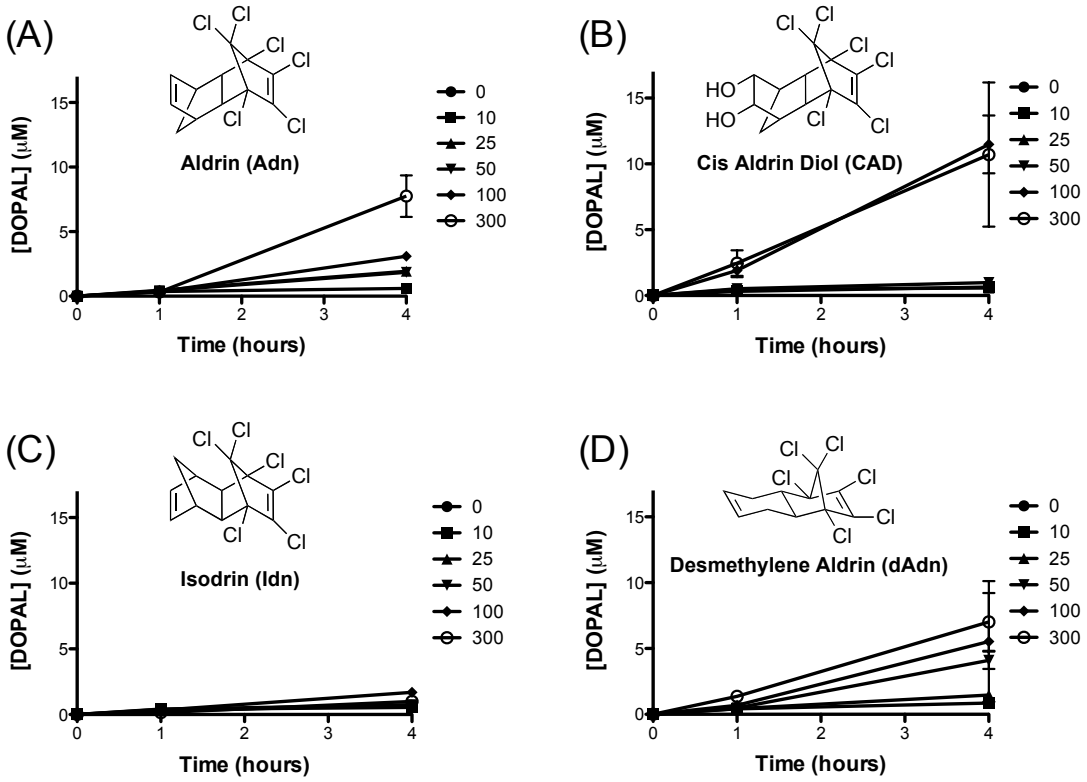


Figure 6.3. Quantification of the extracellular concentration of DOPAL from PC6-3 cells pre-incubated with dopamine (100 μM) and then incubated for 0-4 h with compounds from groups 2 or 3 (A) aldrin, (B) cis aldrin diol, (C) isodrin, or (D) desmethylene aldrin. The values shown represent the mean ±SD (*n* = 3, except for controls where *n* = 21), where peak area was converted to concentration using a standard curve.

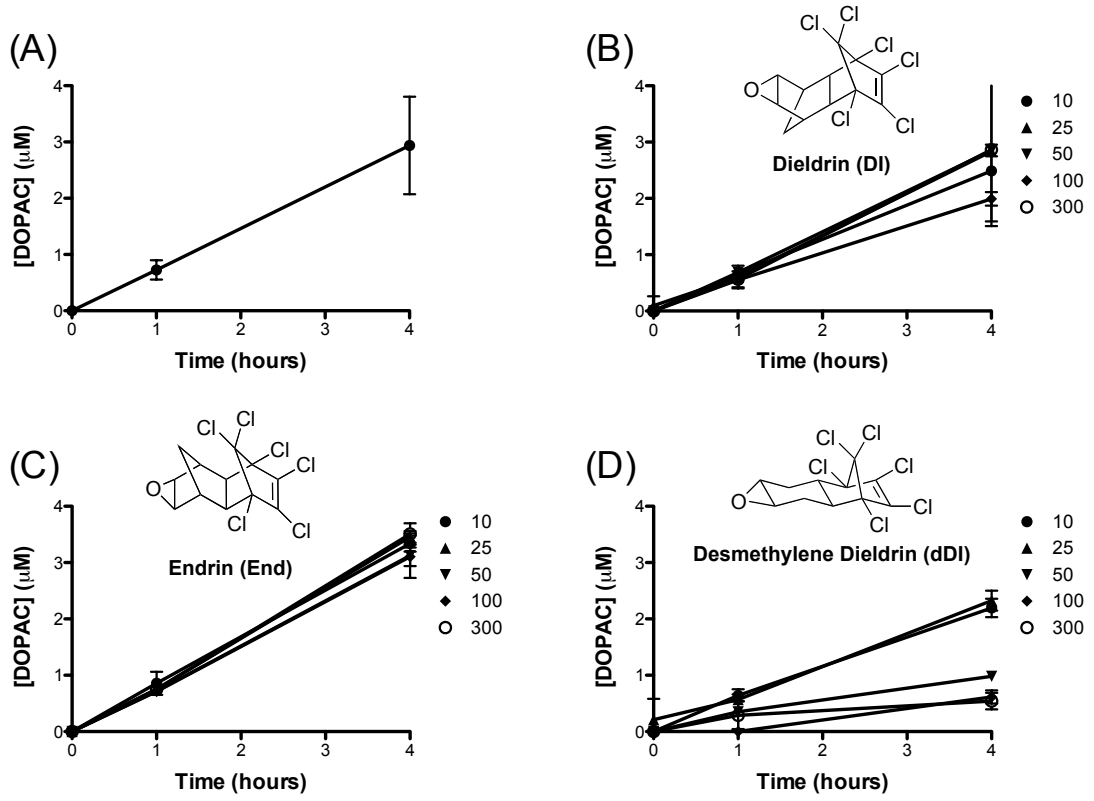


Figure 6.4. Quantification of the extracellular concentration of DOPAC from PC6-3 cells pre-incubated with dopamine (100 μM) and then incubated for 0-4 h with (A) vehicle control (0.6% DMSO), (B) dieldrin, or group 1 compounds (C) endrin, or (D) desmethylene dieldrin. The values shown represent the mean ±SD (*n* = 3, except for controls where *n* = 21), where peak area was converted to concentration using a standard curve.

Reactive Oxygen Species Production

Next, the effect of nontoxic concentrations (< 20% cell death) of each dieldrin analog on the production of reactive oxygen species was analyzed. The intracellular concentration of both superoxide anions and hydrogen peroxide were monitored in the PC6-3 cells using flow cytometry. Two fluorophores (H_2DCFDA and DHE) were used to quantify the changes in fluorescence due to accumulation of intracellular hydrogen peroxide (H_2O_2) and superoxide anions ($O2^{\bullet -}$), respectively. An increase in either of these reactive compounds is indicative of an increase in oxidative stress and/or in the disruption of the normal processing of these reactive oxygen species.

When H_2O_2 production was quantified, a significant elevation was observed upon treatment with each of the compounds studied (endrin was not included in this experiment), Figure 6.5. The dose of the compounds used were chosen based on > 80% cell viability in the MTT assay, therefore the concentrations varied slightly between analogs relative to their toxicity. This elevation in intracellular H_2O_2 indicates an increase in oxidative stress. When superoxide anions were monitored, no significant change from control was observed, Figure 6.5; indicating the accumulation of reactive oxygen species from dieldrin analog exposure is specific for H_2O_2 in the PC6-3 cells.

Table 6.1. Concentration of dopamine metabolites (DOPAL, DOPAC, or DOPET).

Compound	**Group**[a]	**[DOPAL] µM**	**[DOPAC] µM**	**[DOPET] µM**
Control		0.76 ± 0.21	2.94 ± 0.87	1.67 ± 0.32
Dieldrin		1.53 ± 0.25 ***	2.87 ± 1.36 ^{NS}	1.58 ± 0.49 ^{NS}
Endrin	1	0.60 ± 0.09 ^{NS}	3.51 ± 0.19 ^{NS}	1.78 ± 0.14 ^{NS}
Des Dieldrin	1	7.79 ± 2.49 ***	0.54 ± 0.15 ***	1.16 ± 0.27 *
Aldrin	2	7.75 ± 1.61 ***	1.15 ± 0.20 *	1.24 ± 0.11 *
Cis Aldrin Diol	2	10.7 ± 5.48 ***	0.53 ± 0.11 ***	0.58 ± 0.06 ***
Isodrin	3	1.00 ± 0.19 ^{NS}	2.76 ± 0.95 ^{NS}	2.12 ± 0.44 *
Des Aldrin	3	7.02 ± 2.19 ***	0.23 ± 0.20 ***	0.85 ± 0.06 ***

Note: Concentrations of dopamine metabolites (± SD, n = 3, except for controls where n = 21) after 4h treatment with 300µM of each compound or vehicle control (0.6% DMSO). Significance from vehicle control was determined using two-tailed *t* test; difference was significant with $p < 0.05$ (*) or $p < 0.0001$ (***), or not significant with $p > 0.05$ (^{NS}).

[a]See Figure 2 for group assignments and structures.

Lipid Peroxidation Products

Lipid peroxidation is another indicator of oxidative stress, and results in the formation of reactive aldehydes, i.e. 4-hydroxynonenal (4HNE) and malondialdehyde

(MDA). These aldehydes are capable of covalently modifying proteins, disrupting dopamine metabolism and resulting in cellular damage (*44, 46, 56-58, 242, 243*). Proteins modified by 4HNE in the PC6-3 cells upon treatment with each dieldrin analog were quantified using western blot analysis, Figure 6.6. Each of the compounds investigated caused 4HNE protein modification, indicated by an increase in relative band density with respect to control, Table 6.2. This is an indicator of oxidative stress and the formation of lipid peroxidation products, specifically 4HNE.

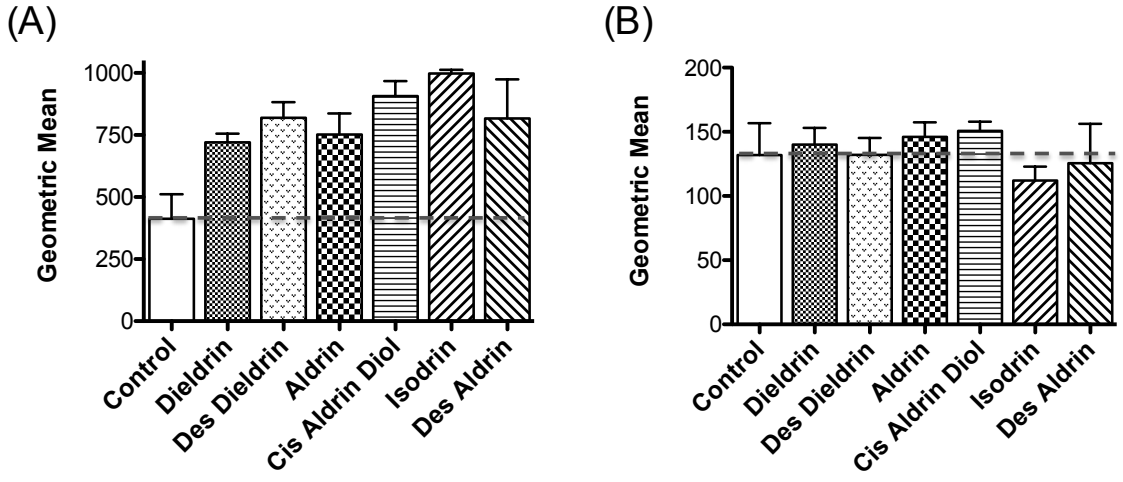


Figure 6.5. Production of reactive oxygen species monitored via flow cytometry after 1h treatment with nontoxic concentrations of each compound (10 μM for desmethylene aldrin and desmethylene dieldrin, 25μM for dieldrin, aldrin, and cis aldrin diol, and 100 μM for isodrin). Results depicted as geometric mean (± SD, n = 3) for (A) H_2DCFDA (H_2O_2), or (B) DHE ($O_2^{\bullet -}$). Change in geometric mean upon treatment with each compound in (A) for H_2DCFDA fluorescence is significantly different from control ($p < 0.005$), using a two-tailed t test. Whereas in (B) for DHE fluorescence, no significant change was observed with any of the compounds ($p > 0.1$), using a two-tailed t test.

Discussion

Dieldrin has been associated with the disruption of many key processes in dopaminergic neurons, including the metabolism of the neurotransmitter, dopamine; mitochondrial function; and the balance of oxidative stress. Misregulation of these processes, and exposure to dieldrin has been correlated to PD (*114, 122-125, 182-184*). Due to the diversity of adverse effects of dieldrin exposure, it is possible there may be multiple protein targets within the susceptible dopaminergic neurons. By examining the effect of structural modifications to dieldrin on various facets of oxidative insult in a dopaminergic model, the structure-activity relationship defined in terms of toxicity will be examined in light of these endpoints. In order to test the hypothesis of this study, that the toxicity profile established in Chapter 5, also applies to the ability of these compounds to disrupt the oxidative balance within dopaminergic cells, the six analogs used in the previous chapter was assessed with respect to their effect on dopamine metabolism, production of reactive oxygen species, and protein modification by 4HNE.

