Synthesis of a Paramagnetic Myeloperoxidase Substrate with Superoxide Dismutase Activity

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Abstract

A scheme was constructed for synthesizing a magnetic resonance imaging (MRI) contrast agent with both myeloperoxidase (MPO) sensing and superoxide dismutase (SOD) therapeutic ability. This contrast agent was designed by modifying a previously published paramagnetic SOD mimetic with therapeutic ability to contain a substrate group for MPO. This allows the contrast agent to accumulate in sites of high MPO concentration, increasing MRI signal intensity.

The scheme relies on economic dipicolinic acid as a starting material for the functionalized ligand. The reaction intended to be utilized to attach the MPO substrate is an environmentally-friendly "click chemistry" reaction. Both synthetic building blocks for the click chemistry reaction were synthesized and characterized and a new, final scheme was devised in order to increase yield and convenience.

Acknowledgments

I would like to thank Worcester Polytechnic Institute Professor Destin Heilman for setting me up on-campus with my project and really getting me started with things. I would also like to thank WPI Professor Drew Brodeur for his excellent assistance and consistent help throughout the year whenever I needed to meet with him.

Above all I would like to thank University of Massachusetts Medical School Professor Alexei Bogdanov and everyone in his molecular imaging laboratory. Dr. Bogdanov was the inspiration for this project and provided invaluable assistance on a day-to-day basis. Anyone in the lab would always be willing to assist me with a procedure or answer my questions, even the most trivial.

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Introduction

Contrast agents in Magnetic Resonance Imaging (MRI) are important tools in medical diagnostics; they are used to enhance the image or highlight specific areas in the body. MRI can generate high resolution images of complex anatomical structures. Differences in signal intensity are visualized as contrast, which causes some areas to appear highlighted. Some pathological conditions cause a measurable change in the local signal intensity, however many do not[1]. Contrast agents interact with water molecules to alter the signal intensity in the local area. Some contrast agents, known as sensing contrast agents (SCAs), also interact with specific biological markers that can be monitored to track the progression of various diseases. This allows MR images to show the presence of these molecules of interest that would normally blend in with the background because they either lack the ability to alter water relaxation times (i.e. non-para- or superparamagnetic) or lack highly mobile protons that can be detected by chemical saturation-transfer techniques (CEST)[2].

Myeloperoxidse (MPO) is an enzyme of interest that plays a critical role in inflammation. MPO is found in the azurophilic granules of neutrophils, which are the most abundant leukocytes in humans[3]. Its total activity in humans is therefore very high and the presence of MPO in vascular lesions and in circulation has been shown to correlate with the outcome of stroke and other cardiovascular disease[4]. When released as a result of the activation and subsequent degranulation of neutrophils, MPO reacts with endogenous H_2O_2 and chloride ions or aromatic alcohol residues to produce highly reactive molecular species such as hypochlorite ions and tyrosyl radicals (*though tyrosine is much less active as MPO reducing molecule than 5-hydroxytryptamine (serotonin)).* These reactive molecular species can go on to stimulate cellular inflammatory signaling cascades. Imaging of MPO activity has been achieved by substituting one or more hydroxyindole groups onto a ligand that can then be chelated to a paramagnetic metal center. The radicals that form when these contrast agents interact with MPO go on to interact with proteins or other molecules of contrast agent, leading to cross-linking and oligomerization, respectively[5]. Both of these processes lead to a change in the signal from water molecules in the local environment.

One source of H2O2 is the dismutation of the superoxide anion (O_2^-, which is usually produced by NAPH oxidase of phagocytes and endothelial cells) by superoxide dismutase (SOD). Superoxide is a free radical produced as a byproduct of normal respiration in the mitochondria. In mice, Mn-SOD deficiency has been shown to exacerbate cerebral infarction following ischemia, which suggests that excess superoxide plays a key role[6]. A family of paramagnetic manganese macrocycles has been shown to mimic SOD activity without producing measurable toxic effects. One of such compounds, M40401, has a catalytic rate exceeding that of the native enzyme[7]. M40401 can be potentially used in MRI to enhance signal in ischemic areas of the brain because the Mn center of this SOD mimetic is a paramagnetic metal cation.

Dr. Bogdanov's laboratory of Molecular Imaging Probes at UMASS Medical School has been working toward synthesizing a functionalized paramagnetic SOD mimetic that also acts as a substrate for MPO (**2**). This compound can be potentially used to detect the activity of MPO *in vivo* using MRI because: 1) it is paramagnetic and therefore detectable by MRI; 2) it reacts with the superoxide anion to produce hydrogen peroxide, which is the primary substrate of MPO; 3) it carries a reducing moiety that converts oxidized MPO into a reactive state during every enzymatic cycle.

Background

The goals of this project were to synthesize **2** and examine the kinetic properties of the compound *in vitro*. The synthetic strategy will be accomplished in the future by converting one of the ligands to an azide and using the Cu(I) catalyzed Alkyne-Azide 4+2 cycloaddition to join the ligand onto the 5-hydroxyindole (5-HT) group.

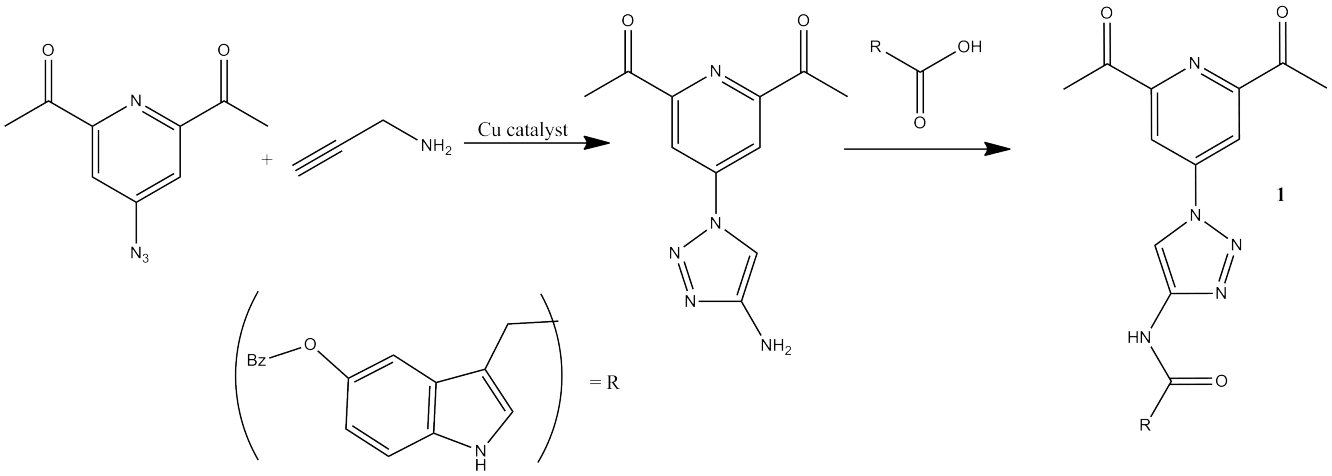


Figure 1 Scheme for functionalization of ligand

This reaction falls into the category of “click chemistry”, a subset of reactions with many favorable characteristics which include high yield, mild conditions, and little to no purification[8]. The final macrocyclic product bearing a reducing 5-HT moiety has not yet been tested in this project. However, a synthetic strategy has been developed that yielded a precursor building block carrying the reactive azide that will be used for further condensation reaction yielding the final product.

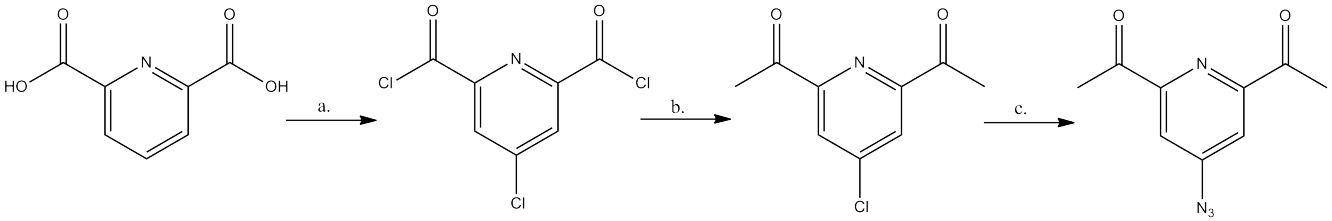


Figure 2 Scheme for synthesis of azide

MRI

MR Images are generated by exposing samples to an external magnetic field, $\boldsymbol{B_0}$, and an induced magnetic field, $\boldsymbol{B_1}$. When a sample is placed in the $\boldsymbol{B_0}$ field, some nuclei interact with the field and orient themselves in discrete directions relative to the $\boldsymbol{B_0}$ field. This is because those atoms possess a quantum mechanical property called nuclear spin, given by the quantum number *I*. Nuclear spin is a consequence of the individual protons and neutrons in the nucleus rotating along an axis, and can be visualized as a vector, ***I***, parallel to the axis of rotation of the nucleus as a whole. The formula for ***I*** is given thus:

$$|\boldsymbol{I}| = \frac{hI(I+1)}{2}$$

The quantum mechanical energy levels, *mI*, are restricted to 2*I* + 1 discrete values (-*I* , -*I* + 1, …, *I*), each corresponding to an ***I*** vector with a different orientation relative to the B_0 field. Because protons are positively charged, spinning protons in the nucleus generate a dipolar magnetic moment vector, ***μ***, that is parallel to the ***I*** vector. The magnitude of *μ* is proportional to the value of *I* based on a constant called the gyromagnetic ratio, *γ*, which is unique for each isotope. Transitions between nuclear energy levels are dependent on *γ*, and excitation of a sample occurs when a rotating $\boldsymbol{B_1}$ field is generated perpendicular to the $\boldsymbol{B_0}$ field by RF pulses of a certain frequency (called the Larmor frequency, a constant proportional to *γ*).

^{1}H nuclei, commonly referred to as protons, have a spin number of ½ and are almost exclusively the target used to generate a signal in MRI. Nuclei with a spin number of ½ are restricted to two quantum mechanical spin states (*mI* = ½ and *mI* = -½). The sum of all the magnetic moment vectors in a sample of protons gives rise to a macroscopic magnetization vector, ***M***. The $\boldsymbol{B_0}$ field is conventionally oriented along the *z*-axis if a coordinate plane is assigned to the sample space. In an unexcited sample, the *x* and *y* components of ***u*** are randomly distributed around the $\boldsymbol{B_0}$ field, meaning the *x* and *y* components of ***M*** are zero. The *z* component of ***M***, however, is proportional to the $\boldsymbol{B_0}$ field, resting at an equilibrium when a sample is in the ground state. When a sample is excited, the

transition to a higher energy state raises the value of M_z , and the rotating $\boldsymbol{B_1}$ field pulls $\boldsymbol{M}$ off the *z* axis and into the *x,y* plane. When the $\boldsymbol{B_1}$ field is turned off, $\boldsymbol{M}$ will still have some non-zero component in the *x,y*-plane, causing it to rotate around the B_0 field[9].

There are two main reference parameters in MRI. Both rely on the concept of relaxation, or the return of the components of $\boldsymbol{M}$ to their equilibrium values. The return of M_z to its equilibrium value, called spin-lattice relaxation, depends on the transition of excited nuclei back to their ground state and is characterized by a time constant, T_1. Processes that generate large amounts of magnetic noise, such as electronic dipole interactions, can stimulate an excited nucleus to return to ground state faster and lower the T_1 value. Spin-spin relaxation, or T_2 relaxation, is the process where the *x* and *y* components of $\boldsymbol{M}$ decay to zero. This is generally caused by interference from the random motion of the molecules in a sample[1]. T_1 values vary more depending on the sample, but T_2 values are lower than T_1. There is a third parameter that can be used to affect images in MRI called ρ, or proton density, but this value is not affected by contrast agents and thus not of interest in this study.

Spatial information is recorded by using a gradient $\boldsymbol{B_1}$ field. With the gradient applied, only a specific slice of the $\boldsymbol{B_1}$ field has the correct energy to excite a sample. Signal intensity, generated from the relaxation and proton density values, is plotted against the spatial information to generate a two or three dimensional image.

Contrast Agents

Radionuclide-based imaging methods such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) rely on radioactive contrast agents to generate signals. These methods often require scans from multiple imaging types in order to generate a higher resolution background image around signals from specific interactions. MRI has the advantage of generating a high resolution background image as well as highlighting specific biochemical interactions. Contrast agents in MRI work by reducing the relaxation times of surrounding protons. This allows for a shorter time between RF pulses without saturating the signal, leading to a higher resolution image[9]. Relaxivity (*R*) values are used to describe the efficacy of a contrast agent and are

inversely proportional to T_1 and T_2 values. Highly paramagnetic contrast agents more strongly affect the T_1 values while Fe3O4 based contrast agents are T_2 based[1]. Gd(III) and Mn(II), with seven and five unpaired electrons, respectively, are the most commonly used species for T_1 contrast agents. This study focuses on T_1 contrast agents so those based on Fe3O4 will not be discussed.

The R_1 value of a T_1 contrast agent is based on two types of interactions, inner sphere (IS) and outer sphere (OS). IS interactions focus on the water molecules (usually 1 or 2) directly coordinated to the metal center of the contrast agent. These molecules are aligned in a specific orientation and can interact with other nearby water molecules, which accounts for the OS interactions.

