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SYNTHESIS AND CHARACTERIZATION OF A NOVEL VERDAZYL SPIN PROBE

A Thesis

Presented to

The Faculty of the Department of Chemistry

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

Allisa Jayne Clemens

May 2020

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The Designated Thesis Committee Approves the Thesis Titled

SYNTHESIS AND CHARACTERIZATION OF A NOVEL VERDAZYL SPIN PROBE

by

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SAN JOSE STATE UNIVERSITY

May 2020

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ABSTRACT

SYNTHESIS AND CHARACTERIZATION OF NOVEL VERDAZYL SPIN PROBE

By Allisa Jayne Clemens

The use of probe molecules has been integral to the understanding of protein structure and dynamics. Spin labels are commonly used as probes on molecules, such as phospholipids, peptides, proteins, or drugs in vivo. The probes enable the precise and quantitative measuring of the movements of these molecules which has led to the elucidation of function. Nitroxides are the most widely studied and employed spin probes, despite being reduced to hydroxylamines by ascorbic acid in vivo in a matter of minutes. Verdazyls provide comparable applications to nitroxide radicals, with the added benefits of having tunable conformation and hydrophobicity by altering the R1/R5 ligand groups. Verdazyls are defined by their 6-membered ring containing four nitrogen atoms, and are notable for their exceptional stability and high variability in substitution. The radicals are stable in solutions over a wide range of pH, resistant to reduction by ascorbic acid, and can quench the fluorescence of organic dyes. We have synthesized a chloromethyl verdazyl as a possible spin probe for biological molecules, as well as a fluorescence quenching agent. The precursor tetrazane species was definitively identified by NMR spectroscopy, and the novel verdazyl by EPR, UV, and IR spectroscopy. Replacement of chlorine with iodide in the chloromethyl verdazyl demonstrated its ability to undergo S_N2 nucleophilic substitution. The chloromethyl verdazyl can be incorporated onto a peptide as a side chain attached label via an S_N2 nucleophilic substitution reaction.

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Finally, I would like to dedicate my thesis to my Grandmother Alice, who allowed me as a six-year-old to turn her kitchen into my first chemistry lab. She never seemed to grow tired of talking about how proud she was of this work, and unfortunately passed away before she could see me complete it. I could never begin to repay her for her wisdom and unwavering belief in me, and I can only hope to one day emulate her grace, strength and unconditional love. I am honored to be her namesake.

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LIST OF ABBREVIATIONS

5-MSL- 3-maleimido-proxyl
aaRS- aminoacyl transfer ribonucleic acid synthetase enzyme
EPR- electron paramagnetic resonance
DEER- double lectron electron resonance
DMPO- 5,5-dimethyl-1-pyrroline N-oxide
DNA- deoxyribonucleic acid
FRET- fluorescence resonance energy transfer/ Förster resonance energy transfer
mRNA- messenger ribonucleic acid
NAC- non-amyloid- β component
NMR- nuclear magnetic resonance
ROS- reactive oxygen species
SDM- site directed mutagenesis
SOMO- single occupied molecular orbital
tRNA- transfer ribonucleic acid
TTOP- 3,5,5-trimethyl-3-(2,6,6-trimethyl-3-oxopiperazin-2-yl)piperazin-2-one

Chapter 1. Introduction

1.1 Introduction

The use of protein modification to study a protein's structure enables the elucidation of its function.¹ A variety of procedures for protein modification were developed long before there was any understanding of amino acids, or protein structure and function. The use of formaldehyde for both the chemical fixation of leather in the tanning industry and the method of preparation of toxoids from bacterial toxins developed in 1922 by Ramon, which was used to inoculate people against bacteria such as *Clostridium tetani*, was developed entirely on empirical observation.^{2,3} WWII brought about peculiar interest in the use of protein modification as a means to elucidate the mechanism of action of neurotoxins being used as chemical weapons.¹ As our interest in protein function has evolved, so have the methods of chemical modifications to study proteins. There are now a variety of methods for the modification of amino acids, as well as the placement of unnatural amino acids at specific sites within a peptide or protein. In 1965 McConnell et al. presented the concept of employing spin labels to detect local environments in biological systems. Spin labels are radical complexes that can be used to study dynamic function of the complexes to which they are attached.^{1,4,5} McConnell's spin label provided the first evidence of the fluidity of biological membranes.¹

More recently, spin labels were used to analyze the role of α -synuclein in the pathogenesis of Parkinson's disease. Nitroxide spin labels strategically placed within the

α -synuclein protein by site-directed mutagenesis allowed for the use of paramagnetic relaxation enhancement nuclear magnetic resonance (NMR) spectroscopy to examine the intermolecular interactions of this intrinsically disordered protein. By employing ensemble molecular dynamics simulations, Dobson *et al.* were able to render these interactions into a high-resolution topological model of the protein.⁶ It was hypothesized that amyloid fibrils are caused by aggregation of the hydrophobic non-amyloid- β component (NAC) region of the protein. The model was able to demonstrate that the native structure shields the exposed region of the protein with a highly charged C-terminus, and that reducing or removing the shielding of the C-terminal region, either by shortening the C-terminus or exposing it to Ca^{2+} ions, heavy metals, and polyamines which can bind to the C-terminus, greatly accelerates aggregation. This finding is consistent with the idea that the aggregation of α -synuclein is inhibited by the existence of long-range interactions within the native structure. The technique gives some insight to the role that long-range interactions play in the formation of amyloid fibrils.⁶

1.2 Background and History of Free Radicals

A spin label is a radical compound. A radical is a molecule with a single unpaired electron. When a radical reacts, it can act as an electron donor, giving away its unpaired electron, or it can act as an electron acceptor, filling the orbital of the unpaired electron. The variability of free radicals makes them valuable in a variety of applications.⁷

Radicals play an important role in sustaining combustion reactions. Controlling the concentration of radicals within a combustion reaction by using radical scavengers or

increasing the formation of radicals can be a useful tool for controlling the rate of burn or extinguishing a flame. By changing the water concentration from 0.1% to 0.2% of a carbon-monoxide-oxygen flame the speed can be doubled, since the water provides a source of hydroxyl and hydrogen radicals.⁸ See Figure 1.

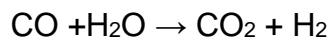
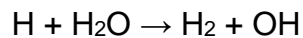
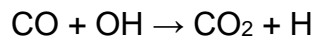


Figure 1. Formation and function of hydrogen and hydroxyl radicals in a carbon monoxide-oxygen flame.

Halogenated hydrocarbons, such as carbon tetrachloride, can be employed as chemical fire extinguishers. Substitution reactions of the halide leaving group with the radical products inhibits propagation of the chain reactions, since, relative to an oxygen, hydroxyl, or hydrogen radical, chlorine or bromine atom radicals are relatively stable and unreactive.⁸

Radical polymerization reactions are an invaluable method of making polymers. Radical polymerization begins with a two-step initiation, during which one or two radicals ($\text{R}\cdot$) are formed from the initiating molecule (RX), forming an active center which then reacts with a monomer forming a larger radical. The chain reaction is propagated until all the monomers are used up, or until termination occurs through various possible side reactions with the radical.⁹ See Figure 2.

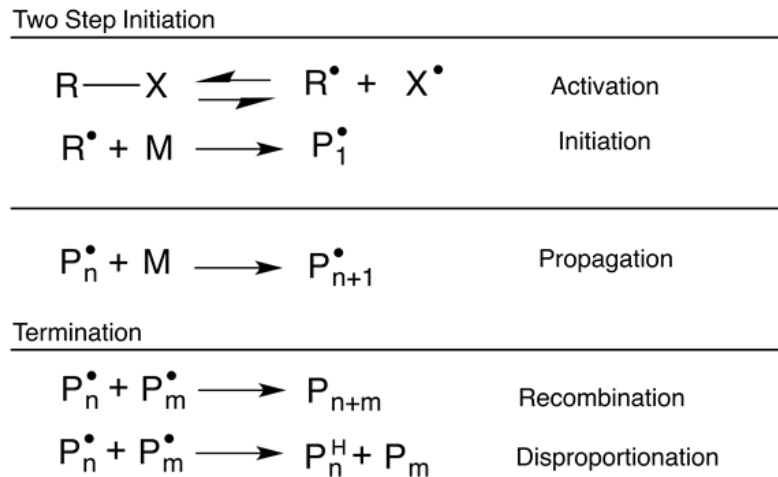


Figure 2. Radical Polymerization. In this figure RX is the initiating molecule, M is the Monomer, and P_1^\bullet is the Active center.

