

**AUTOXIDATION AND ITS INHIBITION IN BOTH INDUSTRIAL AND
BIOLOGICAL CONTEXTS: NEW MOLECULES, METHODS &
MECHANISMS**

by

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Abstract

Autoxidation, a radical chain reaction, is largely responsible for the degradation of most man-made and biological materials. These include chemical products such as lubricants, plastics and rubber; as well as biological molecules and membranes within our bodies. The development of means to hinder this process has been a major focus of the petroleum, chemical, pharmaceutical and biotechnology industry over the past century. The two most common strategies to emerge from these efforts have been the use of compounds that either prevent the initiation of autoxidation or trap the propagating radicals, so-called radical-trapping antioxidants (RTAs).

Herein, we describe our efforts towards the design and development of extremely potent heterocyclic diarylamine RTAs, and their activity in a variety of applications ranging from isotropic organic solution to mammalian cells. We have elucidated the important structural motifs and mechanistic considerations necessary for the development of next-generation arylamine RTAs. Some of the substituted heterocyclic diarylamines analogs we disclose are among the best inhibitors of high temperature autoxidations described to date. Alongside, we developed novel analytical tools to facilitate the studies and acquisition of results for characterizing RTA activity in organic solutions and lipid bilayers. These fluorescent probes are highly relevant and allow for the determination of hydroperoxide and acid concentrations rapidly, as well as screen (or counter-screen) RTAs in liposomal membranes. Our methodologies address numerous drawbacks from frequently used ‘plug-and-play’ assays and we anticipate they will fill a current unmet need in both industrial and academic laboratories worldwide.

Moreover, the recent characterization of ferroptosis – a novel regulated necrotic-like cell-death pathway associated with the accumulation of lipid hydroperoxides – has paved a way forward for studying oxidation induced damage in a biological context. Utilizing our expertise in lipid peroxidation and inhibition, we elucidated the prominent role of autoxidation in the execution of ferroptotic cell death. Alongside, our analytical tools and RTAs have also enabled the identification and characterization of

novel ferroptosis inhibitors. Furthermore, this has prompted the development of a correlation to predict anti-ferroptosis activity of small-molecules using simple spectrophotometric assays.

Statement of Originality

I hereby certify that all of the work described within this thesis is the original work of the author, with exceptions noted in the preface of each chapter. Any published (or unpublished) ideas and/or results from the work of others are fully acknowledged in the references.

Ronak Shah

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List of Abbreviations

AA	arachidonic acid
AAPH	2,2'-azobis-(2-amidinopropane) monohydrochloride
Ar ₂ NH	diarylamine
ArOH	phenol
BDE	bond-dissociation enthalpy
BODIPY	boron-dipyrromethene
CoQ ₁₀	coenzyme Q ₁₀
DPPH	2,2-diphenyl-1-picrylhydrazine
DTUN	di- <i>tert</i> -undecyl hyponitrite
Fer-1	ferrostatin-1
FSP	ferroptosis-suppressing protein
GPX	glutathione-peroxidase
GSH	glutathione
HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high-pressure liquid chromatography
KO	knockout
LA	linoleic acid
Lip-1	lipoxstatin-1
LOH	lipid alcohol
LOOH	lipid hydroperoxide
LOX	lipoxygenase
MeOAMVN	2,2'-azobis-2(4-methoxy-2,4-dimethylvaleronitrile)
PC	phosphatidylcholine
PCET	proton-coupled electron transfer

PL	phospholipid
PLOOH	phospholipid hydroperoxide
PMC	2,2,5,7,8-pentamethyl-6-chromanol
PUFA	polyunsaturated-fatty acid
ROOH	alkylhydroperoxide
ROS	reactive-oxygen species
RTA	radical-trapping antioxidant
TAM	4-hydroxytamoxifen
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxyl
THN	tetrahydronaphthyridinol
TTBP	2,4,6-tri- <i>tert</i> -butylpyridine
α -TOH	α -tocopherol

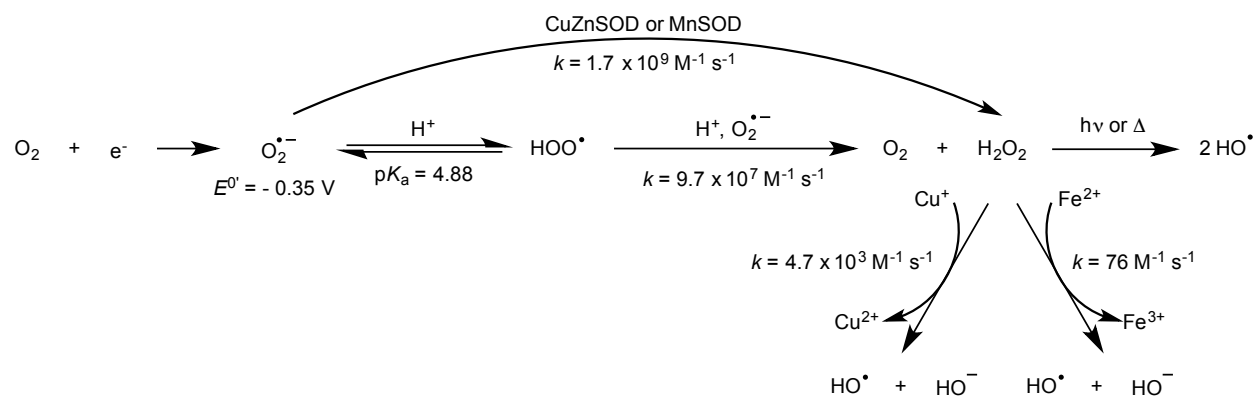
Chapter 1: Background and Significance

Virtually everything is exposed to molecular oxygen, and it is a necessity of life for many organisms.¹ However, oxygen is not innocuous as it reacts with most organic materials spontaneously, although fortunately relatively slowly. It is the reason that in air plastics discolour and disintegrate, rubber cracks, metals rust and animal tissue slowly decays.² Molecular oxygen in its ground state is in a triplet rather than a singlet state. This paramagnetic state (i.e. $^3\text{O}_2$) is characterized by two unpaired electrons with parallel spins, and although the oxygen atoms are highly electronegative, its divalent reduction is difficult because it would violate Pauli's exclusion principle, where the simultaneous addition of a pair of electrons would result in two electrons of the same spin occupying the same orbital.³ This attenuation in reactivity is bypassed in one of three ways:³

- 1) The input of 23 kcal/mol of energy excites one of the unpaired electrons into a higher energy orbital, which is associated with the inversion of its spin, producing singlet oxygen, $^1\text{O}_2$.
- 2) Coordination of oxygen to a metal atom such as iron, which contains its own unpaired electron resulting in direct divalent reduction of oxygen.
- 3) The addition of electrons one at a time which allows for bypassing of the spin restriction.

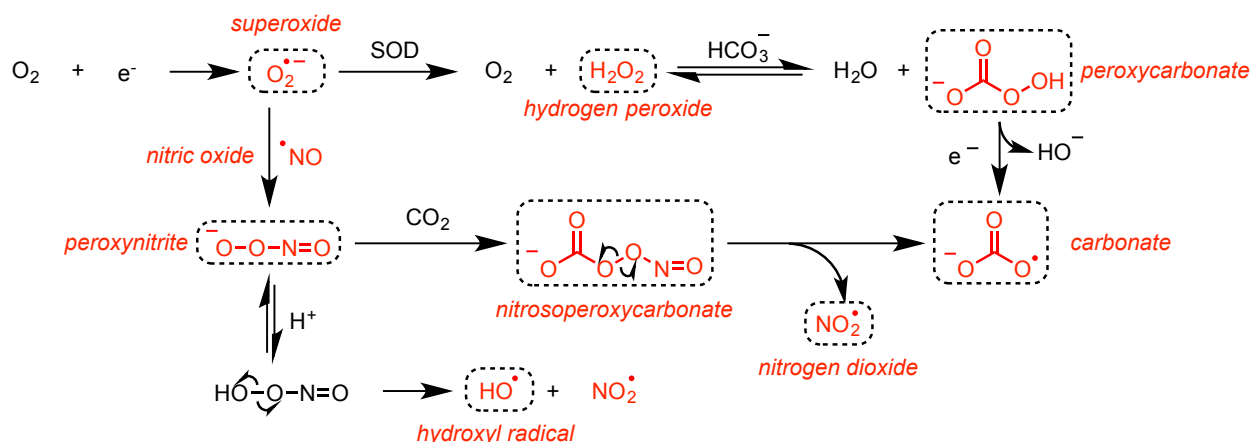
1.1 Reactive oxygen species

The detrimental effect of oxygen is often the result of its transformation to more reactive species, commonly denoted as 'reactive oxygen species' (ROS).⁴ ROS is often used as collective term for any species derived from oxygen that is more reactive than oxygen itself. The one electron reduction of oxygen produces superoxide ($\text{O}_2^{\bullet-}$), which itself is not generally directly responsible for the toxic effect of oxygen⁵ – superoxide is actually a good reductant and not an oxidant.⁴ However, superoxide can undergo several reactions to yield highly oxidizing species shown in Scheme 1.1.^{6,7}



Scheme 1.1 Reaction of superoxide to produce highly reactive oxidizing species.

Furthermore, ‘reactive’ is a relative term, some species like superoxide and hydrogen peroxide are very selective in their reactions, while the hydroxyl radical is indiscriminate. Therefore, an effort should be made to distinguish the reactive species rather than generalize them all as ‘ROS’. Likewise, contrary to common misconception, all oxygen radicals are ROS, but not all ROS are oxygen radicals (Scheme 1.2). Low concentration of superoxide is deliberately produced *in vivo* by the action of several enzymes such as aldehyde oxidase, prostaglandin synthase, cytochrome P450, but predominantly by the accidental electron ‘leakage’ from the NADH-coenzyme Q reductase complex of the electron-transport chain.⁸ As such, superoxide is rapidly converted by superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2) and oxygen.⁹ Hydrogen peroxide can be reduced by low valent metals (Fe^{2+} or Cu^+) to the extremely reactive hydroxyl radical ($\text{HO}^{\bullet}$), which reacts with virtually all biomolecules at rate constants $> 10^8 \text{ M}^{-1} \text{ s}^{-1}$.^{10,11} Fortunately, H_2O_2 is rapidly decomposed to oxygen and water by catalase, which is found in all tissues.¹² Additionally, the family of glutathione peroxidase (GPX) enzymes are also capable of reducing hydrogen peroxide to water.¹³ Therefore, SOD, catalase and GPXs all work in conjunction to reduce the overall levels of ‘ROS’. However, as mentioned previously, neither superoxide nor hydrogen peroxide are directly responsible for oxygen’s toxicity.² It mainly results from the production of hydroxyl radicals, and they are the main culprits for initiating oxidation of biomolecules such as DNA, proteins and lipid membranes. While superoxide itself is a good reductant, once protonated, it is converted into an efficient oxidant to generate the hydroperoxyl radicals (Scheme 1.1).



Scheme 1.2 Different types of radical and non-radical ROS produced upon reaction of superoxide

Oxidation of lipids, nature's hydrocarbons, is a common biological occurrence that is extremely debilitating due to its impact on the lipid bilayer.^{14,15} Phospholipids that surround our cells and organelles are particularly susceptible to peroxidation due to the presence of polyunsaturated-fatty acid (PUFA) side-chains that are present in all membrane structures.¹⁶ The primary products of lipid peroxidation are lipid hydroperoxides, and they are responsible for decreasing membrane fluidity, increasing membrane permeability, impairment of membrane enzymes and eventually leading to cell-death. Recently, the accumulation of lipid hydroperoxides has been implicated in a newly characterized cell-death pathway, termed ferroptosis, and this may be the ultimate link between lipid peroxidation and disease.¹⁷⁻¹⁹ Lipid peroxidation primarily occurs *via* two main pathways: an iron-catalyzed peroxy radical-mediated process called autoxidation¹⁴ and enzyme-mediated processes catalyzed by non-heme iron-dependent lipoxygenases (LOXs).²⁰

1.1.1 (Lipid) Autoxidation

Autoxidation is the spontaneous reaction of oxygen with hydrocarbons which can convert them to their corresponding hydroperoxides.²¹ While the substrates in an industrial setting are petroleum derived products such as lubricants, polymers and commodity chemicals, in a more biological setting, it is the lipid membrane surrounding our cells and organelles.^{16,22} Hydroperoxides cause degradation of industrial products, and are toxic *in vivo*. They can also undergo further decomposition to produce secondary-

oxidation products such as aldehydes, ketones, carboxylic acids or alcohols, some of which have harmful physiochemical properties, as well. Autoxidation is an archetype free-radical chain reaction that formally inserts a molecule of oxygen into a C-H bond of a hydrocarbon and proceeds by formal initiation, propagation and termination reactions (Figure 1.1A).²¹

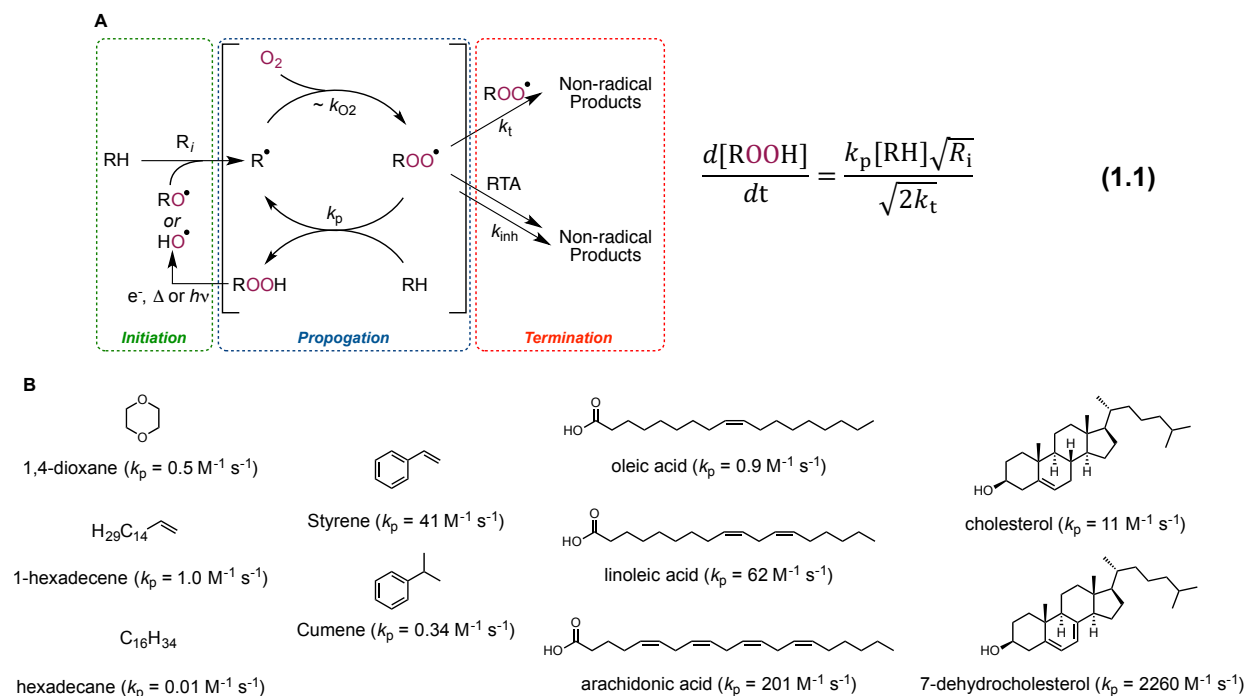


Figure 1.1 (A) Hydrocarbon autoxidation, the equation to derive rate of hydroperoxide formation (Eq 1.1) and (B) various autoxidizable substrates along with their rate constants of propagation. Certain lipids (e.g. sterols can also propagate by the addition of peroxy radicals. See, e.g. Zielinski and Pratt. *J. Am. Chem. Soc.* **2019**, *141*, 3037.

Initiation occurs by the abstraction of a hydrogen atom from a hydrocarbon (RH) by highly reactive oxidizing species to yield a carbon centered radical ($\text{R}^\bullet$). The abstraction of H-atom correlates with the bond dissociation enthalpy (BDE) of the C-H bond.²³ For example, in unsaturated substrates such as PUFAs, the presence of double bonds lowers the BDE of the (bis)allylic hydrogens to ~ 78 kcal/mol. As such, $\text{HO}^\bullet$, $\text{HOO}^\bullet$, $\text{RO}^\bullet$ and $\text{ROO}^\bullet$ radicals are all capable of oxidizing PUFAs at the (bis)allylic hydrogens. The chain initiating species for autoxidation reactions often result from thermal, photochemical or transition metal catalyzed reduction of weak bonds (e.g. decomposition of azo compounds, reduction of ROOH to $\text{RO}^\bullet$ or $\text{HO}^\bullet$ etc.).²⁴ The resulting carbon-centered radical reacts with

molecular oxygen with a rate constant near diffusion ($k_{O_2} = \sim 3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) to yield a chain-carrying peroxy radical ($ROO\bullet$).²⁵ The peroxy-radical propagates the reaction by abstracting a H-atom from another molecule of substrate to produce a hydroperoxide and another alkyl radical, thus continuing the chain reaction. The propagation reaction is much slower compared to the other reactions and as such it is the rate limiting step of autoxidation, characterized by the second-order rate constant k_p . Finally, chain termination occurs when two peroxy radicals react with one another to yield non-radical products, and it is quantified by the rate constant k_t .^{26,27} Termination typically occurs between two peroxy radical due to the facile addition of oxygen to carbon centered radicals, resulting in a low steady state concentration of $R\bullet$ relative to $ROO\bullet$.²³

Even though the rate constant of termination is very high, the low steady state concentration of peroxy radicals ensures that the rate of termination is quite low, resulting in the potential for the formation of a large quantity of $ROOH$ from a single initiating radical. In order for a reaction to be considered a proper autoxidation (i.e. a chain reaction), it is imperative that the rate of propagation (R_p) is higher than the rate of initiation (R_i). Therefore, the number of chains propagated by one initiating radical can be calculated as the kinetic chain length ($\nu = \frac{R_p}{R_i}$). In certain scenarios, such as high temperatures or in the presence of low-valent metals, autoxidation is further exacerbated because the accumulation of hydroperoxides leads to the initiation of more radical chains due to their decomposition into initiating radicals, producing an autocatalytic reaction.^{29,30} In the absence of autocatalysis, the kinetics of autoxidation are very well defined, and utilizing the steady state approximation for peroxy radicals the rate of hydroperoxide formation can be defined by Eq. 1.1.³¹

In a biological setting, the presence of multiple bis-allylic sites on PUFAs suggests that the initial H-atom abstraction can occur at multiple different points on the carbon backbone. Thus, lipid peroxidation of PUFA results in numerous hydroperoxides, all formed as a mixture of regio- and stereoisomers, since autoxidation is not regio- or stereospecific. For example, arachidonic acid (AA) autoxidation produces six different regio-isomeric hydroperoxides called hydroperoxyeicosatetraenoic

acid (HPETE) and depending on the concentration of oxygen, also results in several complicated products including cyclic and bicyclic peroxides (Figure 1.2).³² Lipid autoxidation induced cellular damage has been documented for decades, although a cell-death pathway associated with an accumulation of hydroperoxides was only elucidated recently.^{17–19,33}

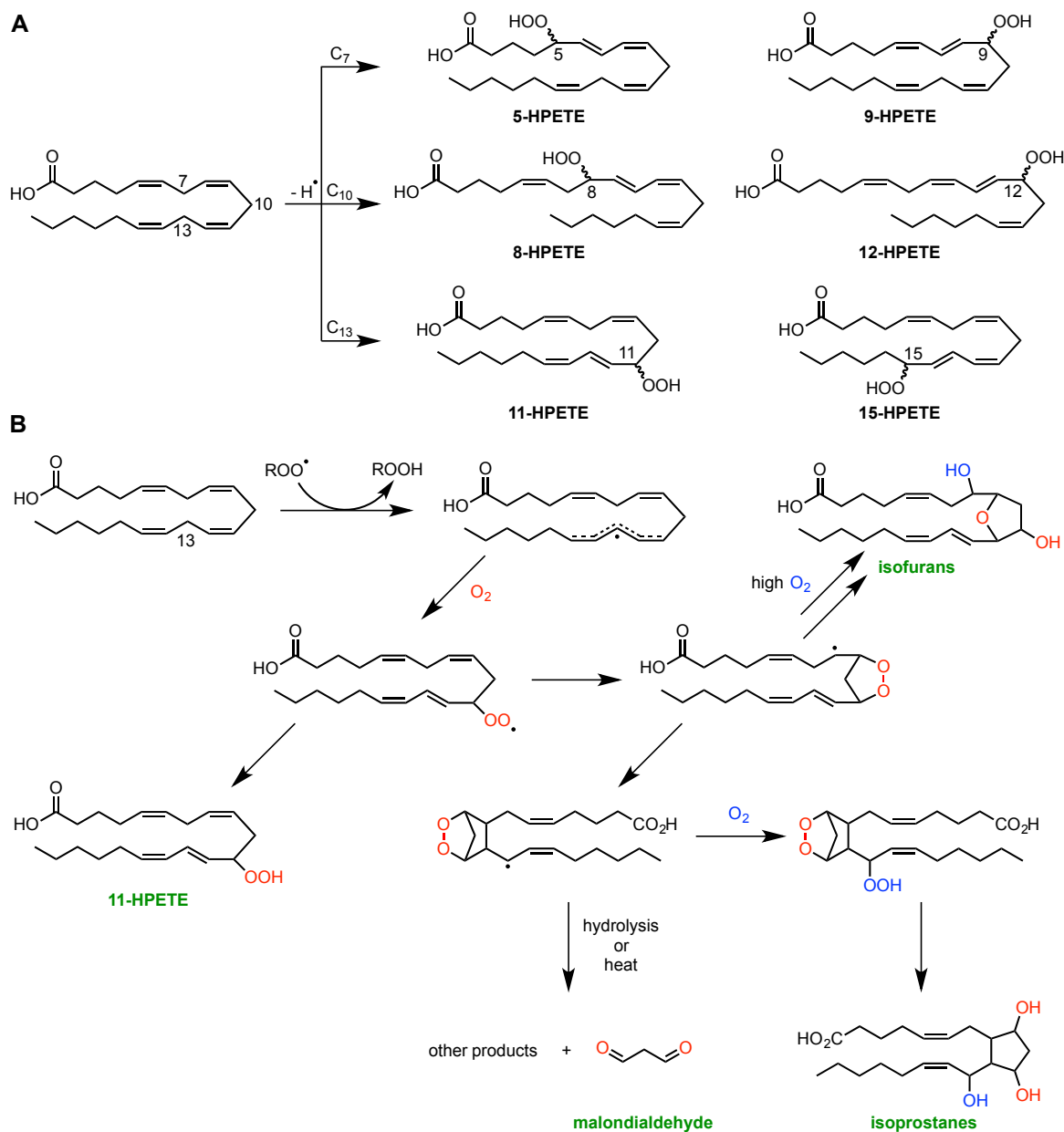


Figure 1.2 (A) Different HPETEs and (B) oxidation products arising from the peroxidation of arachidonic acid

As a result, one would anticipate that autooxidation-promoted increase in lipid hydroperoxide products would lead to the induction of ferroptosis. However, it is purported that 15-HPETE is specifically responsible for the execution of cell-death, leading to speculation on the of the exact role of autooxidation in ferroptosis.³⁴ As mentioned earlier, hydroperoxides can also decompose to form downstream products such as aldehydes which are extremely toxic due to their propensity to electrophilically modify DNA and proteins. Thus, autooxidation and their downstream products are responsible not only for the degradation of industrial products but also in various pathologies such as cancer, atherosclerosis and neurodegenerative diseases.³⁵

1.1.2 Non-enzymatic lipid peroxidation

While lipid peroxidation can occur spontaneously, it also occurs from the reaction of PUFAs and oxygen by a highly regulated family of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes.³⁶ The oxidation products derived from these enzymes are very complex and they have anti- and pro-inflammatory (e.g. prostaglandins, leukotrienes etc.) properties.²⁰ The AA oxidation products are known as eicosanoids and they mediate a wide array of biological functions including pain, fever and inflammation.³⁷ LOXs are a family of non-heme iron-dependent dioxygenases which introduce molecular oxygen antarafacially into PUFAs to generate hydroperoxides (Figure 1.3). There are six different LOX isoforms (5-LOX, 15-LOX-1, 15-LOX-2, 12-LOX-1, 12-LOX-2 and eLOX-3) in humans which are classified based on the distinct regio- and stereo-specific hydroperoxides that they generate.²⁰

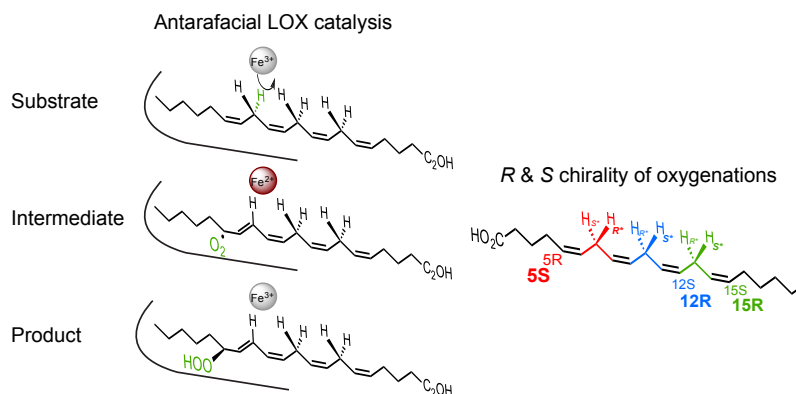


Figure 1.3 Antarafacial hydrogen abstraction and oxygen addition in LOX-enzymes, and the different possible positions of oxygenation in arachidonic acid

The active-site of LOX enzymes is highly conserved and contains a non-heme iron in an octahedral geometry coordinated to a hydroxide ligand and five amino acid side chains (Figure 1.4A). The hydroxide ligand is crucial for the functionality of the enzyme. The mechanism of action involves a regio- and stereo-specific proton-coupled electron transfer (PCET) reaction, where the H-atom from the PUFA transfers onto the hydroxide ligand while the electron reduces the ferric iron to ferrous iron. The resulting pentadienyl radical is delocalized and adds oxygen in an antarafacially but regio-specific manner governed by the LOX isoform to yield a peroxy radical. The peroxy radical is then reduced to the hydroperoxide by another PCET reaction, wherein the concerted reduction of the peroxy radical by ferrous iron and protonation by the water molecule regenerates the enzyme (Figure 1.4A).^{36,38}

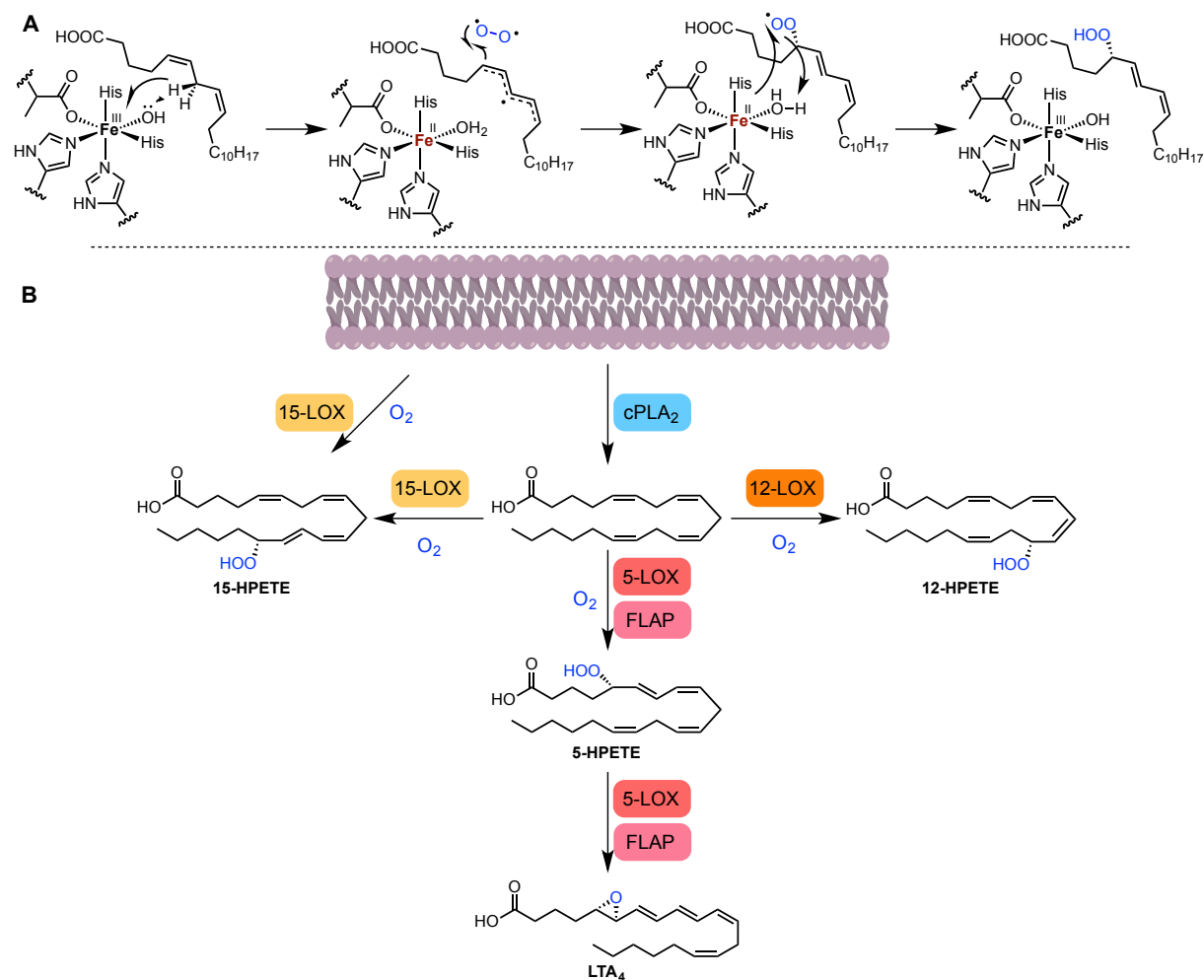


Figure 1.4 (A) Mechanism of LOX enzymes. 5-LOX catalysis is shown as an example (B) arachidonic acid peroxidation and the products formed by different LOX isoforms.

Of the six different isoforms found in mammals, 5-LOX, 12-LOX-1 and 15-LOX-1 have heavily been linked with disease outcomes. Human 5-LOX is constitutively expressed in leukocytes and in conjunction with 5-lipoxygenase activating protein (FLAP) catalyzes the key step in the oxidation of AA to produce leukotriene A₄ (LTA₄) via the 5-HPETE intermediate (Figure 1.4B).³⁹ The metabolic products of LTA₄ are implicated in many inflammatory diseases and for causing host-immune response leading to the pathogenesis of asthma.²⁰ While 5-LOX catalyzes the formation of LTAs, 15-LOX is implicated in a variety of cardiovascular diseases including atherosclerosis.^{40,41} In addition, 15-LOX-1 is the only LOX isoform capable of metabolizing intact phospholipids in addition to free fatty acids, and is responsible for the oxidation of low-density lipoproteins (LDL).^{42,43} The oxidized LDL particles are the main culprits behind the transition of macrophages into foam cells found in atherosclerotic lesions and for the initiation of atherogenesis. The role of 12-LOX-1 is not fully elucidated but it is speculated that its products may be responsible for promoting platelet aggregation and in certain cardiovascular diseases, as well.⁴⁴ Due to their involvement in many pathophysiological diseases, LOX-catalysis and its inhibition were attractive research targets in the past, but little progress was made.

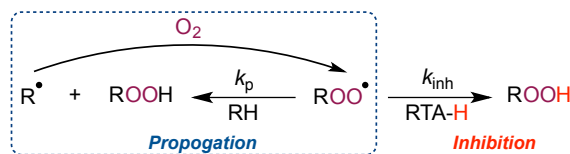
Recently, there has been a renewed interest in LOX-catalysis due to the implication of 15-LOX derived 15-HPETE as the key cell-death marker in cells undergoing ferroptosis.^{34,45} However, no single lipoxygenase isoform has been convincingly implicated in ferroptosis till date. For instance, genetic studies have shown that deficiency in 15-LOX, cannot rescue loss of glutathione peroxidase-4 (Gpx4).⁴⁶ Nevertheless, loss of 15-LOX could rescue male subfertility in heterozygous Gpx4 mice, thus suggesting a complex tissue specific interplay between LOXs and Gpx4.^{46,47} Whether this is a general phenomenon across all cell-types or if other LOXs are involved still remains to be elucidated. Moreover, the interplay between enzymatic and non-enzymatic lipid peroxidation and its role in driving ferroptotic cell-death is still a matter of intense debate.

1.2 Inhibition of lipid peroxidation

Since autoxidation as a whole is responsible for causing oxidative degradation of a wide range of materials, significant effort has been employed towards identifying inhibitors to mitigate this process. Lipid peroxidation inhibitors can broadly be defined as any compounds that are capable of significantly preventing, delaying or repairing the oxidative damage. Given that lipid peroxidation can be triggered through enzymatic and non-enzymatic pathways, strategies aiming to halt this process can be divided into two major groups: lipid autoxidation inhibitors (e.g. radical-trapping antioxidants) and lipoxygenase inhibitors.

1.2.1 Radical-Trapping Antioxidants

Autoxidation can be inhibited, i.e. prevented or retarded, by the use of two important classes of antioxidants: preventative and radical-trapping.^{31,48} Preventative antioxidants exert their action by ‘quenching’ the source of initiating radicals in order to reduce the rate of initiation (R_i). Industrially, compounds such as phosphines or sulfides are used to reduce ROOH to ROH,⁴⁹ while *in vivo* enzymes such as catalase and GPXs are responsible for reducing R(H)OOH to R(H)OH.^{12,13} In contrast, radical-trapping antioxidants (RTAs) – also referred to as chain-breaking antioxidants – are molecules which react with chain-carrying peroxy radicals, i.e. they compete with the slow chain-propagation step to terminate the autoxidation chain reaction (Scheme 1.3).^{31,48}



Scheme 1.3 Radical-trapping antioxidants compete for the peroxy radical to inhibit the chain-propagation reaction.

The most classical RTAs include hydrogen-atom donors such as phenols and diarylamines (Figure 1.5A) and they have long been included as additives to petroleum-derived products (e.g. oils, fuels, plastics, rubbers) to inhibit their autoxidation.⁵⁰ The recognition of their role in protecting biological hydrocarbons (i.e. lipids) from autoxidation is comparatively recent, ushered in largely based on

characterization of the RTA activity of α -tocopherol (the most biologically-active form of Vitamin E) as among the best ever documented.⁵¹ Diarylamines such as dialkyldiphenylamines serve to protect petroleum derived products and improve the longevity of engine oils and lubricants that are exposed to extremely harsh conditions at elevated temperatures. The mechanism of these RTAs involves the transfer of a hydrogen atom to a chain carrying peroxy radical ($\text{ROO}\cdot$) through a proton-coupled electron transfer (PCET)^{52,53} reaction to yield a ROOH and an antioxidant-derived radical (Figure 1.5B). The resulting RTA-derived radical is resonance stabilized, preventing it from reacting with the substrate or oxygen. The RTA-derived radical can further react with another peroxy radical to yield non-radical products.

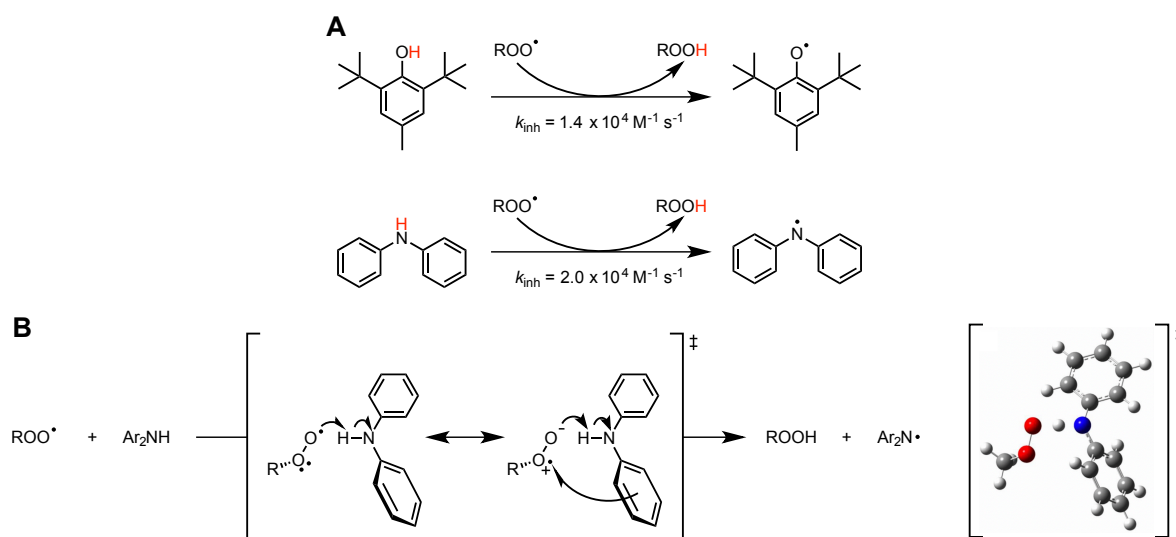


Figure 1.5 (A) Reaction of a peroxy radical with either BHT (top) or diphenylamine (bottom). (B) Reaction of a diphenylamine and peroxy radical proceeding via a proton-coupled electron transfer (PCET).

The rate constant which characterizes the reaction of an RTA and peroxy radical is known as the inhibition rate constant (k_{inh}), and it is probably the most important parameter when considering the activity of antioxidants.⁴⁸ The reactivity of RTAs is predicated on both thermodynamic and kinetic parameters. An excellent Evans-Polanyi relationship exists between the BDE of either the O-H or N-H bonds and the k_{inh} , suggesting that the weaker the bond, the faster the kinetics of the H-atom transfer to a peroxy radical (Figure 1.6A).^{54,55} Furthermore, RTAs are very susceptible to substituent effects and there is a very clear Hammett Correlation when employing the electrophilic parameter σ_p^+ . The reaction

constant for diarylamines is $p = -1.59$. The negative slope signifies that the electron-donating groups stabilize the transition state leading to the production of RTA derived electrophilic radical, resulting in an enhancement of the rate constant of inhibition (Figure 1.6B). Therefore, the addition of electron-donating groups in the *para*-position of either the phenols or diarylamines weaken the O-H or N-H BDEs, leading to extremely reactive compounds.^{56,57} Under constant R_i , the inhibition rate constant can be calculated from the rate of hydroperoxide formation using Eq 1.1. Subsequently, the number of peroxy radicals an antioxidant can trap is known as the stoichiometric factor ' n ' and an important consideration while studying their activity. It can be calculated using Eq. 1.2.³¹

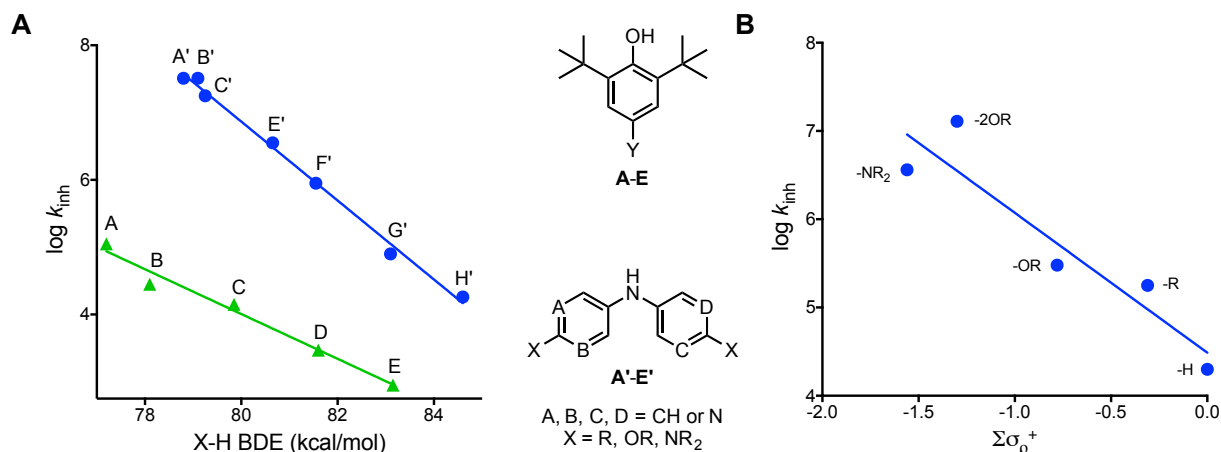


Figure 1.6 (A) The Evans-Polanyi relationship between BDE and k_{inh} for a variety of diarylamines (●, blue) and phenols (▲, green) Legend for Y: A = methoxy; B = thiomethyl; C = methyl; D = H; E = cyano. (B) Linear-free energy relationship of $\Sigma\sigma_p^+$ and k_{inh} for 4,4'-substituted diarylamines ($p = -1.59$).

Under ambient temperatures phenols typically trap two peroxy radicals, i.e. $n = 2$,^{58,59} but this decreases with increasing temperatures, presumably due to the propensity of the peroxidic adduct to undergo O–O bond homolysis and yield chain-initiating radicals (Figure 1.7A). Similarly, most diarylamines also trap two peroxy radicals at ambient temperatures.^{28,60} However, a major difference between the two arises at higher temperatures. For example, in the inhibited autoxidation of paraffin oil at 130°C, stoichiometric factors as high as $n = 40$ have been reported.⁶¹ This observation would suggest that diarylamine antioxidants or the oxidation product derived therefrom are regenerated in situ. The pioneering study by Korcek *et al.* demonstrated that in hexadecane autoxidation at 160°C, the addition of

Ar₂NO•, an intermediate formed upon oxidation of diarylamine, was converted back to the parent compound, Ar₂NH.⁶² This suggested that a reaction pathway existed to rapidly convert the nitroxides back to their respective diarylamine. Since nitroxides are capable of reacting with alkyl radical at rates near diffusion^{63,64} they invoked the involvement of alkoxyamines in their mechanism. It was observed that O-alkylbis(4-octylphenyl) alkoxyamine decomposed at high temperatures to the parent diarylamine, leading to a proposed mechanism of diarylamine regeneration (Figure 1.7B). The rate-determining step in the mechanism involved a postulated N-O bond homolysis from the alkoxyamine, which was followed by an in-cage disproportion to yield the amine and a carbonyl compound.⁶² This was the first report where the catalytic inhibition of diarylamines was rationalized, providing excellent insight for further optimization of diarylamine scaffold in order to exploit the catalytic regeneration behavior. Recently, we utilized computational and experimental approaches to demonstrate that the key step in the proposed catalytic cycle proceeds by a concerted retro-carbonyl-ene reaction (Figure 1.7C).⁶⁵

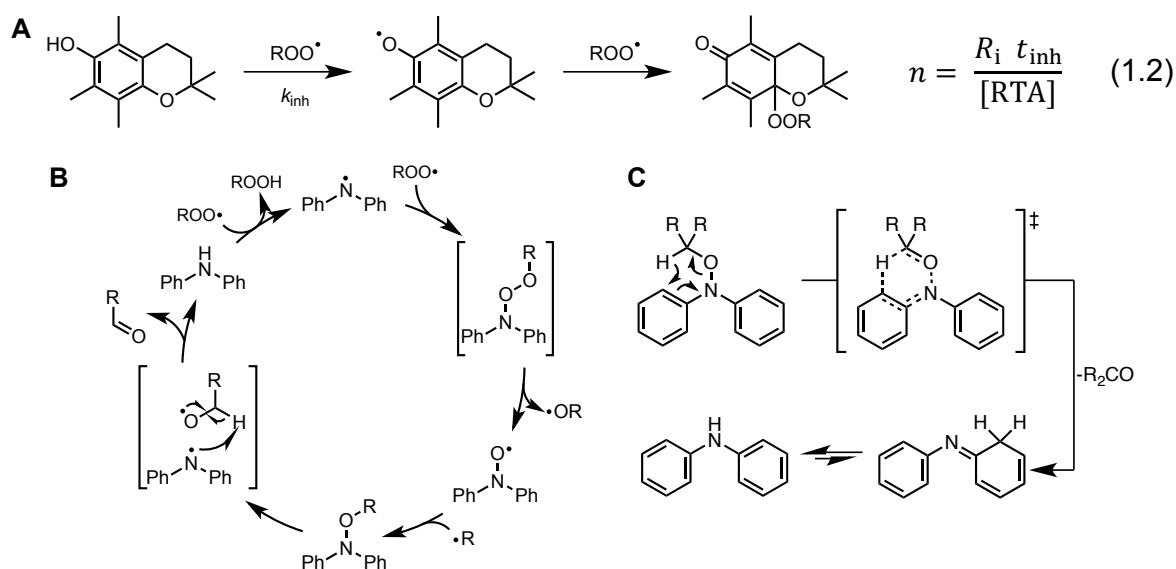


Figure 1.7 (A) Phenols trap two peroxy radicals as illustrated by PMC and the equation utilized to calculate stoichiometry ‘n’ – the number of peroxy radicals trapped by a given RTA. (B) Catalytic mechanism of regeneration for diarylamine RTAs as suggested by Korcek *et al.* (C) Concerted retro-carbonyl-ene reaction for diarylamine regeneration.

A facile method to enhance the reactivity of diarylamines is either the weakening of the N-H bonds to make them more reactive towards peroxy radicals; or by weakening the N-O bonds in the alkoxyamine to allow for more efficient regeneration. A strategy that benefits both pathways is the attachment of electron-donating groups in the *para* position of the aromatic rings. While the inclusion of EDGs helps lower the BDE of the N-H or N-O bond, it also significantly reduces their oxidation potential (Figure 1.8A).^{54,66} The resultant compounds readily undergo single-electron oxidation in the presence of molecular oxygen or hydroperoxides to generate initiating radicals. This is because the radical cation derived from diarylamines is greatly stabilized by the EDGs. Unfortunately, this causes the antioxidant to act as a pro-oxidant and promote oxidation (Figure 1.8B).

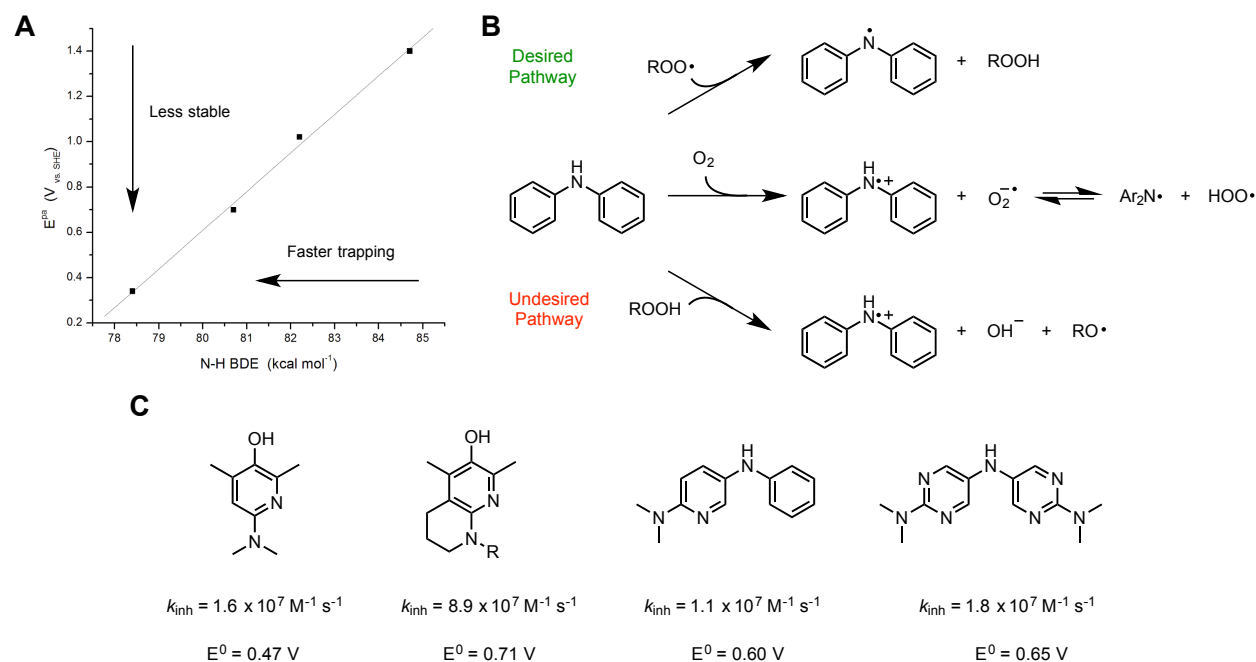


Figure 1.8 (A) Plot showing the decrease in E_{pa} upon weakening of the N-H bonds by the addition of EDG groups to RTA. Plot generated using data found in the supporting information of ref. 54 (B) Scheme depicting the desired and undesired reaction of diarylamine RTA (C) Inhibition rate constants (k_{inh}) and oxidation potential (E^0) of several heterocyclic RTAs.

A solution to this problem was reported by Pratt *et al.*, where they discovered the addition of heteroatoms in the aromatic rings of RTAs results in a dramatic increase of its oxidation potential (E^0) relative to the BDEs (Figure 1.8C).^{66–68} This can be rationalized by considering the electronic effects that

dictate the BDEs and E^0 – the BDE is influenced by resonance π -interaction with *para* and *ortho* substituents, while E^0 is affected by σ -induced electron density in the aromatic system. By the addition of nitrogen in the form of pyridine or pyrimidine rings, both BDE and E^0 are increased, but the increase in E^0 is greater because of the destabilizing effect nitrogen has on the radical cation formed by one-electron oxidation. This was initially demonstrated by the preparation of *ortho*- and *para*-substituted pyridinols and pyrimidinols, which showed high air stability despite having low BDEs.^{66,68,69}

Given the success of pyridinol and pyrimidinol antioxidants, this concept was extended towards diarylamine antioxidants to design extremely reactive, air-stable compounds in order to develop a structure-activity relationship. Compared to the heterocyclic phenols, nitrogen atoms can be incorporated into the diarylamine scaffold in a versatile manner to generate symmetrical or unsymmetrical compounds with differing *ortho/para* substituents. Utilizing this approach, a multitude of heterocyclic diarylamines were prepared, and reactivity of up to 100-times that of dialkyldiphenylamines was observed at ambient temperatures.^{54,70}

1.2.2 LOX Inhibitors

LOX inhibitors can be divided into five major groups based on their mechanism of action: 1) redox inhibitors that maintain the active site iron atom in its (inactive) reduced form; 2) iron chelators which inactivate the enzyme by removal of the active site iron; 3) competitive inhibitors that can occupy the active site, precluding binding of the substrate; 4) suicide substrates which irreversibly inactivate the enzyme; and 5) allosteric inhibitors which affect enzyme activity by binding to a site remote to the substrate binding pocket.⁷¹ Since there are six different isoforms of LOX in humans, a major challenge in the field is the design of isoform specific inhibitors.²⁰ This has been compounded by the lack of three-dimensional structural information of the human LOXs beyond 5-LOX.⁷² Even the 5-LOX crystal structure does not represent the native protein as it was extensively truncated and engineered to confer rigidity for crystallization attempts. Nevertheless, researchers have gone to great lengths to develop isoform-specific inhibitors (Figure 1.9).

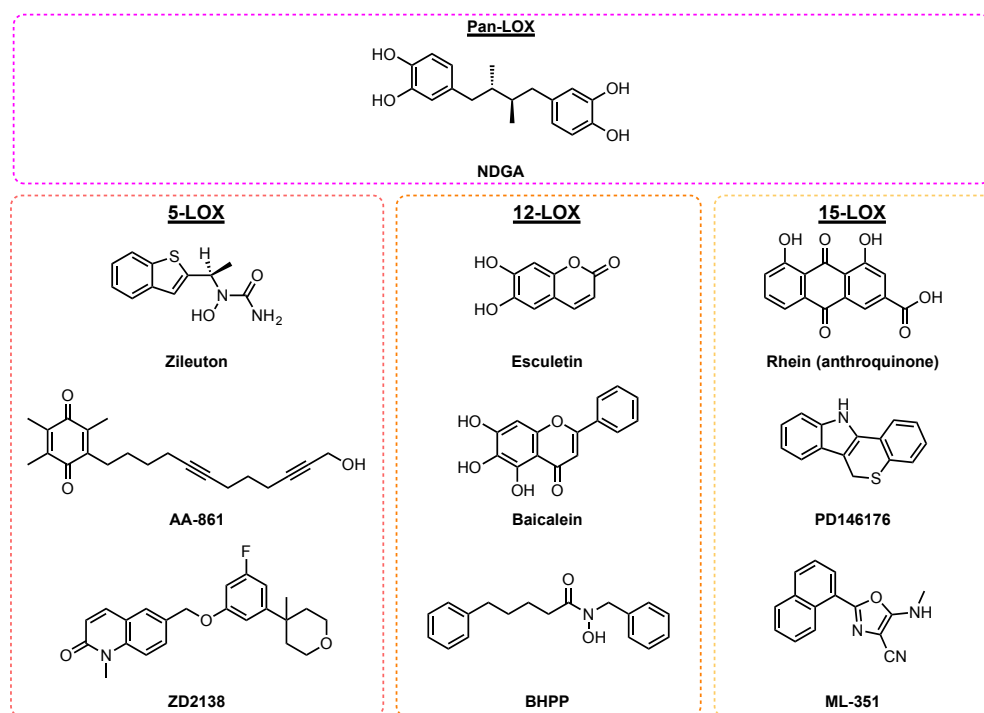


Figure 1.9 The most popular LOX inhibitors used in the literature.

Since 5-LOX is involved in leukotriene biosynthesis, and many of its products are involved in bronchoconstriction leading to asthma, it has been an attractive drug target.²⁰ Unfortunately, most of the drugs that have been developed only target the specific LTA receptor rather than directly inhibit 5-LOX. For example, Singulair, a commonly prescribed anti-asthma drug inhibits the binding of LTD₄ to its receptor, causing reduction in bronchoconstriction.⁷³ The only 5-LOX specific inhibitor that has been FDA approved is Zileuton. It is believed to inhibit 5-LOX by acting as an iron chelator via its N-hydroxyurea moiety.⁷⁴ However, the hydroxamic acid is oxidatively unstable leading to a very low half-life *in vivo*, necessitating administration of very high doses.⁷⁵ Due to the lack of structural information on human 12- and 15-LOX, very few inhibitors exist and none have made it to the market. This is unfortunate due to their implication in various diseases, most importantly atherosclerosis.⁴¹ Several attempts have been made to design isoform specific inhibitors and many compounds have been identified through rational design and high-throughput screens.^{76–78} However, a large majority of compounds are redox active polyphenols that are oxidatively unstable and leading to many off-target effects in cell-

culture or *in vivo*. Nonetheless, the most commonly used standard in the field of LOX inhibition is the pan-LOX inhibitor nordihydroguaiaretic acid (NDGA).

With the recent characterization of the role of LOX in ferroptosis, there is certainly a need for isoform-specific redox-inactive inhibitors. Interestingly a few reports have indicated that multiple LOX inhibition presents a stronger protective effect when comparing with single LOX targeting.^{18,45} Thus, it remains to be seen whether the development of specific LOX or pan-LOX inhibitors are a suitable and effective path for novel drugs aimed at efficiently inhibiting ferroptosis – or diseases that are associated therewith.

1.3 Techniques for Measuring RTA activity

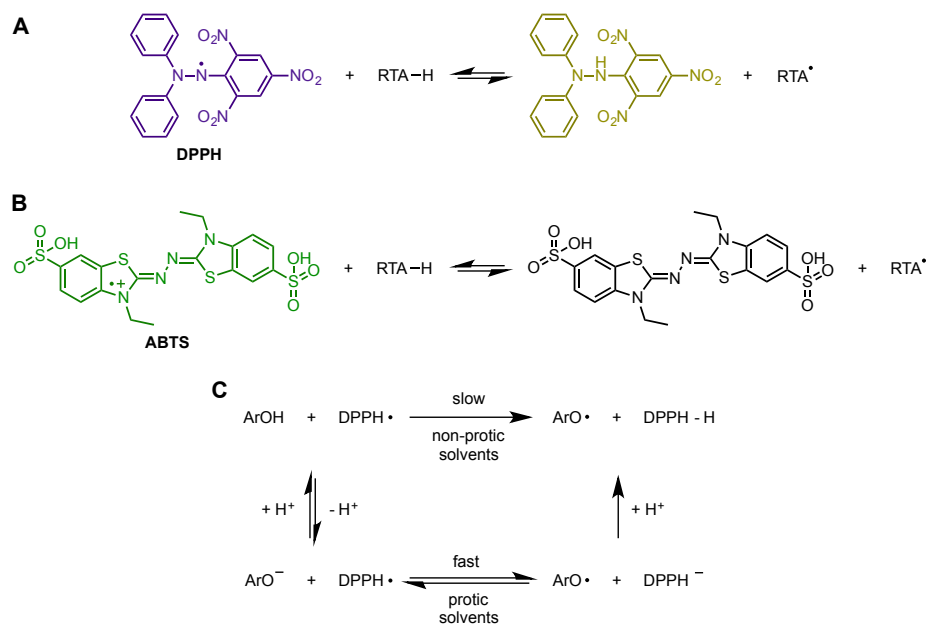
Most common methods to assess the effectiveness of a RTA includes measuring the change in absorbance or fluorescence of a colourimetric probe upon reaction with the putative antioxidant. Although simple, a disadvantage is that some of the most common techniques (including FRAP, DPPH, ORAC, and ABTS)^{79–82} fail to provide accurate information about the reactivity of RTAs towards kinetically relevant oxidizing species – peroxy or hydroperoxy radicals.⁸³ The best and the most effective way to measure RTA efficacy is by inhibited autoxidation

1.3.1 Competitive Probe Reactions

The most prominently used methods to measure the activity of RTAs rely on a simple premise, the reaction of a highly colourimetric probe and RTA, monitored either by changes in absorbance (e.g. DPPH)⁸⁴ or fluorescence (e.g. ORAC). Despite their popularity, most of these assays do not involve substrate autoxidation and offer little to no information on the kinetics of RTA activity. This is because the frequently employed probes are oxidizing agents that undergo a redox reaction with the RTA, a reducing agent. As a result, the assay simply involves titrating the reducing equivalents in the probe solution (Scheme 1.4).

The popularity of these experiments stems from their simplicity and commercial availability in the form of easy-to-use kits. They are amenable to high-throughput screening approaches which increase

their utility to researchers around the world. However, these approaches suffer from some significant shortcomings: 1) The results simply report on the position of the redox equilibrium and not on the kinetics of an RTA with a kinetically relevant oxidizing species. 2) They are carried out in highly polar ionizing solvents which are not often representative of the application of the RTA.



Scheme 1.4 The DPPH (**A**) and ABTS (**B**) assay are some of the most commonly used probes to determine antioxidant efficacy. (**C**) Dependence of DPPH reactivity on the reaction medium

The most commonly used probe in the literature is DPPH (Scheme 1.4A). It is isoelectronic with a peroxy radical, but much more persistent, and is often the desired choice of assay to test the effectiveness of RTAs. Frequently utilized protocol involves incubating several concentrations of a test RTA in the presence of DPPH for an arbitrary amount of time in methanol.⁸⁵ The decrease in the absorbance of DPPH is monitored and used to construct an IC₅₀ curve, which is used to calculate the efficacy of the test RTA.⁸⁶ The results from these experiments simply report on the total reducing equivalents of a given RTA or a crude extract but lack quantitative measure because they fail to account for chemical and physical properties of the inhibitors and the reaction medium. Ingold *et al.* have demonstrated that the reaction medium has a strong influence on the kinetics of RTA towards peroxy radicals, and can even lead to changes in the mechanism of their reaction.^{87,88} As such, if used correctly,

the DPPH assay can provide information on the kinetics of RTA in nonprotic solvents (Scheme 1.4C). DPPH reacts with phenols several orders of magnitude slower compared to peroxy radicals, but their relative rate constants parallel those of peroxy radicals for a series of phenols, allowing for the estimation of their antioxidant activity. This was reported by Ingold, wherein the rate constants for a series of phenols with DPPH were measured accurately, but required the use of specialized equipment such as a stopped-flow spectrophotometer.⁸⁹

1.3.2 Inhibited autoxidations

The most informative strategies to monitor antioxidant activity involves the kinetic study of inhibited autoxidations, these methods analyze the reactivity of the RTA in an environment representative of their application with peroxy radicals as the chain-carrying species. The kinetics of this process can be monitored either by the disappearance of starting materials (RH or O₂) or by the formation of oxidation products (usually hydroperoxides).

Oxygen uptake is one of the most venerable methods of determining RTA efficacy, where the autoxidation of an oxidizable substrate is carried out within a closed system, and involves monitoring the consumption of oxygen by changes in differential pressure (Figure 1.10A).⁴⁸ The experiments are typically initiated using azo-compounds (Figure 1.10C), which decompose at a continuous rate to initiate the autoxidation reaction. Subsequently, oxygen is consumed at a constant rate until termination occurs and can be monitored by the pressure changes in the closed vessel. Because the method measures changes in differential pressure, RTA is added to one of the flasks in order to retard or inhibit the oxidation reaction. In the absence of any RTA, the oxygen consumption continues linearly over time and the initial rates from RTA inhibited autoxidations can be used to calculate the kinetics of antioxidant inhibition (Figure 1.10B). A drawback to this method is that it requires the use of specialized equipment and also an extremely high R_i to ensure that the change in oxygen consumption is sufficient to be measured over time.

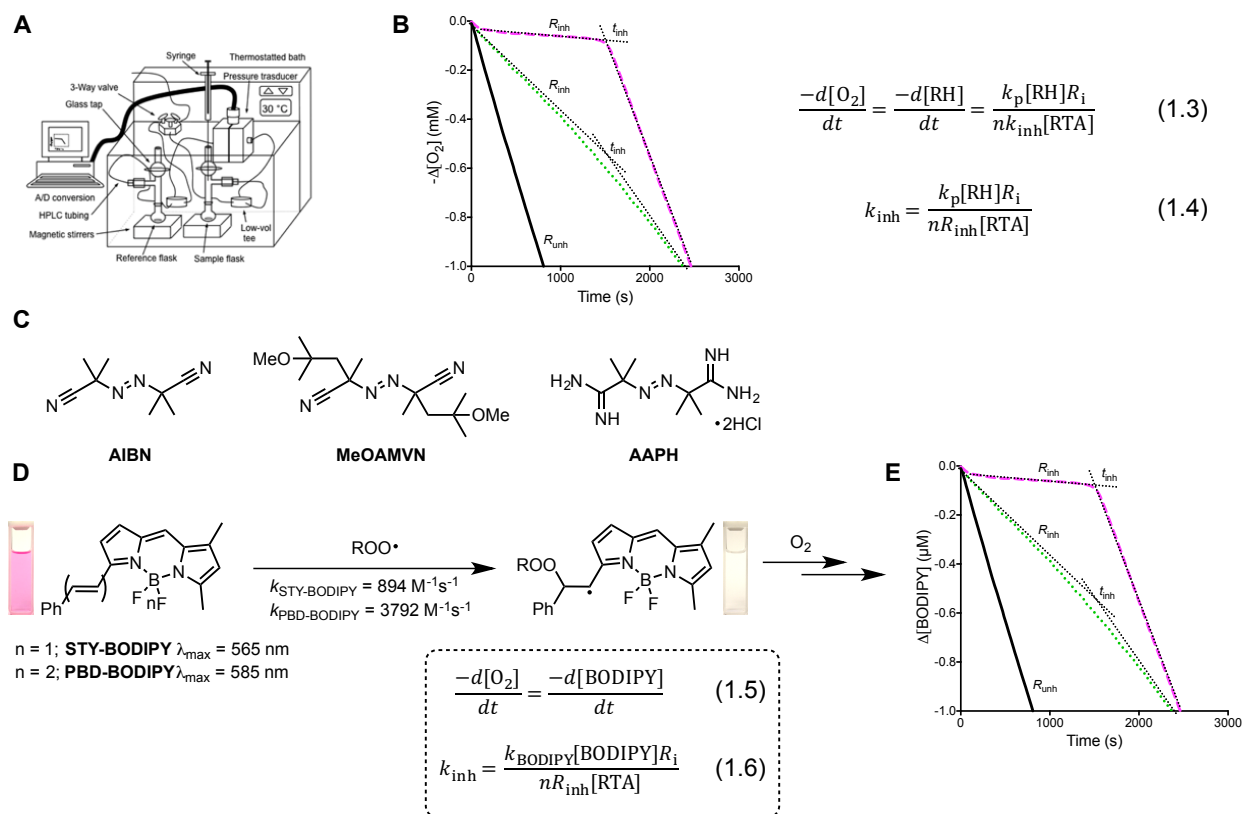


Figure 1.10 (A) Apparatus used to measure the differential pressure-changes in oxygen consumption. Reproduced with permission from John Wiley and Sons. (B) Representative oxygen consumption trace that follows well-defined kinetics and allows for the determination of k_{inh} using Eq 1.4. (C) Common azo-initiators (D) STY- and PBD-BODIPY probes that can be used as colourimetric reporters in co-oxidations as surrogates for oxygen-consumption experiments. This methodology yields almost indistinguishable results (E). The autoxidations follow the same steady-state approximations (Eq. 1.5) and can be utilized to derive k_{inh} from BODIPY consumption experiments (Eq. 1.6).

In order to facilitate acquisition of results and avoid the use of specialized equipment, our group recently introduced an approach that relies on the use of a BODIPY probe.⁹⁰ This system utilizes a conjugated BODIPY-probe that undergoes co-oxidation with the substrate, and the loss in its conjugation is measured overtime by classical spectrophotometry (Figure 1.10D). This assay affords analogous results to the highly specialized and laborious oxygen consumption method. However, rather than directly measure the changes in oxygen-consumption, the indirect changes in BODIPY concentration is used as a surrogate reporter of the autoxidation process (Figure 1.10E). Moreover, this process is more versatile as it can be carried out in a cuvette, in the presence of a variety of substrates, solvents and temperatures.^{91,92}

The BODIPY methodology provides a very facile method to assess the kinetics of antioxidant efficacy (k_{inh} , n).

As mentioned earlier, inhibited autoxidations can also be measured by the production of hydroperoxides. This is usually a discontinuous process and requires the analysis of aliquots taken at different time points. The classical methods include iodometric titrations (Figure 1.11A),⁹³ analysis of products by HPLC,^{94,95} and recently, the use of pro-fluorescent probes that react with hydroperoxides.⁹⁶ HPLC analysis of hydroperoxides is a very powerful quantitative tool, but its use is limited to substrates that yield products which are comparatively simple and whose standards can be easily synthesized. Iodometric titration is advantageous as the identity or the structure of hydroperoxide is not taken into account and it is frequently used in industry, but it is a laborious process and requires large amounts of sample for analysis. Recently, the use of pro-fluorescent phosphine probes that undergo reaction with hydroperoxides have seen an increase in popularity (e.g. Figure 1.11B).^{96–98} These methods utilize a small amount of sample, are insensitive to the hydroperoxide structure and are amenable to high-throughput analysis on a microplate reader. An example is shown, wherein the pseudo-first order rate constant of the reaction of phosphine and hydroperoxide is used to measure the concentration of hydroperoxide (Figure 1.11C).⁹⁶ This approach enables quantitation of more complex experiments such as high-temperature autoxidations that are otherwise difficult to carry out in a laboratory setting. High-temperature autoxidations typically involve placing an oxidizable substrate in a stirred-flow reactor to continuously oxygenate the system in order to prevent mass-transfer of oxygen from becoming rate limiting (Figure 1.11D).⁹⁹ RTAs and other additives can be added to this reaction to assess their impact on the autoxidation, and the aliquots taken at different intervals can be analyzed using the pro-fluorescent phosphine. High-temperature autoxidations seldom offer quantitative information due to the ever increasing R_i from the decomposition of the product hydroperoxides, resulting in an autocatalytic reaction.²⁹

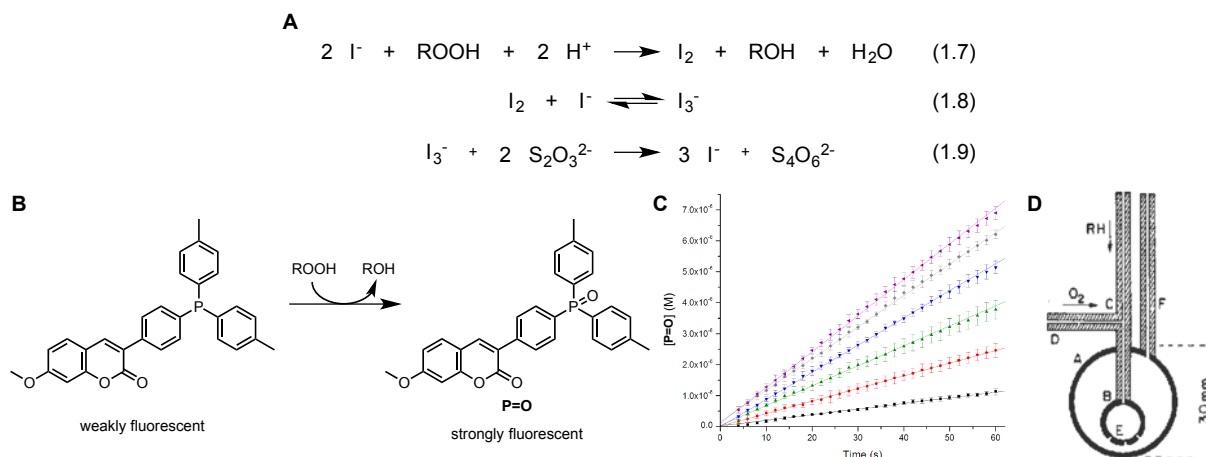


Figure 1.11 (A) Iodometric titrations used to quantify hydroperoxide concentrations. (B) Pro-fluorescent phosphine probe that undergoes fluorescent enhancement upon reaction with hydroperoxides. (C) The pseudo-first order rate constant between the phosphine and hydroperoxide can be used to quantify the concentration in samples.⁹⁶ Reprinted with permission from *Chem. Commun.* **2012**, 48, 10141. Copyright 2012 Royal Society of Chemistry. (D) Stirred-flow reactor utilized for high-temperature autoxidations wherein oxygen is continuously bubbled through the samples.⁹⁹ Reprinted with permission from *J. Am. Chem. Soc.* **1979**, 101, 7574. Copyright 1979 American Chemical Society.

1.4 Ferroptosis¹⁰⁰

The highly orchestrated process through which cells die, and its profound implications in physiology was first realized in 1966.¹⁰¹ The idea of programmed cell death suggested that specific machinery responsible for regulating such processes existed whereby its dysregulation may lead to disease due to improper or insufficient cell death.¹⁰² On the contrary, this behavior also indicated that the organized death of cells responsible for pathologies can be perturbed or altered therapeutically.¹⁰³ Apoptosis remains as the first, well-characterized form of cell death that plays an important role in the homeostasis of biological systems which can be intrinsically triggered without any external stimulus.¹⁰⁴ Continued work in the field of cell death has enabled classification of modalities into three general categories: apoptosis, autophagy and necrosis.¹⁰⁵ Necrosis has been commonly considered to be a very chaotic and an accidental, un-regulated form of death that leads to the rupture of the cellular membrane. Recently, it was demonstrated that the general necrosis phenotype is common among many cell death routines and includes cellular volume increase (oncosis), oxidative stress and organelle swelling.¹⁰⁶ The examination of regulated necrosis at a

biochemical, morphological and genetic level by the modulatory profiling of the cell landscape death led to the identification of ferroptosis.¹⁷ Ferroptosis is an oxidative, iron-dependent form of cell death tightly regulated by pathways converging on a selenoprotein responsible for detoxifying phospholipid hydroperoxides (PLOOH), glutathione peroxidase 4 (GPX4).^{18,107} Hallmarks of ferroptosis are very distinct from other cell death pathways,¹⁷ and include morphological changes such as decreased mitochondrial size, increased membrane density and outer membrane rupture at the ultrastructural level.¹⁸ Biochemically, the increase in the labile iron pool and lipid peroxidation are distinctive events. As such, in addition to identifying novel ferroptosis inducers, early ferroptosis research has been functionally characterized using lipid peroxidation inhibitors and iron chelators. Ferroptosis has emerged as a key player in a myriad of pathologies including tumor suppression,¹⁰⁸ antiviral immunity¹⁰⁹, neurodegeneration¹¹⁰ and ischemia/reperfusion injury (IRI).¹⁸

Recently, the events leading to ferroptosis were shown to be extremely specific and involve oxidation of PUFAs in phosphatidylethanolamine (PE) by 15-LOX-1.³⁴ Since lipid peroxidation is a pre-requisite for cell death, several subsequent studies have associated lipoxygenases in its execution mechanism due to observations that LOX inhibitors halt the cell death process.^{34,45,111,112} However, lipid peroxidation can also occur in a non-enzymatic fashion by a free-radical chain reaction called autoxidation.¹⁴ Understanding which lipid peroxidation mechanism plays a role in the execution of ferroptosis is still unclear and a matter of intense debate. Moreover, as indicated by its name, iron is also critical and an absolute requirement for the cell death induction but its actual nature, form and localization is not well understood and remains to be elucidated. What remains universally accepted is the requirement for PLOOH accumulation and the ablation of GPX4 activity for ferroptosis induction.¹⁹ Therefore, modulation of GPX4 either directly or indirectly is a common strategy used to regulate ferroptosis. Direct inhibition or degradation of GPX4 offers a facile strategy to study ferroptosis in a cellular context,^{113–115} while indirect perturbation of GPX4 can be achieved by limiting the levels of intracellular glutathione

(GSH), a reducing co-factor for the enzyme.¹³ As such, the dysfunction of either cystine metabolism, transsulfuration pathway or glutathione biosynthesis are common avenues for ferroptosis induction.

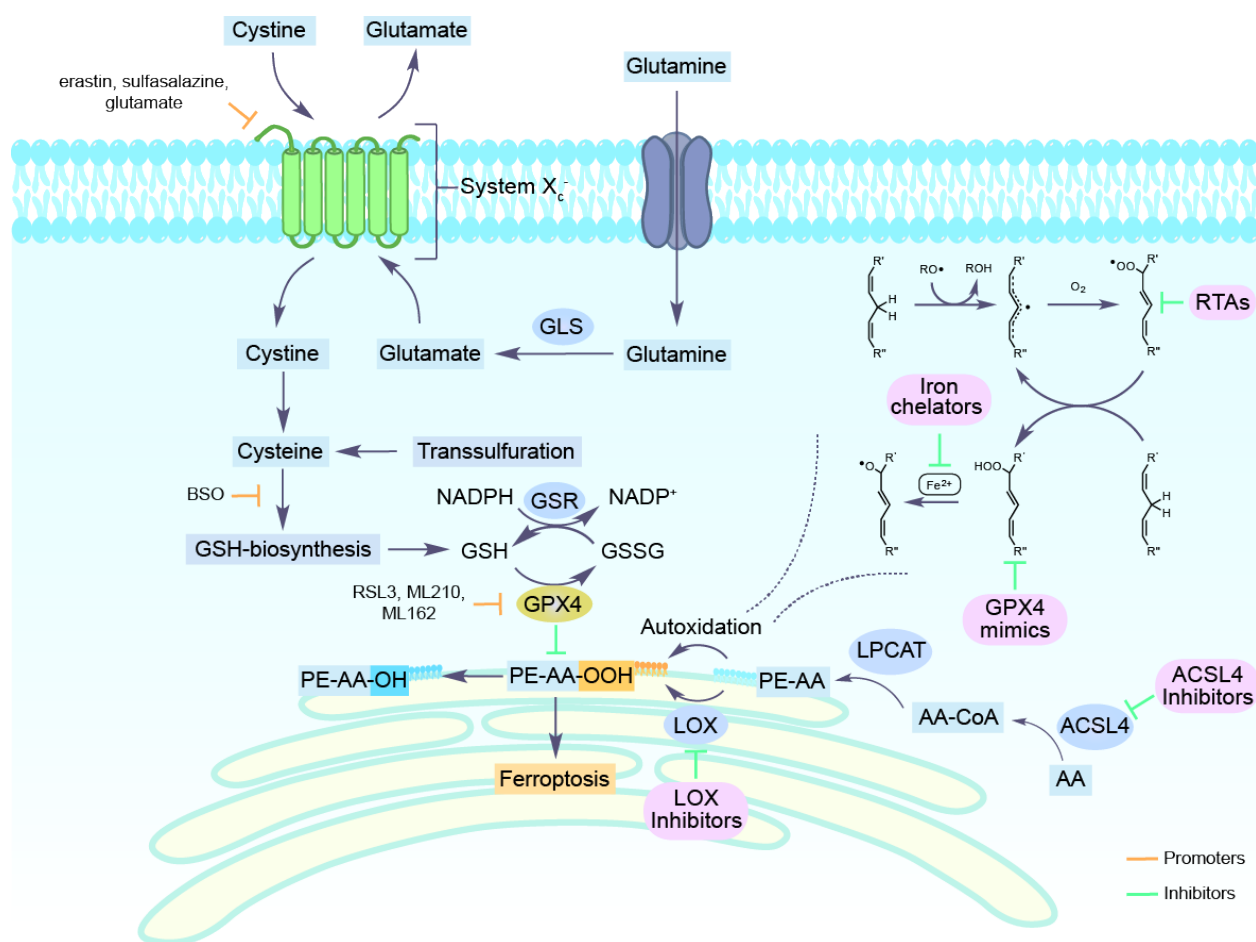


Figure 1.12 Fatty acids (e.g. AA) are activated by acyl-CoA synthetase long chain family member 4 (ACSL4) to generate AA-CoA, which are esterified by lysophosphatidylcholine acyltransferase (LPCAT3)-dependent mechanism into phospholipids. These serve as excellent substrates for lipid peroxidation *via* a LOX- or autooxidation-dependent mechanism, driving ferroptosis execution. Ferroptosis is restrained by the heterodimeric cystine–glutamate antiporter system x_c , which exchanges one molecule of extracellular cystine for a molecule of intracellular glutamate. Once in the cells, cystine is reduced and subsequently used for glutathione (GSH) biosynthesis. Glutathione peroxidase 4 (GPX4) is the key GSH-utilizing enzyme responsible for preventing lipid peroxidation by preferentially reducing phospholipid hydroperoxides to their corresponding alcohols. Arachidonic acid is shown as a representative PUFA and phosphatidylethanolamine as a model phospholipid. Ferroptosis inducers and inhibitors as well as their affected pathways are represented on the figure.

1.4.1 Physiological context of ferroptosis

Understanding cell death mechanisms lends opportunities for therapeutically manipulating such pathways to overcome disease where they are implicated. For example, a classical hallmark of several cancers is the resistance to apoptosis.¹¹⁶ However, because ferroptosis is genetically, morphologically and biochemically orthogonal to apoptosis, leveraging its induction may be a viable strategy to target certain cancer cells for death.¹⁷ GPX4 inhibitor, RSL3, was effective in reducing the tumor size in mouse xenograft models of cancer¹⁰⁷ and recent reports have targeted persister and therapy resistant cancer cells by taking advantage of their vulnerability to lipid peroxidation by triggering ferroptosis.^{117,118}

While there is no evidence that ferroptosis is intrinsically induced and shown to function during development or tissue homeostasis, it is increasingly implicated in pathogenesis of degenerative diseases where PLOOH accumulation has been invoked. The association of lipid peroxidation with human pathological conditions has long been appreciated, however, whether lipid peroxidation plays a causal role in disease onset or progression remains to be directly demonstrated. Ferroptosis may be the ultimate link between lipid peroxidation and disease development. These include several pathologies of the central nervous system (CNS), such as Alzheimer disease (AD),¹¹⁹ Parkinson's disease (PD),¹²⁰ Friedrich Ataxia (FD)¹²¹ and Huntington's disease (HD).¹²² It is also associated with acute renal failure, synchronized renal tubular cell death and ischemia-reperfusion injury.^{18,123} Therefore, ferroptosis inhibitors may be a viable strategy to provide new therapeutic opportunities for these often poorly treatable diseases. This was demonstrated by two independent reports where tissue damage in a model of ischemia/reperfusion injury (IRI) in kidney¹²³ and liver¹⁸ was mitigated by the use of liproxstatin-1 (Lip-1) and ferrostatin-1 (Fer-1) – two archetypical ferroptosis inhibitors.

1.4.2 Ferroptosis execution and inhibition

With the increasing recognition of the essential role of lipid peroxidation in ferroptosis execution, recent emphasis has been on trying to elucidate the execution mechanism of cell death. Lipid-derived peroxides can be formed via autoxidation, as well as by enzyme-catalyzed processes. However, to date, only the

LOX family of enzymes have been associated with ferroptotic cell death.^{45,114,124} Thus, whether autoxidation plays a role in the execution of ferroptosis needs to be clarified.

Due to the implication of ferroptosis in degenerative disease, strategies aiming at the inhibition of lipid peroxidation are re-emerging as attractive cytoprotective strategies. As such, a simple strategy aiming to halt this process can be divided into two simple groups: lipid autoxidation inhibitors (RTAs) and LOX-inhibitors. However, other pathways that regulate ferroptosis can also be targeted for therapeutic intervention and should not be discounted (*cf.* Figure 1.12).

1.4.2.1 Ferroptosis Execution

In order to better understand the signaling pathways involved in ferroptosis execution and inhibition, several genetic and pharmacological tools aiming to induce ferroptosis have been developed. Majority of the strategies aim to inhibit GPX4 (Figure 1.13A), the master regulator of ferroptosis by direct and indirect methods.^{18,125} Since GPX4 is the only enzyme capable of detoxifying PLOOH to their benign alcohols, its inhibition or degradation affords an appealing avenue for ferroptosis induction. Some of the most common methods employed include:

- 1) Direct inhibition of GPX4 using covalent small-molecule inhibitors such as (*1S,3R*)-RSL3,¹¹³ ML210 or ML162.¹²⁶ These compounds are highly reactive electrophiles that irreversibly modify the active site selenocysteine rendering the enzyme inactive (Figure 1.13B).
- 2) Genetic knockout using tamoxifen-inducible expression of Cre-recombinase to delete the *loxP*-flanked GPX4 allele¹¹⁴ or targeting the GPX4 transcript for degradation using RNA interference methods (Figure 1.13C).¹²⁵
- 3) Depletion of glutathione, the reducing cofactor of GPX4, by inhibition of the enzyme gamma-glutamylcysteine synthase involved in its biosynthesis using an irreversible inhibitor such as BSO (Figure 1.13D).¹²⁵

4) Indirect inhibition of GPX4 by depleting the substrates necessary for glutathione biosynthesis.

This is often achieved upstream by the inhibition of the cystine/glutamate antiporter, System Xc.

Starving the cell of cystine, effectively precludes glutathione biosynthesis. Frequently used small-molecules include erastin,¹¹⁵ sulfasalazine,¹⁷ sorafenib¹²⁷ and glutamate (Figure 1.13E).¹²⁸

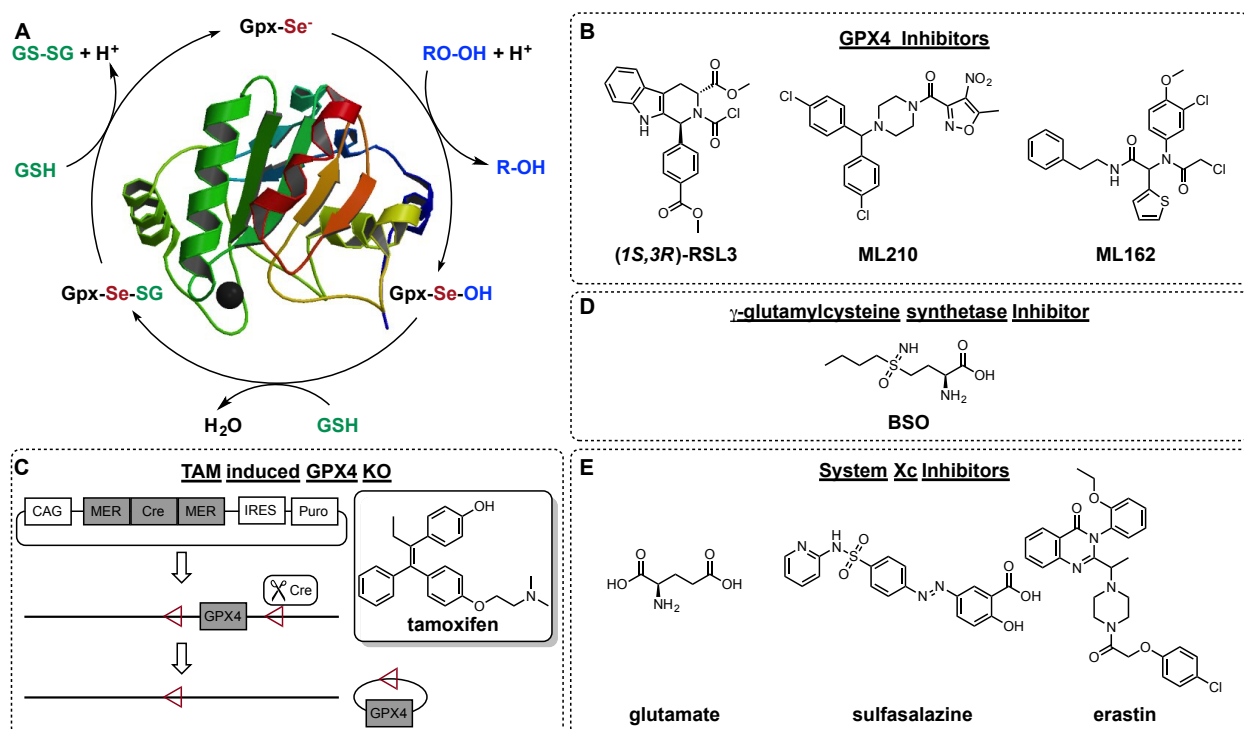


Figure 1.13 (A) Mechanism of action of GPX4 and various strategies and inhibitors used to induce ferroptosis in cellular models (B-E).

1.4.2.2 Ferroptosis Inhibition

1.4.2.2.1 Vitamin E – Nature’s Most-Efficient Ferroptosis Inhibitor

The term Vitamin E refers to one or more structurally related phenolic compounds – four (α -, β -, γ -, and δ -) tocopherols and four (α -, β -, γ -, and δ -) tocotrienols.⁵¹ The discovery of endogenous phenols such as α -tocopherol, the most biologically active form of vitamin E (α -tocopherol, α -TOH), and their ability to hinder the autoxidation *in vivo* is relatively recent.⁵⁸ All congeners of Vitamin E have RTA activity, but α -TOH is both the most reactive *in vitro*⁵¹ and possesses the greatest biological activity *in vivo* (Figure 1.14). The greater biological activity of α -TOH is due to the presence of α TPP, a transport protein that

preferentially binds α -TOH and redistributes it to tissues precluding its metabolism and excretion.¹²⁹ The tocopherols ‘trap’ two peroxy radicals – that is the stoichiometric factor for peroxy radical capture, n , is 2.0.⁵¹ α -TOH fulfills the classical role as an RTA because it efficiently reacts with peroxy radical with a rate constant $k_{inh} = 3.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, efficiently suppressing the oxidation of PUFAs by peroxy radicals. Importantly, it was recently suggested that vitamin E might have an alternative function – direct inhibition of lipoxygenases (15-LOX-1).³⁴ This is proposed to proceed via a ‘corking’ mechanism where different Vitamin E analogs compete for the substrate-binding site of lipoxygenase, thus suppressing the formation of putative lipid ‘death signals’ (Figure 1.14 bottom).³⁴

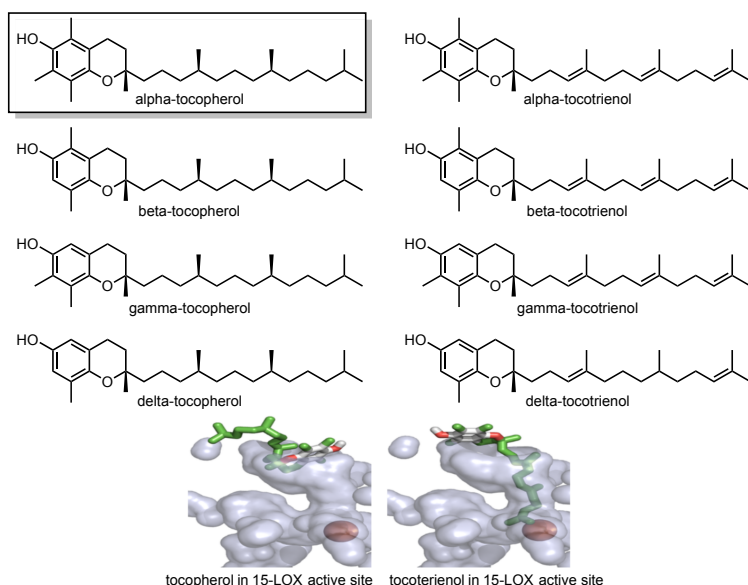


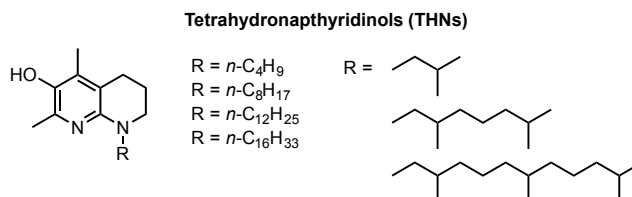
Figure 1.14 Different forms of Vitamin E (tocopherols and tocotrienols) found in vivo. α -TOH is the most potent form of Vitamin E. Molecular modeling of purported inhibition of 15-LOX-1 by tocopherol and tocotrienols (bottom).³⁴ Docking model was reprinted with permission from *Nat. Chem. Biol.* **2017**, *13*, 81. Copyright 2017 Springer Nature.

Nonetheless, the importance of α -TOH in ferroptosis has been demonstrated by several groups who have shown that α -TOH suppresses ferroptosis *in vitro*.^{17,18,130} Whether these effects are due to its RTA activity or its recently proposed lipoxygenase inhibitory effect remains to be seen. Years of research efforts on α -TOH have shown that natural antioxidants do not offer therapeutic potential and have failed in clinical trials aimed at arresting oxidative stress-related disease progression.¹³¹ These outcomes suggest

that RTAs do not function therapeutically by reversing the onset of disease, rather they are preventative agents whose function is to decrease or delay the onset. As such, it will be interesting to observe the effects of α -TOH in animal models of ferroptosis.

1.4.2.2.2 Vitamin E Analogs

Tetrahydronaphthyridinol (THNs) are an extremely potent class of synthetic RTAs derived from structure-activity relationship inspired by Nature's premier lipid-soluble RTA – α -TOH.⁶⁷ Their structures were engineered following extensive studies of heterocyclic phenolic RTAs, which revealed that incorporation of nitrogen atoms in the aromatic rings at the 3 and/or 5 positions relative to the hydroxyl group greatly stabilizes electron-rich phenols against autoxidation.^{66,69} Substitution of these compounds with strongly electron-donating groups (e.g., *N,N*-dialkylamino) substantially weakens the O-H bond and accelerates their rates of reaction towards peroxy radicals without comprising their resistance to autoxidation. The THNs are a specific subset of aza-phenols that possess the best balance of reactivity and stability; they react almost 30-fold faster with peroxy radicals than α -TOH in organic solution and liposomes,¹³² making them among the most potent RTAs reported to date.

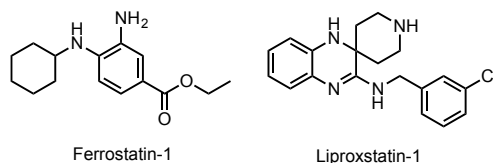


The excellent RTA activity of these compounds and the recognition that lipid peroxidation is essential to ferroptosis posed the logical question whether THNs might also suppress ferroptosis. Indeed, when a small library of THNs with different chain lengths were assayed it was shown that THNs with aliphatic-chains ranging from 12 to 15 carbons conferred an optimal inhibitory effect. Some compounds were even slightly more potent than the classical ferroptosis inhibitors Fer-1 and Lip-1.¹³⁰ Another interesting aspect of THNs is their ability to efficiently bind to α -TOH transfer protein (α TPP),¹³³ whereby – in theory – they should become protected from liver metabolism, allowing efficient tissue

distribution. Therefore, it is anticipated that the efficacy of THNs will translate to *in vivo* ferroptosis and disease-relevant models.

1.4.2.2.3 Lipoxstatins and Ferrostatins

Lip-1 and Fer-1 were identified by high-throughput screening efforts aimed to discover compounds able to efficiently inhibit ferroptosis. Lipoxstatins were identified in a phenotypic screen using compounds that suppress cell death in an inducible Gpx4 KO mouse cell line (Figure 1.13C),¹⁸ while ferrostatins were identified in a model where ferroptosis was elicited by inhibiting cystine uptake by erastin (Figure 1.13E).¹⁷ Lip-1, the first identified molecule of the lipoxstatin class, possessed the ability to inhibit ferroptosis in the low nM concentration range and demonstrated good pharmacological properties in plasma.¹⁸ Importantly, this molecule was applied *in vivo* to ameliorate acute renal failure in a genetic model of Gpx4 deficiency. Similarly, modifications to the original Fer-1 scaffold using classical medicinal chemistry led to second-generation compounds with sufficient *in vivo* stability.¹²³ Importantly, both types of compounds preserved tissue function in both liver and kidney IRI models.

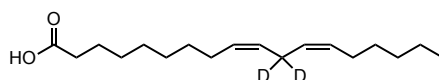


Although very little was initially known regarding their mechanism of action, initial investigations suggested that Fer-1 may function as an RTA.¹³⁴ However, several speculative mechanisms were put forth to account for their action, including their propensity to act as iron chelators and inhibitors of LOX catalysis. To better understand their mechanism, we recently explored their RTA activity and were able to demonstrate that they are intrinsically less reactive towards peroxy radicals in comparison to α -TOH. This is remarkable because lipoxstatins/ferrostatins are at least an order of magnitude more potent at inhibiting ferroptosis relative to α -TOH. The differential activity arises because the compounds are excellent RTAs in the lipid bilayers. The change in reactivity from isotropic solution to lipid membranes is because the aromatic amines are poorer H-bond donors, making them insensitive to interactions with phosphatidylcholine headgroups and/or aqueous surroundings at the interface, enhancing

their relative activity compared to phenols.¹³⁰ Moreover, both Fer-1 and Lip-1 have better dynamics in the lipid bilayer since they are not anchored by a long lipophilic chain. Additionally, Fer-1 and Lip-1 possess a greater radical-trapping capacity than typical phenolic RTAs, which we hypothesized to be due to their subsequent transformation to nitroxides. Although nitroxides are commonly thought to exhibit SOD activity in cells, we have proposed that nitroxides are good RTAs and regenerated by hydride-transfer reactions from water soluble reductants such as NAD(P)H.^{92,135} Recent work by Wipf and Stockwell¹³⁶ has also shown that they can indeed inhibit ferroptosis in *in vitro* cellular systems.

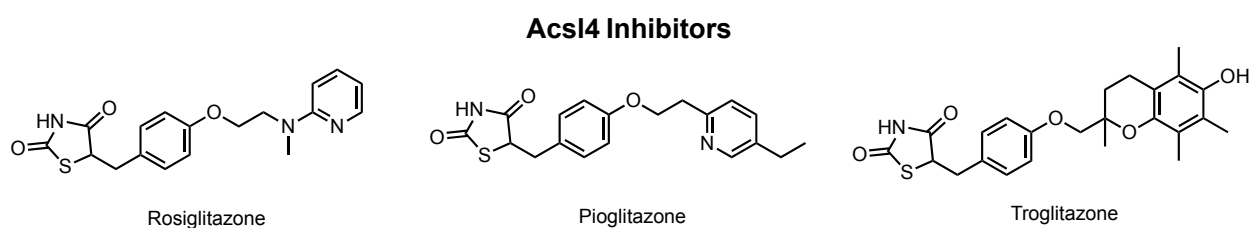
1.4.2.2.4 Non-canonical Ferroptosis Inhibitors

The purported role of LOX-catalysis in ferroptosis execution has not only seen a surge in the use of LOX inhibitors as anti-ferroptosis agents but also certain un-natural fatty acid substrates.⁴⁵ The use of PUFAs where the methylene groups have been replaced with D-atoms is elegant as it assumes that prohibiting the oxidation of the bis-allylic sites by LOX would reduce the overall peroxide tone in the cells. This is based on the well-established role of quantum mechanical tunneling in enzyme catalysis observed for the oxidation of linoleic acid (LA) by soybean lipoxygenase, evident by large kinetic-isotope values of ~ 65 because the deuterated LA is a significantly poorer substrate than the naturally occurring isotopologue.¹³⁷ It was recently shown that tunneling is also likely key to the rapid propagation step of PUFA autoxidation: the propagation step of the autoxidation of 11,11-*d*₂-LA is ~ 13 -fold slower than for LA.¹³⁸ Moreover, evidence suggests that only 20% supplementation of D-PUFAs is sufficient to completely suppress lipid autoxidation, by a yet unknown mechanism.^{139–141} Based on these observations, supplementation of cell media with deuterated LA presented a marked resistance to ferroptosis. The protective effect of D-PUFAs has been shown in multiple models of oxidative stress related disease. Since 20% supplementation is sufficient to suppress lipid peroxidation, it remains to be ascertained whether the administration of deuterated PUFAs is a viable intervention *in vivo*.



11,11-*d*₂-Linoleic Acid

It was recently demonstrated that ferroptosis occurs with the preferential oxidation of PE containing arachidonic (AA) and adrenic acid (AdA).³⁴ Through a genetic screen, the enzyme responsible for activating fatty acids for acylation to PE was identified to be ACSL4.¹⁴² This was supported by direct genetic evidence showing that *Acsl4*/ACSL4-deficient cells are resistant to ferroptosis induced by inactivation of GPX4.^{142,143} This introduced a novel mechanism for ferroptosis inhibition. The suggestion that ACSL4 may be a viable target was demonstrated by the use of thiazolidinediones (TZNs), which specifically inhibit ACSL4 over other ACSL isoforms.^{142,144}



Interestingly, troglitazone is the most potent inhibitor of ferroptosis despite having the lowest ACSL4 inhibition activity. This is presumably due to the bi-functional nature of its inhibition stemming from the presence of the chromanol ring affording it RTA activity. Another inhibitor, rosiglitazone was successfully utilized *in vivo* to mitigate and delay the mortality rate of mice due to acute renal failure in a genetic model of ferroptosis. Thus, pharmacological inhibition of ACSL4 provides a new target in preventing ferroptosis by precluding the incorporation of oxidizable substrate.

1.5 Research Objectives

1.5.1 Develop a facile method to quantify multiple hydrocarbon autoxidation products

High-temperature autoxidation of hydrocarbons produces a complex mixture of products. The primary products, hydroperoxides, can be quantified and we previously reported a pro-fluorescent phosphine probe that undergoes fluorescence enhancement to rapidly quantify hydroperoxide concentration in our samples as an alternative to other laborious methods such as HPLC, GC or iodometric titrations. Hydroperoxides can also undergo further decomposition to produce secondary-oxidation products such as

carboxylic acids that can alter properties of hydrocarbon-based products. Moreover, we have also demonstrated that acids attenuate the reactivity of our heterocyclic diarylamine antioxidants by protonating them. As a result, monitoring acid concentration in addition to hydroperoxide formation can offer insight towards the extent of oxidation in hydrocarbon samples. The objectives of this project were:

- 1) Design and synthesize an acid-sensitive pro-fluorescent probe that undergoes fluorescence enhancement upon reaction with weak acids.
- 2) Characterize the reactivity of the probe in order to quantify acid production from autoxidation of unknown samples
- 3) Design, synthesize and characterize a probe with orthogonal reactivity to simultaneously measure acid and hydroperoxide concentration from autoxidation samples
- 4) Develop a screening method for the evaluation of RTA activity in high-temperature autoxidations

1.5.2 Evaluation of heterocyclic diarylamines in high temperature autoxidations

The development of heterocyclic diarylamine antioxidants has resulted in an enhancement of their reactivity towards chain-carrying peroxy radicals. This has been successfully demonstrated at ambient temperatures where they react at diffusion-controlled rates. However, the activity of diarylamines under conditions where they are actually used industrially, such as engine lubricants, jet fuels and rubber has yet to be studied. Whether the reactivity of these compounds will translate to real-world applications needs to be tested in high-temperature autoxidations of heavy hydrocarbons. To this end, the objectives of this project were:

- 1) Synthesize heterocyclic diarylamine radical-trapping antioxidants with lipophilic carbon chains to ensure solubility in hydrocarbon-rich environment
- 2) Evaluate their activity in high-temperature autoxidations that mimic industrial conditions and characterize their stability under autoxidative conditions experienced in the stirred-flow reactor

- 3) Understand and evaluate the structure-performance relationship to enable the design of next-generation of compounds

1.5.3 Optimization of the structure of heterocyclic diarylamine RTAs

Diarylamine RTAs exhibit catalytic inhibition in high-temperature autoxidations. Our group previously discovered that substituted-pyrimidines demonstrate an unprecedented ability to inhibit hexadecane autoxidations at high-temperatures. Given the foregoing, we sought to improve the activity of the ‘lead’ compound by varying the substitutions on the aromatic rings to establish a structure-performance relationship. The specific objectives include:

- 1) Synthesize substituted pyrimidines with varying alkyl groups on the phenyl ring
- 2) Characterize the reactivity of the compounds at different temperatures and in different substrates
- 3) Utilize structure-performance relationships to provide guidelines for the design of highly reactive diarylamine RTAs

1.5.4 Deciphering the mode of ferroptotic cell-death: Autoxidation or LOX catalysis?

Ferroptosis has been a hot topic in cell and chemical biology since its relatively recent characterization by Stockwell in 2012. Of particular recent interest has been the apparent role of lipoxygenase-catalyzed polyunsaturated fatty acid oxidation in the execution of ferroptosis. Although these assertions are not reconciled by the observations that the most potent ferroptosis inhibitors are all RTAs, bringing into question whether autoxidation plays a role. Understanding the execution mechanism that leads to cell death can help bolster our understanding of cell death pathway. Unfortunately, there are no reports which systematically evaluate the role of enzymatic and non-enzymatic lipid peroxidation in ferroptosis. To provide insight on this point we sought to the objectives of this study were:

- 1) Generate and characterize LOX-overexpressing stable cell-lines to study their involvement in ferroptosis

- 2) Evaluate various RTAs and LOX inhibitors for their antioxidant, LOX-inhibition and anti-ferroptosis activity in different cell lines utilizing different modes of ferroptosis induction
- 3) Delineate the contributions of LOX-catalysis and autoxidation in ferroptosis induction

1.5.5 From solution to cells: evaluation of heterocyclic RTAs as ferroptosis inhibitors

Ferroptosis and its inhibition offers an attractive therapeutic avenue to target diseases in which ferroptosis has been implicated. Since the execution mechanism of ferroptosis is not well understood, rational inhibitor design has been hindered. Ferrostatin-1 and liproxstatin-1, two of the most potent inhibitors were identified by a high-throughput screen and their mechanism of inhibition was recently shown to proceed by inhibiting autoxidation. Since the role of autoxidation in ferroptosis is highly contested, we sought to utilize our rationally-designed heterocyclic diarylamine RTAs to shed light on both the mechanism of ferroptotic cell death and its inhibition by small-molecules and to provide insight whether it is driven by lipoxygenase catalysis or lipid peroxidation. Hence, we aimed to:

- 1) Evaluate the kinetics of various arylamines in organic and liposomal solutions
- 2) Evaluate the anti-ferroptosis activity of the arylamines under different modes of ferroptosis induction (GPX4 inhibition, GSH depletion and genetic knockout)
- 3) Offer insight into the relationship between autoxidation inhibition and ferroptosis inhibition.

1.5.6 Development of a method to screen RTAs as ferroptosis inhibitors

Since their popularization in the early 2000s, investigators have relied almost entirely on simple surrogate ‘antioxidant’ assays, such as the DPPH assay, to provide insight on the potential of compounds to inhibit lipid peroxidation (or hydrocarbon autoxidation, in general) directly by reacting with chain-carrying radicals. The quantification of radical-trapping antioxidant activity is also of central importance in the food, cosmetics, fragrances, polymer and lubricant industries – and all of these fields make routine use of the DPPH assay and related spectrophotometric assays for assessing antioxidant ‘activity’. As such, utilizing appropriate assays to study RTA activity is important not only in the context of ferroptosis and

related forms of oxidative cell death, but also in various other pathological contexts. We sought to develop a microplate-based assay to determine the reactivity of inhibitors of lipid peroxidation that would be operationally similar to the simple ‘antioxidant’ assays, but provide meaningful insight on the reactivity of RTAs.

