Abstract

4-(4-nitrobenzyl)pyridine (NBP) is a colorimetric indicator compound for many types of carcinogenic alkylating agents. It is used in the toxicological screening of pharmaceutical compounds, detection of chemical warfare agents, environmental hygiene technology, and other chemical analyses.

We report a synthesis of NBP-derivatives that allow for the covalent incorporation of NBP into a solid state sensor. These derivatives have been tested in solution and found to be superior in the colorimetric assay of the alkylating agent cyclophosphamide. The derivatives have also been integrated into a polymeric silica material which changes color upon the exposure to dangerous alkylating agents like iodomethane. This material modernizes the NBP assay from a time consuming laboratory analysis to a real-time solid state sensor, which requires neither solvent nor additional reagents, and can detect gas and solution-phase alkylating agents.

The NBP assay is used extensively in preliminary tests for determining toxicology profiles and mutagenicity of medicinal compounds because of its similar reactivity to guanine in DNA. The use of NBP as a DNA model suffers from the compound’s low solubility in water, its lack of reactive oxygen sites and dissimilar steric encumbrance compared to DNA. The compounds synthesized in this report attempt to address several of these problems associated with the use of NBP as a DNA model: (1) A water soluble derivative of NBP has been synthesized, and thus an aqueous assay may more closely reflect in vivo conditions, which is an important property in toxicology testing; (2) NBP-derivatives synthesized herein have reactive oxygen sites, which also improves the similarity of these compounds to DNA, since many hard carbocations react at oxygen in DNA; and (3) the polymeric nature of the solid sensors synthesized may more well reflect the sterics of DNA, which is a polymer.
Preface

All of the work shown in this thesis was performed in the laboratory of Dr. Jennifer Love at the University of British Columbia. I have composed the entirety of this thesis. All of the work outlined in sections 2.1 and 2.2 was conducted by me. I prepared the substrates and carried out all of the reactions therein. Work outlined in sections 2.3 was carried out by me with aid by undergraduate student Brenda Leong. I designed all of the reactions and performed all new procedures. Brenda Leong prepared starting materials under my supervision. Dr. Brian Patrick, the departmental expert in crystallography, conducted the acquisition and analysis of all solid-state structural data obtained by X-ray crystallography as shown in section 2.3.4. All of the work performed in chapters 3 and 4 were carried out by me.
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<th>Description</th>
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<tbody>
<tr>
<td>4-OH-CP</td>
<td>4-Hydroxy-Cyclophosphamide</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
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<tr>
<td>Ac</td>
<td>Acetyl</td>
</tr>
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<td>Anal.</td>
<td>Analysis</td>
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<tr>
<td>AP</td>
<td>Aldophosphamide</td>
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<td>API</td>
<td>Active Pharmaceutical Ingredient</td>
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<td>Ar</td>
<td>Aryl</td>
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<td>aromatic</td>
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<tr>
<td>APTES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated Total Reflectance</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>bn</td>
<td>Billion</td>
</tr>
<tr>
<td>Bn</td>
<td>Benzyl</td>
</tr>
<tr>
<td>br</td>
<td>Broad Signal</td>
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<tr>
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</tr>
<tr>
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<td>Calculated</td>
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<tr>
<td>CDI</td>
<td>Carbonyldiimazole</td>
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<tr>
<td>(CD$_3$)$_2$CO</td>
<td>Deuterated Acetone</td>
</tr>
<tr>
<td>CIDNP</td>
<td>Chemically Induced Dynamic Nuclear Polarization</td>
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<tr>
<td>CP</td>
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<td>Doublet of Doublets</td>
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<tr>
<td>DSSC</td>
<td>Dye Sensitized Solar Cell</td>
</tr>
<tr>
<td>ε</td>
<td>Molar Absorptivity (L mol$^{-1}$ cm$^{-1}$)</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
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<td>EWG</td>
<td>Electron Withdrawing Group</td>
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<tr>
<td>FL</td>
<td>Fluorophore</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared</td>
</tr>
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<td>G</td>
<td>Guanine Nucleobase</td>
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<td>GC-MS</td>
<td>Gas Chromatography-Mass Spectrometry</td>
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<tr>
<td>η</td>
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</tr>
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<td>hν_{ex}</td>
<td>Exciting Light</td>
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<tr>
<td>hν_{em}</td>
<td>Emission Light</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HRMS</td>
<td>High Resolution Mass Spectrometry</td>
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<tr>
<td>Hz</td>
<td>Hertz, s^{-1}</td>
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<td>ITO</td>
<td>Indium Tin Oxide</td>
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<tr>
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<td>Infrared</td>
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<td>'Pr</td>
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<tr>
<td>'PrOH</td>
<td>iso-propanol</td>
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<tr>
<td>J</td>
<td>Coupling Constant</td>
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<tr>
<td>λ</td>
<td>Wavelength</td>
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<tr>
<td>λ_{anal}</td>
<td>Analytical Wavelength</td>
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<tr>
<td>λ_{max}</td>
<td>Wavelength of Maximum Absorbance</td>
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<tr>
<td>L</td>
<td>Organic Linker Group</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
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<tr>
<td>m</td>
<td>Multiplet</td>
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<td>m/z</td>
<td>Mass to Charge Ratio (Mass Spectrometry)</td>
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<tr>
<td>Me</td>
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</table>
MeLi  Methyl Lithium
MeOH  Methanol
mg  Milligram
MHz  Megahertz
min  Minutes
mL  Milliliter
mmol  Millimole
MOA  Mechanism of Action
MS  Mass Spectrometry
NaOAc  Sodium Acetate
"Bu  n-Butyl (Linear)
NBP  4-(4-nitrobenzyl)pyridine
NIOISH  National Institute for Occupational Safety and Health
NIR  Near Infrared
nm  Nanometers
nM  Nanomolar
NMR  Nuclear Magnetic Resonance
NOR  Bis(2-chloroethyl)amine
N7  Nitrogen, Position Seven
N7-G  Nitrogen, Position Seven on Guanine
NI  Naphthalene Imide
O/N  Overnight
o-  ortho-Substitution
O6-G  Oxygen, Position Six on Guanine
O4-T  Oxygen, Position Four on Thymine
OMs  Methanesulfonate
ORTEP  Oakridge Thermal Ellipsoid Plot
OTf  Trifluoromethanesulfate (Triflate)
OTs  Toluene sulfonate (Tosylate)
p-  para-Substitution
p-TsOH  para-Toluenesulfonic Acid
P450  Cytochrome P450 Superfamily
PAM  Phosphoramidate Mustard
PCA  Principle Component Analysis
<table>
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<tr>
<td>Pd(dba)$_2$</td>
<td>bis(dibenzylideneacetone)palladium(0)</td>
</tr>
<tr>
<td>PET</td>
<td>Photoinduced Electron Transfer</td>
</tr>
<tr>
<td>Ph</td>
<td>Phenyl</td>
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<tr>
<td>PhMe</td>
<td>Toluene</td>
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<tr>
<td>PhMgCl</td>
<td>Phenylmagnesium Chloride</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts Per Million</td>
</tr>
<tr>
<td>ppb</td>
<td>Parts Per Billion</td>
</tr>
<tr>
<td>PyBOP</td>
<td>Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>q</td>
<td>Quartet</td>
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<tr>
<td>R, R$^1$, R$^2$</td>
<td>Organic Group</td>
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<tr>
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</tr>
<tr>
<td>'BuLi</td>
<td>sec-Butyl Lithium</td>
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<tr>
<td>Sc(OTf)$_3$</td>
<td>Scandium (III) Trifluoromethanesulfonate</td>
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<tr>
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<tr>
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<td>dicyclohexyl(2',6'-dimethoxy-[1,1'-biphenyl]-2-yl)phosphane</td>
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<td>'Bu</td>
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<td>TDPSCI</td>
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<tr>
<td>TEOS</td>
<td>Tetraethylorthosilicate</td>
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<td>TFP</td>
<td>Tris(2-furanyl)phosphine</td>
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<td>THF</td>
<td>Tetrahydrofuran</td>
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<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<td>TMP</td>
<td>2,2,6,6-tetramethylpiperidyl</td>
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<td>Trimethylsilyl</td>
</tr>
<tr>
<td>TMSCl</td>
<td>Trimethylsilyl Chloride</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of Flight (Mass Spectrometry)</td>
</tr>
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<td>UV/Vis</td>
<td>Ultraviolet/Visible</td>
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<tr>
<td>$V_{ds}$</td>
<td>Drain-Source Voltage</td>
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<td>$V_g$</td>
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<tr>
<td>X</td>
<td>Halogen of Heteroatom</td>
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</table>
Acknowledgements

I would like to offer my gratitude for Professor Dr. Jennifer Love for supporting me through my masters degree and allowing me to work in her laboratory. I would like to thank undergraduate student Brenda Leong for aiding me in my work and for collecting column fractions late into the evening. Thanks go to past and present graduate students within the Love lab for their support in the lab. In particular I would like to thank Addison Desnoyer and Eric Bowes for editing my thesis and Dr. Paul Bichler for his endless advice. Thanks go to Dr. Jay Wickenden for his editing of my thesis, Matty Jeronimo for lending me lab equipment, and Jason Hwang for the great discussions about organic chemistry. I would like to thank my committee members Dr. Mike Wolf and Dr. Chris Orvig. Thanks go to Dr. Pierre Kennespolh for running DFT computations for me. I would like to thank the UBC Chemistry department support staff including Dr. Yun Ling for mass spectrometric data and microanalysis, Dr. Brian Patrick for X-Ray Crystallography and Dr. Maria Ezhova for NMR Spectroscopy training.

I could not have completed this degree without the support of my family and their unending encouragement. They have supported me both emotionally and financially throughout the years. I would like to make a special thanks to my mother who has always believed I could do anything I set my mind to and always being there when times were tough. I would like to thank my partner Niusha Mahmoodi her love and support over this degree and for the editing of my thesis.
Dedicated to my family, friends and Niu
Chapter 1  Introduction to Alkylating Agents and their Sensors

Alkylating agents are broadly used as active pharmaceutical ingredients (APIs), agrochemicals, industrial and laboratory reagents and solvents, and chemical warfare agents. Alkylating agents can be toxic, highly mutagenic and/or carcinogenic because they form covalent bonds with endogenous nucleophilic compounds like DNA, proteins and other nucleophilic biomolecules. While alkylating agents have the potential to be toxic, the covalent modification of biomolecules can be a powerful treatment for a wide variety of maladies, from headaches to cancer.

Chemotherapy drugs are some of the most insidious alkylating agents because they are designed to access DNA and mutate it in order to kill rapidly dividing cells. Though the curative properties of chemotherapy drugs outweigh their carcinogenicity and mutagenicity for cancer patients, non-therapeutic exposure is deleterious to healthy people. Those at highest risk to exposure are healthcare workers because they may be chronically exposed to antineoplastic drugs throughout the course of their duties. Antineoplastic drugs are defined as agents that prevent the growth or spread of tumors or malignant cells, and are a specific class of chemotherapeutics. Occupational exposure to chemotherapy drugs leads to skin rashes, liver toxicity, adverse reproductive outcomes, leukemia and cancer.1,2 In 2004 the National Institute for Occupational Safety and Health (NIOSH) reported that the number of exposed healthcare workers may exceed 5.5 million in the United States, and thus it may be estimated that over half a million workers in Canada may be similarly compromised.3 Additionally, previous findings suggest that cleaning protocols for surfaces contaminated with antineoplastic drugs spread the contamination over a larger area.3 In some cases the cleaning agents may react with the drugs to generate more toxic species.4 NIOSH reports that healthcare workers may be exposed to hazardous drugs in the air, on work surfaces, clothing, and medical equipment and in patient urine and feces. In order to limit exposure to antineoplastic drugs and alkylating agents, the need for a real-time sensor for alkylating agents to assess safety in workplaces is apparent.
Towards the synthesis of an alkylating agent sensor, we envisioned grafting a nucleophilic molecule to a porous material or surface, as pictorialized in Figure 1. Covalent anchoring of the indicator molecule would prevent leaching of the dye during solution sensing. Reactions of this molecule with electrophilic alkylating agents could then be detected by changes in physical, chemical, or electrical properties of the bulk material, leading to a solid state sensor. Reactivity of the small molecule should mimic human physiological responses, so we first examined the chemistry and mechanism of action (MOA) of the model alkylating agent/chemotherapeutic cyclophosphamide (CP).

![Figure 1: Schematic Design of an Alkylating Agent Sensor](image_url)

1.1 Cyclophosphamide

Cyclophosphamide 1, as shown in Figure 2 is used for the treatment of lymphoma, leukemias, multiple myeloma, mycosis fungoides, neuroblastoma, retinoblastoma, cancers of the breast and ovary, and some autoimmune diseases. The antitumor effect of nitrogen mustards was first discovered in the 1940s by Louis S. Goodman and Alfred Gilman while studying chemical warfare reagents like sulfur mustard gas (2). We chose CP as our model compound because it is one of the most potent and widely used chemotherapy drugs. CP is an alkylating agent in the “nitrogen mustard” category (Figure 2).
Figure 2: Cyclophosphamide (CP) and Sulfur Mustard Gas

CP inhibits the replication of DNA in cells, and this inhibition predominantly affects cancer cells because they replicate more rapidly and with less error-correcting. Targeting rapidly replicating cells is a classic mechanism of many different chemotherapy drugs.
Figure 3: Cyclophosphamide Mechanism of Action
The active species of CP is phosphoramide mustard (PAM, 5), which is generated through metabolism by p450 enzyme CYP 2B6 (Figure 3). 5 may then undergo hydrolysis to 7. Both 5 and 7 may form highly reactive aziridinium intermediates (9) which then alkylate guanine in DNA (10) at the N7 position. After one alkylation event, the second chloroethylamine group on the nitrogen mustard may hydrolyze to the hydroxyethylamine species 12 or activate to the aziridinium species 13 and alkylate other nearby DNA bases leading to the crosslinked species 14.

