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Chemoenzymatic synthesis of NAADP derivatives : probing the unknown NAADP receptor

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A Dissertation

Entitled

Chemoenzymatic Synthesis of NAADP Derivatives: Probing the Unknown NAADP Receptor

By

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Submitted as partial fulfillment of the requirements for the

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An Abstract of

Chemoenzymatic Derivatives of NAADP: Probing and Possible Characterization of the Unknown NAADP Receptor

Christopher J. Trabbic

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is the most potent, yet least well characterized of known 2nd messenger responsible for Ca^{+2} mediated signaling. The identity of NAADP binding protein and details concerning its metabolism are subjects for debate. Previous studies uncovering the structure activity relationship (SAR) have provided valuable information regarding ligand-binding interactions. We further this work by demonstrating the amount of substitution at specific positions on NAADP which retain both binding and activity to the sea urchin NAADP receptor. Specifically, the 5 nicotinyl, as well as the 8 adenosyl positions have been identified as "structurally neutral" sites for chemical substitution. The NAADP analogs were synthesized by a chemoenzymatic approach. Nicotinic acid derivatives with chemical substitution at the 5 position were often synthesized by the Sonogashira reaction and provided a set of unique pyridine bases with various functional groups attached. NAD and NADP analogs with substitution at the 8 position were also synthesized. These derivatives can be subjected to an enzyme catalyzed base exchange reaction to form various mono-substituted NAADP derivatives at the 5 or 8 position, as well as di-substituted NAADP derivatives with

substitution at both the 5 and 8 positions. The NAADP analogs were evaluated in a sea urchin homogenate system and assayed for their ability to release Ca^{2+} from intracellular stores and to compete with radiolabeled NAADP in competition-ligand binding experiments. Ligand-receptor binding was also measured by desensitization experiments. Ultimately, substitution at either the 5 or 8 position on NAADP was well tolerated by the NAADP binding protein. Furthermore, di-substituted compounds were also well recognized.

The two-pore channel (TPC) was shown to be controlled by NAADP and was considered a possible NAADP receptor. Recently, photoaffinity labeling (PAL) studies suggested that the NAADP receptor was not the TPC, but instead a novel set of proteins that were not identified through mass spectrometry, suggesting the need for further purification. Based upon the structure activity relationship observed with di-substituted NAADP analogs, we could synthesize a bifunctional NAADP derivative using a chemoenzymatic approach. Containing an aromatic azide for PAL and an aliphatic azide to be utilized as a "click" purification tag, we propose a compound that could possibly label and aid in purification of the NAADP receptor, leading to eventual identification.

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List of Abbreviations

AcOH	acetic acid
ACN	acetonitrile
ADPRC	ADP-ribosyl cyclase
ADP-ribose	adenine dinucleotide phosphate ribose
ADPRP	adenine dinucleotide phosphate ribose phosphate
aq.	aqueous
cADPR	cyclic ADP-ribose
cADPRP	cyclic-ADP-ribose phosphate
CCK	cholecystokinin
CICR	calcium induced calcium release
DAG	diacylglycerol
DCM	dichloromethane
Da	Dalton
DIPEA	diisopropylethyl amine
DMSO	dimethylsulfoxide
EC_{50}	half maximal effective concentration
ER	endoplasmic reticulum
ESI	electrospray ionization
EtOAc	ethyl acetate
EtOH	ethanol
h	hours
HPLC	high-performance liquid chromatography

IC_{50}	half maximal inhibitory concentration
IP3	inositol trisphosphate
MALDI	matrix assisted laser desorption ionization
MeOH	methanol
mm Hg	millimeters of mercury
MMPP	magnesium monoperoxypthalate
m.p.	melting point
NA	nicotinic acid
Nic	nicotinamide
PLC	phospholipase C
SR	sarcoplasmic reticulum
SAR	structure activity relationship
TXPTS	tris-(2,4-dimethyl-5-sulfophenyl)-phenyl phosphine trisodium salt
TEA	triethylamine
TLC	thin-layer chromatography
Tosyl	*p*-toluene sulfonyl
UV	ultraviolet

Chapter 1

Introduction

Calcium ion (Ca^{2+}) is a versatile, ubiquitous intracellular signal responsible for controlling a number of cell processes, including but not limited to fertilization, apoptosis, cell differentiation and proliferation, learning and memory, secretion, and contraction.[1] Stimulation of Ca^{2+} release from intracellular stores is accomplished by intracellular 2nd messengers which interact with specific receptors to control Ca^{2+} selective ion-channels. In response to extracellular stimuli, these messenger molecules are released and initiate a complex intracellular signal, ultimately converting extracellular stimulation into a highly regulated spatio-temporal physiological response.[2] Unlike many 2nd messengers, calcium ion is not made by *de novo* synthesis, nor is it metabolized into an inactive byproduct. Rather, it is sequestered through the process of active transport and released from intracellular stores in response to specific signals. The cell's intrinsic ability to respond to changes in intracellular calcium ion concentrations is of paramount importance for calcium dependent processes and cell survival. Simply stated, at resting state intracellular Ca^{2+} concentrations are low (100 nM) and rise upon activation (1000 nM), enabling physiological Ca^{2+} dependent processes to occur.

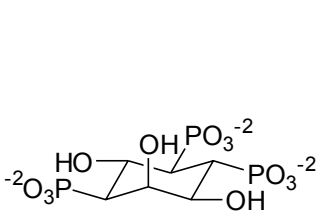
In response to specific stimuli, intracellular calcium ion is mobilized, thereby increasing the intracellular Ca^{2+} concentration leading to activation of calcium sensitive enzymes. A variety of mechanisms, including G-protein coupled receptors, Ca^{2+} binding

proteins, calcium buffers, and channels, such as voltage-operated channels (VOC's) and receptor-operated channels (ROC's), mediate Ca^{2+} dependent processes. Once Ca^{2+} has carried out its signaling function, it is rapidly removed from the cytoplasm to restore the low intracellular Ca^{2+} concentration. This is accomplished by a variety of pumps and exchangers, including the plasma membrane Ca^{2+}-ATPase pump and Na^{+}/Ca^{2+} exchangers which expel intracellular Ca^{2+} to the outside, as well as the sarco-endoplasmic reticulum ATPase pumps (SERCA) which return Ca^{2+} to internal stores.[3] Ca^{2+} signaling can be described as a spatio-temporal dependent process. Depending on the location and type of cell, the increase in Ca^{2+} concentration occurs in prolonged spikes or oscillations, as is observed following fertilization, or in rapid, short bursts as seen with muscle contraction. Three known endogenous Ca^{2+} mobilizing second messengers are known and are well-characterized: D-myo-inositol trisphosphate (IP3), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP). Each utilizes a unique receptor and each is capable of producing different time dependent Ca^{2+} signals.[4,5] Many different calcium signaling pathways coexist to regulate a multitude of different functions within humans. Discovery of the underlying mechanisms regulating the release and reuptake of Ca^{2+} will identify targets for drug discovery and will provide physiological insight onto the inner workings of the human body at the most elementary level.

The best characterized and the first discovered of the three known endogenous Ca^{2+} mobilizing 2^{nd} messengers, IP3 (**Figure 1**) serves as an excellent model and a paradigm for the development of drug therapies. Metabolism of IP3 occurs through activation of G-protein coupled receptors which in turn activate phospholipase C (PLC),

Activated PLC cleaves the membrane associated phosphoinositidyl diphosphate yielding two products: IP3 and diacylglycerol (DAG). The former binds to the IP3 receptor, a tetrameric protein complex, mobilizing intracellular Ca^{2+} from endoplasmic reticulum (ER) stores, or the muscle equivalent sarcoplasmic reticulum (SR).[6] More recently, Ca^{2+} mobilizing 2nd messengers related to pyridine dinucleotides, specifically cADPR (**2**) and NAADP (**3**), were discovered during studies on the events following fertilization of sea urchin eggs. This discovery was unanticipated, and pyridine dinucleotides were previously best known as coenzymes in a variety of metabolic pathways. Similar to IP3, cADPR (**Figure 1**) also stimulates Ca^{2+} release from ER stores, but through a different receptor and by regulating ryanodine sensitive Ca^{2+} channels. The ryanodine receptor also responds to increases in Ca^{2+} concentrations by releasing additional Ca^{2+}, a process called Ca^{2+} induced Ca^{2+} release (CICR). Once synthesized by the enzyme ADPRC, cADPR mobilizes Ca^{2+} from intracellular stores through modulation of the ryanodine receptor. It is unclear whether cADPR binds directly to the ryanodine receptor, binds to a protein which regulates the ryanodine receptor through protein-protein interactions, or regulates its activity through CICR. Additionally, various proteins may be required for full activity. As exemplified by eukaryotic calcium binding protein calmodulin, this protein has been shown to act[7] in conjunction specifically with cADPR to mediate Ca^{2+} release from intracellular stores. Further evidence suggests cADPR enhances the Ca^{2+} sensitivity of the ryanodine receptor producing prolonged Ca^{2+} waves through CICR. cADPR mobilizes Ca^{2+} through an IP3-independent mechanism since it is not affected by the competitive IP3R antagonist, heparin. Likewise, selective antagonists such as 8-NH_2-cADPR and 8-Br-cADPR block Ca^{2+} release from cADPR sensitive stores, but do not

affect IP3 induced Ca^{2+} release,[5] proof that these molecules act at pharmacologically different molecular targets.



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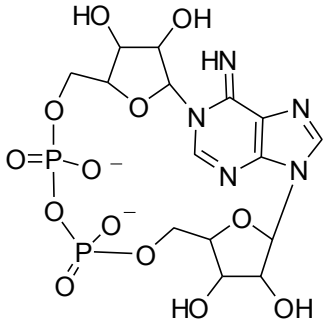

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Figure 1: Structures of 2nd messengers IP3 (**1**) and cADPR (**2**) regulating calcium ion release

NAADP (**Figure 2**) is the most potent, yet the least well characterized of known endogenous 2nd messengers capable of eliciting Ca^{2+} release from intracellular stores. It was first identified as a Ca^{2+} mobilizing agent in sea urchin eggs.[8] It was discovered when preparations of NADP were found to elicit Ca^{2+} release from sea urchin egg homogenates. Further studies revealed that highly pure NADP was inactive, but that a contaminating trace amount of NAADP, a hydrolysis product of NADP, was responsible for this Ca^{2+} release. NAADP was found to be extremely potent (IC_{50} ~20 nM), and small quantities contaminating preparations of NADP were sufficient to elicit Ca^{2+} release. Soon after, NAADP mediated activity was observed in mammalian cells, including but not limited to pancreatic acinar cells[9] and pancreatic β-cells, brain microsomes,[10] and T-lymphocytes.[11] NAADP mobilizes intracellular Ca^{2+} from Ca^{+2} stores different from that utilized by either cADPR or IP3, both of which mobilize Ca^{2+} from ER/SR stores. NAADP induced Ca^{2+} release is unaffected by addition of selective antagonists to

ryanodine and IP3 receptor mediated release and was inhibited by L-Type Ca^{2+} channel blockers (low potency antagonists) which did not affect either IP3 or cADPR, indicating that NAADP operates a distinct Ca^{2+} release mechanism.[8,12] Moreover, the sub-cellular location of the channels binding NAADP was different from IP3 receptor and the RyR. By fractionation of sea urchin egg homogenates using a Percoll gradient,[13] NAADP was shown to release Ca^{2+} from a distinct store which moved to a different position in the gradient than did the Ca^{2+} stores sensitive to either IP3 or cADPR. Further evidence provided by Galione et al. demonstrated that in sea urchin egg homogenates thapsigargin, a SERCA pump inhibitor, prevented Ca^{2+} release from IP3/cADPR stores but did not affect Ca^{2+} release mediated by NAADP.[14]

The initial surge in results following discovery subsided as more difficult questions concerning this novel molecule were addressed. The calcium ion store which was controlled by NAADP and its subcellular location were questioned. Initial evidence from the human cell line Jurkat suggested that the ryanodine receptor might be a potential NAADP target.[2] It seems unlikely that NAADP-mediated Ca^{2+} release originates through the RyR in sea urchin, yet the possibility still remains that within certain mammalian cells, NAADP releases Ca^{2+} through control of the ryanodine sensitive channel.[15-17] This observation may result from NAADP initiated CICR. A more likely candidate involving the sub-cellular Ca^{2+} store released by NAADP is the lysosome or a lysosome–related compartment. In egg homogenates Galione and Churchill[18] demonstrated that NAADP specific intracellular Ca^{2+} mobilization is reliant on a proton gradient maintained by an ATP-dependent vacuolar-type proton pump. Evidence also indicated that the store is a reserve granule, the sea urchins functional equivalent to the lysosome. This was

significant as NAADP appears to release Ca^{2+} from a non-mitochondrial, non-ER/SR store, making NAADP a novel Ca^{2+} regulatory mechanism distinct from cADPR and IP3.

Additionally, the sea urchin NAADP receptor displays another distinct biological aspect: treatment of the receptor with sub-threshold concentrations of NAADP for several minutes can fully suppress subsequent activation of the receptor on treatment with otherwise stimulatory concentrations of NAADP.[19] This self-desensitization, discovered in sea urchin homogenates, suggests NAADP mediated Ca^{2+} release must be carefully regulated. It further suggests that the receptor may contain two binding sites, a high affinity inhibitory site and a low affinity stimulatory site. This novel self-regulation was paramount in distinguishing NAADP sensitive processes from other Ca^{2+} dependent messengers and demonstrates the importance of NAADP in regulating Ca^{2+} homeostasis. Because this self-desensitization property of the NAADP receptor can skew biological results, the use of caged NAADP (**4**) has been previously been reported.[20,21] The chemoenzymatic synthesis of caged NAADP has been reproduced and is an additional tool at our disposal.

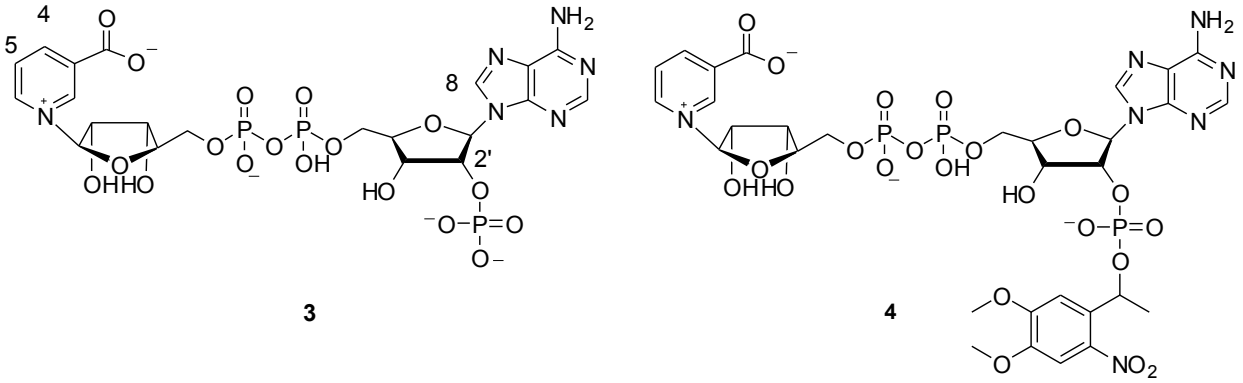


Figure 2: Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP, **3**) and caged NAADP (**4**)

NAADP is believed to play a significant physiological role in initiating the preliminary, highly localized calcium "spark", which is amplified and propagated as Ca^{2+} global waves by CICR involving previously discussed calcium signaling pathways. All evidence suggests that NAADP is acting as a 2nd messenger, so the natural question becomes "what are possible first messengers"? Cholecystokinin (CCK) is one example of a hormone responsible for the production of NAADP in mammalian pancreatic acinar cells.[9,22] Both CCK and acetylcholine (Ach) are important hormones responsible for stimulation of digestive enzymes resulting from 2nd messenger dependent Ca^{2+} signaling. At high physiological concentration (100 μM), both hormones elicit global Ca^{2+} waves, yet low, suprathreshold concentrations produce local Ca^{2+} spikes.[23] Interestingly, in the case of CCK, the local spikes were transformed into global Ca^{2+} waves. Using patch-clamp measurements, NAADP demonstrated the specific ability to transform CCK induced spikes into long lasting Ca^{2+} waves,[22] as prior desensitization with NAADP abolished this activity. Conversely, Ach induced Ca^{2+} sparks were not propagated by NAADP, yet IP3 transduced the initial calcium ion signal into prolonged global waves, suggesting NAADP interacts specifically with CCK, and not Ach in relaying Ca^{2+} signals to a global level. Additionally, the role of various 2nd messengers involved in intracellular Ca^{2+} release appear to interact producing a highly regulated Ca^{2+} response. Pancreatic β-cells respond to glucose by NAADP mediated intracellular Ca^{2+} release. High concentrations of NAADP appear to inhibit glucose induced Ca^{2+} signaling in a time and concentration dependent manner,[24] a characteristic of NAADP receptor mediated calcium release in mammalian cells. Perhaps this desensitization process plays a role in insulin regulation providing new insights into diabetes.[24] Lastly, endothelin-1, a vasoconstrictor

hormone, elicits NAADP production and subsequent calcium ion mobilization associated with lysosomes in coronary arterial myocytes.[25]

1.2 Metabolism of NAADP

Metabolism of NAD and NADP (**Figure 3**) *in vivo* occurs through the actions of NAD glycohydrolases, ADP-ribosyl cyclases (ADPRC) and NAD kinases, yielding various dinucleotides, some of which play critical roles in cell signaling, specifically 2nd messengers cADPR (**2**) and NAADP (**3**). Because of its ability to synthesize these Ca^{2+} signaling molecules, ADPRC, as well as its mammalian homolog CD38, appear to be involved in calcium ion regulation through the synthesis of cADPR and NAADP. The enzyme is believed to be activated by a number of mechanisms, including G-coupled proteins, phosphorylation events, or regulation by ATP. Whether or not this enzyme is the lone source for dinucleotides capable of intracellular Ca^{2+} release remains to be determined.

It is noteworthy to point out that ADPRCs are a general term applied to a superfamily of enzymes that recognize NAD (**5**) or NADP (**6**) as substrates and yield hydrolysis (**8**) and cyclization (**2** ,**7**) products. The term NAD-glycohydrolase refers to a version of ADPRC that catalyzes the formation of a small amount of cyclized product, but is mainly selective for the production of the linear hydrolysis product. *Aplysia* cyclase, on the other hand, forms cADPR as the major product. Depending on the isotype of enzyme, the ability to partition between various dinucleotide products can vary. It is logical to assume ADPRCs contribute significantly to Ca^{2+} signaling since they catalyze

the formation of bioactive 2nd messengers (**2**, **4**) and they are ubiquitous, occurring in a variety of organisms.

ADPRC, and its mammalian homolog CD38,[26] operate using the same mechanism and forms a set of related dinucleotide products. The relative proportions of these products are dependent on conditions (**Figure 4)**. These enzymes bind NAD or NADP and catalyze pyridinium-ribose bond cleavage. The nicotinamide departs with its electrons leaving behind an electron deficient enzyme-ADP-ribose intermediate (**9**). This intermediate could be an enzyme stabilized oxocarbenium ion[27] or a covalent enzyme-ADP-ribose intermediate.[28] The enzyme-ADPR complex can react with available nucleophiles in the environment (water, niacin, or an exogenously added alcohol) partitioning into various dinucleotide products. For example, reaction of the ADP-ribosyl-enzyme intermediate with water results in ADP-ribose (**8**) while attack of the oxygen of ethanol produces β-ethyl ADP-ribose (**10**). Intramolecular attack of the adenosyl N-1 results in cyclization and the formation of cADPR (**2**). If NADP is the substrate, the analogous set of products will be formed but will contain the 2'-phosphate. Not all of the products formed from NAD or NADP are physiologically active. Although cADPR (**2**) and NAADP (**3**) are active 2nd messengers, cADPRP (**7**) and ADPRP (**8a**) are not known to be biologically active. However, linear ADP-ribose (**8**) has been shown to specifically gate the calcium permeable cation channel, LTRPC2[29] indicating that many products of NAD metabolism have physiological roles, specifically in calcium ion regulation. In the presence of exogenous pyridine base, ADPRC can catalyze a trans-glycosidation reaction (**Figure 4**, labeled **Base-exchange product**). NAADP can be

formed by this mechanism when nicotinic acid is present and NADP is the substrate, represented as an equilibrium process.

The relative amounts of the various dinucleotide products formed by the action of ADPRC on NAD or NADP are dependent on conditions and on the concentrations of added nucleophiles. The pH of the media governs the rate of product formation and the product distribution produced by the enzyme. NAD or NADP are either cyclized or hydrolyzed at neutral or alkaline pH, forming cADPR or cADPRP, respectively. At acidic pH and in the presence of nicotinic acid, ADPRC catalyzes a reaction, in which nicotinamide in NADP is "base-exchanged" with nicotinic acid, forming NAADP. The mild acidic conditions (pH = 4), ensures that the neutral nicotinic acid species exists. It is this neutral species that participates in base-exchange as ionized nicotinic acid species are not substrates. Base-exchange reactions can occur at basic pH if the pyridine base remains unionized. For example, 3-methylpyridine exchanges at pH = 8 because the molecule is neutral under the conditions.

Residues involved with binding at the active-site have been identified in both CD38 and the related isotype *Aplysia californica* (known as *Aplysia* cyclase) through site-directed mutagenesis.[30] In *Aplysia*, four amino acids contribute to the active site. Two tryptophans (Trp140 and Trp77) aid in positioning the pyridine dinucleotide substrate while two glutamyl residues (Glu 98 and Glu 179) stabilize the cationic intermediate. In CD38, similar residues (Trp125, Trp 189, Glu146, Glu226) were identified to participate in catalysis. Additionally, the crystal structure for *Aplysia* cyclase has recently been solved.[31]

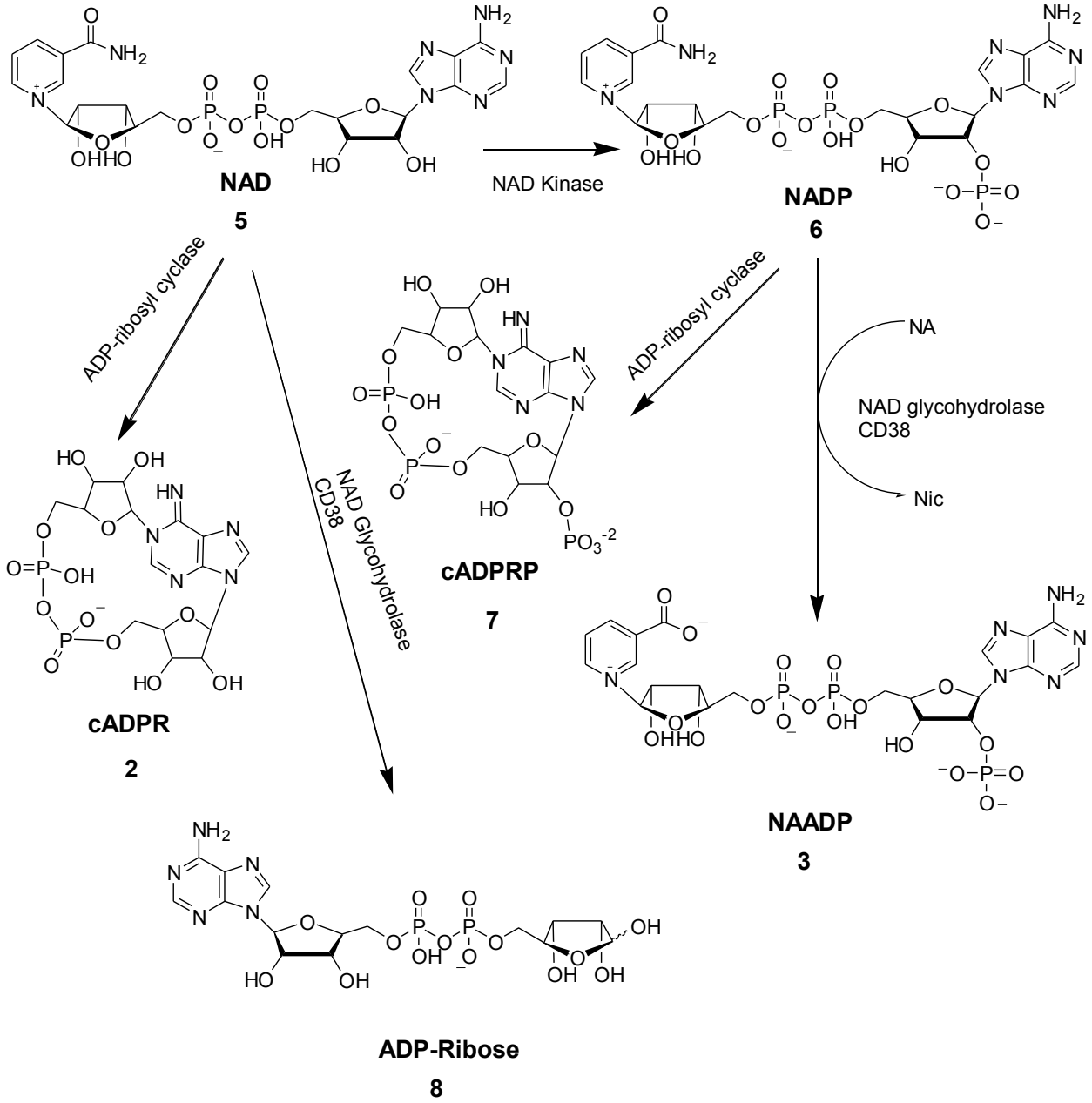


Figure 3: Various *in vivo* metabolic pathways of NAD leading to a variety of dinucleotides

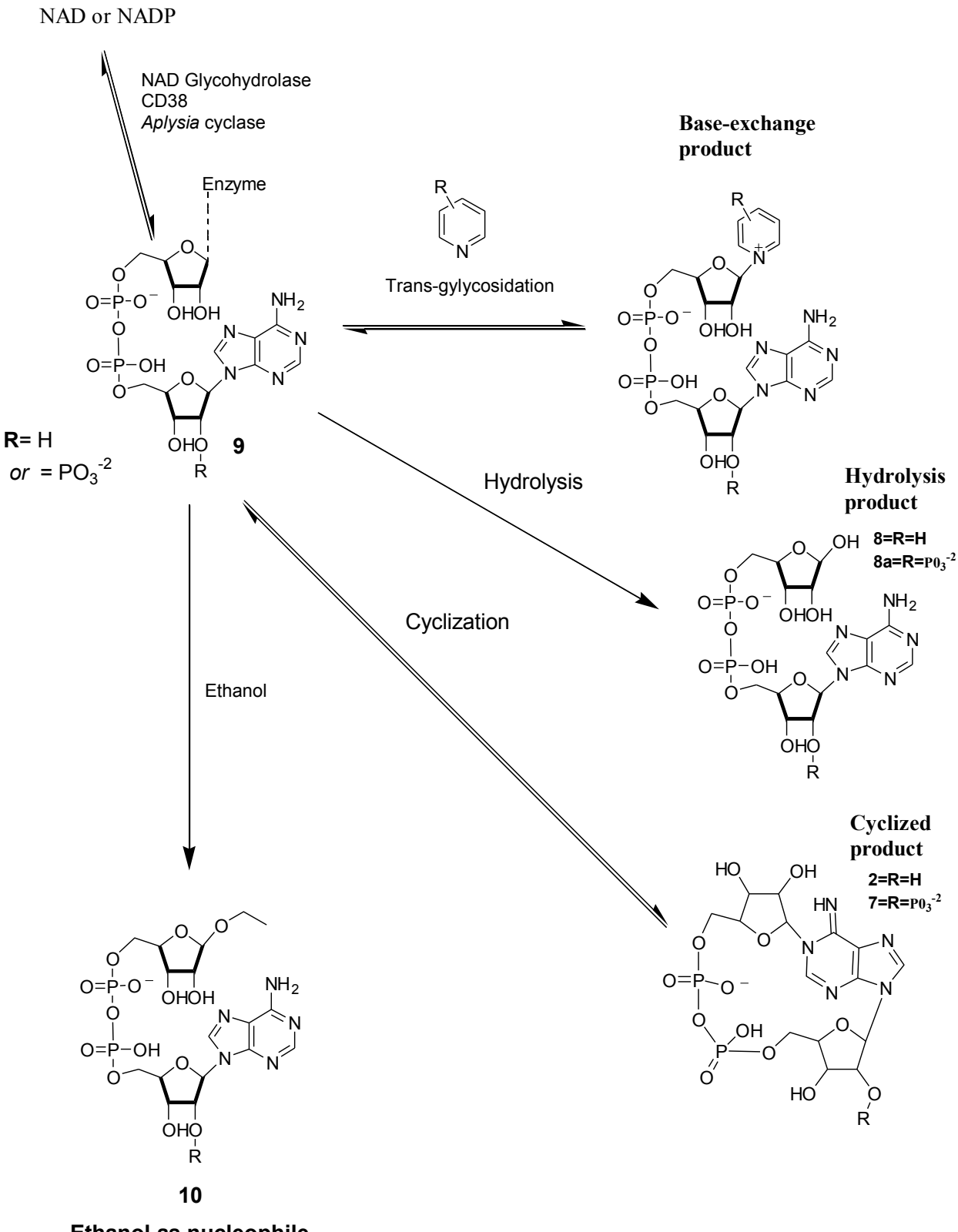


Figure 4: Mechanism of ADPRC catalyzed formation of dinucleotides from NAD or NADP

1.3 Structure-activity relationships NAADP

Before its biological significance was known, Carl Bernofsky described the chemoenzymatic synthesis of NAADP through the base exchange of nicotinic acid catalyzed by calf spleen NAD glycohydrolase.[32] This particular chemoenzymatic synthesis of NAADP has proved crucial in uncovering structure activity relationships and proves to be efficient and strategic in our work. Studies done by Lee *et al*. [33] and Billington *et al*. [34] delved into the importance of location, charge, and size tolerated by the NAADP binding protein. Both studies used *Aplysia* ADP ribosyl-cyclase to chemoenzymatically synthesize NAADP analogs from derivatives of NADP and nicotinic acid. Structure-activity relationships from these analogs identified three critical requirements necessary for receptor mediated response in sea urchins (**Figure 5**). The 3 position on the pyridine must contain a carboxylic acid (**red**) which will be charged at physiological pH-a necessity for activity. The 6-amino group of the adenine moiety (**blue**) as well as the 2'-phosphate on adenosine (**purple**) are additional requirements for recognition by the receptor. The uncharged carbinol-NAADP derivative (**3b**) has no activity, nor does NAADP analogs in which the carboxylate was moved from the 3 position to the 4 position of the pyridine ring. The carboxylate must be attached directly to the pyridine ring, as adding a one carbon spacer (**3a**) between the ring and the carboxylate resulted in significant loss of binding relative to parent ligand, presumably due to sterics. Boronic acid compound (**3e**) does not carry charge at physiological pH; therefore, does not elicit a receptor-mediated response. Substitution with a phosphonate (**3c**) resulted in significant loss of binding affinity to the receptor, suggesting extra negative charge is not accomodated. Interestingly, the phosphino compound (**3d**) has

much greater binding affinity and biologic response, suggesting the receptor prefers functional groups similar to a carboxylate. The 1-H-tetrazole derivative (**3g**) has only a 4-fold decrease in binding affinity to the receptor relative to NAADP further illustrating the importance of charge, as tetrazole carries a stabilized negative charge throughout the ring, which preserves the zwitterionic nature of pyrdinium ring. Also, the tetrazole substituent demonstrates the receptors tolerance for steric bulk. The 6-amino group of adenine (**blue**) tolerates no modification. 6-Deamino-NAADP requires concentrations as high as 5 μM for activity, much higher than the low nanomolar concentrations NAADP exhibits. Substitution of the amine with a hydroxyl decreased potency by 1000 fold. The third critical determinant, the 2'-phosphate on adenosine (**purple**) tolerates some modification. For example, moving the phosphate to the adjacent 3' position on adenosine, or forming the 2',3'-cyclic phosphate results in lower binding affinity, but the compounds remain biologically active. Complete removal of the phosphate results in complete loss of activity.

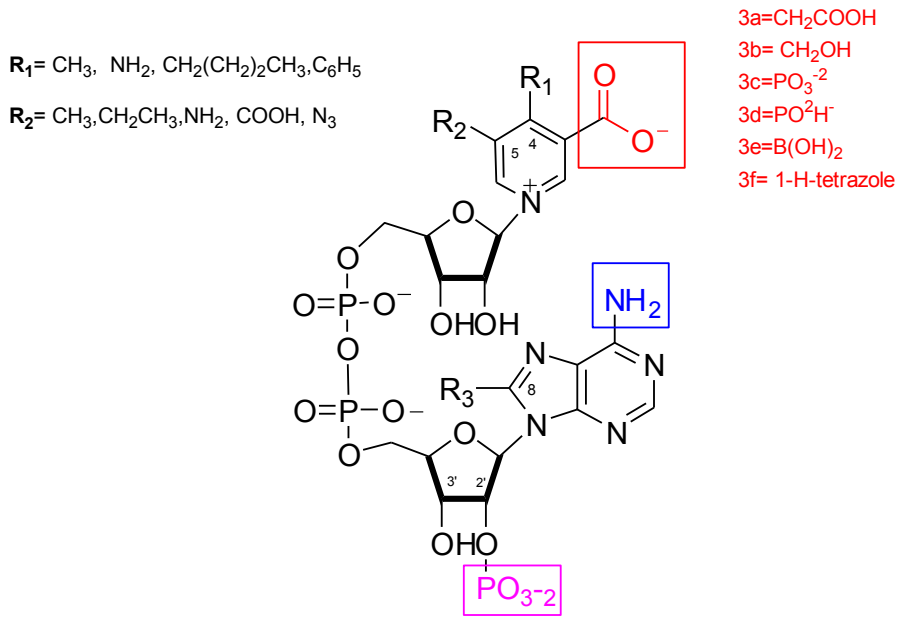


Figure 5: Critical requirements (colored) and recent analogs (R_1,R_2, R_3) of NAADP

The structure activity relationship was further examined where individual chemical substitution at the 4 (R_1) and 5 (R_2) positions on the pyridinium ring was investigated.[35] Clearly, the pyridine ring on NAADP contributes to binding affinity to the receptor[34]. Through a chemoenzymatic synthesis, NAADP derivatives with small substitutions on the nicotinic acid ring were evaluated in sea urchin eggs. Testing these substituted NAADP derivatives identified a "structurally neutral" position at the 5 position of nicotinic acid (**Figure 5**). "Structurally neutral" positions on NAADP refer to sites on the molecule in which chemical substitution has occurred, but no significant loss of binding or activity is observed. Compounds with small substitutions at the 4 position (methyl, amino) of nicotinic acid could be classified as low potency agonists. Larger groups (butyl, phenyl) were active only at high concentration. Conversely, small substitutions at the 5 position (methyl, amino, ethyl, azido) were well tolerated. These molecules demonstrated similar measured Ca^{2+} release (EC_{50}) and competitive binding (IC_{50}) to the parent receptor.

1.4 Photoaffinity labeling and the two pore channel

1.4.1 The two pore channel

A set of organelles have been recently classified as acidic calcium stores termed the endo-lysosome system. These include acidocalcisomes (protists), vacuoles (protists, yeasts, plants), lysosome, lysosome related organelles and secretory granules (mammalian), and the Golgi complex (eukaryotic).[36] Because NAADP acts at a novel channel within the endo-lysosomal system, these targets gained interest. Proposed targets

of NAADP-receptor mediated activity include a family of ion-gated channels that are among the best characterized out of any of the targets within the endo-lysosomal system. Transient receptor potential-mucolipins (TRPML),[37] so named because gene mutations in the founding member TRPLM1 resulted in lysosomal storage disease mucolipidosis IV, consist of three isoforms in mammals (TRPML1-3). Of these, TRPML1-2[38,39] have been identified as possible ion channels involved in NAADP activity, although substantial, definitive and reproducible evidence is lacking. Yet, dismissing these reports is naïve and these ion channels may play a signaling role in NAADP mediated calcium regulation.

The most accepted and likely target at which NAADP eventually acts is the two-pore channel (TPC). TPCs are poorly characterized ion channels originally identified by the sequence similarity to Na^+ and Ca^{2+} voltage-gated ion channels. As newest members of this superfamily of voltage-gated ion channels, TPCs share architectures common to many voltage-gated ion channels.[40] The full length protein is segregated into 4 domains of six TM each with an intervening pore loop between the 5th and 6th (EF Hands).[41] The 4 domains form a tetrameric complex acting as a cation "pore" The EF hands act as small sensors, or "grabbers" of cations, shuttling them into the cytoplasm. Instead of 4 TM domains, a hallmark of Ca^{2+} and Na^+ voltage–gated ion channels, TPCs have 2 homologous domains, hence the name two pore channel. A more interesting difference between TPC and related channels is that the TPCs are not expressed on plasma membranes and most likely localized to the endo-lysosomal system. It is noteworthy to point out that three non-allelic TPC genes (TPC1, TPC2, TPC3) are present in sea urchins and most vertebrate species with TPC3 absent in some rodents and primates.[42] This may

point to an evolutionary redundancy and may indicate that this ion channel may not always be necessary for cell survival

Previous reports have suggested that NAADP mediated Ca^{2+} signals occur through the TPC. For example, northern analysis shows expression of TPC2 mRNA in human tissue, with higher concentrations in the liver and kidney.[33,36] Overexpression[43,44] of TPCs enhance NAADP mediated Ca^{2+} signals, although the increase in binding of radiolabeled NAADP was much lower than the increase in TPC2 mRNA levels (3-fold vs ~250-fold).[45] Knockdown[43] of TPCs resulted in diminished NAADP mediated Ca^{2+} signaling. A highly conserved leucine amino acid residue located in the putative helix of the pore-forming region of TPCs was identified. Mutation of the leucine residue to a helix-breaking proline residue abolished NAADP-mediated signaling providing further evidence that NAADP regulates Ca^{2+} release through the TPC. Likewise, mutation of the lysosomal targeting sequence located near the N terminus of TPC2 allows the TPC2 to be redirected to the plasma membrane. With the TPC2 now expressed on the plasma membrane, normal trafficking of the TPC to the lysosome cannot occur. This uncoupling of the TPC abolished NAADP mediated Ca^{2+} release, which was measured through electrophysiological analysis,[46] further demonstrating that the TPC is the primary target for NAADP, and not the ryanodine receptor.

Circumstantial evidence was accumulating pointing to TPCs as the sought after NAADP receptor, yet, no direct proof had been offered. In 2009, direct evidence was finally provided by a collaborative effort supporting the hypothesis that NAADP does mobilize calcium ion from acidic organelles through control of the two-pore channel.[45] This conclusion was based upon cloning and expressing highly purified TPC proteins

(TPC1, TPC2, TPC3) and studying their dependence on NAADP. Demonstrating that TPC was a target for NAADP was a conclusion made from various experiments. Initially, the authors confirmed the subcellular location of TPC-NAADP mediated response to the endolysosomal system by expressing haemagglutinin tagged TPC2 cells. Tagged cells colocalized with lysosome-membrane associated protein markers (LAMP2) but not with markers for early or late endosomes, ER, Golgi or mitcohondria. Similarly, fluorescent indicators related to the lysosome demonstrated accumulation in vesicles surrounding tagged TPC2 cells. On the other hand, TPC1 and TPC3 were found to be predominantly expressed in endosomes and unidentified intracellular compartments. TPC2 is specifically targeted to lysosomal membranes. Moving forward, TPC2 was considered a likely candidate for the NAADP receptor, although both TPC1 and TPC3 cannot be precluded. NAADP demonstrated unambiguous calcium ion release from the lysosome and exemplified characteristic Ca^{2+} transients through electrophysiological methods. Disrupting the proton gradient of the lysosome with bafilomycin A1 abolished NAADP mediated signaling. Concentration-response relationships for NAADP against TPC2 cells were in accordance with previous studies. Ultimately, NAADP acts through TPC2 as a trigger for a highly localized calcium spark that can be propagated to global Ca^{2+} signals by other discussed Ca^{2+} releasing pathways, such as IP3R and RyR. Although the target has been identified, the question if NAADP directly binds to the TPC remained.

1.4.2 Photoaffinity Labeling

Techniques ranging from X-ray crystallography to spectroscopic analysis have been employed to locate active sites and binding sites of ligand-receptor interactions. Of

these, photoaffinity labeling (PAL) is among the most useful, well documented procedures. Since the technique was advanced by Westheimer et al.,[47] successful labeling of enzymes, membranes,[48] protein structures,[49] neural receptors, and RNA and DNA structures have been accomplished.[50] PAL studies contain the same general experimental elements. The labels are ligands with inherent affinity for a binding site, bind reversibly and posses biological activity, but also contain a photolyzable functional group, which upon irradiation, can form a covalent bond with the protein of interest.[48] Photoprobes will usually be radioactively labeled, facilitating tracking of the tagged binding site[49]. Demonstration of specificity of the photolabeling process is essential. The rate and extent of covalent insertion should be lessened if photoactivation is performed in the presence of competing ligand. Lastly, proteins should only photolabel in the presence of light and photoprobe.

Design of the photoprobes begins with consideration of the SAR for the parent ligand. Ideally, the photoprobe should be bioactive in the same concentration range as the parent compound, but compounds with as much as 1000 fold higher disassociation constants have proven to be useful. Common photoaffinity labels used with success include aryl azides, benzophenone derivatives, and trifluoromethylphenyl diazirine.[51] An excellent example of PAL pertaining to calcium signaling was identification of IP3 binding proteins,[52] achieved by labeling the receptor with a radioactive and photoactive analog of IP3. More recent studies utilized this same methodology by creating a panel of photoactive inositiol polyphosphates to determine binding specificity between different targets that are important in cytoskeletal remodeling, signal transduction, endo and exocytosis and vesicular trafficking.[53] Equally relevant to our anticipated attempts at

characterizing the NAADP receptor is the well documented use of azidodinucleotides in PAL of various targets. Radiolabeled 2-N_3-ATP,[54,55] 2-N_3-ADP,[56,57] 8-N_3-ATP,[58] 8-N_3-GTP,[59] and 8-N_3-cAMP[60] have served as successful photoprobes elucidating and further defining ligand-receptor interactions. These studies suggest that photoactive derivatives of NAADP could be used to label and ultimately to identify NAADP binding proteins.

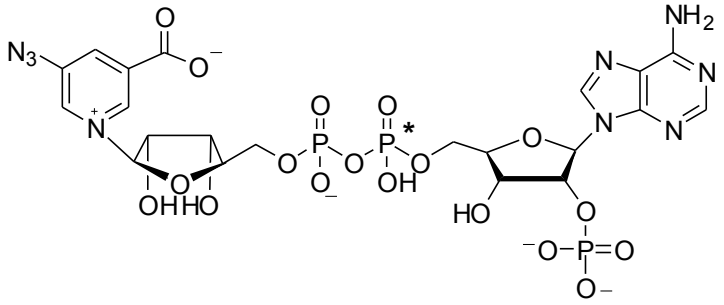


Figure 6: Structure of the photoprobe [^{32}P]-5-N_3-NAADP. The * denotes location of radiolabel

Using this methodology, an innovative discovery was made concerning the NAADP receptor. In a recent publication, Walseth *et al.*[61] demonstrated in sea urchin egg homogenates that NAADP photolabels bands that were different from the two pore channel. It appears that NAADP, and its binding protein, form a complex which results in eventual Ca^{2+} release via the TPC. Photolysis of [^{32}P]-5-N_3-NAADP (**Figure 6**) preincubated with sea urchin egg homogenates specifically labeled 3 proteins (45, 40, and 30 kDa) which were of significant lower molecular weight than TPCs (**Figure 7, Lane 1**). Immunoblots (**Figure 7**, **Lanes 2-4**) were performed under the same conditions as PAL experiments against affinity purified TPC antibodies. Anti-TPC antibodies did not comigrate with proteins observed in PAL experiments (45, 40 kDa bands), further strengthening the hypothesis that NAADP does not bind directly to the TPC.

Additionally, immunoprecipitation experiments using anti-TPC antibodies suggest that photolabeled proteins associate with sea urchin TPC1 and TPC3, providing further evidence that these proteins bind NAADP as a high affinity binding receptor that complex with TPCs leading to eventual Ca^{2+} release. In a companion study, Lin-Moshier *et al.*[62] utilized this same methodology to photolabel proteins in mammals finding similar results, except that the molecular weights of the labeled proteins were 22/23 kDa. The specific [^{32}P]-5-N_3-NAADP labeled bands exhibited a similar pattern seen in Walseth's study and were of lower weight compared to TPC. The photoderivative of NAADP has become a very useful probe in uncovering NAADP mediated biological response. Unfortunately, initial mass spectroscopy experiments designed to identify the photoderivatized bands were futile, as samples were apparently insufficiently pure. The main goal of the research presented in this dissertation was to chemoenzymatically synthesize analogs containing functionalities to aid in purification of photolabeled NAADP binding proteins.

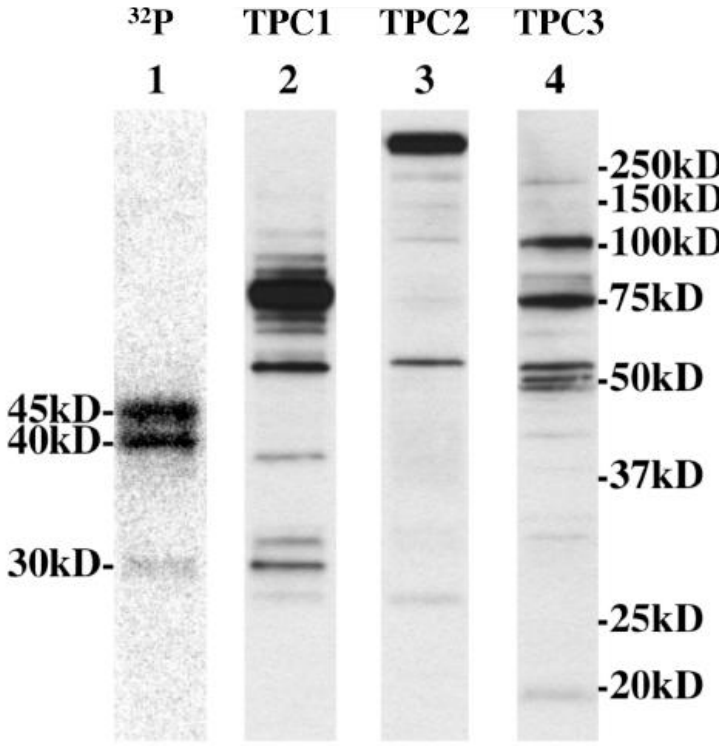


Figure 7: The NAADP receptor is not the two-pore channel. Photolabeled proteins by [^{32}P]-5-N_3-NAADP were of lower molecular compared to TPCs (**Lane 1**). Immunoblots (**Lanes2-4**) against affinity purified anti-TPC1-3 antibodies further demonstrate that the NAADP binding protein is not the TPC.

Chapter 2

Results and Discussion

NAADP has recently emerged as a significant player in complex Ca^{2+} signaling pathways, but the identity of the NAADP receptor remains unknown. Thus, identifying the NAADP receptor will prove to be significant in unlocking unknown cell mechanisms and provide a potential therapeutic target. When approaching the problem of receptor identification from a chemical stand point, clarifying the SAR for NAADP binding was necessary; therefore, identifying "structurally neutral" positions within NAADP was our initial goal. Mono substitution at the 5 position on nicotinic acid with methyl, amino, and ethyl groups was shown to be tolerant to receptor-mediated intracellular Ca^{2+} release. However, the ability to introduce larger and functionalized substituents had not been demonstrated. Mono substitution at the 8 adenosyl position has been hardly investigated. Furthermore, di-substituted NAADP derivatives have not been synthesized and tested at the beginning of the project. We hoped to identify 2 sites that would tolerate chemical substitution. If successful, we could go on to propose chemoenzymatically synthesized derivatives that can be used as bifunctional probes and aid in receptor identification. This will consist of an aromatic azide at one position to be used for PAL, and a second site aiding in purification through click chemistry.

