Chemiluminescent Agents for In Vivo Imaging

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CHEMILUMINESCENT AGENTS FOR IN VIVO IMAGING

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CHEMILUMINESCENT AGENTS FOR IN VIVO IMAGING

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Chemiluminescence is the light production directly from a highly exothermic chemical reaction by generating electronically excited species, and the light will be generated upon relaxation to the ground state. We utilize this light as the readout to construct imaging agents for reactive species. Chemiluminescence as an imaging modality has many advantages compared with current widely used imaging modalities, such as fluorescence and bioluminescence. The main advantages are the elimination of extraneous light excitation or genetic modification, which are required for fluorescence and bioluminescence imaging. Therefore, chemiluminescence as a imaging modality holds great potential for in vivo imaging.

In this dissertation, based on different reactive species, several reaction-based imaging agents have been designed, synthesized, characterized and applied for cellular and in vivo studies. Structural modifications have been conducted to obtain better imaging agents. For example, the introduction of ortho-chloro substitution to the reactive handle lowers the pKₐ of the chemiluminescent scaffold, and the introduction of electron withdrawing groups at the para position helps to increase chemiluminescence emission. CHS-3, HyCL-2, and PNCL are chemiluminescent probes for hydrogen sulfide (H₂S), peroxynitrite (ONOO⁻) and hypoxia and tissue oxygenation. All of them demonstrate high sensitivity and selectivity towards the analyte
of interest. **CHS-3** is the first reaction-based chemiluminescent probe demonstrating that chemiluminescence can be used as an imaging modality for *in vivo* imaging in wild-type animals. **HyCL-2** with the hypoxia responsive *para*-nitrobenzyl alcohol functionality, has the ability to differentiate different tissue oxygenation and image tumor hypoxia. **PNCL** is an enhancer free chemiluminescent probe that features a *ortho*- acrylonitrile group to the isatin for enhanced luminescence emission and an isatin functionality as a ONOO$^-$ specific handle. **PNCL** has been applied for the detection of endogenously generated ONOO$^-$ in RAW 264.7 macrophages. Finally, we are expanding upon the **HyCL** probe series with green and near-infrared emitting modifications. These chemiluminescent scaffolds are in the process of characterization.
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<td>ACT</td>
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</tr>
<tr>
<td>CBS</td>
<td>Cystathionine beta synthase</td>
<td>-</td>
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<td>CCD</td>
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<td>DMEM</td>
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**Chemical Structures**

![Chemical Structure](image)

**Abbreviations**

- ICT: Intramolecular charge transfer
- IEF-PCM: Integral equation formalism polarizable continuum model
- iNOS: Inducible nitric oxide synthase
- i.p.: Intraperitoneal
- LPS: Lipopolysaccharide
- Mn(III)TMPyP: Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride
- MRI: Magnetic resonance imaging
- MSOT: Multispectral optoacoustic tomography
- 3-MST: 3-Mercaptopyruvate sulfurtransferase
NADH  Nicotinamide adenine dinucleotide

NF-κB  Nuclear factor kappa-light-chain-enhancer of activated B cells

NIR  Near-infrared

NIST  National Institute of Standards and Technology

NMR  Nuclear magnetic resonance

PAG  DL-propargylglycine

PET  Positron emission tomography

PNCL  Chemiluminescent probe for peroxynitrite

PPh₃  Triphenylphosphine

PTSA  p-Toluenesulfonic acid

RSON  Reactive sulfur, oxygen, and nitrogen
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<td>Severe combined immunodeficiency / an albino, laboratory-bred strain of the house mouse</td>
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<td>SIN-1</td>
<td>3-Morpholino-sydnonimine</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
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For my parents.
CHAPTER 1

INTRODUCTION

Let there be light!

Chemiluminescence is the light production from a highly exothermic reaction that
generates electronically excited species. Upon relaxation to the ground state, the light emission
can be released. It is different from a conventional thermal reaction which normally releases
energy in the form of heat. A chemiluminescent reaction has to be highly exothermic to raise the
transition state higher than its electronically excited state to promote surface crossing (Figure 1-1).
Light from this unique reaction is sometimes called cold light which is emitted without the
release of heat. Chemiluminescence exists in our daily life in various formats, such as glow
sticks, bioluminescent bays, and fireflies (the latter two are characterized as bioluminescence
because the processes take place in biological systems). As chemists, this glowing phenomenon
is of particular interest. Chemiluminescence was first described by Eilhard Wiedemann in 1888
as "Das bei chemischen Prozessen auftretende Leuchten würde Chemilumineszenz genannt",\(^1\)
which can be translated as “light emission from a chemical reaction should be called
chemiluminescence”. This unique light production directly from a chemical reaction would come
to offer us some useful applications.
Figure 1-1. Potential energy diagrams for (a) an exothermic reaction and (b) a chemiluminescent reaction.\(^2\) (S = starting material, I = reactive intermediate or transition state, P = product).

1.1 Chemiluminescent systems

Several chemiluminescent systems have been reported and some of them have been used for bioassays. In this dissertation, a brief summary will be given of some of the most used chemiluminescent systems and how light is generated from these systems. Luciferin/luciferase, luminol, acridinium esters, peroxoxalates, and 1,2-dioxetanes will be discussed.

1.1.1 Luciferin/luciferase system

Luciferin/ luciferase systems are used by living organisms such as fireflies, bacteria and marine organisms to generate light,\(^3\) which is termed as bioluminescence. Bioluminescence has already been applied for the study of many biological processes both \textit{in vitro} and \textit{in vivo}.\(^4\) Even though there are millions of luciferase enzymes expressed by various species, only a few native luciferin/luciferase pairs have been identified and applied for bioimaging, including D-luciferin/firefly luciferase (Fluc),\(^5\) Coelenterazine/Renilla luciferase (Rluc) or Gaussia luciferase (Gluc),\(^6\) and Vargula luciferase (Vluc).\(^7,8\) Although these bioluminescent systems differ in structures and compositions, they share the same light generation mechanism by generating...
unstable intermediates. In the firefly system, the enzyme luciferase catalyzes the oxidation of D-luciferin in the presence of ATP, Mg$^{2+}$ and O$_2$ to generate an unstable highly energetic 1,2-dioxetanone intermediate$^9$ that decomposes to an electronically excited species (Scheme 1-1a). The emission of light is accompanied with the relaxation of this excited species. This system brought from nature has inspired scientists to adopt it as an analytical tool for biological research.

D-luciferin is the most commonly used bioluminescent substrate for in vitro and in vivo imaging. Firefly luciferase catalyzes the light production from D-luciferin with light emission centered around 560 nm. Compared with fluorescence imaging, bioluminescence doesn’t require an external light excitation, which significantly reduces background emission and autofluorescence. In the last two decades, scientists have made significant progress in the development of better agents for bioluminescence imaging. Chemical modifications, genetic mutations and directed evolutions of bioluminescent substrates have been investigated to offer imaging agents with wavelengths ranging from green to the NIR range,$^{10}$ and orthogonal luciferin/luciferase pairs can be obtained to image multiple analytes or multiple biological processes.$^{11}$ The bioluminescence phenomenon has also inspired chemists to develop small-molecule systems to mimic luciferin/luciferase oxidation and decomposition processes.$^{12}$

Although bioluminescence as an analytical tool has been frequently applied for biological research and in vivo imaging, some key obstacles that it faces are the limited number of luciferin/luciferase pairs that are available, and genetic modification to express luciferase enzyme is required which complicates experimental procedures and could potentially interfere with the biological processes of living systems.
1.1.2 Luminol

Apart from luciferin/luciferase, luminol is one of the most used compounds for the generation of chemiluminescence. It has been utilized for crime scene investigation due to its reactivity towards iron in hemoglobin.\textsuperscript{13} Scientists also used this molecule for biological assays. Chemiluminescence can be triggered when it mixes with an appropriate oxidizing agent in basic conditions (Scheme 1-1b). The detailed process starts from the deprotonation of luminol, followed by one electron oxidation to generate luminol radical anion. This one electron oxidation can be achieved by the addition of a one-electron oxidant such as horseradish peroxidase, metal ions, or free radicals. The luminol radical anion can be further oxidized by reactive oxygen species, such as H$_2$O$_2$, and superoxide anion to form a hydroperoxide adduct. Subsequently an intramolecular reaction leads to the formation of an endoperoxide which can emit light upon decomposition. This system has been applied for designing a reaction-based probe for \textit{in vitro} H$_2$S detection by Pluth and coworkers.\textsuperscript{14}

1.1.3 Acridinium esters and peroxyoxalates

Similar to luminol, light can also be generated from acridinium esters and peroxyoxalates (Scheme 1-1c,d). Light emission from an acridinium ester is triggered with the addition of alkaline hydrogen peroxide to form a four-membered peroxide ring, followed by the decomposition to initiate the light production. This light emitting system has been used for diagnostics and biomolecular labelling.\textsuperscript{15} As for peroxyoxalate, first reported by Rauhut in 1967, the light usually is generated as fluorescent emission by the dye added into this system.\textsuperscript{16} First, an activated oxalate reacts with H$_2$O$_2$ under basic conditions to generate a highly energetic intermediate, 1,2-dioxetanedione. This highly energetic intermediate decomposes with the
release of energy which can be absorbed by a fluorescent dye incorporated into this system. This system has been used for the development of glow sticks and widely applied for analytical detection. Our laboratory has used this system as a platform to monitor \( \text{H}_2\text{O}_2 \) in human exhaled breath condensates using a smart phone camera.\(^{17}\) Although these systems can be tuned for efficient luminescent production, their biological applications are limited. The main disadvantage of these systems is the requirement of additional reagents such as \( \text{H}_2\text{O}_2 \) and a base, which can complicate the detection procedures and interfere with biological processes.

1.1.4 1,2-dioxetanes

Kopecky and Mumford reported the first synthesized 1,2-dioxetane, 3,3,4-trimethyl-1,2-dioxetane, in 1969 (Scheme 1-1e).\(^{18}\) Luminescence emission was observed during its thermal decomposition at 60 °C in a benzene solution. A wealth of 1,2-dioxetane derivatives has been synthesized and reported since then.\(^{19}\) The thermal stability of these 1,2-dioxetanes differs tremendously from each other. 3,3,4-trimethyl-1,2-dioxetane decomposes quickly at room temperature.\(^{18}\) Among them, the most stable 1,2-dioxetane was synthesized by Wieringa in 1972 from adamantylidenadamantane using sensitized oxygenation.\(^{20}\) It has been well accepted that bulky structures such as adamantane are important to stabilize the highly restrained dioxetane by preventing the elongation of O–O and C–C bonds.\(^{21}\) The thermal decomposition energy of this sterically stabilized structure is 37 kcal/mol and its half-life at 20 °C is more than 20 years.\(^{22}\) Initial applications of these 1,2-dioxetanes were mainly limited by their thermal decompositions.\(^{23}\)

A great number of methods for the preparation of 1,2-dioxetane have been reported over the last five decades. The most widely used methods generally fall into two categories,
cyclization of halogen hydroperoxides by base or silver ion\textsuperscript{18} and oxidation of alkenes with singlet oxygen (Scheme 1-2). The singlet oxygen can be generated by either photosensitizer such as Rose bengal or methylene blue with light irradiation or a phosphite ozonide which can be prepared by bubbling ozone into phosphite solution at \(-78 \, ^\circ\text{C}\).\textsuperscript{24}

\textbf{Scheme 1-1.} Some examples of chemiluminescent systems.
1.2 Mechanistic insight of the chemiluminescent decomposition of 1,2-dioxetanes

1.2.1 Thermal decomposition

The thermal fragmentation of a 1,2-dioxetane proceeds through a cycloelimination process by generating two ketone compounds, a fragment of which is in its excited state.\textsuperscript{25} The mechanism of the unimolecular and uncatalyzed decomposition of 1,2-dioxetanes has been thoroughly studied and three different mechanistic schemes have been proposed based on experiments and theoretical simulations.\textsuperscript{26,27} They are the concerted, the biradical, and the merged mechanism (Scheme 1-3). The O–O and C–C bonds break at the same time for the concerted process, which was proposed by McCapra and Turro.\textsuperscript{28,29} The biradical mechanism suggested by O’Neal and Richardson starts with the cleavage of the O–O bond as the rate limiting step to form a dioxy biradical as the real intermediate, followed by the subsequent C–C-bond cleavage, eventually leading to the formation of two carbonyl fragments.\textsuperscript{30,31} Even though the biradical mechanism has been tested to be more compatible with most experimental data in this process\textsuperscript{32} and fits the \textit{ab initio} calculation results,\textsuperscript{33} the concerted mechanism was claimed to be more accurate to account for the excited state formation and the ratio of singlet and triplet quantum yields in this transformation.\textsuperscript{27,34} Based on calculations, a merged mechanism has been proposed recently, where O–O and C–C bonds don’t cleave in a simultaneous fashion.\textsuperscript{35,36}
O–O bond breaks first but the difference is small enough that no biradical intermediate forms. The uncatalyzed thermal decomposition of 1,2-dioxetanes leads to efficient formation of triplet excited carbonyl compounds.\textsuperscript{37,27} Therefore, low chemiluminescence emission was observed in this process.

\begin{equation*}
\begin{aligned}
\text{(a)} & \quad \text{concerted} \\
\text{(b)} & \quad \text{biradical}
\end{aligned}
\end{equation*}

\textbf{Scheme 1-3.} Proposed mechanisms for unimolecular decomposition of 1,2-dioxetane, (a) the concerted mechanism, and (b) the biradical mechanism.\textsuperscript{25}

\subsection*{1.2.2 Catalyzed decomposition}
Catalyzed decomposition is different from thermal uncatalyzed fragmentation which starts by promoting the electron transfer to the dioxetane either by an intermolecular activator (ACT) or an intramolecular electron rich substituent. The singlet product is dominant for catalyzed decomposition and as a result, the chemiluminescence emission is much more efficient.\textsuperscript{38} Although the detailed mechanism of catalyzed decomposition is not perfectly understood, one that is well accepted is chemically initiated electron exchange luminescence (CIEEL) mechanism which was originally proposed by Koo and Schuster.\textsuperscript{39,40} Wilson and McCapa refined this mechanism later by replacing a full one electron with partial charge transfer.\textsuperscript{41} Recently Isobe and coworkers reported charge transfer donor molecule exists together with 1,2-dioxetane in a solvent cage.\textsuperscript{42} The ACT is essential in the intermolecular energy transfer system. The ACT forms a charge-transfer complex with the dioxetane within a solvent cage to
initiate the electron transfer from the ACT to the O–O σ* orbital of the dioxetane, and subsequently the O–O and C–C bonds break to generate a carbonyl radical anion and the radical cation of the ACT inside the solvent cavity. Then a back-electron transfer process between them leads to the formation of ACT in its electronically excited state. Upon relaxation to its ground state, the chemiluminescence emission is released (Scheme 1-4).

Scheme 1-4. Chemically initiated electron exchange luminescence (CIEEL) mechanism for activator (ACT) catalyzed intermolecular decomposition of 1,2-dioxetane.

Baader and coworkers reported that introducing electron donating groups to the dioxetane can significantly increase the chemiluminescent intensity,21 which was further confirmed by Schaap and Gagnon using a phenol substituted dioxetane, 1-(4-hydroxyphenyl)-6-phenyl-2,5,7,8-tetraoxobicyclo [4.2.0]octane (HPTBO).43 Chemiluminescence emission was greatly enhanced by removing the phenolic proton, and light can be triggered without the addition of ACT. It is believed that the charge transfer from an intramolecular electron rich group, such as a phenolate, initiates the cleavage of the O–O and C–C bonds of the four-membered dioxetane ring. A back-electron transfer step then produces the excited species which relaxes with the emission of light. The energy of this excited intermediate can be transferred to other energy acceptors, such as fluorescent dye, which results in a fluorescent emission. The detailed process has been outlined with the example of spiroadamantane-1,2-dioxetane (Scheme 1-5).43 While there have been many
recent advances in the mechanistic studies of 1,2 dioxetanes, the exact mechanism of light emission has not yet been elucidated.

Scheme 1-5. CIEEL mechanism for the catalyzed intramolecular decomposition of spiroadamantane-1,2-dioxetane.

Based on this discovery, coupled with the stable effect of a bulky adamantane group, Schaap and coworkers developed some 1,2-dioxetane derivatives containing masked phenolate groups (Scheme 1-6).\textsuperscript{44,45} Efficient chemiluminescence can be triggered by the deprotection of the phenolate masking group by the action of specific chemical or enzymatic deprotecting agents. A simple example is chemiluminescence that can be produced by using tetrabutyl ammonium fluoride to de-mask the tert-butylsilyl from a phenolate. Other masking groups such as acetate and phosphate have also been reported by the same group.\textsuperscript{44,45}
1.3 Triggered chemiluminescence from 1,2-dioxetanes

Schaap and coworkers pioneered this area with the first report of chemical and enzymatic triggering chemiluminescence from 1,2-dioxetanes in 1987.44,45 Tremendous progress and numerous applications in this field have occurred since then. The intrinsic low background signal of this imaging modality in combination with high specificity of the target recognition unit catered for a specific target have dramatically expanded the use of chemiluminescence for analytical and biomedical programs.

These strategies of chemically or enzymatically46 deprotecting the phenolate forms the basis of the notion that by changing substituents on phenoxy-dioxetanes, efficient chemiluminescence can be triggered by target molecules through desired transformations with high specificity. It also laid the foundation for the preparation of target-directed chemiluminescent probes for bio-imaging. Furthermore, a 1,2-dioxetane bearing a meta-substituent on phenoxy-dioxetanes gives better chemiluminescent efficiency.47,48 Even though triggered 1,2-dioxetane seems an ideal platform for the design and preparation of chemiluminescent probes for in vivo imaging, the low chemiluminescent quantum yield of this scaffold under physiological conditions has largely hindered its further biological applications. Scientists have invested considerable efforts in the development of better scaffolds and systems to translate this imaging modality into living systems during last two decades. Initial attempts to
apply this scaffold for biological applications was realized by the incorporation of a polymeric enhancer solution to improve chemiluminescent intensity by creating a hydrophobic pocket to reduce the water initiated quenching effect and a fluorescent dye to red-shift luminescence emission.

Shabat and coworkers recently reported several methods to increase the chemiluminescent quantum yield and red-shift the scaffold emission using intramolecular energy transfer and direct chemical modifications of the core structure. Their methods centered around the introduction of electron withdrawing groups, efficient fluorophores, and direct NIR dye conjugations to provide new ways to increase the chemiluminescence quantum yields and prepare bright chemiluminescent agents for biological applications without any other additives. With the introduction of an electron withdrawing group at the ortho-position to the reactive site, the chemiluminescent intensity was significantly increased. Particularly, with the ortho-acyronitrile substituent, they increased the chemiluminescent quantum yield by 3000-fold in aqueous solution and the chemiluminescence emission reached approximately the same level compared with the enhancer incorporated systems. Based on these structural advancements, several in vivo or cellular reaction-based chemiluminescent probes have been reported, including β-galactosidase, H₂O₂, Cathepsin B, and peroxynitrite (Scheme 1-7). With these improvements and further structural and system optimizations, we are optimistic about the future application of this imaging modality.
Scheme 1-7. Some examples of reaction-based chemiluminescent agents.

1.5 Reaction-based probes for interrogating biological species

Living systems are a complex collection of an enormous variety of biomolecules including organic compounds, inorganic species, and polymers that together maintain a dynamic environment with a great number of transformations. An overarching goal in biology is to decipher detailed functions of each compound in living systems. To this end, scientists have proposed many specific methods to monitor the behavior of biomolecules. Among them, optical imaging methods have received tremendous attention during the last three decades, which provide powerful tools to interrogate biological systems with high spatiotemporal resolution. Molecular imaging techniques have tremendously advanced our understanding of the dynamics of biomolecules in a complex environment. Reaction-based probes, which can be defined as molecules that react specifically with a target biomolecule to induce a concomitant
photochemical change have stimulated an explosion of biological discoveries over the past three decades. Roger Tsien and coworkers pioneered this field by the development of reaction-based probes for metal cations especially calcium cation (Ca$^{2+}$). Their design strategy was to link a Ca$^{2+}$ chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid, to a fluorophore which displayed a wavelength change upon Ca$^{2+}$ binding, enabling a ratiometric readout.$^{53}$ A lot of photophysical processes have been explored to construct these imaging agents, such as fluorescence resonance energy transfer (FRET)$^{54}$, intramolecular charge transfer (ICT)$^{55}$ and photo-induced electron transfer.$^{56}$ Therefore, a considerable amount of fluorescent on-off and ratiometric chemosensors for pH and metal cations, such as zinc, iron and copper have been reported since then. Later, a myriad of organic transformations has been applied for the development of reaction-based probes. Nagano and co-workers used cycloaddition for singlet oxygen ($^1$O$_2$) detection,$^{57}$ and the same group discovered that aromatic vicinal diamines can react with nitric oxide (NO) in the presence of oxygen, resulting the development of diaminofluoresceins.$^{58}$ Chang and coworkers utilized H$_2$S mediated azide reduction for H$_2$S detection$^{59}$ and they also pioneered H$_2$O$_2$ imaging using oxidative cleavage of boronic ester.$^{60}$ Reaction-based probes have also been used to monitor enzymes such as galactosidase,$^{61}$ and reductive enzymes$^{62}$ (Scheme 1-8). These reaction-based probes help us better understanding the dynamics of these species in living systems. Significantly, the existence and dynamics of some transient species, such as reactive sulfur, oxygen, and nitrogen (RSON) species have been visualized for the first time, which helps us better understand their detailed productions, distributions and functions in health and pathological state. Trying to reveal more biological details of these reactive species motivates chemists to make state-of-the-art imaging probes with high sensitivity and specificity. There are several stringent criteria that need to be fulfilled for
reaction-based probe development. (1) The reaction should be specific and selective between probe and target, which means that the probe should respond selectively to its intended target in a complex biological system without interference from any other species. (2) Reasonable reaction kinetics under physiological conditions between probe and target is required. (3) The probe and product produced from this reaction have to be biocompatible. Relying on the specific reactivity between an organic functionality and an analyte of interest and various methods to tune chemical reactivity, many probes have been used to interrogate the biological details of biomolecules, which as a result has significantly advanced biology research and also laid the foundation for subfields of chemical biology research.
Scheme 1-8. Examples of some reaction-based probes for (a) Ca$^{2+}$, (b) pH, (c) NO, (d) $^{1}$O$_{2}$, (e) H$_{2}$S, (f) H$_{2}$O$_{2}$, (g) galactosidase, and (h) quinone oxidoreductase-1.

1.6 In vivo imaging

Seeing is believing. Optical imaging methods have greatly enabled us to “see” the biological processes at the cellular and molecular level. Among them, magnetic resonance imaging (MRI), positron emission tomography (PET), fluorescence and bioluminescence$^{63}$ are commonly used imaging modalities for in vivo imaging both in clinical and academic settings.
1.6.1 PET/MRI

PET is an imaging technique that detects the signal from radiotracer to observe metabolic processes in the body. MRI uses magnetic resonance techniques to form anatomic images. Both modalities have been used in clinical settings for diagnostic purposes. PET imaging gives high sensitivity and molecular information and MRI provides high spatial and contrast resolution information. In a clinical setting, these two methods have been combined in a frequent fashion to improve diagnostic performance. These two imaging modalities are primarily used for revealing anatomical, physiological and metabolic parameters, and are not quite suitable as laboratory tools to explore cellular or molecular details. Furthermore, despite their great potential in clinical studies, these two methods normally require expensive instrumentations and highly trained personnel, which limits their access to basic research programs.

