DESIGN AND SYNTHESIS OF MODULAR REAGENTS FOR CHEMICAL BIOLOGY

by

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Bereket-Ab Mehari, titled Design and Synthesis of Modular Reagents for Chemical Biology and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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DEDICATION

This dissertation is dedicated to my family.

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List of Abbreviations

AIBN	Azobisisobutyronitrile
BARAC	Biarylazacyclooctynone
BH_3	Borane
BnN_3	Benzyl azide
CD	Cyclodextrin
Ch_2Cl_2	Dichloromethane
CuAAC	Copper-catalyzed azide-alkyne cycloaddition
D2O	Deuterium oxide
DABCO	1,4-diazabicyclo[2.2.2]octane
DCC	N,N'-dicyclohexylcarbodiimide
DIC	N,N'-diisopropylcarbodiimide
DIFO	Difluorinatedcyclooctyne
DIMAC	Dimethoxyazacyclooctyne
EDC	(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
Et_2O	Diethylether
GFP	Green fluorescent protein
FRET	Fluorescence resonance energy transfer
HCl	Hydrochloric acid

ICT	Intramolecular charge transfer
Ka	Association constant
t-BuOK	Potassium tert-butoxide
$Na_2S_2O_4$	Sodium dithionate
NaH	Sodium hydride
NaN_3	Sodium azide
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
n-BuLi	*n*-Butyllithium
$NH_2OH{\cdot}HCl$	Hydroxylammonium chloride
NHC	N-heterocyclic carbene
NMR	Nuclear magnetic resonance
$Pd(OAc)_2$	Palladium(II) acetate
PEG	Polyethylene glycol
$Rh_2(AcO)_4$	Rhodium(II) acetate
RT	Room tempreature
$[Ru(bpy)_3]Cl_2$	Tris(bipyridine)ruthenium(II) chloride
$SnCl_2{\cdot}5H_2O$	Tin(IV) chloride pentahydrate
SPAAC	Strain promoted alkyne-azide cycloaddition
THF	Tetrahydrofuran
TPPTS	3,3′,3″-Phosphanetriyltris(benzenesulfonic acid) trisodium salt

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Abstract

Bioconjugation strategies for chemical modification of biomolecules play an important role in gaining greater understanding of biological processes. A number of chemical reactions have been developed for labeling biomolecules in biologically relevant conditions. In an effort to expand the chemical tools available for biological studies, the Jewett group is working towards developing new and improving current bioconjugation strategies to address questions in chemical biology.

The traceless Bertozzi-Staudinger reaction is one of the most selective bioconjugation reactions. However, its utility is limited due to poor solubility in aqueous conditions and challenges in the synthesis of functionalized variations of the reagent. A modular one-pot synthetic strategy has been developed. It was also demonstrated that this method can be used to introduce aldehyde and azide functionalities as chemical handles for modification of these reagents.

In addition, the design and synthesis of a traceless Bertozzi-Staudinger reagent that has been functionalized with a triazabutadiene probe is described. The orthogonal nature of the traceless Bertozzi-Staudinger and triazabutadiene moieties was established. The utility of the bifunctionalized reagent as a tool for attaching a cargo onto the traceless Bertozzi-Staudinger reagent by utilizing the reactivity of the triazabutadiene moiety and vice versa was demonstrated using a model compound. It was also shown that the reagents that have been modified using this strategy retained their chemical reactivity.

In conclusion, this work describes the design and synthesis of bioconjugation reagents that can expand the toolbox of reagents available for the study of biological process.

Chapter 1 : Biomolecule labeling in chemical biology

1.1 Introduction

The ability to modify biomolecules is an important tool in chemical biology enabling either detection or the ability to influence biological processes, to gain greater insight into physiological and pathological events. Having a selective labeling capability is key to implementing this strategy. Two main approaches which are complementarily utilized in this field are genetic incorporation of probes and chemical modification of biomolecules.

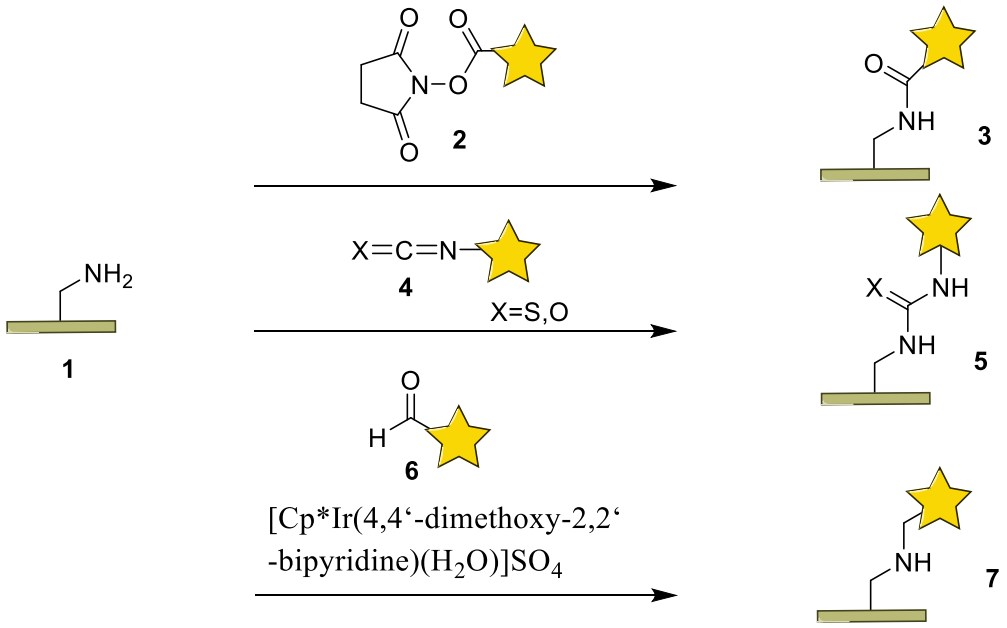
The development of a methodology to genetically encode green fluorescent protein (GFP) reporters has revolutionized our ability to study proteins in complex systems.[1] The potential perturbative effect of the size of these modifications could be drawbacks in their utilization. To address some of the drawbacks with the more traditional fluorescent protein strategy, site specific genetic modifications have been developed. A classic example of this strategy is the tetracysteine-biarsenical method, where an artificial peptide is encoded within a target molecule, to serve as a unique tag for further modifications.[2] However, genetic strategies are geared towards protein modification and are not amendable for the modification of other biomolecules such as lipids and glycans. Chemical modification on the other hand can offer approaches to supplement genetic modification strategies by addressing some of the limitations.

Chemical modification strategies can be grouped into two main classes: a) Selective site specific modification on native biomolecules; and, b) Selective modification of an unnatural reporter incorporated into biomolecules.

1.2 Site specific modification on native biomolecules

Chemical modification of native biomolecules requires identification of a functional group within said biomolecule with a relatively unique reactivity profile. Being the most numerous of all biomolecules and because of the importance they play in cellular processes most reagents developed in this field have targeted protein residues. The reactions for this purpose will be most useful if they are specific for a residue in the presence of multiple competing side chains of the unprotected polypeptide. The reaction needs to be compatible with conditions that allow for the maintenance of the protein structure. This includes being able to be performed in aqueous conditions while maintaining biologically relevant pH and temperature.[3] Most classical conjugation techniques take advantage of the nucleophilic nature of the lysine and cysteine residues. The reactivity profile of the hard amine nucleophile on the lysine sidechains and the softer nucleophilic sulfhydryl group on the cysteine allows for selective targeting of these functionalities.

Lysine residues are prevalent and are commonly solvent exposed. This allows for a generalizable non-specific labeling of proteins. Protocols for labeling lysine residues involve electrophilic acylation and alkylation regents as well as reductive amination with aldehydes (**Scheme 1.1**).[4] Traditional methods tend to result in a statistical mixture of conjugated species on a given protein.[5] However, recent needs of well-defined therapeutic proteins have promoted extensive work on protocols that enable access to pure constructs. A consideration that has to be taken when labeling lysine residues is the effect of the loss of the charged ammonium functionality upon conjugation can significantly alter the solubility profile of the protein.



Scheme 1.1 Bioconjugation reactions for lysine labeling

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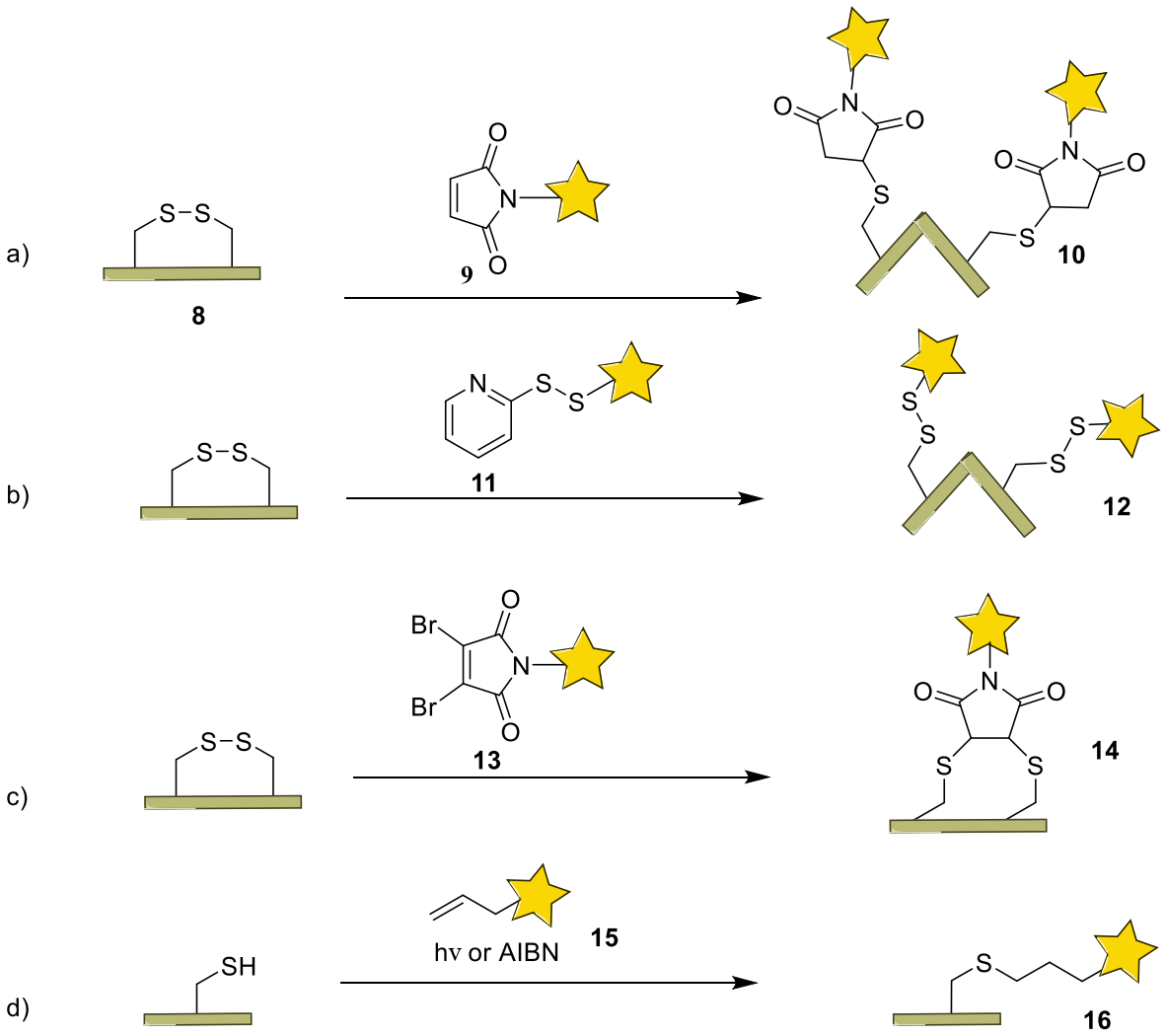
Conjugation protocols for cysteine residues tend to involve an electrophilic reagent that reacts with a free thiol of the side chain (**Scheme 1.2**). Unlike lysine residues, cysteines are much less prevalent in solvent exposed positions. A high level of selectivity has been attained by labeling cysteine residues that have been mutated, leaving single sites for conjugation.[6] Naturally occurring cysteine residues, when present tend to be disulfides. Many of the protocols developed to label these residues require the reduction of the disulfide bond (**Scheme 1.2 a** and **b**). The reduction of the disulfide bridges, which can be essential for structure, shape and stability, in these reactions can lead to loss of activity and/or integrity of the protein of interest. Conjugating strategies have been developed to address this limitation. In this strategy, even though the disulfide bond is reduced a carbon bridge is formed between the thiol groups maintaining the

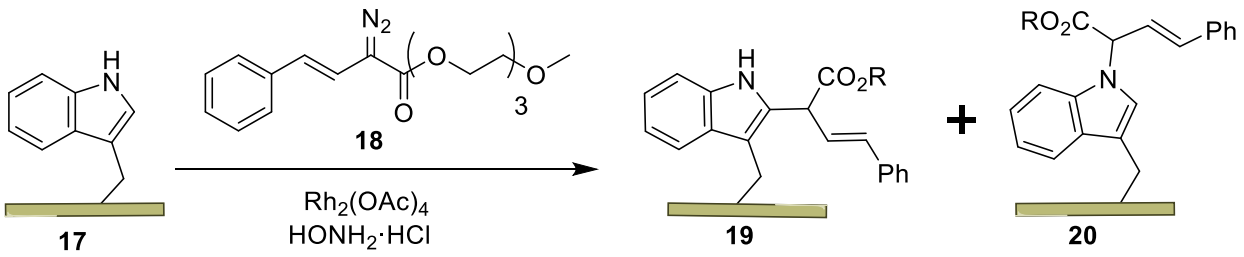
covalent bond between them, (**Scheme 1.2 c**).[7,8] A reaction that has recently attracted considerable attention for the modification of peptides and proteins is the thiol–ene. This radical reaction can be initiated photochemically or by use of a radical initiator such as AIBN, (**Scheme 1.2 d**). [9]



Scheme 1.2 Bioconjugation reactions for cysteine labeling

To address some of the limitations discussed above and have additional site of modification, significant attention has been paid to the aromatic amino acid side chains. In 2004,

Francis and coworkers published a reagent for selective modification of tryptophan residues using Rhodium catalyzed metallocarbenoids (**Scheme 1.3**).[10]



Scheme 1.3 Selective tryptophan labeling with rhodium carbenoids

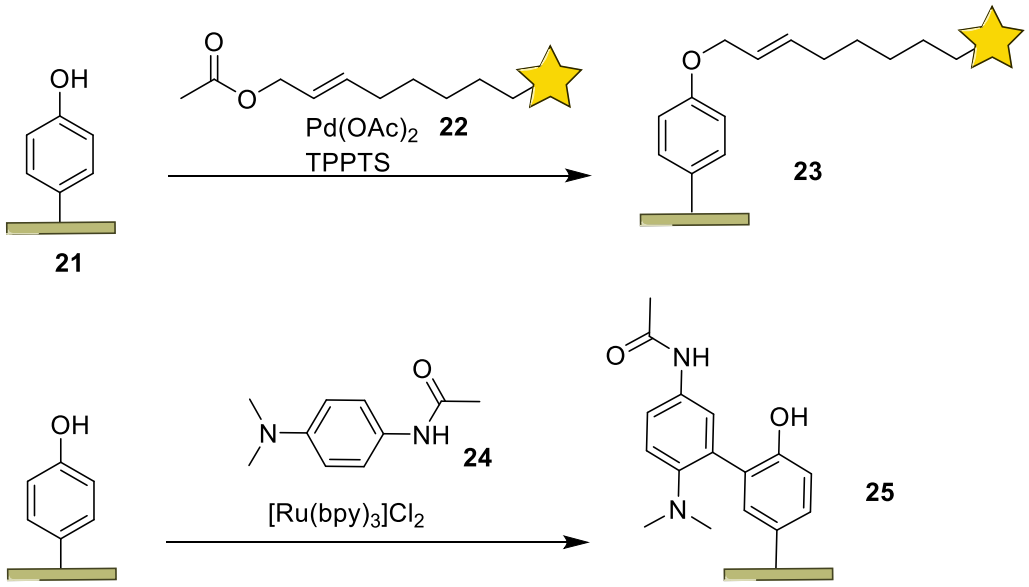
Another approach that takes advantage of rhodium as well as ruthenium complexes to label aromatic side chains has also been reported.[11] The reactions reported resulted in a direct complex of the metals with the amino acid. The product of the conjugation can be conveniently studied in biological media by various spectroscopic methods. Of all the aromatic side chain containing amino acids, tyrosine has received particular attention. The following section discusses the advantages offered by tyrosine for bioconjugation and work that has been done to selectively label the residue.