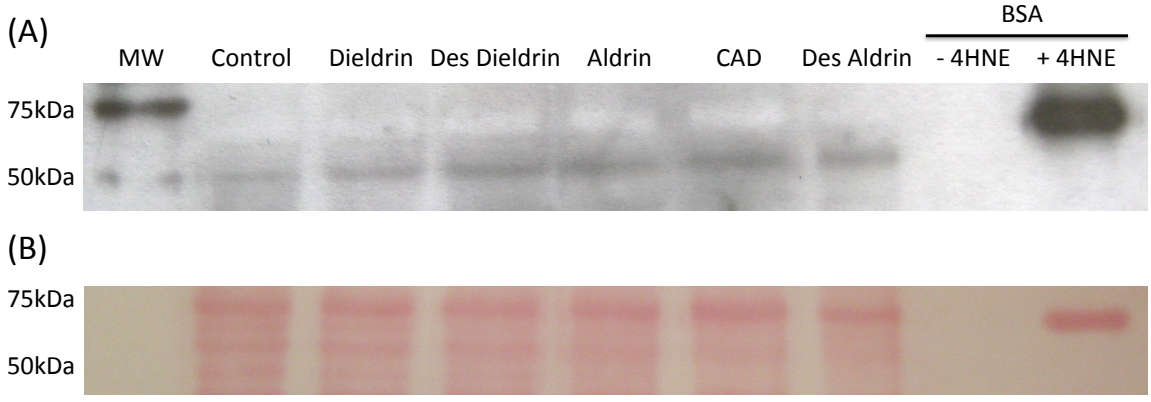


Figure 6.6. Detection of 4HNE-modified proteins in PC6-3 cell lysate by western blot analysis. Lysate was collected from cells pretreated with dopamine (100 µM) and then incubated for 4 h with 300 µM (A) of each compound (dieldrin, desmethylene dieldrin (Des Dieldrin), aldrin, cis aldrin diol (CAD), and desmethylene aldrin (Des Aldrin). Also included is a BSA negative and positive control ± 4HNE. (B) Ponceau S stain of the membrane is also included to demonstrate equal protein loading.

Exposure to dieldrin has been shown to cause depletion of dopamine in both ring doves and mallards (*233, 234*). This effect has also been shown to be specific to dopamine, with no corresponding inhibition of GABA (*235*). When this effect was further investigated in mice dosed with dieldrin, a decrease in DOPAC, the carboxylic acid metabolite of dopamine, was observed. This was coupled with a corresponding increase in cysteinyl-dopamine and cysteinyl-DOPAC and elevated levels of protein carbonyls. This indicates a disruption in dopamine metabolism and trafficking, but instead of seeing elevated levels of the dopamine metabolites, increased protein modification was observed (*21*). In a similar study, dieldrin was found to cause the release of striatal dopamine and DOPAC in mice (*236*). Developmental exposure to dieldrin in mice has also been shown to have long-term, adverse effects on dopamine metabolism. An increase in DOPAC was observed in the striatal region of these mice after they were aged to 12 weeks, and they were much more sensitive to MPTP toxicity (*22*). In a cellular model similar to the one used in the current study, treatment with dieldrin resulted in decreased intracellular dopamine, with a corresponding increase in extracellular dopamine and DOPAC. This effect was attenuated with a monoamine oxidase inhibitor (deprenyl), an enzyme responsible for the metabolism of dopamine (*186*).

These previous studies mainly focused on dopamine and DOPAC, but there is a critical metabolite in between these two compounds, DOPAL. This aldehyde metabolite is produced through the reductive deamination of dopamine by monoamine oxidase. DOPAL is an endogenous neurotoxin that is capable of covalently modifying proteins (*44, 45, 57, 119*). Aberrant levels of this reactive compound have been associated with an increased risk for Parkinson's disease (*42, 43, 119, 120, 244*). In the current study, the compounds that exhibited the greatest potency in cellular toxicity in Chapter 5, caused the largest increase in DOPAL (desmethylene aldrin, desmethylene dieldrin, and cis aldrin diol). The least toxic compounds (endrin and isodrin), showed the least effect on DOPAL concentration, and minimal effect on the concentration of the other dopamine

metabolites quantified (DOPAC and DOPET). This indicates a correlation between an increase in the extracellular concentration of DOPAL and increased toxicity in PC6-3 cells.

Table 6.2. The relative band density of 4HNE protein modification in cell lysate.

Compound	Relative Density
Control	1.00
Dieldrin	1.67
Desmethylene Dieldrin	2.04
Aldrin	1.83
Cis Aldrin Diol	2.02
Desmethylene Aldrin	1.70

Note: Relative density calculated from western blot in Figure 6.6a using ImageJ software.

The effect of dieldrin treatment on reactive oxygen species production has also been previously assessed in two different cellular models. The first one, PC12 cells, are rat pheochromocytoma cells, the parent cell line of the PC6-3 cells (used in the current study). The other cell line that has been used to assess reactive oxygen species production from dieldrin exposure are N27 cells, immortalized rat mesencephalic cells. All three cell lines (PC12, PC6-3, and N27) are dopaminergic, containing many key metabolic and transport proteins. The main difference between them is that the N27s are considered neuronal, whereas the PC12s and PC6-3s were isolated from the adrenal gland and then are differentiated with nerve-growth factor so they assume a neuronal phenotype. Previous studies have shown that dieldrin treatment results in an increase in reactive oxygen species (superoxide anions and H_2O_2) in both the PC12 and N27 cells

(*105, 186, 188, 221*). The production of reactive oxygen species in these studies was attenuated with pretreatment with a tyrosine hydroxylase inhibitor (α-MPT), a monoamine oxidase inhibitor (deprenyl), superoxide disumutase (SOD, an enzyme capable of detoxifying superoxide anions), a superoxide dismutase mimetic (MnTBAP), or *N*-acetyl-Cys (*105, 186, 188, 221*).

In the current study, treatment of the PC6-3 cells with nontoxic doses of each of the organochlorines resulted in a significant increase in H_2O_2 levels. This increased production of reactive oxygen species was not always indicative of toxicity, such as in the case of isodrin which caused the largest increase in reactive oxygen species, but showed the least amount of toxicity (Chapter 5). A similar trend has been observed for the organophosphates, another class of synthetic pesticides that includes compounds such as chlorpyrifos and malathion (*3, 245*). It was initially thought that all of the organophosphates had the same mechanism of action, irreversible inhibition of acetylcholinesterase; while there were some observed differences in relative potency, these could be easily attributed to structural changes (*3*). It was later determined that there were exceptions to this original hypothesis. It was observed that organophosphates could produce similar levels of oxidative stress, but the cellular compensation and/or reactions to this insult was very different depending on the toxicant (*223, 225, 246-252*). Therefore, the same degree of initial insult can result in a varied degree of endpoint toxicity (*225*), as observed with the dieldrin analogs investigated here.

The similar increase in ROS production for each compound, with a disconnect in the corresponding endpoint toxicity implies there are different protein targets dependent on the structural characteristics of the pesticide, and/or differences in the mechanism of toxicity following the production of H_2O_2. If the increase in H_2O_2 was strictly due to MAO activity alone (corresponding to the increased concentration of extracellular DOPAL), then isodrin, which resulted in elevated levels of H_2O_2, should

have resulted in an increase in DOPAL. This effect was not observed, with no significant change to DOPAL concentration with isodrin treatment. Since isodrin resulted in an increase in H_2O_2 but not was not accompanied by an increase in DOPAL, it indicates there may be other sources of H_2O_2 production apart from monoamine oxidase activity, or that there are other factors involved in the elevation of this reactive oxygen species.

The last parameter of oxidative insult that was assessed in the current study was lipid peroxidation. Products of lipid peroxidation, such as 4-hydroxynonenal (4HNE) and malondialdehyde (MDA), are two biomarkers of oxidative stress. Elevated levels of these two reactive aldehydes have been linked to a disruption in the metabolism of dopamine, and found in Parkinson's disease patients (*253*). Increased concentrations of these lipid peroxidation products have been observed in the midbrain of mice exposed to dieldrin (*236*), as well as in the livers of rats dosed orally with dieldrin (*254*). In cellular models, dieldrin treatment was found to increase lipid peroxidation products dose-dependently (*186, 223*). In the current study, lipid peroxidation was assessed by quantifying 4HNE-modified proteins. 4HNE is a highly reactive aldehyde capable of modifying proteins such as aldehyde dehydrogenase (*44, 46, 56-58, 242, 243*). When the PC6-3 cells were treated with various dieldrin analogs, an increase in protein modification by 4HNE was observed via Western blot analysis, Figure 6.6 and Table 6.2. These results correlate with the increase in reactive oxygen species production, Figure 6.5.

Conclusions

It was determined that dieldrin analogs disrupt dopamine metabolism in a manner consistent with their previously established toxicity profiles in a dopaminergic system. The most toxic compounds (desmethylene aldrin, desmethylene dieldrin, and cis aldrin diol) resulted in the highest level of DOPAL, a known neurotoxin. In contrast, there was no structure-activity relationship observed with respect to reactive oxygen species production. All of the compounds tested resulted in an increase in H_2O_2,

indicating there are additional mechanisms and protein targets ultimately contributing to the toxicity of these organochlorines. Treatment with dieldrin analogs also resulted in an increase in 4HNE-modified proteins, another indicator of oxidative insult.

Future studies investigating the effect of dieldrin exposure in mice on dopamine metabolism, with respect to changes in DOPAL concentration, as well as 4HNE-modified proteins will be very important. While previous studies have focused on the concentrations of dopamine and DOPAC in the brains of mice treated with dieldrin, due to the correlation of elevated levels of DOPAL and cellular toxicity, it is important to compare this to *in vivo* studies. Additional experiments investigating the disparity between the level of reactive oxygen species produced as compared to the disruption in dopamine metabolism will also be investigated. This will be accomplished using inhibitors of dopamine metabolism (including deprenyl and MDA) as well as ROS scavengers, i.e. catalase and MnTBAP. The effect of each of the dieldrin analogs on specific aspects of the apoptotic pathway, such as on the activation of caspase-3 and caspase-9, will also be completed in an effort to provide important information regarding possible differences in the mechanism of toxicity for each of these organochlorines.

CHAPTER SEVEN. RESEARCH SUMMARY

> I contend…that we have allowed these chemicals to be used with little or no advance investigation of their effect on soil, water, wildlife, and man himself. Future generations are unlikely to condone our lack of prudent concern for the integrity of the natural world that supports all life.
>
> Rachel Carson, *Silent Spring*, p13

Introduction

Pesticides have been used for thousands of years, without much thought on the long-term effects on human health and on the environment (*5*). Exposure to various pesticides has been associated with the development of diseases such as Parkinson's disease, Alzheimer's disease, and many forms of cancer (*3*). While the use of many pesticides has been reevaluated and/or banned by the United States Environmental Protection Agency (US EPA), there is still a significant risk for exposure due to the environmental persistence of many of these compounds. Also, these regulations do not necessarily guarantee that the pesticides currently used are safer than those that have already been banned.

The use of pesticides is necessary to maintain the large demand on food production while the amount of land available for farming decreases. Due to the actions necessary to meet these demands and maintain effective farming practices, infestation of pests is imminent, requiring the use of toxic compounds. There are alternatives to pesticide use, which are used in the farming of organic produce, however these practices are significantly more expensive, require more land, and result in higher food prices. Pesticides are also commonly used for lawncare and in/around houses to control pests such as dandelions and termites. Given the high usage and varied applications of pesticides, it can be assumed that each person in the United States is exposed to pesticides throughout their life, from conception until death. As Rachel Carson stated, "if we are are going to live so intimately with these chemicals – eating and drinking

them, taking them into the very marrow of our bones – we had better know something about their nature and their power" (*5*).

While the government is doing its best to regulate the use of pesticides and determine the adverse effects and mechanism of toxicity resulting from exposure to these compounds, it is a very large and complicated task to complete. The target in insects can be very different than the one observed in mammals, as observed with dieldrin. There can also be many variations in adverse effects depending on the dose, exposure route, co-exposure to additional pesticides, and individual susceptibility, i.e. polymorphisms. Despite the many factors that complicate an already multifaceted situation, there is a desperate need for additional research on the health effects and mechanism of toxicity of these pesticides, particularly with respect to human diseases.

Two pesticides of interest were investigated in this project, the first being molinate, a thiocarbamate herbicide known to cause neurotoxicity (*25, 33-36*), and the other dieldrin, an organochlorine pesticide linked to the development of Parkinson's disease (*139, 182-184*). The central hypothesis of this study was that specific structural modifications affect the toxicity profiles of pesticides, i.e. dieldrin and molinate, and can be used to define the reactivity and possible protein targets of these compounds. This hypothesis was tested using four aims: Specific Aim 1, determine the relative inhibitory potency of molinate and its metabolites with ALDH2; Specific Aim 2, define the protein reactivity of molinate and its metabolites; Specific Aim 3, examine the localization of dieldrin and the toxicity of dieldrin analogs; and Specific Aim 4, assess the impact of dieldrin analogs on oxidative insult.