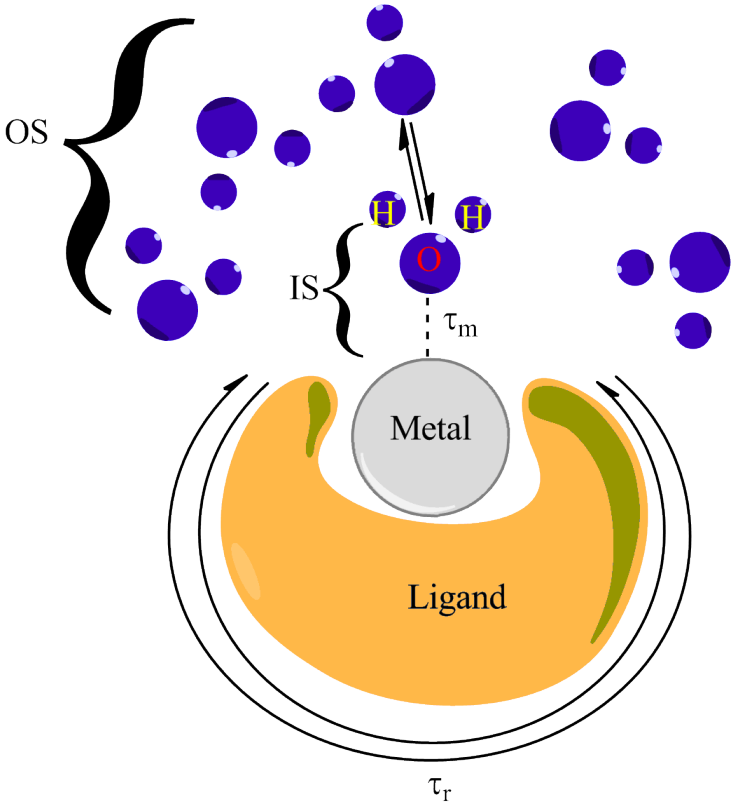


Figure 3 Contrast Agent interactions

The exchange rate of coordinated water molecules, τ_m, reflects the amount of time a molecule stays bound to the metal center, while the reorientation time, τ_r, expresses how much the complex tumbles in solution. An increase in τr or a decrease in τ_m will lead to a higher R_1 value[10].

Smart or sensing contrast agents (SCAs) respond to a biological stimulus that leads to a

measurable change in relaxivity. This can be a general stimulus such as a change in pH or a more specific mechanism such as an enzymatic reaction[11]. SCAs that respond to general stimuli (environmental SCAs) can detect a variety of different changes. One way to generate a change in relaxivity is to create an SCA where the exposure of the metal center to the bulk water can be changed. This has been accomplished in the past by attaching Gd-chelating groups that change affinity when protonated or have a higher affinity for other metals such as Ca or Zn[12][13]. This allows changes in pH or ion concentration to be seen in vivo as a change in MRI signal intensity.

Enzyme-sensing SCAs are especially promising because specific enzymes can be used as biomarkers for a variety of diseases. Early enzymatic SCAs focused on utilizing enzymes' ability to cleave chemical bonds[14]. Enzymes such as phosphatases, hydrolases, and peptidases all cleave a specific type of chemical bond. Functional groups that can compete with water for free chelating spots on the metal center can be attached to the ligand and cleaved off by these specific enzymes. This allows for a large increase in relaxivity by exposing more water molecules to the electronic effects of the metal center. Another method for detecting enzymatic activity in MRI is to design SCAs that are susceptible to enzymatic oxidation and/or polymerization. Many enzymes do not catalyze cleavage of a specific bond, but instead convert the substrate into a more reactive intermediate which can form new bonds without further catalysis. Contrast agents attached to substrates for such enzymes can show a significant change in relaxivity after catalysis and subsequent reactions[15]. One reason for this is that bulkier products such as oligomers and proteins bound to contrast agent have a much larger τ_r. Bulkier products often remain in the body for a longer period of time, resulting in a retention of the local increase in signal intensity. This provides a way to check for the presence of specific enzymes in different areas of the body.

MPO

Myleoperoxidase (MPO) is a protein released from neutrophils during a process known as degranulation. Degranulation happens during phagocytosis when granules in the neutrophils release their contents into the phagosome. The products of degranulation can also escape the phagocyte and

go on to cause damage to normal cell tissue. Reactive species generated by MPO are normally responsible for killing invasive species in the body, meaning they have the potential to cause some pathological conditions[16]. Accumulation of MPO can cause serious complications, especially with atherosclerosis, a heart disease caused by accumulation of plaque on the inside of the arteries. Numerous studies have shown that elevated levels of MPO correlate to adverse effects brought on by atherosclerosis[4][17][18]. One possible explanation for this is an attack on the more pathogenic plaque by neutrophils. This would cause a release of MPO that could cause oxidative damage and trigger more adverse effects from the arterial plaque. The products created by MPO can also stimulate inflammation pathways. The benefits of detecting MPO have clear benefits in diagnostic and preventative medicine.

In order to detect MPO, it is necessary to understand the mechanism of the enzymatic reaction. The active site of MPO consists of a heme group with a coordinated metal ion, Fe(III). This ion is oxidized by a molecule of H_2O_2 to generate a free radical Fe(IV) intermediate (MPO-I)[19]. To regenerate the active site, this intermediate can react with a Cl^- ion to generate the much more reactive ClO^- ion or it can react with two tyrosine residues to create free radicals that can cause cross-linking of proteins[20].

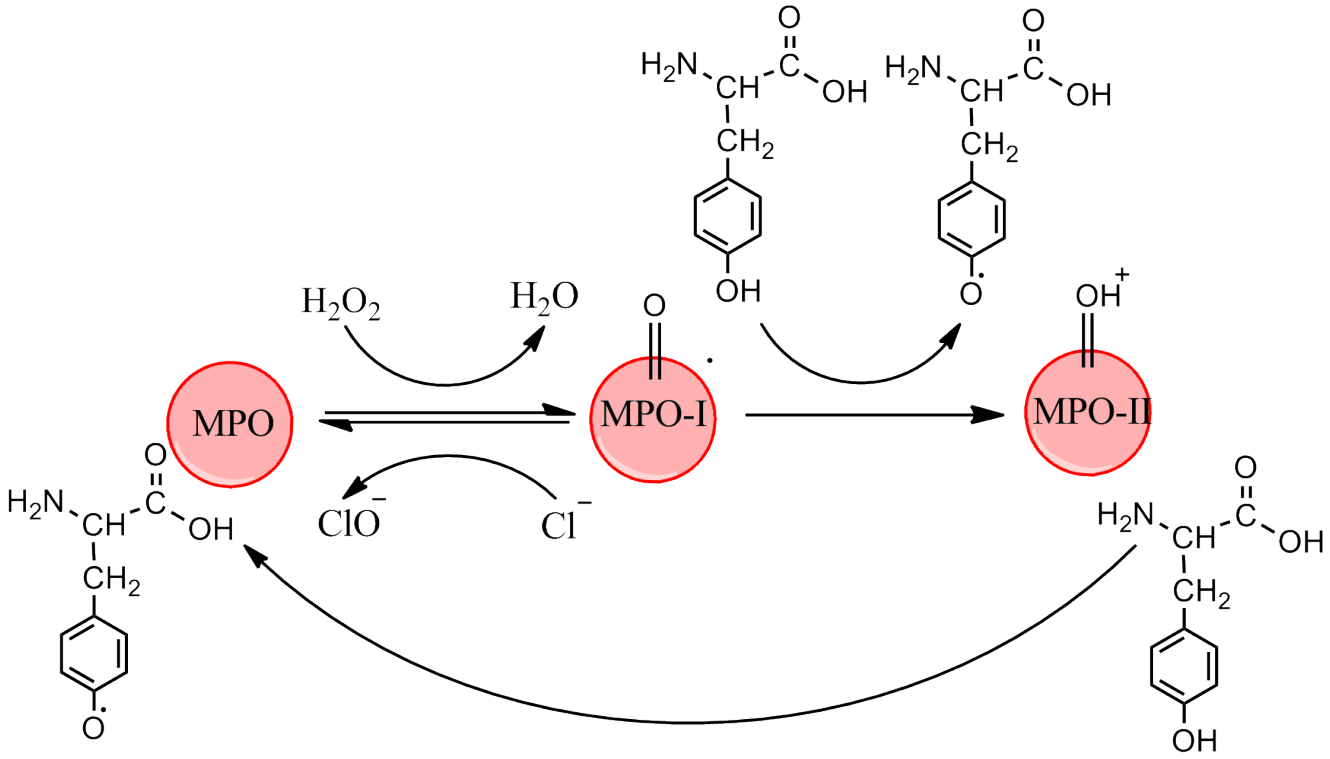


Figure 4 MPO mechanism

MPO-I can also react with other aromatic alcohol (AOH) groups such as 5-hydroxytryptophan to generate reactive species that can oligomerize or also cause cross-linking[21].

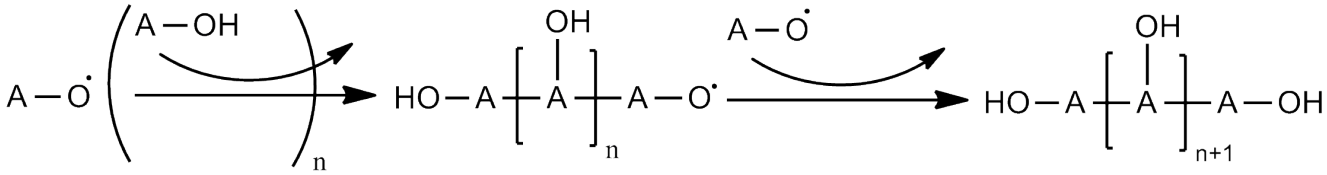


Figure 5 Oligomerization

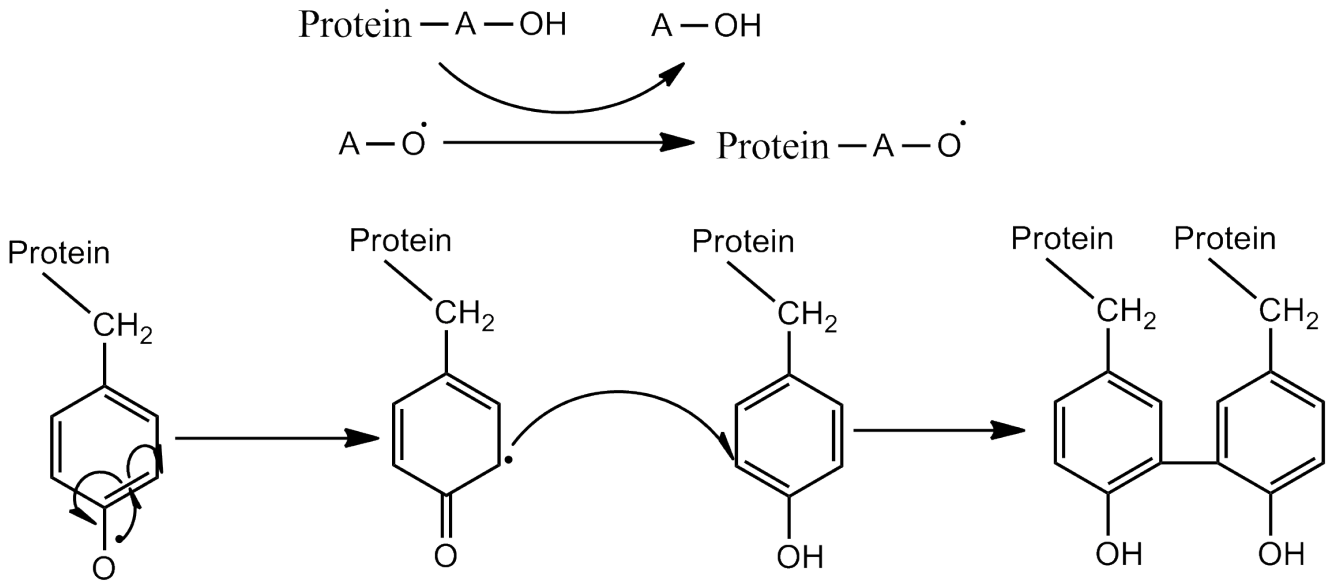


Figure 6 Cross-Linking

Kinetic studies have also shown that molecules containing 5-hydroxytryptamide (5-HT) groups can outcompete chloride anions as a substrate for MPO[22].

Detection of MPO has traditionally been achieved by running *in vitro* tests on cell samples samples taken from patients[4]. Monitoring MPO with MRI has potential for faster and more specific detection. Contrast agents have been synthesized that can accumulate in sites with high concentrations of MPO[23]. The products that form when this contrast agent is oxidized by MPO

accumulate in tissue samples much longer than the free contrast agent, leading to an increase in signal intensity for several days. These contrast agents are synthesized by chemically modifying the ligand around the Gd(III) center to contain one or more A-OH groups[24].