1.2.1 Tyrosine residues for selective bioconjugation

Tyrosine residues provide a particularly attractive target, as the reactivity of the phenolic side chain is largely orthogonal to that of cysteine, lysine, and carboxylate-containing residues.

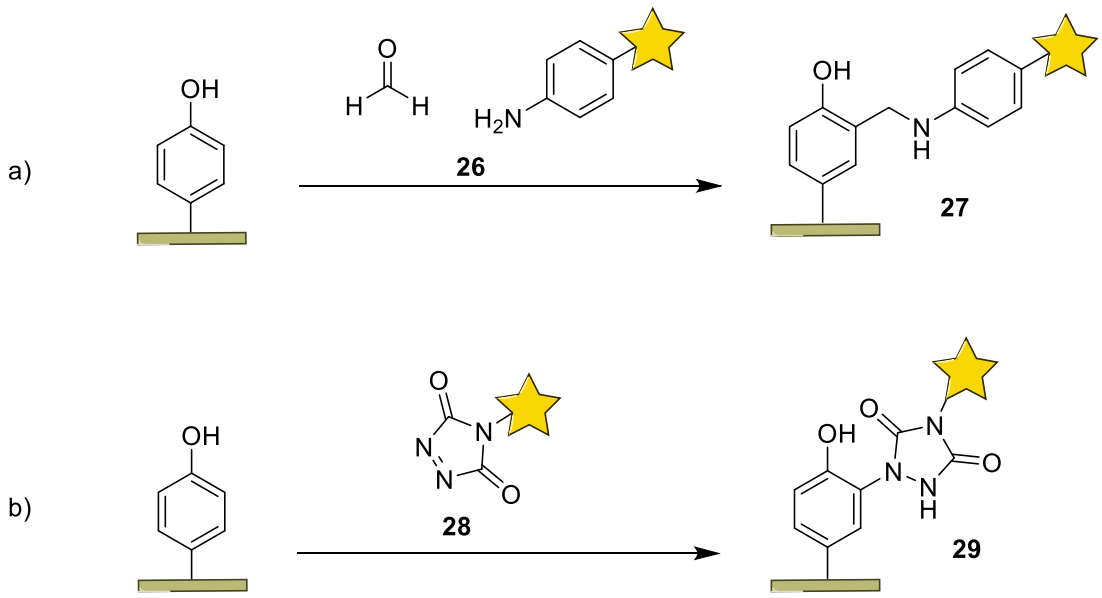
The lack of charge on the tyrosine residue means modification of them is inherently less disruptive to protein structure than those with charged side chains. The abundance of lysine residues in a typical protein or antibody significantly complicates control of the stoichiometry and specificity for bioconjugation reactions. Cysteines, although less abundant, require reductive pre-treatment before bioconjugation. In addition, modification of cysteine may alter the stability and function of an antibody or other protein wherein disulfide linkages are key to stability.[7] Tyrosine residues are not as common as lysine residues and have the added advantage of being relatively prevalent at 'hot spots' of proteins as compared to their overall presence in the protein surface offering an opportunity for active site selective bioconjugation.[12]

The electron rich character of the tyrosine sidechain has long been known to offer a selective site for bioconjugation with the first example reported in 1915 by Pauly.[13] Even though the nucleophilic phenol group has been utilized for alkylation[14] and the relative selectivity by which a tyrosine residue can be oxidized has led to a variety of metal catalyzed single-electron oxidation coupling reactions[15] (**Scheme 1.4**), this section will focus on metal free tyrosine labeling by electrophilic aromatic substitution reactions.



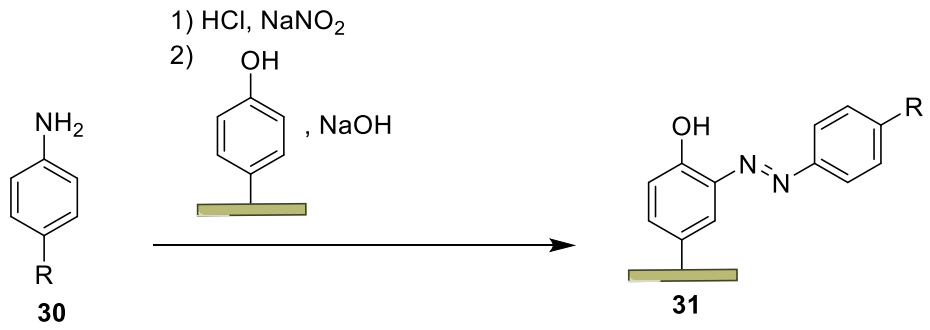
Scheme 1.4 Examples of metal catalyzed conjugation strategies for tyrosine

A three-component Mannich-type reaction for tyrosine bioconjugation was reported by the Francis group in 2004. This reaction involves the formation of a Schiff base by an aldehyde and functionalized aniline, followed by the electrophilic aromatic addition to the ortho position of the tyrosine phenol side chain. The reaction can be carried out under mild conditions that make it compatible with a wide range of functionalities and protein targets, (**Scheme 1.5 a**).[16] Another reaction that capitalizes on the electron rich character of the tyrosine side chain was developed by the Barbas group in 2009. The strategy involves the use of reactive diazodicarboxylate-related molecules to create an efficient aqueous ene-type reaction. This reaction was much faster than the Mannich-like reaction (**Scheme 1.5 b**).[17]



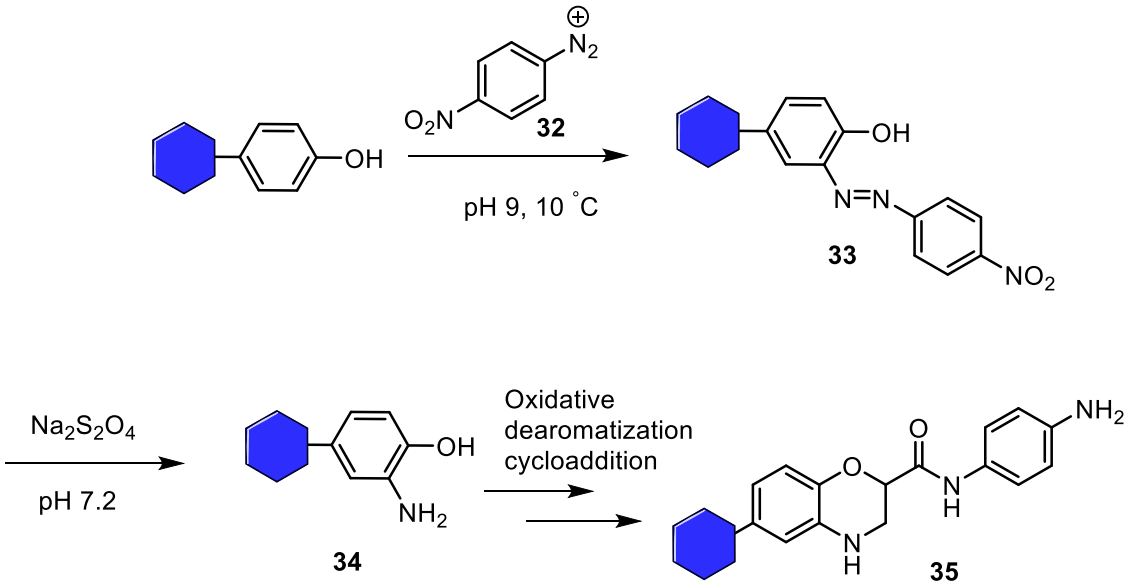
Scheme 1.5 a) Mannich type reaction to label tyrosine. b) ene-type reaction to label tyrosine

The earliest and still widely used method for bioconjugation of tyrosine residues is through a diazonium reaction forming an azo product. The utility of this strategy is severely hampered by the harsh conditions required to generate the diazonium reagent and the hydrolytic instability of the diazonium species, requiring generation of the diazonium ion *in situ*. The instability also prevented the use of common labeling tools, such as fluorophores because of the requirement of the harsh conditions (**Scheme 1.6**).



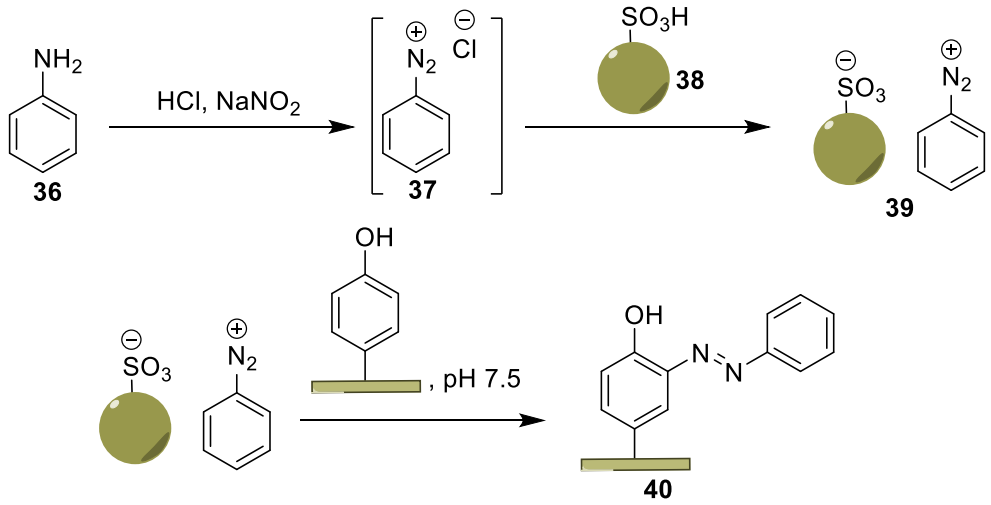
Scheme 1.6 Diazonium reaction with tyrosine

This strategy was reinvigorated by the work of the Francis group. A seminal work was published by the group in 2004, in which tyrosine residues on a viral capsid were conjugated using a diazonium reaction.[9] The azo bond formed upon conjugation **33** (**Scheme 1.7**) was reduced to the aminophenol **34** (**Scheme 1.7**) which was oxidized to enable labeling through a Diels-Alder reaction **35** (**Scheme 1.7**). This multistep process allowed for the addition of a fluorophore under mild conditions by bypassing the initial harsh conditions required for generating the diazonium ion (**Scheme 1.7**).



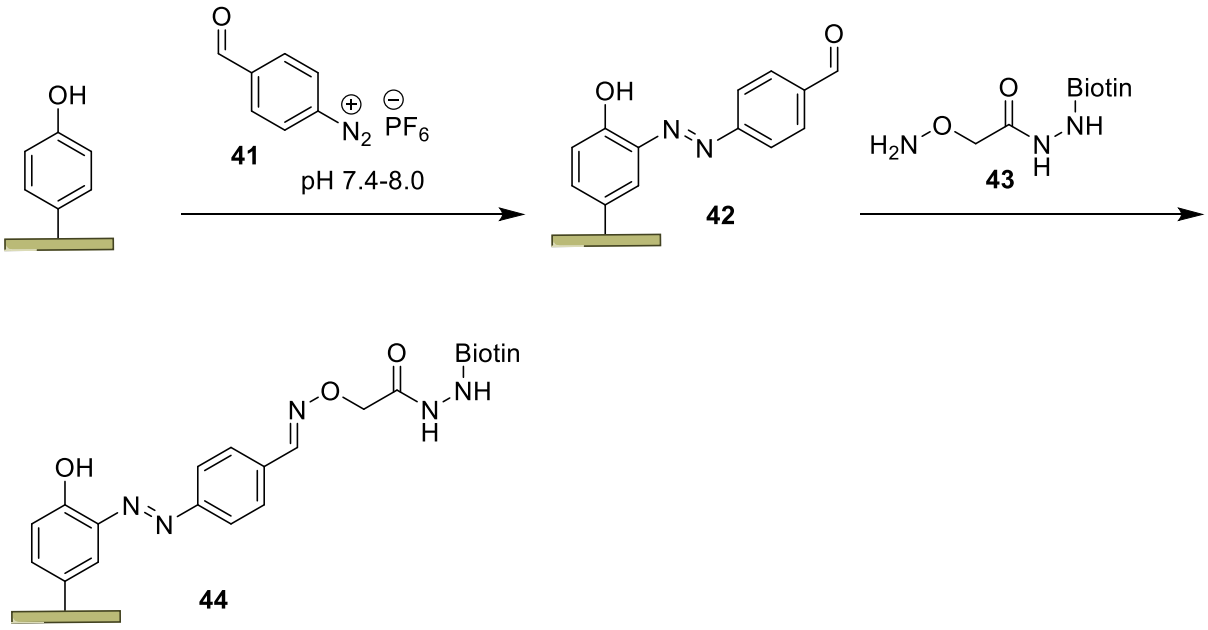
Scheme 1.7 Multistep strategy for labeling of tyrosine using diazonium ion chemistry

Another approach that was first reported in 1965 involved the generation of the diazonium salt followed by swapping the counter ion to a more stabilizing one.[18] In this work the authors generated an unstable diazonium salt with a chloride counter ion **37** (**Scheme 1.8**) which was swapped with sulfonic acid **39** (Scheme **1.8**) and carboxylate cation exchange resins. The generated diazonium-exchange resin had a significantly improved stability profile and the azo coupling reaction could be carried out at pH 7.5, **40** (**Scheme 1.8**).



Scheme 1.8 Resin stabilized diazonium strategy for labeling tyrosine residues

In 2012, The Barbas group used a similar approach to design a bifunctionalized diazonium reagent.[19] The diazonium synthesized was stabilized with hexafluorophosphate, **41** (**Scheme 1.9**) or tetrafluoroborate counter ion. The advantage of this compound over the previous work was that it incorporated an aldehyde handle that allowed for the convenient labeling of the azo product by an alpha effect nucleophile, **44** (**Scheme 1.9**).



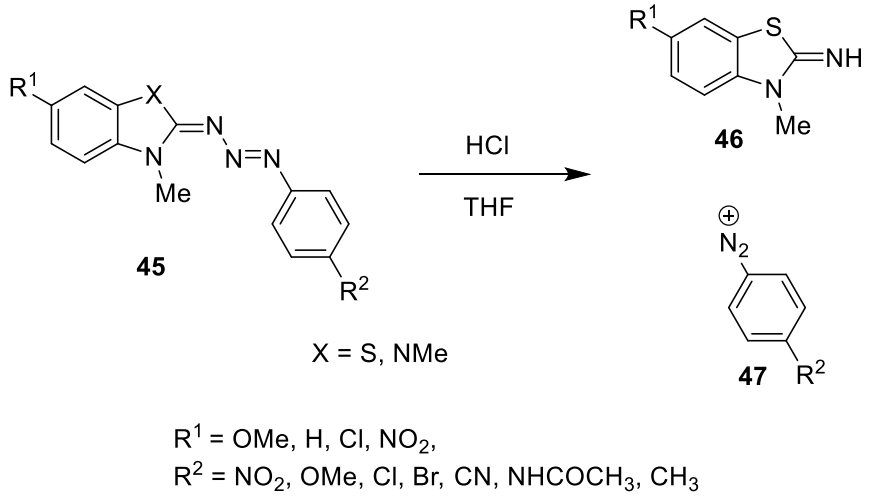
Scheme 1.9 A bifunctional diazonium reagent for labeling tyrosine residues

1.2.2 Masked diazoniums for bioconjugation

The stabilized diazonium species discussed earlier offered the opportunity to perform the azo coupling reaction at biologically relevant conditions. However, these strategies still did not allow for controlled release of the diazonium ion. In our lab, we envisioned a masked diazonium system that can generate the diazonium warhead upon activation by selected physiological triggers.