1.6.2 Fluorescence

With the discovery and development of green fluorescent protein (GFP) from jellyfish, Aequoria Victoria, fluorescence imaging has significantly contributed to the advancement of biological research. GFP emits at a wavelength of ~510 nm when irradiated at ~470 nm. GFP has been introduced into many systems as a fusion tag to study biological processes such as gene expression and protein localization and movement. The important contribution of GFP to biological study was recognized by the 2008 Nobel Prize in chemistry. Small fluorescent dyes, such as fluorescein and rhodamine have also facilitated the study of biological systems. Target-specific fluorescent probes have enabled us to monitor the detailed biological processes of single species with high specificity and resolution. The general concept behind these fluorescent proteins and small fluorophores are that these molecules are emissive upon irradiation at specific
wavelength. The imaging can be formed by collecting the emission wavelength from these molecules or by calculating the ratio at two different wavelengths. Under some circumstances, small fluorescent agents are favored compared to GFP because (1) their small size would cause small interference in living systems; (2) they are easier to implement without the requirement of gene engineering; and (3) they can be easily modified to tune the wavelength, sensitivity and selectivity. Tsien, Nagano, Chang and numerous other labs have greatly advanced in vivo reaction-based fluorescence imaging, resulting in a great amount of state-of-the-art reaction-based fluorescent probes for reactive species, metal ions, and enzymes. Some of these probes have been successfully applied for elucidating new biological details in vivo. These reaction-based fluorescent agents have become indispensable tools in modern biology because they provide dynamic information regarding the quantity and localization of an analyte of interest. In order to translate this imaging modality for deeper tissue imaging, there has been an explosion of reporters of NIR fluorescent probes due to the beneficial effect of red light. NIR fluorophores normally emit above 650 nm. The light in this range is less scattered and less absorbed by tissue and biomolecules. With less light scattering and autofluorescence, it can be applied for deep tissue imaging (> 0.5 cm). NIR fluorescence image-guided surgery is becoming a popular area.70 Although fluorescence imaging has revolutionized basic research and the NIR fluorescence imaging has increased tissue penetration, signal-to-noise ratios and decreased the light scattering and autofluorescence, they unfortunately suffer some disadvantages associated with the excitation which still generates light scattering, and phototoxicity. Furthermore, it requires researchers to know when and where to shine light first, which makes it challenging to explore unknown biological processes.
1.7 Research objectives

This dissertation aims to solve the problems associated with current imaging modalities and establish chemiluminescence as a versatile imaging modality for in vivo study. Our approach involves the design and synthesis of reaction-based chemiluminescent probes for defined analytes. The light generated directly from chemical reactions offers some unique advantages, such as low background emission and reduced phototoxicity. We have reported CHS-3 for H₂S, HyCL-2 for hypoxia and both of them demonstrate great potential for in vivo applications. Furthermore, CHS-3 is the first demonstration of using chemiluminescence for imaging analytes in wild-type animals in this field. Recently we have reported a ONOO⁻ probe PNCL using a selective oxidative decarbonylation reaction. PNCL has been successfully applied for detecting endogenously produced ONOO⁻ in enhancer free conditions. Overall, the goal is to demonstrate that chemiluminescence holds great potential for in vivo imaging and we are confident that with the continuous improvement of this technique, it will be widely applied for visualization of biological processes without problems associated with fluorescence and bioluminescence imaging methods.
1.8 References


CHAPTER 2
CHEMILUMINESCENT PROBES FOR IMAGING H₂S IN LIVING ANIMALS

2.1 Introduction

Hydrogen sulfide (H₂S) with the odor of rotten egg has long been viewed as a toxic gas to biological systems.¹ However, accumulating evidence revealed that in addition to being a toxic geological and environmental pollutant, H₂S is also produced and distributed in mammalian systems in a controlled fashion,² indicating its underestimated but broad biological functions in mammalian physiology and pathology.³ H₂S is a weak acid with pKₐ¹ and pKₐ² of 6.9 and >12 and HS⁻ is the main species at physiological conditions (pH=7.4).⁴ The two electron redox potential of H₂S is +0.17 V at pH 7.0 coupled to a standard hydrogen electrode (SHE).⁵ The general realization of the biological functions of H₂S started in the 1990s, when Abe and Kimura reported that H₂S functions as a neuromodulator in hippocampal long-term potentiation.⁶ Since then, H₂S received tremendous attention in scientific research fields. However, the biological concentration of H₂S remains controversial and has over a 10⁵-fold range with reported studies.⁷ H₂S is mainly produced by two general pathways in physiological conditions. One involves non-enzymatic processes where H₂S can be generated from sulfur containing compounds including glucose, glutathione (GSH), inorganic and organic
polysulfides. The other involves enzymatic processes and three enzymes, cystathionine gamma lyase (CSE), cystathionine beta synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) are involved in the generation of endogenous H$_2$S by utilizing sulfur containing biomolecules such as cysteine and homocysteine (Hcy) (Scheme 2-1). These enzymes have been reported to be organ specific, and H$_2$S has been found in most tissues of living systems, including vasculature, brain, lungs, liver, and pancreas, revealing its widespread physiological roles in living systems. For example, it can be produced in neural systems to mediate neural communication, and it also plays active role in the cardiovascular system and redox regulation (Figure 2-1). However, misregulated H$_2$S production and distribution are associated with various diseases, such as hypertension, diabetes, Down’s syndrome, Alzheimer’s disease and cancers. Considering the multifaceted biological roles of H$_2$S and physiological implications of H$_2$S, new biocompatible tools for detection and monitoring the production, trafficking, and consumption of this gasotransmitter in living cells and animals are needed.
Scheme 2-1. Enzymatic pathways for H$_2$S generation.

Figure 2-1. General biological functions of H$_2$S.
Traditional methods for H$_2$S detection, including the methylene blue assay, high performance liquid chromatography, mass spectrometry, gas chromatography, and electrochemical methods are all unable to provide real time measurement of H$_2$S in intact living organisms with high sensitivity. Over the past decade, reaction-based fluorescent probes for H$_2$S detection have received tremendous attention, which enables us to detect H$_2$S in live cells with higher spatiotemporal resolution and greater selectivity. These reaction-based methods make use of the chemical reactivity of H$_2$S, including nucleophilicity, metal precipitation and reductivity. However, the drawbacks of fluorescence imaging associated with autofluorescence, light scattering, phototoxicity, and probe kinetics impedes its application for study H$_2$S in living animals. A chemiluminescent platform could provide a unique solution to these problems. Direct light production from a chemical reaction provides this imaging modality with some unique advantages. In order to expand the toolbox for reaction-based chemiluminescent probes for H$_2$S and overcome the limitations of current H$_2$S detection methods, we developed three chemiluminescent probes for rapid and selective imaging of H$_2$S in living animals.

The chemiluminescence emission can be initiated by the encounter of the probe and the analyte of interest. Monitoring the generation and intensity of the chemiluminescence emission enables us to determine the presence and concentration of the target molecule and provides a highly sensitive and selective readout. Unlike fluorescent detection methods, no external light excitation is needed for triggering chemiluminescence emission, which can drastically reduce background from autofluorescence and light scattering. Bioluminescence, a subtype of chemiluminescence,
has received widespread attention for its potential value for \textit{in vivo} imaging. Although a number of bioluminescent probes for living animal imaging have been reported to date\textsuperscript{30}, their potential applications are largely hindered by the need of enzymatic additive or genetic modification\textsuperscript{31}.

While chemiluminescent methods have been extensively applied for immunoassays\textsuperscript{32}, enzymatic analyte detection\textsuperscript{33}, reaction-based chemiluminescent probes for \textit{in vivo} imaging remain rare. Herein, we report three reaction-based chemiluminescent \( \text{H}_2\text{S} \) probes, \textbf{CHS-1}, \textbf{CHS-2}, and \textbf{CHS-3} based on spiroadamantane 1,2-dioxetane. The design principle, synthetic strategies, chemiluminescent response and selectivity to \( \text{H}_2\text{S} \), cellular \( \text{H}_2\text{S} \) detection, and living animal imaging will be outlined in this chapter.

\begin{center}
\textbf{Scheme 2-2.} Mechanism of \( \text{H}_2\text{S} \) mediated aromatic azide reduction in aqueous solution.
\end{center}
2.2 Results and discussion

2.2.1 Design principle and synthesis of CHS-1, CHS-2, and CHS-3

CHS probes were designed by linking a chemiluminescent scaffold, spiroadamatane 1,2-dioxetane with phenyl azide through a carbonate linkage. H₂S first reduces the azide. The mechanism of this H₂S mediated azide reduction has been reported by the Pluth group. This reaction is first-order for both reactants and two equivalents of HS⁻ are needed (Scheme 2-2). After reduction, a self-immolative carbonate cleavage yields the free phenolate. Chemiluminescence emission can be triggered upon decomposition of this phenolate through CIEEL mechanism (Scheme 2-3). This newly generated chemiluminescence emission can be detected directly or by donating its energy to other molecules such as quantum dots or fluorescent dyes. Emerald II Enhancer, containing a cationic polymer and a dye, was adapted in our study as an energy acceptor, which not only red shifts the luminescence emission but also increase luminescent intensity by creating a hydrophobic environment within the cationic polymer.
We achieved a modular synthetic route to access the probes CHS-1, CHS-2 and CHS-3 with some optimizations of a literature procedure\(^\text{39}\) (Scheme 2-4). The synthesis started with treating unsubstituted, fluorinated, and chlorinated 3-methoxybenzaldehyde derivatives 1a–c with trimethyl orthoformate in the presence of \(p\)-toluenesulfonic acid to afford acetals 2a–c. These acetals reacted with triethyl phosphite and boron trifluoride diethyl etherate to give the diethyl methoxy (3-methoxyphenyl) methyl phosphonates 3a–c. Then phosphonates 3a-c went through Horner–Wadsworth–Emmons reaction with \("\text{BuLi}\) and 2-adamantanone to afford enol ethers 4a–c.\(^\text{40}\) Nucleophilic demethylation using sodium ethanethiolate gave the phenols 5a–c. The activated ester 6, prepared according to a
literature procedure,$^{41,42}$ was coupled to the phenols to afford 7a–c. Finally, a [2+2] cycloaddition was conducted by bubbling oxygen through a solution of 7a–c and the sensitizer Rose bengal with light irradiation, giving the final chemiluminescent probes CHS-1, CHS-2, and CHS-3.

2.2.2 Response and selectivity

After obtaining these chemiluminescent probes, we started to characterize their luminescent responses and sensitivity towards H$_2$S using an F-7000 Hitachi fluorometer. Each probe (40 µM) displayed instantaneous light production after treating with H$_2$S in the presence of 20% Emerald II Enhancer in 20 mM HEPES buffer (pH 7.4). The luminescence emission gradually increased over a time course of 10 minutes in a dose-dependent manner (Figure 2-2). The light emission could last for more than an hour (Figure 2-3). The emission spectra showed two peaks. The major peak centred at 545 nm corresponding to the dye in the Emerald II Enhancer, and the small one at 450 nm was from the emission of the decomposition of phenolate (Figure 2-1, insets), indicating that the luminescence mostly donates to the acceptor. We observed that CHS-1, CHS-2 and CHS-3 give 5-fold, 4-fold, and 12-fold luminescence turn-on respectively using integrated luminescent intensity under physiologically relevant conditions. No background corrections were performed in our study in order to accurately compare the luminescent response between probes. The chemiluminescent response towards H$_2$S at pH 10 was also investigated. CHS-1 displayed the highest chemiluminescence emission, resulting in a 7-fold turn-on in 10 minutes (Figure 2-4), while CHS-2 and CHS-3 gave 2-
fold and 3-fold luminescent turn-on respectively. This experiment demonstrated that pH is an important trait in regulating the luminescence emission.

**Figure 2-2.** Time scans of the chemiluminescence emission at 545 nm from (a) 40 µM CHS-1, (b) 40 µM CHS-2, or (c) 40 µM CHS-3 and 0, 5, 10, 20, 40, 80, 100, 150, 200 µM Na₂S in 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer. Insets are chemiluminescence spectra of (a) 40 µM CHS-1, (b) 40 µM CHS-2, and (c) 40 µM CHS-3 and 200 µM Na₂S, acquired immediately after adding probes.
Figure 2-3. Long time scan of the chemiluminescence emission at 545 nm of 40 µM CHS-3 to 200 µM H₂S in 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer.

Figure 2-4. Time scans of the chemiluminescence emission at 545 nm from (a) 40 µM CHS-1, (b) 40 µM CHS-2, or (c) 40 µM CHS-3 and 0 µM (red) or 200 µM (black) H₂S in 100 mM glycine buffer (pH 10.02) containing 20% Emerald II Enhancer.

After demonstrating that CHS probes can sensitively detect H₂S at physiologically relevant pH, we next tested the selectivity of CHS-1, CHS-2, and CHS-3 for H₂S against other biologically relevant RSON species. The selectivity test was carried out by treating 40 µM CHS-1, CHS-2 and CHS-3 with different amount of RSON species in 20 mM HEPES buffer (pH 7.4). 5 mM reduced GSH, 1 mM L-cysteine and Hcy, and 200 µM of S-nitrosglutathione, sulfite (SO₃²⁻), hydrogen peroxide (H₂O₂), hypochlorite (OCl⁻), tert-butyl hydroperoxide (tBuOOH), nitroxyl (HNO), nitric oxide (NO), and nitrite (NO₂⁻) were used for evaluation. None of the other species tested displayed significant increases in luminescence intensity over the blank control over a time course of 10 minutes.
Furthermore, the response to Na$_2$S was also tested in the presence of GSH, L-cysteine, and Hcy. No significant chemiluminescent change was observed (Figure 2-5). These selectivity data showed that CHS-1, CHS-2, and CHS-3 are capable of detecting H$_2$S selectively without interference from completing analytes.

**Figure 2-5.** Chemiluminescent responses of (a) 40 µM CHS-1, (b) 40 µM CHS-2, or (c) 40 µM CHS-3 to biologically relevant RSON species in 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer. Bars represent chemiluminescence emission at 545 nm and 30, 60, 120, 240, 360, 480 and 600 second after addition of RSON species. Data shown are for 5 mM GSH, 1 mM cysteine and Hcy, and 200 µM for other RSON species. Legend: (1) Na$_2$S; (2) Na$_2$S, GSH, L-cysteine, and Hcy; (3) GSH; (4) S-nitrosoglutathione; (5) L-cysteine; (6) Hcy; (7) HNO; (8)
NO; (9) NaNO₂; (10) Na₂SO₃; (11) H₂O₂; (12) NaClO; (13) tBuOOH; (14) Blank. Error bars are ± S.D.

2.2.3 Mechanistic studies of chemiluminescent H₂S detection

In order to gain insight into the structure details regarding the chemiluminescent intensity and guide us to prepare better chemiluminescent probes, we started to study the equilibrium of phenol/phenolate using pKₐ of phenol, 2-fluorophenol, and 2-chlorophenol as an approximation due to the difficulty of measuring the pKₐ of these phenoxy-dioxetanes. We integrated chemiluminescence emission of CHS-1, CHS-2, and CHS-3 in response to 200 µM Na₂S at pH 7.4 and pH 10 over a time course of 10 min (Figure 2-6a,b). We plotted the integrated chemiluminescent intensity against the pKₐ values of phenol, 2-fluorophenol, and 2-chlorophenol (Figure 2-6c,d). We observed that at pH 7.4 the chemiluminescent intensity increased when lowering the pKₐ which corresponds to higher ratio of phenolate to phenol (Figure 2-6c), while no direct correlation was observed between pKₐ and the chemiluminescent intensity at pH 10 (Figure 2-6d). To further investigate this correlation, we performed a quantum chemical calculation by calculating the phenolate structures from CHS-1, CHS-2, and CHS-3 (Tables S2-1–S2-3). Density functional theory (DFT) at the B3LYP/6-311+G(d,p) level of the theory was applied to optimize the geometries, and the charge on each atom was calculated by electrostatic potential (ESP) model at the M06/6-311+G(d,p) level of theory. The solvent effect of water was modeled by the integral equation formalism polarizable continuum model (IEF-PCM) using Gaussian 09 package. The computational calculation results indicated that the charge on the phenolic oxygen (O₈) determines the chemiluminescent intensity at pH 10 (Figure 2-6f), while there was no such correlation at pH 7.4 (Figure 2-6e). This result was further verified by ESP charge calculations performed with B3LYP and ωB97XD functions (Figure 2-11). These data
indicated that at lower pH, the chemiluminescence emission was determined by the ratio of phenolate and phenol consistent with the CIEEL mechanism. However, the negativity of O8 is responsible for the chemiluminescent intensity at pH 10, which can be explained by the fact that negative charge promotes the electron transfer step of CIEEL.44

Figure 2-6. Analysis of chemiluminescent responses. (a)–(b) Bar graphs for the integrated chemiluminescence emission over 10 min at (a) pH 7.4 or (b) pH 10 of CHS-1, CHS-2, and CHS-3 to 0 µM Na₂S (grey bars) and 200 µM Na₂S (colored bars). (c)–(d) Plot of the integrated chemiluminescence emission over 10 min at (c) pH 7.4 or (d) pH 10 of 200 µM Na₂S and 40 µM CHS-1 (red), CHS-2 (green) and CHS-3 (blue) versus experimental pKa values of model compounds phenol, 2-fluorophenol, and 2-chlorophenol. (e)–(f) Plot of the integrated chemiluminescence emission over 10 min at (e) pH 7.4 or (f) pH 10 of 200 µM Na₂S and 40 µM CHS-1 (red), CHS-2 (green) and CHS-3 (blue) versus the calculated atomic charge on the phenolate oxygen (O8). All luminescent measurements were acquired in 20 mM HEPES buffer (pH 7.4) or 100 mM glycine buffer (pH 10) containing 20% Emerald II Enhancer. The reported values are averages of the integrated emission intensities over 10 min (n = 4–7). Error bars represent ± S.D. The ESP atomic charges were calculated at the M06/6-311+G(d,p) level of the theory using geometries optimized at the B3LYP/6-311+G(d,p) level of theory. Calculations
were carried out with the IEF-PCM water solvation model using the Gaussian 09 program package. (Computational work was conducted by Dr. Tao and coworkers)

2.2.3 Using CHS-3 to detect cellular H\textsubscript{2}S

We next applied our probe to detect cellularly generated H\textsubscript{2}S in human lung adenocarcinoma epithelial cells (A549). CHS-3 was chosen to perform cellular H\textsubscript{2}S detection due to its most robust luminescence emission at physiological pH. A549 expresses CSE\textsuperscript{45} which can utilize Hcy to produce H\textsubscript{2}S. Before the cell experiment, we first used CHS-3 to detect exogenous H\textsubscript{2}S using a plate reader by loading a 96 well plate with different amounts of H\textsubscript{2}S (Figure 2-7a). The luminescent intensity increased with increasing concentration of H\textsubscript{2}S, demonstrating that CHS-3 is capable of sensitively detecting Na\textsubscript{2}S in a multi-well plate reader format. The detection limit was estimated to be 5.4 µM.

A549 cells were first incubated with Hcy, followed by the addition of CHS-3 and Emerald II Enhancer, and then the plate was subjected to plate reader test. The cells treated with Hcy resulted in ~10% luminescent increase (n = 12, p = 0.044) compared with DI-H\textsubscript{2}O treated control (Figure 2-7b). An enzyme inhibition experiment was also conducted by treating the cell with DL-propargylglycine (PAG), a CSE inhibitor, 20 minutes prior to Hcy addition. A slightly decreased luminescence emission was observed, confirming that CHS-3 can be applied for detecting cellularly generated H\textsubscript{2}S.
Figure 2-7. Detection of cellular H\textsubscript{2}S using a multi-well plate reader. (a) Luminescent responses of 40 µM CHS-3 and 0, 25, 50, 100, and 200 µM Na\textsubscript{2}S in 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer. (b) A549 cells were treated with a vehicle control (DI-H\textsubscript{2}O), 200 µM Hcy, or 200 µM Hcy after being pre-treated with 200 µM PAG (Hcy + PAG) for 20 min. 20 min after incubating with Hcy or vehicle, the cells were washed and treated with 40 µM CHS-3 and 125 µL Emerald II Enhancer. The reported values represent the average luminescent intensity of replicate experiments (n = 12, p = 0.044). Error bars represent ± S.E.M.

2.2.4 In vivo imaging of H\textsubscript{2}S using CHS-3

After demonstrating that CHS-3 is capable of detecting cellular H\textsubscript{2}S, we started to evaluate the ability of CHS-3 to image H\textsubscript{2}S in living animals using an IVIS Spectrum. We first applied CHS-3 to image H\textsubscript{2}S at physiological pH using an opaque 96-well plate by loading with different amount of Na\textsubscript{2}S in 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer. A dose dependent luminescent response was observed (Figure 2-8a) with good linearity in the range of 0–200 µM of H\textsubscript{2}S (Figure 2-8b). After knowing CHS-3 is capable of sensitive and accurate imaging of H\textsubscript{2}S by IVIS Spectrum, we next determined if light production from CHS-3 was sufficient to be observed through mammalian tissue in a mouse carcass model. CHS-3 was injected into the peritoneal cavity of the sacrificed SCID/BALB-C mice without H\textsubscript{2}S (Figure 2-9a,b) as a vehicle control (DI-H\textsubscript{2}O) or with 0.4 µmol H\textsubscript{2}S (Figure 2-9c,d). The carcass injected with H\textsubscript{2}S showed a clear and significant luminescent increase (Figure 2-9e). This important experiment demonstrated that luminescence generated from CHS-3 was sufficient enough to
penetrate mammalian tissue. Finally, we proceeded to apply **CHS-3** to image H$_2$S in living animals. A mixture of 0.08 µmol **CHS-3** and 0.4 µmol Na$_2$S in HEPES buffer containing 20% Emerald II Enhancer was administrated via i.p. injection into the peritoneal cavity of C6 brown mice (Figure 2-10a). For a vehicle control, Na$_2$S was replaced with same volume of DI-H$_2$O (Figure 2-10b). Images were acquired 30 second after injecting. The mice treated with Na$_2$S displayed ~4-fold luminescent increase compared with vehicle control (Figure 2-10c), demonstrating that **CHS-3** is capable of detecting H$_2$S in living mice.

![Figure 2-8](image)

**Figure 2-8.** Imaging H$_2$S using **CHS-3**. (a) Images 30 sec after adding 40 µM **CHS-3** to 0, 25, 50, 100, and 200 µM Na$_2$S in 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer (n = 3). (b) Plot of total photon flux versus H$_2$S concentration for the experiments described in (a).
Figure 2-9. Imaging H₂S in SCID/BALB-C mouse carcasses using CHS-3. Images were obtained 30 seconds after administering an i.p. injection of 0.08 µmol CHS-3 and (a–b) vehicle control (DI-H₂O) or (c–d) 0.4 µmol Na₂S in 100 µL 20 mM HEPES at pH 7.4 containing 20% Emerald II Enhancer. (e) Quantification of the total photon flux from experiments described in (a)–(d). Error bars are ± S.D.
Figure 2-10. (a)–(b) Images of living C6 brown mice 30 second after administering i.p. injections of 0.08 µmol CHS-3 (and (a) vehicle control (DI-H2O) or (b) 0.4 µmol Na2S in 100 µL 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer. (c) Quantification of the total photon flux from three replicates of the experiments described in (a) and (b). Statistical analyses were performed with a two-tailed Student's t-test (n = 3, p = 0.025). Error bars are ± S.D.

