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Protein reactivity of 3,4-dihydroxyphenylacetaldehyde, an endogenous, potential neurotoxin relevant to Parkinson's disease

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PROTEIN REACTIVITY OF 3,4-DIHYDROXYPHENYLACETALDEHYDE, AN ENDOGENOUS, POTENTIAL NEUROTOXIN RELEVANT TO PARKINSON'S DISEASE

by

Jennifer Nicole Rees

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Pharmacy
in the Graduate College of
The University of Iowa

July 2009

Thesis Supervisor: Assistant Professor Jonathan A. Doorn

ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder characterized by two pathological hallmarks, selective loss of dopaminergic neurons and intraneuronal protein aggregation. The presence of an endogenous neurotoxin has been implicated in the pathogenesis of the disease, to explain the observed neurodegeneration. Dopamine (DA) has been indicated to be an endogenous neurotoxin as DA readily undergoes auto-oxidation to an o-quinone capable of protein modification. However, DA is metabolized by monoamine oxidase to form the intermediate 3,4-dihydroxyphenylacetaldehyde (DOPAL) and several studies have demonstrated DOPAL to be orders of magnitude more toxic than DA. An accumulation of DOPAL may cause dopaminergic cell death via the formation of free radicals, inhibition of the mitochondrial transition pore or protein modification. The hypothesis of this work is that DOPAL, a potential endogenous neurotoxin relevant to PD, is capable of protein modification and protein cross-linking through reactivity with amine and thiol nucleophiles. Results demonstrate that elevated DOPAL concentrations in striatal synaptosomes will yield considerable protein modification. In addition, DOPAL was demonstrated to be highly reactive towards amine nucleophiles in comparison to thiol nucleophiles. However, DOPAL was demonstrated to mediate protein cross-linking through reactivity with protein thiols subsequent to modification of amines, indicating DOPAL to be a bifunctional electrophile. Furthermore, a novel isolation procedure was developed, and through a proteomics-based approach, twelve proteins were identified to be relevant to PD and susceptible to DOPAL modification. This research demonstrates increased concentrations of DOPAL lead to significant cellular consequences (i.e. protein modification) and implicate DOPAL as a potential neurotoxin relevant to the pathogenesis of PD.

Abstract Approved: _____
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Graduate College
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CERTIFICATE OF APPROVAL

PH.D. THESIS

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

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has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Pharmacy at the July 2009 graduation.

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Michael W. Duffel

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Kevin G. Rice

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Larry W. Robertson

To Hudson

A discovery is like falling in love and reaching to the top of a mountain after a hard climb all in one, an ecstasy induced not by drugs, but by the revelation of a face of nature that no one has seen before.

Max F. Perutz
Nobel Prize in Chemistry, 1962

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder characterized by two pathological hallmarks, selective loss of dopaminergic neurons and intraneuronal protein aggregation. The presence of an endogenous neurotoxin has been implicated in the pathogenesis of the disease, to explain the observed neurodegeneration. Dopamine (DA) has been indicated to be an endogenous neurotoxin as DA readily undergoes auto-oxidation to an o-quinone capable of protein modification. However, DA is metabolized by monoamine oxidase to form the intermediate 3,4-dihydroxyphenylacetaldehyde (DOPAL) and several studies have demonstrated DOPAL to be orders of magnitude more toxic than DA. An accumulation of DOPAL may cause dopaminergic cell death via the formation of free radicals, inhibition of the mitochondrial transition pore or protein modification. The hypothesis of this work is that DOPAL, a potential endogenous neurotoxin relevant to PD, is capable of protein modification and protein cross-linking through reactivity with amine and thiol nucleophiles. Results demonstrate that elevated DOPAL concentrations in striatal synaptosomes will yield considerable protein modification. In addition, DOPAL was demonstrated to be highly reactive towards amine nucleophiles in comparison to thiol nucleophiles. However, DOPAL was demonstrated to mediate protein cross-linking through reactivity with protein thiols subsequent to modification of amines, indicating DOPAL to be a bifunctional electrophile. Furthermore, a novel isolation procedure was developed, and through a proteomics-based approach, twelve proteins were identified to be relevant to PD and susceptible to DOPAL modification. This research demonstrates increased concentrations of DOPAL lead to significant cellular consequences (i.e. protein modification) and implicate DOPAL as a potential neurotoxin relevant to the pathogenesis of PD.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

LIST OF SCHEMES

Scheme

LIST OF ABBREVIATIONS

3,4-Dihydroxyphenylacetaldehyde ..... DOPAL

3,4-Dihydroxyphenylacetic acid..... DOPAC

3,4-Dihydroxyphenylalanine..... L-DOPA

3,4-Dihydroxyphenylethanol..... DOPET

3,4-Dimethoxyphenylacetaldehyde ..... DMPAL

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

4-Hydroxy-2-nonenal 4HNE

3-Methoxy-4-hydroxyphenylacetaldehyde. ..... MOPAL

1-methyl-4-phenyl-4-propionoxypiperidine ..... MPPP

1-Methyl-4-phenylpyridinium. ..... MPP^+

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine..... MPTP

Acetonitrile..... ACN

Aldehyde dehydrogenase ..... ALDH

Aldehyde or aldose reductase..... AR

Aminophenylboronic acid resin ..... APBA

Blood brain barrier. ..... BBB

Bovine serum albumin ..... BSA

Catechol-o-methyl transferase ..... COMT

Collision induced dissociation. ..... CID

Dihydroxyacetone phosphate. ..... DHAP

Dopamine. ..... DA

Dopamine transporter ..... DAT

Endoplasmic reticulum. ..... ER

Glyceraldehyde-3-phosphate. ..... GAP

Glyceraldehyde-3-phosphate dehydrogenase..... GAPDH

Glutathione. ..... GSH

Glutathione S-transferase. ..... GST

Heat shock protein. ..... HSP

Ion trap mass spectrometer. ..... ITMS

Leucine-rich repeat kinase 1. ..... LRRK2

Malondialdehyde ..... MDA

Matrix assisted laser desorption/ionization time-of-flight mass spectrometry ..... MALDI-TOF-MS

Monoamine oxidase ..... MAO

N-acetyl-cysteine ..... NAC

N-acetyl-lysine ..... NAL

Nerve growth factor. ..... NGF

Neuromelanin. ..... NM

Nitroblue tetrazolium ..... NBT

Paraquat ..... PQ

Parkinson's disease. ..... PD

Phenylacetaldehyde ..... PAL

Reactive oxygen species ..... ROS

Substantia nigra. ..... SN

Sodium cyanoborohydride ..... $NaCNBH_3$

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

Tensin homologue-induced putative kinase 1. ..... PINK1

Tetrahydropyranyl ..... THP

Tris-buffered saline with Tween. ..... TBS-T

Trifluoroacetic acid ..... TFA

Tyrosine hydroxylase ..... TH

CHAPTER ONE

INTRODUCTION

> Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured.
>
> James Parkinson, Essay on the Shaking Palsy

The Shaking Palsy

Parkinson's disease (PD) was first described by James Parkinson in 1817 and is currently the second most-prevalent neurodegenerative disorder after Alzheimer's disease. PD affects about 1% of the general population and over a million individuals in North America alone with an incidence of new diagnoses of 50,000 per year (*3, 8*). Following Arvid Carlsson's 1958 discovery of dopamine (DA) in the mammalian brain (*2*), it was determined that the central pathological feature of PD is the selective loss of dopaminergic neurons in the pars compacta region of the substantia nigra (SN). Further studies also demonstrated the presence of intraneuronal protein deposits in the SN. These protein aggregates are termed Lewy bodies (LBs) and consist of a variety of proteins such as neurofilaments, ubiquitin and α-synuclein (*9*).

Dopaminergic neurons compose several distinct DA pathways in the brain. Two of these pathways, the mesolimbic/mesocortical pathway and the nigrostriatal pathway, are of particular interest because of their potential involvement in the disease process (*Figure 1.1*) (*3*). The mesolimbic/mesocortical pathway projects from the ventral tegmental area to the limbic system and frontal cortex. This pathway modulates behavior response to feelings of reward and is associated with the process of drug addiction. The nigrostriatal pathway projects from the SN to the striatum and is involved in the

regulation of motor function. The degeneration of the nigrostriatal pathway, as a result of the loss of striatal DA and the destruction of SN neurons, leads to the alteration of neural signaling that controls movement (*Figure 1.2*) (*10*). In the normal striatum, DA modulates the activity of inhibitory GABAergic neurons, which in turn modulate neuronal signaling to the motor cortex via "direct" and "indirect" pathways (*Figure 1.3*) (*3*). The direct pathway contains primarily excitatory DA D_1-type receptors that when stimulated increase the excitatory outflow to the motor cortex. The indirect pathway is predominantly inhibitory D_2-type receptors and will reduce the excitatory outflow to the motor cortex. In normal physiological conditions, DA released from the striatum tends to increase the activity of the direct pathway and decrease the activity of the indirect pathway. Individuals with PD have deficient levels of striatal DA, which leads to diminished activity of the direct pathway and increased activity of the indirect pathway. Overall, this results in decreased excitatory input to the motor cortex. The clinical manifestations of PD become apparent when striatal DA concentrations are reduced by approximately 80% and include symptoms such as resting tremor, muscle rigidity, slowness of movement (bradykinesia), sleep deficit and depression (*8*).

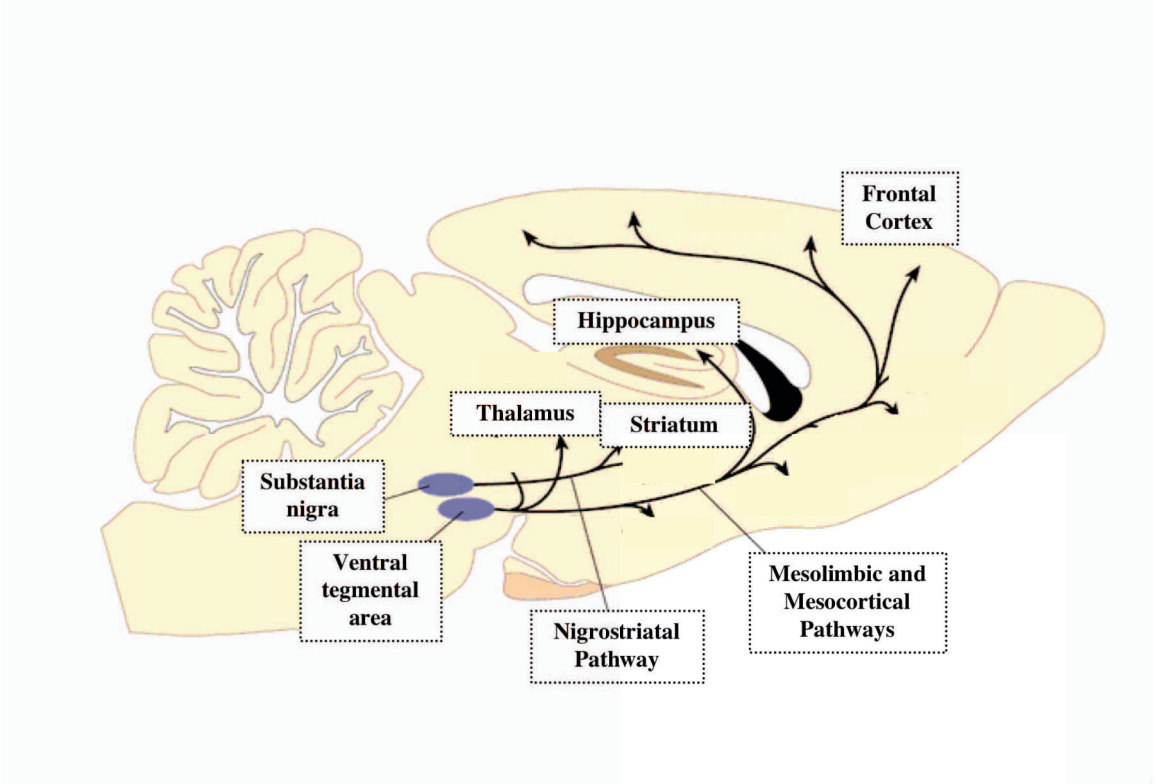


Figure 1.1 An illustration of some of the dopaminergic pathways in the brain. PD is a progressive degeneration of the nigrostriatal pathway, which is associated with the regulation of movement. Figure is adapted from (*1*).

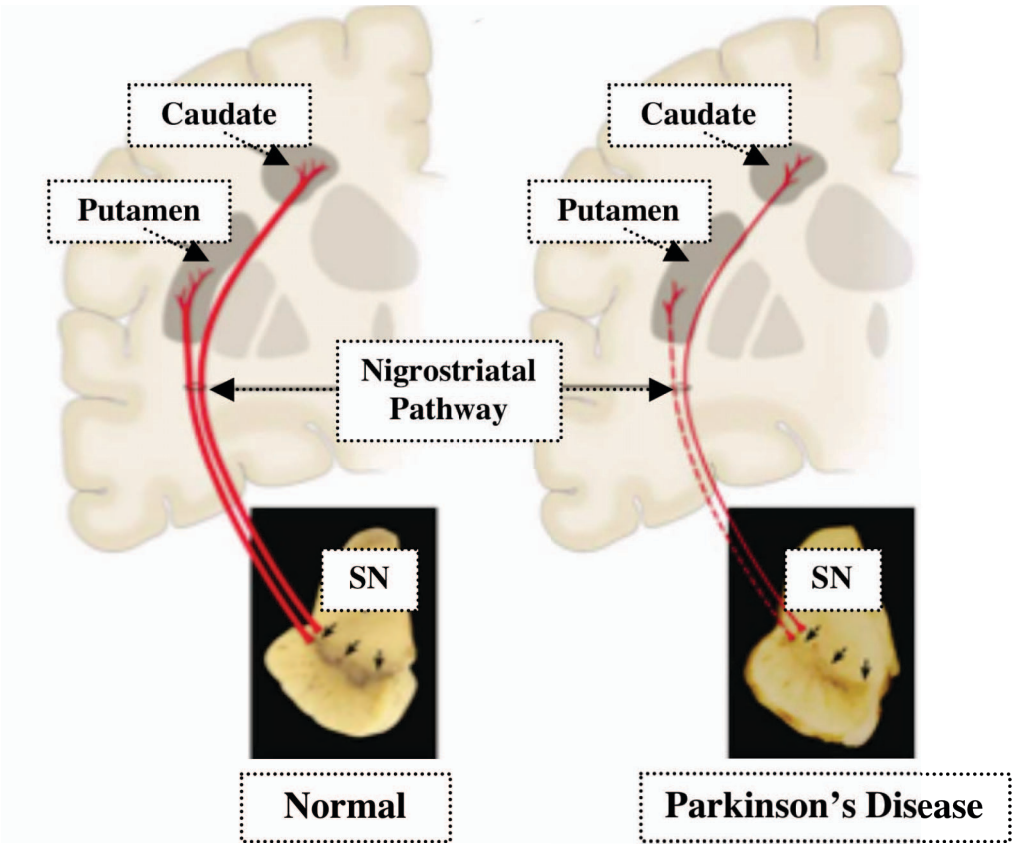


Figure 1.2 Schematic representation of the normal and diseased nigrostriatal dopaminergic pathways. The diseased pathway contains a marked loss in dopaminergic neurons projecting from the SN to the caudate and putamen (the striatum) as represented by the thin and dashed line, respectively. Figure is adapted from (*2*).

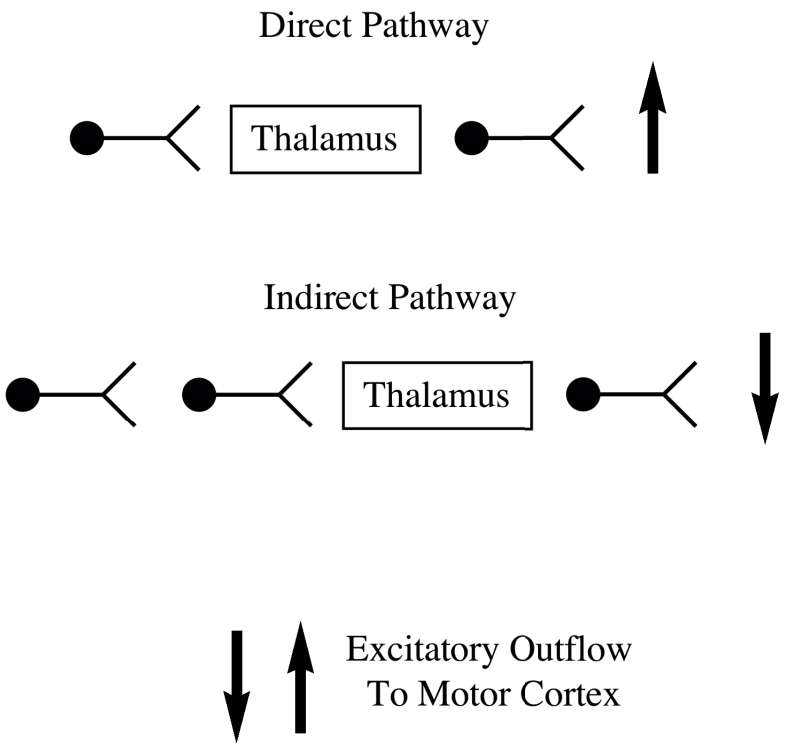


Figure 1.3 An abbreviated representation of the direct and indirect pathways. Stimulation of these pathways by striatal DA regulates the thalamus excitatory outflow to the motor cortex. The direct pathway consists primarily of D_1-type receptors while D_2-type receptors are involved in the indirect pathway. Figure is adapted from (*3*).

Pharmacotherapy of Parkinson's Disease

In 1961, Birkmayer and Hornykiewicz were the first to demonstrate that symptoms of PD can be partially alleviated though the administration of levodopa, an exogenous dopamine precursor (*11*). Levodopa can easily cross the blood brain barrier, unlike DA itself, and is metabolized within dopaminergic neurons to form DA. The increased concentration of DA within the brain after levodopa treatment provides relief from PD symptoms such as rigidity and bradykinesia (*3*). While their discovery was initially met with skepticism,

levodopa therapy was soon recognized as a major breakthrough for the treatment of PD.

Following the introduction of levodopa, alternative treatments were developed to overcome the deficiency of striatal DA by one or more of the following means: increase the synthesis of brain DA, encourage DA release from neurons, stimulate DA receptors or decrease DA catabolism (*3*). These treatments included the use of DA receptor agonists, monoamine oxidase-B (MAO-B) inhibitors and catechol-o-methyl-transferase (COMT) inhibitors (*12*). DA receptor agonists such as cabergoline and bromocriptine will stimulate DA receptors (i.e. D_1-type receptors) in the absence of DA. DA receptor agonists do not require functioning dopaminergic nerve terminals, as does levodopa, which are less numerous in brains affected by PD due to the degeneration of the nigrostriatal pathway. MAO-B inhibitors, rasagiline and selegiline, will prevent the catabolism of DA thereby raising the levels of DA in the brain. COMT inhibitors including entacapone and tolcapone are often prescribed with levodopa treatment to prevent the metabolism of levodopa before it reaches its site of action (i.e. dopaminergic neurons), thus prolonging the action of levodopa. Treatment with DA receptor agonists, MAO-B inhibitors and COMT inhibitors often result in severe side effects, therefore levodopa currently remains the most effective treatment for managing the symptoms of PD.

The majority of patients treated with levodopa, however, also experience a variety of side effects including significant motor complications within five years of treatment (*13*). These complications include fluctuations in motor abilities corresponding to the plasma levels of levodopa throughout the day, termed the "wearing-off effect", and sudden changes from superior symptomatic control to re-emerging parkinsonism, termed the "ON/OFF effect" (*11, 14*). These

complications appear to result from the loss of dopaminergic receptors and the impaired capacity to maintain concentrations of levodopa within the striatum (*14*).

Levodopa and other dopamine replacement treatments, while effective for short-term relief of motor symptoms, do not to provide long-term success nor do they alter or halt the progressive and selective neurodegeneration characteristic of the disease. It is of importance to further examine the mechanisms of disease initiation and progression to further understand the pathogenesis of PD. In this manner, therapeutic strategies that are highly effective, while maintaining no adverse side effects, may be developed for the treatment of this debilitating neurodegenerative disease.

CHAPTER TWO

PARKINSON'S DISEASE

Etiology

Currently, the mechanism of onset for Parkinson's disease (PD) is unknown, however factors such as heredity or exposure to environmental agents are hypothesized to play a role in disease pathogenesis.

Genetic Susceptibility

A genetic component is thought to be involved in a number of PD cases and researchers have discovered several genes with mutations in individuals that correlate with the characteristics of sporadic PD (*2, 15-18*). Studies have identified six mutated genes that are hypothesized to be linked to PD, including α*-synuclein*, leucine-rich repeat kinase 2 (*LRRK2*), *parkin*, phosphatase and tensin homologue-induced putative kinase 1 (*PINK1*), *DJ-1*, and *ATP13A2*. α*-Synuclein* is widely expressed in the nervous system and is a major component of Lewy bodies (LBs). Point mutations (Ala53Thr, Ala30Pro, Glu46Lys) and multiplications of α*-synuclein* can cause an alteration of gene expression that produces detrimental effects for dopaminergic neurons. There exist multiple mutations for the *LRRK2* gene; however, the two most frequent point mutations include Gly2019Ser (found in 30-40% of patients with PD from North African and Middle Eastern populations) and Arg1441Cys (found in several populations). *LRRK2* mutations account for the most common genetic correlation of the disease and patients have clinical symptoms nearly identical to those of classical PD, however the cellular function of the gene stills needs to be elucidated. Mutations in *parkin*, *PINK1*, *DJ-1* and *ATP13A2* are typically associated with early onset parkinsonism. *Parkin* and *PINK1* mutations significantly affect mitochondrial function and cellular susceptibility to oxidative

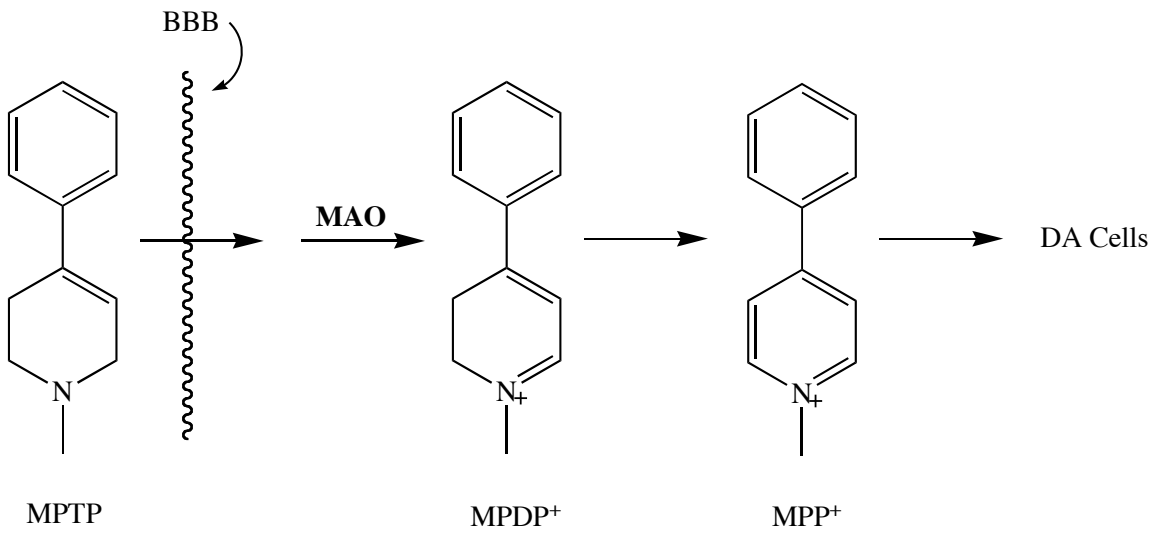
stress as *parkin*, in particular, is involved in the ubiquitin proteasomal pathway. The exact cellular roles of *DJ-1* and *ATP13A2* are still unknown, however, *DJ-1* is thought to be involved in the oxidative stress response as a redox-dependant chaperone and loss of functional *ATP13A2* may lead to lysosomal dysfunction.

Environmental Factors

Several genes with mutations have been identified that correlate with PD and results from a twin study indicate an increased incidence of early-onset PD (< 50 years of age) between twins (*19*). This study, however, also demonstrated a lack of concordance of sporadic, late-onset PD between monozygotic twins (*19*). These data suggest that genetic factors are not a major component of sporadic PD, which represents the vast majority (> 90%) of all cases. It is important, therefore, to identify other risk factors, such as pesticide exposure and metal toxicity, which may lead to the onset of the neurodegenerative disease.

Pesticide Exposure

In the early 1980s, in an attempt to illicitly synthesize the narcotic 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), a group of young adult drug users produced the impurity 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a by-product (*20*). The inadvertent exposure to the MPTP impurity produced Parkinson-like symptoms in these individuals. This discovery triggered a variety of research to identify the correlation between pesticide exposure and PD, as the metabolite of MPTP is “structurally similar” to paraquat (PQ), a widely used herbicide (*20-22*) (*Figure 2.1*). Once an individual is exposed to MPTP, the compond is selectively transported across the blood brain barrier (BBB) and is metabolized to the toxic metabolite, 1-4-methyl-4-phenylpyridinium (MPP^+), a potent mitochondrial complex I inhibitor in dopaminergic cells (*Scheme 2.1*).



Scheme 2.1 An abbreviated scheme demonstrating the pathway of MPTP toxicity. MPTP can cross the BBB and is metabolized by MAO to form $MPDP^+$ and MPP^+. MPP^+ will be transported into dopaminergic neurons via the dopamine transport system. MPP^+ formed elsewhere in the body is unable to cross the BBB. Figure is adapted from (*5*).

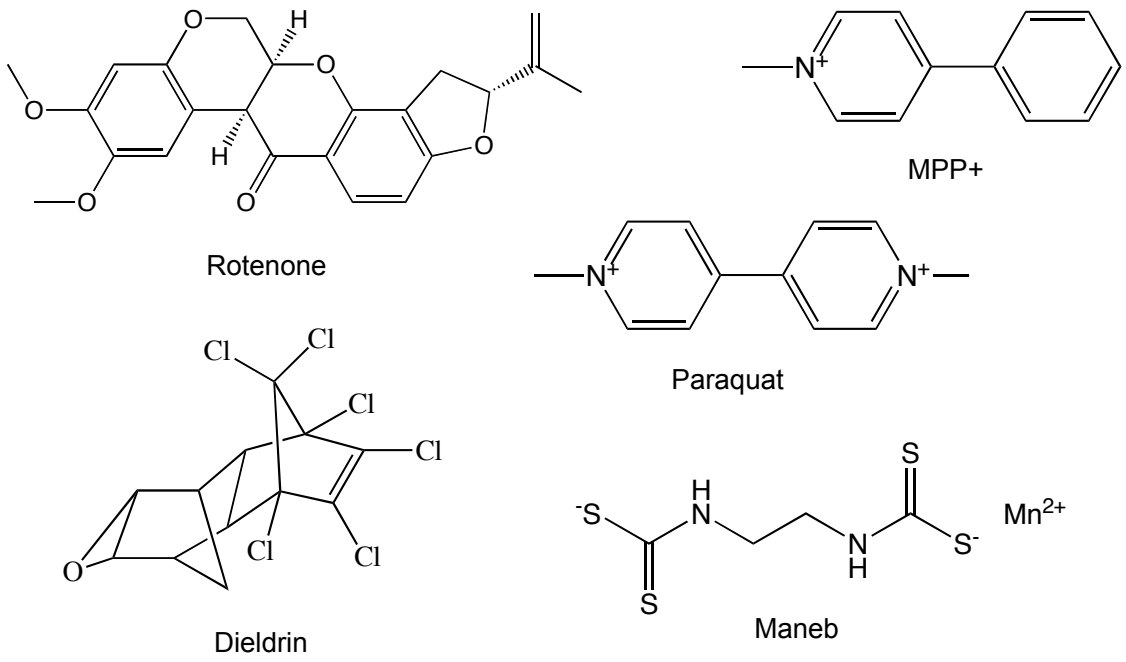


Figure 2.1 Chemical structures of rotenone, MPP^+, PQ, dieldrin and maneb. These compounds are hypothesized to induce PD.

Since the discovery of MPP^+ in the early 1980s, numerous studies have suggested pesticides such as rotenone, dieldrin, PQ and maneb are associated with the onset of PD (*Figure 2.1*) (*23-27*). The mechanism of toxicity of rotenone is analogous to MPP^+, and involves the inhibiton of complex I of the mitochondrial respiratory chain (*26*). Rotenone is a natural product extracted from tropical plants and is used primarily as an insecticide. The insecticide can easily cross the BBB and, unlike MPP^+, does not require the dopamine transporter (DAT) to enter dopaminergic neurons. Rotenone will accumulate within cellular organelles, and recent studies have demonstrated rotenone will cause α-synuclein cytoplasmic lesion in rats, similar to the characteristic Lewy bodies found in humans with PD (*23*).

Dieldrin is another pesticide hypothesized to induce parkinsonism in individuals. Dieldrin was widely used as an insecticide beginning in 1950,

however due to potential carcinogenic action and bioaccumulation, the Environmental Protection Agency banned its use in the United States in 1987. Significant levels of the pesticide are still detectable in the environment however, as it has a half-life of 25 years (*28*). Dieldrin is listed as one of most hazardous pesticides for human exposure and detectable levels of the pesticide can be observed in brain tissue among individuals exposed to the pesticide (*28*). Dieldrin has been demonstrated to play a role in dopaminergic cell death via depletion of dopamine (DA), increased DA release and generation of reactive oxygen species (ROS) (*28*). In addition, it is suggested that exposure to dieldrin during development leads to alterations of the dopaminergic system rendering these neurons more susceptible to toxins later in life, including enhanced neurotoxicity after MPTP exposure (*29*).

PQ, structurally similar to MPP^+, is one of the most commonly used herbicides in the world. PQ is a fast-acting, nonselective herbicide that kills green plant tissue on contact and its principal use is for weed control. The herbicide has been demonstrated to cause pulmonary toxicity (*30*) however, its role in neurotoxicity is of question since studies have demonstrated mixed results regarding whether PQ can cross the BBB and affect dopaminergic neurons (*23, 30, 31*). It is suggested that PQ's toxicity, in regards to PD, is related to its ability to redox cycle (*32*).

Maneb has also been hypothesized to a play a role in the pathogenesis of PD. Maneb, a dithiocarbamate compound, is a fungicide used to control field crop pathologies. Acute exposure of mice to maneb demonstrates the compound to have a depressant-like effect on the central nervous system and the dopaminergic pathway leading to decreased locomotor activity (*23*). The fungicide is also suggested to contribute to ROS production by inhibiting mitochondrial complex III (*25, 26*).

The onset of PD could also be due to a combined exposure of a mixture of pesticides. Maneb and PQ have a geographical overlap of areas of use in the United States and studies have shown mice exposed to a combination of these pesticides have decreased motor activity and reduced striatal DA levels (*33*).

Metal Toxicity

In addition to pesticide exposure, metals including copper, iron and manganese are also implicated as factors that may lead to the onset of PD. Several studies have observed increased concentrations of metal ions in the substantia nigra (SN) of individuals with PD (*34, 35*). An accumulation of metal ions in the SN could lead to the excessive production of ROS, which may play a role in the degeneration of the dopaminergic pathway by inducing neuronal death (*36, 37*). Studies have also indicated increased levels of metal ions could lead to oxidative modification of protein (*38*) and encourage the formation of protein aggregates of α-synuclein (*39*), the main component of LBs.

Copper has been demonstrated to play a role in inducing protein aggregation of α-synuclein (*40*) and research has demonstrated increased concentrations of iron in mice to lead to oxidative stress (*41*), decreased striatal dopamine and impaired motor function (*42*). Exposure to manganese, one of the most prevalent metals, appears to cause toxicity in different brain areas than those affected in PD, however, manganese is suggested to be a risk factor for the development of the disease (*43*).

Summary

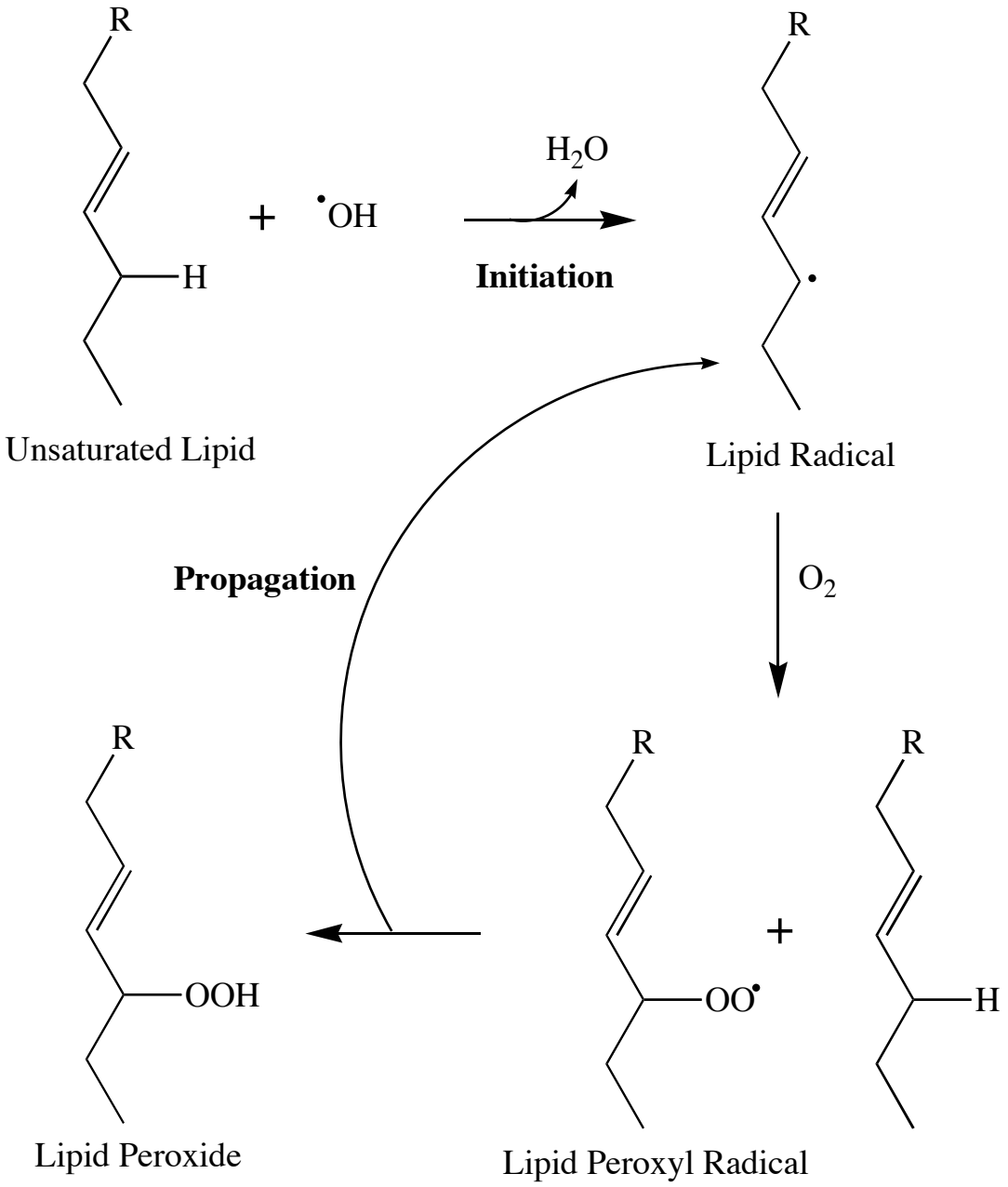
Studies have demonstrated that the mechanism of onset of PD could include either exposure to environmental agents (pesticides, metals) or genetic susceptibility. Regardless of the different factors that could be involved in the initiation of the disease, both forms of PD (familial and sporadic) share the same

basic biochemical hallmark (i.e. loss of dopaminergic neurons) and respond to levodopa (L-3,4-dihydroxyphenylalanine, L-DOPA) treatment. This would appear to indicate that they are similar forms of the disease and suggest they share a common final pathway of neurodegeneration (*18*). Recent evidence suggests oxidative stress as a link between disease initiation and progression, implying increased oxidative stress could play a significant role in the pathogenesis of PD (*25, 44-46*).

Pathogenesis of Parkinson's Disease

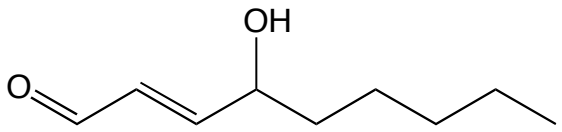
Oxidative Stress and Lipid Peroxidation

Oxidative stress is caused by an imbalance between the production and detoxification of reactive oxygen intermediates. An accumulation of ROS can cause toxic cellular effects such as the production of peroxides and free radicals, which can exert damage on proteins, lipids and DNA. The brain is highly susceptible to oxidative stress because of its high metabolic rate, poor concentrations of antioxidants and high levels of polyunsaturated lipids (*37*). Research has observed that individuals with PD have increased concentrations of iron and decreased levels of antioxidants as compared to controls (*44*). These conditions are favorable for lipid peroxidation, the oxidative degradation of lipids (*Scheme 2.2)*. The lipid peroxidation process is a free radical chain reaction mechanism whereby free radicals acquire electrons from cell membrane lipids. The reaction consists of three steps: initiation, propagation and termination. The initiation step produces a lipid radical as a result of a reaction between a ROS and an unsaturated lipid. The lipid radical will react with molecular oxygen to form a lipid peroxyl acid radical, which will then react with another unsaturated lipid thus propagating the chain reaction. The reaction will terminate when two radicals react and produce a non-radical species.

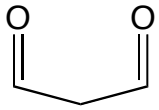


Scheme 2.2 Lipid peroxidation refers to the oxidative degradation of lipids. The process is a free radical chain reaction mechanism consisting of three major steps: initiation, propagation and termination. Lipid peroxidation can result in damage to cell membranes, which consist primarily of lipids.

Two major products of lipid peroxidation include 4-hydroxy-2-nonenal (4HNE) and malondialdehyde (MDA) (*Figure 2.2*). These compounds are considered to be two common components of oxidative stress and studies have observed elevated concentrations of 4HNE in brains of individuals with PD (*47, 48*). Accumulation of these reactive lipid aldehydes can be detrimental as 4HNE and MDA can cause modification of proteins and inhibition of cellular enzymes (*49, 50*).



4-Hydroxy-2-nonenal



Malondialdehyde

Figure 2.2 Chemical structures of the lipid peroxidation products 4HNE and MDA. These reactive lipid aldehydes are considered to be two common components of oxidative stress.

Aldehyde Dehydrogenase

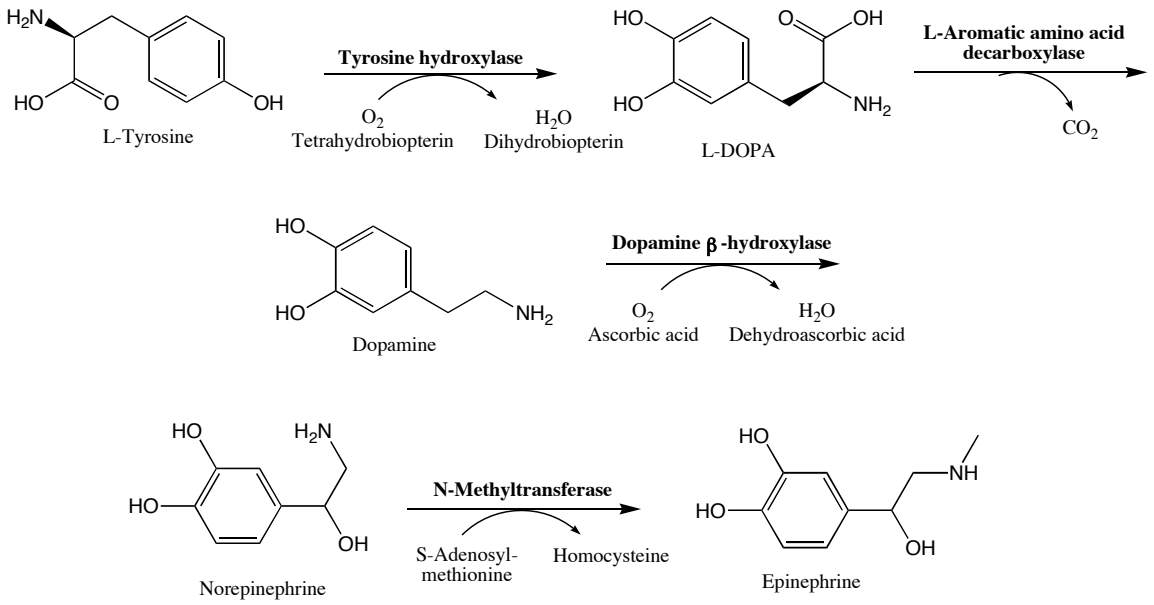
One particular enzyme susceptible to inhibition by 4HNE and MDA is aldehyde dehydrogenase (ALDH). ALDH enzymes catalyze the irreversible oxidation of a variety of aliphatic and aromatic aldehydes to their respective carboxylic acids. The human ALDH gene superfamily contains 19 isoforms (*51*) and resides in numerous subcellular locations including cytosol, mitochondria, endoplasmic reticulum and nucleus (*52*).

4HNE inhibits mitochondrial ALDH at the enzyme active site (Cys 302), displaying a mixed-type inhibition (K_i = 0.5 μM), appearing reversible at low concentrations and irreversible at higher concentrations (*53-56*). At low

micromolar concentrations, MDA will also irreversibly inhibit the mitochondrial enzyme (*57*). ALDH is particularly important in the catabolism of DA; therefore enzyme inhibition could have significant cellular consequences (*52*).

