Bifunctional Reagents for Oxime Ligation

by

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Abstract

Oxime ligation is an effective conjugation strategy for biomolecules and polymers in aqueous solution. The reaction is chemoselective, joining an aldehyde or ketone electrophile with an aminooxy nucleophile. The oxime linkage is thermally and hydrolytically stable under most biological conditions. The most common problem with oxime ligation is that the reaction rate is relatively slow at biological pH. Several groups have improved the rates of oxime ligation using organic catalysts, such as aniline. These catalysts are often more effective at low pH, and can substantially improve the rates of conjugation.

In this thesis, we explore modified organic catalysts that contain an aniline nucleophile and an aminooxy nucleophile on the same scaffold. These compounds were designed to act as ligation reagents that could form an oxime linkage in the absence of an exogenous catalyst. We synthesized the ligation reagents, and measured their rate of reaction with benzaldehyde as a model substrate. We find that the ligation reagents can achieve as much as five-fold acceleration relative to the un-catalyzed reactions, and are between 2-3 fold faster than catalysis with aniline alone. We propose a rationale for these enhanced rates, and propose that these reagents could be used to improve protein bioconjugation strategies.

Preface

This thesis is an original work by Arpan Dandapat. No part of this thesis has been previously published.

To my parents who supported me in every situation of my life

Acknowledgements

I would like to thank several people who have provided me with support and guidance in my studies. First and foremost, I would like to thank my supervisor Dr. Christopher Cairo for his guidance and support throughout my graduate school studies and for all the patience he had shown throughout my investigation.

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Thanks to all of my friends for their wonderful support and sincere suggestion during my studies. I owe special thanks to my parents and my sister for their constant encouragement, inspiration, affection and emotional support.

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List of Abbreviations

Ac	acetyl
CuAAC	copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition
DCM	dicholoromethane
DIAD	diisopropyl azodicarboxylate
DMF	*N,N'*-dimethylformamide
EI	electron ionization
ESI	electrospray ionization
Et	ethyl
FGE	formylglycine generating enzyme
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
LAH	lithium aluminium hydride
5-MA	5-methoxyanthranilic acid
Me	methyl
NBD	nitrobenzoxadiazole
NBS	*N*-bromosuccinimide
NHPI	*N*-hydroxyphthalimide
NMR	nuclear magnetic resonance
PEG	polyethylene glycol
m-PDA	*m*-phenylenediamine
p-PDA	*p*-phenylenediamine

Ph	phenyl
RNA	ribonucleic acid
RP	reverse phase
rt	room temperature
SM	starting material
THF	tetrahydrofuran
TLC	thin layer chromatography

Chapter 1

Bioconjugation via an oxime linkage under aqueous conditions

1.1 Introduction

Bioorthogonal chemistry can be used to selectively react two functional groups in the presence of functional groups commonly found in biological systems.[1-7] This field of chemistry has become an essential tool for chemical biology in the last decade.[1-7] Examples of bioorthogonal reactions that have become popular include the Staudinger ligation,[8-20] azide/alkyne cycloadditions,[21-33] inverse-electron-demand Diels-Alder (tetrazine) reactions,[34-40] and hydrazone and oxime formation.[41-59] These approaches have been applied effectively and efficiently for the introduction of tags, labels, and other markers including fluorophores, biotin, and radiolabels to biomolecules.[1-7] These strategies can also be used to join two large biomolecules together.[60,61] The most selective and general chemistries may also find application in other fields, such as polymer chemistry, dynamic combinatorial chemistry, and reaction development.[62-74]

Oxime and hydrazone formation are some of the earliest known bioorthogonal reactions, although the term was not coined until the last decade.[75] The formation of an oxime from an aldehyde electrophile and an aminooxy nucleophile is an effective way to label biomolecules using mild conditions in aqueous solvent. The reaction is highly chemoselective, and oximes are thermally and hydrolytically stable under biological conditions.[55,76-80] Also, these substrates are readily available and synthetically accessible compared to more complex substrates required in other bioorthogonal methods.[1,41] Moreover, incorporation of an aldehyde or ketone into biomolecules is often straightforward (Figure **1.1**).[78,81-88] Importantly, both the electrophile and nucleophile are relatively inert towards reaction with other biological functional groups under neutral or mildly acidic aqueous conditions.[48]

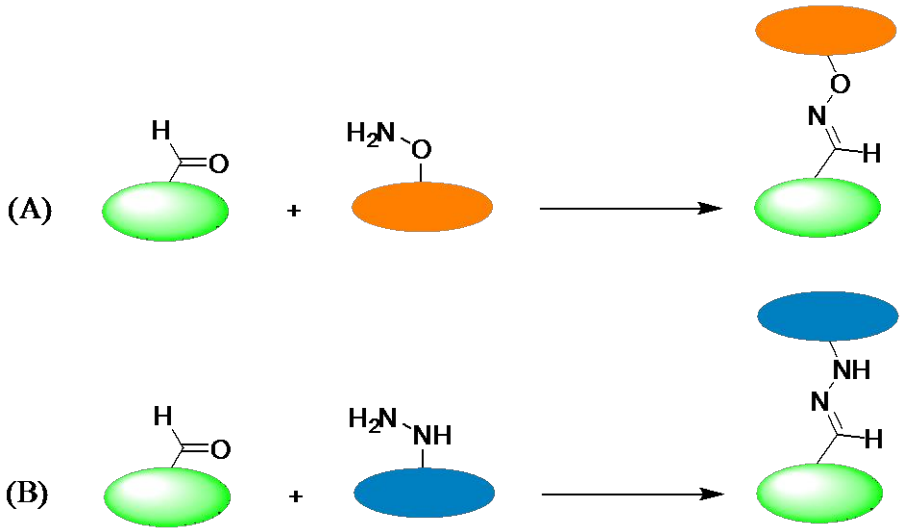


Figure 1.1 Representative example of (A) oxime formation between an aldehyde modified biomolecule (green) and aminooxy probe (orange), and (B) hydrazone formation between an aldehyde modified biomolecule (green) and hydrazine probe (blue).

As aldehyde and ketone groups are relatively rare in biological systems, there are relatively few applications of hydrazone and oxime formation in live cells (Figure **1.1**).[41,42,52] However, in most of these cases these applications have required the *in vitro* preparation of modified biomolecules for conjugation.[69,89-91] The use of oxime or hydrazone chemistry on the cell surface is well known due to the complete absence of aldehyde and ketone groups.[1] Sialic acid present on the cell surface can be easily oxidized by using sodium periodate to generate aldehyde groups which can be subsequently labeled via oxime or hydrazone chemistry.[49,92] Additionally, protein engineering using non-canonical amino acid incorporation has been employed for protein modification via hydrazone or oxime formation both *in vivo* and *in*

vitro.[41,42,47] Cysteine residues can be converted to formylglycine using the formylglycine generating enzyme (FGE) recognition sequence (LCTPSR).[85,93]

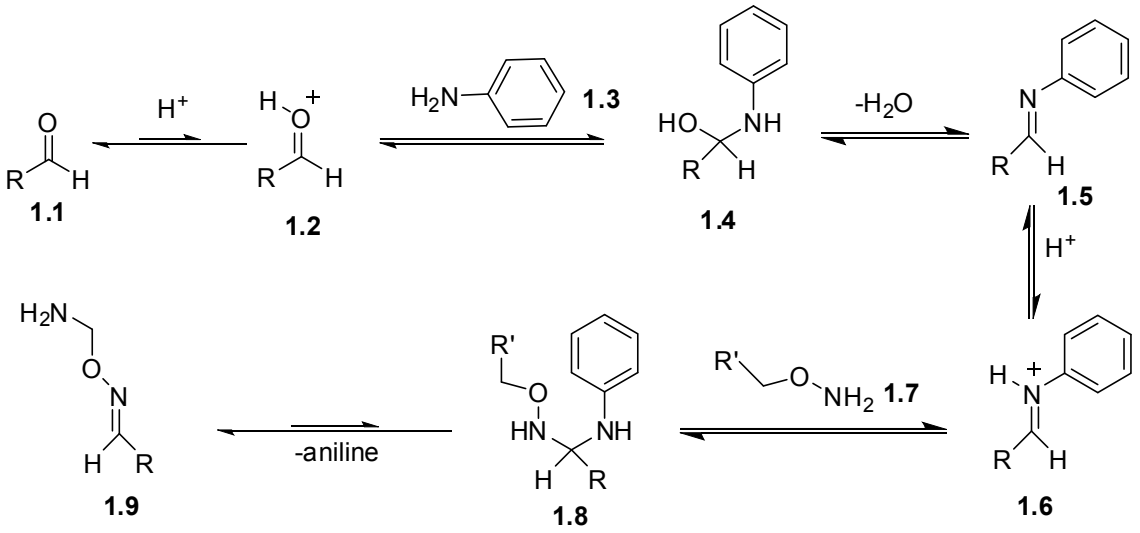
As a result of these favorable attributes, the oxime ligation reaction has been widely used in bioconjugation. However, several aspects of the chemistry are being actively investigated to improve the efficiency of the reaction. The standard aldehyde-aminooxy reaction proceeds at only moderate reaction rates, and requires acidic pH.[48,51,94,95] The primary strategy which has been used to improve the efficiency of the ligation is the use of a nucleophilic catalyst, first introduced by Jencks, and applied to oxime ligation by Dawson et al.[48,53,54,56-59,94,95]

1.2 Nucleophilic catalysis of oxime formation by aniline

Aniline (pK_a 4.6) is a mild and fairly unreactive nucleophile.[48] Jencks first introduced aniline as a nucleophilic organocatalyst in semicarbazone and oxime formation reaction in the 1960s.[94] Four decades later; Dawson and co-workers revisited the earlier study and elegantly applied this concept in bioconjugation.[48] Dirksen et al. demonstrated the ligation of two peptides with an oxime bond using aniline as nucleophilic catalyst. The uncatalyzed reaction had a $t_{1/2}$ of 310 minutes (k_{obs} = 0.057 ± 0.010 M^{-1} sec^{-1}) with 1 mM substrate. The rates were significantly enhanced by using 10 mM aniline at pH 4.5, reducing the $t_{1/2}$ to 25 min (k_{obs} = 0.68 ± 0.05 M^{-1} sec^{-1}) with 1 mM substrate. The uncatalyzed reaction had a $t_{1/2}$ of 8200 minutes (k_{obs} = 0.020 ± 0.001 M^{-1} sec^{-1}) with 0.1 mM substrate. In presence of 10 mM aniline $t_{1/2}$ was reduced to 270 min (k_{obs} = 0.62 ± 0.03 M^{-1} sec^{-1}) and at 100 mM anilinium acetate buffer (pH 4.6) it was further reduced to just 19 minutes (k_{obs} = 8.6 ± 2.0 M^{-1} sec^{-1}). This rate enhancement was explained by considering the increased *in situ* generation of a highly reactive electrophile, the aniline Schiff

base **1.6**, in the presence of aniline **1.3** (Scheme **1.1**). The same group also reported a method to effectively label cell-surface sialylated glycoproteins on live cells by taking advantage of aniline-catalyzed oxime ligation.[49]

In spite of having all these advantages, aniline catalyzed oxime ligations suffers from some difficulties for application in cells or organisms. The ligation rate is still slower in biological conditions which are closer to neutral pH. High aniline concentration can increase the ligation rate. However, it can have toxic effects on the system.[25] Current solutions use a slightly acidic pH and short incubation time and via this approach incomplete reactions are accepted.[49]



Scheme 1.1 Proposed mechanism of aniline-catalyzed oxime ligation.[96]

1.3 Improvements to the nucleophilic catalyst

Extensive efforts have been made to improve the catalytic activity of the nucleophilic catalyst in oxime ligation reactions over the last few years. These include the improvement of the aqueous solubility and tuning the pK_a of the catalysts.[48,53,54,56,95] All of these cases aniline

derivative were used as the starting point. The structural features of both the aldehyde and amine partner of the oxime and hydrazone formation reaction have also been studied.[58,59]

1.3.1 Catalysts with increased anilinium pK_a

Dirksen et al. used *p*-methoxyaniline as a catalyst to perform oxime ligation at elevated pH because of its higher pK_a of 5.3.[48] Two peptides were ligated together at pH 7.0 using 100 mM *p*-methoxyaniline as a catalyst. The uncatalyzed reaction had a $t_{1/2}$ of 11000 minutes (k_{obs} = 0.0015 ± 0.0001 M^{-1} sec^{-1}) with 1 mM substrate. In the presence of 100 mM *p*-methoxyaniline, the reaction $t_{1/2}$ was reduced to 280 min (k_{obs} = 0.061 ± 0.006 M^{-1} sec^{-1}). However, in this study the ligation rate at pH 7.0 was not compared directly to that of aniline.

1.3.2 Phenylenediamines

m-Phenylenediamine (*m*-PDA) **1.43** and *p*-phenylenediamine (*p*-PDA) **1.44** have been used as improved catalysts for oxime-based bioconjugation.[56,95] An electron withdrawing group on the aromatic ring is responsible for an increase in the basicity of the corresponding Schiff base. Also, two amine groups on the same aromatic ring are statistically more favorable towards the formation of an intermediate Schiff base. In addition, the aqueous solubility of these two phenylenediamines is more than that of aniline.

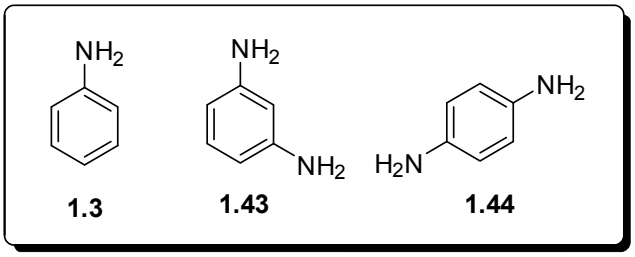


Figure 1.2 Structure of various catalysts used in the oxime-based bioconjugation studies.

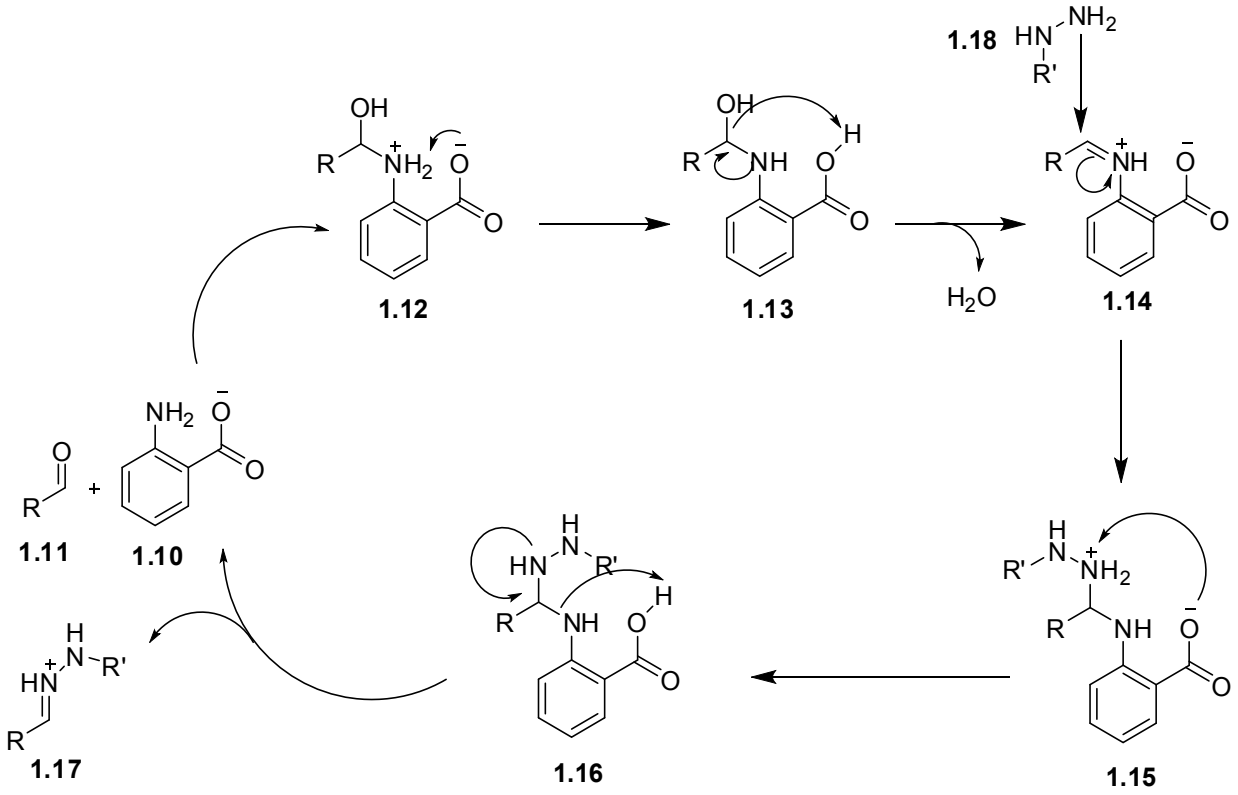
Rashidian et al. discovered the usefulness of *m*-PDA (pK_a 4.88) in oxime ligation and hydrazone-oxime exchange reaction. *m*-PDA (k_{obs} = 0.20 ± 0.0016 M^{-1} sec^{-1}) showed 2.41 times faster oxime formation rate compare to aniline (k_{obs} = 0.082 ± 0.0007 M^{-1} sec^{-1}) at pH 7.3 using 100 mM of catalyst. Also, they demonstrated a kinetic analysis of oxime formation on a GFP-CIVA protein, where, *m*-PDA exhibited ~2.5 times faster kinetics than aniline at the same catalyst concentration (100 mM). *m*-PDA was also used to purify and label proteins using a capture and release strategy from crude cell extracts. In this case, *m*-PDA showed better activity than aniline, and *m*-PDA was not found to have any harmful effect on protein structure and enzymatic activity.

The pK_a of *p*-PDA is 6.08 which is higher than the pK_a of aniline. Therefore, *p*-PDA could act as better catalyst at higher pH as the corresponding Schiff base might also exhibit an increased pK_a. Wendeler et al. studied the advantage of *p*-PDA over aniline in oxime ligation reactions at pH 7.0. Moreover, *p*-PDA showed improved catalytic activity throughout the pH range of 4-7. Oxidized T3 protein (1 mg/mL) was treated with 5 equivalents of aminoxy-PEG at pH 7 and ambient temperature. Catalyst at 10 mM was used for this study. In this reaction condition, *p*-PDA (k_{obs} = 0.29 ± 0.029 M^{-1} sec^{-1}) exhibited 17-times faster ligation rates compare

to aniline (k_{obs} = 0.017 ± 0.003 M^{-1} sec^{-1}) and 48-times faster rates compare to uncatalyzed (k_{obs} = 0.006 ± 0.0012 M^{-1} sec^{-1}) ligation reaction.

1.3.3 Water soluble ortho-proton donors as an organocatalyst for oxime ligations

The requirement of aqueous conditions for bioorthogonal conjugation is often problematic. The Kool group recently discovered improved catalysts, with improved aqueous solubility for oxime and hydrazone ligation.[53,54] They found 5-methoxyanthranilic acid (5-MA) **2.19** and 3,5-diaminobenzoic acid **2.20** (1 mM; k_{obs} = 3.2 ± 0.3 M^{-1} min^{-1}) was a better organocatalyst for hydrazone and oxime formation. At low catalyst concentration **2.19** (1 mM; k_{obs} = 6.6 ± 0.2 M^{-1} min^{-1}) showed 6-fold greater rates of reaction when compared to aniline **1.3** (k_{obs} = 1.1 ± 0.2 M^{-1} min^{-1}) while at higher concentration (10 mM) it provided 100-fold increases in the second-order rate constant at pH 7.4. In this study it was found that acidification of the buffer had a minor role in increasing the rate of the reaction. However, the presence of the *ortho*-acid group played a major role in the rate of catalysis.



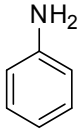
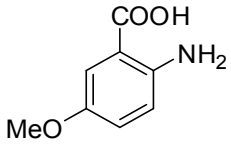
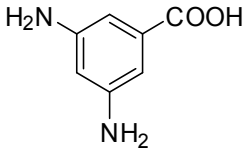
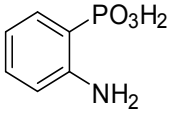
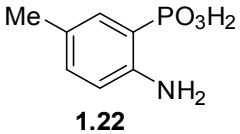
Scheme 1.2 Proposed intramolecular mechanism of anthranilic acid catalyzed hydrazone formation under acidic conditions.[105]

The intermediate imine **1.14** is formed by breaking down the tetrahedral intermediate **1.13**, which is generated from the reaction between catalyst **1.10** and carbonyl compound **1.11**. Iminium formation is the rate determining step for oxime or hydrazone ligation. In this case, the *ortho* carboxylic acid **1.10** helps to from the iminium **1.14** more rapidly than aniline through an intramolecular reaction. The *ortho* carboxylic acid **1.10** likely acts as a general acid-base by providing a proton to the hemiaminal tetrahedral intermediate **1.13** to aid in the formation of the iminium intermediate **1.14** which can lead to the formation of hydrazone product **1.17** (Scheme

1.2).

Kool and coworkers also studied alternative *ortho*-proton donor molecules with varying pK_a.[54] They found that the best catalysts had an approximate match between the pK_a of the *ortho*-proton donor with the buffer pH, without reducing the pK_a of aniline. The 2-aminobenzenephosphonic acid **1.21** (1mM, $k_{obs} = 4.1 \pm 0.2$ M^{-1} min^{-1}) derivatives performed as a better catalyst for hydrazone formation with simple aldehyde substrate at a pH of 7. While 5-methyl-2-aminobenzenephosphonic acid **1.22** (1 mM, $k_{obs} = 8.5 \pm 0.5$ M^{-1} min^{-1}) afforded an 8-fold rate enhancement over aniline (1 mM, $k_{obs} = 1.1 \pm 0.1$ M^{-1} min^{-1}) (Table **1.1**). In contrast, the presence of bulky substituent near the carbonyl moiety hindered the catalytic activity of the phosphonic acid group. In this situation anthranilic acids are preferable as catalysts instead of 2-aminobenzenephosphonic acids.

Table 1.1 Observed second-order rate constants k_{obs} (M^{-1} min^{-1}) for hydrazone formation with different catalysts at pH 7.4.[a]

Catalyst	pK_a	k_{obs} (M^{-1} min^{-1})	Rel. to aniline rxn.
 **1.3**	4.6	1.1 ± 0.2	1
 **1.19**	-	6.6 ± 0.2	6
 **1.20**	-	3.2 ± 0.3	3
 **1.21**	7.29	4.1 ± 0.2	4
 **1.22**	-	8.5 ± 0.5	8

[a]Conditions: 18 μM nitrobenzoxadiazole (NBD) hydrazide, 1 mM 4-nitrobenzaldehyde, 1 mM catalyst in phosphate buffered saline (pH 7.4) containing 10% DMF. Conversion was monitored by increase in

absorbance at 504 nm.[53,54]

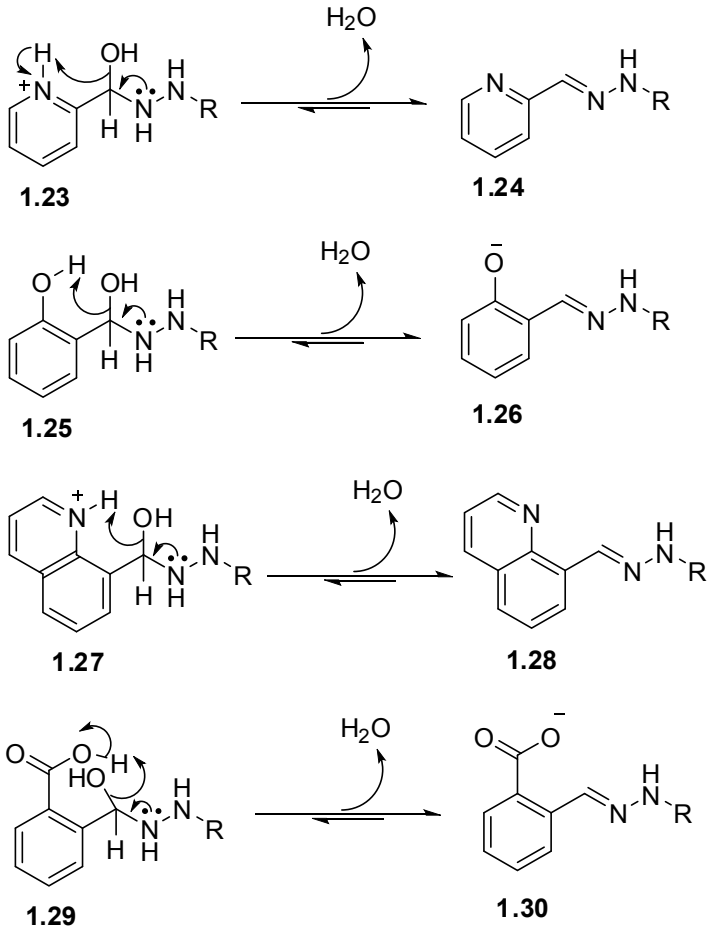
1.4 Substrate effects

Although the presence of catalyst accelerates the oxime or hydrazone formation reaction, it can also introduce complexity and, in some cases, toxicity.[25] For this reason Kool and co-workers studied the structural features of both the aldehyde and amine partner of the oxime and hydrazone formation reaction to increase the reaction rate without using any catalyst.[58,59]

Phenylhydrazine was used as a standard hydrazine and reacted with various carbonyl groups. Rates were measured under pseudo-first-order conditions. Simple alkyl aldehydes showed the fastest rate among all substrates. Butyraldehyde exhibited the highest rate for hydrazone formation reaction with phenylhydrazine. In general, ketones showed slower reaction rates than comparable aldehydes. For example, butyraldehyde ($k_{2(app)}$ = 9.7 ± 2.3 M^{-1} sec^{-1}) showed 44-fold faster reaction rate than 2-butanone ($k_{2(app)}$ = 0.22 ± 0.07 M^{-1} sec^{-1}). Another general trend for this kind of reaction is that electron-deficient aldehyde and ketone substrates favor the reaction compared to electron-rich ones. The 4-methoxybenzaldehyde substrate was the slowest-reacting aldehyde ($k_{2(app)}$ = 0.033 ± 0.008 M^{-1} sec^{-1}). Steric crowding has a notable effect on the hydrazone formation rate. However, in this study steric effects on reaction rate were inconclusive.

The *ortho*-substitution effect on the hydrazone formation rate enhancement is a known phenomenon.[57,97,98] Kool and coworkers have studied this effect of with various groups including imino, hydroxy, and carboxy substituents near the reactive carbonyl center. It was found that all of these groups give rate enhancements for hydrazone formation reactions. For example, quinoline-8-carboxaldehyde ($k_{2(app)}$ = 0.83 ± 0.07 M^{-1} sec^{-1}) exhibits an 8.3-fold rate increase

relative to 1-napthaldehyde ($k_{2(app)} = 0.10 \pm 0.03\ M^{-1}\ sec^{-1}$). Water elimination which occurs with the breakdown of the tetrahedral intermediate is the rate-limiting step for hydrazone formation reactions at neutral pH.[94] Therefore, intramolecular proton transfer to the leaving hydroxy group could accelerate the reaction rate.[54] It has been proposed that these compounds could lose water from the tetrahedral intermediate via 5-, 6-, or 7-membered ring transition sates (Scheme **1.3**).



Scheme 1.3 Proposed mechanisms for intramolecular rate enhancement by an acid/base group in proximity to the reactive carbonyl center.[58]

Kool and co-workers also investigated variation of the rate of ligation due to structural differences on the α-nucleophiles in oxime or hydrazone formation reactions. The 2-formylpyridine substrate was used to study coupling of a range of alkyl hydrazines. Electron-rich hydrazines displayed a higher reaction rate than electron-poor ones. For example, the electron deficient pentafluorophenylhydrazine **1.31** ($k_{2(app)} = 0.12 \pm 0.02\ M^{-1}\ sec^{-1}$) and diphenylhydrazine **1.33** ($k_{2(app)} = 0.14 \pm 0.02\ M^{-1}\ sec^{-1}$) showed the slowest rates, while the electron-rich methoxy- and methyl-substituted aryl-hydrazines exhibited increased reaction rates. Interestingly, two acid/base groups containing hydrazine showed the highest rate for hydrazone formation. The *ortho*-carboxyphenylhydrazine **1.32** ($k_{2(app)} = 1.6 \pm 0.2\ M^{-1}\ sec^{-1}$) and 2-(dimethylamino)ethylhydrazine **1.35** ($k_{2(app)} = 2.8 \pm 0.2\ M^{-1}\ sec^{-1}$) nucleophiles showed 13-fold and 23-fold faster reactivity than the slowest, **1.31**. These two reactive hydrazines were tested with various aldehydes and ketones. Among aryl substrates carbonyl compounds containing proximal imino-groups such as 2-acetylpyridine **1.36** reacted more rapidly with both hydrazines. Additionally, alkyl aldehydes increased the rate of reaction by as much as 100-fold relative to the slowest reactions with **1.31**. The dimethylaminoethyloxyamine **1.37** nucleophile also showed a 3-fold rate enhancement than methoxyamine **1.34**. Therefore, dimethylaminoethyl has a favorable effect in this kind of reaction.

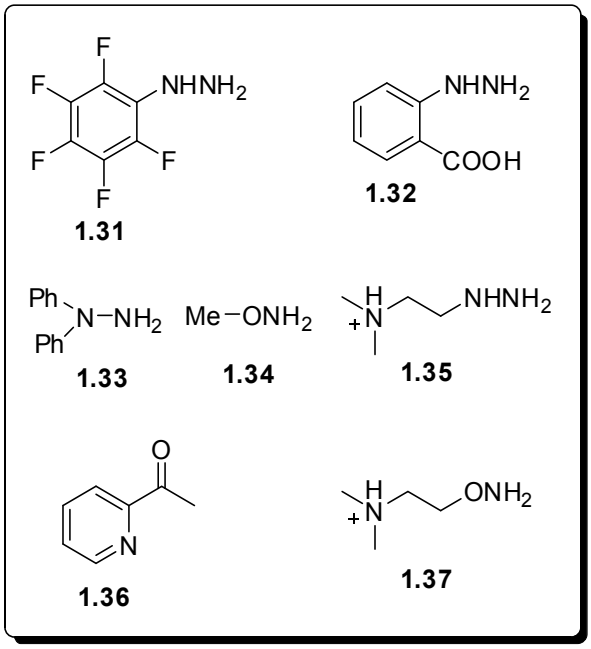


Figure 1.3 Structure of various compounds used in the substrate study in hydrazone and oxime ligation.

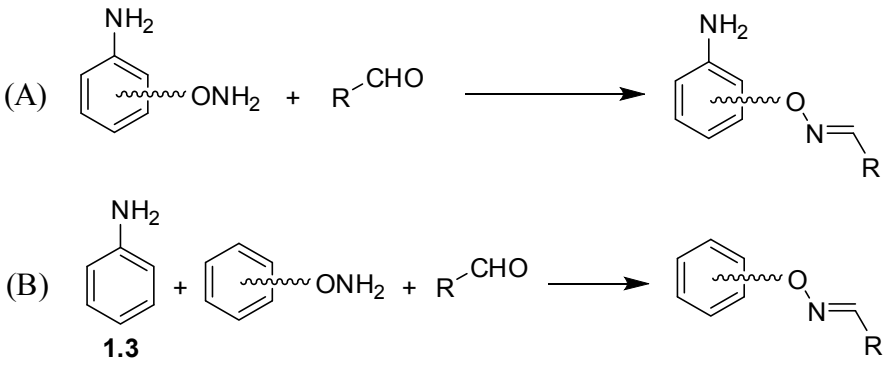
Careful choice of both partners for hydrazone or oxime reaction could potentially eliminate the use of the catalyst. However, all reported improvements in the activity of the nucleophilic catalysts require the use of a stoichiomentric excess of the catalyst to achieve improvements in the rate. As a result, to use these methods in biological systems would require three separate components – increasing the likelihood of toxicity or side reactions. Methods that eliminate the need for three separate components (nucleophile, electrophile, and organocatalyst) would have substantial advantages for biological application.

1.5 Project hypothesis

Although there are many reports which use oxime ligation in bioconjugation, all of these

have some drawbacks. Disadvantages include sluggish reaction rates, high concentration of the catalyst, complexity and toxicity of the catalyst, and excess use of the reagents.[41,53,54,58,59,72,99]

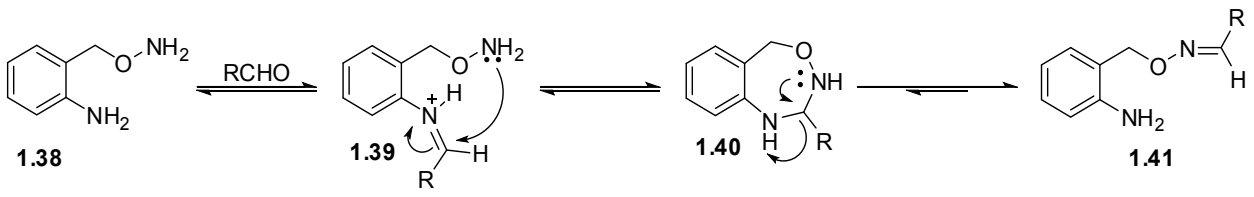
For all these reasons we initiated studies to improve the efficiency using oxime ligation by eliminating the need for a catalyst while still achieving improved rates. We hypothesized a novel pathway for oxime ligation would merge the nucleophilic catalyst and the aminooxy nucleophile on the same scaffold. This configuration would convey two specific advantages: first, there would be no need for an external catalyst (such as aniline); and second, the reaction could be accelerated if the two groups were arranged in such a way that an intramolecular mechanism could be favored (Scheme **1.4**).



Scheme 1.4 A general representation of oxime formation (A) in presence of nucleophile catalyst containing aminooxy group and (B) in presence of aniline and aminoxy compound.

A specific example was envisioned in Scheme **1.5**, where an aniline nucleophile and a benzylic aminooxy nucleophile are *ortho* to each other on an aromatic ring **1.38**. In this example, the reaction could proceed via a 7-*endo*-*trig* cyclization, which is favored by Baldwin's rules.[100-104] It is possible that a ligation reagent of the form of **1.38** would show accelerated rates and

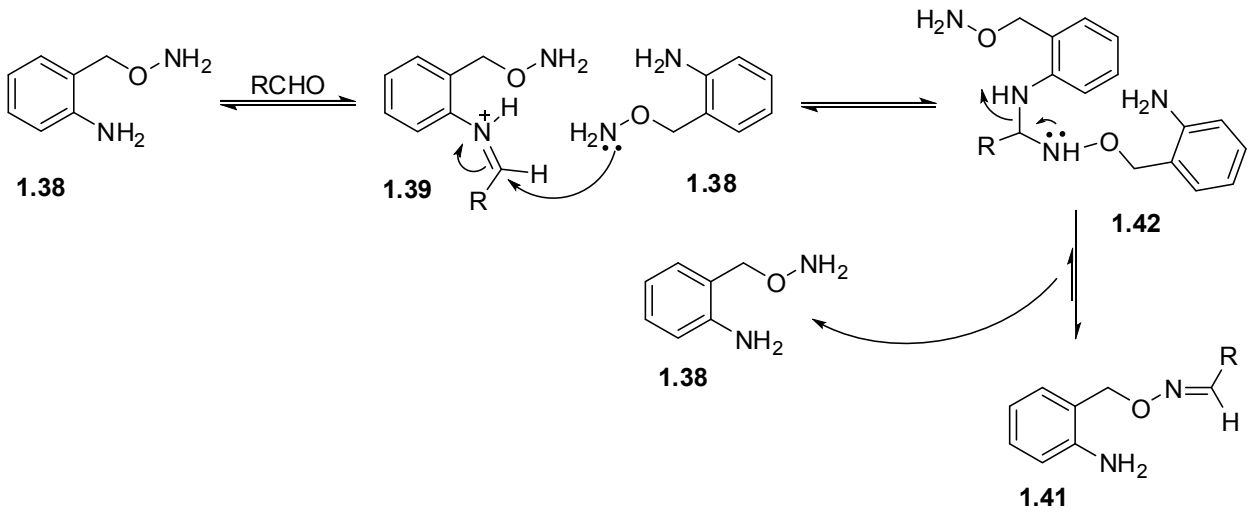
could be used at equimolar stoichiometry to the carbonyl electrophile in water, greatly simplifying the bioconjugation reaction. If the intramolecular pathway shown in Scheme **1.5** were operative, this may also increase the rate of reaction.



Scheme 1.5 Proposed mechanism for the intramolecular oxime formation via 7-*endo-trig* cyclilization.

Alternatively, the mechanism for the oxime formation could occur via an intermolecular pathway (Scheme **1.6**). If this were the case, the reaction would still proceed as a bimolecular ligation reaction, but may not benefit from an enhancement from the intramolecular cyclization pathway.

This thesis explores the hypothesis that compound **1.38** can act as a ligation reagent for oxime bioconjugation. Additionally, using a series of analogs of compound **1.38**, we explore the mechanism of the ligation reaction. Chapter 2 will outline the synthesis and kinetic study of these compounds.



Scheme 1.6 Proposed mechanism for the intermolecular oxime formation.

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Chapter 2

Synthesis and kinetic study of bifunctional oxime ligation reagents

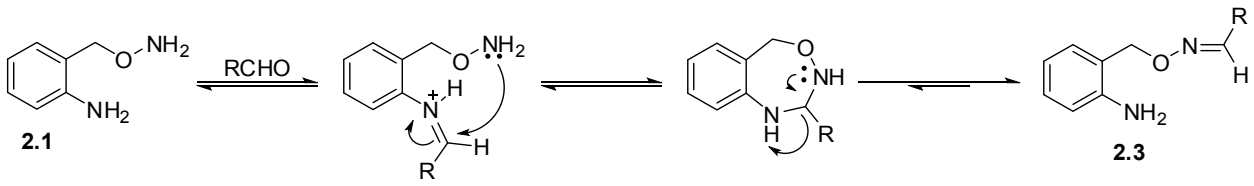
2.1 Introduction

The formation of an oxime from an aldehyde and an aminooxy substrate is an effective way to label and modify biomolecules site-specifically.[1-19] The reaction is highly chemoselective, and oximes are thermally and hydrolytically stable under biological conditions.[15,20-24] Jencks first introduced aniline as a nucleophilic catalyst for oxime or hydrazone formation.[25] Many decades later, Dawson and coworkers demonstrated that the use of an excess of aniline greatly improved the efficiency of the reaction, increasing its use in bioconjugation.[8] Aniline and its derivatives are known to accelerate oxime formation by acting as a nucleophilic catalyst.[8,11,13,14,25,26] The starting materials used in oxime chemistry are less expensive when compared to many bioorthogonal reactions,[1,40] and modification of biomolecules to incorporate aldehyde or ketone groups is fairly simple.[1,2,41,42] Many groups have developed applications of aniline-catalyzed oxime ligation in bioconjugation, polymer chemistry, and combinatorial chemistry. [13,14,15-19,27-39] Although the use of nucleophilic catalysts has provided substantial improvements in coupling efficiencies, two problems stand out which require improvement for application in biological systems: the requirement of low pH and the large excess of the catalyst.

