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Synthesis of Azulenylsilane Nitrones as Diagnostic Tools for Superoxide Detection

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SYNTHESIS OF AZULENYLSILANE NITRONES AS DIAGNOSTIC TOOLS FOR SUPEROXIDE DETECTION

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Relina Tamrakar

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To: Dean Kenneth Furton
College of Arts and Sciences

This dissertation, written by Relina Tamrakar, and entitled Synthesis of Azulenylsilane Nitrones as Diagnostic Tools for Superoxide Detection, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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David Becker, Major Professor

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The dissertation of Relina Tamrakar is approved.

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Dean Kenneth Furton
College of Arts and Sciences

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Florida International University, 2011

DEDICATION

I dedicate this dissertation to my late father, Padma Bir Singh Tamrakar and my mother, Keshari Tara Tamrakar who taught me the value of education; my husband Gyanu Sthapit who has shown exemplary patience while I completed my dissertation. I am deeply indebted to them for their support, encouragement, strength and unconditional love. I also dedicate this dissertation to my wonderful daughter Jasmine Sthapit for being the best new-born at a critical time. I am indeed blessed to have them in my life.

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ABSTRACT OF THE DISSERTATION

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Professor David Becker, Major Professor

The superoxide radical is considered to play important roles in physiological processes as well as in the genesis of diverse cytotoxic conditions such as cancer, various cardiovascular disorders and neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Alzheimer's disease (AD). The detection and quantification of superoxide within cells is of critical importance to understand biological roles of superoxide and to develop preventive strategies against free radical-mediated diseases. Cyclic nitrone spin traps such as DMPO, EMPO, DEPMPO, BMPO and their derivatives have been widely used in conjunction with ESR spectroscopy to detect cellular superoxide with some success. However, the formation of unstable superoxide adducts from the reaction of cyclic nitrones with superoxide is a stumbling block in detecting superoxide by using electron spin resonance (ESR). A chemiluminescent probe, lucigenin, and fluorogenic probes, hydroethidium and MitoSox, are the other frequently used methods in detecting superoxide. However, luceginen undergoes redox-cycling producing superoxide by itself, and hydroethidium and MitoSox react with other oxidants apart from superoxide forming red fluorescent products

contributing to artefacts in these assays. Hence, both methods were deemed to be inappropriate for superoxide detection.

In this study, an effective approach, a selective mechanism-based colorimetric detection of superoxide anion has been developed by using silylated azulenyl nitrones spin traps. Since a nitrone moiety and an adjacent silyl group react readily with radicals and oxygen anions respectively, such nitrones can trap superoxide efficiently because superoxide is both a radical and an oxygen anion. Moreover, the synthesized nitrone is designed to be triggered solely by superoxide and not by other commonly observed oxygen radicals such as hydroxyl radical, alkoxyl radicals and peroxyl radical. *In vitro* studies have shown that these synthesized silylated azylenyl nitrones and the mitochondrial-targeted guanylhydrazone analog can trap superoxide efficiently yielding UV-vis identifiable and even potentially fluorescence-detectable orange products. Therefore, the chromotropic detection of superoxide using these nitrones can be a promising method in contrast to other available methods.

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LIST OF ABBREVIATIONS

AD……..Alzheimer's disease

AGE…… advanced glycation end

ALS…….Amyotrophic lateral sclerosis

ATP…….adenosine triphosphate

AZN……azulenyl nitrone

BMPO…….5-tert-butoxycarbonyl-5-methyl-1-pyroline *N*-oxide

CLA……cypridina luceferin analogue

cGMP…….cyclic guanosine monophosphate

DBA^{++}……*N*,*N*-dimethylbiacridinium

DDQ……...2,3-dichloro-5,6-dicyanobenzoquinone

DEPMPO……5-diethyoxyphosphoryl-5-methyl-1-pyroline *N*-oxide

DHE……dihydroethidium

DIPPMPO……5-diisopropyloxyphosphoryl-5-methyl-1-pyroline *N*-oxide

DMAD……dimethyl acetylenedicarboxylate

DMF…… *N*,*N*-dimethylformamide

DMPO…….5,5-dimethyl-3,4-dihydro-2 *H*-pyrrole *N*-oxide

DMSO…….dimethyl sulfoxide

DNA……deoxyribonucleic acid

EMPO…….5-ethoxycarbonyl-5-methyl-1-pyroline *N*-oxide

EPO…….eosinophil peroxidase

EPR…….electron paramagnetic resonance

ESR…….electron spin resonance

ETC…….electron transport chain

GPx……..glutathione peroxidase

GSH…….glutathinone

GTP……. guanosine triphosphate

HNE…….hydroxynonenal

HE……hydroethidium

HO•……..hydroxyl radical

HOCl……hypochlorous acid

HOO•……hydroperoxyl radical

HPLC……...high performance liquid chromatography

HRMS……..high resolution mass spectrometry

LDA…….lithium diisopropylamide

LDL……. low-density lipoprotein

LRMS……low resolution mass spectrometry

MCLA……..methoxy cypridina luceferin analogue

MDA……....malondialdehyde

MPO……myeloperoxidase

NADH…….....nicotinamide adenine dinucleotide hydride

NADHP……nicotinamide adenine dinucleotide phosphate

NBT……nitro blue tetrazolium

NMR……..nuclear magnetic resonance

NOS…….nitric oxide synthase

$^1O_2$……. singlet oxygen

$O_2^{\bullet-}$…….superoxide radical anion

$ONOO^-$…….peroxynitrite anion

PBN…… α-*N*-phenyl-*N*-*tert*-butylnitrone

PD……Parkinson's disease

PTPs…….protein tyrosine phosphatase

PyOBN…… α-pyridyl-1-oxide *N*-tert-butyl nitrones

RO•……..alkoxyl radical

ROOH…….alkyl hydroperoxide

ROO•……..peroxyl radical

ROS…….reactive oxygen species

SOD……superoxide dismutase

STAZN……stilbazulenyl nitrone

TBDMSCl……...tert-butyldimethylsilyl chloride

THF…….tetrahydrofuran

TLC…….thin layer chromatography

TMSCl…….....trimethylsilyl chloride

UV-vis…….ultraviolet visible

[1] INTRODUCTION

Increasing evidence of involvement of superoxide radical anion ($O_2^{\bullet -}$) as a mediator of various pathological diseases,[1-3] and its importance in physiological processes led to the development of methods for detecting superoxide radical anion over the past decades. By far, the most commonly used probes include cyclic nitrones, hydroethidine, nitro blue tetrazolium, and other chemiluminescent and fluorescent species have received a criticism for various reasons.[4-7] To overcome the existing problem associated with these probes, an effective approach, a selective, mechanism-based colorimetric detection of superoxide anion has been developed by using a novel silylated azulenyl nitrone spin trap. The synthesized nitrones were found to trap superoxide anion, and hence, can be the potential biomarkers for superoxide production. The synthesized spin traps could offer a highly convenient method compared to other available colorimetric detection methods. My research describes the design, synthesis, chemical properties, testing and potential use of new silylated azulenyl nitrone compounds.

[1.1] Historical Background

Over the past decades, it has been established that radicals are ubiquitous in nature, and have a large impact on our lives. They are present in our bodies, produced in the atmosphere and during some important chemical reactions essential to life. Although, more than a century ago Gomberg had established the existence of a relatively stable carbon-centered free radical, the triphenyl methyl radical,[8] it was not until five decades later that considerable importance of free radicals in chemistry and biology was recognized. In 1954, Gershman reported that oxygen poisoning damage is related to the

formation of free radicals during the reduction of oxygen.[9] Soon after her publication, in the same year, free radicals were first detected in lyophilised biological materials by Commoner et al. using ESR.[10] With this breakthrough discovery, investigations into the role of free radicals in biological systems were spurred. In 1956, Harman proposed his free radical theory of aging,[11] in which it was suspected that aging and degenerative diseases were associated with the activity of free radicals. Gradually, in 1969, with the discovery of an enzyme called superoxide dismutase (SOD), McCord and Fridovich solidified the importance of free radicals in the life sciences.[12] They demonstrated that in biological systems, widely distributed superoxide dismutase catalyzes the dismutation of superoxide free radical anion ($O_2^{\bullet -}$) to yield oxygen and hydrogen peroxide and might play a vital role in protecting the organism against the detrimental effects of the superoxide radical anion. Free radicals are considered to be reactive intermediates and involved in many biologically destructive processes. Free radicals are able to damage macromolecules such as cellular lipids, proteins, carbohydrates and nucleic acids by reacting with them and disabling their normal functions. Once viewed as exclusively harmful species to organisms, beneficial roles of free radicals in physiological processes were first recognized in the 1970s. Work conducted by Millar and Murad indicated that the formation of hydroxyl radical (HO•) from superoxide ion and hydrogen peroxide actually stimulates activation of guanylate cyclase which catalyzed the formation of the "second messenger" 3',5'-cyclic guanosine monophosphate (cGMP) from guanosine triphosphate GTP.[13] During the oxidative burst of phagocytic cells, free radicals are sequentially generated to attack invasive microorganisms.[14,15] It has also been known that reactive oxygen species play important roles in the regulation of cardiac and vascular cell

functioning.[16] Moreover, free radicals increase the production of the T-cell growth factor interleukin-2, an immunologically important T-cell protein.[17]

Although the existence of free radicals is essential in maintaining cell viability in living organisms, their accidental damage to cells is inevitable. The overproduction of free radicals results in the formation of highly oxidizing species resulting in cellular dysfunction.[18] Ultimately, the oxidative stress on cells is thought to make a significant contribution to a variety of diseases such as ischemia-reperfusion injury,[19] cancer,[20,21] acute stroke,[22] Alzheimer's disease,[23,24] cardiovascular disease,[25] neurodegenerative disease,[26] Down's syndrome, and diabetes.[27-29] More importantly, it seems that cells maintain a careful balance between beneficial and deleterious effects of free radicals, and this mechanism is obviously a critical aspect of our life. It becomes imperative to have "real-time" monitoring and measurement of these free radicals within cell for further investigation of their physiological and pathological roles that may ultimately lead to the development of new strategies for treatment.

[1.2] Free Radicals

A free radical is defined as an atomic, molecular or ionic species which possesses one or more unpaired electrons in its outermost shell.[30] The presence of an unpaired electron sharply enhances their reactivity with other atoms or molecules especially in hydrogen atom abstraction, in the addition of double bonds in organic compounds and in a variety of other reactions to achieve a stable configuration. Free radicals can have positive, negative or neutral charge. In the biosciences, two types of radicals are commonly encountered, the carbon-centered radical (•R) in which the unpaired electron resides on the carbon atom, and the oxygen-centered radical (•O) in which the unpaired

electron resides on the oxygen atom. Oxygen-centered radicals are among a group of molecules known as reactive oxygen species (ROS). Of the two aforementioned radical types, oxygen-centered radicals such as superoxide anion radical, hydroxyl radical, and peroxyl radicals are believed by many to be the major cause of vascular diseases and pathological conditions in biological systems.

[1.3] Superoxide radical anion

Among the reactive oxygen species, superoxide radical anion ($O_2^{\bullet -}$) is the most important species because it is a progenitor of other reactive oxygen species in biological systems. A ground state oxygen molecule dissolved in aprotic solvents undergoes electrochemical reduction by gaining one electron to form superoxide radical anion based on the work of Sawyer and his co-worker[31,32] according to the following reaction.

$$O_2 + e^- \longrightarrow O_2^{\bullet -} \qquad (1)$$

With one electron in its outermost shell, superoxide radical anion is paramagnetic. The bond distance between O-O is 1.33^{o}A. In aqueous media, superoxide radical anion is always in equilibruim with the hydroperoxyl radical as shown in equation 2.[33]

$$H_2O + O_2^{\bullet -} \rightleftharpoons HOO\bullet + {}^-OH \qquad (2)$$

The electronic structure of superoxide anion contains three electrons in two separate antibonding orbitals (π^*, 2p), one with paired and another with unpaired electrons.[34] Hence, $O_2^{\bullet -}$ is both a radical and anion species. When dissolved in organic solvents, it is very reactive.[35] In water, superoxide anion has been demonstrated to be a strong proton acceptor with at least four molecules of water through hydrogen bonding.[36]

It is unstable and disproportionates in water and readily forms oxygen and hydrogen peroxide. Superoxide radical anion can act as an one electron acceptor as well as one electron donor depending on the nature of substrate with which it is reacting.[37] Therefore, it becomes involved in both oxidation and reduction reactions. If a substrate has strong electron affinity, it undergoes superoxide-mediated one electron reduction. However, with an electron-deficient substrate, superoxide can act as a nucleophile or undergo one-electron transfer to give molecular oxygen.

[1.4] Formation of superoxide radical in biological systems

In biological systems, the superoxide radical anion, $O_2^{\bullet-}$, is not a very reactive damaging agent but plays a critical role in the formation of other harmful reactive oxygen species (ROS) that have been implicated in the genesis of various pathological conditions. These reactive oxygen species include, reactive radicals such as hydroxyl radicals (HO•), hydroperoxyl radicals (HOO•), peroxyl radicals (ROO•), alkoxyl radicals (RO•), the carbonate radical anion ($CO_3^{\bullet-}$) and non-radicals such as hydrogen peroxide (H_2O_2), alkyl hydroperoxides (ROOH), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$) and peroxynitrite anion ($ONOO^-$). Secondary reactive oxygen species are also produced from singlet oxygen, ionizing radiation[38] and peroxynitrite anion, but with less frequency.[39] Superoxide radical anion productions can be both enzymatically and non-enzymatically mediated.

[1.4.1] Electron transport chain as a source of superoxide radical

Molecular oxygen is an essential component for living organisms to survive, and is widely distributed. Aerobic cells utilize oxygen to oxidize organic substrates for energy generation. During cellular respiration, oxygen, by accepting electrons, is consumed

along with metabolic substrates that undergo oxidation to produce ATP, water and carbon dioxide. The cellular respiration takes place in mitochondria via a pathway known as the electron transport chain (ETC). The ETC consists of several electron acceptor enzyme complexes in the inner membrane of the mitochondrion.[40] In this mechanism, electrons are passed from one molecule to another producing a proton gradient across the mitochondrial membrane each time until electrons are passed to oxygen to produce water and energy (ATP). The electrons from oxidation reduce oxygen to water in myocardial cells via two pathways.[41] The mitochondrial electron transport chain reduces ~95 % of oxygen to water by the tetravalent pathway.[41] The reduction of oxygen requires four electrons to each oxygen molecule as shown in the reaction 3 where E^{φ} is the standard reduction potential.[42]

$$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O \qquad E^{\varphi}=+0.82V \qquad (3)$$

It is believed that under normal conditions, molecular oxygen metabolism proceeds via a series of four sequential univalent reduction reactions (four-one electron addition reactions) to form two molecules of water that also results in the formation of two free radicals and one hydrogen peroxide molecule as shown in the overall process.[43] The pKa of the equilibrium between superoxide anion and its conjugated base $H_2O\bullet$ is 4.8 implying that $O_2^{\bullet-}$ is the dominant species at physiological pH and mediates the further steps.

$O_2 + e^- \longrightarrow O_2^{\bullet-} (+ H^+ \rightleftharpoons H_2O\bullet)$ $E^o = -0.33V$ $E' = +0.28V$ (4)

$O_2^{\bullet-} + e^- \longrightarrow O_2^{--} (+ 2H^+ \longrightarrow H_2O_2 + O_2)$ $E^o = +0.87V$ $E' = +0.63V$ (5)

$H_2O_2 + e^- + H^+ \longrightarrow HO\bullet + H_2O$ $E^o = +0.38V$ $E' = +0.73V$ (6)

$HO\bullet + e^- + H^+ \longrightarrow H_2O$ $E^o = +2.33V$ $E' = +1.56V$ (7)

The thermodynamic parameters of this process are reported by Fee and Valentine[44] where E^o is the standard cell potential and E' is the potential at the physiological conditions (pH 7, 25°C) of the specific intermediate. To reduce oxygen by accepting one electron to form superoxide anion is thermodynamically very unfavorable compared to the other reductions. However, once the first step takes place, the remaining steps will occur spontaneously giving minimal chance for the occurrence of only partial univalent reduction of oxygen. The entire process takes place in the enzyme active site where oxygen is incorporated into protein and bound to it so that it cannot leave until it is converted into water. However, it is estimated that ~ 2 % [45] to ~5 % (in the myocardium)[41] of the electron flow through the electron transport chain, is subjected to one univalent reduction of molecular oxygen generating superoxide radical anion. The partial univalent pathway occurs when electrons are leaked from the electron transport chain during the terminal enzyme complex reduction in mitochondria. Molecular triplet-state oxygen then accepts these leaked electrons to form superoxide radical anion[46] and once in its anionic form, it can easily cross the inner mitochondrial membrane.

[1.4.2] Enzymatic source of superoxide radical

In phagocytes, the superoxide radical anion is also produced by the leukocyte

enzyme NADPH oxidase.[47] Phagocytic cells such as neutrophils or macrophages are capable of recognizing the invading microorganisms, and play a role in the immune defense mechanism by oxidative activity called the respiratory burst.[48] During the respiratory burst, the consumption of oxygen is considerably increased compared to normal conditions.[49] When there is the initial inflammatory response, neutrophils are shifted to the site of injury which activates NADH or NADPH oxidases and transfers electrons originating from NADH or NADPH to oxygen, thus generating superoxide radical to kill oxygen dependent microorganisms in the plasma membrane or on its outer surface.[50] Similar enzymes that produce $O_2^{\bullet-}$ are also present in cells other than leukocytes. They prefer NADH to NADPH.[51]

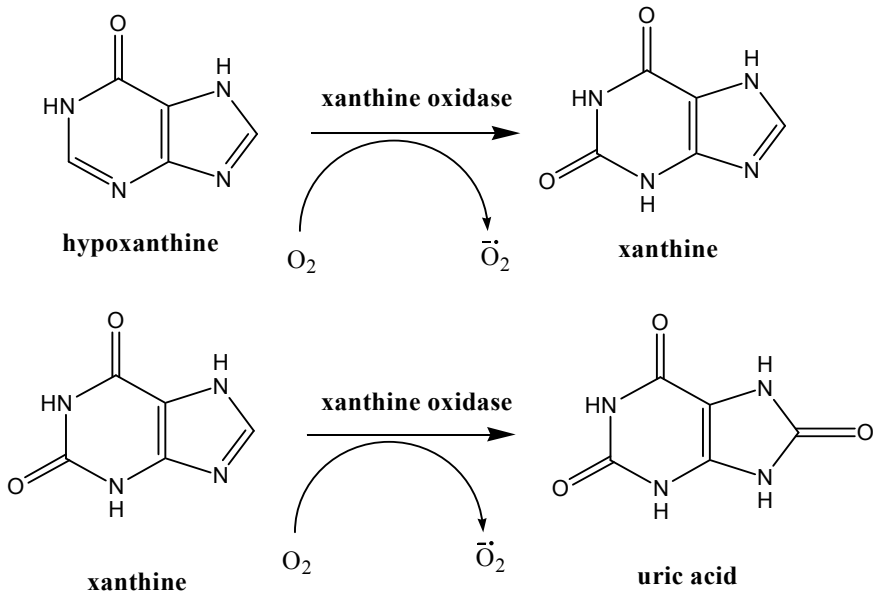


Figure 1. Formation of superoxide by xanthine and hypoxanthine

Xanthine oxidase is another popular enzyme that can also produce superoxide. Xanthine oxidase is widely distributed in human with relatively high activity in liver and

intestine.[52,53] Xanthine oxidase is an inter-convertible form of xanthine dehydrogenase. Under normal conditions, xanthine oxidase exists in the form of xanthine dehydrogenase that uses NAD+ as electron acceptor, thus producing none or very insignificant amounts of superoxide anion.[54] However, upon depletion of ATP, xanthine oxidase is formed as a result of proteolytic cleavage by a calcium-dependent protease.[55] The enzyme then sequentially catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid with a large production of superoxide contributing to oxidative damages during certain disease conditions such as ischemic-reperfusion. The superoxide anion is generated in both oxidation steps.

Peroxidases can also generate the superoxide anion. Evidence shows that peroxidase catalyzes the oxidation of substrates like NADH or NADPH to NAD radical (NAD•) by using hydrogen peroxide. The superoxide anion is apparently produced by the reduction of molecular oxygen by NAD•.[56] Furthermore, the presence of certain phenols stimulates NADH oxidation which increases the rate of the formation NAD•, thus increasing the production of superoxide anion.[57]

The superoxide anion is also produced from oxidation of NADH in the presence of a catalase.[58,59] In this process, catalase probably catalyzes the oxidation of NADH to its free radical in the presence of hydrogen peroxide. The resulting NADH free radical (NAD•) then transfers an electron to molecular oxygen thereby producing superoxide anion.

$$\mathrm{NADH} \xrightarrow[\mathrm{H_2O_2}]{\text{Catalase}} \mathrm{NAD\bullet} + \mathrm{H_2O} \qquad (8)$$

$$NAD\bullet + O_2 \longrightarrow NAD^+ + O_2^{\bullet -} \quad (9)$$

Lipoxygenase also contributes to the formation of superoxide anion.[60] The oxidation of purine neucleotides (NADH or NADPH) catalyzed by lipoxydase in the presence of linoeic acid lead to the generation of superoxide and might cause oxidative stress in cells.[61]

Other enzymes that may be responsible for superoxide anion generation include PGH synthase[62] and cycloxygenase.[63] The mechanism for these processes is similar to that for lipoxygenase and peroxidase.

Monoamine oxidase has shown to be a superoxide anion generator in Parkinson's disease.[64]

[1.4.3] Non-enzymatic source of superoxide radical

Thiols may be responsible for the production of superoxide radical anion non-enzymatically. In biological systems, thiols such as glutathione, ethyl mercaptan, 2-mercaptoethanol and dithiothreitol have shown to inhibit SOD reduction of nitro blue tetrazolium (NBT) suggesting the formation of superoxide anion.[65]

Thiols undergo one-electron oxidation with the formation of thiyl radicals. The oxidation reaction is catalyzed by transition metal ions.[66] Thiyl radicals apparently react with thiolate anion producing disulfide radical anion ($RSSR^{\bullet -}$) which loses an electron to dioxygen yielding superoxide anion and disulfides.[67]

[1.4.4] Microsomes as source of superoxide radical

Superoxide anion production by microsomes has been implicated in *in vivo* studies.[68] Two major components in microsomes are NADPH- cytochrome 450 reductase

and cytochrome 450 which cause the generation of superoxide. Microsomes stimulate the NADPH-dependent SOD inhibitable oxidation of epinephrine to adrenochrome[69] and the reduction of nitro blue tetrazolium (NBT) in rat liver suggesting the formation of superoxide anion.[70] Furthermore, corticosteroids has shown to enhance the activity of NADPH-cytochrome reductase implying the increased formation of superoxide anion.[71]

[1.4.5] Cell nuclei as a source of superoxide radical

Cell nuclei may also be the source of superoxide anion formation. The generation of superoxide anion by cell nuclei was based on the oxidation of epinephrine in the presence of NAHD-cytochrome reductase which was inhibitated by superoxide dismutase (SOD).[72,73] On the basis of the location of cell nuclei, superoxide formation is NADH- or NADPH-dependent. The generation of superoxide anion by the nuclear membrane may cause human malignant brain tumors.[74]

[1.4.6] From nitric oxide synthase

Superoxide anion is also generated by nitric oxide synthase (NOS) in the presence of calcium/calmodulin. Upon binding of calcium/calmodulin to NOS, electrons derived from NADPH transfer from flavin-containing reductase to heme-containing oxidase domains of NOS to activate the enzyme. However, dissociation of ferrous-dioxygen complex, formed by binding of oxygen to the heme group, may occur to generate superoxide anion instead of forming water.[75]

[1.5] Formation of secondary free radicals

Superoxide can undergo spontaneous dismutation by non-enzymatic or enzymatic pathways to hydrogen peroxide which is less reactive and stable than superoxide anion. Superoxide dismutase (SOD) is an enzyme which catalyzes the dismutation of superoxide

anion in biological systems at the rate that is four orders of magnitudes.

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (10)$$

Superoxide dismutase (SOD) contains certain metals such as copper, zinc and manganese which act as a catalyst for the SOD-mediated disproportionation of $O_2^{\bullet-}$ in the cytosol by Cu/Zn-SOD enzyme, and mitochondria by Mn-SOD enzyme.[76] Hydrogen peroxide is known for its capacity to cross the cellular membrane and to diffuse.

Hydrogen peroxide is also produced by peroxisomes particularly in liver under physiological conditions.[77] Damage to fatty acids has been attributed to the hydrogen peroxide production other than in liver.

The resulting hydrogen peroxide is then removed by three general mechanisms.

In the cytosol, two enzymes named catalase and glutathione (GSH) peroxidase are known to catalyze the dismutation of hydrogen peroxide into water and oxygen[78] while in the mitochondria glutathione peroxidase alone accelerates this process:

$$2H_2O_2 \longrightarrow H_2O + O_2 \quad (11)$$

Catalase resides within the microbodies like peroxisomes which contains four porphyrin heme (iron) groups that catalyzes the enzyme-mediated degradation of the hydrogen peroxide primarily produced from fatty acids and amino acids.

Glutathione peroxidase requires reduced-glutathione, a tripeptide (N,N-L-γ-glutamyl-cysteinglycine (GSH), to dismutate hydrogen peroxide into water and oxygen which is then oxidized to disulfides GSSG.[79] The oxidized form of glutathione is then reduced back to GSH by the enzyme glutathione reductase in the presence of NADH

which is supplied by a metabolic source. The process of reduction can be classified as a detoxification mechanism.

$$2\ GSH + 2\ H_2O_2 \xrightarrow{\text{glutathione peroxidase}} 2\ H_2O + O_2 + GSSG \qquad (12)$$

In the second mechanism, hydrogen peroxide can undergo Fenton-like reactions to produce the hydroxyl radical (HO•) in the presence of a transition metal such as iron or copper.[80] The hydroxyl radical reacts rapidly with biomolecules resulting in the formation of carbon-centered radical which is more stable and longer-lived. Normally, iron exists predominantly in its oxidized ferric state form (Fe^{3+}). The reduction of Fe^{3+} to Fe^{2+} is a very important biochemical step to initiate the Fenton-like reaction.[81,82] In the first step, superoxide radical anion acts as a reducing agent for reduction of coordinated Fe^{3+} to Fe^{2+}. In the second step, this reduced form catalyzes the decomposition of hydrogen peroxide into hydroxyl radical. The overall reaction is also called the Haber-Weiss reaction.[83]

Fenton reaction:

$$O_2^{\bullet-} + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \qquad (13)$$

$$Fe^{2+} + H_2O_2 \longrightarrow HO\bullet + Fe^{3+} + OH^- \qquad (14)$$

Haber-Weiss reaction or net reaction:

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe^{2+}} O_2 + HO\bullet + OH^- \qquad (15)$$

The iron cations are strong oxidants which govern iron reactivity in the Fenton reaction, and contribute to the final oxidative damages in biological systems.[84,85] Because of the toxicity of iron, living organisms carefully sequester iron in safe complex forms and carefully release it as a micronutrient.

In the presence of chloride, hydrogen peroxide is also decomposed by an enzyme, myeloperoxidase (MPO) into hypochlorous acid (HOCl). The decomposition process occurs in neutrophils[86] and in monocytes. Hypochlorous acid is a strong oxidant that can damage biomolecules directly and by decomposing to chlorine. Therefore, this mechanism is considered to be a process leading to the formation of a toxic physiological agent. The resulting hypochlorous acid reacts with hydrogen peroxide to form a non-radical oxidant such as singlet oxygen and water.[87,88] Similarly, hydrogen peroxide can form hypobromous acid by the action of the enzyme eosinophil peroxidase (EPO)[89]. These hypohalous acids are capable to produce hydroxyl radical by reacting with the superoxide radical anion:

$$H_2O_2 + X^- \longrightarrow HOX + H_2O \quad (16)$$

$$H_2O_2 + HOX \longrightarrow H_2O + H^+ + X^- + {}^1O_2 \quad (17)$$

$$HOX + O_2^{\bullet -} \longrightarrow HO\bullet + X^- + O_2 \quad (18)$$

Apart from these reactive oxygen species, another biologically significant free radical is nitric oxide (NO•). Nitric oxide (NO) is produced in biological tissue by the semi-essential amino acid L-arginine via the action of nitric oxide synthase (NOS) by a five electron oxidative reaction to form L-citrulline.[90] In the cardiovascular system, NO is

produced primarily by endothelial cells while certain nerves, blood cells, and vascular smooth muscle cells are also capable of producing nitric oxide. The activity of NO is controlled by cytokines which connect the immune system with the cardiovascular, nervous and endocrine systems. Therefore, when there is an urgent need for cell defense, immune cells can make large amounts of nitric oxide.[91]

Nitric oxide (NO) is a very unstable molecule and has a half-life of only a few seconds under physiological conditions. During the oxidative burst, NO reacts spontaneously with superoxide radical anion produced by immune cells to generate a more oxidatively active molecule, the peroxynitrite anion ($ONOO^-$). The formation of peroxinitrite is regulated by the concentration of NO. It is produced when the level of NO is very high and dismutation by SOD is inhibited. In acidic medium, peroxynitrite anion is protonated to afford peroxynitric acid which can react with organic molecules. Spontaneous decay of ONOOH by heterolytic cleavage generates hydroxyl radical (HO•) and nitrogen dioxide radical (NO_2•).[92] The decomposition reaction is catalyzed by the transition metal of SOD and myeloperoxidase which continues to give nitrates as a final product:[93]

$$NO\bullet + O_2^{\bullet -} \longrightarrow ONOO^- \quad (18)$$

$$ONOO^- + H^+ \longrightarrow ONOOH \quad (19)$$

$$ONOOH \longrightarrow HO\bullet + NO_2\bullet \quad (20)$$

$$HO\bullet + NO_2\bullet \longrightarrow NO_3^- + H^+ \quad (21)$$

Peroxinitrite and its protonated form, (ONOOH), can react with selective biomolecules such as protein thiols, zinc fingers, and membrane lipids by a one or two electron oxidation process and eletrophilic nitration.[94] This reaction is accelerated by carbon dioxide.[95] Moreover, the formation of hydroxyl radical by peroxynitrite has a minor role *in vivo* because of an affinity of peroxynitrite towards carbon dioxide. Peroxynitrite reacts rapidly with carbon dioxide to form an unstable molecule, nitrosoperoxycarbonate ($ONOOCO_2$-), which spontaneously homolysis into carbonate radical anion ($CO_3^{\bullet-}$) and nitrogen dioxide radical ($NO_2\bullet$).[96]

[1.6] Mechanism of free radical-mediated tissue damage

When the balance between the rate of formation of free radicals and the rate of their scavenging is disturbed, the damage of tissues and their structures is inevitable. In particular, hydroxyl radical is the most powerful reactive species of oxygen and can react with lipids, DNA and proteins. Because of its strong reactivity, tissue damages occur at the site of its formation.