If the DNA base ordering follows GXC (G being guanine, X being any DNA base, and C being cytidine), there would be another reactive guanine nucleotide diagonal to an alkylated guanine, since the complementary strand follows CXG. Previously this crosslink was predicted to occur at GC/CG, where guanines would be directly diagonal and at a seemingly optimal 7-8 Å apart. Subsequent research has shown that crosslinking occurs across GXC/CXG sequences due to distortions in the DNA structure upon the first alkylation event. 9 These crosslinks prevent DNA polymerase from copying DNA and may cause double strand breaks, and so can induce the onset of apoptosis. 10 Bis-alkylating species are much more cytotoxic because of their cross-linking ability. 9 Additionally, the presence of the CP metabolite acrolein (see Figure 3), in concert with DNA crosslinks has been shown to cause single strand scission, another factor in the genotoxicity of cyclophosphamide. 11

![Figure 4: Crosslinking of DNA by Nitrogen Mustards](image-url)

R = H (7) or P(O)(NH2)(OH) (5)
1.2 Covalent Bond Forming Drugs

In addition to CP, many chemotherapy drugs act on guanine in DNA as alkylating agents. There exists a large array of nitrogen mustards like melphalan (15), chlorambucil, ifosfamide and bendamustine (Figure 5). Other classes of DNA targeting agents are cisplatin (16), aziridines such as thio-TEPA (17) and mitomycin, and nitrosoureas such as streptozotocin (18), lomustine and temozolomide; these classes of drugs form strong bonds with DNA.

![Chemical structures](image)

**Figure 5: Alkylating Agents Used as Pharmaceuticals**

Many drugs operate by the covalent modification of endogenous and exogenous nucleophilic targets other than DNA as well, so called “covalent drugs”. β-Lactams comprise a broad class of antibiotics which covalently bind to penicillin-binding proteins in gram-positive bacteria. Carbamate containing compounds, such as Rivastigmine are being actively researched as cholinesterase inhibitors for Alzheimer’s disease and other forms of dementia. Oxiranes are being aggressively developed for a variety of maladies. Zafgen’s Beloranib (19, Figure 6) is quite astonishing since it contains two oxiranes and a Michael-acceptor and this drug recently passed phase IIb trials for the treatment of obesity. New oxirane and vinyl sulfone compounds are being developed towards the treatment of Chagas’ disease, and often the mechanism of action includes formation of a covalent bond with cysteine proteases or polo-like kinases. Indeed, the Love group recently developed a strategy for the synthesis of analogues of the vinyl sulfone protease inhibitor K777 (20).
Oxirane inhibitors of the popular target protein tyrosine phosphatase are being developed as treatments for a wide range of diseases such as diabetes, obesity, autoimmune disease, and cancer.\textsuperscript{18}

\begin{center}
\begin{tabular}{cc}
\textbf{Beloranib (19)} & \textbf{K777 (20)} \\
\end{tabular}
\end{center}

\textbf{Figure 6: Pharmacophores with Covalent Mechanisms of Action}

Development of drugs that form covalent bonds with biological targets has long been hampered by the greater risk of idiosyncratic life threatening events that may occur from reaction with off-target biomolecules.\textsuperscript{20} However, high profile medicinal chemists have been recently encouraging their field to explore the use of covalent bond forming functionalities in drug design and to target a covalent bond forming mechanism of action during molecular design.\textsuperscript{20bd} The main advantage of covalent drugs is that much lower doses can achieve similar results as irreversibly binding drugs since the covalently bonded drug has extremely low off-rates.

MOA’s of reversible drugs involve supramolecular interactions like hydrogen bonding, dipole interactions, and London dispersion forces; these interactions are quantified by pharmacokinetic analyses, and contribute to the drug’s overall efficiency as a binding ligand. For covalent drugs, target specificity can more important than ligand efficiencies, since off-target covalent interactions can be disastrous. The withdrawal of Rofecoxib (Vioxx\textsuperscript{TM}, Merck), a nonsteroidal anti-inflammatory drug, from the market is probably the most catastrophic recent example where off-target covalent interactions led to idiosyncratic life threatening events.\textsuperscript{19} Though off-mechanism covalent interactions can be extremely harmful, many common drugs like acetylsalicylic acid (aspirin) and penicillin have modes of action that include covalent bond forming events. Indeed, recent estimates put that one third of all MOA’s involve covalent bond forming events (see Figure 7), and yet still medicinal chemists balk at the development of covalent drugs.\textsuperscript{20}
Figure 7: Blockbuster “Covalent Drugs” their worldwide 2009 sales in USD

Off-target interactions with alkylating agents that target DNA may lead to cancers, because often the off-target reaction happens somewhere else in DNA. Most agents that target DNA generally give similar alkylation yields at N7-G (28), which is the target of alkylating agents like CP, but the major difference in their carcinogenicity lies in the different levels of O6-G and O4-T alkylations (see Figure 8).21 O-Alkylations (29-32) in DNA have been studied extensively since they seem to give the greatest base mispairing and mutagenicity.22 Often the CP metabolite acrolein O-alkylates DNA nucleosides, and this is one of the possible carcinogenic modes of action by CP.23 O6-G and O4-T alkylations can lead to carcinogenic effects while N7-G alkylations lead to cytotoxicity and apoptosis of the cell.
Figure 8: Reactive Positions of Guanine and Thymine and their Alkylation Products

We see potential growth in the treatment of diseases with drugs that form covalent bonds with their targets as part of the MOA. Many of these compounds may exhibit toxicity—especially carcinogenicity—and we must thus be able to detect them in order to limit non-medicinal exposures to these agents. Detecting these compounds in real world conditions is important, but due to the increase in research in the area of covalent bond forming drugs, also important is the ability to quickly assess the reactivity of the compound. Quickly and cheaply assessing the reactivity of a drug may enable the medicinal chemist to more rapidly screen a large array of compounds for toxicities without the need to try their hand at biologically based assays like the Ames test.24 The Ames test is a bioassay for the toxicological screening of drug metabolites, and it is very expensive and time consuming. Potentially, a sensor could be designed that may both detect low levels of compound, and may also rapidly and affordably assess the reactivity profile of a compound.
1.3 Alkylating Agent Sensors

Development of alkylating agent sensors has burgeoned in the past two decades. The need for such devices has arisen due to greater concern for occupational and environmental health as well as chemical weapons use. Though quantitative analysis of alkylating agents is generally performed in the lab by chromatographic/mass spectrometric means or assay technology, lab analysis is costly, slow, and cannot provide real-time data. Sensors may provide a real-time signal when and where such detection may be required. A sensor can also provide sufficiently quantitative analysis as to obviate the need for lab analysis. There have been many approaches towards the creation of sensor compounds and materials towards devices for the assessment of alkylating agents. Some focus only on the detection of highly reactive chemical warfare reagents, some seek to determine the agent’s identity, while still others give an ensemble signal of “total alkylating agents”. Alkylating agent sensors have been made that operate by color-change, fluorescence turn-on and -off, or changes in electrical properties of the sensor.
The Strano group has presented the application of guanine towards the detection of alkylating agents with single walled carbon nanotubes (SWCNT) soaked in DNA solutions (Figure 9). The researchers were able to observe the alkylation events by melphalan (15) as changes in the near IR (NIR) spectrum of the carbon nanotube. While not truly feasible in a solid state sensor, this work from the Strano group demonstrates the viability of DNA and guanine as a sensor.

The Strano group reported that the guanine based sensor operates by detecting shifts in the photoluminescent spectra of single walled carbon nanotubes, and they claimed that “single molecule detection” is possible by use of these biosensors. In the detection of trace analytes, it is misleading to call a
sensor capable of single molecule detection. The Weissman group at Rice University has previously reported the theory behind “single molecule detection” with SWCNT: they show that stepwise fluorescence quenching is indeed due to single molecular events. However, in order to view these events the Strano group’s sensor was exposed to 0.5 mM concentrations of the alkylating agent melphalan. While the sensor works very well at these millimolar concentrations, melphalan is dosed into the human body at sub-micromolar concentrations, and even then melphalan has 25-89% bioavailability, so at present this biosensor appears to be incompatible with real world biological conditions. Furthermore, a sensor is superior when it detects “turn-on” signals rather than “turn-off” signals, and this sensor operates by the detection of fluorescence quenching, a commonly detected “turn-off” signal. “Turn-off” sensors indicate the presence of analytes by a reduction in signal, while “turn-on” sensors report the presence of analytes by the increase of a signal. A “turn-on” sensor is more specific and easier to detect than its “turn-off” counterpart. Finally, this sensor is pH sensitive, which is a drawback in unpredictable biological conditions since it may give different responses under varying conditions or may give “false positives”.

In 2006, the Eichen group from Technion published a sensitive turn-on sensor for the detection of alkylating agents based on photoinduced electron transfer (PET). The sensor is composed of a naphthalene imide (NI) core with a pendant nucleophilic amine. In the free, non-alkylated form, the lone pair of the amine quenches the natural fluorescence of the NI core, as depicted in Figure 10. Upon alkylation, the amine lone pair lowers in energy and no longer interacts with the NI fluorophore, resulting in photoluminescence which indicates the presence of the guest.
The Eichen group was able to obtain x-ray diffraction single crystal structures for the alkylated and non-alkylated forms. The conformation of the structures generally follow the cartoon picture, where the alkylated form exists in an “unfolded” state, and the non-alkylated form in a “folded” state. The sensor molecule may be impregnated into a filter that allows for the gas phase detection of alkylating agents, and the detection limit is in the micromolar range, so it is fairly sensitive. The major drawback for this sensor is that the sensor molecule responds in the same way towards acidic protons and metal ions as it does towards electrophiles. Avoidance of “false-positives” such as these is essential in the development of sensors.

The Eichen group solved the sensor molecule’s acid-sensitivity issue by mixing base in with the alkylating agent analyte, which resolves the acid sensitivity, but seems ungainly and inelegant. They claimed that metal ions can be distinguished by lowering the sample concentration and observing whether the sensing is in equilibrium. Since sensing of alkylating agents occurs by covalent bond formation and metal ion sensing may be in dynamic equilibrium, lowering the concentration of an alkylating agent solution should not affect
the sensor signal, while lowering the concentration of a metal ion solution should yield a concomitant drop in signal. They do not describe a practical method for distinguishing metal ions and alkylating agents in real sensing applications.

A number of groups have utilized PET quenching towards an alkylating agent sensor. In 2005, the Rebek group presented a sensor for organophosphorus-based nerve agents like Sarin. The idea behind the PET sensing is very much like the Eichen group’s sensor, but this sensor detects much more reactive and oxophilic phosphorus agents. As shown in Figure 11, diethyl chlorophosphate (35) reacts rapidly with the alcohol moiety (34). The newly formed phosphate is now in close proximity to the free amine, which displaces diethylphosphate yielding fluorophore (36). They tested a large array of fluorophores and linker lengths to discover that a one methylene linker length between the amine and the pyrene fluorophore gave the greatest intensification of fluorescence upon exposure to analytes. They do not mention whether acids, metal ions or polar solvents impede the sensing, though the mechanism of sensing does not seem refractory to their interference.

**Figure 11: PET Based Sensing of Organophosphorus Nerve Agents**

The Swager group from MIT also published a sensor for organophosphorus nerve agents utilizing a similar mechanism. The sensor (37) is comprised of a pyridine unit bound directly to a fluorophore with a nearby alcohol or silyl ether group. As seen in the Rebek sensor, activation of the hydroxy functionality by
the organophosphorus nerve agent (38) and subsequent quaternization of the pyridine nitrogen (39) leads to strong fluorescence (40). Interestingly, HCl produces minimal fluorescence response in solution, although the sensing is appreciably interfered when the compound is impregnated into cellulose acetate.

![Reaction scheme](image)

"Low Fluorescence"

Y = H, iBuMe₂Si

"High Fluorescence"

**Figure 12: Swager Group PET Sensing of Organophosphorus Nerve Agents**

In 2008, the Smith group from Notre Dame reported a turn-on PET based sensor for micromolar levels of chloroalkanes which utilizes both the alkylation of an amine and the complexation of the chloride ion as recognition for chloroalkanes (Figure 13).³⁴

![Recognition diagram](image)

"low fluorescence"

"high fluorescence"

**Figure 13: Recognition of Alkyl Chlorides by a Macrocycle**

The macrocycle (41) is a very powerful sensor for chloroalkanes (42), with the detection limit of chloromethyl methyl ether reported as low as 10 μM.³⁵ The analyte half-life is two minutes, which represents the time required to half signal after exposure to analyte. A two minute half-life, while not rapid, is certainly
faster than laboratory analysis. The sensing mechanism operates by the alkylation of the free amine. When this amine is free and not quaternized (41), it quenches the fluorescence of the naphthalene moiety. The sensor does not work with gas phase analytes, and so they propose a device wherein the gas phase analytes may be bubbled into a solution of the sensing molecule by an air pump, comprising an impinger. Solution based sensing in this way is ungainly and refractory to miniaturization. Also, use of volatile solvents requires that a commercial device be carefully sealed so that no vapors may escape, which seems prohibitory to an impinger use.

The sensing capability of the molecule is based partly on halide recognition, and so the sensor from the Smith group does not respond as strongly towards alkyl iodides and bromides.\textsuperscript{36} Alkyl iodides and bromides are often more dangerous alkylationg agents than their chloride counterparts, and thus are desirable analytes to detect. Finally, a small presence of any polar solvent like water, methanol, DMSO or acid like HCl shuts down the reactivity of the sensor, which is a significant drawback. Some effort was made to use the sensor in micellar solution in order to detect alkylationg agents when polar solvent is around, which seemed to avoid this issue.\textsuperscript{37}

One of the most selective and powerful sensors came from a separate publication in the Eichen group. The sensor was composed of a large array of nucleophilic molecules and polymers. These compounds, as depicted in Figure 14, changed color upon reaction with an alkylationg agent. Each sensing material was spotted onto paper, and exposed to individual alkylationg agents, and the changes in the colors could be graphed against each other to determine the identity of the alkylationg agent. Due to its ability to identify the compounds, the authors dubbed the sensor an “artificial nose”.
The process of identification requires complex data analysis, called principle component analysis (PCA). Application of PCA to the “artificial nose” involves a multivariable graphing of the different sensor molecule responses and can differentiate a wide variety of alkylating agents, as shown in Figure 15. The molecule responses are measured by the change in RGB values.
Figure 15: Responses of an "artificial nose" to Various Alkylating Agents. Small numbers near the color blocks are the RGB values for the color.