SOD

Superoxide (O_2^-) is a reactive oxygen species possessing an unpaired electron. It is produced as a byproduct of normal cellular respiration in the mitochondria and also generated by neutrophils to aid in the phagocytosis process[25]. This free-radical can cause significant oxidative damage when it is present in excess. Excess O_2^- is present in the same situations where excess neutrophils are present, such as inflamed or ischemic regions of tissue or in patients suffering from an autoimmune disorder. O_2^- decays naturally into hydrogen peroxide and molecular oxygen, but this does not happen fast enough to prevent damage[26]. Superoxide dismutase (SOD) is an essential enzyme present in multiple areas of the cell in different forms. Mn-SOD is present in the mitochondria while various Cu/Zn-SODs are present outside the mitochondria both in plasma and extracellularly. SOD deficiencies have been shown to play a critical role in ALS and many other diseases[27].

The evident importance of superoxide as a target for therapeutic agents has sparked decades of research into low molecular weight mimetics for SOD. Low molecular weight agents with SOD activity are able to diffuse into intracellular space and potentially permeate cell membranes better than the native enzyme. They also have the potential to have a much higher half life in the blood than the native enzyme ($t_{1/2}$ ≈ minutes). Rational design of pharmaceutical agents also allows chemists to design a drug that will have a higher affinity for either an aqueous or lipid medium, allowing for more selective delivery depending on the composition of the area with an overproduction of O_2^-[7]. Ions of Cu, Fe, and Mn have been shown to posses SOD mimetic activity. Early reports of metal complexes with SOD mimetic activity mainly focused on Cu complexes. Iron SOD mimetics have also been developed but the toxicity of both free metal ions limits the pharmaceutical potential of such complexes. Manganese complexes have a much higher therapeutic potential because of the reduced toxicity of free Mn ions compared to other candidates[27]. Manganese complexes also have the

advantage of being paramagnetic, meaning their distribution can be monitored by MRI. Mn(II) has five unpaired *d* electrons, making its complexes excellent candidates for T_1 contrast agents.

In order to design more efficient SOD mimetics, an understanding of the mechanism of metal-induced superoxide dismutation is required. Studies have shown that the rate determining step in Mn(II)-mediated O_2^- dismutation is the oxidation of Mn(II) to Mn(III). This means that higher catalytic rates can be achieved by designing a Mn(II) complex whose geometry closely resembles the favored geometry of the corresponding Mn(III) complex. One research group has used computer modeling programs to find such a complex, M40401, off of the parent complex M40403[7]. The *S,S*-stereochemistry of M40401 holds it in a geometry that resembles the favored pseudooctahedral geometry of the oxidized Mn(III) form. M40401 has been shown to have a catalytic rate that is two orders of magnitude greater than the parent complex, and even exceeds the rate of the native Mn-SOD enzyme. The *R,R*-stereoisomer of M40401 is constrained to a much different geometry that does not favor the folding associated with oxidation. Because of this, the *R,R*-isomer is catalytically inactive.

The family of SOD mimetics containing coordinated manganese cations belong to a family of theranostic agents. Theranostics is usually understood as a combination of the diagnostic study and therapeutic application of the same compound. It involves imaging chemical agents in order to deliver appropriate therapies based on specific chemical interactions and a generation of specific signal detectable by imaging instruments. In this regard, M40401 ideally fits into this category as a contrast agent and an SOD mimetic, i.e. a compound with a theranostic potential. The initial tests that involved injection of M40401 encapsulated into liposomes into mice showed signal enhancement in the brain for at least 4 days post injection. MRI has shown that the enhancement of T_1-weighted images (i.e. enhanced as a result of Mn(II) presence) returned to the background levels after 9 days in the cortical, subcortical, and hippocampal regions, suggesting that liposomes carrying M40401 release this compound and that it transiently accumulates in the neural tissue and is slow to be cleared away. Therefore, M40401 was clearly visible in vivo using a 3T clinical MR magnet[28].

We envision combining M40401 with an MPO-sensing substrate that will result in a theranostic

agent. This agent will enable the use of MRI for determining regions of ongoing inflammation in the vascular wall. This is feasible because MRI has a very high anatomical resolution and can utilize a variety of imaging sequences to tell the flowing blood from the blood vessel wall (which will appear stationary and enhanced if this new theranostic compound is present). The detection of inflamed areas in blood vessels is very important for assessing which areas could potentially trigger vascular wall instability, rupture, and potential formation of a blood clot. At the same time, the Mn(II) center in M40401 (that will stay immobilized in the vascular wall due to MPO activation and MPO-mediated binding to the cellular and extracellular components) will function as a SOD mimetic, decreasing the local damaging concentration of superoxide radicals and helping to temporarily alleviate the ongoing inflammation.

Click Chemistry

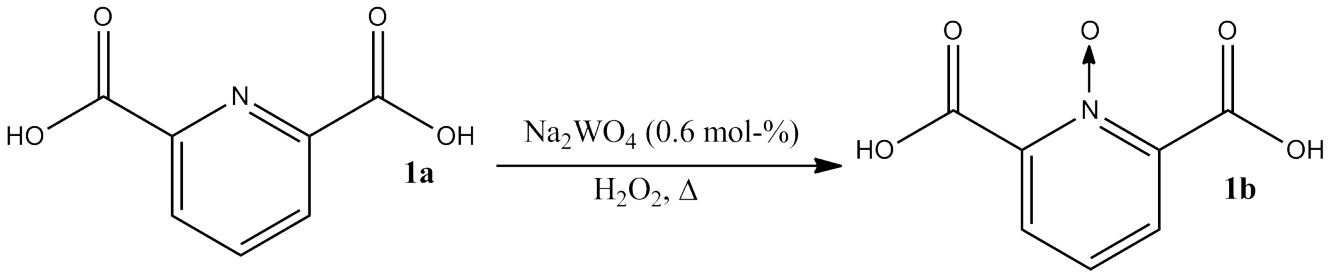
"Click chemistry" is a term used to describe an attractive new field of research. It entails generating a series of molecular building blocks that react easily and reliably. A review published by the Sharpless research group defines the following set of criteria for a click reaction: 1) modular; 2) wide in scope; 3) very high yields; 4) generates inoffensive byproducts; 5) stereospecific (not necessarily enantiomerically selective); 6) simple reaction conditions; 7) readily available starting materials; 8) no solvent or use of a benign solvent (such as water or alcohol); and 9) simple product isolation (no need for preparative chromatography)[8]. By focusing synthetic strategies on reactions that fit these criteria, chemists can greatly reduce the cost and environmental impact of their syntheses.

One reaction that fits the criteria of click chemistry is the Cu(I) catalyzed Huisgen dipolar-1,3-cycloaddition of azides and alkynes. This reaction produces 1,3-substituted triazoles linking two molecules using two functional groups that are easy to introduce. Azides are generally synthesized from alkyl halides in high yields via nucleophilic substitution reactions. Alkynes bearing amino or alcohol groups can also be purchased from most commercial vendors and linked onto another building block.

Methodology

All reagents were purchased from Sigma-Aldrich, Strem Chemicals, Fisher scientific, or VWR and used as received unless indicated. Dichloromethane and pyridine were dried and distilled over CaH_2 and KOH respectively. Reactions were performed in open air unless otherwise specified. TLC analysis was performed on silica gel plates or Reverse Phase-C18 plates. The stationary phase for column chromatography was 60-240 mesh activated silica gel. NMR spectra were recorded on a 400mHz machine. Melting point analysis was taken on a standard MelTemp apparatus.

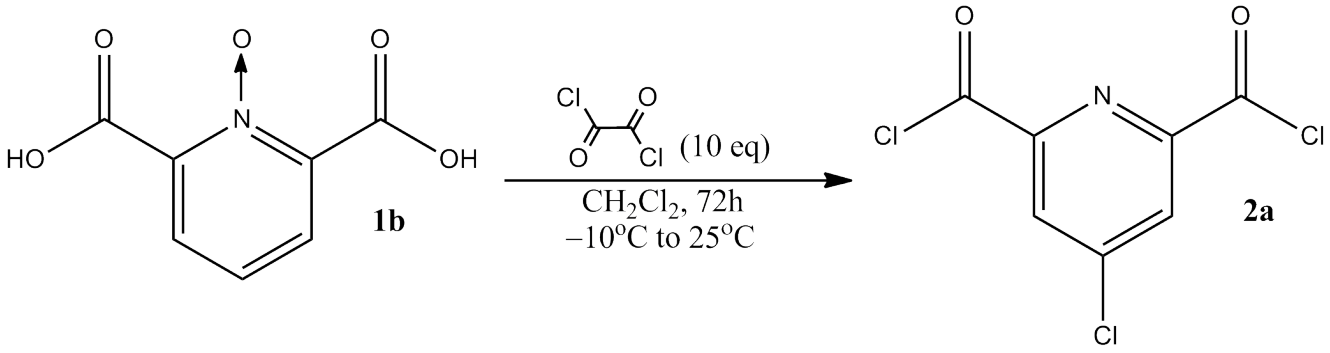
2,6-dipicolinic acid N-oxide



A suspension of 2,6-dipicolinic acid, **1a**, (15.05g, 0.090 mol) was prepared in 30% H_2O_2 (52.5 ml) and cooled to 0 °C in an ice bath. Na_2WO_4 (0.14g, 0.54 mmol, 0.6 mol-%) was added in one portion and the suspension was heated to 100 °C for 2h with stirring. The suspension was cooled to room temperature and a second portion of H_2O_2 was added (105 ml). The reaction mixture was heated at 100 °C overnight. During this time the white solids dissolved to indicate product formation. The reaction mixture was cooled to 0 °C to precipitate the product which was isolated by vacuum filtration and washed with ice water (2 x 10 ml). The filtrate was extracted with CH_2Cl_2 (3 x 50 ml) and combined organic extracts were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Product crops were combined, recrystallized from hot H_2O and dried by lyophilization to

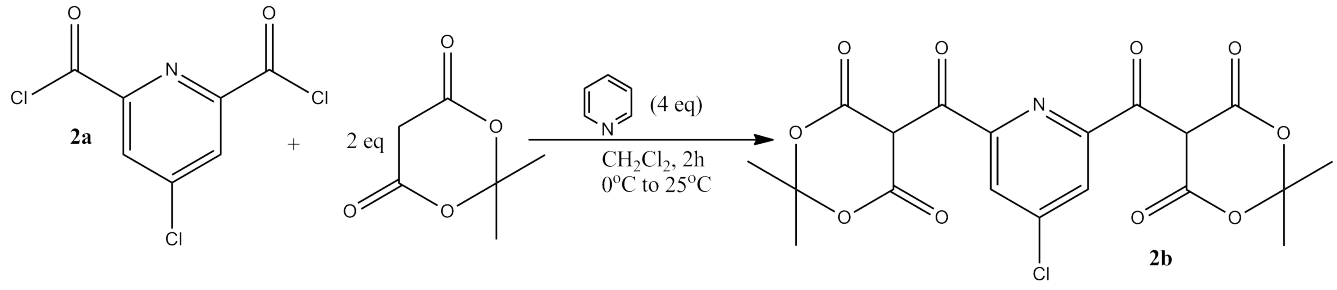
yield 9.18g (56%) of dipicolinic acid-N-oxide, **1b.** Mp: 160-162 °C.

4-chloro-2,6-pyridinedicarbonyl dichloride



N-oxide **1b** (9.18g, 0.050 mol) was suspended in freshly distilled CH_2Cl_2 (100 ml) and cooled to -10 °C by suspending over liquid N_2. The suspension was purged with Ar and oxalyl chloride (42.88 ml, 0.50 mol) was added dropwise with vigorous stirring. The reaction mixture was stirred for 10 min then 2 drops of anhydrous DMF were added and a fizzing sound was observed. The reaction mixture was allowed to warm to room temperature after 8h and then stirred under Ar for an additional 3 days. Solvent and oxalyl chloride were carefully removed under reduced pressure to yield crude 4-chloro-2,6-pyridinedicarbonyl dichloride, **2a** (12.32g). The product was dried in vacuum and used without further purification.

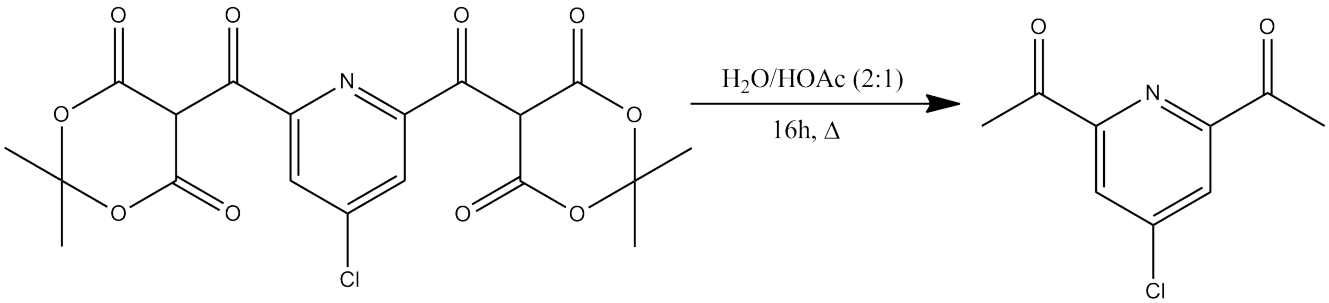
Meldrum's Intermediate



Meldrum's acid (4.61g, 0.032 mol) was dissolved in distilled CH_2Cl_2 (20 ml) and cooled in a

salt/ice bath. Freshly distilled pyridine (5.16 ml, 0.064 mol) was added and the solution was purged with Ar. Acid chloride **2a** (3.83g*, 0.016 mol) was dissolved in distilled CH_2Cl_2 and added dropwise with stirring. The reaction mixture was stirred for 1h in the salt/ice bath then warmed to room temperature and stirred for an additional 1h. The brown solution was washed with 2N HCl (2 x 25 ml) then water (4 x 25 ml), dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield Meldrum's adduct **2b**. The product was used in the next reaction without further purification.