Discussion

Specific Aim 1: Determine the relative inhibitory potency of molinate and its metabolites with ALDH2.

Previous studies have implicated ALDH2 as a target of molinate, but identification of which compound, molinate or one of its sulfoxidation metabolites, is responsible for this enzyme inhibition had not been previously determined. It was found that molinate sulfone was the most potent inhibitor of ALDH activity, using hALDH2 and mouse striatal synaptosomes. This indicates that metabolism of molinate to molinate sulfoxide, and then to molinate sulfone leads to increased inhibitory potency of this pesticide towards ALDH2.

Specific Aim 2: Define the protein reactivity of molinate and its metabolites.

The goal of this aim was to establish a reactivity profile for all three compounds, molinate, molinate sulfoxide, and molinate sulfone, against various protein nucleophiles, i.e. Cys and Lys. The only reaction observed was between molinate sulfone and Cys, and was determined to involve the nucleophilic attack of the thiol to the carbonyl moiety of molinate sulfone. This resulted in the formation of a 126 Da carbamate adduct, based upon LC/MS and MS/MS results. In order to determine if this metabolite covalently modified hALDH2, site-directed mutagenesis and mass spectrometry were used. A single 125 Da adduct was also found on Cys302 of hALDH2 upon treatment with molinate sulfone. From these results, it was inferred that molinate sulfone covalently modifies the active-site Cys residue of ALDH2, which results in the inhibition of this critical enzyme.

Specific Aim 3: Examine the localization of dieldrin and the toxicity of dieldrin analogs.

The purpose of this aim was to determine if dieldrin was capable of covalently modifying proteins and to assess the structure-activity relationship of dieldrin in regards to toxicity in a dopaminergic system. It was determined that dieldrin was not capable of covalently modifying proteins (via nucleophilic attack of Cys or Lys residues). This conclusion supports previous assertions that while dieldrin "binds" to proteins, these interactions are noncovalent (*227, 228*).

The structure-activity relationship of dieldrin was investigated using compounds targeting three different structural aspects of this organochlorine, including the 3-dimensional structure and role of the epoxide. It was determined that the desmethylene compounds, lacking a methano bridge, were much more toxic than dieldrin. The isomers of dieldrin and aldrin, endrin and isodrin, respectively, were determined to be the least toxic analogs, despite their reported toxicity in insects (*202*). It was also determined that the identity of the polar moiety (epoxide, olefin, or cis diol) was important for toxicity, but was not as critical as the 3-dimensional orientation of the methano bridge. Interestingly, the order of potency of these compounds in insects varied greatly from what was observed in PC6-3 cells. This indicates that the mechanism of toxicity in insects and dopaminergic cells is quite different and that there may be variances in the processing of these compounds between these systems as well.

Specific Aim 4: Assess the impact of dieldrin analogs on oxidative insult.

The correlation between increased oxidative stress and Parkinson's disease is well documented, but the causes, i.e. exposure to pesticides, are not well understood. This last aim employed the dieldrin analogs used in Chapter 5 to determine their dopaminergic toxicity, and investigated the role of their relative toxicity relative to their

ability to cause oxidative insult. It was determined that dieldrin analogs disrupt dopamine metabolism in a manner consistent with their previously established toxicity profiles in a dopaminergic system. The most toxic compounds (desmethylene aldrin, desmethylene dieldrin, and cis aldrin diol) resulted in the highest level of DOPAL, a known neurotoxin. In contrast, there was no structure-activity relationship observed with respect to reactive oxygen species production. All of the compounds tested resulted in an increase in H_2O_2, indicating reactive oxygen species production is not the only mechanism contributing to the toxicity of these organochlorines. An increase in 4HNE protein modification was also observed upon treatment with dieldrin, desmethylene dieldrin, aldrin, cis aldrin diol, isodrin, and desmethylene aldrin. This indicates an increase in products of lipid peroxidation and protein damage from the elevated levels of 4HNE.

Conclusions

The results of this project indicate that the structure of these pesticides is critical for its toxicity, and that the importance of these structural features changes depending on the model system, i.e. synaptosomes vs recombinant protein, or immortalized cell line vs insects. While this conclusion may not be surprising, the information achieved from these studies is very important, such as the identification of the metabolite of molinate responsible for the covalent modification of ALDH2. It was also determined that the structure-activity relationship of dieldrin in dopaminergic cells is very different from the one observed in insects, an observation that will be very useful in achieving a better understanding of how exposure to dieldrin increases a person's vulnerability to develop Parkinson's disease.

The identification of the structural features important for toxicity in these model systems is not only important to understand their toxicity in humans, but it is also critical to the development of new pesticides. This is in an effort to increase their

selective toxicity for insects and minimizing their adverse/off-target effects. This can lead to the development of safer, more effective pesticides that will be essential for future human and environmental health.

Future Directions

The next step of the molinate project is to identify proteins that are covalently modified by its metabolites *in vivo*, focusing mainly on ALDH within the brain of animals dosed with molinate. It will also be important to investigate the reactivity of molinate and its metabolites towards other isoforms of ALDH, i.e. ALDH1 or ALDH3 and comparing the relative rates of inhibition of ALDH from various species due to the potency differences depending on the model system used (mouse striatal synaptosomes vs. human recombinant protein). This information will provide a more detailed understanding of molinate's inhibition of ALDH, and may account for the differences observed between model systems and species.

With respect to dieldrin, future work will include analysis of the metabolism and stability of dieldrin and each of the analogs in a dopaminergic system, i.e. PC6-3 cells and mice. It is already known that dieldrin accumulates in the brain, but information regarding stability and metabolism specifically within the central nervous system, has not been investigated. This information can then be compared to the previous results obtained in rat liver microsomes (*219, 231, 232*) to investigate differences between how this pesticide may be processed in the brain as compared to hepatic metabolism pathways.

An investigation between the structure-activity relationship observed in this dopaminergic system will also be compared to their potency as GABA antagonists. The GABA receptor is the known target in insects, however these pesticides do not seem to affect this receptor in humans (*193, 194, 199-201*). Additional studies regarding the effect of dieldrin exposure on dopamine metabolism, with respect to changes in DOPAL

concentration and 4HNE-modified proteins in an animal model will also be important. This will be useful to validate the results observed from acute dieldrin exposure in a cellular model and to take into consideration the metabolism and accumulation of dieldrin in a chronic study.

Another direction for future study is the disparity between the level of reactive oxygen species produced as compared to the disruption in dopamine metabolism. This will be investigated using inhibitors of dopamine metabolism (including deprenyl and MDA) as well as ROS scavengers, i.e. catalase and MnTBAP. This will allow for the more precise identification of differences in toxicity, such as increased activity of MAO, inhibition of ALDH, or if a decrease in reactive oxygen species corrects the disruption in dopamine metabolism. The investigation of the effect of each of the dieldrin analog on specific aspects of the apoptotic pathway, such as on the activation of caspase-3 and caspase-9, will also be completed. This will provide information regarding differences in the activation of the apoptotic pathway between each of the analogs, which may account for the toxicity differences observed.

The results outlined here as well as the future directions are in line with the long-term goals of this project, to provide a complete structure-activity relationship for both molinate and dieldrin, that will be useful in determining their respective mechanisms of toxicity in mammals. Additional goals include the identification of specific protein targets of dieldrin, elucidation of therapeutic targets for dieldrin-induced PD, determination of biomarkers for earlier detection of dieldrin exposure, as well as the development of a course of action to prevent the adverse effects caused by dieldrin and molinate. While the results of this project have made significant progress towards the ultimate goals of this project, millions of tons of pesticides are applied across the United States each year, without much thought as to the long-term effects of exposure and use of these compounds, with respect to human disease and insect resistance. Hopefully, through the diligent investigation of the toxic mechanisms of

these compounds and re-evaluation of the regulation of their use, the adverse effects of pesticides on the environment and human health can be minimized, with the idealistic goal of eliminating the need for pesticides altogether.