[1.6.1] Free radicals in lipid peroxidation

Polyunsaturated fatty acids are among the major targets of radical-mediated damages among othose that involve free radical chain reactions. The presence of a methylene group between double bonds makes polyunsaturated fatty acids susceptible to oxidation with the formation of a conjugated diene bond. The oxidation reaction first generates a second radical, which in turn reacts with another macromolecule and continues the chain reaction.

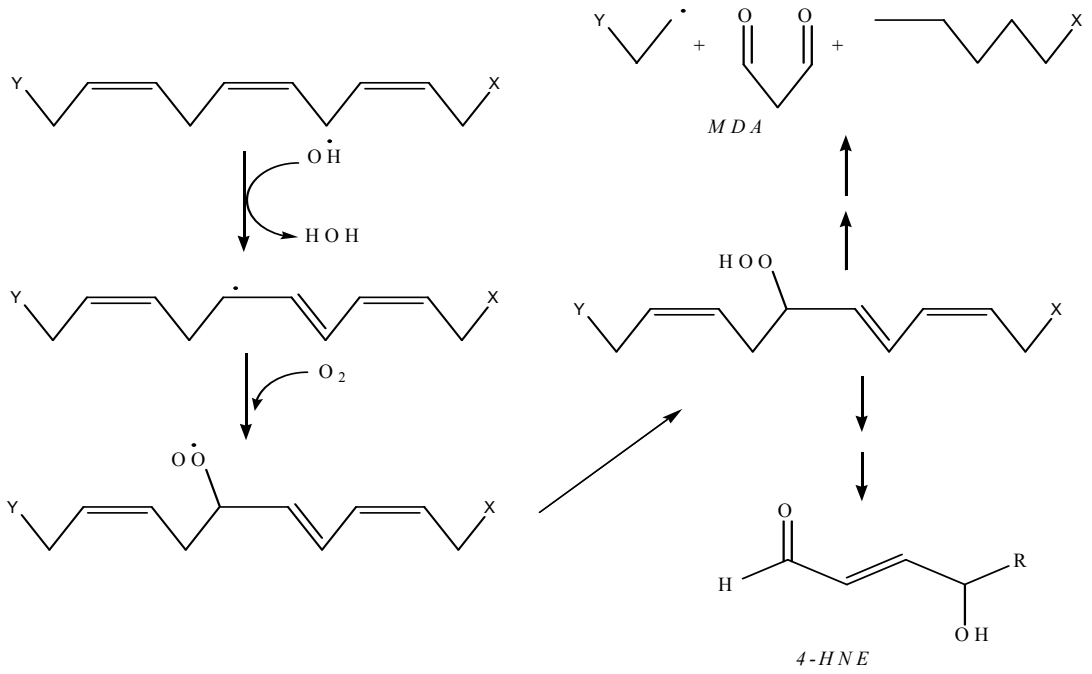


Figure 2. Schematic mechanism of lipid peroxidation of polyunsaturated fatty acids

The hydroxyl radical first abstracts a hydrogen atom from polyunsaturated fatty acids of cell membranes and initiates the chain reaction of lipid peroxidation by generating an alkyl radical (figure 1). The alkyl radical then reacts with oxygen to form an alkyl peroxyl radical, which in turn, abstracts a hydrogen atom from another fatty acid to propagate the chain reaction. A number of products are formed from lipid peroxidation once newly formed lipid peroxides react with metals such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE)[97]. These aldehyde products are electrophilic and can react with nucelophilic sulfhydryl moieties like those in cysteine, and amino acids residues such as histidine and lysine.[98] Lipid peroxidation modifies both the structure and function of cell membranes.[99]

The hydroperoxyl radical (HOO•), the conjugate acid of superoxide anion, may also participate in fatty acid peroxidation initiation *in vivo* by abstracting a hydrogen atom from the methylene group of unsaturated fatty acids.[100]

[1.6.2] Free radicals in DNA damage

The hydroxyl radical can also react with DNA by addition to double bonds and abstracting hydrogen atoms especially from bases. It can also react with the deoxyribose backbone. The consequences of these reactions are modification of individual nucleotide bases, single-strand breakage, DNA-protein cross links, and chromosomal rearrangement. Addition of hydroxyl radical to pyrimidines[101] (thymine and cystosine) and purines[102,103] forms hydroxyl pyrimidines and hydroxyl purines respectively. Hydroxyl radical reacts with guanine bases to form 8-oxo-7-hydo-29-deoxyguanosine.[104,105] Malondialdehyde (MDA), one of the products of lipid peroxidation, can also react with DNA bases causing mutation and cancer.[106]

The peroxynitrite anion also causes oxidative DNA damage as well as nitration of DNA bases.

[1.6.3] Free radicals in protein damage

Proteins are also sensitive to oxidation reactions especially with hydroxyl radicals formed upon exposure to ionization. Abstraction of an alpha hydrogen atom by hydroxyl radical from any one of the amino acid residues or the peptide backbone leads to carbon-centered radicals (alkyl radical), which upon addition of oxygen under aerobic conditions forms alkylperoxyl radicals. The alkyperoxyl radical then interacts with the protonated form of superoxide radical anion (HO_2•) thus leading to an alkyl peroxide that, in turn, further reacts with HO_2• to give a protein alkoxyl radical which can cause peptide bond

cleavage.[107] These newly formed protein alkyl-, alkylperoxyl- and alkoxyl- radicals can abstract hydrogen atoms from amino acid residues in the same or different protein and continue the same reaction.[107] The hydroxyl radical generated by the Fenton reaction can also initiate the same reaction in the absence of ionizing radiation causing loss of histidine residues, [108] loss of tyrosine residues, and bityrosine cross-links.[109] On the other hand, protein-peroxyl radicals can undergo scission and produce carbonyl compounds.[110,111] Peroxynitrite anion is another reactive species that can react with amino acids residues of proteins and modify their structures and thus affect their functions.[112,113]

[1.7] Biological defense against free radicals

The toxic action of free radicals from a variety of sources has led to the development of a series of defense mechanisms within organisms to decrease or eliminate the damage they inflict.[114] Defense mechanisms against the negative influence of free radicals include, mechanisms preventing the formation of free radicals, repair mechanisms of damaged cells, physical defenses, and antioxidant defenses. From the biological standpoint, an antioxidant is any substance that prevents oxidation of other molecules by oxidants while it is itself oxidized to a relatively stable compound either in a radical or non-radical form, that can stop the propagation radical reaction. Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are enzymatic antioxidants. Glutathione (GSH) is a non-enzymatic antioxidant.

When the level of superoxide anion is elevated, cells rapidly synthesize SOD enzymes to dismutate superoxide radical anion. Manganese superoxide dismutase (MnSOD) is associated with DNA which would behave like a “tethered antioxidant.”

Hence, MnSOD would enhances DNA protection from oxidative events and even inhibits carcinogenesis.[115] Copper-zinc superoxide dismutase (CuZnSOD) is unusually stable and is the most important intracellular antioxidant. Among metals, copper appears to be involved in the dismutation process by oxidation and reduction reactions whereas zinc helps in the enzyme stabilization but does not participate in dismutation.[116]

$$\text{Enzyme-Cu}^{2+} + O_2^{\bullet-} \longrightarrow \text{Enzyme-Cu}^{+} + O_2 \qquad (22)$$

$$\text{Enzyme-Cu}^{+} + O_2^{\bullet-} + 2H^{+} \longrightarrow \text{Enzyme-Cu}^{2+} + H_2O_2 \qquad (23)$$

Deficiency of SOD increases the risk of tissue damages and causes morphological disorders. On the other hand, increased SOD expression also has a deleterious effect on mammalian cells.

In physiological conditions, free radical and non radical species exist at low and measurable concentration.[117] Cells maintain the concentrations of these free radical species by establishing equilibrium between the rate of their generation and removal.[118] Each cell function is characterized by the oscillation of electrons stored (redox state) in many cellular constituents.[119] Under oxidative stress conditions, glutathione peroxidase (GPx) removes hydrogen peroxide thereby forming glutathione disulfide at the expense of glutathione (GSH); this in turn, increases the formation of protein mixed disulfide.[120] Oxidized glutathione is then stored inside cells and this will increase the ratio of reduced (GSH) to oxidized glutathione (GSSG). However, too high concentrations of GSSG may damage many enzymes oxidatively. Cells will then remove oxidized glutathione disulfide by the activity of glutathione reductase in the presence of NADPH to maintain the redox

state as represented by the oxidized (GSSG)/ reduced (GSH) ratio.[121] The ratio of oxidized and reduced glutathione is a measure of oxidative stress within an organism.

$$GSSG + NADPH + H^{+} \longrightarrow 2GSH + NADP^{+} \quad (24)$$

The Nernst equation can be used as a tool to estimate a reduction potential from reducing capacity, which is the concentration of the reduced species. Both of these factors are important to determine the redox state of the cell.[119]

Catalase, similar to glutathione peroxidase, eliminates hydrogen peroxide generated from dismutation of superoxide anion. In addition to its role as a cofactor of glutathione peroxidase, glutathione is involved in many other metabolic phenomena. Gluthathione inhibits the ability of copper ion to generate free radicals by chelation.[122] It can quench certain free radicals and non- radicals such as HO•, RO•, RO_2•, R•, and peroxynitrite anion. However, the reaction with a radical would generate the thiyl radical (GS•) which may generate superoxide anion. In such case, SOD might assist GSH in the elimination of free radicals. Moreover, GSH reacts with peroxynitrite anion to form nitrosothiol, which leads to the regeneration of nitric oxide. This is an important process in the protection of cells from deleterious effects of peroxynitrite.

Similarly, the formation of NO can act as direct feedback inhibition of NOS for further synthesis of NO.[123]

[1.8] Role of free radicals in various diseases

Oxidative stresses caused by free radicals have been implicated in various pathological conditions. The involvement of free radicals in disease is triggered by various factors such as genetic, environmental and disturbance of metabolic systems.

Free radicals produced in mitochondria are widely believed to be the major cause of manifestation of a pro-oxidative shift in the plasma thiol/disulfide redox state which impairs glucose tolerance. These conditions are generally observed in cancer[124] and diabetes.[125] As in the other cases, the enhanced activity of NAD(P)H oxidase and xanthine-oxidase are the major cause of ROS generation derived from superoxide anion. These conditions are normally observed in chronic inflammation[126] and ischemia/ reperfusion injury respectively.

An alteration of cellular thiol/disulfide redox state as a result of oxidative stress has been found in many cancer cells. Damage to DNA, occurs because free radical-induced permanent modification of genetic materials, thereby leading to cell mutagenesis and carcinogenesis.[127] An increased level of DNA damage has also been found in tumors which suggest the involvement of oxidative stress in the etiology of cancer. If DNA is severely damaged, then affected cells are selectively eliminated by the process called apoptosis.[128] Apoptosis, a normal biological process, is a programmed cell death that destroys the damaged cells that could otherwise cause a threat to the integrity of organisms by leading to many morphological changes.[129] However, uncontrolled apoptosis can lead to the destruction of healthy cells.[130] The consequences of DNA damage are arrest or induction of transcription, induction of signal transcription pathways, genomic instability, and replication error; all of them are associated with carcinogenesis.[131,132] The formation of the DNA oxidation product, 8-oxo-7-hydo-29-deoxyguanosine (8-oxo-dG), has been studied most extensively as a potential biomarker of carcinogenesis because it is easily detectable. 8-oxo-dG was first detected in urine by

Shigenaga et al[133] Tobacco smoking is one of the sources of carcinogenic reactive oxygen species.[134]

Free radicals are also involved in diabetes mellitus.[29,135] Hyperglycemia is the common characteristic of both type-1 diabetes mellitus (insulin-dependent) and type-2 diabetes mellitus (non-insulin-dependent). Hyperglycemia is the condition in which an excessive amount of glucose is circulated in the blood plasma. Increased glucose levels are shown to increase the production of free radicals from various sources. The major site of superoxide anion formation in diabetes is mitochondrial complex II[136] which is different from the sites mitochondrial complex I and the ubiquinone-complex III that generate superoxide anion under normal conditions. Glucose auto-oxidation has been shown to produce superoxide anion in diabetic patients[137,138] The advanced glycation end (AGE) products are the result of glucose oxidation which interact with specific cell surface binding proteins with subsequent generation of reactive oxygen species (ROS).[139] The increased level of ROS may cause the development of diabetic complications such as accelerated atherosclerosis[140] and other vascular pathologies.[141]

Various inflammatory diseases are associated with the elevated production of reactive oxygen species.[142] Atherosclerosis is one of the chronic inflammatory diseases that is characterized by hardening and thickening of the arterial walls that develop in the inner coat.[143] In the presence of hyperlipidemia, oxidative stress may activate the genes that induce an inflammatory response to the vascular endothelium. The injured endothelial cells then stimulate the recruitment of mononuclear cells into the arterial walls which bind oxidized low-density lipoprotein (LDL) resulting in abnormal macrophages, that subsequently undergo apoptosis. The massive apoptosis of

macrophages causes the formation of atherosclerotic plaque.[144] Moreover, experimental data shows that hypertension may cause an intensification of atherosclerosis.[145]

A role for oxidative stress in hypertension has been supported by the study of an animal model of hypertension by Vaziri et al. via *in vivo* glutathione depletion.[146] Similarly, a potential causative role for oxidative stress is also found in patients with essential hypertension.[147,148] The mechanism that seems to contribute to hypertension is increased generation of superoxide anion in the vessel wall followed by the formation of peroxinitrite anion,[149] which in turn, increases vasoconstriction.[150] *In vivo* studies in animals demonstrated that angiotensin II is the main factor that is involved in the oxidative stress that leads to chronic high blood pressure.[151,152]

It is suggested that oxidative stress is also involved in several neurodegenerative diseases. Nervous tissue suffers more oxidative damages than other tissue types.[153] Although the brain contributes only a few percent of all tissue mass, it is the most large amount of ATP is required to maintain normal neuronal functions. Brain has a high iron content[154] that can be released as ions during brain injury, and these ions catalyze free radical reactions resulting in HO• formation which thereby accelerates lipid peroxidation and autoxidation of neurotransmitters.

Alzheimer's disease (AD) is a neurodegenerative disease characterized by impairment of higher cognitive functions and extensive neuronal loss. Two pathological features found in AD patients are the accumulation of amyloid plaques (amyloid-β peptide) in the brain and the presence of neurofibrillary tangles and neurophil threads.[155] A progressive cerebral accumulation of amyloid-β peptide is the major event in the development of Alzheimer's disease. The accumulation of peptides causes neurofibrillary

damage in neurons and neuronal cell death. A postmortem study of Alzheimer's disease brains indicated the reduction of various neurotransmitters.[156] These phenomena are the major cause of loss of cognitive functions.[116] The evidence of increased lipid peroxidation and decreased polyunsaturated fatty acids content, increased protein and DNA oxidation, increased formation of 4-hydroxynonenal (product of lipid peroxidaiton) in AD ventricular fluids, the presence of advanced glycation end products, malondialdehyde, nitrotyrosine, and peroxynitrite in neurofibrillary tangles supports the involvement of oxidative stress in the brains of Alzheimer's disease victims.[155,157] The significant amount of lipid peroxidation has been implicated by the increased level of 4-hydroxynonenal in the postmortem cerebrospinal fluid of Alzheimer's disease patients.[158] Most individuals with Down's syndrome have been shown to develop Alzheimer's disease as they get older.[159] The higher risk of Alzheimer's disease in individuals with Down's syndrome might be as a result of the extra copy of chromosome 21 which leads to increased production of amyloid beta. Down's syndrome is a genetic disease that results from three copies of chromosome 21. The syndrome leads to mental retardation, often ranging from mild to moderate disability. A small number of individuals with Down's syndrome have a severe to high degree of intellectual disability. There is evidence of over-expression of Cu/ZnSOD in Down's syndrome that may result in increased production of hydrogen peroxide.[28] Overproduction of Cu/ZnSOD in transfected cells appears to promote increased lipid peroxidation. [160]

Parkinson's disease (PD) is characterized by a problem in controlling movement that includes slow movement of foot and hand when they are at rest and muscle rigidity. Parkinson's disease (PD) is associated with a selective loss of neurons in a part of the

midbrain called the substantia nigra.[98] The cells of the substantia nigra produce dopamine, a neurotransmitter-chemical messenger between brain and nerve cells, which communicates with cells in another region of the brain. It is believed that oxidative stress is an initiator of dopaminergic cell degeneration which is also associated with the presence of Lewy bodies in the substantia nigra and elsewhere on the basis of the postmortem study.[64,161] Lewy bodies are small spherical proteins which affect the brain's normal functions and interrupt the action of important chemical messengers.

Similarly, in amyotrophic lateral sclerosis (ALS), motor neurons are the main targets affected in the motor cortex, spinal cord and brain stem[162] which leads to muscle tone impairment, respiratory failure, paralysis, and often death. Multiple mutations are associated with familial amyotrophic lateral sclerosis. Studies show that 20 % of familial ALS patients carry mutations in the Cu/ZnSOD gene, suggesting the involvement of free radicals.[163] One hypothesis concerning the etiology of ALS is that metals at the active sites of mutant Cu/ZnSODs allow these enzymes to catalyse oxidation reactions via peroxynitrite-generated nitrating species.[164] Several lines of transgenic mice overexpressing mutant Cu/ZnSOD have shown to develop a motor neuron disease, whose pathology and mode of progression resemble familial ALS patients. Gurney, Becker et al. used azulenyl nitrone (AZN) to suggest oxidative stress in SOD mutant transgenic mice.[165] Recently, Siddque's team discovered mutation in ubiquilin 2 gene in the brains and in the spinal cords of ALS victims.[166] The mutant ubiquilin 2 gene causes the accumulation of damaged protein contributing several forms of amyotrophic lateral sclerosis.

Friedreich's ataxia is an autosomal recessive genetic disorder which is caused by

a defect in gene called Fractaxin.[167] The clinical features consist of loss of position and vibration sense in the lower limbs and dysarthria, loss of deep tendon reflexes, and progressive limb and gait ataxia. Evidence of oxidative stress and therapeutic benefit from antioxidants have been reported.[168]

Huntington's disease (HD) is a neurodegenerative disease of autosomal dominant inheritance which causes both movement disorder and dementia. There is evidence of elevated levels of oxidative damage products such as malondialdehyde (MDA) and leukocyte-8-hydroxydeoxyguanosine (8-OHdG) in Huntington's disease patients.[169]

Multiple sclerosis (MS) is an inflammatory disease which affects the brain and spinal cord. Multiple sclerosis is caused by damage to the myelin sheath which is the protective covering of nerve cells.[170] Post-mortem analysis of the brains of the MS victims indicated the presence of nitrotyrosine which may be produced by reaction between superoxide and nitric oxide.[171]

Neuronal ceroid lipofuscinoses (NCL) are a group of neurodegenerative lysosomal storage disorders which is characterized by the progressive and permanent loss of motor and physiological ability.[172,173] It involves the progressive accumulation of the pigments called lipofuscins in cells of the brain and other tissues. Clinically, NCLs are defined by their age of onset and symptoms: infantile NCL (Santavuori-Haltia), late-infantile NCL (Jansky-Bielschowsky), juvenile NCL (Batten disease, Spielmeyer-Vogt) and adult NCL (Kuf's disease) and epilepsy NCL (progressive epilepsy with mental retardation). Study on cultured cell derived from patients with NCL indicated that oxidative stress is common manifestation of neuronal ceroid lipofuscinoses.[174]

Injury to heart, brain, lungs, intestine, liver, kidney and skeletal muscle resulting

from ischemic/reperfusion may present in a variety of clinical conditions such as organ transplantation, stroke, myocardial infractions, and other situations.[175-177] On the basis of hospital discharge records, 5 million patients (16.2 %) were diagnosed with ischemic cardiovascular events and such events were the major cause of death (38.7 %) in the United States.[178] Occurrence of tissue damage is attributed to the large amount of ROS production during ischemia/reperfusion.[179] Under the ischemic condition (oxygen deprivation), there is depletion of adenosine triphosphate (ATP) which results in the conversion of xanthine dehydrogenase is into xanthine oxidase that utilizes oxygen. At the same time, cellular ATP is catabolized to hypoxanthine which accumulates in ischemic tissue. Upon subsequent reperfusion (reoxygenation), xanthine oxidase uses the influx of oxygen to oxidize hypoxanthine into xanthine with rapid generation of superoxide anion and hydrogen peroxide.[180] Treatment with a synthetic SOD mimetic has been shown to attenuate tissue damage in a rat model of ischemia/reperfusion injury.[181]

Obstructive sleep apnea is a breathing disorder among adults, characterized by an abnormal pause in breathing during sleep.[182] The resulting repeated hypoxia and reoxygenation is similar event to the conditions of ischemia/reperfusion. Untreated and severely affected individuals often develop cardiovascular diseases such as coronary artery disease, arterial hypertension and cerebrovascular disease[183] that are associated with increased mortality.[184] The involvement of free radicals in obstructive sleep apnea, which could be the cause of the associated cardiovascular events, has been implicated by the detection of increased production of superoxide anion from polymorphonuclear neutrophils in affected patients after exposure to various stumuli.[185]

Aging is a process of progressive loss in the efficiency of physical and cognitive

functions of an organism after the reproductive phase of life.[107] The fact that free radicals cause deleterious damage to cells over time which results in aging was proposed by Harman et al.[11] Several studies also support his theory by showing progressive generation of free radicals and almost exponential increases in accumulation of oxidatively damage proteins, lipids and DNAs during aging.[186-188] Mitochondria are the primary sites of generation of oxidants during the aging process. Much of the oxidized DNAs, especially the nuclear DNA, are repaired by the cell but the mitochondrial DNA receives only low repair activity.[189] Over time, the damaged mitochondrial DNA accumulates and slowly shuts down mitochondria causing cell to age and eventually die.

[1.9] Free radicals in important physiological processes

In addition to the detrimental effects of free radicals, they are also well recognized for their importance in vital physiological activities. It is well established that free radicals play a role in protective signaling and regulation within the cells. Cells in organisms communicate with each other and respond to extracellular stimuli via a signaling mechanism called a signal transduction.[190] With this biological mechanism, cells convey the information from the outside of the cell to various functional agents inside the cell. Signals are transferred within cells when cells are stimulated by reactive oxygen species. Signal transduction is initiated by extracellular signals such as growth factors, neurotransmitters, hormones and cytokines.[48] The signal transduction processes can leads to the activation of a class of proteins called transcription factors which are responsible for the expression of genes. Through the free radicals, which are generated in low concentration by stimulation of growth factors and hormones, the signal is transmitted. For example, hydrogen peroxide, produced by growth factors, is found to be

necessary for the proper functioning of signal transduction[191,192] and acts as a secondary messenger.[192] However, superoxide anion appears to regulate protein tyrosine phosphatase (PTPs) activity more effectively than hydrogen peroxide via a cysteine residue with the formation of the cysteine disulfide.[193] The formation of the cysteine disulfide results in inactivation of the protein, which is reactivated by a specific enzyme that reduces cysteine disulfide. The reversible regulation of PTPs indicates that free radicals play an important cell signaling role in controlling redox balance.

Free radicals and ROS also regulate immune responses. Superoxide anion and nitric oxide generated in neutrophils and macrophages during the inflammatory condition plays a major role in cell defense. During inflammation, peroxynitrite anion may form from superoxide and nitric oxide which participates in the CNS inflammatory response of rat infected with Borna disease virus and in the host immune response to Coxsackievirus infection.[194]

Superoxide and other ROS produced by NADPH oxidase of vascular cells appear to regulate the vascular and cardiac cell functioning in rat by activating intracellular signaling pathways.[16] Free radicals are shown to be involved in the stimulation of guanylate cyclase which catalyzes the formation of 3',5'-cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). The resulting cGMP regulates vascular smooth muscle tone and inhibits platelet adhesion.[195]

Free radicals as signaling factors also regulate the monitoring of oxygen tension in the control respiratory ventilation.[196] Changes in oxygen concentration are sensed by the regulated production of hormones such as erythropoietin and IGF-II (insulin-like growth factors) which are controlled by hypoxia-inducible factor.[196]

The involvement of free radicals in a signal preconditioning has been an interesting subject in many studies aimed at understanding the natural protective mechanism for ischemia in minimizing sudden cardiac death.[197-200]xx Preconditioning, a condition whereby a signal increases the tolerance of tissue to ischemia/reperfusion injury, such that a brief episode of non-lethal ischemia confers long and short term protection against the lethality of a prolonged ischemic insult.[201] Evidence shows the beneficial effect of preconditioning in a variety of organ systems including the circulating system.

[1.10] Detection of free radicals

Detection of free radicals began to develop soon after the discovery of the biological importance of superoxide anion in physiology.[202] Several *in vivo* studies on the action of antioxidants suggested the role of free radicals and ROS as a stimulator of inflammatory signaling and promoter of growth stimulatory signals which can lead to several disorders.[203] Therefore, in biomedical research, identification of ROS and their sources is crucial to the development of novel drugs that function as both radical scavenger and non-steroidal anti-inflammatory agents (which target the sites of free radical generation). The identification of radicals will allow investigator to understand their exact role in specific diseases as well as their formation sites and their molecular targets. However, problems exist in the detection and quantification of intracellular superoxide radical anion. Spectroscopic technique known as electron spin resonance (ESR) otherwise known as electron paramagnetic resonance (EPR) were developed.

Electron spin resonance has been a powerful tool for the detection of unpaired electrons within free radicals. Free radicals can have either + ½ or – ½ spin and therefore

act as a small magnet.[116] Electron spin resonance is the resonant absorption of microwave radiation by paramagnetic species under the influence of an external applied magnetic field.[204] The electron magnetic moment of the unpaired electron can align itself either parallel or anti-parallel to the field creating two energy levels which vary with magnetic flied strength. When the applied microwave energy matches the energy level separation, energy is absorbed and causes movement of the electron from the lower energy level to the upper one. The absorption is monitored and a spectrum is obtained usually in the microwave region of the electromagnetic spectrum. The degree of absorption is proportion to the number of free radical in the substance.[205] The equation to obtain absorbance is:

$$\Delta E = g\beta H \qquad (22)$$

where ΔE is a the energy gap between the two energy levels of the electron, g is a gyromagnetic ratio of the electron whose value for the free electron is 2.00232 (most biological free radicals have value close to this), β is the Bohr magneton and H is the applied magnetic field. The number of lines in the ESR spectrum of a radical is known as the hyperfine structure. The presence of many nuclei in radicals makes it often very complicated. Generally, a radical is identified by calculating the *g* value, hyperfine structure and line shape.

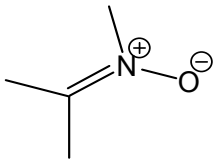
Although a promising instrument, the ESR is insensitive for direct detection of free radical such as superoxide radical anion and hydroxyl radical in living system. Direct detection of intracellular superoxide radical by spectroscopic observation is difficult or virtually impossible because of its exceptionally short lifetime, low concentration and

rapid intracellular reactions. Direct detection of biological free radicals is only possible with high concentrations of free radicals or if low temperature is used to minimize their reactions. For example, at exceptionally low temperature, ESR was able to detect the hydroperoxyl radical (HO_2•) in argon at 4.2°K directly.[206] The hydroperxyl radical was generated by addition of hydrogen atom to oxygen. In another experiment, Saito[207] and Czapski[208] have reported using ESR to detect HO_2• by forming a metal complex. However, these approaches limit the detection of biologically generated free radicals. To overcome this difficulty, the spin trapping approach was developed. In this technique, a radical is allowed to react with a spin trap molecule to form a more stable radical product, detectable by ESR, which provides a spectrum characteristic for the particular free radical that is trapped. The spin trapping method to detect short-lived radicals was first pioneered by Janzen et al.[209] With the advent of ESR spectroscopy and the spin trapping method, it is now possible to identify free radicals produced at physiological temperatures. Spin trapping methods have proved to be a very useful tool in detecting superoxide radical anion both *in vivo* and *in vitro* detection. In addition to spin trapping, other techniques have also been employed for the detection of free radicals particularly superoxide radical anion, which will be discussed in a subsequent section.

[1.11] Nitrones

The term "nitrone" is a concise word that describes compounds derived from nitrogen ketone compounds. The name nitrone was first given by Pfeiffer in 1916 to a class of compounds containing a nitrogen-oxygen linkage (N→O) in order to indicate the chemical similarity to carbonyl compounds.[210] Later, in 1938, Smith [211] reviewed those compounds and redefined nitrone as a class of compounds for both open and closed

chains containing the group below:



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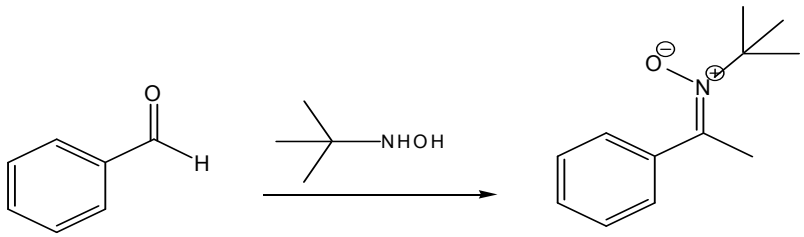
There are a variety of methods to synthesize these nitrones such as direct alkylation of oximes, oxidation of N,N-disubstituted hydroxylamines, thermal isomerization of oxaziranes, and from carbonyl and nitroso compounds.[212] Nitrones have a remarkable ability to undergo nucleophilic addition [213] and 1,3-dipolar cycloaddition reactions.[214] Because of these properties, nitrones have received special attention as building blocks in the synthesis of various natural and biologically active compounds as well as for their application in radical trapping studies (including those that takes place in biological systems).[215]

The compounds containing the nitrone moiety have demonstrated a potential therapeutic activity in both *in vivo* and *in vitro* studies.[216,217] In addition, nitrones have also been useful for detection of free-radical mediated oxidative stress in biological systems.[217] Up until now, many nitrone-containing molecules have been developed, and examined for their efficacy as antioxidants and as potential tools for superoxide anion radical detection.