Using the *Aplysia californica* cADPR synthase[63] (a version of NAD glycohydrolase), multiple substituted NAADP analogs could be prepared that would otherwise take an extreme amount of time using total synthetic methodologies.[64] Illustrated in **Figure 8**, the enzyme base-exchange reaction was the foundation of this work. In the presence of NADP (or 8-substituted NADP at $\mathbf{R_2}$), 5-substituted nicotinic acid ($\mathbf{R_1}$), and water (pH = 4), the enzyme *Aplysia* cyclase catalyzes the base exchange of nicotinamide for 5 substituted nicotinic acid. Using this methodology, various derivatives were chemoenzymatically synthesized and evaluated in sea urchin egg homogenates. A limitation of the chemoenzymatic synthesis was that the enzyme must recognize the substrate (nicotinic acid) to afford NAADP analogs. Previous experience suggests that nicotinic acids containing certain substituents (phenyl, acetylenes) attached directly to the 5 position were not recognized.[65] Another limitation of the enzyme catalyzed base-exchange is solubility of the added pyridine base; if the pyridine base becomes too hydrophobic and therefore insoluble in water, exchange is not possible. To combat this, a cosolvent was sometimes used to help solubilize the substrate. DMSO (up to 25%) was found to be satisfactory, but in certain cases even this was insufficient to obtain a successful reaction

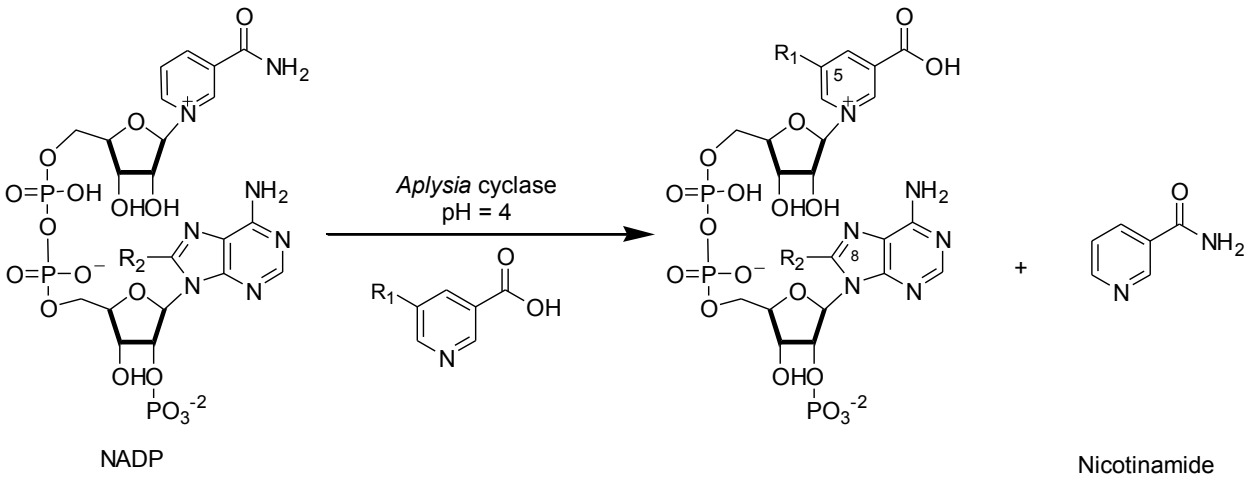


Figure 8: Base-exchange illustration

2.1 Novel 5-substituted nicotinic acid analogs

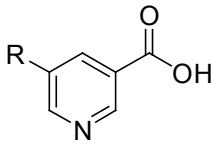
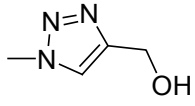
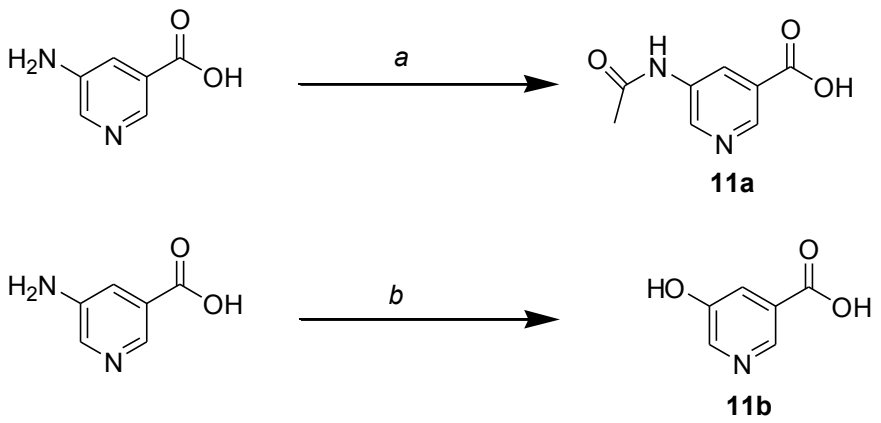
Structure	Cmpd	Substituent	Base-exchange product detected
 **11a-11m**	**11a**	-$NHCOCH_3$	Yes
	11b	-OH	Yes
	11c	-$CH_2(CH_2)_2NHCOCH_3$	Yes
	11d	-$CH_2(CH_2)_2NHC(O)O(CH_3)_3$	Yes
	11e	-$CH_2(CH_2)_2NH_2$	Yes
	11f	-$CH_2(CH_2)_2OH$	Yes
	11g	-$CH_2(CH_2)_2N_3$	Yes
	11h	-SCH_3	Yes
	11i	-$S(O)CH_3$	No
	11j	-$CH_2CH_2C_6H_5$	No
	11k	-*p*-chlorophenyl	No
	11l		No
	11m	-$CH_2(CH_2)_6CH_3$	No

Table 1: Results of synthesized 5 substituted nicotinic acids (**11a-11m**) and their ability as substrates for base-exchange reaction

Various 5 substituted nicotinic acid analogs were synthesized (**Table 1**), many of which are new compounds which are described for the first time in this work. Incorporation of 5 substituted nicotinic acid derivatives into NAADP through the enzyme catalyzed base-exchange reaction provided functionalized 5 substituted NAADP derivatives used in probing the NAADP binding protein. Our main approach in achieving compounds with various functional groups at the 5 nicotinyl position was through the Sonogashira reaction. This reaction efficiently coupled a convenient starting material (5-bromonicotinic acid ethyl ester) with various terminal alkynes which introduced a number of side chains attached to the nicotinic acid 5 position. Attaching branched side chains with various functional groups at the 5 position on NAADP were used to determine binding potency to the unknown NAADP receptor.

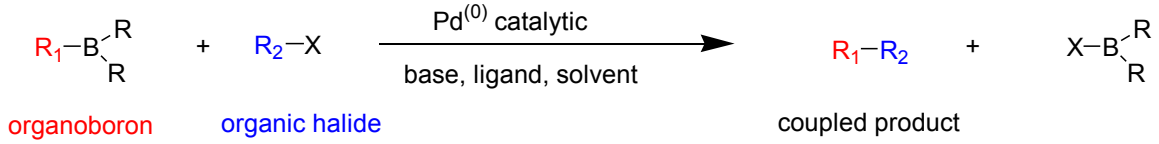
Preliminary studies began with the synthesis of two simple nicotinic acid derivatives (**Scheme 1**) from commercially available 5-aminonicotinic acid, which could be acetylated (**11a**) using acetic anhydride or subjected to diazotization, and further transformed to 5-hydroxynicotinic acid (**11b**) in boiling H_2SO_4 (aq). To make more elaborate substitutions, it became apparent that a common precursor combined with an efficient approach capable of retaining some hydrophilicity was necessary.



Scheme 1: Synthesis of 5-acetamidonicotinic acid and 5-hydroxynicotinic acid a) acetic anhydride, pyridine, reflux, 22 h, 39% b) aqueous H_2SO_4 (20 % v/v), $NaNO_2$, 0 ^oC, then aqueous H_2SO_4 (60 % v/v), 100 ^oC 1 h, 40%

Carbon-carbon bond formation using palladium catalysis has advanced significantly over the past decade and constitutes a relatively effective way to accomplish an otherwise difficult reaction. Suzuki cross-couplings[66,67] can be utilized to form carbon-carbon bonds between organoboron compounds and organic halides through a palladium catalyzed reaction (**Figure 9**). In the presence of an organoboron compound, which in our experiments was *p*-chlorophenylboronic acid, and an aryl halide, palladium$^{(0)}$ catalyzes a carbon-carbon bond formation (**coupled product**). Conversely, the Sonogashira[68,69] cross-coupling reaction couples an aryl halide to a terminal alkyne in the presence of catalytic amounts of Cu^{+1} and $Pd^{(0)}$, and excess base (**Figure 9**). Products of this reaction could be further manipulated to form novel, small molecules. Examples of these reactions in this work are **Schemes 7** and **Scheme 9** (Suzuki), as well as **Schemes 4-6** and **Scheme 8** (Sonogashira).

Suzuki cross-coupling



Sonogashira cross-coupling

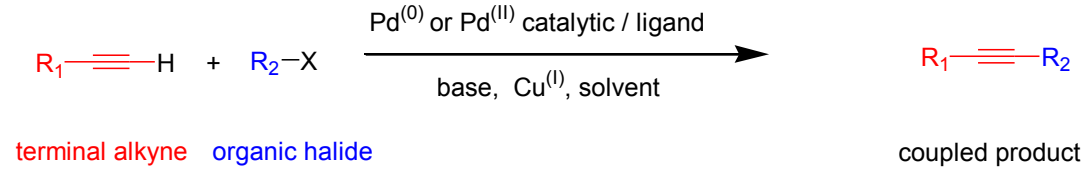
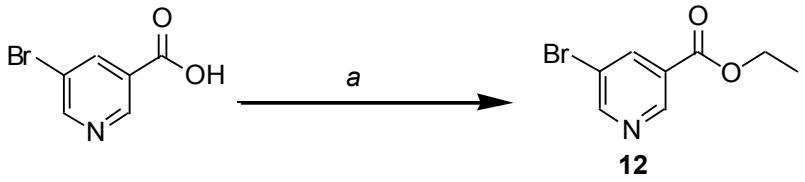


Figure 9: Palladium cross coupling reactions used in the synthesis of substituted nicotinic acid derivatives.

Utilizing this methodology, various substitutions at the 5 position on nicotinic acid have been produced. Suzuki and Sonogashira reactions, using the convenient and commercially available starting material 5-bromonicotinic acid, yielded messy reactions requiring tedious purifications. Simple protection of the bromonicotinic acid with an ethyl ester resulted in the production of fewer side products and produced compounds that were more easily purified. Using Fisher esterification (**Scheme 2**), 5-bromonicotinic acid was converted to the ethyl ester **12** in the presence of H^+ in ethanol in acceptable yields. Compound **12** was used as the substrate in most subsequent palladium catalyzed reactions.

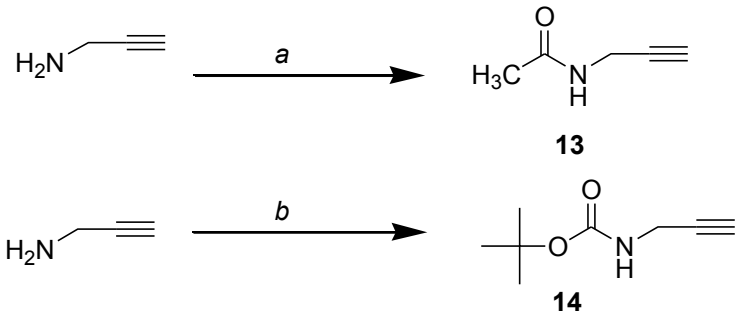


Scheme 2: 5-Bromonicotinic acid ethyl ester a) i) 5N HCl in dioxane, EtOH, reflux, 48 h, 88% ii) NaOH

The following palladium catalyzed reactions were divided into two series of compounds based upon their targeted functionalities: 1) The aminopropyl series and 2) the azidopropyl series. A third series, the thiomethyl series, resulted from nucleophilic aromatic substitution. The last category describes compounds that were not substrates for base- exchange, illustrating limitations to our chemoenzymatic methodology.

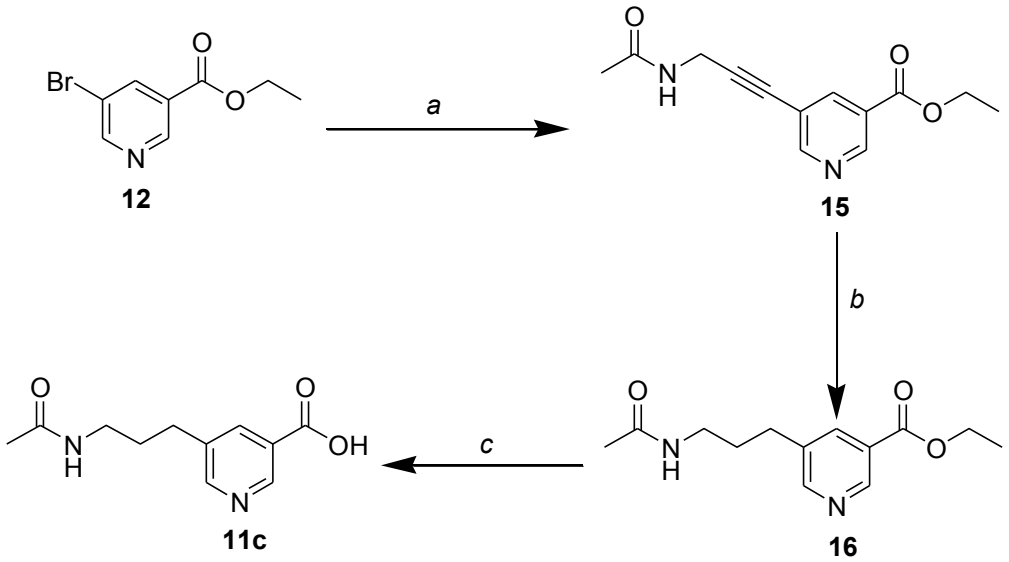
2.1.1 Aminopropyl series

From commercially available propargyl amine, two amide derivatives (**Scheme 3**) were synthesized as reactants for Sonogashira reaction. Treatment of propargyl amine with acetic anhydride yielded desired 3-acetamidoprop-1-yne (**13**), while di-*t*-butyl-dicarbonate effectively protected the amine (**14**).



Scheme 3: Synthesis of terminal alkynes for aminopropyl series a) acetic anhydride, TEA, 24 h, 58% b) *tert*-butyldicarbonate, DCM, 0 ^{o}C 16 h, 56%

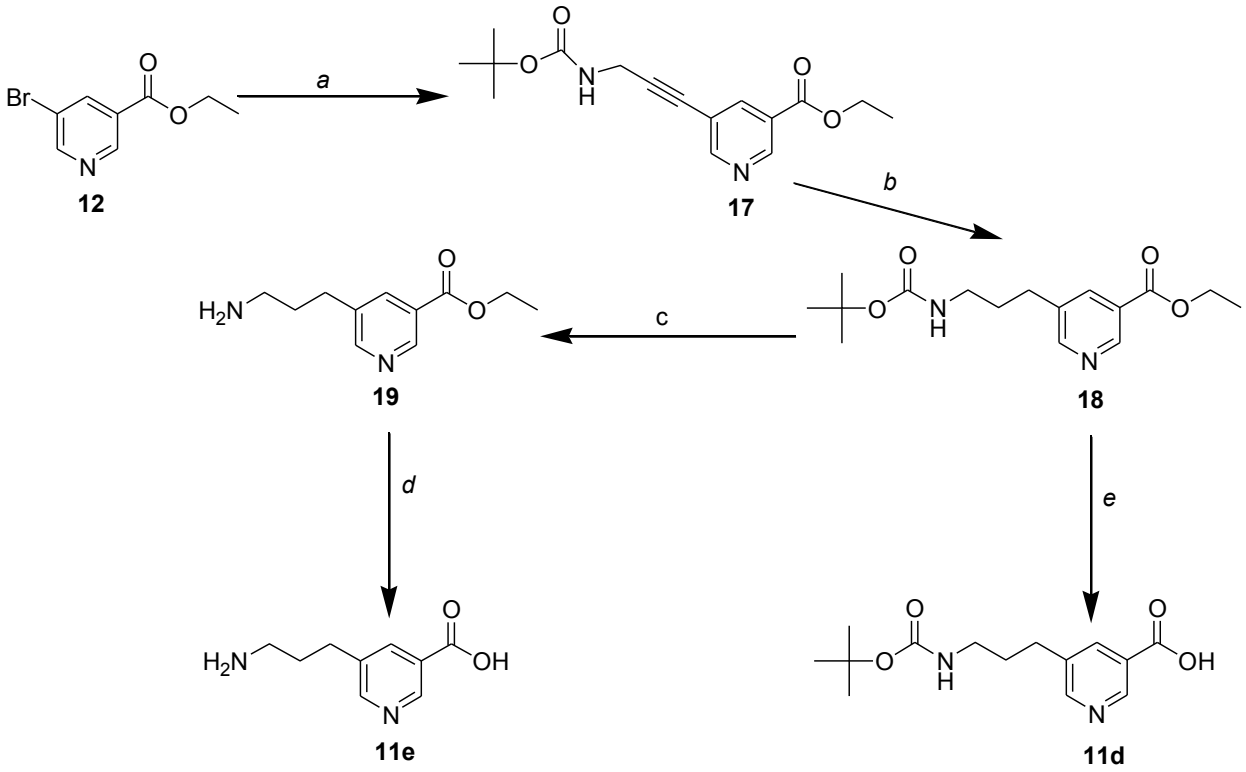
These simple alkynes were then coupled to 5-bromonicotinic acid ethyl ester (**12**) providing a set of unique compounds with alkyne moieties at the 5 position. In **Scheme 4**, starting material (**12**) was coupled to **13** under Sonogashira coupling conditions. The product was then reduced by hydrogenation (**16**), then de-esterified (**11c**) with aqueous base.



Scheme 4: Synthesis of 5-(3-acetamido)-nicotinic acid a) **13**, $Pd(PPh_3)_2Cl_2$, CuI, DIPEA, ACN reflux, 18 h, 60% b) H_2 (g), Pd/C, MeOH, 8 h, 91% c) i) NaOH, MeOH, 3.5 h ii) HCl, 89%

Synthesis of target molecule 5-(3-aminopropyl)nicotinic acid (**11e**) was accomplished by coupling protected propargyl amine to common building block (**12**) yielding alkyne (**17**) under Sonogashira conditions (**Scheme 5**). Standard hydrogenation conditions provided reduced compound (**18**), while removal of the *t*-Boc protecting group with TFA followed by alkaline treatment afforded desired aliphatic amine (**11e**).

Compound **18** proved to be an important compound in the aminopropyl series as hydrolysis afforded acid (**11d**). The steric bulk at this position, as well as its unexpected solubility in water, proved to be a valuable in identifying the tolerance of *Aplysia* cyclase for large hydrophobic substituents placed at the 5 position of nicotinic acid.

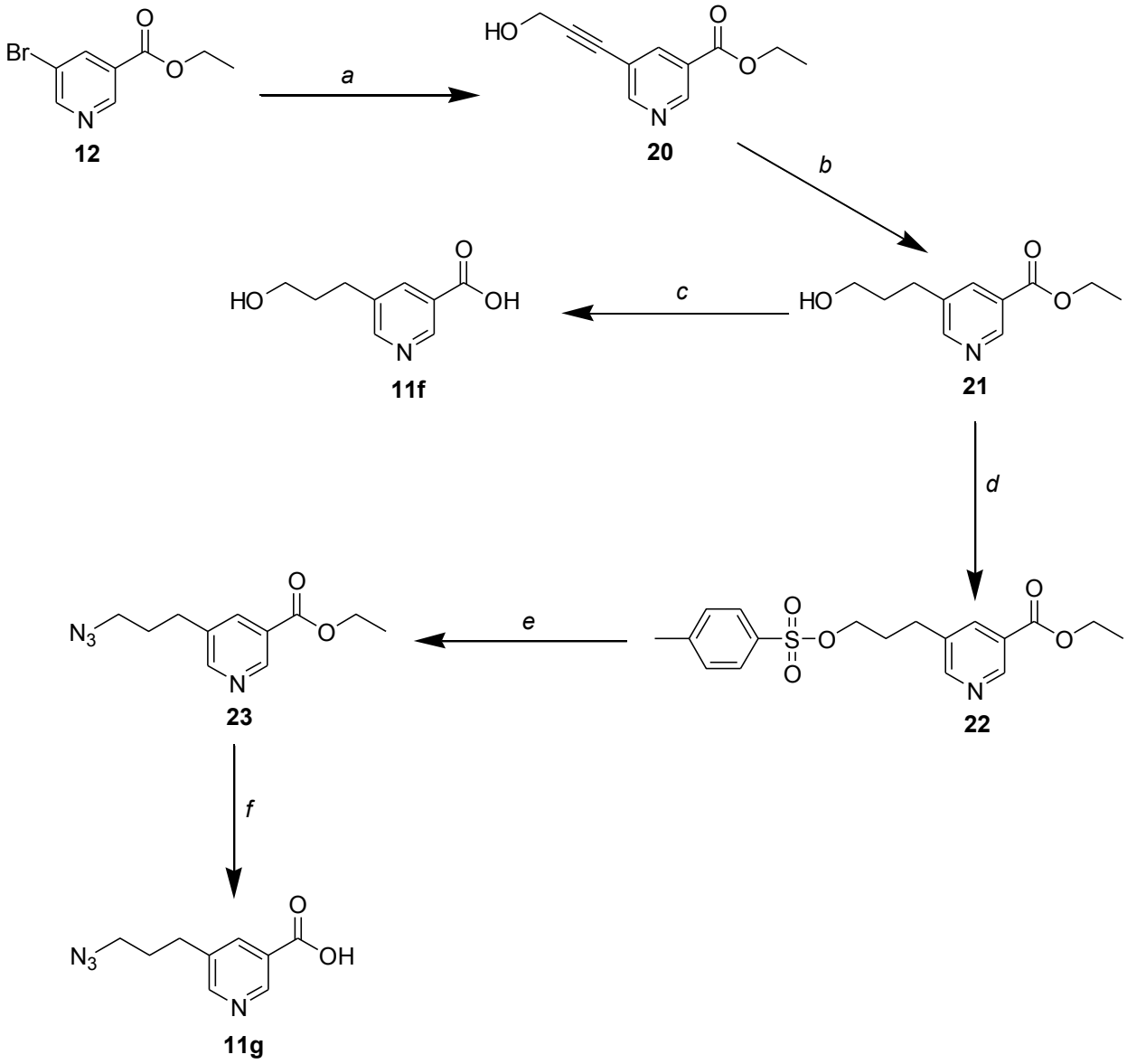


Scheme 5: Aminopropyl series a) **14**, $Pd(PPh_3)_2Cl_2$, CuI, DIPEA, ACN, reflux, 6 h, 61% b) H_2 (g), Pd/C, MeOH , 6 h, 93% c) TFA, MeOH, 37 ^oC, 2 h d) NaOH, MeOH, 8 h, 89% e) i) NaOH, MeOH, 37 ^oC, 4 h ii) HCl, 83%

2.1.2 Azidopropyl series

Similar to the above methodology, palladium catalyzed couplings with propargyl alcohol (**scheme 6)** and **12** provided another route to unique nicotinic acid derivatives. Coupled alkyne (**20**) was reduced, forming intermediate compound (**21**). The free acid analog (**11f**) contains a primary alcohol and added depth to the series. The hydroxyl

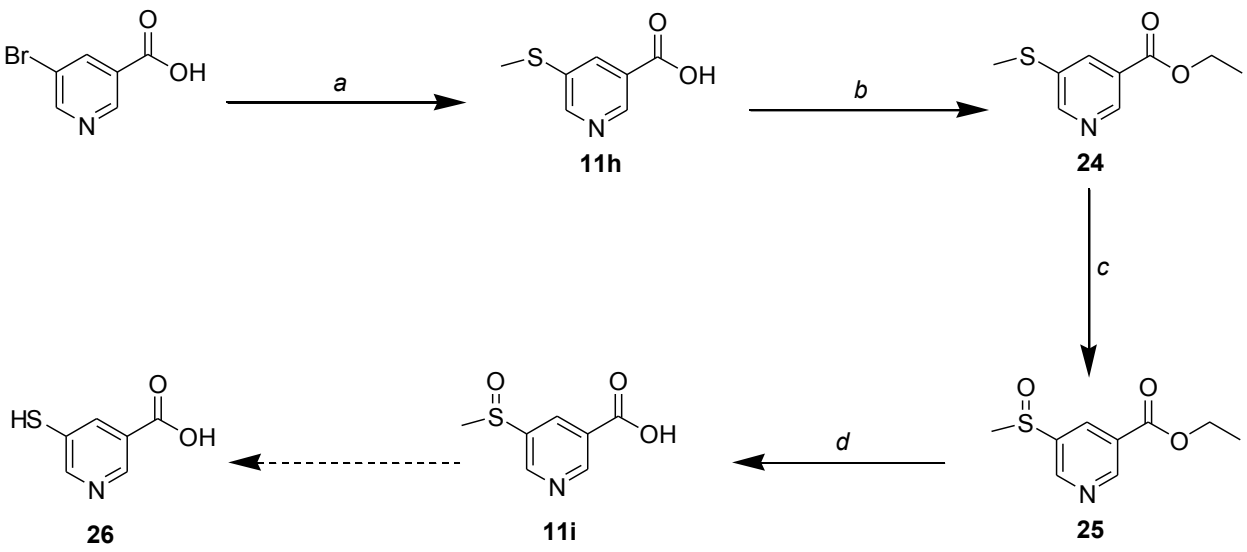
group also served as a site of functionalization, as activation with tosyl chloride (**22**) and subsequent nucleophilic attack with NaN_3 afforded **23**. Hydrolysis of the ester gives 5-(3-azidopropyl)nicotinic acid (**11g**). The primary azide, when introduced into an NAADP analog, will offer a site at which the side-chain can be modified using "click chemistry". Additional derivatives can be synthesized through intermediate **22** and a diverse set of functionalities can potentially be introduced by displacement of the tosyl group with a variety of nucleophiles.



Scheme 6: Azidopropyl series a) propargyl alcohol, $Pd(PPh_3)_2Cl_2$, CuI, DIPEA, ACN, reflux, 16 h, 71% b) H_2 (g), Pd/C, MeOH, 10 h, 89% c) i) NaOH, MeOH, 37 ^oC, 4 h ii)HCl, 96% d) Tosyl chloride, TEA, DCM, reflux 28 h, 51% e) NaN_3. DMF, 60 ^oC, 18 h, 73% f) i) NaOH, MeOH, 37 ^oC, 3 h ii) HCl, 91%

2.1.3 Thiomethyl series

Envisioning the eventual production of thiol compound **26**, a set of sulfur containing molecules were developed (**scheme 7**). The reactive sulfur was predicted to be advantageous because synthesis of a variety of thioether analogs could be achieved by alkylation of the –SH using a variety of alkyl halides. Starting from 5-bromonicotinic acid, a nucleophilic aromatic displacement reaction occurred with sodium thiomethoxide[70,71] at high temperature microwave heat. It was difficult to push the reaction to completion and starting material and product possess similar physical properties making purifications difficult. Normally, protecting the acid as an ethyl ester will make compounds less polar and easier to purify. It was quickly realized that sodium thiomethoxide quickly and effectively removed ethyl ester, resulting in the production of free acid. Carrying out the synthesis in this particular order was important. O-Ethyl-N,N'-diisopropyl pseudourea was synthesized according to the literature[72] and used as a reagent for esterification forming **24**. MMPP, a peroxyacid compatible in sulfoxide formation,[73] efficiently formed sulfoxide derivative[74] **25**. To produce 5-thionicotinic acid, we envisioned use of the Pummerer rearrangement[75] of either **25** or **11i**. Unfortunately, all attempts were unsuccessful. Formation of 5-thionicotinic acid was possibly accomplished through Sandmeyer conditions[76] using sodium hydrosulfide as a nucleophile, but the method was so inefficient and the product isolated in small amounts and was never able to be fully characterized.

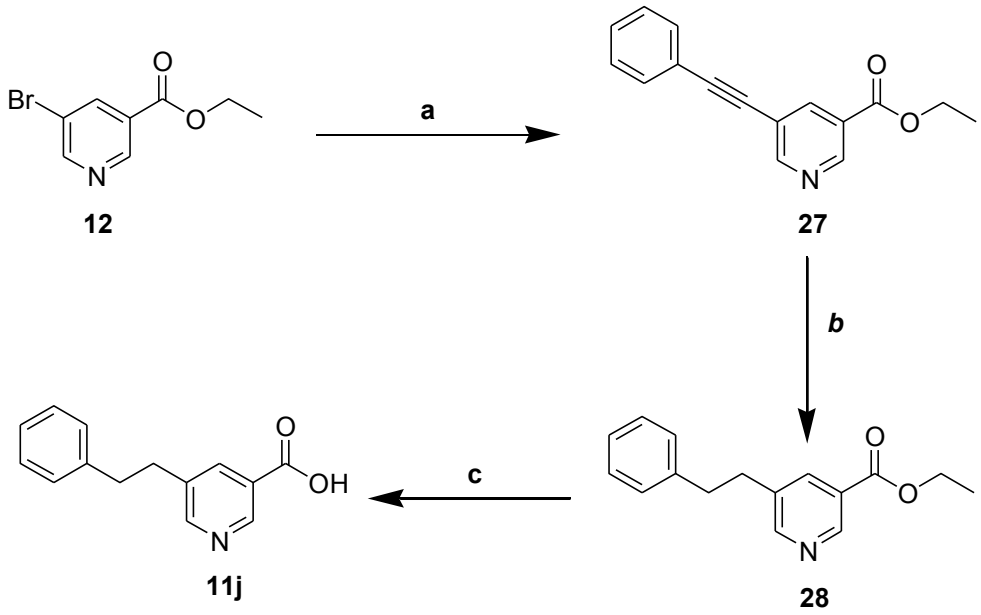


Scheme 7: Thiomethyl series a) $NaSCH_3$, DMF, mw 200 ^oC, 48 h, 57% b) O-ethyl-N,N'-diisopropyl pseudourea, DMF, 80 ^oC, 16 h, 86% c) MMPP, MeOH, 0 ^oC, 2 h, 76% d) i) NaOH, MeOH, 37 ^oC, 3 h ii) HCl, 75%

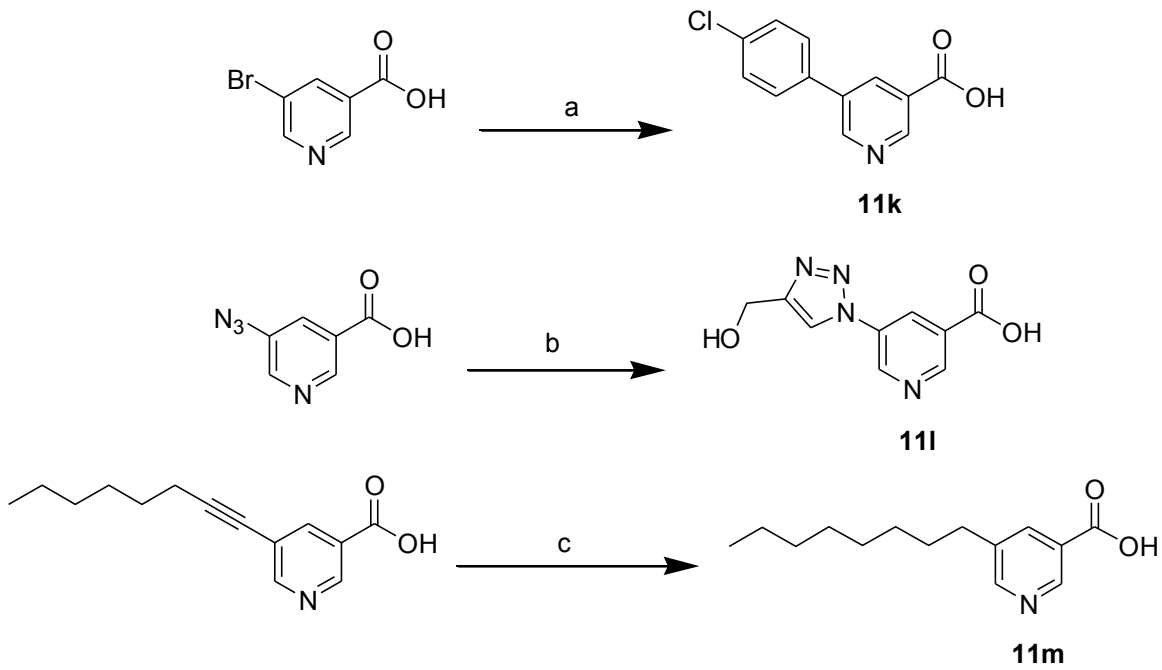
2.1.4 Non-exchangeable derivatives

Compounds described in this section unable to be made into NAADP analogs and were not always fully characterized as our emphasis remained in probing the NAADP receptor. Compounds in **Scheme 8** were used as model chemistries for previously described Sonogashira reactions **(Schemes 4-6**). These compounds were synthesized and screened for optimal Sonogashira conditions. Phenethyl compound (**11j**) was synthesized by coupling to phenylacetylene under Sonogashira conditions, hydrogenated, and made the free acid with aqueous base. **Scheme 9** further illustrates nicotinic acid analogs that were not base-exchange substrates. Compound **11m** was synthesized by reduction of the commercially available alkyne by hydrogenation. Triazole (**11l**) was synthesized by click chemistry involving a [1,3]-dipolar cycloaddition reaction.[77] The Cu^{+1} catalyst explains

the regioselectivity of the reaction and affords the 1,4- substituted product.[78] Compound **11k** was synthesized by Suzuki conditions and ultimately served as a model for Suzuki reactions on NAD or NADP in water (**Scheme 11**). Summary of 5 substituted nicotinic acid derivatives and their ability to serve as substrates in the base-exchange reaction are shown in **Table 1**.



Scheme 8: Synthesis of 5-phenethylnicotinic acid a) phenylacetylene, $Pd(PPh_3)_2Cl_2$, CuI, DIPEA, ACN, reflux, 16 h b) H_2 (g), Pd/C, MeOH, 10 h c) NaOH, MeOH, 37 ^oC, 10h

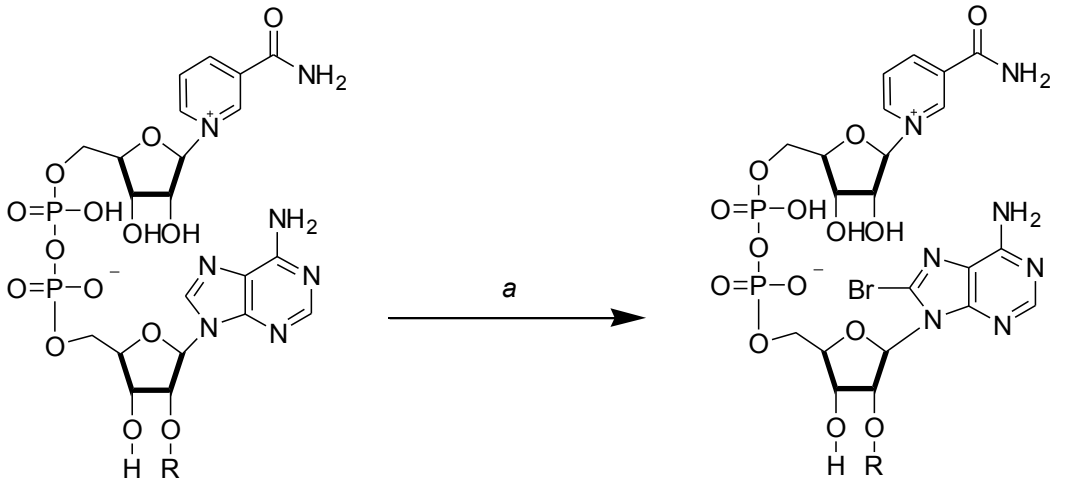


Scheme 9: Other non soluble/non exchangeable derivatives a) *p*-chlorophenyl boronic acid, $Pd(OAc)_2$, TXPTS, K_2CO_3, H_2O, 50 ^oC, 12 h b) propargyl alcohol, $CuSO_4$, sodium ascorbate, 2 h c) Pd/C, EtOH, 20 h.

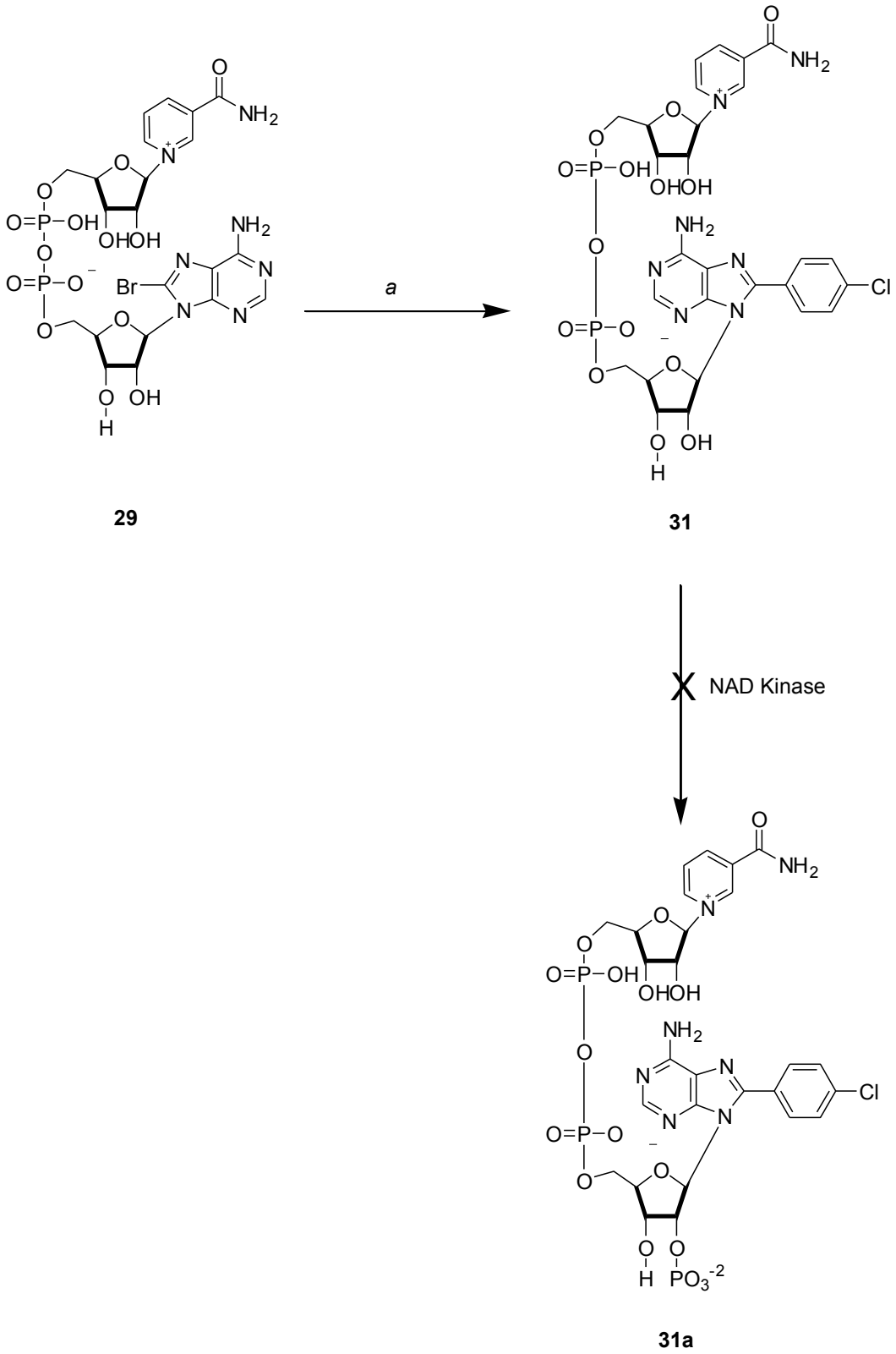
2.2 Synthetic strategies for modification of the 8 position of NAD and NADP

Discovering a second "structurally neutral" site was necessary, as our approach to identifying the NAADP receptor requires bifunctional probes. 8-Br-NAD (**5**) and 8-Br-NADP (**6**) were deemed to be strategic precursors to 8-substiuted NAADP derivatives. Illustrated in **Scheme 10**, bromine successfully halogenated the 8 position of the adenine ring. This reaction was based upon known brominations of nucleotides.[79,80] With a reactive 8-bromo group capable of various known reactions, further functionalization at the 8-adenosyl position was achieved. Substituents such as and *p*-chlorophenyl (**31**) and azido (**33a**) were introduced. **Scheme 11** highlights palladium catalyzed reactions in water.[81,82] By using a water soluble palladium catalyst ($Pd(OAc)_2$) and a water soluble

ligand (TXPTS), Suzuki coupling to *p*-chlorophenylboronic acid can be accomplished. This success implies that Sonogashira type coupling of **29** or **30** to an alkyne might be used to synthesize NAD or NADP derivatives containing 8-alkynyl substituents. These methods eliminate multi-step, low-yielding dinucleotide procedures, and if optimized, could be an extremely useful technique. Unfortunately, 8-*p*-chlorophenyl NAD (**31**) was not a substrate for NAD kinase (**Scheme 11**), and therefore could not be converted into an NADP analog. Because the coupled derivative could not be phosphorylated enzymatically (**31a**), couplings must be accomplished on NADP derivatives (**30**) in which as the phosphate is already present. Preliminary data for Suzuki couplings between 8-Br-NADP (**30**) and *p*-chlorophenylboronic acid indicate the reaction works in low yields based on HPLC and ^{1}H NMR data, but the product **31a** has not yet been isolated pure and characterized. It will be interesting to see if this compound will be recognized by *Aplysia* cyclase. 8-Azido-NAD (**33**) has been previously synthesized.[83] Subsequent enzymatic phosphorylation utilizing NAD kinase and ATP (**Scheme 12**) afforded 8-azido-NADP (**33a**).

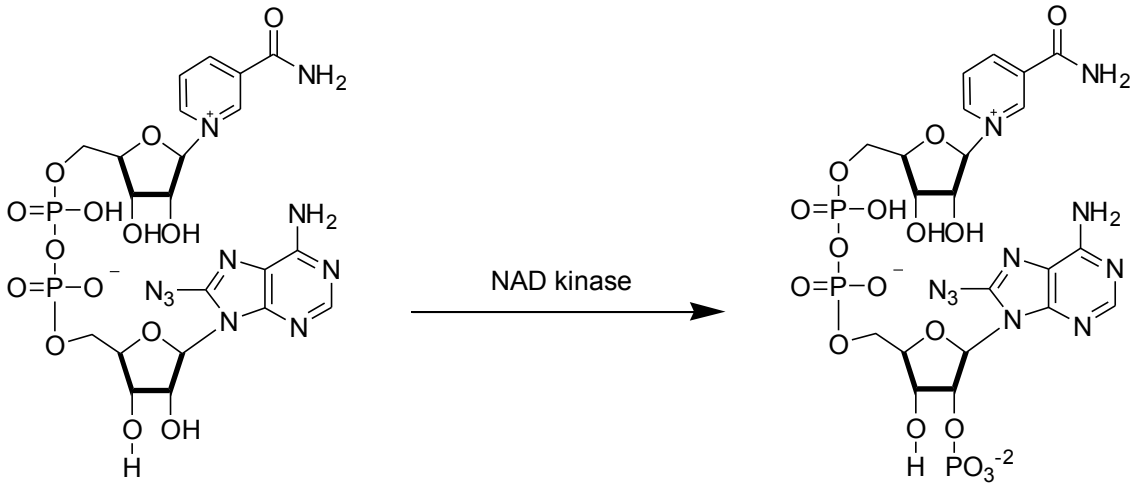


Scheme 10: Bromination of NAD or NADP a) Br_2, 0.5M NaOAc (pH = 4.8), 2 h



29 **31** **31a**

Scheme 11: Palladium catalyzed coupling in water a) *p*-chlorophenylboronic acid, $Pd(OAc)_2$, TXPTS, K_2CO_3, H_2O, 40 ^oC, 1.75 h



8-N_3-NAD

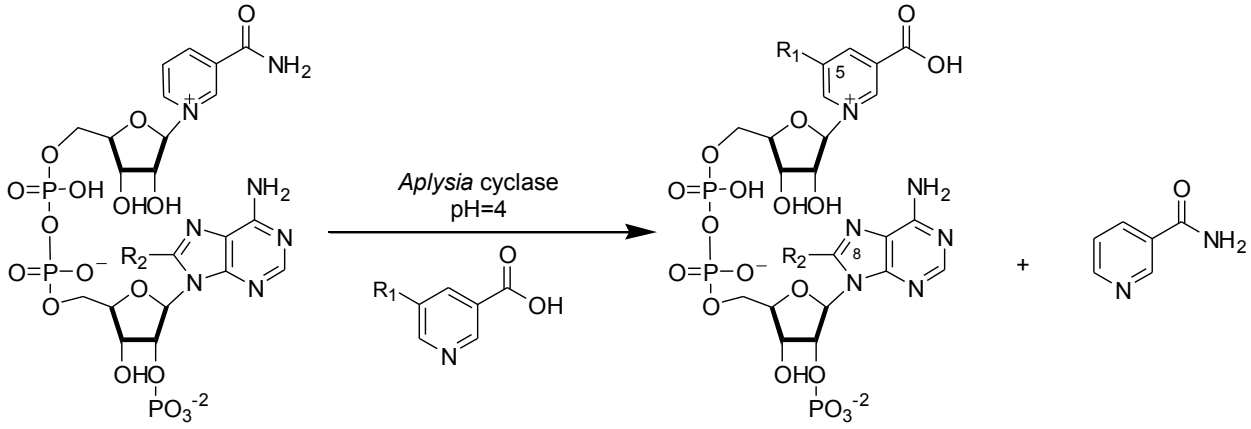
33

8-N_3-NADP

33a

Scheme 12: Phosphorylation of 8-azido-NAD by NAD kinase

2.3 Novel substituted NAADP analogs



NADP or
8 substituted NADP

Nicotinamide

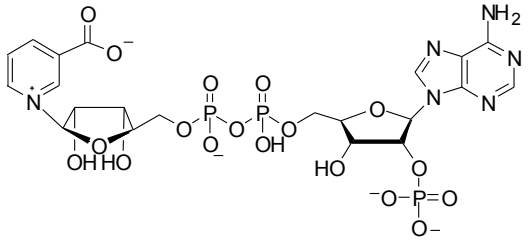
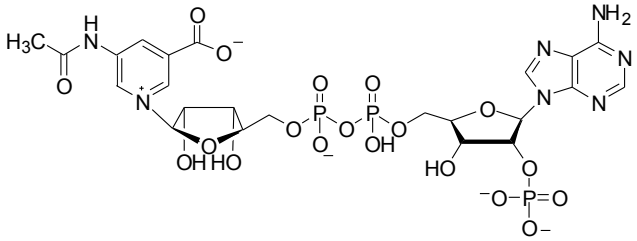
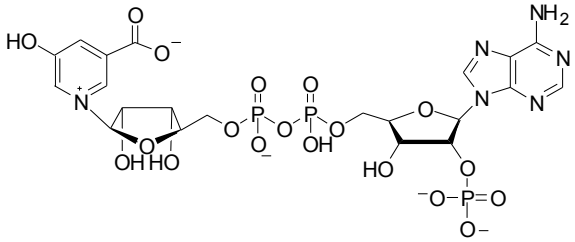
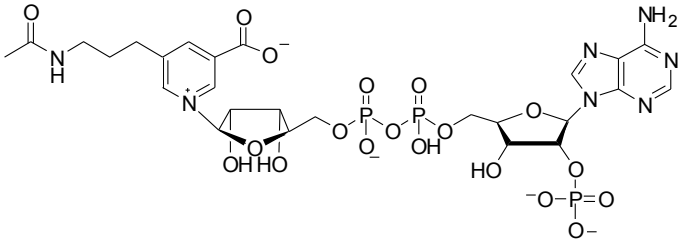
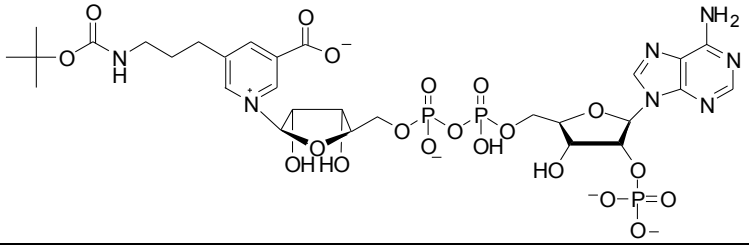
Figure 10: Base-exchange illustration for the chemoenzymatic synthesis of NAADP analogs.

In the presence of NADP (or 8 substituted NADP, $\mathbf{R_2}$), 5 substituted nicotinic acid ($\mathbf{R_1}$), and water (pH = 4), the enzyme *Aplysia* cyclase catalyzes the base exchange of

nicotinamide for 5 substituted nicotinic acid. With precursors containing either substitution at the 5 position or the 8 position, NAADP derivatives were synthesized as either mono or di-substituted analogs. Nicotinic acid derivatives (**11a-h**) were substrates for the base exchange reaction (**Figure 10, Table 1**) producing various 5 substituted NAADP analogs (**Table 2)**. These analogs illustrate classical medicinal chemistry techniques, as SAR studies were undertaken to probe receptor tolerance to various functionalities, including an aliphatic amine (**32e**), primary hydroxyl group (**32f**), amide derivatives and steric bulk (**32c, 32d**), and general bioisosteric replacement (**32a, 32b, 32h**). As our current approach intends on utilizing click chemistry to aid in purification of photoinserted NAADP, the aliphatic azide (**32g**) has more utility than simply furthering the SAR. Not all synthesized 5 substituted nicotinic derivatives were substrates for the base exchange reaction. Synthetic strategies for nicotinic acid analogs described in **Scheme 8** and **Scheme 9** were not base exchange substrates. In the case of phenylethyl compound (**11j**), this derivative exhibited poor water solubility and was therefore not a base exchange substrate. Similarly, compound **11m** was poorly soluble in water and was not a base exchange substrate. Alkynes and aromatic substituents attached directly to the 5 position are not known to be exchange substrates[65] as seen with triazole (**11l**) and chlorophenyl derivative (**11k**). Presumably, these substituents decrease the nucleophilicity of the pyridine nitrogen thereby diminishing their reactivity in the base-exchange reaction

With a number of 5 substituted NAADP derivatives synthesized, our focus turned to synthesis of 8 substituted NAADP derivatives, which uncovered a second site for functionality tolerating receptor mediated binding and activity. Using compounds (**30,**

33a) described in **Schemes 10** and **Scheme 12**, a set of 8-adenosyl substituted NAADP derivatives has been synthesized using base exchange reaction. 8-Br-NAADP (**32i**) and 8-azido-NAADP (**32j**) afforded preliminary analogs determining receptor tolerance at this position.