Radicals are intrinsic in many metabolic reactions, serving a role in normal physiological functions and abnormal pathological functions. The formation of reactive oxygen species during oxidative phosphorylation is an example of the “Oxygen Paradox.” Molecular oxygen, being a strong oxidant, makes for an ideal terminal electron acceptor, and essential for aerobic respiration, but is also equivalently involved in the pathogenesis of numerous diseases and degenerative states caused by reactive oxygen species (ROS).¹⁰

Side reactions of the mitochondrial electron transport chain, caused by electrons following an unintended pathway of reacting with molecular oxygen directly, generate reactive oxygen species (ROS), including superoxide ion ($O_2^{\bullet-}$), peroxide (H_2O_2) and hydroxyl radical (OH^\bullet). About 1-2% of the overall oxygen consumption *in vitro* is converted into superoxide anion radicals.¹¹ Superoxide forms when molecular oxygen gains a single electron. The superoxide anion radical can then react with hydronium to

dismutate and form hydrogen peroxide (H_2O_2), which can react further to generate the hydroxyl radical ($\text{HO}\cdot$) See Figure 3. Due to the contiguity of the formation of these highly reactive compounds, mitochondrial deoxyribonucleic acid (DNA) and proteins are particularly susceptible to denaturation by ROS. For example, hydroxyl radicals can reduce disulfide bonds in proteins, disrupting the tertiary structure of the protein. The hydroxyl radical can also oxidize DNA by reacting with guanine to produce 8-hydroxy-2'-deoxyguanosine. In an effort to maintain homeostasis against attack by ROS, biological systems deploy free radical scavengers, which act as antioxidants to hinder or halt the (often chain) reactions of ROS.¹²

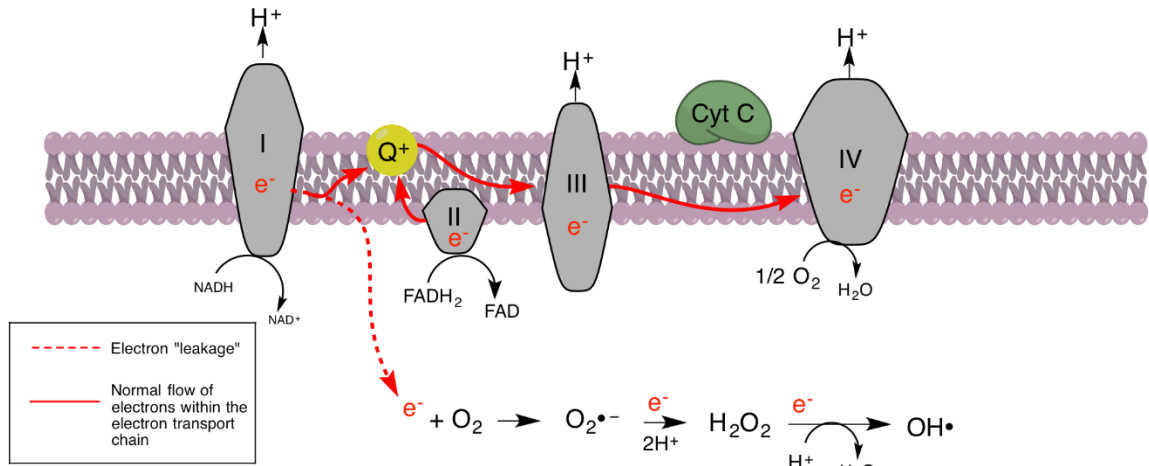


Figure 3. Side reactions of the mitochondrial electron transport chain. Reduction pathway of molecular oxygen by the electron transport chain and by side reactions to form a hydroxyl radical.

Despite the unique variability in function the existence of radicals remained disputed by the scientific community until the early 1930s.⁷ Scientists hypothesized that there could be a free radical containing carbon, but after many failed attempts to isolate the

radical, the consensus view was that carbon can only be tetravalent. The first free radical wasn't discovered until 1900 with Moses Gomberg and the synthesis of trimethylphenyl. Gomberg was trying to synthesize hexaphenylethane, in a reaction of triphenylchloromethane with zinc under atmospheric CO₂. When he inadvertently synthesized triphenylmethyl, which was a highly reactive, unstable substance, Gomberg knew he had not made hexaphenylethane because it reacted with iodine and atmospheric oxygen, which was not expected to occur. He recognized it as a free radical and published his findings in 1900: "An Instance of Trivalent Carbon: Triphenylmethyl".¹³ Despite Gomberg's publication, the existence of the persistent radical, triphenylmethyl, and other organic free radicals remained in dispute for many decades before being accepted by mainstream organic chemistry.¹⁴

The discovery of electron paramagnetic resonance (EPR) in 1944, by Zavoisky enabled the direct observation of radical compounds.¹⁵ The technique is similar to nuclear magnetic resonance spectroscopy, with the difference that it focuses on the interaction of an external magnetic field with the unpaired electron or electrons in a molecule, instead of the nuclei of individual atoms in a molecule. EPR is complimentary to NMR and fluorescence labeling. Electron spin resonance works on a timescale of nanoseconds, while nuclear magnetic resonance works of a timescale of microseconds.

Many of the benefits of using a spin probe, particularly for molecules in solution, are owed to the increased sensitivity of electron spin resonance spectroscopy over other types of resonance detection, such as nuclear magnetic resonance.⁴ The faster time-scale of

electron spin resonance facilitates greater temporal resolution of molecular motions. The use of EPR spectroscopy also enables the measuring of spin-spin distances longer than direct nuclear magnetic resonance measurements and shorter distances than that which fluorescence resonance energy transfer is useful.^{4, 16}

1.3 Radical Stability

Free radicals vary in reactivity. Typical descriptors include “highly reactive”, “persistent”, or “stable”. Highly reactive radicals lack the stabilization and steric hindrance needed to resist reaction. Persistent radicals generally exhibit more steric hindrance surrounding the unpaired electron, essentially shielding the radical from reacting. Stable radical compounds are able to resist chemical reaction by resonance stabilization, steric hindrance, or both.

Stability of an organic radical is determined by the geometry and the substituents of the compound. Generally, the more conjugated a compound, the more stable the radical electron, since the single electron is delocalized over a greater area. However, the difference in stability between di-tert-butyl nitroxide and diphenyl nitroxide makes a good example of how delocalization does not always equate to greater stability, as the resonance of the phenyl groups makes the unpaired electron “more available” to react. See Figure 4.

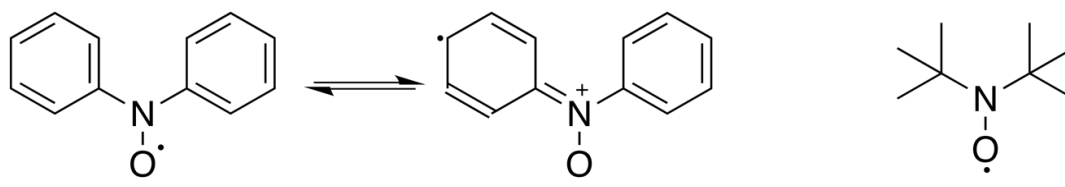


Figure 4. Resonance Structure of diphenyl nitroxide and di-tert-butyl nitroxide

Steric hindrance can also contribute to radical stabilization by hindering access of the radical to potential reactants. Alkyl or aryl substituents are often used to increase the radical electron stability through delocalization or steric hindrance, as a result, most stable organic radicals are relatively nonpolar.¹⁷ See Figure 5.

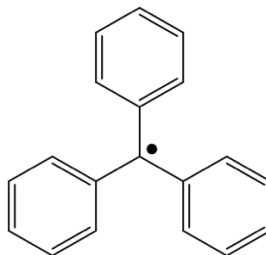


Figure 5. Triphenylmethyl, the first identified radical. Synthesized by Moses Gomberg in 1900: “An Instance of Trivalent Carbon: Triphenylmethyl” is an example of persistent radical. The steric crowding by the phenyl ligands provides protection for the radical electron resulting in the compound being resistant to chemical reaction.

The captodative effect can also influence radical stability by enhancing delocalization. “Capto” meaning to catch or borrow, refers to a group on the compound which stabilizes the single electron by withdrawing electron density, and “dative” which means to give, refers to a group on the compound that alleviates the electron deficiency of the single electron by sharing its electron density.¹⁸ Delocalization has a stabilizing effect as it spreads the electron density over a greater area and helps to satisfy the

molecules valence orbitals, which can lower the potential energy of the compound and making it less reactive.

An example of the captodative effect can be seen 3,5,5-Trimethyl-3-(2,6,6-trimethyl-3-oxopiperazin-2-yl)piperazin-2-one (TTOP). The homolytic bond dissociation of TTOP is favorable due to the synergistic captodative substitution pattern of the compound.¹⁷ See Figure 6.

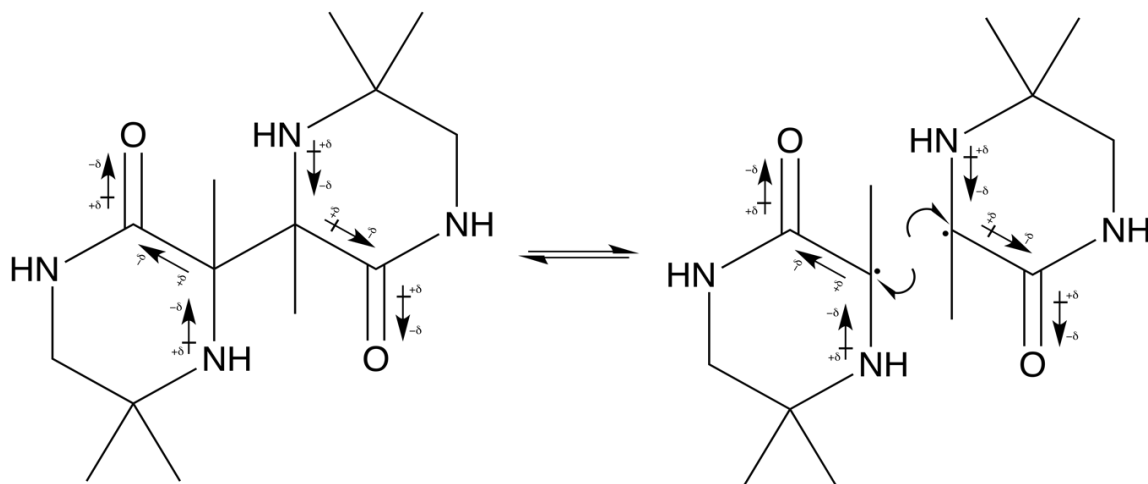


Figure 6. The captodative substitution pattern of the compound resulting in the homolytic bond dissociation of 3,5,5-Trimethyl-3-(2,6,6-trimethyl-3-oxopiperazin-2-yl)piperazin-2-one into a radical compound.

1.3.1 Reactive Radicals

The hydroxyl radical is an example of a highly reactive radical. The lack of steric hindrance, resonance stabilization, and delocalization of the unpaired electron make reactions with the hydroxyl radical extremely favorable.¹² See Figure 7.

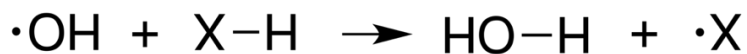


Figure 7. Substitution reaction of a hydroxyl radical and a hydrogen halide to form a more stable radical and water.