- 1) Design a high-throughput assay that is practically simple but offers information into the mechanisms and kinetics of RTAs
- 2) Characterize a model liposomal system to correlate and predict the RTA activity in liposomes to their ability to inhibit ferroptosis in cell-models
- 3) Provide insight on the mechanisms of ferroptosis modulators and the important properties in the design of novel inhibitors of lipid peroxidation and/or ferroptosis

1.5.7 Elucidate mechanism of a non-canonical enzymatic ferroptosis inhibitor

Commonly utilized ferroptosis inhibitors include small molecules that inhibit lipid peroxidation by functioning as RTAs or LOX inhibitors. Iron chelators are also capable of inhibiting ferroptosis by sequestering the low-valent iron responsible for catalyzing the Fenton reaction. However, limited strategies outside of small-molecule inhibitors exist to inhibit ferroptosis. The recent identification of ferroptosis-suppressing protein-1 (FSP1) may offer a novel pathway towards modulating ferroptosis. Since, it is a non-characterized protein that offers significant cytoprotection and even compensates for Gpx4 loss we sought to:

- 1) Characterize the functional activity of the protein by determining enzyme kinetics
- 2) Design an *in vitro* model system that mimics the results obtained in a cellular context
- 3) Elucidate the mechanism of action as it pertains to ferroptosis inhibition

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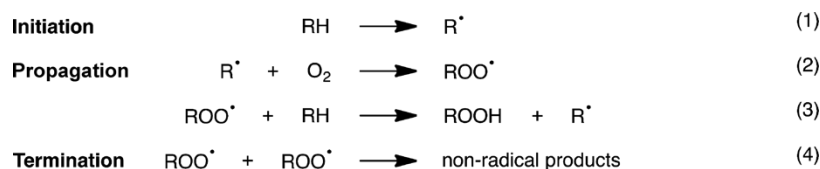
Chapter 2: Determination of Key Hydrocarbon Autoxidation Products by Fluorescence

2.1 Preface

Hydroperoxides and carboxylic acids are key primary products that arise in the autoxidation of hydrocarbons. We have developed a simple approach to rapidly and simultaneously determine both types of products using hydroperoxide- and acid-sensitive moieties conjugated to nonpolar coumarin- and BODIPY-based fluorophores. The coumarin- and BODIPY-conjugated amine probes described here undergo 38- and 8-fold enhancement, respectively, upon protonation in a solvent system compatible with heavy hydrocarbons. The latter can be used directly with our previously described hydroperoxide-sensitive coumarin-conjugated phosphine probe to enable rapid quantification of both carboxylic acids and hydroperoxides in hydrocarbon samples. The utility of the approach is illustrated by the ready determination of the differing relative rates of hydroperoxide and acid formation with changes in hydrocarbon structure (e.g., *n*-hexadecane vs 1-hexadecene vs a lubricant base stock). The method offers significant versatility and automation compared with common but laborious titration approaches, and greatly improves screening efficiency and accuracy for the identification of novel radical-trapping antioxidants for high temperature applications. This application was demonstrated by the automated analysis of hydroperoxides and carboxylic acids (by microplate reader) in samples from 24 inhibited autoxidations of a lubricating oil, which were carried out on a parallel synthesizer at 160 °C in triplicate in a single day. Reprinted (adapted) with permission from: Shah, R.; Pratt, D. A. *J. Org. Chem.* **2016**, *81*, 6648. Copyright 2016 American Chemical Society.

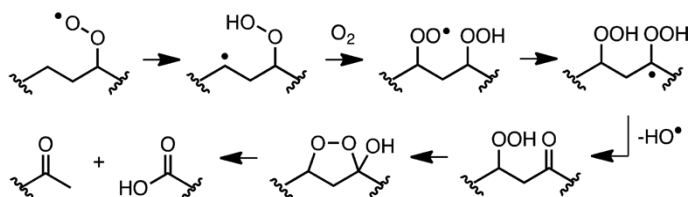
2.2 Introduction

Autoxidation is the spontaneous radical-mediated process that converts hydrocarbons into their corresponding hydroperoxides (Scheme 2.1). It is primarily responsible for the degradation of all hydrocarbon-based products such as plastics, rubbers, and lubricants as well as specialty chemicals and biotechnology products.¹



Scheme 2.1 Hydrocarbon autoxidation

The sequence of reactions in Scheme 2.1 (in particular eqs. 2 and 3) constitutes a generalized but also greatly simplified mechanism of hydrocarbon autoxidation. In fact, the primary products of autoxidation are not simply monohydroperoxides. In fact, as first demonstrated by Korcek and co-workers for the autoxidation of *n*-hexadecane, ketohydroperoxides (of which the γ -isomer is predominant) are formed competitively from very early on and carboxylic acids and methyl ketones shortly thereafter.² The formation of each of these products can be rationalized by the mechanism in Scheme 2.2.³



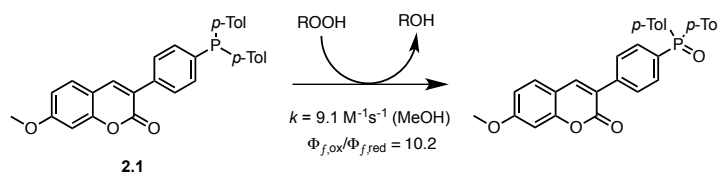
Scheme 2.2 Proposed Mechanism of Acid Formation in *n*-Alkane Autoxidation

The accumulation of autoxidation products (or subsequent decomposition products thereof) can alter the properties of hydrocarbon-based products or materials that come into contact with them. For instance, autoxidation of engine lubricants increases their viscosity and the production of acids leads to corrosion of metallic engine parts. As such, antioxidants are key additives to essentially all hydrocarbon-based materials. Radical-trapping antioxidants (RTAs, A–H in eq 5), such as phenols and aromatic amines, intervene directly in the radical chain-reaction depicted in Scheme 2.1 by reducing the chain-carrying peroxy radicals by H-atom transfer, thereby precluding chain propagation.^{4,5}



In an attempt to speed up the identification and assessment of the new chemical entities as RTAs, we recently reported a strategy to monitor the production of hydroperoxides using fluorogenic phosphine **2.1**.⁶ This probe enables rapid quantification of hydroperoxides by measuring the pseudo-first order rate of fluorescence increase in a microplate assay instead of more conventional methods, such as iodometric

titration (i.e., ASTM method D3703-13) or lengthy HPLC or GC analyses.⁷ This method has proven highly useful for quantifying hydroperoxides⁸⁻¹¹ but ignores the acids that are produced. In addition to their role in advancing corrosion, carboxylic acids can have a profound impact on both the kinetics and mechanisms of the RTAs added to protect the substrate from autoxidation (e.g., diarylamines⁹ and hindered amine light stabilizers¹⁰). Thus, we have extended our work on **2.1** (Scheme 2.3) to the development of an analogous strategy to monitor the production of carboxylic acids in hydrocarbon autoxidations, which are conventionally determined by acid–base titration (i.e., ASTM method D664-11a).¹²



Scheme 2.3 Reaction of Fluorogenic Coumarin-Triarylphosphine **2.1** with Hydroperoxides

Although acid-sensitive fluorescent probes have been developed for the real-time monitoring of intracellular pH by flow cytometry or fluorescence microscopy, they are unsuitable for monitoring acid production in the current context for solubility reasons.¹³⁻²¹ Moreover, the probes reported to date are relatively complex, and we envisioned simply using the same trivially accessed 7-methoxycoumarin core as in hydroperoxide probe **2.1**. We surmised that a similar probe wherein the triarylphosphine moiety was replaced with a basic trialkylamine unit would undergo fluorescence enhancement with increasing acidity of the medium, similarly to the response of **2.1** to increasing hydroperoxide concentration. The electron-rich trialkylamine is expected to quench the fluorescence of the coumarin by photoinduced electron transfer (PeT), while protonation would preclude PeT.²²

2.3 Results and Discussion

Coumarin **2.2** was easily prepared (Figure 2.1A, see Experimental Section), and as expected, its fluorescence increased significantly upon protonation (Figure 2.1B). Determination of the fluorescence of

2.2 as a function of pH yielded a sigmoidal profile whose midpoint indicates a $s\text{p}K_a$ of 7.9 ± 0.1 (Figure 2.1C). The experiments were carried out in 4:1 MeOH/i-PrOH (v/v) to ensure a balance between solubility of hydrocarbon samples (vide infra), efficient quenching of coumarin fluorescence by PeT, and ionizability of **2.2**. It should be noted that this solvent mixture is characterized by an autoprotolysis constant of 15.3 and associated neutrality at $s\text{pH} = 9.2$.²³ Under these conditions, the ratio of fluorescence quantum yields of **2** and its conjugate acid (2-H^+) was determined to be 38.

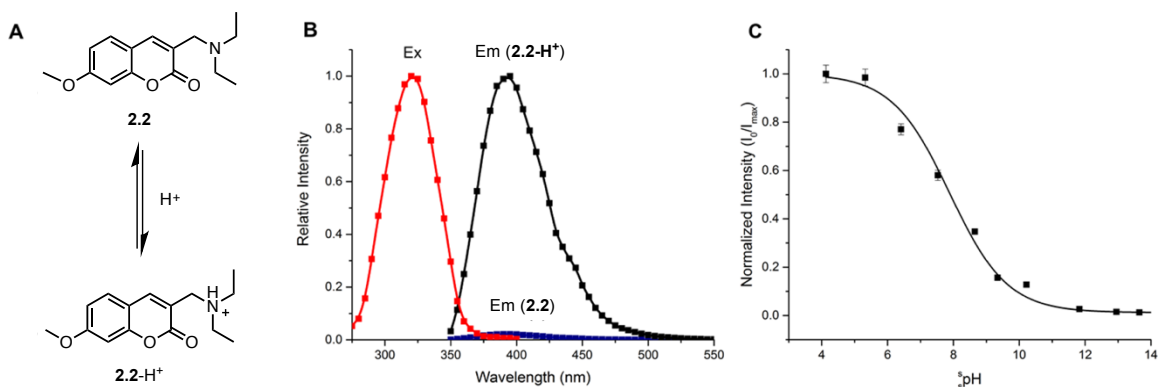


Figure 2.1 (A) The relevant equilibrium of the coumarin–trialkylamine **2.2** and its conjugate acid 2-H^+ . (B) Excitation and emission spectra of **2.2** and 2.2-H^+ . (C) Fluorescence of **2.2** ($\lambda_{\text{ex}} = 315$ nm; $\lambda_{\text{em}} = 395$ nm) as a function of pH in 4:1 MeOH/i-PrOH.

The foregoing suggested that carboxylic acid formation could be monitored in autoxidation samples simply by determination of their fluorescence following addition of **2.2** and dissolution in the 4:1 MeOH/i-PrOH solvent system.²⁴ Figure 2.2A reveals the linear relationship between the fluorescence of **2.2** and added palmitic acid, a representative lipophilic carboxylic acid. Based on this standard, carboxylic acid formation in the initial stages of an *n*-hexadecane autoxidation (carried out at 160 °C in a stirred flow reactor)^{2,6,25} was readily determined and yielded similar results to those obtained previously by (negative ion) electrospray mass spectrometric analysis (Figure 2.2B).⁹ The previous results involved determination of each individual isomer of carboxylic acid formed in the *n*-hexadecane autoxidations from the mass spectra and then summing them in order to determine the total acid concentration.^{26,27} It should be noted

that **2.2** does not react with hydroperoxides (see the Supporting Information for the results of the control experiments).

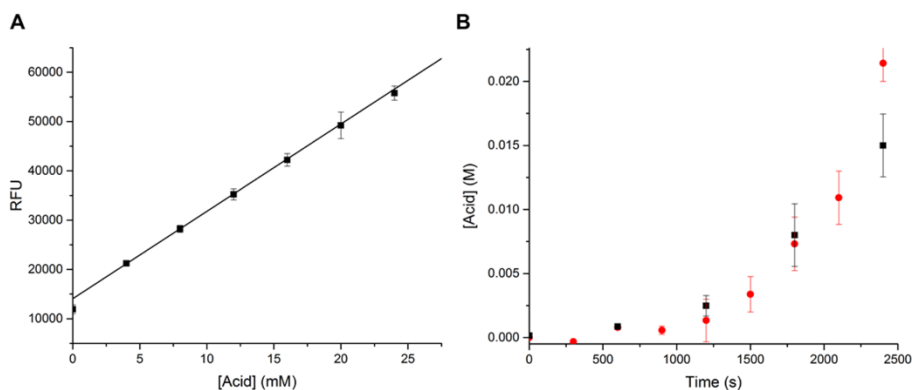


Figure 2.2 (A) Fluorescence of **2** ($\lambda_{\text{ex}} = 315 \text{ nm}$; $\lambda_{\text{em}} = 395 \text{ nm}$) determined as a function of palmitic acid concentration in 4:1 MeOH/i-PrOH. (B) Acid formation in the initial stages (<2% conversion) of an n-hexadecane autoxidation at 160 °C determined directly by ESI-MS (■) or indirectly by the fluorescence of **2.2** (red ●).

n-Hexadecane is a convenient model for heavy hydrocarbon autoxidation because it produces the simplest possible product distribution. In fact, industrial or commercially relevant hydrocarbon-based products are usually mixtures of compounds and constitutional isomers. Since the current method does not require knowledge of the precise structure of each of the carboxylic acid products that are formed, acid production can easily be determined in these complex mixtures. An example is shown in Figure 2.3B where acid formation was determined in the initial stages of the autoxidation of a lubricant basestock (an American Petroleum Institute Group III base oil) at 160 °C.²⁸ The hydroperoxides were determined in parallel (using **2.1**) and are shown on the same plot alongside the corresponding data for an n-hexadecane autoxidation (Figure 2.3A).

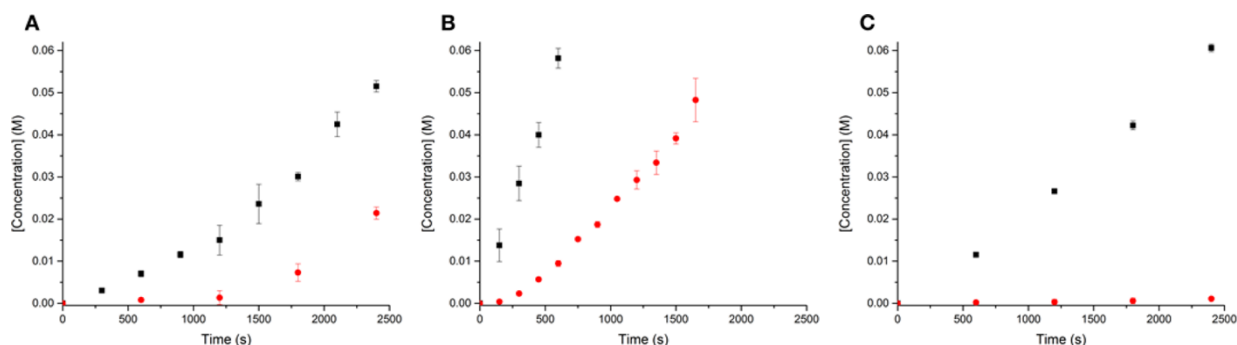


Figure 2.3 Formation of hydroperoxides (■) and acids (red ●) in the initial stages (<2% conversion) of the autoxidation of either *n*-hexadecane (**A**) or a API Group III base oil (**B**) at 160°C or 1-hexadecene (**C**) at 120°C. Autoxidations of *n*-hexadecane and base oil were initiated with tetralin hydroperoxide at 10 and 5 mM, respectively, while the 1-hexadecene autoxidations were initiated with 10 mM dicumyl peroxide.

The data reveal that the base oil is more easily oxidized than *n*-hexadecane. However, less acid is produced relative to hydroperoxide at these early stages of the autoxidation. These results can be rationalized on the basis that the base oil contains branched hydrocarbons and that H-atom abstraction from tertiary positions of hydrocarbons by peroxy radicals is faster than from secondary positions (i.e., $k = 7.0 \times 10^{-3}$ and $1.5 \times 10^{-4} \text{ M}^{-1}\text{s}^{-1}$ for H-atom abstraction from the tertiary and secondary positions of 2-methylbutane, respectively, at 30 °C),²⁹ and this is the rate-determining propagation step (eq. 3, Scheme 2.1) in the autoxidation. Moreover, the initially formed tertiary peroxy radicals cannot undergo the sequence of reactions to form carboxylic acids as in Scheme 2.2; only the fraction of secondary peroxy radicals that are formed, and that undergo intramolecular H-atom abstraction from a secondary site in lieu of intermolecular propagation by abstraction at a tertiary site, can contribute via this pathway. Of course, it must be acknowledged that acids may arise from other mechanisms.

The results of corresponding autoxidations of 1-hexadecene, a model α -olefin, are shown in Figure 2.3C. These autoxidations were carried out at 120 °C since 1-hexadecene is an unsaturated hydrocarbon that oxidizes much more rapidly than saturated ones, such as *n*-hexadecane (i.e., $k_p = 1 \text{ M}^{-1}\text{s}^{-1}$ for 1-octene vs $k_p = 0.0015 \text{ M}^{-1}\text{s}^{-1}$ for *n*-octane at 30 °C).^{30,31} Very little acid is produced relative to hydroperoxide in the early stages of these autoxidations, presumably because intermolecular propagation is much faster than intramolecular H-atom abstraction. The production of acid via the mechanism in Scheme 2.2 requires the intramolecular H-atom abstraction from an unactivated position to compete with the intermolecular H-atom abstraction from an allylic position, which is unlikely. Indeed, ESI-MS measurements of samples from the 1-hexadecene autoxidation (Figure 2.4B) reveal a very different profile of acids that are formed compared with that observed from samples of the *n*-hexadecane autoxidation (cf. Figure 2.4A), implying that the mechanism of acid formation in 1-hexadecene

autoxidations is very different than that in autoxidations of linear saturated hydrocarbons. The two predominant peaks in the mass spectra are consistent with the molecular formulas of hexadeca-2-enoic acid and *n*-pentadecanoic acid, which may derive from the expected allylic hydroperoxide arising in the autoxidation of 1-hexadecene as is shown in Scheme 2.4.³²

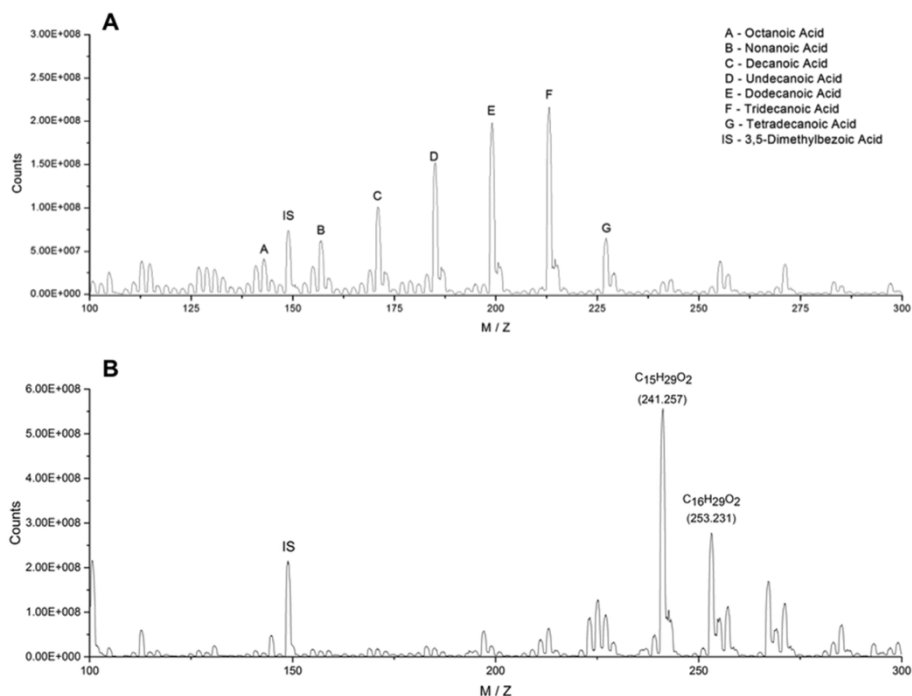
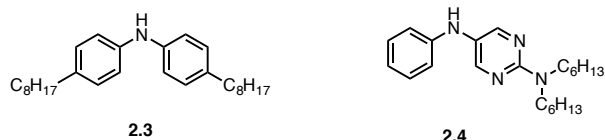


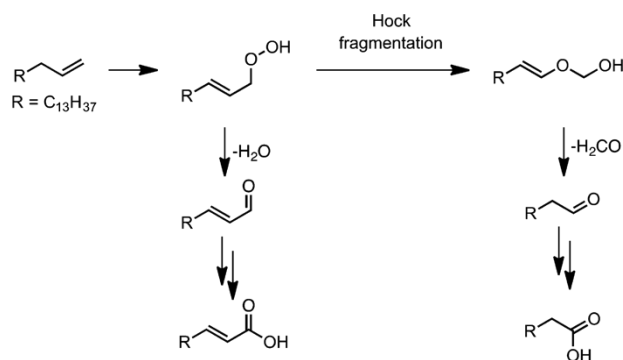
Figure 2.4 Representative negative ion electrospray mass spectra from the initial stages of autoxidations of either *n*-hexadecane (**A**) or 1-hexadecene (**B**) at 160 and 120 °C, respectively. Samples (50 μ L) were withdrawn 2400 or 4800 s into the autoxidation and diluted with 4.95 mL of a 20% *i*-PrOH/80% MeOH solution containing 3,5-dimethylbenzoic acid (10.5 μ M) as an internal standard.



Our motivation for the development of rapid means to quantify hydroperoxides⁶ (and now acids) in hydrocarbon autoxidations was the ability to assess the efficacy of RTAs. In Figure 2.5, we show the results of inhibited autoxidations of the same three model hydrocarbons (*n*-hexadecane, the API Group III base oil, and 1-hexadecene) in the presence of diarylamines **2.3** and **2.4**. Compound **2.3** is an alkylated diphenylamine representative of catalytic diarylamine antioxidants found in a variety of hydrocarbon-based products (e.g., lubricating oils and rubber), and compound **2.4** is representative of a recently

developed class of heterocyclic diarylamines that have been shown to better inhibit hydrocarbon autoxidation compared with industry standards.^{9,33-36}

The increased reactivity of the heterocyclic diarylamine **2.4** relative to **2.3** is readily observed in Figure 2.5A,B, where **2.4** was able to suppress acid formation to a greater extent than **2.3** in each of *n*-hexadecane and the API Group III base oil, respectively. The same trend is observed in the inhibition of 1-hexadecene autoxidation (Figure 2.5C), but because acid formation lags considerably behind hydroperoxide formation in 1-hexadecene (*cf.* Figure 2.4), resolution of the difference requires running the autoxidation to much higher conversion.



Scheme 2.4 Possible Mechanisms for the Formation of Hexadeca-2-enoic Acid and *n*-Pentadecanoic Acid in the Autoxidation of 1-Hexadecene

The corresponding hydroperoxide analyses of the inhibited autoxidation samples using the fluorogenic phosphine **2.1** are shown in Figure 2.5D–F. Interestingly, the heterocyclic diarylamine **2.4** appears to be more effective than the diphenylamine standard **2.3** at inhibiting hydroperoxide formation in the *n*-hexadecane autoxidation relative to the base oil autoxidation, even though the profiles are similar when one considers acid formation only. Since the base oil autoxidizes so rapidly, it is more difficult for the antioxidants to compete with propagation (at this antioxidant loading). However, since acid formation is relatively slower (*cf.* Figure 2.4), the differences in the antioxidant efficacy are better resolved.

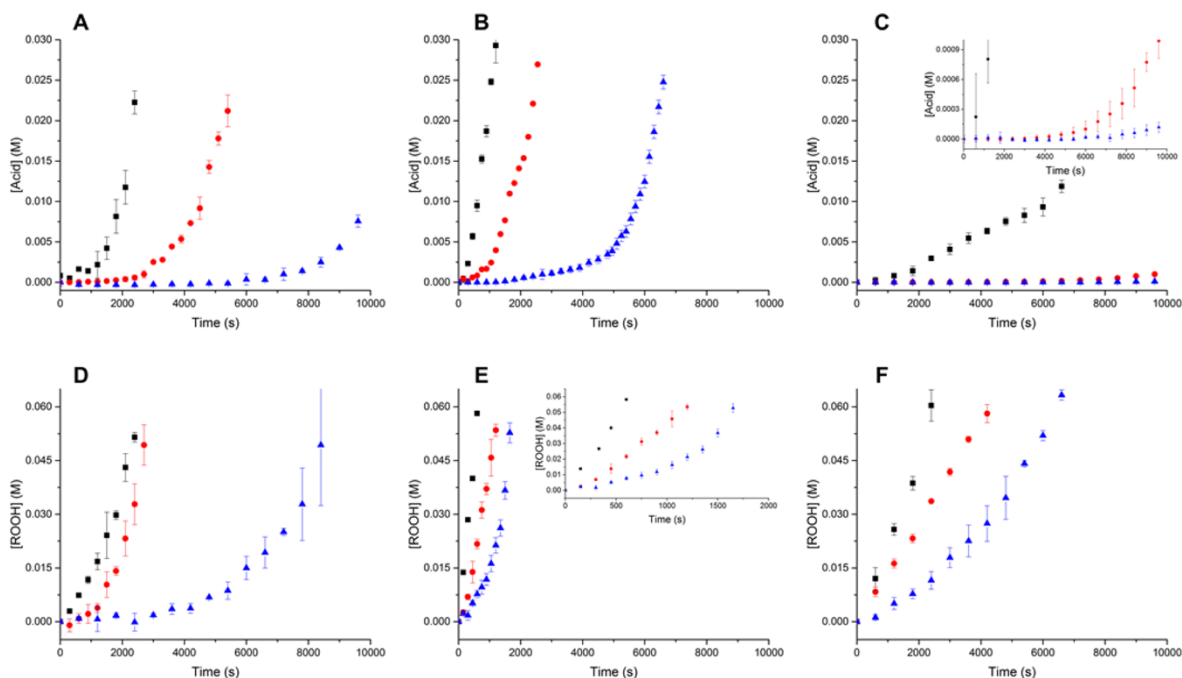


Figure 2.5 Formation of carboxylic acids (A–C) and hydroperoxides (D–F) in the autoxidation of either *n*-hexadecane (A,D) or a API Group III base oil (B,E) at 160°C or 1-hexadecene (C,F) at 120°C (■) and corresponding data for autoxidations inhibited by either **2.3** (red ●) or **2.4** (blue ▲) at 40 μM in *n*-hexadecane and 1-hexadecene and 100 μM in base oil. Autoxidations of *n*-hexadecane and base oil were initiated with tetralin hydroperoxide at 10 and 5 mM, respectively, while the 1-hexadecene autoxidations were initiated with 10 mM dicumyl peroxide. Autoxidations were carried out in the presence of 2,4,6-tri-*tert*-butylpyridine (1 mM) in order to prevent deactivation of **2.4** by acid formed during the autoxidation (see ref 9).

In the foregoing experiments, hydroperoxides and acids were determined in parallel. That is, duplicate samples were withdrawn from the hydrocarbon autoxidation, added to separate wells of a microplate and solutions of either **2.1** or **2.2** were added to one or the other and the fluorescence recorded. For operational expediency, and to limit the amount of material that must be used for analysis, it would be desirable to carry out both measurements on a single sample. However, since the fluorophores in **2.1** and **2.2** have practically identical properties, this is not possible. Hence, we prepared the BODIPY-substituted trialkylamine **2.5** (Figure 4.6) and examined its potential as an alternative to **2.2**.^{13,37} Although the fluorescence enhancement observed upon protonation was less than that for **2.2** (i.e., $\Phi_{5-H^+}/\Phi_5 = 8$ vs Φ_2 -

$H^+/\Phi_2 = 38$), it could be used interchangeably (i.e., $s\text{p}K_a = 8.1 \pm 0.2$ for **2.5** vs 7.9 ± 0.1 for **2.2** in in 4:1 MeOH/*i*-PrOH) and yielded essentially identical results for the foregoing experiments (see Supporting Information for the data).

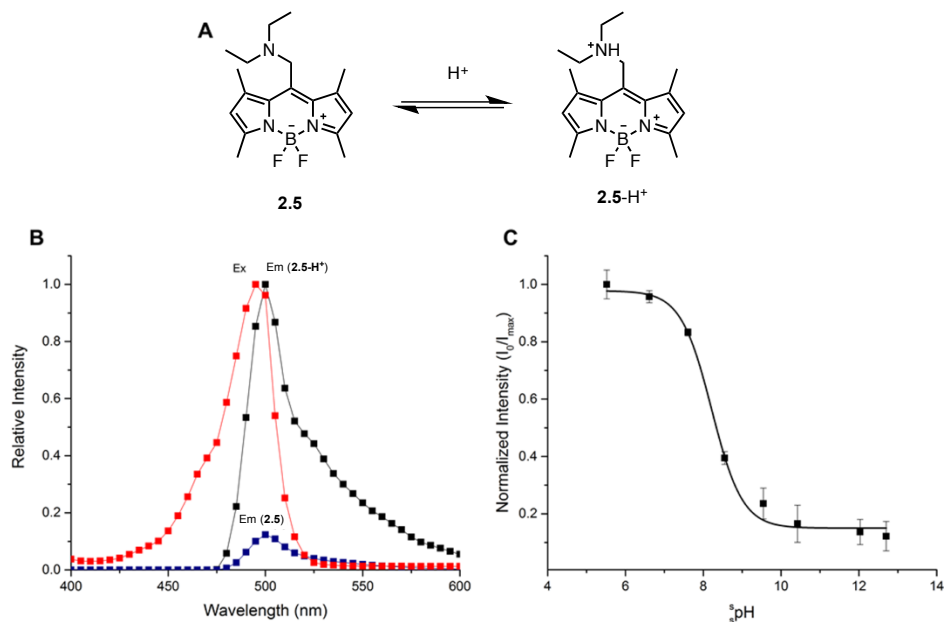


Figure 2.6 (A) The relevant equilibrium of the BODIPY–trialkylamine **2.5** and its conjugate acid **2.5-H⁺**. (B) Excitation and emission spectra of **2.5** and **2.5-H⁺**. (C) Fluorescence of **2.5** ($\lambda_{\text{ex}} = 495$; $\lambda_{\text{em}} = 500$) as a function of pH in 1:4 isopropanol/methanol.

To illustrate how these fluorogenic probes can be used to rapidly screen antioxidant efficacy, we carried out 24 inhibited autoxidations of base oil at 160 °C simultaneously in a commercial parallel reactor system that was adapted in-house to enable continuous oxygenation of all 24 reaction vessels. The reactions were supplemented with either of 12 different aromatic amines (**2.6**–**2.17**) or 12 different phenols (**2.18**–**2.29**). The aromatic amines were assessed at lower loadings than the phenols (250 μM vs 1 mM) since they are known to trap multiple radicals at elevated temperature due to a catalytic mechanism wherein the substrate can be used to regenerate the amine.^{8,38} An aliquot from each reaction vessel was taken 1 h post-initiation, and acid and hydroperoxide concentrations were determined using probes **2.1** and **2.5** simultaneously using a microplate reader. The entire experiment was completed in triplicate in a single day.

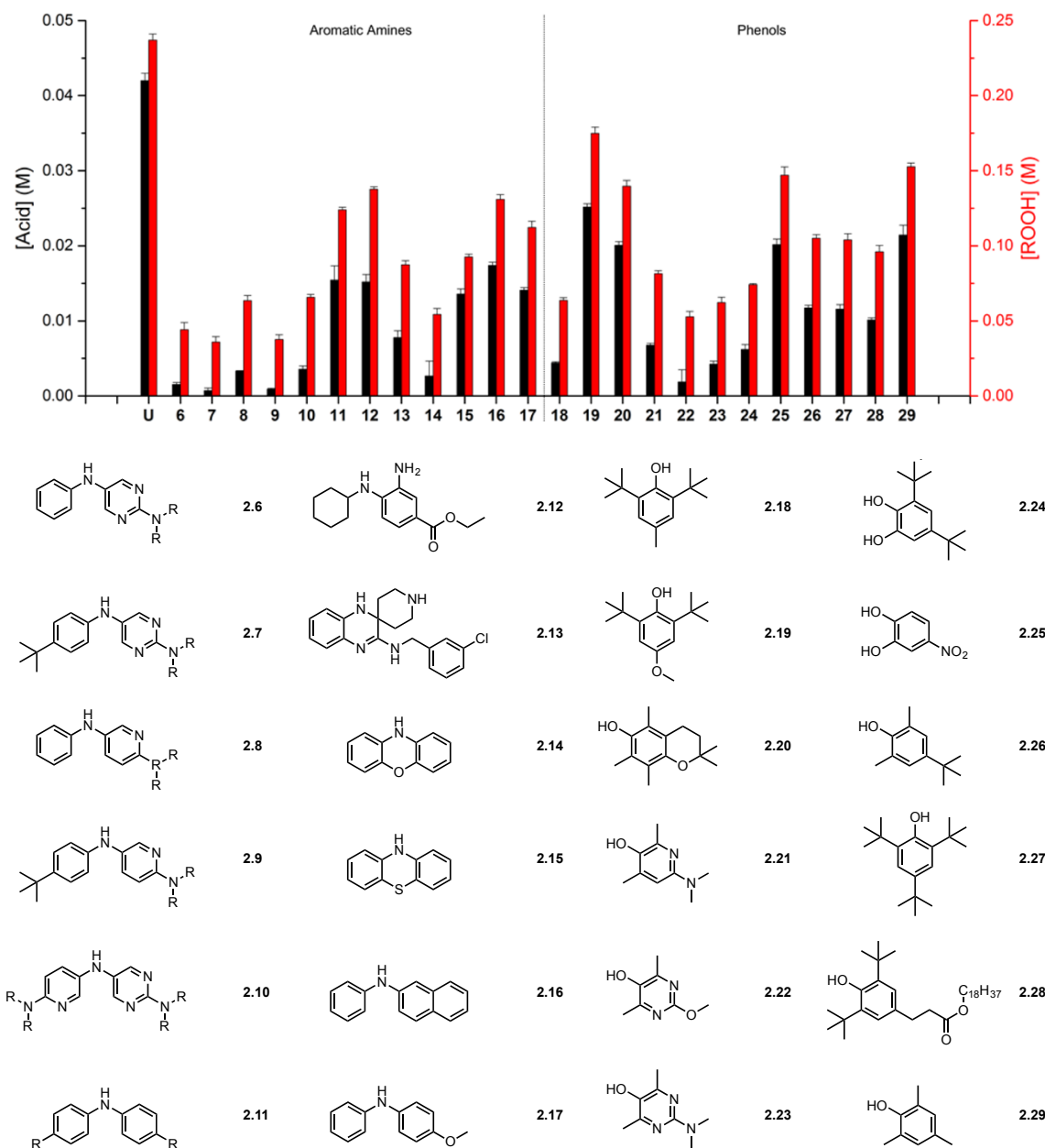


Figure 2.7 Formation of carboxylic acids (black) and hydroperoxides (red) upon autoxidation of a API Group III base oil for 1 h at 160 °C (U = uninhibited) and corresponding data for autoxidations inhibited by either 250 μ M of aromatic amines **2.6–2.17** or 1 mM of phenols **2.18–2.29**. Autoxidations were initiated with 5 mM tetralin hydroperoxide and carried out in the presence of 2,4,6-tri-tert-butylpyridine (1 mM).⁴¹

The data in Figure 2.7 indicate that, consistent with our previous (significantly lower throughput) studies,⁹ the heterocyclic diarylamines **2.6–2.10** all outperform the representative industrial standard dialkylated diphenylamine **2.11**. Similarly reactive to the heterocyclic diarylamines is phenoxazine (**2.14**), which was significantly better than its sulfur analog phenothiazine (**2.15**). Interestingly, liproxstatin-1 (**2.13**), one of the most potent inhibitors of cellular lipid peroxidation,³⁹ is also particularly effective at inhibiting hydrocarbon autoxidations at high temperatures, while ferrostatin-1 (**2.12**), a similarly potent antioxidant in cell culture,⁴⁰ is noticeably less effective.

On the other hand, of the phenolic antioxidants we investigated, only pyrimidinols **2.22** and **2.23** suppressed autoxidation to a larger extent compared with the representative industrial standard BHT, **2.18**, which was similar to the di-tert-butylated catechol **2.24**. Despite the exciting reactivity of pyrimidinols as radical-trapping antioxidants at ambient temperatures,^{42,43} they have yet to be studied at elevated temperatures. This result and those obtained for phenoxazine and liproxstatin-1 underscore the value of this rapid assessment of high temperature RTA activity for further, more detailed investigations of promising compounds or classes of compound. Given that these analyses are generally carried out in both industry and academic laboratories by laborious iodometric and acid–base titrations, respectively, we anticipate that this will be a widely adopted screening tool for the identification of useful compounds and unanticipated conceptually novel chemistry.

2.4 Experimental

2.4.1 General Experimental

Reagents were purchased from commercial suppliers and used without further purification, unless otherwise indicated. Column chromatography was carried out using flash silica gel (40–63 μm , 230–400 mesh). ^1H and ^{13}C NMR were recorded on a spectrometer operating at 400 and 100 MHz, respectively, unless specified otherwise. High-resolution mass spectra were obtained on a magnetic sector electron impact mass spectrometer.

2.4.2 Synthetic Procedures

3-((Diethylamino)methyl)-7-methoxy-2H-chromen-2-one (2.2). To a solution of 3-(bromomethyl)-7-methoxy-2H-chromen-2-one⁴⁵ (0.53 g, 2.0 mmol), KI (0.033 g, 0.2 mmol), and K₂CO₃ (0.4 g, 3 mmol) in THF (20 mL), diethylamine (0.24 mL, 2.4 mmol) was added dropwise. The reaction was stirred overnight at room temperature until completion as determined by TLC. The mixture was added to a separatory funnel and extracted using Et₂O and water. The organic layer was dried over MgSO₄ and filtered, and the solvent was evaporated under vacuum. Column chromatography (25% Et₂O/ hexanes eluent) afforded pure product. Yield: 0.45 g, 86%. Brown oil. ¹H NMR (400 MHz; acetone-d₆): δ 7.90 (s, 1H), 7.59 (d, J = 8.5 Hz, 1H), 6.93–6.88 (m, 2H), 3.91 (s, 3H), 3.41 (d, J = 1.3 Hz, 2H), 2.58 (q, J=7.1Hz,4H), 1.05 (t, J=7.1Hz,6H). ¹³CNMR(100MHz; acetone-d₆): δ 163.0, 161.6, 155.6, 139.8, 129.6, 124.9, 114.0, 112.9, 101.0, 56.2, 52.6, 48.2, 12.6. HRMS: *m/z* calcd C₁₅H₁₉NO₃ 261.1365; found 261.1369.

7-Methoxy-3-methyl-2H-chromen-2-one. The precursor to 3-(bromomethyl)-7-methoxy-2H-chromen-2-one⁴⁵ used in the synthesis of **2.2** was prepared using a previously reported procedure with slight modifications.⁴⁴ Ethyl 2-(triphenylphosphoranylidene)propanoate (0.9 g, 6.0 mmol) and 2-hydroxy-4-methoxybenzaldehyde (2.1 g, 6.0 mmol) were stirred in a sealed tube and heated to 200 °C overnight. The reaction was cooled, and the crude product was recrystallized from chloroform and hexanes. Yield: 1.05 g, 92%. Brown needles. ¹H NMR (400 MHz; acetone-d₆): δ 7.69 (s, 1H), 7.49 (d, J = 8.5 Hz, 1H), 6.91–6.86 (m, 2H), 3.90 (s, 3H), 2.09 (s, 3H). NMR spectra were consistent with previously reported data.⁴⁴

10-((Diethylamino)methyl)-5,5-difluoro-1,3,7,9-tetramethyl-5H-dipyrrolo[1,2-c:2',1'-

ff][1,3,2]diazaborinin-4-ium-5-uide (2.5). To a solution of 10-(chloromethyl)-5,5-difluoro-1,3,7,9-tetramethyl-5H-4 λ⁴,5 λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinine¹³ (0.1 g, 0.3 mmol), KI (0.005 g, 0.03 mmol), and K₂CO₃ (0.062 g, 0.45 mmol) in THF (3 mL) diethylamine (0.037 mL, 0.36 mmol) was added dropwise. The reaction was stirred overnight at room temperature until completion as determined by TLC. The mixture was added to a separatory funnel and extracted using Et₂O and water. The organic

layer was dried over MgSO_4 and filtered, and the solvent was evaporated under vacuum. Column chromatography (25% Et_2O / hexanes eluent) afforded pure product. Yield: 0.042 g, 42%. Orange solid. ^1H NMR (400 MHz; acetone- d_6): δ 6.45 (s, 1H), 6.17 (s, 1H), 3.83 (s, 2H), 2.66 (s, 3H), 2.58 (q, $J = 7.1$ Hz, 4H), 2.48 (s, 3H), 2.45 (s, 6H), 1.04 (t, $J = 7.1$ Hz, 6H). ^{13}C NMR (76 MHz; acetone- d_6): δ 158.5, 154.0, 143.8, 142.4, 142.1, 132.7, 122.0, 120.8, 51.8, 48.7, 17.5, 17.3, 16.7, 14.4, 12.6. HRMS: m/z calcd $\text{C}_{18}\text{H}_{26}\text{BF}_2\text{N}_3$ 333.2188; found 333.2189.

2.4.3 General Procedure for Hydrocarbon Autoxidations

n-Hexadecane, 1-hexadecene, or the base oil (100 mL) was thoroughly degassed with argon and then heated to 160 °C in a stirred flow reactor. Once the temperature stabilized, inhibitor **2.3** or **2.4**, base (2,4,6-tri-*tert*-butylpyridine, 1 mM final concentration), and an appropriate amount of initiator (see text) were added to the solution, and the flow of argon was replaced with O_2 . Aliquots (0.5 mL) were withdrawn every 5 min and allowed to cool to room temperature for analysis.

2.4.4 General Procedure for Determining Hydroperoxide Concentration

Four replicates (30 μL) of each sample were loaded into separate wells of a 96-well microplate, and the automated reagent dispenser of the microplate reader was used to dilute each sample with *tert*-amyl alcohol (200 μL) and a solution of a fluorogenic phosphine dye **2.1** (20 μL of a 250 μM stock solution in acetonitrile) immediately before reading. The plate was stirred for 8 s and allowed to rest for 2 s, and the fluorescence of each well was measured every second for 60 s (excitation wavelength = 340 nm; emission wavelength = 425 nm). The concentration of hydroperoxide was determined from the rate of phosphine oxidation using the rate constant for the reaction of the dye with secondary hydroperoxides in *tert*-amyl alcohol ($k = 1.2 \text{ M}^{-1} \text{ s}^{-1}$) assuming pseudo-first-order kinetics.

2.4.5 General Procedure for Determining Acid Concentration

Four replicates (1.2 μL) of each sample were loaded into separate wells of a 96-well microplate, the automated reagent dispenser of the microplate reader was used to dilute each sample with 20% isopropyl

alcohol in methanol (280 μL), and the plate was stirred for 15 s. Afterward, a solution of fluorogenic amine **2** (20 μL of a 630 μM stock in methanol) was added. The plate was stirred for 8 s, the fluorescence of each well was measured every second for 10 s (excitation wavelength = 315 nm; emission wavelength = 395 nm), and the acid concentration was determined from the average fluorescence reading ($\text{RFU} = 1771[\text{Acid}] + 14065$).

2.4.6 Procedure for Simultaneous Determination of Hydroperoxide and Acid Concentration

Four replicates (5 μL) of each sample were loaded into separate wells of a 96-well microplate, the automated reagent dispenser of the microplate reader was used to dilute each sample with 20% isopropyl alcohol in methanol (280 μL), and the plate was stirred for 15 s. Afterward, a solution containing fluorogenic phosphine dye **2.1** and fluorogenic amine **2.5** (20 μL of a solution containing 250 μM of probe **1** and 630 μM of probe **5** in acetonitrile) was added. The plate was stirred for 8 s, and the fluorescence of each well was measured every 5 s for 60 s (excitation wavelength = 340 nm, emission wavelength = 425 nm for probe **2.1** and excitation wavelength = 495 nm, emission wavelength = 500 nm for probe **2.5**). The acid concentration was determined from the average fluorescence reading over 10 s ($\text{RFU} = 352[\text{Acid}] + 34925$). The concentration of hydroperoxide was determined from the rate of phosphine oxidation using the rate constant for the reaction of the dye with secondary hydroperoxides in 20% isopropyl alcohol in methanol ($k = 5.1 \text{ M}^{-1} \text{ s}^{-1}$) assuming pseudo-first-order kinetics.

2.4.7 Screening of High Temperature Antioxidant Activity

Base oil (3 mL) was added to test tubes in a 24-place parallel synthesizer, thoroughly degassed with argon, and then heated to 160 $^{\circ}\text{C}$. Once the temperature stabilized, an appropriate amount of inhibitor **2.6–2.29** (see text), base (2,4,6-tri-*tert*-butylpyridine, 1 mM final concentration), and 5 mM tetralin hydroperoxide were added to the test tubes, and the flow of argon was replaced with O_2 . An aliquot (0.1 mL) was withdrawn 1-h post-initiation and allowed to cool to room temperature for analysis.

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2.6 Supporting Information

2.6.1 Reactivity of Probe 2.2 with Palmitic Acid and Tetralin Hydroperoxide

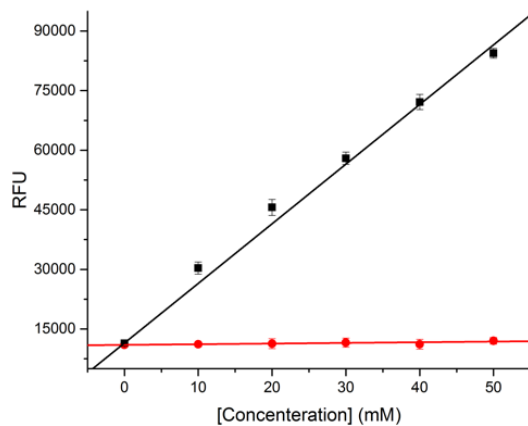


Figure S2.1 Increase in fluorescence of probe **2.2** as a function of an increasing concentration of either palmitic acid (■) or tetralin hydroperoxide (●).

2.6.2 Reactivity of Probe 2.1 with Palmitic Acid and Tetralin Hydroperoxide

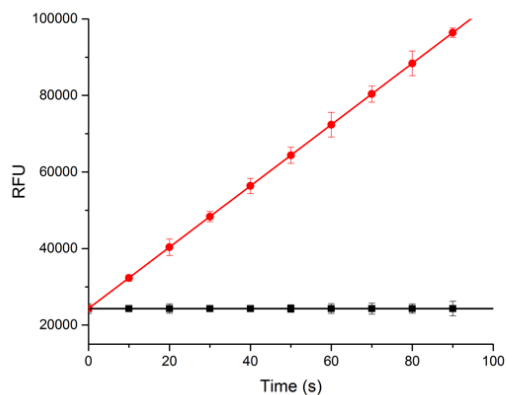


Figure S2.2 Increase in fluorescence of probe **2.1** as a function of time in the presence of either 20mM palmitic acid (■) or 20mM tetralin hydroperoxide (●).

2.6.3 Hydroperoxide and Acid Concentration using Probes 2.1 and 2.5 Simultaneously

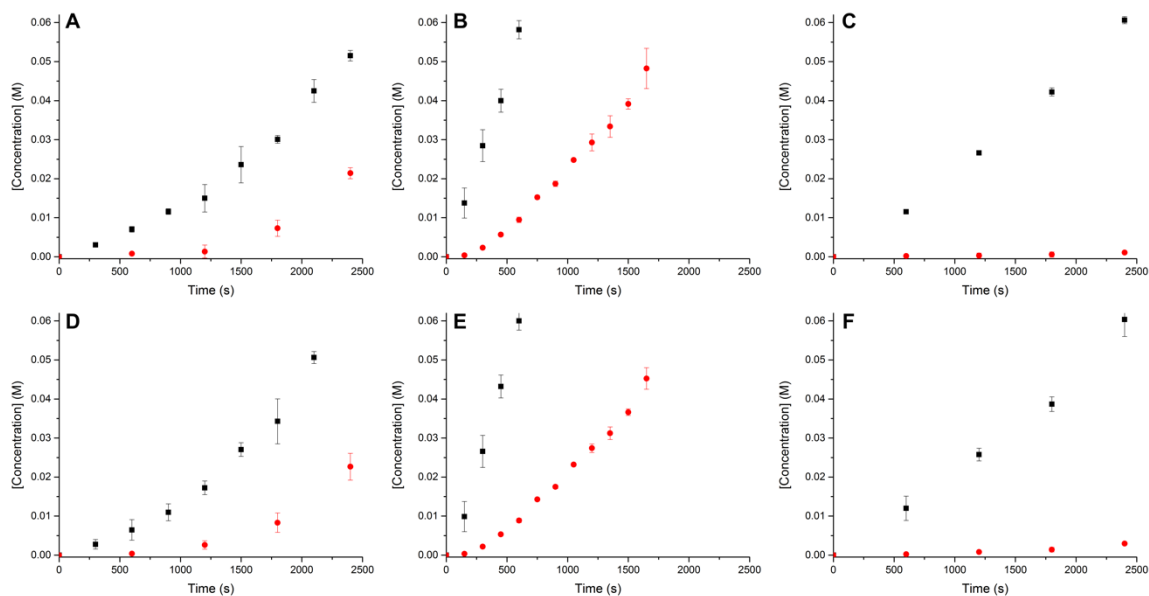


Figure S2.3 (A-C) Formation of hydroperoxides (■) and acids (●) in the initial stages (<2% conversion) of the autoxidation of *n*-hexadecane, a API Group III base oil and 1-hexadecene respectively using probes 2.1 and 2.2 separately. (D-E) Formation of hydroperoxides (■) and acids (●) in the initial stages (<2% conversion) of the autoxidation of *n*-hexadecane, a API Group III base oil and 1-hexadecene respectively using probes 2.1 and 2.5 simultaneously

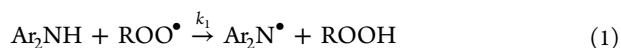
Chapter 3: Unprecedented Inhibition of Hydrocarbon Autoxidation by Diarylamine Radical-Trapping Antioxidants

3.1 Preface

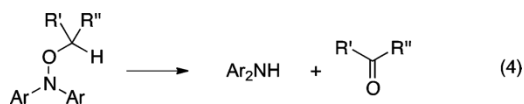
The reactivities of novel heterocyclic diarylamine radical-trapping antioxidants (RTAs) are profiled in a heavy hydrocarbon at 160 °C, conditions representative of those at which diphenylamine RTAs are used industrially. While carboxylic acids produced during the autoxidation are shown to deactivate these more basic RTAs, the addition of a sacrificial base leads to efficacies that are unprecedented in the decades of academic and industrial research in this area. The work was performed with assistance from fellow graduate student, Evan Haidasz, who assisted with a portion of the hexadecane autoxidations. Reprinted (adapted) with permission from: Shah, R.; Haidasz, E. A.; Valgimigli, L.; Pratt, D. A. *J. Am. Chem. Soc.* **2015**, *137*, 2440. Copyright 2015 American Chemical Society.

3.2 Results and Discussion

Diarylamine (Ar_2NH) radical-trapping antioxidants (RTAs) are key additives to many types of petroleum derived products because of their high inherent reactivities toward peroxy radicals (eq. 1) and the persistence of the resultant radicals toward both O_2 and nonradical species.^{1,2} Instead, the diarylaminy radical reacts with another peroxy radical to yield a nitroxide (eq. 2), which can either react with a peroxy radical on one of the aryl rings or combine with an alkyl radical to give an alkoxyamine (eq. 3). Either way, a single Ar_2NH molecule inhibits the propagation of two autoxidative chain reactions and gives rise to a so-called stoichiometric factor of 2.



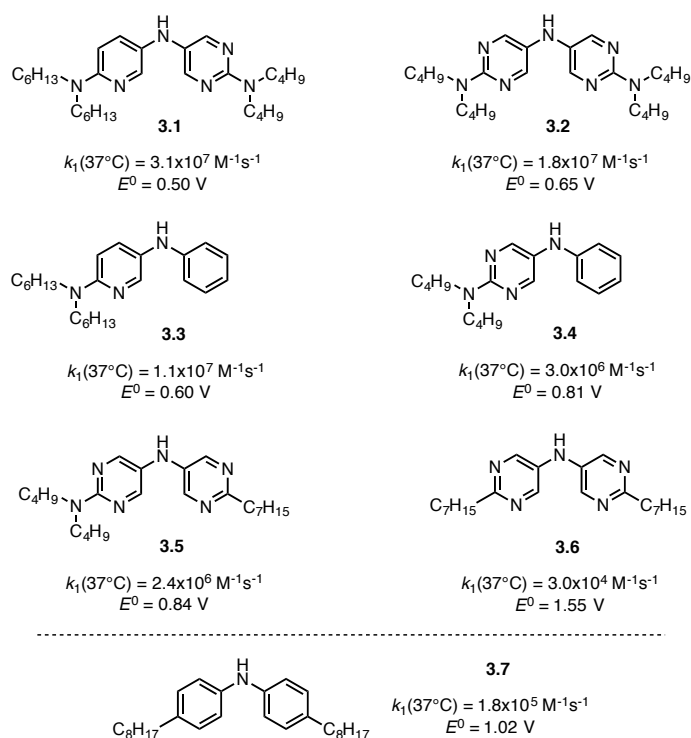
At elevated temperatures, such as those to which engine lubricants are exposed and at which polymers are processed, much greater stoichiometric factors have been reported. For example, a remarkable stoichiometric factor of 52 was determined for a 4,4'-dialkyldiphenylamine in paraffin oil at 130 °C.⁴ The apparent catalytic antioxidant behavior of diphenylamines results from regeneration of the diphenylamine from the N,N-diarylalkoxyamine (eq. 4), as first suggested by Korcek⁵ and recently investigated by our group.⁶ This transformation, which occurs either by N–O dissociation and in-cage disproportionation or a retro-carbonyl-ene reaction, is believed to underlie the unique efficacy of diphenylamine RTAs in high-temperature applications.



Over the years we have shown that the incorporation of nitrogen atoms into the aromatic rings of phenolic⁷⁻¹¹ and diphenylamine¹²⁻¹⁴ RTAs enables the design of compounds with outstanding reactivities toward peroxy radicals (i.e., $\Delta H_1^\ddagger \approx 0$). Herein we report preliminary results of the reactivity of these heterocyclic diarylamine RTAs at 160 °C, a temperature more representative of their potential “real world” applications and at which the reaction in eq. 4 may be expected to occur. The compounds in Chart 3.1 were selected for study on the basis of their reactivity toward peroxy radicals (high k_1 , i.e., **3.1–3.3**), stability toward oxidation (high E^0 , i.e., **3.6**), or a balance of these two characteristics (i.e., **3.4** and **3.5**). In fact, they are similar to the compounds we initially reported,¹²⁻¹⁴ but are substituted with longer alkyl chains in order to ensure their solubility in heavy hydrocarbons and low volatility at elevated temperatures (see the Supporting Information for details of their preparation). Diarylamine **3.7** is representative of industry-standard alkylated diphenylamines, therefore providing a basis for comparison.

To evaluate the high-temperature RTA activities of **3.1–3.7**, autoxidations of *n*-hexadecane were carried out at 160 °C in a stirred-flow reactor, where a constant positive pressure of O₂ was used to stir the medium and prevent mass transfer from becoming rate-limiting, which is otherwise a problem at this temperature.^{3,15} Tetralin hydroperoxide (10 mM) was used as a radical initiator since inhibited

autoxidations in the absence of an initiator were very slow (see Supporting Information). At regular intervals, aliquots of the reaction mixture were removed and cooled, and the concentration of hydroperoxide products were determined by reaction with a fluorogenic phosphine developed in our laboratory.¹⁶ The autoxidations were followed for the first 2% of the reaction (based on hydroperoxide formation), since beyond ca. 3% conversion the hydroperoxide yield begins to decrease and highly complex mixtures of ketones, alcohols, carboxylic acids, and esters arise.¹⁵ The results are shown in Figure 3.1.



Scheme 3.1 Some Diarylamines and Their Relevant Properties.²⁷

In the absence of an RTA additive, the rate of hydroperoxide formation increases with time because of the autocatalytic nature of the reaction: the product hydroperoxides undergo O–O cleavage, leading to radicals that initiate new chain reactions.¹⁵ The industry standard **3.7** inhibits the autoxidation for ca. 900 s, after which a profile roughly identical to that for the uninhibited autoxidation is observed, while the heterocyclic diarylamines **3.1–3.6** show varied behavior. The data obtained with the most

reactive compounds (**3.1**, **3.2**, and **3.3**) are shown in the top panel of Figure 3.1. Of them, only the bis(pyrimidine) **3.2** inhibits the autoxidation more effectively than **3.7**, and the difference seems inconsistent with the 100-fold difference in k_1 determined at 37 °C (cf. Chart 3.1). Although the pyridine-containing diarylamines **3.1** and **3.3** display poor inhibitory activity in the early going, they appear to slow the rate of autoxidation with time, suggesting that one or more of their oxidation products are inhibitors. The data obtained with the less reactive compounds (**3.4**, **3.5**, and **3.6**) are shown in the bottom panel of Figure 3.1. Compound **3.4** has a similar effect on the autoxidation as bis(pyrimidine) **3.2**, both of which exhibit superior activity compared with the industry standard **3.7**; in contrast, bis(pyrimidine) **3.5** starts off well but appears to be entirely consumed in the first 1500 s, and bis(pyrimidine) **3.6** is ineffective, consistent with its lower k_1 .

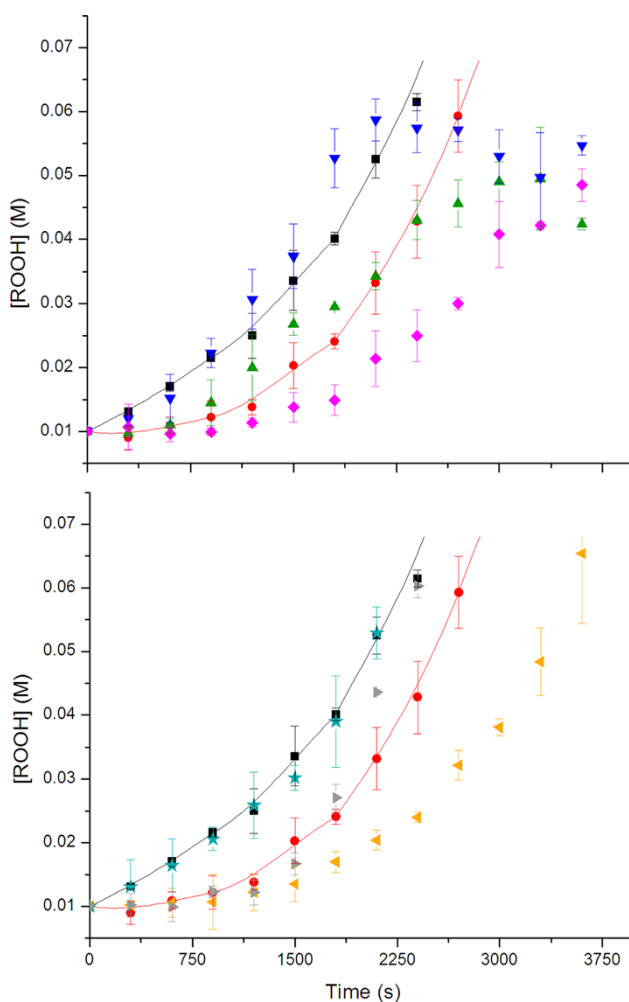
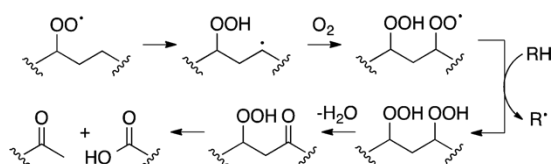


Figure 3.1 Hydroperoxide formation in the autoxidation of *n*-hexadecane at 160 °C initiated by 10 mM tetralin hydroperoxide in the absence of an RTA (black ■) or in the presence of 40 μM **3.1** (blue ▼), **3.2** (magenta ◆), **3.3** (green ▲), **3.4** (orange ◀), **3.5** (gray ►), **3.6** (aqua ★), or **3.7** (red ●).

Beyond the results obtained with bis(pyrimidine) **3.6**, the data in Figure 3.1 are impossible to reconcile in light of the values of k_1 given in Chart 3.1. Since the reactions have very small activation enthalpies, raising the temperature has only a small effect on the magnitude of k_1 (e.g., in the case of **3.7**, for which $\log A = 6.9$ and $E_a = 2.5$ kcal/mol,¹⁴ $k_1 = 4.4 \times 10^5$ M⁻¹ s⁻¹ at 160 °C, which is only 2.4-fold higher than at 37 °C). On this basis it was expected that the significant differences in the reactivities of **3.1–3.6** and **3.7** found at ambient temperature (37 °C) should translate to elevated temperatures (160 °C). Moreover, the rate of regeneration of the diarylamine from the corresponding alkoxyamine (i.e., eq. 4) should increase with the stability of the diarylaminy radicals derived from **3.1–3.5** (e.g., N–H bond dissociation energy (BDE) = 79.2 kcal/mol for **3.2** compared with that derived from **3.7** (N–H BDE = 82.2 kcal/mol)).¹⁴

Given the foregoing, we wondered whether the heterocyclic diarylamines, while stable toward O₂ at ambient temperatures, were not so at elevated temperatures. In fact, when each of **3.1–3.6** was heated to 160 °C in *n*-hexadecane under O₂ in the absence of an initiator, the most oxidizable compounds (**3.1**, **3.2**, and **3.3**; $E^0 = 0.5$ – 0.65 V) decomposed steadily over 1 h ($t_{1/2} = 19$, 24, and 22 min, respectively) while compounds **3.4**, **3.5**, and **3.6** ($E^0 > 0.8$ V) did not decompose to any significant extent, similarly to **3.7** (see the Supporting Information for the data). Therefore, the poor reactivity of the pyridine-containing compounds as RTAs at 160 °C can be readily explained by the fact that they autoxidize under the reaction conditions. However, since the pyrimidine-containing compounds are stable toward O₂, something else must account for their poorer-than-expected RTA activities.



Scheme 3.2 Proposed Mechanism of Acid Formation in the Early Stages of Hydrocarbon Autoxidations

We next considered whether the heterocyclic diarylamines were sufficiently basic to interact with carboxylic acids that are formed in the autoxidation. Hydrocarbon autoxidation is known to produce carboxylic acids from the very early stages of the reaction in addition to hydroperoxides. Korcek and co-workers suggested that they arise primarily from fragmentation of γ -hydroperoxy ketones by a concerted mechanism recently investigated by Green, Truhlar, and co-workers (cf. Scheme 3.1).¹⁷ In fact, when acid formation was monitored over the initial period (the first 2400 s) of an autoxidation of *n*-hexadecane directly by electrospray ionization mass spectrometry, the total acid concentration was already roughly 25% of the hydroperoxide concentration (~ 15 mM vs ~ 60 mM; see the Supporting Information). Protonation of the diarylamines by these acids would lead to much poorer RTAs since the diarylaminy radicals are already inherently electron-poor.^{18,19} For example, the N-H BDE of **3.4** (calculated using the reliable CBS-QB3 method)²⁰ increases from 81.8 to 86.0 kcal/mol upon protonation of the ring.²¹

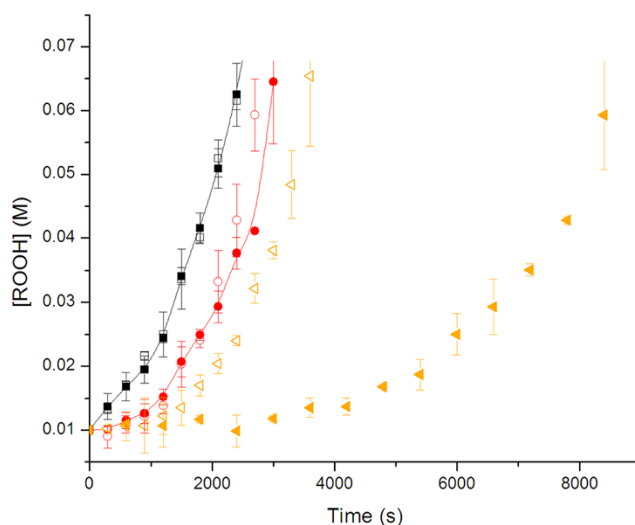


Figure 3.2 Hydroperoxide formation in the autoxidation of *n*-hexadecane at 160 °C initiated by 10 mM tetralin hydroperoxide in the absence of RTA (black ■) or inhibited by 40 μ M **3.4** (gold ◄) or **3.7** (red ●) in the presence of TTBP (1 mM). Open symbols correspond to the data in Figure 3.1 (i.e., in the absence of TTBP).

To assess whether acid suppresses the reactivity of the heterocyclic diarylamines, palmitic acid, a non-volatile saturated carboxylic acid, was added to an autoxidation inhibited by **3.4**. In the event, as little as 1 mM acid was found to disrupt the inhibitory activity of **3.4**, while that of the expectedly less basic

diphenylamine **3.7** was unchanged, as was the course of the uninhibited oxidation (see the Supporting Information for the data). In light of these results, a base was added to an autoxidation inhibited by **3.4** in an attempt to “rescue” its RTA activity. Gratifyingly, in the presence of the non-volatile non-nucleophilic base 2,4,6-tri-*tert*-butylpyridine (TTBP) (1 mM), an unprecedented inhibited period was observed (Figure 3.2). Importantly, the addition of base had no effect on the activity of diphenylamine **3.7** (or the uninhibited rate of oxidation), suggesting that the acid produced early in the autoxidation was sufficient to deactivate heterocyclic diarylamine **3.4**, but not diphenylamine **3.7**. The activity of **3.4** could also be rescued using cesium carbonate or the *tert*-alkylated primary amine Primene 81-R, underscoring that the inactivation of **3.4** is due to protonation (see the Supporting Information for the data).²²

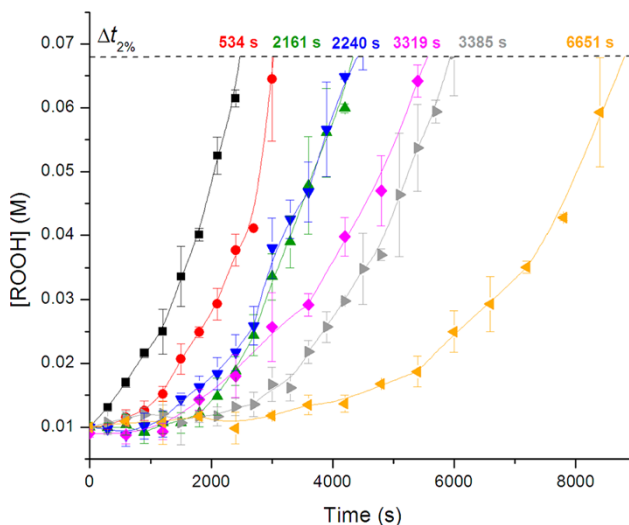


Figure 3.3 Hydroperoxide formation in the autoxidation of *n*-hexadecane at 160 °C initiated by 10 mM tetralin hydroperoxide in the absence of RTA (black ■) or inhibited by 40 μM **3.1** (blue ▼), **3.2** (magenta ◆), **3.3** (green ▲), **3.4** (gold ◀), **3.5** (gray ▶), or **3.7** (red ●) in the presence of TTBP (1 mM). The delay in the time required to reach [ROOH] = 68 mM (=2%) in the presence of each of **3.1**–**3.5** or **3.7** is given as $\Delta t_{2\%}$.

Consistent with the foregoing, the pK_a of the conjugate acid of **3.4** was determined to be almost 4 units greater than that of the conjugate acid of **3.7** (8.2 ± 0.2 vs 4.6 ± 0.3 in 4:1 $\text{CH}_3\text{CN}/\text{H}_2\text{O}$; see the Supporting Information for the data). In fact, each of the reactive heterocyclic diarylamines (**3.1**–**3.5**) were found to be significantly more basic than the diphenylamine, with pK_a 's of the corresponding

conjugate acids of 9.8 ± 0.1 (**3.1**), 8.8 ± 0.1 (**3.2**), 8.4 ± 0.1 (**3.3**), and 8.3 ± 0.1 (**3.5**).²³ Accordingly then, autoxidations were much better inhibited by these compounds in the presence of added base (Figure 3.3).²⁴ Interestingly, even the pyridine-containing compounds **3.1** and **3.3**, which were found to be relatively unstable toward O₂ at 160 °C, were superior to **3.7**. This is presumably because the balance of either **3.1** or **3.3** remains over the course of the autoxidation is highly potent. The pyrimidine-containing compounds **3.2**, **3.4**, and **3.5** are much better overall, presumably because of a compromise between stability and reactivity, which is best demonstrated by **3.4**.

The unprecedented inhibited periods observed for the heterocyclic diarylamines in the presence of base must result from increased stoichiometric factors for the reactions of these amines with peroxy radicals,²⁵ arising from one or both of the following: faster/more efficient regeneration of the diarylamine from its corresponding alkoxyamine and/or fewer deleterious off-cycle reactions. We recently investigated the mechanism of diphenylamine regeneration,⁶ which revealed that either a retro-carbonylene reaction or N–O dissociation/disproportionation pathway operates depending on the structure of the diarylamine and/or substrate. Those studies were enabled by the synthesis of authentic alkoxyamines derived from diphenylamine and 4,4'-di-tert-butyl diphenylamine. Unfortunately, attempts to prepare authentic alkoxyamines derived from **3.1–3.5** have not been successful, precluding determination of the kinetics of diarylamine regeneration for these compared with **3.7**. However, CBS-QB3 computations²⁰ of the N–O BDEs in model (O–CH₃) alkoxyamines derived from **3.4** and **3.7** suggest that the regeneration of diarylamine **3.4** requires 2.3 kcal/mol less energy than the regeneration of **3.7**, which corresponds to a 14-fold increase in rate at 160 °C – in good agreement with the observed 12.5-fold difference in $\Delta t_{2\%}$ (moreover, in the presence of **3.4**, the maximum oxidation rate has still not been reached at 2% conversion; cf. Figure 3.3).²⁶

The foregoing results provide a clear path forward for the development of more effective additives for preserving petroleum-derived products than those that are currently in use. Although we have demonstrated that carboxylic acids that are produced in the autoxidation of hydrocarbons can mask

the greater efficacy of the heterocyclic diarylamines, this can be easily unmasked by addition of base to the autoxidizing hydrocarbon. In practice, bases are universally added to petroleum-derived products that come into contact with metal surfaces in order to minimize corrosion. Of the wide variety of applications wherein the compounds described above may have a significant impact, the most tangible would be increasing maintenance intervals on automobile and jet engines and enabling the development of more efficient combustion engines, which necessarily run at higher temperatures and require lubricants containing more effective RTAs.

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- (21) Protonation of the ring would also be expected to slow the regeneration of the amine from the corresponding alkoxyamine (i.e., eq 4) via N–O bond homolysis/disproportionation, since the same diarylaminyll must be formed, or via the retro-carbonyl–ene reaction, since the highest-occupied molecular orbital of the aryl ring will decrease in energy (*cf.* ref 6). In fact, the O–C BDE in a O-methyl alkoxyamine derived from **3.4** calculated using CBS–QB3 increases from 39.0 to 43.2 kcal/mol upon protonation of the ring.
- (22) Addition of more base did not further extend the inhibited period.
- (23) For reference, the pK_a of diphenylamine in water is 0.79. See: Pankratov, A. N.; Shchavlev, A. E. *J. Anal. Chem.* **2001**, *56*, 123.
- (24) Addition of base did not improve the activity of bis(2-alkylpyrimidine)amine **2.6**, consistent with the fact that its conjugate acid ($pK_a = 4.2 \pm 0.1$) is stronger than the conjugate acid of **3.7** ($pK_a = 4.6 \pm 0.3$).
- (25) Unfortunately, it is not possible to accurately determine the stoichiometry of the reaction of the diarylamines with peroxy radicals at this temperature since the autocatalytic nature of the reaction

means that R_i changes as the reaction proceeds. As a result, we have “quantified” the efficacy of the inhibition of the heterocyclic diarylamines relative to the industry standard **3.7** simply by comparing the times required for 2% of the substrate to be autoxidized ($\Delta t_{2\%}$).

- (26) We acknowledge that this may be entirely fortuitous, as it assumes that the off-cycle reactions of the intermediate diarylaminyll and diarylnitroxide radicals are essentially the same for **3.4** and **3.7**, which may not be the case.
- (27) The data shown are from ref. 14 and were obtained with the analogous compounds with shorter alkyl side chains, except for the data for **3.5**, which are reported for the first time here (see the Supporting Information).
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3.4 Supporting Information

3.4.1 General Experimental

Reagents were purchased from commercial suppliers and used without further purification, unless otherwise indicated. The diarylamines **3.1-3.4** were synthesized according to our previously published report¹³ with slight modifications. Diarylamine **3.5** was prepared described as described below and **3.6** was prepared exactly as previously described.¹³ Column chromatography was carried out using flash silica gel (40-63 μm , 230-400 mesh). ^1H and ^{13}C NMR were recorded on a Bruker AVANCE spectrometer at 400 MHz and 100 MHz respectively, unless specified otherwise. High-resolution mass spectra were obtained on a Kratos Concept Tandem mass spectrometer.

3.4.2 Synthetic Procedures

3.4.2.1 General method for the preparation of 5-bromo-N,N-dialkylaminopyri(mi)dines.

To a solution of 5-bromo-2-aminopyri(mi)dine (1.0 mmol) in dry THF (2 mL) at 50°C, NaH (2.2 mmol) was added slowly and the mixture was stirred until H_2 evolution ceased. Alkylbromide (2.1 mmol) was then added and the reaction was refluxed overnight. The reaction was cooled, quenched with MeOH and

extracted with Et₂O. The combined organics were washed with brine and dried over MgSO₄. The oil obtained was passed through a plug of silica (Et₂O/hexanes) to obtain pure products.

5-Bromo-*N,N*-dihexylpyridin-2-amine (3.8). Yield: quantitative. Colourless Oil. ¹H NMR (400 MHz; CDCl₃): δ 7.28 (s, 1H), 6.52 (d, *J* = 9.1 Hz, 2H), 3.24 (t, *J* = 7.7 Hz, 4H), 1.57 (t, *J* = 6.1 Hz, 4H), 1.35 (m, 12H), 0.93 (t, *J* = 6.7 Hz, 6H). ¹³C NMR (100 MHz; CDCl₃): δ 14.18, 22.83, 26.95, 27.19, 31.86, 51.27, 106.86, 113.42, 114.29, 131.88, 147.22, HRMS: *m/z* Calc: C₁₇H₂₉BrN₂: 340.1514 Found: 340.1515.

5-Bromo-*N,N*-dibutylpyrimidin-2-amine (3.9). Yield: 92%. Colourless Oil. ¹H NMR (400 MHz; CDCl₃): δ 8.20 (s, 2H), 3.46 (t, *J* = 7.5 Hz, 4H), 1.57-1.49 (m, 4H), 1.30 (dt, *J* = 14.6, 7.3 Hz, 5H), 0.90 (dd, *J* = 7.2, 6.5 Hz, 7H). ¹³C NMR (100 MHz; CDCl₃): δ 159.7, 157.6, 104.6, 77.4, 77.1, 76.7, 47.8, 29.8, 20.2, 14.0, HRMS: *m/z* Calc: C₁₂H₂₀BrN₃: 285.0840 Found: 285.0841.

5-Bromo-*N,N*-dihexylpyrimidin-2-amine (3.10). Yield: 92%. Colourless Oil. ¹H NMR (400 MHz; CDCl₃): δ 8.24 (s, 2H), 3.48 (t, *J* = 7.6 Hz, 4H), 1.57 (s, 5H), 1.30 (t, *J* = 3.3 Hz, 12H), 0.89 (d, *J* = 2.5 Hz, 6H). ¹³C NMR (100 MHz; CDCl₃): δ 159.8, 157.7, 104.7, 48.2, 31.8, 27.6, 26.8, 22.8, 14.2, HRMS: *m/z* Calc: C₁₆H₂₈BrN₃: 340.1466 Found: 340.1466.

5-Bromo-2-hexylpyrimidine (3.11). *n*-Hexylmagnesium bromide was prepared prior to use. Mg (1.0 mmol) in dry THF was activated 4 times with I₂. *n*-Hexyl bromide (1.0 mmol) in dry THF (10 mL) was added slowly to maintain the solution near reflux. After addition was complete the reaction was heated to 60 °C for 1 hour. ZnCl₂ (1.25 mmol) solution was added dropwise to Hexylmagnesiumbromide (1.0 mmol) in THF (12.5 mL) and stirred for 10 minutes. 5-Bromo-2-iodopyrimidine (0.5 mmol) and catalytic Pd(PPh₃)₄ was added and stirred overnight. The reaction quenched with water, and extracted with Et₂O. The combined organics were washed with brine, and dried over MgSO₄. Column chromatography (10% Et₂O/hexanes eluent) afforded pure products. Yield: 61%. White Powder. ¹H NMR (400 MHz; CDCl₃): δ 8.69 (s, 2H), 2.90 (t, *J* = 7.7 Hz, 2H), 1.78 (dt, *J* = 14.7, 7.5 Hz, 2H), 1.31 (dd, *J* = 5.9, 3.6 Hz, 7H), 0.87

(t, $J = 7.0$ Hz, 3H). ^{13}C NMR (100 MHz; CDCl_3): δ 170.0, 157.7, 117.7, 39.0, 31.7, 29.1, 28.7, 22.7, 14.2, HRMS: m/z Calc: $\text{C}_{10}\text{H}_{15}\text{BrN}_2$: 242.0418 Found: 242.0419.

5-Benzylamino-*N,N*-dibutylpyrimidin-2-amine (3.12). To a sealed tube was added **3.9** (1.0 mmol), CuI (0.2 mmol), L-proline (0.4 mmol), and K_2CO_3 (1.5 mmol). The flask was backfilled with argon before DMSO (1.5 mL) and water (1.0 % v/v) was added. After a few minutes of stirring, benzylamine (1.8 mmol) was added and the reaction was heated to 80°C until completion, as determined by TLC. The reaction was cooled, quenched with water, and extracted with Et_2O . The combined organics were washed twice with water, washed with brine, and dried over MgSO_4 . Column chromatography (40% Et_2O /hexanes eluent) afforded pure products. The compound was recrystallized from hexanes to analytical purity. Yield: 83%. Yellow Needles. ^1H NMR (400 MHz; CDCl_3): δ 7.91 (s, 2H), 7.35-7.26 (m, 5H), 4.23 (s, 2H), 3.47 (t, $J = 7.5$ Hz, 4H), 1.59-1.51 (m, 4H), 1.33 (dq, $J = 15.1, 7.5$ Hz, 4H), 0.93 (t, $J = 7.3$ Hz, 6H). ^{13}C NMR (100 MHz; CDCl_3): δ 157.3, 145.1, 139.3, 132.5, 128.8, 127.72, 127.52, 77.5, 77.2, 76.8, 50.1, 47.8, 30.3, 20.4, 14.2, HRMS: m/z Calc: $\text{C}_{23}\text{H}_{36}\text{N}_4$: 368.2940 Found: 368.2939.

***N*²,*N*²-Dibutylpyrimidine-2,5-diamine (3.13).** To a solution of **3.12** (1.0 mmol) in degassed MeOH (5 mL) were added 10% Pd/C (10 wt %) and ammonium formate (3.0 mmol). The flask was backfilled with argon and the solution was heated to reflux until completion, as determined by TLC. When complete, the solution was cooled and filtered through a pad of celite. The solvent was evaporated and the resulting solid was re-dissolved in a 40% Et_2O /hexanes solution. The precipitate formed was filtered off through another pad of celite and washed with ether. The solvent was evaporated to obtain the desired compound without any residual ammonium formate. No further purification was attempted because of oxidative instability and difficulty associated with chromatographing this compound.

3.4.2.2 General method for the preparation of diarylamines.

To a flame-dried Schlenk flask were added Pd_2dba_3 (0.01 mmol) and BippyPhos (0.04 mmol). The flask was evacuated and backfilled with argon before *tert*-amylalcohol (1 mL) was added, followed by KOH (1.5 mmol) and H_2O (1.0% v/v) and the solution was stirred for 20 minutes. Aryl bromide (1.0 mmol) and

arylamine (1.1 mmol) were then added, and the reaction was heated to 110°C. Once complete, the mixture was concentrated under vacuum and loaded on a silica column. Column chromatography (Et₂O/hexanes containing 1–5% Et₃N depending on substrate) afforded pure products.

***N*²,*N*²-Dibutyl-*N*⁵-(6-(dihexylamino)pyridin-3-yl)pyrimidine-2,5-diamine (3.1).** Yield: 67%. Brown Oil. ¹H NMR (300 MHz; CDCl₃): δ 8.09 (s, 2H), 7.83 (d, *J* = 2.7 Hz, 1H), 7.07 (dd, *J* = 9.0, 2.9 Hz, 1H), 6.35 (d, *J* = 8.9 Hz, 1H), 4.75 (s, 1H), 3.58 (q, *J* = 7.0 Hz, 4H), 3.36 (t, *J* = 7.6 Hz, 4H), 1.57-1.52 (m, 4H), 1.29-1.26 (m, 20H), 1.17 (t, *J* = 7.0 Hz, 6H), 0.87 (t, *J* = 6.7 Hz, 6H). ¹³C NMR (75 MHz; CDCl₃): δ 157.9, 154.2, 151.0, 138.9, 130.6, 129.0, 128.8, 106.0, 49.1, 42.2, 32.0, 29.7, 29.5, 27.9, 27.3, 22.8, 14.2, 13.3, HRMS: *m/z* Calc: C₂₉H₅₀N₆: 482.4097 Found: 482.4098.

***N*²,*N*²-Dibutyl-*N*⁵-(2-(dibutylamino)pyrimidin-5-yl)pyrimidine-2,5-diamine (3.2).** Yield: 65%. Yellow Powder. ¹H NMR (300 MHz; CDCl₃): δ 7.95 (s, 4H), 3.47 (t, *J* = 7.5 Hz, 8H), 1.60-1.50 (m, 9H), 1.33 (dt, *J* = 15.0, 7.4 Hz, 9H), 0.92 (t, *J* = 7.3 Hz, 12H). ¹³C NMR (75 MHz; CDCl₃): δ 157.7, 147.0, 129.4, 47.8, 30.2, 20.4, 14.2, HRMS: *m/z* Calc: C₂₄H₄₁N₇: 427.3423 Found: 427.3422.

***N*²,*N*²-Dihexyl-*N*⁵-phenylpyridine-2,5-diamine (3.3).** Yield: 74%. Brown Oil. ¹H NMR (400 MHz; CDCl₃): δ 8.03 (d, *J* = 2.7 Hz, 1H), 7.31 (dd, *J* = 9.0, 2.8 Hz, 1H), 7.19-7.15 (m, 2H), 6.78-6.75 (m, 3H), 6.43 (d, *J* = 9.0 Hz, 1H), 5.25 (s, 1H), 3.42 (t, *J* = 7.7 Hz, 4H), 1.62-1.56 (m, 4H), 1.35-1.32 (m, 12H), 0.90 (t, *J* = 6.8 Hz, 6H). ¹³C NMR (100 MHz; CDCl₃): δ 14.22, 22.86, 27.01, 27.83, 31.94, 49.10, 105.76, 114.26, 118.71, 126.69, 129.40, 134.36, 144.46, 147.00, 155.58, HRMS: *m/z* Calc: C₂₃H₃₅N₃: 353.2831 Found: 353.3428.

***N*²,*N*²-Dihexyl-*N*⁵-phenylpyrimidine-2,5-diamine (3.4).** Yield: 81%. Brown Oil. ¹H NMR (400 MHz; CDCl₃): δ 8.22 (s, 2H), 7.20-7.16 (m, 2H), 6.79 (t, *J* = 7.3 Hz, 1H), 6.72 (d, *J* = 7.6 Hz, 2H), 5.13 (s, 1H), 3.54 (t, *J* = 7.6 Hz, 4H), 1.63-1.58 (m, 4H), 1.33 (d, *J* = 3.7 Hz, 12H), 0.90 (t, *J* = 6.6 Hz, 6H). ¹³C NMR (100 MHz; CDCl₃): δ 159.5, 155.4, 146.8, 129.5, 124.9, 119.1, 114.0, 48.2, 31.9, 27.9, 26.9, 22.8, 14.2, HRMS: *m/z* Calc: C₂₂H₃₄N₄: 354.2783 Found: 354.2790.

*N*²,*N*²-Dibutyl-*N*⁵-(2-hexylpyrimidin-5-yl)pyrimidine-2,5-diamine (**3.5**). Yield: 84%. Yellow Powder. ¹H NMR (400 MHz; CDCl₃): δ 8.19 (s, 2H), 8.15 (s, 2H), 5.23-5.16 (m, 1H), 3.53 (t, *J* = 7.5 Hz, 4H), 2.83 (t, *J* = 7.7 Hz, 2H), 1.74 (quintet, *J* = 7.1 Hz, 2H), 1.59 (quintet, *J* = 7.3 Hz, 4H), 1.37-1.29 (m, 10H), 0.94 (t, *J* = 7.3 Hz, 6H), 0.86-0.84 (m, 3H). ¹³C NMR (100 MHz; CDCl₃): δ 162.4, 159.8, 155.4, 142.7, 138.6, 123.1, 48.0, 38.6, 31.8, 30.0, 29.18, 29.11, 22.7, 20.4, 14.20, 14.16, HRMS: *m/z* Calc: C₂₂H₃₆N₆: 384.3001 Found: 384.3099.

3.4.3 Hexadecane Autoxidations.

n-Hexadecane (100 mL) was thoroughly degassed with argon and heated to 160°C while argon was continuously bubbled through the liquid. Once the temperature stabilized, 0.04 mmol of inhibitor (**3.1-3.7**) and 164 mg (1.0 mmol) of tetralin hydroperoxide were added to the solution and the flow of argon was replaced with O₂. Aliquots (0.5 mL) were removed every 5 minutes, and allowed to cool to room temperature for analysis. Four duplicates (30 µL) of each sample were loaded into separate wells of a 96-well microplate and the automated reagent dispenser of the microplate reader was used to dilute each sample with *tert*-amyl alcohol (200 µL) and a solution of fluorogenic phosphine dye solution (20 µL of a 250 µM stock solution in acetonitrile) immediately before reading. The plate was stirred for 8 seconds, allowed to rest for 2 more seconds, and the fluorescence of each well was measured every second for 60 seconds (absorption at 340; emission at 425). The concentration of hydroperoxide was determined from the rate of phosphine oxidation using the rate constant for the reaction of the dye with secondary hydroperoxides in *tert*-amyl alcohol (*k* = 1.2 M⁻¹s⁻¹) assuming pseudo-first-order kinetics.

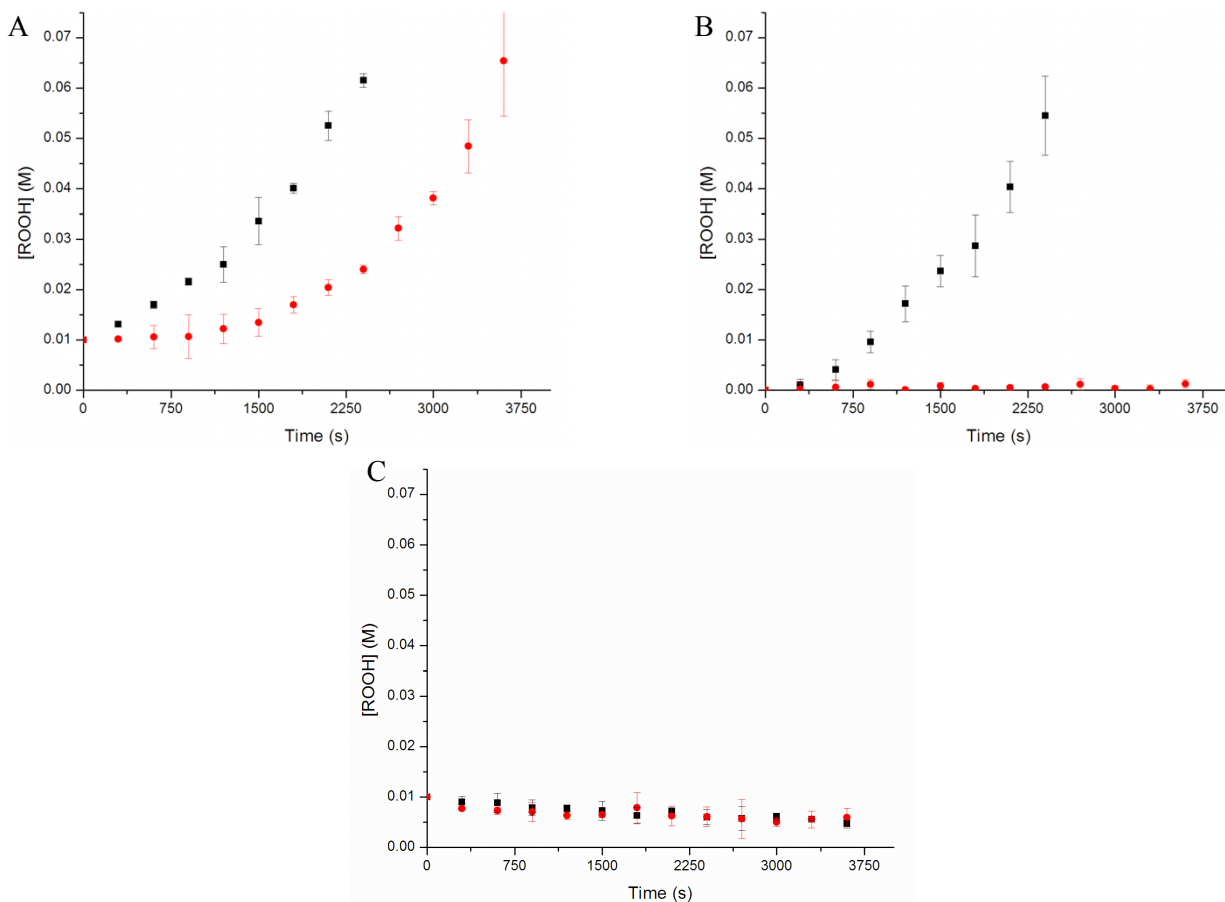


Figure S3.1 Hydroperoxide formation in the autoxidation of *n*-hexadecane at 160°C (A) initiated by 10 mM tetralin hydroperoxide (black, ■), and with an added 40 μM of 3.4 (red, ●), (B) uninitiated (black, ■) and with an added 40 μM of 3.4 (red, ●), and (C) in the presence of 10 mM tetralin hydroperoxide (black, ■) and with an added 40 μM of 3.4 (red, ●), but in the absence of O₂.

3.4.4 Diarylamine Stability to Hydroperoxides.

Degassed *n*-hexadecane (1.0 mL) was added to 2.5 mmol of diarylamines **3.1-3.7** that were weighed into a glass vial under argon. A magnetic Teflon coated stir bar was added, the vial was capped with a rubber septum and fitted with an argon balloon. The suspension was stirred for a few minutes to dissolve the solid. The vial was then placed in a heating block at 160°C for 30 seconds before 10 μL of a 1.0 M solution of tetralin hydroperoxide solution in ethyl acetate was added. The solution was stirred for 30 seconds and the first sample was taken. Subsequent 100 μL aliquots were removed at regular intervals and added to 900 μL of a 2.2 mM solution of benzyl alcohol (internal standard) in 2%

isopropanol/hexanes (HPLC grade). Samples were analysed using a Waters 2695 Alliance HPLC at a flow rate of 2.1 mL min⁻¹ (8.0% *i*PrOH/hexanes) on a Sunfire Silica column (5µm, 4.6 x 250 mm) and determined by their UV absorbance. The data are shown in Figure S2.

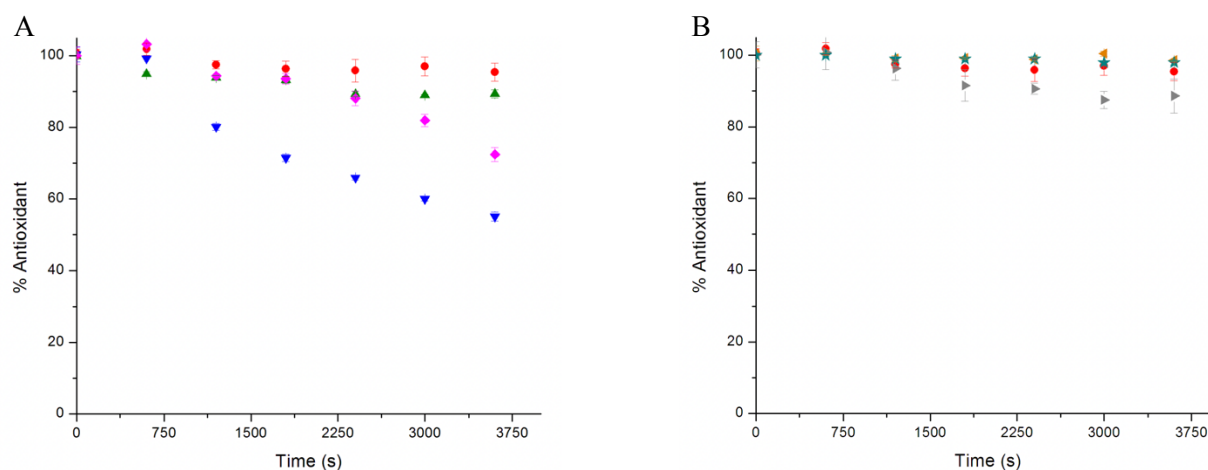


Figure S3.2 Decomposition of diarylamines at 160°C in the hexadecane in the presence of 10 mM tetralin hydroperoxide under argon: (A): **3.1** (▼), **3.2** (◆), **3.3** (▲) and **3.7** (●); (B): **3.4** (▶), **3.5** (►), **3.6** (★) and **3.7** (●).

3.4.5 Diarylamine Stability to Oxygen.

n-Hexadecane (100 mL) was thoroughly degassed with argon and then heated to 160 °C while argon was continuously bubbled through the liquid. Once the temperature stabilized, 2.5 mmol of diarylamines **3.1**-**3.7** were added to the solution and the flow of argon was replaced with O₂. Aliquots (100 µL) were removed every 10 minutes, and added to 900 µL of a 2.2 mM solution of benzyl alcohol (internal standard) in 2% isopropanol/hexanes (HPLC grade). Samples were analysed using a Waters 2695 Alliance HPLC at a flow rate of 2.1 mL min⁻¹ (8.0% *i*PrOH/hexanes) on a Sunfire Silica column (5µm, 4.6 x 250 mm) and determined by their UV absorbance. The data are shown in Figure S3.3.

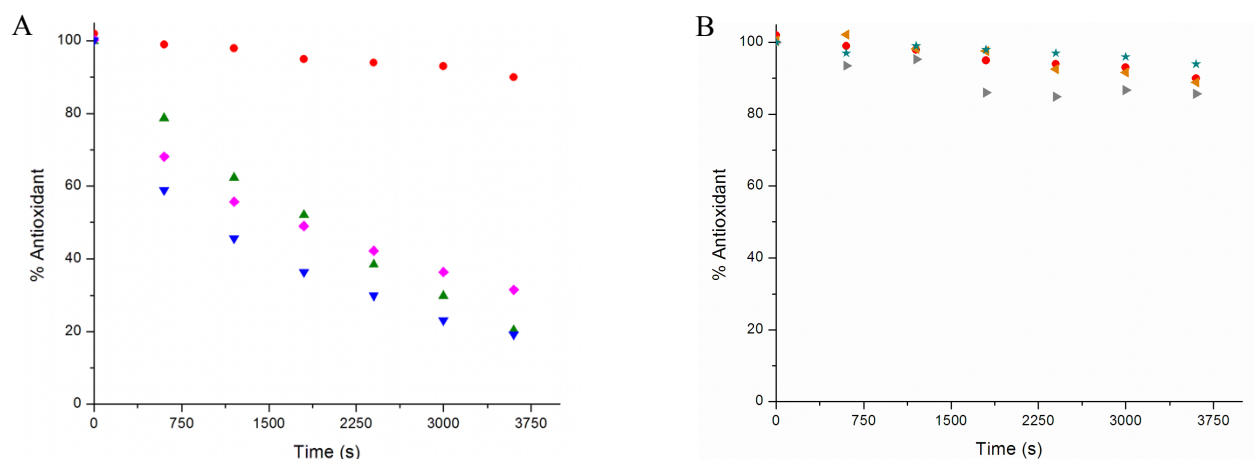


Figure S3.3 Decomposition of antioxidants at 160 °C under oxygen (A): 3.1 (▼), 3.2 (◆), 3.3 (▲) and 3.7 (●); (B): 3.4 (◀), 3.5 (▶), 3.6 (★) and 3.7 (●).

3.4.6 Determination of Carboxylic Acids in a Hexadecane Autoxidation.

Aliquots (50 μ L) were removed from uninhibited hexadecane autoxidations carried out as described above. The samples were diluted into 4.95 mL of a 20% iPrOH in MeOH solution containing 3,5-dimethylbenzoic acid (10.5 μ M, internal standard). The samples were infused directly onto the TQ mass detector of a Waters Acuity H-Class UPLC-MS. The negative ion mass spectrum (from 50-400 m/z) of each sample was recorded three times, for 2 minutes per read, with a scan time of two seconds. Instrument parameters are as follows: capillary voltage = 3.2 kV, cone voltage = 40 V, extractor voltage = 3V, source temperature = 150 °C, desolvation temperature = 250 °C, desolvation gas flow = 500 L/hr, cone gas flow = 50 L/hr. Standards were prepared from 2.0 mM solutions of octanoic, nonanoic, decanoic, undecanoic, dodecanoic, tridecanoic, and tetradecanoic acids (in the same hexadecane solution), and diluted in the same way as described above.

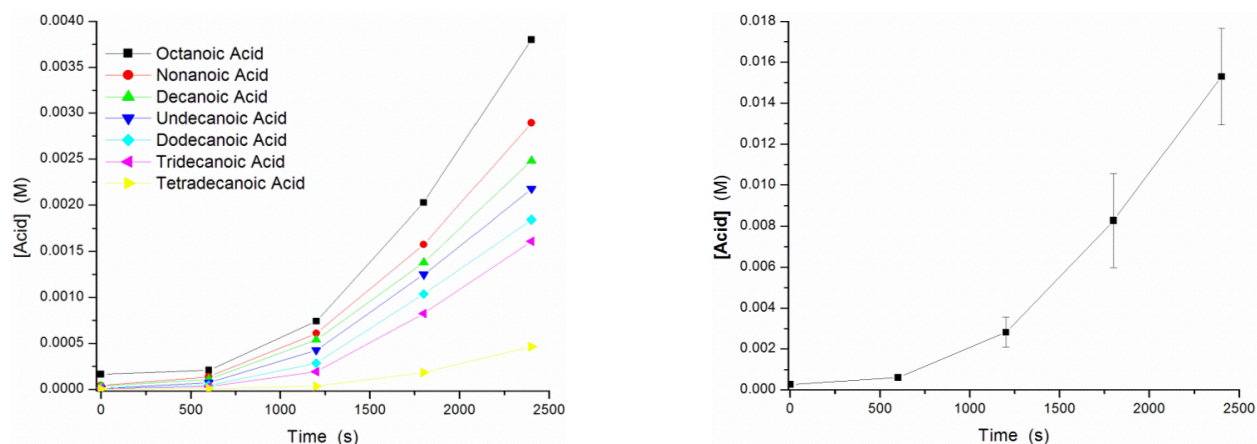


Figure S3.4 (left) Concentration of C₈ to C₁₄ carboxylic acids determined in the first 40 minutes of an uninhibited autoxidation of *n*-hexadecane at 160 °C initiated by 10 mM tetralin hydroperoxide. (right) Total carboxylic acid production.

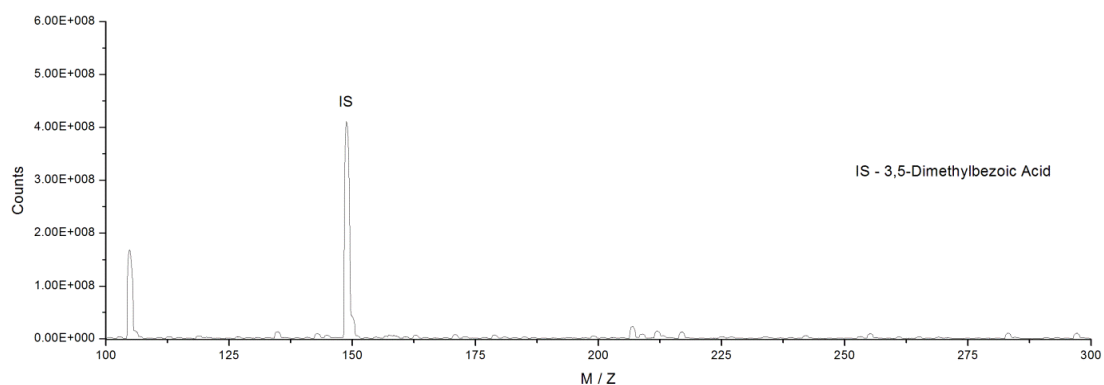


Figure S3.5 Mass spectrum of a 50 μ L aliquot from an uninhibited autoxidation of hexadecane at 160 °C ($t = 0$ s) diluted with 4.95 mL of a MeOH/*i*PrOH (4:1) containing 3,5-dimethylbenzoic acid (10.5 μ M).

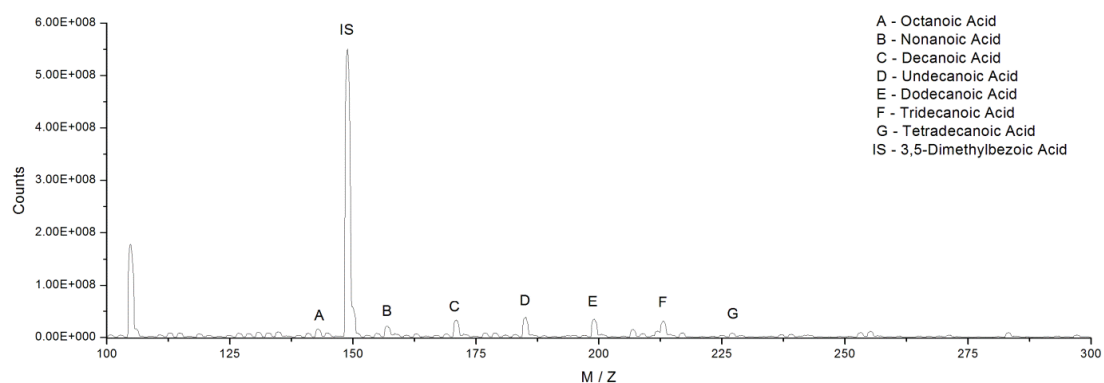


Figure S3.6 Mass spectrum of a 50 μL aliquot from an uninhibited autoxidation of hexadecane at 160 $^{\circ}\text{C}$ ($t = 600$ s) diluted with 4.95 mL of a MeOH/*i*PrOH (4:1) containing 3,5-dimethylbenzoic acid (10.5 μM).

