Chemiluminescence imaging probes for quantification of HNO and pH

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Chemiluminescence imaging probes for quantification of HNO and pH
Chemiluminescence imaging probes for quantification of HNO and pH

A Dissertation Presented to the Graduate Faculty of

Dedman College

Southern Methodist University

in

Partial Fulfillment of the Requirements

for the degree of

Doctor of Philosophy

with a

Major in Chemistry

by

Weiwei An

B. E. Beijing Institute of Graphic Communication, Beijing

August 6, 2019
ACKNOWLEDGEMENTS

I can still remember that day, the Spring Festival of 2014. In the morning when I was checking my emails as usual, I saw an email from Dr. Lippert that lead me to here and start the life of graduate school. Five and half years has passed, I’m working on my thesis now. I’m always thinking that I’m such a lucky person since I’ve got the chance to become a member of the Lippert group and a graduate student in the Chemistry department of SMU. All of this is because I have such a patient, supportive and wise advisor Alex Lippert. He’s one of the smartest people I’ve met ever and I can’t accomplish all of my projects without his scientific advice and insightful discussions. He’s always patient and supportive when I feel depressed about the failure of the experiments. I can feel his care for us since we have individual meetings at least once a semester and group meetings every week. Each time after I talked with him from these meetings, I felt more enthusiastic about my projects. He’s really willing to spend his time with students. Every time before my department presentations, he’ll spend hours with me and help me to practice the presentation and go through every single slide with me. I can’t have any progress in scientific presentations and writing without his guidance. He really sets a good example as an advisor. Scientific writing and presenting skills, patience and support to students and critical thinking, all I’ve learned from him will always be my guide in the future when I start to work as an independent researcher.

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Chemiluminescence imaging probes for quantification of HNO and pH

Advisor: Professor Alexander R. Lippert

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Dissertation completed July 30, 2019

Chemiluminescence is the light emission from a chemical reaction. The excited state is accessed in the course of a chemical reaction and relaxes to the ground state with the emission of a photon. Independence from the need for light excitation gives chemiluminescence several advantages over fluorescence including better signal-to-noise ratio and reduced background at deeper tissue imaging depth. As a result, the sensitivity of chemiluminescent probes for biological analyte detection has been improved significantly. Because of the sensitivity of chemiluminescent probes, we are planning to use this technique to quantify important factors for biological activities.

In this dissertation, two quantification methods to study biological activities will be introduced in detail. The first one is a pH quantification system based on a pH sensitive chemiluminescent system. In aqueous solution, the light emission from the chemiluminescent probe will transfer to a ratiometric pH sensitive dye carboxy-SNARF-1 in the presence of a commercially available enhancer. By plotting out the light emission ratio of the dual emission peaks of carboxy-SNARF-1 with various pH, we can use this system as an optical pH detection method.

The second project in this dissertation is the development of a chemiluminescent HNO probe. Pharmacological HNO is related to a wide range of biological activities, but its detection is difficult because of its instability and activity towards biological molecules. Based on a 1,2-spirodoxetane structure, with the acrylonitrile on the ortho position of the phenolate, we synthesized the first HNO chemiluminescent probe, HNOCL-1. The chemiluminescent intensity of the scaffold has been improved significantly and is capable of HNO detection in the nanomolar scale. With HNOCL-1, we successfully detected and quantified the HNO generated from the reaction between NO and H2S, which is one of the possible endogenous sources of HNO.
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<tr>
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<th>Name</th>
<th>Structure</th>
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<td>AIDH</td>
<td>Aldehyde dehydrogenase</td>
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</tr>
<tr>
<td>DIPEA</td>
<td>N, N-Diisopropylethylamine</td>
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</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
<td><img src="image" alt="Chemical structure" /></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Media</td>
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<tr>
<td>DMF</td>
<td>N, N-Dimethylformamide</td>
<td><img src="image" alt="Chemical structure" /></td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
<td><img src="image" alt="Chemical structure" /></td>
</tr>
<tr>
<td>DN</td>
<td>DEA NONOate</td>
<td><img src="image" alt="Chemical structure" /></td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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19
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>EDC</td>
<td>N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>ET</td>
<td>Electron transfer</td>
</tr>
<tr>
<td>Et₃N</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>EtSNa</td>
<td>Sodium ethanethiolate</td>
</tr>
<tr>
<td>F-1,6-bisP</td>
<td>Fructose-1,6-bisphosphatase</td>
</tr>
<tr>
<td>F-6-P</td>
<td>Fructose-6-phosphate</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fe-MGD</td>
<td>N-methyl-D-glucamine dithiocarbamate iron</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography–mass spectrometry</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td><strong>GSNO</strong></td>
<td>S-Nitrosoglutathione</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------</td>
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<th><strong>GTP</strong></th>
<th>Guanosine triphosphate</th>
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<th><strong>HBTU</strong></th>
<th>O-benzotriazole-(N,N',N''N'')-tetramethyl-uranium hexafluorophosphate</th>
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<th>HNO chemiluminescent probe</th>
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</tbody>
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<table>
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<tr>
<th><strong>HPLC-MS</strong></th>
<th>High pressure liquid-chromatography mass spectrometry</th>
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<tr>
<th><strong>Hycl-2</strong></th>
<th>Hypoxia Probe 2</th>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>Hycl-4-AM</td>
<td>Hypoxia Probe 4</td>
</tr>
<tr>
<td>IVIS</td>
<td>In vivo imaging system</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>MCT1</td>
<td>Monocarboxylate–H(^+) efflux Cotransporter 1</td>
</tr>
<tr>
<td>MCT4</td>
<td>Monocarboxylate–H(^+) efflux Cotransporter 4</td>
</tr>
<tr>
<td>MOM</td>
<td>Methoxymethyl</td>
</tr>
<tr>
<td>N(_2)</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl cysteine</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NHE</td>
<td>Na(^+)–H(^+) exchanger</td>
</tr>
<tr>
<td>NIS</td>
<td>N-Iodosuccinimide</td>
</tr>
<tr>
<td>Symbol</td>
<td>Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>Pd(OAc)₂</td>
<td>Palladium(II) acetate</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>pHₑ</td>
<td>Extracellular pH</td>
</tr>
<tr>
<td>pHᵢ</td>
<td>Intracellular pH</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein Kinase G</td>
</tr>
<tr>
<td>PNCL</td>
<td>ONOO⁻ Probe</td>
</tr>
<tr>
<td>PPh₃</td>
<td>Triphenylphosphine</td>
</tr>
<tr>
<td>PET</td>
<td>Photoinduced electron transfer</td>
</tr>
<tr>
<td>RSON</td>
<td>Reactive oxygen and nitrogen species</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylyl cyclase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SNARF</td>
<td>Semi naphtharhodafluor</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TRPA1</td>
<td>Transient receptor potential cation channel, subfamily A, member 1</td>
</tr>
<tr>
<td>XF2</td>
<td>HNO fluorescent probe</td>
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</tbody>
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Chapter 1 Introduction to Chemiluminescence

1.1 Chemiluminescence

From the fireflies in the woods, luminescent bacteria in the lakes, and jellyfish deep under the sea, the splendid light emission of light is a beautiful part of the natural world. Back to the city, people use glow sticks for parties, concerts and celebrations for festivals. Detectives and police used luminol to track the hint of wiped blood. This chemical based light emission, which is called chemiluminescence, is almost everywhere is people’s life. It is the remarkable production of light from exothermic chemical reactions that can be understood using a Jablonski diagram describing the interactions between light and molecules. The ground state electron in $S_0$ will transit to the

**Scheme 1 - 1.** Jablonski Diagram for Chemiluminescence Mechanism.
excited state $S_1$ upon obtaining a sufficient amount of energy, which could come from the light absorbance or a chemical reaction. For chemiluminescence, the energy is obtained from the chemical reaction. Internal conversion happens when the electron relaxes to a lower energy state without photon emission. The relaxation of the electron to the ground state with the release of a photon will cause the light emission (Scheme 1-1). For fluorescence, the energy to excite the electron is from external light. Intersystem crossing can also happen in this process, which is the electron transition to the triplet excited state $T_1$ before relaxation to the ground state with a slower transition rate in a process called phosphorescence.$^1$ Compared with fluorescence, light emission from chemiluminescence does not rely on external light sources, which significantly increases the tissue depth that can be adequately imaged when applied in a biological system. Meanwhile, chemiluminescence will give a lower background in biological system due to reduced autofluorescence and light scattering. Hence chemiluminescence is very useful for analytical chemistry and has been used in a wide range of applications.

The application of chemiluminescence as an analytical method has been studied for a long time since the concentration for most endogenous compounds have a low concentration and are hard to detect.$^1$ Before chemiluminescence methods, radiolabeling detection was a leading way to trace analytes in biological systems, but has several drawbacks.$^2$ Chemiluminescence provides a less complicated and cheaper way compared to radiolabeling procedures. Studies on luminol,$^4$ acridinium,$^5-6$ peroxyoxalate,$^7$ luciferin and luciferase$^8$ and sterically stabilized 1,2-dioxetanes$^1$ are the most commonly reported systems.

1.2 Introduction of chemiluminescent systems

1.2.1 Luminol and its derivatives

![Scheme 1 - 2. Chemiluminescence of luminol.](attachment:Scheme_1-2.png)
The principle of light emission is the oxidation of the heterocyclic ring by a series of oxidants to yield an important intermediate α-hydroxyperoxide, which decomposes to yield the excited aminophthalate ion which relaxes back to the ground state and emits light.\(^4\) The oxidization of luminol in protic solution needs oxidants that can include molecular oxygen, peroxides or superoxide anion, but this reaction requires the presence of catalysis such as enzyme and metals complexes.\(^5\)

### 1.2.2 Acridinium and its derivatives

The basic chemiluminescence mechanism for acridinium and its derivatives is shown in Scheme 1-3. With the hydroperoxide and a strong base, the acridinium will decompose and release the acridone in its excited state, which will release a bright blue emission when it relaxes to the ground state.\(^6\)-\(^7\) It has been used as an efficient tool to label DNA strands without reacting with thiols and sulfites.\(^1\)

**Scheme 1 - 3.** Chemiluminescence of acridinium.
1.2.3 Peroxyoxalate and its derivatives

Peroxyoxalate and its derivatives have an aryl oxalate ester structure, which can react with hydrogen peroxide to form the dioxetanedione with high energy. The dioxetanedione can chemically excite the fluorophore to the S<sub>1</sub> state and trigger the luminescence process. However, the low stability and solubility in water limit the application of peroxyoxalic compounds in biological systems.

1.2.4 Luciferin and luciferase

The basic light emission mechanism for luciferin and luciferase chemiluminescent system is shown in Scheme 1-5. In the presence of ATP, O<sub>2</sub> and Mg<sup>2+</sup>, luciferin will be catalyzed by luciferase and turn into an intermediate with the 1,2-dioxetane, which will decompose into the oxyluciferin in its excited state and release visible light when it relaxes into ground state. The luciferase and luciferin bioluminescent system has a broad range of application. With various luciferases, the light emission could change from 480 nm to 612 nm. It has been used to detect a
series of biological activities including viral infection, host response, antiviral therapy and real time monitoring and quantification of small molecule analytes.\textsuperscript{11}

\subsection{1.2.5 1,2-dioxetane}

\textbf{Scheme 1 - 6.} Chemiluminescence of 1,2-dioxetane.

The chemiluminescence mechanism of 1,2-dioxetane is shown in Scheme 1-6. There are two distinct pathways for its decomposition, thermal and chemical decomposition. The thermal decomposition of the 1,2-dioxetane will lead to the formation of the diradical intermediate and release the decomposition product ketone in its triplet state ($T_1$), which will be rapidly quenched in aqueous solution. The chemical decomposition pathway will go through the chemically initiated electron exchange luminescence (CIEEL) process, and lead to the generation of a ketone in its singlet state ($S_1$), which will emit light upon relaxing to the ground state. The generation of the singlet intermediate makes the chemical decomposition for 1,2-dioxetane more efficient in aqueous solution, and as a result, it will be a useful scaffold as tool to detect biological activities.\textsuperscript{1}
1.3 Development and Application of 1,2-spirodioxetane

1.3.1 Development of 1,2-spirodioxetane

Scheme 1 - 7. Development of 1,2-dioxetane as a chemiluminescent probe.

Hundreds of molecules have been synthesized based on the 1,2-dioxetane structure.\(^1\) When the 1,2-dioxetane moiety was first synthesized in 1969 (Scheme 1-7), it had several drawbacks including thermal instability and aqueous quenching.\(^1\) In 1972, more stable structures of 1,2-dioxetane with a 1,1′-biadamantyl group as stabilizer were developed, which have a higher decomposition temperature and a longer half-life.\(^12\) The asymmetric structure of 1,2-dioxetane was synthesized later. The chemiluminescence process would be triggered once the analyte-responsive protecting group is removed.\(^13\-14\) Schaap and his group developed several structures with a silylated or ester group as protecting groups, which could react with fluoride anions or arylesterase and initiate chemiluminescent processes in organic solvents (Scheme 1-7).\(^15\-17\) Later, Bronstein and colleagues developed (3-(2,7-spiroadamantane)-4-methoxy-4-(3-phosphoryloxy)phenyl-1,2-dioxetane disodium salt also named 3-4-methoxyspiro(1,2-dioxetane-3,27-tricyclo[3.3.1.1.3,7]-decan)-4-yl phenylphosphate disodium salt (AMPPD), the solubility in water and comparatively stability at room temperature make it one of the most common structure for chemiluminescent probes.\(^13\) Recently Shabat’s group develop several powerful chemiluminescent probes. By attaching the acrylate or acrylonitrile electron-withdrawing group to the benzyl moiety, the quantum yield and chemiluminescent intensity are increased dramatically.\(^18\)
1.3.2 Chemically initiated electron exchange luminescence

CIEEL is light emission from electron-transfer chemistry, which is part of the chemiluminescent process. Intermolecular electron transfer or intramolecular electron transfer of thermostabilized spiroadamantyl-substituted dioxetanes is the main reason for CIEEL. When the protecting group is removed from the scaffold, it will release the phenolate anion, which would lead to the decomposition of spiroadamantyl-substituted dioxetane and initiate the light emission.

Two alternative decomposition mechanisms have been proposed for CIEEL process. The cleavage of the phenolate anion will lead to the intramolecular electron transfer (ET) from the phenolate moiety to the antibonding σ* orbital with the cleavage of O-O bond. This is the rate-limiting step for CIEEL since it need energy for the electron transfer from the phenolate to the peroxide bond. The excited methyl m-oxybenzoate ion could be formed from the direct electron transfer, but the research evidence support the alternative way, which is the electron back transfer (BET). BET happens in the solvent cage between the radical fragments from the decomposition of spiroadamantyl-substituted dioxetanes phenolate, which has been demonstrated by the solvent viscosity dependence of the chemiexcitation yield of the system. This phenomenon has been demonstrated that the chemiexcitation yield is rising with the increasing viscosity. Finally, the excited methyl m-oxybenzoate ion relaxes to the ground state and leads to the light emission.
1.3.3 Probes based on 1,2-spirodioxetane in aqueous solution

Scheme 1 - 9. Probes based on 1,2-spirodioxetane scaffold.