1.3.2 Persistent Radicals

Persistent radicals usually resist reaction through steric shielding, making them less likely to react. The increased stability of these compounds makes persistent radicals useful in spin trapping, in which the persistent radical is produced by a reactive radical forming a covalent bond with the spin trap, reducing the spin trap to form a more stable radical adduct. For example, in Figure 8. The nitron spin trap 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) forms a covalent bond with the reactive radical ($R\cdot$), the product of this reaction is a more persistent radical. This analytical technique facilitates the detection of radicals that cannot persist in a radical state long enough to detect by EPR.

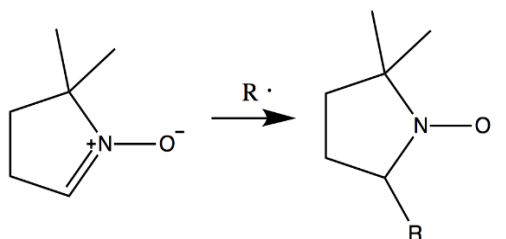


Figure 8. Spin-trapping involving the addition of radical to a nitron spin trap resulting in a spin adduct of a nitroxide based persistent radical.

1.3.3 Stable Radicals

1.3.3.1 Nitroxides

Nitroxides are the most widely studied and employed stable radicals.⁴ The stability of the nitroxide radical is due in part to the non-bonding electrons on the nitrogen atom, which form a partial bond between nitrogen and oxygen.

The TEMPO radical is a type of nitroxide radical. The stability of the TEMPO radical is produced by the resonance provided by the nitroxide functional group, as well as the

methyl groups adjacent to the nitroxyl group which provide steric shielding of the radical.

See Figure 9.

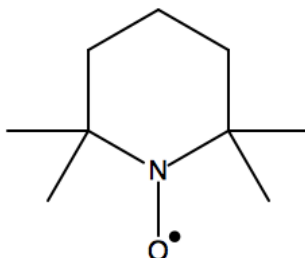


Figure 9. Structure of the TEMPO stable radical.

1.3.3.2 Verdazyls

Verdazyls are another type of stable radical. Verdazyls are defined by their 6-membered ring containing four nitrogen atoms. See Figure 10. Verdazyls are notable for their exceptional stability and high variability in substitution. Verdazyls are generally more resistant to reduction than nitroxides.¹⁹ The first verdazyl radical was synthesized in 1963, by Kuhn and Trischmann.²⁰ Methylation of triphenylformazan resulted in a cyclic compound that could be oxidized by atmospheric oxygen to form the verdazyl radical. It was found that the cyclic radicals could remain stable even after being treated with mineral acid and boiled with concentrated acetic acid or sodium methoxide for long periods of time. The compounds EPR spectra contained nine lines, indicating the ring contained four equivalent nitrogen atoms.

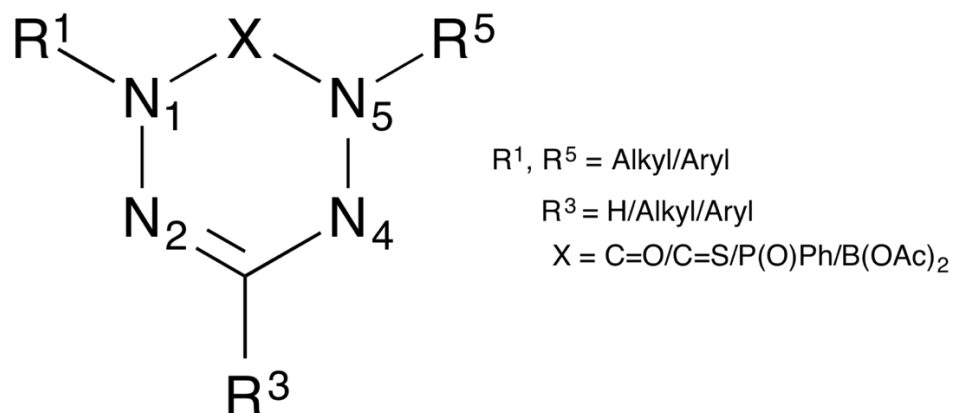
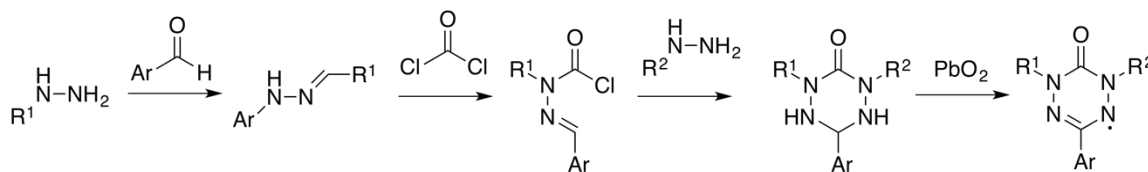


Figure 10. Verdazyl Structure.

Neugebauer and Fischer reported the first synthesis of an oxoverdazyl [$X = \text{C=O}$] in 1980.²¹ Reaction between an alkyl hydrazine and phosgene result in a bis-hydrazide. Condensation of the resultant bis-hydrazine with an aldehyde yielded a tetrahydrotetrazane. Oxidation of the tetrazane with potassium ferricyanide, silver oxide, or lead oxide gave the stable oxoverdazyl.²¹ See Figure 10, $X = \text{C=O}$.

Both of these synthesis methods yield a compound with a mirror plane across the C(3)C(6) plane; limiting the variability of the verdazyl. In 1994, Milcent and Barbier resolved the symmetry restraint with their method for the synthesis of an asymmetric verdazyl.²² See Scheme 1.

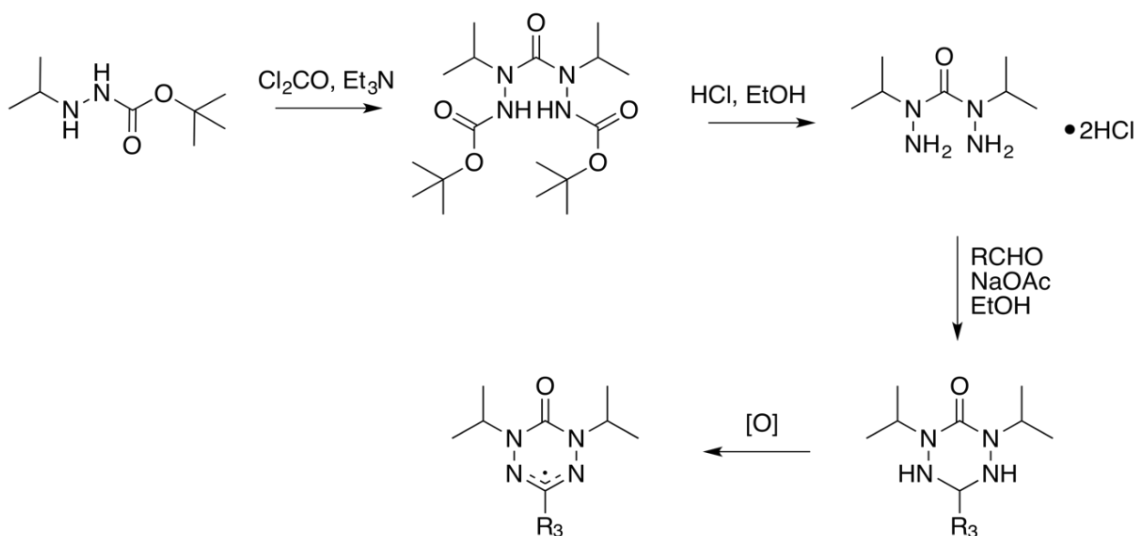


Scheme 1: Asymmetric synthetic of a verdazyl radical.

A synthesis for a verdazyl radical was published by Brook et al. in 2005, introducing another variability of the R^1 and R^5 ligand groups. Synthesis begins with the reaction of

a BOC protected isopropyl hydrazine with phosgene to form a bis-hydrazide.

Condensation of the bis-hydrazide with an aldehyde attaches the R3 group to form a tetrazane. Oxidation of the tetrazane gives the verdazyl radical.²³ See Scheme 2.



Scheme 2: Synthesis of a diisopropyl verdazyl radical.

Synthetically, the R3 position is the easiest site to vary, since it is the final group to be added to the compound, and the addition of the corresponding R3 aldehyde group facilitates the ring closure. This position on the ring is also the site of a node in the single occupied molecular orbital (SOMO) in which the radical electron resides. See Figure 11. A node is a site in which the wave function of the electron is equal to zero, giving zero probability of finding an electron, and as a result that position does not have a great role in the stability of the radical electron.²³ So theoretically, the substituent at the R3 position can be altered without altering the stability of the radical greatly.

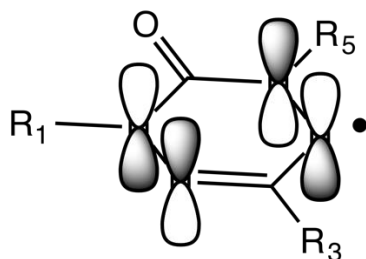


Figure 11. Lewis structural approximation of the SOMO orbitals of the radical verdazyl. Note that this SOMO has a node at the carbon corresponding to the attachment of R3.

1.4 Radicals as Spin labels

Spin labels are a type of stable radical. The unpaired electron of the spin label provides a unique electronic paramagnetic resonance signal. The unique EPR signal of the spin label will vary depending on the nuclear spin of the molecules in the compound that contribute to the single electron stability.⁴ The unique EPR signal enables the label to act as reporter groups in EPR spectroscopy. As the unpaired electron in the stable free radical interacts with its immediate environment, the unique EPR signal is influenced and can provide information about the motion, distance, and orientation of the compound to which it is attached. Many spin labels are composed of a nitroxide moiety, but there are quite a few other types, and they vary in stability and structure.^{1, 4, 16}

1.5 EPR Interpretation of Spin Label Attached Proteins

The EPR spectra of the label attached to a protein will be influenced by the rotational motion on the label relative to the peptide backbone, rotation of the label at the α -carbon backbone, and the rotational diffusion/motion of the protein.⁴ Depending on what the label is being used to examine, experimental conditions can help to isolate these variables. Addition of a solute, such as 30% sucrose or glycerol, increases viscosity and

acts to reduce the rotational diffusion of the peptide/protein.⁵ Effects caused by rotation at the α -carbon can be mitigated by sterically hindering the flexibility of the side chain, essentially immobilizing the labels conformation on the peptide backbone.^{1,4} See Figure 12.

