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Julia Donovan March 17, 2023

Asparagine and Glutamine Bioconjugation

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Chemistry

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Abstract

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Bioconjugation is a method that joins together bioactive and small molecules and has applications for activity-based protein profiling, chemoproteomics, and probing proteins. While there are bioconjugation methods for the selective modification of nucleophilic amino acids, there are no methods for the bioconjugation of weak nucleophiles- glutamine and asparagine. This study focuses on the development of a bioconjugation method for the chemoselectivity of asparagine and glutamine. Specifically, this study highlights the synthesis of dihydroxyl amine hydrocarbon and polyethylene glycol linkers to staple asparagine and glutamine residues on peptides. The impact of the results is a method to selectively label asparagine and glutamine, which can be applied to chemoproteomics labeling, stability of peptide drugs, and the discovery of new proteins containing asparagine and glutamine at the active site through flurosequencing. The impact of stapling asparagine and glutamine residues on a peptide will increase peptide rigidity, which improves biophysical properties to increase binding ability.

Asparagine and Glutamine Bioconjugation

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Acknowledgments

I would first like to thank Dr. Monika Raj for her support and guidance throughout my time as an undergraduate in her lab. She's not only supported me through all my research challenges but also in my pursuit of graduate school. My time in her lab has allowed me to grow as a chemist.

I would also like to thank Dr. Jose Soria who sparked my interest in organic synthesis through his lab class freshman year. His continued guidance as my chemistry advisor has been invaluable.

I would also like to thank Dr. Matthew Weinschenk. Taking his Advanced Organic class solidified my love for organic synthesis. I very much enjoyed attending his office hours to talk more about chemistry and life.

Finally, I would like to thank everyone in the Raj lab, especially John Talbott and Benjamin Emenike for their guidance and support on my thesis project. Thank you so much to all the grad students, post-docs, and undergrads for listening and helping with my research problems.

Contents

Introduction:

Bioconjugation chemistry aims to form a covalent bond between bioactive molecules. Bioconjugation can also be used to link biomolecules to small molecules, such as dyes, ligands, or drugs. Bioconjugation is a useful tool in probing proteins, chemoproteomics labeling, the discovery of new proteins, and activity-based protein profiling.[1]

A specific application of bioconjugation is to stapling peptides. The addition of a staple locks the peptide into an exact conformation, which increases biophysical properties like conformational and proteolytic stability, cellular uptake, binding affinity and specificity for biological target.[17] There are two types of stapled peptides. The first has the reactive residues in the middle of the peptide so that when the linker is added, the staple forms between the two residues to form a linear stapled peptide. The second type has the reactive residues on both ends of the peptide, so that when the linker is added, it forms a stapled cyclic peptide or macrocyclic peptide. The addition of the staple increases rigidity of the peptide by locking the peptide into an α -helical conformation. Chemists are able to chemically change the framework of the peptide to stabilize its degradation and mimic binding sites of proteins, making stapled peptides a useful tool for the developed of peptide-based drugs. The macrocyclization of a peptide further increases its rigidity which increases binding affinity even more and makes the peptide resistant to hydrolysis (Figure 1).[18]

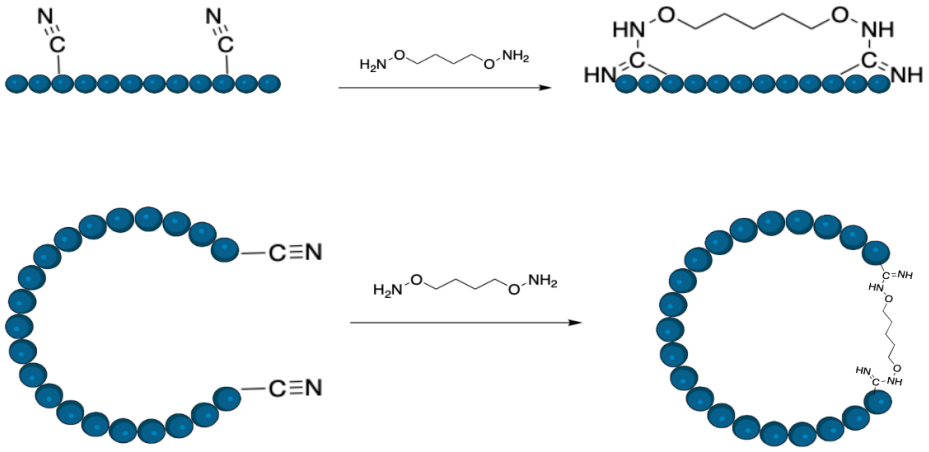


Figure 1 Stapled Peptides

Bioconjugation of asparagine (Asn) and glutamine (Gln) is especially important due to their function in metabolic functions of endothelial cells and supporting growth and survival of some cancer cells.[2, 3] Asn and Gln are fairly abundant amino acids. Gln is the most abundant amino acid in the body and is found to make up 20% of the total free amino acids in the blood and 40-60% of free amino acids in liver and skeletal muscles.[4] Many methods have been developed (DIANA and PrionW) to identify Asn and Gln residues in proteins due to their correlation with prion formation.[5]

However, despite the wide applications of bioconjugation, the prevalence of Asn and Gln and need for biological tools to identify Asn and Gln, there exist no methods for the bioconjugation of Asn and Gln. There are no peptide examples for side-chain modification of Asn and Gln in arylation, halogenation, oxidation, 1,4-addition, cross-coupling, pericyclic reactions, photoreactions, and radical reactions with some preliminary results in alkylation, acylation,

condensation, and transition-metal functionalization (Figure 2).[15] This is likely due to the presence of more nucleophilic and oxidizable amino acids that outcompete Gln and Asn.

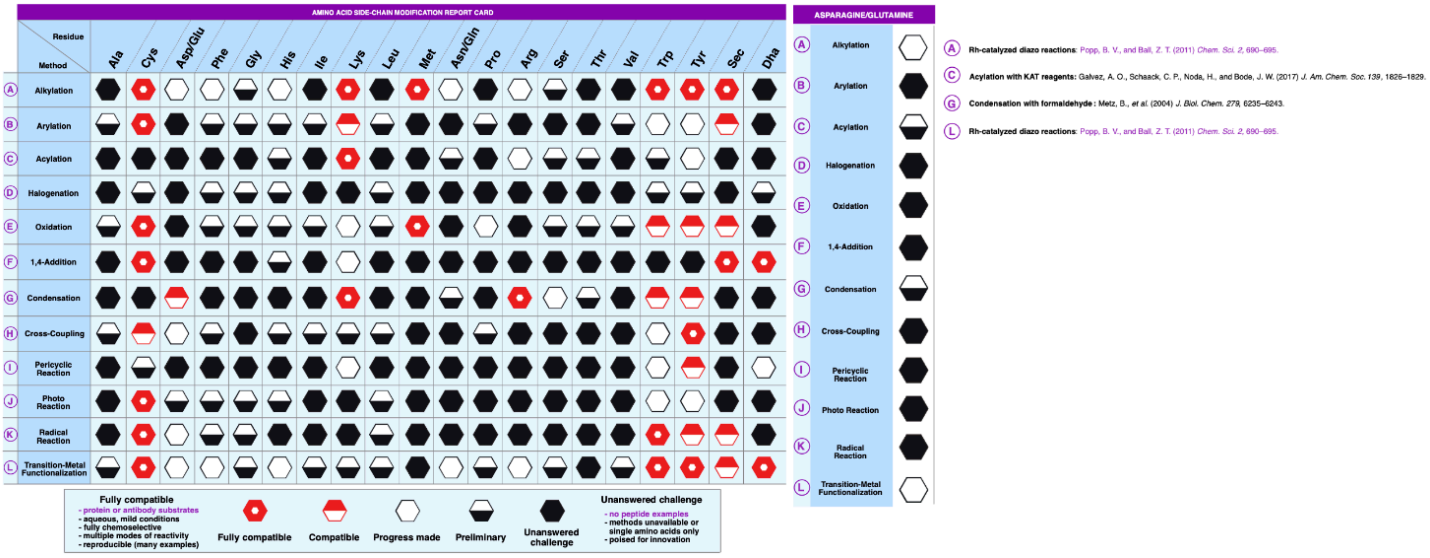


Figure 2 deGruyter Bioconjugation Chart

In 2011, Popp et. al. reported an example of modified Gln and Asn with a dirhodium metallopeptide catalyst (Figure 3).[16] Gln was converted at a rate greater than 95% in 6 h whereas Asn was converted at a rate of 70% in 24 hr. While an advancement in bioconjugation of Asn and Gln, full conversion was not observed, the catalyst used is not commercially available, and the system is not compatible with proteins.

