4-27-2017

Investigation and Synthesis of Novel Aromatic Azo Inhibitors of LMW-PTP Isoform B

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Chem. 360

5/10/17

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Abstract

The goal of this investigation was to synthesize novel aromatic azo inhibitors based on an aromatic azo inhibitor discovered from *in silico* screening against Low Molecular Weight Protein Tyrosine Phosphatase (LMW-PTP). These inhibitors were then tested *in vitro* aginst LMW-PTP Isoform B. The synthesis of the inhibitor of interest was achieved via a Sandmeyer reaction, utilizing p-aminobenzoic acid. The nucleophile used in the synthesis, 3-hydroxy-2-naphthoic acid, was linked with p-aminobenzoic acid, and the final product was purified via recrystallization. Characterization techniques included ^{1}H-NMR, ^{13}C-NMR, COSY, and HMQC. This newly synthesized product was an analogue to a previously synthesized competitive inhibitor, 156563, which had an inhibitor dissociation constant of 33.5µM. By synthesizing an analogue to inhibitor 156563, our group hopes to probe the carboxylic acid chain length as a function of inhibition.

Introduction

It is essential that the body regulates signal transduction methods for cellular communication. Some of these signal transduction methods may include paths that control cell growth and proliferation, motility and even gene expression. The body regulates signal transduction through post-translational modifications, and one of the key post-translation modifications involves phosphorylation—and dephosphorylation—of residues in proteins in a process known as reversible post-translational phosphorylation[1]. Phosphorylation is the addition of a phosphoryl group (PO_3^-) to a molecule. An extensive amount of proteins, sugars, lipids, and other molecules are often temporarily phosphorylated in order to carry out vital functions[2]. Phosphorylation and dephosphorylation, the opposite of phosphorylation, work hand-in-hand in regulating critical cellular processes. These steps often occur on Tyrosine side chains, and to a lesser extent Serine/Threonine side chains, in close proximities to the molecules of interest.

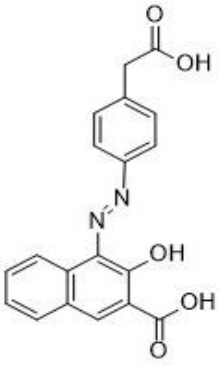
The phosphorylation state of Tyrosine is controlled mainly by two groups of enzymes, Protein Tyrosine Kinases (PTKs) and Protein Tyrosine Phosphotases (PTPs). These two work in a delicate balance to regulate proteins by activating/deactivating them through phosphorylation. Protein Tyrosine Kinases regulate proteins by transferring a phosphoryl group from ATP to the desired protein. Protein Tyrosine Phosphatases work by removing the phosphoryl groups from the phosphorylated tyrosine resides on proteins[3].

Among the Protein Tyrosine Phosphatases there are a total of 107 different enzymes that are classed into four subgroups: classical pTyr specific, dual specificity, Cdc25, and Low Molecular Weight PTP (LMW-PTP)[1]. Low Molecular Weight Protein Tyrosine Phosphatases

exist in two active isoforms and two inactive isoforms. The two active isoforms are Isoform A and Isoform B, and the two inactive isoforms are Isoform C and SV3. Isoform A and Isoform B have very slight differences. When superimposed on top of each other, their structures look identical. These PTPs all share a common active site phosphate binding P-loop (CXR(S/T)), but differ otherwise in sequence. This variation determines the specificity of binding to substrates.

Low Molecular Weight PTPs were chosen as proteins of interest because they have emerged as a novel drug target based on their involvement in metabolic and tumor regulation[4]. There have been strong links between human LMW-PTPs and cancer; LMW-PTPs have been found to be active in the signaling pathways of growth factors[5-8]. In addition, LMW-PTP mRNA and protein levels have been shown to be significantly increased in human breast, colon, bladder, and kidney tumor samples[6]. Over expression of Isoform B, specifically, has been associated with the development of cancer and diabetes. It has actually been discovered the two active isoforms play opposite roles in the early tumorigenic process, where Isoform A is anti-oncogenic and Isoform B is oncogenic[9]. This, therefore, emphasizes the importance of identifying potent inhibitors of LMW-PTPs.