We hypothesized that one way to overcome the need for stoichiometric excess of the catalyst relative to the aminooxy nucleophile would be to combine these two moieties onto a single scaffold. The resulting bifunctional structure would act as a ligation reagent, and would not require the addition of a separate nucleophilic catalyst. If designed appropriately, the compound could even accelerate the rate of reaction and could react at comparable rates to the three-component strategy introduced by Dawson and coworkers.[8]

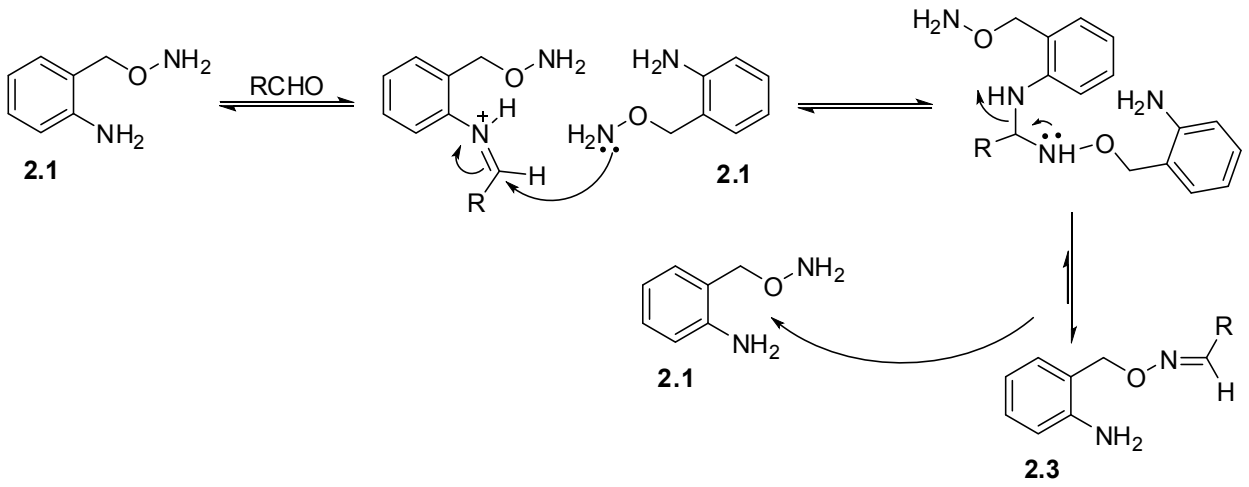
A specific example was envisioned in Scheme **2.1**, where an aniline nucleophile and a

benzylic aminooxy nucleophile are *ortho* to each other on an aromatic ring (**2.1**). In this example, the reaction could proceed via a 7-*endo-trig* cyclization, which is favored by Baldwin's rules.[55-59] It is possible that a ligation reagent of the form of **2.1** would show accelerated rates and would require no additional reagents in the presence of a carbonyl electrophile in water, greatly simplifying the bioconjugation reaction. If the intramolecular pathway shown in Scheme **2.1** were operative, this may also increase the rate of reaction. Alternatively, the mechanism for the oxime formation could occur via an intermolecular pathway. If this were the case, the reaction would still proceed as a bimolecular ligation reaction, but may not benefit from an enhancement from the intramolecular cyclization pathway.



Scheme 2.1 Proposed mechanism for the intramolecular oxime formation via 7-*endo-trig* cyclilization.

Alternatively, the mechanism for the oxime formation could occur via an intermolecular pathway (Scheme **2.2**). If this were the case, the reaction would still proceed as a bimolecular ligation reaction, but may not benefit from an enhancement from the intramolecular cyclization pathway.



Scheme 2.2 Proposed mechanism for the intermolecular oxime formation.

This chapter will focus on the synthesis of bifunctional oxime ligation reagents (**2.1** and **2.2**) and kinetic studies of their ability to form the oxime linkage using HPLC. We have used several other compounds for the kinetic studies (Figure **2.1**).

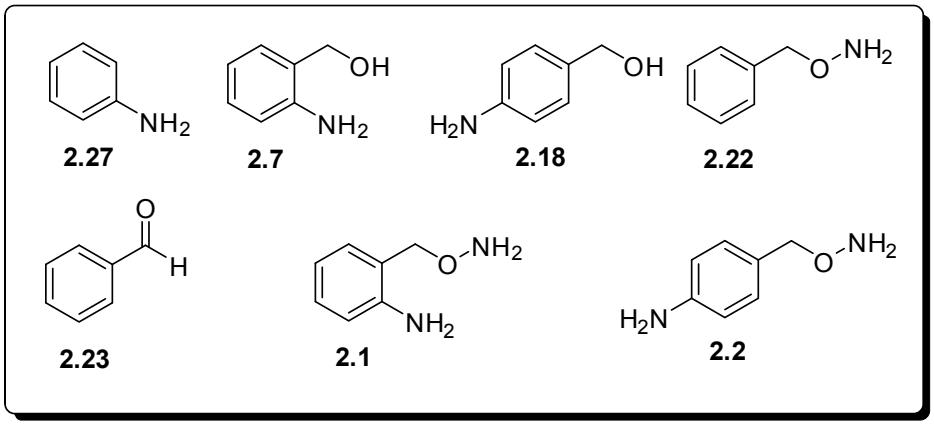


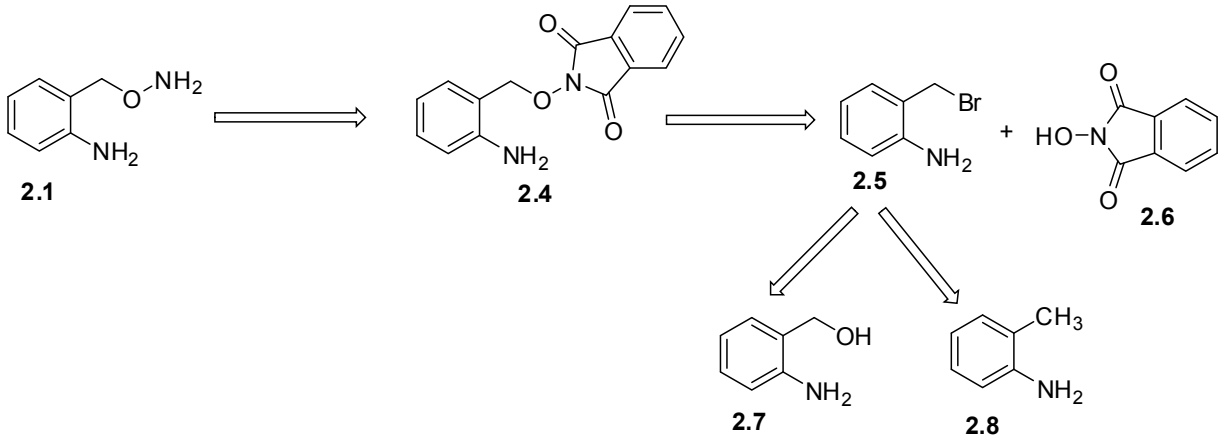
Figure 2.1 Targeted bifunctional aminoxy compounds and other compounds used in kinetic studies.

2.2 Synthesis of 2-[(aminooxy)methyl]-benzenamine

We set out to synthesize two key compounds to test in kinetic studies that could be used to test our central hypothesis. The structures of compounds **2.1** and **2.2** are shown in Figure **2.1**, and contain both a nucleophilic catalyst (aniline) and an aminooxy nucleophile on the same scaffold.

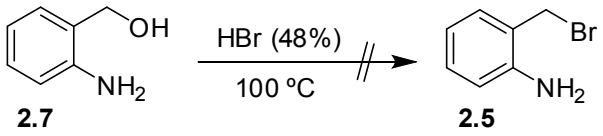
The most challenging component of this structure is the formation of the N-O bond. Typical strategies involve displacement with an amine-protected aminooxy derivative. Therefore, we considered using *N*-hydroxyphthalimide (NHPI) **2.6** as a latent source of the N-O bond, and subsequent deprotection would provide the target aminooxy group.

Our first plan for the synthesis of compound **2.1** is shown in Scheme **2.3**. The key N-O bond of **2.1** would be formed by an S_N2 reaction with NHPI **2.6**, followed by deprotection of **2.4**. The required *o*-amino-benzylbromide **2.5** should be easily generated by displacement of a benzylic alcohol **2.7** or benzylic bromination of an *o*-amino-toluene **2.8** (Scheme **2.3**).



Scheme 2.3 Retrosythetic analysis of compound **2.1**.

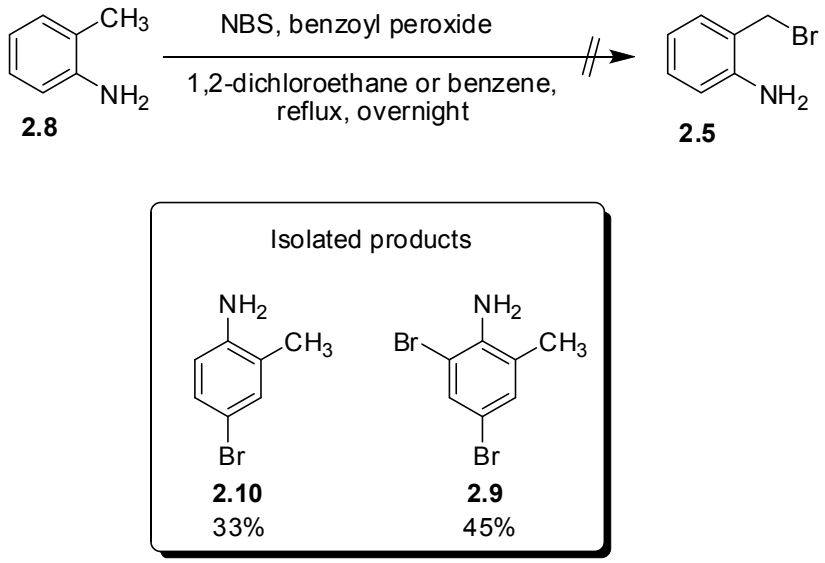
2-Aminophenylmethanol **2.7** was treated with HBr (48%) at 100 ºC to obtain 2-(bromomethyl)-benzenamine **2.5**.[43] However, this reaction was not successful and we observed the degradation of starting materials (by TLC) (Scheme **2.4**).



2.7 **2.5**

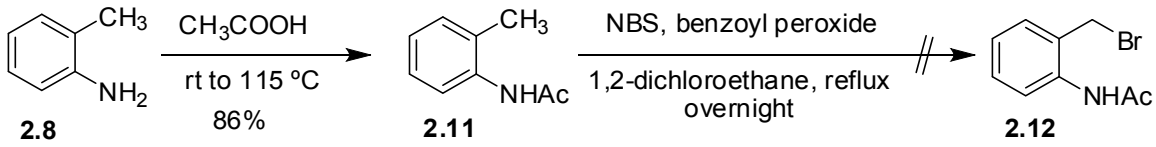
Scheme 2.4 Attempted synthesis of **2.5**.

We next sought to generate the *o*-aminobenzylbromide **2.5**, via radical halogenation with *N*-bromosuccinimide (NBS) in 1,2-dichloroethane solvent.[44] Unfortunately, instead of the desired product, we observed the formation of two undesired products, compounds **2.9** and **2.10** with 45% and 33% isolated yields respectively (Scheme **2.5**). This observation is likely due to electrophilic aromatic substitution which results from the increased activation of the aromatic ring due to the amino substituent. Replacing the solvent with benzene did not alter the outcome.



Scheme 2.5 Attempted synthesis of **2.5**.

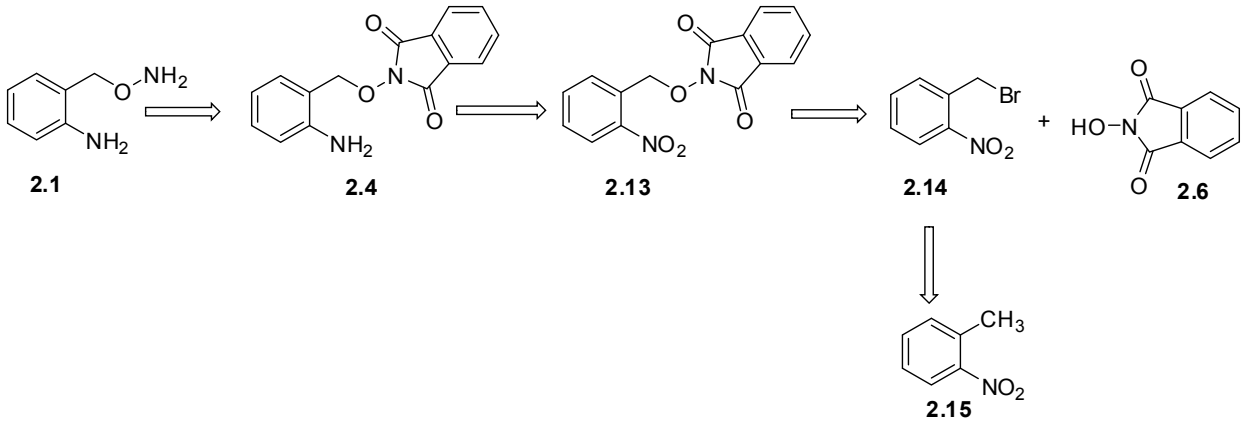
Attempts to reduce the nucleophilicity of the aromatic ring by protecting the amino group as an amide[45] were also unsuccessful. Compound **2.11** was unreactive in the benzylic bromination reaction with the NBS/benzoyl peroxide system, and only starting material was isolated (Scheme **2.6**).



Scheme 2.6 Attempted synthesis of **2.12**.

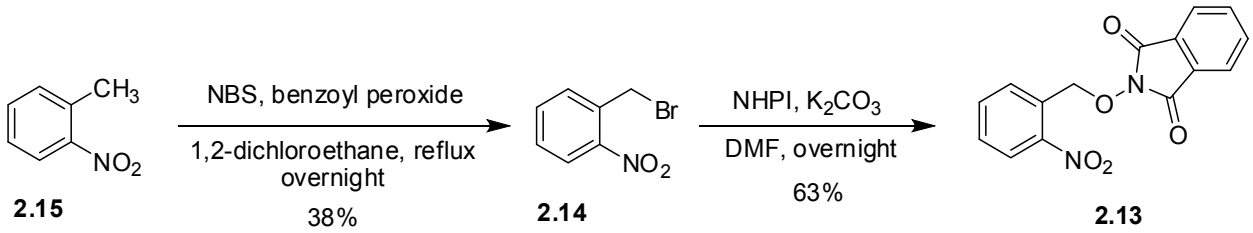
We next examined an alternative approach that would avoid the use of an activated aromatic ring. Starting from compound **2.15**, we hoped to conduct a bromination reaction on the

deactivated ring that would be selective for the benzylic position. Subsequent reduction of the nitro group would then allow us to obtain the desired compound **2.1** (Scheme **2.7**).



Scheme 2.7 Retrosynthetic analysis of compound **2.1**.

Radical halogenation of **2.15** was successful, although in moderate yield.[46] Displacement of the benzylic bromide with NHPI **2.6** proceeded in better yield to give compound **2.13** (Scheme **2.8**).[47]

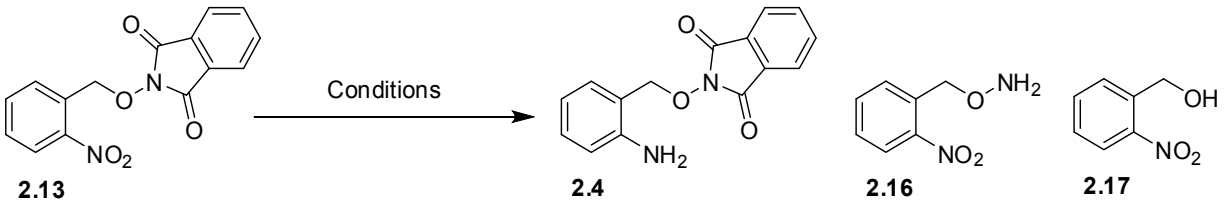


Scheme 2.8 Synthesis of compound **2.13**.

At this point we attempted to reduce the nitro group of compound **2.13** to generate the desired intermediate **2.4**.[48-52] We examined a variety of possible reduction conditions, which are

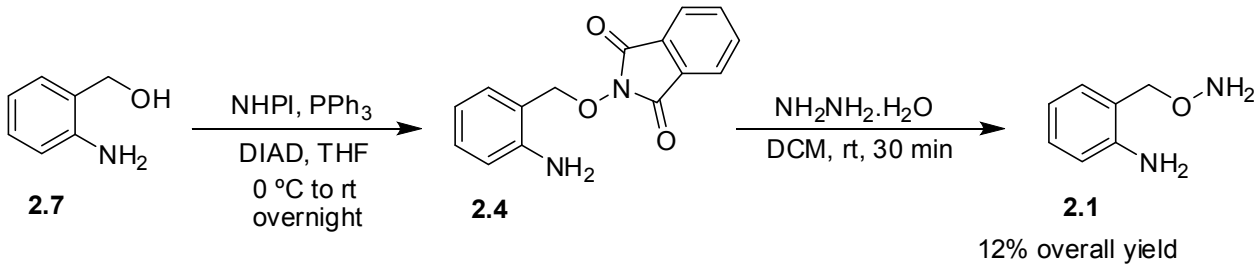
summarized in Table **2.1**. Unfortunately, none of these methods provided the desired target, and we sought out an alternative route.

Table 2.1 Attempted reduction of the nitro group of **2.13**.



Entry	Conditions	Outcome
a	Zn, AcOH MeOH	SM recovered
b	Fe powder, NH_4Cl EtOH:H_2O (1:1) rt to 90 ºC	SM recovered
c	$Na_2S_2O_4$ EtOH, 80 ºC	SM recovered
d	Pd/C, $NH_2NH_2.H_2O$ (2 eqv.) EtOH	2.16 isolated
e	Pd/C, $NH_2NH_2.H_2O$ (10 eqv.) EtOH	2.16 isolated
f	Fe powder EtOH:AcOH (1:1) 79 ºC	SM recovered
g	Ni EtOH:AcOH (1:1) 79 ºC	SM recovered
h	Pd/C H_2 (60 psi)	2.17 isolated
i	Pd/C H_2 balloon EtOH	2.17 isolated
j	Pd/C H_2 balloon MeOH	2.17 isolated

We envisioned that compound **2.1** could be easily accessed by a Mitsunobu reaction between commercially available compound **2.7** and NHPI **2.6** (Scheme **2.9**).[53] The formation of **2.4** using Mitsunobu conditions was successful, and deprotection of the phthalimide group was achieved using hydrazine monohydrate at room temperature.[47] Although we were able to obtain the target, the purification of compound **2.1** was challenging. Attempts to isolate the desired product employing column chromatography using silica as the stationary phase proved to be fruitless and led to complete decomposition. We speculate that the reason behind this failure is the extreme polarity of compound **2.1**. Preparative TLC was used to obtain the compound **2.1** in moderate yields (Scheme **2.9**).

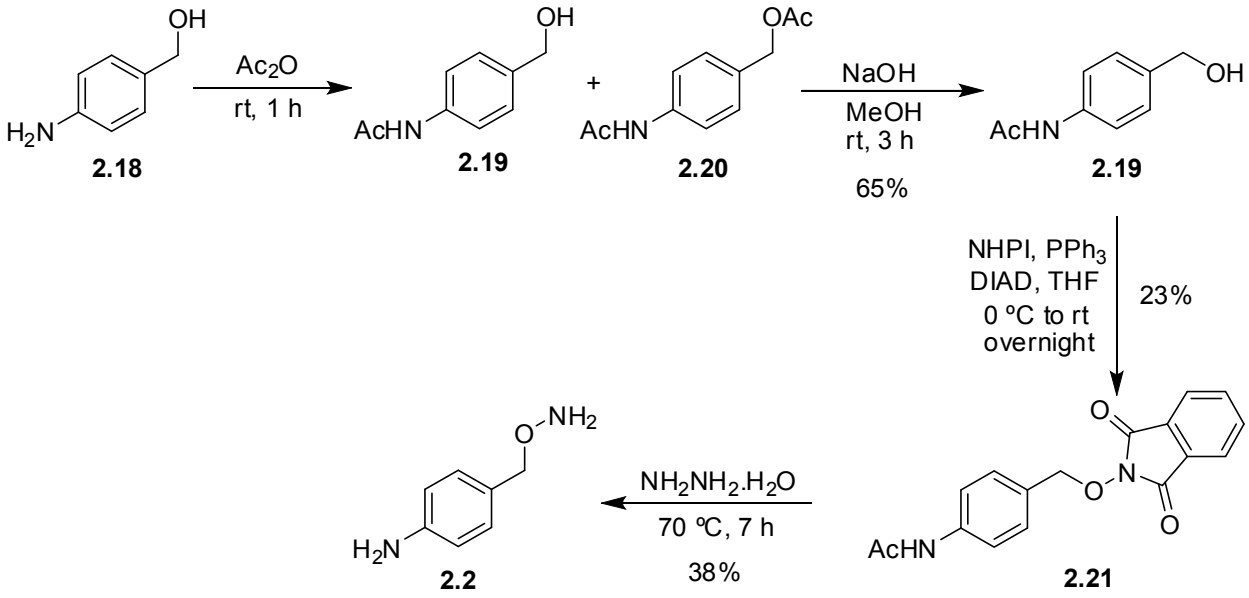


Scheme 2.9 Synthesis of 2-[(aminooxy)methyl]-benzenamine **2.1**.

2.3 Synthesis of 4-[(aminooxy)methyl]-benzenamine

We next turned our attention to the isomer of compound **2.1**, **2.2**, which would be an important control for the investigation of the reaction mechanism. Mitsunobu reaction of compound **2.18** resulted in a complex mixture (as indicated by TLC). With the hope of achieving a cleaner and more efficient reaction, the amino group was protected as an amide by treatment with acetic anhydride. However, product **2.20** was also isolated along with the desired **2.19**. This

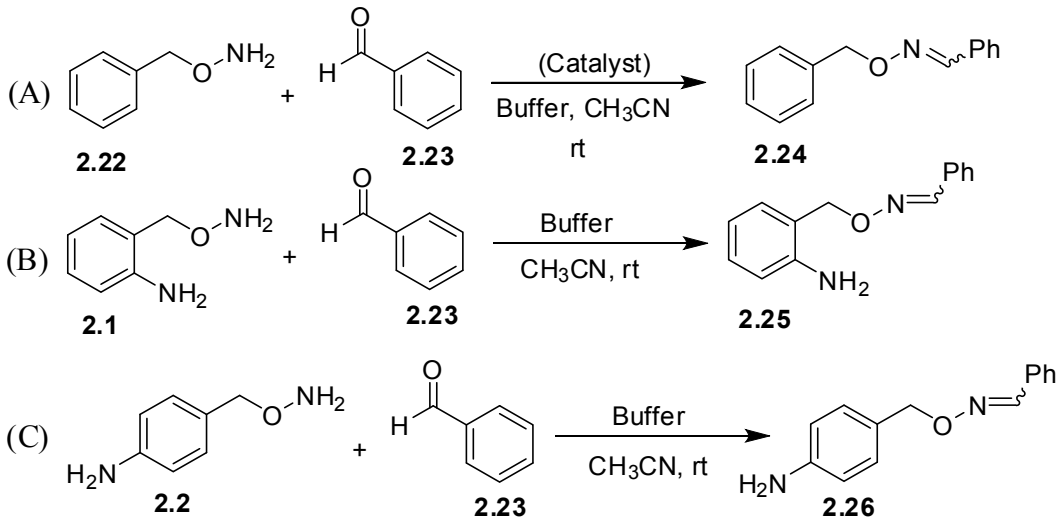
reaction mixture, without further purification, was treated with NaOH in MeOH to allow the deprotection of the O-acetate group present in **2.20**. After these two steps, compound **2.19** was obtained in 65% combined yield. To attain the desired final compound **2.2**, compound **2.21** was generated via a Mitsunobu reaction[53] followed by deprotection with hydrazine to obtain the desired target.[47] The product was again purified by preparative TLC (Scheme **2.10**).



Scheme 2.10 Synthesis of 4-[(aminooxy)methyl]-benzenamine **2.2**.

2.4 Kinetic studies of ligation reagents

With compounds **2.1** and **2.2** in hand, we sought to test the rates of reaction of each compound in oxime formation (Scheme **2.11**).



Scheme 2.11 Schematic representation of ligation reactions, studied by HPLC (A) in presence or absence of different catalysts, (B) with compound **2.1**, and (C) with compound **2.2**.

We used three different catalysts for oxime formation to compare their rate with the rate of compound **2.1** and **2.2**. The list of the compounds used in this study is shown in Figure **2.2**.

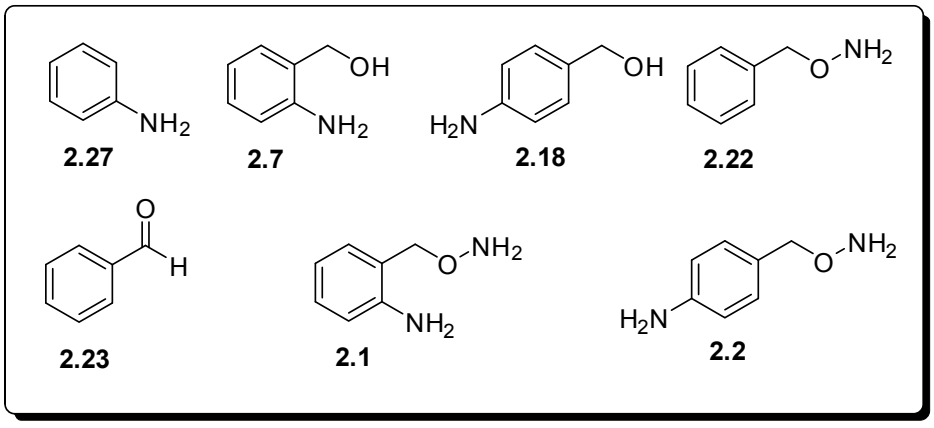
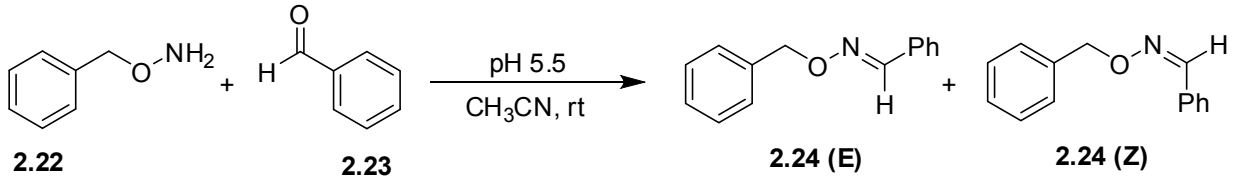


Figure 2.2 Compounds used in oxime formation kinetic study.

We employed an HPLC-based assay for following the rate of reaction, using benzaldehyde **2.23** as the electrophilic partner. To provide points of comparison, we needed to test the uncatalyzed reaction, using *o*-benzylhydroxylamine **2.22** as the nucleophile; as well as the aniline-catalyzed reactions, again with **2.22** as the nucleophile. Uncatalyzed and aniline-catalyzed ligation reaction would form **2.24**. We hoped that compounds **2.1** or **2.2** would form the desired oxime product **2.25** or **2.26** in the absence of catalyst, but sought to determine if the rate of the reaction was improved for the bifunctional oxime ligation reagents. Reactions were carried out with 1 mM of the electrophile (defined as 1 equiv below) at room temperature. The medium used for the reaction was an acetate buffered solution (0.1 M) at the pH indicated. Rates of reaction were compared based on relative k_{obs} rates (see Experimental methods).

In this HPLC-based assay we used an isocratic condition (65% acetonitrile and 35% water) for analysis of all 1 mM and 2 mM oxime ligation reactions. For reactions at 10 mM (aniline-catalyzed and uncatalyzed reactions) we used the same condition. However, for 10 mM reactions with compounds **2.1** and **2.2** we used an isocratic condition with 40% acetonitrile and 60% water. Each time point was acquired with a 0 μL injection of the reaction mixture using a 25 μL glass syringe. We measured the oxime product formation (**2.24**, **2.25**, and **2.26**) with time. The ligation products were quantitated by integration of the absorbance signal at 254 nm.



Scheme 2.12 Oxime ligation between **2.23** (1 mM) and **2.22** (1 mM) at pH 5.5.

HPLC chromatograms of oxime ligation between **2.23** (1 mM) and **2.22** (1 mM) at pH 5.5 over times (Scheme **2.12**) are shown in Figure **2.3**. Retention times for starting materials **2.22** and **2.23** are 1.99 min and 2.31 min respectively. Oxime products are less polar than both the starting materials. Retention times for **2.24 (Z)** and **2.24 (E)** are 3.91 min and 4.37 min respectively. All the peaks were well separated.

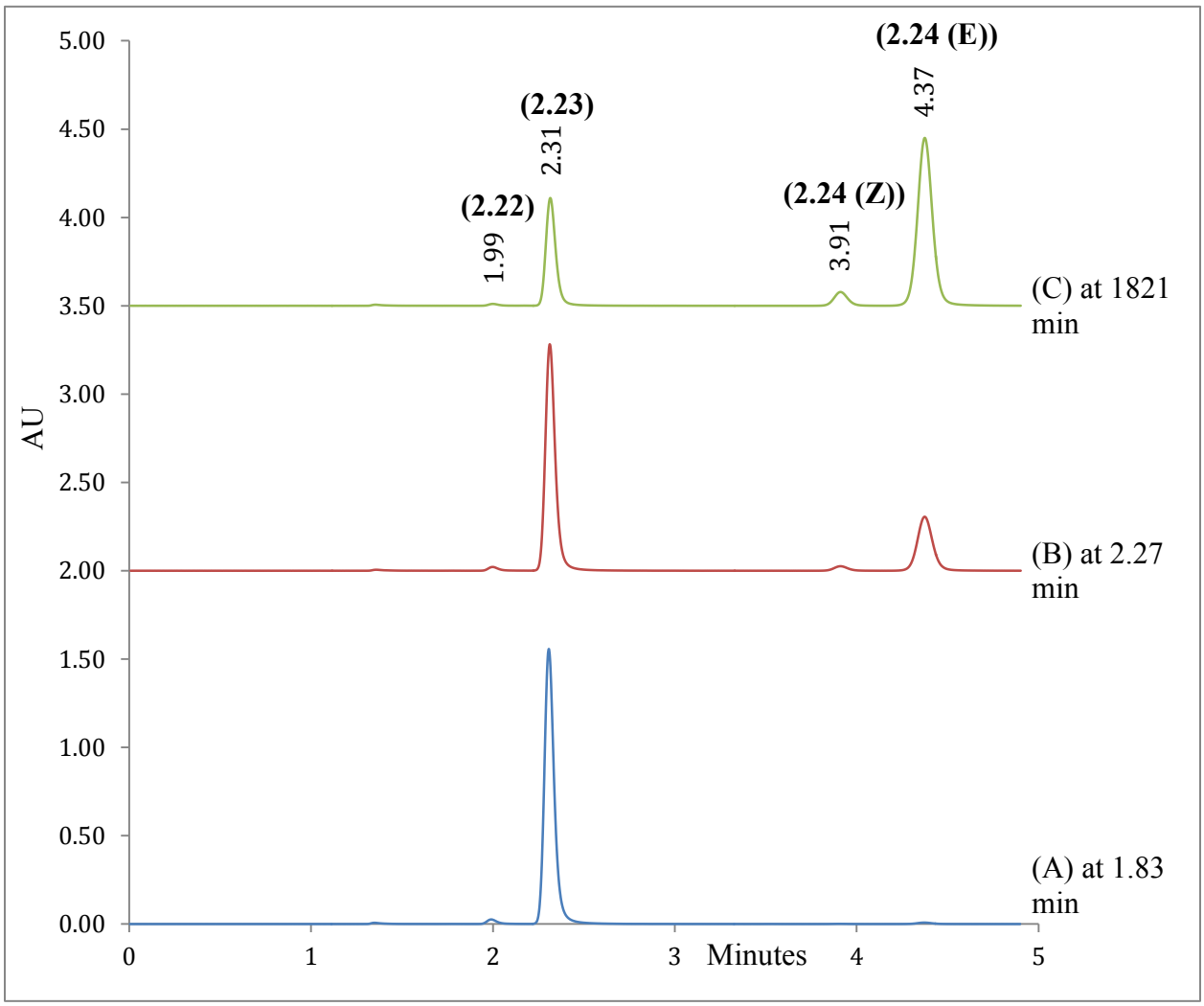


Figure 2.3 HPLC chromatograms of oxime ligation between **2.23** (1 mM) and **2.22** (1 mM) at pH 5.5 at the indicated times.

We found out that oxime ligations produced two isomers (E and Z) of the product. In all of these cases, the E isomer was formed in large excess relative to the Z isomer (likely due to the E isomers increased stability). We also found out that rate of formation of E isomer was much faster than that of Z isomer. These results are summarized in Table **2.2**. E and Z isomer were observed in 12:1, 19:1, and 13:1 ratios for **2.22**, **2.1**, and **2.2** respectively. However, column chromatography and preparative TLC purification of the oxime products showed different ratios for E and Z (see Experimental methods).

Table 2.2 Observed second-order rate constants k_{obs} (M^{-1} min^{-1}) for the formation of E and Z isomers of oxime at different pH 5.5 and the concentration of catalyst and nucleophiles are 1 mM.

Nucleophile	Catalyst	k_{obs} (M^{-1} min^{-1})	Isomer
2.22	-	1.32 ± 0.06	E
2.22	-	0.03 ± 0.00	Z
2.22	**2.27**	2.66 ± 0.04	E
2.22	**2.27**	0.03 ± 0.01	Z
2.1	-	1.92 ± 0.11	E
2.1	-	0.02 ± 0.00	Z
2.2	-	5.7 ± 0.3	E
2.2	-	0.04 ± 0.01	Z

As the formation of Z isomer is substantially less than that of the E isomer, in the rest of this thesis we calculated the k_{obs} values by considering both the isomers as one product.

We first screened the rate of oxime formation over a wide range of pH values from 6.5 down to 4.5 (Table **2.3**). Compound **2.1** showed faster ligation rates (k_{obs}) at pH 4.5, 5.5 and 5.7 than the uncatalyzed reaction; whereas, aniline showed the best ligation rates at all tested pH values. We were encouraged that, at pH 5.5, the ligation rate for compound **2.1** showed a substantial increase from the uncatalyzed ligation (1.5-fold). We chose to examine reactions at pH 5.5 and at pH 5.7 in further studies.

Table 2.3 Observed second-order rate constants k_{obs} (M^{-1} min^{-1}) for oxime formation at different pH.

Catalyst/amino-oxy compound	pH 4.5	Rel. k_{obs}	pH 5.3	Rel. k_{obs}	pH 5.5	Rel. k_{obs}	pH 5.7	Rel. k_{obs}	pH 6.5	Rel. k_{obs}
No catalyst	7.2 ± 0.1	1.0	0.64 ± 0.01	1.0	1.53 ± 0.03	1.0	0.63 ± 0.03	1.0	0.41 ± 0.01	1.0
Aniline (1 mM)	11.90 ± 0.08	1.7	0.82 ± 0.01	1.3	2.85 ± 0.06	1.9	0.75 ± 0.05	1.2	0.59 ± 0.01	1.4
Compound **2.1** (1 mM)	8.1 ± 0.1	1.1	0.69 ± 0.00	1.1	2.26 ± 0.07	1.5	0.67 ± 0.02	1.1	0.36 ± 0.01	0.9

Interestingly, we observed that in the pH range of 5.5-5.7, compound **2.2** showed the fastest ligation rates. The second order rate constants for **2.2** were approximately 2-fold (k_{obs} =

1.14 ± 0.04 M^{-1} min^{-1}) and 5-fold (k_{obs} = 6.66 ± 0.04 M^{-1} min^{-1}) faster relative to uncatalyzed ligation at pH 5.7 and pH 5.5, respectively at 1 mM concentration. Previous studies have examined the rate of oxime ligations with varying concentration of aniline and aminoxy nucleophiles.[8] We found that 2 mM aniline resulted in enhanced rates of reaction of between 1.3- and 1.6-fold over uncatalyzed reactions at pH 5.7 and 5.5, respectively (Table **2.4**). At 10 equivalents of aniline, the reaction rate at pH 5.7 was 1.5-fold faster than the uncatalyzed reaction. Compound **2.1** gave comparable rates of reaction when compared to identical concentrations of aniline at both pH 5.5 and 5.7, but none of these showed an enhancement over aniline itself. In contrast, compound **2.2** gave substantial rate enhancements over aniline at all concentrations tested and at both pH ranges. The best condition was found to be pH 5.5, with 2 equivalents of compound **2.2** which gave a 3-fold rate enhancement over the uncatalyzed reaction and a 2-fold enhancement over the same concentration of aniline (Table **2.4**).

Table 2.4 Second-order rate constants k_{obs} (M^{-1} min^{-1}) for different nucleophiles and catalysts at pH 5.5 and 5.7.

pH	Nucleophile	Nucleophile Conc^{n} (mM)	Catalyst	Catalyst Conc^{n} (mM)	k_{obs} (M^{-1} min^{-1})	Rel. to uncat. rxn.
5.5	**2.22**	2	-	-	4.6 ± 0.2	1.0
	2.22	2	**2.27**	2	7.4 ± 0.5	1.6
	2.22	2	**2.7**	2	7.9 ± 0.5	1.7
	2.22	2	**2.18**	2	10.02 ± 0.69	2.2
	2.1	2	-	-	6.4 ± 0.2	1.4
	2.2	2	-	-	14.0 ± 0.7	3.0
5.7	**2.22**	2	-	-	0.99 ± 0.10	1.0
	2.22	2	**2.27**	2	1.3 ± 0.1	1.3
	2.22	2	**2.7**	2	0.87 ± 0.03	0.9
	2.22	2	**2.18**	2	1.16 ± 0.06	0.9
	2.1	2	-	-	1.12 ± 0.07	0.9
	2.2	2	-	-	1.7 ± 0.1	1.3
	2.22	10	-	-	2.03 ± 0.15	1.0
	2.22	10	**2.27**	10	3.1 ± 0.3	1.5
	2.1	10	-	-	2.3 ± 0.2	1.1
	2.2	10	-	-	5.4 ± 0.5	2.7

To provide some insight into the increased rates observed for compound **2.2**, we tested *o*- and *p*-amino-methanol compounds **2.7** and **2.18** in the same assay. Although these compounds should have similar electronic effects on the aniline nucleophile, they cannot form the oxime product. Benzylic alcohols **2.7** and **2.18** were both active in promoting the rate of oxime formation with activities over the aniline catalyst of as much as 1.4-fold (Table **2.4**).

2.5 Discussion

Our aim was to incorporate aniline and the aminoxy group onto a single scaffold to construct a bifunctional ligation reagent for oxime ligation. We thought if the intramolecular pathway were operative, this may also increase the rate of reaction. Ideally, this strategy would eliminate the need for any external catalyst or additional reagents.

We found out from our kinetic study that **2.1** was able to increase the ligation rate when compared to an uncatalyzed reaction. Additionally, ligation rates were comparable for aniline and **2.1**. Interestingly, **2.2** increased the ligation rate compare to both uncatalyzed and aniline catalyzed reaction. Most importantly, in all of the cases we obtained the desired oxime target at accelerated rates without using the need for an external catalyst.