[1.11.1] Synthesis of biologically important nitrone-based spin traps

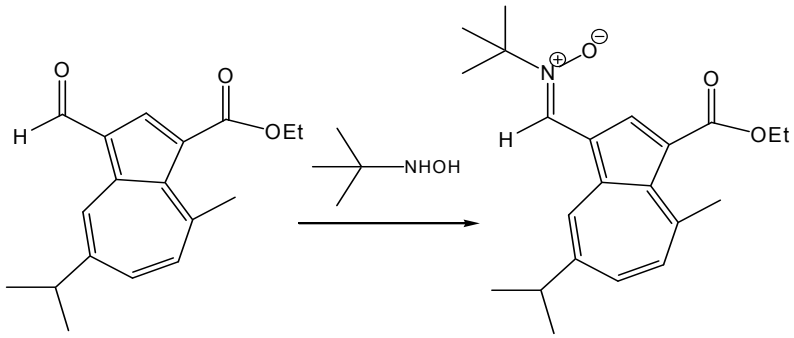
Acyclic nitrones can be synthesized directly by condensation of *N*-

monosubstituted hydroxylamines with carbonyl compounds. The most widely tested nitrone, α-*N*-phenyl-*N*-*tert*-butylnitrone (PBN), and its derivatives have been synthesized by using this method of preparation.[218]



2 PBN

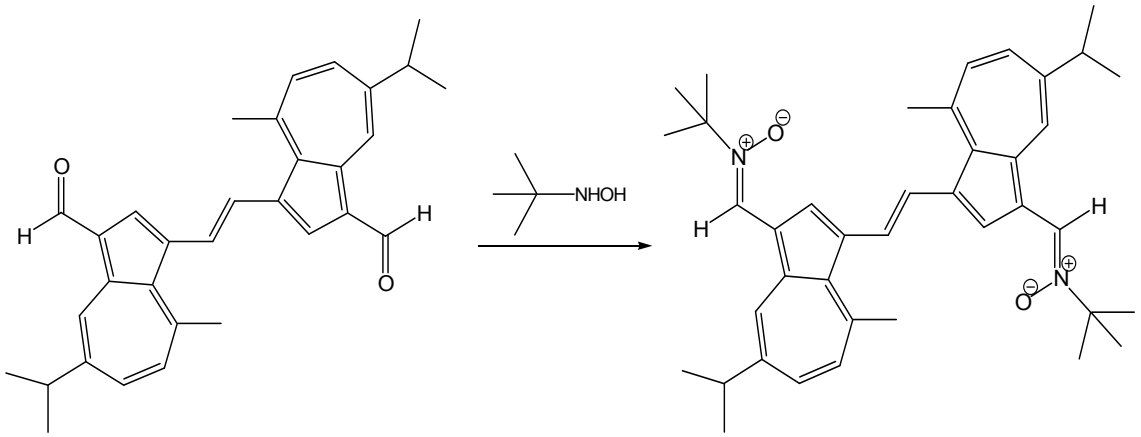
Azulenyl nitrone (AZN) is a new class of acyclic nitrone first prepared by Becker et al. by condensing N-*tert*-butyl hydroxylamine with an azulenyl aldehyde derived from guaiazulene.[219] Studies of AZN in animal models showed a therapeutic benefit against a number of diseases and utility as biomarker of free radical events.[165,217,220]



3 AZN

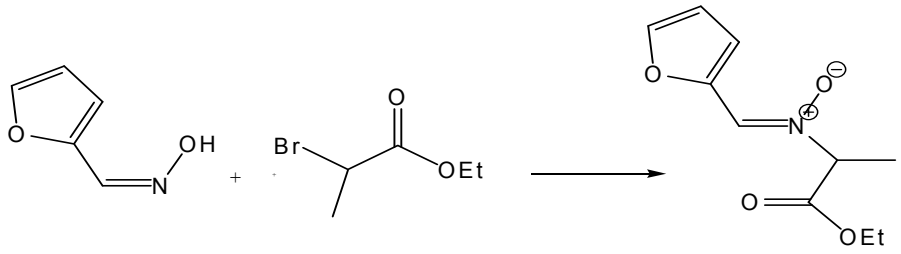
Using the same protocol, STAZN, a second generation azulenyl nitrone was synthesized.[221] Recently, in studies conducted by researchers at the Salk Institute, STAZN was deemed an emerging therapy for ischemic stroke. Its mode of action,

according to these researchers, may be pleiotropic.[222] Ginsberg and Becker et al. reported several papers that documented the beneficial effects of STAZN for stroke in animal models.[223-226]



4 STAZN

Heteroaryl-nitrone molecules, in which the carbon of the nitrone moiety has a heteroaromatic substituent, can be prepared by various methods[227] including *N*-alkylation of oximes,[228] and condensation of active methylene compounds with nitroso compounds.[229]



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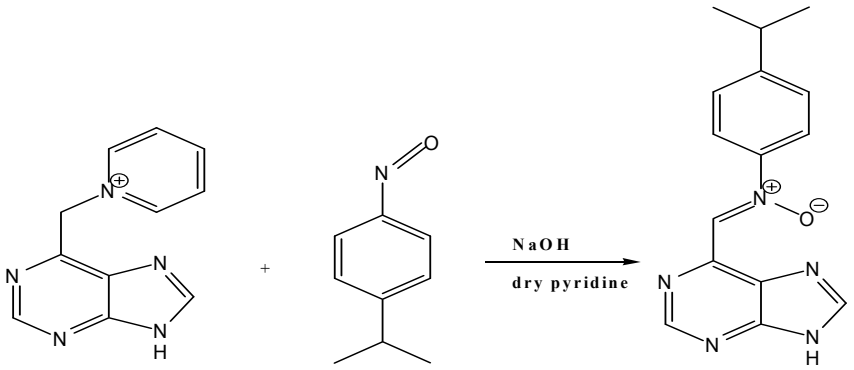

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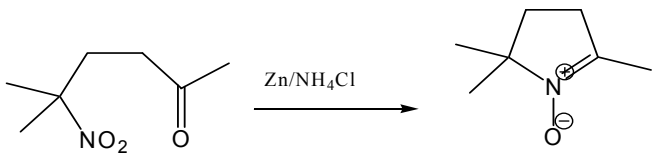
Cyclic nitrones have also received much attention-specially for the superoxide detection. Cyclic nitrones are usually prepared by reductive cyclization between carbonyl and nitro functional groups. First, the nitro group is reduced to the hydroxylamine with zinc and aqueous ammonium chloride, which is followed by intramolecular condensation with the carbonyl moiety form a ring.[230] Many pyrroline *N*-oxide derivatives were made by this method.[231-233]



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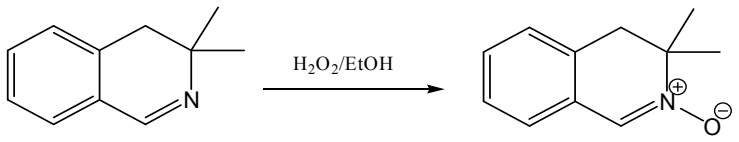
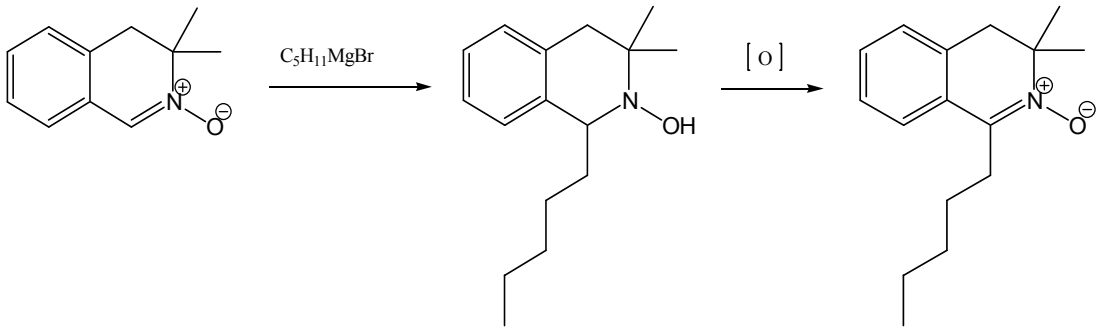
It is also possible to prepare nitrones by oxidizing amines and imines. Bernotas et al. have made a cyclic analog of PBN, MDL-101,002, and other derivatives, by catalytic oxidation of imines with hydrogen peroxide.[234,235]



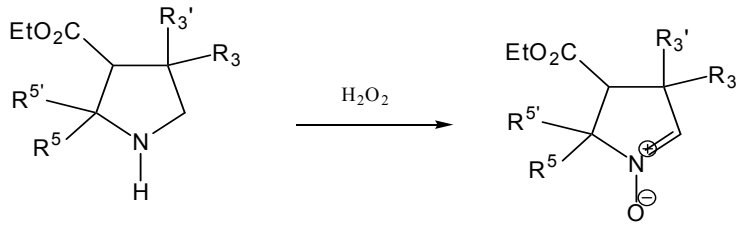
8 (MDL-101,002)

The subsequent nitrone can be a precursor for the synthesis of another nitrone. For instance, MDL-108,881 was prepared by nucleophilic addition followed by oxidation.[236]



9 (MDL-108,881)

Some highly substituted nitrones have been synthesized by oxidation of amines.[237]



10

[1.12] Methods for superoxide radical detection

[1.12.1] Nitrones as spin traps for superoxide radical detection

The spin trapping technique commonly consists of using one of two classes of compounds; namely, nitroso compounds and nitrones. Reaction of unstable radicals with nitroso compounds and nitrones often formed a long-lived nitroxide radical which is also called an aminoxyl radical or spin adduct. The nitroxide adducts so formed are detectable by ESR spectroscopy.

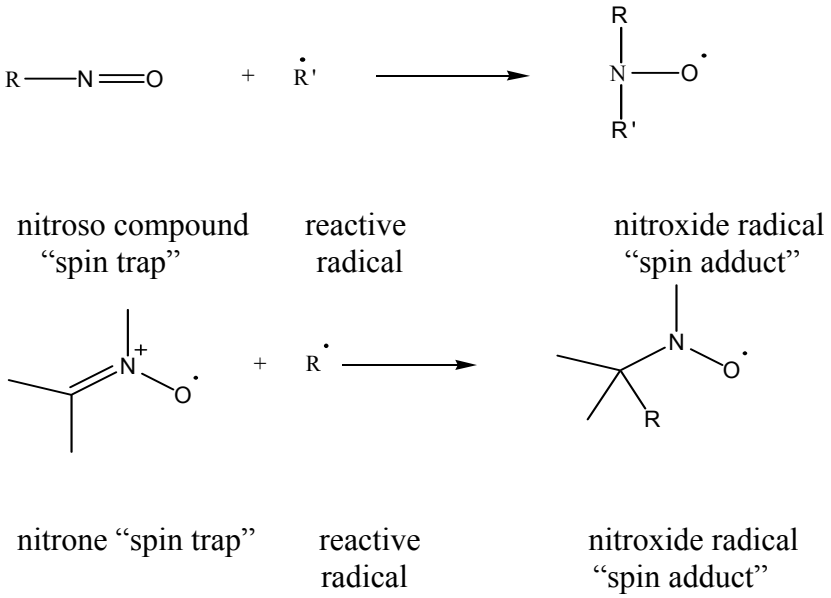


Figure 3. Spin trapping of a free radical with nitrone and nitroso compound

Nitroxide radicals are fairly stable because of the delocalization of the electron between the nitrogen and oxygen.

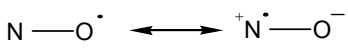
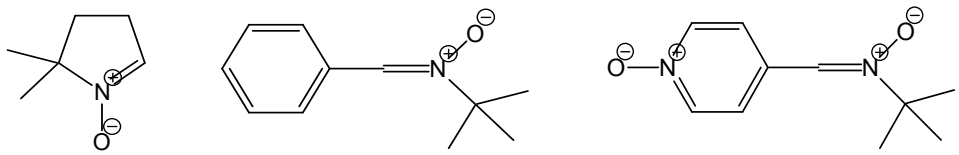


Figure 4. Resonance stablization of spin adduct

In nitroso compounds, the free radical directly adds to the nitrogen atom. The resulting spin adducts displays hyperfine nitrogen splittings because of the trapped radical whereas, in nitrone spin traps, the radical detected by ESR is influenced by the carbon adjacent to the nitrogen atom (because the free radical adds to the adjacent carbon). However, spin adducts obtained from nitroso compounds are often less stable, especially when oxygen-centered radicals are trapped compared to those obtained from nitrones. Furthermore, nitroso compounds are toxic are are thus less frequently used in biological systems. As a result of undesirable features, nitrones are preferred in biological systems despite the fact that spin adduct from nitroso compounds give better structural informations from ESR than do those from nitrones.

In early work, two kinds of spin traps have been developed, the five membered cyclic nitrone, 5,5-dimethyl-3,4-dihydro-2 *H*-pyrrole *N*-oxide (DMPO), and linear nitrones such as α-*N*-phenyl-*N*-tert-butylnitrone (PBN) and *N*-[(1-oxidopyridin-1-ium-4-yl)-methylidene]-1,1-isopropylamine (PyOBN). Of these, the linear nitrones PBN and PyOBN were found to be very inefficient in detecting superoxide anion by ESR.[238,239] α-*N*-phenyl-*N*-tert-butylnitrone (PBN) traps superoxide anion to form the superoxide spin adduct (PBN-OOH), which is highly unstable and decays into its corresponding hydroxyl adduct (PBN-OH) which also suffers a decomposition reaction. The kinetic studies showed that the rate of decay was first-order.[240] The half-life of the hydroxyl adduct is 38 s while that of the superoxide adduct is even less than that at neutral pH.[241] Therefore, the ESR spectra of these superoxide spin adducts are not very characteristic of the radical trapped. To improve their efficiency, efforts were made by elaborating phosphorylated analogues and replacing methyl group with ester moieties.[242,243] However, no better

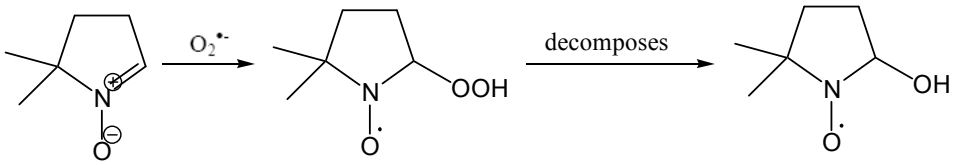
results have been observed in trapping superoxide with these derivatives despite the fact that they are efficient at trapping carbon-centered radicals. A kinetic study conducted by Tuccio et al demonstrated that cyclic nitrones are incomparably better at detecting superoxide anion that was than the linear nitrones.[244]



11 DMPO 12 PBN 13 PyOBN

Figure 5. Structures of DMPO, PBN and PyOBN

5,5-dimethyl-3,4-dihydro-2 *H*-pyrrole *N*-oxide (DMPO) is a cyclic nitrone the first to be used. It has been used as such for decades. Bonnett et al. first prepared DMPO as a model molecule towards the synthesis of corrins, which is the parent macrocyclic component of vitamin B_{12}.[245] The spin trapping property of DMPO with carbon-centered radicals was first examined by Iwamura et al. in 1967.[246] The spin adduct formed from the reaction was found to be very unstable. Later, Janzen et al. demonstrated the ability of DMPO to trap oxygen-centered radicals.[247] It was Harbour et al. who first brought attention to the use of DMPO for the detection of superoxide radical.[248] DMPO indeed can trap superoxide radical to produce a relatively stable DMPO-superoxide adduct (DMPO-OOH).

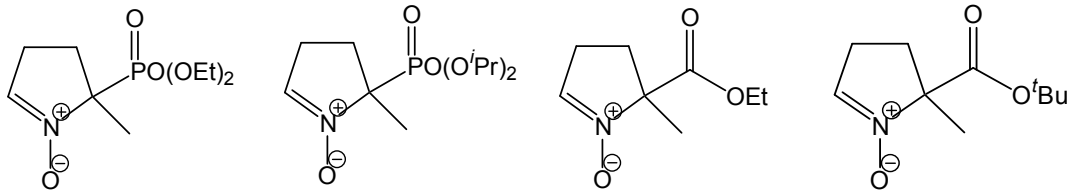


11 DMPO DMPO-OOH DMPO-OH

Figure 6. Reaction of DMPO with superoxide radical

The ESR spectrum of the spin adduct consists of hyperfine splitting because of the β–hydrogen and the nitroxide nitrogen. The hyperfine coupling constant of DMPO-OOH was reported as: A_N = 14.34 G, A_H^{β} = 11.7 G, A_H^{γ} = 1.25 G (gauss).[249] However, the DMPO-OOH adduct was found to be unstable and spontaneously decomposes into the DMPO-hydroxyl adduct (DMPO-OH) and a non radical species.[238] The stability of DMPO-OOH is pH dependent. Butterner and Oberley have reported the half-life of DMPO-OOH in the range of 27s and 91 s at pH 9 and pH 5 respectively.[250] It has been reported that a cyclic nitroxide radical possessing an α-hydrogen atom tends to disproportionate rapidly.[251] The hyperfine coupling constant of DMPO-OH is A_N = 14.874 G and A_H = 14.81 G (gauss). On the basis of a kinetic study, the second order rate constant for the reaction of DMPO with superoxide is only 10 $M^{-1}s^{-1}$ while that with hydroxyl radical is approximately 2 x 10 $M^{-1}s^{-1}$.[252] Glutathione peroxidase, which is present in most cells rapidly catalyzes the decomposition reaction. As a result, the superoxide dismutase-inhibitable formation of DMPO-OH was monitored for superoxide detection. However, the detection of DMPO-OH leads to misinterpretation of the free radical actually trapped because DMPO efficiently reacts with hydroxyl radicals to produce DMPO-OH adducts. Hence, the detection of DMPO-OH does not unequivocally

provide evidence for the identification of the hydroxyl radical either. Reaction of DMPO with neutrophils superoxide anion gave a number of EPR spectra including that of DMPO-OH while the experimental study demonstrated no evidence of hydroxyl radical in human neutrophil.[253] To overcome these drawbacks of DMPO, several DMPO-type analogues have been developed. These spin traps include the alkoxy-phosphorylated analogues: 5-diethyoxyphosphoryl-5-methyl-1-pyroline *N*-oxide (DEPMPO)[254] and 5-diisopropyloxyphosphoryl-5-methyl-1-pyroline *N*-oxide (DIPPMPO).[255] As expected, the spin adduct DEPMPO-OOH and DIPPMPO-OOH did not decompose into the corresponding hydroxyl adduct and have higher persistency compared to DMPO-OOH.



14 DEPMPO 15 DIPPMPO 16 EMPO 17 BocMPO (BMPO)

Figure 7. Structures of DEPMPO, DIPPMPO, EMPO and BocMPO

The first ESR evidence of superoxide generation, in ischemia/reperfusion of isolated rat myocardium, has been detected by using the DEPMPO spin trap.[256] However, superoxide detection using DEPMPO and DIPPMO remains limited in biological systems because of the confounding ESR spectrum because of an additional ^{31}P hyperfine coupling, the existence of diastereomers, and formation of ESR silent products.

Since the presence of an electron withdrawing dialkoxy-phosphoryl moiety in the

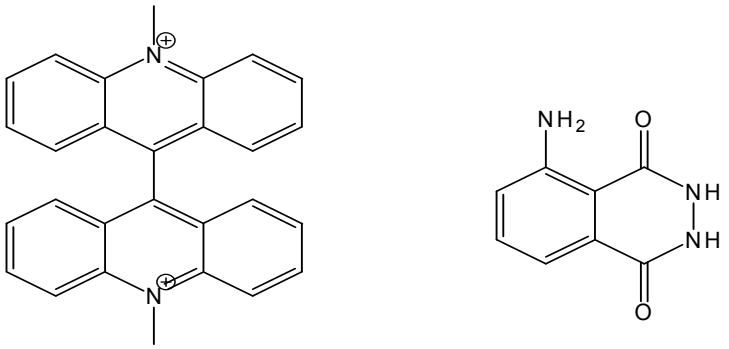
β-position of the nitrone function stabilizes the superoxide adduct, investigators have turned to the synthesis of alkoxy-carbonyl nitrones, 5-ethoxycarbonyl-5-methyl-1-pyroline *N*-oxide (EMPO),[257] its isotpoically labeled nitrone (^{15}N) EMPO,[232] and 5-tert-butoxycarbonyl-5-methyl-1-pyroline *N*-oxide (BocMPO or BMPO). The presence of an ester function in EMPO and (^{15}N) EMPO improved the ESR signal to noise ratio and increased the stability of its superoxide adduct (EMPO-OOH) compared to that of DMPO-OOH. Moreover, EMPO-OOH did not decompose into its corresponding adduct EMPO-OH. However, the major disadvantage of EMPO is that EMPO-OOH decays faster than DEPMPO-OOH. The relative half-life of EMPO-OOH is 8 min while that of DEPMPO-OOH is 14 min. Steric hindrance may play a role in the stability of nitroxide molecule.[258] In terms of stability of the superoxide adduct, BMPO-OOH is slightly more stable than EMPO-OOH. Similar to EMPO-OOH, BMPO-OOH does not decay non-enzymatically to the BMPO-OH adduct. The time-dependent change in ESR spectra demonstrated that BMPO-OOH adduct could be detected even up to 35 min while the EMPO-OOH signal intensity was not detectable at this time-point. The BMPO-derived adduct also provided a higher signal to noise ratio in biological systems. Even with these ideal characteristics, it has some limitations. For instance, the enzyme glutathione peroxidase can readily decompose BMPO-OOH into BMPO-OH adduct, making the ESR spectrum difficult to interpret in biological systems. The rate constant for the superoxide trapping reaction of EMPO was reported three times higher than BMPO at pH 7.2 which indicates that EMPO is superior to BMPO.[243] At the same pH, the rate constant of BMPO is fairly equal to DEPMPO, but at pH 7.4, the rate constant of DEPMPO is twice as much as BMPO[259] suggesting DEPMPO is twice as efficient as BMPO at trapping

superoxide. One of the problems of using DEPMPO is that it decomposes on heating which makes its preparation in high purity very difficult.

Despite the tremendous efforts in developing new ESR suitable nitrone spin traps to improve spin trapping of superoxide, the intracellular detection of superoxide using ESR spectroscopy remains a big challenge. Several groups have turned to other techniques for superoxide detection.

[1.12.2] Chemilumigenic probes for detecting superoxide radical

In the chemiluminescence-based technique, a chemiluminescent probe, upon exposure to superoxide anion, emits a photon, which, in turn, can be detected by a luminometer or scintillation counter. The sensitive measurement of the emitted photons reflects the biological production of superoxide. The possibility of detecting superoxide



18 Lucigenin (DBA^{++}) 19 Luminol

Figure 8. Structures of lucigenin and luminol

by use of a chemiluminescent probe came from Totter et al.,[260] who postulated that oxygen radicals in the reaction caused emission of light. Later, Fridovich[261] demonstrated the involvement of superoxide anion in the system of xanthine oxidase/xanthine and a chemiluminescent compound leading to the emission of light.

The most widely used compound in this study was bis-*N*-methylacridimium nitrate (lucigenin or DBA^{++}) because of its sensitivity. Lucigenin is cell permeable, hence, detected superoxide reflects both intracellular and extracellular superoxide production. Lucigenin-derived chemiluminescence has been used in the specific detection of superoxide formation by *in vitro* isolated enzymes, vascular tissue[262] and inflammatory cells, such as neutrophils and macrophages.[263] It has also been used as an indicator of superoxide production in numerous disease conditions such as various form of hypertensions,[264] diabetes mellitus,[265] and hypercholesterolemia.[266] It is also possible to detect mitochondrial superoxide production by using lucigenin.[267]

Other chemiluminescent compounds include 5-amino-2,3-dihydroxy-1,4-phthalazinedione (luminol) and cypridina luceferin analogs such as 2-methyl-6-phenyl-3,7-dihydroimidazo (1,2-α)-pyrazin-3- one (CLA), 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo (1,2-α)-pyrazin-3-one (MCLA), and 2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl) 8-benzyl-3,7-dihydroimidazol[1,2-α]pyrazin-3-one (coelenterazine).[268] Luminol is oxidized by various ROS such as hydrogen peroxide, hydroxyl radical, peroxynitrite, and hypochlorous acid besides superoxide anion. MCLA has also shown to emit light in response to hydroxyl radical and other ROS.[269] Thus, these molecules are not specific for detecting superoxide anion.

The potential mechanism of reaction of lucigenin with superoxide anion is illustrated in Fig. 2. Lucigenin first undergoes one electron reduction by superoxide anion

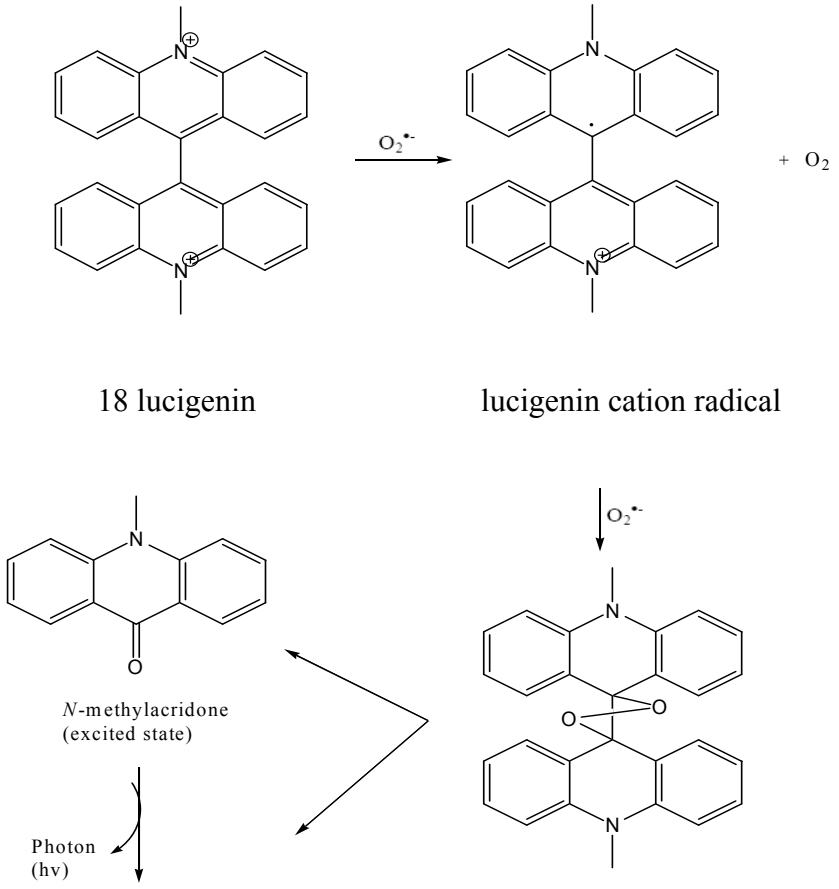


Figure 9. Reaction between lucigenin and superoxide radical

to produce lucigenin cation radical which, in turn, reacts with a second molecule of superoxide anion to yield a highly unstable energy-rich lucigenin dioxetane intermediate.[270] The lucigenin dioxetane finally decomposes to produce two molecules of *N*-methylacridone, one in an exicited state, which relaxes to the ground state by emitting photons. By measurement of the photon emission, superoxide production in biological systems can be detected.

However, the credibility of lucigenin-derived chemiluminescence for biological superoxide detection has been criticized because superoxide production might be incorrectly detected as a result of a phenomenon called redox cycling.[4] It turned out that in *in vitro* enzymatic systems, lucigenin itself can produce superoxide via autoxidation of lucigegnin cation radical[5] (Fig. 3). The reaction is accelerated by flavin containing enzymes such as xanthine oxidase, endothelial NO synthase, and cytochrome P450 monooxygenases. Furthermore, the autoxidation reaction depends on the concentration of superoxide anion. If the biological superoxide anion level is low, the relative contribution of superoxide via redox cycling to the measured superoxide anion will be higher than the contribution when biological superoxide level is higher. Several groups reported that redox cycling depends on the concentration of lucigenin. At higher concentration, lucigenin reacted with several flavin containing enzymes to generate superoxide anion but at low concentration it failed to stimulate superoxide generation. If lucigenin has to be used to detect superoxide anion under a particular condition, a safe non- redox cycling concentration of lucigenin has to be determined by either the measurement of stimulation of oxygen consumption by oxygen polarography or detection of stimulation of superoxide production by the DEPMPO spin trap.[271] Thus, this experiments suggests that to validate the result obtained from lucigenin-derived chemiluminescence, a second method has to be used for confirmation.