Name	Structure
NAADP (**3**)	
5-acetamido-NAADP (**32a**)	
5-hydroxy-NAADP (**32b**)	
5-(3-acetamidopropyl)-NAADP (**32c**)	
5-(3-t-boc-aminopropyl)-NAADP (**32d**)	

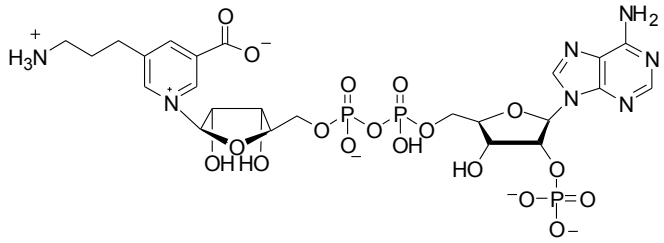
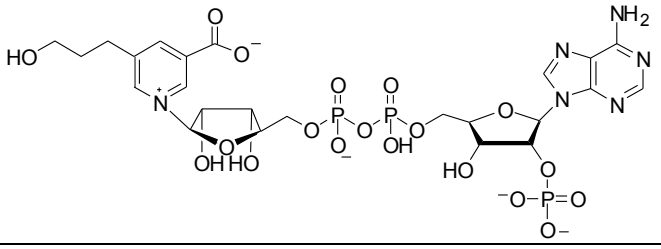
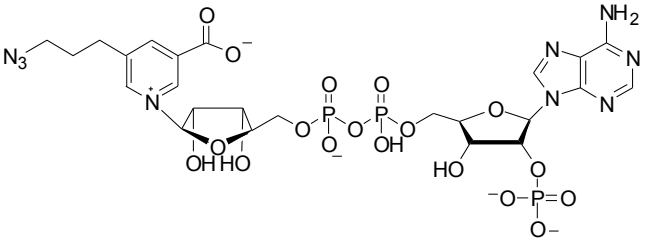
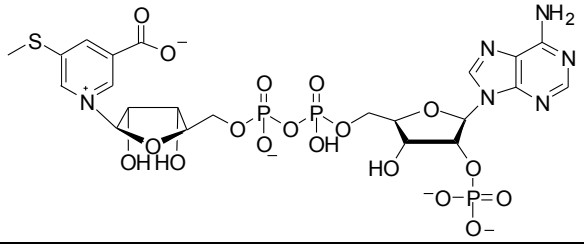
5-(3-aminopropyl)-NAADP (**32e**)	
5-(3-hydroxypropyl)-NAADP (**32f**)	
5-(3-azidopropyl)-NAADP (**32g**)	
5-thiomethyl-NAADP (**32h**)	

Table 2: Summary of 5 substituted NAADP analogs

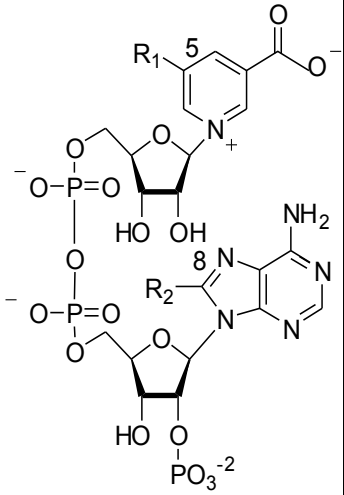
Structure	R_1	R_2	#	Name	Yield
	- $NHCOCH_3$	-H	**32a**	5-acetamido-NAADP	47%
	-OH	-H	**32b**	5-hydroxy-NAADP	63%
	- $CH_2CH_2CH_2$-NH-Ac	-H	**32c**	5-(3-acetamidopropyl)-NAADP	58%
	- $CH_2CH_2CH_2$-NH-*t*-Boc	-H	**32d**	5-(3-*t*-Boc-aminopropyl)-NAADP	42%
	- $CH_2CH_2CH_2$ -NH_3^+	-H	**32e**	5-(3-aminopropyl)-NAADP	66%
	-$CH_2CH_2CH_2$-OH	-H	**32f**	5-(3-hydroxypropyl)-NAADP	59 %
	-$CH_2CH_2CH_2$-N_3	-H	**32g**	5-(3-azidopropyl)-NAADP	52%
	- SCH_3	-H	**32h**	5-(thiomethyl)-NAADP	51%
	-CH_2CH_2-C_6H_5	-H		Not formed	0%
	-$SOCH_3$	-H		Not formed	0%
	-H	-Br	**32i**	8-bromo-NAADP	60%
	-H	-N_3	**32j**	8-azido-NAADP	N/A
	-N_3	-Br	**32k**	5-azido-8-bromo-NAADP	35%
	-N_3	-N_3	**32l**	5-azido-8-azido-NAADP	N/A

Table 3. Summary of novel mono and di-substituted NAADP analogs at the 5 ($\mathbf{R_1}$) position and 8 position ($\mathbf{R_2}$)

One important goal of this study was to synthesize bifunctional NAADP analogs. To accomplish this, di-substituted NAADP derivatives were synthesized and tested for recognition by the NAADP receptor. Technically, a "bifunctional" compound can be classified as a di-substituted NAADP analog bearing two differently functionalized substitutents. The difference is their eventual use. Di-substituted compounds help further the SAR and increase general knowledge, while a bifunctional compound was designed for a specific application which will utilize the attached functionalities. Subjecting 5-azidonicotinic acid and either 8-bromo-NADP or 8-azido-NADP (**Table 3**) to base-exchange conditions (**Figure 10**) resulted in di-substituted NAADP derivatives **32k** and

32l. Due to our interest in PAL, derivatives containing the 5-azido moiety were synthesized, as this has proven to be a successful photoprobe.

2.4 Characterization of NAADP analogs

All NAADP derivatives were characterized by ^{1}H NMR, ^{31}P NMR, high resolution mass spectrometry (MALDI-TOF), and HPLC (anion exchange chromatography). In all cases the spectra and the observed molecular mass supported the assigned structure. Synthesis of NAADP was repeated and values were not taken directly from the literature.[8,64,84] Previously reported assignments were consistent with our observations. NMR experiments were performed under neutral conditions (pH = 7). For successful mass spectrometric determinants, it was necessary to desalt analogs in order to observe the molecular ion. HPLC purifications were performed on resins that were appropriately prepared, then packed into a glass column that could be fitted to an HPLC system (see Experimental section).

Compounds selected (**32e**, **32i**) were chosen to illustrate proton chemical shift differences between 5 and 8 substituted analogs (**Table 4**). As a comparison, NAADP was included. Splitting patterns observed in NAADP, specifically the nicotinic acid moiety, were not present in the 5-substituted analogs as the 5-pyridinyl proton was absent. Instead, the pyridine protons appear as singlets. When the 8-position was modified, one of the adenosyl protons was replaced with either a bromo or azido group. The corresponding 8-adenosyl proton would now be absent. ^{1}H NMR was very useful in determining the structure of NAADP derivatives, as aromatic (H_N2-6, H_A2, 8) and anomeric (H_N1', H_A1") signals provide initial structural evidence, as well as provide a

preliminary indication of sample purity. Spectra consisting of extraneous anomeric signals suggest the material is contaminated with other dinucleotide impurities. The two sets of ribosyl peaks were difficult to assign individually and were often observed as a "clump" of peaks reported as a multiplet (m) spanning approximately 0.2 parts per million (ppm). ^{31}P data was used to prove the general structure of dinucleotides. Peaks resonating at approximately at 0.5 ppm (2'-adenosyl phosphate) and -10 ppm (pyrophosphate) were present. In the case of NAD derivatives **29** and **31**, the 2'-adenosyl phosphate was absent.

The Experimental section thoroughly describes the HPLC methods. NAADP derivatives were subjected to anion exchange chromatography which effectively purifies compounds based on charge. Nucleotides produced as side products in the *Aplysia* catalyzed base-exchange reaction, as well as stating material NADP, were easily distinguished from NAADP (**Figure 11**). The same compounds described in **Table 4** are used for HPLC illustration (**3**, **32e**, **32i**). NADP (**6**) was also included, as this was the starting material for base-exchange reaction. The primary amine (**32e**) was protonated at physiological pH allowing for a less negatively charged species, explaining why the compound eluted from the column earlier than other NAADP derivatives (t = 6.93). NAADP derivatives (**3**, **32i**) demonstrate typical elution times.

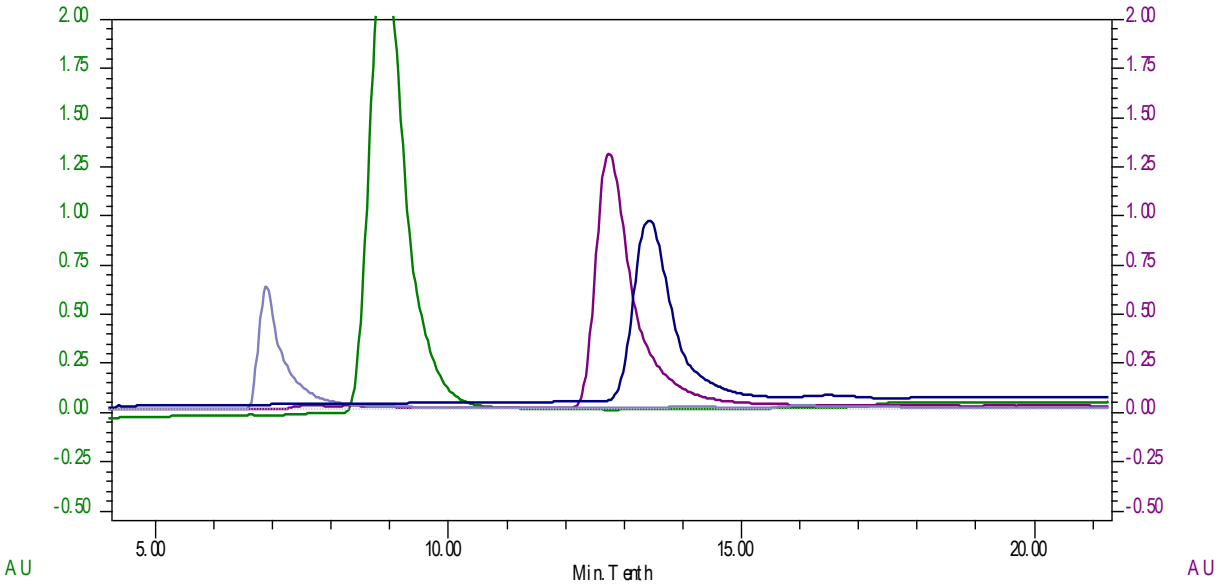
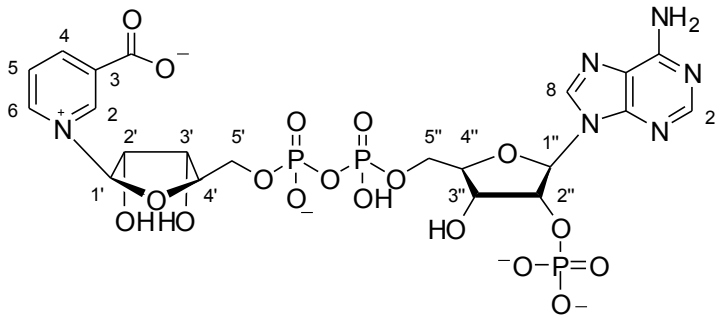


Figure 11: HPLC (0-50% 100 mM TFA) comparative traces of various dinucleotides (from left to right). 5-(3-aminopropyl)-NAADP (**32e**, light blue, t = 6.93 min), NADP (**6**, green, t = 8.96 min), NAADP (**3**, purple, t = 12.8 min), 8-bromo-NAADP (**32i**, dark blue, t = 13.47 min).



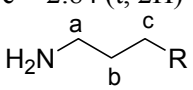
Proton location	NAADP (**3**)	5-(3-propylamino)-NAADP (**32e**)	8-bromo-NAADP (**32i**)
Nicotinate ring protons			
H_N2	9.12 (s, 1H)	8.94 (s, 1H)	9.22 (s, 1H)
H_N4	8.75 (d, 1H, *J* = 8 Hz)	8.60 (s, 1H)	8.89 (d, 1H, *J* = 8)
H_N5	8.07 (m, 1H)	aminopropyl	8.18 (m, 1H)
H_N6	9.01 (d, 1H, *J* = 6 Hz)	8.80 (s, 1H)	9.01 (d, 1H, *J* = 6.4)
Adenine ring protons			
H_N2	8.14 (s, 1H)	8.06 (s, 1H)	8.25 (s, 1H)
H_N8	8.44 (s, 1H)	8.34 (s,1H)	bromo
Anomeric protons			
H_N1'	6.04 (d, 1H, *J* = 3.2 Hz)	5.96 (d, 1H, *J* = 4)	6.04 (d, 1H, *J* = 5.2)
H_A1''	6.18 (d, 1H, *J* = 7.8 Hz)	6.06 (d, 1H, *J* = 4.8)	6.21 (d, 1H, *J* = 6.4)
Pyridinium-ribose protons			
H2'	4.23-4.41 (m, 1H)	4.13-4.32 (m, 1H)	4.31-4.49 (m, 1H)
H3'	4.23-4.41 (m, 1H)	4.13- 4.32 (m, 1H)	4.31-4.49 (m, 1H)
H5'	4.23-4.41 (m, 2H)	4.13- 4.32 (m, 2H)	4.31-4.49 (m, 2H)
H4'	4.5 (s, 1H)	4.44 (s, 1H)	4.31-4.49 (m, 1H)
Adenine-ribose protons			
H2''	5.11 (m, 1H)	4.89 (m, 1H, buried in HDO)	5.51 (m, 1H)
H3''	4.64 (m,1H)	4.54 (m, 1H)	4.91 (m, 1H)
H4''	4.23-4.41 (m,1H)	4.13-4.32 (m, 1H)	4.31-4.49 (m, 1H)
H5''	4.23-4.41 (m,2H)	4.13-4.32 (m, 1H)	4.31-4.49 (m, 2H)
Propyl group		a = 3.02 (t, 2H) b = 1.97 (m, 2H) c = 2.84 (t, 2H) 	

Table 4: ^{1}H NMR characterization for select 5 and 8-substituted NAADP derivatives relative to NAADP

2.5 Biological activity

NAADP analogs were biologically tested in sea urchin egg homogenates performed by a collaborating investigator Tim Walseth. It is noteworthy to mention he was also responsible for synthesis of 8-azido-NAADP (**32j**) and the corresponding precursors. Sea urchin egg homogenates are considered to be the "gold standard" for measuring intracellular Ca^{2+} release, as IP3 and cADPR receptor-mediated activity were discovered using this system. The eggs are easy to work with and provide robust Ca^{2+} signals. Invertebrate systems will serve as a model for NAADP mediated activity in mammalian cells. Currently, studies are underway trying to correlate data from sea urchin eggs to mammalian cell preparations.[35] Measuring Ca^{2+} release in mammalian cells is obviously desired, but the process is difficult.

The ability of NAADP analogs (**32a-l**) to elicit Ca^{2+} release from sea urchin egg homogenates was tested using a fluorometric assay. Calcium ion was allowed to sequester to intracellular stores driven by the presence of ATP. Upon addition of NAADP analog, Ca^{2+} was released and binds to Ca^{2+} indicator, Fluo-3, which itself is weakly fluorescent. Upon chelation with released Ca^{2+}, Fluo-3 fluorescence was enhanced. The increase in fluorescence was proportional to the released Ca^{2+} and can be measured using a fluorescence plate reader (excitation 490 nm and emission 535 nm). These experiments were performed in triplicate at a variety of agonist concentrations and EC_{50} values were determined (**Table 5**). Receptor binding studies using radiolabeled NAADP were performed on all analogs in 96-well filter plates. The competitive binding experiments used a constant concentration of ^{32}P-NAADP (0.2 nM) and were incubated simultaneously (90 min) with NAADP analog. Seven concentrations of each analog were

tested to determine IC_{50} values. The homogenate was filtered and washed. The radioactivity retained on the filter and was determined by liquid scintillation (**Table 6**). A final assay of synthesized NAADP analogs determines receptor recognition by a second, non-radioactive method. The sea urchin NAADP receptor was desensitized upon pretreatment with sub threshold concentrations of NAADP; Subsequent additions of NAADP were incapable of releasing Ca^{2+} from intracellular stores. Therefore, if the sea urchin NAADP receptor was incubated for several minutes with chemoenzymatically synthesized NAADP analogs, the receptor should be inactivated if the analogs are well recognized and further challenge by NAADP will be incapable of Ca^{2+} release from intracellular stores. Pretreatment (7 min incubation) with varying concentrations of synthesized NAADP analog (**32a-l**) followed by addition of NAADP (1 μM) then fluorometric measurement of Ca^{2+} release were reported in terms of IC_{50} values (**Table 7**). Analogs that were well recognized should not respond to NAADP challenges as the receptor will be desensitized. Experiments were performed in triplicate. **Figure 12** illustrates typical concentration response relationships measured by the above assays (Ca^{2+} release, competition-ligand binding, and desensitization). Both compounds demonstrate high potency to the sea urchin NAADP receptor. 5-Thiomethyl-NAADP was considered a full agonist.

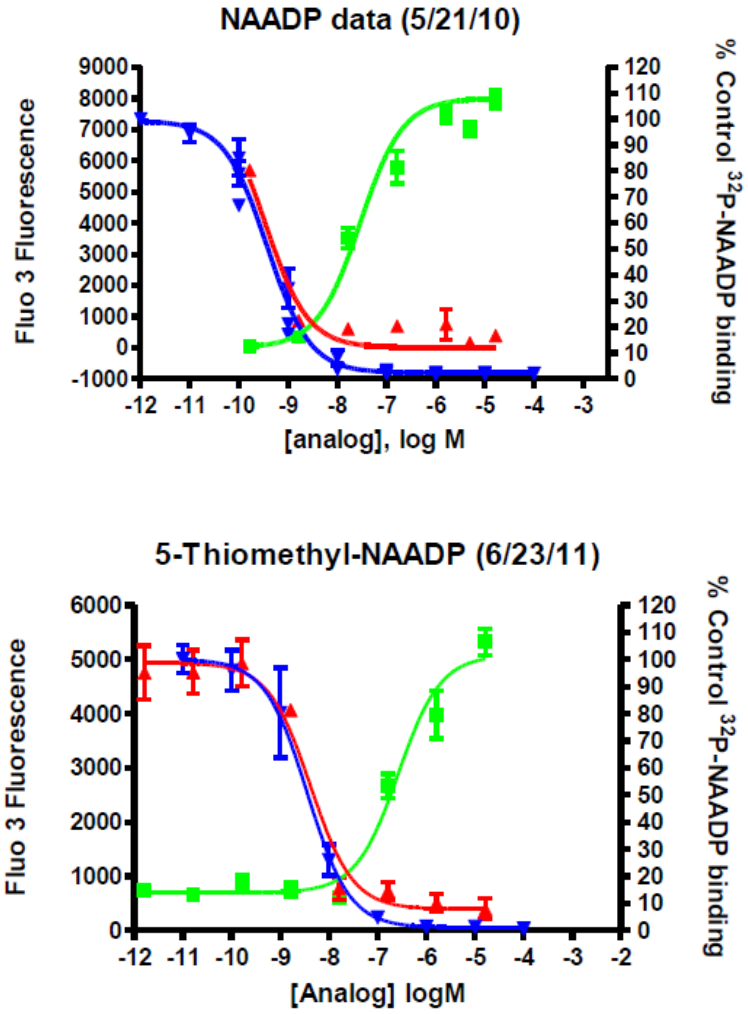


Figure 12: Typical concentration response curves for NAADP (**3**, top) and analog 5-thiomethyl-NAADP (**32h**, bottom). The experiments were performed in sea urchin egg homogenates. The green line represents Ca^{2+} release. The blue line corresponds to competition-ligand binding experiments. The red line is measuring Ca^{2+} release after desensitization. This data is illustrative of potent agonists to the NAADP receptor.

Previously, compounds containing small substituents (methyl, amino, ethyl) at the 5 position of NAADP were shown to be agonists, with binding potency comparable to

that of the parent molecule (measured as relative fold increases in IC_{50} and EC_{50}). This was further confirmed in our study. Thiomethyl compound (**32h**) was an agonist with an EC_{50} for Ca^{2+} release 5-fold higher relative to NAADP (**Table 5, Figure 12**) and with an IC_{50} value for competitive ligand binding 10 fold higher than that of NAADP (**Table 6, 7**). Structurally similar compound 5-ethyl-NAADP (EC_{50} = 18 fold increase IC_{50} = 23 fold increase) was slightly less potent, suggesting that replacing carbon with sulfur offers a slight advantage. Additional small substitutions at the 5 position, exemplified by acetamido (**32a**) and hydroxyl (**32b**), were also shown to be high potency agonists. The slight loss of activity in **32a** (EC_{50} = 20.9 fold increase), as well as its binding (IC_{50} = 42.9 fold increase for competitive binding, IC_{50} = 51.2 fold increase for desensitization) could be explained by the increased branching. 5-Hydroxy-NAADP (**32b**) demonstrated unexpected loss of activity. For example, the EC_{50} value for the isosteric 5-amino-NAADP was measured as a 1.5 fold increase relative to parent ligand (**3**). Substitution with hydroxyl group (**32b**) measured a 64 fold loss of potency. This relative loss of activity was unexpected and can best be attributed to the ionization of the phenolic group at physiological pH, suggesting the receptor prefers the pyridinium functionality to be zwitterionic, but to possess no net charge. This proposal was further supported by the results of testing aliphatic amine (**32e**). The primary amine will be protonated at physiological pH, affording a net +1 charge on the pyridinium ring explaining loss of potency. Apparently, receptor-mediated events require analogs that preserve the zwitterionic nature of the pyridinium ring. Additionally, 5-carboxy-NAADP was previously synthesized by subjecting NADP and 3,5-pyridine-dicarboxylate to the base exchange reaction (**Figure 10**).[65] This particular NAADP analog carries a net -1 charge

on the pyridinium moiety at physiological pH. The 5-Carboxy-NAADP was not recognized by the sea urchin NAADP receptor, supported by its inability to release Ca^{2+} from intracellular stores and its inability to compete with radiolabeled NAADP in competition ligand-binding experiments. It is becoming increasingly apparent that extra charge is not accommodated by the receptor. Ultimately, the pyridinium moiety must be zwitterioninc and possess no net charge for receptor-mediated events to occur.

Compound	EC_{50} ± s.e.m., nM (n)	Fold increase in EC_{50}
NAADP (**3**)	30 ± 6.9 (15)	1
5-hydroxy-NAADP (**32b**)	1932.3 ± 440.1 (3)	64.4
5-acetamido-NAADP (**32a**)	626.2 ± 46.1 (7)	20.9
5-thiomethyl-NAADP (**32h**)	151.3 ± 22.8 (3)	5.0
5-(3-hydroxypropyl)-NAADP (**32f**)	4433.0 ± 578.7 (7)	147.8
5-(3-azidopropyl)-NAADP (**32g**)	40840 ± 23350 (3)	1361.3
5-(3-*t*-Boc-aminopropyl)-NAADP (**32d**)	160021 ± 54720 (9)	5334
5-(3-aminopropyl)-NAADP (**32e**)	>1000000 (3)	>33333
5-(3-acetamidopropyl)-NAADP (**32c**)	41718 ± 7097 (6)	1468.9
8-bromo-NAADP (**32i**)	709.1 ± 63.6 (3)	23.6
8-azido-NAADP (**32j**)	47.7 ± 1.6 (3)	1.6
5-azido-8-bromo-NAADP (**32k**)	>10000 (3)	>333
5-azido-8-azido-NAADP (**32l**)	4489.0 ± 529.5 (3)	149.6

Table 5: EC_{50} values for Ca^{2+} release induced by NAADP (**3**) and NAADP analogs (**32a-l**) measured fluorometrically from Ca^{2+} loaded sea urchin egg homogenates.

Compound (structure #)	IC_{50} ± s.d., nM (n)	Fold increase in IC_{50}
NAADP (**3**)	0.35 ± 0.15 (11)	1
5-hydroxy-NAADP (**32b**)	9.7 ± 0.0 (1)	27.7
5-acetamido-NAADP (**32a**)	15.0 ± 3.0 (4)	42.9
5-thiomethyl-NAADP (**32h**)	3.6 ± 1.4 (2)	10.3
5-(3-hydroxypropyl)-NAADP (**32f**)	49.0 ± 15.2 (4)	140.0
5-(3-azidopropyl)-NAADP (**32g**)	52.3 ± 13.2 (2)	149.4
5-(3-*t*-Boc-aminopropyl)-NAADP (**32d**)	109.1 ± 0.0 (1)	311.7
5-(3-aminopropyl)-NAADP (**32e**)	590000 ± 7950 (2)	1685714
5-(3-acetamidopropyl)-NAADP (**32c**)	4200 ± 0.0 (1)	12000
8-bromo-NAADP (**32i**)	12.0 ± 0.0 (1)	34.3
8-azido-NAADP (**32j**)	0.7 ± 0.2 (2)	2
5-azido-8-bromo-NAADP (**32k**)	371.6 ± 0.0 (1)	1062
5-azido-8-azido-NAADP (**32l**)	10.6 ± 0.5 (3)	30.2

Table 6. IC_{50} values determined for compeition ligand binding between [^{32}P]NAADP and NAADP analogs (**32a-32l)**

Compound (structure #)	IC_{50} ± s.e.m., nM (n)	Fold increase in IC_{50}
NAADP (**3**)	0.42 ± 0.08 (5)	1
5-hydroxy-NAADP (**32b**)	**23.1 ± 7.7 (3)**	55
5-acetamido-NAADP (**32a**)	21.5 ± 7.9 (3)	51.2
5-thiomethyl-NAADP (**32h**)	4.2 ± 0.6 (3)	10
5-(3-hydroxypropyl)-NAADP (**32f**)	122.0 ± 21.8 (3)	290.4
5-(3-azidopropyl)-NAADP (**32g**)	52.5 (1)	125
5-(3-*t*-Boc-aminopropyl)-NAADP (**32d**	942 ± 359 (7)	2242
5-(3-aminopropyl)-NAADP (**32e**)	674000 (1)	154143
5-(3-acetamidopropyl)-NAADP (**32c**)	1230.6 ± 174.6 (6)	2930
8-bromo-NAADP (**32i**)	15.3 ± 5.4 (3)	36.4
8-azido-NAADP (**32j**)	0.6 ± 0.2 (2)	1.4
5-azido-8-bromo-NAADP (**32k**)	575.5 ± 57.4 (1)	1370.2
5-azido-8-azido-NAADP (**32l**)	56.5 ± 11.2 (2)	134.5

Table 7: IC_{50} values determined for desensitization of calcium release induced by 1μM NAADP after a 7 minute preincubation with varying concentrations of NAADP analogs

One of the goals of this project was to produce NAADP analogs possessing a practical utility, particularly for a receptor purification and characterization. Derivatives with a 3-carbon spacer arm attached to various functional groups (**32c-g**) were synthesized and evaluated. Acetamidopropyl **(32c**), *t*-Boc-aminopropyl (**32d**), hydroxypropyl (**32f**), and azidopropyl (**32g**) were developed as an analogous series of compounds. Both **32c** and **32d** illustrated the upper limits of substitution as the

compounds are highly branched, and in the case of *t*-Boc derivative (**32d**), contain a relatively extreme amount of steric bulk. This derivative demonstrated a 5000 fold loss of potency. Conversely, the competitive-ligand binding studies illustrate only a 300 fold loss of potency. Compound **32d** exhibits activity comparable to a partial agonist, because the measured competition ligand binding predicts higher potency for Ca^{2+} release (comparable to **Figure 13**). Hydroxypropyl derivative (**32f**) demonstrated modest potency and competed relatively well with radiolabeled NAADP, although the IC_{50} for desensitization experiments was slightly higher than expected (**Table 7**, IC_{50} = 290 fold increase). In this particular case, the difference in experimental conditions may explain this result. Azidopropyl analog (**32g**) displayed interesting data in that the compound was not very effective in Ca^{2+} release (EC_{50} = 1300 fold increase) yet binding assays (**Tables 6, 7**) suggest this compound binds fairly well relative to similar substituted compounds containing substituted n-propyl chains at the 5 position. Demonstrated in **Figure 13**, compound **32g** binds and activates the receptor, but only partial effectiveness (Ca^{2+} release) was observed relative to NAADP. From this data, **32g** can be classified as a partial agonist to the NAADP receptor.

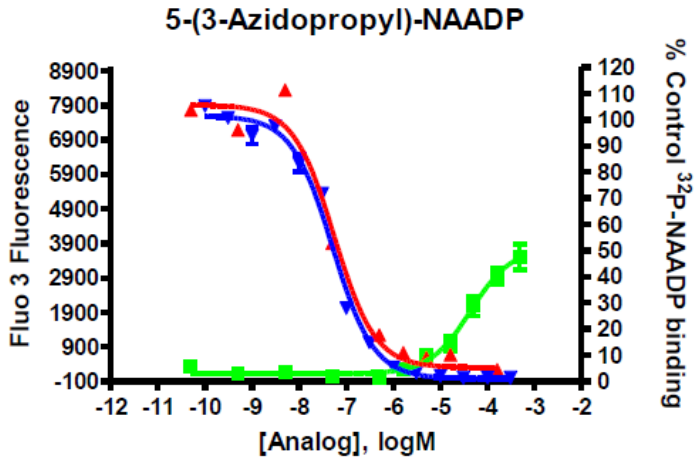


Figure 13: Concentration response curves for 5-(3-azidopropyl)-NAADP. The experiments were performed in sea urchin egg homogenates. The green line represents Ca^{2+} release. The blue line corresponds to competition-ligand binding experiments. The red line is measuring Ca^{2+} release after desensitization. This data is illustrative of a partial agonist to the NAADP receptor.

The data in **Tables 5-7** clearly indicate that NAADP analogs with substituents at the 8-adenosyl position were recognized as high potency agonists. 8-Azido-NAADP (**32j**) was shown to be a high potency agonist, as both the EC_{50} and IC_{50} were no more than 2 fold higher than NAADP. 8-Bromo-NAADP (**32i**) was found to be slightly less potent, as its EC_{50} was approximately 20 fold higher than that of NAADP. Binding studies measured IC_{50} values to be approximately 35 fold higher than NAADP. Together, the 8-adenosyl position has been identified as a "structurally neutral" position. Next, di-susbtituted NAADP derivatives have been synthesized and tested in the sea urchin egg system (**Tables 5-7**). Comparing 8-bromo-NAADP (**32i**) with 8-azido-NAADP (**32j**), it was obvious that azido analog was more potent. It comes as no surprise that di-

substituted 5-azido-8-azido-NAADP (**32l**) was better recognized by the receptor than was 5-azido-8-bromo-NAADP (**32k**), as the difference in 8-substituents clearly influences potency. Compound **32k** was recognized by the receptor, but as a low potency partial agonist, as IC_{50} and EC_{50} were greater than 1000 fold less potent than parent ligand. On the other hand, di-substituted azido compound was considerably more potent (**Tables 5-7**), suggesting that bifunctional compounds with substituents on the nicotinic acid 5 position and on the 8 adenosyl position can be developed to support future studies on receptor isolation.

2.6 First generation bifunctional NAADP derivative

Based upon the high potency of 8-azido-NAADP (**32j**) and the modest potency exhibited by 5-(3-azidopropyl)-NAADP (**32g**), it was postulated that a di-substituted compound containing this substitution could serve as an effective tool with which to probe the identity of the NAADP receptor. At this point, describing this compound as bifunctional would be appropriate, because different substitutions will be introduced at the nicotinic acid 5-position and the adenosyl 8-position for different roles in receptor identification. Photoaffinity labeling (PAL) was previously discussed (page 18) using 5-azido-NAADP. To reiterate, all attempts to gather sequence information from PAL studies were futile. The desired proteins are likely rare, and a method of purification needs to be developed to identify the receptors. PAL of the receptor using 8-azido-NAADP has been accomplished and the bands detected were identical to those in the previous study.[85] 5-(3-Azidopropyl)nicotinic acid-8-azidoadenosyl-NAADP (**32m**) is currently undergoing testing to determine binding potency and can be synthesized using

the base exchange reaction (**Figure 14**) from 8-azido-NADP (**33a**) and 5-(3-azidopropyl)nicotininc acid (**11g**). We predict this analog to be recognized based upon the extensive SAR we have presented.

2.6.1 Proposed purification and identification of the NAADP binding protein

The concept of photoaffinity labeling a protein, then using a second, distinct functionality to aid in further characterization has been previously utilized.[86-89] For example, protein binding partners for phospatidylinositol 3,4,5-trisphosphate have been identified with use of a bifunctional probe, which in this case, was a photoanalog of PIP_3 with a corresponding terminal acetylene as a "clickable" detection tag. Through this methodology, PIP3 binding proteins were identified and further characterized.[88] Our proposed attempts at further identifying the NAADP binding protein are illustrated in **Figure 15**. Upon photoinsertion of the azide at the 8-adenosyl position to the protein of interest, the clickable tag will distinguish photolabeled proteins from unlabeled ones offering a possible site for purification. The purification of the receptor complex will be dependent on the azidopropyl group by "click" chemistry to a modified biotin. Photolyzation of aromatic azide (**32m**) produces a very reactive nitrene intermediate (**34**) that reacts with proteins associated with the NAADP receptor, forming a covalently bound ligand-receptor complex (**35**). The aliphatic azide connected to a 3-carbon atom spacer arm can be "clicked" linking it to biotin functionalized with an alkyne (**36**), aiding in purification of the biotin-triazole-ligand-receptor complex (**37**). It is well known that biotin and streptavidin form a tightly bound complex. Through binding and elution on

streptavidin based resins, we propose to purify the NAADP receptor and provide evidence of its protein sequence through mass spectrometry.

Technology utilizing palladium-catalyzed reactions in water was developed for a specific purpose (**Scheme 11**). Illustrated in **Figure 16**, future strategies forming a bifunctional analog with 8-substituted alkynes and 5-azidonicotininc acid by base-exchange may be necessary, as there are no guarantees 1st generation bifunctional compounds will succeed. It is reasonable to expect coupling of 8-bromo-NADP (**30**) to propargyl alcohol under water soluble Sonogashira conditions ($Pd(OAc)_2$, TXPTS, $CuSO_4$, ascorbic acid) will yield desired propargyl NADP derivative (**38**), which can then be reacted with 5-azidonicotinic acid by base exchange reaction forming bifunctional compound (**39**). The purpose of sodium ascorbate is to generate Cu^{+1} catalyst *in situ* by reduction of $CuSO_4$. In the case of the 2nd generation bifunctional probe (**39**) azido biotins will be used opposed to alkynyl biotin derivatives (**Figure 16**) in order to click the molecule to a purification tag.

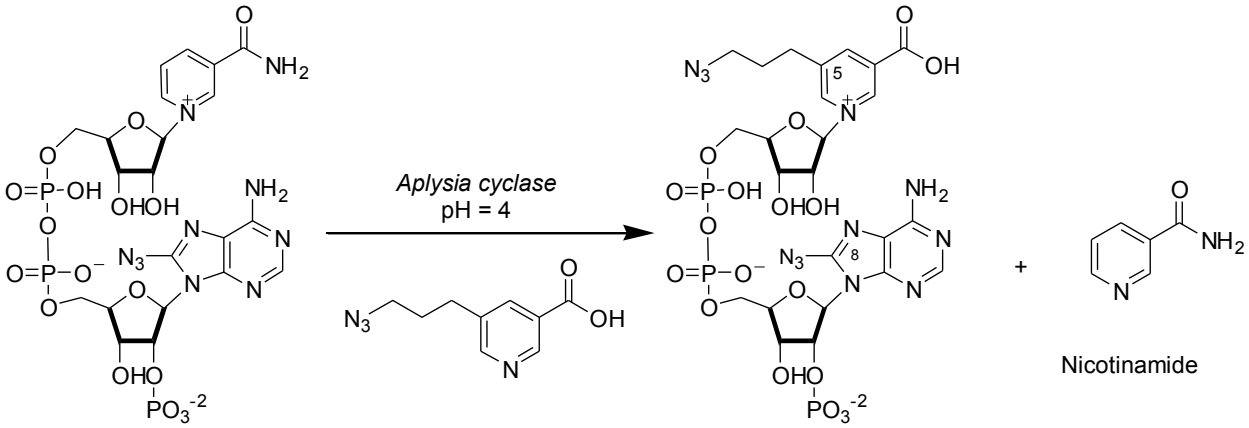


Figure 14: Proposed bifunctional compound 5-(3-azidopropyl)nicotinic acid-8-azidoadenosyl-NAADP (**32m**)

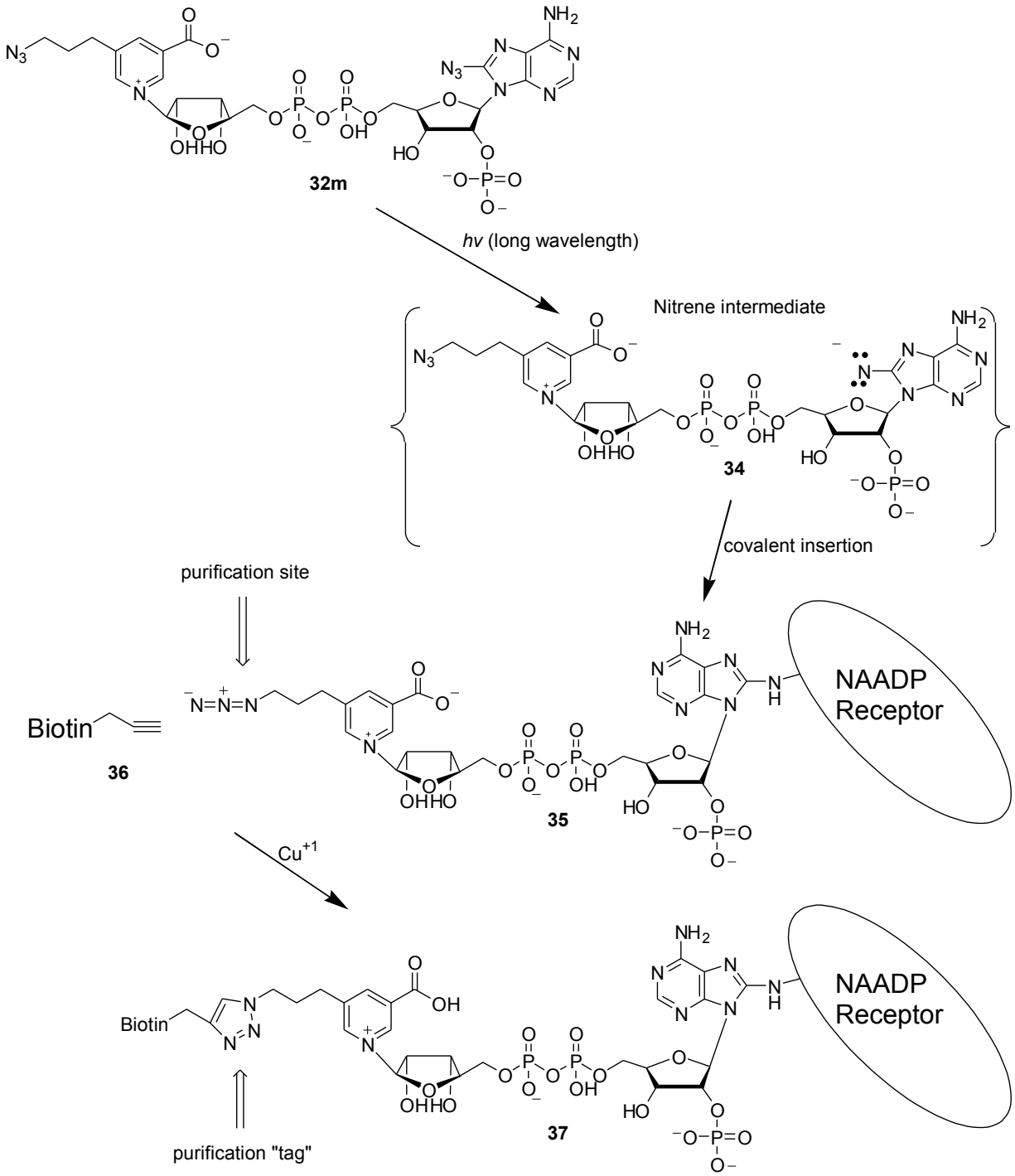


Figure 15: Proposed utilization of bifunctional probe (**32m**) in NAADP receptor labeling and purification.

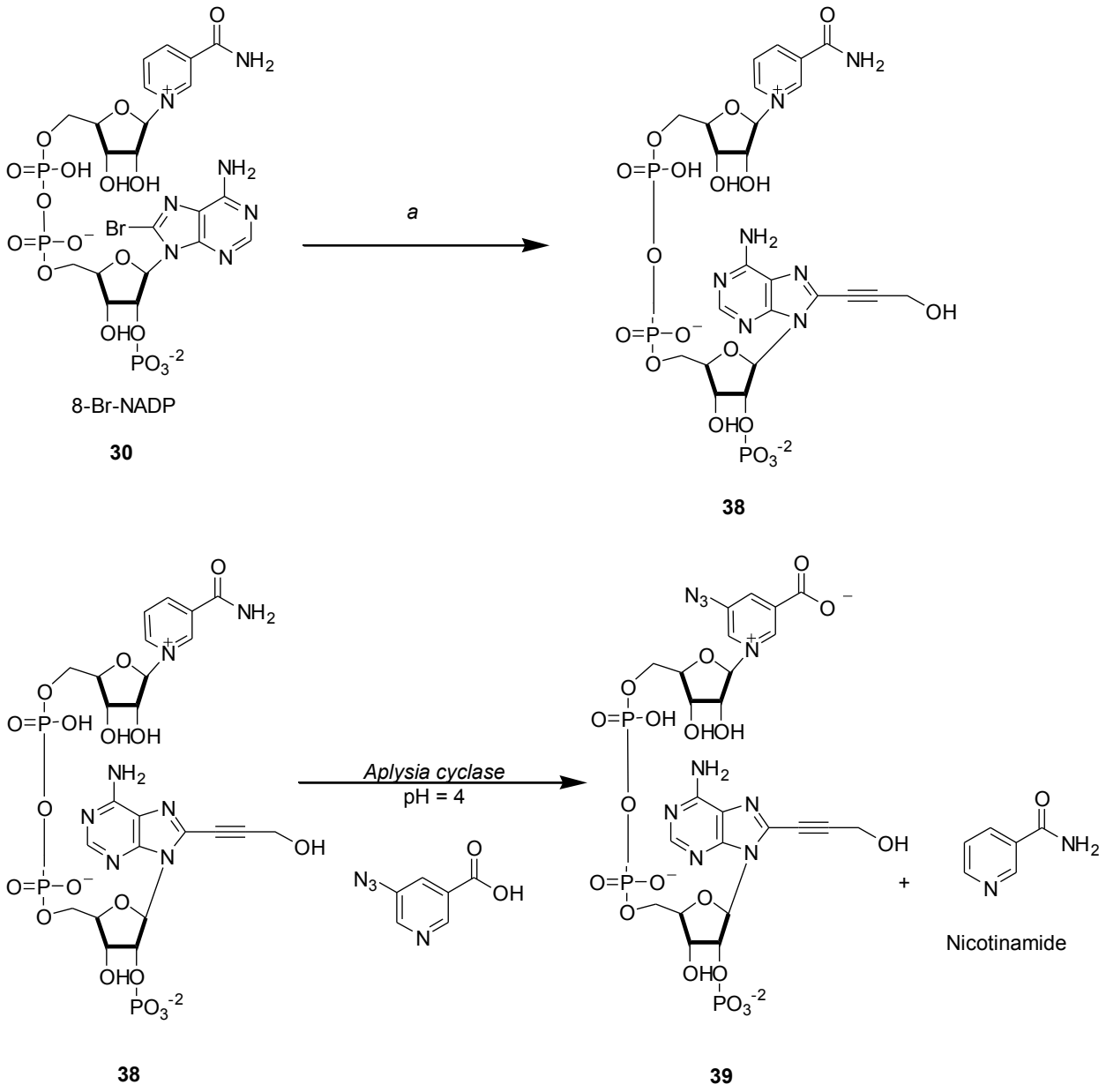


Figure 16: Proposed 2nd generation bifunctional probe (**39**): a) propargyl alcohol, $Pd(OAc)_2$, TXPTS, $CuSO_4$, sodium ascorbate

Chapter 3

Experimental Sections

3.1 General description

The following procedures were used in all reactions unless otherwise noted. Reactions were performed in washed and oven-dried glasswares (≥ 110 ^{o}C). Sensitive compounds were protected from exposure to moisture and oxygen by replacement of the air in the reaction with nitrogen using an N_2 line. One manifold is connected to vacuum, while the other is connected to N_2 (g). Repeated vacuum and N_2 (g) treatments (3x) ensures an inert atmosphere for reactions to occur. Air sensitive reactions were protected from the atmosphere under a slight positive pressure of N_2 (g). Sensitive and anhydrous liquids and solutions were transferred by syringe under a positive pressure of N_2 (g) through a rubber septum. Reactions were stirred using a magnetic stirring apparatus with Teflon-coated stir bars. Reagents were generally obtained from either Acros Organics or Sigma-Aldrich, and purified when necessary according to instructions in Perrin's Purification of Laboratory Chemicals.[90] Anhydrous solvents were generally purchased from Sigma-Aldrich or purified according to the procedures described in Perrin's Purification of Laboratory Chemicals. All reagent grade solvents (acetone, DCM, MeOH, ethyl acetate, and hexane) were purchased from Pharmco-AAPER or EMD chemicals. Proton (^{1}H) NMR and carbon (^{13}C) NMR were determined using either a Unity-400 spectrophotometer (400 mHz) or a Varian Inova-600 spectrophotometer (600 mHz).

