# Development of 1,10-Phenanthroline-based Nek2 Biosensor for Live Cell Enzymology

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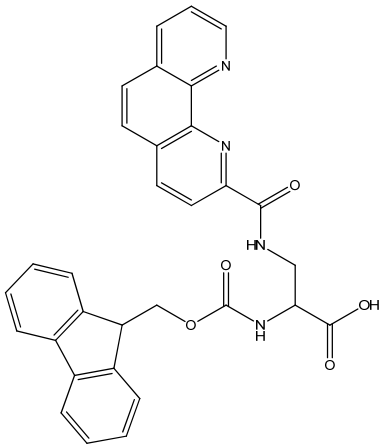
## Abstract



**FMOC-3-(1,10-phenanthrolino-2-carboxamido)-2,3-diaminopropanoic acid "PCAP"**

Nek2 is a centrosomal serine/threonine kinase that is overexpressed in many forms of human cancer. However, despite its involvement in cancer, much of the Nek2 biology remains unknown. This is primarily due to the lack of available chemical tools (e.g. biosensors) needed for *in vivo* study of this enzyme. We plan to develop a novel Nek2 biosensor utilizing 1,10-phenanthroline as a fluorophore. To accomplish this objective, the synthesis of the PCAP fluorophore (Fmoc-3-(1,10-phenanthroline-2-carboxamido)-2,3-diaminopropanoic acid) is undertaken in this study. The bidentate properties of 1,10-phenanthroline allows it to participate in chelation-enhanced fluorescence (CHEF). The PCAP fluorophore will be subsequently utilized to develop fluorescence-based Nek2 biosensors for studying the role of Nek2 kinase in living cells.

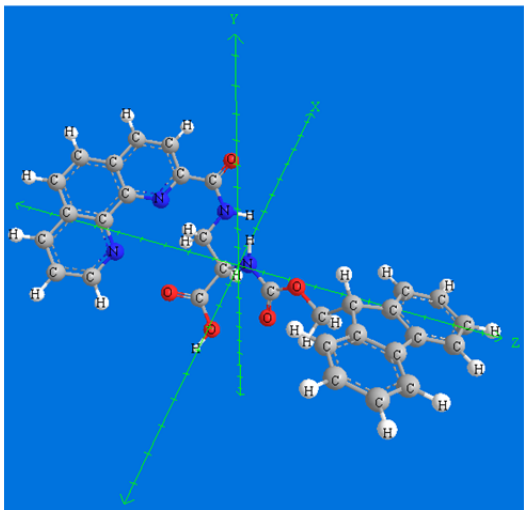


## Introduction

Accurate determination of enzymatic activity is critical in the development of inhibitors and activators. Standard methodology typically follows radiometric procedures, which can be costly and difficult to handle. Other techniques do include fluorometric means. This does provide a more stable and non-radioactive alternative, but can be costly and difficult to synthesize. Therefore, it is a primary focus of this investigation to develop an Fmoc-protected fluorescent amino acid that can be coupled to any peptide chain using established Fmoc-based solid-phase peptide synthesis procedures.

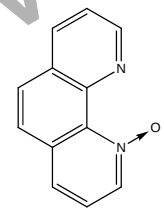
1,10-phenanthorline is a bidentate aromatic fluorescent molecule. Furthermore, studies have shown this moiety to chelate polyvalent metal cations. This, including its wide availability, made 1,10-phenanthroline an excellent candidate to incorporate into the development of a synthetic fluorescent amino acid; one that would take part in chelation-enhanced fluorescence (CHEF). The concept behind CHEF is to append a fluorophore into a substrate of a target enzyme. In the case of kinases, a phosphate will be attached to the hydroxyl group of its target residues. Upon phosphorylation, the fluorophore and the phosphorylated residue will form a coordination compound with polyvalent metal cations already in solution. The extensive conjugation in conjunction with the chelated complex results in a notable increase in fluorescence as well as a shift in the fluorescence to longer wavelengths of light. This effect is visualized in the future work section of this paper.