Initial research found three compounds from the National Cancer Institute (NCI) Diversity Set I that could serve as potent inhibitors for LMW-PTP Isoform B via *in silico* docking methods[10]. This research group decided to expand the search and screen Diversity Set II and III, which composed of 1356 compounds and 1597 compounds, respectively. For the *in silico* docking method, which was used to find compounds for the best general fit for the enzyme, all of the compounds were compared to an already known potent inhibitor, pyridoxal 5'-phosphate (PLP). This inhibitor is a competitive inhibitor for LMW-PTP with a K_{is} value of 7.6μM (pH 5.0). All compounds that were chosen had to have comparable or better docking score to PLP. This searched resulted in 47 total compounds from both sets (24 compounds from Diversity Set II and 23 compounds from Diversity Set III), and these compounds were requested from the NCI and evaluated *in vitro*[1]. Ten of the inhibitors showed strong inhibition with K_{is} values less than 100μM. Out of those inhibitors the inhibitor of interest for this study was inhibitor 156563.



Inhibitor 156563

Figure 1. Previously discovered NCI Diversity Set II inhibitor of LMW-PTP Isoform B with NSC identifier number.

This amino-based azo inhibitor from Diversity Set II had a K_{is} value of 33.5±8.2μM. This group aims to modify this inhibitor by shortening the chain length connection of a carboxylic acid group, so that the carboxylic acid in the para position from the nitrogen will be attached directly to the ring. With this enzyme, named (*E)*-4-((4-carboxyphenyl)diazenyl)-3-hydroxy-2-naphthoic acid according to IUPAC naming, this group aims to optimize the binding in the enzyme, maximize bond interactions, and increase competitive inhibition of the inhibitor.

Experimental

Materials

Hydrochloric acid, water, p-aminobenzoic acid, sodium nitrite, sodium hydroxide, and 3-hydroxy-2-naphthoic acid were obtained from the stockroom in Ardolf Science Center (ASC).

Synthesis of (E)-4-((4-carboxyphenyl)diazenyl)-3-hydroxy-2-naphthoic acid. Hydrochloric acid (HCl, 1.37 mL, 1 M) and water (21.9 mL) were placed together in a 100 mL beaker. P-aminobenzoic acid ($C_7H_7NO_2$, 0.615g, 0.0045 mol) was added to this beaker and cooled over ice. While this was cooling, sodium nitrite ($NaNO_2$, 0.315g, 0.0045 mol) and DI water (2.2 mL) were combined in a 50mL beaker. The contents of the 50mL beaker was added slowly to the 100mL beaker, and the solution was left to stir for 30 minutes. An alkaline solution of 3-hydroxy-2-naphthoic acid ($C_{11}H_8O_3$, 0.849g, 0.0045 mol) and NaOH (3 mL 2.5 M) was added to the mixture after the first mixture had stirred for 30 minutes. A thick, maroon paste began to form and the mixture was left to stir overnight. The next morning, the paste had thickened. The solution was acidified dropwise with dilute H_2SO_4 (3 mL) and boiled for five minutes on a hot plate at 75°C. The mixture was filtered using a Hirsch funnel. The product was then recrystallized in acetonitrile and dried in a vacuum oven. The dried product was a dark maroon powder. ^{1}H-NMR analysis of the product in $CDCl_3$ displays shifts at 10.16 ppm (singlet, 1H), 8.58 ppm (singlet, 1H), 8.0 ppm (doublet, 2H), 7.8 ppm (doublet, 1H), 7.7 ppm (doublet, 1H), 7.5 ppm (triplet, 1H), and 7.36 ppm (triplet, 1H), 7.32 ppm (sing, 1H), 6.8 ppm (doublet, 2H). ^{1}H-NMR analysis of the product in DMSO-d_6 displays shifts at 10.2 ppm (singlet, 1H), 8.59 ppm (singlet, 2H), 8.49 ppm (doublet, 2H), 8.43 ppm (doublet, 2H), 8.0 ppm (doublet, 2H), 7.92 ppm (doublet, 1H), 7.7 ppm (mult.), 7.5 ppm (triplet, 1H), and 7.31 ppm (triplet, 1H), 7.27 ppm (d. of sing, 1H), 6.78 ppm (doublet, 2H).

In vitro kinetic assays. After synthesis of the product, kinetic studies were conducted on the product using pNPP, a compound used mainly in the study of kinetics of reactions catalyzed by phosphate enzymes. The red color of the product presented a problem since a baseline fluorescence was needed. The product was soluble in small concentrations in DMSO. 10mg of the compound was dissolved in 10mL of DMSO before the compound could be fully dissolved.

Results & Discussion

Spectral analysis

From the synthesis of this new inhibitor, the ^{1}H-NMR spectrum should essentially be a combination of the ^{1}H-NMR spectra for both p-aminobenzoic acid and 3-hydroxy-2-naphthoic acid, with slight differences due to the absence of certain protons that were lost due to the new bond formed. The figures below show the ^{1}H-NMR spectrum for both p-aminobenzoic acid and 3-hydroxy-2-naphthoic acid, respectively.