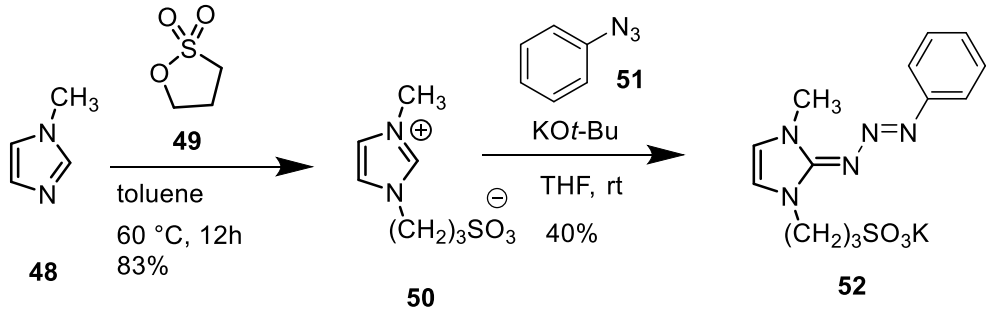
The approach, which was pioneered in our lab by Dr. Kimani, built on the research on triazabutadiene systems **45** (**Scheme 1.10**) carried out by the Fanghänel group. In their work, they were able to demonstrate that compounds with this motif degrade under highly acidic

conditions releasing a diazonium species **47** (**Scheme 1.10**). They were also able to demonstrate the rate of degradation was dependent on the structure and electronics of the system.[20]



Scheme 1.10 Generation of diazonium from triazabutadiene under highly acidic condition

Dr. Kimani was able to build on this work and design a water soluble triazabutadiene reagent that was capable of generating a diazonium ion under physiologically relevant acidic conditions, **52** (**Scheme 1.11**). The reagent was synthesized by reaction of an *in situ* generated water-soluble *N*-heterocyclic carbine with phenyl azide (**51**, **Scheme 1.11**).[21]



Scheme 1.11 Synthesis of a water-soluble triazabutadiene

In her work, Dr. Kimani was able to show that the by tuning the electronics it was possible to change the stability of the compound. Another level of stability control can also be afforded by changing the sterics of the reagent. In work conducted by Dr. He in our lab, he was able to demonstrate that changing the size and nature of the substituent on the Zwitterionic N-heterocyclic carbene precursor could significantly change the stability of the reagent.[22]

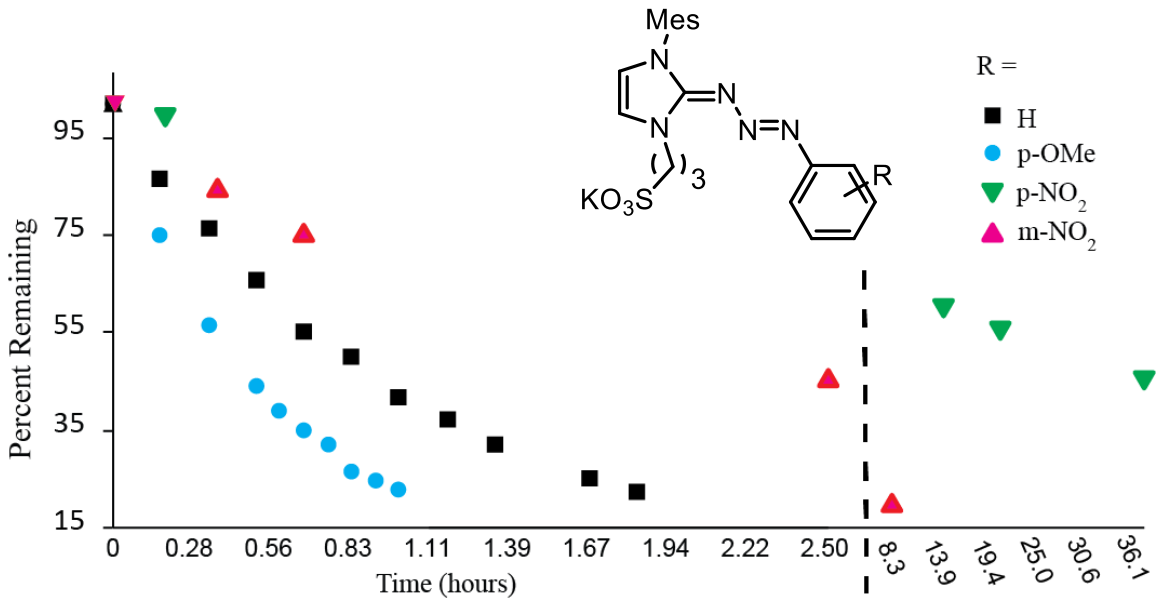


Figure 1.1 Effect of substituent on the rate of decomposition of triazabutadiene

The utility of this system for biological labeling has been demonstrated by labeling Bovine serum albumin[23] and viral surfaces[24] in our lab and genetically encoded noncanonical amino acid, 5-hydroxytryptophan by the Chatterjee group.[25]

1.3 Site specific unnatural modification for bioconjugation

Bioconjugation reactions that target native residues are very popular techniques; however the fact that these strategies target functionalities that occur in nature in multiple copies limits their utility for *in vivo* experiments. Another challenge with the strategy is that these reactions are generally aimed towards protein modification and have limited application in labeling other biomolecules. An approach that utilizes a reporter with an abiotic functionality incorporated into biomolecule of interest to be probed by a label with selective reactivity for that functionality can address these limitations. The term Bioorthogonal reaction was coined by Carolyn Bertozzi to describe bioconjugation methods that can occur between an abiotic reporter and probe pair with high efficiency, selectivity, low toxicity and high biological tolerance under mild aqueous conditions (**Fig. 1.2**).[26] The fact that the earliest works that were conducted in the Bertozzi lab involved the labeling of metabolically modified cell surface oligosaccharides demonstrated the utility of this strategy to label a wide range of biomolecules.[27]

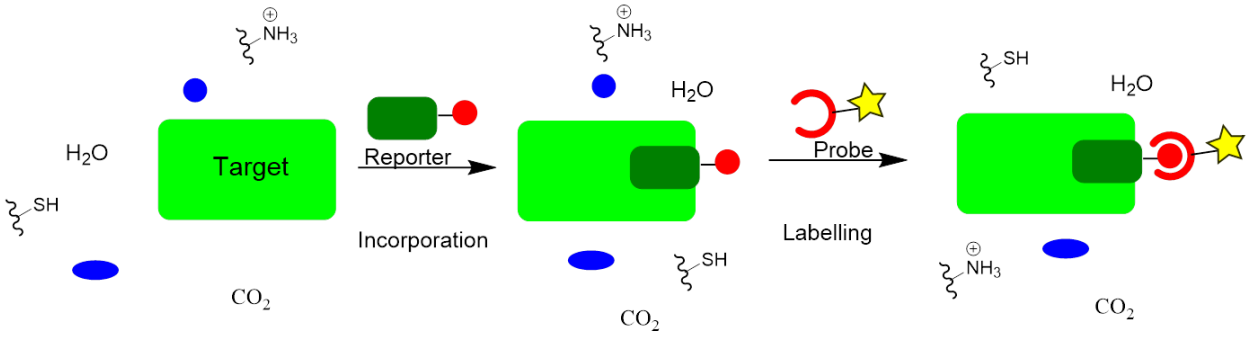
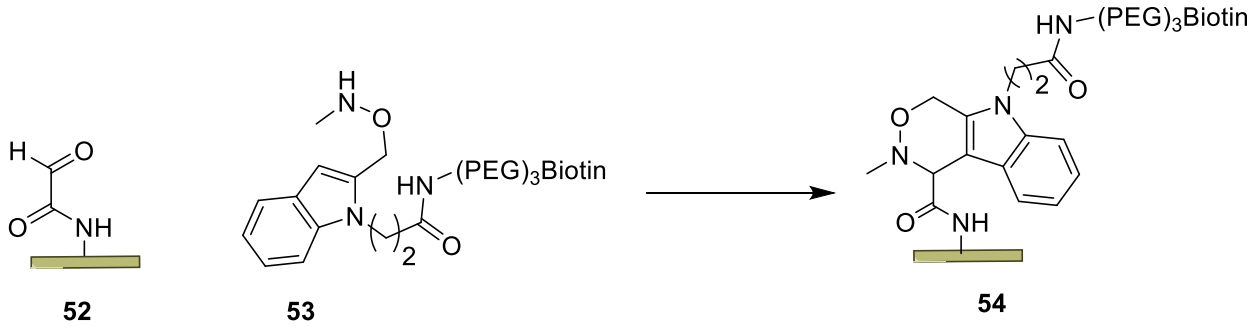


Figure 1.2 Bioorthogonal strategy for reaction with an unnatural reporter

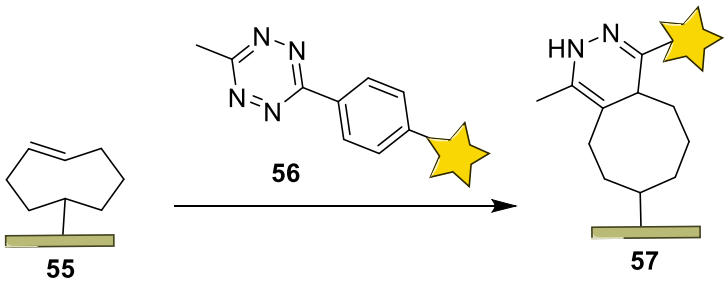
A number of important parameters have to be taken into considerations when designing a reporter and probe pair. As already mentioned previously, the reporter-probe pair should be abiotic (or nearly so). Ideally, the reaction pair must react robustly with one another in aqueous environments, yet remain inert to all other cellular functionality. To avoid interference with the biological processes a small sized reporter is advantageous. These tasking requirements have meant that currently the number of 'privileged' reaction pairs that have achieved wide utilization is limited.[28]

The first reporter that was utilized for selective reaction *in vivo* conditions was an aldehyde modification.[29] Even though not fully abiotic, the aldehyde functionality is rare in cell surfaces and extracellular spaces. The small size of this reporter makes it amenable to incorporation into biomolecules. In one of the most significant works in this field by the Bertozzi group, an unnatural ketone derivative of *N*-acetylmannosamine was successfully metabolically incorporated followed by labeling of the unnatural metabolite on the cell surface.[27] Other methods of incorporation have also been reported including genetic incorporation[30] and enzymatic ligation.[31] Aldehydes and ketones perform a selective reaction with α-effect nucleophiles. However the product of these reactions tends to be reversible limiting their utility. To address this limitation, a modified Pictet-Spengler reagent has been synthesized that greatly improves the stability of the conjugation product.[32]



Scheme 1.12 Pictet-Spengler ligation for protein chemical modification

Some of the most impressive reaction rates to be reported between privileged reactant pairs have been inverse electron demand reactions between tetrazines and strained alkenes. First reported in 2008, this interchangeable reporter and probe pair have been shown to be highly tunable. Reaction rates as high as 3,300,000 $M^{-1}s^{-1}$ with a modified *trans*-cyclooctene have been reported.[33]

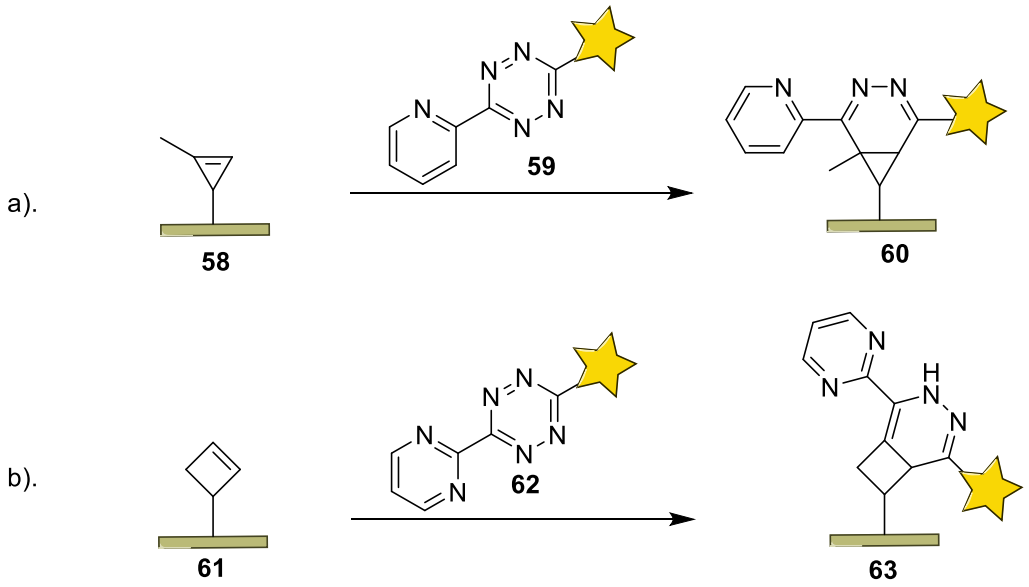


Scheme 1.13 Reaction of *trans*-cyclooctene with tetrazine

Even though the strained *trans*-cyclooctene system has the obvious advantage in rate, its bulk and hydrophobicity as well as incompatibility with many endogenous biosynthetic

pathways can limit its application. Smaller strained alkenes like cyclopropene, (**Scheme 1.14 a**) and cyclobutene, (**Scheme 1.14 b**) have been synthesized to address these issues. Even though significantly slower than *trans*-cyclooctene, cyclopropene modified biomolecules were shown to be able to be metabolically incorporated into cell surface glycans.[34]

Arguably, the functionality most closely associated with being a reporter in bioorthogonal chemistry is the organic azide. The azide functionality is abiotic except for the unique case of (*S*)-2-amino-3-azidopropanoic acid in Salmonella grown in the presence of azide.[35] The small size of the azide, its innocuous nature and its demonstrated unique reactivity makes it a powerful tool in bioconjugation. Its stability and size allows azide modifications of biomolecules relatively easily by chemical and biological methods.[36] The next section will discuss the use of the azides in bioorthogonal labeling and the reaction partners that are used to probe biomolecules for this functionality.



Scheme 1.14 Reaction of tetrazine with a) cyclopropene and b) cyclobutene

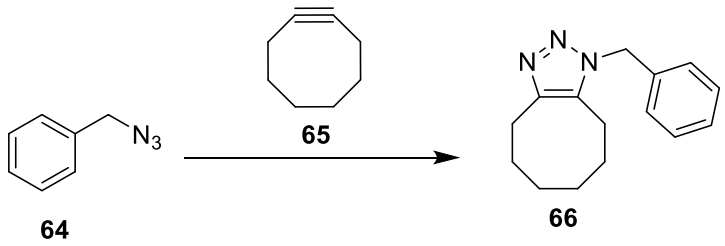
1.3.1 Organic azides in bioortogonal chemistry

In the seminal paper, credited with heralding the field of bioorthogonal chemistry, published in 2000 by the Bertozzi group, an azide reporter was metabolically installed in the surface of Jurkat cells.[26a] Even though a number of reporters have been developed since then, organic azides remain as one of the most important functionalities in chemical biology. Two main groups of probes have been developed as reaction partners to the azide moiety, namely alkynes and Staudinger reagents.

Even though the reaction of terminal alkynes with azides was first observed some 60 years previously, it was the insights into the mechanism by Rolf Huisgen that was instrumental in the wider application of the cycloaddition reaction that bears his name.[37] A copper-catalyzed azide-alkyne cycloaddition (CuAAC) was first reported in 2002.[38] Because of its usefulness and reliability, Prof. Barry Sharpless referred to this reaction as the "cream of the crop" when it comes to click reactions.[39] This reaction has become so closely associated with the concept of click reactions that a common misnomer for the CuAAC is simply referring to it as 'the click reaction'. CuAAC has been widely utilized for *in vitro* conjugation with the azide and alkyne functionalities being used as reporter and probe interchangeably, however its utility in *in vivo* is severely hampered by the toxic nature of the copper catalyst required for this reaction.[40] The advent of this reaction and its demonstrable reliability has led to the development of strategies for copper free versions of this reaction.

A copper free variant of the azide alkyne Huisgen cycloaddition reaction where a strained cyclooctyne was observed to react with phenyl azide was first reported in 1953 by Blomquist and Liu (**Scheme 1.15**).[41] They reported that the reaction occurred explosively to give an unidentified

viscous liquid. The reaction is generally referred to as strain promoted alkyne-azide cycloaddition (SPAAC).