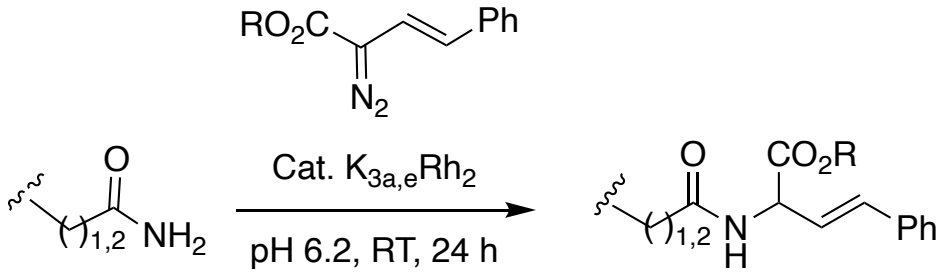
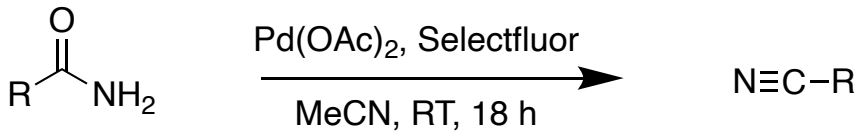


Figure 3 Popp and Ball Asn and Gln Chain Modification Method

The Raj lab set out to develop a chemoselective method for Asn and Gln that proceeds in a physiological pH, aqueous solution, and room temperature. The Raj lab envisioned converting the primary amide side chain on Asn and Gln to a nitrile. Several papers have reported this reaction. In 2015, Cheng et. al. reported a palladium-catalyzed intermolecular aminocarbonylation with iodine. They also observed that the fastest reaction was the side reaction of carbon monoxide reducing Pd(II) to Pd(0), thus the reduction of the palladium catalyst had to be slowed down, which was done by increasing the Lewis acidity of palladium.[7] In 2018, Al-Huniti et. al. presented a reaction that used a palladium (II) catalyst with Selectfluor to chemoselectively convert primary amides into nitriles, and in 2019, Okabe et. al. reported a palladium-catalyzed dehydration of primary amides to nitriles in aqueous conditions using dichloroacetonitrile as a water acceptor (Figure 4).[8,9]

A. Al-Huniti et. al.



B. Okabe et. al.

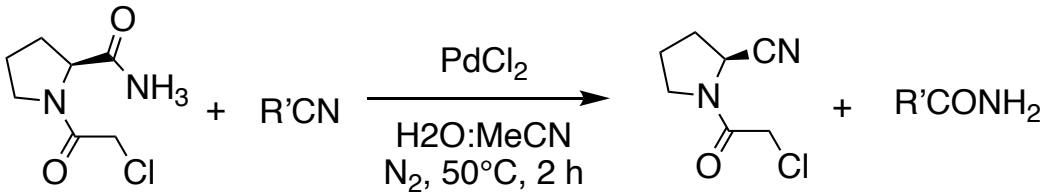


Figure 4 Previous Reports of Primary Amides to Nitriles

These reactions inspired the Asn and Gln bioconjugation project and were used as a starting point for the reaction development. The envisioned idea involved using a modified catalytic Appel reaction as a redox-neutral dehydration of primary amides that became known as redox neutral bioconjugation reaction (RN-BioCoRe).

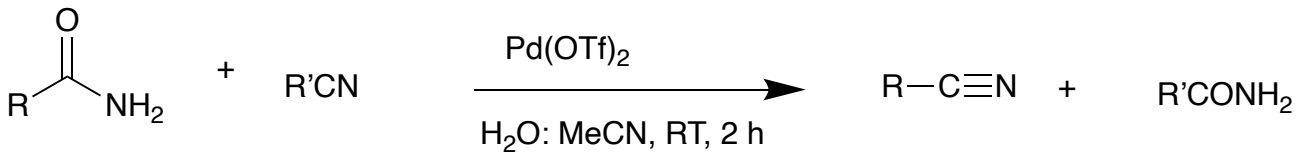


Figure 5 RN-BioCoRe Conditions

John Talbot of the Raj lab optimized reaction conditions to discover that a solvent system of 1:1 water: acetonitrile with $Pd(O_2CCF_3)_2$ as the catalyst yielded the best results with complete conversion in 2 hours.

Optimization of Pd Catalyst						
Pd type (1 equiv.)	1 hour	2 hours	4 hours	8 hours	12 hours	24 hours
$PdCl_2$ (II)	20%	-	-	67%	-	94%
$PdOAc_2$ (II)	10%	-	-	25%	-	63%
$Pd(O_2CCF_3)_2$ (II)	80%	>99%	-	>99%	-	-
$PdACN_2Cl_2$ (II)	-	15%	-	65%	-	-

Table 1 Palladium Catalyst Optimization

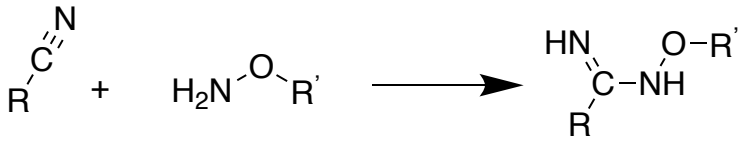
Due to the acidic nature of the ligands, buffer systems were tested and overall had little effect on the conversion rate and time.

Percent Conversion at Room Temperature with $Pd(Otf)_2$		
	½ hour	2 hours
pH 8.0	91%	>99%
pH 10.0	93%	>99%

Table 2 Buffer Trials

The last step in the methodology development was to test the chemoselectivity ability of RN-BioCoRe. The system was discovered to be 100% chemoselective for Asn and Gln, which was expected since Asn and Gln are the only amino acids that have an amide sidechain.

Having a developed a method to convert the sidechain of Asn and Gln to a reactive functional group, the envisioned idea was to trap the nitrile with a hydroxylamine. The rational is that the hydroxylamine would be nucleophilic enough to attack the nitrile. In addition, many commercially available protein dyes contain a hydroxylamine, so it would be easy to attach different R groups to the hydroxylamine such as affinity tags, fluorophore, drugs, and peptide protein complexes (Figure 6).[6]



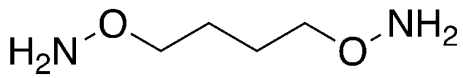
R'= Affinity tags such as alkynes and Biotin
=Fluorophore
=Drugs
=Peptide Protein Complexes

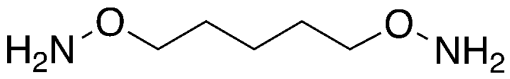
Figure 6 Envisioned Asn/Gln Nitrile Trapping

Having developed RN-BioCoRe, this study focuses on the development of the linkers that will be used to form stapled and macrocyclic peptides.

Results:

The plan was to develop three dihydroxyl amine hydrocarbon linkers with varying lengths of the hydrocarbon chain, specifically, a length of four, five, and six carbons, as these linkers would be water-soluble and potentially useful in cells as well as allow asparagine and glutamine to be stapled at varying residues apart (Figure 7).





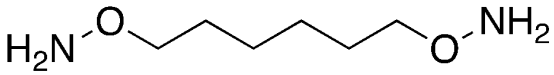
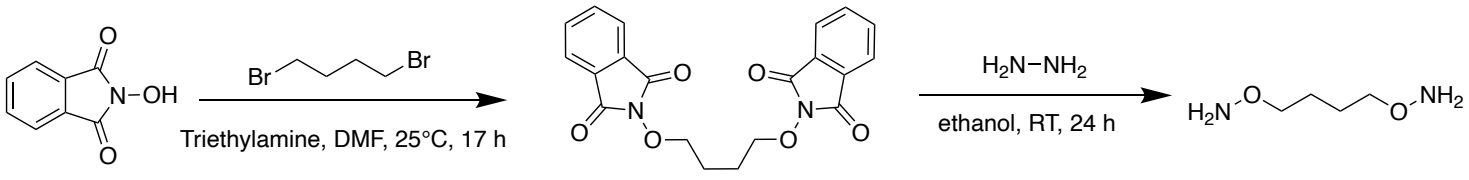


Figure 7 proposed linker structures

The developed reaction scheme proposed two steps to obtain the linker (Scheme 1).



Scheme 1 Proposed Synthesis of Dihydroxyl Amine Hydrocarbon Linkers

The first proposed step formed a white precipitate after 17 hours (Figure 8).[10] According to the paper, the precipitate contained the desired product, but both were analyzed as a precaution.

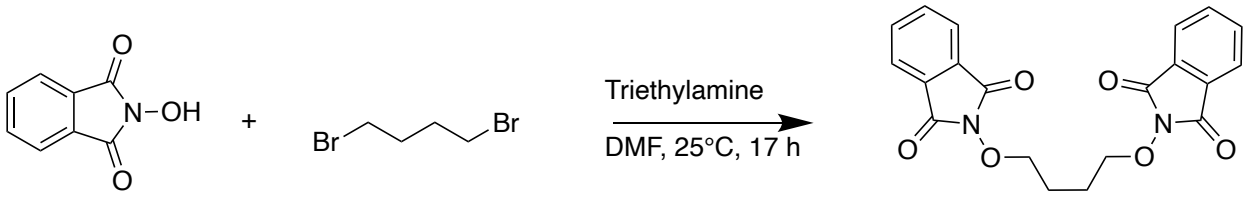


Figure 8 First Reaction Step for Dihydroxyl Amine Hydrocarbon Linker

The filtrate was purified to obtain three different compounds, none of which lined up with the mass. The white precipitate was not dissolvable in any deuterated solvents (chloroform, DMSO, and deuterated water). However, it was dissolvable in a mixture of organic solvents and a mass was able to be obtained.

The mass of the desired product (380 plus sodium) was seen as was a mass of 319. Based on the mass, the other major product forming was believed to be a nucleophilic attack from triethylamine (Figure 9). Without an NMR to prove that the desired product and side product were forming, a recrystallization was attempted in 100°C, but the crude product still would not dissolve.