APPENDIX

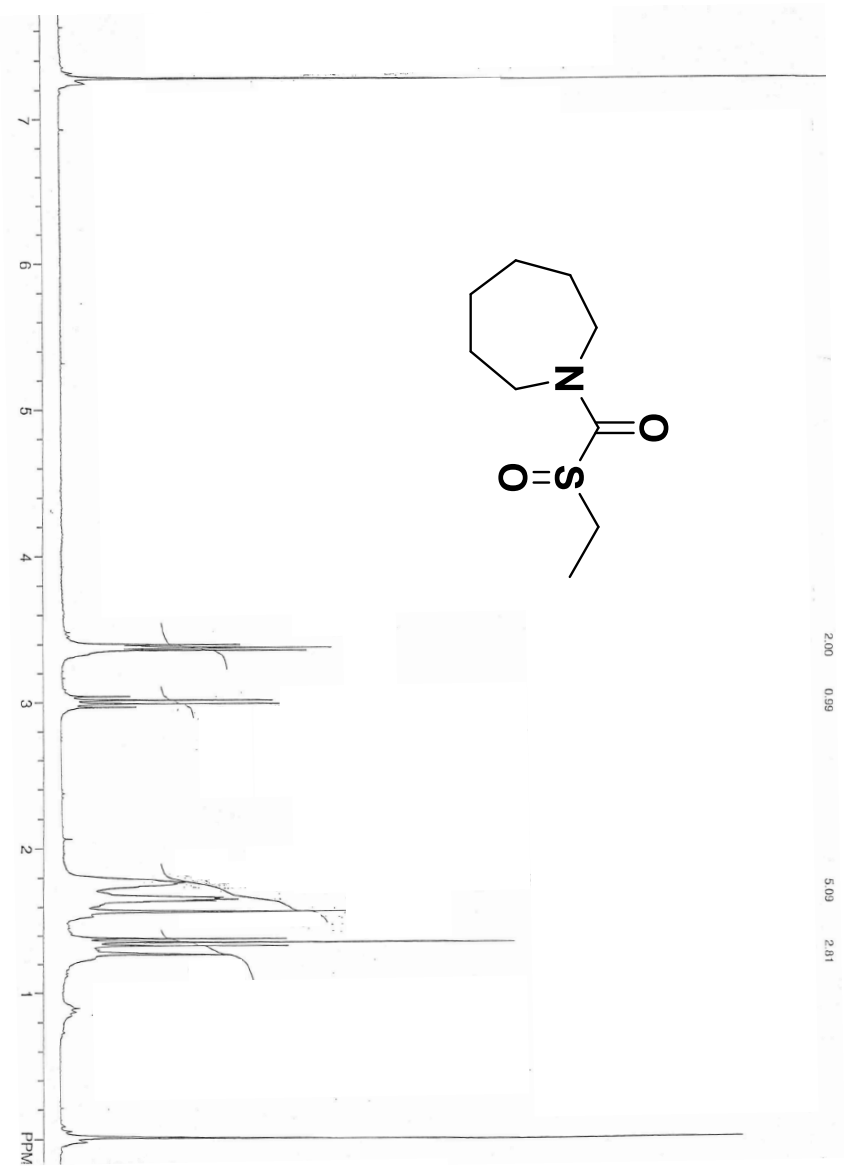


Figure A.1. ^{1}H NMR Spectra (300 mHz, $CDCl_3$) of molinate sulfoxide

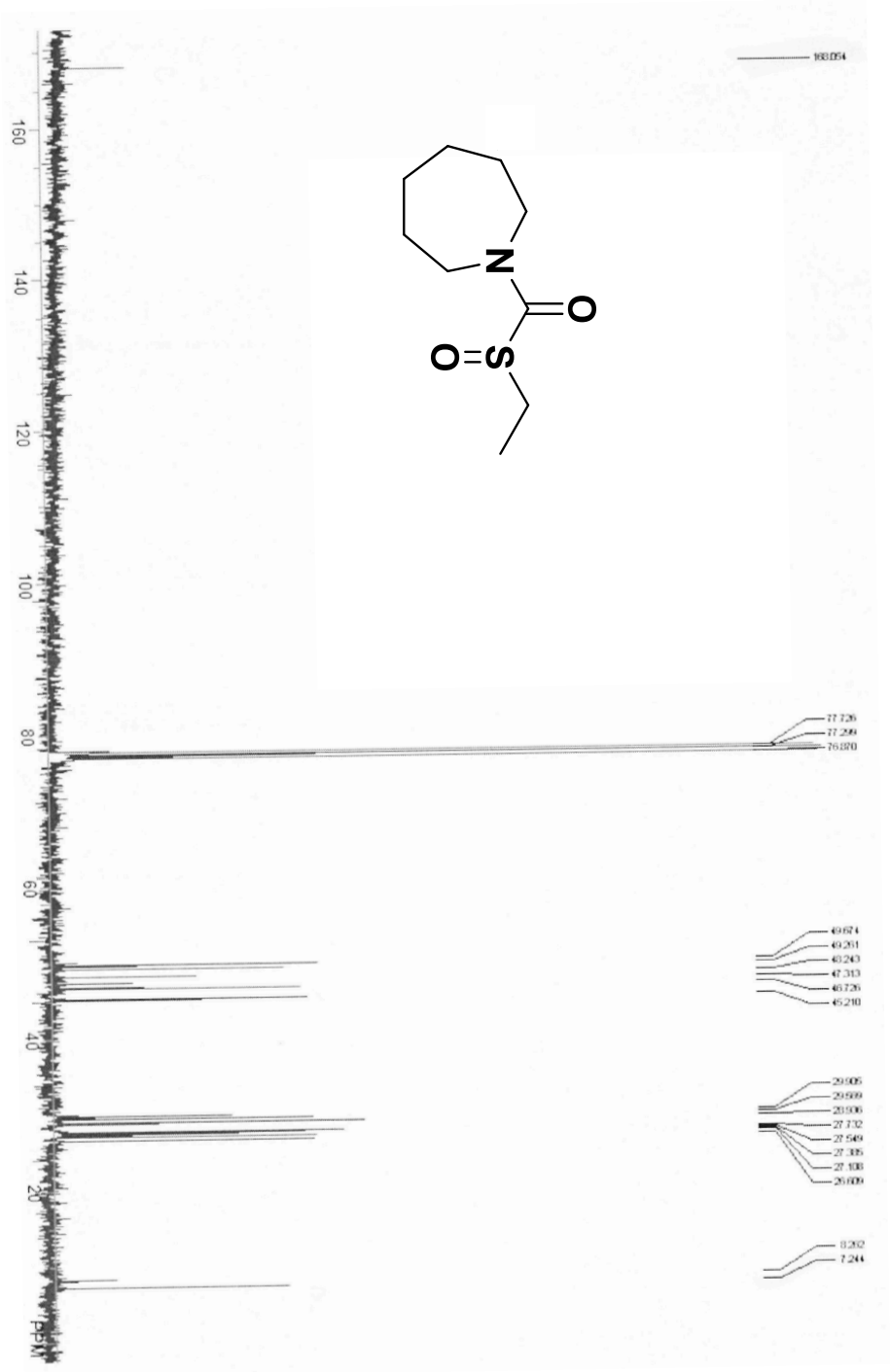
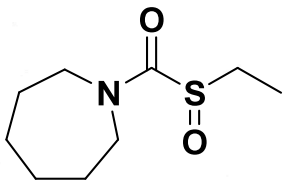


Figure A.2. ^{13}C NMR Spectra (300 mHz, $CDCl_3$) of molinate sulfoxide

Elemental Composition Report

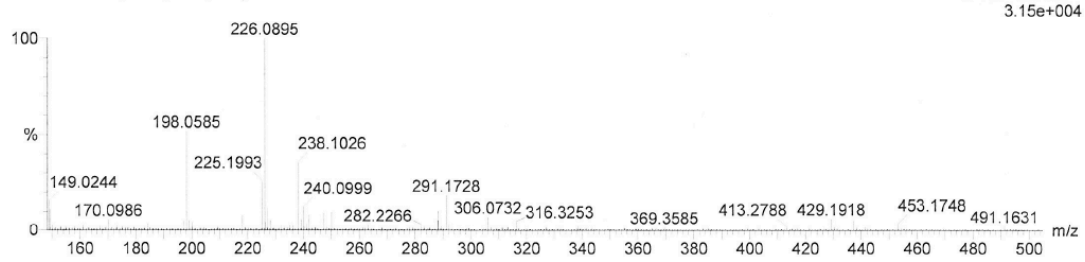


Single Mass Analysis
Tolerance = 3.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
276 formula(e) evaluated with 2 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-500 H: 0-1000 N: 0-6 O: 0-10 S: 0-1 Na: 1-1

Molinate Sulfoxide Allen/Doorn
T05051046 51 (2.089) Cm (8:66)

4
1: TOF MS ES+
3.15e+004



Minimum:				-1.5			
Maximum:		3.0	20.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
226.0895	226.0878	1.7	7.5	1.5	123.3	0.0	C9 H17 N O2 S Na
	226.0916	-2.1	-9.3	2.5	141.1	17.8	C6 H13 N5 O3 Na