3D Structure of the PCAP Fluorophore

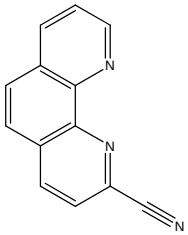


During the course of this study, 2-carboxy-1,10-phenanthroline was synthesized from 1,10-phenanthroline according to published procedures. Afterwards, several attempts were made to couple the synthesized carboxylic acid to an amine in order to form an amide bond. The end goal was to find a method of coupling that would be applicable to couple our 2-carboxy-1,10-phenanthroline to Fmoc-DAP-OH. However, both liquid-phase and solid-phase coupling, using several methodologies failed to yield positive results. Additional efforts will include conversion of the carboxylic acid into an acyl chloride to form the amide bond with Fmoc-DAP-OH. A planned backup synthesis involving reductive amination may also be explored. However, the resulting final structure is slightly modified. If the reductive amination procedure is successful, the end coupling would be an amine bond instead of an amide bond. This is illustrated in the experimental methodology roadmap section of this paper.

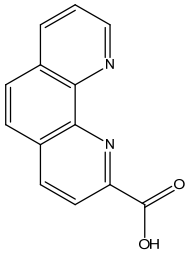
## Experimental Methodology – Organic Synthesis



**Synthesis of 1,10-phenanthroline-1-oxide**. 3.0g of 1,10-phenanthroline was completely dissolved in glacial acetic acid (AcOH) under magnetic stirring. Then 2.0mL of 30% hydrogen peroxide ($H_2O_2$) was added drop-wise to the reaction chamber and refluxed for 6 hours. Halfway through the reflux, an addition 2mL of $H_2O_2$ was added drop-wise to the reaction chamber. After cooling, the mixture was neutralized (≈pH 9-10) with a saturated solution of potassium hydroxide (KOH). At this time, a heavy brown precipitate formed and was extracted repeatedly with chloroform until no longer precipitate remained in solution. The chloroform fractions were combined and rotary evaporated to yield dark and light brown solids.

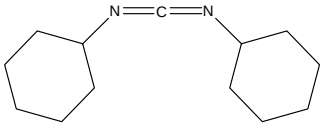


**Synthesis of 2-cyano-1,10-phenanthroline**. A solution of 1.2g of 1,10-phenanthroline-1-oxide and 1.2g of potassium cyanide (KCN) in a solution of 15mL of deionized water was added drop-wise to a sample of 1.7mL of benzoyl chloride under magnetic stirring. The solution was stirred for a total of 3 hours and vacuum filtered to yield a brown solid.



**Synthesis of 2-carboxy-1,10-phenanthroline**. 1.13g of sodium hydroxide (NaOH) in 8mL of deionized water was added to a solution of 1.16g of 2-cyano-1,10-phenanthroline in 10mL of ethanol. The mixture was refluxed for 2 hours and concentrated via rotary evaporation. The remaining solution was then acidified with dilute hydrochloric acid (HCl). The solid was isolated and recrystallized from water and acetone to yield very hygroscopic light tan fiber-like needles, which were dried via vacuum desiccation.

**Attempted synthesis of PCAP via DCC coupling**. A solution of 149mg of N,N'-dicyclohexylcarbodiimide (DCC) and 80mg of N-hydroxysuccinimide (NHS) in 2.0mL of dry dimethylformamide (DMF) was added drop-wise to a solution of 105mg of 2-carboxy-1,10-phenanthroline in 2.4mL of dry DMF in an ice water bath under argon atmosphere. The mixture was allowed to stir overnight. The following morning, the mixture was immediately added to a reaction vessel containing Fmoc-protected 2,3-diaminopropanoic acid (Fmoc-DAP-OH), 200µL of triethylamine ($Et_3N$), and trace amounts of water in 2.0mL of tetrahydrofuran (THF). This mixture was stirred at 35°C for 5 hours.




## Experimental Methodology - Purification

Several attempts to recrystallize the 2-carboxy-1,10-phenanthroline from ethanol resulted in failure, contrary to the literature. Thus, the carboxylic acid was dissolved in water and vacuum filtered and evaporated. The resultant solid was then dissolved in hot ethanol and immediately vacuum filtered. This removed many of the water and ethanol insoluble impurities, such as the sodium chloride and certain unreacted precursors.

## Results

2-carboxy-1,10-phenanthroline was synthesized from 1,10-phenanthroline using established methodologies. However, attempts to couple Fmoc-DAP-OH and 2-carboxy-1,10-phenanthroline, using liquid-phase DCC coupling as well as Fmoc-based solid-phase peptide synthesis procedures proved to be unsuccessful.

