SYNTHESIS OF NOVEL TRIAZINES,

CHEMICAL PROPERTIES AND THEIR APPLICATIONS IN BIOCHEMISTRY

By

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Abstract

Aryl diazonium ions serve as a powerful tool in bioconjugation, protein functionalization, and numerous applications present in biochemical literature dating over a century. The synthesis and utility of these reagents is typically restricted to in vitro environments due to the biologically incompatible nature of their generation, as well as their stability. As a result, protection mechanisms, like triazabutadiene scaffolds have been synthesized and further functionalized to release aryl diazonium ions selectively *in vivo*.[1] Here, we detail the generation and study of novel triazines for use in protecting aryl diazonium ions, synthesized by addition of morpholine and piperazine, and capable of initiated diazonium delivery via UV irradiation. One of our goals is to determine how protecting one of the piperazine amines affects the nucleophilicity of the remaining amine group of piperazine.

Introduction

Aryl diazonium ions have been popularly used as a means of protein bioconjugation due to the high electrophilicity of the diazonium ion. Specifically, the mechanism of bioconjugation occurs through electrophilic aromatic substitution, and allows for the substitution of a diazonium into the ortho-position of the tyrosine residue's side chain.This biochemical capability has varied and wide-reaching applications, including biotinylation and PEGylation. Moreover, the diazonium can be specifically functionalized, including diazotization of a fluorophore to confer fluorescence to a protein for imaging, or diazotization of a molecular crosslinker to attach "cargo" to a given protein through various orthogonal chemistries including "click chemistry"[2].

However, one challenge with diazonium bioconjugation and labeling includes the limited stability and robust reactivity of diazonium ions. Diazonium ions are known to react with many biological nucleophiles, including tyrosine and histidine. Moreover, diazonium ions have been known to react with biological thiols, and potentially a variety of other biologically relevant molecules, including nucleobases[3]. This reactivity hinders targeted and selective use of the bioconjugation tool *in vivo*, and thus chemical protection is used to prevent hydrolytic degradation and undesired side reactions of the diazonium ions. One such form of chemical protection is a particular class of π-conjugated triazines called triazabutadienes (TBD), that have previously been shown to reversibly protect diazonium ions in the presence of acid, or in some cases UV irradiation. TBDs are easily functionalized and can be designed to readily release diazonium ions in a pH-dependent manner.[1] Such pH responsiveness allows them to release specific diazonium cargo within targeted chemical environments, thereby increasing selecitivity. Though useful, triazabutadiene synthesis and functionalization is nontrivial and occurs in multiple synthetic steps, including the synthesis of critical azide intermediates. Fortuitously, we encountered a simpler triazine scaffold within the literature that allows for quick and efficient synthesis of protected aryl

diazonium requiring only diazotization of aniline starting material and coupling to secondary amines of choice.

Past work showed triazine synthesis with a wide scope of commercially available secondary amine compounds, including piperidine, pyrrolidine, and morpholine [4]. Importantly, acidification results in the release of the diazonium ion for subsequent bioconjugation and labeling. Piperidine and morpholine constructs stemmed intrigue, but also prompted interest in the related molecule piperazine, which contains two secondary amine groups potentially capable of triazenation via reaction with diazonium ions.

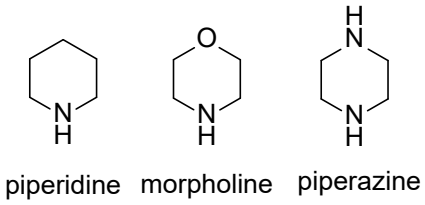


Figure 1. Various cyclic secondary amine nucleophiles (piperidine, morpholine, and piperazine) used for triazenation.

Regarding piperazine, we aimed to investigate the synthesis of 'single' and 'double addition' products, or simultaneous protection of multiple diazonium ions. Moreover, the conditions to selectively synthesize one, or the other were not yet established. The nucleophilicity and pK_a of the nitrogen groups within the molecule is furthermore hypothesized to vary as a result of a single protection reaction. This could provide a mechanism with which to tune the pH conditions under which a second protected diazonium could be released, allowing us greater control over cargo release or labeling.

The newly proposed triazines represent new opportunities for diazonium protection, as well as specification of the protecting group for more selective and functionalized release. Herein we describe the work done to best understand how to utilize the different potential protectors.

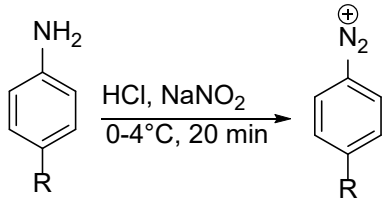
Methods and Materials

Synthesis and Purification of Novel Triazines

Standard diazotization conditions were used in synthesizing aryl diazonium ions to be used for triazenation. 50 mg of aryl precursor was introduced to 5 mL of H_2O, and 100 μL concentrated HCl. The mixture was stirred until dissolution. Five equivalencies of sodium nitrite ($NaNO_2$) were added dropwise to the reaction solution. The reaction was allowed to stir at 0° C for 20 minutes. *Table 1* enumerates the three aryl precursors of interest.

Table 1. Aniline precursors for diazotization and triazenation with piperazine and morpholine.

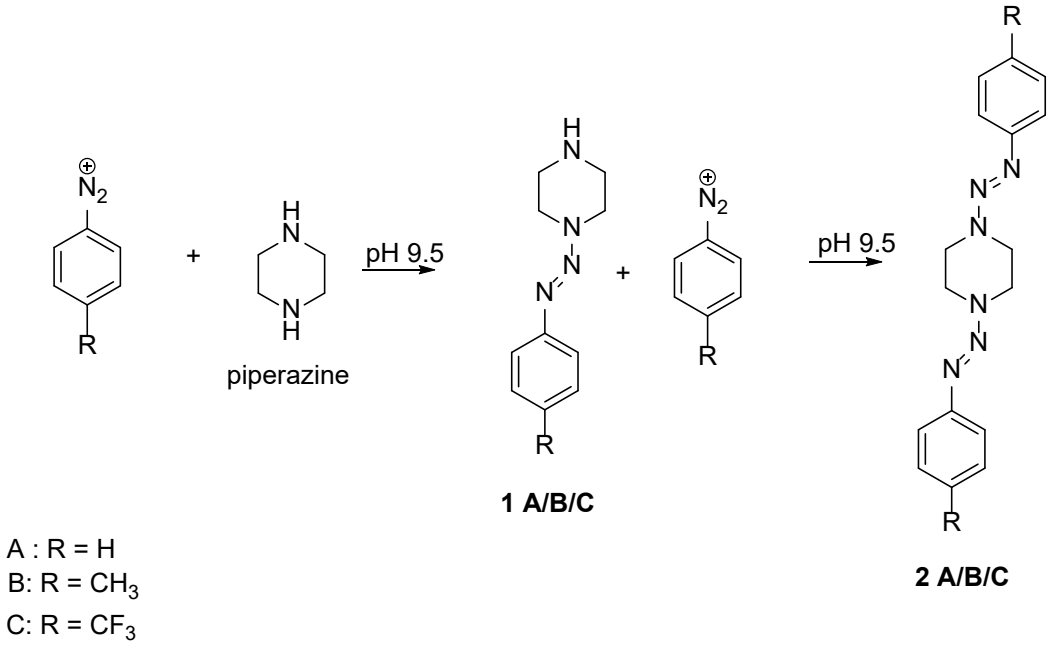
Name	*Molecular Weight (g/mol)*
Aniline HCl	129.59
4-methyl-aniline	107.15
4-triflouromethyl-aniline	161.12



Scheme 1 Synthesis of an aryl diazonium from a precursor aniline.