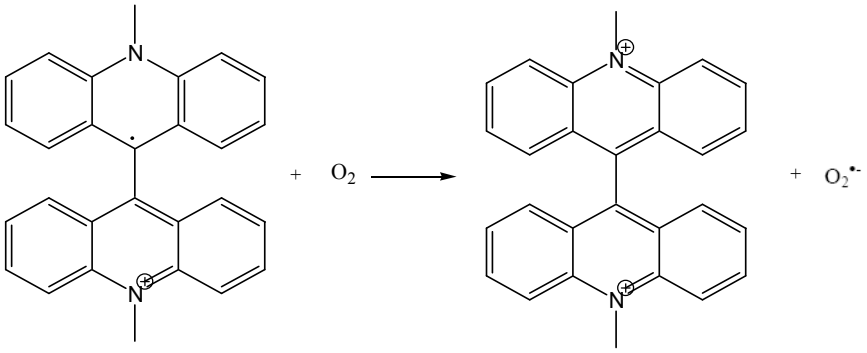
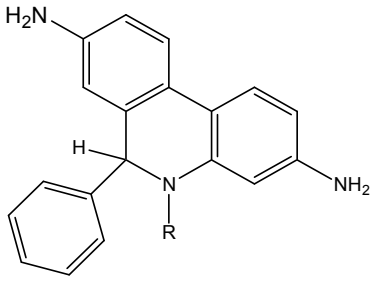


Figure 10. Formation of superoxide anion from lucigenin cation radical

[1.12.3] Fluorescent probes for detecting superoxide radical

The fluorescence-based technique has been widely used for *in vitro* and *in vivo* detection of superoxide anion. Recently, the most popular fluorogenic probe used for detecting intracellular superoxide production is hydroethidine (HE), also known as dihydroethidium (DHE) and its mitochondria-targeted analogue (MitoSox red), in which the ethyl group of hydroethidine was replaced by a triphenylphosphonium moiety to enhance the accumulation of the molecule into mitochondria. Although the intracellular distribution of HE and MitoSox red is different, their chemical reactivities in redox reactions are quite similar.[272]

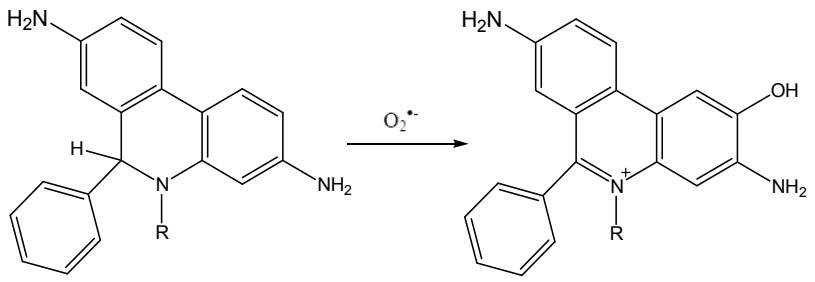


R= $-C_2H_5$ 20 HE
$-(CH_2)_6P^+(Ph)_3$ 21 MitoSox red

Figure 11. Structure of hydroethidine and MitoSox red

Initially, it was assumed that hydroethidine, upon exposure to superoxide anion, formed the two-electron-oxidized product ethidium cation (E^+),[6] which would, in turn, intercalate with DNA due to its charge,[273] yielding red fluorescence (exicitation ~ 500 nm and emission ~ 600 nm), and the intensity of the signal would reflect the level of superoxide production. However, recent publications suggest that hydroethidine reacts with superoxide anion to form a specific product, 2-hydroxyethidium (2-OH-E^+). The mass spectrometric analysis indicated that the molecular weight of the product is 16 U greater than E^+ which is consisted with 2-OH-E^+ (Fig. 4).[274]

Detection of superoxide using hydroethidine has been executed in many biological systems including whole cells and whole organs in live animals over the past two decades.[275] Red fluroresence derived from the reaction of hydroethidine and MitoSox red with superoxide anion have been observed in many studies such as neurodegeneration and neuroprotection,[276] neurosignaling,[277] ischemia and reperfusion,[278] diabetes,[279] hypertension,[280] oxidative burst in leukocytes,[281] vascular signaling and pathology,[282] renal function and pathology,[283] the anti and proapoptotic action of drugs,[284] and mitochondrial and radiation-induced damages.[285] All of these studies demonstrated the involvement of superoxide anion in many physiological and pathological conditions. Using HE, Sorescu et al[286] described the dramatic increased production of superoxide anion in human atherosclerotic lesion, especially plaques of the shoulder.



R= -C_2H_5 20 HE 2-OH-E^+

-$(CH_2)_6P^+(Ph)_3$ 21 MitoSox red 2-OH-Mito-E^+

Red fluorescence

Figure 12. Reaction of hydroethidine and MitoSox red with superoxide anion

Many researchers found the fluorescence-based assay using hydroethidine and MitoSox more convenient compared to the ESR technique which requires specific training. Currently, MitoSox red is commercially available for research purposes.

In a recent review, the detection of intracellular superoxide anion through observation of red fluorescence derived from hydroethidine and MitoSox red was described as an unreliable approach for superoxide detection.[275,287] Hydroethidine and MitoSox red are prone to poor specificity for detecting superoxide anion because they can be easily oxidized (via loss of the elements of a hydride anion) by other oxidants besides superoxide anion in biological systems, and some of which yield a red fluorescence product different from 2-OH-E^+ and 2-OH-Mito-E^+. Generally, the reaction between HE and superoxide anion, generated by potassium superoxide, produces the increased red fluorescence intensity, however, some reporters observed the decreased fluorescence intensity in the presence of a higher concentration of superoxide anion. The decreased fluorescence intensity could be because of the oxidation reaction between HE and other oxidants. Earlier it was reported that HE does not readily react with hydrogen

peroxide.[288,289] However, in the presence of metal ions such as iron or copper[290] and horseradish perxodase (HRP)[291] HE is easily oxidized by hydrogen peroxide and organic peroxide with the formation of E^+ along with nonfluoresent products. Since ferricytochrome c (cyt c^{3+}) can react with HE with the formation ferrocytochrome c (cyt c^{2+}),[6] complexes of iron and/or cytochromes may accelerate the reaction of HE and peroxides in the biological systems. The same review reported the reduction of up to four molecules of cyt c^{3+} by one molecule of HE to form E^+ and, most likely, the dimeric products. HPLC analysis of the products from the reaction between HE and cyt c^{3+} indicated the formation of E^+, E^+-E^+ , HE-HE and HE-E^+.[272] Similar reaction has been observed from MitoSox red with the mitochondrial cytochrome forming Mito-E^+ and several dimeric products.

In addition to this, HE has also been found to react with hydroxyl radical and with H_2O_2 under Fenton conditions to provide the fluorescent product 2-OH-E^+.[291,292] However, other publication indicated the formation of HE radical cation from the reaction of HE with hydroxyl radical by direct monitoring of pulse radiolysis.[293] Some evidences indicated the reaction of HE with NO radical, and nitrogen dioxide radical ($^{\bullet}NO_2$) yielding flurescent products.[290] Other oxidants that can oxidize HE include peroxynitrite with[274] or without CO_2,[291] hypochlorous acids and singlet oxygen.[294] Upon irradiation with UV light, HE undergoes photooxidation in the presence of oxygen with the formation of 2-OH-E^+, which further undergoes oxidation to form E^+.[295]

Given that the excitation light between 350 and 400 nm represent 2-OH-E^+ and 2-OH-Mito-E^+ but not E^+ or Mito- E^+ , E^+ can still absorb light at this wavelength and emit fluorescence. On the other hand, since the level of detected E^+ in the biological systems is

almost 10 fold higher than that of 2-OH-E^+, the fluorescence intensity could be significantly contributed by E^+.[296] These drawbacks emphasize the present limitations of using fluorescence-based detection of superoxide in intact cells or tissue. Several groups find that high performance liquid chromatography (HPLC) is the only currently available methodology to separate HE and its oxidation products including 2-OH-E^+ thus allowing unambiguous determination of the origin of the red fluorescence. Up until now, HPLC based assay is the only method of choice for superoxide detection using MitoSox red. Some drawbacks of using HPLC could be the lengthy analysis time and the optimization of the sample extraction method.

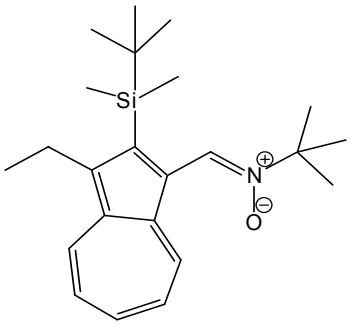
[2] RESEARCH OBJECTIVE

The objectives of this research are to design and synthesize various novel silylated azulenyl nitrones as spin traps for chromotropic detection of superoxide anion radical, to study their chemical properties and to study their reaction with superoxide anion. Towards this aim, the following nitrones were pursued.

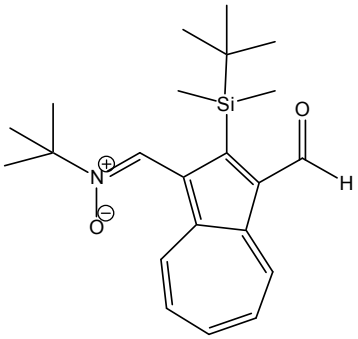
[2.1] Silylated azulenyl nitrones:

Nitrones are known as good candidates to react with superoxide anion by spin trapping yielding detectable diamagnetic spin adducts. A silyl substituent, a non-polar, oxophilic, electropositive group, has a strong propensity to react with an oxygen anion such as that which is present in a superoxide spin adduct. Hence, the presence of a nearby silyl group in nitrone molecules is expected to confer distinctive reaction pathways with superoxide that are not available in traditional nitrones. Moreover, because of colorimetric properties of azulene compounds, the end products of the spin trapping would likely yield characteristically colored molecules. Hence, the following silylated

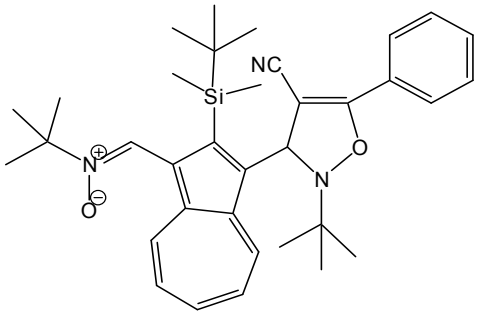
azulenyl nitrones 22, 23 and 24 are putatively equipped specifically for superoxide anion detection by likely yielding characteristically colored end products with a characteristic UV-VIS spectral profile upon reaction with superoxide anion.



Compound 22



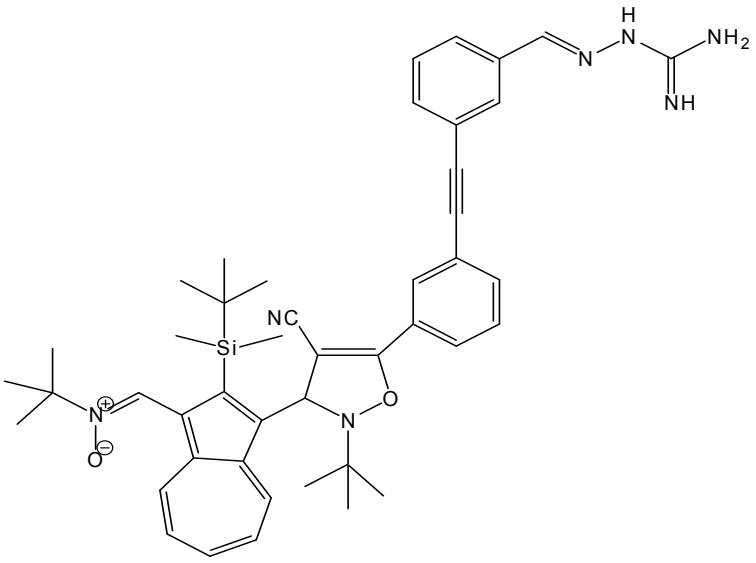
Compound 23



Compound 24

[2.2] Mitochondria-targeted silylated azulenyl nitrone:

Mitochondria are the major sites of superoxide production. To detect mitochondrial superoxide, the chemical structure of compound 24 was modified by incorporation of a guanylhydrazone moiety. Since guanylhydrazones are protonated at physiological pH, the attachment of this moiety to compound 24 is expected to increase the permeability of compound 25 into the mitochondrial membrane to reach sites of superoxide anion generation. The structure of compound 25 is given below:



Compound 25

[3] RESULT AND DISCUSSION

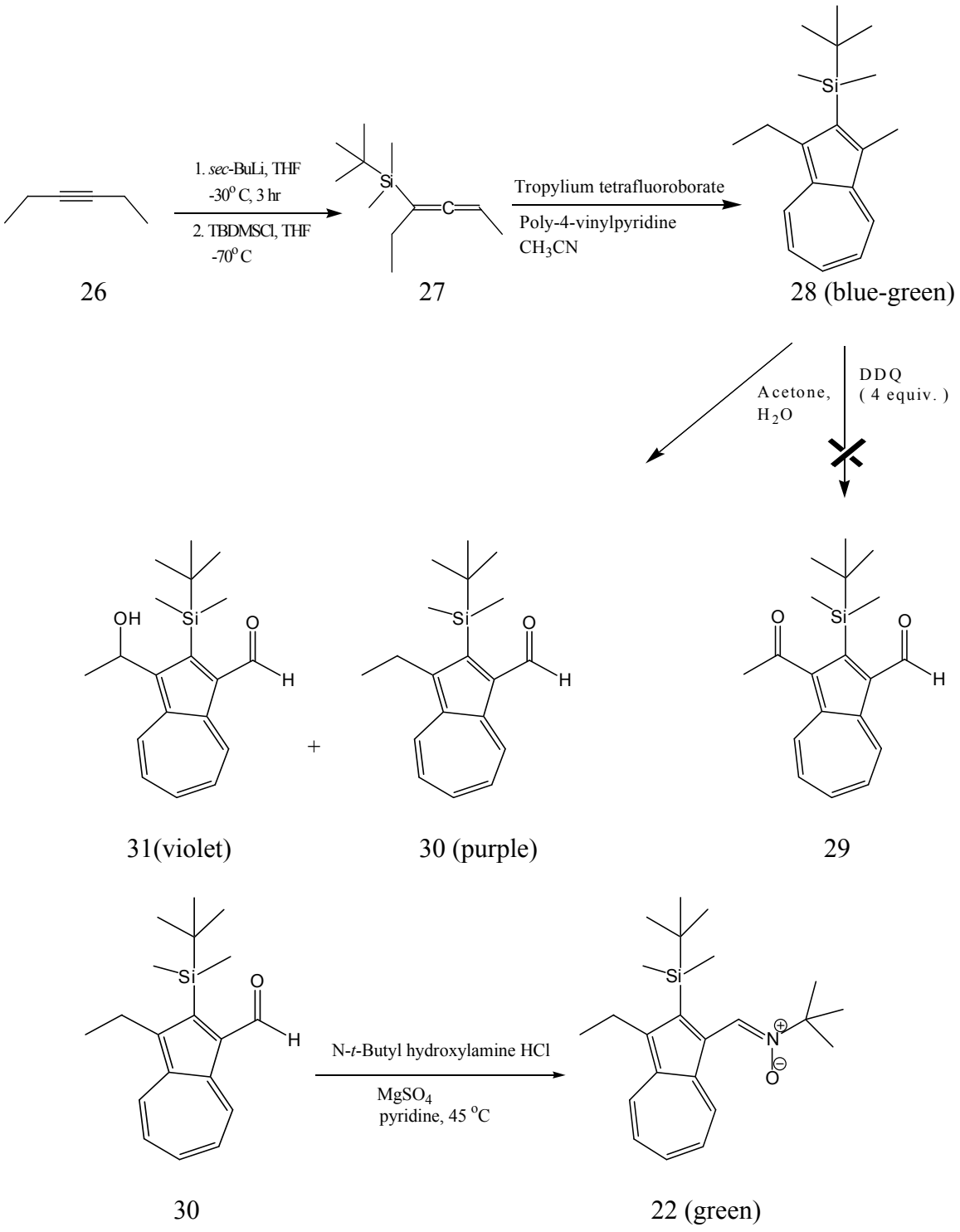
[3.1] Synthesis of azulenylsilane nitrones:

Nitrones have been shown to exhibit an efficient antioxidant activity *in vitro* and likely *in vivo* by trapping reactive free radicals.[223,297] Because of their spin trapping properties, numerous nitrones have been developed for detecting superoxide anion in biological systems in assays that require ESR spectroscopy. Superoxide adducts thereby formed were found to be very unstable and this reduced persistence complicates their detection by ESR. The research project described here was to synthesize silylated azulenyl nitrones for superoxide detection that could selectively yield a characteristic product after the oxidation reaction with superoxide anion, which could be detected by UV-VIS and simple colorimetric techniques without using ESR spectroscopy.

[3.1.1] Synthesis of ethylated azulenylsilane mononitrone 22:

Towards our goal, the first synthetic effort in the preparation of ethylated

azulenylsilane nitrone (22) involved four efficient steps starting from commercially available 3-hexyne as illustrated in scheme 1. Allenylsilane (27) was prepared in one step employing a metalation reaction according to the method reported in the literature.[298] The metalation reaction was carried out by the addition of 3-hexyne to a cooled sec-BuLi solution in THF and left for 3 hr which formed the monolithiated product. The metalation mixture was then silylated by treatment with excess *tert*-butyldimethylsilylchloride at -70^{o}C and then left overnight at room temperature (10 %). Danheiser and Becker reported the preparation of several azulenylsilanes via cyclization reaction between allenylsilanes and tropylium cations.[299] Following the same protocol, azulenylsilane (28) was synthesized by addition of 2.2 equiv. of tropylium tetrafluoroborate and 3.3 equiv. of poly-4-vinylpyridine in acetonitrile (53 %) which underwent (3 + 2) annulation involving rearrangement and cyclization. Attempted oxidation of the 1-ethyl and 3-methy group of azulene (28) by treatment with 4 equiv. of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ)[300] failed to give the expected ketoaldehyde (29). Instead the unexpected product ethyl aldehyde (30) in 53 % yield and the by-product alcohol (31) were obtained from NMR data. It is obvious from the product that partial oxidation might have taken place due to steric hindrance of the silyl group and its substituents. It was possible to optimize the yield of ethylaldehyde (30) from 53 % to 70 % using only 2 equiv. of DDQ for oxidation reaction. In one attempt, during the preparation of aldehyde (30), approximately 2.5 % of bis-aldehyde (32) was also recovered. Condensation of



26 27 28 (blue-green)

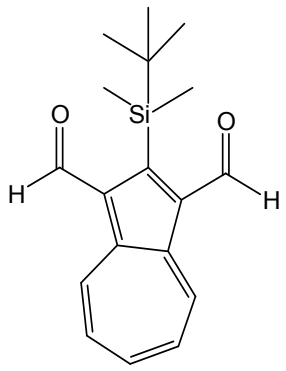
31(violet) 30 (purple) 29

30 22 (green)

Scheme 1. Synthesis of azulenylsilane ethylnitrone 2

ethylaldehyde (30) with N-*tert*-butylhydroxylamine hydrochloride in pyridine at 45^{o}C ethylated azulenylsilane nitrone (22) in 47 % yield.

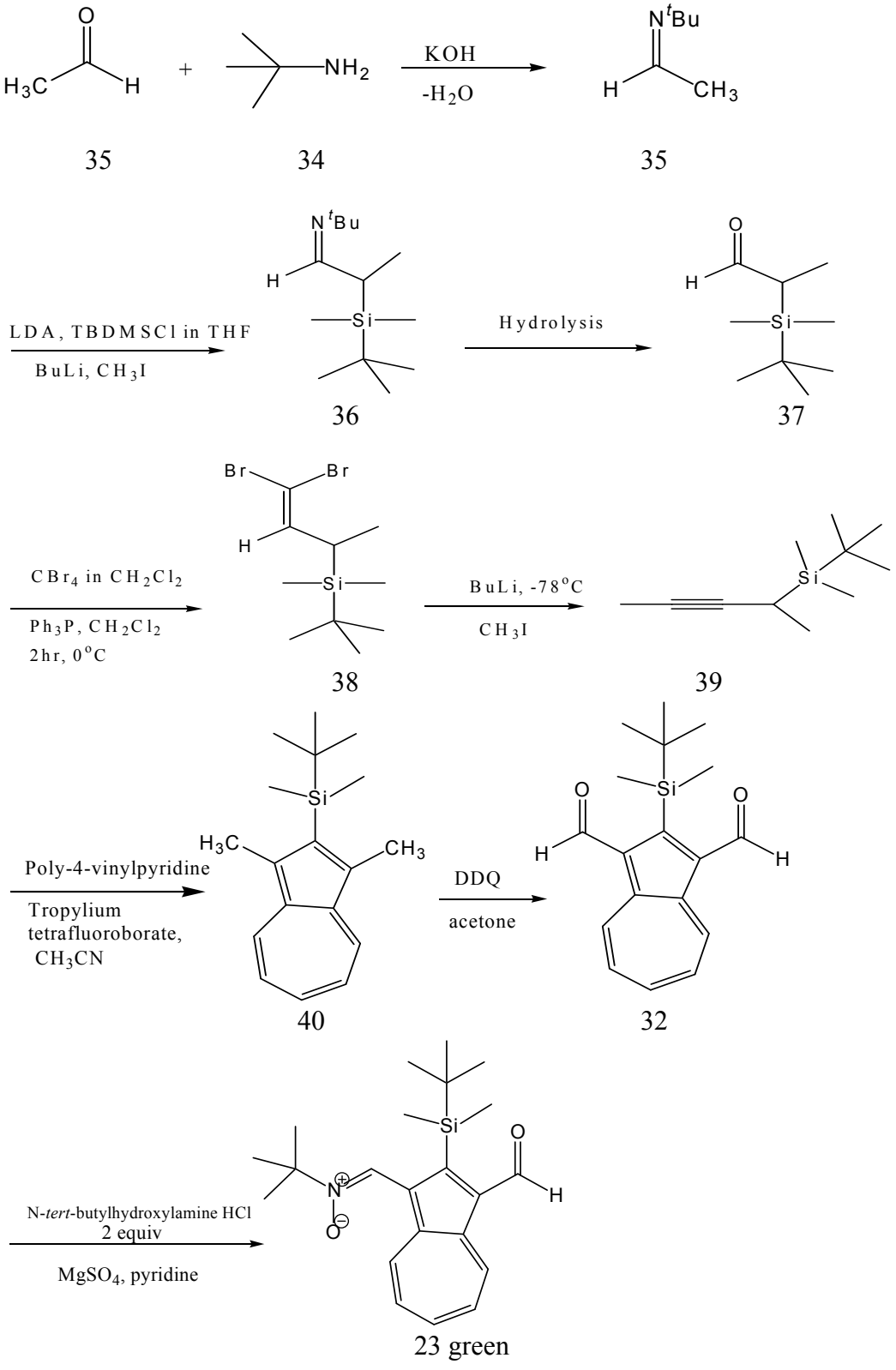
Synthetically, bis-aldehyde 32 was a desirable intermediate for the synthesis of other compounds. For example, compound 32 would prove to be a useful precursor for the preparation of other nitrones that might have significance in trapping superoxide anion (vide infra). Keeping this in mind, efforts were made to synthesize 32 efficiently.



32

[3.1.2] Synthesis of azulenylsilane carboxaldehyde nitrone:

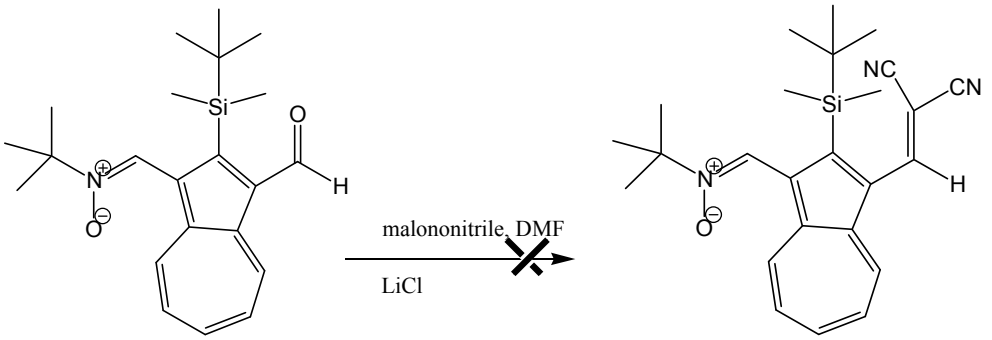
In an attempt to synthesize mononitrone (23) from the red aldehyde 32, readily available acetaldehyde (33) was used as the starting material. N-*tert*-butylimine (35) was prepared from addition of acetaldehyde to tert-butylimine (34) at 0^{o}C (without any solvent) followed by distillation according to the modified method of Chancel[301,302] as illustrated in scheme 2. The N-*tert*-butylimine (35) was then deprotonated with LDA and silyated by using TBDMSCl followed by repeated deprotonation with BuLi, and methylation with iodomethane which afforded mono methylated α-silylimine (36) in a one-pot process reported by Tietze.[303] The methylated silylimine (36) is quite unstable and was easily hydrolyzed by washing through a silica gel column using petroleum ether



Scheme 2. Synthesis of azulenylsilane carbaldehydenitrone 23

and ethylacetate which gave aldehyde (37). A Corey-Fuchs reaction involving the treatment of aldehyde (37) with a mixture of triphenylphosphine and tetrabromomethane in CH_2Cl_2 at 0^oC provided dibromoalkene (38) via a Wittig-like reaction. Subsequently, treatment of dibromoalkene (38) with BuLi followed by exposure to iodomethane yielded alkynylsilane (39) via the generation of a bromoalkyne intermediate that formed in a process consisting of dehydrohalogenation and metal-halogen exchange. A (3+2) annulation reaction between alkyneylsilane (39) and tropylium tetrafluoroborate in acetonitrile in the presence of excess poly-4-vinylpyridine resulted in formation of blue azulenylsilane (40).[299] Oxidation of two methyl groups of azulenylsilane (40) with four equivalents of DDQ[300] in the presence of acetone afforded bis-aldehyde (32) which was converted into the ethylated azulenylsilane mononitrone (23) by reaction with two equivalents of N-*tert*-butylhydroxylamine hydrochloride in pyridine at 45^oC.

It was presumed that the incorporation of electron withdrawing group such as the cyano moiety in nitrone 23 might produce a stable end product generated from the spin trapping reaction. Spin trapping of superoxide anion by nitrone 23 would produce an unstable intermediate, containing oxygen with a negative charge at the 2- position, which can undergo nucleophilic attack at the cyano group forming a stable six membered α-pyrone ring, diagnostic for the superoxide detection, with a characteristic UV-vis spectral profile.

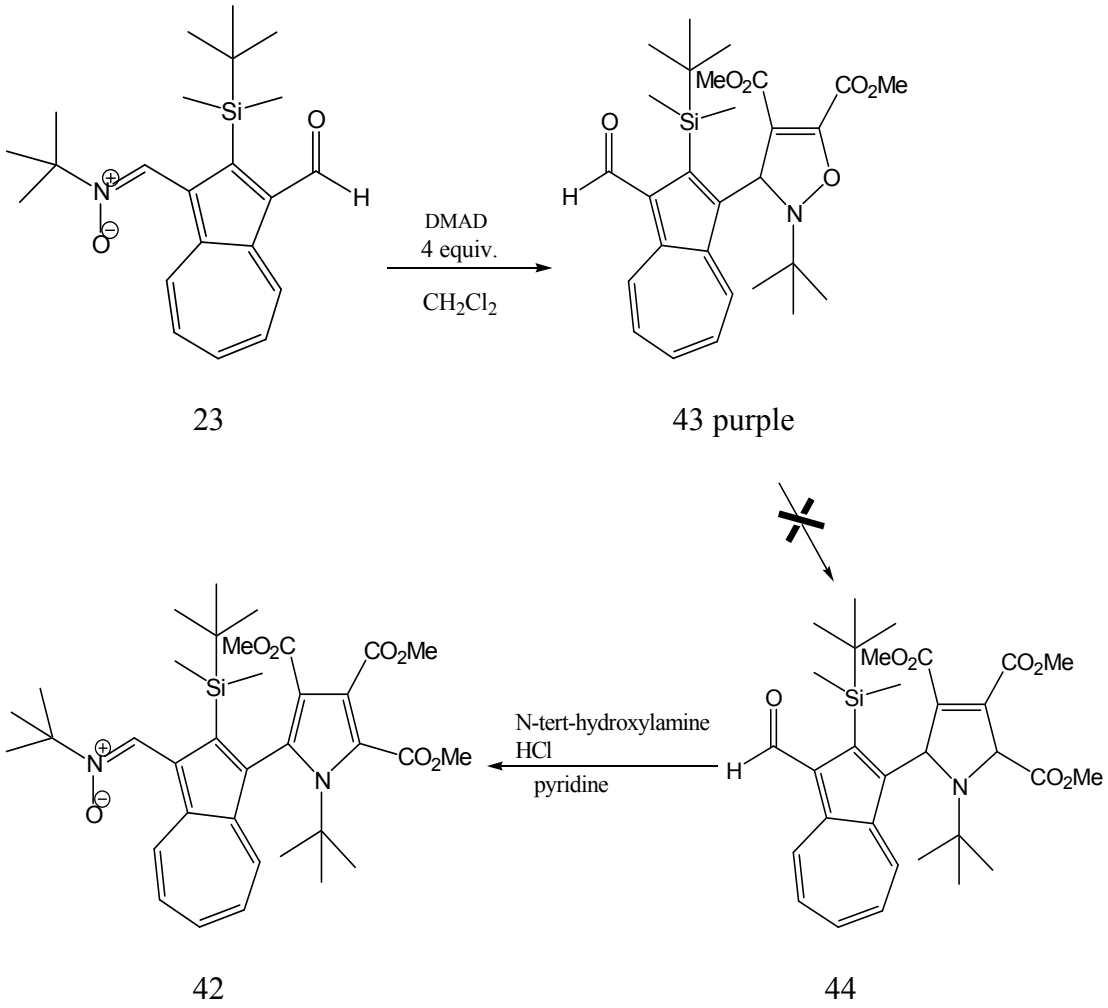


23 41

Scheme 3. Attempted synthesis of malononitrile derivative 41

However, several attempts to synthesize the malononitrile derivative 41 either by using weak lewis acid in DMF[304] or weak basic condition with malonitrile failed (scheme 3). The NMR analysis of the product indicated the absence of silyl methyl-protons and the silyl tert-butyl protons. Failure to form the desired malononitrile derivative could be the result of the fact that alkoxide might have attacked the silyl group which was eventually cleaved from the azulene ring.[305]

After failure to synthesize compound 41, other attempts were made to incorporate an ester group to mononitrone 23 in hopes of preparaing tricarboxylicacid triester nitrone 42 via 1,3-cycloaddition reaction. For example, compound 42 might undergo a spin trapping reaction with superoxide anion to produce a stable six membered α-pyrone ring system, which might have a characteristic UV-VIS spectral profile. Nitrones are well known to undergo 1,3-dipolar cycloaddtion reaction efficiently with a variety of alkynes to afford 4-isoxazoline.[306,307] It is known that nitrones react with DMAD (dimethyl acetylenedicarboxylate) to give 4-isoxazolines which undergo rearrangement to afford azomethine ylides via the isomerization to acylaziridines.[308] Generated azomethine ylides

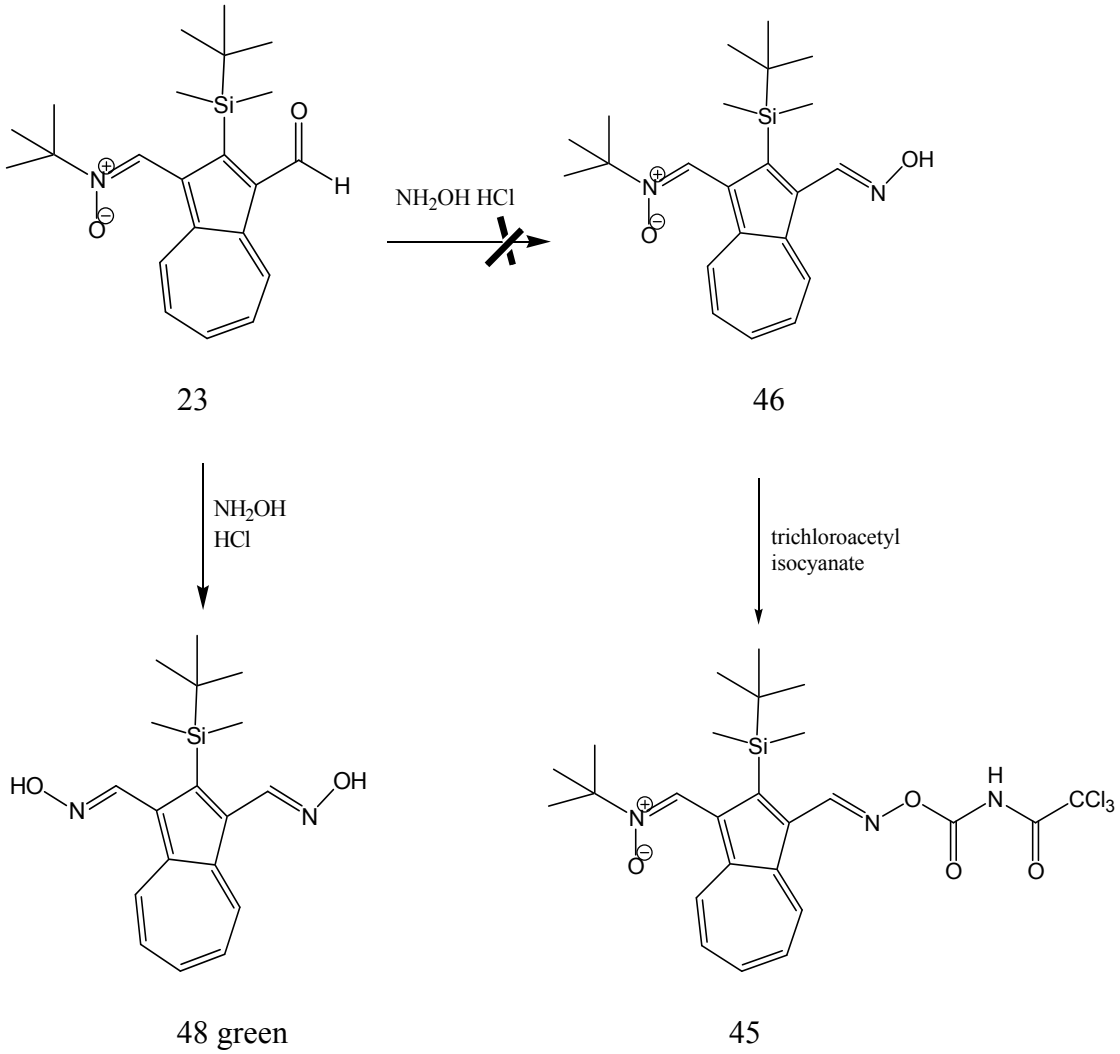


23 43 purple

42 44

Scheme 4. Attempted synthesis of tricarboxylic acid triester derivative 42

can be trapped by a second cycloaddition with DMAD to potentially obtain compound 44.