While quite brilliant in design and execution, Eichen’s “artificial nose” suffers from several major drawbacks, most importantly that it is unable to determine the reactivity of the analyte. The “artificial nose” would be employed in non-ideal situations where many different, and potentially harmful analytes, may be present at one time. This complex mixture of compounds could limit the overall utility of this promising sensor.

The Eichen group also developed a separate sensor to give kinetic data on alkylating agents. Kinetic data on the reactivity of an analyte can be invaluable because it enables the danger assessment of unknown compounds. The device utilizes an organic field effect transistor based on a polythiophene block co-polymer with pendant pyridines. As the polymer becomes alkylated at the pyridine side-chain, the material becomes more conductive, which causes an increase in the source-drain current. The initial diffusion of the compound causes a drop in this current, and only when reactions begin does the current rise. This allows for the differentiation of diffusion time and reactivities. However, like other polythiophenes, this polymer is also
sensitive to oxidation,\textsuperscript{39} so reaction with oxidants like I\textsubscript{2} can give a false-positive. While acutely sensitive to oxidants like I\textsubscript{2}, slow oxidation of polythiophenes in air may degrade the sensing capability of the polymer. The authors do not comment on the shelf life of the sensor, or how long it takes for atmospheric O\textsubscript{2} to oxidize the polymer.

**Figure 16: An Organic Field Effect Sensor for the Reactivity of Alkylating Agents**

Review of the current alkylating agent sensors has inspired ideas about what properties a good sensor should have. The sensors should be a turn-on sensor that detects alkylating agents at very low concentrations, preferably in the nanomolar range. The mechanism of sensing ought to be prohibitive of common interferents like polar solvents, acids, and oxidants. In order to analyze compounds and samples of unknown composition, it might be better not to specifically identify a compound, but rather to identify the concentration of an alkylating agent ensemble and its reactivity.
1.4 Rational Design of an Alkylating Agent Sensor

Figure 17: 4-(4-nitrobenzyl)pyridine (NBP)

4-(4-nitrobenzyl)pyridine, (NBP, 62), is an important indicator that is used to both detect trace alkylating agents present in the surrounding environment, as well as the biological activity of analytes. NBP was first used in the detection of mustard gas agents by Koenigs et al. in 1925. Further work by Epstein et al. improved this compound’s versatility to include quantitative determinations. We sought to further improve the utility of this class of chemical sensor by grafting NBP to a solid surface, whereby the products of an alkylation could be detectable via analysis of the NBP-solid surface following an alkylation event (Figure 18).

Figure 18: Schematic Representation of an Alkylating Agent Sensor and Sensing Events

NBP had been previously impregnated into a material by simply soaking a porous cellulose based tape in a solution of NBP and base. This was found to be a good sensor for gaseous phosgene down to 6 ppb. Nakano et al. found that the tape was sensitive only to very reactive species like phosgene, but not relatively
unreactive carcinogenic agents like benzyl chloride.\textsuperscript{42,43} Additionally, the monitoring tape was found to lose 10\% of its activity after exposure to air for three months.

In order to link NBP to a surface, the structure needed to be modified so that it would bind to the material. We wanted to devise an molecule with improved sensing capabilities based on the NBP motif with (1) greater range of reactivity, (2) better sensitivity, (3) improved stability (both of the indicating dye and the initial compound), and (4) similarity to biological systems.

To determine the best way to covalently incorporate NBP into a material, we examined the mechanism of dye formation, which had been first posited by the US military prior to 1955. The suggested mechanism follows an \(S_N2\) displacement of a halide by the nucleophilic pyridine, resulting in conversion of 62 to 63 in acetone/water (Figure 19). Upon addition of base like triethylamine or \(\text{NaOH}\), one of the activated acidic methylene protons is removed, effectively yielding the carbanion 64.\textsuperscript{44} Resonance forms of carbanion 64 are neutral form 65 and betaine 66, and thus this type of structure is called an anionoid in literature.\textsuperscript{108}
As can be seen from the mechanism, NBP is not sensitive towards the presence of Brønsted-Lowry acids, because a subsequent basification is required, which would also deprotonate any protonated NBP. Furthermore, the conditions are very broad, not requiring very specific buffered conditions like guanine/DNA, and the sensing can take place in a wide variety of solvents. Furthermore, NBP interactions with alkylating agents can be observed directly by UV/Vis spectroscopy. Observation of alkylation events of guanine/DNA requires the presence of carbon nanotubes to generate fluorescent signal. NBP provides the required function of guanine/DNA while minimizing complexity of the system, analogous to function oriented synthesis.\textsuperscript{45}

With the mechanism of dye formation in mind, we set about designing an NBP derivative that could link to a bulk material. The linker should be bound through the carbon network of the NBP scaffold while: (1) not interfering with or enhancing the nucleophilicity of the pyridine and (2) maintaining or increasing the stability of the delocalized electrons in the conjugated system of the dye form. The nucleophilicity of the pyridine nitrogen should not be changed as it closely matches the nucleophilicity of the N7 position of
guanine, so we did not envision substituting the pyridine at the D or E positions (Figure 20). The Swain-Scott nucleophilicity constants for NBP and guanine are both 3.5, so we did not want to alter this reactivity of NBP.\textsuperscript{46,47} Due to the extensive research performed on the reactivity of NBP and protocols set for analysis of alkylating agents, we wanted the reactivity of our compound to be similar in order to match the already utilized relationships between NBP and alkylating agents.\textsuperscript{48} In fact, reactivity of NBP with epoxides has been shown to correlate with the carcinogenicity of the epoxide, so NBP can be a good mimic for biological systems.\textsuperscript{49}

**Figure 20: Potential Designs of an Alkylating Agent Sensing Molecule**

To generate a sensor molecule consistent with NBP applications and reactivity, the best position for the linker should be A, B, or C. In order to best lower the ground state energy of the anionoid dye structure, the linker should be comprised of an unsaturated electron-withdrawing functionality so it can further delocalize the free electrons (Figure 21). In the A position, an electron withdrawing group could only stabilize the carbanion through induction, while in the B and C positions a conjugated electron withdrawing group could also stabilize through resonance (72 and 74). When the linker is an amide such as 69 (or ketone, ester,
phosphonate, other unsaturated conjugated electron withdrawing group) the dye form should benefit from greater resonance stability, so we thought these could be promising targets. Using a carbonyl functionality such as an amide for installation of a linker molecule should allow for testing of many types of linkers. The resonance structures of the anionoid chromophores are drawn in Figure 21.

![Resonance structures of anionoid chromophores](image)

**Figure 21: Stabilization of Methine Carbanions by an Amide Functionality**

In 2013 the Detty group at the University at Buffalo employed this strategy of stabilizing a similar chromophoric motif with a conjugated electron withdrawing functionality. They synthesized a number of chalcogenopyrylium monomethine dyes with pendant aryl phosphonates (75) for the dual purpose of stabilizing the dye and enabling the binding of the molecule to nanocrystalline TiO$_2$ towards the synthesis of a dye sensitized solar cell (Figure 22).
While 75 was successful in its application to a dye sensitized solar cell, we thought that an amide would also allow for attachment to a support. Targeting an amide should also allow for the transformation into an ester or imine, so parallel or divergent oriented synthesis would be possible. While alkyl or alkoxy groups in the A position should also work, we thought they might not significantly stabilize the anionoid chromophore. Thus we decided to try to place an amide linker in the B position, since an amide bond should be robust and have good versatility in synthesis of different linkers.

Furthermore, when looking at the mechanism of action of carcinogenicity as discussed previously, (Section 1.2 Covalent Bond Forming Drugs) carcinogenic alkylation events at DNA are O-alkylations. The presence of an amide functionality in the sensing molecule makes the molecule a greater mimic of DNA, since the most often O-alkylated positions in DNA are amides. Casado et al. point out in a review of NBP literature that NBP will not be a good model for DNA because no oxygens are present in the molecule, and so our idea of amide installation may increase the guanine likeness.

If an amide can be accessed then the carboxylic acid moiety would likely be synthesized as an intermediate. A carboxylic acid derivative of NBP may have water solubility, which may allow for in vivo sensing. Casado et al. point out that if aqueous assay conditions could be used then it would be more similar to human physiological conditions. Thus a water soluble NBP derivative would be a major step forward in development of the NBP system as a DNA model.
The NBP-material linker extending from the amide nitrogen could be any length organic fragment that terminates in a reactive functionality. Common reactive functionalities include trisalkoxysilanes, thiols, carboxylic acids, diazonium salts, and others. Invariably, the choice of the reactive functionality should be determined by the material chosen for incorporation. Thiols are often chosen for the generation of functionalized gold thin films due to the strong gold-sulfur bond and useful electrical and physical properties of gold.\textsuperscript{52} Oxygen based functionalities like hydroxy, siloxy, and carboxy are often incorporated into silicas because of the high oxophilicity of silicon.\textsuperscript{54,55} If the sensor was going to be based on the NBP sensor motif, the response should be colorimetric in nature, and so we thought an optically transparent material would be the best choice. Polymeric silica networks known as sol-gels have the potential to be transparent if they are amorphous. On the other hand, indium tin oxide (ITO) has the dual advantage of being transparent and electrically conducting. An electrically conducting material would have good sensing properties, since small changes in the electrical properties of the material could be easily detected.\textsuperscript{38}

\begin{chemfig}

[...]

\end{chemfig}

**Figure 23: Sol-Gel Formation**

Sol-gels are amorphous or structured silica films usually generated from acid or base hydrolysis of tetraalkylorthosilicates (Figure 23). The most common starting material for silica gels is tetraethylorthosilicate (TEOS, 76). Sol-gels have a few advantages as a sensor based material: (1) they are easily synthesized, (2)
they can be mesoporous, which leads to rapid uptake of analytes, (3) they can have high surface area, offering many spots for a reaction to take place, and (4) they can facilitate proton transfers in the solid state.\textsuperscript{56,57}

**Figure 24: Impregnation of Triethoxysilanes and Carboxylic Acids into a Sol-Gel**

The incorporation of organic fragments into the polymeric silica network is facile: the organic fragment may be a carboxylic acid or organotrisalkoxysilane (81, 82) and is simply stirred with the orthosilicate over the course of hydrolysis (Figure 24).\textsuperscript{55}
**Chapter 2  Preparation of an Alkylating Agent Sensor**

![Chemical Structures]

**Figure 25: Envisioned NBP/Sol-Gel based Sensor for Alkylating Agents**

Synthetic plans were then devised towards 84 (Figure 26), leading to the sensor material shown in Figure 25. Though 62 is available commercially, functionalization of the molecule’s benzene ring is difficult. Access to the arene carboxylic acid by lewis-acid catalyzed Friedel-Crafts acylation or Vilsmeier-Haack formylation is not possible because the nitrobenzene ring is electron poor and NBP is sensitive to strong lewis bases and high temperatures (Figure 26). If the acylation was attempted without the nitro group in place, the ring would likely be sufficiently electron rich, but the acylation would occur in the para position due to steric effects. The pyridine ring is significantly slower in electrophilic aromatic substitution reactions due to, again, its electron poor nature.
Reduction of the nitro group in NBP to 86 should yield a ring sufficiently electron rich to undergo Vilsmeier-Haack formylation, but the electron rich amine functionality would likely point the formylation to the ortho position (Figure 27, 87).

We therefore looked at synthesizing 85 from readily available 4-picoline derivatives (87) and 2-halobenzoic acids (88, 89). By retrosynthetic analysis, we imagined some type of C-C coupling to generate the ortho-carbonyl substituted 4-picolylnitrobenzene 85 (Figure 28). The nitro group could be installed pre- or post-coupling, depending on the sensitivity of coupling towards the nitro group.
Figure 28: Retrosynthetic Analysis of Carboxylic Acid 85

2.1 4-Picoline as Electrophile

The synthetic strategy, as outlined in Figure 29, hinged on the key step of carbon-carbon bond formation through a nitroaryl cuprate as reported by Knochel in 2005. Metalated nitroaryl species are difficult to synthesize because of their sensitivity to reduction by electron transfer, as exemplified in the Bartoli indole synthesis. Thus their preparation has only been reported a handful of times. Most reports for the synthesis of metalated nitroaryl species utilize lithium metal at very low temperatures (-100 °C), and further functionalized metalated nitroaryl species had not been reported before the Knochel publication in 2005. Nitro groups are often refractory to transition metal facilitated cross-couplings because strong coordination of the nitro group can poison a catalyst. The metalated aryl depicted here is further complicated by the presence of an ester, which can undergo addition by the metal transfer reagent (PhMgCl in this case) to eject ethoxide and generate a diarylketone. The method was likely successful because the rate of bromide/magnesium exchange was so rapid, with completion observed by Knochel et al. within 30 seconds at -40 °C by GC-MS.
Figure 29: Synthetic Plans Towards 93: The Key Step

Preparation of the iodobenzene starting material went relatively smoothly. Towards the nitration of 2-iodobenzoic acid we found that the procedure outlined by Miyata in 2010 did not furnish 5-iodo-2-nitrobenzoic acid in our hands as reported. Miyata reported that nitration of 2-iodobenzoic acid was carried out by stirring the starting material in nitric acid/sulfuric acid at 0 °C and then warming to room temperature overnight. No nitration products were observed by disappearance of a signal in the aromatic region of the ¹H NMR spectrum, but only a shift by a few ppm, which still has not been explained. The Knochel group reported that ethyl-2-iodobenzoate was nitrated by heating the reaction to 75 °C for twelve hours. We found that this procedure yielded 2-iodo-5-nitrobenzoic acid with a 95% yield when applied to 2-iodobenzoic acid (Figure 30).