A range of probes based on the 1,2-spirodioxetane scaffold for reactive oxygen and nitrogen species and reactive biological analyte detection has been synthesized recently in our lab (Scheme 1-9). CHS-1-3 are the first generation of these probes. The benzyl azide will react with H₂S and yield an aniline, which will lead to the generation of the phenolate ion and CIEEL. Although among these probes, CHS-3 gives the strongest light emission, it still relies on the addition of a commercially available enhancer, Emerald II to increase light emission.²¹ Later, the
chlorinated 1,2-dioxetane **Hycl-2** was synthesized as a tool to detect hypoxia. The nitrobenzene will be reduced to aniline under hypoxic conditions by mammalian reductive enzymes and release the phenolate ion to initiate the CIEEL process. This scaffold still relies on the addition of enhancers to magnify the emission intensity.\(^{22}\) A pH quantification system with the addition of the enhancer was developed later after **Hycl-2**.\(^{23}\) With the attachment of an acrylate or acrylonitrile electron-withdrawing group to the aromatic moiety, the chemiluminescence emission intensity of the 1,2-spirodioxetane has been significantly improved because the quantum yield of the fluorophore released from the decomposition of the dioxetane has increased.\(^{18}\) As a result, probes based on the acrylate and acrylonitrile dioxetane do not require the addition of enhancers for reactive oxygen and nitrogen species detection. **PNCL** and **HNOCL-1** were synthesized later to detect ONOO\(^{-}\) and HNO respectively and gave a high sensitivity towards the analytes.\(^{24-25}\)

Recently, our group found with the addition of the acetoxymethyl ester to the benzoic moiety could increase the cell trapability of the 1,2-dioxetane. Upon reaction with intercellular esterases when treating the cells with the probe, the acrylate carboxylic acid intermediate will be released from the system and increase the solubility of the scaffold. As a result, light emission will be improved significantly. Based on this structure, our group developed the second-generation probes for hypoxia detection **Hycl-3** and **Hycl-4-AM** that gave us a much brighter response towards hypoxia upon adding to the cells compared with other probes.\(^{26}\)

### 1.4 Research objectives

This dissertation aims to quantify factors that are related to certain biological activities. The aim for the pH quantification project is using a chemiluminescent probe as the light source to transfer light emission to a ratiometric pH sensitive fluorophore **carboxy-SNARF-1**. By establishing a standard graph of the relationship between pH and light emission ratio, it can be used to detect the pH using an optical method.\(^{27}\) The HNO quantification project aims to development a highly selective and sensitive chemiluminescent probe **HNOCL-1** to detect HNO at nanomolar concentrations. The HNO detection and quantification has been successfully conducted *in vitro* to provide a quantification of HNO generated. Hence, **HNOCL-1** could provide an efficient way to study the pathways and functions of HNO in biological systems in the future.
1.5 References


Chapter 2 Detection of pH with a chemiluminescent probe

2.1 Introduction

2.1.1 Misregulated pH in cancer cells

Regulated pH is an important factor to keep the balance of the human body. Normal cells have a lower intracellular pH (pH\textsubscript{i}) of 7.2 compared to the extracellular pH (pH\textsubscript{e}) of 7.4, which is buffered by HCO\textsubscript{3}\textsuperscript{–} (Figure 2-1).\textsuperscript{1} This characteristic is important for normal adult cell metabolism and apoptosis.\textsuperscript{2} Cancer cells have a higher intracellular pH and lower extracellular pH compared to normal adult cells. The increased production of lactic acid and the change of the expression of the cell membrane transporters and pumps lead to this abnormal pH condition of cancer cells.\textsuperscript{3} The fast proliferation rate of cancer cells means that the normal cellular metabolism in healthy adult cells cannot meet the requirement for energy.\textsuperscript{4} As a result, cancer cells resort to another way of energy production, which involves the breakdown of glucose in cytosol with the generation of

\textbf{Figure 2-1.} Misregulated pH in cancer and normal cells.
lactic acid.\textsuperscript{4} Meanwhile, a change in the expression of ion pumps and transporters on the cell membrane speeds up the H\textsuperscript{+} efflux and leads to the increase of extracellular H\textsuperscript{+} concentration.\textsuperscript{5}

2.1.2 High intracellular pH increases proliferation

The higher intracellular pH (pH\textsubscript{i} > 7.2) is a key factor for cancer cell proliferation due to the inhibition of cell acidification controlled apoptosis.\textsuperscript{6} The function of cyclin-dependent kinase 1 (CDK1), the key complex for promoting mitosis, is limited under lower pH\textsubscript{i}.\textsuperscript{7} However, a higher intracellular pH facilitates the CDK1 activities and promotes G2/M process entry and cell mitosis.\textsuperscript{7} Also, pH dynamics can change the activity of certain proteins and inhibit several processes of cell apoptosis.\textsuperscript{8} In acidic cytosol, a pre-apoptosis protein induces pore formation on the mitochondria membrane and releases another pre-apoptosis protein cytochrome c, which is important to facilitate the activity of caspases and at pH 6.8, efficiency of these caspases reaches maximum.\textsuperscript{8} With a higher pH\textsubscript{i} the function of cytochrome c will be suppressed and the cell death process will be inhibited.\textsuperscript{9} Moreover, the function of lactate dehydrogenase, a key enzyme for pyruvate conversion of lactate, is maximal at pH 7.5. Furthermore, the activity of the most important glycolysis rate regulated enzyme phosphofructokinase 1 is increased with an alkaline pH shift.\textsuperscript{10}

2.1.3 Low extracellular pH increases invasion and metastasis

Meanwhile, a lower pH\textsubscript{e} is the main reason for extracellular matrix (ECM) remodeling and acid-activated protease activities enhance and promote tumor cell invasion and metastasis.\textsuperscript{11} An ECM degradation enzyme, matrix metalloproteinase 3 is more active at lower pH\textsubscript{e}.\textsuperscript{12} However, the reason for the dysfunction is not well understood. Hence, it’s important to monitor the real time pH dynamics to facilitate studying and understanding the mechanisms behind these organs' misbehaviors.
2.1.4 pH measurement by magnetic resonance

Magnetic resonance is a key technique for measuring pH in tumors and measurement methods include $^{31}$P MRS, $^{13}$C MRS, $^{19}$F MRS, magnetization transfer and pH-dependent chemical reagents. Some of these methods are aimed at testing the endogenous resonance while other require exogenous reagent injection. $^{31}$P MRS is used to detect the resonance of endogenous phosphate $P_i$, but for necrosis cells, the extracellular $P_i$ is also visible on the spectra, which can cause the appearance of complicated resonances. Another method is to measure the extracellular pH by injecting 3-aminopropylphosphate (3-APP), which is membrane impermeable and non-toxic, and measures the pH-dependent shift of $^{31}$P. Another pH measurement by MRS is $^{19}$F MRS, which has a high gyromagnetic ratio and is not be influenced by endogenous sources of $^{19}$F, which are very low in tissue. By injecting exogenous reagents, it is possible to obtain the spectra with large chemical shift dispersion. $^1$H MRS can also provide highly sensitive spectra with a stable, downfield region with slight interferences. Even though some endogenous molecules can also have H-resonance responses, these compounds are too dilute to be observed from the spectra. Hyperpolarized $^{13}$C MRS is also an innovative method to detect the intercellular pH by intravenous injection of hyperpolarized bicarbonate $H^{13}CO_3^−$.

2.1.5 Ratiometric pH sensitive fluorescent probe: carboxy-SNARF-1

![Scheme 2 - 1. Structure and dual emission of carboxy-SNARF-1 at different pH.](image)

Scheme 2 - 1. Structure and dual emission of carboxy-SNARF-1 at different pH.
Numerous pH sensitive fluorescent probes have been synthesized and reviewed.\textsuperscript{19-20} The light emission of these probes will shift with the change of pH. It is because of protonation or deprotonation of a certain function group.\textsuperscript{19-20} **Carboxy-SNARF-1** is a commonly used ratiometric dye that has a clear isosbestic point at around 600 nm. Its pKa is around 7.4, which is appropriate as a pH detector. The single excitation and dual emission of **carboxy-SNARF-1** make it a convenient dye for pH detection in various instruments.\textsuperscript{21,23}

2.1.6 pH measurement by optical methods

Optical pH measurement is another commonly used strategy to detect pH. With the existence of a ratiometric pH sensitive fluorescent dye – **carboxy-SNARF-1** and cell membrane permeable fluorescein, the pH of mitochondria has been detected by high resolution confocal scanning laser microscopy (CSLM) and multiphoton laser scanning microscopy (MPLSM).\textsuperscript{22} CSLM is able to detect emission signals at different wavelengths at the same time. This property enables the detection of the two emission peaks of **carboxy-SNARF-1** in various pH microenvironments.\textsuperscript{23} Another pH detection method is using pH (Low) Insertion peptide–pHLIP.\textsuperscript{24} The peptide is a water-soluble polypeptide and would form a transmembrane alpha helix with the drop of intercellular pH from 7.4 to around 6.5–7.0. By this way, cargo molecules can attach to the cell membrane surface or the membrane-impermeable molecules and will enter into the cell plasma.\textsuperscript{24}

2.1.7 Enhancers

The CIEEL is generated with the decomposition of the deprotonated 1,2-spirodioxetane phenolate ion. The decomposition rate of phenolate ion is $4.4 \times 10^6$ faster than the protonated phenol.\textsuperscript{25} The fluorophore released from decomposition of the probe is in a charge transfer state, which can be quenched by proton transfer in the protic solution. Fortunately, the quenching process can be prevented by the hydrophobic microenvironment formed by the surfactant from the enhancer. With the addition of the enhancer, the chemiluminescence emission intensity will be improved significantly.\textsuperscript{26} Meanwhile, the micelle formed from the added surfactant would attach the fluorophores to the system and increase the energy transfer efficiency.\textsuperscript{27} In our lab, we used the commercially available enhancers Emerald II and Sapphire II to improve the chemiluminescent emission intensity. There is also a fluorophore in the enhancers that can be excited by the CIEEL and lead to the red-shift of the emission. Poly (vinylbenzyltrimethylammoniumchloride) (TMQ),
poly (vinylbenzyltributylammonium chloride) (TBQ) and poly (vinylbenzyltrimethylbenzylammonium chloride) (BDMQ) are the surfactants include in the enhancers. By surrounding the 1,2-dioxatane structure into the hydrophobic microenvironment, they keep the protonation of the 1,2-dioxetane and enhance the chemiluminescence of the probe.

2.2 Results and discussion

2.2.1 Design and synthesis of pH sensitive probe and ratiometric pH sensitive fluorophore

The synthesis procedure of the 1,2-dioxetane probe is adopted from previous work in our laboratory. First, we treated the 4-chloro-3-methoxybenzaldehyde 2 with trimethyl orthoformate in MeOH in the presence of p-toluenesulfonic acid to yield acetal 3. The acetal 3 was treated with triethyl phosphite and boron trifluoride diethyl etherate to obtain the diethyl methoxy (3-methoxyphenyl) methyl phosphonate 4. We ran the Horner–Wadsworth–Emmons reaction with the phosphonate 4, n-BuLi and 2-admantanone to yield the enol ether 5. Next, the enol ether 5 would go through a demethylation reaction upon treatment with sodium ethanethiolate and cesium carbonate to obtain the phenol 6. Later, I protected the phenol with a MOM protecting group through the addition of chloromethoxymethane to yield the MOM protected enol ether 8. The MOM protected enol ether underwent reaction with Rose bengal as the catalyst and O2 bubbled through the system, and this procedure obtained the 1,2-dioxetane structure MOM protected product 8. The MOM protecting group was removed by adding p-toluenesulfonic acid to yield the pH sensitive probe Compound-1.
2.2.2 Synthesis and isolation of carboxy-SNARF-1


The synthesis of carboxyl-SNARF-1 was adapted from the procedure published by Dr. Christopher J. Chang in 2011.\textsuperscript{30} 1,3-Dihydro-1,3-dioxo-5-isobenzofuran carboxylic acid mixed with 3-dimethylaminophenol was heated up to 110°C and stirred overnight in toluene to yield the product 9. The product 9 was mixed with 1,6-naphthalenediol in pure MeSO\textsubscript{3}H and stirred for 1 hour and obtain the product 10 as a purple solid.\textsuperscript{30}

2.2.3 Mechanisms of pH quantification

Scheme 2 - 4. Energy transfer chemiluminescence for ratiometric pH imaging.\textsuperscript{31}
Because of the low background and less light scattering chemiluminescence, our lab developed a pH quantification system based on a chemiluminescent scaffold with the addition of a ratiometric pH sensitive dye. Scheme 2-4 shows the mechanism of the system. Light emission from the chemiluminescent compound 1 in the Emerald II or Sapphire II enhancer solution will be transferred to a ratiometric pH sensitive fluorophore carboxy-SNARF-1. The light emission will alter from 585 nm in the protonated form at lower pH to 650 nm in the deprotonated form at higher pH. The ratio of the light emission from these two peaks will be a constant number at a given pH, which will enable the pH quantification in an aqueous solution.

2.2.4 Chemiluminescence response towards pH in different enhancer

Figure 2-2. The pH dependent (A) emission spectrum and (B) ratio of the chemiluminescence emission intensities at 650 nm and 585 nm of 60 µM 1 and 80 µM carboxy-SNARF-1 in aqueous buffer (pH 5.99–10) containing 6% Sapphire II Enhancer. The pH dependent (C) emission spectrum and (D) ratio of the chemiluminescence emission intensities at 650 nm and 585 nm of 60 µM 1 and 80 µM carboxy-SNARF-1 in aqueous buffer (pH 5.99–10) containing 6% Emerald II Enhancer.

To obtain the pH sensitivity properties of 1 and the ratiometric pH sensitive dye carboxy-SNARF-1, we first characterized the chemiluminescence result using a F-7000 Hitachi
fluorometer. Luminescence response toward the change of pH was tested in PBS (0.02 M, pH 6.0–8.0) buffer and sodium bicarbonate (0.01 M, pH 9.2–10.8) buffer solution. We developed the system with two different commercially available enhancers Emerald II and Sapphire II. With a tetra-alkyl ammonium polymer in the Sapphire II (6%) that encapsulates the dioxetane 1 and the carboxy-SNARF-1, the energy transfer efficiency has been significantly improved. There are two main emission peaks in the Sapphire II enhancer solution. The one at 467 nm is the light emission from the decomposition of 1 and the rising peak at 650 nm with the increasing pH is from the deprotonated carboxy-SNARF-1. We plotted out the ratio of light emission respectively at 650 nm and 585 nm, which are the two emission peaks for protonated and deprotonated carboxy-SNARF-1, and a pH dependent graph was constructed. The emission peak at 585 nm is masked by the peak at 467 nm, but this is still the peak at which the maximum change is observed. From this graph, we can see a 5.6-fold increase in the ratio with increasing pH from 5.99 to 10.02 (Figure 2-2, A-B), which confirms that the system we have established has the ability to quantify the pH in an aqueous system. We observed similar results with another commercially available enhancer Emerald II. In the 6% Emerald II buffer solution, there are 3 emission peaks at 467 nm, 540 nm and 650 nm, which are respectively from the light emission of dioxetane 1, the fluorophore included in the enhancer and the deprotonated carboxy-SNARF-1. After plotting out the ratio of chemiluminescence at 650 nm and 585 nm, we can see a 5.7-fold increase in the ratio with the increasing pH from 5.99 to 10.02 in the graph (Figure 2-2, C-D).
2.2.5 Evaluation of signal sensitivity to time and probe concentration

![Graphs showing emission intensity and ratio vs. pH and concentration](image)

**Figure 2-3.** The independence of chemiluminescence emission on the concentration of 1. (A) Emission spectra at pH 7.42 and (B) ratio of the chemiluminescence emission intensity at 650 nm and 585 nm of 20–200 µM 1 and 80 µM carboxy-SNARF-1 in aqueous buffers (pH 5.99, 7.42, and 8.01) containing 6% Sapphire II Enhancer. (C) Emission spectra at pH 7.42 and (D) ratio of the chemiluminescence emission intensities at 650 nm and 585 nm of 20–200 µM 1 and 80 µM carboxy-SNARF-1 in aqueous buffers (pH 5.99, 7.42, and 8.01) containing 6% Emerald II Enhancer.

After confirming the pH quantification ability of our probe, we proceeded to test the consistency of the ratiometric signal with other varying factors. The concentration of the dioxetane is one of the key factors that could change the light emission intensity from the system. The chemiluminescent emission becomes stronger with the increasing concentration of the dioxetane 1. In the Sapphire II enhancer solution, we keep the concentration of carboxy-SNARF-1 and enhancer constant and vary the concentration of dioxetane 1 from 20 µM to 200 µM. After we plot out the ratio of chemiluminescence intensity from 650 nm to 585 nm, even though the light emission gets stronger with the rising concentration of dioxetane 1, the ratio does not change. Similar results were also found with the Emerald II enhancer solution. The ratio of emission at 650 nm to 585 nm stays constant from 20 µM to 100 µM, and only a slight increase is observed from 100 µM to 200 µM.
We also conducted this test with different pH buffers, and no matter how the time changes, the ratio stays the same. In this way, the time independence of this pH quantification system has been demonstrated.
2.2.6 In vitro testing in more complex systems

![Graphs](image)

**Figure 2- 5.** Ratiometric pH measurement in different environments. (A) Ratio of the chemiluminescence emission intensities at 650 nm and 585 nm of 60 µM 1 and 80 µM **carboxy-SNARF-1** in pH 7.42 buffers containing 6%–14% Sapphire II Enhancer. (B) Ratio of the chemiluminescence emission intensities at 650 nm and 585 nm of 60 µM 1 and 80 µM **carboxy-SNARF-1** in pH 7.42 buffers containing 6%–14% Emerald II Enhancer. (C) Ratio of the chemiluminescence emission intensity at 650 nm and 585 nm of 60 µM 1 and 80 µM **carboxy-SNARF-1** in 20 mM PBS buffered to pH 7.4 containing 6% Emerald II Enhancer in the absence and presence of 10% fetal bovine serum (FBS).

The percentage of the enhancer also plays an important role in this system since it is a key factor for the energy transfer from the dioxetane 1 to **carboxy-SNARF-1**. As a result, it could potentially alter the ratio of CL$_{650\text{nm}}$/CL$_{585\text{nm}}$. The pH of the buffer would not change with varying percentage of the enhancer, which has been confirmed by pH meter. We observed a slight decrease of the CL$_{650\text{nm}}$/CL$_{585\text{nm}}$ ratio with the increasing percentage of the Sapphire II enhancer. A similar result has been observed with Emerald II enhancer and the decrease in the ratio of CL$_{650\text{nm}}$/CL$_{585\text{nm}}$ is a little bit more intense than the ratio of Sapphire II enhancer solution, which could because the 585 nm peak is overlaid with the shoulder of the fluorophore in the Emerald II enhancer and increasing the enhancer will lead to the rising the fluorophore peak, and as a result, the ratio will slightly decrease. Our goal for this project is using this established system to quantify pH in a biological system. The ability to keep the ratio constant in more complicated systems is important.
for this approach. FBS (fetal bovine serum) is the main nutrition for the cell in mammalian cell maintenance, and it contains a variety of proteins. We can imitate the biological system by adding FBS to the PBS buffer and test our system in this solution. Constant pH with the addition of 10% FBS to the PBS buffer has been confirmed with a pH meter. We tested our system with Emerald II enhancer. The results show that the addition of FBS doesn’t cause any change to the ratio of $\text{CL}_{650\text{nm}}/\text{CL}_{585\text{nm}}$ in our system, which demonstrates that we can used this system in a more complicated biological system.

2.2.7 In vitro testing under IVIS spectrum

![Chemiluminescence images of the pH dependent emission of 30 μM 1 and 40 μM carboxy-SNARF-1 in aqueous buffers (pH 5.99–8.01) containing 6% Sapphire II Enhancer using a (A) 580 nm filter or (B) 640 nm filter in an IVIS Spectrum.](image)

**Figure 2-6.** Chemiluminescence images of the pH dependent emission of 30 μM 1 and 40 μM carboxy-SNARF-1 in aqueous buffers (pH 5.99–8.01) containing 6% Sapphire II Enhancer using a (A) 580 nm filter or (B) 640 nm filter in an IVIS Spectrum.
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well results in a pH-dependent curve that shows an increase in plate.