These results led us to conclude that the intermolecular pathway of the ligation is most likely the major pathway. Our kinetic results suggest that enhancement observed for the bi-functional oxime ligation reagent, compound **2.2**, is due to electron donation by the benzylic aminooxy functional group. We suspect that the benzylic aminooxy group's electron withdrawing effect on the aromatic ring is responsible for increased basicity of the corresponding Schiff base, thus increasing the reaction rate.[16]

Our kinetic data also suggest that the *ortho* arrangement of the amino and aminooxy groups found in compound **2.1** reduces the reactivity of this compound due to steric crowding of one or both nucleophilic sites.

We found that the **2.2** was a substantially improved catalyst as compared to aniline, showing an approximately 2–3-fold increase in the k_{obs} (at 2 mM) relative to uncatalyzed and aniline catalyzed ligations, respectively. Dawson and co-workers showed a 12-fold greater

kinetic rate using 10 mM aniline at pH 4.5 on a peptide.[8] The same group also demonstrated *p*-PDA can act as catalyst and can improve the ligation rate 19-fold over aniline using 10 mM catalyst at pH 7.0 during protein PEGylation.[26] Distifeno and co-workers achieved 2.5-fold faster oxime ligation over aniline, using 100 mM *m*-PDA as catalyst at pH 7.3 on GFP-CVIA protein.[16] Kool and co-workers found that 5-methyl-2-aminobenzenephosphonic acid can improve the hydrazone formation rate 8-fold over aniline using 1mM of catalyst at pH 7.4.[14] The same group also obtained ligation rate enhancement by choosing appropriate carbonyl and hydrazine substrate structure at biological pH without using any catalyst. However, Kool and coworkers used a large excess (50-55 times) of one component, which is not appropriate in case of very costly materials or even *in vivo* reaction.[18,19]

Although our bifunctional aminoxy compounds did not show a very large increases in rate, they illustrate that the combined functionality of a nucleophilic catalyst and a reactive nucleophile on the same scaffold can result in improved rates over uncatalyzed reactions. Importantly, this strategy has allowed us to achieve accelerated rates with a stoichiometric amount of the nucleophilic catalyst alone.

2.6 Conclusions

In this study we found out that bifunctional aminooxy compounds can be effective for oxime ligation. Ligation rates of **2.1** were comparable to that of aniline, while **2.2** showed enhanced ligation rates relative to aniline. Both of these bifunctional compounds exhibited better rates than that of uncatalyzed ligation reaction. Our results also allow us to conclude that both **2.1** and **2.2** operate via intermolecular pathway to form an oxime product. We suspect the lower

ligation rate of **2.1** is due to steric hindrance of the ortho group. Most importantly, our results show that we can diminish the use of catalyst by employing bifunctional aminooxy regents for oxime ligation. Future applications will test this strategy on biomolecules.

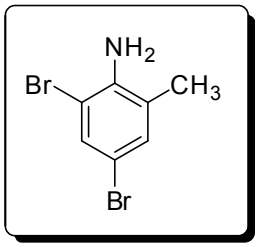
2.7 Experimental methods

2.7.1 General

All reagents were purchased from commercial sources and used without purification unless otherwise stated. Anhydrous solvents used in the reaction were purified by successive passage through columns of alumina and copper under argon. If reactions were run under an inert environment using anhydrous solvents, it is stated. The reactions were monitored by analytical thin layer chromatography (TLC) using silica gel (60-F_{254}, 0.25 mm, Silicycle, Quebec, Canada) as a medium and the spots were visualized under ultraviolet light (254 nm) or stained by charring with potassium permanganate solution. Organic solvents were evaporated under reduced pressure, and the products purified by column chromatography are indicated using silica gel (230-400 mesh, Silicycle, Quebec, Canada). All NMR spectra were obtained using Varian instruments. ^{1}H NMR spectra were performed at 400, 500 or 600 MHz at room temperature as indicated. ^{13}C NMR spectra were performed at 125 MHz. Electrospray mass spectra were recorded on Agilent Technologies 6220 TOF using CH_2Cl_2, MeOH or H_2O as solvent with added NaCl. All the ligation reactions were analyzed on a high pressure liquid chromatography (Waters® XTerra®) system coupled to an UV-Vis detector probing at 254 nm using a RP C18 analytical column, particle size: 18.3 μm, column dimensions: 4.8*150 mm.

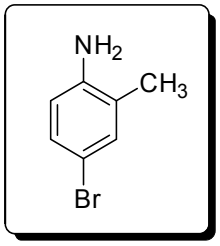
2.7.2 Synthetic methods

2,4-Dibromo-6-methyl-benzenamine (2.9)



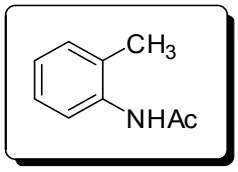
1, 2-Dichloroethane (4 mL) was added to a flask charged with NBS (356 mg, 2 mmol). O-Toluidine (214 μL, 0.2 mmol) and benzoyl peroxide (4 mg) were added to the reaction mixture. The color of the reaction mixture turned from clear to pink, and was refluxed overnight. The progress of the reaction was monitored by TLC (30% ethyl acetate/hexanes). Solvent was evaporated by under reduced pressure. The crude reaction mixture was purified by column chromatography to provide compound **2.9** (28.5 mg, 0.09 mmol, 45% yield) as a reddish solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.45 (s, 1H, ArH), 7.14 (s, 1H,ArH), 4.07 (brs, 2H, NH_2), 2.19 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 4 .6 (ArC-NH_2), 132.1 (ArC-H), 132.0 (ArC-H), 125.0 (ArC-CH_3), 109.5 (ArC-Br), 109.2 (ArC-Br), 18.2 (CH_3). EI HRMS calcd. for C_7H_7NBrBr (M+) 264.8925, found 264.8916.

4-Bromo-2-methyl-benzenamine (2.10)



Compound **2.10** was isolated as a byproduct in the synthesis of compound **2.9** discussed above, as a greenish liquid (12.3 mg, 0.066, 33% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.20 (d, *J* = 2.5 Hz, 1H, ArH), 7.15 (dd, *J* = 8.5, 2.5 Hz, 1H, ArH), 6.58 (d, *J* = 8.0 Hz, 1H, ArH), 2.17 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 43. 6 (ArC-NH_2), 132.9 (ArC-H), 129.6 (ArC-H), 124.4 (ArC-CH_3), 116.3 (ArC-H), 110.1 (ArC-Br), 17.2 (CH_3). EI HRMS calcd. for C_7H_8NBr (M+) 186.9820, found 186.9824.

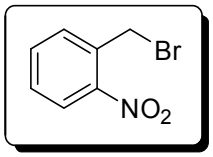
N-(2-methylphenyl)-acetamide (2.11)



Acetic acid (4 mL) was added to o-toluidine **2.8** (1 g, 9.4 mmol) at room temperature. The reaction mixture was heated to 115 °C for 12 h. Progress of the reaction was monitored by TLC (30% ethyl acetate/hexane). The acid was evaporated by using reduced pressure. The crude reaction mixture was purified by column chromatography to provide compound **2.11** (1.2 g, 8.04

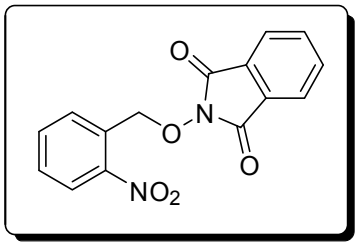
mmol, 86% yield) as a pale pink solid. Spectral data for this compound matched literature reports.[45]

2-Nitrobenzyl bromide (2.14)



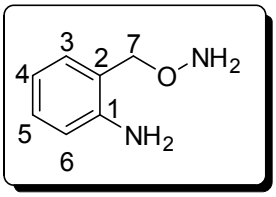
o-Toluidene **2.15** (50 mg, 0.37 mmol), N-bromosuccinimide (65 mg, 0.37 mmol) and benzoyl peroxide (1.0 mg) were dissolved in 1,2-DCE (0.8 mL) and heated under reflux for 3 days. Although the reaction did not go to completion, the mixture was reduced and compound **2.14** (30 mg, 0.14 mmol, 38%, yield) was isolated by flash chromatography. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 8.08 (d, J = 8.5 Hz, 1H, ArH), 7.67-7.61 (m, 2H, ArH), 7.55-7.51 (m, 1H, ArH), 5.89 (s, 2H, CH_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 48. (ArC-NO_2), 133.7 (ArC-H), 132.9 (ArC-CH_2Br), 132.6 (ArC-H), 129.6 (ArC-H), 125.5 (ArC-H), 28.8 (CH_2). EI HRMS calcd. for $C_7H_6NO_2$ 136.0400, found 136.0403; calcd. for C_7H_5O 105.0340, found 105.0344; calcd. for C_6H_6N 92.0500, found 92.0495.

2-(2-Nitrobenzyloxy)isoindoline-1,3-dione (2.13)



Potassium carbonate (45 mg, 0.32 mmol) was added to a solution of N-hydroxyphthalimide (34.7 mg, 0.21 mmol) in DMF (10 mL), causing the appearance of a reddish brown color. 2-Nitrobenzyl bromide **2.14** (46 mg, 0.21 mmol) was then added to the solution, followed by heating to 50 °C under a cold water condenser. After 3 h, the solution became a clear yellow and was evaporated and then diluted with dichloromethane. The reaction mixture was washed with water (3X10 mL), sodium bicarbonate (2X10 mL) and again with water (2X10 mL). The organic layer was dried over Na_2SO_4, filtered through a plug of cotton, and concentrated under reduced pressure. The crude product was purified by recrystallization using dichloromethane as solvent to afford **2.13** (40 mg, 0.13 mmol, 63% yield) as a crystalline yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 8. 8 (dd, *J* = 8.5, 1.0 Hz, 1H, ArH), 8.05 (dd, *J* = 7.8, 1.0 Hz, 1H, ArH), 7.89-7.86 (m, 2H, ArH), 7.82-7.78 (m, 2H, ArH), 7.77-7.74 (m, 1H, ArH), 7.59-7.56 (m, 1H, ArH), 5.69 (s, 2H, CH_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 63.3 (CO), 147.5 (ArC-NO_2), 134.7 (ArC-H), 133.9 (ArC-H), 130.8 (ArC-CO), 129.9 (ArC-H), 129.3 (ArC-H), 128.8 (ArC-CH_2O), 125.0 (ArC-H), 123.7 (ArC-H), 75.9 (CH_2). ESI HRMS calcd. for $C_{15}H_{10}N_2NaO_5$ (M+Na)+ 321.0482, found 321.0489.

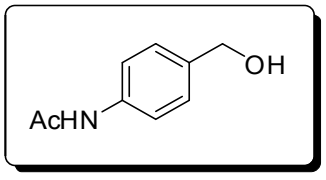
2-[(Aminooxy)methyl]-benzenamine (2.1)



(2-Aminophenyl)methanol **2.7** (300 mg, 2.44 mmol), N-hydroxyphthalimide **2.6** (397.5 mg, 2.44 mmol) and triphenyl phosphine (770 mg, 2.93 mmol) were dissolved in dry THF (5 mL)

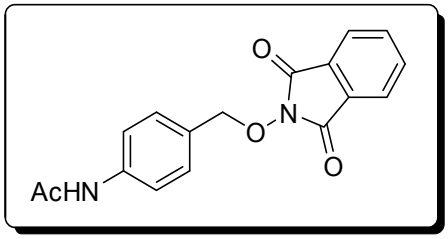
under an argon atmosphere and cooled to 0 °C. A solution of THF (5 mL) and diisopropyl azodicarboxylate (590 mg, 2.93 mmol) was added drop-wise to the mixture. The reaction was stirred for 12 h. After the reaction appeared to be complete by TLC, the solvent was evaporated under reduced pressure and the compound was isolated by flash chromatography. Crude product was dissolved in dichloromethane and hydrazine monohydrate (118.4 μL, 2.44 mmol) was added and stirred for 30 min. The reaction mixture was evaporated and redissolved in dichloromethane and undissolved solids were were filtered through a plug of cotton. The solvent was evaporated to provide 178.5 mg (53%) of crude product. Using preparative TLC, 50 mg of the crude product was purified, and neutralized by the eluent (25% ethyl acetate/dichloromethane with 2% triethylamine). The TLC plate was eluted twice in the same eluent. The desired product was isolated by extraction from silica with ethyl acetate. After vacuum filtration, the solvent was evaporated under reduced pressure to provide **2.1**(11.7 mg, 0.085 mmol, 12% yield) as a pale white solid. FTIR (neat) 3442 (NH_2), 3360 (NH_2), 3234 (NH_2), 3025 (ArC-H), 2918 (Alkyl C-H), 2850 (Alkyl C-H), 1622 (N-H), 1282 (C-N), 1204 (C-O), 984 (O-N) cm^{-1}; ^1H NMR (600 MHz, D_2O) δ = 7. 5 -7.10 (m, 2H, H_5, H_3), 6.77-6.73 (m, 2H, H_6, H_4), 4.61 (s, 2H, H_7). ^{13}C NMR (125 MHz, D_2O) δ = 4 6.6 (C_1), 132.2 (C_3), 131.2 (C_5), 122.4 (C_2), 120.3 (C_4), 118.0 (C_6), 76.5 (C_7). ESI HRMS calcd. for $C_7H_{11}N_2O$ (M+H)+ 139.0866, found 139.0863.

***N*-[4-(Hydroxymethyl)phenyl]-acetamide (2.19)**



(4-Aminophenyl)methanol **2.18** (400 mg, 3.25 mmol) was dissolved in acetic anhydride (10 mL) and stirred for 1 h. After completion (as observed by TLC), the solvent was evaporated under reduced pressure and dissolved in methanol (5 mL). Sodium hydroxide (97.7 mg, 2.4 mmol) was added followed by stirring for 3 h. The solvent was evaporated and the crude reaction mixture was purified by column chromatography to give **2.19** (350 mg, 2.12 mmol, 65% yield) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ = 7.50-7.47 (m, 2H, ArH), 7.28-7.25 (m, *J* = 2H, ArH), 4.52 (s, 2H, CH_2), 2.09 (s, 3H, CH_3). ^{13}C NMR (125 MHz, CD_3OD) δ = 7 .6 (CO), 139.0 (ArC-CH_2OH), 138.5 (ArC-NH), 128.6 (ArC-H), 121.1 (ArC-H), 64.9 (CH_2), 23.8 (CH_3). ESI HRMS calcd. for $C_9H_{11}NNaO$ (M+Na)+ 188.0682, found 188.0684.

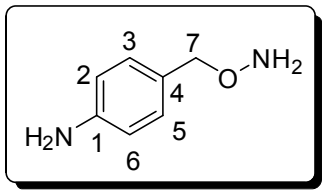
***N*-[4-[[(1,3-dihydro-1,3-dioxo-2*H*-isoindol-2-yl)oxy]methyl]phenyl]-acetamide (2.21)**



N-[4-(Hydroxymethyl)phenyl]-acetamide **2.19** (350 mg, 2.12 mmol), *N*-Hydroxyphthalimide **2.6** (346 mg, 2.12 mmol) and triphenyl phosphine (671 mg, 2.55 mmol) were dissolved in dry THF (20 mL) under argon atmosphere and cooled to 0 °C. A solution of diisopropyl azodicarboxylate (503 µL, 2.55 mmol) in THF (10 mL) was added drop-wise to this mixture. The reaction was stirred for 12 h. The solvent was evaporated and the crude reaction mixture was purified by column chromatography to give **2.21** (153 mg, 0.49 mmol, 23% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ = 0.0 (s, N) , 7.84-7.82 (m, 4H, ArH), 7.59-7.56 (m, 2H, ArH), 7.40-7.38 (m, 2H,

ArH), 5.07 (s, 2H, CH_2), 2.02 (s, 3H, CH_3). ^{13}C NMR (125 MHz, DMSO-d_6) δ = 68.9 ($NHCOCH_3$), 163.6 (Ar-CO), 140.5 (ArC-CH_2), 135.3 (ArC-NH), 131.0 (ArC-CON), 129.0 (ArC-H), 128.9 (ArC-H), 123.7 (ArC-H), 119.1 (ArC-H), 79.3 (CH_2), 24.5 (CH_3). ESI HRMS calcd. for $C_{17}H_{14}N_2KO_4$ (M+K)+ 349.0585, found 349.0584.

4-[(Aminooxy)methyl]-benzenamine (2.2)



Hydrazine monohydrate (5 mL) was added to compound **2.21** (135 mg, 0.435 mmol) and stirred for 6 h at 70 °C. The reaction mixture was reduced and redissolved in dichloromethane, and undissolved solids were filtered through a plug of cotton. The crude reaction mixture was purified using preparative TLC after neutralization by the eluent (25% ethyl acetate/ dichloromethane with 2% triethylamine). The TLC plate was eluted twice in the same eluent. The desired product was isolated by treating silica with ethyl acetate. After vacuum filtration, the solvent was evaporated under reduced pressure to provide **2.2** (22.8 mg, 0.165 mmol, 38% yield) as a yellowish white solid. FTIR (neat) 3425 (NH_2), 3353 (NH_2), 3224 (NH_2), 3032 (ArC-H), 2916 (Alkyl C-H), 2852 (Alkyl C-H), 1612 (N-H), 1284 (C-N), 1177 (C-O), 988 (O-N) cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ = 7.20-7.17 (m, 2H, H_2, H_6), 6.70-6.68 (m, 2H, H_3, H_5), 4.60 (s, 2H, H_7). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 46.5 (C_1), 130.2 (C_3, C_5), 127.0 (C_4), 115.0 (C_2, C_6), 78.0 (C_7). ESI HRMS calcd. for $C_7H_{11}N_2O$ (M+H)+ 139.0866, found 139.0865.

2.7.3 Kinetic analysis of oxime ligation rates

We used an isocratic condition (65% acetonitrile and 35% water) for analysis of all 1 mM and 2 mM oxime ligation reactions. For reactions at 10 mM (aniline catalyzed and uncatalyzed reactions) we used the same condition. However, for 10 mM reactions with compounds **2.1** and **2.2** we used an isocratic condition with 40% acetonitrile and 60% water. Each time point was acquired with a 10 μL injection of the reaction mixture using a 25 μL glass syringe. All the ligation reactions were analyzed on a high pressure liquid chromatography (Waters® XTerra®) system coupled to an UV-Vis detector probing at 254 nm using a RP C18 analytical column, particle size: 18.3 μm, column dimensions: 4.8*150 mm and the volume of the injector loop was 20 μL.

2.7.4 1 mM Oxime ligation at pH 5.5

For the ligation reaction performed in the absence of aniline, 00 μL of benzaldehyde **2.23** solution in acetonitrile (4 mM) was added to a mixture of 00 μL of benzylhydroxylamin **2.22** solution in acetonitrile (4 mM) with 120 μL of acetonitrile and 80 μL of 0. M ammonium acetate buffer (pH 5.5). The final concentrations of both benzaldehyde and **2.22** were 1 mM.

For the ligation reactions performed in the presence of aniline, 00 μL of benzaldehyde solution in acetonitrile (4 mM) was added to a mixture of 00 μL of aniline solution in acetonitrile (4 mM), 00 μL of benzylhydroxylamine **2.22** solution in acetonitrile (4 mM), and 20 μL of acetonitrile and 80 μL of 0. M ammonium acetate buffer (p 5.5). The final concentrations of all three components (benzaldehyde, **2.22** and aniline) were 1 mM.

For the ligation reactions performed in the presence of **2.1**, 00 μL of benzaldehyde solution in acetonitrile (4 mM) was added to a mixture of 25 μL of **2.1** solution in acetonitrile (16 mM), 95 μL of acetonitrile, and 80 μL of 0. M ammonium acetate buffer (p 5.5).The final concentrations of both benzaldehyde and **2.1** were 1 mM.

For the ligation reactions performed in the presence of **2.2**, 00 μL of benzaldehyde solution in acetonitrile (4 mM) was added to a mixture of 25 μL of **2.2** solution in acetonitrile (16 mM), 95 μL of acetonitrile, and 80 μL of 0. M ammonium acetate buffer (p 5.5). The final concentration of both benzaldehyde and **2.2** were 1 mM.

2.7.5 2 mM Oxime ligation at pH 5.5

For the ligation reaction performed in the absence of aniline, 00 μL of benzaldehyde solution in acetonitrile (4 mM) was added to a mixture of 00 μL of benzylhydroxylamine **2.22** solution in acetonitrile (8 mM), 20 μL of acetonitrile and 80 μL of 0. M ammonium acetate buffer (pH 5.5). The final concentrations of benzaldehyde and **2.22** were 1 mM and 2 mM respectively.

For the ligation reactions performed in the presence of aniline, 00 μL of benzaldehyde solution in acetonitrile (4 mM) was added to a mixture of 00 μL of aniline solution in acetonitrile (8 mM), 00 μL of benzylhydroxylamine **2.22** solution in acetonitrile (8 mM), and 20 μL of acetonitrile and 80 μL of 0. M ammonium acetate buffer (p 5.5). The final concentrations of benzaldehyde, **2.22** and aniline were 1 mM, 2 mM, and 2 mM respectively.

For the ligation reactions performed in the presence of **2.1**, 00 μL of benzaldehyde solution in acetonitrile (4 mM) was added to a mixture of 50 μL of **2.1** solution in acetonitrile (16

mM), 70 μL of acetonitrile and 80 μL of 0. M ammonium acetate buffer (p 5.5). The final concentrations of benzaldehyde and **2.1** were 1 mM and 2 mM respectively.

For the ligation reactions performed in the presence of **2.2**, 00 μL of benzaldehyde solution in acetonitrile (4 mM) was added to a mixture of 50 μL of **2.2** solution in acetonitrile (16 mM), 70 μL of acetonitrile and 80 μL of 0. M ammonium acetate buffer (p 5.5). The final concentrations of benzaldehyde and **2.2** were 1 mM and 2 mM respectively.

2.7.6 10 mM Oxime ligation at pH 5.7

For the ligation reaction performed in the absence of aniline, 00 μL of benzaldehyde solution in acetonitrile (4 mM) was added to a mixture of 00 μL of benzylhydroxylamine **2.22** solution in acetonitrile (40 mM), and 20 μL of acetonitrile and 80 μL of 0. M ammonium acetate buffer (pH 5.7). The final concentrations of benzaldehyde and **2.22** were 1 mM and 10 mM respectively.

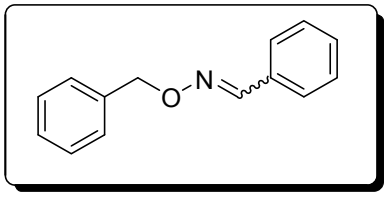
For the ligation reactions performed in the presence of aniline, 00 μL of benzaldehyde solution in acetonitrile (4 mM) was added to a mixture of 00 μL of aniline solution in acetonitrile (40 mM), and 00 μL of benzylhydroxylamine **2.22** solution in acetonitrile (40 mM), 20 μL of acetonitrile and 80 μL of 0. M ammonium acetate buffer (p 5.7). The final concentrations of benzaldehyde, **2.22** and aniline were 1 mM, 10 mM, and 10 mM respectively.

For the ligation reactions performed in the presence of **2.1**, 20 μL of benzaldehyde solution in acetonitrile (20 mM) was added to a mixture of 250 μL of **2.1** solution in acetonitrile (16 mM), 50 μL of acetonitrile, and 80 μL of 0. M ammonium acetate buffer (p 5.7). The final concentrations of benzaldehyde and **2.1** were 1 mM and 10 mM respectively.

For the ligation reactions performed in the presence of **2.2**, 20 μL of benzaldehyde solution in acetonitrile (20 mM) was added to a mixture of 250 μL of **2.2** solution in acetonitrile (16 mM)**,** 50 μL of acetonitrile, and 80 μL of 0. M ammonium acetate buffer (p 5.7). The final concentrations of benzaldehyde and **2.2** were 1 mM and 10 mM respectively.

2.7.7 Characterization of oxime products

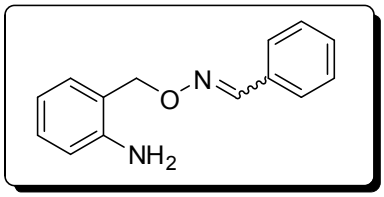
Benzaldehyde *O*-benzyl oxime (2.24)



Ligation product **2.24** was purified by column chromatography. E and Z isomer were observed in 40:1 ratio. Literature report showed that the alkene proton shift for E isomer is higher than that of Z isomer.[54]

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8. 7 (s, , CH), 7.62-7.60 (m, 2H, ArH), 7.46-7.32 (m, 8H, ArH), 5.32 (s, 0.06H, CH_2), 5.24 (s, 2H, CH_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 49. (HC=N), 137.5 (ArC-CN), 132.3 (ArC-CH_2O), 129.9 (ArC-H), 128.7 (ArC-H), 128.5 (ArC-H), 128.4 (ArC-H), 128.0 (ArC-H), 127.1 (ArC-H), 76.4 (CH_2). EI HRMS calcd. for $C_{14}H_{13}ON$ (M+) 211.0997, found 211.0999.

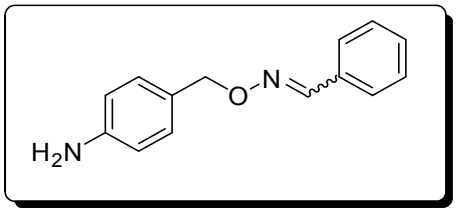
Benzaldehyde *O*-2-aminobenzyl oxime (2.25)



Ligation product **2.25** was purified by preparative TLC. Trace amount of Z isomer found.

^{1}H NMR (500 MHz, $CDCl_3$) δ = 8. 2 (s, , CH), 7.59-7.57 (m, 2H, ArH), 7.40-7.38 (m, 3H, ArH), 7.23 (d, *J* = 7.5 Hz, 1H, ArH), 7.18 (t, *J* = 7.5 Hz, 1H, ArH), 6.78 (t, *J* = 7.5 Hz, 1H, ArH), 6.74 (d, *J* = 8.0 Hz, 1H, ArH), 5.20 (s, 2H, CH_2), 4.19 (brs, 2H, NH_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 49. (HC=N), 146.3 (ArC-NH_2), 132.0 (ArC-CN), 131.2 (ArC-H), 130.0 (ArC-H), 129.9 (ArC-H), 128.7 (ArC-H), 127.1 (ArC-H), 121.8 (ArC-CH_2O), 118.3 (ArC-H), 116.0 (ArC-H), 74.8 (CH_2). ESI HRMS calcd. for $C_{14}H_{15}ON_2$ (M+H)+ 227.1178, found 227. 1176.

Benzaldehyde *O*-4-aminobenzyl oxime (2.26)



Ligation product **2.26** was purified by preparative TLC. E and Z isomer were observed in 16:1 ratio.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.10 (s, 1H, CH), 7.59-7.56 (m, 2H, Ar-H), 7.38-7.34 (m, 3H, Ar-H), 7.24 (dt, *J* = 8.4, 2.4 Hz, 2H, ArH), 6.68 (dt, *J* = 8.4, 2.4 Hz, 2H, ArH), 5.14 (s, 0.11H, CH_2), 5.09 (s, 2H, CH_2), 3.70 (brs, 2H, NH_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 148.7 (HC=N),

146.4 (ArC-NH_2), 132.4 (ArC-CN), 130.3 (ArC-CH_2O), 129.7 (ArC-H), 128.6 (ArC-H), 127.2 (ArC-H), 127.0 (ArC-H), 115.0 (ArC-H), 76.5 (CH_2). ESI HRMS calcd. for $C_{14}H_{15}ON_2$ (M+H)+ 227.1178, found 227. 1179.

2.7.8 Fitting of kinetic HPLC data

All the reactions are bimolecular reactions following second order reaction kinetics. The reaction rate was calculated from the disappearance of benzaldehyde (B) in time, by fitting [B] (M) against *t* (min) to equation (1)[8] using Sigma Plot. Unit for second-order rate constant k_{obs} is M^{-1} min^{-1}.

$$[B]_t = \frac{[B]_0}{1+[B]_0 t k_{obs}} \quad (1)$$

2.8 References

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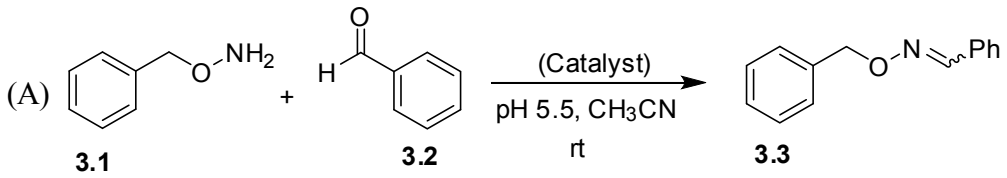
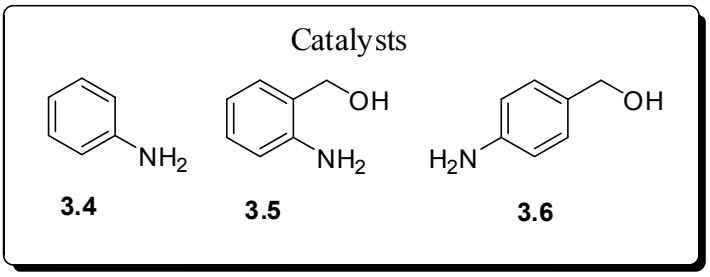
Chapter 3

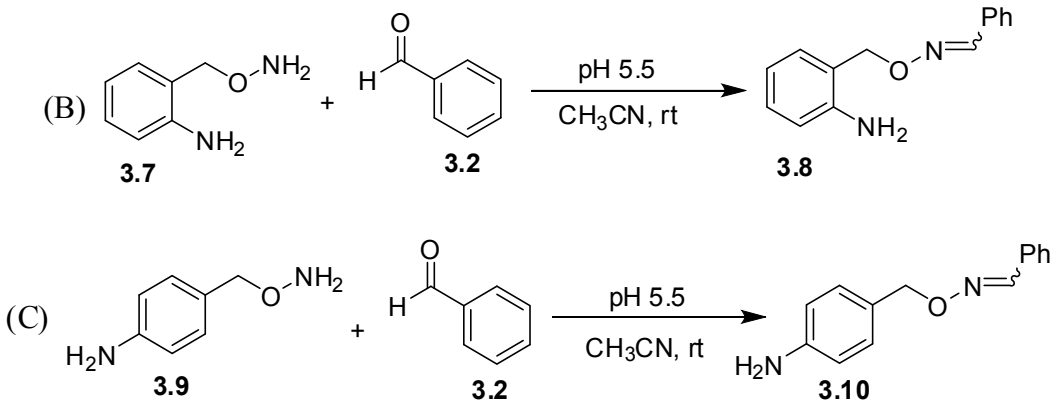
Summary and future directions

3.1 Summary

In this project we designed and synthesized aminoxy compounds that combine the functionality of the nucleophilic catalyst with that of the nucleophile for an oxime ligation. The rate of ligation for the key reagents, 2-[aminooxy)methyl]-benzenamine **3.7**, 4-[aminooxy)methyl]-benzenamine **3.9**, with a benzaldehyde **3.2** substrate were determined and compared to reactions without catalyst, or at various concentrations of aniline. These reagents were confirmed to react in a bimolecular reaction with aldehyde substrates at improved rates relative to the uncatalyzed reaction, confirming the first goal of our study. Although the rate enhancements are somewhat modest, we propose that this study provides validation that ligation reagents can be developed that will not require a separate catalyst for oxime ligation reactions (Scheme **3.1**).







Scheme 3.1 Schematic representation of ligation reactions, studied by HPLC (A) in presence or absence of different catalysts, (B) with compound **3.7**, and (C) with compound **3.9**.

To gain an understanding of the role of the aminooxy moiety, we compared these reactions to those with **3.7** and **3.9**. We found that the **3.9** was a substantially improved catalyst as compared to aniline, showing an approximately 3- and 2-fold increases in the k_{obs} (at 2 mM) relative to uncatalyzed and aniline catalyzed ligation, respectively. These results allowed us to conclude that the ligation pathway for this compound follows an intermolecular pathway and that the enhancements observed for the reaction are due to the electron donating nature of the aminoxy group. On the other hand, steric crowding near the amine group is likely responsible for the reduction on the ligation rate for **3.7** as compared to aniline **3.4**. These observations have been also supported by the study of two aniline derivatives, (2-aminophenyl)methanol **3.5** and (4-aminophenyl)methanol **3.6**, used as catalysts for oxime ligation (Table **3.1**).

Table 3.1 Second-order rate constants k_{obs} (M^{-1} Min^{-1}) for different nucleophiles and catalysts at pH 5.5 and at 2 mM concentrations of catalysts and nucleophiles.

Nucleophile	Catalyst	k_{obs} (M^{-1} Min^{-1})	Rel. k_{obs}
3.1	-	4.6 ± 0.2	1.0
3.1	**3.4**	7.4 ± 0.5	1.6
3.1	**3.5**	7.9 ± 0.5	1.7
3.1	**3.6**	10.02 ± 0.69	2.2
3.7	-	6.4 ± 0.2	1.4
3.9	-	14.0 ± 0.7	3.0

3.2 Future directions

We propose that the results here will be useful for the design of future bioconjugation strategies. Bifunctional aminooxy compounds can be used as a potential reactant in oxime ligation. In this case we do not have to use any additional nucleophilic catalyst. It will reduce the complexity and any potential side reactions in bioconjugation.

It may be possible to improve the rates of reaction for the bifunctional ligation oxime reagents. There are some catalysts reported for oxime or hydrazone formation reaction in the literature.[1-4] We can design a possibly better bifunctional reagent for oxime ligation by applying this idea on those molecules (Figure **3.1**).

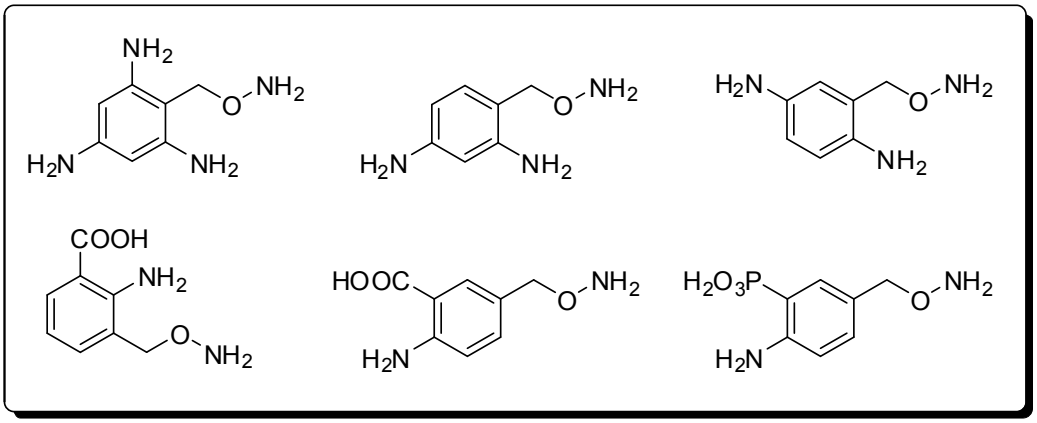
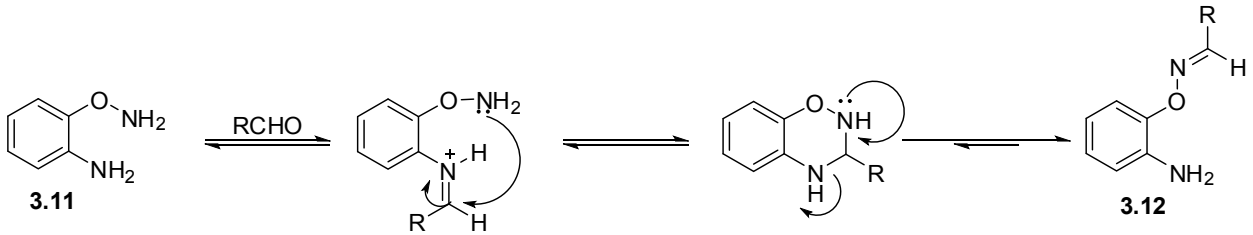


Figure 3.1 Potential bifunctional aminooxy reagents.

Other structure tuning can be yielding faster oxime ligation rate. Also, careful introducing other electron donating group on the aromatic ring could be useful for oxime ligation rate enhancement. These aminoxy derivatives with higher pK_a value will be helpful to form oxime bond at higher or even neutral pH values. Also, intramolecular oxime formation mechanism could be possible if it goes via the formation of six membered ring. We can think about 2-(aminooxy)-benzenamine **3.11** as a potential substrate of this kind (Scheme **3.2**).



Scheme 3.2 Proposed mechanism for the intramolecular oxime formation via 6-*endo*-*trig* cyclilization.

Our yields for the Mitsunobu reactions were low. Reports in the literature have improved stereochemical control of Mitsunobu reactions by performing them at low temperature (-78 °C).[5,6] Therefore, low temperature Mitsunobu reaction conditions may reduce side-reactions.

Any successful bifunctional oxime reagents will become far more useful as trifunctional derivatives that include a handle for further functionalization. Copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) is a well-known reaction for bioconjugation.[7-13] We could take advantage of CuAAC to conjugate the bifunctional aminooxy compound with a corresponding alkyne or azide tag (Figure **3.2**).

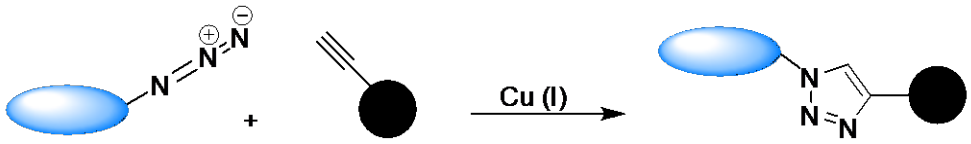
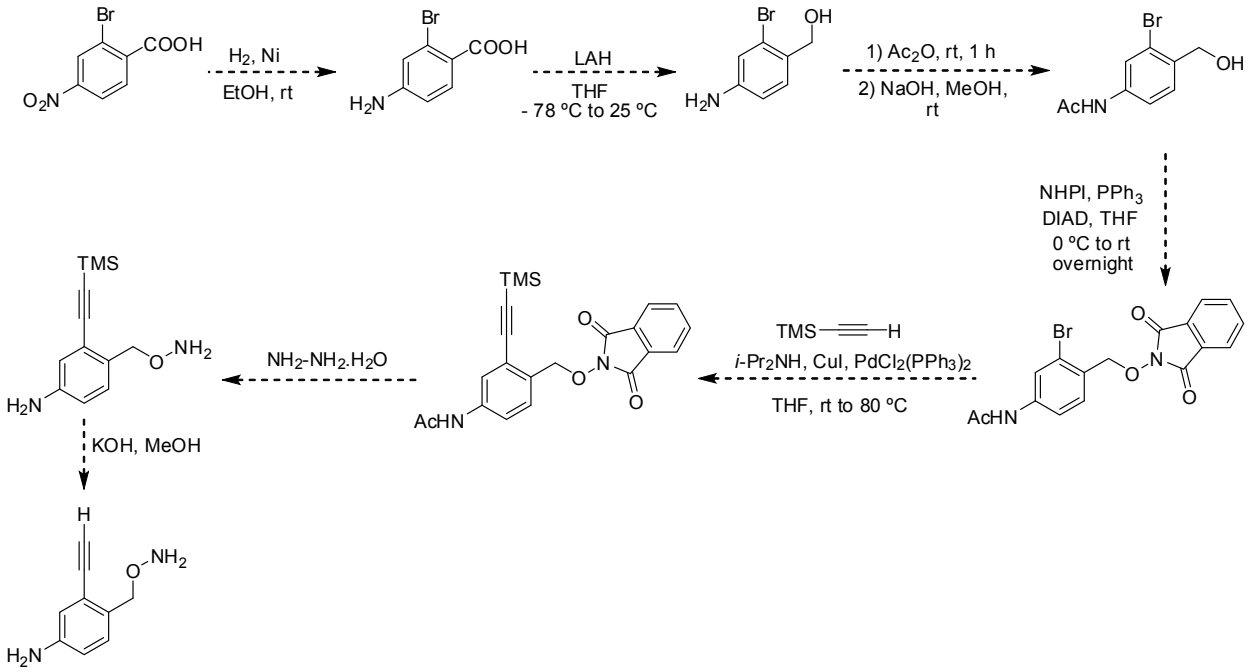


Figure 3.2 Representative example of copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) between an azide group containing tag molecule (blue) and alkyne group containing molecule (black).