Scheme 1.15 Strain-promoted alkyne-azide reaction between cyclooctyne and benzyl azide

The Bertozzi lab was able to build on this and later works to develop the first iteration of this compound for biological labeling referred to as OCT.[42] This reagent was shown to have improved reaction kinetics as compared to the linear alkynes. A number of versions of the cyclooctyne reagents have since been synthesized to increase aqueous solubility[43] and increase rate with the fastest version referred to as BARAC achieving second rate constant of 0.96 M^{-1} s^{-1}.[44]

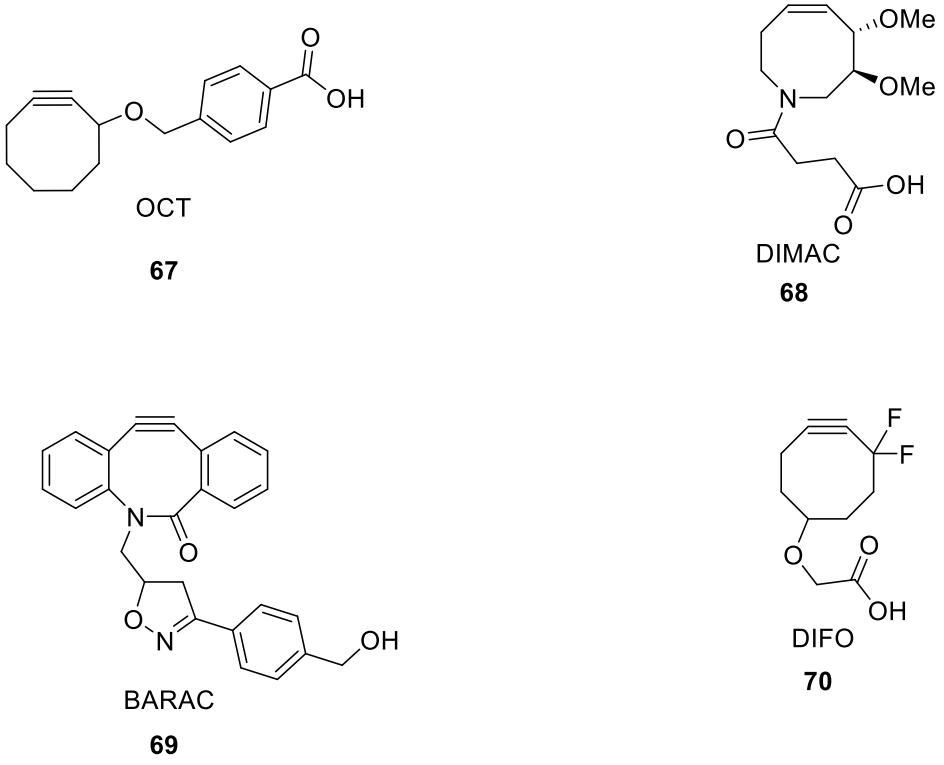
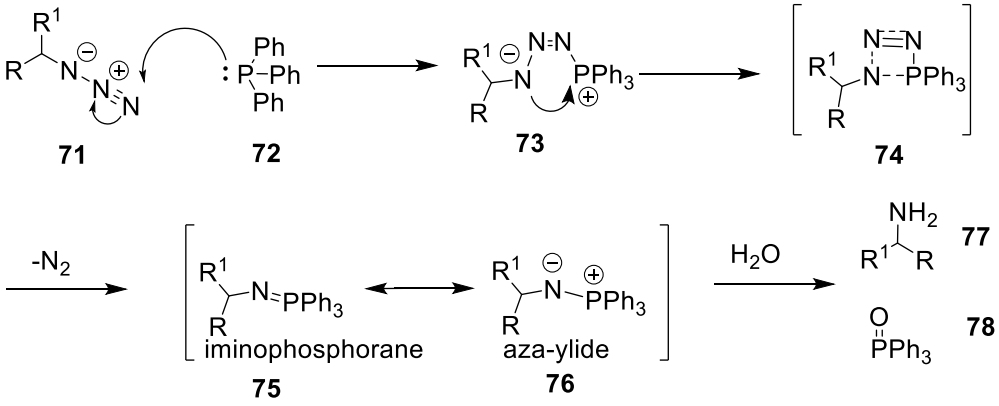


Figure 1.3 Examples of cyclooctyne reagents for SPAAC

Another group of reagents that are a reaction pair to azides take advantage of the affinity of the azide molecule to phosphines and phosphites. These reagents are used ubiquitously in chemical biology. This group of probes, referred to as Staudinger reagents for the purpose of this work, will be discussed in the next section.

1.3.2 Staudinger reagents in bioconjugation

In 1919, Hermann Staudinger and Viktor Meyer described a reaction of organic azides with triaryl phosphine to generate an iminophosphorane with a loss of N_2.[45] A mechanistic study of the reaction demonstrated the formation of the iminophosphorane goes through a four member transition state. When the reaction was conducted in the presence of water, a hydrolytic cleavage of the phosphane imines occurs with the end product being a primary amine and phosphine oxide. The formation of a highly stable oxygen phosphorus double bond is the driving force for this reaction, which is classically known as Staudinger reduction.[46]



Scheme 1.16 Staudinger reduction mechanism

The iminophosphoranes generated by the reaction of the phosphine and azide, which is in equilibrium with the aza-ylide, was shown to be highly nucleophilic. Staudinger was able to demonstrate that when reaction is conducted in the presence aldehydes or ketones imines are

formed.[47] Less reactive carbonyl electrophiles, such as amides or esters, also undergo reaction with the iminophosphorane, especially if the electrophilic attack proceeds in an intramolecular fashion.[48]

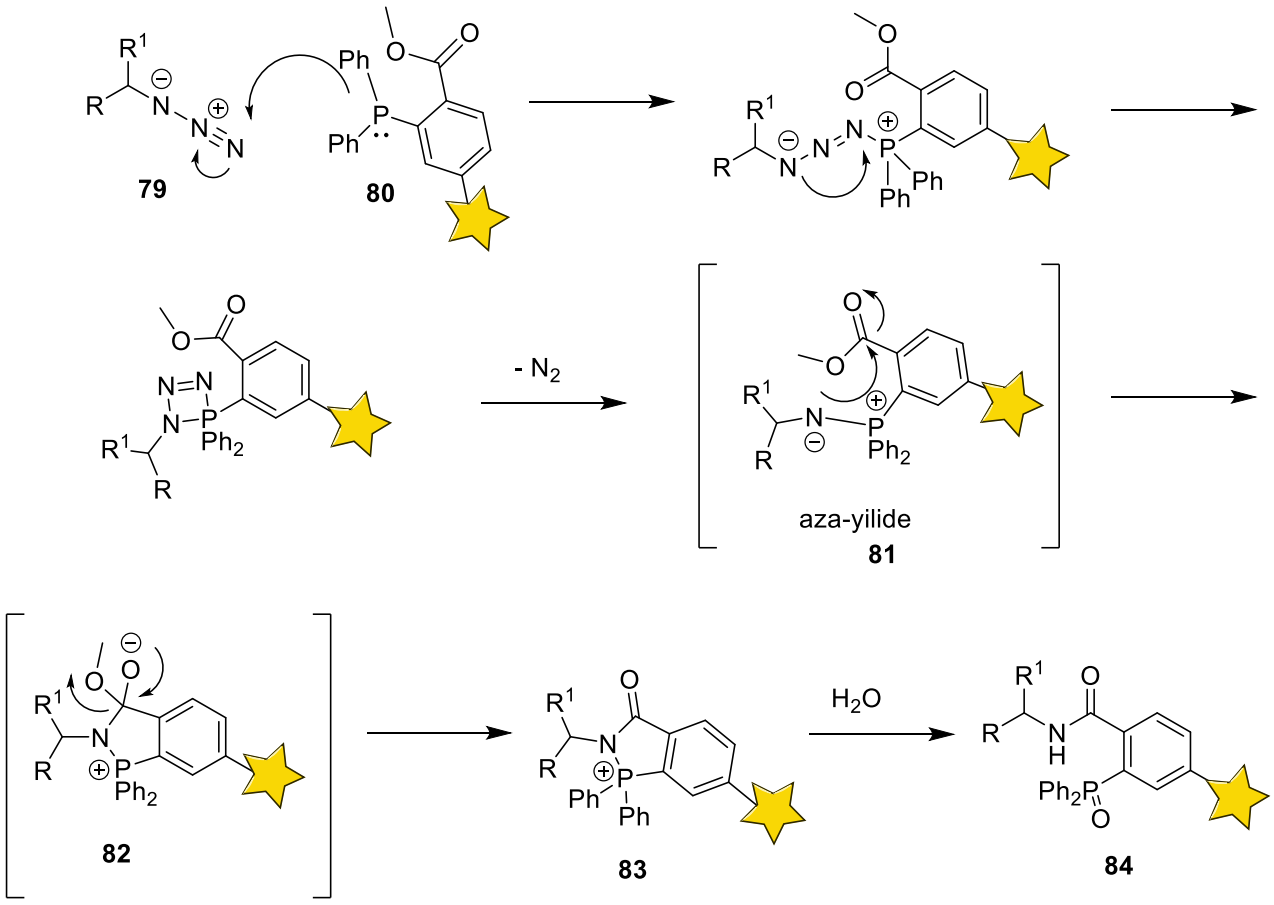
A. The Bertozzi-Staudinger ligation

The ability of the iminophosphorane to react with esters was an inspiration for the design of a three component reaction, to form an amide bond between a reduced azide and an intramolecular ester. This reaction was first reported by the Bertozzi lab in 2000, and used for the modification of a cell surface.[26a] As previously stated in this chapter, this work was pivotal in the introduction of the bioorthogonal approach in bioconjugation.

The key to this reaction was the understanding that by having an electrophilic group close to the location of the iminophosphorane generated by the reaction of a phosphine and azide, it was possible to trap the aza-ylide as an amide before the competing hydrolysis could occur.

The rate of the Bertozzi-Staudinger ligation calculated at 2.5×10^{-3} $M^{-1}s^{-1}$ is sluggish[49] even as compared to the other azide labeling strategies with $k2$ that can reach 200 $M^{-1}s^{-1}$ for CuAAC[38a] and 0.96 $M^{-1}s^{-1}$ for SPAAC, which was developed much later.[44] Attempts to increase the rate of the reaction by increasing the electron density on the phosphorus atom led to substrates which were more prone to air oxidation.[50] The hydrophobic nature of the relatively large triphenylphosphine and the fact that the triphenylphosphine oxide remained in labelled biomolecules was another limitation of this reagent.[51] In spite of these drawbacks, the exquisite selectivity and biological environment compatibility of this reagent has currently made the

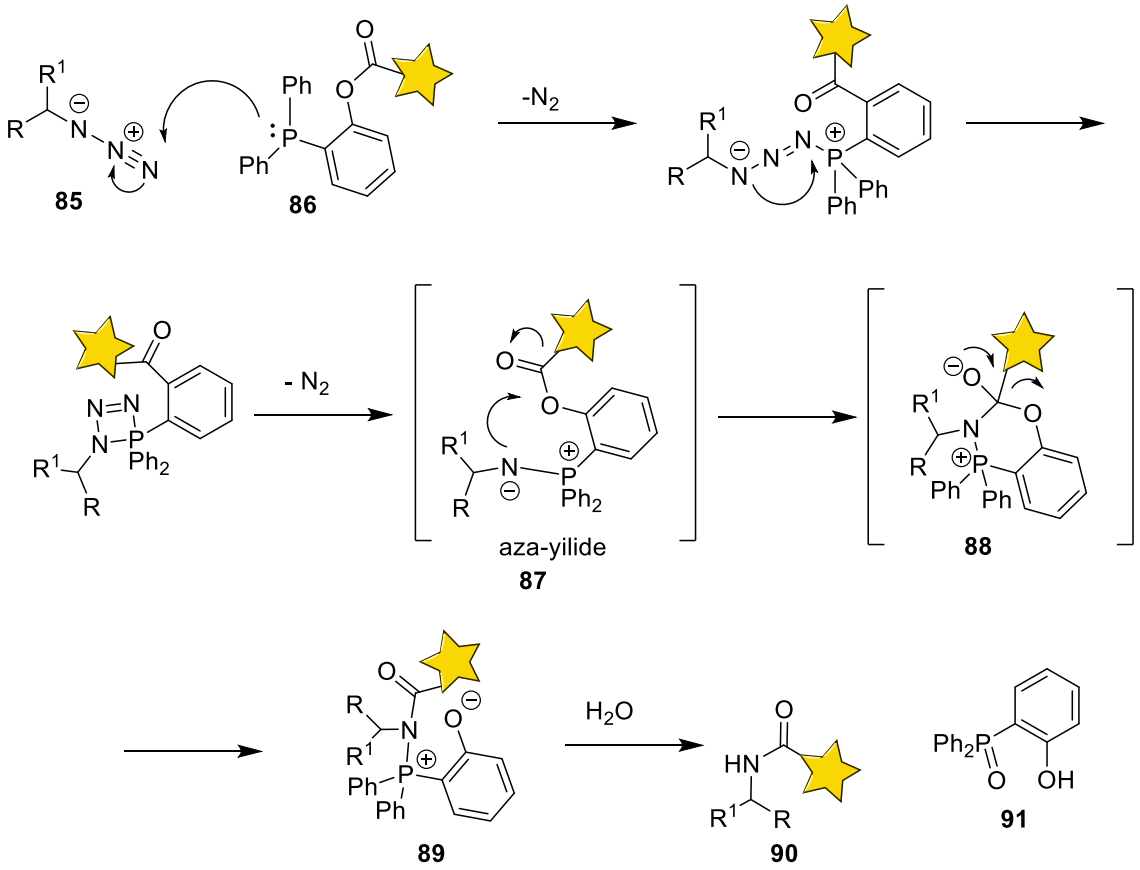
Bertozzi-Staudinger reaction highly useful and a classic in the field of bioorthogonal chemistry.[52]



Scheme 1.17 Bertozzi-Staudinger ligation mechanism

B. The traceless Staudinger reaction

Shortly after the introduction of the Bertozzi-Staudinger ligation, the Bertozzi[53] and Raines[54] groups working separately developed a 'traceless' version of this probe. In both of these strategies, the bulky phosphine oxide was no longer part of the final bioconjugation product. The Bertozzi group maintained the ester motif while the Raines group utilized a thioester approach. These reagents maintained the exquisite selectivity towards azides of the original Bertozzi-Staudinger ligation reagent.