One equivalency of piperazine was dissolved in 20 mL borate buffer at pH 9.5. The diazonium reaction solution was added slowly, and the pH checked such that the solution remained above pH 9. The combined solution was stirred at 0° C for 30 minutes.

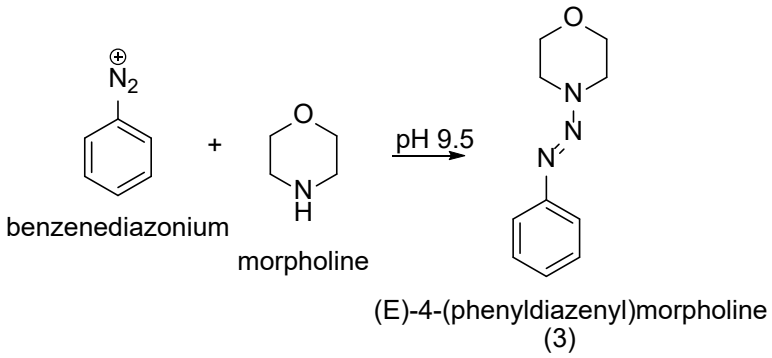
Gravity filtration with Whatman 1 paper was done and the filtered product was dried overnight in the the open within a fume hood. The filtrate was extracted using three washes of dichloromethane, which was thereafter evaporated using air stream. proton and carbon NMR was performed on the resulting products in deuterated chloroform at 400 MHz. The process was repeated for each aryl precursor at 1:1 equivalency.



Scheme 2 The reaction scheme for triazenation reactions.

Note the triazenation synthesis was also done at a 5:1 4-triflouromethyl-benzenediazonium to piperazine molar equivalency ratio to recover **2C** effectively.

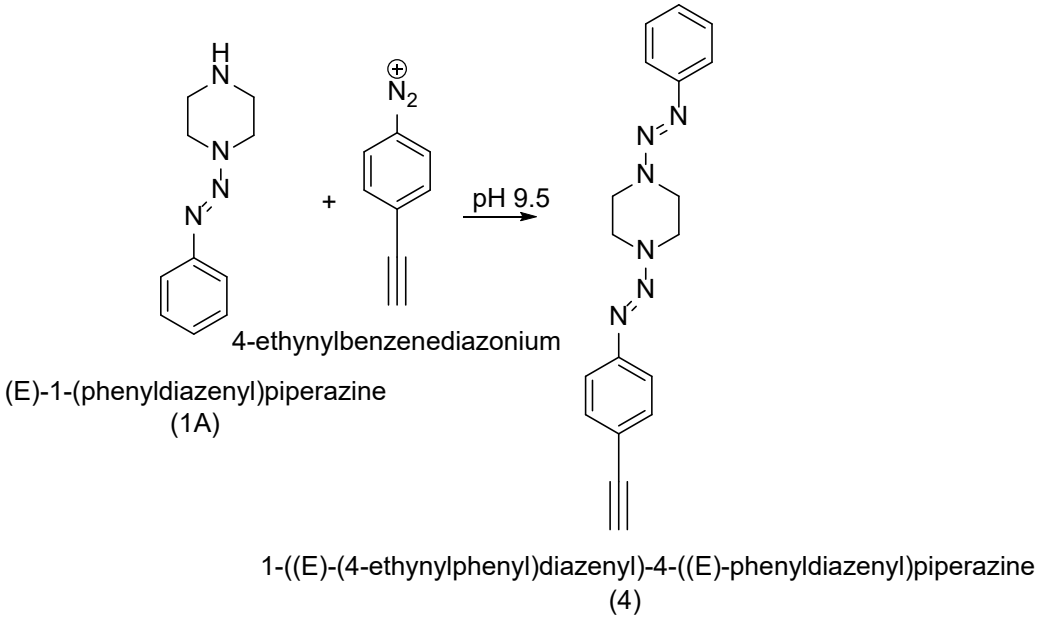
Finally, morpholine was used as a substitute for piperazine to synthesize a triazine with benzenediazonium at a 1:1 equivalency.



Scheme *3* Reaction of benzenediazonium with morpholine to form a novel triazine.

Synthesis of a Hetero-Addition Triazine

The extracted product from synthesis of the aniline-piperazine triazine, also known as *(E)*-1-phenyldiazenyl piperazine, was weighed and dissolved in 20 mL borate buffer at pH 9.5. 1 molar equivalent of 4-propargyl aniline was diazotized using the same diazotization reaction. The 4-propargyl aryl diazonium was slowly pipetted into the borate buffer solution and stirred at 0° C for 30 minutes.



Scheme 4 Hetero-triazenation of piperazine.

Precipitation was subsequently filtered off via gravity filtration with Whatman 1 paper. Filtered product was allowed to dry overnight. The resulting product was characterized via ^{1}H-NMR.

Results

Following purification of the triazine synthesis reactions, all triazetized products were weighed. Product yields are included in *Table 2.*

Table 2. Product yields of each product produced in the given reaction schemes.

Reaction Scheme	*Product*	*Mmol Recovered*	*Product yield*	*1:2 Molar Ratio*	*Overall Product Yield By Diazonium*	*Overall Product Yield By Piperazine*
Scheme 2A	1A	$1.31 * 10^{-1}$	34.1%	1.54:1	78.1%	56.1%
	2A	$8.49 * 10^{-2}$	22.0%			
Scheme 2B	1B	$9.79 * 10^{-2}$	20.9%	1:2.05	46.5%	31.2%
	2B	$4.77 * 10^{-2}$	10.2%			
Scheme 2C	1C	$2.78 * 10^{-2}$	9.0%	3.17:1	62.5%	37.5%
	2C	$8.83 * 10^{-2}$	28.4%			
Scheme 3	3	$2.53 * 10^{-1}$	65.9%	-	-	-
Scheme 4	4	$5.97 * 10^{-2}$	31.1%	-	-	-

Additionally, triazine synthesis was done at 5:1 equivalency of 4-trifluoromethyl benzenediazonium to piperazine. Product **1C** could not be recovered, although 20mg of product **2C** was recovered for a product yield of 75%. Each product was characterized using ^{1}H NMR and ^{13}C NMR when possible. The annotated NMR spectra can be found in *Appendix A.*

Discussion

Synthesis of Novel Triazines

Synthesis of the triazines followed a standard procedure. In *Schemes 2A-C,* shortly following addition of the diazonium reaction mixture to the piperazine solution, precipitation was observed in the reaction mixture. The solution was allowed to react for 30 minutes before filtration. Notably, when the reaction was filtered before sufficient time had passed, precipitation was observed to continue forming in the filtrate. This was potentially indication that a nontrivial amount of triazenation was occurring throughout the allotted time frame. Alternatively, this could also be the result of delayed precipation.

Schemes 2A and *2B* yielded opague foggy precipitation in solution, while *Scheme 2C* yielded a foggy pink precipitant. Upon filtration, the filtered product of *Reactions 2* and *3* turned a brown color, while the filtered product of *Scheme 2C* remained milky pink. Importantly, the H-NMRs of these products are found in *Spectra 2, 4,* and *6* in *Appendix A.* The spectra reflect that the products filtered from solution

are **2A**, **2B**, and **2C**, the result of two sequential triazenation reactions on the piperazine molecule. The resulting product is insoluble in water, which bears intuitive chemical sense, as the number of hydrogen bonds possible diminishes significantly in the 'double addition' product.

The observation of sequential triazenation reactions in a one-to-one equivalency reaction is of nontrivial significance. Triazine synthesis occurs as a result of nucleophilic attack by the piperazine amine on the electrophilic nitrogen of the diazonium ion. A second sequential triazenation reaction indicates out-competition of the piperazine amines by the amine on products **1A**, **1B**, and **1C**. Such an observation indicates variable nucleophilicity as a direct result of the first triazenation reaction, suggesting the nucleophilicity of the piperazine amine groups can be tuned via one triazenation reaction.