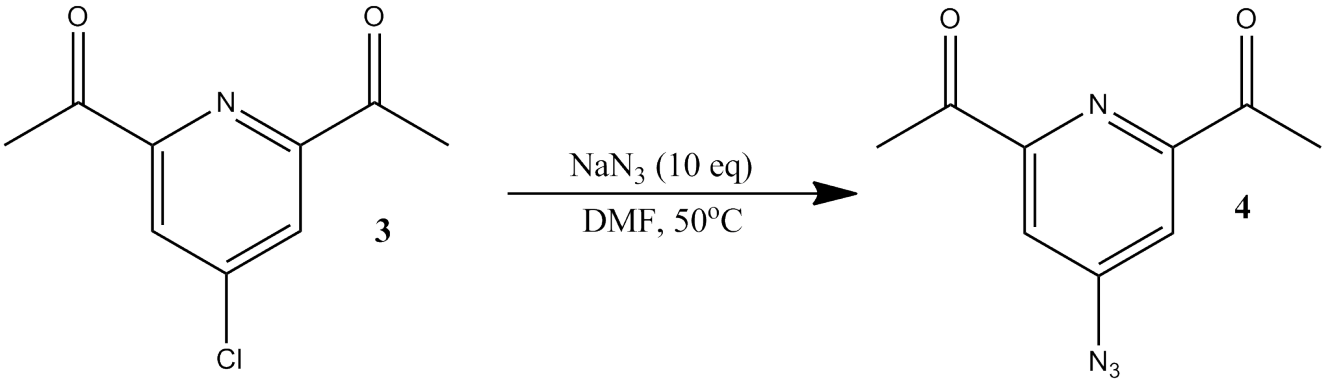
4-chloro-2,6-diacetylpyridine



2b **3**

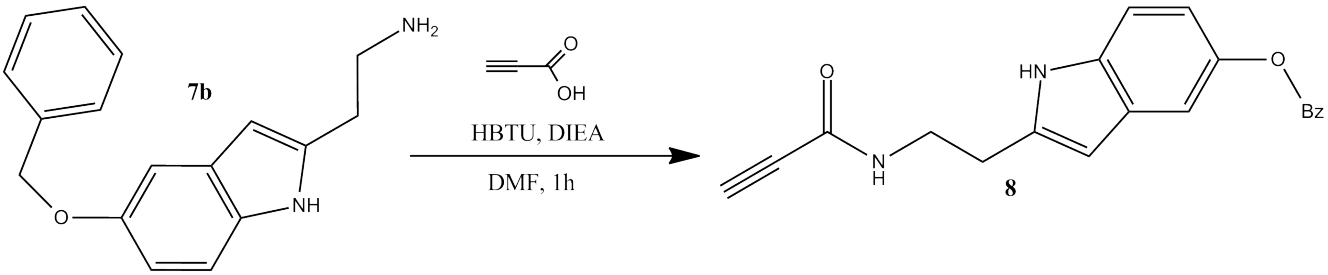
Meldrum's condensation product **2b** (0.016 mol calcd. from previous reaction) was refluxed in H_2O/glacial acetic acid (100 ml, 2:1) for 5h until product formation stopped on TLC. The reaction mixture was extracted with CH_2Cl_2 (4 x 100 ml) and the organic phase was filtered then washed with 5% aqueous $NaHCO_3$ (2 x 100 ml) and H_2O (2 x 50 ml). The organic phase was dried over Na_2SO_4, filtered, and concentrated to yield a brown crude product. The crude product was purified via flash chromatography on activated silica (0 - 10% EtOAc in Hexane) to yield pure 4-chlorodiacetylpyridine, **3** (0.45g, 14% over 2 steps).

4-azido-2,6-diacetylpyridine



Aryl chloride **3** (0.88g, 4.4mmol) was dissolved in anhydrous DMF. Oxygen was removed by three cycles under high vacuum and replaced with Ar. NaN_3 (2.86g, 44 mmol) was added in one portion and the bright yellow solution was heated at 50 °C overnight under a slow stream of Ar. A white solid (NaCl) had fallen out of solution the next day. The reaction mixture was cooled to room temperature and poured into cold water (35 ml) to precipitate a yellow solid. The solid was examined on TLC (EtOAc/Hexane, 1:1) to reveal the starting material **3** as the major component and a smaller presence of a new product suspected to be the azide, **4**. The solid was purified by flash chromatography. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.83 (2H, s) 2.80 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 26 (s), 115 (s), 154.5 (s), 197 (s).

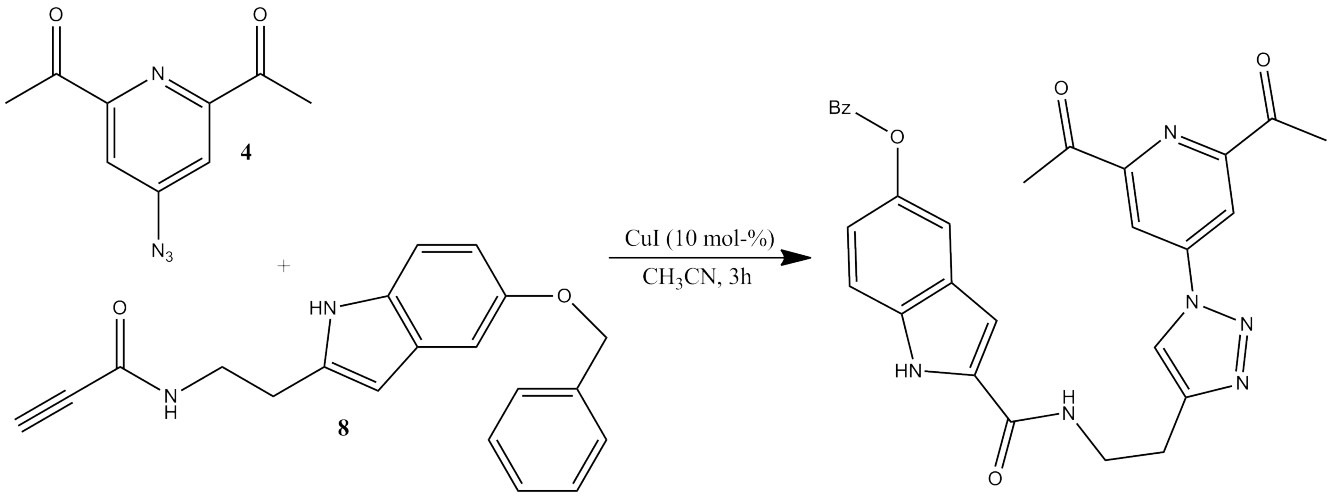
2-(2-propiolamidoethyl)-1H-indol-5-yl benzoate



Amine **7b** (0.27g, 1 mmol) was dissolved in anhydrous DMF (3 ml) and propiolic acid (0.093 ml, 1.5 mmol) was added in one portion. HBTU (0.569g, 1.5mmol) was suspended in DMF (1 ml) and

added slowly. DIEA (0.435 ml, 2.5 mmol) was added and the reaction mixture turned pale yellow. The mixture was stirred at room temperature for one hour and examined on TLC (EtOAc/Hexane, 1:1) which showed consumption of starting material propiolic acid. The reaction mixture was partitioned between EtOAc (150 ml) and 1N HCl (150 ml) and the organic phase was washed with 1N HCl (2 x 50 ml), sat. $NaHCO_3$ (2 x 50 ml), and brine (2 x 40 ml). The organic portion was dried over Na_2SO_4, filtered and concentrated under reduced pressure to yield a golden brown oil. The crude product was purified via flash chromatography on activated silica (10 - 67% EtOAc in Hexane) to yield amide **8** (0.26g, 82%) as a yellow oil. The product was analyzed by NMR spectroscopy.^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.73 (1H, s), 2.95 (2H, t), 3.61 (2H, q), 5.11 (1H, s), 7.32 (9H, m), 8.18 (1H, s).

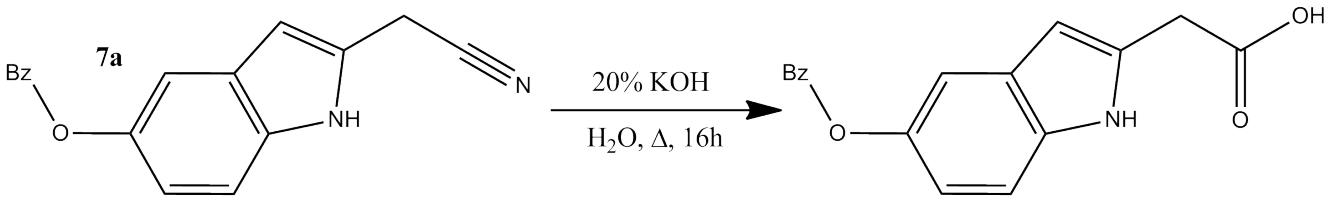
2-((2-(1-(2,6-diacetylpyridin-4-yl)-1H-1,2,3-triazol-4-yl)ethyl)carbamoyl)-1H-indol-5-yl benzoate



Alkyne **8** (0.22g, 0.68mmol) was dissolved in CH_3CN (2 ml) and **4** (0.14g, 0.68mmol) was added in one portion. The solution was purged with Ar and purified CuI (0.02g, 0.068mmol) was added. The reaction mixture was stirred for 3h at room temperature under a cushion of Ar. The reaction was diluted in EtOAc (50 ml) and washed with sat. aqueous NH_4Cl/NH_4OH (1:1, 2 x 30 ml). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield

an orange solid. Analysis on the solid was inconclusive.

2-(5-(benzoyloxy)-1H-indol-2-yl)acetic acid



Nitrile **7a** (2.05g, 7.8 mmol) was suspended in 20% aqueous KOH (20 ml) and refluxed overnight. The next day, all reagents had dissolved to produce a brown solution. Concentrated HCl (10N, ~8 ml) was slowly added with stirring until the solution was neutral to precipitate the product as a pinkish solid. The solid was stored under Ar at 4 °C for further analysis.

Discussion

The initial component of our synthetic scheme required the search for a convenient and inexpensive approach to the synthesis of the initial precursor: 4-chloropyridine dicarbonyl dichloride. The synthesis of this precursor was commonly achieved in the past by chlorination of commercially available chelidamic acid[29][30].

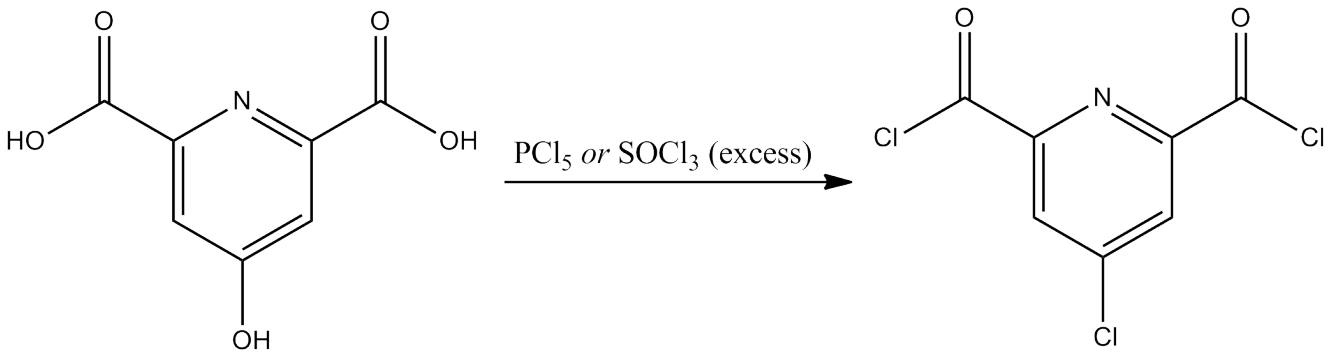


Figure 7 Traditional starting material and functionalization method

The high cost of this reagent made this method undesirable, so an alternative synthesis was sought.

A recent publication describes a synthesis of 4-chloropyridine dicarbonyl dichloride from inexpensive dipicolinic acid[31]. Conversion of the dipicolinic acid into an n-oxide activates the para position of the pyridine ring toward electrophilic substitution. Treatment of the n-oxide with 10 equivalents of oxalyl chloride over three days resulted in a high yield conversion into the desired product. It must be noted that the diacid dichloride is moisture sensitive and should be reacted in the next step as soon as possible. Oxalyl chloride is an extremely corrosive reagent and great caution must be taken when removing excess from the reaction mixture.