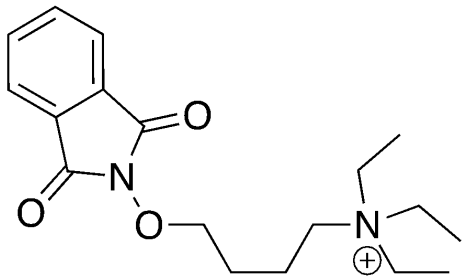


Figure 9 Proposed Triethylamine Impuritiy

Since it was believed that the crude product would not dissolve due to the triethylamine impurity, the reaction was rerun with a change from six equivalents of triethylamine to two and four equivalents of triethylamine. The idea was triethylamine was added in the exact amount needed to deprotonate 2-hydroxyisoindoline-1,3-dione and not added in excess to hopefully avoid the formation of the impurity. After leaving overnight with heat for over a week, no precipitate was seen in either reaction flask and the resulting solutions had no desired product. The second step of the reaction contained hydrazine and it was proposed that perhaps the hydrazine makes the crude compound soluble. The reaction was run in ethanol with hydrazine (Figure 10).[11] While the compound appeared to dissolve, the resulting reaction turned into one clump of white precipitate that was unable to dissolve in any solution of organic solvents and thus unable to be analyzed in any way. The reaction was run with hydrazine as the solvent, which did allow the crude compound to dissolve. After purification and analysis via NMR, the compounds did not match the desired product.

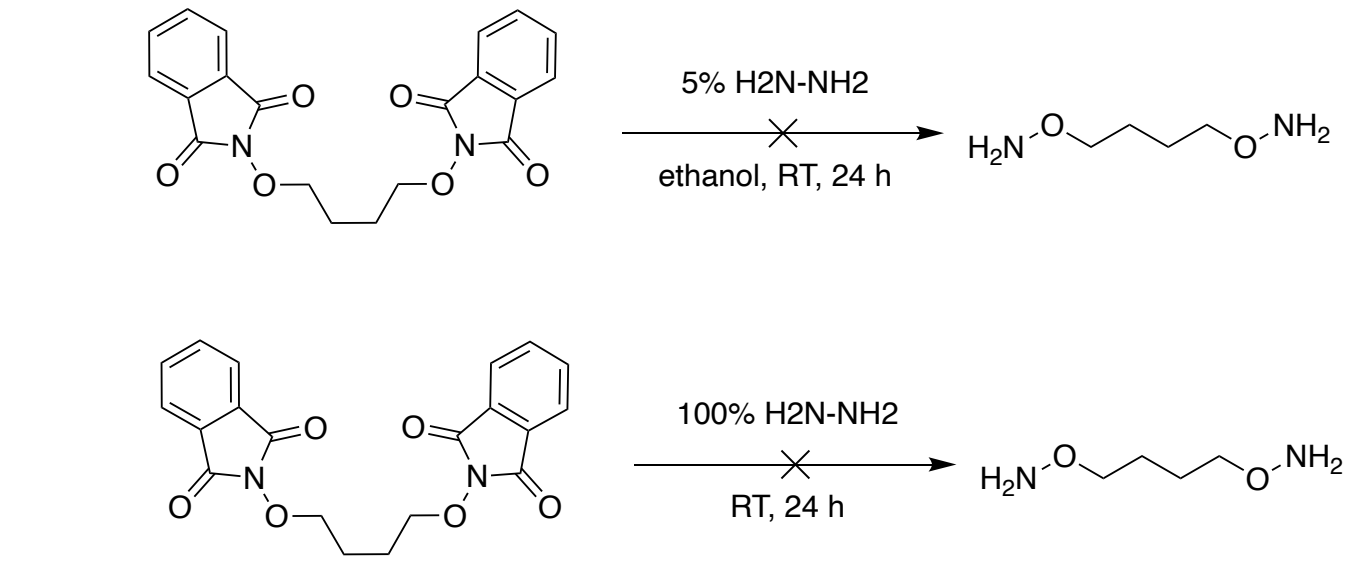


Figure 10 Second Reaction Step for Dihydroxyl Amine Hydrocarbon Linker

Since triethylamine appeared to cause issues in the reaction, it was swapped out in favor of DBU (Figure 11).[12] A precipitate was formed and while washing with cold acetonitrile, the precipitate dissolved. After lyophilizing, no solid was reobtained.

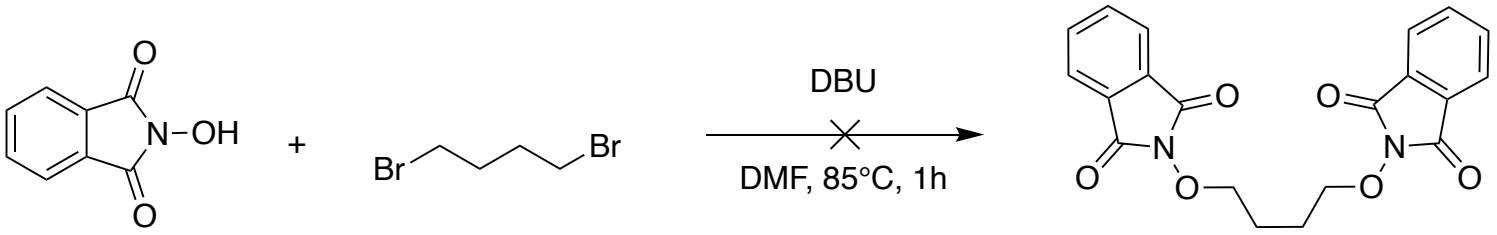
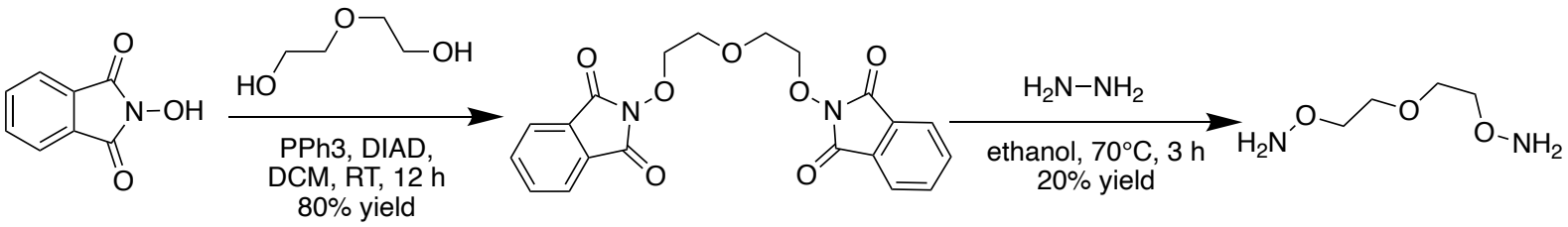


Figure 11 DBU Reaction Step for Dihydroxyl Amine Hydrocarbon Linker

Since a purified dihydroxyl amine hydrocarbon linker would not be obtained due to solubility issues, the focus was shifted to the development of a water-soluble dihydroxyl amine

polyethylene glycol (PEG) linker as the addition of heteroatoms would increase the linker's solubility. The proposed reaction scheme was very similar to the hydrocarbon linker (Scheme 2).



Scheme 2 Proposed Synthesis of Water-Soluble Dihydroxyl Amine

Following the procedure for the first step (Figure 12),[13] the NMR showed the formation of the desired product along with an impurity. This crude product was used in the second step as the procedure stated but was unable to be fully purified.

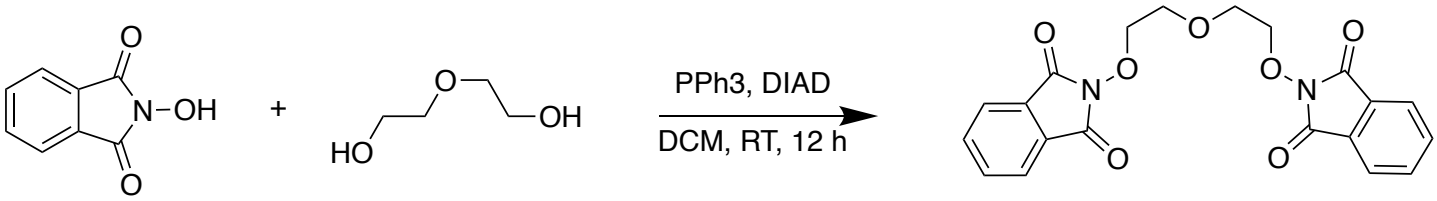


Figure 12 First Reaction Step of Water-Soluble Dihydroxyl Amine Linkers

The second reaction step showed the same impurities as the first reaction, so it was believed that purification of the first step would grant the pure PEG linker (Figure 13).

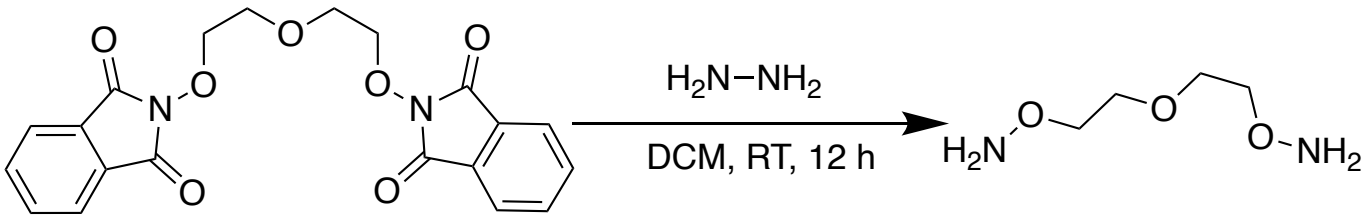


Figure 13 Second Reaction Step of Water-Soluble Dihydroxyl Amine Linkers

The first step of the PEG linker was purified by column.[14] While some impurities were removed, the NMR still showed the product peaks and another impurity. This impurity was identified as the known hydrazine byproduct from a Mitsunobu reaction (Figure 14).