Dopamine Biosynthesis and Metabolism

At the nerve terminal the synthesis of DA begins with hydroxylation of the amino acid L-tyrosine to L-DOPA via the enzyme tyrosine hydroxylase (TH) and the cofactor tetrahydrobiopterin (*Scheme 2.3*) (*4*). L-DOPA is metabolized to DA by the enzyme L-aromatic amino acid decarboxylase with the cofactor pyridoxal phosphate. TH is the rate-limiting step in the synthesis of DA and can be regulated by a variety of factors including activation of synthesis-modulating autoreceptors and end-product inhibition by intraneuronal dopamine. Subsequent to the biosynthesis of DA, the neurotransmitter is taken up into vesicles by the vesicular monoamine transporter, thus decreasing the cytoplasmic concentration of DA and preventing the metabolism of DA to reactive intermediates. The DAT is responsible for transporting DA into or out from the terminal depending on the concentration gradient (*Figure 2.3*). DA can also be further metabolized to the neurotransmitters norepinephrine and epinephrine.



Scheme 2.3 The biosynthetic pathway of dopamine and the neurotransmitters norepinephrine and epinephrine.

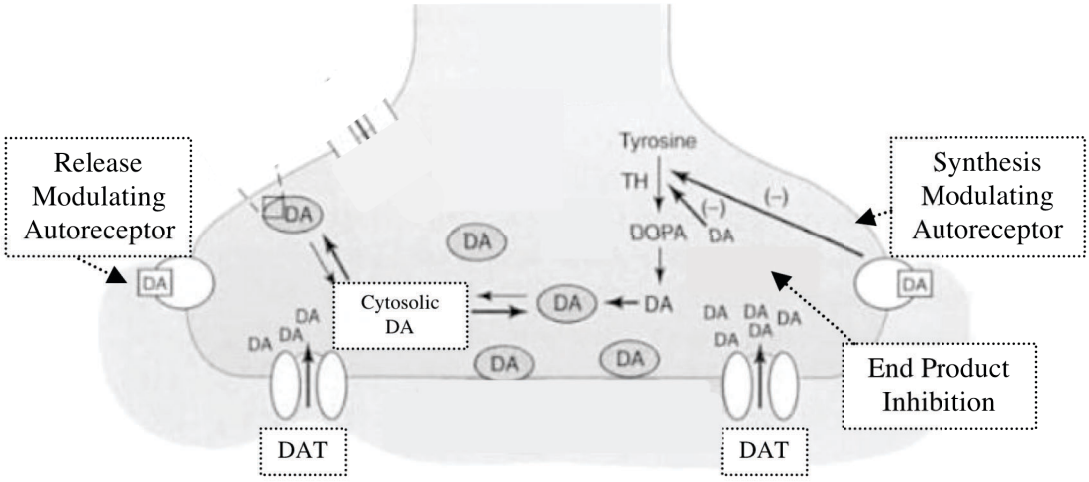
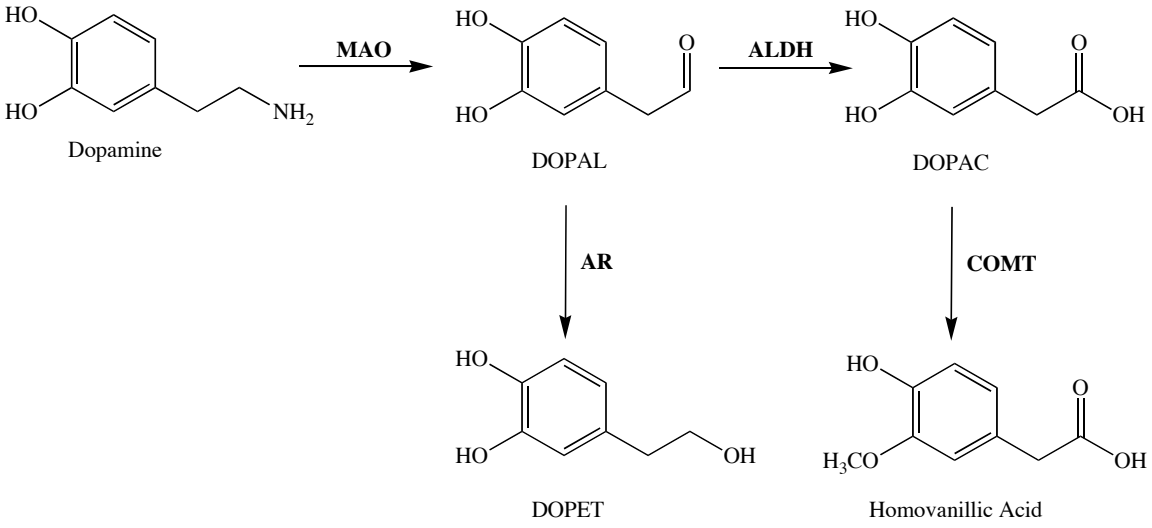


Figure 2.3 A diagram of a dopaminergic nerve terminal depicting the metabolic pathway of DA and the mechanisms that modulate its synthesis, release and storage. Figure is adapted from (*4*).

After reuptake by DAT from the synapse, cytosolic DA undergoes oxidative deamination catalyzed by the enzyme monoamine oxidase (MAO) to yield 3,4-dihydroxyphenylacetaldehyde (DOPAL) (*4*). MAO, located on the outer mitochondrial membrane, exists in two isoforms: MAO-A and MAO-B. MAO-A primarily deaminates serotonin, noradrenaline, and adrenaline while MAO-B deaminates dopamine, β-phenylethylamine and benzylamine (*58*). MAO requires molecular oxygen as a cofactor and produces hydrogen peroxide as a byproduct.

Subsequent to the oxidative deamination of DA to the aldehyde intermediate, DOPAL undergoes oxidation to an acid (3,4-dihydroxyphenylacetic acid, DOPAC) via mitochondrial ALDH and the cofactor NAD. The specific ALDH isoform involved in the metabolism of DOPAL has yet to be determined (*52*), however, numerous studies indicate the particular isoform to be ALDH2 (*59-62*).

Alternatively, DOPAL could also be reduced to the alcohol (3,4-dihydroxyphenylethanol, DOPET) via aldehyde/aldose reductase (AR) (*63*). However, the first step of DA catabolism (i.e. oxidative deamination via MAO) occurs within the mitochondria and AR enzymes reside in the cytosol (*64, 65*). Therefore, the primary pathway for DOPAL metabolism is oxidation via mitochondrial ALDH to DOPAC followed by methylation via the enzyme catechol-O-methyl transferase (COMT) to homovanillic acid, which can be easily removed from the body (*Scheme 2.4*).



Scheme 2.4 The metabolic pathway for DA. DA will undergo deamination via MAO to DOPAL, an aldehyde intermediate, which is primarily oxidized by ALDH to DOPAC.

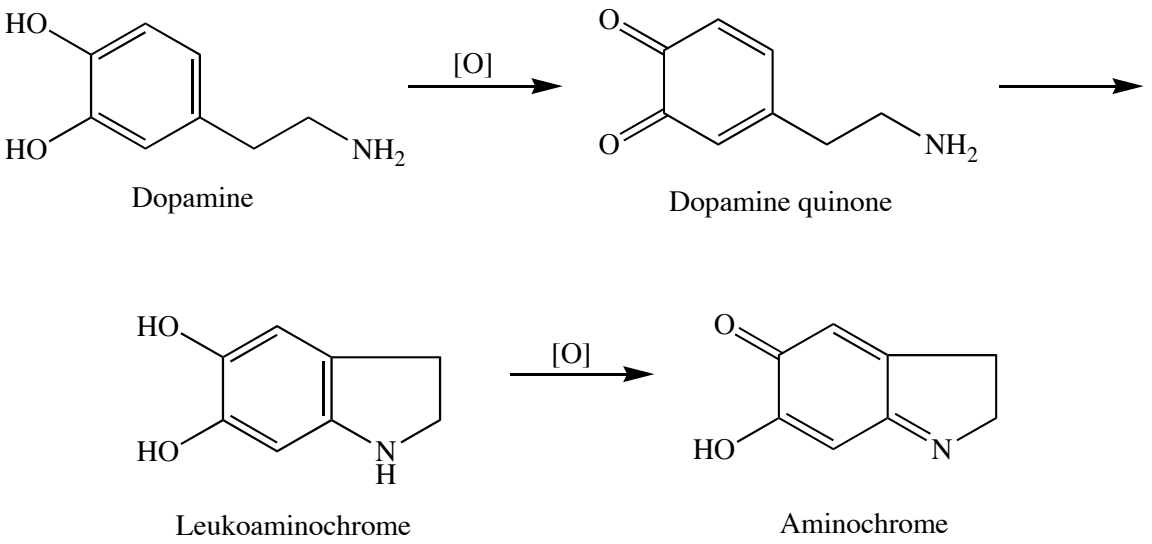
An Endogenous Neurotoxin

The selective degeneration of dopaminergic neurons, a fundamental characteristic of PD, suggests a neurotoxin endogenous to dopaminergic cells may play a role in the pathogenesis of the neurodegenerative disease (*66-68*). DA was exclusively considered an intermediate in the synthesis of the

catecholamines norepinephrine and epinephrine until the 1950s when DA was discovered in both tissue and brain (*4*). Several studies indicate DA to be an endogenous neurotoxin as the catecholamine can exert cellular toxicity through the generation of ROS (*69*) and its ability to form a reactive DA quinone (*Scheme 2.5*) (*70, 71*). In addition, the DA quinone is a soft electrophile and is capable of protein modification through reactivity with soft nucleophiles such as thiols. Several studies have demonstrated the reactivity of the DA quinone toward protein Cys residues (*72, 73*) and have indicated the presence of cysteinyl-DA adducts in post-mortem studies of individuals with PD (*74*).

The DA quinone, however, rapidly undergoes intramolecular rearrangement to the leukoaminochrome followed by further auto-oxidation to aminochrome (*Scheme 2.5*) (*75*). This demonstrates the DA quinone is highly unstable and suggests its rearrangement and subsequent oxidation will predominate over reactivity with Cys residues provided the thiol is not located near the site of quinone formation.

Several alternative studies have suggested the aldehyde metabolite of DA, DOPAL, to be an endogenous neurotoxin. Research has demonstrated DOPAL to be 100-fold more toxic in vitro and 1000-times more toxic in vivo as compared to DA (*8, 76*). In addition, several studies have demonstrated DOPAL can induce cell death at low micromolar concentrations (~ 6 μM) (*77, 78*). Measured physiological levels of DOPAL are approximately 2-3 μM (*79*), therefore a slight increase in DOPAL concentration could produce detrimental effects within dopaminergic neurons.



Scheme 2.5 Upon oxidation of DA, the reactive ortho-quinone is generated which will subsequently cyclize to form the leukochrome. Oxidation of leukochrome yields aminochrome.

Elevated Levels of 3,4-Dihydroxyphenylacetaldehyde

As indicated in *Scheme 2.4*, DOPAL is generated as a normal intermediate in the metabolism of DA; therefore it is of interest to determine the mechanisms that could lead to increased concentrations of the reactive aldehyde. One such mechanism of interest includes the inhibition of the ALDH enzyme by products of lipid peroxidation, specifically 4HNE. Normal cellular concentrations of 4HNE are between 0.1-1.0 μM, however under conditions of oxidative stress, levels of 4HNE can reach in excess of 10 μM (*49, 80*). Recent research has determined that 4HNE effectively inhibits ALDH activity in several model systems including human recombinant ALDH2, rat brain mitochondrial lysate and isolated rat brain mitochondria, yielding an accumulation of the reactive aldehyde, DOPAL (*59*). In addition, AR activity toward DOPAL was not observed (*65*) and 4HNE was demonstrated to have no effect on MAO, indicating

that the lipid peroxidation product directly inhibits ALDH leading to elevated DOPAL concentrations.

Summary

Oxidative stress is suggested to play a role in the pathogenesis of PD (*25, 44-46*). The two common components of increased oxidative stress are the lipid peroxidation products, 4HNE and MDA. Several studies have demonstrated that these reactive lipid aldehydes are present in individuals with PD (*47, 48*) and could cause detrimental cellular effects such as enzyme inhibition (*49, 50*). Current research has determined 4HNE will cause inhibition of ALDH, a critical enzyme for DA catabolism, and demonstrated inhibition of this enzyme to lead to elevated concentrations of DOPAL (*59*). The cellular consequence of this result, however, has yet to be determined. An accumulation of the reactive aldehyde, DOPAL, may cause cellular damage and dopaminergic neuronal death, potentially involving the formation of free radicals (*82*), the mitochondrial transition pore (*77*) and protein modification (*83-86*). Determining the capability of DOPAL to initiate or propagate toxicity towards dopaminergic neurons will elucidate the role of this potential endogenous neurotoxin in the pathogenesis of PD.

CHAPTER THREE

STATEMENT OF THE HYPOTHESIS

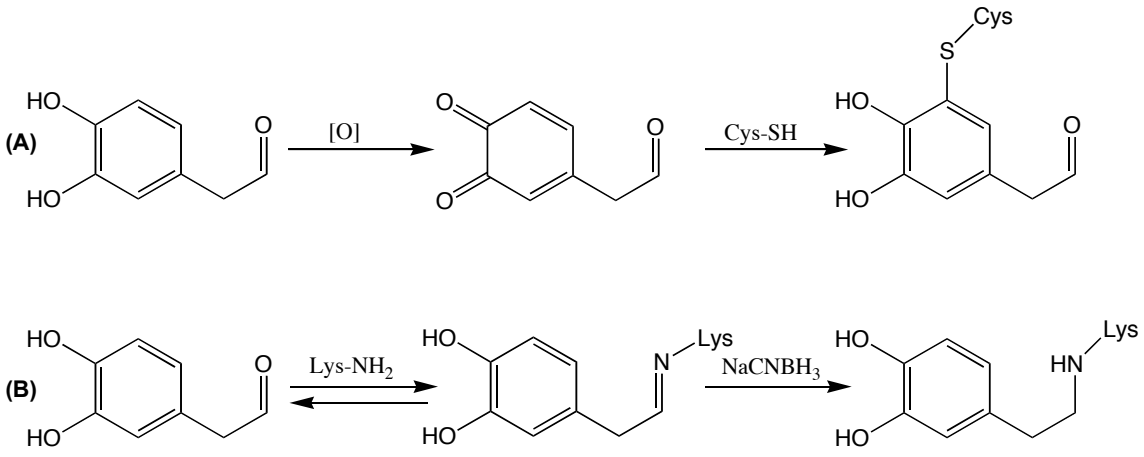
Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by the cell death of dopaminergic neurons and the formation of Lewy bodies (*8*). While the factors of disease onset (i.e. environmental or genetic) are currently unknown, the progression of the disease is hypothesized to involve oxidative stress (*25, 44-46*) and the generation of products of lipid peroxidation such as 4-hydroxy-2-nonenal (4HNE) and malondialdehyde (*47, 48*). 4HNE can cause inhibition of cellular enzymes including mitochondrial aldehyde dehydrogenase (ALDH) (*53-56*), an enzyme critical for dopamine (DA) catabolism. ALDH catalyzes the oxidation of 3,4-dihydroxphenylacetaldehyde (DOPAL), a reactive aldehyde, to 3,4-dihydroxyphenylacetic acid, a stable acid.

DOPAL has been implicated to be an endogenous neurotoxin relevant to the pathogenesis of PD. The aldehyde intermediate has been determined to cause cellular toxicity at low micromolar concentrations (*77, 78*) and, in comparison with DA, is more toxic in vitro and in vivo (*8, 76*). In addition, research has demonstrated DOPAL levels can be elevated as a result of 4HNE inhibition of ALDH (*59*). It is of interest, therefore, to identify the cellular consequence of increased concentrations of the reactive DA intermediate.

A number of studies have hypothesized the mechanism of DOPAL toxicity to involve protein modification (*83-86*), however, the reactivity of DOPAL towards specific protein nucleophiles (i.e. amines vs thiols) is of question. Furthermore, DOPAL is predicted to be a bifunctional electrophile as it contains two reactive functional groups (i.e. aldehyde and catechol); therefore this DA intermediate could be capable of inducing protein cross-linking. Oxidation of the catechol to

form DOPAL quinone could yield protein adducts via reaction with thiols and forming a thioether linkage. The aldehyde could subsequently react with protein amines to yield protein cross-linking. Alternatively, after formation of a Schiff base between DOPAL and an amine, DOPAL could undergo oxidation to the quinone and react with a thiol to yield protein cross-linking. The goal of this study is to demonstrate the mechanism of DOPAL-mediated protein modification. Specifically, determining if DOPAL protein adduction occurs via oxidation of the catechol to yield a Cys-reactive quinone (which is known to occur for DA (*70, 71*)) or if protein adduction occurs via the aldehyde, forming adducts with Lys residues (*Scheme 3.1*).



Scheme 3.1 Possible mechanisms for DOPAL-mediated protein modification include (A) auto-oxidation of the catechol followed by reaction with protein thiols or (B) reaction with protein amines via the aldehyde. The Schiff base in (B) may require reduction for stability.

Hypothesis

3,4-Dihydroxyphenylacetaldehyde, an intermediate of dopamine catabolism and potential endogenous neurotoxin relevant to Parkinson's disease,

is capable of protein modification and protein cross-linking through reactivity with amine and thiol nucleophiles.

Specific Aims

To test the central hypothesis of this research, the following Specific Aims were developed.

Specific Aim I. Demonstrate the ability of DOPAL to modify proteins. Previous work has observed increased DOPAL concentrations upon inhibition of DA catabolism by products of lipid peroxidation, therefore, completion of this aim will determine if protein modification is a consequence of elevated DOPAL levels.

Specific Aim II. Determine the reactivity of DOPAL towards protein nucleophiles. Completion of this aim will demonstrate both qualitatively and quantitatively the reactivity of DOPAL with proteins, specifically determining amine vs thiol adduction.

Specific Aim III. Identify proteins of dopaminergic cells susceptible to adduction by DOPAL and relevant to PD. Identification of protein targets, that upon modification by DOPAL, leads to dopaminergic cell death would aid in determining the role of DOPAL in the pathogenesis of PD. Completion of this aim will utilize a novel technique to isolate DOPAL-modified protein from unmodified protein in PC6-3 cell lysate.

CHAPTER FOUR
3,4-DIHYDROXYPHENYLACETALDEHYDE: A REACTIVE ALDEHYDE INTERMEDIATE

Abstract

Recent work indicates oxidative stress to be a factor in Parkinson's disease (PD); however, it is unknown how this condition causes selective dopaminergic cell death. The neurotransmitter dopamine (DA) has been implicated as an endogenous neurotoxin to explain the selective neurodegeneration. DA undergoes catabolism by monoamine oxidase (MAO) to the reactive intermediate 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is further oxidized to 3,4-dihydroxyphenylacetic (DOPAC) acid via mitochondrial aldehyde dehydrogenase (ALDH). Previous studies found DOPAL to be more toxic than DA and that the major lipid peroxidation products, i.e. 4-hydroxynonenal (4HNE) and malondialdehyde (MDA), potently inhibit ALDH. The hypothesis of this work is that lipid peroxidation products inhibit DOPAL oxidation, yielding aberrant levels of the reactive aldehyde intermediate. Treatment of striatal synaptosomes with 2 – 100 μM 4HNE or 2 – 50 μM MDA impaired DOPAL oxidation, resulting in elevated DOPAL concentrations. The aberrant concentration of DOPAL yielded an increase in protein modification by the DA-derived aldehyde, evident via staining of proteins with nitroblue tetrazolium (NBT). Pretreatment of synaptosomes with an MAO inhibitor significantly decreased NBT staining. Based on NBT staining, the order of protein reactivity for DA and metabolites was found to be: DOPAL >> DOPAC > DA. Mass spectrometric analysis of a model peptide reacted with DOPAL revealed the adduct to be a Schiff base product. In summary, these data demonstrate the sensitivity of DA catabolism to the lipid peroxidation products 4HNE and MDA

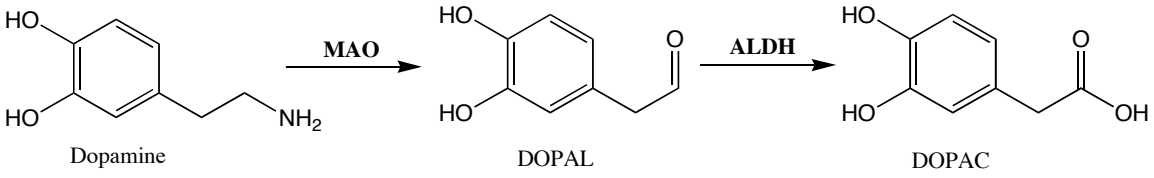
even at low, physiologic levels and suggest a mechanistic link between oxidative stress and generation of aberrant levels of an endogenous and protein reactive dopaminergic toxin relevant to PD.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by dopaminergic cell death. The mechanism of pathogenesis is currently unknown; however, results of recent studies indicate exposure to environmental agents and oxidative stress to be significant factors in the disease process (*27, 44, 45*). Previous studies demonstrated that individuals with PD have increased oxidative burden as compared to controls, including elevated levels of iron and decreased concentrations of antioxidants (*45*). These conditions are favorable for lipid peroxidation, and earlier reports have indicated increased oxidative decomposition of lipids in PD brains (*47, 48*). Two major products of lipid peroxidation are 4-hydroxy-2-nonenal (4HNE) and malondialdehyde (MDA), which are considered to be two common components of oxidative stress (*49*). At high enough concentrations, these lipid aldehydes can cause cellular damage by modifying proteins and inhibiting enzymes (*49, 50*).

Aldehyde dehydrogenase (ALDH) found in the mitochondria is particularly susceptible to inhibition by 4HNE and MDA given that these compounds are aldehyde-substrates. 4HNE was demonstrated to be both a substrate and potent reversible inhibitor of the enzyme, displaying mixed-type inhibition (K_i = 0.5 μM) (*53-56*), and a previous study found that ALDH is irreversibly inactivated by MDA at low micromolar concentrations (*57*). Of note, the enzyme is critical to the dopamine (DA) catabolic pathway by catalyzing oxidation of a reactive aldehyde intermediate (*Scheme 4.1)* (*52*). Monoamine oxidase (MAO), located on the outer mitochondrial membrane, mediates oxidative deamination of DA to an

aldehyde intermediate, 3,4-dihydroxyphenylacetaldehyde (DOPAL) (*63*). Subsequently, DOPAL undergoes oxidation to 3,4-dihydroxyphenylacetic acid (DOPAC; *Scheme 4.1*) in the mitochondrial matrix via ALDH and the cofactor NAD (*52, 63, 87, 88*). Therefore, even low micromolar 4HNE or MDA, known to be generated during periods of oxidative stress, will potently inhibit ALDH, yielding an increase in cellular levels of DOPAL. Aldehyde metabolism via reductases (i.e. aldehyde reductase) may be able to compensate for aberrant concentrations of DOPAL (*81*); however, the DA-derived aldehyde is not a good substrate for reductases, and these enzymes reside in the cytoplasm, leaving the mitochondria vulnerable (*64, 89*). Indeed, a recent report demonstrated that physiologically relevant levels of 4HNE (i.e. ~10 μM) impair DOPAL oxidation (i.e. ALDH) in rat brain mitochondria (*59*). However, the study utilized whole rat brains, and therefore, it is not known whether this effect can be extended to dopaminergic regions. In addition, the paper did not characterize the resulting increase in DOPAL concentration and subsequent protein modification.



Scheme 4.1 The primary pathway for the metabolism of DA involves oxidative deamination of DA to DOPAL followed by reduction to DOPAC.

Previous work showed DOPAL to be reactive toward cellular nucleophiles and harmful to cells, and therefore, dysregulation of its metabolism is predicted to yield cytotoxicity. DA has been implicated as an endogenous neurotoxin; however, for comparison, DOPAL was shown to be more toxic in vitro (i.e. 100-

fold) and in vivo (i.e. ~1000-fold) (*8, 76*). Furthermore, previous studies have demonstrated the DA-derived metabolite to be reactive towards proteins (*83-85*). These results indicate DOPAL is an endogenous neurotoxin and suggest the DA-derived aldehyde to be a factor in PD-related neuronal degeneration.

The current study was conducted to test the hypothesis that the characteristic products of oxidative stress, 4HNE and MDA, inhibit the ALDH-mediated metabolism of DOPAL thus yielding an accumulation of the toxic DA-derived aldehyde. To evaluate the effect of 4HNE and MDA on DA catabolism, isolated nerve terminals (i.e. synaptosomes) were prepared from rat striatal tissue and used as a model for DA catabolism. DOPAL, an ALDH substrate (*52*), was generated in situ via addition of DA to synaptosomes, and its metabolism in the presence of physiologic concentrations of 4HNE or MDA was determined using an HPLC method allowing simultaneous measurement of the aldehyde and its oxidized or reduced metabolites. Additionally, a consequence of elevated DOPAL concentrations may include protein modification. Therefore, the presence of DOPAL-protein adducts in rat striatal synaptosomes was demonstrated with nitro blue tetrazolium (NBT), a redox dye that is sensitive to catechol-protein adducts (*38, 90*). Such work is of importance as it may elucidate a mechanistic link between oxidative stress and the generation of an endogenous neurotoxin at elevated levels, with implications for PD pathogenesis.

Experimental Procedures

Materials

4HNE was synthesized as previously described in (*91*). MDA was synthesized by acidification of 1,1,3,3-tetramethoxypropane with hydrochloric acid (1:1) and diluting into 50 mM sodium phosphate buffer (pH 7.4). Concentrations of 4HNE and MDA were determined spectrophotometrically.

DOPAL was biosynthesized via an established procedure involving enzyme-catalyzed conversion of DA to DOPAL by rat liver MAO (*84*). The DOPAL concentration was determined using an aldehyde dehydrogenase (ALDH) assay with NAD (*85*) and high performance liquid chromatography (HPLC) analysis, as described below. Such a procedure provided an approximate 70% yield of DOPAL. 3,4-Dihydroxyphenylethanol (DOPET) was obtained via reduction of DOPAL with a 10-fold excess of sodium borohydride. DA, DOPAC and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Preparation of Synaptosomes

[Collaboration with Virginia Florang]

Rat striatal synaptosomes were prepared according to previously established procedures (*92*) from Sprague-Dawley rats (200-250g) euthanized with sodium pentobarbital. Briefly, striatal tissue was removed from one rat and homogenized in media (4 °C) containing 0.32 M sucrose, 10 mM Tris, and 0.5 mM EDTA (pH 7.5) at 10% (w/v). The homogenate was centrifuged at 800 g for 10 min and the resulting pellet was discarded. Supernatant was mixed (1:1) with 1.2 M sucrose solution to yield a 0.8 M final concentration, and the solution was then spun at 18,000 g for 25 min. The supernatant was then discarded and the pellet was resuspended in HEPES-buffered synaptosomal media, containing 136 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.4 mM $CaCl_2$, 10 mM glucose, 140 µM EDTA and 20 mM HEPES, pH 7.4. This solution was further diluted to 100% of the original volume. The Bradford Assay was used to determine the protein concentration. It should be noted that such a preparation would contain both whole and lysed synaptosomes and free mitochondria.

Treatment of Synaptosomes with 4-Hydroxy-2-Nonenal and Malondialdehyde

[Collaboration with Virginia Florang]

Prepared synaptosomes were diluted in HEPES-buffered media (pH 7.4) to a final protein concentration of 0.5 mg/mL. To initiate DA catabolism and generate DOPAL in situ, DA was added to striatal synaptosomes (final concentration of 100 μM) and incubated for 15 min at 37 °C. After this pre-incubation period, 4HNE (final concentrations of 2, 5, 10, 20 and 100 μM) or MDA (final concentrations of 2, 5, 10, 20 and 50 μM) from stock solutions in 50 mM sodium phosphate buffer was added to the synaptosome suspensions. A contemporaneous control was included in all experiments that did not contain any 4HNE or MDA. The synaptosome suspensions were incubated at 37 °C (pH 7.4). Aliquots were removed at 0, 5, 10, 15, 30, 45 and 60 min and placed in microcentrifuge tubes containing perchloric acid (5% v/v final concentration) to terminate the reaction and precipitate protein. Precipitated protein was removed via centrifugation at 10,000 g for 10 min. The supernatant was stored at -20 °C until analysis could be performed. DA and metabolites were fractionated and detected using the HPLC method described below.

To determine whether an additive effect exists for lipid peroxidation products used in the study, an additional experiment was performed as described above with both 4HNE and MDA. 10 μM of 4HNE and MDA were simultaneously added to the synaptosome samples (37 °C, pH 7.4) after a 15 min pre-incubation with 100 μM DA. Aliquots were removed at 0, 5, 10, 15, 30, 45 and 60 min and the reaction was terminated by protein precipitation with 5% perchloric acid (v/v) as above.

High Performance Liquid Chromatography

An Agilent 1100 Series Capillary HPLC system was used for separation and quantification of DOPAL and its oxidized and reduced metabolites, DOPAC and DOPET, respectively. This unit is equipped with a sample cooler, capable of maintaining a temperature of 4 °C in the autosampler tray. Briefly, 10 µL of sample solution (i.e. supernatant) was injected and separation was achieved using a Phenomenex C18 Luna microbore column (1 × 150 mm, 100Å) with isocratic conditions consisting of 0.1% trifluoroacetic acid (v/v) in HPLC-grade water and 6% acetonitrile (v/v) at a flow rate of 50 µL/min. DOPAL and metabolites were detected with a photo-diode array detector using absorbance set to 202 and 280 nm for quantitation. Retention times for DA, DOPAL, DOPET and DOPAC were determined to be 3.8, 7.3, 8.8 and 12.3 min, respectively. Calibration curves were generated using standards (i.e. DA, DOPAL, DOPET and DOPAC) and utilized to convert peak area to concentration units. No other metabolic products were detected (i.e. methylation of DA or DOPAC from catechol-*O*-methyl transferase activity).

Measurement of Aldehyde Dehydrogenase Activity and 3,4-Dihydroxyphenylacetaldehyde Concentration

[Collaboration with Virginia Florang]

The initial linear slopes for the time-dependent formation of DOPAC were calculated via GraphPad Prism 4.0 (GraphPad Prism Software, San Diego, CA). Comparison of initial linear slopes for synaptosomes treated with 4HNE of MDA to those of the contemporaneous control yielded the ALDH activity relative to a control (i.e. % Control Activity (DOPAC)). The change in concentration of DOPAL from 0 to 60 min (∆ [DOPAL] (60 min)) was determined from $[DOPAL]_{60\ min} - [DOPAL]_{0\ min}$.

Linear Regression and Statistical Analysis

All linear regression and statistical analyses were performed using the software GraphPad Prism 4.0. For ALDH activity (i.e. DOPAL oxidation), data were compared and significant differences determined using one-way ANOVA with the Tukey's post-test ($p < 0.05$). To determine whether the change in [DOPAL] over the 60 min timeframe (Δ [DOPAL] (μM)) was significantly different from the control (0 μM 4HNE or MDA), a pair-wise two-tailed t-test was performed ($p < 0.05$).

Reactivity of 3,4-Dihydroxyphenylacetaldehyde Toward a Model Peptide

[Collaboration with Dr. Jonathan Doorn]

100 μM DOPAL was incubated with 10 μM RKRSRAE peptide in 10 mM tricine buffer, pH 7.4, for 4 hrs at 37 °C. As a control, the peptide was incubated with DA for 4 hrs using the same conditions. RKRSRAE treated with DOPAL and DA was analyzed via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Briefly, 1 μL of sample was diluted 1:10 with 0.1% trifluoroacetic acid and mixed with 1 μL 1:1 acetonitrile:water saturated with α-cyano-4-hydroxycinnaminic acid on a plate. The mixture was allowed to air-dry and analyzed using an Applied Biosystems MALDI-TOF-MS in linear mode using an acceleration voltage of 20 kV.

Determination of 3,4-Dihydroxyphenylacetaldehyde Protein Reactivity

The extent of DOPAL-protein modification was measured using the redox-cycling dye, NBT (*90*), which has been previously utilized to demonstrate DA-adducts on proteins (*38*). NBT stains blue-purple on nitrocellulose membranes in the presence of catechols, which are redox-sensitive functional groups.

To determine the reactivity of DOPAL towards proteins, rat striatal synaptosomes (1.3 mg/mL protein) in HEPES media (37 °C, pH 7.4) were pre-incubated for 15 min with 100 μM DA to generate DOPAL in situ. After the pre-incubation period, 4HNE was added to give a final concentration of 5, 10, 50 or 100 μM. A control was included in experiments containing no 4HNE. An additional control was utilized to determine the dependence of observed protein modification on MAO activity, specifically, 100 μM pargyline (an MAO inhibitor) was included in the 15 min pre-incubation with DA before the addition of 0 or 50 μM 4HNE. Samples were incubated for 3 h at 37 °C and stored at -20 °C until analysis.

A 7% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was used for separation of protein samples (60 μg protein) treated with sodium cyanoborohydride (5 mM) to stabilize adducts. Upon completion of SDS-PAGE, gels were placed in Bjenum and Schafer-Nielsen transfer buffer (37 °C, pH 7.4) for 10 min and transferred to a nitrocellulose membrane utilizing a semi-dry transfer apparatus (70 min, 20 V). The nitrocellulose membrane was placed in 0.24 mM NBT with 2 M potassium glycine buffer (pH 10) and allowed to incubate overnight at 4 °C. The membranes were rinsed with 0.1 M sodium borate (pH 10) and stored in 5 mL distilled water (4 °C) (*90*).

To compare the reactivity of DA and its metabolites to adduct protein, 100 μM of DA, DOPAL, DOPAC or 3,4-dihydroxyphenylalanine (L-DOPA) was incubated with a 1.2 mg/mL solution of bovine serum albumin (BSA) for 3 h (37 °C, 50 mM sodium phosphate buffer, pH 7.4). As described above, a 10% gradient SDS-PAGE gel was used for protein separation of samples (50 μg protein) treated with excess sodium cyanoborohydride (5 mM). The gel was transferred to a nitrocellulose membrane and incubated overnight with 0.24 mM

NBT in 2 M potassium glycine buffer (pH 10) at 4 °C. The membranes were rinsed with 0.1 M sodium borate (pH 10) and stored in 5 mL distilled water (4 °C). The integrated density of each lane was quantified using the program NIH ImageJ version 1.37 (http://rsb.info.nih.gov/ij/) after conversion of figures to 32-bit format and subtraction of the background.

Results

Inhibition of Dopamine Catabolism by Products of Lipid Peroxidation

As shown in *Figure 4.1A and B*, treatment of rat striatal synaptosomes with 4HNE even at low concentrations (i.e. 2-10 μM), yielded a decrease in the time-dependent formation of DOPAC (i.e. rate of DOPAL oxidation to DOPAC). This effect was found to be apparently dose-dependent in regards to 4HNE (2-100 μM), and all concentrations of the lipid aldehyde resulted in impairment of DOPAC formation (*Figure 4.1A and B*). These results demonstrate that 4HNE is a strong inhibitor of DOPAL oxidation as ALDH activity is significantly reduced compared to control. Interestingly, even low micromolar 4HNE (2-10 μM) significantly impairs DA catabolism.

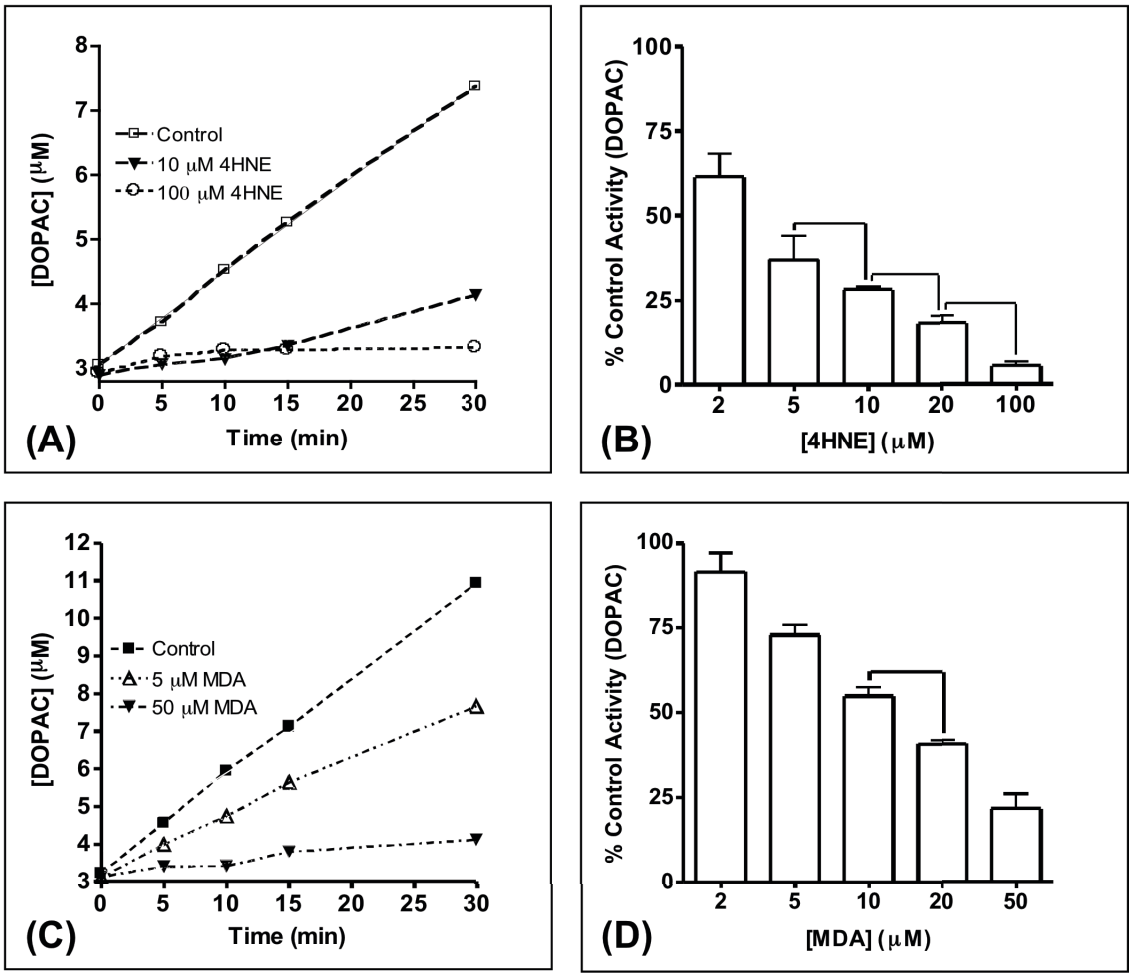


Figure 4.1 Treatment of synaptosomes with 4HNE or MDA yields inhibition of ALDH. (A) Representative time course demonstrating inhibition of DOPAL oxidation (i.e. DOPAC production) in the presence of 4HNE. (B) 4HNE inhibits DOPAL oxidation in a dose-dependant manner. (C) Representative time course demonstrating inhibition of DOPAL oxidation (i.e. DOPAC production) in the presence of MDA. (D) MDA inhibits DOPAL oxidation in a dose-dependant manner. Values shown represent means ± SEM (n = 4, except 2 μM 4HNE, where n = 3) and are significantly different unless connected by a bar ($p < 0.05$).

A decrease in DOPAL oxidation (10-70%) was also observed when rat striatal synaptosomes were treated with MDA (2-50 μM) as shown in *Figure 4.1B and C*. In the presence of all concentrations of MDA, the rate of DOPAC formation was decreased. As with 4HNE, MDA-mediated impairment of DOPAL oxidation was apparently dose-dependent; however, MDA was not as effective as an inhibitor compared to 4HNE.

Interestingly, the combination of 4HNE and MDA (i.e. 10 μM each) produced ALDH inhibition greater than 10 μM MDA alone ($p < 0.001$) but slightly higher, although not significantly, than 10 μM 4HNE by itself ($p > 0.05$). For rat striatal synaptosomes incubated with the combination of 10 μM 4HNE and 10 μM MDA ($n = 2$), DOPAL oxidation was reduced to 19.9 (± 1.76)% of control activity, compared to 54.9 (± 2.51)% and 28.1 (± 0.731)% for MDA and 4HNE, respectively.

As indicated in *Figure 4.1A and B*, the rate of DOPAC production (i.e. DOPAL oxidation) was impaired by 4HNE even at low micromolar concentrations. Such inhibition corresponded to increased DOPAL concentrations (i.e. 150-210%) after 60 min incubation as shown in *Figure 4.2A and B*. Furthermore, rat striatal synaptosomes treated with MDA resulted in a 125-225% increase in DOPAL concentration as compared to controls (*Figure 4.2C and D*) after 60 min incubation. In the control, steady-state DOPAL concentration is typically in the range of 8-10 μM; however, after treatment with MDA or 4HNE, levels of DOPAL increased in a non-linear manner (*Figure 4.2*). Treatment of synaptosomes with 10 μM 4HNE plus 10 μM MDA yielded a 196 (± 13.4) % increase ($n = 2$) in DOPAL concentration as compared to control. These results demonstrate that in the presence of even low micromolar lipid peroxidation products (i.e. 4HNE and/or MDA) at low or physiologic levels, DOPAL oxidation is inhibited, yielding elevated concentrations of DOPAL.