Sapphire II Enhancer (Fig. 5) or Emerald II Enhancer (Fig. 6) described ratiometric imaging protocol.

locations using this chemiluminescent substrate that the pH can be quantified at defined spatial

the Sapphire II Enhancer (Fig. 7A) and from 0.7 to 2.1 when containing 6% carboxy-SNARF-1, Spectrum. Images were rapidly acquired using either a 580 nm filter or a 640 nm filter. Carboxy-SNARF-1, SNARF-1 was synthesized according to a literature procedure to the ratiometric pH sensitive dye

at 650 nm and 585 nm (CL

absence and presence of 10% fetal bovine serum (FBS).

650 nm and 585 nm of 60 µM

filter or a 640 nm filter. Carboxy-SNARF-1, SNARF-1.

- –

Figure 2- 7. Chemiluminescence images of the pH dependent emission of 30 µM 1 and 40 µM carboxy-SNARF-1 in aqueous buffers (pH 5.99–8.01) containing 6% Emerald II Enhancer using a (A) 580 nm filter or (B) 640 nm filter in an IVIS Spectrum.

Figure 2- 8. The pH dependent chemiluminescence emission ratio of the emission at 640 nm to 580 nm of 30 µM 1 and 40 µM carboxy-SNARF-1 in aqueous buffers (pH 5.99–8.01) containing (A) 6% Sapphire II Enhancer or (B) 6% Emerald II Enhancer in an IVIS Spectrum.

Finally, we proceeded to test the system in an IVIS Spectrum. The IVIS Spectrum is an ultra-sensitive camera that can capture the light emission at a very dim scale. It has filters with different wavelength in front of the camera that can control the wavelength of the light that the instrument will capture. We prepared the system in aqueous enhancer solution at pH 6, 6.4, 6.8,
7.2, 7.4, 7.6, 7.8, or 8.0 in PBS buffer with 6% Sapphire II enhancer, 30 μM dioxetane 1 and 40 μM carboxy-SNARF-1. The IVIS Spectrum could capture the picture of light emission at 580 nm and 640 nm. In the Sapphire II enhancer solution, the light emission intensity at 580 nm is much lower than 640 nm (Figure 2-6, A-B). Each pH has 6 replicates in this test. The computer program can calculate the sum of light emission from each well, and by plotting out the ratio of CL<sub>640nm</sub>/CL<sub>580nm</sub>, a graph has been obtained and a 4-fold increase of CL<sub>640nm</sub>/CL<sub>580nm</sub> ratio from pH 6-8 has been observed (Figure 2-8, A). Similar results have been observed with Emerald II as the enhancer (Figure 2-7, A-B) and according to the graph (Figure 2-8, B), a 3-fold increase of CL<sub>640nm</sub>/CL<sub>580nm</sub> ratio from pH 6-8 is obtained.

2.3 Conclusions

Herein, we have realized quantitative ratiometric chemiluminescence imaging of pH via the transfer of energy from a chemiluminescent excited state of a phenolate derived from the decomposition of 1 to the ratiometric pH sensitive dye carboxy-SNARF-1. The synthesis and isolation of 1 was of key importance in providing a bright chemiluminescent system to ensure excitation of carboxy-SNARF-1. The system provides a reliable ratiometric response to variable pH, which is independent of confounding variables such as time and the concentration of the chemiluminescent probe. The system also provides an accurate measurement of pH in the presence of fetal bovine serum, demonstrating operational compatibility with complex biological fluids. Protocols have been established for the quantification of pH using chemiluminescence imaging on an IVIS Spectrum. Due to increased luminescence emission in the range between 585 nm and 650 nm, the system containing the Emerald II Enhancer provides greater signal-to-noise for pH measurement. On the other hand, the system containing the Sapphire II Enhancer provides a higher magnitude change in the ratiometric signal. Some fluctuations are seen when the volumes of the Enhancer solutions are altered, a problem that is more severe in the case of the Emerald II Enhancer. The formulation of systems with stable polymeric encapsulation or covalent linkage of components to ensure consistent stoichiometry could provide solutions to this issue. Nevertheless, quantitative imaging of pH using this ratiometric chemiluminescent system has been achieved. We ultimately anticipate that similar strategies will be compatible with the current library of known ratiometric fluorescent probes to provide a powerful new toolkit for ratiometric chemiluminescence imaging.
2.4 Synthesis Procedures

2.4.1 General materials and methods

All reactions were performed in dried glassware under an atmosphere of dry N\textsubscript{2}. 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. \textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectra for characterization of new compounds and monitoring reactions were collected in CDCl\textsubscript{3} (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.

![image]

Diethyl((4-chloro-3-methoxyphenyl)(methoxy)methyl)phosphonate  (4). 4-Chloro-3-methoxybenzaldehyde (1502 mg, 8.8 mmol, 1 equiv) was dissolved with 5 mL MeOH in a dry flask filled with N\textsubscript{2}. Trimethyl orthoformate (0.96 mL, 9.3 mmol, 1.1 equiv) and p-Toluenesulfonic acid (147 mg, 0.9 mmol, 0.1 equiv) was added to the flask. The mixture was stirred for 24 hours at room temperature under N\textsubscript{2}. After that the mixture was neutralized by NEt\textsubscript{3}. Pour 30 mL
NaHCO$_3$ to the mixture and extract with 3 x 30 mL EtOAc. The organic layer was dried by Na$_2$SO$_4$, filtered and concentrated under reduced pressure to obtain crude acetal 3 (1897.4 mg). Compound 3 was dissolved in 5 mL DCM at 0°C. Triethyl phosphite (1.65 mL, 10.2 mmol, 1.2 equiv) and boron trifluoride etherate (1.13 mL, 9.00 mmol, 1.0 equiv) were added to the flask at 0°C. The reaction was raised to 30°C and stirred for 1 hour. The reaction was quenched by 30 mL NaHCO$_3$, extracted by 3 x 30 mL EtOAc and dried by Na$_2$SO$_4$ and filtered. Evaporate the EtOAc under reduced pressure and purify by column chromatography (1:15 EtOAc/Hexane) yield the product as a pale yellow oil (13389 mg, 47%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 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); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 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 C$_{13}$H$_{20}$ClO$_5$P (M+Na$^+$) 345.0629, found 345.0624.

(1r,3r,5R,7S)-2-((4-chloro-3-methoxyphenyl)(methoxy)methylene)adamantine (5). Compound 4 (1339 mg, 4.2 mmol, 1.0 equiv) was dissolved in 5 mL anhydrous THF in a dry flask under N$_2$ and cooled to −78 °C by adding dry ice to acetone. 2-Adamantanone (786 mg, 5.2 mmol, 1.2 equiv) was dissolved in 5 mL anhydrous THF and cooled to −78 °C by the same way. Add 5 mL 1.6 M n-BuLi to the compound 4 solvent at −78 °C and stirred for 6 min. Add the cooled 2-adamantanone solvent to the flask and raise the temperature of the reactant to 30 °C. The mixture was stirred for 2 hours and the temperature was raised to 90 °C under reflux. After 1 hour reflux, the reaction was quenched by 30 mL NH$_4$Cl and extracted by 3 x 30 mL EtOAc. Purification by chromatography (1:15 EtOAc/Hexane) yield Compound 5 as colorless oil (1351 mg, >95%). $^1$H 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$_{22}$ClO$_2$ (M+H$^+$) 319.1459, found 319.1461.
5-(((1R,3R,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-chlorophenol (6). Compound 5 (1351 mg, 4.2 mmol, 1.0 equiv) was dissolved in 14.4 mL anhydrous DMF at room temperature in a dry flask under N₂. Cs₂CO₃ (3436 mg, 10.5 mmol, 2.5 equiv) and EtSNa (867 mg, 10.3 mmol, 2.5 equiv) was added to the flask. The temperature of the mixture was raised to 90 °C under reflux for 16 hours. The reaction was quenched by 30 mL of NH₄Cl, extracted by 3 x 30 mL, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by column chromatography (1:15 EtOAc:Hexane) yield compound 6 as a white solid (954 mg, 74%). ¹H NMR (500 MHz, CDCl₃) δ 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); ¹³C NMR (125 MHz, CDCl₃) δ 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 C₁₈H₂₁ClO₂ (M–H⁺) 303.1157, found 303.1150.

(1R,3R,5R,7S)-2-((4-chloro-3-methoxymethylphenyl)(methoxy)methylene)adamantine (7). DIPEA (1.00 mL, 5.7 mmol, 1.8 equiv) was added to a solution of compound 6 (954 mg, 3.1 mmol, 1.0 equiv) in 10 mL DCM at room temperature under N₂. Chloromethoxymethane (0.47 mL, 6.2 mmol, 2.0 equiv) was added dropwise to the solvent at 0 °C. After the addition of chloromethoxymethane, the reaction was raised to room temperature and stirred for 2.5 hours. Quench the reaction with 30 mL saturated aq NH₄Cl and the mixture was extracted with 3 x 30 mL DCM and evaporated under reduced pressure. Purification by chromatography (1:12 EtOAc/Hexane) yielded compound 7 as a colorless oil (662 mg, 61%). ¹H NMR (500 MHz, CDCl₃) δ 7.30 (d, 1H, J=10.0 Hz), 7.12 (s, 1H), 6.90 (dd, 1H, J=10 Hz), 5.21 (s, 2H), 3.55 (s, 3H), 3.28 (s, 3H), 3.22 (s, 1H), 2.62 (s, 1H), 1.69-1.95 (m, 12H); ¹³C NMR (125 MHz, CDCl₃) δ 151.48, 135.64, 129.02, 121.20, 111.53, 95.57, 50.02, 47.10, 39.38, 36.43, 34.82, 33.21, 32.34, 31.75, 31.61, 31.04, 27.90, 26.07, 25.94. (M–H⁺), found 381.1463.
Compound 7 (208 mg, 0.6 mmol, 1.0 equiv) and Rose Bengal (21.2 mg, 0.02 mmol, 0.03 equiv) were added into a dry two-neck flask and dissolved in 5 mL THF. O₂ was bubbled through the solvent while illuminated with a 120W light bulb (Home Depot, Dallas, TX) at 0–5 °C. Monitor the reaction by TLC. After 4 hours, the mixture was concentrated at 0 °C. Purification by silica column chromatography (1:15 EtOAc/Hexane) delivered compound 8 as a yellow oil (206 mg, 91%). ¹H NMR (500 MHz, CDCl₃), δ 7.01–7.64 (m, 3H, br), 5.28 (m, 2H), 3.49 (s, 3H), 3.20 (s, 3H), 3.01 (s, 1H), 2.12 (s, 1H), 1.45-1.90 (m, 12H); ¹³C NMR (125 MHz, CDCl₃) δ 152.62, 134.80, 130.13, 125.03, 111.61, 95.47, 56.30, 49.98, 39.50, 35.43, 33.24, 33.11, 31.71, 31.58, 30.30, 26.08, 26.00. MS not found.

Compound 8 (651 mg, 1.7 mmol, 1.0 equiv) was dissolved in 10 mL MeOH at room temperature. p-toluenesulfonic acid (37.3 mg, 0.2 mmol, 0.1 equiv) was dissolved in 10 mL MeOH at room temperature. The p-toluenesulfonic acid solvent was added to the compound 7 drop by drop. After addition of p-toluenesulfonic acid, the mixture was raised to 65 °C and stirred for 6.5 hours. The mixture was quenched by 50 mL brine and extracted by 3 x 50 mL EtOAc and evaporated under reduced pressure. Purification was by silica column chromatography (1:20 EtOAc/Hexane). Compound 1 was obtained as a white solid (303 mg, 53%). ¹H NMR (500 MHz, CDCl₃) δ 7.02–7.53 (m, 3H, br), 5.81 (s, 1H), 3.22 (s, 3H), 3.05 (s, 1H), 2.17 (s, 1H), 1.43–1.83 (m, 12H); ¹³C NMR (125 MHz, CDCl₃) δ 151.42, 135.70, 121.20, 111.52, 95.71, 50.04, 47.05, 39.20, 36.44, 34.82, 33.21, 33.18, 32.50, 31.75, 31.61, 26.07, 25.94. (M–H⁺) found 349.1565.
4-[4-(dimethylamino)-2-hydroxybenzoyl]- 1,3-Benzenedicarboxylic acid (9).

1,2,4-Benzenedicarboxylic anhydride (2105 mg, 11.0 mmol, 1.0 equiv) and 3-(Dimethylamino)phenol (1505 mg, 11.0 mmol, 1.0 equiv) were added to a dry flask and dissolved with 30.0 mL toluene. The mixture was raised to 120 °C and stirred overnight. The mixture was washed by toluene 3 x 30 mL, 3 x 50 mL DCM and 3 x 50 mL EtOAc and filter the product and vacuum dry. Purification was by silica gel column chromatography (1:18 MeOH:DCM). Compound 9 was obtained as a dark green solid (3126 mg, 79%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 12.36 (s, 1H), 12.33 (s, 1H), 6.76–8.50 (m, 6H), 6.07 (s, 1H), 2.97 (d, 6H, $J$=2.5).

1,3-Benzenedicarboxylic acid, 4-[4-(dimethylamino)-2-hydroxybenzoyl] (2657 mg, 8.1 mmol, 1.0 equiv) and 1,6-naphthalenediol (1383 mg, 8.6 mmol, 1.1 equiv) were added to a dry, vacuum and N$_2$ filled flask. Later 5 mL of MeSO$_3$H was added to the flask and stirred for 3 hrs. The mixture was washed by water 3 x 30mL, EtOAc 3 x 30 mL and DCM 30 mL. Purification was by column chromatography (1:4 MeOH:DCM) and yielded carboxy-SNARF-1 as a purple solid (340 mg, 9%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 10.35 (s, 1H), 10.21 (s, 1H), 6.56–8.42 (m, 11H), 6.96 (s, 1H), 3.32 (s, 6H).

2.4.2 Imaging experiments with Fluorescence Spectroscopy

Chemiluminescent responses were acquired with a Hitachi F-7000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) using the luminescence detection mode. The Spectrophotometer scan mode was set as wavelength scan with the range of emission wavelength from 400 nm to 900 nm and scan speed was set to 1200 nm / min. Response time was set to 2.0 s.
pH 5.5 was made from 0.2 M Citric Acid – Sodium Citrate Buffer Solutions while pH 6.0 – 8.0 was made from 0.2 M PBS buffer solution and pH 9.2 – 10 was made from 0.1 M Sodium Carbonate – Sodium Bicarbonate Buffer Solutions.

2.4.3 pH dependence property

463 µL buffer, 30 µL of Emerald II enhancer or Sapphire II enhancer, 4 µL carboxy-SNARF-1 solvent (10mM) in DMSO and 3 µL 10mM Compound-1 in DMSO were added to a quartz cuvette (Starna, Atascadero, CA). The cuvette was shaken gently to assure mixing. The cuvette was placed in the middle of the Spectrophotometer and obtained the wavelength scan graph. The procedure was repeated with all the buffers in different pH.

2.4.4 Time independence property

463 µL PBS buffer solution, 30 µL of Emerald II enhancer or Sapphire II enhancer, 4 µL carboxy-SNARF-1 solvent (10mM) in DMSO and 3 µL Compound-1 solution (10mM) in DMSO were added to a quartz cuvette (Starna, Atascadero, CA). The cuvette was shaken gently to mixed. The cuvette was placed in the spectrophotometer and the wavelength scan mode was run every 3 mins for ten times.

2.4.5 Probe independence property

465 µL 0.2 M pH 7.4 PBS buffer solution, 30 µL Emerald II enhancer or Sapphire II enhancer, 4 µL carboxy-SNARF-1 solution (10 mM) in DMSO and 1 µL Compound-1 solution (10 mM) in DMSO were added to a quartz cuvette (Starna, Atascadero, CA). The cuvette was shaken gently to assure mixing. The cuvette was placed in the center of spectrophotometer and obtained the wavelength scan graph and repeat the experiment with increasing amount of Compound-1 solution from 1 µL to 10 µL (1 µL each time) and decreasing amount of buffer solution from 465 µL to 456 µL (1 µL each time).

2.4.6 Imaging experiments with IVIS Spectrum

Chemiluminescent responses were acquired with an IVIS Spectrum (Caliper, Waltham, MA) using the “Luminescent” and “Photograph” mode. The exposure time was set as 2 seconds, and the binning was set to small. The F/stop was set to 4, and the FOV was set to C, which means the field
of view was set to 13 cm. With these settings, images could be acquired with 267 μM spatial resolution. The height of each photograph was 1.5 cm. The excitation was blocked and a sequence was set for the emission mode. The sequence was set as 580 nm, 640 nm, 580 nm, 640 nm, 580 nm and 640 nm. 231.5 μL aliquots of PBS buffers with pH from 5.99–8.01 were added to the wells on the 96-well plate from A1 to A8. Later 2 μL of a 5 mM stock solution of carboxy-SNARF-1 (80 μM final concentration) in DMSO was added to each well followed by the addition of 15 μL Emerald II Enhancer or Sapphire II Enhancer. Next 1.5 μL of a 5 mM stock solution of 1 in DMSO was added to each well after the adding of the buffer, enhancer and carboxy-SNARF-1. This was repeated in groups A1 to A8 for 6 times on each plate. The sequence described above was acquired and the images were analyzed using the Living Image software.