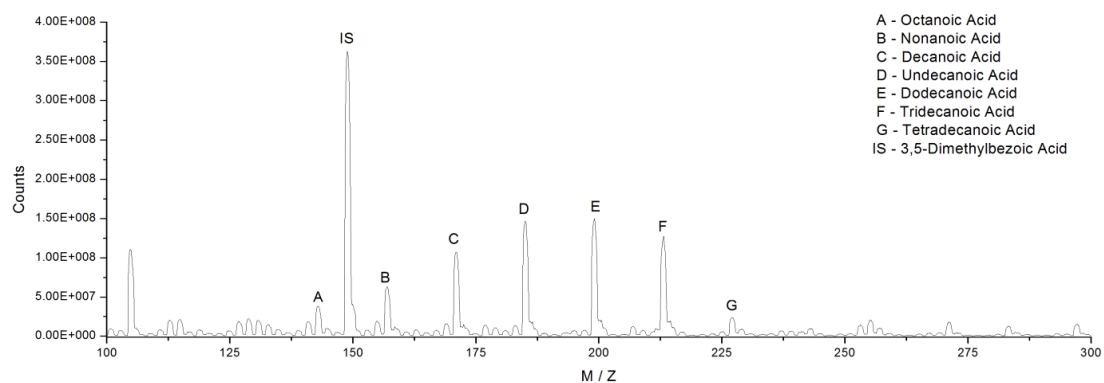


Figure S3.7 Mass spectrum of a 50 μL aliquot from an uninhibited autoxidation of hexadecane at 160 $^{\circ}\text{C}$ ($t = 1200$ s) diluted with 4.95 mL of a MeOH/*i*PrOH (4:1) containing 3,5-dimethylbenzoic acid (10.5 μM).

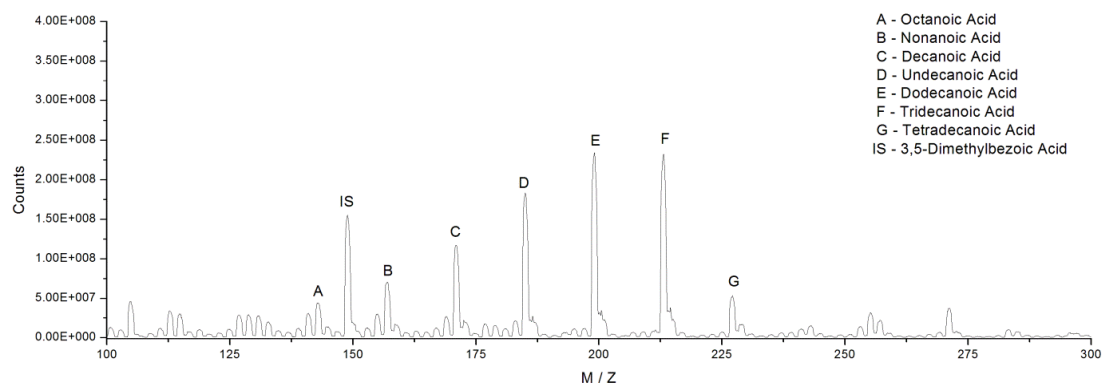


Figure S3.8 Mass spectrum of a 50 μL aliquot from an uninhibited autoxidation of hexadecane at 160 $^{\circ}\text{C}$ ($t = 1800$ s) diluted with 4.95 mL of a MeOH/*i*PrOH (4:1) containing 3,5-dimethylbenzoic acid (10.5 μM).

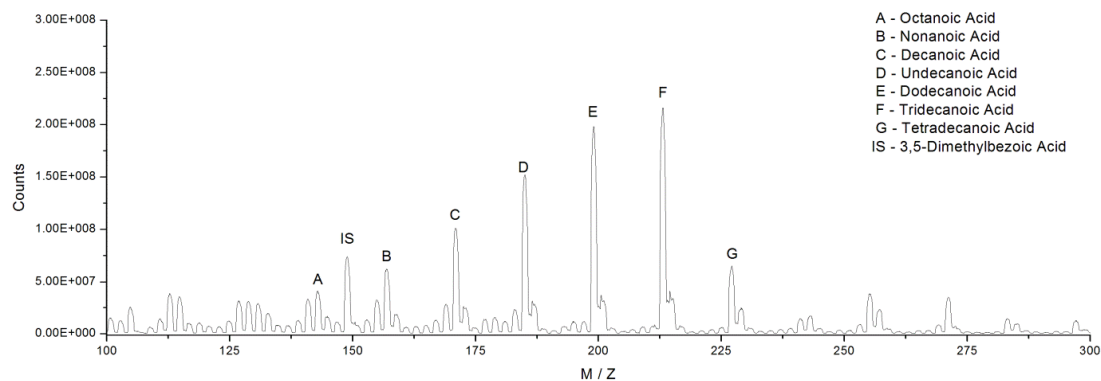


Figure S3.9 Mass spectrum of a 50 µL aliquot from an uninhibited autoxidation of hexadecane at 160 °C ($t = 2400$ s) diluted with 4.95 mL a MeOH/*i*-PrOH (4:1) containing 3,5-dimethylbenzoic acid (10.5 µM).

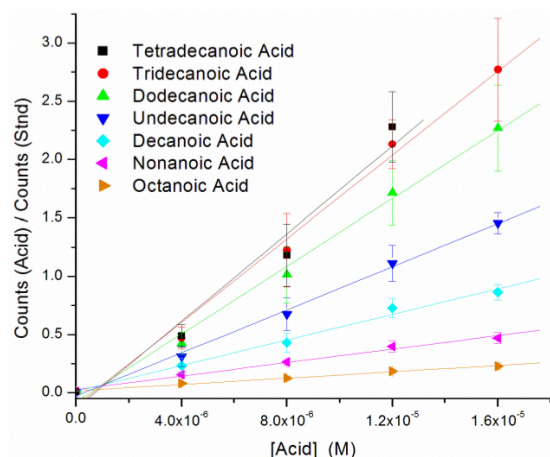


Figure S3.10 Standard curves for determinations of carboxylic acids (0-16 µM) versus 3,5-dimethylbenzoic acid (10.5 µM).

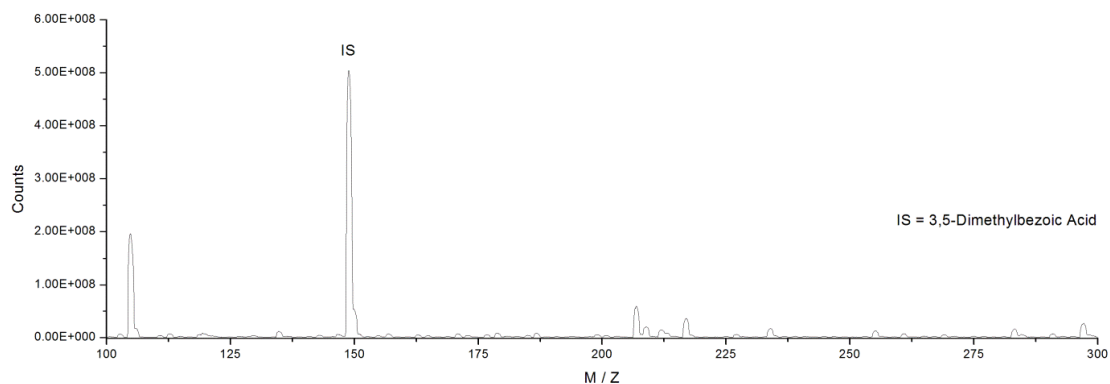


Figure S3.11 Mass spectrum of a standard solution (1% hexadecane, 20% *i*PrOH, 79% MeOH) containing no carboxylic acids and 3,5-dimethylbenzoic acid (10.5 µM).

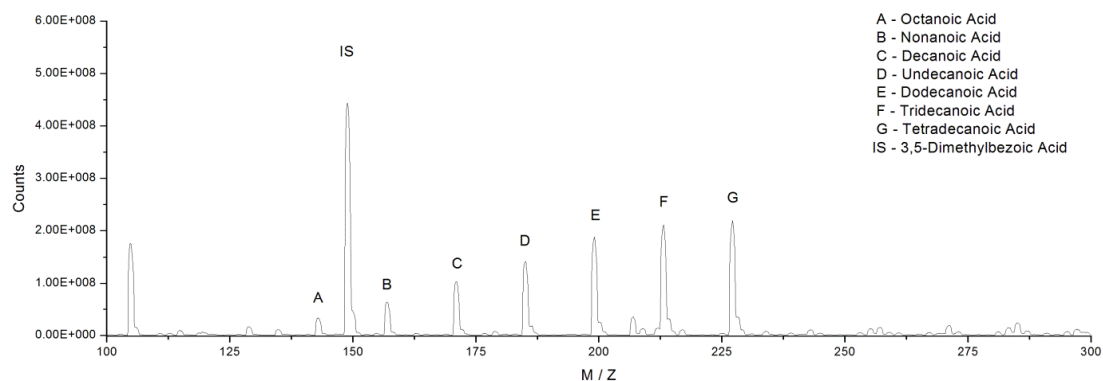


Figure S3.12 Mass spectrum of a standard solution (1% hexadecane, 20% iPrOH, 79% MeOH) containing the indicated carboxylic acids (4.0 μM) and 3,5-dimethylbenzoic acid (10.5 μM).

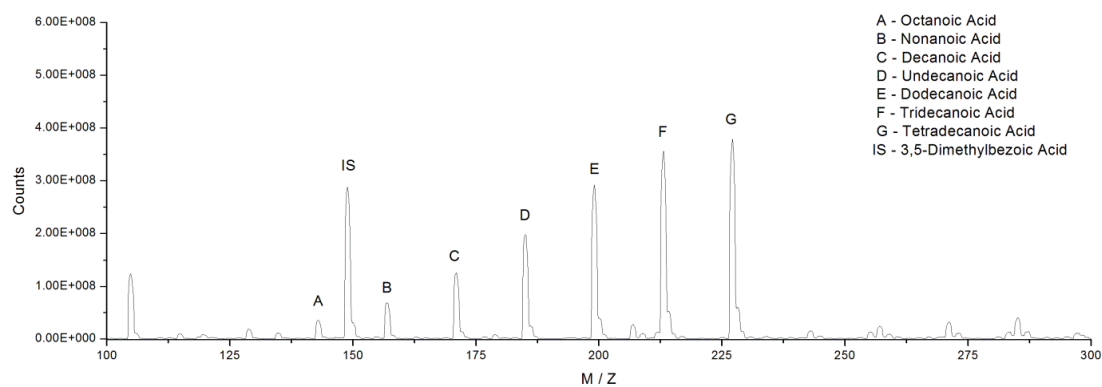


Figure S3.13 Mass spectrum of a standard solution (1% hexadecane, 20% iPrOH, 79% MeOH) containing the indicated carboxylic acids (8.0 μM) and 3,5-dimethylbenzoic acid (10.5 μM).

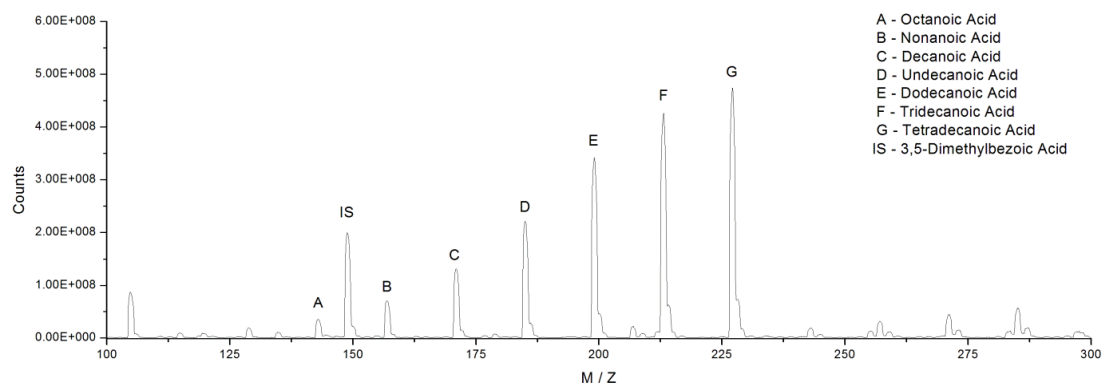


Figure S3.14 Mass spectrum of a standard solution (1% hexadecane, 20% iPrOH, 79% MeOH) containing the indicated carboxylic acids (12.0 μM) and 3,5-dimethylbenzoic acid (10.5 μM).

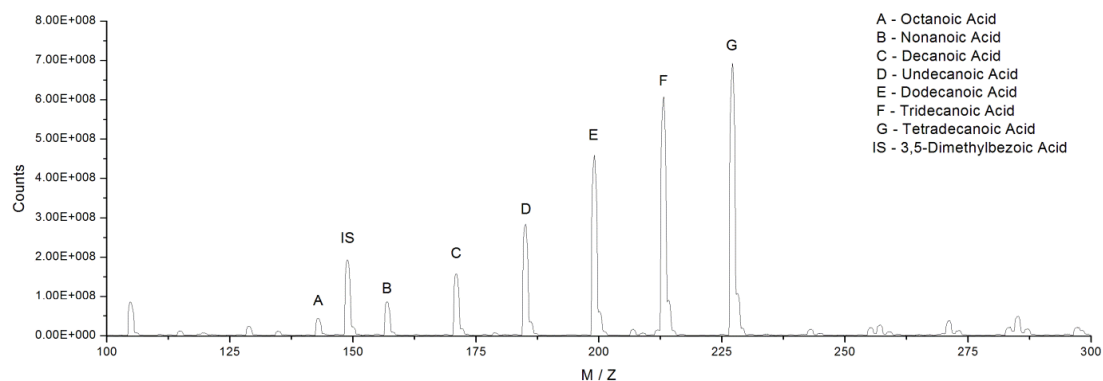


Figure S3.15 Mass spectrum of a standard solution (1% hexadecane, 20% iPrOH, 79% MeOH) containing the indicated carboxylic acids (16.0 μM) and 3,5-dimethylbenzoic acid (10.5 μM).

3.4.7 Hexadecane Autoxidation in the Presence of Palmitic Acid.

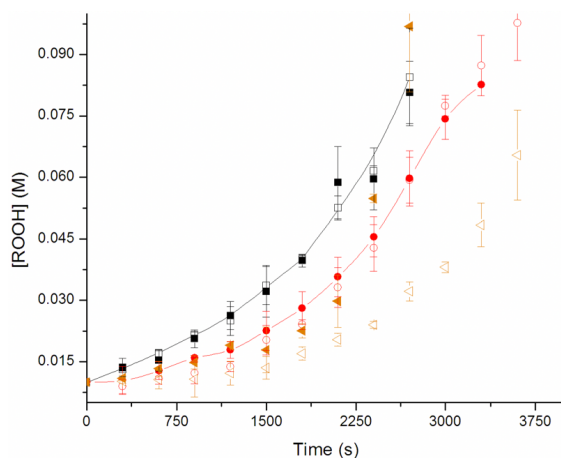


Figure S3.16 Hydroperoxide formation in the autoxidation of hexadecane at 160 °C initiated by 10 mM tetralin hydroperoxide (■) and inhibited by 40 μM of either 3.7 (●) or 3.4 (▲) in the presence of palmitic acid (1 mM). Empty symbols depict autoxidations carried out in the absence of acid.

3.4.8 Hexadecane Autoxidation in the Presence of Cesium Carbonate and a Tertiary Alkyl Primary Amine (Primene 81-R)

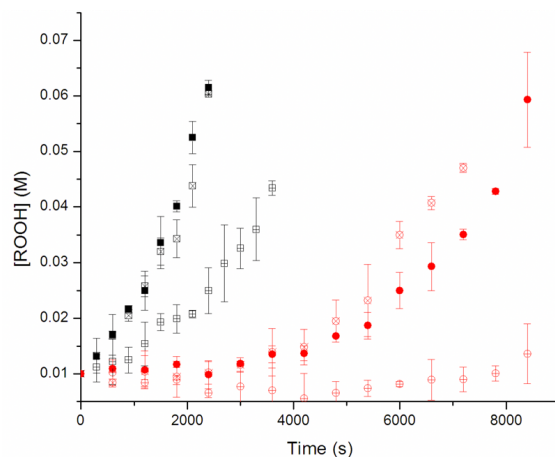


Figure S3.17 Hydroperoxide formation in the autoxidation of *n*-hexadecane at 160 °C initiated by 10 mM tetralin hydroperoxide in the presence of 1.0 mM TTBP (■), 1.0 mM Cs₂CO₃ (□), and *ca.* 1.0 mM Primene 81-R (molecular weight estimated at 190 g/mol) (⊠) and inhibited by 40 μM of **3.4** in the presence of 1.0 mM TTBP (●), 1.0 mM Cs₂CO₃ (◐), and *ca.* 1.0 mM DOW Primene 51 (⊗).

3.4.9 pK_a Measurements with Diarylamines 3.1-3.7

Solutions of the hydrochloride salts of diarylamines **3.1-3.7** (1 mM) were made up in 4:1 CH₃CN:H₂O (3.0 mL) and the pH was measured using an Accumet Electrode microbe referenced to Ag/Ag⁺ as a function of added volumes of NaOH (10 mM). The *s*pH in 4:1 (v:v) CH₃CN/H₂O was obtained by adding 0.897 to the measured value as suggested in reference 27. The titration curves for the diarylamines are shown in Figure S3.18 (averages of triplicate experiments), and the pK_as reported in the manuscript correspond to the pH when 0.5 molar equivalents of NaOH were added to the solution.

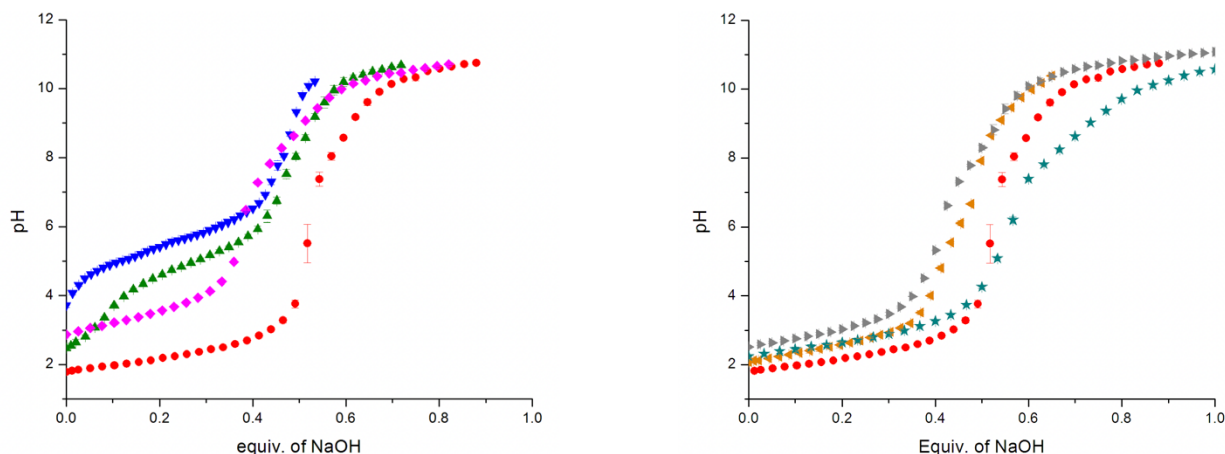


Figure S3.18 Titrations of solutions of diarylamines (1 mM) in 4:1 CH₃CN:H₂O with 10 mM NaOH. (left): **3.1** (▼), **3.2** (◆), **3.3** (▲) and **3.7** (●); (right): **3.4** (◀), **3.5** (▶), **3.6** (★) and **3.7** (●).

3.4.10 Peroxyl Radical Clock Kinetics with Diarylamine 3.5

Peroxyl radical clocking experiments (to determine the inhibition rate constant k_{inh}) for compound **3.5** were carried out according to our previously published reports.¹³ Inhibition rate constants of the other compounds are reported in a previous publication.¹⁴

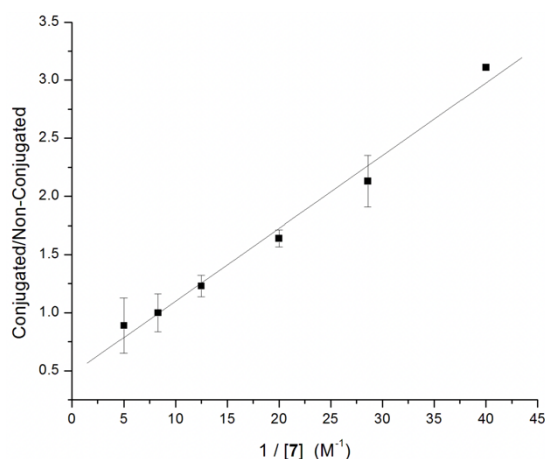


Figure S 3.19 Double reciprocal plot used to obtain $k_{\text{inh}} = 2.4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ for compound **3.5**.

3.4.11 Electrochemical Studies with Diarylamine 3.5

A standard potential for diarylamine **3.5** was measured from its cyclic voltammogram obtained using a BASi potentiostat with a glassy carbon working electrode, a platinum counter electrode and a Ag/AgNO₃ reference electrode. The diarylamine was dissolved in degassed acetonitrile containing 0.1 M tetrabutylammonium hexafluorophosphate as supporting electrolyte and the current recorded in the range of 0-1 V at a scan rate of 200 mV/s. Standard potentials of the other compounds are reported in a previous publication.¹⁴

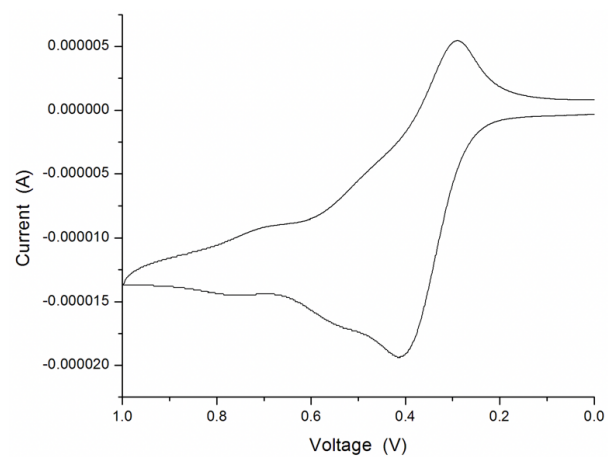


Figure S3.20 Cyclic voltammogram of diarylamine 3.5 (1 mM in CH₃CN containing 0.1 M Bu₄NPF₆) vs. Ag/Ag⁺. $E^0 = 0.84$ V vs. NHE.

Chapter 4: Heterocyclic Diarylamines Offer Insight on the Temperature and Medium-Dependence of the Catalytic Mechanisms of Diarylamine Radical-Trapping Antioxidants

4.1 Preface

The development of processes to slow hydrocarbon autoxidation in order to maximize their longevity and performance of products derived therefrom has been a major focus of chemical industries over the past century. The main strategy to emerge from these efforts was the use of compounds that trap the chain-propagating radicals, so-called radical-trapping antioxidants (RTAs). Recently, we reported our efforts towards the design of significantly reactive compounds, more specifically, substituted diarylamines (Ar_2NH) containing pyridine and pyrimidine rings. These compounds displayed unprecedented inhibition of a model hydrocarbon at 160 °C and provided a clear path forward towards the development of next-generation of diarylamine RTAs. From our preliminary studies, it was immediately clear that the best diarylamines were ones where there was a balance between reactivity and stability. Herein, we report our efforts towards improving the activity of our most efficacious compound by investigating the effects of alkyl substituents on the unsubstituted phenyl ring. Through this endeavor we discovered the excellent beneficial effects of benzylic positions on the aromatic rings. Alongside, the effect of different substrates and temperatures on the reactivity of our compounds was also evaluated. The structure-performance relationship established herein suggests future optimization of diarylamines should be centered on substituted-pyrimidines with benzylic sites. Lastly, our results offer insight into the multifaceted activity of nitroxides – they behave catalytically in unsaturated substrates but adversely affect the regeneration in saturated hydrocarbons. Calculations shown in Scheme 4.1 and 4.4 were carried out by Dr. Evan Haidasz and Dr. Jia-Fei Poon, respectively.

4.2 Introduction

Hydrocarbon autoxidation, the archetype free radical chain reaction, results in the oxidative degradation of virtually all hydrocarbons including oils, lubricants, plastics and biological membranes (Figure 4.1A).^{1,2} The key propagation step comprises the addition of O₂ to a substrate-derived carbon-centered radical to form a peroxy radical and the subsequent abstraction of a H-atom from the hydrocarbon substrate. The chain reaction is initiated in a number of ways, but arguably the most relevant are either thermolysis of hydroperoxides, or their heterolysis by dissociative electron transfer from low-valent metal atoms to give hydroxyl and/or alkoxy radicals (Figure 4.1A). Radical trapping antioxidants (RTAs) such as hindered phenols (e.g. butylated hydroxytoluene, BHT) and diarylamines (e.g. dialkyldiphenylamines, 4.1) inhibit this process by reacting with the intermediate chain carrying peroxy radicals by transferring a hydrogen atom through a proton-coupled electron transfer (PCET) reaction.³

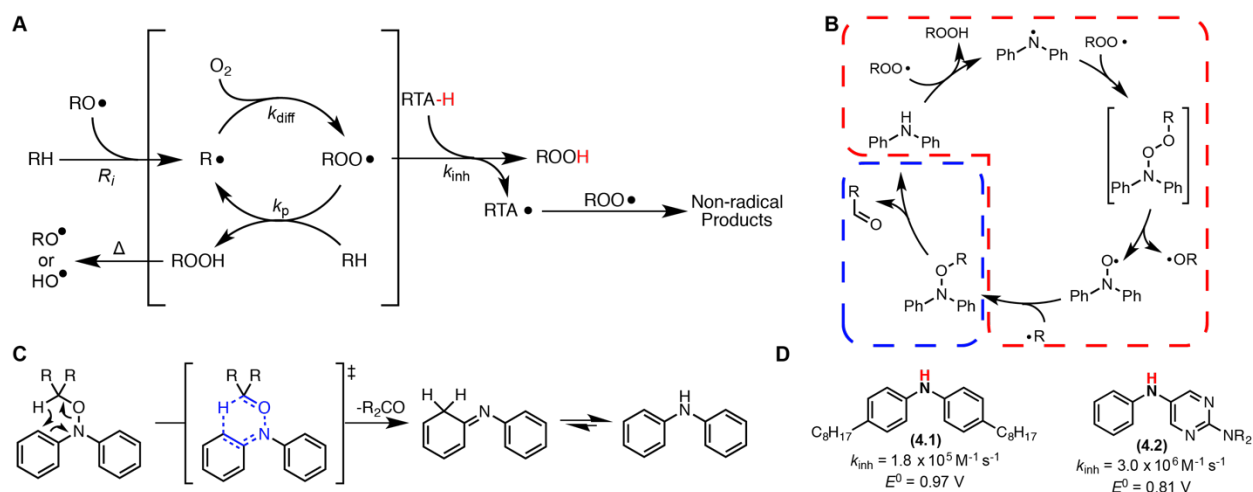


Figure 4.1 (A) Simplified mechanism of hydrocarbon autoxidation. (B) Mechanism of diarylamine regeneration proposed by Korcek et al. (C) Retro-carbonyl-ene (RCE) reaction for the regeneration of diarylamines. (D) Commonly used industrial antioxidant (4.1) in high temperature applications, and the best Ar₂NH reported to date (4.2) to inhibit hydrocarbon autoxidations at elevated temperatures.

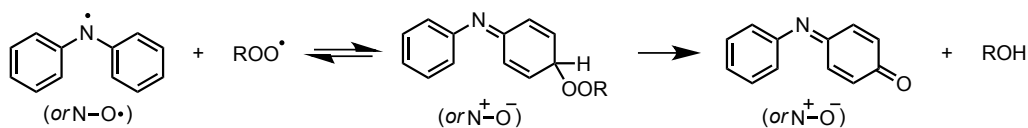
Phenols, such as BHT, trap two peroxy radicals, i.e. they have a stoichiometric number, $n = 2$.⁴ However, at elevated temperatures (e.g. lubricating oils used in internal combustion engines, polymer extruders), BHT and other phenols trap less than one radical because the peroxidic adduct decomposes to yield chain-initiating radicals.⁵ These facts lead to the development of diarylamines (Ar₂NH) as the

additives of choice for high temperature applications. While Ar₂NH are no more reactive to peroxy radicals than BHT and most industrial phenolic RTAs (i.e. they have similar values of k_{inh}) the reactivity of intermediate diarylaminy radicals differs considerably from their phenoxyl counterparts. In the subsequent step, rather than reacting with peroxy radicals to produce non-radical products, the diarylaminy radical undergoes formal oxygen atom transfer from the peroxy radical to yield a nitroxide (Figure 4.1B, red).^{6,7} The second radical is trapped by the reaction of a peroxy radical with the aromatic ring or by the coupling of alkyl radicals with the nitroxides to form alkoxyamines. We recently reported that at ambient temperatures and under specific conditions, nitroxides can exhibit catalytic inhibition both in organic and aqueous solutions either by a cross-dismutation or hydride-transfer, respectively.^{8,9}

The most outstanding reactivity of Ar₂NH arises at high temperatures (>100°C), where their stoichiometric values have been reported to reach as high as $n = 40$ due to the regeneration of the parent Ar₂NH antioxidant.¹⁰ This makes Ar₂NH extremely remarkable as one molecule is capable of trapping > 2 peroxy radicals at elevated temperatures, where they are of most industrial importance. A catalytic mechanism of regeneration for the diarylamines was proposed to account for such high values, where the diarylamines are regenerated *in situ* from a substrate-derived alkoxyamine intermediate (Figure 4.1B, blue).⁷ We recently reported the results of computational and experimental studies designed to elucidate the mechanism of this catalytic activity which revealed that the key step in the proposed catalytic cycle – decomposition of the alkoxyamine derived from capture of a substrate-derived alkyl radical with a diarylamine-derived nitroxide – proceeds by an unprecedented concerted retro-carbonyl-ene reaction (Figure 4.1C).¹¹

We previously described a strategy to improve the stability and reactivity of diarylamines by the incorporation of ring nitrogens, and results revealed that these novel heterocyclic diarylamines were resistant to one-electron oxidation while being up to 200 times more reactive compared di-*n*-octyldiphenylamine (**4.1**), an *n*-substituted analog of the prototypical industry standard.^{12–14} Following up on these preliminary results and exploring their reactivity at high temperatures revealed unprecedented

inhibited periods resulting from the use of heterocyclic diarylamines compared to **4.1**.¹⁵ We quantified the amount of acid produced during autoxidation and its negative effect on our extremely basic heterocyclic diarylamines. The addition of an external base to react with the acid led to the ‘unmasking’ of the true reactivity of our compounds.¹⁶ The results obtained from these studies helped establish an excellent structure-activity profile. Herein, we attempt to optimize the structure of our compounds to further enhance the reactivity exhibited by **4.2**.



Scheme 4.1 Addition of peroxy radicals on the aromatic ring of a diarylaminyll or diarylnitroxide radical leads to the production of quinone-imines or quinone-*N*-oxides.

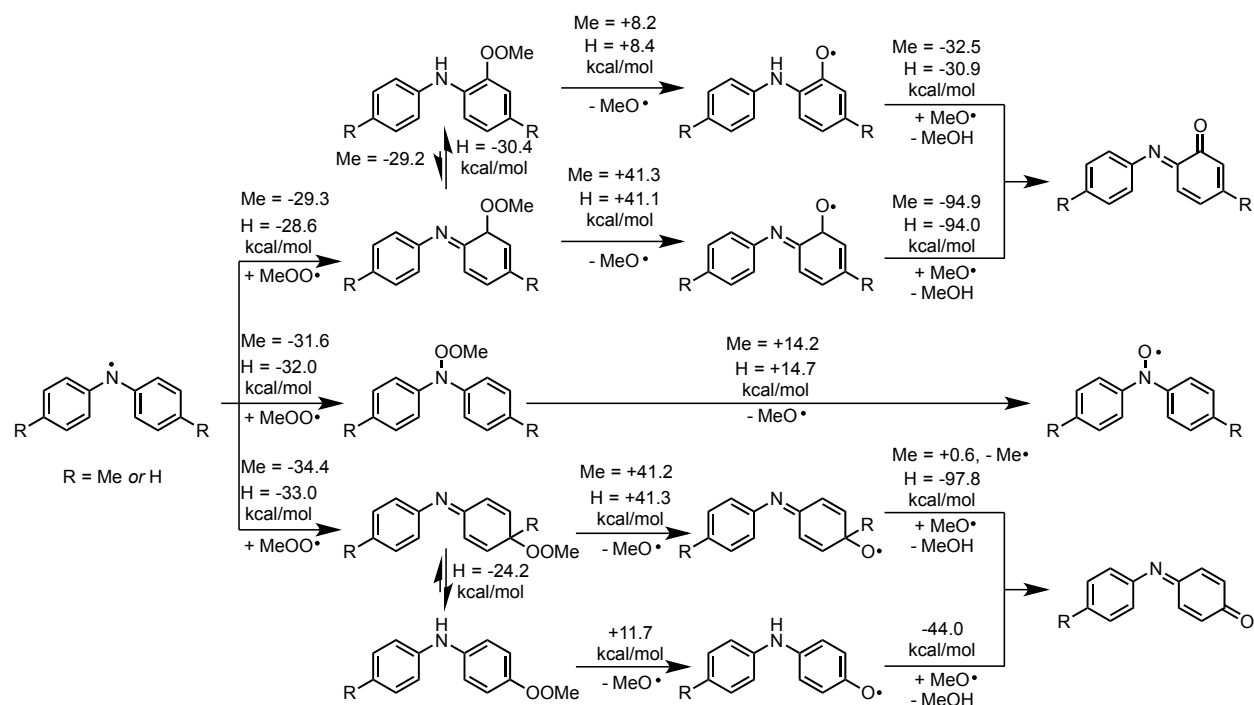
Looking at industrial diphenylamines, we noticed that their aromatic rings are commonly substituted with tertiary-alkyl chains. There are many reasons for this, including a higher molecular weight to decrease compound volatility, increased lipophilicity to enhance miscibility with lubricating oils and enhancement of their activity compared to the unsubstituted compounds. The increased activity was first quantitated by Bolsman *et al.*, and higher stoichiometries were reported for alkyl substituted diphenylamines compared to the unsubstituted analog.¹⁷ This was speculated to be a result of improved stability of the diarylamine against deleterious side-reactions resulting from the addition of a peroxy radical onto the aromatic ring of the diarylnitroxide. Moreover, our recent computational investigation suggested that the side-reactions may also occur from the diarylaminyll radical, effectively leading to the destruction of the RTA (Scheme 4.1).¹¹ Similar addition reactions on the *ortho*- or *para*-positions are well preceded for a variety of aromatic amines to yield quinone-type compounds.^{18,19} Therefore, we anticipated that adopting a similar strategy and substituting the phenyl ring of **4.2** with alkyl groups could circumvent the off-cycle pathways (Scheme 4.1), making the compounds more resistant to the deleterious reactions and increasing their efficacy.

4.3 Results

4.3.1 Computational Studies on Potential Fates of the Radicals Derived from 4.2

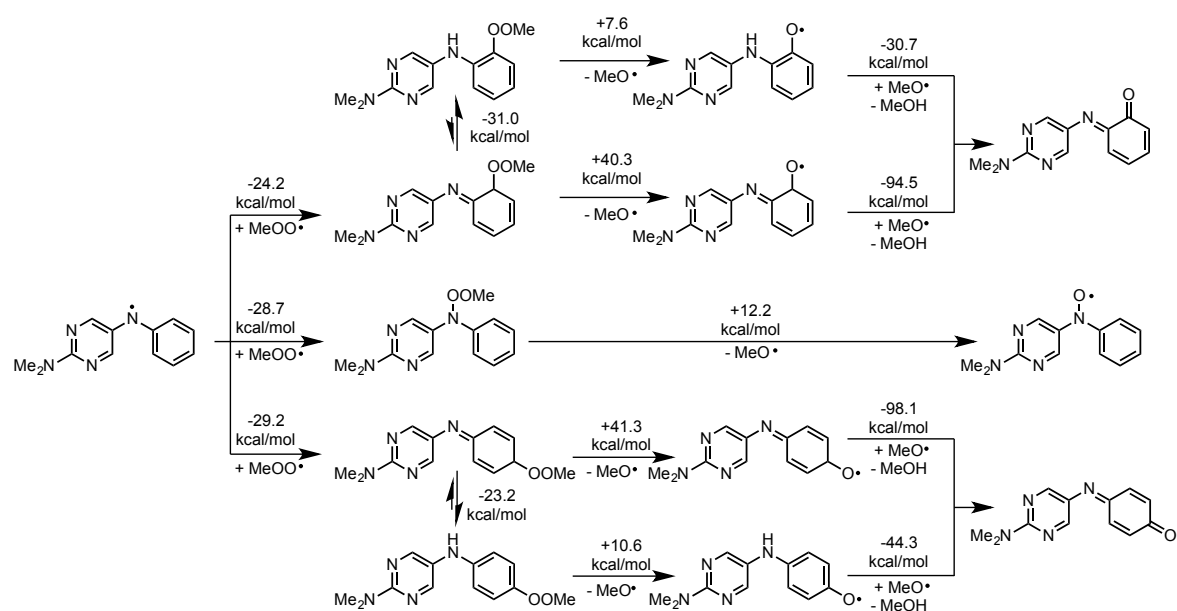
In earlier work, we used quantum chemical calculations to probe the extent to which nitroxide formation was preferred over peroxy radical addition to the aromatic rings of the diphenylaminyl radical.¹¹ CBS-QB3 calculations suggested that addition of peroxy radicals to the *para* position relative to the aminyl nitrogen is preferred by ~ 1 kcal/mol over formation of the peroxyamine precursor to the diphenylnitroxide (Scheme 4.2). In the peroxyamine intermediate the O–O bond is much weaker than the N–O bond (14.7 vs 32.0 kcal/mol), cleaving to yield the nitroxide and alkoxy radical, whereas in the *para*-coupled adduct, the C–O bond is much weaker than the O–O bond (33.0 vs 41.3 kcal/mol). Similarly, the *ortho*-adduct also has a weaker C–O bond compared to the O–O bond (28.6 vs 41.1 kcal/mol) suggesting that the intermediates will be funneled toward the thermodynamic product: the nitroxide. As such, the role of ring substitution of diphenylamines is, in part, to favour the formation of nitroxide over ring addition products. We have since found that peroxy radical addition is predicted to be *more* favourable when the aryl rings are alkylated – at least with a methyl group (see also Scheme 4.2). Thus, it would appear that alkylation must disfavour a follow-up reaction. Given that the O–O BDEs in the adducts are roughly the same (~ 41.2 kcal/mol), we must conclude that alkylation prevents tautomerization of peroxy ring adduct to an arylperoxide, which would rapidly decompose and undergo in-cage disproportionation to yield quinone-imines. Although, the O–O BDE of alkylperoxy-intermediate is relatively high (~ 41 kcal/mol), in the absence of an alkyl group, the tautomerization reaction is thermodynamically favourable (-30.4 and -24.2 kcal/mol for *ortho*- and *para*-adducts) and is likely a viable pathway that competes with nitroxide formation to generate the decomposition products.

Indeed, results imply that blocking the *para* position with alkyl substituents prevents the key tautomerization step, forcing the intermediate through the O–O bond homolysis (BDE = 41.2 kcal/mol) pathway, which would instead fragment back to yield the aminyl radical due to the lower C–O BDE of the alkylperoxy intermediate (BDE = 34.4 kcal/mol).



Scheme 4.2 Competing pathways and Associated ΔH values for aminyl (top) and nitroxide (bottom) derived from diphenylamine or ditolylamine.

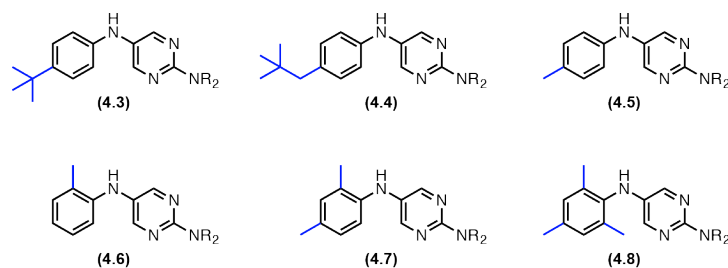
Along those lines we subsequently carried out analogous computations on the diarylaminy radical derived from **4.2** and found a similar preference (Scheme 4.3). Again, it seems that the addition of the peroxy radical and its tautomerization is a thermodynamically feasible pathway that can compete with the production of the nitroxide. The theoretical insights predict that several non-productive reactions compete with the regeneration of the diarylamines, namely by the addition of peroxy radicals on the vacant *ortho*- and *para*-sites on the phenyl ring. Therefore, blocking these positions with alkyl substituents should confer a protective effect by prohibiting the decomposition steps and consequently direct the intermediates into more productive reactions.



Scheme 4.3 Competing pathways and associated ΔH values for aminyl + $\bullet\text{OOMe}$ derived from **4.2**.

4.3.2 Synthesis of substituted heterocyclic diarylamines

Compounds in Scheme 4.4 were anticipated to afford protection from the aforementioned decomposition reactions. The propensity of the peroxy radicals to be directed at the *para* position led to the design of **4.3** – **4.5**. Since *ortho* sites are also subject to addition chemistry, analogs **4.6** – **4.8** were anticipated to offer a compromise at blocking both the *ortho* and *para* sites. Bulky groups were restricted to the 4'-position due to the steric effects with the reactive N-H bond as well as distortion of the planarity of the aromatic rings precluding radical delocalization. The steric hindrance due to the close proximity of the *o*-methyl substituents to the labile H-atom is evident in **4.6-4.8** and the presence of two *o*-methyl groups (**4.8**) forces the angle of the rings to be 88.3° , making them virtually orthogonal to one another (Figure S4.7).



Scheme 4.4 Proposed compounds based on the scaffold of **4.2** to perturb deleterious reactions resulting from peroxy-radical addition to *ortho*- and *para*-position

The diarylamines (**4.2-4.8**) were prepared in a similar manner as described in our recent manuscripts.^{12,15} We envisioned that a modular strategy where the ultimate step catalyzed by a Pd-catalyzed Buchwald-Hartwig amination between alkyl substituted anilines and *N,N*-dialkylamino-substituted pyrimidiyl bromide would allow us to quickly build a library of compounds (Figure 4.3). The latter was prepared from 2-aminopyrimidine by bromination and then alkylation, we mainly used octyl- or hexyl- groups as alkyl substituents to ensure solubility of the diarylamines in hydrocarbon rich environment. The final step was catalyzed using Pd₂(dba)₃ and BippyPhos as the ligand. This procedure was easily scalable, allowing us to obtain large quantities (> 10g) of our compounds in excellent yields. Complete details are available in the supporting information.

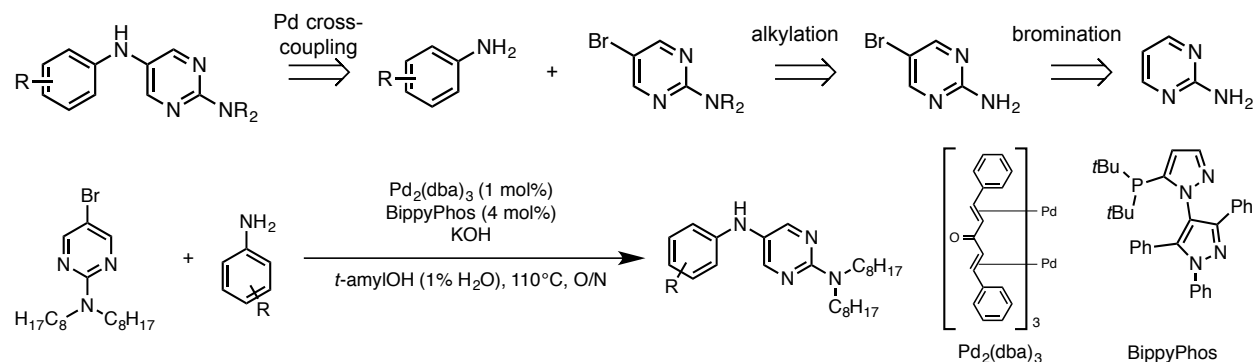


Figure 4.2 Synthesis of diarylamines **4.2-4.8**.

4.3.3 Inhibited Autoxidations at Ambient Temperatures

The kinetics of the newly synthesized diarylamines were quantified using a recently reported method by our laboratory. This involves the addition of a highly colourimetric probe (e.g. PBD-BODIPY), which undergoes co-oxidation in the presence of an autoxidizable hydrocarbon (Figure 4.3B).²⁰ The absorbance of the probe is monitored using a conventional spectrophotometer to track changes in its concentration over time. Using this approach, the rate constant of inhibition can be calculated from the initial rates of inhibited autoxidations (Eq. 4.1), and the stoichiometry '*n*' can be derived from the inhibition time (Eq. 4.2). Since recent work has shown that mechanism of diarylamines is substrate-dependent,^{8,9} the preliminary experiments were carried out with 1,4-dioxane and styrene as the representative saturated and unsaturated substrates.

The second order rate constants of the diarylamines with peroxy radicals were measured by autoxidation of 1,4-dioxane, which is an excellent substrate to study autoxidations of saturated hydrocarbons at ambient temperatures as its oxidation kinetics are well characterized.²¹ It must be acknowledged that in the presence of a H-bond accepting solvent, the reactivity of RTAs is attenuated since they cannot react with peroxy radicals when engaged in a H-bond.^{22,23} This kinetic solvent effect (KSE) is well understood and the rate constant in the absence of an H-bonding solvent can be calculated based on the equations outlined by Snelgrove *et al.* (Eq. 4.3).²² The α_H^2 and β_H^2 parameters represent the hydrogen bond donating ability of the RTA and the hydrogen bond accepting ability of the solvent, respectively.^{24,25} The α_H^2 values were measured using the NMR approach developed by Abraham *et al* and used to calculate the rate constants in the absence of a H-bonding solvents.²⁶ The autoxidations were initiated using AIBN and the results are compared alongside 4,4'-di-*tert*-butyldiphenylamine (**4.9**) and 4,4'-di-methyldiphenylamine (**4.10**) in Table 4.1 (see representative data, Figure 4.3A and S4.1).

Rate constants for industrial standards **4.9** and **4.10** were consistent with the literature and measured to be $2.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $2.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, respectively.¹³ The reactivity of all the heterocyclic diarylamines were relatively similar and only varied 3-fold, ranging from 2.3×10^6 to $7.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. Compounds **4.3-4.5** experienced a modest ~1.5-fold increase in reactivity relative to **4.2**,¹⁵ likely resulting from a slight reduction of their BDE by the addition of alkyl substituents in the *para* position. Alternatively, compounds **4.6-4.9** were found to be the slowest of the set, presumably due to the steric hinderance owing to the *ortho* methyl groups. This observation is also borne out in the low α_H^2 values measured for the same compounds as the *ortho* substituents hinder the formation of a H-bond with the solvent. The stoichiometries for all compounds were *ca.* 2, suggesting that there was no observable regeneration occurring at ambient temperature in saturated hydrocarbons. This is consistent with the high activation energies required for efficient turnover of the alkoxyamine into the parent diarylamine through the Korcek cycle (*cf.* Figure 4.1C).¹¹ Moreover, these experiments did not offer insights on the benefits provided by substituting the aromatic ring with alkyl groups to prevent the off-cycle reactions.

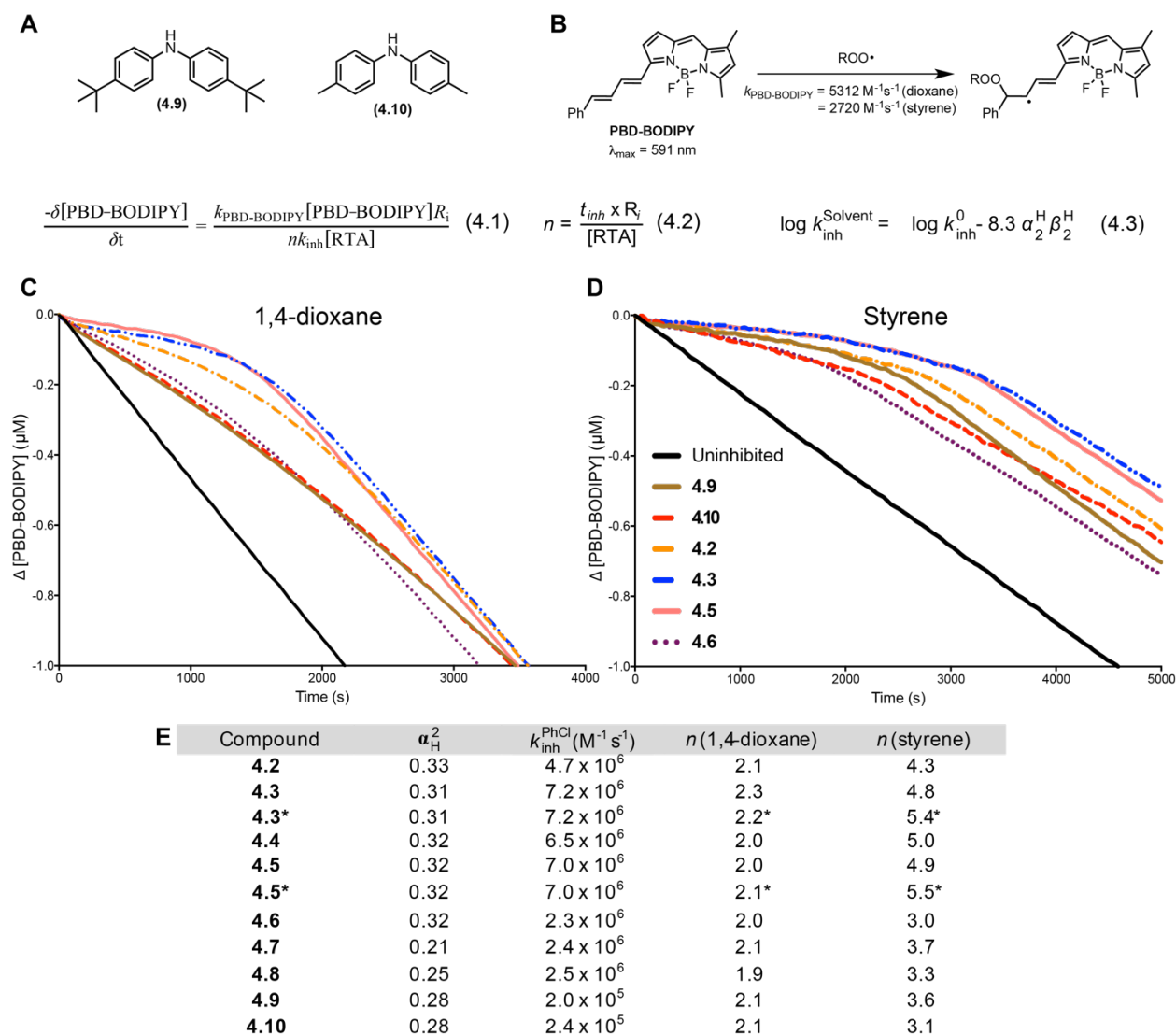


Figure 4.3 (A) Structures of dialkyldiphenylamines commonly used in the industry (B) PBD-BODIPY serves as the signal carrier in 1,4-dioxane and styrene autooxidations, respectively, enabling determination of inhibition rate constants (k_{inh} , Eq. 4.1) and stoichiometries (n , Eq. 4.2) for reactions of inhibitors with chain-carrying peroxy radicals. Co-autoxidation of 1,4-dioxane (2.9 M) and PBD-BODIPY (10 μ M) initiated by AIBN (6 mM) in PhCl at 37°C monitored at 587 nm ($\epsilon = 123,023 \text{ M}^{-1}\text{cm}^{-1}$) or styrene (4.3M) and PBD-BODIPY (10 μ M) initiated by AIBN (6 mM) in PhCl at 37°C monitored at 591 nm ($\epsilon = 139,000 \text{ M}^{-1}\text{cm}^{-1}$). Uninhibited (black), and inhibited with 2 μ M of **4.9** (brown), **4.10** (red), **4.2** (orange), **4.3** (blue), **4.5** (pink) or **4.6** (purple). (E) Abraham H-Bond acidity parameters, Chlorobenzene inhibition rate constants, and Stoichiometries for various RTAs in 1,4-dioxane and styrene. Compounds denoted with * were tested with $\frac{1}{2} R_i$ (i.e. 3 mM AIBN)

We wondered whether heterocyclic diarylamines might regenerate at ambient temperatures in styrene autoxidations by the intervention of nitroxides through our recently reported mechanism (Scheme 4.5). It would also be an indirect approach to probe whether nitroxides are formed from heterocyclic diarylamines as all attempts to isolate authentic nitroxides derived from **4.2-4.8** have been unsuccessful. We anticipated that *in situ* formation of the nitroxides may be sufficient to recycle the compounds, allowing us to evaluate the influence of the alkyl substituents (Figure 4.3D, S4.2). Since nitroxide regeneration would compete with the deleterious reactions, compounds more resistant to degradation should yield higher stoichiometries relative to **4.2**. The stoichiometry for compounds **4.3**, **4.4** and **4.5** saw a modest increase from 4.3 to ca. 5, highlighting that efforts to reduce these deleterious pathways might yield stable compounds capable of undergoing catalytic regeneration. Compounds **4.6**, **4.7** and **4.8** had lower stoichiometry (~ 3.5 vs 4.3) likely due to the decreased reactivity towards peroxy radicals from the sterics in the *ortho* position. These results offer indirect support of nitroxide formation under these reaction conditions. The foregoing experiments at ambient temperatures suggest that attempts to mitigate the non-productive pathways from the addition of peroxy radicals on diarylamines should be mainly focused on *para* substituents as it results both in an enhanced reactivity towards peroxy radicals and a higher stoichiometry compared to the *ortho* substituents.

4.3.4 Inhibited autoxidation at 100°C

Having characterized the efficacy of the compounds at ambient temperature and demonstrated their ability to regenerate catalytically, we employed a similar co-autoxidation method using PBD-BODIPY to carry out autoxidations of 1-hexadecene at elevated temperature. We utilized 100°C as an intermediate temperature to ascertain whether the trends observed at 37°C translate to higher temperatures (Figure 4.5A). Most rate constants measured for compounds at 100°C were similar to ambient temperatures (Figure 4.5B). This can be readily explained due to the small activation enthalpies for the reaction between the diarylamines and peroxy radicals, whereby raising the temperature only has a small effect on the magnitude of k_{inh} (e.g. $\log A = 6.9$ and $E_a = 2.5$ kcal/mol for **4.10**).¹³ Initially we expected the

regeneration to be limited by the turnover of the alkoxyamine, which should increase with the stability of the diarylaminy radicals derived from **4.2-4.8** (N-H BDE ~ 77 kcal/mol) compared to **4.9-4.10** (N-H BDE = 82 kcal/mol).¹³ However, the apparent rate constant of inhibition for the industrial standards **4.9** and **4.10** were an order of magnitude higher at 100°C ($\sim 2.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$). The rate constants are very similar to those of the heterocyclic analogs, suggesting that the values weren't reporting on the reaction between a diarylamine (N-H) and a peroxy radical. Instead, the rate constants are consistent with the reaction of 4,4'-di-*tert*-butyldiphenylnitroxide (TBPNO) and peroxy radicals in olefinic substrates.⁸ This is not surprising given the tendency of 1-hexadecene to regenerate nitroxides to their corresponding hydroxylamines by the cross-dismutation of hydroperoxy radicals (Scheme 4.5A,B). Moreover, the comparable rate constants imply that the regeneration of the compounds does not significantly depend on the structure of the RTA but rather on the structure of the substrate. The small changes in k_{inh} likely result from the minor structure variations of the parent diarylamine.

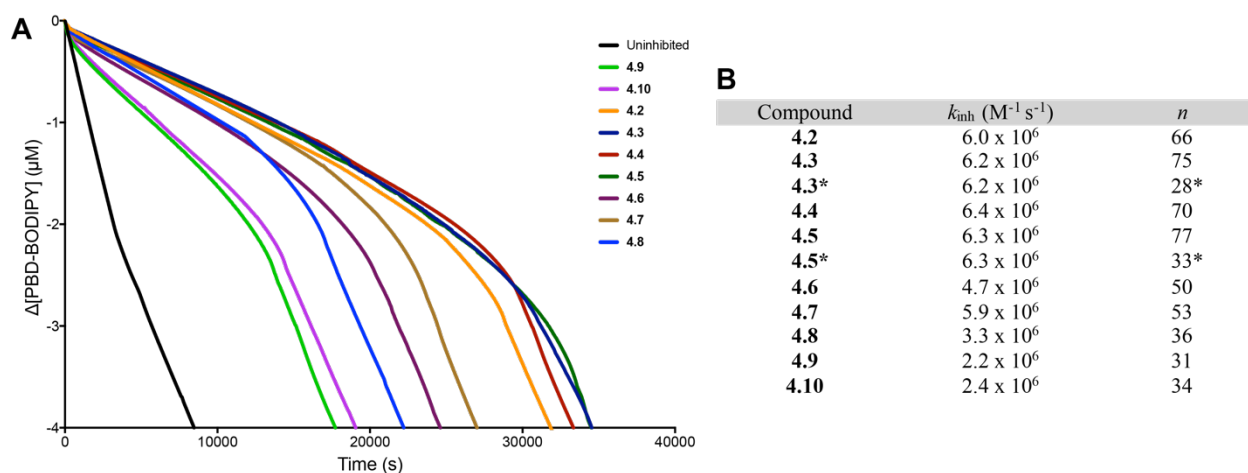


Figure 4.4 (A) Co-autoxidation of 1-hexadecene (2.8 M) and PBD-BODIPY (10 μM) initiated by dicumyl peroxide (1 mM) in PhCl at 100°C, monitored at 587nm ($\epsilon = 131,972 \text{ M}^{-1} \text{ cm}^{-1}$). Uninhibited (black), or inhibited with 2 μM of **4.2** (orange), **4.3** (navy blue), **4.4** (red), **4.5** (dark green), **4.6** (purple), **4.7** (brown), **4.8** (blue), **4.9** (green) or **4.10** (magenta). (B) Inhibition rate constants and stoichiometries for compounds **4.2 – 4.10**. Compounds denoted with * were tested with 2 R_i (i.e. 2 mM dicumyl peroxide)

There was a general correlation between the rate constants and stoichiometries where they roughly increased with respect to one another. The calculated values were some of the largest ever

measured, with **4.3** and **4.5** reaching 75 and 77, respectively. There was also a greater resolution of the stoichiometries at this temperature, where all *para* substituted compounds (**4.3-4.5**) had greater values compared to the *ortho* substituted analogs (**4.6-4.9**) ($n = 77$ for **4.5** vs $n = 36$ for **4.8**). However, the increase in stoichiometries were relatively minor compared to the unsubstituted compound **4.2** ($n = 66$) arguing whether alkyl substitution really do offer any benefits under these conditions. Nevertheless, the stoichiometry values suggest that the compounds are effective inhibitors and reasonably stable towards off-cycle reactions under these conditions. The results indicate that the compounds regenerate by nitroxide mediated cross-dismutation of hydroperoxyl radicals, similar to styrene autoxidations. Since the compounds do not inhibit indefinitely, the decomposition pathway likely stems from the nitroxide intermediate. The positive trends prompted us to evaluate the compounds in high temperature autoxidations.

4.3.5 High Temperature Autoxidations at 160°C

Majority of diarylamines are used in applications over 100°C to take advantage of their catalytic behavior (Figure 4.1B).⁷ Diarylamines **4.2-4.10** were assayed for their ability to inhibit the oxidation of *n*-hexadecane by monitoring the production of hydroperoxides upon heating to 160°C under a constant positive pressure of O₂. This ensures that the mass transfer of oxygen does not become rate limiting which can affect the kinetics of the autoxidations.^{27,28} The experiments were carried out in our newly developed parallel stirred-flow reactor where aliquots were removed at pre-determined intervals.¹⁶ Primene was added as a base to react with the excess acid that is produced and known to decrease the reactivity of heterocyclic diarylamines.¹⁵ Hydroperoxides were determined using the recently developed coumarin-conjugated triarylphosphine which undergoes a significant fluorescence enhancement upon oxidation with a hydroperoxide, since the phosphine oxide cannot quench the fluorescence of the coumarin (Figure 4.5A).^{16,29} Because the rate constant for the oxidation of the phosphine by secondary hydroperoxides is known, the hydroperoxide concentration can be estimated from the initial rate (Eq. 4.4). The results are shown in Figure 4.5.

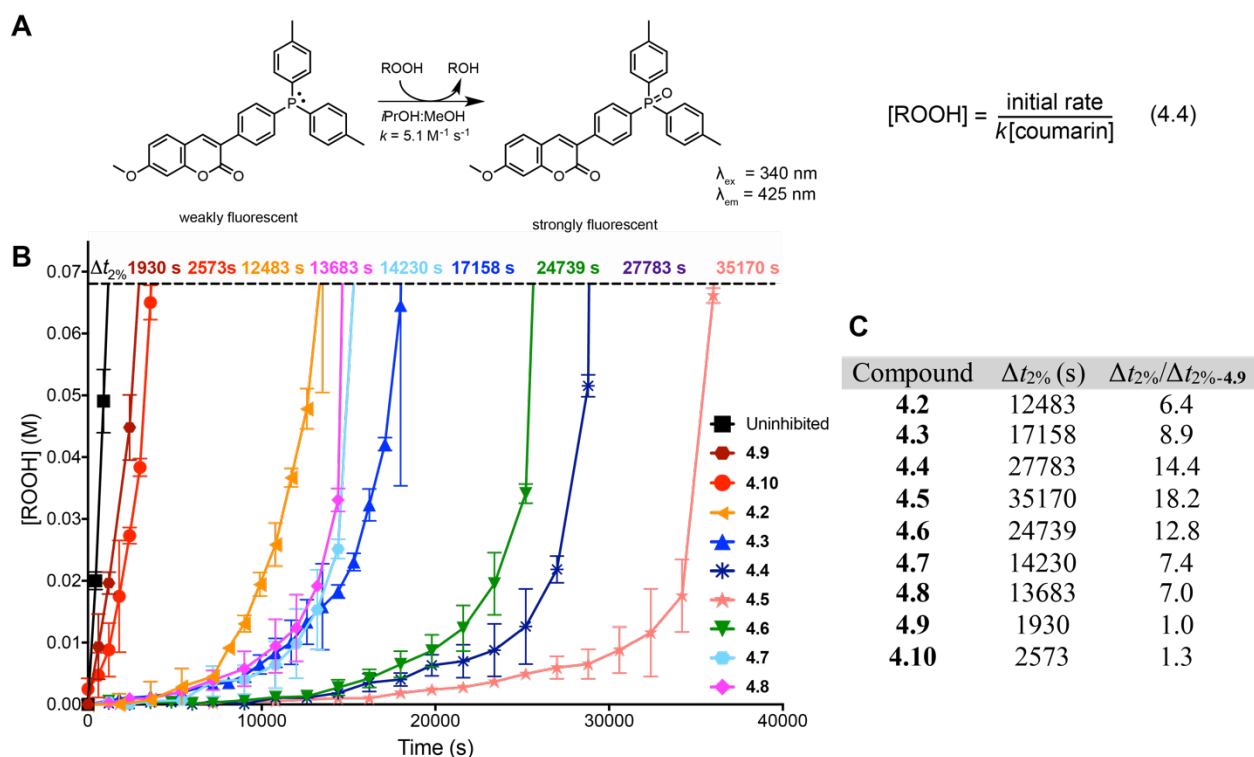


Figure 4.5 (A) Coumarin-conjugated triarylphosphine enables the quantification of hydroperoxides in *n*-hexadecane autoxidations using Eq. 4.4. (B) Hydroperoxide formation during the inhibited autoxidation of *n*-hexadecane at 160°C, no initiator, uninhibited (black), or inhibited with 200 μM of **4.9** (maroon), **4.10** (red), **4.2** (orange), **4.3** (blue), **4.4** (navy blue), **4.5** (pink), **4.6** (green), **4.7** (cyan), or **4.7** (maroon). All autoxidations are carried out in the presence of 2 mM Primene-81R (C) Absolute and relative inhibition times of different heterocyclic diarylamine RTAs.

The autoxidations were followed for the first 2% of the reaction (68 mM based on hydroperoxide yield), since beyond ca. 3% conversion the hydroperoxides begin to decompose to yield a mixture of ketones, alcohols, carboxylic acids, and esters (Figure 4.5B).^{5,27} Moreover, at this temperature the hydroperoxides commonly undergo homolysis to generate initiating radicals which gradually increases the rate of initiation, resulting in an autocatalytic reaction. As such, it is not possible to quantitatively determine the stoichiometry (n) and inhibition rate constants (k_{inh}) of the reaction of the diarylamines with peroxy radicals since the autocatalytic nature of the reaction means that R_i changes as the reaction proceeds.⁵ Thus, we “quantified” the efficacy of the inhibition of the heterocyclic diarylamines relative to

the industry standard **4.9** by comparing the times required for 2% of the substrate to be autoxidized ($\Delta t_{2\%}$, Figure 4.5C).

All compounds were effective inhibitors of hexadecane and compared favourably to the two industrial standards. It should be noted that these reactions are carried out in 95% *n*-hexadecane, where the rate of initiation is higher than in the 99% *n*-hexadecane used in our previous studies. Compound **4.2** which was 12-fold better under our older reaction condition was only found to be 6.4-fold more potent under these conditions.¹⁵ There was significant difference in the inhibition times with **4.4** and **4.5** preventing the autoxidation 18.2 and 14.2-fold better than the industrial standards. The results obtained were slightly different than those obtained at lower temperatures as the two best performing compounds were **4.4** and **4.5** as opposed to **4.3** and **4.5**. Remarkably, all compounds performed better than **4.2**, demonstrating that shielding the *ortho* or *para* position to develop more stable compounds was successful. Even **4.7** and **4.8**, delayed the reaction for 14230 s and 13683 s, respectively, compared to 12483 s for **4.2**. The longest inhibition time was for **4.5**, with a simple methyl group in the *para* position of **4.2**, and it afforded a 2.8-fold increase relative to the unsubstituted compound. Analogous compound **4.3** with the *tert*-butyl group was only 1.3-fold better, perhaps due to the lability of *tert*-alkyl groups at high temperatures.³⁰

Most importantly, the collective trends show that the best compounds were ones with exposed benzylic positions. Since saturated hydrocarbons do not produce significant quantities of hydroperoxyl radicals (see Scheme 4.5) we considered the source of the catalytic activity. We quantified the concentrations of diarylamines from the autoxidation mixture over time by measuring their inherent fluorescence. Compounds **4.3**, **4.5** and **4.10** are shown in Figure 4.6A (and Figure S4.3) for ease of comparison. It is clear that the depletion of the diarylamine coincides with a rapid increase in hydroperoxides. However, for **4.3** and **4.10**, the hydroperoxide concentration increased even when approximately 40% of the compound was still present, while autoxidation was completely suppressed until **4.5** was entirely consumed. There was also a difference in the rate of consumption of the two heterocyclic

compounds. The concentration of **4.3** gradually decreased while that of **4.5** was relatively constant throughout the inhibited period, suggesting that **4.3** (and **4.10**) or intermediates derived therefrom were not effective inhibitors of autoxidation. This cannot be ascribed to their reactivity towards the peroxy radicals because both diarylamines have similar inhibition rate constant (*cf.* Table 4.1).

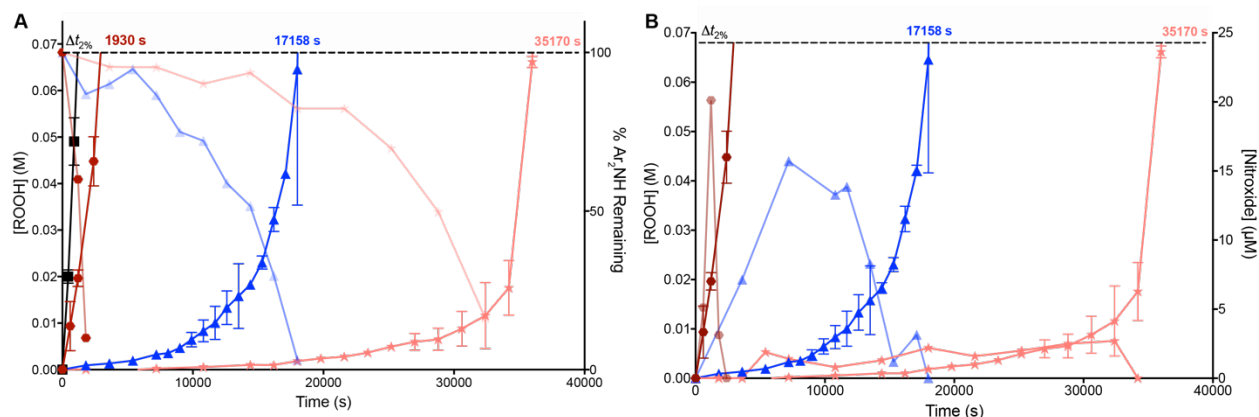


Figure 4.6 (A) Percentage of **4.9** (faint-maroon), **4.3** (faint-blue) and **4.5** (faint-pink) remaining over time, overlaid with their corresponding inhibitory activity. (B) Concentration of nitroxide formed from **4.9** (faint-maroon), **4.3** (faint-blue) and **4.5** (faint-pink) during inhibited autoxidations.

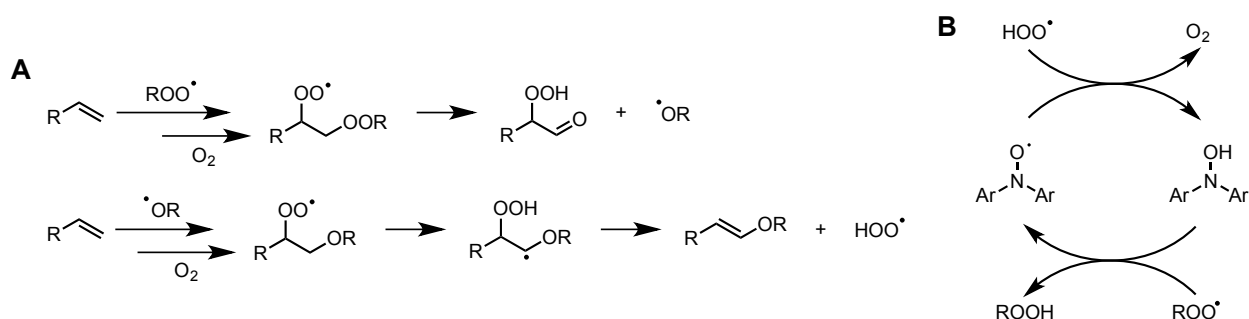
Since hydroperoxyl radicals are not formed in saturated hydrocarbons, it would suggest that Korcek mechanism would be the predominant pathway by which compounds regenerate. However, the key step in the Korcek cycle requires the formation of alkoxyamine by a competition between a nitroxide and oxygen for alkyl radicals. This is difficult given that the reaction of nitroxides with alkyl radicals is at least an order of magnitude slower^{31,32} than that of O_2 ³³ and would require the concentration of nitroxide to be far higher than oxygen (~ 2 mM).³⁴ To gain insight into this paradox, we measured the formation of nitroxides in our samples (Figure 4.6B, S4.4). We observed that **4.3** led to a rapid increase in nitroxide concentration that peaked at $15 \mu M$ at approximately 7200s, after which there was a drop in its concentration overlapping with an increase in the hydroperoxides concentration. Instead, **4.5** had a very low ($\sim 2 \mu M$) but steady concentration of nitroxide throughout the inhibited phase. Similarly, it was realized that compounds with poor inhibition times (**4.3**, **4.7** and **4.8**) were ones where there was an accumulation of nitroxide followed by its decomposition, indicating that in saturated hydrocarbons nitroxide accumulation is detrimental to the regeneration of diarylamines.

4.4 Discussion

Application of novel RTAs in ‘real-world’ requires them to be significantly superior than the existing industrial standards, as such it is desirable to optimize the structure of our heterocyclic compounds to increase their efficacy. From our understanding of the mechanism of diarylamine RTAs, it is anticipated that the reactions that limit the effectiveness of **4.2** is the addition of peroxy radicals to the phenyl ring of either the diarylaminyll intermediate (*cf.* Scheme 4.2 and 4.3), or the diarylnitroxide intermediate (see Scheme 4.7).¹¹ Both peroxidic adducts are expected to decompose very rapidly to yield a mixture of non-RTA products. We expected that substituting the phenyl ring of **4.2** with either a *t*-alkyl substituent (**4.3**), a primary *t*-alkyl substituent (**4.4**) or a primary alkyl group (**4.5**) would slow addition of peroxy radicals to the *para* position, favouring the desired reaction pathway. This was certainly the case in autoxidations of styrene and 1-hexadecene. The compounds were slightly better than **4.2**, and they saw an increase in both their k_{inh} and n values. Since addition of the peroxy radical to one of the *ortho* positions of the phenyl ring may also take place, **4.6**, **4.7** and **4.8** were designed. We observed the *ortho* substituted compounds to have diminished reactivity relative to **4.2**. There was a decrease in their k_{inh} and n values, likely because the *ortho* substituents increase the steric hindrance near the labile N-H bond and adversely affect their reactivity by forcing them to adopt an unreactive conformation wherein the two aromatic rings are orthogonal to each other (Figure S4.7).³⁵

We were excited to observe the reactivity of the new compounds translate well from 37°C to 160°C. This was also the first evidence of heterocyclic diarylamines catalytically trapping peroxy radicals at temperatures lower than 120°C, suggesting it was through an alternative pathway to the Korcek cycle. The mechanism of diarylamines is reliant on the nature of the substrate and nitroxides have been invoked as catalytic agents in unsaturated hydrocarbons (Scheme 4.5).⁸ However, since persistent nitroxides of the compounds have yet to be isolated, we considered that their transient production may be responsible for the high stoichiometric numbers. Thus, we quantified their concentration in styrene and 1-hexadecene autoxidations for the two best compounds, **4.3** and **4.5**, and observed a low but steady concentration of nitroxide throughout the inhibited period for styrene autoxidations (Figure S4.5).

However, a significant amount was converted to nitroxide (e.g. 1.2 μM for **4.3** and 0.8 μM for **4.5**) in 1-hexadecene, and their concentrations tracked well with the inhibited period (Figure 4.7A). These results explain why a longer inhibition period is observed in 1-hexadecene relative to styrene. Moreover, the results in both olefinic substrates are consistent with the regeneration of nitroxides to their corresponding hydroxylamine by the cross-dismutation of hydroperoxyl radicals (Scheme 4.5). Compounds **4.6-4.8** had lower reactivity and stoichiometries because the *ortho* methyl groups hinder the reactivity with the nitroxide centre, and in **4.8** it reduces the planarity of the molecule resulting in decreased conjugation with the radical centre, preventing spin delocalization (Figure S4.7).³⁵



Scheme 4.5 (A) Formation of hydroperoxyl radicals from the addition of alkoxy radicals to olefinic substrates. (B) Cross-dismutation of hydroperoxyl radicals to catalytically regenerate nitroxides to their corresponding hydroxylamines

The increase in efficacy of **4.3-4.5** relative to **4.2** strongly supported the notion that compound modifications were in the right direction. The steady state concentration of nitroxide in unsaturated substrates prevents them from undergoing the deleterious reactions and funnels the intermediates into the productive pathway, effectively ‘protecting’ the compounds. As such, the catalytic activity is less reliant on the structure of the RTA (or the intermediate nitroxide) because the facile regeneration via hydroperoxyl radicals enables efficient trapping of peroxy radicals. However, the nitroxides must eventually undergo non-productive reactions explaining why we do not observe indefinite inhibition by these compounds. To validate our theory, we doubled the rate of initiation to observe whether the higher concentration of peroxy radicals would result in more off-cycle reactions and yield lower stoichiometries. This was indeed the case, whereby increasing the rate of initiation resulted in significantly reduced

stoichiometries ($n = 28$ and 33 for **4.3** and **4.5**, Figure 4.3E, 4.7B) compared to the original conditions (where $n = 75$ and 77 for **4.3** and **4.5**, cf. Figure 4.4B). Both compounds have vacant *ortho* sites and likely undergo decomposition by pathways shown in Scheme 4.7, which are analogous to the diarylaminyll side-reactions discussed previously (cf. Scheme 4.3), but involve a nitroxide instead. It is interesting to observe that **4.3** which accumulates a larger concentration of nitroxide, had a corresponding lower stoichiometry ($n = 28$ vs 75) under high rates of initiation, supporting our rationale that nitroxides regeneration is in constant competition with the off-cycle reactions. Subsequently, when the rate of initiation was decreased by half in styrene autoxidations, the stoichiometries for **4.3** and **4.5** increased to 5.4 and 5.5 from 4.8 and 4.9 , respectively (Figure S4.4B, 4.3E). The foregoing results establish nitroxides as important intermediates in the regeneration of diarylamines, where their accumulation is also responsible for the decomposition of the diarylamines. Consistently, in the autoxidation of dioxanes, which does not involve the recycling of nitroxides, the stoichiometries remained at ~ 2.0 even when the rate of initiation was halved. The results corroborate our computational insights on the off-cycle pathways contributing to diarylamine decomposition. As mentioned previously, this likely occurs by the energetically favourable tautomerization of the alkylperoxy intermediate since the direct O–O homolysis reaction is highly unfavourable.

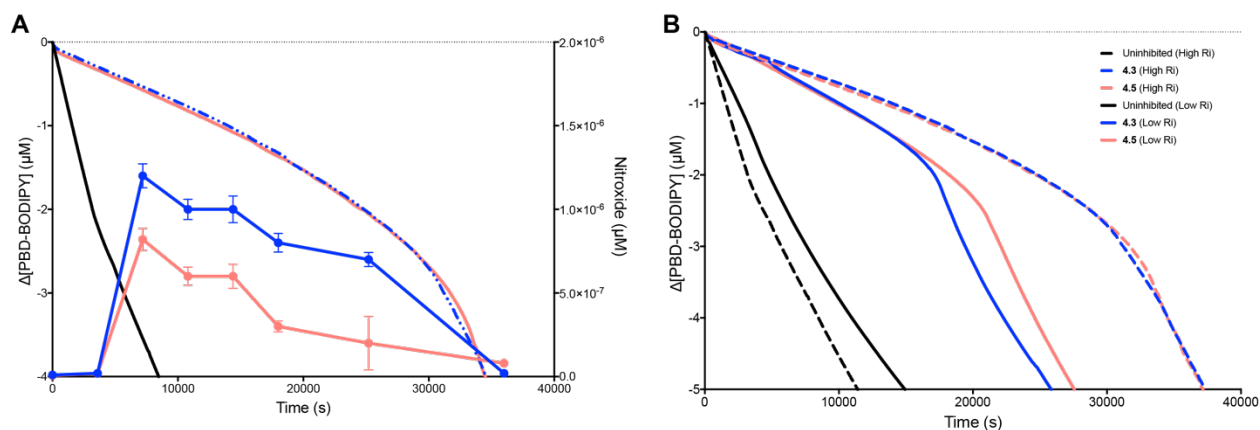
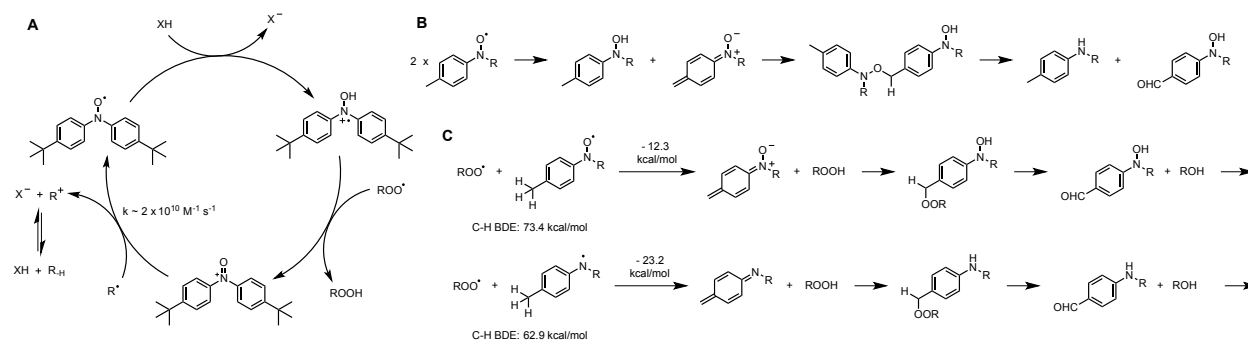


Figure 4.7 (A) Concentration of Ar_2NO determined by EPR during **4.3** and **4.5**-inhibited co-autoxidations of hexadecene. (B) Co-autoxidation of 1-hexadecene (2.8 M) and PBD-BODIPY (10 μM) initiated by dicumyl peroxide (1 mM or 2 mM) in PhCl at 100°C , monitored at 587nm ($\epsilon = 131,972 \text{ M}^{-1}\text{cm}^{-1}$). Uninhibited (black), or inhibited with 2 μM of **4.3** (blue) or **4.5** (pink). Data obtained with 1 mM

dicumylperoxide is denoted in dashed lines, while corresponding data with 2 mM dicumylperoxide is shown with solid lines.

These findings are also corroborated in high-temperature autoxidations. When comparing **4.3** and **4.5**, we observe a significant increase in nitroxide concentration for **4.3** (15 μM vs 2 μM), which results in an overall decreased inhibition for **4.3**. All heterocyclic compounds can be categorized in two groups, ones that accumulate nitroxides and ones that have a low steady concentration. Compounds **4.3**, **4.7** and **4.8** accumulated large concentrations but had relatively low inhibition times compared to **4.4**, **4.5** and **4.6** which demonstrated opposite trends. The discrepancy between inhibition time arises because hexadecane is not activated for the production of hydroperoxyl radicals (Scheme 4.5), as such the more persistent nitroxides have higher susceptibility to undergo the off-cycle reactions. This is exacerbated at higher temperatures because the rate of initiation gradually increases due to autocatalysis as opposed to the constant rate of initiation observed in styrene and 1-hexadecene autoxidations, where diarylamines with a low steady state concentration of the nitroxide are ‘protected’ from the decomposition reactions. However, these results are not consistent with the Korcek cycle which requires nitroxides to react with alkyl radicals to form alkoxyamines – already a losing competition with oxygen. Hence, if nitroxide accumulation is detrimental then how are the alkoxyamines formed, if at all, and what is the real mechanism of regeneration in saturated hydrocarbons at elevated temperatures?



Scheme 4.6 (A) Proposed mechanism of acid-catalyzed RTA activity of nitroxides in saturated hydrocarbons (B) Nitroxide disproportionation, followed by substitution and fragmentation to yield amine and hydroxylamine. Calculated values represent the ΔH of the reactions. (C) H-atom abstraction from benzylic position on diarylnitroxide (top) or diarylaminyl (bottom) radical to generate amines or hydroxylamines.

It should be recognized that small quantities of alkoxyamines may form, but it is not sufficient to rationalize the elongated inhibition periods observed for the diarylamines. Perhaps, different mechanisms should be considered. We previously reported a mechanism to support the regeneration of hindered amine light stabilizers (HALS) which involved the acid-catalyzed reaction of a nitroxide with a peroxy radical to yield an oxoammonium ion, followed by an electron-transfer from an alkyl radical to the oxoammonium ion to reform the nitroxide.³⁶ We envision, such a mechanism may still account for the regeneration of diarylnitroxides, especially ones that are formed in large quantities (**4.3**, **4.7** and **4.8**) (Scheme 4.6A). The reaction would be facilitated by carboxylic acids that are produced *in situ* in high-temperature autoxidations.^{15,16} Moreover, the electron-transfer from alkyl-radical to TEMPO-oxoammonium ion was calculated to be $\sim 2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$, and while diaryloxoammonium ion is a weaker oxidant, the high rate constant may still enable it to compete with oxygen.⁹ While this mechanism could be relevant for **4.4**, **4.5** and **4.7**, it is difficult to reconcile since they do not accumulate large amounts of nitroxides. Forrester *et al.* has previously shown that nitroxides with benzylic positions are extremely reactive and undergo disproportionation to yield a hydroxylamine and the corresponding imine-*N*-oxide, which dimerize and fragment to produce a hydroxylamine and the amine (Scheme 4.4B).^{37,38} These compounds can further trap more peroxy radicals. However, the low steady-state concentration of nitroxides in solution may prevent such a reaction from occurring, but a similar reaction with peroxy radical can be anticipated (Scheme 4.6C, top).

Preliminary computations suggest that the low BDE of the benzylic hydrogens and the exothermicity of the reaction may make it a viable pathway. In fact, this reaction is not only limited to nitroxides but may also occur with the intermediate diarylaminy radical as evident by an even lower BDE and a more exothermic reaction (Scheme 4.4C, bottom). These reactions are in constant competition with the addition pathway, and it appears that the energetics of the benzylic H-atom abstraction from the aminyl radical ($\Delta H = -23.2 \text{ kcal/mol}$) compared to the addition of peroxy radical in the *para*-position ($\Delta H = -34.4 \text{ kcal/mol}$) are less favourable. However, at elevated temperatures the addition is likely

reversible and fragments to give the aminyl radical, while the H-atom abstraction to yield the imine-methide would be irreversible. The alkylperoxy adduct also cannot undergo tautomerization and subsequent O–O bond cleavage to yield the imino-quinones, further driving the intermediates back to the aminyl radical. It should be noted that while the *ortho*-sites are still vacant and can undergo the addition-tautomerization-disproportionation steps, the reversibility at high temperatures likely funnels the intermediates back to the aminyl radical which can undergo an irreversible H-atom abstraction to yield the imine-methides. While, the fates of the corresponding nitroxide intermediate are very similar, the enthalpy of reaction is less favourable and further computations are required to accurately compare them against the analogous addition reactions.

The resultant amines/hydroxylamines derived the benzylic H-abstraction pathway can trap more peroxy radicals, but only accounts for the additional inhibition of 1-2 radical chains, and does not justify the elongated inhibition periods that are observed. Perhaps, other mechanisms responsible for the inhibition exist that have yet to be identified. Investigations to probe the importance of the benzylic substituents are underway and it is anticipated they will shed insight into the extended inhibition observed for the benzylic compounds.

4.5 Conclusion

Attempts to bolster the reactivity and stability of the best performing heterocyclic diarylamine **4.2** was successful. We have demonstrated for the first time that heterocyclic diarylamines form nitroxides *in situ* and are capable of undergoing catalytic regeneration in unsaturated substrates by the cross-dismutation of hydroperoxyl radicals. This resulted in some of the highest stoichiometries that have been reported till date. Moreover, our results shed light on the dual function of nitroxides – while they are excellent inhibitors in unsaturated substrates, their accumulation is the predominant decomposition pathway in saturated substrates. These problems can be circumvented in high temperature autoxidations of saturated hydrocarbons by utilizing compounds with *para* benzylic positions to the reactive N-H. These compounds demonstrate unparalleled inhibition times in saturated and unsaturated substrates, and are a substantial

improvement compared the previously reported ‘lead’ RTA. Overall, our results demonstrate that future efforts aimed towards improvement of diarylamine RTAs should be concentrated on pyrimidine-scaffolds with exposed benzylic sites in the *para* position or analogs thereof in order to maintain low steady-state concentration of nitroxide to limit the deleterious reactions.

4.6 References

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4.7 Experimental

4.7.1 General method for the preparation of 5-bromo-N,N-dialkylaminopyrimidines

To a solution of 5-bromo-2-aminopyrimidine (1.0 mmol) in dry THF (2 mL) at 50°C, NaH (2.2mmol) was added slowly and the mixture was stirred until H₂ evolution ceased (~ 20 minutes). Octylbromide (2.1 mmol) was then added and the reaction was refluxed overnight. The reaction was cooled, quenched with MeOH and extracted with Et₂O. The combined organics were washed with brine and dried over MgSO₄. The solvent was evaporated and the oil obtained was passed through a plug of silica (20% Et₂O/Hexanes) to obtain the product in analytical purity. NMR spectra were in accordance to literature.

4.7.2 General Procedure for Buchwald-Hartwig Cross-Couplings

To a Schlenk flask were added Pd₂dba₃ (0.01mmol) and BippyPhos (Pd/L = 1:4). The flask was evacuated and backfilled with argon before *tert*-amylalcohol (1 mL) was added, followed by KOH (1.5 mmol) and H₂O (1.0% v/v), and the solution was stirred for 20 minutes. After 20 min, ArBr (1.0 mmol) and ArNH₂ (1.2 mmol) were added, and the reaction was heated to 110°C. Once the reaction was judged complete by TLC analysis (~ 36 hours), the solvent was removed under reduced pressure and the residue loaded on a silica gel flash column. Column chromatography (Et₂O/hexanes eluent with 1-3% Et₃N) afforded the product in analytical purity.

***N*²,*N*²-dioctyl-*N*⁵-phenylpyrimidine-2,5-diamine (4.2)** Yield: 89%. Yellow Oil. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.19 (s, 2H), 7.52 (s, 1H), 7.11 (dd, *J* = 8.5, 7.2 Hz, 2H), 6.70 (dt, *J* = 7.7, 1.1 Hz, 2H), 6.68

– 6.63 (m, 1H), 3.54 – 3.43 (m, 4H), 1.54 (t, $J = 7.2$ Hz, 4H), 1.34 – 1.15 (m, 21H), 0.89 – 0.81 (m, 6H).

^{13}C NMR (101 MHz, DMSO- d_6) δ 158.45, 153.65, 147.05, 129.64, 126.47, 118.22, 113.77, 47.73, 31.69, 29.31, 29.14, 27.76, 26.89, 22.54, 14.40. HRMS: m/z Calc: $\text{C}_{22}\text{H}_{36}\text{N}_6$: 410.6500 Found: 410.6505.

N^5 -(4-(*tert*-butyl)phenyl)- N^2,N^2 -dioctylpyrimidine-2,5-diamine (4.3) Yield: 92%. Brown Oil. ^1H NMR (400 MHz, DMSO- d_6) δ 8.17 (s, 2H), 7.38 (s, 1H), 7.18 – 7.09 (m, 2H), 6.65 (d, $J = 8.7$ Hz, 2H), 3.53 – 3.42 (m, 4H), 1.54 (t, $J = 7.2$ Hz, 4H), 1.35 – 1.22 (m, 20H), 1.21 (s, 9H), 0.89 – 0.79 (m, 6H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 153.11, 126.19, 113.69, 47.69, 34.03, 31.84, 31.71, 29.33, 29.16, 27.80, 26.91, 22.55, 14.40. HRMS: m/z Calc: $\text{C}_{22}\text{H}_{36}\text{N}_6$: 466.7580 Found: 466.7582.

N^2,N^2 -dibutyl- N^5 -(4-neopentylphenyl)pyrimidine-2,5-diamine (4.4) Yield: 91%. Yellow Oil. ^1H NMR (400 MHz, DMSO- d_6) δ 8.19 (d, $J = 0.7$ Hz, 2H), 7.40 (s, 1H), 6.88 (d, $J = 8.4$ Hz, 2H), 6.64 (d, $J = 8.3$ Hz, 2H), 3.57 – 3.42 (m, 4H), 2.32 (s, 2H), 1.59 – 1.48 (m, 4H), 1.29 (h, $J = 7.4$ Hz, 4H), 0.91 (t, $J = 7.3$ Hz, 7H), 0.83 (s, 9H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 158.34, 153.19, 144.91, 131.39, 128.99, 127.00, 113.42, 49.14, 47.49, 31.89, 30.06, 29.56, 20.19, 14.37. HRMS: m/z Calc: $\text{C}_{22}\text{H}_{36}\text{N}_6$: 368.5690 Found: 368.5691.

N^2,N^2 -dioctyl- N^5 -(*p*-tolyl)pyrimidine-2,5-diamine (4.5) Yield: 88%. Clear Oil. ^1H NMR (400 MHz, DMSO- d_6) δ 8.16 (s, 2H), 7.36 (s, 1H), 6.93 (d, $J = 8.1$ Hz, 2H), 6.64 (d, $J = 8.4$ Hz, 2H), 3.48 (dd, $J = 8.7, 6.3$ Hz, 4H), 2.16 (s, 3H), 1.62 – 1.46 (m, 4H), 1.36 – 1.15 (m, 21H), 0.88 – 0.78 (m, 6H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 152.88, 144.43, 130.04, 126.87, 114.16, 47.71, 31.70, 29.33, 29.15, 27.80, 26.91, 22.55, 20.57, 14.40. HRMS: m/z Calc: $\text{C}_{22}\text{H}_{36}\text{N}_6$: 424.6770 Found: 424.6777.

N^2,N^2 -dioctyl- N^5 -(*o*-tolyl)pyrimidine-2,5-diamine (4.6) Yield: 96%. Clear Oil. ^1H NMR (400 MHz, DMSO- d_6) δ 8.15 (s, 2H), 7.11 – 7.02 (m, 1H), 6.95 (t, $J = 7.7$ Hz, 1H), 6.71 (s, 1H), 6.64 (td, $J = 7.5, 1.1$ Hz, 1H), 6.54 (d, $J = 8.2$ Hz, 1H), 3.49 (t, $J = 7.5$ Hz, 4H), 2.20 (s, 3H), 1.54 (p, $J = 7.5, 7.0$ Hz, 4H), 1.30 – 1.20 (m, 20H), 0.88 – 0.82 (m, 6H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 158.47, 154.14, 145.28, 130.98, 127.03, 126.94, 118.75, 112.54, 47.69, 31.69, 29.31, 29.14, 27.77, 26.88, 22.54, 18.29, 14.40.

HRMS: m/z Calc: $\text{C}_{22}\text{H}_{36}\text{N}_6$: 424.6770 Found: 424.6775.

*N*⁵-(2,4-dimethylphenyl)-*N*²,*N*²-dihexylpyrimidine-2,5-diamine (4.7) Yield: 82% Brown Oil. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.11 (s, 2H), 6.89 (d, *J* = 2.0 Hz, 1H), 6.82 – 6.74 (m, 1H), 6.59 (s, 1H), 6.51 (d, *J* = 8.1 Hz, 1H), 3.48 (dd, *J* = 8.5, 6.4 Hz, 4H), 2.16 (d, *J* = 3.4 Hz, 6H), 1.55 (d, *J* = 7.8 Hz, 4H), 1.33 – 1.19 (m, 12H), 0.91 – 0.78 (m, 6H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 158.22, 153.07, 142.54, 131.70, 127.78, 127.68, 127.34, 124.93, 113.60, 47.69, 31.63, 27.81, 26.60, 22.59, 20.57, 18.23, 14.38. HRMS: *m/z* Calc: C₂₂H₃₆N₆: 382.5960 Found: 382.5962.

*N*²,*N*²-dihexyl-*N*⁵-mesitylpyrimidine-2,5-diamine (4.8) Yield: 73% Brown Oil. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.61 (s, 2H), 6.89 (s, 2H), 6.62 (s, 1H), 3.44 – 3.35 (m, 4H), 2.22 (s, 3H), 2.08 (s, 7H), 1.53 – 1.40 (m, 4H), 1.24 (s, 13H), 0.84 (q, *J* = 5.0, 3.5 Hz, 6H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 156.43, 144.28, 136.60, 134.46, 133.96, 132.12, 129.50, 47.64, 31.62, 27.91, 26.60, 22.57, 20.92, 18.49, 14.36. HRMS: *m/z* Calc: C₂₂H₃₆N₆: 396.6230 Found: 396.6233.

4.7.3 Inhibited Autoxidation of 1,4-Dioxane.

A 3.5 mL quartz cuvette was loaded with unstabilized 1,4-dioxane (0.625 mL) along with PhCl (1.805 mL). The cuvette was then preheated to 37 °C for approximately 5 min. The cuvette was blanked and 12.5 μL of 2 mM PBD-BODIPY in 2,3,5-trichlorobenzene was added followed by 50 μL of 0.3 M AIBN in chlorobenzene followed by a thorough mixing. After 10 min, an aliquot of antioxidant stock solution (1 mM) in chlorobenzene was added, and the loss of absorbance at 587 nm was followed. The inhibition rate constant (*k*_{inh}) and stoichiometry (*n*) were determined for each experiment according to the equations in Figure 4.2. Rate constants and standard derivations are derived from three independent experiments.

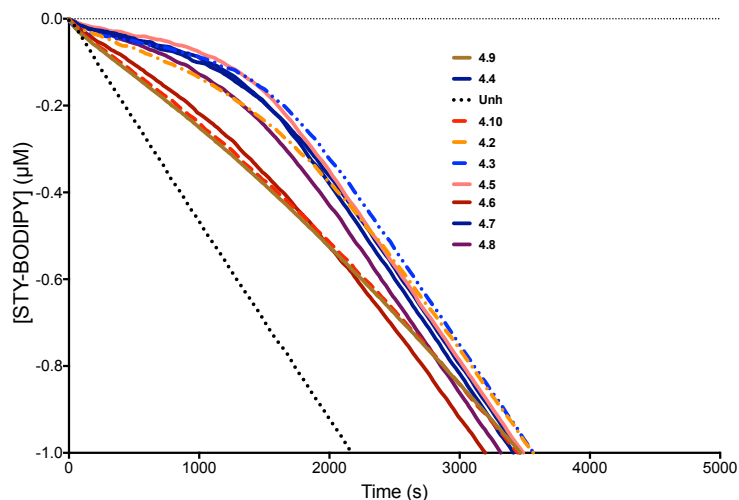


Figure S4.1 PBD-BODIPY serves as the signal carrier in 1,4-dioxane, enabling determination of inhibition rate constants (k_{inh}) and stoichiometries (n) for reactions of inhibitors with chain-carrying peroxy radicals. Co-oxidation of 1,4-dioxane (2.9 M) and PBD-BODIPY (10 μM) initiated by AIBN (6 mM) in PhCl at 37°C monitored at 587 nm ($\epsilon = 123,023 \text{ M}^{-1} \text{ cm}^{-1}$). Uninhibited (black), and inhibited with 2 μM of **4.2** – **4.10**.

4.7.4 Inhibited Autoxidation of Styrene

Styrene was washed thrice with 1 M aqueous NaOH, dried over MgSO_4 , filtered, distilled under vacuum and purified by percolating through silica, then basic alumina. To a cuvette containing 1.25 mL styrene was added 1.18 mL chlorobenzene and the solution equilibrated for 5 minutes at 37 °C. The cuvette was blanked and 12.5 μL of 2 mM PBD-BODIPY in 2,3,5-trichlorobenzene was added followed by 50 μL of 0.3 M AIBN in chlorobenzene and the solution was thoroughly mixed. After 40 minutes, an aliquot of RTA stock solution (1 mM) in chlorobenzene was added and the loss of absorbance at 591 nm followed. The inhibition rate constant (k_{inh}) and stoichiometry (n) was determined for each experiment according to equations in Figure 4.2. Kinetic data are given as averages of three independent measurements.

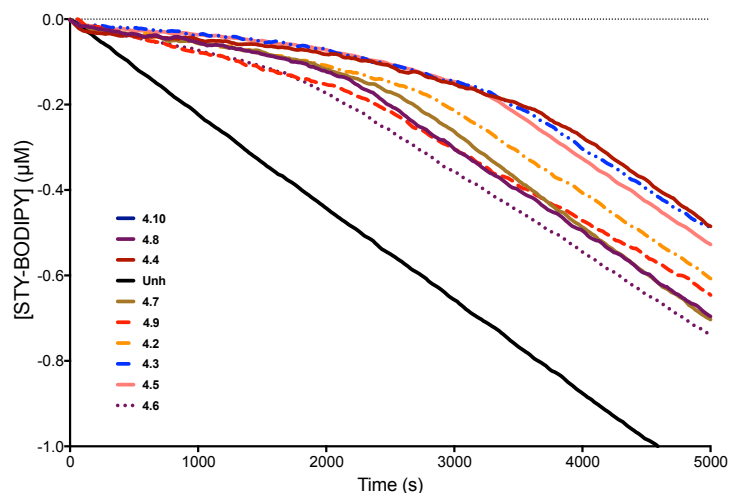


Figure S4.2 PBD-BODIPY serves as the signal carrier in styrene autoxidations, enabling determination of inhibition rate constants (k_{inh}) and stoichiometries (n) for reactions of inhibitors with chain-carrying peroxy radicals. Co-autoxidation of styrene (4.3M) and PBD-BODIPY (10 μM) initiated by AIBN (6 mM) in PhCl at 37°C monitored at 591 nm ($\epsilon = 139,000 \text{ M}^{-1}\text{cm}^{-1}$). Uninhibited (black), and inhibited with 2 μM of **4.2** – **4.10**.

4.7.5 Hexadecane Autoxidations at 160 °C

Hexadecane (10 mL) was added to test tubes in a 24-place parallel stirred-flow reactor, thoroughly degassed with argon, and then heated to 160°C. Once the temperature stabilized, 300 μM of inhibitors **4.2**–**4.10**, 2 mM Primene base, were added to the test tubes, and the flow of argon was replaced with O_2 . An aliquot (0.1 mL) was withdrawn at pre-determined intervals and allowed to cool to room temperature for analysis.

4.7.6 Quantifying Hydroperoxide Concentration

A small volume (5 μL) of each sample was loaded into the wells of a 96-well microplate and diluted with 2-propanol and methanol (1 : 4.215 mL) using the automated reagent dispenser of the microplate reader. Next, 30 μL of a solution containing the fluorogenic coumarin phosphine dye (100 mM) in acetonitrile was added to each well again using the automated reagent dispenser. The plate, incubated at 37°C, was stirred for 30 seconds, and after a 5 second delay, the fluorescence of each well was measured every

second for 60 seconds (coumarin phosphine dye: absorption 340 nm, emission 425 nm). The concentration of hydroperoxide in each well was determined from the rate of phosphine oxidation using the rate constant for the reaction of the dye with secondary hydroperoxides ($k = 5.1 \text{ M}^{-1} \text{ s}^{-1}$), assuming pseudo-first-order kinetics.

4.7.7 Determining diarylamine concentration

A small volume (5 μL) of each sample was loaded into the wells of a 96-well microplate and diluted with 2-propanol and methanol (1 : 4.215 mL) using the automated reagent dispenser of the microplate reader. Next, fluorescence spectra of each diarylamine was recorded between 300-700 nm by exciting at 360 nm. The emission maximum at 460 nm was used to calculate the percentage of diarylamine content by setting $t = 0$ as 100% diarylamine (300 μM).

4.7.8 Nitroxide Quantification by EPR

Electron paramagnetic resonance (EPR) spectra were recorded on a Bruker EMXplus (X-band) spectrometer equipped with an ER 4119HS cavity. Samples (100 μL) obtained from hexadecane, styrene or 1,4-dioxane autoxidations were loaded in a thin glass capillary and placed in an EPR tube. The radical concentration was determined at ambient temperatures using the quantitative EPR package of the Bruker Xenon software.

4.7.9 Calculations

All calculations were carried out using the CBS-QB3³⁹ complete basis set method as implemented in the Gaussian 09 suite of programs unless otherwise noted.⁴⁰ The DFT-optimized structures of compounds in Figure S4.7 were calculated using B3LYP level of theory.

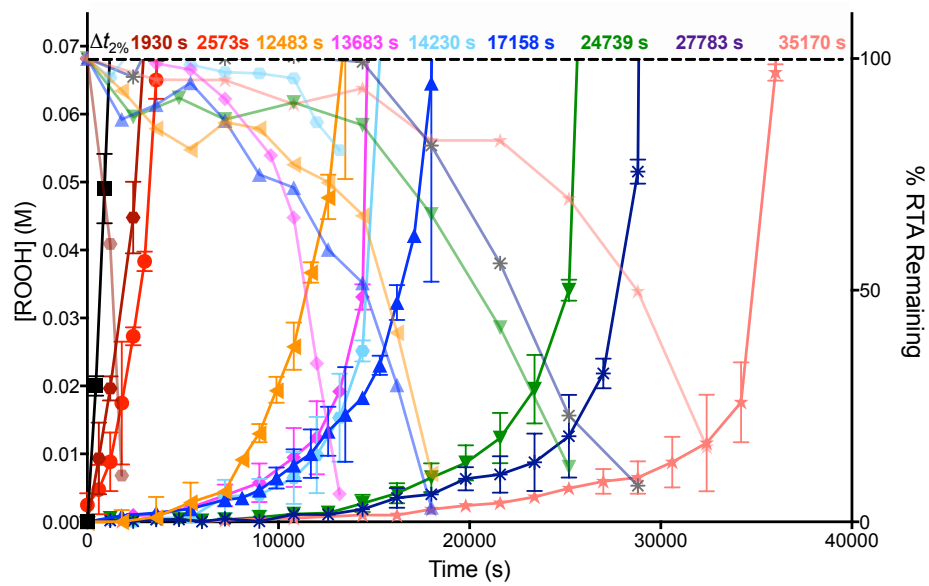


Figure S4.3 Percentage of diarylamines 4.2-4.10 in autoxidation mixture over time.