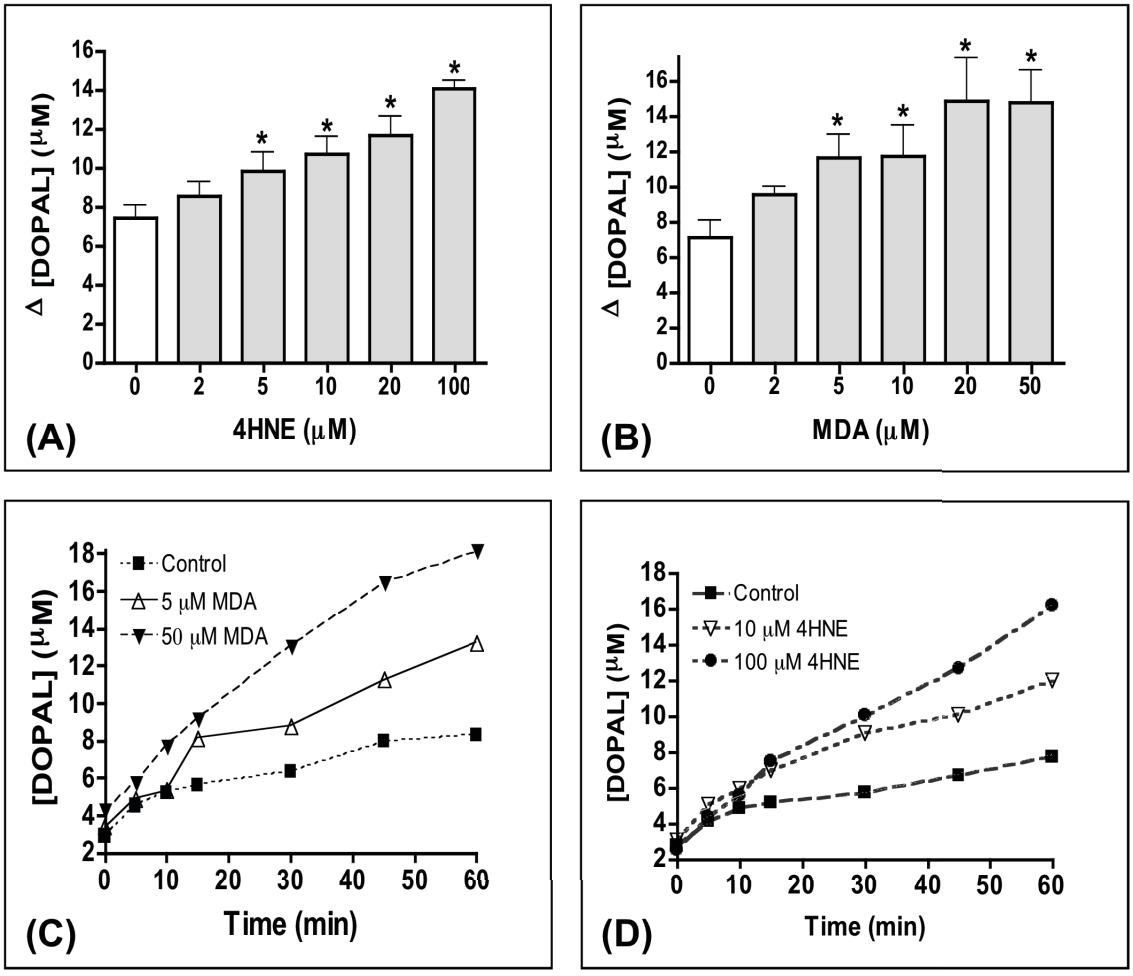


Figure 4.2 Treatment of synaptosomes with 4HNE or MDA yields inhibition of ALDH resulting in elevated [DOPAL]. (A) Representative time-course demonstrating 4HNE-mediated increase in [DOPAL]. (B) Inhibition of DOPAL oxidation by 4HNE results in an increase in [DOPAL] over the 60 min time frame. (C) Representative time-course demonstrating MDA-mediated increase in [DOPAL]. (D) Inhibition of DOPAL oxidation by MDA results in an increase in [DOPAL] over the 60 min timeframe. Δ [DOPAL] (μM) represents the change in [DOPAL] after 60 min and was calculated as follows: $[DOPAL]_{60\ min} - [DOPAL]_{0\ min}$. Values shown represent mean ± SEM (n = 4, except for 2 μM 4HNE where n = 6 and 10 and 50 μM MDA where n = 3) and are not significantly different from the control (0 μM 4HNE or MDA) unless denoted with an * ($p < 0.05$).

Modification of a Model Peptide by 3,4-Dihydroxyphenylacetaldehyde

Results of previous work suggested DOPAL to be reactive towards proteins (*62, 77, 78, 83-85*). Modification of proteins by DOPAL could be the result of reaction of primary amines (e.g. Lys) with the aldehyde, yielding a Schiff base. In addition, catechols can auto-oxidize to an ortho-quinone and react with protein nucleophiles via Michael addition. To verify the protein reactivity of DOPAL and determine the type of adduct (e.g. Schiff base), a Lys-containing peptide (i.e. RKRSRAE) was incubated with DOPAL. Interestingly, treatment of the peptide (measured MH^+ at *m/z* 903.4) with DOPAL yielded a new peak at *m/z* 1037.7, corresponding to a 134.3 Da adduct and Schiff base formation, i.e. peptide with a DOPAL adduct minus water *(Figure 4.3*). No adducts representing Michael addition (i.e. 152 Da) were observed for DOPAL (*Figure 4.3B*) or DA (*Figure 4.3C*) under conditions utilized. In addition, treatment of the peptide with DOPAL yielded a new peak at *m/z* 1172.0 corresponding to two adducts (134.3 Da). Based on signal intensity, the majority of peptide was modified by DOPAL (i.e. 67%).

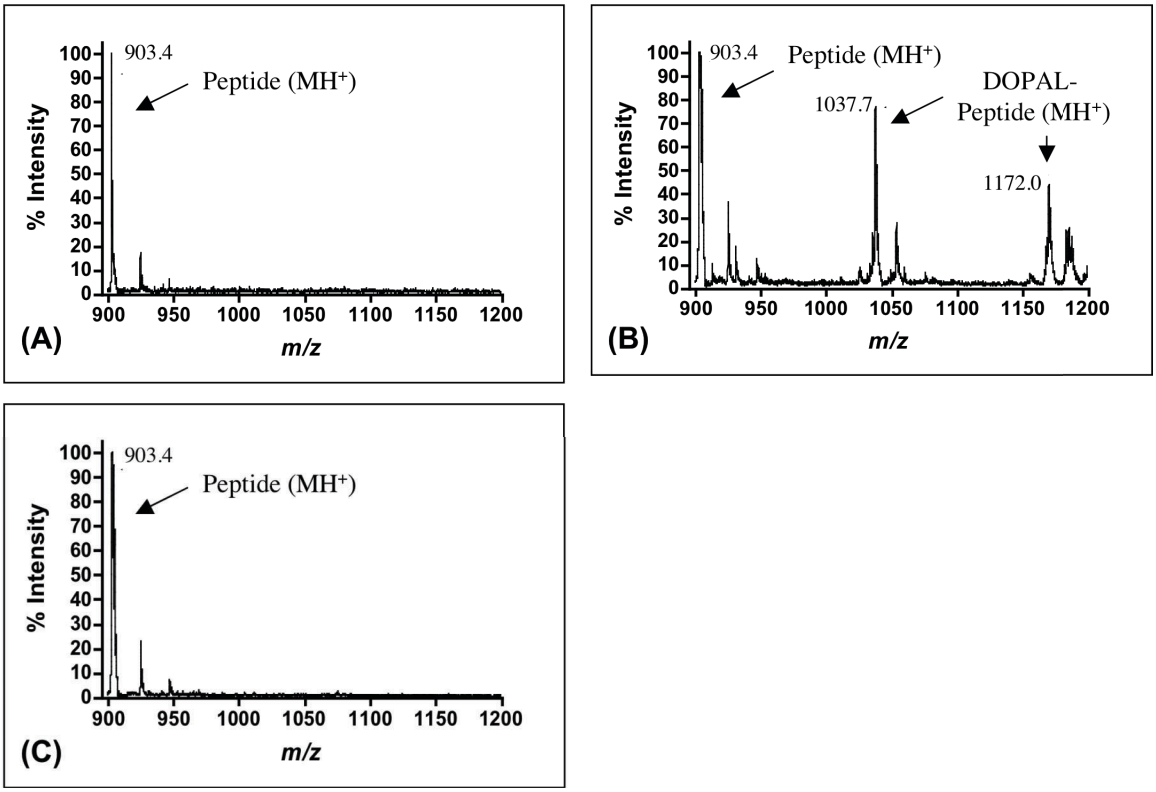


Figure 4.3 MALDI-TOF-MS of the peptide RKRSRAE treated with DOPAL or DA, demonstrating modification of the peptide by DOPAL but not DA. (A) Control, (B) 100 μM DOPAL, and (C) 100 μM DA.

3,4-Dihydroxyphenylacetaldehyde Protein Modification in Rat Striatal Synaptosomes

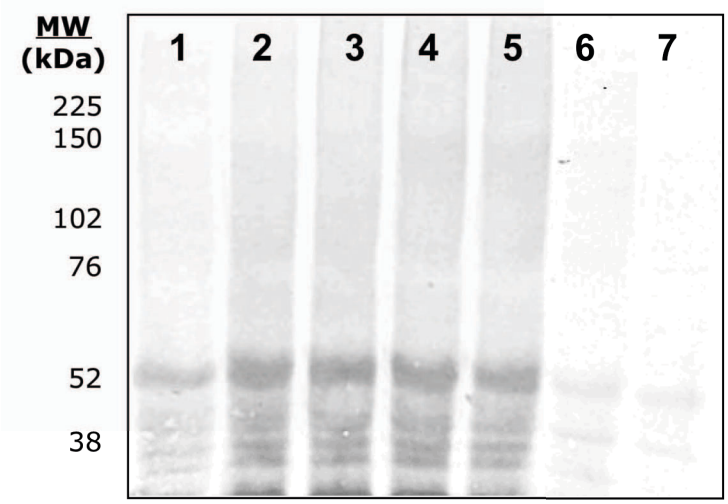
An elevated level of DOPAL, resulting from the presence of lipid peroxidation products, is predicted to yield increased protein modification. To evaluate this prediction, a redox-cycling dye (i.e. NBT) previously shown to be sensitive to catechol-protein adducts was used to detect DOPAL-protein modification in rat striatal synaptosomes (*38, 90*). With the control and all concentrations of 4HNE (5-100 μM), NBT staining was apparent for total synaptosomal protein; however, an increase in NBT reactivity was observed for samples treated with 5-100 μM 4HNE (*Figure 4.4*). Densitometry analysis

revealed a >2-fold increase in staining for lanes corresponding to samples treated with 4HNE.

Synaptosomes pretreated with the MAO inhibitor pargyline (100 μM) plus 0 or 50 μM 4HNE exhibited a marked reduction in NBT staining (*Figure 4.4*). Quantification of the integrated lane density showed a 50-70% decrease in NBT reactivity when pargyline was included in samples. Such a result demonstrates the dependence of NBT staining on MAO activity, suggesting that protein adduction is primarily due to DOPAL. It should be noted that pargyline can be metabolized to propiolaldehyde, a potent ALDH inhibitor (*93*). However, it does not appear that this is the case given the observed results.

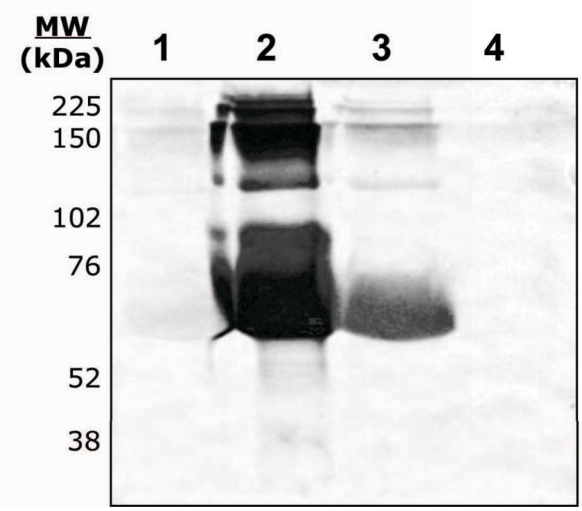
Modification of Bovine Serum Albumin by Dopamine and its Metabolites

As shown in *Figure 4.5*, treatment of BSA with DA, DOPAL, DOPAC or L-DOPA (100 μM) for 3 hrs at 37 °C resulted in varying levels of NBT reactivity and protein modification. Based on quantification of NBT staining as described in the Materials and Methods section, the order of reactivity was determined to be DOPAL >> DOPAC > DA ≥ L-DOPA. Given this evidence with BSA, and that the MAO inhibitor pargyline significantly inhibits NBT reactivity when synaptosomes are treated with DA as shown in *Figure 4.4*, it is likely that the majority of observed NBT staining is due to DOPAL.



Sample	Lane	% Control
0 μM 4-HNE	1	100
5 μM 4-HNE	2	227
10 μM 4-HNE	3	243
50 μM 4-HNE	4	238
100 μM 4-HNE	5	213
0 μM 4HNE + 100 mM pargyline	6	49.8
50 μM 4HNE + 100 mM pargyline	7	32.6

Figure 4.4 Modification of total synaptosomal proteins by DOPAL as determined via NBT reactivity. Treatment of striatal synaptosomes with 4HNE (5-100 μM) resulted in an increase in catechol adducts (i.e. NBT staining of total proteins). Synaptosomes incubated with 4HNE, but pretreated with the MAO inhibitor paragyline (100 μM), demonstrate MAO dependence for protein modification (i.e. NBT reactivity of total proteins). Such a result indicates that the majority of staining is due to DOPAL. % Control refers to the integrated staining density for the control sample (no 4HNE or pargyline).



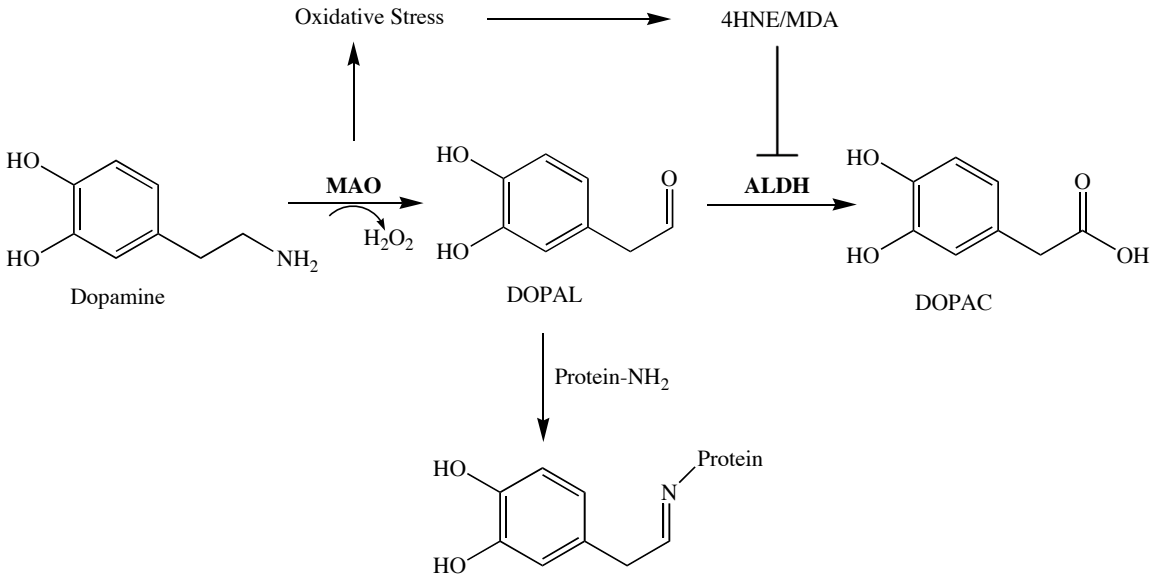
Sample	Lane	% Control
DA	1	7.9
DOPAL	2	100
DOPAC	3	28
L-DOPA	4	3.2

Figure 4.5 Treatment of BSA with 100 μM DA, DOPAL, DOPAC, or L-DOPA at 100 μM yielded varying degrees of NBT staining as shown in lanes 1-4. Such a result demonstrates the order of reactivity to be DOPAL >> DOPAC > DA ≥ L-DOPA. % Max signal refers to the integrated staining density for a given lane relative to that determined for lane 2 (BSA + DOPAL), which had the highest staining intensity.

Discussion

In the current study, it was demonstrated that the two common components of oxidative stress, 4HNE and MDA, impair DA catabolism in rat striatal synaptosomes even at very low concentrations (e.g. 2-10 μM). Specifically, the lipid peroxidation products inhibit the second step of the DA catabolic pathway, i.e. DOPAL oxidation by ALDH, without significantly affecting MAO activity (*63*) (*Scheme 4.2*). Concentrations of 4HNE and MDA used in experiments are considered to be in the physiological range (*49, 80*), and therefore, oxidative stress and lipid peroxidation experienced in dopamine neurons are predicted to yield inhibition of DOPAL oxidation (*Scheme 4.2*). Such findings are consistent with earlier studies showing both 4HNE and MDA to be potent inhibitors of ALDH, respectively (*53-55, 57*). In addition, these results are

in agreement with a recent report demonstrating impairment of DOPAL metabolism in rat brain mitochondria by 4HNE (*59*).



Scheme 4.2 Inhibition of DOPAL metabolism via 4HNE and MDA, products of oxidative stress, yields an increase in DOPAL-protein adduction.

It should be noted that both 4HNE and MDA were added exogenously to synaptosomes as a one-time dose, thus requiring the lipid peroxidation products to traverse various membrane barriers before coming in contact with enzymes involved in DOPAL oxidation, such as the mitochondrial ALDH (*63*). Therefore, mitochondrial oxidative stress yielding 4HNE and/or MDA in proximity of matrix ALDH enzymes is likely to result in greater inhibition. In addition, persistent oxidative stress yielding lipid peroxidation may yield sustained inhibition of DOPAL oxidation.

Interestingly, a slight but not statistically significant additive effect for inhibition was observed when synaptosomes were treated with both 4HNE and

MDA. Under conditions of oxidative stress, it is likely that a mixture of aldehydic compounds are generated, with 4HNE and MDA being the primary products (*49*). Therefore, it was of interest to determine whether such a physiologically relevant combination of lipid peroxidation-derived compounds would have an additive effect. The combination of 4HNE and MDA produced inhibition greater than MDA alone ($p < 0.001$) but slightly higher, although not significantly, than 4HNE alone ($p > 0.05$). Mitochondria are known to contain several ALDH's (*94, 95*), and such a result suggests that 4HNE and MDA may target the same enzymes involved in DOPAL oxidation.

As shown in *Figure 4.2*, inhibition of DOPAL oxidation, i.e. time-dependent formation of DOPAC, yields a corresponding increase in [DOPAL] that is apparently dose-dependent on 4HNE or MDA. While the batch to batch variability was substantial (*Figure 4.2B and D*) such that averaging the % Control [DOPAL] yielded significant deviation, the overall trend was consistent. Specifically, within each batch of synaptosome, increasing 4HNE or MDA concentrations yielded an apparent dose-dependent elevation of DOPAL concentration. It should be noted that the % Control [DOPAL] shown in *Figure 4.2B and 4.2D* were determined from the DOPAL concentrations at the 60 min timepoint, and longer incubations may yield even higher levels of the DA-derived aldehyde. Therefore, it is conceivable that sustained ALDH inhibition, perhaps from persistent oxidative stress, could result in DOPAL several-fold higher than basal levels.

A previous study suggested that alternative metabolic pathways (e.g. aldehyde reductases) may compensate if ALDH is saturated (*81*); however, no significant amount of the reduced product, DOPET, was observed. In addition, cytosolic ALDH's that could participate in DOPAL oxidation also appear to be inhibited by the lipid peroxidation products. As a result, the net effect of 4HNE

and MDA on the DA catabolic pathway is predicted to be an accumulation of the DOPAL intermediate.

Given previous reports on the high toxicity of DOPAL to cells, an accumulation of the endogenous neurotoxin is significant. Physiologic concentrations of DOPAL are estimated to be around 2-3 μM (*79*); however, an earlier study found that the DA-derived aldehyde at concentrations as low as 6 μM, just above basal level, can cause cell death over time (*77, 78*). Therefore, the 2- to 3-fold elevation in DOPAL concentration observed in the current study due to the presence of lipid peroxidation products may be damaging to dopaminergic cells, potentially involving the formation of free radicals (*82*), mitochondrial pore transition (*77*), release of DA (*96*) or protein modification (*83-85*).

Protein modification may be due to reaction of amines (e.g. Lys) with the aldehyde of DOPAL, yielding a Schiff base adduct. As shown in *Figure 4.3*, MALDI-TOF-MS analysis revealed two new peaks with mass corresponding to the peptide with one or two Schiff base adducts. Such evidence indicates the reactivity of the DOPAL aldehyde toward the Lys nucleophile under physiological conditions. It should be noted that while the mass of the adduct (134 Da) is consistent with a Schiff base and the reaction of primary amines with aldehydes generally produces a Schiff base, it cannot be ruled out that the modification is an enamine or other chemical species that is more stable.

It is conceivable that DOPAL may oxidize to an ortho-quinone, as observed for DA (*70, 71*), yielding a bifunctional electrophile that could modify proteins. Given the active site thiols of ALDH (*97*), the enzyme could be a target for the ortho-quinone of DOPAL. Work is in progress to characterize the ortho-quinone of DOPAL (generated via sodium periodate and tyrosinase). In the current study, the DOPAL ortho-quinone was not observed during time courses

(60 min); however, given longer incubations or higher DA concentration, such an electrophile might be formed to a significant degree. In addition, incubation of a model peptide with DOPAL yielded the Schiff base modification and no ortho-quinone adducts were observed *(Figure 4.3*).

The finding that DOPAL forms adducts on proteins *(Figure 4.4*) is in agreement with several previous studies (*83-85*). Treatment of synaptosomes with 4HNE yielded an increase in NBT staining *(Figure 4.4*), evidence of elevated DOPAL-protein modification. Synaptosomes were used in experiments to generate the data shown in *Figure 4.4*, and therefore, the primary subcellular location, i.e. cytoplasm or mitochondria, of DOPAL-adducts is not known at this point. Future work will entail determining the subcellular location of DOPAL adducts and identifying the protein targets.

It might be argued that the NBT staining is indicative of adduction of proteins by DA given that this redox-cycling dye detects catechols (*38*), and numerous studies have demonstrated the reactivity of DA toward proteins (*69, 72, 98*). However, two key pieces of evidence in the current study show that the majority of NBT reactivity is due to DOPAL-protein adducts. First, inclusion of the MAO inhibitor pargyline (100 μM) resulted in a marked decrease in staining by the redox-sensitive dye. Such a result demonstrates that the NBT reactivity is highly dependent on MAO activity, i.e. production of DOPAL.

Second, as shown in *Figure 4.5*, DOPAL is far more reactive toward protein (i.e. BSA) than DA or DOPAC. This finding is not a surprise given that DOPAL already contains an electrophilic aldehyde group but DA requires oxidation to an orthortho-quinone before it can modify proteins (*70, 71*). In addition, the electrophilic form of DA (i.e. quinone) is short-lived as it can rapidly rearrange via intramolecular nucleophilic addition to leukoaminochrome and then aminochrome (*70, 71*).

Reactive oxygen species (ROS) are produced via the MAO-catalyzed oxidative deamination of DA, namely, H_2O_2. It might be argued that ROS could oxidize DA to the reactive quinone or inhibit ALDH via oxidative modifications, thus explaining results shown in *Figure 4.4*. However, it should be noted that the first step of the DA catabolic pathway, i.e. MAO, is insensitive to 4HNE (*59*). Therefore, DA deamination will be comparable between control and treated samples, yielding similar levels of H_2O_2.

Elevation of DOPAL concentration and levels of DOPAL-protein modification due to products of oxidative stress may be highly significant to neurodegenerative disease. Recent work has implicated oxidative stress and lipid peroxidation, possibly resulting from exposure to environmental agents, as a factor in PD (*27, 44*). The relationship between general oxidative stress and PD pathogenesis, which involves selective degeneration of dopamine neurons, is not known (*44*). However, DOPAL could represent such a mechanistic link. In the current study, physiological levels of two common components of oxidative stress, i.e. 4HNE and MDA, were shown to inhibit DOPAL oxidation, yielding aberrant levels of this neurotoxin endogenous to DA neurons. Chronic exposure to environmental agents that induce oxidative stress may cause persistent dysregulation of DA catabolism and protein modification as shown in *Scheme 4.2*.

At this point, it is not known how aberrant levels of DOPAL may participate in neurodegeneration relevant to PD pathogenesis, however, it could occur over the course of many years and involve several mechanisms (e.g. protein modification) as described above. Future studies are warranted to answer such questions. Potentially, such work may prove DOPAL to be a biomarker of neurodegenerative disease or a novel therapeutic target, thus justifying use of "carbonyl scavengers" (*99-101*) in treating or preventing PD pathogenesis.

CHAPTER FIVE
REACTIVITY OF 3,4-DIHYDROXYPHENYLACETALDEHYDE TOWARDS PROTEINS

Abstract

Dopamine (DA) has been implicated as an endogenous neurotoxin to explain the selective neurodegeneration as observed for Parkinson's disease (PD). However, previous work demonstrated 3,4-dihydroxyphenylacetaldehyde (DOPAL) to be more toxic than DA. DOPAL is generated as a part of DA catabolism via the activity of monoamine oxidase and the mechanism of DOPAL toxicity is proposed to involve protein modification. Previous studies have demonstrated protein reactivity via the aldehyde moiety; however, DOPAL contains two reactive functional groups (catechol and aldehyde) both with the potential for protein adduction. The goal of this work was to determine whether protein modification by DOPAL occurs via a thiol-reactive quinone generated from oxidation of the catechol, which is known to occur for DA, or if the aldehyde forms adducts with amine nucleophiles. To accomplish this objective, the reactivity of DOPAL towards N-acetyl-lysine (NAL), N-acetyl-cysteine (NAC) and two model proteins was determined. In addition, several DOPAL analogues were obtained and used for comparison of reactivity. Results demonstrate that at pH 7.4 and 37°C, the order of DOPAL reactivity is NAL >> NAC and the product of NAL and DOPAL is stable in the absence of reducing agent. Moreover, DOPAL will react with model proteins, but in the presence of amine-selective modifiers citraconic anhydride and 2-iminothiolane hydrochloride, the reactivity of DOPAL towards the proteins is diminished. In addition, DOPAL-mediated protein cross-linking is observed when a model protein or a protein mixture (i.e. mitochondria

lysate) are treated with DOPAL at concentrations of 5-100 μM. Protein cross-linking was diminished in the presence of ascorbate, suggesting the involvement of a quinone in DOPAL-mediated protein modification. These data indicate DOPAL to be highly reactive towards protein nucleophiles with the potential for protein cross-linking.

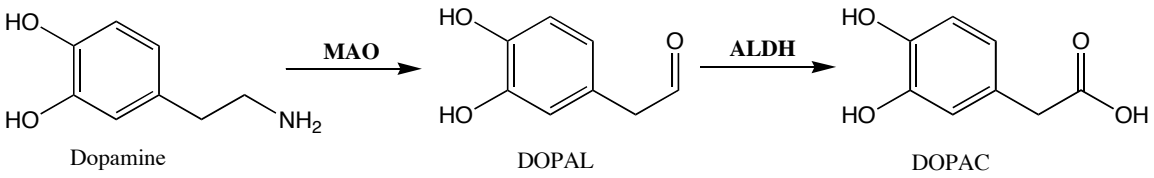
Introduction

Oxidative stress is hypothesized to play a significant role in Parkinson's disease (PD), a condition involving dopaminergic cell death (*45, 46*); however, the exact mechanisms underlying the pathogenesis of this common neurodegenerative disorder are currently unknown. It has been proposed that a toxin endogenous to dopamine neurons is generated subsequent to disease insult and this neurotoxin mediates neurodegeneration (*76, 102-104*).

Studies have demonstrated that dopamine (DA) is an endogenous neurotoxin that readily undergoes auto-oxidation to an ortho-quinone (*70, 71*) capable of protein modification (*69, 72, 98*). Subsequent to biosynthesis, cytosolic DA is rapidly sequestered into vesicles and many reports have shown that dysregulation of DA trafficking and storage, such as toxicant-mediated release from vesicles, yields cell toxicity (*105-107*).

However, previous work has demonstrated that the aldehyde metabolite of DA, 3,4-dihydroxyphenylacetaldehyde (DOPAL), which results from the activity of monoamine oxidase (MAO) (*Scheme 5.1*), is orders of magnitude more toxic in vitro and in vivo than DA (*8, 76*). Physiologic concentrations of DOPAL are around 2-3 μM (*79*) and controlled via the action of aldehyde reductases and several aldehyde dehydrogenases (ALDHs), both cytosolic and mitochondrial (*52*). When levels of the aldehyde intermediate are only slightly elevated (6 μM), cell death is observed (*77, 78*). Such evidence indicates DOPAL to be toxic, and

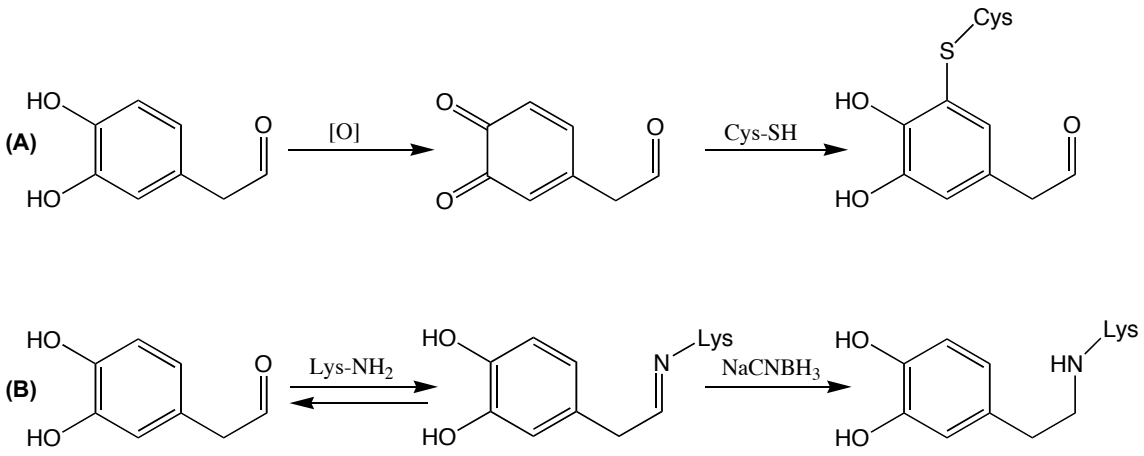
therefore, inhibition of its metabolism could be extremely harmful to cells and play a significant role in the onset of PD.



Scheme 5.1 The primary pathway for DA metabolism involves oxidative deamination via MAO to an aldehyde intermediate followed by oxidation to an acid product.

Several studies have demonstrated adduction of proteins by DOPAL (*84-86, 102*), and recent work has shown that inhibition of DOPAL oxidation and/or reduction via oxidative stress/lipid peroxidation products yielded an increase in DOPAL concentration and protein modification (*59, 102*). However, protein residues susceptible to modification by DOPAL, that is, Cys or Lys, have not been conclusively identified. Oxidation of the catechol yields a Cys-reactive quinone, which is known to occur for DA (*70, 71, 75*); however, the aldehyde may form adducts with Lys residues (*102*) (*Scheme 5.2*). In addition, the stability of each adduct under physiologic conditions is of question, especially for Lys as Schiff base products often require reduction for stability. It is also conceivable that DOPAL is a bifunctional electrophile which could cause extensive protein cross-linking. Therefore, the current study was undertaken to identify the mechanism by which DOPAL modifies proteins as the DA-derived aldehyde contains two reactive functional groups, i.e. aldehyde and catechol. DOPAL and structural analogues of DOPAL, including the DA metabolite 3-methoxy-4-hydroxyphenylacetaldehyde (MOPAL), were obtained and reacted with protein

nucleophiles, including amino acids and two model proteins. For the first time, the protein reactivity of DOPAL was determined both qualitatively and quantitatively.



Scheme 5.2 Possible mechanisms for DOPAL-mediated protein modification include (A) auto-oxidation of the catechol followed by reaction with protein thiols or (B) reaction with protein amines via the aldehyde. The Schiff base in (B) may require reduction for stability.

Experimental Procedures

Materials

DOPAL was biosynthesized via an established procedure involving enzyme-catalyzed conversion of DA to DOPAL by rat liver MAO (*84*) and its concentration was determined using an ALDH assay with nicotinamide adenine dinucleotide (NAD) (*85*) and high performance liquid chromatography (HPLC) analysis, as described below. 3,4-Dihydroxyphenylethanol (DOPET) was obtained via reduction of DOPAL with a 10-fold excess of sodium borohydride. The DOPAL quinone was generated via reaction of DOPAL with a 2-fold excess

of sodium metaperiodate in 50 mM sodium phosphate buffer, pH 7.4, and used as a standard for HPLC analysis.

3,4-Dimethoxyphenylacetaldehyde (DMPAL) was synthesized via alkene oxidation of eugenol methyl ether (Sigma-Aldrich) using 3.5 mol% $RuCl_3$ and 4 equiv $NaIO_4$ [3 h in 6:1 acetonitrile:water; 40% yield; ^{1}H NMR ($CDCl_3$) δ 3.60 (d, 2H), 5.93 (s, 2H), 6.65-6.82 (m, 3H), 9.71 (t, 1H); ^{13}C NMR ($CDCl_3$) δ 50.23, 56.12 (2C), 111.82, 112.84, 122.03, 124.38, 148.55, 149.48, 199.68] (*108*).

MOPAL was biosynthesized via a similar procedure as outlined for DOPAL above, namely, rat liver MAO was used to convert 3-methoxytyramine to MOPAL. DA, 3,4-dihydroxyphenylacetic acid (DOPAC), bovine serum albumin (BSA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Evaluation of the Reactivity of 3,4-Dihydroxyphenylacetaldehyde

[Collaboration with Laurie Eckert]

To determine the reactivity between DOPAL and Lys or Cys residues, 100 µM DOPAL was incubated with either N-acetyl-Lysine (NAL, 1, 2.5, 5, 7, 10 mM) or N-acetyl-Cysteine (NAC, 10 mM) (37°C, 50 mM sodium phosphate buffer, pH 7.4). Aliquots were taken periodically throughout the 90 min time course and the reaction was quenched by a 1:10 dilution with 1% trifluoroacetic acid (TFA) followed by storage at -20°C. The amount of DOPAL consumed during the course of the experiment was determined via HPLC as described below. In addition, a control was analyzed via HPLC to account for any spontaneous loss of DOPAL (i.e. oxidation to the acid, DOPAC).

Reactivity studies were also completed with reagents that were structurally similar to that of DOPAL. Specifically, NAL at varying concentrations was incubated for 3.5 h with 100 μM of either DMPAL, MOPAL or phenylacetaldehyde (PAL) in both the presence and absence of 5 mM sodium cyanoborohydride ($NaCNBH_3$) reducing agent (50 mM sodium phosphate buffer, pH 7.4, 37°C). Aliquots were quenched by a 1:10 dilution with 1% TFA and quantified by HPLC.

High Performance Liquid Chromatography

Quantification of DMPAL, DOPAL, and PAL at each time point of the reactivity study was performed using an Agilent 1100 Series Capillary HPLC system with a photodiode array detector set to absorbance at 202 and 280 nm. Briefly, 10 *μ*L of sample solution was injected and separation was achieved using a Phenomenex C18 Luna microbore column (1 × 150 mm, 100 Å) and a mobile phase consisting of 0.1% TFA (v/v) in HPLC-grade water and 6% acetonitrile (v/v) at a flow rate of 50 *μ*L/min. MOPAL was analyzed using a mobile phase consisting of 0.1% TFA (v/v) in HPLC-grade water and 10% acetonitrile (v/v) at a flow rate of 50 *μ*L/min.

Comparison of Protein Reactivity, 3,4-Dihydroxyphenylacetaldehyde vs. 3-Methoxy-4-Hydroxyphenylacetaldehyde

[Collaboration with Laurie Eckert]

BSA (1 mg/mL) was treated with either 50 μM DOPAL, 50 μM MOPAL, 1:1 DOPAL:MOPAL (50 μM each), 1:2 DOPAL:MOPAL (50 μM and 100 μM) or 1:10 DOPAL:MOPAL (50 μM and 500 μM). In the presence of reducing agent (5 mM $NaCNBH_3$), samples were incubated for 4 h (37 °C, 50 mM sodium phosphate buffer, pH 7.4).

Catechol-containing protein adducts were detected using the redox-cycling dye nitroblue tetrazolium (NBT) (*38, 90*) or coomassie blue. While coomassie blue stain detects all proteins, NBT is selective for catechols, which are redox-sensitive functional groups. Therefore, NBT will selectively stain proteins containing such adducts (*38, 90*). A 7.5% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was used for protein separation of samples (5 µg). SDS-PAGE gels were placed in Bjenum and Schafer-Nielsen transfer buffer (37 °C, pH 7.4) for 10 min before being transferred to a nitrocellulose membrane utilizing a semi-dry transfer apparatus (70 min, 20 V). The nitrocellulose membrane was placed in 0.24 mM NBT with 2 M potassium glycine buffer (pH 10) and allowed to incubate overnight at 4 °C. The membranes were rinsed and stored with 5 mL distilled water (4 °C). The integrated density of each lane was quantified using the program NIH ImageJ version 1.37 (http://rsb.info.nih.gov/ij/).

To further verify the compounds were binding to the protein during the course of the reaction, the aforementioned BSA samples treated with DOPAL and MOPAL were analyzed by HPLC using the procedure described above. In addition, a model peptide (10 µM RKRSRAE) was incubated with 100 µM of DOPAL or MOPAL (10 mM tricine buffer, pH 7.4, 4 h at 37 °C) and analyzed via matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Briefly, 1 µL of sample was diluted 1:1 with 0.1% TFA and mixed with 1 µL of water saturated with α-cyano-4-hydroxycinnaminic acid on a plate. The mixture was allowed to air dry and analyzed using a Bruker Bioflex MALDI-TOF-MS in reflectron mode. Calibration was performed using the peptides angiotensin I and bradykinin.

Detection of 3,4-Dihydroxyphenylacetaldehyde Adducts on Proteins Treated with a Lysine or Cysteine Modifier

The model proteins BSA (14 μM) and GAPDH (27.7 μM) were treated for 45 min at 37°C (50 mM sodium phosphate buffer, pH 7.4) with one of the following protein modifiers at concentrations of 0, 1, 5 and 10 mM; citraconic anhydride (modifies primary amines), 2-iminothiolane hydrochloride (Traut's reagent, converts amines to thiols) or iodoacetic acid (modifies thiol residues). To verify the amine to thiol conversion, samples were titrated with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and absorbance at 412 nm was determined. Following reaction with the modifiers, the proteins were incubated with 100 μM DOPAL for 4 h at 37 °C (50 mM sodium phosphate buffer, pH 7.4).

Additionally, BSA modified by citraconic anhydride (0, 1, 5, 10 mM) for 45 min at 37 °C was treated with 1 M HCl to acidify the sample (pH 3) and incubated for 2 h to remove the citraconic anhydride adduct (Sigma Aldrich technical bulletin). It should be noted that citraconic anhydride can react with thiols; however, the resulting adduct cannot be removed via acid hydrolysis. Therefore, the ability to restore DOPAL reactivity following acid hydrolysis of the citraconic anhydride modification allows discrimination between Lys and Cys adducts (*109, 110*). Samples were subsequently treated with 100 μM DOPAL and incubated overnight (37 °C, 50 mM sodium phosphate buffer, pH 7.4). As described above, gradient SDS-PAGE gels (7.5% for BSA samples and 10% for GAPDH samples) and NBT were used for protein separation and staining.

Determination of Catechol-Adducts on a Model Protein

To compare the reactivity of DA and its metabolites to adduct proteins, 100 μM DA, DOPAL, DOPAC, or 3,4-dihydroxyphenylalanine (L-DOPA) was incubated with 1 mg/mL (27.7 μM) GAPDH for either 3 or 6 h (37 °C, 50 μM sodium phosphate buffer, pH 7.4). A 10% gradient SDS-PAGE gel and NBT were used for protein separation and staining.

Presence of Glyceraldehyde-3-Phosphate Dehydrogenase Protein Cross-Linking upon Treatment with 3,4-Dihydroxyphenylacetaldehyde

[Collaboration with Virginia Florang]

A 0.3 mg/mL solution of GAPDH (8.3 μM) was treated with DOPAL (0, 5, 50 and 100 μM) and incubated at 37°C for either 2 or 4 h (50 mM sodium phosphate buffer, pH 7.4). For comparison, the experiment was repeated using 0 and 100 μM MOPAL along with 0 and 100 μM DOPAL. Proteins were separated via SDS-PAGE (7.5% acrylamide) and stained with Coomassie Blue.

Additionally, a 0.3 mg/mL solution of GAPDH was treated with either DOPAL (0 and 50 μM), DOPAL (0 and 50 μM) with 5 mM $NaCNBH_3$ or DOPAL (0 and 50 μM) with 5 mM sodium ascorbate and incubated at 37 °C for 4 h (50 mM sodium phosphate buffer, pH 7.4). A 10% gradient SDS-PAGE gel and coomassie blue staining were used for protein separation and staining.

3,4-Dihydroxyphenylacetaldehyde Protein Cross-Linking in a Protein Mixture

[Collaboration with Virginia Florang]

Mouse liver mitochondrial lysate was obtained from a 20-25 g Swiss Webster (CD1 IGS) mouse. Briefly, mice were euthanized via 150 mg/kg sodium

pentobarbital administered via intraperitoneal injection and the liver was quickly removed. Tissue was homogenized in 10 mM tris buffer (pH 7.4) containing 0.25 M sucrose solution at 4 ºC, and samples were centrifuged at 1000 g for 10 min. The supernatant was collected and spun at 12,000 g for 10 min, and the pellet was reconstituted in homogenizing media (4 ºC). This step was repeated for a total of three spins, yielding mitochondria. The mitochondria were reconstituted in 50 mM sodium phosphate buffer (pH 7.4) lysed via sonication and membranes pelleted via centrifugation at 100,000 g for 30 min (4 ºC). Mouse liver mitochondrial lysate (0.5 mg/mL) was incubated with DOPAL (0 and 10 μM) at 37°C for 2, 4 or 6 h (50 mM sodium phosphate buffer, pH 7.4). To samples containing an initial DOPAL concentration of 10 μM, additional DOPAL was added every 1 h (5 μM final). A 7.5% gradient SDS-PAGE gel and coomassie blue were used for protein separation and staining. To determine the extent of DOPAL metabolism during the course of the reaction, the 6 h sample was analyzed by HPLC after treatment with perchloric acid (5% v/v) to precipitate protein.

Results

3,4-Dihydroxyphenylacetaldehyde is More Reactive Towards Lysine than Cysteine Residues

To determine the ability of DOPAL to modify amine vs thiol nucleophiles, the reactivity of DOPAL towards NAL and NAC was monitored using HPLC over a 1.5 h time course. As demonstrated by data in *Figure 5.1A*, no observable reaction occurred between DOPAL and NAC under conditions used; however, DOPAL showed considerable reactivity towards NAL. Slopes (-*k*′) for decreasing [DOPAL] were log linear and correlated with the concentration of NAL. The rate

constant for the DOPAL-NAL reaction was calculated to be 2.0 $M^{-1}min^{-1}$ (*Figure 5.1B*).