Products **1A**, **1B**, and **1C** were soluble in water and recovered in the subsequent extraction of the filtrate. Refer to *Spectra 1, 3,* and *5* for the corresponding H-NMR spectra, respectively. Note from *Table 2*, the recovered product, specifically the ratio of moles of single to double triazenation, varies as a result of the diazonium used. Note products formed with use of benzene diazonium, **1A**, and **2A**, were recovered in a 3:2 ratio, favoring the single triazenation product. Products **1B** and **2B** were synthesized with use of methylbenzene diazonium; returning a molar product ratio of 2:1, favoring the single addition slightly more. Finally, products **1C** and **2C** were synthesized using 4-triflouromethylbenzene diazonium, returning a molar product ratio of roughly 1:3, heavily favoring the double triazenation.

The variable molar ratios further suggest that the nucleophilicity and reactivity of piperazine's second amine can further be tuned by the specific chemical character of the diazonium used in the first triazenation. Specifically, the empirical data supports the notion that electron donating diazonium ions, like that of methylbenzene diazonium, will subsequently result in a higher ratio of single triazenation reactions. Electron withdrawing diazonium ions, like that of 4-trifluoromethyl-benzenediazonium, will conversely result in a higher ratio of double triazenation reactions.

This can be explained by the shifting electron density around the second piperazine amine as a result of the first triazenation reaction. Withdrawing diazonium ions likely lessen electron density via induction effects, and thus the deprotonation step of the triazenation mechanism is more likely. Conversely, donating diazonium ions increase electron density around the amine nitrogen via inductive effects, and thus the deprotonation step is less likely, making the second triazenation less likely.

Morpholine Triazine, and Other Synthetic Observations

Product **3** was successfully synthesized and characterized in *Spectra 7.* The triazine was used as a synthetic test case. Only a single triazenation reaction was possible per morphaline molecule, and yet both

filtration and extraction were performed. Both filtered and extracted products returned very similar spectra, indicating that **3** was marginally soluble in water.

Additionally, several key observations were made in the course of synthesizing triazines with p-trifluoromethyl-benzenediazonium. As earlier discussed, the reaction heavily favors synthesis of product **2C** over **1C**. Filtration of the reaction solution and subsequent drying failed, however, to purify product **2C** as it had for products **2A** and **2B**. ^{1}H NMR spectra revealed residual product **1C** in the filtered product. To attempt to purify, the filtered product was mixed with water to form a suspension that was again filtered. The technique again produced impure filtered product. This was likely the result of hydrogen bonding of the amine hydrogen of product **1C** and the fluorine atoms of product **2C**. To negate this effect, the filtered product was again mixed with 10% NaOH w/v solution to deprotonate amine groups and thereby remove hydrogen bond donators. The resulting suspension was again filtered. The filtered product was dried and ^{1}H NMR was taken. Signals observed from **1C** were significantly diminished but not entirely absent.

To obtain a pure spectrum of **2C**, the reaction was redone using 5:1 diazonium to piperazine equivalency. Interestingly, the resulting *Spectra 6* is free of impurities, and no single triazenation product was able to be recovered from the reaction. This is preliminary evidence that the number of triazenation reactions that proceed can be manipulated via tuning starting equivalencies of the initial reactants.

The final observation made in the synthesis of the various products is the stability of the triazines. Dry triazines were observed to remain intact when kept at room temperature and in the dark for extended periods of time. Importantly, single triazenation products were noted to decompose when kept in aqueous solution for greater than two days. This stability is promising for piperazine triazines as a means to protect diazonium ions for long periods of time via more straightforward and relatively inexpensive process.

Synthesis of the Hetero-Addition Triazine and Its Implications

Spectra 8 confirms successful synthesis of a so called ‘hetero-triazenation’ or successive triazenation and protection of different diazonium ions. Due to the relationship between the electronic composition of the molecule and the diazonium ions protected, it is hypothesized that triggered release of the protected diazonium ions favors one diazonium over the other. The implications of this proposed mechanism include more selective release of a diazonium for labelling or cargo delivery.

Further study is required to learn more about this system. Experiments are proposed using UV irradiation as a means to trigger diazonium release and monitoring the subsequent released diazonium via addition of resorcinol. Thin layer chromatography can first be used to verify that initial release is selective

for a single diazonium, and NMR can be used to characterize the reacted diazonium and resorcinol species.

Future Directions

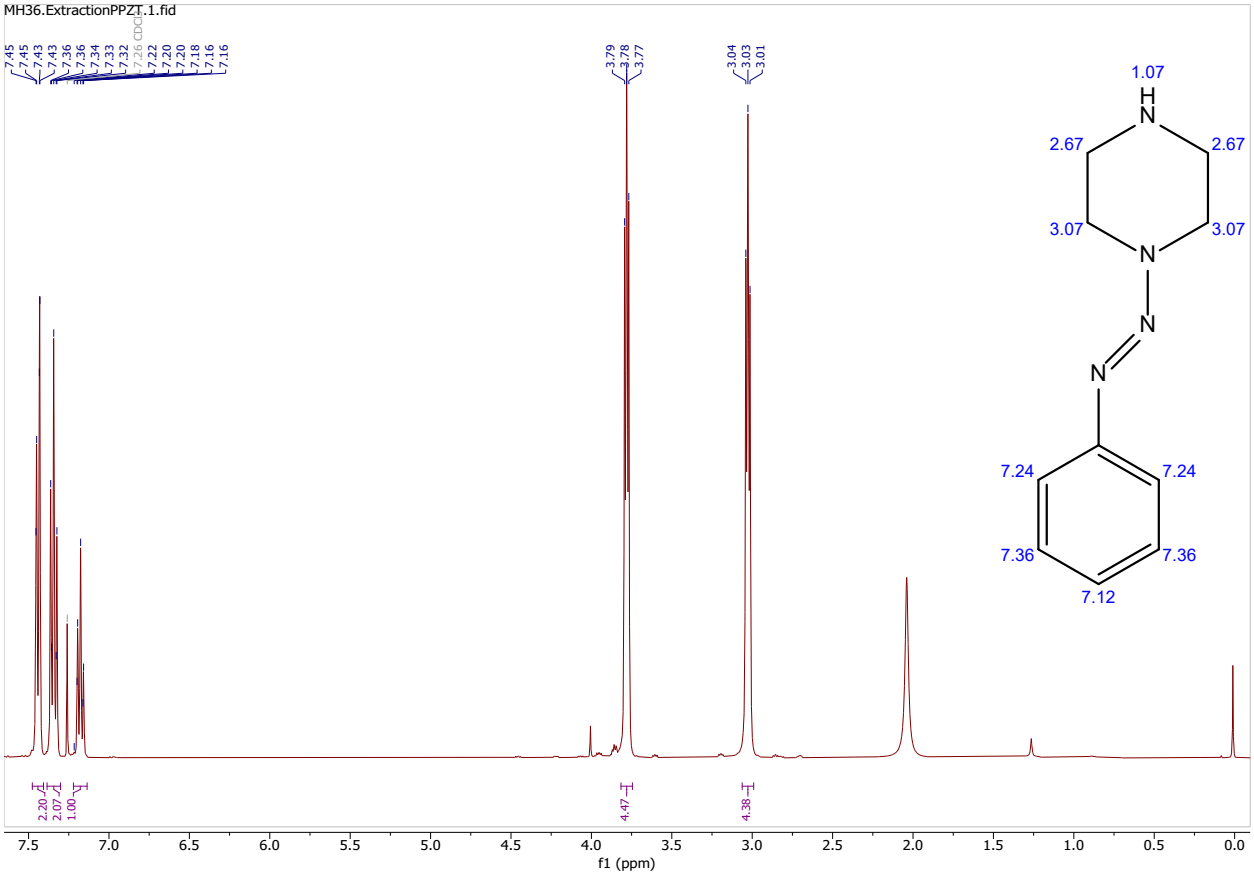
Conclusions supported by synthesis yield data can be further investigated by studying the pK_a of the remaining amine unconjugated in products **1A**, **1B**, and **1C**. The acidity of the amine group provides insight into its relative nucleophilicity, and thereby provides information about its reactivity in protection reactions. Experiments are ongoing to quantify the pK_a of the aforementioned products and include generation of titration curves.