The two-step conversion of 4-chloropyridine dicarbonyl dichloride into 4-chloro-2,6-diacetylpyridine with Meldrum's acid did not proceed exactly as described in the literature[32]. Extraction and isolation of product in both steps proved to be exceedingly difficult and the yields for these steps were lower than those previously reported. The reported yields for this reaction were already low (~33%), so alternatives were sought from the beginning. The Gilman reagent, $(CH_3)_2CuLi$, was examined as an alternative, but the limited solubility of the acid chloride in THF or Et_2O at the low

temperatures required for the reaction resulted in low yields. Following a literature recommendation, an attempt was made to increase the yield of the initial two-step process. The commercially available Meldrum's acid was recrystallized before use and the pyridine and dichloromethane were distilled. The effect of the purified reagents was not established because of problems during extractions were encountered that may have lowered the yield. Hydrolysis of the acid chloride was suspected to be a negative factor affecting the reaction yield, which is why the trichloride should be reacted as soon as possible.

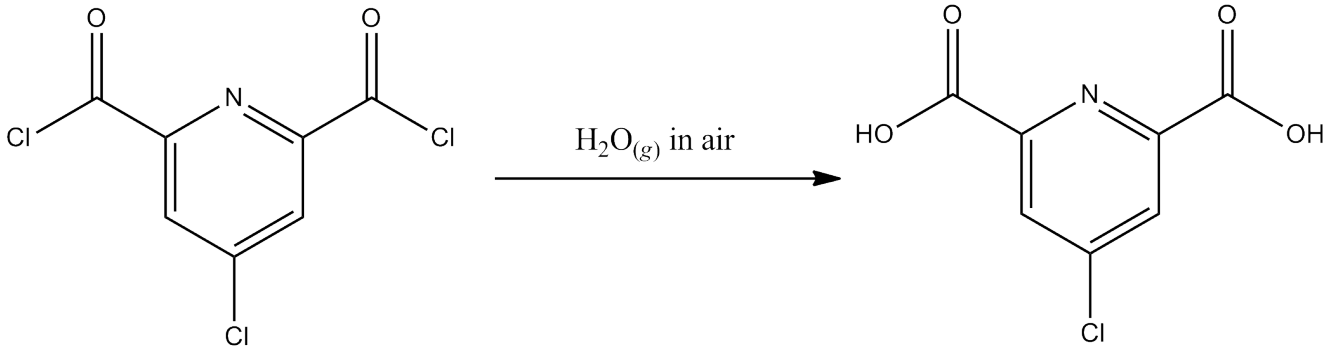


Figure 8 Hydrolysis

The sub-optimal extraction conditions that are recommended in the literature for isolating the products in both steps contributed to the undesirable lower yield as well. A plausible alternative to this reaction has been identified that also uses a two step process. A recent paper[35] describes a conversion of para-substituted dicarbonylpyridine dichlorides into diketones by first converting the acid chloride into a secondary amide and then treating the amide with commercially available MeMgCl.

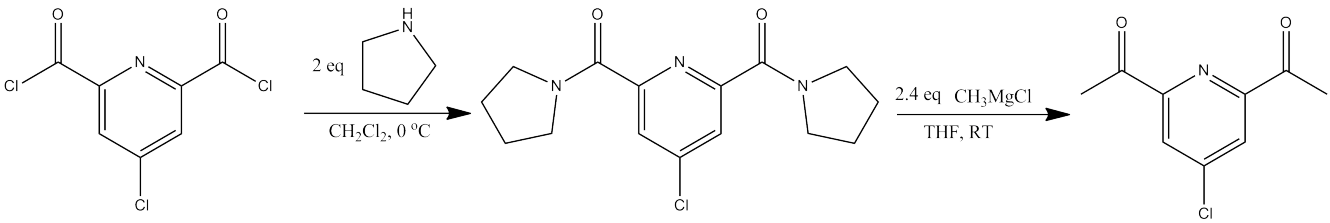


Figure 9 New ketone synthesis

This reaction will be attempted in the future in order to avoid all the problems associated with the meldrum's reaction.

Conversion of 4-chloro-2,6-diacetylpyridine into the respective azide was accomplished

following a synthesis designed for the 2,6-diester derivative[33]. Yields were lower than those reported in the literature and the product was contaminated with starting material. The para-chloride group on the diester is suspected to be more reactive toward nucleophilic substitution than in the diketone, which would mean more vigorous reaction conditions are required for complete conversion of the diketone. In the future, temperature will be increased and the reaction will be monitored by TLC until complete consumption of the starting material is observed. Another problem with this synthesis was the formation of condensation by-products, indicated by a red-orange color in the reaction mixture. Eliminating oxygen from the reaction mixture in the future will hopefully reduce the formation of by-products in the more vigorous conditions than need to be employed.

Dr. Bogdanov's laboratory has done research in the past with contrast agents containing 5-hydroxytryptamine groups, so there was a readily available supply of benzyl-protected 5-hydroxytryptamine. This indole amine was conjugated with commercially available propiolic acid using HBTU as a coupling reagent. This reaction produced a gold colored oil that turned brown if exposed to light or air for too long. The high sensitivity of this compound made it difficult to isolate. The literature also has few examples of propiolic acid derivative being used for Cu(I) cycloadditions. The conjugation of the carbonyl carbon with the triple bond is suspected to be a factor. A new alkyne, propargylamine, was proposed for the Cu(I) cycloaddition which would eliminate the problems previously encountered with isolation of the alkyne and lead to a product with a free amino group. This product can then be conjugated to an indole acetic acid that can be synthesized in good yields in one step from the corresponding nitrile.

The click chemistry intermediates, 4-azido-2,6-diacetylpyridine and 2-(2-propiolamidoethyl)-1H-indol-5-yl benzoate, were synthesized and characterized by NMR spectroscopy.

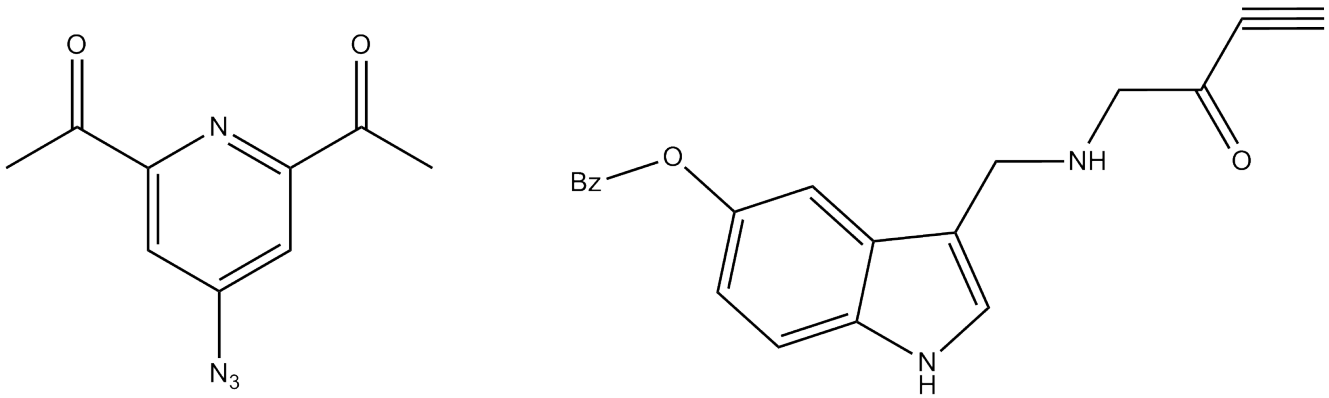


Figure 10 Click chemistry reagents

These reagents were mixed with a Cu(I) catalyst in acetonitrile, but the reaction proceeded very slowly as monitored by TLC. Analysis on this reaction mixture was inconclusive, and a literature analysis showed that some research groups were having similar problems with heterocycles as click chemistry intermediates[34]. Extra lone pairs of electrons, such as the lone pair on the pyridine nitrogen, can coordinate and scavenge free copper catalyst. This results in longer reaction times and reduced yields. One way of solving this problem is to use a Cu(I) catalyst bound to a ligand to eliminate the possibility of the heterocycle coordinating to free catalyst. A new scheme has been devised that uses a ligand-bound Cu(I) catalyst and replaces the 2-(2-propiolamidoethyl)-1H-indol-5-yl benzoate with propargylamine. The indole substrate will be linked via a peptide bond after the Cu(I) catalyzed cycloaddition.

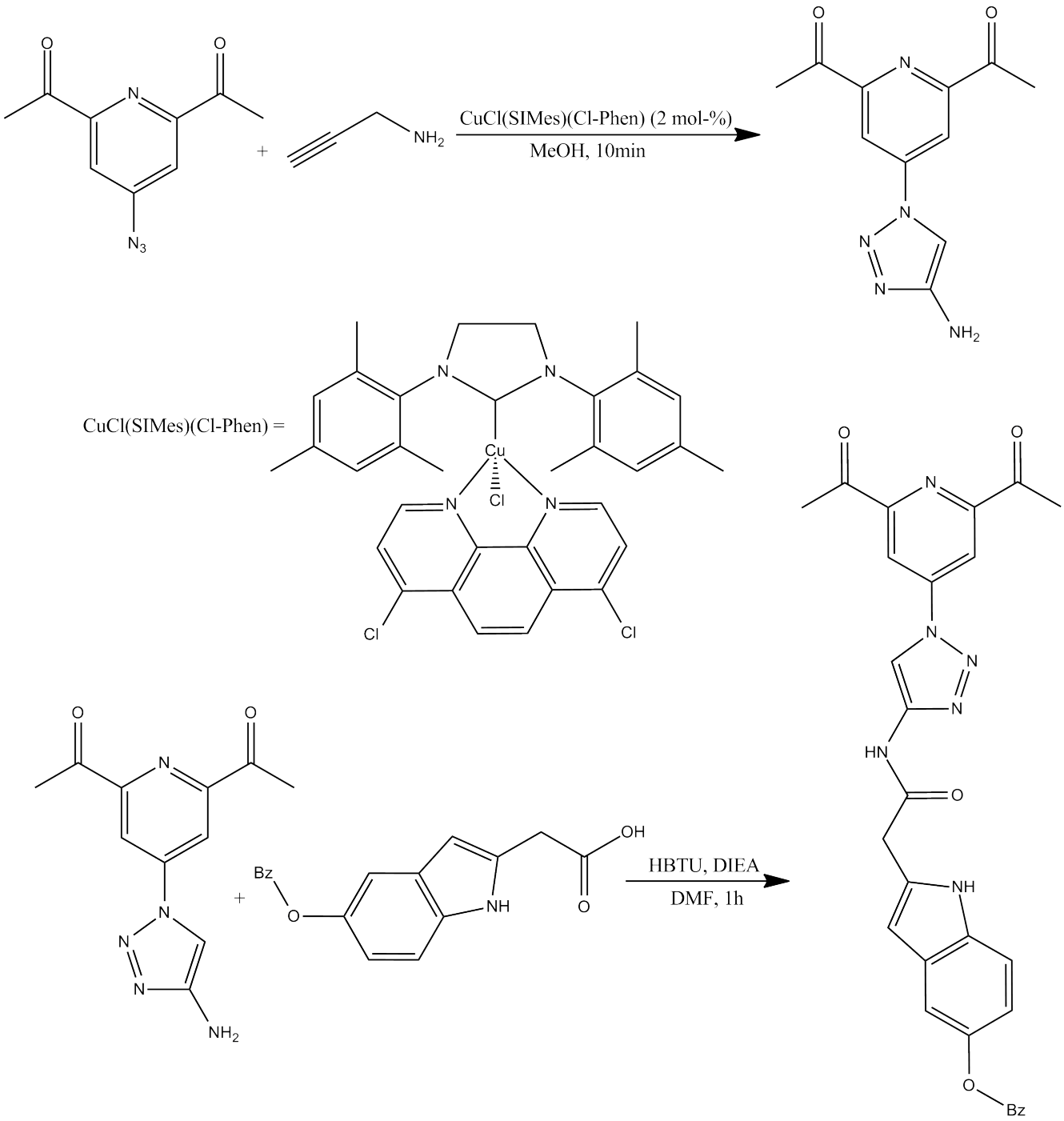


Figure 11 Click chemistry scheme

This new scheme will hopefully result in higher yields and shorter reaction times.

The catalyst for the cycloaddition will be synthesized in two steps from Cu_2O and two commercially available ligands.