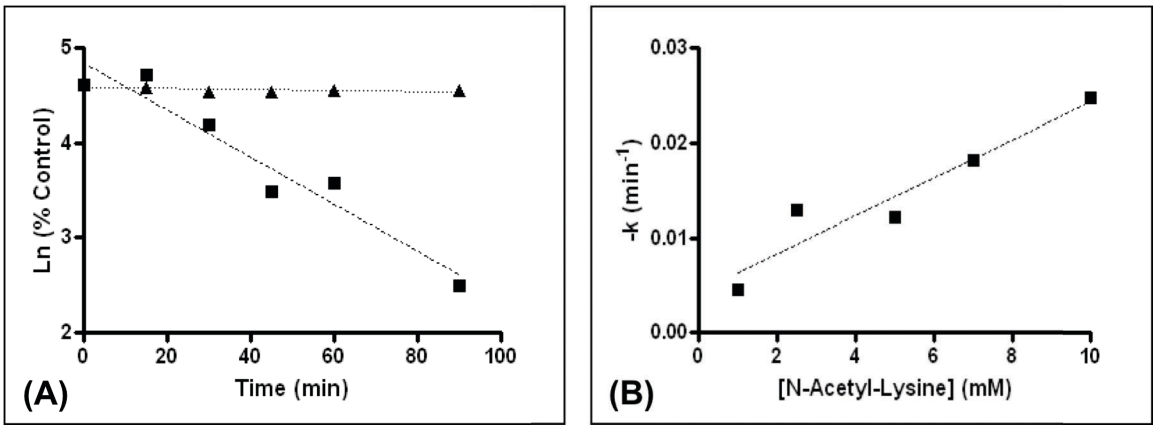


Figure 5.1 (A) No apparent reaction occurs between DOPAL (100 µM) and NAC (▲) (10 mM) over the indicated time course whereas a considerable reaction appears to occur between DOPAL (100 µM) and NAL (■) (10 mM). Such a result indicates that DOPAL is more reactive with amine nucleophiles than thiol nucleophiles. (B) Treatment of NAL at various concentrations with 100 µM DOPAL yields a second order rate constant of 2.0 $M^{-1}min^{-1}$.

Both the Catechol and Aldehyde Functional Groups are Required for Protein Modification

Compounds structurally analogous to DOPAL were obtained and utilized to study the reaction between DOPAL and NAL (*Figure 5.2* and *Table 5.1*). These analogues either lack a catechol or contain a protected catechol. In the absence of reducing agent (*Figure 5.2A*), DOPAL was observed to be 5-fold more reactive towards NAL than MOPAL and 10-fold more reactive than PAL. In the presence of $NaCNBH_3$, the reactivity of MOPAL and PAL towards NAL is increased however, it is still significantly less than the reaction between DOPAL and NAL reaction (*Figure 5.2B*). Such a finding indicates DOPAL to be highly

reactive towards amine nucleophiles, more so than DMPAL, MOPAL or PAL. In addition, DOPAL will form a stable adduct such that reduction to an amine is not necessary, suggesting the formation of a reversible Schiff base-type adduct.

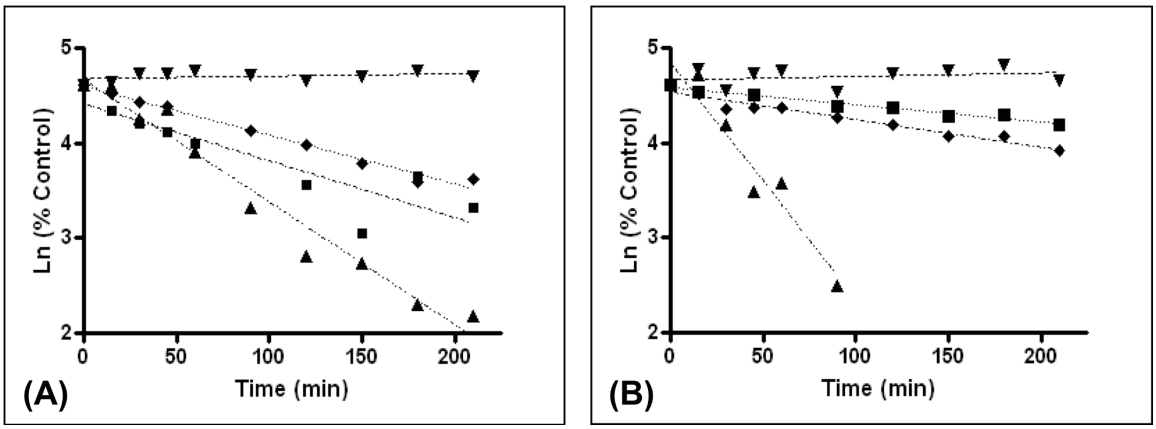
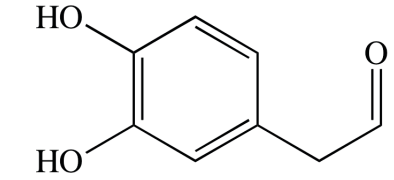
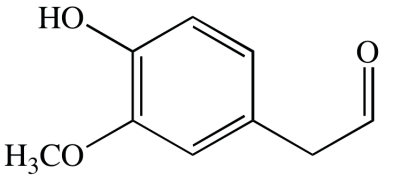
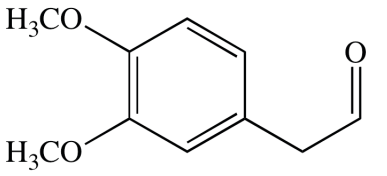
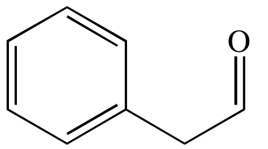


Figure 5.2 Reaction between NAL and DOPAL (▲) and NAL with various structural analogous of DOPAL (DMPAL (▼), MOPAL (◆) and PAL (■)), indicate a catechol is essential for enhanced reactivity with amine nucleophiles. In the (A) absence and (B) presence of 5 mM $NaCNBH_3$, DOPAL is highly reactive towards NAL and will form a stable adduct such that reduction to an amine is not necessary. The results demonstrate the order of reactivity towards NAL to be DOPAL >> PAL, MOPAL > DMPAL and are further summarized in *Table 5.1*.

Chemical Structure	Compound	k ($M^{-1}min^{-1}$)
	DOPAL	2.0 ± 0.04[a]
	MOPAL	0.42 ± 0.04[b]
	DMPAL	ND[c]
	PAL	< 0.2[d]

[a]Absence of $NaCNBH_3$

[b]Presence of $NaCNBH_3$; without reducing agent reactivity very low, k< 0.40

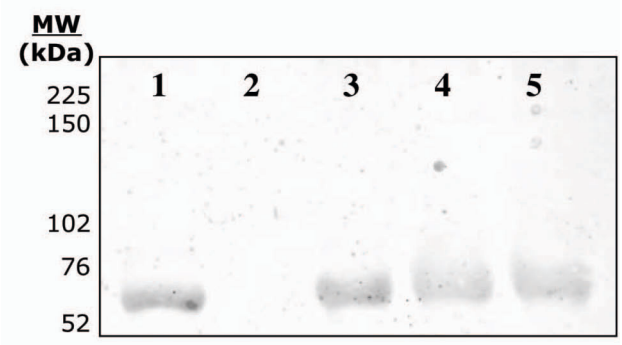
[c]No significant reaction detected over time period

[d]Estimated value based on experimental conditions

Table 5.1 DOPAL was demonstrated to be more reactive towards NAL than the three DOPAL analogs, MOPAL, DMPAL and PAL, with an observed rate constant of 2.0 $M^{-1}min^{-1}$.

Additionally, a comparison of the extent of protein modification by DOPAL versus MOPAL (*Figure 5.3*) further demonstrates the greater reactivity of DOPAL than MOPAL and it's ability to form stable protein adducts. In the presence of increased MOPAL concentration (i.e. 1:2 and 1:10 ratios), the percent of NBT staining as compared to the control decreased only slightly indicating that there was little competition for protein amines between DOPAL and MOPAL. If MOPAL was competing for Lys residues, NBT staining would be significantly

diminished as NBT detects the presence of catechols and MOPAL's structure does not contain a catechol. To further study the competition of DOPAL and MOPAL for protein nucleophiles, the samples were analyzed via HPLC to measure changes in DOPAL and MOPAL concentrations. It was determined that MOPAL did not positively or negatively influence DOPAL adduction during the course of the reaction.



Sample	Lane	% Control
DOPAL	1	100
MOPAL	2	0
1:1 DOPAL:MOPAL	3	100
1:2 DOPAL:MOPAL	4	76.79
1:10 DOPAL:MOPAL	5	87.85

Figure 5.3 DOPAL-mediated protein modification is not hindered in the presence of MOPAL. % Control refers to the integrated staining density for the control sample (BSA + DOPAL).

To confirm protein/amine reactivity of MOPAL, a model peptide was incubated with MOPAL and analyzed via MALDI-TOF-MS (*Figure 5.4*). Treatment of RKRSRAE (measured *m/z* at 901.974) with MOPAL yielded new peaks corresponding to the peptide with 150.00 Da adducts (i.e. *m/z* at 1051.946, 1201.946). The shift in mass of 150.00 Da corresponds to a MOPAL adduct generated via reaction of MOPAL with Lys followed by reduction of the imine to an amine (theoretical mass for adduct = 150.06 Da).

Treatment of DOPAL with the peptide RKRSRAE yielded a new peak at *m/z* 1037.7 corresponding to a 134.3 Da adduct and Schiff base formation as previously described (*102*).

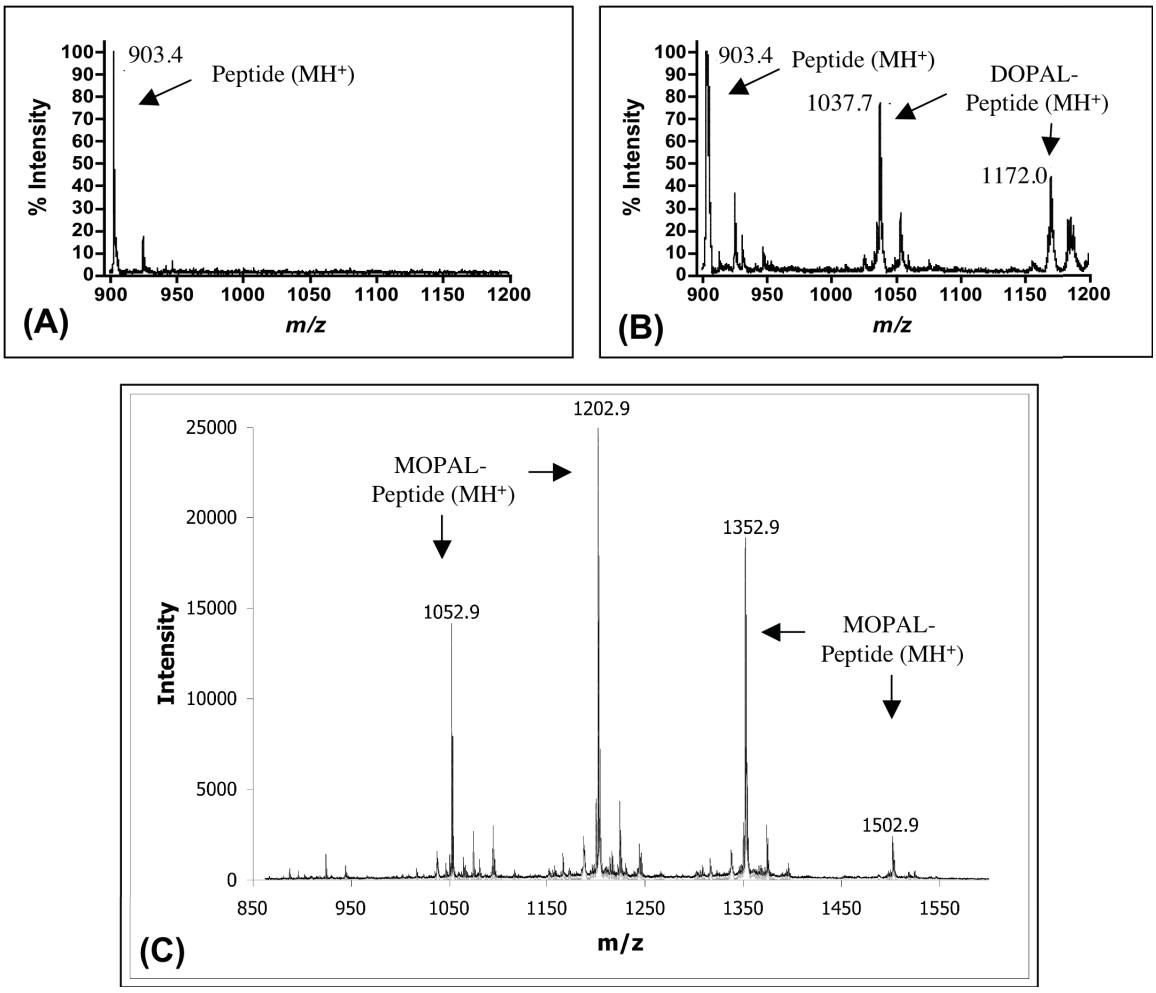


Figure 5.4 MALDI-TOF-MS of the peptide RKRSRAE treated with DOPAL or MOPAL, demonstrating the reactivity of both DOPAL and MOPAL towards the peptide. (A) Control, (B) 100 μM DOPAL, and (C) 100 μM MOPAL.

Reactivity of 3,4-Dihydroxyphenylacetaldehyde Towards Proteins is Diminished When Proteins are Pre-Treated with Amine Modifiers

As summarized in Table 5.2 and determined by the data in *Figures 5.5* and *5.6*, pre-treatment of BSA and GAPDH with the protein modifiers citraconic anhydride (modifies primary amines), Traut's reagent (converts amines to thiols) and iodoacetic acid (modifies thiol residues) further demonstrates the reactivity of DOPAL towards amine nucleophiles. Treatment of BSA with increasing concentrations of citraconic anhydride reduced the number of adducts on BSA, as demonstrated in *Figure 5.5A*. Upon removal of the citraconic anhydride adduct, DOPAL reactivity was restored (*Figure 5.5B*) thus further indicating DOPAL protein modification is dependent upon the availability of amine residues. When BSA was pre-treated with Traut's reagent, DOPAL-protein adducts were significantly diminished (*Figure 5.5C*), whereas, pre-treatment with iodoacetic acid, did not significantly affect DOPAL's ability to modify the protein (*Figure 5.5D*). To verify the effect of Traut's reagent on the protein, samples were titrated with DTNB and an increase in free thiol concentration was observed, indicating an amine to thiol conversion (data not shown).

In line with such findings, data in *Figure 5.6A* demonstrate that pre-treatment of GAPDH with Traut's reagent diminished the reactivity of DOPAL towards the protein. When GAPDH was pre-treated with iodoacetic acid, protein modification via DOPAL was not significantly affected unless high concentration (i.e. 10 mM) of iodoacetic acid was used (*Figure 5.6B*).

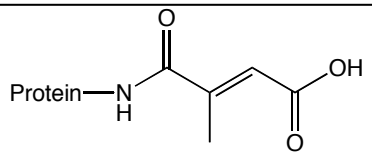
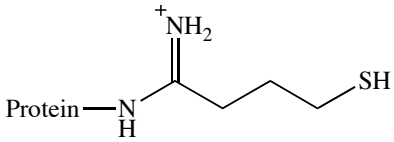
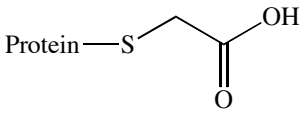
Protein Modifier	Reaction with Modifier	DOPAL-Protein Reactivity?
Citraconic Anhydride (modifies Lys residues)		DOPAL does *not* react with protein
Traut's Reagent (converts amines to thiols)		DOPAL does *not* react with protein
Iodoacetic Acid (modifies Cys residues)		DOPAL *will* react with protein

Table 5.2 Protein modification with either citraconic anhydride or Traut's reagent will diminish the ability for DOPAL to adduct with a model protein while modification by iodoacetic acid does not hinder DOPAL reactivity.

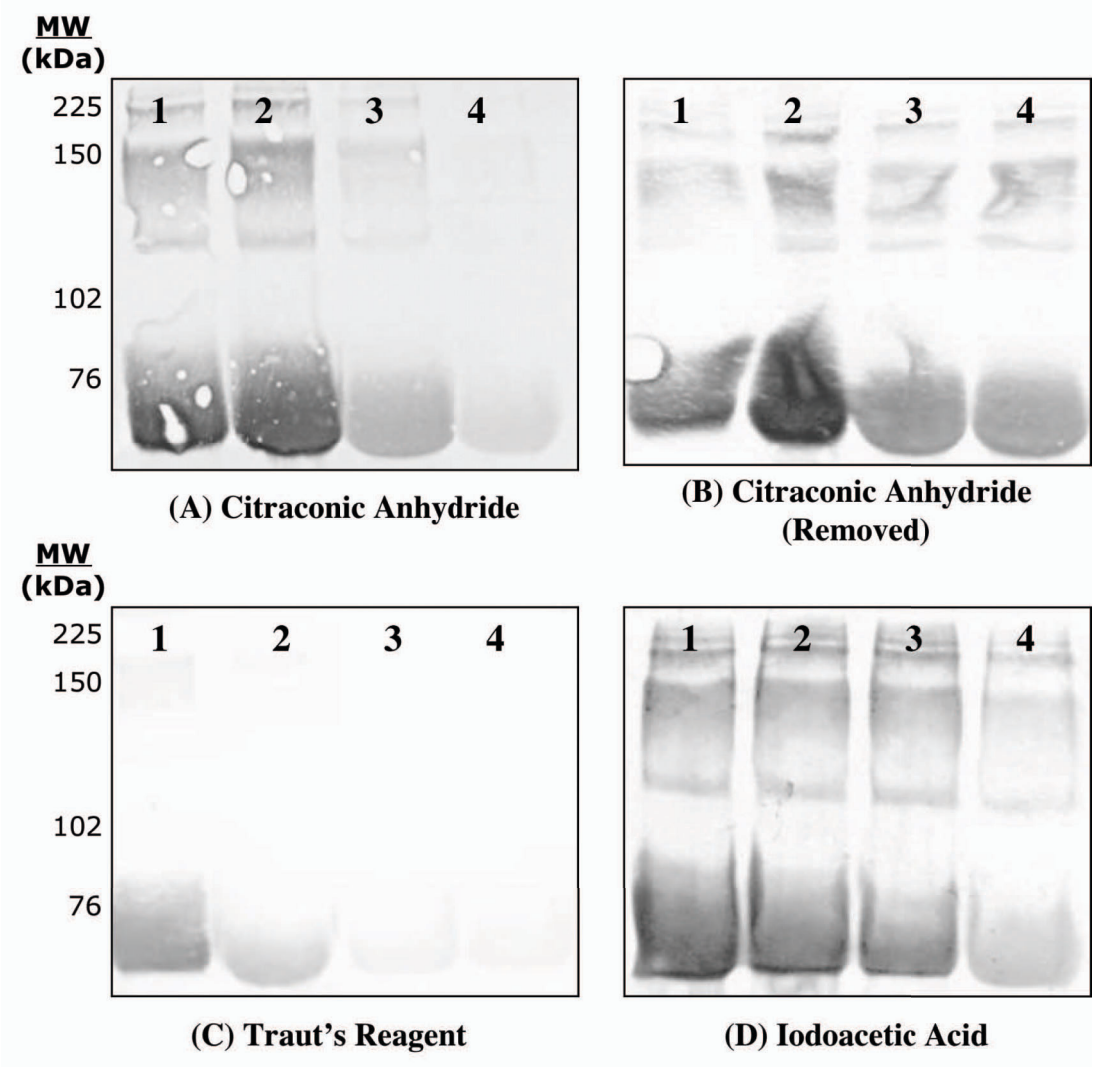
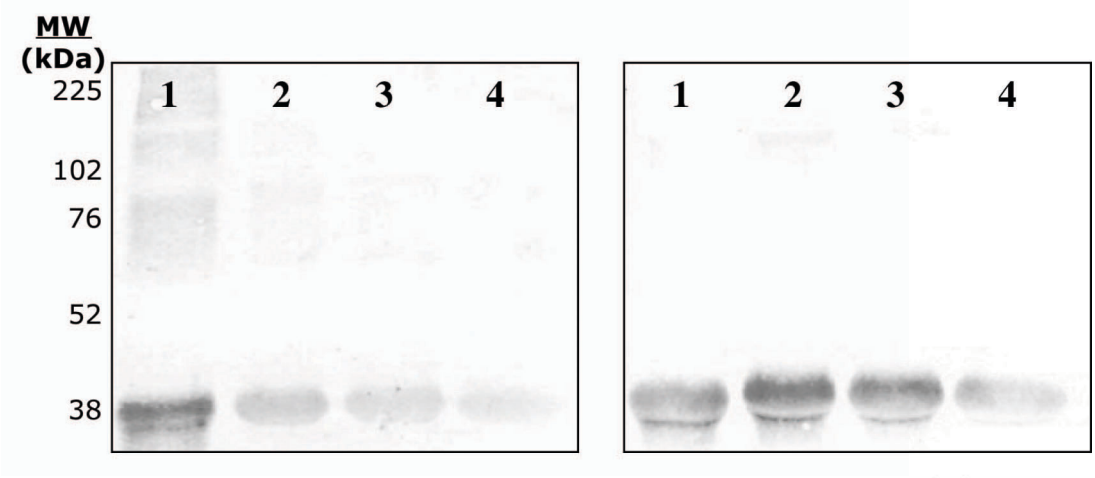


Figure 5.5 The reactivity of DOPAL towards BSA is diminished when the model protein is pre-treated with (A) citraconic anhydride (modifies amines) however; reactivity of DOPAL is restored when the (B) citraconic anhydride moiety is removed. Additionally, DOPAL reactivity is reduced when BSA is pre-treated with (C) Traut's reagent (converts amines to thiols) but is not affected when BSA is pre-treated with (D) iodoacetic acid (modifies thiols). These results indicate that DOPAL modifies amine residues. For all lanes, the concentration of the protein modifiers are 0 mM (lane 1), 1 mM (lane 2), 5 mM (lane 3) and 10 mM (lane 4). Results are further summarized in *Table 5.3*.

Gel	Concentration	Lane	% Control
(A) Citraconic Anhydride *(modifies primary amines)*	0 mM	1	100
	1 mM	2	110
	5 mM	3	47
	10 mM	4	26
(B) Citraconic Anhydride *(removed)*	0 mM	1	100
	1 mM	2	134
	5 mM	3	87
	10 mM	4	73
(C) Traut's Reagent *(converts amines to thiols)*	0 mM	1	100
	1 mM	2	33
	5 mM	3	5.4
	10 mM	4	4.6
(D) Iodoacetic Acid *(modifies thiols)*	0 mM	1	100
	1 mM	2	99
	5 mM	3	94
	10 mM	4	53

Table 5.3 Modification of BSA with citraconic anhydride and Traut's reagent will diminish the ability for DOPAL to react with the model protein as evidenced by decreased NBT staining intensity; the % control refers to the integrated staining density for the control sample (untreated BSA).



(A) Traut's Reagent **(B) Iodoacetic Acid**

Gel	Concentration	Lane	% Control
(A) Traut's Reagent *(converts amines to thiols)*	0 mM	1	100
	1 mM	2	29
	5 mM	3	18
	10 mM	4	8.6
(B) Iodoacetic Acid *(modifies thiols)*	0 mM	1	100
	1 mM	2	134
	5 mM	3	99
	10 mM	4	38

Figure 5.6 The reactivity of DOPAL towards GAPDH is diminished when the protein is pre-treated with (A) Traut's reagent (converts amines to thiols) however; when GAPDH is pre-treated with (B) iodoacetic acid (modifies thiols), protein modification via DOPAL is not affected. For all lanes, the concentrations to the protein modifiers are 0 mM (lane 1), 1 mM (lane 2), 5 mM (lane 3) and 10 mM (lane 4). % Control refers to the integrated staining density relative to that of the control sample (untreated GAPDH).

Modification of Glyceraldehyde-3-Phosphate Dehydrogenase by Dopamine and its Metabolites

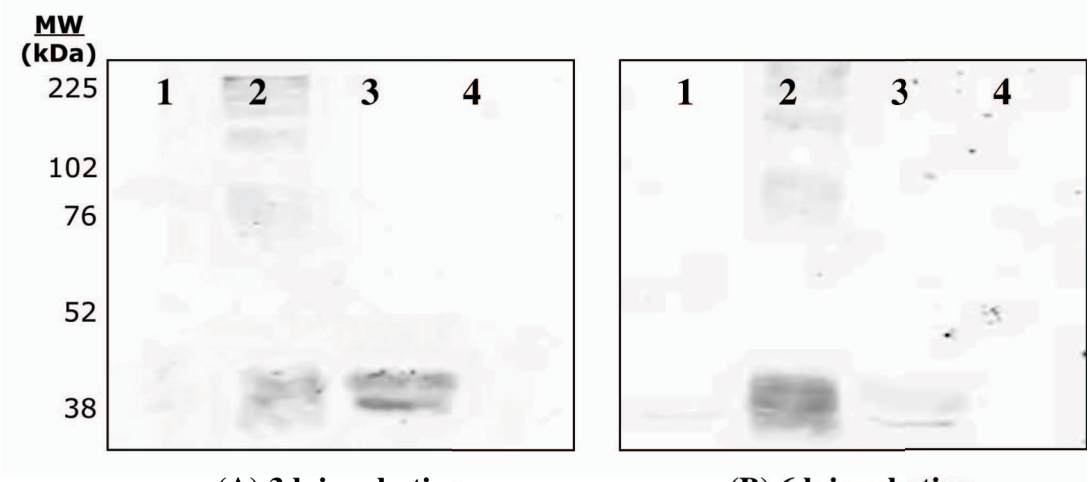
As shown in *Figure 5.7A,B*, treatment of GAPDH with DA, DOPAL, DOPAC or L-DOPA (100 μM) resulted in varying levels of NBT reactivity and protein modification. The order of reactivity, based on NBT staining, was

determined to be DOPAL > DOPAC > DA, L-DOPA. Additionally, these results indicate that DOPAL (Lane 2) crosslinks GAPDH as demonstrated by the presence of higher molecular weight protein bands, indicative of GAPDH oligomers such as the tetramer (i.e. 146 kD tetramer).

3,4-Dihydroxyphenylacetaldehyde Mediates Glyceraldehyde-3-Phosphate Dehydrogenase Protein Cross-linking

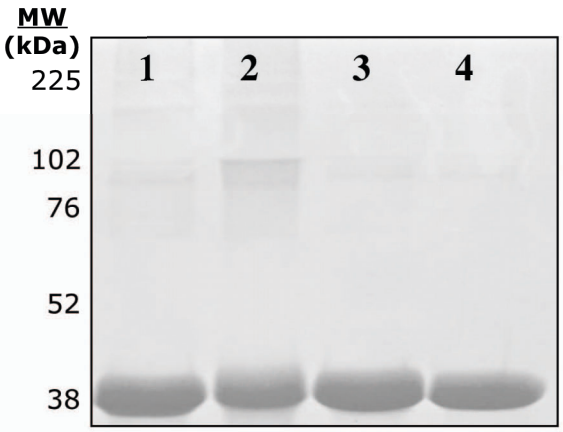
Protein cross-linking was evident for GAPDH treated with DOPAL at various concentrations. As shown *in Figure 5.8A*, control samples at 2 and 4 h contain a protein band at 36 kDa, corresponding to the molecular weight of the GAPDH monomer. However, in samples treated with DOPAL (5-50 μM), higher molecular weight protein bands became apparent. Specifically, protein treated with 100 μM DOPAL yielded protein bands in excess of 200 kDa while the band corresponding to the 36 kDa monomer is reduced in staining intensity at both 2 and 4 h (37 and 86%, respectively).

When GAPDH is treated with DOPAL in the presence of $NaCNBH_3$ (imine and quinone reducing agent) and ascorbate (quinone reducing agent), protein cross-linking/aggregation was significantly diminished (*Figure 5.8B*). Reducing agents appear to offer protection from protein cross-linking via DOPAL, with ascorbate being the most effective. Interestingly, GAPDH treated with 100 μM MOPAL did not yield any noticeable protein cross-linking, in contrast to that observed for GAPDH incubated with DOPAL.



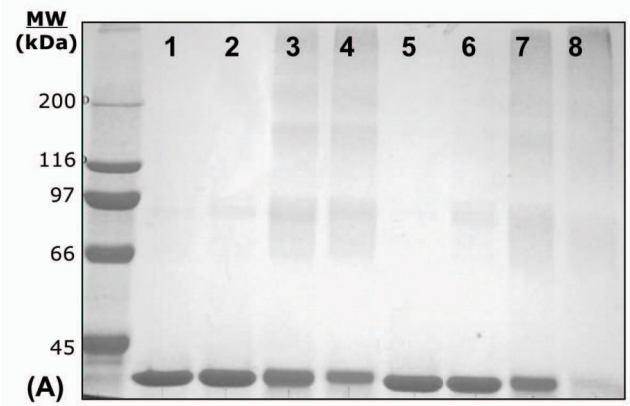
(A) 3 h incubation (B) 6 h incubation

Gel	Metabolite	Lane	% Control
(A) 3 h incubation	DA	1	1.5
	DOPAL	2	100
	DOPAC	3	13
	L-DOPA	4	5.7
(B) 6 h incubation	DA	1	3.5
	DOPAL	2	100
	DOPAC	3	73
	L-DOPA	4	1.2



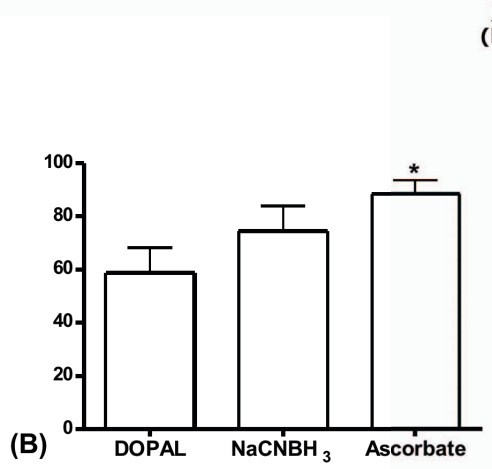
(C) 6 h incubation

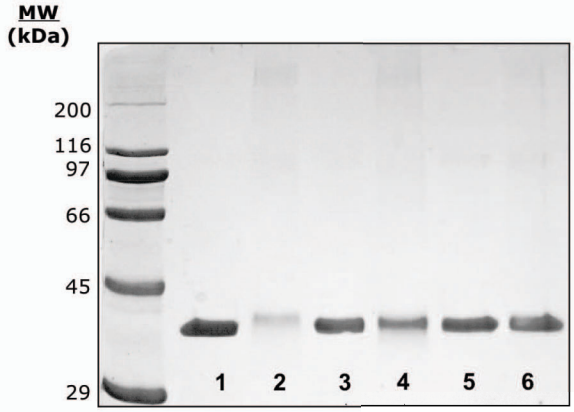
Figure 5.7 GAPDH incubated with 100 μM of DA, DOPAL, DOPAC or L-DOPA for (A) 3 h and (B) 6 h yielded varying degrees of reactivity towards the model protein. Results demonstrate the order of reactivity to be DOPAL > DOPAC > L-DOPA, DA. In addition, protein cross-linking of GAPDH by DOPAL was observed using two staining methods: (A), (B) NBT and (C) Coomassie. % Max signal refers to the integrated staining density for a given lane relative to that determined for lane 2 (GAPDH + DOPAL), which had the highest staining intensity.



Lane	Incubation	Concentration	% Control
1	2 h	0 μM	100
2		5 μM	103
3		50 μM	91
4		100 μM	63
5	4 h	0 μM	100
6		5 μM	94
7		50 μM	66
8		100 μM	14

37 kDa GAPDH Monomer



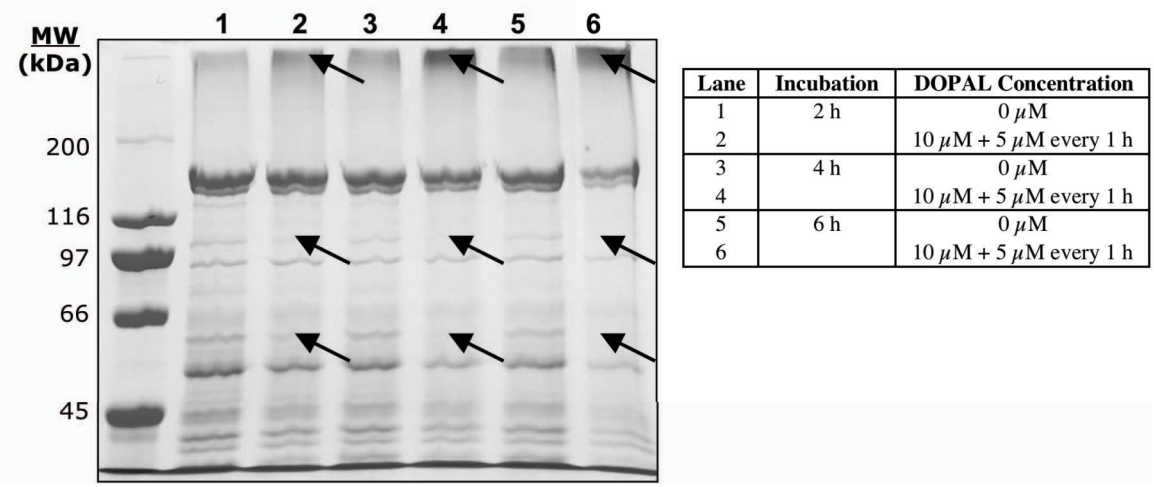


	Lane	% Control
Control	1, 3, 5	100
DOPAL	2	58 +/- 9.4
DOPAL + $NaCNBH_3$	4	76 +/- 9.3
DOPAL + Ascorbate	6	88 +/- 5.2

Figure 5.8 (A) Treatment GAPDH with various concentrations of DOPAL (5-100 μM) demonstrate the ability for DOPAL to cause protein cross-linking of GAPDH monomers. % Control refers to the integrated staining density for the GAPDH monomer (37 kD) relative to that of the control samples (untreated protein). (B) The presence of 5 mM $NaCNBH_3$ (lane 4) and 5 mM ascorbate (lane 6) will protect GAPDH against DOPAL-induced protein cross-linking. % Control refers to the integrated staining density relative to that of the control samples (untreated GAPDH). The band shown represents the 37 kD GAPDH protein. Values shown represent means ± SEM (n = 4). *, significantly different from GAPDH treated with DOPAL (p < 0.05), determined using a two-tailed t-test.

3,4-Dihydroxyphenylacetaldehyde Mediated Protein Cross-linking in Protein Mixture

When a mixture of proteins (i.e. mouse liver mitochondrial lysate) was treated with DOPAL, protein modification was observed at all three time points; 2, 4 and 6 h (*Figure 5.9*). High molecular weight protein bands (i.e. in excess of 200 kDa) increased in staining, while proteins at lower molecular weights (i.e. 57, 67 and 90 kDa) decreased in signal as compared to controls. Such evidence indicates the ability for DOPAL to cause protein cross-linking. Analysis by HPLC found the concentration of DOPAL in the 6 h sample to be 12 μM.



Lane	Incubation	DOPAL Concentration
1	2 h	0 μM
2		10 μM + 5 μM every 1 h
3	4 h	0 μM
4		10 μM + 5 μM every 1 h
5	6 h	0 μM
6		10 μM + 5 μM every 1 h

Figure 5.9 Lysate from mouse liver mitochondria (0.5 mg/mL) treated with DOPAL resulted in time-dependent protein cross-linking. Arrows denote a change in protein band intensity (increase or decrease) in comparison with control protein incubated without DOPAL at each incubation length (i.e. 2, 4 and 6 h).

Discussion

The DA intermediate, DOPAL, has been implicated as an endogenous neurotoxin relevant to the pathogenesis of PD (*71, 72*). Studies have

demonstrated DOPAL to be toxic towards dopaminergic cells via various mechanisms including production of oxidative stress, induction of the mitochondrial transition pore and protein modification (*8, 62, 76, 82*). Recent work has shown that products of lipid peroxidation (i.e., 4-hydroxy-2-nonenal and malondialdehyde) will inhibit DA catabolism, specifically, the aldehyde oxidation step catalyzed by one or more ALDHs. Such inhibition leads to increased concentrations of DOPAL using rat brain mitochondria and striatal synaptosomes as model systems (*59, 102*). Evidence of protein modification via DOPAL was demonstrated including indication that DOPAL has a significantly greater reactivity towards proteins than DA or DOPAC (*102*).

The current study further extends these previous findings with the goal of identifying the mechanism by which DOPAL modifies protein. Data are presented demonstrating the reactivity between DOPAL and Lys is greater than that for the reactivity between DOPAL and Cys; however, protein cross-linking, mediated by a DOPAL-quinone, was observed in experiments utilizing model proteins and mouse liver mitochondria.

DOPAL is predicted to be a bifunctional electrophile as it contains two reactive functional groups (i.e. aldehyde and catechol). Therefore, it was of interest to determine if DOPAL-mediated protein modification occurs by oxidation of the catechol to yield a Cys-reactive quinone (which is known to occur for DA (*70, 71*)) or if the aldehyde forms adducts with Lys residues. As demonstrated by data in *Figure 5.1*, the reaction of DOPAL with NAL was found to be significant, as demonstrated by the time-dependent loss of DOPAL concentration. However, there was no significant reaction observed between DOPAL and NAC under the conditions used. Such a finding indicates that amine adduction may represent a significant pathway for DOPAL-protein modification. Although ortho-quinones are highly reactive toward protein thiols, it should be noted that

oxidation/activation of the catechol is first required and is rapidly followed by rearrangement or intracyclization (*70, 71, 75*). In contrast, the aldehyde moiety does not require activation or oxidation before protein modification. During the course of our reactions, DOPAL was not oxidized to an ortho-quinone as determined using HPLC and a DOPAL ortho-quinone standard generated via tyrosinase or sodium metaperiodate. However, under significant oxidative conditions, such as that found in the substantia nigra during DA catabolism (i.e. hydrogen peroxide production), or perhaps in the presence of prostaglandin-H-synthase, catechol oxidation could occur to a greater extent (*111, 112*).

While the aldehyde appears to be more reactive than the catechol toward protein nucleophiles under the conditions used, modification of the catechol greatly alters DOPAL reactivity, indicating that the ability of DOPAL to react with amine nucleophiles is dependent on the catechol (*Figure 5.2* and *Table 5.1*). The structurally analogous compounds MOPAL, DMPAL and PAL were incubated with NAL, and it was found that DOPAL is much more reactive toward NAL, even in the presence of $NaCNBH_3$. It is apparent from these results that the reactivity of these compounds toward NAL is dependent upon the structural differences of the catechol moiety, that is, aryl substituents. This raises the question of how do the aryl substituents enhance the reaction rate? It is conceivable that the observed effect of the catechol substitutions is due to (1) increased aldehyde reactivity or (2) increased stability of the product.

Interestingly, the reactivity of MOPAL and PAL toward NAL increases in the presence of reducing agent while the reactivity of DOPAL is slightly diminished. $NaCNBH_3$ is an imine and quinone reducing agent; therefore, the enhanced reactivity of MOPAL/PAL in the presence of $NaCNBH_3$ is predicted to stem from Schiff base (unstable) reduction to an amine (stable). In contrast, the DOPAL adduct could oxidize to a quinone that rearranges to a more stable form

(i.e. indole), via reaction of the amine with the quinone. Therefore, a reducing agent is expected to preclude oxidation of the catechol and subsequent rearrangement to a stable adduct. In support of this assertion, it was found that inclusion of 5 mM ascorbate in incubations of DOPAL with NAL also significantly decreased the apparent rate of the reaction. Such evidence implicates a role for a quinone in DOPAL-protein modification and indicates that the reaction of DOPAL with protein amines is dependent on catechol oxidation. Future work will include experiments to chemically characterize the structure of DOPAL-amino acid conjugates.

To further investigate the reactivity of DOPAL and the structurally analogous compound, MOPAL, with protein nucleophiles, BSA was treated with varying ratios of MOPAL and DOPAL. It should be noted that MOPAL is an endogenous product of DA catabolism and is found within dopaminergic cells via activity of catechol-O-methyl transferase (COMT) with DOPAL and from MAO with 3-methoxytyramine (*113*). Because of the simultaneous generation of these two compounds in the cell, it is predicted that MOPAL competes for protein binding sites with DOPAL. While the reaction rate for DOPAL with NAL was found to be much higher than that for MOPAL (*Figure 5.2* and *Table 5.1*), an additional experiment was performed to show conclusively that DOPAL is more reactive than MOPAL. Using a model peptide containing primary amines, it was found that indeed, MOPAL readily reacts with Lys to form a Schiff-base product. The data in *Figure 5.3*, however, demonstrate that MOPAL does not significantly interfere with DOPAL-protein adduction. Specifically, DOPAL is able to cause protein modification to a similar extent in both the absence and excess of MOPAL (i.e. 1:10 ratio). In addition, these findings suggest that methylation of DOPAL by COMT may be a detoxification step.