Figure 30: Nitration of 2-iodobenzoic Acid

Esterification of acid 94 was performed by formation of the acid chloride by heating 2-iodobenzoic acid to reflux in thionyl chloride. Thionyl chloride was removed in vacuo, and subsequently the acid chloride
was heated to reflux in ethanol. 95 precipitated out after cooling to room temperature and was isolated in 72% yield.

![Reaction Scheme](image)

**Figure 31: Esterification of 2-iodo-5-nitrobenzoic Acid**

Preparation of the pyridine electrophilic coupling partner was problematic. Treatment of the commercially available 4-pyridinemethanol (96) with p-toluenesulfonyl chloride resulted in an expected dark red solution. Aqueous workup and extraction with dichloromethane (DCM) led to, as expected, a red solution in DCM. Upon removal of the DCM a dark red tar was isolated—this seemed reasonable since similar compounds are reported to be red oils; however, the tar was found to be insoluble in DCM suggesting that workup led to polymerization (99). Polymerization reoccurred when care was taken to avoid applying any heat to the compound while removing solvent during workup. Thus it was likely that the polymerization was due to concentration of the material. Furthermore, attempts to similarly generate the less reactive methanesulfonate (98) led to the same polymerized tar (Figure 32).

![Reaction Scheme](image)

**Figure 32: Tosylation and Mesylation of 4-pyridinemethanol**

This polymerization problem was exacerbated in the preparation of the even less reactive bromide derivative 100 (Figure 33). Heating 4-pyridinemethanol in dioxane with phosphorus tribromide gave an
intractable tar. It seemed that polymerization (101) occurred in this reaction as a consequence of heating to 40 °C. The brominated product 100 was not observed, only the insoluble red tar.

![Chemical structure of 4-pyridinemethanol bromination](image)

**Figure 33: Bromination of 4-pyridinemethanol**

Since it seemed possible to transiently generate the tosylated pyridine 97 as in Figure 32, it was thought that the 97 could be utilized without isolation. A by-product of the tosylation is H₂O, so the reaction was stirred with 4 Å molecular sieves in order to remove the evolved H₂O. Additionally, KOH was used in a stoichiometry of unity rather than excess. Subsequently the dark red reaction mixture was transferred by filter-cannula into the coupling reaction (Figure 34).

![Chemical structure of copper mediated coupling](image)

**Figure 34: Copper Mediated Coupling of a Grignard Reagent and an Aryl Halide**

No product was observed by ¹H NMR spectroscopy or mass spectrometry. The major problem was likely crude 97 used, so attempts were made to add the generated aryl Grignard reagent into 4-formylpyridine (104) (Figure 35). The lactone 105 was isolated in only trace amounts and was found to be resistant to ring opening and reduction. Trace amounts of the dehalogenated starting material 106 was also observed. Bulkier protection with an isopropyl ester 103 yielded the same results.66
Figure 35: Lactone Formation with 4-formylpyridine

As shown in Figure 36, synthesis of “dianions” 109 and 110 of N-alkyl 2-iodo-5-nitrobenzamide and N-methyl-benzamide was also attempted in order to limit the formation of the lactone. This strategy was pursued under the assumption that deprotonated amides could not undergo addition by an alkoxide.

Figure 36: Dianion Formation and Frustration of Lactonization

Synthesis of a Grignard dianion 112 from N-alkyl-2-iodo-5-nitrobenzamide (111) is complicated by the rapid iodide/magnesium exchange and inter-complex quenching of the arylmagnesium specie (113).67 Often metalated acid-bearing arenes will quench themselves, leading to unproductive reactivity, as shown in Figure 37. Thus it is not possible to generate the 2° amide-Grignard reagent (112) by the addition of two or more equivalents of the metal transfer agent PhMgCl.
Figure 37: Self-Quenching in Dianion Formation of Grignard Reagents of Acid Bearing Arenes

The secondary amide 111 was prepared through the acid chloride in 40% yield. As shown in Figure 38, the amide was initially treated with sodium hydride to generate the sodium amidate 112, which would then react with PhMgCl to generate the aryl Grignard species. Since no product (amide/alcohol or lactone) was observed, the Grignard was likely not formed. Potentially, the sodium amidate prohibited the metal transfer reaction.

Figure 38: Dianion Formation by Sodium Hydride and Halogen-Magnesium Exchange

N-methyl-benzamide (114) is known to form the dilithiate (115) by directed ortho-metalation (Figure 39). Synthesis of 115 was facile: slow addition of "BuLi yielded a colorless crystalline solid which precipitated out of THF completely after the first equivalent of "BuLi, indicating formation of the monolithiated amide. As the second equivalent of "BuLi was added, this salt reacted rapidly and dissolved as a yellow-brown solution, demonstrating formation of the dilithiate (115). Frustratingly, however, 115 generated a complex mixture upon trapping with 4-formylpyridine at -78 °C, which ultimately yielded only trace product. Likely the dilithiate was too reactive, potentially ionizing the aldehyde or adding into the pyridine ring.68
2.2 4-Picoline as Nucleophile

Due to the myriad difficulties associated with the approach based on electrophilic picoline derivatives, the roles of the benzene and picoline derivatives were switched from nucleophile and electrophile to electrophile and nucleophile, respectively. This approach was inspired by a later Knochel report utilizing their previously developed hindered base TMPZnCl·2LiCl (TMP = 2,2,6,6-tetramethylpiperidyl) to directly generate a zincated picoline and perform Negishi type couplings (Figure 40).

Although there are a number of strategies for the arylation of 2-picoline, the Knochel report details the first and only known arylation of 4-picoline. Arylation of picolines is complicated by a number of factors, as shown in Figure 41. 1) 2-picoline, though directing, form strong chelates with palladium (119) that are reluctant to reductively eliminate; 2) in metalated 4-picoline the equilibrium favors the quinoidal structure.
(121); and (3) though the pKₐ of the methyl group is relatively low (~32), metalation requires specific choice in base because ring-addition can often occur with strong bases (122). Knochel et al. employed the Lewis acid Sc(III) triflate to occupy the nitrogen in the picoline by coordination, which presumably limited pyridine N-complexation of the palladium catalyst and encouraged reductive elimination.

**Figure 41: Problems with Benzylic Couplings of 4-Picoline**

Knochel was able to demonstrate the benzylic coupling of picolines with a variety of aryl bromides with good to excellent yields (Figure 42). The aryl bromides used had a variety of electron donating and withdrawing groups, and it was found that the added Lewis acid most substantially enhanced coupling with electron-poor aryl bromides. This was relevant to our synthesis so we imagined that this method could be applied towards the synthesis of our target compound.
Figure 42: Previous and Proposed Coupling Reactions of Zincated 4-Picoline

Knochel’s work only explored one *ortho*-substituted aryl bromide, which was an electron rich arene. No work has been done on electron poor *ortho*-substituted aryl bromides or aryl bromides with two electron withdrawing groups. Additionally, only aryl bromides were studied and no comment was made concerning aryl iodides. However, we believed the difference in our substrates and the substrates used successfully by the Knochel group would not prohibit the use of this method.

The metalated TMP base was prepared by the treatment of 126 with "BuLi in THF and hexanes as per the published procedure. The "BuLi solution was titrated with iodine in a saturated solution of LiCl in THF, as per a modified procedure also published by the Knochel group. The Knochel group reported that 128 was titrated by benzoic acid with 4-(phenylazo)diphenylamine as an indicator. This titration procedure was found to be imprecise because the purple-to-yellow endpoint was difficult to observe exactly (Figure 43).
Figure 43: Preparation of TMP-Zn and Zincation of 4-picoline

Due to the difficulties associated with the yield of 128, a GC-MS procedure was developed to determine the concentration of metalated TMP and 4-picoline over the course of the transformations. As each metalated compound was formed, two small aliquots were taken and quenched separately over deuterium oxide and iodine. GC-MS was used to monitor the metalated species by the H/D isotopic ratios. Initially we thought that quenching 123 on solid I$_2$ would yield the iodo derivative but it was never observed. However, on occasion $n$-iodobutane could be observed from reaction of $n$BuLi with I$_2$, which was useful because it further indicated how successful the initial deprotonation of 126 was. Though the yield of 128 was reasonable, we were never able to get a solution as concentrated as reported by Knochel (reported: 1.3 M, found: 0.4 M). With the lower concentration 128, the zincation of 4-picoline took overnight rather than one hour, as reported by Knochel. Interestingly, if extra $n$BuLi was present, the oxidative coupling of $n$BuLi and 123 yielded 4-$n$-pentylypyridine, which was possibly induced by iodine. Since most iodine facilitated oxidative couplings are either between enolates or constitute intermolecular C-N bond formation, our finding could yield a significant result in cross-coupling if optimized.

Using GC-MS to monitor each reaction allowed for optimization of the synthesis of 123. 123 was then screened as a participant in various coupling reactions, as shown in Table 1, but was not found to be amenable to any of the palladium catalyzed procedures. Furthermore, this reaction was unsuccessful with our other proposed substrates. This might be due to issues with the ortho-ester or contaminants. These reactions also did not work with Pd(dba)$_2$/TFP catalyst as reportedly used by Knochel for the cross-coupling of functionalized nitroaryl magnesium halides.
We thought that there might be a problem with the 128, especially since the desired concentration of the 128 could not be achieved, and thus excess 126 would be present, potentially inhibiting coupling. Previous reports71 have shown that, while "BuLi, 'BuLi and other very strong bases will add into the pyridine ring, MeLi has a 95% preference for metalation of the 4-picoline methyl group. If MeLi works as well as 128 in the deprotonation of 4-picoline, it should improve the metalation/coupling procedure by obviating the tedious preparation of 128. The metalation of 4-picoline was achieved by treatment with MeLi at 0 °C, utilizing GC-MS to monitor the metalation and transmetalation of picoline by analysis of quenched products. Salt metathesis of lithiated 4-picoline with ZnCl₂ in THF afforded 123, which was tested under the Knochel group’s coupling conditions against our aryl halide coupling partners as in Table 1; however, the expected products were never observed by MS or 1H NMR spectroscopy.

The final coupling experiment tried was with a substrate from the Knochel 2011 publication, 4-bromo-N,N-dimethylaniline (129) (Figure 44). This reaction was successful to some extent, which indicated that the conditions employed were not altogether refractory to the arylation of 4-picolines, and that likely our

<table>
<thead>
<tr>
<th>Entry</th>
<th>Y</th>
<th>X</th>
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<th>Yield</th>
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<td>Et</td>
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<tr>
<td>2</td>
<td>NO₂</td>
<td>I</td>
<td>iPr</td>
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<td>H</td>
<td>I</td>
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<td>4</td>
<td>H</td>
<td>Br</td>
<td>Et</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 1: Benzylic Coupling of Zincated 4-Picoline
previous attempts at cross-coupling were foiled by the presence of the *ortho*-ester functionality in the aryl halide substrates.

![Chemical structure](image)

**Figure 44: Repeating a Knochel Substrate**

While a coupling method was never developed successfully, this work did succeed in exploring the metalation of 4-picoline. The results of this study are shown in Figure 45. Previously mentioned work\(^7^1\) utilized only NMR techniques to determine the metalation/ring addition chemistry of strong bases acting upon 4-picoline, while this work utilizes arguably more quantitative GC-MS methods. Concerning the chemistry of strong bases and picolines, there is significant disagreement between results by various researchers about the ratios of products achieved and whether they are metalated or ring-addition products.\(^7^7,^7^1\) This work is in general agreement with the work by Kaiser *et al.*, but we found more ring-addition products with methyl lithium at room temperature than they describe.
Figure 45: Metalation and Quenching Reactions of 4-picoline

Though the Knochel report on benzylic coupling of 4-picolines was thorough in method development, we were not able to make it work after extensive troubleshooting. This failure may have sprung from the inability to generate solution with the same properties as reported by Knochel. Additionally, the coupling method does not work when there is an ester ortho to the bromide, which significantly limits the scope of this
methodology. Extrapolation of Knochel’s results to further access desirable picoline derivatives is unpredictable.

2.3 C-C Bond Formation by Rearrangement

Since an ionic strategy appeared refractory towards the synthesis of our target molecule, we decided to look towards more unusual strategies to achieve the core 4-(2’-carbonyl-benzyl)pyridine motif. In 2008 the Kumar group from Guru Jambheshwar University of Science and Technology reported that polystyrene-supported hypervalent iodine can induce an oxidative rearrangement to the diacylbenzene derivative 142, as shown in Figure 46.\(^78\)

![Figure 46: PhI(OAc)\(_2\) Mediated Oxidative Rearrangement](image)

While the rearrangement of this type has been known for some time, Kumar’s report represents the first time applying the procedure to a pyridine derivative. From the diacylbenzene derivative in Figure 47, a few adjustments of oxidation states and a nitration should lead us to our desired product.

![Figure 47: Functional Group Transformations Required to Product](image)
This rearrangement of hydrazides can be performed with a few oxidizing agents: lead tetraacetate (LTA),\textsuperscript{79} (diacetoxy)iodobenzene,\textsuperscript{80} and sodium hypochlorite.\textsuperscript{81} The oxidative rearrangement was first discovered with LTA, and this reagent has since been used for the synthesis of boron-containing near-IR fluorescent dyes,\textsuperscript{82,83} diarylisobenzofurans,\textsuperscript{84} 7,8- and 3,4-diacylcoumarins,\textsuperscript{85,86} 6- substituted dibenzazepin-11-ones,\textsuperscript{87} thiophene bearing monomers for polymeric photovoltaics,\textsuperscript{88} 2-acylbenzoyl bromides,\textsuperscript{89} and others.