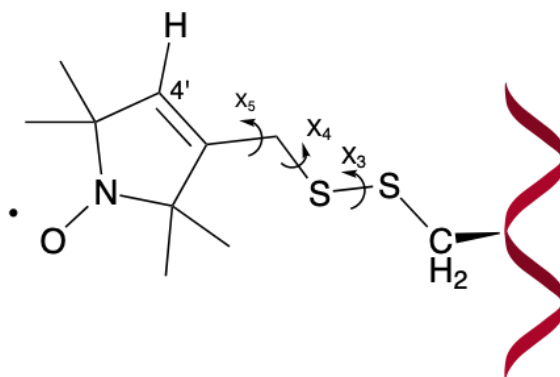


Figure 12. Nitroxide spin label attached to a cysteine side chain showing rotation about the X₃, X₄, and X₅ bonds; addition of a bulky constituent at the 4' position can act to sterically hinder rotation about the axis.

1.6 Spin Label Incorporation to Peptides and Proteins

Attachment of the spin labels on peptides and proteins can be achieved in several ways. Spin label incorporation by chemical attachment to a native moiety, such as a nucleophilic side chain, C-terminus, or N-terminus, on the peptide or protein can be done.⁴ The method is relatively straightforward, but it is constrained by the availability and placement of the native moieties accessible for attachment. The native protein structure may have more than one binding site for the label to attach, resulting in more than one label attaching to a single compound, or it may have none.^{1,4}

Site directed mutagenesis (SDM) allows for the placement of the label at a specific site on along the peptide sequence. The SDM method requires the modification of a native amino acid in the protein. Modification can be done *in vitro* or *in vivo*.^{1, 4}

For example, Zioudrou developed a method for the chemical conversion of serine to cysteine on a protein *in vitro*, providing the earliest example of site-directed protein mutagenesis, and additional available sites for chemical attachment of labels.²⁴ Chemical attachment is usually facilitated by a nucleophilic side chain; the thiol functional group in cysteine is commonly used, as well as the amine functional group on lysine, as both can act as a nucleophile in an S_N2 nucleophilic substitution.^{1, 4} See Figure 13.

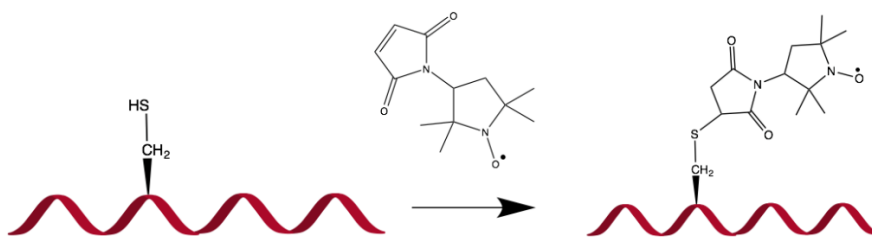


Figure 13. Chemical attachment of a spin label 3-maleimido-proxyl (5-MSL) to a cysteine side chain.

Chemical attachment is limited to the accessible amino acid side chains on the exterior of the protein. The label must come into contact with the binding moiety, so the side chain site must be on the exterior of the protein or peptide as dictated by the tertiary structure of the folded protein.⁴ Additionally, care should be taken that the chemical attachment does not disrupt or distort the native structure of the protein such that it does not function properly. For example, placing a nonnative cysteine proximally to any native cysteine risks forming mutagenic, nonnative disulfide bonds.^{4, 16} Disrupting the tertiary

structure of the protein may result in the undesirable effect of altering the function of the protein. Chemical treatment also poses a risk of disrupting any native bonds of the protein by the addition of the label.^{4, 16}

Both of these methods bind the label to an amino acid side chain. As a result, the EPR spectra will be influenced by the flexibility of the side chain to which it is attached, as well as the flexibility of the label molecule itself. The possible variability in position of the label on the protein must be taken into account when using the spin label to examine the peptide backbone and protein conformation.⁴

The nonnative amino acid can also be incorporated into the protein or peptide by site directed mutagenesis and nonsense suppression *in vivo* to create a binding site at a specific location on a protein.^{1, 4} The nonnative amino acid can be a natural occurring amino acid or an unnatural amino acid.

The site directed mutagenesis technique modifies the mRNA sequence coding for a protein of interest and employs the cells native transcription and translation systems to place the desired nonnative amino acid at a specific site of a single protein within a cell. The mutation is facilitated by exchanging the native codon within the mRNA for a codon of the desired amino acid. The tRNA is altered so that its anticodon specifically recognizes the nonsense codon.²⁵ See Figure 14.

		Second Letter					
		Uracil	Cytosine	Adenine	Guanine		
First Letter	Uracil	UUU } Phe UUC } UUA } Leu UUG }	UCU } Ser UCC } UCA } UCG }	UAU } Tyr UAC } UAA } STOP UAG }	UGU } Phe UGC } UGA } STOP UGG } Trp	Third Letter	U C A G
	Cytosine	CUU } Leu CUC } CUA } CUG }	CCU } Pro CCC } CCA } CCG }	CAU } Phe CAC } CAA } Phe CAG }	CGU } Arg CGC } CGA } CGG }		U C A G
	Adenine	AUU } Ile AUC } AUA } Met AUG }	ACU } Thr ACC } ACA } ACG }	AAU } Phe AAC } AAA } Phe AAG }	AGU } Ser AGC } AGA } Arg AGG }		U C A G
	Guanine	GUU } Val GUC } GUA } GUG }	GCU } Ala GCC } GCA } GCG }	GAU } Phe GAC } GAA } Phe GAG }	GGU } Gly GGC } GGA } GGG }		U C A G

Key:
Ala: Alanine
Arg: Arginine
Asn: Asparagine
Asp: Aspartate
Cys: Cysteine
Gln: Glutamine
Glu: Glutamate
Gly: Glycine
His: Histadine
Ile: Isoleucine
Leu: Leucine
Lys: Lysine
Met: Methionine
Phe: Phenylalanine
Pro: Proline
Ser: Serine
Thr: Threonine
Trp: Tryptophan
Tyr: Tyrosine
Val: Valine

Figure 14. Codons with their corresponding amino acids.

Unnatural amino acids can be incorporated by implementing a nonsense codon, such as the amber (UAG), ochre (UAA) or opal (UGA). These are codons which no standard tRNA molecule exists within the host system and signal the ribosome to terminate translation. A tRNA is evolved such that its anticodon specifically recognizes the nonsense codon and acts to control the termination by artificially suppressing the nonsense codon. A modified aminoacyl-tRNA synthetase enzyme (aaRS) acylates the altered tRNA with the desired amino acid. The artificial suppressor tRNA can then carry the amino acid in the activated form to the cells ribosomes for protein synthesis. Incorporation of a nonnative amino acid by nonsense suppression can match the cells natural level of translational precision, provided that the tRNA and aaRS used are orthogonal to the host system. Orthogonal, in this case, meaning the modified tRNA and aaRS are complimentary to the host system, and capable of functioning within the host

system with each other, but not complementary to the host, such that the modified tRNA and aaRS only recognize each other and do not interact with the host tRNA and aaRS. Thus, the interaction is controlled and leaves endemic cellular tRNA systems unaffected.²⁵

The nonnative amino acid incorporated into the peptide by site directed mutagenesis and nonsense suppression can be an unnatural amino acid with a side chain that can act as a spin label, incorporating the spin label directly into the backbone of a protein.^{25, 27} By binding the spin label directly into the backbone of the protein it can provide direct conformational properties of the protein backbone, without the variability information of the position of the side chain and spin label attachment. Attaching the spin label directly to the peptide backbone has been shown to mitigate many of the difficulties presented in chemical attachment of spin labels. The label 2,2,6,6-tetramethyl-piperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) is an unnatural paramagnetic amino acid, and the first spin label incorporated in peptides by means of a peptide bond.²⁸ TOAC is a nitroxyl radical amino acid that has been shown to preferentially adopt a helical conformation when incorporated into a protein by crystallographic studies.^{24, 25} The helical conformation torsion angles restrict the rotation of the protein backbone. The preferential conformation is beneficial, if the amino acid is being inserted into a helical site on the protein. Otherwise, the torsion strain could potentially hinder protein function by inhibition of conformational changes needed to function. The preferential conformation of the TOAC spin labels makes them disruptive in beta sheet, beta barrels, or any other site

where the nonnative amino acid would act to distort the secondary structure and function of the protein.⁵ Each unnatural amino acid comes with its' own set of limitations and advantages. For a protein modification method to be relevant, it must be biologically benign and function in the atmosphere in which it will be placed, without destroying the protein structure or function in the process. The usefulness of a particular type of label will depend on what it is being used to investigate and the chemistry available to employ it.

1.7 Employment of Spin Probes

1.7.1 Small Molecule Tracking by Electron Spin Resonance

Spin labels can also be used in track and identify compounds in vivo by in electron spin resonance by utilizing the labels' unique electron spin resonance spectra.