Furthermore, experiments are also proposed into **4**, the triazine species acting as chemical protection for two different diazonium ions. The experiments include short term irradiation and sequential NMR evaluation of **4** and other synthesized 'hetero-triazenation' products. It is hypothesized that the diazonium released first will be the more electron donating of the two species based on similar experiments conducted using piperadine and monitored over time.

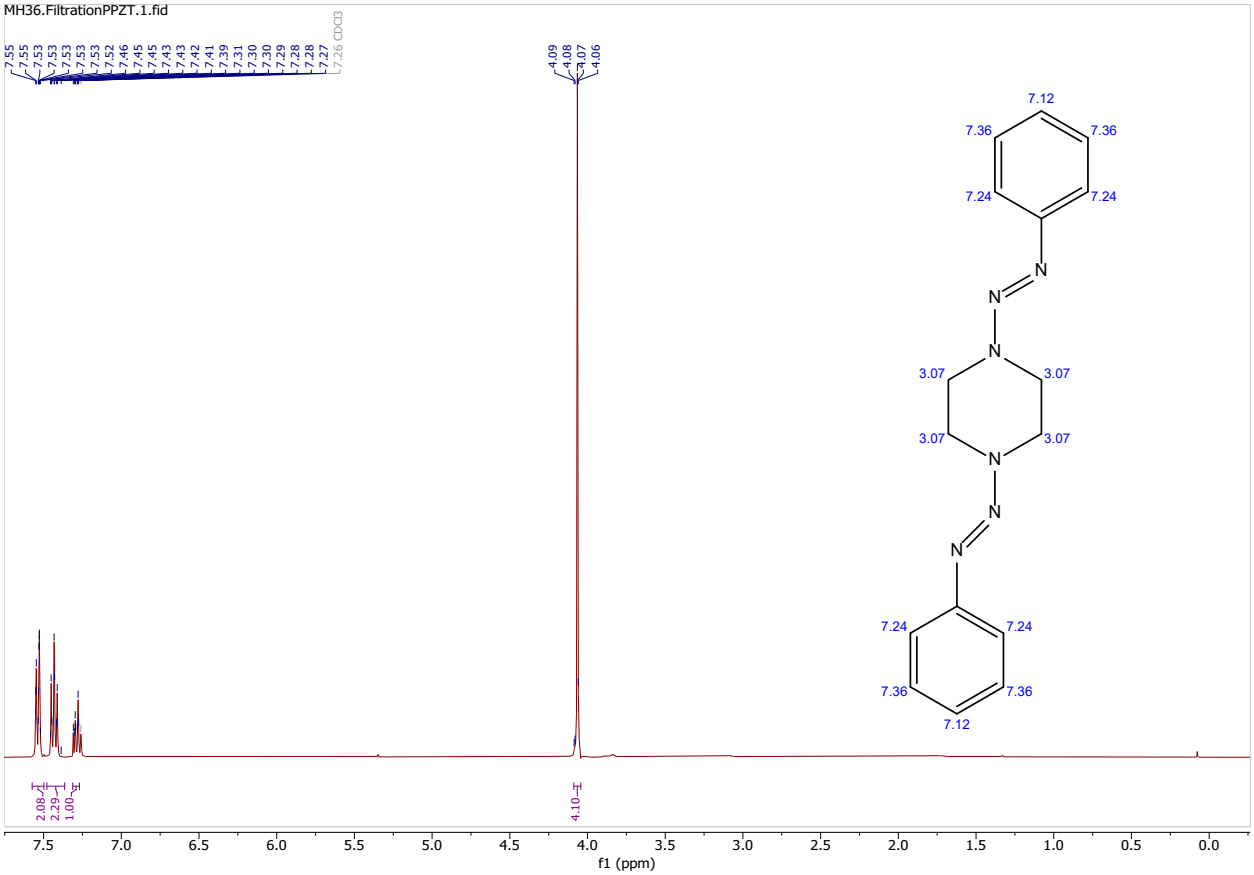
Finally, further investigation is required into the precise biochemical applications of the newly formed triazines. This is proposed by triazenation of 4-propargylbenzene diazonium and treatment of the triazine to the bovine serum albumin (BSA) protein. The mixture would subsequently be exposed to UV irradiation to trigger diazonium release, and the newly bioconguated protein can be loaded on to an SDS gel for fluorescent imaging. Furthermore, other experiments testing homo-dimer ethynyl triazine may show utility for this compounds as protein crosslinkers that can be reversed upon irradiation with UV light.

Synthesis of these novel triazines is a significant addition to the library of chemical protections available for diazonium ions. Ease of synthesis makes these scaffolds easily accessible, and synthesis is ostensibly customizable to favor single or double protection products through equivalency manipulation, or by selection of the diazonium to be protected. The potential ability to provide for more selective release demands further study, though evidence supports piperazine as a viable protection alternative for diazonium ions.