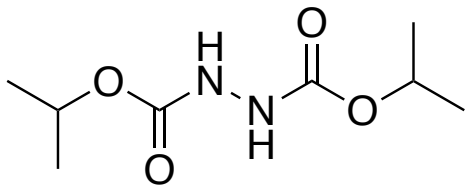


Figure 14 Second Step of Water-Soluble Dihydroxyl Amine Linker Impurity

Running a reaction to cleave the nitrogen-nitrogen bond was considered but a considerable worry was selective cleavage of the nitrogen-nitrogen bond over cleavage of the nitrogen-oxygen bond in the desired product. Due to the large differences in polarity between the product and impurity, another column purification appeared to be the solution. Through various solvent systems and staining with phosphomolybdic acid, a solvent system was developed that removed all the impurities. The pure product was used for the second step. While product formation was seen in the second step, new impurities also appeared. A crude TLC was stained with ninhydrin to see the product spot on baseline regardless of solvent system

used. Lacking the proper equipment to run a reverse-phase column, the crude product was salted out with HCl. When no solid was observed, the crude in HCl was put in the fridge overnight to yield no precipitate. Another attempt at purification used a workup with water and then lyophilizing the water layer to yield a white solid that was not the desired product. Using heating, a white solid formed within 3 h and was purified through extraction to yield the pure 4-carbon dihydroxyl amine PEG linker. Currently, the first step for the 6-carbon dihydroxyl amine PEG has been successfully run and characterized.

Conclusion:

Through the development of Rn-BioCoRe, the Raj group was successfully able to chemoselectively convert the primary amide of Asn and Gln to a nitrile. Currently, we have synthesized the 4-carbon PEG linker and the first step of the 6-carbon PEG linker. The second step of the 6-carbon PEG linker and both steps of the 8-carbon PEG linkers have not been run yet. Once the pure linkers have been obtained, they will be reacted with containing Asn and Gln residues in an attempt to form stapled linear peptides and macrocyclic peptides[*, **] with the conversion rate being measured.

Supplemental Information:

All reactions were carried out in a regular atmosphere. Solvents for workup, extraction, and column chromatography were used as received from commercial suppliers without purification.

All other chemicals were purchased from Sigma-Aldrich, Oakwood Chemicals, or Alfa Aesar and were all used without further purification.

$_1$H nuclear magnetic resonance (NMR) spectra were taken on the following: Bruker 600 spectrometer (600 MHz) and Varian Inova 600 spectrometer (600 MHz). All were taken at room temperature in $CDCl_3$ with an internal $CHCl_3$ as the reference (7.26 ppm) unless otherwise state. Chemical shifts (δ values) were reported in parts per million (ppm). Multiplicity is indicated through standard abbreviations (s= singlet, d=doublet, t=triplet, q=quartet, qn=quintet, m=mulitplet). Analytical thin layer chromatography (TLC) was performed on precoated aluminum-backed 20 by 20 cm sheets and were visualized with UV light sometimes with a phosphomolybdic acid (PMA) stain.

*The following peptides have been synthesized by the Raj lab and have the following sequences.

QYKNFHLFK

QYKLFHNFK

**The peptides are purchased bioactive peptides and have the following sequences.

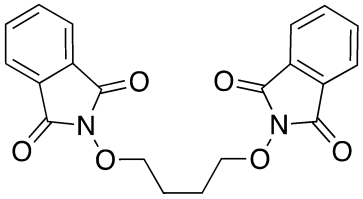
Adrenomedullin: YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY

Alpha endorphin:YGGFMTSEKSQTPLVT

GTP-binding fragment:CGAGESGKSTIVKQMK

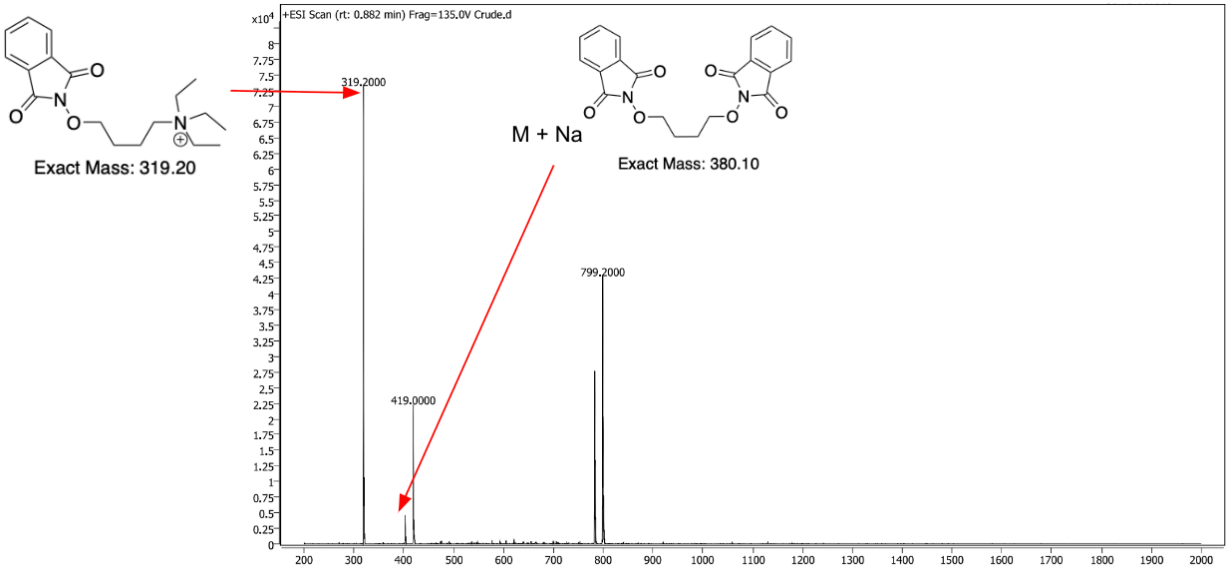
Aviptadil: HSDAVFTDNYTRLRKQMAVKKYLNSILN

Parathyroid hormone: SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF



2,2'-(butane-1,4-diylbis(oxy))bis(isoindoline-1,3-dione) Procedure 1

2-hydroxyisoindoline-1,3-dione(2 equiv), 1,4-dibromobutane (1 equiv), and triethylamine (6 equiv) were dissolved in DMF. The solution immediately turned red with the addition of the bromobutane. The reaction stirred for 25°C for 17 hours after which a white precipitate was formed. The precipitate was filtered off then washed with water. The filtrate was concentrated under vacuum and purified on column (5% MeOH/DCM). The white precipitate was analyzed via a mass spectrograph due to its inability to dissolve.



2,2'-(butane-1,4-diylbis(oxy))bis(isoindoline-1,3-dione) Procedure 2

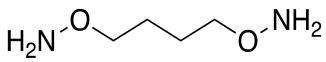
2-hydroxyisoindoline-1,3-dione(2 equiv), 1,4-dibromobutante (1 equiv), and triethylamine (2 equiv) were dissolved in DMF. The reaction stirred for 25°C overnight. After no formation of precipitate, the reaction mixture was heated to 50°C and left overnight. The next morning, there was still no precipitate, so the reaction was put in the fridge for an hour. After failing to crash out any precipitate, the reaction stirred for 80°C for 48 more hours. After a total of 72hours with no precipitate, the reaction was removed.

2,2'-(butane-1,4-diylbis(oxy))bis(isoindoline-1,3-dione) Procedure 3

The procedure for this remains the same as procedure 2 with the exception of adding 4 equiv of triethylamine. No product was formed.

2,2'-(butane-1,4-diylbis(oxy))bis(isoindoline-1,3-dione) Procedure 4

2-hydroxyisoindoline-1,3-dione(1 equiv) was dissolved in 30mL of DMF and DBU (2 equiv) was added dropwise. Thereafter, dibromobutane was added. The mixture was heated to 85°C for 1 hour. The resulting solution was then poured into ice and the precipitate was filtered and washed with 13mL of cold water followed by 8mL of cold ACN. With the addition of ACN, the crude dissolved and was lyophilized to form no product.



***O,O*'-(butane-1,4-diyl)bis(hydroxylamine) Procedure 1**

The crude from procedure 1 of 2,2'-(butane-1,4-diylbis(oxy))bis(isoindoline-1,3-dione(1 equiv) was added to a flask with ethanol. Hydrazine hydrate (2 equiv) was added dropwise and the