Fuchs et al. demonstrated the use of a spin probe acting as reporter groups in a living system. Using a nitroxide labelled estradiol. See Figure 15. The epidermal barrier function toward the estradiol was investigated.²⁶

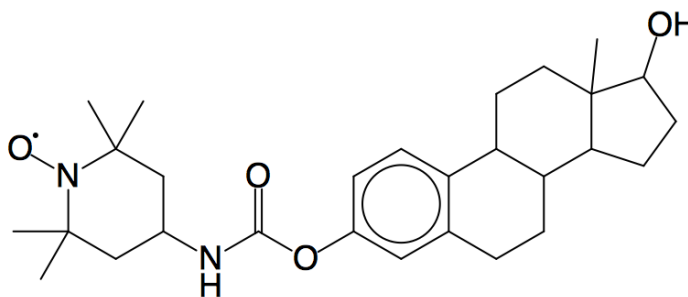


Figure 15. Nitroxide labelled estradiol

The spin labelled estradiol was left to absorb onto the skin of hairless mice to determine the rate of absorbance. The function of the spin label was to act as a reporter in

the EPR image to reveal how well the estradiol was able to absorb. After a predetermined amount of time, the epidermis of the hairless mice was biopsied. The resulting EPR provided information describing the depth of absorption of the labelled estradiol with relation to time, with spatial resolution much higher than could be obtained using NMR or ultrasound.²⁶

The biochemical and biophysical information describing the pharmacokinetics of the spin labelled estradiol also revealed that the degree of influence the nitroxide spin label had on the physiochemical properties of molecules it is attached to is related to size. It was found that smaller molecules experience more of an effect from the attached label than larger molecules.²⁶

Another method of using spin labels to study protein structure and dynamics involves observing the EPR signal of a peptide fitted with two labels at low temperatures and is called double electron electron resonance (DEER). DEER, uses EPR spectroscopy to measure the strength of the interactions between the two spin labels. The resulting measurements can be used to calculate the distances between the two labels. The technique can be used to aid in the resolution of the secondary structure of the peptide.^{4, 27}

1.7.2 Fluorescence Quenching and Resonance Energy Transfer

Spin probes attached to the peptide of interest can be used in a variety of capacities, one of which is fluorescence quenching. Fluorescence quenching is a process that decreases the intensity of the fluorescence emission, by rerouting the energy of the

excited singlet state into some other mode of relaxation/decay (Figure 16). Fluorescence quenching can occur through collisional or non-collisional transfers of energy.²⁷

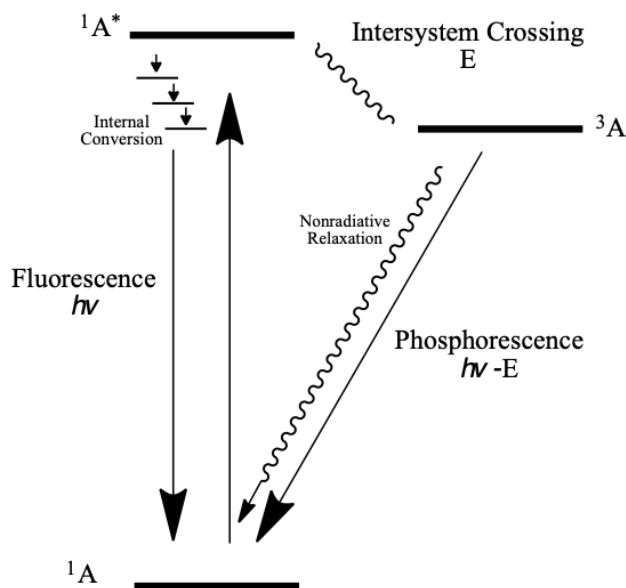


Figure 16. Jablonski diagram depicting different pathways of electron relaxation. Fluorescence quenching the intensity of the fluorescence emission, by rerouting the energy of the excited singlet state into some other mode of relaxation/decay

Fluorescence resonance energy transfer, or FRET, which is also referred to as Förster resonance energy transfer, is a non-collisional transfer of energy between a donor fluorophore and an acceptor chromophore. In FRET, the donor is excited at a specific wavelength and then transfers its energy to an acceptor chromophore through resonance; thus, the acceptor chromophore quenches the fluorescence of the donor, and the acceptor then relaxes by non-radiative internal conversion or radiative relaxation at a different wavelength.²⁷

Resonance energy transfer is analogous to the behavior of coupled oscillators, such as a pair of tuning forks vibrating at the same frequency. If one tuning fork is resonating

close enough to another tuning fork of the same frequency, both tuning forks will begin to resonate.

In the presence of a stable radical quencher the angular momentum of the donor-acceptor system is conserved and it becomes more favorable for the donor fluorophore to undergo intersystem crossing and enter its excited triplet state. The donor fluorophore can then relax by nonradiative decay in which the energy is released as phonons, more commonly known as heat.²⁷ See Figure 17. By conserving the angular momentum of the donor-acceptor system, the stable radical makes intersystem crossing more favorable.

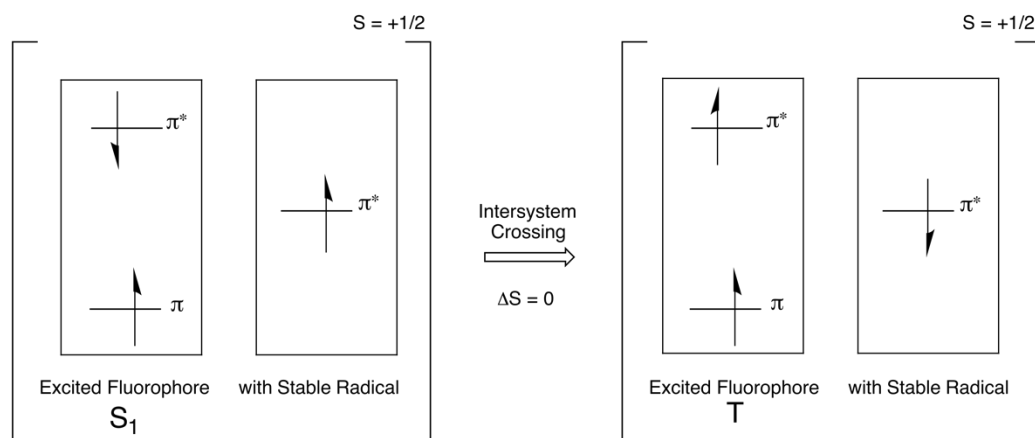


Figure 17. Fluorescence quenching mechanism of an excited fluorophore-stable radical system by intersystem crossing.

FRET is dependent on the dipole-dipole interactions between the transition dipoles of the donor and acceptor. There are four conditions that must occur between the donor fluorophore and the acceptor fluorophore for fluorescence resonance energy transfer to occur. The first is that the fluorescence emission spectrum of the donor molecule must overlap the excitation spectrum of the acceptor molecule. The second is that the donor

and acceptor must be in close proximity to one another; typically, 1 to 10 nanometers. The third is that the transition dipole of the donor and acceptor must be approximately parallel to each other. The fourth condition is that the fluorescence lifetime of the donor molecule must be long enough for the energy transfer to occur.⁵ Solvent, temperature, and concentration can also affect the rate of FRET, once all these requirements are met. Of these conditions, FRET is most influenced by distance, which is why FRET is sometimes referred to as a molecular ruler. The strict proximity dependence of the method makes it a useful way of measuring distance precise enough to measure molecular changes.²⁷ See Figure 18.

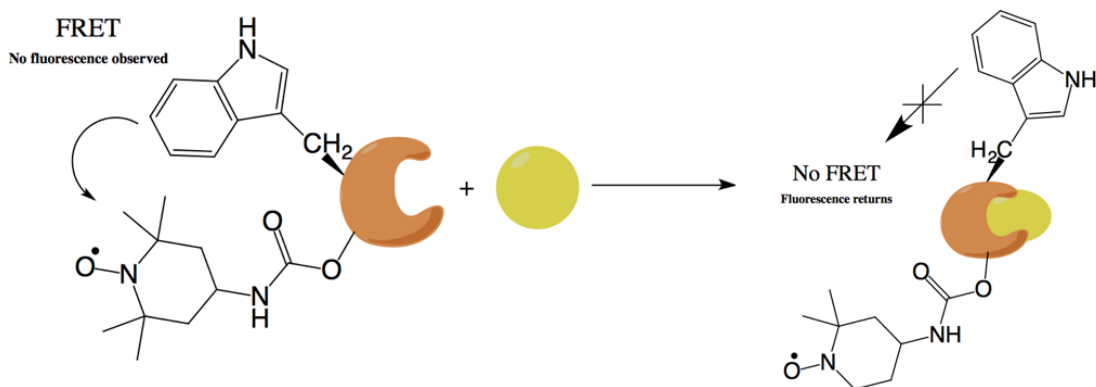


Figure 18. Model of a molecule undergoing a structural change by binding to a substrate resulting in the loss of FRET, due to the increased distance between the donor and acceptor, and the return of fluorescence.

Another proposed mechanism of relaxation of the fluorophore system is through electron transfer. Electron transfer results in fluorescence quenching through collisional transfer of energy. Since the radical species can act as an electron donor or an electron acceptor, the radical can accept the excited electron of the donor fluorophore, then relax

through electron transfer back to the donor. Electron transfer occurs through the overlap of the molecular orbitals of the fluorophore (donor) and the radical (quencher). The radical can also donate its unpaired electron to the excited donor fluorophore, which would then undergo fluorescence or nonradiative decay before transferring an electron back to the radical species. The distance which allows for electron transfer to occur is typically on the scale of around 1nm, shorter than the typical FRET process.²⁷ See Figure 19.

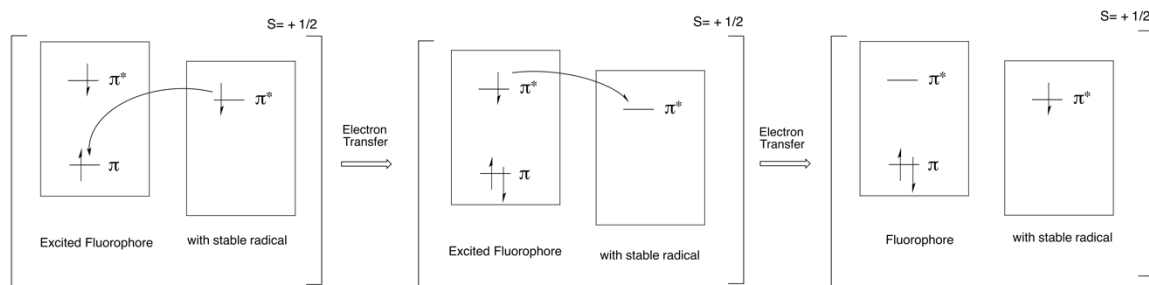


Figure 19. Quenching mechanism of an excited fluorophore by electron transfer with a stable radical.