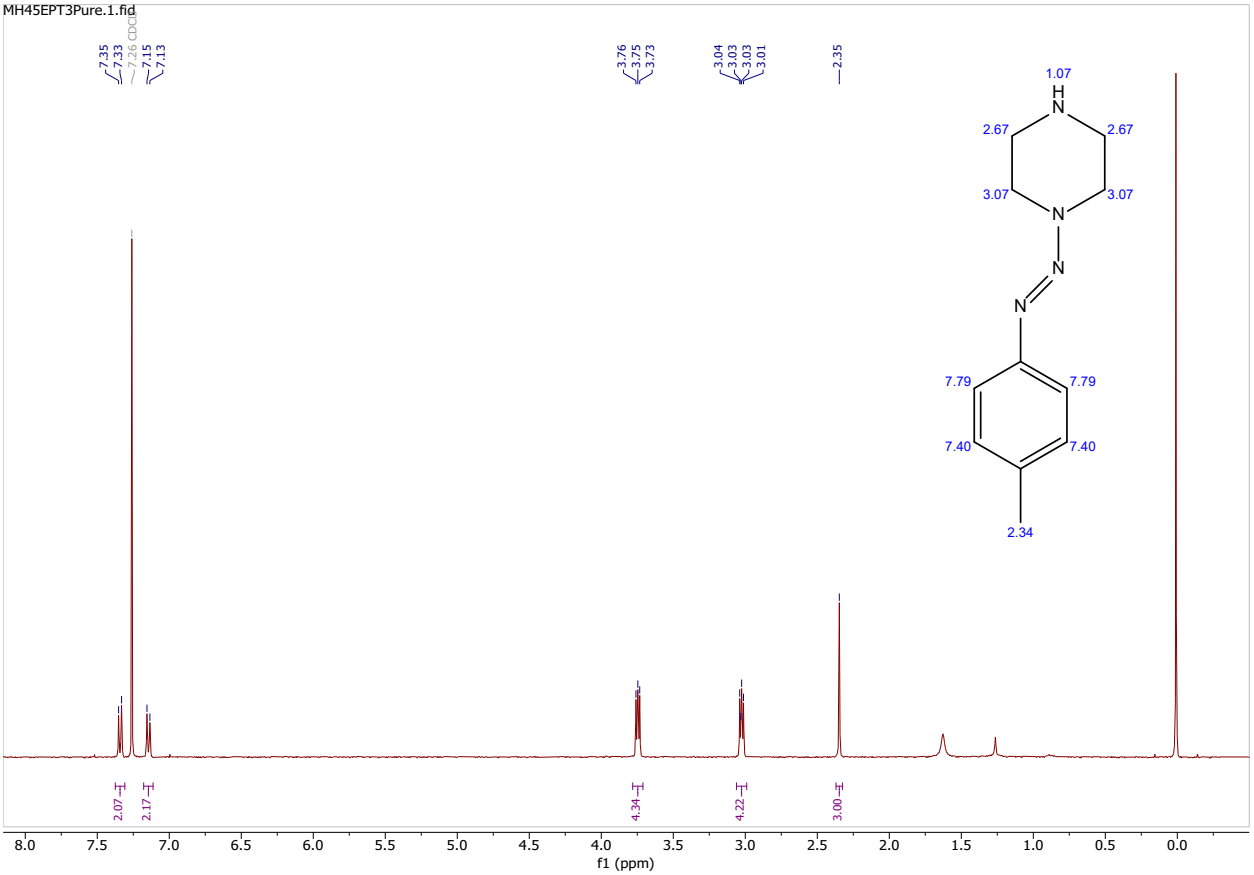
Appendix A – NMR Spectra



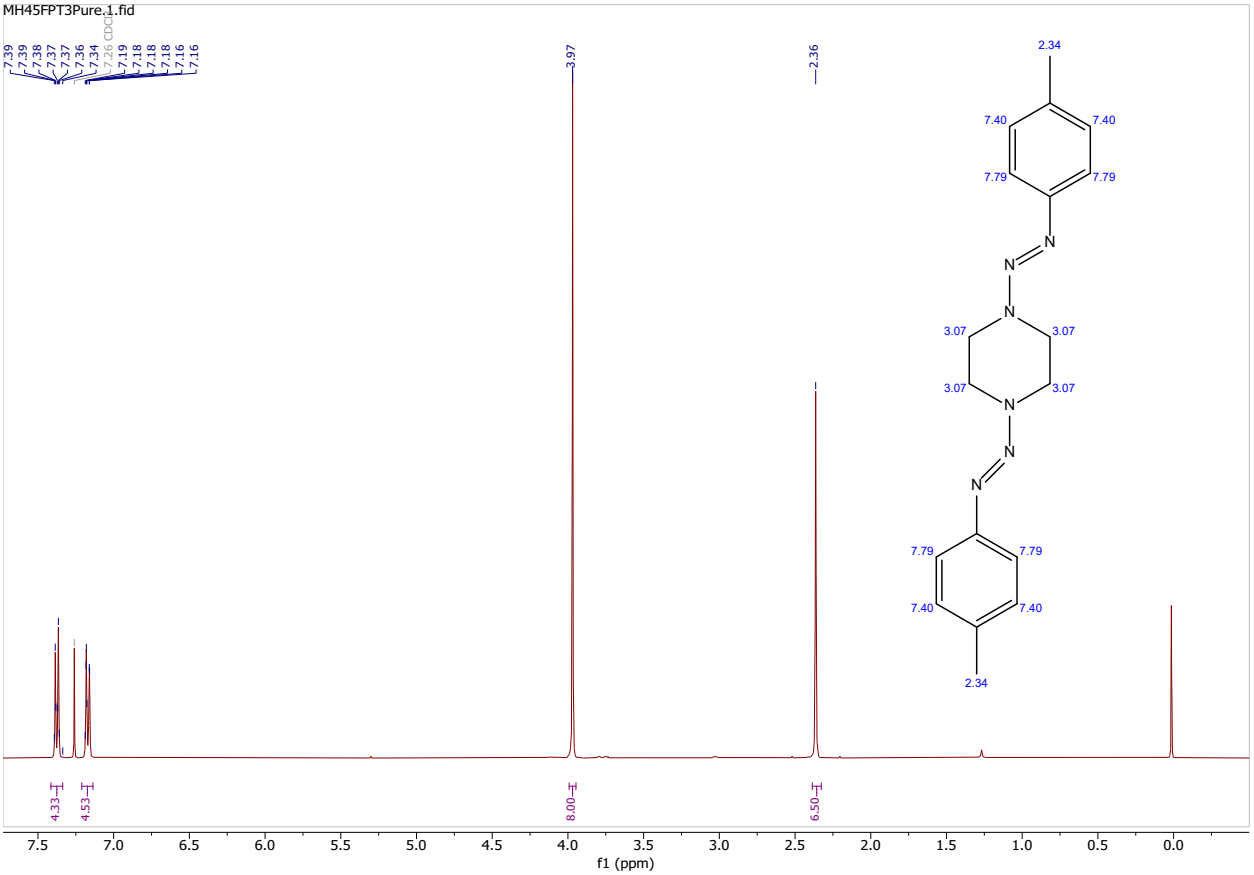
Spectra 1 (E)-1-(phenyldiazenyl)piperazine. (1A). ^{1}H NMR ($CDCl_3$, 400MHz) δ 3.03 (t, 4H), 3.78 (t, 4H), 7.18 (t, 1H), 7.34 (t, 2H), 7.44 (d, 2H). Note, ^{1}H-NMR predicted shifts are generated by ChemDraw and demonstrated on the structure of (1A).



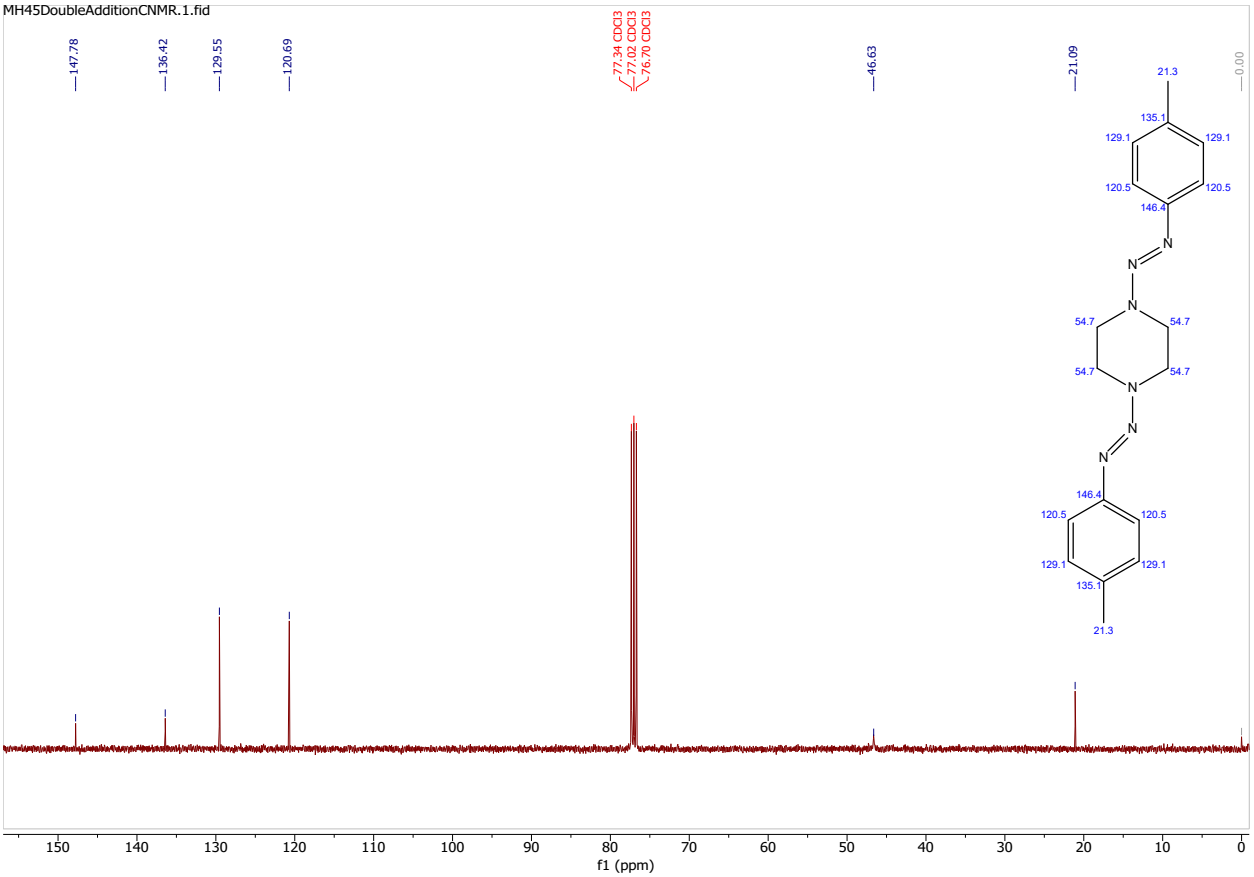
Spectra 1 1,4-bis(E)-1-(phenyldiazenyl) piperazine. (2A). ^{1}H NMR ($CDCl_3$, 400MHz) δ 4.07 (s, 8H), 7.29 (t, 2H), 7.43 (t, 4H), 7.54 (d, 4H). Note, ^{1}H-NMR predicted shifts are generated by ChemDraw and demonstrated on the structure of (2).