2.3 Conclusions

Three reaction-based 1,2-dioxetane chemiluminescent H2S probes, CHS-1, CHS-2, and CHS-3 have been designed and synthesized and all of them displayed instant light production when reacting with H2S at physiological pH with good sensitivity and selectivity. These reagents have been applied for H2S detection in vitro via spectrophotometers, multi-well plate readers, and IVIS Spectrum instruments. By collaborating with Dr. Tao, we have developed a general platform for the prediction of the performance of spiroadamantane stabilized phenoxy-dioxetane probes based on the pKₐ of the phenol and the negative charge on the phenolic oxygen (O8). Finally, CHS-3
has been successfully applied for cellular H₂S detection and has been demonstrated to be capable of imaging H₂S in living animals.

2.4 Experimental section

2.4.1 Synthetic procedures

All reactions were performed in dried glassware under an atmosphere of dry N₂. Silica gel P60 (SiliCycle) was used for column chromatography and SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick) was used for analytical thin layer chromatography. Plates were visualized by fluorescence quenching under UV light or by staining with iodine. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), EMD Millipore (Billerica, MA), Oakwood Chemical (West Columbia, SC), and Cayman Chemical (Ann Arbor, MI) and used without further purification. ¹H NMR and ¹³C NMR spectra for characterization of new compounds and monitoring reactions were collected in CDCl₃ (Cambridge Isotope Laboratories, Cambridge, MA) on a JEOL 500 MHz spectrometer in the Department of Chemistry at Southern Methodist University. All chemical shifts are reported in the standard notation of parts per million using the peak of residual proton signals of the deuterated solvent as an internal reference. Coupling constant units are in Hertz (Hz) Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; dt, doublet of triplets. High resolution mass spectroscopy was performed on a Shimadzu IT-TOF (ESI source) and low resolution mass spectroscopy was performed on a Shimadzu LCMS-8050 Triple Quadrupole LCMS (ESI source) or a Shimadzu Matrix Assisted Laser Desorption/Ionization MS (MALDI) at the Shimadzu Center for Advanced Analytical Chemistry at the University of Texas, Arlington.
**Diethyl(methoxy(3-methoxyphenyl)methyl)phosphonate (3a).** 3-Methoxybenzaldehyde (1.83 mL, 15.0 mmol, 1.0 equiv), trimethyl orthoformate (1.65 mL, 15.0 mmol, 1.0 equiv) and p-toluenesulfonic acid (258 mg, 1.50 mmol, 0.10 equiv) were added to a dry round-bottom flask and flushed with N₂. The reaction contents were dissolved in 6.0 mL of MeOH. The reaction proceeded for 24 h at rt, and was then neutralized with NEt₃. After neutralization, the crude mixture was poured into 30 mL saturated aq NaHCO₃. The layers were separated and the aqueous layer was washed with an additional 2 x 40 mL EtOAc. The combined organic layers were collected and dried over Na₂SO₄, filtered, and concentrated to yield the crude acetal 2a (2.52 g). Compound 2a (1.94 g, 10.7 mmol, 1.0 equiv) was dissolved in 10.0 mL CH₂Cl₂, and triethyl phosphite (1.89 mL, 11.0 mmol, 1.03 equiv) and boron trifluoride etherate (1.38 mL, 11.0 mmol, 1.03 equiv) were added dropwise at 0 °C. Once all the reagents were added, the reaction was heated to 30 °C and allowed to react for 1 h under N₂ atmosphere. The reaction was quenched with 20 mL saturated aq NaHCO₃, extracted with 2 x 30 mL EtOAc, and evaporated under reduced pressure. Purification by column chromatography (40%–100% EtOAc/hexanes) afforded 3a as a pale yellow oil (2.332 g, 89%). ¹H NMR (500 MHz, CDCl₃) δ 7.26 (t, 1H, J = 8.0 Hz), 6.97–7.02 (m, 2H), 6.85 (d, 1H, J = 8.0 Hz), 4.47 (d, 1H, J = 15.5 Hz), 3.90–4.13 (m, 4H), 3.81 (s, 3H), 3.38 (s, 3H), 1.20–1.29 (m, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 159.63, 135.94, 129.31, 120.43, 114.31, 112.96, 80.52, 79.71, 63.10 (d, J = 7.2 Hz), 62.93 (d, J = 7.2 Hz), 58.76 (d, J = 14.3 Hz), 55.22, 16.40 (d, J = 5.9 Hz), 16.33 (d, J = 5.9 Hz); HRMS calcd for C₁₃H₂₁O₅P (M+Na⁺) 311.1019, found 311.1013.
Diethyl((4-fluoro-3-methoxyphenyl)(methoxy)methyl)phosphonate (3b). 4-Fluoro-3-methoxybenzaldehyde (1.0 g, 6.5 mmol, 1.0 equiv), trimethyl orthoformate (0.71 mL, 6.5 mmol, 1.0 equiv) and p-toluenesulfonic acid (111.9 mg 0.6498 mmol, 0.10 equiv) were dissolved in 4.0 mL of MeOH. The reaction proceeded for 24 h at rt, and was then neutralized with NEt₃. After neutralization, the crude mixture was poured into 30 mL saturated aq NaHCO₃. The layers were separated and the aqueous layer was washed with an additional 2 x 40 mL EtOAc, the combined organic layers were collected and dried over Na₂SO₄, filtered, and concentrated to yield crude acetal 2b (1.25 g). Compound 2b (1.25 g, 6.25 mmol, 1.0 equiv) was dissolved in 6.0 mL CH₂Cl₂, and triethyl phosphite (1.10 mL, 6.44 mmol, 1.0 equiv) and boron trifluoride etherate (0.80 mL, 6.4 mmol, 1.0 equiv) were added dropwise at 0 °C. Once all the reagents were added, the reaction was heated to 30 °C and allowed to react for 1 h under N₂ atmosphere. The reaction was quenched with 20 mL saturated aq NaHCO₃, extracted with 2 x 30 mL EtOAc, and evaporated under reduced pressure. Purification by column chromatography (40%–100% EtOAc/hexanes) afforded 3b as a yellow oil (1.247 g, 66%). ¹H NMR (500 MHz, CDCl₃) δ 6.92 (dt, 1H, J = 8.6, 2.3 Hz), 6.83 (dd, 1H, J = 10.9, 2.3 Hz), 6.71–6.75 (m, 1H), 4.26 (d, 1H, J = 15.5 Hz), 3.80–3.91 (m, 4H), 3.68 (s, 3H), 3.17 (s, 3H), 0.95–1.10 (m, 6H); ¹³C NMR (125 MHz, CDCl₃) 152.58, 150.65, 147.02 (d, J = 10.7 Hz), 130.20, 119.90 (d, J = 7.2 Hz), 114.95 (d, J = 19.1 Hz), 112.11, 79.84, 78.49, 62.41 (d, J = 7.2 Hz), 62.18 (d, J = 7.2 Hz), 58.32 (d, J = 14.3 Hz), 55.36, 15.70 (d, J = 5.9 Hz), 15.64 (d, J = 5.9 Hz); HRMS calcd for C₁₃H₂₀FO₅P (M+Na⁺) 329.0925, found 329.0931.
Diethyl((4-chloro-3-methoxyphenyl)(methoxy)methyl)phosphonate (3c). 4-Chloro-3-methoxybenzaldehyde (400 mg, 2.34 mmol, 1.0 equiv), trimethyl orthoformate (256 µL, 2.34 mmol, 1.0 equiv) and p-toluenesulfonic acid (40.4 mg 0.234 mmol, 0.10 equiv) were added to a dry flask and flushed with N₂. The reaction contents were dissolved in 1.5 mL of MeOH. The reaction proceeded for 24 h at rt, and was then neutralized with NEt₃. After neutralization, the crude mixture was poured into 20 mL saturated aq NaHCO₃. The layers were separated and the aqueous layer was washed with an additional 2 x 30 mL EtOAc, the combined organic layers were collected and dried over Na₂SO₄, filtered, and concentrated to yield crude acetal 2c (474 mg). Compound 2c (474 mg, 2.19 mmol, 1.0 equiv) was dissolved in 2.0 mL CH₂Cl₂, and triethyl phosphite (0.39 mg, 2.3 mmol, 1.0 equiv) and boron trifluoride etherate (0.28 mL, 2.3 mmol, 1.0 equiv) were added dropwise at 0 °C. Once reagents were added, the reaction was heated to 30 °C and allowed to react for 1 h under N₂ atmosphere. The reaction was quenched with 20 mL saturated aq NaHCO₃, extracted with 2 x 30 mL EtOAc, and evaporated under reduced pressure. Purification by column chromatography (40%–100% EtOAc/hexanes) afforded 3c as a pale yellow oil (598 mg, 83%). ¹H NMR (500 MHz, CDCl₃) δ 7.23 (d, 1H, J = 8.1 Hz), 6.98 (t, 1H, J = 2.0 Hz), 6.83 (dt, 1H, J = 6.3, 2.0 Hz), 4.37 (d, 1H, J = 16.1 Hz), 3.86–4.00 (m, 4H), 3.81 (s, 3H), 3.29 (s, 3H), 1.11–1.26 (m, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 154.82, 134.40, 129.72, 122.29, 120.74, 111.12, 80.44, 79.43, 63.05 (d, J = 10.7 Hz), 62.81 (d, J = 10.7 Hz), 58.65 (d, J = 14.3 Hz), 55.97, 16.26 (d, J = 5.9 Hz), 16.18 (d, J = 5.9 Hz); HRMS calcd for C₁₃H₂₀ClO₅P (M+Na⁺) 345.0629, found 345.0624.
(1r,3r,5R,7S)-2-(methoxy(3-methoxyphenyl)methylene)adamantane (4a). Compound 3a (1.27 g, 4.47 mmol, 1.0 equiv) and 2-adamananone (939 mg, 6.25 mmol, 1.4 equiv) were dissolved separately in 15 mL and 8 mL anhydrous THF under N₂ atmosphere and cooled to –78 °C by mixing dry ice with acetone. n-Butyl lithium (3.91 mL, 6.25 mmol, 1.4 equiv) was then added dropwise to the solution of compound 3a at –78 °C to form the phosphonate carbanion. After 5 min, the 2-adamananone solution was slowly added. The reaction was slowly warmed to rt and heated to 35 °C for 2 h and then refluxed for 1 h. After the reaction mixture was cooled to rt, it was quenched with 20 mL saturated aq NH₄Cl. The mixture was then extracted with 2 x 30 mL EtOAc and evaporated under reduced pressure. Purification by column chromatography (1:20 EtOAc/hexanes) delivered 4a as a colorless oil (1.2482 g, 85%). ¹H NMR (500 MHz, CDCl₃) δ 7.25 (t, 1H, J = 8.0 Hz), 6.89–6.92 (m, 2H), 6.83 (dd, 1H, J = 8.0, 3.0 Hz), 3.81 (s, 3H), 3.31 (s, 3H), 3.27 (s, 1H), 2.66 (s, 1H), 1.71–1.98 (m, 12H); ¹³C NMR (125 MHz, CDCl₃) δ 159.45, 143.45, 136.95, 131.74, 128.96, 122.05, 114.74, 113.07, 57.82, 55.27, 39.30, 39.16, 37.29, 32.34, 30.29, 28.42; HRMS calcd for C₁₉H₂₄O₂ (M+H⁺) 285.1849, found 285.1853.

(1r,3r,5R,7S)-2-((4-fluoro-3-methoxyphenyl)(methoxy)methylene)adamantane (4b). Compound 3b (1.17 g, 3.87 mmol, 1.0 equiv) and 2-adamananone (730 mg, 4.86 mmol, 1.3 equiv) were dissolved separately in 13 mL and 6 mL anhydrous THF under N₂ atmosphere and cooled to –78 °C by mixing dry ice and acetone. n-Butyl lithium (3.0 mL, 4.86 mmol, 1.3 equiv)
was then added dropwise to the solution of compound 3b at −78 °C to form the phosphonate carbanion. After 5 min, the 2-adamantanone solution was slowly added. The reaction was slowly warmed to rt and heated to 35 °C for 2 h and then refluxed for 1 h. After the reaction mixture was cooled to rt, the reaction mixture was quenched with 20 mL saturated aq NH₄Cl. The mixture was then extracted with 2 x 30 mL EtOAc and evaporated under reduced pressure. Purification by column chromatography (1:20 EtOAc/hexanes) delivered 4b as a colorless oil (1.12 g, 85%).

1H NMR (500 MHz, CDCl₃) δ 6.99–7.03 (m, 1H), 6.94 (dd, 1H, J = 8.0, 2.0 Hz), 6.79–6.82 (m, 1H), 3.87 (s, 3H), 3.29 (s, 3H), 3.23 (s, 1H), 2.60 (s, 1H), 1.58–1.97 (m, 12H); 13C NMR (125 MHz, CDCl₃) δ 152.58, 150.62, 147.18 (d, J = 10.7 Hz), 142.81, 131.93, 122.12 (d, J = 7.2 Hz), 115.40 (d, J = 19.1 Hz), 114.22, 57.79, 56.26, 39.24, 39.11, 37.23, 32.38, 30.30, 28.36; HRMS calcd for C₁₉H₂₃ClO₂ (M+H⁺) 303.1755, found 303.1762.

(1r,3r,5R,7S)-2-((4-chloro-3-methoxyphenyl)(methoxy)methylene)adamantane (4c).

Compound 3c (365 mg, 1.13 mmol, 1.0 equiv) and 2-adamantanone (215 mg, 1.43 mmol, 1.3 equiv) were dissolved separately in 4 mL and 2 mL anhydrous THF under N₂ atmosphere and cooled to −78 °C by mixing dry ice and acetone. n-Butyl lithium (0.89 mL, 1.4 mmol, 1.3 equiv) was then added dropwise to the solution of compound 3c at −78 °C to form the phosphonate carbanion. After 5 min reaction, the 2-adamantanone solution was slowly added. The reaction was slowly warmed to rt and heated to 35 °C for 2 h and then refluxed for 1 h. After the reaction mixture was cooled to rt, it was quenched with 10 mL saturated aq NH₄Cl. The mixture was then extracted with 2 x 20 mL EtOAc and evaporated under reduced pressure. Purification by column chromatography (1:20 EtOAc/hexanes) delivered 4c as a colorless oil (294 mg, 78%). 1H NMR
(500 MHz, CDCl$_3$) $\delta$ 7.30 (d, 1H, $J = 8.0$ Hz), 6.91 (d, 1H, $J = 1.8$ Hz), 6.83 (dd, 1H, $J = 8.0$, 1.8 Hz), 3.89 (s, 3H), 3.30 (s, 3H), 3.24 (s, 1H), 2.62 (s, 1H), 1.58–1.97 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 154.84, 142.77, 135.44, 132.56, 129.62, 122.54, 121.41, 112.82, 57.90, 56.16, 39.24, 39.11, 37.21, 32.43, 30.35, 28.34; HRMS calcd for C$_{19}$H$_{23}$ClO$_2$ (M+H$^+$) 319.1459, found 319.1461.

3-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)phenol (5a). Sodium ethane thiolate (769 g, 9.14 mmol, 2.5 equiv) and cesium carbonate (2.98 g, 9.14 mmol, 2.5 equiv) were added to a dry round bottle flask containing compound 4a (1.04 g, 3.66 mmol, 1.0 equiv) dissolved in 30 mL anhydrous DMF under N$_2$ atmosphere. After refluxing overnight, the reaction mixture was partitioned between EtOAc and NH$_4$Cl, dried over Na$_2$SO$_4$ and evaporated under high vacuum to remove residual DMF. Purification by column chromatography (1:15–1:10 EtOAc/hexanes) afforded 5a as white solid (620.9 mg, 63%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.17–7.21 (m, 1H), 6.99 (s, 1H), 6.80–6.94 (m, 3H), 3.35 (s, 3H), 3.23 (s, 1H), 2.67 (s, 1H), 1.63–1.98 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 155.92, 142.62, 136.48, 132.63, 129.11, 121.70, 115.86, 114.71, 57.73, 39.06, 38.93, 37.03, 32.20, 30.26, 28.16. LRMS (MALDI) calcd for C$_{18}$H$_{22}$O$_4$ (M$^+$) 270.1620, found 270.1815.

5-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-fluorophenol (5b). Sodium ethane thiolate (215 mg, 2.56 mmol, 1.5 equiv) and cesium carbonate (834 g, 2.56 mmol, 1.5
equiv) were added to a dry round bottle flask filled with N\textsubscript{2} containing compound 4b (516 mg, 1.71 mmol, 1.0 equiv) dissolved in 10 mL of anhydrous DMF. After refluxing overnight, the reaction mixture was partitioned between EtOAc and NH\textsubscript{4}Cl, dried over Na\textsubscript{2}SO\textsubscript{4} and evaporated under high vacuum to remove residual DMF. Purification by column chromatography (1:15 EtOAc/hexanes) afforded 5b as white solid (323 mg, 62%). \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \( \delta \) 7.00–7.05 (m, 1H), 6.95–6.98 (m, 1H), 6.78–6.82 (m, 1H), 6.74 (m, 1H), 5.47 (s, 1H), 3.30 (s, 3H), 3.23 (s, 1H), 2.59 (s, 1H), 1.66–1.96 (m, 12H); \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) \( \delta \) 156.56, 142.77, 138.58, 135.43, 132.64, 121.73, 117.53, 115.30, 57.89, 39.16, 39.02, 37.10, 32.18, 30.78, 28.23; HRMS calcd for C\textsubscript{18}H\textsubscript{21}FO\textsubscript{2} (M–H\textsuperscript{+}) 287.1453, found 287.1456.

5-(((1\textsubscript{r},3\textsubscript{r},5\textsubscript{R},7\textsubscript{S})-adamantan-2-ylidene)(methoxy)methyl)-2-chlorophenol (5c). Sodium ethane thiolate (204 mg, 2.43 mmol, 2.5 equiv) and cesium carbonate (791 mg, 2.43 mmol, 2.5 equiv) were added to a dry round bottle flask containing compound 4c (309 mg, 0.971 mmol, 1.0 equiv) dissolved in 9.0 mL anhydrous DMF under N\textsubscript{2} atmosphere. After refluxing overnight, the reaction mixture was partitioned between EtOAc and NH\textsubscript{4}Cl, dried over Na\textsubscript{2}SO\textsubscript{4} and evaporated under high vacuum to remove residual DMF. Purification by column chromatography (1:12 EtOAc/hexanes) afforded 5c as white solid (232 mg, 79%). \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \( \delta \) 7.28 (d, 1H, \( J = 8.0 \) Hz), 6.98 (d, 1H, \( J = 3.5 \) Hz), 6.83 (dd, 1H, \( J = 8.0, 3.5 \) Hz) 5.95 (s, 1H), 3.30 (s, 3H), 3.22 (s, 1H), 2.62 (s, 1H), 1.55–1.96 (m, 12H); \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) \( \delta \) 151.12, 142.18, 135.88, 132.79, 128.57, 122.38, 118.84, 116.97, 57.80, 39.08, 38.94, 37.04, 32.22, 30.21, 28.17; HRMS calcd for C\textsubscript{18}H\textsubscript{21}ClO\textsubscript{2} (M–H\textsuperscript{+}) 303.1157, found 303.1150.
4-azidobenzyl(2,5-dioxopyrrolidin-1-yl)carbonate (6). N,N'-Disuccinimidyl carbonate (830 mg, 3.25 mmol, 1.5 equiv) was added to a solution of 4-azidobenzyl alcohol (323 mg, 2.16 mmol, 1.0 equiv) in 5.0 mL CH2Cl2, followed directly by the addition of NEt3 (0.91 mL, 6.5 mmol, 3.0 equiv). The reaction was stirred for 4 h at rt. The reaction was quenched with 20 mL 1 M NaHCO3, extracted with 2 x 30 mL EtOAc, washed with 10 mL brine, dried over Na2SO4, filtered, and concentrated to yield 6 (628.7 mg) as an orange oil and used without further purification. 1H NMR (500 MHz, CDCl3) δ 7.38 (d, 2H, J = 8.6 Hz), 7.03 (d, 2H, J = 8.6 Hz), 5.29 (s, 2H), 2.82 (s, 4H).

3-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)phenyl(4-azidobenzyl)carbonate (7a). Compound 5a (77 mg, 0.29 mmol, 1.0 equiv) was dissolved in 1.5 mL 4:1 THF:CH2Cl2 in a dry flask under N2 atmosphere. Compound 6 (126 mg, 0.44 mmol, 1.5 equiv) was added as a solution in 1.5 mL CH2Cl2. DMAP (53 mg, 0.44 mmol, 1.5 equiv) and NEt3 (124 µL, 0.899 mmol, 3.1 equiv) were then added in succession. After 14 h of stirring at rt, the mixture was poured into a separatory funnel containing 20 mL CH2Cl2 and 15 mL DI-H2O and extracted with 3 x 20 mL CH2Cl2. The organic layer was washed with 10 mL brine, dried over Na2SO4, filtered, and concentrated. Purification by silica column chromatography (1:15 EtOAc/hexanes) afforded 7a as a clear oil (84 mg, 62%). 1H NMR (500 MHz, CDCl3) δ 7.43 (d, 2H, J = 8.6 Hz), 7.03 (d, 2H, J = 8.6 Hz), 5.29 (s, 2H), 2.82 (s, 4H).
3.29 (s, 3H), 3.24 (s, 1H), 2.64 (s, 1H), 1.65–2.10 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 153.51, 150.84, 142.52, 140.61, 137.13, 132.78, 131.36, 130.30, 128.94, 126.97, 121.68, 119.82, 119.23, 69.66, 57.90, 39.11, 38.99, 37.07, 32.09, 30.22, 28.19; HRMS calcd for C$_{26}$H$_{27}$N$_3$O$_4$ (M+H$^+$) 446.2074, found 446.2073.