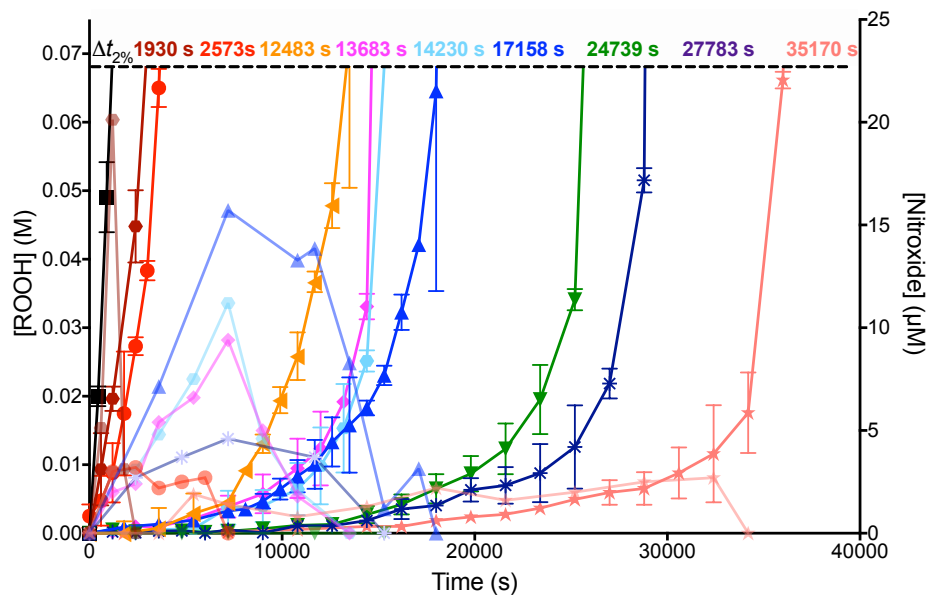


Figure S4.4 Concentration of nitroxides formed from 4.2-4.10 in autoxidation mixture over time

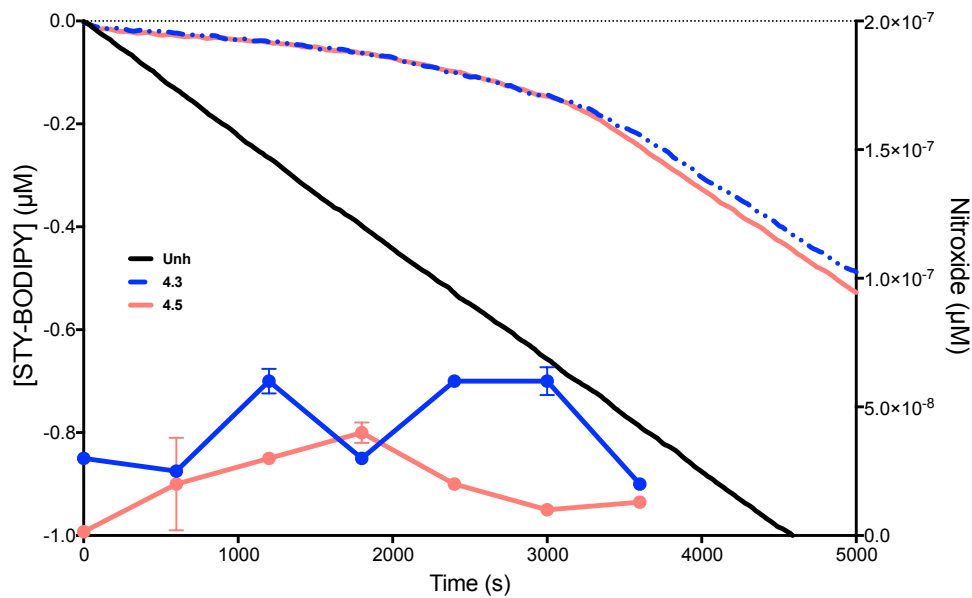


Figure S4.5 Nitroxide formation in styrene autoxidations for compounds **4.3** and **4.5** overlaid with their inhibition activity.

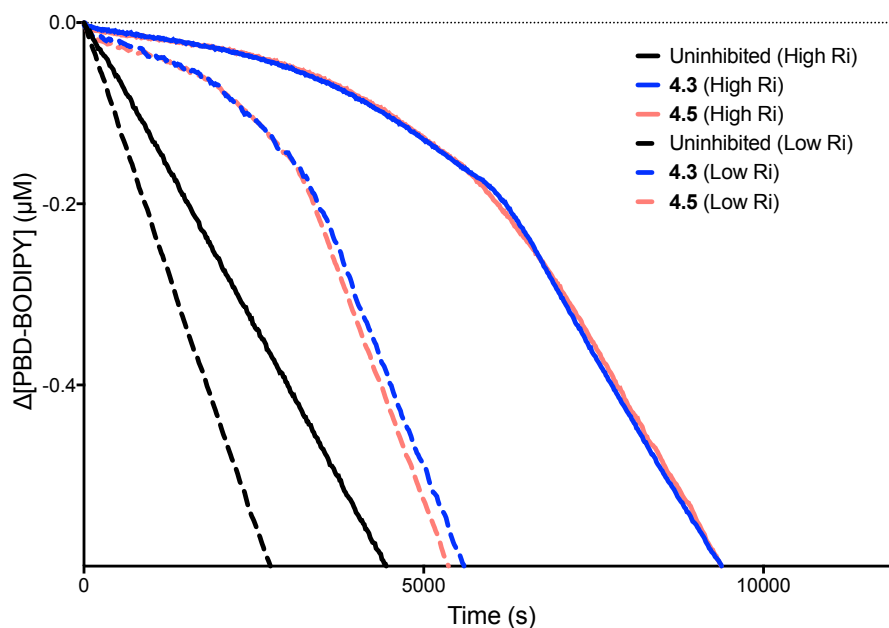


Figure S4.6 Co-autoxidation of styrene (4.3 M) and PBD-BODIPY (10 μM) initiated by AIBN (3 mM or 6 mM) in PhCl at 37°C, monitored at 591 nm ($\epsilon = 139,000 \text{ M}^{-1}\text{cm}^{-1}$). Uninhibited (black), or inhibited with 2 μM of **4.3** (blue) or **4.5** (pink). Data obtained with 6 mM AIBN is denoted in dashed lines, while corresponding data with 3 mM AIBN is shown with solid lines.

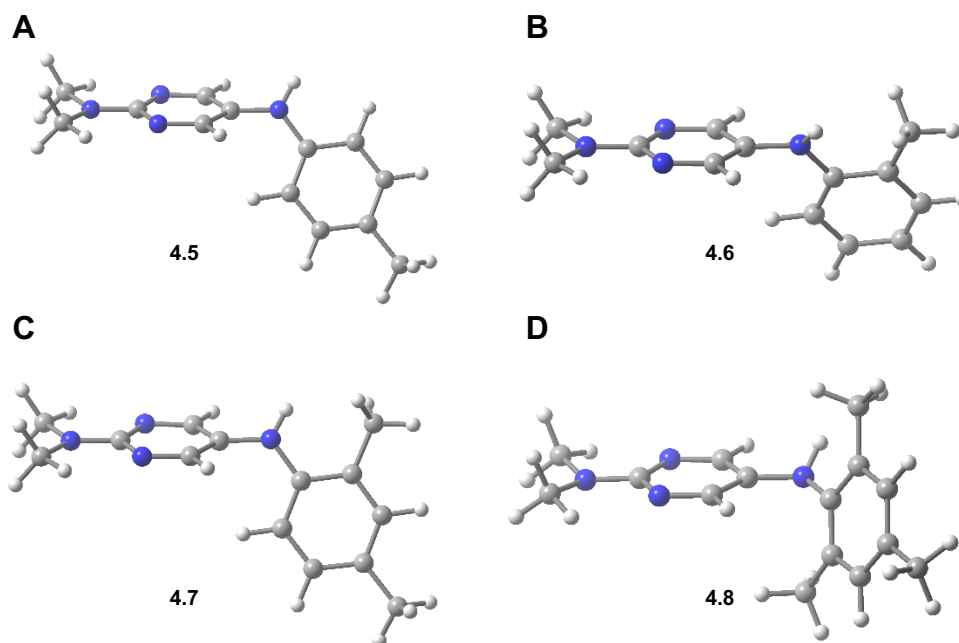
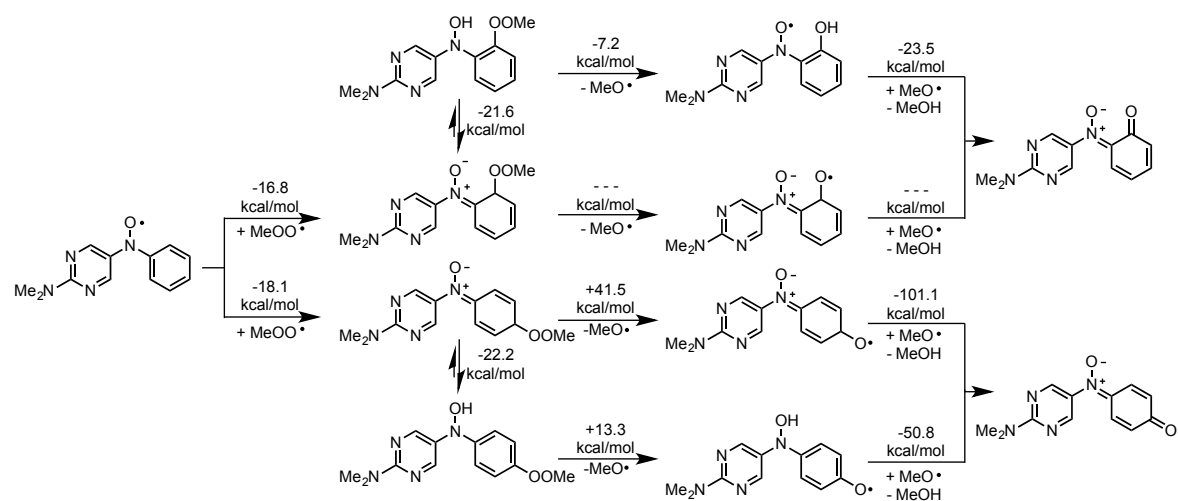


Figure S4.7 DFT-optimized structures of compounds **4.5-4.8** computed using DFT. The dihedral angles between the two aromatic rings are 53.5° , 57.5° , 56.4° and 88.3° , respectively.



Scheme 4.7 Competing pathways and associated ΔH values for nitroxide + $\bullet\text{OOMe}$ derived from **4.2**.

Chapter 5: Resolving the Role of Lipoxygenases in the Initiation and Execution of Ferroptosis

5.1 Preface

Lipoxygenases (LOXs) have been implicated as central players in ferroptosis, a recently characterized cell death modality associated with the accumulation of lipid hydroperoxides: the products of LOX catalysis. To provide insight on their role, human embryonic kidney cells were transfected to overexpress each of the human isoforms associated with disease, 5-LOX, p12-LOX, and 15-LOX-1, which yielded stable cell lines that were demonstrably sensitized to ferroptosis. Interestingly, the cells could be rescued by less than half of a diverse collection of known LOX inhibitors. Furthermore, the cytoprotective compounds were similarly potent in each of the cell lines even though some were clearly isoform-selective LOX inhibitors. The cytoprotective compounds were subsequently demonstrated to be effective radical-trapping antioxidants, which protect lipids from autoxidation, the autocatalytic radical chain reaction that produces lipid hydroperoxides. From these data (and others reported herein), a picture emerges wherein LOX activity may contribute to the cellular pool of lipid hydroperoxides that initiate ferroptosis, but lipid autoxidation drives the cell death process. Mikhail Shchepinov supplied the deuterated fatty acids. Reprinted (adapted) with permission from: Shah, R.; Shchepinov, M. S.; Pratt, D. A. *ACS Cent. Sci.* **2018**, *4*, 387. Copyright 2018 American Chemical Society.

5.2 Introduction

Lipid peroxidation has long been implicated in a staggering array of human pathologies.¹ However, only recently has a direct connection between lipid peroxidation and a specific mode of cell death been recognized. Ferroptosis – coined for this modality – was introduced in 2012.²⁻⁷ It was initially ascribed to the caspase-independent cell death resulting from glutathione depletion by the system x_c^- inhibitor erastin.⁸ Glutathione depletion starves glutathione peroxidase 4 (Gpx4) of its reducing cosubstrate, preventing the detoxification of (phospho)lipid hydroperoxides (LOOH) by reduction to the

corresponding (phospho)lipid alcohols (LOH). Ferroptosis can also be induced by pharmacological inhibition of Gpx4 (e.g., with RSL3),⁹ or via inducible deletion of the gene encoding Gpx4.¹⁰ Changes to lipid metabolism that increase the pool of oxidizable polyunsaturated (phospho)lipid or which disrupt iron homeostasis also sensitize cells to ferroptosis.^{11,12}

The formation of cellular LOOH is known to occur by two primary mechanisms: an iron-catalyzed spontaneous peroxy radical-mediated chain reaction called autoxidation,¹³ and an enzyme-mediated process catalyzed by (non-heme) iron-dependent lipoxygenases (LOXs) (Figure 5.1A).¹⁴ Several reports have implicated LOXs (the 15-LOX-1 isoform, in particular) as a key regulator in ferroptotic cell death.^{15,16} Evidence for the involvement of LOX in ferroptosis is based largely on the observation that pharmacological inhibition of LOX is cytoprotective.¹⁵⁻¹⁷ Although it has also been demonstrated that cells are resistant to ferroptosis when LOX activity is knocked down using siRNAs,¹⁶ only pharmacological inhibition of LOX has been shown to rescue gpx4^{-/-} knockout mice;¹⁰ disruption of the gene encoding 15-LOX-1 (*alox15*) failed to do so.¹⁸

The identification of specific molecular and genetic inducers of ferroptosis enabled the Stockwell and Conrad groups to identify ferrostatin-1 (Fer-1)¹⁹ and liproxstatin-1 (Lip-1)¹⁷ as potent inhibitors of ferroptosis from high-throughput screens – the former based on GSH depletion with erastin and the latter by gpx4 knockout with Cre-recombinase. The potencies of Lip-1 and Fer-1 compare very favorably to cytoprotection afforded by known LOX inhibitors such as zileuton (5-LOX inhibitor), PD146176 (15-LOX-1 inhibitor), and NDGA (a pan-LOX inhibitor). We recently demonstrated that while Fer-1 and Lip-1 are poor inhibitors of 15-LOX-1, they are excellent inhibitors of lipid peroxidation (autoxidation) owing to their reactivity as radical-trapping antioxidants (RTAs),²⁰ compounds which reduce chain-carrying peroxy radicals.²¹ In fact, both Fer-1 and Lip-1 are more effective RTAs in lipid bilayers than α -TOH, Nature's premier lipophilic RTA.²² This observation is fully consistent with their greater potency as inhibitors of ferroptosis compared to α -TOH.²⁰ Moreover, highly reactive designer RTAs, including lipophilic naphthyridinols and diarylamines, are potent inhibitors of ferroptosis.^{23,24} Thus, it follows that

either LOX inhibition or RTA activity can subvert ferroptosis, suggesting that both LOX-catalyzed lipid oxygenation and lipid autoxidation contribute to the execution of ferroptosis and preventing one or the other can subvert it. Alternatively, the LOX inhibitors that have been demonstrated to suppress ferroptosis may simply do so as RTAs. Although it is known that zileuton, NDGA, and other redox-active LOX inhibitors have “antioxidant” properties,²⁵ to the best of our knowledge, their reactivity as RTAs have yet to be investigated. Given the confounding observations surrounding the role of LOX in the execution of ferroptosis (vide supra), we have generated cells that have been stably transfected to overexpress each of the three isoforms most commonly associated with disease (5-LOX, platelet-type 12-LOX, and 15-LOX-1) and characterized both their sensitivity to ferroptosis-inducing compounds and the ability of LOX inhibitors and RTAs to rescue them from cell death. These data suggest that although LOX activity may sensitize cells to ferroptosis by contributing to the cellular pool of hydroperoxides, lipid autoxidation clearly drives cell death.

5.3 Results

5.3.1 LOX Overexpression Sensitizes Cells to Ferroptosis.

To provide insight on the potential role of different LOX isoforms in the regulation of ferroptosis, LOX-overexpressing HEK293-derived cell lines were generated by stable transfection with recombinant pcDNA3/*alox5*, *alox12*, and *alox15* constructs.²⁶ Overexpression of each LOX was verified by immunoblotting (Figure 5.1B), and enzymatic activity was confirmed by determination of the corresponding arachidonic acid oxygenation products (hydroperoxyeicosatetraenoic acids, HPETEs) by UPLC/ESI-MS/MS (Figure 5.1C,D). LOX expression was not detected in the wild-type cells, and LOX overexpression did not affect Gpx4 levels (Figure S5.1A–C).

of 5-LOX, platelet 12-LOX, and 15-LOX-1 in HEK293 cells. (C) MS/MS transitions for the 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETEs) produced by the overexpressed LOXs. HETEs were analyzed following reduction of the corresponding hydroperoxyeicosatetraenoic acids (HPETEs) with TCEP. (D) LC/MS/MS chromatograms of organic extracts of cell lysates following incubation with 70 μ M arachidonic acid for 10 min. (E, F) RSL3- and erastin-induced ferroptosis in each cell line following 4 and 24 h incubation, respectively. Data represent the mean $\pm$ SD of 3 independent experiments ($P < 0.0001$ and $P \leq 0.001$ for LD₅₀ values of RSL3 and erastin as determined by one-way ANOVA followed by Dunnett's multiple comparisons test). (G) Flow cytograms of C₁₁-BODIPY-treated cells supplemented with 70 μ M AA.

The sensitivity of the LOX-overexpressing cells to ferroptosis was assessed by Gpx4 inhibition with (1*S*,3*R*)-RSL3⁹ or GSH depletion with erastin.⁸ The transfected cell lines were sensitized to ferroptosis induced by either agent, albeit to slightly different extents (Figure 5.1E,F). LOX overexpression had a more dramatic effect on RSL3-induced ferroptosis, dropping the LD₅₀ from 6.8 μ M in the wild-type cells to 0.6, 0.4, and 0.5 μ M in the 5-LOX-, p12-LOX-, and 15-LOX-1-overexpressing cells, respectively. Erastin treatment was characterized by LD₅₀s of 6.6 μ M in the wild-type cells and 1.4, 0.9, and 1.7 μ M in the 5-LOX-, p12-LOX-, and 15-LOX-1-overexpressing cells, respectively. A similar sensitization of the LOX-overexpressing cells to the purported Gpx4 inhibitor ML210²⁷⁻²⁹ was also observed (Figure S5.1D).

Insight on the origin of the sensitization of the transfected cells to ferroptosis is evident in the chromatograms shown in Figure 5.1D. Although LOX overexpression results in an abundance of the specific regioisomeric H(P)ETE derived from LOX catalyzed oxygenation of AA, the amount of nonspecific regioisomeric H(P)ETEs also increase relative to the nontransfected cells. It should be noted that several other regioisomers (i.e., 8-, and 11-H(P)ETE) are also formed in these reactions (Figure S5.1E–G). Thus, the higher sensitivity of LOX-overexpressing cells to ferroptosis may be ascribed to an initial increase in the concentration of specific LOOH which can subsequently decompose to yield initiating species (alkoxyl and/or hydroxyl radicals by the Fenton reaction) for non- enzymatic lipid peroxidation (autoxidation). To corroborate that the LOX overproducing cells experience a higher rate of

(nonregiospecific) lipid autoxidation concomitant with the increase in regiospecific LOOH formation, C₁₁-BODIPY was used to report on the formation of autoxidation chain-carrying lipid peroxy radicals.³⁰ Indeed, as is shown in Figure 5.1G, C₁₁-BODIPY oxidation was significantly higher in LOX-overexpressing cells compared to the nontransfected cells, suggesting that, even in the presence of functional Gpx4, the LOX-overexpressing cells undergo a higher rate of lipid peroxidation. This difference was further exaggerated when Gpx4 was inhibited using RSL3 (Figure S5.1H–K).

5.3.2 LOX-Overexpressing Cells Are Rescued by Some LOX Inhibitors and All Good RTAs.

Given the foregoing results, we sought to delineate the role of LOX-catalyzed lipid peroxidation and autoxidation in ferroptosis by quantitating the potency of both LOX inhibitors (Figures 5.2A–C, S5.2A–E) and RTAs (Figures 5.2D–F, S5.2F–J) in a systematic manner. The pan-LOX inhibitor NDGA was an effective inhibitor of RSL3-induced cell death across all LOX-overproducing cell lines (EC_{50} = 387–437 nM). Interestingly, it was similarly potent in the wild-type cells that were devoid of LOX activity. Perhaps more surprisingly, zileuton, a 5-LOX-selective inhibitor, was not only a moderately potent inhibitor of ferroptosis in 5-LOX overproducing cells (EC_{50} = 1.6 μ M), but it was similarly potent in the nontransfected cells as well as in cells transfected to overproduce p12-LOX and 15-LOX-1. Investigations with PD146176, a 15-LOX-1 selective inhibitor, produced similar results, in that the compound was equally potent in all cell lines. In contrast, 5-LOX inhibitors CAY10649 and CJ-13610^{31,32} were ineffective inhibitors of ferroptosis in all cell lines.³³ From these results, it is clear that LOX inhibitory activity is not at all correlated with antiferroptotic potency.

Lip-1 and Fer-1, the archetypal ferroptosis inhibitors, rescued the transfected cells from RSL3-induced ferroptosis with very similar potencies to those determined in the wild-type cells. For example, in the wild-type cells the EC_{50} for Lip-1 was 8.9 nM, while it was 12, 20, and 15 nM for the 5-LOX, p12-LOX, and 15-LOX-1 overproducing cells, respectively. Similar results were obtained with Fer-1. Recent results from our group have demonstrated that both Lip-1 and Fer-1 inhibit ferroptosis via their RTA

activity, which is excellent in lipid bilayers.²⁰ Consistent with the foregoing results, α -tocopherol (α -TOH) and the improved aza-tocopherol analogue (C₁₅-THN) were also similarly potent in each of the cell lines.

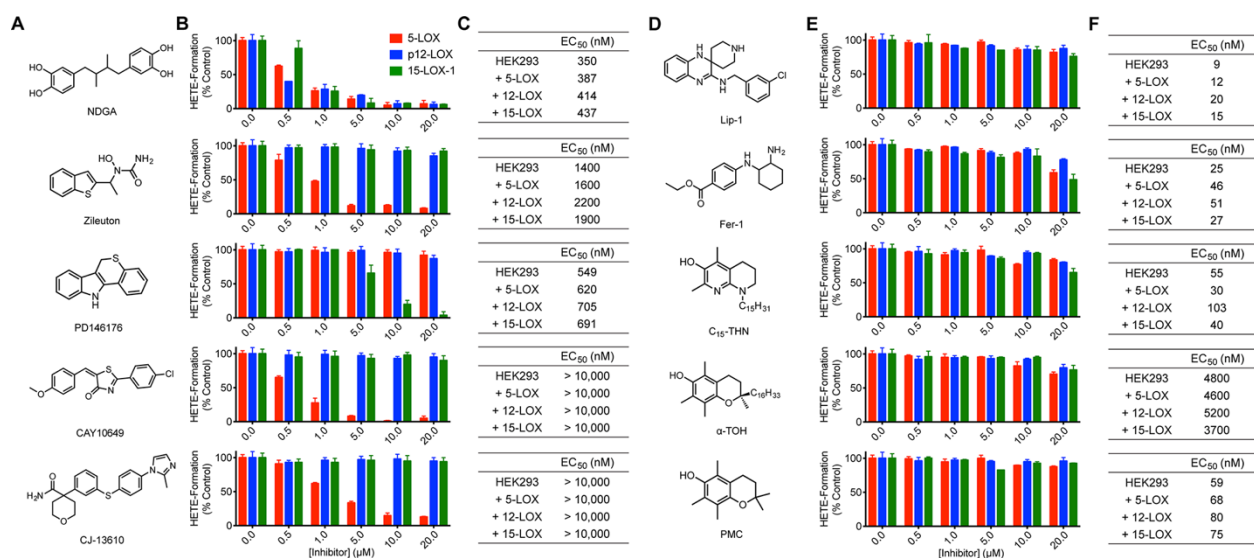


Figure 5.2 Structures of some LOX inhibitors (**A**), their isoform-selectivity (**B**), and potency as inhibitors of RSL3-induced ferroptosis in the four different cell lines (**C**). Structures of common RTAs (**D**), their activity as inhibitors of the various LOX isoforms (**E**), and potency as inhibitors of RSL3-induced ferroptosis in the four different cell lines (**F**).

It should be noted that these results are inconsistent with the suggestion that the anti-ferroptotic activity of α -TOH is due to its ability to inhibit 15-LOX-1 (by inserting its lipophilic side chain into the active site and precluding access of the substrate).¹⁵ To provide further insight on this point, 2,2,7,8-pentamethyl-6-chromanol (PMC, Figure 5.2D), a truncated analogue of α -TOH lacking the lipophilic side chain, was also assayed and was shown to be an excellent inhibitor of ferroptosis in all cell lines, suggesting that the lipophilic side chain of α -TOH is not only unnecessary for its inhibitory activity, but also reduces its potency (e.g., EC₅₀ = 59 nM in the nontransfected cells compared to 4.8 μ M for α -TOH). These results are fully consistent with the greater RTA activity of PMC compared to α -TOH in lipid bilayers, which can be ascribed to improved dynamics.³⁴ Furthermore, we found that O-methylated α -TOH (α -TOMe) was not only devoid of LOX inhibitory activity (Figure S5.3C), but was unable to rescue any of the cells from ferroptosis at concentrations up to 10 μ M (Figure S5.3D). These results are

consistent with a previous report wherein it was shown that α -TOH does not inhibit 15-LOX-1 at concentrations where it is effective at subverting ferroptosis.²⁰

5.3.3 Cytoprotective LOX Inhibitors Are Also Good RTAs.

The ability of isoform-selective LOX inhibitors to inhibit ferroptosis in all cell lines with similar potency led us to speculate whether these compounds have off-target effects that underlie their cytoprotective properties. In particular, we wondered if the cytoprotective LOX inhibitors were RTAs, while those LOX inhibitors devoid of antiferroptotic activity were not RTAs. Thus, we determined the RTA activity of NDGA, zileuton, PD146176, CAY10649, and CJ-13610 in inhibited autoxidations of Cumene,³⁵ as well as in egg phosphatidylcholine unilamellar liposomes.^{20,24} Reaction progress was monitored by the consumption of STY-BODIPY, a highly absorbing autoxidizable cosubstrate (Figure 5.3A). The rate constant for peroxy radical-trapping (or the inhibition rate constant, k_{inh}) was determined from the initial (inhibited) rate of dye consumption as in eq 1, whereas the reaction stoichiometry (how many peroxy radicals are trapped by each molecule of RTA) is given from the length of the inhibited period as in eq 2. The autoxidation traces are shown in Figures 5.3B and 5.3C, with the kinetic data tabulated below them.

The results for the known RTAs are fully consistent with previous reports. They are good to excellent inhibitors of cumene autoxidation (Figure 5.3B, Figure S5.3A), with k_{inh} varying from as low as $2.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for Lip-1 to as high as $8.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for C₁₅-THN. The cytoprotective LOX inhibitors NDGA, zileuton, and PD146176 clearly display reactivity as RTAs, ranging from moderately reactive (PD146176, $k_{inh} = 6.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) to highly reactive ($k_{inh} = 1.7 \times 10^6$ and $1.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for NDGA and zileuton, respectively). On the other hand, the LOX inhibitors which were devoid of antiferroptotic activity were unable to inhibit the autoxidation of cumene. The reactivity of NDGA, zileuton, and PD146176 is explained by the presence of labile phenolic O–H bonds in NDGA,³⁶ the hydroxamic acid O–H bond in zileuton,³⁷ and arylamine N–H bond in PD146176. In contrast, CAY10649 and CJ-13610 do not possess labile H-atoms that could reduce autoxidation chain-carrying peroxy radicals.

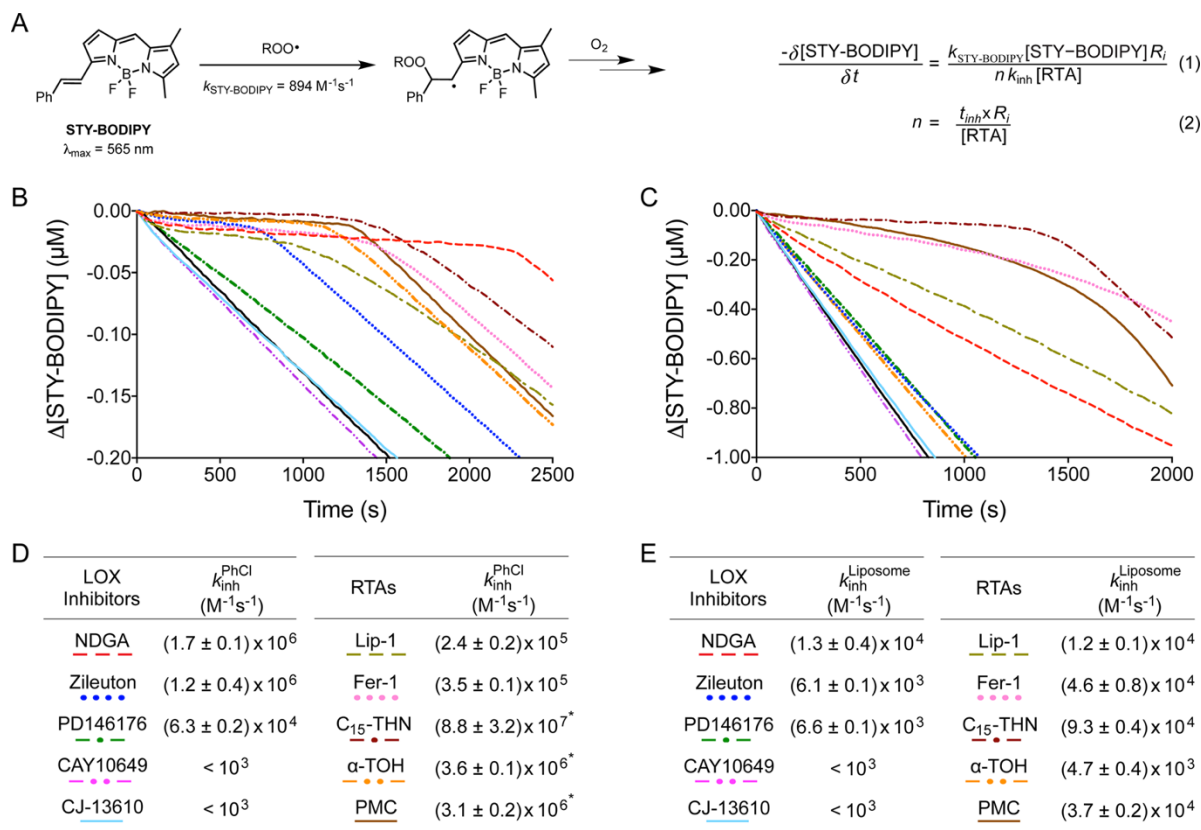


Figure 5.3 STY-BODIPY (**A**) serves as the signal carrier in inhibited autoxidations, enabling determination of inhibition rate constants (k_{inh}) and stoichiometries (n) for reactions of inhibitors with chain-carrying peroxy radicals (eqs 1 and 2, respectively). Coautoxidations of cumene (3.6 M) and STY-BODIPY (10 μM) initiated by AIBN (6 mM) in chlorobenzene at 37 °C (black) and inhibited by 2 μM of NDGA, zileuton, PD146176, CAY10649, CJ-13610, Lip-1, Fer-1, C₁₅-THN, α -TOH, and PMC (**B**). Coautoxidations of egg phosphatidylcholine liposomes (1.0 mM) and STY-BODIPY (10 μM) suspended in phosphate-buffered saline (10 mM) at pH 7.4 initiated by MeOAMVN (0.2 mM) at 37 °C (black) and inhibited by 2 μM of NDGA, zileuton, PD146176, CAY10649, CJ-13610, Lip-1, Fer-1, C₁₅-THN, α -TOH, and PMC (**C**). Inhibition rate constants obtained from coautoxidations of cumene (**D**) or phosphatidylcholine liposomes (**E**) with STY-BODIPY at 37 °C. *Rate constants were determined from styrene/ PBD-BODIPY co-autoxidations, see ref. 20.

Similar results were obtained in the more biologically relevant model system of egg phosphatidylcholine liposomes (Figure 5.3C, Figure S5.3A), albeit where the differences in RTA activity between the compounds were suppressed due to limited diffusion and H-bonding interactions.^{34,38} All cytoprotective compounds had apparent k_{inh} ranging from $4.7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ to $9.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. Again, α -

TOMe (Figure S5.3E,F) and the LOX inhibitors which were devoid of antiferroptotic activity (CAY10649 and CJ-13610) were unreactive.

5.3.4 Only Arachidonic Acid Deuterated at All Bis-Allylic Positions Protects Cells from Ferroptosis.

It was recently shown that cells grown in media supplemented with 11,11- d_2 -linoleic acid (d_2 -LA) are refractory to ferroptosis induced with either erastin or RSL3.¹⁶ Following on this observation, if LOX-catalyzed oxidation of AA – its native substrate – plays a key role in ferroptosis execution, cells grown in media supplemented with AA labeled at the bis-allylic positions from which each LOX isoform regiospecifically abstracts a H-atom should be protected from ferroptosis. The use of regiospecifically labeled substrates³⁹ as isoform-selective inhibitors of LOX (Figure 5.4A, Figure S5.4A,B) removes any uncertainty regarding off-target effects of inhibitors or interactions of inhibitors with the agents used to induce ferroptosis. As expected, 7,7- d_2 -AA inhibited 5-LOX activity, but not p12-LOX or 15-LOX-1 activity (Figure 5.4B), 10,10- d_2 -AA inhibited p12-LOX activity, but not 5-LOX or 15-LOX-1 activity (Figure 5.4C), and 13,13- d_2 -AA inhibited 15-LOX-1 activity, but not 5-LOX or p12-LOX activity (Figure 5.4D). These results are consistent with the very large kinetic isotope effects reported for LOX-catalyzed oxidation of AA by p12-LOX and 15-LOX-1.⁴⁰

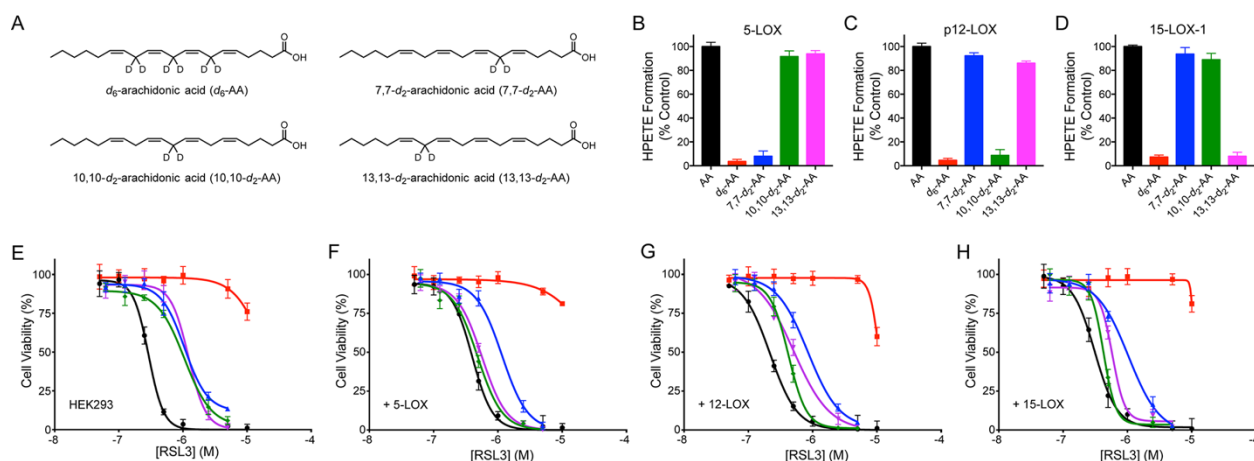


Figure 5.4 (A) Structures of deuterated arachidonic acids. HPETE formation in cells overexpressing 5-LOX (B), p12-LOX (C), and 15-LOX-1 (D) incubated with 70 μ M AA (black), 7,7,10,10,13,13- d_6 -AA (red), 7,7- d_2 -AA (blue), 10,10- d_2 -AA (green), and 13,13- d_2 -AA (magenta). RSL3-induced ferroptosis in nontransfected HEK293 cells (E) and transfected cells overexpressing 5-LOX (F), p12-LOX (G), and 15-

LOX-1 (H) preincubated with 40 μ M arachidonic acid (black, ●), 7,7- d_2 -AA (blue, ▲), 10,10- d_2 -AA (green, ◆), 13,13- d_2 -AA (magenta, ▼), and 7,7,10,10,13,13- d_6 -AA (red, ■).

Despite their ability to specifically inhibit a given LOX isoform, the addition of the d_2 -AAs had no significant cytoprotective effect on the corresponding LOX-overexpressing cells (Figures 5.4E–H). However, consistent with the observation that the addition of d_2 -LA is cytoprotective against ferroptosis, a d_6 -AA where all abstractable (bis-allylic) H-atoms were replaced with deuterium atoms was strongly cytoprotective. This observation is consistent with the large kinetic isotope effects reported for hydrocarbon autoxidation,⁴¹ and specifically H-atom transfer from polyunsaturated lipids.⁴²

5.3.5 Induction of Ferroptosis Does Not Require Specific Hydroperoxide(s)

Given the foregoing, we wondered if the H(P)ETEs produced by LOX activity simply contribute to the cellular pool of LOOH to accelerate initiation of massive (nonspecific) autoxidation that drives cell death. Interestingly, when the concentrations of 5-, 12-, and 15-H(P)ETE were monitored in the four cell lines postinduction with RSL3 (Figure 5.5A–D), the concentration of total LOOH peaked at ca. 20 μ M, suggesting that, once a critical threshold of LOOH is achieved, ferroptosis is triggered. To corroborate that the overall concentration of hydroperoxide is far more relevant than its structure, we investigated if cells were similarly sensitized to RSL3 induced ferroptosis upon addition of exogenous LOOH. Four compounds that would not be formed endogenously by LOX catalysis were investigated: 10-hydroperoxy oleic acid (oleic 10-OOH), phosphatidylcholine esterified 10-hydroperoxyoleate (PC-oleate-10-OOH), cholesterol-7-hydroperoxide, and a generic abiotic lipophilic hydroperoxide (tert-dodecyl-OOH). The data in Figure 5.5F show that the exogenous LOOH sensitize the cells to RSL3 induced ferroptosis to a similar extent. Moreover, the concentration of added LOOH necessary to achieve cell death is strikingly similar to the amount of HPETE produced endogenously in the LOX-overexpressing cells that is associated with cell death. A similar lack of structural dependence on the ability of hydroperoxides to sensitize cells to RSL3-induced cell death was also observed in Pfa1 mouse embryonic fibroblasts (MEFs). These cells have been studied extensively and have been reported to be highly sensitive to ferroptosis.^{6,17} Similar to HEK293 cells, the MEFs do not express detectable amounts of 5-LOX, p12-

LOX, or 15-LOX-1 (Figure S5.5A–C) and, consistent with their greater sensitivity to ferroptosis (compare LD₅₀s of 38 and 113 nM for RSL3 and erastin, respectively, to 6.8 and 6.6 μ M in HEK293 cells, Figure S5.5D), lower concentrations of added hydroperoxide sensitized them to cell death (Figure S5.5F).

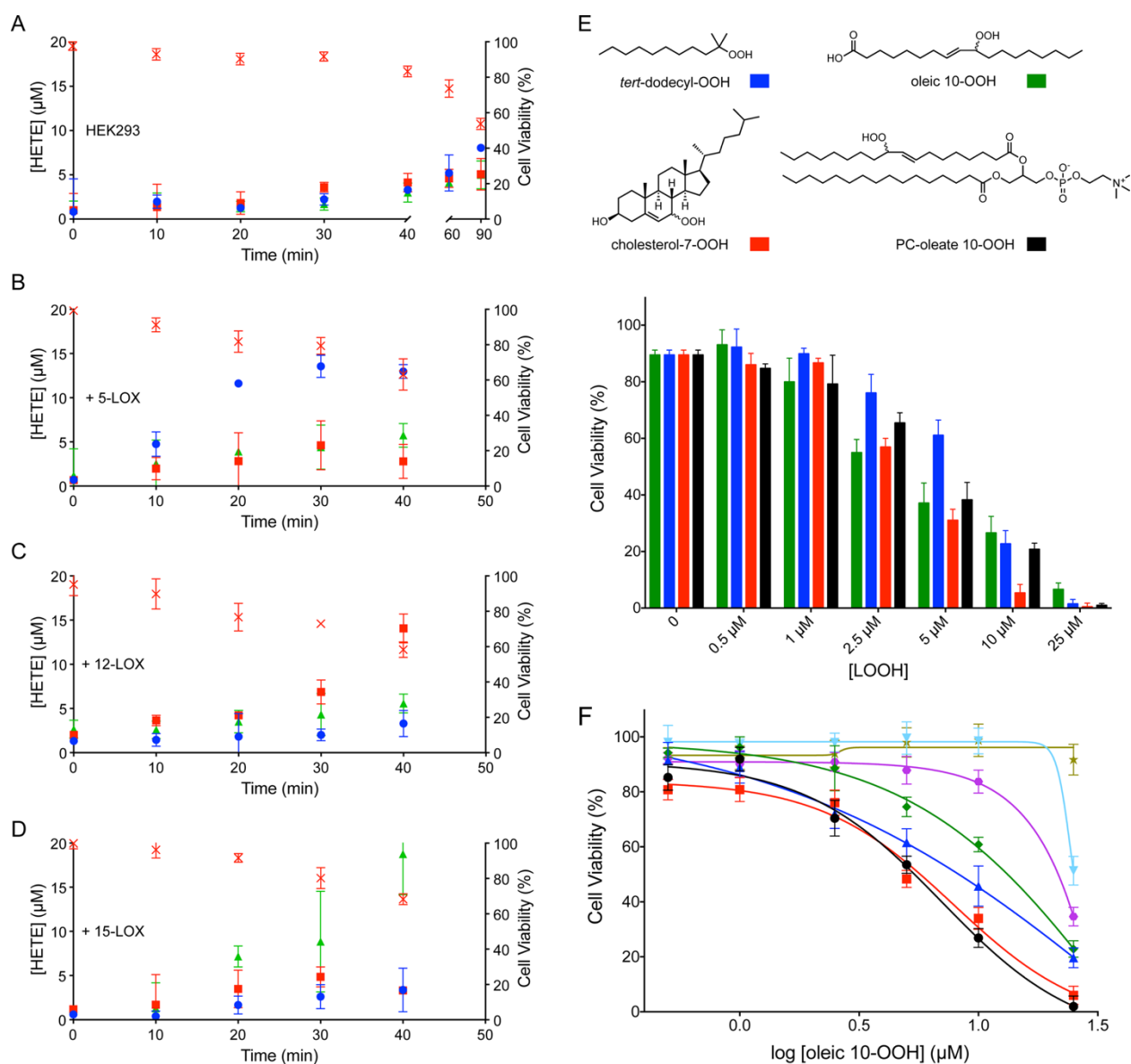


Figure 5.5 Formation of 5-HETE (blue, ●), 12-HETE (red, ■), and 15-HETE (green, ▲) in nontransfected HEK293 cells (A), and cells overexpressing 5-LOX (B), p12-LOX (C), and 15-LOX-1 (D) treated with 5 μ M RSL3 and 40 μ M arachidonic acid. Cell viability is overlaid on each graph (red ×). (E) Sensitization of wild-type cells to ferroptosis with 0.5 μ M RSL3 in the presence of various concentrations of

exogenously prepared hydroperoxides: oleic 10-OOH (green), cholesterol-7-OOH (red), tert-dodecyl-OOH (blue), or PC-oleate 10-OOH (black). (F) Viability of HEK293 cells exposed to oleic-10-OOH in the presence of 0.5 μ M RSL3 (black, ●) inhibited with 3.1 (red, ■), 6.3 (blue, ▲), 12.5 (green, ◆), 25 (magenta, ●), 50 (cyan, ▼), or 100 (yellow, ★) nM Lip-1.

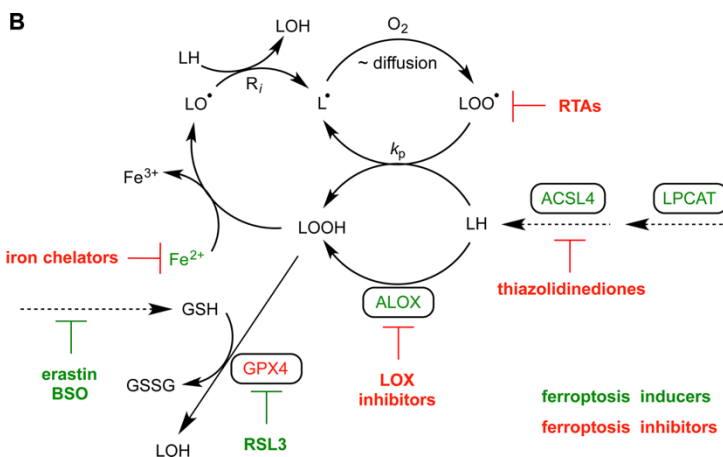
If LOX catalysis serves simply to supply hydroperoxides to initiate massive autoxidation that drives ferroptosis execution, it follows that as long as there is a sufficiently high concentration of RTA to limit the propagation of autoxidation, higher concentrations of LOOH – endogenous or exogenous – will be tolerated by the cell. This appears to be the case, an example of which is shown in Figure 5.5F wherein the concentration of oleic hydroperoxide added to wild-type cells was increased in the presence of increasing amounts of Lip-1. While the entire cell population is dead upon incubation with 25 μ M oleic 10-OOH, cell viability was increased in a dose-dependent manner with added Lip-1, with full viability being restored at 100 nM Lip-1. It is important to note that Lip-1 does not react directly with oleic 10-OOH under these conditions (Figure S5.3G).

5.4 Discussion

Polyunsaturated fatty acids are integral structural components of biological membranes and serve as precursors to a diverse array of signaling molecules that are essential to maintaining cellular and tissue homeostasis as well as the control of cell proliferation and differentiation. The unsaturation that imparts the molecular geometry necessary to contribute to both a dynamic lipid bilayer and structural diversity of signaling molecules makes PUFAs particularly sensitive to the same deleterious reactions responsible for the degradation of all hydrocarbon-derived products: autoxidation. Autoxidation is the free radical chain reaction that formally inserts a molecule of oxygen into the C–H bond of an organic substrate to yield a hydroperoxide. The hydroperoxide can undergo several possible reactions, including dehydration to yield a carbonyl compound or acid-catalyzed Hock fragmentation to yield a pair of carbonyl compounds (or their equivalent).^{43,44} In a biological context, lipid-derived carbonyls have long been directly implicated in cell death through caspase-dependent apoptotic processes,⁴⁵ but only recently have lipid hydroperoxides themselves been implicated directly in regulated cell death, via ferroptosis.²

Lipoxygenase-catalyzed oxidation of PUFAs – primarily arachidonic acid – have been proposed to steer cells toward ferroptosis.^{15,16} However, the foregoing results clearly demonstrate that although LOX-catalyzed oxygenation of AA may sensitize cells to ferroptosis (induced by the most commonly employed agents RSL3 and erastin),³³ it is not strictly required. Accordingly, although the inhibition of LOX catalysis cannot rescue cells from ferroptosis induced by the most common ferroptosis-inducing agents, radical-trapping antioxidants can, implying a central role for autooxidation in the execution of ferroptosis.

The well-established kinetics of hydrocarbon autooxidation^{46,47} enable the rationalization of many observations associated with the mechanism of ferroptosis. The expression that generally describes these kinetics – which was first derived from experiments in homogeneous organic solution, but which has also been shown to apply in lipid bilayers⁴⁸ – is shown in eq 3 in Figure 5.6A. It indicates that the rate of hydroperoxide formation is proportional to the concentration of the substrate (L–H), the rate constant for H-atom abstraction from the substrate by a chain-carrying peroxy radical (k_p), the rate of initiation (R_i), and the rate constant for termination of the chain reaction by the reaction of two chain-carrying peroxy radicals (k_t). Since autooxidation is, in a sense, auto-catalytic, i.e. the reaction product can initiate new chain reactions (Figure 5.6B), the rate of initiation (R_i) isn't constant, but increases with the conversion of lipids to their corresponding hydroperoxides. Thus, any process that contributes to the cellular pool of LOOH – such as LOX catalysis – will increase R_i and therefore the overall rate, sensitizing cells to ferroptosis. In contrast, gene products that decrease the overall pool of LOOH, such as Gpx4, will decrease R_i and suppress ferroptosis. Moreover, any process that increases the pool of autoxidizable lipid (i.e., [LH]), such as acyl-CoA synthetase long chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), will increase the overall rate and sensitize cells to ferroptosis activity. In contrast, supplementing cells with less autoxidizable lipids (e.g., D-PUFAs) or with inhibitors of ACSL4 or LPCAT3,¹² will suppress ferroptosis.

$$\text{uninhibited autoxidation} \quad \frac{d[\text{LOOH}]}{dt} = k_p [\text{LH}] \left(\frac{R_i}{2k_t} \right)^{\frac{1}{2}} \quad (3)$$
$$\text{inhibited autoxidation} \quad \frac{d[\text{LOOH}]}{dt} = \frac{k_p [\text{LH}] R_i}{n k_{\text{inh}} [\text{RTA}]} \quad (4)$$


In homogeneous solution and lipid bilayers, the kinetics of the inhibited autoxidation of hydrocarbons is generally described by eq 4 in Figure 5.6A.^{47,48} Thus, compounds with a sufficiently high rate constant (k_{inh}) or stoichiometry (n) for reactions with peroxy radicals (that do not generate a chain-initiating or chain-propagating radical) will inhibit autocatalytic LOOH formation. These RTA compounds can be either highly reactive or have a high radical-trapping capacity or, ideally, both. α -TOH, Nature's major lipophilic RTA,²² is only moderately reactive in lipid bilayers,^{34,48} but its radical-trapping capacity can be bolstered by its regeneration by coantioxidants, such as ascorbate and reduced co-Q10.^{49,50} The aza-TOH analogue, C₁₅-THN, is much more reactive in lipid bilayers and is also regenerable by coantioxidants,²³ which translates to high potency in cells. Lip-1 and Fer-1 also have good activity in lipid bilayers. It remains unclear whether these compounds are regenerable, but it would seem that they have higher radical-trapping capacity in lipid bilayers than either α -TOH or C₁₅-THN, possibly due to the formation of nitroxides,²⁰ which can act as catalytic RTAs.⁵¹ The LOX inhibitors NDGA, Zileuton, and PD146176 also clearly display this activity, explaining why they inhibit ferroptosis in all four cell lines with similar potencies. Most importantly, LOX inhibitors devoid of any RTA activity (CAY10649 and CJ-13610) do not. It should be noted that other LOX inhibitors have been reported to inhibit ferroptosis, specifically, baicalein,^{16,52,53} CDC,¹⁶ and AA-861.^{16,52} We have confirmed that these

compounds also have significant RTA activity (see Figure S5.6A–D), which can be ascribed to the labile H-atoms found on their respective redox-active functionalities: CDC contains a lipophilic catechol, baicalein a lipophilic pyrogallol, and AA-861 is a lipophilic quinone analogous to coenzyme Q10 that can be reduced in situ to yield a hydroquinone.²⁵ These results serve to caution investigators to avoid making conclusions regarding the involvement of LOX-catalyzed lipid oxidation in cell death on the basis of inhibition by (redox-active) LOX inhibitors, including (but not limited to) NDGA,^{53–55} Zileuton,^{16,56,57} PD146176,^{10,15,16,55,58,59} baicalein,^{16,52,53,59} CDC¹⁶ and AA-861.¹⁶

Since hydrocarbon autoxidation can be autocatalytic, the rate of initiation (R_i) is not constant, but ever-increasing, depending on the LOOH concentration and the availability of low valent metals (e.g., Fe^{2+}) and/or other one-electron-reducing agents capable of converting hydroperoxides to chain-initiating species (i.e., hydroxyl and/or alkoxyl radicals). As R_i increases, the cell's intrinsic RTA defenses are rapidly depleted and, with the accumulation of LOOH left unabated, cell death is inevitable. In HEK293 cells (transfected to overexpress LOX or not) the LOOH concentration at which this takes place appears to be ca. 20 μM . This “threshold” is likely to vary with cell type, metabolic state, and/or specific culture conditions that can affect either R_i or the intrinsic RTA complement/activity of the cell. Indeed, we have shown that cells that are more sensitive to ferroptosis, such as the well-studied mouse embryonic fibroblasts,¹⁷ are characterized by a significantly lower threshold, and supplementing cells with additional RTA can significantly raise this threshold.⁶⁰

Although the results presented above establish autoxidation as the central process in the execution stage of ferroptosis, it must be stressed that the events leading up to it – events that enable the initial buildup of LOOH to a level where R_i will overwhelm a cell's limited intrinsic RTA capacity – remain unclear. It is at this initiation stage that LOXs may play a role, and their expression levels – as well as those of proteins/ enzymes that may be associated with their activity (e.g., PEBP1, a scaffolding protein that was recently reported to assist 15-LOX-1 in acquiring phosphatidylethanolamine-esterified AA for oxygenation,⁶¹ Figure S5.7) – may contribute to the sensitivity of a given cell type to ferroptosis. It is also

at this stage where pharmacological intervention to suppress lipid oxidation with LOX inhibitors or deuterated PUFAs⁶² may be useful in pathological contexts. Consistent with this view, 5-LOX-overexpressing cells passaged in the presence of CJ-13610 (the non-redox-active 5-LOX selective inhibitor) were desensitized to RSL3-induced ferroptosis, while the p12-LOX- and 15-LOX-1-overexpressing cells remained sensitized (Figure S5.4C,D). Although RTA treatment is clearly effective in subverting ferroptosis in cell culture, it targets the downstream process (autoxidation), and once the RTA is exhausted, if the underlying condition remains (e.g., ineffective Gpx4 and/or GSH insufficiency), the cells will die. Indeed, Gpx4^{-/-} cells were viable in α -TOH-enriched media (passaged 20 times), but underwent cell death within 24 h in its absence.¹² In vivo, where accumulation of initiating LOOH may be slower, and induction of ferroptosis correspondingly less acute, LOX inhibition may ultimately prove effective.

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5.6 Supporting Information

5.6.1 General Experimental

C₁₅-THN¹, Fer-1², Lip-1³, RSL3^{2,3}, erastin^{4,5}, and STY-BODIPY⁶, Oleic 10- hydroperoxide⁷, Cholesterol 7-hydroperoxide⁸ were synthesized according to literature procedures. Egg phosphatidylcholine, AIBN, MeOAMVN, BODIPY-C11^{581/591}, NDGA, Zileuton, PD146176, CAY10649, CJ-13610, α -TOH, ML210, baicalein, cinnamyl-3,4- dihydroxy- α -cyanocinnamate, MEM media with/without phenol red, Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), penicillin-streptomycin, Antibodies, AquaBluer and reagents for synthesis were purchased from commercial sources and used as received.

5.6.2 Inhibited Autoxidation of Cumene.

Cumene was washed thrice with 1 M aqueous NaOH, dried over MgSO₄, filtered, distilled under vacuum and purified by percolating through silica, then basic alumina. To a cuvette containing 1.25 mL cumene was added 1.18 mL chlorobenzene and the solution equilibrated for 5 minutes at 37 °C. The cuvette was blanked and 12.5 μ L of 2 mM PBD-BODIPY in 2,3,5-trichlorobenzene was added followed by 50 μ L of 0.3 M AIBN in chlorobenzene and the solution was thoroughly mixed. After 20 minutes, an aliquot of LOX inhibitors or RTA stock solution (1 mM) in chlorobenzene was added and the loss of absorbance at 591 nm followed. The inhibition rate constant (k_{inh}) and stoichiometry (n) was determined for each experiment according to Eq. (1) and (2), respectively. Kinetic data are given as averages of three independent measurements.

5.6.3 Inhibited Autoxidation of Egg-PC Liposomes.

To a cuvette of 2.34 mL of 10 mM PBS at pH 7.4 was added liposomes (125 μ L of 20 mM stock in PBS at pH 7.4) and the solution equilibrated for 5 minutes at 37 °C (liposome formation from egg-PC was described elsewhere).¹ The cuvette was blanked and 10 μ L of 2 mM STY-BODIPY in DMSO was added followed by 10 μ L of 0.05 M MeOAMVN in acetonitrile and the solution was thoroughly mixed. After 5

minutes, an aliquot of LOX inhibitors or RTA stock solution (1 mM) in DMSO was added and the loss of absorbance at 565 nm followed. The inhibition rate constant (k_{inh}) and stoichiometry (n) was determined for each experiment according to Eq. (1) and (2), respectively. Kinetic data are given as averages of three independent measurements. Indistinguishable results were obtained in select control experiments where the antioxidant was added prior to liposome extrusion.

5.6.4 Cell Culture.

All cell lines were cultured at 37 °C in a 5% CO₂ atmosphere unless otherwise indicated. HEK-293 cells were cultured in MEM with 10% FBS, 1% 100× non-essential amino acid solution, 1 mM sodium pyruvate and 1% penicillin-streptomycin. Cells were passaged by dissociation with 0.05% trypsin and 0.2% EDTA every two to three days. LOX overexpressing stable cell lines were cultured in MEM with 10% FBS, 1% 100× non-essential amino acid solution, 10 mM glutamine and 1 g/L geneticin. Pfa1 cells were cultured in DMEM with 10% FBS, 1% 100× non-essential amino acid solution, 1 mM sodium pyruvate and 1% penicillin- streptomycin. All cell experiments were carried out as a minimum of three biological replicates.

5.6.5 Overexpression of 5, 12- and 15-LOX in HEK 293 cells.

Cells were plated to 30% confluency and were transfected with a recombinant pcDNA3/*alox5*, *alox12* and *alox15* constructs using LipoJet reagent according to the manufacturer's recommendations. The cells were passaged to 35 mm plates after 1 day and colonies were screened using selection medium containing 1 g/L geneticin. Stable expression was verified every few passages.

5.6.6 Immunoblotting.

Protein extracts were prepared by lysing either HEK293 cells, LOX overexpressing cells or Pfa1 cells in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein loading buffer (62.5 mM Tris [pH 6.8], 25% glycerol, 2% SDS, 0.1% bromophenol blue, 5% 2-mercaptoethanol). After separation by 10% PAGE, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immuno-Blot PVDF; Bio-Rad Laboratories), and blocked in 5% dry milk, Tris-buffered saline. The

membrane was probed with an antibody specific for 5, 12- and 15-LOX, GPX4 and β -actin (Novusbio). Binding of the primary antibody was detected using a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Novusbio) and visualized by ECL chemiluminescence reaction (Pierce ECL, Thermo Scientific).

5.6.7 Determination of H(P)ETEs by UPLC/MS/MS for Enzyme Activity.

LOX overexpressing cells were harvested and lysed in Tris-HCl [pH 7.4] in the presence of 1% protease inhibitor cocktail (Sigma Aldrich). The residue was vortexed at 10,000 rpm for 10 minutes and the supernatants were incubated with 70 μ M arachidonic acid (AA, Nu-Chek Prep) at 37 °C for 10 min. The reactions were terminated with 1 volume of methanol and 1.5 mg/mL solution of TCEP (Sigma Aldrich). The samples were stored at room temperature for 90 min to ensure complete reduction of the HPETEs to HETE takes place. For inhibition studies, incubations with inhibitors were carried out for 10 min on ice prior to the addition of AA. Briefly, 10 μ M prostaglandin B₂ (Cayman Chemical) was added as internal standard along with 15 μ l of 1M HCl for acidification followed by extraction using C-18 solid phase extraction columns (Thermo Scientific) as per manufacturer's protocol. The eluent in methanol was evaporated to dryness and resuspended in 100 μ l methanol. Aliquots (5 μ l) were injected on a Waters Acquity C₁₈ column (2.1 mm $\times$ 50 mm, 1.7- μ m particle size) in a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system. HETEs were eluted isocratically with a mixture of acetonitrile, methanol, water and acetic acid (42:25:33:0.007) and detected using MS/MS analysis. Eicosanoids were detected in the negative ion mode by multiple reaction monitoring of m/z 333 $\rightarrow$ 235 for PGB₂, m/z 319 $\rightarrow$ 115 for 5-HETE, m/z 319 $\rightarrow$ 179 for 12-HETE, and m/z 319 $\rightarrow$ 219 for 15-HETE. Each experiment was performed in technical triplicate and reproduced independently three times, error bars represent SD from the mean.

5.6.8 Determination of H(P)ETEs by UPLC/MS/MS.

LOX overexpressing cells were plated in a 10-cm plate and allowed to adhere overnight. The next day, media was removed, the cells were washed with PBS and suspended in media containing 5 μ M RSL3 and

40 μ M AA. The cells were harvested using a cell scraper at appropriate time points and flash frozen in liquid nitrogen. After thawing, the cells were treated with 5 mL of MeOH containing the internal standard, 1 mg/mL BHT and 1.5 mg/mL TCEP, followed by sonication to lyse the cells. BHT was added in order to suppress any air oxidation of the PUFAs present in the sample. The mixture was left on a shaker for 30 minutes at room temperature to ensure complete reduction of HPETEs. 10 mL of chloroform and 1 mL of 0.9% NaCl solution was added followed by shaking for 1 hour to ensure maximal extraction of lipids. The tubes were centrifuged to separate the organic and aqueous layers. The lipids were recovered by removing the bottom chloroform layer. The mixture was re-extracted using 5 mL chloroform and the two organic layers were combined. The combined chloroform layers were dried in a vacuum dryer at 20 °C. The lipids were re-suspended in 500 μ L MeOH containing BHT, followed by the addition of 500 μ L KOH (1.5%) solution and incubated at 60 °C for 30 minute to saponify all the phospholipids. After incubation, the solution was acidified using 500 μ L HCl (2 M) solution. The mixture was purified using the above protocol and analyzed on Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system.

5.6.9 Induction of Ferroptosis Induced by Gpx4 Inhibition with (1*S*,3*R*)-RSL3.

HEK293 cells, the LOX-overexpressing cells and Pfa1 cells (3,000 in 100 μ L) were seeded in 96-well plates and incubated overnight. The next day the media was removed, the cells were washed twice with PBS and the cells were suspended in new media for 30 minutes before addition of (1*S*, 3*R*)-RSL3 in a final volume of 100 μ L. Cell viability was assessed 4 hours later using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Experiments are carried out with six-technical replicates (n = 6 wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

5.6.10 Induction of Ferroptosis Induced by GSH Depletion with Erastin.

HEK293 cells, the LOX- overexpressing cells and Pfa1 cells (3,000 in 100 μ L) were seeded in 96-well plates and incubated overnight. The next day the media was removed, the cells were washed twice with

PBS and the cells were suspended in new media for 30 minutes before addition of erastin in a final volume of 100 μ l. Cell viability was assessed 24 hours later using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Experiments are carried out with six-technical replicates (n = 6 wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

5.6.11 Induction of Ferroptosis Induced by ML210.

HEK293 cells and the LOX-overexpressing cells (3,000 in 100 μ L) were seeded in 96-well plates and incubated overnight in the presence or absence of 40 μ L LA. The next day the media was removed, the cells were washed with PBS and the cells were suspended in new media containing 10 μ M ML210 in a total volume of 100 μ l. Cell viability was assessed at either 24 hours (LA-treated cells) or 48 hours (LA-untreated cells) using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Experiments are carried out with six-technical replicates (n = 6 wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

5.6.12 Cellular Lipid Peroxidation.

HEK293 and LOX overexpressing cells were cultured in 12-well plates (1×10^5 cells/mL) overnight. The media was removed and the cells were washed with PBS three times and then treated with 1 μ M BODIPY-C11^{581/591} in media and incubated at 37 °C in dark for 30 minutes after which the cells were trypsinized and AA (70 μ M) in DMSO was added. Cells were then analyzed by flow cytometry (λ_{ex} = 488 nm; λ_{em} = 525 $\pm$ 25 nm). Cells treated with DMSO as vehicle were used as negative control. Experiments are carried out with three-technical replicates and performed independently with a minimum of three biological replicates.

5.6.13 Inhibition of Ferroptosis Induced by Gpx4 Inhibition with (1*S*,3*R*)-RSL3.

HEK293, LOX overexpressing cells or Pfa1 cells (3,000 in 100 μ L) were seeded in 96-well plates and cultured overnight. The next day the media was removed, the cells were washed twice with PBS and the

cells were suspended in new media for 30 minutes before addition of RTAs and LOX inhibitors. Ferroptosis was induced using (*1S,3R*)-RSL3 (10 μ M) 30 minutes after incubation of compounds. Cell viability was assessed 6 hours later using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Experiments are carried out with six-technical replicates ($n = 6$ wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

5.6.14 Inhibition of Ferroptosis Induced by ML210.

HEK293 and LOX overexpressing cells (3,000 in 100 μ L) were seeded in 96-well plates and cultured overnight in the presence of 40 μ M LA. The next day the media was removed, the cells were washed with PBS and the cells were suspended in new media for 30 minutes before addition of RTAs and LOX inhibitors. Ferroptosis was induced using ML210 (10 μ M) concomitant with the administration of compounds. Cell viability was assessed 24 hours later using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Experiments are carried out with six-technical replicates ($n = 6$ wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

5.6.15 Inhibition of LOX Overexpressing Cells Using Deuterated AAs.

Cells were plated in a 96- well plate and allowed to adhere overnight. The next day, media was removed and the cells were washed with PBS. Clear media containing the coumarin-triarylphosphine probe (10 μ M) was added and allowed to incubate for 15 minutes. After incubation, a solution of AA was added and the fluorescence of the probe was measured over time in a plate reader at 37 $^{\circ}$ C (excitation wavelength = 340 nm; emission wavelength = 425 nm). The initial rates were used to determine the rate of product formation. Experiments are carried out with three-technical replicates ($n = 3$ wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

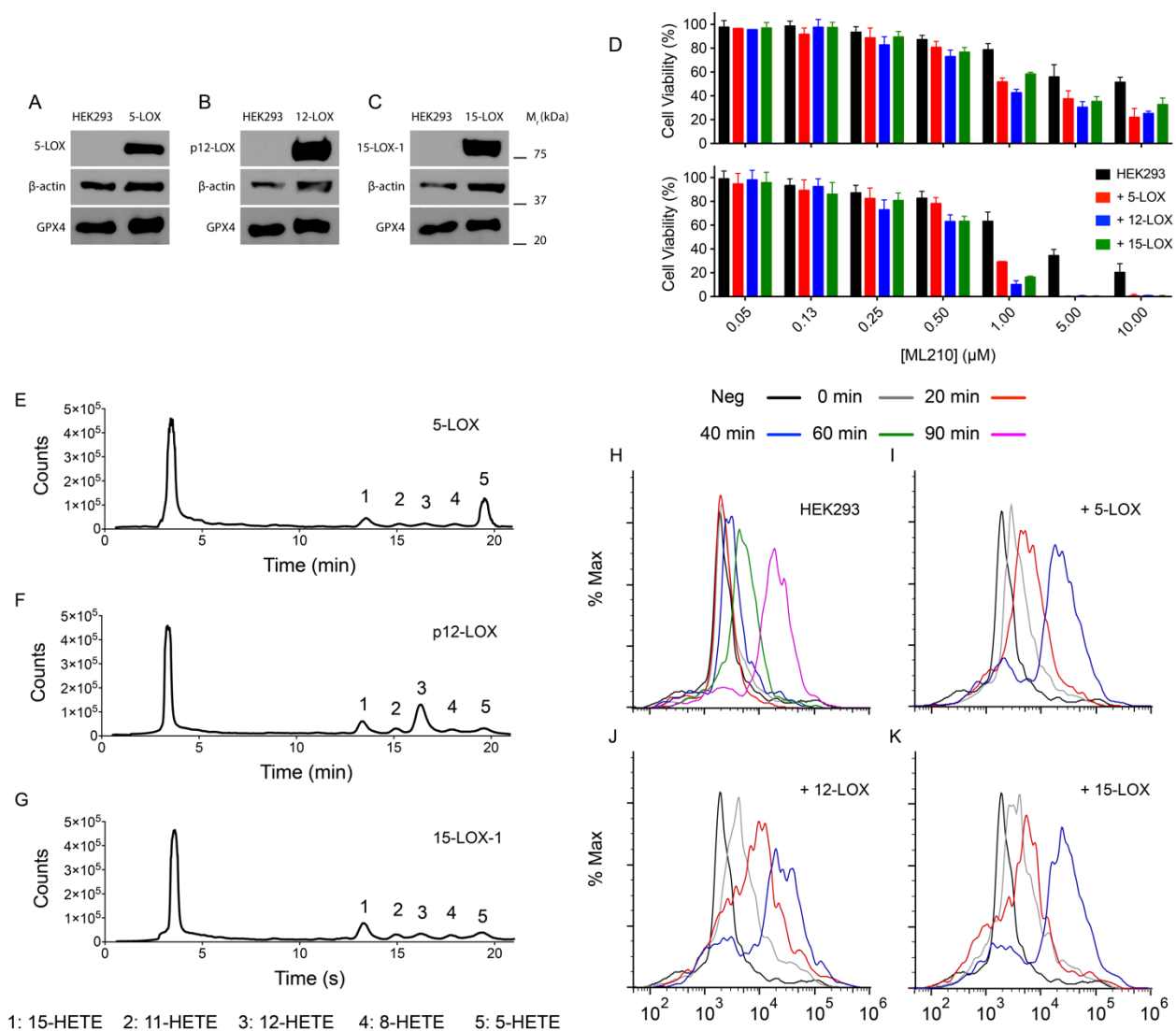


Figure S5.1 Western blots showing GPX4 expression in HEK293, 5-LOX (A), 12-LOX (B) and 15-LOX (C) cells. (D) ML210 induces ferroptosis in each of the cell lines following 48-hour incubation (top) and 24-hour incubation (bottom) in the presence of 40 μ M linoleic acid. LC/MS chromatograms of lipid extracts of lysates from 5-LOX (E), p12-LOX (F) and 15-LOX-1 (G) following incubation with 70 μ M arachidonic acid for 10 minutes. Flow cytograms of C_{11} -BODIPY treated cells supplemented with 5 μ M RSL3 in HEK293 (H), 5-LOX (I), 12-LOX (J) and 15-LOX (K) cell lines.

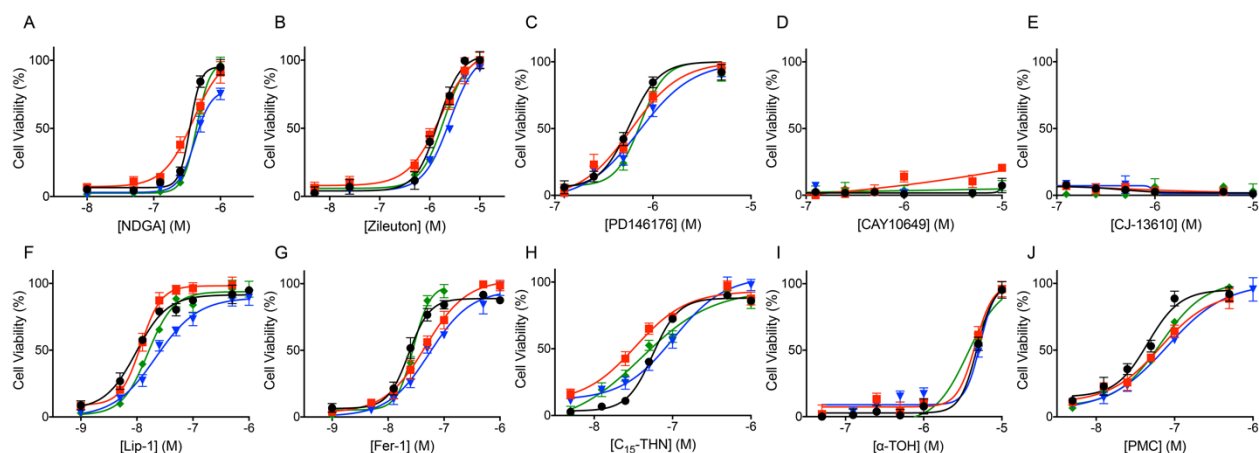


Figure S5.2 Dose-response curves for inhibition of RSL3-induced ferroptosis by various LOX inhibitors: (A) NDGA, (B) Zileuton, (C) PD146176, (D) CAY10649, (E) CJ-13610 and RTAs: (F) Lip-1, (G) Fer-1, (H) C₁₅-THN, (I) α-TOH, (J) PMC in HEK293 (●), + 5-LOX (■), + 12-LOX (▼) and + 15-LOX (◆) cells.

Table S5.1 Potency of representative RTAs and LOX inhibitors as inhibitors of ML210-induced ferroptosis in HEK293, + 5-LOX, + 12-LOX and + 15-LOX cells.

	EC ₅₀ (nM)			
	HEK293	+ 5-LOX	+ 12-LOX	+ 15-LOX
NDGA	1041	1181	922	1057
Zileuton	887	1454	1402	1089
PD146176	158	253	188	210
CJ-13610	> 10,000	> 10,000	> 10,000	> 10,000
Lip-1	11	14	24	14
Fer-1	20	48	35	32

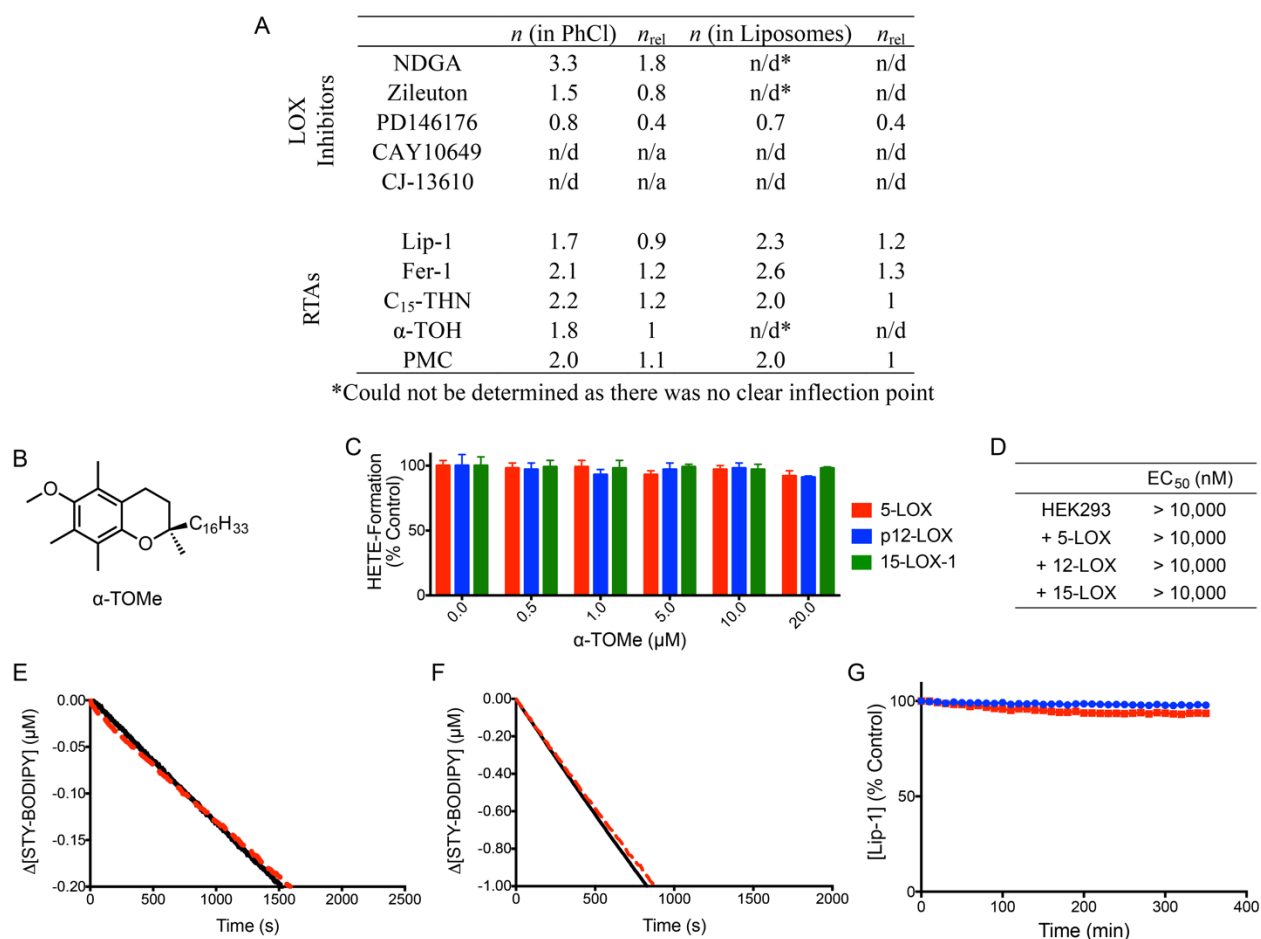


Figure S5.3 Reaction stoichiometry (n) for LOX inhibitors and RTAs in cumene and liposome autoxidations determined using Eq. 2 from Figure 5.3 (A); relative values of n are given for easy comparison to α -TOH. Structure of α -TOME (B), its inhibitory activity against 5-LOX, p12-LOX and 15-LOX-1 (C), and potency as inhibitor of RSL3-induced ferroptosis in the four different cell lines (D). Co- autoxidations of cumene (3.6 M) and STY-BODIPY (10 μM) initiated by AIBN (6 mM) in chlorobenzene at 37°C (black) and inhibited by 2 μM of α -TOME (red,) (E). Co- autoxidations of egg phosphatidylcholine liposomes (1.0 mM) and STY-BODIPY (10 μM) suspended in phosphate-buffered saline (10 mM) at pH 7.4 initiated by MeOAMVN (0.2 mM) at 37°C (black) and inhibited by 2 μM of α -TOME (red,) (F). Stability of Lip-1 (10 μM) in the absence (●) or presence (■) of oleic 10-hydroperoxide (50 μM) in PBS buffer at 37°C monitored by the loss in absorbance of Lip-1 by UV-Vis spectroscopy (G).

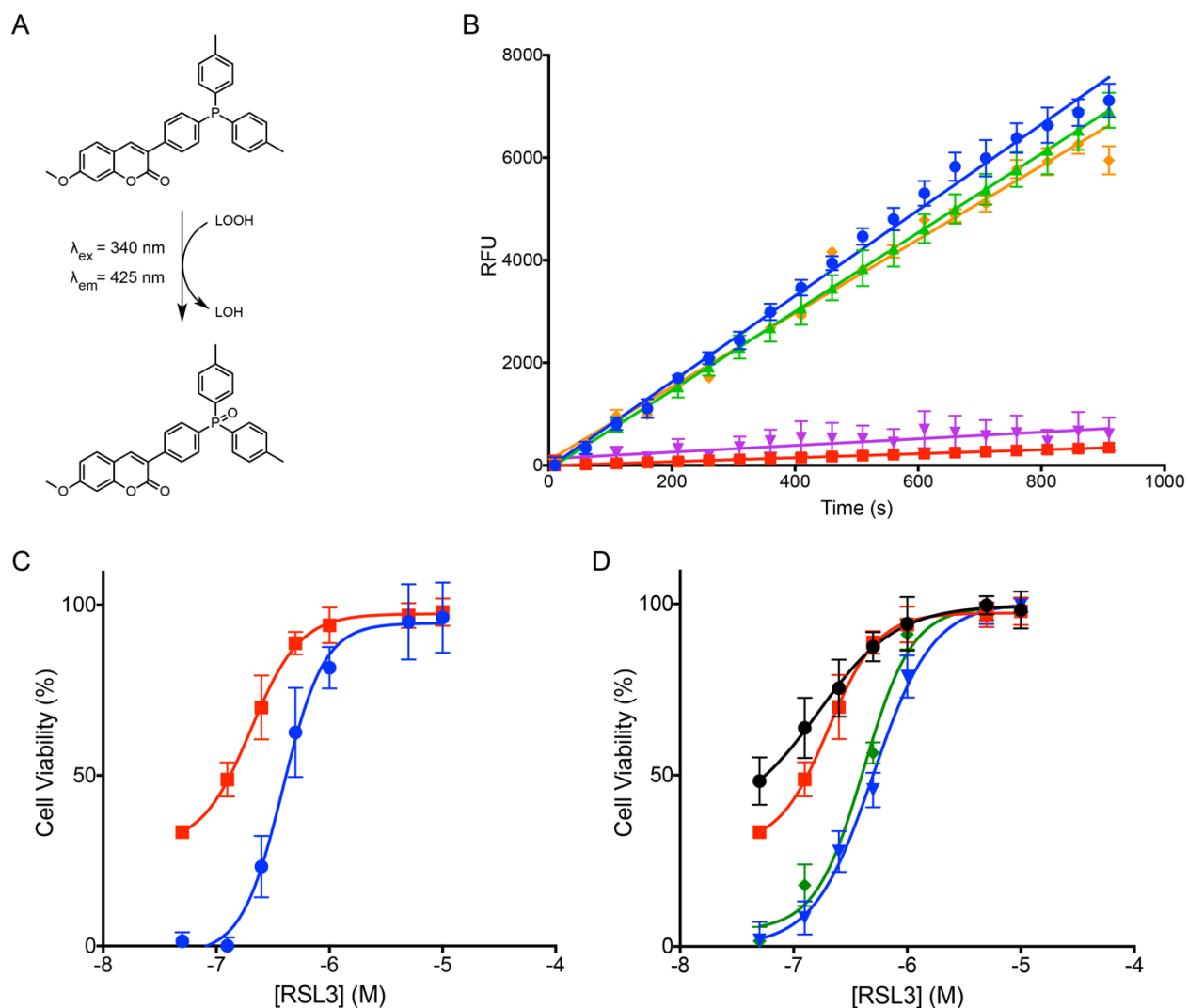


Figure S5.4 (A) Reaction of fluorogenic coumarin-triarylphosphine conjugate with lipid hydroperoxides. (B) Representative data for LOX-activity obtained from 12-LOX cells determined using the coumarin-triarylphosphine probe (10 μM) in the presence of 70 μM of either AA ($\bullet$), d₆-AA ($\blacksquare$), 7,7-d₂-AA ($\blacktriangle$), 10,10-d₂-AA ($\blacktriangledown$) and 13,13-d₂-AA ($\blacklozenge$). (C) Cell viability determined for 5-LOX cells with varying concentrations of RSL3, cultured in the absence ($\bullet$) or presence of 10 μM CJ-13610 ($\blacksquare$) using AquaBluer 4-hours post induction. (D) Cell viability determined for HEK293 ($\bullet$), 5-LOX ($\blacksquare$), 12-LOX ($\blacktriangledown$) and 15-LOX ($\blacklozenge$) cells with varying concentrations of RSL3 cultured in the presence of 10 μM CJ-13610 using AquaBluer 4-hours post induction.

The production of LOOH from LOX cells in the presence of different PUFAs (Figure S5.4B, C and D) was determined using our coumarin-triarylphosphine probe. The probe undergoes fluorescence enhancement upon reaction with hydroperoxides which can be monitored using a microplate reader.⁹⁻¹¹

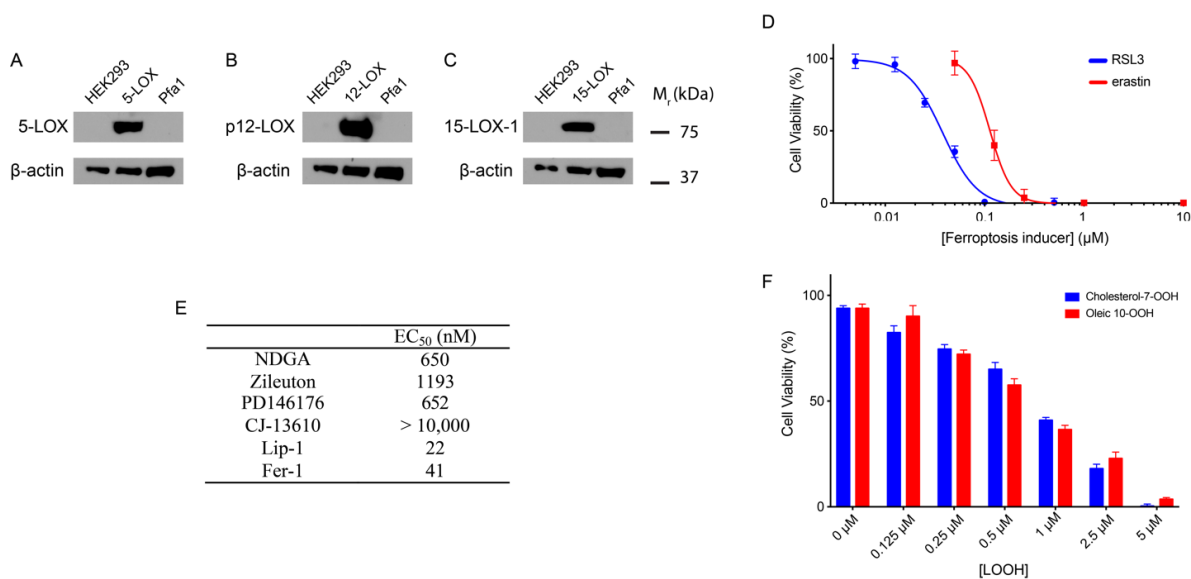


Figure S5.5 Western blots showing expression levels of 5-LOX (A), p12-LOX (B) and 15-LOX-1 (C) in Pfa1 cells compared alongside HEK293 and LOX-overexpressing cells. (D) Cell viability LD₅₀ curves for ferroptosis initiated with RSL3 (blue) and erastin (red) in Pfa1 cells. (E) EC₅₀ values obtained from RSL3 induced ferroptosis in Pfa1 cells, inhibited by RTAs and LOX inhibitors. (F) Sensitization of Pfa1 cells to ferroptosis with 0.05 μ M RSL3 in the presence of various concentrations of exogenously prepared cholesterol-7-OOH (blue) or oleic 10-OOH (red).

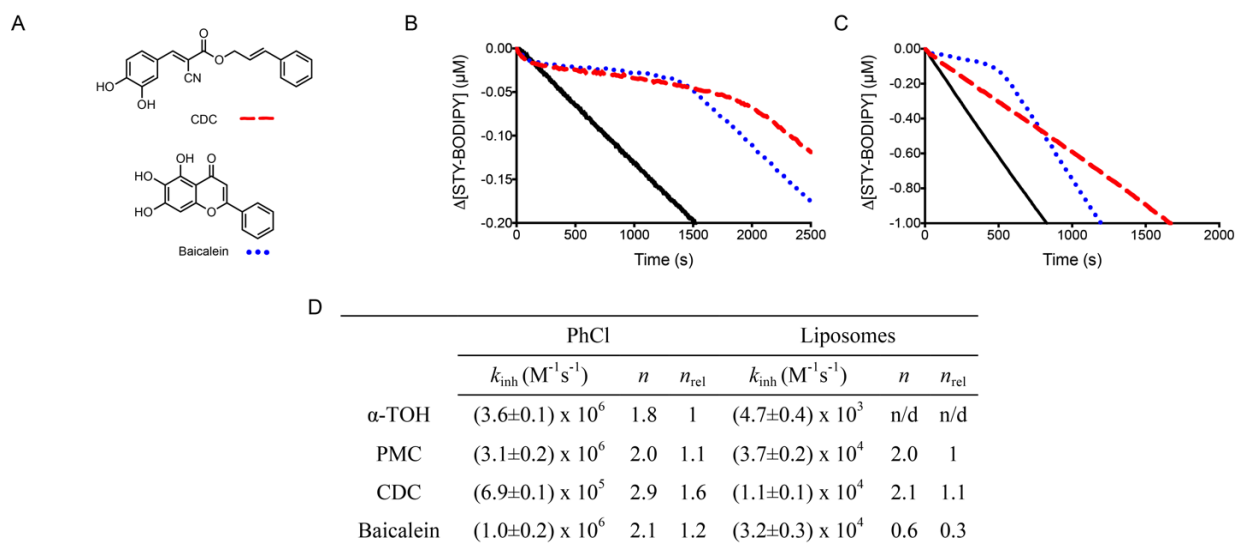


Figure S5.6 (A) Structures of commonly used LOX inhibitors, CDC and Baicalein. (B) Co- autoxidations of cumene (3.6 M) and STY-BODIPY (10 μ M) initiated by AIBN (6 mM) in chlorobenzene at 37°C

(black) and inhibited by 2 μ M of CDC (red,) and Baicalein (blue, • • •) (C) Co-oxidations of egg phosphatidylcholine liposomes (1.0 mM) and STY-BODIPY (10 μ M) suspended in phosphate-buffered saline (10 mM) at pH 7.4 initiated by MeOAMVN (0.2 mM) at 37°C (black) and inhibited by 2 μ M of CDC (red, – – –) and Baicalein (blue, • • •). (D) Rate constants (k_{inh}) and reaction stoichiometry (n) determined for CDC and Baicalein in cumene and liposome autoxidations compared along with α -TOH and PMC.

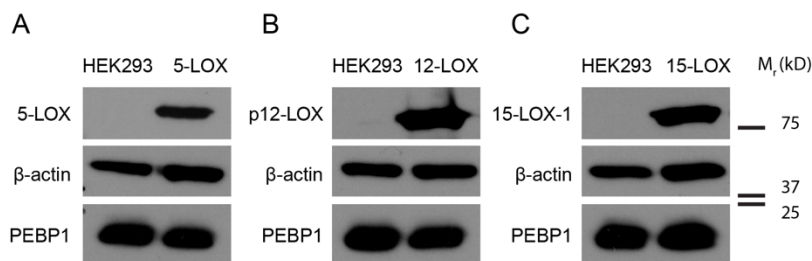


Figure S5.7 Western blots showing expression levels of PEBP1 in 5-LOX (A), 12-LOX (B) and 15-LOX (C) overexpressing cells compared alongside HEK293 cells.

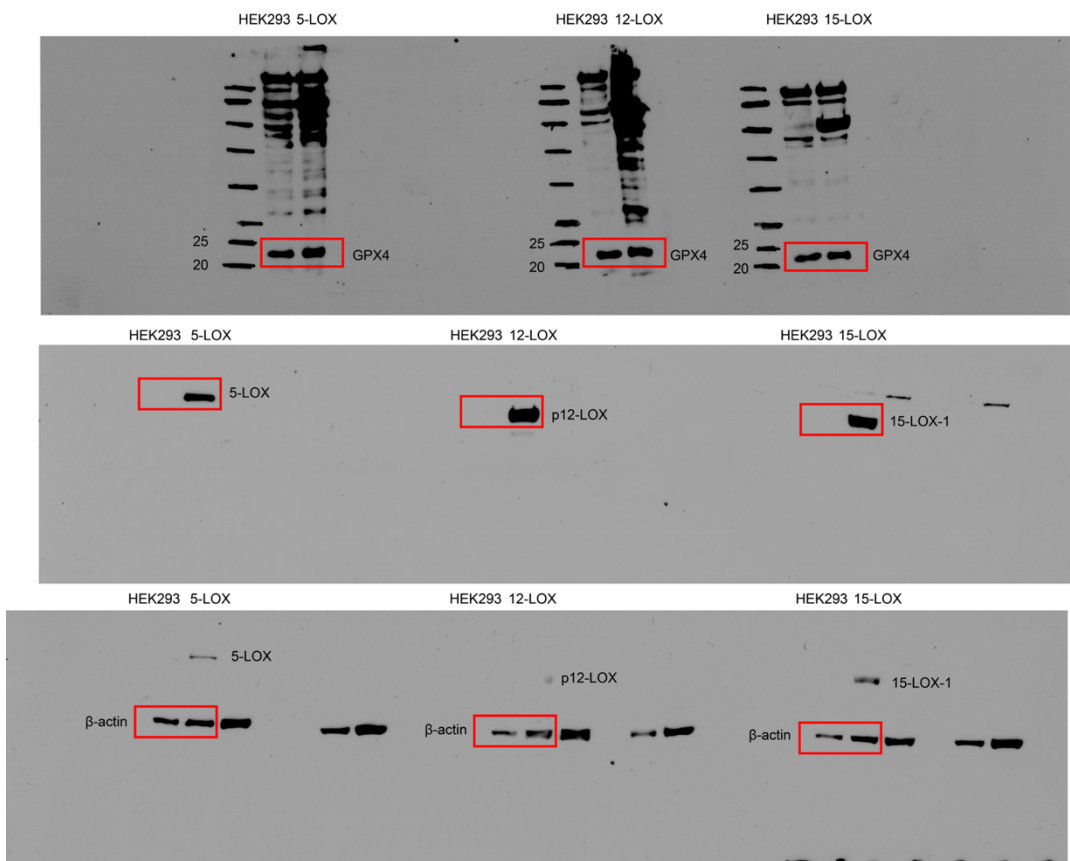


Figure S5.8 Uncropped blots.

5.6.16 Supporting Information References:

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Chapter 6: The Potency of Diarylamine Radical-Trapping Antioxidants as Inhibitors of Ferroptosis Underscores the Role of Autoxidation in the Mechanism of Cell Death

6.1 Preface

Two aromatic amines (ferrostatin-1 and liproxstatin-1) were recently identified from high-throughput screening efforts to uncover potent inhibitors of ferroptosis, the necrotic-like cell death induced by inhibition of glutathione peroxidase 4 (GPX4), deletion of the corresponding *gpx4* gene, or starvation of GPX4 of its reducing cosubstrate, glutathione (GSH). We have since demonstrated that these two aromatic amines are highly effective radical-trapping antioxidants (RTAs) in lipid bilayers, suggesting that they subvert ferroptosis by inhibiting lipid peroxidation (autoxidation) and, thus, that this process drives the execution of ferroptosis. Herein, we show that diarylamine RTAs used to protect petroleum-derived products from autoxidation can be potent inhibitors of ferroptosis. The diarylamines investigated include representative examples of additives to engine oils, greases and rubber (4,4'-dialkyldiphenylamines), core structures of dyes and pharmaceuticals (phenoxazines and phenothiazines), and aza-analogues of these three classes of compounds that we have recently shown can be modified to achieve much greater reactivity. We find that regardless of how ferroptosis is induced (GPX4 inhibition, *gpx4* deletion or GSH depletion), compounds which possess good RTA activity in organic solution ($k_{\text{inh}} > 10^5 \text{ M}^{-1} \text{ s}^{-1}$) and lipid bilayers ($k_{\text{inh}} > 10^4 \text{ M}^{-1} \text{ s}^{-1}$) are generally potent inhibitors of ferroptosis (in mouse embryonic fibroblasts). Likewise, structural analogs that do not possess RTA activity are devoid of anti-ferroptotic activity. These results further support the argument that lipid peroxidation (autoxidation) plays a major role in the mechanism of cell death induced by either GPX4 inhibition, *gpx4* deletion, or GSH depletion. Moreover, it offers clear direction that ongoing medicinal chemistry efforts on liproxstatin and ferrostatin derivatives, which have been proposed as lead compounds for the treatment and/or prevention of ischemia/reperfusion injury, renal failure, and neurodegeneration, can be widened to

include other aminic RTAs. To aid in these efforts, some relevant structure–reactivity relationships are discussed. The preliminary data in this work was generated by Kaitlyn Margison. Reprinted (adapted) with permission from: Shah, R.; Margison, K. D.; Pratt, D. A. *ACS Chem. Biol.* **2017**, *12*, 2538. Copyright 2017 American Chemical Society.

6.2 Introduction

Ferroptosis is a recently characterized form of regulated necrosis in which the iron-dependent accumulation of (phospho)lipid hydroperoxides (LOOH) has been demonstrated to play a key role.^{1,2} As such, ferroptosis has attracted considerable attention for its potential involvement in the pathophysiology of degenerative diseases wherein lipid oxidation has been implicated, such as cardiovascular disease and neurodegeneration.^{3–5} Glutathione peroxidase 4 (GPX4), the only enzyme known to be capable of reducing phospholipid hydroperoxides to their corresponding alcohols (Figure 6.1),⁶ has been identified as a key regulator of ferroptosis. Inhibition of GPX4 via pharmacological intervention (e.g., with RSL3⁷) or genetic deletion (using the Cre/recombinase approach⁸) has been shown to induce ferroptosis. Likewise, depletion of cellular glutathione (GSH) by either inhibition of the acquisition of cystine from the extracellular medium (e.g., erastin inhibition of the system x_c[−] antiporter⁹) or inhibition of its biosynthesis (e.g., buthionine sulfoximine (BSO) inhibitor of γ -glutamylcysteine synthetase¹⁰) induces ferroptosis.

The accumulation of cellular LOOH is known to occur by two primary mechanisms: an iron-catalyzed peroxy radical-mediated process called autoxidation^{11,12} (Figure 6.1A) and enzyme-mediated processes catalyzed by nonheme iron-dependent lipoxygenases^{13,14} (LOXs; Figure 6.1B). Thus, compounds that inhibit either or both processes may provide important leads for therapeutic and/or preventive agents. Arachidonic acid is the native substrate of LOXs and is also the most abundant readily autoxidizable polyunsaturated fatty acid found in most cell types. The requirement for arachidonic acid (AA)-esterified phospholipids in the execution of ferroptosis is underscored by the fact that cells deficient in either ACLS4 or LPCAT3, the enzymes responsible for catalyzing the activation of AA to AA-CoA and transferring the AA from CoA to a lyso-phospholipid, respectively, are resistant to ferroptosis.^{15–17}

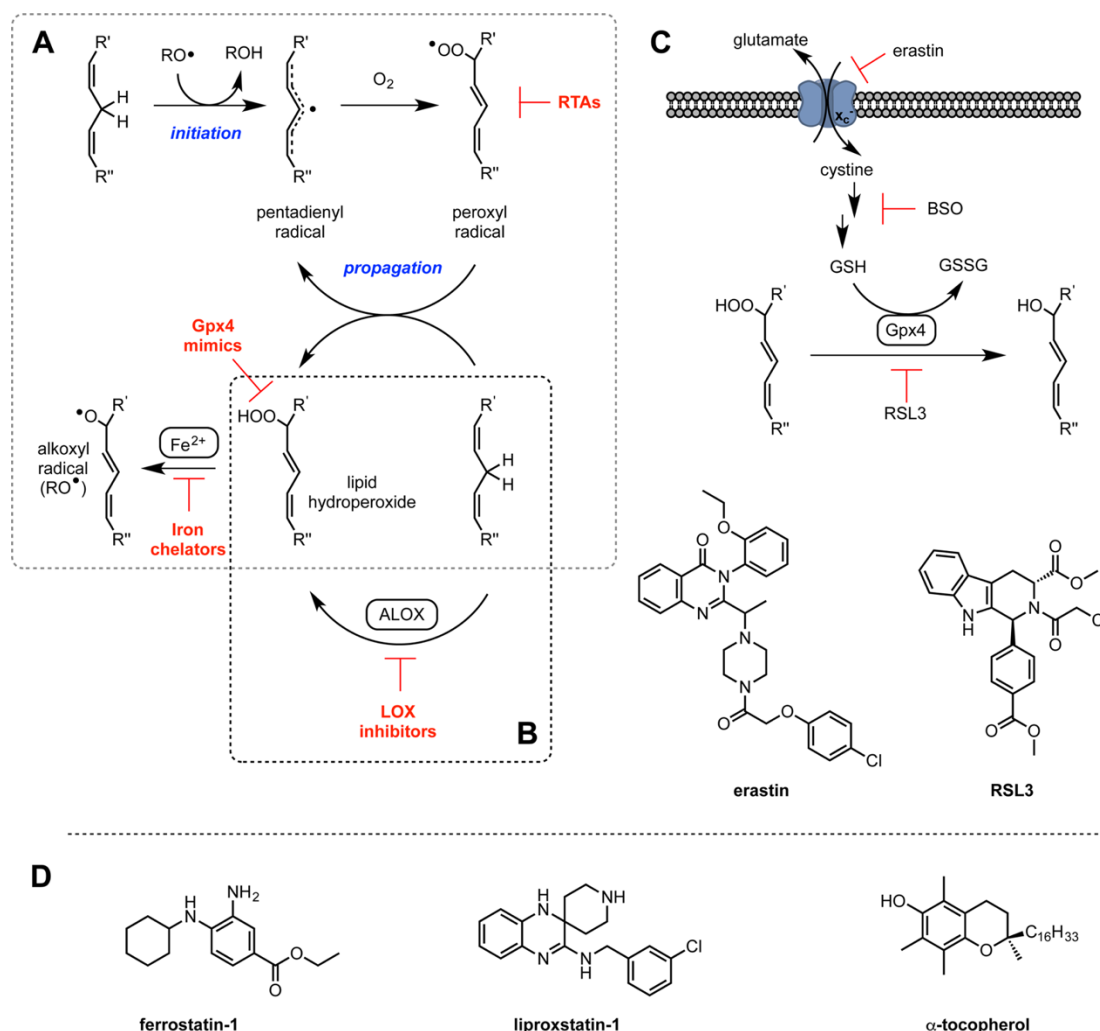


Figure 6.1 Generation of lipid hydroperoxide (LOOH) either by iron-accelerated autoxidation (**A**) or lipoxygenase catalyzed oxidation of polyunsaturated fatty acids (**B**). (**C**) Inhibition of either Gpx4 using RSL3 or cystine uptake by erastin leads to the induction of ferroptosis by increasing the concentration of lipid hydroperoxides in the cell. (**D**) Structures of potent inhibitors of ferroptosis.

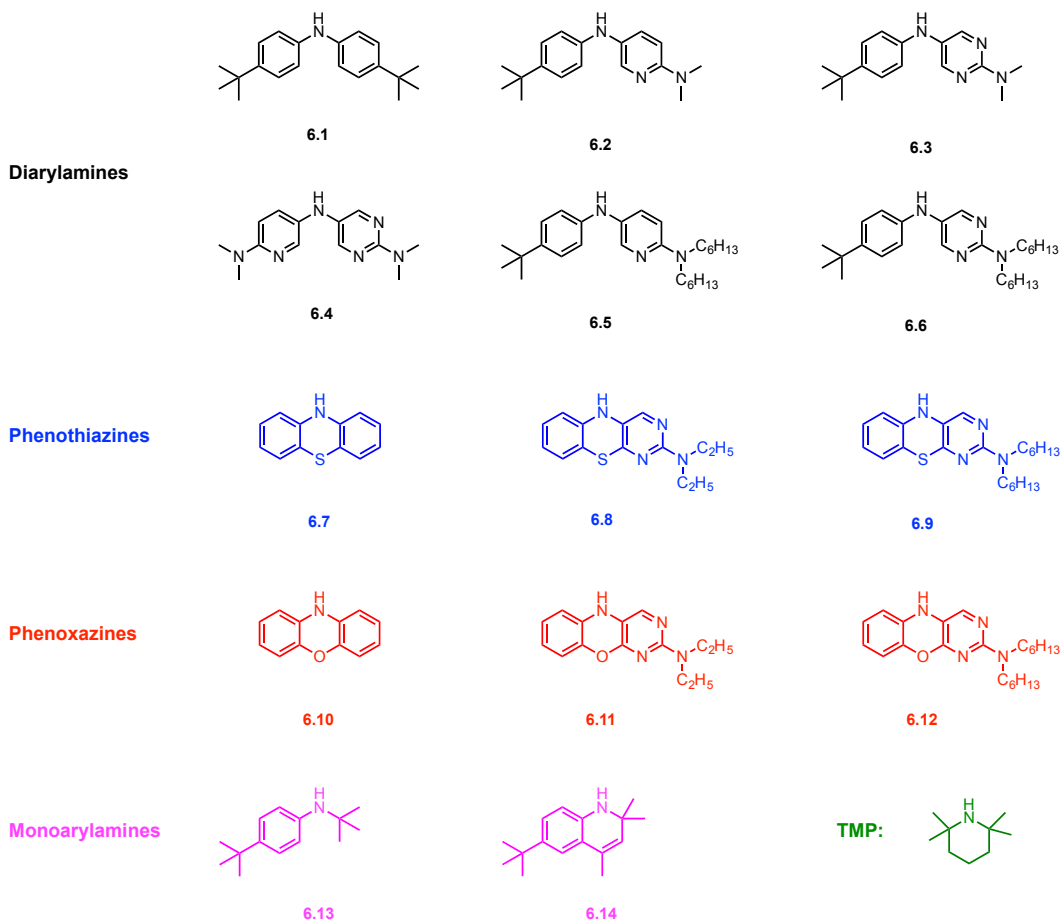
Ferrostatin-1 and lipoxstatin-1 (Fer-1 and Lip-1, respectively, see Figure 6.1D) were identified by the Stockwell and Conrad groups, respectively, as the first highly potent inhibitors of ferroptosis.^{1,6} Fer-1 and Lip-1 were discovered using a high-throughput screen for compounds capable of subverting ferroptosis initiated by either induced deletion of the *gpx4* gene in mouse embryonic fibroblasts (MEFs) or depletion of GPX4's reducing cosubstrate, GSH, by erastin in human fibrosarcoma cells. Although the cytoprotective activities of Fer-1 and Lip-1 were clearly associated with a diminution in cellular lipid oxidation (as determined by both BODIPY-C₁₁ staining and lipidomic analyses^{1,6}), it was unclear whether

this was due to inhibition of autoxidation or LOX catalysis. We recently showed that Lip-1 and Fer-1 are not effective inhibitors of human 15-LOX-1, but are highly efficient inhibitors of lipid autoxidation.¹⁸ Experimental and computational evidence were consistent with the rapid transfer of a H-atom from the arylamine moieties of Fer-1 and Lip-1 to autoxidation chain-carrying lipid peroxy radicals. This reactivity is characteristic of compounds generally referred to as radical-trapping antioxidants (RTAs), such as α -tocopherol (α -TOH), nature's premier RTA and also a good inhibitor of ferroptosis (albeit considerably less potent than Fer-1 and Lip-1).^{15,19}

Diarylamines are one of three classes of RTA that are routinely used to protect petroleum-derived products from oxidative degradation the others being phenols (such as α -TOH) and hindered aliphatic amines. Diarylamines are the additives of choice to inhibit the autoxidation of hydrocarbons at elevated temperatures (e.g., engine lubricants, fuels, and rubbers) due to a catalytic reactivity wherein the diarylamine is regenerated from an oxidation product in situ using the substrate as the stoichiometric reductant.^{20,21} Given that the inherent reactivity of Fer-1 and Lip-1 toward peroxy radicals ($k = 3.5$ and $2.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, respectively, at 37 °C in chlorobenzene)¹⁸ is similar to that of common industrial antioxidants (4,4'-dialkyldiphenylamines, for which $k = 1.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ under the same conditions),²⁵ we wondered if these industrial additives would be similarly potent inhibitors of ferroptosis. Moreover, we wondered if heterocyclic diarylamines – which we have shown to be up to 2000-fold more reactive than 4,4'-dialkyldiphenylamines^{22,25,38} – would be even more potent. Such a realization would not only provide alternative, and perhaps better, leads to preventive and/or therapeutic agents than Fer-1 and Lip-1, but would also contribute to resolving the relevance of autoxidation in the execution of ferroptosis.