2.5 References


3.1 Introduction

3.1.1 What is HNO

Nitroxy (HNO) is the reductive product of NO with the addition of one electron and one proton. NO$^-$ is isoelectronic to molecular oxygen and has a triplet ground state, which raises the pKa of HNO to 11.\textsuperscript{1} HNO will dimerize and eliminate water to rapidly form nitrous oxide (N$_2$O) ($k = 8 \times 10^6$ M$^{-1}$s$^{-1}$ at 23 °C).\textsuperscript{2} At pH > 11, the $^3$NO$^-$ will react with $^3$O$_2$ and yielded ONOO$^-$.\textsuperscript{3,4} Thiols\textsuperscript{5} and iron in heme-containing proteins\textsuperscript{6} are two main biological targets for HNO.

3.1.2 Biological and pharmacological functions of HNO

NO has been recognized as a vasodilator and endothelium-derived relaxing factor (EDRF) since the 1980s. NO can activate the heme protein soluble guanylyl cyclase (sGC) and can lead to the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), a key factor to initiate a cascade of biological activities that result in vasodilation.\textsuperscript{7} Studies have been done with isolated sGC exposed to donors like HNO, NO and NO$^+$, and the results showed that the sGC will be activated only when exposed to NO.\textsuperscript{8} This result implies that the HNO induced vasodilation is because of the oxidation of HNO to NO in tissue that leads to the generation of cGMP and result in the sGC activating.\textsuperscript{9} A range of biological reagents could help HNO to convert to NO including SOD, metHP and flavins.\textsuperscript{10} However only small portion (<5%) of HNO could be converted to NO in biological conditions,\textsuperscript{10} which leads to the question that if HNO itself could directly lead to the vessel relaxation. Recent evidence supports the possibility that HNO could regulate vascular tone and blood flow through an HNO/TRPA1/CGRP pathway.\textsuperscript{11,12} Pharmacological HNO can be used as a pain reducer in animal models through a cGMP/PKG/K$_{ATP}$ pathway.\textsuperscript{13} Pharmacological HNO is being investigated as a treatment for heart failure. The release
of CGRP has been observed from rat aortic strips upon exposure to HNO, which is a key factor to cause visodilation.\textsuperscript{14} Cyanamide has been used as an alcohol deterrent agent. With the existence of catalase and H\textsubscript{2}O\textsubscript{2}, cyanamide will release HNO as an aldehyde dehydrogenase (AIDH) inhibitor.\textsuperscript{15} HNO can also be cytotoxic since it will affect the cellular function by changing redox status of cell, but this process will be protected by GSH that works as an HNO scavenger.\textsuperscript{16}

3.1.3 Possible endogenous HNO resources

A range of endogenous biomolecules can lead to the generation of HNO. Endogenous generation of HNO could result from the reaction between H\textsubscript{2}S with NO has been suggested to have a biological role in a HNO/TRPA1/CGRP pathway.\textsuperscript{11-12} The reaction between NH\textsubscript{2}OH and NO could also be an endogenous resource for HNO, even with a low generation rate \((5 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1} \text{ at } 25 \text{ °C}, \text{ pH } 7.3).\textsuperscript{17-18}\) Although free Fe (II) has a low concentration in biological systems, it is still capable of reducing NO to HNO due to the stoichiometric conversion.\textsuperscript{19-20} The S-nitrosothiol is another source for endogenous HNO upon the reaction with excess endogenous thiols at a comparability low reaction rate \((8.3 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}).\textsuperscript{21}\) Meanwhile, the reaction of GSNO and GSH\textsuperscript{22}, GSNO and NADH\textsuperscript{23} also contribute to the generation of endogenous HNO. However, even though the generation of HNO has been confirmed with isolated donors, the low reaction rate and low concentration of the reactant limited the possibility of occurrence in biological system. As a result, HNO detection methods have started to attract attention from researchers.

3.1.4 HNO detection methods and difficulties

The fast dimerization rate \((k = 8 \times 10^{6} \text{ M}^{-1} \text{ s}^{-1} \text{ at } 23 \text{ °C})\textsuperscript{2}\) and rapid reaction rate with molecular oxygen (range from \(10^{3}\) to \(10^{4} \text{ M}^{-1} \text{ s}^{-1}\))\textsuperscript{5, 24} for HNO in biological systems make its detection challenging. Researchers have developed various methods to detect the biological HNO reaction products instead of directly detecting HNO due to its instability. The dimerization product N\textsubscript{2}O is a good indicator for HNO generation, which has been successfully detected by gas chromatography and with electron paramagnetic resonance (EPR) spectroscopy trapping by N-methyl-D-glucamine dithiocarbamate iron (Fe-MGD) to produce ERP active molecules.\textsuperscript{25} Even though the detection of N\textsubscript{2}O is applicable in solution, but application of this method in biological systems is limited. Glutathione sulfonamide, the product of HNO and GSH, can also be used as a biomarker for HNO detection with high pressure liquid-chromatography mass spectrometry
However, sensitive tools for direct detection of HNO are needed to study potential endogenous biological pathways and functions of HNO as well as measure HNO released from pharmacological donor compounds.

3.1.5 Current HNO probes

A series of fluorescent HNO probes have been reported recently including copper-based fluorescent probes, phosphine-based fluorescent probes, TEMPOL-based fluorescent probes and 2-mercapto-2-methylpropionic acid-based probes.

![Scheme 3 - 1. Copper-based HNO fluorescent probe Cu(BOT1).](image)

The first small molecule fluorescent HNO probe was synthesized by Lippard’s group in 2010 based on the Cu
II
cation (Scheme 3-1). When Cu
II
is bounded to the tripodal dipicolylamine-appended receptor, the fluorescence will be quenched. The Cu
II
can quench the fluorescence of the molecule by photoinduced electron transfer (PET) of the singlet excited state of BODIPY. Cu(BOT1) has an 4-fold turn on towards 1000 equivalents of Angeli’s salt and a good selectivity toward HNO. Later a series of probes based on copper has been synthesized with a turn-on range of 5-fold to 15-fold at different emission wavelengths. Another type of HNO probe has been synthesized with phosphine as the protecting group. The fluorescence of the fluorophore is quenched or diminished with the attachment of phosphine, and once reacted with HNO, the phosphine protecting group will be removed and released the fluorophore with a higher fluorescent emission. A series of probes based on this mechanism have been synthesized with different emission wavelengths. Compared with Cu-based probes, phosphine-based probes present a better fluorescent response. The metal free and reductant resistant trigger give it a better selectivity for HNO detection in biological systems (Scheme 3-2). TEMPOL-based fluorescent probes and 2-mercapto-2-methylpropionic acid-based fluorescent probes also have been reported before.
However, because of the instability of HNO in biological systems, a more sensitive tool for HNO detection is needed to study the biological pathways and function of HNO. Compared with fluorescent probes, chemiluminescent probes have a better signal-to-noise ratio, which could significantly increase the detection sensitivity. Our lab designed and synthesized the first chemiluminescent HNO probes, which have an over 800-fold increase toward 20 equivalents of Angeli’s salt compared with the control. We successfully quantified the HNO generated from H$_2$S and NO and achieved HNO monitoring in biological systems.$^{46}$ These results will be presented and discussed in this chapter.

**Scheme 3 - 2.** HNO detection mechanism for phosphine-based probes.$^{28}$

3.2 Results and discussion

3.2.1 Design and Synthesis of HNOCL-1

Our lab aimed to develop highly sensitive probes as a powerful tool to detect and study the generation, pathways and biological function of reactive oxygen and nitrogen species, and a series of chemiluminescent probes based on 1,2-spirodioxetane have been synthesized and characterized before$^{1-4}$. Phosphine has been used as the HNO detection group for a series of probes$^{5-10}$. The design of **HNOCL-1** consists of linking a triaryl phosphine group to the sterically stabilized 1,2-dioxetane. By reacting with HNO, it will form a self-cleavable intermediate and release the
phenolate. In aqueous solution, the phenolate will trigger Chemically Initiated Electron Exchange Luminescence (CIEEL) mechanism and emit light.

**Scheme 3 - 3. Design and synthesis of HNOCL-1.**

The synthesis of HNOCL-1 started with 2-chloro-3-methoxybenzaldehyde. First, we treated the 4-chloro-3-methoxylbenzaldehyde 11 with trimethyl orthoformate the presence of p-toluenesulfonic acid in methanol to yield the acetal 12. The acetal 12 was treated with triethyl phosphite and boron trifluoride diethyl etherate to obtain the diethyl methoxy phosphonate 13. We ran the Horner–Wadsworth–Emmons reaction with phosphonate, where we treated 12 with n-BuLi and 2-admantanone to yield the enol ether 14. The enol ether 14 would go through a demethylation reaction via nucleophilic substitution with sodium ethanethiolate and cesium carbonate to obtain the phenol 15. The enol ether was treated with N-iodosuccinimide, which yielded the iodide phenol 16. The iodide phenol 16 went through a Heck reaction and yielded the acrylate phenol 17. The acrylate phenol 17 was dissolved with THF and Rose Bengal was added to the solvent as a catalyst. O2 was then bubbled through the system and the 1,2-spirodioxetane phenol 18 was obtained. Finally, the 1,2-spirodioxetane phenol 18 was treated with EDC, DMAP and 2-diphenylphosphinobenzoic acid and yielded HNOCL-1 as product.
3.2.2 Mechanism of HNO detection

Scheme 3 - 4. Proposed mechanism for the reaction of the triaryl phosphine in HNOCL-1 with HNO.

Once reacted with HNO, the HNOCL-1 will turn into a self-cleavable intermediate azaylide which will lead to the ester cleavage and release the phenol. In aqueous solution, the phenol will be deprotonated and yield the phenolate, which would decompose and trigger CIEEL. The light emission for this compound is at 525 nm.47

3.3.3 Response and selectivity

Figure 3- 1. Chemiluminescence response of HNOCL-1 and Angeli’s salt, Na₂N₂O₃ (AS). (A) Chemiluminescence emission spectra at 525 nm of 20 μM HNOCL-1 and 0 (blue trace), 50, 100, 150, and 200 μM (red trace) AS. (B) Time course of chemiluminescence emission of 0 (blue trace), 5, 25, 50, and 100 μM (red trace) AS. Experiments were performed in 20 mM PBS (pH 7.4), containing ≤1% DMSO.
The response towards HNO was collected later after obtaining HNOCL-1. 20 µM HNOCL-1 was treated with 0 - 200 µM Angeli’s salt and the emission peak was observed at 525 nm (Figure 3-1, A). The time scan at 525 nm with 0 µM, 5 µM, 25 µM, 50 µM and 100 µM of Angeli’s Salt shows a dose dependent response for HNOCL-1, which demonstrates the high sensitivity of the probe. The emission reaches the maximum emission at around 15 mins and starts to decay with a slower rate over the time course of 40 min (Figure 3-1, B).

![Chemiluminescent responses of 20 µM HNOCL-1 and 200 µM reactive oxygen and nitrogen species.](image)

**Figure 3-2.** Chemiluminescent responses of 20 µM HNOCL-1 and 200 µM reactive oxygen and nitrogen species. Legend: 1. AS, 2. GSH (2 mM), 3. GSNO, 4. H₂O₂, 5. KO₂, 6. Cys (1 mM), 7. Na₂S, 8. Na₂S₂O₃, 9. NaN₂O₂, 10. HO⁺, 11. ONOO⁻, 12. 'BuOOH, 13. OCl⁻, 14. DEA NONOate, 15. Blank. Experiments were performed in 20 mM PBS (pH 7.4), containing ≤1% DMSO.

We proceeded to evaluate the selectivity of HNOCL-1 towards HNO compared with other biologically related reactive oxygen and nitrogen species. We treated 20 µM HNOCL-1 in PBS buffer (pH=7.4, 20 mM) and added different reactive oxygen and nitrogen species including 2 mM GSH, 1 mM cysteine and 200 µM other reactive oxygen and nitrogen species. HNOCL-1 has a significant increase of chemiluminescent intensity upon the addition of Angeli’s salt compared with other reactive oxygen and nitrogen species. The NO donor, DEA NONOate caused a slight increase when tested in 20 mM HEPES buffer but nothing happened in PBS buffer. The reason for the increase could be the reduction of NO by HEPES to yield a small amount of HNO.

**3.3.4 Comparison with HNO fluorescent probe**

![Chemical structure of the fluorescent probe.](image)
Scheme 3 - 5. Principle of HNO detection with XF2.

XF2 is a fluorescent probe previously synthesized by our lab to detect HNO. The fluorescence is quenched by attaching the diphenylphosphino group. Once reacted with HNO, the fluorophore will be released and the fluorescence emission will increase.

![Chemiluminescence response of XF2 and Angeli’s salt, Na2N2O3 (AS). (A) Time course of chemiluminescence emission of 10 μM HNOCL-1 and 0 (blue trace) or 200 μM (red trace) AS. (B) Fluorescence emission spectra of 10 μM XF2 after reacting with 0 (blue trace) or 200 μM (red trace) AS for 25 minutes. Experiments were performed in 20 mM PBS (pH 7.4), containing ≤1% DMSO.](image)

Figure 3- 3. Chemiluminescence response of HNOCL-1 and Angeli’s salt, Na2N2O3 (AS). (A) Time course of chemiluminescence emission of 10 μM HNOCL-1 and 0 (blue trace) or 200 μM (red trace) AS. (B) Fluorescence emission spectra of 10 μM XF2 after reacting with 0 (blue trace) or 200 μM (red trace) AS for 25 minutes. Experiments were performed in 20 mM PBS (pH 7.4), containing ≤1% DMSO.

Compared with XF2, HNOCL-1 has a significantly improved signal-to-noise ratio response towards the same amount of HNO with the integrated data at the time point 25 min. After the control is normalized to 1, the emission from HNOCL-1 reacted with HNO lead to an 833-fold turn-on response. With the same concentration of probe and HNO, the XF2 only has a 20-fold turn-on response.

3.3.5 Quantification of HNO generated from NO and H2S

\[ [\text{HNO}] = 1/(k_i[\text{HNOCL-1}]) \cdot (k_3[1] + d[1]/dt) \]

Eq. 3-1. Equation to quantify the concentration of HNO. [HNOCL-1] is the concentration of HNOCL-1, [1] is the concentration of the phenol. \( k_i = 20,433 \text{ M}^{-1}\text{s}^{-1} \) at 25 °C, pH 7.4 according to the method developed by Miranda and Wink\(^5\). \( k_3 = 5.92 \times 10^{-4} \text{ s}^{-1} \) at 25 °C, pH 7.4 according to measurements of the exponential decay of isolated 1.

Because of the sensitivity of HNOCL-1, we were able to quantify the concentration of HNO generated from the system. The derivation of Eq. 3-1 is given in the Experimental Methods section. [HNOCL-1] is the amount of the probe that we added to the system. [1] is the
concentration of the phenol, which could be determined by isolated 1 decay. The [1] could be determined by chemiluminescent emission intensity. The chemiluminescent emission could be converted to [1] with a carefully calibrated graph with varying concentration of 1 and chemiluminescent emission intensity (Figure 3-16). \( k_1 = 20,433 \text{ M}^{-1}\text{s}^{-1} \) at 25 °C, pH 7.4 and \( k_3 = 5.92 \times 10^{-4} \text{ s}^{-1} \) at 25 °C.

![Figure 3-4](image)

**Figure 3-4.** Concentration of HNO generated from AS (A) measured from the raw chemiluminescence emission of 20 µM HNOCL-1 or (B) computationally simulated. Experiments were performed in 20 mM PBS (pH 7.4), containing ≦1% DMSO.

We then measured the concentration of HNO generated from various concentrations of Angeli's salt according to Eq 3-1 from Figure 3-1, B. The results are shown on Figuer 3-4, A. The results matched the simulated results determined from numerical solution of the differential equation modeling HNO generation from Angeli's salt (Figure 3-4, B).

![Figure 3-5](image)

**Figure 3-5.** (A) Time course of chemiluminescent emission and (B) integrated emission intensity of 20 µM HNOCL-1 alone (blue trace), 200 µM Na₂S alone or with 0.2, 0.5, and 1 mM (red trace) DN, and 200 µM Na₂S, 1 mM DN, and 2 mM N-acetyl cysteine (NAC). (C) Concentration of HNO produced from 20 µM HNOCL-1 alone (blue trace), and 200 µM Na₂S and 0.2, 0.5, and 1 mM (red trace) DN as determined from Eq. (1) and the data shown in (A). All experiments were
performed in 20 mM HEPES or PBS (pH 7.4), containing ≤1% DMSO. Error bars are + S.D. from n = 3 – 6 replicates.

With HNOCL-1, we successfully confirmed and quantified the generation of HNO from H₂S and NO, which is one of the possible endogenous sources of HNO. With 200 µM Na₂S and 20 µM HNOCL-1, a significant rise in chemiluminescent emission appeared with the addition of DEA NONOate. It shows a dose-dependent manner with different concentrations of DEA NONOate. With the increasing concentration of DEA NONOate, the maximum emission increased and it takes shorter time to reach the emission peak (Figure 3-5, A). Once we added the N-acetyl cysteine (NAC), an HNO scavenger, the light emission is quenched and the signal disappeared (Figure 3-5, B). When we applied the light emission intensity to Eq. 3-1, the intensity of HNO was calculated (Figure 3-5, C).

3.3.6 HNO detection in living cells

![Chemiluminescence measurement of HNO in living cells](image)

**Figure 3-6.** Chemiluminescence measurement of HNO in living cells. (A) Time course of chemiluminescent emission and (B) integrated emission intensity of A549 cells incubated with 20 µM HNOCL-1 for 30 minutes, washed and treated with AS. Error bars are +S.D. from n = 9 wells across 3 biological replicates.