Chemical shifts are reported in ppm (δ) and were referenced to the residual proton signal of the the deuterated solvent. When TMS is present ($CDCl_3$ and D_6-DMSO), the residual peak at zero ppm was used as the reference. ^{31}P NMR was recorded on a Unity-400 spectrophotometer (162 mHz) with 85 % H_3PO_4 as an external reference. The chemical shifts for ^{1}H NMR were reported to the second decimal place while ^{13}C chemical shifts were reported to the first decimal place. When appropriate, 2 decimal places are reported. The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, q= quartet, m = multiplet, dd = doublet of doublets. Coupling constants (*J*) are reported in Hertz (Hz). Melting points were determined on a MEL-TEMP II apparatus and are uncorrected. Melting point determinations were performed in duplicate to ensure accurate measurement. Mass spectral analysis of substituted nicotinic acid derivatives, as well as dinucleotides, were performed at The Ohio State Mass Spectrometry & Proteomics Facility. High resolution mass spectrometry (HRMS) of substituted nicotinic acid derivatives were obtained by TOF-ES^+ mass spectrometry, while dinucleotides were measured using MALDI-TOF mass spectrometry. Analytical TLC was performed on Baker-flex TLC plates (2.5 x 7.5 cm) with a 254 nm fluorescent indicator (IB-F). Plates were developed in a covered chamber, usually with 5-10 mL of mobile phase, and visualized by UV-light, exposure to I_2 vapor, sulfuric acid charring, or ninhydrin stain. Flash chromatography was done using Fisher Silica Gel 60, 200-425 mesh (40-60 mm) as a stationary phase. Flash columns were packed as described in the literature[91] and generally require about 50 times the amount of compound to be purified. Dry silica gel, tightly packed into a glass column, was washed with approximately 5 column volumes (CV) of non-polar solvent, and then sample was applied. Another 3 CVs

of non-polar solvent was used to wash away any highly non-polar compounds. The column was then run as a linear gradient from 0% polar solvent to the reported value of elution over the course of 20 CVs. Alternatively, flash chromatography could be accomplished using a Combiflash Rf system (Teledyne- Isco). Normal phase silica columns (12 g or 40 g) and C_{18} columns (15.5 g) were purchased from Teledyne-Isco and were made specifically to fit this particular apparatus. Exact gradients from open column flash chromatography (normal phase) could be applied to the Combiflash Rf system and often provides a better separation and consumes less time. HPLC purifications were performed on a Bio-Rad BioLogic Duo Flow system equipped with a 254 nm UV-detector. Solvent A consists of water and Solvent B consists of 100 mM TFA. Analytical anion-exchange columns were purchased from Bio-Rad (Uno-Q, 7 x 35 mm, 1.3 mL). Preparative sized columns were packed in an empty OMNI glass column with appropriate resins and were made to be fitted to the HPLC using a BioLogic Duo Flow System Fittings Kit. Columns were packed by taking resins with the appropriate counter-ion and added them to the empty glass column via pipette. Once packed, the column was attached to the pump head and water was allowed to flow into a waste beaker. The pressure compresses the resin producing a void volume, which was then refilled with wet resin. This process was repeated to avoid any dead volume at the top of the column. HPLC methods were reported as follows: 1) Injection of sample on attached column via injection loop; 2) Wash with solvent A to remove unionized compounds; 3) Applying linear gradient of 100 mM TFA to purify dinucleotides; 4) Wash with eluting buffer (solvent B) to remove any highly charged compounds; 5) Wash with solvent A for future use. Solvent evaporation for volatile solvents was done under reduced pressure using a

Heidolph rotary evaporator (Hei-VAP Value, "The Collegiate") connected to either a water aspirator or a non-oil driven vacuum pump (Heidolph Rotovac valve tec). In experimental descriptions, reduced pressures in mbar are often stated. This refers to the apparatus which includes a Heidolph rotevaporator, a non-oil driven vacuum pump (Rotovac valve tec), and a pressure regulator (Heidolph, Vac Control Automatic). For less volatile solvents, traditional 2-stage oil-driven vacuum pump connected to a Buchi rotary evaporator and fitted with a heating bath was used. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA) and were regarded as being acceptable when the result was within ±0.4 % of the theoretical values. The yields of dinucleotides were based initially on physical weight determined using an analytical balance (when desalted) and confirmed by measuring the absorbance and calculating the exact weight using Beers Law, using the extinction coefficient of NADP. The conditions of reactions performed in a microwave synthesizer (Biotage Initiator 2.5) were reported in terms of the instruments' adjustable settings: time, temperature, pre-stirring, vial type, absorption level, and fixed hold time. The vials, caps and stir bars that are made for this system were purchased from Biotage. Determination of pH was made using a PHM82 Standard pH Meter (Radiometer America, Cleveland OH). UV/Vis spectrophotometry was performed on a Shimadzu Bio Spec 1601. Notable compounds not purchased from Acros Organic/Sigma-Aldrich were: 5-aminonicotinic acid (AK Scientific, Union City CA), 5-hydroxynicotinic acid (TCI, Tokyo Japan), NAD (Roche Diagnostics, Indianapolis, IN) and NADP as mono sodium tetra-hydrate MW=837.4 g/mol (Research Products International, Mt. Prospect IL). All forms of MnO_2, which we purchased from various commercial sources, were found to be unreactive for the oxidation of hydrazone to diazo

compound. Collaborator Tim Walseth provided a satisfactory grade that accomplished the oxidation of hydrazone in the caged NADP reaction.[20] Boronic acids for Suzuki reaction were purchased from Sigma-Aldrich.

Preparation of the *Aplysia* cyclase[63] was as follows. Aliquots (10 mg/mL, 100 μL) were received from Tim Walseth. Aliquots not used were stored at -80 ^{o}C. An aliquot was taken as needed and diluted to 5 mL with 20 mM HEPES (4.900 mL, pH = 7.5). The final concentration is 0.2 mg/mL. The sample was mixed thoroughly and aliquoted into 60 μL portions. Normally, 3 aliqouts are used in the base exchange of NADP (0.018 mmol). Diluted samples are stored at -80 ^{o}C and are dethawed before use.

3.1.1 Resin Preparations

50 g of DEAE Cellulose (DE53, product #4058200; Whatman Inc, Florham Park, NJ USA) was added to a stirred solution of 1.5 M ammonium bicarbonate (300 mL) and thoroughly mixed for 4 hours. Stirring was stopped and the resin was allowed to settle overnight, at which time, excess liquid was decanted and a second portion of 1.0 M ammonium bicarbonate (250 mL) was added and stirred for 2 hours. Once the resin settled, excess liquid was decanted and then resuspended and stirred (30 min) in water (300 mL). After decanting, the resin was washed twice more with water as above, for a total of three water washes. At this point, a sample of the supernatant should be close to neutral, even slightly acidic on pH paper. Repeated washes with water are necessary until the pH is neutral. Unused resin that is not to be used within 2 weeks was stored at 4 ^{o}C in 100 mM ammonium bicarbonate.

The preparation of Bio-Rad AG-MP1 (Bio-Rad Laboratories, Hercules, CA USA) was modified from a previously described procedure.[92] AG-MP1 (30 g) was added to a stirred solution of 4 N NaOH (200 mL) and thoroughly mixed for 6 hours. Stirring was stopped and the resin was allowed to settle overnight, at which time, excess liquid was decanted and a second portion of 2 N NaOH (200 mL) was added and stirred for 3 hours. Once the resin settled, excess liquid was decanted and then resuspended and stirred (30 min) in water (250 mL). After decanting, the resin was washed twice more with water as above, for a total of three water washes. We needed to monitor the presence of chloride anion during the washes with hydroxide anion, since it is the hydroxide anion that displaces the chloride anion. A portion of resin free supernatant (1 mL) was added to a test tube, acidified with concentrated aqueous nitric acid (7 drops), and then assayed with silver nitrate. If chloride ion was present, insoluble silver chloride precipitates out of solution. The first two washes with NaOH indicated Cl^- was present, while the third was Cl^- free, indicating that displacement of Cl^- with OH^- was complete. Washings with water were then conducted until supernatants are silver chloride negative and the pH of the water wash was neutral. If silver chloride continues to precipitate out of solution, the procedure must be repeated to appropriately convert the resin to the ^-OH form. Upon testing negative for silver chloride, the resin was decanted and resuspended in 2 M TFA (150 mL). This final mixture is stirred for 30 minutes before storage at 4 ^oC and was ready for use.

Amberchrom resins (CG71M, Dow Chemical Company, product #10235577; Copenhagen, Denmark) are used in reverse phase applications. Resins were purchased as suspensions in ethanol and water. The resin was degassed by placing the wet resin in a

500 mL capped filtration flask and attaching it to a water aspirator for 30 minutes. The resin then was placed in a desiccator and the desiccator evacuated using a 2-stage oil pump for 15 minutes. The wet resin was poured into an econo column (Bio-Rad) and washed with 10 column volumes (350 mL) of 20% ethanol in water. Before sample application, the resin was washed with 10 column volumes (350 mL) of water. Typically, desalting and purification procedures use water isocratically, but sometimes application of a gradient of 0-20% alcohol (MeOH, EtOH) was necessary for purification of some of the more hydrophobic polar compounds (**11c**,**11d**, **11f**). Resins were stored in 15% MeOH. The column was equipped with fraction collector, peristaltic pump, and UV detector (254 nm). Appropriate fractions were combined and dried.

Reverse phase C18 columns for the Combiflash Rf system were purchased from Teledyne Isco (Lincoln, NE) and were used to polish substituted nicotinic acids and as a desalting procedure. The method consisted of running a gradient from 0-80 % MeOH in water. The product generally elutes before the gradient starts and the rest of the method can be considered a wash and storage procedure. Compounds may not bind tightly to the C18 resin, but interact enough to desalt.

3.2 5-Nicotinic acid analogs

5-(Acetamido)nicotinic acid (11a). The purification of **11a** was modified from the original procedure.[93] 5-Aminonicotinic acid (0.250 g, 1.81 mmol), acetic anhydride (1.11g, 11 mmol) and pyridine (0.72 g, 10 mmol) were added to a round bottom flask and refluxed for 22 hours. According to the original procedure, a precipitate would form and this was recrystallized from ethanol. This did not work in our hands. Instead, solvents were removed by distillation *in vacuo*. The sample was dissolved in DMSO and purified

by column chromatography on silica gel in butanol: acetic acid: water (5:2:3). This gave a mixture of two compounds. The sample was further purified on silica gel (90:9:1 DCM: MeOH: acetic acid). Removal of solvents by distillation under reduced pressure afforded a yellowish-brown solid (126 mg, 39%): TLC R_f 0.73 in butanol: acetic acid: water (5:2:3), TLC R_f 0.36 in DCM: MeOH: acetic acid (90:9:1; the R_f value was recorded following 2 developments of the TLC plate), ^{1}H NMR (400 mHz, d_6-DMSO) δ 10.37 (bs, 1H, -NH), 8.88 (s, 1H), 8.74 (s, 1H), 8.58 (s, 1H), 2.11 (s, 3H). This material was identical to its original description.[92]

5-Hydroxynicotinic acid (11b). The purification of **11b** was modified from the original procedure[93]. To an ice cooled solution of 5-aminonicotinic acid (500 mg, 3.62 mmol) in concentrated aqeous H_2SO_4 (20 % v/v), a solution of sodium nitrite (275 mg, 4 mmol) in water (1 mL) was added. The mixture was stirred for 10 minutes at 0 ^oC and then added over a period of 10 minutes to a boiling solution of concentrated aqueous H_2SO_4 (60 % v/v). Boiling was continued for 1 hour and evolution of N_2 (g) was observed. The volume of the mixture was reduced to half of its original volume, then neutralized with 5N NaOH. After storing at 4 ^oC for 3 hours, a precipitate formed, which was collected, dissolved into a minimal amount of DMSO, and purified by column chromatography on silica gel (5:2:3 butanol: acetic acid: water). Removal of solvents by distillation under reduced pressure afforded a yellowish-white powder (200 mg, 40 %): TLC R_f 0.78 in butanol: acetic acid: water (5:2:3) ^{1}H NMR (400 mHz, d_6-DMSO) δ 8.53 (s, 1H), 8.32 (s, 1H), 7.60 (s, 1H); ^{13}C NMR (100 mHz, d_6-DMSO) δ 166.6, 153.6, 141.8, 140.9, 127.6,122.1. ESI-MS calcd for $C_6H_5NO_3$: 139.03, Found *m/z*: 139.30. This material was identical to its original description.[92]

5-(3-Acetamidopropyl)nicotinic acid (11c). Compound **16** (104 mg, 0.42 mmol) was dissolved in MeOH (2 mL), 4 N NaOH (2 mL) was added and stirred at ambient temperature for 3.5 hours. The solvent was removed under reduced pressure and the residue extracted 5 times with hot MeOH (10 mL portions). The resulting 50 mL extract was filtered through Celite, and the filtrate dried *in vacuo*. The yellow powder was purified by reverse phase chromatography by using a C18 cartridge (15.5 g, isocratic. water) using Combiflash Rf system. The product eluted shortly after the void volume (13.5 mL), which upon distillation of solvents *in vacuo* afforded an oily white solid (83 mg, 89%): mp 268-270 ^{o}C; TLC R_f 0.53 in butanol: acetic acid: water (5:2:3), ^{1}H NMR (400 mHz, CD_3OD) δ 8.89 (s, 1H), 8.42 (s, 1H), 8.18 (s, 1H), 3.22 (t, 2H, J = 6 Hz), 2.72 (t, 2H, J = 7.6 Hz), 1.94 (s, 3H), 1.85 (m, 2H); ^{13}C NMR (100 mHz, CD_3OD) δ 173.4, 172.8, 151.1, 149.0, 138.69, 138.58, 135.0, 40.0, 31.8, 31.1, 22.7. HRMS calcd for $C_{11}H_{14}N_2O_3$: 245.0902 (M+Na), Found *m/z*: 245.0893 (M+Na).

5-(3-*t*-Boc-Aminopropyl)nicotinic acid (**11d**). Compound **18** (100 mg, 0.324 mmol) was dissolved in MeOH (2 mL) and 4 N NaOH (1mL) and stirred at 37 ^{o}C for 4 hours. After this time, the solvent was removed and the product was dried *in vacuo* then neutralized by dissolving in 1 N HCl. The water was removed by lyophilization then the product was immediately purified by column chromatography on silica gel (90:9:1 DCM: MeOH: acetic acid) affording a yellowish-white solid (81 mg, 89%): mp 225-227 ^{o}C, TLC R_f 0.47 in DCM: MeOH: acetic acid (90:9:1). TLC R_f 0.79 in butanol: acetic acid: water (5:2:3), ^{1}H NMR (400 mHz, CD_3OD) δ 8.89 (s, 1H), 8.41 (s, 1H), 8.17 (s, 1H), 3.10 (t, 2H, J = 6.4 Hz), 2.71 (t, 2H, J = 8 Hz), 1.82 (m, 2H), 1.43 (s, 9H); ^{13}C NMR (100

mHz, CD_3OD) δ 172.8, 158.6, 151.1, 148.9, 138.8, 138.7, 134.9, 80.0, 40.9, 32.5, 31.0, 28.9.

5-(3-Aminopropyl)nicotinic acid (**11e**). Compound **18** (100 mg, 0.324 mmol) was dissolved in water (2 mL) and TFA (1 mL) and was stirred at ambient temperature for 8 hours. The solvents were evaporated *in vacuo*. The crude residue was examined and NMR verified the removal of the *t*-Boc group, while the ethyl ester was intact. The sample was then dissolved in MeOH (4 mL) and 4 N NaOH (2.0 mL) and was allowed to stir at ambient temperature for 8 hours. The reaction mixture was neutralized with 1 N HCl and solvents were removed *in vacuo.* The relatively pure mixture which resulted was polished and desalted by reverse phase chromatography on a C18 cartridge (15.5 g, 13.5 mL void volume) using a Combiflash Rf system. The product eluted between column volumes 3 and 4 in an isocratic (water) system. Removal of water through lyophilization yielded a white solid (48 mg, 83%): mp 270-272 ^oC; TLC R_f 0.33 in butanol: acetic acid: water (5:2:3), δ 8.59 (s, 1H), 8.23 (s, 1H), 7.87 (s, 1H), 2.9 (t, 2H, J = 6.4 Hz), 2.59 (t, 2H, J = 8 Hz), 1.85 (m, 2H); ^{13}C NMR (100 mHz, D_2O) δ 172.9, 150.2, 147.0, 137.2, 136.3, 132.0, 38.9, 28.8, 28.0. HRMS calcd for $C_9H_{12}N_2O_2$: 181.0977 (M+H), Found *m/z*: 181.0981 (M+H).

5-(3-Hydroxypropyl)nicotinic acid (11f). Compound **21** (156 mg, 0.746 mmol) was dissolved in MeOH (2 mL) and 4 N NaOH (1mL) and stirred at 37 ^oC for 4 hours. After this time the solvent was evaporated *in vacuo*, the residue dissolved in water and the alkali neutralized using 1 N HCl. After the water was removed by lyophilization, the residue was applied to a combiflash C18 cartridge (15.5 g) and eluted isocratically with water. The mixture was concentrated *in vacuo* and applied to Amberchrom resin, which

sufficiently desalted the mixture (isocratic, water). Upon drying, a yellowish-white solid was recovered (129 mg, 96 %): mp 216-218 ^{o}C; TLC R_f 0.16 in DCM: MeOH: acetic acid (90:9:1), TLC R_f 0.69 in butanol: acetic acid: water (5:2:3). ^{1}H NMR (400 mHz, CD_3OD) δ 8.90 (s, 1H), 8.42 (s, 1H), 8.19 (s, 1H), 3.59 (t, 2H, J = 6.4 Hz), 2.77 (t, 2H, J = 8 Hz), 1.87 (m, 2H); ^{13}C NMR (100 mHz, CD_3OD) δ 173.0, 151.2, 148.9, 139.0, 138.7, 135.0, 61.9, 35.0, 30.1. HRMS calcd for $C_9H_{11}NO_3$: 182.0817 (M+H), Found *m/z*: 182.0821 (M+H).

5-(3-Azidopropyl)nicotinic acid (11g). Compound **23** (50 mg, 0.21 mmol) was dissolved in methanol (1mL) and 4N NaOH (0.5mL) and stirred for 3 hours at 37 ^{o}C. Upon completion, solvents were removed *in vacuo* and the residue was neutralized with 1 N HCl. The sample was concentrated to a volume of 0.5 mL then applied to a C18 cartridge (15.5 g) which was then attached to a Combiflash Rf system. The product eluted in water in the 2nd CV. Water was removed by lyophilization to yield a white solid (39 mg, 91%): mp 77-78 ^{o}C, TLC R_f 0.40 in DCM: MeOH: acetic acid (95:4:1), TLC R_f 0.81 in butanol: acetic acid: water (5:2:3) ^{1}H NMR (400 mHz, CD_3OD) δ 8.97 (s, 1H), 8.62 (s, 1H), 8.28 (s, 1H), 3.37 (t, 2H J = 6.4 Hz), 2.83 (t, 2H, J = 7.6 Hz), 1.94 (m, 2H); ^{13}C NMR (100 mHz, CD_3OD) δ 168.0, 153.8, 149.1, 139.1, 128.7, 51.8, 31.3, 30.7. HRMS calcd for $C_9H_{10}N_4O_2$: 207.0882 (M+H), Found *m/z*: 207.0881 (M+H).

5-Thiomethylnicotinic acid (11h). It is notable that sodium thiomethoxide purchased from Sigma-Aldrich was the only grade of material that worked in our hands. 5-Bromonicotinic acid (1 g, 5 mmol), sodium thiomethoxide (385 mg, 5.5 mmol) and DMF (10 mL) were added to a 10-20 mL Biotage microwave reactor vial. The vial was properly capped and allowed to react for 24 hours at 200 ^{o}C. After this time, TLC clearly

revealed the presence of starting material. Another portion of sodium thiomethoxide (385 mg, 5.5 mmol) was added and allowed to react for 24 hours at 200 ^{o}C. The reaction was now shown to be complete and the crude material was filtered through Celite. The Celite was washed with 100 mL of 50% methanol in DCM. The combined filtrates were combined and the solvent was evaporated *in vacuo*. The resulting residue was purified by column chromatography on silica gel (97:2:1 DCM:MeOH: acetic acid) affording a white solid (480 mg, 57 %): TLC R_f 0.49 in DCM: MeOH: acetic acid (95:4:1), ^{1}H NMR (400 mHz, CD_3OD) δ 8.85 (s, 1H), 8.60 (s, 1H), 8.22 (s, 1H), 2.59 (s, 3H); ^{13}C NMR (100 mHz, CD_3OD) δ 151.1, 147.4, 135.9, 15.2. HRMS calcd for $C_7H_7NO_2S$: 170.0276 (M+H), Found *m/z*: 170.0278 (M+H).

5-(Methylsulfinyl)nicotinic acid (11i). 5-Thiosulfonylmethylnicotinic acid ethyl ester (**25**) (50 mg, 0.23 mmol) was dissolved in MeOH (2.5 mL) and 4 N NaOH (1 mL) and allowed to stir at 37 ^{o}C for 3 hours at which time the solvent was removed *in vacuo*. The resulting residue was dissolved in water (3 mL) and the pH was adjusted to 7. The water was removed by lyophilization. The residue was applied to a C18 cartridge (15.5 g) and was fitted to a Combiflash Rf system. The product eluted between the 2nd and 3rd column volume (27 - 40.5 mL) when the chromatography was developed with water. After removal of water through lyophilization, the product was obtained as a white solid (32 mg, 75 %): ^{1}H NMR (400 mHz, CD_3OD) δ 9.32 (s, 1H), 9.10 (s, 1H), 8.77 (s, 1H), 3.24 (s, 3H).

5-Bromonicotinic acid ethyl ester (12). To a solution of 5-bromonicotinic acid (5 g, 28.4 mmol) in absolute ethanol (50 mL) was added 5 N HCl in dioxane (10 mL). The reaction mixture was refluxed for 48 hours. Solvents were removed *in vacuo* then

water (75 mL) was added and neutralized with 5 N NaOH. Upon neutralization, the solution was extracted 3x with ethyl acetate. Ethyl acetate was distilled under reduced pressure to give an off-white solid which was recrystallized twice from water affording a white solid (5.7 g, 88%): mp 39-40 ^{o}C; TLC R_f 0.52 in 20% ethyl acetate: hexane, ^{1}H NMR (600 mHz, $CDCl_3$) δ 9.13 (s, 1H), 8.84 (s, 1H), 8.43 (s, 1H), 4.43 (q, 2H, J = 7.2 Hz), 1.42 (t, 3H, J = 7.2 Hz); ^{13}C NMR (100 mHz, $CDCl_3$) δ 164.0, 154.4, 148.8, 139.5, 127.6, 120.6, 62.0, 14.2. The characterization of this material was consistent with previous reports in the literature.[94]

3-Acetamidoprop-1-yne (13). To a solution of propargylamine (500 mg, 9.1 mmol) and triethylamine (1.37 g, 13.7 mmol) in DCM (5 mL) at 0 ^{o}C was added acetic anhydride (1.11 g, 11 mmol). The mixture was stirred for 1 hour at 0 ^{o}C, then left stirring for an additional 23 hours at room temperature. Solvent was distilled *in vacuo* and then purified by bulb-to-bulb distillation (0.3 mm Hg, 110-130 ^{o}C) affording a clear oil which was precipitated with hexanes, then recrystallized from ether/DCM to afford white crystals (510 mg, 58 %): m.p. 77-78 ^{o}C ; ^{1}H NMR (400 mHz, $CDCl_3$) δ 5.91 (bs, 1H – NH), 4.05 (q, 2H), 2.24 (s, 1H), 2.02 (s, 3H); ^{13}C NMR (100 mHz, $CDCl_3$) δ 169.7, 79.5, 71.6, 29.2, 23.0. The characterization of this material was consistent with previous reports in the literature.[95]

***tert*-Butyl-prop-2-ynylcarbamate (14)**. To a solution of propargylamine (750 mg, 14.0 mmol) in methylene chloride (5 mL) at 0 ^{o}C was added a solution of *tert*-butyldicarbonate (3.36 g, 15.4 mmol) in DCM (6 mL) over a period of 20 min. After this 20 minute period, the solution was allowed to warm to room temperature and stirred for an additional 48 hours. Solvent was distilled *in vacuo* and the resulting yellow oil was

stored at 4 ^{o}C for 16 hours. A yellowish-white precipitate formed which was collected, dried under reduced pressure, and recrystallized from ether/DCM affording white crystals (1.5 g, 56%): mp 37-39 ^{o}C; ^{1}H NMR (400 mHz, $CDCl_3$) δ 4.79 (bs, 1H, -NH), 3.93 (s, 2H), 2.22 (s, 1H), 1.46 (s, 9H), ^{13}C NMR (100 mHz, $CDCl_3$) δ 155.4, 80.27, 80.05, 71.3, 30.4, 28.4. The characterization of this material was consistent with previous reports in the literature.[96]

5-(3-Acetamidoprop-1-yne)nicotinic acid ethyl ester (15). To a solution of 5-bromonicotinic acid ethyl ester (250 mg, 1.09 mmol), $Pd(PPh_3)_2Cl_2$ (38 mg, 0.0545 mmol) and DIPEA (423 mg, 3.27 mmol) in acetonitrile (5 mL) were added **13** (127 mg, 1.31 mmol) and CuI (15 mg, 0.0763 mmol) while stirring under a N_2 atmosphere. After the addition is complete, the reaction mixture was refluxed for 10 hours. At the end of this time TLC indicated that the starting material was consumed. Solvents were removed *in vacuo* and the residue partitioned between water and chloroform. The chloroform was distilled *in vacuo* and the residue was purified by column chromatography on silica gel (0-5 % DCM/methanol) affording a yellow solid (161 mg, 60%). For analytically pure samples, the yellow solid was recrystallized from ether/DCM affording a white powder: mp 82-84 ^{o}C; TLC R_f 0.63 in 5% MeOH/ DCM, ^{1}H NMR (400 mHz, $CDCl_3$) δ 9.11 (s, 1H), 8.78 (s, 1H), 8.29 (s, 1H), 6.82 (bs, 1H, -NH), 4.42 (q, 2H, J = 7.2 Hz), 4.32 (d, 2H, J = 5.2 Hz), 2.08 (s, 3H), 1.41 (t, 3H, J = 7.2 Hz); ^{13}C NMR (100 mHz, $CDCl_3$) δ 170.0, 164.5, 155.3, 149.4, 139.6, 125.9, 119.8, 90.0, 78.7, 61.8, 29.8, 23.0, 14.2; Analysis calcd. for $C_{13}H_{14}N_2O_3$: C 63.40, H 5.73, N 11.38, found; C 63.57, H 5.80, N 11.20; HRMS calcd for $C_{13}H_{14}N_2O_3$: 246.1004, Found *m/z*: 269.0891 (M+Na).

5-(3-Acetamidopropyl)nicotinic acid ethyl ester (16). Compound **15** (260 mg, 1.06 mmol) was dissolved in MeOH (7 mL) and transferred to a glass hydrogenation flask and cooled in dry ice/acetone for 5 minutes. Pd/C (52 mg, 20% w/w) was added, then the flask was attached to a PARR hydrogenator and shaken for 8 hrs at 30 PSI of H_2. Upon completion, the mixture was filtered through Celite and solvent was distilled *in vacuo*, and the residue then partitioned between water and chloroform. The phases were separated and the chloroform was distilled *in vacuo*. This residue was purified by column chromatography on silica gel (0-10 % MeOH/ DCM) affording a white powder (240 mg, 91%): mp 50-52 ^{o}C; TLC R_f 0.55 in 10% MeOH/DCM, δ 8.92 (s, 1H), 8.61 (s, 1H), 8.23 (s, 1H), 4.40 (q, 2H, J = 7.2 Hz), 3.21 (t, 2H, J = 6.8 Hz), 2.75 (t, 2H, J = 8 Hz), 1.94 (s, 3H), 1.84 (m, 2H), 1.40 (t, 3H, J = 7.2 Hz); ^{13}C NMR (100 mHz, CD_3OD) δ 173.3, 166.4, 154.2, 148.8, 139.5, 138.6, 128.0, 62.8, 40.0, 31.8, 31.0, 22.7, 14.7. HRMS calcd for $C_{13}H_{18}N_2O_3$ 273.1215, Found *m/z*: 273.1215 (M+Na).

5-(*t*-Butyl-prop-2-ynylcarbamate)nicotinic acid ethyl ester (**17**). To a solution of **12** (250 mg, 1.09 mmol), $Pd(PPh_3)_2Cl_2$ (38 mg, 0.0545 mmol) and DIPEA (423 mg, 3.27 mmol) in acetonitrile was added **14** (203 mg, 1.31 mmol) and CuI (15 mg, 0.0763 mmol) while stirring under an N_2 atmosphere. After the addition was complete, the reaction mixture was refluxed for 6 hours. At this time, TLC indicated complete consumption of starting material. The solvent was removed by distillation *in vacuo* and the residue partitioned between DCM and water. The phases were separated and the solvent was distilled under reduced pressure. The resulting residue was purified by column chromatography on silica gel (0-33% ethyl acetate/ hexane) affording a yellow oil which upon freezing at -20 ^{o}C for 16 hours produced a yellowish-white solid (202 mg,

61%): mp 54-56 ^{o}C; TLC R_f 0.52 in hexane/ethyl acetate (1:1), ^{1}H NMR (400 mHz, $CDCl_3$) δ 9.12 (s, 1H), 8.79 (s, 1H), 8.30 (s, 1H), 5.52 (bs, 1H, -NH) 4.42 (q, 2H, *J* = 7.2 Hz), 4.21 (s, 2H), 1.48 (s, 9H), 1.41 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (100 mHz, $CDCl_3$) δ 164.5, 155.4, 149.4, 139.5, 125.8, 119.9, 90.5, 80.0, 78.6, 61.7, 31.0, 28.4, 28.0.; Analysis calcd. for $C_{16}H_{20}N_2O_4$: C 63.14, H 6.62, N 9.20, found; C 61.83, H 6.42, N 8.60. HRMS calcd for $C_{11}H_{15}NO_3$: 327.1321, Found *m/z*: 327.1315 (M+Na).

5-(3-*t*-Boc-Aminopropyl)nicotinic acid ethyl ester (**18**). Compound **17** (100 mg, 0.346 mmol) was dissolved in MeOH (5 mL) and transferred to a glass hydrogenation flask, which was capped and cooled in dry ice/acetone for 5 minutes. Pd/C (20 mg, 20 % w/w) was added and mixed and the flask was connected to a PARR hydrogenator and shaken for 6 hrs at 35 PSI of H_2. Upon completion, the mixture was filtered through Celite. The solvent was removed by distillation *in vacuo* and the residue partitioned between water and DCM. The phases were separated and the DCM was concentrated *in vacuo*. The resulting residue was purified by column chromatography on silica gel (1:1 hexane:ethyl acetate), which upon distillation of the solvents *in vacuo* afforded a yellowish-white solid (99 mg, 93%). TLC R_f 0.31 (1:1 hexane:ethyl acetate). mp 59-60 ^{o}C. ^{1}H NMR (400 mHz, $CDCl_3$) δ 9.06 (s, 1H), 8.61 (s, 1H), 8.13 (s, 1H), 5.07 (bs, 1H, -NH), 4.42 (q, 2H, *J* = 7.2 Hz), 3.21 (q, 2H, *J* = 6.4 Hz), 2.73 (t, 2H, *J* = 8 Hz), 1.87 (m, 2H), 1.45 (s, 9H), 1.42 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (100 mHz, $CDCl_3$) δ 165.4, 156.0, 153.4, 148.5, 136.8, 136.6, 126.0, 79.2, 61.4, 40.0, 31.4, 30.0, 28.4, 14.3. HRMS calcd for $C_{16}H_{24}N_2O_4$: 331.1634 (M+Na), Found *m/z*: 331.1646 (M+Na).

5-(3-Aminopropyl)nicotinic acid ethyl ester (**19**). Compound **18** (97.5 mg, 0.316 mmol) was dissolved in water (1 mL) and TFA (1 mL) and stirred for 2 hours at 37

^{o}C. The solvent was removed *in vacuo* forming a deeply yellow colored oil: TLC R_f 0.67 in butanol: acetic acid: water (5:2:3), TLC R_f 0.12 in DCM: MeOH: acetic acid (90:9:1), ^{1}H NMR (400 mHz, CD_3OD) δ 9.17 (s, 1H), 8.90 (s, 1H), 8.77 (s, 1H), 4.47 (q, 2H, *J* = 7.2), 3.05 (t, 2H, *J* = 7.2 Hz), 2.99 (t, 2H, *J* = 8 Hz), 2.08 (m, 2H), 1.42 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (100 mHz, CD_3OD) δ 164.3, 148.5, 144.7, 144.4, 141.7, 130.5, 63.7, 40.0, 30.3, 29.5,14.6. The compound was immediately taken to the next step in the formation of **11e**.

5-(3-Hydroxy-1-propynyl)nicotinic acid ethyl ester (**20**). To a solution of **12** (250 mg, 1.09 mmol), $Pd(PPh_3)_2Cl_2$ (38 mg, 0.0545 mmol) and DIPEA (423 mg, 3.27 mmol) in acetonitrile (5 mL) was added propargyl alcohol (73 mg, 1.31 mmol) and CuI (15 mg, 0.0763 mmol) while stirring under a N_2 atmosphere. After the addition is complete, the reaction mixture was refluxed for 16 hours. At this time, TLC indicated that the starting material was consumed. Solvents were removed *in vacuo* and the residue partitioned between water and chloroform. The phases were separated and chloroform was concentrated *in vacuo*. The resulting oil was purified by column chromatography on silica gel (1:1 hexane/ethyl acetate) affording a yellow solid (160 mg, 71%) that was pure by NMR. The product was further purified by recrystallizing from ether/DCM to produce white crystals: mp 87-88 ^{o}C, ^{1}H NMR (400 mHz, $CDCl_3$) δ 9.13 (s, 1H), 8.86 (s, 1H). 8.8.33 (s, 1H), 4.53 (d, 2H, *J* = 6 Hz), 4.42 (q, 2H, *J* = 7.2 Hz), 1.41 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (100 mHz, $CDCl_3$) δ164.5, 155.3, 149.4, 140.0, 126.0, 120.0, 92.5, 81.0, 61.8, 51.2, 14.2. Analysis calcd. for $C_{11}H_{11}NO_3$: C 64.38, H 5.40, N 6.83, found; C 64.43, H 5.28, N 6.78. HRMS calcd for $C_{11}H_{11}NO_3$: 228.0637, Found *m/z*: 228.0634 (M+Na).

5-(3-Hydroxypropyl)nicotinic acid ethyl ester (21). Compound **20** (270 mg, 1.32 mmol) was dissolved in MeOH (8 mL) and transferred to a glass hydrogenation flask and cooled in dry ice/acetone for 5 minutes. Pd/C (54 mg, 20 % w/w) was added and the flask was attached to a PARR hydrogenator and shaken for 10 hrs at 30 PSI of H_2. Upon completion, the mixture was filtered through Celite and solvents were removed from the filtrate *in vacuo*. The resulting residue partitioned between water and chloroform. The chloroform was concentrated under reduced pressure and was purified by column chromatography on silica gel (5% MeOH/DCM) affording a yellow oil (245 mg, 89%): TLC R_f 0.37 in 5% MeOH/DCM, ^{1}H NMR (400 mHz, $CDCl_3$) δ 9.03 (s, 1H), 8.61 (s, 1H), 8.17 (s, 1H), 4.41 (q, 2H, *J* = 7.2 Hz), 3.71 (t, 2H, *J* = 6.4 Hz), 3.62 (bs, 1H, -OH), 2.83 (t, 2H, *J* = 7.6 Hz), 1.93 (m, 2H), 1.42 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (100 mHz, $CDCl_3$) δ 165.4, 153.3, 148.2, 137.4, 137.0, 126.1, 61.5, 61.1, 33.7, 29.0, 14.4. HRMS calcd for $C_{11}H_{15}NO_3$: 232.0950, Found *m/z*: 232.0946 (M+Na).

5-(3-Toluenesulfonyl)propylnicotinic acid ethyl ester (22). Compound **21** (107 mg, 0.51 mmol) was dissolved in dry DCM (5 mL). To this solution was added *p*-toluenesulfonyl chloride (292 mg, 1.5 mmol) and triethylamine (114 mg, 0.1.12 mmol). The mixture was refluxed for 16 hours. Upon TLC analysis in hexane/ethyl acetate (1:1), starting material was still present. Additional *p*-toluenesulfonyl chloride (50 mg, 0.26 mmol) and triethylamine (15 mg, 0.15 mmol) were added to the mixture and allowed to stir at reflux for an additional 12 hours. This pushed the reaction to completion and the product was isolated by removal of solvents by distillation and the residue partitioned between saturated sodium bicarbonate (50 mL) and DCM (50 mL). The organic layer was further washed with brine (3x). The phases were separated and the DCM was evaporated

in vacuo. The crude material was purified by chromatography on silica gel in hexane/ethyl acetate (1:1) to obtain a colorless oil (94 mg, 51%): TLC R_f 0.27 in hexane/ethyl acetate (1:1), ^{1}H NMR (400 mHz, $CDCl_3$) δ 9.06 (s, 1H), 8.54 (s, 1H). 8.07 (s, 1H), 7.79 (d, 2H), 7.35 (d, 2H), 4.41 (q, 2H, *J* = 7.2 Hz), 4.06 (t, 2H, *J* = 6 Hz), 2.75 (t, 2H, *J* = 8 Hz), 2.46 (s, 3H), 2.00 (m, 2H), 1.42 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (100 mHz, $CDCl_3$) δ165.4, 153.6, 149.0, 145.2, 136.8, 135.8, 133.0, 130.11, 129.92, 128.0, 126.3, 125.3, 69.2, 61.7, 30.2, 28.7, 21.8, 14.4.

5-(3-Azidopropyl)nicotinic acid ethyl ester (23). To a solution of **22** (47 mg, 0.13 mmol) in dry DMF (2 mL) was added sodium azide (17 mg, 0.26 mmol). The reaction mixture was stirred and heated for 18 hours at 60 ^{o}C. Upon completion, solvents were distilled under reduced pressure and the residue purified by column chromatography on silica gel in hexane:ethyl acetate (1:1) yielding a yellow oil (22 mg, 73%): TLC R_f 0.49 in hexane: ethyl acetate (1:1), TLC R_f 0.77 in DCM: MeOH: acetic acid (95:4:1), ^{1}H NMR (400 mHz, $CDCl_3$) δ 9.08 (s, 1H), 8.62 (s, 1H), 8.13 (s, 1H), 4.42 (q, 2H, *J* = 7.2 Hz), 3.35 (t, 2H, *J* = 6.4 Hz), 2.79 (t, 2H, *J* = 8 Hz), 1.95 (m, 2H), 1.42 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (100 mHz, $CDCl_3$) δ165.4, 153.5, 148.8, 136.7, 136.1, 126.1, 61.5, 50.4, 30.05, 29.69, 14.3. HRMS calcd for $C_{11}H_{14}N_4O_2$: 257.1014, Found *m/z*: 257.1011 (M+Na).

5-Thiomethylnicotinic acid ethyl ester (24). Compound **11h** (480 mg, 2.8 mmol) was dissolved in DMF (10 mL). Directly to this solution was added O-ethyl-N,N'-diisopropyl pseudourea[72] (782 mg, 3.1 mmol). After addition was complete, the reaction mixture was heated at 80 ^{o}C for 16 hours. After this time, the DMF was distilled under reduced pressure. The product was extracted from the crude material into 100 mL of 10%

MeOH in DCM by stirring for 15 minutes. The DCM/MeOH solution was removed and filtered through a bed of Celite. The filtrate was collected and the solvent was removed *in vacuo*. The resulting residue was purified by column chromatography on silica gel (1:1 hexane:ethyl acetate), and upon removal of solvent yielded a yellow oil (472 mg, 86 %): TLC R_f 0.57 in hexane/ethyl acetate (1:1), ^{1}H NMR (400 mHz, $CDCl_3$) δ 8.95 (s, 1H), 8.63 (s, 1H). 8.11 (s, 1H), 4.42 (q, 2H, *J* = 7.2 Hz), 2.56 (s, 3H), 1.42 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (100 mHz, $CDCl_3$) δ 164.9, 150.9, 146.9, 136.0, 134.1, 126.2, 61.6, 15.4, 14.3.

5-(Methylsulfinyl)nicotinic acid ethyl ester (25). Compound **24** (134 mg, 0.68 mmol) was dissolved in MeOH (5 mL) at 0 ^{o}C, and then MMPP (403 mg, 0.82 mmol) was added in one portion. The mixture reacted for 2 hours, and then was immediately filtered through a bed of Celite. The Celite was washed with one portion of MeOH (50 mL) and the washings were combined. The methanol was distilled *in vacuo* and the residue was purified by column chromatography on silica gel (0-70% ethyl acetate/hexane) affording an off-white solid (110 mg, 76 %): TLC R_f 0.29 in hexane/ ethyl acetate (1:1), ^{1}H NMR (400 mHz, $CDCl_3$) δ 9.47 (s, 1H), 9.31 (s, 1H). 8.82 (s, 1H), 4.48 (q, 2H, *J* = 7.2 Hz), 3.18 (s, 3H), 1.45 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (100 mHz, $CDCl_3$) δ163.4, 154.9, 151.6, 137.02, 136.36, 126.9, 62.4, 44.8, 14.2.

3.3 General procedure for the base exchange reaction

Substituted nicotinic acid (10-20 equiv.) was dissolved in water (6 mL) and stirred for 30 minutes at 37 ^{o}C. After this time period, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 minutes. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has

stabilized. After the pH has stabilized, NADP was added and stirred for 5 minutes at 37 ^{o}C. Next, *Aplysia californica* ADP-ribosyl synthase (180 µL, 36 ng) was added and the reaction was stirred at 37 ^{o}C for 2 hours. Upon time completion, HPLC confirmed consumption of starting material (**HPLC Method 1**: NADP t = 8.96 min, 13% 100 mM TFA). The sample was then injected onto the preparative HPLC column and the product was purified by anion-exchange chromatography using a linear gradient formed between water (solvent A) and 100 mM TFA (solvent B). For this separation, the chromatography was developed using a linear gradient in solvent A from 0% solvent B to 50% solvent B. After the product was collected, the combined fractions were added to a 125 mL separatory funnel and extracted 3x with DCM (35 mL portions) to remove TFA. The phases were separated and the aqueous layer was then distilled *in vacuo* at 25 mbar for 10 minutes to remove any residual DCM and TFA. The pH of this resulting solution was adjusted between 7.2-7.4 with 1 M NaOH. The aqueous solution was allowed to freeze at -80 ^{o}C then lyophilized. This substance was then dissolved in water (2 mL) and applied to 3 mL column of DEAE cellulose according to **Method 6**. The DEAE cellulose chromatography was developed by applying a 0-500 mM ammonium bicarbonate gradient and the product eluted into ca. 225 mM ammonium bicarbonate. The product showed UV absorbance at 254 nm; Fractions absorbing at this wavelength were collected and combined. The combined fractions were then lyophilized until a foamy white residue appeared. The sample was redissolved in water (2 mL) and lyophilized. This process was repeated twice more affording pure substituted NAADP derivative.

3.3.1 Purification procedures (All detections used 254 nm light)

Method 1:Analytical method:

A Bio-Rad Uno-Q column (7 x 35 mm, 1.3 mL of anion-exchange resin) was fitted to a Bio-Rad BioLogic Duoflow HPLC apparatus. Injection loops with volumes of either 50 µL or 250 µL were used. In these particular descriptions, a 250 µL injection loop was used in monitoring these reactions. NAADP derivatives (**32a-l**) were determined to be pure using this methodology. Concentrations of substituted NAADP derivatives were approximately 1 mg/ml. Solvent A: H_2O; Solvent B: 100 mM aqeous TFA. The flow rate throughout was 3 mL/min. 1) Load/inject sample, 1 mL; 2) Solvent A, 10 mL; 3) Linear gradient, (0-50% solvent B) formed over 60 mL; the percentage on this step relates to elution of dinucleotide. 4) Solvent B, 11 mL; 5) Solvent A, 11 mL. The total volume of this procedure is 93 mL.

Method 2: Analytical method

A Bio-Rad Uno-Q column (7 x 35 mm, 1.3 mL of anion exchange resin) was used with a Bio-Rad BioLogic Duoflow HPLC apparatus. Injection loops with volumes of 50 µL or 250 µL were used. In these particular descriptions, a 50 µL injection loop was used in monitoring these reactions. NAADP derivatives (**32a-j**) were determined to be pure using this methodology. Concentrations of substituted NAADP samples were ca. 1 mg/ml. Solvent A: H2O; Solvent B: 100 mM TFA. The flow rate throughout was 3 mL/min. 1) Load/inject sample, 0.8 mL; 2) Solvent A, 9 mL; 3) Linear gradient, (0-15 % solvent B) formed over 120 mL; the reported percentage on this step relates to elution of dinucleotide. 4) Solvent B, 9 mL; 5) Solvent A, 12mL. The total volume of this procedure is 150.8 mL.

Method 3: Prep-scale HPLC (0-50% Solvent B)

A glass column (Omni, 1.5 x 11.5 cm) filled with Bio-Rad AG-MP1 ion exchange resin (trifluoroacetate form, see resin preparations) was connected to a Bio-Rad BioLogic Duoflow HPLC apparatus. For preparative scale work, injection loops of 2 mL or 5 mL were used. In these particular descriptions, a 5 mL injection loop was used for purification of these compounds. Concentrations of NAADP derivatives were ca. 3 mg/mL. Solvent A: H_2O; Solvent B: 100 mM TFA. The flow rate throughout was 5 mL/min. 1) Load/inject sample, 7 mL; 2) Solvent A, 20 mL; 3) Linear gradient, (0-50% Solvent B) formed over 160 mL; 4) Solvent B, 25 mL; 5) Solvent A, 35 mL. The total volume of this procedure is 247 mL. Excess TFA was removed by extraction with DCM (3x). Following the extraction, residual DCM and TFA were removed under reduced pressure for 10 minutes at 25 mbar.

Method 4: Prep-Scale HPLC (0-20% Solvent B)

A glass column (Omni, 1.5 x 11.5 cm) filled with Biorad AG-MP1 ion exchange resin (trifluoroacetate form) was connected to a Bio-Rad BioLogic Duoflow HPLC apparatus. Injection loops with volumes of 2 mL or 5 mL were used. In these particular descriptions, a 5 mL injection loop was used for purification of these compounds. Concentrations of dinucleotide samples were ca. 3 mg/mL. Solvent A: H_2O; Solvent B: 100 mM TFA. The flow rate throughout was 5 mL/min. 1) Load/inject sample, 9 mL; 2) Solvent A, 15 mL; 3) Linear gradient, (0-20% Solvent B) formed over 225 mL; 4) Solvent B, 40 mL; 5) Solvent A, 40 mL. The total volume of this procedure was 329 mL. Excess TFA was removed by extraction with DCM (3x). Following the extraction, residual DCM and TFA were removed under reduced pressure for 10 minutes at 25 mbar.

Method 5: Prep-Scale HPLC (0-8% Solvent B)

A glass column (Omni, 1.5 x 11.5 cm) filled with Bio-Rad AG-MP1 ion exchange resin (trifluoroacetate form) was equipped to a Bio-Rad BioLogic Duoflow HPLC apparatus. Injection loops with volumes of 2 mL or 5 mL were used. In these particular descriptions, a 5 mL injection loop was used for purification of these compounds. Concentrations of dinucleotide samples were ca. 3 mg/mL. Solvent A: H_2O; Solvent B: 100 mM TFA. The flow rate throughout was 5 mL/min. 1) Load/inject sample, 9 mL; 2) Solvent A, 15 mL; 3) Linear gradient, (0-8% Solvent B) formed over 190 mL; 4) Solvent B, 40 mL; 5) Solvent A, 40 mL. The total volume of this procedure is 294 mL. Excess trifluoroacetic acid is removed by extraction with DCM (3x). Following the extraction, residual DCM and TFA were removed under reduced pressure for 10 minutes at 25 mbar.

Method 6: Desalting of NAADP derivatives on DEAE cellulose

A Bio-Rad Econo-Column (2.5 x 8 cm) was filled with DE53 cellulose (DE53, 4058200; Whatman Inc, Florham Park, NJ USA; 3 cm wet resin). Water (25 mL) was passed through the column until the pH was neutral. The sample of dinucleotide was applied to the column as a dilute solution at pH $\geq$ 7.5 and properly adsorbed. Using a gradient former, water (150 mL) and 0.5 M ammonium bicarbonate (150 mL) are added with a slight positive pressure resulting from a peristaltic pump. Forming a gradient (0-0.5 M ammonium bicarbonate) allows proper binding to the column. At low ionic concentrations the dinucleotide remains bound to the resin, allowing salts such as NaCl, ammonium chloride and sodium trifluoroacetate to elute. The dinucleotide elutes from the column at >200 mM ammonium bicarbonate concentration. Fractions can be collected automatically with the aid of a fraction collector (Teledyne Isco, Retreiver 500, 100

drops/tube) and the absorption of the effluent monitored continuously with a UV detector (Isco UA-6 UV/Vis Detector). A peristaltic pump (Teledyne Isco, TRIS) was used to maintain a constant flow. The appropriate fractions exhibiting absorbance at 254 nm were combined, frozen, and lyophilized. Repeated lyophilization of the sample from water ensures complete removal of volatile ammonium bicarbonate, leaving pure, salt free dinucleotide.

Method 7: DEAE Cellulose. Alternate preparative purification to HPLC

An alternate method used when the Bio-Rad BioLogic DuoFlow apparatus was not available consisted of packing glass Bio-Rad Econo-Columns (2.5 x 50 cm) with appropriately prepared DE-52 cellulose (42 cm wet resin). Water (100 mL) was passed through the column as a precautionary measure and to ensure the pH was neutral. The dinuclotide sample was applied to the column and properly adsorbed. Using a gradient former, water (350 mL) and 0.6 M ammonium bicarbonate (350 mL) are added with a slight positive pressure resulting from the use of a peristaltic pump. A linear gradient was formed from 0-0.6 M ammonium bicarbonate and allows purification of compounds based on charge. NAADP derivatives usually elute into 320 mM ammonium bicarbonate. With the aid of a fraction collector (Teledyne Isco, Retreiver 500, 100 drops/tube), UV detector (Isco UA-6 UV/Vis Detector) and peristaltic pump (Teledyne Isco, TRIS) the appropriate fractions (activity at 254 nm) are combined, frozen, and lyophilized. Repeated lyophilization of the sample from water ensures complete removal of volatile ammonium bicarbonate, leaving pure, salt free dinucleotide.