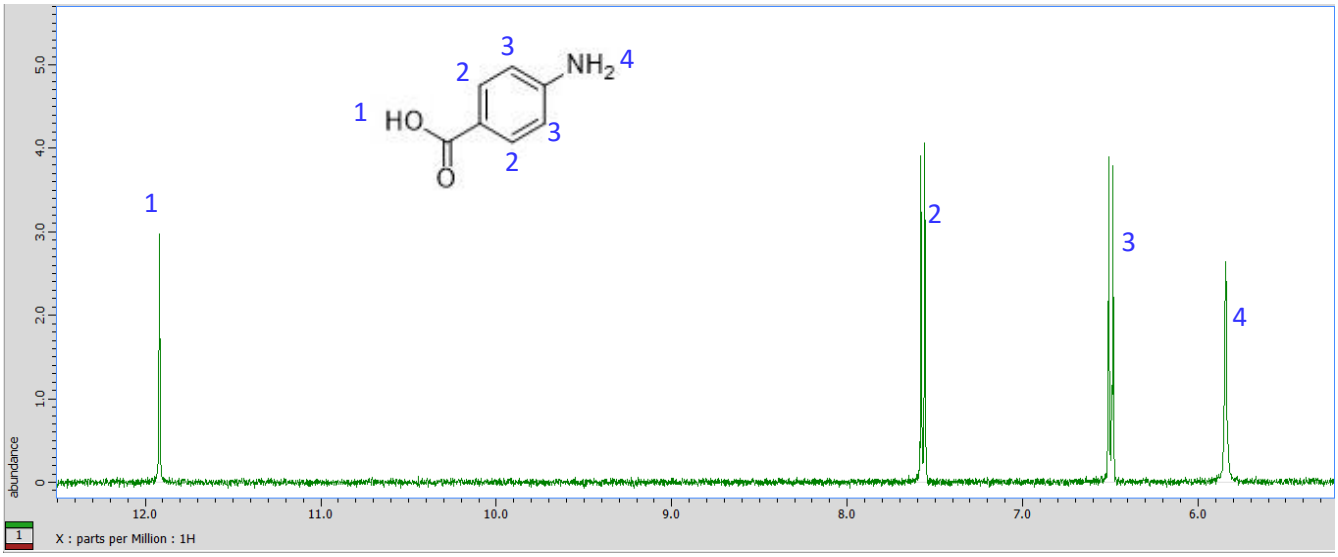


Figure 2. ^{1}H-NMR of p-aminobenzoic acid in DMSO-d_6 with corresponding ^{1}H peaks labeled. Analysis of spectrum displays shifts at 11.92 ppm (singlet, 1H), 7.56 ppm (doublet, 2H), 6.49 ppm (doublet, 2H), 5.84 ppm (singlet, 3H).