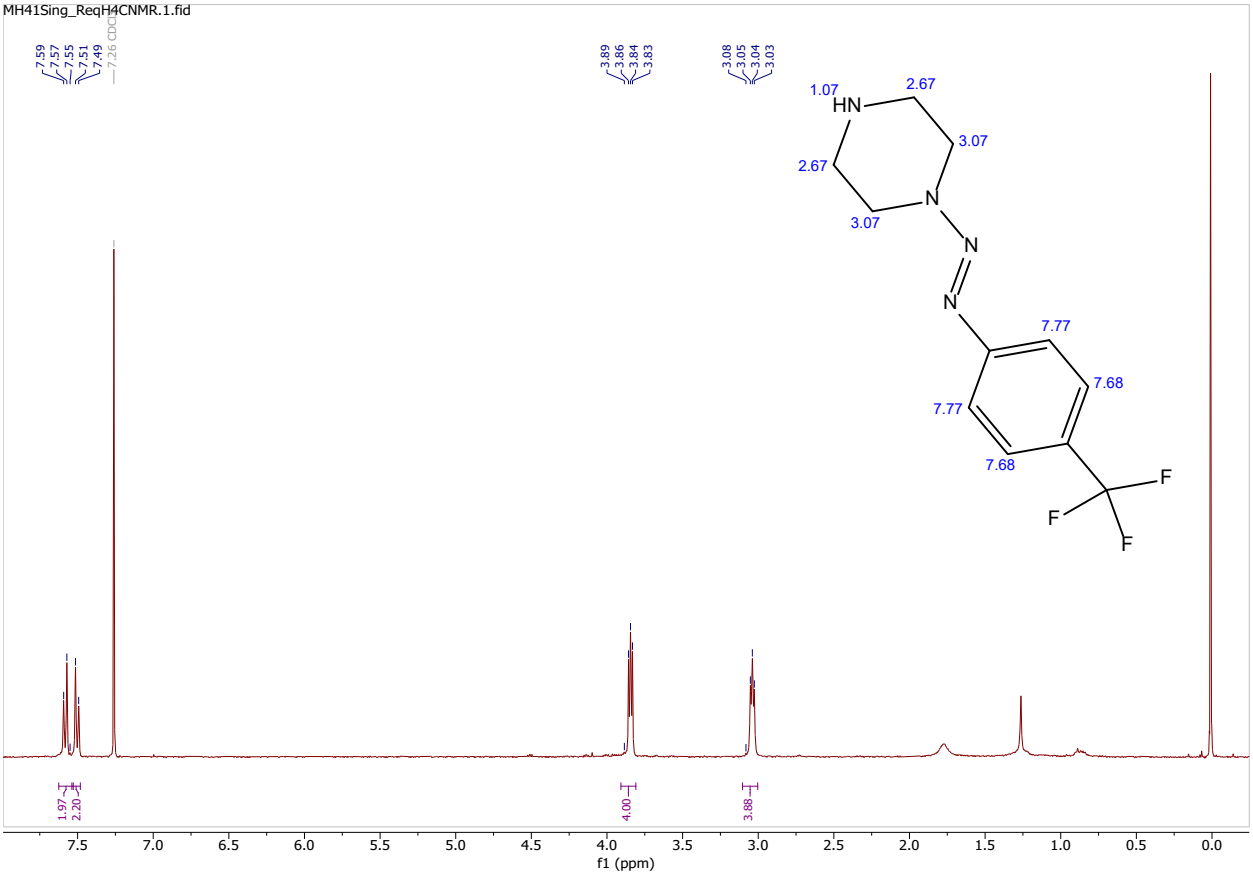
Spectra 2 (E)-1-(p-tolyldiazenyl) piperazine. (1B). ^{1}H NMR ($CDCl_3$, 400MHz) δ 2.35 (s, 3H), 3.03 (t, 4H), 3.75 (t, 4H), 7.14 (d, 2H), 7.34 (d, 2H). Note, ^{1}H-NMR predicted shifts are generated by ChemDraw and demonstrated on the structure of (1B).