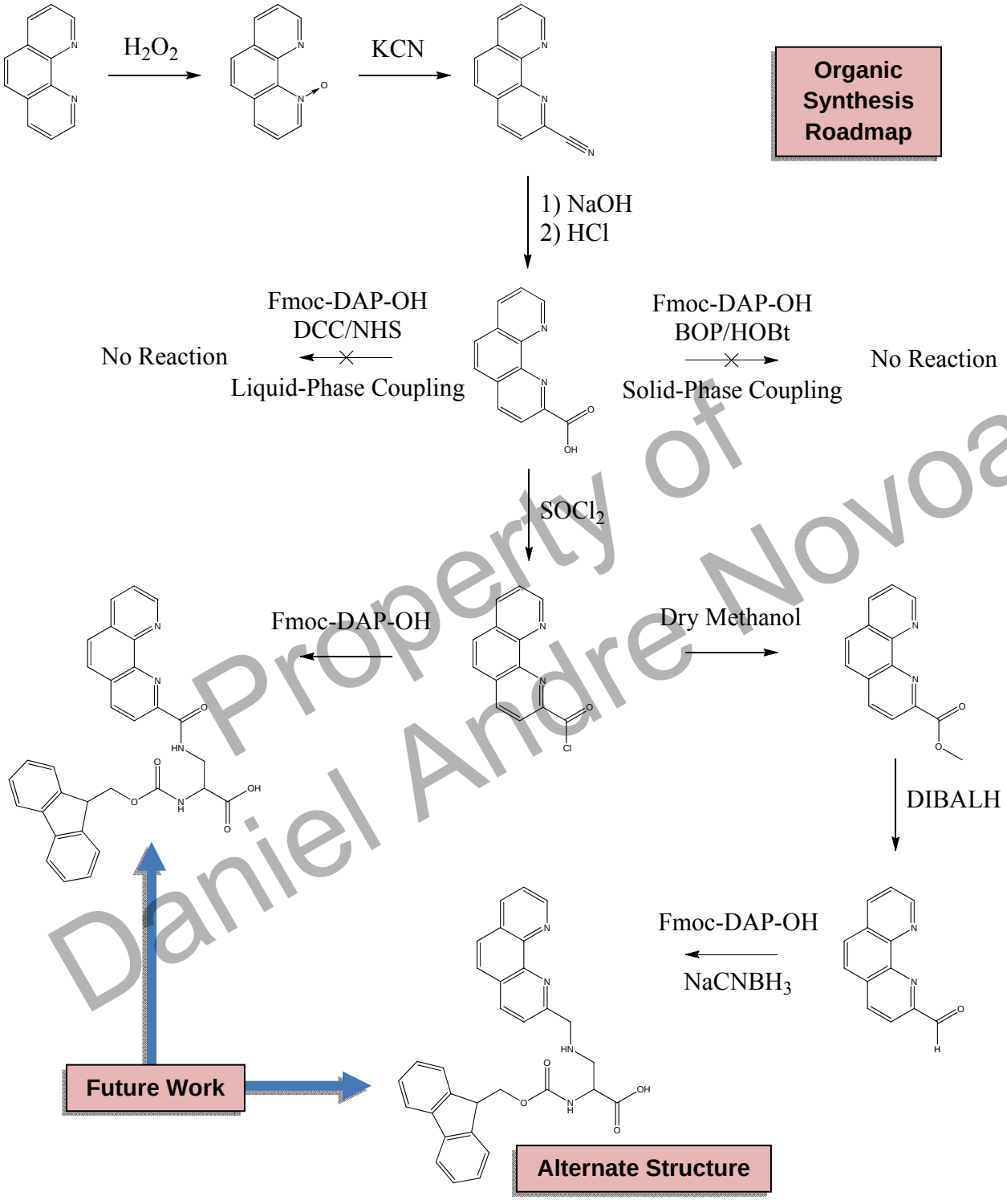
## Discussion

Synthesis of the PCAP fluorophore remains a work in progress. Two methods of incorporating the 1,10-phenanthroline motif into an Fmoc-protected amino acid include an acyl chloride coupling and reductive amination. Even though reductive amination would result in a slightly altered structure of our target compound, the end goal of this investigation would still be met. The altered structure is graphically represented in the experimental methodology roadmap section of this paper.