The mechanism is somewhat unusual, and has not been completely elucidated, but the Kotali group has done several experiments in support of the following mechanism with LTA as shown in Figure 48: upon protonolytic coordination of the lead reagent with the NH hydrazide in 143, one of the acetate ligands migrates into the hydrazone double bond (144), inducing a cyclization and further acetate migration to the 1,3,4-oxadiazoline (146). At this stage in the mechanism, the 1,3,4-oxadiazoline 146 may be isolated,\textsuperscript{90} however, if the benzene ring is ortho-hydroxy-substituted, then displacement of the acetate group by the hydroxy functionality generates a tricyclic 1,3-dioxane 147. Rapid extrusion of dinitrogen and decomposition of the resulting strained ring system in 148 yields the diacylbenzene product 149. Migration and displacement of acetate shown in 145 and 146 are unexpected, since a mechanism could be drawn without these high energy transition states, so possibly a carbocation is involved.

![Mechanism of Lead Tetraacetate Induced Oxidative Rearrangement](image)

**Figure 48: Mechanism of Lead Tetraacetate Induced Oxidative Rearrangement**
Kinetic studies show that the rate determining step is the protonolysis of LTA in the first step and that intermolecular acetoxy uptake follows this as shown in 144. Electron spin resonance spectroscopy (ESR) and chemically induced dynamic nuclear polarization (CIDNP) studies suggest that a radical species is not present in the reaction, and that likely it proceeds by a polar mechanism, unlike some other LTA reactions.18O Labelling studies unequivocally demonstrate the origins of the oxygen atoms in the product. Finally, acid catalysis was discounted because the reaction was not slowed in the presence of triethylamine.

2.3.1 Synthesis of the NBP-Carbonyl Core

\(N\)-Pyridoylehydrazone 152 was synthesized by stirring salicylaldehyde and isoniazid in refluxing isopropanol at 65% yield, which is in agreement with the literature procedure. After optimization, this reaction went to near completion (97%) when ethanol was utilized as solvent (Figure 49). The product was less soluble in ethanol than isopropanol, so precipitation of the product likely drove the reaction to higher yields. This reaction was scaled up to yield 80 grams of product without affecting the yield or procedure.

![Figure 49: Formation of Hydrazide 3](image)

The \(N\)-pyridinoylehydrazone 152 was then subjected to oxidative rearrangement conditions, wherein freshly recrystallized LTA was added slowly to a suspension of 152 in dry THF (Figure 50). Later, the recrystallization of LTA was found not to be necessary or favorable, since recrystallization removed the acetic acid additive which stabilized the reagent while weighing under ambient atmospheric conditions.
Figure 50: Oxidative Rearrangement Induced by Lead Tetraacetate

Though Kumar et al. reportedly isolated 142 by oxidative rearrangement induced by (diacetoxy)iodobenzene, our $^1$H NMR spectroscopy measurements in CDCl$_3$ differ significantly from those reported by Kumar et al.$^78$ They reported a multiplet for C5-H from 7.04-7.7 ppm (Figure 51), which is consistent with the $^1$H NMR spectrum of a by-product that we isolated but have not succeeded in elucidating its structure. This by-product requires very good separation by column chromatography to remove. In our $^1$H NMR measurements of pure 142, this proton (C5-H) appears at 7.50 ppm, and no protons appear below 7.50 ppm. Furthermore, their reported signals for carbonyls in $^{13}$C NMR spectroscopy appeared at 161 and 165 ppm, which is an unlikely region for carbonyls to appear in $^{13}$C NMR. We found that these carbon signals appear at 190.7 and 195.7 ppm, which tends to agree with literature values for carbonyl containing organic compounds. Their erroneous $^{13}$C NMR signals are, again consistent with signals found in the by-product. They reported IR stretches at 1726 and 1645 cm$^{-1}$, while we found only overlapping CO stretches at 1678 cm$^{-1}$. Perhaps most damning is that they report a melting point of 115 °C of 142 while the compound is in fact an oil at room temperature.$^78$ This evidence is cause for suspicion because they report that their elemental analysis is within 0.04% for carbon, nitrogen and hydrogen, which means that the compound was especially pure. We can be fairly definite about the identity of the compound 142 we isolated since we have single crystal x-ray analysis of a later intermediate in our synthesis, so potentially the Kumar report$^78$ had incorrectly characterized a mixture of product and impurity.
While the by-product was never fully identified, we suspect that it may be some intermediate. Figure 52 shows a possible identity of this impurity, 153. This compound is plausible because, if the hydroxy moiety were replaced by a hydrogen, then this would be the final product of the oxidative rearrangement, as can be deduced from the mechanism and has been isolated previously in related systems. It would make sense that 153 would be difficult to completely isolate because of its highly reactive nature and potential degradation through dinitrogen extrusion.
Initially, we had hoped to avoid extensive column chromatography of compound 142, so a simple silica plug was utilized to reduce the quantity of impurities. This purification was suitable for the next reaction, wherein the aldehyde was oxidized directly to the ester by Oxone®, a potassium peroxymonosulfate triple salt (Figure 53). This reaction putatively goes by a Baeyer-Villiger type mechanism. Initially, we used isopropyl alcohol as the solvent, but the product was not observed, only some other oxidation product. By electrospray ionization-mass spectrometry we observed that the other oxidation product might be the isopropyl carbonate (156), as a Dakin oxidation intermediate. The methyl ester 155 was afforded from oxidation in methanol in moderate yields (60%), so we moved forward with the deoxygenation of the ketone.

![Figure 52: Structure of Suspected Impurity](image)

Initially, we had hoped to avoid extensive column chromatography of compound 142, so a simple silica plug was utilized to reduce the quantity of impurities. This purification was suitable for the next reaction, wherein the aldehyde was oxidized directly to the ester by Oxone®, a potassium peroxymonosulfate triple salt (Figure 53). This reaction putatively goes by a Baeyer-Villiger type mechanism. Initially, we used isopropyl alcohol as the solvent, but the product was not observed, only some other oxidation product. By electrospray ionization-mass spectrometry we observed that the other oxidation product might be the isopropyl carbonate (156), as a Dakin oxidation intermediate. The methyl ester 155 was afforded from oxidation in methanol in moderate yields (60%), so we moved forward with the deoxygenation of the ketone.

![Figure 53: Direct Access to the Ester 155 from the Aldehyde 154](image)
2.3.2 Deoxygenation

Deoxygenation was attempted via a method reported by the Merck Research Labs, utilizing $\text{H}_3\text{PO}_2$ as the hydrogen source in a sodium iodide catalyzed reduction of di(hetero)aryl ketones.\textsuperscript{97,98} As before, the lactone \textbf{160} was a very stable intermediate, but continuous application of heat yielded the diarylmethane species \textbf{157}, as observed by mass spectrometry (Figure 54). This product proved difficult to isolate because in aqueous solution it is present as a zwitterion, likely only isolable by crystallization. This was not possible in our case since we were working on a very small scale.

![Scheme 15: Deoxygenation of Lactone 160 to Diarylmethane 157](image)

\begin{center}
\begin{align*}
\text{(158)} & \quad \xrightarrow{\text{Rapid}} \quad \text{(159)} & \quad \xrightarrow{\text{O/N}} \quad \text{(160)} & \quad \xrightarrow{\text{4 days}} \quad \text{(157)} \\
&m/z \ 227+H & m/z \ 211+H & m/z \ 213+H
\end{align*}
\end{center}

\textbf{Figure 54: Deoxygenation by Hypophosphoric Acid}

While working on the deoxygenation by hypophosphorous acid, another route towards the diarylmethane core was explored using a Wolff-Kishner reduction. Instead of directly oxidizing the aldehyde, an acetal protecting group was installed on the aldehyde by acid catalysis and removal of water \textit{via} a Dean-Stark apparatus (Figure 55). This reaction required extensive purification of the starting material by column chromatography, otherwise the compound decomposed under the high temperatures employed.
Figure 55: Acetal Protecting Group Installation

Transformation of 142 to 161 represents the first synthesis of the 1-(1,2-dioxolanyl)-2-carbonyl-benzene motif from 1,2-(dicarbonyl)-benzene starting material (Figure 56). Previous reports generally utilize addition of a Grignard reagent into an aldehyde and subsequent oxidation of the alcohol (Figure 56). While we are not totally certain why three equivalents of ethylene glycol were required for the protection, use of 1.2 equivalents of ethylene glycol gave a 50% conversion. Possibly the ethylene glycol-toluene azeotrope came into effect, where ethylene glycol boiled off with the toluene, and was subsequently removed from the system because it separated into the water layer in the Dean-Stark collector. Interestingly, no reaction at the ketone was observed, possibly due to the large steric hindrance. After conditions were optimized, this reaction required no column chromatography to purify the products. This reaction was scaled to 15 grams starting material without effect on the procedure or yield.

Figure 56: Access to 1-(1,2-dioxolane)-2-carbonyl-benzene Motif

Wolff-Kishner deoxygenation of the acetal protected diaryl ketone was facile. As seen in Figure 57, treatment of the ketone 161 with distilled hydrazine (35% in H2O) and subsequently with KOH under strict exclusion of air at 135 °C in a sealed vessel, yielded compound 164 in excellent yields. No
hydrazinylpyridines or hydroxypyridine products were observed as result of ring-addition. The acetal protecting group was then quantitatively removed under aqueous acidic conditions by warming for three hours and then stirring at room temperature overnight following a previously reported procedure. The deoxygenation and deprotection steps were scaled to 18 and 16 gram scales, respectively, without change in yield or procedure.

![Figure 57: Optimized Deoxygenation and Deprotection](image)

2.3.3 Nitration

Stirring 165 in sulfuric and nitric acid yielded the appropriately substituted nitro derivative 166 in good yields, with no nitration of the pyridine or over-nitration products detected (Figure 58). The position of the nitro group was determined by the multiplicity of signals in the $^1$H NMR spectrum, and subsequently by X-ray crystallography.

![Figure 58: Nitration of 165](image)
Thus the desired carbonyl substituted NBP core was obtained in 59% yield over six steps with only one step requiring column chromatography, and was scaled to furnish over 10 grams of 166. At this point in the synthesis, the molecule became quite sensitive to Lewis acids bases and oxidants. Initially KOH pellets were used to adjust the pH of the concentrated acid reaction mixture, but this resulted in a broad mixture of unidentified products. Dropwise addition of a saturated solution of sodium bicarbonate to a stirring reaction mixture while cooling the flask periodically resulted in a clean product after extraction. Likely addition of the nitro group increased the lability of the methylene protons, and deprotonation by concentrated KOH led to undesirable by-products. Additionally, the nitro compound was first extracted with either chloroform or dichloromethane, which led to a bluish colored product. Compound 166 may be alkylated by halogenated solvents, and under the basic extraction conditions, the dye may form. The impurity of this dye could not be observed in NMR spectroscopy, but the coloration of the compound compromised the color purity of 166. Precipitation of 166 from ethyl acetate by the addition of petroleum ether yielded the orange, visually pure product. Extraction with ethyl acetate did not yield any colored impurities.

While requiring more steps, the sequence of reactions leading to 166 compares favorably to the reported synthesis of di- and tri-nitrobenzylpyridines by Herges et al., wherein nucleophilic aromatic substitution (S_NAr) of 2,4,6-trinitrofluorobenzene (168) with 4-TMS-methyl-pyridine (167) gave significantly lower yields (Figure 59).¹⁰¹ Herges et al. also reported difficulty in transition metal catalyzed coupling reactions and Grignard reactions to achieve the nitrobenzylpyridine motif. The overall synthesis leading to 166 is shown in Figure 60.
Figure 59: Nucleophilic Aromatic Substitution of 2,4,6-trinitrofluorobenzene with 2-(trimethylsilylmethyl)pyridine

Previously Reported

![Chemical Structures]

Figure 60: Overall Synthesis of 166

![Overall Synthesis Diagram]
2.3.4 Growing Crystals

In order to perform X-ray crystallography, a single crystal of **166-HCl** was grown from the slow generation of HCl in chloroform. As posited in *Path A* of Figure 61, the action of imidazole on tert-butyldiphenylsilyl chloride (TBPSCl) at room temperature very slowly generates HCl, which can be trapped by the pyridine functionality. Alternatively, the silylimidazole salt **167** could directly protonate the pyridine in **166**, as per *Path B*. While the formation of a related imidazole trialkysilane has been reported, no report of this practice has been reported in growing HCl-adduct crystals. This method yielded a slow formation of the HCl salt, which gave single crystal x-ray crystallographic quality crystals of **169**. HCl-adduct single crystals of 4-(4-nitrobenzyl)pyridine were also be grown by this method. **169** has similar bond lengths and angles as reported in related structures.

![Figure 61: Proposed Mechanism of the Formation of 169 by TDPSCl and Imidazole](image-url)
Figure 62: ORTEP Diagram of 169

*Tert*-butyldiphenylsilyl chloride gave the favorable slow formation of crystalline material, while treatment of 166 with trimethylsilyl chloride (TMSCl) resulted in immediate generation of dye 170 and rapid decomposition and precipitation. In addition to producing a slow infusion of HCl, the steric hindrance of TBPSCl prevented attack of the silane by the pyridine nitrogen (Figure 63).
To probe the effects of the oxidation state of the carbonyl substituent on 166, the carboxylic acid and alcohol were prepared by oxidation and reduction of the aldehyde, respectively. The carboxylic acid could not be isolated from oxidation by KMnO$_4$, so a Pinnick oxidation was attempted as shown in Figure 64. Under Pinnick conditions, the carboxylic acid 85 precipitated out cleanly, but one half of the starting material was over-oxidized to the dicarbonyl derivative, 171. Though benzylic oxidations are known to occur with sodium chlorite, they generally require an additional oxidant like tert-butylhydroperoxide or a transition metal catalyst, so this result was unexpected. 104
This reaction was optimized to yield the carboxylic acid preferentially by reducing equivalents of sodium chlorite and using less water in the reaction to decrease the solubility of the mono-oxidized product. These efforts led to moderate yields of 76% of the desired carboxylic acid 85.