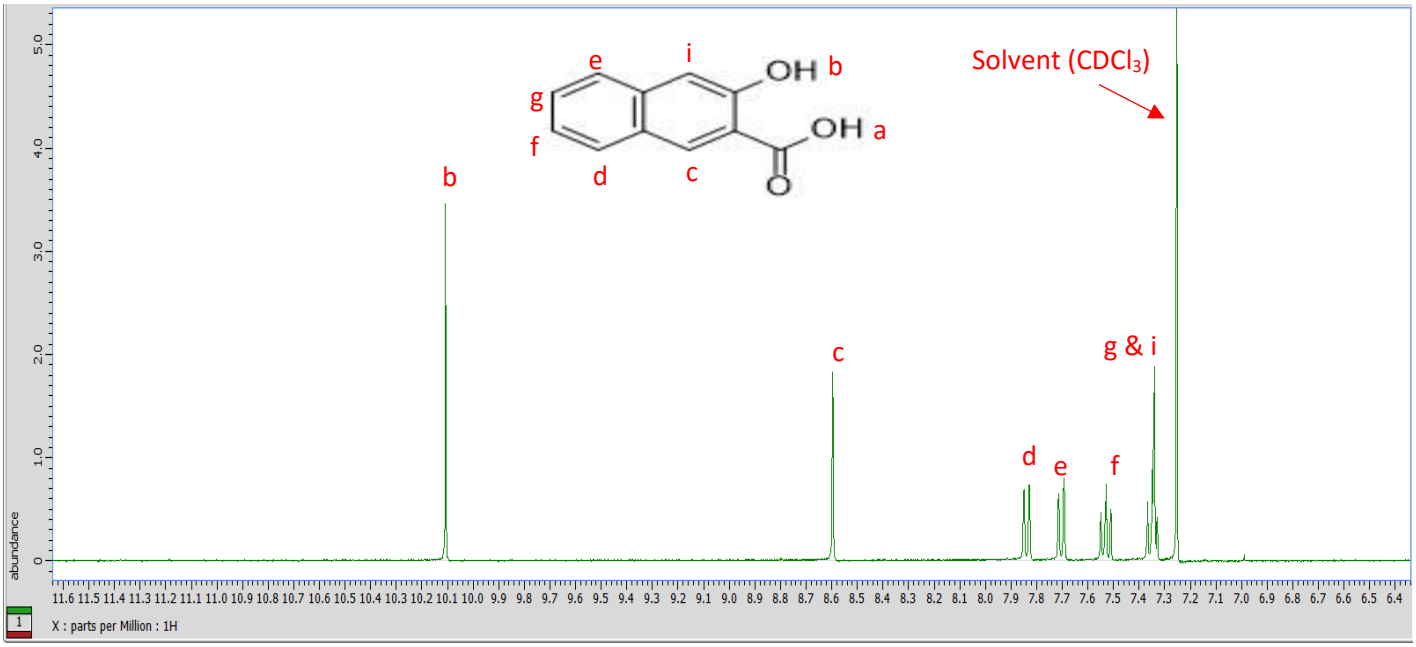


Figure 3. ^{1}H-NMR of 3-hydroxy-2-naphthoic acid in $CDCl_3$ with corresponding ^{1}H peaks labeled. Analysis of spectrum displays shifts at 10.11 ppm (singlet, 1H), 8.60 ppm (singlet, 1H), 7.8 ppm (doublet, 1H), 7.7 ppm (doublet, 1H), 7.5 ppm (triplet, 1H), and 7.36 ppm (triplet, 1H), 7.32 ppm (sing, 1H).

Figures 2 and 3 show the ^{1}H-NMR spectra of p-aminobenzoic acid in DMSO-d_6 and 3-hydroxy-2-naphthoic acid in $CDCl_3$, respectively. The corresponding ^{1}H peaks for has been labeled accordingly, so when these two compounds are brought together, the ^{1}H spectrum for the product should essentially be a ^{1}H spectrum that includes all but two of the peaks shown; peak *5*

of p-aminobenzoic acid and peak *i* of 3-hydroxy-2-naphthoic acid should disappear because the reaction mechanism essentially eliminates all of those protons through deprotonation or proton tautomerization to form the final product. The ^{1}H spectrum of the final product, however, showed almost all of these predictions with a few differences.