We can incorporate a alkyne group on **3.10** to do CuAAC reaction with a azide containing tag molecule. A proposed synthetic route to make **3.14** is shown below (Scheme **3.3**).



Scheme 3.3 Synthetic plan to incorporate alkyne group on **3.21**.

It is very simple to introduce aldehyde and ketone group in native biomolecules. Sialic acid present on the cell surface can be easily oxidized by using periodate to generate aldehyde.[14,15] Peptide and protein can be readily oxidized at N-terminal serine residue by using periodate oxidation.[16,17] Similarly, RNAs can generate aldehyde on their 3′ end in one step.[18] Designing a similar aminoxy compound attached with a useful label will help to introduce those labels in modified biomolecules via oxime conjugation (Figure **3.3**).

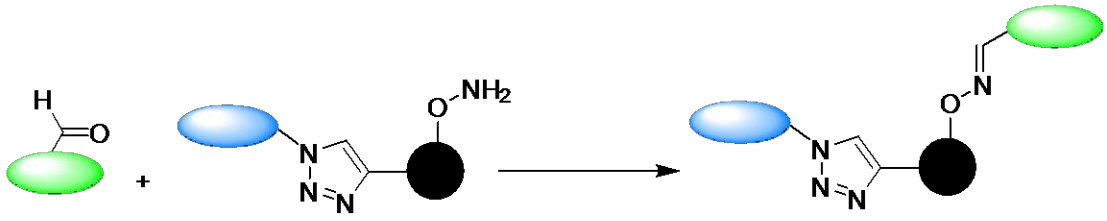


Figure 3.3 Representative example of an oxime ligation between an aldehyde modified biomolecule (green) and bifunctional aminooxy reagent (black) tagged with a biomarker (blue).

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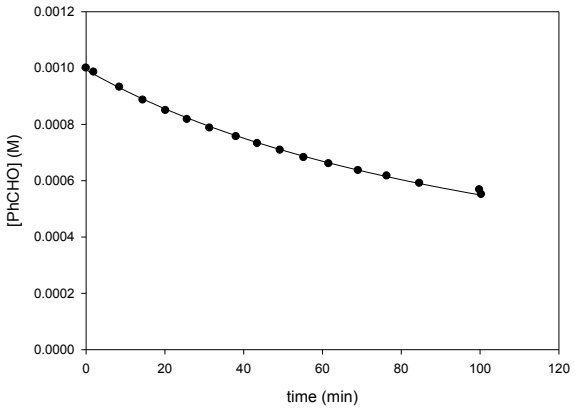
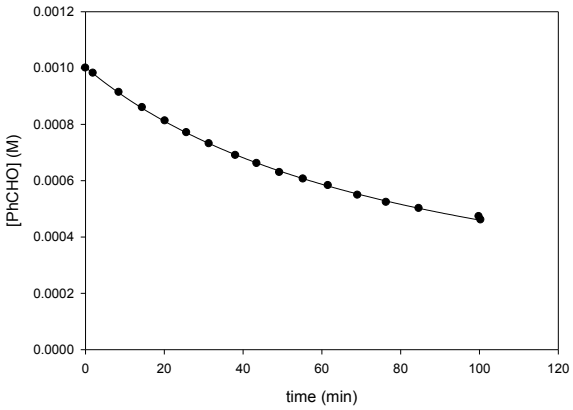
Appendix

Plots for consumption of benzaldehyde (M) over time (min) to form oxime in different conditions.

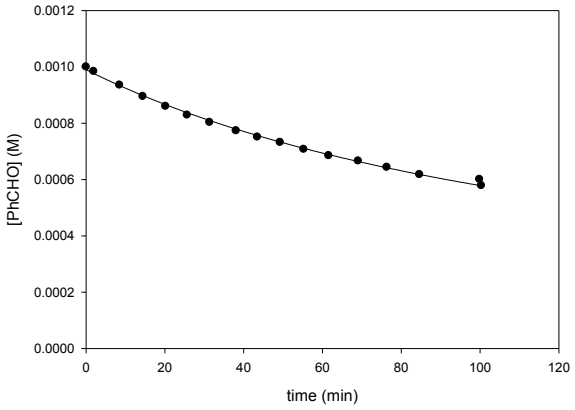
1. 1 mM **2.1** at pH 4.5



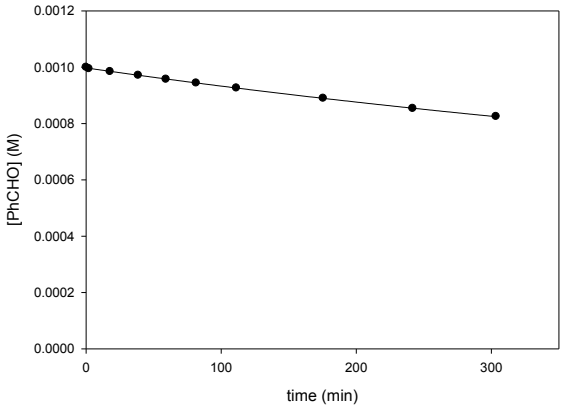
2. 1 mM aniline at pH 4.5



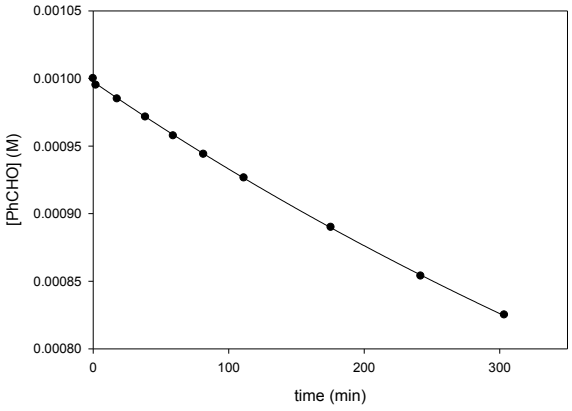
3. 1 mM uncatalyzed at pH 4.5



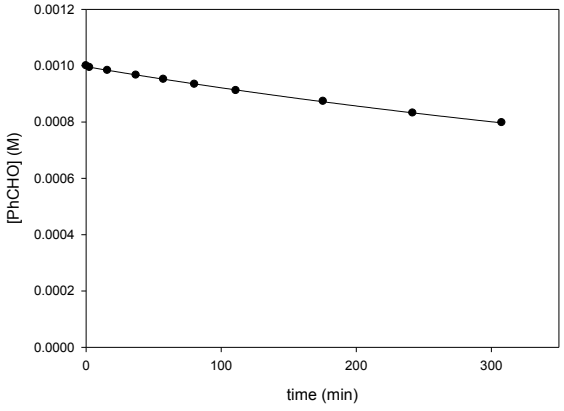
4. 1 mM **2.1** at pH 5.3



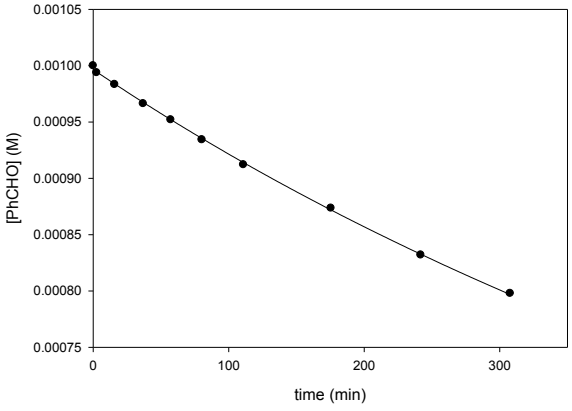
5. 1 mM **2.1** at pH 5.3 (expanded)



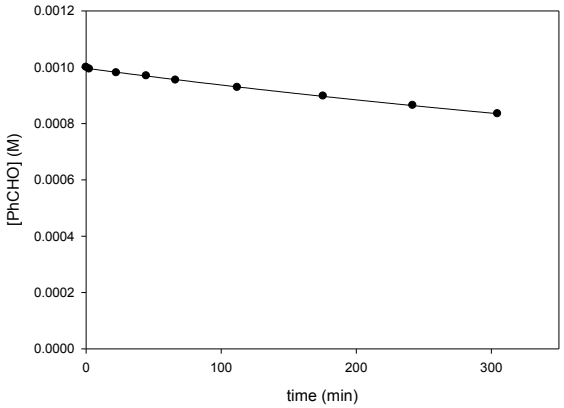
6. 1 mM aniline catalyzed at pH 5.3



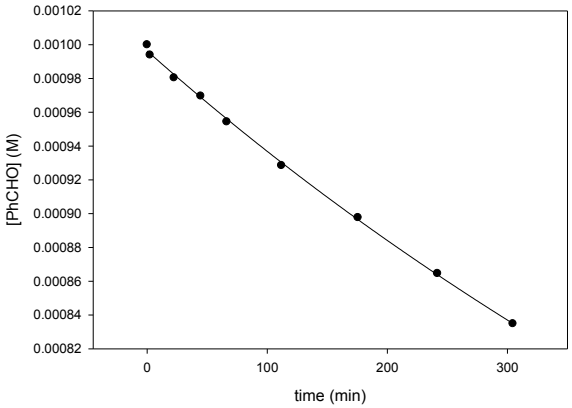
7. 1 mM aniline catalyzed at pH 5.3 (expanded)



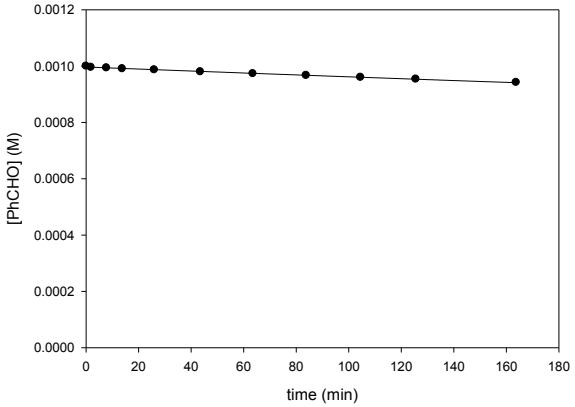
8. 1 mM **2.22** at pH 5.3



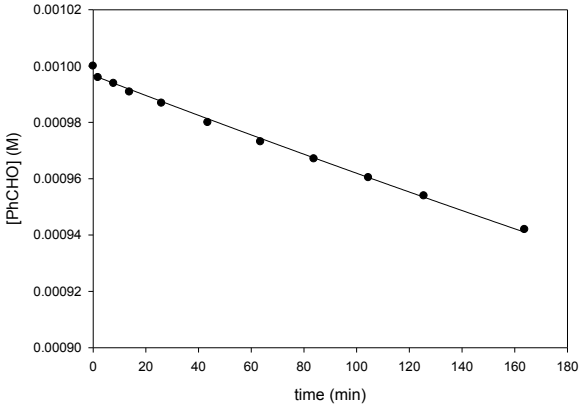
9. 1 mM **2.22** at pH 5.3 (expanded)



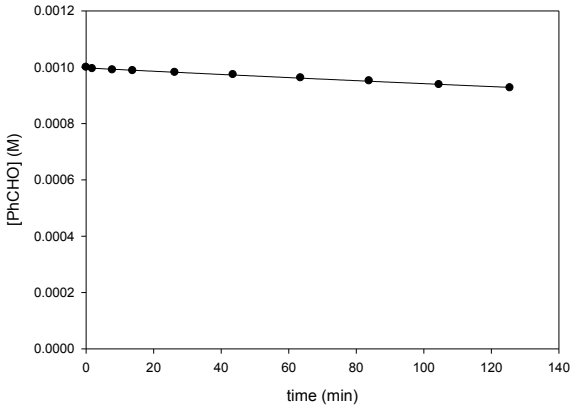
10. 1 mM **2.1** at pH 6.5



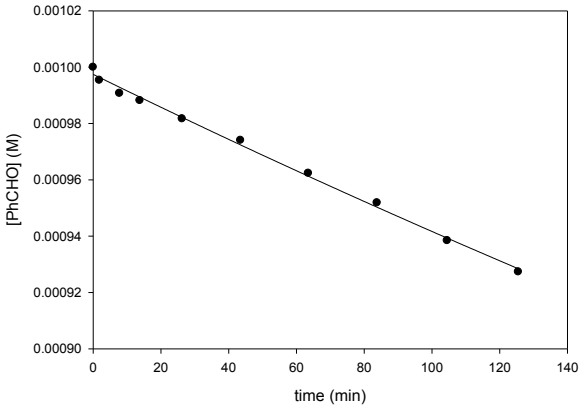
11. 1 mM **2.1** at pH 6.5 (expanded)



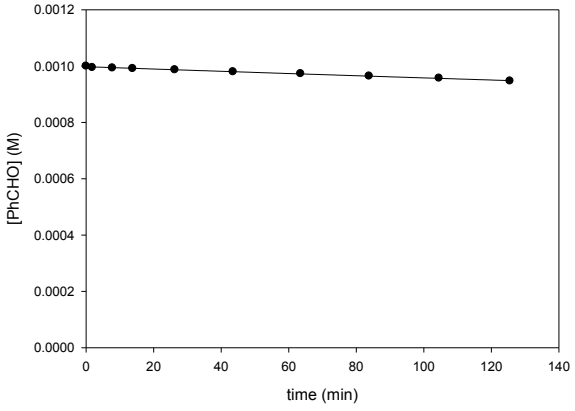
12. 1 mM aniline catalyzed at pH 6.5



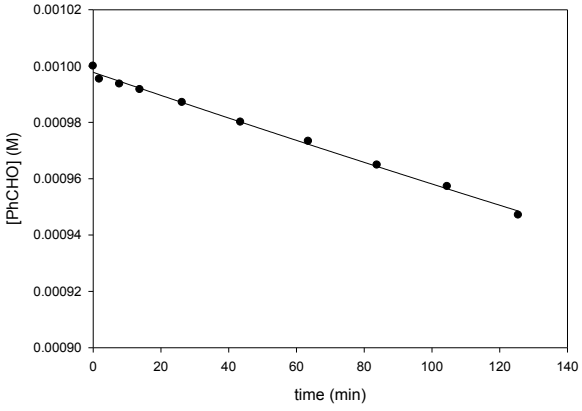
13. 1 mM aniline catalyzed at pH 6.5



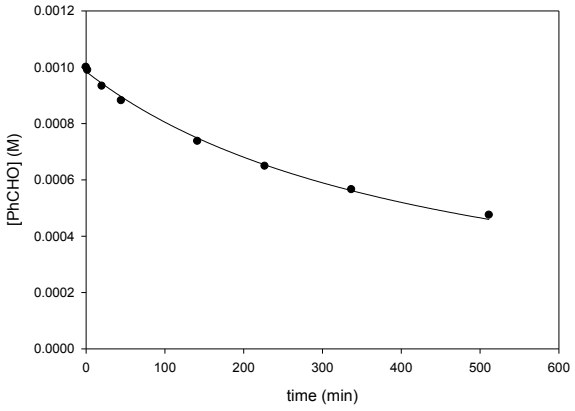
14. 1 mM **2.22** at pH 6.5



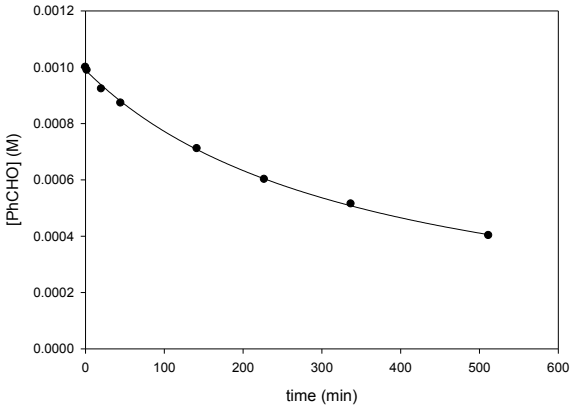
15. 1 mM **2.22** at pH 6.5 (expanded)