6.3 Results and Discussion

The selection of arylamines investigated here is given in Scheme 6.1. Some are commercially available (**6.1**, **6.7**, and **6.10**), while the others were synthesized as we have described previously (see Supporting Information for specific details). They are grouped into four categories: diarylamines, phenothiazines, phenoxazines, and monoarylamines.



Scheme 6.1 Amines Investigated in This Work

The diarylamines include 4,4'-di-tert-butyl-diphenylamine (**6.1**), which is representative of the alkylated diphenylamines that are added to lubricants, fuels, and rubbers, and a variety of aza derivatives (**6.2–6.6**) that we have recently shown to be more reactive.²² Phenothiazine (**6.7**) has long been known to possess potent RTA activity,²³ and despite the fact that it is not generally used as an RTA, phenoxazine (**6.10**) has been shown to be more reactive still.²⁴ Again, we have shown that nitrogen incorporation into these compounds further enhances their reactivity toward peroxy radicals and have chosen some of those derivatives for study here (**6.8**, **6.9**, **6.11**, and **6.12**).²⁵ We also studied monoarylamines **6.13** and **6.14**, as well as 2,2,6,6-tetramethylpiperidine (TMP), which is representative of the structure of hindered amine light stabilizers, a common antioxidant additive to plastics and coatings.

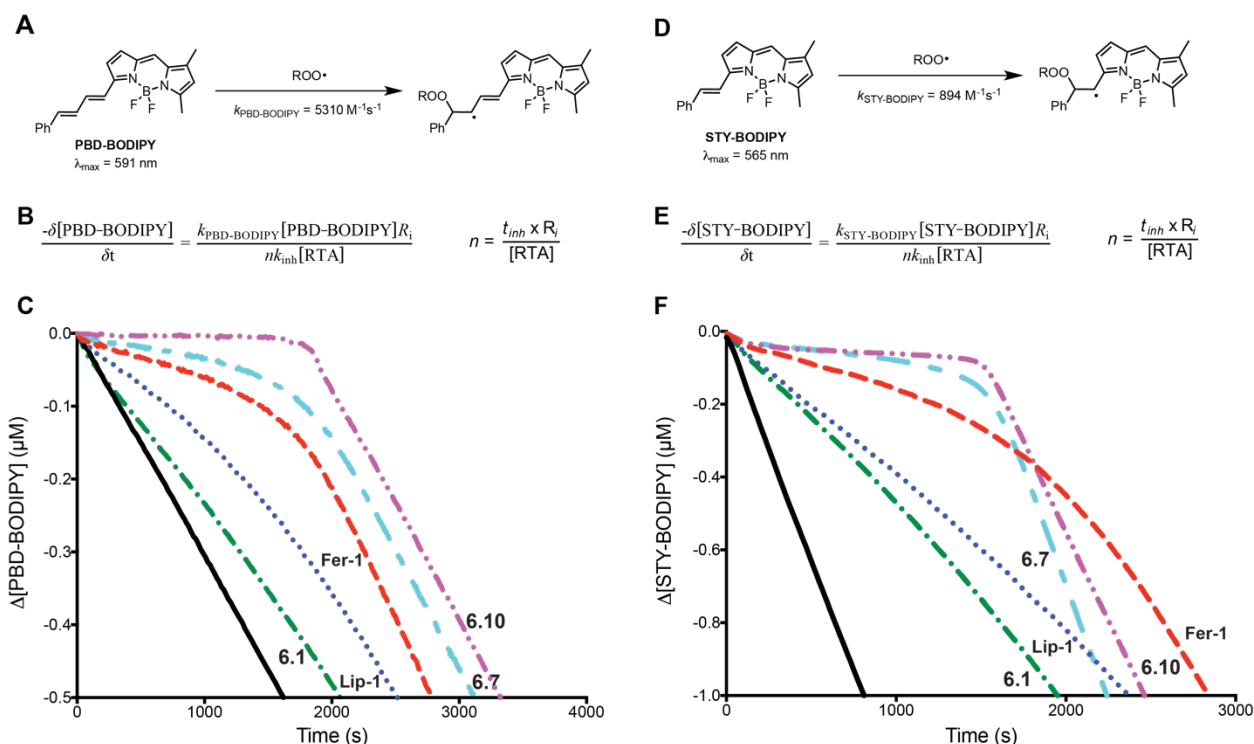


Figure 6.2 PBD-BODIPY (**A**) and STY-BODIPY (**D**) serve as the signal carriers in 1,4-dioxane and egg phosphatidylcholine autoxidations, respectively, enabling determination of inhibition rate constants (k_{inh}) and stoichiometries (n) for reactions of inhibitors with chain-carrying peroxy radicals (**B**, **E**). Representative co-autoxidations of 1,4-dioxane and PBD-BODIPY (10 μM) initiated by AIBN (6 mM) in chlorobenzene at 37 °C (black solid line) and inhibited by 2 μM of Lip-1 (blue •••), Fer-1 (red —), 6.1 (green —•—), 6.7 (cyan — —), and 6.10 (purple —••—) (**C**). Representative co-autoxidations of egg phosphatidylcholine liposomes (1.0 mM) and STY-BODIPY (10 μM) suspended in phosphate-buffered saline (10 mM) at pH 7.4 initiated by MeOAMVN (0.2 mM) at 37 °C (black solid line) and inhibited by 2 μM of Lip-1 (blue •••), Fer-1 (red —), 6.1 (green —•—), 6.7 (cyan — —), and 6.10 (purple —••—) (**F**).

To set a baseline for comparison, we first evaluated the inherent RTA activity of the test compounds by determining the kinetics of their reactions with peroxy radicals in inhibited autoxidations in organic solution under identical conditions. Although these experiments have long been carried out by the venerable O₂ consumption methodology, a recently developed spectrophotometric equivalent employing a highly oxidizable and highly absorbing cosubstrate (PBD-BODIPY) enables the experiments to be carried out far more quickly and reproducibly.²⁶ The approach is summarized in Figure 6.2, which includes the key reaction (**A**), how kinetic data are derived (**B**), and some representative results (**C**).

Table 6.1 Inhibition Rate Constants^a Obtained from Inhibited Co-Autoxidations of 1,4-Dioxane with PBD-BODIPY or Phosphatidylcholine Liposomes with STY-BODIPY at 37 °C^b

RTA	$k_{inh}^{PbCl} (M^{-1}s^{-1}) / n$	k_{rel}	$k_{inh}^{Liposome} (M^{-1}s^{-1}) / n$	k_{rel}
Fer-1	$(3.5 \pm 0.1) \times 10^5 / 2.0$	1.8	$(4.6 \pm 0.8) \times 10^4 / 3.1$	1.8
Lip-1	$(2.4 \pm 0.2) \times 10^5 / 1.9$	1.2	$(1.2 \pm 0.1) \times 10^4 / 3.5$	0.48
<i>Diarylamines</i>				
6.1	$(2.0 \pm 0.1) \times 10^5 / 1.0$	1	$(2.5 \pm 0.1) \times 10^4 / 1.8$	1
6.2	$(1.1 \pm 0.2) \times 10^7 / 2.2^c$	55	$(5.7 \pm 0.1) \times 10^5 / 3.2$	23
6.3	$(6.8 \pm 0.9) \times 10^6 / 1.9$	34	$(8.2 \pm 0.7) \times 10^4 / 2.1$	3.3
6.4	$(3.5 \pm 0.1) \times 10^7 / 2.3^c$	>250	$(7.7 \pm 0.1) \times 10^5 / 2.9$	31
6.5	$(1.1 \pm 0.2) \times 10^7 / 2.2^c$	55	$(6.8 \pm 0.1) \times 10^4 / 0.9$	2.7
6.6	$(6.8 \pm 0.9) \times 10^6 / 1.9$	34	$(1.3 \pm 0.1) \times 10^4 / 2.0$	5.2
<i>Phenothiazines</i>				
6.7	$(8.0 \pm 0.4) \times 10^6 / 2.1$	40	$(2.1 \pm 0.3) \times 10^5 / 1.9$	8.4
6.8	$(1.3 \pm 0.3) \times 10^8 / 2.1^c$	650	$(5.7 \pm 0.1) \times 10^5 / 1.8$	23
6.9	$(1.3 \pm 0.3) \times 10^8 / 2.1^c$	650	$(3.6 \pm 0.1) \times 10^5 / 1.8$	14
<i>phenoxazines</i>				
6.10	$(4.1 \pm 0.1) \times 10^7 / 2.3^c$	205	$(4.5 \pm 0.5) \times 10^5 / 1.9$	18
6.11	$(5.2 \pm 0.1) \times 10^8 / 1.8^c$	2600	$(6.2 \pm 0.2) \times 10^5 / 1.8$	25
6.12	$(5.2 \pm 0.1) \times 10^8 / 1.8^c$	2600	$(4.1 \pm 0.1) \times 10^5 / 1.6$	16
<i>monoarylamines</i>				
6.13	$(8.9 \pm 0.4) \times 10^4 / nd$	0.45	$(1.0 \pm 0.1) \times 10^4 / nd$	0.4
6.14	$(2.4 \pm 0.1) \times 10^5 / 1.7$	1.2	$(9.0 \pm 0.5) \times 10^4 / 2.1$	3.6
<i>aliphatic amine</i>				
TMP	too slow		too slow	

^aAverage relative rate constants (referenced to 1) are given alongside. ^cMeasurements carried out in the presence of 25% DMSO. DMSO engages the amine in a H-bond, precluding the transfer of the H-atom to peroxy radicals. The observed rate constant can be corrected to reveal its value in the absence of DMSO by assuming pre-dissociation kinetics and measuring the equilibrium constant for H-bond formation; see ref 27.

Briefly, the rate constant for the reaction of the amine with a peroxy radical (generally referred to as the inhibition rate constant, k_{inh}) is derived from the initial rates of the inhibited autoxidations, while the stoichiometry of the reaction (n) is given by the length of the inhibited period (τ_{inh}). An autoxidizable cosubstrate (e.g., styrene, cumene, or 1,4- dioxane) is essential to the quantification of this reactivity since it ensures that the autoxidation is a chain reaction, with a constant steady state concentration of peroxy

radicals for which the expressions in Figure 6.2B are derived. The results obtained for compounds **6.1–6.14** are given along with those determined for TMP, Fer-1, and Lip-1 in Table 6.1. The inherent reactivity of these compounds to peroxy radicals spans over 4 orders of magnitude ($k_{\text{inh}} \sim 10^4$ to $10^8 \text{ M}^{-1} \text{ s}^{-1}$). All the results are consistent with literature data (determined by O_2 consumption, PBD-BODIPY coautoxidation, or peroxy radical clocks) wherever comparison is possible. Overall, the aliphatic amine (TMP) is not an inhibitor, monoarylamines are poor inhibitors, diarylamines are good inhibitors, phenothiazines are excellent inhibitors, and phenoxazines are the best. Fer-1 and Lip-1, being monoarylamines, are among the least reactive compounds. They react more quickly than the simplest monoarylamines, since their aromatic rings are substituted with electron-donating groups (amine in Fer-1, amidine in Lip-1) which weaken the aminic N–H bond, but are less reactive than all the diarylamines except the alkylated diphenylamine **6.1** (slightly).

Moving toward cell models of ferroptosis, the inherent reactivities of the amines were evaluated in the lipid bilayers of unilamellar liposomes prepared from egg phosphatidylcholine (PC). Liposome autoxidations were monitored in a similar manner to the solution autoxidations described above, but with the more slowly autoxidizing cosubstrate STY-BODIPY (Figure 6.2D–F). Although the trends observed in solution are largely preserved in lipid bilayers, i.e., aliphatic amines are not inhibitors and monoarylamines are less effective than diarylamines, the absolute values of the apparent rate constants are lower by 1 to 3 orders of magnitude. Indeed, it is quite clear that the lipid bilayers afford a “leveling effect” on the reactivity of the amines with peroxy radicals; that is, where the span in k_{inh} in chlorobenzene is 10^4 to $10^8 \text{ M}^{-1} \text{ s}^{-1}$, in lipid bilayers it is only 10^4 to $10^5 \text{ M}^{-1} \text{ s}^{-1}$.

The diminution in the rate constants for the reaction between RTAs and peroxy radicals upon moving from organic solution to lipid bilayers is well-established for phenolic RTAs.^{28,29} For example, the reactivity of the most thoroughly studied natural RTA, α -TOH, drops from $k_{\text{inh}} = 3.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ in chlorobenzene to $4.7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ in egg phosphatidylcholine liposomes (Table 6.1). The reasons are two-fold: (1) H-bonding interactions between the H-atom donor and H-bond acceptors at the phospholipid/water interface preclude H-atom transfer from the RTA to peroxy radicals,²⁸ (2) increased

van der Waals contacts between aliphatic side chains on the RTA and the lipids results in poor dynamics,²⁹ slowing the encounter with radicals – be it at the interface or deep within the lipid bilayer. The importance of the latter is clear upon comparing the reactivity of the pairs of diarylamines that differ only in alkyl side chain substitution (**6.2/6.5** and **6.3/6.6**); despite having indistinguishable reactivity in solution, **6.2** and **6.3** have 8.4- and 6.3-fold higher reactivity in lipid bilayers than **6.5** and **6.6**, respectively. Nevertheless, if RTA activity in lipid bilayers is predictive of anti-ferroptotic potency, these data predict that diphenylamine **6.1**, representative of the alkylated diphenylamines used to preserve lubricants, fuels, and rubbers, should possess similar potency to Fer-1 and Lip-1. Moreover, the results suggest that phenoxazine, phenothiazine and many of the heterocyclic diarylamines we have designed will be even more potent than Fer-1 and Lip-1.

Table 6.2 Anti-Ferroptotic Activity and Cytotoxicity of Compounds **6.1–6.14** in Mouse Embryonic Fibroblasts and HepG2 Cells, Respectively^c

RTA	GPX4 inhibition with RSL3 ^a EC ₅₀ (nM)	<i>gpx4</i> deletion with TAM ^b EC ₅₀ (nM)	GSH depletion with erastin ^c EC ₅₀ (nM)	Cytotoxicity in HepG2 ^d TC ₅₀ (μM)
Fer-1	45±2	38±1	48±2	126±11
Lip-1	35±2	26±3	27±1	14.2±2.1
<i>Diarylamines</i>				
6.1	80±1	57±1	74±4	69.8±2.8
6.2	36±1	30±2	31±2	> 100
6.3	43±2	35±1	42±1	77.9±3.1
6.4	103±7	85±5	122±2	> 100
6.5	31±3	22±2	34±1	> 100
6.6	37±2	23±4	41±2	> 100
<i>Phenothiazines</i>				
6.7	22±1	20±2	24±1	> 100
6.8	153±4	172±8	152±5	> 100
6.9	15±1	21±2	21±1	91.8±3.7
<i>Phenoxazines</i>				
6.10	9±2	6±1	10±2	> 100
6.11	70±1	67±3	70±6	> 100
6.12	11±2	13±2	17±1	98.3±13.3
<i>Monoarylamines</i>				
6.13	4800±180	7100±228	11800±386	44.8±1.9
6.14	85±2	71±3	70±4	51.9±2.6
<i>aliphatic amine</i>				
TMP	>20000	>20000	>20000	> 100

^aFerroptosis was induced with RSL3 (100 nM), and cell survival was determined 6 h postinduction. ^bFerroptosis was induced with tamoxifen (1 μ M), and cell survival was determined 48 h postinduction. ^cFerroptosis was induced with erastin (1 μ M), and cell survival was determined 24 h postinduction. ^dCytotoxicity was determined by AquaBluer assay following 48 h incubation and was corroborated by LIVE/DEAD cell assays (see Supporting Information for the results). ^eAll data are reported as the mean $\pm$ SD of six analytical replicates of a minimum of three independent experiments

Accordingly, we carried on to determine the ability of the various arylamines to prevent ferroptosis in a variety of cell- based assays. We took advantage of the best-established methods available to induce ferroptosis in mouse embryonic fibroblasts (Pfa1 cells),⁸ beginning with inhibition of GPX4 using RSL3, which covalently modifies the enzyme, rendering it inactive.^{7,19} Compounds were preincubated with the cells for 30 min prior to RSL3 treatment, and cell viability was determined 6 h postinduction by Aqua-Bluer assay. The data are shown in Table 6.2. In general, the potency of the compounds paralleled the RTA activities determined in liposomes. Perhaps most interestingly, the potency of diphenylamine **6.1** was within a factor of 2 of Fer-1 and Lip-1 (80 nM versus 45 and 35 nM, respectively). Moreover, the heterocyclic diarylamines **6.2**, **6.3**, **6.5**, and **6.6** were similarly potent or slightly more so than Fer-1 and Lip-1. Phenoxazines **6.10** and **6.12** were the most potent inhibitors of ferroptosis, with EC₅₀ values of 9 and 11 nM, respectively, followed closely by phenothiazines **6.7** (22 nM) and **6.9** (15 nM). Interestingly, despite their impressive RTA activity in solution and in liposomes, compounds **6.4**, **6.8**, and **6.11** were comparatively poor inhibitors of ferroptosis.

Indirect inhibition of GPX4 through limitation of the supply of its reducing cosubstrate (GSH) is also a common model of ferroptosis. Depletion of GSH can be achieved in a number of ways, but inhibition of system x_c⁻, an antiporter that mediates the exchange of intracellular glutamate for extracellular cystine, is a particularly useful strategy because it does not rely on the depletion of GSH by added electrophilic reagents that may (likely) have other cellular targets. Thus, MEFs were seeded overnight in 96-well plates, followed by concomitant administration of the system x_c⁻ inhibitor erastin and the test compounds, and cell viability was measured 24 h after induction. The data are collected alongside the results of direct inhibition of GPX4 with RSL3 in Table 6.2. Similar trends were observed overall,

with compounds **6.2**, **6.3**, **6.5**, and **6.6** being similarly potent to Lip-1 ($EC_{50} = 27$ nM) and Fer-1 ($EC_{50} = 48$ nM); compounds **6.7**, **6.9**, **6.10**, and **6.12** being slightly more potent; and compounds **6.4**, **6.8**, and **6.11** being less so.

To avoid any potential artifacts arising from the interaction of the test compounds with either RSL3 or erastin, an inducible genetic knockout model was used to introduce GPX4 deficiency in MEFs. Pfa-1 MEFs feature *gpx4* genes flanked with *loxP* sites, which direct deletion of the gene by Cre recombinase upon the addition of tamoxifen.⁶ Thus, cells were plated in the presence of tamoxifen in order to induce knockout of *gpx4*. The test compounds were added 24 h later, and cell viability was assessed 48 h postinduction by AquaBluer assay. The data are tabulated in Table 6.2 alongside those for RSL3 and erastin treatment of the same MEFs. The results are fully consistent with the foregoing experiments. It is gratifying and somewhat remarkable that all three models of ferroptosis, executed with different reagents and over different time periods, yield the same trends in the potencies of the test compounds. It should be pointed out that this is not the only report that demonstrates aromatic amines can be highly potent inhibitors of oxidative cell death. In 2001, Moosmann and co-workers investigated the protective activity of aromatic amines and imines against cell death in HT-22 mouse hippocampal cells induced by either glutamate challenge (inhibition of system x_c^-), BSO administration, or H_2O_2 administration.³⁰ Although their contribution predates the characterization of ferroptosis, and some of the investigated conditions may have instead led to apoptosis, our results are fully consistent with Moosmann's earlier results in particular, that phenoxazine and phenothiazine are highly potent cytoprotective compounds.

At first glance, our results suggest that the cytoprotective ability of aromatic amines can be improved by optimization of their RTA reactivity. However, when their potency is plotted against their RTA activity in each of solution and lipid bilayers (Figure 6.3A and B, respectively), some important considerations come to light. The plots in Figure 6.3 include data for α -TOH and its sidechain-truncated analog 2,2,5,6,8-pentamethyl-7-hydroxychroman (PMHC), as well as C_{15} -THN, a potent aza-tocopherol derivative.¹⁸ To rule out coincidental correlation of RTA activity and cytoprotection in the arylamines

studied here, we synthesized *N*-methylated analogs of diphenylamine **6.1** and phenoxazine **6.10**, which we anticipated would be devoid of RTA activity owing to the lack of a labile arylamine H-atom. Indeed, STY-BODIPY/egg-PC co-autoxidations were not inhibited by these compounds, and they were also completely ineffective at subverting ferroptosis induced by GPX4 inhibition, *gpx4* deletion, or GSH depletion (data provided in the Supporting Information).

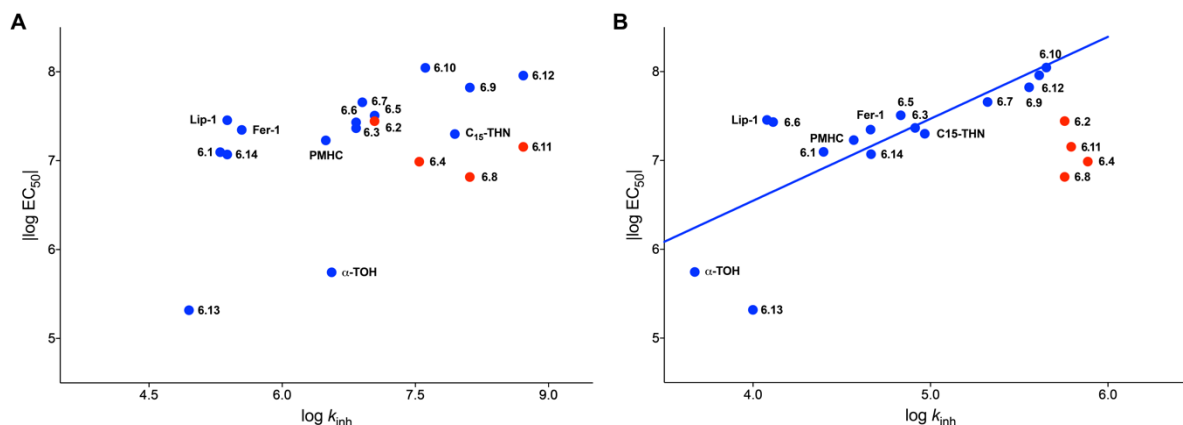


Figure 6.3 Correlations of the cytoprotective potency of RTAs against RSL3-induced ferroptosis in mouse embryonic fibroblasts and their inhibition rate constants derived from inhibited co-autoxidations in either chlorobenzene (**A**) or egg phosphatidylcholine liposomes (**B**).

Figure 6.3A clearly illustrates that the potency of the compounds does not correlate well with the solution-phase kinetic data. However, the majority of the data in Figure 6.3B lie on a correlation that relates the potency of the compounds to their RTA activity in liposomes. Four diarylamines are exceptions: **6.2**, **6.4**, **6.8**, and **6.11** (data indicated in red in Figure 6.3). We surmised that this was due to the ease with which they autoxidize (e.g., compare $E^\circ = 0.54$ and 0.59 V for **6.8** and **6.11** with $E^\circ = 0.87$ and 0.85 V for phenoxazine and phenothiazine, respectively, and $E^\circ = 0.50$ for **6.4**) particularly in aqueous environs where the intermediates formed upon electron transfer to O₂ are better solvated. However, we found these compounds to be stable under the assay conditions (see Supporting Information). Instead, since these compounds are significantly more basic compared to the (di)phenylamines (pK_a's of 9.8 and 8.4 have been measured for the conjugate acids of **6.2** and **6.4**; compare to 4.6 for the conjugate acid of **6.1** under the same conditions),²² it seems likely that protonation

will reduce the concentration of the compound in the lipid, where it is required to intercept chain-propagating lipid peroxy radicals. Appending longer alkyl side chains to these compounds (as in **6.5**, **6.9**, and **6.12**) aids in their partitioning to lipid regions, which is consistent with the fact that these derivatives better fit the correlation. Although partitioning of the RTAs between lipid and aqueous phases is relevant in both cells and liposomes, it is likely that this has a more significant impact in cells given that the compounds must traverse the plasma membrane and distribute themselves within the cell in competition with deleterious interactions with components in the cell culture medium to which they are exposed for significantly longer. Taken together, these results highlight the importance of sufficient lipid solubility to ensure rapid cell entry and/or localization as well as to minimize oxidative degradation but not too much lipid solubility such as to lead to poor radical-trapping reaction dynamics in lipid bilayers (*vide supra*).

The inherent reactivity of the arylamine to peroxy radicals is almost certainly not the only factor underlying their potency in cells. For example, Lip-1 is more potent than what is predicted solely based on its k_{inh} . However, it is characterized by a greater capacity for trapping peroxy radicals in liposome oxidations (*cf.* Table 6.1), implying that an oxidation product derived therefrom is still able to inhibit lipid peroxidation. The origin of this reactivity remains under investigation, but as we recently reported,¹⁸ it may involve the intervention of nitroxide intermediates. Alternatively, mechanisms wherein the RTA is recycled from its oxidation products may lead to greater potency than that expected from its k_{inh} alone. Thus, not only do the foregoing results suggest that STY-BODIPY/egg-PC coautoxidations are useful for screening the activities of potential ferroptosis inhibitors, but the identification of compounds with enhanced cell potency relative to RTA reactivity may suggest that additional mechanisms contribute to cytoprotection.

To complement our studies of the antiferroptotic potential of diarylamine RTAs, we carried out preliminary studies of their cytotoxicity again, in comparison with data obtained with Fer-1 and Lip-1. We chose HepG2 cells in lieu of the MEFs used in the foregoing experiments since they are a better model for liver metabolism and toxicity of arylamines.^{31,32} Like Fer-1 and Lip-1, the test compounds are

cytotoxic only at concentrations that are significantly (up to 10^5 -fold) higher than the concentrations at which they are cytoprotective, with most displaying no significant diminution in cell viability up to 100 μ M. Thus, like Fer-1 and Lip-1 which have already undergone some testing in animal models of degenerative disease^{6,33,34} it would appear that the diarylamines offer an attractive therapeutic window.

6.4 Conclusions

Fer-1 and Lip-1 were identified from high-throughput screening of commercial libraries using cell-based assays of ferroptosis, and subsequent medicinal chemistry efforts have aimed to improve the activity of the ferrostatin and liproxstatin scaffolds.^{6,35,36} The foregoing results clearly indicate that potent ferroptosis inhibition can be achieved by good diarylamine RTAs, in general, thereby opening the structural space for ferroptosis inhibitor development well beyond that of the ferrostatin and liproxstatin scaffolds. Furthermore, our recent demonstration that the naphthyridinols are also potent ferroptosis inhibitors suggests that the rational design of RTAs is arguably the most promising lead-generating strategy for ferroptosis inhibition and underscores that lipid peroxidation by autoxidation drives the execution of ferroptosis.

6.5 Experimental

6.5.1 General Experimental

Fer-1,³⁵ Lip-1,¹⁸ arylamines **6.1–6.14**,^{22,37,38} PBD-BODIPY and STY-BODIPY,²⁶ RSL-3,⁶ and Erastin⁹ were synthesized according to literature procedures. Egg phosphatidylcholine, MeOAMVN, MEM with/without phenol red, DMEM with/without phenol red, Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), penicillin-streptomycin, Aquabluer, and reagents for synthesis were purchased from commercial sources and used as received.

6.5.2 Inhibited Autoxidation of 1,4-Dioxane.

A 3.5 mL quartz cuvette was loaded with unstabilized 1,4-dioxane (0.625 mL) along with PhCl (1.805 mL). Experiments carried out in the presence of DMSO contained 0.625 mL along with 1,4-dioxanes (0.625 mL) and PhCl (1.18 mL). The cuvette was then preheated to 37 °C for approximately 5 min. The

cuvette was blanked and 12.5 μ L of 2 mM PBD-BODIPY in 2,3,5-trichlorobenzene was added followed by 50 μ L of 0.3 M AIBN in chlorobenzene followed by a thorough mixing. After 20 min, an aliquot of antioxidant stock solution (1 mM) in chlorobenzene was added, and the loss of absorbance at 587 nm was followed. The inhibition rate constant (k_{inh}) and stoichiometry (n) were determined for each experiment according to the equations in Figure 2B. Rate constants and standard derivations are derived from three independent experiments.

6.5.3 Inhibited Autoxidation of Egg-PC Liposomes.

To a 3 mL cuvette, 2.34 mL of 10 mM PBS at pH 7.4 was added followed by Egg-PC unilamellar liposomes (125 μ L of 20 mM stock solution in PBS at pH 7.4) and the solution equilibrated for 5 min at 37 °C. The cuvette was blanked, and 12.5 μ L of 2 mM STY-BODIPY in DMSO was added followed by 10 μ L of 0.05 M MeOAMVN in acetonitrile. The solution was thoroughly mixed. After 5 min, an aliquot of antioxidant stock solution (1 mM) in DMSO was added, and the loss of absorbance at 565 nm followed. The inhibition rate constant (k_{inh}) and stoichiometry (n) were determined for each experiment according to the equations in Figure 6.2E. Rate constants and standard derivations are derived from three independent experiments.

6.5.4 Cell Culture.

All cells were cultured at 37 °C in a 5% CO₂ atmosphere unless otherwise indicated. Mouse embryonic fibroblasts (Pfa1 cells) were cultured in DMEM-High Glucose containing 10% FBS, 10 mM glutamine, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. Cells were passaged by dissociation with 0.05% trypsin and 0.2% EDTA every other day. HepG2 cells were cultured in MEM containing 10% FBS, 10 mM glutamine, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. Cells were passaged by dissociation with 0.05% trypsin and 0.2% EDTA every three to four days.

6.5.5 Inhibition of Ferroptosis Induced by Gpx4 Inhibition with (1*S*,3*R*)-RSL3.

Pfa-1 cells (3000 in 100 μ L) were seeded in 96-well plates and incubated overnight. The next day the media was removed. The cells were washed twice with PBS, and suspended in new media and treated

with different compounds for 30 min before the addition of (*1S,3R*)-RSL3 (100 nM) to a final volume of 100 μ L. Cell viability was assessed 6 h later using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Each experiment was carried out in six analytical replicates per concentration of RTA and repeated independently at least three times.

6.5.6 Inhibition of Ferroptosis Induced by Erastin.

Pfa-1 cells (3000 in 100 μ L) were seeded in 96-well plates and cultured overnight. The next day 1 μ M erastin was added to each well followed by treatment with the test compounds. Cell viability was assessed 24 h later using the AquaBluer assay according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Each experiment was carried out in six analytical replicates per concentration of RTA and repeated independently at least three times.

6.5.7 Inhibition of Ferroptosis Induced by *gpx4* Deletion.

Tamoxifen-inducible Gpx4^{-/-} Pfa-1 cells (1000 in 100 μ L) were seeded in 96-well plates and cultured for 48 h in the presence of 1 μ M tamoxifen. After 24 h, the test compounds were added. Cell viability was determined 48 h after ferroptosis induction using the Aquabluer assay according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Each experiment was carried out in six analytical replicates per concentration of RTA and repeated independently at least three times.

6.5.8 Cytotoxicity.

HepG2 cells (3000 in 100 μ L) were seeded in 96-well plates and cultured overnight. The next day, stock concentrations of several arylamines were added and incubated for 48 h. Cell viability was assessed 48 h later using the AquaBluer assay according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Each experiment was carried out in six analytical replicates per concentration of RTA and repeated independently at least three times.

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6.7 Supporting Information

6.7.1 Synthesis

Compounds **6.2**, **6.3**, **6.5** and **6.6** were prepared according to our previously reported procedure.¹ Compounds **6.9** and **6.12** were also prepared according to recently reported procedure.² *N*-methyl derivatives, compounds **6.1A**³ and **6.10A**⁴ were synthesized according to previously reported procedure.

*N*⁵-(4-(*tert*-butyl)phenyl)-*N*²,*N*²-dimethylpyrimidine-2,5-diamine (**6.2**). Yellow Solid, 84%. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.22 (s, 2H), 7.46 (s, 1H), 7.19 – 7.12 (m, 2H), 6.73 – 6.62 (m, 2H), 3.10 (s, 6H), 1.22 (s, 9H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 158.6, 152.2, 143.7, 140.2, 126.9, 125.7, 113.3, 36.9, 33.5, 31.3. HRMS: *m/z* Calc: C₁₆H₂₂N₄: 270.1844 Found: 270.1849.

*N*⁵-(4-(*tert*-butyl)phenyl)-*N*²,*N*²-dimethylpyridine-2,5-diamine (**6.3**). Brown Solid, 87%. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.93 (d, *J* = 2.8 Hz, 1H), 7.48 (s, 1H), 7.34 (dd, *J* = 8.9, 2.9 Hz, 1H), 7.14 (d, *J* = 8.3 Hz, 2H), 6.72 (d, *J* = 8.2 Hz, 2H), 6.62 (d, *J* = 9.0 Hz, 1H), 2.97 (s, 6H), 1.22 (s, 9H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 155.3, 143.7, 140.6, 139.9, 131.3, 129.0, 125.6, 113.6, 106.1, 38.1, 33.5, 31.3. HRMS: *m/z* Calc: C₁₇H₂₃N₃: 269.1892 Found: 269.1890.

*N*⁵-(4-(*tert*-butyl)phenyl)-*N*²,*N*²-dihexylpyrimidine-2,5-diamine (**6.5**). Yellow Solid, 78%. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.17 (s, 2H), 7.39 (s, 1H), 7.20 – 7.09 (m, 2H), 6.71 – 6.59 (m, 2H), 3.48 (t, *J* = 7.5 Hz, 4H), 1.60 – 1.48 (m, 4H), 1.28 – 1.23 (m, 12H), 1.21 (s, 9H), 0.90 – 0.81 (m, 6H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 158.3, 153.1, 144.4, 140.5, 127.0, 126.1, 113.6, 47.7, 34.0, 31.8, 31.7, 27.8, 26.9, 22.5, 14.4. HRMS: *m/z* Calc: C₂₇H₄₃N₃: 410.3409 Found: 409.3404.

N⁵-(4-(tert-butyl)phenyl)-N²,N²-dihexylpyridine-2,5-diamine (6.6). Orange Solid, 69%. ¹H NMR (400 MHz, DMSO- *d*₆) δ 7.37 (s, 1H), 7.09 (d, *J* = 8.7 Hz, 2H), 6.95 – 6.84 (m, 2H), 6.73 (d, *J* = 8.7 Hz, 2H), 6.55 (d, *J* = 9.0 Hz, 2H), 3.13 (t, *J* = 7.5 Hz, 4H), 1.43 (h, *J* = 6.9 Hz, 4H), 1.24 (d, *J* = 3.1 Hz, 12H), 1.19 (s, 9H), 0.89 – 0.78 (m, 6H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 144.0, 143.7, 140.0, 132.6, 125.9, 121.7, 114.4, 113.7, 51.2, 34.0, 31.8, 31.6, 31.6, 27.3, 26.7, 22.64, 14.3. HRMS: *m/z* Calc: C₂₇H₄₃N₃: 409.3457 Found: 409.3461.

N,N-dihexyl-5H-benzo[b]pyrimido[5,4-*e*][1,4]oxazin-2-amine (6.9). Yellow Solid, 80%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.97 (s, 1H), 7.45 (s, 1H), 6.85 – 6.68 (m, 2H), 6.59 – 6.40 (m, 2H), 3.35 (d, *J* = 9.0 Hz, 4H), 1.47 (t, *J* = 7.2 Hz, 4H), 1.24 (d, *J* = 8.5 Hz, 12H), 0.93 – 0.80 (m, 6H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 157.8, 156.7, 141.7, 140.6, 132.5, 125.4, 119.7, 116.3, 115.6, 113.9, 47.5, 31.6, 27.8, 26.5, 22.5, 14.3. HRMS: *m/z* Calc: C₂₇H₄₃N₃: 368.2576 Found: 368.2577.

N,N-dihexyl-5H-benzo[b]pyrimido[5,4-*e*][1,4]thiazin-2-amine (6.12). Yellow Solid, 82%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.17 (s, 1H), 7.51 (s, 1H), 7.00 – 6.81 (m, 2H), 6.71 – 6.52 (m, 2H), 3.38 (d, *J* = 7.3 Hz, 4H), 1.47 (t, *J* = 7.3 Hz, 4H), 1.25 (q, *J* = 8.0, 6.3 Hz, 12H), 0.93 – 0.80 (m, 6H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 156.8, 151.9, 140.7, 140.3, 127.9, 126.5, 125.1, 120.6, 114.2, 114.0, 47.0, 31.0, 27.2, 26.0, 22.0, 13.9. HRMS: *m/z* Calc: C₂₇H₄₃N₃: 384.2348 Found: 384.2346.

6.7.2 Inhibition of Ferroptosis Induced by Gpx4 Inhibition with (1S,3R)-RSL3.

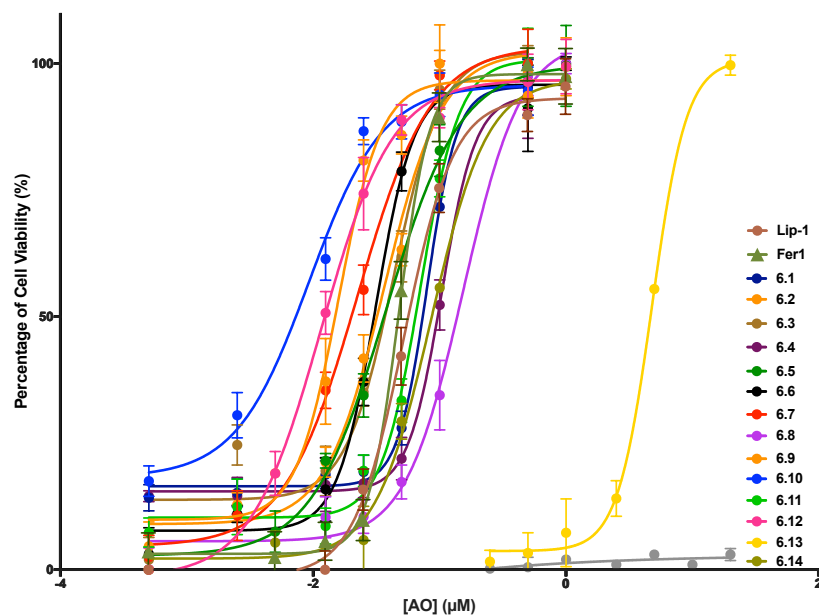


Figure S6.1 RSL3 induced ferroptosis inhibited by several arylamine radical-trapping antioxidants.

6.7.3 Inhibition of Ferroptosis Induced by Erastin.

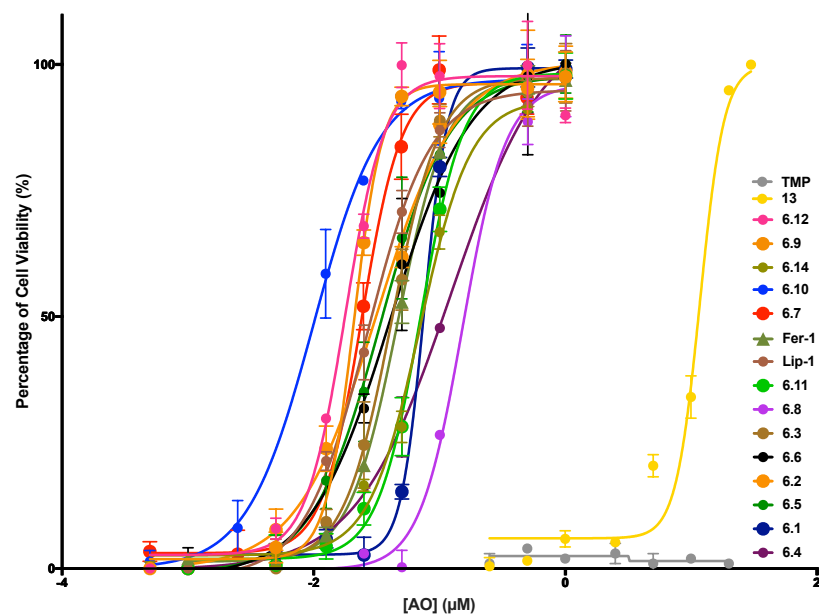


Figure S6.2 Erastin induced ferroptosis inhibited by several arylamine radical-trapping antioxidants.

6.7.4 Inhibition of Ferroptosis Induced by gpx4 Deletion.

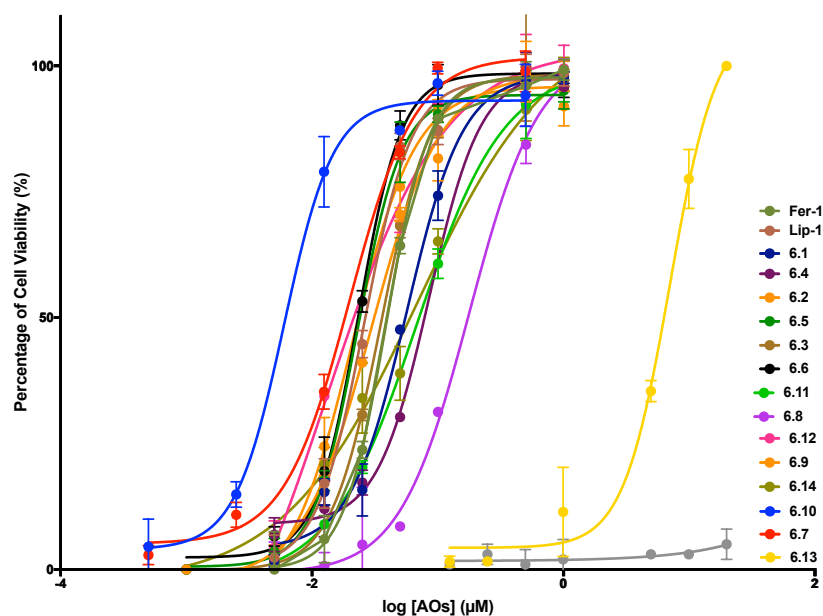


Figure S6.3 TAM induced ferroptosis inhibited by several arylamine radical-trapping antioxidants.

6.7.5 Cytotoxicity assay using HepG2.

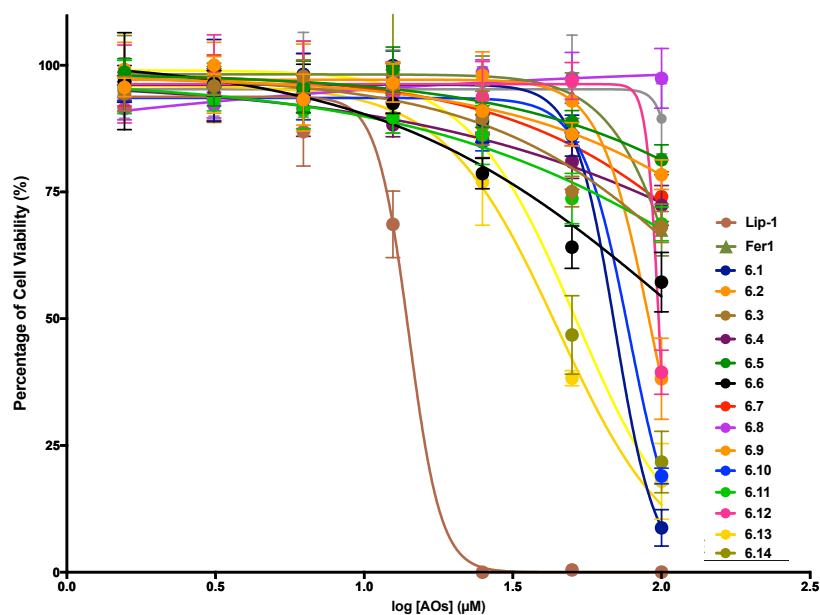


Figure S6.4 Cytotoxicity data of several arylamine radical-trapping antioxidants.

6.7.6 Live/Dead cell assay.

HepG2 cells (3,000 in 100 μL) were seeded in 96-well plates and cultured overnight. The next day stock concentrations of several arylamines was added and incubated for 48 hours. The relative percent of live and dead cells were determined using the Live/Dead cell staining kit II as per manufacturer's protocol (Promokine).

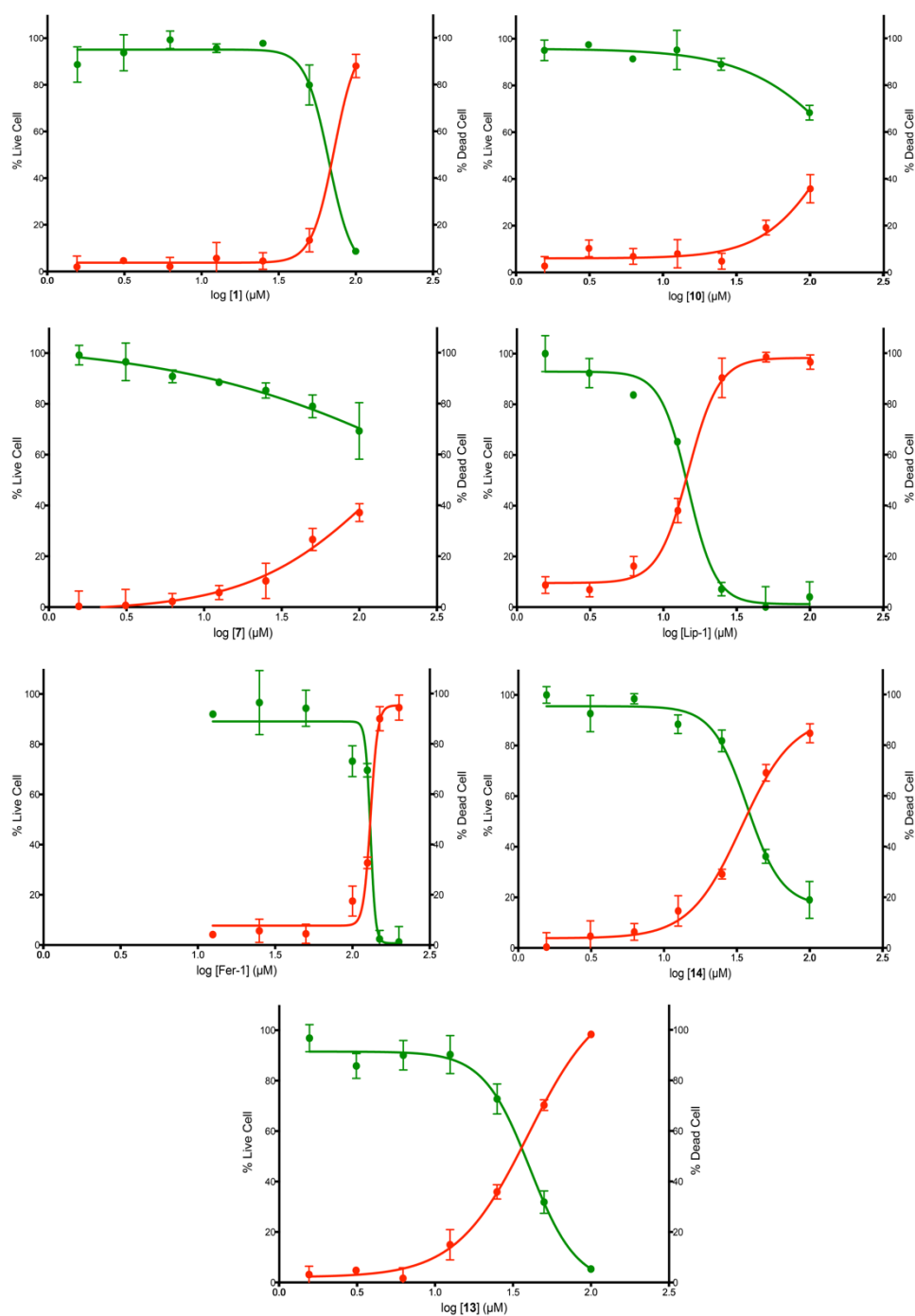


Figure S6.5 The relative percent of live and dead cells determined by the Live/Dead cell staining kit for several different arylamines. Live cells were determined by an increase in fluorescence due to the metabolism of Calcein-AM dye at 515 nm and the dead cells were determined by monitoring the enhancement of fluorescence of Ethidium Homodimer-III at 635 nm.

6.7.7 Effect of *N*-Methyl derivatives of compound **6.1** and **6.10**:

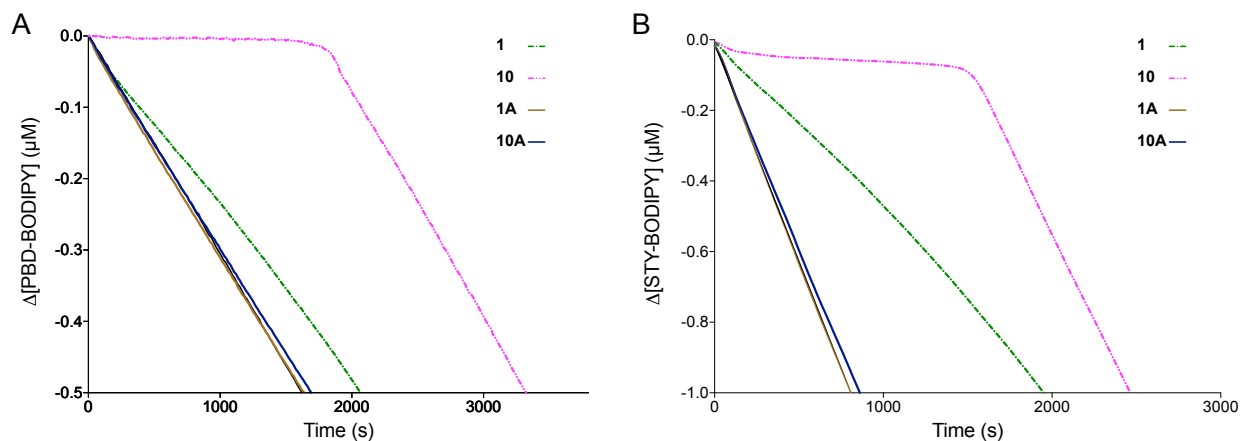


Figure S6.6 (A) Co-oxidations of 25% 1,4 dioxane and PBD-BODIPY (10 μ M) initiated by AIBN (6 mM) in chlorobenzene at 37°C (black) and inhibited by 2 μ M of either **6.1** or **6.10** and their *N*-Methyl analogs **6.1A** and **6.10A**. (B) Co-oxidations of egg phosphatidylcholine liposomes (1.0 mM) and STY-BODIPY (10 μ M) suspended in phosphate-buffered saline (10 mM) at pH 7.4 initiated by MeOAMVN (0.2 mM) at 37°C (black) and inhibited by 2 μ M of either **6.1** or **6.10** and their *N*-Methyl analogs **6.1A** and **6.10A**

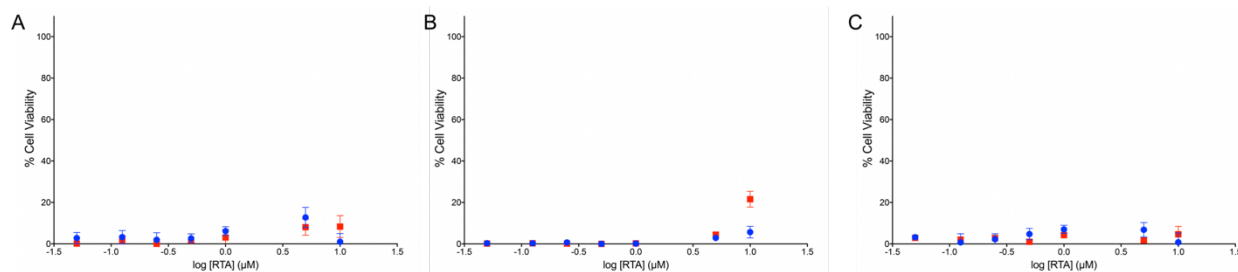


Figure S6.7 Compounds **6.1A** and **6.10A** exhibit no anti-ferroptotic activities in Pfa1 cells as high as 10 μ M when ferroptosis is induced via either RSL3 (A), Erastin (B) or TAM (C)

6.7.8 Stability of different RTAs in lipid bilayers of Egg-PC liposomes

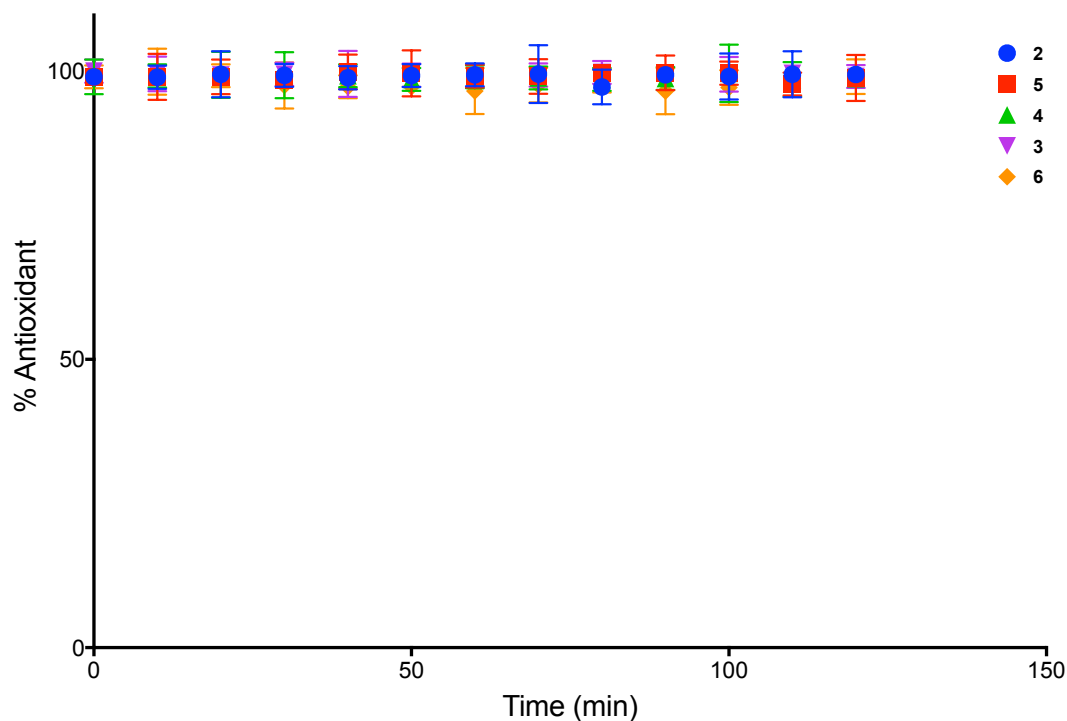


Figure S6.8 Decomposition of different RTAs (10 μ M) in Egg-PC liposomes at 37 $^{\circ}$ C observed by monitoring the loss in absorbance of compounds **6.2** (220 nm), **6.3** (222 nm), **6.4** (220 nm), **6.5** (223 nm) and **6.6** (223 nm).

6.7.9 Supporting Information References.

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Chapter 7: Beyond DPPH – Use of Fluorescence-ENabled Inhibited AutoXidation (FENIX) to Predict Oxidative Cell Death Rescue

7.1 Preface

‘Antioxidant activity’ is an often invoked, but generally poorly characterized, molecular property – particularly in biological contexts where antioxidants are often hailed as proverbial silver bullets against diseases in which lipid peroxidation has been implicated. Several assays exist to determine ‘antioxidant activity’, the most popular of which is based upon the ability of a putative antioxidant to reduce the persistent radical DPPH. Herein we show that the capacity of a molecule to reduce DPPH does not at all correlate with its ability to rescue cells from ferroptosis, the cell death modality which results directly from lipid peroxidation. We subsequently describe our efforts to develop an approach, dubbed FENIX (Fluorescence-ENabled Inhibited AutoXidation), that quantifies the reactivity of putative antioxidants with the (phospho)lipid peroxy radicals that propagate (phospho)lipid peroxidation. The development of the assay is relayed to demonstrate the importance of an appropriate medium, substrate concentration and initiator of lipid peroxidation. The results obtained with this approach afford an excellent correlation with anti-ferroptotic potency, which facilitates mechanistic characterization of ferroptosis inhibitors, and reveals the importance of H-bonding interactions between antioxidant and phospholipid that underlie both the lacklustre antioxidant activity of phenols under physiologically-relevant conditions and the emergence of arylamines as inhibitors of choice. The predictive implementation of the assay was enabled by the synthesis of a new initiator of phospholipid peroxidation, di-*tert*-undecylhyponitrite, which produces lipophilic alkoxyl radicals that more closely resemble the initiating radicals that derive from the lipid peroxidation products that seed ferroptotic cell death. The work was performed in conjunction with Omkar Zilka, Luke A. Farmer and Antonious T. M. Van Kessel. O.Z. carried out the synthesis and characterization of DTUN; LF was responsible for the synthesis of the phenols and characterizing their reactivity in different H-bonding solutions; ATMVK provided preliminary data. Reprinted (adapted) with

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7.2 Introduction

Lipid peroxidation, the radical-mediated chain reaction that converts lipids into lipid hydroperoxides (and a myriad of other oxidation products) has been implicated in the pathogenesis of virtually every type of degenerative disease.¹ The current molecular level understanding of lipid peroxidation (simplified mechanism, Figure 7.1A)^{2,3} has been established largely on the basis of detailed chemical studies of hydrocarbon autoxidation, the process primarily responsible for the oxidative degradation of petroleum-derived products.^{4,5} As such, it has long been thought that radical-trapping antioxidants (RTAs),^{6,7} which are preservatives added to essentially every type of hydrocarbon-based commercial and/or industrial product, may slow the onset and development of degenerative disease. Despite considerable investigation, the utility of RTAs in commercial and/or industrial applications has not translated to results in the clinic. Put simply, there has been overwhelming evidence against antioxidant intervention as a useful strategy for degenerative disease treatment and/or prevention in humans.⁸

At the root of many clinical trials of antioxidant treatment and/or supplementation is the characterization of potential antioxidants using assays of ‘antioxidant activity’. The most common assays involve the decolourization of a highly-absorbing oxidant by reduction with a putative antioxidant. Examples include DPPH,⁹ FRAP,¹⁰ ORAC,¹¹ and ABTS.¹² The limitations of these assays have been elaborated upon elsewhere.¹³ Most importantly, they fail to provide information about the reactivity of a potential inhibitor towards the kinetically relevant oxidizing species – a propagating lipid peroxy radical (i.e. k_{inh} , Figure 7.1A).^{6,7} This is where the divergence between industrial compound development and potential preventive/therapeutic compounds originates. Industrial antioxidants, such as hindered phenols and aromatic amines, are developed and tested in a context that is highly similar to their eventual application – and where the experimental methods measure relevant properties, such as oxidation product formation or viscosity changes.^{14–17} This underscores the need for methods which assay RTA activity

under physiologically relevant conditions. Of course, it must be acknowledged that modeling a lubricant or polymer is far easier than a cell, tissue or whole organism.

The recent characterization of ferroptosis,^{18–20} a form of regulated necrosis associated with the accumulation of (phospho)lipid oxidation products, and the development of new cell-based tools to study it, have provided an unprecedented capability to investigate the link between lipid peroxidation and disease. Ferroptosis can be induced in a variety of ways, but the quintessential – and arguably most direct – method is to interfere with the cell's ability to detoxify (phospho)lipid hydroperoxides. Left to accumulate, the hydroperoxides can undergo one-electron reduction by ferrous iron, or other one electron reductants, to produce lipid-derived alkoxyl radicals that can initiate further lipid peroxidation chain reactions. Glutathione peroxidase 4 (GPX4), a selenoprotein that catalyzes the glutathione-mediated reduction of hydroperoxides to alcohols, is the unique enzyme that can operate directly on phospholipid hydroperoxides – and is therefore generally considered the master regulator of ferroptosis.^{19,21,22}

The inhibition of GPX4 (e.g. with RSL3^{18,23} or ML210,^{24,25} Figure 1B), deletion of the gene encoding it^{19,26} or suppression of its expression, present the ideal basis for cell-based assays of inhibitors of lipid peroxidation. Indeed, such assays have been used to screen compound libraries to identify some of the most potent ferroptosis inhibitors to date (e.g. ferrostatin-1^{18,27} and liproxstatin-1,¹⁹ Figure 1E). However, because cellular lipid peroxidation can be modulated in others ways (e.g. the incorporation of (non-)oxidizable polyunsaturated fatty acids in the lipid bilayer),^{28,29} these cell-based assays need to be complemented by a method that can unambiguously resolve intervention in the propagation of lipid peroxidation. Such a method would enable resolution of inhibitors that do not act as antioxidants, providing insight on other mechanisms that may contribute to ferroptosis and/or its regulation. Building upon previous work wherein we showed that a small amount of an autoxidizable chromophore (i.e. STY-BODIPY or PBD-BODIPY) can be used to monitor hydrocarbon autoxidations and their inhibition by RTAs,³¹ we now report a microplate-based assay that enables screening for, and assessment of, RTA activity in phospholipid bilayers. We relay its development in an effort to highlight the important aspects that must be considered to accurately determine antioxidant efficacy in a cellular context. These aspects

clarify why phenolic antioxidants are not particularly effective under physiologically-relevant conditions, why aromatic amines are strongly preferred, and serve to guide future design and/or optimization of ferroptosis inhibitors.

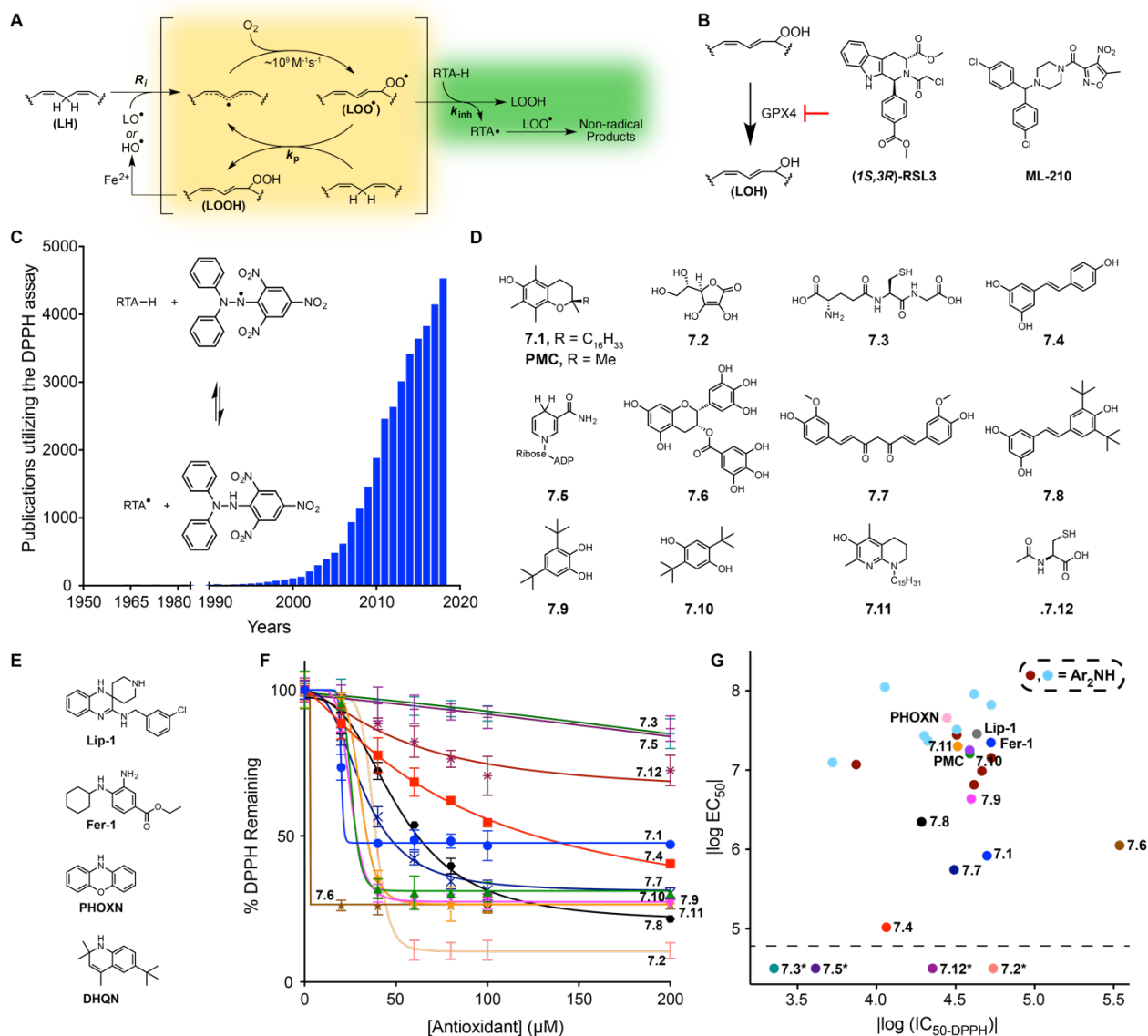


Figure 7.1 (A) Simplified mechanism of lipid peroxidation (autoxidation) and its inhibition by RTAs. (B) Inhibition of GPX4 leads to the induction of ferroptosis. (C) The DPPH assay (inset) and trends in publications with keywords 'DPPH' in either the article title or abstract.³⁰ (D) Structures of purported natural product 'antioxidants' and some related derivatives used in this study. (E) Structures of potent ferroptosis inhibitors. (F) The titration of DPPH (100 μM) with compounds 7.1-7.12. (G) Plot of IC_{50} for DPPH quenching versus EC_{50} for inhibition of RSL3-induced ferroptosis in Pfa1 mouse embryonic

fibroblasts (MEFs). Data in light blue and brown correspond to the diarylamines (Ar_2NH) in Figure S7.1A ($R^2 = 0.01$). Data points labeled with an asterisk did not inhibit ferroptosis up to 10 μM .

7.3 Results

7.3.1 The DPPH Assay Fails to Predict Cytoprotective Potency of Putative Antioxidants.

Among the aforementioned measures of ‘antioxidant activity’, the DPPH assay (Figure 7.1C) is arguably the most popular method. Introduced in 1922,³² DPPH was mainly used in EPR spectroscopy as a calibrant and standard. It wasn’t until 1958 that it was utilized to determine antioxidant activity.⁹ Ever since, the use of DPPH to determine antioxidant activity has seen a dramatic increase that roughly coincides with the recognition of ‘oxidative stress’ as being relevant in human health.³⁰ Despite the well-documented shortcomings of the DPPH assay,¹³ its popularity for the assessment of antioxidant activity prompted us to assess its utility for the prediction of ferroptosis inhibitor potency. In fact, others have used the results of DPPH assays to provide insight on the mechanism of inhibition of ferroptosis by small molecules.^{18,28,29,36,37} Our test set included ferrostatin-1 (Fer-1) and liproxstatin-1 (Lip-1), the two quintessential ferroptosis inhibitors, a variety of diarylamines (Figure 1E, S1A) that we recently demonstrated to be equivalent to – or better than – Fer-1 and Lip-1,³⁸ some representative phenolic antioxidants including Nature’s premier RTA, α -tocopherol (α -TOH),³⁹ and the related, but much more potent tetrahydronaphthylidol derivative.^{40,41} We also included a variety of purported antioxidants of varying hydrophilicity/hydrophobicity, including ascorbic acid, glutathione, N-acetylcysteine, NADPH, a hydroquinone, a catechol, curcumin, resveratrol and epigallocatechin gallate, the so-called ‘green tea polyphenol’ (Figure 7.1D).

The DPPH assay was carried out in a 96-well microplate wherein various concentration of compounds were incubated with 100 μM DPPH in ethanol for 30 minutes prior to determination of the absorbance at 517 nm using a microplate reader.⁴² The IC_{50} values were calculated by fitting the percentage of the DPPH radical remaining after the incubation period, as is customary (Figure 7.1F, Figure S7.1B-C).^{9,43,44} The most potent RTA in this assay was the polyphenolic natural product

epigallocatechin gallate (**6**), with an IC_{50} of 2.9 μ M, while glutathione (**7.3**) was the least potent with an IC_{50} of 445 μ M. These results are consistent with literature precedent, wherein it is reported that the efficacy for DPPH quenching increased in the following order: glutathione (**7.3**) > hydroquinone (**7.10**) > catechol (**7.9**) > ascorbic acid (**7.2**) $\approx$ epigallocatechin gallate (**7.6**).⁴⁵ Surprisingly, we found that the IC_{50} values for glutathione (**7.3**) and N-acetylcysteine (**7.12**) were 10-fold different, despite the fact that both molecules have the same redox-active thiol moiety. It is reported that natural product resveratrol (**7.4**) and its corresponding *tert*-butylated analog (**7.8**) have different RTA activities, yet they had very similar IC_{50} values,⁴⁶ suggesting that the assay is not sensitive enough to discern alkyl substituent effects. It was also difficult to observe any trends in results with arylamines – the IC_{50} were in a very narrow range of 18-35 μ M despite the fact that they are reported to have vastly different RTA activities.³⁸

The potencies of these compounds as ferroptosis inhibitors were then determined in mouse embryonic fibroblasts¹⁹ treated with the GPX4 inhibitor RSL3²³ (Figure S7.1D). The EC_{50} values are plotted alongside the IC_{50} values obtained from the DPPH assay in Figure 7.1G, wherein no correlation is evident. Most naturally-occurring phenolic antioxidants (**7.1**, **7.4**, **7.6**, **7.7**) were modest inhibitors of cell death with EC_{50} > 1 μ M, while the synthetic derivatives (**7.8-7.11**) were more potent with EC_{50} values < 450 nM. As expected from previous work, aromatic amines, including Fer-1, Lip-1 and phenoxazine (PHOXN) were the most potent compounds. On the other hand, water soluble compounds (**7.2**, **7.3**, **7.5** and **7.12**) failed to rescue cells from ferroptosis (up to 10 μ M), presumably due to their inability to traverse the lipid bilayer ($ClogP$ < -0.5). Of course, this is not captured in the DPPH assay. Overall, it is clear that despite its popularity, the DPPH assay is a poor predictor of ferroptosis inhibition as evidenced by the clear lack of a correlation in Figure 1G ($R^2 = 0.01$).

7.3.2 Initial Efforts to Develop a Predictive Assay.

The rate-limiting step in the propagation of the lipid peroxidation chain reaction involves the abstraction of a H-atom from the lipid by a peroxyl radical^{2,3,47} (Figure 7.1A). An effective RTA must compete with phospholipids for this peroxyl radical and yield an RTA-derived radical that is persistent (i.e. does not

propagate the chain reaction).^{6,7} Thus, an assay that is based upon this *kinetic competition* is expected to be better able to predict the potency of putative RTAs in cells. A short time ago, we developed the basis for such an assay. The experiment involves the competition of an RTA and a highly-coloured and highly-oxidizable probe for the peroxy radicals that propagate the autoxidation of a hydrocarbon substrate. Thus, by adding a small amount of this probe molecule (i.e. STY-BODIPY or PBD-BODIPY, Figure 7.2A) to an autoxidizable substrate, the progress of the reaction and its inhibition by RTAs can be readily monitored by conventional spectrophotometry.³¹ Moreover, since the rate constant for the reaction of the probe molecule and a peroxy radical can be independently determined, the rate constant for the reaction of the RTA and a peroxy radical (k_{inh}) can be derived from the initial rate of the inhibited autoxidation where this competition takes place (Eq 7.1, see supplemental information for derivation of k_{inh}).

In preliminary work, we used this method to determine the apparent inhibition rate constants (k_{inh}) for Lip-1, Fer-1 and a variety of diarylamines that were found to be potent inhibitors of ferroptosis.^{38,41} We have since expanded the set of antioxidants investigated with this approach to those with which we obtained the above data for reactions with DPPH. Representative data from co-autoxidations of PBD-BODIPY and 1-hexadecene in chlorobenzene are included in Figure 7.2B (rate constants are tabulated in Figure S7.1E). It is important to note that the autoxidations were initiated with AIBN and that the rate of initiation (R_i), which is important in determining the stoichiometry of radical-trapping (or the number n of peroxy radicals trapped per molecule of RTA, Eq. 7.2, see SI for derivation), was determined using the inhibition time of PMC, the truncated analog of Vitamin E, for which $n = 2$.³⁹ The kinetic data roughly trend with the cytoprotective potencies of the test compounds, although the correlation is rather poor, with a significant amount of scatter still evident in Figure 7.2C ($R^2 = 0.29$). Therefore, although these experiments report on the kinetics of reactions of antioxidants with peroxy radicals they still lack predictive capacity when it comes to the potency of antioxidants as ferroptosis inhibitors.

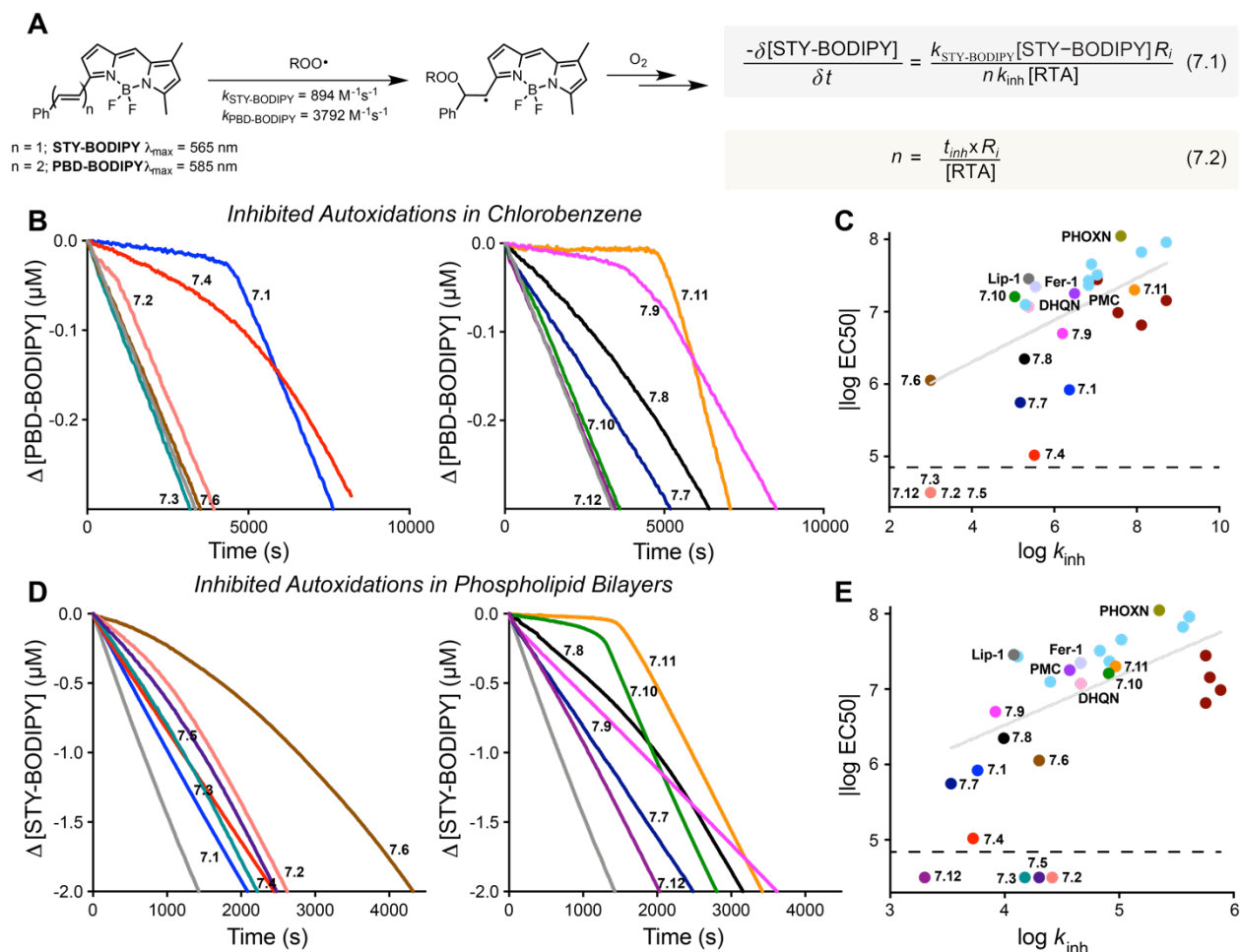


Figure 7.2 (A) The reaction of STY-BODIPY and PBD-BODIPY with peroxy radicals can be used as a signal-carrier in inhibited co-autoxidations to obtain inhibition rate constants (k_{inh}) and stoichiometries (n) of added RTAs. (B) Representative co-autoxidations of PBD-BODIPY (10 μM) and 1-hexadecene (2.8 M) initiated by AIBN (6 mM) in chlorobenzene at 37 $^{\circ}\text{C}$ (grey) and inhibited by 2 μM of compounds 1-12. (C) Correlation of inhibition rate constants determined in chlorobenzene and EC_{50} values from RSL3-induced ferroptosis in Pfa1 MEFs. $R^2 = 0.29$ (D) Representative co-autoxidations of STY-BODIPY (10 μM) embedded liposomes of egg phosphatidylcholine (1 mM) suspended in phosphate-buffered saline (10 mM) at pH 7.4 initiated by MeOAMVN (0.2 mM) at 37 $^{\circ}\text{C}$ (grey) and inhibited by 2 μM of compounds 7.1-7.12. (E) Correlation of inhibition rate constants determined in liposomes and EC_{50} values from RSL3-induced ferroptosis in Pfa1 MEFs ($R^2 = 0.42$). Compounds below the dashed line did not inhibit ferroptosis up to 10 μM .

In the aforementioned preliminary work, a slightly better correlation between k_{inh} and cytoprotective potency was obtained when the kinetics were determined from MeOAMVN-initiated co-oxidations of STY-BODIPY and the polyunsaturated lipids of unilamellar liposomes of egg phosphatidylcholine – conditions expected to more closely mimic the cellular membrane and the membranes of subcellular compartments (Figure 7.2D). However, once again, an expansion of the initial data set to include all of the compounds investigated above did not lead to a substantial improvement (Figure 7.2E, $R^2 = 0.42$). Interestingly, while the data for many compounds seem to correlate better, most of the ‘outliers’ segregate into two distinct groups: water-soluble compounds that were measured to be good RTAs in liposomes, but are ineffective in cells (i.e. 7.2, 7.3 and 7.5) and water-soluble diarylamines that are among the most potent of RTAs, but are less potent in cells than expected (brown circles). These results suggest that the assay conditions developed to date are still not fully representative of the lipid peroxidation that drives ferroptotic cell death. Since we anticipated that efforts to optimize assay parameters using the current protocol could prove prohibitively time consuming, we first sought to develop a high-throughput version of our co-oxidation approach to enable us to more rapidly survey reaction conditions.

7.3.3 Optimization of Co-oxidation Conditions.

Until now, reaction progress in STY-BODIPY (or PBD-BODIPY) co-oxidations has been monitored by the decrease in absorbance of the chromophore at its λ_{max} as it reacts with peroxyl radicals (Figure 7.3A). However, we anticipated that a fluorescence-based method would be better suited for a more high-throughput microplate- based assay. The narrow stokes shift in STY-BODIPY prompted to test several excitation wavelengths to determine which would afford optimal increase in green fluorescence. We settled on excitation at 488 nm and emission at 518 nm (Figure 7.3B).

Expectedly, the fluorescence of the probe increased linearly over time when it was added as a signal carrier during the autoxidation of 1 mM egg PC liposomes (extruded to 100 nm). To enable quantitation of the amount of STY-BODIPY (0.2 – 1 μ M) that had been oxidized, we determined the plateau in

fluorescence as a function of [STY-BODIPY]. The plateau represents the point at which the styryl sidechain has undergone oxidation, but the slower reaction of peroxy radicals with the BODIPY core has yet to take place (at which point the fluorescence decreases, see Figure S7.2A).⁴⁸ Plotting the maximal fluorescence as a function of dye concentration resulted in a response factor of 7.5×10^4 RFU/ μ M (Figure S7.2B). This enabled the determination of the concentration of oxidized probe as a function of time, which is necessary to derive k_{inh} from Eq 7.1. With a microplate-based method available to follow reaction progress in liposome autoxidations, we surveyed a variety of conditions to establish a model capable of predicting the potency of ferroptosis inhibitors in cells using the apparent inhibition rate constants in liposomes. To expand on our preliminary work, we tested both water-soluble (AAPH, 1 mM) and lipid-soluble (MeOAMVN, 0.2 mM) initiators in 1 mM egg PC liposomes (Figure 7.3C and 7.3D, respectively). Initially, autoxidations inhibited using either PMC (4 μ M), PHOXN (4 μ M), GSH (10 μ M), ascorbic acid (10 μ M) or NAC (50 μ M) (Figure 7.3E, left and 7.3F, left) were carried out. Surprisingly, although the values of k_{inh} determined for the lipophilic RTAs (PMC and PHOXN) varied with initiator, those of the water soluble RTAs (GSH, NAC and ascorbic acid) did not (cf. Table S7.1). Since AAPH is water-soluble, it is expected that the water-soluble RTAs inhibit STY-BODIPY oxidation by reacting directly with the initiator-derived radicals prior to initiating lipid peroxidation, whereas the lipophilic RTAs require import of the radicals from the aqueous phase to the lipid phase in order to react, and therefore compete with lipids for peroxy radicals. MeOAMVN, on the other hand, is considered to be a lipid-soluble initiator, so it is expected that the efficacy with which water-soluble RTAs inhibit the autoxidation would be substantially worse than in the AAPH-initiated autoxidations. This was obviously not the case. Since small unilamellar vesicles have large curvature resulting in increased dynamics of the bilayer, we wondered whether this effect was simply due to the facile diffusion of water-soluble RTAs into the liposomal membrane. However, a similar experiment with 200 nm liposomes afforded equivalent results, wherein water soluble RTAs were still highly potent in MeOAMVN-initiated autoxidations (Figure S7.2E).

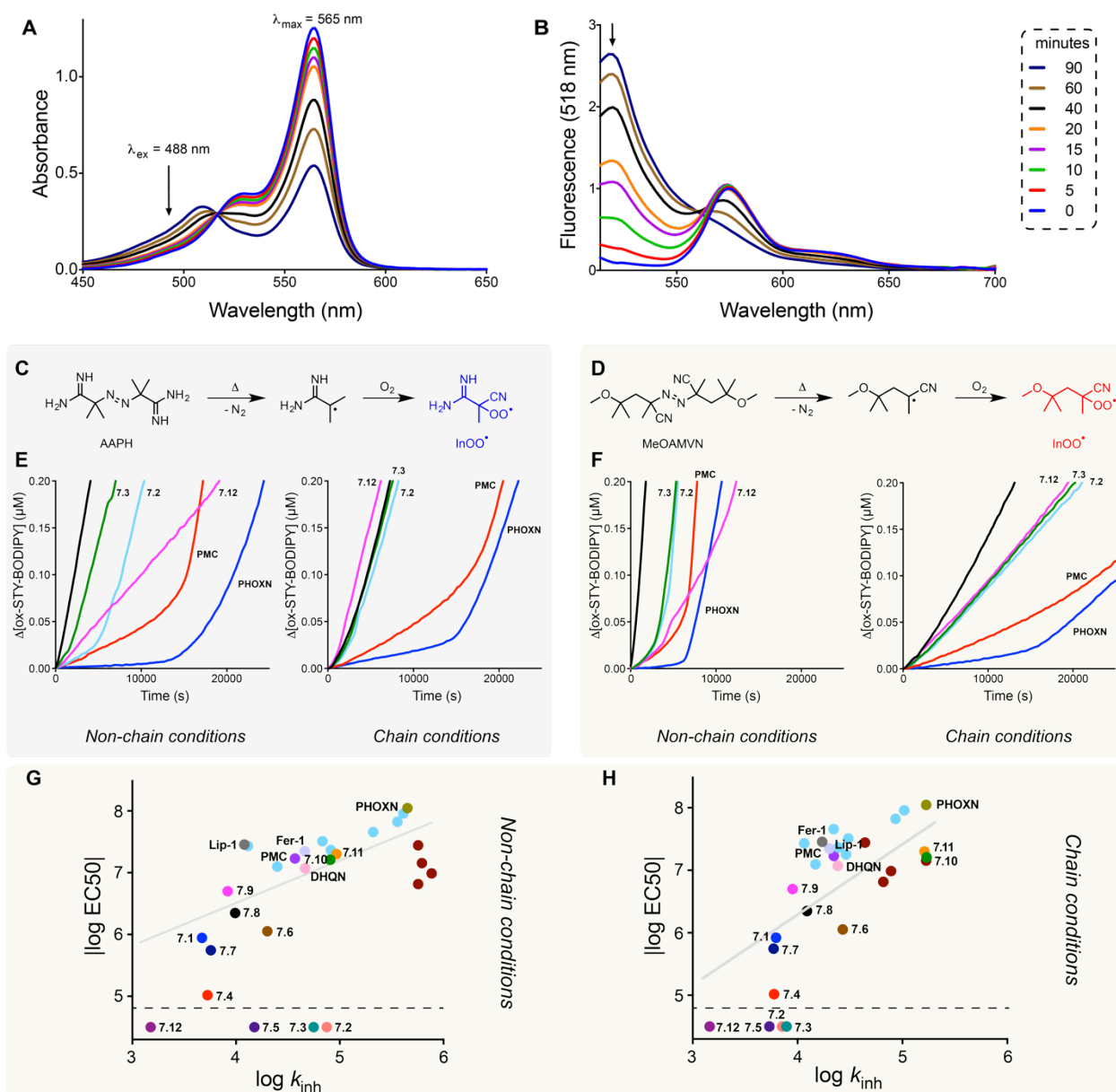


Figure 7.3 Co-autoxidation of STY-BODIPY (1 μM) embedded liposomes of egg PC (1 mM) suspended in phosphate-buffered saline (10 mM) at pH 7.4 initiated with MeOAMVN (0.2 mM) monitored by either absorbance (A) or fluorescence (B). AAPH (C) and MeOAMVN (D) radical initiators. Representative co-autoxidations of STY-BODIPY (1 μM) embedded liposomes of egg PC (1 or 20 mM) initiated by either (E) AAPH (1 mM, black) or (F) MeOAMVN (0.2 mM, black) and inhibited by PMC (4 μM, red), phenoxazine (4 μM, blue), ascorbic acid (10 μM, cyan), glutathione (10 μM, green) or NAC (50 μM, magenta). Correlation of inhibition rate constants determined in either 1 mM, R² = 0.40 (G) or 20 mM, R² = 0.58 (H) liposomes and EC₅₀ values from RSL3-induced ferroptosis in Pfa1 MEFs. Compounds below the dashed line did not inhibit ferroptosis up to 10 μM.

These findings suggested that the water-soluble RTAs were reacting directly with MeOAMVN-derived radicals at the lipid-aqueous interface, such that these RTAs compete directly with initiation of lipid peroxidation. Expanding the test set of compounds investigated to the ‘full’ set examined in Figures 1 and 2, reinforced the trends observed with the small test set *and* our preliminary efforts – particularly, that water-soluble compounds appear to be reacting directly with initiator-derived radicals (Figure 7.3G, $R^2 = 0.40$).³⁸ Indeed, when we calculated the kinetic chain lengths for both reaction systems – even in the absence of RTA – they were found to be less than 1 (Table S7.2), suggesting that the oxidations were not radical chain reactions.

To ensure that the oxidations were being carried out under radical chain condition (i.e. rate of oxidation $\gg$ rate of initiation), such that they can be considered *bona fide* autoxidations, we increased the lipid concentration to 20 mM in egg PC while keeping the rate of initiation the same (Figure 7.3E, right and F, right). Doing so gave kinetic chain lengths of 8 and 12 for the AAPH- and MeOAMVN-initiated oxidations, respectively. Under these conditions, the water-soluble RTAs were far less effective than under non-chain conditions, consistent with the phase-separation of the lipid peroxy radicals from the water-soluble antioxidants. Encouraged by these results, we carried out inhibited autoxidations with the full test set of compounds under these conditions (Figure 7.3H). Surprisingly, the resultant correlation of EC_{50} with k_{inh} improved only marginally ($R^2 = 0.58$) relative to that obtained when using data from the oxidations carried out under non-chain conditions (Figure 7.3G), suggesting that interception of initiator-derived radicals by water-soluble or amphiphilic RTAs may still be a problem. Indeed, we found that no consumption of STY-BODIPY took place in MeOAMVN-treated liposomes which were subsequently purified by size-exclusion chromatography (see Figure 7.4B), indicating that MeOAMVN is poorly incorporated into lipid bilayers and must reside primarily in the interfacial region.⁴⁹

7.3.4 Use of a Lipophilic Hyponitrite Initiator Completes a Predictive Assay.

Hyponitrites, such as di-*tert*-butyl hyponitrite (DTBN), were often used to initiate lipid autoxidation^{50,51} prior to the widespread adoption of MeOAMVN.⁵² The commercial availability of MeOAMVN, its well

characterized kinetics of radical generation in multiple systems and ease of handling (it is crystalline at room temperature) has led to its adoption as the initiator of choice for lipid peroxidation studies. Since hyponitrites decompose to yield alkoxyl radicals, which are the initiating radicals when lipid hydroperoxides decompose, we reasoned that a lipophilic analog of DTBN may be more suitable for controlled initiation of lipid peroxidation in liposomes. Therefore, we synthesized di-*tert*-undecyl hyponitrite, hereafter DTUN (Figure 7.4A), by alkylation of silver hyponitrite with *tert*-undecyl iodide (see Supporting Information for details, Figure S7.3A). DTUN was confirmed to be an efficient initiator (see Figure S7.3B-D); the inhibition periods of PMC-inhibited autoxidations of 1-hexadecene yielded rates of radical initiation from which $ek_d = 8.3 \times 10^{-6} \text{ s}^{-1}$ was derived – which was within experimental error of the value of $6.7 \times 10^{-6} \text{ s}^{-1}$ we found for DTBN. The value for MeOAMVN under the same conditions was measured to be $2.4 \times 10^{-5} \text{ s}^{-1}$, in good agreement with the literature (Figure S7.3E).⁵² In liposomes, $ek_d = 1.2 \times 10^{-6} \text{ s}^{-1}$, expectedly slower than in solution due to the increased microviscosity of the medium, and similar to that measured for MeOAMVN ($1.0 \times 10^{-6} \text{ s}^{-1}$) (Figure S3F-G). Interestingly, like MeOAMVN, DTBN was not efficiently incorporated into liposomes, as we found substantially less consumption of STY-BODIPY in DTBN-treated liposomes which were subsequently purified by size-exclusion chromatography (see Figure S3H-J). In contrast, DTUN-treated liposomes oxidized to a similar extent before or after purification (Figure 7.4B).⁵³

DTUN-initiated co-autoxidations of STY-BODIPY and egg phosphatidylcholine were carried out initially with the same ‘training set’ of compounds from Figure 3E-F under both chain and non-chain conditions (Figure 7.4C). In contrast to the results of the AAPH- and MeOAMVN-initiated autoxidations, the water soluble RTAs did not inhibit autoxidation to a significant extent under either set of conditions. These results are similar to those obtained from MeOAMVN-initiated autoxidations carried out under chain conditions. Autoxidations under chain conditions, which require a large amount of lipid, are both expensive and more challenging to carry out due to the increased optical density of the solution, which lowers signal quality. Since the foregoing suggested that the RTA kinetics determined from the DTUN-

initiated autoxidations are effectively independent of chain length (lipid concentration), we assayed the full test set of compounds in 1 mM PC liposomes.⁵⁴ The correlation of the k_{inh} values determined under these conditions and the EC_{50} values obtained in cells was excellent (Figure 7.4D, $R^2 = 0.84$).

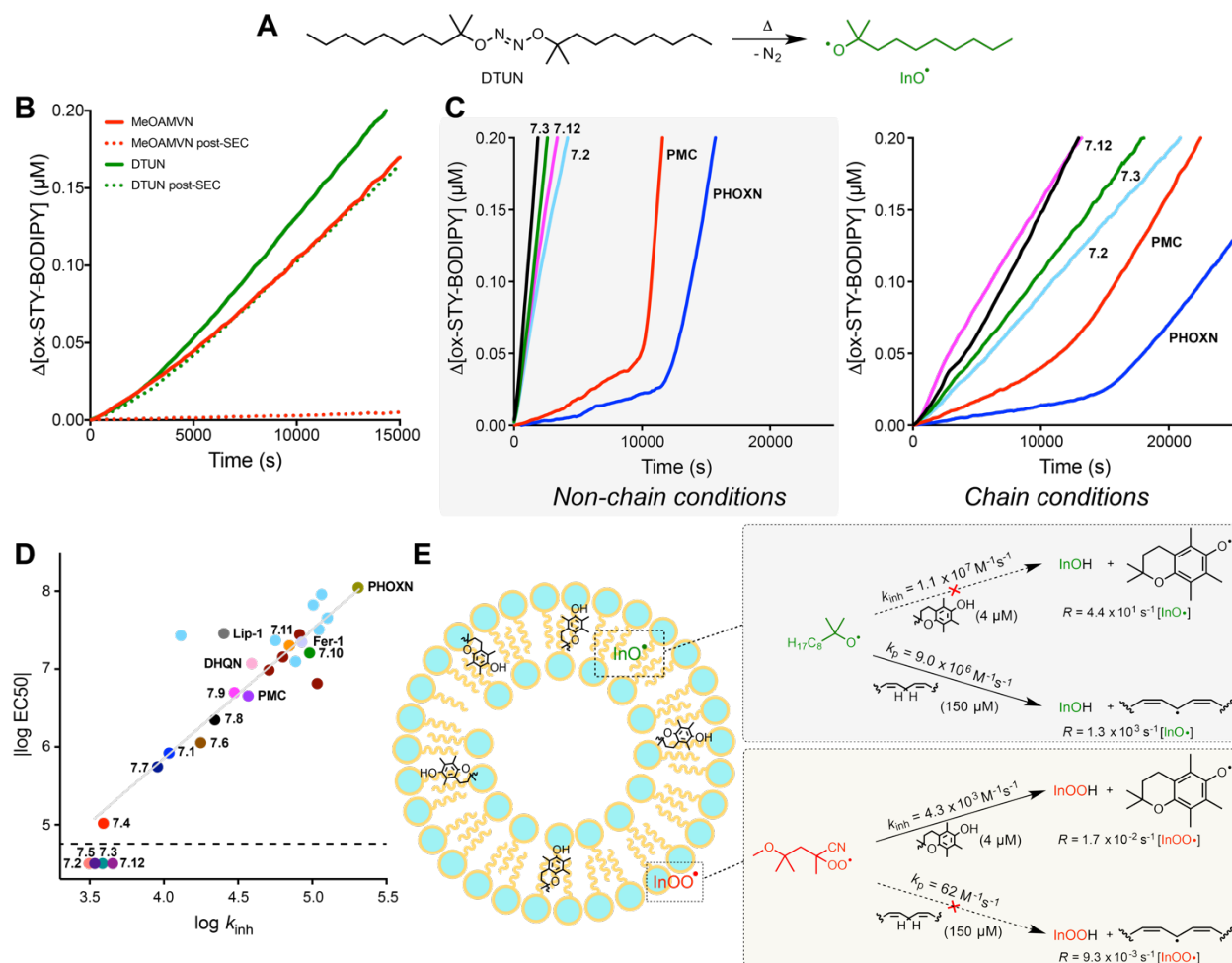


Figure 7.4 (A) The DTUN radical initiator. (B) Purification of liposomes by size exclusion chromatography following initiator additions removed MeOAMVN, but not DTUN, indicating that MeOAMVN is not efficiently incorporated into the liposome. (C) Representative co-autoxidations of STY-BODIPY (1 μM) embedded liposomes of egg PC (1 or 20 mM) initiated by DTUN (0.2 mM, black) and inhibited by either PMC (4 μM , red), phenoxazine (4 μM , blue), ascorbic acid (10 μM , cyan), glutathione (10 μM , green) or NAC (50 μM , magenta). (D) Correlation of inhibition rate constants determined in liposomes and EC_{50} values from RSL3-induced ferroptosis in Pfa1 MEFs, $R^2 = 0.84$. Compounds below the dashed line did not inhibit ferroptosis up to 10 μM . (E) Model of liposome and associated kinetics accounting for the difference in reactivity of MeOAMVN- and DTUN-derived initiating radicals.

The fact that DTUN-initiated autoxidations yield similar data at either high or low concentrations of lipid can be rationalized on the basis of available kinetic data (Figure 7.4E). Using PMC to illustrate this point, alkoxyl radicals abstract bis-allylic H-atoms with $k = 9.0 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$,⁵⁵ while they abstract an H-atom from PMC with $k = 1.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$.^{56,57} Taking into account the relative concentration of PMC (4 μM) and polyunsaturated lipid (150 μM ; linoleate makes up ca. 15% of the lipid in egg phosphatidylcholine, which is 1 mM), the kinetics predict that the DTUN-derived alkoxyl radicals will react with lipid 30-fold faster than with PMC.

7.3.5 H-Bonding of RTAs and Phospholipid Headgroups Attenuates Reactivity and Potency in a Predictive Fashion.

The significant improvement in the cell potency vs. RTA activity correlation observed when the kinetics were determined in liposomes instead of solution prompted us to consider the origin of this difference in more detail. We anticipated that it must be related to the dramatic suppression of reactivity observed upon moving from solution to liposomes, e.g. for PMC, $k_{\text{inh}} = 3.8 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $2.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ in chlorobenzene and egg PC liposomes, respectively. Although other suggestions have been made to account for this observation (*vide infra*), we hypothesized that H-bonding between the RTA and the phospholipid headgroup renders it unreactive to propagating lipid peroxy radicals (Figure 7.5A).

Ingold has clearly shown that H-bonding can slow the rate of H-atom transfer reactions because, while engaged in an H-bond, the H-atom is inaccessible to abstraction by radical species (Figure 7.5B).^{57,58} Thus, the kinetics of H-atom transfer in H-bonding media follow a simple pre-dissociation model and can be expressed as in Eq. (7.3). This expression is often shown as a linear free energy relationship, wherein the rate constant of the H-atom transfer in a given solvent (k_{inh}^S) is related to the rate constant in a non-H-bonding solvent (k_{inh}^0) by a term composed of the product of Abraham's H-bond acidity (α_2^H)⁵⁹ and basicity (β_2^H)⁶⁰ parameters of the H-atom donor and solvent, respectively (Eq. 7.4).⁵⁷

The H-bond accepting ability of egg phosphatidylcholine was determined by the ¹⁹F NMR method devised by Taft *et al.* (Eq. 7.5, Figure S7.4A),⁶¹ who showed that the chemical shift difference between 4-

fluorophenol and 4-fluoroanisole in a non-H-bonding solvent (e.g. CCl₄) is related to the formation constant of the H-bonded dimer between 4-fluorophenol and an added H-bond acceptor (Figure 7.5C). This can subsequently be recast in terms of β_2^H in order to facilitate comparison with other H-bond acceptors without needing to remeasure their individual equilibrium constants. The resultant value of $\beta_2^H = 0.79$ is quite large – for comparison, $\beta_2^H = 0.38$ and 0.45 for water and an ester, respectively – the other possible H-bond acceptors in the liposome system. Moreover the determined β_2^H is consistent with that of phosphate ester (0.77) as reported by Abraham *et al.*⁶⁰ This value, when used with Eq. 4, predicts a drop in k_{inh} for PMC ($\alpha_2^H = 0.38$) on going from chlorobenzene ($\beta_2^H = 0.09$) to egg PC bilayers ($\beta_2^H = 0.79$) of 161-fold, in very good agreement with the experimental observation of a 213-fold decrease in reactivity.⁶¹

To demonstrate how profoundly the H-bonding interaction between an RTA and phospholipid head group can impact RTA kinetics, we explored the medium dependence of the reactivity of a group of structurally-related phenols (Figure 7.5D) that differ slightly in their inherent H-atom transfer reactivity, but due to differing steric environments of the reactive phenolic H-atom, exhibit substantial differences in their H-bond acidity (α_2^H range from 0.59 to 0.16, Fig. 7.5E and Figure S7.5I). All of the compounds are lipophilic with $\log P > 1.5$,⁶² which ensures that the reactivity differences do not derive simply from different partitioning between the lipid and aqueous phases. The results are shown in Figure 7.5F (See Figure S4B for data).