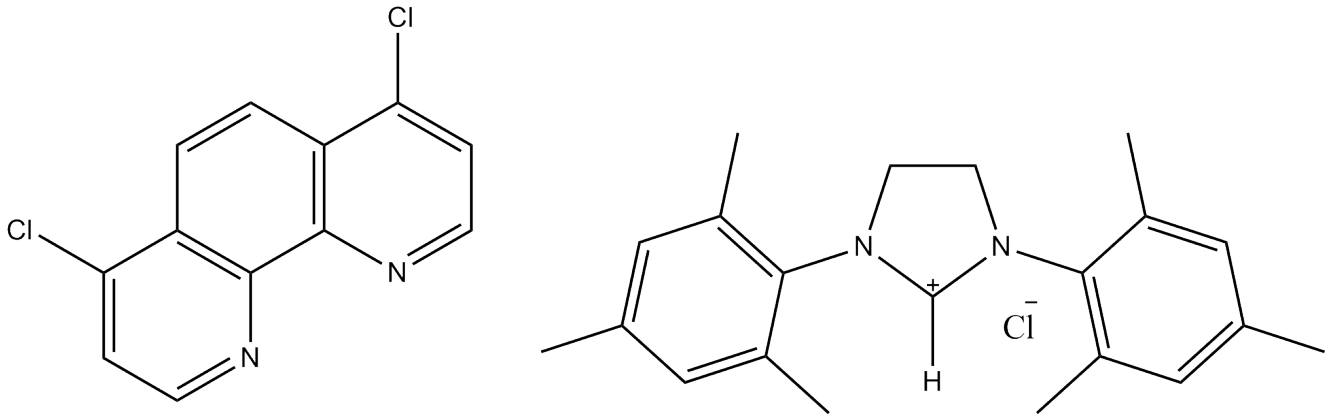


Figure 12 Ligands 4,7-dichloro-1,10-phenanthroline and SIMes Hcl

An attempt was made to synthesize the first step of this catalyst with the N-heterocyclic carbene ligand, 1,3-bis(2,4,6-trimethylphenyl)imidazolinium chloride. During the synthesis we encountered the problems with the resultant product crystallization. Deviations from the literature procedure are suspected to be the cause, and the procedure will be repeated more carefully this summer.

The synthesis of the end product, i.e. a Mn(II) coordinating macrocycle will proceed as described previously in the literature for M40401 using the 5-HT functionalized ligand in place of 2,6-diacetylpyridine[7]. This synthesis is a two-step procedure that involves refluxing the ligands with ultra-pure $MnCl_2$ in anhydrous ethanol then reducing the double bonds that form with a heterogeneous Pd/C and NH_4COH system in anhydrous methanol. The heterogeneous reduction is essential in order to form a product with the correct stereochemistry. Using a homogeneous catalyst system, such as $NaBH_4$, for reduction produces the catalytically inactive *R,R*-stereoisomer. The reduction with Pd/C is also expected to be a convenient approach for removing the benzyl protecting group from the indole substituent.

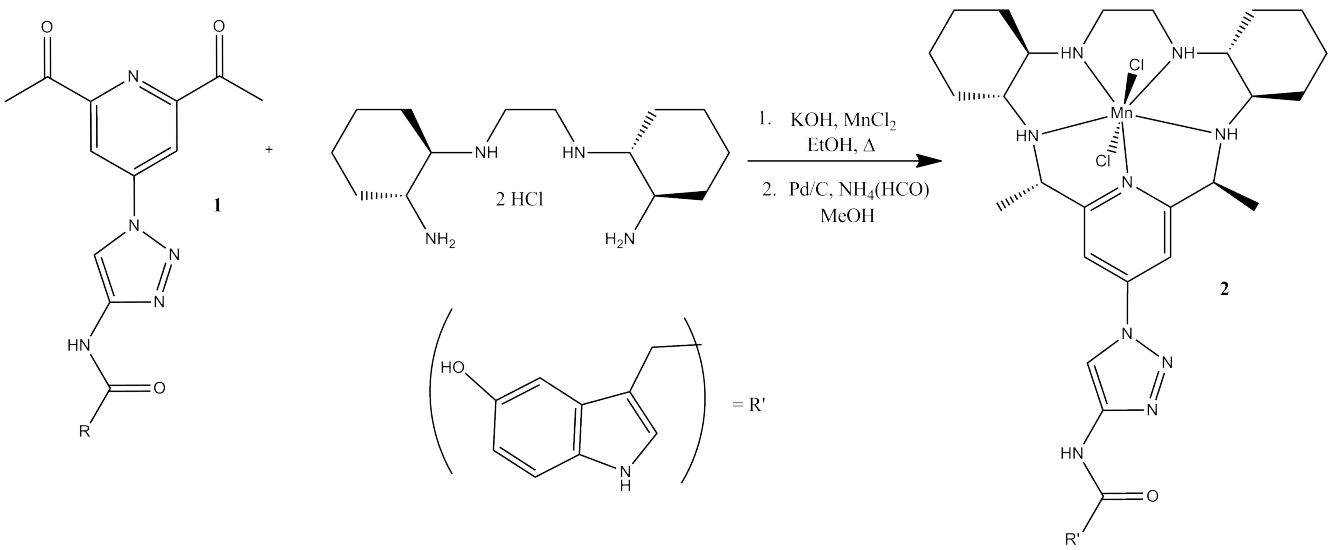


Figure 13 Macrocycle scheme

Conclusion

A plausible scheme has been devised for synthesizing a theranostic MRI contrast agent that possesses both superoxide dismutase activity and the ability to detect myeloperoxidase. This scheme relies on readily available starting materials and utilizes the environmentally friendly and economical click chemistry reaction, the Cu(I) catalyzed Huisgen dipolar-1,3-cycloaddition of azides and alkynes.

The following was concluded during the course of the project in order to improve the initial scheme: 1) Dipicolinic acid is an economical, commercially available starting material. 2) The Meldrum's acid synthesis is both inconvenient and low-yielding, but can be replaced with a 2-step amide/Grignard Reagent reaction. 3) Condensation product formation must be monitored and prevented during the ketone synthesis. 4) Heterocyclic reactants posses low reactivity toward free copper catalyst due to scavanging of Cu(I) by lone pairs or electrons. 5) The conjugated product of propiolic acid and the indole substrate demonstrated low reactivity in the click reaction, and 6) Starting materials for a new, more reactive scheme can be synthesized from readily available reagents.

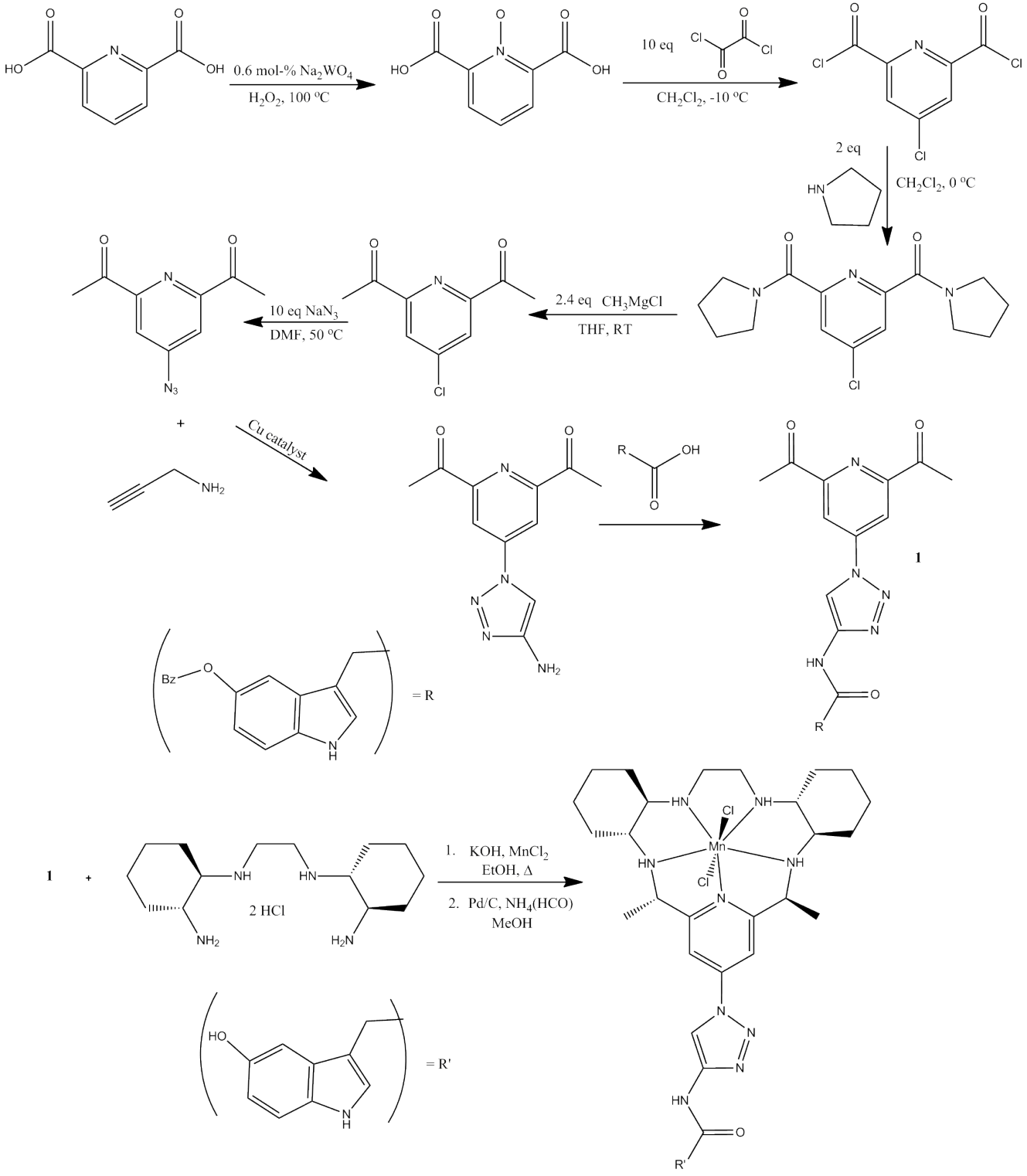


Figure 14 Total scheme

Bibliography

1: Lauffer RB, Paramagnetic Metal Complexes as Water Proton Relaxation Agents for NMR Imaging: Theory and Design. *Chemical Reviews*, *1987*, 87, 901-927
2: Querol M and Bogdanov AB, Amplification strategies in MR Imaging: Activation and Accumulation of Sensing Contrast Agents (SCAs). *Journal of Magnetic Resonanance Imaging*, *2006*, 24, 971-982
3: Rausch PG, Pryzwansky KB, Spitznagel JK, Immunocytochemical identification of azurophilic and specific granule markers in the giant granules. *New Englang Journal of Medicine*, *1978*, 268, 693-698
4: Brennan MB, Penn MS, Van Lente F, Nambi V, Shishehbor MH, Aviles RJ, Goormastic M, Pepoy ML, McErlean ES, Topol EJ, Nissen SE, Hazen SL, Prognostic Value of Meyloperoxidase in Patients with Chest Pain. *New England Journal of Medicine*, *2003*, 349, 1596-1604
5: Chen JW, Sans MQ, Bogdanov AB, Weissleder R, Imaging of Myeloperoxidase in Mice by Using Novel Amplifiable Paramagnetic Substrates. *Radiology*, *2006*, 240(2), 473-481
6: Kim Gw, Kondo T, Noshita N, Chan PH, Manganese Superoxide Defficiency Exacerbates Cerebral Infarction after Focal Cerebral Ischemia/Reperfusion in Mice: Implictions for the Production and Role of Superoxide Radicals. *Stroke*, *2002*, 33(3), 809-815
7: Aston K, Rath N, Naik A, Slomczynska U, Schall OF, Riley DP, Computer-aided design(CAD) of Mn(II) complexes: superoxide dismutase mimetics with catalytic activity exceeding the native enzyme. *Inorganic Chemistry*, *2001*, 40(8), 1779-1889
8: Kolb HC, Finn MG, Sharpless KB, Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angewandte Chemie International Edition*, *2001*, 40(11), 2004-2021
9: Merbach AE, Toth E, .,2001,,
10: Zhang S, Merritt M, Woessner DE, Lenkinski RE, Sherry AD, PARACEST Agents: Modulating MRI Contrast via Water Proton Exchange. *Accounts of Chemical Research*, *2003*, 36, 783-790
11: Lowe MP, Parker D, Reany O, et al, pH Dependent modulation of relaxivity and luminescence in macrocyclic gadolinium and europium complexes based on reversible intramolecular sulfonamide ligation. *Journal of the Americal Chemical Society*, *2006*, 123, 7601-7609
12: Li W-H, Fraser SE, Meade TJ, A calcium-sensitive magnetic resonance imaging contrast agent. *Journal of the Americal Chemical Society*, *1999*, 121, 1413-1414
13: Hanaoka K, Kikuchi K, Urano Y, et al, Design and synthesis of a novel magnetic resonance imaging contrast agent for selective sensing of zinc ion. *Chemical Biology*, *2002*, 9, 1027-1032
14: Moats RA, Fraser, SE, Meade TJ, A "smart" magnetic resonance imagin agent that reports on specific enzymatic activity. *Angewandte Chemie International Edition*, *1997*, 36, 726-728
15: Bogdanov Jr A, Matuszewski L, Bremer C, Petrovsky A, Weissleder R, Oligomerization of paramagnetic substrates result in signal amplification and can be used for MR imagin of molecular targets. *Molecular Imaging*, *2002*, 1, 16-23
16: Klebanov SJ, Myeloperoxidase: friend and foe. *Journal of Leukocyte Biology*, *2005*, 77, 598-625
17: Young JL, Libby P, Schonbeck U, Cytokines in the pathogenesis of atheroschlerosis. *Thrombosis and Haemostasis*, *2002*, 88(4), 554-567
18: Naruko T, Ueda M, Haze K, et al, Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation*, *2002*, 106(23), 2894-2900
19: Allegra M, Furtmuller PG, Regelsberger G, et al, Mechanism of reaction of melotonin with human myeloperoxidase. *Biochemical and Biophysical Research Communications*, *2001*, 282, 380-386
20: DeLeo MJ 3rd, Gounis MJ, Hong, B, et al, Carotid artery brain aneurism model: in vivo molecular enzyme-specific MR imaging of active inflammation in a pilot study. *Radiology*, *2009*, 252, 696-703
21: Rodriguez E, Nilges M, Weissleder R, et al, Activatable macnetic resonance imaging agents for myeloperoxidase sensing: mechanism of activation, stability, and toxicity. *Journal of the American Chemical Society*, *2009*, 132, 168-177
22: Dunford HB, Hsuanyu Y, Kinetics of oxidation of serotonin by myeloperoxidase compounds I and