3.4 Mono and di-substituted NAADP analogs

NAADP (3). Nicotinic acid (40 mg, 0.325 mmol) was suspended in water (6 mL) and stirred for 30 minutes at 37 ^{o}C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 minutes. The pH was measured again and readjusted to pH = 4 with 0.5 N NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (20 mg, 0.024 mmol) was added and stirred for 5 minutes at 37 ^{o}C. Next, *Aplysia* cyclase (180 µL, 36 ng) was added and the reaction was stirred at 37 ^{o}C for 2 hours. After 2 hours, analysis by HPLC confirmed consumption of starting material (NADP). The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water/TFA gradients according to **HPLC Method 3**. NAADP eluted between 22.85 and 28.40 min (27-36%). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2-7.4 with 1 M NaOH. The aqueous solution was frozen then lyophilized producing a white, amorphous solid. The sample was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose according to **Method 6**. The product **3** eluted into 225 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved in water (2 mL) and again lyophilized, affording pure NAADP as a white powder: ^{1}H NMR (400 mHz, D_2O) δ 9.12 (s, 1H), 9.01 (s, 1H), 8.75 (s, 1H), 8.44 (s, 1H), 8.14 (s, 1H), 8.07 (s, 1H), 6.18 (s, 1H), 6.04 (s, 1H), 5.12 (s, 1H), 4.64 (s, 1H), 4.5 (s, 1H), 4.41 (s, 3H), 4.32- 4.21 (m, 4H). HRMS calcd for $C_{21}H_{28}N_6O_{18}P_3^+$: 745.067, Found

m/z: 744.897 (M+); **HPLC Method 1**: t = 12.80, 23 % 100 mM TFA. **Method 7**: 300 mM ammonium bicarbonate.

8-Br-NAD (29). To a stirred solution of NAD (100 mg, 0.15 mmol) in 0.5 M sodium acetate (1.5 mL, pH = 4.75), was added Br_2 (0.2 mL, 4 mmol). The reaction was stirred vigorously using a magnetic stirrer for 3 hours while wrapped in aluminum foil to protect from light. Upon completion, the mixture was diluted with water (8.5 mL) and DCM (20 mL) was added. The reaction mixture was transferred to a separatory funnel and the organic layer was discarded. The aqueous layer was further extracted 5 times with DCM (20 mL). The pH was adjusted to 7 with 4 N NaOH and was concentrated *in vacuo* until the total volume was ca. 10 mL. The crude product was purified by anion exchange chromatography according to **HPLC Method 5** in 5 mL portions. 8-Br-NAD eluted between 30.83 and 35.09 min (5-6% 100 mM TFA). Fractions exhibiting UV absorption at 254 nm were combined and extracted 3 times with DCM (10 mL). The aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The resulting solution was neutralized with 0.5 M NaOH then lyophilized. The amorphous solid was dissolved in water (2 mL) and purified on DEAE cellulose according to **Method 6**. The product **29** eluted into ca. 175 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording a slightly tinted yellow-white solid (50 mg, 45%): ^{1}H NMR (400 mHz, D_2O) δ 9.37 (s, 1H), 9.20 (d, 1H, J = 6 Hz), 8.91 (d, 1H, J = 8 Hz), 8.38 (s, 1H), 8.25 (t, 1H), 6.11 (d, 1H, J = 5.2 Hz), 6.05 (d, 1H, J = 5.2 Hz), 5.18 (t, 1H, J = 5.6 Hz), 4.63 (t, 1H, J = 4.8 Hz), 4.49 (d, 2H), 4.39 (m, 1H), 4.28-4.19 (m, 5H); ^{31}P NMR (162 MHz, D_2O) -10.64 (dd). HRMS calcd for

$C_{21}H_{27}BrN_7O_{14}P_2^+$: 742.027, Found *m/z*: 742.075 (M+). **HPLC Method 2**: t = 12.08 min, 3% 100 mM TFA.

8-Br-NADP (30). To a stirred solution of NADP (100 mg, 0.12mmol) in 0.5 M sodium acetate (1.5 mL, pH = 4.75) was added Br_2 (0.2 mL, 4 mmol). The reaction was stirred vigorously using a magnetic stirrer for 3 hours while wrapped in aluminum foil to protect from light. Upon completion, the mixture was diluted with water (8.5 mL) then DCM (20 mL) was added. The reaction mixture was transferred to a separatory funnel and the organic layer was discarded. The aqueous layer was further extracted 5 times with DCM (20 mL). The pH was adjusted to 7 with 4 M NaOH and was concentrated *in vacuo* until the total volume was ca. 10 mL. The crude product was purified by anion exchange chromatography according to **HPLC Method 4** in 5 mL portions. 8-Br-NADP eluted between 29.44 and 37.39 min (11-15% 100 mM TFA). Fractions exhibiting UV absorption at 254 nm were combined and extracted 3 times with DCM (10 mL). The aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The resulting solution was neutralized with 0.5M NaOH then lyophilized. The amorphous solid was dissolved in water (2 mL) and applied to 3 mL column of DEAE cellulose according to **Method 6**. The product **30** eluted into 175 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 8-Br-NADP as a yellowish-white solid (56 mg, 51%): ^{1}H NMR (400 mHz, D_2O) δ 9.37 (s, 1H), 9.18 (s, 1H), 8.94 (d, 1H, *J* = 7.6 Hz), 8.31 (s, 1H), 8.20 (s, 1H), 6.21 (s, 1H), 6.11 (s, 1H), 5.53 (s, 1H), 4.5-4.22 (9H); ^{31}P NMR (162 MHz, D_2O) 2.09, -10.79; **HPLC Method 2**: t = 22.72 min, 7% 100 mM TFA.

8-*p*-Chlorophenyl-NAD (31). 8-Br-NAD (50 mg, 0.067 mmol) and K_2CO_3 (14 mg, 0.1 mmol) were dissolved in degassed water (1.5 mL) and transferred to a 2-5 mL Biotage microwave vial and the pH checked to ensure it was between (9-9.6). To this vial was added *p*-chlorophenyl boronic acid (13 mg, 0.081 mmol), $Pd(OAc)_2$ (1.5 mg, 0.0067 mmol) and TXPTS (9 mg, 0.014 mmol). The vial was appropriately capped and attached to a Biotage Initiator microwave synthesizer at the following settings: time: 1 hour 45 min; Temp: 40 ^oC; Pre-stirring: 1 min,; Vial type: 2-5 mL; Absorption level: high; fixed hold time: on. Upon completion, the vial removed and the reaction mixture was filter through Celite. The Celite was washed with water (3.5 mL) affording a final volume of 5 mL. The sample was purified by anion exchange chromatography according to **HPLC Method 5**. 8-*p*-Chlorophenyl-NAD eluted between 23.57 and 27.57 min (4-5%). Fractions exhibiting UV absorption at 254 nm were combined and extracted 3 times with DCM (10 mL). The aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The resulting solution was neutralized with 0.5 M NaOH then lyophilized. The amorphous solid was dissolved in water (2 mL) and purified on DEAE cellulose according to **Method 6**. The product **31** eluted at ca. 80 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 8-*p*-chlorophenyl-NAD as a brownish-white powder (23 mg, 44%): TLC R_f = 0.47 in butanol: acetic acid: water (5:2:3), ^1H NMR (400 mHz, D_2O) δ 9.04 (s, 1H), 8.87 (d, 1H), 8.55 (t, 1H), 7.97 (m, 1H), 7.92 (s, 1H), 7.18 (dd, 4H, J = 8.4 Hz), 5.76 (d, 1H, J = 5.2 Hz), 5.55 (d, 1H, J = 5.2 Hz), 4.96 (t, 2H), 4.32 (t, 1H), 4.19-3.90 (7H); ^{31}P NMR (162 mHz, D_2O)

-10.68 (dd). HRMS calcd for $C_{27}H_{31}ClN_7O_{14}P_2^+$: 774.109, Found *m/z*: 774.096 (M+). **HPLC Method 2**: t = 12.35 min, 3% 100 mM TFA.

5-(Acetamido)-NAADP (32a). 5-(Acetamido)nicotinic acid (35 mg, 0.194 mmol) was suspended in water (6 mL) and stirred for 30 minutes at 37 ^oC. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 minutes. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 minutes at 37 ^oC. Next, *Aplysia* cyclase (180 µL, 36 ng) was added and the reaction was stirred at 37 ^oC for 4 hours. At the end of 4 hours, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water/TFA gradients according to **HPLC Method 3**. 5-(Acetamido)-NAADP eluted between 24.12 and 27.91 min (31-37%). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2-7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **32a** eluted into 225 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(acetamido)-NAADP as a white powder (7.45 mg, 47%): ^1H NMR (400 mHz, D_2O) δ

9.24 (s, 1H), 9.16 (s, 1H), 8.88 (s, 1H), 8.47 (s, 1H), 8.36 (s, 1H), 6.18 (s,1H), 6.12 (s, 1H), 5.06 (s, 1H), 4.59 (s, 1H), 4.52 (s, 1H), 4.42-4.23 (7H), 2.18 (s, 3H); ^{31}P NMR (162 mHz, D_2O) δ 0.35, -10.56; **HPLC Method 1**: t = 13.68 min, 25 % 100 mM TFA. **Method 7**: 340 mM ammonium bicarbonate.

5-Hydroxy-NAADP (32b). 5-Hydroxynicotinic acid (35 mg, 0.252 mmol) was suspended in water (6 mL) and stirred for 30 minutes at 37 ^{o}C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 minutes. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 minutes at 37 ^{o}C. Next, *Aplysia* cyclase (180 µL, 36 ng) was added and the reaction was stirred at 37 ^{o}C for 2 hours. At the end of 2 hours, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water/TFA gradients according to **HPLC Method 3**. 5-Hydroxy-NAADP eluted between 25.60 and 29.84 min (32-38%). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The pH of this solution was adjusted to between 7.2-7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **32b** eluted into 240 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was

redissolved and again lyophilized, affording pure 5-hydroxy-NAADP as a white powder (9.55 mg, 63%): ^{1}H NMR (400 mHz, D_2O) δ 8.43-8.39 (d, 3H), 8.13 (s, 1H), 7.96 (s, 1H), 6.12 (d, 1H, J = 4.8 Hz), 5.82 (s, 1H, J = 4.8 Hz), 5.01 (m, 1H), 4.58 (t, 1H), 4.39 (s, 1H), 4.34-4.15 (6H); ^{31}P NMR (162 mHz, D_2O) δ 0.91, -10.8. HRMS calcd for $C_{21}H_{28}N_6O_{19}P_3^+$: 761.062, Found *m/z*: 761.153 (M+). **HPLC Method 1**: t = 14.13 min, 26 % 100 mM TFA. **Method 7**: 350 mM ammonium bicarbonate.

5-(3-Acetamidopropyl)-NAADP (32c). 5-(3-Acetamidopropyl)nicotinic acid (37.5 mg, 0.169 mmol) was suspended in water (6 mL) and stirred for 30 minutes at 37 ^{o}C. The pH initially was 8.4 and was adjusted to 4 with 1 N HCl and stirred for 30 minutes. The pH was measured again and readjusted to pH = 4 with 1 N HCl. After this time, the pH stabilized. NADP (15 mg, 0.018 mmol) was added and stirred for 5 minutes at 37 ^{o}C. Next, *Aplysia* cyclase (180 µL, 36 ng) was added and the reaction was stirred at 37 ^{o}C for 5 hours. At the end of 5 hours, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water/TFA gradients according to **HPLC Method 3**. 5-(3-Acetamidoaminopropyl)-NAADP eluted between 21.87 and 26.24 min (26-33%). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The pH of the resulting solution was then adjusted to between 7.2-7.4 with 1M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to

Method 6. The product **32c** eluted into 225 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(3-acetamidopropyl)-NAADP as an off-white powder (9.7 mg, 58 %): ^{1}H NMR (400 mHz, D_2O) δ 9.28 (s, 1H), 9.12 (s, 1H), 8.89 (s, 1H), 8.58 (s, 1H), 8.40 (s, 1H), 6.26 (d, 1H, *J* = 4.8 Hz), 6.11 (d, 1H, *J* = 4.8 Hz), 5.09 (m, 2H), 4.63-4.23 (9H), 3.18 (t, 2H, *J* = 6.4 Hz), 2.97 (t, 2H, *J* = 7.6 Hz), 1.93 (s, 5H), ^{31}P NMR (162 mHz, D_2O) δ 0.39, -10.68. HRMS calcd for $C_{26}H_{37}N_7O_{19}P_3^+$: 844.136, Found *m/z*: 844.217 (M+). **HPLC Method 1**: t = 12.45 min, 22% 100 mM TFA.

5-(3-*t*-Boc-Aminopropyl)-NAADP (32d). 5-(3-*t*-Boc-Aminopropyl)nicotinic acid (35 mg, 0.125 mmol) was suspended in water (5 mL) and DMSO (1 mL) and stirred for 30 minutes at 37 ^{o}C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 minutes. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 minutes at 37 ^{o}C. Next, *Aplysia* cyclase (180 μL, 36 ng) was added and the reaction was stirred at 37 ^{o}C for 6 hours. At the end of 6 hours, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water/TFA gradients according to **HPLC Method 3**. 5-(3-*t*-Boc-Aminopropyl)-NAADP eluted between 22.57 and 27.75 min (27-34%). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to

remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2-7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **32d** eluted into 215 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(3-*t*-Boc-aminopropyl)-NAADP as a white solid (7.6, 42%): ^{1}H NMR (400 mHz, D_2O) δ 8.96 (s, 1H), 8.79 (s, 1H), 8.62 (s, 1H), 8.42 (s, 1H), 8.09 (s, 1H), 6.11 (s, 1H), 5.96 (s, 1H), 4.60-4.10 (10H), 3.06 (s, 2H), 2.79 (s, 2H), 1.79 (s, 2H), 1.34 (s, 9H); ^{31}P NMR (162 mHz, D_2O) δ 0.44, -10.39. HRMS calcd for $C_{29}H_{43}N_7O_{20}P_3^+$: 902.178, Found *m/z*: 902.278 (M+); **HPLC Method 1**: t = 12.75 min, 23 % 100 mM TFA.

5-(3-Aminopropyl)-NAADP (32e). 5-(3-Aminopropyl)nicotinic acid (35 mg, 0.194 mmol) was dissolved in water (6 mL) and stirred for 30 minutes at 37 ^{o}C. After this time, the pH was adjusted to 4 with 1 M HCl and stirred for 30 minutes. The pH was measured again and readjusted to pH = 4 with 1 M HCl. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 minutes at 37 ^{o}C. Next, *Aplysia* cyclase (180 μL, 36 ng) was added and the reaction was stirred at 37 ^{o}C for 6 hours. At the end of 6 hours, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water/TFA gradients according to **HPLC Method 3.** 5-(3-Aminopropyl)-NAADP eluted between 12.83 and 15.44 min (12-16%). The combined

aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2-7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **32e** eluted into 140 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(3-aminopropyl)-NAADP as a white powder (10.5 mg, 66%): ^{1}H NMR (400 mHz, D_2O) δ 8.94 (s, 1H), 8.80 (s, 1H), 8.60 (s, 1H), 8.34 (s, 1H), 8.06 (s, 1H), 6.06 (d, 1H, J = 4.8 Hz), 5.96 (d, 1H, J = 4 Hz), 4.89 (partially buried in HDO peak, 1H), 4.54 (m, 1H), 4.44 (s, 1H), 4.32-4.13 (6H), 3.02 (t, 2H, J = 6.8 Hz), 2.84 (t, 2H, J = 7.6 Hz), 1.97 (m, 2H); ^{31}P NMR (162 mHz, D_2O) δ 4.22, -10.5 (d), -11.14 (d). HRMS calcd for $C_{24}H_{35}N_7O_{18}P_3^+$: 802.125, Found *m/z* 802.182 (M+). **HPLC Method 1**: t = 6.93 min, 11 % 100 mM TFA

5-(3-Hydroxypropyl)-NAADP (32f). 5-(3-Hydroxypropyl)nicotinic acid (35 mg, 0.193 mmol) was suspended in water (6 mL) and stirred for 30 minutes at 37 ^{o}C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 minutes. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 minutes at 37 ^{o}C. Next, *Aplysia* cyclase (180 μL, 36 ng) was added and the reaction was stirred at 37 ^{o}C for 4 hours. At the end of 4 hours, analysis by HPLC confirmed that the starting material (NADP) had been

consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water/TFA gradients according to **HPLC Method 3**. 5-(3-Hydroxypropyl)-NAADP eluted between 21.73 and 26.53 min (26-34%). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2-7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **32f** eluted into 225 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(3-hydroxypropyl)-NAADP as a white powder (9.5 mg, 59%): ^{1}H NMR (400 mHz, D_2O) δ 8.96 (s, 1H), 8.82 (s, 1H), 8.65 (s, 1H), 8.42 (s, 1H), 8.14 (s, 1H), 6.13 (d, 1H, J = 5.6 Hz), 5.96 (d, 1H, J = 4.4 Hz), 5.03 (m, 2H), 4.6 (t, 1H), 4.45 (s, 1H), 4.38-4.18 (6H), 3.59 (t, 2H, J = 6.4 Hz), 2.86 (t, 2H, J = 8.4 Hz), 1.86 (m, 2H); ^{31}P NMR (162 mHz, D_2O) δ 0.55, -10.64). HRMS calcd for $C_{24}H_{34}N_6O_{19}P_3^+$: 803.109, Found *m/z* 803.214 (M+). **HPLC Method 1**: t = 12.70 min, 23% 100 mM TFA. **Method 7**: 310 mM ammonium bicarbonate.

5-(3-Azidopropyl)-NAADP (32g). 5-(3-azidopropyl)nicotinic acid (35 mg, 0.170 mmol) was suspended in water (5mL) and DMSO (1 mL) and stirred for 30 minutes at 37 ^{o}C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 minutes. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if

necessary. This procedure is repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 minutes at 37 ^{o}C. Next, *Aplysia* cyclase (120 µL, 12 ng) was added and the reaction was stirred at 37 ^{o}C for 5 hours. At the end of 5 hours, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water/TFA gradients according to **HPLC Method 3.** 5-(3-Azidopropyl)-NAADP eluted between 23.46 and 27.03 min (30-35%). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2-7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **32g** eluted into 225 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(3-azidopropyl)-NAADP as a white powder (8.5 mg, 52%): ^{1}H NMR (400 mHz, D_2O) δ 9.30 (s, 1H), 9.12 (s, 1H), 8.91 (s, 1H), 8.57 (s,1H), 8.38 (s, 1H), 6.25 (d, 1H, J = 5.6 Hz), 6.10 (d, 1H, J = 5.2 Hz), 5.08 (s, 1H), 4.62 (s, 1H), 4.56 (s, 1H), 4.53-4.23 (7H), 3.38 (t, 2H, J = 6.4 Hz), 3.02 (t, 2H, J = 7.2 Hz), 1.98 (m, 2H); ^{31}P NMR (162 mHz, D_2O) δ 0.39, -10.40. HRMS calcd for $C_{24}H_{33}N_9O_{18}P_3^+$: 828.116, Found *m/z*: 828.214 (M+). **HPLC Method 1**: t = 13.17 min, 24% 100 mM TFA

5-Thiomethyl-NAADP (32h). 5-Thiomethylnicotinic acid (35 mg, 0.207 mmol) was suspended in water (5mL) and DMSO (1 mL) and stirred for 30 minutes at 37 ^{o}C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 minutes. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 minutes at 37 ^{o}C. Next, *Aplysia* cyclase (120 µL, 24 ng) was added and the reaction was stirred at 37 ^{o}C for 2 hours. At the end of 2 hours, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water/TFA gradients according to **HPLC Method 3**. 5-Thiomethyl-NAADP eluted between 23.60 and 27.89 min (29-35%). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2-7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6.** The product **32h** eluted into 225 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-thiomethoxy-NAADP as a white powder (8 mg, 51%). ^{1}H NMR (400 mHz, D_2O) δ 9.08 (s, 1H), 8.88 (s, 1H), 8.72 (s, 1H), 8.57 (s, 1H), 8.40 (s, 1H), 6.25 (s, 1H), 6.07 (s, 1H), 5.08 (s, 1H), 4.62-4.20 (9H), 2.65 (s, 3H); ^{31}P NMR (162 mHz, D_2O) δ 0.31, -10.72.

HRMS calcd for $C_{22}H_{30}N_6O_{18}P_3S^+$: 791.055, Found *m/z*: 791.139 (M+). **HPLC Method 1**: t = 13.44 min, 25 % 100 mM TFA.

8-Bromo-NAADP (32i). Nicotinic acid (35 mg, 0.207 mmol) was dissolved in water (5mL) and DMSO (1 mL) and stirred for 30 minutes at 37 ^oC. After this time period, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 minutes. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, 8-Br-NADP (15 mg, 0.018 mmol) was added and stirred for 5 minutes at 37 ^oC. Next, *Aplysia* cyclase (180 µL, 36 ng) was added and the reaction was stirred at 37 ^oC for 2 hours. . At the end of 2 hours, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water/TFA gradients according to **HPLC Method 3.** 8-Br-NAADP eluted between 23.00 and 27.97 min (28-35%). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 minutes to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2-7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **32i** eluted into 220 mM ammonium bicarbonate. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 8-Br-NAADP as a yellowish-white powder (8 mg, 51%). ^1H NMR (400 mHz, D_2O) δ 9.22 (s,

1H), 9.01 (d, 1H, *J* = 6.4 Hz), 8.89 (d, 1H, *J* = 8 Hz), 8.25 (s, 1H), 8.18 (m, 1H), 6.21 (d, 1H, *J* = 4 Hz), 6.04 (d, 1H, *J* = 5.2 Hz), 5.49 (m, 1H), 4.91 (m,1H), 4.49-4.31 (8H); ^{31}P NMR (162 mHz, D_2O) δ 5.17, -9.91. HRMS calcd for $C_{21}H_{27}BrN_6O_{18}P_3^+$: 822.978, Found *m/z*: 823.208 (M+). **HPLC Method 1**: t = 13.47 min, 25 % 100 mM TFA.

Chapter 4

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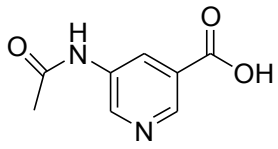
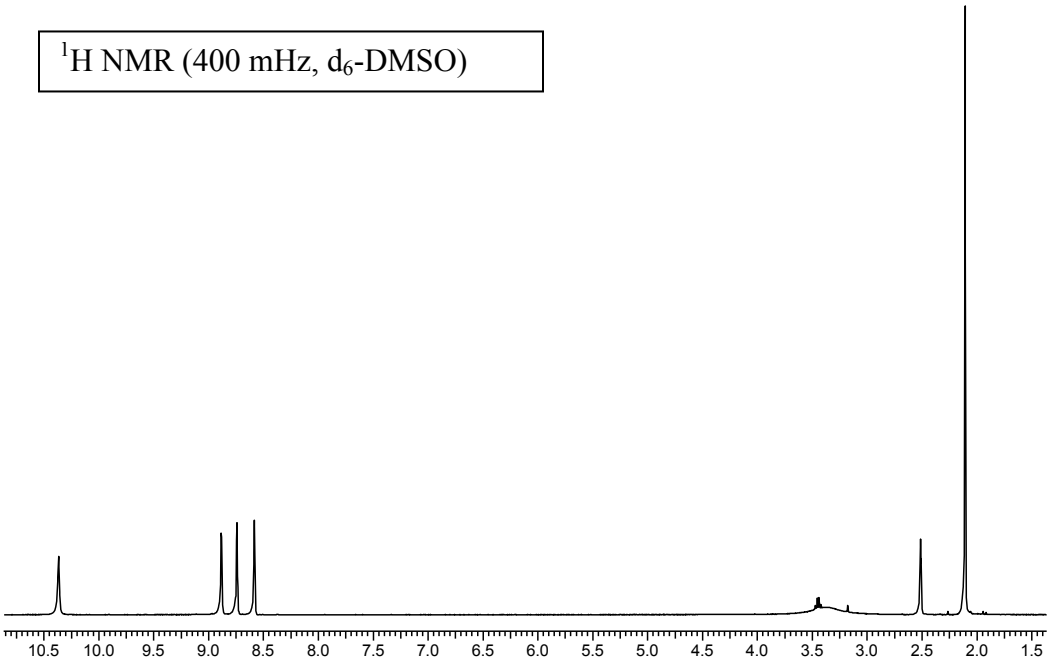
Chapter 5

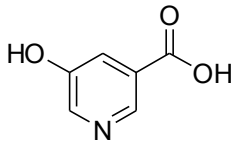
Appendix



11a

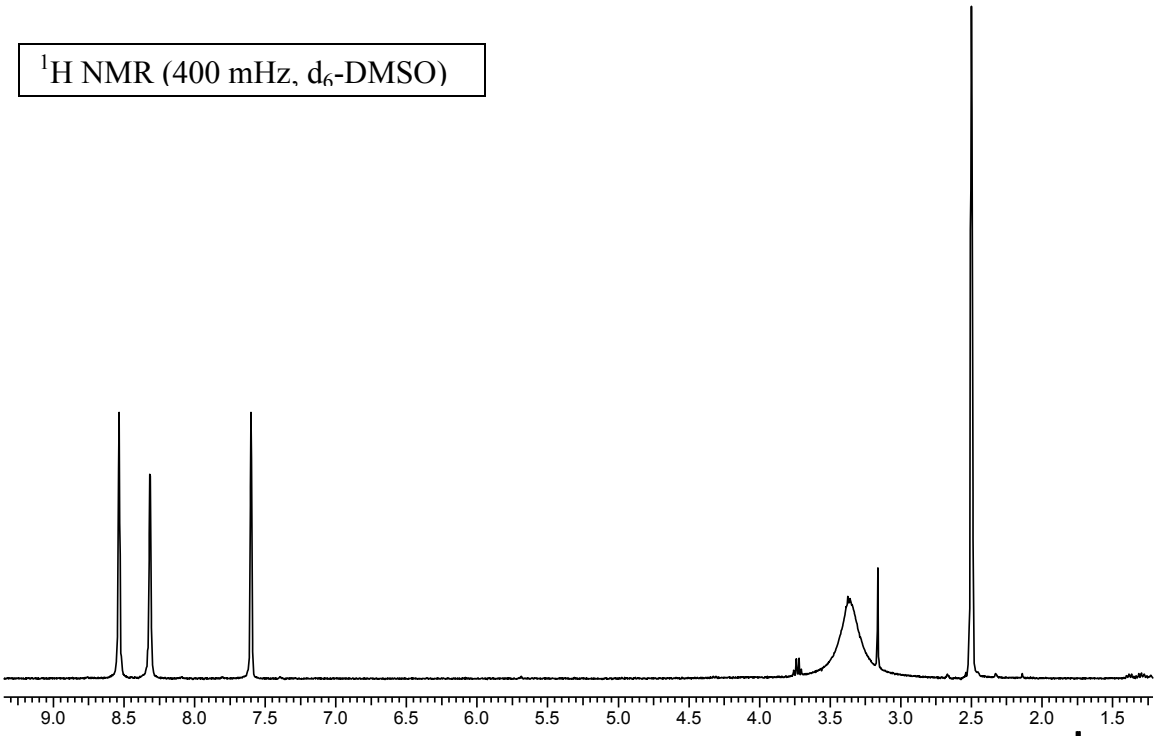
^{1}H NMR (400 mHz, d_6-DMSO)



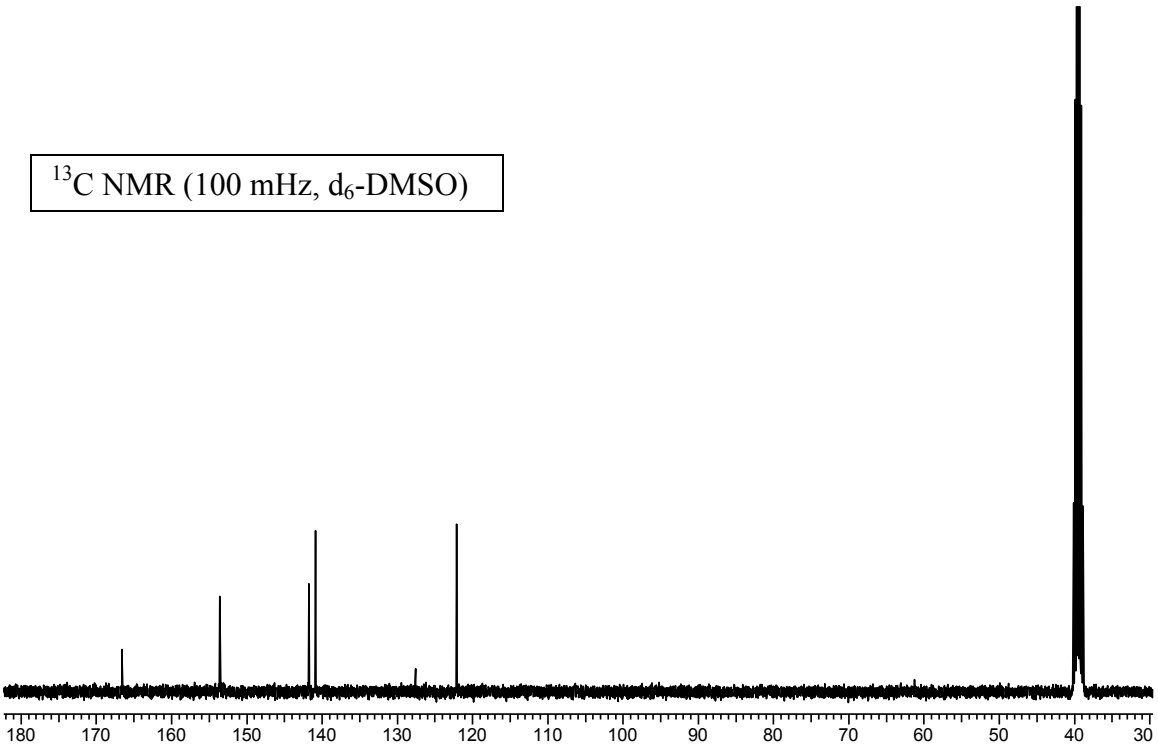


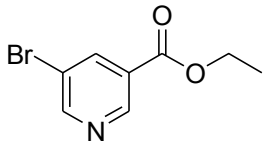
11b

^{1}H NMR (400 mHz, d_6-DMSO)



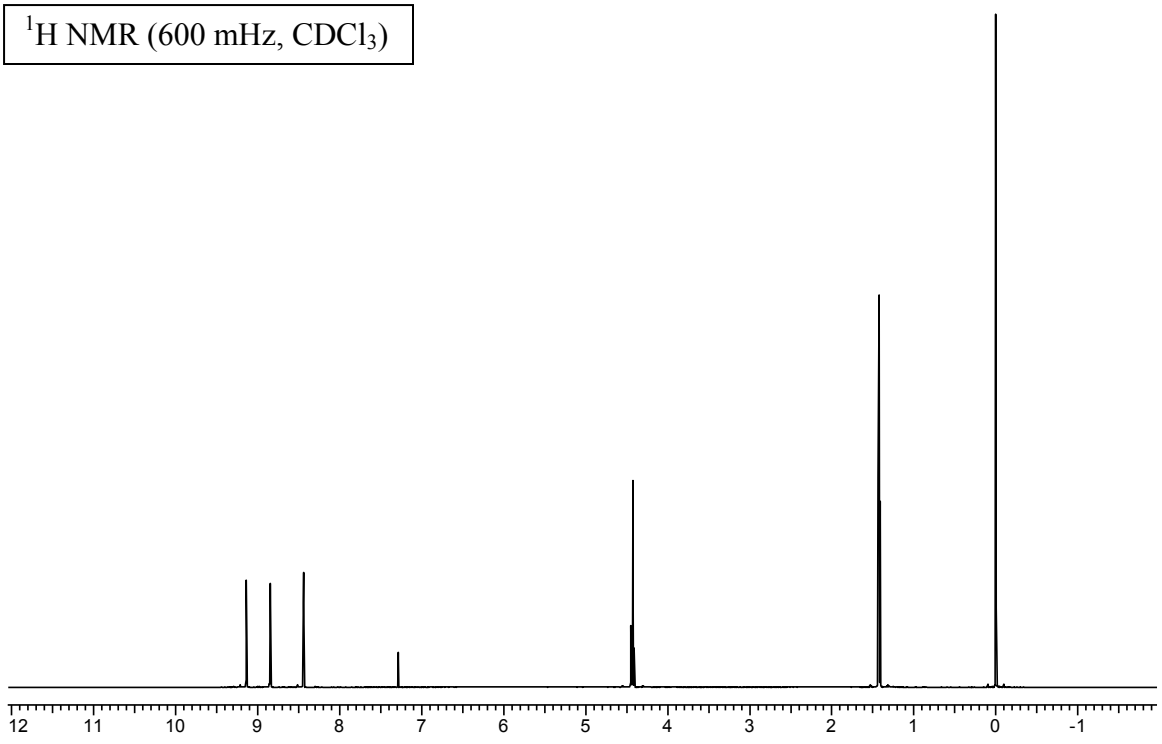
^{13}C NMR (100 mHz, d_6-DMSO)



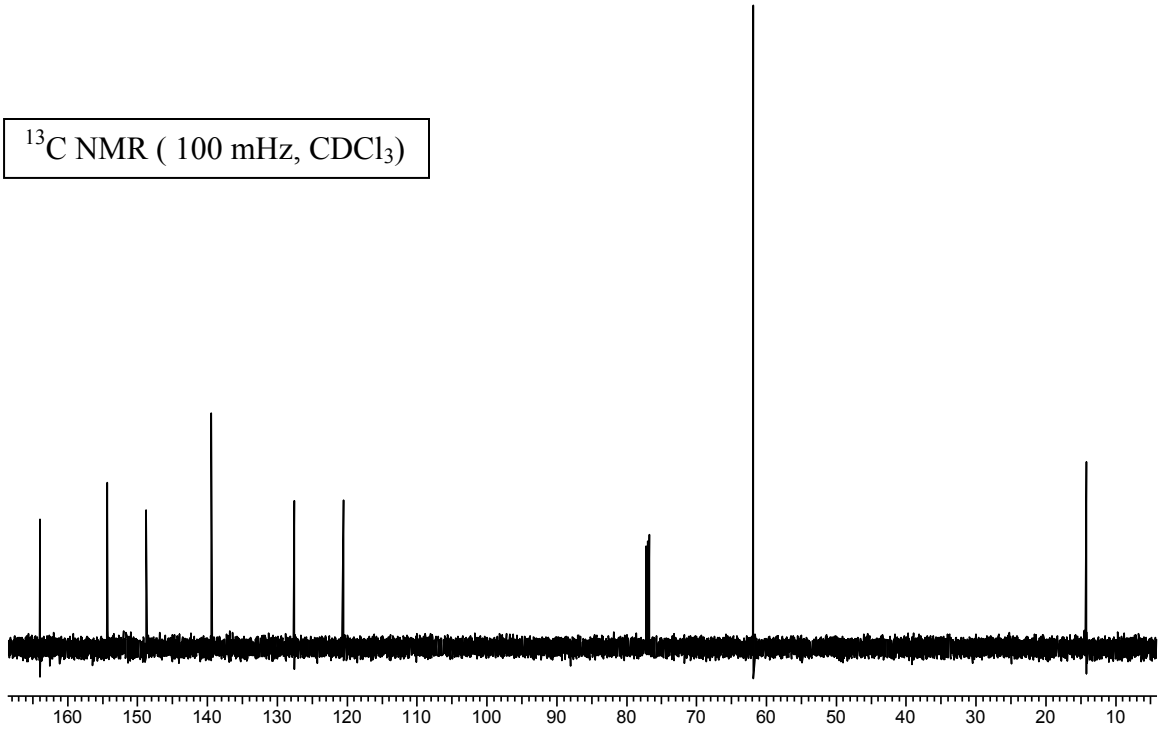


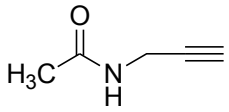
12

^{1}H NMR (600 mHz, $CDCl_3$)



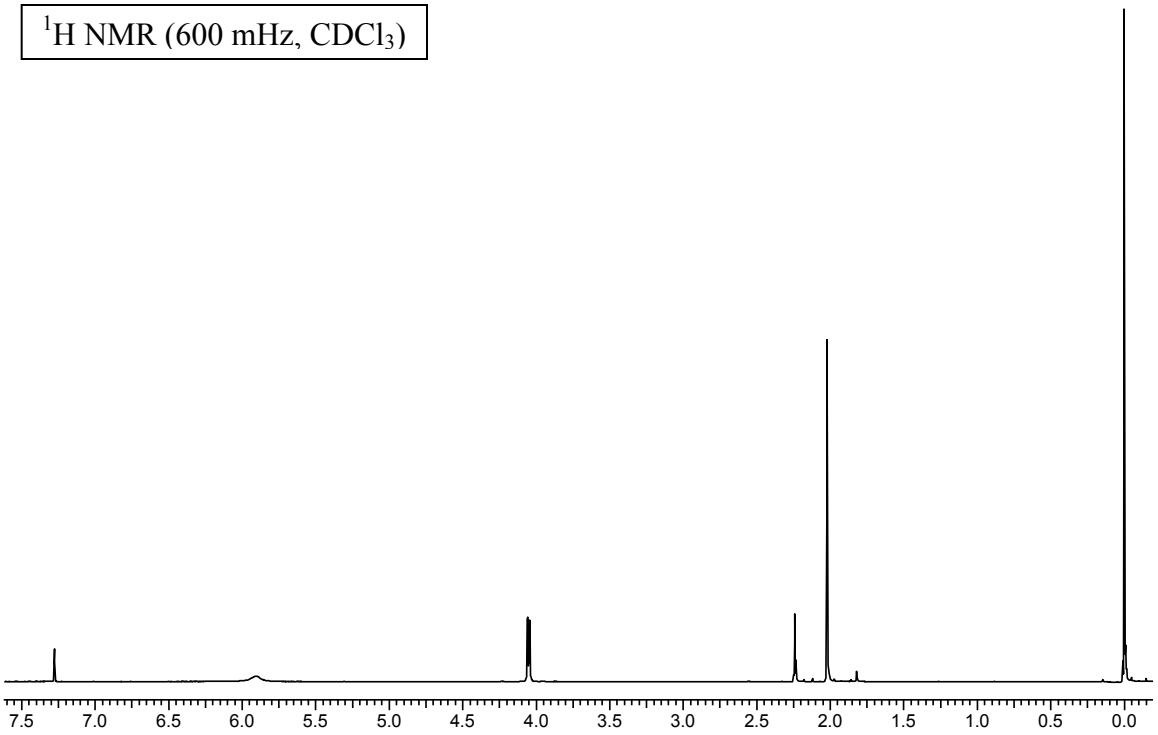
^{13}C NMR (100 mHz, $CDCl_3$)



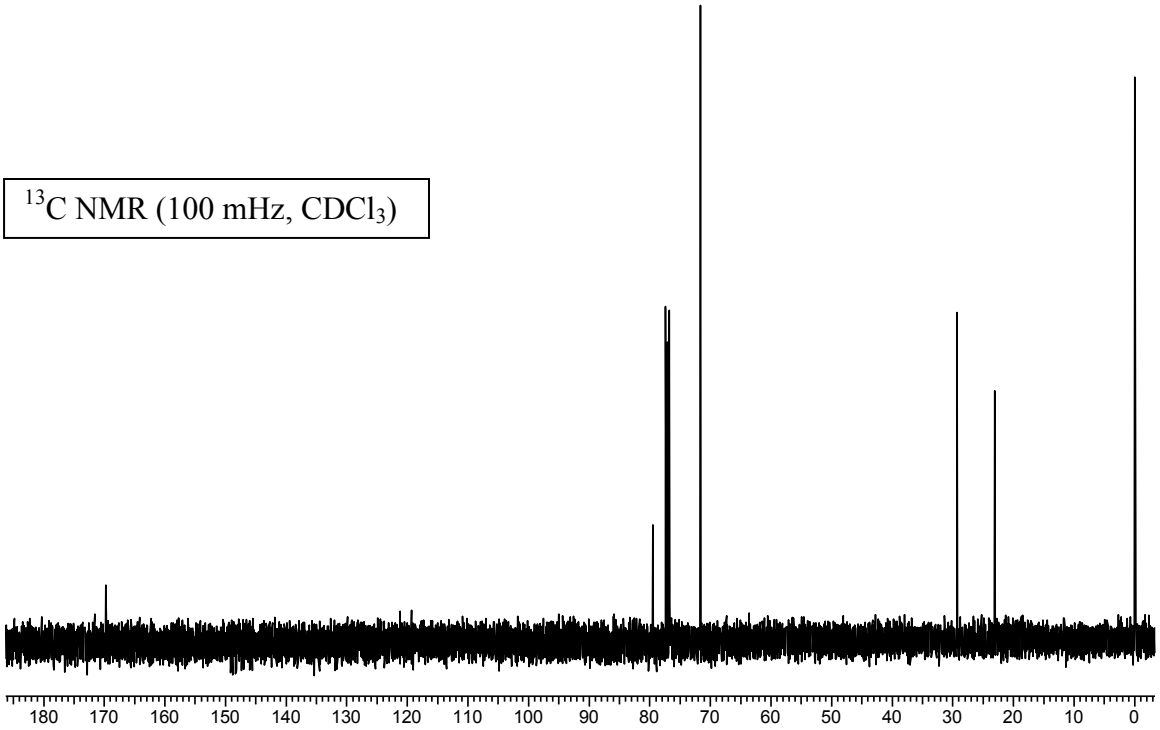


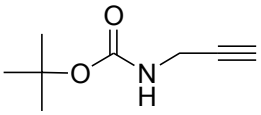
13

^{1}H NMR (600 mHz, $CDCl_3$)



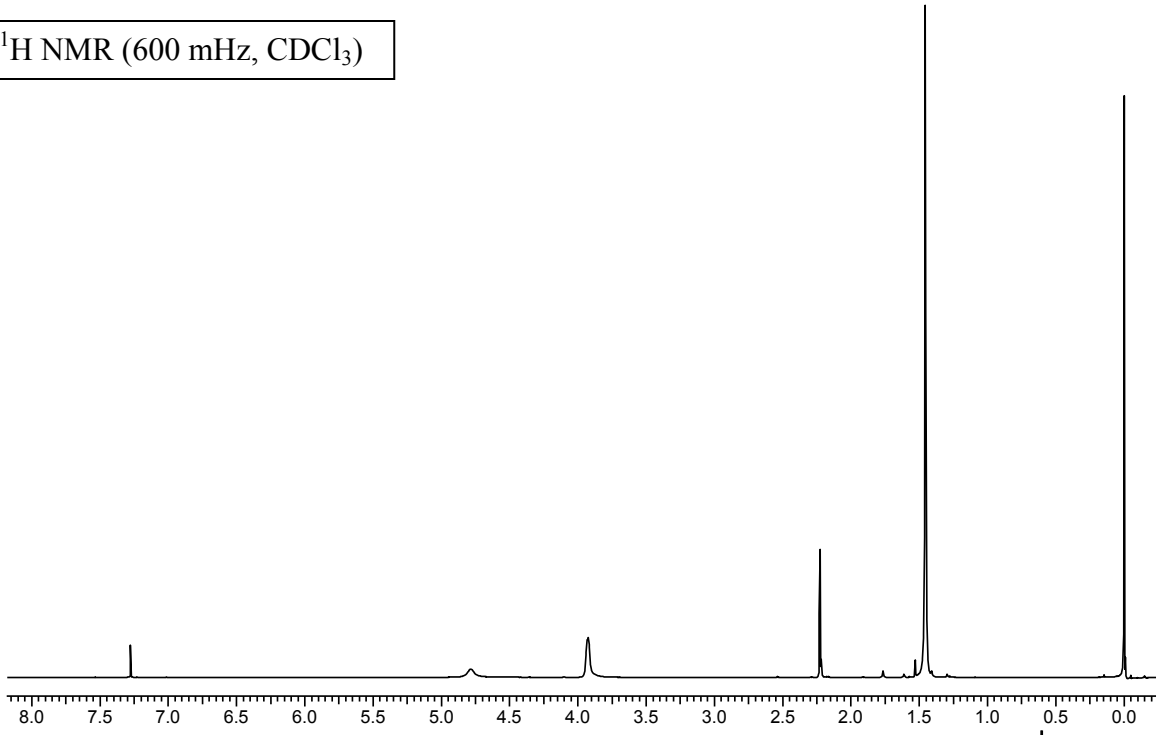
^{13}C NMR (100 mHz, $CDCl_3$)



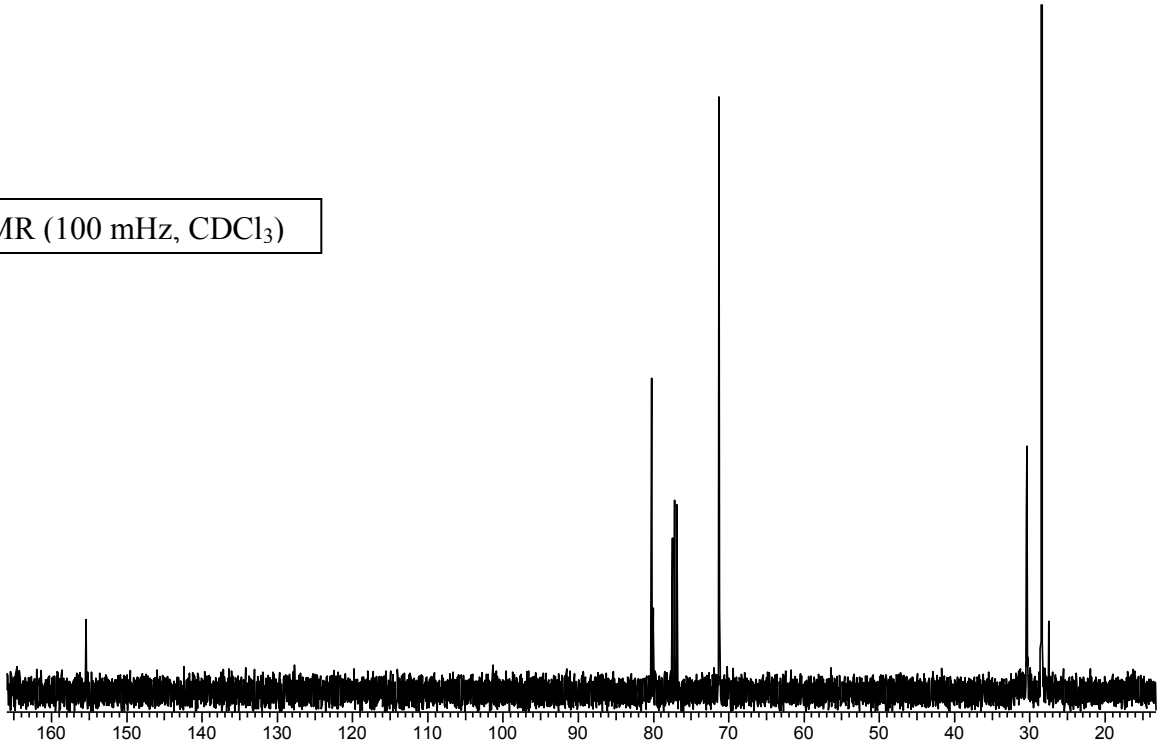


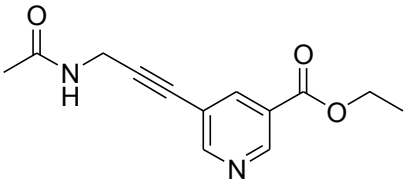
14

^{1}H NMR (600 mHz, $CDCl_3$)



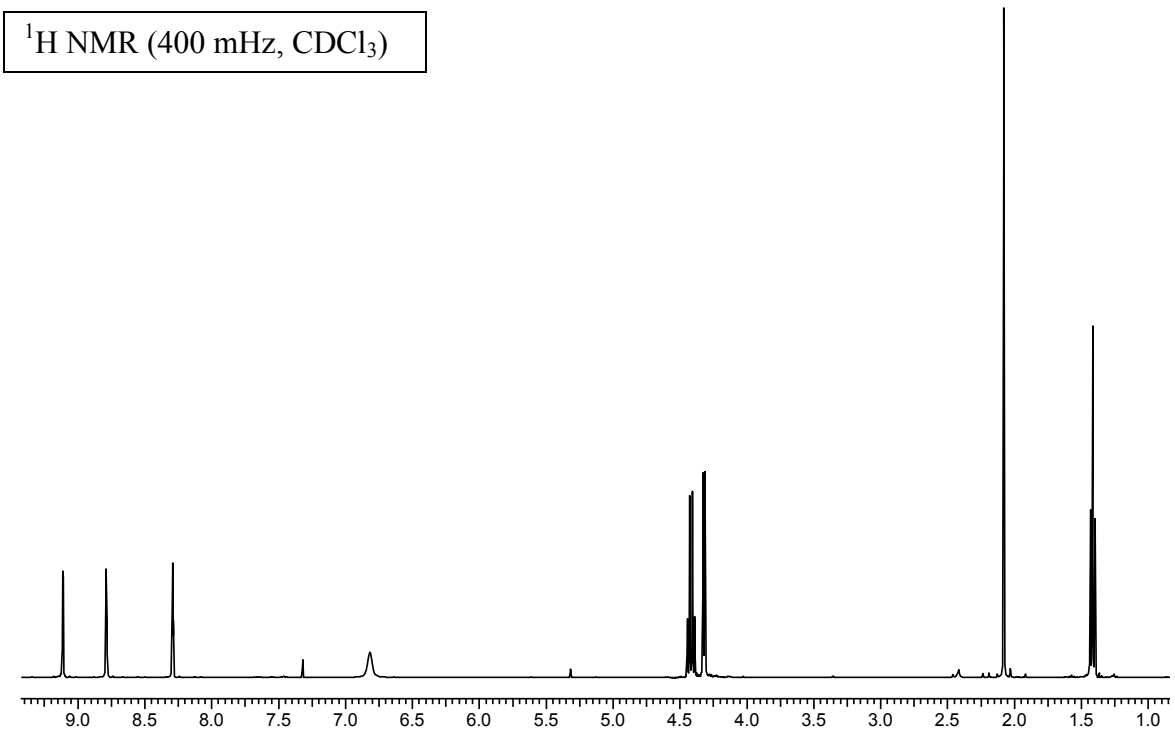
^{13}C NMR (100 mHz, $CDCl_3$)