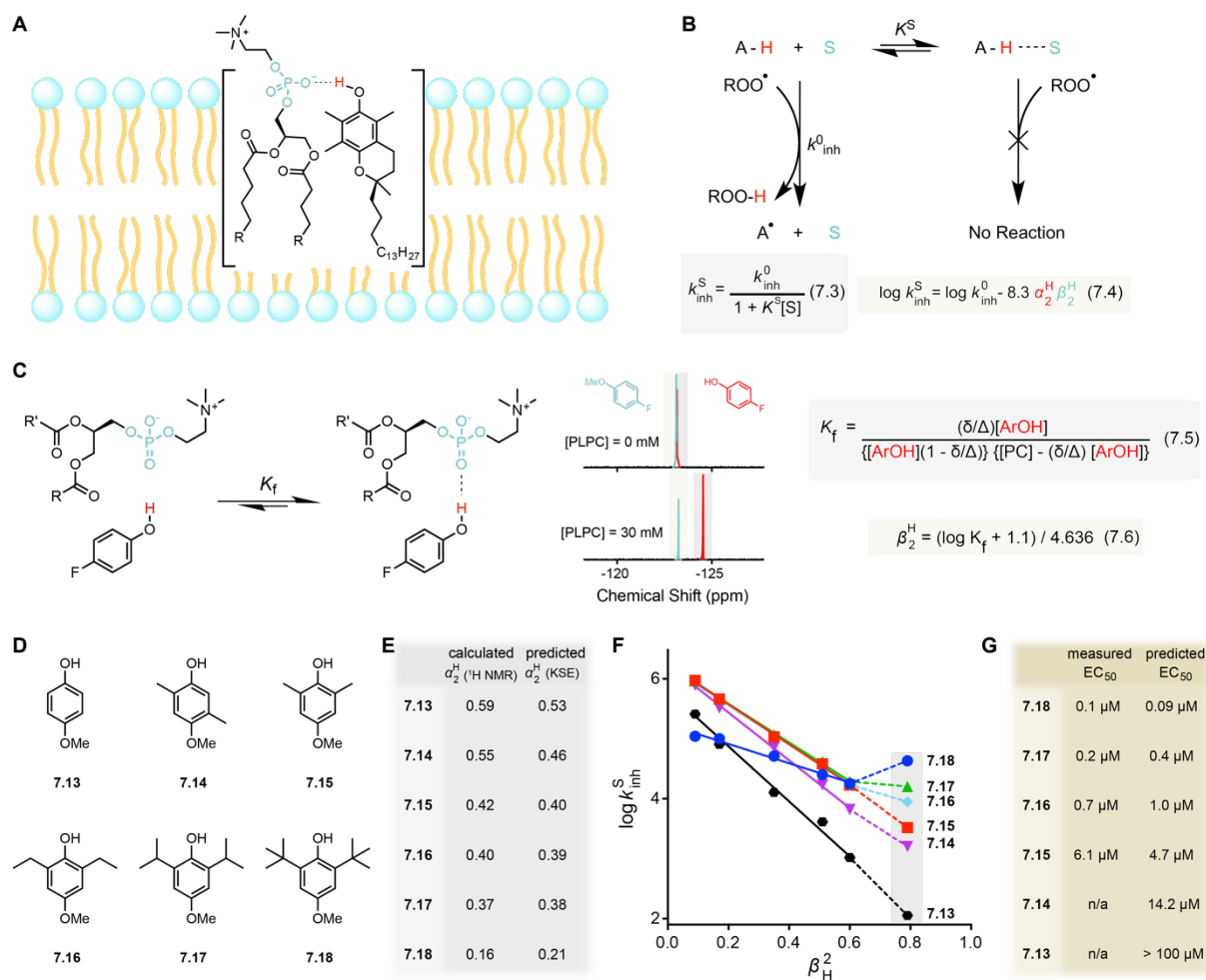


Figure 7.5 (A) Formation of H-bonded complexes between α -TOH and the phosphatidylcholine headgroup are proposed to slow its reaction with peroxy radicals in lipid bilayers. (B) The equilibrium between “free” RTA (A-H) and RTA participating in a 1:1 hydrogen-bond complex with hydrogen bond accepting solvent S. Assuming the 1:1 complex does not react with peroxy radicals, the kinetics of radical-trapping can be described as in equation 3 or 4.⁵⁷ (C) The equilibrium between “free” 4-fluorophenol and 4-fluorophenol participating in a 1:1 hydrogen-bond complex with headgroup of phosphatidylcholine along with representative ^{19}F NMR spectra of 4-fluorophenol and 4-fluoroanisole (0.01 M) in CCl_4 in the presence of none (top) or 30 mM (bottom) egg PC used to derive β_2^H of the phospholipid. Equations 5 and 6 can be used to determine the β_2^H where A_0 is the concentration of 4-fluorophenol, B_0 is the concentration of egg PC, Δ is the difference in chemical shift seen between 4-fluorophenol and 4-fluoroanisole at a concentration of HBA saturation ($B_0 = 0.20$ M) and δ is the difference in chemical shift seen between 4-fluorophenol and 4-fluoroanisole at the dilute concentrations of egg PC. (D) Structures of differently substituted phenols along with their calculated (^1H NMR) and

predicted α_2^H values (E). (F) Correlation of $\log k_{inh}^S$ and β_2^H for compounds **7.13** (●), **7.14** (▼), **7.15** (■), **7.16** (◆), **7.17** (▲) and **7.18** (●) in solvents with increasing hydrogen-bond basicity. (G) Predicted and measured EC₅₀ values from RSL3-induced ferroptosis in Pfal MEFs for phenols **7.13-7.18**. Predicted EC₅₀ values were obtained using the line of best fit from Figure 4C: $|\log EC_{50}| = 1.529 (\log k_{inh}) - 0.0509$.

The medium effects are dramatic – and clearly depend linearly on the β_2^H of the solvent as predicted by Ingold (See Figure S4B). Moreover, they clearly also depend on the H-bond donating ability of the phenol (α_2^H). For example, while 4-methoxyphenol ($\alpha_2^H = 0.59$) and 2,6-dimethyl-4-methoxyphenol ($\alpha_2^H = 0.42$) differ in reactivity by a factor of ~3.5 in chlorobenzene, the difference in egg PC liposomes is nearly 30-fold. According to Eq. 7.4, the slopes of the $\log k_{inh}/\beta_2^H$ correlations should correspond to $-8.3\alpha_2^H$. Indeed, we generally find good agreement between the α_2^H values derived from the slopes and the values we determined directly by NMR spectroscopy (Figure 7.5E), demonstrating the robustness of Ingold's relationship.

Interestingly, while the kinetic data obtained for the unhindered phenols **7.13**, **7.14** and **7.15** in liposomes falls on the $\log k_{inh}/\beta_2^H$ correlation, as the bulk around the phenolic O-H increases further as in **7.16**, **7.17** and **7.18**, the deviation from the correlation increases. Indeed, the most hindered phenol (**7.18**) is almost as reactive in the egg PC liposomes as in chlorobenzene – a stark difference from the dramatic drop in reactivity of the unhindered compounds in the different media (~3 orders of magnitude). This represents the limitation to this very useful linear free energy relationship: since the α_2^H of the phenols were determined using an unhindered H-bond acceptor (DMSO) and the β_2^H of PLPC was determined with an unhindered H-bond donor (4-fluorophenol), approximation of the H-bonding equilibrium between a hindered H-bond donor and a hindered H-bond acceptor is likely to be overestimated. Nevertheless, as expected based upon the data in Figure 7.4C, the kinetics determined in liposomes using the DTUN initiator correctly predict the potency of these compounds in cells (Figure 7.5G, Table S7.5J).

7.4 Discussion

Lipid peroxidation has been implicated in disease pathogenesis for decades, but attempts to identify, design or develop compounds to target aberrant lipid peroxidation for therapeutic or preventive purposes have largely failed. This has prompted many to believe that lipid peroxidation is not a part of disease etiology, but instead simply a hallmark of pathogenesis. Alternatively, it could be argued that the ‘antioxidant’ compounds that have been the subject of the great multitude of clinical trials aimed at suppressing ‘radical damage’, such as dietary (poly)phenols, were not good choices, simply convenient ones.

Lipids are the primary cellular target of ‘radical damage’ due to both their high reactivity toward H-atom abstraction and their high local concentration in lipid bilayers, which facilitate propagation of the autoxidative chain reaction. Therefore, assessment of putative ‘antioxidants’ should be made on the basis of their ability to compete with *lipids* for *peroxyl radicals* in *lipid bilayers*. Unfortunately, this is not common. Instead, surrogate experiments have been developed to assess ‘antioxidant activity’, such as the DPPH assay.^{63,64} Although the DPPH radical is isoelectronic with a peroxyl radical, the IC₅₀ values obtained from this assay are clearly a poor predictor of the ability of a given compound to inhibit lipid peroxidation in cells (*cf.* Figure 7.1G). Indeed, in addition to being the wrong type of radical, the kinetics of H-atom transfer from the antioxidant to the radical are not determined; the IC₅₀ values obtained from this assay simply reflect the position of the H-atom transfer equilibrium between DPPH and the test compound. The assay can be rendered more informative by determining the kinetics of the reaction, but this requires specialized equipment (a stopped-flow spectrophotometer).⁶⁵ Moreover, because these measurements are carried out in homogenous solution, and the identity of the solvent can dramatically influence both the rates and the mechanism of the reaction,^{58,66} it is unlikely that the results will be predictive of what goes in a cell or tissue.

Our recently implemented co-autoxidation approach utilizing STY-BODIPY as a signal carrier served as an ideal starting point for the implementation of a far more predictive alternative ‘antioxidant activity’ assay to DPPH (and presumably related existing methods, although this was not rigorously

investigated). It should be pointed out that the assay can also be carried out with the commercially available C₁₁-BODIPY^{581/591} cellular lipid peroxidation reporter⁶⁷ – which served as the inspiration for the initial development of PBD-BODIPY – and which yields essentially indistinguishable results (see Supporting Information for examples, Figure S7.2C-D, F). Our efforts to generate a model system capable of predicting the potency of RTAs in cells highlights the importance of selecting both an appropriate reaction environment and an appropriate radical initiator. Indeed, the results of experiments initiated by MeOAMVN, the conventional choice as a lipid-soluble initiator, fail to provide a good correlation with cell-based results – yielding data which overestimate the reactivity of water-soluble or amphiphilic RTAs. This is a particularly significant problem at low concentrations of lipid, where there is no radical chain reaction, such that the added RTAs simply react with initiator-derived radicals (Figure 7.3G). When the concentration of lipid is increased such that a radical chain reaction takes place in the absence of RTA, uncertainty remains over the precise location of the somewhat amphiphilic – and not at all lipid-like – initiator. As such, some apparent inhibition of lipid peroxidation by water-soluble or amphiphilic RTAs may still derive simply from direct reactions with initiator-derived radicals. This is implied from the poor correlation of water-soluble RTA activity and anti-ferroptotic activity (Figure 7.3H).

The newly-developed lipophilic hyponitrite initiator DTUN addresses the shortcomings of the assay based upon MeOAMVN. The lengthy hydrocarbon sidechains ensures that radical generation occurs exclusively in the lipid, and the relatively indiscriminant reactivity of the alkoxyl radicals derived therefrom ensures they react with the lipids, which are in far greater abundance than the RTA (Figure 7.4E). As such, the inhibition observed in the presence of added RTAs – and apparent k_{inh} derived therefrom – results from their reaction with lipid peroxy radicals. These advantages are clearly borne out in the excellent correlation between the $\log k_{inh}$ values determined using these conditions and the potency of the compounds in cells (Figure 7.4C).⁶⁸ A further advantage of the use of DTUN to initiate lipid peroxidation is its mechanistic similarity to what presumably takes place during ferroptosis, wherein (phospho)lipid hydroperoxides that escape detoxification by GPX4 can undergo Fenton-type reactions with low-valent iron (or other one-electron reductants). Although different in the structure of the alkyl

sidechains, the lipid-derived alkoxyl radicals which result are expected to have essentially identical reactivity to the alkoxyl radicals that result from the decomposition of the hyponitrite.

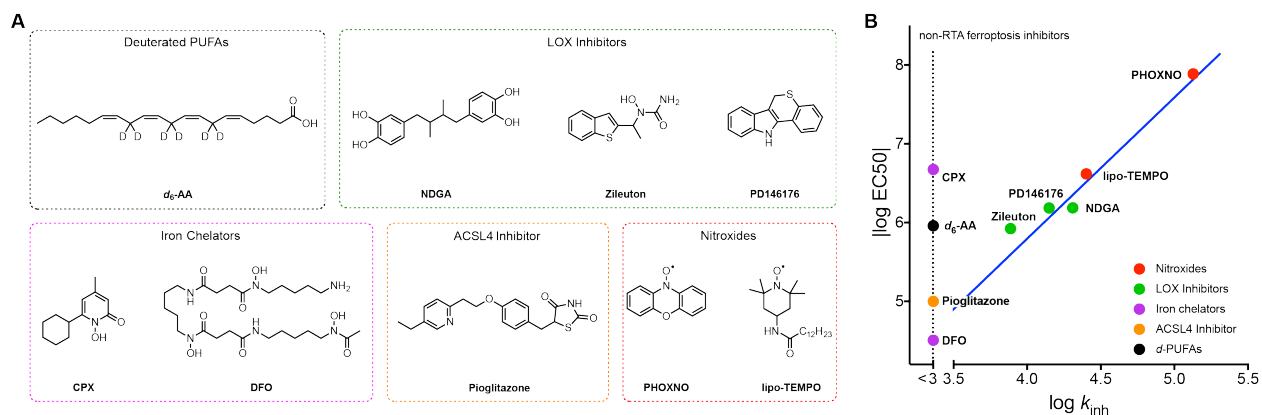


Figure 7.6 (A) Structures of various classes of ferroptosis inhibitors and **(B)** their potency to inhibit RSL3-induced ferroptosis in Pfa1 MEFs and reactivity as inhibitors of lipid peroxidation overlaid on the correlation of EC₅₀ and k_{inh} from Figure 7.4C.

In addition to offering a more rigorous high-throughput alternative to existing ‘antioxidant activity’ assays, this approach enables researchers to reliably rule out RTA activity when attempting to elucidate the mode of action of compounds which protect against ferroptosis or related forms of oxidative cell death (e.g. oxytosis). A recent example that demonstrates this point relates to the role of lipoxygenase-catalyzed lipid peroxidation in ferroptosis. Lipoxygenases (LOXs) had been implicated in ferroptosis based largely upon the observation that LOX inhibitors are able to subvert ferroptosis. Characterization of the RTA activity of these LOX inhibitors enabled us to conclude that LOX activity could only sensitize cells to ferroptosis, since LOX inhibitors lacking RTA activity had no effect on cell survival – even in LOX-overexpressing cells.⁶⁹ Indeed, when the k_{inh} is determined for these redox-active LOX inhibitors (NDGA, Zileuton and PD146176) using the optimized assay protocol which afforded the EC₅₀ versus log k_{inh} correlation in Figure 7.4, we find that these data also lie on the correlation (Figure 7.6) – fully consistent with our assertion that they protect cells from ferroptosis simply as RTAs.^{69,70} In addition to LOX inhibitors, we also assayed the nitroxides lipo-TEMPO and phenoxazine-*N*-oxyl (PHOXNO),⁶³ since nitroxides have been suggested to inhibit ferroptosis via a mechanism involving mitochondrial ROS.⁷¹ Again, we find these to be competent RTAs, with k_{inh} values that correlate with

their potencies in cells, implying that they act merely as RTAs to prevent ferroptosis. In contrast, when other known ferroptosis inhibitors, such as the iron chelators desferrioxamine (DFO) and ciclopiroxamine (CPX), the deuterated polyunsaturated fatty acid *d*₆-arachidonic acid and a thiazolidinedione ACSL4 inhibitor, were assayed, no RTA activity could be detected, consistent with the fact that they act by other mechanisms.

At first glance, moving from homogenous organic solution to lipid bilayers to assay RTA activity seems like an obvious and necessary step to obtain data that would be useful in predicting cell potency. Barclay first quantitated the difference in the rate constants for the reaction between RTAs and peroxy radicals upon moving from organic solution to lipid bilayers.^{55,72,73} He, and others, generally attributed this observation to one of three factors: 1) the localization of the RTA, particularly its partitioning between the lipid and aqueous phase, 2) increased van der Waals interaction between aliphatic sidechains on the RTA and the lipids, slowing their encounter with propagating radicals,⁷⁴ and 3) H-bonding between the RTA and water at the lipid-aqueous interface. The importance of localization is abundantly clear from the low reactivity of water-soluble compounds (**7.2**, **7.3**, **7.5** and **7.12**) towards lipid peroxy radicals. The role of dynamics is evident upon comparing the reactivity of compounds that differ only in alkyl substitution, e.g. **7.1** and PMC: despite having similar reactivity in solution, PMC has a 2.6-fold higher reactivity in lipid bilayers than **7.1**. Although H-bonding between the RTA and water is insufficient to explain the drop in reactivity, we have now shown H-bonding to the phospholipid headgroup is far stronger, and accounts for the massive drop in reactivity upon moving from solution to liposomes. These results highlight an element that is overlooked in the design of RTAs and/or anti-ferroptotic agents: H-bond acidity. Furthermore, it enables the prediction of the cytoprotective potencies of putative RTAs from the results of inhibited autoxidations or organic substrates in homogenous organic solvents – no cell culture required! H-Bond acidity underlies the lackluster activity of phenols, the quintessential naturally-derived antioxidants of much fanfare, as RTAs in a cellular context, and may contribute to the disappointing results of phenolic antioxidants in the many clinical trials in which they have been

examined as potential therapeutic or preventive agents. Moreover, it underpins the identification of aromatic amines such as Fer-1 and Lip-1 in high-throughput screens of compound libraries for inhibitors of ferroptosis, despite the fact that they are far generally far less inherently reactive than many phenols (including α -tocopherol); aromatic amines are simply far less H-bond acidic than phenols. It will be interesting to see how compounds which are excellent RTAs under relevant conditions (including cell models of ferroptosis) fare in clinical trials.

7.5 References

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7.6 Supporting Information

7.6.1 General Experimental

C₁₅-THN¹, Fer-1², Lip1³, arylamines (S7.1-S7.12)⁴, Phenols (7.14, 7.15 & 7.16)⁵⁻⁷, RSL3^{2,3}, PBD-BODIPY⁸, and STY-BODIPY⁸, were synthesized according to literature procedures. The synthesis of DTUN is reported here. Any other compounds, DMEM media with/without phenol red, Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), penicillin-streptomycin, AquaBluer and reagents for synthesis were purchased from commercial suppliers and used as received unless stated otherwise.

7.6.2 DPPH Assay

Test compounds (or vehicle) at various concentrations were dissolved in ethanol and added to clear bottom 96-well plates. To this was added a solution of DPPH dissolved in ethanol to achieve a final concentration of 100 μ M. Samples were placed in the BioTek Synergy H1 plate reader and mixed vigorously for 60 seconds. The samples were incubated at room temperature for 30 minutes and the absorbance at 517 nm was measured.⁹ Samples were subtracted for background (ethanol only) and normalized to starting DPPH absorbance. The IC₅₀ values were obtained by fitting the results to a dose-response curve in GraphPad Prism. The results represent the mean $\pm$ S.E. of three independent experiments.

7.6.3 Inhibited Autoxidation of 1-hexadecene.

A 3.5-mL quartz cuvette was charged with PhCl (0.44 mL), 1-hexadecene (2.00 mL). The cuvette was preheated to 37 °C in a thermostatted sample holder of a UV-vis spectrophotometer and allowed to equilibrate for approximately 15 min. To the cuvette was added PBD- BODIPY (12.5 μ L of a 2.00 mM stock solution in 1,2,4-trichlorobenzene) and AIBN (50 μ L of a 300 mM stock solution in chlorobenzene). The solution was thoroughly mixed prior to monitoring the uninhibited co-autoxidation via the disappearance of the PBD-BODIPY probe at 588 nm for 10 min to ensure the reaction was proceeding at a constant rate. Finally, the antioxidant under investigation was added (10.0 μ L of a 1.0 mM solution in chlorobenzene), the solution was mixed thoroughly, and the absorbance readings were resumed. The resulting Abs vs time data were processed as previously reported.⁸ The rate of initiation ($R_i = 1.3 \times 10^{-9} \text{ M s}^{-1}$) and second order rate constant for propagation for the dye ($k_{\text{PBD-BODIPY}} = 3792 \text{ M}^{-1} \text{ s}^{-1}$) necessary to compute stoichiometric data (n) and inhibition rate constants (k_{inh}) were determined using PMC as a standard, which has an established stoichiometry of 2.

7.6.4 Inhibited Autoxidation of Egg-PC Liposomes on UV-Vis.

To a cuvette of 2.34 mL of 10 mM PBS at pH 7.4 was added liposomes (125 μ L of 20 mM stock in PBS at pH 7.4) and the solution was equilibrated for 5 minutes at 37°C (liposome formation from egg PC was

described elsewhere).¹ The cuvette was blanked and 10 μL of 2 mM STY-BODIPY in DMSO was added followed by 10 μL of 0.05 M MeOAMVN in acetonitrile and the solution was thoroughly mixed. After 5 minutes, an aliquot of RTA stock solution in DMSO was added and the loss of absorbance at 565 nm followed. The inhibition rate constant (k_{inh}) and stoichiometry (n) was determined for each experiment according to Eq. (1) and (2), respectively. The resulting Abs vs time data were processed as previously reported.⁸ The rate of initiation ($R_i = 2.5 \times 10^{-9} \text{ M s}^{-1}$) and second order rate constant for propagation for the dye ($k_{\text{STY-BODIPY}} = 894 \text{ M}^{-1} \text{ s}^{-1}$) necessary to compute stoichiometric data (n) and inhibition rate constants (k_{inh}) were determined using PMC as a standard, which has an established stoichiometry of 2. Kinetic data are given as averages of three independent measurements.

7.6.5 Inhibited autoxidation of Egg-PC liposomes on plate reader.

To a 96-well black polypropylene plate (Nunc) was added liposomes (1 mM for non-chain; 20 mM for chain conditions) and either one of STY-BODIPY (1 μM) or C₁₁-BODIPY (1 μM) in PBS at pH 7.4 to a final volume of 295 μL . This was followed by the addition of RTAs (2 μL aliquots) at desired concentrations. The plate was incubated for 10 minutes at 37°C in the BioTek Synergy H1 plate reader followed by a vigorous mixing protocol for 5 minutes. The plate was ejected from the plate reader and the autoxidation was initiated by the addition of a 3 μL aliquot of either AAPH (1 mM), MeOAMVN (0.2 mM) or DTUN (0.2 mM), followed by another mixing protocol for 5 minutes. The plate was incubated for an additional 10 minutes to equilibrate at 37°C before data was acquired by excitation of the probes at 488 nm and emission was measured at 518 nm. The data was transformed by dividing the raw RFU values by the response factor of $7.49 \times 10^4 \text{ RFU}/\mu\text{M}$ or $1.93 \times 10^4 \text{ RFU}/\mu\text{M}$ for STY-BODIPY and C₁₁-BODIPY, respectively. The data shown is an average of three independent experiments.

7.6.6 Cell Culture.

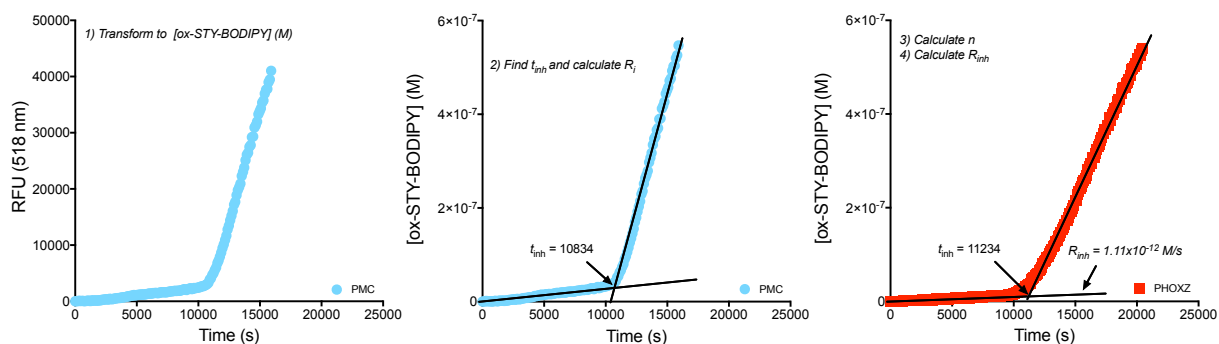
All cell lines were cultured at 37 °C in a 5% CO₂ atmosphere unless otherwise indicated. Pfa1 cells were cultured in DMEM with 10% FBS, 1% 100× non-essential amino acid solution, 1 mM sodium pyruvate

and 1% penicillin-streptomycin. Cells were passaged by dissociation with 0.05% trypsin and 0.2% EDTA every two to three days. All cell experiments were carried out as a minimum of three biological replicates.

7.6.7 Inhibition of Ferroptosis Induced by Gpx4 Inhibition with (1S,3R)-RSL3.

Pfa1 cells (3,000 in 100 μ L) were seeded in 96-well plates and cultured overnight. The next day the media was removed, the cells were washed twice with PBS and the cells were suspended in new media for 30 minutes before addition of RTAs and LOX inhibitors. Ferroptosis was induced using (1S, 3R)-RSL3 (10 μ M) 30 minutes after incubation of compounds. Cell viability was assessed 6 hours later using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Experiments are carried out with six-technical replicates ($n = 6$ wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

7.6.8 How to Calculate k_{inh} and n using FENIX



Variables

$$k_{\text{STY-BODIPY}} = 894 \text{ M}^{-1} \text{ s}^{-1}$$

[STY-BODIPY] = dye concentration at $t = 0$ (M)

n = stoichiometry

R_i = calculated as shown

[RTA] = antioxidant concentration (M)

R_{inh} = rate of inhibition, (M/s)

t_{inh} = inhibition time (s)

1) Covert all RFU into [ox-STY-BODIPY] ($[\text{ox-STY-BODIPY}] = \frac{RFU}{7.5 \times 10^{10} RFU/M}$)

2) Calculate the rate of initiation ($R_i = \frac{[RTA] \cdot n}{t_{inh}}$) by standardizing using PMC

$$[PMC] = 4\mu M, n = 2, t_{inh} = \text{time of inhibition} = 10885 \text{ s} \therefore R_i = \frac{[4 \times 10^{-6} M] \cdot 2.0}{10885} = 7.40 \times 10^{-10} \text{ s}^{-1}$$

3) Calculate stoichiometry using Eq. 2 ($n = \frac{t_{inh} \cdot R_i}{[RTA]}$)

$$\text{for PHOXZ, } t_{inh} = 11234 \text{ s} \therefore n = \frac{11234 \cdot 7.40 \times 10^{-10}}{[4 \times 10^{-6} M]} = 2.1$$

4) Determine R_{inh} from the rate of change of the inhibited period

$$\text{for PHOXZ } R_{inh} = 1.11 \times 10^{-12} \text{ M/s}$$

5) Calculate k_{inh} using rearranged Eq. 1 ($k_{inh} = -\frac{k_{STY-BODIPY} \cdot [STY-BODIPY] \cdot R_i}{n \cdot [RTA] \cdot R_{inh}}$)

$$\text{For PHOXZ } \therefore k_{inh} = -\frac{894 \text{ M}^{-1} \text{ s}^{-1} \cdot [1 \times 10^{-6} M] \cdot 7.40 \times 10^{-10} \text{ s}^{-1}}{2.1 \cdot [4 \times 10^{-6} M] \cdot 1.11 \times 10^{-12} \text{ M s}^{-1}} = 7.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$$

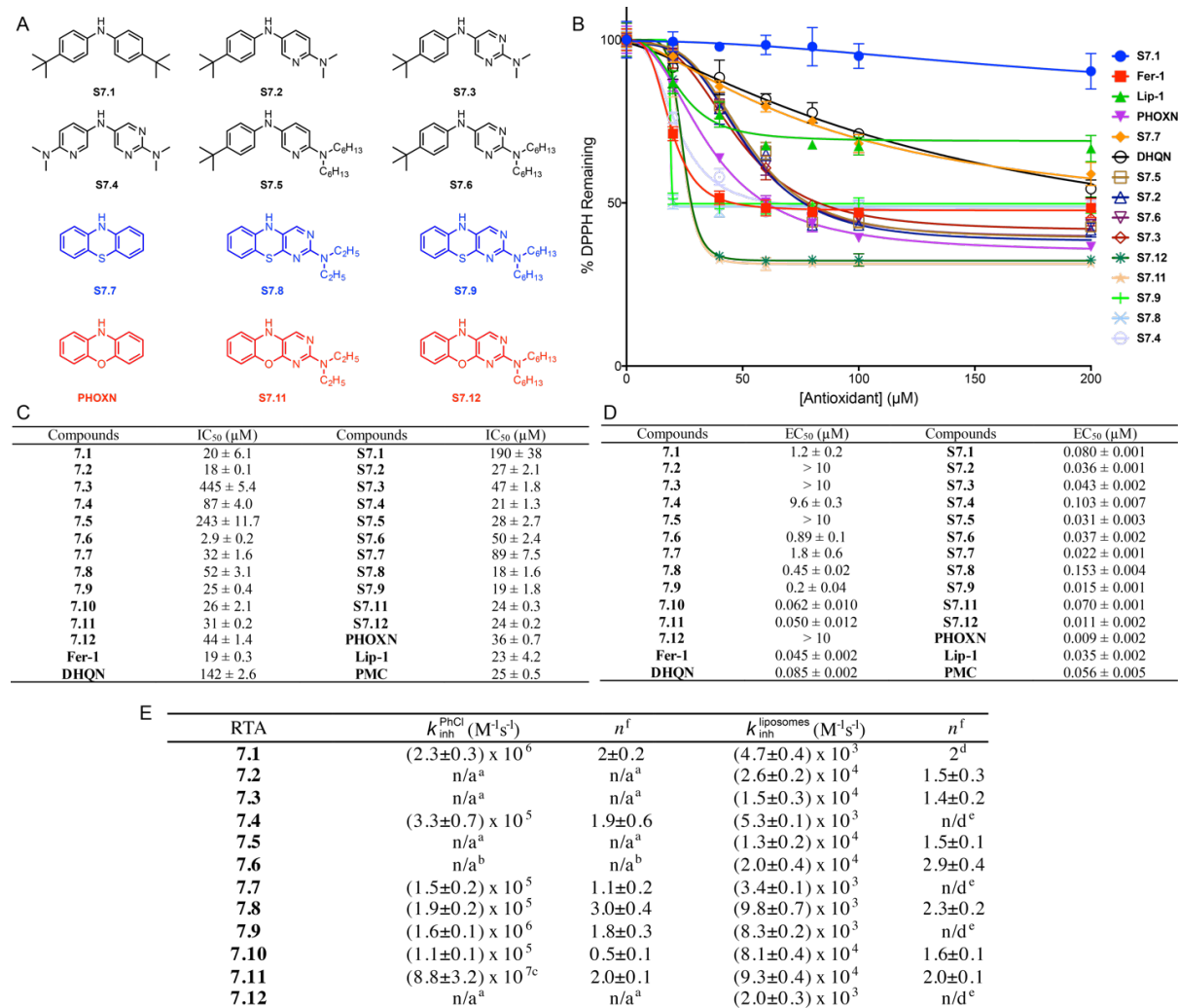


Figure S7.1 (A) Structures of different arylamines used in this study and labelled as 'Ar₂NH' in the main manuscript. (B) Raw data from the quenching of DPPH by various arylamines. (C) IC₅₀ values obtained

from the DPPH assay for compounds used in this study. **(D)** EC₅₀ values obtained from the RSL3 induced ferroptosis for compounds used in this study. **(E)** Inhibition rate constants and stoichiometries (*n*) obtained from inhibited co-oxidations of 1-hexadecene with PBD-BODIPY or egg phosphatidylcholine liposomes with STY-BODIPY at 37 °C using UV-Vis. ^aNo observed reactivity in organic solution. ^bReactivity could not be measured as EGCG is not soluble in organic solutions. ^cInhibition rate constant obtained from ref. 1; the rate constant is greater than what can be determined from this data. ^dCould not be determined and was assumed to be 2. ^eCannot be determined due to a lack of a distinct inhibition period.

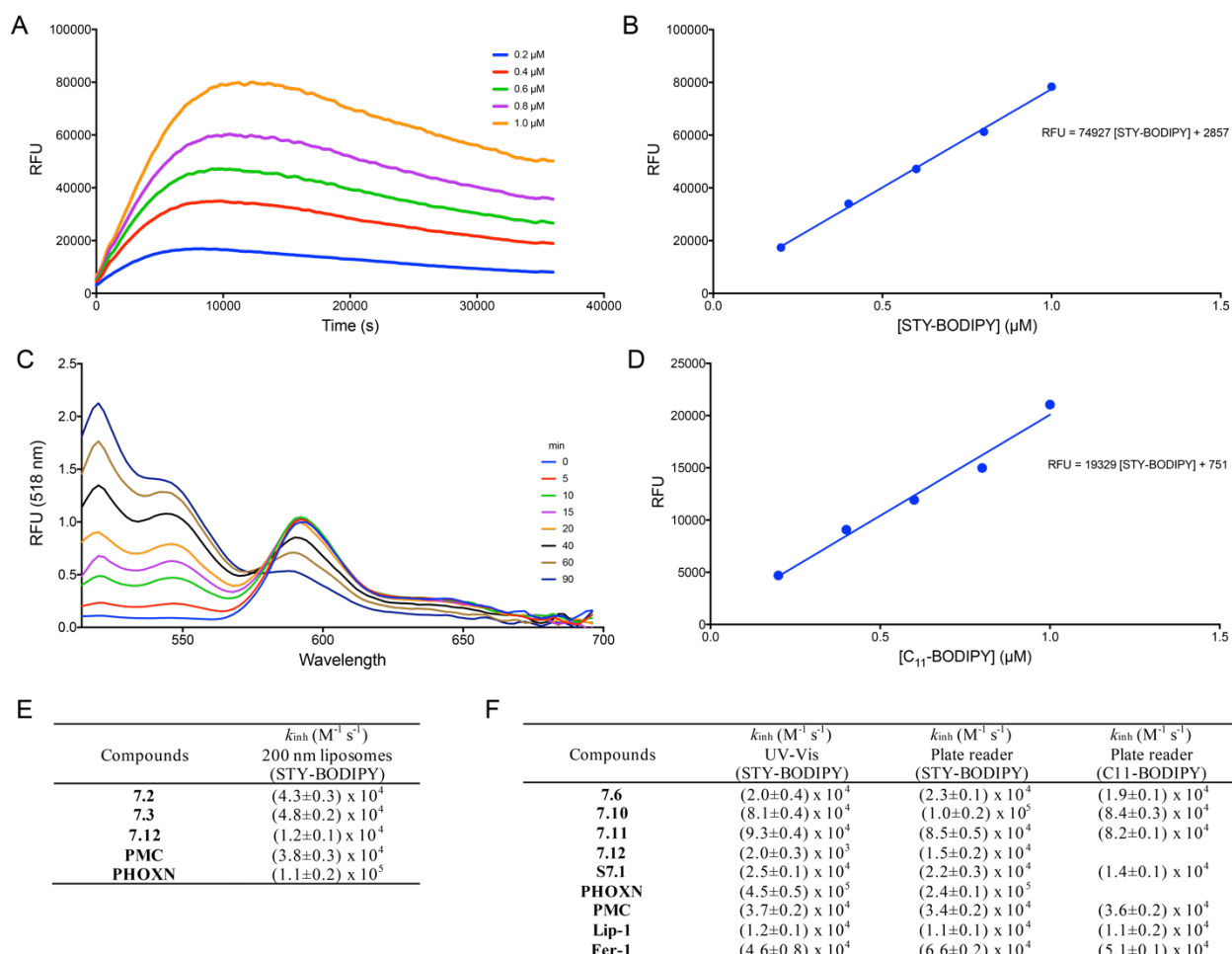


Figure S7.2 **(A)** Increase in fluorescence of various concentrations of STY-BODIPY over time depicting the maximum enhancement of fluorescence prior to oxidative degradation of the BODIPY core. **(B)** Response factor of STY-BODIPY used to convert RFU into μM of ox-STY-BODIPY. **(C)** Co-oxidation of C₁₁-BODIPY (1 μM) in the presence of 1 mM EggPC liposomes initiated using MeOAMVN (0.2 mM) monitoring the emission over time. **(D)** Response factor of C₁₁-BODIPY used to convert RFU into μM of ox-C₁₁-BODIPY. **(E)** Co-oxidation of 200 nm liposomes (1 mM) initiated

using MeOAMVN (0.2 mM) (F) Representative values comparing inhibition rate constants obtained using different methods initiated using 0.2 mM MeOAMVN.

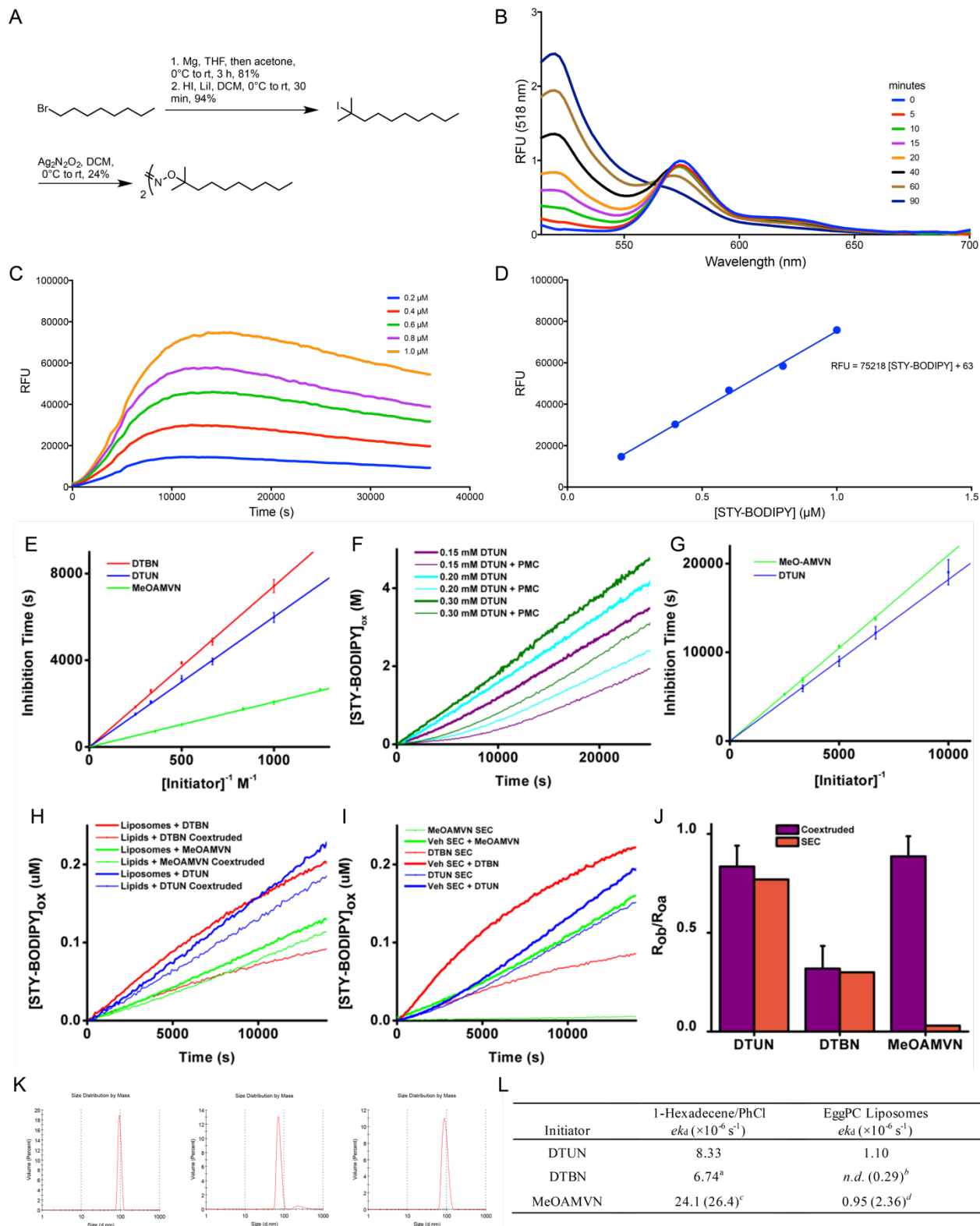


Figure S7.3 (A) Synthetic scheme for DTUN. (B) Co-oxidation of STY-BODIPY (1 μM) in the presence of 1 mM EggPC liposomes initiated using DTUN (0.2 mM) monitoring the emission over time. (C) Increase in fluorescence of various concentrations of STY-BODIPY over time depicting the maximum enhancement of fluorescence prior to oxidative degradation of the BODIPY core. (D) Response factor of STY-BODIPY used to convert RFU into μM of ox-STY-BODIPY. Co-oxidation of PBD-BODIPY and 1-hexadecene in chlorobenzene at 37°C inhibited by 50 μM PMHC (E). Co-oxidations of STY-BODIPY in 20 mM Egg-PC liposomes pH 7.4 PBS buffer at 37 °C initiated with DTUN and inhibited by 2 μM PMHC (F) and in comparison to MeOAMVN (G). Comparison of STY-BODIPY and Egg-PC liposome co-oxidation profiles between either coextrusion of initiator with the initial liposome dispersion (H) or separation by size exclusion chromatography (SEC) on Sephadex-G25 columns (I) shown along with the corresponding traces obtained when initiator was added after the step. Comparison of the initial STY-BODIPY coautoxidation rates between either coextruded initiator or SEC isolated liposomes (R_{ob}) with the corresponding initial rate obtained when initiator was added after the step (R_{oa}) (J). (K) Liposome particle size measured using dynamic-light scattering in the presence of vehicle + 1 mM liposomes (left), 0.2 mM DTUN + 1 mM liposomes (middle) and 0.2 mM DTUN + 20 mM liposomes (right). (L) Comparison of radical generation efficiency (ek_d) of lipophilic initiators in this work.

7.6.9 Determining kinetic chain length:

$$\text{Kinetic Chain Length} = \frac{R_p}{R_i} \times \frac{k_p[\text{substrate}]}{k_p[\text{Dye}]}$$

$$\text{Non-chain conditions (AAPH)} = \frac{5.1 \times 10^{-11} \text{M/s}}{5.8 \times 10^{-10} \text{M/s}} \times \frac{62 \text{ M}^{-1} \text{s}^{-1} [1.5 \times 10^{-4} \text{M}]}{894 \text{ M}^{-1} \text{s}^{-1} [1.0 \times 10^{-6} \text{M}]}$$

$$\text{Chain conditions (AAPH)} = \frac{3.3 \times 10^{-11} \text{M/s}}{4.6 \times 10^{-10} \text{M/s}} \times \frac{62 \text{ M}^{-1} \text{s}^{-1} [3.0 \times 10^{-3} \text{M}]}{1703 \text{ M}^{-1} \text{s}^{-1} [1.0 \times 10^{-6} \text{M}]}$$

Table S7.1 Kinetic chain length under chain (20 mM liposomes) and non-chain (1 mM liposomes) conditions for different initiators

Initiators	Kinetic chain length	
	Non-chain condition	Chain condition
AAPH	0.9	7.9
MeOAMVN	0.9	12.4
DTUN	1.1	8.4

Table S7.2 Comparison of radical generation efficiency (ek_d) of lipophilic initiators in this work.

Initiator	1-Hexadecene/PhCl	EggPC Liposomes
	$ek_d (\times 10^{-6} \text{ s}^{-1})$	$ek_d (\times 10^{-6} \text{ s}^{-1})$
DTUN	8.33	1.10
DTBN	6.74 ^a	<i>n.d.</i> (0.29) ^b
MeOAMVN	24.1 (26.4) ^c	0.95 (2.36) ^d

^aLiterature value = $1.76 \times 10^{-6} \text{ s}^{-1}$ was calculated using: $e \approx 0.55$ and $k_d = 3.2 \times 10^{-6} \text{ s}^{-1}$ in neat tetralin at 30°C.¹⁰ ^bLiterature value = $0.13 \times 10^{-6} \text{ s}^{-1}$ was calculated using: $e \approx 0.09$ and $k_d = 3.2 \times 10^{-6} \text{ s}^{-1}$ in aqueous egg lecithin at 30°C.¹⁰ ^cLiterature value = $26.4 \times 10^{-6} \text{ s}^{-1}$ in acetonitrile.¹¹ ^dLiterature value = $2.36 \times 10^{-6} \text{ s}^{-1}$ in soybean PC liposomes.¹¹

Table S7.3 Apparent inhibition rate constants ($\text{M}^{-1}\text{s}^{-1}$) and stoichiometry (n) determined for selected compounds from co-autoxidations of STY-BODIPY-embedded liposomes of egg phosphatidylcholine initiated by AAPH, MeOAMVN or DTUN and carried out under non-chain (1 mM lipid) and chain (20 mM lipid) conditions.

	Non-chain conditions			Chain conditions		
	AAPH	MeOAMVN	DTUN	AAPH	MeOAMVN	DTUN
PHOXN	$(1.6 \pm 0.3) \times 10^5$ /2.1±0.2	$(2.4 \pm 0.1) \times 10^5$ /1.9±0.1	$(7.1 \pm 0.2) \times 10^4$ /2.1±0.3	$(2.8 \pm 0.3) \times 10^4$ /1.8±0.2	$(7.2 \pm 0.3) \times 10^4$ /1.8±0.1	$(4.4 \pm 0.2) \times 10^4$ /2.2±0.3
PMC	$(1.8 \pm 0.4) \times 10^4$ /2.0±0.1	$(3.4 \pm 0.2) \times 10^4$ /2.0±0.2	$(2.9 \pm 0.1) \times 10^4$ /2.0±0.2	$(1.0 \pm 0.1) \times 10^4$ /2.0±0.2	$(2.2 \pm 0.1) \times 10^4$ /2.0±0.1	$(1.6 \pm 0.3) \times 10^4$ /2.0±0.2
Ascorbate (2)	$(4.4 \pm 0.2) \times 10^4$ /0.2±0.01	$(7.5 \pm 0.1) \times 10^4$ /0.3±0.02	$< 10^3$	$< 10^3$	$(2.0 \pm 0.4) \times 10^3$ /n.d	$(2.4 \pm 0.3) \times 10^3$ /n.d
GSH (3)	$(1.1 \pm 0.1) \times 10^4$ /0.1±0.02	$(5.6 \pm 0.5) \times 10^4$ /0.3±0.01	$< 10^3$	$< 10^3$	$(1.8 \pm 0.2) \times 10^3$ /n.d	$(3.5 \pm 0.3) \times 10^3$ /n.d
NAC (12)	$(4.8 \pm 0.3) \times 10^3$ /0.2±0.01	$(1.5 \pm 0.2) \times 10^3$ /0.3±0.08	$< 10^3$	$< 10^3$	$< 10^3$	$< 10^3$

A

B_0 [PLPC] (M)	Chemical Shift α, α, α - trifluorotoluene (ppm)	Chemical Shift 4- Fluoroanisole (ppm)	Chemical Shift 4- Fluorophenol (ppm)	Normalized δ (ppm)	K_f	β_2^H
0	-62.60	-123.23	-123.20	-	-	-
0.005	-62.60	-123.24	-124.60	1.36	524	0.79 ± 0.02
0.010	-62.60	-123.24	-125.39	2.15	385	
0.015	-62.60	-123.24	-125.83	2.59	346	
0.020	-62.60	-123.24	-126.10	2.86	341	
0.030	-62.60	-123.25	-126.25	3.14	353	
0.200	-62.60	-123.33	-126.89	3.56	-	-

B

	PhCl ($\beta_2^H = 0.09$) [M ⁻¹ s ⁻¹]	m-Xylene ($\beta_2^H = 0.17$) [M ⁻¹ s ⁻¹]	Dioxane/PhCl ($\beta_2^H = 0.35$) [M ⁻¹ s ⁻¹]	THF ($\beta_2^H = 0.51$) [M ⁻¹ s ⁻¹]	Dioxane/DMSO ($\beta_2^H = 0.60$) [M ⁻¹ s ⁻¹]
PMHC	(3.8×10^6)	$1.3 \pm 0.2 \times 10^6$	$3.9 \pm 0.4 \times 10^5$	$1.4 \pm 0.2 \times 10^5$	$6.1 \pm 0.8 \times 10^4$
13	$2.6 \pm 0.2 \times 10^5$	$8.1 \pm 0.1 \times 10^4$	$1.3 \pm 0.1 \times 10^4$	$4.1 \pm 0.3 \times 10^3$	$1.1 \pm 0.2 \times 10^3$
14	$7.9 \pm 0.7 \times 10^5$	$3.3 \pm 0.3 \times 10^5$	$7.1 \pm 0.1 \times 10^4$	$1.7 \pm 0.1 \times 10^4$	$6.6 \pm 0.3 \times 10^3$
15	(9.4×10^5)	$4.6 \pm 0.3 \times 10^5$	$1.1 \pm 0.1 \times 10^5$	$3.8 \pm 0.3 \times 10^4$	$1.7 \pm 0.1 \times 10^4$
16	$9.1 \pm 0.7 \times 10^5$	$4.4 \pm 0.1 \times 10^5$	$1.1 \pm 0.1 \times 10^5$	$3.9 \pm 0.2 \times 10^4$	$1.7 \pm 0.1 \times 10^4$
17	$9.3 \pm 0.8 \times 10^5$	$4.6 \pm 0.2 \times 10^5$	$1.2 \pm 0.2 \times 10^5$	$4.2 \pm 0.4 \times 10^4$	$1.9 \pm 0.1 \times 10^4$
18	(1.1×10^5)	$1.0 \pm 0.1 \times 10^5$	$5.2 \pm 0.3 \times 10^4$	$2.5 \pm 0.1 \times 10^4$	$1.8 \pm 0.1 \times 10^4$

Figure S7.4 Summary of K_f and β_2^H measurements of PLPC by ¹⁹F NMR. Summary of the inhibition rate constants measured in organic solution.

7.6.10 Determination of α_2^H by Kinetic Solvent Effect

Utilizing the Ingold-Abraham relationship (Eq. S1) in the form of Eq. S2, α_2^H is determined as the intercept of Eq. S3

$$\log k_{inh}^0 = \log k_{inh}^S + 8.3\alpha_2^H \beta_2^H \quad \text{Eq. S7.1}$$

$$\log k_{inh}^{PhCl} + 8.3\alpha_2^H \beta_2^{H(PhCl)} = \log k_{inh}^S + 8.3\alpha_2^H \beta_2^{H(S)} \quad \text{Eq. S7.2}$$

$$\frac{\log k_{inh}^{PhCl}}{8.3(\beta_2^{H(S)} - \beta_2^{H(PhCl)})} = \frac{\log k_{inh}^S}{8.3(\beta_2^{H(S)} - \beta_2^{H(PhCl)})} + \alpha_2^H \quad \text{Eq. S7.3}$$

7.6.11 Determination of α_2^H by ¹H NMR

Functional groups which engage significantly as hydrogen bond donors (having measurable α_2^H values) demonstrate substantially lower-field chemical shifts in a strongly hydrogen bonding solvent (DMSO-d₆) compared to a weakly hydrogen bonding solvent (CDCl₃ or Benzene-d₆). The correlation between α_2^H

values and the $\Delta\delta$ of DMSO-d6 and CDCl_3 was described by Abraham *et al* to afford the empirical relationship Eq. S7.4. In a previously published paper we utilized benzene-d6 instead of CDCl_3 (due to an unusual incompatibility between CDCl_3 and several of our phenoxazines/phenothiazines) which afforded a similar correlation rendering Eq. S7.5.

$$\alpha_2^H = 0.133\Delta\delta_{\text{DMSO-CDCl}_3} + 0.0066 \quad \text{Eq. S7.4}$$

$$\alpha_2^H = 0.134\Delta\delta_{\text{DMSO-Ben}} - 0.1087 \quad \text{Eq. S7.5}$$

The H-bond accepting solute (PLPC) was diluted in CCl_4 to render five dilute solutions of differing concentrations and one 0.20M solution. To these were added 4-fluorophenol and 4-fluoroanisole for a final concentration of 0.01M of each. The solutions were transferred into NMR tubes containing seal capillaries of benzene-d6 with α,α,α -trifluorotoluene, after which their ^{19}F spectra were recorded with a Bruker Avance II 300 equipped with a thermostat (sample kept at 37°C). The 1:1 H-bond formation constant (K_f) of 4-fluorophenol and the H-bond acceptor(s) can be determined with by Eq. 7.6 as described by Taft and co-workers:

$$K_f = \frac{\left(\frac{\delta}{\Delta}\right)A_0}{\left\{A_0\left[1-\left(\frac{\delta}{\Delta}\right)\right]\right\}\left[B_0-\left(\frac{\delta}{\Delta}\right)A_0\right]} \quad \text{Eq. S7.6}$$

Where A_0 is the concentration of 4-fluorophenol, B_0 is the concentration of the H-bond acceptor (PLPC), Δ is the difference in chemical shift between 4-fluorophenol and 4-fluoroanisole sufficient to saturate H-bonding with 4-fluorophenol (maximum δ , $[\text{PLPC}] = 0.20\text{M}$), and δ is the observed difference in chemical shift between 4-fluorophenol and 4-fluoroanisole at the corresponding B_0 and A_0 . The β_2^H can be calculated from the measured K_f using Eq. 7.7.

$$\beta_2^H = (\log K_f + 1.1)/4.636 \quad \text{Eq. S7.7}$$

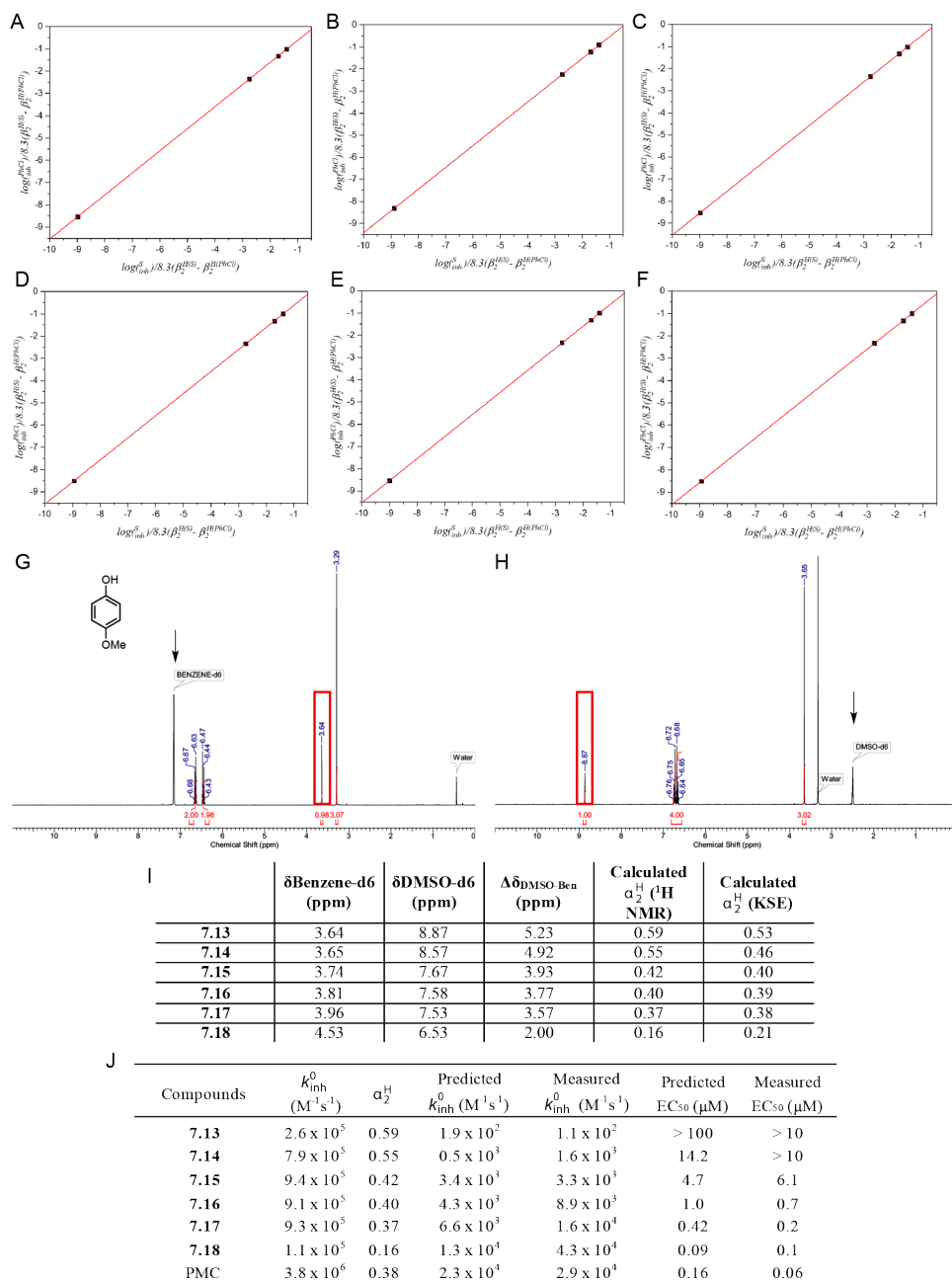


Figure S7.5 (A) Linear regression of Eq. 7.3 for **7.13**: $y=0.972x+0.532$; $R^2 = 1.000$; ($\alpha_2^H=0.53$). (B) Linear regression of Eq. 7.3 for **7.14**: $y=0.987x+0.460$; $R^2 = 1.000$; ($\alpha_2^H=0.46$). (C) Linear regression of Eq. 7.3 for **7.15**: $y=0.993x+0.400$; $R^2 = 1.000$; ($\alpha_2^H=0.40$). (D) Linear regression of Eq. 7.3 for **7.16**: $y=0.994x+0.393$; $R^2 = 1.000$; ($\alpha_2^H=0.39$). (E) Linear regression of Eq. 7.3 for **7.17**: $y=0.991x+0.383$; $R^2 = 1.000$; ($\alpha_2^H=0.38$). (F) Linear regression of Eq. 7.3 for **7.18**: $y=1.020x+0.207$; $R^2 = 1.000$; ($\alpha_2^H=0.21$). (G, H) Representative ^1H NMR for the α_2^H determination of **7.13** in benzene- d_6 (G) and DMSO- d_6 (H), the phenolic O-H is highlighted. (I) Summary of α_2^H values determined by ^1H NMR and by kinetic solvent

effect. (J) Rate constants in organic solutions, eggPC liposomes and EC₅₀ values from RSL3 induced ferroptosis for phenols 7.13 – 7.18.

7.6.12 Synthesis and Characterization

7.6.12.1 Synthesis of DTUN

2-methyldecan-2-ol. 1-bromooctane (19.3 g, 100 mmol, 1 eq) was added dropwise to magnesium turnings (3.04 g, 125 mmol, 1.25 eq, activated dry with iodine) suspended in anhydrous THF (100 mL) under N₂ atmosphere at a rate that maintained a gentle reflux. Upon completion of the reaction, the solution was transferred via cannula to a dry RBF under N₂, cooled thoroughly in an ice bath, and acetone (7.0 mL, 95 mmol, 0.95 eq, dried overnight on 3A sieves) was added dropwise over about 30 min. The mixture was allowed to warm to room temperature over 3 h, then re-cooled on ice, quenched with 6 mL acetic acid and concentrated *in vacuo*. The residue was vigorously stirred with 100 mL 3:1 chloroform:isopropanol and 100 mL water for 10 min before extracting the aqueous layer 2 x 50 mL 3:1 chloroform:isopropanol. The combined organics were washed 3x 100mL 10% aqueous sodium chloride, 1x 100 mL brine, dried over Na₂SO₄, filtered and concentrated to dryness. The residual oil was distilled with a short path fractionating distillation apparatus collecting the middle fraction of distillate to afford 2-methyldecan-2-ol (13.95 g, 81%) as a colourless oil. Spectra were in agreement with literature values.¹²

2-iodo-2-methyldecane. An oven dried 25 mL round-bottom flask and stir bar were cooled under N₂ atmosphere, wrapped completely in foil, charged with concentrated HI (6.7 mL, *ca.* 2 eq) and then cooled in an ice bath. Lithium iodide (3.36 g, 25 mmol, 1 eq) was added in portions (caution: exotherm/fumes with powdered LiI; beads are preferred). A solution of 2-methyldecan-2-ol (4.31 g, 25 mmol, 1 eq.) in methylene chloride (5 mL) was added slowly by syringe with vigorous stirring under nitrogen atmosphere. The ice bath was then removed and the biphasic mixture stirred for about 10 minutes, at which point the alcohol was completely consumed by ¹H NMR. The mixture was carefully transferred to separatory funnel and the bottom aqueous layer cautiously extracted twice with methylene chloride (10 mL). The combined organic layers were washed once with 1:1 brine:water containing drops of sodium bisulfite solution until the mixture was mostly colourless. The organic extracts were fully protected from

light while drying over Na₂SO₄, filtering and concentrating on a rotary evaporator followed by hovac (flask backfilled with nitrogen) to yield **IMDa** as a pale oil (6.63 g, 94%). The product was pure by ¹H NMR without distillation and was stored at -20°C in a foil-wrapped vial, ideally backfilled with nitrogen. ¹H NMR (300 MHz, CDCl₃) δ 1.92 (m, 6H), 1.65-1.22 (m, 14H), 0.88 (t, *J* = 6.9 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 53.2, 50.7, 38.2, 32.0, 29.6, 29.5, 29.4, 28.6, 22.8, 14.2. HRMS: *m/z* Calc: C₁₁H₂₃ (ESI: M-I)⁺ 155.1800 Found: 155.1817.

bis-2-methyldecane hyponitrite (DTUN). An oven dried 25 mL round-bottom flask and stir bar were cooled under N₂ atmosphere and charged with **IMDa** (1.44 g, 6 mmol) followed by degassed anhydrous methylene chloride (10 mL). The flask was completely wrapped in aluminum foil to exclude light and then cooled thoroughly in an ice bath. Freshly dried silver hyponitrite (1.24 g, 4.5 mmol) was added in three portions over 5 min with vigorous stirring. The ice bath was then removed and NMR analysis indicated consumption of the tertiary iodide usually within 10-20 min. The reaction mixture was then re-cooled in an ice bath and diluted with cold pentane (10 mL) before filtering over celite. The filtrate was concentrated on a rotary evaporator without heating followed by hovac for about 5 min while on ice. The residual oil was crystallized overnight from methanol (*ca.* 20 mL, N₂ purged) on dry ice. The majority of supernatant was then removed by syringe, discarded, and the solid washed with three portions of cold methanol in the same manner. The solid was allowed to thaw into a small volume of methanol (*ca.* 5 mL) until a solution formed. The product was promptly isolated thereafter while being kept cold on ice at all times. The solution was extracted with three portions of cold pentane (5 mL), the combined pentane extracts washed once with 1:1 methanol:water, dried over Na₂SO₄, filtered, concentrated on a rotary evaporator without heating followed by hovac to yield **DTUN** (270 mg, 24%) as a colourless wax. The product when free from residual solvent is solid on ice and rapidly melts when allowed to warm to ambient temperature. The solid was aliquoted by Pasteur pipette into tared vials and the vials were stored at -78°C. Stocks up to 0.2 M were prepared in ethanol or chlorobenzene and held on ice or dry ice just prior to use. ¹H NMR (600 MHz, CDCl₃) δ 1.65 (m, 2H), 1.34 (s, 6H), 1.34-1.24 (m, 12H), 0.88 (t, *J* = 6.6

Hz, 3H). ^{13}C NMR (151 MHz, CDCl_3) δ 83.4, 40.6, 32.0, 30.1, 29.6, 29.4, 25.9, 24.0, 22.8, 14.2. FTIR (ATR) ν : 988, 1147, 1368, 1384, 1466, 2854, 2923 cm^{-1} . HRMS: m/z Calc: $\text{C}_{11}\text{H}_{23}$ (EI: $\text{M}-\text{C}_{11}\text{H}_{23}\text{N}_2\text{O}_2$) $^{+}$ 155.1794 Found: 155.1787.

7.6.12.2 Synthesis of Phenols

2,6-Diethylhydroquinone. A 100mL double neck round bottom flask was charged with glacial acetic acid (35 mL), concentrated sulfuric acid (0.4 mL) and 2,6-diethylphenol (3.89 g; 25.9 mmol). With mixing, 30% H_2O_2 (13 mL) was added by dropping funnel. While monitoring the temperature of the solution with an alcohol thermometer (affixed through side neck) the mixture was gradually heated to 50°C (heat gun) after which the reaction was sufficiently exothermic to maintain its temperature. The reaction was kept between 55-65°C for ~30 minutes after which the heat of the reaction subsided until cooling to room temperature. The reaction was poured into water and extracted with ethyl acetate three times. The combined organic extracts were rinsed three times with 1M NaOH solution, and subsequently dried with MgSO_4 , filtered, and concentrated under reduced pressure. The residue was dissolved into ether (40 mL) and transferred to a 250 mL round bottom flask charged with methanol (25 mL), water (90 mL), and sodium dithionite (13 g). The flask was sealed, evacuated, and backfilled with N_2 and left to stir overnight. The ether was then separated, and the aqueous phases was extracted with ether. The combined organic solutions were subsequently dried with MgSO_4 , filtered, and concentrated under reduced pressure. The product was isolated by column chromatography (30% EtOAc in hexanes) and recrystallized out of hexanes affording 2,6-diethylhydroquinone as white needles (1.00 g, 23.2%); mp 90-92 °C; ^1H NMR (400 MHz; $\text{DMSO}-d_6$): δ 8.49 (s, 1H), 7.32 (s, 1H), 6.32 (s, 2H), 2.48 (q, J = 7.5 Hz, 4H), 1.08 (t, J = 7.5 Hz, 6H); ^{13}C NMR (101 MHz; $\text{DMSO}-d_6$): δ 150.2, 144.2, 131.93, 112.7, 23.0, 14.38; HRMS (EI, magnetic sector): calcd for $\text{C}_{10}\text{H}_{14}\text{O}_2$ 166.0993, found 166.1007.

2,6-Diethyl-4-methoxyphenol (7.14). Methanol (15 mL) and 2,6-diethylhydroquinone (0.95 g; 5.7 mmol) were placed in a 100 mL round bottom flask equipped with a stir bar and reflux condenser. Carefully, 4.5 mL of concentrated sulfuric acid was added and the reaction mixture was heated to reflux

for ~4 hours. Once cooled the, the reaction mixture was poured into water followed by extraction with ether (three times). The combined organic extracts were dried with MgSO₄, filtered, and concentrated under reduced pressure. The product was isolated by column chromatography (10% EtOAc in hexanes). Two recrystallizations out of hexanes (minimum of hexanes at room temperature, followed by chilling on dry ice resulted in rapid crystallization) afforded the 2,6-diethyl-4-methoxyphenol as white needles (0.39 g, 38%); mp 34-36 °C; ¹H NMR (400 MHz; DMSO-d₆): δ 7.58 (s, 1H), 6.49 (s, 2H), 3.65 (s, 3H), 2.54 (q, *J* = 7.5 Hz, 4H), 1.10 (t, *J* = 7.5 Hz, 6H). ¹³C NMR (101 MHz; DMSO-d₆): δ 152.5, 145.6, 132.0, 111.6, 55.1, 23.2, 14.4; HRMS (EI, magnetic sector): calcd for C₁₁H₁₆O₂ 180.1150, found 180.1140.

2,6-Diisopropylhydroquinone. The same general procedure for the preparation of 2,6-diethylhydroquinone was used in the preparation of 2,6-diisopropylhydroquinone from 2,6-diisopropylphenol (6.04g; 33.9mmol). The product was isolated by column chromatography (30% EtOAc in hexanes) and recrystallized out of hexanes affording 2,6-diisopropylhydroquinone as white leaflets (1.34g, 20.4%); mp 94-96 °C; ¹H NMR (400 MHz; DMSO-d₆): δ 8.49 (s, 1H), 7.26 (s, 1H), 6.37 (s, 2H), 3.23 (sept., *J* = 6.9 Hz, 2H), 1.10 (d, *J* = 6.9 Hz, 12H); ¹³C NMR (101 MHz; DMSO-d₆): δ 150.7, 142.75, 136.9, 109.4, 26.2, 23.0; HRMS (EI, magnetic sector): calcd for C₁₂H₁₈O₂ 194.1306, found 194.1297.

2,6-Diisopropyl-4-methoxyphenol (7.17). Methanol (20 mL) and 2,6-diisopropylhydroquinone (1.25 g; 6.4 mmol) were placed in a 100 mL round bottom flask equipped with a stir bar and reflux condenser. Carefully, 6 mL of concentrated sulfuric acid was added and the reaction mixture was heated to reflux for ~4 hours. Once cooled the, the reaction mixture was poured into water followed by extraction with ether (three times). The combined organic extracts were dried with MgSO₄, filtered, and concentrated under reduced pressure. The product was isolated by column chromatography (10% EtOAc in hexanes). After drying under vacuum, the residue was chilled (-20 °C) affording 2,6-diisopropyl-4-methoxyphenol as a beige crystalline solid (1.06 g, 79.5%); mp 48-50 °C; ¹H NMR (400 MHz; DMSO-d₆): δ 7.53 (s, 1H), 6.51 (s, 2H), 3.66 (s, 3H), 3.28 (sept., *J* = 6.9 Hz, 2H), 1.13 (d, *J* = 6.9 Hz, 12H); ¹³C NMR (101 MHz; DMSO-

d₆): δ 153.0, 144.2, 136.9, 108.3, 55.0, 26.4, 22.9. HRMS (EI, magnetic sector): calcd for C₁₃H₂₀O₂ 208.1463, found 208.1487

7.6.13 Supporting Information References

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Chapter 8: The Molecular Mechanism of Ferroptosis Suppressor Protein 1

8.1 Preface

Ferroptosis, an iron-dependent form of oxidative cell death is triggered as a consequence of lipid hydroperoxide accumulation by the peroxidation of phospholipids. It is exclusively regulated by glutathione peroxidase 4 (Gpx4), the enzyme responsible for reducing phospholipid hydroperoxides to their corresponding alcohols and the ablation of which leads to rapid cell-death. The most common strategies to combat Gpx4 loss include small molecules that either preclude the incorporation of oxidizable lipids or prevent the initiation (iron-chelators, LOX inhibitors) and propagation of lipid peroxidation (radical-trapping antioxidants). Several studies have reported metabolic regulators of ferroptosis and recognized genes associated with lipid metabolism such as ACSL₄, LPCAT and HMG-CoA reductase responsible for promoting ferroptosis. Recent reports on the vulnerability of persister and therapy-resistant cancer cells towards the lipid peroxidation pathway has prompted for an investigation of mechanisms that confer ferroptosis resistance to certain cell lines. Our collaborators sequenced ferroptosis resistant cells to identify genes able to complement the loss of GPX4. The results revealed a previously uncharacterized protein, “apoptosis inducing factor mitochondria-associated 2 (AIFM2)”, so called due to its close sequence homology to AIFM1. Now renamed to “ferroptosis-suppressor-protein 1” (FSP1), its expression overlapped with ferroptosis resistance in multiple cancer cells and strong cytoprotective activity when over-expressed in ferroptosis susceptible cell lines. Here, we elucidate the mechanism of action of FSP1 and demonstrate that it acts on a molecular level by reducing lipid peroxidation and the accumulation of hydroperoxides. Our results establish that FSP1 is a NAD(P)H dependent oxidoreductase that regenerates the endogenous antioxidant pool through exerting its action on coenzyme Q₁₀ and α -tocopherol. Moreover, we relay the importance of protein myristoylation and its critical role on the function of native FSP1. Part of the results presented in this chapter were published as part of a preliminary study in Nature (doi:10.1038/s4158-019-1707-0). Sebastian Doll and Pedro Friedmann Angeli developed the cell lines and provided the recombinant (m)FSP1 protein used in the studies.

8.2 Introduction

A novel form of regulated necrosis, termed ferroptosis, was recently characterized as a cell-death routine that is morphologically, biochemically and genetically distinct from other well-established cell-death modalities.¹⁻³ Ferroptosis has attracted considerable attention due to its implication in several pathophysiological conditions such as cardiovascular disease, neurodegeneration and cancer.^{1,4,5} Due to its diverse applications, the current field is mainly separated in two areas: 1) aiming to subvert ferroptosis and provide therapeutic opportunities for poorly treatable diseases⁶ and 2) trying to recruit ferroptosis to selectively induce cancer cell-death.^{7,8} Establishing the execution mechanism of ferroptosis, which occurs by an iron-catalyzed radical-chain reaction called autoxidation was the first step towards understanding the downstream events leading to cell-death.^{9,10} As such, inhibitors of this process, radical-trapping antioxidants (RTAs), have provided the most effective strategy for ferroptosis inhibition.^{6,11-14} Recent reports have shown that certain therapy-resistant cancer cells are particularly susceptible to Gpx4 inactivation, signifying their dependence on the lipid peroxidation pathway.^{7,8} Conversely, some cells are extremely resistant even without the supplementation of canonical ferroptosis inhibitors, which has sparked an interest in mechanisms that confer inherent endogenous resistance. Recently, an expression cloning approach was undertaken to identify genetic regulators of ferroptosis able to compensate for the loss of Gpx4.¹⁵

Transduction of ferroptosis sensitive Pfa1 cells with lentiviral particles containing a cDNA library derived from MCF7 ferroptosis-resistant cell line^{9,16} yielded 14 single cells that were sequenced to yield several clones with a high expression of AIFM2 (Figure 8.1A). This was an unexpected finding because AIFM family of genes are labeled as pro-apoptotic factors due to close sequence homology with the well characterized, apoptosis-inducing factor, mitochondria-associated, 1 (AIFM1).¹⁷ To validate the results, AIFM2 was overexpressed in Pfa1 cells¹⁸ (Figure 8.1B) and in human fibrosarcoma HT1080 cells (data not shown). Cells expressing AIFM2 were not sensitized to apoptosis (data not shown) but were strongly

protected against pharmacological and genetic inducers of ferroptosis (Figure 8.1C).¹⁹ These cells also exhibited an impressive ability to proliferate indefinitely without the requirement of exogenous RTAs. To date, only regular supplementation with RTA has allowed cells to proliferate in the absence of Gpx4, but only for limited passages.^{2,18} Since AIFM2 is the first reported enzymatic system capable of complementing the loss of GPX4, it was renamed to “Ferroptosis-Suppressor-Protein 1 (FSP1)”.¹⁵

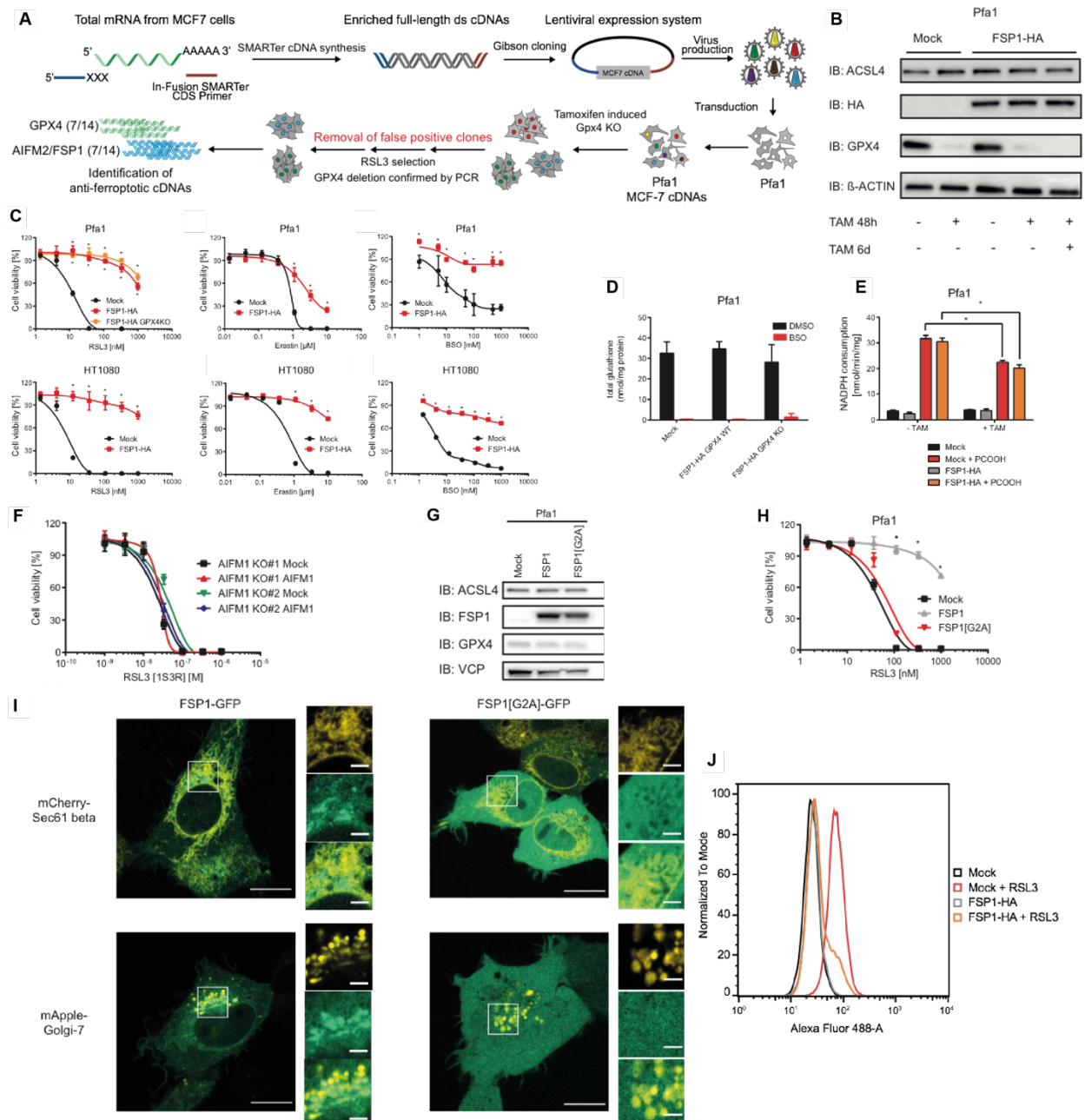


Figure 8.1 (A) Scheme depicting the generation of the lentiviral overexpression library using the total mRNA from MCF7 cells. This library was applied to Pfa1 cells, GPX4 KO was induced by TAM and cDNAs with anti-ferroptotic properties were identified in the surviving single cell clones. (B) Immunoblot analysis of ACSL4, HA-tag, GPX4 and β -ACTIN of Pfa1 cells infected with lentiviral particles containing transfer plasmids (C) Dose-dependent toxicity of (1*S*,3*R*)-RSL3 (RSL3), erastin and L-buthionine sulfoximine (BSO) in Pfa1 and HT1080 cells expressing Mock or FSP1-HA. (D) Measurement of total glutathione levels in Pfa1 Mock, Pfa1 FSP1 and Pfa1 FSP1 GPX4 KO cells treated with or without the L-buthionine sulfoximine (BSO). (E) Determination of the NADPH consumption by glutathione reductase as an indirect measure of the GPX4 activity. (F) Dose-dependent toxicity of RSL3, erastin and BSO in Pfa1_AIFM1KO cell clones (#1 and #2) overexpressing Mock or AIFM1. (G) Immunoblot analysis of ACSL4, FSP1, GPX4 and VCP of Pfa1 cells infected with lentiviral particles containing the transfer plasmids (H) Dose-dependent toxicity of RSL3 in Pfa1 cells with lentiviral overexpression of p442-Mock, p442-FSP1-HA or p442- FSP1[G2A]-HA. (I) Enhanced resolution confocal microscopy of HT1080 cells (FSP1-GFP and FSP1[G2A]-GFP) overexpressing ER localization or Golgi localization. GFP is displayed in green, while ER and Golgi localization fluorescence are pseudo-colored in yellow. (J) Flow cytometry analysis of BODIPY 581/591 C₁₁ oxidation in Pfa1 cells with lentiviral overexpression of mock or FSP1-HA induced by RSL3 (300 nM for 3 h).

Furthermore, there is no interplay between the common ferroptosis modulators such as GSH levels, GPX4 activity or ACSL4 expression and FSP1 (Figure 8.1D-E), suggesting that it is a parallel system that does not affect or is effected by the common ferroptosis regulators. Independent corroboration has shown that its homologue, AIFM1, does not offer any protection from ferroptosis (Figure 8.1F). The N-terminus of FSP1 is highly conserved as it contains a myristoylation motif,²⁰ whereupon tagging or mutating the predicted myristoylation site from [G2A] results in complete abolition of its anti-ferroptosis activity (Figure 8.1G, H). The myristoylation presumably allows FSP1 to interact closely with the lipid bilayer,²¹ implying that it may be closely associated near sites of lipid peroxidation. To probe its subcellular localization, C-terminus tagged GFP-fusion proteins of FSP1 and G2A-FSP1 were transfected into HT1080 cells. FSP1-GFP was found to localize to unspecified perinuclear membrane compartments, and partially overlap with markers of the ER and Golgi (Figure 8.1I). In contrast, G2A-FSP1-GFP was distributed throughout the cell, supporting the notion that lipid membranes are the primary sites of ferroptosis induced damage and why the G2A mutant does not provide ferroptosis resistance. This was

also apparent by the low of C₁₁-BODIPY^{581/591} oxidation in FSP1 overexpressing Pfa1 cells treated with a lethal concentration of RSL3 (Figure 8.1J). The suppression of (phospho)lipid peroxidation suggested that the foundation of FSP1 protection resulted from either a direct or an indirect inhibition of the autoxidation pathway.¹⁵

Herein, we validate that similar with the other AIF enzymes, FSP1 is a NAD(P)H dependent oxidoreductase capable of regenerating quinones of various chain lengths. The enzymatic activity of both native and G2A-FSP1 is characterized using a novel pro-fluorescent ubiquinone probe designed to undergo enhancement upon reduction to ubiquinol. Using the recently developed FENIX methodology, the potent anti-ferroptosis function of FSP1 was discovered to act *via* the CoQ₁₀- α -tocopherol (α -TOH) antioxidant system in the cells. We conclusively demonstrate that FSP1 effectively converts the water-soluble reducing equivalents from NAD(P)H to regenerate the lipophilic RTAs, which inhibit lipid peroxidation. Moreover, enzyme myristoylation and its importance for the mechanism of FSP1 is also addressed.

8.3 Results and Discussion

8.3.1 Characterizing kinetics parameters of FSP1 activity

To supplement previous results that FSP1 reduces global lipid peroxidation (Figure 8.1J) and ascertain whether this directly translates to lower levels of (phospho)lipid oxidation products, FSP1-overexpressing and wild-type cells were incubated with arachidonic acid where ferroptosis was induced using RSL3. The concentrations of 5-, 12- and 15-H(P)ETE were significantly lower in FSP1 overexpressing cells compared to the wild-type control (Figure 8.2A). These results firmly establish FSP1 as the only enzyme identified to date capable of compensating for the loss of GPX4.

With increasing evidence that FSP1 offers a cytoprotective role by reducing the overall levels of PLOOH in cells, we sought to fully elucidate its mechanism of action. Members of the AIF family have been shown to possess NADH:ubiquinone oxidoreductase activity,²² and we speculated that FSP1 might suppress lipid peroxidation by regenerating lipophilic quinones in a NAD(P)H dependent manner (Figure

8.2B). The reduced form of coenzyme Q₁₀ (CoQ₁₀-H₂) has been demonstrated to be a good RTA in phospholipids and lipoproteins,²³ but is believed to be of limited value beyond mitochondria because an efficient recycling mechanism is hitherto unknown. To study the effects of FSP1 on CoQ₁₀ and determine kinetic parameters for the enzyme, recombinant non-myristoylated and myristoylated FSP1 (mFSP1) were purified from bacterial (DH5α) and insect cells (Sf9), respectively.

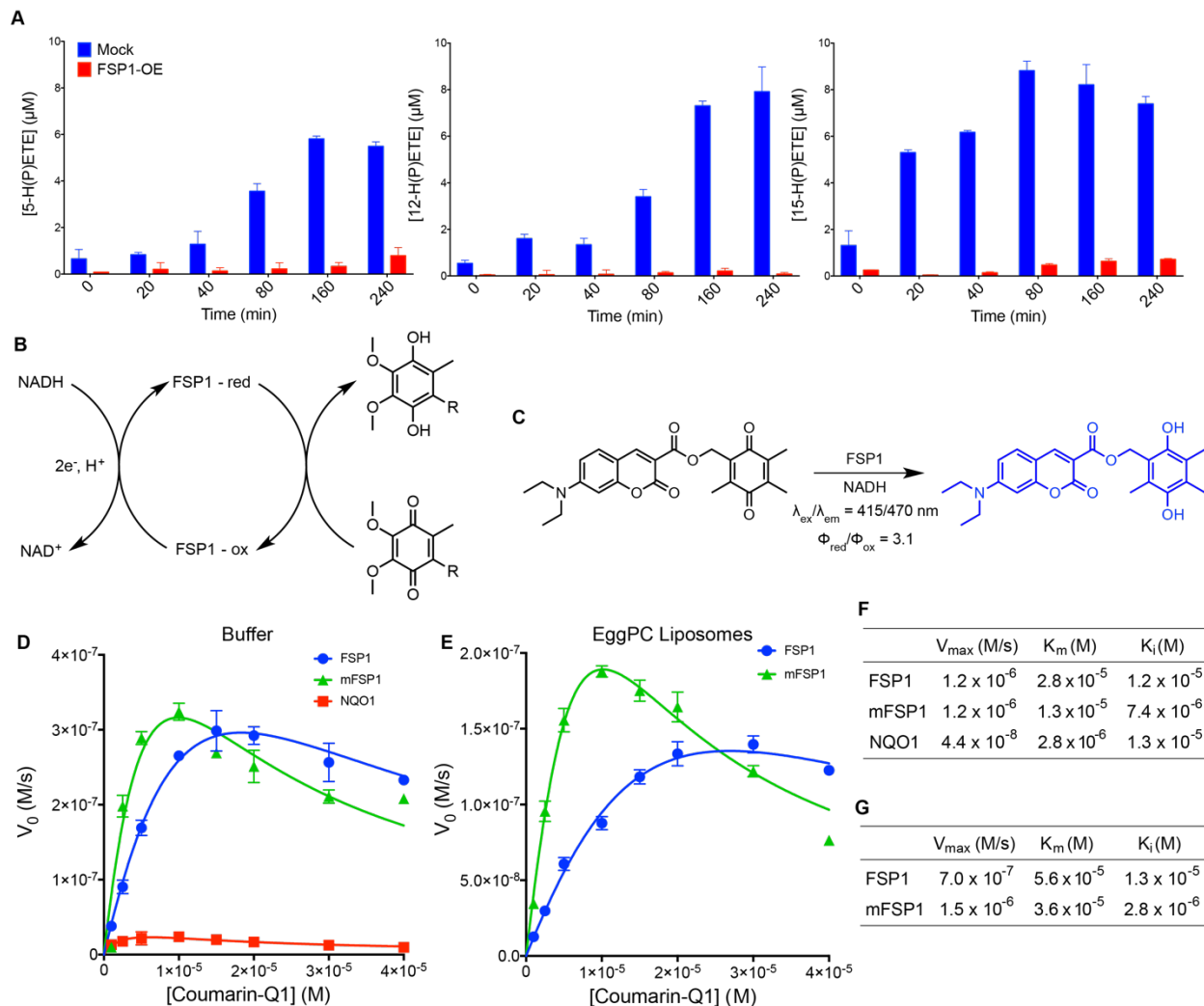


Figure 8.2 (A) Formation of 5-H(P)ETE (MRM: 319→115), 12-H(P)ETE (MRM: 319 →179) and 15-H(P)ETE (MRM: 319→219) in either mock (blue) or FSP1-HA overexpressing (red) Pfa1 cells treated with 0.2 μM RSL3 and 40 μM arachidonic acid. Hydroperoxides were analyzed as their alcohols following reduction with PPh₃ in methanol. (B) Proposed mechanism of FSP1 activity (C) Coumarin-quinone conjugate that undergoes fluorescence enhancement upon reduction to the coumarin-hydroquinone (ex = 415 nm, em = 470 nm). Kinetic parameters for the reduction of coumarin-quinone by

FSP1 (50 nM, blue), mFSP1 (50 nM, red) and NQO1 (50 nM, red) in TBS (10 mM, pH 7.4) (**D**, **F**) or egg PC liposomes (**E**, **G**) in the presence of NADH (200 μ M) at 37°C. Initial rates were determined from the fluorescence of the product hydroquinone. The data was fit to a standard substrate inhibition model.

Kinetic parameters such as K_m and V_{max} were determined using an ubiquinone appended coumarin fluorophore (Figure 8.2C). The probe, which was synthesized as described below, was designed to undergo fluorescence enhancement upon reduction to the hydroquinone. NADH alone was unable to reduce the probe to the hydroquinone under the reaction conditions. However, it was swiftly reduced in the presence of FSP1. The kinetics revealed much higher V_{max} for both FSP1 and mFSP1 compared to classical oxidoreductases (e.g. NQO1), along with typically observed substrate inhibition (Figure 8.2D-G).²⁴ The activities of FSP1 and mFSP1 were very similar in TBS buffer (Figure 8.2D,F) with a V_{max} of 1.2×10^{-6} M/s. More importantly, we observed that the maximal velocity in egg phosphatidylcholine (egg PC) liposomes was 2-fold higher for mFSP1, indicating the importance of myristoylation. Myristoylation ensures that the enzyme is closely associated with the lipid bilayer and enabling more efficient reduction of the lipophilic coumarin-quinone probe (Figure 8.2E,G) – and presumably other lipophilic quinone substrates. The K_m of FSP1 and mFSP1 was also an order of magnitude higher than NQO1 ($K_m = 2.8 \times 10^{-5}$ M), suggesting that FSP1 is rather promiscuous and accepts a broad range of substrates, consistent with its capacity to reduce resazurin as well as quinones of various chain lengths.¹⁵

8.3.2 Characterization of the Functional Role of FSP1 in a Model System

To interrogate the hypothesis that FSP1 suppresses lipid peroxidation by maintaining CoQ₁₀ in its reduced state (which in turn reacts with chain-carrying peroxy radicals), we utilized FENIX to carry out co-oxidations of egg PC liposomes²⁵ using the lipophilic alkoxyl radical initiator, DTUN (Figure 8.3A-B). The reactions were followed by monitoring the increase in ox-STY-BODIPY fluorescence. We verified there were no artefacts from fluorescence quenching due to the presence of FSP1 and NADH with a simple BODIPY-scaffold (Figure S8.1). First, we evaluated whether FSP1 or mFSP1 would directly react with the chain-carrying peroxy radicals by electron-transfer to inhibit the propagation of lipid peroxidation. It was anticipated that mFSP1 would exhibit better inhibition activity in egg PC liposomes

due to the myristoyl anchor placing it in close proximity of the lipid bilayer. In our system, neither FSP1 or mFSP1 alone or in combination of their reducing co-substrate, NADH, were able to suppress lipid peroxidation effectively (Figure 8.3C,F). The results imply that in lipid bilayers, (m)FSP1 cannot directly react with peroxy radicals to inhibit lipid peroxidation. This is presumably because the enzyme is separated from the lipid peroxy radicals and electron transfers are not facile in hydrocarbon-rich environments.

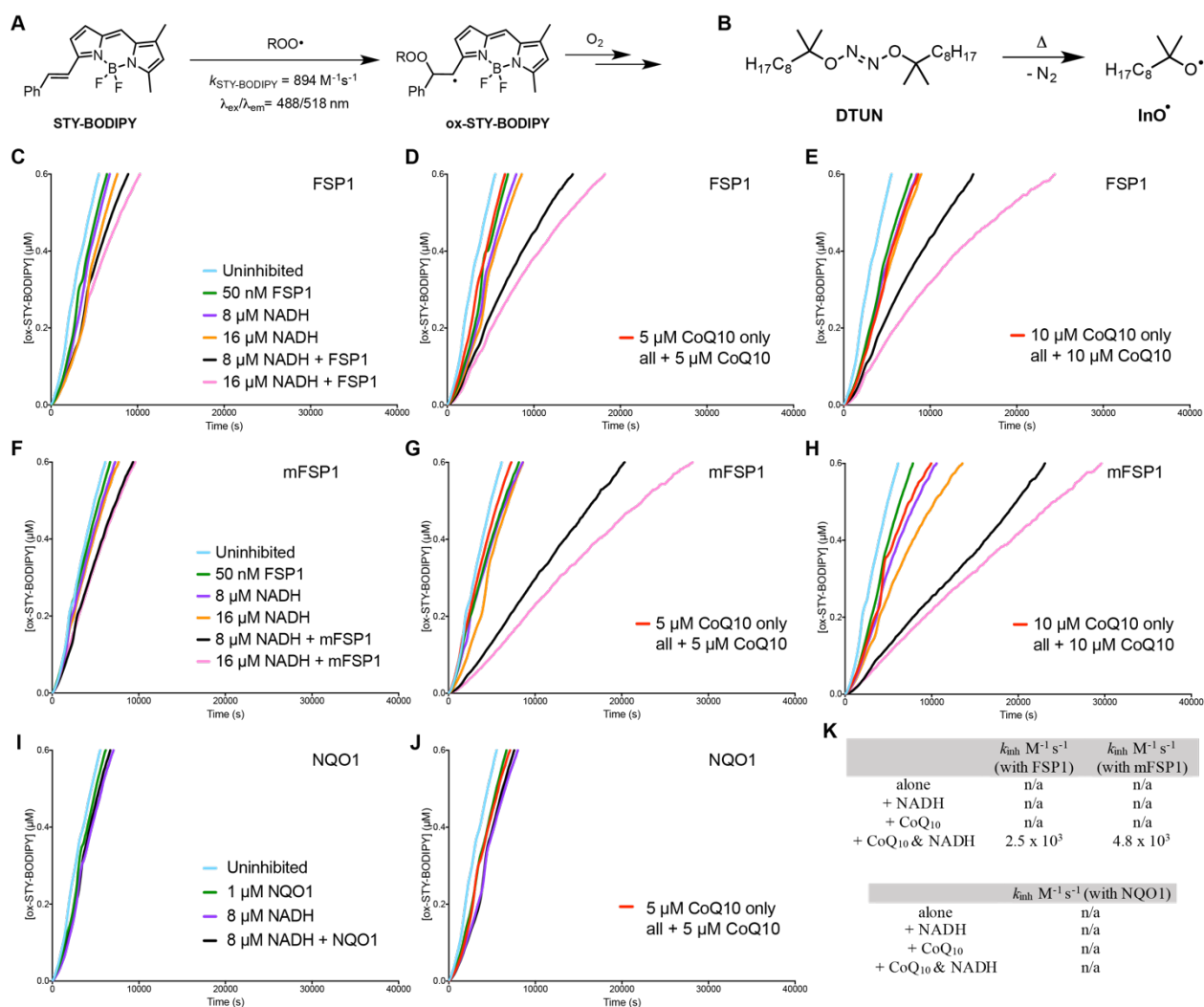
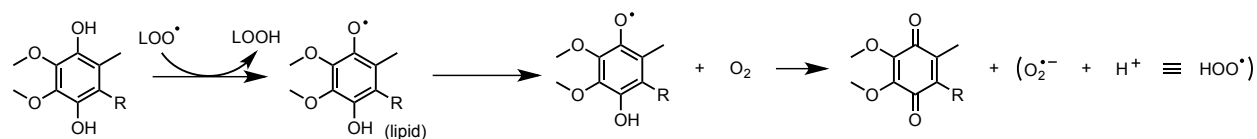


Figure 8.3 Co-oxidations of STY-BODIPY (1 μ M) (A) and egg PC liposomes (1 mM). The increase in fluorescence of ox-STY-BODIPY is monitored over the course of the autoxidation, which is initiated using DTUN (0.2 mM) (B). Representative autoxidations inhibited by either 50 nM (m)FSP1 (green), 8 μ M NADH (purple), 16 μ M NADH (orange), 50 nM (m)FSP1 and 8 μ M NADH (black) or 50 nM (m)FSP1 and 16 μ M NADH (blue) (C,F). Analogous representative autoxidations inhibited to which 5

μM CoQ₁₀ (D, G), or 10 μM CoQ₁₀ (E, H) were added. Recombinant NQO1 failed to suppress autooxidations in a similar way (I, J). (K) Rate constant of inhibition (k_{inh}) for various conditions in C-J.

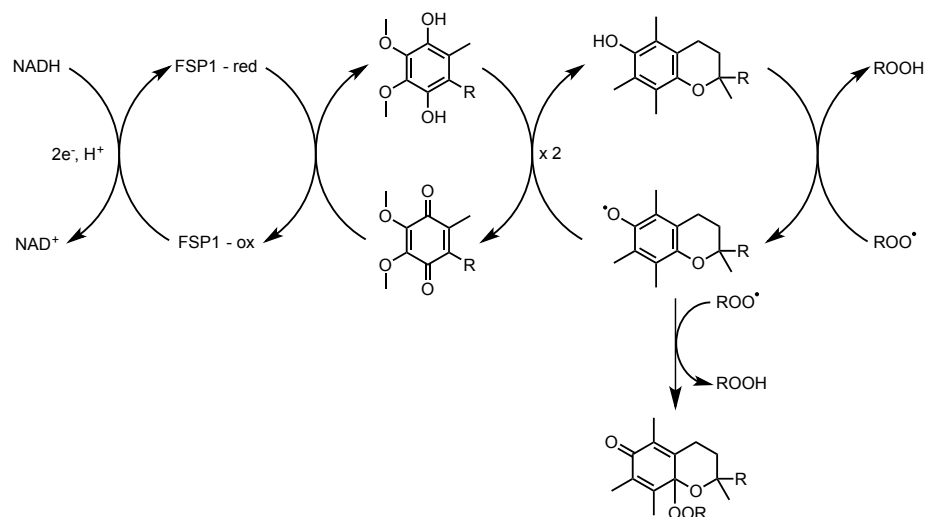
Immediately upon addition of CoQ₁₀, the autooxidation was retarded in a dose-dependent manner (Figure 8.3D-E, G-F). Unfortunately, the inhibitory activity of CoQ₁₀ was lacklustre, which can be readily explained due to its reduced mobility resulting from its lipophilic side chain. The van der Waal interactions with membrane lipids decreases its dynamics in the bilayer precluding reaction with peroxy radicals.²⁶



Scheme 8.1 Proposed pro-oxidant induced behavior of CoQ₁₀H₂

Moreover, CoQ₁₀H₂ is known to undergo background autooxidation to generate initiating radicals, mainly by the production of superoxide (and consequently hydroperoxyl radicals),²⁷ which negatively impacts its inhibition activity²⁸ as the hydroperoxyl radicals can go on to initiate new radical-chain reactions (Scheme 8.1). Due to the complexity of the system, parameters of RTA efficacy are difficult to delineate in the presence of the enzyme and are reported as apparent rate constants of inhibition ($k_{\text{inh}}^{\text{app}}$). The $k_{\text{inh}}^{\text{app}}$ for CoQ₁₀ was $2.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $4.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ with FSP1 and mFSP1, respectively. This rate constant confirms speculation established on qualitative data stating that k_{inh} of CoQ₁₀H₂ is expected to be similar with that of α -TOH (lit. $k_{\text{inh}} = 5.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$)²⁹ in lipid bilayers,³⁰ but this is the first report where a rate constant for CoQ₁₀H₂ has been measured independently. Incidentally, the 2-fold increase in rate constant is consistent with a 2-fold higher V_{max} of mFSP1 compared to FSP1, due to its close interaction with the liposomal membranes (Figure 8.3G-H). There was also increased inhibition time when mFSP1 was utilized, however due to the lack of distinct inflection point the stoichiometry could not be calculated.

The results indicate that via (m)FSP1, CoQ₁₀ aids in importing the reducing equivalents from NAD(P)H into the lipid bilayer to inhibit propagation of lipid peroxidation. Consistent with the activity assays presented earlier, NQO1 was unable to serve in the same capacity as FSP1 in these assays (Figure 8.3I-J).



Scheme 8.2 Proposed activity of FSP1. The reduction of CoQ₁₀ to CoQ₁₀H₂ by FSP1 is coupled to the regeneration of α -TOH, which reacts with the chain-carrying peroxy radicals and inhibits the accumulation of hydroperoxides.

Since CoQ₁₀ itself is such a poor inhibitor, we speculated whether the cytoprotective activity of FSP1 observed in cells might be due to the involvement of α -tocopherol (α -TOH), where following its reaction with a lipid-derived peroxy radical, α -TOH could be regenerated by reduced CoQ₁₀ (Scheme 8.1).³¹ Indeed, this appears to be the case, the addition of CoQ₁₀ in the presence of (m)FSP1 coupled to the regeneration of α -TOH inhibited lipid peroxidation to a significant extent. The combined activity of both antioxidants appears to be synergistic when present together. The increased activity is due to α -TOH acting as the primary chain-breaking antioxidant and its resultant phenoxyl radical is recycled by either CoQ₁₀H₂ or CoQ₁₀H• (Scheme 8.1). This was first demonstrated by Niki in the autoxidations of soy phosphatidylcholine liposomes by ESR spectroscopy, where the concentration of α -TOH remained constant until CoQ₁₀H₂ was consumed, whereby α -TOH started to be depleted.³⁰ Moreover, the

superoxide induced pro-oxidant behaviour of CoQ₁₀H₂ is likely averted in the presence of α -TOH derived-phenoxyl radical, which can be reduced by superoxide to regenerate α -TOH. The synergistic behavior of CoQ₁₀/ α -TOH is well documented,³¹ and is often invoked as a protective mechanism in LDL particles. The presence of CoQ₁₀H₂ in LDL is extremely important to reduce the α -TOH derived phenoxyl radical. In its absence, α -TOH acts as a chain-transfer agent rather than a RTA, where the intermediate phenoxyl radical abstracts a H-atom from the lipid before encountering another peroxy radical due to poor chain propagation in LDL.²⁷ However, this is the first demonstration where such a system accounts for increased resistance to cell-death. The k_{inh}^{app} for α -TOH with 10 μ M CoQ₁₀ and 16 μ M NADH was calculated to be $2.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $4.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of FSP1 and mFSP1, respectively. This is in line with previous findings which suggest that mFSP1 is 2-fold more efficient at reducing lipophilic antioxidants, which was also reflected in the increased inhibition time of 26641 s from 11745 s. Moreover, *in vitro*, α -TOH was regenerated directly by FSP1 without the need for CoQ₁₀. This is presumably due to a direct electron transfer from the enzyme to the intermediate phenoxyl radical to regenerate α -TOH. The k_{inh}^{app} of α -TOH only experienced a modest increase in the presence of FSP or mFSP1 and was measured at $8.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $1.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. However, inhibition time for α -TOH increased by 1.7-fold in the presence of mFSP1. These results explain why NAD(P)H abundance is a good predictor of ferroptosis³² sensitivity as it provides the water-soluble reducing equivalents for the enzyme to regenerate lipophilic antioxidants.

To establish that the inhibition was solely due to subverting lipid peroxidation and not off-target reactions in the presence of STY-BODIPY, we corroborated the results by directly monitoring the formation of PLOOH in autoxidations of liposomes made of linoleate-rich soy lecithin. Similar to the STY-BODIPY data, we did not observe any meaningful suppression of autoxidation by (m)FSP1 and NADH directly. The use of CoQ₁₀ in the presence of FSP1 decreased the accumulation of PLOOH, which was further suppressed in the presence of both CoQ₁₀ and α -TOH (Figure 8.4). Once again, the inhibition was more potent in the presence of mFSP1, which associates closely with the lipid bilayer and can reduce

the lipophilic antioxidants efficiently. The results are in agreement with the cell data where native FSP1 overexpressing cells were significantly more resistant to ferroptosis than the G2A mutant (*cf* Figure 8.1H), underscoring the importance of myristoylation. Thus, our liposomal system closely mimics the cell model and offers an excellent method to evaluate the differences in the activity of FSP1 and mFSP1.

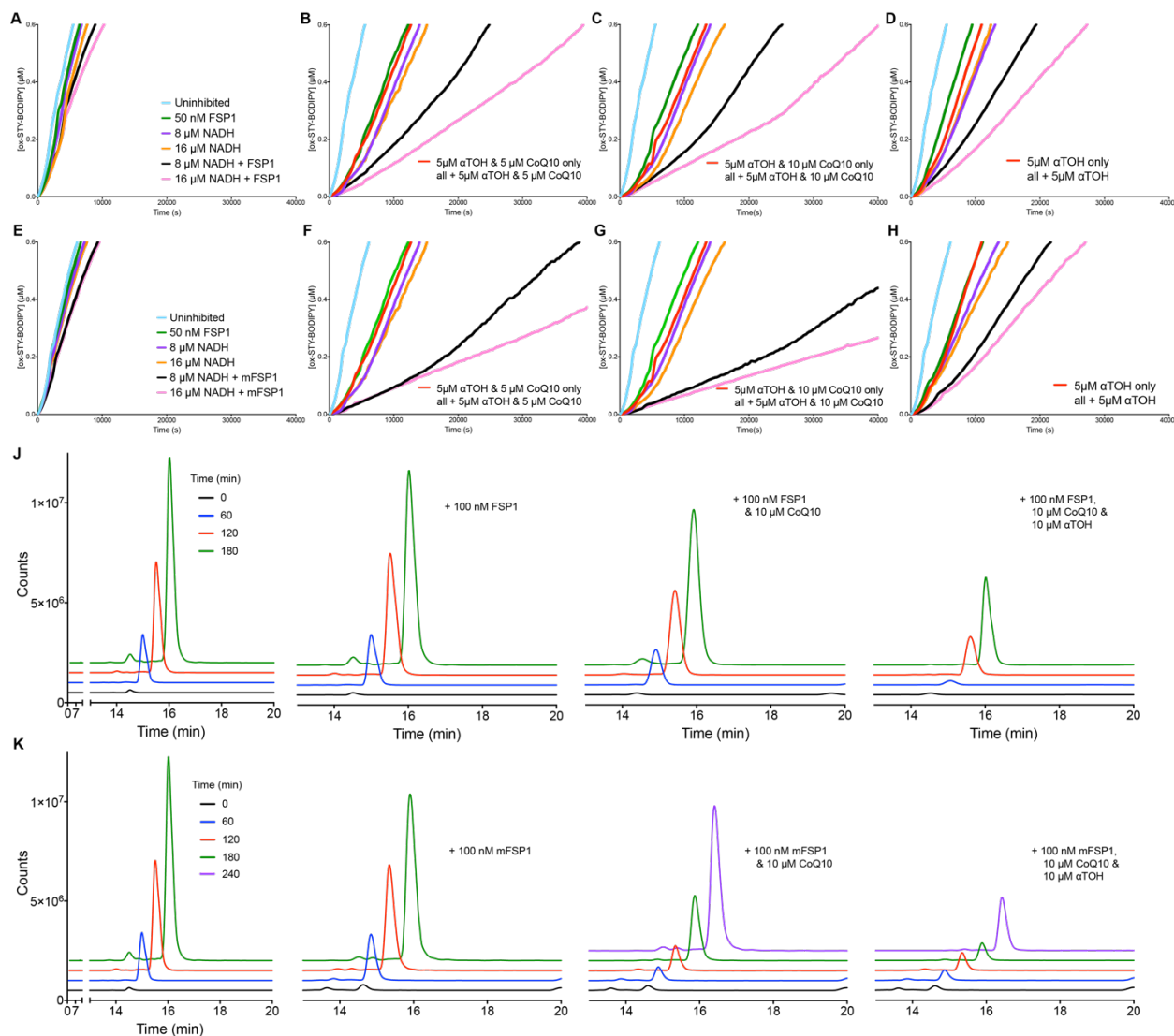


Figure 8.4 Co-oxidations of STY-BODIPY (1 μM) and egg PC liposomes (1 mM). The increase in fluorescence of ox-STY-BODIPY is monitored over the course of the autoxidation, which is initiated using DTUN (0.2 mM). Representative autoxidations inhibited by either 50 nM (m)FSP1 (green), 8 μM NADH (purple), 16 μM NADH (orange), 50 nM (m)FSP1 and 8 μM NADH (black) or 50 nM (m)FSP1 and 16 μM NADH (blue) (A,E). Analogous representative autoxidations inhibited to which αTOH and CoQ₁₀ (B-C, F-G), or αTOH (D-H) were added. (J-K) PLPC-OOH produced from the autoxidation of

soy lecithin liposomes (13.3 mM), inhibited by (m)FSP1 alone, or in the presence of either 10 μ M CoQ₁₀ or 10 μ M α TOH and 10 μ M CoQ₁₀. PLPC-OOH was measured 0, 60, 120, 180 and 240 min after autoxidation was induced using LC-MS/MS (MRM: 790 $\rightarrow$ 184).