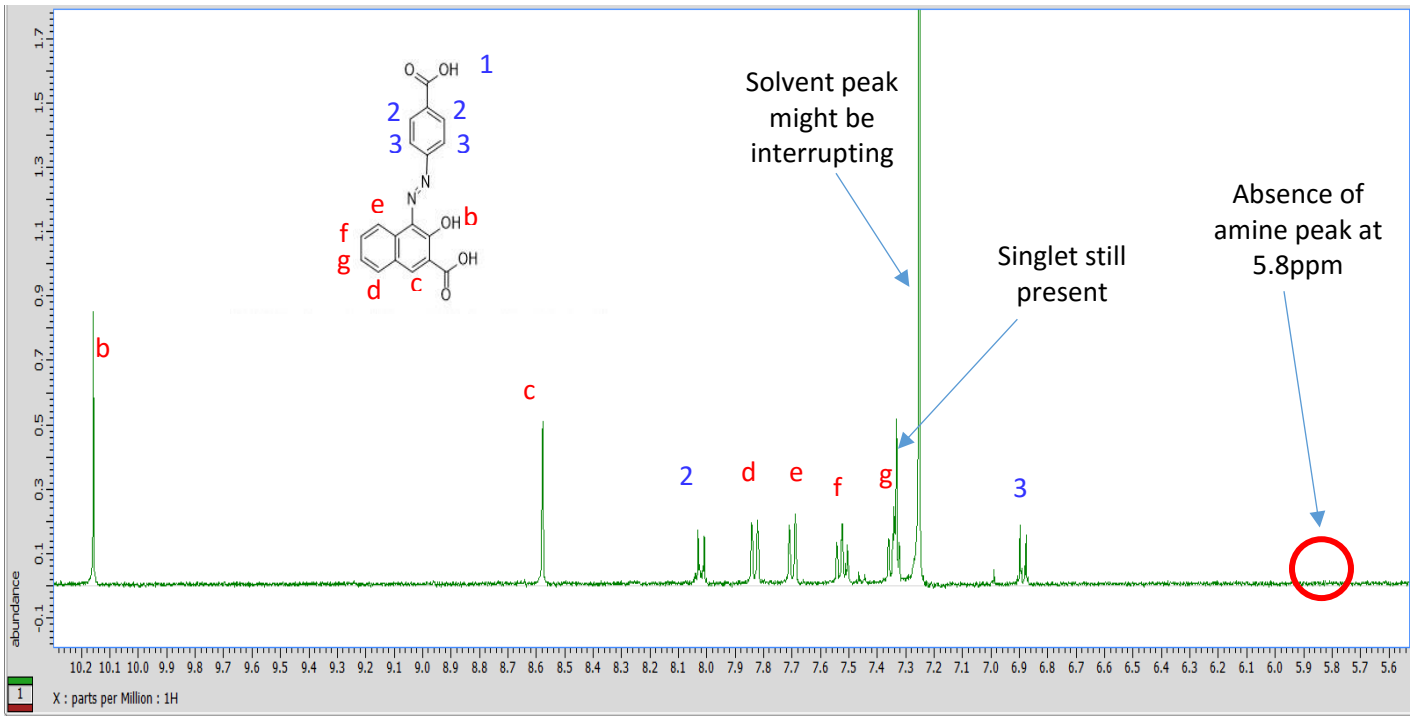


Figure 4. ^{1}H-NMR of product in $CDCl_3$ with corresponding ^{1}H peaks labeled. Analysis of spectrum displays shifts at 10.16 ppm (singlet, 1H), 8.58 ppm (singlet, 1H), 8.0 ppm (doublet, 2H), 7.8 ppm (doublet, 1H), 7.7 ppm (doublet, 1H), 7.5 ppm (triplet, 1H), and 7.36 ppm (triplet, 1H), 7.32 ppm (sing, 1H), 6.8 ppm (doublet, 2H).

A notable difference from the expected spectrum include a missing ^{1}H peak for the carboxylic acid group of the p-aminobenzoic acid portion (label *1*). Another difference is the downfield shift of the other ^{1}H peaks of the p-aminobenzoic (^{1}H peaks *2* and *3*). Peak *2* shifted from 7.56ppm in its original structure to 8.0ppm and peak *3* shifted from 6.49ppm in its original structure to 6.8ppm. The third noticeable difference is the presence of the singlet on the 3-hydroxy-2-napthoic acid structure that was initially label *i*. A possible explanation for this could be that some of the product may contain the intermediate preceding the final product. That intermediate undergoes proton tautomerization where proton *i* is cleaved off to get to the final product structure. The final noticeable difference is the absence of the amine peak at 5.8ppm. This is a keen observation because it helps to confirm that an azo bond has been formed between the two initial reagents. A significant comment worth mentioning is that throughout this process, all ^{13}C-NMR spectra were inconclusive because the only peaks present were solvent peaks. Therefore, results were based on ^{1}H-NMR and COSY. Efforts were carried out to run the ^{1}H-NMR for the product in a different solvent (DMSO-d_6) in an attempt to achieve better resolution.