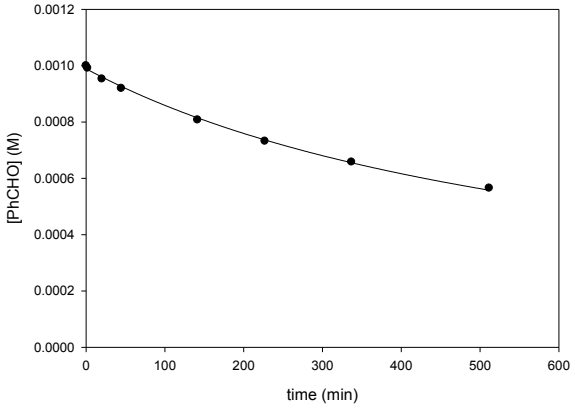
16. 1 mM **2.1** at pH 5.5



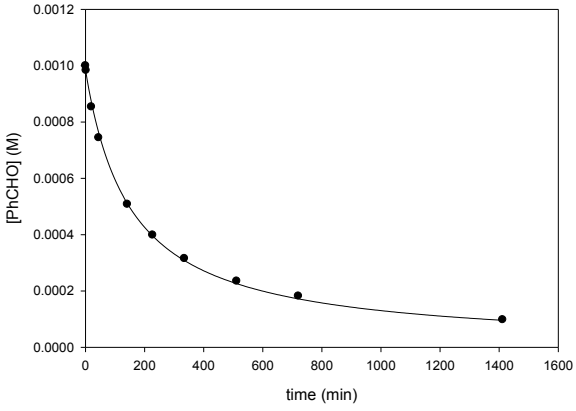
17. 1 mM aniline catalyzed at pH 5.5



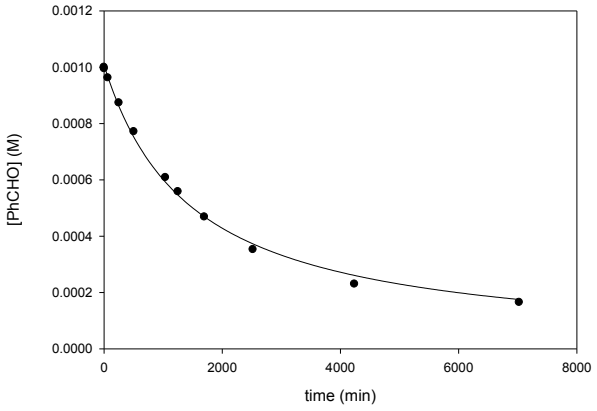
18. 1 mM **2.22** at pH 5.5



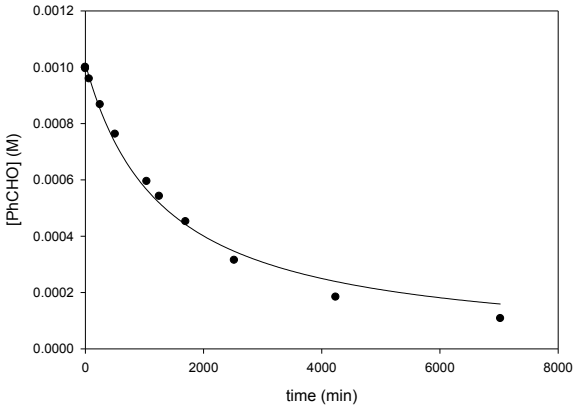
19. 1 mM **2.2** at pH 5.5



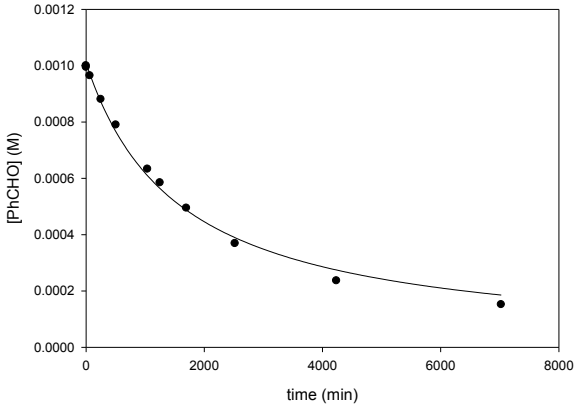
20. 1 mM **2.1** at pH 5.7



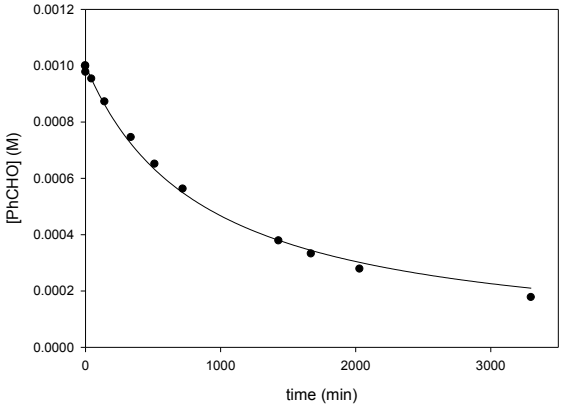
21. 1 mM aniline catalyzed at pH 5.7



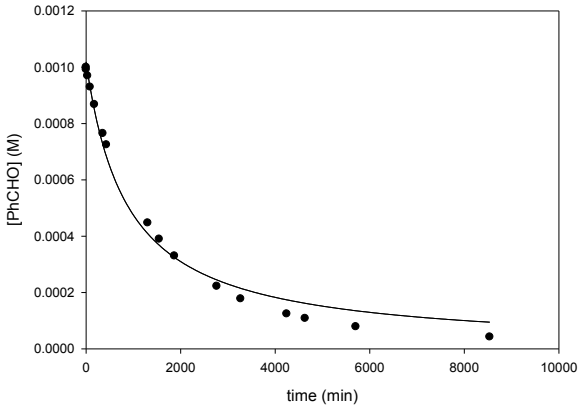
22. 1 mM **2.22** at pH 5.7



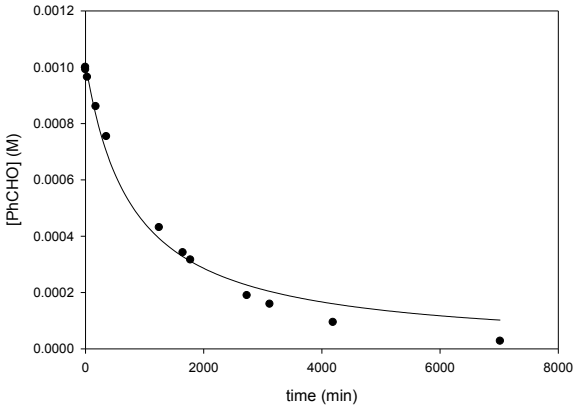
23. 1 mM **2.2** at pH 5.7



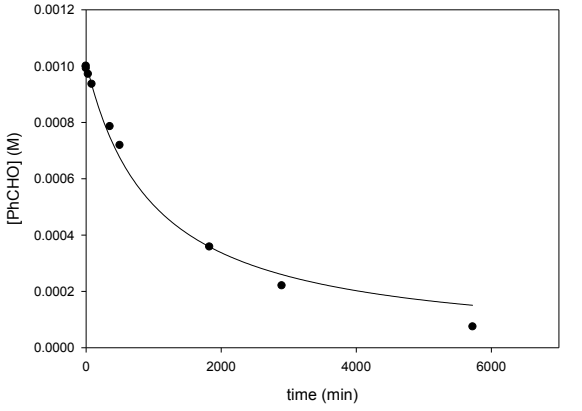
24. 2 mM **2.1** at pH 5.7



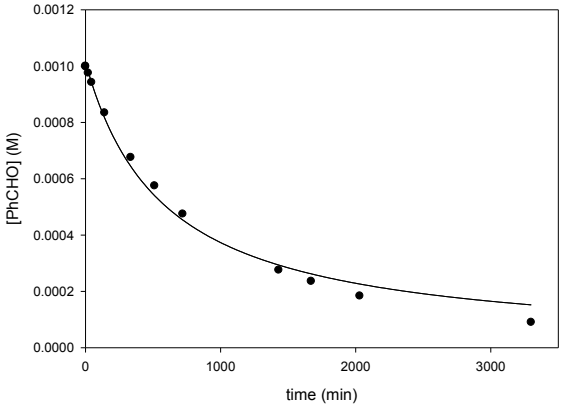
25. 2 mM aniline catalyzed at pH 5.7



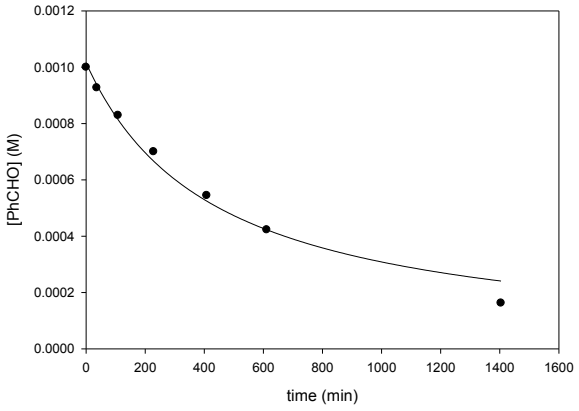
26. 2 mM **2.22** at pH 5.7



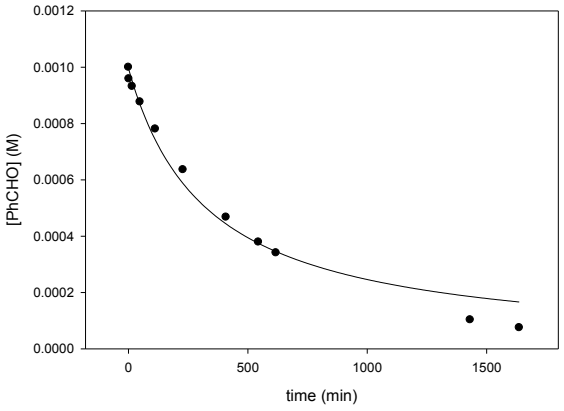
27. 2 mM **2.2** at pH 5.7



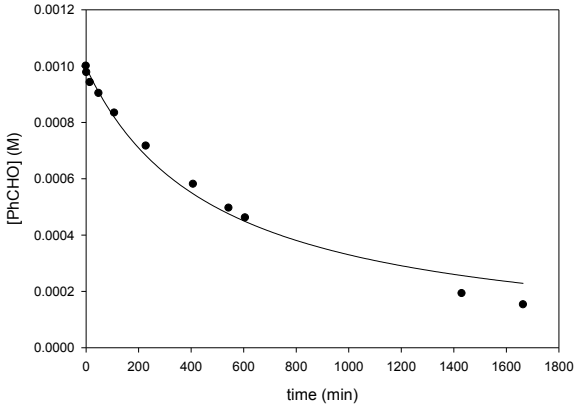
28. 10 mM **2.1** at pH 5.7



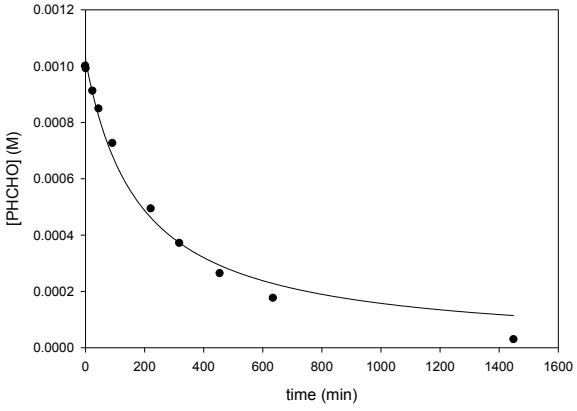
29. 10 mM aniline catalyzed at pH 5.7



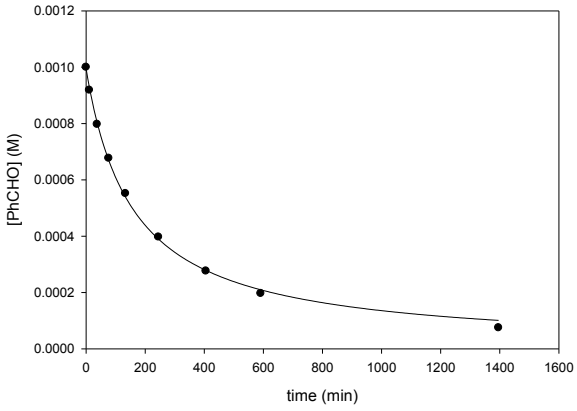
30. 10 mM **2.22** at pH 5.7



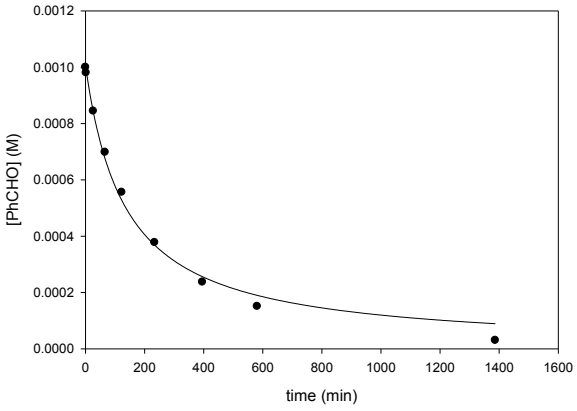
31. 10 mM **2.2** at pH 5.7



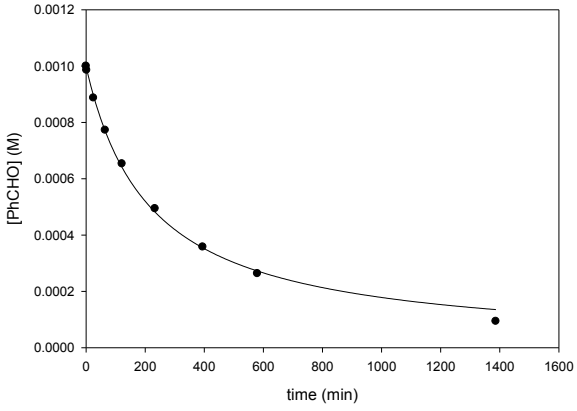
32. 2 mM **2.1** at pH 5.5



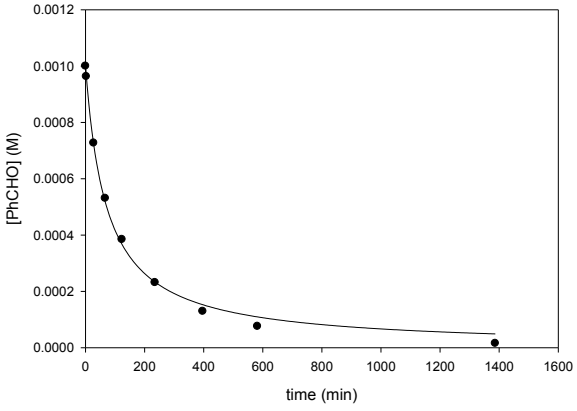
33. 2 mM aniline catalyzed at pH 5.5



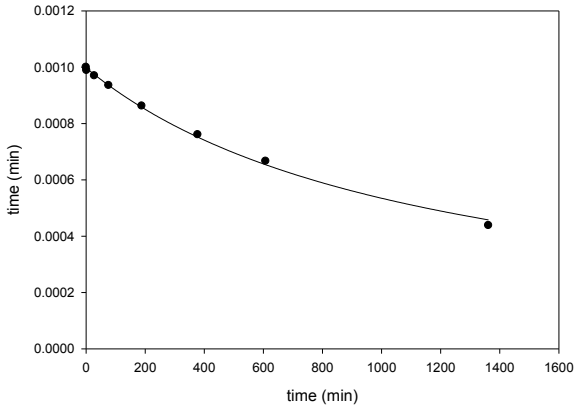
34. 2 mM **2.22** at pH 5.5



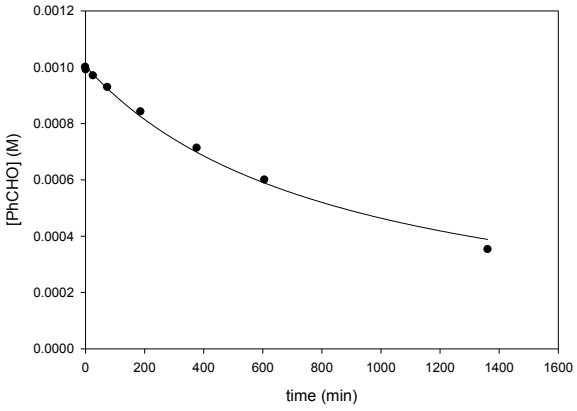
35. 2 mM **2.2** at pH 5.5



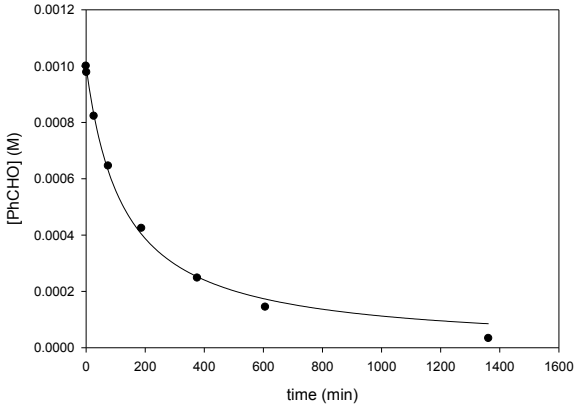
36. 2 mM **2.7** catalyzed at pH 5.7



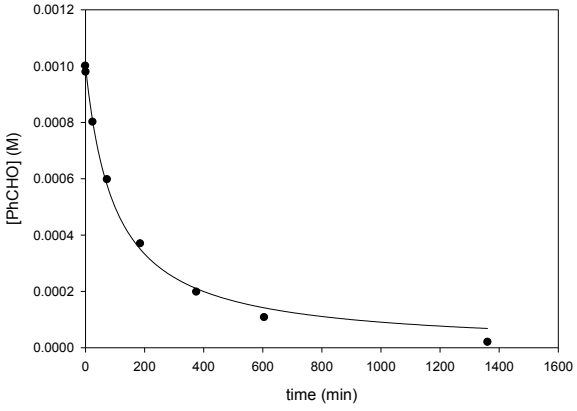
37. 2 mM **2.18** catalyzed at pH 5.7



38. 2 mM **2.7** catalyzed at pH 5.5

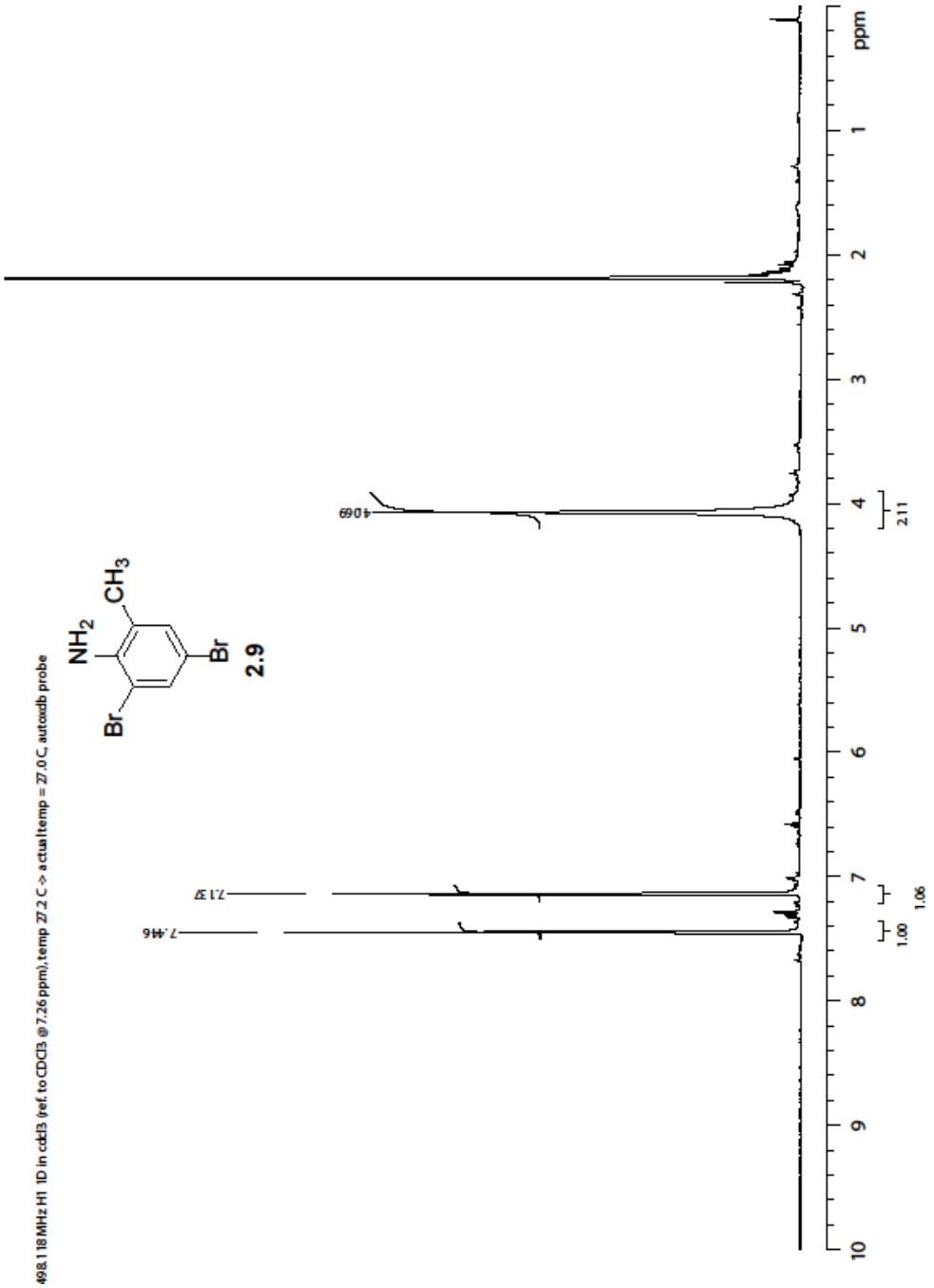


39. 2 mM **2.18** catalyzed at pH 5.5

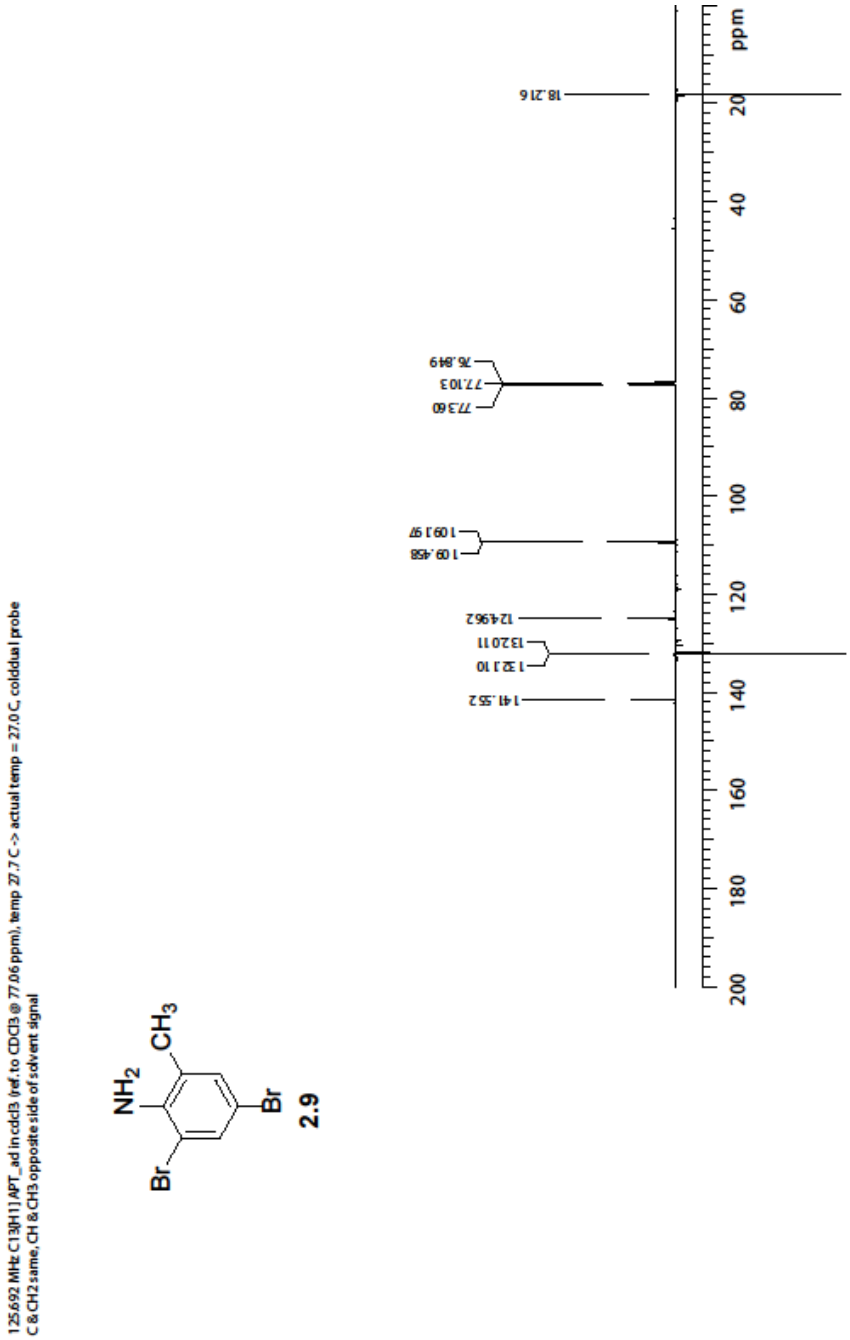