Given the foregoing, we wondered if the regeneration behaviour was specific to α -TOH/CoQ₁₀ or whether other RTAs may be used instead. We utilized the canonical ferroptosis suppressors Lip-1 and Fer-1 along with PMC, the truncated form of α -TOH (See Figure S8.2). Under the reaction conditions we observed that all RTAs inhibited lipid peroxidation, and were regenerated to varying extents by FSP1 and mFSP1 from a direct electron transfer to the intermediate aminyl or phenoxyl radical. Surprisingly, there was no clear dependence on the nature of the enzyme as there were no enhancements in rate constants or inhibition times in the presence of mFSP1 over FSP1 (Figure 8.5A, red). PMC, Lip-1 and Fer-1 inhibited autoxidation 1.8, 1.3 and 1.4-fold longer relative to the control where no enzyme was added (Figure 8.5A, blue). Furthermore, there was no synergism when CoQ₁₀ was present in the system (See Figure S8.2).

8.3.3 Electron Transfer from FSP1 Inhibits Autoxidation

To establish that electron transfer from the enzyme plays a role in the regeneration of RTAs, autoxidations of egg PC liposomes were carried out with MeOAMVN, an azo-compound that initiates near the aqueous/lipid interface. Since MeOAMVN derived peroxy radicals are known to react with water-soluble RTAs and prevent chain propagation, it was expected that the initiating radicals may be directly reduced by the enzyme if an electron transfer was taking place. Although direct inhibition was not observed with DTUN (*cf.* Figure 8.3C, F) due to phase separation of FSP1 and lipid peroxy radicals, we anticipated that the identical localization of the enzyme and MeOAMVN derived peroxy radicals in the aqueous compartment might facilitate this process. Indeed, autoxidation was completely suppressed in the presence of FSP1 and NADH (Figure S8.3A). The pronounced inhibited period allowed for the calculation of k_{inh}^{app} and n , which were $8.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and 5.0, respectively. This is possibly the highest rate constant ever reported for the inhibition of lipid peroxidation, and the first demonstration of an enzyme catalyzed-electron transfer to peroxy radicals. It must be acknowledged that under these conditions the second-

order rate constant simply reports on the reaction with a MeOAMVN derived peroxy and not a lipid peroxy radical. While this model does not directly report on lipid peroxidation inhibition, it was utilized to further investigate certain observations made previously. For example, the pro-oxidant tendency of CoQ₁₀H₂ was invoked for its poor RTA activity due to the production of hydroperoxy radicals (*cf.* Scheme 8.1). This was apparent from the inverse relationship between inhibition time and the concentration of CoQ₁₀ as evidenced by the stoichiometry, $n = 2.2, 1.9$ and 1.7 for $2, 4$ and $6 \mu\text{M}$ of CoQ₁₀, respectively (Figure S8.3B). The pro-oxidant behaviour was also noticeable with co-antioxidants PMC and α -TOH, where the inhibition time deteriorated in a concentration dependent manner compared to just the RTAs themselves ($n = 2.6$ vs 5.3 for PMC and $n = 2.5$ and 3.3 for α -TOH, in the presence and absence of Q₁₀) (Figure S8.3C, D). The k_{inh}^{app} of α -TOH and FSP1 ($1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) was similar to that of the enzyme itself ($8.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$), while the k_{inh}^{app} of PMC and FSP1 ($5.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) was the same as PMC ($4.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$). This is likely due to the reduced dynamics of α -TOH, which prevents interception of MeOAMVN derived radicals near the interface and thus they are rapidly reduced by a direct electron transfer from FSP1. However, PMC is not restricted in the lipid and can directly capture the initiating radicals while being regenerated by FSP1 (Figure S8.3E, F).

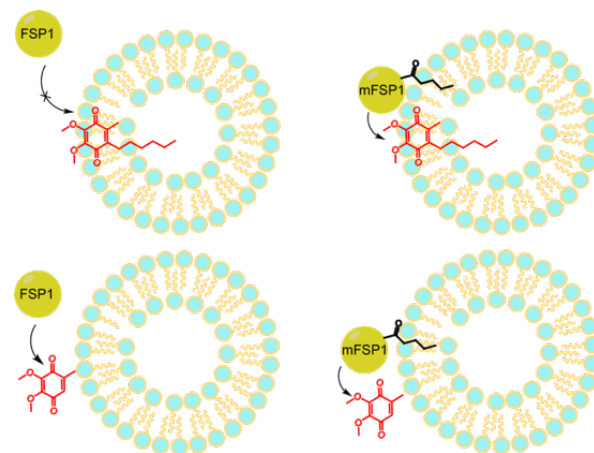
The role of electron transfer was also independently corroborated in an aqueous system consisting of TBS buffer to which 25% THF was added to carry the autoxidation-chain reaction. We confirmed that the enzyme activity was not affected substantially under the reaction conditions. In aqueous environment, once again, FSP1 alone in the presence of NADH was a potent inhibitor of THF autoxidation ($k_{inh}^{app} = 2.5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) (Figure S8.4A). Consistent with the other results, FSP1 also directly regenerated Trolox, a water soluble analog of PMC and our designer α -TOH mimetic, C₁₅-THN (Figure S8.4B-D). The addition of CoQ₁₀ did not afford synergism but reduced the inhibited period relative to the compounds themselves. The results highlight that while the enzyme cannot inhibit lipid peroxidation directly, the direct reaction of the enzyme with peroxy radicals is observed in aqueous environments or near the aqueous/lipid interface where electron transfers are accelerated. A potential advantage of this system may be to reduce

water-soluble oxidants before they can enter lipid bilayers to initiate peroxidation. The electron transfer behaviour of FSP1 underscores its ability to regenerate RTAs and needs to be corroborated directly. Protein film voltammetry (PFV) can be utilized to determine the electrochemical properties of FSP1 and measure its reduction potential.

To validate that electron transfer is selective to antioxidants and does not promote oxidation by catalyzing heterolysis of hydroperoxides, we tested the propensity of FSP1 to reduce oleic acid-hydroperoxide. Utilizing a pro-fluorescent phosphine probe, very little decomposition (< 15%) of hydroperoxides was detected over two hours, confirming that electron transfer is unlikely to play a role in the initiation of autoxidation (Figure S8.4) largely due to the phase separation of lipid hydroperoxides and the enzyme. It should also be noted that while 15% of the hydroperoxide underwent decomposition, 200 μ M of oleic-hydroperoxide was present in the lipids and this concentration far exceeds levels that would accumulate in egg PC liposomes ($\sim$ 15 μ M).

8.3.4 Probing the Importance of Myristoylation on Lipophilic Antioxidants

Having demonstrated the electron transfer capability of FSP1, we sought to rationalise the lack of increased efficacy of Lip-1, Fer-1 and PMC in the presence of mFSP1, and understand the discrepancy compared to α -TOH. This prompted the investigation of antioxidant lipophilicity and its dependency on enzyme myristoylation. Given the foregoing, it was hypothesized that non-lipophilic antioxidants that are not anchored in lipids can be reduced to a similar extent by both FSP1 and mFSP1. The myristoyl tag is essential to enhance activity of lipophilic antioxidants that have reduced dynamics and benefit from the increased proximity to mFSP1 (Scheme 8.2). Since Lip-1, Fer-1 and PMC can easily transverse the lipid bilayer, their regeneration should not be limited by accessibility to FSP1 or mFSP1.



Scheme 8.3 Depicting the importance of enzyme myristoylation for lipophilic antioxidants

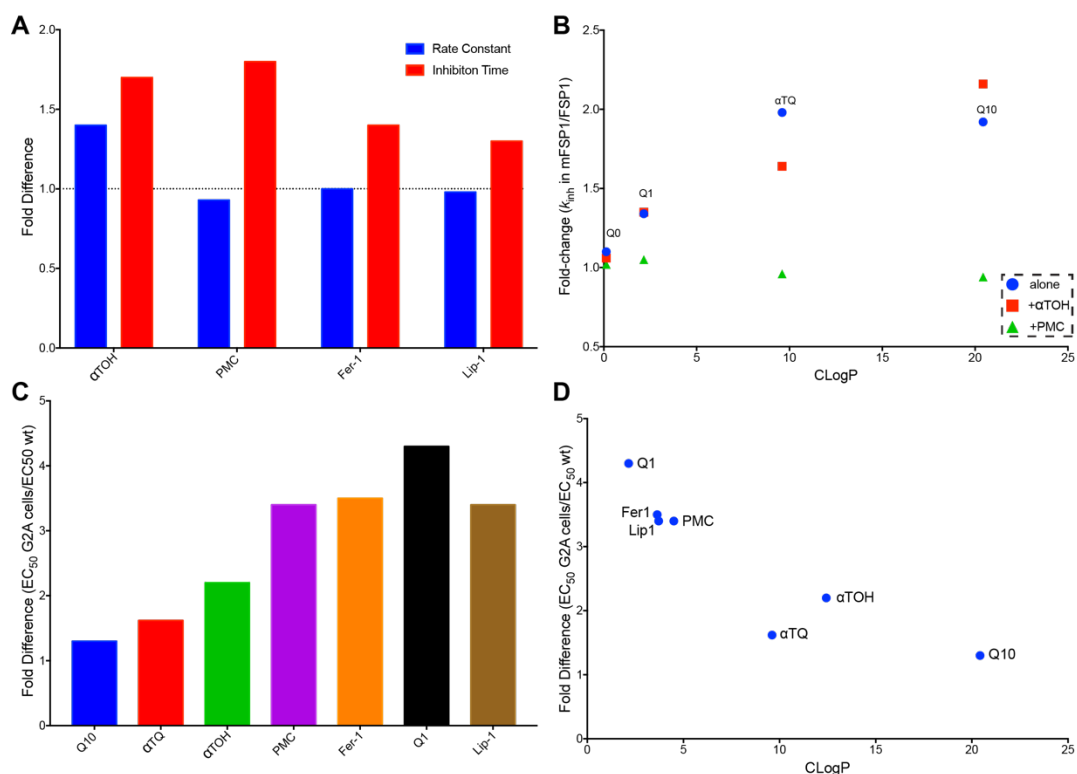


Figure 8.5 (A) Enhancement in k_{inh}^{app} for α -TOH, PMC, Fer-1 and Lip-1 compared to mFSP1 and FSP1 (blue) and increase in inhibited period for the same compounds in the presence of (m)FSP1 relative to control where no enzyme is utilized. (B) Correlation between CLogP of different quinones and their dependence on the nature of the enzyme evaluated as a ratio of k_{inh}^{app} for mFSP1 and FSP1. PMC and α -TOH are added as co-antioxidants. (C) Relative ratios of EC_{50} values for various RTAs in G2A cells compared to the wild-type control (D) Correlation between CLogP of different RTAs and their relative efficacy in G2A cells vs wild-type control.

This model was verified by utilizing quinones of varying chain lengths in liposome autoxidations. Consistent with the expectation, increasing inhibition activity from $\text{CoQ}_0 < \text{CoQ}_1 < \text{CoQ}_{10} \approx \alpha\text{-TOH}$ derived quinone (αTQ) in the presence of mFSP1 was observed. The enhancement in k_{inh}^{app} was linearly dependent on the CLogP of the quinones, and increased by 1.1, 1.3, 1.9 and 2-fold for CoQ_0 , CoQ_1 , CoQ_{10} and αTQ , respectively (Figure 8.5B, S8.5). The relative increase appeared to plateau at 2-fold, which coincides with the V_{max} of the mFSP1 being ~2-fold greater. Unsurprisingly, this difference was entirely abolished when PMC was added as a co-antioxidant. Because the mobility of PMC is not restricted within the lipid bilayer, it is directly regenerated by FSP1 or mFSP1, as such its activity is less dependent on recycling by quinones (Figure 8.5B). In the co-antioxidant system with lipophilic quinones (CoQ_{10} and αTQ) and $\alpha\text{-TOH}$, we observed a prolonged inhibition period and k_{inh}^{app} enhancement with mFSP1. Both quinones and $\alpha\text{-TOH}$ have reduced dynamics and benefit from the proximity of mFSP1 to the liposomes. This improvement was largely lost when CoQ_0 and CoQ_1 were employed to recycle $\alpha\text{-TOH}$, as they can be reduced to the hydroquinone with equal efficacy by both FSP1 and mFSP1 (Figure 8.5B). Overall, these observations readily explain why FSP1 overexpressing cells are extremely resistant to ferroptosis, where the close proximity of the enzyme to lipid bilayers and its ability to regenerate $\text{CoQ}_{10}/\alpha\text{-TOH}$ affords an excellent antioxidant protection system.

It is now clear that the G2A mutant cells were not exceedingly resistant to ferroptosis because of their inability to recycle the endogenous lipophilic antioxidants (i.e. $\text{CoQ}_{10}\text{H}_2$ and αTOH) effectively. We sought to recapitulate the liposomal results in the G2A mutant cells to verify whether our insights would translate to the cellular model. We expected to observe an opposite trend in the G2A cells, where the lipophilic RTAs and quinones would be relatively less potent compared to their non-lipophilic analogs. The exogenous addition of CoQ_1 , CoQ_{10} and αTQ all had cytoprotective effects in G2A over-expressing cells when ferroptosis was induced using RSL3. While CoQ_{10} did not completely rescue the cells due to its limited solubility, we did observe cytoprotection. Similarly, Lip-1, Fer-1, PMC and $\alpha\text{-TOH}$ also inhibited cell-death, corroborating the results from our liposomal system. All quinones and RTAs had

higher potencies in G2A-FSP1 overexpressing cells compared to the wild-type control because of G2A-FSP1 activity, even though it is not membrane bound. However, the difference in efficacy of lipophilic vs non-lipophilic compounds is evident in their relative potencies. The non-lipophilic compounds were comparatively more potent than the lipophilic analogs in G2A-FSP1 overexpressing cells, consistent with their propensity be regenerated by readily accessing the cytosolic G2A-FSP1 (Figure 8.5C). For example, α -TOH was only 2.2-fold more potent in the G2A overexpressing cells relative to PMC which was 3.4-fold more potent. Similarly, the efficacy of α TQ was only enhanced by 1.6-fold while Q₁ increased by 4.3-fold. Once again, a clear trend was visible with CLogP values of the compounds and their relative potencies towards ferroptosis suppression in G2A cells. These experiments are in agreement with our hypothesis that mFSP1 acts by regenerating lipophilic antioxidants by direct electron transfer or by the mediation of CoQ₁₀ (and other quinones). However, cytosolic FSP1 is capable of halting lipid peroxidation by the regeneration of non-lipophilic quinones or RTAs, and lipophilic ones to a smaller extent. As such, the G2A cells is an excellent model to evaluate regeneration of small-molecule RTAs. Overall, our findings reveal that FSP1, with or without myristoylation, is a potent NAD(P)H oxidoreductase whose function is to subvert ferroptosis by regenerating the endogenous antioxidant pool to inhibit lipid peroxidation induced accumulation of PLOOH.

It would be ideal to survey the relative potencies in the native FSP1-overexpressing cells but due to their increased resistance to ferroptosis it is very difficult to induce cell death. However, to conclusively support our ideas, cell lines that overexpress both native FSP1 and G2A-FSP1, and are devoid of 4-hydroxybenzoate polyprenyltransferase (CoQ2), the gene responsible for the first step in the biosynthesis of CoQ₁₀ need be evaluated. Inhibiting the biosynthesis of endogenous CoQ₁₀ would be an excellent avenue to perturb FSP1 function and probe the effects of different RTAs/quinones, and their synergism with α -TOH. Moreover, the G2A mutant cells can be utilized to identify antioxidants that are capable of undergoing regeneration very effectively and develop novel ferroptosis inhibitors.

8.4 Conclusion

Our results establish that FSP1 is an NADH oxidoreductase which functions as a potent suppressor of (phospho)lipid peroxidation and ferroptosis. Using *in vitro* kinetics and cell assays we determined that FSP1 exerts its anti-ferroptotic activity by maintaining reduced CoQ₁₀ and regenerating α -TOH, which can suppress lipid peroxidation even in the absence of GPX4. The data also provides justification for the extra-mitochondrial CoQ₁₀ pool and supports that its antioxidant activity should be rationalized alongside FSP1 in models of oxidative cell-death rescue. Moreover, the enzyme is capable of reducing other small-molecule RTAs either directly by electron transfer, or indirectly by recycling quinones. We also clarify the role of enzyme myristoylation, which enables FSP1 to closely associate with lipid bilayers in order to exhibit its beneficial effects. However, the G2A mutant lacking the myristoyl motif also rescues the cells from ferroptosis but necessitates the use of non-lipophilic RTAs or quinones. This discovery explains why NAD(P)H and defects in the HMG-CoA pathway predict sensitivity to ferroptosis and could be potentially exploited to selectively induce ferroptosis in certain cell lines.

8.5 References

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8.6 Supporting Information

8.6.1 Synthesis of coumarin-quinone conjugate

((2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)methyl 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylate) Briefly, 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylic acid (1.0 mmol), DCC (1.1 mmol) and DMAP (0.1 mmol) were added to a solution of DCM (3.5 mL). To this, was added 2-(hydroxymethyl)-3,5,6-trimethylcyclohexa-2,5-diene-1,4-dione (1.0 mmol) and the solution was stirred overnight at room temperature. The product precipitated out, it was filtered, washed with ethanol and dried thoroughly. Yellow powder, 83%. ¹H NMR (400 MHz, CDCl₃) δ 8.36 (s, 1H), 7.33 (d, *J* = 9.0 Hz, 1H), 6.59 (dd, *J* = 9.0, 2.5 Hz, 1H), 6.44 (d, *J* = 2.4 Hz, 1H), 5.24 (s, 2H), 3.44 (q, *J* = 7.1 Hz, 4H), 2.20 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 1.23 (t, *J* = 7.1 Hz, 6H). ¹³C NMR (76 MHz, CDCl₃) δ 187.5, 185.9, 163.7, 158.6, 158.1, 153.1, 149.5, 145.1, 141.1, 140.8, 136.9, 131.3, 109.7, 108.1, 107.8, 96.9, 58.0, 45.3, 12.6, 12.6, 12.5. HRMS: *m/z* Calc: C₂₄H₂₅NO₆: 423.1682 Found: 423.1677.

8.6.2 Reduction of a coumarin-quinone conjugate by FSP1.

The kinetic parameters were determined by the addition of varying amounts of Coumarin-Q1 probe to 50 nM FSP1 and 200 μM NADH in TBS (pH 7.4) at 37°C. The initial rates of the reaction were obtained by monitoring the increase in the fluorescence upon the reduction of the quinone to the hydroquinone on a plate reader (ex/em = 415/470 nm). The kinetic parameters were obtained by fitting the data to a standard substrate inhibition model in GraphPad Prism. Each experiment was performed independently three times, error bars represent SD from the mean.

8.6.3 Inhibited autoxidation of egg PC liposomes.

To a 96-well polypropylene plate (Nunc) was added egg PC liposomes (1 mM) and STY-BODIPY (1 μM) in TBS at pH 7.4. This was followed by the addition of either FSP1 (50 nM), NADH (8 or 16 μM), CoQ10 (5 or 10 μM), αTOH (5 or 10 μM) or a combination thereof. The plate was incubated for 10 minutes at 37°C in the BioTek Synergy H1 plate reader followed by a vigorous mixing protocol for 5

minutes. The plate was ejected from the plate reader and the autoxidation was initiated by the addition of 3 μ L C₉-HN (0.2 mM) in EtOH, followed by another mixing protocol for 5 minutes. Data was acquired by excitation of STY-BODIPY at 488 nm and emission was measured at 518 nm. The data was transformed into [ox-STY-BODIPY] by taking the raw RFU values and dividing them by the response factor of 7.49×10^{10} . The data shown is an average of three independent experiments.

8.6.4 LC-MS analysis of 5-H(P)ETE/12-H(P)ETE/15-H(P)ETE formation.

Mock and FSP1 overexpressing Pfa1 cells were plated on a 10-cm plate and allowed to adhere overnight. On the next day, media was removed, the cells were washed with PBS and suspended in media containing 0.2 μ M RSL3 and 40 μ M AA. The cells were harvested using a cell scraper at appropriate time points and flash frozen in liquid nitrogen. After thawing, the cells were treated with 5 mL of MeOH containing the internal standard, 1 mg/mL BHT and 1.5 mg/mL triphenylphosphine, followed by sonication to lyse the cells. BHT was added in order to suppress any air oxidation of the PUFAs present in the sample. The mixture was left on a shaker for 30 minutes at room temperature to ensure complete reduction of HPETEs. 10 mL of chloroform and 1 mL of 0.9% NaCl solution was added followed by shaking for 1 hour to ensure maximal extraction of lipids. The tubes were centrifuged to separate the organic and aqueous layers. The lipids were recovered by removing the bottom chloroform layer. The mixture was re-extracted using 5 mL chloroform and the two organic layers were combined. The combined chloroform layers were dried in a vacuum dryer at 20 °C. The lipids were re-suspended in 500 μ L MeOH containing BHT, followed by the addition of 500 μ L KOH (1.5%) solution and incubated at 60°C for 30 minute to saponify all the phospholipids. After incubation, the solution was acidified using 500 μ L HCl (2 M) solution. The mixture was purified using the C-18 solid phase extraction columns (Thermo Scientific) as per manufacturer's protocol. The eluent in methanol was evaporated to dryness and resuspended in 100 μ L methanol. Aliquots (5 μ L) were injected on a Waters Acquity C₁₈ column (2.1 mm $\times$ 50 mm, 1.7- μ m particle size) in a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system. H(P)ETEs were eluted isocratically with a mixture of acetonitrile, methanol, water and acetic acid (42:25:33:0.007)

and detected using MS/MS analysis. Eicosanoids were detected in the negative ion mode by multiple reaction monitoring of m/z 333 $\rightarrow$ 235 for PGB₂, m/z 319 $\rightarrow$ 115 for 5-HETE, m/z 319 $\rightarrow$ 179 for 12-HETE, and m/z 319 $\rightarrow$ 219 for 15-HETE. Each experiment was performed independently three times, error bars represent SD from the mean.

8.6.5 Determination of PLPC-OOH from autoxidation of soy lecithin liposomes.

Autoxidations of 13.3 mM soy-lecithin liposomes were carried out at 37°C and initiated using C₉-HN (1 mM). The autoxidations were inhibited using NADH (25 μ M) and FSP1 (100 nM) in the presence of either CoQ10 (10 μ M), α TOH (10 μ M) or a combination thereof. Aliquots (250 μ L) of the autoxidation mixtures were taken every hour and diluted using a solution of 1% BHT in MeOH (500 μ L) in order to lyse the liposome particles. The BHT was added in order to prevent further autoxidation of PUFAs. Aliquots (10 μ L) were injected on a ThermoFisher Hypersil Gold C₁₈ column (4.6 mm $\times$ 250 mm, 5.0- μ m particle size) in a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system. PLOOH were eluted with a gradient of 1.0 mL/min gradually changing to 1.1 mL/min at 12 minutes. The solvent system changed from 15:85 $\rightarrow$ 1:99 for the mixture of 10 mM NH₄OAc (in H₂O) and methanol (15:85), respectively. The overall run time was 20 minutes and PLOOH were detected in positive ion mode by multiple reaction monitoring m/z 790 $\rightarrow$ 184.

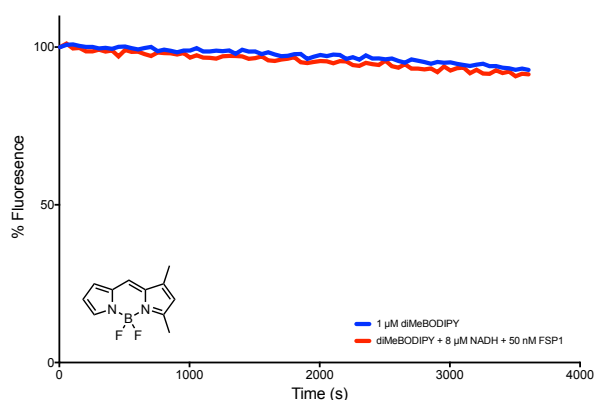


Figure S8.1 Fluorescence quenching of the BODIPY scaffold by FSP1 and NADH.

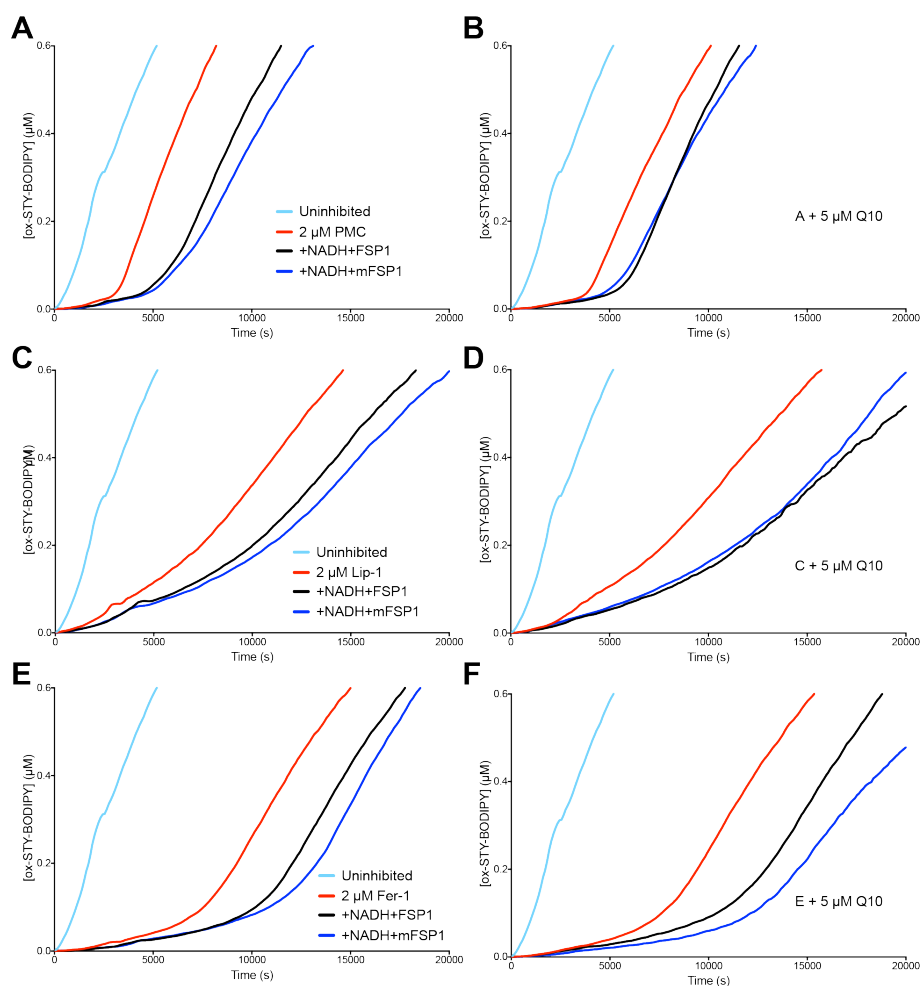


Figure S8.2 Co-oxidations of STY-BODIPY (1 μM) and egg PC liposomes (1 mM). The increase in fluorescence of ox-STY-BODIPY is monitored over the course of the autoxidation, which is initiated using DTUN (0.2 mM). Representative autoxidations inhibited by either 2 μM PMC (A) , Lip-1 (B) or Fer-1 (C) to which 50 nM FSP1 (black) and 50 nM mFSP1 (blue) were added. Analogous representative autoxidations inhibited to which 5 μM CoQ₁₀ (D, E or F) was added.

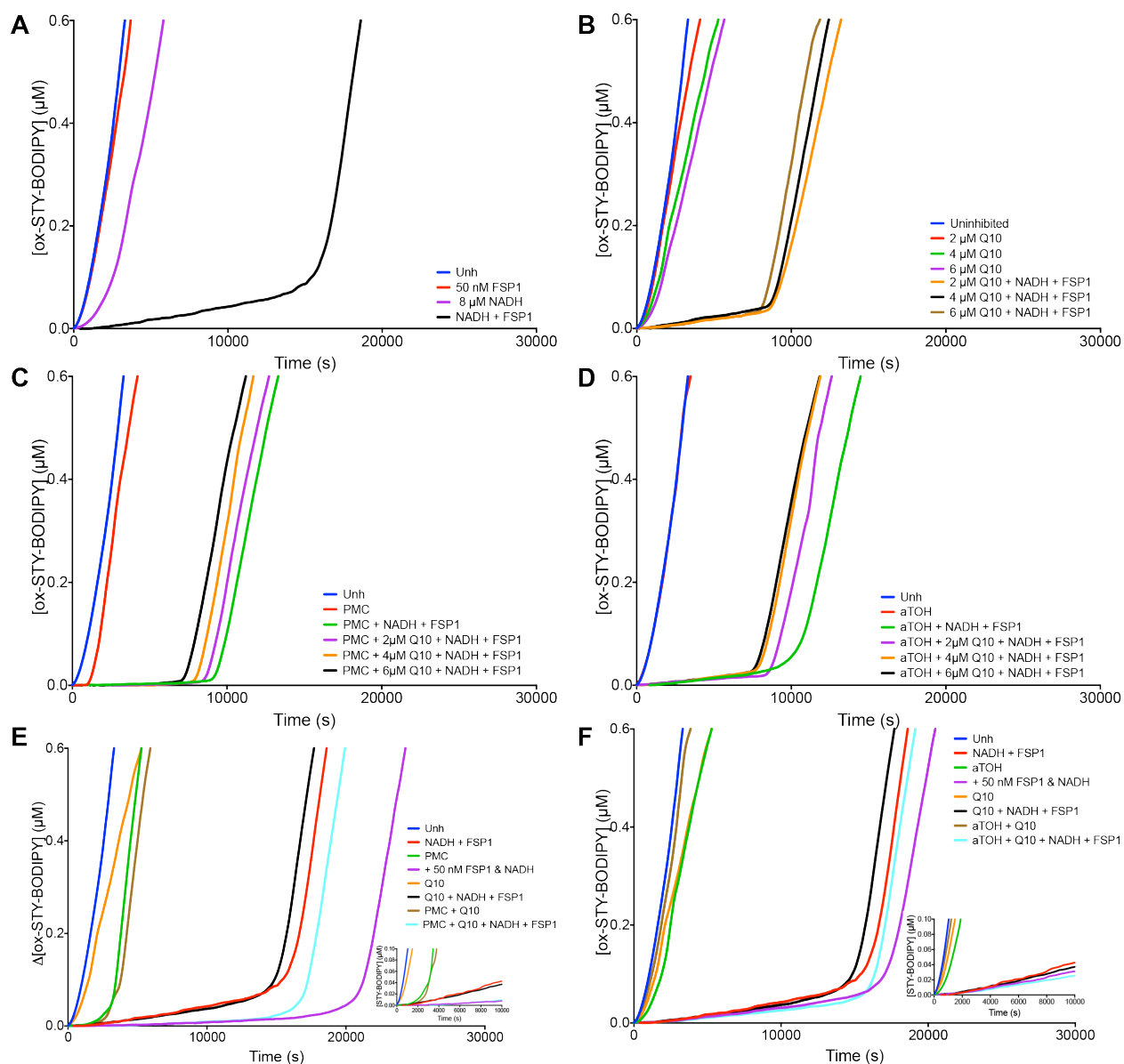


Figure S8.3 Co-autoxidations of STY-BODIPY (1 μM) and egg PC liposomes (1 mM). The increase in fluorescence of ox-STY-BODIPY is monitored over the course of the autoxidation, which is initiated using MeOAMVN (0.68 mM). Representative autoxidations inhibited by either 50 nM FSP1 (red), 8 μM NADH (purple), or 50 nM FSP1 and 8 μM NADH (black) (**A**). Analogous representative autoxidations inhibited to which CoQ₁₀ (**B**), PMC and CoQ₁₀ (**C**, **E**) or α TOH and CoQ₁₀ (**D**, **F**) were added.

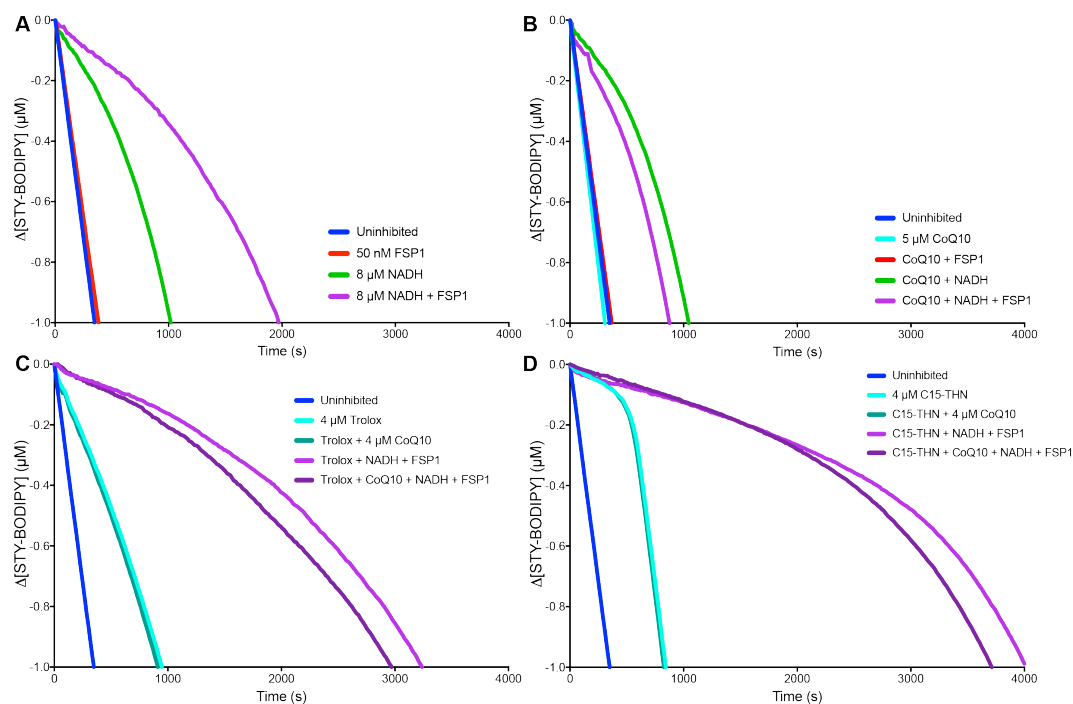


Figure S8.4 Co-oxidation of THF (3.1 M) and STY-BODIPY (10 μM) in phosphate buffer (100 mM, pH 7.4) at 37 °C (blue). The decrease in absorbance of STY-BODIPY is monitored over the course of the autoxidation at 562 nm ($\epsilon = 132,261 \text{ M}^{-1} \text{ cm}^{-1}$), which is initiated using AAPH (6 mM). Representative autoxidations inhibited by either 50 nM FSP1 (red), 8 μM NADH (green), or 50 nM FSP1 and 8 μM NADH (purple) (A). Analogous representative autoxidations inhibited to which CoQ₁₀ (B), Trolox and CoQ₁₀ (C) or C₁₅-THN and CoQ₁₀ (D) were added.

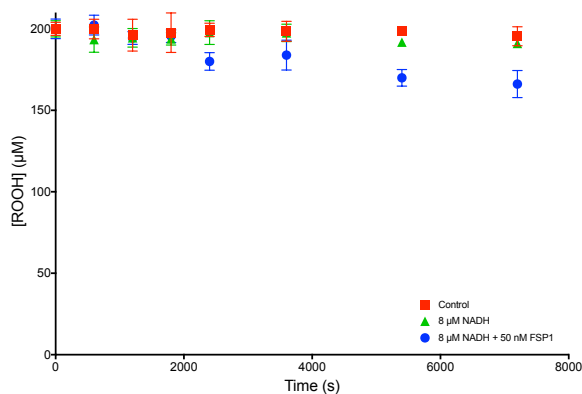


Figure S8.5 Decomposition of 200 μM oleic acid-hydroperoxide incorporated into 1 mM egg PC liposomes in the presence of 8 μM NADH or NADH and 50 nM FSP1.

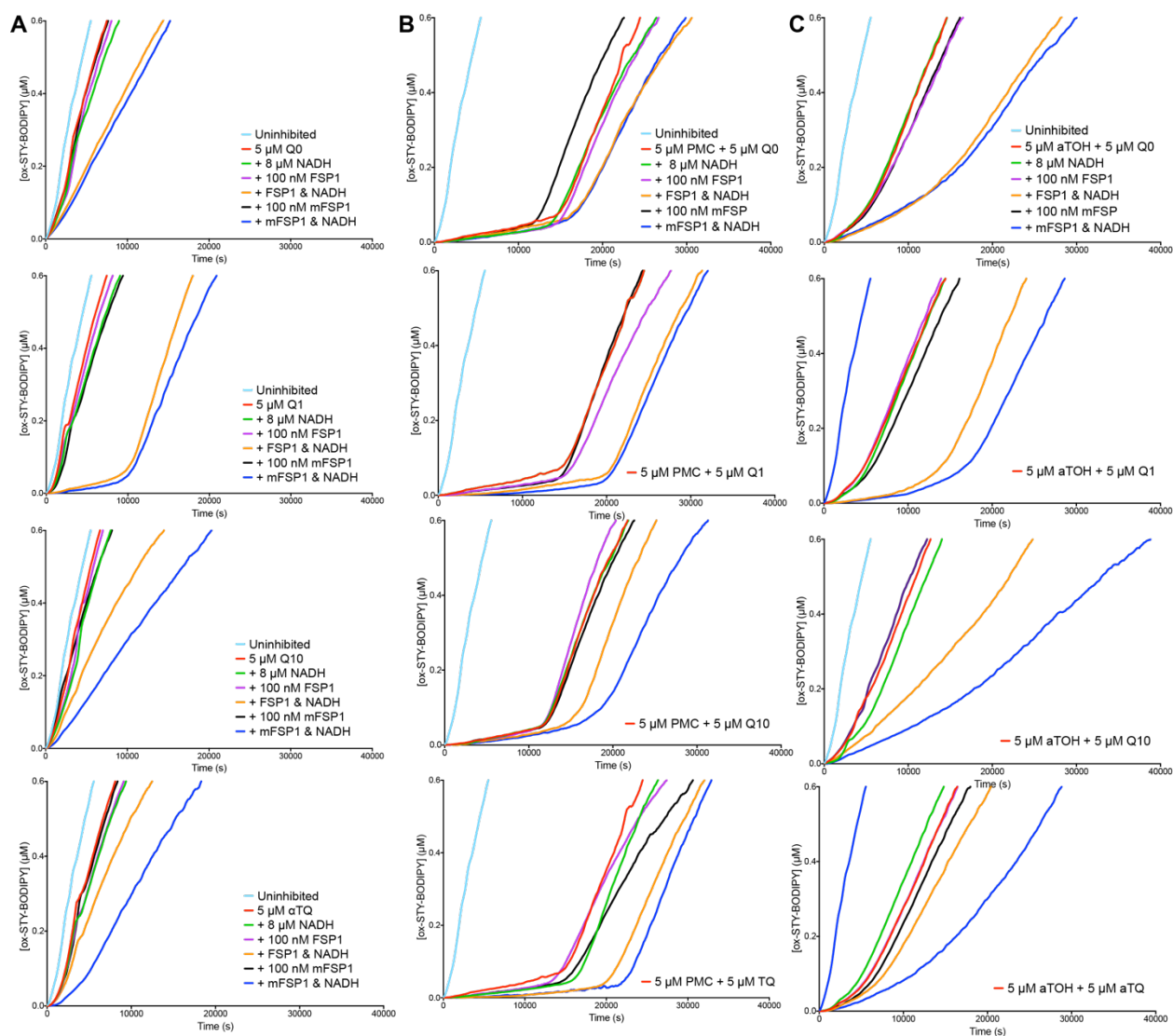


Figure S8.6 Co-autoxidations of STY-BODIPY (1 μM) and egg PC liposomes (1 mM). The increase in fluorescence of ox-STY-BODIPY is monitored over the course of the autoxidation, which is initiated using DTUN (0.2 mM). Representative autoxidations inhibited by either 5 μM Q₀, Q₁, Q₁₀ or αTQ (A) to which 8 μM NADH (green), 50 nM FSP1 (purple), 50 nM mFSP1 (black) or 50 nM FSP1 & 8 μM NADH (orange) and 50 nM mFSP1 & 8 μM NADH (blue) were added. Analogous representative autoxidations inhibited to which 5 μM PMC (B) or $\alpha\text{-TOH}$ (C) were added.

Chapter 9: Summary and Prospectus

This dissertation describes significant advances towards a better understanding of processes that govern autoxidation-mediated degradation and its inhibition for industrial and biological applications. The design of novel fluorescent probes (to detect product hydroperoxides and acids) enabled rapid acquisition of data and analysis of results^{1,2} to create structure-performance relationships for the next-generation radical-trapping antioxidants (RTAs) for use in high-temperatures environments.³ Not only did these compounds demonstrate significant potential for industrial use, some of them were also outstanding inhibitors of ferroptosis, a recently characterized oxidative cell-death process.^{4,5} Guided by fundamental knowledge and understanding of lipid autoxidation, its critical role in the execution of ferroptosis was uncovered.⁶ Moreover, to offer mechanistic insights on lipid peroxidation inhibitors, by measurement of their kinetics and prediction of their potencies in cell models of ferroptosis, we developed the fluorescence enabled inhibited autoxidation (FENIX) methodology.⁷ This assay was successfully applied to elucidate the mechanism of FSP1, a previously uncharacterized ferroptosis suppressing protein.⁸

The study of inhibited autoxidation typically requires precise instrumentation to quantify the concentrations of so called ‘degradation’ products, consisting primarily of alkyl hydroperoxides.⁹ Our group previously disclosed a pro-fluorescent phosphine probe to measure the levels of hydroperoxides in bulk solution.² The influence of secondary decomposition products such as acids and their role in attenuating the activity of heterocyclic RTAs led to the development of acid responsive probes in Chapter 2.^{1,3} Introduction of the simultaneous acid/hydroperoxide quantification system and a novel parallel stirred flow-reactor offers a unique ability to perform multiple autoxidations and their analysis in a facile manner. The dual probe approach circumvents laborious titrations frequently used to quantify hydroperoxides and acids, and was instrumental for sample analysis in Chapters 3 and 4, as well as several publications arising from our laboratory.^{1,3,10–12} The utility of this system was demonstrated by screening the efficacy of multiple aminic and phenolic RTAs as inhibitors of hydrocarbon autoxidation. The reaction conditions utilized to assess RTA performance in the laboratory are very simplistic and

consist of a RTA and an autoxidizable substrate. The implementation of this method allows assessment of various additives commonly used in fully formulated base-stocks in the oil and lubricant industry. Currently, the effects of several additives such as over-based detergents, anti-wear agents, viscosity modifiers and sulfurized olefins are being evaluated in different substrates for their ability to interfere or synergize with our designer RTAs. It is envisioned that the results obtained from these studies will aid in the identification of an excellent additive package to improve the longevity of oil and lubricant products. Moreover, these probes can also serve as reporters in cellular environments should measurements such as superoxide content or pH be necessary.

The effectiveness of highly reactive heterocyclic diarylamines in high-temperature applications was explored in Chapter 3. The majority of efforts towards potent diarylamines have relied on weakening the N-H bond, making the compounds susceptible to background oxidations. The heterocyclic analogs offer a compromise by increasing the oxidation potential without affecting the BDE, allowing for the addition of electron-donating groups to weaken the N-H bond without sacrificing compound stability.¹³ Unfortunately, some of the substituted pyridine- and pyrimidine-analogs based on the diphenylamine scaffold that were previously shown to be 200-fold more reactive at ambient temperatures did not fully translate to 160°C.^{3,14} After establishing a structure-performance relationship, the importance of achieving a balance between stability and reactivity was immediately clear. The negative effects of carboxylic acids produced in high-temperature autoxidations on the heterocyclic diarylamines RTAs were discovered and the use of a non-nucleophilic, non-participating base yielded to some of most efficacious compounds reported to date. The ‘lead’ compound identified through our efforts (**9.2**) inhibited autoxidation of a model hydrocarbon 12-fold better than the current industrial standard (**9.1**) and provided a scaffold for further optimization. Moreover, this compound was also superior in preventing oxidation of ‘real-world’ substrates such as base-oils (Figure 9.1).

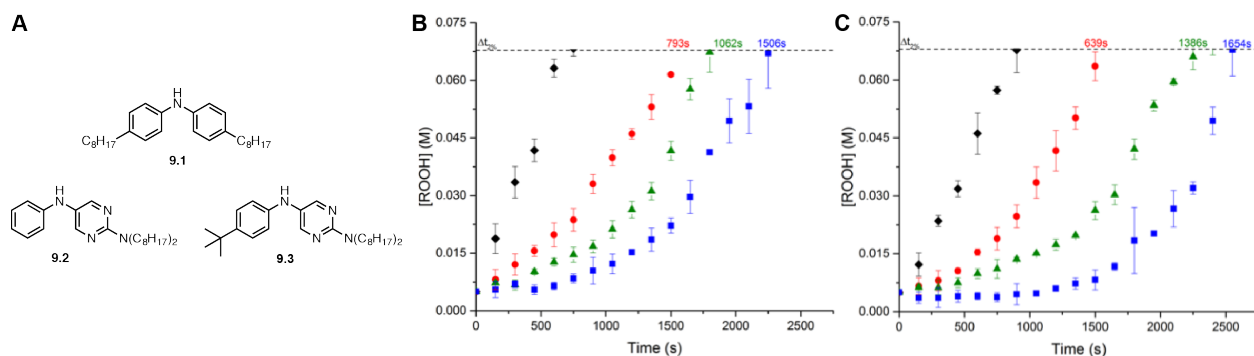
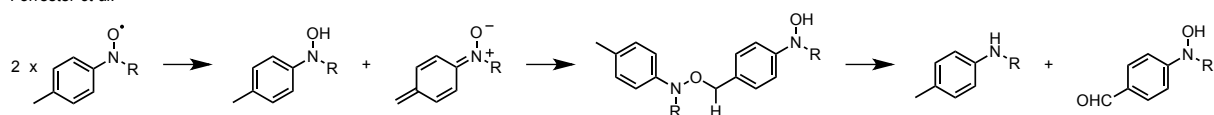


Figure 9.1 (A) Industrial standard (9.1), 'lead' compound (9.2) and its *tert*-butyl substituted analog (9.3). Inhibited Autoxidations of Group III (B) and IV (C) base stocks at 160°C initiated with tetralin hydroperoxide (5 mM) in the presence of 100 μ M of either 9.1 (●), 9.2 (▲), 9.3 (■) or no additive (◆) in the presence of 1 mM Primene 81-R.

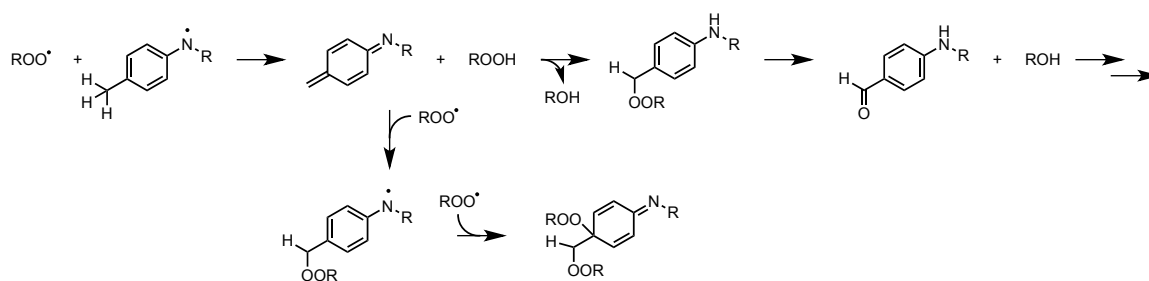
Unfortunately, the compound was not 12-fold better under conditions which were more similar to those under which they would see their greatest use (Figure 9.1B,C). As such, it was clear that a pathway existed through which diarylamines underwent decomposition.¹² The substitution of 9.2 with a *tert*-alkyl group in the *para* position resulted in improved inhibition times and computational investigation revealed that the reactions responsible for compound decomposition included the addition of peroxy radicals in the *ortho* or *para* position of the aromatic ring. This led to the design of the second-generation diarylamine RTAs in Chapter 4, where the benefits of non-tertiary alkyl substitution was discovered. These compounds exhibited regeneration as low as 37°C in styrene autoxidation, and which became more pronounced with increasing temperature. While the results with the new panel of diarylamines are remarkable, their catalytic mechanism is not immediately clear and requires further investigation. Previous work by Forrester *et al.* provided precedence on the participation of benzylic positions in the regeneration mechanism *via* the nitroxide intermediate to produce a hydroxylamine and an amine (Scheme 9.1A).¹⁵ Unfortunately, the low steady-state concentration of nitroxides preclude such self-reactions. Although similar reactions in the presence of peroxy radicals may be envisioned to yield intermediates with potential RTA activity (Scheme 9.1B). Future work includes probing potential pathways indirectly by deuteration of the benzylic position to ascertain whether H-atom transfers are involved in the regeneration reactions. In parallel, autoxidations on preparative scale need to be carried

out to analyze samples as a function of time by high pressure liquid chromatography (HPLC) coupled with mass spectrometric (MS) detection. It is anticipated that separation of the oxidation products and determination of their mass spectra will aid in understanding the fate of the RTA throughout the autoxidation. An attempt should be made to isolate the predominant products by preparative HPLC in order to unambiguously determine their structures. Furthermore, kinetics of the isolated intermediates (e.g. formylated amine, quinone imines and imine-N-oxides) towards peroxy radicals and their catalytic activity under different conditions should be evaluated.

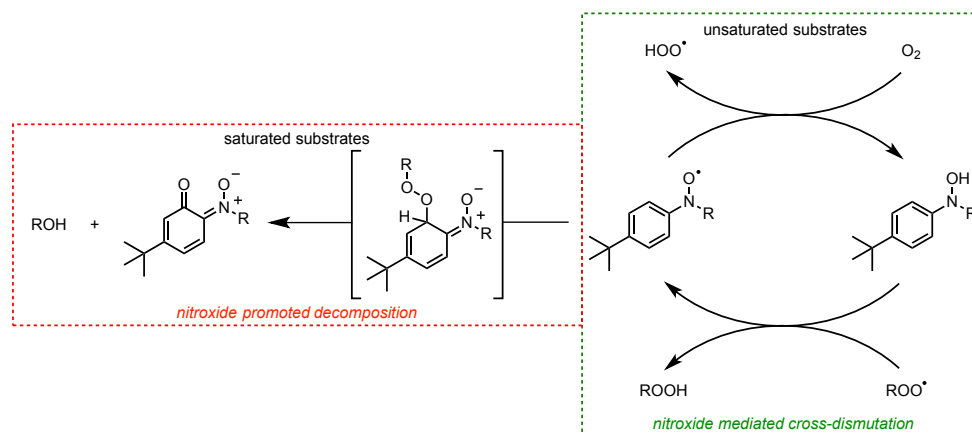
A Forrester *et al.*



B



C



Scheme 9.1 (A) Mechanism by Forrester *et al.* for the dismutation of nitroxides followed by a substitution-elimination reaction to generate an amine and a hydroxylamine. (B) Proposed reactions of the aminyl radical resulting from benzyl substituted aromatic amines to account for their catalytic regeneration. (C) The dual-faceted nature of nitroxides, catalytic regeneration in unsaturated hydrocarbons by the cross-dismutation of hydroperoxyl radicals and peroxy radical mediated decomposition in saturated substrates.

Simultaneous with compound optimization, the results in Chapter 4 provided several indirect mechanistic insights on the catalytic mechanism of diarylamines. The dual-functionality of nitroxides was realized for the first time, where their accumulation is advantageous in unsaturated substrates¹⁶ but provides a pathway for decomposition in saturated hydrocarbons (Scheme 9.1C). Therefore, future aminic RTAs should be designed to maintain a low steady-state concentration of nitroxide to ensure efficient regeneration in unsaturated substrates as well as provide protection from decomposition pathways in saturated ones. Overall, the discrepancy in inhibition times between benzylic and tertiary-alkyl substituted diarylamines along with the differences in their nitroxide yields suggest that they regenerate by separate mechanisms in saturated hydrocarbons.

The observations from Chapter 4 also brought the generally accepted Korcek cycle into question,¹⁷ which invokes the decomposition of an alkoxyamine as the rate-limiting step in the regeneration of diarylamines. Unfortunately, it overlooks simple kinetics where the low concentration of nitroxides (low μM) would not allow them to compete with oxygen ($\sim 2\text{ mM}$) for alkyl radicals. To understand this paradox, exploratory experiments were carried out with a model nitroxide, TBPNO, in the presence of an azo-initiator to monitor alkoxyamine formation and its subsequent decomposition (Figure 9.2A). TBPNO serves as an ideal surrogate for heterocyclic compounds that produce nitroxides in high-temperature autoxidations. Under an atmosphere of O_2 , low amounts of alkoxyamine (TBPNO R) was formed, suggesting that even at higher concentrations (2 mM) nitroxides can't outcompete oxygen for alkyl radicals. The concentration of TBPNO R also did not increase significantly under inert conditions. Similarly, the concentration of TBPNO H appeared to be independent of the reaction conditions, which is surprising given the tendency of the amine to react with initiator derived peroxy radicals. The results are difficult to reconcile because alkoxyamine decomposition is relatively slow at 80°C ($k_{\text{dec}} = 2.5 \times 10^{-6}\text{ s}^{-1}$, Figure 9.2B)¹² and production of $\sim 400\text{ }\mu\text{M}$ TBPNO H would necessitate the decomposition of $\sim 12\text{ mM}$ TBPNO R over the time scale of the reaction. It should be noted that in all instances the mass balance of the reaction was not constant, which is consistent with the appearance of multiple unidentified products

on the HPLC traces. Control experiments in the absence of azo-initiator revealed that TBPNO alone decomposed to yield amine without the intervention of alkoxyamine by an unknown mechanism. Moreover, the faster rate of nitroxide loss in the absence of an initiator suggests some form of interaction with the peroxy radicals, which is not immediately clear. Forrester *et al.* had provided a mechanism whereby aromatic nitroxides react together to yield quinone-imine-N-oxides and aromatic amines¹⁸ (Figure 9.2C). However, a deuterated analog of TBPNO also decomposed at similar rates (Figure 9.2D, KIE = 1.4), suggesting that the reaction does not proceed through this pathway. Instead, N-O homolysis followed by in-cage disproportionation could occur, but this is essentially what is invoked in the Korcek mechanism, and which we have deemed to be too slow to compete at this temperature (*vide supra*). Further investigations under relevant conditions (low steady state nitroxide concentration relative to peroxy radicals) are underway to fully elucidate this mechanism.

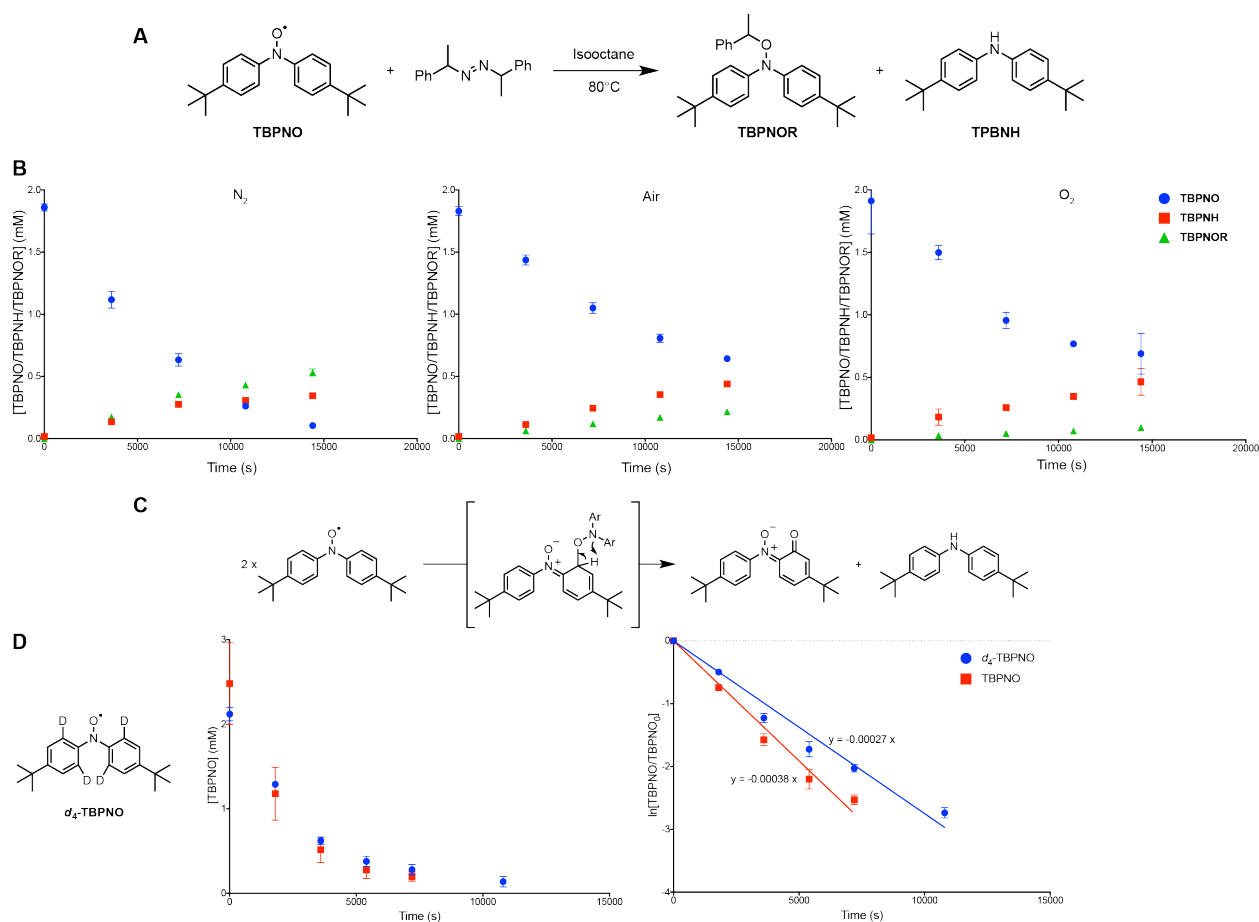


Figure 9.2 (A) Reaction scheme of TBPNO (2 mM) with 2,2'-azobis(2-phenylethane) (1 mM) in isooctane at 80°C. The reaction was carried out to probe whether nitroxides can compete with oxygen to produce TBPNO. (B) HPLC analysis of products from reaction in (A) under atmosphere of N₂, air or O₂. Very little alkoxyamine was formed, even under N₂ where it is the expected product. (C) Proposed mechanism of amine regeneration by Forrester *et al.*¹⁸ (D) HPLC analysis of the self-reaction between 2 mM TBPNO or *d*₄-TPBNO at 80°C in isooctane reveals no difference in rate constants.

Oxidation of nature's hydrocarbons (lipids) has been subject to many studies aiming to understand their role in various pathologies.¹⁹ High levels of lipid hydroperoxides and their downstream aldehydes have been assumed to play a role in cardiovascular and neurodegenerative diseases.²⁰ However, whether lipid peroxidation is a cause or consequence of disease remains unclear. Interest into the mechanisms of lipid peroxidation has regained momentum with the discovery that it is a hallmark of ferroptosis, a regulated cell-death modality distinct from other well-recognized cell-death routines.^{4,21–23} There has been much confusion regarding the mode of lipid peroxidation in the ferroptotic cell death process. Several studies cite the decisive role of polyunsaturated fatty acid dioxygenases (lipoxygenases or LOX) in the execution mechanism of cell death, largely brought about by using so-called "lipoxygenase-specific" inhibitors.^{24–29} But the results are difficult to rationalize in light that cell-lines with extremely low LOX expression still undergo cell death. As shown in Chapter 5, stable cell-lines overexpressing different LOX-isoforms simply sensitized cells to ferroptosis by 'seeding' the lipid autoxidation process and providing hydroperoxides for initiation. However, it was ultimately the uncontrolled lipid autoxidation that drove the cell-death process. Moreover, it was established that common LOX-specific inhibitors were non-specific RTAs, which may have been responsible for the incorrect conclusions regarding their involvement in ferroptosis.⁶ Inhibition of lipid autoxidation is currently the most effective strategy to prevent ferroptosis in both cells and animal models of disease.^{22,23} Understanding the underlying execution mechanism also enables rational design of ferroptosis inhibitors. Incidentally, common RTA additives in oils, lubricants and rubbers are excellent inhibitors (equal to or better than the archetype inhibitors Lip-1 and Fer-1 discovered by high-throughput screening) as evidenced by potencies of heterocyclic diarylamines (Chapter 6) where ferroptosis was induced using

different mechanisms.⁵ While a lot of data has been gathered with HEK293 and MEF cell lines, what remains to be seen is whether our findings translate to other cell models. As such, additional cell lines need to be evaluated to observe whether the role of autoxidation is generalizable. The results need to be bolstered by using LOX inhibitors devoid of RTA activity and measuring their efficacy in these cell lines. In order to conclusively support the ideas, a correlation between critical hydroperoxide concentration, LOX expression and potencies of RTA/non-RTA compounds using different ferroptosis inducers need to be constructed. This would be the first step in predicting susceptibility of different cell lines towards ferroptosis, especially in pathologies where ferroptosis is implicated and would benefit from therapeutic intervention. Work in this direction is well underway in the group by fellow graduate students Katie Shirley and Spencer Short.

New biochemical pathways in ferroptosis signaling and regulation are constantly being discovered and elucidation of its execution mechanism will certainly further our understanding of the cell-death process. It is accepted that (phospho)lipid hydroperoxides lead to cell-death, but what still remains elusive is the biophysical changes that occur upon hydroperoxide accumulation. Molecular dynamics simulations have suggested that the hydroperoxide content increases the membrane curvature, leading to increased accessibility by oxidants, eventually forming membrane pores and causing micellization.³⁰ Experimental evidence is necessary to corroborate these observations, but are difficult to execute in a cellular model. A liposomal system was envisioned to tackle this problem, where lipid bilayers loaded with a self-quenching fluorophore would be oxidized and the rate of dye release correlated to peroxide content to determine the critical hydroperoxide threshold required for membrane destabilization (Figure 9.3A,B). Primary experiments were carried in conjunction with Honours student Aidan Pezacki. Carboxyfluorescein release was evident by the increase in fluorescence due to its dilution upon leakage from the liposomes (Figure 9.3C). Moreover, there was an excellent concentration dependence on the initiator concentration and the rate of dye release (Figure 9.3D). The peroxide content in liposome was measured using the phosphine-conjugated coumarin and also correlated well with the initiator

concentration (Figure 9.3E). The preliminary results establish that higher rates of oxidation and hydroperoxide formation lead to faster membrane permeability (Figure 9.3F). Future work in this area requires determining a relationship between hydroperoxide concentration and the onset of membrane pore formation. As evidenced by the raw data, it can be seen that the dye release appears to be biphasic. There is a slow rate of dye release, followed by an exponential increase which plateaus when all fluorophore is released. The data implies that once a critical threshold of hydroperoxide is reached, dye release is independent of oxidation due to the leakiness of the membrane. Of course, further validation is necessary, but these experiments provide an excellent foundation for future investigations. The findings from the liposomal model will be translated to a cellular environment and membrane impermeable fluorophores will be used to track permeability as a function of hydroperoxide concentration.

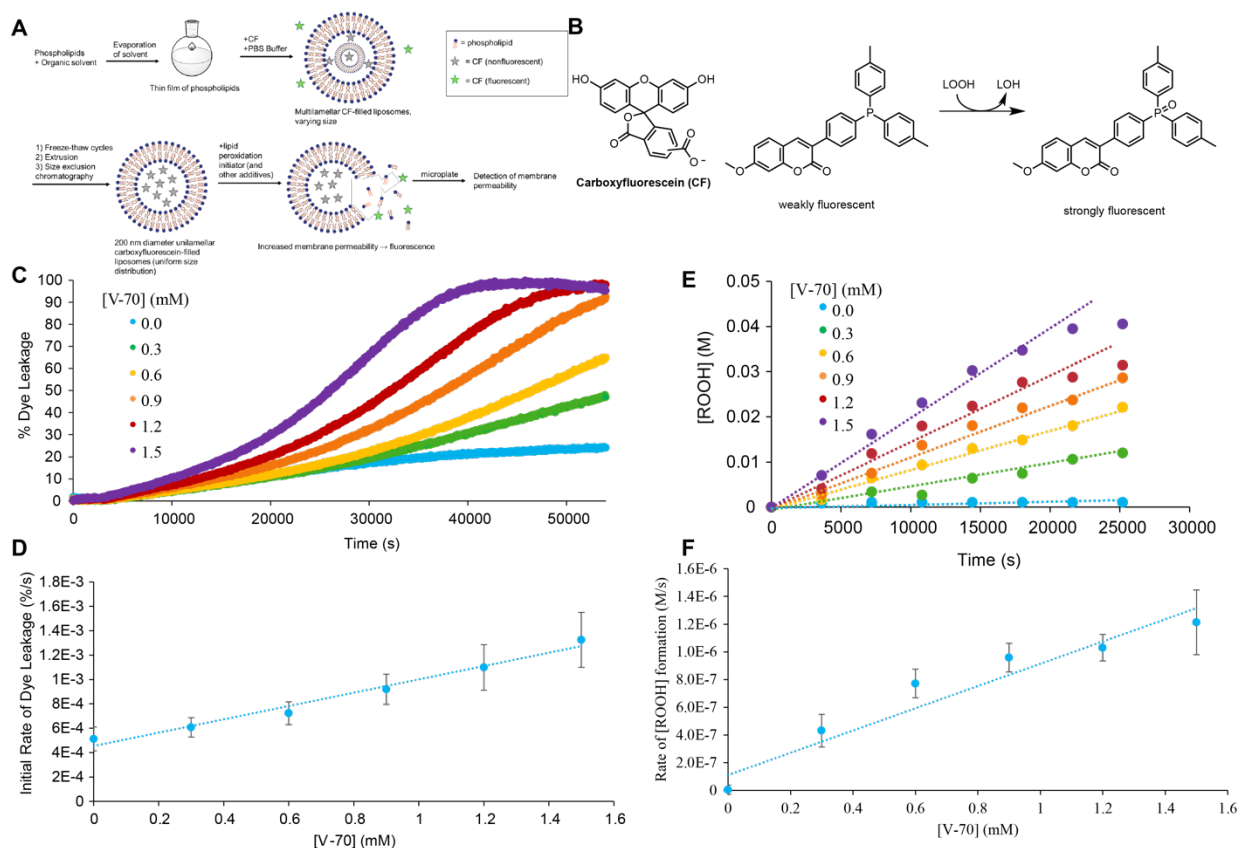


Figure 9.3 (A) Protocol for oxidation induced dye-leakage model of egg PC liposomes. (B) Carboxyfluorescein (CF), a self-quenching membrane impermeable dye that is loaded into the liposomes

and the coumarin-phosphine conjugate utilized to measure hydroperoxides in liposomal samples. (C) Percentage of dye leakage from liposomes as a function of MeOAMVN concentration over time (D) The initial rates of dye leakage plotted as a function of initiator concentration. (E) Hydroperoxide formation in egg PC liposomes over time when autoxidation is initiated with different concentrations of MeOAMVN (F) Rate of hydroperoxide formation as a function of initiator concentration.

The majority of work in this thesis underscores the importance of carrying out inhibited autoxidations to inform on important parameters of RTA efficacy such as their inherent reactivity towards peroxy radicals (k_{inh}) and the number of radicals they trap (n). While several rigorous methods such as oxygen consumption and HPLC analysis of methyl linoleate autoxidations already exist in the chemical literature, they are often carried out in isotropic organic solutions.^{31–33} The recently developed co-autoxidation approach by our laboratory offers a spectrophotometric variant where a small amount of probe is added to autoxidation and enables derivation of kinetic parameters for RTA activity. It was applied to organic solutions,^{16,34–36} aqueous buffers³⁷ and model liposomal membranes.^{5,6,37,38} This technique was indispensable in elucidating the mechanism of Lip-1 and Fer-1, as well as the role of LOX-inhibitors as RTAs. However, this methodology has not been adopted beyond our laboratory. The fields of lipid peroxidation, redox biology, food and agriculture additives routinely carry out assessments of antioxidant efficacy, but prefer simple assays that can be purchased as ready-to-use kits and carried out in multi-well plates.³⁹ In order to facilitate the wide-spread use of our assay and characterize lipid peroxidation inhibitors we adopted it to a microplate reader. As outlined in Chapter 7, the fluorescence enabled inhibited autoxidation (FENIX) is a simple to use protocol that is amenable to high-throughput screening, and allows for the characterization of novel RTAs (in multi-well plates) without compromising on the kinetic data it provides. Moreover, a lipophilic hyponitrite initiator has enabled the development of a correlation between k_{inh} obtained in liposomes and efficacy of RTAs as ferroptosis inhibitors. The simplicity and precision of FENIX should allow users to easily measure kinetics and stoichiometry, obtain mechanistic insights and counter screen inhibitors to further validate hits. An additional advantage of FENIX is the ability to carry out parallel experiments in H₂O and D₂O buffers to verify whether H-atom transfer from an inhibitor is involved in the rate-limiting step (Figure 9.4A).

FENIX was also applied to counter-screen ~ 650 ferroptosis inhibitors identified from a phenotypic screen of 40,000 compounds. Autoxidations in egg PC liposomes enabled the separation of compounds into RTA and non-RTA classes. Arguably, the differentiation of non-RTA compounds is more interesting as they act on pathways independent from lipid peroxidation, where their mechanism of action might provide novel insights into other ferroptosis modulators. Several potent compounds with unexpected RTA activity (non-classical scaffolds) were also identified (Figure 9.4B). Conventional inhibitors included analogs of Lip-1 (**9.4**), dihydroperimidines (**9.5**) and diarylamino-thiazoles (X = S, **9.6**). Of these, several compounds derived from **9.6** are being evaluated for their *in vivo* efficacy towards ferroptosis related pathologies. Non-classical derivatives of the edaravone (**9.7**) family are FDA approved drugs (for the treatment of ALS and recovery following stroke) with purported antioxidant activity which needs further validation. It is anticipated that the efforts towards the design and identification of novel inhibitors will be applicable in various models of oxidative cell-death.

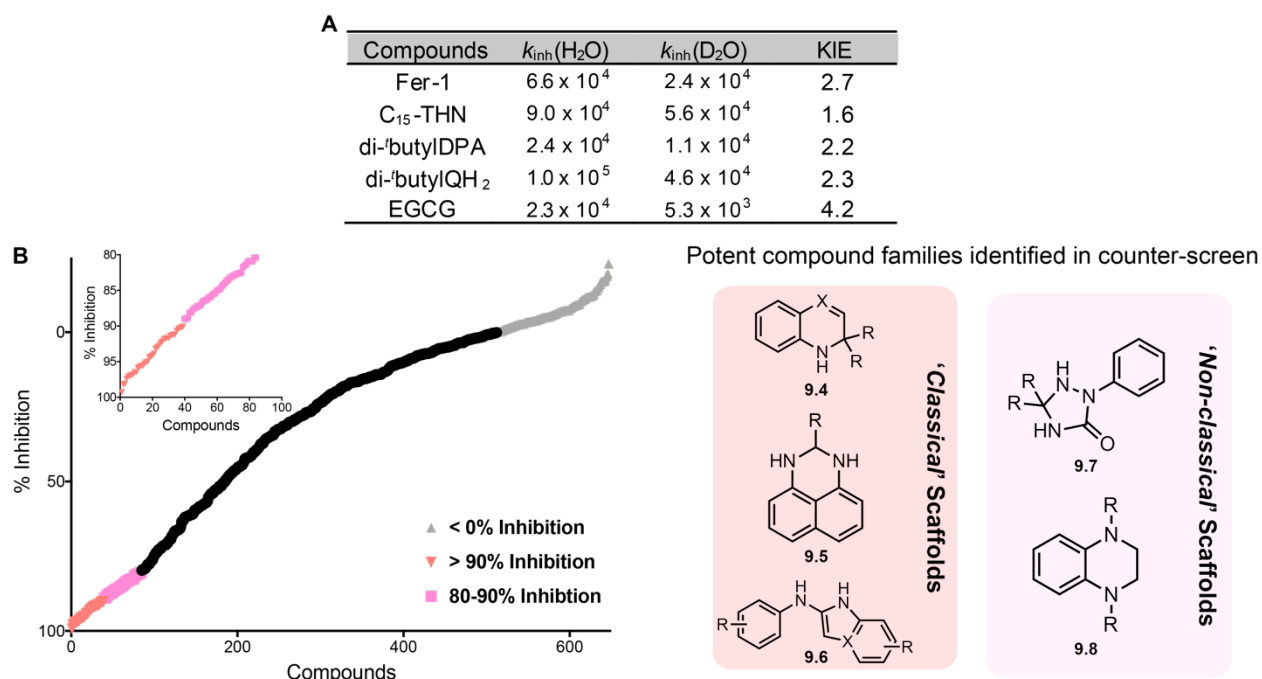


Figure 9.4 (A) Rate constants of inhibition obtained in H₂O and D₂O buffers in egg PC liposome autoxidation with various inhibitors. The KIE is calculated as a ratio of $k_{\text{H}}/k_{\text{D}}$. (B) Counter-screen of 647 ‘ferroptosis inhibitors’ identified from a library of 40,000 compounds for their RTA activity. Percent

inhibition is calculated as a ratio of their initial rate relative to the uninhibited control. Scaffolds of some highly potent compounds with RTA activity identified from the screen.

The versatility of FENIX was further utilized to characterize a protein shown to confer cytoprotection from ferroptosis. The protein, now denoted as ferroptosis suppressor protein-1 (FSP1), is a NAD(P)H:oxidoreductase that regenerates endogenous lipophilic antioxidants to inhibit lipid peroxidation and associated accumulation of hydroperoxides (Chapter 8). Moreover, using model lipid membranes it was shown that myristoylation of the native protein is critical for its function, and abolition of the myristylation motif requires the use of non-lipophilic RTAs or quinones to obtain the same level of protection. The discovery of an inherent mechanism able to regenerate RTAs introduces yet another aspect when considering the design of inhibitors: regeneratability. The model system of (m)FSP1 and egg PC liposomes can be employed to identify such compounds, where compound regeneration is evaluated simultaneously with their inherent RTA activity. While the work outlined in this thesis has focused on ferroptosis inhibition, the analytical tools can also be applied towards the identification of ferroptosis sensitizers. Recent reports on the vulnerability of therapy-resistant cancer cells on the lipid peroxidation pathway and the tendency of certain cells to overexpress FSP1 affords an excellent opportunity to explore these avenues. Both FENIX and the coumarin-ubiquinone probe can be used to screen FSP1 inhibitors in order to suppress the antioxidant regeneration machinery and synergize with GPX4 inhibition. Moreover, the varying genetic and protein expression in different cells, and the rapid accessibility to genetic screening approaches (such as CRISPR/Cas9 or RNASeq) will ultimately guide future discoveries of other regulators that protect from ferroptosis. The adaptability of FENIX should enable elucidation of the mechanism of action of such ‘anti-ferroptosis’ gene products.

Overall, it is anticipated that the analytical tools disclosed in this dissertation will facilitate the development of novel RTAs both in academic and industrial laboratories worldwide. Insights from the structure-performance relationships on heterocyclic diarylamines provide a clear path for further optimization to develop extremely potent compounds. The elucidation of the execution mechanism of

ferroptosis and development of FENIX will accelerate studies of lipid peroxidation and the discovery of inhibitors in pathologies where it is implicated.

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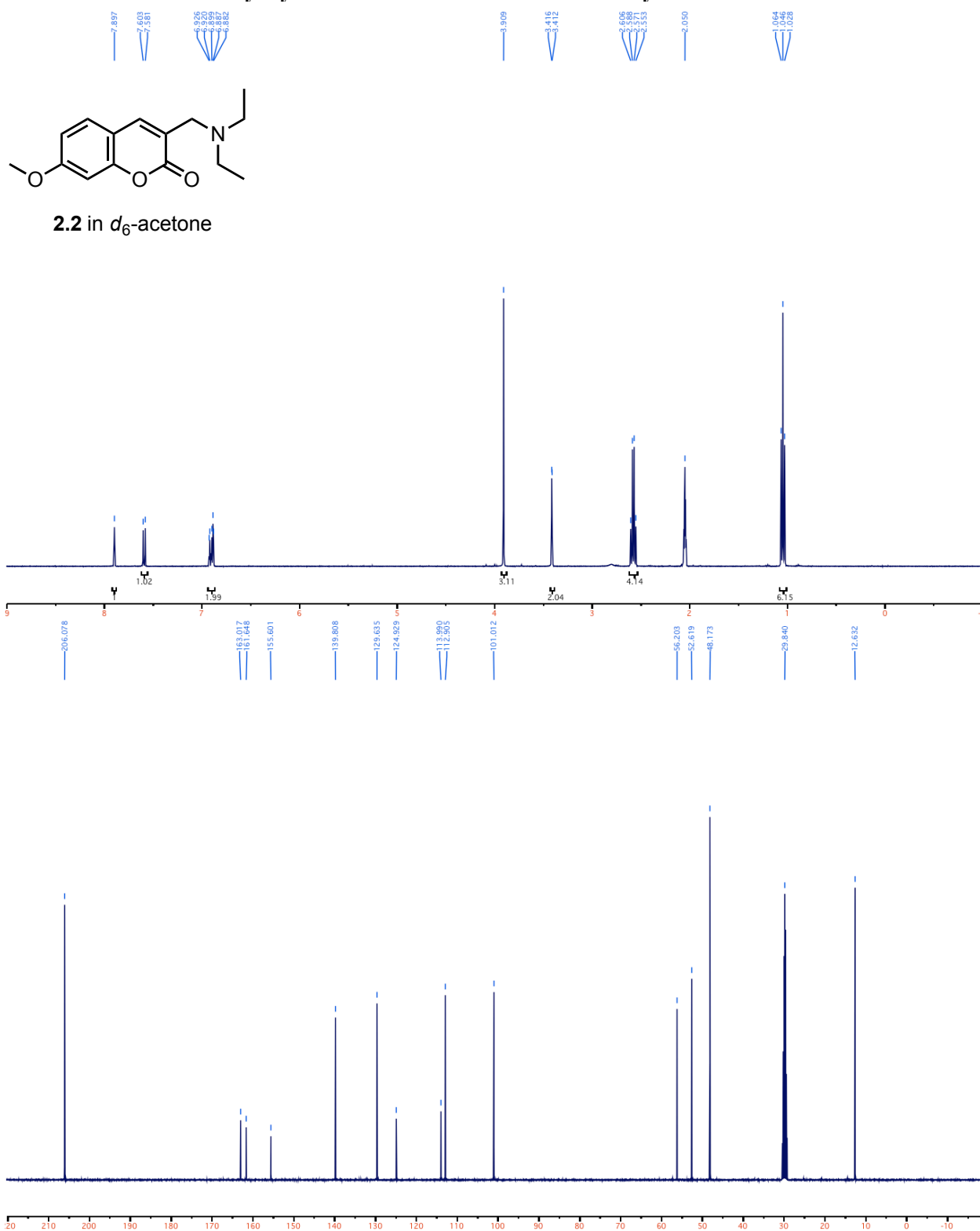
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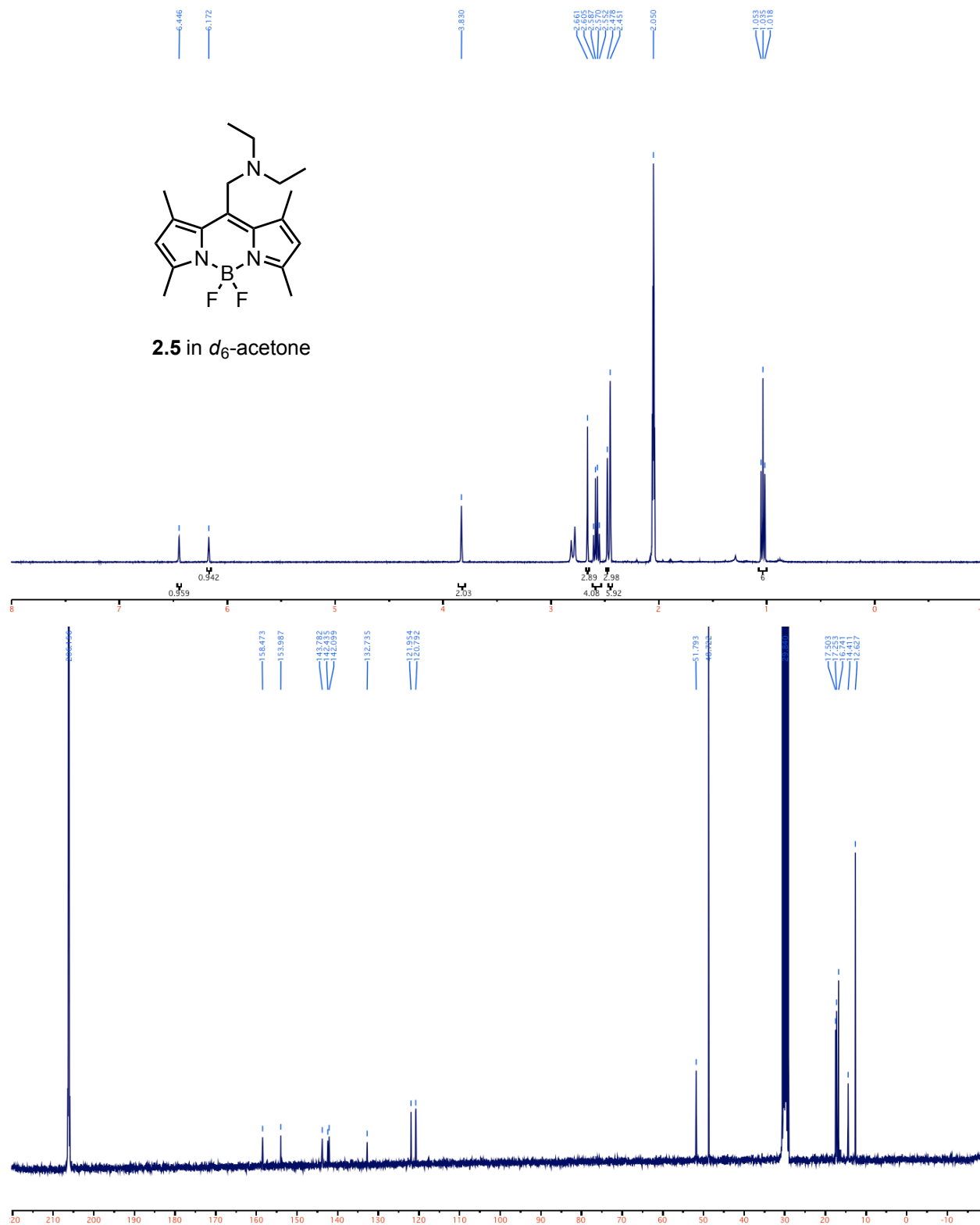
Appendix A: Publication List

1. **The Catalytic Mechanism of Diarylamine Radical-Trapping Antioxidants**
Evan A. Haidasz, Ron Shah and Derek A. Pratt. *Journal of the American Chemical Society* **2014**, 136, 16643 (featured in *JACS* spotlight)
2. **Unprecedented Inhibition of Hydrocarbon Autoxidation by Diarylamine Radical-Trapping Antioxidants.**
Ron Shah, Evan A. Haidasz, Luca Valgimigli and Derek A. Pratt. *Journal of the American Chemical Society* **2015**, 137, 2440 (featured in *JACS* spotlight)
3. **Rapid Determination of Key Hydrocarbon Autoxidation Products by Fluorescence.**
Ron Shah, and Derek A. Pratt. *Journal of Organic Chemistry* **2016**, 81, 6649
4. **On the Mechanism of Cytoprotection by Ferrostatin-1 and Liproxstatin-1 and the Role of Lipid Peroxidation in Ferroptotic Cell Death.**
Omkar Zilka, Ron Shah, Bo Li, José Pedro Friedmann Angeli, Markus Griesser, Marcus Conrad and Derek A. Pratt. *ACS Central Science* **2017**, 3, 232 (highlighted in *Cell Chemical Biology*)
5. **Ferroptosis Inhibition: Mechanisms and Opportunities.**
José Pedro Friedmann Angeli, Ron Shah, Derek A. Pratt and Marcus Conrad. *Trends in Pharmacological Sciences* **2017**, 38, 489
6. **The Potency of Diarylamine Radical-Trapping Antioxidants as Inhibitors of Ferroptosis Underscores the Role of Autoxidation in the Mechanism of Cell Death.**
Ron Shah, Kaitlyn Margison and Derek A. Pratt. *ACS Chemical Biology* **2017**, 12, 2538
7. **Resolving the Role of Lipoxygenases in the Initiation and Execution of Ferroptosis.**
Ron Shah, Mikhail S. Shchepinov and Derek A. Pratt. *ACS Central Science*, **2018**, 4, 387 (selected for a *ACS Central Science* First Reactions)
8. **The Catalytic Reaction of Nitroxides with Peroxyl Radicals and its Relevance to their Cytoprotective Properties.**
Markus Griesser, Ron Shah, Antonius T. Van Kessel, Omkar Zilka, Evan A. Haidasz, Derek A. Pratt. *Journal of the American Chemical Society* **2018**, 140, 3798
9. **FSP1 is a glutathione-independent ferroptosis suppressor**
Sebastian Doll, Florencio Porto Freitas, Ron Shah, Maceler Aldrovandi, Milene Costa Silva, Irina Ingold, Andrea Goya Grocin, Thamara Nishida Xavier da Silva, Elena Panzilius, Christina Scheel, André Mourão, Katalin Buday, Mami Sato, Jonas Wanninger, Thibaut Vignane, Vaishnavi Mohana, Markus Rehberg, Andrew Flatley, Aloys Schepers, Andreas Kurz, Daniel White, Markus Sauer, Michael Sattler, Edward William Tate, Werner Schmitz, Almut Schulze, Valerie O'Donnel, Bettina Proneth, Grzegorz M. Popowicz, Derek Pratt, José Pedro Friedmann Angeli, Marcus Conrad. *Nature* **2019**, Accepted (doi:10.1038/s41586-019-1707-0) (highlighted in News & Views)
10. **Peroxyl Radical-Trapping Kinetics of Inhibitors of Phospholipid Peroxidation Enables Prediction of Oxidative Cell Death Rescue**
Ron Shah, Luke A. Farmer, Omkar Zilka, Antonius T. M. Van Kessel and Derek A. Pratt. *Cell Chemical Biology* **2019**, 26, 1 (highlighted in *Cell Chemical Biology* Perspective)

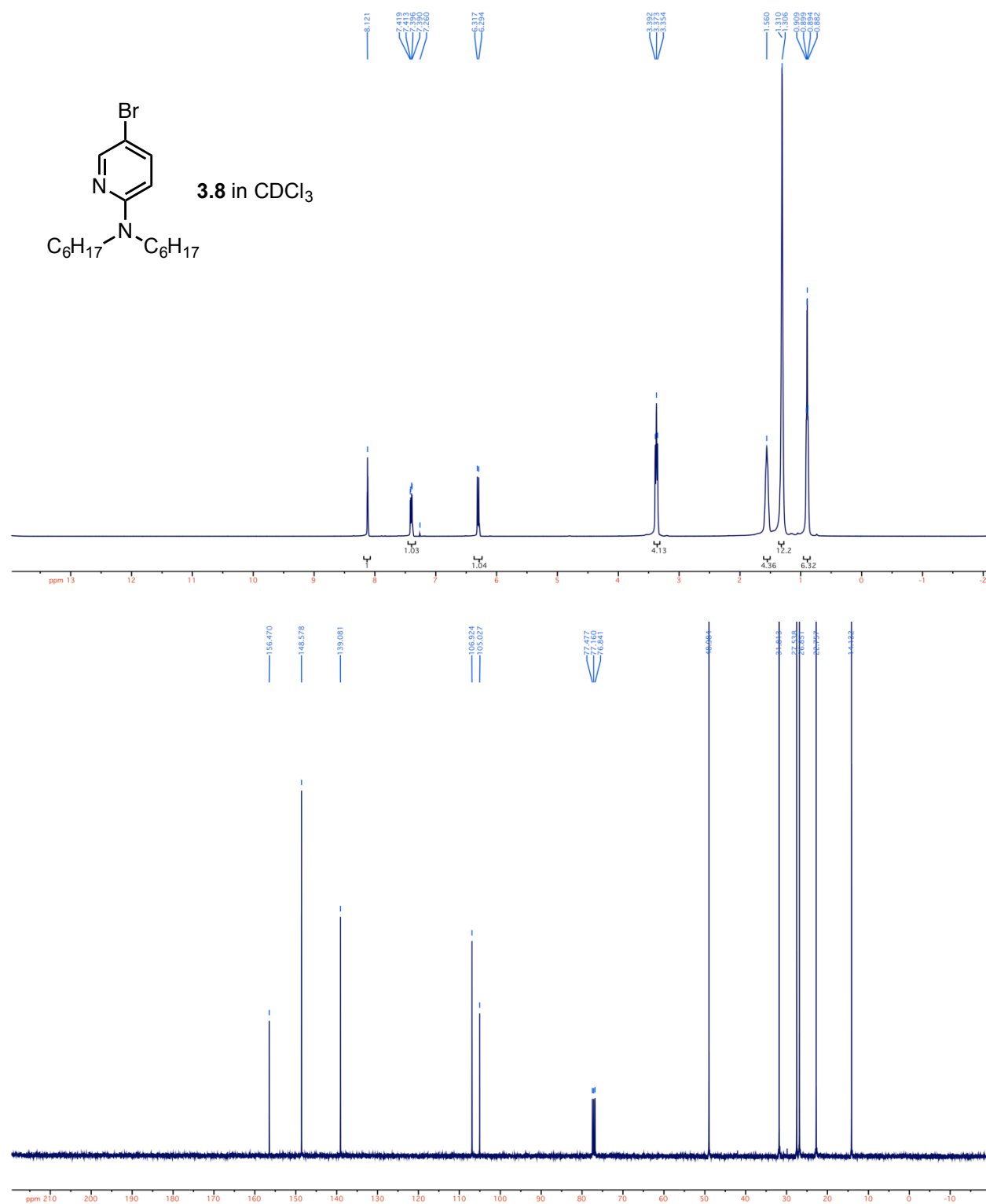
Appendix B: NMR Spectra

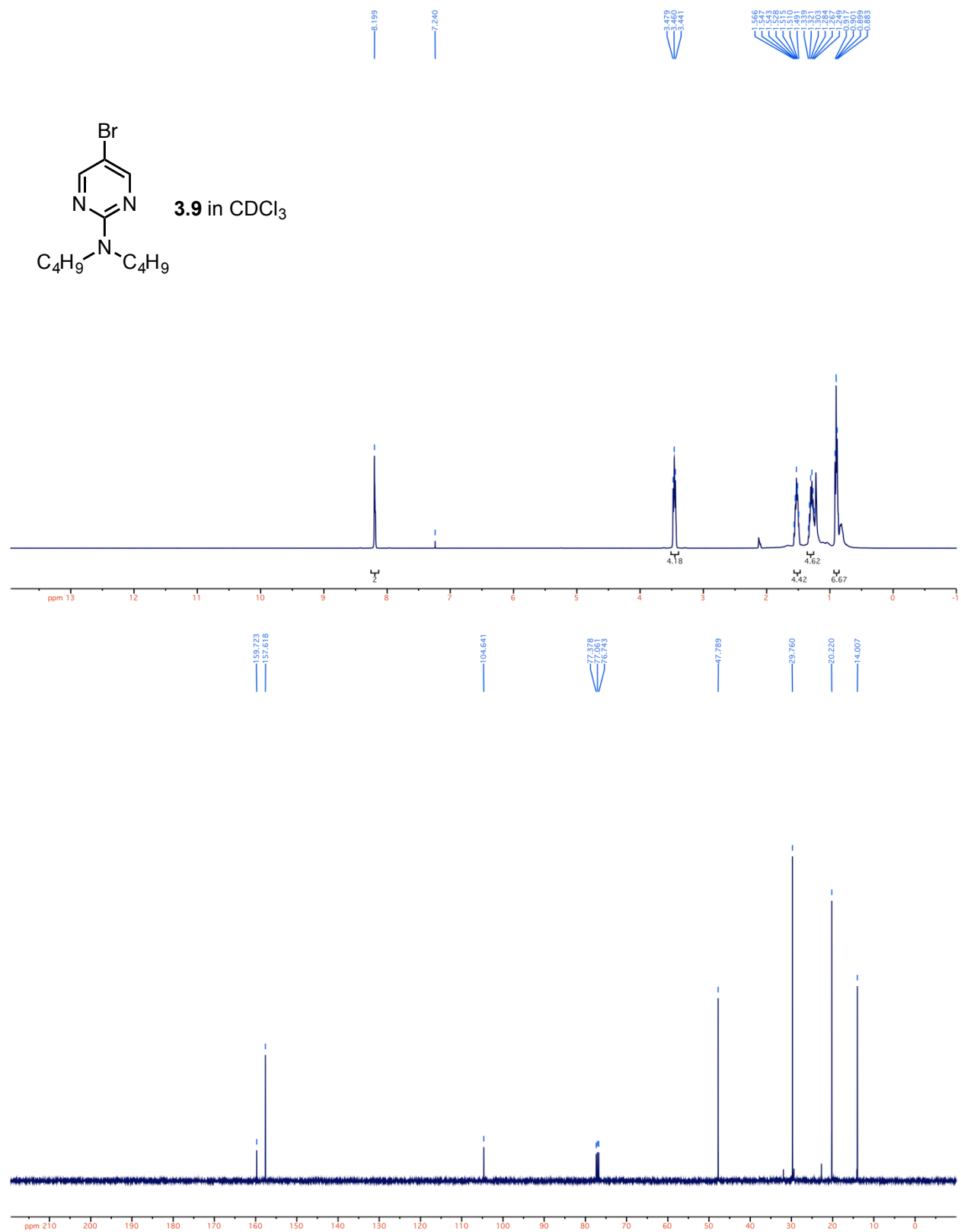
B.2 Determination of Key Hydrocarbon Autoxidation Products by Fluorescence

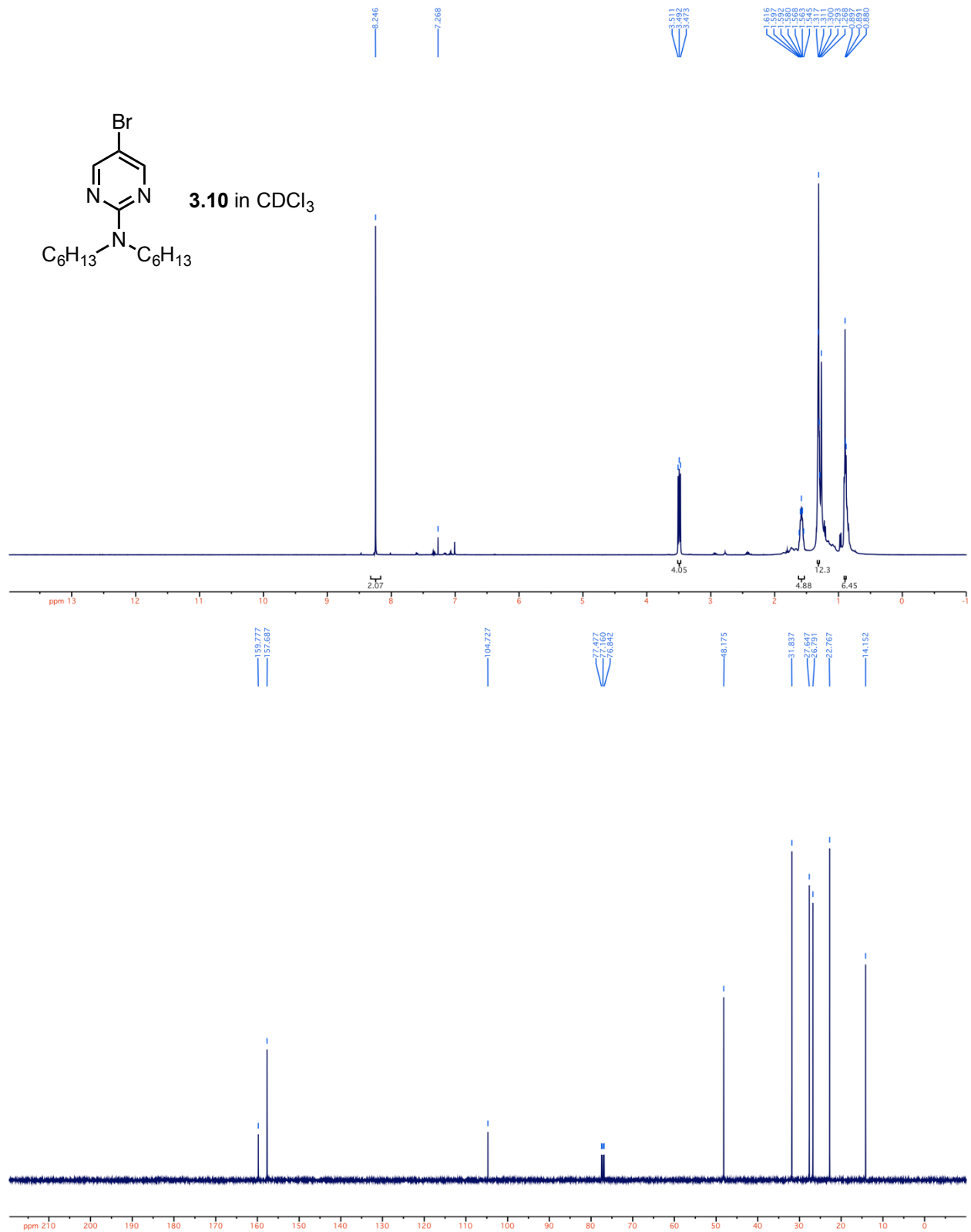


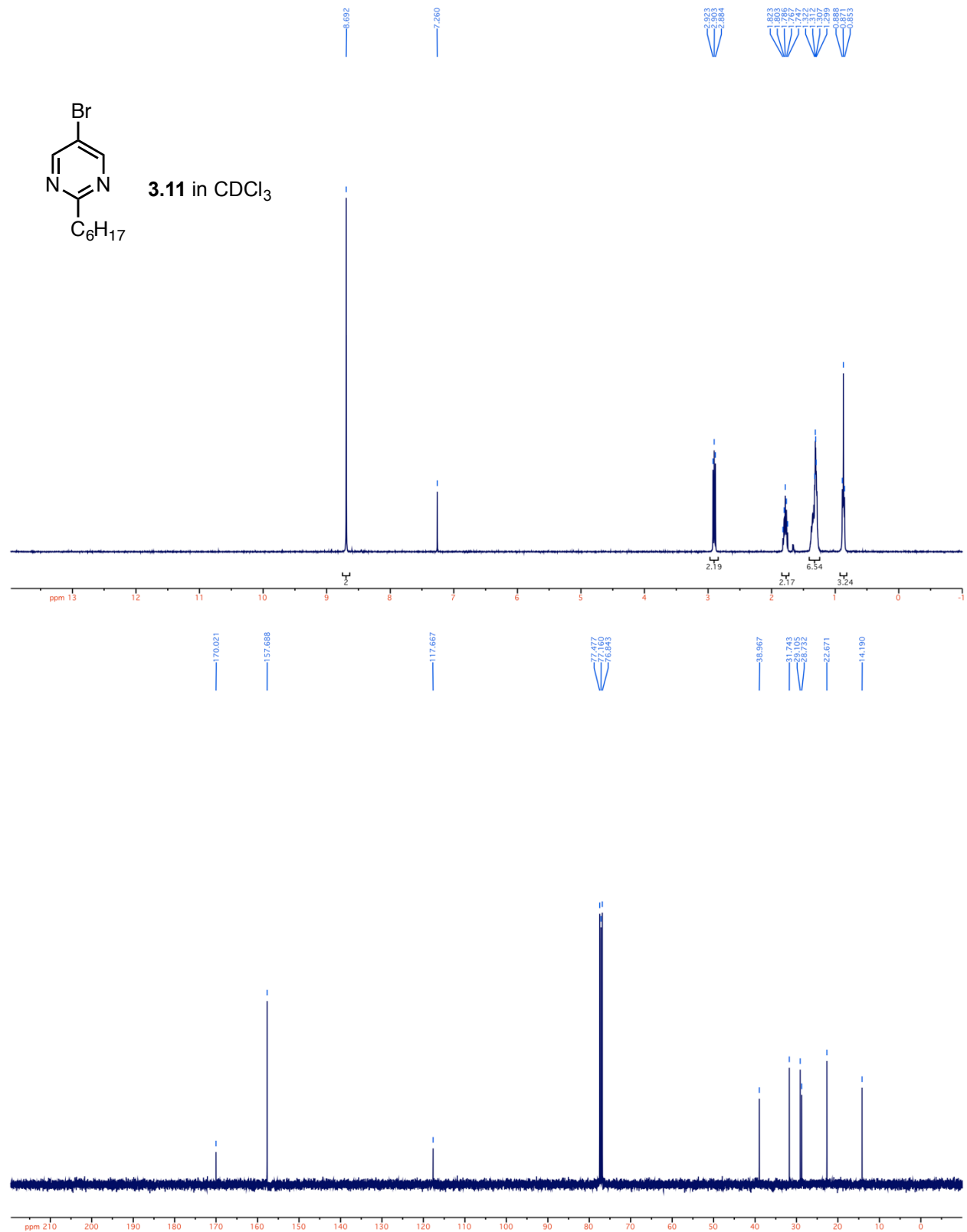


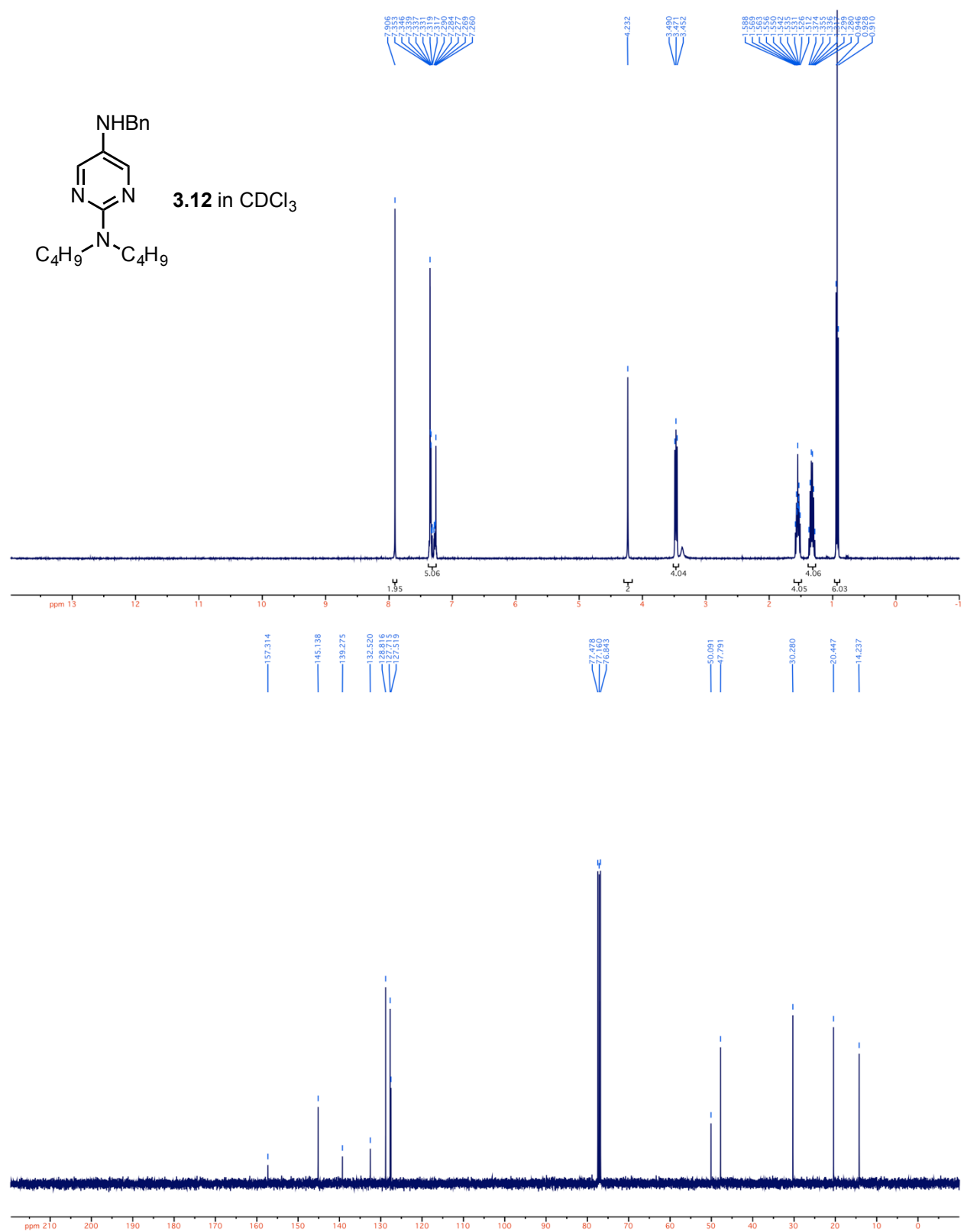
B.3 Unprecedented Inhibition of Hydrocarbon Autoxidation by Diarylamine Radical-Trapping Antioxidants