We proceeded to test if HNOCL-1 is capable of detecting pharmacological HNO in biological systems. A549 lung cancer cells were incubated with 20 µM HNOCL-1 for 30 mins and were thoroughly washed with PBS buffer to remove all the extracellular probes. The cells were treated with various amounts of Angeli’s salt afterwards. The signal from the cells increased with a higher concentration of Angeli’s salt (Figure 3-6). The signal started to max out from 500 µM to 1 mM Angeli’s Salt, probably due to the dimerization of HNO.
**Figure 3-7.** Chemiluminescent measurement of HNO in living cells. (A) Time course of chemiluminescence emission of A549 cells incubated with 20 µM HNOCL-1 for 30 minutes, washed and incubated without (blue trace) or with 1 mM DEA NONOate (DN) and 200 µM Na2S (red trace). (B) Integrated emission intensity of A549 cells incubated with 20 µM HNOCL-1 for 30 minutes, washed and treated as indicated with H2S as 200 µM Na2S, NO as 1 mM DN, and NAC at 2 mM. Error bars are ±S.E. from n = 6–11 wells and 2–4 biological replicates. Statistical significance was assessed using a two-tailed student's t-test. **p<0.01, *p<0.05. All cellular experiments were performed at 37 °C.

We then continued to test if HNOCL-1 had the ability to detect the HNO generated from the reaction between H2S and NO in biological system. After 30 mins incubation with HNOCL-1, the Na2S and DEA NONOate were added to the A549 lung cancer cells. The experimental group showed a slight rise compared to the control group. Upon the addition of N-acetyl cysteine (NAC) to the cells treated with Na2S and DEA NONOate, there was a significant decrease in the emission. These results demonstrated that HNOCL-1 has the ability to detect the HNO generated from Na2S and NO in a biological system.
3.3.7 HNO in vivo detection

![Images of BALB/c nude mice 2 minutes after IP injection with 40 µM HNOCL-1 and (A) vehicle control or (B) 1 mM Angeli's salt in 20 mM PBS (pH 7.4) containing 5% DMSO.]

**Figure 3-8.** Images of BALB/c nude mice 2 minutes after IP injection with 40 µM HNOCL-1 and (A) vehicle control or (B) 1 mM Angeli's salt in 20 mM PBS (pH 7.4) containing 5% DMSO.

We also investigated the ability for HNOCL-1 to detect HNO *in vivo*. We conducted the experiments in the BALB/c nude mice. From the results, the chemiluminescence emission from the experimental group with 1 mM AS and 40 µM HNOCL-1 increase 300-fold compared with the vehicle control. This result shows that HNOCL-1 has promise for pharmacological HNO detection *in vivo*.

### 3.3 Conclusions

In summary, we have described the first chemiluminescent probe for real-time monitoring of HNO in living cells. The rapid and sensitive response has enabled an innovative kinetics-based approach that provides quantitative estimates of HNO concentration. To date, this has only been accomplished using electrodes and a xerogel optical sensor film, which are inherently limited to extracellular monitoring. Chemiluminescent probes like HNOCL-1 can be loaded into cells, and we anticipate that expansion of this kinetics-based approach will lead to a unique method for quantification of HNO in living cells. This approach is generalizable to other reaction-based probes as long as one can achieve high sensitivity, mechanistic understanding, and evaluation of kinetic parameters. The cellular data shows that HNOCL-1 provides a method to reliably monitor the time-course of HNO delivery from donor systems and preliminary experiments in living mice demonstrate that the high photon flux from HNOCL-1 is sufficient for imaging HNO in animal tissue. We anticipate that HNOCL-1 will find use in understanding HNO biology and the development of new HNO-based therapeutics.
3.4 Experimental Methods

3.4.1 General synthetic materials and methods.

All reactions were performed in dried glassware under an atmosphere of dry N\textsubscript{2}. Silica gel P60 (SiliCycle) was used for column chromatography and Analytical Chromatography TLC Silica gel 60 F\textsubscript{254} (Merck Millipore, Darmstadt, Germany) was used for analytical thin layer chromatography. Plates were visualized by fluorescence quenching under UV light or by staining with iodine. N,N-Diisopropylethylamine was distilled from potassium hydroxide, but all other reagents were used without further purification. Anhydrous dimethylformamide was used in all reactions. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), EMD Millipore (Billerica, MA), Oakwood Chemical (West Columbia, SC), or TCI (Tokyo, Japan) and used without further purification. \textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectra for characterization of new compounds and monitoring reactions were collected in CDCl\textsubscript{3} (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. Chemical abbreviations are used as follows: CH\textsubscript{2}Cl\textsubscript{2}, dichloromethane; EtOAc, ethyl acetate; THF, tetrahydrofuran; DMF, dimethylformamide; H\textsubscript{2}O, water; HBTU, O-benzotriazole-N,N,N’,N’-tetramethyl- uronium-hexafluoro- phosphate; DIPEA, N,N-diisopropylethylamine; KOH, potassium hydroxide; DMSO, dimethylsulfoxide N\textsubscript{2}, nitrogen. 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.
(12). 2-Chloro-3-methoxybenzaldehyde (9900 mg, 58.6 mmol, 1 equiv) was dissolved with 80 mL MeOH in a dry flask filled with N₂. Trimethyl orthoformate (6.4 mL, 59 mmol, 1.0 equiv) and p-Toluenesulfonic acid (1009 mg, 5.9 mmol, 0.1 equiv) was added to the flask. The mixture was stirred for 24 hours at room temperature under N₂. After that the mixture was neutralized by NEt₃. 300 mL NaHCO₃ was poured into the mixture and extract with 3 x 300 mL EtOAc. The organic layer was dried by Na₂SO₄, filtered and concentrated under reduced pressure to obtain crude acetal 11 (1897 mg). Compound 11 was dissolved in 20 mL DCM at 0 °C. Triethyl phosphite (10.5 mL, 61.1 mmol, 1.0 equiv) and boron trifluoride etherate (7.7 mL, 61.1 mmol, 1.0 equiv) were added to the flask at 0 °C and stir for 20 mins. The reaction was raised to 30 °C and stirred for 2 hours. The reaction was quenched by 50 mL NaHCO₃, extracted by 3 x 50 mL EtOAc and dried by Na₂SO₄ and filtered. The EtOAc was evaporated under reduced pressure and purified by column chromatography (1:15 EtOAc/Hexane) yield the product as a pale yellow oil 12 (1339 mg, >95 %).

1H NMR (500 MHz, CDCl₃) δ 7.29 (m, 2H), 6.91 (m, 1H), 5.19 (d, 1H, J = 15.0 Hz), 4.17–4.21 (m, 4H), 4.16 (s, 3H), 3.34 (s, 1H), 1.31–1.35 (t, 3H, J = 6.8 Hz), 1.19–1.24 (t, 3H, J = 6.8 Hz); 13C NMR (125 MHz, CDCl₃) δ 156.41, 135.67, 128.82, 124.33, 122.63, 113.16, 77.04, 64.66, 64.57, 60.26, 57.73, 17.91, 17.80.

(13). Compound 12 (19351 mg, 60 mmol, 1.0 equiv) was dissolved in 30 mL anhydrous THF in a dry flask under N₂ and cooled to −78°C by adding dry ice to acetone. 2-Adamantanone (11174 mg, 72.0 mmol, 1.2 equiv) was dissolved in 30 mL anhydrous THF and cooled to −78 °C. 30.0 mL 1.6 M n-BuLi was added to the compound 12 solvent at −78 °C and stirred for 20 min. The cooled 2-adamantanone solution was added to the flask and the temperature of the reactants was raised to 30 °C. The mixture was stirred for 2 hours and the temperature was raised to 90 °C under reflux. After 1 hour reflux, the reaction was quenched by 100 mL NH₄Cl and extracted by 3 x 100 mL EtOAc. Purification by chromatography (1:20 EtOAc/Hexane) yielded compound 13 as a colorless
oil (17320 mg, 91%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.25 (t, 1H, \(J = 7.6\) Hz), 6.89–6.94 (m, 2H), 3.95 (s, 3H), 3.34 (s, 3H), 3.32 (s, 1H), 1.62–2.10 (m, 12H); \(^1^3\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 155.28, 139.91, 136.12, 131.17, 126.80, 124.09, 123.16, 111.24, 57.05, 56.27, 47.06, 39.35, 38.71, 37.24, 37.06, 34.68, 33.07, 32.91, 29.67.

\(\text{(14). Compound 13 (4662 mg, 14.6 mmol, 1.0 equiv) was dissolved in 35.0 mL anhydrous DMF at room temperature in a dry flask under N}_2\). Cs\(_2\)CO\(_3\) (5781 mg, 17.6 mmol, 1.2 equiv) and EtSNa (1496 mg, 17.6 mmol, 1.2 equiv) was added to the flask. The temperature of the mixture was raised to 90 °C under reflux for 18 hours. The reaction was quenched by 200 mL of NH\(_4\)Cl, extracted by 3 x 200 mL. The mixture was washed with 3 x 200 mL brine to remove the extra DMF and the organic layer was dried over Na\(_2\)SO\(_4\) and evaporated under reduced pressure. Purification by column chromatography (1:19 EtOAc:Hexane) yielded compound 14 as a white solid (3657 mg, 82%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.14 (t, 1H, \(J = 8\) Hz), 6.98 (dd, 1H, \(J_1 = 10.0\) Hz, \(J_2 = 2.5\) Hz), 6.82 (dd, 1H, \(J_1 = 7.5\) Hz, \(J_2 = 1.15\) Hz), 3.30 (s, 3H), 3.27 (s, 1H), 1.70–1.93 (m, 12H); \(^1^3\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 151.79, 139.79, 135.06, 131.91, 127.38, 123.96, 120.64, 115.54, 57.16, 47.04, 39.35, 38.71, 37.18, 36.37, 34.61, 33.24, 32.89, 29.72.

\(\text{(15). Compound 14 (4584 mg, 15.0 mmol, 1 equiv) was dissolved with 70 mL toluene at \(-5\) °C. The NIS (3391 mg, 15.0 mmol, 1 equiv) was added to the solvent later and the mixture was stirred at 0 °C for 50 mins. The mixture was washed with 100 mL brine to quench the reaction and extract with 3 x 200 mL EtOAc. Several pieces of Na\(_2\)S\(_2\)O\(_3\) crystals were added to the mixture to remove the extra iodine. The color of the mixture was changing during the work-up. It turned from red to brown, later to orange and finally the organic layer ended up with light yellow. The organic layer was later dried through Na\(_2\)SO\(_4\) and the solvent was removed under reduced pressure. A pale dark yellow oil was obtained and purified by washed with pure Hexane. A white solid will be obtained}
after wash (3818 mg, 59%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.60 (d, 1H, $J = 8$ Hz), 6.09 (d, 1H, $J = 8.0$ Hz), 3.30 (s, 3H), 3.25 (s, 1H), 2.08 (s, 1H), 1.72–1.94 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 177.03, 151.19, 144.48, 139.23, 136.78, 135.77, 132.69, 130.38, 127.38, 125.28, 120.07, 99.03, 82.23, 57.31, 37.11, 32.93, 29.66, 28.40.

(16). Compound 15 (300 mg, 0.7 mmol, 1 equiv), and Pd(OAc)$_2$ (7.8 mg, 0.04 mmol, 0.05 equiv) was added to the microwave reaction tube and fill the tube with N$_2$. Dessolve the mixture with 1.0 mL MeCN. Later TEA (0.15 mL, 1.0 mmol, 1.5 equiv) was added to the tube and all the solid was dissolving upon the addition. Acrylonitrile (0.14 mL, 2.1 mmol, 3 equiv) was added to the mixture later. The tube was put into the microwave and set the temperature to 120 °C for 70 mins. When the reactants cooled down to the room temperature, add 30 mL saturated NH$_4$Cl to it. The 3 x 30 mL EtOAc was added to the mixture to extract the product. The organic layer was later dried through Na$_2$SO$_4$ and the solvent was removed under reduced pressure. A brown oil was obtained after the evaporation. Purification of the product is through the column chromatography with pure DCM and a light yellow solid was obtained. (101 mg, 41%). $^1$H NMR (400 MHz, CDCl$_3$), $\delta$ 7.60 (d, 1H, $J = 16$ Hz), 7.30 (d, 1H, $J = 8$ Hz), 6.91 (d, 1H, $J = 4$ Hz), 6.38 (s, 1H), 6.20 (s, 1H, 1H, $J = 16$ Hz), 3.33 (s, 3H), 3.29 (s, 1H), 2.12 (s, 1H), 1.98–1.78 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 150.45, 145.26, 139.08, 137.43, 133.36, 126.71, 123.74, 120.96, 118.51, 98.59, 57.38, 39.15, 39.01, 38.84, 38.71, 38.57, 36.93, 32.87, 29.69, 28.19.

(17). Compound 16 (101 mg, 0.3 mmol, 1.0 equiv) and Rose Bengal (22.2 mg, 0.02 mmol, 0.07 equiv) were added into a dry two-neck flask and dissolved in 5 mL THF. O$_2$ was bubbled through the solvent while illuminated with a 120W light bulb (Home Depot, Dallas, TX) at 0–5°C. Monitor the reaction by TLC. After 1 hour, the mixture was concentrated at 0 °C. Purification by silica column chromatography (1:19 EtOAc/Hexane) delivered compound 17 as a light yellow solid (71.8 mg, 65 %). $^1$H NMR (400 MHz, CDCl$_3$), $\delta$ 7.73 (d, 1H, $J = 4$ Hz), 7.63 (d, 1H, $J = 16$ Hz),
7.45 (d, 1H, \(J = 4\) Hz), 6.59 (s, 1H), 6.26 (d, 1H, \(J = 16\) Hz), 3.24 (s, 3H), 3.04 (s, 1H), 2.05 (s, 1H), 1.89–1.59 (m, 12H). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 150.69, 144.77, 134.74, 126.82, 124.84, 122.80, 118.18, 111.40, 99.99, 96.28, 49.68, 36.42, 34.01, 33.42, 32.79, 32.08, 31.49, 26.04, 25.70.\(^{54}\)

2-Chloro-6-(((\(E\))-2-cyanovinyl)-3-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-(1,2)dioxetan]-4'-yl)phenyl 2-(diphenylphosphaneyl)benzoate (HNOCL-1). The compound 2-(diphenylphosphino) benzoic acid (12.4 mg, 0.039 mmol, 1 equiv) was added to a dry, N\(_2\) filled flask and dissolved with 5.0 mL CH\(_2\)Cl\(_2\). Dimethylaminopyridine (6.0 mg, 0.048 mmol, 1.25 equiv) and \(N\)-(3-dimethylaminopropyl)-\(N'\)-ethylcarbodiimide hydrochloride (8.0 mg, 0.039 mmol, 1.0 equiv) were added to the flask. The mixture was stirred at room temperature for 5 min. The compound 17 (15.0 mg, 0.039 mmol, 1.0 equiv) was dissolved in 2.0 mL CH\(_2\)Cl\(_2\) and added to the mixture. The reaction was stirred at ambient temperature for 2 hr. The reaction was quenched by 30 mL saturated NH\(_4\)Cl and extracted with 3 x 30 mL CH\(_2\)Cl\(_2\). The organic layer was dried with Na\(_2\)SO\(_4\), filtered and concentrated. Purification by silica column chromatography (10:1 Hexane:EtOAc) yielded HNOCL-1 (12.9 mg, 49\%) as a white solid. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.39–8.38 (m, 1H),) 7.94 (d, 1H, \(J = 8.5\) Hz), 7.46–7.44 (m, 4H), 7.28–7.18 (m, 10H), 6.96–6.95 (m, 1H), 5.85 (d, 1H, \(J = 17.1\) Hz), 3.06 (s, 3H), 2.91 (s, 1H), 1.85–1.35 (m, 13H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 146.48, 143.08, 137.04, 136.96, 136.00, 134.54, 134.23, 134.05, 133.98, 133.91, 133.82, 133.66, 133.51, 132.10, 130.63, 130.04, 129.02, 128.64, 128.59, 128.46, 127.33, 124.09, 117.29, 111.39, 100.86, 96.36, 49.80, 39.22, 36.49, 33.80, 33.54, 32.09, 31.54, 31.47, 30.92, 26.08, 25.63; HRMS calcd for C\(_{40}\)H\(_{25}\)NO\(_2\)PCl [M+Na]\(^+\) 698.1834 m/z, found 698.1854.

Ethyl 2-((3'-hydroxy-3-oxo-3H-spiro [isobenzofuran-1,9'-xanthen]-6'-yl)oxy)acetate (3).