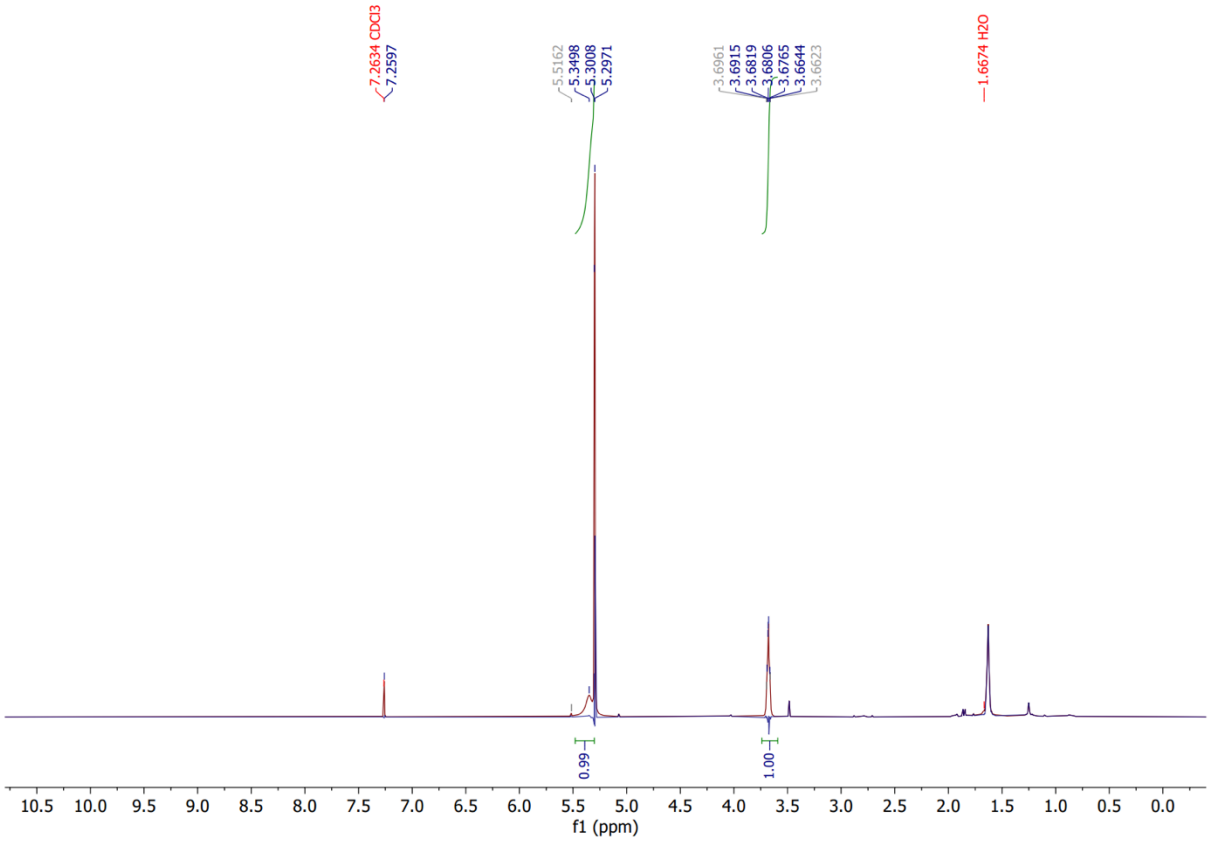
mixture stirred at room temperature for 24 hours. At this point, the reaction mixture had amalgamated to a white precipitate that would not dissolve in any solvent.

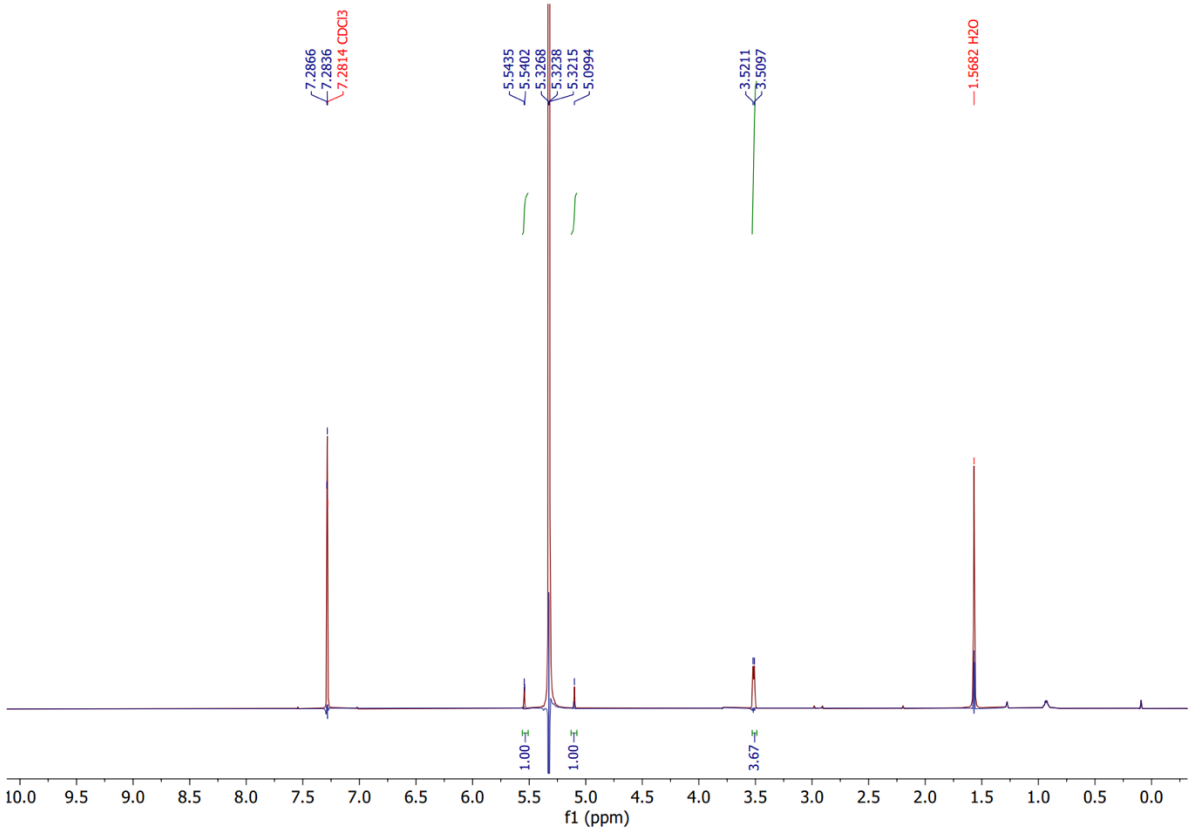
_O,O_'-(butane-1,4-diyl)bis(hydroxylamine) Procedure 2

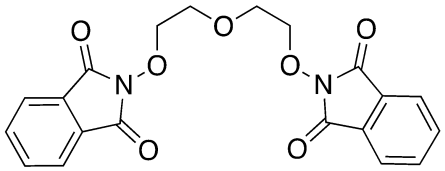
The crude from procedure 1 of 2,2'-(butane-1,4-diylbis(oxy))bis(isoindoline-1,3-dione was dissolved in 100% hydrazine and stirred overnight at room temperature. The resulting mixture was purified on column (5% MeOH/DCM) to afford two unidentifiable compounds both as oil.

$_1$H NMR($CDCl_3$, 600MHz), δ 5.39-5.29 (m, 1H), 3.68 (t, 1H) ppm.

$_1$H NMR($CDCl_3$, 600MHz), δ 5.54 (s, 1H), 5.09 (s, 1H), 3.52(4H, d) ppm.