Figure A.3 Elemental composition and mass spectra (ESI^+) of molinate sulfoxide

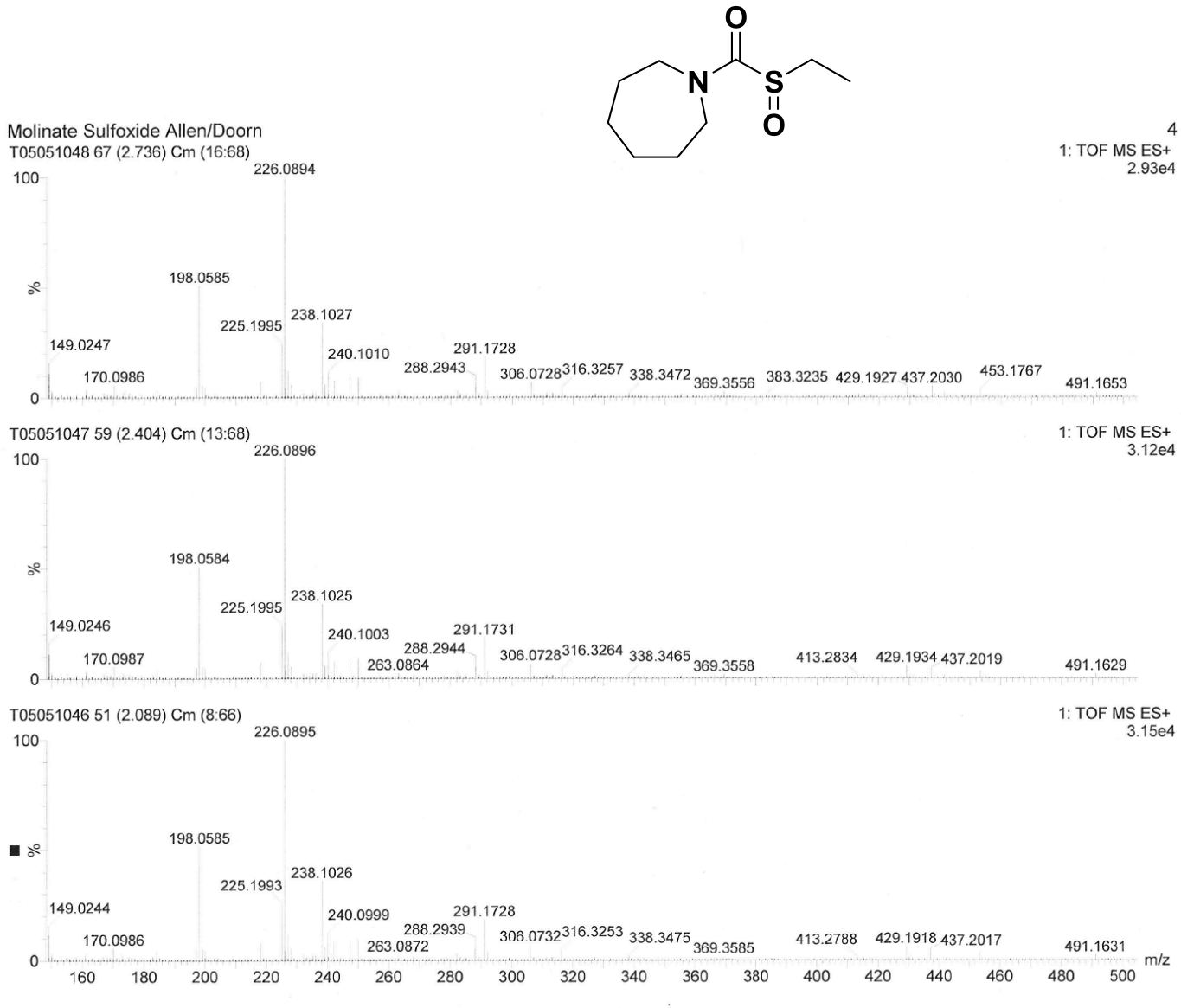


Figure A.4. Mass Spectra (ESI^+) of molinate sulfoxide

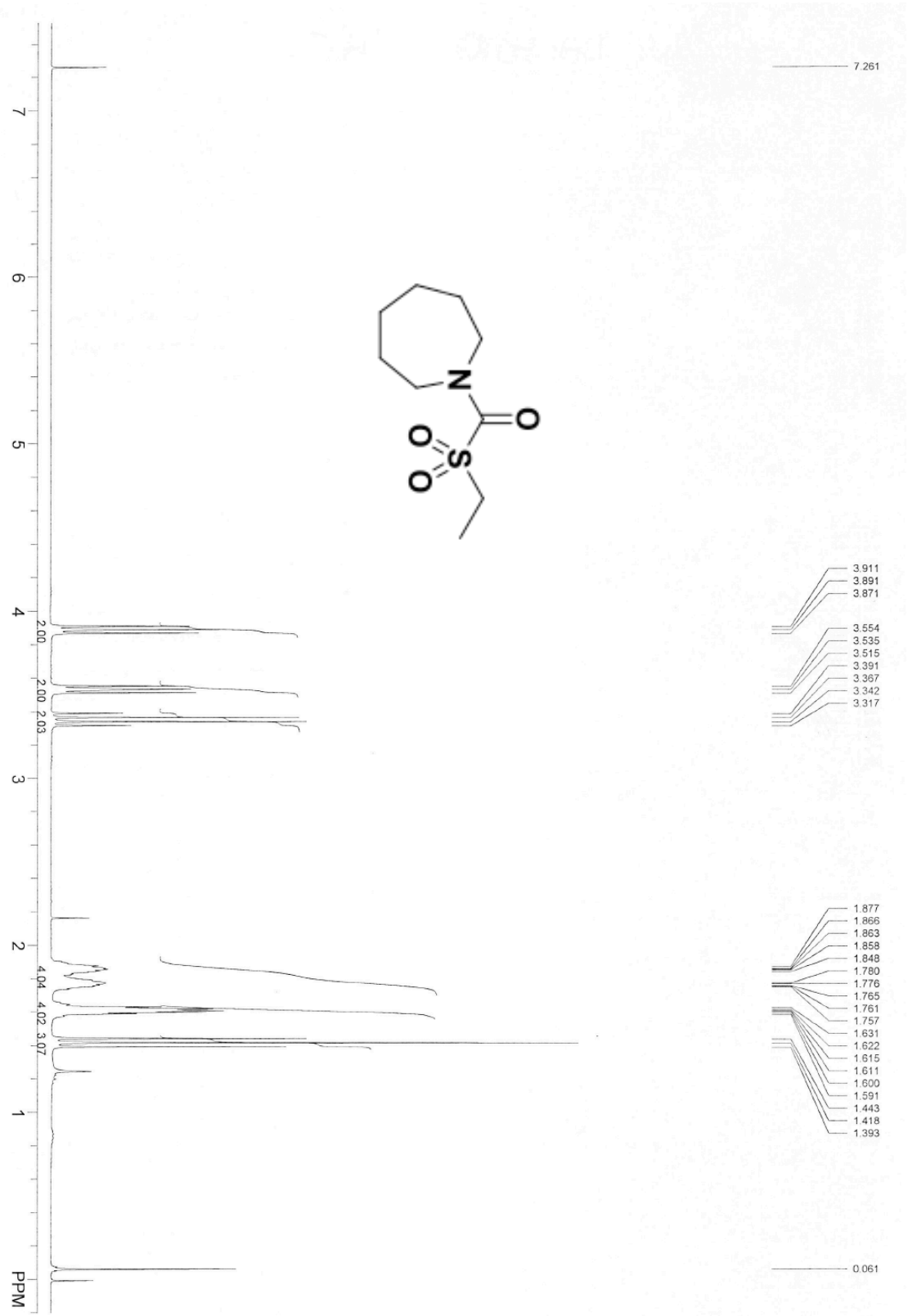


Figure A.5. ^{1}H NMR Spectra (300 mHz, $CDCl_3$) of molinate sulfone

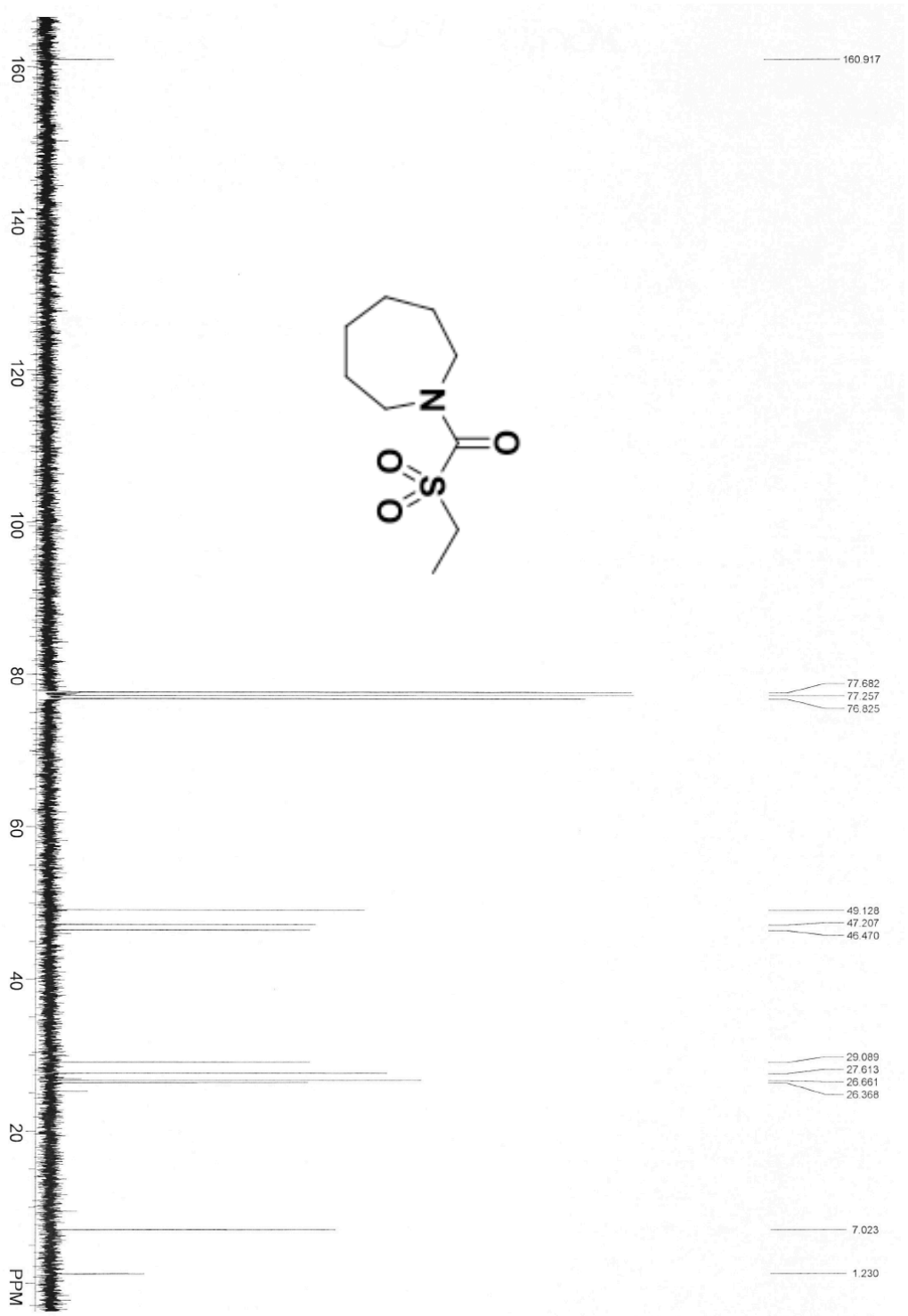


Figure A.6. ^{13}C NMR Spectra (300 mHz, $CDCl_3$) of molinate sulfone

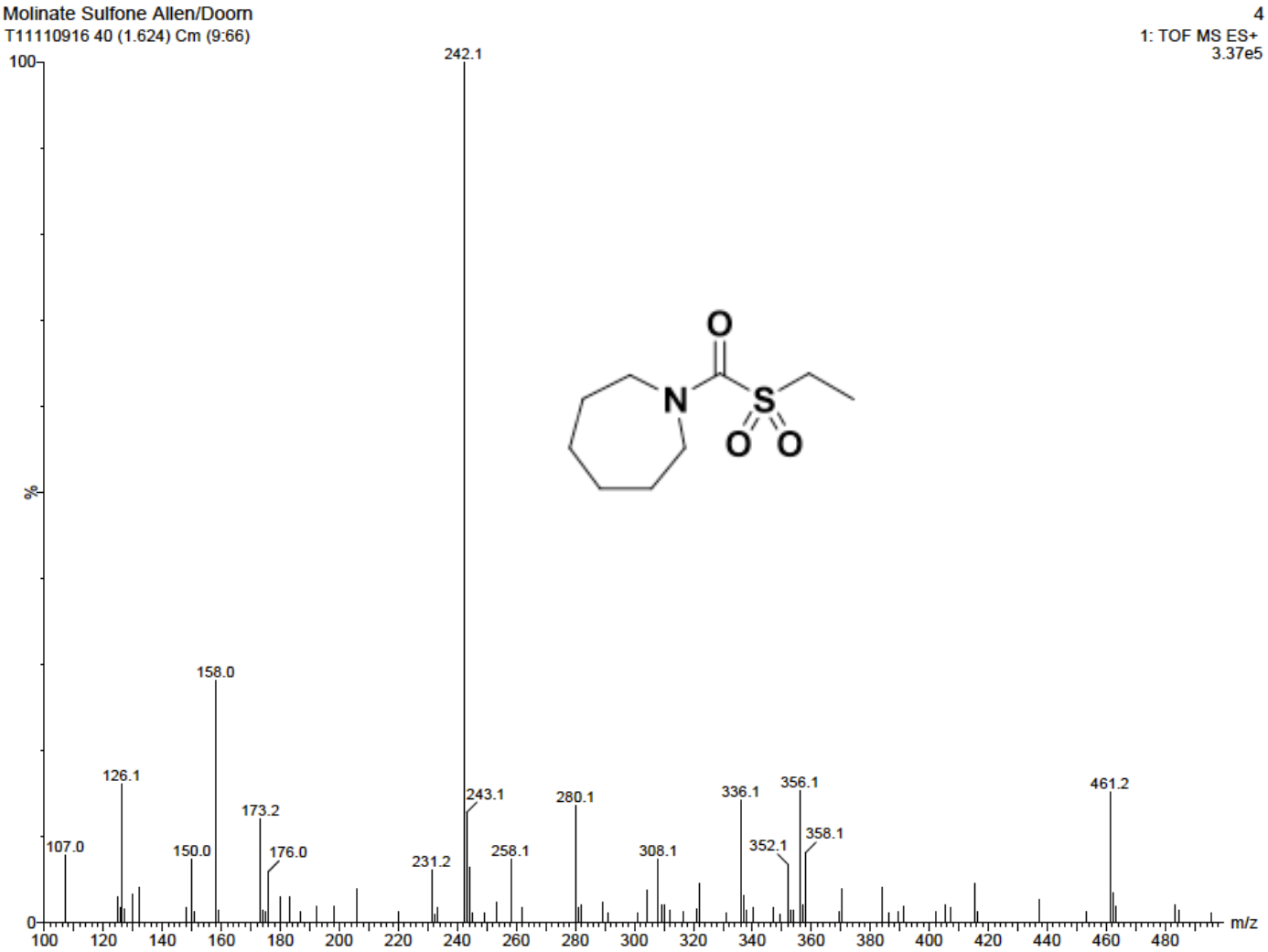


Figure A.7. Mass spectra (ESI^+) of molinate sulfone

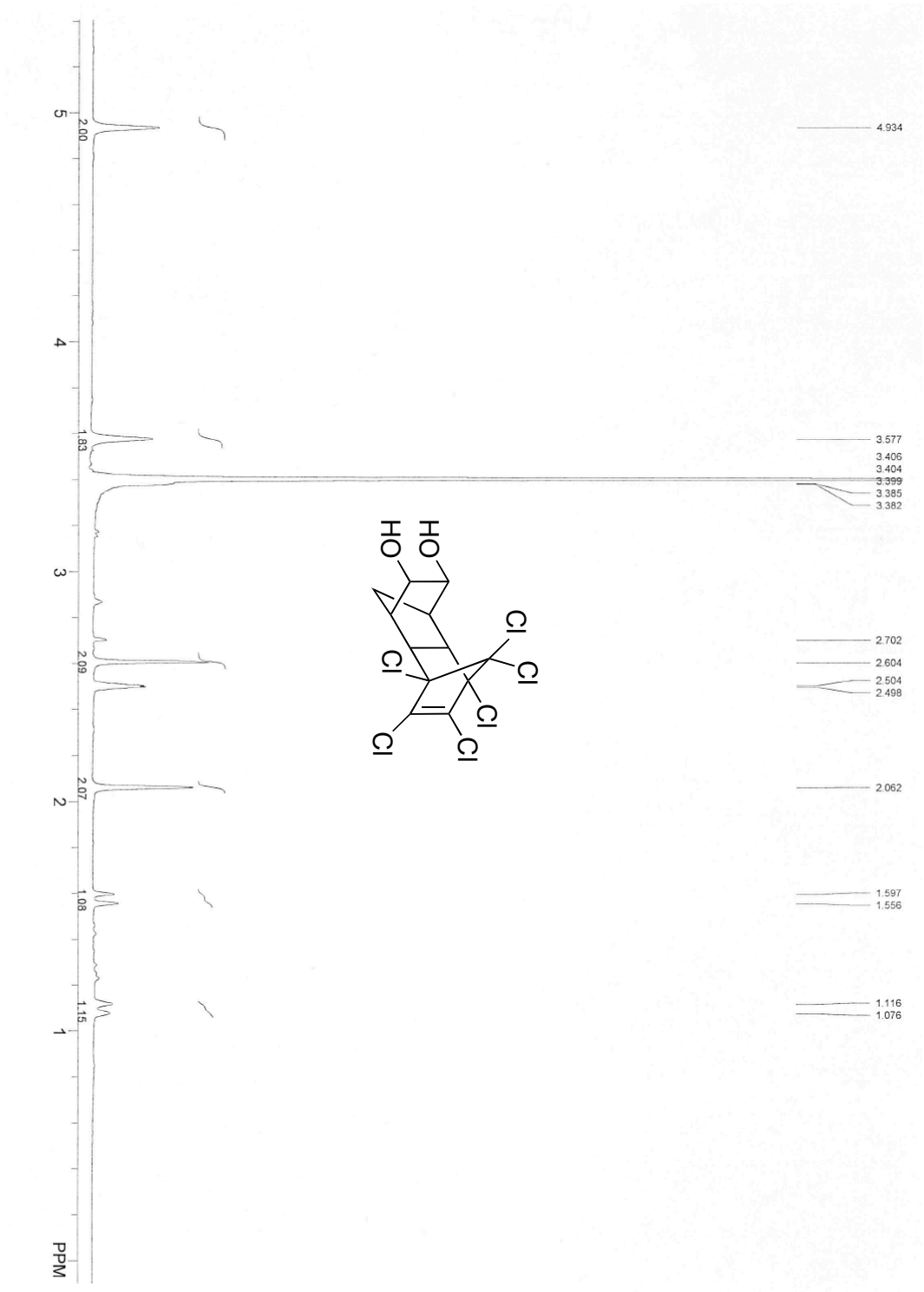


Figure A.8. ^{1}H NMR Spectra (300 mHz, DMSO) of cis aldrin diol.

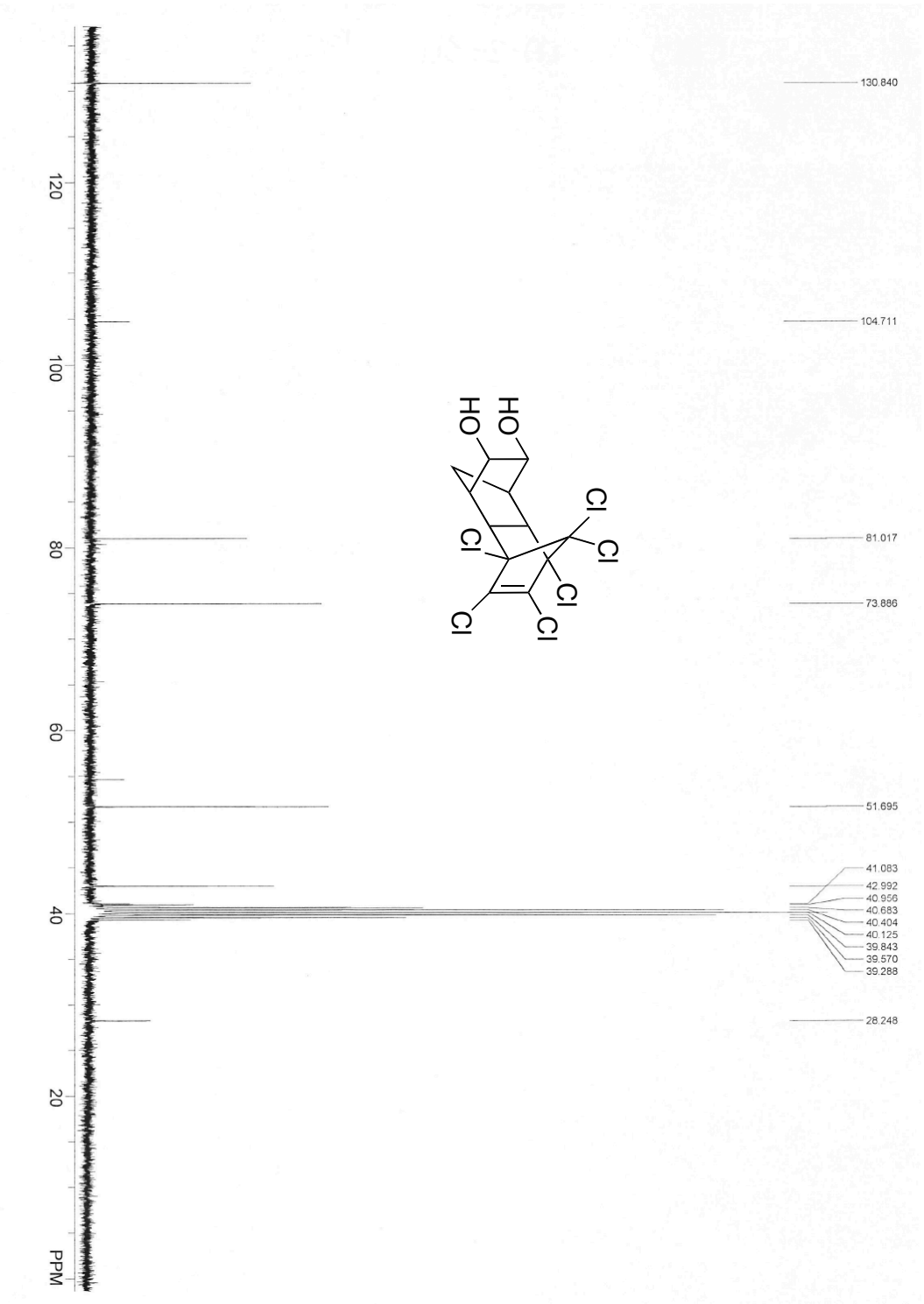


Figure A.9. ^{13}C NMR Spectra (300 mHz, DMSO) of cis aldrin diol.