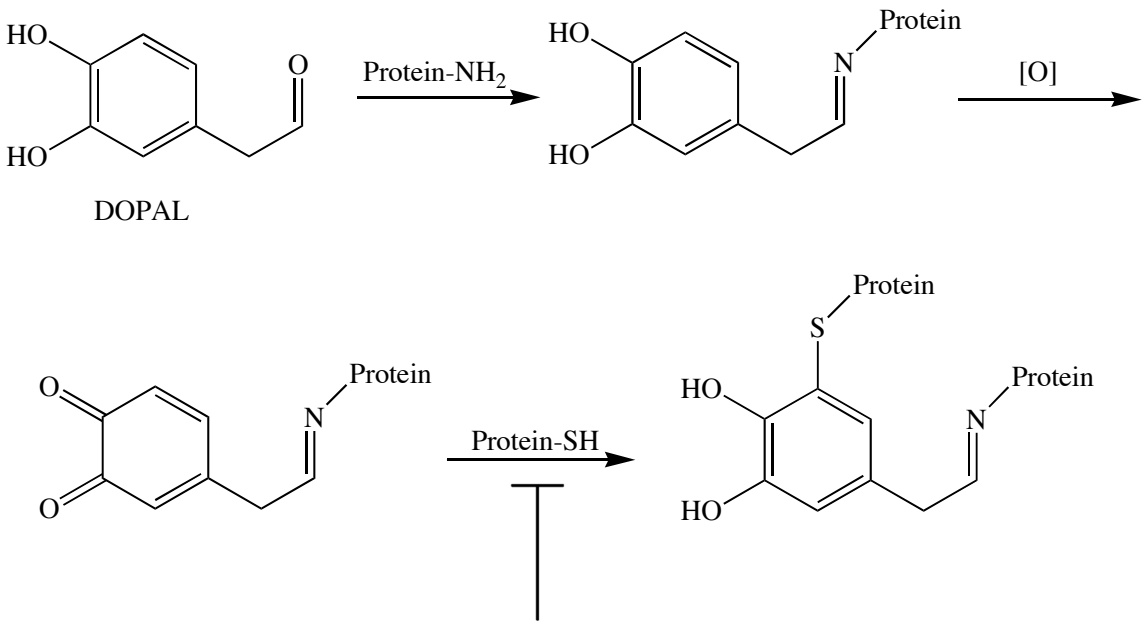
Use of NAL and NAC as models for protein nucleophiles provided evidence that amines are reactive toward DOPAL; however, it was of interest to determine the extent to which amines are responsible for DOPAL adducts on proteins. Two model proteins were utilized for this work, BSA and GAPDH. As shown in *Figures 5.5* and *5.6*, when primary amines were selectively modified (citraconic anhydride) and/or converted to thiol residues (Traut's reagent), the reactivity of DOPAL towards BSA or GAPDH was significantly diminished, as judged via NBT staining. Removal of the citraconic anhydride adduct (via acid) restored protein reactivity toward DOPAL, indicating involvement of Lys (*109, 110*). In addition, if Cys were the primary target of DOPAL, then conversion of Lys to Cys via the Traut's reagent would yield an increase in DOPAL-protein modification. In contrast, when thiol residues were selectively modified (i.e. iodoacetic acid) no significant change in protein modification was observed except at high concentrations of iodoacetic acid (10 mM). These results further demonstrate that reaction of DOPAL with proteins involves Lys/primary amines to a significant extent.

In a previous study, it was determined that DOPAL is more reactive towards BSA than DA or DOPAC (*102*). In line with such findings, the data in *Figure 5.7* demonstrate a similar result for the model protein GAPDH. Specifically, DOPAL was found to be more reactive towards GAPDH than DOPAC, DA or L-DOPA. In addition, treatment of protein with DOPAL for 3 or 6 h, resulted in higher molecular weight protein bands corresponding to various oligomers and aggregates of GAPDH that were resistant to the reducing and denaturing conditions used in SDS-PAGE. This result was observed only for DOPAL and not the other related compounds (i.e., L-DOPA, DA, DOPAC). It should be noted that such a finding is similar to that reported by Burke et al., who

demonstrated that incubation of DOPAL with α-synuclein resulted in significant protein aggregation (*114*).

In vivo, GAPDH can exist as a tetramer containing 37 kDa subunits, as a dimer or as the 37 kDa monomer, depending on the specific function that is needed (*115*). In the presence of DOPAL, significant cross-linking between GAPDH subunits was demonstrated (*Figure 5.8A*). This result was not observed for DA, DOPAC, L-DOPA or MOPAL, with the latter suggesting that protein aggregation may be dependent on the catechol. Evidence of protein cross-linking becomes more apparent with increasing DOPAL concentration and length of incubation time.

Of question then, is how is DOPAL cross-linking GAPDH? As noted above, while MOPAL is protein-reactive, treatment of GAPDH with the DOPAL analogue (i.e. protected catechol) did not result in protein aggregation. Therefore, it was hypothesized that DOPAL-mediated cross-linking occurs via Lys modification followed by catechol oxidation and Cys adduction. To test this hypothesis, GAPDH was treated with both DOPAL and the quinone-reducing agent, ascorbate (*116*). It was found that the presence of ascorbate afforded protection against DOPAL-mediated protein aggregation as shown in *Figure 5.7B*, and such a result indicates the involvement of a quinone in DOPAL-mediated protein cross-linking (*Scheme 5.3*). Interestingly, $NaCNBH_3$ also protected against protein cross-linking, which is different than predicted given that the reducing agent will stabilize the DOPAL adduct (i.e. reduce imine to amine); however, $NaCNBH_3$ can also reduce quinones (*117*). These results implicate the possible involvement of catechol oxidation in the mechanism of DOPAL protein modification and cross-linking.



Scheme 5.3 DOPAL will react with protein amines to form a Schiff base product. DOPAL can subsequently undergo oxidation of the catechol to yield the quinone and react with protein thiols leading to protein cross-linking. Quinone reducing agents (ascorbate and $NaBNBH_3$) will diminish the ability for DOPAL to form protein cross-links.

To determine the extent of protein cross-linking in a complex mixture of proteins, mitochondrial lysate was obtained and incubated with DOPAL. As shown in *Figure 5.8*, a shift from lower to higher molecular weight bands was observed as compared to control. Specific proteins decreased in staining intensity over time in the presence of DOPAL, and in particular, a large band appeared at approximately the 200 kDa molecular weight marker.

An increase in DOPAL concentration could therefore be detrimental to cells in regards to protein modification/cross-linking. Physiological concentrations of DOPAL are estimated to be around 2-3 μM (*79*) and several studies have demonstrated that the DA-derived aldehyde at concentrations as low as 6 μM, just above basal level, can cause cell death over time (*69, 72*). The

data in Figure 8, demonstrate a potential consequence of DOPAL at low micromolar in vitro.

In summary, the present study qualitatively and quantitatively demonstrated the reactivity of DOPAL towards protein nucleophiles. Specifically, it was determined that DOPAL is significantly more reactive towards Lys than Cys. Such evidence demonstrates aldehyde reactivity, however, the catechol greatly influences Lys modification. In addition, using model proteins (BSA, GAPDH) and a protein mixture (mitochondria lysate) treated with DOPAL (5-100 μM), considerable protein cross-linking was demonstrated. These data demonstrate DOPAL to be a bifunctional electrophile involving both the aldehyde and catechol in its mechanism of protein modification. These results may prove useful for predicting target proteins and sites of adduction on proteins.

CHAPTER SIX
ISOLATING AND IDENTIFING 3,4-DIHYDROXYPHENYLACETALDEHYDE MODIFIED PROTEINS

Abstract

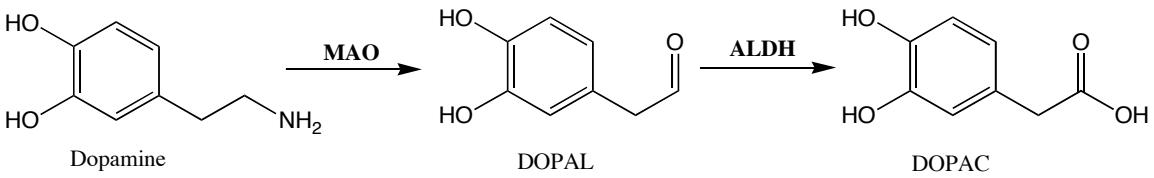
The formation of an endogenous neurotoxin has been suggested to explain the selective neurodegeneration as observed for Parkinson's disease. Based on data from recent studies, 3,4-dihydroxyphenylacetaldehyde (DOPAL), an intermediate of dopamine (DA) catabolism, was hypothesized to be an endogenous neurotoxin. Elevated concentrations of the reactive aldehyde resulted from inhibition of the DA metabolic pathway and DOPAL was found to cause protein modification, including protein cross-linking, in dopaminergic model systems (i.e. striatal synaptosomes). The goal of this work was to isolate and identify proteins that are susceptible to adduction by DOPAL to further characterize the role of DOPAL in the pathogenesis of PD. To accomplish this objective, an aminophenylboronic acid resin was utilized to separate DOPAL-modified protein from unmodified protein in PC6-3 cell cytosol. A proteomics-based approach was used to identify twelve different proteins, involved in a variety of cellular functions, to be vulnerable to modification by DOPAL. These proteins include chaperones, detoxification enzymes, metabolic proteins, ER proteins and inflammatory proteins. One of the proteins identified, tyrosine hydroxylase (TH), was of particular interest as this enzyme is involved in the biosynthesis of DA. The identity of TH was verified via a proteomics-based approach and Western blot using anti-TH antibodies. Results of this study will help to elucidate the consequence of an accumulation of DOPAL-modified protein in regards to the progression of PD.

Introduction

Parkinson's disease (PD) is a prominent neurodegenerative disorder characterized by the selective cell death of dopaminergic neurons and the presence of intraneuronal protein aggregates termed Lewy bodies. While the mechanism of disease onset remains elusive, the pathogenesis of the disease is hypothesized to involve oxidative stress (*45, 46*) and an accumulation of a neurotoxin endogenous to dopaminergic cells (*76, 102-104*).

3,4-Dihydroxyphenylacetaldehyde (DOPAL), a reactive aldehyde intermediate of dopamine (DA) catabolism (*Scheme 6.1*), was hypothesized to be an endogenous neurotoxin relevant to PD. Several studies have determined DOPAL to be orders of magnitude more toxic in vitro and in vivo as compared to DA (*8, 76-78*). In addition, in the presence of the lipid peroxidation product 4-hydroxy-2-nonenal (4HNE), DOPAL concentrations are elevated while 3,4-dihydroxyphenylacetic acid (DOPAC) levels are decreased (*59, 102*). Such evidence indicates DOPAL to be a toxic aldehyde intermediate.

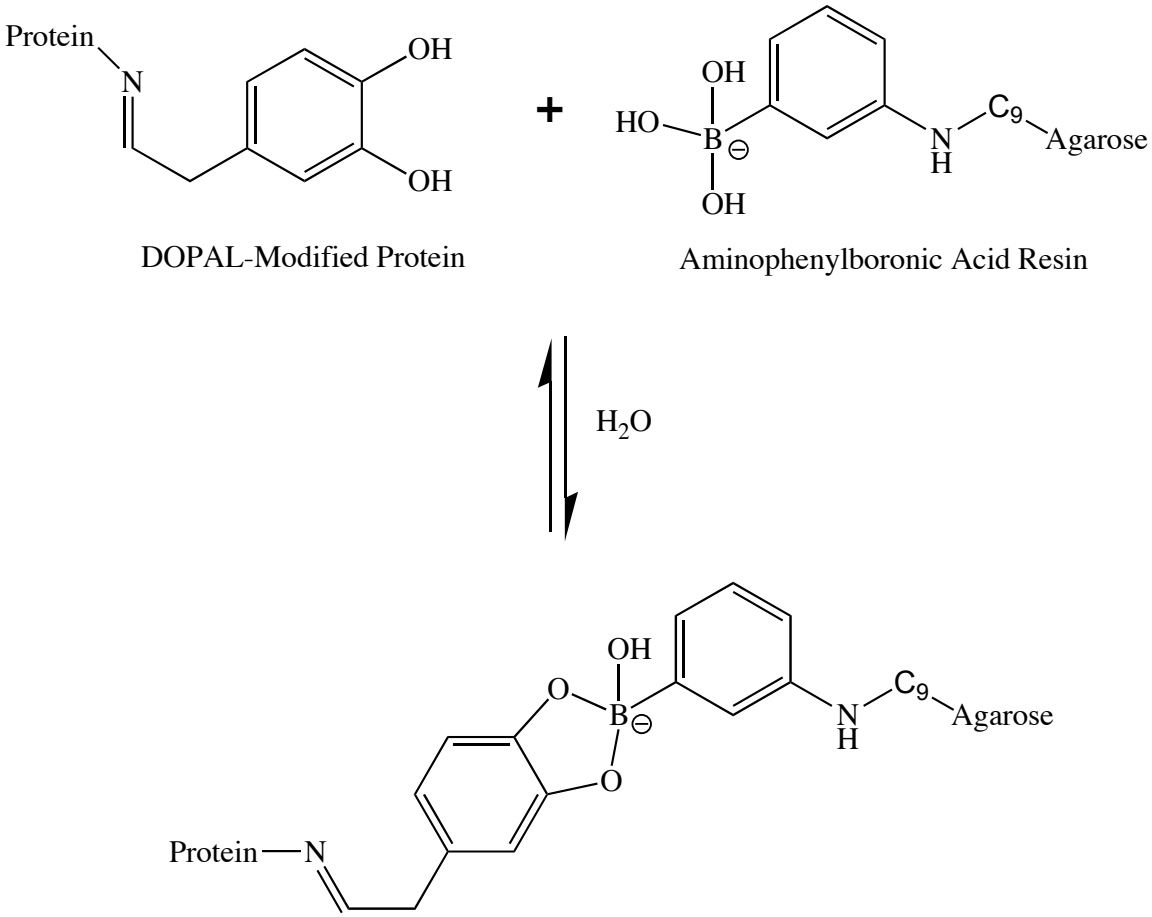
The mechanism of DOPAL toxicity, in regards to dopaminergic cell death, is hypothesized to involve protein modification (*84-86, 102*). Recent work has demonstrated DOPAL to be reactive toward protein amines with the ability to cause extensive protein cross-linking for both model proteins and a protein mixture (i.e. mouse liver mitochondrial lysate) (*118*). This study, however, did not identify cellular proteins susceptible to modification by DOPAL. Identifying proteins vulnerable to modification would help to elucidate the role of DOPAL in this progressive neurodegenerative disease.



Scheme 6.1 The metabolism of DA involves MAO catalyzed deamination of DA to DOPAL, a reactive aldehyde, followed by the subsequent oxidation to DOPAC, a stable acid.

The current study was undertaken to selectively isolate and identify DOPAL-modified proteins in DA cells. A technique was developed utilizing an aminophenylboronic acid (APBA) resin, which has been previously used to isolate a DA-modified protein (*72*). As demonstrated in *Scheme 6.2*, the boronic acid complex of the APBA resin will bind to the catechol moiety of the DOPAL-modified protein. After the unmodified protein has been removed (i.e. washing the resin), DOPAL-bound protein can be released from the resin under acidic conditions. The APBA resin technique was utilized to isolate DOPAL-modified protein from both model proteins and PC6-3 cells. PC6-3 cells are a subline of PC12 cells and are useful as a model dopaminergic system (*119*). This cell line is not of neuronal origin, however, it is considered to be an appropriate model for the metabolism of DA, assumes a homogenous population and has been utilized to demonstrate mechanisms of toxicity linked to dopaminergic toxicity (*105, 120, 121*). Twelve different proteins from PC6-3 cells, including tyrosine hydroxylase (TH), were identified to be vulnerable to DOPAL adduction. Modification of these proteins may result in a variety of cellular consequences including protein misfolding, an accumulation of toxic metabolites, decreased ATP concentrations and disruption of the DA biosynthetic pathway. Identification of proteins susceptible to DOPAL adduction will aid in determining the biological or

pathological effect of increased concentrations of the reactive aldehyde, as has been observed under conditions of oxidative stress (*59, 102*).



Scheme 6.2 A technique involving an APBA resin will allow for the separation of DOPAL-modified protein from unmodified protein. The boronic acid complex of APBA will bind to the catechol moiety of DOPAL.

Experimental Procedures

Materials

DOPAL was biosynthesized via an established procedure involving enzyme-catalyzed conversion of DA to DOPAL by rat liver MAO (*84*) and its

concentration was determined using an aldehyde dehydrogenase (ALDH) assay with nicotinamide adenine dinucleotide (*85*) and high performance liquid chromatography (HPLC) analysis, as described below. 4HNE was synthesized as previously described in (*91*) and its concentrations was determined spectrophotometrically (224 nm, ε = 13,750 $cm^{-1}M^{-1}$).

Trypsin was purchased from Promega Corporation (Madison, WI) and antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Bovine serum albumin (BSA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle, APBA resin and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

High Performance Liquid Chromatography

An Agilent 1100 Series Capillary HPLC system was used for quantification of DOPAL. This unit is equipped with a sample cooler, capable of maintaining a temperature of 4 °C in the autosampler tray. Briefly, 10 µL of sample solution was injected and separation achieved using a Phenomenex C18 Luna microbore column (1 × 150 mm, 100Å) with isocratic conditions consisting of 0.1% trifluoroacetic acid (v/v) in HPLC-grade water and 6% acetonitrile (ACN) (v/v) at a flow rate of 50 µL/min. DOPAL was detected with a photo-diode array detector using absorbance set to 202 and 280 nm for quantitation. Retention time for DOPAL was determined to be 7.3 min. A calibration curve was generated using a DOPAL standard and utilized to convert peak area to concentration units.

Cell Culture

[Collaboration with Dr. Yunden Jinsmaa]

PC6-3 cells were cultured in RPMI 1640 medium supplemented with heat-inactivated 10% horse serum, 5% fetal bovine serum, and penicillin (10 IU/mL)

and streptomycin (10 mg/mL). Cells were grown in 100 mm^2 tissue culture dish at 37 ^oC in a humid atmosphere containing 5% CO_2. PC6-3 cells (6 x 10^4) were seeded into six-well plates at final volume of 2 mL/well and were incubated at 37 ^oC for 3 days in 5% of CO_2. Nerve growth factor (NGF) was added into each well at a final concentration of 50 ng/mL for cell differentiation and plates were maintained for four days before being used for studies. For experiments involving treatment of cells with 4HNE, the media was removed 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 mM NaH_2PO_4 and 15 mM HEPES (pH 7.4).

Treatment of Cells with 4-Hydroxy-2-Nonenal

[Collaboration with Dr. Yunden Jinsmaa]

NGF-differentiated PC6-3 cells (6 × 10^4 per well) in HEPES-buffered media (pH 7.4) were pretreated with 100 μM DA for 15 min at 37 °C to initiate DA catabolism. After the pre-incubation period, 50 μM 4HNE was added to the cells and the control group did not contain any 4HNE. All groups (i.e. control and treated) were incubated at 37 °C for 4 hr. The media was removed and the cells were rinsed once with HEPES-buffer media. The cells were lysed using 10 mM potassium phosphate (pH 7.4) containing 1% triton X-100 (v/v) and sonicated for 15 sec on ice using an ultrasonicator. The cell lysate was centrifuged at 700 x g for 10 min (4 °C). The supernatant was removed and centrifuged at 10,000 x g for 30 min (4 °C). The resultant supernatant was kept and used in the APBA resin experiments. Protein concentration was determined via the BCA assay.

Identifying 3,4-Dihydroxyphenylacetaldehyde Modified Proteins via an Aminophenylboronic Acid Resin

Model Proteins (Bovine Serum Albumin and Glyceraldehyde-3-Phosphate Dehydrogenase)

APBA resin (50 µL) was washed three times with 50 µL of 50 mM sodium phosphate buffer (pH 7.4), centrifuging after each wash (8,300 x g, 2 min). Three samples containing model proteins (1 mg/mL) were prepared including, BSA, GAPDH and a mixture of BSA and GAPDH. Each sample, BSA (60 µg), GAPDH (60 µg) and a mixture of BSA and GAPDH (30 µg each), was diluted with 50 mM sodium phosphate buffer (50 µL, pH 7.4) and added to the APBA resin. The samples were allowed to incubate with the resin for 3 h on a shaker table (room temperature). After incubation, the samples were centrifuged (8300 rpm, 2 min) and the supernatant was removed and kept. The resin was subsequently washed with 1:1 ACN : 50 mM sodium phosphate buffer (pH 7.4) (twice, 50 µL each) and distilled water (twice, 50 µL each), centrifuging after each wash fraction (8300 rpm, 2 min). To elute any protein that was bound to the APBA resin, the resin was washed with 1% TFA (twice, 50 µL each) and 0.05% TFA (twice, 50 µL), centrifuging after each release fraction (8300 rpm, 2 min). The pH of each release fraction was neutralized with 5 µL of 1 M sodium phosphate buffer (pH 7.4). A 10% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and coomassie blue staining were used for protein separation and staining.

To determine if the APBA resin could be used to isolate DOPAL-modified proteins, BSA and GAPDH were treated with 100 µM DOPAL for 4 h (37 °C, 50 mM sodium phosphate buffer, pH 7.4). The DOPAL treated samples, BSA (60 µg), GAPDH (60 µg) and a mixture of BSA and GAPDH (30 µg each), were

allowed to incubate with the APBA resin for 3 h at room temperature. The experiment proceeded as stated previously. A 10% gradient SDS-PAGE gel and coomassie blue staining were used for protein separation and staining.

PC6-3 Cells (Cytosol)

The resin was also used to isolate DOPAL-modified protein from PC6-3 cells treated with 50 μM 4HNE. After the cells were lysed, the cytosol fraction was further treated with 100 μM DOPAL for 4 h at 37 °C to ensure a higher concentration of DOPAL-adducted proteins. PC6-3 cells not treated with 4HNE or DOPAL were used for control. Cell samples (180 μg protein) were allowed to incubate with the APBA resin for 3 h (room temperature) and the experiment proceeded as stated previously. A 10% gradient SDS-PAGE gel and coomassie blue staining were used for protein separation and staining.

After separation by gel electrophoresis, an in-gel tryptic digestion was performed on the protein bands. Briefly, individual protein bands were excised and washed for 10 min each with 100 mM ammonium bicarbonate buffer (1 x 50 μL, pH 7.4), a solution of 1:1 acetonitrile: 50 mM ammonium bicarbonate buffer (4 x 200 μL) and cold acetonitrile (1 x 200 μL). The supernatant was removed after each wash. The protein sample was allowed to air dry and reconstituted in a solution of 50 mM ammonium bicarbonate buffer (24 μL, pH 7.4) and acetonitrile (2 μL). Trypsin (1 μL of a 0.5 mg/mL solution) was added and the sample was allowed to rehydrate for 30 min at 4 °C. An additional 250 μL of 50 mM ammonium bicarbonate buffer was added and the samples were digested overnight using a water bath (37 °C, 18 h). The resulting digestion mixture was subject to mass spectrometry at the University of Iowa Proteomics Facility (Iowa City, IA) using a Thermo LTQ XL ion trap mass spectrometer

(ITMS) with a collision induced dissociation (CID) ion source. The SEQUEST database search engine was used to match peptides to known proteins.

Western Blot Analysis

[Collaboration with Lydia Mexas]

To confirm the identity of the protein band at 56 kDa, the protein was probed via Western blot analysis using anti-TH antibodies. The SDS-PAGE gel was placed in Bjenum and Schafer-Nielsen transfer buffer (37 °C, pH 7.4) for 10 min and then transferred to a nitrocellulose membrane utilizing a semi-dry transfer apparatus (70 min, 20 V). The membrane was incubated for 18 h at 4 °C with a solution of 3% BLOTTO in tris-buffered saline with 0.1% Tween 20 (v/v) (TBS-T). The blocking solution was removed and the membrane was placed in 8 mL of primary antibody solution (rabbit anti-TH) diluted 1:1000 in BLOTTO and incubated for 3 h on a shaker table (room temperature). After incubation, the membrane was washed with TBS-T for five minutes (3x's, 5 mL each). The membrane was placed in 10 mL of secondary antibody solution (goat anti-rabbit) diluted 1:10,000 in BLOTTO and incubated for 2 h on a shaker table (room temperatureerature). After incubation, the membrane was washed with TBS-T for five minutes (4x's, 5 mL each). An enhanced chemiluminescence (ECL) kit (Amersham Biosciences-GE Healthcare) was used to determine the presence of TH protein. Briefly, in the darkroom, the TBS-T solution was removed and the membrane was incubated with the ECL solution for 5 min. The membrane was exposed to film for 1 min and subsequently developed using a developer and fixer solution.

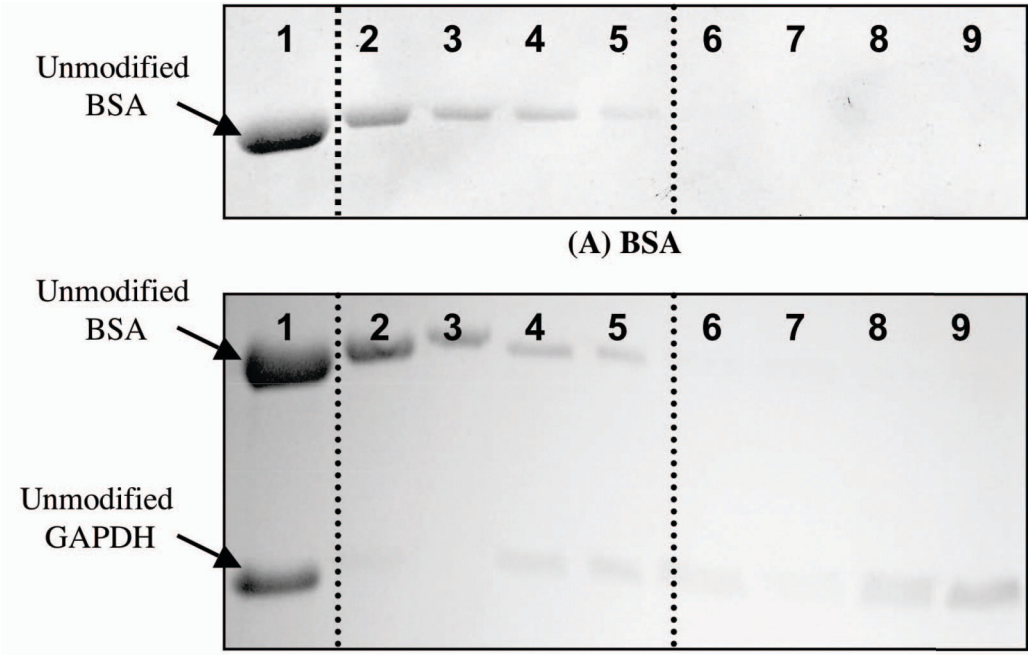
Results

Aminophenylboronic Acid Resin and Model Proteins

A protein separation technique utilizing an APBA resin was developed to isolate DOPAL-modified protein effectively from unmodified protein. As demonstrated by the data in *Figure 6.1*, unmodified model protein, BSA and GAPDH, primarily eluted in the supernatant and wash fractions, demonstrating only proteins with a DOPAL-bound adduct will bind to the resin. DOPAL-modified protein, however, will bind to the APBA resin and can be eluted from the resin via acidic conditions as demonstrated by the data in *Figure 6.2*. These data demonstrate DOPAL-modified protein can be isolated from samples containing one protein as well as protein mixture.

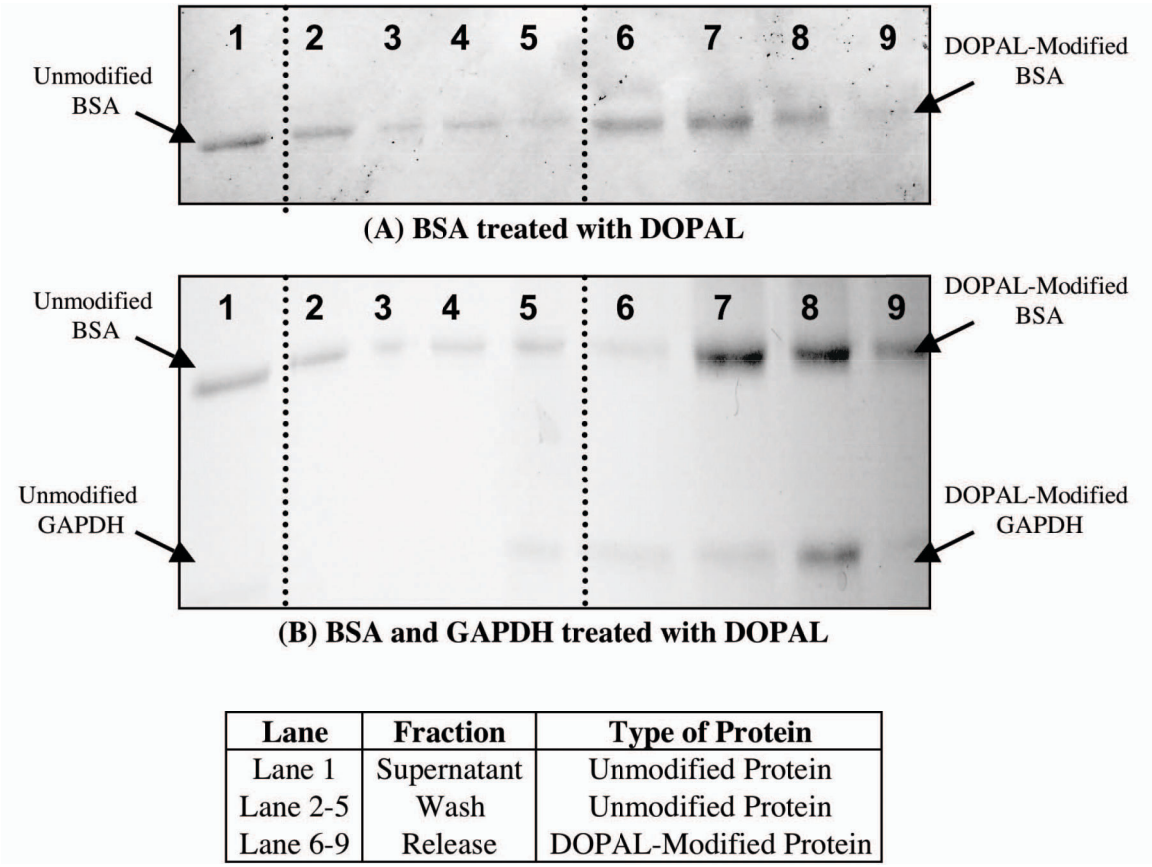
Aminophenylboronic Acid Resin and PC6-3 Cells

When PC6-3 cell lysate was subjected to the APBA resin, four protein bands were observed in the release fractions indicative of DOPAL-modified protein (*Figure 6.3*). These four bands corresponded to proteins with molecular weights of 25, 56, 65, and 76 kDa as determined by SDS-PAGE. The control, PC6-3 cells not treated with 4HNE or DOPAL, demonstrated no evidence of DOPAL-modified protein in the APBA release fractions (*Figure 6.4*).



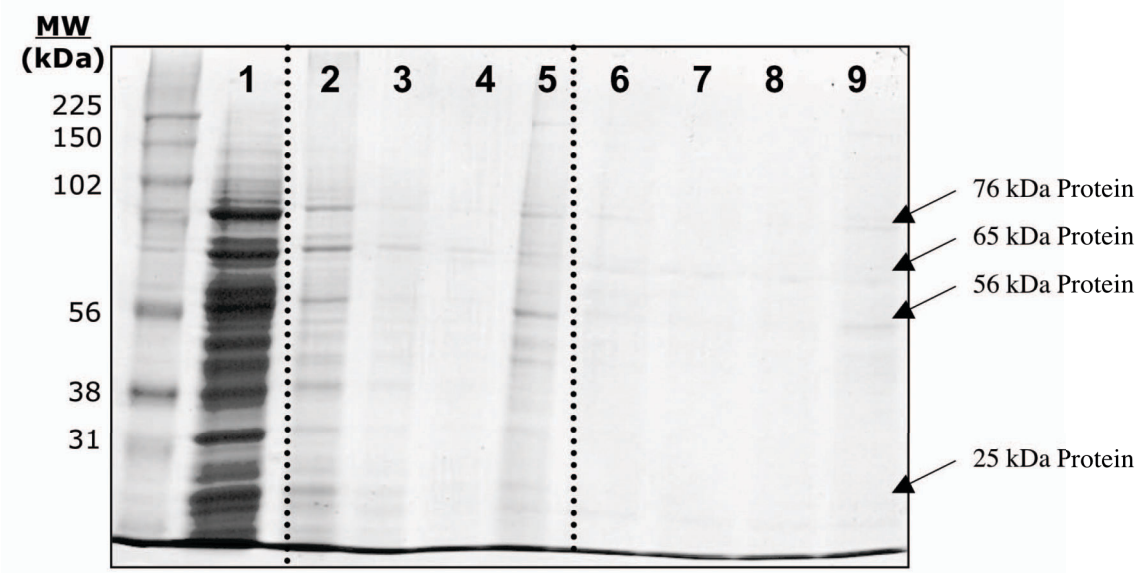
Lane	Fraction	Type of Protein
Lane 1	Supernatant	Unmodified Protein
Lane 2-5	Wash	Unmodified Protein
Lane 6-9	Release	DOPAL-Modified Protein

Figure 6.1 Proteins that do not contain a DOPAL adduct will not bind to the APBA resin. As the data in this figure demonstrate, the unmodified model proteins (i.e. BSA and GAPDH) primarily elute in the supernatant and wash fractions (Lanes 1-5).



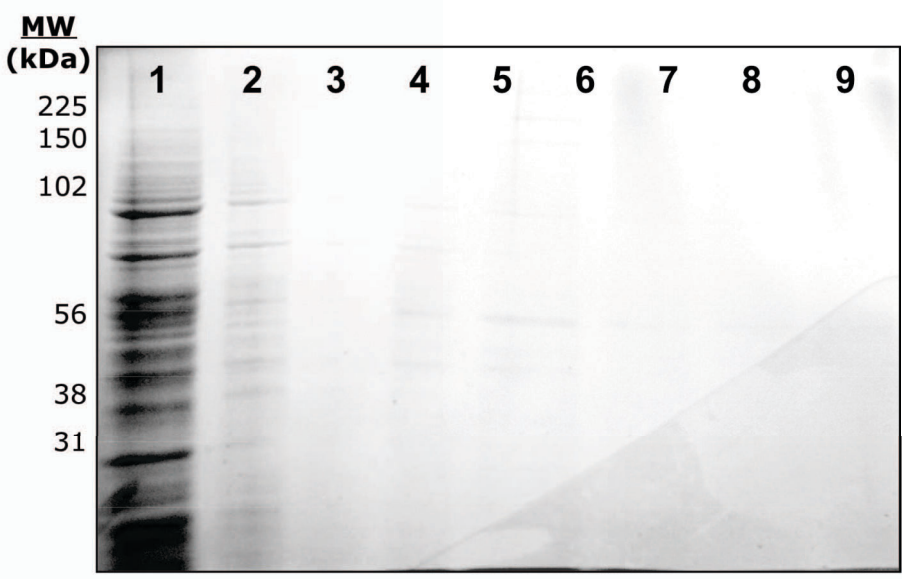
Lane	Fraction	Type of Protein
Lane 1	Supernatant	Unmodified Protein
Lane 2-5	Wash	Unmodified Protein
Lane 6-9	Release	DOPAL-Modified Protein

Figure 6.2 Proteins that contain a DOPAL adduct will bind to the APBA resin. As the data in this figure demonstrate, the DOPAL-modified model proteins (i.e. BSA and GAPDH) primarily elute in the release fractions (Lanes 6-9).



Lane	Fraction	Type of Protein
Lane 1	Supernatant	Unmodified Protein
Lane 2-5	Wash	Unmodified Protein
Lane 6-9	Release	DOPAL-Modified Protein

Figure 6.3 Proteins that do not contain a DOPAL adduct elute in the supernatant (Lane 1) and wash fractions (Lane 2-5). DOPAL-modified protein will bind to the APBA resin and are eluted under acidic conditions (Lane 6-9). Four protein bands are observed in the release fractions when the cytosol from PC6-3 cells is subjected to the APBA resin. These bands at 25, 56, 65 and 76 kDa may represent proteins vulnerable to DOPAL modification and relevant in the pathogenesis of PD.



Lane	Fraction	Type of Protein
Lane 1	Supernatant	Unmodified Protein
Lane 2-5	Wash	Unmodified Protein
Lane 6-9	Release	DOPAL-Modified Protein

Figure 6.4 As the data in this figure demonstrate, protein from the unmodified PC6-3 cells (i.e. not treated with 4HNE or DOPAL) elute in the supernatant and wash fractions (Lanes 1-5). No protein is observed in the APBA release fractions.

To identify the isolated proteins from the 4HNE treated PC6-3 cells, an in-gel tryptic digestion was performed on the protein bands and the samples were subjected to mass spectrometry using ITMS. A SEQUEST database search engine was used to match peptides to known proteins. The SEQUEST results were filtered according to two parameters of rigorous criteria, the Delta Cn value and the Xcorr value. The Delta Cn value, an indicator of the separation between the peptide with the best correlation and the peptide with the second best correlation, was required to be greater than 0.1. The Xcorr value, the cross correlation score, was required to be greater than 1.9, 2.7 and 3.5 for peptides that were single, double and triple charged, respectively. When comparing the

MS data with the protein database, several additional criteria were used to further refine the list of protein candidates found by database searching: 1) the molecular weight of the protein had to match the molecular weight estimated from the position of the band on the gel and 2) there had to be at least two peptides generated by trypsin digestion that matched the database. The search and comparison led to 12 different classes of proteins (*Table 6.1*) involved in a variety of cellular functions (*Figure 6.4*).

The protein band at approximately 56 kDa, as determined by SDS-PAGE, was of particular interest as the molecular mass of TH is 60 kDa. TH plays an important role in the biosynthesis of DA as the enzyme catalyzes the conversion of L-tyrosine to L-DOPA, the precursor to DA. Western blot analysis (*Figure 6.5)* demonstrated that TH is present in the release fractions of the APBA resin, indicating TH could be susceptible to DOPAL-modification. These preliminary results indicate the APBA resin could be used to effectively isolate DOPAL-modified protein from a dopaminergic model system.

Protein Category	Molecular Weight (Da)	Peptides Matched
Chaperone		
Heat shock protein 1, alpha	84761	28
Heat shock protein 1, beta	83264	25
Heat shock protein 2	69486	8
Heat shock protein 8	70816	8
Tumor rejection antigen gp96	74161	6
Detoxication		
Aldehyde dehydrogenase, family 2	56452	6
Aldehyde dehydrogenase, family 3	54047	4
Glutathione S-transferase, mu 1	25897	9
Glutathione S-transferase, mu 2	25624	2
Glutathione S-transferase, mu 4	25626	4
Metabolism		
3-Phosphoglycerate dehydrogenase	56457	4
Triosephosphate isomerase	26849	11
Triosephosphate isomerase	26847	6
ER Protein		
Calnexin	67212	8
Ribophorin 1	68262	11
Ribophorin 2	69018	3
Inflammatory		
Leukotriene A4 hydrolase	69045	3
DA Synthesis		
Tyrosine hydroxylase	55931	3

Table 6.1 Proteins isolated from PC6-3 cell lysate that were identified via ITMS and a SEQUEST database search.

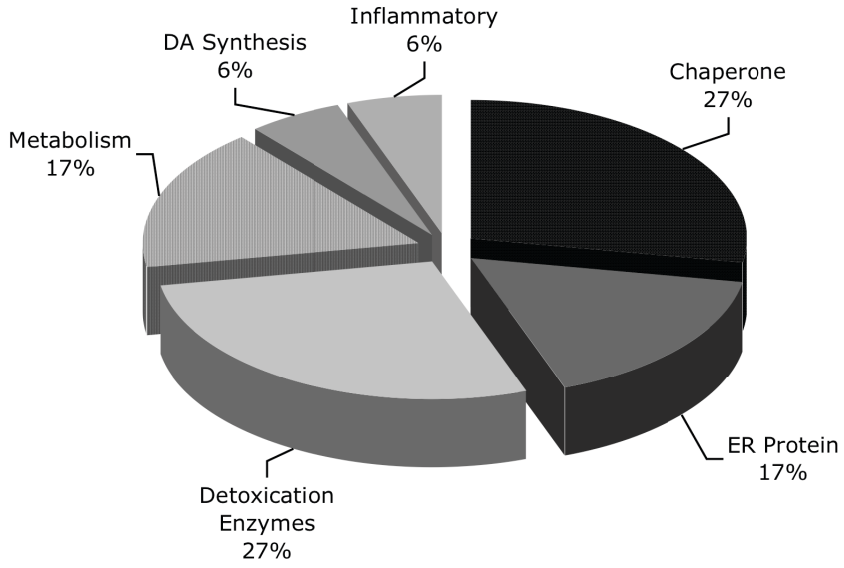
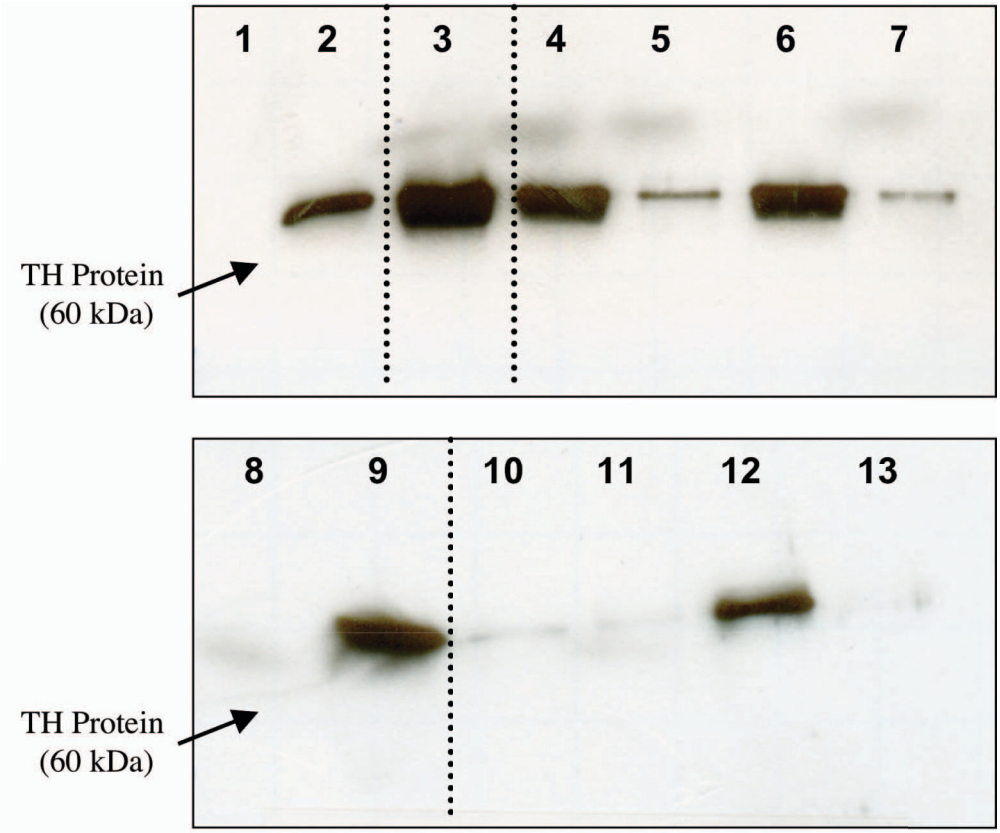


Figure 6.5 The functional classification of the predicted proteins susceptible to DOPAL modification. The proteins were classified into six groups; chaperone, endoplasmic reticulum (ER) protein, detoxication enzymes, metabolism, DA synthesis and inflammatory.