Scheme 1.18 The traceless Bertozzi-Staudinger mechanism

The Raines reagent was primarily utilized for peptide synthesis. This was possible with the traceless reagents because the final product was a simple, naturally occurring amide bond. The ligation of the reagent with azido amino acids was shown to maintain the stereochemistry at the α-carbon except in the case of azido glycine.[55]

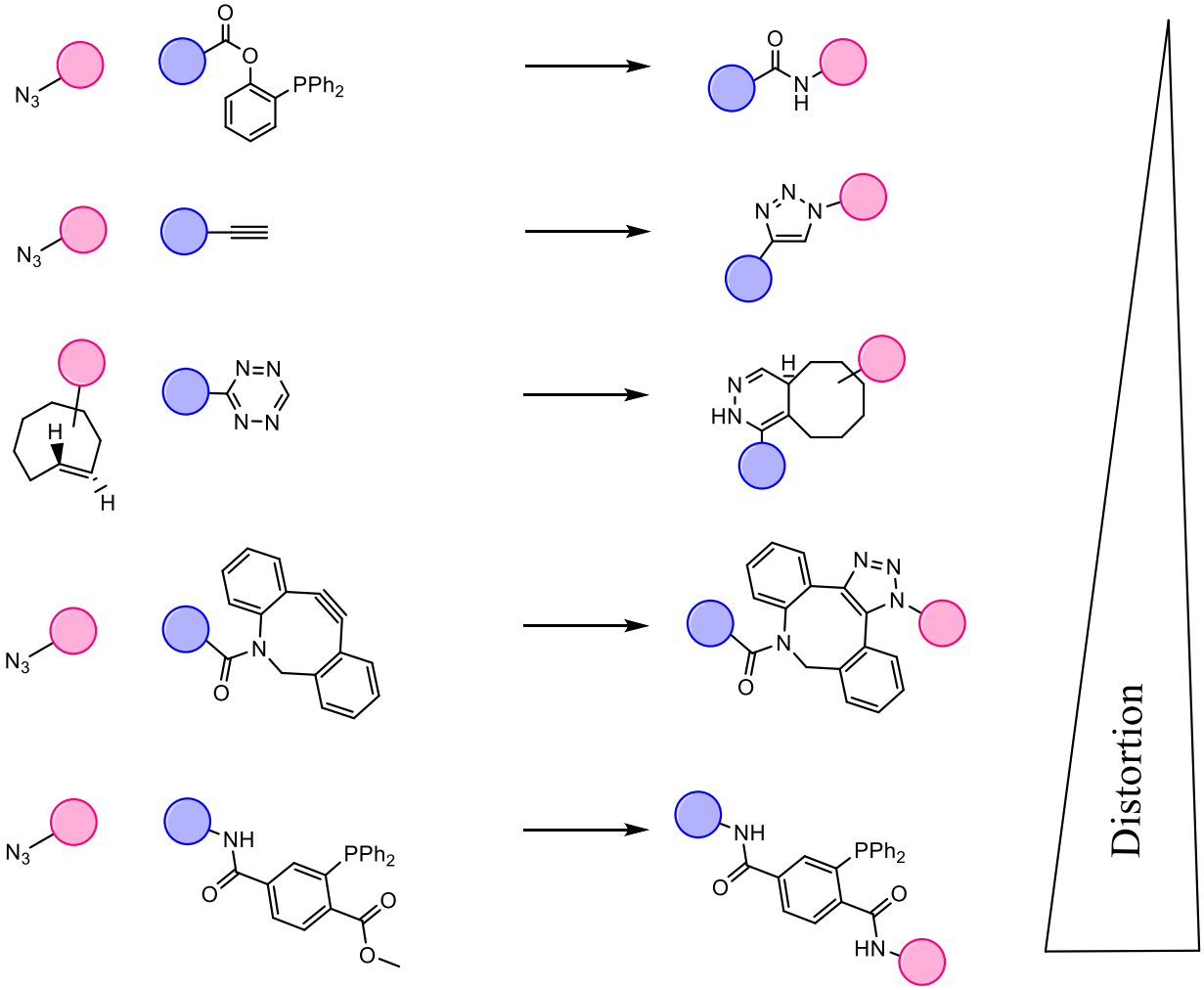
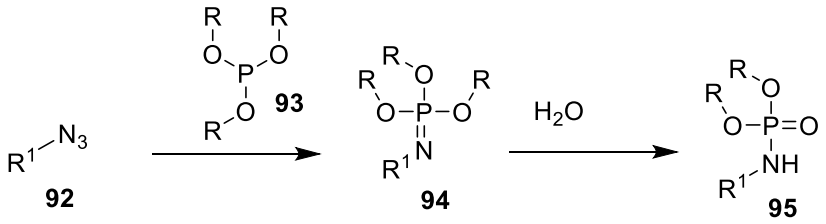


Figure 1.4 Overview of ligation reactions in relation to the mass of product

The small size of the final ligation product offers advantages not only over the non-traceless form of the reagent but also other widely used bioconjugation strategies (**Fig. 1.4**).[56] This is especially useful where distortion caused by size of the ligation product can interfere with the study being performed. In our lab we were able to demonstrate the utility of this reagent by using it to install minimally perturbing diazirine functionality at sites of azide protein modifications.[57]

C. The Staudinger-Phosphite reaction

In 1956 Kabachnik and Gilyarov first reported that the iminophosphorane that is formed upon the reaction of phosphites and organic azides did not lead into an azide upon hydrolysis but rather in the formation of a phosphoramidates with the loss of an alcohol group.[58] The Hackenberger group has utilized this reaction to label azide modified biomolecules.[59] The phosphite reagents are significantly easier to access synthetically than the phosphine reagents required for the other Staudinger methods. In one of their earliest papers describing this strategy the Hackenburger group showed that this strategy could be used to introduce a potentially biological relevant functional group. By building in functionalities that allowed for the light saponification of the phosphodiester bond of the phosphoramidate, they were able to obtain a charged phosphoroamidate that closely resembles phosphorylated residues.[59a]



Scheme 1.19 The Staudinger phosphite reaction

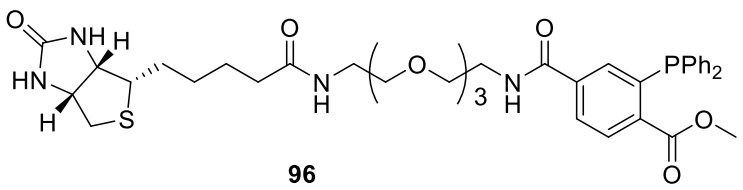
The bioconjugation strategies discussed in this chapter have contributed towards a greater understanding of biological processes. However, research into developing new bioconjugation strategies continues. Reagents that can be used to address new questions or modifications on current technologies to increase their utility, is a growing field of study. In the following chapters some of the work that was done in this regard is discussed.

Chapter 2 : Modular traceless Bertozzi-Staudinger reagents for biological labeling

2.1 Introduction

Since its introduction in the year 2000, the Bertozzi-Staudinger ligation[26a] and its traceless variants have been widely used in biological chemistry and synthesis.[60] Even though much faster conjugation reactions have been reported, including with azides, the exquisite selectivity and biological tolerance of these reagents have ensured their continuous utility in this field.[52] A drawback that was identified early on with this reagent was their highly hydrophobic nature. To overcome this limitation, a water soluble version of the original Bertozzi-Staudinger ligation reagent **96** (**Fig. 2.1**) was synthesized with a solubilizer group forming a link between the reagent and the probe. Synthetically, this approach had the advantage of not requiring additional steps involving the oxidation prone phosphine reagent as the solubilizer could be conjugated with the cargo prior to its reaction with the phosphine. The increased solubility of this reagent allowed for its use in aqueous conditions, but also resulted in increasing the already significant size and molecular weight of the ligation product.

The strategy of attaching the solubilizer to the probe would however not be optimal when it comes to the traceless versions of the Staudinger ligation. The advantage of these reagents over the non-traceless version is that they are capable of delivering a probe with the smallest ligation size reported. This has allowed them to be utilized in peptide synthesis[61] as well as delivering cargo in a biological system where maintaining a small non-perturbing size is essential.[23]



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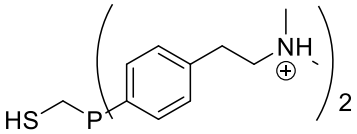

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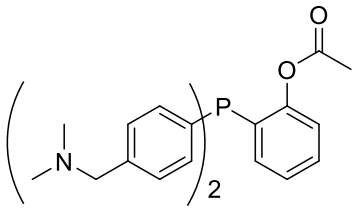


98

Figure 2.1 Water soluble Staudinger reagents

The solubility issue has been extensively investigated by the Raines group in regard to the phosphinothioester traceless reagent that was developed in their lab.[61] In their approach they developed a wide variety of acid and base functionalized reagents which would be ionized in aqueous solution to yield water soluble salts, **97** (**Fig. 2.1**). The Raines group as able to shown that this approach allowed for the synthesis of peptides in biologically relevant conditions.[62]

Another approach towards a traceless Staudinger reagent was developed by the Bertozzi group.[53] In this strategy, discussed in Section 1.3.2 of this work, the triphenylphosphine core of the Bertozzi-Staudinger reagent contains the phenol portion of the ester that is formed with the probe being attached. This allows for a traceless conjugation with an azide moiety; however, electron rich phosphines are even more prone to oxidation, which could significantly increase the synthetic challenge associated with these reagents.[50] To the best of our knowledge only one published work has shown a strategy to synthesize a water soluble version of this reagent, **98** (**Fig. 2.1**).[63]

Another challenge encountered in bioorthogonal fluorophore labeling, especially in reagents with slower reaction rates such as the Staudinger reagents, is background fluorescence of unreactive probes. The amount of excess of the fluorescent label that is needed for labeling is directly proportional to the reaction rate of the applied bioorthogonal chemistry strategy. Where larger concentration of the probe is utilized several washing step cycles are required when possible to remove excess unreacted probe to minimize background fluorescence.[64] However, washing steps are not possible when *in vivo* studies are being conducted, conditions where the Staudinger reagents selectivity is highly advantageous. An approach that has been widely employed to minimize background fluorescence is the use of fluorogenic probes. These probes have the advantage of displaying increased fluorescence upon reaction with the appropriate reporter. Fluorogenic probes have been developed for some of the most commonly utilized bioorthogonal reactions including copper catalyzed azide alkyne reactions, **a** (**Fig. 2.2)**[65]**,** strain promoted alkyne-azide cycloaddition, **b** (**Fig. 2.2**)[66] and tetrazine-*trans*-cyclooctene reactions, **c**(**Fig. 2.2**).[67]

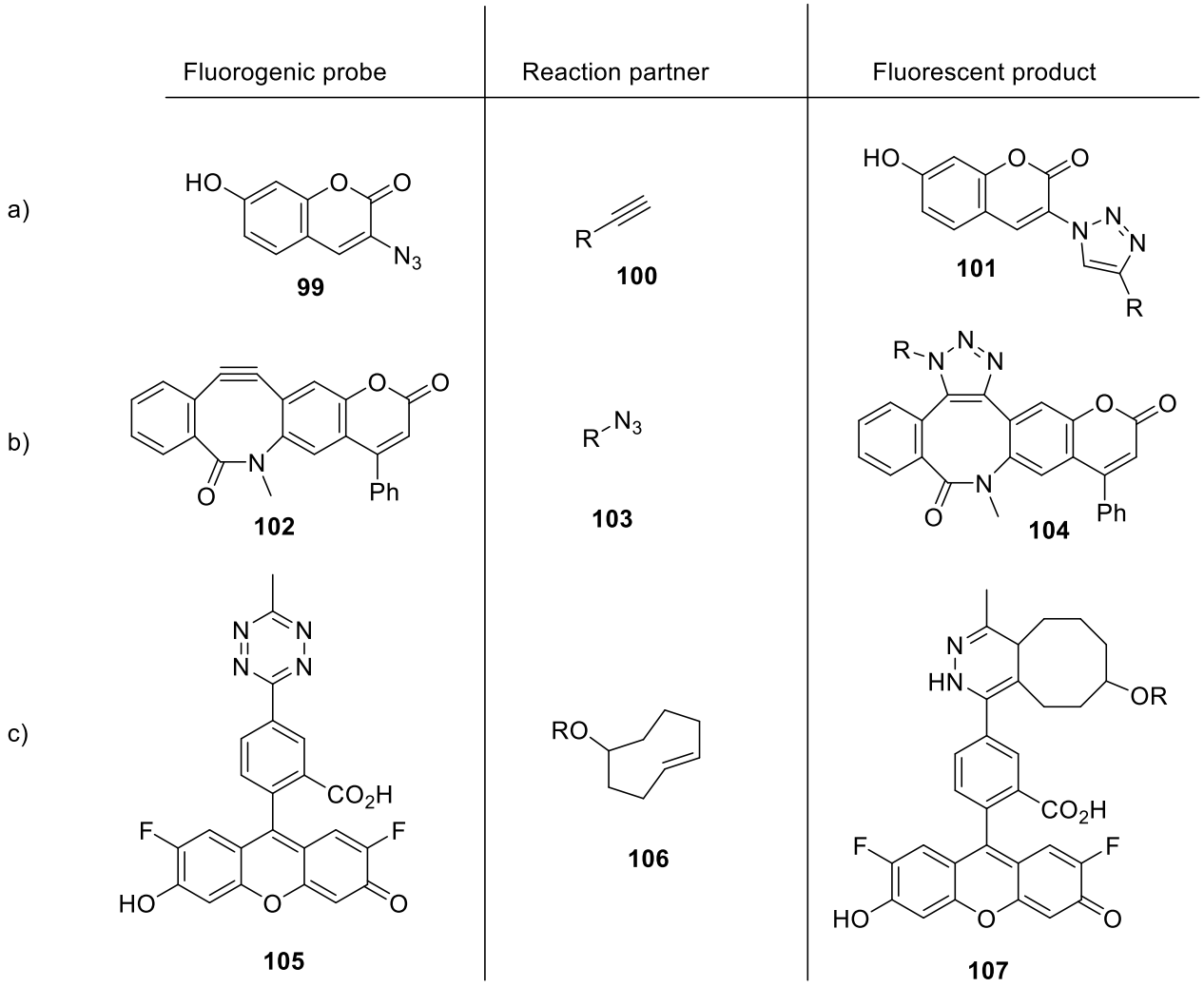
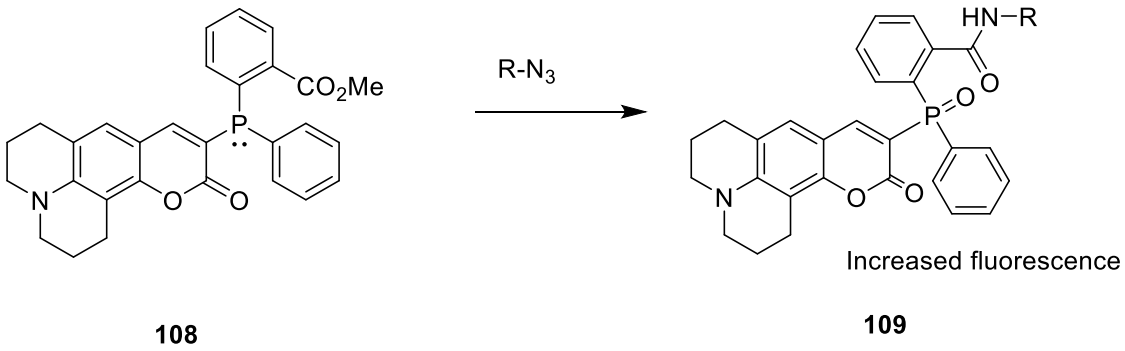


Figure 2.2 Examples of fluorogenic probes for biological labeling

Fluorogenic versions of the Bertozzi-Staudinger ligation reagent have also been synthesized with varying success. The first version of these reagents, synthesized by the Bertozzi group, exploited the difference in electronics between the phosphine of the reagent and the phosphine oxide product of the conjugation reaction with azides.[68] This reagent consisted of a coumarin fluorophore appended to the phosphine such that the lone pair of electrons of the phosphine was at the 3 position of the coumarin, **108** (**Scheme 2.1**). The intramolecular charge

transfer (ICT) from the lone pairs of the phosphine was able to quench the fluorescence of the coumarin. Upon oxidation, the phosphine oxide is no longer able to donate electrons and the coumarin attains fluorescence, **109** (**Scheme 2.1**). With this strategy, a 60-fold increase of fluorescent signal was attained. Unfortunately, this reagent still exhibited large background fluorescence. This was due to the fact that the increase in fluorescence was from the oxidation of the phosphine rather than the conjugation of the azide. This meant that if the phosphine was oxidized prior to the azide reaction, which it is prone to do in biological settings, the increase in fluorescence would still be observed.