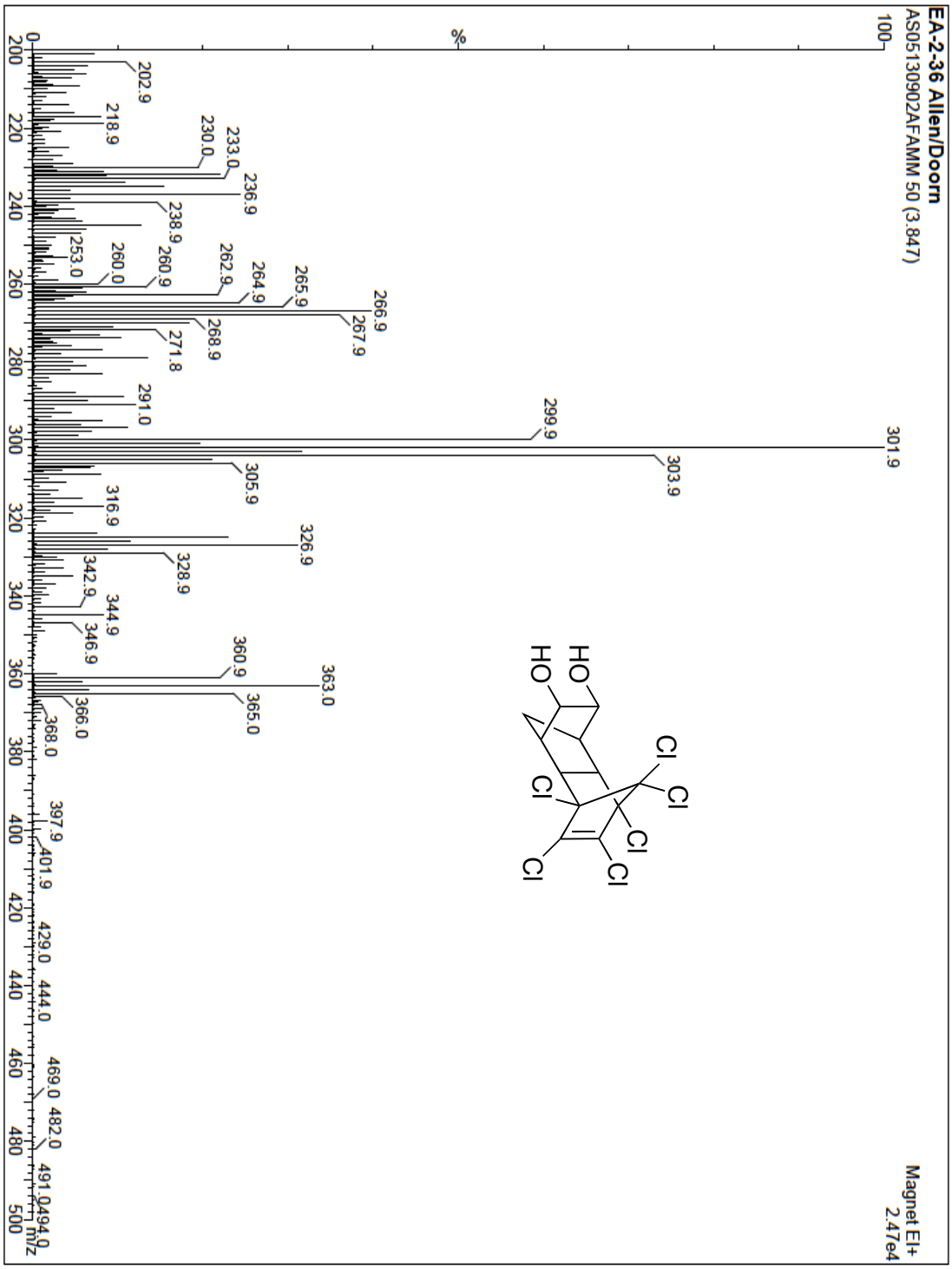


Figure A.10. Mass Spectra (EI^+) of cis aldrin diol.

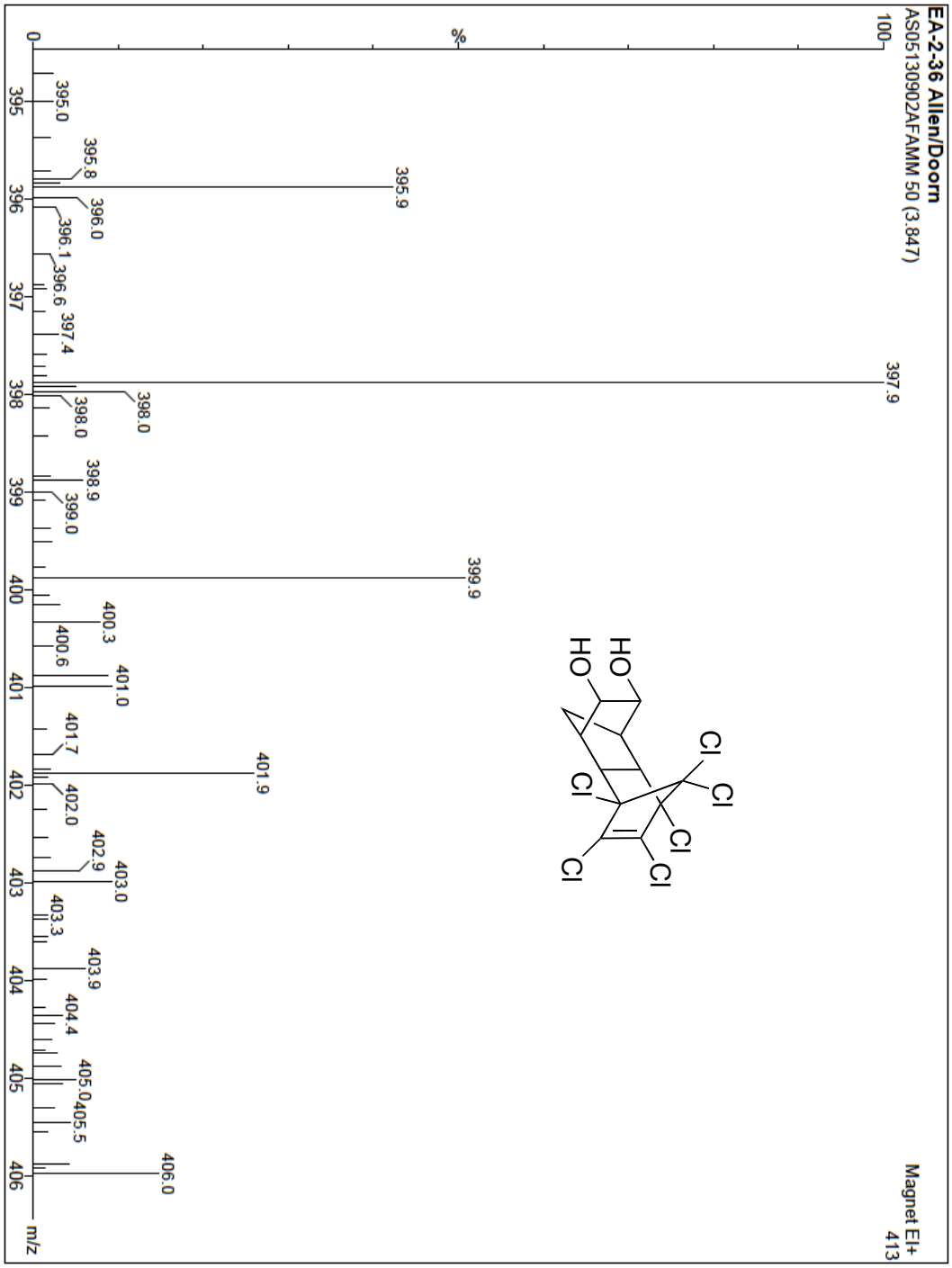


Figure A.11. Expansion of *m/z* 355-406 of cis aldrin diol mass spectra (EI+).

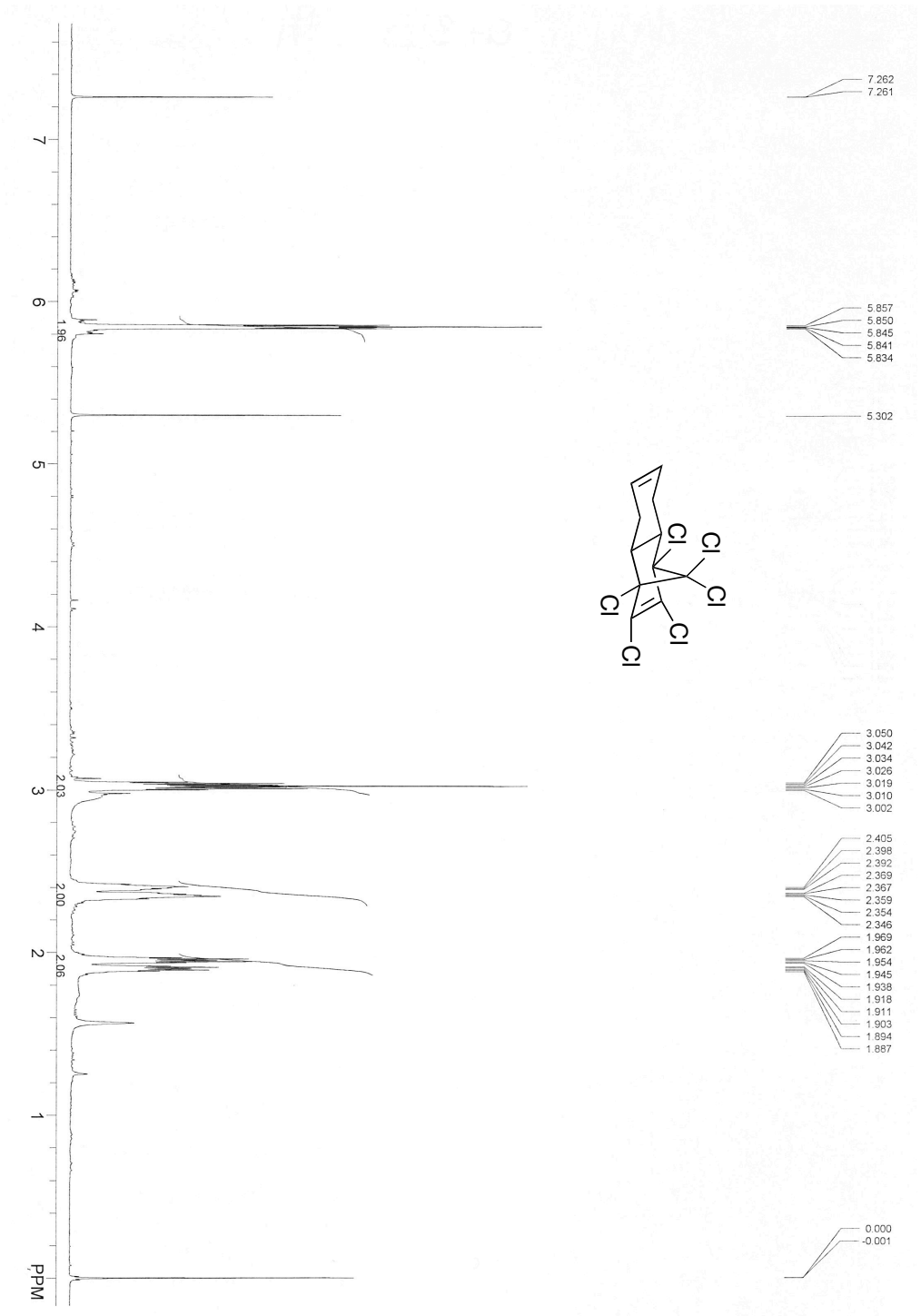


Figure A.12. ^{1}H NMR Spectra (300 mHz, $CDCl_3$) of desmethylene aldrin.