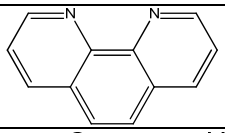
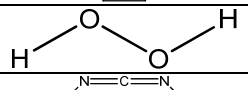
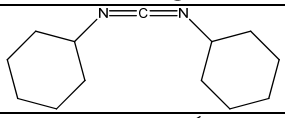
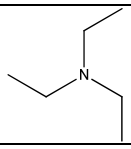
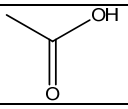
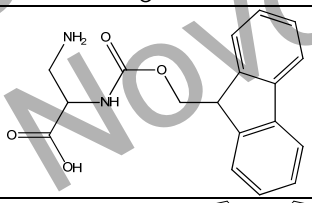
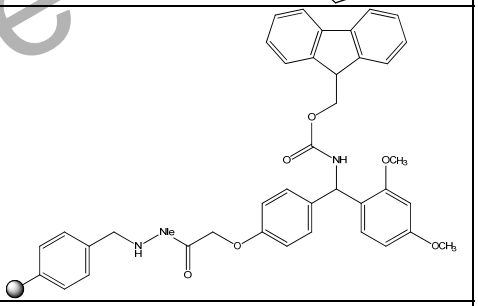
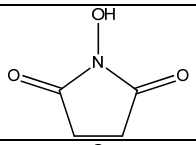
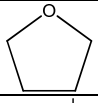
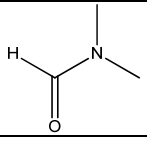
Illustrated in the future work section of this paper is the expanded structural formula of an established Nek2 recognition motif (IRRLSTRRR). Appended into the peptide sequence is the synthetic amino acid PCAP, a primary intention of this research. Although the image illustrates that PCAP is inserted into the primary structure only one amino acid away from the phosphorylatable residue, multiple peptides would have to be synthesized with PCAP in various locations to determine the optimal distance from the phosphate group in order to give the most prominent increase in fluorescence.

Upon phosphorylation of the serine residue and addition of divalent metal cations, PCAP and the phosphoserine form coordination bonds with the positively charged metal ion. The complex structure greatly increases the fluorescence of an already fluorescent molecule, upon application of UV-light. This process has been well established in literature and is referred to as chelation-enhanced fluorescence (CHEF). It also typically results in a shift in the fluorescence to longer wavelengths of light. Additionally, it is worth noting that the coordination bonds would not only take place between magnesium ions. In fact, many polyvalent metal cations can be used, including ionic zinc, calcium, and cadmium.