Reduction of 166 to the alcohol 172 proceeded smoothly by treatment with sodium borohydride in ethanol using a procedure previously reported (Figure 65). Fortuitously, the nitro and methylene groups were unaffected over the course of this reaction.

Additionally, the benzyl imine 173 was generated by condensation of 166 with benzylamine in quantitative yields as per a modified procedure from Organic Syntheses (Figure 66). This preparation of an imine from an aldehyde and a primary amine is very mild and proceeds to completion with no starting material or by-products detected. The condensation reaction is presumably driven by the trapping of water as the magnesium sulfate hydrate. Filtering the resultant mixture through anhydrous sodium sulfate and removal
of the solvent under reduced pressure gives the desired imine product. We found that this procedure may be used with ethyl acetate, ethyl ether, dichloromethane or chloroform as the solvent depending on the sensitivity and solubility of the starting materials. As expected, if the imine condensation of 166 is performed in dichloromethane then visible darkening is observed, which is consistent with previously observed interaction of the pyridine with DCM and resultant dye formation.

Figure 66: Formation of Imine 16
Chapter 3  Assays of Cyclophosphamide by NBP and its Derivatives

Figure 67: Synthesized NBP Derivatives

After the core of the NBP was attained by synthesis, cyclophosphamide was assayed with the varying NBP compounds (Figure 67) to compare their molecular sensing capabilities directly with our previous results from the parent compound, 4-(4-nitrobenzyl)pyridine. The assay method was modified from a previous report.\textsuperscript{107}

1. Generate a 105-6740 μM (27.5-1827 ppm) calibration curve of cyclophosphamide in water by serial dilutions and three blanks, with all samples being 1 mL.
2. Cool samples to 0 °C
3. Add 1 mL 0.2 M NaOAc buffer pH 4.5
4. Add 0.75 mL 3.3% (w/v) NBP-X in solvent
   a. For NBP, 166, and 173, the solvent was acetone
   b. For 85, the solvent was H₂O, with saturated KOH added until the potassium salt dissolved
   c. For 172, the solvent was dimethylformamide
5. The samples were heated to 100 °C for twenty minutes in closed vials
6. After cooling samples to RT, 2.5 mL 1:1 triethylamine:acetone was added
7. For 85, the dye did not form on triethylamine addition. 200 µL saturated aqueous KOH was added to achieve dye formation.

8. Samples were shaken and UV/Vis spectra were taken within an hour.

Some coloration occurred in the blank samples, so the UV/Vis spectra were taken in scan mode and the blank sample spectra were subtracted from the sample spectra. The method required no avoidance of light or extraction of the dye by organic solvent. The assay was run once for each NBP derivative with the same stock solution of cyclophosphamide. The same stock solution was used across all assays, ensuring that the data are internally consistent. Our assay technology is shown to detect down to nearly 100 µM without optimization, and considering cyclophosphamide is dosed at approximately 192-239 µM with 75% bioavailability, the assay could be useful in biological applications.
Figure 68: Calibration Curves from the Assay of Cyclophosphamide with NBP and its Derivatives

As can be seen from the above graph (Figure 68), the NBP compounds differed significantly over the tested series. The molar absorptivities of the dyes resulting from reaction of the NBP series with CP followed the trend $85 > 166 \approx 173 > \text{NBP} \approx 172$, as shown in Table 2.
Table 2: Photophysical Properties of NBP Based Dyes

The analytical wavelength ($\lambda_{\text{anal}}$) was taken as the wavelength of highest intensity in the 400-700 nm range after background corrections were made. This wavelength correlated somewhat to the molar absorptivities among the dyes; however, most interesting was the correlation between the identity of the functional group $R$ and the analytical wavelength. It is not unreasonable to predict that the structures of the dyes should be more twisted (Figure 69) at the methine position as the $R$ group becomes more sterically bulky. This twist would result in a less conjugated chromophore which would absorb a shorter wavelength; however, this rationalization does not fit the experimental trend where the most sterically demanding benzyl imine and carboxylate groups absorbed at the longer wavelengths.

![Diagram of NBP dye structures](image)

Table: Photophysical Properties of NBP Based Dyes

<table>
<thead>
<tr>
<th>$R$</th>
<th>$\varepsilon$ (L mol$^{-1}$cm$^{-1}$)</th>
<th>$\lambda_{\text{anal}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_2$OH</td>
<td>0.46 x 10$^6$</td>
<td>570</td>
</tr>
<tr>
<td>H</td>
<td>0.55 x 10$^6$</td>
<td>575</td>
</tr>
<tr>
<td>CHNBn</td>
<td>0.77 x 10$^6$</td>
<td>581</td>
</tr>
<tr>
<td>CHO</td>
<td>0.89 x 10$^6$</td>
<td>587</td>
</tr>
<tr>
<td>COOK</td>
<td>2 x 10$^6$</td>
<td>603</td>
</tr>
</tbody>
</table>

Figure 69: "Flat" and "Twisted" Conformation of NBP Dyes

The best explanation of the molar absorptivity and absorbance wavelength trends seems to be a correlation to the oxidation state of the benzylic carbon in the synthesized NBP series. By assigning oxidation
state values to carboxylic acid $85$, aldehyde $166$, imine $173$, and alcohol $172$ as $+3$, $+2$, $+2$, and $+1$, respectively, we can graph these values against the light absorbance values from Table 2 and see a decent correlation (Figure 70).

![Benzylic Oxidation State Correlation to $\lambda_{\text{max}}$ and Molar Absorptivity](image)

**Figure 70: Correlation of Benzylic Oxidation state to light absorbance values**

In 2001 the Pagani group in Milan published a comparison of a wide range of quinoid/zwitterion dyes shown in Figure 71.$^{108}$ Utilizing $^{15}$N and $^{13}$C NMR spectroscopy they were able to determine the relative neutral-quinoid or zwitterionic-aromatic nature of the dyes across the series. They did so by determining the anisochrony of the chemical shifts of the heterocycle ring, which arose from greater double bond character of the heterocycle-bridging atom bond. Between $176$ and $179$, which were the closest to the compounds studied in this work, the more electron-poor dinitrophenyl ring stabilized the carbanion better by inducing a more...
zwitterionic/aromatic character. 179 absorbs a longer wavelength than 176, which compares to our results where an electron withdrawing ring substituent causes an increase in the absorbance wavelength. Stabilization of the carbanion should generate a larger chromophore by increasing resonance into the electron-poor benzene ring.

Study of the photophysics in our system is ongoing. Solvatochromic studies of these compounds suffer from the fact that they are not soluble in the same solvents, and the UV-Vis spectra the dyes generated in other ways give extremely broad peaks which are difficult to interpret. We did observe qualitatively that the more stabilized carbanion exhibits the greater stability as a dye. The dye generated from 172 and cyclophosphamide was stable for only approximately thirty minutes, while the dye generated from the potassium salt of 85 exhibits no degradation after sitting at room temperature under ambient conditions in DMSO for over six months.

Figure 71: Characterizing a Push-Pull Chromophore
Aldehyde **166** was found to not react with the Lewis acidic metal ions Sc(III) triflate or zinc acetate. Brønsted acids were ruled out since generation of the compound is controlled by pH adjustments. **166** reacted and produced dyes with electrophiles in unbuffered solvent systems successfully; however, these conditions did not appear to be optimal spectroscopically since the peaks in the UV-Vis spectra were very broad.

*Figure 72: Relative Wavelength Absorptions of NBP Based Dyes*
Chapter 4  Installing a Linker and Materials Synthesis

After discovering that the synthesized series of NBP derivatives show favorable assay properties, we set about trying to install a linker to the three molecules with the greatest sensing ability, 85, 166, and 173. The first linker installed was via an imine condensation because this was considered to be the easiest reaction. Stirring 3-aminopropyltriethoxysilane (APTES) with 166 in ethyl acetate with two equivalents anhydrous magnesium sulfate at room temperature overnight yielded 184 quantitatively.

\[
\text{NO}_2 \quad \text{H}_2\text{N} - \text{Si(OEt)}_3 \\
\text{O} \quad \text{H}_2\text{N} - \text{Si(OEt)}_3 \\
\text{N} \quad \text{H}_2\text{N} - \text{Si(OEt)}_3
\]

\(2\text{ equiv. MgSO}_4\)

EtOAc, RT, O/N

\(>99\%\) Yield

Figure 73: APTES Linker Installation by Imine Bond Formation

Previously, we had installed the APTES linker into 2-iodo-5-nitrobenzoic acid via the acid chloride (Figure 74), so we imagined that the linker could be installed similarly into 85.

\[
\text{O}_2\text{N} \quad \text{I} \quad \text{OH} \\
\text{O}_2\text{N} \quad \text{N} - \text{Si(OEt)}_3
\]

\(\text{SOCl}_2\)

reflux, 3 hr

DCM, reflux, O/N

40\% Yield

Figure 74: Silane Linker Installation into 2-iodo-5-nitrobenzoic Acid

However, after heating 85 in thionyl chloride, apparent oxidation of the benzylic carbon to a ketone 185 was observed, which was somewhat surprising, but consistent with our previous observations that this
benzylic position is sensitive to oxidation (Figure 75). A handful of reports of oxidation by thionyl chloride have been reported,\textsuperscript{109} but few lead to the direct installation of oxygen.

![Figure 75: Apparent Oxidation of 14 in Thionyl Chloride](image)

In order to probe this unusual reactivity of the benzylic group on 4-picoline, we tried reacting other picolines with thionyl chloride. After heating the picoline containing starting material in thionyl chloride, the thionyl chloride was removed \textit{in vacuo} and the resulting species quenched with wet methanol. Similarly, 4-(4-nitrobenzyl)pyridine was oxidized to the diaryl ketone; however, reaction of thionyl chloride with 4-picoline led to 4-trichloromethylpyridine (82%) and methyl isonicotinate (18%) as determined by GC-MS. The trichlorination of the 4-picoline methyl group and its subsequent hydrolysis to the carboxylic acid has been reported previously,\textsuperscript{110,111} however, the direct conversion to the ester is novel upon searching literature. Direct access to an ester from an alkane in this way would be advantageous to multi-step syntheses. Furthermore, this testing with 4-picoline suggests that chlorination/hydrolysis is the likely mechanism of the apparent oxidation of 4-(4-nitrobenzyl)pyridine and 85 by thionyl chloride (Figure 76). In fact, we were able to isolate and characterize the trichlorinated form of 85 (compound 189 in Experimental and Appendix) evinced by the shift of the methylene carbon signal from 37.7 ppm to 96.6 ppm in \textsuperscript{13}C NMR spectroscopy.
Thus we looked towards gentler amide coupling reactions. Treatment of 85 with carbonyldiimidazole (CDI) at room temperature in dry DMF results in an 85-imidazole complex which underwent amide coupling (Figure 77). The product was observed by mass spectrometry, but decomposed on a silica column. Upon addition of CDI to 85, the solution turned bright red, which is reminiscent of the color reported for the reaction of NBP with phosgene, so potentially some dye formation occurs upon reaction with CDI, as shown in Figure 77. While this undesired reactivity led to a complex mixture, the desired product was observed as the dominant specie in high resolution mass spectrometry.

**Figure 76: Mechanism of Apparent Oxidation of 4-picoline**

![Mechanism of Apparent Oxidation of 4-picoline](image-url)
Though amide coupling with CDI led to a broad mixture of products, the method was superior to coupling with PyBOP, which gave a dark purple dye immediately upon mixing with 85 in DMF, and led to a complex mixture of products. Sensitivity of carboxylic acid 85 to the Lewis acidic phosphonium moiety of PyBOP was surprising, since this reagent is considered very mild and is often employed to avoid racemization in amide bond forming reactions. Since 193 could not be cleanly isolated, the reaction mixture was added directly to a sol-gel forming reaction after coupling with CDI.

**Figure 77: Possible Reactions in CDI Coupling**

Though amide coupling with CDI led to a broad mixture of products, the method was superior to coupling with PyBOP, which gave a dark purple dye immediately upon mixing with 85 in DMF, and led to a complex mixture of products. Sensitivity of carboxylic acid 85 to the Lewis acidic phosphonium moiety of PyBOP was surprising, since this reagent is considered very mild and is often employed to avoid racemization in amide bond forming reactions. Since 193 could not be cleanly isolated, the reaction mixture was added directly to a sol-gel forming reaction after coupling with CDI.
4.1 Sol-Gels

Three different moieties were applied in sol-gel synthesis and cursorily tested for their alkylating agent sensing capabilities. Potassium salt of carboxylic acid 85, imine 184 and amide 193 were stirred with ten equivalents of tetraethylorthosilicate in ethanol/water with sodium carbonate as catalyst, as per a modified procedure. Most sol-gels are formed under acid catalysis, but we imagined that a basified sol-gel might facilitate the dye forming mechanism. Gratifyingly, exposure of these materials to iodomethane vapors gave a colored response without requirement for additional base. The carboxylate 85 based sol-gel gave the most intensely purple response, while interestingly the imine linked 184 sol-gel gave a lighter green response (Figure 78). The sol-gels formed by acid catalysis showed no response, even after subsequent addition of base.