Fluorescein (600 mg, 1.81 mmol, 1.0 equiv) was dissolved in 5 mL of DMF. DIPEA (0.94 mL, 5.4 mmol, 3.0 equiv) was added and the reaction mixture was stirred for five minutes. Ethyl bromoacetate (0.40 mL, 3.6 mmol, 2.0 equiv) was added. The reaction was stirred at rt for 5 h. The reaction mixture was poured into a separatory funnel containing 100 mL of CH₂Cl₂, washed with 85 mL H₂O and 40 mL of brine. The organic layer was dried over sodium sulfate, filtered, and concentrated. The resulting product was purified by silica gel column chromatography (1:1 Hexanes:EtOAc) to afford the ethyl acetate fluorescein 3 (182 mg, 24% yield). ¹H NMR (500 MHz, CDCl₃) δ 8.03 (d, 1H, J = 6.3 Hz), 7.67 (td, 1H, J = 6.3, 1.2 Hz), 7.62 (t, 1H, J = 7.4 Hz), 7.16 (d, 1H, J = 7.4 Hz), 6.75–6.25 (m, 5H), 6.53 (dd, 1H, J = 8.6, 2.9 Hz), 5.21 (s, 1H), 4.65 (s, 2H), 4.29 (q, 2H, J = 6.8 Hz), 1.31 (t, 3H, J = 6.7 Hz); ¹³C NMR (125 MHz, CDCl₃) δ 169.95, 168.61, 159.47, 157.97, 153.17, 152.47, 135.24, 129.86, 129.43, 126.76, 125.13, 124.01, 112.47, 112.37, 111.77, 103.22, 102.06, 65.48, 61.75, 14.25; HRMS calcd for C₂₄H₁₈O₇ [M+Na]⁺ 441.0945, found 441.0928.
3′-(2-Ethoxy-2-oxoethoxy)-3-oxo-3H-spiro[isobenzofuran-1,9′-xanthen]-6′-yl 2-(diphenylphosphanyl) benzoate (XF2). 2-(diphenylphosphino)benzoic acid (74.7 mg, 0.244 mmol, 1.0 equiv) and HBTU (111 mg, 0.293 mmol, 1.2 equiv) were dissolved in 5 mL of DMF. DIPEA (0.190 mL, 1.01 mmol, 4.1 equiv) was added and the reaction was stirred for 5 min. Compound 3 (99.3 mg, 0.243 mmol, 1.0 equiv) was added and the reaction was stirred at rt for 2.5 h. The reaction mixture was poured into a separatory funnel containing 50 mL of CH$_2$Cl$_2$, washed with 3 x 50 mL H$_2$O and 50 mL of brine. The organic layer was dried over sodium sulfate, filtered, and concentrated. The resulting product was purified by silica gel column chromatography (3:2 Hexanes:EtOAc) to afford XF2 (37.3 mg, 29% yield) as a clear oil. $^1$H NMR (500 MHz, CDCl$_3$) δ 8.26–824 (m, 1H), 8.02 (d, 1H, $J = 7.5$ Hz), 7.67 (td, 1H, $J = 7.5$, 1.2 Hz), 7.62 (t, 1H, $J = 7.5$ Hz), 7.48–7.36 (m, 2H), 7.37–7.26 (m, 10H), 7.14 (d, 1H, $J = 7.4$ Hz), 7.01–6.98 (m, 1H), 6.96 (d, 1H, $J = 2.3$ Hz), 6.76–6.61 (m, 5H), 4.64 (s, 2H), 4.29 (q, 2H, $J = 6.9$ Hz), 1.31 (t, 3H, $J = 6.9$ Hz); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 169.28, 168.30, 164.70, 159.49, 152.95, 152.12, 151.77 151.64, 141.70, 141.47, 137.44, 137.36, 135.15, 134.49, 134.08, 133.90, 133.02, 132.76, 131.40, 129.87, 129.27, 128.89, 128.84, 128.61, 128.55, 128.35, 126.34, 125.08, 124.06, 117.60, 116.57, 112.02, 110.47, 101.90, 82.23, 65.42, 61.61, 14.16; HRMS calcd for C$_{43}$H$_{31}$O$_8$P [M+H]$^+$ 707.1829, found 707.1831.

3.4.2 Chemiluminescence response of HNOCL-1.

Chemiluminescence responses and time scans were acquired with a Hitachi F-7000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan, Figures 3-1 A, 3-1, 3-3 B) using the luminescence detection module or a Cytation 5 BioTek plate reader (Winooski, VT, Figures 3-1 B, 3-3 A, 3-4 A, 3-6 A, 3-7 A) using the luminescence detection method, endpoint read type, and setting gain to 135 and operating at ambient temperature (25–28 °C). 20 mM PBS buffer (pH 7.4), 5 mM HNOCL-1 stock solution in DMSO (20 µM final concentration) and Angeli’s salt were added to a quartz cuvette (Starna, Atascadero, CA) or multi-well plate in sequence. Samples were shaken
gently to assure mixing. For spectral scans, 20 μM HNOCL-1 was treated with 0, 50, 100, 150
and 200 μM Angeli’s salt and measured in a fluorescence spectrometer. Time scans were acquired
using the plate reader with 0, 5, 25, 50, and 100 μM Angeli’s salt. The time between adding
Angeli’s salt and beginning the measurement was recorded and was between 20 and 100 seconds.

3.4.3 GC/MS determination of HNOCL-1 products.
A 5.1 mM stock solution of Angeli’s salt was made by dissolving Angeli’s salt in 0.01 mM NaOH,
and the final concentration was confirmed by UV/Vis by measuring the absorbance at 237 nm (ε
= 6100 M\(^{-1}\) cm\(^{-1}\)). 1.3 mg (0.0019 mmol) of HNOCL-1 was dissolved in 10.0 mL acetone in a 20
dram vial. 9.62 mL of 20 mM PBS buffer (pH = 7.4) and 0.38 mL of 5.1 mM Angeli’s salt solution
were added to make a final concentration of 95 μM HNOCL-1 with 95 μM Angeli’s salt. A stir
bar was added, and the solution was capped and stirred at room temperature for 24 hours. The
solution was poured into a separatory funnel, washed with brine, and the organic layer was
extracted with 3 x 15 mL EtOAc. The organic layer was evaporated under reduced pressure, and
the leftover contents were dissolved in 1.5 mL CH\(_2\)Cl\(_2\) and pipetted into a GC/MS vial. GC/MS
was conducted immediately using a 6850 Series GC/MS (Agilent Technologies, Santa Clara, CA).
Mass spectra were averaged across the major peaks in the chromatogram and molecular ions for
m/z = 150.1 (Figure 3-9) and m/z = 304.1 (Figure 3-10) were found. The spectrum of 2-
adamantanone was matched against the NIST standard, which can be found via web at:
https://webbook.nist.gov/cgi/cbook.cgi?ID=C700583&Units=CAL&Mask=200#Mass-Spec,
accessed September 26, 2018.
Figure 3-9. Mass spectrum of the peak assigned to adamantane. Inset is the NIST standard.

Figure 3-10. Mass spectrum of the peak assigned to the acyl iminophosphorane.
3.4.4 Solubility and stability studies.
Solutions of 20, 40, 60, 80, and 100 µM HNOCL-1 were prepared in 20 mM PBS (pH = 7.4) containing 1% DMSO. The precipitation was evaluated by measuring the raise in the baseline absorbance at 750 nm (Figure 3-11). In order to evaluate stability, 20 µM HNOCL-1 was pre-incubated in 20 mM PBS (pH 7.4) containing ≤ 1% DMSO for 0, 60, or 120 minutes before treating with 200 µM Angeli's salt. The time-course of the chemiluminescence emission was measured using a Cytation 5 BioTek plate reader (Figure 3-12).

**Figure 3-11.** Solubility of HNOCL-1. (A) Photographs of cuvettes containing 20, 60, and 100 µM HNOCL-1 in 20 mM PBS buffer containing ≤ 1% DMSO. (B) Absorbance at 750 nm of 20–100 µM HNOCL-1 in 20 mM PBS buffer containing ≤ 1% DMSO. Error bars are ± S.D. from n = 3 replicates.

**Figure 3-12.** Stability of HNOCL-1. 20 µM HNOCL-1 was pre-incubated in 20 mM PBS (pH 7.4) containing ≤ 1% DMSO for 0 (blue trace), 60 (black trace), or 120 (red trace) minutes before treating with 200 µM Angeli’s salt. Error bars are ± S.D. from n = 3 replicates

3.4.5 Determination of the detection limit for HNOCL-1
Chemiluminescence 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 30 °C. A 0.3016 mM stock solution of Angeli’s salt was made by dissolving Angeli’s salt with 0.01 mM NaOH, and the final concentration was confirmed by UV/Vis by measuring the absorbance at 237 nm ($\varepsilon = 6100 \text{ M}^{-1} \text{ cm}^{-1}$). An aliquot of 1 µL of a 5 mM HNOCL-1 stock solution (final concentration of 20 µM) was added to each well, filled with the appropriate volume of 20 mM PBS buffer for a final volume of 250 µL in each well. Finally, Angeli's salt was added to each well to give final concentrations of 0, 0.25, 0.5, 1, 1.5, and 2 µM Angeli's salt. The emission was integrated over 20 minutes and the calibration curve was constructed using experiments replicated on two different days, for a total of 6 replicates for each data point (3 replicates for 0.25 and 2 µM). The detection limit (3σ/k) was determined as 138 nM, which, according to kinetic simulations (see section 4. Kinetics), generates concentrations of less than 50 pM HNO.

![Graph A](image1)

**Figure 3-13.** Determination of detection limit. (A) Chemiluminescence emission intensity of 20 µM HNOCL-1 and 0 (blue trace), 0.25, 0.5, 1, 1.5, and 2 µM (red trace) Angeli's salt. (B) Calibration plot of integrated emission intensity versus [AS]. Error bars are ± S.D. from n = 3–6 replicates.

### 3.4.6 Response and selectivity for XF2

![Graph B](image2)
Figure 3-14. (A) Fluorescence response of 10 µM XF2 to 200 µM Angelis' salt. Time points represent 0, 1, 5, 10, 15, 20, and 25 min after addition of 200 µM Angelis' salt. (B) Fluorescence response of 10 µM XF2 to 200 µM biologically relevant reactive oxygen and nitrogen species. Emission was collected from 508 and 650 nm. Legend: (1) AS; (2) H2S; (3) ONOO−; (4) NaOCl; (5) H2O2; (6) GSH (5 mM); (7) GSNO; (8) DEA NONOate; (9) tBuOOH. Data were acquired in 20 mM HEPES buffer (pH 7.4) at 21 °C with excitation at λex = 488 nm. Bars represent fluorescent emission at 508 nm, and 1, 5, 10, 15, 20, 25, and 30 minutes after addition of the analyte. Error bars are ± S.D. from n = 3 replicates.

Selectivity for XF2 was performed using an F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) and monitored every 5 minutes for 30 minutes. UV/Vis measurements were acquired on a DU-800 spectrophotometer (Beckman Coulter, Brea, CA). Reactions were performed as triplicates. All probe aliquots were prepared as 5 mM stock solutions in DMF and the selectivity reactions were performed in 20 mM HEPES (pH 7.4).

Blank: 2 µL of 5 mM XF2 was added to 998 µL of 20 mM HEPES (10 µM final concentration).

AS: 2 µL of 5 mM or XF2 was added to 978 µL of 20 mM HEPES (10 µM final concentration), and 20 µL of 10 mM AS (confirmed by UV-Vis) was added to the mixture (200 µM final concentration).

H2S: 2 µL of 5 mM XF2 was added to 978 µL of 20 mM HEPES (10 µM final concentration), and 20 µL of 10 mM Na2S in H2O was added to the mixture (200 µM final concentration).

NaOCl: 2 µL of 5 mM XF2 was added to 978 µL of 20 mM HEPES (10 µM final concentration), and 20 µL of 10 mM NaOCl in H2O was added to the mixture (200 µM final concentration).

H2O2: 2 µL of 5 mM XF2 was added to 978 µL of 20 mM HEPES (10 µM final concentration), and 20 µL of 10 mM H2O2 was added to the mixture (200 µM final concentration).

Glutathione: 2 µL of 5 mM XF2 was added to 898 µL of 20 mM HEPES (10 µM final concentration), and 100 µL of 50 mM glutathione was added to the mixture to make final concentration of 5 mM for glutathione.
S-nitrosoglutathione: S-nitrosoglutathione (GSNO) was synthesized and characterized using a literature procedure.\textsuperscript{48} Approximately 1 mg of GSNO was dissolved in 1 mL DI H\textsubscript{2}O, and the precise concentration was measured by UV/Vis using the absorptions at 335 nm ($\varepsilon = 922$ M\textsuperscript{-1} cm\textsuperscript{-1}) or 545 nm ($\varepsilon = 15.9$ M\textsuperscript{-1} cm\textsuperscript{-1}). The solution was made on a daily basis and kept at 0 °C. 2 μL of 5 mM XF\textsubscript{2} was added to 982.1 μL of 20 mM HEPES (10 µM final concentration), and 15.9 μL of 12.58 mM S-nitrosoglutathione was added to the mixture (200 µM final concentration).

ONOO\textsuperscript{–}: ONOO\textsuperscript{–} was synthesized based on an adaptation of a literature procedure,\textsuperscript{49} by adding 0.24 mL of H\textsubscript{2}O\textsubscript{2} (35% wt. in H\textsubscript{2}O, 2.8 mmol, 1.4 equiv) to a round bottom flask containing 4.5 mL of 0.55 M NaOH and 5 mL isopropyl alcohol. Isoamyl nitrite (0.27 mL, 2.0 mmol, 1.0 equiv) was added, and stirred at rt for 15 minutes. MnO\textsubscript{2} (10 mg, 0.12 mmol) was added to the reaction and stirred for extra 5 minutes to decompose excess H\textsubscript{2}O\textsubscript{2}. The reaction mixture was filtered to remove MnO\textsubscript{2} and washed four times with 10 mL of DCM in a separatory funnel. The aqueous layer was removed with a Pasteur pipet to avoid having any DCM. The concentration was determined by UV/Vis using the absorption at 302 nm ($\varepsilon = 1670$ M\textsuperscript{-1} cm\textsuperscript{-1}), and was diluted to make a 10 mM stock solution. 2 μL of 5 mM XF\textsubscript{2} was added to 978 μL of 20 mM HEPES (10 µM final concentration), and 20 μL of 10 mM ONOO\textsuperscript{–} was added to the mixture (200 µM final concentration).

DEA NONOate: A stock solution of DEA NONOate (Cayman Chemical, Ann Arbor, Michigan) was prepared in 0.01 M NaOH and the precise concentration was measure by UV/Vis using the absorption at 250 nm ($\varepsilon = 6500$ M\textsuperscript{-1} cm\textsuperscript{-1}), and diluted to a final concentration of 10 mM. 2 μL of 5 mM XF\textsubscript{2} was added to 978 μL of 20 mM HEPES (10 µM final concentration), and 20 μL of 10 mM DEA NONOate was added to the mixture (200 µM final concentration).

tBuOOH: 2 μL of 5 mM XF\textsubscript{2} was added to 978 μL of 20 mM HEPES (10 µM final concentration), and 20 μL of 10 mM tBuOOH was added to the mixture (200 µM final concentration).
3.4.7 Kinetics

**General methods.** Chemiluminescent responses were measured using a Cytation 5 BioTek plate reader (Winooski, VT) using the luminescence detection mode, end point read type. Measurements were made at ambient temperature (25–28 °C), the gain was set at 135, and the height was set at 4.5 mm.

**Derivation of equation (1).** The reactions between HNOCL-1 and HNO used to model triggered chemiluminescence are shown in Scheme 3-8 and the derivation of equation (1) is given in reactions (2) – (4) and equations (5) – (12).

![Scheme 3-7](image)

**Scheme 3 - 7.** Reactions for chemiluminescence emission of HNOCL-1.

\[
[HNOCL-1] + [HNO] \xrightarrow{k_1} [2] \tag{2}
\]

\[
[2] \xrightarrow{k_2} [1] \tag{3}
\]

\[
[1] \xrightarrow{k_3} h\nu + \text{products} \tag{4}
\]

\[
d[HNO]/dt = -k_4[HNOCL-1][HNO] \tag{5}
\]

\[
d[2]/dt = k_1[HNOCL-1][HNO] - k_2[2] \tag{6}
\]

\[
d[1]/dt = k_2[2] - k_3[1] \tag{7}
\]

\[
[HNO] = \frac{1}{(k_1[HNOCL-1])} \left( k_2[2] + d[2]/dt \right) \tag{8}
\]

\[
[2] = \frac{1}{k_2} (k_3[1] + d[1]/dt) \tag{9}
\]

\[
d[2]/dt = \frac{1}{k_2} (k_3d[1]/dt + d^2[1]/dt^2) \tag{10}
\]

\[
[HNO] = \frac{1}{(k_1[HNOCL-1])} \left( k_3[1] + d[1]/dt + \frac{1}{k_2} (k_3d[1]/dt + d^2[1]/dt^2) \right) \tag{11}
\]
The reaction between the triaryl phosphine in HNOCL-1 and HNO forms an azaylide intermediate 2 in reaction (2) with a rate defined by equation (6). While it takes two equivalents of probe to form product, we use an observed second order rate constant, $k_1$, determined as described below. Since the concentration of probe is constant throughout all experiments in this manuscript, the observed second order rate constant will be consistent with this model. The nucleophilic nitrogen of 2 mediates ester cleavage to form the phenol 1 in the intramolecular reaction (3) with a rate defined by equation (7). Finally, 1 decomposes via a chemically initiated electron exchange luminescence (CIEEL) mechanism in reaction (4) with a rate also defined by equation (7).

Equation (6) can be reorganized into equation (8) to provide [HNO] in terms of [2] and $d[2]/dt$ and equation (7) can be reorganized into equation (9) to provide [2] in terms of [1] and $d[1]/dt$, which is differentiated to give equation (10). Substitution of equations (9) and (10) into equation (8) provides equation (11), which can be reorganized into equation (12). Here, we make two key assumptions:

1. If $k_3 \ll k_2$ (this is true if the chemiluminescent decomposition is rate-limiting) and the second derivative $d^2[1]/dt^2$ is small, then equation (12) simplifies to equation (1).

2. [HNOCL-1] >> [HNO], and so [HNOCL-1] can be approximated as constant over the course of the reaction.

In order to use equation (1) to obtain [HNO] from the relative chemiluminescent emission we need to know $k_1$, $k_3$, [1], and $d[1]/dt$.