Lane	Fraction	Type of Protein
Lane 1, 8	BSA	Control
Lane 2, 9	Cell Lysate	Control
Lane 3	Supernatant	Unmodified Protein
Lane 4-7	Wash	Unmodified Protein
Lane 10-13	Release	DOPAL-Modified Protein

Figure 6.6 The APBA resin fractions of PC6-3 cell lysate were further analyzed via Western blot to determine the presence of TH. Unmodified TH is present in the wash fractions (Lane 4-7), however TH also appears in the release fractions (Lane 10-13) indicating that the protein may be susceptible to DOPAL modification.

Discussion

An endogenous neurotoxin has been implicated in the pathogenesis of PD to explain the characteristic, selective neurodegeneration of dopaminergic neurons (*76, 102-104*). Numerous studies have indicated DOPAL, an

intermediate of DA catabolism, to be the endogenous neurotoxin as these studies have demonstrated DOPAL to be toxic to DA cells (*8, 76-78*). In addition, under conditions of oxidative stress (i.e. the presence of products of lipid peroxidation) DOPAL concentrations are observed to be elevated (*59, 102*). DOPAL is predicted to cause toxicity via modification of essential cellular proteins (*84-86, 102*). Results of recent work have demonstrated that DOPAL displays considerable reactivity toward amine nucleophiles and will cause protein cross-linking as observed with model proteins and a protein mixture (i.e. mitochondrial lysate) (*118*).

To further understand DOPAL's mechanism of toxicity towards dopaminergic cells, it is important to determine proteins targets of DOPAL. Knowledge of the proteins vulnerable to modification will aid in elucidating the mechanism of DOPAL in the pathogenesis of PD. The current study identifies a novel technique to isolate DOPAL-modified protein from protein that do not contain a DOPAL adduct. Data are presented that demonstrate the use of an APBA resin, in combination with proteomic techniques, to be a viable method to identify DOPAL-modified protein.

As demonstrated by the data in *Figure 6.1 and 6.2*, the APBA resin can be used to separate DOPAL-modified protein from samples containing only one model protein as well as a mixture of two model proteins. Unmodified BSA and GAPDH (i.e. not treated with DOPAL) were primarily found in the supernatant and wash fractions of the experiment (*Figure 6.1*). DOPAL-modified BSA and GAPDH bound to the APBA resin and could be eluted in the release fractions under acidic conditions (*Figure 6.2*). These results indicate the APBA resin procedure developed could be used towards isolating DOPAL-modified proteins from samples containing a mixture of proteins.

Preliminary work using the APBA resin to isolate protein from PC6-3 cells treated with 4HNE identified four protein bands correlating to DOPAL-adducted protein (*Figure 6.3)*. Analysis of these bands via ITMS and a SEQUEST database search indicated a variety of proteins to be susceptible to DOPAL modification (*Table 6.1* and Appendix B, *Figures B1-B6*). To further verify the identity of these proteins, future work of this project would involve protein separation by 2D SDS-PAGE before analysis via ITMS. A 2D SDS-PAGE technique will allow the proteins to be separated by isoelectric focusing (charge of the protein) in addition to the molecular weight. This would allow for improved isolation of individual protein bands and lead to enhanced mass spectrum analysis. Nonetheless, DOPAL modification of any of these proteins identified by the current study could lead to a variety of cellular consequences and offers insight as to the potential mechanism of DOPAL toxicity in regards to dopaminergic cells. These proteins were determined to be involved in a broad range of cellular functions, including chaperones, detoxication, metabolism, protein synthesis, inflammation and DA synthesis (*Figure 6.5*).

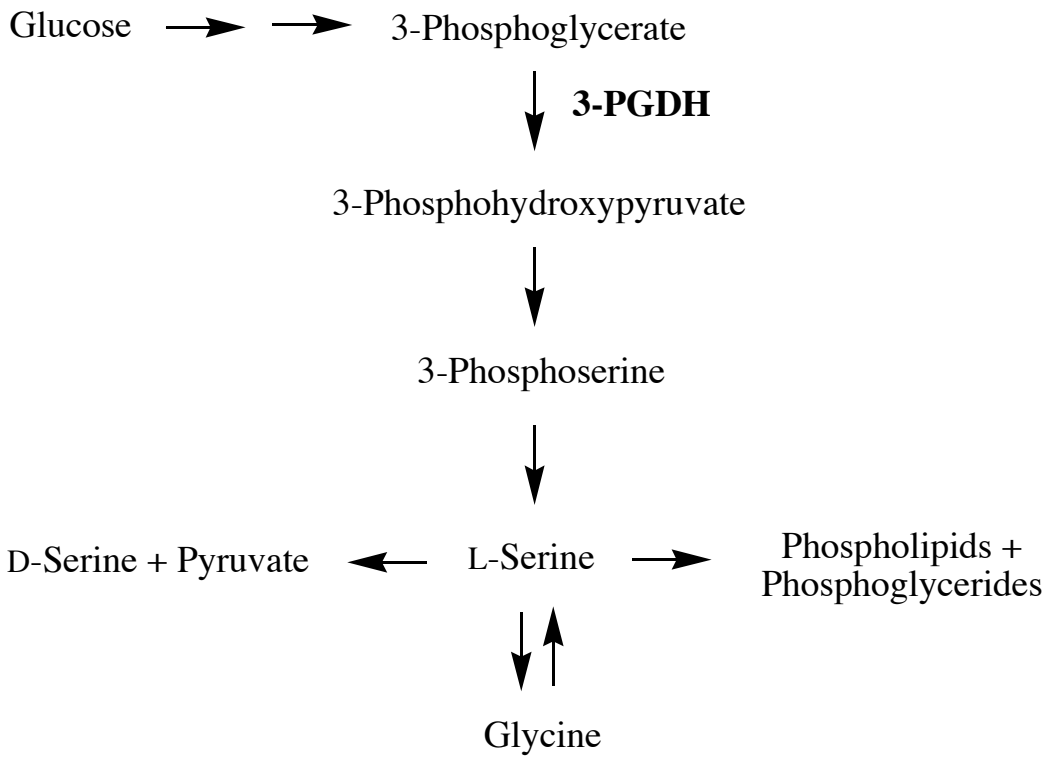
Heat shock proteins (Hsps) are proteins that are upregulated when cells are exposed to elevated temperature or environmental stress (inflammation, toxins, hypoxia, etc) (*122*). These proteins function as intracellular chaperones and will repair protein damage caused by exposure to heat. Specifically, these chaperones will protect against protein aggregation, assist in protein folding and target damaged proteins for degradation (*122*). Hsps 1, 2, 8 and gp96 have been predicted to be susceptible to DOPAL modification and various studies have hypothesized that these proteins play a role in neurodegenerative disorders (*122-124*). Research has demonstrated significantly decreased concentrations of Hsps in the substantia nigra of individuals with PD (*125*) and various chaperone-type proteins are present in the composition of LBs (*126*). Protein

modification of Hsps could lead to an accumulation of misfolded proteins and increased production of reactive oxygen species, eventually leading to cell apoptosis and the further progression of neurodegenerative diseases (*124*).

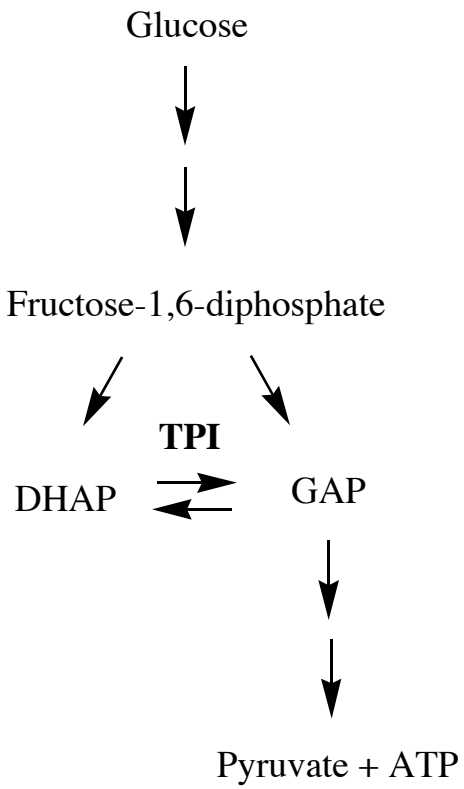
Glutathione S-transferases (GSTs) and ALDHs are key enzymes involved detoxication. The enzymatic detoxication pathway consists of two steps and involves the biotransformation of lipophilic xenobiotics into water-soluble and less toxic metabolites which can then be easily eliminated from the body (*127*). GSTs can catalyze a variety of reactions including nucelophilic aromatic substitutions, Michael additions, and epoxide ring opening reactions, all of which result in the formation of glutathione conjugates that can be removed via a variety of transport mechanisms (*128*). GSTs enzymes are largely cytosolic and are divided into eight distinct classes (based on the primary structure of the N-terminus) termed alpha, kappa, mu, omega, pi, sigma, theta and zeta (*128*). DOPAL modification of GSTs could result in a disruption of the detoxification pathway and an accumulation of xenobiotics within a cell. Several studies have shown evidence of increased risk of disease, including PD, with decreased concentrations of detoxication enzymes (*129*).

ALDHs catalyze the oxidation of a variety of endogenous and exogenous aldehydes to their respective carboxylic acids which are less reactive metabolites (*130*). Modification of these enzymes could result in an accumulation of reactive aldehydes leading to an array of disease states (*130*). Furthermore, ALDH is an important enzyme in DA catabolism (*Scheme 6.1*), catalyzing the reduction of DOPAL, a reactive aldehyde, to DOPAC, a stable acid. Several studies have indicated DOPAL could positively influence its own cellular concentration by inhibiting ALDH (*59, 131*), thus yielding increased concentrations of DOPAL and further progression of PD.

The metabolic enzymes 3-phosphoglycerate dehydrogenase (3-PGDH) and triosephosphate isomerase (TPI) have also been predicted to be susceptible to DOPAL modification. 3-PGDH is involved in L-serine biosynthesis and catalyzes the conversion of 3-phosphoglycerate to 3-phosphohydroxypyruvate (*Scheme 6.3*) (*132*). L-Serine plays an important role in brain function, as it is a precursor for the synthesis of nucleotides, phospholipids, D-serine and glycine, a major inhibitory neurotransmitter involved in the regulation of movement. L-Serine deficiency, as a result of modifications or mutations of 3-PGDH, has been associated with severe neurological symptoms (*132*). TPI is a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP) (*Scheme 6.4)* (*133, 134*). Glucose is the main source for ATP production in the brain, therefore, modification of TPI by DOPAL could lead to the disruption of the glycolysis pathway and decreased availability of ATP. Several studies have suggested TPI to be associated with progressive neurological dysfunction as the protein has been demonstrated to be susceptible to oxidation in individuals with Alzheimer's disease (*134*) and will bind to the surface of microtubules, the main component of axons (*133*).



Scheme 6.3 An abbreviated scheme for the synthesis of L-serine and glycine. 3-Phosphoglycerate is oxidized to 3-phosphohydroxypyruvate by the enzyme 3-PGDH. Modification of 3-PGDH could lead to deficiencies in the overall concentration of L-serine.



Scheme 6.4 An abbreviated scheme of the glycolysis pathway. TPI is involved in the interconversion of DHAP and GAP. Modification of TPI by DOPAL could lead to decreased concentrations of cellular ATP.

Ribophorins and calnexin are both endoplasmic reticulum (ER) proteins identified to be vulnerable to modification. Ribophorins are transmembrane glycoproteins of the rough ER and are involved in the binding of ribosomes (*135, 136*). Ribosomes are involved in protein biosynthesis and translate RNA to build proteins by assembling amino acids to form the appropriate polypeptide chain. Protein modification of ribophorins may impede ribosome binding to the ER or interfere with protein synthesis leading to mutations or decreased concentrations of various proteins. Mutations, such as ribosomal frameshifting, have been suggested to lead to an accumulation of aberrant protein and may be involved in the progression of neurodegenerative diseases (*137*). Calnexin is an ER stress

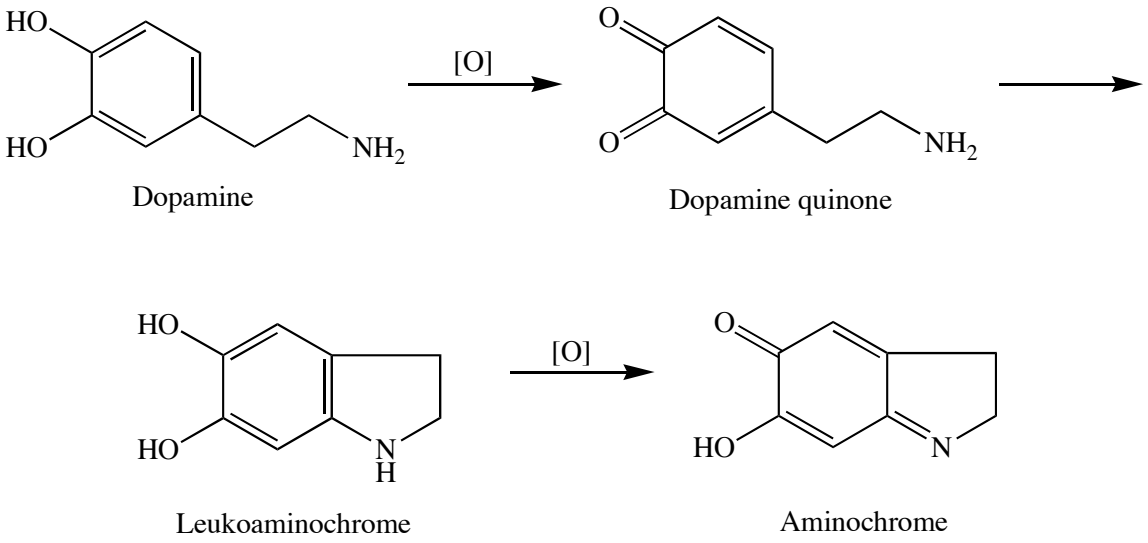
protein and belongs to a family called ER chaperones. While the protein has not been definitively linked to PD pathogenesis, ER stress has been demonstrated to play a role in the progression of the disease (*138*). Several studies have identified calnexin to be associated with α-synuclein (*138*) and present in neuromelanin (NM) granules (*139*). NM granules are dark polymer pigments located in the substantia nigra and their presence appears to decrease upon the onset of PD (*139*).

LTA_4-h is a bifunctional enzyme, which displays both epoxide hydrolase and aminopeptidase activities (*140*). The enzyme plays a role in inflammatory reactions therefore inhibition of LTA_4-h could lead to a decreased biological response to the onset of disease.

TH was identified by both ITMS and Western blot to be susceptible to DOPAL modification (*Table 6.1* and *Figure 6.6*). TH, the rate-limiting enzyme in catecholamine synthesis, catalyzes the conversion of L-tyrosine to L-DOPA, which is the precursor to DA (*4*). Several studies have demonstrated decreased activity of TH and low levels of all four human TH isoform mRNAs in the striatum of individuals with PD (*141*). In addition, research has demonstrated decreased TH activity early in the progression of PD, suggesting the inhibition of TH occurs prior to dopaminergic cell death (*142*). The decreased activity of the enzyme has been indicated to be due to peroxynitrite-mediated nitration of the enzyme active site (*142*), however the current results imply TH could also be inactivated through protein modification by DOPAL.

It might be argued that the proteins isolated in the release fractions of the APBA resin to be DA-modified proteins as numerous studies have demonstrated the reactivity of DA toward proteins (*69, 72, 98*). However, DA requires oxidation to an ortho-quinone before it can modify proteins (*70, 71*). The electrophilic form of DA (i.e. quinone) is short-lived as it rapidly rearranges via intramolecular

nucleophilic addition to leukoaminochrome and then aminochrome (*70, 71*) rendering it unable to modify proteins (*Scheme 6.5*). In addition, staining of the SDS-PAGE gel with nitroblue tetrazolium blue (NBT), a redox cycling dye which detects catechols (*38*) will further verify the isolated proteins contain a DOPAL adduct. Based on quantification of NBT staining, recent studies have determined DOPAL is far more reactive toward protein (i.e. BSA and GAPDH) than either DA or DOPAC (*102, 118*).



Scheme 6.5 Upon oxidation of DA, the reactive ortho-quinone is generated which will subsequently cyclize to form the leukochrome. Oxidation of leukochrome yields aminochrome.

In summary, this study has identified a technique to isolate DOPAL-modified proteins using an APBA resin. Results have demonstrated that the APBA resin will bind model proteins (i.e. BSA and GAPDH) containing a DOPAL adduct thus allowing for the separation of modified and unmodified proteins. Preliminary work using the APBA resin and PC6-3 cells treated with 4HNE has

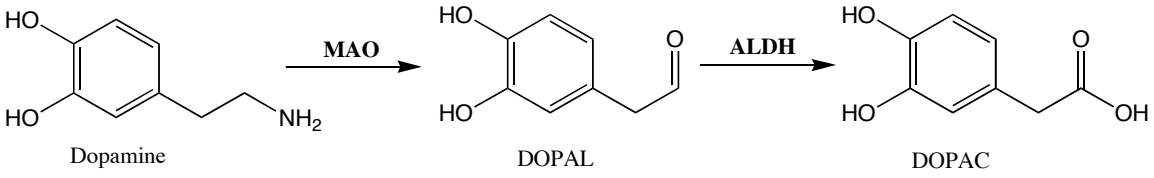
demonstrated a variety of proteins to be susceptible to DOPAL modification (*Table 6.1*). These proteins are involved in an assortment of cellular functions including detoxification, protein folding and synthesis, the cellular metabolic pathway, inflammatory and the biosynthesis of DA (*Figure 6.6*). These findings demonstrate increased DOPAL concentrations could yield modification of various enzymes and proteins, thus leading to oxidative stress, deficiencies in various metabolites or protein aggregation. Identification of proteins vulnerable to DOPAL adduction will aid in elucidating the role of DOPAL in the progression of PD in addition to determining the mechanism of the selective neurodegeneration of dopaminergic cells. Potentially, such work may facilitate the development of effective therapeutics for the treatment of the disease.

CHAPTER SEVEN

RESEARCH SUMMARY

Discussion

Parkinson's disease (PD) is a neurodegenerative disease characterized by two pathological hallmarks, selective degeneration of dopaminergic neurons and formation of intraneuronal protein aggregates, termed Lewy bodies (LBs) (*8*). Reasons as to the onset of PD are currently unknown, however oxidative stress has been indicated to play a role in the progression of the disease (*25, 44-46*). Two common components of increased oxidative stress are the formation of the lipid peroxidation products 4-hydroxy-2-nonenal (4HNE) and malondialdehyde (MDA) (*47, 48*). Elevated levels of 4HNE and MDA can be detrimental to cells as they can inhibit and modify cellular enzymes (*49, 50*). An accumulation of these reactive lipid aldehydes in the brain has been observed in individuals with PD (*47, 48*). One particular enzyme susceptible to both 4HNE and MDA inhibition is mitochondrial aldehyde dehydrogenase (ALDH) (*53-57*), an enzyme critical for dopamine (DA) catabolism. ALDH catalyzes the oxidation of 3,4-dihydroxphenylacetaldehyde (DOPAL), a reactive aldehyde, to 3,4-dihydroxyphenylacetic acid (DOPAC), a stable acid (*Scheme 7.1*).



Scheme 7.1 The metabolic pathway for DA. DA undergoes oxidative deamination via MAO to form the aldehyde intermediate DOPAL. DOPAL is oxidized to DOPAC by the mitochondrial enzyme ALDH.

Results of recent research indicate the involvement of an endogenous neurotoxin to explain the selective neurodegeneration as observed in the pathogenesis of PD (*76, 102-104*). Several studies have indicated DA to be an endogenous neurotoxin, as it will readily undergo auto-oxidation to an ortho-quinone (*70, 71*) capable of protein modification (*69, 72, 98*). Subsequent to biosynthesis, cytosolic DA is rapidly sequestered into vesicles and many reports have shown that dysregulation of DA trafficking and storage, such as toxicant-mediated release from vesicles, yields cell toxicity (*105-107*).

Results from other studies, however, have demonstrated the aldehyde metabolite of DA, DOPAL, to be orders of magnitude more toxic in vitro and in vivo (*8, 76*) as compared to DA. DOPAL cytotoxicity is determined to be dose-dependant and DOPAL concentrations slightly above normal physiological levels (i.e. > 2-3 μM) have been observed to cause cellular toxicity and induce cell death (*77, 78*). The generation of elevated DOPAL levels is indicated to be a result of the inhibition of DA catabolism by oxidative stress, specifically the presence of products of lipid peroxidation (*59*). Consequently, it is hypothesized that DOPAL, and not DA, is an endogenous neurotoxin relevant to the pathogenesis of PD.

It is of question, however, as to the cellular consequence of increased concentrations of DOPAL. The hypothesis of this research was DOPAL, an intermediate of DA catabolism and potential endogenous neurotoxin relevant to PD, is capable of protein modification and protein cross-linking through reactivity with amine and thiol nucleophiles. To test this hypothesis, three Specific Aims were developed and accomplished: Specific Aim I, demonstrate the ability of DOPAL to modify proteins; Specific Aim II, determine the reactivity of DOPAL towards protein nucleophiles and Specific Aim III, identify proteins of dopaminergic cells susceptible to adduction by DOPAL and relevant to PD.

Specific Aim I: Demonstrate the Ability of 3,4-Dihydroxyphenylacetaldehyde to Modify Proteins

As demonstrated by the data in *Figure 4.1* and *Figure 4.2*, the lipid peroxidation products 4HNE and MDA will inhibit ALDH yielding decreased levels of DOPAC and elevated concentrations of DOPAL in rat striatal synaptosomes. Several studies have demonstrated the ability for DOPAL to react with a variety of proteins (*84-86, 102*), therefore one consequence of an accumulation of the DA intermediate could be protein modification. In agreement with these studies, this work has observed that upon inhibition of DA catabolism and elevated DOPAL concentrations, the reactive aldehyde will cause protein modification in rat striatal synaptosomes (*Figure 4.4*). Furthermore, it was determined that DOPAL is considerably more reactive towards a model peptide and protein than DA, DOPAC or L-DOPA (*Figure 4.3* and *Figure 4.5*).

Specific Aim II: Determine the Reactivity of 3,4-Dihydroxyphenylacetaldehyde Towards Protein Nucleophiles

DOPAL is predicted to be a bifunctional electrophile as it contains two reactive functional groups (i.e. aldehyde and catechol). Two potential mechanisms for DOPAL protein modification include oxidation of the catechol to an ortho-quinone and reactivity with Cys residues, which is known to occur for DA (*70, 71, 75*) or adduction with Lys residues via the aldehyde. Results from this study determined that DOPAL is considerably more reactive toward Lys nucleophiles than Cys residues (*Figure 5.1*). This observation was further demonstrated by the data in *Figure 5.5* and *Figure 5.6*, specifically, the reactivity of DOPAL towards a model protein was dependent upon the availability of amine

nucleophiles. Furthermore, the ability of DOPAL to cause protein modification was not affected when thiol nucleophiles were modified/protected.

Results, however, demonstrated that the catechol of DOPAL does play a role in protein modification via enhancing the reactivity of the aldehyde and contributing to protein cross-linking. In comparison with various DOPAL analogs that contain differences of the catechol moiety (i.e. DMPAL, MOPAL and PAL), DOPAL was determined to be considerably more reactive towards Lys residues (*Figure 5.2, Figure 5.3* and *Table 5.1*). In addition, protein cross-linking was demonstrated in model proteins and rat liver mitochondrial lysate upon prolonged treatment (> 2 h) with DOPAL (*Figure 5.7, Figure 5.8A* and *Figure 5.9*). In the presence of quinone-reducing agents, evidence of protein cross-linking was diminished (*Figure 5.8B*). These results indicate DOPAL-mediated protein modification involves reaction with amine nucleophiles via the aldehyde in addition to protein cross-linking via the catechol moiety.

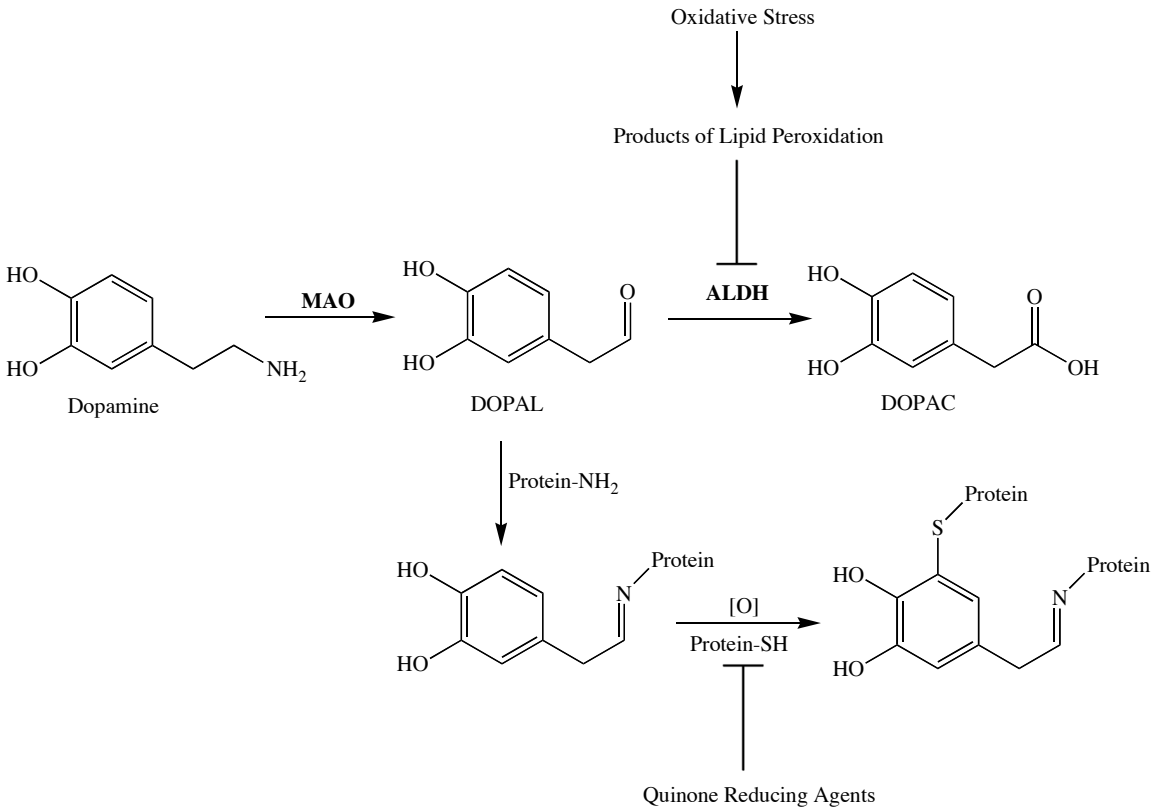
Specific Aim III: Identify Proteins of Dopaminergic Cells Susceptible to Adduction by 3,4-Dihydroxyphenylacetaldehyde and Relevant to Parkinson's Disease

To determine the cellular consequence of the presence of DOPAL-modified protein, a technique involving an aminophenylboronic acid (APBA) resin was developed. As the data in *Figure 6.1* and *Figure 6.2* demonstrate, proteins (i.e. BSA and GAPDH) require a DOPAL adduct in order to bind with the APBA resin. Thus, this technique allows for the separation between modified and unmodified proteins. Preliminary work using the APBA resin with PC6-3 cells treated with 4HNE determined a number of proteins to be susceptible to DOPAL modification (*Figure 6.3* and *Table 6.1*). Twelve different classes of proteins

were identified and they are involved in a variety of cellular functions including detoxication, protein folding, metabolism and the biosynthesis of DA (*Figure 6.5*). Identification of proteins vulnerable to DOPAL adduction will aid in elucidating the mechanism of DOPAL toxicity in regards to the progressive and selective neurodegeneration of dopaminergic neurons.

Conclusion

PD is characterized by two pathological hallmarks, selective cell death of dopaminergic neurons and protein aggregation (i.e. LBs). A neurotoxin endogenous to dopaminergic cells is hypothesized to be involved in the progression of this neurodegenerative disorder (*76, 102-104*). As demonstrated in *Scheme 7.2*, the results of this study suggest DOPAL, the aldehyde intermediate of DA catabolism, to be an endogenous neurotoxin. Upon increased oxidative stress, products of lipid peroxidation (i.e. 4HNE and MDA) have been demonstrated to inhibit mitochondrial ALDH yielding elevated concentrations of DOPAL. DOPAL has been demonstrated to be reactive towards proteins; therefore, prolonged exposure of DOPAL to dopaminergic cells could lead to extensive protein modification and protein cross-linking. Preliminary work using an APBA resin to isolate DOPAL-modified proteins demonstrated numerous proteins could be susceptible to modification including those involved in detoxication, protein folding and biosynthesis of DA. These results indicate DOPAL to be a highly reactive DA intermediate and relevant to the pathogenesis of PD. Potentially, such work may direct future research for novel therapeutics for this debilitating neurodegenerative disorder such that the disease can be effectively treated or possibly prevented.



Scheme 7.2 DOPAL is proposed to be an endogenous neurotoxin relevant to the pathogenesis of PD. Lipid peroxidation products, as a result of oxidative stress, have been observed to inhibit ALDH leading to increased concentrations of DOPAL. DOPAL is a reactive DA intermediate that can form adducts with protein amines and cross-linking with protein thiols. Evidence of protein cross-linking, however, is reduced in the presence of quinone reducing agents.

Future Direction of the Project

This current study determined protein modification and protein cross-linking to be a consequence of elevated DOPAL concentrations. DOPAL was demonstrated to react with amine nucleophiles via the aldehyde, though, would also react with thiol nucleophiles, yielding protein cross-linking. It has yet to be established, however, the structure of DOPAL when bound to a protein (i.e. formation of a cyclization product). In addition, preliminary studies have determined a number of proteins to be susceptible to DOPAL adduction;

however, further research in this regard will elucidate with more certainty the cellular consequence of protein modification via DOPAL within dopaminergic neurons. Additional studies examining these questions could yield a better understanding as to the mechanism of DOPAL toxicity in regards to the pathogenesis of PD.

Identifying the Protein Adduct of 3,4-Dihydroxyphenylacetaldehyde

DOPAL contains two reactive functional groups, aldehyde and catechol. Results from this study determined DOPAL is more reactive towards Lys than Cys, demonstrating that the DA intermediate is a highly reactive aldehyde. The catechol moiety, however, does appear to be involved in protein modification, especially in regards to protein cross-linking. To further examine the mechanism of DOPAL protein modification, it would be of interest to determine the structure of the DOPAL adduct. Specifically, does DOPAL form an indole-like structure when bound to a protein, similar to the formation of the DA aminochrome? Furthermore, after modification of all available Lys residues, will DOPAL modify other protein amines (i.e. Arg, Asn, Gln, His) to a lesser extent?

Recent results demonstrated that DOPAL, and not DA, will react with an amine-containing peptide (i.e. RKRSRAE, *Figure 4.3*) and additional studies were conducted to further examine the reactivity between DOPAL and Lys-containing peptides (Appendix B, *Figures B7-B9*). The N-acetyl-peptides utilized include myelin (ASQKRPSQRHG, MW 1293.4), myelin basic protein (MBP, QKRPSQRSKYL, MW 1432.7) and melanocyte stimulating hormone (MSH, SYSMEHFRWGKPV, MW 1664.9). Each model peptide (10 μM) was incubated with 100 μM of DOPAL (10 mM tricine buffer, pH 7.4, 4 h at 37 °C). Samples were further incubated with 1 mM $NaCNBH_3$ (2 h at 37 °C) and analyzed via

matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Briefly, 1 µL of sample was diluted 1:1 with 0.1% trifluoroacetic acid and mixed with 1 µL of water saturated with α-cyano-4-hydroxycinnaminic acid on a plate. The mixture was allowed to air dry and analyzed using a Bruker Bioflex MALDI-TOF-MS in reflectron mode. Calibration was performed using the peptides angiotensin I and bradykinin.

As determined by the data in Appendix B (*Figures B7-B9*), DOPAL will react with the model peptides to form adducts of m/z 136, corresponding to the formation of a reduced Schiff base. Specifically, the spectra demonstrate myelin (one Lys residue) to contain two DOPAL adducts (m/z 1429.5 and 1563.5), MBP (two Lys residues) to contain one DOPAL adduct (m/z 1569.6) and MSH (one Lys residue) to contain one DOPAL adduct (m/z 1800.7). Of note, the peaks at m/z 1680.6 and 1816.7 correspond to oxidized MSH (i.e. Met) and oxidized MSH with a DOPAL adduct, respectively.

These preliminary results appear to indicate DOPAL will react with proteins via formation of a Schiff base. Furthermore, it is predicted that DOPAL may react with protein amines other than Lys (i.e. myelin contains two adducts). To further determine the structure and location of the DOPAL protein adduct, mass spectrometry with fragmentation analysis could be utilized. It would also be of interest to determine the effect of quinone-reducing agents on the reactivity of DOPAL with various amine and thiol-containing peptides. This would further demonstrate the role of the quinone in DOPAL protein modification/cross-linking.

Identifying Proteins Susceptible to Adduction by 3,4-Dihydroxyphenylacetaldehyde

DOPAL modification of proteins of dopaminergic neurons could lead to significant cellular consequences. Preliminary research using PC6-3 cells

treated with 4HNE (*Figure 6.3*) demonstrated twelve classes of proteins susceptible to DOPAL modification, including chaperones, ER proteins, detoxication enzymes, metabolic enzymes, enzymes involved in DA synthesis and inflammatory proteins. Modification of these proteins could result in oxidative stress, increased presence of toxic metabolites and protein aggregation.

To further determine the cellular consequence of DOPAL-modified proteins, it is of interest to continue to identify proteins vulnerable to adduction by DOPAL. Mitochondrial proteins may be uniquely susceptible to DOPAL as DA catabolism (i.e. the biosynthesis of DOPAL) occurs in the mitochondrial matrix. Modification of these proteins could affect mitochondrial function including signaling, the electron transport chain and ATP generation. Extensive modification of ubiquitin, which tags misfolded proteins for proteasomal degradation, could also lead to a considerable accumulation of damaged proteins. In addition, recent research has demonstrated DOPAL may cause protein aggregation of α-synuclein (*114*), a primary component of LBs.

Various methodologies may be used to isolate proteins susceptible to DOPAL modification including APBA resin (as previously described, Chapter Six), immunoprecipitation with DOPAL antibodies or methodology involving fluorescent labeling or radiolabeling. A proteomics-based approach, utilizing 2D SDS-PAGE and mass spectrometry, may be used to identify DOPAL-modified proteins and determine what effect modification of these proteins may have on dopaminergic cells.

Identifying proteins vulnerable to DOPAL modification in addition to demonstrating the structure of the DOPAL protein adduct will help to further elucidate the mechanism of DOPAL as a potential endogenous neurotoxin relevant to PD. Potentially, such work may determine DOPAL to be a biomarker

for PD or a novel therapeutic target, thus carbonyl scavengers (*99-101*) could be used to treat or prevent the pathogenesis of this debilitating neurodegenerative disease.

APPENDIX A
THE SYNTHESIS OF
3,4-DIHYDROXYPHENYLACETALDEHYDE

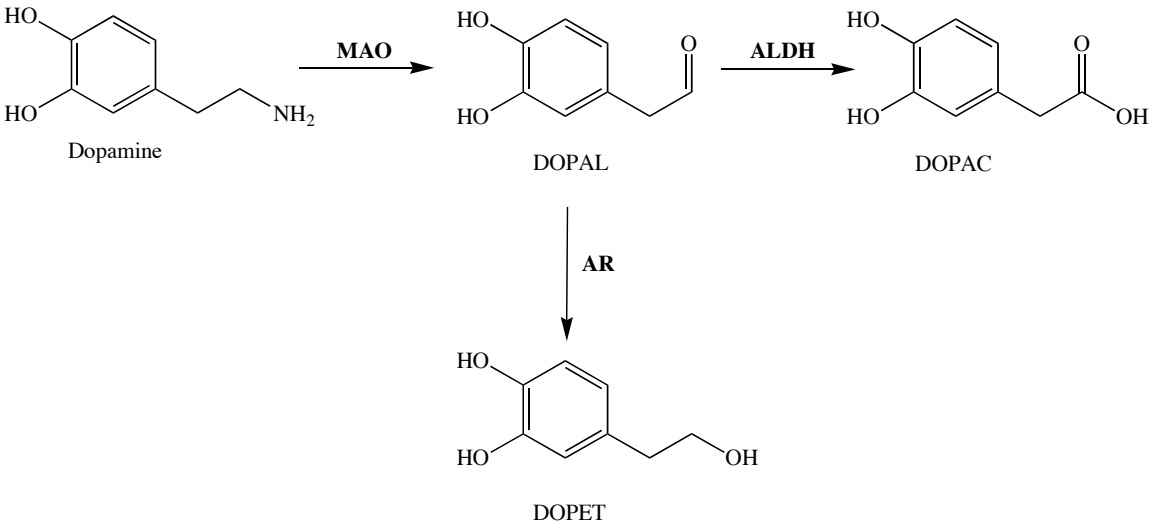
Abstract

3,4-Dihydroxyphenylacetaldehyde (DOPAL), an intermediate of dopamine metabolism, has been implicated as an endogenous neurotoxin to explain the progressive neurodegeneration as observed for Parkinson's disease (PD). Several studies have indicated the mechanism of DOPAL toxicity to involve protein modification, however it has yet to be established with certainty the rationale for the progressive and selective cell death of dopaminergic neurons. The goal of this work was to develop an improved synthesis for DOPAL in order to obtain a standard that can be used to further elucidate the role of DOPAL in PD. This study describes four novel synthetic routes for the synthesis of DOPAL. These schemes afford the desired product in three to five steps with an overall yield between 4-16%.

Introduction

3,4-Dihydroxyphenylacetaldehyde (DOPAL) is a reactive aldehyde intermediate of dopamine (DA) catabolism formed by the oxidative deamination of DA by monoamine oxidase (MAO) (*63*). DOPAL is primarily oxidized to 3,4-dihydroxyphenylacetic acid (DOPAC), however the aldehyde could also be reduced to 3,4-dihydroxyphenylethanol (DOPET) (*Scheme A.1*). Developing an efficient synthesis for DOPAL is important for a variety of reasons as DOPAL is hypothesized to be an endogenous neurotoxin relevant to Parkinson's disease (PD) (*8, 59, 76-78, 102*). PD is a common neurodegenerative disorder and is characterized by selective neurodegeneration in addition to an accumulation of intraneuronal protein aggregates (i.e. Lewy bodies). The mechanism of DOPAL

toxicity, in regards to dopaminergic cell death, is hypothesized to involve protein modification (*84-86, 102, 118*). Developing an efficient synthesis to acquire DOPAL for use as a standard will allow for an accurate measurement of DOPAL concentrations in dopaminergic systems and aid in determining the involvement of DOPAL in the pathogenesis of PD.

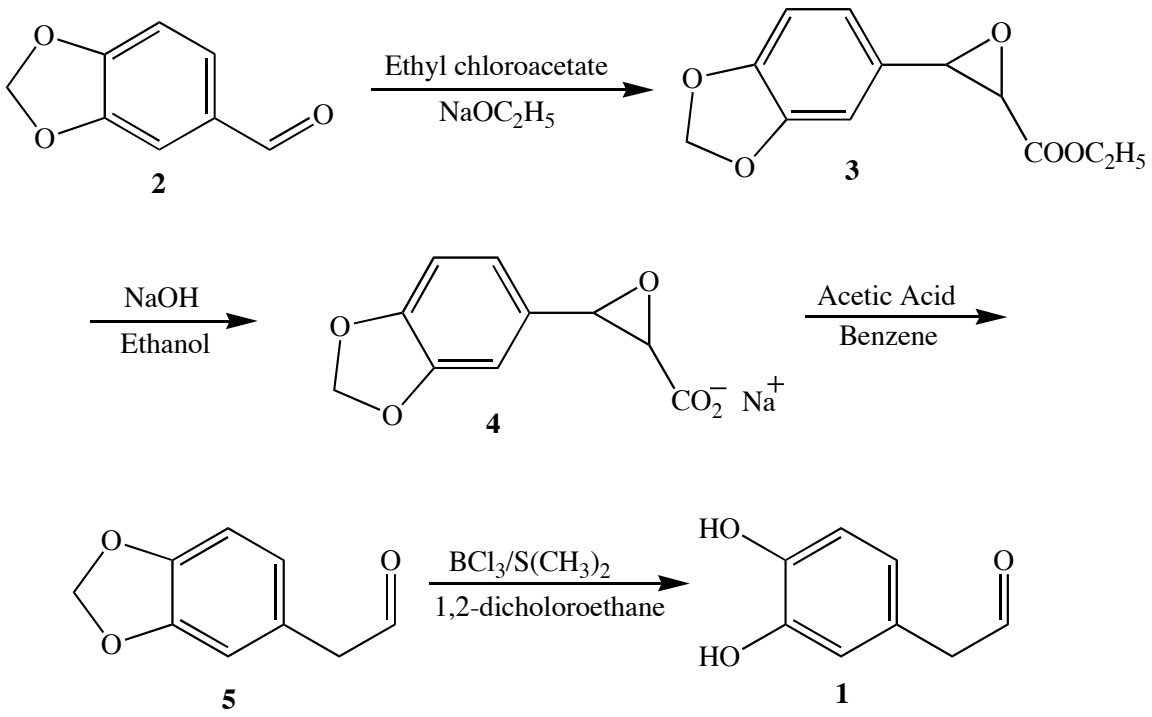


Scheme A.1 DOPAL is an intermediate of DA catabolism. DA undergoes oxidative deamination to form the reactive aldehyde that is subsequently oxidized by aldehyde dehydrogenase (ALDH) to DOPAC. A minor pathway is the reduction of DOPAL to DOPET by aldose/aldehyde reductase (AR).