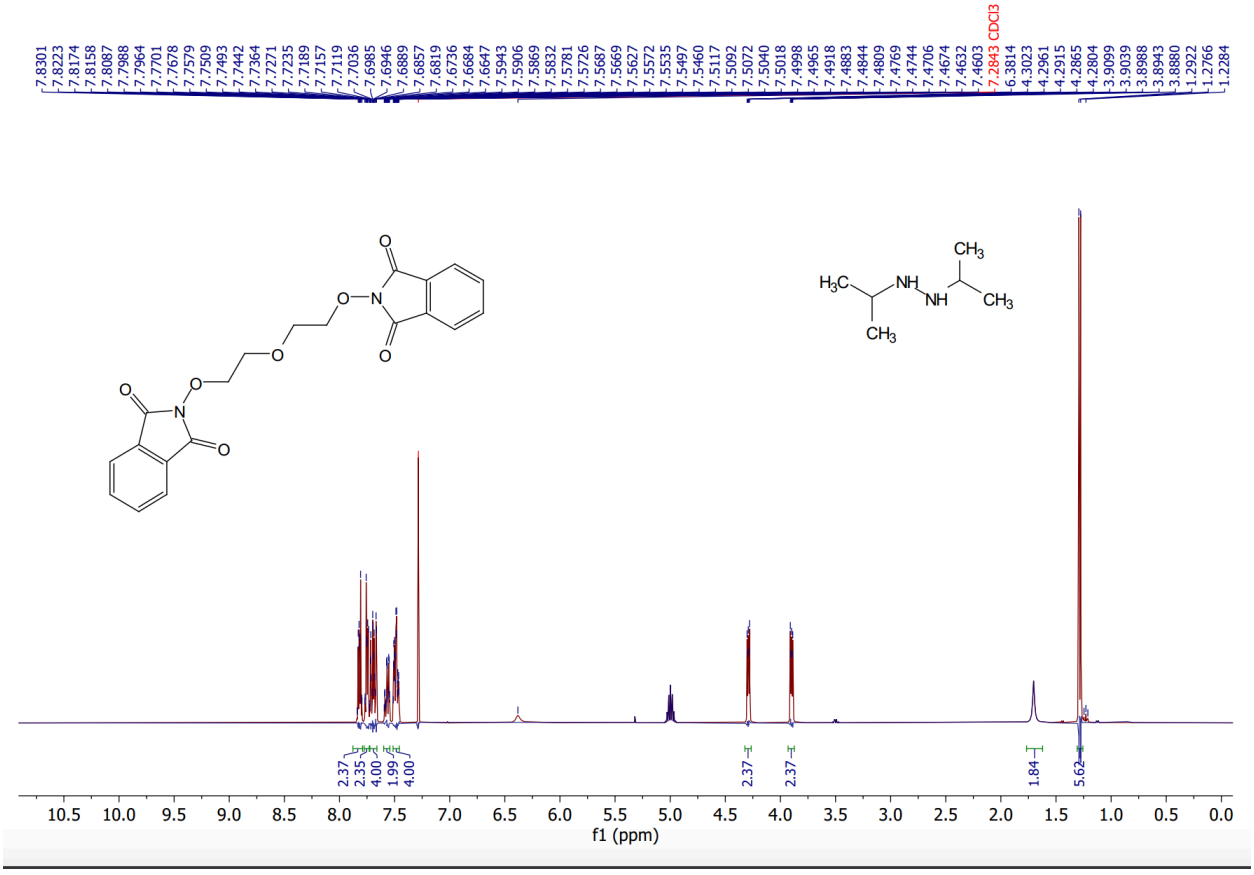




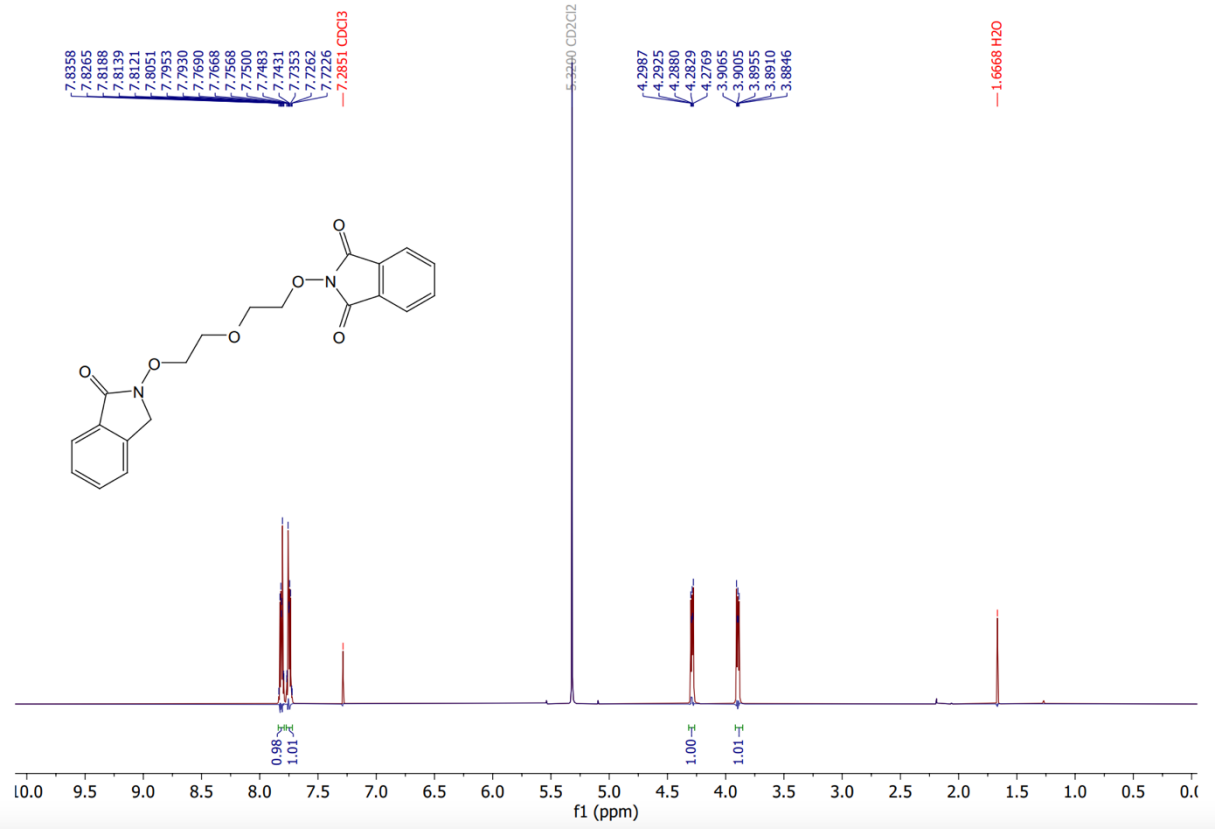


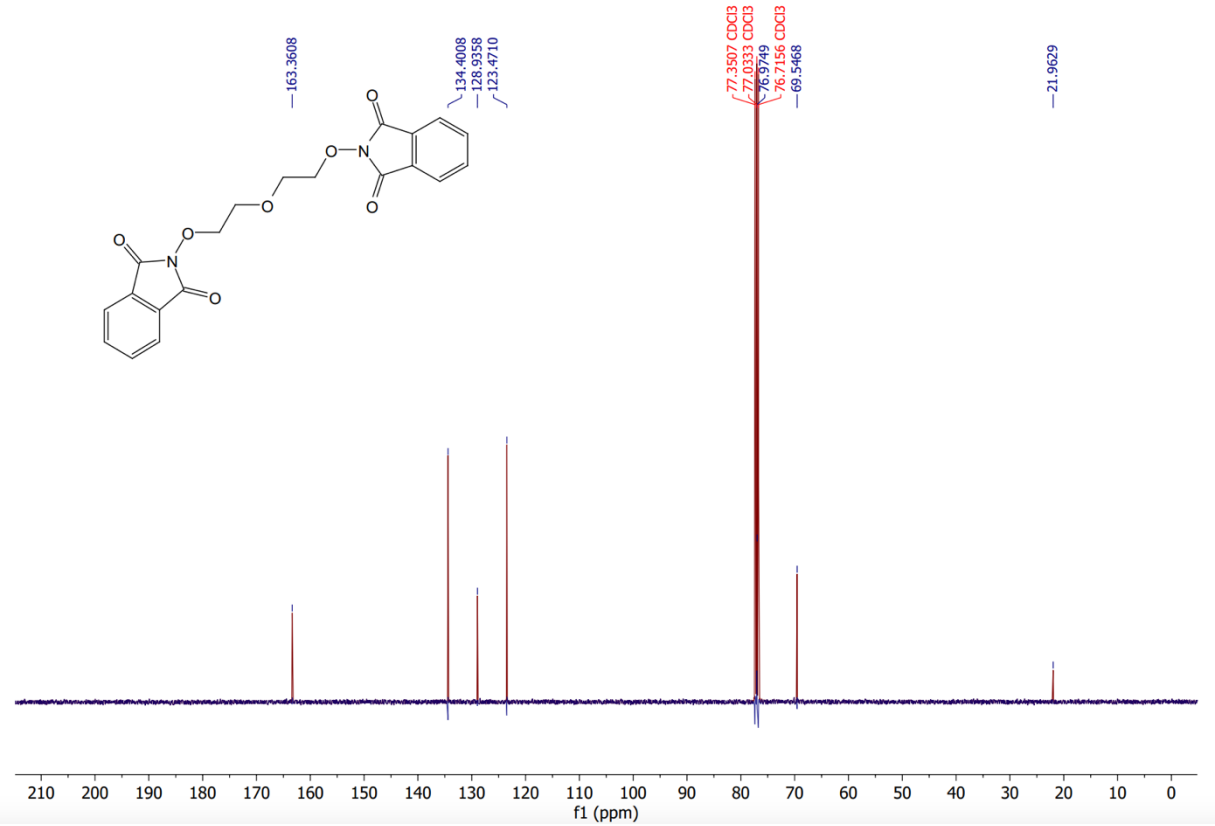
2,2'-((oxybis(ethane-2,1-diyl))bis(oxy))bis(isoindoline-1,3-dione) Procedure

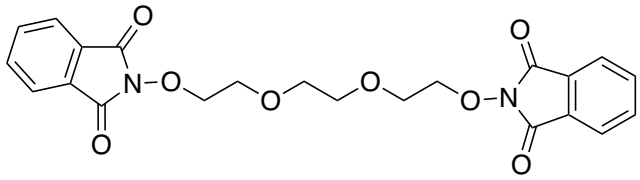
A round bottom flask equipped with a stir bar was charged with 2-hydroxyisoindoline-1,3-dione (2.2 equiv), triphenylphosphine (2.1 equiv) and butane-1,4-diol (1 equiv). The solids were dissolved in DCM. After 15 minutes, the mixture was treated in small portions with DIAD (2.2 equiv) allowing the orange color to fade between each addition. After 12 hours of stirring, the mixture was concentrated in vacuo and diluted with 100mL of diethyl ether and stirred at 4°C for 30 minutes. The white precipitate was collected via filtration and purified via column (7:3 DCM/ethyl acetate) to afford the title compound and 1,2-diisopropylhydrazine. $_1$H: NMR($CDCl_3$, 600MHz), δ7.8307.79 (m, 2H), 7.76-7.72 (m, 2H),7.74-7.65 (m,4H), 7.60-7.55 (m, 2H), 7.51-7.46(m, 4H), 4.30-4.28 (m, 2H), 3.91-3.88 (m, 2H), 1.74 (s, 2H) 1.29-1.21 (d, 6H) ppm.



To remove all the impurities, the crude product was purified on column (5% MeOH/DCM) to give the pure title compound and spots were visualized with PMA. $_{1}$H NMR($CDCl_3$, 600MHz), δ 7.83-7.79 (m, 2H), 7.76-7.72 (m, 2H), 4.29-4.27 (m, 2H), 3.91-3.88 (m, 2H) ppm. $_{13}$C NMR($CDCl_3$, 600MHz), δ 163.36, 134.40, 128.93, 123.47, 76.75, 69.54, 21.96.