II. *Biochemistry and Cell Biology*, *1999*, 77(5), 449-457
23: Chen JW, Phan W, Weissleder R, Bogdanov Jr. A, Human myeloperoxidase: a potential target for molecular MR imaging in atherosclerosis. *Magnetoc Resonance in Medicine*, *2004*, 52, 1021-1028
24: Shazeeb MS, Yang X, Gupta S, Bogdanov Jr. AA, A novel paramagnetic substrate for detecting myeloperoxidase activity in vivo. *Molecular Imaging*, *2012*, 11(5), 433-443
25: Mruk DD, Silvestrini B, Mo M, Cheng CY, Antioxidant superoxide dismutase - a review: its function, regulation in the testis, and role in male fertility. *Contraception*, *2002*, 64(4), 305-311
26: Miller FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL, Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. *Circulation Research*, *1998*, 82(12), 1298-1305
27: Riley DP, Functional mimetic of superoxide dismutase enzymes as therapeutic agents. *Chemical Reviews*, *1999*, 99, 2573-2587
28: Shazeeb MS, Feula G, Bogdanov Jr. AA, Liposome-encapsulated superoxide dismutase mimetix:theranostic potential of an MR detectable and neuroprotective agent. *In Press*, *2013*, ,
29: Chaubet F, van Duong MN, Gref A, Courtieu J, Crumbliss AL, Gaudemer A, The Design of Magnetic Resonance Contrase Agents: New Iron(III) Dihydroxamate Complexes. *Thetrehedron Letters*, *1990*, 31(40), 5729-5732
30: Dumont A, Jacques V, Desreux JF, New Synthons for the Synthesis of Lanthanide Containing Macrocyclic Schiff Bases Featuring Sunstituents Available for Tethering. *Tetrahedron*, , 54(14), 2043-2052
31: Mitsui K, Parquette JR, Convenient multigram synthesis of 4-chloropyridine-2,6-dicarbonyl dichloride. *Synthesis*, *2009*, 5, 713-714
32: Fallahpour R-A, Constable EC, Novel synthesis of substituted 4'-hydroxy-2,2':6',2''-terpyridines. *Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry*, *1997*, 16, 2263-2264
33: Huang B, Prantil MA, Gustafson TL, Parquette JR, The Effect of Global Compaction on the Local Secondary Structure of Folded Dendrimers. *Journal of the American Chemical Society*, *2003*, 125(47), 14518-14530
34: Teyssot M-L, Nauton L, Canet J-L, Cisnetti F, Chevry A, Gautier A, Aromatic nitrogen donors for efficient copper(I)-NHC CuAAC under reductant-free conditions. *European Journal of Organic Chemistry*, *2010*, 18, 3507-3515