The response was fairly slow, with all the sensors requiring a few hours to exhibit a signal upon exposure to gaseous iodomethane. While this might preclude the development of these sensors as a quick-check exposure sensor, they may work as a badge to measure overall exposure to alkylating agents over a longer period of time. Iodomethane was detected more rapidly in solution, with a response observed in less than ten minutes for carboxylate 85 based sol-gel.
<table>
<thead>
<tr>
<th>Sol-Gel Additive</th>
<th>Material Before MeI Exposure</th>
<th>Material After MeI Exposure</th>
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**Figure 78:** Responses of Sol-Gels to MeI vapors
Chapter 5 Conclusions

In conclusion, a range of novel sensor molecules based on the NBP motif were synthesized, characterized and tested in solution and solid state for detection of the alkylating agents cyclophosphamide and iodomethane. Key intermediate 166 was achieved in six steps in 59% yield overall and was scaled to yield over ten grams of the aldehyde derivative 166. Also reported is an X-ray crystal structure of the 166·HCl adduct 169. The synthesis represents a new method in the limited repertoire for the generation of 4-benzylpyridine derivatives, specifically with an ortho carbonyl group on the benzene ring. Of these compounds synthesized, the potassium salt of carboxylic acid derivative 85 was found to be the most efficacious in sensing alkylating agents both in solution and in the solid state. The potassium salt of 85 has significant advantage over state of the art compound NBP. The compound is soluble in water, which increases in vivo likeness of sensing conditions while also producing a four-fold stronger color when in the dye state. Containing a carbonyl, 85 has the potential to sense O-alkylating agents in addition to N-alkylating agents. Finally, the potassium salt of 85 may be impregnated directly into a sol-gel towards as solid state sensor.
Chapter 6  Experimental

6.1  General Procedures

Reactions under N₂ gas were performed by standard Schlenk techniques with a Chemglass dry N₂/vacuum manifold. NMR spectra were recorded on Bruker Avance 300 or Bruker Avance 400 spectrometers. ¹H NMR spectra are reported in parts per million and were referenced to residual solvent: CDCl₃ δ = 7.27 ¹H NMR, 77.00 for ¹³C NMR, for DMSO-d₆ δ = 2.50 ¹H NMR, 39.51 for ¹³C NMR, for (CD₃)₂CO δ = 2.05 ¹H NMR, 206.68 for ¹³C NMR. The multiplicities are abbreviated as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, and br = broad signal. Coupling constants were measured in Hz and abbreviated as J. All spectra were obtained at 25 °C. Mass spectra were recorded on Waters/Micromass LCT mass spectrometer. IR spectra were obtained in thin film on PerkinElmer Frontier FT-IR spectrometer equipped with diamond Attenuated Total Reflectance. UV/Vis spectroscopy was done with a Varian Cary 5000 UV-Vis-NIR spectrophotometer. Thin layer chromatography (TLC) plates were stained with the Dragendorff stain.¹ All single crystal x-ray measurements were made on a Bruker APEX DUO diffractometer with graphite monochromated Mo-Kα radiation.

6.2  Materials and Methods

When use of dry solvents is indicated, THF and methylene chloride (DCM) were purified via passage through solvent purification columns. Hydrazine (50-60% in H₂O) was distilled at 10 mTorr. Nitric acid (70%) and ethylene glycol were distilled at ambient pressure. All other reagents and solvents were obtained from commercial sources and used as received.

¹ The Dragendorff stain is prepared as follows: two solutions are first prepared (1) 3.4 g bismuthyl nitrate in 200 mL 20% acetic acid solution, and (2) 40 g KI in 200 mL deionized water. The stain itself is prepared by mixing solution (1) (2 mL) and solution (2) (2 mL) into a mixture of 8 mL glacial acetic acid and 28 mL deionized water. The resulting solution was stored at room temperature and was prepared fresh every two weeks or so.
**N’-(2-hydroxybenzylidene)isonicotinohydrazide (152):** Isoniazid (20 g, 146 mmol), salicylaldehyde (15.5 mL, 146 mmol) and ethanol (700 mL) were combined in a round bottom flask and heated to reflux overnight while stirring. The title compound formed a white precipitate, which was filtered and washed with chilled ethanol (33.14 g, 97%): $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta = 12.29$ (s, 1 H), $11.08$ (s, 1 H), $8.80$ (d, $J = 5.8$ Hz, 2 H), $8.68$ (s, 1 H), $7.92—7.75$ (m, 2 H), $7.62—7.57$ (m, 1 H), $7.32$ (s, 1 H), $6.99—6.87$ (m, 2 H) $^{13}$C{$_1$H} NMR (101MHz, DMSO-d$_6$) $\delta = 161.3, 157.4, 150.4, 148.9, 140.0, 131.7, 129.2, 121.5, 119.4, 118.7, 116.4$; HRMS (ESI+ TOF MS) $m/z$ 242.0930 (calcd for C$_{13}$H$_{11}$N$_3$O$_2$ + H$^+$ 242.0930)

![N’-(2-hydroxybenzylidene)isonicotinohydrazide](image)

**2-isonicotinoylbenzaldehyde (142):** Compound 3 (25 g, 103.7 mmol) was dissolved in dry THF (625 mL) in a two liter flame dried two necked flask. Lead tetraacetate (50.5 g, 113.9 mmol) was added slowly under nitrogen while vigorously stirring, resulting in a strong effervescence. The vessel was then closed with a ground glass stopper, though kept open to the Schlenk line in order to allow for escaping gas. The mixture was stirred for three hours at room temperature. The THF was removed under reduced pressure and the mixture was dissolved in ethyl acetate. The resulting slurry was then filtered through a silica plug. The title compound was purified by gradient column chromatography (loading with 1:1 ethyl acetate:light petroleum ether and gradient to neat ethyl acetate) yielding a light yellow oil (16.6 g, 76%): IR (ATR) 1678.81 cm$^{-1}$ (overlapping C=O); $^1$H NMR (400MHz, CDCl$_3$) $\delta = 10.02$ (s, 1 H), $8.80$ (d, 2 H, $J = 5.8$ Hz), $8.05—8.01$ (m, 1 H), $7.79—7.74$ (m, 2 H), $7.57—7.56$ (m, 2 H, $J = 4.4, 1.7$ Hz), $7.53—7.48$ (m, 1 H); $^{13}$C{$_1$H} NMR (101MHz, CDCl$_3$) $\delta = 195.7, 190.5, 150.4, 142.7, 138.9, 135.2, 133.7, 131.6, 131.1, 128.6, 122.0$; HRMS (ESI+ TOF MS) $m/z$ 212.0711 (calcd for C$_{13}$H$_9$NO$_2$ + H$^+$ 212.0712)
Methyl 2-isonicotinoylbenzoate (155): 90 mg of 142 (0.43 mmol) was dissolved in 4.3 mL MeOH in a 1 mL round bottom flask, to which was added 262 mg Oxone® triple salt (KHSO₅·½KHSO₄·½K₂SO₄) (0.85 mmol). The slurry was stirred at reflux overnight and then filtered through Celite®. The solvent was removed under reduced pressure and the product was purified via gradient column chromatography (1:1 light petroleum ether to neat ethyl acetate) to yield a colorless residue (62 mg, 60%): ¹H NMR (400MHz, CDCl₃) δ = 8.81 (br., 2 H), 8.09 (dd, J = 8 Hz, 1 H), 7.60-7.73 (m, 3 H), 7.55 (br., 2 H), 7.42 (dd, J = 1.2, 7.3 Hz, 1 H), 3.66 (s, 3 H); ¹³C{¹H} NMR (101MHz, CDCl₃) δ = 196.4, 166.3, 151.0, 143.6, 140.7, 133.2, 130.7, 130.6, 129.4, 128.0, 52.7, 1.3. HRMS (ESI+ TOF MS) m/z 242.0816 (calcd for C₁₄H₁₂NO₃ + H⁺ 242.0817)

(2-(1,3-dioxolan-2-yl)phenyl)(pyridin-4-yl)methanone (161): Compound 142 (14.6 g, 69.2 mmol) was dissolved in toluene (511 mL). p-Toluenesulfonic acid (0.4 g, 2.1 mmol) and distilled ethylene glycol (8.76 mL, 157 mmol) were then added to the solution. The solution was heated to reflux overnight with a Dean-Stark apparatus installed. After cooling the reaction to room temperature, saturated aqueous sodium bicarbonate (50 mL) was added. The toluene layer was separated, washed with saturated aqueous sodium bicarbonate (50 mL) and washed with brine (50 mL). The organic layer was dried over anhydrous Na₂SO₄ and subsequently filtered. Removal of toluene under reduced pressure afforded the title compound as a yellow oil (17.4 g, 98%): IR (ATR) 1675.73 cm⁻¹ (C=O); ¹H NMR (400MHz, CDCl₃) δ = 8.84—8.72 (m, 2 H), 7.70 (d, J = 7.5 Hz, 1 H), 7.60—7.53 (m, 3 H), 7.45 (t, J = 7.5 Hz, 1 H), 7.30 (d, J = 7.5 Hz, 1 H), 6.02 (s, 1 H), 3.91—
3.80 (m, 4 H); \(^{13}\text{C}\{^1\text{H}\} \text{NMR (101MHz, CDCl}_3 \delta = 196.1, 166.0, 150.7, 143.3, 140.4, 132.8, 130.3, 130.2, 129.1, 127.7, 52.4, 1.0; HRMS (ESI+ TOF MS) m/z 256.0978 (calcd for C\(_{15}\)H\(_{13}\)NO\(_3\) + H\(^+\) 256.0974)

4-(2-(1,3-dioxolan-2-yl)benzyl)pyridine (164): Compound 161 (17.7 g, 69.4 mmol) was dissolved in a minimum amount of methylene chloride (DCM) and transferred into a flame dried double walled schlenk bomb under nitrogen gas. The DCM was removed \textit{in vacuo} and distilled hydrazine (140 mL, 50-60\% in H\(_2\)O) was added \textit{via} syringe with a septum in place. The flask was sealed by a Teflon stopper, heated to 135°C, and stirred vigorously overnight. Upon heating the starting material became soluble in the aqueous hydrazine mixture. The flask was then allowed to cool to room temperature while stirring under ambient conditions and opened under nitrogen gas. Powdered KOH (19.14 g, 341 mmol) was added, the tube was resealed, and heated to 135°C overnight with vigorous stirring. After cooling, glass precipitate was observed and the reaction was quenched with 80:20 DCM:isopropanol (10 mL). Deionized water (10 mL) was added and the mixture was extracted with DCM (3 x 100 mL). The organic layer was washed with brine, dried over anhydrous sodium sulfate and the DCM was removed under reduced pressure to yield the title compound as a yellow oil (15.9 g, 95\%): IR (ATR) 1597.81 cm\(^{-1}\) (arom.); \(^{1}\text{H} \text{NMR (400MHz, CDCl}_3 \delta = 8.55—8.41 (m, 2 H), 7.65—7.59 (m, 1 H), 7.34—7.30 (m, 2 H), 7.14—7.09 (m, 1 H), 7.08 (d, J = 5.8 Hz, 2 H), 5.87 (s, 1 H), 4.17 (s, 2 H), 4.15—3.97 (m, 4 H); \(^{13}\text{C}\{^1\text{H}\} \text{NMR (75MHz, CDCl}_3 \delta = 149.8, 139.8, 137.0, 135.6, 130.8, 129.4, 127.0, 126.7, 124.2, 101.9, 65.2, 37.3; HRMS (ESI+ TOF MS) m/z 242.1179 (calcd for C\(_{13}\)H\(_{13}\)NO\(_2\) + H\(^+\) 242.1181)
2-(pyridin-4-ylmethyl)benzaldehyde (165): Aqueous 3M HCl (561 mL) was added to compound 164 (11.5 g, 48 mmol) in a one liter round bottomed flask and stirred at 50°C for three hours. After heating the reaction for three hours, the heating bath was removed and the flask was allowed to cool to room temperature, at which temperature the reaction was stirred overnight. The reaction mixture was made basic by addition of KOH pellets while stirring. The mixture was extracted with DCM (4 x 60 mL), the organic layer was washed with brine, dried with anhydrous sodium sulfate and the solvent was removed under reduced pressure to yield the title compound quantitatively as a bright green viscous oil (9.4 g): IR (ATR) 1697.22 (C=O), 1597.49 (arom.) cm⁻¹; ¹H NMR (300MHz, CDCl₃) δ = 10.14 (s, 1 H), 8.49 (d, 2 H, J = 4.3 Hz), 7.87 (dd, 1 H, J = 7.5, 1.4 Hz), 7.61—7.48 ppm (m, 2 H), 7.29 (d, 1 H, 8.7 Hz), 7.07 (d, 2 H, 5.3 Hz), 4.46 (s, 2 H); ¹³C{¹H} NMR (101MHz, CDCl₃) δ = 192.5, 149.7, 149.3, 140.3, 134.1, 133.95, 133.93, 131.9, 127.6, 124.0, 37.7; HRMS (ESI+ TOF MS) m/z 198.0916 (calcd for C₁₃H₁₁NO + H⁺ 198.0919)

5-nitro-2-(pyridin-4-ylmethyl)benzaldehyde (166): After charging into a 350 mL round bottomed flask, compound 165 (10.5 g, 53.3 mmol) was cooled to -10 °C in a dry ice/ethylene glycol bath and concentrated H₂SO₄ (52.6 mL) was added to form a thick red tar. A 1:2 HNO₃/H₂SO₄ mixture (52.61 mL) was then slowly added, and the reaction was stirred overnight by spinning the flask mechanically in the cooling bath. The resulting orange solution was neutralized by dropwise addition of saturated aqueous sodium
bicarbonate solution and periodically cooling the flask with cold water. Excessive foam from carbonate neutralization at this time required that the reaction be split into four two liter round bottom flasks. The resulting aqueous solutions were extracted individually with ethyl acetate (5 x 100 mL), combined, and then the organic layers were washed with brine, dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The compound was taken up in chloroform and precipitated out by the addition of light petroleum ether to yield the product as an orange crystalline material. The solution over the material was first removed by Pasteur pipette and the residual solvent was removed from the solid product in vacuo (11.1 g, 86%): IR (ATR) 1702.29 (C=O), 1599.72 (arom.), 1522.17 (NO₂, as) cm⁻¹; 1H NMR (300MHz, CDCl₃) δ = 10.16 (s, 1 H), 8.63 (d, J = 2.3 Hz, 1 H), 8.44 (br., 2 H), 8.32 (dd, J = 2.5, 8.5 Hz, 1 H), 7.46 (d, J = 8.5 Hz, 1 H), 7.01 (d, J = 3.88 Hz, 2 H), 4.50 (s, 2 H). ¹³C{¹H} NMR (101MHz, CDCl₃) δ = 190.1, 150.1, 147.4, 147.3 (overlapping), 147.2, 134.6, 133.1, 128.3, 127.9, 124.0, 37.6; HRMS (ESI+ TOF MS) m/z 243.0773 (calcd for C₁₃H₁₀N₂O₃ + H⁺ 243.0770)