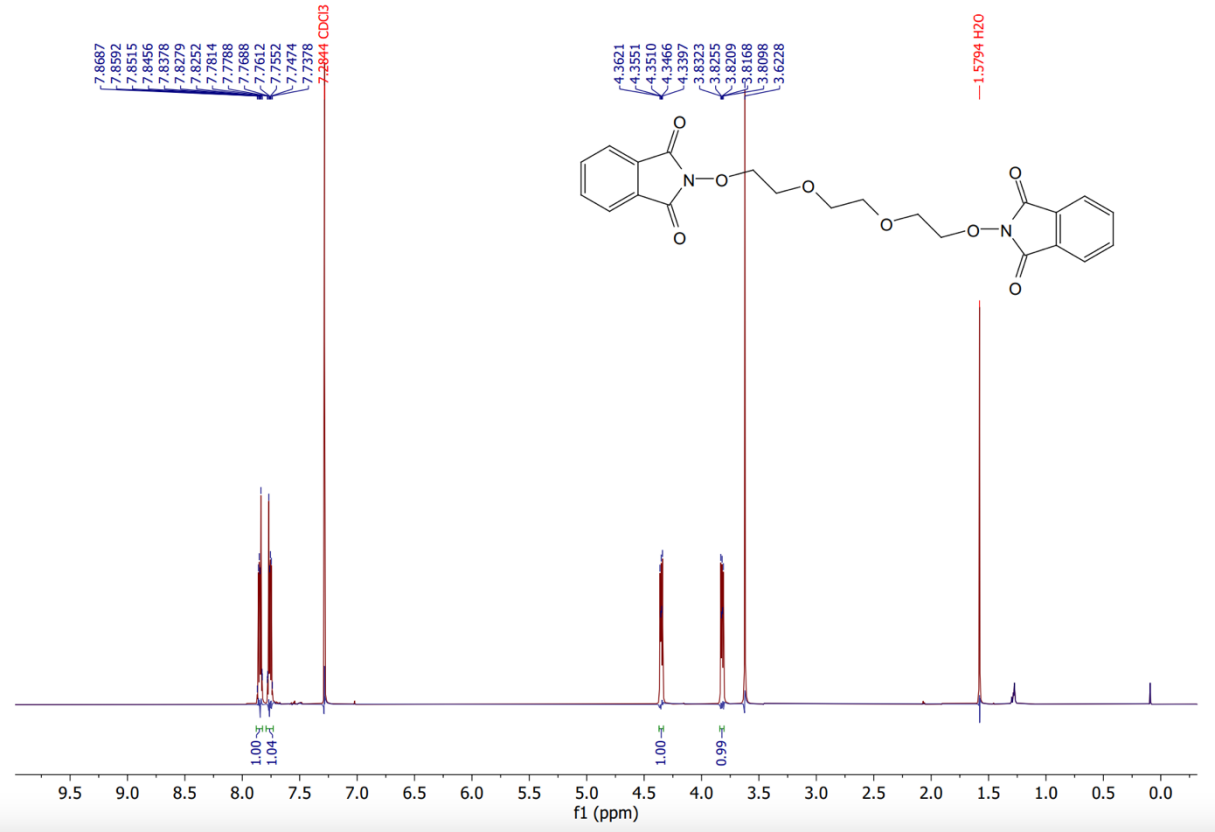


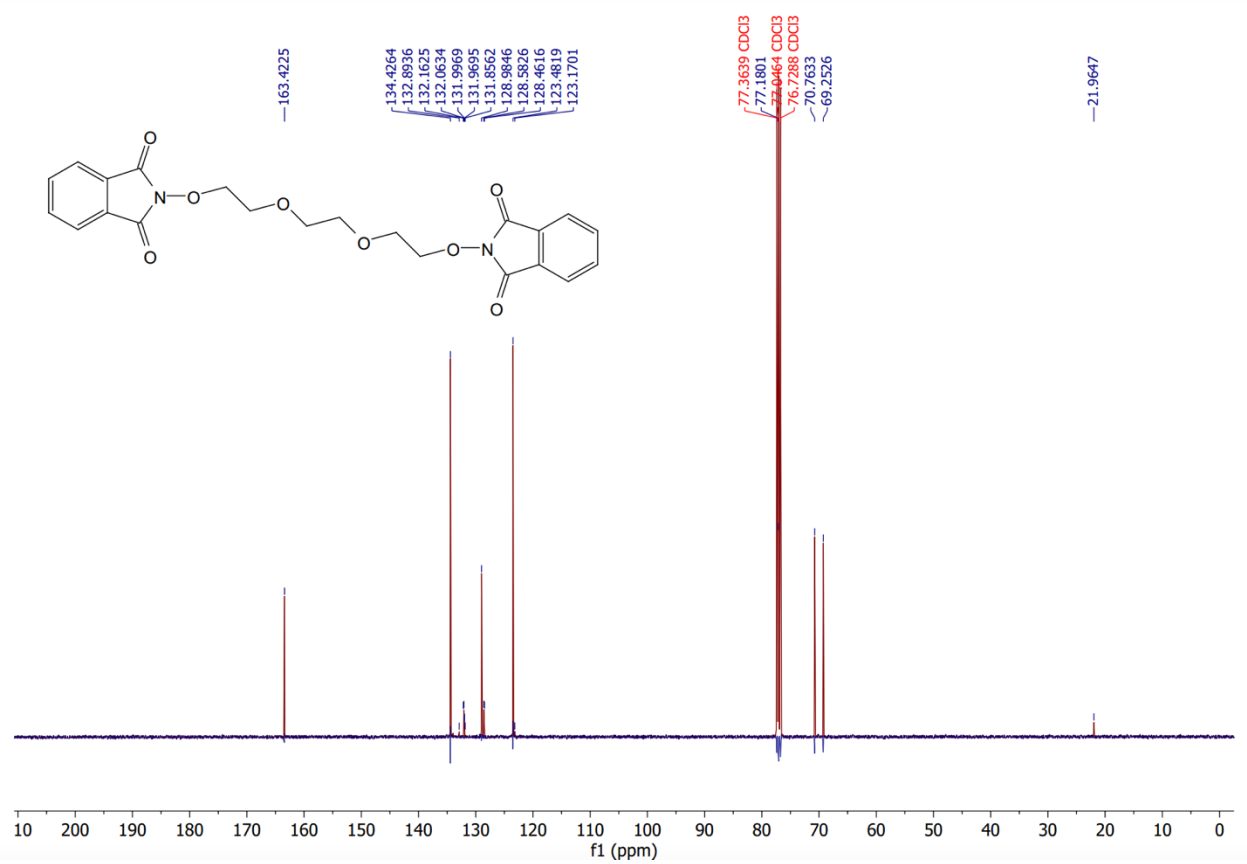


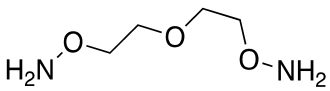
2,2'-(((ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl))bis(oxy))bis(isoindoline-1,3-dione)

A round bottom flask equipped with a stir bar was charged with 2-hydroxyisoindoline-1,3-dione (2.2 equiv), triphenylphosphine (2.1 equiv) and butane-1,4-diol (1 equiv). The solids were dissolved in DCM. After 15 minutes, the mixture was treated in small portions with DIAD (2.2 equiv) allowing the orange color to fade between each addition. After 12 hours of stirring, the mixture was concentrated in vacuo and diluted with 100mL of diethyl ether and stirred at 4°C for 30 minutes. The white precipitate was collected via filtration and purified on column (5% MeOH/DCM) to give the pure title compound and spots were visualized with PMA. $_1$H

NMR($CDCl_3$, 600MHz), δ7.87-7.82 (m, 2H), 7.78-7.74 (m, 2H), 4.36-4.39 (m, 2H), 3.83-3.62 (m, 2H) ppm. $_{13}$C NMR($CDCl_3$, 600MHz), δ163.42, 134.43, 128.98, 128.58, 77.18, 70.76, 69.25 ppm.