5-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-fluorophenyl(4-azidobenzyl)carbonate (7b). Compound 5b (123 mg, 0.427 mmol, 1.0 equiv) was dissolved in 3.5 mL 4:1 THF:CH$_2$Cl$_2$ in a dry flask under N$_2$ atmosphere. Compound 6 (186 mg, 0.641 mmol, 1.5 equiv) was added as a solution in 3.3 mL CH$_2$Cl$_2$, followed directly by the addition of DMAP (78.3 mg, 0.641 mmol, 1.5 equiv) and NEt$_3$ (185 µL, 1.32 mmol, 3.1 equiv). After 14 h of stirring at rt, the mixture was poured into a separatory funnel containing 30 mL CH$_2$Cl$_2$ and 20 mL DI-H$_2$O and extracted with 3 x 30 mL CH$_2$Cl$_2$. The organic layer was washed with 10 mL brine, dried over Na$_2$SO$_4$, filtered, and concentrated. Purification by silica column chromatography (1:15 EtOAc/hexanes) afforded 7b as a clear oil (125 mg, 63%). $^1$H NMR (500 MHz, CDCl$_3$) δ 7.43 (d, 2H, J = 8.0 Hz), 7.10–7.20 (m, 3H), 7.05 (d, 2H, J = 8.0 Hz), 5.25 (s, 2H), 3.28 (s, 3H), 3.22 (s, 1H), 2.60 (s, 1H), 1.53–2.10 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 153.98, 152.66, 141.64, 140.69, 132.98, 132.29, 131.12, 130.25, 128.16, 123.89, 119.25, 116.38, 116.24, 70.18, 57.90, 39.08, 38.96, 37.02, 32.11, 30.24, 28.14; LRMS (MALDI) calcd for C$_{26}$H$_{26}$ClN$_3$O$_4$ (M$^+$) 463.1907, found 463.3052.
5-(((1r,3r,5r,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-chlorophenyl(4-azidobenzyl)carbonate (7c). Compound 5c (66.6 mg, 0.218 mmol, 1.0 equiv) was dissolved in 1.5 mL 4:1 THF: CH₂Cl₂ in a dry flask under N₂ atmosphere. Compound 6 (76.6 mg, 0.262 mmol, 1.2 equiv) was added as a solution in 1.2 mL CH₂Cl₂. DMAP (40.0 mg, 0.327 mmol, 1.5 equiv) and NEt₃ (95 µL, 0.68 mmol, 3.1 equiv) were then added in succession. After 14 h of stirring at rt, the mixture was poured into a separatory funnel containing 20 mL CH₂Cl₂ and 10 mL DI-H₂O and extracted with 3 x 20 mL CH₂Cl₂. The organic layer was washed with 10 mL brine, dried over Na₂SO₄, filtered, and concentrated. Purification by silica column chromatography (1:15 EtOAc/hexanes) afforded 7c as a clear oil (53 mg, 51%). ¹H NMR (500 MHz, CDCl₃) δ 7.43 (d, 2H, J = 8.6 Hz), 7.39 (d, 1H, J = 8.1 Hz), 7.15–7.17 (m, 2H), 7.05 (d, 2H, J = 8.6 Hz), 5.26 (s, 2H), 3.30 (s, 3H), 3.22 (s, 1H), 2.63 (s, 1H), 1.56–2.00 (m, 12H); ¹³C NMR (125 MHz, CDCl₃) δ 152.53, 146.75, 141.60, 140.66, 135.76, 133.75, 131.22, 130.24, 129.86, 128.13, 125.37, 123.76, 119.23, 70.12, 58.04, 39.09, 38.07, 37.00, 32.15, 30.31, 28.14; LRMS (MALDI) calcd for C₂₆H₂₆ClN₃O₄ (M⁺) 479.1612, found 479.2970.

4-azidobenzyl(3-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'yl)phenyl)carbonate (CHS-1). Compound 7a (75 mg, 0.17 mmol, 1.0 equiv) and Rose bengal (8.5 mg, 0.0087 mmol, 0.051 equiv) were added into a dry flask and dissolved in 5 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 3.5 h of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum at 0 ºC and the residue was purified by the silica column chromatography (1:15 EtOAc/hexanes) to deliver CHS-1 as a white solid (56.4 mg,
70%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.42–7.92 (br m, 3H), 7.42–7.44 (m, 2H), 7.23 (dd, 1H, $J$ = 9.2, 2.3 Hz), 7.05 (d, 2H, $J$ = 8.6 Hz), 5.23 (s, 2H), 3.21 (s, 3H), 3.02 (s, 1H), 2.12 (s, 1H), 1.20–1.90 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 153.43, 151.08, 140.82, 136.73, 131.34, 130.42, 129.33, 122.10, 119.39, 111.58, 95.52, 69.94, 50.13, 36.44, 34.74, 32.94, 32.82, 32.35, 31.77, 31.50 25.93, 25.81.

![Image of a molecular structure](image)

4-azidobenzyl(2-fluoro-5-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)phenyl)carbonate (CHS-2). Compound 7b (47.6 mg, 0.103 mmol, 1 equiv) and Rose bengal (7 mg, 0.007 mmol, 0.07 equiv) were added into a dry flask and dissolved in 5.0 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0–5 °C. After 6 h of reaction, the mixture was concentrated under vacuum at 0 °C. Purification by silica column chromatography (1:15 EtOAc/hexanes) provided CHS-2 as a white solid (32.5 mg, 66%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.40–7.91 (br m, 2H), 7.41–7.44 (m, 2H), 7.20–7.24 (m, 1H), 7.04–7.06 (d, 2H, $J$ = 8.6 Hz), 5.25 (s, 2H), 3.21 (s, 3H), 3.01 (s, 1H), 2.07 (s, 1H), 0.90–2.00 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 155.47, 153.46, 152.48, 140.79, 138.33, 131.61, 130.98, 130.26, 119.29, 116.82, 116.67, 111.06, 95.37, 70.34, 50.00, 39.25, 36.26, 34.78, 33.13, 32.87, 32.19, 31.67, 31.50, 25.93, 25.78.

![Image of a molecular structure](image)

4-azidobenzyl(2-chloro-5-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)phenyl)carbonate (CHS-3). Compound 7c (37.3 mg, 0.0777 mmol, 1 equiv) and Rose
bengal (6 mg, 0.006 mmol, 0.08 equiv) were added into a dry flask and dissolved in 4.0 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0–5 °C. The reaction was monitored by TLC. When TLC showed no starting material, the mixture was concentrated under vacuum at 0 °C. The residue was purified by silica column chromatography (1:15 EtOAc/hexanes). Compound CHS-3 was obtained as white solid (36.1 mg, 91%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.10–7.89 (br m, 2H), 7.49–7.56 (m, 1H), 7.42–7.44 (m, 2H), 7.03–7.07 (m, 2H), 5.26 (s, 2H), 3.21 (s, 1H), 3.01 (s, 1H), 2.07 (s, 1H), 0.96–1.90 (m, 12H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 152.41, 147.07, 140.76, 135.31, 131.07, 130.23, 130.10, 128.12, 119.26, 111.00, 95.38, 70.28, 50.04, 36.25, 34.78, 33.11, 32.93, 32.17, 31.69, 31.50, 25.93, 25.78; LRMS (ESI) calcd for C\(_{26}\)H\(_{26}\)ClN\(_3\)O\(_6\)Na (M+Na\(^+\)) 534.1408, found 534.200.

### 2.4.2 Chemiluminescent response

Chemiluminescent responses and time scans were acquired using a Hitachi F-7000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) using the luminescence detection module and setting emission wavelength at 545 nm. 393 \(\mu\)L of a 20 mM HEPES buffered to pH 7.4 (Figure 1-1) or 100 mM glycine buffered to pH 10.02, 100 \(\mu\)L Emerald II Enhancer (Life Technologies, Carlsbad, CA), 5 \(\mu\)L of a 20 mM Na\(_2\)S stock solution in DI-H\(_2\)O and 2 \(\mu\)L of a 10 mM stock solution of CHS probes in CH\(_3\)CN were added to a quartz cuvette (Starna, Atascadero, CA). Samples were shaken gently to assure mixing. Then chemiluminescent spectra were acquired immediately after adding the probes. Time scans were acquired using the time scan module. 40 \(\mu\)M CHS-1, CHS-2, and CHS-3 were treated with 0, 5, 10, 20, 40, 80, 100, 150, and 200 \(\mu\)M Na\(_2\)S, and 10 min or 120 min time scans were measured 1 min after adding probes.
2.4.3 Selectivity tests

Selectivity for CHS-1, CHS-2, and CHS-3 was measured by monitoring the time-dependent chemiluminescence emission at 545 nm. All assays were performed in 20 mM HEPES buffered to pH 7.4 with 20% Emerald II Enhancer.

H₂S: 5 µL of a 20 mM stock solution of Na₂S in DI-H₂O was added to a solution of 393 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM CHS-1, CHS-2, or CHS-3 in CH₃CN was added into this mixture.

H₂S, GSH, cysteine and Hcy: 5 µL of a 20 mM stock solution of Na₂S in DI-H₂O, 25 µL of a 100 mM stock solution of GSH in 20 mM HEPES buffer, 5 µL of a 100 mM stock solution of L-cysteine in DI-H₂O and 5 µL of a 100 mM stock solution of Hcy in DI-H₂O were added to a solution of 358 µL HEPES and 100 µL Emerald II Enhancer, mixed them well and then 2 µL of 10 mM CHS-1, CHS-2, or CHS-3 in CH₃CN was added into this mixture.

GSH: 25 µL of a 100 mM stock solution of GSH in 20 mM HEPES buffer was added to a solution of 373 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM CHS-1, CHS-2, or CHS-3 in CH₃CN was added into this mixture.

S-nitrosoglutathione: 2 µL of a 50 mM stock solution of S-nitrosoglutathione in DI-H₂O was added to a solution of 396 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM CHS-1, CHS-2, or CHS-3 in CH₃CN was added into this mixture.

Cysteine: 5 µL of a 100 mM stock solution of L-cysteine in DI-H₂O was added to a solution of 393 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM CHS-1, CHS-2, or CHS-3 in CH₃CN was added into this mixture.
**Hcy:** 5 µL of a 100 mM stock solution of Hcy in DI-H$_2$O was added to a solution of 393 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM **CHS-1**, **CHS-2**, or **CHS-3** in CH$_3$CN was added into this mixture.

**HNO:** Angeli’s salt (Na$_2$N$_2$O$_3$) was used to generate HNO. The stock solution was made by dissolving Angeli’s salt in 0.01 M NaOH solution immediately prior to use. The concentration of this alkaline stock solution of Angeli’s salt was measured by UV/Vis using $\varepsilon = 6100$ M$^{-1}$ cm$^{-1}$ at 237 nm. 4 µL of a 25 mM stock solution of Angeli’s salt was added to a solution of 394 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM **CHS-1**, **CHS-2**, or **CHS-3** in CH$_3$CN was added into this mixture.

**NO:** PROLI NONOate was used to generate NO. It was stored at –80 °C and dissolved in 0.01 M NaOH solution immediately prior to use. The concentration of this alkaline stock solution of PROLI NONOate was measured by UV/Vis using $\varepsilon = 8400$ M$^{-1}$ cm$^{-1}$ at 252 nm. 8 µL of a 12.5 mM stock solution of PROLI NONOate was added to a solution of 390 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM **CHS-1**, **CHS-2**, or **CHS-3** in CH$_3$CN was added into this mixture.

**NaNO$_2$:** 1 µL of a 100 mM stock solution of NaNO$_2$ in DI-H$_2$O was added to a solution of 397 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM **CHS-1**, **CHS-2**, or **CHS-3** in CH$_3$CN was added into this mixture.

**Na$_2$SO$_3$:** 1 µL of a 100 mM stock solution of Na$_2$SO$_3$ in DI-H$_2$O was added to a solution of 397 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM **CHS-1**, **CHS-2**, or **CHS-3** in CH$_3$CN was added into this mixture.
H$_2$O$_2$: 0.5 µL of a 200 mM stock solution of H$_2$O$_2$ in DI-H$_2$O was added to a solution of 397.5 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM CHS-1, CHS-2, or CHS-3 in CH$_3$CN was added into this mixture.

OCl$: 1 µL of a 100 mM stock solution of OCl$^{-}$ in DI-H$_2$O was added to a solution of 397 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM CHS-1, CHS-2, or CHS-3 in CH$_3$CN was added into this mixture.

tBuOOH: 1 µL of a 100 mM stock solution of tBuOOH in DI-H$_2$O was added to a solution of 397 µL HEPES and 100 µL Emerald II Enhancer and then 2 µL of 10 mM CHS-1, CHS-2, or CHS-3 in CH$_3$CN was added into this mixture.

Blank: 2 µL of 10 mM CHS-1, CHS-2, or CHS-3 in CH$_3$CN was added to a solution of 398 µL HEPES and 100 µL Emerald II Enhancer.

2.4.4 Computational results

All the geometries were optimized using density functional theory (DFT) with B3LYP$^{46,47,48,49}$ functional and Pople basis set 6-311+G(d,p)$^{50}$ and integral equation formalism of polarizable continuum model (IEF-PCM)$^{51}$ with water as solvent. Atomic charges are calculated using ESP model.$^{52}$ The ESP charges were also calculated at using M06$^{53}$ and ωB97XD$^{54}$ functionals and 6-311+G(d,p) basis set using IEF-PCM with water as solvent at geometries optimized at B3LYP/6-311+G(d,p) level of theory. All the calculations were carried out using Gaussian09.$^{55}$

Table 2-1. Cartesian coordinates and ESP charges of the phenolate released from CHS-1.
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Table 2-2. Cartesian coordinates and ESP charges of the phenolate released from CHS-2.

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Table 2-3. Cartesian coordinates and ESP charges of the phenolate released from CHS-3.

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**Figure 2-11.** Plot of the integrated chemiluminescence emission over 10 min at pH 10 of 200 µM H₂S and 40 µM CHS-1 (red), CHS-2 (green) and CHS-3 (blue) versus the calculated atomic charges on the phenolate oxygen (O8). All luminescent measurements were acquired in 100 mM glycine (pH 10) containing 20% Emerald II Enhancer. The reported values are averages of the integrated emission intensities over 10 min (n = 4–7). Error bars represent ± S.D. Geometries were optimized with B3LYP/6-311G+(d,p) and ESP atomic charges were calculated with (a) B3LYP/6-311+G(d,p) or (b) ωB97XD/6-311+G(d,p). Calculations were carried out with the IEF-PCM water solvation model using Gaussian 09. (Data was collected by Tao group)

### 2.4.5 Cellular experiments

Chemiluminescent response using a multi-well plate reader. Chemiluminescent responses were measured using a BioTek plate reader (Winooski, VT) by using the luminescence detection method, endpoint read type, and setting sensitivity to 135. 120 µL, 119 µL, 119 µL, 118 µL, and
116 μL of 20 mM HEPES buffer (pH 7.4) were added into the wells of a black opaque Corning®
96-well plate from A1 to A5 in sequence, 30 μL Emerald II Enhancer was pipetted into each
well, then different volumes of a 10 mM Na₂S solution (0 μL, 0.38 μL, 0.75 μL, 1.5 μL, 3.0 μL)
were added into each well. 0.60 μL CHS-3 was injected into each well and the luminescent
intensity of the plate was measured every 2 min after addition of probes. The detection limit was
estimated as the amount of Na₂S required to give a chemiluminescent signal above three times
the standard deviation of at least 3 independent experiments with 0 μM Na₂S. The concentration
of Na₂S needed was estimated by fitting a line to the linear region of the curve between the data
points corresponding to 0 μM Na₂S and 25 μM Na₂S.

Cell culture and detecting cellular H₂S using a multi-well plate reader. Human lung
adenocarcinoma epithelial cell (A549) were purchased from ATCC and cultured in F-12K media
supplemented with 10% Fetal Calf Serum (FCS) at 37 °C with 5% CO₂. Two days before the
experiment, cells were passed and plated on Costar® 12-well plates by adding 150K–175K of
A549 cells per well, filling each well up to 600 μL of media with FCS, and aspirating the media
upon 90–95% confluence. Cells were serum-starved for 18 h prior to the experiment. Stock
solutions of 20 mM Hcy and 20 mM DL-propargyl glycine (PAG) were prepared in 20 mM
HEPES buffer (pH 7.4) and 10 mM CHS-3 was prepared in CH₃CN. 6 μL PAG (final
concentration: 200 μM) was added to C1–C3 of the 12-well plate. After 20 min incubation, 6 μL
Hcy (final concentration: 200 μM) was added into B1–B3 and C1–C3 of the 12-well plate, and 6
μL 20 mM HEPES buffer (pH 7.4) as a vehicle control was added to A1–A3. After another 20
min incubation, cells were washed with 1 x PBS. Then 500 μL PBS media was added into each
well after aspirating the media. 125 μL Emerald II Enhancer and 2.7 μL CHS-3 (final
concentration: 40 µM) were added to each well and the luminescent intensity was measured every two minutes for 20 minutes. The experiment was repeated with four independent well plates and the peak value for the luminescence emission for each well at 10–12 minutes was normalized to the average luminescence emission at 10–12 minutes for the control replicates of each plate. A single outlier was rejected according to the extreme studentized deviate method (Grubbs' test, p < 0.01).

2.4.6 Imaging experiments

Chemiluminescence imaging at pH 7.4. Imaging was carried out with a Caliper Xenogen IVIS® Spectrum (Perkin-Elmer, Santa Clara, CA) in black 96-well Costar® plates and all the images were analyzed using Living Image 3.1 software. 10 mM CHS-3 in CH₃CN and 10 mM Na₂S in DI-H₂O were prepared prior to imaging. 199 µL, 198 µL, 198 µL, 197 µL and 195 µL of 20 mM HEPES buffer (pH 7.4) were added into wells from A1 to A5 in sequence, 50 µL Emerald II Enhancer was pipetted into each well, then different volume of Na₂S solution (0 µL, 0.61 µL, 1.25 µL, 2.5 µL, 5.0 µL) were added into each well. 1 µL CHS-3 was injected into the mixture and imaging was performed after 30 seconds using an open filter. All images were acquired with f-stop 1, medium binning, auto exposure and the chamber set to 37 °C.

Imaging H₂S in mouse carcass. A stock solution of 25 mM CHS-3 in DMSO and 50 mM Na₂S in DI-H₂O were prepared in advance. The 50 mM stock solution of Na₂S was diluted to provide a final concentration of 4 mM Na₂S in 100 µL (0.4 µmol) to be injected. Images were acquired 30 second after administering i.p. injections to the carcasses of SCID/BALB-C mice with 0.08 µmol
CHS-3 and either 0.4 µmol Na$_2$S or a vehicle control (H$_2$O) in HEPES buffered at pH 7.4 containing 20% Emerald II Enhancer.

**Imaging H$_2$S in living mice.** The UT Southwestern Institutional Animal Care and Use Committee approved these investigations under APN #2009-0150. A stock solution of 25 mM CHS-3 in DMSO and 50 mM Na$_2$S in DI-H$_2$O were prepared in advance. 68.8 µL of 20 mM HEPES buffer (pH 7.4), 20 µL Emerald II Enhancer, 8 µL Na$_2$S solution and 3.2 µL CHS-3 were intraperitoneally injected into C6 brown mice, lifting the skin to avoid puncture of internal organs. Imaging was performed 30 second after injecting using an open filter to maximize light collection. The vehicle control was conducted by adding 8 µL DI-H$_2$O instead of Na$_2$S solution.
2.5 References


CHAPTER 3
IN VIVO CHEMILUMINESCENCE IMAGING AGENTS FOR NITROREDUCTASE AND TISSUE OXYGENATION

3.1 Introduction

Hypoxia is a pathogenic condition of low tissue oxygenation. It is a characteristic of solid tumors. Cancer cells undergo a variety of biological changes to support their survival in hypoxia or low tissue oxygenation conditions, including proliferation and angiogenesis. The most significant and important biological response to low oxygen conditions is the activation of the transcription factor hypoxia inducible factor 1α (HIF-α). In normoxia, HIF-α is hydroxylated in the presence of oxygen (O₂), iron (Fe²⁺), and prolyl hydroxylase enzymes, increasing its chance of binding to Von Hippel-Lindau, a tumor suppressor protein, which targets its ubiquitination and mediates its degradation. In hypoxia, HIF-α dimerizes with HIF-β, and transcriptionally regulates hypoxia-responsive genes to support cancer cell survival. Some reductive mammalian enzymes, such as xanthine oxidase, NADPH-cytochrome c reductases, and potentially cytosol DT diaphorase, aldehyde oxidase, and lipoyl dehydrogenase have been widely used as biomarkers for hypoxia using selective bioreduction of nitroaromatic compounds under hypoxic conditions (Scheme 3-1). Imaging agents like FMISO and immunohistochemical stains like pimonidazole targets these reductive enzymes. GSH.
thioredoxin, reduced nicotinamide adenine dinucleotide (NADH), and vitamin C are several other important redox systems playing fundamental roles in biological systems.\(^9\)

\[ \text{Normoxia} \quad \xrightarrow{\text{O}_2} \quad \text{Hypoxia} \]
\[ \begin{array}{c}
RNO_2 \\
O_2^- 
\end{array} \quad \xrightarrow{\text{e}^-} \quad \begin{array}{c}
RNO_2 \\
O_2 
\end{array} \quad \xrightarrow{2\text{e}^-} \quad \begin{array}{c}
RNO \\
\xrightarrow{2\text{e}^-} \quad \text{RNHOH}\quad \xrightarrow{2\text{e}^-} \quad \text{RNH}_2 
\end{array} \]

**Scheme 3-1.** Proposed mechanism of bioreduction in hypoxic conditions.

Classic methods for low tissue oxygenation and hypoxia measurement include oxygen electrodes,\(^10\) PET,\(^11\) MRI,\(^12\) and multispectral optoacoustic tomography (MSOT).\(^13\) Even though they offer accurate detection and diagnosis of hypoxia and tissue oxygenation, they are either invasive or require expensive instrumentation and highly trained experts. Over the last three decades, optical imaging techniques have received tremendous attention, and reaction-based probes have been widely used for interrogating biological species. Reductive enzymes are well accepted biomarkers for targeting poorly oxygenated and hypoxic tissue.\(^5\) Reductases, a class of flavin-containing enzymes for the oxygen-dependent reduction of nitroaromatics to amines, have become a well-established method for sensing tissue oxygenation and hypoxia.\(^14\) Recently, several reaction-based fluorescent\(^15\) and bioluminescent probes\(^16\) for nitroreductase and hypoxia have been reported. For fluorescent detection, the dyes must be first excited by an external light source, which generates a significant amount of autofluorescence and light scattering. A genetically modified biological system is required for bioluminescence imaging.\(^31\) To offer better imaging agents for hypoxia, our group initiated a project for detecting nitroreductase and hypoxia using reaction-based chemiluminescent probes.
A spiroadamantane 1,2-dioxetane scaffold was selected to make reaction-based chemiluminescent probes because this structure has already been demonstrated to be capable of 

\textit{in vivo} imaging of galactosidase\textsuperscript{38} and H\textsubscript{2}S.\textsuperscript{17} Our design strategy for reaction-based chemiluminescent probes for hypoxia relies on tethering a \textit{para}-nitrobenzyl moiety to a spiroadamantane 1,2-dioxetane chemiluminescent scaffold via carbonate or ether linkage (Scheme 3-2). The Hypoxia Chemiluminescent Probe 1 (\textbf{HyCL-1}) was synthesized by the dimethylaminopyridine-catalyzed coupling of the previously reported phenol 5c\textsuperscript{17} with compound 8 to provide the precursor 9. This precursor 9 was subjected to [2+2] cycloaddition by bubbling oxygen through the reaction mixture using Rose bengal as a sensitizer with light irradiation to afford \textbf{HyCL-1}. For Hypoxia Chemiluminescent Probe 2 (\textbf{HyCL-2}), the precursor 11 was obtained by Mitsunobu reaction\textsuperscript{18} between phenol 5c and \textit{para}-nitrobenzyl alcohol 10 and then this precursor 11 underwent the same [2+2] cycloaddition to deliver \textbf{HyCL-2} (Scheme 3-3).
3.2 Results and discussion

After obtaining **HyCL-1** and **HyCL-2**, we started to collect their absorption (Figure 3-1) and emission (Figure 3-2) spectra and measure their luminescent responses to nitroreductase from *Escherichia coli* in the presence of NADH. Luminescent response toward nitroreductase was evaluated in PBS (10 mM, pH 7.4) buffer containing 10% Emerald II Enhancer. Treatment of 10 µM of **HyCL-1** and **HyCL-2** with 14 µg/ml nitroreductase, 0.4 mM NADH, and 10% Emerald II Enhancer resulted in instantaneous luminescence emissions centered at 545 nm that increased over a time course of 60 minutes (Figure 3-5), providing a ~5-fold and ~170-fold luminescence increases for **HyCL-1** and **HyCL-2** respectively (Figure 3-3). This luminescent
response is dose-dependent with good linearity in the range of 0–14 µg/mL of nitroreductase (Figure 3-4). Furthermore, the ether-linked **HyCL-2** showed a much lower background emission (Figure 3-3b, red scan) compared with the carbonate linked **HyCL-1** (Figure 3-3a, red scan). To better understand nitro reduction by reductase enzymes, a GC-MS experiment was conducted by analyzing the reaction products between **HyCL-2**, NADH, and nitroreductase, indicating the generation of 2-adamantanone (Figure 3-14) and methyl 4-chloro-3-hydroxybenzoate (Figure 3-15), whose fragmentation patterns match the spectra found in the NIST database. A molecular ion at m/z = 121 was also observed that was assigned to 4-methylenecyclohexa-2,5-dien-1-one oxime (Figure 3-16), consistent with previous reports that the nitro group was reduced to a hydroxylamine, followed by a self-immolative cleavage to generate the dioxetane phenolate.