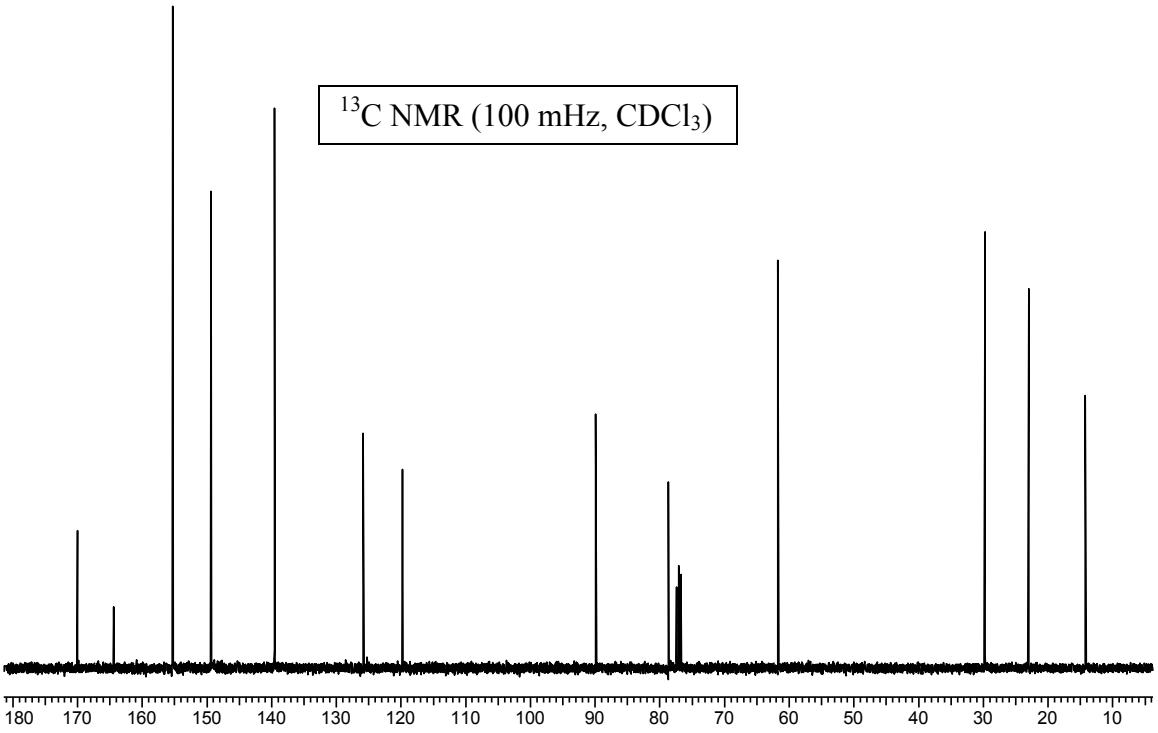


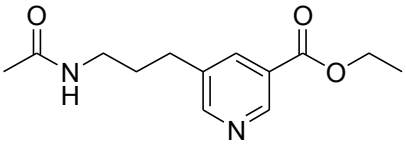
15

^{1}H NMR (400 mHz, $CDCl_3$)



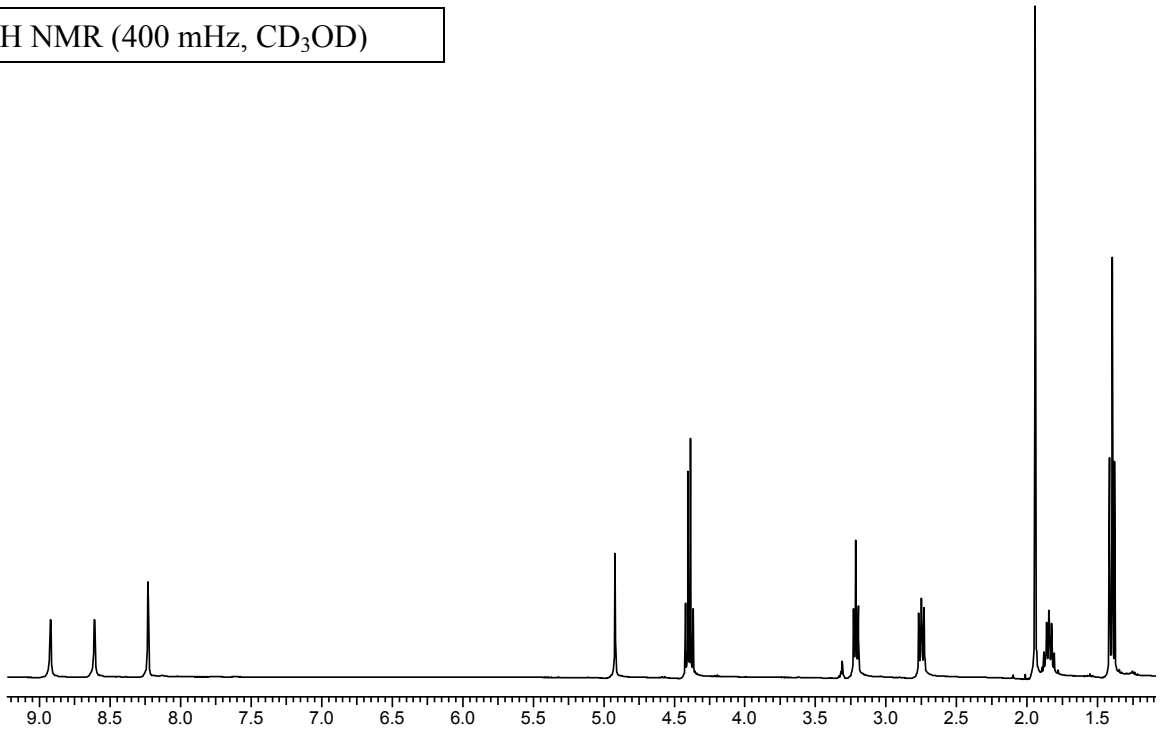
^{13}C NMR (100 mHz, $CDCl_3$)



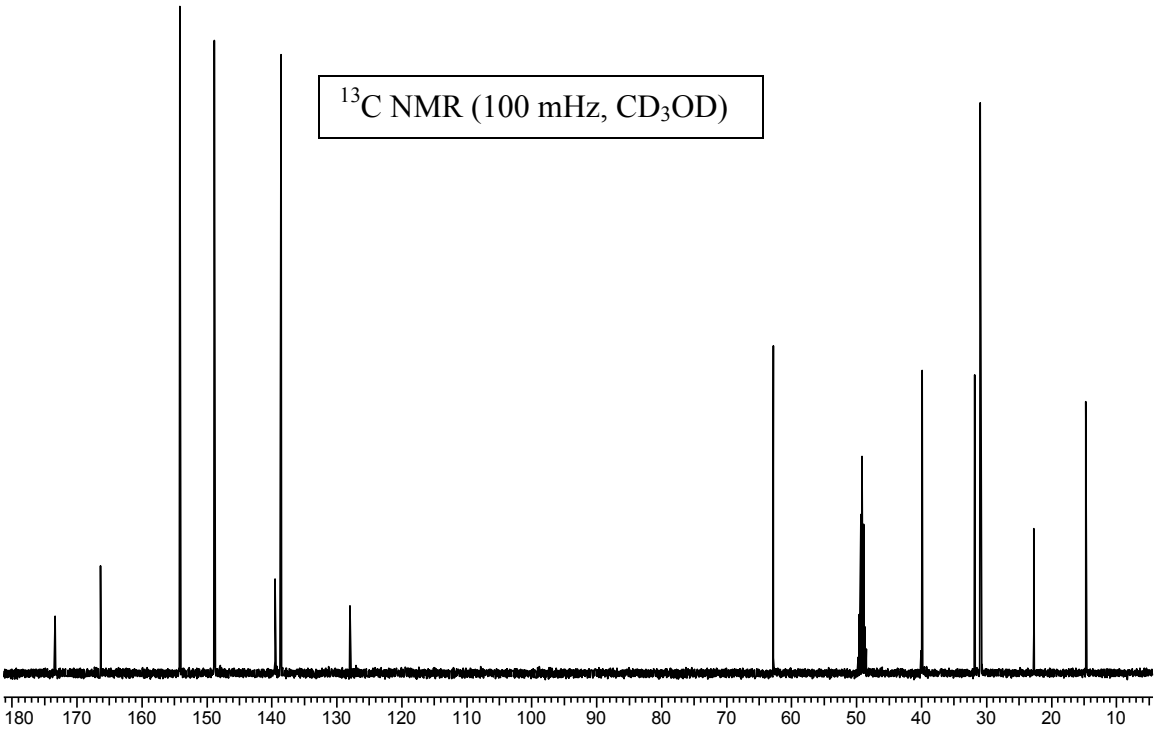


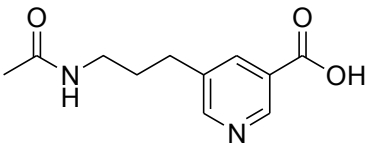
16

^{1}H NMR (400 mHz, CD_3OD)



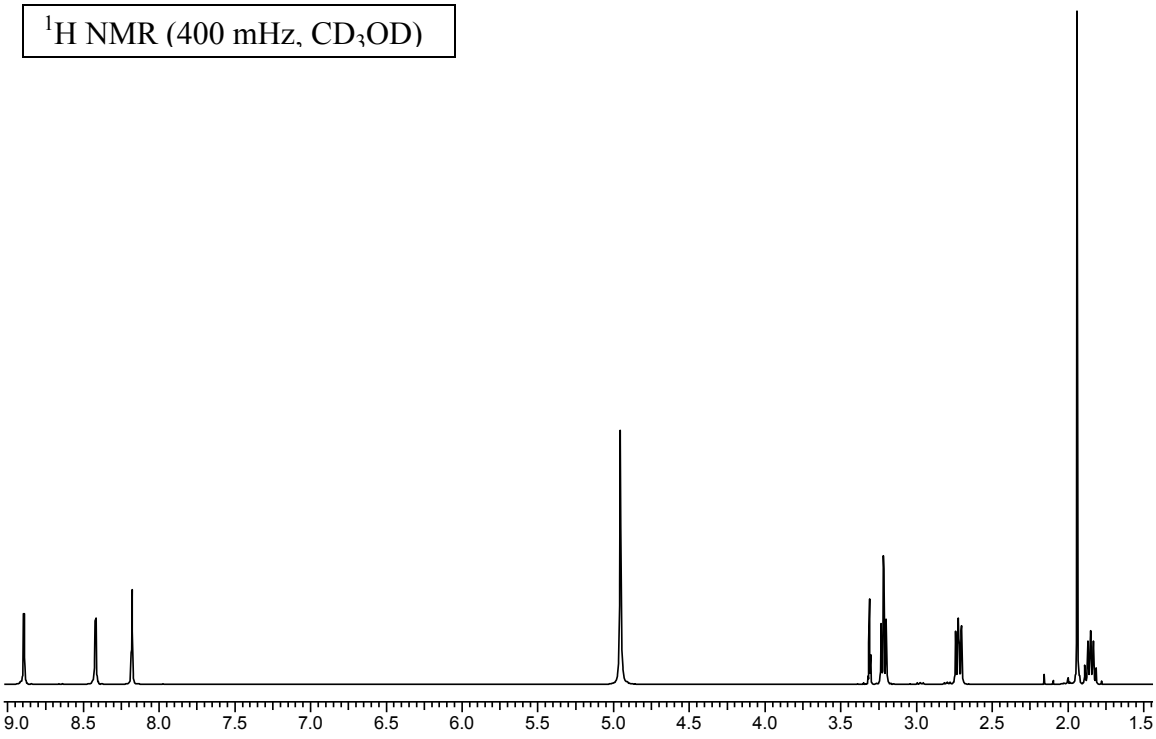
^{13}C NMR (100 mHz, CD_3OD)



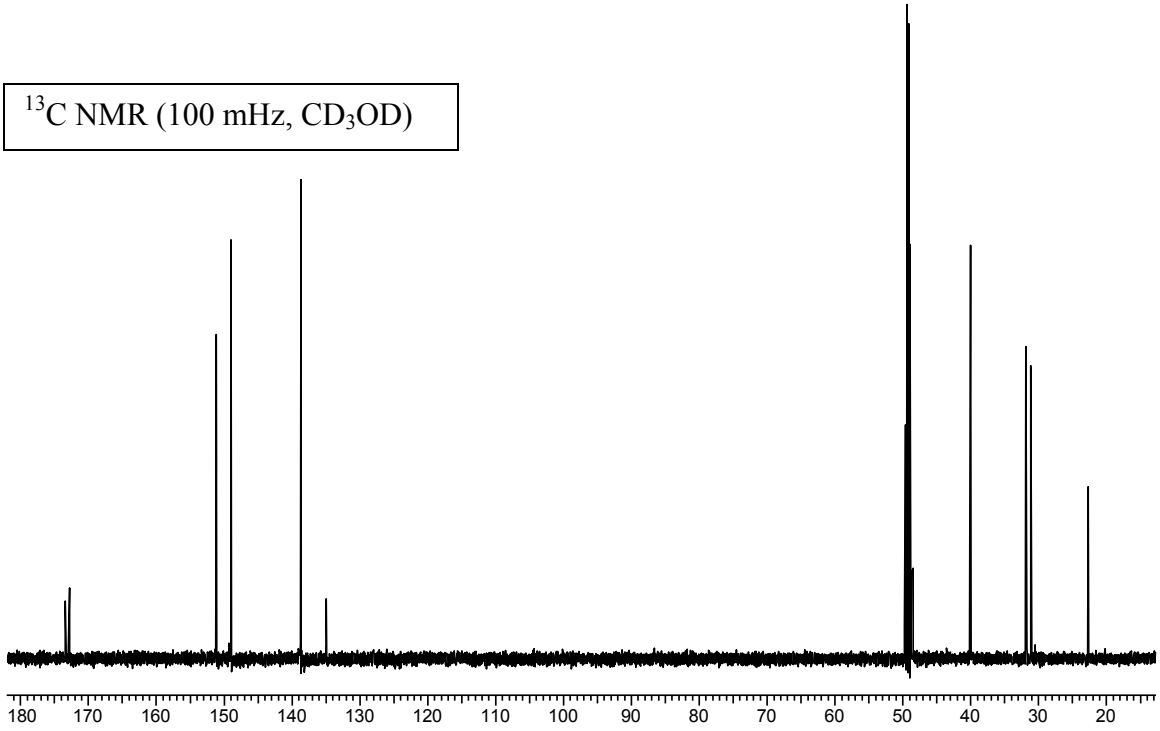


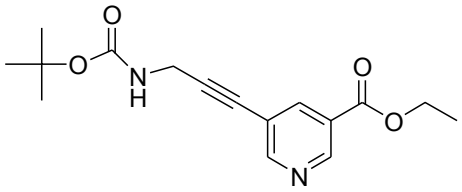
11c

^{1}H NMR (400 mHz, CD_3OD)



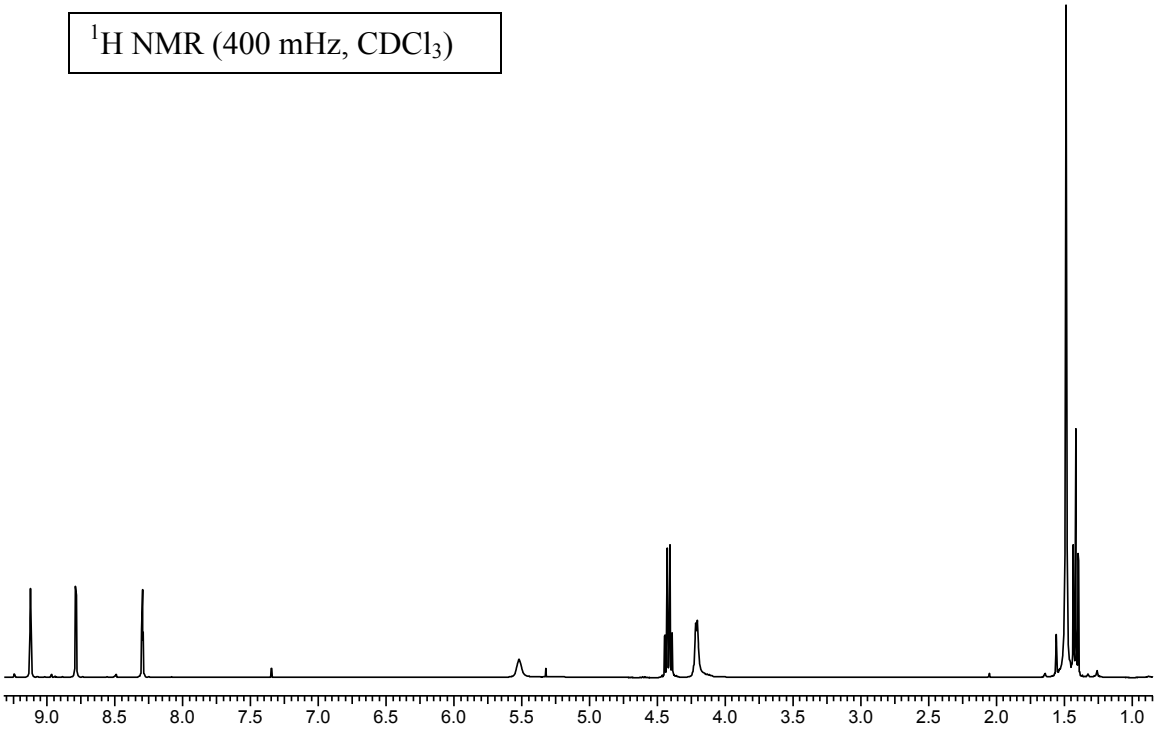
^{13}C NMR (100 mHz, CD_3OD)



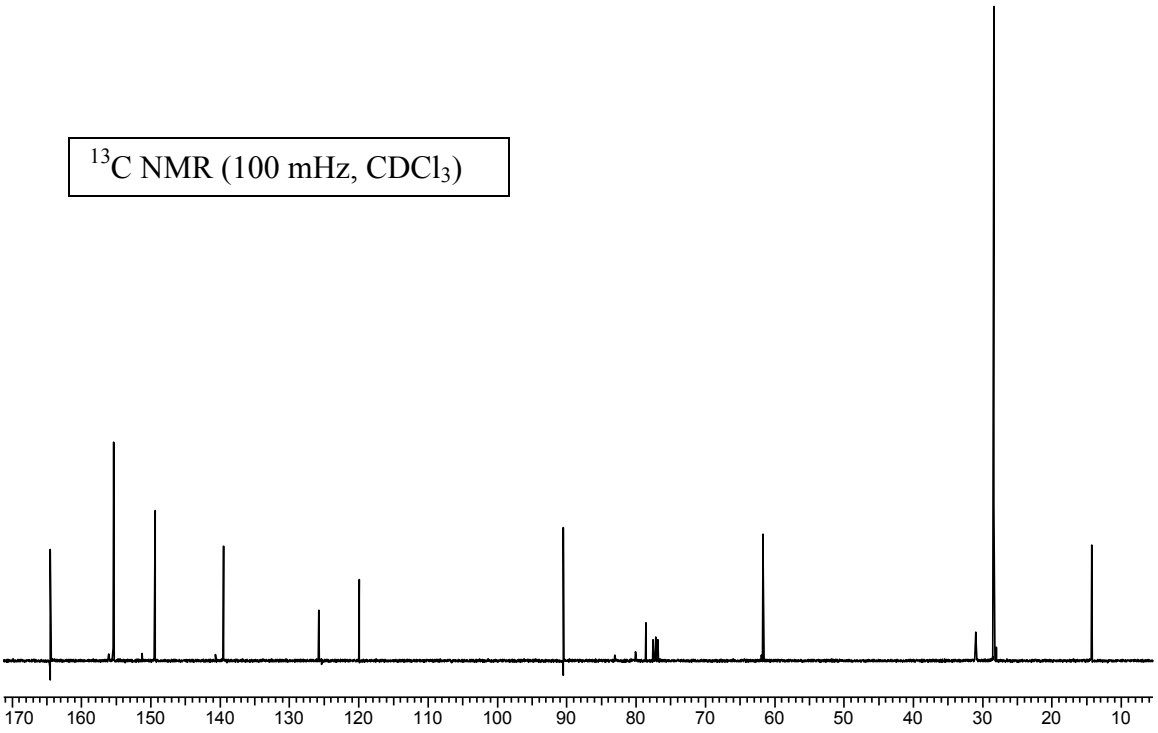


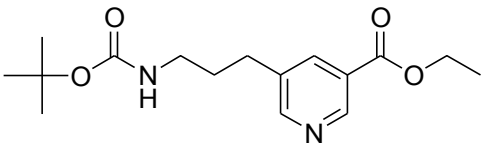
17

^{1}H NMR (400 mHz, $CDCl_3$)



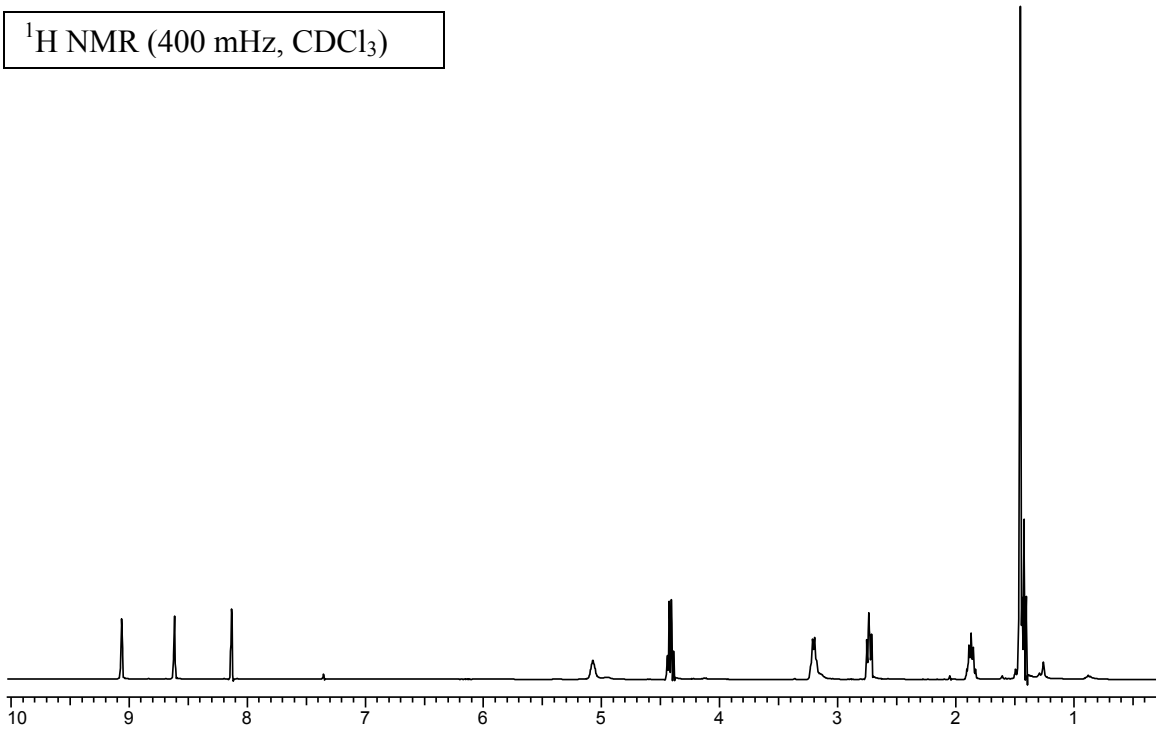
^{13}C NMR (100 mHz, $CDCl_3$)



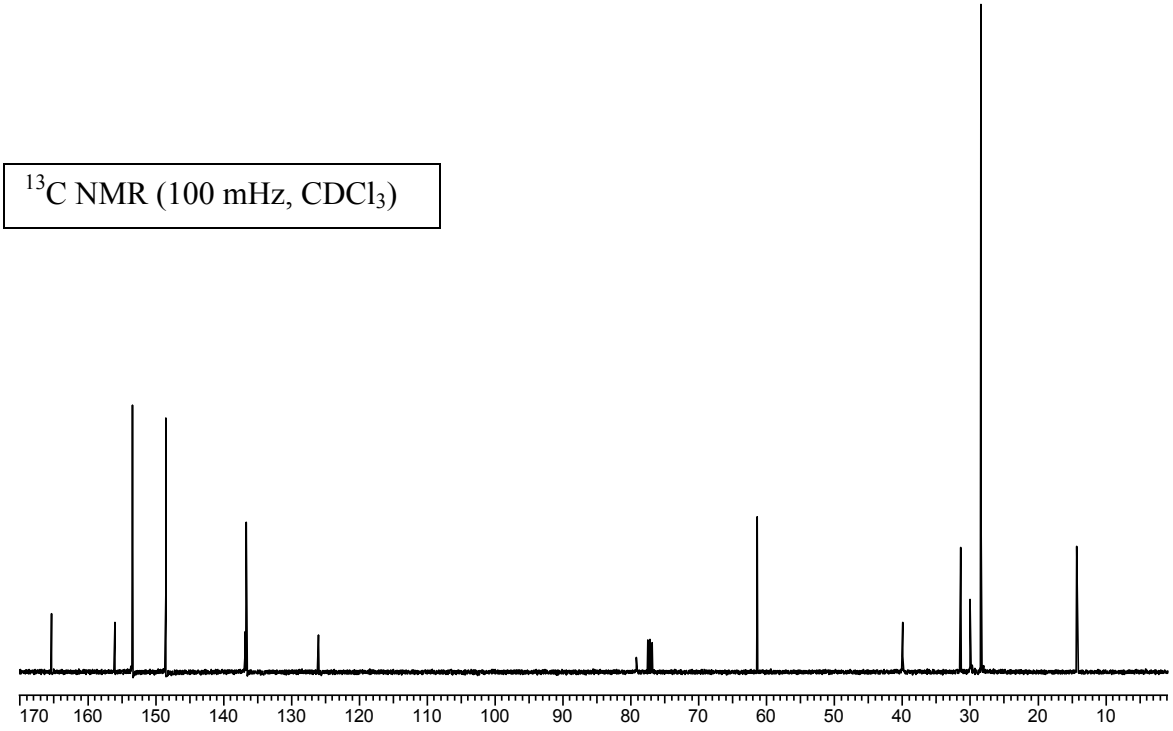


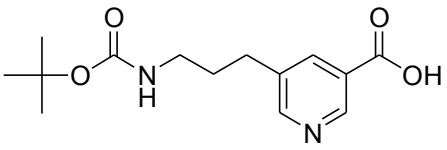
18

^{1}H NMR (400 mHz, $CDCl_3$)



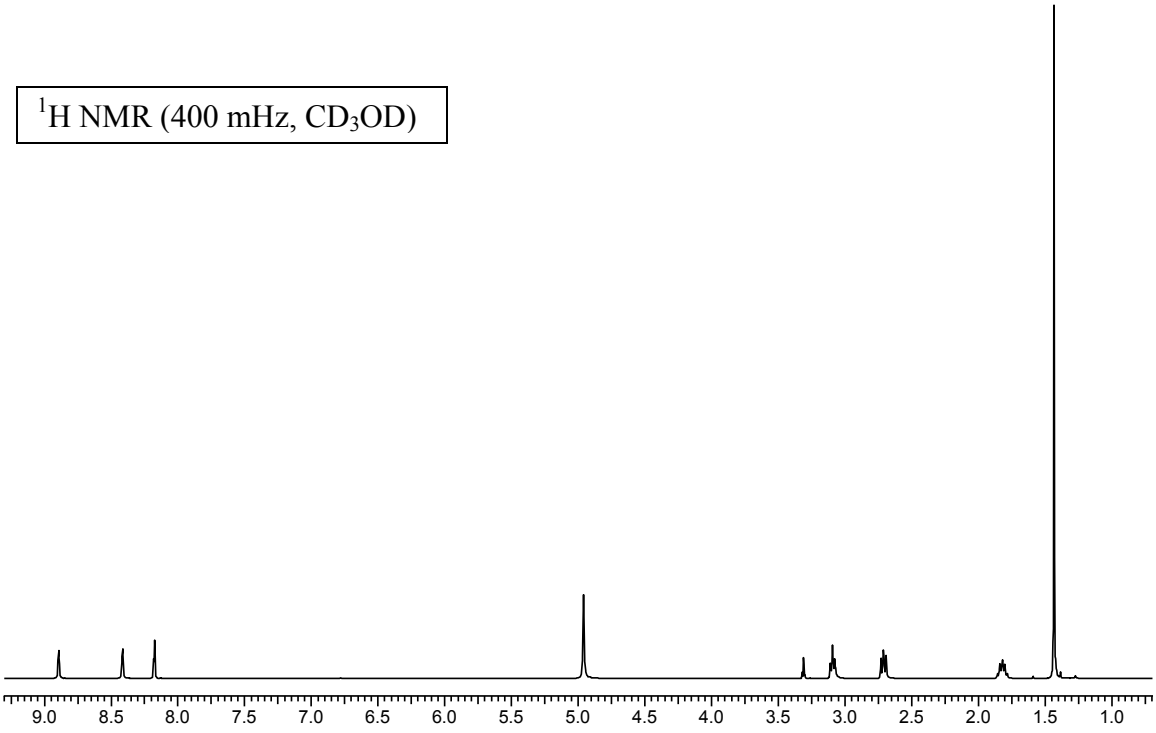
^{13}C NMR (100 mHz, $CDCl_3$)



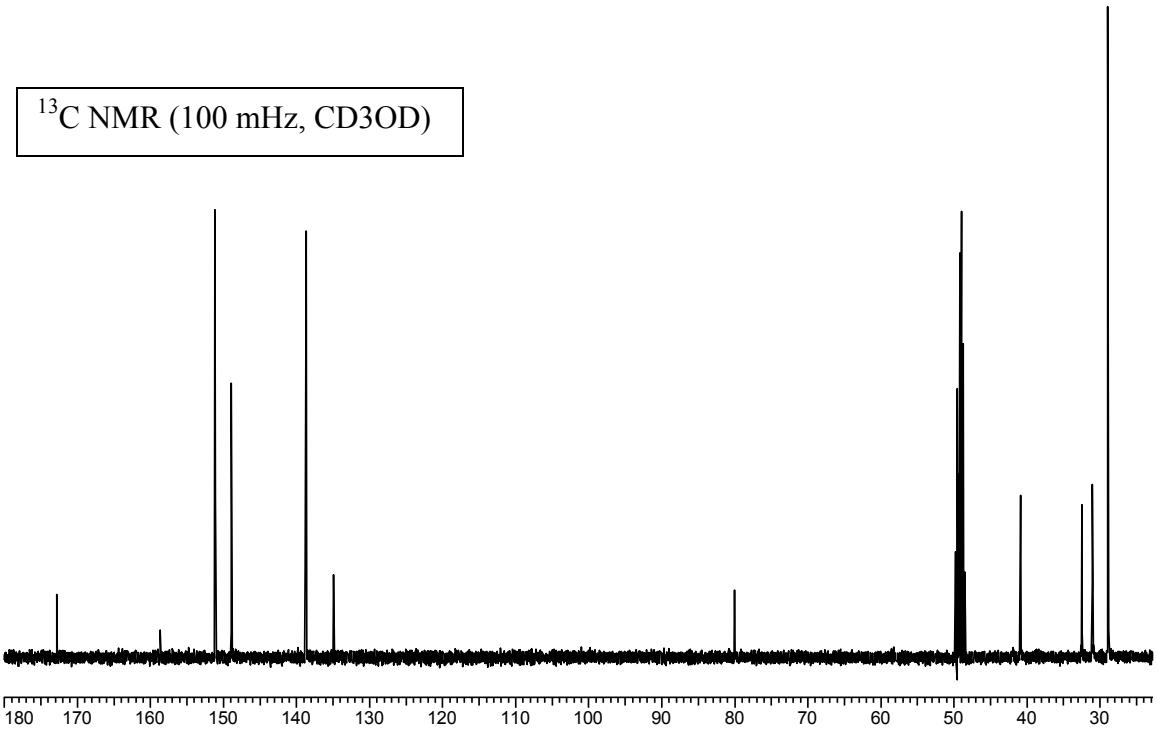


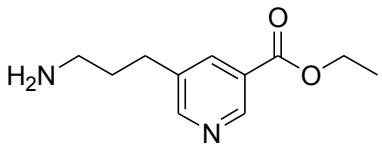
11d

^{1}H NMR (400 mHz, CD_3OD)



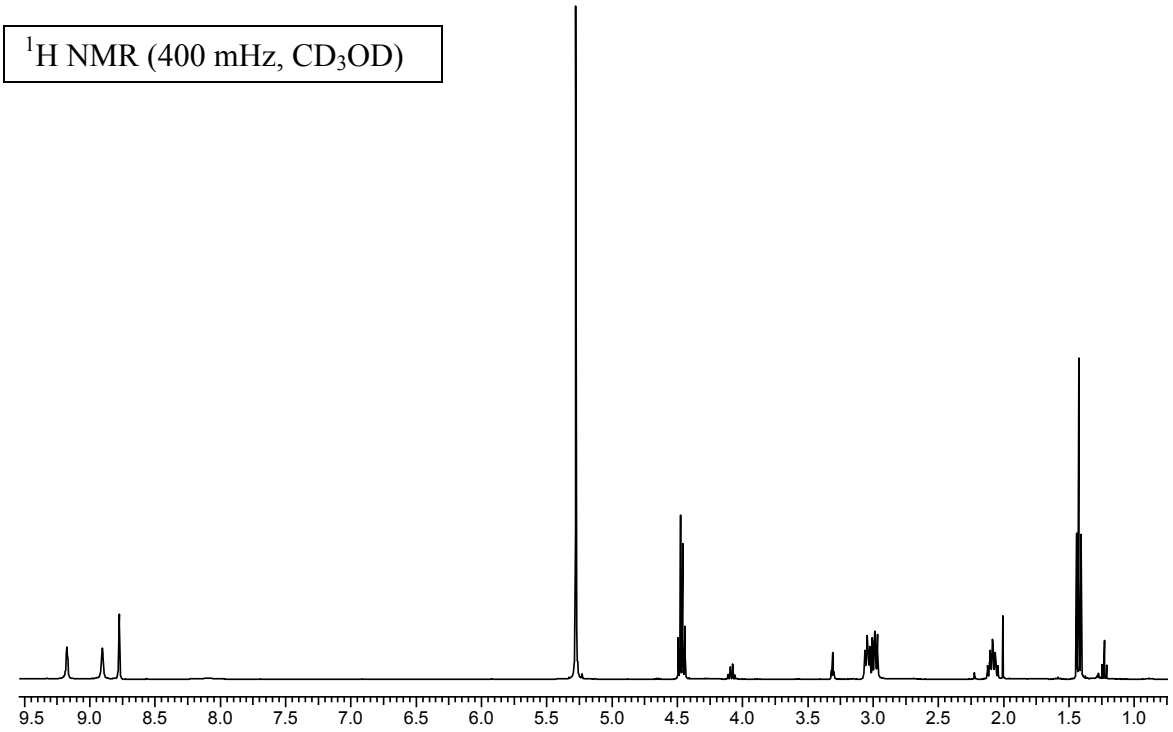
^{13}C NMR (100 mHz, CD3OD)



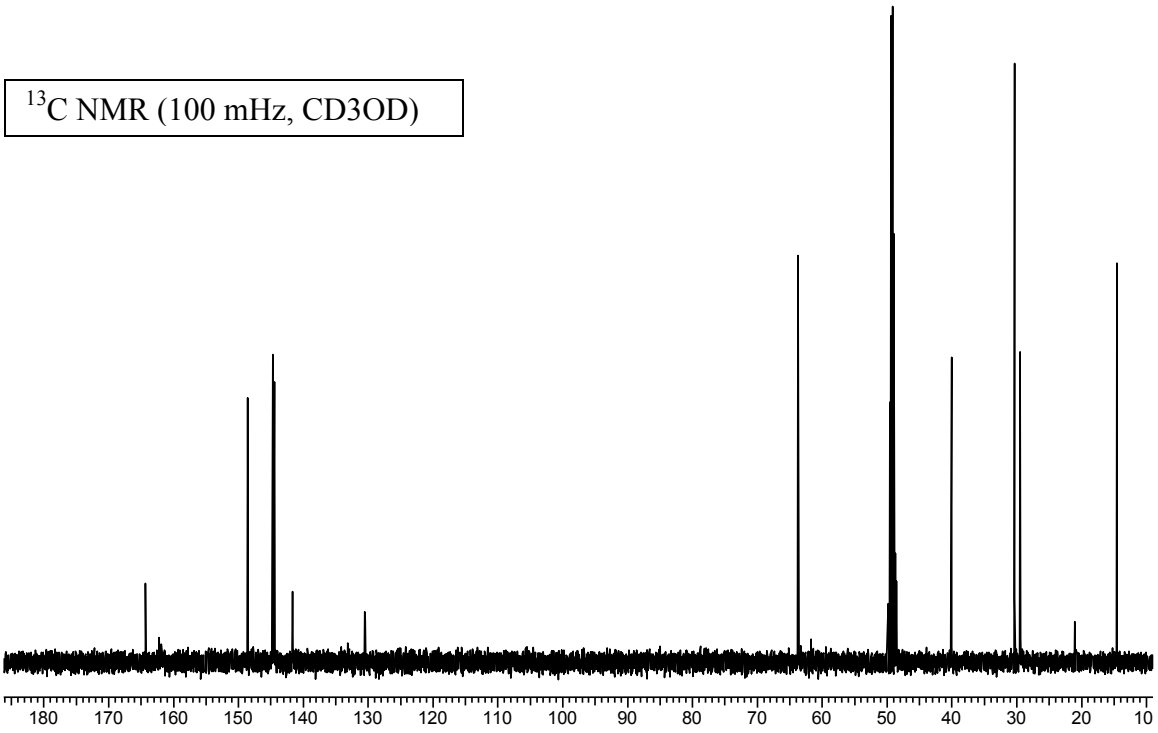


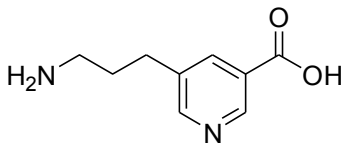
19

^{1}H NMR (400 mHz, CD_3OD)



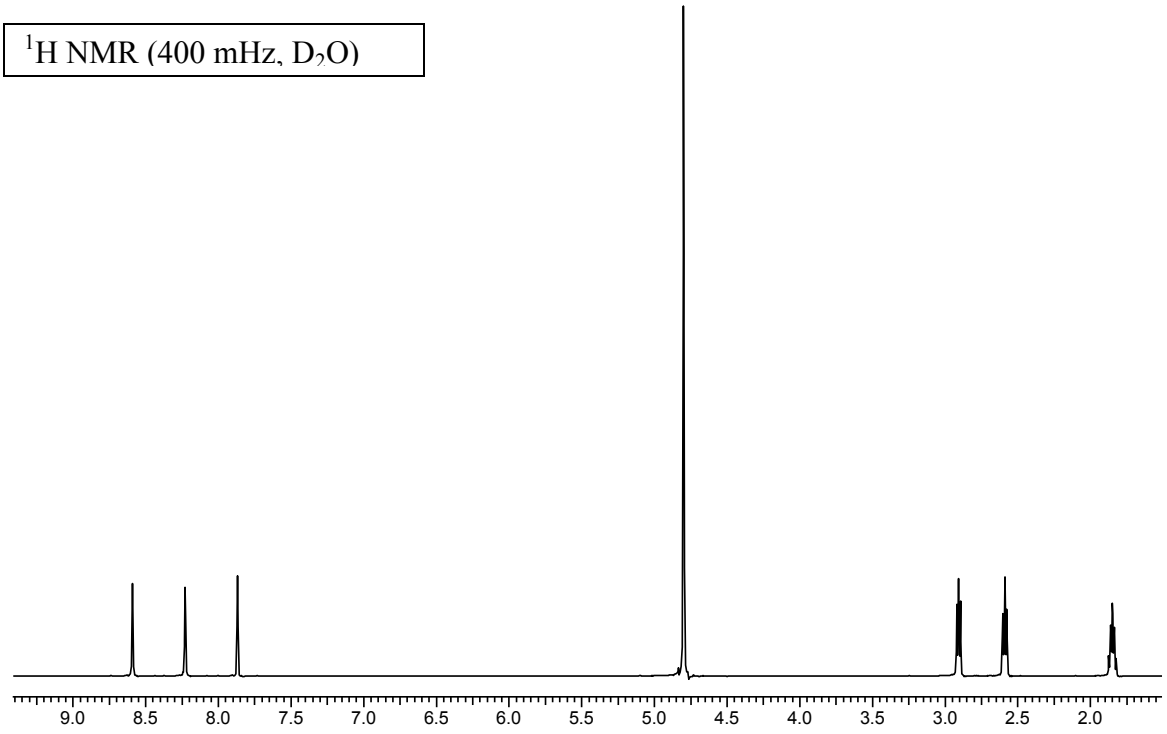
^{13}C NMR (100 mHz, CD3OD)



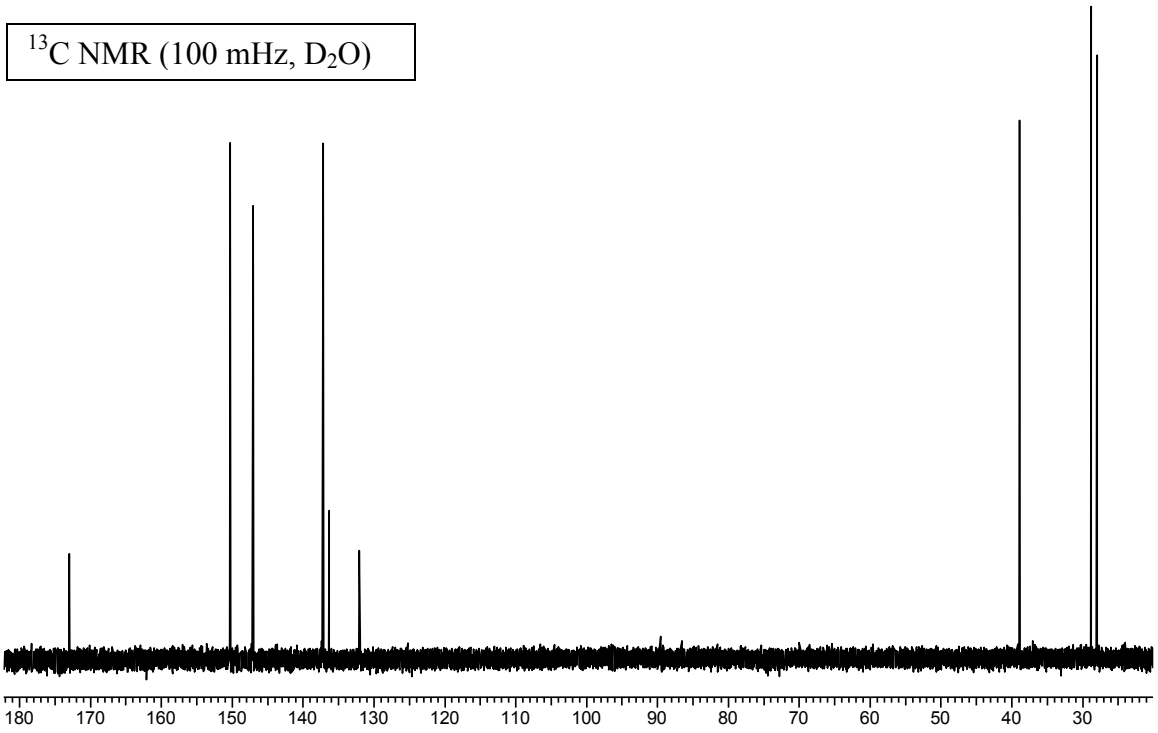


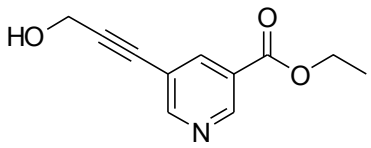
11e

^{1}H NMR (400 mHz, D_2O)



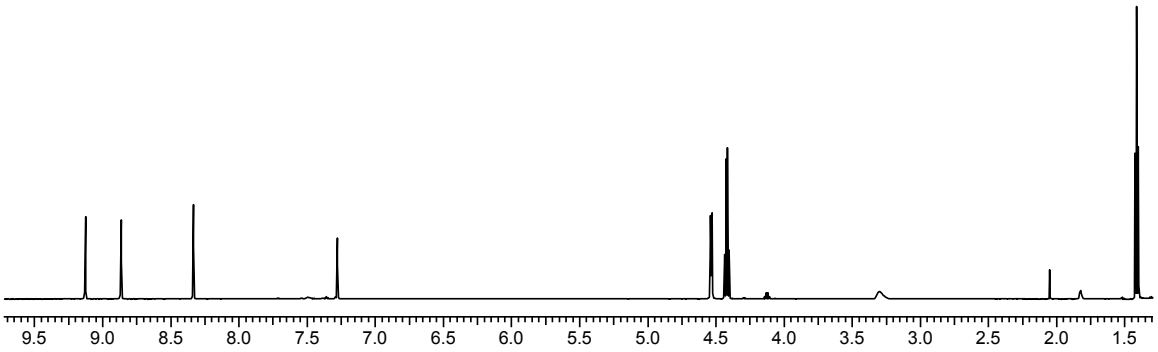
^{13}C NMR (100 mHz, D_2O)



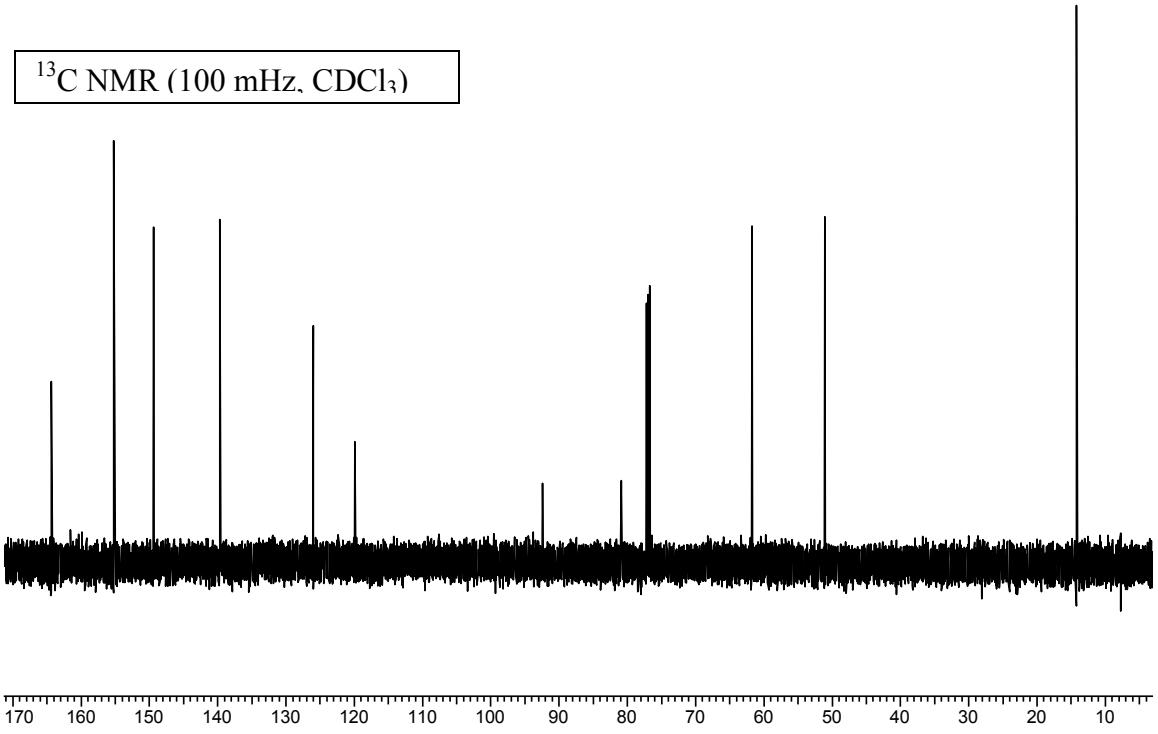


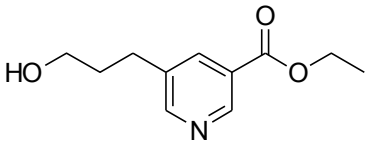
20

^{1}H NMR (600 mHz, $CDCl_3$)