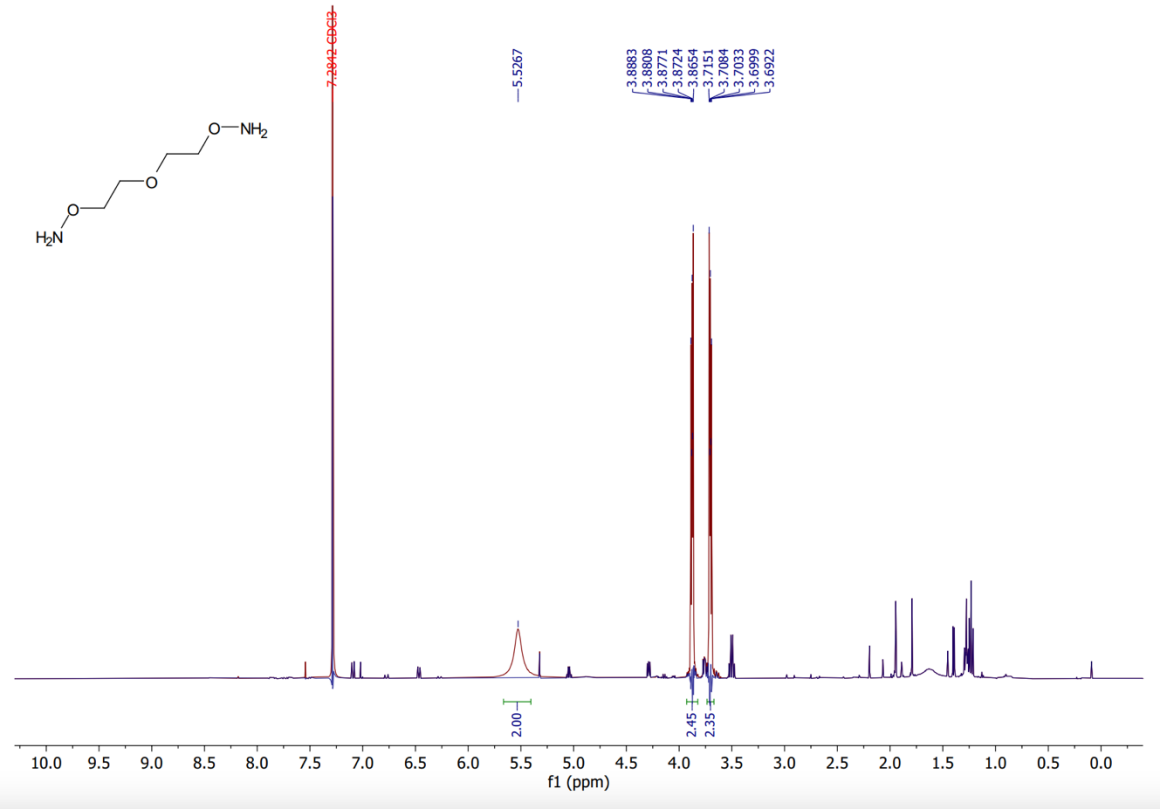






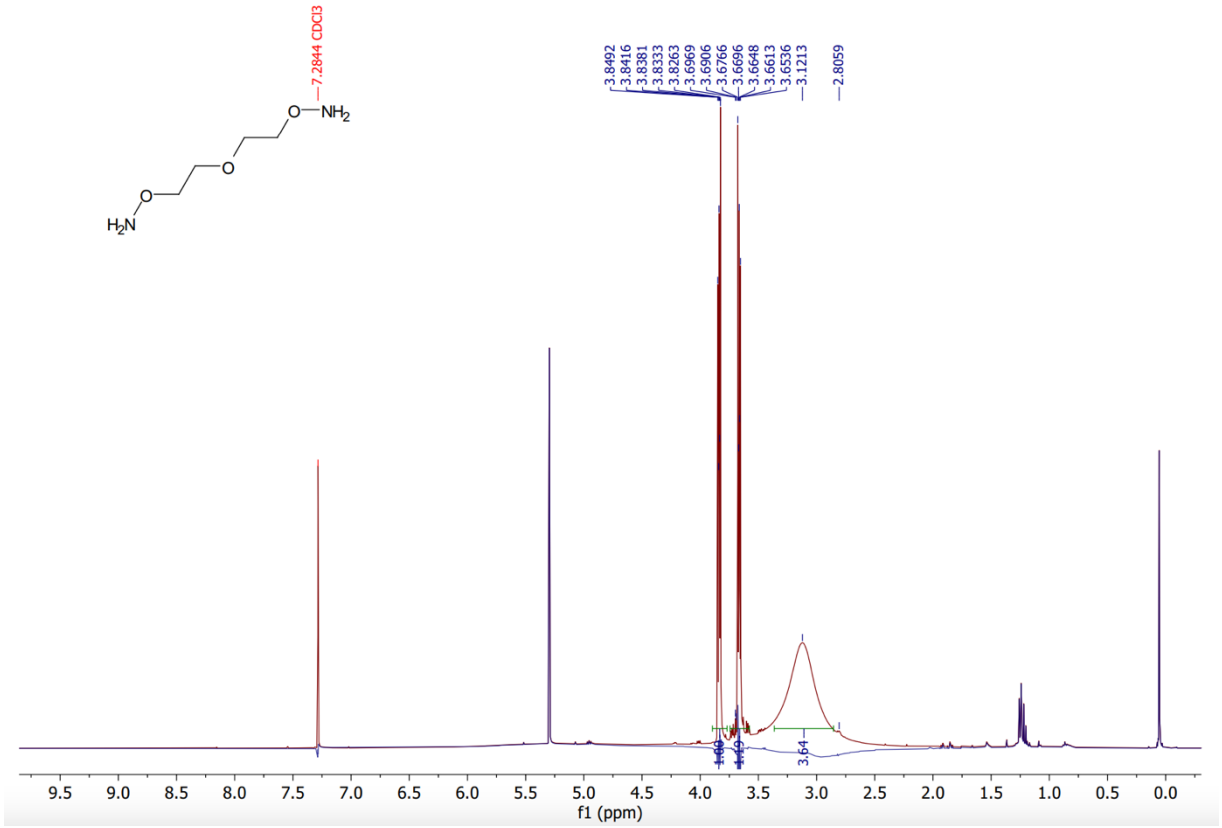
***O,O*'-(oxybis(ethane-2,1-diyl))bis(hydroxylamine) Procedure 1**

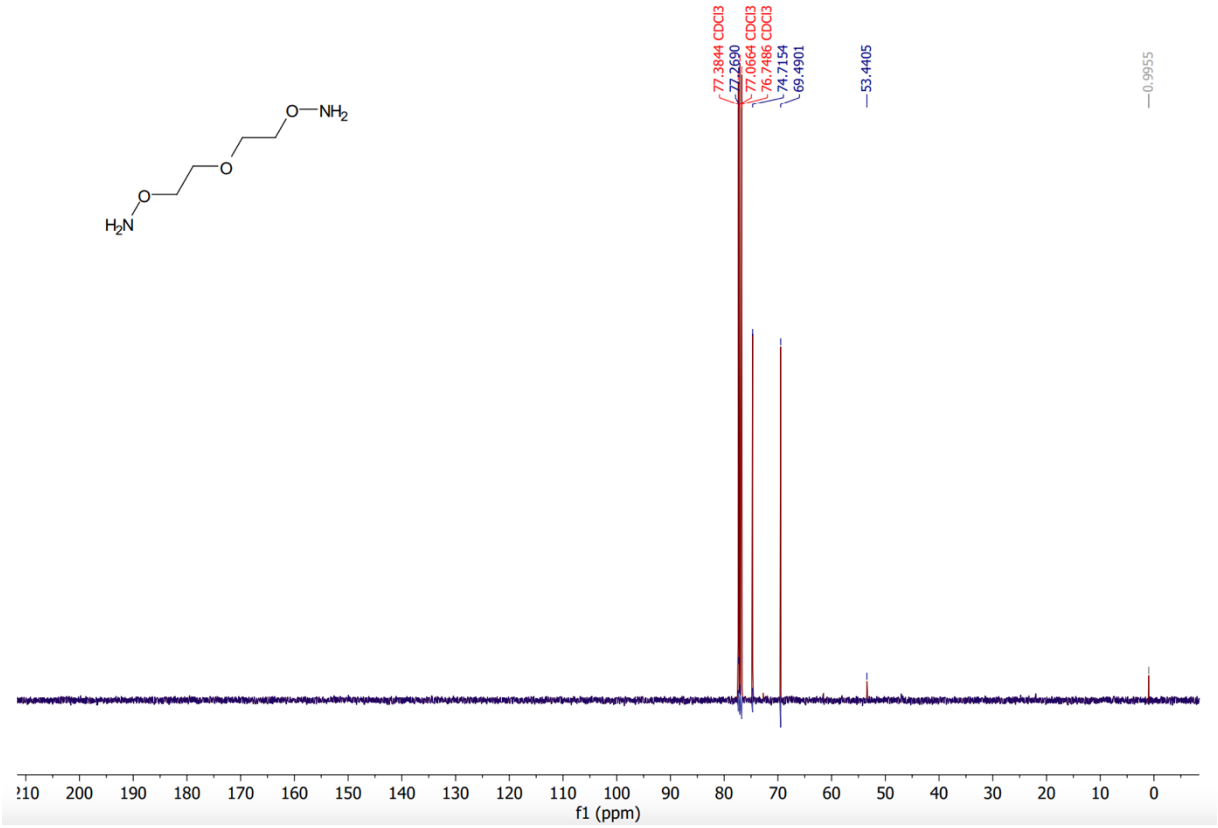
Dissolve the starting material (1 equiv) in DCM. Add hydrazine monohydrate (2.2 equiv) and stir at room temperature for 12 hours. Filter off the solid. Dilute the filtrate with 100mL of diethyl ether. Stir at 4°C for 1 hour. Filter the resulting solution and concentrated the filtrate under vacuum; however no precipitate was observed at 4°C or 0°C. Dissolve in 50mL of chloroform and filter the solution. Concentrate the filtrate under vacuum to yield impure title compound. Column purification not possible as compound is too polar. Attempts to salt the desired compound with HCl yielded no results. Extraction with water also yielded no results. $_1$H NMR($CDCl_3$, 600MHz), δ 5.53 (s broad, 2H), 3.88 (m, 2H), 3.75-3.69 (m, 2H) ppm.



_O,O_'-(oxybis(ethane-2,1-diyl))bis(hydroxylamine) Procedure 2

Dissolve 2,2'-((oxybis(ethane-2,1-diyl))bis(oxy))bis(isoindoline-1,3-dione) (1 equiv) in ethanol. Add hydrazine monohydrate (20 equiv) to solution, which should become clear. Reflux at 70°C for 3 hours. After 1 hour, a white precipitate was observed. After 3 hours, the reaction was filtered and the filtrate was extracted with chloroform then lyophilized to yield the title compound. $_{1}$H NMR($CDCl_3$, 600MHz), δ 3.85-3.83 (1H, m) 3.69-3.65 (1H, m), 3.12-2.81 (4H, s broad). $_{13}$C NMR($CDCl_3$, 600MHz), δ77.27, 74.71, 69.49, 53.44.


7.2844 CDCl3
3.8492
3.8416
3.8381
3.8333
3.8263
3.6969
3.6906
3.6766
3.6696
3.6648
3.6613
3.6536
3.1213
2.8059
3.64
f1 (ppm)


77.3844 CDCl3
77.2690
77.0664 CDCl3
76.7486 CDCl3
74.7154
69.4901
53.4405
0.9955
f1 (ppm)

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