![Figure 3-1](image.png)

**Figure 3-1.** Chemiluminescence emission spectra of (a) 10 µM **HyCL-1** and (b) 10 µM **HyCL-2** with 0 (grey) and 14 (black) µg/mL nitroreductase in the presence of 0.4 mM NADH in 10 mM PBS buffer (pH 7.4) containing 10% Emerald II Enhancer acquired 30 min after adding nitroreductase.
Figure 3-2. Chemiluminescent absorption spectra of (a) 10 µM HyCL-1 and (b) 10 µM HyCL-2 with 10% Emerald II Enhancer (black), or without Emerald II Enhancer (grey) in 10 mM PBS buffer.

Figure 3-3. Time scans of the chemiluminescence emission at 545 nm from (a) 10 µM HyCL-1 and (b) 10 µM HyCL-2 and 0, 2.5, 5, 7.5, 10, 12.5, 14 µg/mL nitroreductase in the presence of 0.4 mM NADH in 10 mM PBS buffer (pH 7.4) containing 10% Emerald II Enhancer.
Figure 3-4. Chemiluminescence emission at 545 nm from (a) 10 µM HyCL-1 and (b) 10 µM HyCL-2 and 0, 2.5, 5, 7.5, 10, 12.5, 14 µg/mL nitroreductase in the presence of 0.4 mM NADH in 10 mM PBS buffer (pH 7.4) containing 10% Emerald II Enhancer.

Figure 3-5. Long-time scan of the chemiluminescence emission at 545 nm of 10 µM HyCL-2 to 14 µg/mL nitroreductase and 0.4 mM NADH in 10 mM PBS buffer (pH 7.4) and 10% Emerald II Enhancer.

We next evaluated the selectivity of HyCL-1 and HyCL-2 against other biologically relevant agents such as thiols which are well known reducing agents in our body involved in various redox cycles.²⁰ HyCL-1 and HyCL-2 (10 µM) were treated with 14 µg/mL nitroreductase and 0.4 mM NADH, 5 mM reduced GSH, 1 mM L-cysteine and Hcy, and 200 µM H₂S, dithiothreitol, citrate, sodium metabisulfite, and L-ascorbic acid in PBS buffer containing 10% Emerald II Enhancer, and the luminescence responses were monitored in a time course of 20 min. Surprisingly, HyCL-1 with the carbonate linker showed poor selectivity towards some
thiol species such as GSH, cysteine, Hcy, and dithiothreitol (Figure 3-6a), whereas the ether linked **HyCL-2** showed excellent selectivity, and none of the species tested displayed significant increases in luminescence intensity over the blank control (Figure 3-6b). Selectivity towards nitroreductase (14 µg/mL) or NADH (0.4 mM) was also studied. None of them induced an observable chemiluminescent increase, suggesting that only nitroreductase and NADH together can produce a luminescent response. Though H$_2$S-mediated reduction of nitro groups to amines has been reported, this synthetic procedures requires a pH in the range of 10.5 to 12.$^{21}$ Due to this requirement for elevated pH, no significant chemiluminescence emission was observed when treating our probes with H$_2$S at pH 7.4, consistent with the previous report from the Pluth group.$^{22}$ To better understand the selectivity of **HyCL-1** with cellular relevant thiols and decipher the poor selectivity of **HyCL-1**, **HyCL-1** was treated with cysteine and the reaction progress was monitored via $^1$H NMR (Figure 3-7). From NMR experiments, we observed that the methylene peak at 5.36 ppm disappeared and a new peak at 5.32 ppm formed quickly within 5 minutes, indicating the reaction between carbonate and thiol, and the peak at 8.18 ppm and 7.56 ppm persisted for more than 25 hours, suggesting that nitro group wasn’t reduced by thiols. Taken together, these results demonstrated that the ether linked **HyCL-2** can be applied for sensitive and selective detection of reductive enzyme in vitro.
Figure 3-6. Chemiluminescent responses of (a) 10 µM HyCL-1 and (b) 10 µM HyCL-2 to biologically relevant analytes in 10 mM PBS buffer (pH 7.4) containing 10% Emerald II Enhancer. Bars represent chemiluminescence emission at 545 nm and 4, 8, 12, 16, 20 min after addition of reducing agents. Data shown are for 5 mM GSH, 1 mM cysteine and Hcy, and 200 µM for other agents. (1) nitroreductase and NADH; (2) GSH; (3) L-cysteine; (4) Hcy; (5) dithiothreitol; (6) nitroreductase; (7) NADH; (8) H₂S; (9) citrate; (10) Na₂S₂O₅; (11) L-ascorbic acid; (12) blank.
We next investigated the ability of HyCL-2 for hypoxia detection using an *in vitro* model. A solution of 10 mM PBS (pH 7.4) containing 10% Emerald II Enhancer was bubbled with nitrogen gas for 60 minutes to interrogate the luminescent response under low oxygen conditions. The mixture was transferred to a capped cuvette, and 10 µM HyCL-2, 0.4 mM NADH and various amounts of nitroreductase were added in a strict nitrogen protected atmosphere. A significant difference of chemiluminescent signal between deoxygenated solution and non-degassed solution was observed. Luminescence emission from the deoxygenated solution was 5-fold higher compared with non-degassed solution (Figure 3-8). A control experiment was conducted by bubbling air through a solution of 10 mM PBS (pH 7.4) containing 10% Emerald II Enhancer for 60 minutes. No significant difference in luminescent intensity was
observed from the experiment conducted under ambient conditions, confirming that the increased signal resulted from deoxygenation and not from a mechanical effect of the bubbling procedure (Figure 3-9). This is surprising because the nitroreductase used in this study is oxygen-insensitive. One possible explanation is that oxygen quenches the triplet state product or one of the radical intermediates of the chemiluminescent reaction of HyCL-2. Taken together, these in vitro experiments confirm that the oxygen level plays a significant role in chemiluminescence emission of HyCL-2, a necessary trait for imaging tumor hypoxia.

**Figure 3-8.** Integrated chemiluminescence emission in the presence (red bars) and absence (green bars) of oxygen over 20 min of 10 µM HyCL-2 and 0, 5, 10, and 12.5 µg mL⁻¹ nitroreductase in 10 mM PBS buffer (pH 7.4) containing 0.4 mM NADH and 10% Emerald II Enhancer (n = 3). Error bars are ±S.D.
Figure 3-9. Integrated chemiluminescence emission at 545 nm from (1) ambient, (2) oxygen bubbled, (3) air bubbled, or (4) N\textsubscript{2} bubbled 10 mM PBS buffer (pH 7.4) containing 10\% Emerald II Enhancer with 12.5 µg/mL of nitroreductase in the presence of 0.4 mM NADPH and 10 µM HyCL-2 integrated over 20 min. Error bars are ± S.D.

![Figure 3-9](image)

Next, we applied HyCL-2 for nitroreductase imaging at physiological pH using an IVIS Spectrum. We added 10 µM HyCL-2, 0, 2.5, 7.5, 10, 12.5 µg/mL nitroreductase, 0.4 mM NADH and 10\% Emerald II Enhancer in PBS buffer (pH 7.4) into a 96 well white plate. The
light intensity clearly increased with the increasing nitroreductase (Figure 3-10a), and a linear response was observed (Figure 3-10b), indicating that **HyCL-2** is capable of accurately and sensitively imaging reductase activity. The detection limit (3 σ) obtained from this experiment was between 2–10 ng/mL. Inspired with the robust chemiluminescent light production from plate experiment, we proceeded to apply **HyCL-2** for nitroreductase imaging in living animals. Anesthetized SCID/BALB-C mice were administered subcutaneous injections with nitroreductase and NADH on the upper side and a control without nitroreductase and NADH was conducted on the lower back (Figure 3-11). The upper side displayed a dramatic increase in chemiluminescence emission compared with vehicle control on the lower back, confirming that the light production from **HyCL-2** was sufficient for nitroreductase imaging in living animal.

![Figure 3-11](image)

**Figure 3-11. In vivo nitroreductase imaging.** (a) Image of a living SCID/BALB-C mouse 30 s after administering subcutaneous injections of 30 µL of a solution containing 40 µM **HyCL-2**, 14 µg mL⁻¹ nitroreductase, 0.4 mM NADH, and 10% Emerald II Enhancer in 10 mM PBS (pH 7.4). In the vehicle control nitroreductase and NADH was replaced with DI-H₂O and 0.01 M
NaOH. (b) Quantification of biological replicates (n = 2 mice). Error bars are high and low values of the two replicates.

After demonstrating that light production from **HyCL-2** was sufficient to be observed through mammalian tissue, we interrogated the ability of **HyCL-2** to image tissue oxygenation and hypoxia in human tumor xenograft models. H1299 lung tumors were planted subcutaneously on the flanks of SCID/BALB-C mice. Mice were treated with either air (21% oxygen) or 100% oxygen before imaging. After several minutes, mice were intratumorally injected with the **HyCL-2** in PBS buffer (pH 7.4) containing 10% Emerald II Enhancer. The mice breathing air showed high chemiluminescent signal compared with the mice breathing pure oxygen, which can be attributed to the different level of tumor oxygenation (Figure 3-13). This tumor imaging experiment is in agreement with the *in vitro* experiment that low oxygenation gives high chemiluminescent signal. We conducted a MSOT imaging experiment to further confirm our results. The concentration of hemoglobin (Hb) and oxygen-bound hemoglobin (HbO₂) of the same mice who underwent chemiluminescence imaging were evaluated by MSOT imaging\textsuperscript{13}. The mice breathing pure oxygen displayed a clearly elevated concentration of HbO₂ (Figure 3-14). Taken together, those experiments validate that **HyCL-2** is capable of imaging tissue oxygenation and tumor hypoxia.
Figure 3-12. Images of living H1299 lung tumor xenografts on SCID/BALB-C mice 1.5 min after administering intratumoral injections of 100 µL of a solution of 40 µM HyCL-2 and 10% Emerald II Enhancer in 10 mM PBS (pH 7.4) while breathing (a) air or (b) 100% oxygen. (c) Quantification of biological replicates (n = 2 mice). Error bars are high and low values of the two replicates.
Figure 3-13. Multi-spectral optoacoustic tomography (MSOT) images of a living H1299 bearing SCID/BALB-C mouse while breathing (a) 16% oxygen or (b) 100% oxygen. Scale bars are 3 mm.

3.3 Concluding remarks

To close, we have designed and synthesized two reaction-based chemiluminescent probes, **HyCL-1** and **HyCL-2**. Both display immediate light emission upon reacting with nitroreductase at physiological pH. **HyCL-2** gave ~170-fold chemiluminescent increase in a time course of 20 min with negligible background emission. Furthermore, **HyCL-2** displayed superior selectivity towards biological relevant thiol species such as GSH and cysteine. These improved properties can be attributed to a more stable ether linkage. **HyCL-2** has been successively applied for the detection and imaging of nitroreductase using an IVIS Spectrum. **HyCL-2** is capable of imaging tissue oxygenation and tumor hypoxia during oxygen challenge experiment.
in H1299 human tumor xenografts grown in a murine model as independently confirmed using optoacoustic imaging.

3.4 Experimental section

3.4.1 Chemical synthesis

![Chemical Structure](image)

4-nitrobenzyl(2,5-dioxopyrrolidin-1-yl)carbonate (8). N,N'-Disuccinimidyld carbonate (1.253 g, 4.89 mmol, 1.5 equiv) was added to a solution of 4-nitrobenzyl alcohol (500 mg, 3.26 mmol, 1.0 equiv) in 10.0 mL CH₂Cl₂, followed directly by the addition of NEt₃ (1.37 mL, 9.79 mmol, 3.0 equiv). The reaction was stirred for 8.5 h at rt. The reaction was quenched with 20 mL 1 M NaHCO₃, extracted with 2 x 30 mL CH₂Cl₂, washed with 10 mL brine, dried over Na₂SO₄, filtered, and concentrated to yield 8 (955.3 mg) as an orange oil and used without further purification. ¹H NMR (500 MHz, CDCl₃) δ 8.26 (d, 2H, J = 8.6 Hz), 7.57 (d, 2H, J = 8.6 Hz), 5.40 (s, 2H), 2.83 (s, 4H).

![Chemical Structure](image)

5-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-chlorophenyl(4-nitrobenzyl)carbonate (9). Phenol 5c (235 mg, 0.77 mmol, 1.0 equiv) was dissolved in 5 mL 4:1 THF:CH₂Cl₂ in a dry flask under N₂ atmosphere. Mixed carbonate 8 (284 mg, 0.92 mmol, 1.2 equiv) was added as a solution in 1.5 mL CH₂Cl₂. DMAP (146 mg, 1.2 mmol, 1.5 equiv) and
NEt$_3$ (322 µL, 2.31 mmol, 3.0 equiv) were then added in succession. After 10 h of stirring at rt, the mixture was poured into a separatory funnel containing 20 mL CH$_2$Cl$_2$ and 15 mL DI-H$_2$O and extracted with 3 x 20 mL CH$_2$Cl$_2$. The organic layer was washed with 10 mL brine, dried over Na$_2$SO$_4$, filtered, and concentrated. Purification by silica column chromatography (1:15 EtOAc/hexanes) afforded 9 as a clear oil (164.8 mg, 44%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.24 (d, 2H, $J = 8.6$ Hz), 7.60 (d, 2H, $J = 8.6$ Hz), 7.39 (d, 1H, $J = 8.0$ Hz), 7.19 (m, 2H), 5.39 (s, 2H), 3.28 (s, 3H), 3.22 (s, 1H), 2.63 (s, 1H), 1.20–2.00 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 152.57, 148.09, 146.76, 141.86, 141.66, 136.03, 134.00, 130.04, 128.60, 128.45, 125.35, 124.00, 123.74, 69.06, 58.14, 39.21, 39.08, 37.10, 32.30, 30.44, 28.24; HRMS calcd for C$_{26}$H$_{26}$NO$_6$Cl (M+Na)$^+$ 484.1521, found 484.1519.

2-chloro-5-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-(1,2]dioxetan]-4'yl)phenyl(4-nitrobenzyl)carbonate (HyCL-1). Enol ether 9 (80 mg, 0.17 mmol, 1.0 equiv) and Rose bengal (8 mg, 0.0079 mmol, 0.046 equiv) were added into a dry flask and dissolved in 7 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 3 h of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum at 0 °C and the residue was purified by the silica column chromatography (1:15 EtOAc/hexanes) to deliver HyCL-1 as a white solid (68 mg, 75%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.27 (d, 2H, $J = 8.6$ Hz), 7.61 (d, 2H, $J = 8.6$ Hz), 7.00–7.80 (br, 3H), 5.40 (s, 2H), 3.21 (s, 3H), 3.01 (s, 1H), 2.06 (s, 1H), 1.00–1.90 (m, 12H); $^{13}$C
NMR (125 MHz, CDCl$_3$) $\delta$ 152.41, 148.17, 146.73, 141.65, 135.57, 130.45, 128.62, 128.12, 124.07, 111.05, 95.48, 69.18, 50.17, 36.33, 34.89, 33.23, 33.05, 32.26, 31.80, 31.60, 26.01, 25.88.

(1$\text{r,3r,5R,7S}$)-2-((4-chloro-3-((4-nitrobenzyl)oxy)phenyl)(methoxy)methylene)adamantane (11). Phenol 5c (230 mg, 0.68 mmol, 1.0 equiv) and triphenylphosphine (214 mg, 0.82 mmol, 1.2 equiv) were dissolved in anhydrous THF. Diethyl azodicarboxylate (128 $\mu$L, 0.82 mmol, 1.2 equiv) was added dropwise over 5 min and then 4-nitrobenzyl alcohol (104 mg, 0.68 mmol, 1.0 equiv) was added immediately. After 1 h of stirring at rt, the mixture was concentrated. Purification by silica column chromatography (1:12 EtOAc/hexanes) afforded 5 as a yellow oil (250 mg, 84%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.24 (d, 2H, $J$ = 8.6 Hz), 7.65 (d, 2H, $J$ = 8.6 Hz), 7.35 (d, 1H, $J$ = 8.0 Hz), 6.91 (m, 2H), 5.25 (s, 2H), 3.26 (s, 3H), 3.21 (s, 1H), 2.54 (s, 1H), 1.20–2.00 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 153.33, 147.65, 144.01, 142.42, 135.54, 132.97, 129.97, 127.50, 123.90, 123.48, 122.21, 114.54, 69.42, 57.90, 39.23, 39.06, 37.13, 32.38, 30.35, 28.27. HRMS calcd for C$_{25}$H$_{26}$NO$_4$Cl (M-H)$^-$ 438.1478, found 438.1466.
(1r,3r,5r,7r)-4′-(4-chloro-3-((4-nitrobenzyl)oxy)phenyl)-4′-methoxyspiro [adamantane-2,3′- [1,2]dioxetane] (HyCL-2). Enol ether 5 (75 mg, 0.17 mmol, 1.0 equiv) and rose bengal (8.5 mg, 0.0084 mmol, 0.049 equiv) were added into a dry flask and dissolved in 5 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 3 h of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum at 0 °C and the residue was purified by the silica column chromatography (1:15 EtOAc/hexanes) to deliver HyCL-2 as a white solid (56.4 mg, 71%). ¹H NMR (500 MHz, CDCl₃) δ 8.25 (d, 2H, J = 8.6 Hz), 7.67 (d, 2H, J = 8.6 Hz), 6.80–7.30 (br, 3H), 5.34 (s, 2H), 3.24 (s, 3H), 2.98 (s, 1H), 2.06 (s, 1H), 1.00–2.00 (m, 13H); ¹³C NMR (125 MHz, CDCl₃) δ 153.33, 147.74, 143.68, 134.98, 127.56, 124.00, 111.57, 95.57, 87.03, 69.48, 50.04, 36.31, 34.83, 33.28, 33.17, 32.28, 31.72, 31.54, 25.97, 25.90; HRMS calcd for C₂₅H₂₆NO₆Cl (M+Na)⁺ 494.1341, found 494.1337.

3.4.2 Spectroscopic measurements

889 µL of a 10 mM PBS buffered to pH 7.4, 2 µL of a 5 mM HyCL-1 or HyCL-2 in DMSO, 8 µL of a 50 mM NADH in 0.01 mM NaOH solution, 1.4 µL of nitroreductase from Escherichia coli  (Sigma-Aldrich, N9284-1MG) in DI-H₂O (1 mg nitroreductase dissolved in 100 µL DI-H₂O, 10 mg/mL), and 100 µL Emerald II Enhancer were added to an Eppendorf tube and samples were shaken gently to assure mixing. The mixed solution was then transferred to a quartz cuvette. Time scans were acquired using the time scan module 1 min after adding probes. For the dose-dependent response to nitroreductase, 10 µM HyCL-1 and HyCL-2 were treated with 8 µL of a 50 mM NADH and 0, 0.5, 0.75, 1.0, 1.25, 1.4 µL 10 mg/mL nitroreductase and 20 min or 120 min scans were measured 1 min after adding probes. Selectivity for HyCL-1 and
HyCL-2 was measured by monitoring the time-dependent chemiluminescence emission at 545 nm. All assays were performed in 10 mM PBS buffered to pH 7.4 with 10% Emerald II Enhancer.

Nitroreductase (14 µg/mL) and NADH (0.4 mM): 889 µL of a 10 mM PBS buffer (pH 7.4), 2 µL of 5 mM HyCL-1 or HyCL-2 in DMSO, 8 µL of 50 mM NADH in 0.01 mM NaOH, 1.4 µL of 10 mg/mL nitroreductase, and 100 µL Emerald II Enhancer were added to an Eppendorf tube and was shaken gently to assure mixing. The mixed solution was then transferred to a quartz cuvette.

GSH (5 mM): 50 µL of a 100 mM stock solution of GSH in DI-H2O was added to a solution of 848 µL 10 mM PBS buffer (pH 7.4) and 2 µL of 5 mM HyCL-1 or HyCL-2 in DMSO and then 100 µL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

L-Cysteine (1 mM): 10 µL of a 100 mM stock solution of L-cysteine in DI-H2O was added to a solution of 888 µL 10 mM PBS buffer (pH 7.4) and 2 µL of 5 mM HyCL-1 or HyCL-2 in DMSO and then 100 µL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

Hcy (1 mM): 10 µL of a 100 mM stock solution of Hcy in DI-H2O was added to a solution of 888 µL 10 mM PBS buffer (pH 7.4) and 2 µL of 5 mM HyCL-1 or HyCL-2 in DMSO and then 100 µL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

Dithiothreitol (200 µM): 2 µL of a 100 mM stock solution of dithiothreitol in DI-H2O was added to a solution of 896 µL 10 mM PBS buffer (pH 7.4) and 2 µL of 5 mM HyCL-1 or HyCL-2 in DMSO and then 100 µL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.
NADH (0.4 mM): 890 µL of a 10 mM PBS buffer (pH 7.4), 2 µL of a 5 mM HyCL-1 and
HyCL-2 in DMSO, 8 µL of a 50 mM NADH, and 100 µL Emerald II Enhancer were added to an
Eppendorf tube and was shaken gently to assure mixing. The mixed solution was then transferred
to a quartz cuvette.

Nitroreductase (14 µg/mL): 897 µL of a 10 mM PBS buffer (pH 7.4), 2 µL of a 5 mM HyCL-1
or HyCL-2 in DMSO, 1.4 µL of nitroreductase, and 100 µL Emerald II Enhancer were added to
an Eppendorf tube and was shaken gently to assure mixing. The mixed solution was then
transferred to a quartz cuvette.

H₂S (200 µM): 10 µL of a 20 mM stock solution of Na₂S in DI-H₂O was added to a solution of
888 µL 10 mM PBS buffer (pH 7.4) and 2 µL of 5 mM HyCL-1 or HyCL-2 in DMSO and then
100 µL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then
transferred to a quartz cuvette.

Sodium Citrate (200 µM): 2 µL of a 100 mM stock solution of sodium citrate in DI-H₂O was
added to a solution of 896 µL 10 mM PBS buffer (pH 7.4) and 2 µL of 5 mM HyCL-1 and
HyCL-2 in DMSO and then 100 µL Emerald II Enhancer was added into this mixture in an
Eppendorf tube and then transferred to a quartz cuvette.

Na₂S₂O₅ (200 µM): 2 µL of a 100 mM stock solution of Na₂S₂O₅ in DI-H₂O was added to a
solution of 896 µL 10 mM PBS buffer (pH 7.4) and 2 µL of 5 mM HyCL-1 or HyCL-2 in
DMSO and then 100 µL Emerald II Enhancer was added into this mixture in an Eppendorf tube
and then transferred to a quartz cuvette.

L-ascorbic acid (200 µM): 2 µL of a 100 mM stock solution of L-ascorbic acid in DI-H₂O was
added to a solution of 896 µL 10 mM PBS buffer (pH 7.4) and 2 µL of 5 mM HyCL-1 or
**HyCL-2** in DMSO and then 100 µL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

**Blank:** 2 µL of 5 mM **HyCL-1 or HyCL-2** in DMSO was added to a solution of 898 µL 10 mM PBS buffer (pH 7.4) and then 100 µL Emerald II Enhancer was added.