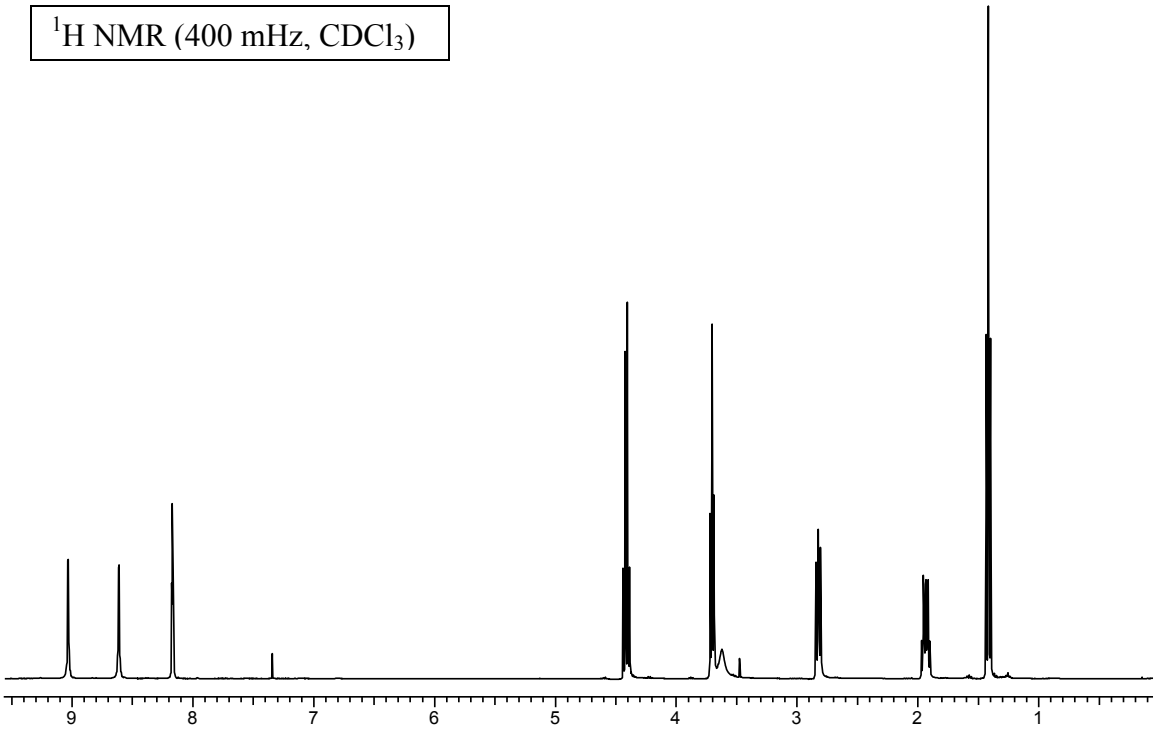
^{13}C NMR (100 mHz, $CDCl_3$)



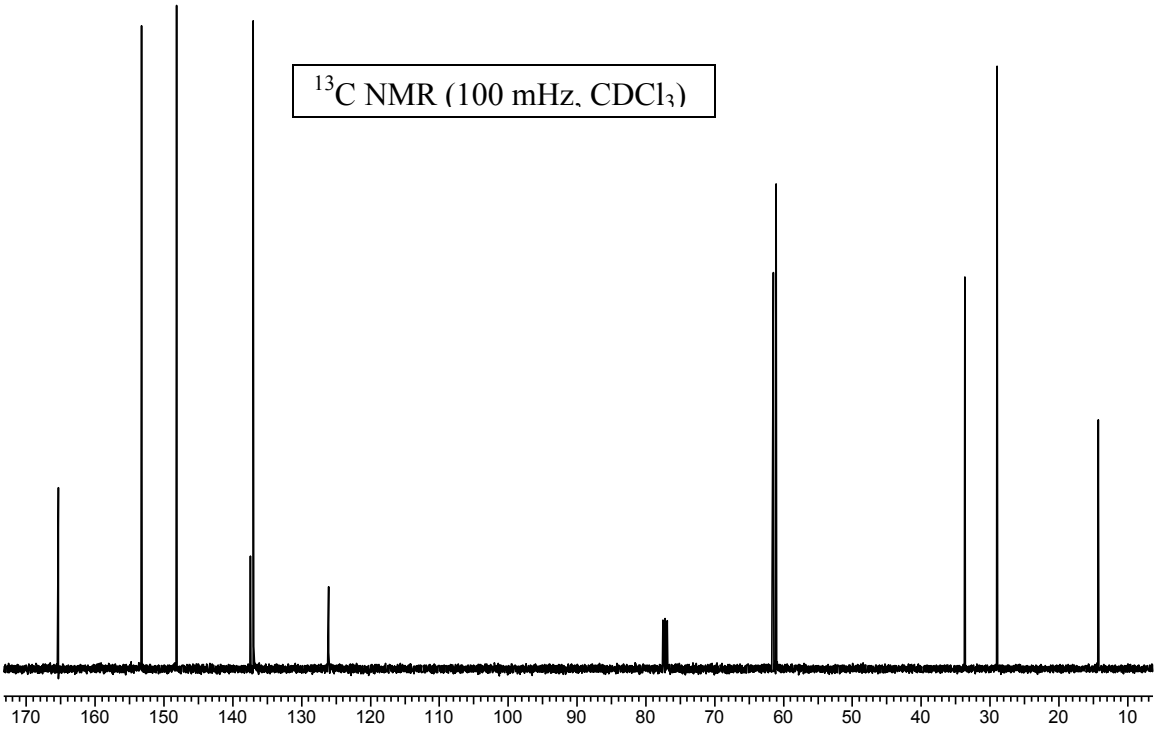


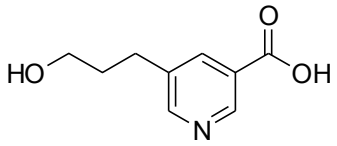
21

^{1}H NMR (400 mHz, $CDCl_3$)



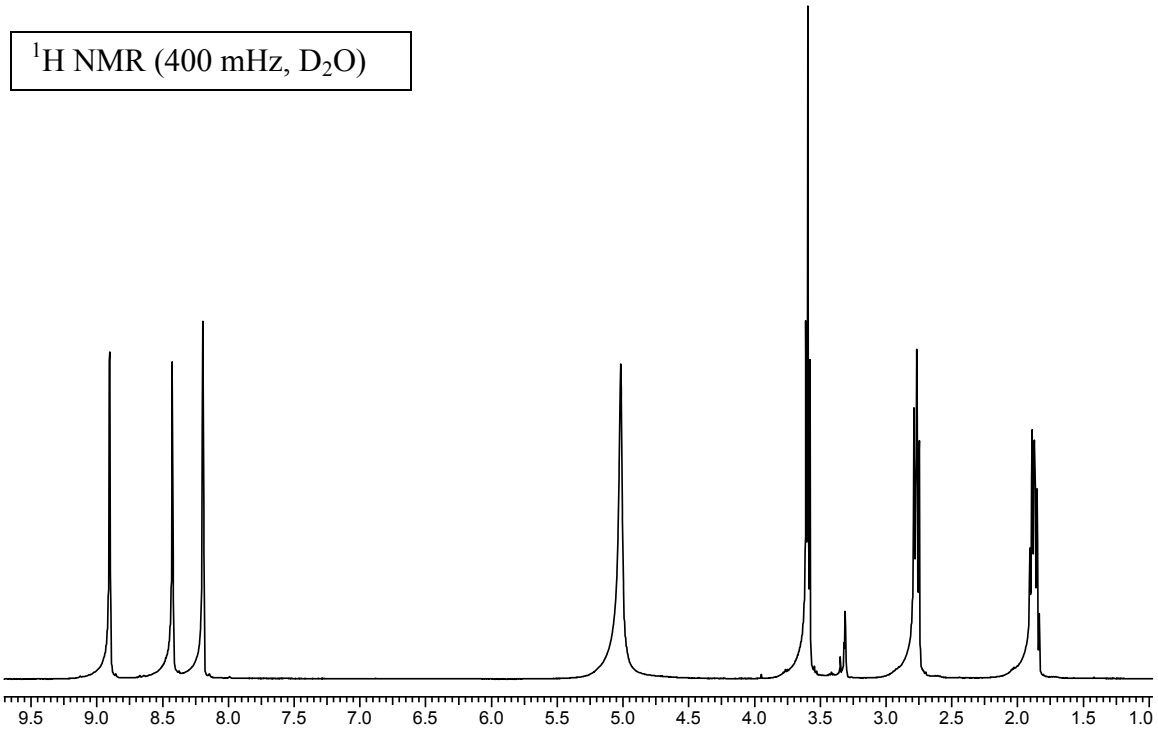
^{13}C NMR (100 mHz, $CDCl_3$)



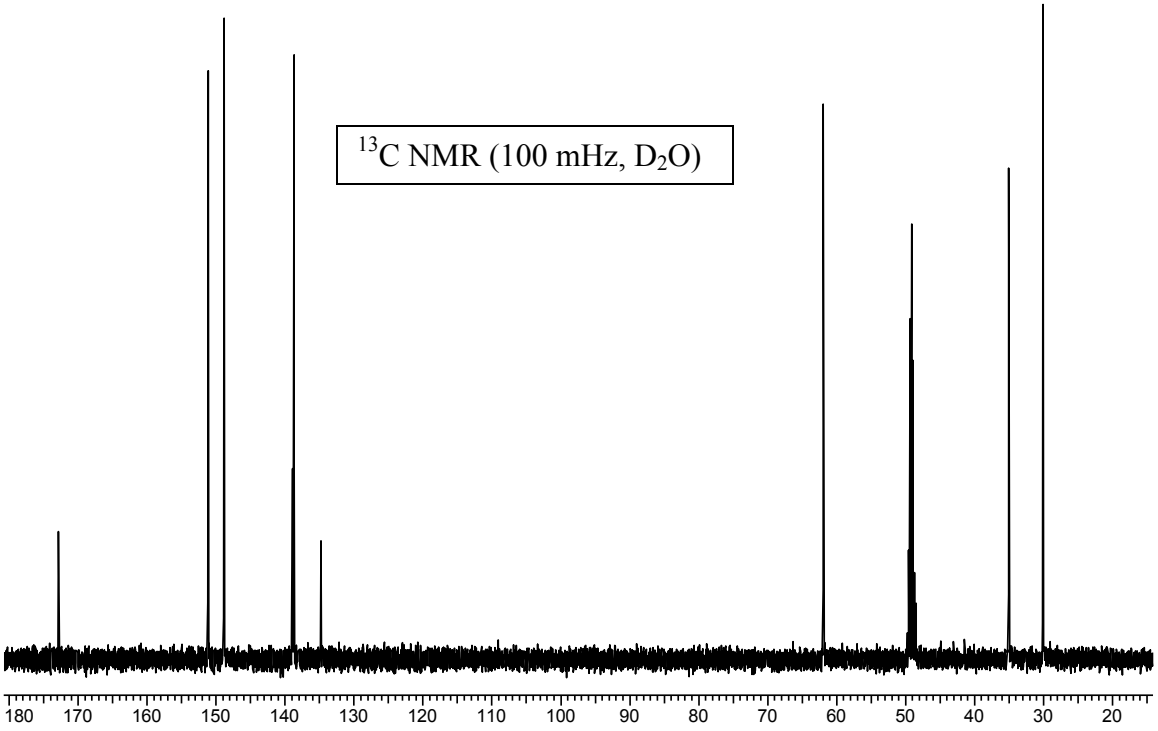


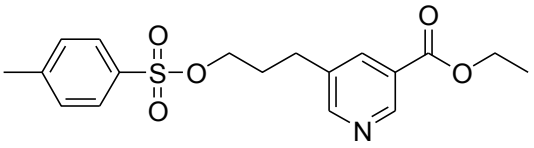
11f

^{1}H NMR (400 mHz, D_2O)



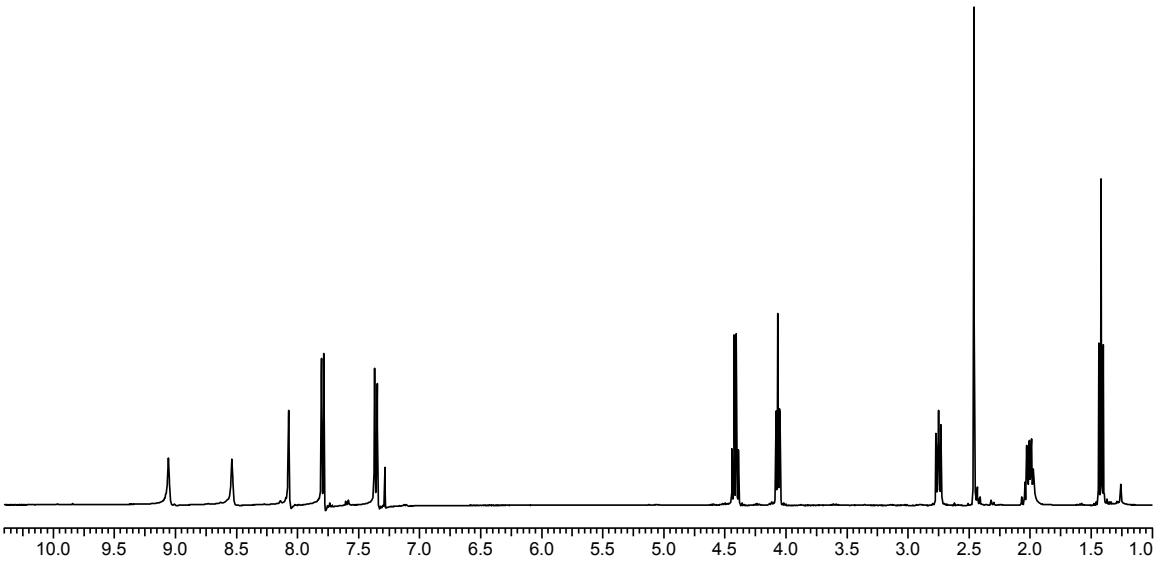
^{13}C NMR (100 mHz, D_2O)



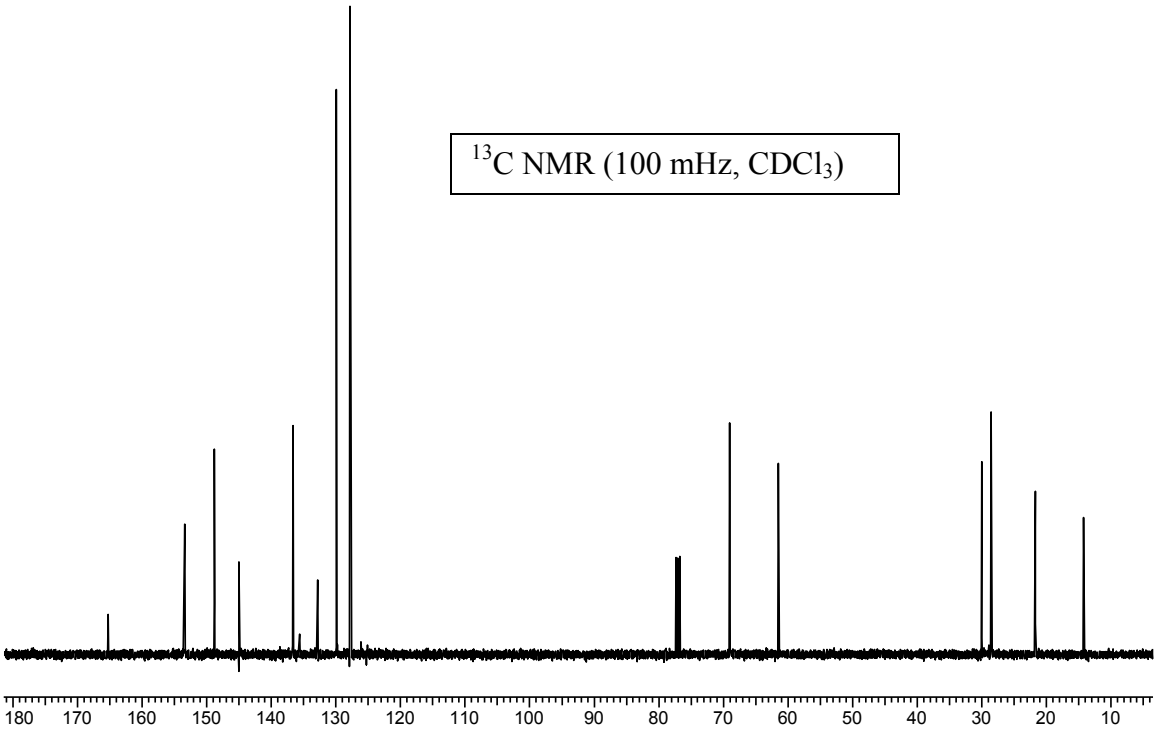


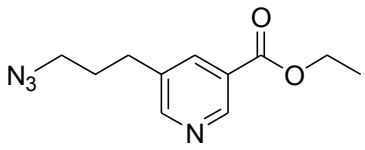
22

^{1}H NMR (400 mHz, $CDCl_3$)



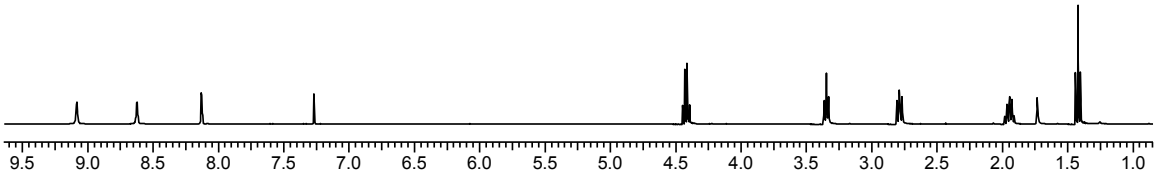
^{13}C NMR (100 mHz, $CDCl_3$)



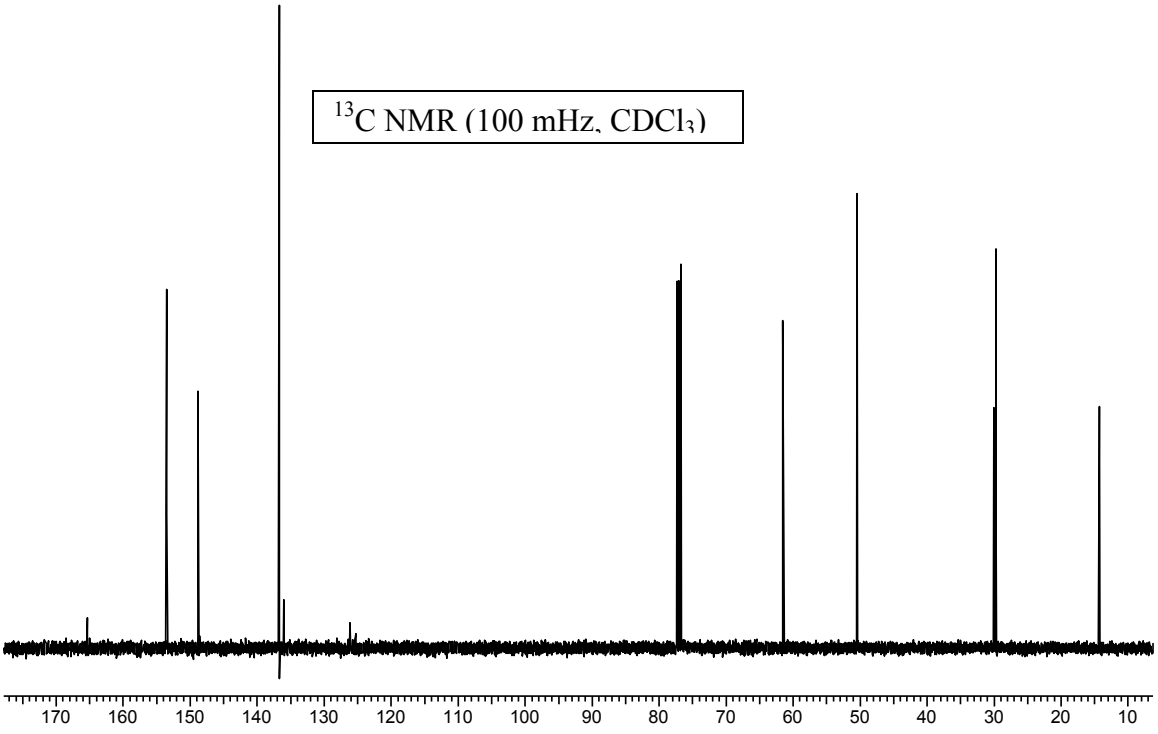


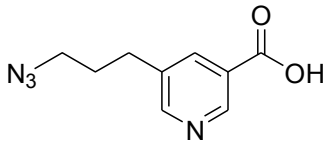
23

^{1}H NMR (400 mHz, $CDCl_3$)



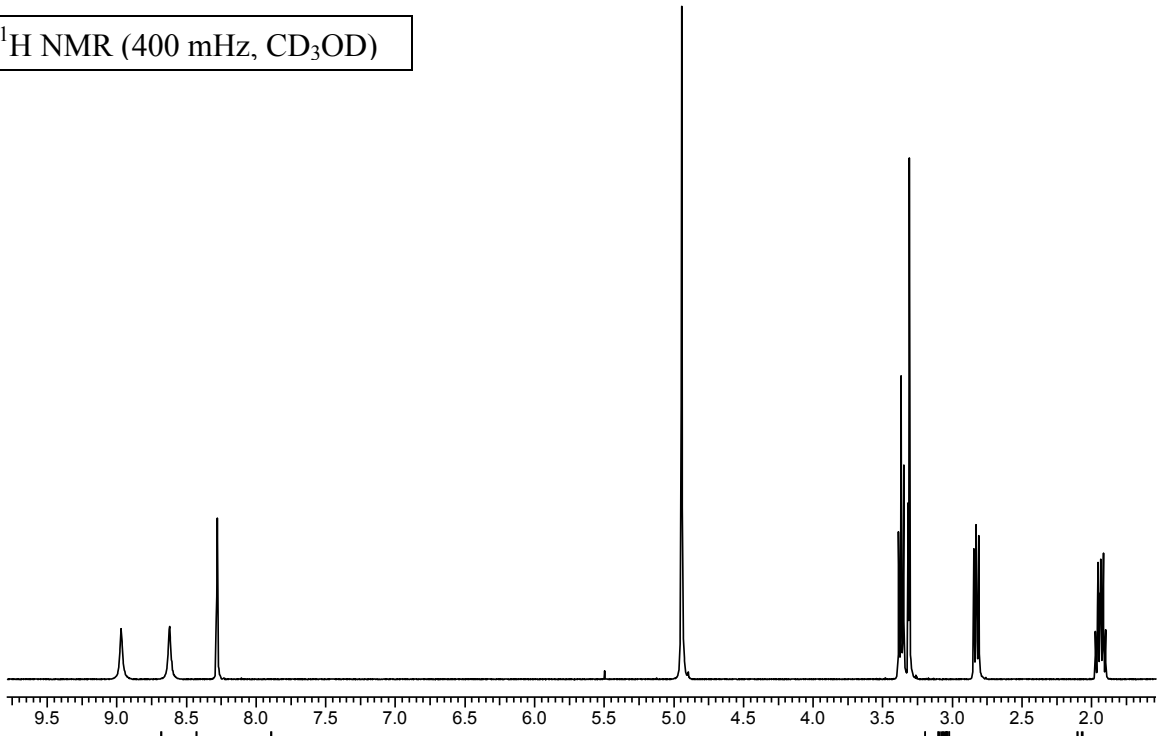
^{13}C NMR (100 mHz, $CDCl_3$)



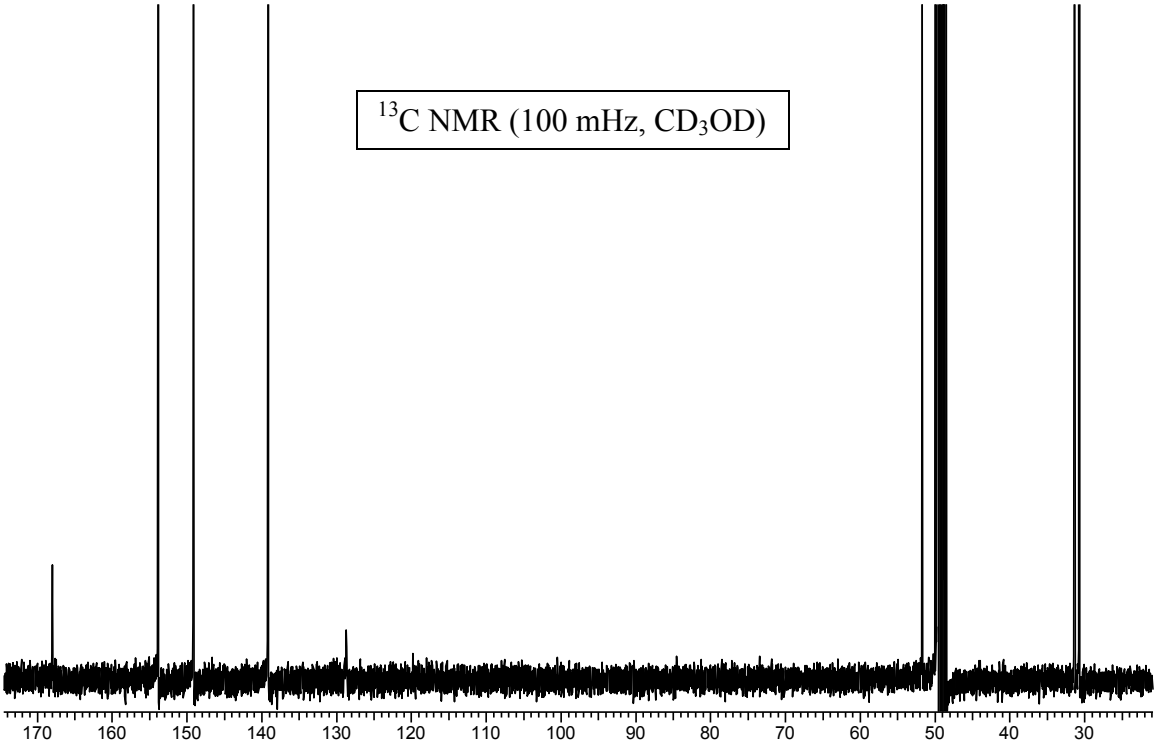


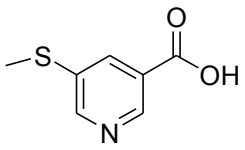
11g

^{1}H NMR (400 mHz, CD_3OD)



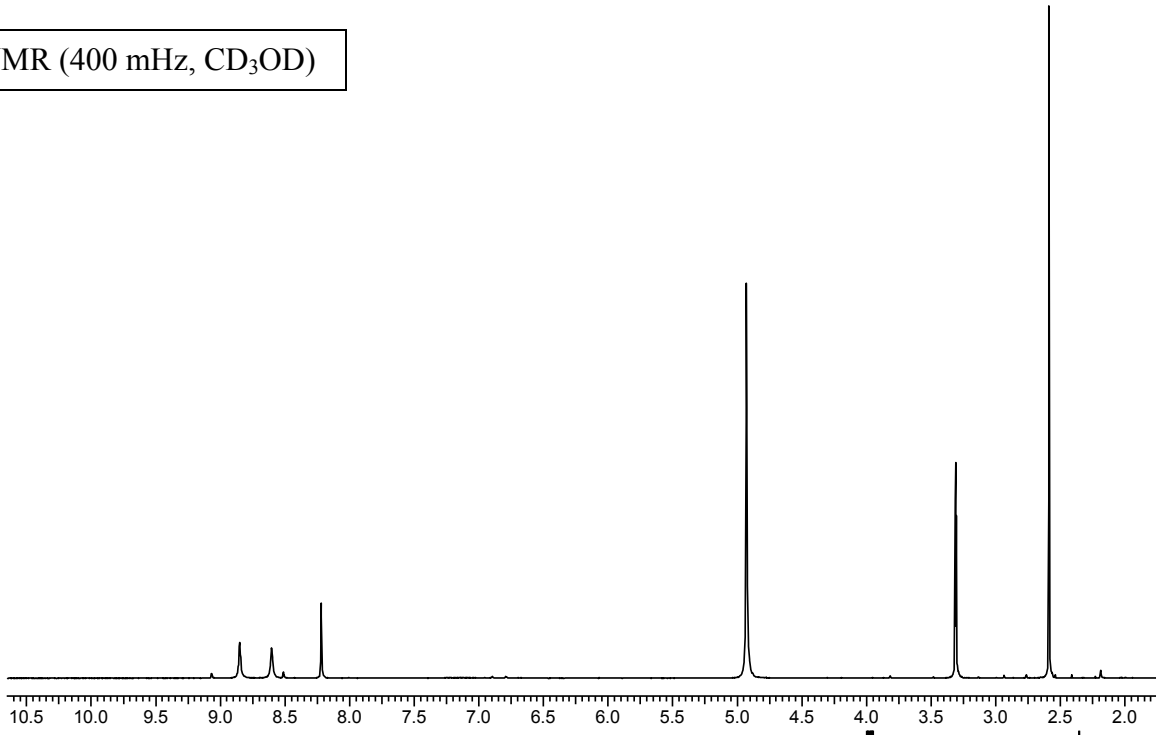
^{13}C NMR (100 mHz, CD_3OD)



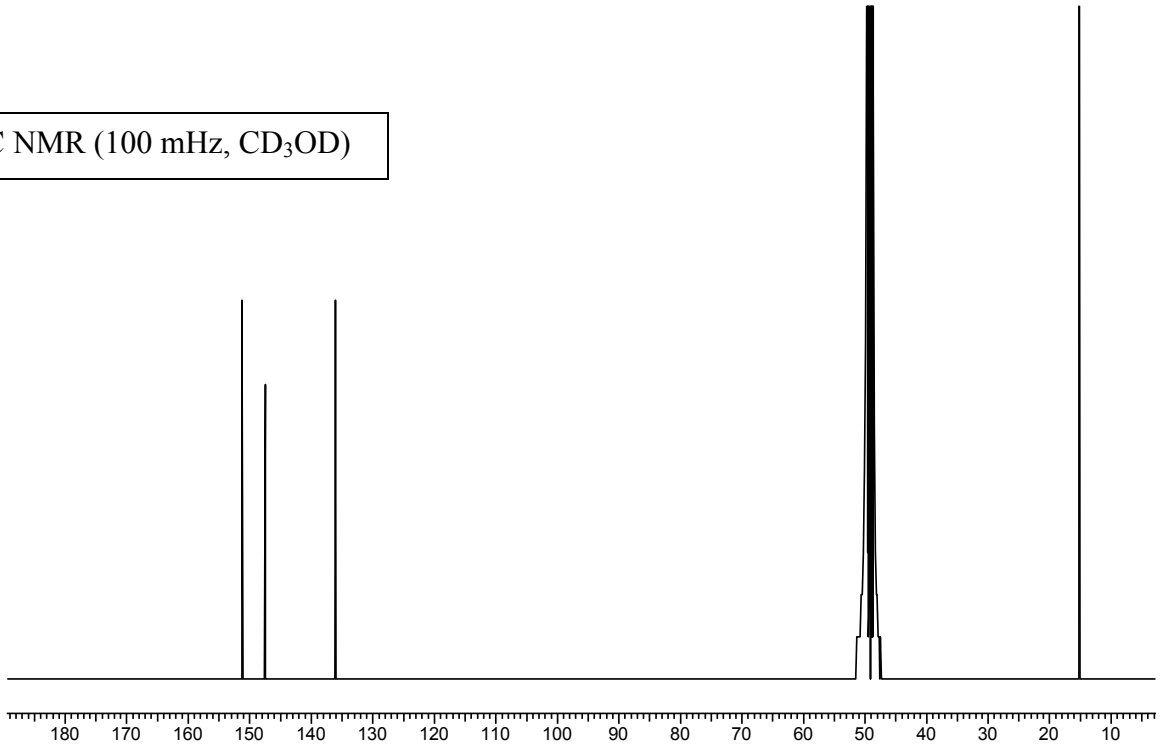


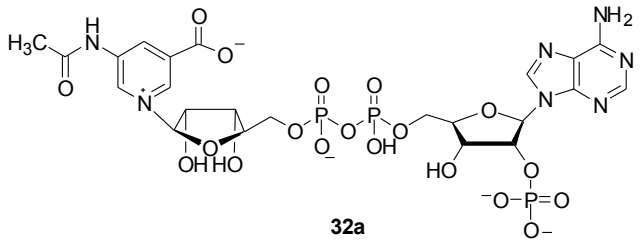
11h

^{1}H NMR (400 mHz, CD_3OD)



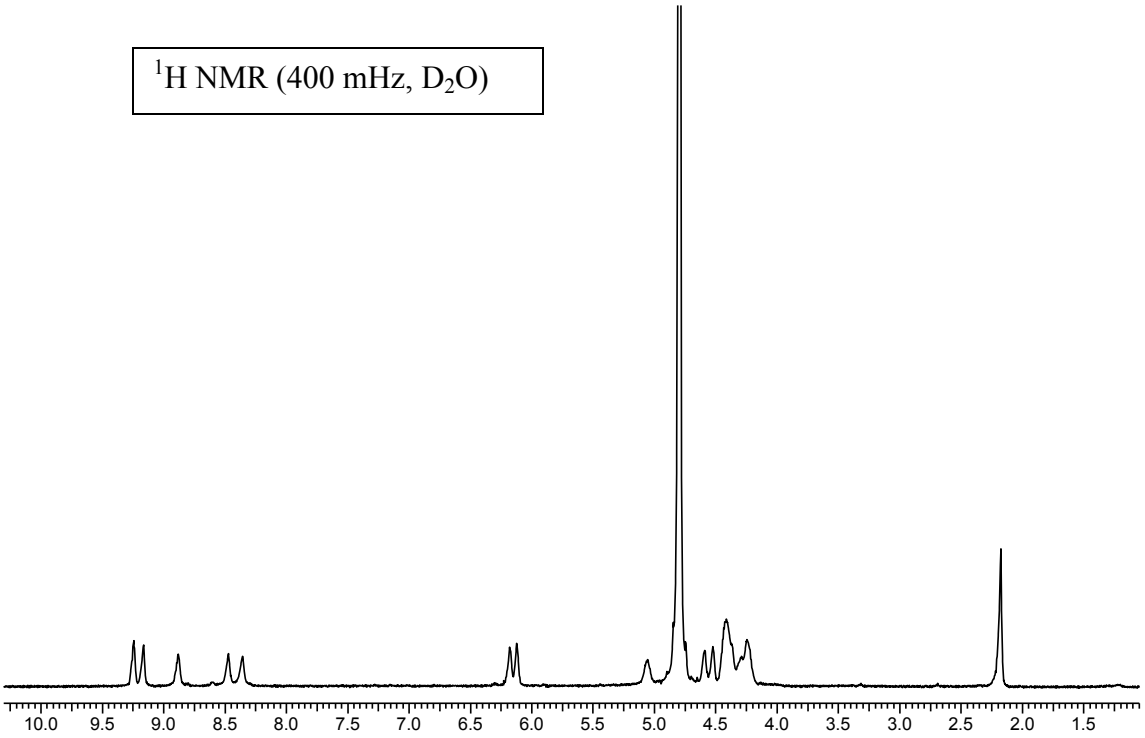
^{13}C NMR (100 mHz, CD_3OD)



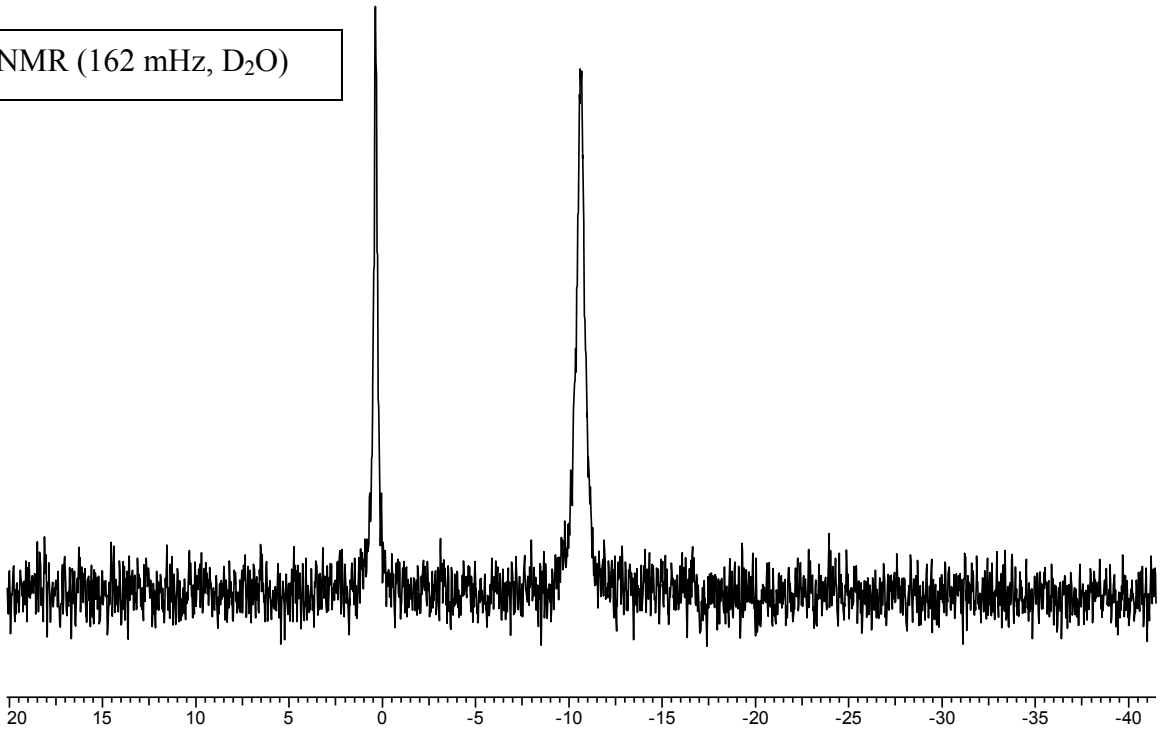


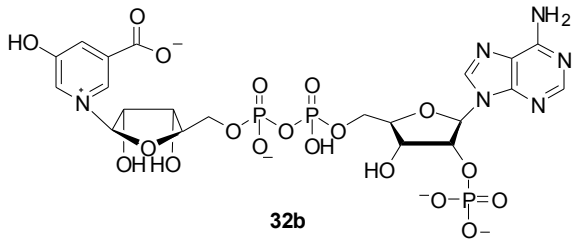
32a

^{1}H NMR (400 mHz, D_2O)



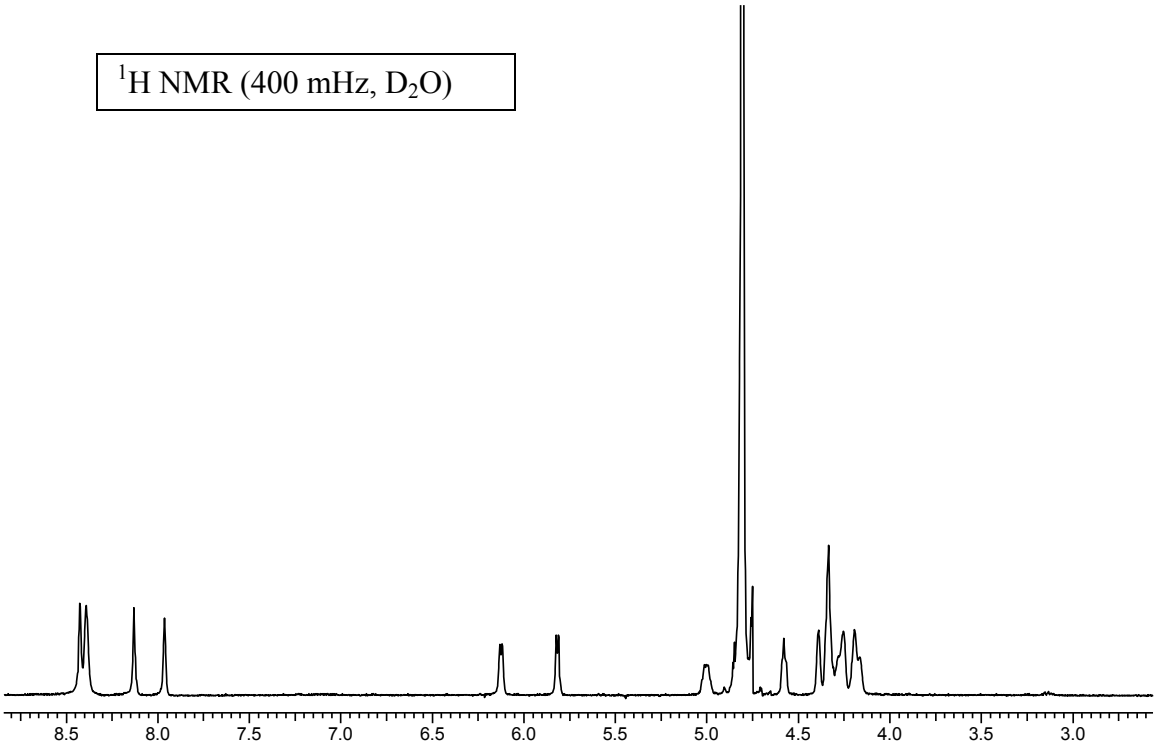
^{31}P NMR (162 mHz, D_2O)



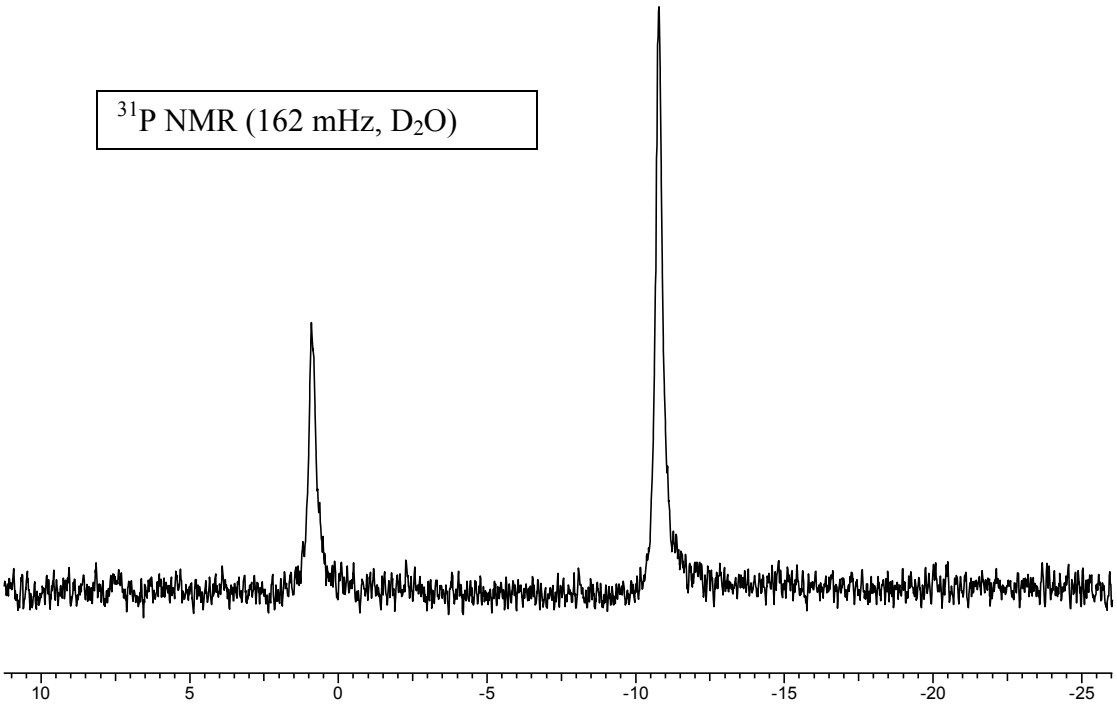


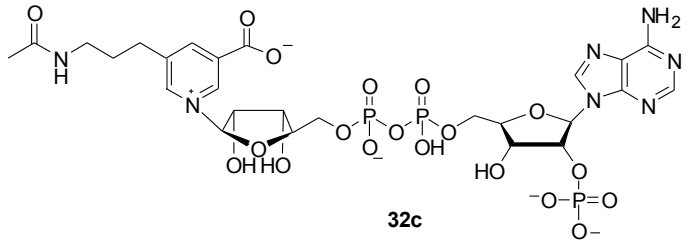
32b

^{1}H NMR (400 mHz, D_2O)



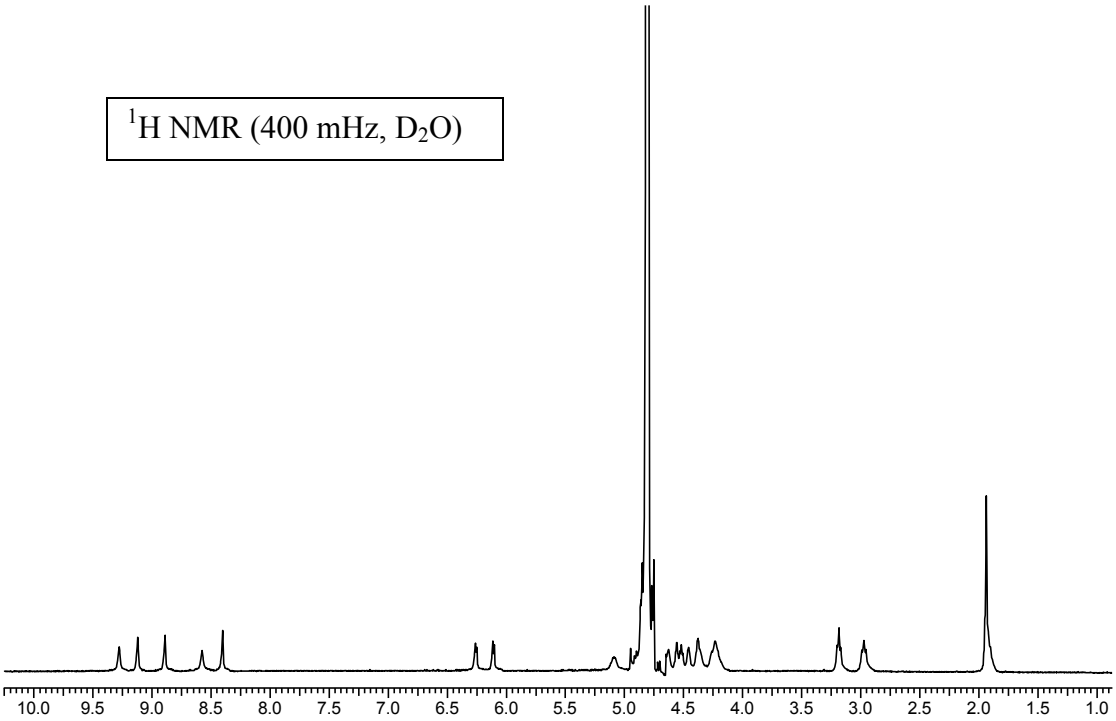
^{31}P NMR (162 mHz, D_2O)



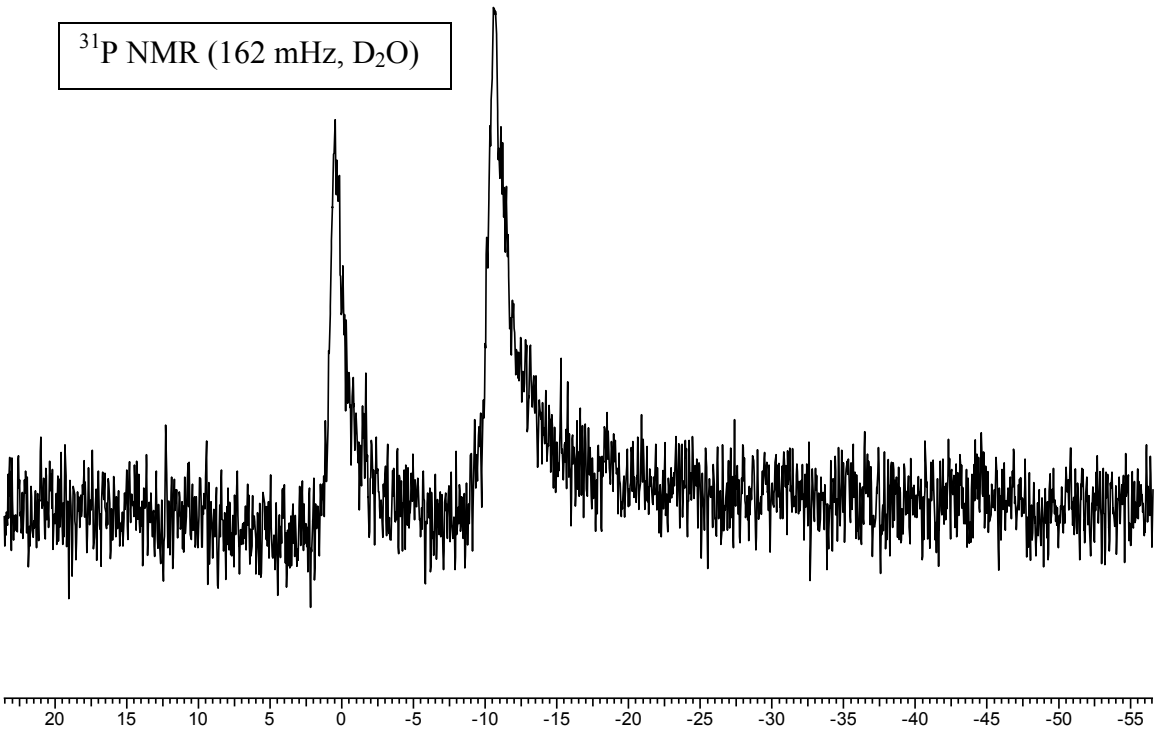


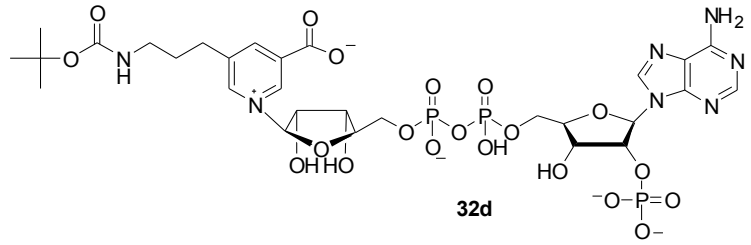
32c

^{1}H NMR (400 mHz, D_2O)