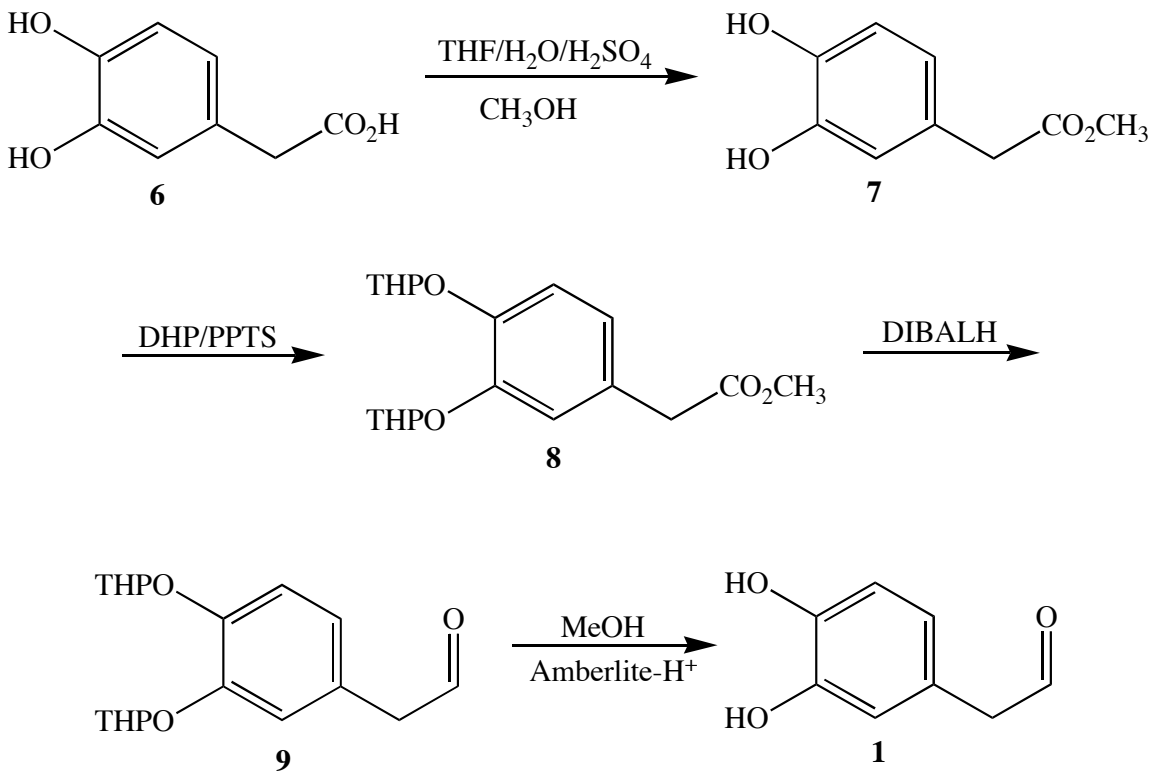
Two synthetic schemes previously outlined in the literature, include a four step synthesis with a 4% overall yield by Li et al. (*6*) and a four step synthesis with a 25% overall yield by Narayanan et al. (*7*). As demonstrated in (*Scheme A.2*), the synthesis developed by Li et al. used piperonal (**2**) as a starting material and achieved ethyl glycidate (**3**) by reaction with ethyl chloroacetate and sodium ethoxide. Compound **3** was hydrolyzed with sodium hydroxide to form the sodium salt (**4**). The aldehyde (**5**) was generated with benzene and acetic acid

and deprotection to the catechol with boron trichloride methyl sulfide afforded DOPAL (**1**) in a 4% overall yield. The synthesis developed by Narayanan et al. (*Scheme A.3*) uses 3,4-dihydroxyphenylacetic acid (**6**) as the starting material and reduced the carboxylic acid to the methyl ester (**7**). The catechol was protected via tetrahydropyranyl (THP) protecting groups (**8**) and the aldehyde (**9**) was generated with DIBAL. Deprotection with amberlite resin afforded DOPAL (**1**) in a 25% overall yield.

While these two syntheses provide a convenient method to obtain DOPAL, the goal of this study was to develop an alternative synthesis that was inexpensive, time efficient and produced a high overall yield.



Scheme A.2 The DOPAL synthesis developed by Li et al. (*6*) affords the aldehyde product in a 4% overall yield with piperonal as the starting material.



Scheme A.3 The DOPAL synthesis developed by Narayanan et al. (*7*), affords the aldehyde product in four steps with an overall yield of 25%.

Experimental Procedures

Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Supelco Discovery DSC-18 Solid Phase Extraction (SPE) Tubes were utilized to isolate DOPAL from the reaction mixture. The SPE tubes contain a matrix of silica gel (50 µM, 70 Å) with octadecyl (18% carbon) bonding and endcapping. Each tube has a volume of 3 mL and a bed weight of 500 mg. NMR spectra of synthetic intermediates were run in $CDCl_3$ on a Bruker 300 MHz spectrometer (Appendix B, *Figures B9-B12*). DOPAL was further characterized

via an Agilent 1100 Series Capillary high performance liquid chromatography (HPLC) system (Appendix B, *Figure B13*).

High Performance Liquid Chromatography

An Agilent 1100 Series Capillary HPLC system was used for quantification of DOPAL. This unit is equipped with a sample cooler, capable of maintaining a temperature of 4 °C in the autosampler tray. Briefly, 10 µL of sample solution (i.e. supernatant) was injected and separation was achieved using a Phenomenex C18 Luna microbore column (1 × 150 mm, 100Å) with isocratic conditions consisting of 0.1% trifluoroacetic acid (TFA) (v/v) in HPLC-grade water and 6% acetonitrile (ACN) (v/v) at a flow rate of 50 µL/min. DOPAL was detected with a photo-diode array detector using absorbance set to 202 and 280 nm for quantitation. Retention time for DOPAL was determined to be 7.3 min. A calibration curve was generated using a DOPAL standard and utilized to convert peak area to concentration units.

Synthetic Methods

Synthesis One

Methyl 2-(3,4-dimethoxyphenyl)acetate (**11**)

3,4-Dimethoxyphenylacetaldehyde (1 g, 5.5 mmole) (**10**) was dissolved in a 5 mL solution of MeOH and 1 M HCl and heated for 2 h (80 °C). The acidic solution was neutralized and the product (**11**) was extracted into ether. The organic layer was dried ($MgSO_4$) and evaporated to afford 0.96 g of the methyl ester (**11**) as a light brown colored oil in a 78% yield. Compound **11** was used in the next step without further purification.

2-(3,4-Dimethoxyphenyl)ethanol (**12**)

Compound **11** (0.3 g, 1.5 mmole) was dissolved in 4 mL dry THF and added dropwise to $LiAlH_4$ (0.15 g, 4 mmoles) under a N_2 environment. The solution was allowed to stir for 1 h in an ice bath. The ice bath was subsequently removed and the solution was allowed to stir for an additional 1 h at room temperature. The reaction was quenched by the dropwise addition of 4 mL cold H_2O. Using a separatory funnel, the product (**12**) was extracted into ethyl acetate (3 x 10 mL) and washed with ammonium chloride (1 x 5 mL). The organic layer was dried ($MgSO_4$) and evaporated via rotary evaporator to give 0.2 g of **12** as an oily residue in a 70% yield. Compound **12** was used in the next step without further purification.

^{1}NMR ($CDCl_3$) δ: 2.65 (t, 2H), 3.67 (t, 2H), 3.71 (s, 3H), 3.74 (s, 3H), 6.61-6.69 (m, 3H).

2-(3,4-Dimethoxyphenyl)acetaldehyde (**13**)

Compound **12** (0.3 g, 1.5 mmole) and Dess Martin periodinane (0.7 g, 1.6 mmoles) were dissolved in 5 mL CH_2Cl_2 and allowed to stir at room temperature for 2 h. The reaction was quenched by addition of 10 mL ether, 5 mL sodium bicarbonate (saturated) and 5 mL sodium thiosulphate (saturated). The solution was stirred vigorously until a color change was observed from cloudy to clear. The product (**13**) was extracted into ether (3 x 10 mL). The organic layer was dried over $MgSO_4$ and evaporated via rotary evaporator to give 0.2 g of **13** as a light colored oil in a 78% yield. Compound **13** was used in the next step without further purification.

^{1}NMR ($CDCl_3$) δ: 3.54 (d, 2H), 3.76 (s, 3H), 3.77 (s, 3H), 6.64-6.80 (m, 3H), 9.63 (t, 1H).

2-(3,4-Dihydroxyphenyl)acetaldehyde (**1**)

Compound **13** (0.01g, 0.06 mmole) was dissolved in 10 mL CH_2Cl_2 and placed under a N_2 environment and in a dry ice bath. BBr_3 (0.3 mL, 3 mmoles) was added dropwise and the solution was allowed to stir for 5 min (*143*). The dry ice bath was subsequently removed and the reaction was stirred at room temperature for 1 h. The reaction was quenched with 20 mL H_2O and stirred for an additional 45 min. Using a separatory funnel, the aqueous and organic layers were separated and the aqueous layer was subject to a SPE column. Briefly, the aqueous layer was divided into 2 mL aliquots and each aliquot was added to a Supelco SPE tube. Compound **1** was eluted from the column using a 0.1% TFA solution. The fractions were collected and analyzed by HPLC (19% yield).

HPLC (6% ACN): 6.94 min.

Synthesis Two

2-(3,4-Dimethoxyphenyl)acetaldehyde (**13)**

4-Allyl-1,2-dimethoxybenzene (**14**) (0.95 mL, 5.5 mmole) was dissolved in a solution of 35 mM $RuCl_3$ (5.6 mL, 3.5 mol %) and 5 mL 6:1 ACN : H_2O (*108*). Over a 5 min period, $NaIO_4$ (2.4 g, 11 mmoles) was added to the solution in small increments. After stirring for 3 h at room temperature, the reaction was quenched with 5 mL sodium thiosulphate (saturated). Using a separatory funnel, compound **13** was extracted into ethyl acetate (3 x 20 mL). The organic layer was dried ($MgSO_4$) and evaporated via a rotary evaporator. The resultant was further purified by column chromatography on silica gel and elution was performed with a 6:1 mixture of hexanes and ethyl acetate. The product (**13**) was obtained as a yellow oil in a 21% yield.

^{1}NMR ($CDCl_3$) δ: 3.54 (d, 2H), 3.76 (s, 3H), 3.77 (s, 3H), 6.64-6.80 (m, 3H), 9.63 (t, 1H).

2-(3,4-Dihydroxyphenyl)acetaldehyde (**1**)

Compound **13** (0.01g, 0.06 mmole) was dissolved in 10 mL CH_2Cl_2 and placed under a N_2 environment and in a dry ice bath. BBr_3 (0.3 mL, 3 mmoles) was added dropwise and the solution was allowed to stir for 5 min (*143*). The dry ice bath was subsequently removed and the reaction was stirred at room temperature for 1 h. The reaction was quenched with 20 mL H_2O and stirred for an additional 45 min. Using a separatory funnel, the aqueous and organic layers were separated and the aqueous layer was subject to a SPE column. Briefly, the aqueous layer was divided into 2 mL aliquots and each aliquot was added to a Supelco SPE tube. Compound **1** was eluted from the column using a 0.1% TFA solution. The fractions were collected and analyzed by HPLC (19% yield).

HPLC (6% ACN): 6.94 min.

Synthesis Three

2-(Benzo[1,3]dioxol-5-yl)acetaldehyde (**16**)

Safrole (**15**) (0.09 mL, 0.6 mmole) was dissolved in a solution of 35 mM $RuCl_3$ (0.75 mL, 3.5 mol %) and 5 mL 6:1 ACN : H_2O (*108*). $NaIO_4$ (0.5 g, 2 mmoles) was added to the solution over a period of 5 min. After stirring for 3 h at room temperature, the reaction was quenched with 5 mL sodium thiosulphate (saturated). Using a separatory funnel, compound **15** was extracted into ethyl acetate (3 x 20 mL). The organic layer was dried ($MgSO_4$) and evaporated via a rotary evaporator. The resultant was further purified by column chromatography on silica gel and elution was performed with a 6:1 mixture of hexanes and ethyl acetate. The product (**16**) was obtained as a yellow oil in a 39% yield.

^{1}NMR ($CDCl_3$) δ: 3.60 (d, 2H), 5.93 (s, 2H), 6.65-6.82 (m, 3H), 9.71 (t, 1H).

2-(3,4-Dihydroxyphenyl)acetaldehyde (**1**)

Compound **16** (0.02 g.12 mmole) was dissolved in 10 mL CH_2Cl_2 and placed under a N_2 environment and in a dry ice bath. BBr_3 (0.04 mL, 0.3 mmoles) was added dropwise and the solution was allowed to stir for 5 min (*143, 144*). The dry ice bath was subsequently removed and the reaction was stirred at room temperature for 1 h. The reaction was quenched with 20 mL H_2O and stirred for an additional 45 min. Using a separatory funnel, the aqueous and organic layers were separated and the aqueous layer was subject to a SPE column. Briefly, the aqueous layer was divided into 2 mL aliquots and each aliquot was added to a Supelco SPE tube. Compound **1** was eluted from the column using a 0.1% TFA solution. The fractions were collected and analyzed by HPLC (27% yield).

HPLC (6% ACN): 6.94 min.

Synthesis Four

2-(Benzo[1,3]dioxol-5-yl)acetaldehyde (**16**)

Safrole (**15**) (0.09 mL, 0.6 mmole) was dissolved in a solution of 35 mM $RuCl_3$ (3.5 mol %) and 5 mL 6:1 ACN : H_2O (*108*). $NaIO_4$ (0.5 g, 2 mmoles) was added to the solution over a period of 5 min. After stirring for 3 h at room temperature, the reaction was quenched with 5 mL sodium thiosulphate (saturated). Using a separatory funnel, compound **15** was extracted into ethyl acetate (3 x 20 mL). The organic layer was dried ($MgSO_4$) and evaporated via a rotary evaporator. The resultant was further purified by column chromatography on silica gel and elution was performed with a 6:1 mixture of hexanes and ethyl acetate. The product (**16**) was obtained as a yellow oil in a 39% yield.

^{1}NMR ($CDCl_3$) δ: 3.60 (d, 2H), 5.93 (s, 2H), 6.65-6.82 (m, 3H), 9.71 (t, 1H).

5-(2,2-Dichloroethyl)benzo[1,3]dioxole (**17**)

Compound (**16**) (0.01 g, 0.08 mmole) was added to PCl_5 (0.1 g, 0.5 mmoles mmoles) and the reaction was stirred neat at room temperature for 30 min to afford the protected aldehyde compound (**17)** (*145*). Compound **17**, a light green solid, was used in the next step without further purification.

^{1}NMR ($CDCl_3$) δ: 3.40 (d, 2H), 5.80 (t, 1H), 5.98 (s, 2H), 6.74-6.78 (m, 3H).

2-(3,4-Dihydroxyphenyl)acetaldehyde (**1**)

Compound **17** was dissolved in 20 mL CH_2Cl_2 and placed under a N_2 environment and in a dry ice bath. BBr_3 (0.02 mL, 0.2 mmoles) was added dropwise and the solution was allowed to stir for 5 min (*143, 144*). The dry ice bath was subsequently removed and the reaction was stirred at room temperature for 1 h. The reaction was quenched with 30 mL H_2O and stirred for an additional 45 min. Using a separatory funnel, the aqueous and organic layers were separated and the aqueous layer was subject to a SPE column. Briefly, the aqueous layer was divided into 2 mL aliquots and each aliquot was added to a Supelco SPE tube. Compound **1** was eluted from the column using a 1% TFA solution. The fractions were collected and analyzed by HPLC (41% yield).

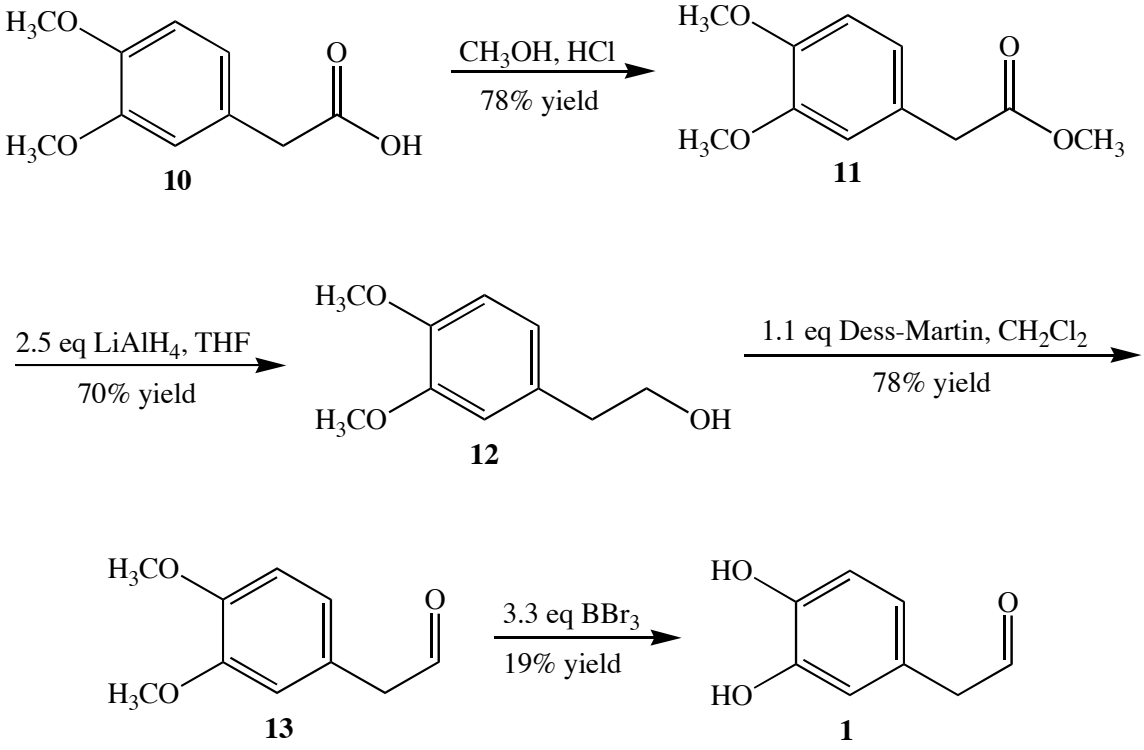
HPLC (6% ACN): 6.94 min.

Results

Synthesis One

As demonstrated in *Scheme A.4*, Synthesis One is a four-step synthesis with an overall yield of 8%. 3,4-Dimethoxyphenylacetic acid (**10**) was heated with MeOH to afford the methyl ester (**11**) and then reduced to the alcohol (**12**). Compound **12** was oxidized to **13** and the catechol was deprotected to afford DOPAL (**1**). Alternative routes to this synthesis to improve the yield included

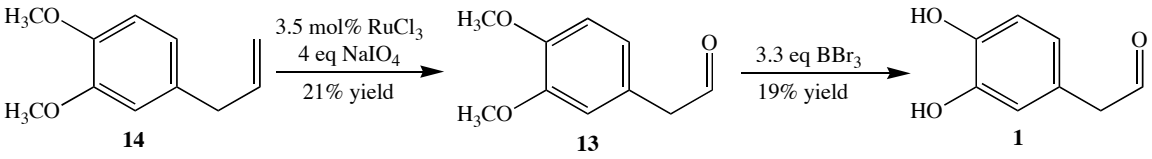
using DIBAL to convert the methyl ester (**11**) to the aldehyde and using CH_3SiI to obtain compound **1** by deprotecting the catechol, however, these methods did not yield the desired products. Additionally, the use of alternative starting materials were explored such as 3,4-dihydroxyphenylethanol (DOPET), eugenol and eugenol methyl ether (4-allyl-1,2-dimethoxybenzene). Oxidization of DOPET to **1** via Dess-Martin periodinane and alkene oxidation of eugenol via the reagents OsO_4, $NaIO_4$ and 2,6-lutidine (*146*) did not yield significant results, however alkene oxidation of eugenol methyl ether lead to **13**, suggesting the eugenol derivative could be used as a starting reagent.



Scheme A.4 Synthesis One begins with the starting material 3,4-dimethoxyphenylacetic acid and affords DOPAL in four steps with an 8% overall yield.

Synthesis Two

Synthesis Two (*Scheme A.5*) used eugenol methyl ether as the starting material and afforded DOPAL (**1**) in two steps with an overall yield of 4.1%. Alkene oxidation of safrole (**14**) via $RuCl_3$ and $NaIO_4$ afforded **13** and deprotection by BBr_3 yielded DOPAL (**1**). Of interest, it was determined during the course of this synthesis that protecting the aldehyde via an acetal would slightly enhance the demethylation reaction with BBr_3 (*147*).



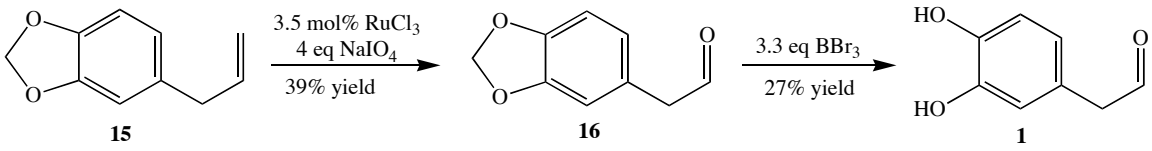
14 **13** **1**

Scheme A.5 Synthesis Two begins with the starting material safrole and affords DOPAL in a two-step synthesis with an overall yield of 4%.

Synthesis Three

Synthesis Three (*Scheme A.6*) is a two-step synthesis that obtained DOPAL (**1**) in a 10% overall yield. Compound **1** was achieved via alkene oxidation of safrole (**15**) and BBr_3 demethylation of compound **16**. In an effort to optimize the reaction, alkene oxidation of safrole (**15**) was attempted via two other methods; OsO_4, $NaIO_4$, 2,6-lutidene (*146*) and $RuCl_3$, Oxone, $NaHCO_3$ (*108*), these alternative methods did not yield compound **16** successfully. Additionally, to achieve DOPAL, $LiAlH_4$ was used to reduce 2-(benzo[1,3]dioxol-5-yl)acetic acid (the carboxylic acid derivate of safrole) to the alcohol (70-90% yield), however neither Swern oxidation (*148*) nor Dess-Martin reagent could yield the desired aldehyde compound. The Weinreb amide of the safrole derivative (i.e. carboxylic acid) could also be easily synthesized (76% yield) (*149*-

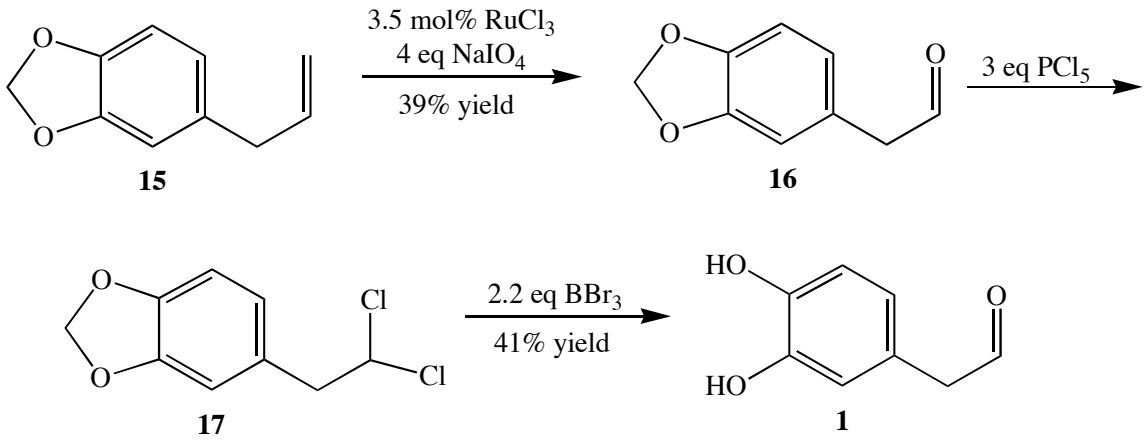
151) although; reduction via $LiAlH_4$ or DIBAL did not afford the aldehyde. Alternative deprotection reaction conditions included $AlBr_3$, EtSH and PCl_5 (*145*). While none of these reagents yielded DOPAL, NMR analysis determined the PCl_5 reagent would chlorinate the aldehyde moiety and not the methylene dioxy group. This result indicated PCl_5 could be used a protecting reagent.



Scheme A.6 Synthesis Three also uses the starting material safrole however, this two-step synthesis affords DOPAL with a 10% overall yield.

Synthesis Four

As demonstrated in *Scheme A.7*, Synthesis Four afforded DOPAL (**1**) in 16% overall yield and three steps utilizing the starting material safrole (**15**). Alkene oxidation of **15** yielded the aldehyde compound **16**. The aldehyde can be protected via PCl_5 and deprotection of **17** yielded the desired product (**1**).



Scheme A.7 Synthesis Four begins with safrole as the starting material and affords DOPAL in three steps with an overall yield 16%.

Discussion

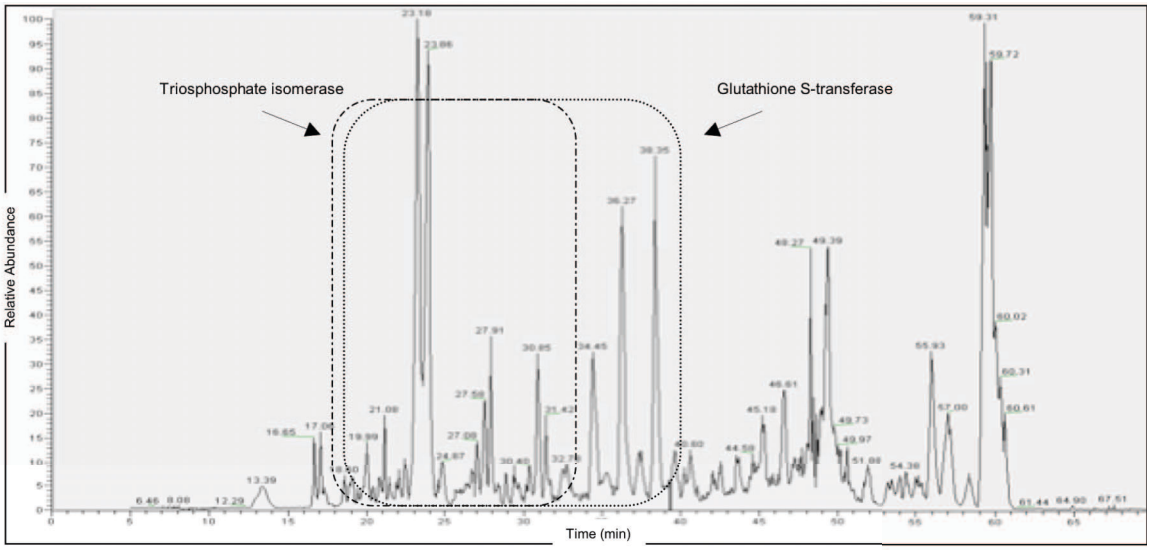
DOPAL is an intermediate of DA catabolism and is hypothesized to play a role in the pathogenesis of PD (*8, 59, 76-78, 102*). To further elucidate the involvement of DOPAL in the progression of this neurodegenerative disease, an efficient synthetic scheme is needed to achieve a DOPAL standard. A total of four DOPAL synthetic schemes were proposed that utilize inexpensive starting materials and reagents and achieve the desired product in three to five steps with 4% - 16% overall yield (*Scheme A.4-A.7*).

During the course of these experiments, DOPAL was determined to be a highly reactive aldehyde such that at high concentrations of the DA intermediate a polymer residue was observed. Due to the reactive nature of the aldehyde, isolating DOPAL from a reaction mixture was proven to be difficult and as a result the technique of SPE was utilized. Characterization of DOPAL could only be achieved with low concentrations (i.e. < 200 μM) therefore HPLC and not NMR was used. It is expectant that future work on the synthesis of DOPAL will identify a more effective method to isolate DOPAL from the reaction mixture and to

achieve a clean NMR spectra. Developing an efficient DOPAL synthesis will be beneficial to a multitude of researchers who are working towards understanding the pathogenesis of PD and developing therapeutics that may halt the progression of the disease.

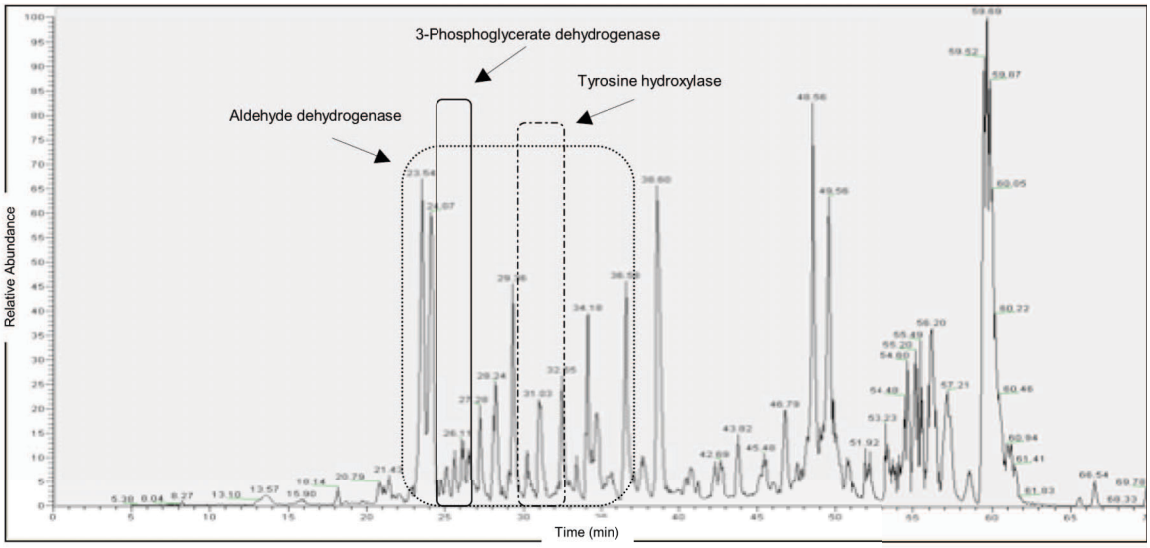
APPENDIX B

SPECTRA



Peptide	Molecular Weight (Da)	Time (min)	Peptide Sequence
Triosephosphate isomerase	26849	30.18	K.DLGATWVVLGHSER.R
		34.28	K.VVLAYEPVWAIGTGK.T
		33.41	K.VVLAYEPVWAIGTGK.T
		32.62	K.DLGATWVVLGHSER.R
		33.43	K.VVLAYEPVWAIGTGK.T
		39.47	K.ELASQPDVDGFLVGGASLKPEFVDIINAK.Q
		16.44	K.TATPQQAQEVHEK.L
		17.31	K.TATPQQAQEVHEK.L
		17.38	K.TATPQQAQEVHEK.L
		28.31	K.FFVGGNWK.M
		37.13	R.LKVPPAINQFTQALDR.Q
Triosephosphate isomerase	26847	24.94	R.RHIFGESDELIGQK.V
		26.23 - 26.23	R.HIFGESDELIGQK.V
		28.32	R.HIFGESDELIGQK.V
		26.25	R.HIFGESDELIGQK.V
		13.05	R.EAGITEK.V
		12.18	R.EAGITEK.V
Glutathione S-transferase, mu 1	25897	40.32	K.LGLDFPNLPYLIDGSR.K
		39.45	K.LGLDFPNLPYLIDGSR.K
		40.45	K.LGLDFPNLPYLIDGSR.K
		39.20	K.LGLDFPNLPYLIDGSR.K
		23.67	R.ADIVENQVMDNR.M
		19.37	R.KITQSNAIMR.Y
		27.58	R.YLSTPIFSK.L
		27.42	R.YLSTPIFSK.L
		19.12	K.LAQWSNK.-
Glutathione S-transferase, mu 2	25624	26.55	K.LYSEFLGK.Q
		26.89	K.LYSEFLGK.Q
Glutathione S-transferase, mu 4	25626	21.88	K.RPWFAGDK.V
		22.43	K.RPWFAGDK.V
		26.55	K.IYSEFLGK.R
		26.89	K.IYSEFLGK.R

Figure B.1 HPLC chromatogram, ITMS and SEQUEST database search results from the analysis of the 25 kDa SDS-PAGE protein band. Triosephosphate isomerase and glutathione S-transferase were identified to be susceptible to DOPAL modification and isolated via an APBA resin from PC6-3 cells treated with 4HNE.



Peptide	Molecular Weight (Da)	Time (min)	Peptide Sequence
Aldehyde dehydrogenase, family 2	56452	32.30	R.TFVQEDVYDEFVER.S
		23.11	R.TEQGPQVDETQFK.K
		37.48	K.VAEQTPLTALYVANLIK.I
		32.59	R.GYFIQPTVFGDVK.D
		28.55	K.YGLAAAVFTK.D
		32.43	R.GYFIQPTVFGDVK.D
Aldehyde dehydrogenase, family 3	54047	25.68	R.FDHILYTGNTAVGK.I
		30.73	K.NVEEAINFINDR.E
		23.23	K.IAFGGETDEATR.Y
		30.67	R.YIAPTILTDVDPNSK.V
3-Phosphoglycerate dehydrogenase	56457	25.21	R.AGTGVDNVDLEAATR.K
		25.19	R.AGTGVDNVDLEAATR.K
		25.33	K.GTIQVVTQGTSLK.N
		25.98	K.ILQDGGLQVVEK.Q
Tyrosine hydroxylase	55931	31.97	R.TGFQLRPVAGLLSAR.D
		30.61	K.LIAEIAFQYK.H
		30.50	K.LIAEIAFQYK.H

Figure B.2 HPLC chromatogram, ITMS and SEQUEST database search results from the analysis of the 56 kDa SDS-PAGE protein band. Aldehyde dehydrogenase, 3-phosphoglycerate dehydrogenase and tyrosine hydroxylase were identified to be susceptible to DOPAL modification and isolated via an APBA resin from PC6-3 cells treated with 4HNE.

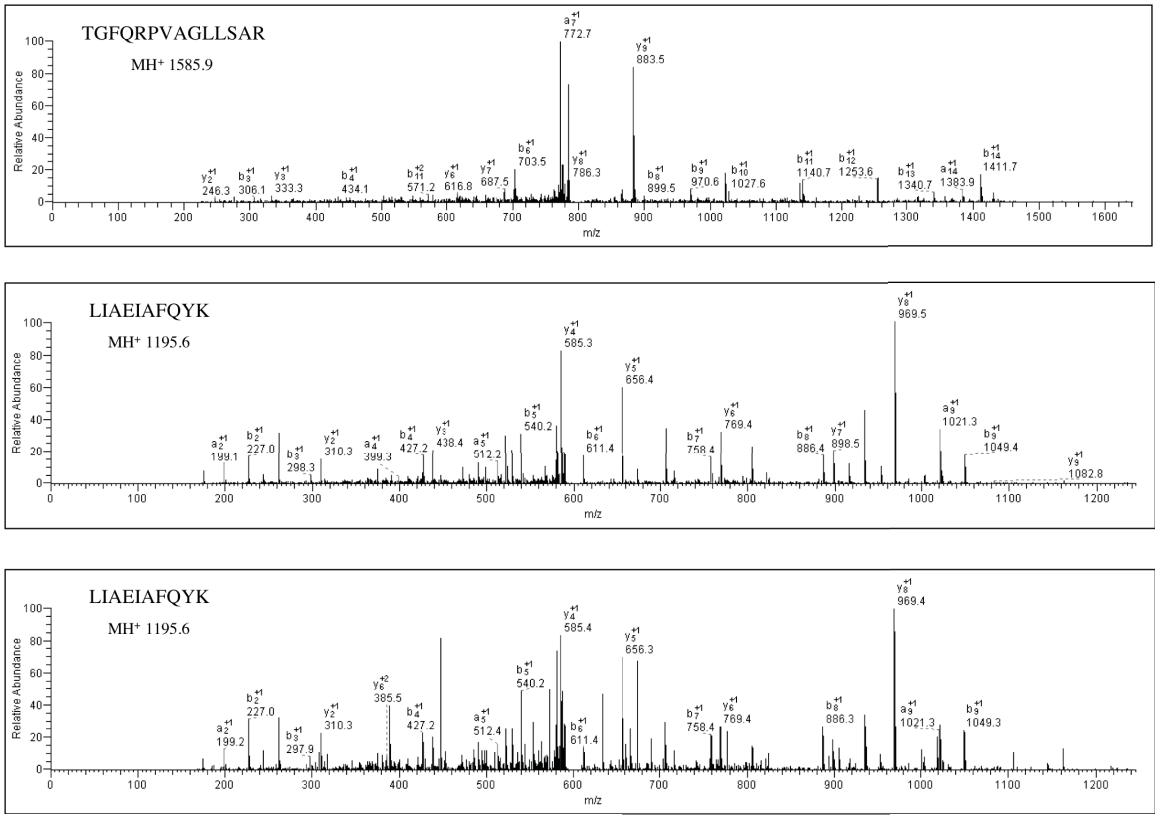
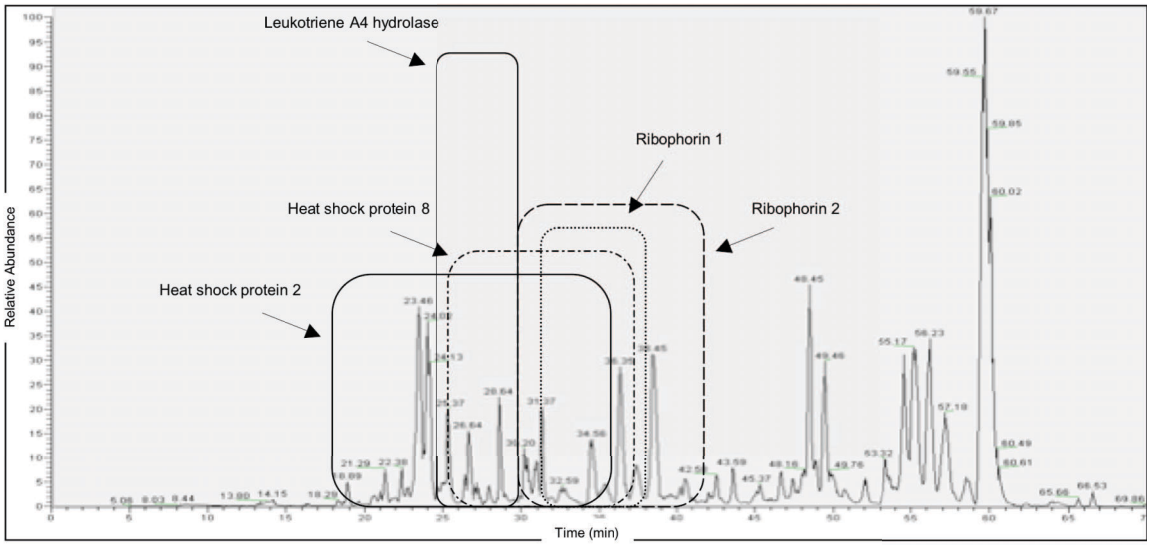


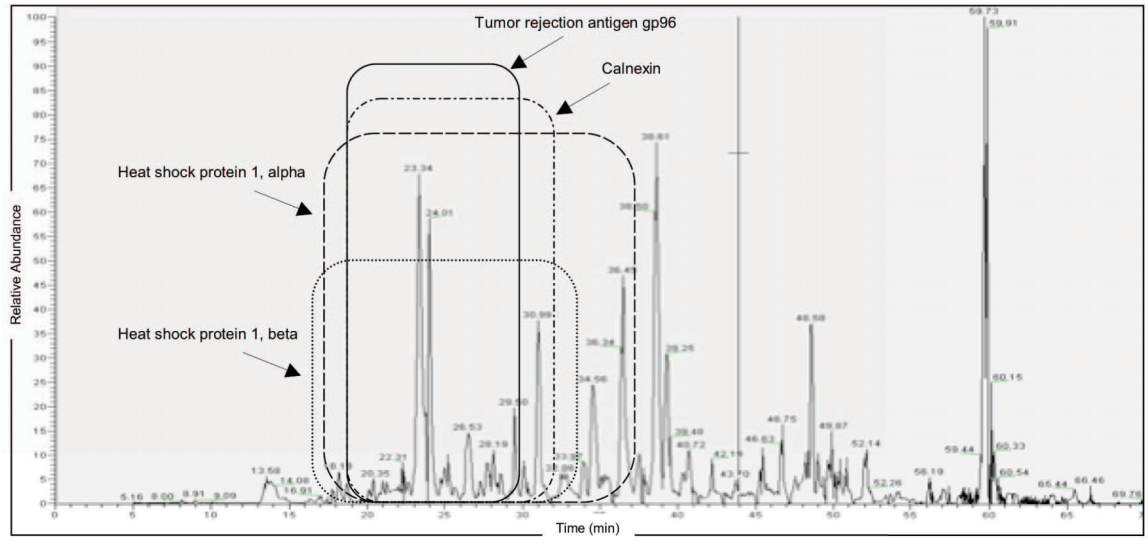
Figure B.3 ITMS product ion spectra of three peptides identified to be from the protein tyrosine hydroxylase by a SEQUEST database search.