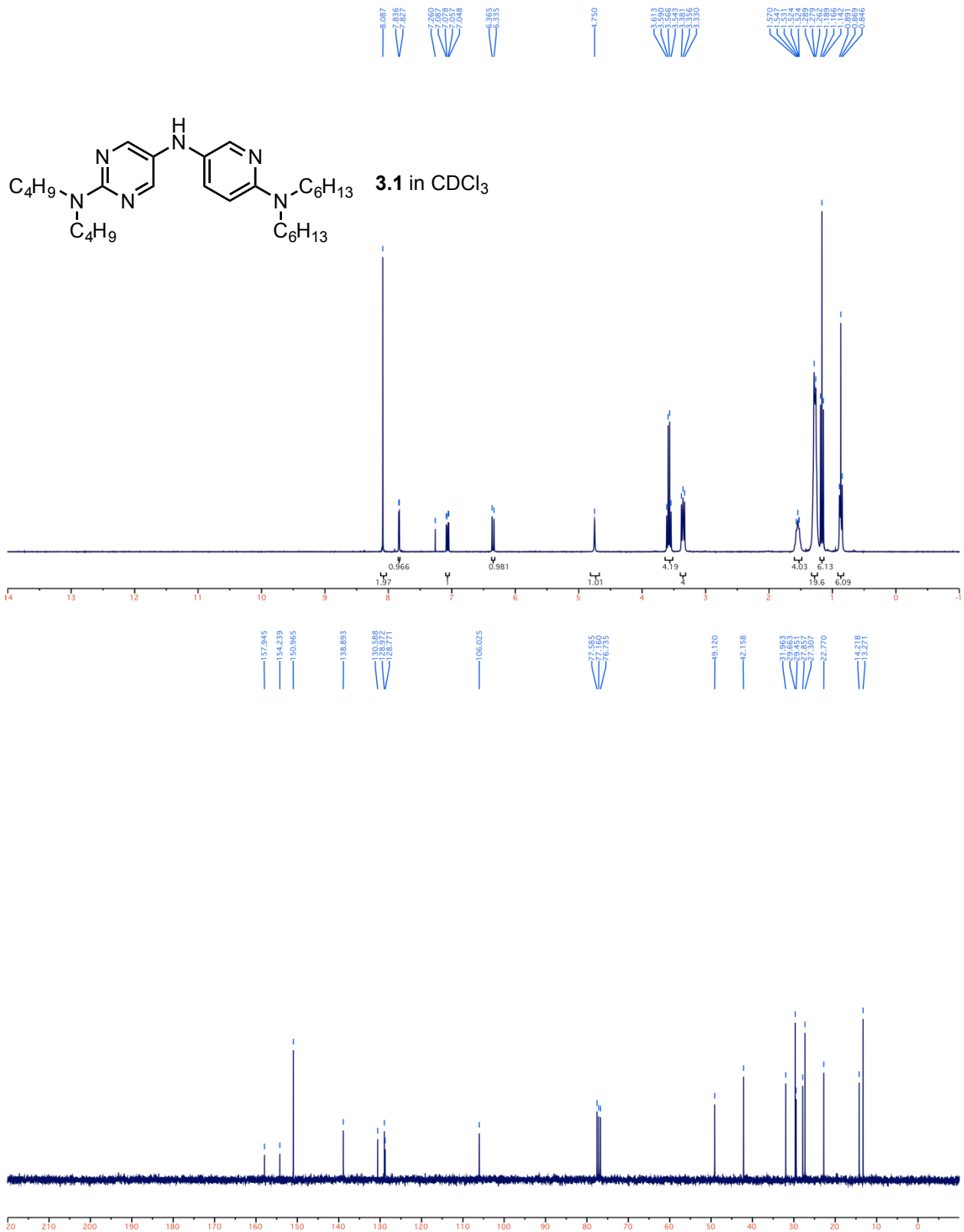


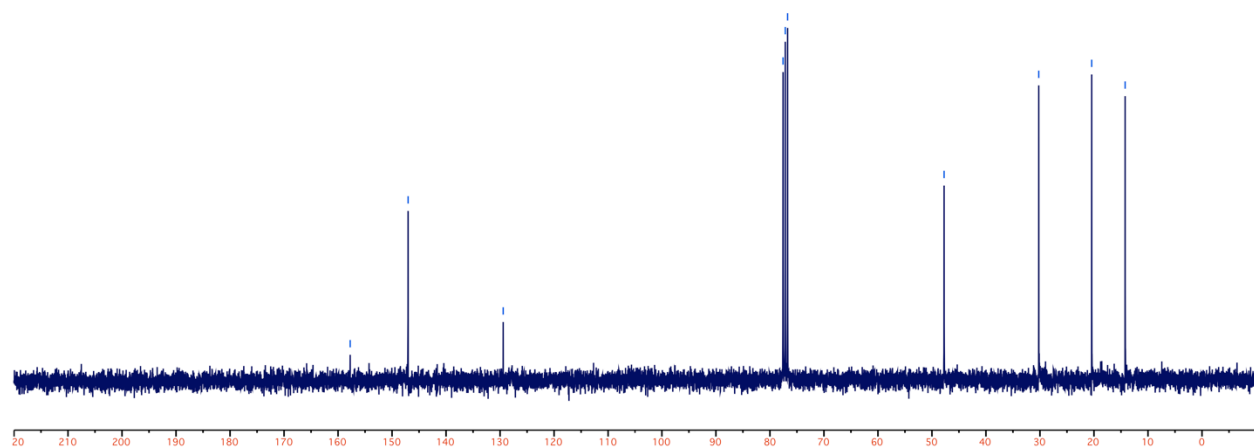
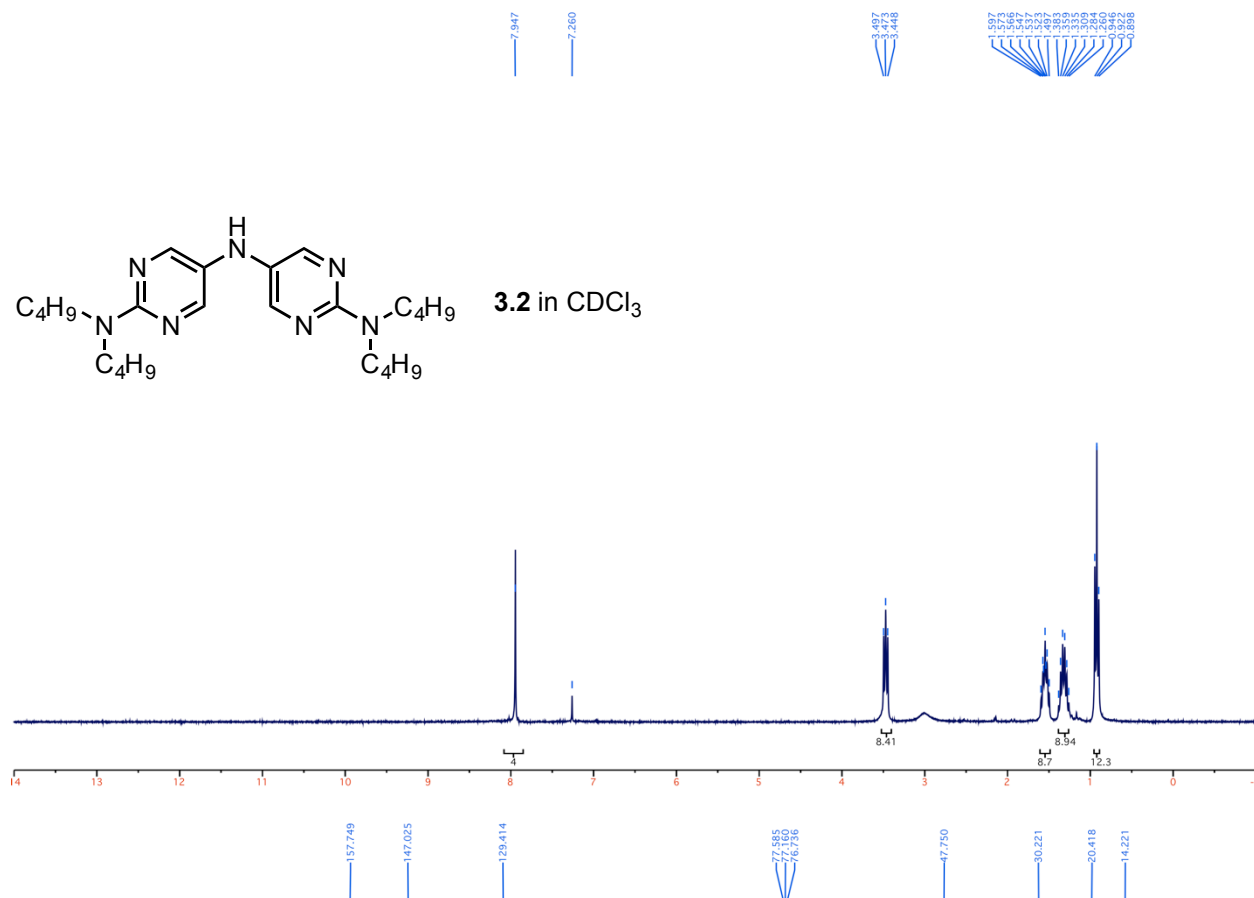


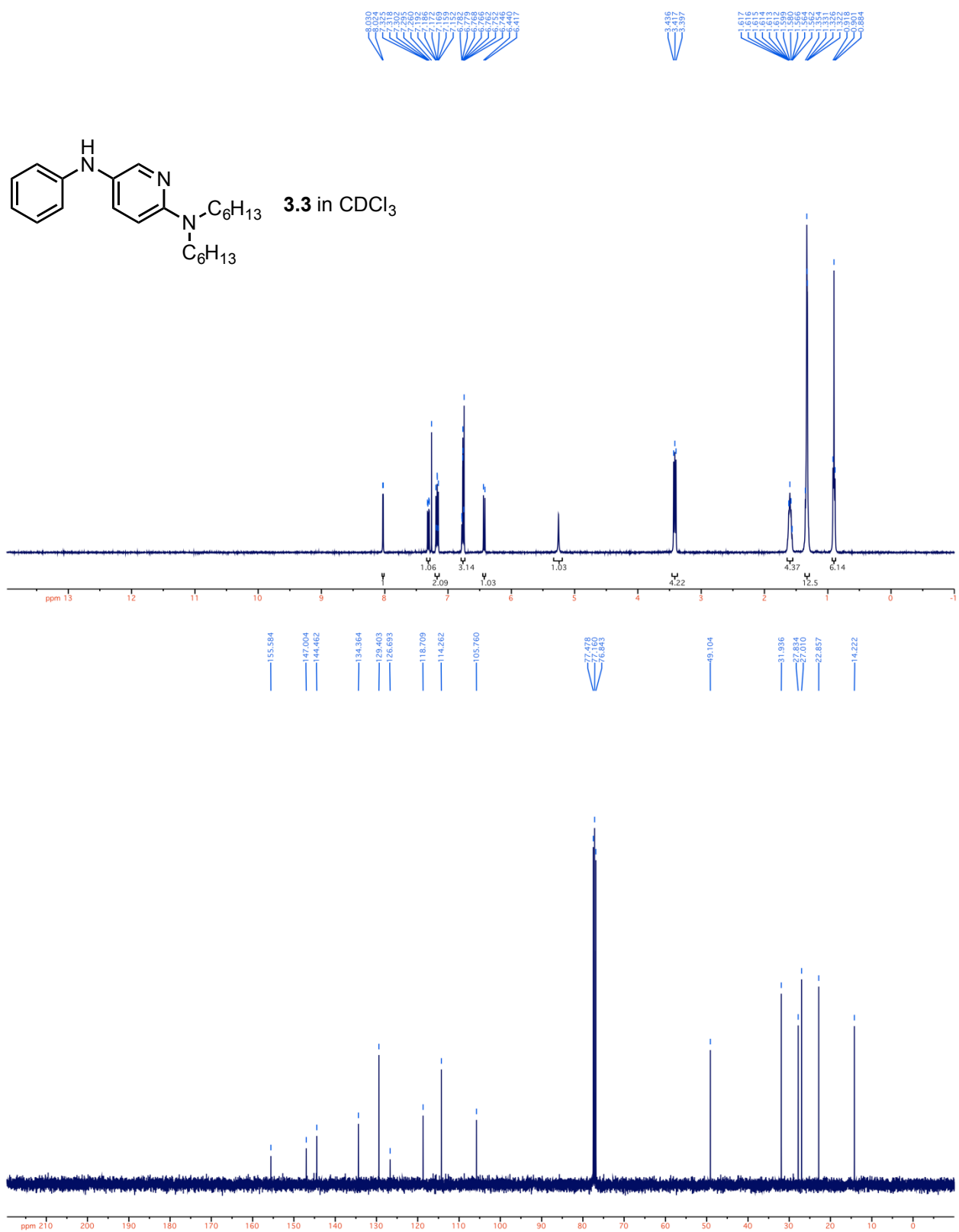


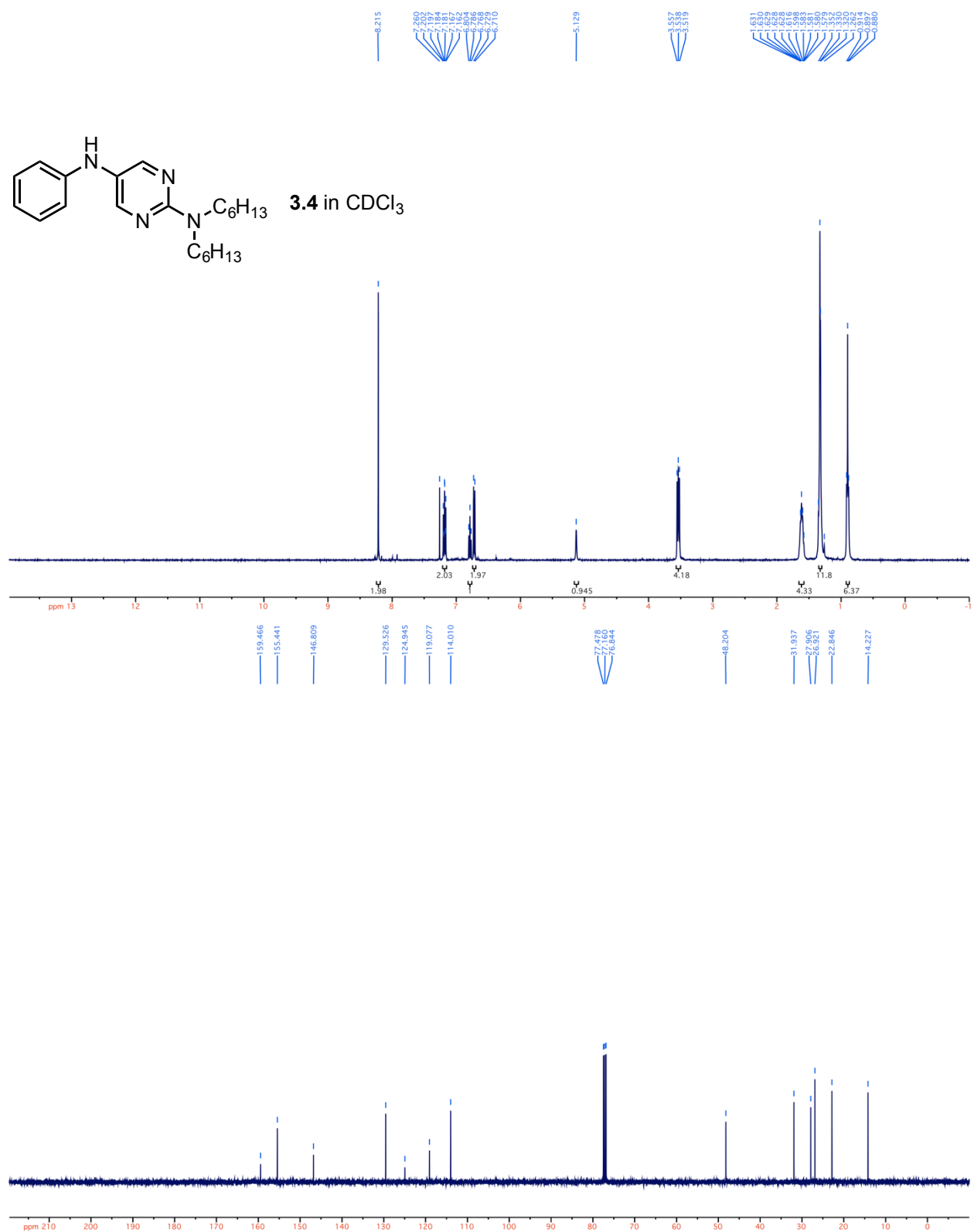


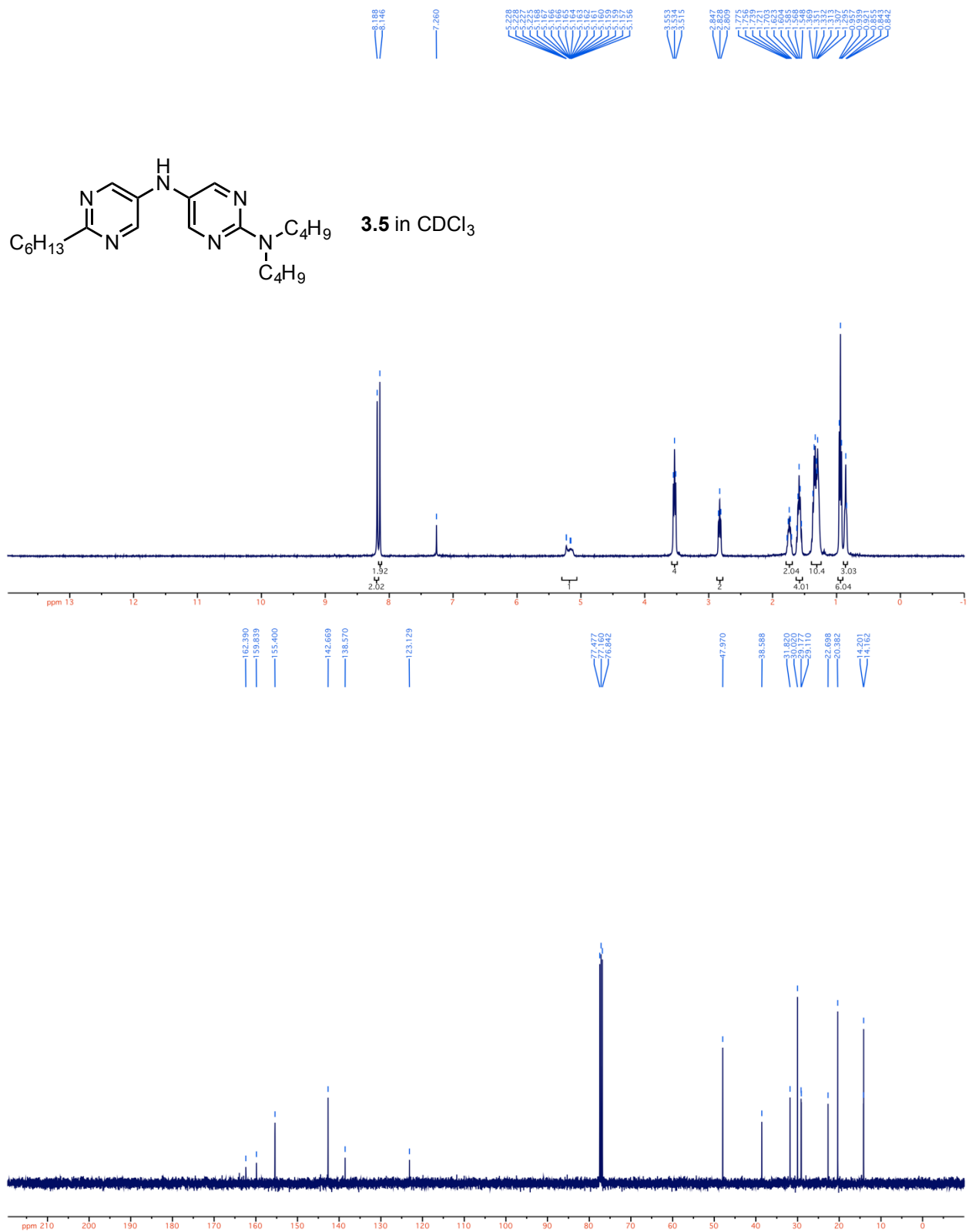




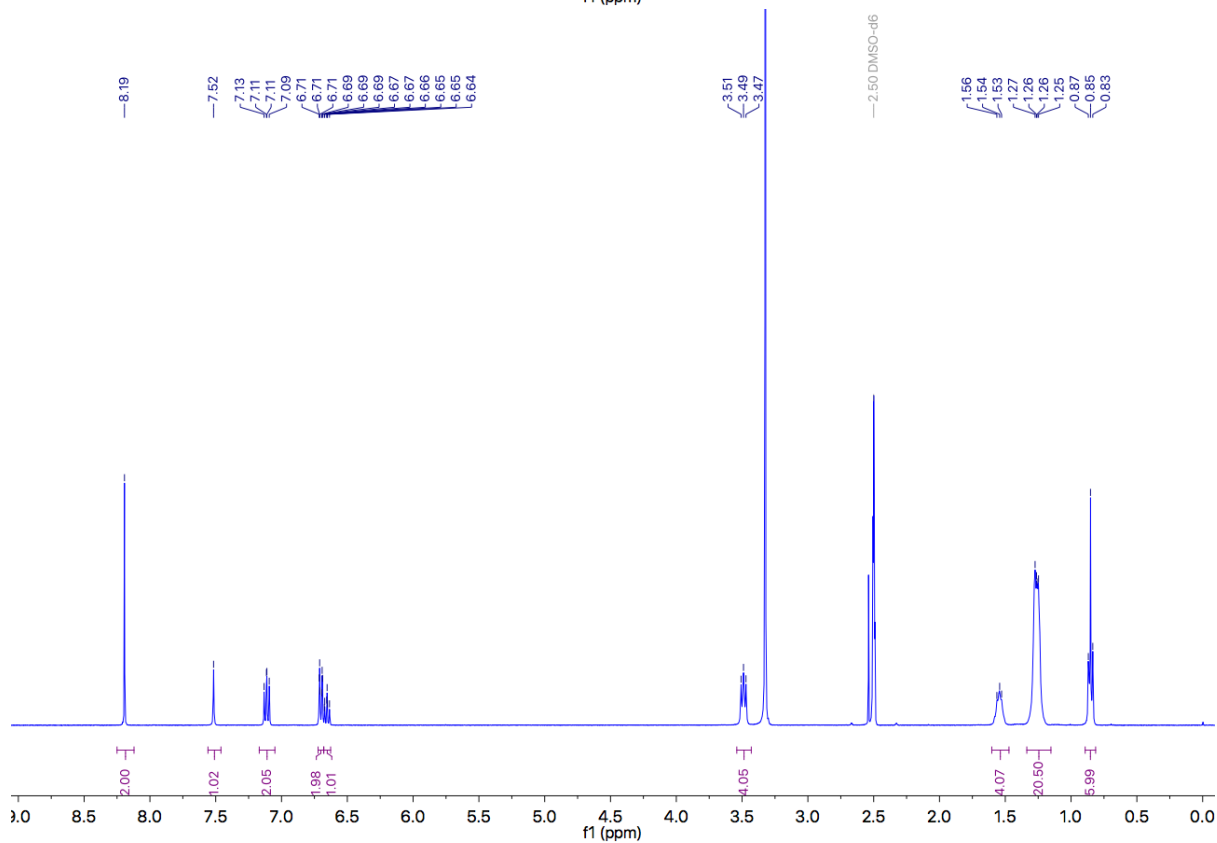
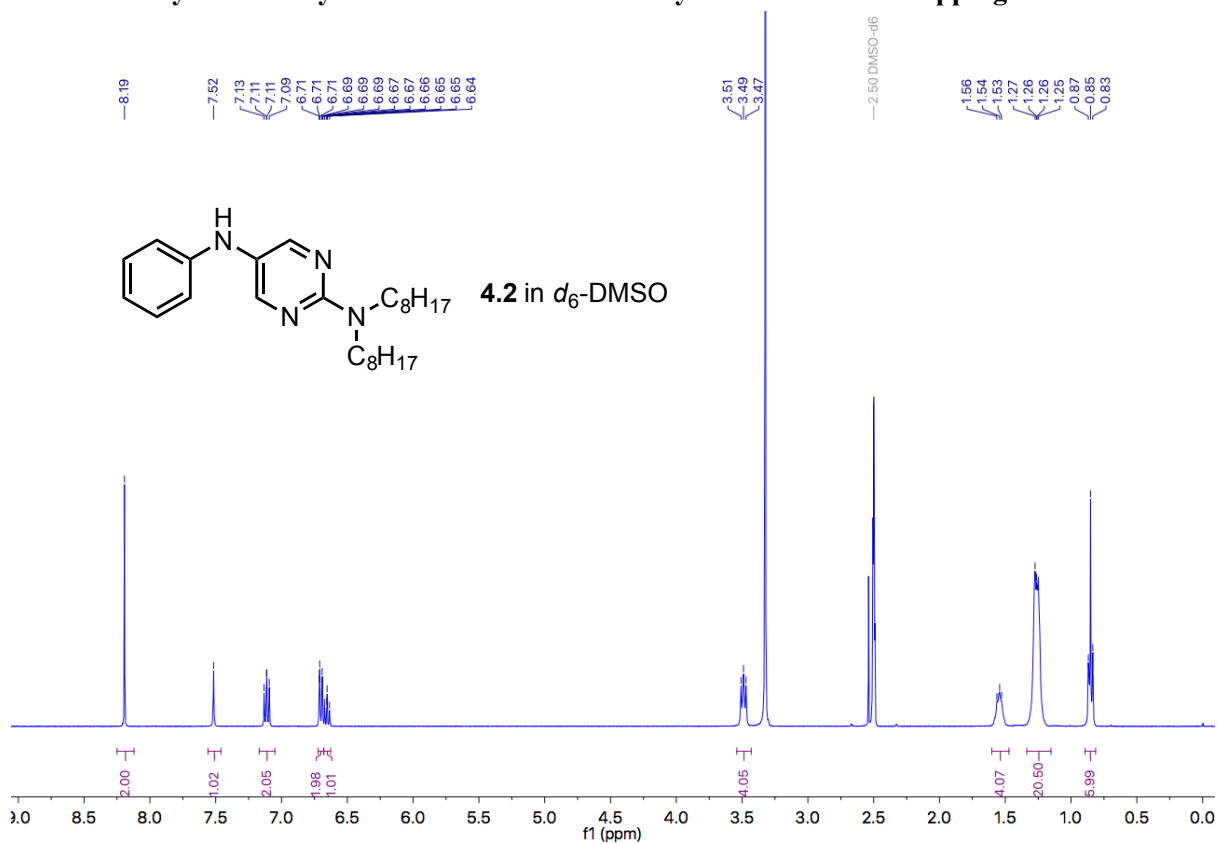


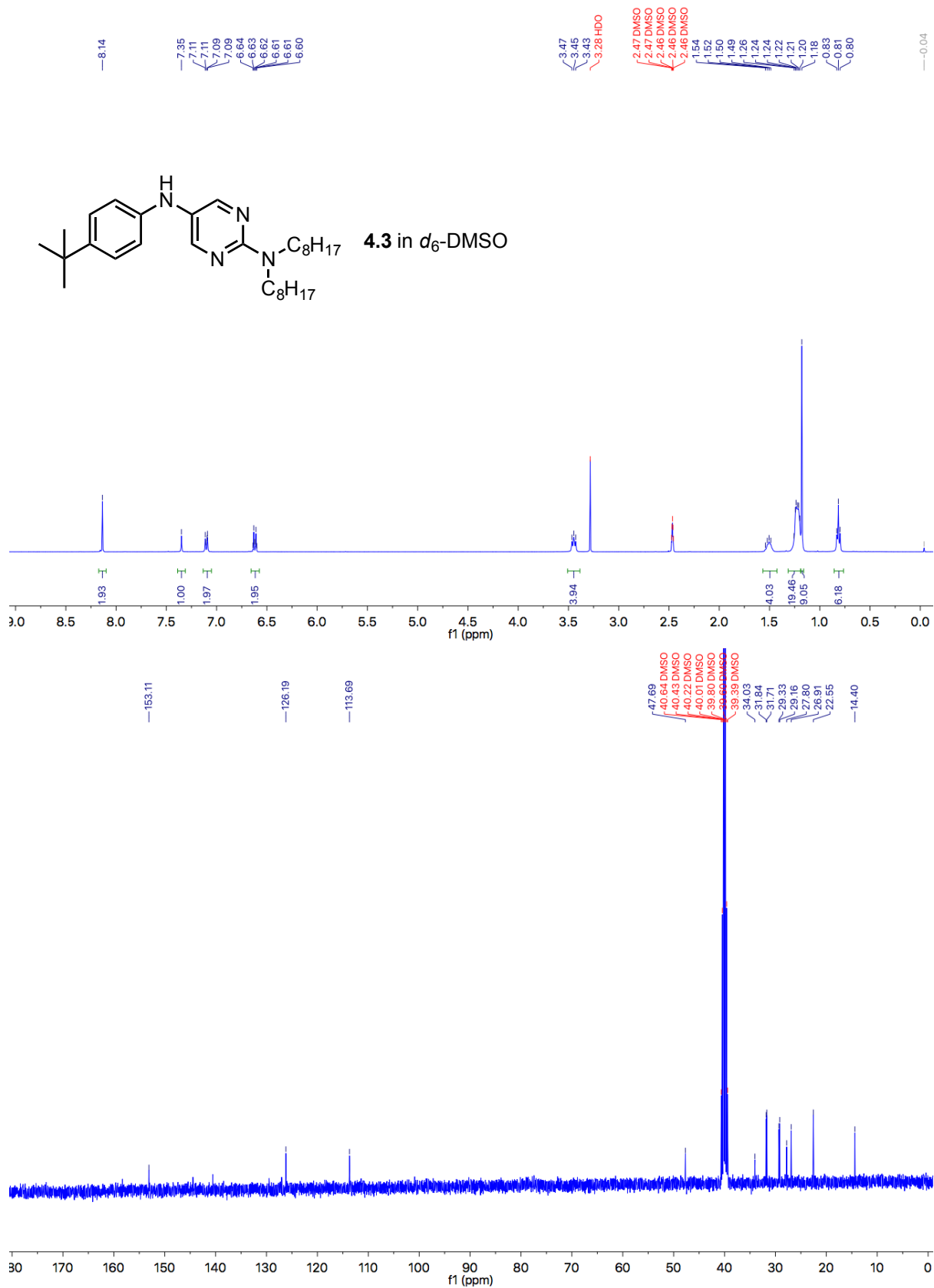


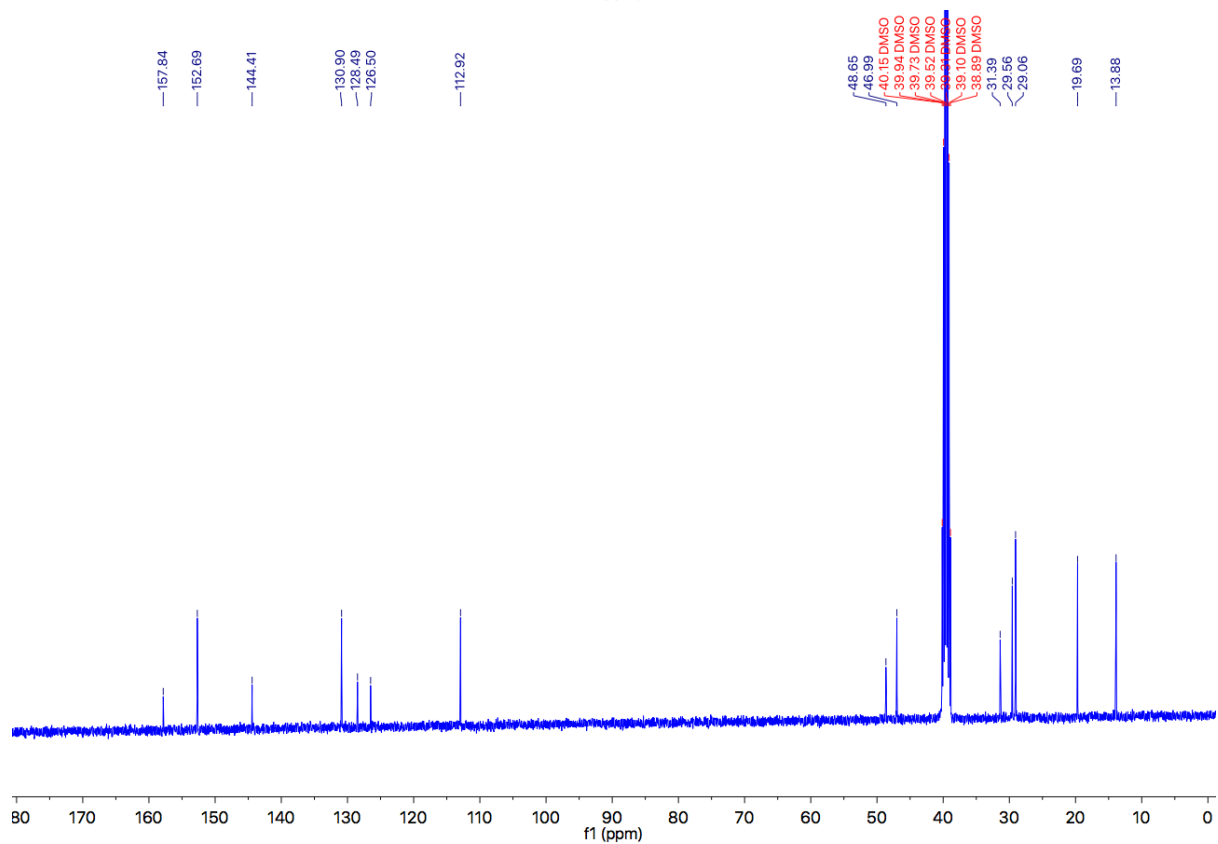
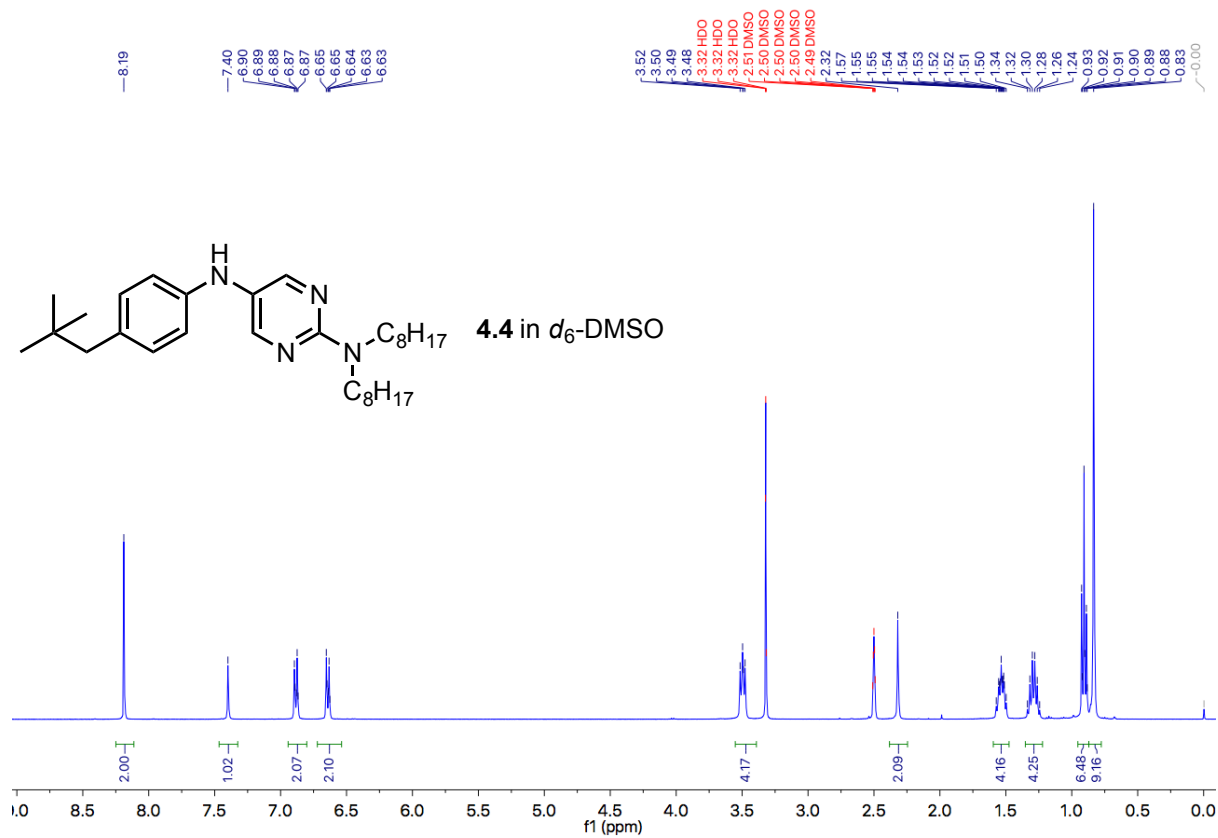


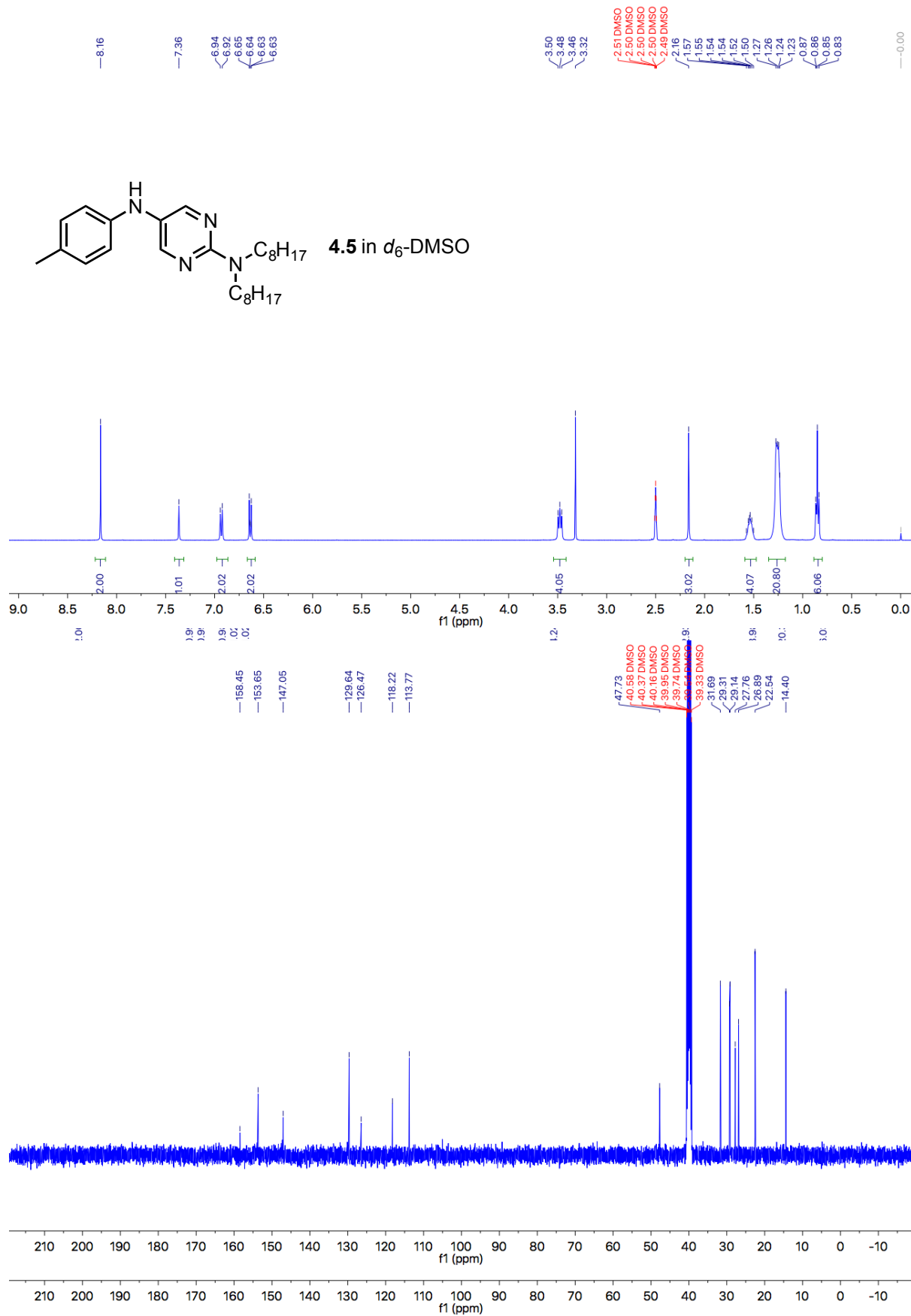


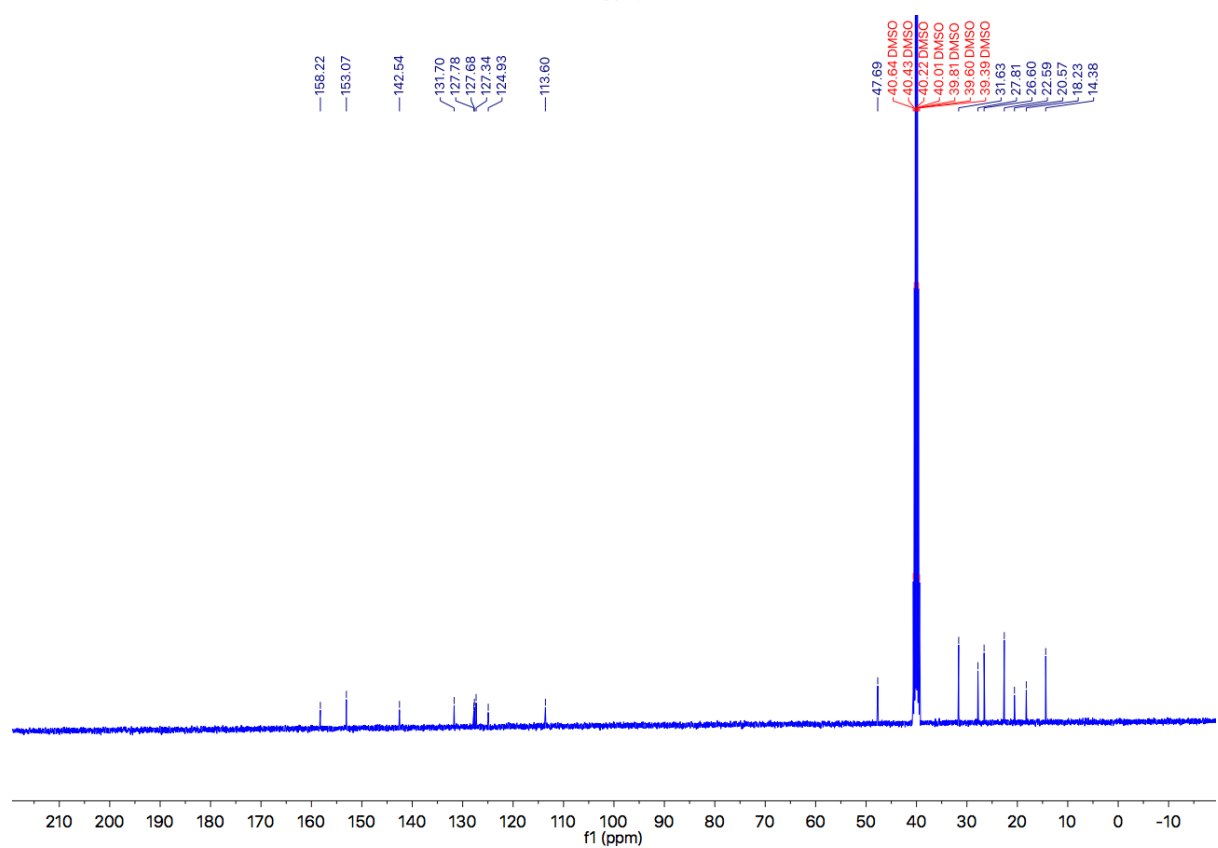
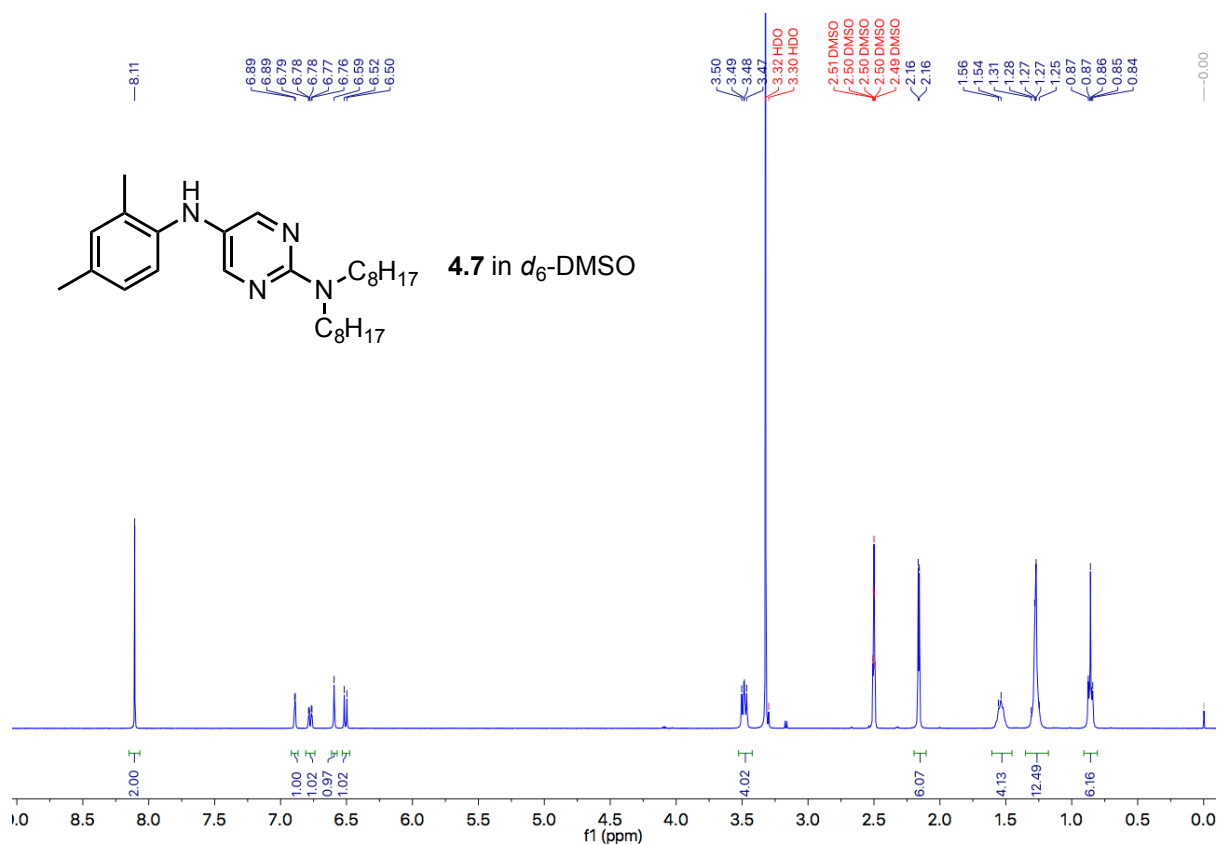
B.4 Reactivity and Catalytic Behaviour of Novel Diarylamine Radical-Trapping Antioxidants

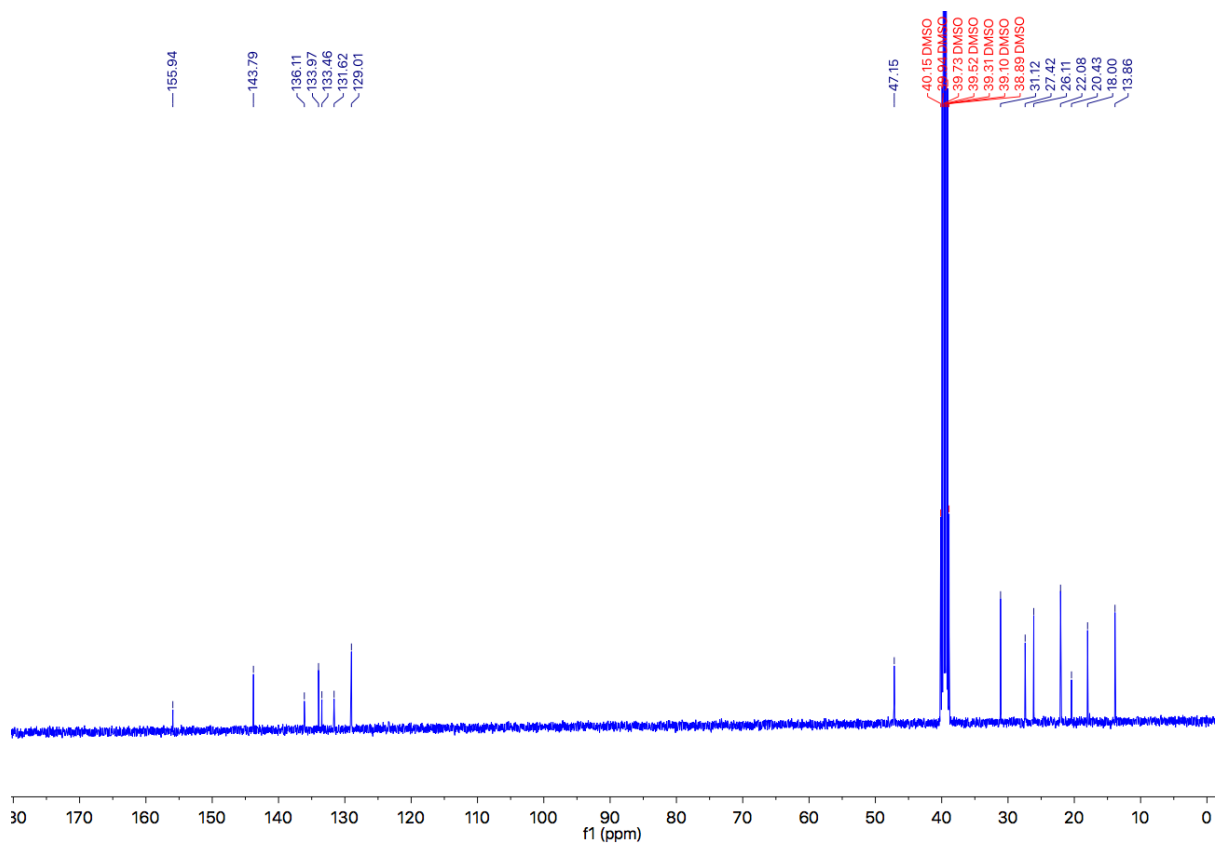
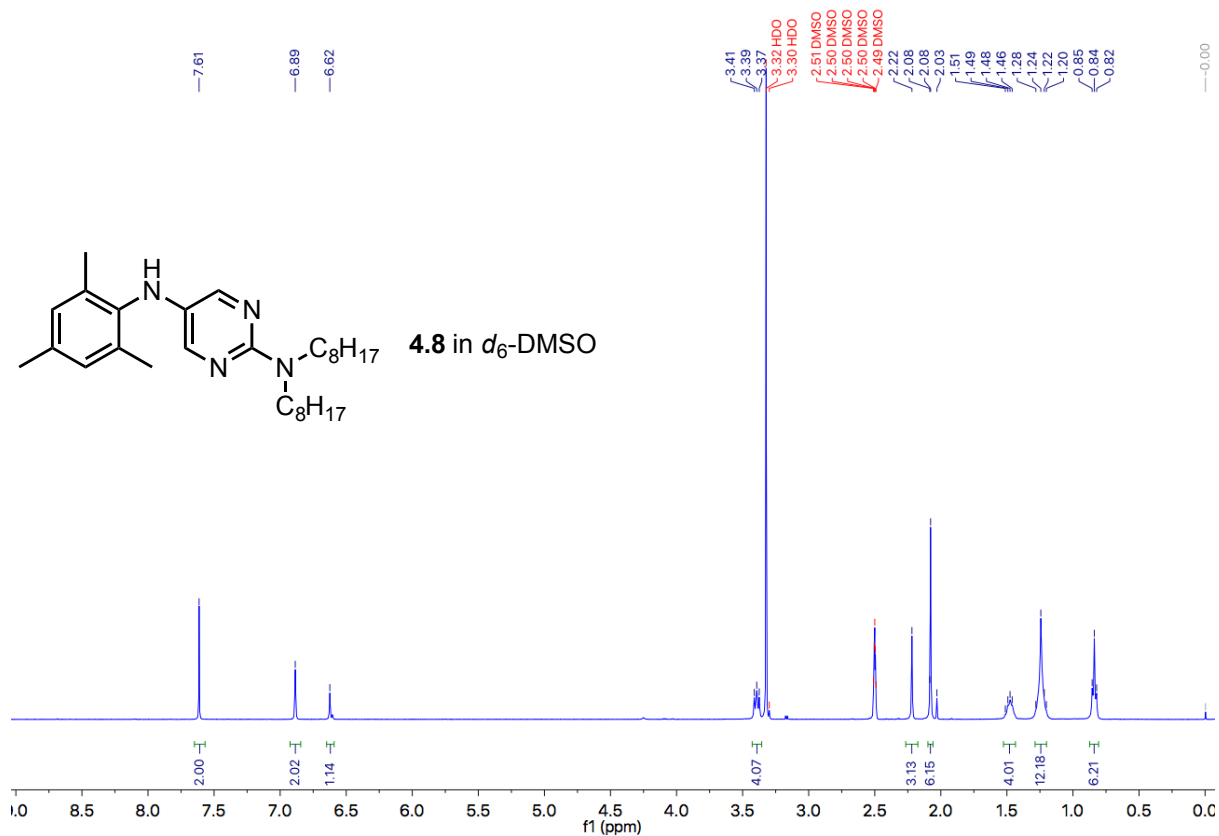




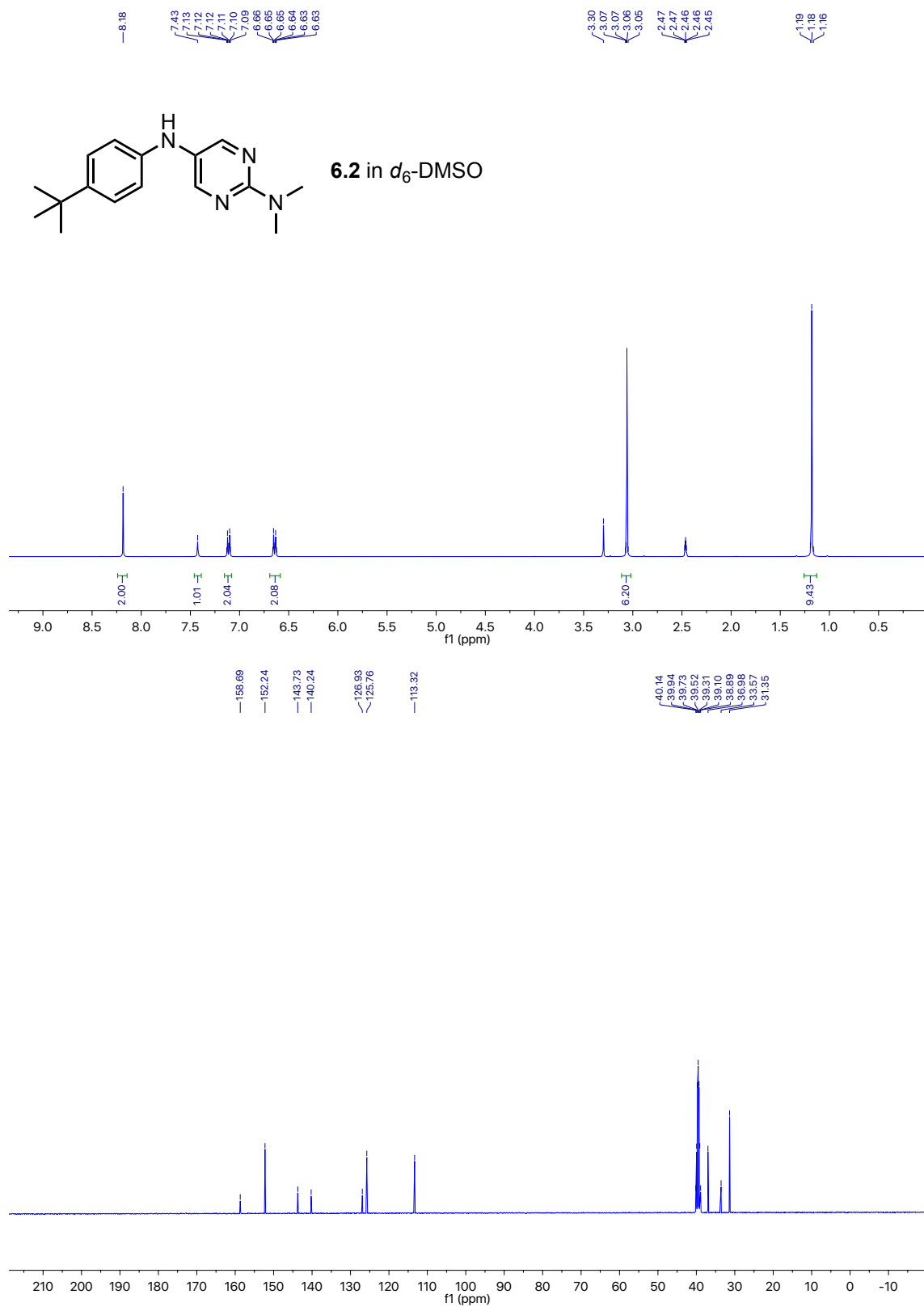


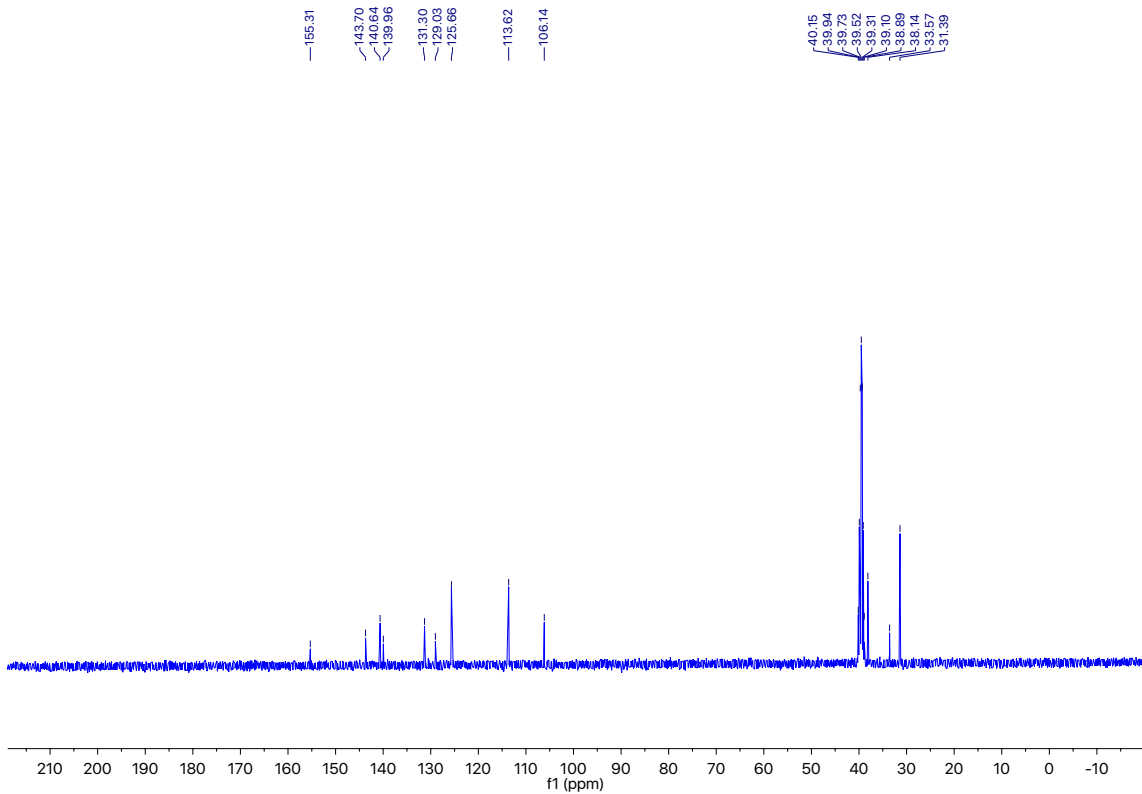
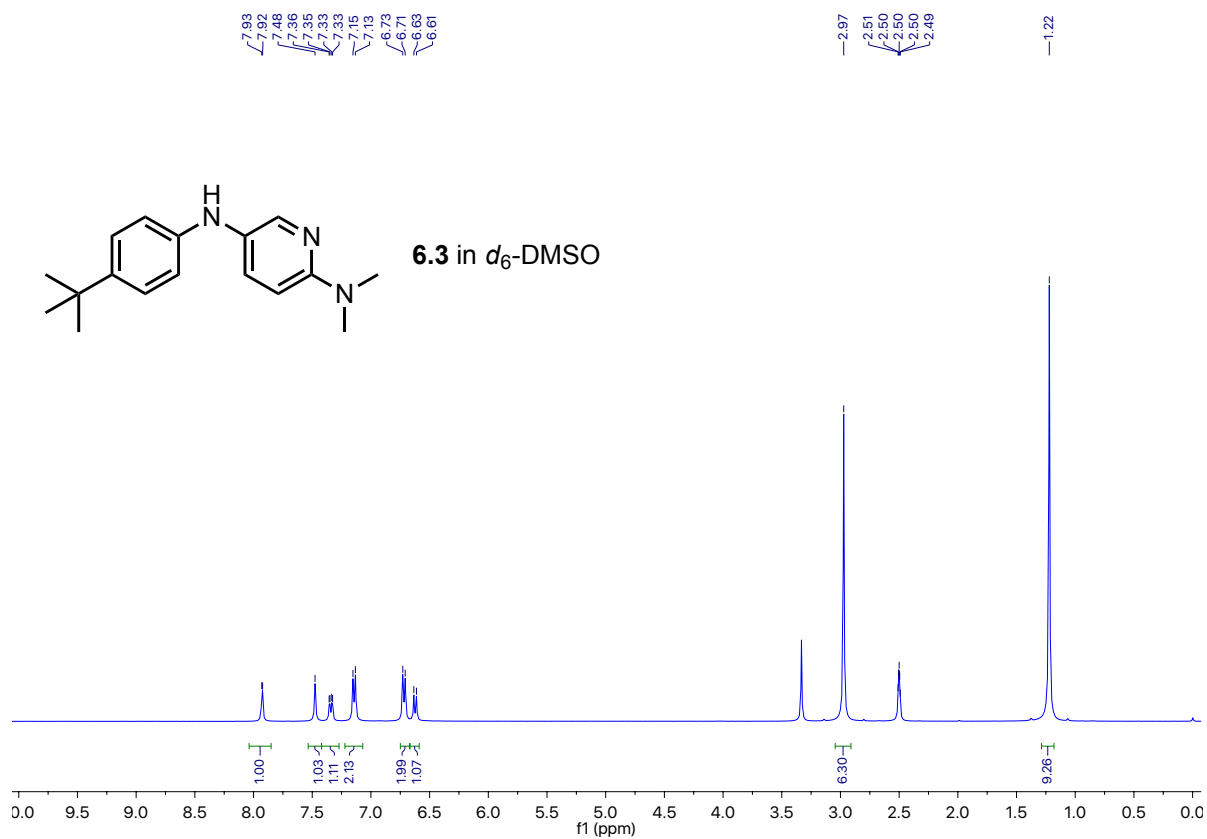


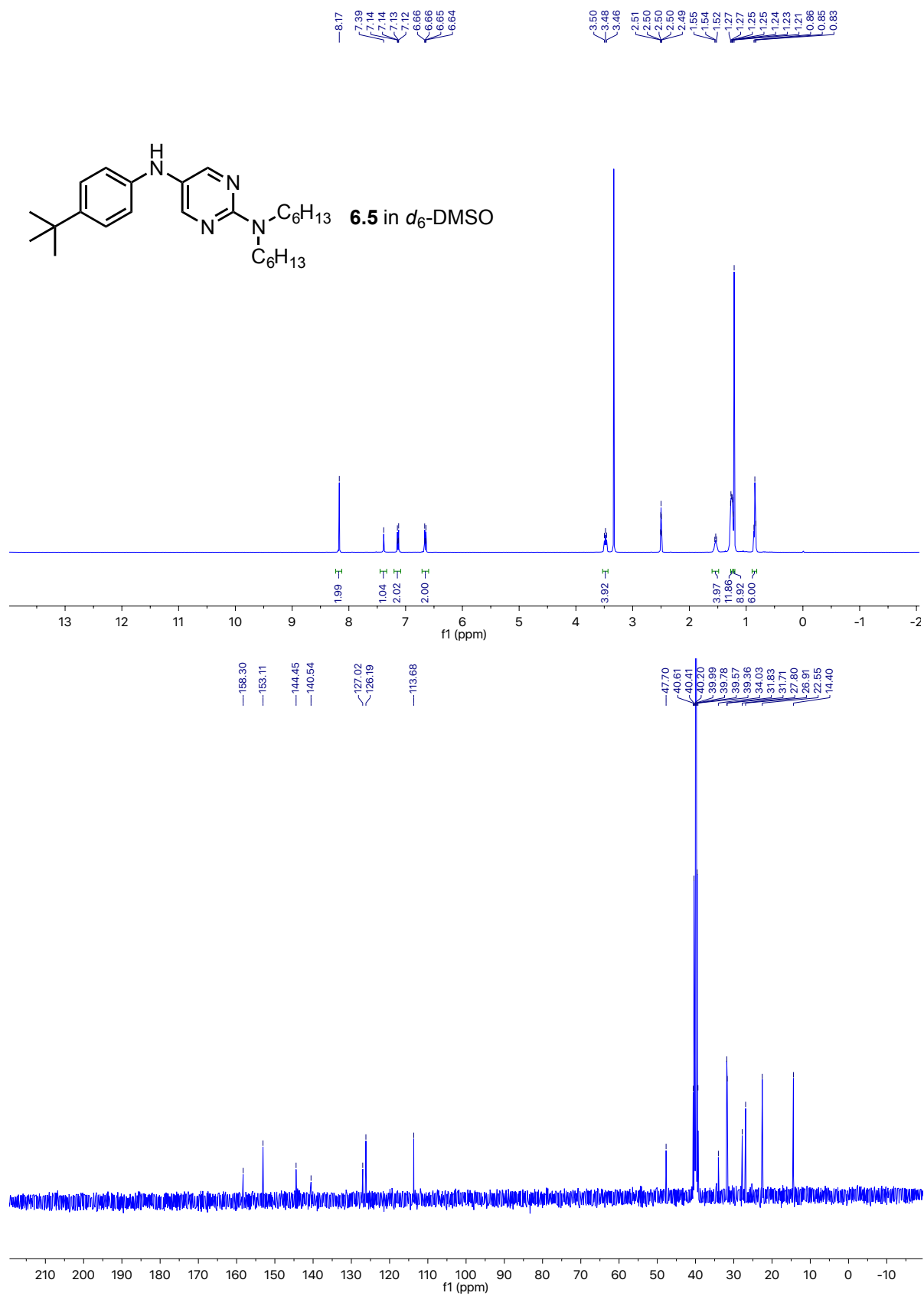


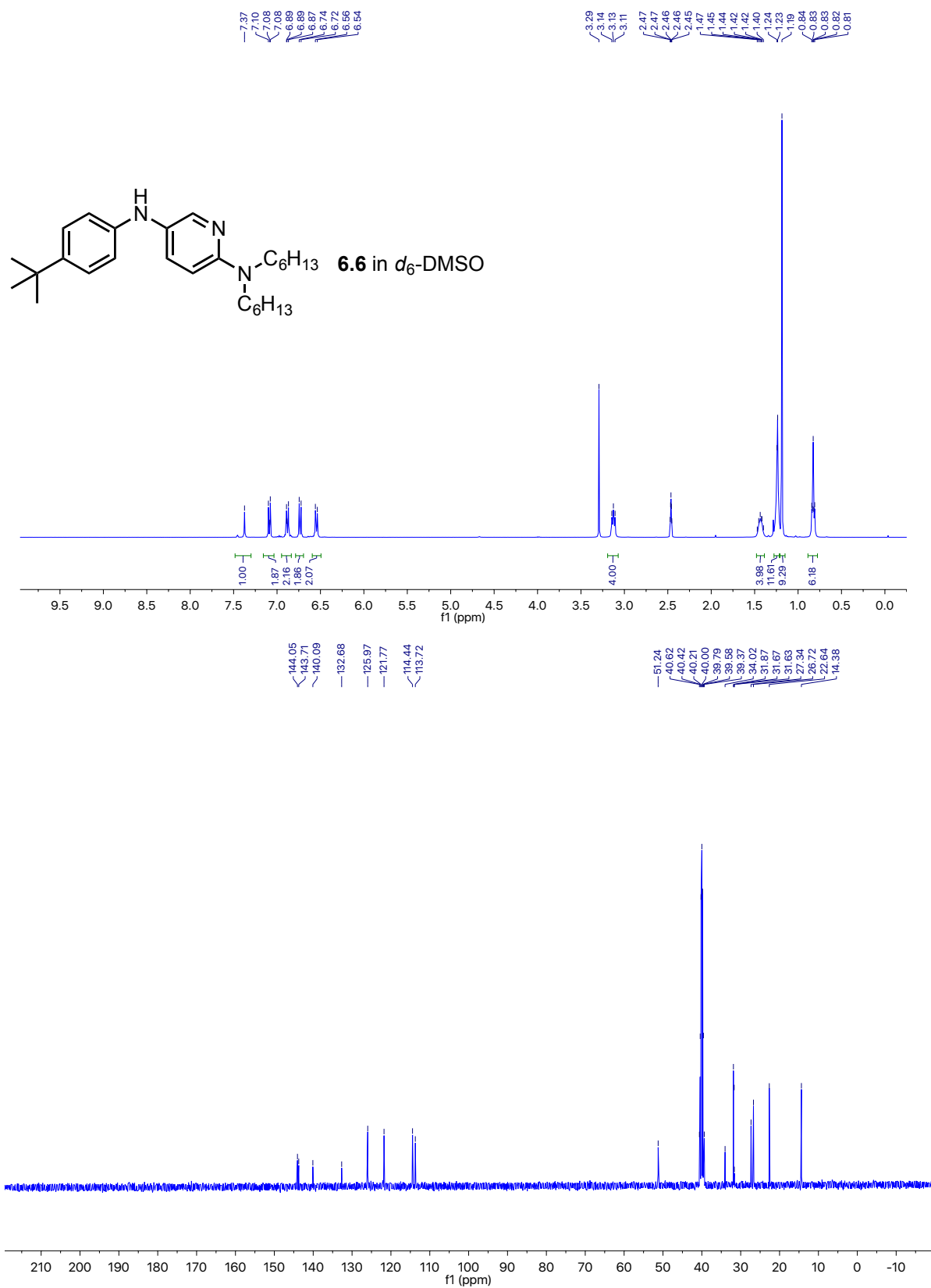


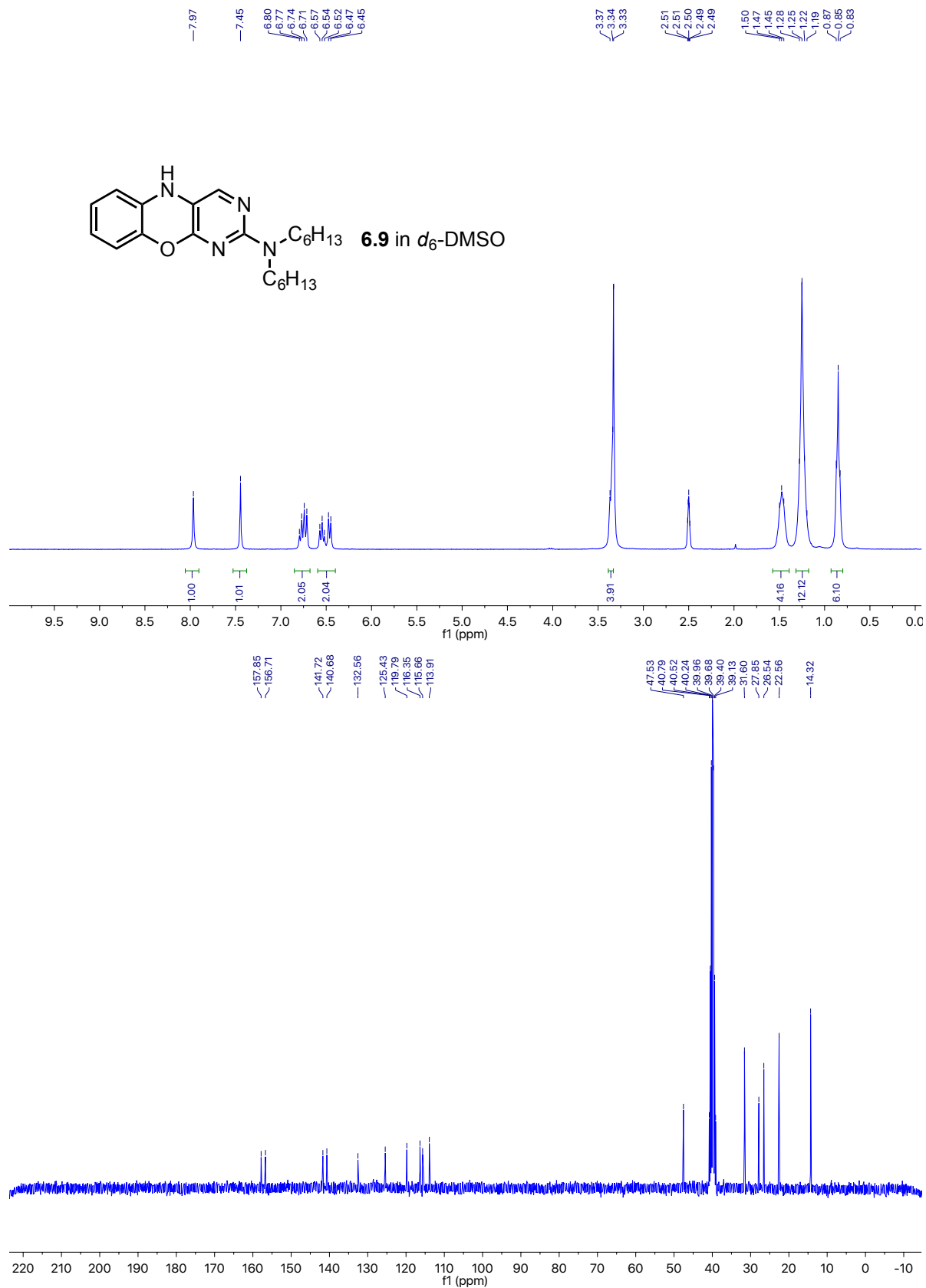
B.6 The Potency of Diarylamine Radical-Trapping Antioxidants as Inhibitors of Ferroptosis Underscores the Role of Autoxidation in the Mechanism of Cell Death

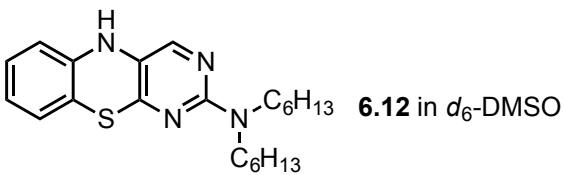




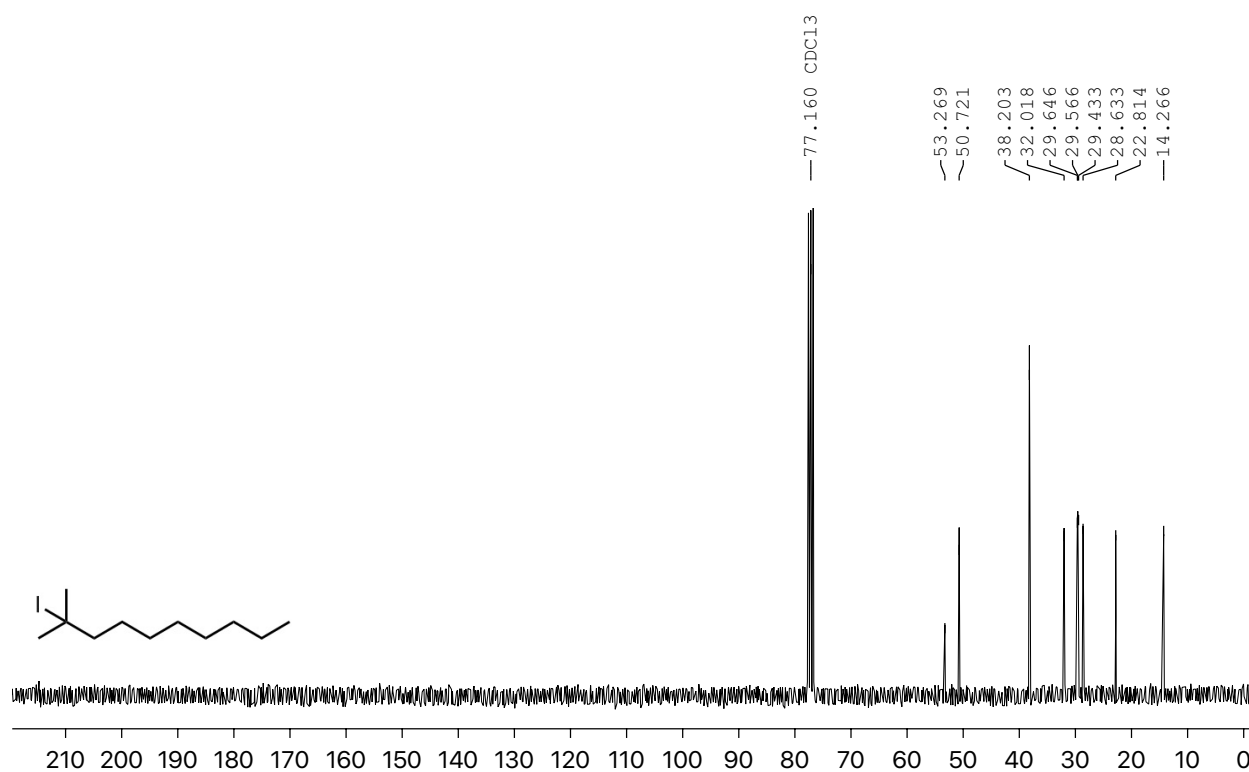
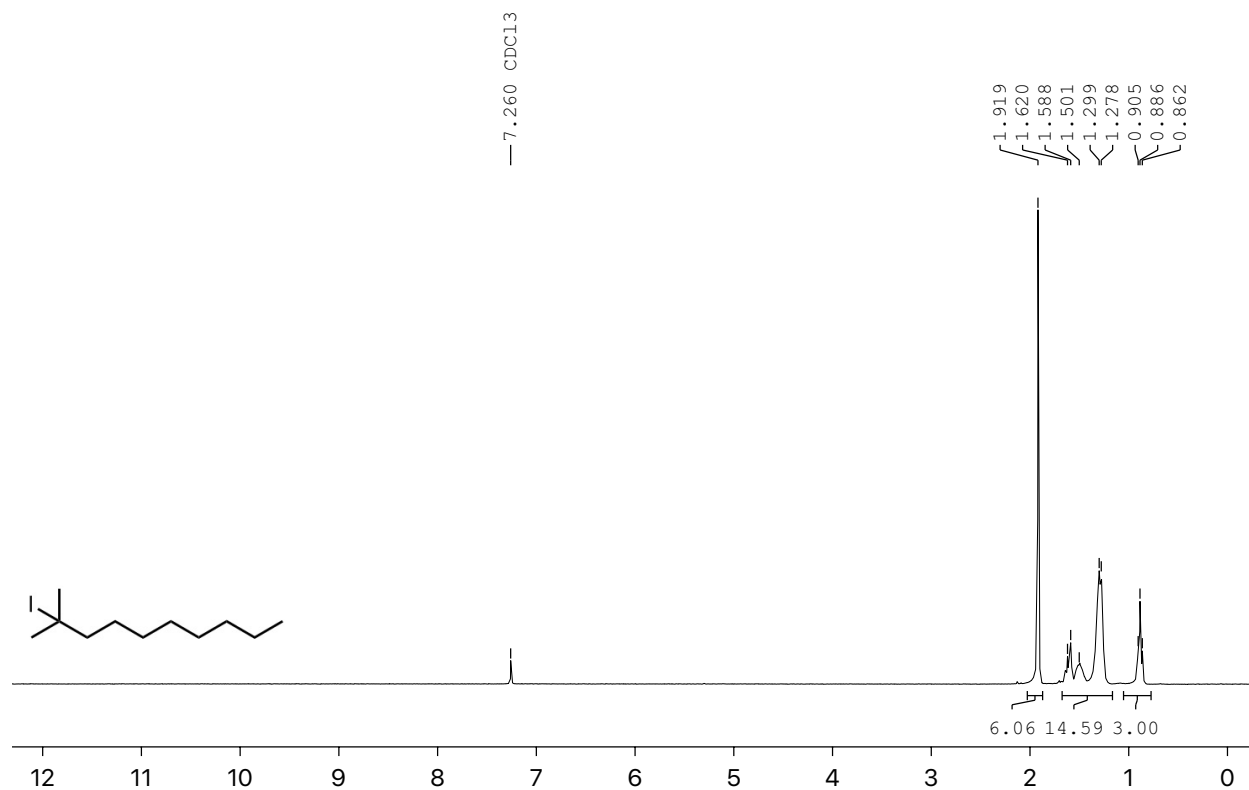


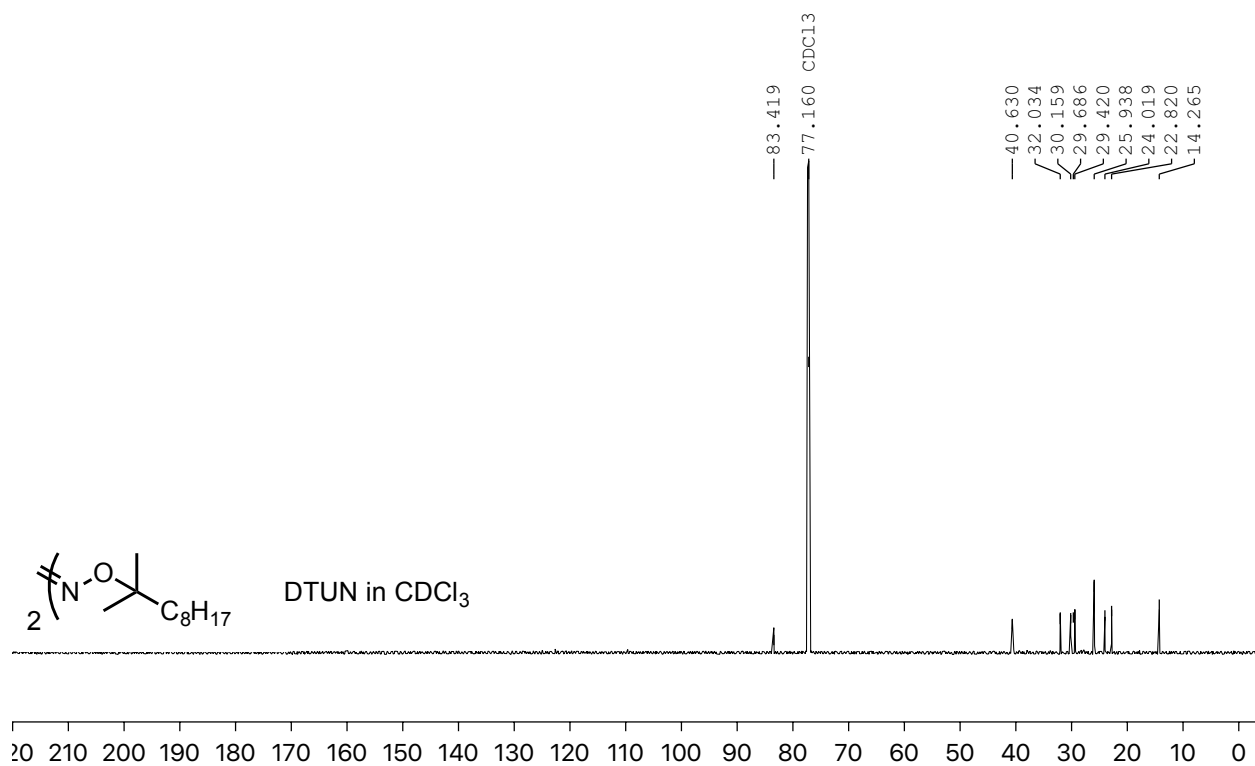
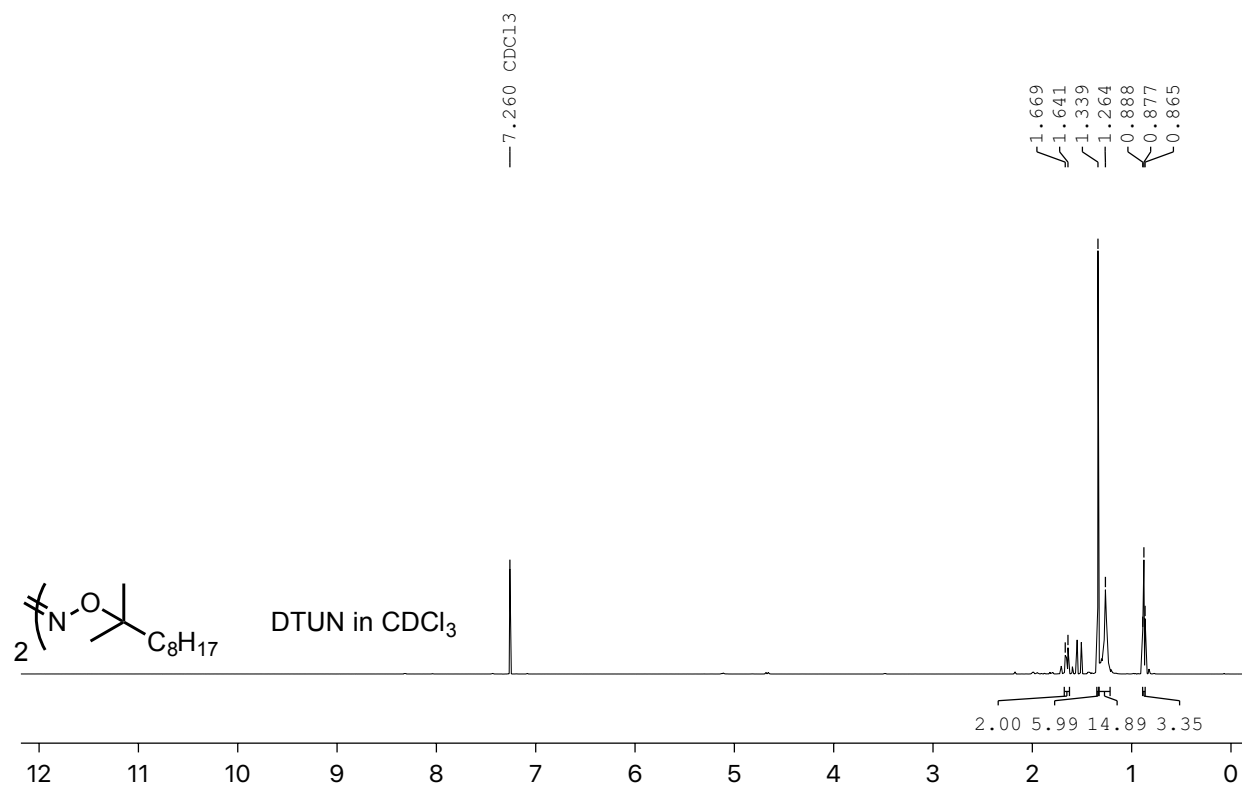


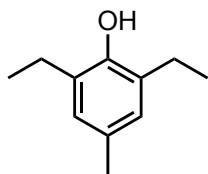




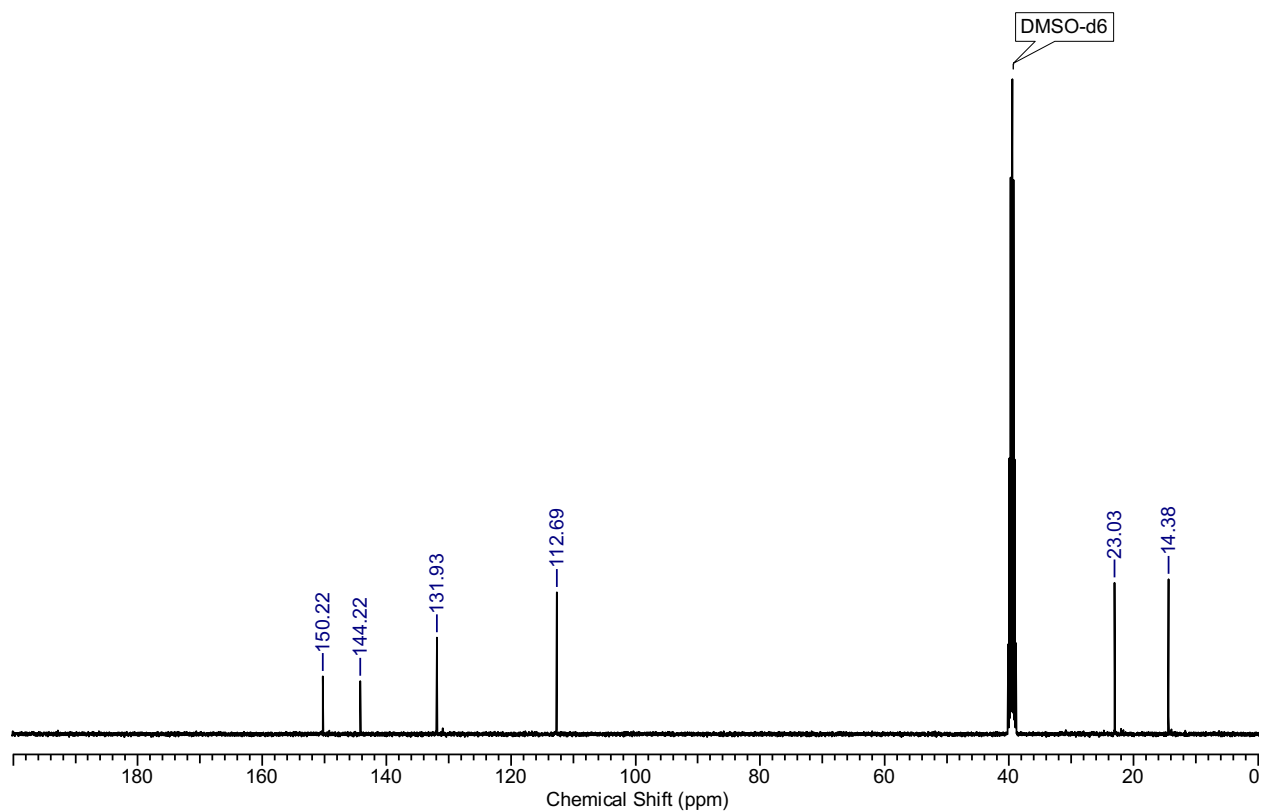
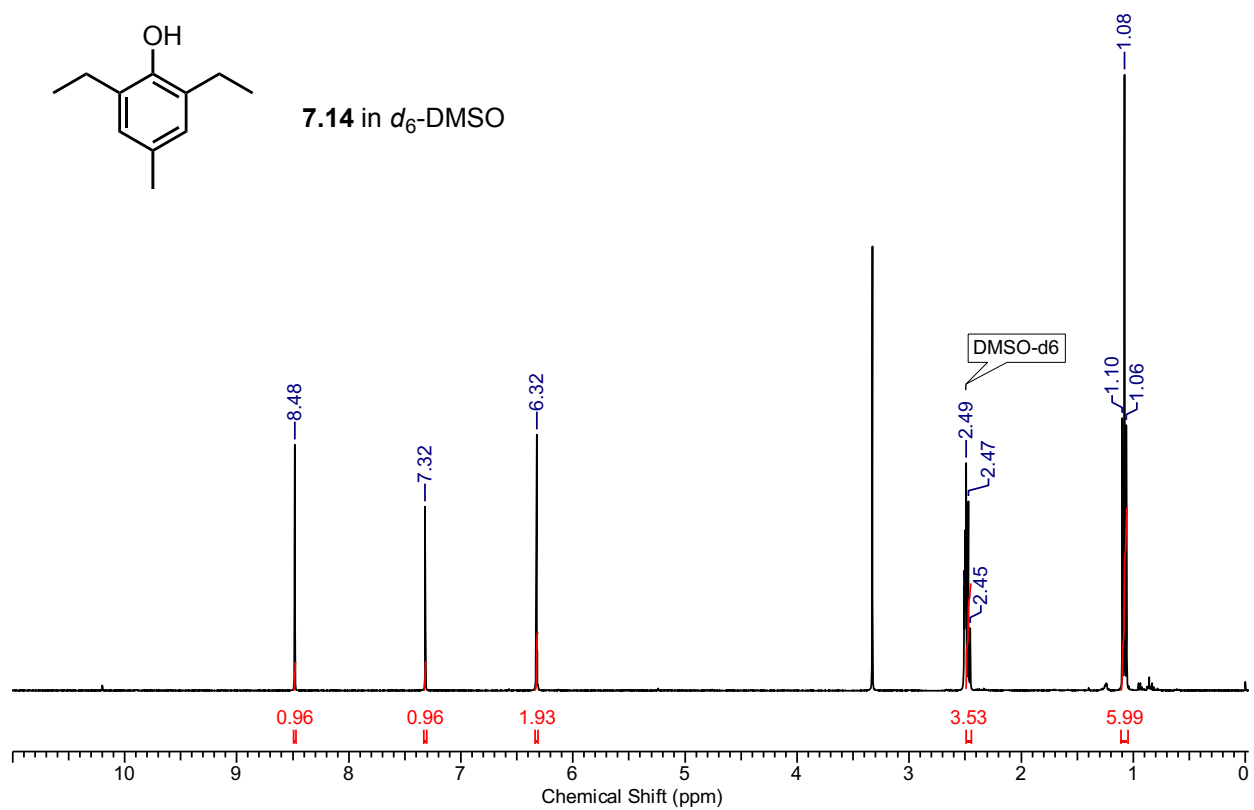
B.7 Beyond DPPH: Use of Fluorescence-ENabled Inhibited AutoXidation (FENIX) to Predict Oxidative Cell Death Rescue

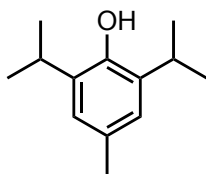




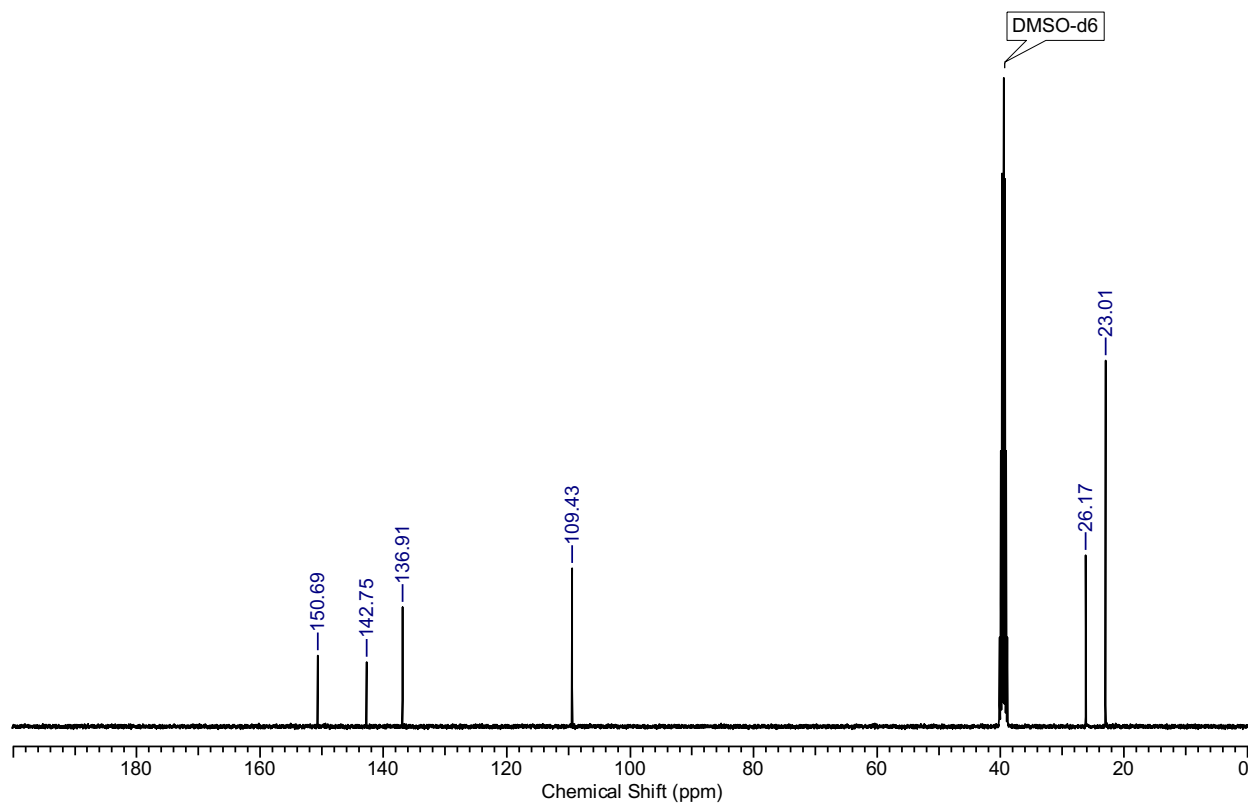
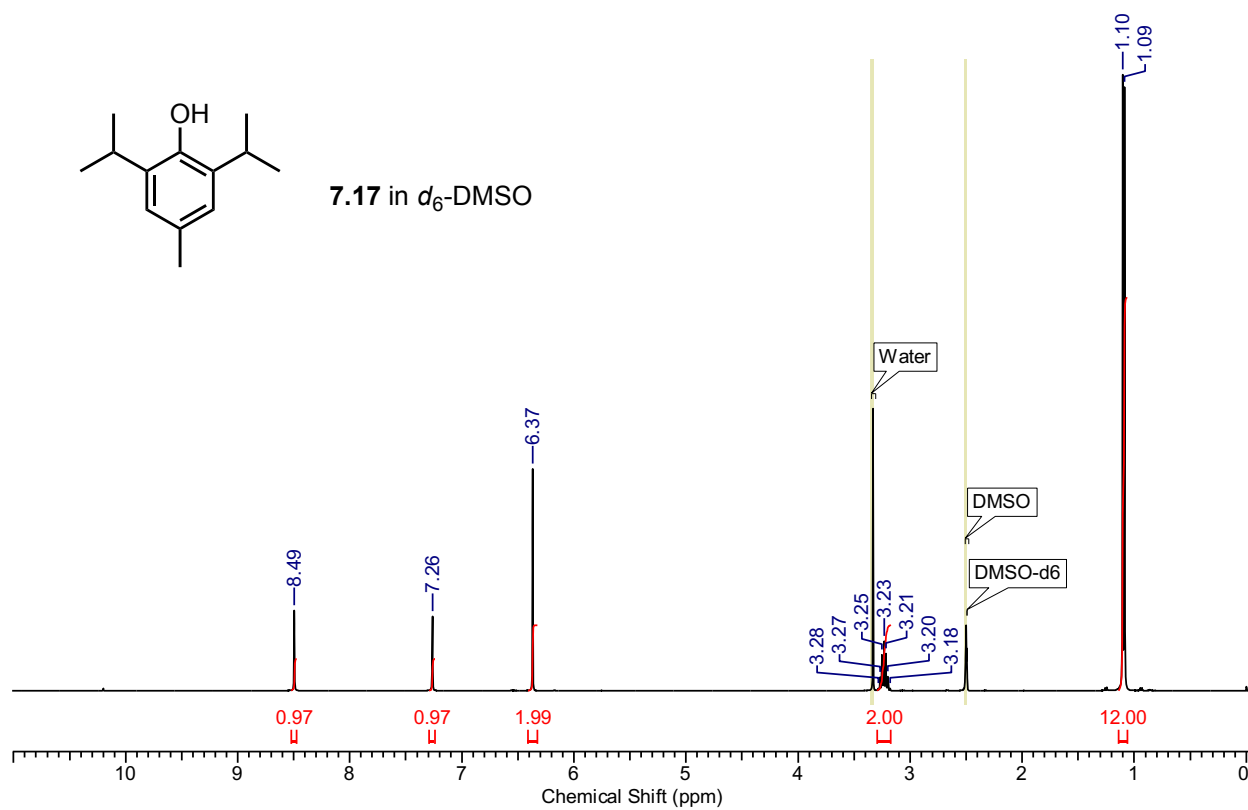


7.14 in d_6 -DMSO





7.17 in d_6 -DMSO



B.8 Mechanism of FSP1 as a glutathione-independent ferroptosis suppressor