1.7.3 Aldose Verdazyls: An Example of the Verdazyl as a Fluorescence Quencher.

Previous research has confirmed verdazyls have the ability to quench the fluorescence of riboflavin.⁵ Condensation of 2,4-diisopropylcarbonobis(hydrazide) bis-hydrochloride with a series of aldoses gives rise to tetrazanes that can be oxidized with potassium ferricyanide to give stable aldose derived verdazyl radicals.¹⁹ See Figure 20.

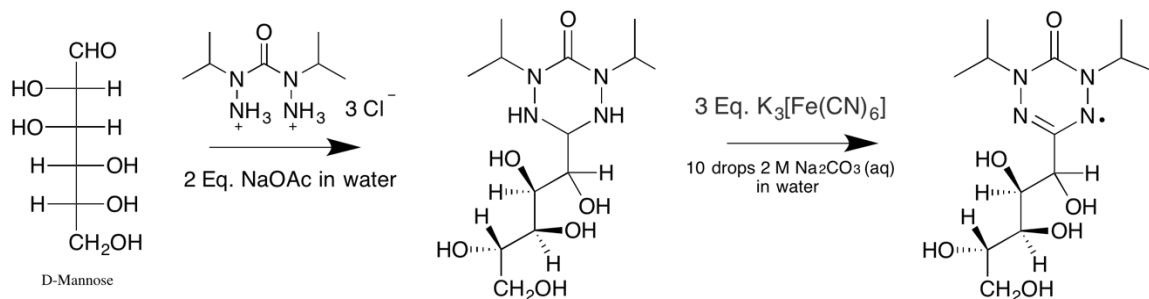


Figure 20. Synthesis of aldose derived verdazyl radical.

In this system, the hydrogen bonding between the aldose side chain and the riboflavin fluorophore mediated the distance requirement for FRET to occur. As a result, when the verdazyl and fluorophore were in the polar methanol solvent very weak quenching of the fluorescence of riboflavin was observed, as the fluorophore interacts with the solvent. When the fluorophore and verdazyl were placed in a less polar solvent, chloroform, the lack of hydrogen bonding with between the solvent the both the fluorophore and quencher enhanced hydrogen bonding between the fluorophore and quencher. The closer proximity allowed for FRET to occur and fluorescence was not observed.¹⁹

Chapter 2: Project Goals

The primary goal of this project was to synthesize a novel verdazyl which could be attached to a peptide and function as possible spin probe for biological molecules, as well as a fluorescence quenching agent. Nitroxides are the most widely studied and employed spin probes, despite being reduced to hydroxylamines by ascorbic acid in-vivo in a matter of minutes.¹⁹ Verdazyls provide comparable applications to nitroxide radicals, with the added benefits of having high variability in conformation and hydrophobicity by altering the R1/R5 ligand groups while maintaining their exceptional stability. The radicals are stable in solutions over a wide range of pH, resistant to reduction by ascorbic acid, and can quench the fluorescence of organic dyes.¹⁹

Another goal of this project was to investigate an alternative abridged synthesis of the verdazyl, by circumventing the need for an aldehyde to facilitate the ring closure during the Brook synthesis. This would enable the label to be attached to sites of a protein containing a carboxylic acid or alcohol containing side chain, since these functional groups are readily converted to a methyl ester attachment site. Methods for these modifications have been established.²³

The target compounds of this project would enable the verdazyl to be attached as a side chain attached label via an S_N2 nucleophilic substitution reaction, or attached directly to the peptide backbone via site directed mutagenesis. See Figure 21 and Figure 22.

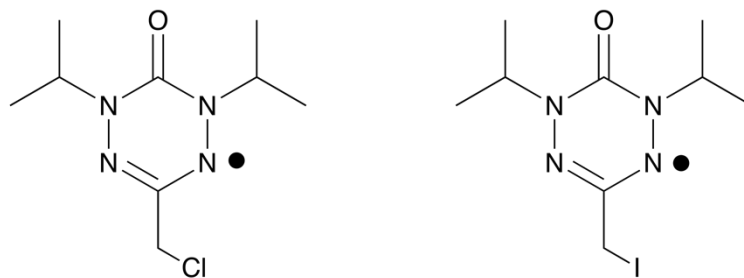


Figure 21. Target compound for SN2 attachment, 1,5-diisopropyl-3-chloromethyl-6-oxoverdazyl (left) and 1,5-diisopropyl-3-iodomethyl-6-oxoverdazyl (right).

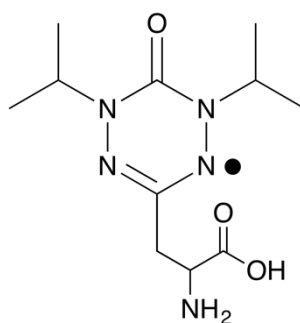
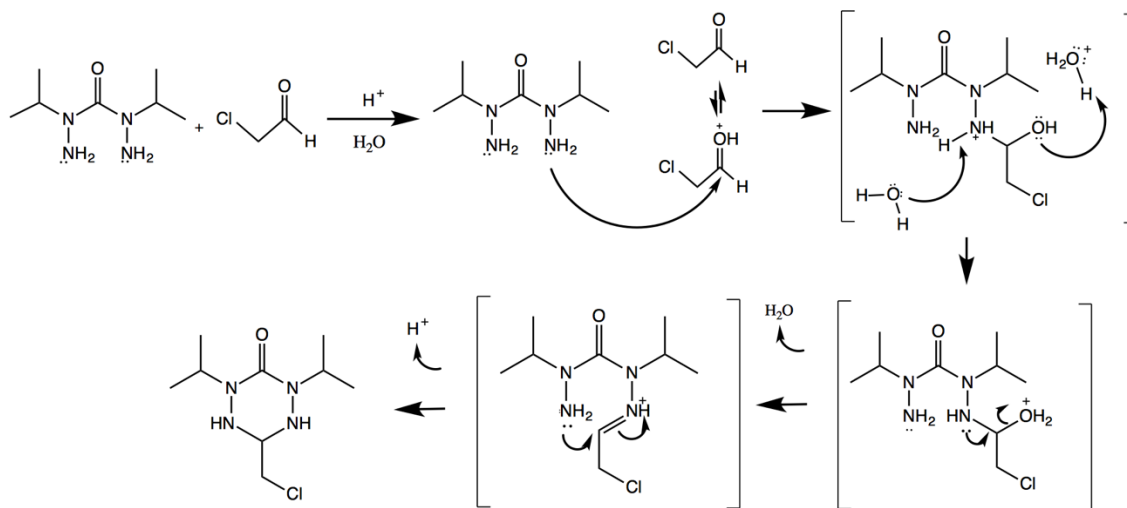


Figure 22. Target compound for attachment directly onto the peptide backbone, 2-amino-3-(1,5-diisopropyl-6-oxoverdazyl) propanoic acid.

Chapter 3: Results

3.1 Attaching the Verdazyl via S_N2

Condensation of chloroacetaldehyde and bishydrazide yields the novel chloromethyl verdazyl. See Scheme 3.



Scheme 3: Proposed mechanism of condensation reaction of bishydrazide and chloroacetaldehyde in water.

A reaction of the novel chloromethyl verdazyl product, shown above in Scheme 3, and excess potassium iodide in methanol demonstrated its ability to undergo S_N2 nucleophilic substitution, as the ESR and mass spectra confirm the replacement of the chlorine ligand with iodine. See Figure 23 and Figure 24.

The novel verdazyls presented in this thesis allow for the attachment of the verdazyl to a molecule of interest via an S_N2 reaction. The halide on the R3 ligand acts as a leaving group during nucleophilic substitution.

Addition of excess potassium iodide may also act to catalyze attachment of the chloromethyl verdazyl label. Iodine, being a soft base and having a bigger atomic radius than chlorine, makes for a more energetically favorable leaving group, and will theoretically more readily react with the nucleophilic attachment site.