### 3.4.3 GC-MS analysis of the reaction of HyCL-2 with nitroreductase

2966 µL of a 10 mM PBS buffer (pH 7.4), 6 µL of 10 mM **HyCL-2** in DMSO, 24 µL of 50 mM NADH in 0.01 mM NaOH, 4 µL of 10 mg/mL nitroreductase were added into a vial, mixed well. Then After 3 h of incubation at rt, the mixture was poured into a separatory funnel containing 10 mL CH$_2$Cl$_2$ and 15 mL DI-H$_2$O and extracted with 3 x 10 mL CH$_2$Cl$_2$. The organic layer was collected, dried over Na$_2$SO$_4$, filtered, and concentrated. Then the solid was re-dissolved in 2 mL CH$_2$Cl$_2$, transferred to a GC-MS vial and GC-MS was conducted immediately using a 6850 Series GC-MS (Agilent Technologies, Santa Clara, CA). As a control experiment, the same procedure was conducted except that **HyCL-2** was replaced with 6 µL DMSO. Mass spectra were averaged across the peak found in the extracted ion chromatogram for m/z = 186 (Figure S2-1), m/z = 150 (Figure S2-2), and m/z = 121 (Figure S2-3). Matches to the NIST database were found using the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library Version 2.0 d, build Dec 2, 2005 installed with the Enhanced Chemstation software.
Figure 3-14. Mass spectrum found for m/z = 186. Inserted is the mass spectra found by NIST Mass Spectral Search Program.

Figure 3-15. Mass spectrum found for m/z = 150. Inserted is the mass spectra found by NIST Mass Spectral Search Program.
Figure 3-16. Mass spectrum found for m/z = 121.

3.4.4 Monitoring reaction of HyCL-1 and nitroreductase by $^1$H NMR

500 µL of 10 mM HyCL-1 in DMSO-d$_6$ and 100 µL of 100 mM cysteine in D$_2$O were mixed well in an Eppendorf tube and then transferred to an NMR tube. The reaction progress was monitored by $^1$H NMR at 500 MHz.

3.4.5 In vitro deoxygenation experiments

10 mM PBS buffer (pH 7.4) and Emerald II Enhancer were mixed with a ratio of 9 to 1 and N$_2$, air or 100% O$_2$ was bubbled through it for 60 minutes. The solution was then transferred into a capped cuvette by syringe, and later HyCL-2, NADH and nitroreductase were added into the cuvette using a syringe. Time scans were performed 1 min after adding nitroreductase. For deoxygenation experiments, PBS buffer (pH 7.4) and Emerald II Enhancer mixture was deoxygenated by bubbling N$_2$ for 60 minutes, and then 889 µL of this deoxygenated mixture was transferred into a capped cuvette by syringe, and later 2 µL of a 5 mM HyCL-2 in DMSO, 8 µL
of a 50 mM NADH in 0.01 mM NaOH solution, 1.25 µL of nitroreductase (10 mg/mL) were added into the cuvette using syringe. Time scans were performed 1 min after adding nitroreductase. The control experiment was carried out under ambient conditions without deoxygenation.

3.4.6 *In vitro* chemiluminescence imaging

Imaging was carried out with a Caliper Xenogen IVIS® Spectrum in black 96-well Costar® plates and all the images were analyzed using Living Image 3.1 software. 1 mM *HyCL-2* in DMSO, 2 mg/mL nitroreductase (10 µL from 1 mg nitroreductase /100 uL DI-H₂O diluted to 50 µL by adding 40 µL DI-H₂O) and 50 mM NADH in 0.01 mM NaOH solution were prepared prior to imaging. 176.4 µL, 176.1 µL, 175.9 µL, 175.5 µL, 175.4 µL and 175.1 µL of 10 mM PBS buffer (pH 7.4) were added into wells from A1 to A6 in sequence, 1.6 µL NADH was pipetted into each well and then different volume of nitroreductase solution (0 µL, 0.25 µL, 0.5 µL, 0.75 µL, 1.0 µL, 1.25 µL) were added into each well. 2 µL *HyCL-2* was added into the mixture and 20 µL Emerald II Enhancer was pipetted into each well, and imaging was performed 3.45 minutes after starting adding reagent into 96 well plate using an open filter. All images were acquired with f-stop 1, medium binning, auto exposure and the chamber set to 37 °C. The detection limit was determined from the linear plot of nitroreductase concentration versus total photon flux as the amount of nitroreductase required to give a chemiluminescent signal above three times the standard deviation of at least 3 independent experiments with 0 µg/mL nitroreductase. These provided detection limits of 9.6 ng/mL with measurements taken 30 second after reagent addition and 1.9 ng/mL with measurements taken 30 min after reagent addition.
3.4.7 General animal protocols

The UT Southwestern Institutional Animal Care and Use Committee approved these investigations under Animal Protocol Number (APN #2009-0150). Hair was removed from area surrounding the tumor using Nair (Church & Dwight Co, Inc, Ewing, NJ), but the skin was left unbroken before injection. Mice were anesthetized and maintained using inhalation of 1.6% isoflurane in 16% oxygen gas.

3.4.8 In vivo CLI of nitroreductase

A stock solution of 5 mM HyCL-2 in DMSO, 1 mg nitroreductase dissolved in 100 µL DI-H₂O and 50 mM NADH in 0.01 mM NaOH solution were prepared in advance. A 30 µL aliquot of this solution was taken out from a mixture of 437 µL 10 mM PBS, 4 µL HyCL-2, 8 µL NADH, 1.25 µL nitroreductase and 50 µL Emerald II Enhancer and images were acquired 30 second after administering i.p. injections to anesthetized (1.6% isoflurane) SCID/BALB-C mice. A vehicle control was conducted in the same way except replacing nitroreductase and NADH with H₂O and 0.01 M NaOH, respectively.

In vivo CLI of hypoxia in tumor xenografts. 100 µL solution was taken out from a mixture of 446 µL of 10 mM PBS, 50 µL Emerald II Enhancer and 4 µL of a 5 mM solution of HyCL-2 and images were acquired immediately and every minute after administering intratumoral injections to H1299 lung tumor xenografts on mice while the animals breathed ambient air. A similar experiment was performed in the same mice, but while breathing 100% oxygen 5 minutes before injection and throughout the course of the imaging experiment. These experiments were reproduced on two different mice (n = 2). Tumor sizes were 9.9 mm x 6.4 mm x 5.5 mm and 9.5 mm x 7.6 mm x 6.0 mm.
Multispectral optoacoustic tomography (MSOT). A thin layer of ultrasound coupling gel provided contact with the membrane and the animal holder was placed in the imaging chamber of the MSOT 256-TF device (iThera Medical Gmbh, Munich, Germany). Multi-spectral optoacoustic tomography (MSOT) imaging was performed in a transaxial section through the center of the tumor using five wavelengths: 715, 730, 760, 800, and 850 nm. Twenty frames per wavelength were acquired and averaged. Initially, the tumor region was imaged while breathing 16% oxygen, which was switched to 100% oxygen. A model-based reconstruction was used prior to multispectral processing to provide relative concentrations of oxyhemoglobin and deoxyhemoglobin. These experiments were reproduced on two different mice (n = 2).
3.5 References


CHAPTER 4
A CHEMILUMINESCENT PROBE FOR CELLULAR PEROXYNITRITE USING A SELF-IMMOLATIVE OXIDATIVE DECARBONYLATION REACTION

4.1 Introduction

Peroxynitrite (ONOO\(^-\)) is a strong reactive oxygen species that can be formed biologically from a fast reaction between superoxide radical anion (O\(_2^-\)) and nitric oxide (NO\(^-\)).\(^1\) ONOO\(^-\) and its protonated species, ONOOH (pKa 6.8) coexist under physiological conditions. There are two distinct pathways for ONOO\(^-\) involved oxidation. ONOO\(^-\) can oxidize target molecules directly through one- or two-electron reactions. The most notable functional groups involved in this chemistry are thiols.\(^2\) The more robust oxidative modifications are mediated by the radicals from its decomposition. The protonated ONOOH decomposes to form nitrogen dioxide (NO\(_2^-\)) and hydroxyl (OH\(^-\)) radicals via homolysis of the O–O bond. ONOO\(^-\) can be consumed rapidly by CO\(_2\),\(^3\) to form ONOOOCO\(_2^-\) which decomposes into carbonate radical (CO\(_3^-\)), and nitrogen dioxide (NO\(_2^-\)) (Scheme 4-1). Due to the abundance of CO\(_2\) in living system, and the fast reactivity, this represents a major pathway for radical generation. These radicals together can mediate a lot of oxidative transformations of biomolecules.
Scheme 4-1. The generation of ONOO\(^{-}\) and its decomposition pathways.

With its strong oxidative properties and the propensity of generating radicals upon decomposition, ONOO\(^{-}\) has long been recognized as a toxic agent, highly associated with oxidative damage and destruction of biomolecules, such as lipids, proteins, and nucleic acids.\(^4\)

Upregulation of ONOO\(^{-}\) was believed to disrupt cellular processes and signaling pathways and contribute to diseases such as ischemia-reperfusion,\(^5\) diabetes,\(^6\) cardiac dysfunction,\(^7\) inflammatory conditions,\(^8\) and autoimmune\(^9\) and neurodegenerative diseases.\(^10\) Recently, ONOO\(^{-}\) has been recognized to play some beneficial roles in certain conditions, such as in the immune system\(^11\) and ischemic preconditioning.\(^12\) ONOO\(^{-}\) can be generated in the immune activation process to attack invading pathogens.\(^13\) The main challenge in revealing its detailed biological roles and differentiating its oxidative damage from its beneficial effects lie in the difficulty of direct monitoring its generation and distribution in living systems. Therefore, the development of new methods and probes for ONOO\(^{-}\) is crucial for better understanding its biological functions.

Nitrotyrosine has been used previously as a biomarker for indirect measurement of ONOO\(^{-}\),\(^14\) but lacks temporal precision and can’t provide direct information on ONOO\(^{-}\). Optical imaging methods have become indispensable tools to monitor the dynamic of biomolecules in
living systems during last three decades and scientists are thrilled about “seeing” biological systems using imaging agents. A myriad of reaction-based fluorescent probes has been reported for ONOO⁻. These imaging agents can be tuned for the selective detection of ONOO⁻ through unique transformations to display an optical change such as the intensity or the wavelength, and many of them have been successfully applied for biological studies in living cells or animals, such as trifluorocarbonyl-based, N-dearylation-based, boronic ester-based. Among them, organoselenium-based, and organotellurium-based probes can be used for monitoring the reversible cycle of the oxidative stress. However, these fluorescent probes have some disadvantages associated with this imaging modality, such as photoscattering, autofluorescence and photobleaching.

In light of the unique advantages of chemiluminescent probes owing to the elimination of excitation light, we and others have previously used chemiluminescence as an imaging modality for designing reaction-based chemiluminescent probes for cellular and live animal imaging. Schaap’s adamantylidene-dioxetane scaffold was adopted by tethering a target responsive functionality for a specific analyte. Chemiluminescence can only be triggered by removal of the functional group by the analyte of interest. Although this design strategy is promising for constructing chemiluminescent agents for a variety of reactive species in living systems, the light production isn’t sufficient, which requires the addition of polymeric enhancer solutions to amplify light emission. Shabat and coworkers have proposed a new molecular methodology to obtain highly emissive chemiluminescent probes by introducing an electron withdrawing group such as an acrylonitrile at the ortho-position of the phenol. As a result, chemiluminescence emission can be observed in aqueous conditions without the addition of enhancer. Chemiluminescent probes for ONOO⁻ detection have been reported by using nanoparticle-based
methods\textsuperscript{27} which respond to the downstream decomposition products HO\textsuperscript{•} and O\textsubscript{2}\textsuperscript{−} to provide an indirect measurement of ONOO\textsuperscript{−}. In order to expand the toolbox for ONOO\textsuperscript{−} detection and create a chemiluminescent probe for direct ONOO\textsuperscript{−} measurement, we report the development and application of a chemiluminescent probe for the detection of ONOO\textsuperscript{−}, PNCL, with both \textit{in vitro} and live cell applications.

\textbf{4.2 Results and discussion}

\textbf{4.2.1 Design and synthesis of PNCL}

Our lab is interested in developing new imaging agents for interrogating the biological functions of RSON\textsuperscript{28,29,30} species. We have previously reported that isatin reacts selectively with ONOO\textsuperscript{−} through an oxidative decarbonylation reaction to generate anthranilic acid.\textsuperscript{31} \textsuperscript{19}F NMR probes, 5-fluoroisatin and 6-fluorisatin, have been developed for selective detection of ONOO\textsuperscript{−} based on this discovery. Initial attempts to expand the toolbox for ONOO\textsuperscript{−} detection centered around the preparation of \textit{N}-aryl isatin derivatives, anticipating a photophysical change upon reacting with ONOO\textsuperscript{−}. However, these attempts didn’t work out the way as we expected.

Mechanistic explorations were conducted to elucidate the structural necessity of this selective transformation. We treated isatin and its derivatives (Figure 4-1A) with SIN-1 (ONOO\textsuperscript{−} donor), revealing that fluorescent emission from \textit{N}-phenyl isatin and a dimethyl acetal derivative was significantly decreased. This important experiment demonstrated that the ketone and NH sites are essential in maintaining the selective reactivity between isatin and ONOO\textsuperscript{−}.
Figure 4-1. Mechanistic and structure-activity studies. (a) Fluorescence emission intensity at 400 nm of 50 µM isatin and 200 µM SIN-1. (b) Fluorescence emission intensity at 400 nm of 200 µM ONOO$^{-}$ and 50 µM of isatin derivatives. Legend: 1. isatin, 2. N-methyl isatin, 3. N-phenyl isatin, 4. isatin dimethyl acetal. (c) Fluorescence emission intensity at 400 nm of 50 µM isatin, 200 µM ONOO$^{-}$, and 0–5 mM NaHCO$_3$. (d) Fluorescence emission intensity at 400 nm of 50 µM isatin, 200 µM ONOO$^{-}$, and 0–5 mM TEMPOL. All experiments were performed in 20 mM HEPES (pH 7.4), containing 1% DMSO with excitation wavelength at 320 nm. (Data was collected by Audrey Reeves)

Scheme 4-2. Proposed mechanism for the reaction between isatin and ONOO$^{-}$. 
To rule out the reactivity between isatin and downstream radicals generated by ONOO−, such as HO•, CO3•− and NO2•, we performed a series of competition experiments. These results showed that the radical scavenger, TEMPO, didn’t interfere with the reaction between isatin and ONOO−, while NaHCO3, which equilibrates with CO2 in solution, inhibited the reaction (Figure 4-1C,D) since ONOO− can rapidly react with CO2. These inhibitor experiments further supported that isatin reacted directly with ONOO− instead of its downstream radicals. One possible mechanism was proposed based on these studies (Scheme 4-2). These mechanistic studies also promoted us to adopt isatin as a self-immolative functionality for making a chemiluminescent probe for ONOO−. The design strategy relies on tethering the isatin moiety to a 1,2-dioxetane chemiluminescent scaffold using an ether linkage (Scheme 4-3). The isatin reacts with ONOO− to generate the anthranilic acid, followed by a self-immolative 1,6-elimination to trigger the chemiluminescence emission through CIEEL. Synthesis of the PeroxyNitrite ChemiLuminescent probe (PNCL) started with the protection of the ketone group of 5-iodoisation 12 with dimethylacetal 13 (Scheme 4-4). A reductive formylation was conducted with palladium acetate as catalyst and N-formyl saccharin as the CO source to convert the dimethylacetal 13 to the aldehyde 14.32 The aldehyde 14 was subjected to the reduction with NaBH4, followed by deprotection to deliver isatin alcohol 15. Next, a Mitsunobu reaction was performed for the preparation of the precursor 17. Finally, [2+2] cycloaddition was conducted using Rose bengal as a photosensitizer by bubbling oxygen into the reaction mixture with light irradiation to provide the 1,2-dioxetane PNCL.
Scheme 4-3. Spiroadamantane 1,2-dioxetane-based probe for chemiluminescent ONOO\(^{-}\) detection.

Scheme 4-4. Synthesis of PNCL.

4.2.2 Response and selectivity of PNCL to ONOO\(^{-}\)

After obtaining PNCL, we started to collect its photophysical properties and test its reactivity towards ONOO\(^{-}\). Chemiluminescence emission was obtained by treating 20 µM PNCL with various amounts of ONOO\(^{-}\) without enhancer. The luminescence emission at 525 nm was increased with increasing the concentration of ONOO\(^{-}\) and it displayed excellent
linearity in the range of 0–200 µM ONOO• (Figure 4-2A,C). Although PNCL was tested in enhancer free conditions, the luminescent intensity was comparable with CHS-3 and HyCL-2, which were tested in the presence of Emerald II Enhancer. A dose dependent manner of the chemiluminescent intensity was also observed in the time scan test (Figure 4-2B), indicating our probe can detect ONOO• with high sensitivity. Chemiluminescence emission reached a maximum immediately after adding ONOO• and was slowly decayed over a time course of 20 min, demonstrating an advantage of this imaging modality for quick detection without extensive incubation. Surprisingly, the luminescent intensity of PNCL can be recovered after several cycles of ONOO• (Figure 4-3). This reversible property of chemiluminescent probe can be applied for monitoring ONOO• fluxes over time. The detection limit of PNCL was 6 nM (Figure 4-4), comparing favorably to most reported fluorescent and chemiluminescent probes (Table 4-1). The reaction between PNCL and ONOO• was further confirmed by GC-MS (Figure 4-5). The reactivity of PNCL towards SIN-1 was also evaluated (Figure 4-2D), demonstrating the ability of PNCL to monitor the slow release of ONOO•. SIN-1 generates ONOO• by simultaneously generating NO• and O2•− (Scheme 4-5).
Scheme 4-5. SIN-1 generates ONOO⁻ by simultaneously generating NO⁺ and O₂⁻.

Figure 4-2. Response of PNCL to ONOO⁻. (A) Chemiluminescence emission spectra of 20 μM PNCL and 0 (blue trace), 5, 10, 20, 40, 80, 100, 200 μM (red trace) ONOO⁻. (B) Time scans of the chemiluminescence emission at 525 nm of 20 μM PNCL and 0 (blue trace), 5, 10, 20, 40, 80, 100, 200 μM (red trace) ONOO⁻. (C) Peak emission intensity at 525 nm of 20 μM PNCL after adding 0–200 μM ONOO⁻. (D) Time scan of the chemiluminescence emission at 525 nm of 20 μM PNCL and 200 μM SIN-1. All experiments were performed in 20 mM HEPES (pH 7.4), containing <1% DMSO.
Figure 4-3. Repeatability of chemiluminescent response. Time course of the chemiluminescence emission at 525 nm of 20 µM PNCL and 200 µM ONOO⁻ in 20 mM HEPES (pH 7.4). Every 20 min, an additional 200 µM ONOO⁻ was added to the solution.

Figure 4-4. Detection Limit of PNCL for bolus ONOO⁻. Integrated chemiluminescence emission intensity of 20 µM PNCL and 0 µM, 0.5 µM, 1 µM, 1.5 µM, and 2 µM of ONOO⁻.

Table 4-1. Detection limits of selected fluorescent and chemiluminescent ONOO⁻ probes.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>LoD</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>Fluorescence</td>
<td>2.5 µM</td>
</tr>
<tr>
<td>35</td>
<td>Fluorescence</td>
<td>0.917 µM</td>
</tr>
<tr>
<td>36</td>
<td>Chemiluminescence</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>37</td>
<td>Fluorescence</td>
<td>50 nM</td>
</tr>
<tr>
<td>38</td>
<td>Fluorescence</td>
<td>35 nM</td>
</tr>
<tr>
<td>39</td>
<td>Fluorescence</td>
<td>10 nM</td>
</tr>
</tbody>
</table>
We proceeded to evaluate the selectivity of PNCL for ONOO\(^{-}\) against other biologically relevant RSON species and metal cations. The selectivity test was carried out by treating PNCL...
(20 μM) with different amount of RSON species and metal cations in HEPES buffer (20 mM, pH 7.4). 5 mM GSH, 1 mM L-cysteine, or 200 μM other reactive species and metal cations were used for evaluation. Most of the other species displayed negligible chemiluminescent increase over the blank control within 20 min (Figure 4-6). However, Angeli’s salt, a nitroxyl (HNO) donor, displayed a small luminescent increase. This was also observed from our previous 19F NMR probes.31

![Chemiluminescence emission at 525 nm of 20 μM PNCL and 200 μM reactively relevant analytes in 20 mM HEPES (pH 7.4). Bars represent chemiluminescence emission at 525 nm at 4, 8, 12, 16, 20 min after addition of reactive species. Legend: 1. ONOO⁻, 2. Cys, 3. DEA NONOate, 4. GSH (5 mM), 5. GSNO, 6. H₂O₂, 7. Angeli’s salt, 8. KO₂, 9. Na₂S, 10. Na₂SO₃, 11. Na₂SO₄, 12. NaNO₂, 13. HO*, 14. OCl⁻, 15 'BuOOH, 16. ¹O₂, 17. Na*, 18. Mg²⁺, 19. K⁺, 20. Ca²⁺, 21. Zn²⁺, 21. Fe²⁺, Blank. Error bars are ± S.D. All experiments were performed in 20 mM HEPES (pH 7.4), containing <1% DMSO. (Data was collected by me and Weiwei An)"

In light of previous reports that HNO reacts with molecular oxygen to form ONOO⁻ at physiological pH,41 and different reactivities of these two species, we designed a series of competition experiments to reveal what caused this luminescent increase. It is known that ONOO⁻ reacts rapidly with CO₂, but slowly with GSH. However, HNO preferentially reacts with thiols such as GSH.42 We performed the experiment by treating PNCL (20 μM) with
ONOO\(^-\) (200 µM) or Angeli’s salt (200 µM) in the presence of various concentration of HCO\(_3^-\) (Figure 4-7) and GSH (Figure 4-8). Chemiluminescent intensity was decreased for both reactions with HCO\(_3^-\) addition, while GSH only quenched the chemiluminescence emission from Angeli’s salt. Interestingly, chemiluminescence emission relapsed after a certain amount time when less than 200 µM GSH was added (Figure 4-8C). This observation is consistent with the facts that GSH functions as the HNO scavenger at physiological condition and Angeli's salt releases HNO with a half-life of 2.3 minutes at 37 °C. Once the GSH was fully consumed, newly released HNO from Angeli’s salt can be oxidized to ONOO\(^-\) to produce a luminescent increase.