5-nitro-2-(pyridin-4-ylmethyl)benzoic acid (85): In a 50 mL round bottomed flask, 1.033 g of 166 (4.3 mmol) was combined with 2-methyl-2-buten (1.3 mL, 12.3 mmol) in 18 mL acetone. NaClO₂ (0.7263 g, 8 mmol) and NaH₂PO₄·H₂O (4.6 g, 33.3 mmol) were dissolved in H₂O (8 mL) by sonication. The solution of NaClO₂ and NaH₂PO₄·H₂O was added to the acetone solution of 166 by Pasteur pipette and this mixture was stirred at room temperature for 45 minutes. A white precipitate formed and was filtered and washed with acetone and water (0.843 g, 76%): IR (ATR) 3084 (OH), 1708 (C=O), 1612 (arom.), 1520 (NO₂, as) cm⁻¹; 1346 cm⁻¹ (NO₂, s); ¹H NMR (300MHz, DMSO-d₆) δ = 8.56 (d, 1 H, J = 2.6 Hz), 8.43 (d, 2 H, J = 5.5 Hz),
8.27 (dd, 1 H, J = 8.5, 2.4 Hz), 7.17 (d, 2 H, J = 5.9 Hz), 4.52 (s, 2 H). $^{13}$C{$^1$H} NMR (101MHz, DMSO-d$_6$) δ = 167.5, 149.5, 149.1, 146.8, 146.0, 134.3, 133.0, 125.4, 124.9, 124.1, 37.7; HRMS (ESI+ TOF MS) m/z 259.0719 (calcd for C$_{13}$H$_{10}$N$_2$O$_4$ + H$^+$ 259.0719); Anal. Calcd. for C$_{13}$H$_{10}$N$_2$O$_4$: C, 60.47; H, 3.90; N, 10.85. Found: C, 60.47; H, 3.90; N 10.85

![Structure](image.png)

(5-nitro-2-(pyridin-4-ylmethyl)phenyl) methanol (172): 166 (0.509 g, 2.1 mmol) was dissolved in ethanol (3 mL) in a 20 mL vial and the solution was warmed to 40 °C while stirring. Solid NaBH$_4$ (0.154 g, 4.05 mmol) was added by spatula over five minutes, resulting in a black solution with some effervescence. The vial was capped and allowed to stir at 40 °C for thirty minutes, after which water (6 mL) was added to the reaction, which induced the precipitation of a yellow-orange solid. The solution was filtered off and the solid was washed with cold water. Residual water was removed from the yellow crystalline material under vacuum in a desiccator to yield the title compound (0.42 g, 82%): IR (ATR) 3162 (OH), 1602 (arom.), 1510 (NO$_2$, as) cm$^{-1}$; 1342 cm$^{-1}$ (NO$_2$, s); $^1$H NMR (400MHz, CDCl$_3$) δ = 8.50 - 8.46 (m, 2 H), 8.40 (d, J = 2.4 Hz, 1 H), 8.14 (dd, J = 2.4, 7.2 Hz, 1 H), 7.31 (d, J = 8.2 Hz, 1 H), 7.05 (d, J = 5.8 Hz, 2 H), 4.72 (s, 2 H), 4.14 (s, 2 H); $^{13}$C{$^1$H} NMR (101MHz, (CD$_3$)$_2$CO) δ = 151.2, 149.6, 148.6, 145.3, 144.2, 132.5, 125.5, 123.2, 122.9, 62.0, 38.2; HRMS (ESI TOF MS) m/z 245.0932 (calcd for C$_{13}$H$_{12}$N$_2$O$_3$ + H$^+$ 245.0926)
N-benzyl-1-(5-nitro-2-(pyridin-4-ylmethyl)phenyl)methanimine (173): Prepared in the same manner as 184 from 166 and benzylamine: IR (ATR) 1640 (C=N), 1599 (arom.), 1521 (NO₂, as), 1345 cm⁻¹ (NO₂, s); ¹H NMR (300MHz, CDCl₃) δ = 8.76 (d, J = 2.1 Hz, 1 H), 8.53 (s, 1 H), 8.48 (d, J = 5.0 Hz, 2 H), 8.23 (dd, J = 2.1, 8.2 Hz, 1 H), 7.42 - 7.28 (m, 4 H), 7.20 (d, J = 7.1 Hz, 2 H), 6.96 (d, J = 5.3 Hz, 2 H), 4.80 (s, 2 H), 4.40 (s, 2 H); ¹³C{¹H} NMR (101MHz, CDCl₃) δ = 158.0, 150.1, 148.3, 147.4, 144.9, 138.4, 135.9, 132.5, 128.8, 128.2, 127.4, 124.8, 124.4, 124.0, 65.5, 38.1; HRMS (ESI+ TOF MS) m/z 332.1395 (calcd for C₂₀H₁₇N₃O₂ + H⁺ 332.1399)

1-(5-nitro-2-(pyridin-4-ylmethyl)phenyl)-N-(3-(triethoxysilyl)propyl)methanimine (184): A flame dried flask was charged with 166 (105 mg, 0.43 mmol), ethyl acetate (1.68 mL) and anhydrous MgSO₄ (105 mg, 0.88 mmol). 3-aminopropyltriethoxysilane (100.8 uL, 0.43 mmol) was added via micropipette, the vial was closed, and the reaction was stirred overnight at room temperature. The reaction mixture was then filtered through a plug of anhydrous sodium sulfate and the solvent was removed under reduced pressure to yield the title compound quantitatively as a dark oil (191 mg); ¹H NMR (300MHz, CDCl₃) δ = 8.71 (d, J = 2.3 Hz, 1 H), 8.52 (d, J = 5.5 Hz, 2 H), 8.47 (s, 1 H), 8.20 (dd, J = 2.4, 8.3 Hz, 1 H), 7.35 (d, J = 8.5 Hz, 1 H), 7.02 (d, J = 5.7 Hz, 2 H), 4.40 (s, 2 H), 3.83 (q, J = 7.1 Hz, 6 H), 3.60 (t, J = 6.76 Hz, 2 H), 1.87 - 1.73 (m, 2

80
H), 1.23 (t, J = 7.0 Hz, 9 H), 0.60 - 0.66 (m, 2 H); $^{13}$C\{$^1$H\} NMR (101MHz, CDCl$_3$) $\delta$ = 157.0, 150.1, 148.0, 147.3, 144.5, 135.9, 132.1, 124.4, 124.2, 123.8, 64.7, 58.4, 37.8, 24.2, 18.3, 8.1; HRMS (ESI+ TOF MS) m/z 446.2115 (calcd for C$_{22}$H$_{32}$N$_3$O$_5$Si + H$^+$ 446.2111)

![Structure](image)

2-iodo-5-nitro-N-(3-(triethoxysilyl)propyl)benzamide (111): 2-iodo-5-nitrobenzoic acid (94) (5 g, 17 mmol) was suspended in 37.5 mL thionyl chloride and heated to reflux for 3 hours. The vessel was then cooled to room temperature and the thionyl chloride was removed in vacuo with a KOH trap in place to yield a yellow oil. 3-Aminopropyltriethoxysilane (3.22 mL, 13.75 mmol) and triethylamine (4.2 mL, 41.3 mmol) were dissolved in 31 mL dry DCM. This mixture was then added slowly via cannula to the previously mentioned yellow oil. The resulting mixture was heated to reflux for twelve hours with a drying tube in place. After the vessel cooled to room temperature the solvent was removed under reduced pressure and the product was isolated by gradient column chromatography (2:1 pentane:ethyl acetate to 1:1 pentane:ethyl acetate) to yield the title compound as a yellow crystalline material (2.73 g, 40%): IR (ATR) 3263 (N-H), 1640 (C=O) cm$^{-1}$; $^1$H NMR (400MHz, CDCl$_3$) $\delta$ = 8.21 (d, J = 2.4 Hz, 4 H), 8.08 (d, J = 8.5 Hz, 4 H), 7.92 (dd, J = 2.7, 8.5 Hz, 4 H), 6.41 (br. s., 4 H), 3.87 - 3.75 (m, 24 H), 3.55 - 3.46 (m, 8 H), 1.87 - 1.75 (m, 8 H), 1.20 (t, J = 7.0 Hz, 36 H), 0.79 - 0.71 (m, 8 H); $^{13}$C\{$^1$H\} NMR (101 MHz, DMSO-d$_6$) $\delta$ = 167.0, 147.1, 144.4, 140.9, 124.5, 121.9, 103.2, 57.7, 41.9, 22.4, 18.2, 7.5
2-(dichloro(pyridin-4-yl)methyl)-5-nitrobenzoyl chloride (189): Compound 85 (60 mg, 0.23 mmol) was suspended in 0.52 mL of thionyl chloride in a 5 mL two-necked round bottom flask and heated to reflux for three hours. After cooling, thionyl chloride was removed in vacuo with a KOH trap in place to yield the title compound as an orange foam (80.4 mg, >99%): IR (ATR) 1806.29 (C=O), 1534.22 (NO₂, as), 1349.58 (NO₂, s) cm⁻¹; ¹H NMR (300MHz, (CD₃)₂CO) δ = 8.94 (d, J = 4.6 Hz, 2 H), 8.81 (dd, J = 2.1, 8.5 Hz, 1 H), 8.77 (s, 1 H), 8.40 (d, J = 8.2 Hz, 1 H), 8.31 (d, J = 5.9 Hz, 2 H); ¹³C{¹H} NMR (101MHz, (CD₃)₂CO) δ = 164.3, 154.7, 153.4, 151.5, 143.6, 132.2, 126.6, 126.0, 125.4, 122.5, 96.6
References


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Appendices

NMR Spectra

Figure 79: (152) $^1$H NMR, DMSO-d$_6$, 400 MHz, 25 °C
Figure 80: (152) $^{13}$C NMR, DMSO-d$_6$, 101 MHz, 25 °C
Figure 81: (142) $^1$H NMR, CDCl$_3$, 400 MHz, 25 °C
Figure 82: (142) $^{13}$C NMR, CDCl$_3$, 101 MHz, 25 °C
Figure 83: (155) $^1$H NMR, CDCl$_3$, 400 MHz, 25 °C
Figure 84: (155) $^{13}$C NMR, CDCl$_3$, 101 MHz, 25 °C
Figure 85: (161) $^1$H NMR, CDCl$_3$, 400 MHz, 25 °C
Figure 86: (161) $^{13}$C NMR, CDCl$_3$, 101 MHz, 25 °C
Figure 87: (164) $^1$H NMR, CDCl$_3$, 400 MHz, 25 °C
Figure 88: (164) $^{13}$C NMR, CDCl$_3$, 101 MHz, 25 °C
Figure 89: (165) $^1$H NMR, CDCl$_3$, 300 MHz, 25 °C
Figure 90: (165) $^{13}$C NMR, CDCl$_3$, 101 MHz, 25 °C
Figure 91: (166) $^1$H NMR, CDCl$_3$, 300 MHz, 25 °C
Figure 92: (166) $^{13}$C NMR, CDCl$_3$, 101 MHz, 25 °C
Figure 93: (85) $^1$H NMR, DMSO-d$_6$, 300 MHz, 25 °C
Figure 94: (85) $^{13}$C NMR, DMSO-d$_6$, 101 MHz, 25 °C
Figure 95: (172) $^1$H NMR, CDCl$_3$, 400 MHz, 25 °C
Figure 96: (172) $^{13}$C NMR, CO(CD$_3$)$_2$, 101 MHz, 25 °C
Figure 97: (173) $^1$H NMR, CDCl$_3$, 300 MHz, 25 °C
Figure 98: (173) $^{13}$C NMR, CDCl$_3$, 101 MHz, 25 °C
Figure 99: (184) $^1$H NMR, CDCl$_3$, 400 MHz, 25 °C
Figure 100: (184) $^{13}$C NMR, CDCl$_3$, 101 MHz, 25 °C
Figure 101: (111) $^1$H NMR, CDCl$_3$, 400 MHz, 25 °C
Figure 102: (111) $^{13}$C NMR, DMSO-$d_6$, 101 MHz, 25 °C
Figure 103: (189) $^1$H NMR, CO(CD$_3$)$_2$, 300 MHz, 25 °C
Figure 104: (189) $^{13}$C NMR, CO(CD$_3$)$_2$, 101 MHz, 25 °C
Comparison of UV-Vis Spectra of NBP-Cyclophosphamide Dyes

UV-Vis Spectra
X-Ray Crystallography Data

Selected Crystallographic Parameters for Compound 169

Dataset ID  jl098
Empirical Formula  C₁₃H₁₁N₂O₃Cl
Formula Weight  278.69
Crystal Colour, Habit  orange, irregular
Crystal Dimensions  0.12 x 0.31 x 0.37 mm
Crystal System  triclinic
Lattice Type  Primitive
Lattice Parameters
a = 7.5626(4) Å  
b = 8.2241(4) Å  
c = 11.3238(6) Å  
α = 92.818(1)°  
β = 104.129(2)°  
γ = 113.802(2)°  
V = 616.18(6) Å³
Space Group  P-1 (#2)
Z value  2
Dcalc  1.502 g/cm³
F000  288.00
μ(Mo-Kα)  3.15 cm⁻¹
Radiation  Mo-Kα (λ = 0.71073 Å)
Data Images  1530 exposures @ 1.5 seconds
Detector Position  38.15 mm
2θmax  60.2°
No. of Reflections (I>0.00σ(I))
Measured  Total: 12030
Unique Reflections  3622 (Rint = 0.019)
Absorption
(Tmin = 0.898, Tmax = 0.963)
Max. and Min. Transmission  0.898, 0.963
R1  0.041
wr2  0.098
Largest Diff. Peak and Hole  0.55 and 0.21 e⁻/Å³
Goodness of Fit Indicator  1.04