^{1}H and ^{13}C NMR spectra for the compounds

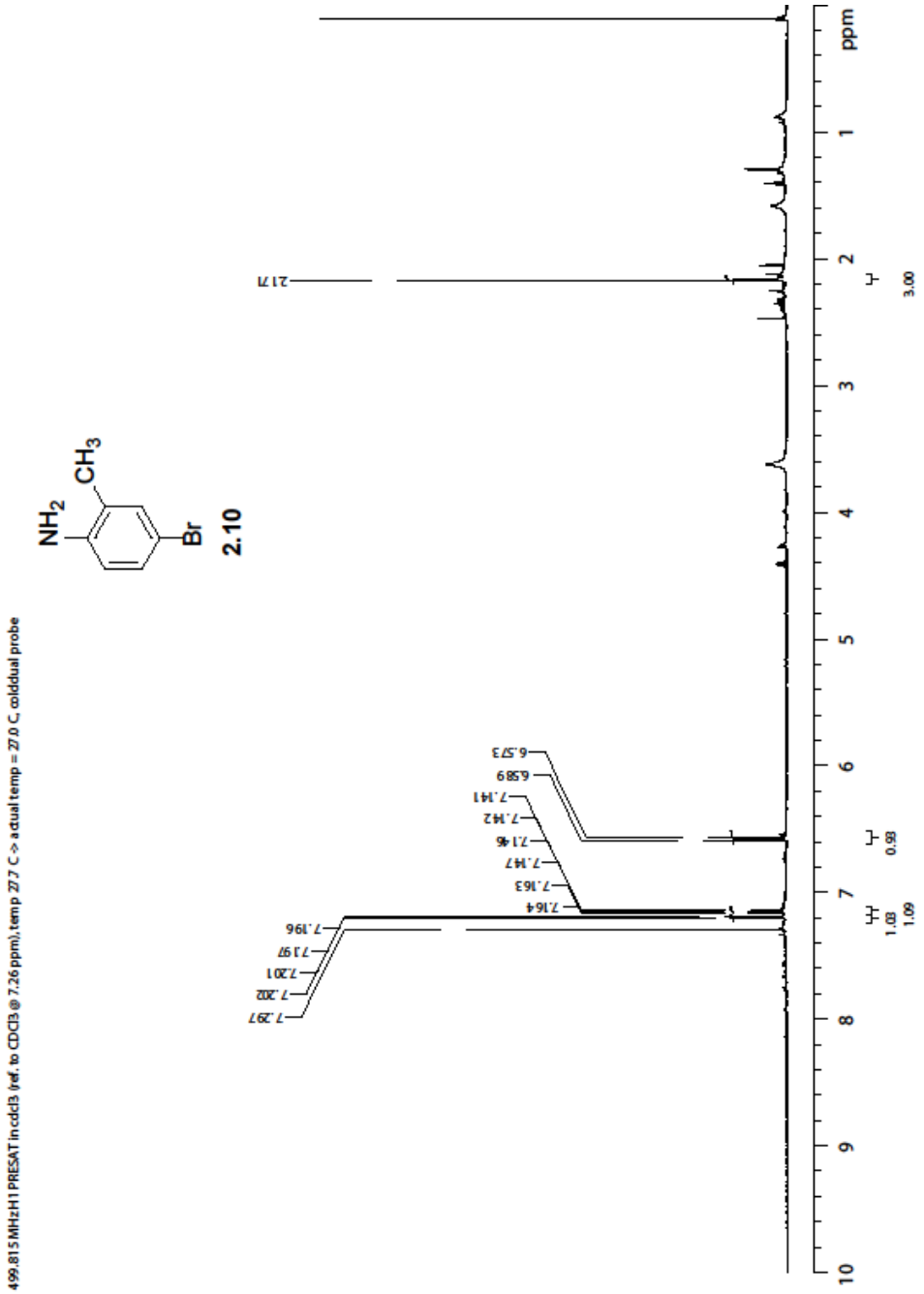
^{1}H NMR, $CDCl_3$, 500 MHz



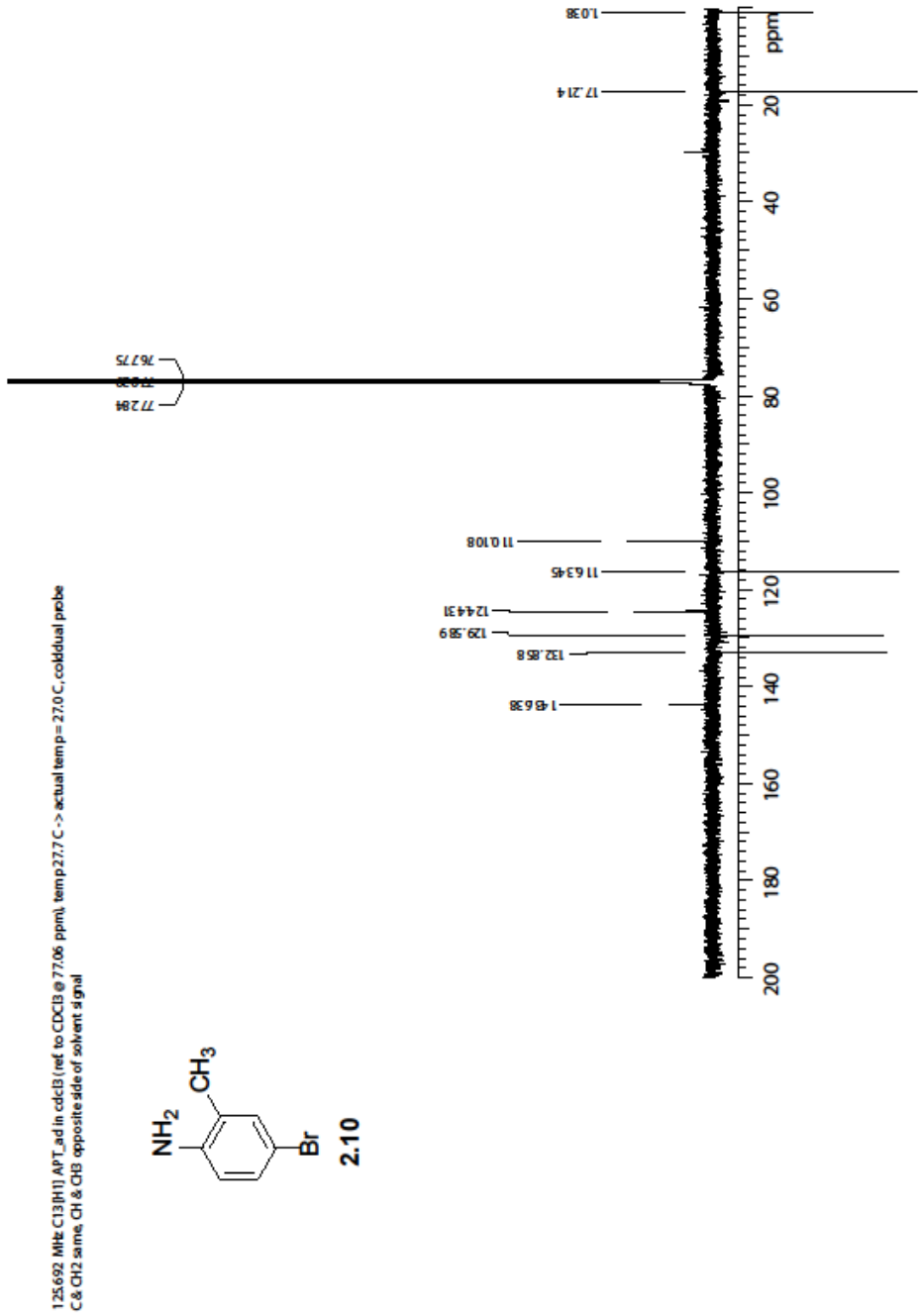
^{13}C NMR, $CDCl_3$, 125 MHz



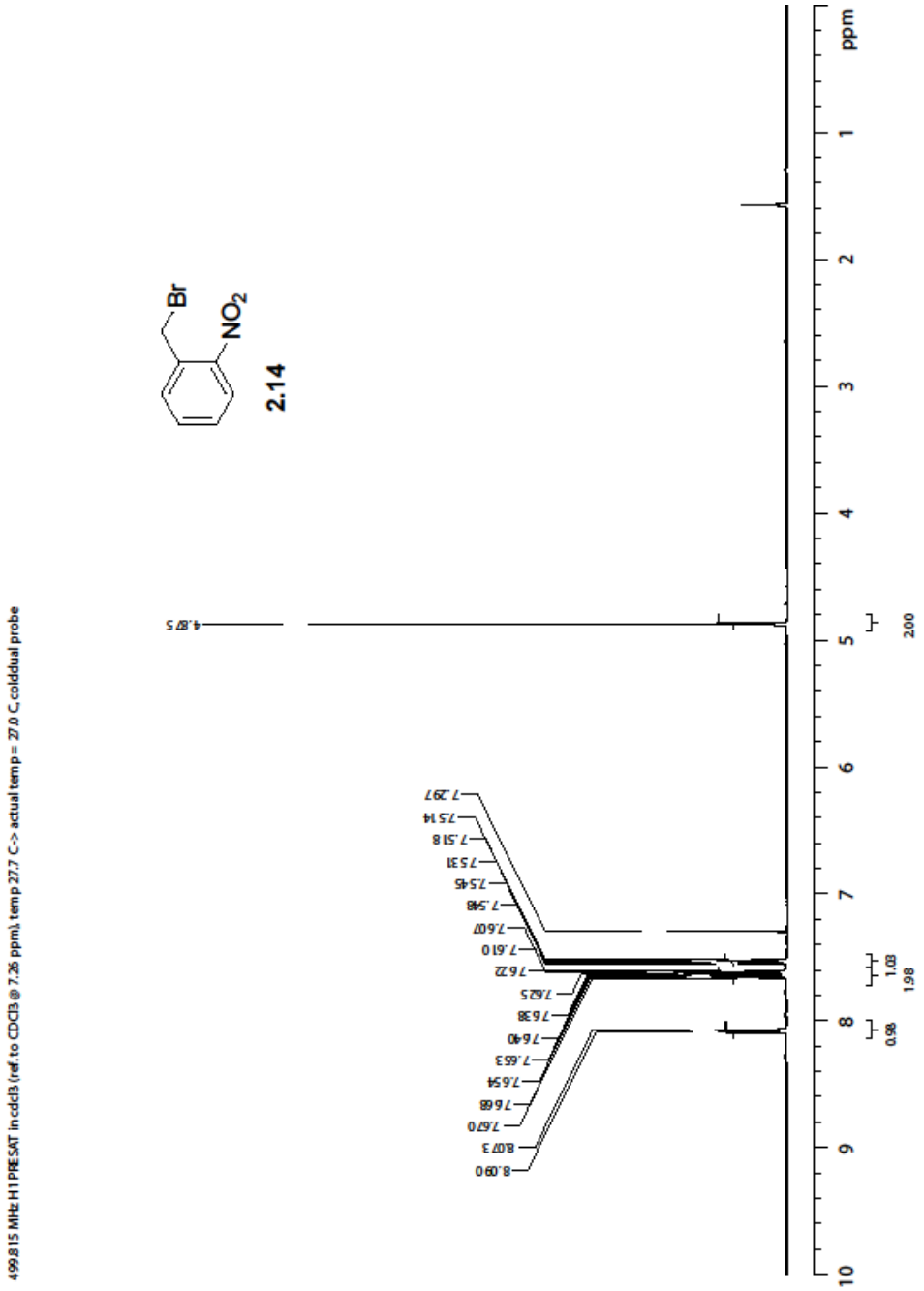
^{1}H NMR, $CDCl_3$, 500 MHz



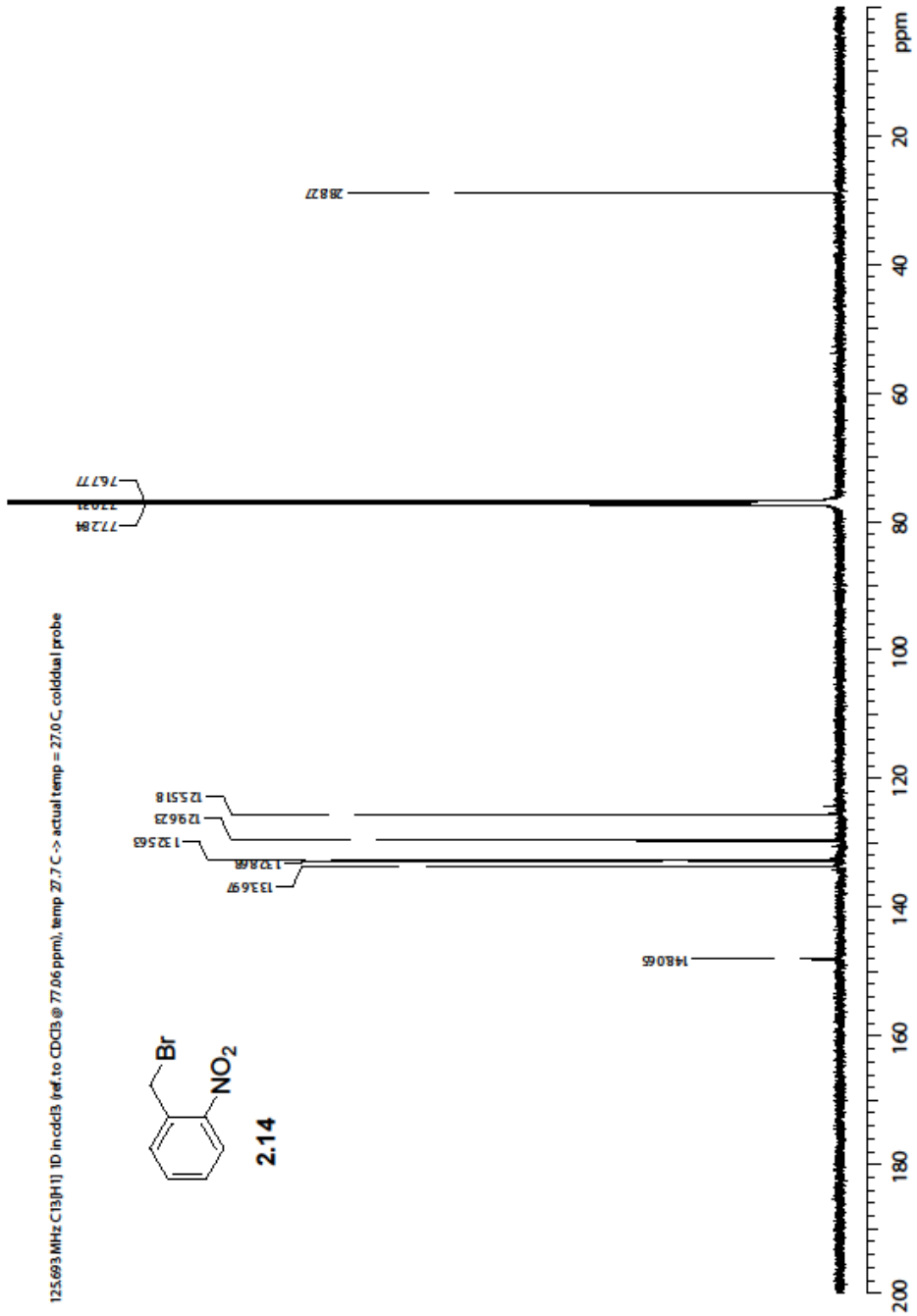
^{13}C NMR, $CDCl_3$, 125 MHz



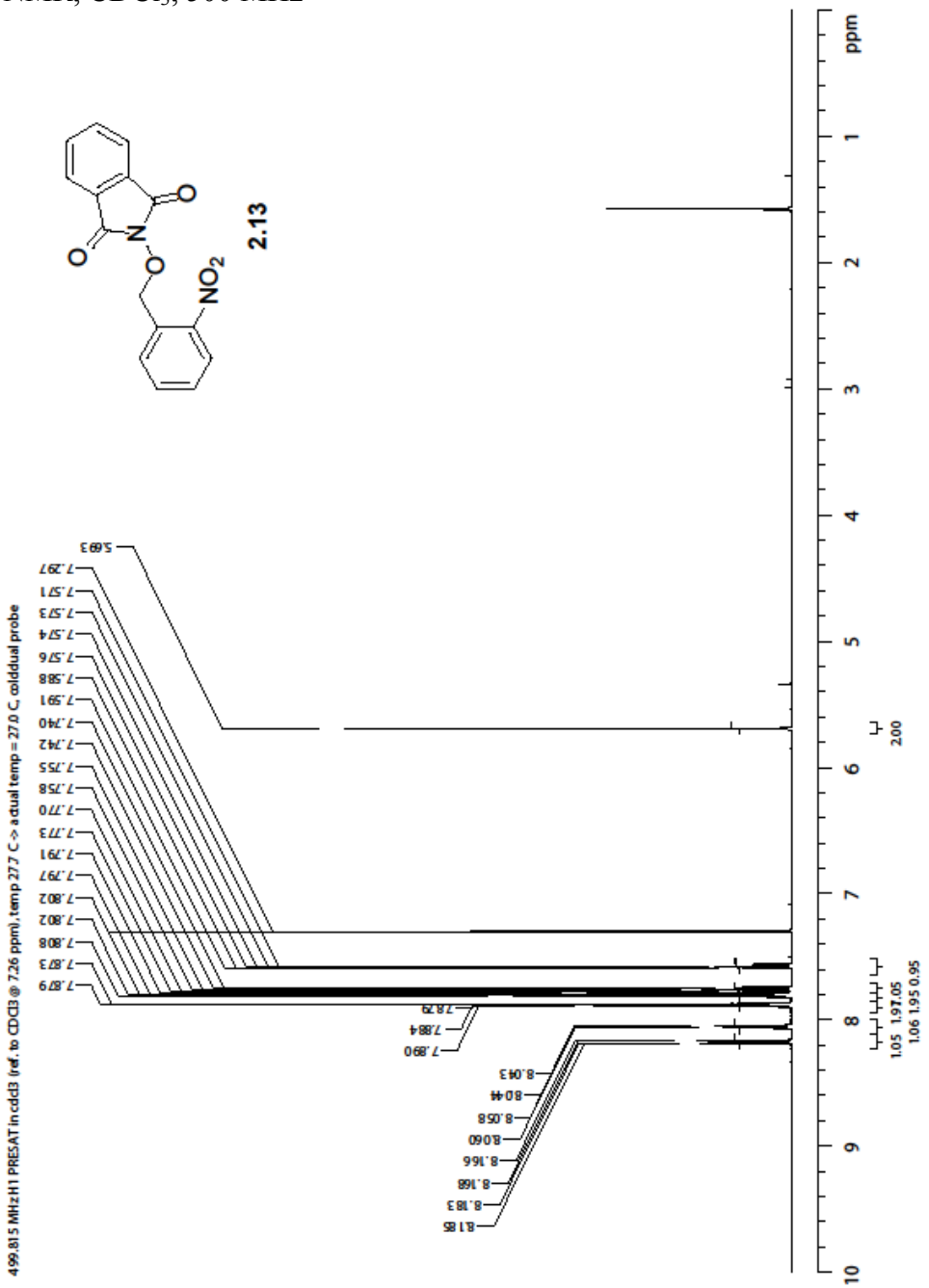
^{1}H NMR, $CDCl_3$, 500 MHz



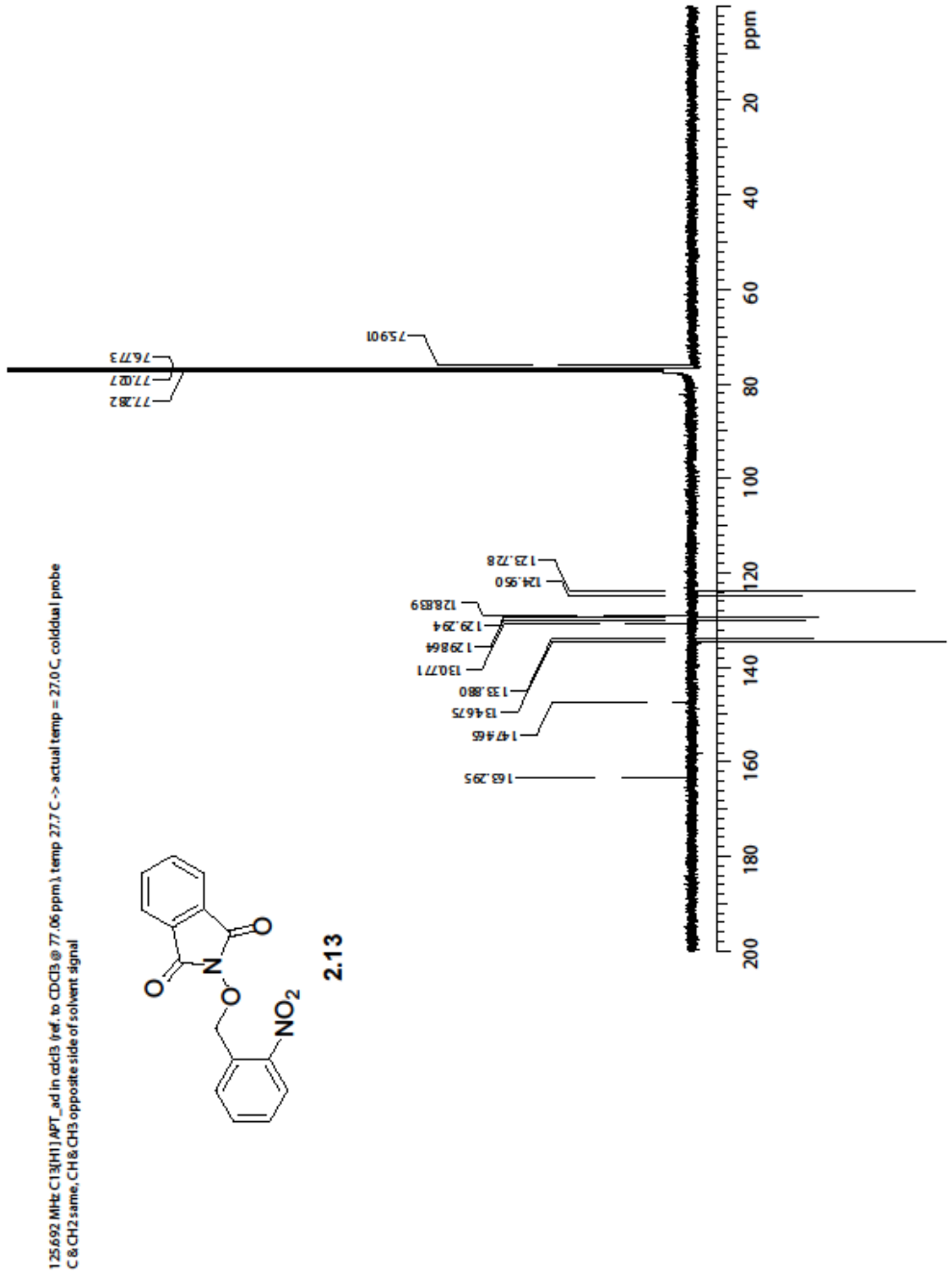
^{13}C NMR, $CDCl_3$, 125 MHz



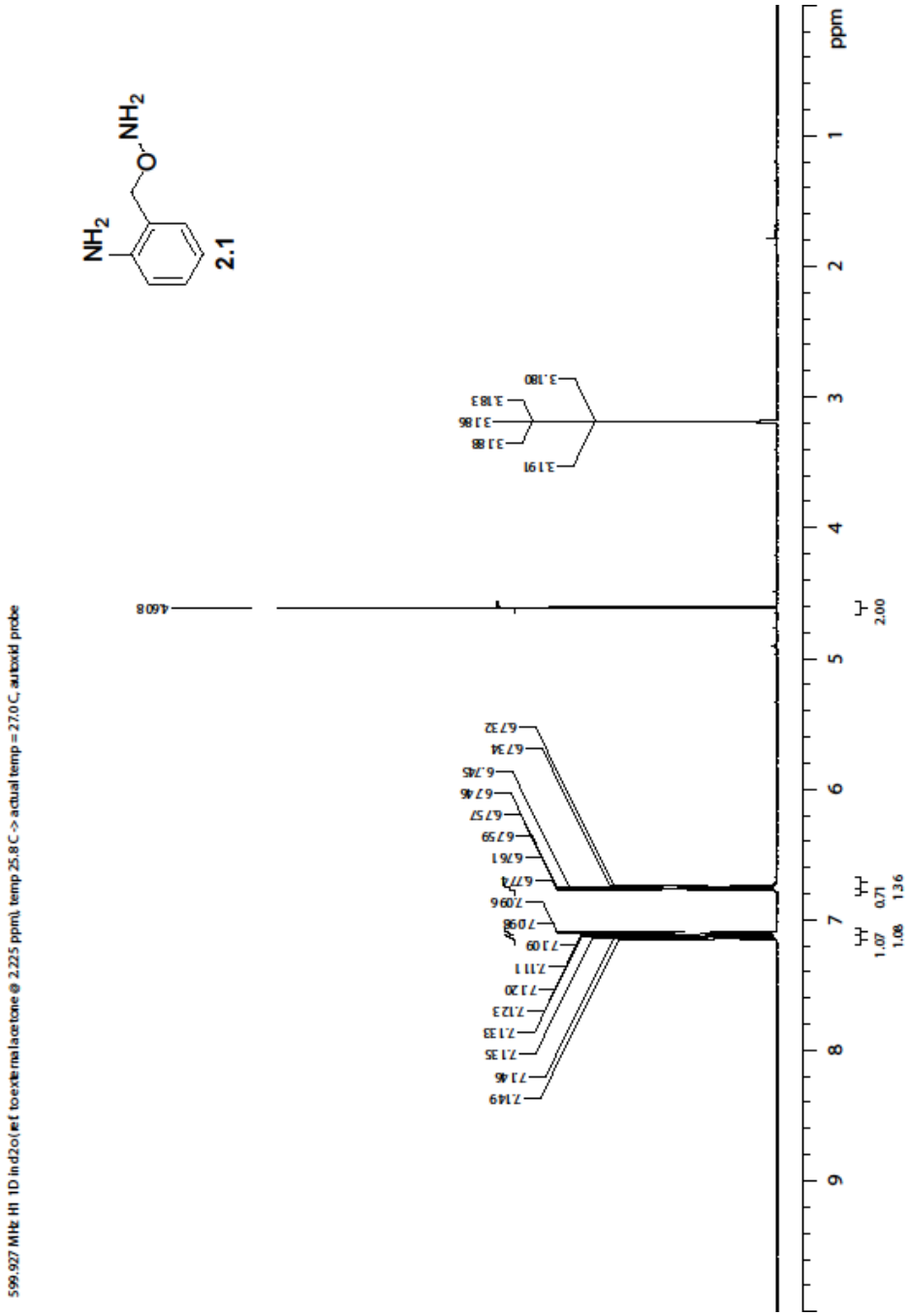
^{1}H NMR, $CDCl_3$, 500 MHz



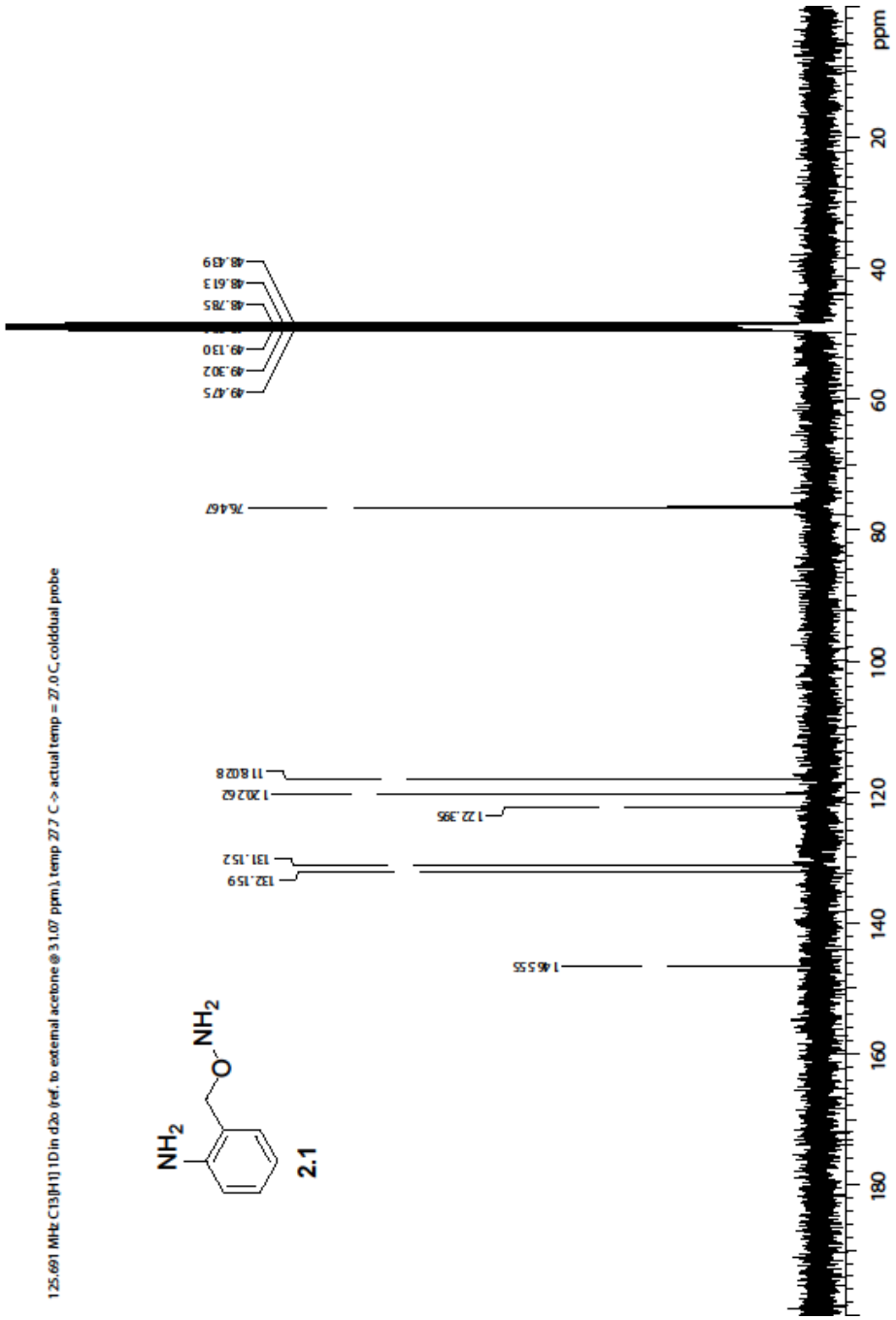
^{13}C NMR, $CDCl_3$, 125 MHz



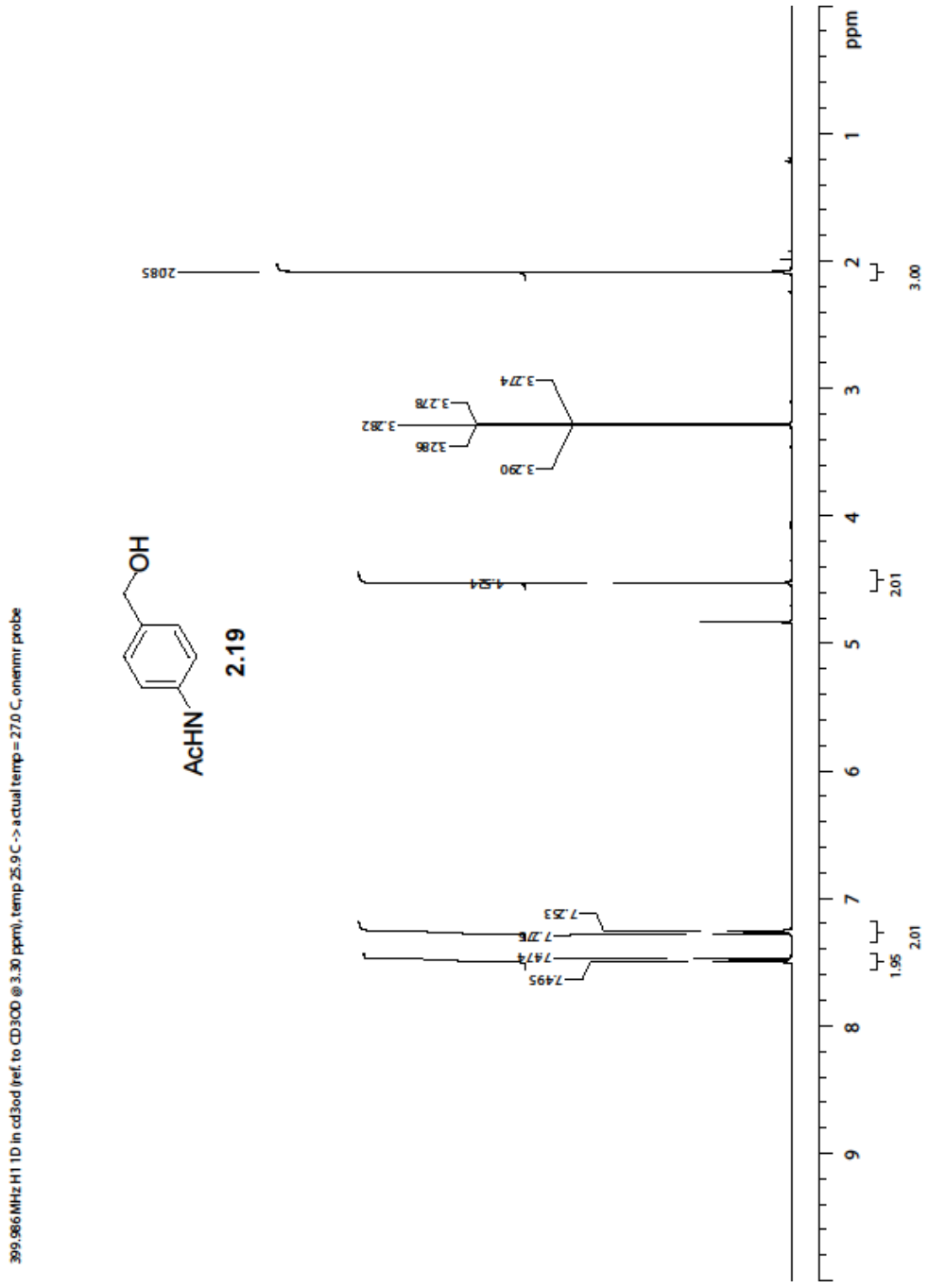
^{1}H NMR, D_2O, 600 MHz



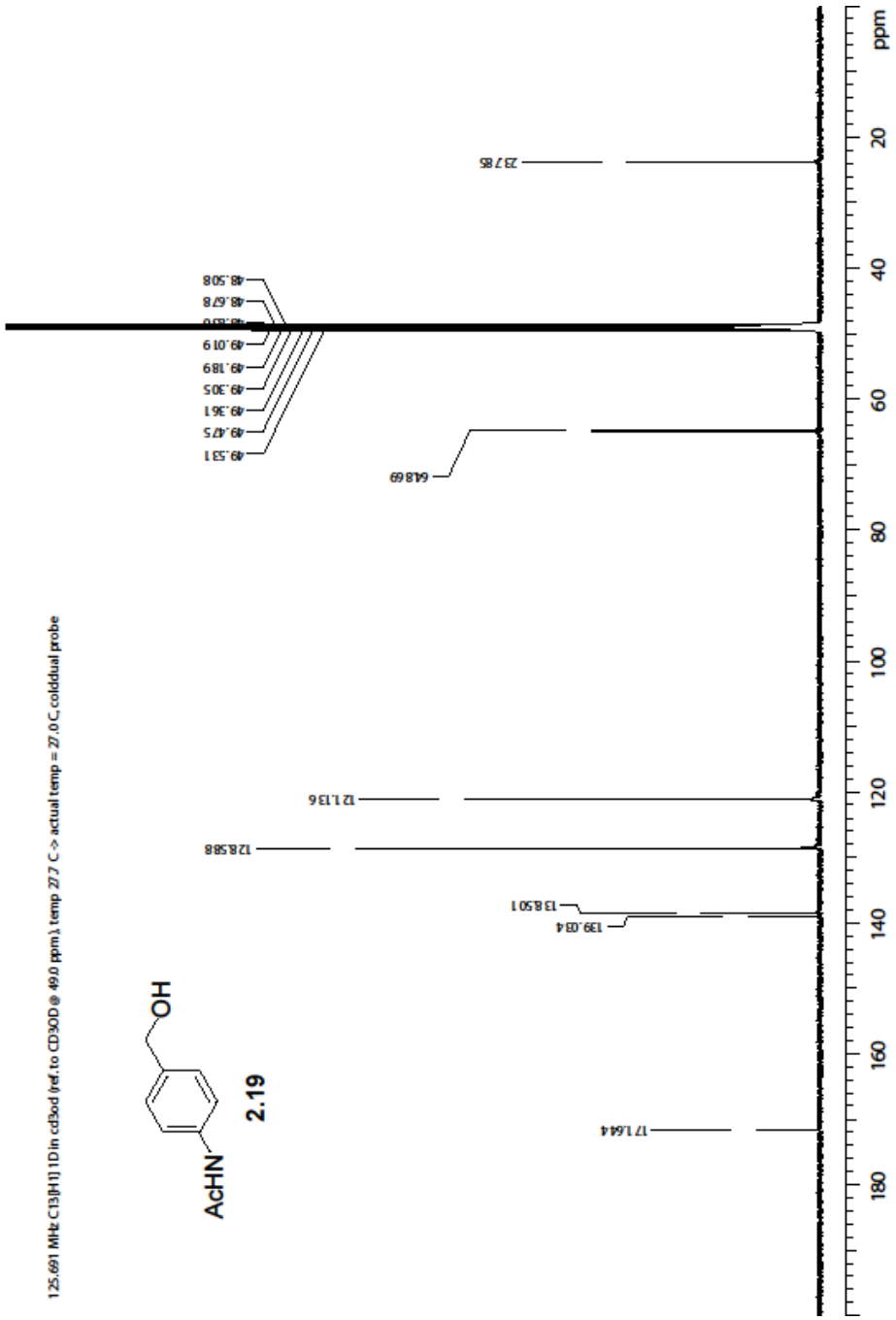
^{13}C NMR, D_2O, 125 MHz



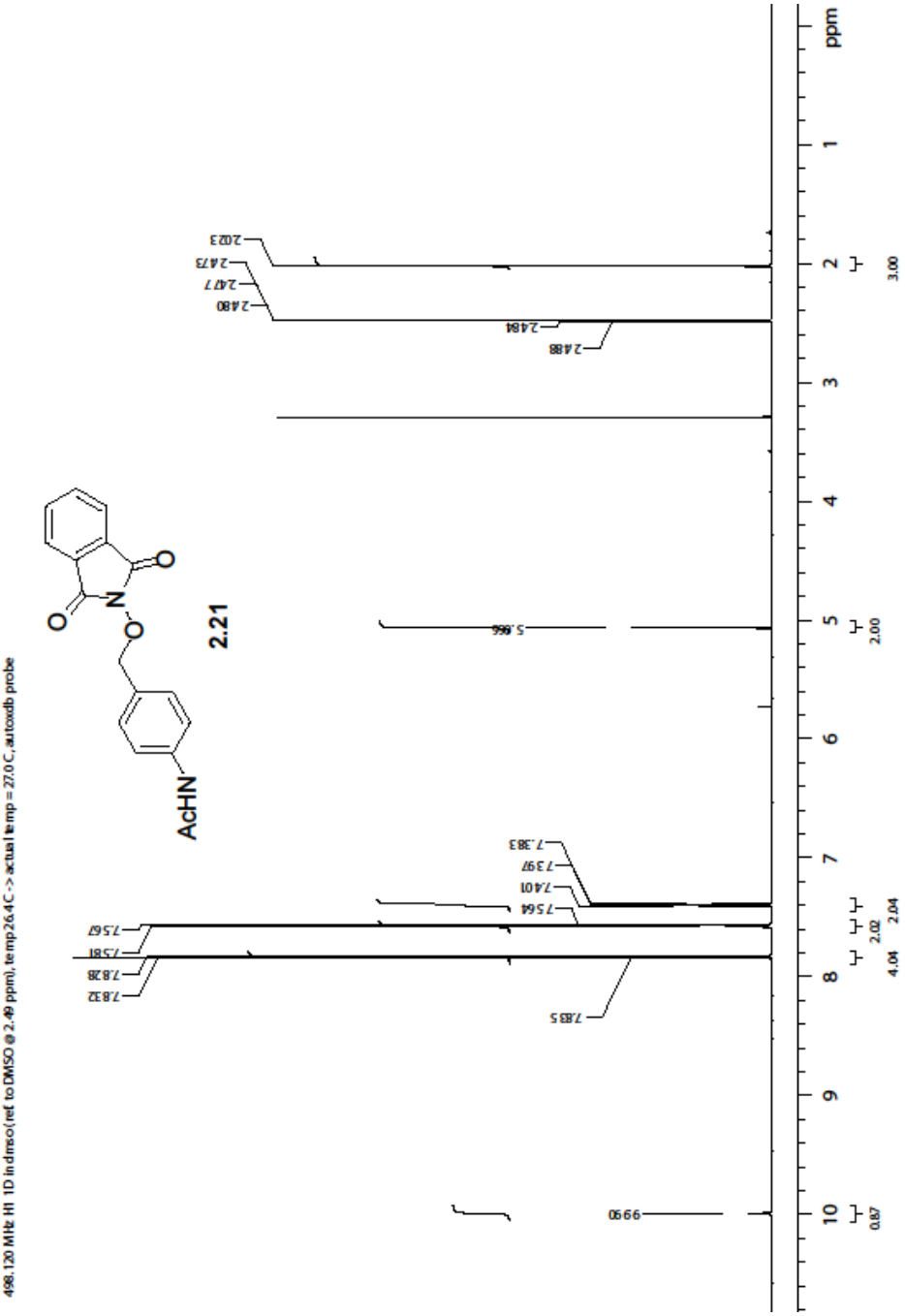
^{1}H NMR, CD_3OD, 400 MHz



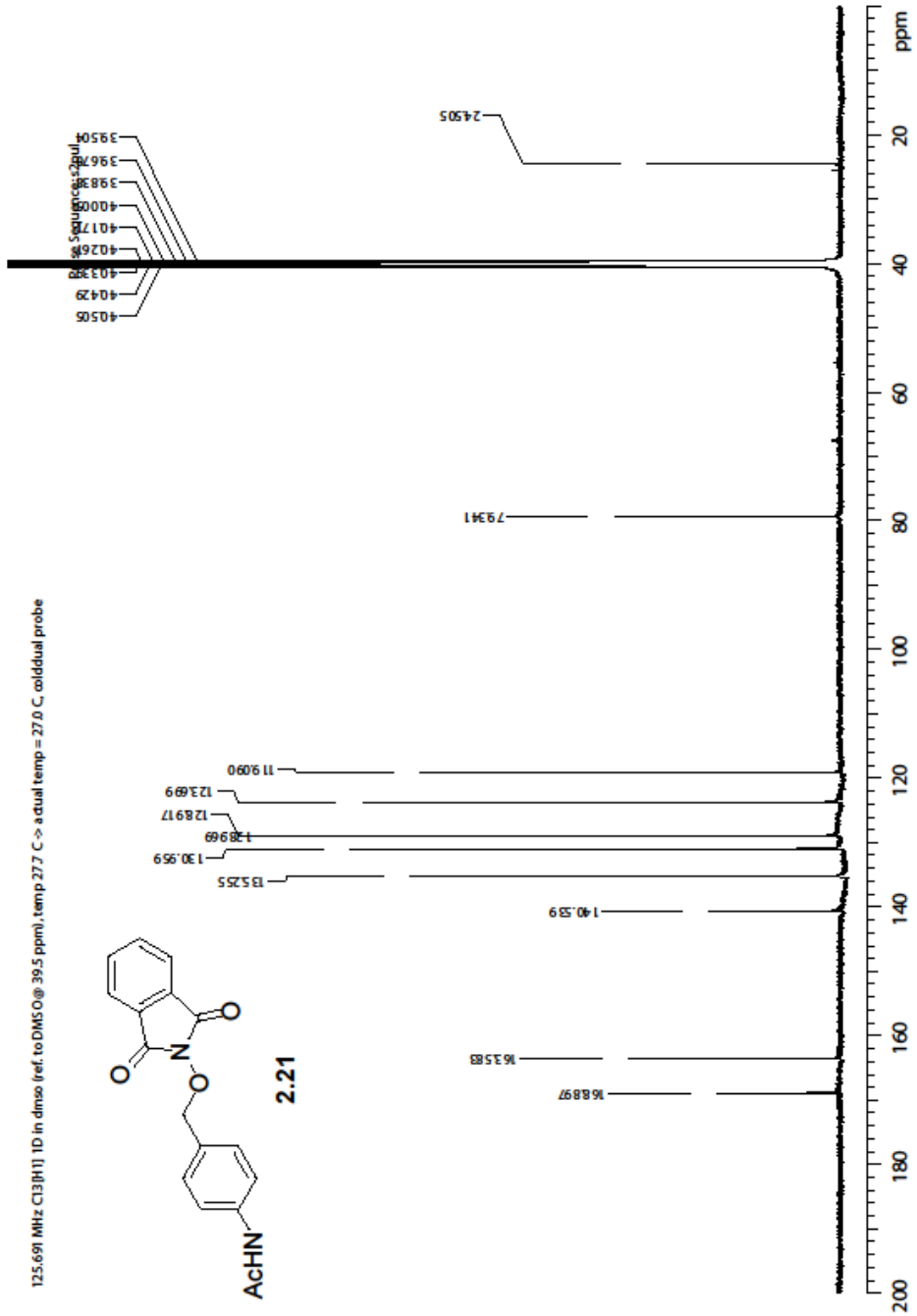
^{13}C NMR, CD_3OD, 125 MHz



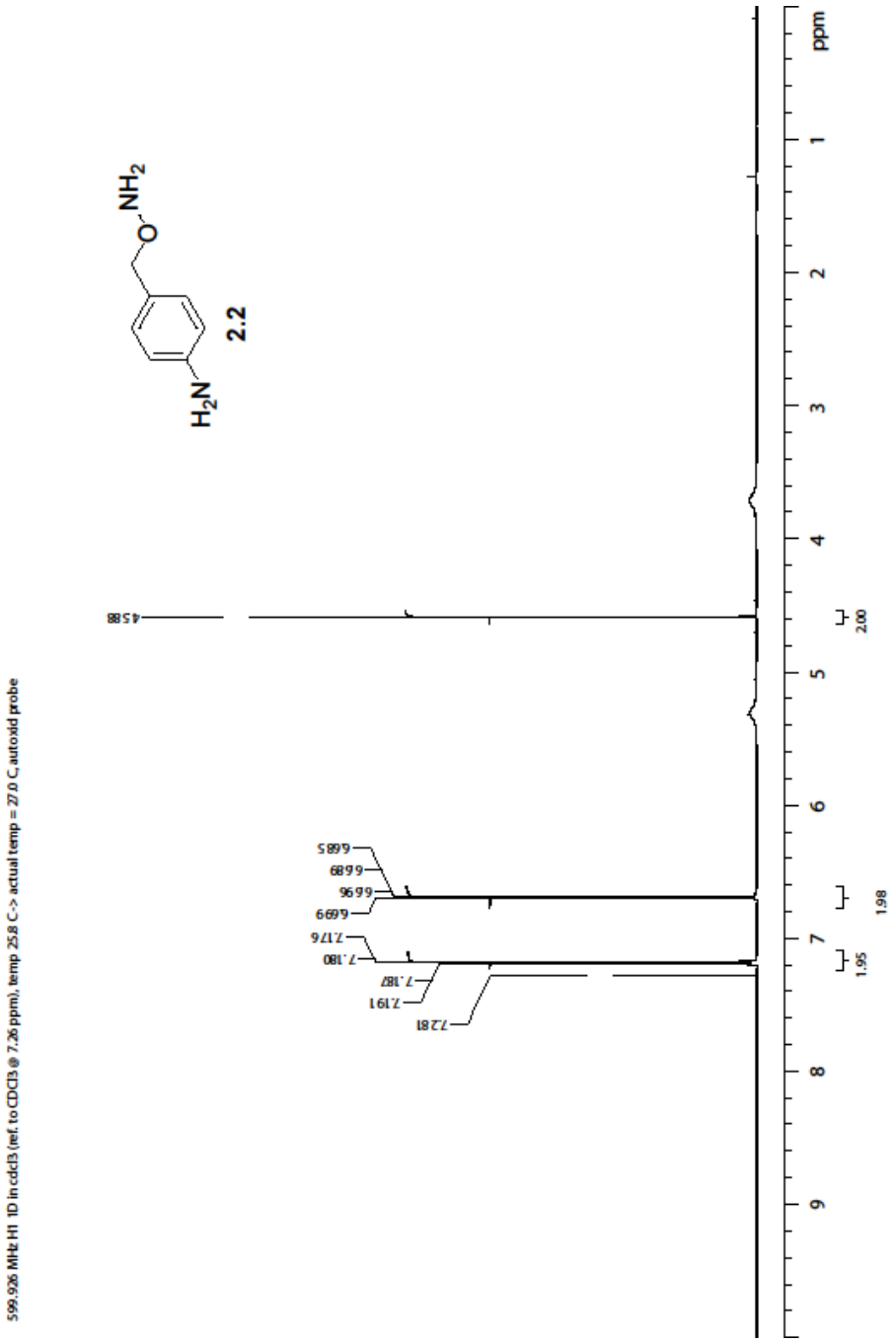
^{1}H NMR, DMSO-d_6, 500 MHz



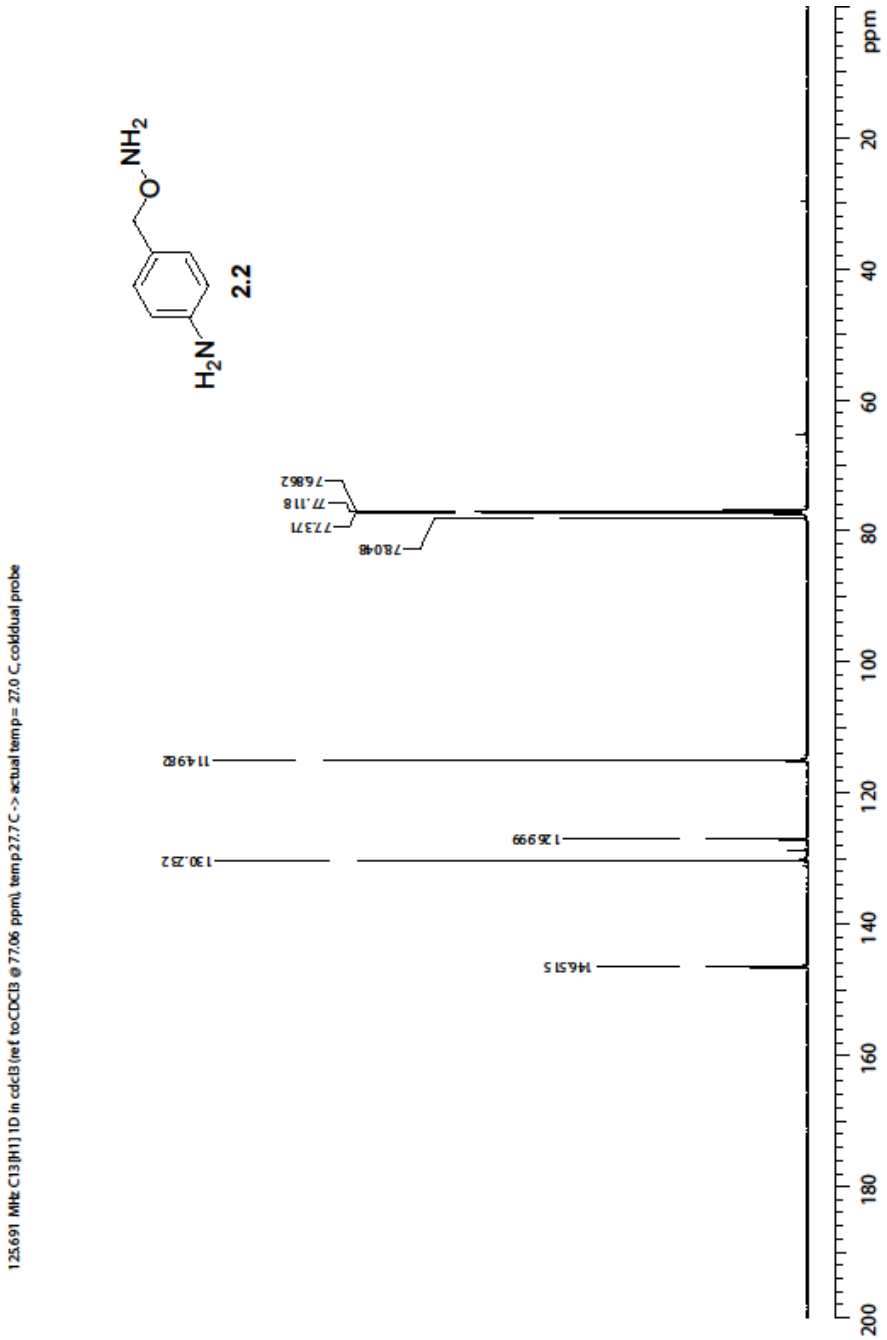
^{13}C NMR, DMSO-d_6, 125 MHz



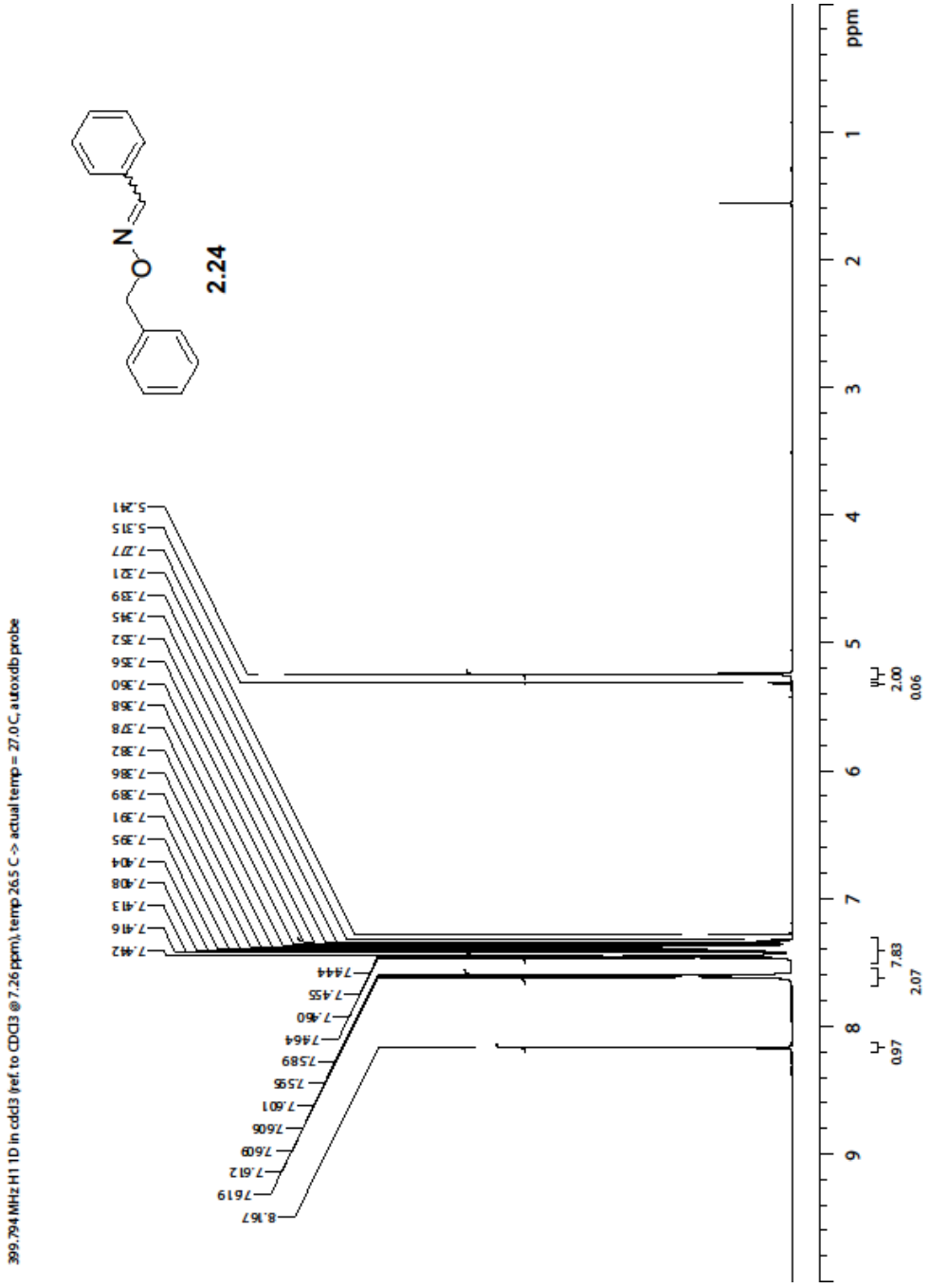
^{1}H NMR, $CDCl_3$, 600 MHz



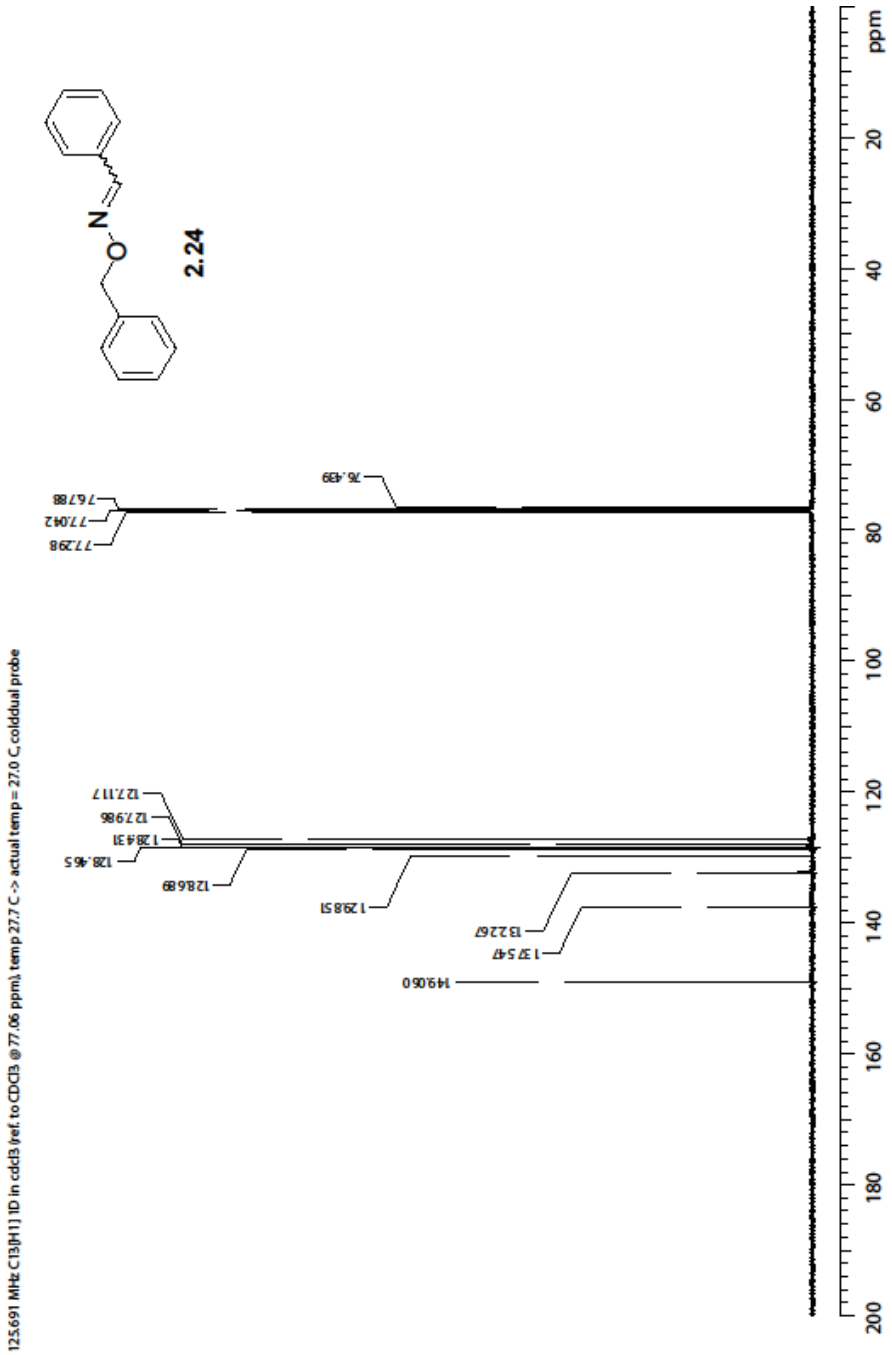
^{13}C NMR, $CDCl_3$, 125 MHz



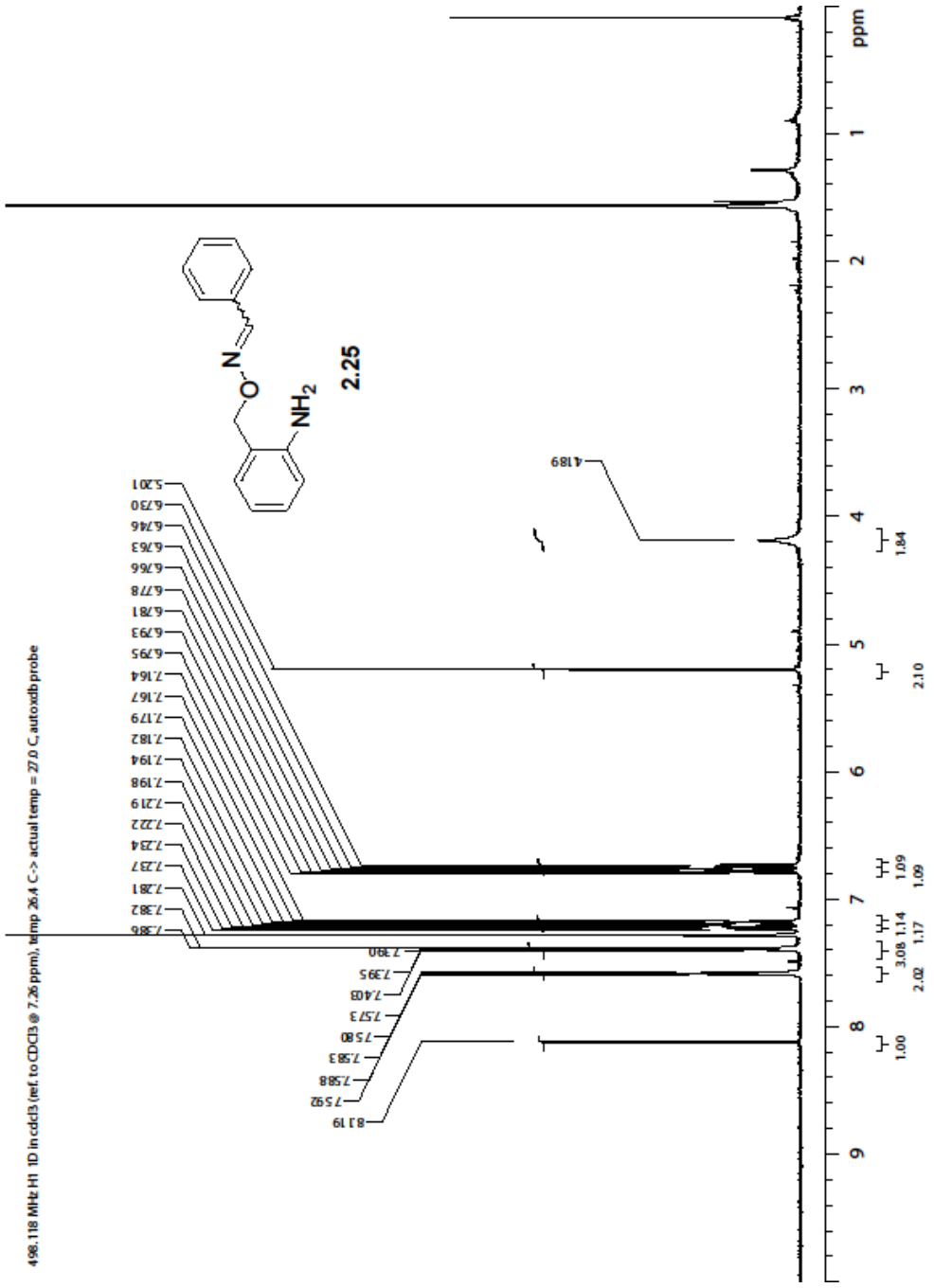
^{1}H NMR, $CDCl_3$, 400 MHz



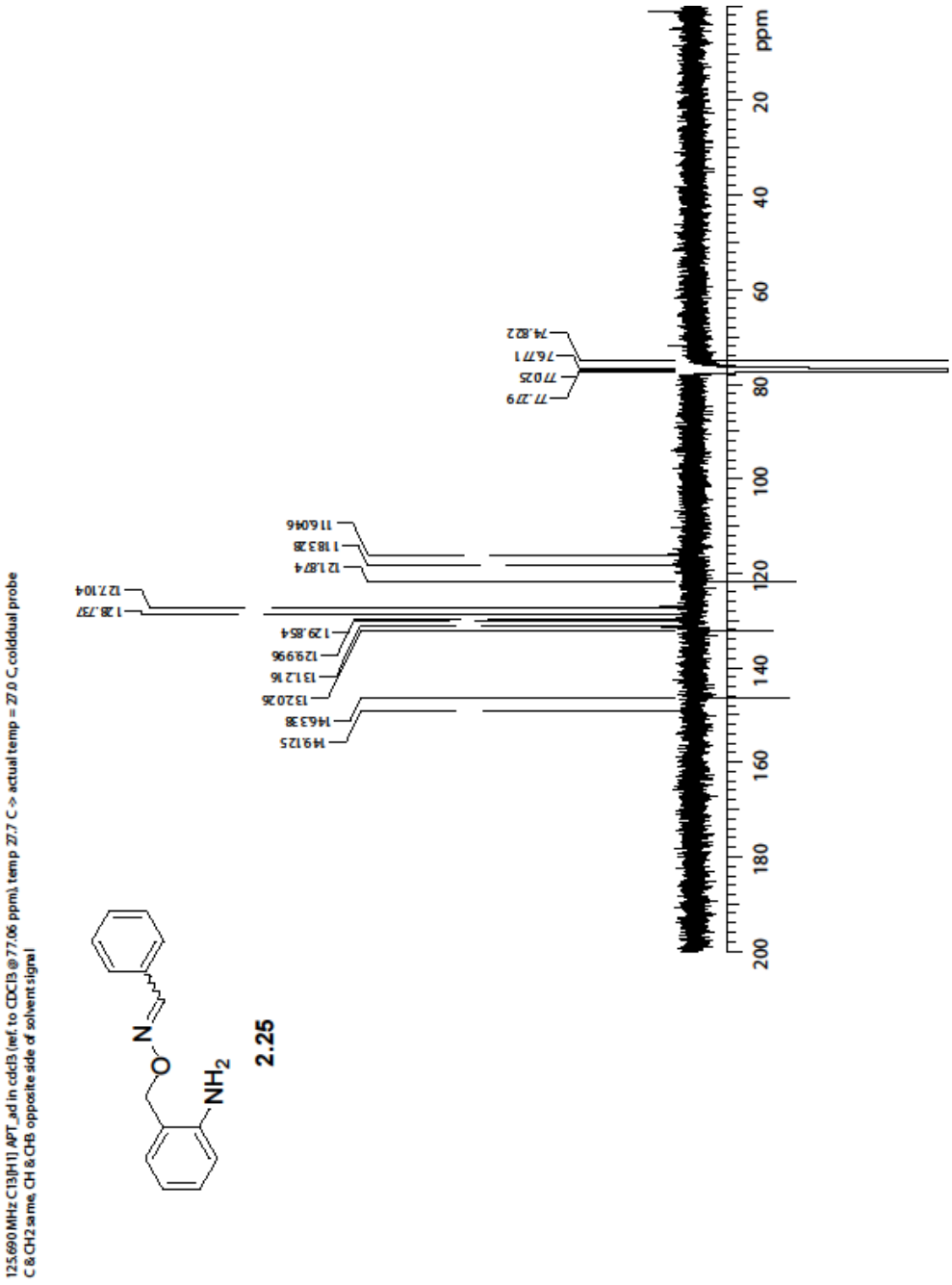
^{13}C NMR, $CDCl_3$, 125 MHz



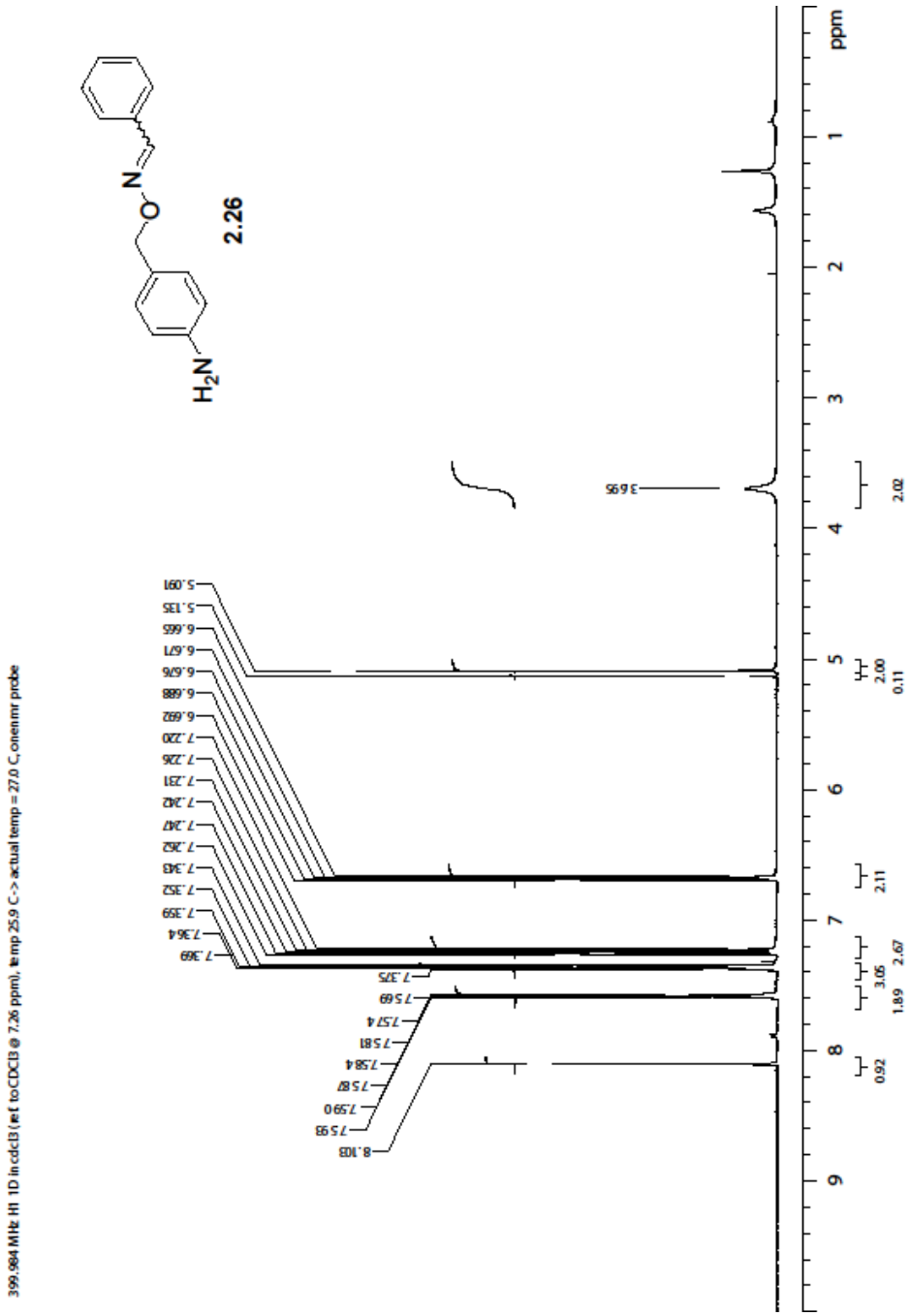
^{1}H NMR, $CDCl_3$, 500 MHz



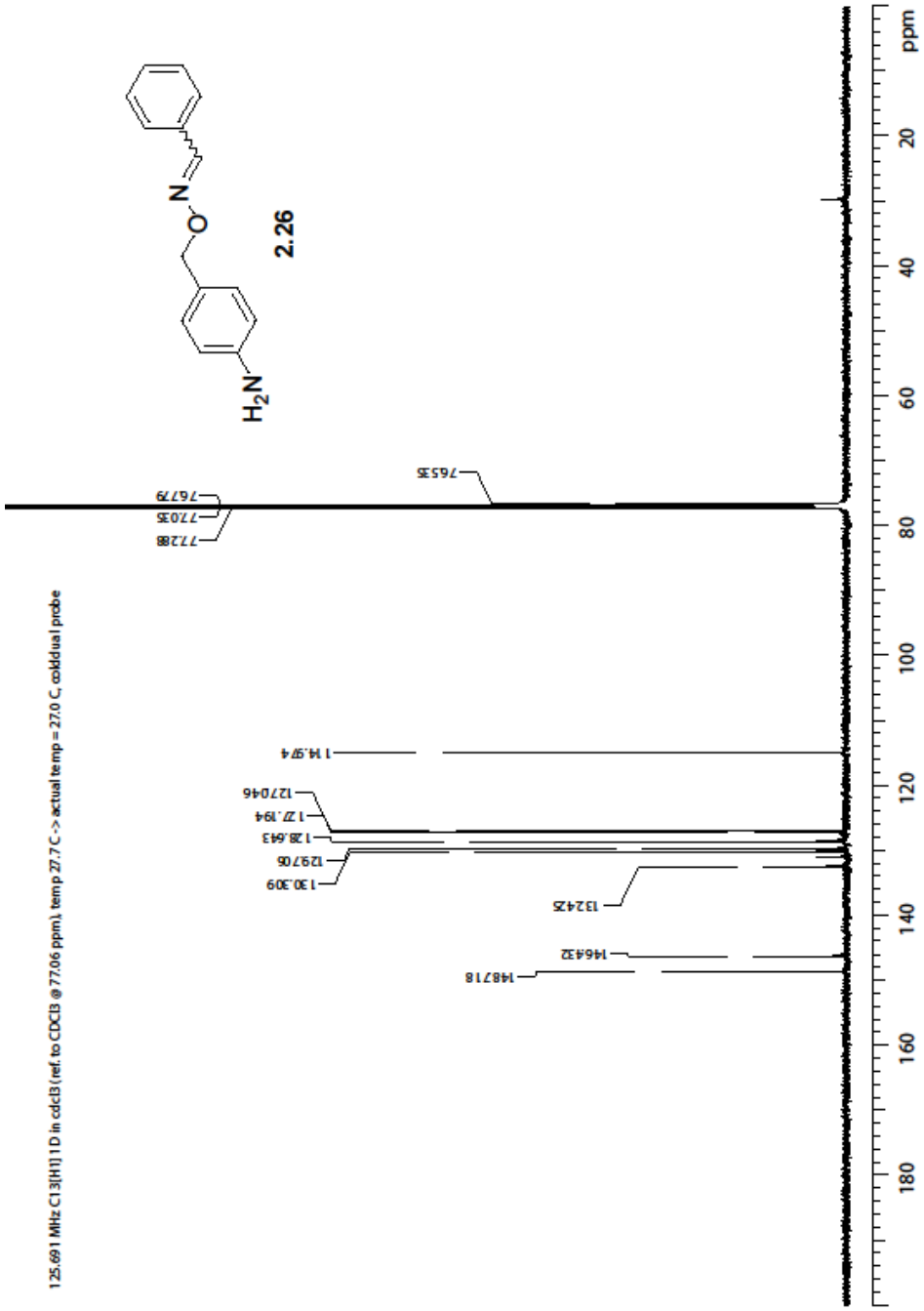
^{13}C NMR, $CDCl_3$, 125 MHz



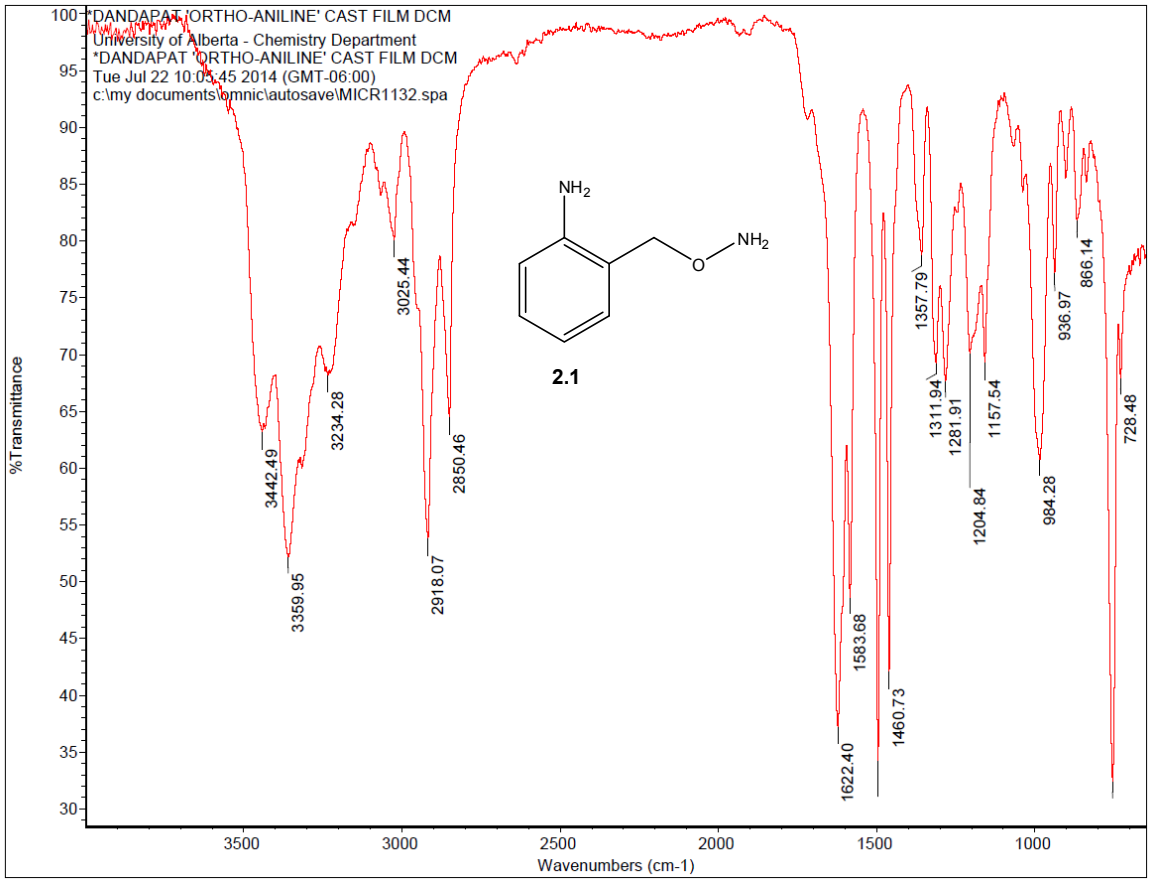
^{1}H NMR, $CDCl_3$, 400 MHz



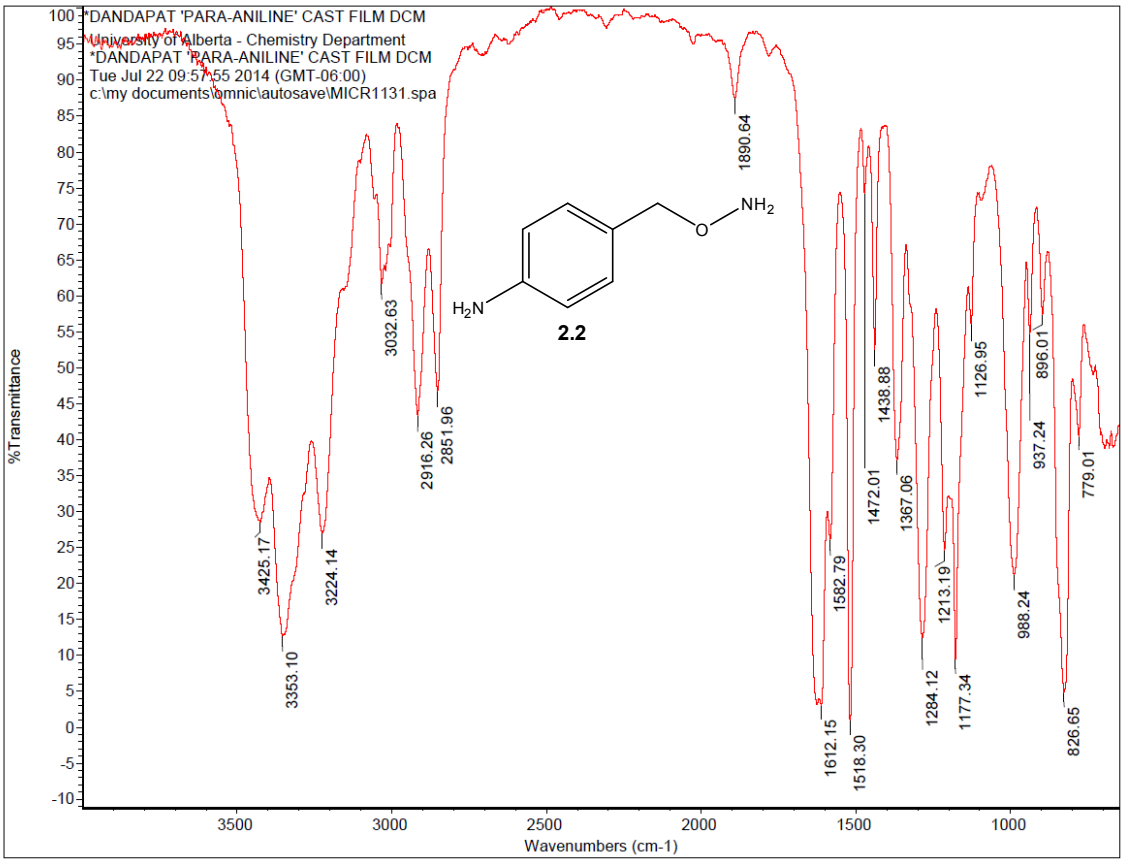
^{13}C NMR, $CDCl_3$, 125 MHz



1. FTIR spectrum for compound **2.1**

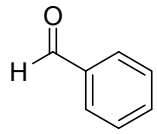
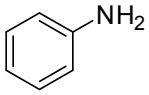
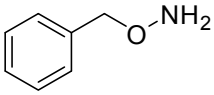
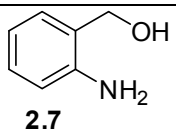