**Measurement of $k_1$.** The value of $k_1$ was measured by a method similar to that of Miranda and Wink by looking at the chemiluminescence response of HNOCL-1 with increasing concentrations of Angeli’s salt. Due to instrumental limitations, experiments were performed under aerobic conditions, so the relevant rate is defined by equation (13), where $k_D$ is the rate of HNO dimerization, $k_1$ is the rate of reaction of the probe with HNO, $k_{O2}$ is the rate of reaction of O$_2$ with HNO, and $k_{AS}$ is the rate of the decomposition of Angeli’s salt (AS).

$$d[HNO]/dt = -2k_D[HNO]^2 - k_1[HNOCL-1][HNO] - k_{O2}[O_2][HNO] + k_{AS}[AS] \quad (13)$$
Using the steady state approximation and applying the quadratic equation gives:

$$[\text{HNO}] = \frac{(k_1[H\text{NOCL-1}] + k_{O2}[O_2]) \pm ((k_1[H\text{NOCL-1}] + k_{O2}[O_2])^2 - 4(-2k_D)k_{AS}[AS])^{1/2}}{2(-2k_D)}$$  \hspace{1cm} (14)

As the concentration of Angeli's salt is increased, dimerization is the main competitive reaction. At the concentration of Angeli's salt that gives 50% product formation versus competitive HNO dimerization, the rates are equal and equation (15) holds as derived by Miranda and Wink.\(^5\)

$$k_1 = -2k_D \frac{[\text{HNO}]}{[H\text{NOCL-1}]}$$ \hspace{1cm} (15)

Substitution into equation (14) gives equation (16), which simplifies (after reorganization and application of the quadratic equation) to equation (17).

$$k_1 = \frac{2k_D}{[H\text{NOCL-1}]} \ast \frac{(k_1[H\text{NOCL-1}] + k_{O2}[O_2]) \pm ((k_1[H\text{NOCL-1}] + k_{O2}[O_2])^2 - 4(-2k_D)k_{AS}[AS])^{1/2}}{2(-2k_D)}$$  \hspace{1cm} (16)

$$k_1 = -\frac{([O_2]k_{O2}) \pm ([O_2]k_{O2})^2 + 16k_Dk_{AS}[AS])^{1/2}}{4[H\text{NOCL-1}]}$$ \hspace{1cm} (17)

The concentration of Angeli's salt needed for 50% product formation was found to be 0.148 mM from the experiment shown in Figure 3-15.

![Figure 3-15](image)

**Figure 3-15.** Initial value of the relative emission intensity of 20 \(\mu\)M \(H\text{NOCL-1}\) and 0, 0.2, 0.5, 1, 2, and 4 mM Angeli's salt in pH 7.4 PBS buffer at 25 °C. Error bars are ± S.D. from \(n = 3\) replicates.

Other values were used as reported in the literature:

- \(k_{O2} = 18,000 \text{ M}^{-1}\text{s}^{-1}\) at 25 °C\(^50\)
- \([O_2] = 225 \mu\text{M}\)
- \(k_{AS} = 0.00084 \text{ s}^{-1}\) at 25 °C\(^50\)
- \(k_D = 8,000,000 \text{ M}^{-1}\text{s}^{-1}\) at 23 °C\(^51\)
Substituting these values into equation (17) gives a rate constant $k_1 = 20,433 \text{ M}^{-1} \text{s}^{-1}$ at 25 °C and pH 7.4.

**Measurement of $k_3$ and calibration of [1].** The rate of chemiluminescent decomposition, $k_3$, was determined from monitoring the chemiluminescence emission of isolated 1 over time to yield a value of $k_3 = 5.92 \pm 0.32 \times 10^{-4} \text{ s}^{-1}$ at 25 °C and pH 7.4 (Figure 3-16, A). The rate was independent of the concentration of 1, and this reported value is the average of the value determined at different concentrations of 1. Plotting the initial value of the chemiluminescence emission versus the concentration of 1 provided a linear calibration plot to convert the relative emission intensity into [1]. The calibration was performed on four separate days and the average values were used to construct the plot in Figure 3-16, B.

**Figure 3-16.** (A) Time course of the chemiluminescence emission of 0 (blue trace), 0.5, 1, 1.5, 2, 2.5, and 3 µM (red trace) 1. (B) Calibration plot of the initial chemiluminescence emission intensity versus concentration of 1. Error bars are ± S.D. from n = 4 replicates.

**Example of converting relative chemiluminescence emission into [HNO].** The raw chemiluminescence emission (Figure 3-17, A) was converted into a plot of [1] using the linear calibration plot in Figure 3-16, B, and the derivative at each point was determined by computing the slope (Figure 3-17, C). In order to reduce noise, [1] was averaged over 30 seconds (2 data points). The concentration of [HNO] was determined using equation (1) and is represented by the black data points in Figure 3-17, D. The simulation was computed by numerical solution of equation (20), derived from equation (13), and is given as the red trace in Figure 3-17, D.
The rate of decomposition of Angeli’s salt follows equation (18), which can be solved analytically to provide equation (19), where \([\text{AS}]_0\) is the initial concentration of Angeli’s salt. Substitution into equation (13) provides equation (20), which was solved numerically using Wolfram Mathematica version 11. Analogous methods were used to generate the plots in Figure 3-5.

\[
\frac{d[\text{HNO}]}{dt} = -2k_0[\text{HNO}]^2 - k_1[\text{HNOCL-1}][\text{HNO}] - k_O[\text{O}_2][\text{HNO}] + k_{AS}[\text{AS}]
\]  

(13)

The rate of decomposition of Angeli’s salt follows equation (18), which can be solved analytically to provide equation (19), where \([\text{AS}]_0\) is the initial concentration of Angeli’s salt. Substitution into equation (13) provides equation (20), which was solved numerically using Wolfram Mathematica version 11. Analogous methods were used to generate the plots in Figure 3-5.

\[
\frac{d[\text{AS}]}{dt} = -k_{AS}[\text{AS}]
\]  

(18)

\[
[\text{AS}] = [\text{AS}]_0e^{-k_{AS}t}
\]  

(19)

\[
\frac{d[\text{HNO}]}{dt} = -2k_0[\text{HNO}]^2 - k_1[\text{HNOCL-1}][\text{HNO}] - k_O[\text{O}_2][\text{HNO}] + k_{AS}[\text{AS}]_0e^{-k_{AS}t}
\]  

(20)

Figure 3-17. Quantification of HNO concentration. (A) Time-course of the chemiluminescence emission intensity of 20 μM \text{HNOCL-1} and 50 μM Angeli’s salt. (B) Conversion from relative emission intensity to \([1]\) using the calibration curve in Figure 3-16, B. (C) Time-course of the derivative determined from the plot in Figure 3-17, B. (D) HNO concentration measured using equation 1 (black data points) and simulated by numerically solving equation 20 (red trace).

3.4.8 Selectivity tests for \text{HNOCL-1}

Selectivity for \text{HNOCL-1} was measured by monitoring the time-dependent chemiluminescent emission at 525 nm with an F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). All assays were performed in 20 mM HEPES buffered to pH 7.4, except for \text{HO}^\cdot, which was performed in 20 mM PBS buffered to pH 7.4. All analytes were tested with final concentration of 200 μM,
with the exception of glutathione (2 mM) and L-cysteine (1 mM).

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/Vis is using $\varepsilon = 6100 \text{ M}^{-1} \text{ cm}^{-1}$ at 237 nm. 13.6 μL of a 14.7 mM stock solution of Angeli’s salt in 0.01 M NaOH was added to a 986 μL solution of 20 μM HNOCL-1 (from 4 μL of a 5 mM stock solution) in HEPES buffer.

ONOO$^-$ (200 μM): 7 μL of 28.5 mM ONOO$^-$ was added to a solution of 989 μL HEPES buffer and 4 μL of 5 mM HNOCL-1 (20 μM final concentration) in DMSO.

NO$_2^-$ (200 μM): 4 μL of 50 mM NaNO$_2$ in DI-H$_2$O was added to a solution of 992 μL HEPES buffer and 4 μL of 5 mM HNOCL-1 (20 μM final concentration) in DMSO.

L-Cysteine (1 mM): 10 μL of 100 mM L-cysteine and 4 μL of 5 mM HNOCL-1 (20 μM final concentration) in DMSO were added to 986 μL HEPES buffer.

GSNO (200 μM): 4 μL of 50 mM GSNO in DI-H$_2$O was added to a solution of 992 μL HEPES buffer and 4 μL of 5 mM HNOCL-1 (20 μM final concentration) in DMSO.

GSH (2 mM): 20 μL of 100 mM GSH in DI-H$_2$O was added to a solution of 976 μL HEPES buffer and 4 μL of 5 mM HNOCL-1 (20 μM final concentration) in DMSO.

H$_2$O$_2$ (200 μM): 4 μL of 50 mM H$_2$O$_2$ in DI-H$_2$O was added to a solution of 992 μL HEPES buffer and 4 μL of 5 mM HNOCL-1 (20 μM final concentration) in DMSO.

‘BuOOH (200 μM): 4 μL of 50 mM ‘BuOOH in DI-H$_2$O was added to a solution of 992 μL HEPES buffer and 4 μL of 5 mM HNOCL-1 (20 μM final concentration) in DMSO.
O$_2^-$ (200 µM): 280 µL of 5 mM HNOCL-1 stock solution (20 µM final concentration) in DMSO was added to 70 mL HEPES buffer, later 1.0 mg KO$_2$ (200 µM final concentration) was added to the mixture.

Na$_2$S (200 µM): 4 µL of 50 mM Na$_2$S in DI-H$_2$O was added to a solution of 992 µL HEPES buffer and 4 µL of 5 mM HNOCL-1 in DMSO (20 µM final concentration).

HO’ (200 µM): 4 µL of 5 mM HNOCL-1 stock solution was added to 988 µL 20 mM PBS buffer (pH = 7.4). Later 4 µL 50 mM Fe(ClO$_4$)$_2$ and 4 µL of 50 mM H$_2$O$_2$ were added to the mixture.

NO (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 M$^{-1}$ cm$^{-1}$ at 250 nm. 2.51 µL of 79.7 mM DEA NONOate in 0.01 M NaOH solution was added to a 993 µL solution of 20 µM HNOCL-1 in HEPES buffer.

OCl$^-$ (200 µM): 4 µL of 50 mM NaOCl in DI-H$_2$O was added to a solution of 992 µL HEPES and 4 µL of 5 mM HNOCL-1 in DMSO (20 µM final concentration).

Blank: 4 µL of 5 mM HNOCL-1 stock solution was added to 996 µL HEPES.

3.4.9 Cell culture and biological studies

Macrophages (RAW 264.7) were purchased from Sigma-Aldrich 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). Human lung adenocarcinoma epithelial cells (A549) cells were purchased from ATCC and cultured in Ham’s F-12K (Kaighn’s) Medium 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% CO$_2$. One or two days before the experiment, cells were passaged and plated on Costar® 12-well plates by adding 150K–200K of cells per well, filling each well up to 1 mL of media. Chemiluminescent responses were measured using a Cytation 5 BioTek plate reader (Winooski, VT). Fluorescent imaging was
conducted using an EVOS-fl fluorescent microscope (Advanced Microscopy Group) equipped with a GFP filter cube.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Human lung adenocarcinoma epithelial cells (A549) were purchased from ATCC and were seeded in a 96-well plate to a total volume of 120 μL/well. The plate was maintained at 37 °C with 5% CO$_2$ for 12 h. The medium was removed upon reaching 70%–80% confluency and the cells were washed with PBS. The cells were incubated for 18 h after adding different concentrations of HNOCL-1 at 0, 0.1, 1, 10, 100, and 200 μM respectively in 125 μL completed F12K media. 10 μL of the MTT reagent (Cayman Chemical, Ann Arbor, MI) was 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 and cell viability was expressed as a percent of the control.

![Graph](image)

**Figure 3- 18.** A549 cells were incubated with 0–100 μM HNOCL-1 for 16 hours and then viability was evaluated using the MTT assay. Error bars are ± S.D. from n = 3 replicates.

Detection of Angeli’s salt-generated HNO in living cells. Human lung adenocarcinoma epithelial cells (A549) or RAW 264.7 macrophages were seeded in a 12-well plate to a total volume of 1 mL/well. Before imaging, the medium was removed upon 90%–95% confluency and the cells were washed with 1 mL PBS. Each well was fill with 996 μL FluoroBrite DMEM or F-12K media. Later, 4 μL of 5 mM HNOCL-1 in DMSO (20 μM final concentration) was added to each well and incubated for 30 minutes. After incubation, the medium was removed and the cells were washed with 2 x 1 mL PBS. Different volumes of 73.3 mM Angeli’s salt in 0.01 M NaOH...
(0 µL, 2.73 µL, 6.8 µL and 13.6 µL) were added to each well. The luminescence was measured with a Cytation 5 BioTek plate reader using the luminescence detection mode directly after the addition of Angeli’s Salt, end point read type. The temperature was set at 37 °C and the CO₂ was set at 5%. Each experiment consisted of three technical replicates for each condition, and each experiment was repeated with three biological replicates on three separate days. The reported integrated chemiluminescence intensity values are the average of a total of nine wells across three biological replicates.

**Figure 3-19.** (A) Time course of chemiluminescent emission and (B) integrated emission intensity of RAW 264.7 macrophages incubated with 20 µM HNOCL-1 for 30 minutes, washed and then treated with 0, 200, 500, and 1000 µM AS. Error bars are ± S.D from n = 9 wells across 3 biological replicates.

**Detection of intracellular HNO formed from NO reduction.** Human lung adenocarcinoma epithelial cells (A549) were seeded in a 12-well plate to a total volume of 1 mL/well. Before imaging, the media was removed upon 90%–95% confluency and the cells were washed with 1 mL PBS. Each well was filled with 996 µL F-12K media. 4 µL of 5 mM HNOCL-1 stock solution in DMSO was added to each well (20 µM final concentration of HNOCL-1) and incubated for 30 minutes. After incubation, the media was removed and the cells were washed with 2 x 1 mL PBS. An aliquot of 4 µL of 50 mM Na₂S in H₂O was added to the appropriate wells. Next, aliquots of 50 mM N-acetyl-L-cysteine in H₂O (2 mM final concentration) were added to the appropriate wells. Finally, aliquots of a 203.8 mM DEA NONOate (1 mM final concentration) were added to the appropriate wells. The luminescence was measured with a Cytation 5 BioTek plate reader using the luminescence detection mode, end point read type (Figure 3-19, A). The temperature was set at 37 °C under ambient atmosphere. The gain was set at 135 and the read height was set at 4.5 mm. Control values are the average of 11 technical replicates from 4 biological replicates. Other
experiments are the average of 5–6 technical replicates from 2 biological replicates. Significant outliers (2 of 36 total data points) were excluded using a Grubb's Test for Outliers with p < 0.05.

Fluorescence Microscopy with XF2. For cellular imaging with XF2, cells were passaged every 3–4 days. One day before imaging, 6-well plates (Falcon) were seeded with 300,000–500,000 cells per well. 6-well plates were incubated in 5% CO₂ and 37 °C overnight to reach ca. 80% confluence. A final concentration of 10 μM XF2 was used for all experiments. Angeli’s salt was used as a nitroxyl donor and the stock solution concentration was determined by UV/Vis prior to use. Fluorescence imaging was performed in PBS or final F12K media as described above using an EVOS-fl inverted fluorescence microscope (Advanced Microscopy Group, Bothell, WA) equipped with a GFP filter cube (470/22 nm Excitation; 510/42 nm Emission) and 40x objective. Images were analyzed in ImageJ by converting the raw image into an 8-bit image, adjusting the threshold and selecting cells using the standard ImageJ Huang⁵² or Otsu⁵³ algorithms, and measuring the mean pixel intensity in the selected areas. All quantification is from 3 fields/well with 3 biological replicates performed on different days (n = 9). The mean value for each set of conditions across all biological replicates was divided by the mean value of the controls across all biological replicates for normalization (n = 9). All images within each experiment were analyzed identically.

Evaluation of background signal. Human lung adenocarcinoma epithelial cells (A549) were seeded in a 12-well plate to a total volume of 1 mL/well. Before imaging, the medium was removed upon 60%–80% confluency and the cells were washed with 1 mL PBS. Three wells of cells were treated with 4 μL of 5 mM HNOCL-1 in DMSO (20 μM final concentration) and incubated for 30 minutes and compared to three wells of cells not treated with HNOCL-1. After incubation, the medium was removed and the cells were washed with 2 x 1 mL PBS. The luminescence was measured with a Cytation 5 BioTek plate reader using the luminescence detection mode, end point read type. The temperature was set at 37 °C under ambient atmosphere. The gain was set at 135 and the read height was set at 4.5 mm. Data are from 3 technical replicates.
**Figure 3-20.** Evaluation of background. Time-course of the chemiluminescence emission intensity of A549 cells incubated in the presence (red trace) or absence (blue trace) of 20 μM HNOCL-1. Error bars are ± S.D. from n = 3 wells of cells.

**Figure 3-21.** Fluorescence microscopy images of HNO detection in live A549 cells using XF2. A549 cells were incubated with 10 μM XF2 for 30 min in F12K media at 37 °C and washed with PBS before adding (A) 0 µM, (B) 50 µM, or (C) 200 µM Angeli's salt in F12K media for the final 30 min. (D)–(E) Brightfield images of the field of cells in (A)–(C). (G) Quantification of the mean pixel intensity of cells of replicate experiments (n = 9; 3 biological replicates with 3 fields of cells in each replicate). (H) Comparison of the normalized fold turn-on of XF2 and HNOCL-1 upon treating cells with 200 μM Angeli's salt (dark grey bars) versus control (light grey bars). Statistical analysis was performed using a two-tailed Student's t-test. ** indicates a p-value < 0.001 with respect to the control. Error bars are ± S.D. from n = 9 fields across 3 biological replicates.
bars for HNOCL-1 are ± S.D. from n = 9 wells across 3 biological replicates. Scale bars represent 50 µm.