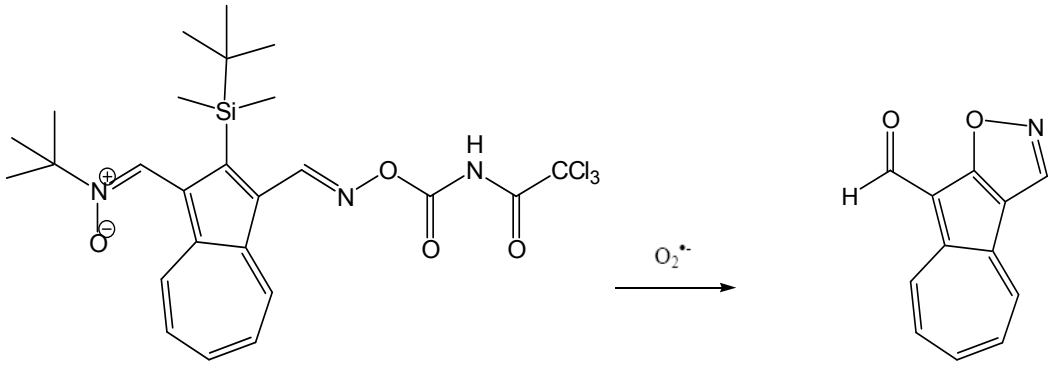
23 46

48 green 45

Scheme 5. Attempted synthesis of nitrone 45

Unfortunately, the reaction failed to give the expected product when nitrone 23 was treated with 4 equivalent of DMAD. Instead, the intermediate product 43 was obtained, illustrated in scheme 4, which was identified by NMR data. Failure to achieve the formation of triester 43 suggests that only one equivalent of DMAD had been consumed in a 1,3-dipolar cycloaddition reaction to afford cyaloaddition adduct 43, which thereafter failed to open the isoxazoline ring to rearrange to azomethine ylide before a second cycloaddtion reaction with another equivalent of DMAD could occur.

Another alternative could be the synthesis of nitrone 45 from nitrone 23. A condensation reaction of nitrone 23 with hydroxylamine hydrochloride was postulated to give oxime 46. Treatment of oxime with trichloroacetyl isocyanate would afford carbamoylaldoxime 45 as described by Stokker et al.[309] (scheme 5). Subsequently, spin trapping of superoxide anion with nitrone 45 would give the end product 47 which would be formed from the intramolecular cyclization by nucleophilic substitution of the *ortho*-negative charge of oxygen to nitrogen as shown in figure 13. The evidence of spontaneous cyclization[309] has been observed by Stokker in his effort to synthesize carbamoylaldoxime from the compound containing a phenolic group and an oxime group in ortho position. Instead, the only product obtained was 1,2-benzisoxazole that appeared to be formed via spontaneous cyclization by attack of the *ortho*-OH group on nitrogen without addition of any base. A similar cyclization mechanism that leads to benzisoxazole was also suggested by Searcey.[310] However, the reaction failed to give nitrone 45. The only product formed from this reaction was dioxime 48 which was identified by its NMR spectrum and its low resolution mass spectrum.

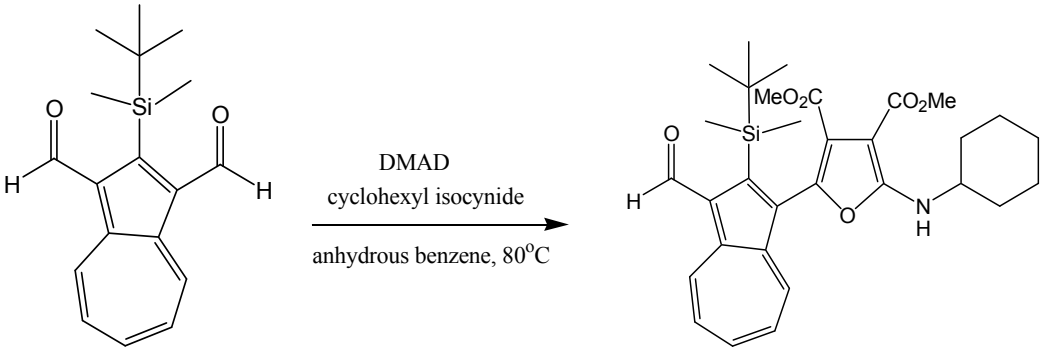


45 47

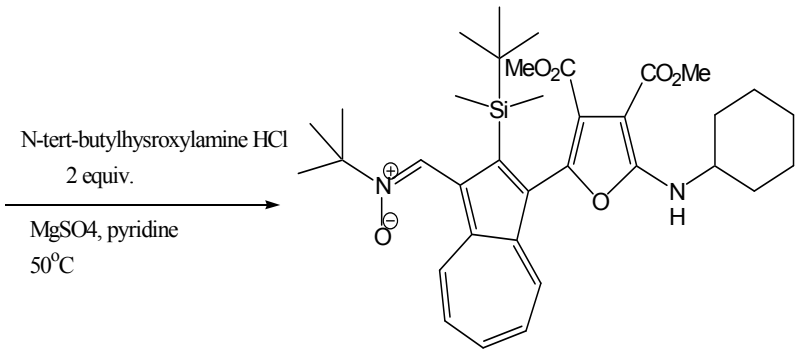
Figure 13. Expected product 47 from spin trapping of superoxide anion with 45

[3.1.3] Synthesis of azulenylsilane aminofuran nitrone:

Unsuccessful synthesis of nitrone 45 led to the development of a synthetic route to aminofuran 49 as illustrated in the scheme 6. A one-pot approach involving the treatment of bis-aldehyde 8 with cyclohexyl isocyanide and DMAD at 80°C in anhydrous benzene provided aminofuran derivative 50. The reaction proceeded with the initial formation of a zwitterionic intermediate derived from isocyanide and DMAD which is trapped by bis-aldehyde 32.[311] The condensation reaction between aminofuran derivative 50 and N-*tert*-butylhydroxylamine hydrochloride in pyridine at 45°C afforded aminofuran nitrone 49.



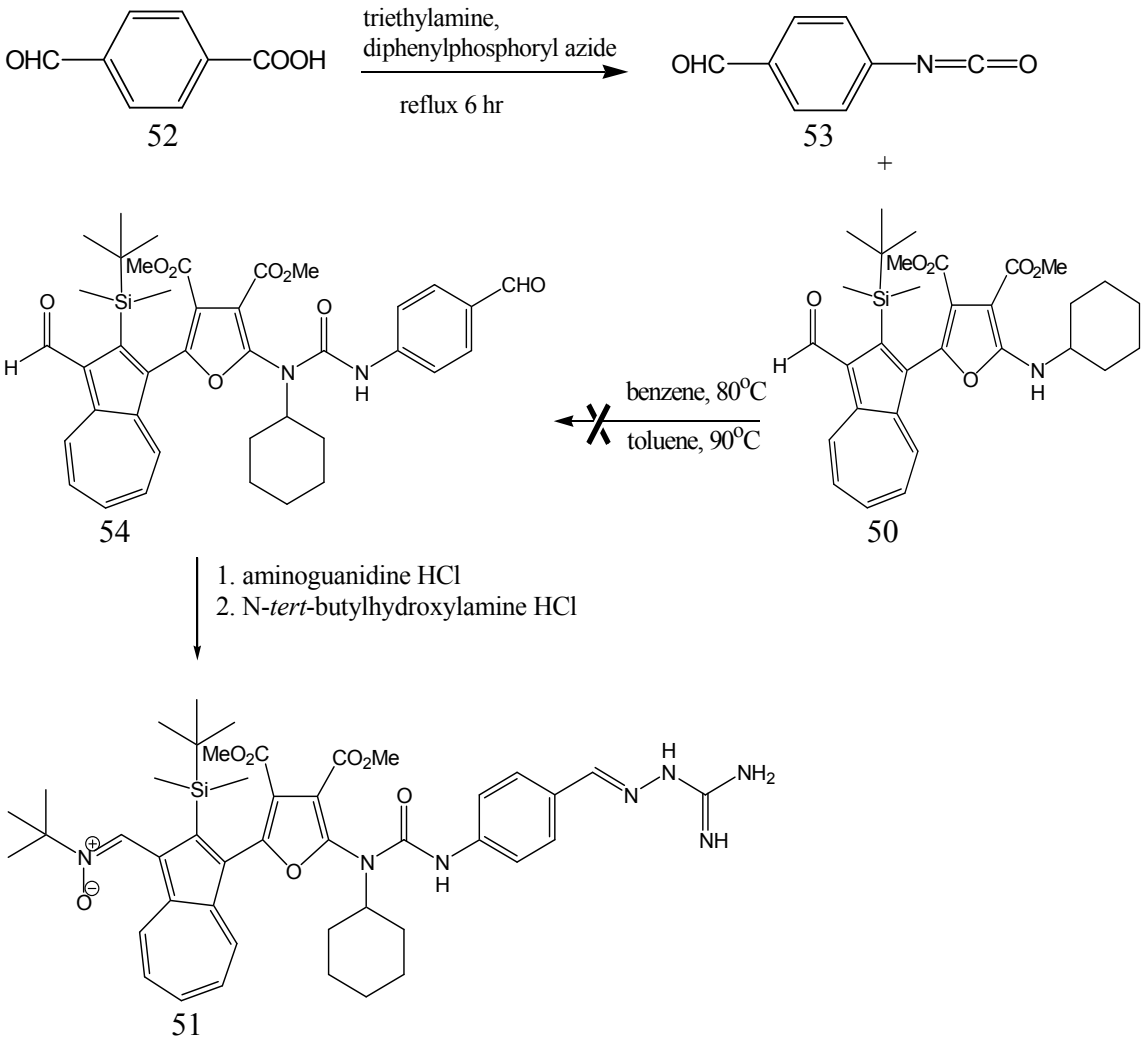
32 50 purple



49 green

Scheme 6. Synthesis of aminofuran nitrone 49

With the successful synthesis of aminofuran nitrone, an attempt was made to improve hydrophilicity by modifying compound 49 in order to detect superoxide generated in mitochondria. The incorporation of a guanylhydrazone moiety was sought as a means of increasing hydrophilicity. Since a guanylhydrazone is positively charged at physiological pH, it may traverse across cellular membranes and therefore aid in the accumulation of nitrone into mitochondria.

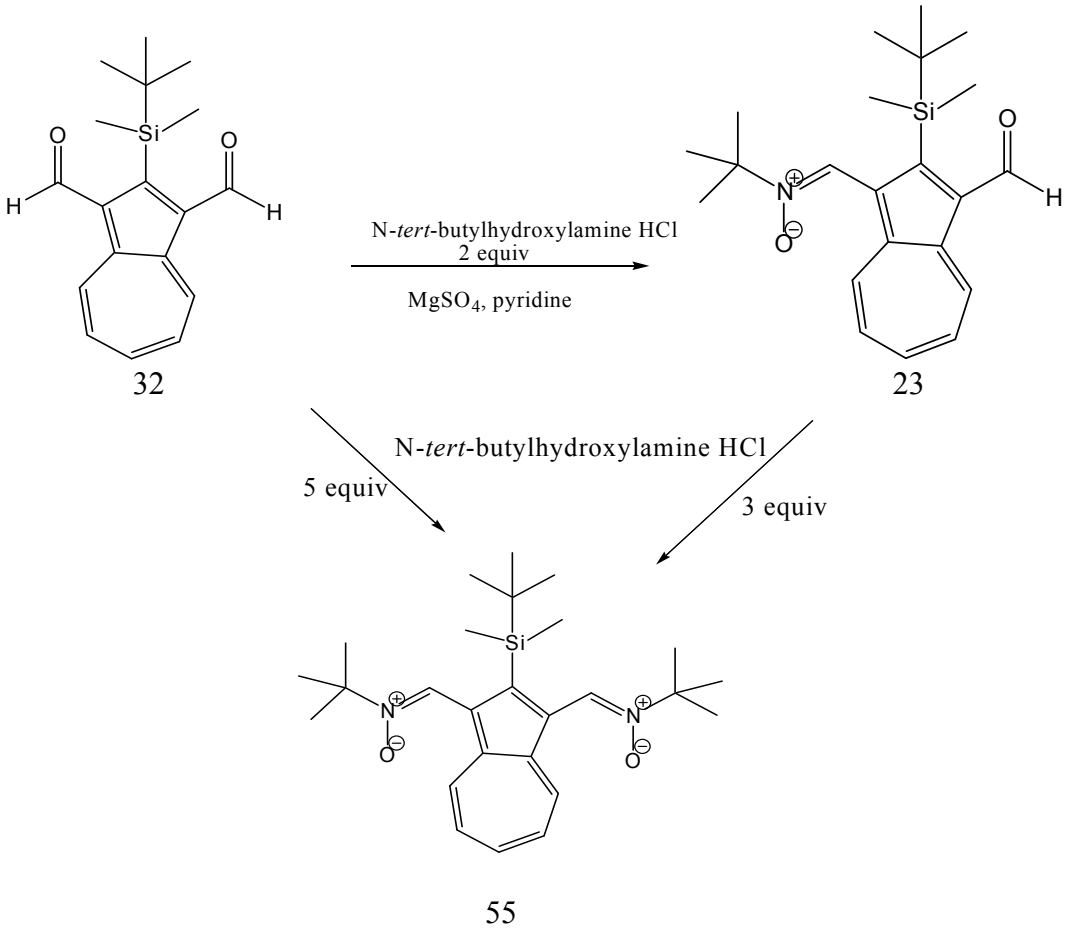


Scheme 7. Attempted synthesis of aminoguanidine hydrazone derivative 51

In an attempt to synthesize mitochondria-targeted nitrone 51, *p*-formylbenzoic acid 52 was first refluxed with triethylamine in the presence of diphenylphosphoryl azide for 6 hr according to the protocol of the modified curtius rearrangement[312] as outlined in scheme 7 which afforded *p*-formylphenyl isocyanate 53 exclusively. However, treatment of isocynate 53 with aminofuran adduct 50,[313] employing Fowler's method, failed to give the desired urea 54 either in anhydrous benzene or in anhydrous toluene which was planned to be transformed into nitrone 51 by treatment wih aminoguanidine hydrochloride followed by treatment with N-*tert*-butylhydroxylamine hydrochloride.

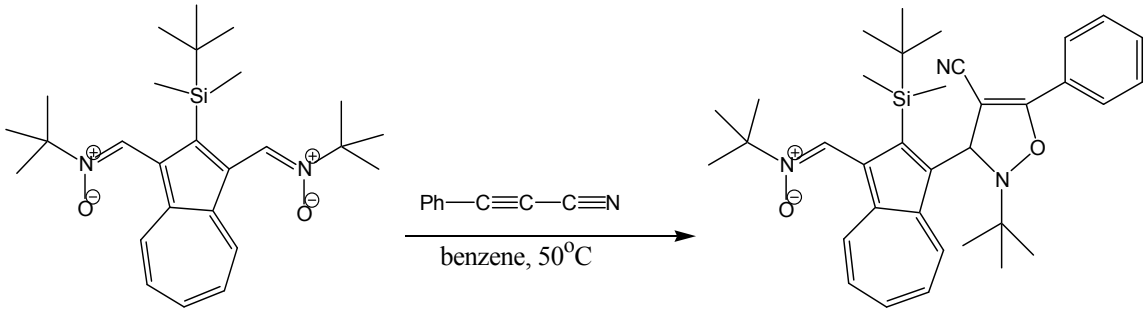
[3.1.4] Synthesis of isoxazoline azulenylsilane nitrone 24:

Azulenylsilane dinitrone 55 was found to be a useful precursor for the synthesis of other nitrones which allowed incorporation of other substituents on either the 1 or 3 position via a cycloaddition appraoch (vide infra). A simple condensation reaction between mononitrone 23 and three equivalents of N-*tert*-butylhydroxylamine hydrochloride in pyridine at 45^{o}C yielded azulenylsilane dinitrone 55. It was also possible to prepare this dinitrone in 33 % yield from bis-aldehyde 32 by using five equivalent of N-*tert*-butylhydroxylamine hydrochloride as outlined in the scheme 8.



Scheme 8. Synthesis of azulenysilane dinitrone 55

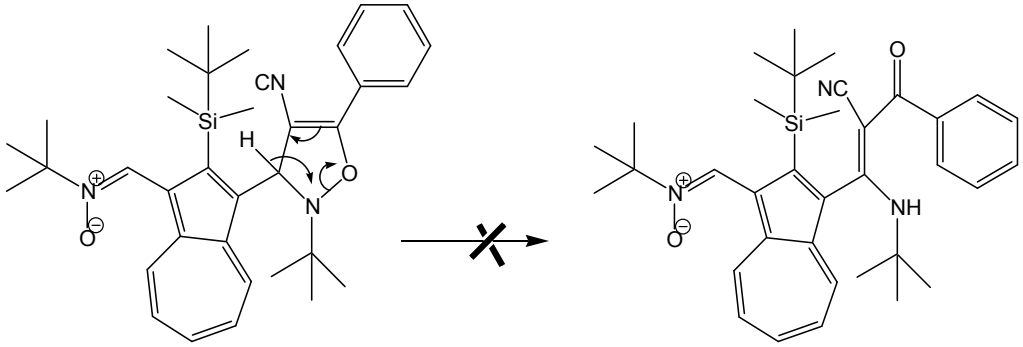
One of the possibilities to introduce an electron withdrawing cyano moiety to the azulenylsilane nitrone is a 1,3-dipolar cylcoaddition approach using dinitrone 55. Coates, who has studied the regioselective cycloaddition between and cyclic nitrones with various alkene nitriles and alkyne nitriles, demonstrated successful formation of cycloadducts using phenylpropiolonitrile.[314] Using the same protocol, isoxazoline nitrone, 24 was prepared regiospecifically from dinitrone 55 and phenylpropiolonitrile in benzene at 50^{o}C for 6 days (scheme 9).



55 24 green

Scheme 9. Synthesis of isoxazoline nitrone 24

Initially, the product nitrone 24 was thought to undergo ring opening of the isoxazoline system to produce enamine derivative 56 on basis of an the earlier study.[315] But such rearrangement was not observed in this case (figure 14).

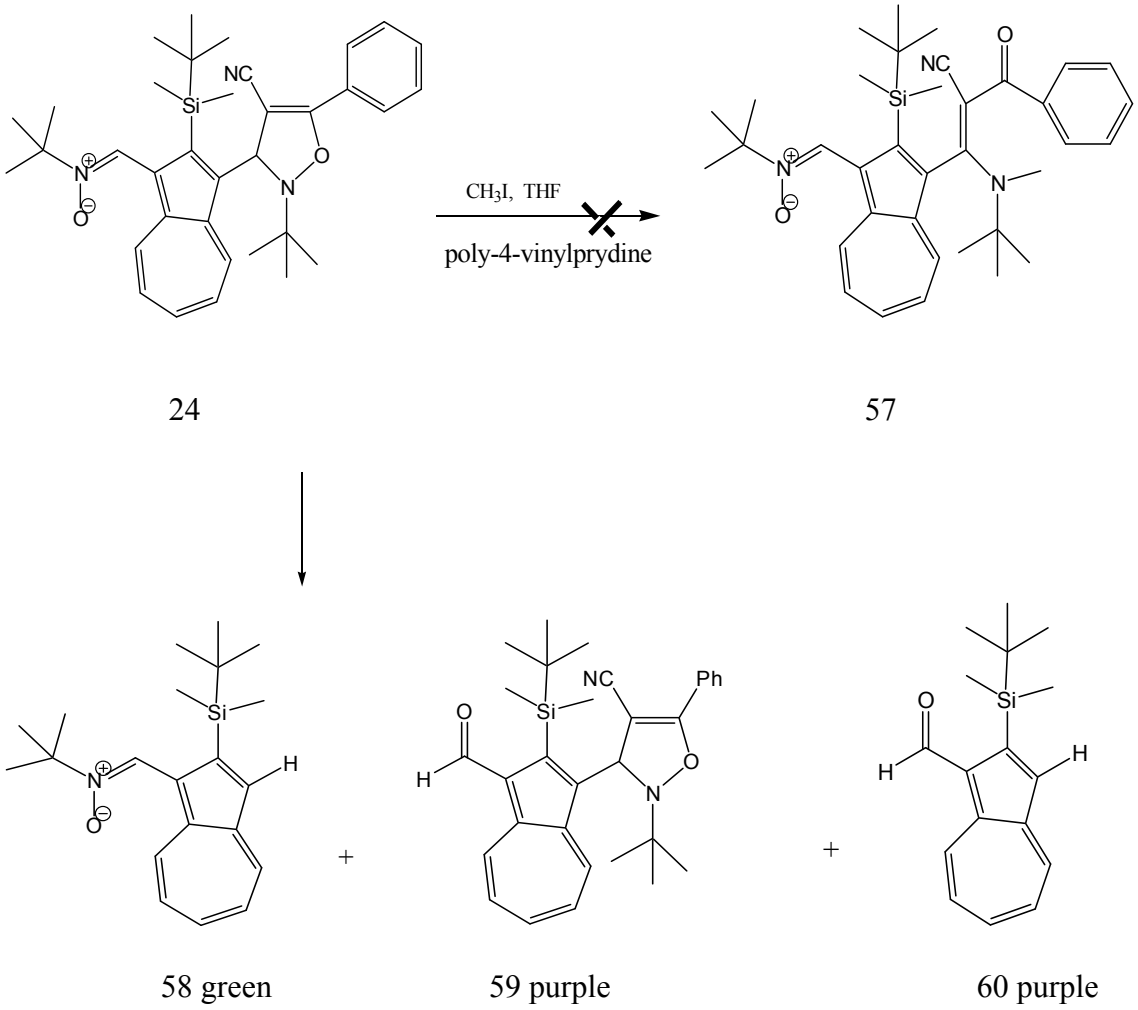


24 56

Figure 14. Expected product 56 via ring opening

Since the expected product 56 was not observed, effort was made to open the isoxazoline ring system. Oxidation of the nitrogen atom of a 2-substituted-4-isoxazoline activates the system to undergo a ring opening reaction. It is reported that using an excess amount of iodomethane improves the lability of the N-O bond thus leading to the formation of an α,β-unsaturated ketone via an isoxazolinium salt intermediate.[316,317]On

the other hand, the presence of an easily abstractable proton at the 3-position in the isoxazoline ring could open the ring to form the corresponding enamino derivative.[318]



24 57

58 green 59 purple 60 purple

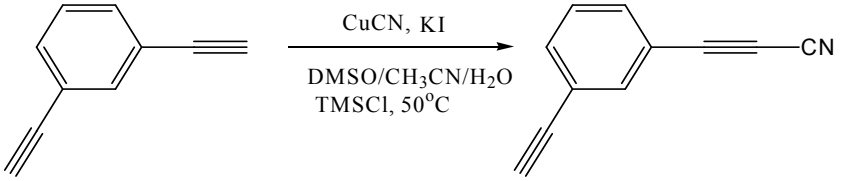
Scheme 10. Attempted synthesis of compound 57

Unfortunately, treatment of nitrone 24 with excess iodomethane and excess poly-4-vinylpyridine did not provide the expected enamine product 57. However, three different unexpected products were observed as shown in figure 2. Their structures are identified as 58, 59 and 60 determined by their molar mass (MH^+) 342, 497 and 271

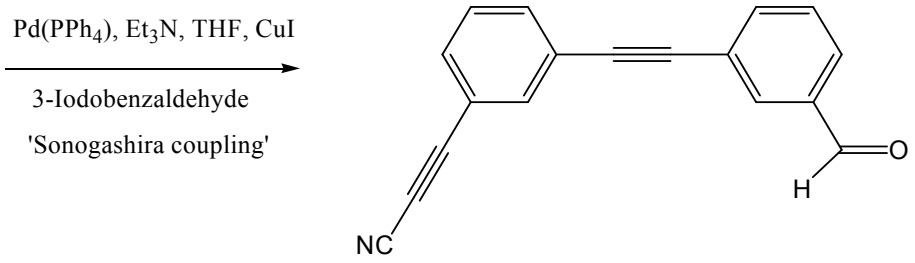
respectively, detected by mass spectrum. The formation of these three products suggests the two possible processes. Hydroiodic acid that has formed under the reaction conditions probably underwent a substitution reaction with the azulene ring to give compound 58, and hydrolysis of the nitrone might have occurred to form aldehyde 59. Both processes could have led to the formation of compound 60. The results are not unexpected due to the fact that azulene, with high electron density on the five membered ring, is liable to electophilic attack at either postions 1 or 3.[319]

[3.1.5] Synthesis of isoxazoline azulenylsilane guanylhydrazone nitrone (25):

Isoxazoline nitrone 24 seemed to be a promising agent for *in vitro* superoxide detection. However, the drawback to this compound is that it is not a lipophilic cation



62 63

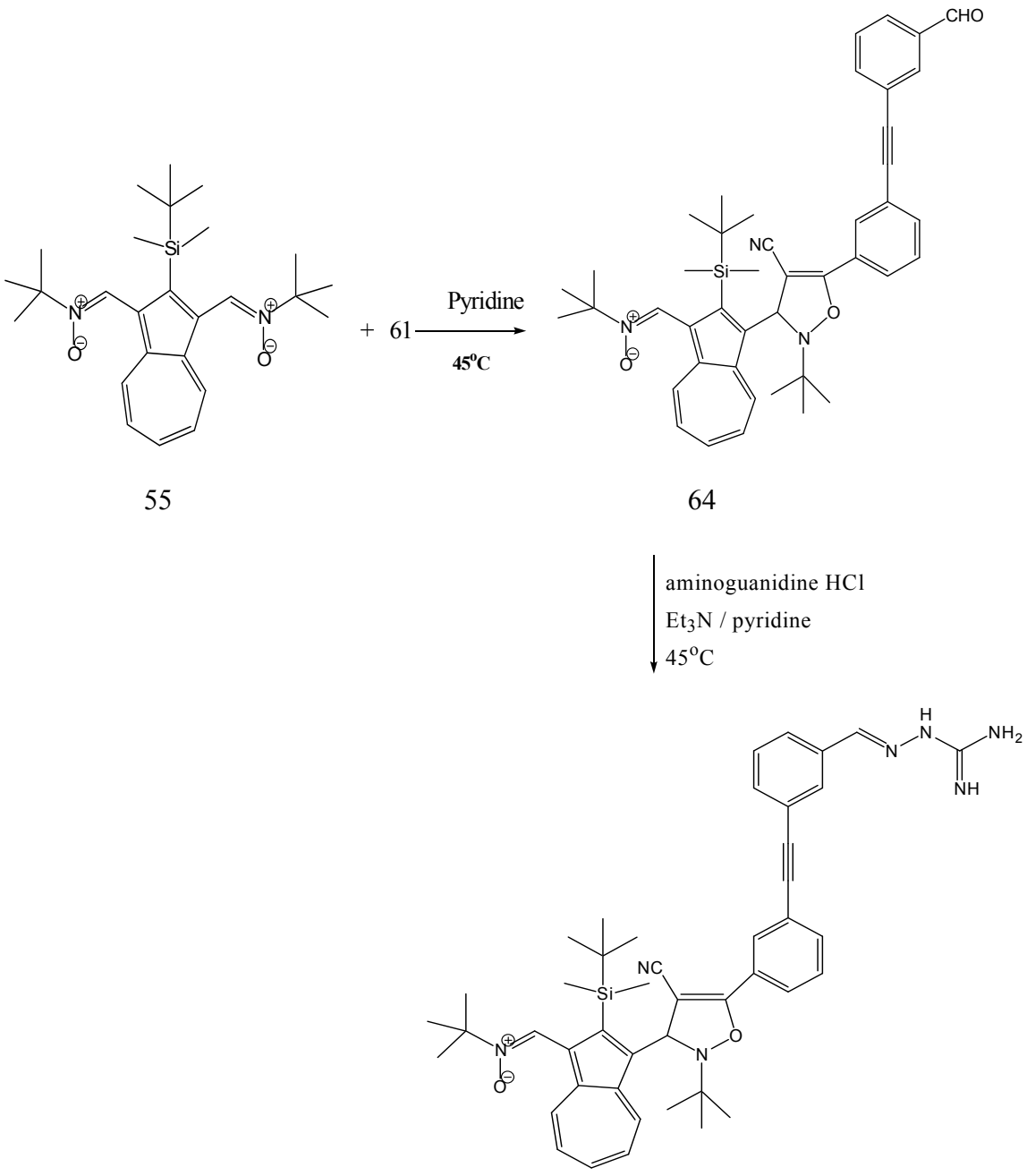


61

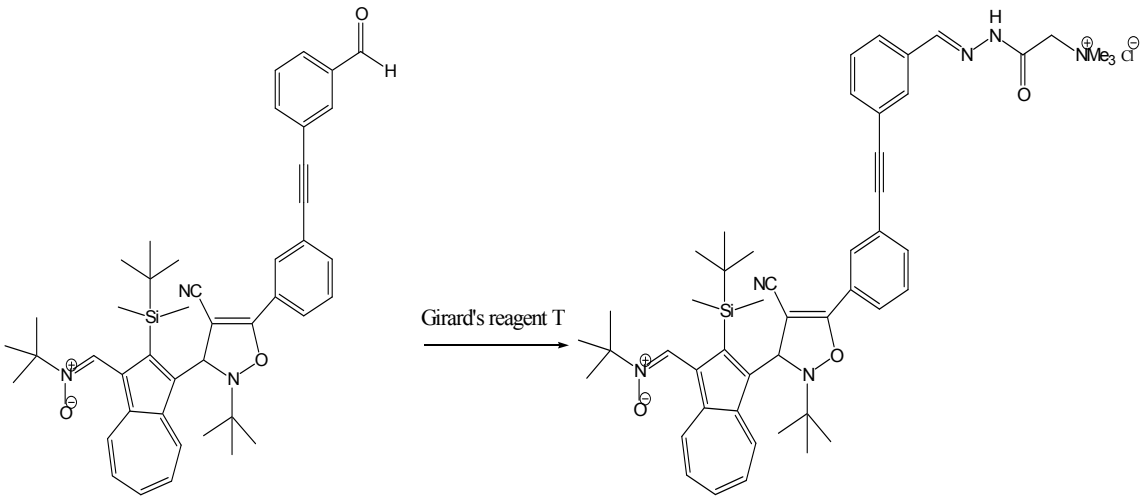
Scheme 11. Synthesis of [3-(3-Formyl-phenylethynyl)-phenyl]-propyne nitrile 61

which limits towards its accumulation in mitochondria. Therefore, effort has been made to enhance its mitochondriotropic activity by incorporating a guanylhydrazone moiety. The effort resulted in the preparation of guanylhydrazone nitrone 25 from [3-(3-Formyl-phenylethynyl)-phenyl]-propyne nitrile 61.