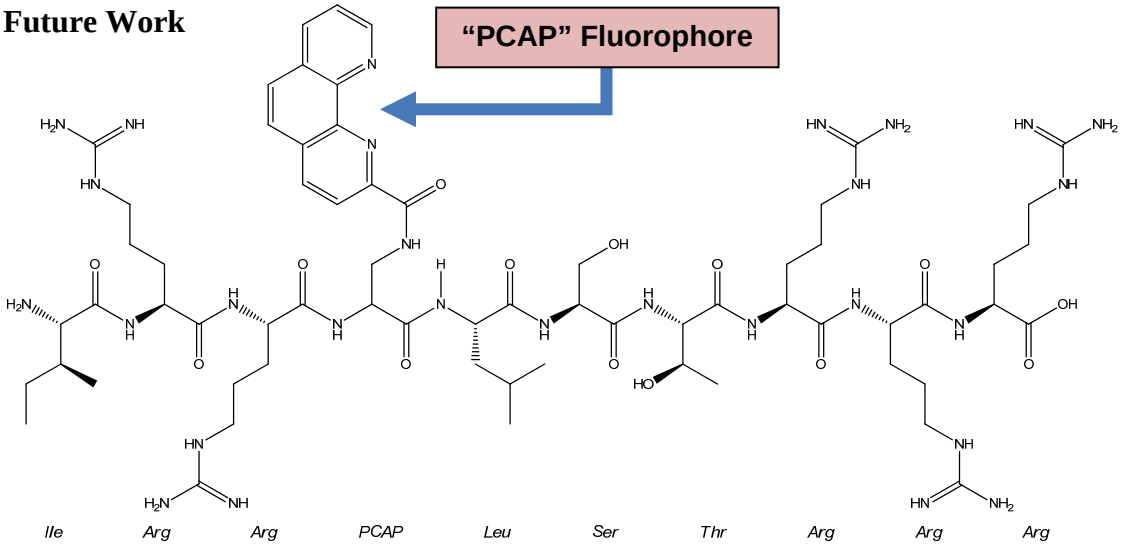
**Experimental Methodology - Summary**

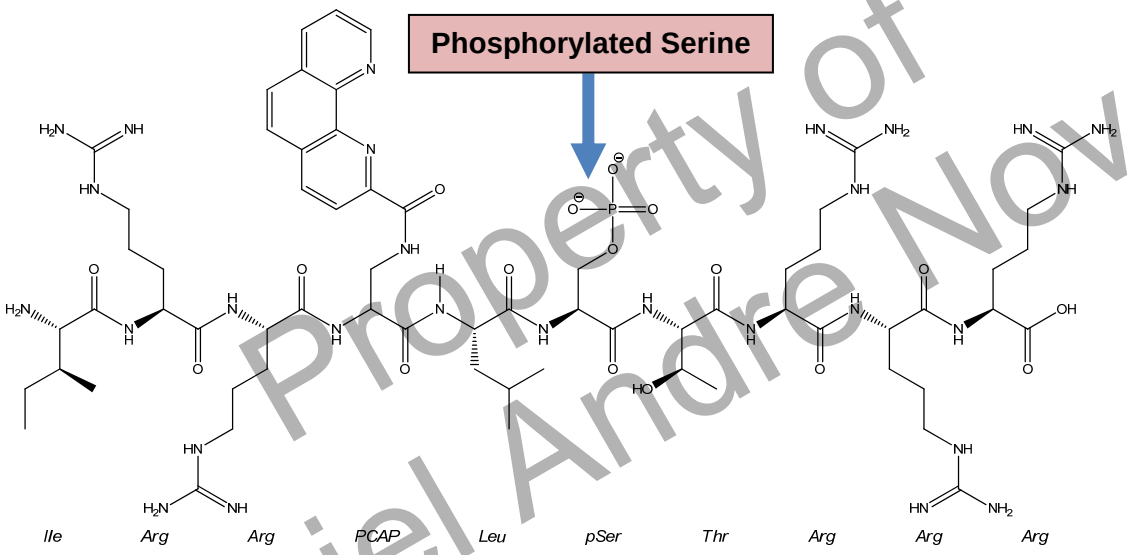


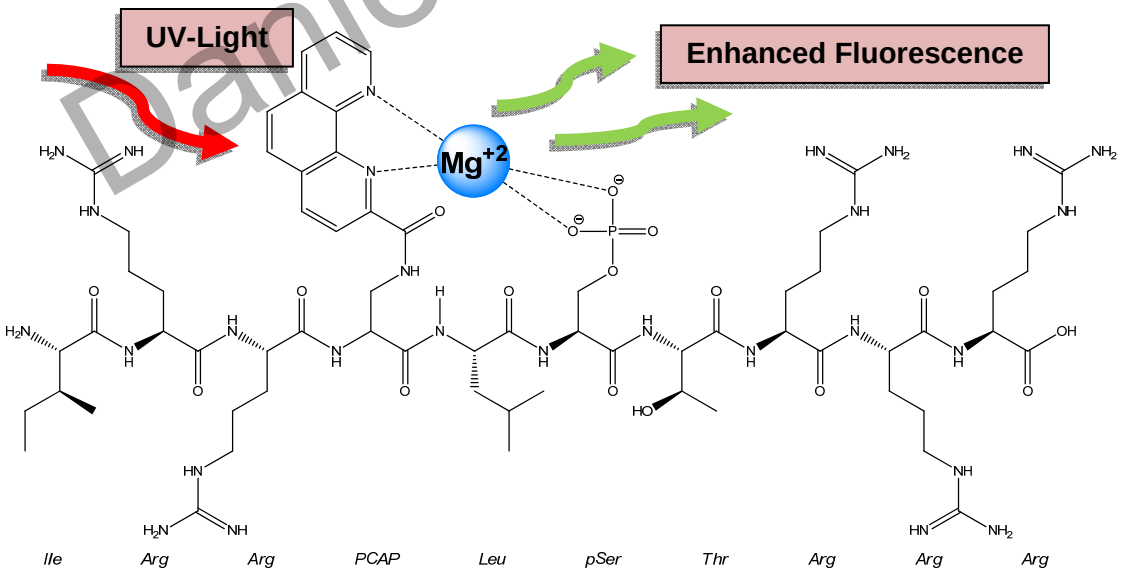
**Materials**

| Chemical Name | Manufacturer | Lot Number | Structure |
|---|---|---|---|
| 1,10-Phenanthroline | Acros Organics | A0310819 |  |
| 30% Hydrogen Peroxide ($H_2O_2$) (08/03/2011) | Fisher Scientific | AD-11207-18 |  |
| N,N'-Dicyclohexylcarbodiimide (DCC) | Sigma Aldrich | 01202AJ |  |
| Triethylamine ($Et_3N$) | Alfa Aesar | E14S031 |  |
| Hydrochloric Acid (HCl) | Pharmco-AAPER | PL001365HAG | HCl |
| Glacial Acetic Acid (AcOH) | Pharmco-AAPER | 3001738AA |  |
| Fmoc-2,3-Diaminopropanoic Acid (Fmoc-DAP-OH) | Bachem Bioscience | 1008986 |  |
| Rink Amide AM Resin (200-400 mesh) loading = 0.71 $\frac{mmole}{g}$ | Novabiochem | S5272104-048 |  |