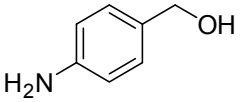
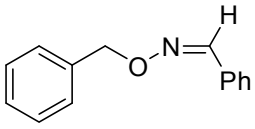
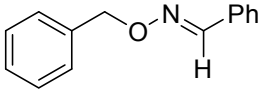


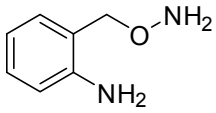
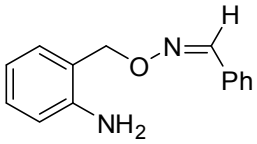
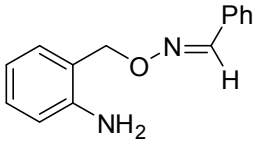
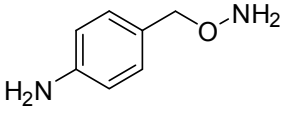
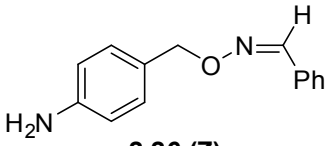
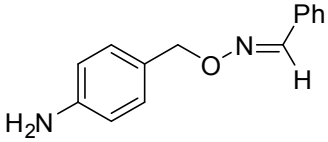
2. FTIR spectrum for compound **2.2**



HPLC Chromatograms

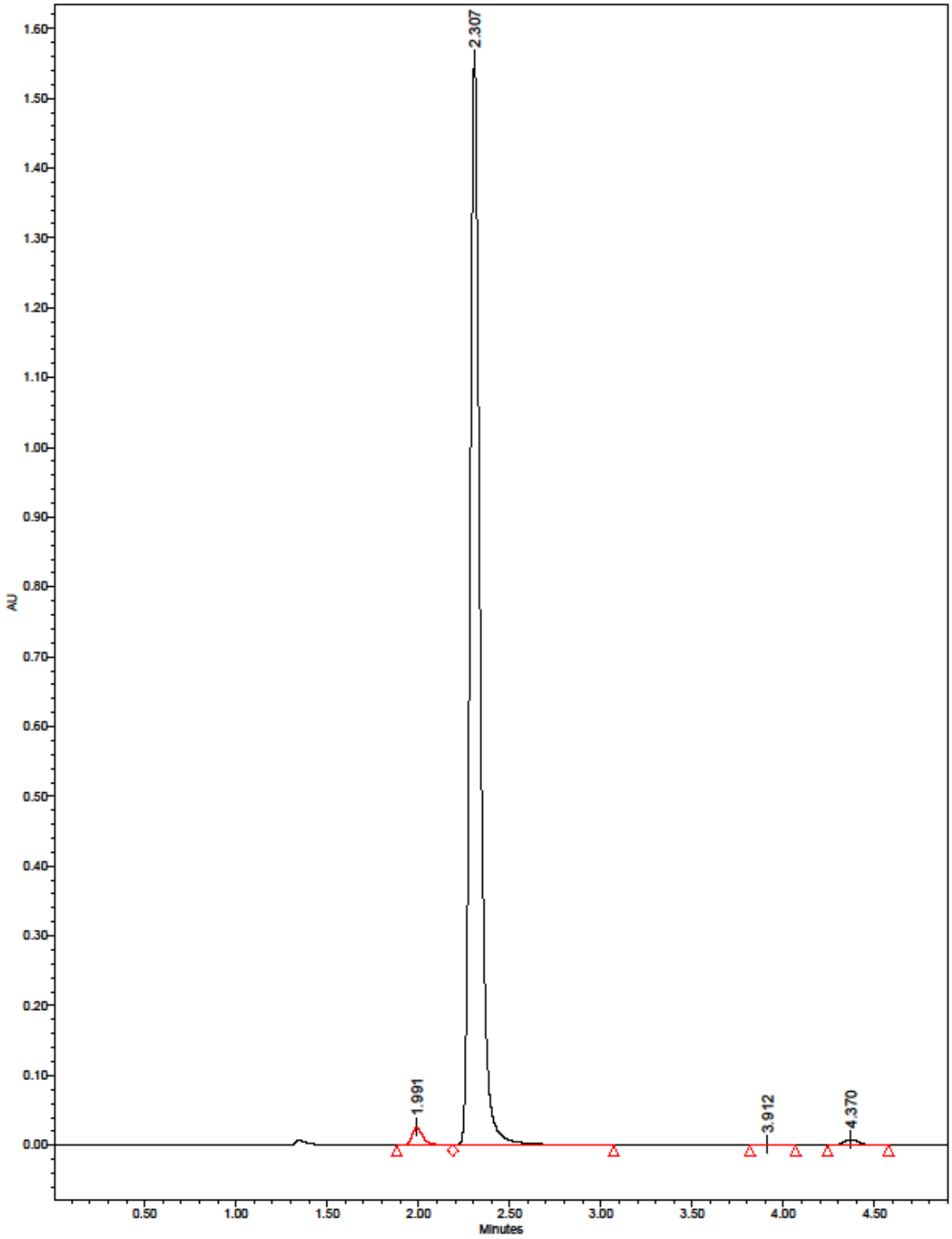
Table: List of molecules with their retention times using an isocratic condition (65% acetonitrile and 35% water).

Molecules	Retention time (min)
 **2.23**	2.3
 **2.27**	2.1
 **2.22**	1.9
 **2.7**	1.8
 **2.18**	1.7
 **2.24 (Z)**	3.9
 **2.24 (E)**	4.3

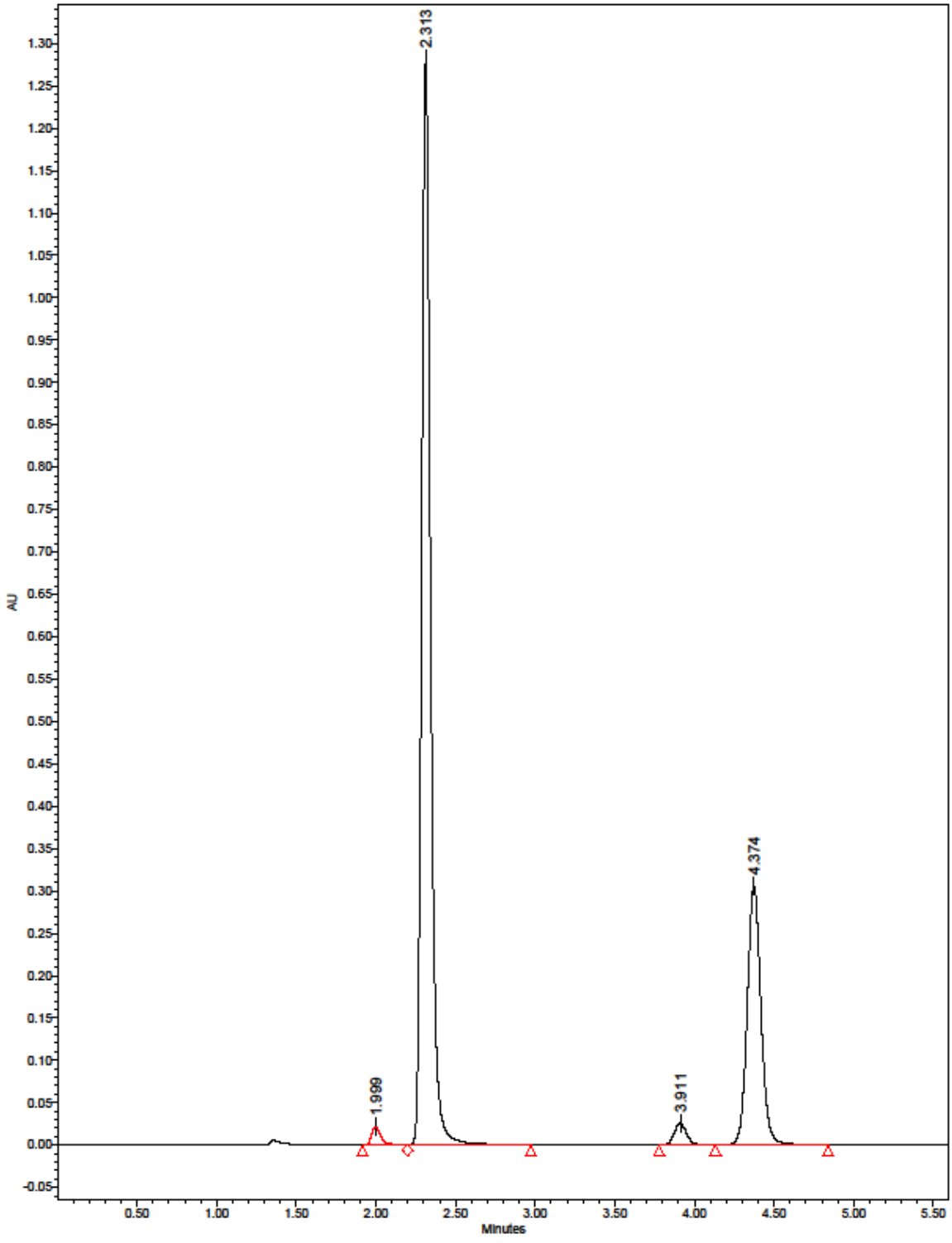
 **2.1**	1.8
 **2.25 (Z)**	3.0
 **2.25 (E)**	3.2
 **2.2**	1.7
 **2.26 (Z)**	2.7
 **2.26 (E)**	2.9

1. HPLC chromatograms of oxime ligation between **2.23** (1 mM) and **2.22** (1 mM) over times.

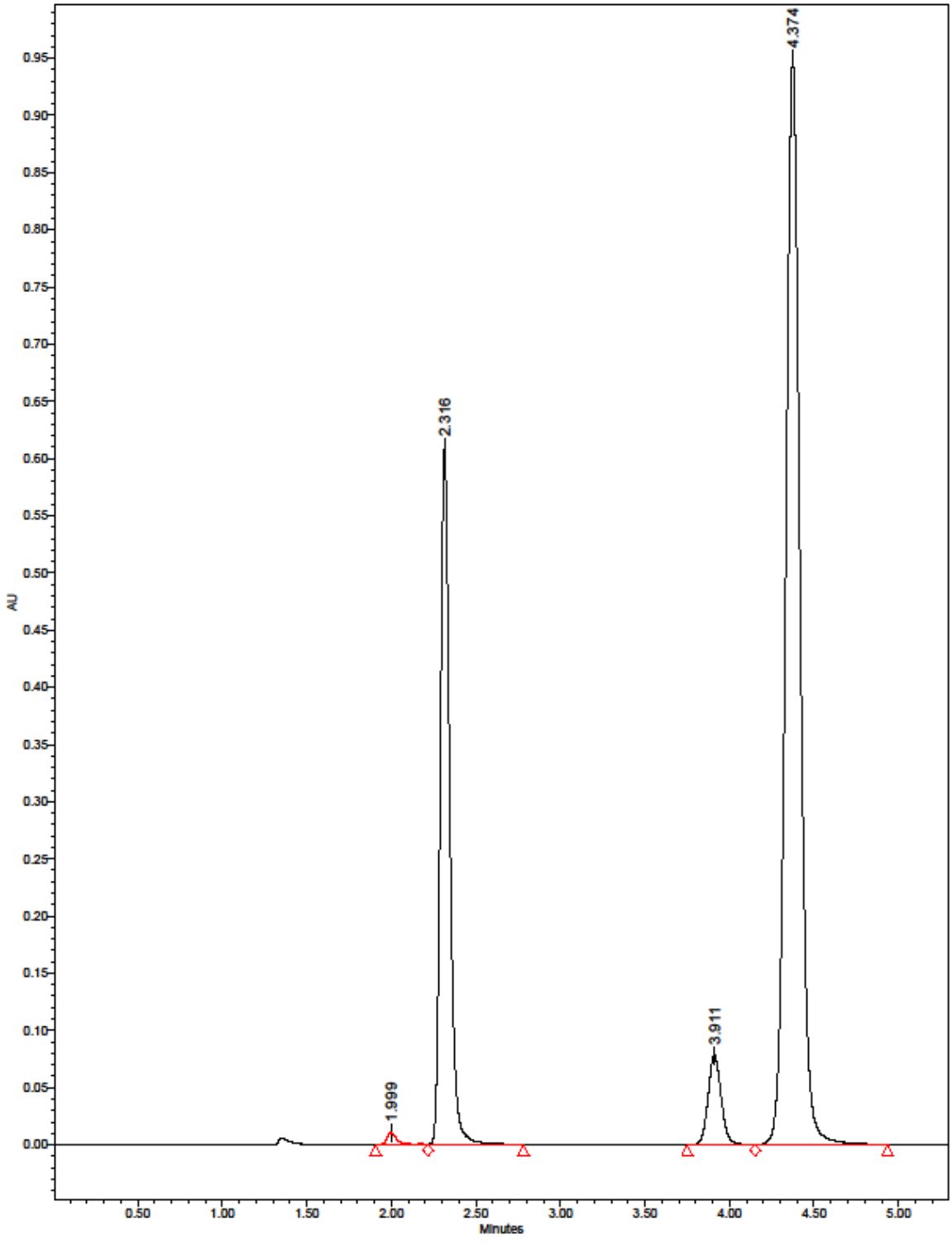
(A) 1.83 min



(B) 2.27 min

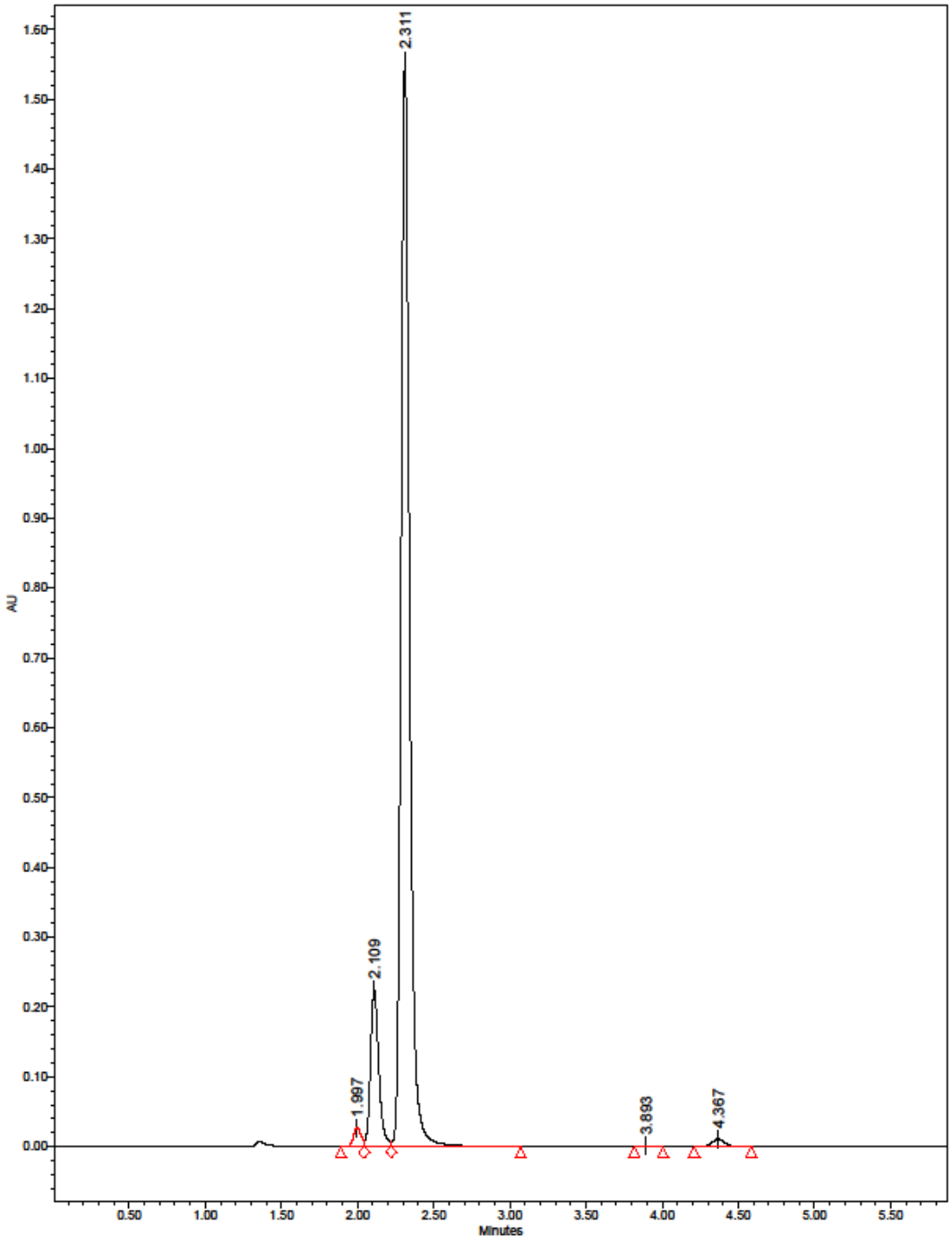

2.313
1.999
3.911
4.374
AU
Minutes
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50

(C) 1821 min

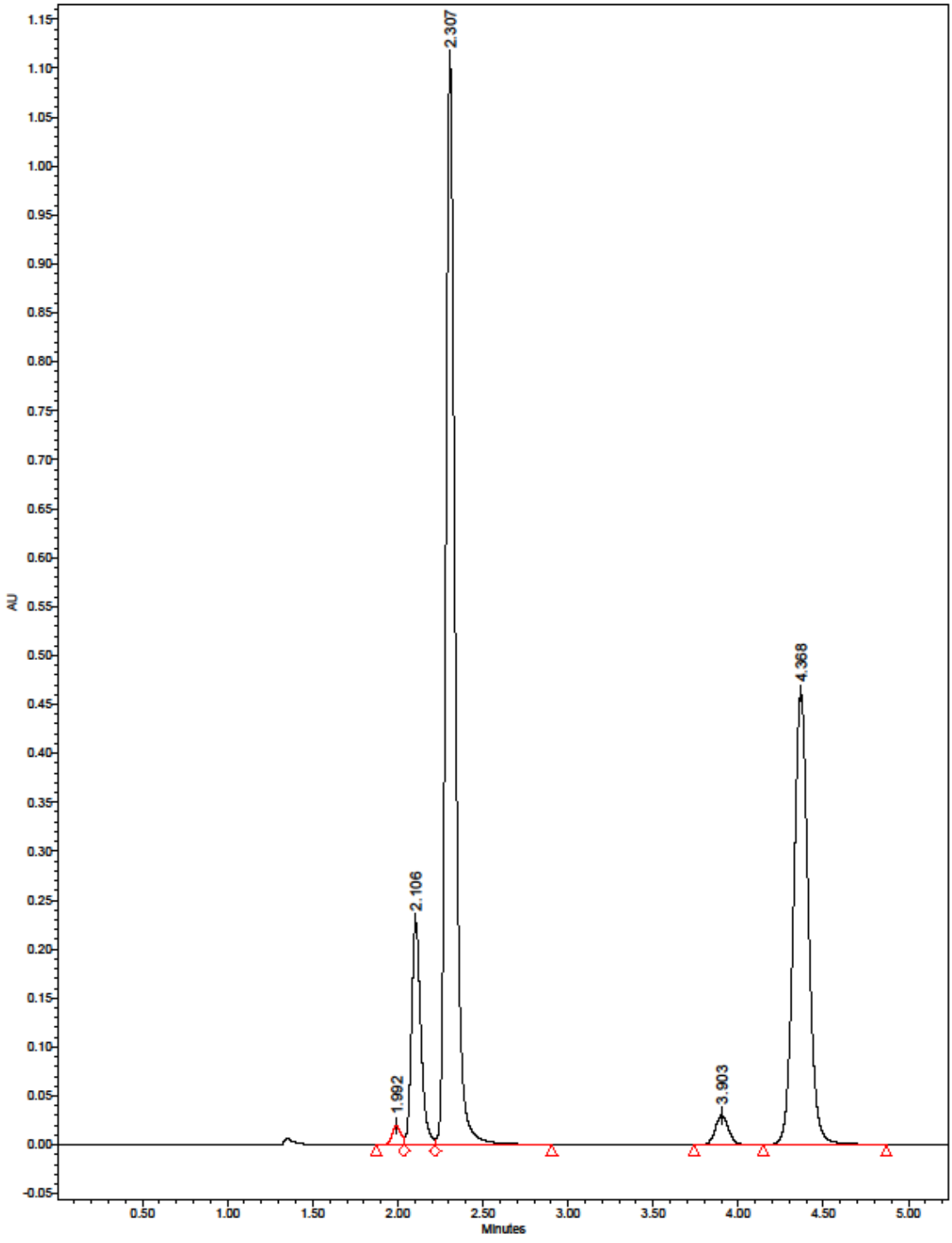

4.374
2.316
3.911
1.999
AU
Minutes
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00

2. HPLC chromatograms of oxime ligation between **2.23** (1 mM) and **2.22** (1 mM) using **2.27** (1 mM) as catalyst over times.

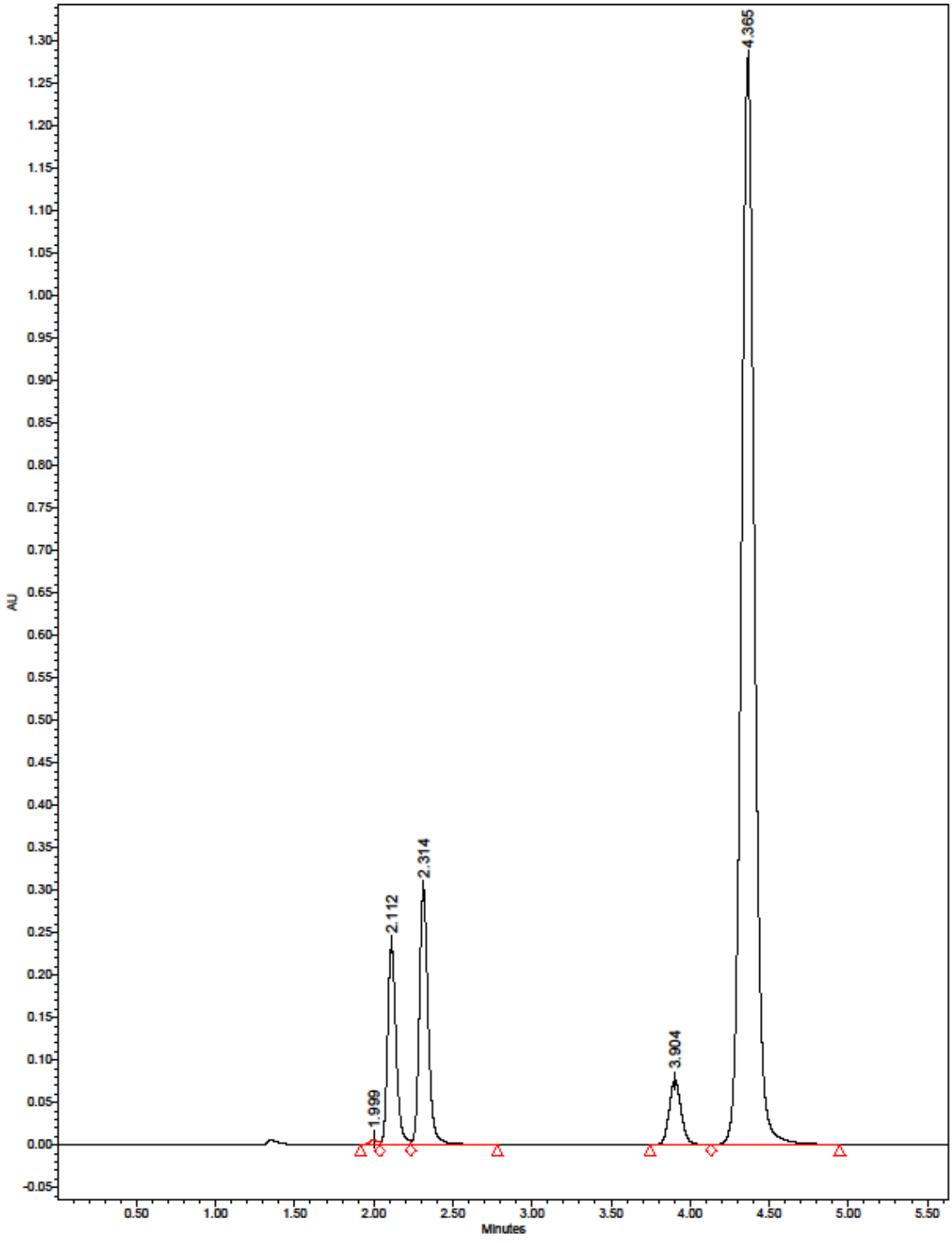
(A) 1.83 min



(B) 2.27 min

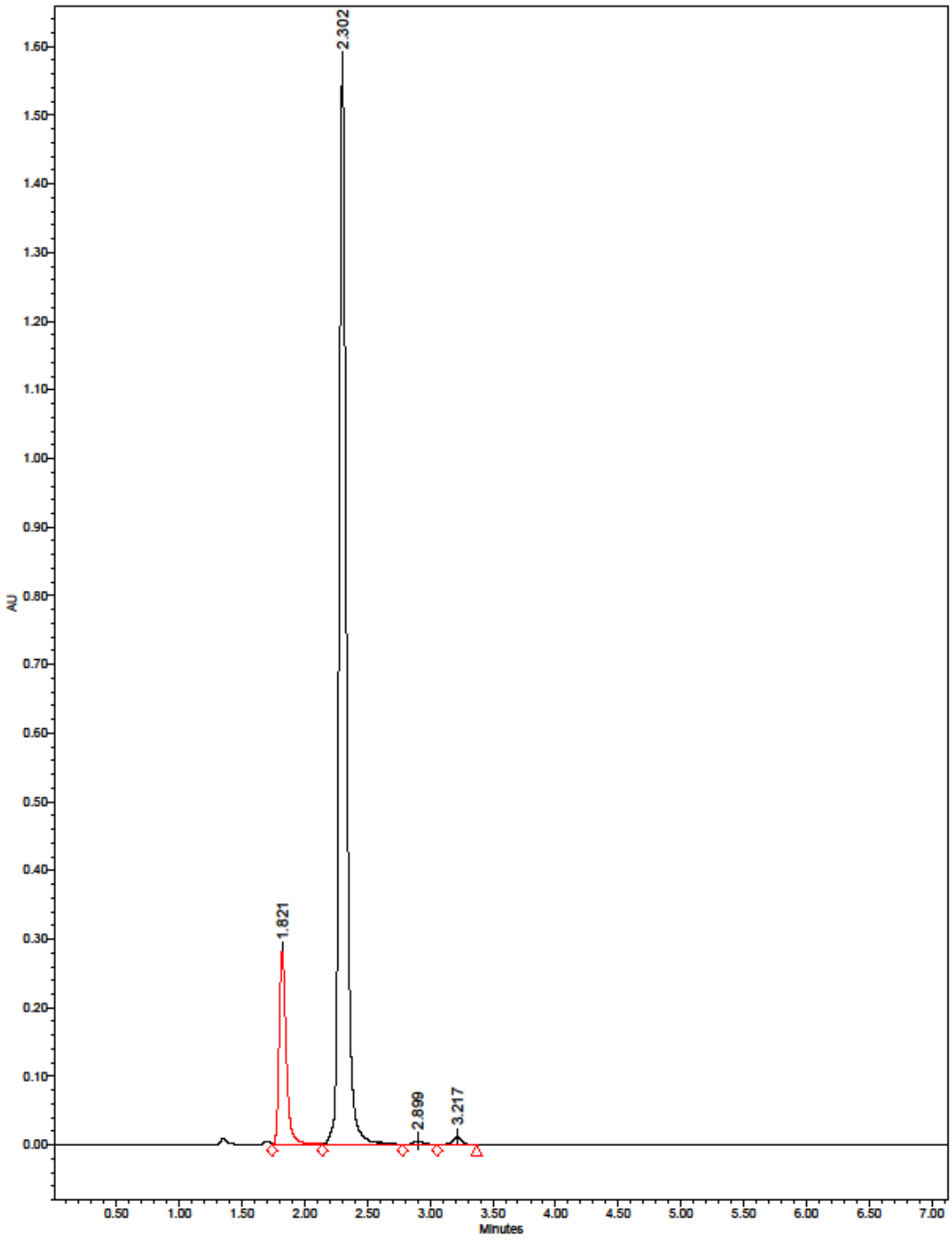

2.307
2.106
1.992
3.903
4.368
AU
Minutes
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00

(C) 1821 min

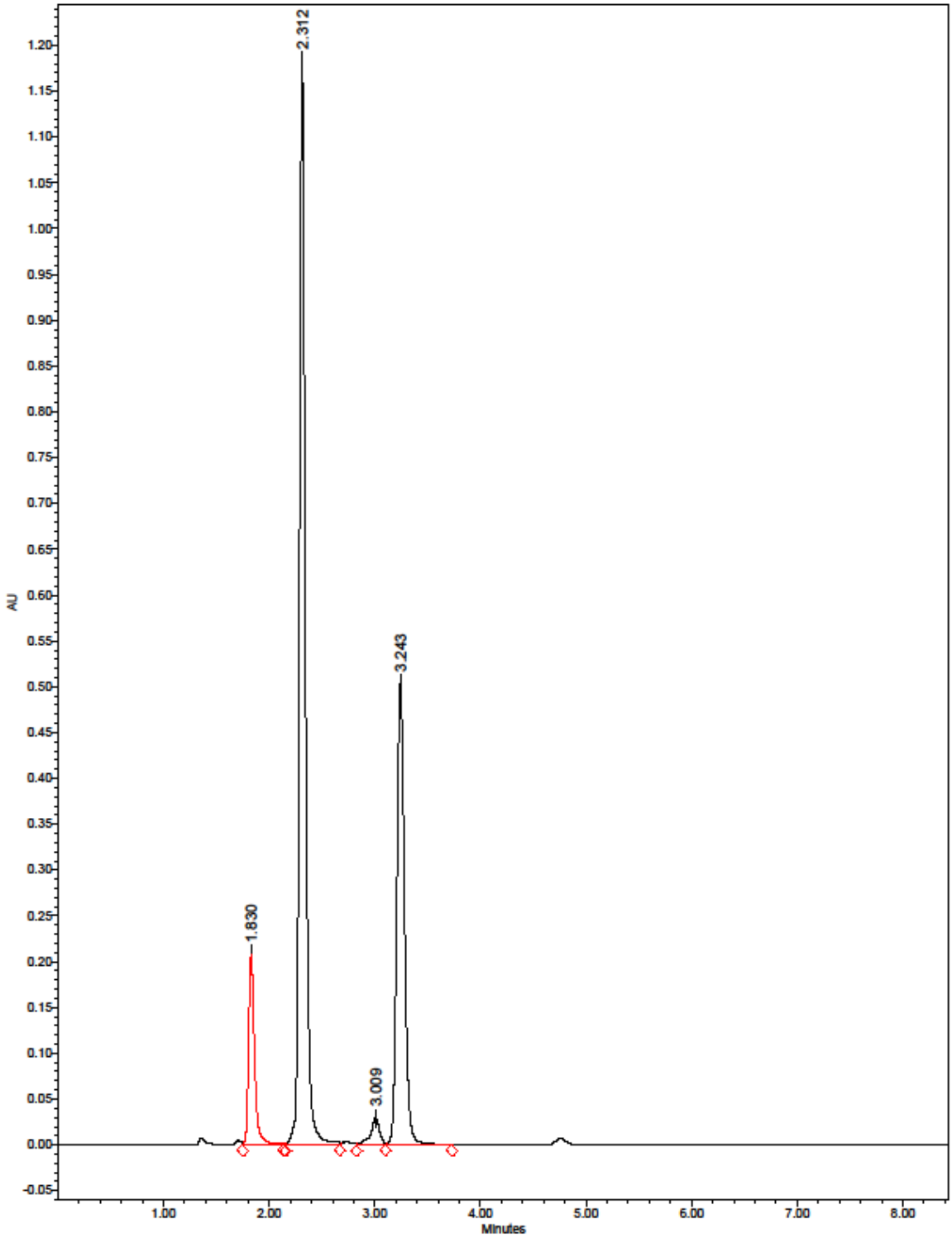

4.365
2.314
2.112
3.904
1.999
AU
Minutes
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50