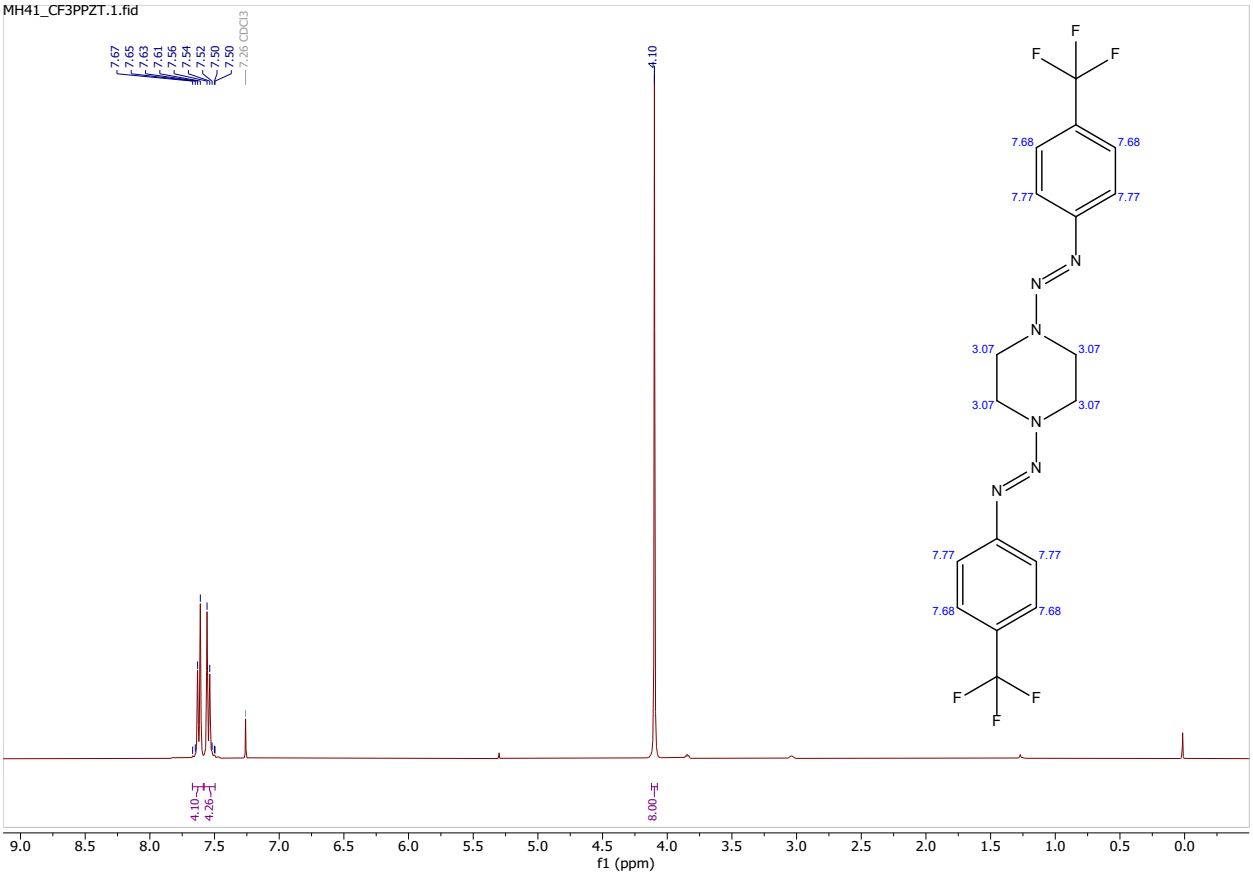
Spectra 3 1,4-bis(E)-1-(p-tolyldiazenyl) piperazine. (2B). ^{1}H NMR ($CDCl_3$, 400MHz) δ 2.36 (s, 6H), 3.97 (s, 8H), 7.17 (d, 4H), 7.38 (d, 4H). Note, ^{1}H-NMR predicted shifts are generated by ChemDraw and demonstrated on the structure of (2B).



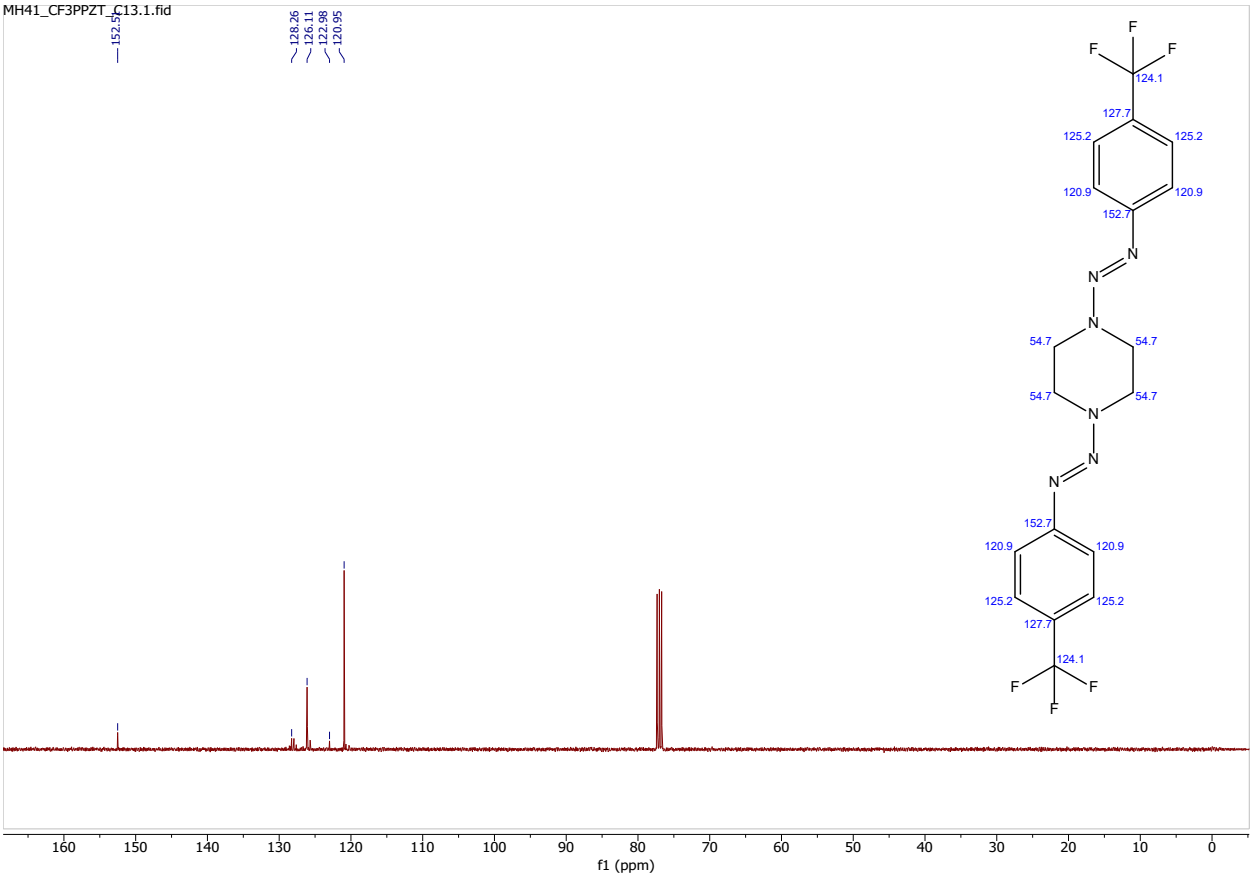
Spectra 4.1 1,4-bis(E)-1-(p-tolyldiazenyl) piperazine. (2B). ^{13}C NMR ($CDCl_3$, 400MHz) δ 21.09, 46.63, 120.69. 129.55, 136.42, 147.78. Note, ^{13}C-NMR predicted shifts are generated by ChemDraw and demonstrated on the structure of (2B).