| Potassium Hydroxide (KOH) | | | KOH |
| Sodium Hydroxide (NaOH) | Sigma Aldrich | MKBG9080V | NaOH |
| N-hydroxysuccinimide (NHS) | | |  |
| Tetrahydrofuran (THF) | Pharmco-AAPER | PL001544THF |  |
| Dimethylformamide (DMF) | Acros Organics | 1013944 |  |

## Future Work

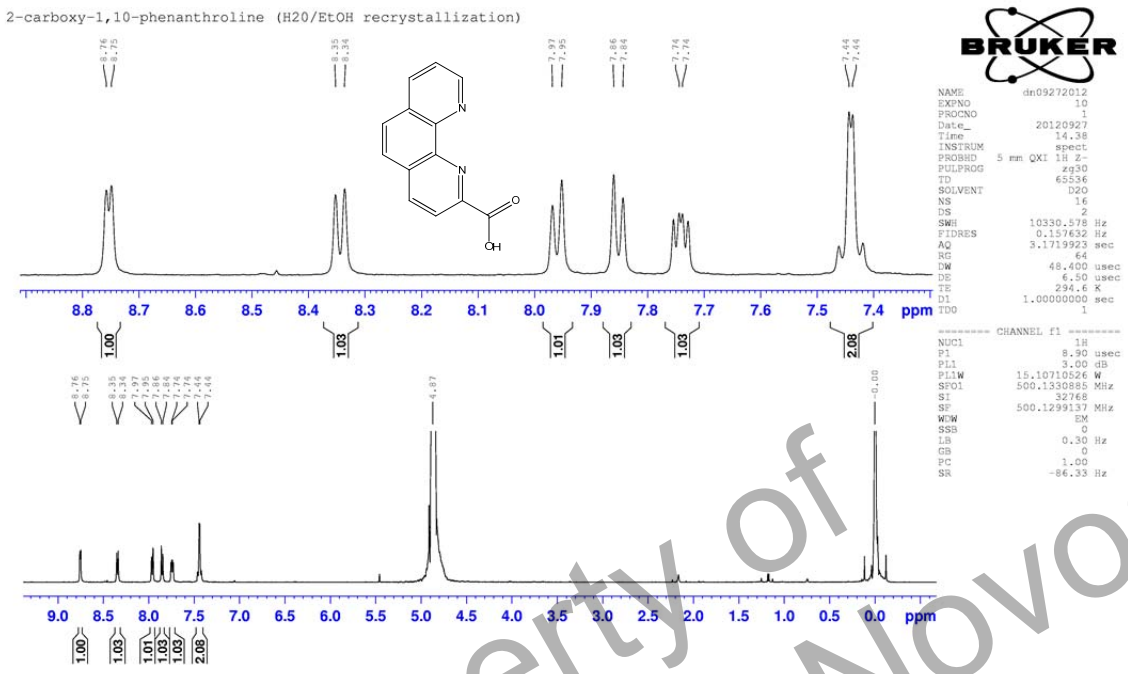


## NMR Spectra

### 2-carboxy-1,10-phenanthroline







## Acknowledgements

The work presented was made possible by a research grant from the National Science Foundation (NSF), under the mentorship of Dr. Sanjai Kumar at Queens College, as a part of the NSF STEP Queensborough Bridge Program under the Student Coordinator Alejandra Castaño. All drawn graphics were created by Daniel Andre Novoa using the ChemBioDraw Suite by Chambridgesoft and Microsoft Office.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