Appendix: NMR Spectra

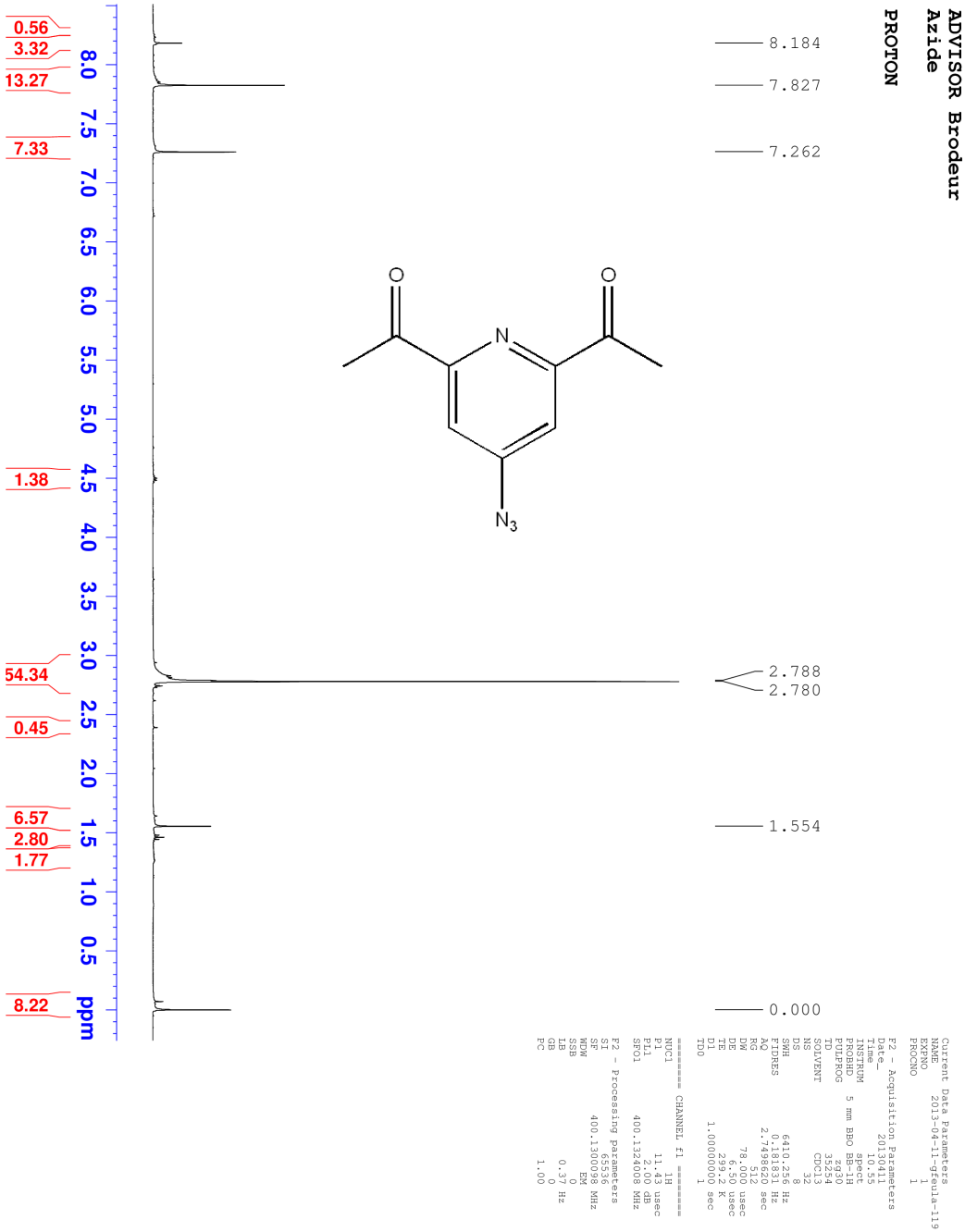


Figure 15 1H NMR Spectrum of 4-azido-2,6-diacetylpyridine

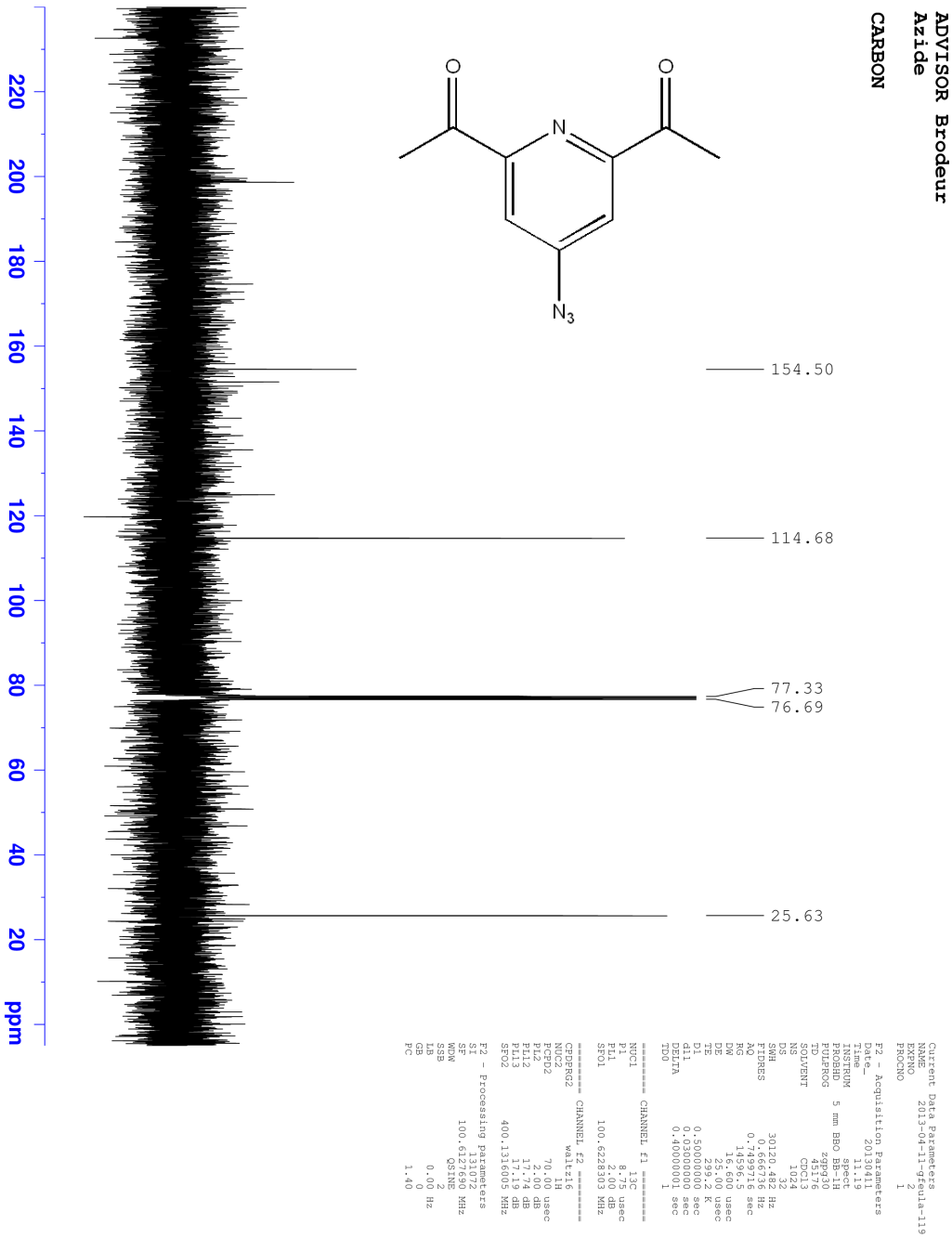


Figure 16 13C NMR Spectrum of 4-azido-2,6-diacetylpyridine

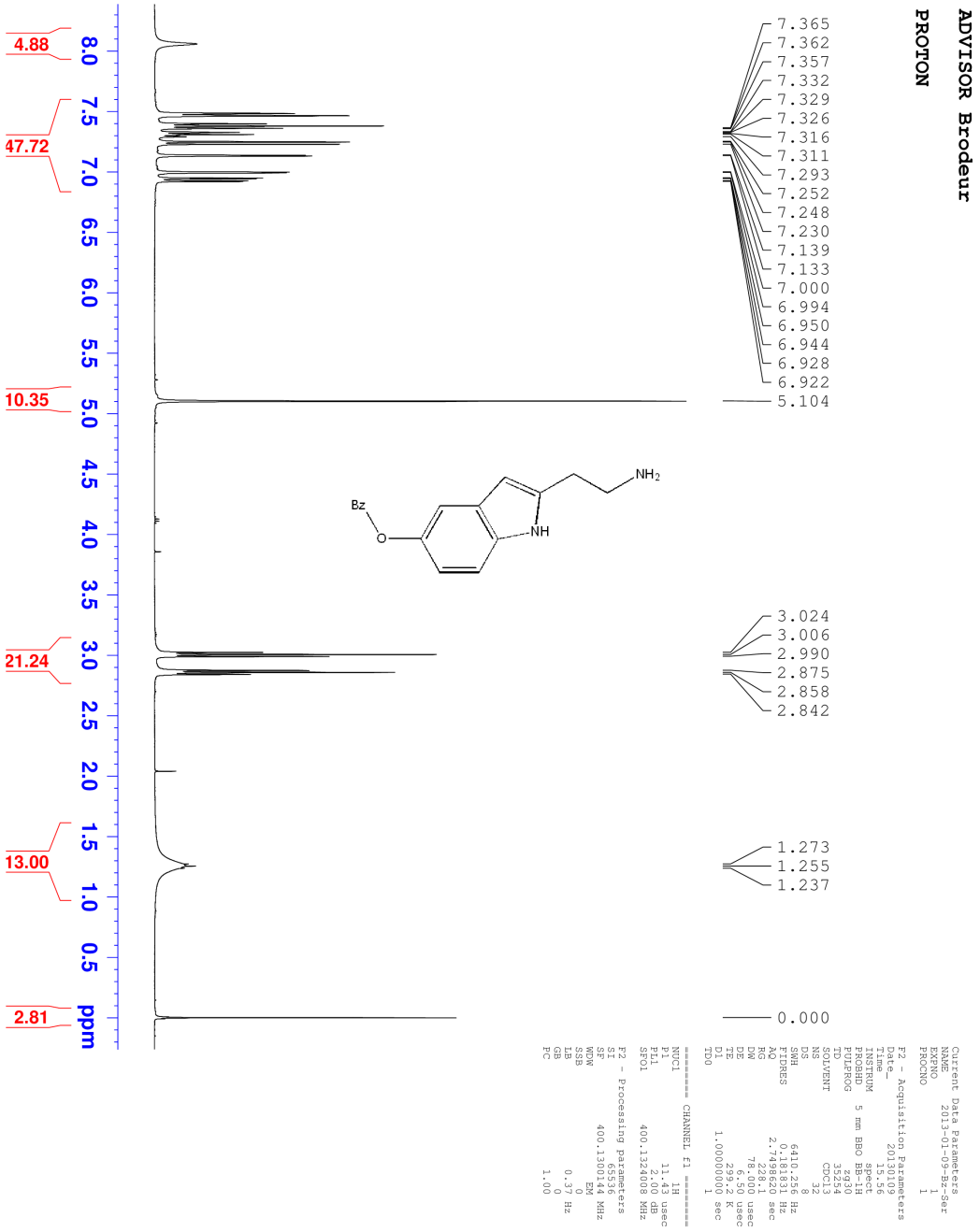


Figure 17 1H NMR Spectrum of 5-Bz-Serotonin

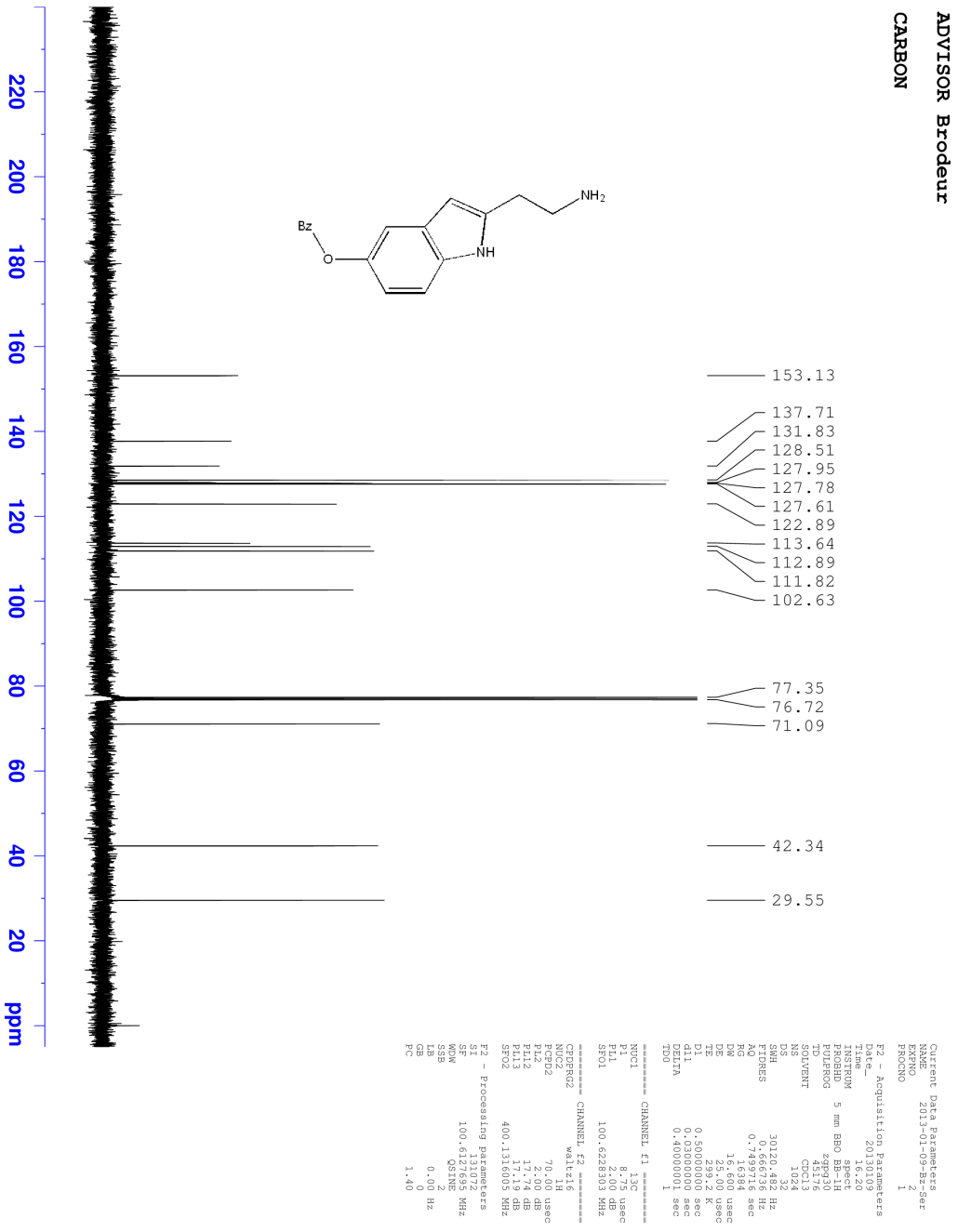


Figure 18 13C NMR Spectrum of 5-Bz-Serotonin

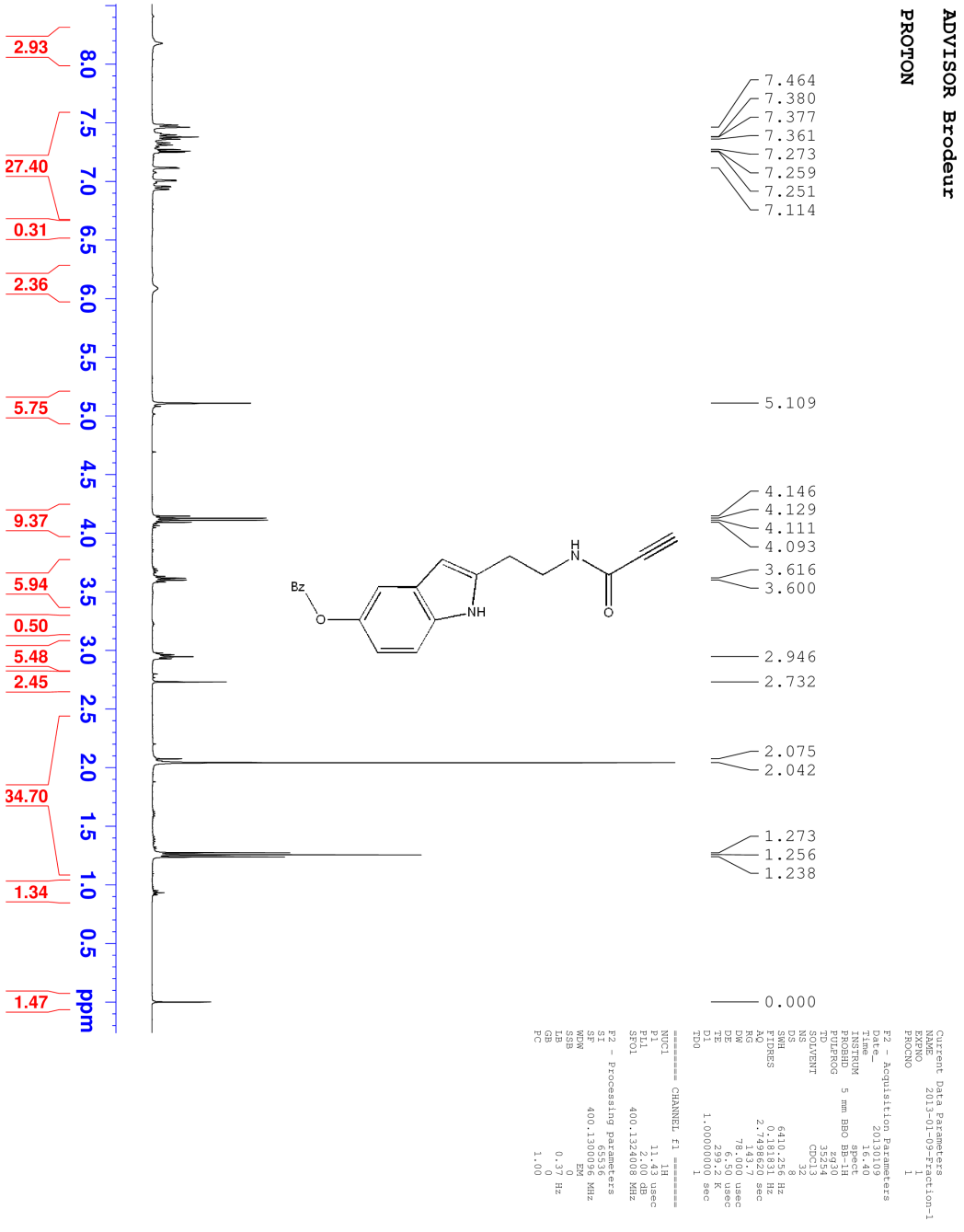


Figure 19 1H NMR Spectrum of 2-(2-propiolamidoethyl)-1H-indol-5-yl benzoate

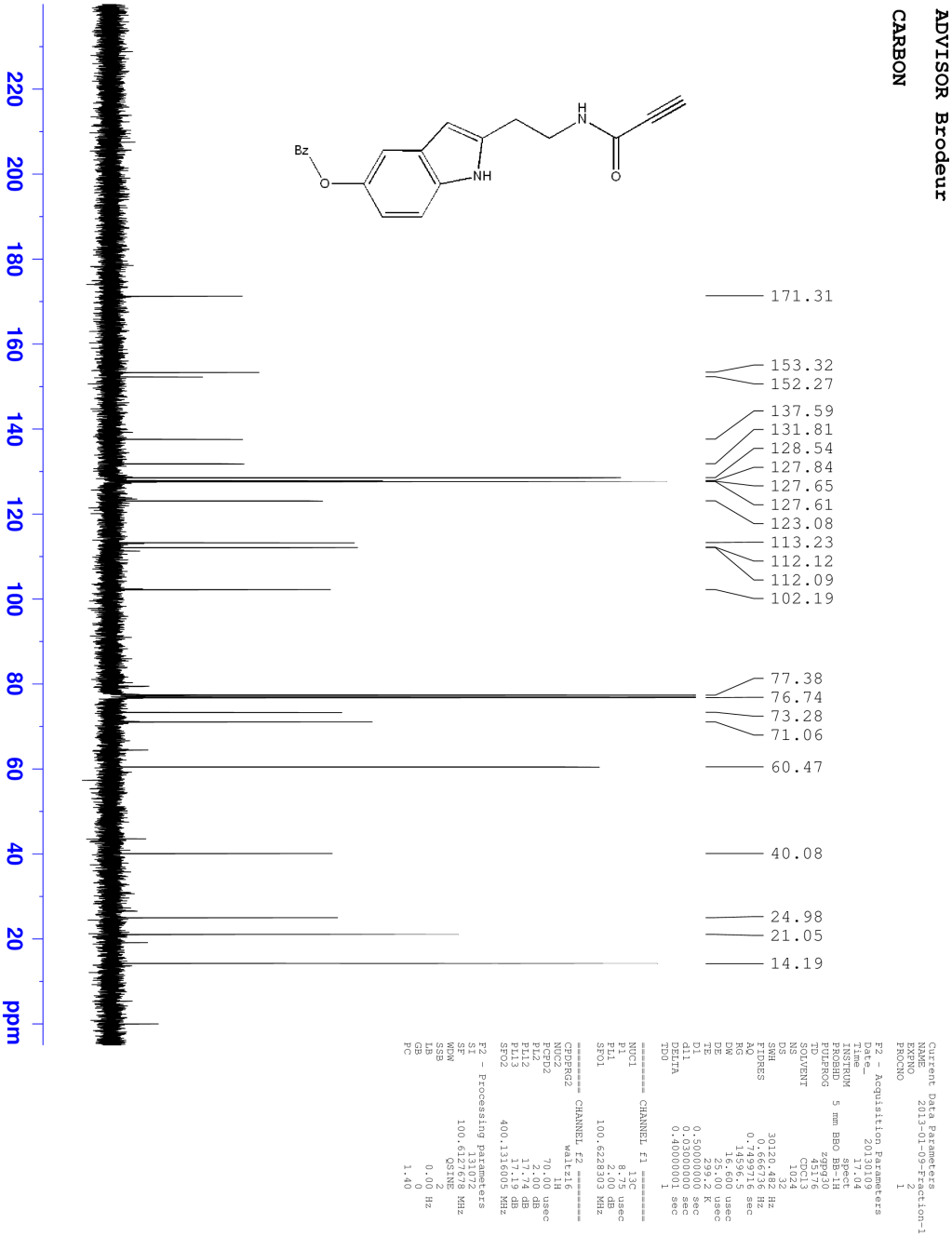


Figure 20 13C NMR Spectrum of 2-(2-propiolamidoethyl)-1H-indol-5-yl benzoate