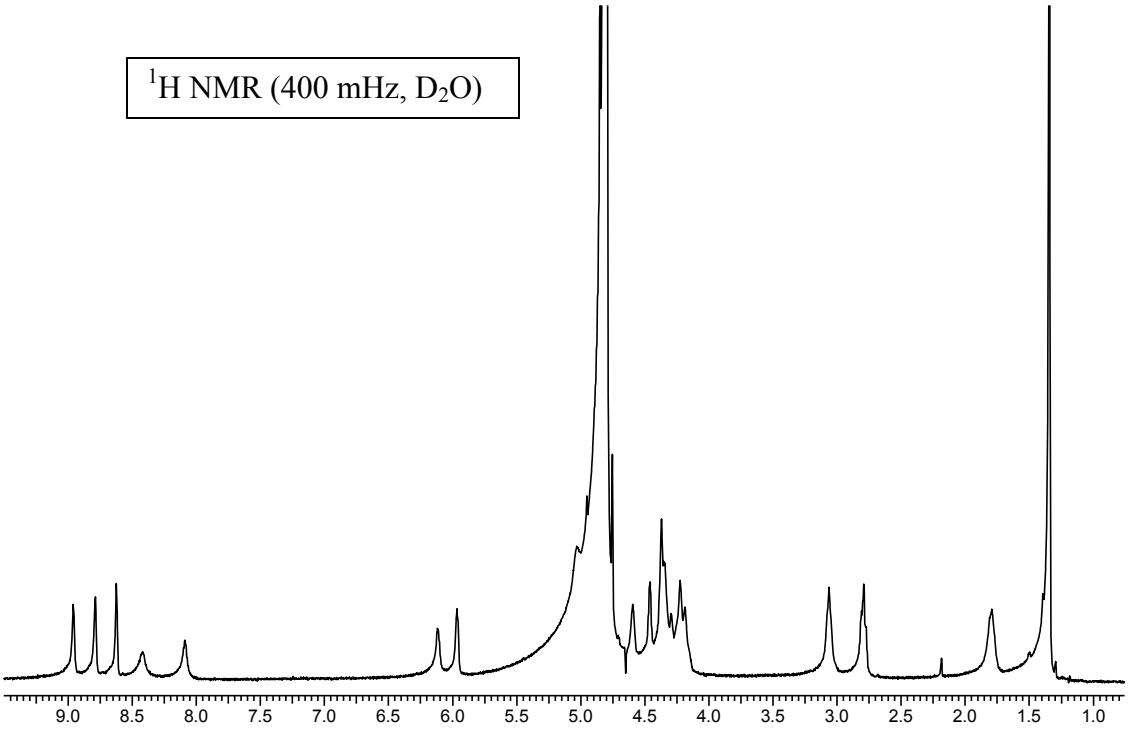
^{31}P NMR (162 mHz, D_2O)



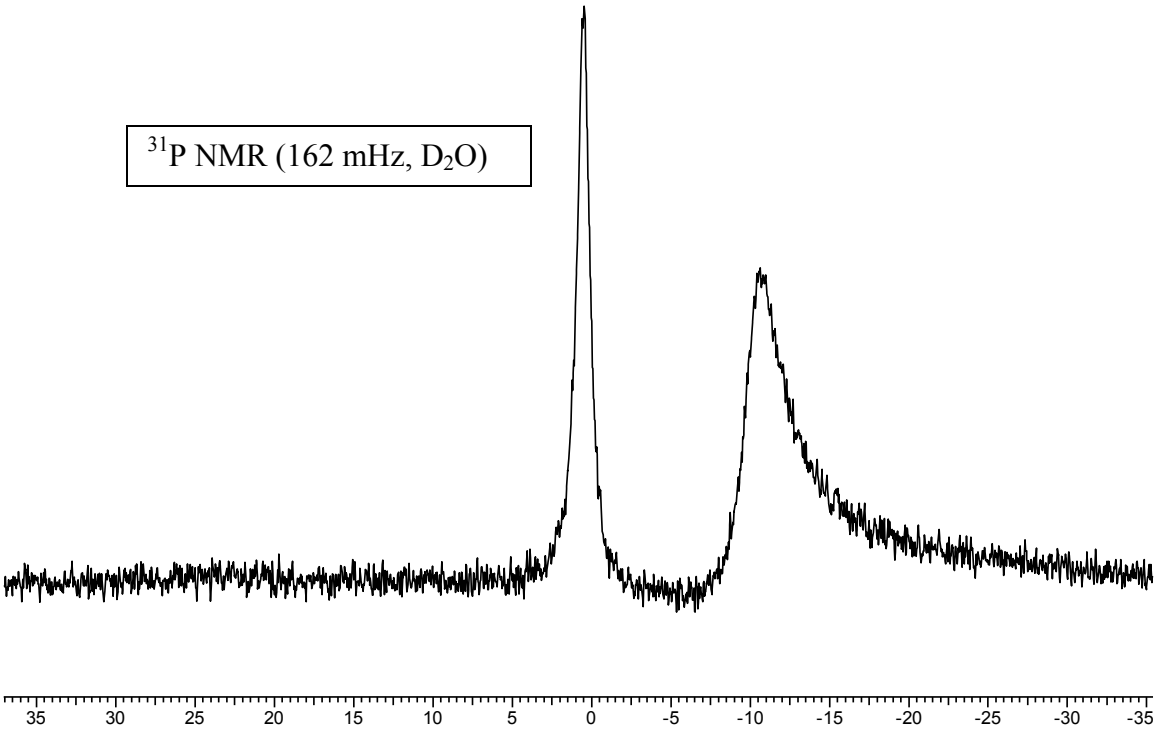


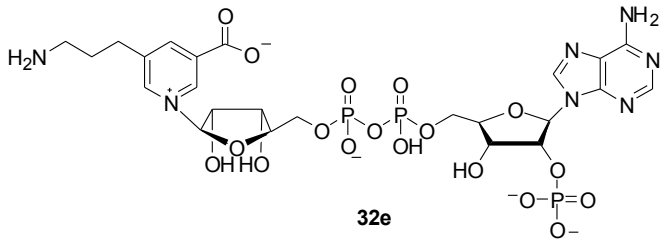
32d

^{1}H NMR (400 mHz, D_2O)



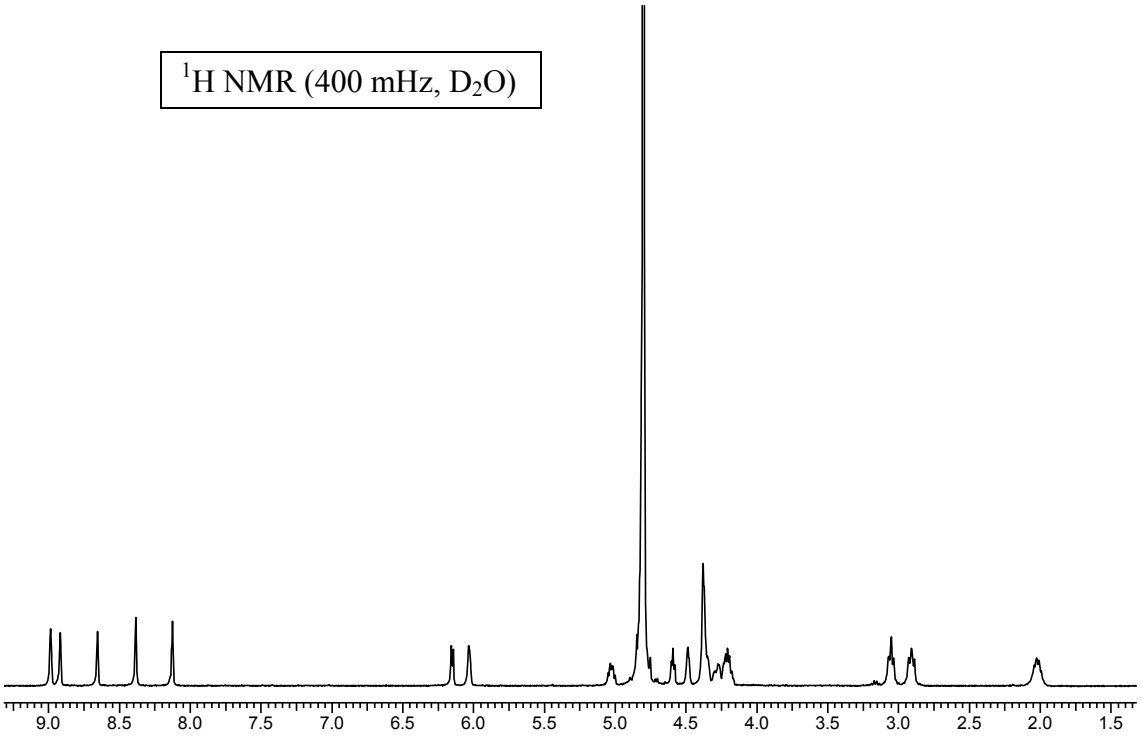
^{31}P NMR (162 mHz, D_2O)



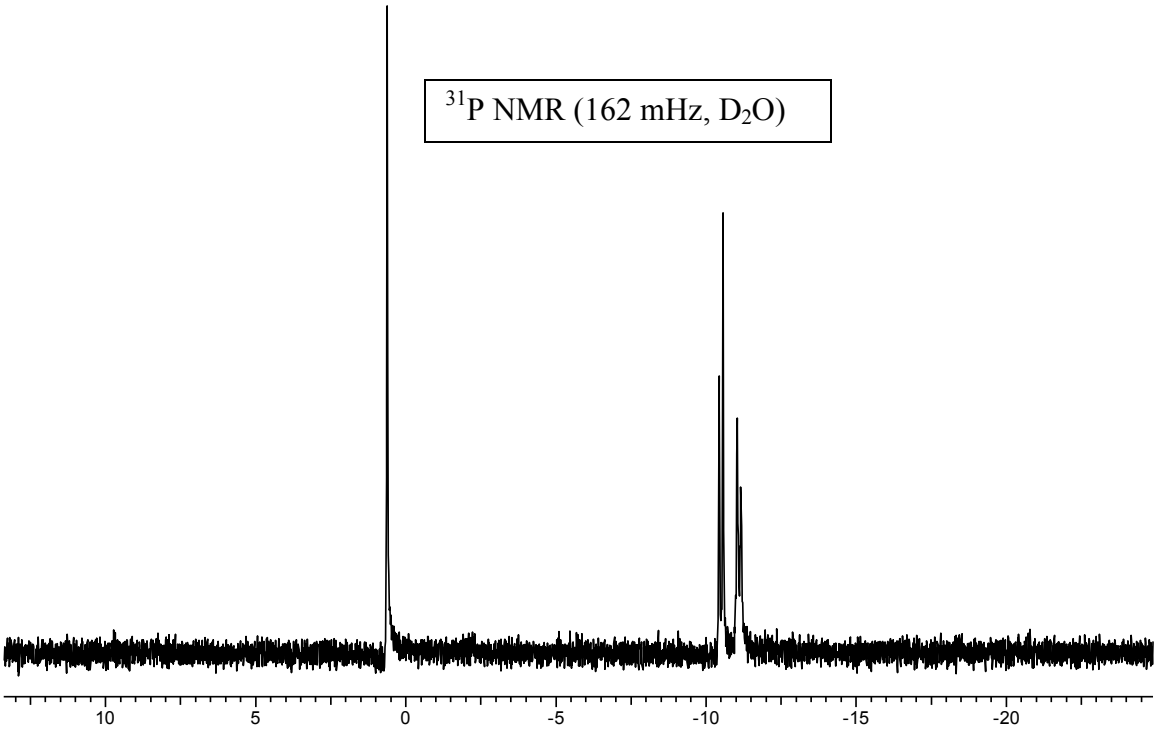


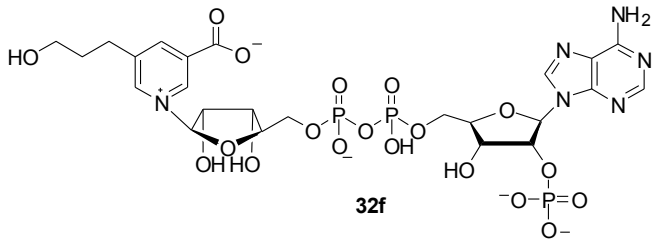
32e

^{1}H NMR (400 mHz, D_2O)



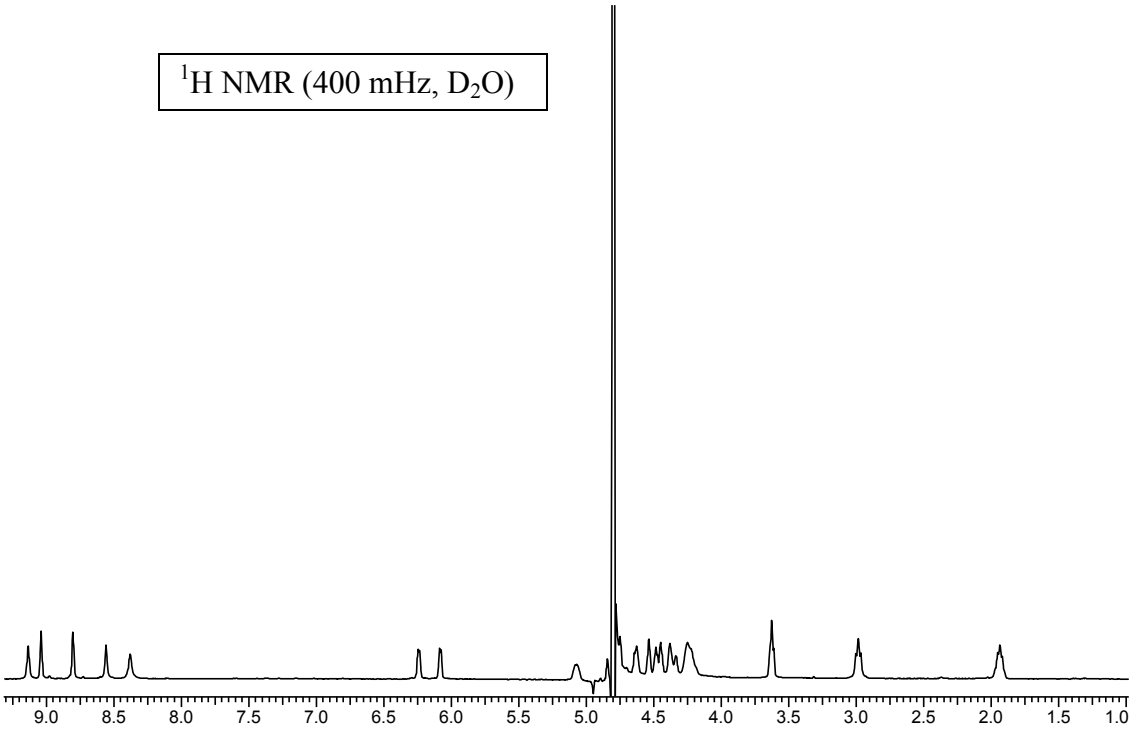
^{31}P NMR (162 mHz, D_2O)



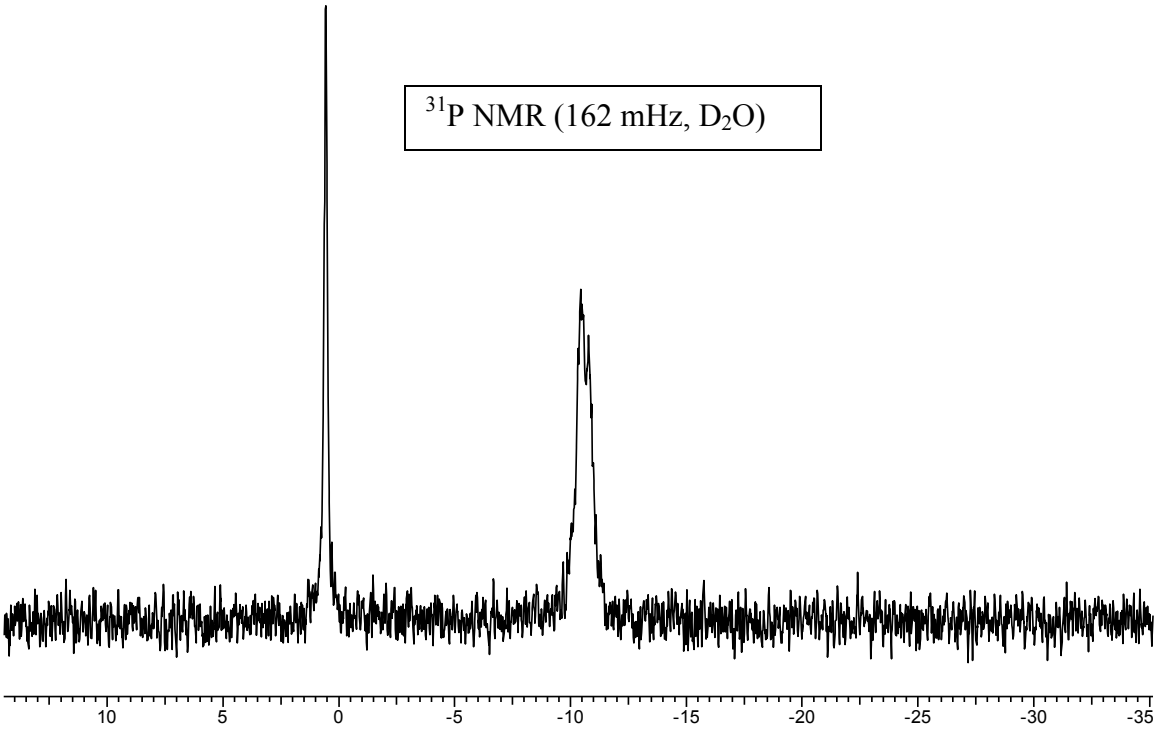


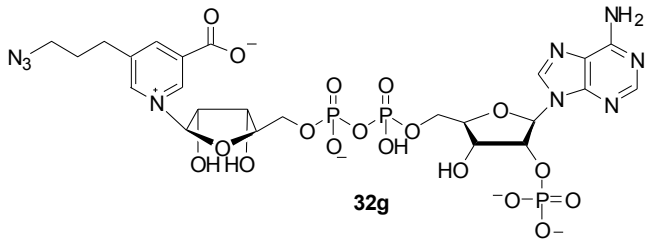
32f

^{1}H NMR (400 mHz, D_2O)



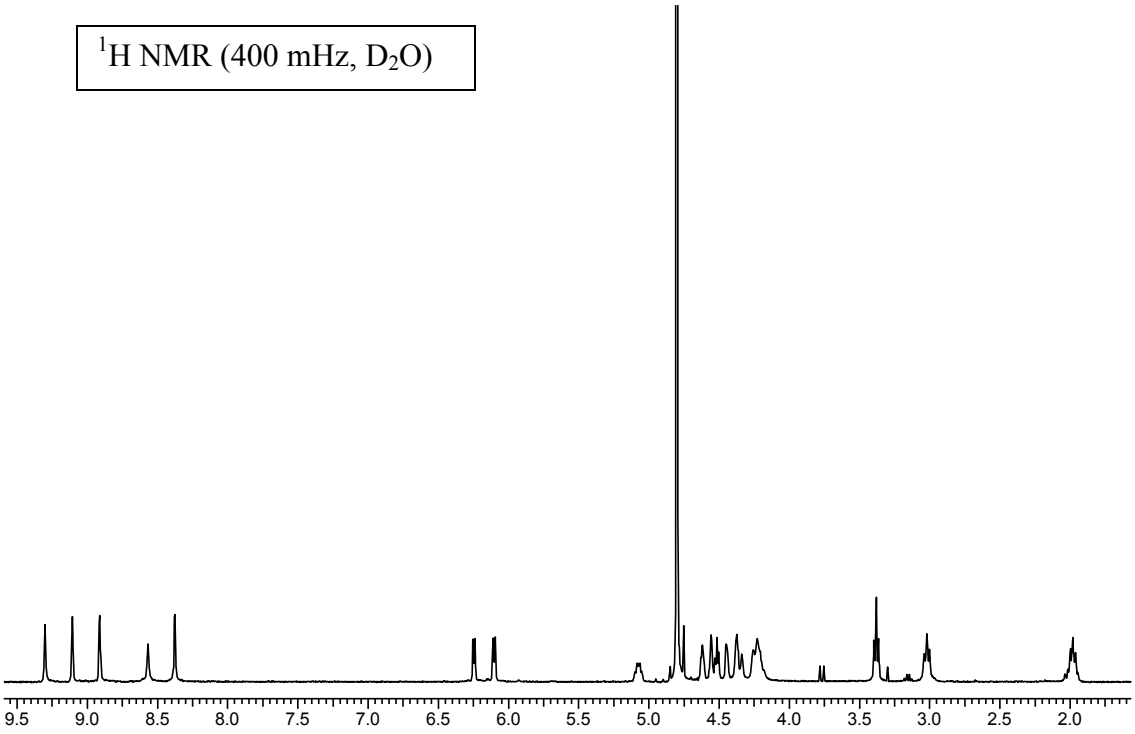
^{31}P NMR (162 mHz, D_2O)



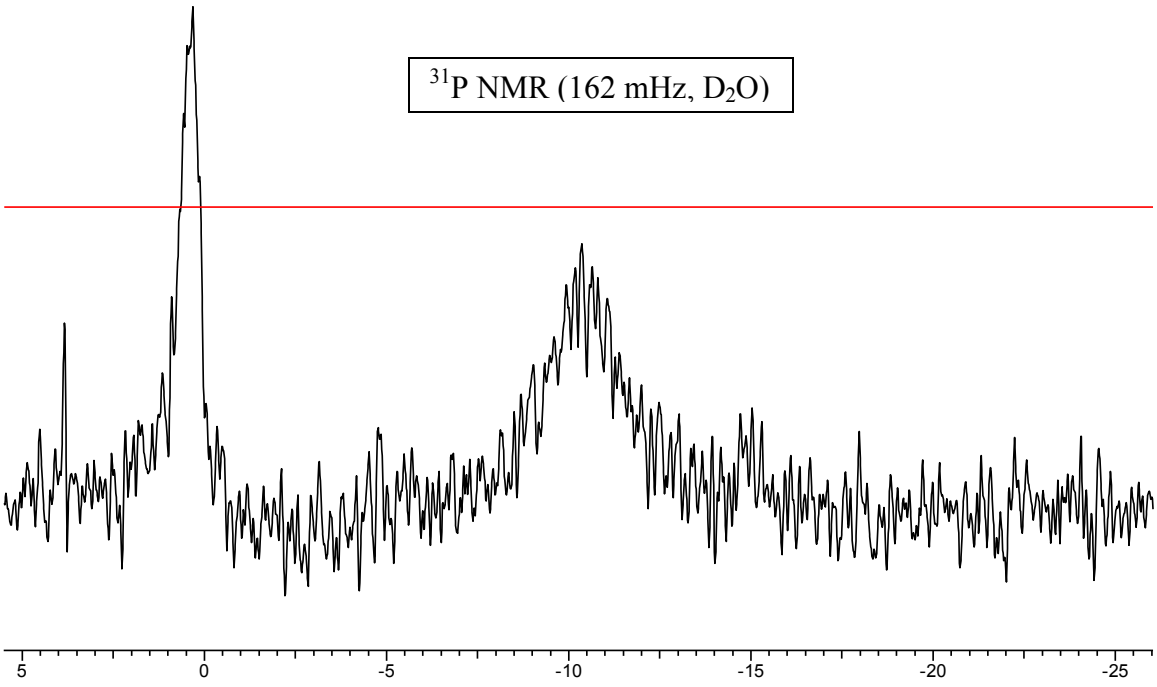


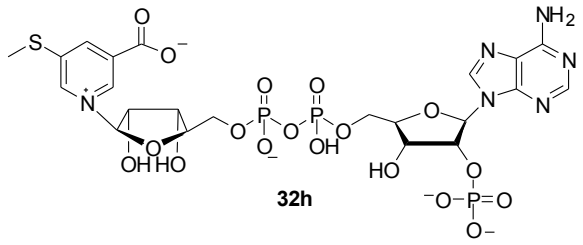
32g

^{1}H NMR (400 mHz, D_2O)



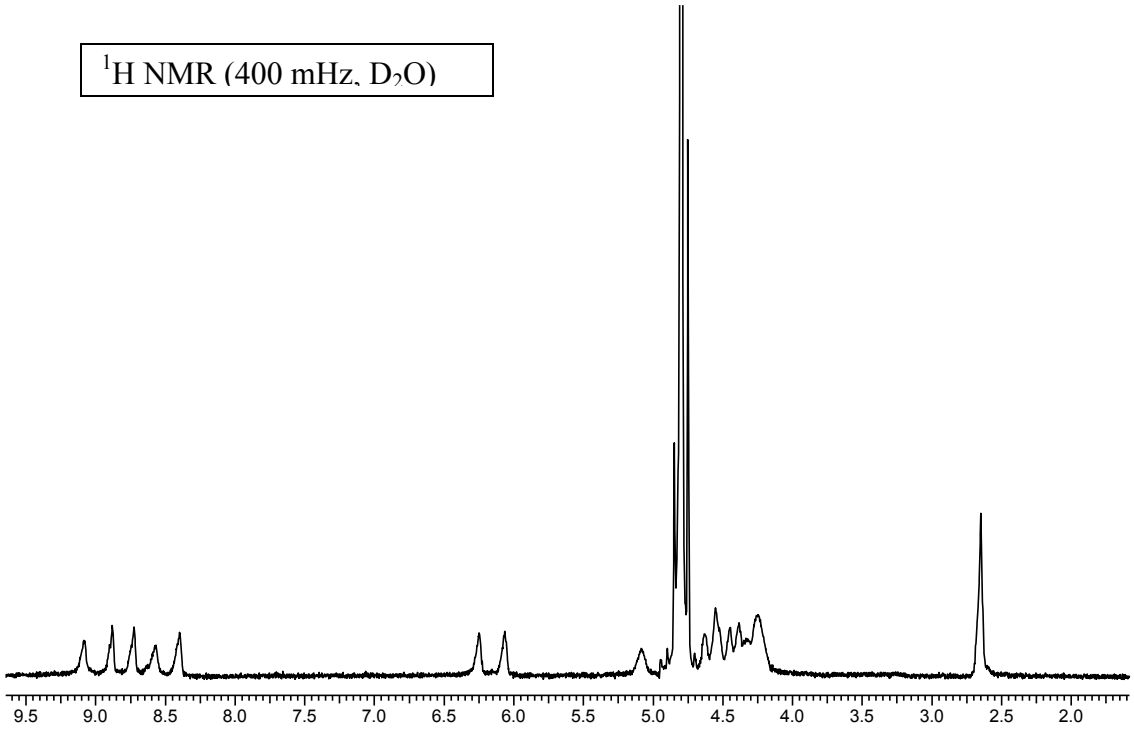
^{31}P NMR (162 mHz, D_2O)



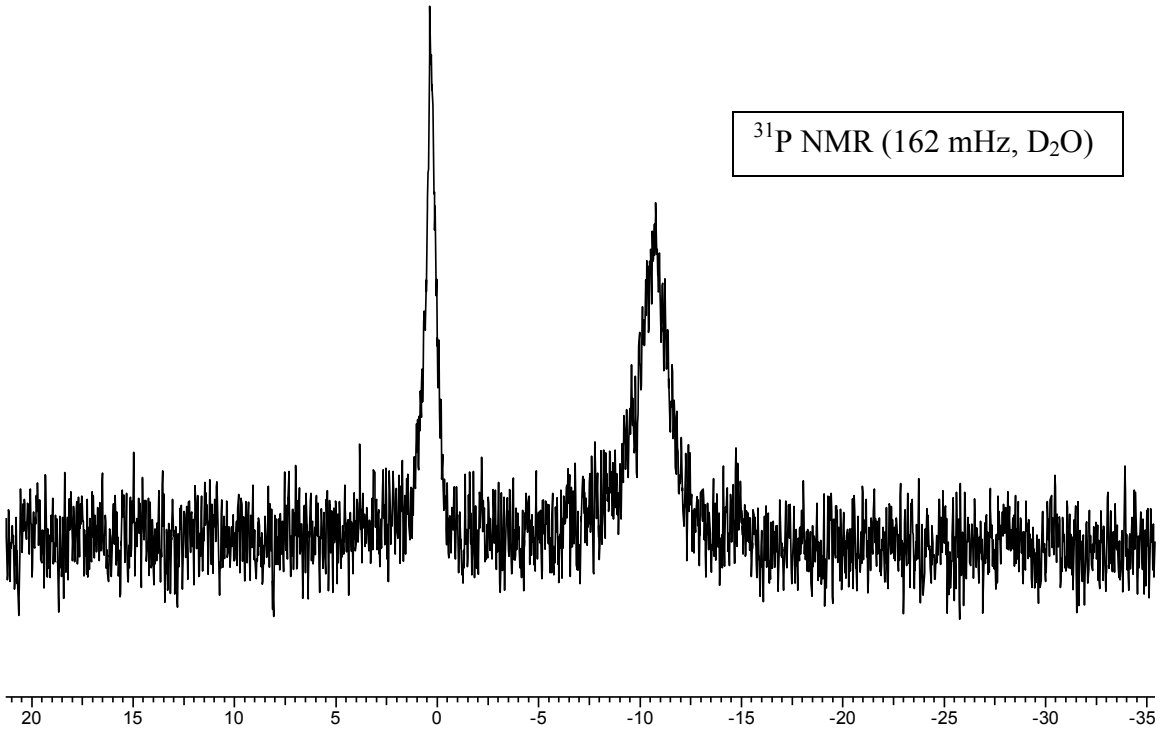


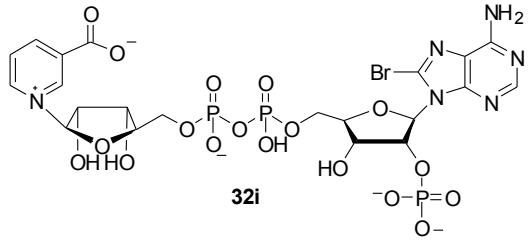
32h

^{1}H NMR (400 mHz, D_2O)



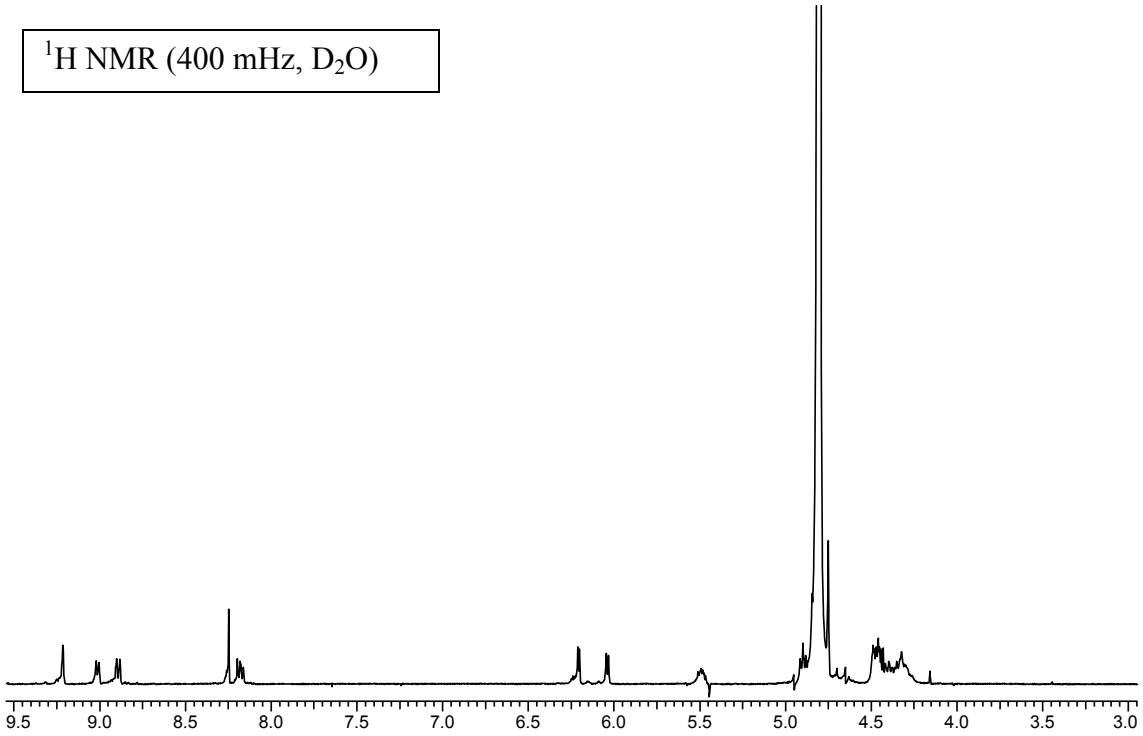
^{31}P NMR (162 mHz, D_2O)



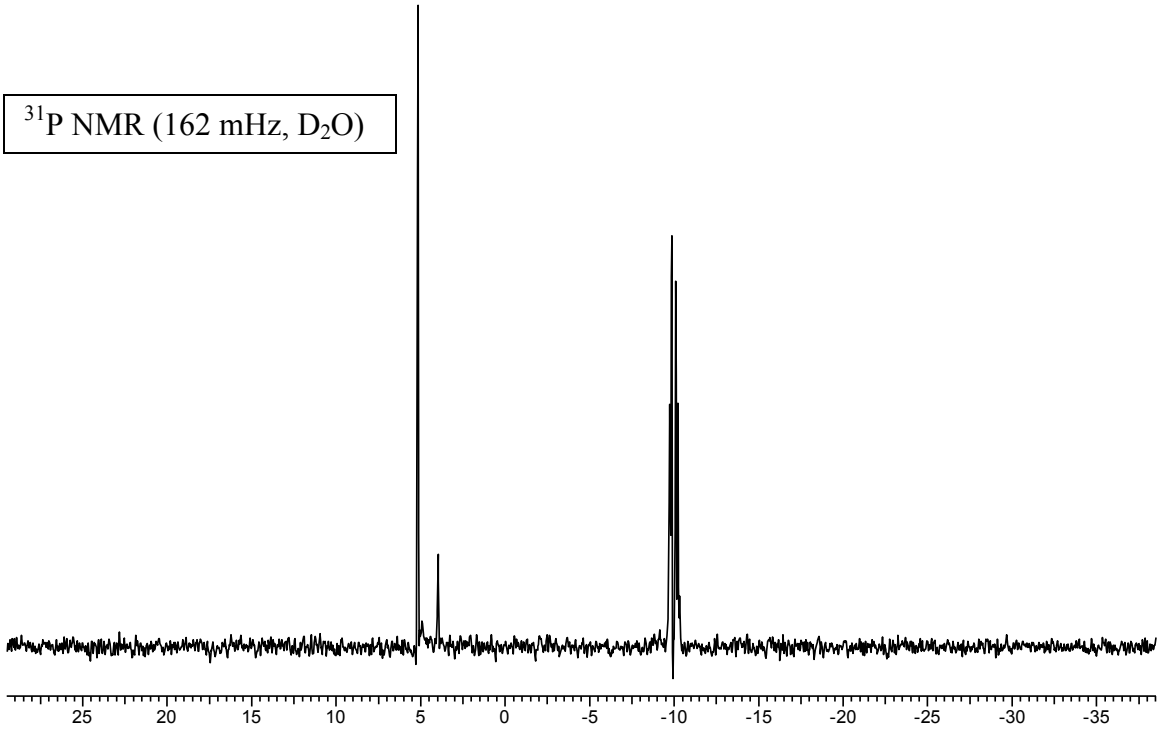


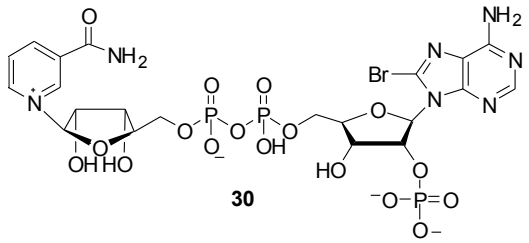
32i

^{1}H NMR (400 mHz, D_2O)



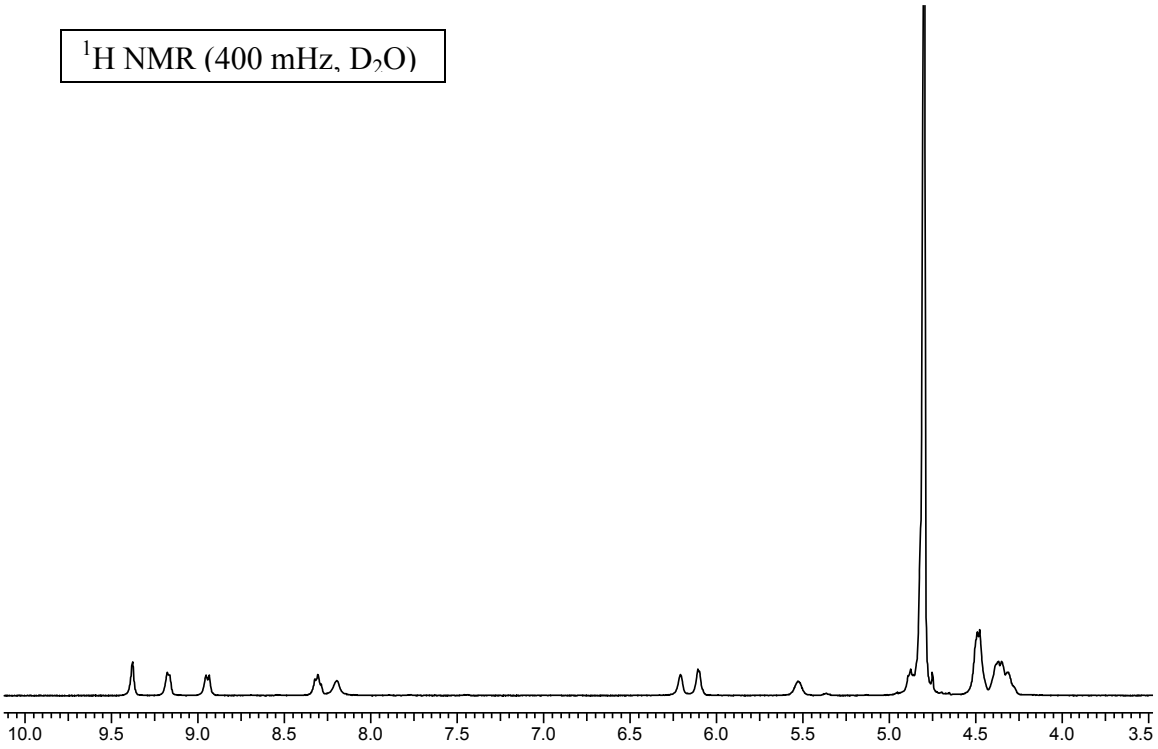
^{31}P NMR (162 mHz, D_2O)

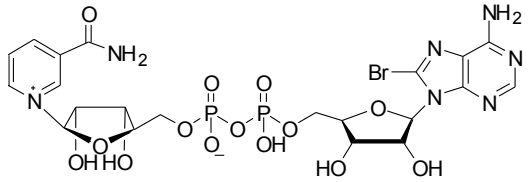




30

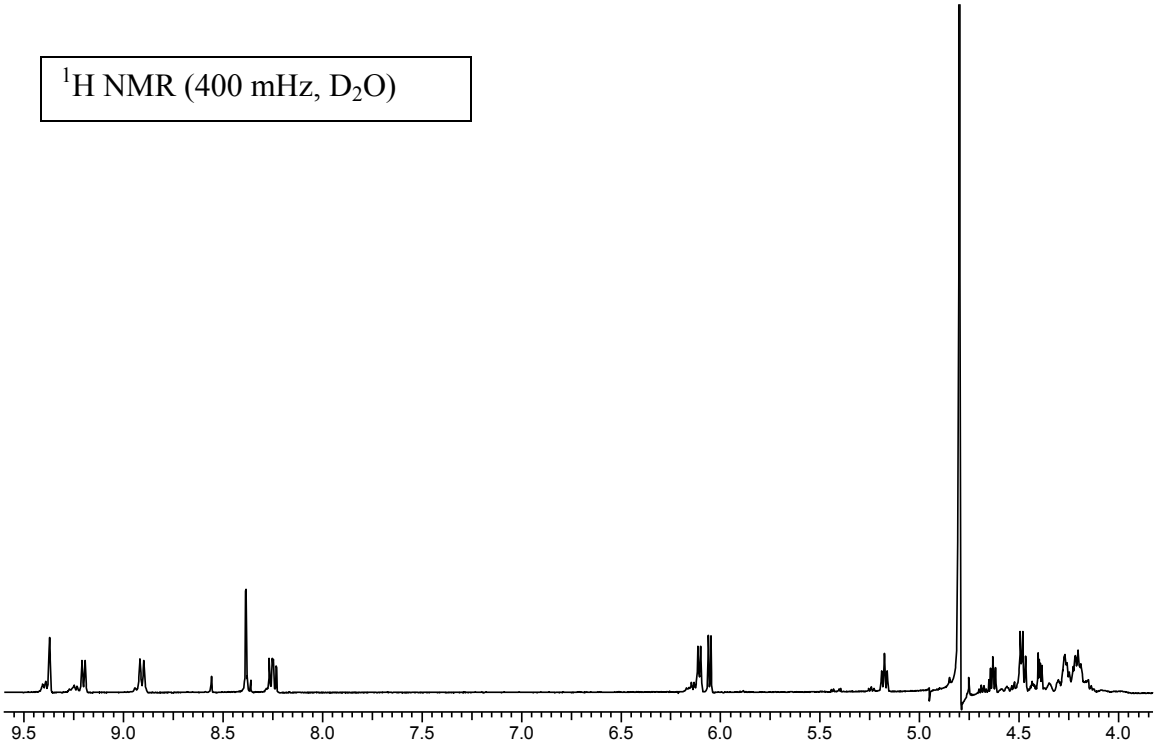
^{1}H NMR (400 mHz, D_2O)



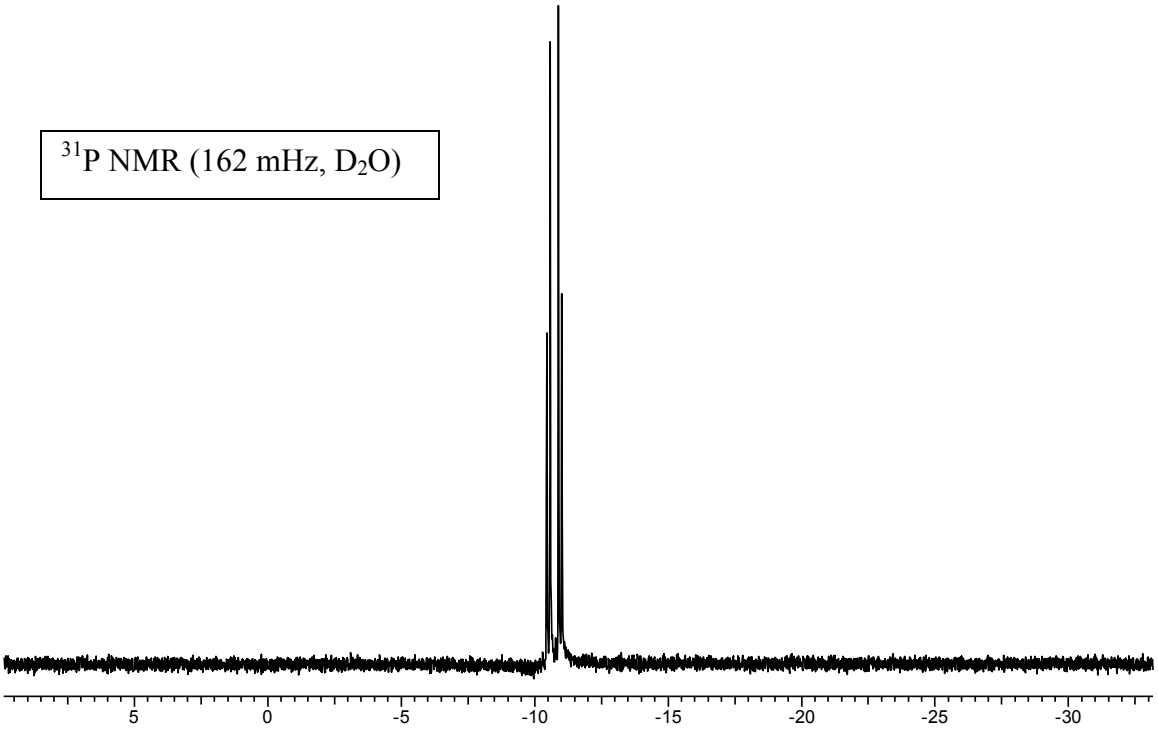


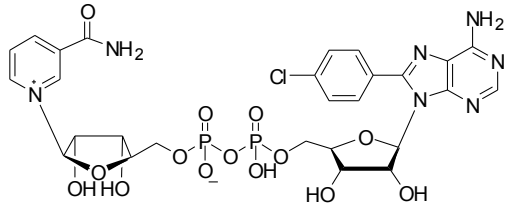
29

^{1}H NMR (400 mHz, D_2O)



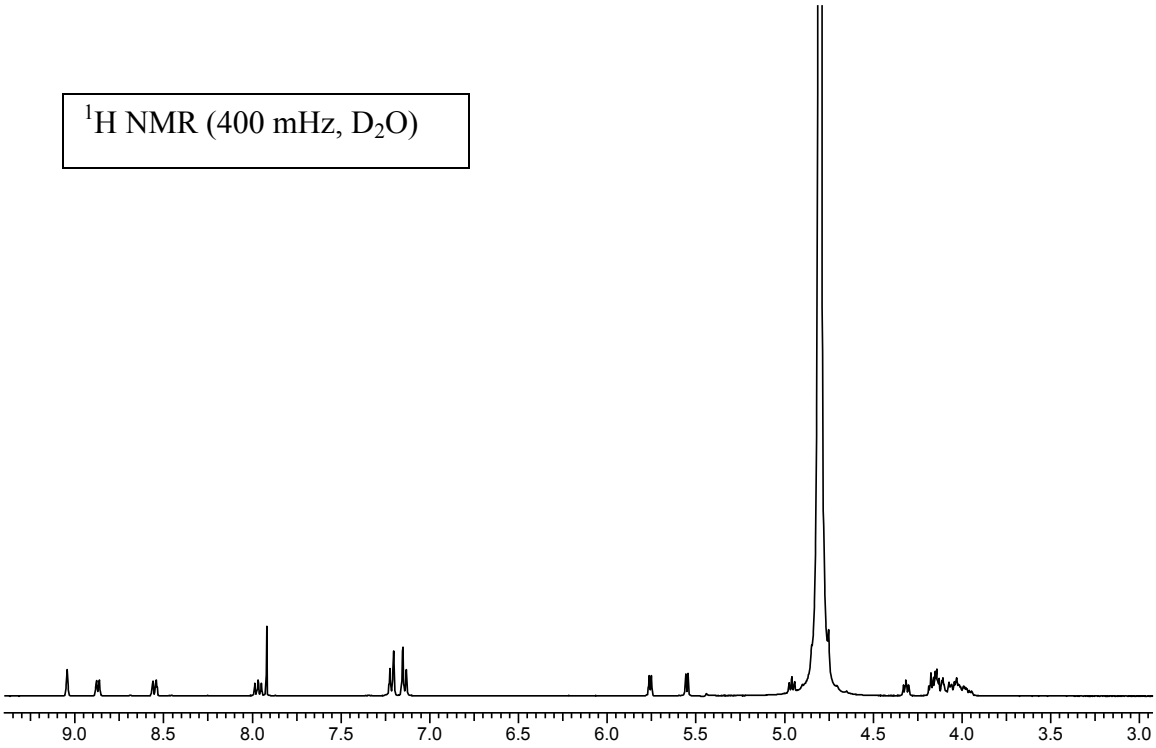
^{31}P NMR (162 mHz, D_2O)





31

^{1}H NMR (400 mHz, D_2O)



^{31}P NMR (162 mHz, D_2O)