Aldehyde 61 was prepared in two steps as shown in scheme 11. According to the method reported by Wang,[320,321] cyanation of one terminal acetylene of 1,3-diethynylbenzene 62, using two equivalents of cuprous cyanide in the presence of TMSCl, water and a catalytic amount of sodium iodide at 50^{o}C afforded nitrile derivative 63. The crucial factor in this cyanation process was the composition of solvent. Thus, DMSO and a co-solvent (CH_3CN) should be in the ratio of 3:1. Sonogashira coupling of compound 63 with 3-iodobenzaldehde[322] in THF using tetrakistriphenylphosphine palladium at room temperature for 24 hour provided [3-(3-Formyl-phenylethynyl)-phenyl]-propyne nitrile 61. Treatment of one equivalent of nitrile 61 with dinitrone 55 in benzene at 50^{o}C engendered 1,3-dipolar cycloaddition that provided adduct 64. A condensation reaction between compound 64 with aminoguanidine hydrochloride in the presence of triethylamine in pyridine at 45^{o}C afforded guanylhydrazone nitrone 25 (scheme 12).



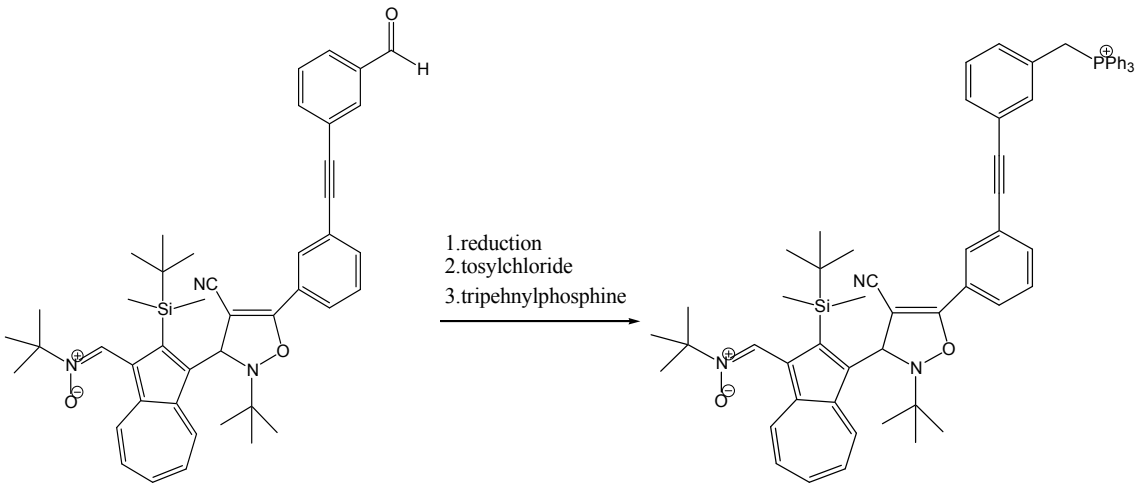
Scheme 12. Synthesis of guanylhydrazone nitrone 25



64 65

Figure 15. Synthetic scheme of compound 65

Unfortunately, nitrone 25, despite the presence of the polar guanylhydrazone, is found to be poorly water soluble (but could still be mitochondriotropic). Another possibility of making mitochondria-targeted compounds involves the synthesis of salts of nitrones 65 and 66. The presence of the aldehyde group in compound 64 is ideal for such a purpose. Nitrone 65 could theoretically be prepared by treatment of aldehyde 64 with Girard's reagent as shown in figure 15. Alternatively, transformation of aldehyde 64 into salt 66 could proceed in several steps as illustrated in figure 16. First, a reduction reaction converts aldehyde 64 into the alcohol derivative which upon treatment with tosylchloride would give the corresponding tosylate congener. Subsquently, S_N2 reaction of the tosylate with triphenylphosphine would afford the potential mitochondriotropic phosphonium salt 66.



64 66

Figure 16. Synthetic scheme of compound 66

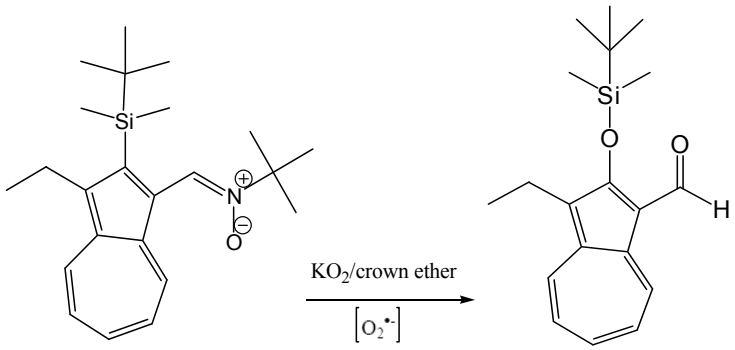
[3.2] The *in vitro* investigation of the reaction of novel nitrones with superoxide radical:

After the successful synthesis and characterization of several nitrones, experiments were conducted to test the properties and potential diagnostic application of these novel nitrones for detecting superoxide radical anion.

[3.2.1] Attempts at *in vitro* detection of superoxide with ethylated azulenylsilane mononitrone 22

The first attempt to detect superoxide involved reaction of nitrone 22 with potassium superoxide in the presence of 18-crown-6 in benzene at room temperature[323] No reaction was observed when nitrone 22 was treated with a solution of one to five equivalents of potassium superoxide and one equivalent of crown ether dissolved in benzene. Thus, the amounts of potassium superoxide and crown ether were increased to 5 equivalents. Still no product was observed. The result could be because of the fact that

not enough superoxide radical anion was present because of poor solubility of potassium superoxide. Therefore, 20 equivalents of potassium superoxide and crown ether were directly treated with nitrone 22. These conditions gave a product in very low yield which was impossible to identify. Finally, nitrone 22 was directly treated using 6 equivalents of potassium superoxide. In this case, oxidation reaction was conveniently followed by TLC by monitoring the appearance of a less polar orange product which was analyzed by ^{1}H NMR spectroscopy. The product was identified as aldehyde 67 by observing the aldehyde-proton signal and the up-field shifting of the ethyl signal of the azulene ring.

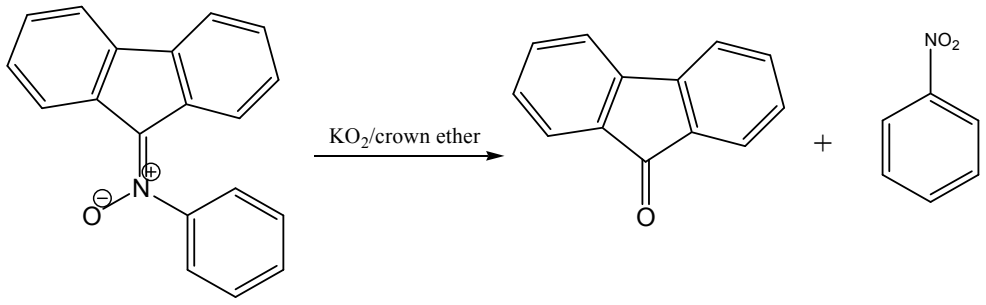


22 67 orange

Figure 17. Formation of aldehyde end product 67

Formation of a ketone from analogous reaction between a ketonitrones and potassium superoxide has been previously reported.[323] Thus, α-diphenylene-N-phenylnitrone 68 was treated with superoxide and ketone 69 was found as a major product as shown in figure 18. Interestingly, aldehyde 67 was found to be very unstable because it decomposed while another orange product was started to appear as the reaction proceeded. As a result of difficulty in purification of aldehyde 67, identification of the second orange product was not possible. The ^{1}H NMR data showed no aldehyde-proton,

and a low resolution mass peak at MH^+ of 663 was observed for this second orange product. The possible explanation could be that the aldehyde product is very liable to oxidation with superoxide anion and oxidized faster than nitrone 22 to form the second product.



68 69 nitrobenzene

Figure 18: Formation of ketone from α-diphenylene-*N*-phenylnitrone with KO_2

[3.2.1.1] Mechanism of superoxide radical anion with nitrone 22

On the basis of the above result, expected aldehyde 67 was observed as an initial orange product which is supported by the proposed mechanism of oxidation of nitrone with superoxide (figure 19). Superoxide radical adds to nitrone 22 by radical addition to form intermediate nitroxide anion 70. Since silicon has high affinity towards oxygen, intramolecular nucleophilic attack of the anionic oxygen on silicon gives rise to an unstable pentavalent intermediate[324] 71 which subsequently undergoes rearrangement by migrating the azulenyl group to the adjacent oxygen as an alkoxide ion departs. Finally, collapse of the tetrahedral intermediate provides aldehyde 67. Such an oxidative desilylation process was initially described by Tamao and Kumada.[325,326]

Information regarding the paramagnetic superoxide spin adduct, an intermediate presumed to be formed from the reaction of nitrone 22 with superoxide anion, remained unknown since ESR measurement was not available. However, similar to the superoxide spin adducts of the conventional acyclic nitrones, such a superoxide spin adduct would be expected to be highly unstable at room temperature. Grulke et al., in his ESR spin trapping study of DMPO with metmyoglobin and hydrogen peroxide at varying temperature, has found the nitrone peroxyl spin adduct unstable even at 0°C.[327]

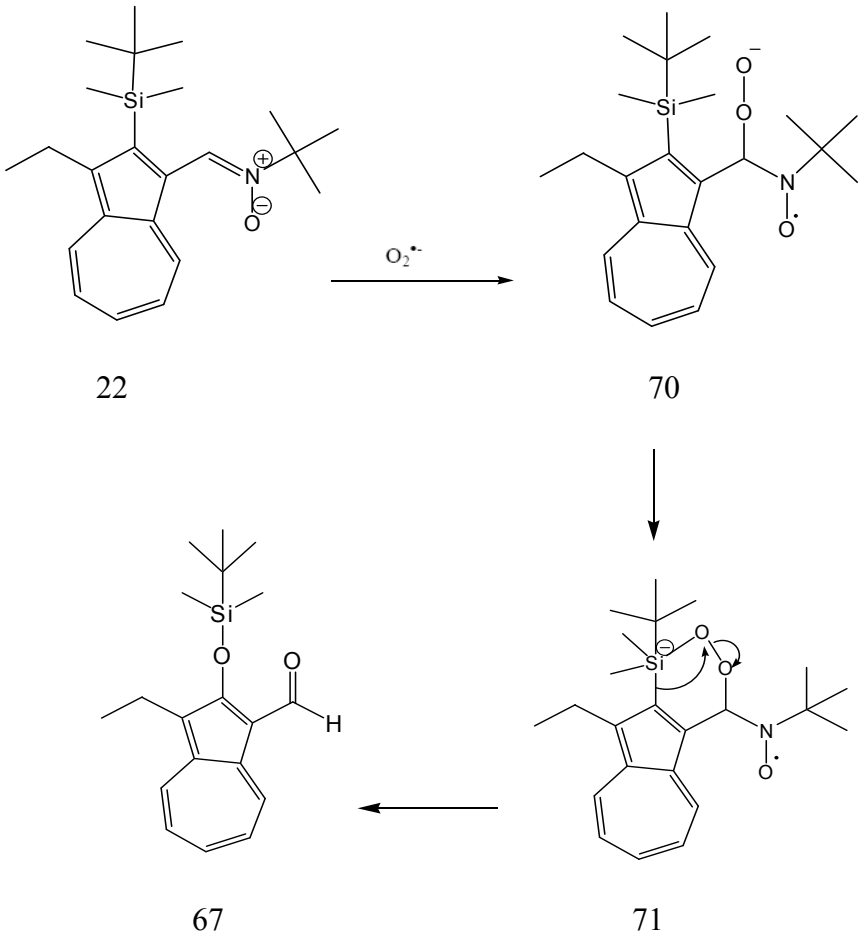
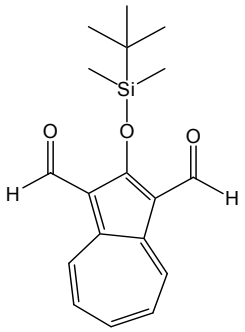


Figure 19. Proposed mechanism of the reaction of nitrone 22 with superoxide radical

[3.2.2] The *in vitro* detection of superoxide with azulenylsilane mononitrone 23

The detection of superoxide with azulenysilane mononitrone 23 was based on the previous method as described (vida supra). Six equivalents of potassium superoxide were used as before. As mentioned earlier, the previous reaction was monitored by observing an orange product on thin layer chromatography (TLC). In this case, the reaction mixture turned completely from green to orange in 90 min. On the basis of the previous mechanism, it was expected to obtain the bis-aldehyde 72 as an end product. However, ^{1}H NMR spectral information of the orange end product indicated the loss of the silyl group, and presence of only the mono-aldehyde. The low resolution mass spectrum detected a MH^{+} of 448. Unfortunately, it was not possible to purify enough for the NMR characterization. Hence, the structure identification of end product remained unsolved. It is believed that the superoxide spin adduct that formed was unstable and fragmented into another compound rather than the expected one. The loss of the silyl group could result after rearrangement via a nucleophilic attack on silicon by a second superoxide anion since an excess of superoxide anion was used and due to the high affinity of silicon towards oxygen.

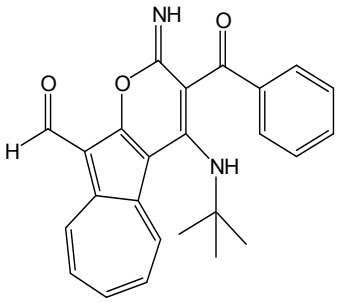


72

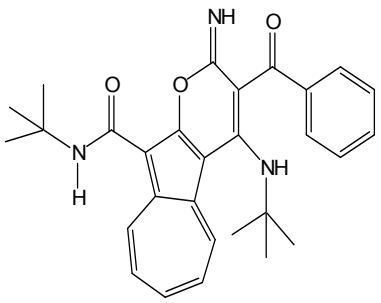
Figure 20. Expected product from the reaction of nitrone 23 with superoxide radical

[3.2.3] The *in vitro* detection of superoxide with isoxazoline azulenylsilane nitrone 24

The unidentified end product from superoxide radical anion with nitrone 23 led me to synthesize an analogue, the isoxazoline azulenylsilanenitrone 24, and test it for its possible utility in superoxide detection. Using the same method as described earlier, superoxide was solubilized in benzene by employing crown ether. The only difference in this case was the use of three equivalents of potassium superoxide instead of six equivalents. The green reaction mixture was completely turned into orange solution. As in the previous case, aldehyde product 73 was expected to be formed. But surprisingly, NMR analysis showed the absence of an aldehydic proton, and a molecular mass of 469 was detected, which did not match with the aldehyde product. A high resolution mass spectrum of the major product was obtained and it was found to possess a molecular formula of $C_{29}H_{31}N_3O_3$. With HRMS and NMR spectral analyses, the orange product was identified as amide 74.



73



74 orange

Figure 21. Structures of the expected product 73 and observed product 74 from the reaction of superoxide radical with nitrone 24

Figure 22 shows the proposed mechanism for the formation of amide 74 from the spin trapping of superoxide radical anion by nitrone 24. The addition of superoxide

radical to the double bond of nitrone 24 leads to radical anion 75, which then suffers nucleophilic attack by the negatively charged oxygen on the silicon to give an intermediate 76 with a six-membered ring.[328] Then subsequent desilylation by migrating the azulenyl group to the adjacent oxygen atom transforms 76 into anion 77 in analogy to the work of Tamao and Kumada.[325] Rearrangement involving a nucleophilic attack of the anionic oxygen on silicon leaves the negative charge on oxygen at the 2-position, which in turn, undergoes a nucleophilic attack on the nitrile moiety thus resulting in the compound 78, a six-membered ring intermediate. This step might also result in the rapid opening of the isoxazoline ring. Meanwhile, oxygen radical may be reduced by disproportionation (or reduced by ascorbic acid in a biological system) to form the corresponding hydroxylamine. Dehydration followed by desilylation affords amide 74. In addition to amide 74, a trace of orange compound 79 was also obtained which is identified by MS data 584 (MH^+). The formation of 79 suggests a divergent mechanism that might have taken place in the reaction. Contrary to the first mechanism, the second mechanism pathway might have involved addition of one molecule of superoxide anion followed by dehydration to give 79.

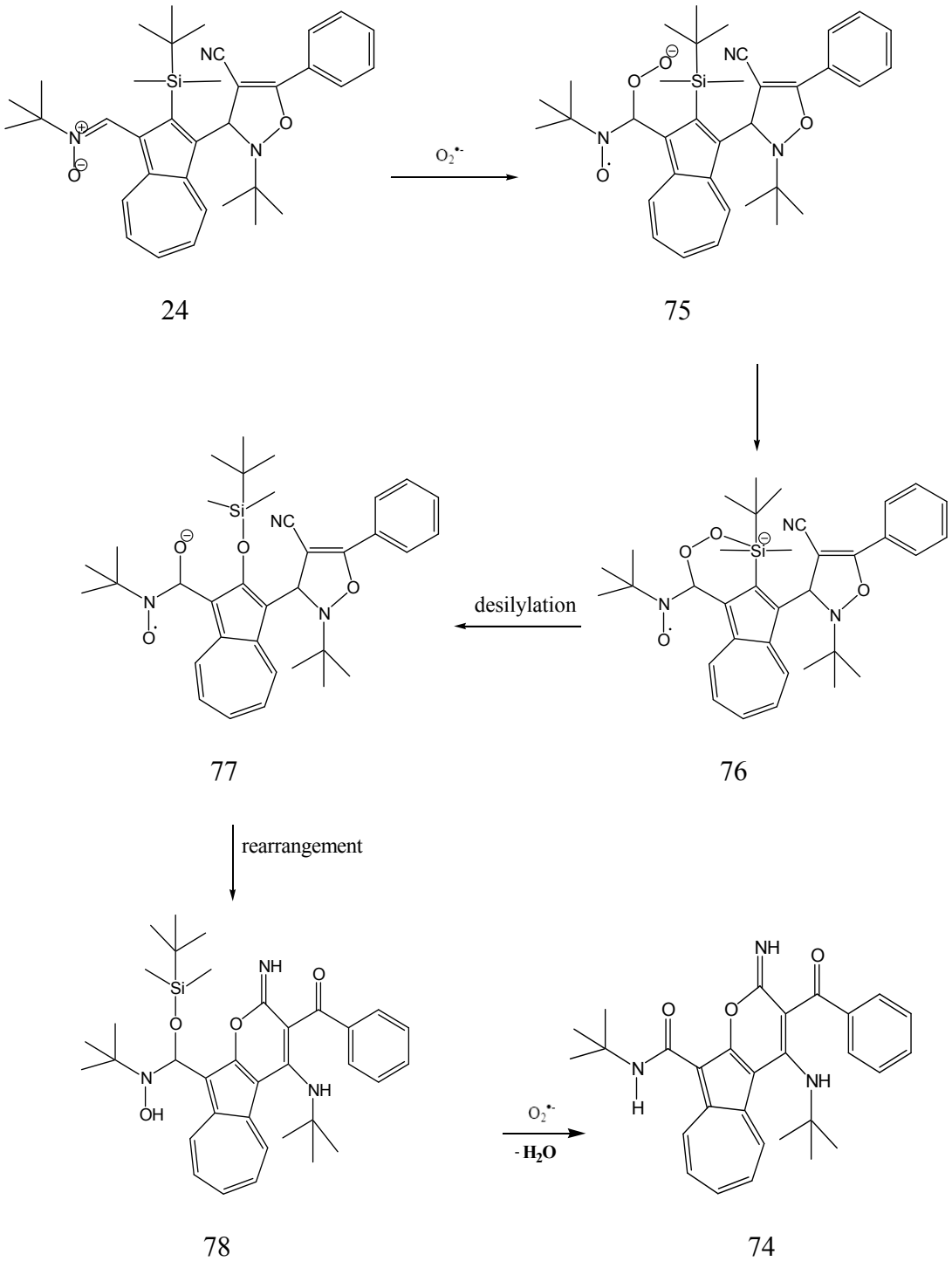
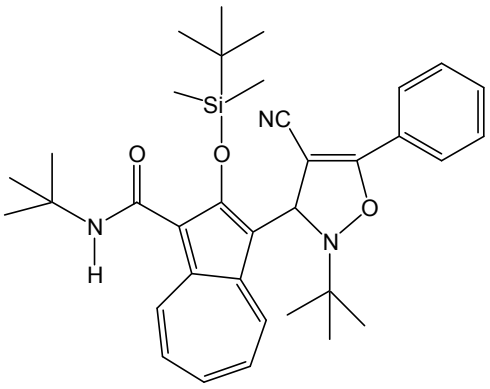


Figure 22. Hypothetical mechanism of the reaction of nitrone 24 with superoxide radical



79 orange

Figure 23. Potential structure of the second product from the reaction of nitrone 24 with superoxide radical

[3.2.3.1] UV-vis and fluorescence of amide 74

The UV-vis (ultra visible) absorption spectrum and fluorescence of chromotropic amide 74 was measured in ethanol. As shown in figure 24, the UV-vis is indicated by the blue signal which consists of two strong absorbances in the range of 250-400 nm and one weak absorbance in the range of 400-500 nm. The fluorescence signal, indicated by red signal, was obtained by irradiating at the excitation wavelength 390 nm as shown in the spectrum. The wavelength dependence of the intensity of emitted light has been recorded at ~490 nm (emission). The quantum yield of amide was measured at 2.7 %. These results suggest that compound 74 has fluorescence, and this probe may possibly be employed to detect superoxide anion in biological system by identifying its orange fluorescence. In addition to its chromotropic behavior, it may bring an advantage over conventional cyclic nitrones which require ESR for superoxide detection.

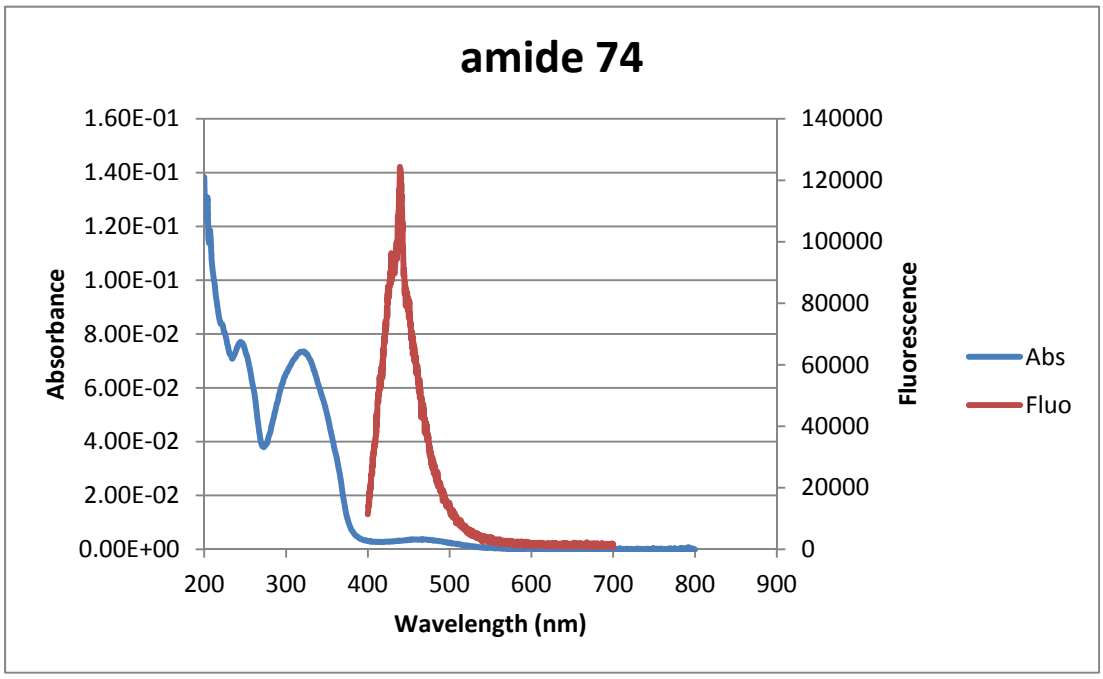


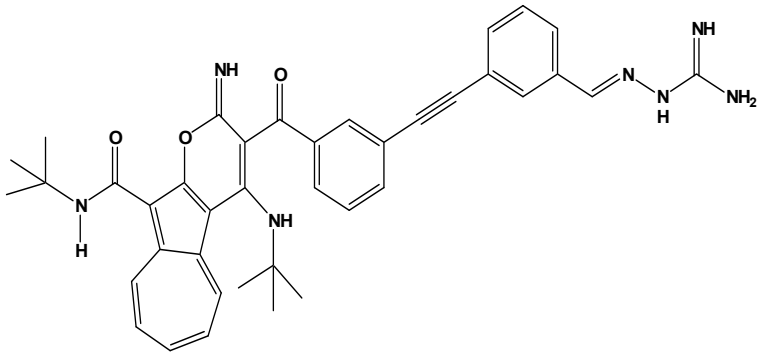
Figure 24. UV-vis absorbance and fluorescence of amide 74 taken in ethanol

One of the advantages of fluorescence detection is the possibility of real time monitoring of the oxidation process.

[3.2.4] The *in vitro* detection of superoxide with azulenylsilane guanylhydrazone nitrone 25

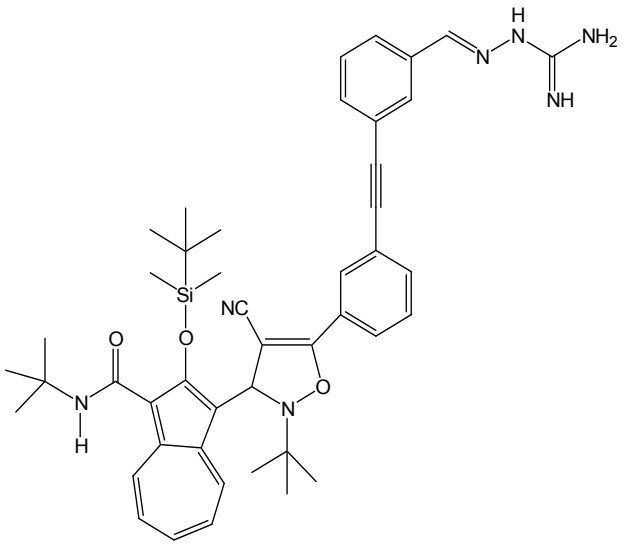
Guanylhydrazone nitrone 25 is a congener of nitrone 24. Incorporation of guanylhydrazone moiety may make it mitochondriotropic, and thereby render it useful to detect superoxide anion present in the mitochondria. As expected, nitrone 25 has also shown to trap superoxide efficiently in analogy to the other aforementioned nitrones. Using the same method as described for the other nitrones, nitrone 40 turned into brownish orange upon reaction with potassium superoxide. The brownish orange end product was isolated and identified as amide 80 using low resolution mass spectrum

(MH^+ = 654) which is analogous to the end product 74. In addition to amide 80, a trace of another orange product was also observed which is identified as amide 81 (MH^+ = 768) analogous to amide 79 end product.



80 brownish orange

Figure 25. Structure of the product amide 80 from the reaction of nitrone 25 with superoxide radical



81 brownish orange (minor product)

Figure 26. Potential structure of the product amide 81

[3.3] The solubility study of nitrone 25 with solutol HS 15

Since nitrone 25 was poorly water soluble, its solubility in an aqueous systems was examined using solutol HS 15 compound. Solutol HS 15 is a non-ionic solubilizer and emulsifying agent produced from 1 mole of 12-hydroxystearic acids and 15 moles of ethylene oxide. It provides excellent solubility to poorly soluble (lipophilic) molecules in water, and hence, it is used for formulation of drugs in the pharmaceuticals industry.