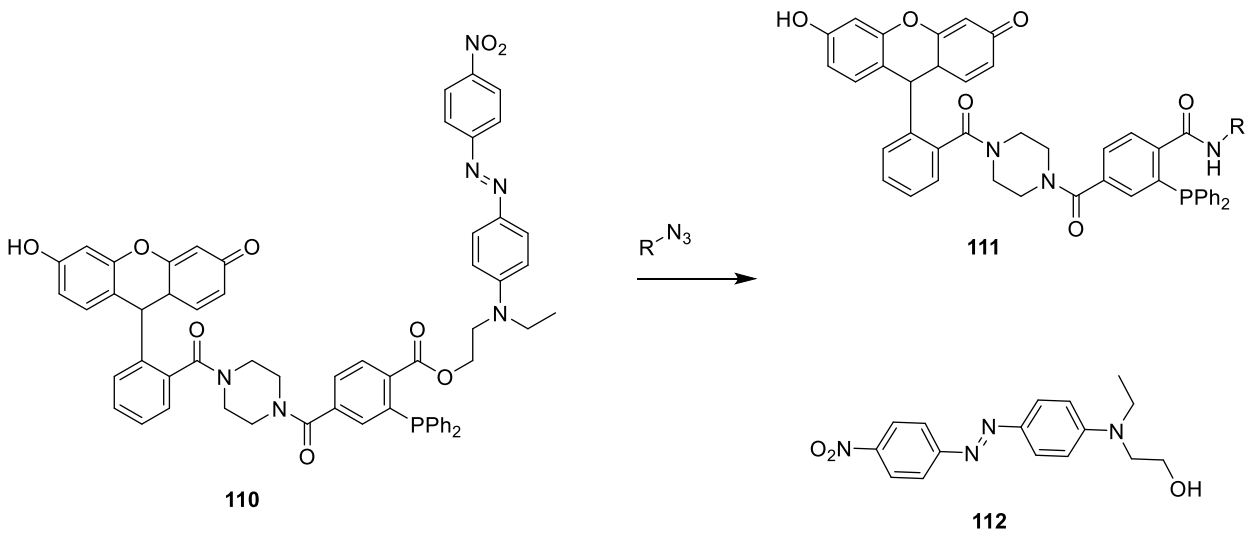


108 **109**

Scheme 2.1 Coumarin based fluorogenic Bertozzi-Staudinger ligation reagent

Another fluorogenic strategy that was utilized by the Bertozzi group was the design of a fluorescence resonance energy transfer (FRET) based system, **110** (**Scheme 2.2**).[69] In this strategy, a quencher and a fluorophore were attached to the reagent. The proximity of the reagent-quencher pair resulted in loss of fluorescence. The design of this reagent was in such a

way that the quencher was released upon the conjugation reaction with an azide. The loss of proximity restored the fluorescence of the fluorophore with a 170-fold signal amplification, **111** (**Scheme 2.2**). Since this strategy is dependent on proximity and not ICT as in the previous case, oxidation of the phosphine did not result in background fluorescence. This highly cited work also showed that this reagent could be used to visualize cell surface glycans. However, to the best of our knowledge, this reagent has not been utilized in another published work which we believe could be attributed to the synthetic challenges associated with these reagents. A traceless version of these fluorogenic systems has not been reported.



Scheme 2.2 A FRET based fluorogenic Bertozzi-Staudinger ligation reagent

The synthetic challenges associated with the Staudinger reagents are mainly due to the difficulties associated with working with phosphines. By designing systems whereby we can reduce the number of steps a free phosphine is involved with can significantly reduce the challenges associated with the synthesis of these reagents. This work focuses on designing

modular systems for the traceless Bertozzi-Staudinger reagent. We believed that designing methodologies for modification of the phosphine core at the end point of the synthesis would allow for the synthesis of a modular system that can address the limitations associated with the Staudinger reagents. In this regard we proposed two strategies; the first approach proposed was using host-guest chemistry to impart the required characteristics on the reagent without the requirement of a covalent modification; and, a second approach whereby a methodology is designed to attach modular handles, that can be used for end point attachment of moieties that can impart the required characteristics, to the phosphine core.

2.2 Host-guest chemistry towards a modular Staudinger probe

As previously stated in this chapter, the challenges associated with the synthesis of Staudinger reagents can be attributed to the instability of the phosphine moiety in solution. To address this, a host-guest approach was proposed where by a traceless Bertozzi-Staudinger core would be synthesized with a guest molecule attachment (**Fig. 2.3**). A host molecule would separately be synthesized with the characteristics that we would want to impart onto the reagent. The inclusion of the guest moiety on the phosphine core would then result in a reagent with the desired attributes. Because this approach requires no covalent modification to the phosphine core, we believed that it would significantly reduce the synthetic difficulties. To address the limitations with these reagents that have already been discussed, we aimed to choose a host molecule that it could be used to increase the aqueous solubility of the overall system. We believed that this strategy would enable us to design a system with mM aqueous solubility for

utilization in biological studies. An ability to functionalize the host molecule would also allow for the attachment of a quencher to enable FRET quenching of the host-guest complex, **115** (**Fig. 2.3**). Fluorescence could then be recovered upon reaction with an azide and the loss of proximity that would follow, **117** (**Fig. 2.3**).

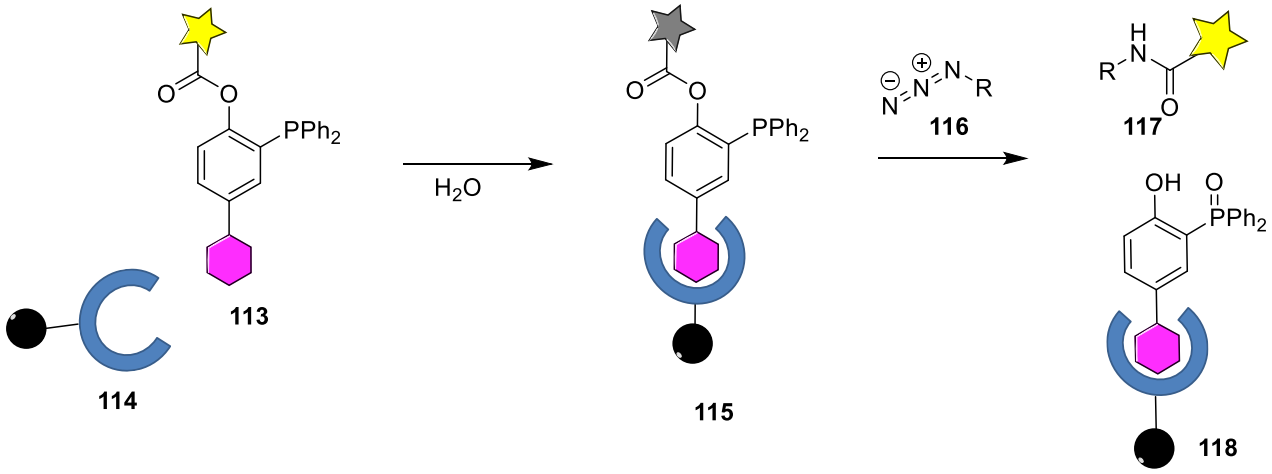


Figure 2.3 Host-guest design for a fluorogenic probe

In deciding the host molecule, we looked into biocompatibility, accessibility, an ability to be functionalized and a known ability to solubilize organic molecules. The cyclomaltooligosaccharides (cyclodextrins) fulfilled all of our requirements. Cyclodextrins are cyclic oligomers of α-D-glucopyranose that can be produced by the transformation of starch by certain bacteria such as *Bacillus macerans*application.[70] The most common forms of cyclodextrins are the alpha-cyclodextrin (5-sugars), beta-cyclodextrin (6-sugars) and gamma-cyclodextrin (7-sugars) (**Fig. 2.4**). They are non-toxic and are widely used in the food industry.[71]

Cyclodextrins have also been used in drug delivery[72] as well as chemical biology for labeling[73], imaging[74] and manipulation of interactions.[75] Selective functionalization was carried out in many of those cases which bodes well for our strategy of attaching additional functionalities.

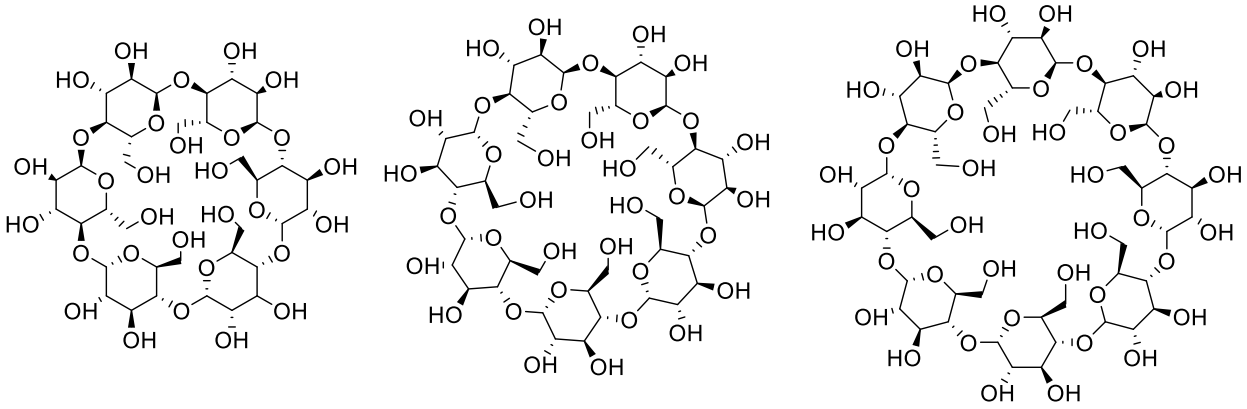


Figure 2.4 Common cyclodextrins

We then looked into the choice of a guest molecule. The interaction of the guest molecule with the host in aqueous conditions needed to be strong enough to ensure the stability of the complex. As much as possible we wanted the guest to be chemically inert to minimize possible side reactions during the synthesis of the phosphine core and during its application in biological studies. Adamantane, a group which has been used in a number of inclusion studies with cyclodextrin, fulfilled the requirements. This non-polar group has been reported to be solubilized in aqueous conditions using cyclodextrins. Adamantane and β-cyclodextrin form one of the strongest binding between a small molecule guest and host pair with a K_a of $3 \times 10^4\ M^{-1}$ in H_2O (**Fig. 2.5**).[76]

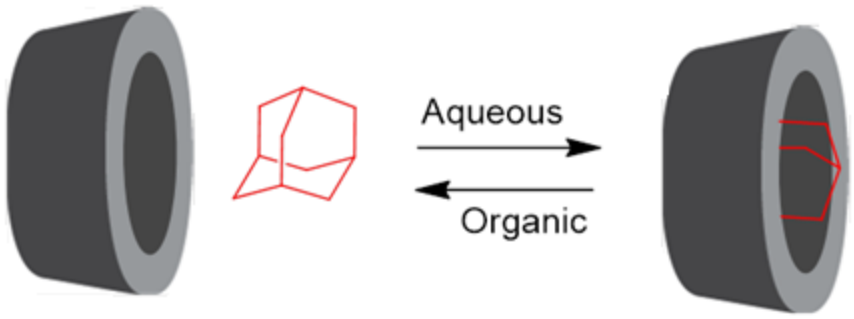
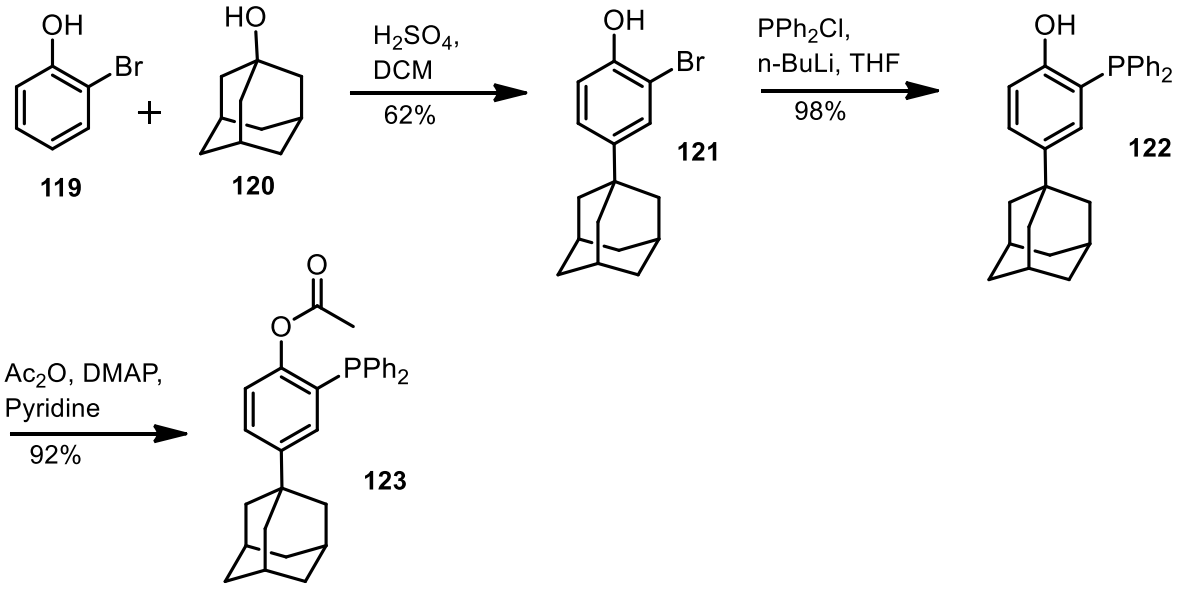


Figure 2.5 Adamantane-cyclodextrin host-guest interaction

With the choice of the guest and host we then synthesized adamantane modified traceless Bertozzi-Staudinger **123** (**Scheme 2.3**) was synthesized.[77]



Scheme 2.3 Synthesis of adamantane modified traceless Bertozzi-Staudinger reagent

Having the modified adamantane guest in hand we then performed inclusion experiments to make the adamantane-cyclodextrin complex. A biphasic ethyl acetate-D_2O solution was prepared and the phosphine and cyclodextrin in a 1:1 ratio were added. Slow removal of the organic layer under reduced pressures with mixing resulted in the formation of a white precipitate. The D_2O layer was then submitted to NMR, however no phosphorus peak could be observed. Attempts to dissolve the precipitate in deuterated organic solvent were also not successful and no phosphorus peak was observed in the NMR spectrum. We concluded that the complex that was formed was not soluble in the D_2O or deuterated organic solvents. Review of literature afforded a possible explanation of these results. It has previously been reported that β-cyclodextrin has a lower solubility than both the α and γ cyclodextrins.[78] This was explained by the fact that β-cyclodextrin induces stronger ordering on the surrounding water in comparison with the others. The enthalpy of solutions of the three CDs is very similar, but the entropy of solution of β-cyclodextrin is lower relative to the others leading to the lower solubility of β-cyclodextrin in water.

Even though β-cyclodextrin forms the strongest interaction with adamantane, α and γ cyclodextrins are also known to form complexes with α-cyclodextrins forming a 2:1 complex with adamantane.[76] However, attempts to form a soluble complex with phosphine **123** resulted in precipitation in a similar manner as that of the β-cyclodextrin.

Our next attempts were with commercially available β-cyclodextrin derivatives with reported higher aqueous solubility than the parent β-cyclodextrin. Randomly methylated-β-cyclodextrin, hydroxypropylated-β-cyclodextrin, and β-cyclodextrin sulfobutyl ethers sodium salts were subjected to inclusion conditions with phosphine **123**. No phosphorous signal could be detected by NMR in the aqueous solution from any of the complexes formed.

To investigate the possibility of using a polymer to solubilize the complex formed, a PEG (750) modified cyclodextrin was subjected to the inclusion conditions with phosphine **123**. A phosphorus peak was observed in D_2O by NMR. Comparing the NMR with that of the adamantane phosphine system that was subjected to the same condition in the absence of the cyclodextrin supported the hypothesis that a host-guest system could be used for solubilizing the traceless Staudinger-Bertozzi reagent. However, because of the low concentration (nM concentration) of phosphorus observed by NMR did not meet our stated goal for aqueous solubility (mM concentration) it was concluded that our second proposed approach of designing a reagent with a modular handle might be more widely useful.