**Cell permeability of HNOCL-1.** Human lung adenocarcinoma epithelial cells (A549) were seeded in a 12-well plate to a total volume of 1 mL/well. Before imaging, the medium was removed upon 60%–80% confluency and the cells were washed with 1 mL PBS. Each well was filled with 996 µL F-12K media. Later, 4 µL of 5 mM HNOCL-1 in DMSO (20 µM final concentration) was added to three wells and incubated for 30 minutes. After incubation, the medium was removed and the cells were washed with 2 x 1 mL PBS. Angeli’s salt (final concentration 200 µM) was added to the wells containing HNOCL-1 and the cells were incubated for 2 hours. Imaging was performed using an EVOS-fl fluorescence microscope containing a GFP filter cube (470/22 nm Excitation; 510/42 nm Emission), 40x objective, with the light intensity set to 70% and the exposure time set to 500 ms. The fluorescence emission from cells treated with probe was compared with the autofluorescence of untreated cells. The brightness and contrast of fluorescence images were identically adjusted with a minimum pixel intensity of 27 and a maximum pixel intensity of 91.

![Figure 3-22](image_url)

**Figure 3-22.** Cell permeability. Fluorescence microscopy images of (A) autofluorescence from untreated A549 and (B) A549 cells incubated with 20 µM HNOCL-1 for 30 minutes, washed and treated with 200 µM Angeli's salt. (C)–(D) Brightfield images of the fields shown in (A)–(B).
**Imaging in Chicken Heart Tissue.** Fresh chicken (*gallus gallus domesticus*) hearts (purchased from the Good Fortune Supermarket, Richardson, TX) were stored at –4 ºC. Control hearts (Figure 3-8 A and Figure 3-12 A, top row) were injected with 20 µM HNOCL-1 in 20 mM PBS (pH 7.4) containing ≤ 1% DMSO and 11.7 µL 0.01 M NaOH as a vehicle control. HNO-treated hearts (Figure 3-8 B and Figure 3-12 A, bottom row) were injected with 20 µM HNOCL-1 in 20 mM PBS (pH 7.4) containing ≤ 1% DMSO and 11.7 µL of 85.5 mM Angeli’s salt in 0.01 M NaOH for a final concentration of 1 mM Angeli’s salt. Images were acquired every 30 seconds using a Caliper Xenogen IVIS Spectrum® (PerkinElmer, Santa Clara, CA) with a 5 second exposure time, large binning, f-stop set to 1, blocked excitation, open emission, FOV set to C, and height set to 1.5.

![Figure 3-23](image)

**Figure 3-23.** Imaging in heart tissue. (A) Images of chicken hearts injected with 20 µM HNOCL-1 and (A) vehicle control or (B) 1 mM Angeli’s salt in 20 mM PBS (pH 7.4) containing ≤ 1% DMSO (n = 3 hearts). (B) Time course of the total flux from ROIs selected around the entire hearts for the control experiment (blue trace) and after injection with Angeli’s salt (red trace). Statistical analysis was performed using a two-tailed Student's t-test. For data points at time 0 and 0.5 min, p < 0.01 with respect to the control. For data points from 1 min to 9 min, p < 0.05 with respect to the control. Error bars are ± S.E. from n = 3 hearts.

**Imaging in Living Mice.** The UT Southwestern Institutional Animal Care and Use Committee approved these investigations under Animal Protocol Number (APN #2017-102329). Mice were anesthetized and maintained using inhalation of 1.6% isoflurane. Anesthetized BALB/c nude were injected in the intraperitoneal (IP) cavity with 40 µM HNOCL-1 and either a vehicle control (0.01...
0.1 M NaOH) or 1 mM Angeli's salt in 20 mM PBS (pH 7.4), containing 5% DMSO. Images were acquired every 30 seconds using a Caliper Xenogen IVIS Spectrum® (PerkinElmer, Santa Clara, CA) with auto exposure, medium binning, f-stop set to 1, blocked excitation, open emission, FOV set to C, and height set to 1.5. Movie S1 was constructed from the frames shown in Figure 3-25 (30 second intervals) and shows a time-lapse of the chemiluminescence imaging of the mouse injected with Angeli's salt.

Figure 3-24. Quantification of live animal imaging experiments. Total photon flux from nude BALB-C mice injected with 40 µM HNOCL-1 and vehicle control (blue trace, n = 1 mouse) or 1 mM Angeli's salt in 20 mM PBS (pH 7.4) containing 5% DMSO (red trace, n = 1 mouse).
Figure 3-25. Frames of the Movie S1. The frames are from images collected at 30 second intervals for a total time-lapse of 30 minutes.

3.5 Reference

APPENDIX

Synthesis of porcine liver esterase probe

\[
\text{(3-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-chloro-6-((E)-2-cyanovinyl)phenoxy)methyl 1-methylcyclopropane-1-carboxylate (19). Chloromethyl 1-methylcyclopropanecarboxylate}^1 \text{ (116 mg, 0.783 mmol, 1.5 equiv) was added to the dry, N}_2 \text{ filled flask, then dissolved with 2.1 mL anhydrous acetone. Nal (129 mg, 0.858 mmol, 1.7 equiv) was added to the solvent and the mixture was stirred for 24 hours at ambient temperature. The reaction was concentrated under reduced pressure. Purification by chromatography (CH}_2\text{Cl}_2 \text{ yielded a pale}
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yellow oil. \((E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-hydroxyphenyl)acrylonitrile^{2,3}\) (184 mg, 0.518 mmol, 1.0 equiv) was dissolved with 2.0 mL anhydrous DMF in an dry, \(N_2\) filled flask and anhydrous \(N,N\)-diisopropylethylamine (DIPEA, 0.19 mL, 1.1 mmol, 2.1 equiv) was added to the solvent. The pale yellow oil was dissolved with 3.0 mL anhydrous DMF and added to the flask. The reaction mixture was stirred for 21 h at ambient temperature. The reaction was concentrated under reduced pressure. Purification by column chromatography (1:20 EtOAc/Hexane) yielded 19 as a white solid (107 mg, 44%). \(^1\)H NMR (500 MHz, CDCl\( _3\)) \(\delta\) 7.63 (d, 1H, \(J = 16.6\) Hz), 7.36 (d, 1H, \(J = 8.1\) Hz), 7.11 (d, 1H, \(J = 8.1\) Hz), 5.95 (d, 1H, \(J = 16.6\) Hz), 5.70 (m, 2H), 3.29 (s, 1H), 3.25 (s, 1H), 2.04 (s, 1H), 1.94–1.63 (m, 12H), 1.28 (s, 3H), 1.23 (m, 2H), 0.77 (m, 2H); \(^1^3\)C NMR (500 MHz, CDCl\( _3\)) \(\delta\) 175.07, 152.02, 144.76, 139.33, 139.03, 133.30, 129.21, 128.62, 128.54, 123.91, 117.83, 98.59, 89.32, 57.44, 39.19, 39.03, 38.61, 38.56, 36.96, 32.91, 31.59, 29.73, 28.29, 28.11, 19.10, 18.54, 17.60; HRMS calcd for C\(_{27}\)H\(_{30}\)ClNO\(_4\) (M+Na\(^+\)) 490.1756, found 490.1755.

Chemilum-CM\(_2\)

(2-chloro-6-((\(E\))-2-cyanovinyl)-3-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)phenoxy)methyl 1-methylcyclopropane-1-carboxylate (Chemilum-CM\(_2\)). Enol ether 19 (107 mg, 0.230 mmol, 1.0 equiv) was dissolved with 5 mL THF at 0 °C in a two-neck flask and rose bengal (12.4 mg, 0.0122 mmol, 0.050 equiv) was added to the solvent. O\(_2\) was bubbled through the solvent when illuminated with a 120W light bulb (Home Depot, Dallas, TX). The reaction was monitored by TLC. After 3 h 15 min, the mixture was concentrated under reduced pressure. Purification by column chromatography (1:20 EtOAc/Hexane) and yield Chemilum-CM\(_2\) as a white solid (76.9 mg, 67%). \(^1\)H NMR (500 MHz, CDCl\( _3\)) \(\delta\) 7.97 (d, 1H, \(J = 8.6\) Hz), 7.66 (d, 1H, \(J = 16.7\) Hz), 7.52 (d, 1H, \(J = 8.6\) Hz), 6.03 (d, 1H, \(J = 17.2\) Hz), 5.67 (dd, 2H), 3.21(s,

3H), 3.01 (s, 1H), 1.96 (s, 1H), 1.85-1.58 (m, 12H), 1.28 (s, 3H), 1.23 (m, 2H), 0.79 (m, 2H); $^{13}$C NMR (500 MHz, CDCl$_3$) δ 175.11, 152.70, 144.42, 136.53, 130.51, 129.80, 127.19, 124.13, 117.63, 111.63, 99.99, 96.48, 89.32, 49.87, 36.57, 33.99, 33.66, 32.67, 32.24, 31.62, 31.58, 29.82, 26.17, 25.83, 19.18, 18.59, 17.74; HRMS calcd for C$_{27}$H$_{30}$ClNO$_6$ (M+Na$^+$) 522.1654, found 522.1654.
Cell Culture Protocol

Media/reagent for cell lines:
Currently, the cell lines being used in the lab are A549 lung cancer epithelium and RAW 264.7 macrophage cells.
For A549: Complete F12K medium is typically used.
For Macrophages: Complete DMEM medium is used.

Preparation of complete medium: 10% FBS and 1% antibiotic in completed media.
Under the hood, open a new 500 mL bottle of the media. Using a 55 mL pipette, remove 55 mL of media into a falcon tube and keep aside. Add 50 mL FBS (filtered if possible, but not totally necessary) and 5 mL of pen/strep antibiotic to the 455 mL medium. Pipette up and down. Label as Opened and complete, with date and initials and store in the refrigerator. FBS is stored in the -80 °C freezer (prepare 4 aliquots of 50 mL in each Falcon tube before storing).

Thawing of vials and seeding:
1. New vials of cells can be ordered through the ATCC website, paying careful attention to cell line/strain description.
2. If thawing a vial that is stored in our liquid nitrogen dewar, keep track of vials used. Make sure to label the removed cells on the Liquid Nitrogen Storage Chart.
3. Thawing: Keep the appropriate media warmed in the water bath to 37 °C and sterilize the hood with UV light for at least 10 mins. With a pipette, pour about 10 mL of warmed completed media into a 15 mL or 50 mL falcon tube. The vial of cells is carefully dipped (not immersed) in water bath until just a tiny portion of the ice is remaining. Remove the vial and wipe with ethanol.
4. Open lid of vial slowly to release pressure and then transfer the contents into the media of the falcon tube with the 1000 µL pipet.
5. Centrifuge for 10 minutes at 1000 rpm.
6. With a pipette, pour 20 mL of warmed completed media into a T75 flask (or 3 mL to a T25 flask). Taking the falcon tube out, aspirate liquid supernatant and disperse the pellet in around 1 mL of fresh completed media. Add all the cells to the T75 flask and distribute.
cells with gently rocking of the flask. Label the flask with date, passage number, cell line and initials and place in the incubator.

7. On the next day, check the cells and remove the media with the aspirator. Add 20 mL new complete media to the T75 flask. The purpose of this step is to remove the dead cells that didn’t survived the freezing and thaw cycle.

**Cell Passaging:**
A549

1. Keep the F12K complete media, PBS and 0.25 % trypsin in the water bath about 30 mins to warm up to 37 °C. Turn on the UV of the hood and leave between 10 – 30 mins.
2. When the bottles are warm, wipe out the water, spray with alcohol and keep ready in the hood, remove the flask from the incubator and aspirate the media. Wash the cells once with about 10 mL PBS. Aspirate the PBS.
3. Add around 2 mL of trypsin to the T75 flask (1 mL for T25). Move the plate around a bit to make sure all the cells are coated with trypsin. Keep the flask in the incubator for 8 to 10 mins. Trypsin breaks the bonding between the cell surface and the bottom of the flask so that cells will detach and float.
4. Add between 10 mL of media to the flask and then transfer all the contents to a falcon tube. Centrifuge 10 mins at 1000 rpm.
5. Aspirate the supernatant. Thoroughly distribute the cells in around 1 mL of media with up and down motions of the pipette.
6. In a T75, pipette in about 20 mL of completed F12K. Depending on how many cells you need, transfer about 150 µL of the cell cocktail to the flask. Distribute thoroughly with gentle rocking of the flask. Label with date, passage number and generation, date and your initial and put back to the incubator.

**Macrophage:**

1. Keep the DMEM complete media, PBS and 0.25 % trypsin in the water bath about 30 mins to warm up to 37 °C. Turn on the UV of the hood and leave between 10 – 30 mins.
2. When the bottles are warm, wipe out the water, spray with alcohol and keep ready in the hood, along with a cell scraper, remove the flask from the incubator and aspirate the media. The cells are washed once with about 10 mL PBS. Aspirate the PBS.

3. Add around 1 mL of 0.25 % trypsin to the T25 flask (The amount of trypsin is depending on the size of the T flask). Using the cell scraper, gently but quickly scrape all the cells off the bottom of the flask. Do not allow the cells to be exposed too long to trypsin, since cells become sticky.

4. Add between 10 mL of completed DMEM media to the flask and then transfer all of the contents to a falcon tube. Centrifuge 10 mins at 1000 rpm.

5. Aspirate the supernatant. Thoroughly distribute the cells in around 1 mL of completed DMEM media with up and down motions of the pipette.

6. In a T75, pipette in 20 mL of DMEM. Then depending on how many cells you need, transfer about 150 µL of the cell cocktail to the flask. Label with date, passage number and generation, date and your initial and put back to the incubator.

**Cell Freezing:**

1. Freezing of cells should be done after about 2 to 3 passages from a fresh thawed vial. Do not allow for more passages to be done before freezing a batch of cells. It is recommended that after thawing of a vial, the cells need to grow enough to make enough vials.

2. Make sure cell confluency is around 80–90% but not overgrown.

3. Cell freezing media needs to be prepared ahead of time. It is 10% cell-culture grade DMSO in complete media.

4. Warm complete media, PBS, trypsin and cell freezing media and make sure hood has been UV treated. Follow all the passaging steps for your cells line up to the pellet formation.

5. Aspirate the media and keep the pellet. From a sufficiently confluent flask you can make around 10 vials of cells. Prepare 10 mL cell freezing media. Add 1 mL of cell freezing media to the pellet first to disperse the cells thoroughly. Later add the rest of the freezing media to the falcon tube. It is better to add 1ml first, then add the rest and mix.

6. The cryopreservation vials are used to freeze cells. Add 1 mL of the cell cocktail into as many vials as possible with pipet. Close the vials tightly, label them with cell type,
generation and passage number, date and initials. Parafilm the lids of all the vials. Store them in a Mr. Frosty freezing container containing ethanol and keep overnight in the –80 °C freezer (Dr. Zoltowski’s lab). Three days later, transfer the vials to the liquid nitrogen dewar, make a note of the date stored, location and number of vials on the Liquid Nitrogen Storage Chart.

Incubator and water bath water maintenance (Monthly)

The incubator needs to be at 37 °C, 5% CO₂ and water filled on the bottom to maintain humidity and temperature. The water to needs be autoclaved before filling and treated with a disinfectant (aquaguard from Sigma or Conflikt from VWR) about once a month. 2% of disinfectant in autoclaved water should be enough. Conflikt is also used to wipe down the incubator every semester on group clean-up day (instead of alcohol). The water in the water bath needs to be changed and treated in the same way (once a semester on group clean-up day).
SCANNED $^1$H NMR AND $^{13}$C NMR SPECTRA
13C NMR

Field_strength = 11.7473767901 [T] (1000 MHz)
X_info: 0.13967927 [Hz]
X_domain = 13C
X_freq = 125.745220764 [MHz]
X_offset = 120 [ppm]
X_points = 12704
X_redundancy = 4
X_resolution = 6.19953034 [Hz]
X_zero = 139.39976143 [mHz]
Xr_domain = 18
Xr_freq = 120.3589331 [MHz]
Xr_offset = 1.3 [ppm]
Clipped = TRUE
Mod_return = 0
Steps = 10
Total_scans = 49

X95 width = 13.41 [mHz]
X95_time = 0.8312792 [s]
X_angle = 30 [deg]
X_axis = 8.14 [deg]
X_axis_domain = 8.14 [deg]
X_axis_freq = 8.14 [Hz]
X_axis_offset = 8.14 [mHz]
Xr_domain = 40
Xr_time = 0.92 [mHz]
Xr_time = 0.92 [s]
Xr_angle = 0
Xr_axis = 0
Xr_axis_domain = 0
Xr_axis_freq = 0
Xr_axis_offset = 0

Pulse_time = 1.0 [s]
Echo_gap = 0
Relaxation_delay = 2 [s]
Expeition_time = 2.8151759 [s]
Total = 30.7 [DC]
$^{13}$C NMR
$^{13}$C NMR
$^{1}H$ NMR
$^{13}$C NMR
$^1$H NMR
$^{13}$C NMR
$^1$H NMR
$^{13}$C NMR
$^1$H NMR
$^{1}H$ NMR
$^{13}$C NMR
$^{13}$C NMR
XF2

^H NMR
Chemilum-CM$_2$