3. HPLC Chromatograms of oxime ligation between **2.23** (1 mM) and **2.1** (1 mM) over times.

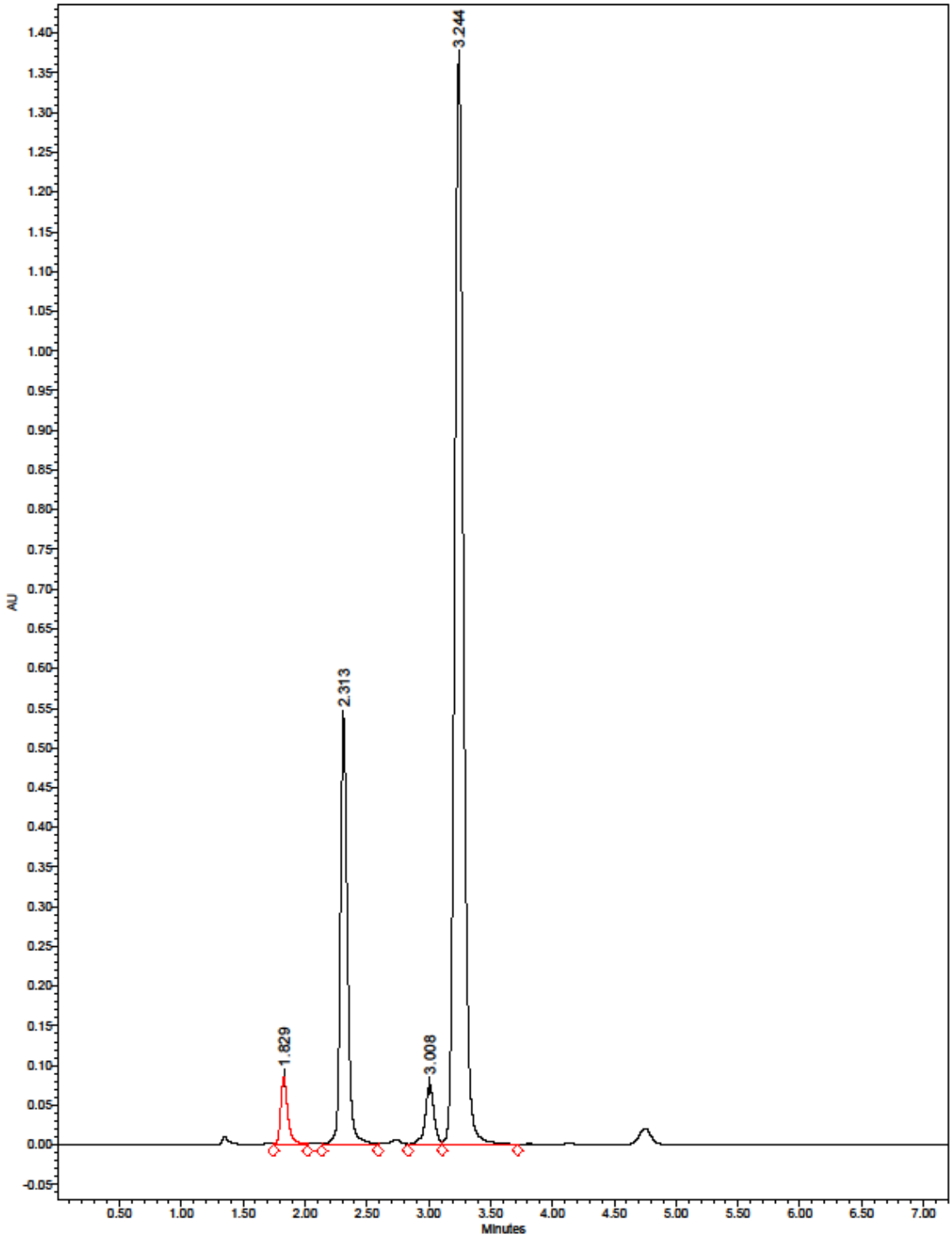
(A) 1.83 min



(B) 2.27 min

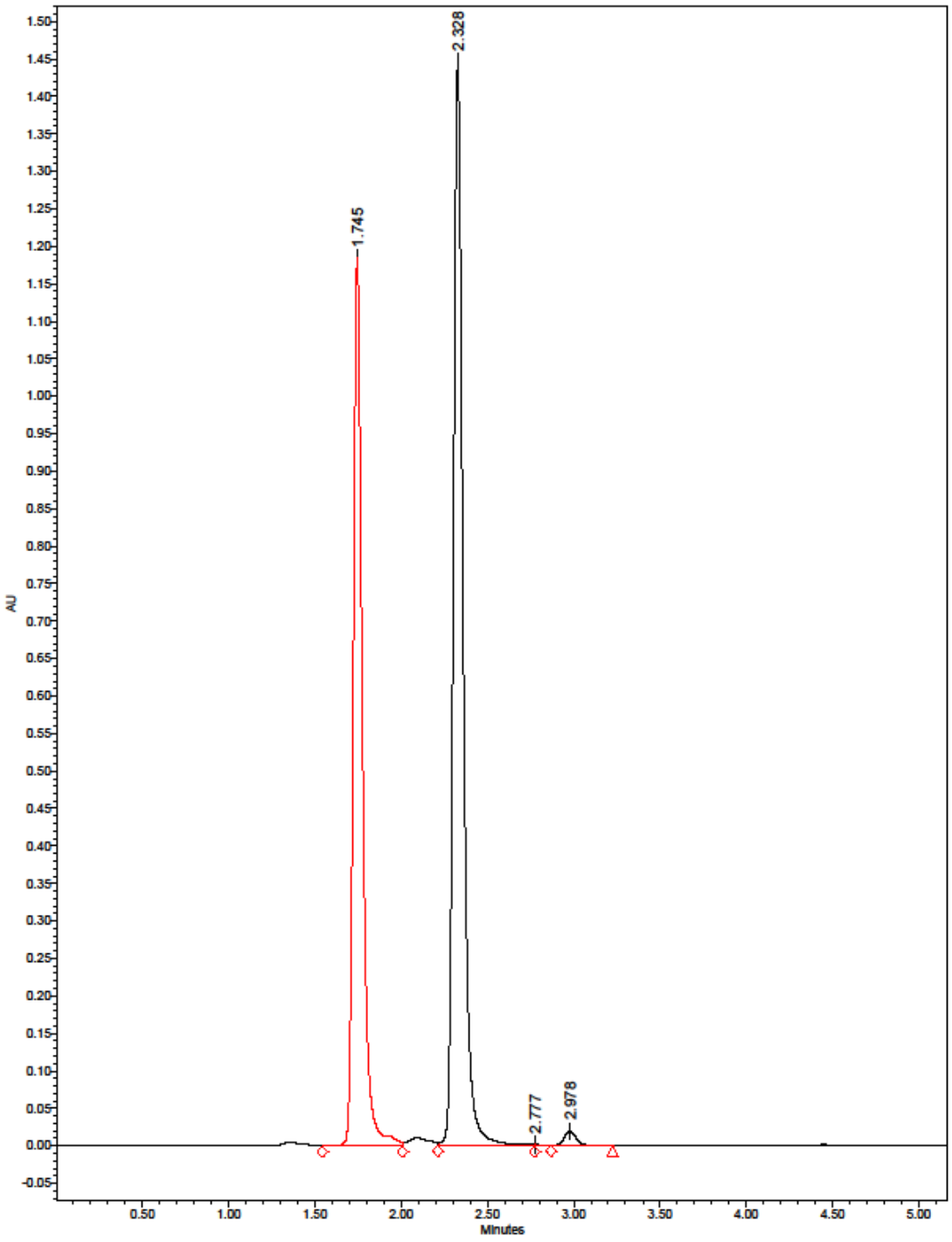

2.312
3.243
1.830
3.009
AU
Minutes
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00

(C) 1821 min

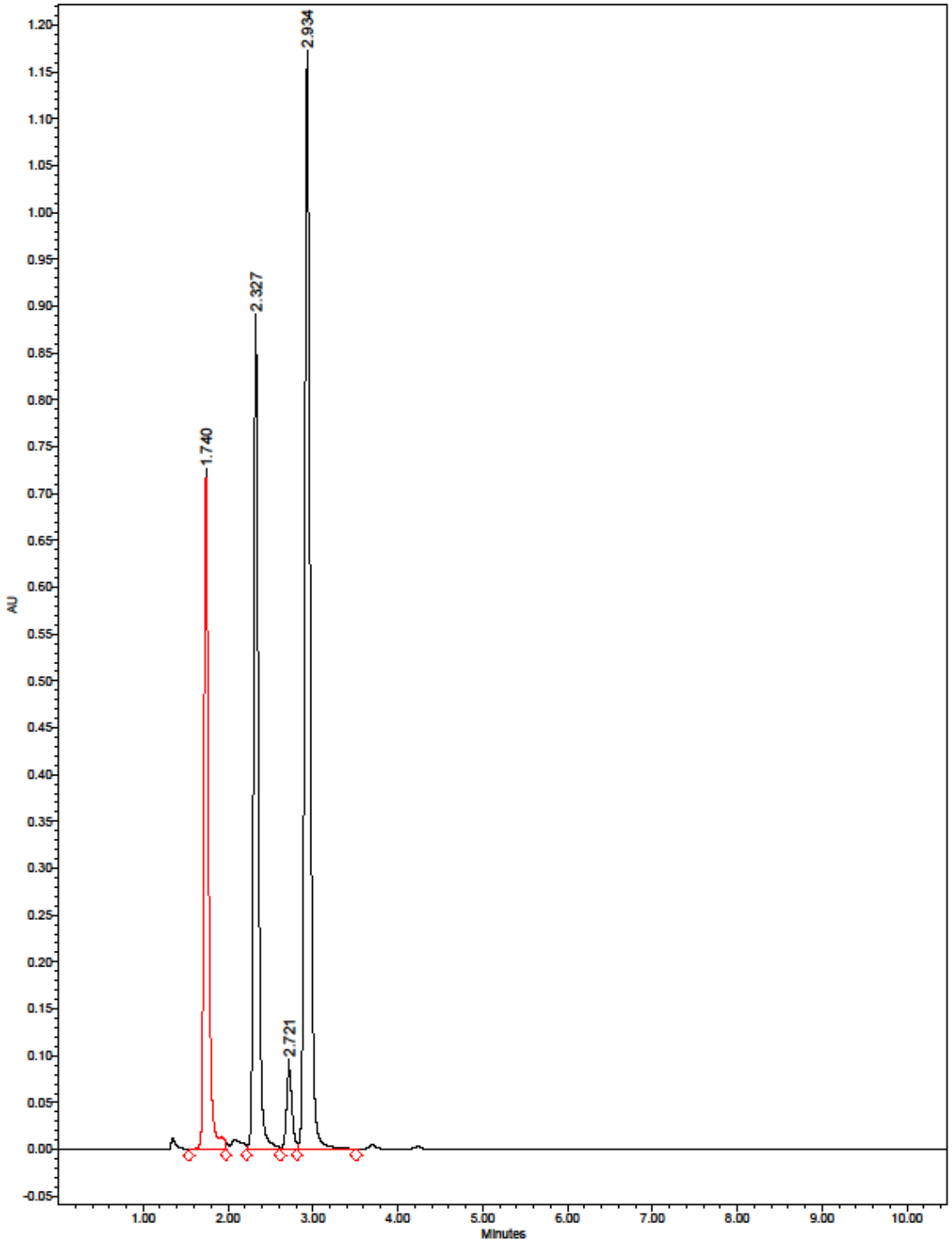

3.244
2.313
1.829
3.008
AU
Minutes
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50
6.00
6.50
7.00

4. HPLC Chromatograms of oxime ligation between **2.23** (1 mM) and **2.2** (1 mM) over times.

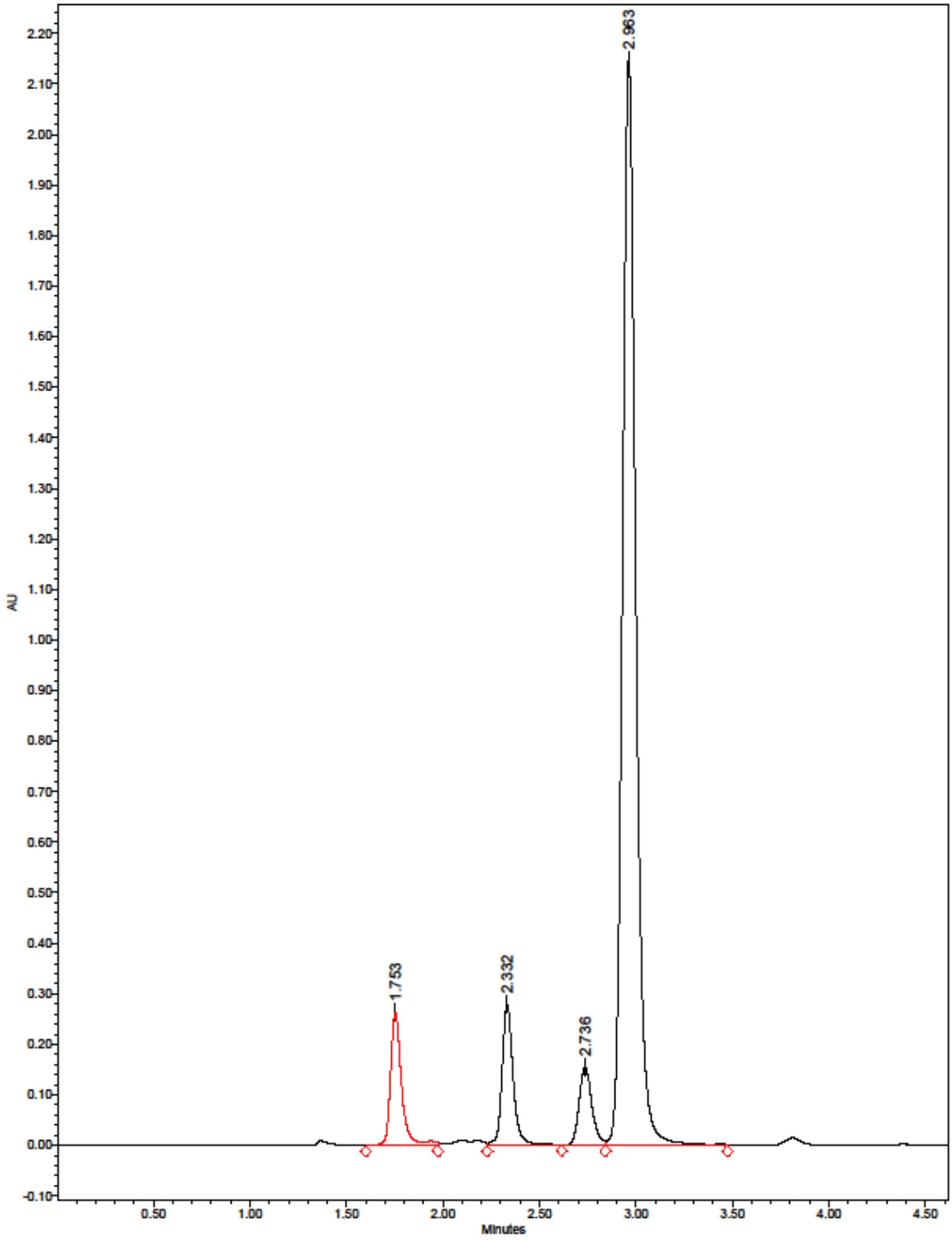
(A) 1.83 min



(B) 227 min

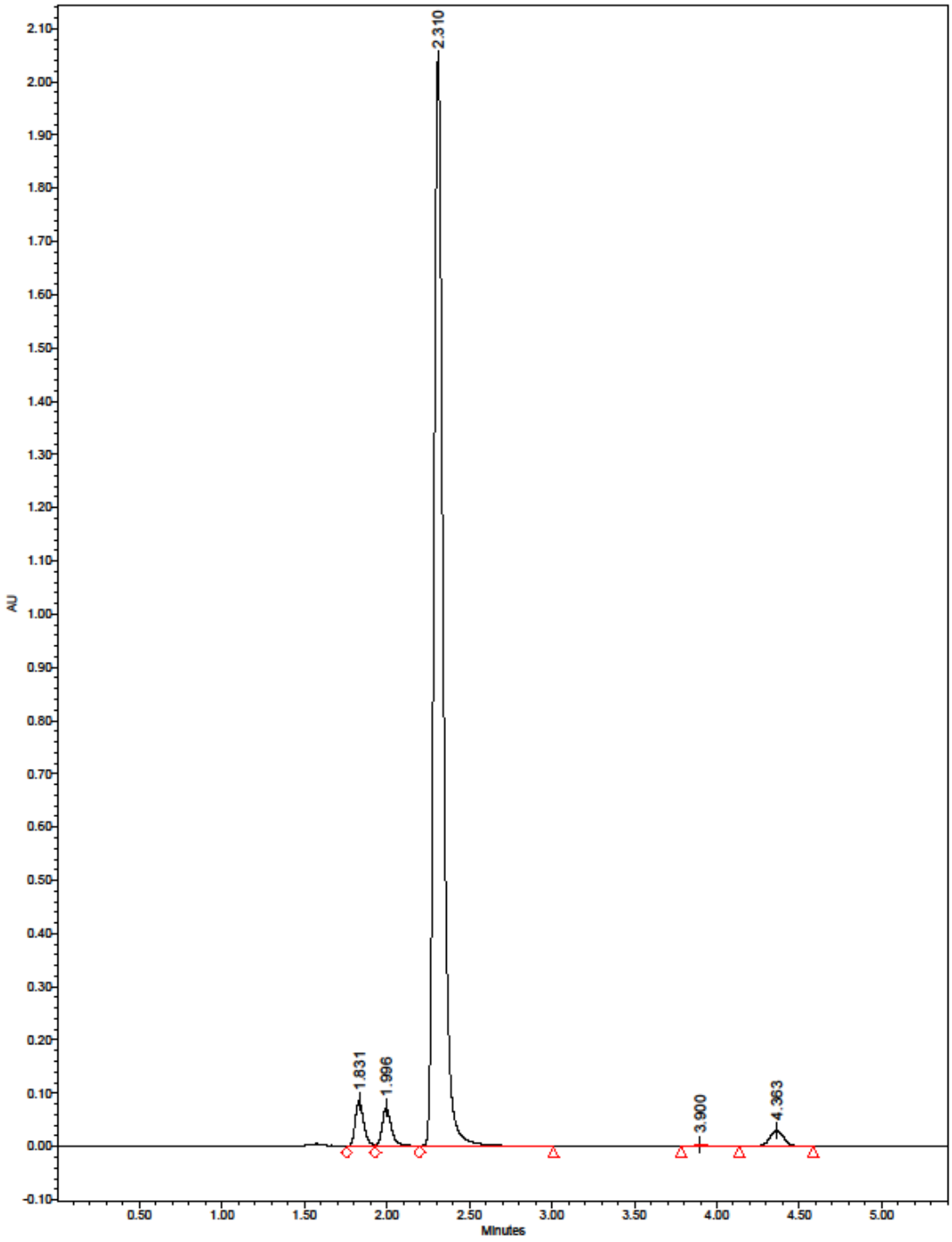

1.740
2.327
2.721
2.934
AU
Minutes
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00

(C) 1411.5 min

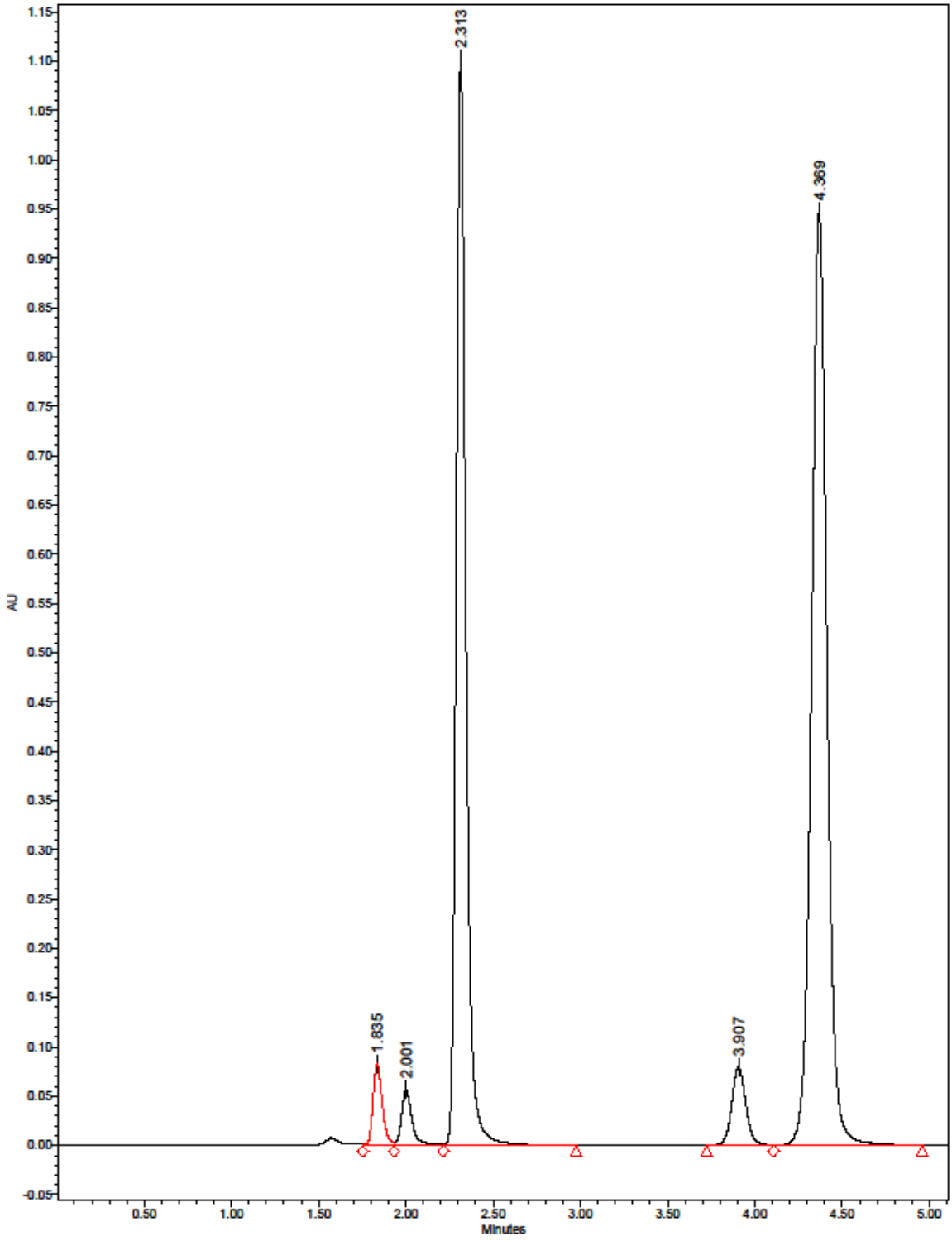

2.963
1.753
2.332
2.736
AU
Minutes
2.20
2.10
2.00
1.90
1.80
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50

5. HPLC chromatograms of oxime ligation between **2.23** (1 mM) and **2.22** (1 mM) using **2.7** (1 mM) as catalyst over times.

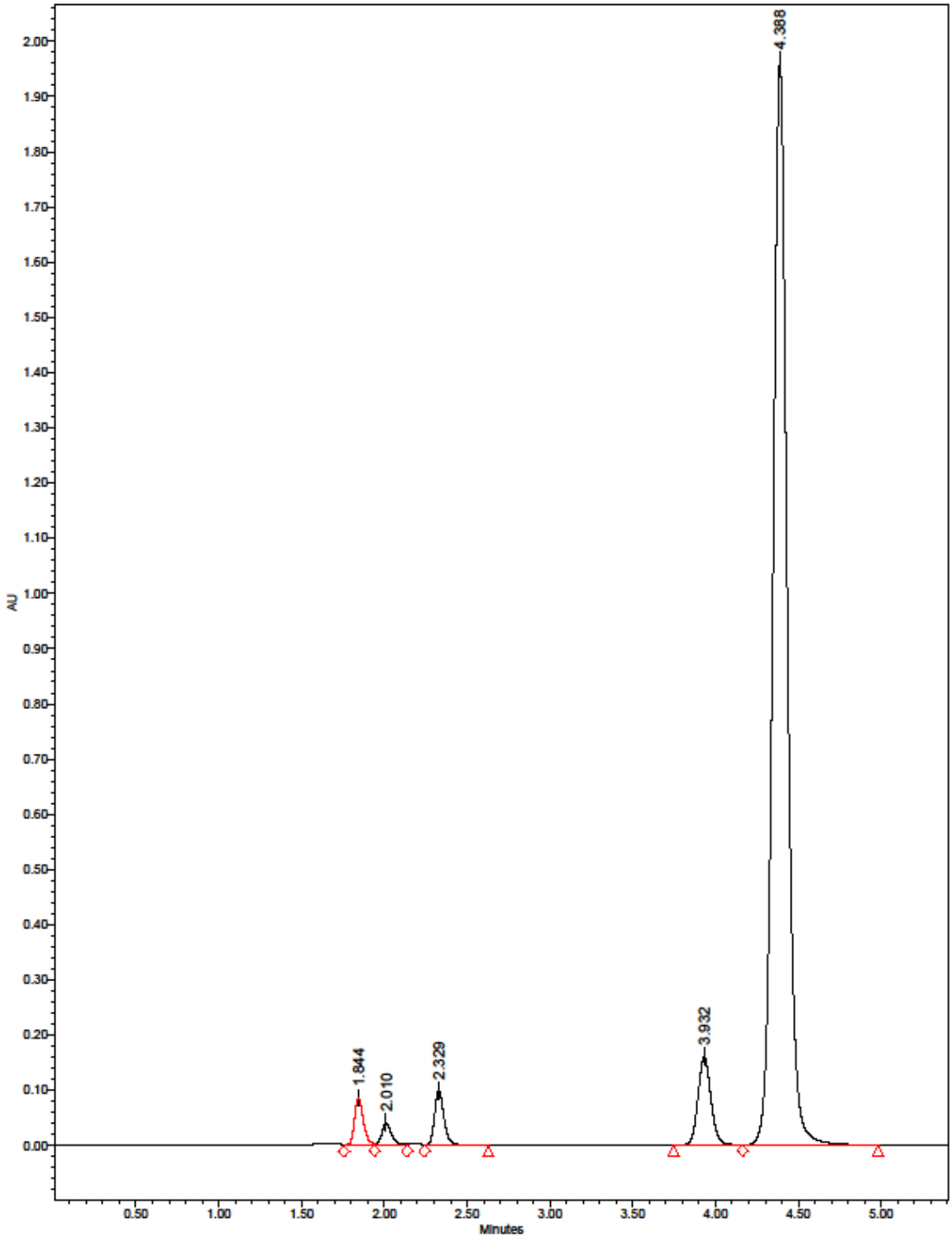
(A) 2.25 min



(B) 57.6 min

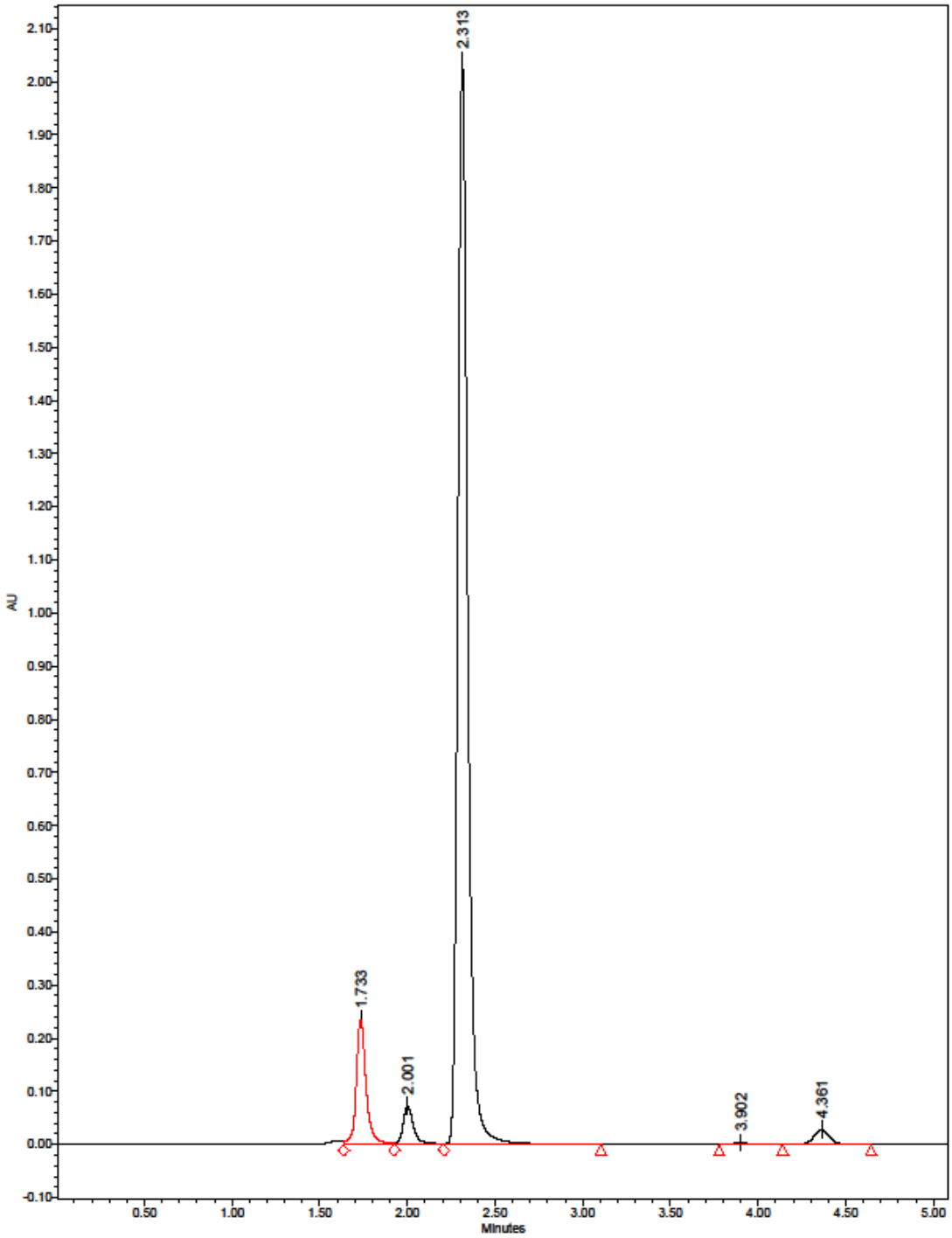


(C) 96.71 min

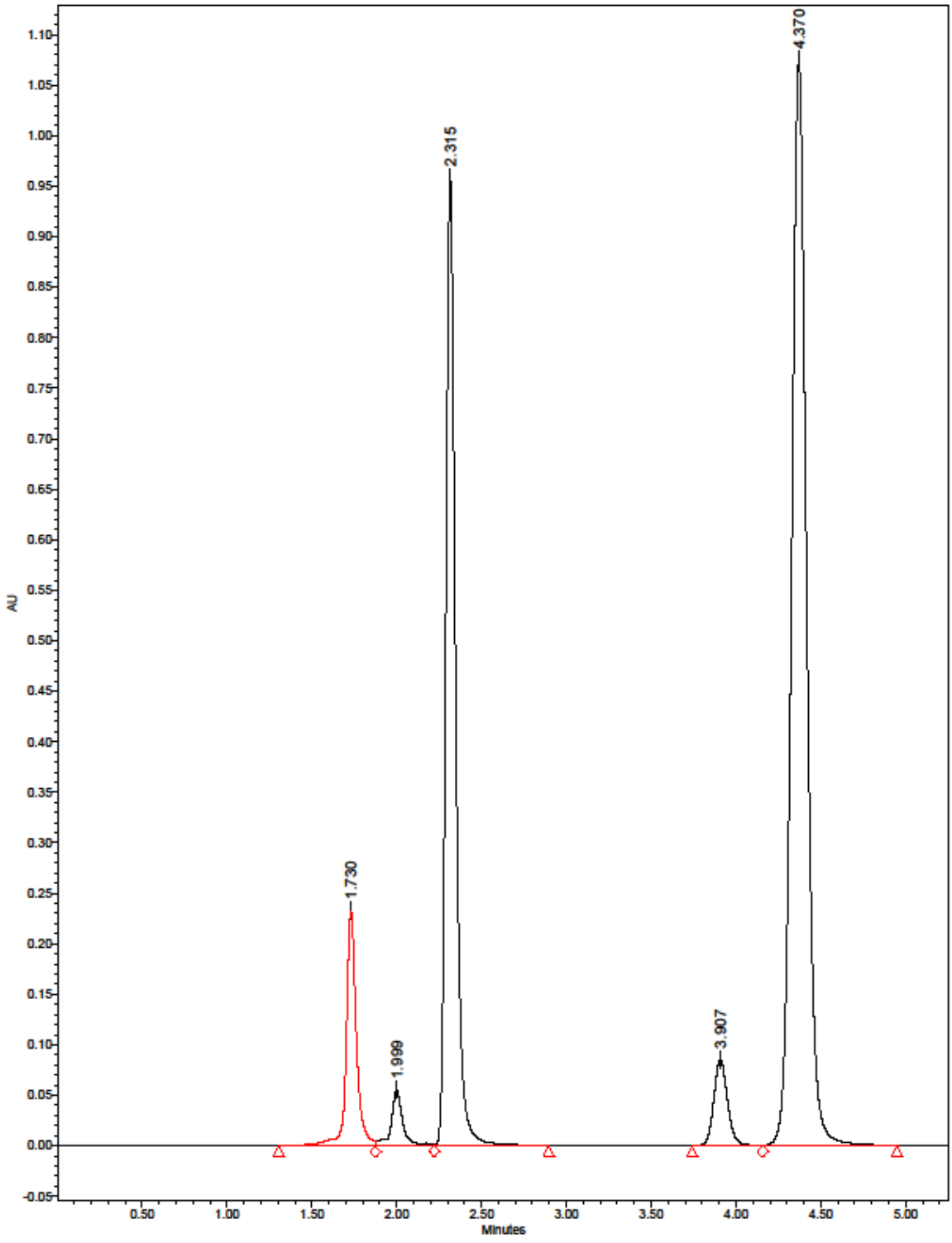

4.388
3.932
2.329
2.010
1.844
AU
Minutes
2.00
1.90
1.80
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00

6. HPLC chromatograms of oxime ligation between **2.23** (1 mM) and **2.22** (1 mM) using **2.7** (1 mM) as catalyst over times.

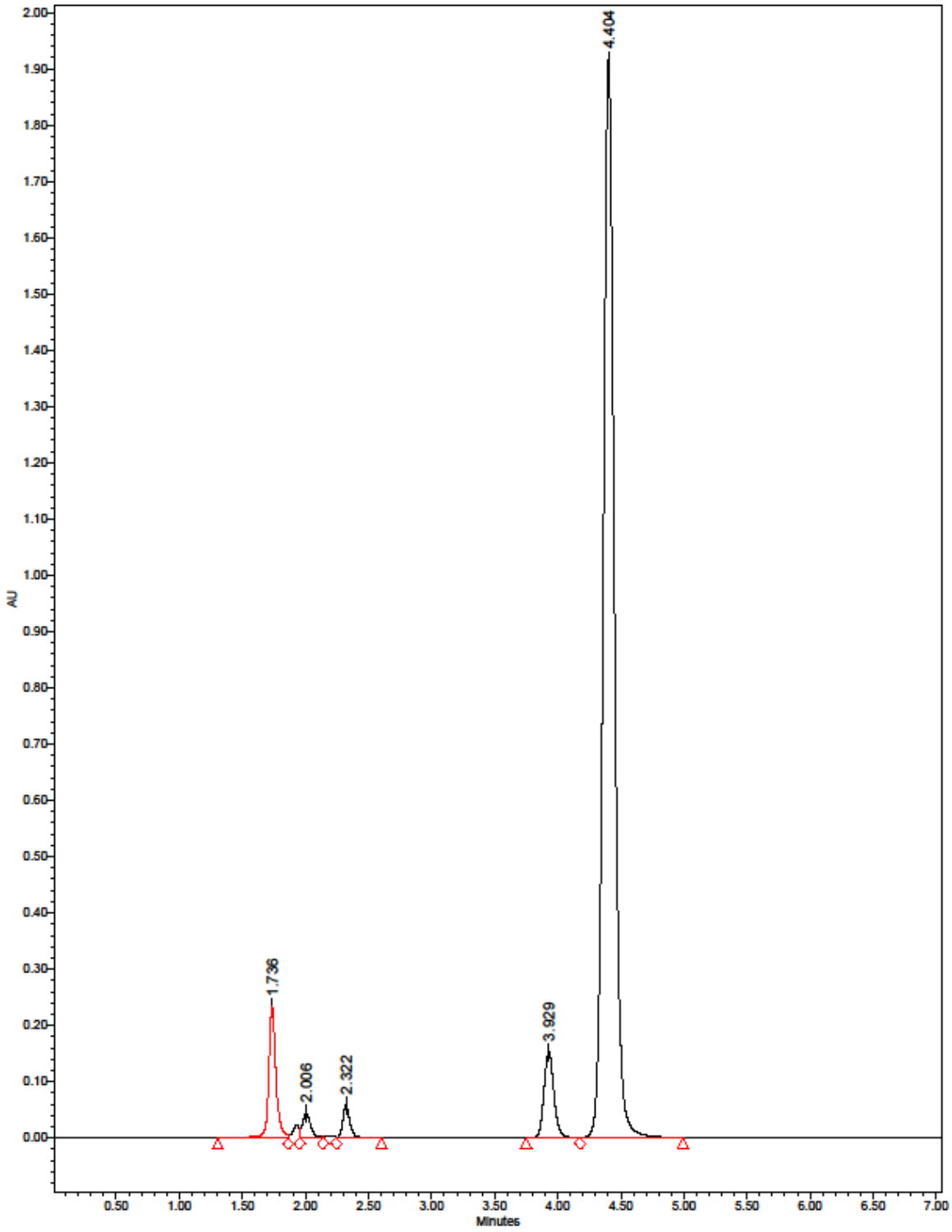
(A) 1.58 min



(B) 186.17 min


4.370
2.315
1.730
1.999
3.907
AU
Minutes
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00

(C) 1361.5 min


4.404
1.736
2.006
2.322
3.929
AU
Minutes
2.00
1.90
1.80
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50
6.00
6.50
7.00