**Figure 4-7.** Inhibition of response by NaHCO\(_3\). (a) Time scans and (b) integrated intensity of the chemiluminescence emission of 20 µM PNCL, 200 µM ONOO\(^-\) and 0 (blue trace), 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM (red trace) NaHCO\(_3\). (c) Time scans and (d) integrated intensity of the chemiluminescence emission of 20 µM PNCL, 200 µM Angeli's salt and 0 (blue trace), 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM (red trace) NaHCO\(_3\). All experiments were performed in 20 mM HEPES (pH 7.4), containing <1% DMSO. (Data was collected by Weiwei An)
Figure 4-8. Inhibition of response by GSH. (a) Time scans and (b) integrated intensity of the chemiluminescence emission of 20 μM PNCL, 200 μM ONOO⁻ and 0 (blue trace), 50 μM, 100 μM, 200 μM, and 1 mM (red trace) GSH. (c) Time scans and (d) integrated intensity of the chemiluminescence emission of 20 μM PNCL, 200 μM Angeli’s salt and 0 (blue trace), 50 μM, 100 μM, 200 μM, and 1 mM (red trace) GSH. All experiments were performed in 20 mM HEPES (pH 7.4), containing <1% DMSO. (Data was collected by Weiwei An)

We also prepared a fluorescent nitroxyl (HNO) probe, XF1⁴³,⁴⁴ (Figure 4-9A), to determine the quenching effect of CO₂ to HNO. XF1 reacts with Angeli’s salt (Figure 4-10 A,B) with a strong fluorescent increase, and XF1 showed good selectivity to HNO from other RSON species (Figure 4-10C). The fluorescence response of XF1 towards Angeli’s salt was completely aborted with 200 μM GSH (Figure 4-9B,C), while no significant fluorescence change was observed from NaHCO₃ treated solution (Figure 4-9B,C), confirming that GSH quenches HNO but HCO₃⁻/CO₂ does not scavenge HNO. Taken together, these results indicate the formation of ONOO⁻ from HNO oxidation and further supports that PNCL is highly selective towards ONOO⁻.
Figure 4-9. HNO scavenging by NaHCO$_3$ and GSH. (a) Design of XF1, a fluorescent probe for HNO. (b) Peak emission intensity of 10 µM XF1, 200 µM Angeli's salt and 0-5 mM NaHCO$_3$. (c) Peak emission intensity of 10 µM XF1, 200 µM Angeli's salt and 0-1 mM GSH. All experiments were performed in 20 mM HEPES (pH 7.4), containing <1% DMSO with $\lambda_{ex} = 488$ nm. (Data was collected by Audrey Reeves)

Figure 4-10. (a) Fluorescence emission of 10 µM XF1 and 200 µM Angeli's salt after reacting for 1, 5, 10, 15, 20, 25, and 30 min in 20 mM HEPES (pH 7.42). (b) Peak emission intensity of 10 µM XF1 and 200 µM Angeli's salt after reacting for 1, 5, 10, 15, 20, 25, and 30 min in 20 mM HEPES (pH 7.42). (c) Selectivity of XF1 versus other reactive sulfur, oxygen, and nitrogen species. Legend: 1. Angeli's salt, 2. Na$_2$S, 3. ONOO$^-$, 4. ClO$^-$, 5. H$_2$O$_2$, 6. GSH (5 mM), 7. GSNO, 8. DEA NONOate, 9. tBuOOH. (Data was collected by Sara Merrikhihaghi Lange)
The depth of tissue penetration of PNCL and its 1,2-dioxetane phenol was determined using IVIS Spectrum. We used the beef bologna (Tom Thumb) as a tissue phantom to mimic the biological tissue and the depth was measured by ruler. The data showed that the chemiluminescent light from 1 mM 1,2-dioxetane phenol in basic conditions (2 mM NaOH) can penetrate up to 2–2.5 cm beef bologna. Furthermore, light from 1 mM PNCL and 2 mM ONOO⁻ can still be visualized through up to 1.5–2 cm beef bologna (Figure 4-11). This experiment demonstrates that chemiluminescence as an imaging modality has great potential to explore biological processes in deep tissue.

![Figure 4-11](image-url)

**Figure 4-11.** Tissue penetration of chemiluminescence of (A) 1,2-dioxetane phenol and (B) PNCL. Beef bologna was used to mimic tissue sample, and the depth was determined with ruler.

### 4.2.3 Using PNCL for ONOO⁻ detection in living cells

PNCL reacts with ONOO⁻ with high sensitivity and selectivity, and the luminescent intensity was strong enough without the addition of a polymeric enhancer solution, which could potentially open a gate for ONOO⁻ detection in living cells. A BioTek Cytation 5 plate reader was used for cell experiments. We first evaluated the cell toxicity of PNCL by the MTT assay.
Data confirmed that PNCL exhibits low toxicity at concentrations less than 100 µM and more than 50% cell survived with 1 mM PNCL for 24-hour incubation (Figure 4-12). Cell permeability was evaluated by imaging the intrinsic fluorescence emission of PNCL using an EVOS microscope, demonstrating good cell permeability (Figure 4-13). We next determined the ability of PNCL to detect ONOO\(^-\) delivered from donor compound, SIN-1. RAW 264.7 macrophages were incubated with 20 µM PNCL for 30 minutes in Dulbecco’s Modified Eagle Medium (DMEM), and then washed twice with PBS to remove excess probe. Luminescence emission was monitored by a plate reader for 8 hours after the addition of 0, 200 µM, 400 µM, 800 µM, 1 mM, or 2 mM SIN-1. A dose dependent luminescent increase was observed and the signal reached a maximum at 40 minutes (Figure 4-15A,B), resulting a ~5-fold luminescent increase with 2 mM SIN-1 over a blank control. Similar result was obtained in A549 cells (Figure 4-14), demonstrating that PNCL can be used for different cell lines. Chemiluminescence emission was decreased by Mn(III)TMPyP, a ONOO\(^-\) scavenger (Figure 4-15), confirming that the increased signal was from ONOO\(^-\).

**Figure 4-12.** MTT assay of RAW264.7 macrophage cells in the presence of different concentrations of PNCL. Error bars are ±S.D. (n = 3).
Figure 4-13. Cellular internalization of PNCL. Normalized fluorescence (A) excitation and (B) emission spectrum of PNCL in 20 mM HEPES (pH 7.4). Confocal fluorescence images of living RAW 264.7 cells in (C) the absence of PNCL or (D) presence of 40 μM PNCL.

Figure 4-14. ONOO⁻ detection in A549 cells. (A) Time scans and (B) integrated intensity of chemiluminescence emission of A549 cells incubated with 20 μM PNCL for 30 minutes, washed and then treated with 0 (blue trace), 200 μM, 400 μM, 800 μM, 1 mM, and 2 mM (red trace) SIN-1. (C) The linear region of the response curve shown in (B).
Figure 4-15. Inhibition of signal from SIN-1 by ONOO− scavenger MnTMPyP. (A) Time scans of RAW 264.7 macrophages incubated with 20 µM PNCL for 30 minutes, washed, and then incubated with (blue trace) vehicle control (DMSO), (red trace) 2 mM SIN-1, or (black trace) 2 mM SIN-1 and 50 µM Mn(III)TMPyP. (B) Integrated intensity of chemiluminescence emission of RAW 264.7 macrophages incubated with 20 µM PNCL for 30 minutes, washed, and then incubated with vehicle control, 2 mM SIN-1, 2 mM SIN-1 and 25 µM MnTMPyP, or 2 mM SIN-1 and 50 µM MnTMPyP. Error bars are S.D. from n = 3 wells. Statistical significance was assessed using a two-tailed student's t-test. *** p < 0.001.

Finally, we determined the ability of PNCL to detect endogenously generated ONOO− in macrophages with lipopolysaccharide (LPS) stimulation. LPS is a characteristic component of the bacterial cell wall and can be used to model the pathogen attack to stimulate an immune response of macrophages. LPS activates the immune response by interacting with the membrane receptors, subsequently activating a cascade of signal transductions, resulting in the high expression of inducible nitric oxide synthase (iNOS), followed by the generation of ONOO− (Scheme 4-6).45 Data showed that macrophages with 1000 ng mL−1 LPS treatment displayed ~36% increase in integrated luminescence intensity (Figure 4-16C), and the increased signal was attenuated after the addition of Mn(III)TMPyP, confirming the increased signal from PNCL resulted from newly generated ONOO−. Chemiluminescent signal was also decreased after the addition of iNOS inhibitor 1400W (Figure 4-17), indicating iNOS is essential for the biosynthesis of ONOO−. These experiments validate the ability of PNCL to detect biosynthetic
processes of ONOO\(^-\). The formation of phagosomes was observed after LPS stimulation (Figure 4-16D).

**Scheme 4-6.** Biosynthetic pathway of the generation of ONOO\(^-\) upon LPS stimulation in macrophages.
Figure 4-16. ONOO\textsuperscript{−} detection in RAW 264.7 macrophages. (a) Time scans and (b) integrated intensity of chemiluminescence emission of RAW 264.7 macrophages incubated with PNCL for 30 minutes, washed and then treated with 0 (blue trace), 200 µM, 400 µM, 800 µM, 1 mM, and 2 mM (red trace) SIN-1. (c) RAW 264.7 macrophages stimulated with (Cont) DI-H\textsubscript{2}O as a vehicle control, (LPS) 1000 ng mL\textsuperscript{−1} LPS, or (LPS, MnTMPyP) 1000 ng mL\textsuperscript{−1} LPS and 25 µM Mn(III)TMPyP. (d) Brightfield images of stimulated RAW 264.7 macrophages. Error bars are S.D. from n = 3–7 wells and 3 biological replicates (n = 10 wells from 6 biological replicates for the control experiment). Statistical significance was assessed using a two-tailed student's t-test. ** p < 0.001, * p < 0.01.

Figure 4-17. Inhibition of iNOS mediated ONOO\textsuperscript{−} production. (A) Time scans of RAW 264.7 macrophages incubated with (blue trace) vehicle control (DI-H\textsubscript{2}O), (red trace) 1000 ng/mL LPS, or (black trace) 1000 ng/mL LPS and 200 µM 1400W for 16 hours, then incubated with 20 µM PNCL and vehicle control or 1400W for 30 minutes, washed and measured. (B) Integrated intensity of chemiluminescence emission of RAW 264.7 macrophages incubated with vehicle control, 1000 ng/mL LPS, or 1000 ng/mL LPS and 200 µM 1400W for 16 hours, then incubated with 20 µM PNCL and vehicle control or 1400W for 30 minutes, washed and measured. Error
bars are S.D. from n = 3 wells. Statistical significance was assessed using a two-tailed student's t-test. *** p < 0.001, * p < 0.05.

4.3 Conclusions

To close, we have designed and synthesized a reaction-based chemiluminescent probe for ONOO\(^-\), termed as PNCL. PNCL was constructed by linking a 1,2-dioxetane chemiluminescent scaffold with isatin through an ether linkage. Mechanistic studies revealed that isatin reacts directly with ONOO\(^-\) instead of its downstream radicals. Isatin was used as a masking group which can be cleaved after reacting with ONOO\(^-\) through an oxidative decarbonylation reaction to initiate the light emission. PNCL responds to ONOO\(^-\) with high sensitivity and selectivity at physiological pH. We also provided some experimental support for the autooxidative product of HNO. PNCL has been applied for ONOO\(^-\) detection from donor compounds in multiple cell lines, as well as cellular ONOO\(^-\) generated by macrophages with LPS stimulation. Taken together, we have reported a useful tool, PNCL, to interrogate the biological functions of ONOO\(^-\).

4.4. Experimental section

4.4.1 Chemical synthesis

![Chemical structure](image)

**5-iodo-3,3-dimethoxyindolin-2-one (13)**. 5-iodoisatin (203.1 mg, 7.43 mmol, 1.0 equiv) was dissolved in 300 mL MeOH, followed directly by the addition of 7.1 mL HCl. The reaction was stirred for 48 h at rt. The reaction mixture was neutralized with saturated NaHCO\(_3\), and then the reaction mixture was poured into a separatory funnel containing 150 mL H\(_2\)O, and extracted with
3 x 100 mL EtOAc. The organic layer was washed with 10 mL brine, dried over Na₂SO₄, filtered, and concentrated. Purification by silica column chromatography (1:4 EtOAc/hexanes) afforded 13 (2.2 g, 93%) as a colorless solid. \(^1\)H NMR (500 MHz, CDCl₃) δ 9.46 (s, 1H), 7.65 (d, 1H, \(J = 1.7\) Hz), 7.58 (dd, 1H, \(J = 8.0, 1.7\) Hz), 6.70 (d, 1H, \(J = 8.0\) Hz), 3.53 (s, 6H); \(^1\)C NMR (125 MHz, CDCl₃) δ 173.02, 140.40, 139.61, 133.84, 127.42, 113.36, 97.42, 85.43, 51.08. HRMS calcd for C₁₀H₁₀INO₃ (M–H⁻) 317.9633, found 317.9638.

3,3-dimethoxy-2-oxindoline-5-carbaldehyde (14). Compound 13 (607.0 mg, 1.90 mmol, 1.0 equiv), N-formyl saccharin (602.8 mg, 2.86 mmol, 1.5 equiv), palladium(II) acetate (12.8 mg, 0.057 mmol, 0.03 equiv), 1,4-bis(diphenylphosphino)butane (36.5 mg, 0.086 mmol, 0.045 equiv), and Na₂CO₃ (302.3 mg, 2.86 mmol, 1.5 equiv) were added to a 10 mL pressure flask. The flask was evacuated and backfilled with N₂ three times. Then, a degassed solution of Et₃SiH (395 µL, 2.47 mmol, 1.3 equiv) and anhydrous DMF (2 mL) were added to the flask under N₂. The pressure flask was sealed immediately and the mixture was stirred for 10 min at rt. Then the mixture was warmed to 80 °C and stirred for 16 h. The reaction was quenched with 100 mL saturated NH₄Cl, extracted with 3 x 80 mL EtOAc, washed with 10 mL brine, dried over Na₂SO₄, filtered, and concentrated. The crude was purified by the silica column chromatography (1:3 EtOAc/hexanes) to deliver compound 14 as a white solid (245.0 mg, 59%). \(^1\)H NMR (500 MHz, CDCl₃) δ 9.90 (s, 1H), 9.33 (s, 1H), 7.93 (d, 1H, \(J = 1.7\) Hz), 7.87 (dd, 1H, \(J = 8.0, 1.7\) Hz), 7.07 (d, 1H, \(J = 8.0\) Hz), 3.59 (s, 6H); \(^1\)C NMR (125 MHz, CDCl₃) δ 190.81, 173.33, 146.20, 134.69, 131.82, 126.25, 125.85, 111.36, 96.60, 51.02. HRMS calcd for C₁₁H₁₁NO₄ (M–H⁻) 220.0615, found 220.0609.
5-(hydroxymethyl)indoline-2,3-dione (15). Compound 14 (160.0 mg, 0.72 mmol, 1.0 equiv) and sodium borohydride (41.9 mg, 1.11 mmol, 1.53 equiv) were dissolved in 12 mL EtOH, and the mixture was stirred at rt for 1 h. The reaction was quenched with 80 mL saturated NH₄Cl, extracted with 3 x 40 mL EtOAc, washed with 10 mL brine, dried over Na₂SO₄, filtered, and concentrated to yield 5-(hydroxymethyl)-3,3-dimethoxyindolin-2-one (162 mg) as a colorless oil and used without further purification. ¹H NMR (500 MHz, CDCl₃) δ 9.05 (s, 1H), 7.39 (s, 1H), 7.24 (d, 1H, J = 8.0 Hz), 6.79 (d, 1H, J = 8.0 Hz), 4.61 (s, 2H), 3.53 (s, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 173.33, 140.14, 135.67, 129.81, 125.43, 124.28, 111.05, 97.41, 64.88, 50.95. HRMS calcd for C₁₁H₁₃NO₄ (M–H⁻) 222.0772, found 222.0778. 5-(hydroxymethyl)-3,3-dimethoxyindolin-2-one (189 mg, 0.85 mmol, 1.0 equiv) was dissolved in 12 mL 1 M HCl solution, and the reaction was stirred at rt for 30 min. The reaction was neutralized with saturated NaHCO₃, and then the reaction mixture was poured into a separatory funnel, and extracted with 3 x 40 mL 3:1 DCM: iPrOH and 3 x 30 mL EtOAc. The organic layer was washed with 10 mL brine, dried over Na₂SO₄, filtered, and concentrated. Purification by silica column chromatography (1:1 to 2:1 EtOAc/hexanes) afforded 15 (132.0 mg, 88%) as an orange solid. ¹H NMR (500 MHz, (CD₃)₂CO) δ 7.58 (d, 1H, J = 8.0 Hz), 7.50 (dd, 1H, J = 8.0, 1.7 Hz), 6.96 (d, 1H, 1.7 Hz), 4.57 (s, 2H); ¹³C NMR (125 MHz, (CD₃)₂CO) δ 184.09, 159.14, 149.60, 137.82, 136.77, 122.77, 118.10, 112.00, 62.81. HRMS calcd for C₉H₇NO₃ (M–H⁻) 176.0353, found 176.0345.
(E)-3-(4-(((1r,3r,5r,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-((2,3-dioxoindolin-5-yl)methoxy)phenyl)acrylonitrile (17). Compound 15 (20 mg, 0.11 mmol, 1.0 equiv), compound 16 (43.8 mg, 0.11, 1.0 equiv), and triphenylphosphine (36.0 mg, 0.13 mmol, 1.2 equiv) were dissolved in 2 mL anhydrous THF, and the reaction mixture was cooled to 0 °C. Diethyl azodicarboxylate (21.3 µL, 0.14 mmol, 1.2 equiv) was added dropwise over 5 min. After 1 h of stirring at rt, the mixture was concentrated. Purification by silica column chromatography (2% MeOH/DCM) afforded 17 as a yellow oil (49.0 mg, 84%). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.56 (s, 1H), 7.71 (dd, 1H, $J$ = 8.0, 1.7 Hz), 7.64 (d, 1H, $J$ = 1.7 Hz), 7.50 (d, 1H, $J$ = 16.8 Hz), 7.12 (d, 1H, $J$ = 8.0 Hz), 7.02 (d, 1H, $J$ = 8.0 Hz), 5.90 (d, 1H, $J$ = 16.8 Hz), 4.98 (m, 2H), 3.32 (s, 3H), 3.27 (s, 1H), 1.64-2.20 (m, 13 H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 182.76, 159.24, 152.97, 149.62, 144.73, 139.66, 139.05, 133.50, 131.91, 129.98, 128.40, 125.88, 124.59, 118.24, 117.98, 112.85, 98.63, 75.11, 57.56, 39.27, 39.12, 38.73, 37.05, 33.11, 29.85, 29.36, 28.38, 28.21. HRMS calcd for C$_{30}$H$_{27}$ClN$_2$O$_4$ (M–H–) 513.1587, found 513.1595.

(E)-3-(3-chloro-2-((2,3-dioxoindolin-5-yl)methoxy)-4-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)phenyl)acrylonitrile (PNCL). Enol ether 17 (40.0 mg, 0.075 mmol, 1.0 equiv) and Rose bengal (9.8 mg, 0.010 mmol, 0.13 equiv) were added into a dry flask and dissolved in 5 mL THF. Oxygen was bubbled through the reaction.
mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 3 h of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum at 0 °C and the residue was purified by the silica column chromatography (10:1 DCM/Ether) to deliver PNCL (33.0 mg, 81%) as a yellow solid. $^1$H NMR (500 MHz, CDCl$_3$) δ 9.02 (s, 1H), 7.97 (d, 1H, $J$ = 8.6 Hz), 7.66-7.70 (m, 2H), 7.25-7.52 (m, 2H), 7.12 (d, 1H, $J$ = 8.0 Hz), 7.04 (d, 1H, $J$ = 8.0 Hz), 5.96 (d, 1H, $J$ = 16.6 Hz), 4.90 (s, 2H), 3.22 (s, 3H), 3.02 (s, 1H), 1.24-2.20 (m, 13H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 182.77, 159.21, 153.45, 149.72, 144.23, 138.94, 136.76, 131.60, 120.50, 129.62, 125.86, 124.77, 118.30, 117.65, 112.92, 111.63, 100.03, 96.49, 75.26, 49.87, 36.58, 33.98, 33.72, 32.79, 32.23, 31.64, 31.05, 29.79, 26.18, 25.87; MALDI-MS calcd for C$_{30}$H$_{27}$ClN$_2$O$_6$ (M+H$^+$) 547.16, found 547.15.

### 4.4.2 Preparation of ONOO$^{-46}$

To a 200 mL flask was added 2.2 mL of 11.6 M H$_2$O$_2$ (26.0 mmol) further diluted with 50 mL DI water. The mixture was allowed to chill to 0 °C before addition of 36 mL of 0.55 M NaOH followed by 5 mL of 0.04 M EDTA. The final mixture was diluted to a total volume of 100 mL with DI water before the addition of 3.4 mL isopentyl nitrite (26.0 mmol). A deep, yellow color indicates formation of peroxynitrite. After 6 hours of stirring at 0 °C, the mixture was washed 3 x 20 mL with DCM. To the aqueous layer was added roughly 20 mg of MnO$_2$. The mixture was allowed to stir until bubbling ceased, upon which it was filtered twice and stored on ice or in the freezer. The concentration was determined using an extinction coefficient of $\varepsilon$ = 1670 M$^{-1}$ cm$^{-1}$ at 302 nm.
4.4.3 GC-MS procedure to monitor reaction intermediates

300 µL of 10 mM PNCL in DMSO (3 mM final concentration), and 125 µL of 48 mM ONOO⁻ (6 mM final concentration) were mixed in an Eppendorf tube, and vortexed for 5 second. The reaction mixture was then poured into a separatory funnel containing 10 mL EtOAc and 15 mL DI-H₂O and extracted with 2 x 10 mL EtOAc. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated. Then the solid was re-dissolved in 2 mL CH₂Cl₂, transferred to a GC-MS vial and GC-MS was conducted immediately.

4.4.4 Chemiluminescent response

20 mM HEPES buffer (pH 7.4), 5 mM PNCL stock solution in DMSO and ONOO⁻ were added to a quartz cuvette in sequences. Samples were shaken gently to assure mixing. 20 µM PNCL was treated with 0, 5, 10, 20, 40, 80, 100 and 200 µM ONOO⁻. Time scans were acquired using the time scan module by setting the emission wavelength to 525 nm. Wavelength scans were acquired by treating 20 µM PNCL with 0 µM to 200 µM ONOO⁻. Time scans and wavelength scans were measured 10 second after adding ONOO⁻.

4.4.5. Determination of the detection limit of bolus ONOO⁻

Chemiluminescent responses were measured using a Cytation 5 BioTek plate reader (Winooski, VT) by using the luminescence detection method, endpoint read type, and setting gain to 135 and temperature to 37 °C. 0.125 mM, 0.25 mM, 0.5 mM, 0.75 mM ONOO⁻ stock solutions were made by dilution of a 16 mM ONOO⁻ solution with 0.01 M NaOH, and the final concentration was also confirmed by UV/Vis. 249 µL of 10 mM PBS buffer (pH 7.4) was added into vehicle well, and 248 µL of 20 mM HEPES buffer (pH 7.4) was added into other wells. 1 µL
of 5 mM PNCL was pipetted into every well, and then 1 µL of different concentrated ONOO− stock solutions were added into wells. The chemiluminescence emission was then measured on the plate reader. The emission was integrated over 30 minutes and the calibration curve was constructed using three replicate experiments. The detection limit was determined as LoD = 3σ/k.

4.4.6 Structure-activity studies

**Fluorescence response.** 5 mM isatin and 30 mM SIN-1 in DMSO were prepared before testing. 10 µL of 5 mM isatin (50 µM final concentration) and 16.7 µL of 30 mM SIN-1 (500 µM final concentration) were added into 973.3 µL of 20 mM HEPES buffer (pH = 7.42), and fluorescence response was taken 1 min after addition with excitation wavelength at 320 nm. Isatin derivatives, N-methyl isatin, N-phenyl isatin, and isatin dimethyl acetal were prepared in DMSO with final concentrations of 5 mM. 10 µL of 5 mM isatin or its derivatives (50 µM final concentration) was added into a cuvette with 983 µL HEPES buffer, and then 6.62 µL of 30.2 mM ONOO− (200 µM final concentration) was added into the mixture. Wavelength spectra were measured 1 min after adding ONOO− with excitation wavelength at 320 nm.