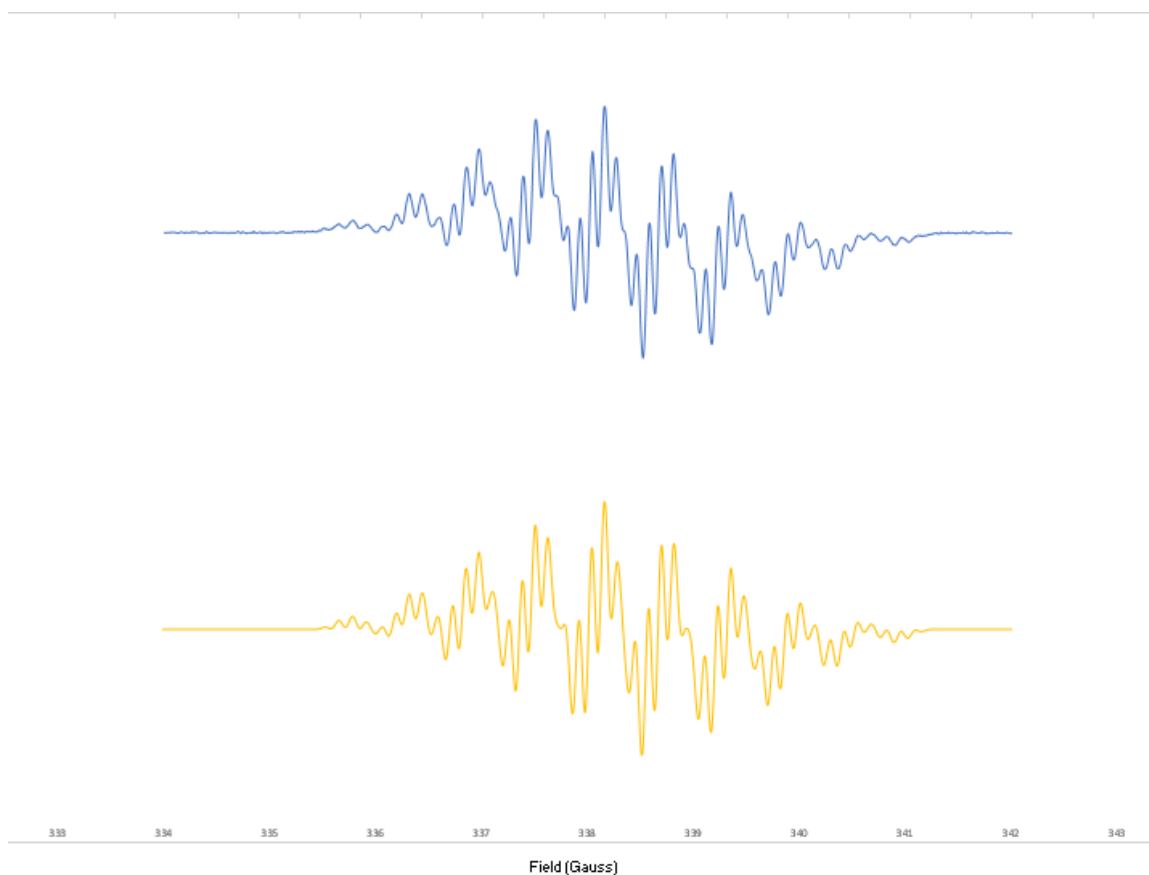


Figure 23. Experimental (top) and simulated (bottom) EPR spectra for Chloromethyl verdazyl. The spectrum was recorded in toluene. The program WINSIM from the public EPR software tools (PEST) was used for simulation. Simulation parameters: $g = 2.0045$, $a_{N1,5} = 6.464$ G, $a_{N2,4} = 5.388$ G, $a_{H(i Pr)} = 1.41$ G, $a_{H(CH2)} = 1.263$ G, $a_{Cl} = 0.933$ G, line width = 0.543 G

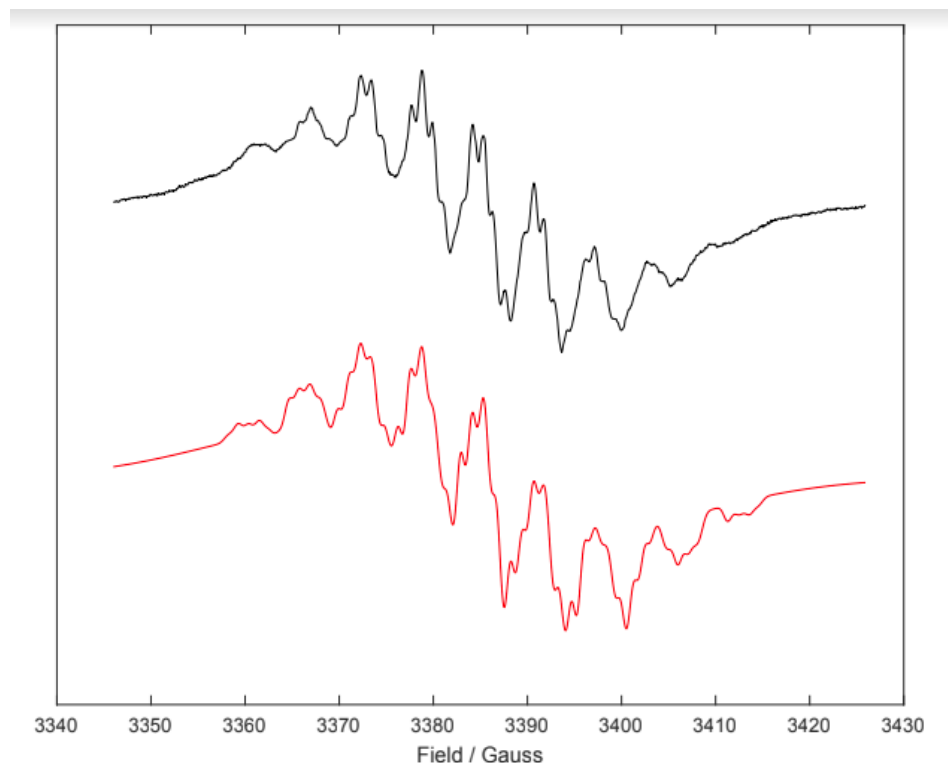
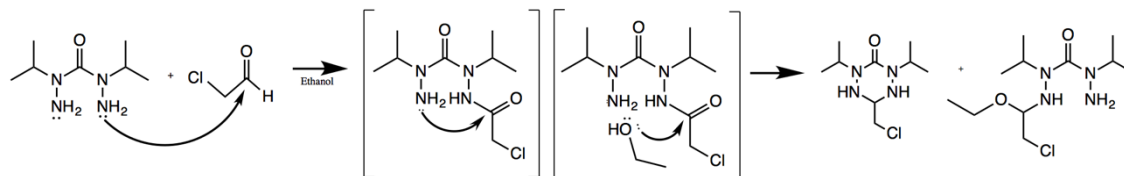


Figure 24. Experiment (top) and simulated (bottom) EPR spectra for Iodomethyl verdazyl. The spectrum was recorded in toluene. The program WINSIM from the public EPR software tools (PEST) was used for simulation. Simulation parameters: $g = 2.0043$, $a_{N1,5} = 5.3$ G, $a_{N2,4} = 6.6$ G, $a_{H(i Pr)} = 0.951$ G, $a_{H(CH_2)} = 2.3$ G, $a_I = 0.4$ G, line width = 0.95 G

It was also determined that nucleophilic solvents can form unintended side products by competing with the second amine attack on the carbonyl carbon of the aldehyde, preventing the ring closure and formation of the tetrazane. See Scheme 4.

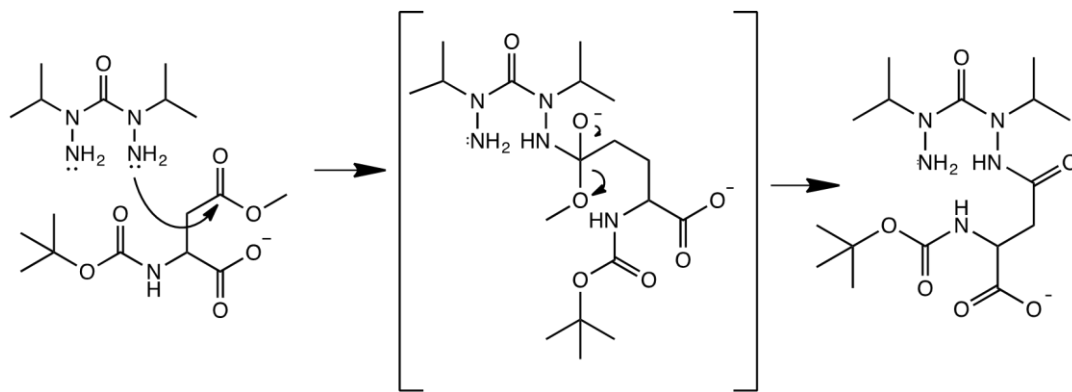


Scheme 4: Proposed mechanism of condensation reaction of bishydrazide and chloroacetaldehyde in ethanol.

3.2 Attempted Alternative Verdazyl Synthesis.

In an attempt to facilitate the ring closure with a methylester, a succession of reactions between various methyl esters and 2,4-diisopropylcarbonohydrazide bishydrochloride was studied. Using a methyl ester would abridge the previously used verdazyl synthesis published by Brook et al. in 2005, in which an aldehyde facilitates the addition of the R3 group in a condensation reaction with 2,4-diisopropylcarbonohydrazide bishydrochloride to yield a tetrazane.

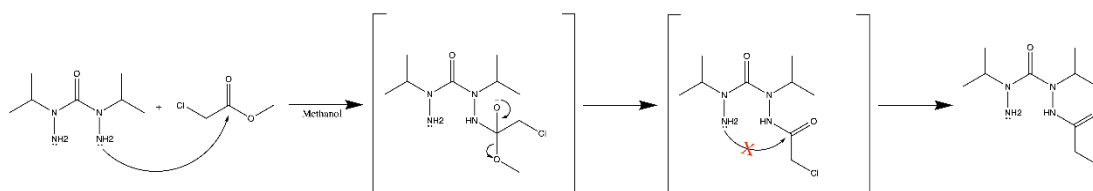
N-(tert-Butyloxycarbonyl)-L-aspartic acid 4-methyl ester was an attractive methyl ester to use to attempt to the ring closure because it would result in a verdazyl with an amino acid R3 ligand. This would enable the incorporation of the verdazyl practically anywhere within a peptide. However, the high number of functional groups within the compound made it difficult to determine what was preventing the reaction from completing. See Scheme 5.



Scheme 5: Probable reaction mechanism between Boc-methyl monoester and N-(tert-Butyloxycarbonyl)-L-aspartic acid 4-methyl ester, yielding the formation of an amide bond and an incomplete ring closure of the tetrazane.

To simplify the reaction and determine if the ring closure could be facilitated by a

methyl ester, without the superfluous functional groups of the boc protected amino acid, a reaction between methyl chloroacetate and 2,4-diisopropylcarbonohydrazide bishydrochloride to yield a tetrazane was attempted. See Scheme 6.



Scheme 6: Proposed mechanism of condensation reaction of bishydrazide and methyl chloroacetate in methanol to form an amide bond.

However, it was determined that this condensation was unsuccessful at completing the ring closure as the first amine attachment to the methyl ester forms an amide bond. See Scheme 5 and 6.