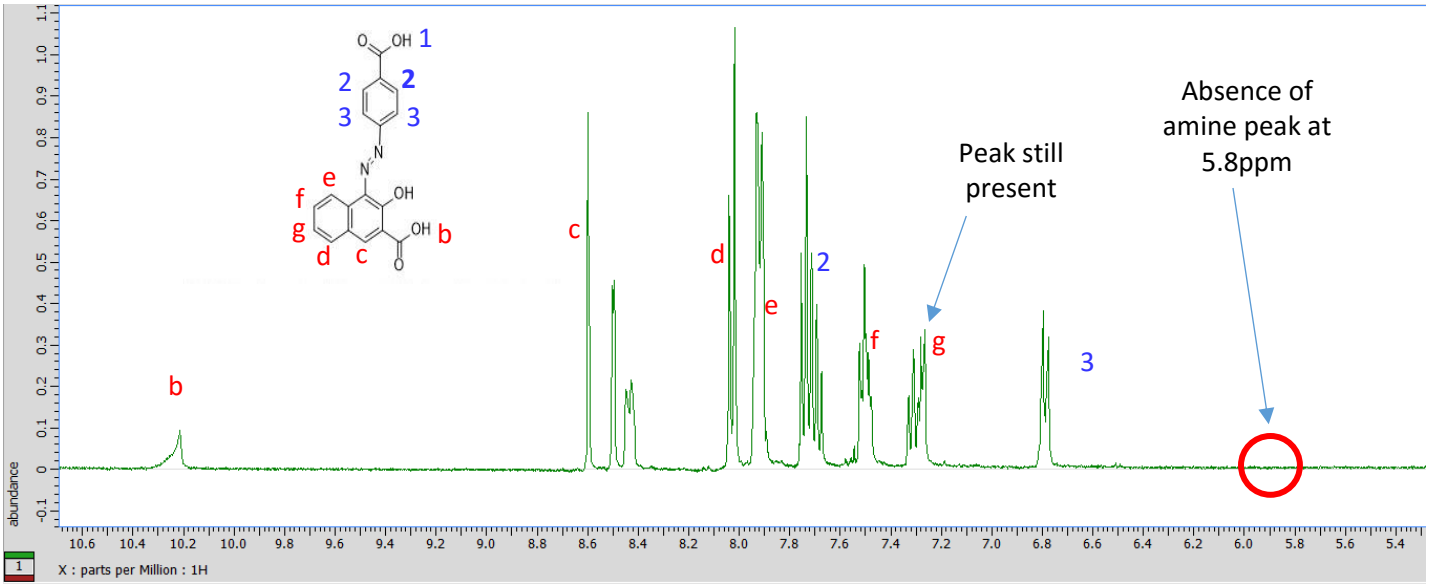


Figure 5. ^{1}H-NMR of product in DMSO-d_6 with corresponding ^{1}H peaks labeled. Analysis of spectrum displays shifts at 10.2 ppm (singlet, 1H), 8.59 ppm (singlet, 2H), 8.49 ppm (doublet, 2H), 8.43 ppm (doublet, 2H), 8.0 ppm (doublet, 2H), 7.92 ppm (doublet, 1H), 7.7 ppm (mult.), 7.5 ppm (triplet, 1H), and 7.31 ppm (triplet, 1H), 7.27 ppm (d. of sing, 1H), 6.78 ppm (doublet, 2H).

Compared to the previous spectrum, a similarity that both spectra of the final product possess is a missing ^{1}H peak for the carboxylic acid group of the p-aminobenzoic acid portion (label *1*). In addition, most of the peaks appeared at the same positions comparatively, and the absence of the amine peak at 5.8ppm remains constant, which again suggests that an azo bond has been formed between the two initial reagents. Another similarity is the presence of the ^{1}H peak on the 3-hydroxy-2-napthoic acid structure that was initially label *i;* however, unlike the previous spectrum ran in $CDCl_3$, this spectrum displays that peak as a doublet of singlets rather than a singlet. The possible explanation for this could be the same reason listed before: some of the product may contain the intermediate preceding the final product. That intermediate undergoes proton tautomerization where proton *i* is cleaved off to get to the final product structure.

A notable difference in this spectrum is an upfield shift of the ^{1}H peak of the p-aminobenzoic labeled *2*, in comparison to the previous spectrum ran in $CDCl_3$. Peak *2* shifted from 8.0ppm in the spectrum ran in $CDCl_3$ to 7.7ppm, which is closer to its original position as predicted by the ^{1}H-NMR of p-aminobenzoic acid ran in DMSO-d_6. The second noticeable difference is the presence of new peaks at 8.43ppm and 8.0ppm, and the increase in multiplicity of the peak labeled *2*. A possible explanation for this could be that the additional peaks may be a result of instability; the final product may be breaking down. A plausible reason for this could be that the product may be photosensitive and hence breaks down over time, mainly at the site of the azo bond. COSY spectra were used to confirm the identity of the different protons by checking which protons were in close proximity to each other.