In this experiment, as expected, solutol HS 15 was found to increase the solubility of nitrone 25 in water. To prepare the concentration of 0.64 mg/ml of nitrone 25 in 3:7 solutol HS 15:1 % saline solution, 1 ml of solutol HS 15 was heated until it turned into a clear liquid and cooled to room temperature. Meanwhile, 2.1 mg of nitrone 25 was crushed to a fine powder and dissolved in 1 ml of clear solutol HS 15. It was then diluted with 2.3 ml of 1 % saline solution in water with frequent shaking and warming. It was

then cool to room temperature to obtained green solution. The green solution was kept in a freezer, and found to be stable. The aqueous formulation of nitrone 25 with solutol HS 15 is believed to be amenable for mitochondrial superoxide detection and other biological studies.

[4] CONCLUSION

Superoxide radical anion generation and its reactions in biological systems have received considerable attention because of the involvement of superoxide in physiological process as well as in the pathogenesis of various diseases. In biomedical research, it is important to characterize the specific free radical and its exact role in diseases which will allow investigators to design molecular or pharmacological approaches to prevent or ameliorate free radical-mediated maladies. But the direct detection of biologically generated superoxide radical anion, the identification of its sites of production, and its quantification are of the major challenges. By their nature, the inherent reactivity of most free radicals makes their direct *in vivo* detection impossible. Because of this difficulty, various techniques based on an indirect detection of superoxide have been developed with some degree of success.

Thus, techniques such as ESR (with cyclic nitrone spin traps), fluorescence, chemiluminescence and cytochrome c reduction have been used to study *in vitro* and *in vivo* detection of superoxide radicals in hopes of gaining insight with regard to cellular metabolism and pathogenesis of diverse cytotoxic events but each exhibits potential pitfalls as well as advantages. The fluoresence-based technique using MitoSox red is considered as a simple and accurate method for estimation of biological superoxide, and hence it has been widely used for this purpose. However, it was found unreliable for

intracellular detection because it does not detect superoxide specifically.

In a search for a more reliable, selective, and convenient method, this research developed structurally diverse novel azulenylsilane nitrones. Structurally, these novel nitrones are set up in such a way that they are specific for superoxide detection. For example, a nitrone moiety and an adjacent silyl group react readily with radicals and oxygen anion respectively. Such nitrones can trap superoxide efficiently because superoxide is both radical and an oxygen anion. Moreover, such nitrones allow chromotropic detection of superoxide by the virtue of their azulene core. Synthesis of dinitrone 55 has made it possible to design and synthesize other nitrone congeners with the incorporation of the electron-withdrawing cyano group, which yields a stable, characteristically colored, end product from its spin trapping reaction with superoxide. As expected, 2-terbutyldimethylsilyl-1ethyl-3-azulenylnitrone (22), 2-*tert*-butyldimethylsilyl-3-azulenecarboxaldehyde1-1nitrone (23), 2-*tert*-butyldimethylsilyl-3-*tert*-butylnitrone-1-(2-tert-butyl-4-cyano-5-phenyl)-azulenylisoxazoline (24) and mitochondria-targeted 2-*tert*-butyldimethylsilyl-1-[5-(3-aminoguanidinyl-phenylethynyl)-phenyl) 2-*tert*-butyl-4-cyano-5-phenyl isoxazole]-3-*tert*-butylazulenylnitrone (25) have been shown to trap superoxide radical anion efficiently yielding UV-VIS identifiable and even potentially fluorescence-detectable orange products. The *in vitro* results suggest that the chromotropic detection of superoxide using these nitrones can be a promising method in contrast to other available methods. Further work involves synthesis of mitochondria-targeted nitrones 65 and 66. Examination of superoxide reaction with nitrone 25 in biological systems encountered is underway. The *in vivo* detection of superoxide radical anion may provide additional information. Thus,

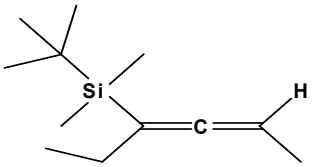
nitrone 25 may be mitochondriotropic, and may have significant potential as a diagnostic tool for biological superoxide detection.

[5] EXPERIMENTAL SECTION

General notes:

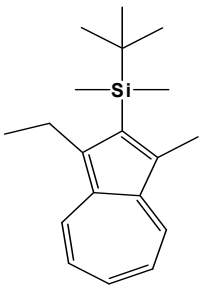
All of the moisture sensitive reactions have been carried out using syringe septum rubber cap technique under N_2 or Ar atmosphere or in a vial by flushing N_2 or Ar gas into it. The reactions at -78^o C have been performed employing compressed CO_2 and acetone bath. All of the reactions were monitored by TLC analysis (Whatman pre-coated silica gel F_{254} plates, 250 μm layer thickness), and visualization was achieved by 254 nm UV light. The crude reaction mixtures have been purified by column chromatography using 60A (200-300 mess) on silica gel. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AVANCE-400 spectrometer in deuterated chloroform. Chemical shifts were measured in parts per million with residual solvent peak used as an internal standard. The ^{1}H NMR spectra were run at 400 MHz and ^{13}C NMR spectra were recorder at 400 MHz with proton decoupled sequence. Coupling constants were measured in Hertz. All low resolution mass spectra were obtained on Finnigan Navigator LC/MS instrument and DECA LC/MS by direct injection into the mass spectrometer and detecting molecular ion with ESI, and GC/MS. The UV-vis spectrum was recorded using a Varian Cary 50 Bio spectrophotometer. The fluorescence (FL) spectrum was obtained using a Fluoromax-3 spectrofluorometer (Jobin Yvon/Habira). For determination of quantum yield (QY), 9,10-bis(phenylethynyl)anthracene (QY=1.0) in cyclohexane was used as a fluorescence standard.

[5.0.1] Synthesis of 1-*tert*-butyldimethylsilyl-1-ethyl-3-methylallene (27)



In a 150 ml three-necked round bottom flask 12.57 ml (17.6 mmol) of sec-butyllithium was cooled to -30^{o}C under argon and stirred with magnetic stirrer. To this was added 0.723 g (8.8 mmol) of 3-hexyne via a syringe and stirred for 3 hours. The reaction mixture was then cooled to -78^{o}C and, 2.6 gm (17.6 mmol) of TBDMSCl in THF was added with a syringe and left the mixture overnight. Subsequently, the reaction mixture was poured on ice and aqueous layer was extracted with ether (3 x 20 ml). The combined ether layers were then washed with 15 ml of saturated sodium chloride, dried over $MgSO_4$ and, concentrated in a rotavap. The resulting residue was distilled (b.p. 90-95^{o}C) and column chromatographed using pentane to yield 172 mg of solution colorless oil containing allenylsilane (27). The crude solution was used to prepare compound 28 without further purification.

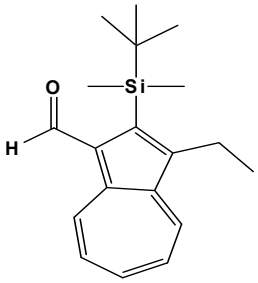
[5.0.2] Synthesis of 2-*tert*-butyldimethylsilyl-1-ethyl-3-methylazulene (28)



In a 250 ml three-necked round bottom flask, fitted with an argon inlet adapter and a magnetic stirring bar, allenylsilane (232 mg, 1.18 mmol) was treated with 10 ml of acetonitrile, and 0.415 g of poly-4-vinylpyridine (3.94 mmol) and stirred for a minute.

Tropylium tetrafluoroborate (0.470 g, 2.63 mmol) was then added to the resulting suspension in one portion. The reaction mixture turned blue in one minute, which was then stirred in the dark for 24 h at the room temperature. The dark blue reaction mixture was filtered, and the solid residue was washed with hexane 100 ml of hexane followed by 10 ml of acetonitrile until washing were clear. The filtrate was transferred into a separatory funnel and the top hexane layer was separated from the bottom acetonitrile layer. The separated acetonitrile layer was further extracted from hexane (3 x 20 ml) until the extract was clear. The combined hexane layers were then washed with 15 ml of saturated sodium chloride, dried over anhydrous magnesium sulfate, filtered, and evaporated in a rotavap. The blue green solid was then purified by chromatography on silica gel (eluent hexane) to give 0.1678 g of azulenylsilane as blue-green crystals. ^{1}H NMR δ 8.11 (d, 1H, J = 9.4), 8.09 (d, 1H,J = 9.5), 7.39 (t, 1H, J = 9.7), 6.87 (t, 2H, J = 9.8), 3.17 (q, 2H, J = 7.5), 2.70 (s, 3H), 1.26 (t, 3H, J = 7.5), 0.91 (s, 9H), 0.5 (s, 6H); ^{13}C NMR δ 147.7, 140.9, 139.3, 138.1, 137.6, 134.2, 134.0, 133.4, 121.9, 121.8, 28.4, 23.0, 20.5, 19.0, 15.8, 0.00. LRMS (LC/MS-ESI, MeOH) *m*/*e* 285 (MH^+)

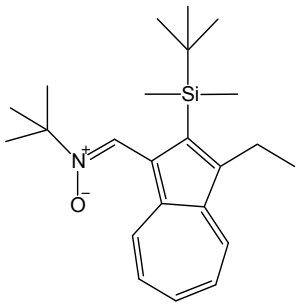
[5.0.3] Synthesis of 2-*tert*-butyldimethylsilyl-1ethyl-3-azulenecarboxaldehyde (30)



In a 250 ml round bottom flask, azulenyl silane (290 mg, 1.021 mmol) was dissolved in 2ml of acetone and was added 2,3-dichloro-5,6-dicyano-1-benzoquinone

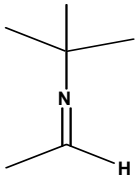
(463.5 mg, 2.042 mmol) and stirred at room temperature for 30 minutes in the dark. The reaction mixture immediately turned from blue to violet color. When all the reactant was gone, 15 ml of saturated sodium thiosulfate was added and stirred for 5 min to remove unreacted DDQ (2,3-dichloro-5,6-dicyano-1-benzoquinone). To this reaction mixture, 15 ml of sodium carbonate was added and stirred for 5 minutes to remove acidic $DDQH_2$ (2,3-dichloro-5,6-dicyanohydroquinone). The resulting reaction mixture was then poured into 250 ml of chloroform and transferred into separatory funnel. The separation of two layers was hard to visualize. The bottom chloroform layer was separated from the top aqueous layer then the aqueous layer was extracted with chloroform (2 x 25 ml). The combined chloroform layers were washed with 15 ml of saturated sodium chloride, dried over anhydrous magnesium sulfate, and concentrated in a rotavap. The residue violet solid was then purified by column chromatograpghy with eluent 2:8 ethyl acetate:hexane to provide 228 mg of azulenylsilane aldehyde (30) in 75 % yield. ^{1}H NMR δ 10.54 (s, 1H), 9.85 (d, 1H, J = 9.85), 8.44 (d, 1H, J = 9.80), 7.79 (t, 1H, J = 9.77), 7.55 (t, 1H, J = 9.86), 7.47 (t, 1H, J = 9.76), 3.18 (q, 2H J = 7.54), 1.30 (t, 3H, J = 7.53), 0.97 (s, 9H) 0.56 (s, 6H); ^{13}C NMR δ 190.16, 153.14, 142.63, 142.42, 142.11, 140.4, 138.69, 135.74, 130.85, 130.15, 127.61, 127.49, 21.64, 18.72, 17.23, 0.00; LRMS (LC/MS-ESI, MeOH) *m*/*e* 299 (MH^+). Elution with 3:7 ethylacetate:hexane gave 32 mg of azulenysilane alcohol-aldehyde (31) in 10 % yield. ^{1}H NMR δ 10.52 (s, 1H), 9.92 (dd, 1H), 9.36 (dd, 1H), 7.86 (tm, 1H), 7.62 (t, 1H), 7.54(t, 1H), 5.72 (q, 1H), 1.95 (s, 1H), 1.77 (d, 3H, J = 6.64) 1.1 (s, 9H), 0.55 (s,3H)).50 (s,3H); ^{13}C NMR δ 189.82, 152.25, 142.83, 141.82, 141.77, 140.76, 139.02, 138.84, 130.36, 130.18, 127.97, 67.81, 27.01, 24.29, 18.16, 0.25, 0.10; LRMS (LC/MS-ESI, MeOH) *m*/*e* 315 (MH^+)

[5.0.4] Synthesis of 2- *tert*-butyldimethylsilyl-1ethyl-3-azulenylnitrone (22)



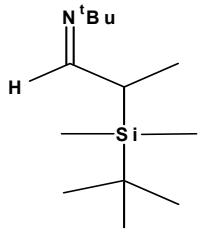
According to Becker et al. protocol, 101 mg (0.34 mmol) of compound 30 was treated with N-*tert*-butyl hydroxylamine (212.84 mg, 1.69 mmol) and anhydrous magnesium sulfate (204 mg, 1.69 mmol) in 2 ml of anhydrous pyridine under argon. The reaction mixture was stirred at 40^{o}C for 21 days. The TLC showed the product as well as some starting material. The reaction mixture was then poured into 20 ml of water and extracted with ether (3 x 25 ml). The ether layer was washed with 10 ml of saturated sodium chloride, dried over magnesium sulfate and concentrated in a rotavap. The residue product was then purified by column chromatography using 7:3 ethylacetate:hexane to provide 58.6 m of nitrone (22) in 47 % yield. ^{1}H NMR δ 8.25 (d, 2H J = 9.75), 8.11 (s, 1H), 7.60 (t, 1H), 7.22 (t, 1H), 7.18 (t, 1H), 3.16 (q, 2H), 1.7 (s, 9H), 1.26 (t, 3H), 0.94 (s, 9H), 0.49(s, 6H); ^{13}C NMR δ 146.71, 141.68, 140.04, 138.54, 137.88, 135.03, 133.63, 129.53, 124.12, 123.93, 123.31, 69.81, 28.46, 26.87, 21.65, 18.77, 17.43, -1.56; LRMS (LC/MS-ESI, MeOH) *m*/*e* 369 (MH^{+})

[5.0.5] Synthesis of acetaldehyde *tert*-butylimine (35)



Following Tieze's method, I placed 16.5 g of *tert*-butylamine (226 mmol) in a three-necked round bottom flask, fitted with a dropping funnel and a magnetic stir bar, (under argon and kept on ice bath). Ten 10 g of acetaldehyde (226 mmol) was introduced dropwise at 0^{o}C into the flask. The mixture was stirred at this temperature for 3 hr, then 600 mg of solid KOH (10.7 mmol) was added and kept in a refrigerator at 4^{o}C for 12 hr to allow two layers to seperate. The bottom aqueous layer was removed using a separatory funnel and the top organic layer was transferred into a flask which was then distilled (bp 66-70^{o}C) to provide 19.9 g of aldiimine (35) in 88% yield as a colorless oil. The product was found identical to an authentic sample by using ^{1}H NMR. ^{1}H NMR δ 7.61 (q, 1H J = 4.8 Hz), 1.90 (dd, 3H, J = 4.8 Hz), 1.10 (s, 9H)

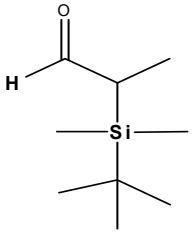
[5.0.6] Synthesis of 2-*tert*-butyldimethylsilyl-propylimine (36)



Following the procedure of Tietze's,[303] 13.4 ml of 1.5 M LDA in THF (20.20 mmol) was treated with 2 g of acetaldehyde aldimine 35 in THF (20.20mmol) at 0^{o}C and the reaction mixture was stirred at this temperature for 30 min under argon. Initially the reaction mixture turned into yellow, and later it turned into red. To this mixture, 3.05 g of

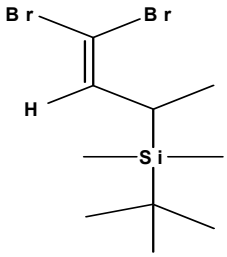
TBDMSCl in THF (20.20 mmol) was added followed by the addition 0.373 g of Bu_4NI (1.01 mmol) at 0^oC. The reaction mixture was then stirred at room temperature for 3 hr. The resulting yellow color solution was then treated with 8.08 ml of BuLi in hexane (20.20 mmol) at 0^oC and stirred for 1 hr at this temperature. Subsequently, 2.86 g of iodomethane (20.20 mmol) was added at this temperature and then allow the mixture to warm up to room temperature over 12 hr. The reaction mixture was then diluted with 40 ml of diethyl ether and poured onto ice-water. The organic layer was separated and the aqueous layer was extracted with diethyl ether (2 x 40 ml). The combined organic layers were washed with saturated NaCl, dried over $MgSO_4$ and concentrated in a vacuo to provide 6.5 g of crude 36 which has been used to prepare compound 37 without further purification.

[5.0.7] Synthesis of 2-*tert*-butyldimethylsilyl-propylaldehyde (37)



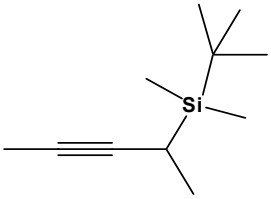
The crude product 36 was hydrolysed by dissolving in 2 ml of petroleum ether/EtOAc (7:1) and filtered through a column of silica gel using petroleum ether/EtOAc (7:1) as eluent to furnish 2.82 g of silylaldehyde (81 %) as an orange oil. The product is identical to the authentic sample. ^1H NMR δ 9.72 (d, 1H, J = 1.6), 2.49 (dq, 1H J = 6.7, 1.6), 1.16(d, 3H, J = 6.7), 0.92 (s, 9H), 0.03 (s, 3H), 0.01(s, 3H); ^{13}C NMR δ 203.22, 40.95, 26.69, 17.50, 8.95, -6.71, -6.88; LRMS (GC-MS) *m*/*e* 172 (M^+)

[5.0.8] Synthesis of 1-dibromo-3-*tert*-butyldimethylsilyl-1-butene (38)



2.7 g of tetrabromomethane (8.14 mmol) was stirred in CH_2Cl_2 (1M) and kept at 0^oC. To this solution was added 4.35 g triphenylphosphine (16.6 mmol) in CH_2Cl_2 (2M) and stirred at this temperature for 30 min. Then the solution of 500 mg of aldehyde 37 (2.90 mmol) in CH_2Cl_2 (0.5M) was added and stirred at 0^oC for 2 hr. The reaction mixture was then treated with 42 ml of ether and the resulting white precipitates were filtered off. The filtrate was then washed with 9 ml of H_2O, 9 ml of saturated $NaHCO_3$, 9 ml of saturated NH_4Cl, 9 ml of saturated NaCl, and dried over $MgSO_4$. The solvent was then evaporated in vacuo and the residue was eluted with 30:1 petroleum ether:ethylacetate in a column chromatography to furnish 733 mg of dibromo compound 38 in 77 % yield as a colorless oil. ^1H NMR δ 6.25 (d, 1H, J = 11.5), 2.08 (dq, 1H, J = 11.5, 7.2), 1.10 (d, 3H, J = 7.2), 0.94 (s, 9H), 0.00 (s, 3H), -0.01(s,3H); ^{13}C NMR δ 142.82, 84.41, 27.04, 26.52, 17.37, 14.79, -7.28, -7.34; LRMS (GC-MS) *m*/*e* 328 (M^+)

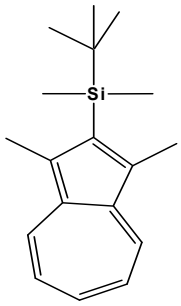
[5.0.9] Synthesis of 4-*tert*-butyldimethylsilyl-1-pentyne (39)



340 mg of 38 (1.07 mmol) was dissolved in THF (0.25M) and kept in dry ice/ acetone water bath. To this solution 1 ml of BuLi (2.28 mmol) was added at -78^oC under

argon and stirred for 30 min. The reaction mixture was then quenched with 0.08 ml (182 mg) of iodomethane (1.28 mmol) and heated at 40^{o}C for 2 hr. After 2 hr stirring, the reaction mixture was brought to room temperature and stirred for another 18 hr. To this reaction mixture 10 ml of 1:1 ether/water was added and then aqueous layer was separated and washed with ether (3x 20 ml). The organic layers were combined and washed with 20 ml of saturated NaCl, drie over $MgSO_4$ and concentrated in rota vap. The residue was then purified by column chromatography using petroleum ether to furnish 160 mg of 39 as pale yellow oil (82 %). ^{1}H NMR δ 1.78 (s, 3 H), 1.75-1.79 (m, 1 H), 1.17 (d, 3 H, J = 7.2), 0.95 (s, 9 H), -0.02(s, 3H), 0.04 (s, 3H); ^{13}C NMR δ 83.20, 74.89, 27.12, 17.40, 16.29, 10.29, 3.61, -7.42, -7.82; LSMS (GC-MS) *m*/*e* 182 (M^{+})

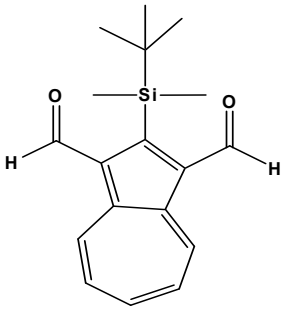
[5.1.0] Synthesis of 2-*tert*-butyldimethylsilyl-1,3-dimethylazulene (40)



In a three necked round bottomed flask, containing a magnet stirrer, 2.13 mg of 38 (11.69 mmol) was treated with 85 ml of acetonitrile and 4.06 g of poly(4-vinylpyridine (38.57 mmol) and stirred under argon at room temperature. To the resulting suspension was added 4.58 g of tropylium tetrafluoroborate (25.72 mmol) in one portion. The reaction mixture was turned into blue in 60 seconds and stirred at room temperature in the dark for 24 h. The dark blue reaction mixture was then filtered, and the solid residue was washed with 300 ml of hexane and 20 ml of acetonitrile until the washing

became clear. The filtrate was transferred into a separatory funnel, shaken vigorously, and the top blue hexane layer was separated from the bottom brown acetonitrile layer. The acetonitrile layer was extracted with hexane (4 x 40 ml). The combined hexane layers were washed with 15 ml of saturated sodium chloride, dried over anhydrous $MgSO_4$, filtered, and evaporated the solvent to obtain blue-green solid. The blue green residue was then purified by column chromatography using hexane to furnish 687 mg of azulene 40 as blue-green crystals in 22% yield. ^{1}H NMR δ 8.18 (d, 2H, J = 9.8), 7.46 (t, 1H, J = 9.8), 6.96 (t, 2H, J = 9.8), 2.81 (s, 6H), 1.05 (s, 9H), 0.6 (s, 6H); ^{13}C NMR δ 147.12, 137.87, 136.89, 132.68, 132.28, 120.38, 26.99, 19.33, 14.38, - 1.46; LRMS (LC/MS-ESI, MeOH) *m*/*e* 271 (MH^+)

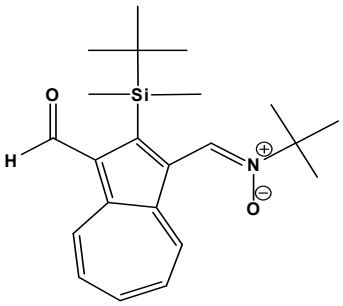
[5.1.1] Synthesis of 2-*tert*-butyldimethylsilyl-1,3-azulenedicarboxaldehyde (32)



90 mg of 40 (0.33 mmol) was dissolved in 5 ml of acetone at room temperature. To this solution was added 359 mg of DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) (1.58 mmol) in one portion. The reaction mixture immediately turned color from blue to red as it was stirred in the dark for 1 hour. When all reactant was gone the reaction mixture was stirred with 15 ml of saturated sodium thiosulfate solution to remove unreacted DDQ for 2 min and then 15 ml of saturated sodium bicarbonate was added, and then it was stirred for 2 min to remove acidic $DDQH_2$ (2,3-dichloro-5,6-

dicyanohydroquinone). The reaction mixture was then extracted with chloroform (5 x 40 ml). The combined bottom red organic phases were subsequently washed with 15 ml of saturated NaCl solution, dried over anhydrous $MgSO_4$, filtered and evaporated. The red residue was then purified by column chromatography using 7:3 hexane:ethylacetate to provide 88.4 mg of bis-aldehyde 32 in 89 % yield as red crystals. ^{1}H NMR δ 10.59 (s, 2H), 10.12 (dd, 2H, J = 10), 8.11 (tt, 1H, J = 10), 7.94 (t, 1H, J = 10), 1.05 (s, 9H), 0.61 (s, 6H); ^{13}C NMR δ 190.28, 162.4, 144.9, 142.75, 140.94, 134.14, 132.73, 27.06, 17.99, 0.98; LRMS (LC/MS-ESI, MeOH) *m*/*e* 299 ($MH^{+)}$

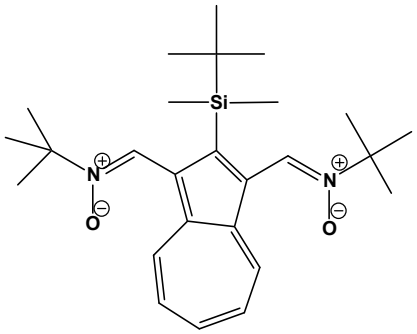
[5.1.2] Synthesis of 2-*tert*-butyldimethylsilyl-3-formyl-azulene-1-nitrone (23)



To the mixture of 50 mg of bis-aldehyde 32 (0.167 mmol), 42.2 mg of *tert*-butylhydroxylamine hydrochloride (0.335 mmol) and 40.4 mg of anhydrous magnesium sulfate (0.335 mmol) was added 1.4 ml of pyridine to dissolve. The reaction mixture was then stirred and heated to 40^{o}C for 1 week under argon. The color of the reaction solution changed from red into green. The crude product was then poured into 5 ml of water and extracted with 5 ml of ether. Then aqueous layer was separated from ether layer. The aqueous layer was further extracted with ether (2 x 5 ml). The combined ether layer was washed with saturated sodium chloride, dried over magnesium sulfate, filtered and, evaporated in a vacuo. The residue was then purified by silica gel column

chromatography using 5:5 hexane:ethylacetate to produce 20.3 mg of mononitrone 23 in 33 % yield as green crystals. ^{1}H NMR δ 10.51 (s, 1H), 9.95 (d, 1H, J = 9.9), 8.27 (d, 1H, J = 9.9), 8.10 (s,1H), 7.94 (t, 1H, J = 9.9), 7.74 (t, 1H, J = 9.9), 7.69 (t, 1H, J = 9.9), 1.71 (s, 9H), 0.99 (s, 9H), 0.53 (s, 6H); ^{13}C NMR δ 189.25, 156.37, 143.34, 140.97, 140.49, 139.84, 139.52, 132.35, 131.69, 129.2, 128.19, 127.83, 70.46, 28.35, 26.81, 18.35, -0.65; LRMS (LC/MS-ESI, MeOH) *m*/*e* 370 (MH^+)

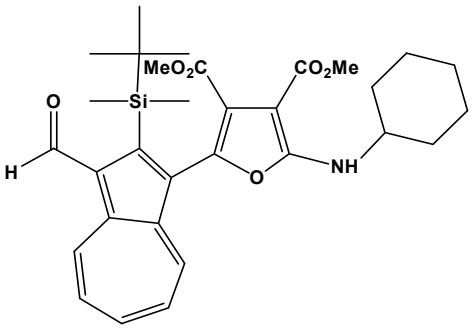
[5.1.3] Synthesis of 2-*tert*-butyldimethylsilyl-1,3-azulenyldinitrone (55)



A solution of 77 mg of dialdehyde 32 (0.26 mmol), 162 mg of tert-butylhydroxylamine hydrochloride (1.3 mmol), 155 mg of anhydrous magnesium sulfate, and 1 ml of pyridine was stirred at 45oC for 2 weeks under argon. The resulting mixture was then pour into 5 ml of water and extracted with 5 ml of ether. The aqueous layer was extracted with (2 x 5 ml) ether. The combined ether layers were washed with saturated sodium chloride, dried over $MgSO_4$, filtered and concentrated in vacuo. The resulting residue was then purified by column chromatography using 100:1 chloroform:methanol as eluenting solvent to furnish 35.4 mg of 55 in 31 % yield as green crystals. Compound 55 was also prepared in 35 % yield by heating 66 mg of mononitrone 23 (0.18 mmol), 67.4 mg of tert-butylhydroxylamine hydrochloride (0.54 mmol), 64.6 mg of anhydrous $MgSO_4$ (0.54 mmol) and 1 ml of pyridine at 45^{o}C for 10 days. ^{1}H NMR δ 8.35 (d, 2H, J =

9.5), 8.07 (s, 2H), 7.76 (t, 1H, J = 9.7), 7.48(t, 2H, J = 9.9), 1.70 (s,18H), 0.95 (s, 9H), 0.48 (s, 6H); ^{13}C NMR δ 149.62, 139.58, 138.71, 138.04 128.74, 126.86, 126.11, 70.08, 28.44, 26.53, 18.70, -2.05; LRMS (LC/MS-ESI, MeOH) *m*/*e* 441 (MH^+)

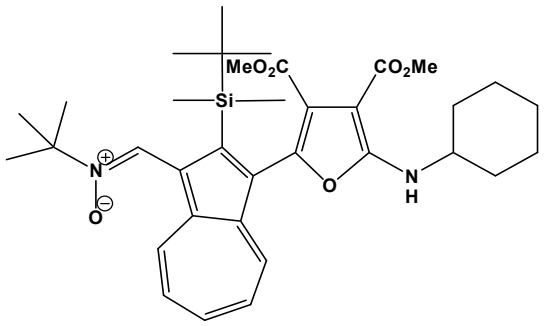
[5.1.4] Synthesis of 2-[2-(*tert*-butyldimethyl-silanyl)-3-formyl-azulen-1-yl]-5-cyclohexylamino-furan-3,4-dicarboxylic acid dimethyl ester (50)



A mixture of 250 mg of bis-dialdehyde 32 (0.84 mmol) and 92 mg of DMAD (dimethyl acetylenedicarboxylate) (0.84 mmol) was dissolved in 8.5 ml of anhydrous benzene and heated at 80^{o}C under argon. To the solution was added 119 mg of cyclohexylisocyanide (0.84 mmol) by syringe, and the heating was continued at 80^{o}C for further 5 days. The color of the reaction changed from red into brown. The solvent was then evaporated in a vacuo, and the crude residue was purified on column chromatography using 8:2 hexane:ethylacetate to obtaine first starting material, and then polarity was increased to 7:3 hexane:ethylacetate to furnish 117 mg of 50 as wine red solid in 25 % yield. ^{1}H NMR δ 10.59 (s, 1H), 9.99 (dd, 1H, J = 9.9, 0.8), 8.40 (dd, 1H, J = 9.9, 0.8), 7.90 (t, 1H, J = 9.8), 7.71 (t, 1H, J = 9.8), 7.40 (t, 1H, J = 9.7), 6.72 (d, 1H, J = 8.9), 3.82 (s, 3H), 3.52-3.365 (m, 1H), 3.49 (s, 3H), 1.20-2.05 (m, 10H), 0.95 (s, 9H),0.34 (s, 3H), 0.23 (s, 3H); ^{13} C NMR δ 189.84, 165.34, 164.39, 162.04, 157.31, 146.20, 141.70, 141.15, 139.83, 139.73, 138.19, 131.24, 130.20, 129.45, 125.96, 118.56, 86.09,