Peptide	Molecular Weight (Da)	Time (min)	Peptide Sequence
Heat shock protein 2	69486	29.23	R.IINEPTAAAIAYGLDKK.G
		30.68	R.IINEPTAAAIAYGLDK.K
		22.11	K.STAGDTHLGGEDFDNR.M
		26.32	R.TTPSYVAFTDTER.L
		31.27	R.FEELNADLFR.G
		20.78	K.VEIIANDQGNR.T
		30.27	K.LLQDFFNGK.E
		18.64	K.VQVEYK.G
Heat shock protein 8	70816	36.65	K.SINPDEAVAYGAAVQAAILSGDK.S
		32.54	K.QTQTFTTYSDNQPGVLIQVYEGER.A
		25.47	K.SQIHDIVLVGGSTR.I
		30.10	K.TVTNAVVTVPAYFNDSQR.Q
		30.05	K.TVTNAVVTVPAYFNDSQR.Q
		30.11	K.TVTNAVVTVPAYFNDSQR.Q
		29.92	K.DAGTIAGLNVLR.I
		30.19	K.DAGTIAGLNVLR.I

Peptide	Molecular Weight (Da)	Time (min)	Peptide Sequence
Ribophorin 1	68262	38.84	K.ISIVVETVYTHVLHPYPTQITQSEK.Q
		37.90	K.NLVEQHIQDIVVHYTFNK.V
		23.07	K.VTAEVVLAHPGGGSTAR.A
		34.88	R.FVDHVFDEQVIDSLTVK.I
		33.83	R.ASSFVLALEPELESR.L
		37.89	K.THYIVGYNLPSYEYLYNLGDQYALK.M
		33.93	R.ASSFVLALEPELESR.L
		26.27	K.NIQVDSPYDISR.A
		28.75	K.TILPAAAQDVYYR.D
		20.90 - 20.91	K.GEDEEDNNLEVR.E
		31.86	R.SEDILDYGPFK.D
Ribophorin 2	69018	30.11	R.YHVPVVVVPEGSASDTQEQAILR.L
		41.77	R.NIVEEIEDLVAR.L
		41.85	R.NIVEEIEDLVAR.L
Leukotriene A4 hydrolase	69045	30.58	K.SSALQWLTPEQTSGK.Q
		28.58	K.FSYQSVTTDDWK.S
		25.56	K.LTYTAEVSVPK.E

Figure B.4 HPLC chromatogram, ITMS and SEQUEST database search results from the analysis of the 60 kDa SDS-PAGE protein band. Heat shock proteins, ribophorin and leukotriene A4 hydrolase were identified to be susceptible to DOPAL modification and isolated via an APBA resin from PC6-3 cells treated with 4HNE.



Peptide	Molecular Weight (Da)	Time (min)	Peptide Sequence
Heat shock protein 1, alpha	84761	26.78	K.VILHLKEDQTEYLEER.R
		27.74 - 27.75	R.NPDDITNEEYGEFYK.S
		26.73	K.VILHLKEDQTEYLEER.R
		26.71	K.VILHLKEDQTEYLEER.R
		37.99	K.HSQFIGYPITLFVEK.E
		29.28	R.GVVDSEDLPLNISR.E
		29.32	R.GVVDSEDLPLNISR.E
		28.58	K.SLTNDWEEHLAVK.H
		28.94	K.HLEINPDHSIIETLR.Q
		34.87	K.HLEINPDHSIIETLR.Q
		30.47	R.GVVDSEDLPLNISR.E
		26.60	R.ELISNSSDALDKIR.Y
		26.15	K.ELHINLIPNKQDR.T
		29.72	K.SLTNDWEEHLAVK.H
		29.61	R.GVVDSEDLPLNISR.E
		21.11 - 21.12	K.YIDQEELNK.T
		29.40	R.TLTIVDTGIGMTK.A
		29.53	R.RAPFDLFENR.K
		22.55	K.EGLELPEDEEEKKK.Q
		25.04	K.HIYFITGETK.D
		31.76	K.HFSVEGQLEFR.A
		22.37	K.DQVANSAFVER.L
		21.15	K.YIDQEELNK.T
		29.79	R.TLTIVDTGIGMTK.A
		25.14	K.EGLELPEDEEEK.K
		17.68	K.LGIHEDSQNR.K
		22.80	K.FYEQFSK.N
		28.47	R.ALLFVPR.R
Tumor rejection antigen gp96	74161	28.11	K.GVVDSDDLPLNVSR.E
		29.09	K.SILFVPTSAPR.G
		25.63	K.FAFQAEVNR.M
		27.95	K.LIINSLYK.N
		19.25	K.LGVIEDHSNR.T
		25.28	R.ELISNASDALDK.I

Peptide	Molecular Weight (Da)	Time (min)	Peptide Sequence
Heat shock protein 1, beta	83264	28.17	R.YHTSQSGDEMTSLSEYVSR.M
		18.89	K.IEDVGS*DEEDDSGKDK.K
		17.24	K.IEDVGSDEEDDSGKDKK.K
		33.81	K.LGLGIDEDEVTAEEPSAAVPDEIPPLEGDEDASR.M
		27.40	K.HLEINPDHPIVETLR.Q
		18.14	K.IEDVGSDEEDDSGKDK.K
		27.34	K.HLEINPDHPIVETLR.Q
		33.93	K.LGLGIDEDEVTAEEPSAAVPDEIPPLEGDEDASR.M
		27.22	K.HLEINPDHPIVETLR.Q
		28.25 - 28.26	K.SLTNDWEDHLAVK.H
		33.92	K.LGLGIDEDEVTAEEPSAAVPDEIPPLEGDEDASR.M
		33.06	K.HLEINPDHPIVETLR.Q
		18.78	K.IEDVGSDEEDDSGK.D
		29.12	K.SLTNDWEDHLAVK.H
		17.90	K.IEDVGS*DEEDDSGKDKK.K
		28.08	R.YHTSQSGDEMTSLSEYVSR.M
		18.37	K.LGIHEDSTNR.R
		29.41	R.RAPFDLFENK.K
		18.61	R.EKEIS*DDEAEEEK.G
		18.89	K.IEDVGSDEEDDS*GKDK.K
		23.00	R.RLSELLR.Y
		22.13	K.EQVANSAFVER.V
		23.04	R.RLSELLR.Y
		29.96	R.ALLFIPR.R
		22.35	K.EQVANSAFVER.V
Calnexin	67212	31.23	K.APVPTGEVYFADSFDR.G
		31.16	K.APVPTGEVYFADSFDR.G
		25.56	K.IADPDAVKPDDWDEDAPSK.I
		19.29	K.TDAPQPDVKDEEGKEEEK.N
		18.99	R.KPEDWDERPK.I
		22.49	K.AEEDEIILNR.S
		30.71	R.GSLSGWILSK.A
		30.64	R.GSLSGWILSK.A

Figure B.5 HPLC chromatogram, ITMS and SEQUEST database search results from the analysis of the 76 kDa SDS-PAGE protein band. Heat shock protein 1, tumor rejection antigen gp96, and calnexin were identified to be susceptible to DOPAL modification and isolated via an APBA resin from PC6-3 cells treated with 4HNE.

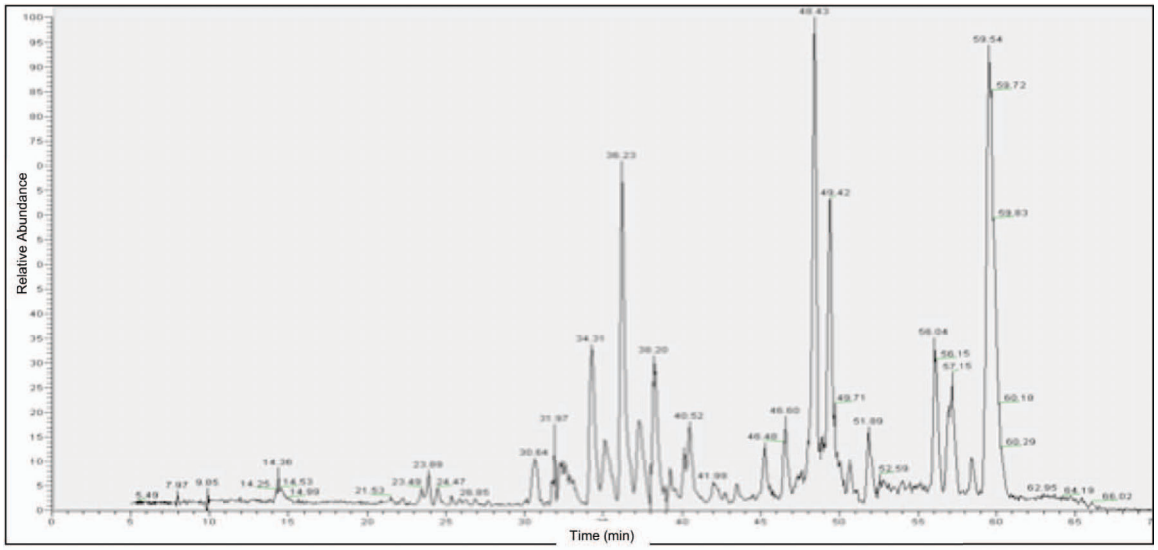


Figure B.6 HPLC chromatogram from the analysis of the SDS-PAGE blank.

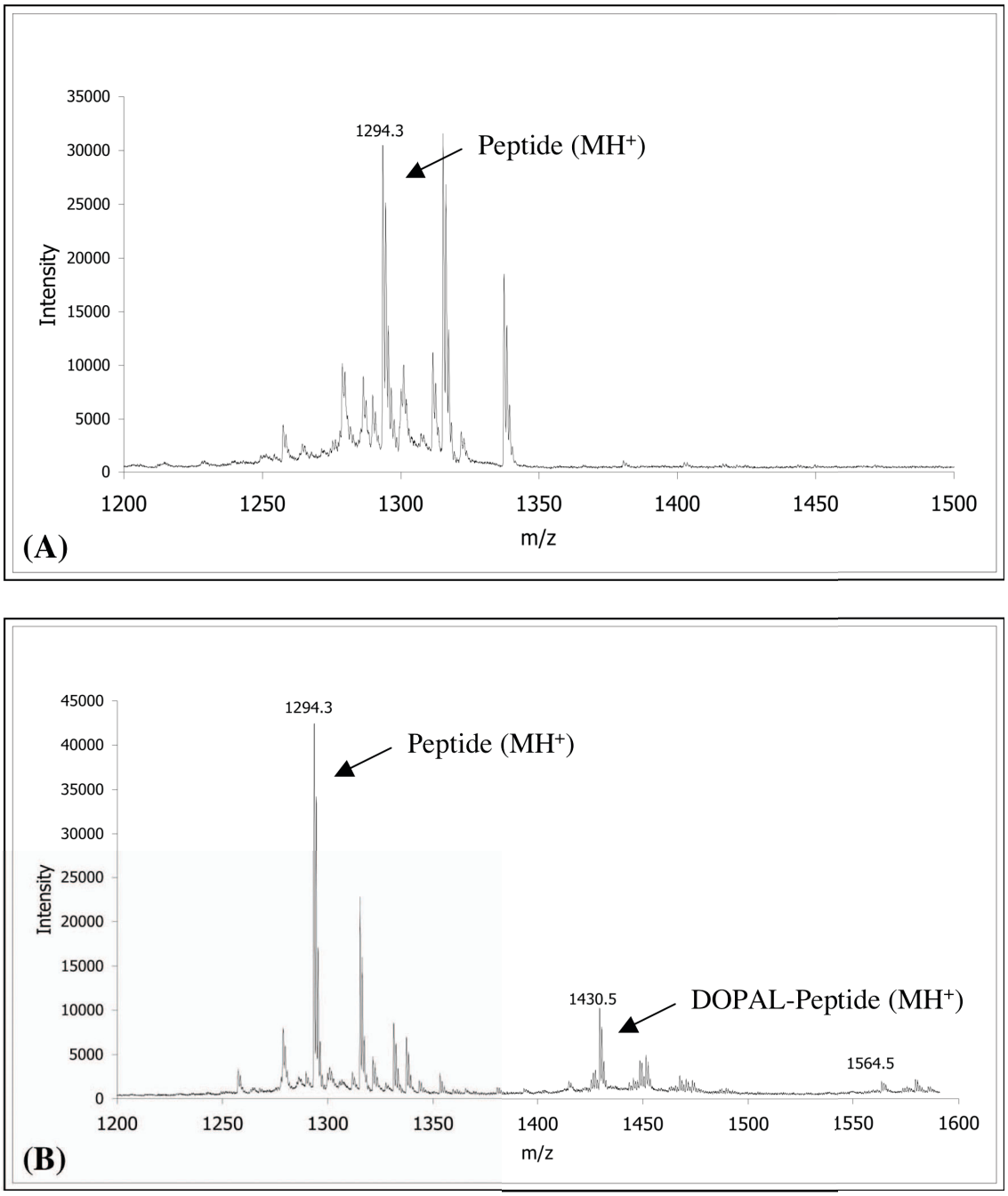


Figure B.7 MALDI-TOF-MS of the peptide Myelin treated with DOPAL demonstrating modification of the peptide by DOPAL; (A) Control and (B) 100 μM DOPAL with 1 mM $NaCNBH_3$.

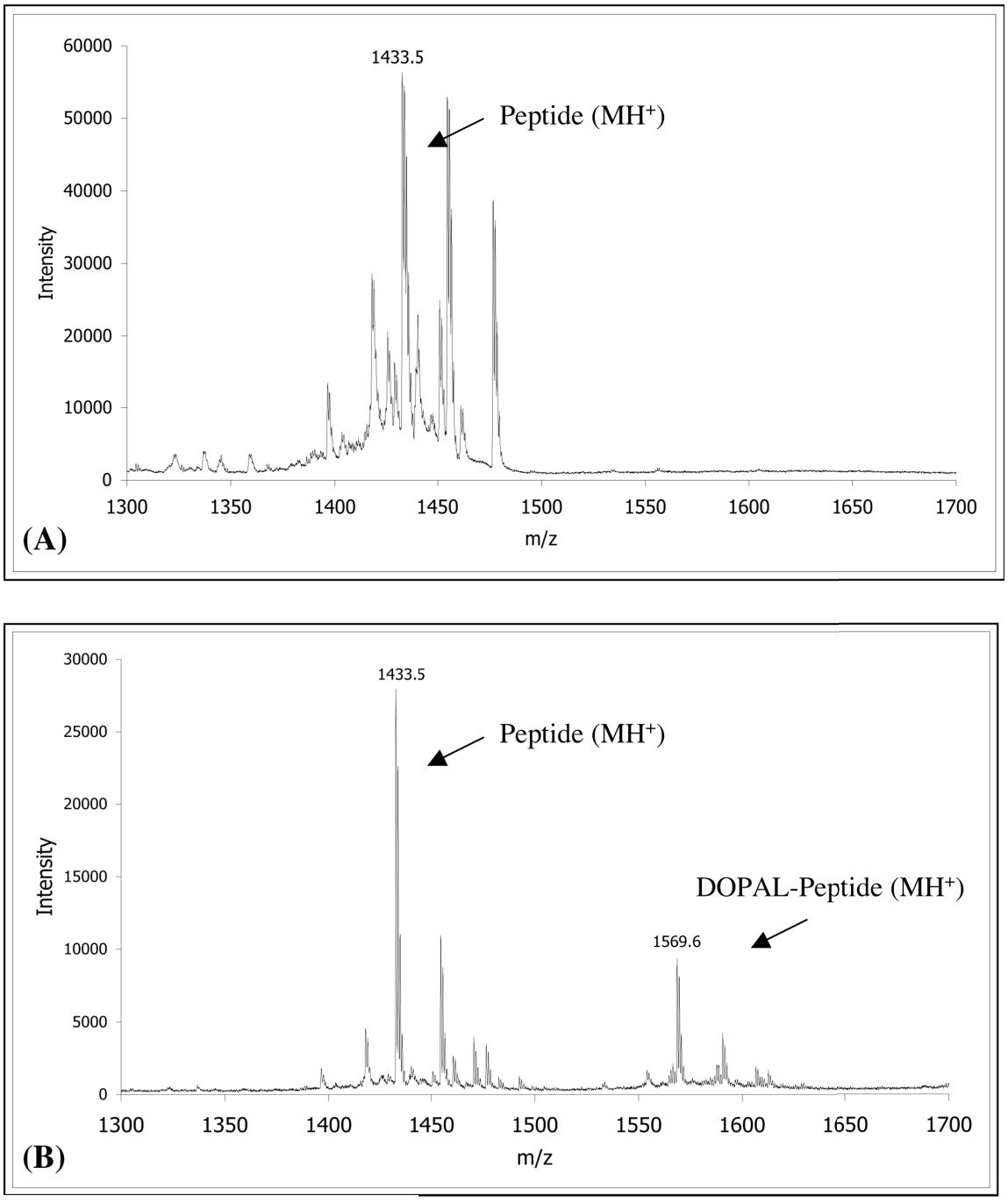


Figure B.8 MALDI-TOF-MS of the peptide MBP treated with DOPAL demonstrating modification of the peptide by DOPAL; (A) Control and (B) 100 μM DOPAL with 1 mM $NaCNBH_3$.

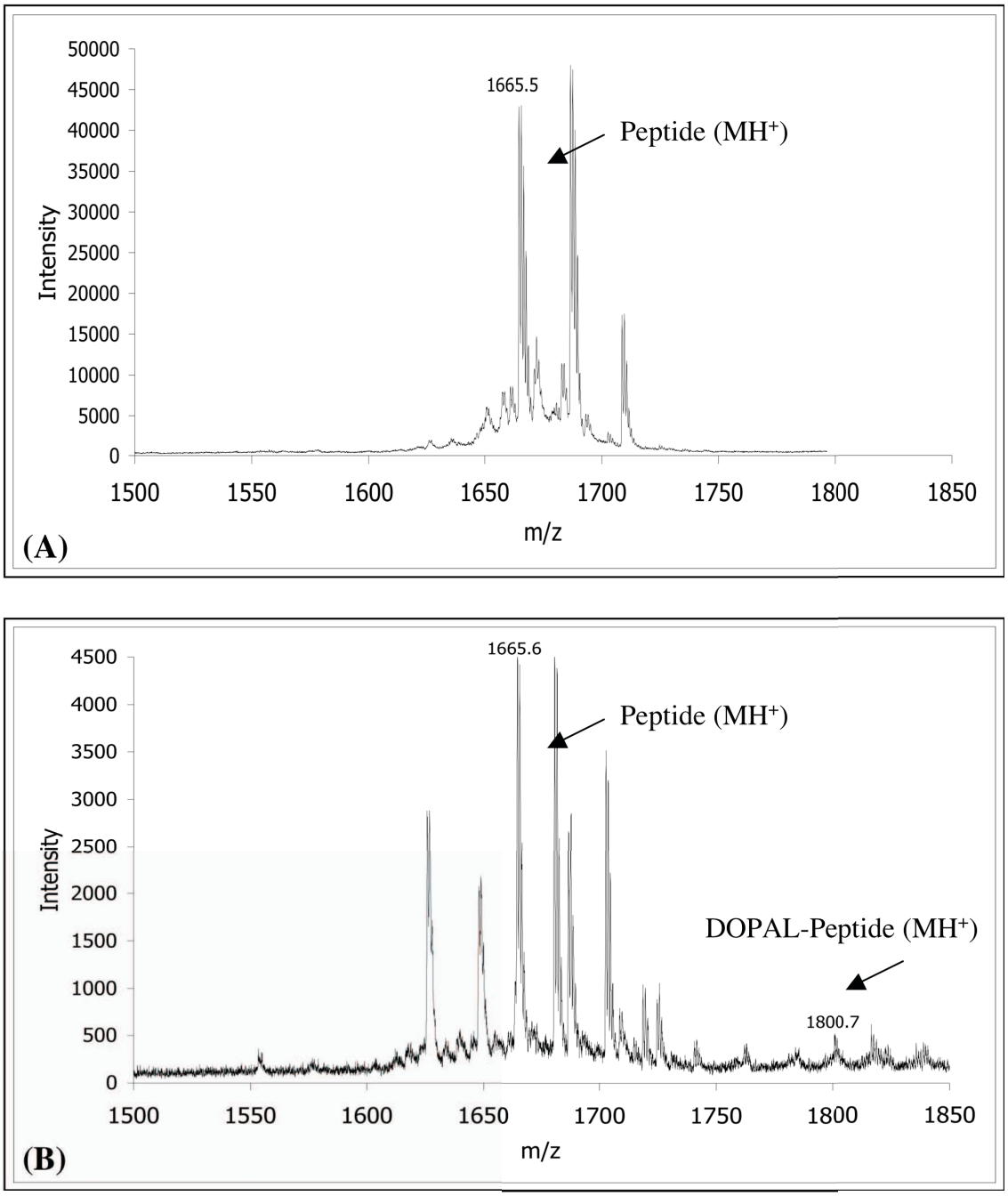


Figure B.9 MALDI-TOF-MS of the peptide MSH treated with DOPAL demonstrating modification of the peptide by DOPAL; (A) Control and (B) 100 μM DOPAL with 1 mM $NaCNBH_3$.

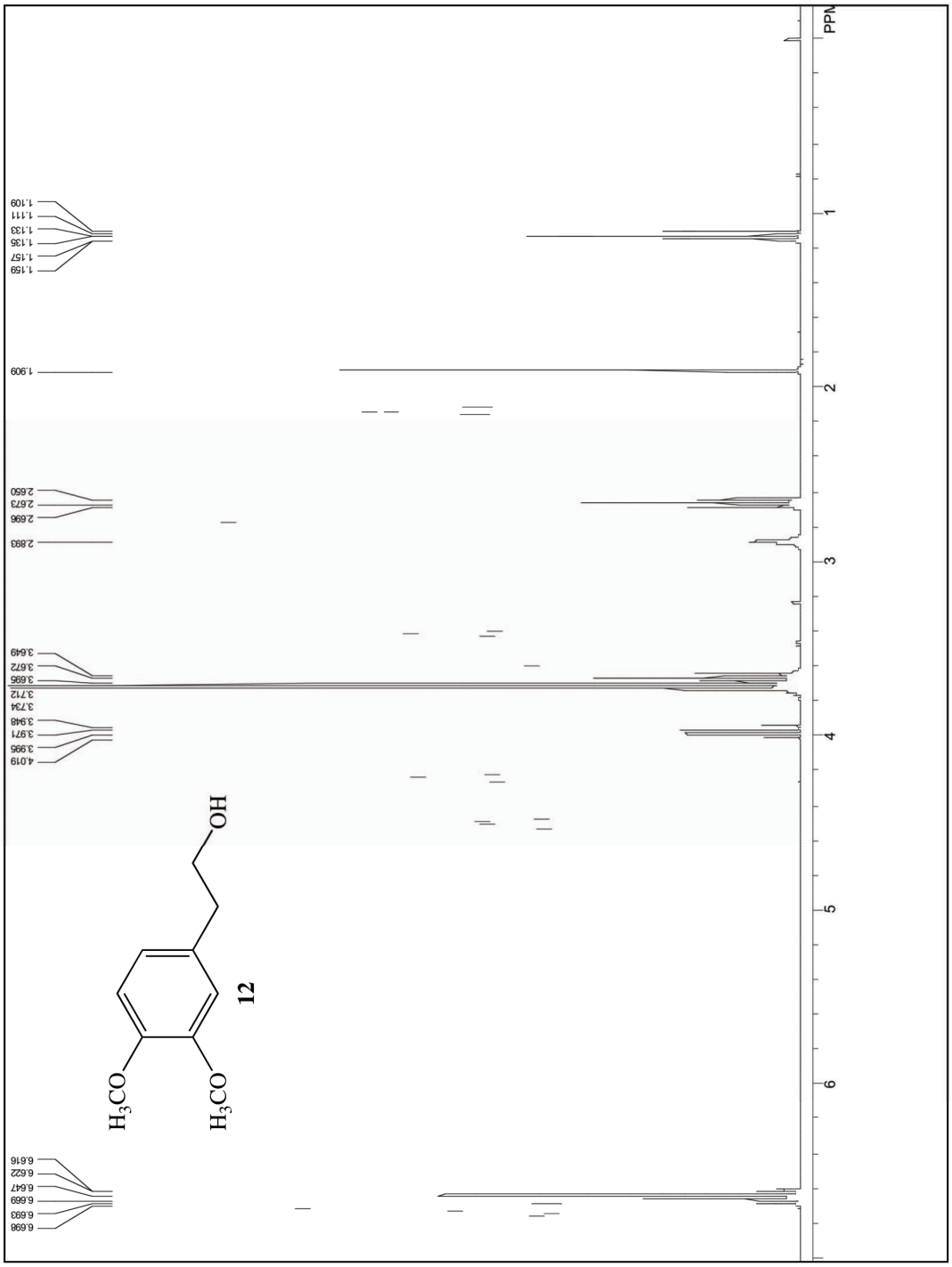


Figure B.10 300 MHz ^{1}H NMR of compound (**12**); 2-(3,4-dimethoxyphenyl)ethanol, ($CDCl_3$).

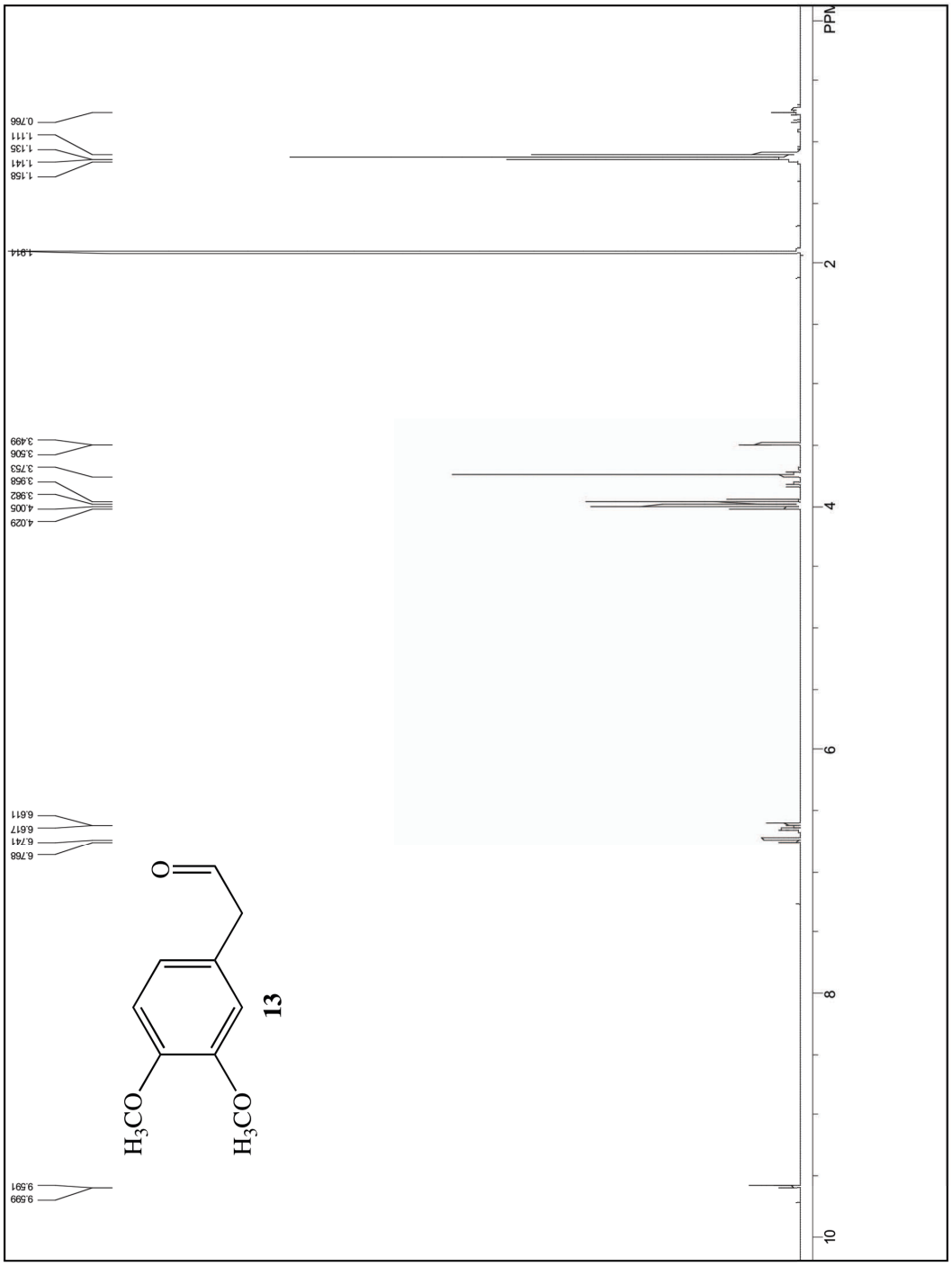


Figure B.11 300 MHz ^{1}H NMR of compound (**13**); 2-(3,4-dimethoxyphenyl)acetaldehyde, ($CDCl_3$).

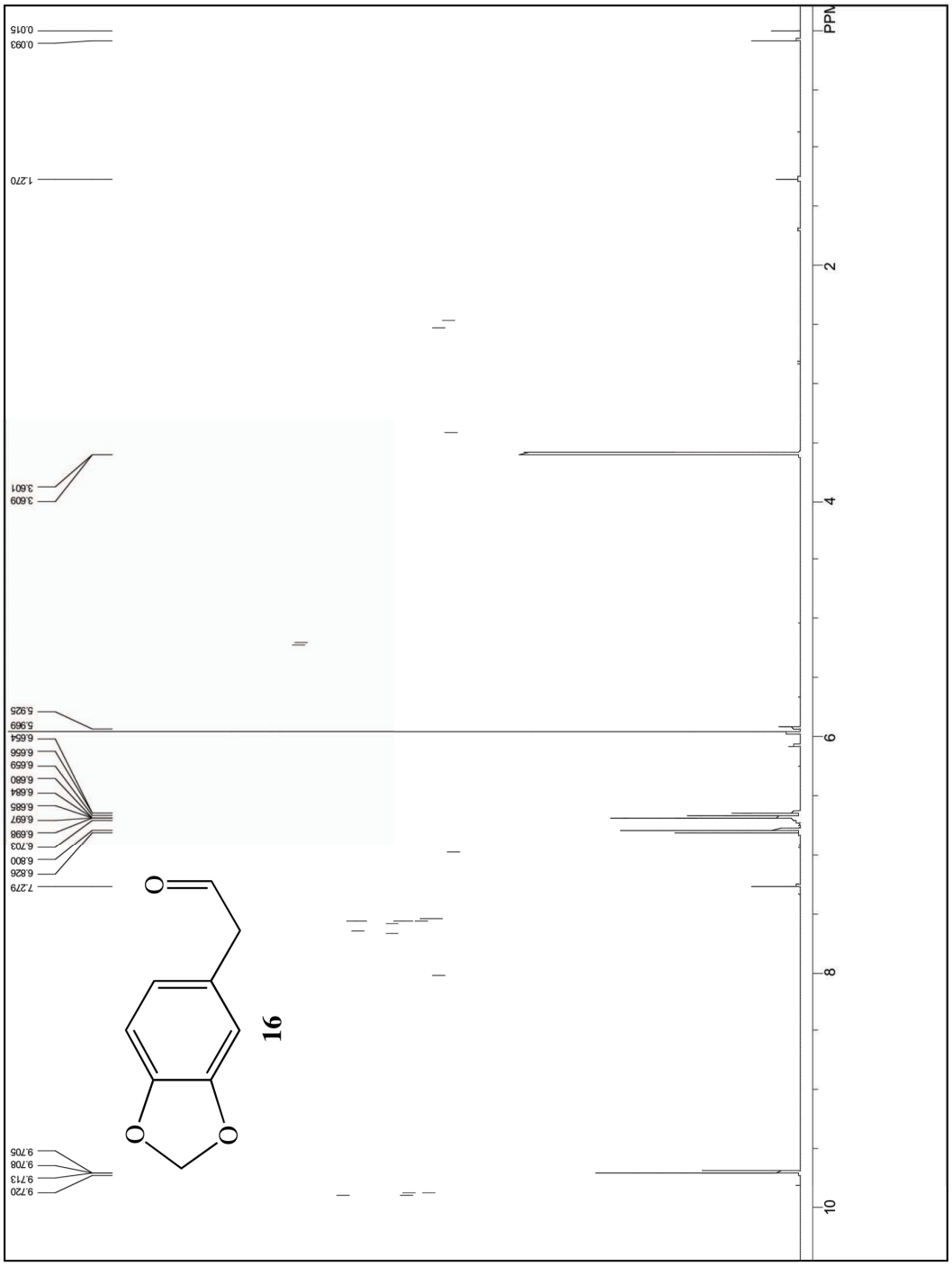


Figure B.12 300 MHz ^{1}H NMR of compound (**16**); 2-(benzo[1,3]dioxol-5-yl)acetaldehyde, ($CDCl_3$).

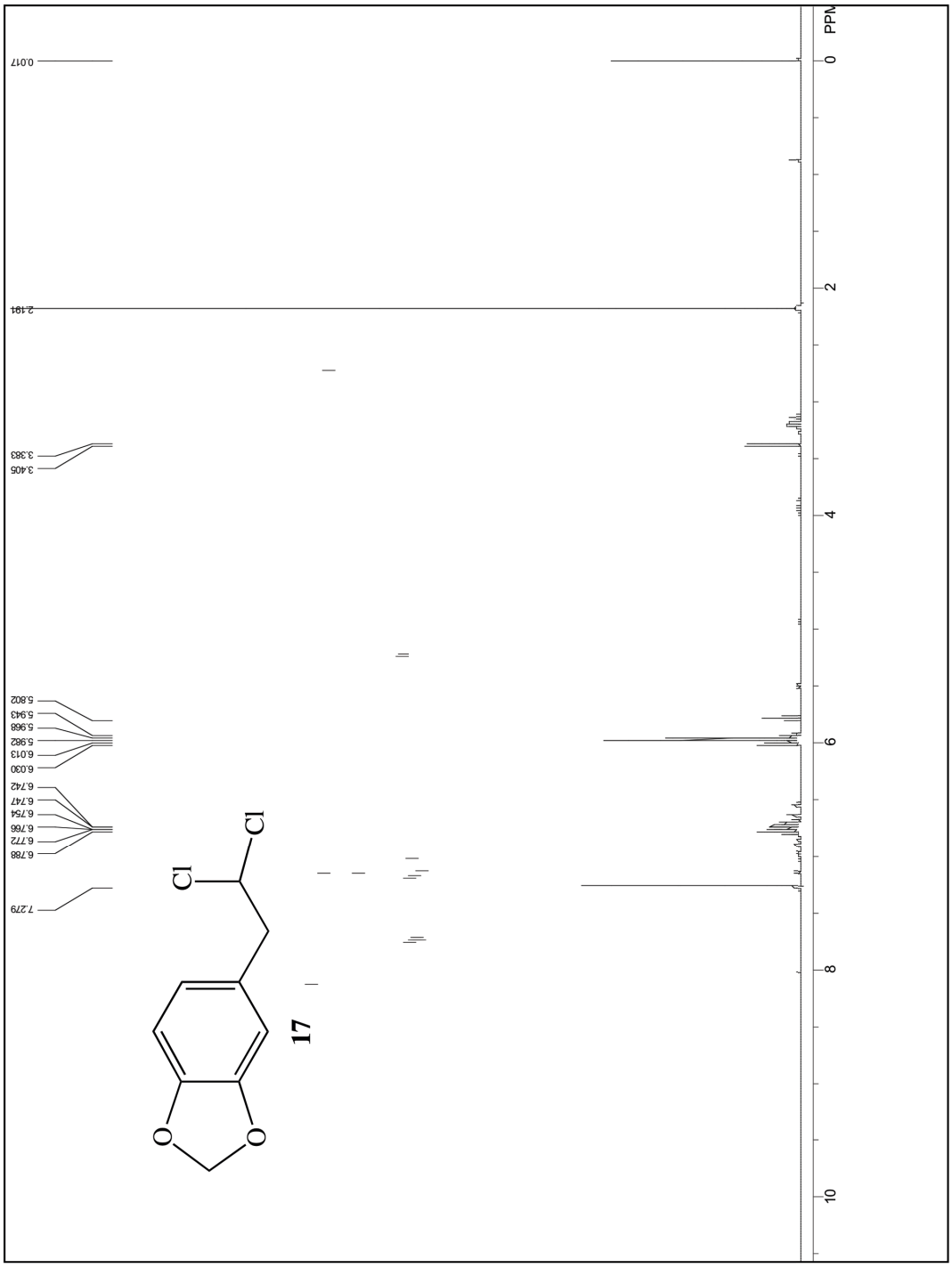


Figure B.13 300 Mhz ^{1}H NMR of compound (**17**); 5-(2,2-dichloroethyl)benzo[1,3]dioxole, ($CDCl_3$).

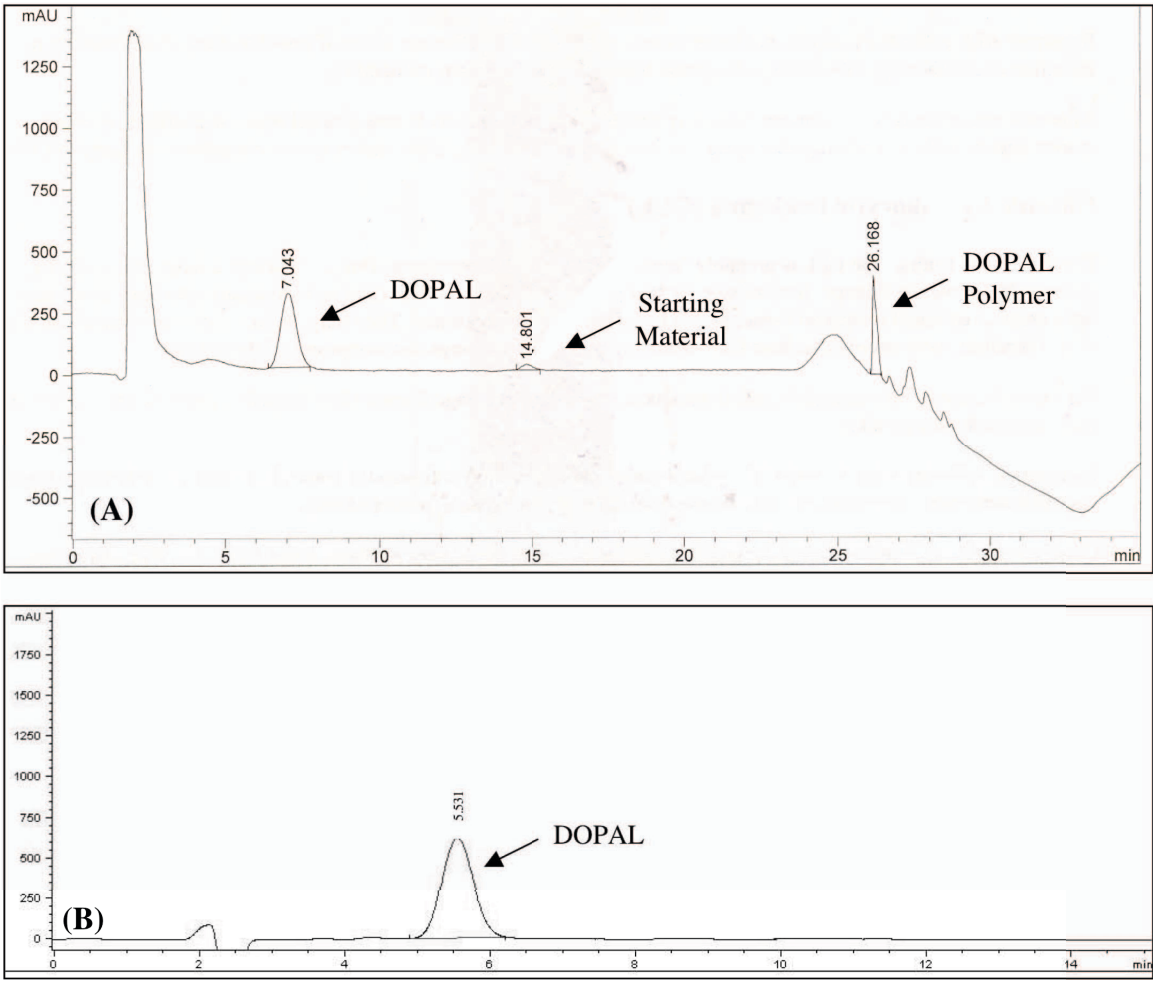


Figure *B.14* HPLC spectra of the aqueous layer from the demethylation reaction with BBr_3 (A) before and (B) after solid phase extraction. (A) Before extraction there are three peaks in the aqueous layer corresponding to DOPAL, starting material and DOPAL-polymer at 7.04, 14.80 and 26.16 min respectively. (B) After extraction only one peak (5.53 min) is present corresponding to DOPAL, compound (**1**).

APPENDIX C

AUTHOR BIOGRAPHY

Jennifer Nicole Rees received her Bachelor of Science degree in Chemistry from Iowa State University (Ames, Iowa). While attending Iowa State, she did undergraduate research studying carbohydrates with Dr. Nicola L. Pohl. Jennifer joined the Division of Medicinal and Natural Products Chemistry at the University of Iowa (Iowa City, Iowa) to pursue her doctorate in August 2004. She subsequently joined the laboratory of Dr. Jonathan A. Doorn as his first graduate student. During the course of her graduate studies, Jennifer published over 20 conference abstracts, four journal articles (including two first author papers) and one book chapter. As a second year graduate student, she was selected to present her research at the 44th Annual MIKI (Minnesota-Illinois-Kansas-Iowa) Medicinal Chemistry Meeting. Jennifer has also presented her work at the University of Iowa 9th Annual James F. Jakobsen Conference in which she received Honorable Mention for the Division of Biological and Health Sciences. Other honors and awards Jennifer has received during her graduate school career include the Society of Toxicology Graduate Student Travel Funds Award, a University of Iowa Student Government Research Grant and several University of Iowa Graduate Student Senate Travel Awards. Furthermore, out of 40 participants, Jennifer received the Usha Balakrishman Prize for Best Poster at the University of Iowa 16th Annual Center for Biocatalysis and Bioprocessing Conference.

In addition to her research achievements, Jennifer was active in the Society of Toxicology and was a member of both the Central States Division and the Neurotoxicology Specialty Section. She held the position of Student Representative for the Central States Division and represented the chapter as a

member of the Regional Chapter Graduate Committee. Jennifer was also an active participant in the annual MIKI Medicinal Chemistry Meetings and was a member of the Planning Committee, holding the position of Fundraising Coordinator, for the 46th Annual MIKI Meeting held in Iowa City.

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