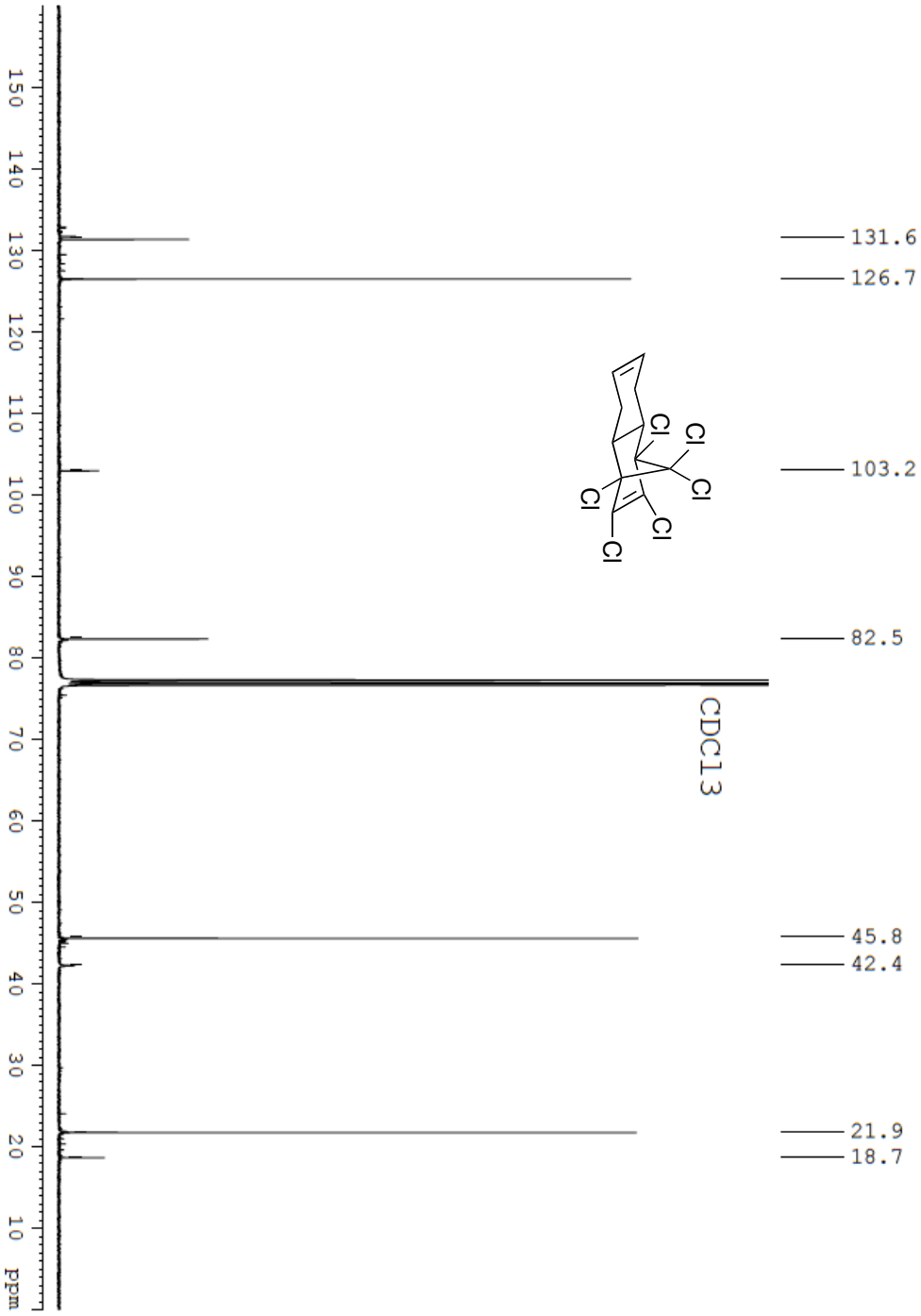
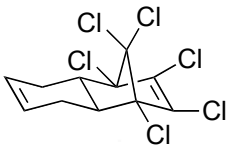


Figure A.13. ^{13}C NMR Spectra (400 mHz, $CDCl_3$) of desmethylene aldrin.

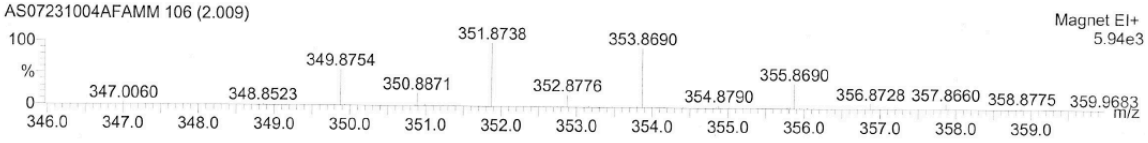


Elemental Composition Report

Multiple Mass Analysis: 4 mass(es) processed - displaying only valid results
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
337 formula(e) evaluated with 6 results within limits (up to 50 closest results for each mass)

EA-3-8 Allen/Doorn
AS07231004AFAMM 106 (2.009)



Minimum:	20.00				-1.5		
Maximum:	100.00		5.0	5.0	50.0		
Mass	RA	Calc. Mass	mDa	PPM	DBE	Score	Formula
355.8690	38.38	355.8669	2.1	6.0	5.0	1	C11 H8 35Cl3 37Cl3
353.8690	93.15	353.8698	-0.8	-2.3	5.0	1	C11 H8 35Cl4 37Cl2
		353.8651	3.9	11.0	0.0	2	C8 H11 35Cl6 37Cl
351.8738	100.00	351.8728	1.0	2.9	5.0	1	C11 H8 35Cl5 37Cl
		351.8775	-3.7	-10.5	10.0	2	C14 H5 35Cl3 37Cl2
349.8754	56.61	349.8757	-0.3	-0.9	5.0	1	C11 H8 35Cl6

Figure A.14. Elemental composition and mass spectra (EI^+) of desmethylene aldrin.

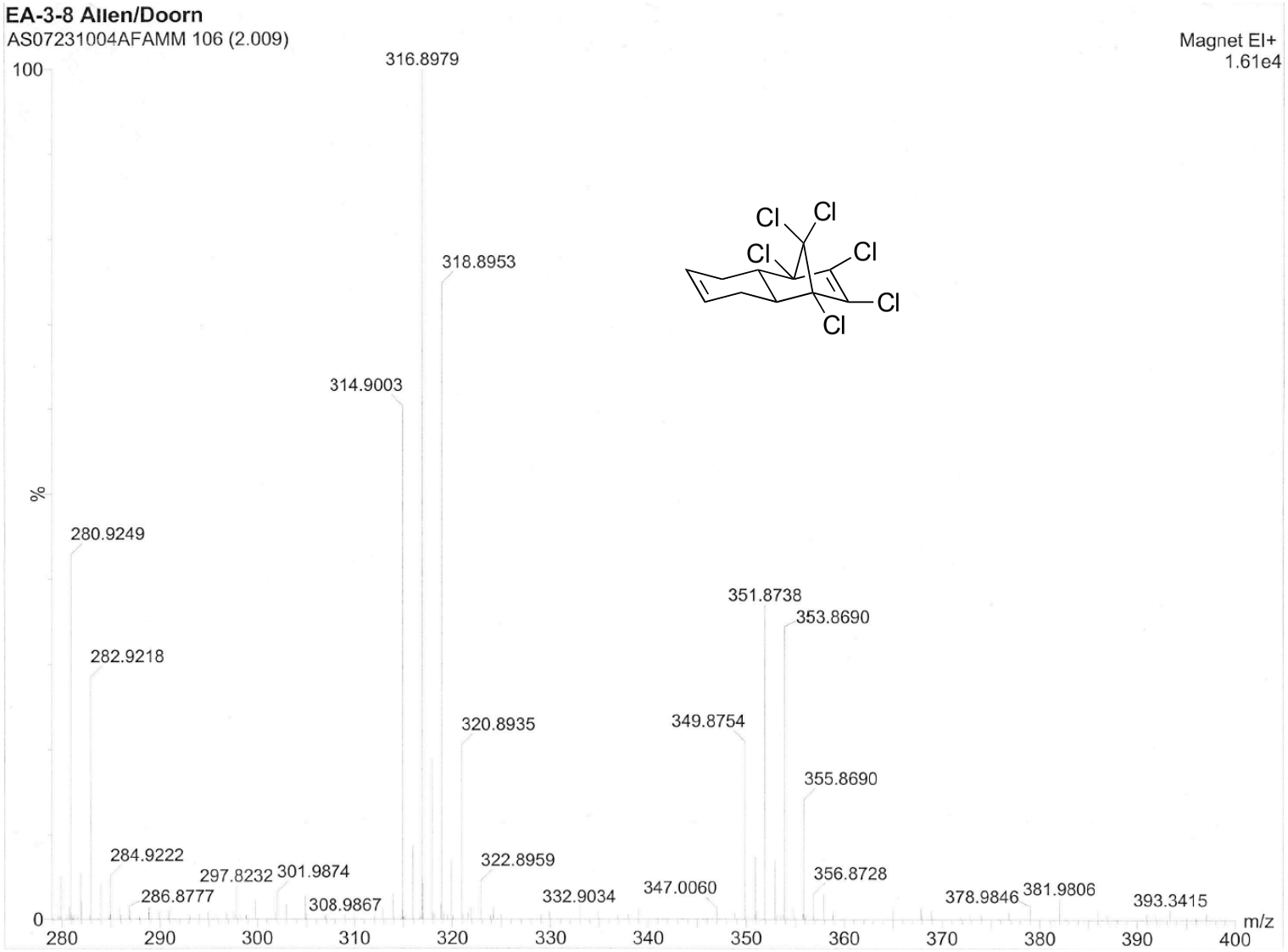


Figure A.15. Mass spectra (EI^+) of desmethylene aldrin.

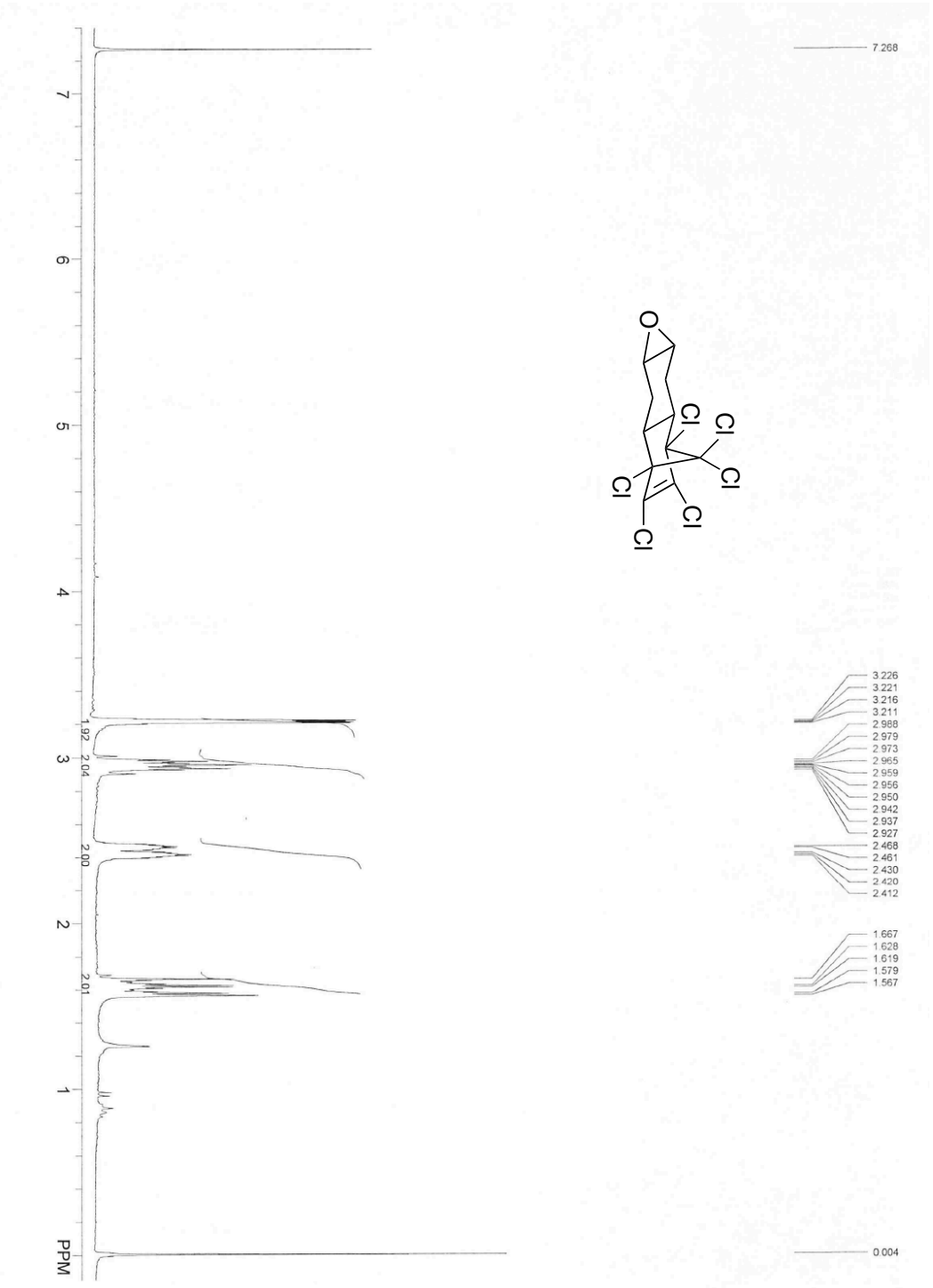


Figure A.16. ^{1}H NMR Spectra (300 mHz, $CDCl_3$) of desmethylene dieldrin.