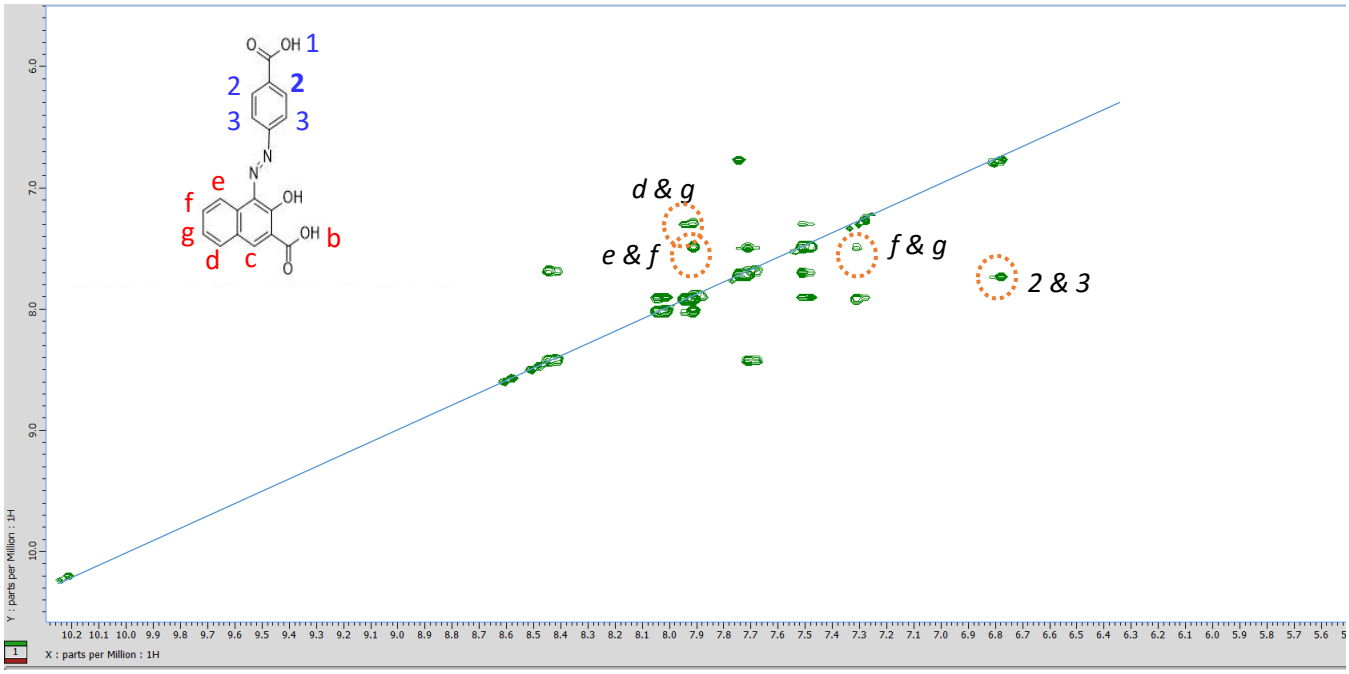


Figure 6. COSY spectrum of product in DMSO-d_6 with corresponding ^{1}H peaks labeled. Analysis of spectrum displays connectivity between protons *e* & *f*, *d* & *g*, *f* & *g*, and *2* & *3*.

By observation of Figure 6, it is apparent that the protons on each of the previous spectrum was labelled correctly because connectivity has been established between select protons. Lines showing connectivity were eliminated to improve the quality of the spectrum image.

In vitro kinetic assays

To begin this analysis, fluorescence spectral analysis had to be conducted in order to determine the excitation wavelength. The excitation wavelength, however, was very close to that of pNPP due to the very bright red color of the compound when dissolved in solution. To combat this, the excitation wavelength for this compound was estimated in order to continue kinetic studies.

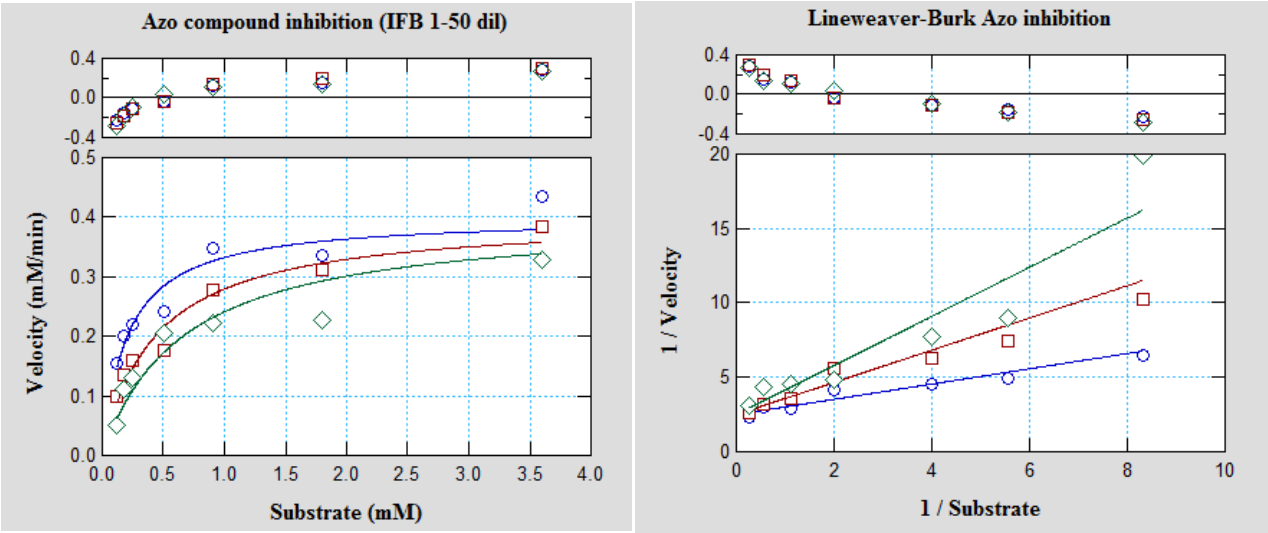


Figure 7. Spectrum of both Michaelis-Menten and Lineweaver-Berk plots for the synthesized product.