Chapter 4. Discussion

This work synthesized and characterized two novel verdazyls and was successful in demonstrating their ability to undergo an S_N2 substitution reaction. See Figure 21. It was also determined that the methyl ester was unable to facilitate the ring closure of the tetrazane reaction. See Scheme 5 and Scheme 6. The chloromethyl verdazyl and the iodomethyl verdazyl offer a novel tool in spin labelling and fluorescence quenching. It is hoped that the vast and unknown potential of these compounds will inspire scientists to re-investigate synthesis pathways to allow for additional methods of attachment of the spin labels, specifically, attachment of the label via a peptide bond. Previous attempts to synthesize a novel amino acid that carries a verdazyl as its side chain were unsuccessful. It has been well established that condensation of the bis-hydrazide with an aldehyde attaches as the R3 group to form a tetrazane; Fmoc-2-amino-4-oxobutanoic acid would serve as an excellent aldehyde to synthesize the verdazyl amino acid. Unfortunately, limited by the chemistry possible with the reagents available, we were unsuccessful in synthesizing the aldehyde necessary for this reaction. However, Fmoc-2-amino-4-oxobutanoic acid is available for purchase.

In addition to synthesizing the proposed verdazyl amino acid, future studies exploring the verdazyl's ability to quench fluorescence either by resonance energy transfer or electron transfer and determine its limitations for each would also be useful.

Chapter 5. Experimental

5.1 General

Experiments were performed in a fume hood. Solvents were used without purification. Triethylamine was distilled from calcium hydride before use. ^1H NMR spectra were recorded at 300 MHz and ^{13}C NMR spectra were recorded at 75.4 MHz on a Bruker instrument. For all NMR spectra, chemical shifts were determined using solvent peaks as a reference. The IR spectra were recorded as thin films on NaCl plates using a Mattson Genesis II FTIR. Electrospray mass spectra were recorded using a PE SCIEX API300 LC/MS/MS system and a Turbo Ionspray. EPR spectra were recorded using a Bruker EMX X-band instrument.

5.2 BOC-protected isopropyl hydrazine.

tert-butylcarbazate (20.54 g, 0.16 mol) was dissolved in minimal deionized water. 25.0mL acetone was added slowly added while stirring. The solution was left to stand overnight. The resultant white crystals were filtered and then dissolved in methanol. To this solution, sodium cyanoborohydride (7.49 g, 0.12 mol) dissolved in methanol was added. Excess acetic acid (0.2 mol) in methanol was added dropwise to the solution in the hood, with constant stirring. To the solution sodium hydroxide in methanol was added dropwise, rapid effervescence was noted, and this was continued until gas formation subsided and the pH was adjusted to 10-11. The solution was allowed to stand overnight, at which point the resultant white crystals were filtered and dried under vacuum. Spectra were consistent with previously synthesized and characterized samples.

5.3 2,4-diisopropylcarbonohydrazide bishydrochloride.

BOC-protected isopropyl hydrazine (13.79 g, 0.08 mol) was dissolved in toluene with triethylamine (8.00 g, 0.08 mol). Triphosgene (3.92 g, 0.013 mol) dissolved in toluene was added drop-wise and allowed to stir at room temperature for 2 hours, filtered and the filtrate evaporated to yield *i-tert*-butyl-2,2' -carbonyl bis-(2-isopropylhydrazine-carboxylate). Treatment with concentrated hydrochloric acid in dry ethanol at 75°C was used to remove the BOC protecting groups. The solution is left to cool overnight. Removal of remaining acid and solvent under vacuum yields bis-(2-isopropylhydrazine-carboxylate). Spectra were consistent with previously synthesized and characterized samples.

5.4 1,5-Diisopropyl-3-chloromethyl-6-oxotetrazane.

Chloroacetaldehyde sodium bisulfite (0.2 g, 1 mmol) was dissolved in minimal deionized water. 2,4-diisopropylcarbonohydrazide bishydrochloride (0.27 g, 1 mmol) and sodium acetate (0.16 g, 2 mmol) was dissolved in minimal water. The two solutions were combined and allowed to stand overnight, at which point the resultant white crystals were filtered and washed with cold deionized water to give the tetrazane. Mp:58°C; ¹H NMR ((D₂O) δ 1.07(q, 6H, CH₃), 1.10 (q, 6H, CH₃), 5.25(s,1H, CH) 9.84(t, 2H, CH₂Cl) 8.67(d, (2H, NH), 10.09 (s, 1H, CH); ¹³C NMR (D₂O) 18.54, 19.33, 40.19, 46.31, 67.40, 153.63; IR (film on NaCl plate) 3263 (NH), 2978 (CH), 1600(CO); GCMS (GC-MS, electron ionization) tR. 10.6 min with MS (EI, m/z) calculated for C₉H₁₆N₄OCl, found

231 (15), 189(23), 147(50), 99(100), tR. 12.2 min with MS EI, m/z) calculated for $C_9H_{19}N_4OCl$, 234(75,M+), 185(85), 143(50), 101(65)).

5.5 1,5-Diisopropyl-3-chloromethyl-6-oxoverdazyl.

1,5-Diisopropyl-3-chloromethyl-6-oxotetrazane (0.12 g, 0.5mmol) was dissolved in minimal water. To this, potassium ferricyanide (0.295g, 0.897 mmol), combined with 10 drops of 2 M sodium carbonate (aq), dissolved in adequate deionized water was added. The mixture was allowed to stir overnight, resulting in a color change from orange to greenish-yellow, indicating that the potassium ferricyanide had been reduced.

Dichloromethane was used to extract the verdazyl radical. GCMS (method conditions) tR. 10.4 min with MS (EI, m/z) calcd for $C_9H_{16}N_4OCl$, found 231 (32,M+), 189(48), 147(100), 98(15))) 98.6% pure. Simulation parameters: $g = 2.0045$, $a_N^{1,5} = 6.464$ G, $a_N^{2,4} = 5.388$ G, $a_H(i\text{ Pr}) = 1.41$ G, $a_H(\text{CH}_2) = 1.263$ G, $a_{Cl} = 0.933$ G, line width = 0.543 G

5.6 1,5-Diisopropyl-3-iodomethyl-6-oxoverdazyl.

1.0g 1,5-Diisopropyl-3-chloromethyl-6-oxoverdazyl with excess potassium iodide was allowed to stir at room temperature in methanol overnight. Slight color change occurred, from yellow to yellow-orange. GCMS (GC-MS, electron ionization) tR. 11.5 min with MS (EI, m/z) calcd for $C_9H_{16}N_4OI$, found 323 (36,M+), 281(53), 239(33), 112(100))) Simulation parameters: $g = 2.0043$, $a_N^{1,5} = 5.3$ G, $a_N^{2,4} = 6.6$ G, $a_H(i\text{ Pr}) = 0.951$ G, $a_H(\text{CH}_2) = 2.3$ G, $a_I = 0.4$ G, line width = 0.95 G

5.7 L-Aspartic acid β -methyl ester hydrochloride.

5.00 g (37.6 mmol) L-aspartic acid was dissolved in adequate dry methanol. 3.8 mL

(41.2 mmol) thionyl chloride was added dropwise -10°C while stirring. The solution was then allowed to stir at room temperature for 2 hours. Evaporation of the solvent revealed white powder.

5.8 *N*-(*tert*-Butyloxycarbonyl)-L-aspartic acid 4-methyl ester.

0.1001g (0.5452 mmol) L-Aspartic acid β -methyl ester hydrochloride and 0.1350g (1.607 mmol) sodium bicarbonate was dissolved in adequate dry methanol and stirred at room temperature. To this solution, 0.169g (0.7743 mmol) di-*tert*-butyl dicarbonate was added and allowed to stir overnight. The methanol was evaporated and resulting in a white powder. To this adequate deionized water added and the pH was neutralized with dilute acetic acid resulting in a white precipitate. Diethyl ether was used to extract the resulting precipitate. ¹H NMR ((MeOD) δ 1.32(9H, s, CH₃) 1.41(H, s, CH₃), 2.72 (1H, septet, CH), 3.58(2H, s, CH₂), 4.38(1H, t, NH); ¹³C NMR ((MeOD) 27.39, 36.74, 52.29, 79.40, 156.23, 172.06, 174.71

5.9 Attempted Condensation of 2,4-diisopropylcarbonohydrazide bishydrochloride with *N*-(*tert*-Butyloxycarbonyl)-L-aspartic acid 4-methyl ester.

0.0132 g (0.5739 mmol) sodium was dissolved in 5 mL methanol. To this solution 0.050g (0.1799mmol) 2,4-diisopropylcarbonohydrazide bishydrochloride was added and allowed to stir at room temperature. To this solution 0.067g (0.00 mmol) *N*-(*tert*-Butyloxycarbonyl)-L-aspartic acid 4-methyl ester dissolve in methanol was added. The solution was then left to stir under reflux overnight. Evaporation the solvent revealed

white crystals. $^1\text{H NMR}$ (CDCl_3) δ 1.16 (12H, d, CH_3), 1.24, (1H, s, NH), 1.41(9H, s, CH_3), 2.80 (2H, s, CH), 3.63(2H, s, CH_2), 3.93(2H, septet, CH), 4.20 (1H, s, NH)

5.10 Attempted condensation of 2,4-diisopropylcarbonohydrazide bishydrochloride with methyl chloroacetate.

0.0132 g (0.5739 mmol) sodium was dissolved in 5 mL methanol. To this solution 0.050g (0.1799mmol) 2,4-diisopropylcarbonohydrazide bishydrochloride was added and allowed to stir at room temperature. To this solution 0.0195g (0.1799 mmol) methyl chloroacetate dissolve in methanol was added. The solution was then left to stir under reflux overnight. Evaporation the solvent revealed white crystals. $^1\text{H NMR}$ (D_2O) δ 1.062 (6H, d, CH_3), 1.116 (6H, d, CH_3), 1.24 (1H, s, NH), 3.856(2H, d, CH_2), 3.915(1H, s, CH), 4.055(2H, d, NH_2), 4.638(1H, s, CH); $^{13}\text{C NMR}$ (D_2O) 18.906, 43.431, 47.428, 153.679, 167.260; IR (ATR) 3263 (NH), 3232 (NH), 2978 (CH), 2933 (CH), 1591 cm^{-1} (C=O)

Chapter 6. References

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