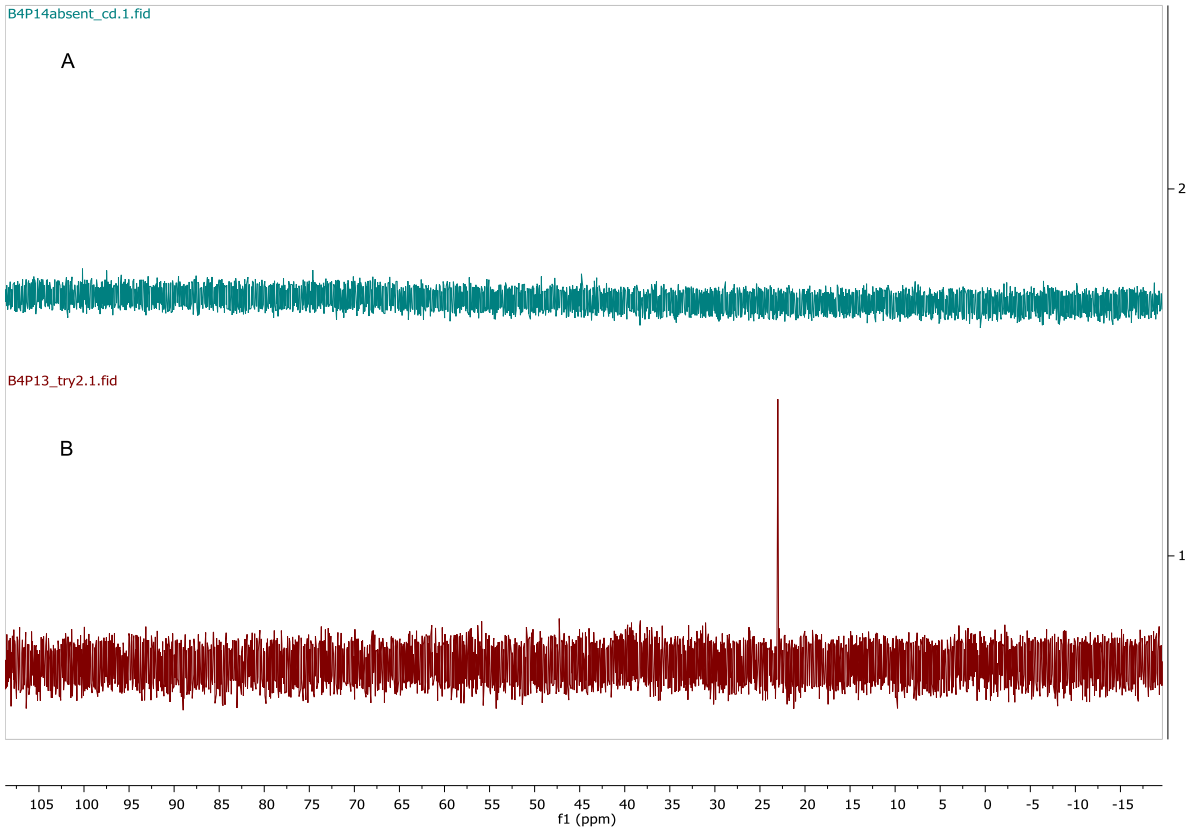
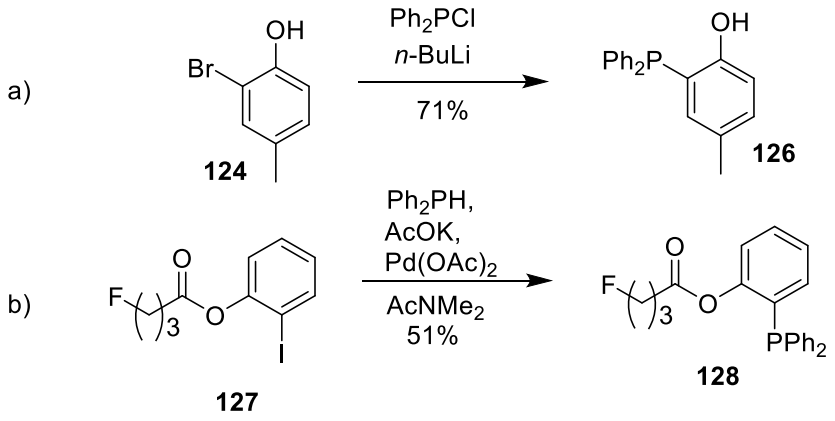


Figure 2.6 ^{31}P (202 MHz, D_2O) signal A) Adamantane functionalized phosphine B) Adamantane functionalized phosphine in the presence of PEG functionalized cyclodextrin

2.3 A method to synthesize a modular traceless Bertozzi-Staudinger reagent

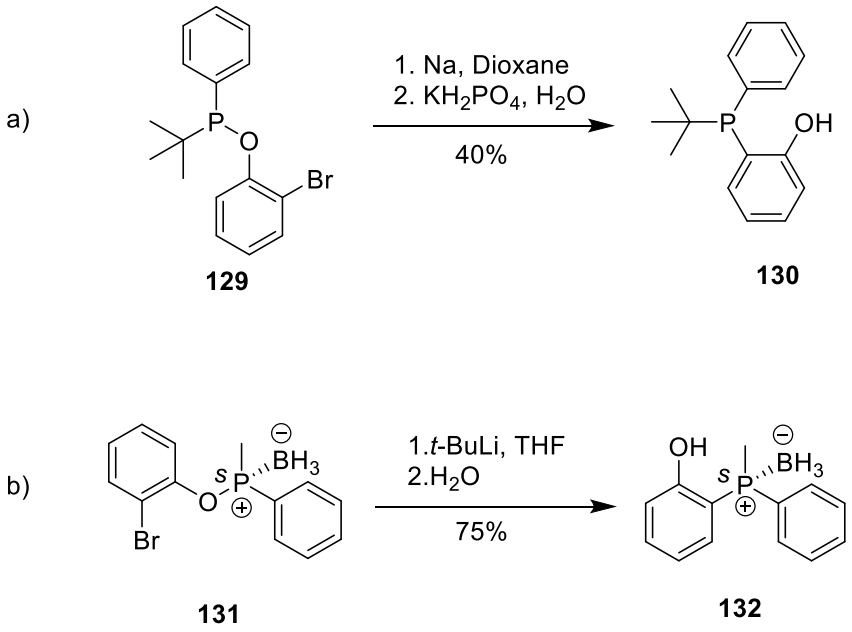
A challenge associated in the synthesis and functionalization associated with the synthesis of Bertozzi-Staudinger reagents especially electron rich ones such as the traceless Bertozzi-Staudinger reagent is the oxidation of the phosphine. To address this issue, we designed a one pot synthesis yielding a borane protected phosphine with a reactive modular handle for functionalization.

Current synthesis strategies for traceless Bertozzi-Staudinger ligation involve the reaction of the *ortho* halophenols, **124**(**Scheme 2.4**) with chlorodiphenylphosphine.[79] Another method that has been employed is the palladium catalyzed reaction of diphenyl phosphane with 2-iodophenol or its ester derivative, **127** (**Scheme 2.14**).[80] Both of these strategies result in the formation of an unmodified reagent core. These reactions also require the maintenance of highly stringent conditions for reaction. Our work with the synthesis of modified and unmodified phosphine cores for the traceless Bertozzi-Staudinger reagent led us to believe that a simplified synthesis with the capability for modular functionalization would be an important contribution to this field.



Scheme 2.4 Current strategies for synthesis of traceless Bertozzi-Staudinger reagents

In our aim to design a simplified synthesis, we were inspired by the work of the Tzschach group to make bis(*o*-hydroxyaryl) phosphine oxides and bis(*o*-hydroxyaryl) phosphonic acid derivatives as chelating ligands.[81] They showed that orthohalophenoxy phosphane and phosphinate compounds can undergo intramolecular *ortho* Fries-like rearrangement upon metalation with Mg, Na or Li (**Scheme 2.5 a**). In 2000, the Jugé group following a similar strategy synthesized P-stereogenic 2-hydroxyarylphosphine ligands where a borane-phosphine complex was used to maintain stereospecificity (**Scheme 2.5 b**).[82]

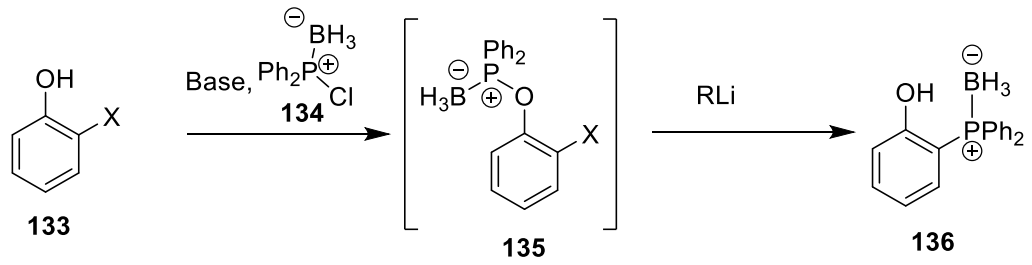


Scheme 2.5 Intramolecular Fries-like rearrangement of orthohalophenoxy phosphorus species

We realized that these rearrangements could yield the phosphine core of the traceless Bertozzi-Staudinger reagent. We were especially interested with the rearrangement yielding a protected phosphine (**Scheme 2.5 b**). Even though we were not concerned about the stereospecific nature of the reaction, the fact that the approach yielded a protected phosphine, which would enable us to do further modifications to the relatively stable phosphine-borane complex, was advantageous. We envisioned that these reactions might also require less stringent conditions than an intermolecular version of a lithium halogen exchange reaction that had been previously utilized to synthesize these reagents.

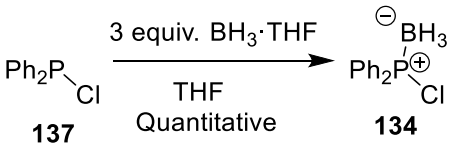
In our proposed reaction scheme, 2-halophenol **133** (**Scheme 2.6**), would be reacted with a borane protected chlorodiphenylphosphine **134** yielding the (2-halophenoxy)diphenylphosphane **135**. We envisioned that the intramolecular rearrangement can

be carried out in the same pot upon the addition of a lithium source giving the final borane protected phosphine core of the traceless Staudinger-Bertozzi reagent **136** in one pot.



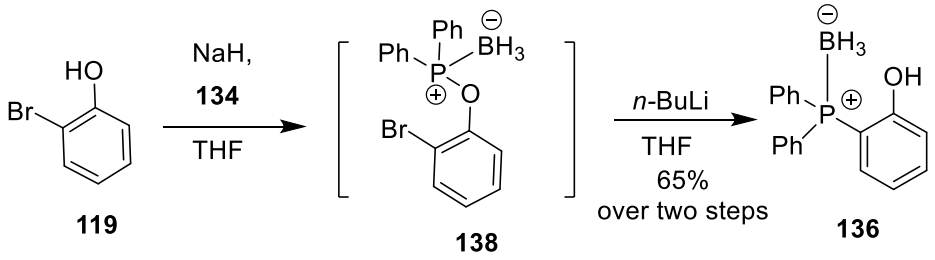
Scheme 2.6 Proposed one pot strategy for synthesis of traceless Bertozzi-Staudinger reagents

As an initial step, we looked into the synthesis of borane protected chlorodiphenylphosphine. Current published procedures involve the acidolysis of aminophosphine boranes.[83] However we believed that a simple reaction between the commercially available chlorodiphenylphosphine with borane would yield the desired product. The reaction was attempted with 3 equivalents of borane added to a solution of chlorodiphenylphosphine. This resulted in the quantitative formation of the desired product (**Scheme 2.7**). Borane complex formed was stored at 4 °C and was found to be stable over a period of a month.



Scheme 2.7 Synthesis of borane protected chlorodiphenylphosphine

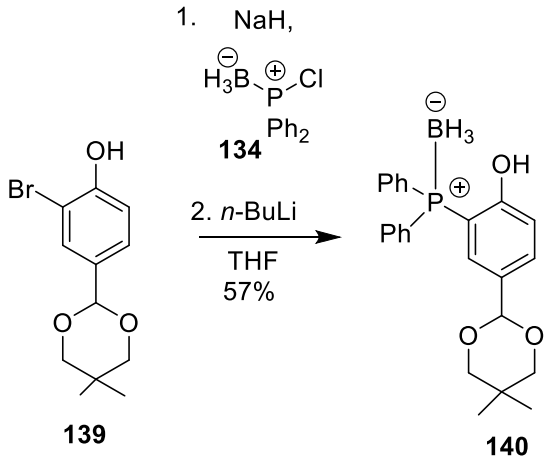
As a proof of concept, we first attempted to synthesize the commercially available unfunctionalized traceless Bertozzi-Staudinger reagent core (2-hydroxyphenyl)diphenylphosphine (**140**, **Scheme 2.8**). Our approach started by first making the phenolate of 2-bromophenol **119** by reaction with NaH. To this salt was added the previously synthesized borane protected chlorodiphenylphosphine **134**. After the completion of the reaction forming (2-bromophenoxy)diphenylphosphane borane **138** was confirmed by ^{31}P NMR. To this was added an equivalent of *n*-BuLi to initiate the rearrangement. Aqueous work up followed by sila gel column chromatography yielded the desired product, **136**, in 65% overall yield.



Scheme 2.8 Synthesis of (2-Hydroxyphenyl)diphenylphosphine

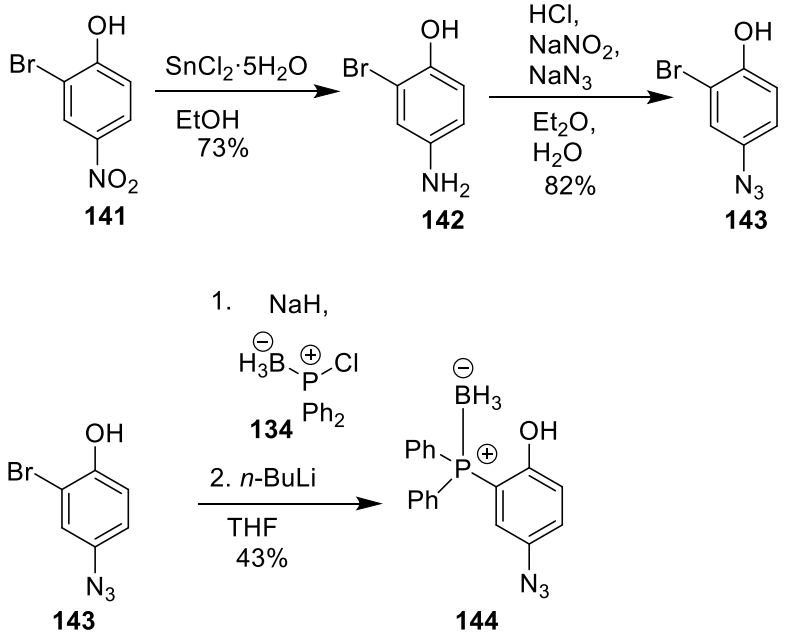
We then wanted to investigate if this approach could be used to install a modular handle to the reagent. For the choice of handle we wanted functionalities with known high fidelity reaction with a reaction partner. Another issue considered was the synthetic accessibility or commercial availability of functionalized versions of the reaction partner that could impart the desired property to the final reagent. With this in mind we looked to bioorthogonal reaction pairs.

The first handle we chose to synthesize was an aldehyde. The reaction of alpha effect nucleophiles and amine functionalities with aldehyde has been discussed in Chapter 1 of this work. These reactions can be carried out under mild conditions and are functional group tolerant. For the synthesis of the aldehyde-modified traceless Bertozzi-Staudinger reagent core, a protected aldehyde functionalized 2-bromophenol (**139**, **Scheme 2.9**), was subjected to the previously established conditions. The reaction yielded the desired protected aldehyde functionalized reagent, **140**, in 57% yield.



Scheme 2.9 Synthesis of aldehyde modified traceless Bertozzi-Staudinger reagent

Another functionality we wanted to investigate as a handle was an azide. Alkyne modified moieties with utility in chemical biology are widely commercially available. It has also been well established that a number of azide specific reactions discussed in chapter 1 of this work proceed in high yield under very mild conditions. The concern associated with this reaction was the known reaction of phosphines with organic azides, in fact the traceless Bertozzi-Staudinger reagent is designed as a reaction partner for this reagent. However, previously published works have shown that borane protected phosphines do not react with organic azides.[84] An azide functionalized 2-bromophenol **143** (**Scheme 2.10**), was synthesized in two steps from the commercially available 2-bromo-4-nitrophenol(**141**, **Scheme 2.10**). The product was then subjected to the established conditions to yield the desired azide modified traceless Bertozzi-Staudinger reagent, **144,** in 43 % yield.



Scheme 2.10 Synthesis of azide modified traceless Bertozzi-Staudinger reagent

Currently we are developing modular phosphine systems using the handles that have been discussed in this section. Current work and future directions will be discussed in greater detail in Chapter 4 of this work.

Chapter 3 : A bifunctionalized traceless Bertozzi-Staudinger-Triazabutadiene reagent

3.1 Introduction

Over the years many bioconjugation strategies have been developed. One of the main requirements for these reactions is the need for these reactions to be efficient and selective under mild conditions. A number of publications have leveraged these characteristics to develop modular systems combining two or more orthogonal systems from these reactions. In biological systems, this strategy has been utilized to incorporate secondary handles for purification and fluorescent labeling after a primary bioconjugation reaction[85] and in synthesis these systems have been used for sequential chemical transformations under mild conditions.[86]

In this work, we envisaged a bifunctionalized system that incorporates two bioconjugation methods that we have previously utilized in our lab, namely the Bertozzi-Staudinger reaction and the triazabutadiene systems (**Fig. 3.1**). We primarily designed this system as a reagent for the mild modification of both the phosphine and triazabutadiene systems. A label of interest, be it a fluorophore or purification handle, could be attached to the triazabutadiene system by the reaction of an azide form of the label with the phosphine of the bifunctionalized reagent. Conversely, the phosphine could be first modified by the reaction of the triazabutadiene system in the bifunctionalized reagent with an electron rich aromatic system containing label after diazonium ion formation (**Fig. 3.1**).