51.74, 51.35, 51.03, 34.01, 33.89, 27.34, 25.27, 24.69, 24.60, 17.95, -1.86, -3.42; LRMS (LC/MS-ESI, MeOH) m/*e* 550 (MH^+)

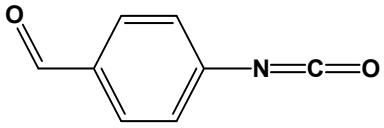
[5.1.5] Synthesis of 2-[2-(*tert*-butyldimethyl-silanyl)-3-*tert*-butylnitrone-azulen-1-yl]-5-cyclohexylamino-furan-3,4-dicarboxylic acid dimethyl ester (49)



A solution of 13.5 mg of aminofuran adduct 50 (0.025 mmol), 9.3 mg of tert-butylhydroxylamine hydrochloride (0.074 mmol), 8.9 mg of magnesium sulfate (0.074 mmol), and 0.3 ml of pyridine was purged with argon and stirred at 50^{o}C for 7 days. The reaction mixture was then poured into 5 ml of water and extracted with ether (3 x 5 ml). The combined ether layers were then washed with 10 ml of saturated sodium chloride, dried over anhydrous magnesium sulfate, filtered, and evaporated in a vacuo. The resulting green residue was then purified by column chromatography using 5:5 ethylaetate/hexane to produce 3 mg of nitrone 49 in 20 % yield. ^{1}H NMR δ 8.48 (d, 1H, d = 9.9), 8.25 (d, 1H, d = 9.2), 8.2 (s, 1H), 7.74 (t, 1H, d = 9.8), 7.44 (t, 1H, d = 9.9), 7.37 (t, 1H, d = 9.8), 6.68 (d, 1H, d = 8.8), 3.81 (s, 3H), 3.50 - 3.57 (m, 1H), 3.49 (s, 3H), 1.85 – 2.00 (m, 2 H), 1.7 (s, 9H), 1.10 – 1.40 (m, 8H), 0.28 (s, 3H), 0..2 (s, 3H); ^{13}C NMR δ 165.47, 164.61, 161.99, 151.19, 144.02, 140.27, 139.60, 139.55, 136.68, 136.10, 129.02, 126.50, 125.40, 125.33, 125.22, 118.07, 86.11, 70.15, 51.11, 51.37, 51.00, 33.97,

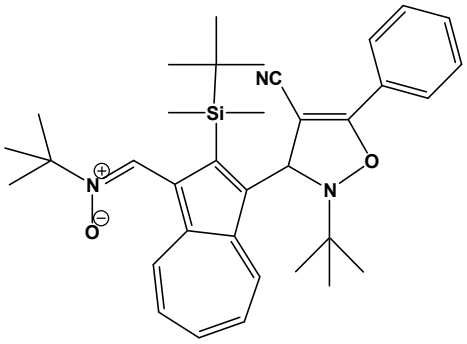
28.47, 27.17, 25.36, 24.79, 24.63, 18.48, 0.00, -3.20, -4.15 ; LRMS (LC/MS-ESI, MeOH) *m*/*e* 621 (MH^+)

[5.1.6] Synthesis of *p*-formylphenyl isocyanate (53)



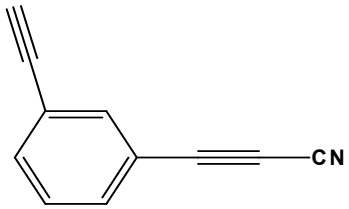
According to the modified method of Yamada, 1 gm of 4-carboxybenxzaldehyde (6.67 mmol) was suspended in 16 ml of anhydrous methylene chloride and added 0.9 ml (0.66 g) of triethylamine (6.67 mmol). After all the solid had dissolved, 1.44 ml of diphenylphosphoryl azide (6.67 mmol) was added dropwise through a syringe. The content was then refluxed for 6 hr. The reaction mixture changed the color from colorless to pale yellow. The solvent was then evaporated in a vacuo. The resulting yellow solid was then dissolved in 40 ml of ether and washed with aqueous sodium bicarbonate at PH 9 (2 x 25 ml). The ether layer was dried over anhydrous magnesium sulfate, filtered, and evaporated ether to provide 1 gm of 53 as a pale yellow solid in 100 % yield. ^{1}H NMR δ 10.12 (s, 1H), 8.20 (d, 2H, J = 8.3), 7.80 (d, 2H, J = 8.3); ^{13}C NMR δ 191.33, 171.73, 140.03, 135.34, 130.05, 129.70; LRMS (GC-MS, CH_2Cl_2) *m*/*e* 147 (M^+), 146, 118, 90, 63, 51; IR (NaCl) 2136 cm^{-1} for isocyanate group.

[5.1.7] Synthesis of 2-*tert*-butyl-3-[2-(*tert*-butyldimethyl-silanyl)-3-nitrone-azulen-1-yl]-5-phenyl-2,3-dihydro-isoxazole-4-carbonitrile (24)



The mixture of 26 mg of dinitrone 55 (0.06 mmol) and 7.5 mg of phenylpropiolonitrile (0.06 mmol) was dissolved in 1 ml of anhydrous benzene and heated at 50^{o}C for 6 days. The solvent was then dried in a vacuo to provide green solid. The resulting green residue was then purified by column chromatography on silica gel using 7:3 hexane:ethylacetate which afforded 25 mg of 24 as green crystals in 75 % yield and 19 % of starting materials. ^{1}H NMR δ 9.22 (d, 1H, J = 9.5), 8.21 (d, 1H, J = 9.5), 8.19 (s, 1H), 7.92 (dd, 2H, J = 8, 1.3), 7.65 (t, 1H, J = 9.7), 7.47 (m, 3H), 7.32 (t, 1H, J = 9.7), 7.25 (t, 1H, J = 9.7), 6.33 (s, 1H), 1.70 (s, 9H), 1.25 (s, 9H), 1.00 (s, 9H), 0.73 (s, 3H), 0.40 (s, 3H); ^{13}C NMR δ 161.62, 146.75, 140.63, 139.34, 138.55, 137.21, 136.95, 135.44, 131.68, 129.30, 128.86, 127.12, 125.87, 125.66, 124.90, 124.63, 116.20, 85.80, 70.06, 65.28, 61.87, 28.38, 28.03, 25.39, 19.18, 0.27, -1.26; LRMS (LC/MS-ESI, MeOH) *m*/*e* 568 (MH^{+})

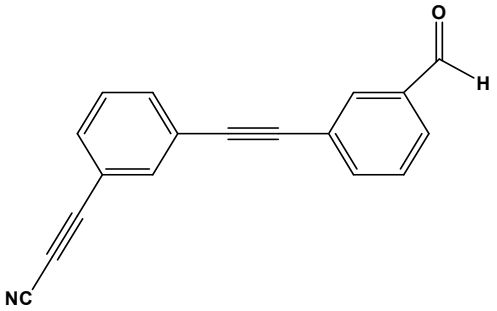
[5.1.8] Synthesis of (3-ethynyl-phenyl)-1-propyne nitrile (63)



Three-necked round bottom flask, fitted with argon inlet and containing a magnetic stirring bar, is charged with 11.3 ml of DMSO, (dimethyl sulfoxide), 3.75 ml of acetonitrile and 0.2 ml of water, and stirred the solution rapidly. To the rapidly stirring solution, 1.35 g of cuprous cyanide (15 mmol) was added very slowly to avoid formation of lumps. Then 113 mg of sodium iodide (0.752 mmol) was added to the reaction mixture, and was stirred for 1 min. Then 1 ml (0.940 g) of 1,3-diethynylbenzene (7.53 mmol) was added slowly via syringe. Subsequently, 2.9 ml (2.45 g) of chlorotrimethylsilane (22.56 mmol) was added dropwise via syringe over 20 min and stirred the reaction mixture at 50^{o}C for 24 hr. The reaction mixture was then cooled to room temperature and then 5 ml of water was added. It was then extracted with ether (5 x 10 ml). The combined ether layers were then washed with 25 ml of saturated sodium bicarbonate, 25 ml of saturated sodium chloride, dried over anhydrous magnesium sulfate, filtered and evaporated the solvent by rotatory evaporation to give crude yellow oil. The crude residue was then purified by silica gel column chromatography using pentane as eluenting solvent to afford 147 mg of (3-ethynyl-phenyl)-1-propyne nitrile as white crystals in 13 % yield. ^{1}H NMR δ 7.72 (sm, 1H), 7.63 (dt, 1H, J = 0.02), 7.58 (dt, 1H, J = 0.02), 7.39 (t, 1H, J = 0.02), 3.16 (s, 1H); ^{13}C NMR δ 136.74, 135.26, 133.39,

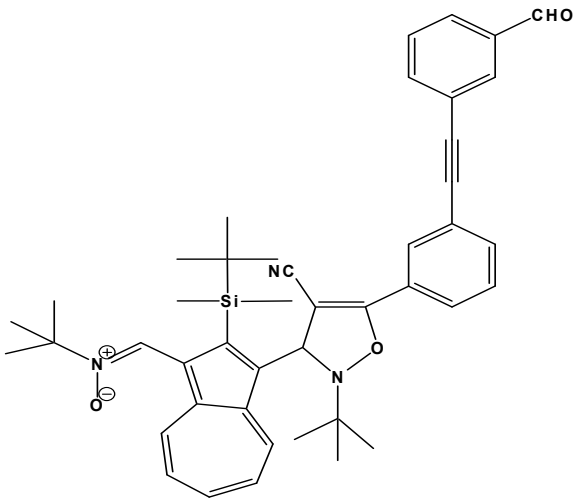
129.03, 123.44, 118.06, 105.19, 81.67, 81.49, 79.26, 63.59; LRMS (GC/MS, CH_2Cl_2) *m*/*e* 151 (M^+)

[5.1.9] Synthesis of [3-(3-formyl-phenylethynyl)-phenyl]-propyne nitrile (61)



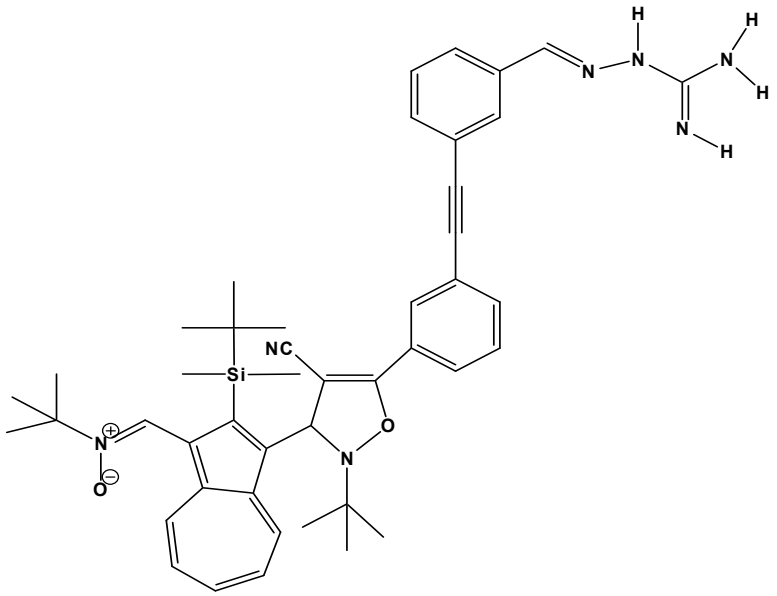
The mixture of 87 mg of (3-ethynyl-phenyl)-1-propyne nitrile 63 (0.576 mmol), 120.3 mg of 3- iodobenzaldehyde, and 0.8 ml triethylamine (58.5 mg, 0.576 mmol) were dissolved in 1.5 ml of anhydrous THF, and the solution was deoxygenated by purging argon in it. Then 22 mg of tetrakistriphenylphosphine palladium (0.019 mmol) was added to the solution, and the reaction was allowed to stir for 20 min at room temperature. After 20 min, 0.9 mg of cuprous iodide (4.5×10^{-3} mmol) was added to the stirring solution and stirred at room temperature for 2 hr, followed by heating further at 50^{o}C for 2 hr. The solvent was then evaporated in a rotatory evaporation to give brown solid. The residue was then purified by silica gel column chromatography using 1:9 dichloromethane:hexane to collect starting material. The polarity was increased to 2:8 dichloromethane:hexane to afford 80 mg of 61 as a golden solid in 55 % yield. ^{1}H NMR δ 10.2 (s, 1H), 8.04 (sm,1H), 7.89 (dm, 1H, J = 7.7), 7.78 (m, 2H), 7.69 (dm, 1H, 7.8), 7.60 (dm, 1H, J = 7.8), 7.56 (t, 1H, J = 7.7), 7.44 (t, 1H, J = 7.8); ^{13}C NMR δ 191.30, 137.13, 136.59, 136.27, 134.81, 133.21, 132.90, 129.61, 129.26, 123.99, 123.69, 118.16, 105.22, 89.72, 88.64, 81.77, 63.62; LRMS (GC/MS, CH_2Cl_2) *m*/*e* 255 (M^+)

[5.2.0] Synthesis of 2-*tert*-butyl-3-[2-(*tert*-butyldimethyl-silanyl)-3-nitrone-azulen-1-yl]-5-[3-(3-formyl-phenylethynyl)-phenyl]-2,3-dihydro-isoxazole-4-carbonitrile (64)



A mixture of 288 mg of dinitrone 55 (0.65 mmol) and 17.4 mg of [3-(3-Formyl-phenylethynyl)-phenyl]-propyne nitrile 63 (0.65 mmol) was dissolved in 8 ml of anhydrous benzene. The reaction mixture was then heated at 50^{o}C for 1 week. The solvent was then dried in a vacuo to afford a green solid which was purified by column chromatography on silica gel .The column was washed with 7:3 hexane:ethylacetate to elute 380 mg of aldehyde 64 as green crystals in 84 % yield with 16 % of starting materials 55. ^{1}H NMR δ 10.02 (s, 1H), 9.23 (d, 1H, J = 9.8), 8.21 (d, 1H, J = 10.1), 8.19, (s, 1H), 8.06 (s, 1H,),8.04 (s, 1H), 7.94 (dm, 1H, J = 8.1), 7.87 (dm, 1H, J = 7.7), 7.80 (dm, 1H, J = 7.7), 7.68 (m, 2H), 7.54 (t, 1H, J = 7.7), 7.47 (t, 1H, J = 7.8), 7.34 (t, 1H, J = 9.8), 7.28 (t, 1H, J = 10.1), 6.36 (s, 1H), 1.71 (s, 9H), 1.27 (s, 9H), 1.00 (s, 9H); ^{13}C NMR δ 191.43, 160.59, 146.86, 140.65, 139.46, 138.62, 137.19, 137.17, 137.02, 136.57, 135.05, 134.61, 133.13, 130.03, 129.37, 129.25, 129.20, 129.12, 127.20, 126.33, 125.72, 124.98, 124.77, 124.02, 123.70, 115.89, 89.64, 89.19, 86.61, 70.12, 65.39, 61.96, 28.39, 28.03, 25.43, 19.20, 0.27, -1.25; LRMS (LC/MS-ESI, MeOH) *m*/*e* 696 (MH^{+})

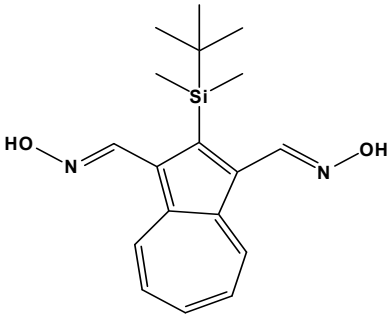
[5.2.1] Synthesis of 2-*tert*-butyl-3-[2-(*tert*-butyldimethyl-silanyl)-3-nitrone-azulen-1-yl]-5-[3-(3-guanylhydrazone-phenylethynyl)-phenyl]-2,3-dihydro-isoxazole-4-carbonitrile (25)



A mixture of 554 mg of 64 (0.797 mmol), 88.2 mg of aminoguanidine hydrochloride (0.797 mmol) and 0.33 ml of triethylamine (2.39 mmol) was dissolved in 1.5 ml of pyridine. The reaction mixture was then stirred for 7 days at 45^{o}C. The resulting green reaction solution was then treated with 5 ml of water and extracted with dichloromethane (6 x 10 ml). The combined organic phases were then washed with 10 ml of saturated sodium chloride, dried over anhydrous magnesium sulfate, filtered, and evaporated in a vacuo to obtain green solid. The green residue was then purified by column chromatography on silica gel. The column was first washed with 60:40 hexane:ethylacetate to collect 10 mg of starting material 64. The polarity of solvent was then increased to 95:5 chloroform:methanol to wash the column to afford 570 mg of nitrone 25 in 95 % yield as a green crystal. ^{1}H NMR δ 9.43 (d, 1H, J = 9.88), 8.33 (s,

1H), 8.01 (s, 1H), 8.00 (d, 1H, J = 9.50), 7.90 (s, 1H), 7.70-7.80 (m, 4H), 7.39 (d, 1H, J = 7.80) 7.58 (d, 1H, J = 8.29), 7.20-7.44 (m, 5H), 6.30 (s, 1H), 1.7 (s, 9H), 1.25 (s, 9H), 1.04 (s, 9H), 0.7 (s, 3H), 0.5 (s, 3H); ^{13}C NMR δ 160.71, 155.80, 147.18, 146.03, 140.26, 140.10, 137.81, 137.75, 136.79, 134.90, 134.26, 133.40, 132.76, 131.74, 130.58, 130.17, 128.97, 128.75, 127.63, 126.47, 126.22, 125.87, 125.39, 124.06, 123.79, 123.02, 116.18, 90.66, 88.85, 86.22, 70.20, 65.40, 61.77, 28.26, 28.01, 25.36, 19.17, -0.08, -1.17**;** LRMS (LC/MS-ESI, MeOH) *m*/*e* 752 (MH^+)

[5.2.2] Synthesis of 2-*tert*-butyldimethylsilyl-1,3-azulenyldioxime (48)



In an attempt to synthesize nitrone oxime 46, 13 mg of mononitrone 23 (0.0352 mmol) was mixed with 2.5 mg of hydroxylamine hydrochloride (0.0352 mmol) and dissolved in 1 ml of pyridine in a small vial under argon. The reaction mixture was heated at 40^{o}C. After 2 hr of stirring no product has been observed by TLC so 1 more equivalent of hydroxylamine hydrochloride was added and stirred the reaction mixture for further 24 hr. When TLC showed no more reactant, 8 ml of water was added to the resulting reaction mixture and extracted with ethylacetate (3 x 10 ml). The combined organic layer was washed with 10 ml saturated sodium chloride solution, dried over anhydrous magnesium sulfate, filtered and concentrated in a vacuo. Purification of the resulting green solid by column chromatography on silica gel using 7:3 hexane:

ethylacetate failed to provide nitrone oxime 46 indicated by NMR and LRMS. Instead 10 mg of dioxime 48 as green crystals in 87 % yield was obtained confirmed by NMR and LRMS. ^{1}H NMR δ 9.22 (d, 2H, J = 9.6), 8.80 (s, 2H), 7.70 (t, 1H, J = 9.8), 7.5 (br s, 2H), 7.39 (t, 1H, J = 10) 0.99 (s, 9H), 0.52 (s, 6H); ^{13}C NMR δ 150.80, 150.05, 140.67, 139.87, 138.51, 127.38, 127.08, 26.98, 18.46, -0.74; LRMS (LC/MS-ESI, MeOH) *m*/*e* 329 (MH^{+})

[5.2.3] Attempted synthesis of 2-*tert*-butyldimethylsilyl-3-nitrone-1-azulenemalonolitrile (41)

A mixture of 15 mg of mononitrone 23 (0.041 mmol) and 2.7 mg of malononitrile (0.041 mmol) in 0.5 ml of dimethylformamide (DMF) was treated with 1.7 mg of LiCl (0.0041 mmol) and stirred either at room temperature for 24 hour. Thin layer chromatography showed one reactant spot and another orange spot. The reaction mixture was then treated with 2 ml of water and extracted with 5 ml ethylacetate several times. The combined ethylacetate layers were dried over magnesium sulfate and concentrated in vacuo. The resulting residue was purified by column chromatography. The column was washed first with 5:5 ethylacetate/ hexane to collect 3.6 mg of starting material then polarity was increased to 80 % to collect 7.3 mg of an orange product. An unidentified orange product has been observed by ^{1}H NMR that contained no *tert*-butyl and methyl groups attached to silyl group. The compound has not been identified yet.

[5.2.4] Attempted synthesis of isoxazole tricarboxylic acid trimethylester (44)

A mixture of 10 mg of mononitrone 23 (0.0271 mmol) and 13.3 µl dimethyl acetylenedicarboxylate (0.108 mmol, 4 equiv.) was dissolved in 0.2 ml of dichloromethane. The reaction mixture was stirred at room temperature for 11 hr.

Evaporation followed by purification on column chromatography using 7:3 hexane:ethylacetate gave 5 mg of purple product. However, the purple compound is identified as an intermediate 43 based on ^{1}H NMR and MS. Therefore, the reaction is further stirred for 7 days at 35^{o}C but only the intermediate compound was recovered.

[5.2.5] Attempted synthesis of 54 from p-formyl isocyanate and aminofuran adduct (50)

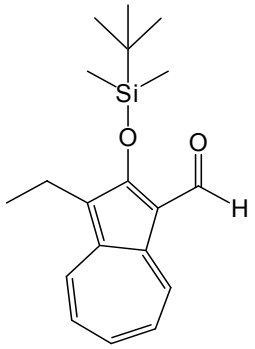
Following the modified method of Fowler,[313] treatment of 3.4 mg of p-formyl isocyanate 53 (0.022 mmol) with 12 mg of aminofuran adduct 50 (0.022 mmol) dissolved either 0.2 ml of benzene or toluene and heated at 90^{o}C for 7 days failed to show any product by TLC. The solvent was then removed by rotator evaporation. Recrystallized from ether gave 12 mg of wine red solid (89 %) which is identical to the starting material 50 confirmed by TLC and ^{1}H NMR.

[5.2.6] Attempted synthesis of α,β–unsaturated ketone derivative of isoxazoline (57)

A mixture of 7.2 mg of carboxynitrile compound 24 (0.0127 mmol), 0.04 ml of iodomethane (0.609 mmol) and 64 mg of poly-4 vilylpyridine (0.609 mmol) was dissolved in 0.5 ml of tetrahydrofuran. The reaction was stirred 40^{o}C for 36 hour. After three product were observed by TLC, the reaction mixture was filtered and washed with 15 ml of hexane:ethylacetate and concentrated in a vacuo. The resulting residue was then chromatographed on silica gel by washing the column with 8:2 hexane:ethylacetate to collect the first two purple products 1.5 mg and 3 mg respectively and then polarity was increased to 5:5 ethylacetate:hexane to collect 1 gm of green product and 0.8 mg of starting material. However, the mixture of products failed to indicate the presence of 57

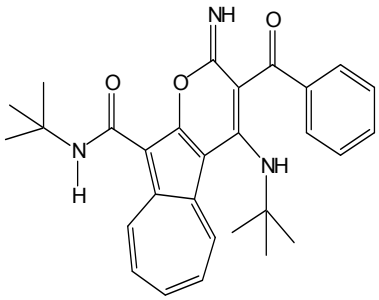
by ^{1}H NMR and LRMS. Three products were presumed to be 58, 59 and 60 based on their LRMS data.

[5.2.7] Aldehyde product (67) of superoxide trapping with nitrone 22

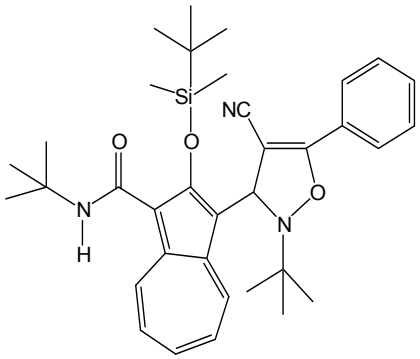


A mixture of 6 mg of nitrone 22 (0.0163 mmol), 6.9 mg of potassium superoxide (0.097 mmol, 6 equiv.) and 8.6 mg of crown ether (0.032 mmol, 2equiv.) was dissolved 1 ml of benzene. The reaction was stirred for 3 hr at room temperature. The product was washed with 10 ml of saturated sodium chloride several times, dried over magnesium sulfate and concentrated. The mixture was purified by column chromatography using 5:5 hexane:ethylacetate to give 2.2 mg of crude 67 (43 %). It was not possible to obtain 67 pure enough for definitive NMR characterization. Nevertheless, the presence of aldehyde-proton and up-field shifting of ethyl-proton in ^{1}H NMR data suggested the compound 67. When the same reaction was stirred for three days, another orange product was observed. The ^{1}H NMR was observed with no aldehyde-proton signal and LRMS detected MH^{+} of 663. It was not possible to isolate the product 67 in a pure form for structure identification.

[5.2.8] Amide products 74 and 79 of superoxide trapping with nitrone 24



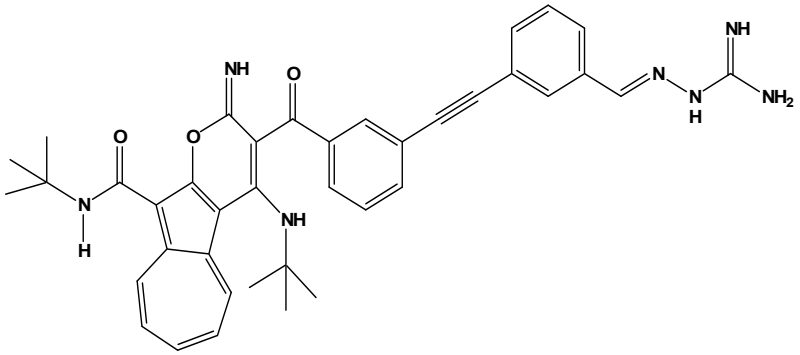
74



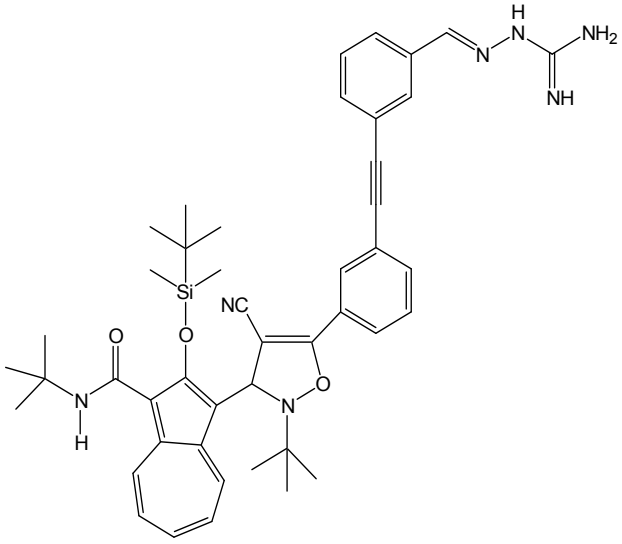
79

A mixture of 20 mg of nitrone 24, 7.5 mg of potassium superoxide and 18.6 mg of crown ether was dissolved in 1 ml of benzene. The reaction was stirred for 16 hr at room temperature. The mixture was then washed with 10 ml of saturated sodium chloride several times, dried and concentrated. Purification of the mixture product by column chromatography using 5:5 ethylacetate:hexane afforded 7.2 mg of orange amide 74 in 44 % yield. ^{1}H NMR δ 8.8 (s, 1H), 7.7 (d, 1H J = 10.3), 7.63 (m, 2H), 7.56 (m, 1H), 7.40 (t, 1H J = 7.3), 7.7.33-7.22 (m, 4H), 7.0 (m, 1H), 6.24 (s, 1H), 1.57 (s, 9H), 1.48 (s, 9H); ^{13}C NMR δ 190.81, 172.62, 168.62, 157.58, 138.93, 138.43, 135.46, 133.20, 131.36, 128.35, 127.83, 127.12, 125.92, 125.11, 124.81, 122.05, 116.53, 115.0, 83.68, 63.42, 52.90, 29.90, 28.99; LRMS (LC/MS-ESI, MeOH) *m*/*e* 470 (MH^+); HRMS calculated for $C_{29}H_{31}O_3N_3$ found 470.2438. In addition to compound 74, trace of amide 79 was also recovered which was not enough for NMR characterization. However, it was possible to obtain consistent MS data: LRMS (LC/MS-ESI, MeOH) *m*/*e* 584 (MH^+).

[5.2.9] Amide products 80 and 81 of superoxide trapping by nitrone 25



80



81

A mixture of 15 mg of nitrone 25 (0.021 mmol), 4.4 mg (3 equiv.) of potassium superoxide and 11.1 mg (2 equiv.) of crown ether was dissolved in 1 ml of benzene and stirred for 19 hr. Work up was done as aforementioned method. The mixture product was separated by column chromatography beginning with 98:2 dichloromethane:methanol and increasing the polarity to 94:6 dichloromethane:methanol to provide crude brownish orange product. LRMS analysis indicated the compound 80 with 654 (MH^+) as a major product and the compound 81 with 768 (MH^+) as a minor product along with some

starting material. It was not possible to separate the mixture pure enough for NMR characterization.

[5.3.0] UV-vis and fluorescence analysis

UV-vis and fluorescence experiments were performed with assistance of Dr. Moon's students. The orange product 74 from the reaction of 2-*tert*-butyldimethylsilyl-3-*tert*-butylnitrone-1-(2-tert-butyl-4-cyano-5-phenyl)-azulenylisoxazoline (24) with superoxide was tested. Compound 74 was dissolved in ethanol for UV-vis absorbance measurement in scan range of 200-400 nm with scan rate of 600 nm/min at average time 0.1 s and data interval of 1.00 nm. Fluorescence signal was obtained in scan range of 400-700 nm using 0.1 s integration time with an increment of 0.1 nm. Both the excitation slit wide and the emission slit wide were selected 1.5 nm with the excitation wavelength fixed at 390 nm.

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PATENT:

Silyated Azulenyl Nitrone Spin Traps as Chromotropic Superoxide Detectors - pending