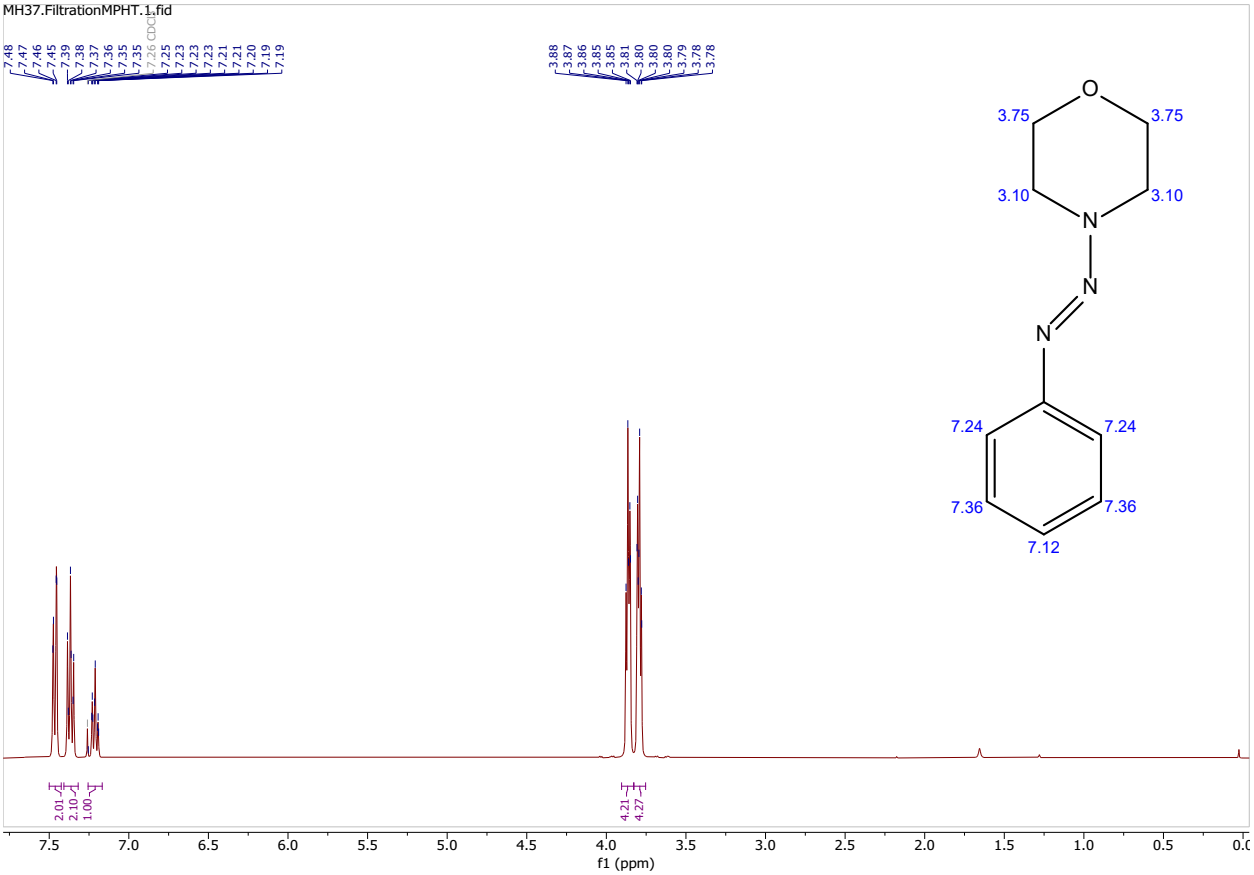
Spectra 5 (E)-1-((4-triflouromethyl) phenyl) piperazine. (1C). ^{1}H NMR ($CDCl_3$, 400MHz) δ 3.04 (t, 4H), 3.84 (t, 4H), 7.50 (d, 2H), 7.58 (d, 2H). Note, ^{1}H-NMR predicted shifts are generated by ChemDraw and demonstrated on the structure of (1C).



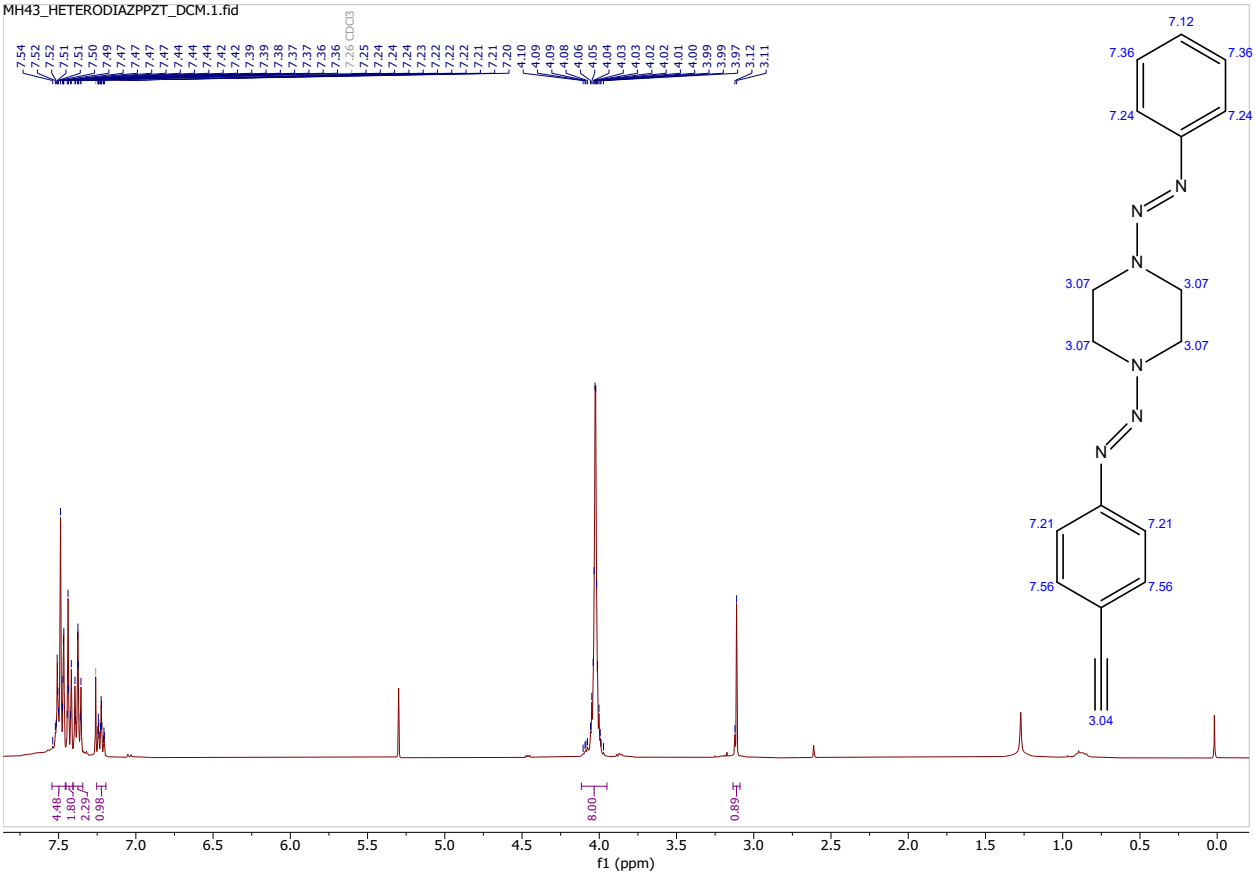
Spectra 6 1,4-bis(E)-1-((4-triflouromethyl)phenyl) piperazine. (2C). ^{1}H NMR ($CDCl_3$, 400MHz) δ 4.10 (s, 8H), 7.55 (d, 4H), 7.62 (d, 4H). Note, ^{1}H-NMR predicted shifts are generated by ChemDraw and demonstrated on the structure of (2C).



Spectra 7.1 1,4-bis(E)-1-((4-triflouromethyl)phenyl) piperazine. (2C). ^{13}C NMR ($CDCl_3$, 400MHz) δ 120.95, 122.98, 126.11, 128.26, 152.61. Note, ^{13}C-NMR predicted shifts are generated by ChemDraw and demonstrated on the structure of (2C).



Spectra 8 (E)-1-(phenyldiazenyl)morpholine. (3). ^{1}H NMR ($CDCl_3$, 400MHz) δ 3.80 (t, 4H), 3.86 (t, 4H), 7.22 (t, 1H), 7.37 (t, 2H), 7.45 (d, 2H). Note, ^{1}H-NMR predicted shifts are generated by ChemDraw and demonstrated on the structure of (3).



Spectra 9 1-((E)-(4-ethynylphenyl)diazenyl)-4-((E)-(phenyldiazenyl)piperazine. (4). ^{1}H NMR ($CDCl_3$, 400MHz) δ 3.12 (s, 1H), 4.02 (m, 8H), 7.22 (t, 1H), 7.37 (t, 2H), 7.43 (d, 2H), 7.48 (t, 4H). Note, ^{1}H-NMR predicted shifts are generated by ChemDraw and demonstrated on the structure of (4). Note, four aromatic hydrogens that theoretically should appear in two different signals appear in one combined signal at 7.48.

References

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