**Inhibition experiments with HCO3− and TEMPOL.** Different volumes of 100 mM HCO3− or TEMPOL in DI-H2O (0 µL, 2 µL, 5 µL, 10 µL and 50 µL) were added to the 20 mM HEPES buffer in the presence of 10 µL of 5 mM isatin (50 µM final concentration) in DMSO, followed by adding 5.29 µL of 37.9 mM ONOO− (200 µM final concentration). The cuvette was shaken gently to assure mixing. Then, fluorescence spectra were acquired 1 min after addition with excitation wavelength at 320 nm.
4.4.7 Selectivity tests

Selectivity for PNCL was measured by monitoring the time-dependent chemiluminescence emission at 525 nm with an F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). All assays were performed in 20 mM HEPES buffered to pH 7.42. All analytes were tested with final concentration of 200 µM, with the exceptions of GSH (5 mM), and L-cysteine (1 mM).

$\text{ONOO}^- (200 \mu M)$: 3.1 µL of 65 mM ONOO$^-$ was added to a solution of 993 µL HEPES buffer and then 4 µL of 5 mM PNCL in DMSO was added to the mixture.

$\text{S}_2\text{O}_5^{2-} (200 \mu M)$: 4 µL of 50 mM Na$_2$S$_2$O$_5$ in DI-H$_2$O was added to a solution of 992 µL HEPES buffer and then 4 µL of 5 mM PNCL in DMSO was added to the mixture.

$\text{tBuOOH} (200 \mu M)$: 4 µL of 50 mM tBuOOH in DI-H$_2$O was added to a solution of 992 µL HEPES buffer and then 4 µL of 5 mM PNCL in DMSO was added to the mixture.

$\text{S-nitrosoglutathione} (200 \mu M)$: 20 µL of 10 mM S-nitrosoglutathione in DI-H$_2$O was added to a solution of 976 µL HEPES buffer and then 4 µL of 5 mM PNCL in DMSO was added to the mixture.

$\text{GSH} (5 \text{ mM})$: 20 µL of 250 mM GSH in DI-H$_2$O was added to a solution of 976 µL HEPES buffer and then 4 µL of 5 mM PNCL in DMSO was added to the mixture.

$\text{L-Cysteine} (1 \text{ mM})$: 4 µL of 250 mM cysteine in DI-H$_2$O was added to a solution of 992 µL HEPES buffer and then 4 µL of 5 mM PNCL in DMSO was added to the mixture.

$\text{NaNO}_2 (200 \mu M)$: 4 µL of 50 mM NaNO$_2$ in DI-H$_2$O was added to a solution of 992 µL HEPES buffer and then 4 µL of 5 mM PNCL in DMSO was added to the mixture.

$\text{Na}_2\text{SO}_4 (200 \mu M)$: 4 µL of 50 mM Na$_2$SO$_4$ in DI-H$_2$O was added to a solution of 992 µL HEPES buffer and then 4 µL of 5 mM PNCL in DMSO was added to the mixture.
\textbf{Na}_2\text{S (200 μM)}: 4 μL of 50 mM \text{Na}_2\text{S} in DI-H\text{O} was added to a solution of 992 μL HEPES buffer and then 4 μL of 5 mM \text{PNCL} in DMSO was added to the mixture.

\textbf{H}_2\text{O}_2 (200 μM): 4 μL of 50 mM H\text{O}_2 in DI-H\text{O} was added to a solution of 992 μL HEPES buffer and then 4 μL of 5 mM \text{PNCL} in DMSO was added to the mixture.

\textbf{NO}^\cdot (200 μM): DEA NONOate was used to generate NO. It was stored at –20 °C and dissolved in 0.01 M NaOH solution immediately prior to use. The concentration of this alkaline stock solution of DEA NONOate was measured by UV/Vis using $\varepsilon = 6500 \text{ M}^{-1} \text{ cm}^{-1}$ at 250 nm. 6.36 μL of 31.45 mM DEA NONOate in 0.01 M NaOH solution was added to a 994 μL solution of 20 μM \text{PNCL} in HEPES buffer.

\textbf{HNO (200 μM)}: Angeli’s salt (Na$_2$N$_2$O$_3$) was used to generate HNO. The stock solution was made by dissolving Angeli’s salt in 0.01 M NaOH solution immediately prior to use. The concentration of this alkaline stock solution of Angeli's salt was measured by UV/V is using $\varepsilon = 6100 \text{ M}^{-1} \text{ cm}^{-1}$ at 237 nm. 6.33 μL of 31.55 mM stock solution of H\text{O}_2 in DI-water was added to a 994 μL solution of 20 μM \text{PNCL} in HEPES buffer.

\textbf{O}_2^\cdot (200 μM): 1 mg KO$_2$ (final concentration 200 μM) was added to a 70 mL solution of 20 μM \text{PNCL} in HEPES buffer.

\textbf{OH}^\cdot (200 μM): 1 mg Fe(ClO$_4$)$_2$ (final concentration 200 μM) was added to a 30 mL solution of 20 μM \text{PNCL} and H\text{O}_2 (final concentration 200 μM) in HEPES buffer.

\textbf{OCl}^\cdot (200 μM): 4 μL of 50 mM NaOCl in DI-H\text{O} was added to a solution of 992 μL HEPES and 4 μL of 5 mM \text{PNCL} in DMSO was added into this mixture.

\textbf{Blank}: 3.1 μL of a 0.20 M NaOH in DI-H\text{O} was added to a solution of 993 μL HEPES and 4 μL of 5 mM \text{PNCL} in DMSO was added into this mixture.
OH' interference: 4 µL of 50 mM FeCl₂ and 4 µL of 50 mM H₂O₂ were added to a solution of 984 µL HEPES buffer containing 20 µM PNCL. 4 µL of 50 mM ONOO⁻ was then added to that solution, and the mixture was shaken gently before testing.

4.4.8 Synthesis of XF₁

3 - Oxo - 3H - spiro [isobenzofuran - 1, 9' - xanthene]- 3', 6' - diyl bis (2 - (diphenylphosphanyl)benzoate) (XF₁). 2-(diphenylphosphino) benzoic acid (336.9 mg, 1.1 mmol, 2.2 equiv) and HBTU (417.2 mg, 1.1 mmol, 2.2 equiv) were dissolved in 5 mL of DMF. DIPEA (0.52 mL, 3.0 mmol, 6.0 equiv) was added and the reaction was stirred for five minutes. Fluorescein (166.2 mg, 0.5 mmol, 1.0 equiv) was added and the reaction was stirred for 24 h. The reaction mixture was concentrated and purified by silica gel column chromatography (1:1 Hexanes/EtOAc) to yield XF₁ (78.4 mg, 17% yield) as a white solid. ¹H NMR (500 MHz, CDCl₃) δ 8.25–8.23 (m, 2H), 8.01 (d, 1H, J = 7.5 Hz), 7.66 (td, 1H, J = 7.5, J = 1.2 Hz), 7.61 (td, 1H, J = 7.5, J = 1.2 Hz), 7.49–7.45 (m, 4H), 7.35–7.25 (m, 20H), 7.12 (d, 1H, J = 7.4 Hz), 7.01–6.98 (m, 2H), 6.90 (d, 2H, J = 2.3 Hz), 6.74 (d, 2H, J = 8.6 Hz), 6.63 (dd, 2H, J = 8.6 Hz, 2.3 Hz); ¹³C NMR (125 MHz, CDCl₃) δ 169.53, 164.86, 153.29, 151.93, 151.48, 137.53, 137.45, 135.35, 134.56, 134.20, 134.04, 133.08, 132.93, 132.86, 131.92, 131.52, 130.06, 129.28, 128.97, 128.89, 128.74, 128.68, 128.45, 126.07, 125.23, 124.24, 117.85, 116.43, 110.59, 81.85; HRMS calcd for C₅₈H₃₈O₇P₂ [M+Na]+ 931.1985, found 931.1990.

4.4.9 XF₁ response and selectivity tests

Response. Wavelength scan of fluorescent emission of 10 µM XF₁ at 488 nm before and after adding 200 µM Angeli's salt were acquired in 20 mM HEPES buffer (pH 7.42). 996 µL HEPES
was added to an Eppendorf tube, then 2 µL of 5 mM XF1, and 2.3 µL of 86 mM Angeli’s salt were added. The mixtures were vortexed for 5 seconds. The reaction was monitored every 5 minutes for 30 minutes.

Selectivity. Selectivity for **XF1** was performed by treating it with various reactive species (5 mM GSH and 200 µM for other species) by monitoring fluorescent change every 5 minutes for 30 minutes with excitation wavelength at 488 nm. Stock solution was prepared as 5 mM in DMSO and the selectivity reactions were performed in 20 mM HEPES (pH 7.42).

### 4.4.10 Inhibition experiments with HCO$_3^-$ and GSH

**Inhibition of response by GSH.** Different volumes of 50 mM GSH in DI-H$_2$O (0 µL, 1 µL, 2 µL, 4 µL and 20 µL) were added to the 20 mM HEPES buffer in the presence of 4 µL of 5 mM **PNCL** in DMSO (20 µM final concentration), followed by adding 5.2 µL of 38.4 mM ONOO$^-$ (200 µM final concentration) or 1.2 µL of 172.7 mM Angeli’s salt (200 µM final concentration). The cuvette was shaken gently to assure mixing. Then time scans were acquired using the time scan module with emission wavelength at 525 nm.

**Inhibition of response by HCO$_3^-$**. Different volumes of 50 mM NaHCO$_3$ in DI-H$_2$O (0 µL, 20 µL, 40 µL, 60 µL, 80 µL and 100 µL) were added to the 20 mM HEPES buffer in the presence of 4 µL of 5 mM **PNCL** in DMSO (20 µM final concentration), followed by adding 5.2 µL of 38.4 mM ONOO$^-$ (200 µM final concentration) or 1.1 µL of 177.4 mM Angeli’s salt (200 µM final concentration). The cuvette was shaken gently to assure mixing. Then time scans were acquired using the time scan module with emission wavelength at 525 nm.

**Inhibition experiments for XF1.** Different volumes of 50 mM GSH in DI-H$_2$O (0 µL, 1 µL, 2 µL, 4 µL and 20 µL) or different volumes of 50 mM NaHCO$_3$ in DI-H$_2$O (0 µL, 20 µL, 40 µL, 60
µL, 80 µL and 100 µL) were added to the 20 mM HEPES buffer in the presence of 2 µL of 5 mM XF1 (10 µM final concentration) in DMSO, followed by adding 1.2 µL of 172.7 mM Angeli’s salt. The cuvette was shaken gently to assure mixing. Then time scans were acquired using the time scan module with emission wavelength at 525 nm.

### 4.4.11 Cell culture

Macrophages (RAW 264.7) were purchased from ATCC and cultured in high glucose Dulbecco’s Modified Eagle Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (penicillin/streptomycin, 100 U/mL). Cells were maintained in a humidified incubator at 37 °C with 5% CO₂. 12 hours before the experiment, cells were passed and plated on multi-well plates by adding 1000K–1500K of macrophages per well, filling each well with 1 mL of media.

Human lung adenocarcinoma epithelial cell (A549) were purchased from ATCC and cultured in F-12K media supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (penicillin/streptomycin, 100 U/mL). Cells were maintained in a humidified incubator at 37 °C with 5% CO₂. Two days before the experiment, cells were passed and plated on Costar® 12-well plates by adding 150K–200K of A549 cells per well, filling each well up to 1 mL of media.

Chemiluminescent responses and MTT absorption were measured using a Cytation 5 BioTek plate reader (Winooski, VT). Fluorescence imaging was conducted using an EVOS-fl fluorescent microscope (Advanced Microscopy Group) equipped with a GFP filter cube.
4.4.12 MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: RAW 264.7 macrophage cells (10^6 cell/mL) were seeded in a 96-well plate to a total volume of 100 µL/well. The plate was maintained at 37 °C with 5% CO₂ for 12 h. Cells were then incubated for 24 h after adding different concentrations of PNCL, 0, 0.1, 1, 10, 100 and 1000 µM respectively. 10 µL of the MTT reagent (Cayman Chemical, Ann Arbor, MI) was then added to each well, and mixed gently. After 4 h incubation, 100 µL of crystal dissolving solution was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm in a Cytation 5 BioTek plate reader.47

4.4.13 Cellular internalization of PNCL

RAW 264.7 macrophage cells (10^6 cell/mL) were seeded in a 12-well plate to a total volume of 1 mL/well. Plate was maintained at 37 °C with 5% CO₂ for 12 h. Prior to imaging, the medium was removed and cells were washed with 2 x 1 mL PBS, and each well was filled with 992 µL of PBS. Then, 8 µL of 5 mM PNCL in DMSO (40 µM final concentration) or 8 µL DMSO was added. The plate was imaged on an EVOS-fl fluorescent microscope using a GFP filter set after 10 min incubation.

4.4.14 Detection of SIN-1 generated ONOO⁻ in living cells

Detection of SIN-1 generated ONOO⁻ in macrophages. RAW 264.7 macrophage cells (10^6 cell/mL) were seeded in a 12-well plate to a total volume of 1 mL/well. Prior to imaging, the medium was removed and cells were washed with 1 x 1 mL PBS. Each well was filled with 996 µL of FluoroBrite DMEM Media. Then, 4 µL of 5 mM PNCL in DMSO (20 µM final
concentration) was added to each well, and incubated for 30 min. After incubation, the media was removed and cells were washed with 2 x 1 mL PBS. Different amount of PBS media was added into each well to make the final volume equal to 1 mL. Different volumes of 50 mM SIN-1 in DMSO (0 µL, 8 µL, 16 µL, 20 µL, and 40 µL) were added into each well. Different volume of DMSO was added into each well to make each well have same amount of DMSO. Then luminescence response was measure by the Cytation 5 BioTek plate reader by using the luminescence detection method, endpoint read type, and setting gain to 135 and temperature to 37 °C.

Detection of SIN-1 generated ONOO− in A549 cells. Human lung adenocarcinoma epithelial cell (A549) were plated in a 12-well plate to a total volume of 1 mL/well. The media was removed upon 90–95% confluence. Each well was washed with 1 x 1 mL PBS, and each well was filled with 996 µL of FluoroBrite DMEM Media. Then, 4 µL of 5 mM PNCL in DMSO (20 µM final concentration) was added to each well, and incubated for 30 min. After incubation, the media was removed and cells were washed with 2 x 1 mL PBS. Different amount of PBS media was added into each well to make the final volume equal to 1 mL. Different volumes of 50 mM SIN-1 in DMSO (0 µL, 8 µL, 16 µL, 20 µL, and 40 µL) were added into each well. Different volume of DMSO was added into each well to make each well have same amount of DMSO. Then luminescence response was measure by the plate reader.

Scavenger experiment in macrophages. Macrophages were washed with 1 x 1 mL PBS, and each well was filled with 996 µL of FluoroBrite DMEM Media. Then, 4 µL of 5 mM PNCL in DMSO (20 µM final concentration) was added to each well, and incubated for 30. The media
was removed after incubation and cells were washed with 2 x 1 mL PBS. Different amount of PBS media was added into each well to make the final volume equal to 1 mL. 40 µL of 50 mM SIN-1 in DMSO, 1.4 µL or 2.8 µL of 2 mg/mL Mn(III)TMPyP were added.

4.4.15 Application of PNCL for detecting endogenous ONOO$^-$

0.1 mg/mL LPS was prepared in DI-H$_2$O, and 2 mg/mL Mn(III)TMPyP solution was made in 20 mM PBS buffer (pH = 7.42) on the same day of the test. Prior to imaging, media was removed from macrophages and macrophages were washed with 1 x 1 mL PBS, and each well was filled with FluoroBrite DMEM Media. 10 µL of LPS (1000 ng/mL final concentration) were added to experimental wells, and 10 µL of DI-H$_2$O was added to control wells. After 16 h incubation, 4 µL of 5 mM PNCL (20 µM final concentration) was added to each well, and incubated for another 30 min. After incubation, the media was removed and cells were washed with 2 x 1 mL PBS, and vehicle or 25 µM Mn(III)TMPyP was added to the appropriate wells. Then the luminescence response was measured every 10 minutes over 4 hours.

4.4.16 iNOS inhibition of the production of ONOO$^-$ in macrophages

0.1 mg/mL LPS and 10 mg/mL 1400W (Cayman Chemical, Ann Arbor, MI) were prepared in DI-H$_2$O on the same day of the test. Prior to imaging, media was removed from macrophages and cells were washed with 1 x 1 mL PBS, and each well was filled with FluoroBrite DMEM Media. 10 µL of LPS (1000 ng/mL final concentration) and 5 µL of 1400W (200 µM final concentration) were added to the appropriate wells. The control was performed by adding 15 µL DI-H$_2$O. After 16 h incubation, 4 µL of 5 mM PNCL (20 µM final concentration) was added to each well, and incubated for another 30 min. The media was removed and cells
were washed with 2 x 1 mL PBS. 5 µL of 1400W were added for the inhibition tests. Then the luminescence response was measured every 10 minutes over 4 hours.


APPENDIX I.

ENHANCER FREE CHEMILUMINESCENT PROBE FOR HYPOXIA

Synopsis: This appendix summarizes the synthesis and *in vitro* characterization of HyCL-3.

Experimental procedure.

Synthesis.

\[
(E)-3-(4-(((1R,3R,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-((4-nitrobenzyl)oxy)phenyl)acrylonitrile (18).
\]

15 (85 mg, 0.218 mmol, 1.0 equiv), triphenyl phosphite (69 mg, 0.262 mmol, 1.2 equiv) and 4-nitrobenzyl alcohol (33 mg, 0.218 mmol, 1.0 equiv) were added to a dry round bottom flask flushed with nitrogen. The reaction contents were dissolved in 3.0 mL THF, and was cooled to 0° C. diethylazodicarboxylate (41 µL, 0.262 mmol, 1.2 equiv) was added dropwise to the solution, and stirred for 1 hr. The crude mixture was combined with 30 mL brine solution, and mixture was washed with 2 x 20 mL EtOAc. The combined organic layers were collected and dried over Na₂SO₄, filtered, and concentrated under reduced pressure. Purification by column chromatography (10% EtOAc/Hexanes) yielded 3 (99
mg, 92%) as a pale yellow solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.29 (d, 2H, $J$ = 8.6 Hz), 7.62 (d, 1H, $J$ = 16.5 Hz), 7.38 (d, 1H, $J$ = 8 Hz), 7.14 (d, 1H, $J$ = 8 Hz), 5.97 (d, 1H, $J$ = 16.5 Hz), 5.03-5.15 (m, 2H), 7.04 (d, 1H, $J$ = 8.0 Hz), 5.96 (d, 1H, $J$ = 16.6 Hz), 5.03-5.15 (m, 2H), 7.04 (d, 1H, $J$ = 8.0 Hz), 5.96 (d, 1H, $J$ = 16.6 Hz), 3.34 (s, 3H), 3.27 (s, 1H), 1.64-2.20 (m, 13H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$CO) $\delta$ 152.95, 147.92, 144.26, 142.93, 139.57, 138.95, 133.51, 129.76, 128.11, 128.26, 124.60, 123.89, 117.80, 99.00, 74.36, 57.59, 39.16, 39.9 9, 38.62, 28.53, 36.91, 32.98, 29.72, 28.24, 28.08.

**HyCL-3.** Compound 18 (40 mg, 0.081 mmol, 1.0 equiv) and Rose bengal (8.5 mg, 0.0087 mmol, 0.107 equiv) were added into a dry flask and dissolved in 5 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 3.5 h of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum at 0 °C and the residue was purified by column chromatography (1:15 EtOAc/hexanes) to deliver **HyCL-3** as a white solid (56.4 mg, 87%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.31 (d, 2H, $J$ = 8.6 Hz), 8.00 (d, 1H, $J$ = 8.6 Hz), 7.67 (d, 2H, $J$ = 8.6 Hz), 7.60 (d, 1H, $J$ = 16.6 Hz), 7.54 (d, 1H, $J$ = 8.0 Hz), 5.02 (s, 2H), 3.22 (s, 3H), 3.02 (s, 1H), 1.24-2.20 (m, 13H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 153.53, 148.11, 143.86, 136.79, 130.33, 129.77, 128.37, 127.94, 124.92, 124.01, 117.55, 111.58, 100.59, 96.44, 74.54, 49.86, 37.03, 36.57, 33.96, 33.73, 32.23, 3 1.61, 26.16, 25.86.
4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-hydroxybenzaldehyde (21). Iodine phenol 20 (400 mg, 0.93 mmol, 1.0 equiv), N-formylsaccharine (296 mg, 1.40 mmol, 1.5 equiv), sodium carbonate (148 mg, 1.40 mmol, 1.5 equiv) palladium acetate (6.2 mg, 0.028 mmol, 0.03 equiv), and 1,4-bis(diphenylphosphino) butane (17 mg, 0.04 mmol, 0.045 equiv) were added to a 10 mL pressure flask, and a small stir bar was added. The flask was evacuated and back-filled with N₂ three times. The contents were dissolved in 4 mL anhydrous DMF. Triethyl silane (193 µL, 1.21 mmol, 1.3 equiv) was then added, and the reaction was immediately sealed and stirred for 10 min. The mixture was brought up to 80 °C and stirred for 16 hr. The reaction was quenched with 50 mL NH₄Cl, and extracted with 3 x 30 mL EtOAc. The combined organic layers were dried with Na₂SO₄, and concentrated under reduced pressure. Column chromatography (10% EtOAc/Hexanes) afforded compound 6 (263 mg, 85 %) as a yellow solid. 

$^1$H NMR (500 MHz, CDCl₃) δ 11.66 (s, 1H), 9.93 (s, 1H), 7.50 (d, 1H, $J = 8.0$ Hz), 7.00 (d, 1H, $J = 8.0$ Hz), 3.36 (s, 3H), 3.32 (s, 1H), 1.24-2.20 (m, 13H); $^{13}$C NMR (125 MHz, CDCl₃) δ 195.83, 157.62, 143.58, 138.95, 133.49, 131.03, 122.99, 120.47, 57.63, 39.32, 39.12, 38.65, 38.55, 37.06, 33.03, 29.82, 28.38, 28.14.
In vitro characterization.

Figure 1A-1. Response of HyCL-3 to nitroreductase. (a) Chemiluminescence emission spectra of 10 µM HyCL-3 before (blue trace) and after (red) trace adding 14 (red trace) µg/mL nitroreductase in the presence of 0.4 mM NADH. (b) Time scans of the chemiluminescence emission at 525 nm of 10 µM HyCL-3 and 0 (blue trace), 5 and 0, 2.5, 5, 7.5, 10, 12.5, 14 (red trace) µg/mL nitroreductase in the presence of 0.4 mM NADH in 10 mM PBS buffer (pH 7.4).

Figure 1A-2. Chemiluminescent responses of 10 µM HyCL-3 to biologically relevant analytes in 10 mM PBS buffer (pH 7.4) containing 10% Emerald II Enhancer. Bars represent chemiluminescence emission at 545 nm and 4, 8, 12, 16, 20 min after addition of reducing agents. Data shown are for 5 mM GSH, 1 mM cysteine and hcy, and 200 µM for other reducing agents. (1) nitroreductase and NADH; (2) GSH; (3) L-cysteine; (4) hcy; (5) nitroreductase; (6) NADH; (7) H₂S; (8) citrate; (9) Na₂S₂O₅; (10) L-ascorbic acid; (11) blank.
APPENDIX II.

SCANNED $^1$H AND $^{13}$C NMR SPECTRA