The Michaelis-Menten plot gave a plot that suggested competitive inhibition, however, this could be confirmed since extra date points were not taken to ensure this. The Lineweaver-Berk plot also suggests competitive inhibition by displaying very similar $1/V_{max}$ values. The K_{is} value obtained from this study was 30.9±16.8µM. Below is a table comparing the inhibitor 156563 to its derivative.

Table 1. *In vitro* activity of inhibitors against LMW-PTP isoform B

Compound (NSC #)	K_{is} (µM)	Diversity Set
156563	33.5±8.2	2
N/A **IUPAC: (*E*)-4-((4-carboxyphenyl)diazenyl)-3-hydroxy-2-naphthoic acid**	30.9±16.8	N/A

The K_{is} value was slightly lower compared to Inhibitor 156563; however, the margin of error does not allow this conclusion to be confidently made. A possible reason for this error could be due to the fluorescence spectral analysis that had to be conducted in order to determine the excitation wavelength. Since the excitation wavelength had to be estimated due to the product having a very close excitation wavelength to that of pNPP, the resulting error could not be avoided. The confidence of these results, nonetheless, is not to be completely disregarded. This is because PLP was tested and the K_{is} value of 8.6µM was comparable to the literature value.

Conclusion

To conclude, phosphorylation of residues in proteins is a key post-translational modification that serves as a common means for the control of cellular activities. Phosphorylation is controlled by two groups of enzymes, Protein Tyrosine Phosphatases (PTPs) and Protein Tyrosine Kinases (PTKs). Protein Tyrosine Phosphatases (PTPs) are a class of 107

enzymes that are divided into four subgroups. One of those subgroups, Low Molecular Weight PTP (LMW-PTP) has two active isoforms (Isoform A and Isoform B) that differ in their p-loop regions. This variation, however, determines the specificity of binding to substrates. It has been discovered that over-expression of LMW-PTP Isoform B is oncogenic while Isoform A has the opposite effect. This then stresses the importance of identifying potent inhibitors of LMW-PTPs, specifically for Isoform B.

Inhibitor 156563, was then chosen in an effort to maximize its binding and inhibition capabilities by modifying its structure, mainly shortening the chain length connection of a carboxylic acid group. The aim after this specific synthesis would then be to test levels of inhibition *in vitro* in hopes of obtaining lower K_{is} values. The spectra for the synthesis showed a missing ^{1}H peak for the carboxylic acid group of the p-aminobenzoic acid portion (label *1*). In addition, the absence of the amine peak at 5.8ppm suggested that an azo bond has been formed between the two initial reagents, while the presence of the ^{1}H peak on the 3-hydroxy-2-napthoic acid structure that was initially label *i* still remains. The presence of new peaks at 8.43ppm and 8.0ppm, and the increase in multiplicity of the peak labeled *2*, also suggested that the final product may be breaking down. A plausible reason for this could be that the product may be photosensitive and hence breaks down over time, mainly at the site of the azo bond. The Michaelis-Menten plot gave a plot that suggested competitive inhibition, and that was affirmed by the Lineweaver-Berk plot that displayed competitive inhibition by through very similar $1/V_{max}$ values. The K_{is} value of 30.9±16.8μM was slightly lower compared to Inhibitor 156563; however, the margin of error does not allow this conclusion to be confidently made. A possible reason for this error could be due to an error in determining the excitation wavelength of the product. Since the excitation wavelength had to be estimated due to the product having a very close excitation wavelength to that of pNPP, the resulting error could not be avoided

In the future, this lab group aims to conduct more tests to determine the success of the synthesis, as well as conduct further *in vitro* kinetic studies on Isoform B. The next goal would then be to determine the *in vitro* inhibition of Isoform A as well to make sure the inhibitor is not a promiscuous inhibitor. Furthermore, the group hopes to continue to synthesize inhibitors from database as well as modify structures of known inhibitors.

Acknowledgements

The author would like to thank Dr. McIntee and Dr. Jakubowski for their guidance and support throughout this project. Furthermore, the author would like to thank Max Olson for his help with the *in vitro* kinetic studies, and anyone who has assisted the author in any way throughout this research process.

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