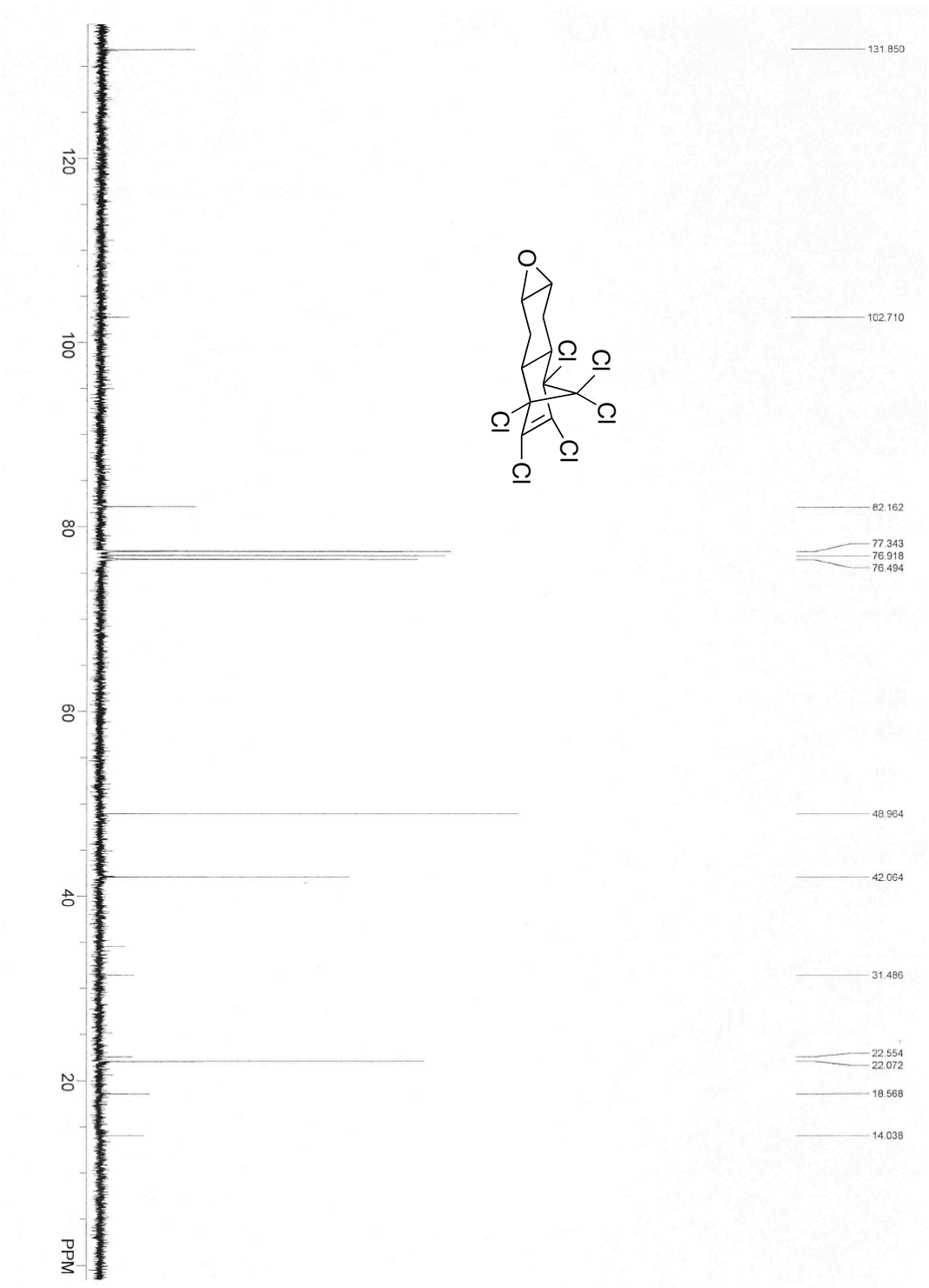
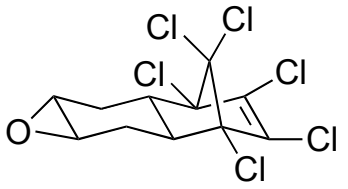


Figure A.17. ^{13}C NMR Spectra (300 mHz, $CDCl_3$) of desmethylene dieldrin.

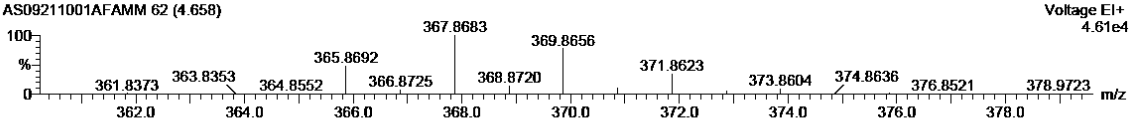


Elemental Composition Report **Page 1**

Multiple Mass Analysis: 4 mass(es) processed - displaying only valid results
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
10756 formula(e) evaluated with 34 results within limits (up to 50 closest results for each mass)

EA-3-70 Allen/Doom
AS09211001AFAMM 62 (4.658)
Voltage EI+
4.61e4



Minimum:	27.00				-1.5		
Maximum:	100.00		5.0	5.0	50.0		
Mass	RA	Calc. Mass	mDa	PPM	DBE	Score	Formula
371.8623	34.79	371.8626	-0.3	-0.8	4.0	n/a	12C10 H8 O2 35Cl5 37Cl
		371.8618	0.5	1.4	5.0	n/a	12C11 H8 O 35Cl3 37Cl3
		371.8628	-0.5	-1.5	9.5	n/a	12C12 13C H4 O2 35Cl3 37Cl2
		371.8603	2.0	5.3	13.5	n/a	12C15 13C H2 35Cl4 37Cl
		371.8601	2.2	6.0	8.0	n/a	12C13 H6 35Cl6
		371.8648	-2.5	-6.7	13.0	n/a	12C16 H3 35Cl4 37Cl
		371.8581	4.2	11.2	4.5	n/a	12C9 13C H7 O2 35Cl5 37Cl
		371.8573	5.0	13.4	5.5	n/a	12C10 13C H7 O 35Cl3 37Cl3
369.8656	77.41	369.8655	0.1	0.1	4.0	n/a	12C10 H8 O2 35Cl6
		369.8658	-0.2	-0.5	9.5	n/a	12C12 13C H4 O2 35Cl4 37Cl
		369.8650	0.6	1.7	10.5	n/a	12C13 13C H4 O 35Cl2 37Cl3
		369.8647	0.9	2.3	5.0	n/a	12C11 H8 O 35Cl4 37Cl2
		369.8677	-2.1	-5.8	13.0	n/a	12C16 H3 35Cl5
		369.8633	2.3	6.3	13.5	n/a	12C15 13C H2 35Cl5
		369.8695	-3.9	-10.4	10.0	n/a	12C14 H5 O 35Cl2 37Cl3
		369.8611	4.5	12.2	4.5	n/a	12C9 13C H7 O2 35Cl6
		369.8703	-4.7	-12.6	9.0	n/a	12C13 H5 O2 35Cl4 37Cl
		369.8705	-4.9	-13.3	14.5	n/a	12C15 13C H O2 35Cl2 37Cl2
367.8683	100.00	367.8679	0.4	1.0	10.5	n/a	12C13 13C H4 O 35Cl3 37Cl2
		367.8687	-0.4	-1.2	9.5	n/a	12C12 13C H4 O2 35Cl5
		367.8677	0.6	1.7	5.0	n/a	12C11 H8 O 35Cl5 37Cl
		367.8669	1.4	3.9	6.0	n/a	12C12 H8 35Cl3 37Cl3
		367.8724	-4.1	-11.2	10.0	n/a	12C14 H5 O 35Cl3 37Cl2
		367.8727	-4.4	-11.8	15.5	n/a	12C16 13C H O 35Cl 37Cl3
		367.8732	-4.9	-13.4	9.0	n/a	12C13 H5 O2 35Cl5
365.8692	47.61	365.8698	-0.6	-1.7	6.0	n/a	12C12 H8 35Cl4 37Cl2
		365.8701	-0.9	-2.4	11.5	n/a	12C14 13C H4 35Cl2 37Cl3
		365.8679	1.3	3.6	2.5	n/a	12C8 13C H9 O2 35Cl3 37Cl3
		365.8706	-1.4	-3.9	5.0	n/a	12C11 H8 O 35Cl6
		365.8709	-1.7	-4.6	10.5	n/a	12C13 13C H4 O 35Cl4 37Cl
		365.8662	3.0	8.3	5.5	n/a	12C10 13C H7 O 35Cl6
		365.8723	-3.1	-8.6	2.0	n/a	12C9 H10 O2 35Cl3 37Cl3
		365.8653	3.9	10.5	6.5	n/a	12C11 13C H7 35Cl4 37Cl2
		365.8651	4.1	11.2	1.0	n/a	12C9 H11 35Cl6 37Cl

Figure A.18. Elemental composition and mass spectra of desmethylene dieldrin.

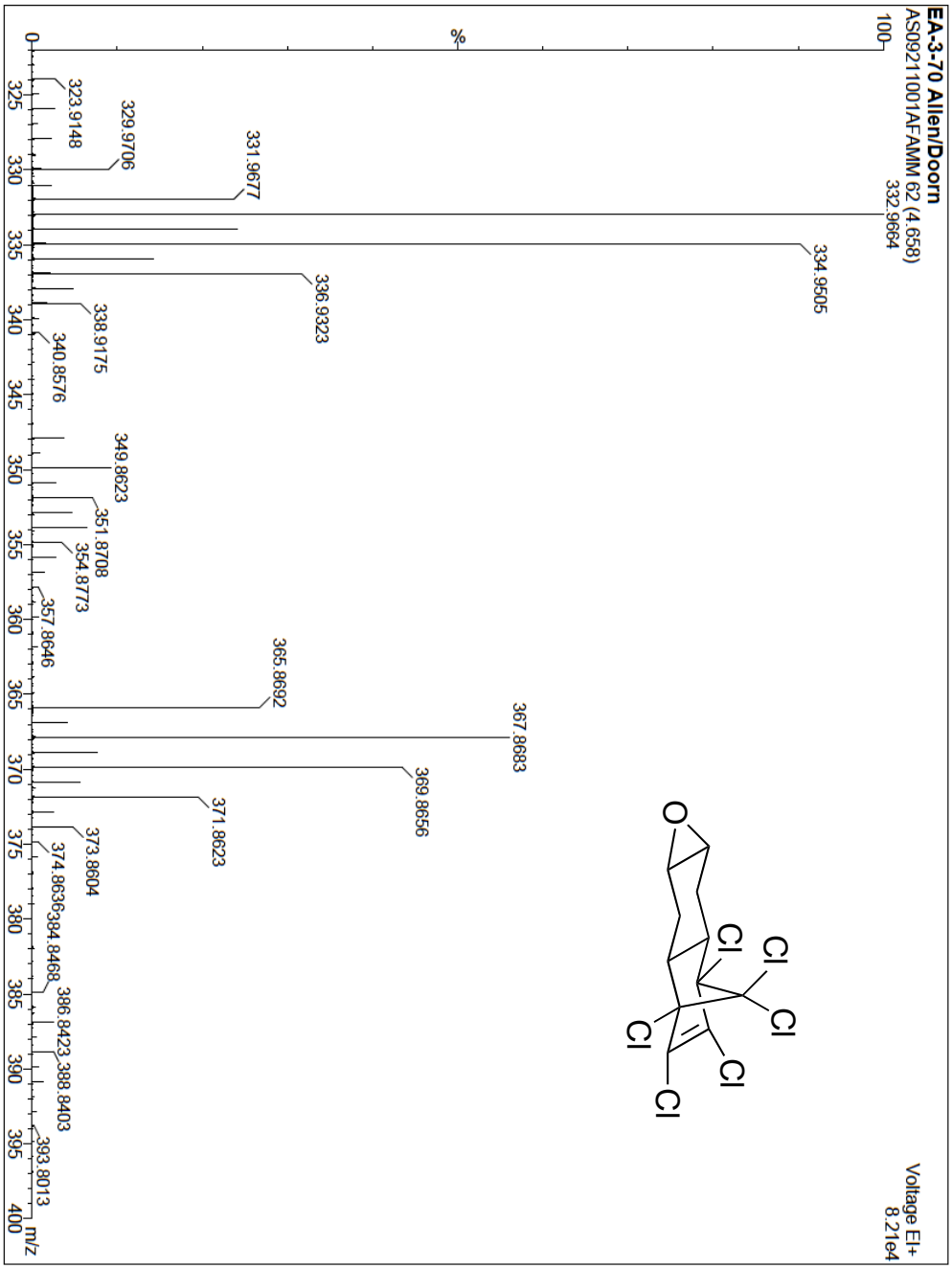


Figure A.19. Mass spectra (EI^+) of desmethylene dieldrin.

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