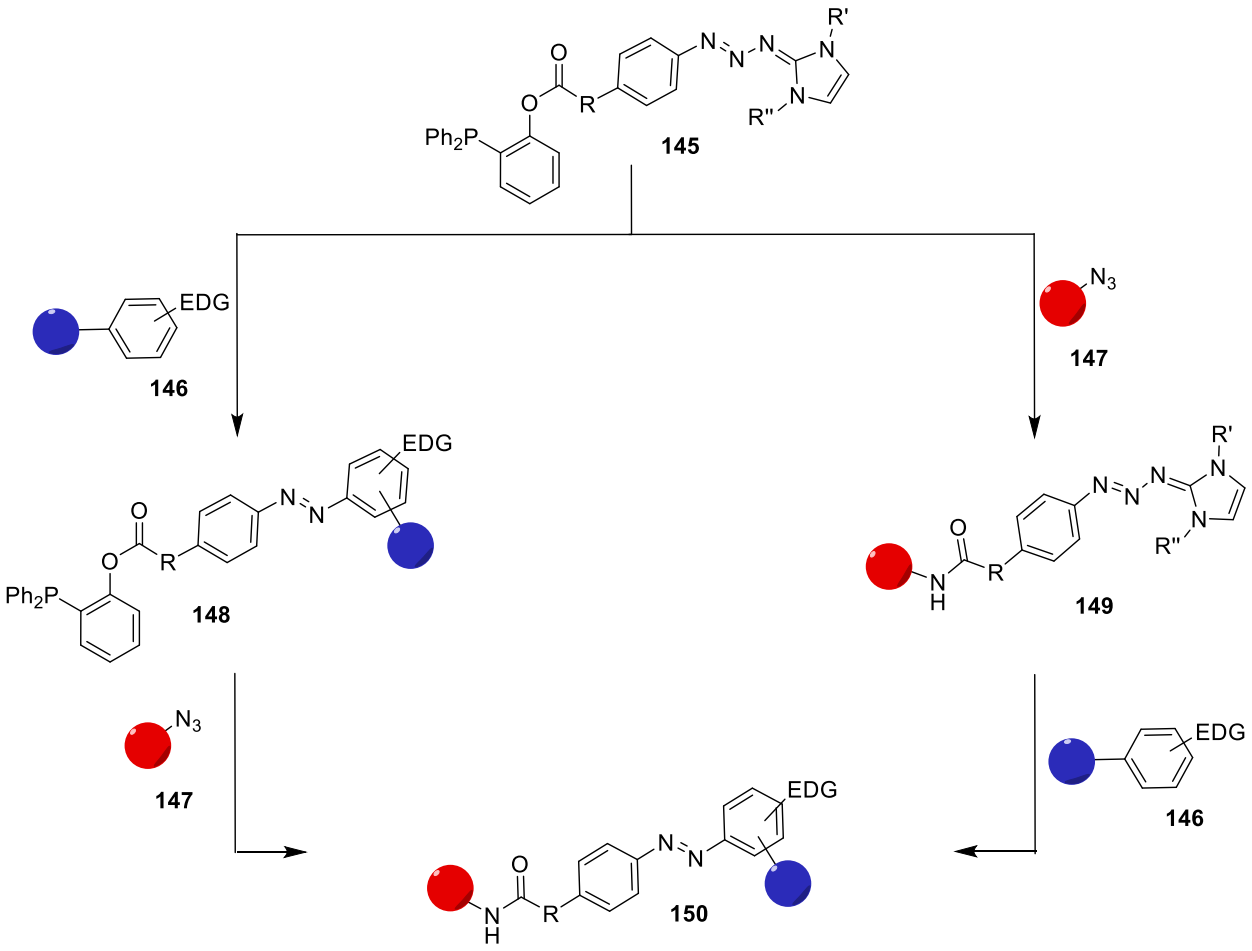
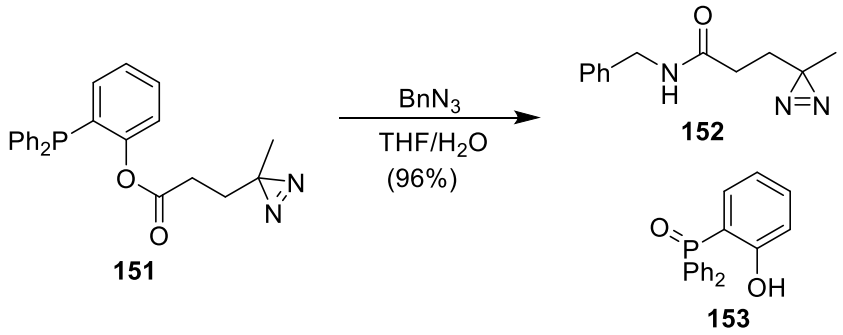


Figure 3.1 A bifunctionalized traceless Bertozzi-Staudinger-Triazabutadiene reagent

We chose the traceless-Bertozzi-Staudinger strategy for the phosphine moiety of our bifunctionalized system. As described in the previous chapter, a number probe partners have been developed to label azides. Our choice of the traceless Bertozzi-Staudinger reagent was due to the small size of the naturally occurring amide ligation product, which reduces the overall size and mass of the cargo, which in turn could reduce the perturbation resulting from the conjugation of the load onto the biomolecules[56]. These advantages have been utilized to deliver fluorescence dyes and radionuclides[87] and in the synthesis of peptides and proteins.[61, 88]

Previous in our lab a bifunctionalized system was designed containing a phosphine moiety and a cross linker, **151** (**Scheme 3.1**). This strategy was used to install an amide-linked diazirine on an azide-modified protein. This allowed us to attain a photo crosslinking capability without having to alter our established azide reporter incorporation strategy.[89]



Scheme 3.1 Model reaction of traceless Bertozzi-Staudinger reagent with a diazirine load with benzyl azide

Our lab has pioneered the work of another type of crosslinker, namely the triazabutadiene, a masked diazonium for labeling or crosslinking with tyrosine residues.[21] The choice of this reagent as one of the moieties in the bifunctionalized reagent gives us a similar capability for incorporation of a crosslinker on our azide-modified proteins as in our previous work. Additionally, this system could be used to attach a traceless Bertozzi-Staudinger moiety onto a tyrosine residue of a protein using the diazonium generated upon activation of the triazabutadiene moiety of the bifunctionalized reagent.

3.2. Synthesis of a bifunctionalized reagent

Two possible approaches for the synthesis of the bifunctionalized system were investigated. In the first approach the two components of the bifunctionalized system, namely the phosphine, **136** (**Fig. 3.2**) and the triazabutadiene, **154**, would be separately synthesized. After which the components would be conjugated as the last step of the synthesis, **155**. We believed that this approach would offer a combination of synthetic ease and a higher yield as it would enable us to reduce the steps that would be required involving the synthetically challenging phosphine moiety, which is prone to oxidation.

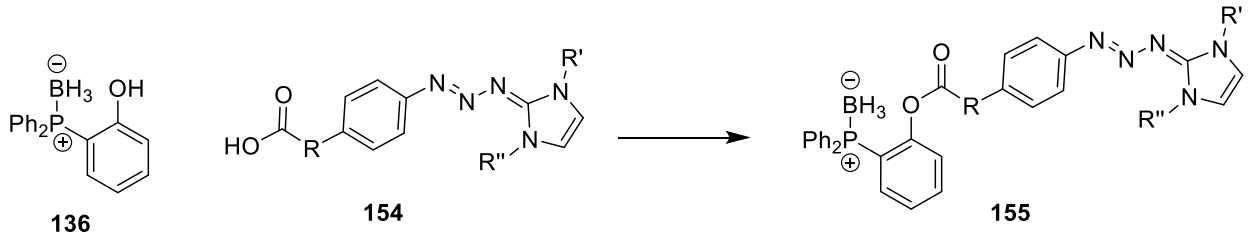


Figure 3.2 Direct conjugation approach to a bifunctionalized reagent

The second possible approach would involve the synthesis of the bifunctionalized reagent in a step wise manner, where by the two moieties would be synthesized from a conjugated core (**Fig. 3.3**).

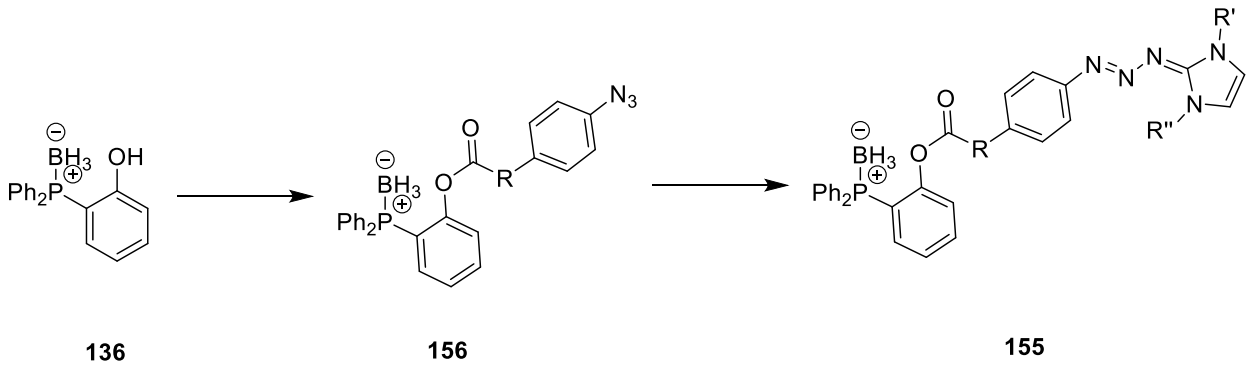
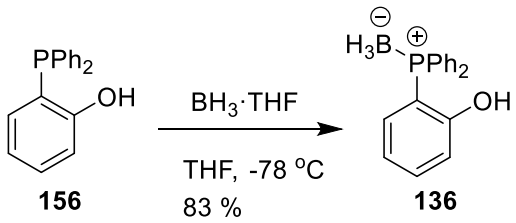


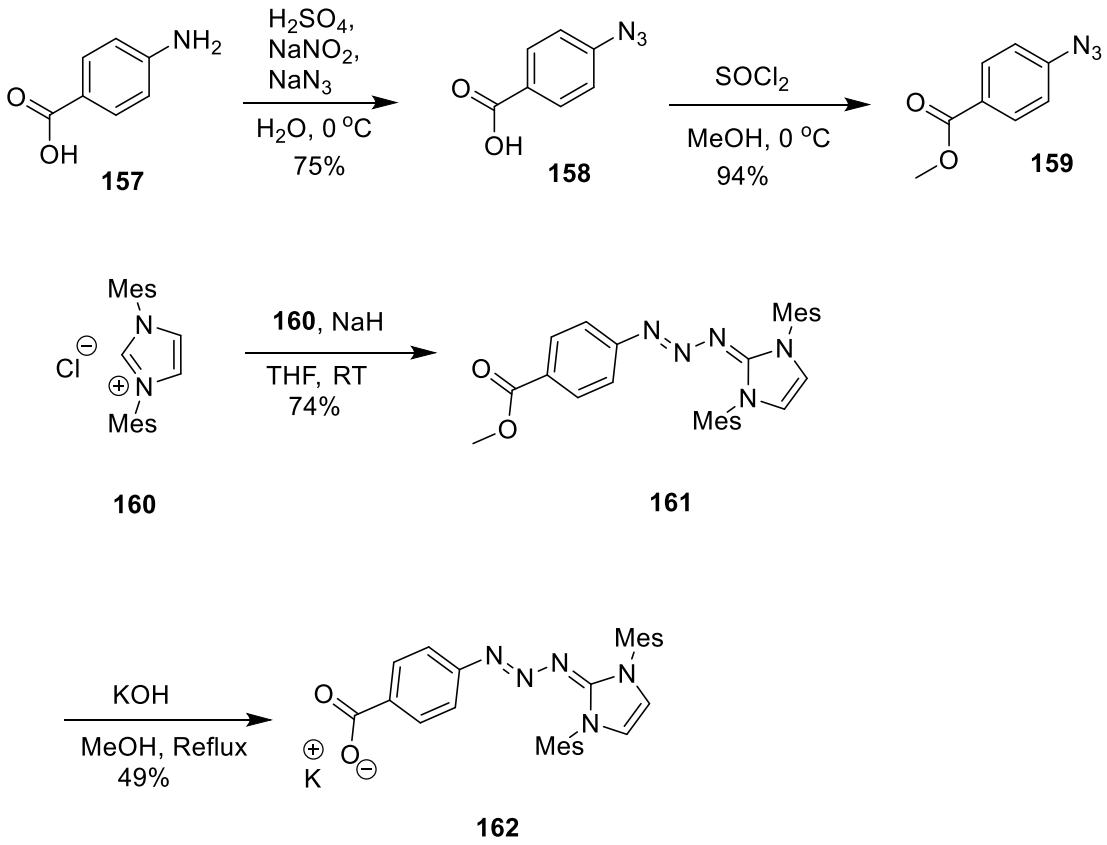
Figure 3.3 A stepwise approach to a bifunctionalized system

In the first approach, the traceless Bertozzi-Staudinger phosphine core of the reagent was initially synthesized by the modular method discussed in Chapter 2. The product was also synthesized by borane protection of the commercially available (2-hydroxyphenyl)diphenylphosphine (**156**, **Scheme 3.2**) following published procedure.[90]



Scheme 3.2 Synthesis of traceless Bertozzi-Staudinger core

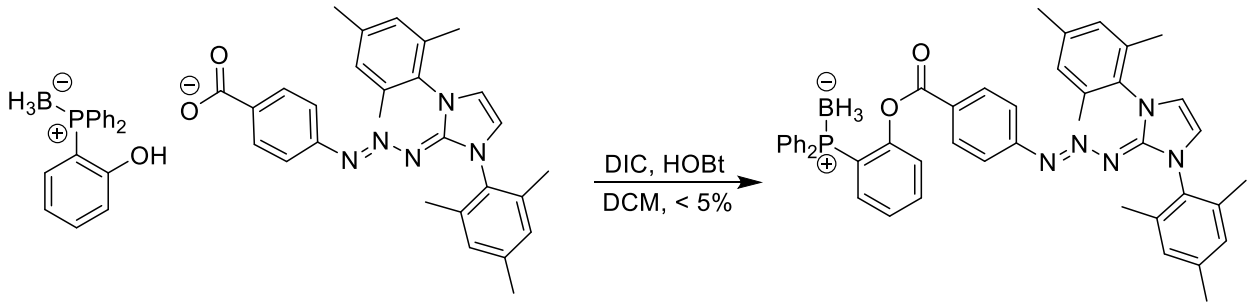
A carboxylic acid functionalized triazabutadiene was chosen as a reacting partner for the traceless Bertozzi-Staudinger core, **136**(**Scheme 3.2**) that was synthesized. 4-aminobenzoic acid (**157**, **Scheme 3.3**) was converted to the azide, **158**, following literature procedure.[91] Because an acidic proton would interfere with the subsequent steps, the benzoic acid was esterified in acidic methanol to give 4-azidomethylbenzoate **159.** The choice of the *N*-heterocyclic carbene (NHC) salt was made based on synthetic and stability concerns. The stability of the triazabutadiene generated would be mainly affected by the choice of the substitutions on the NHC salt.[22] Even though a more reactive triazabutadiene has certain advantages in biological settings, it also presents increased challenges in synthesis. For a proof of concept, it was decided the use of a stable dimesityl substitution would be a better option. The dimesityl substituted NHC salt, **160**, was synthesized following literature procedure.[23] A reaction of the salt with the previously synthesized 4-azidomethylbenzoate **159** yielded a methylbenzoate triazabutadiene, **161**.[23] To couple the triazabutadiene with the phosphine core, it was required that the methylbenzoate be hydrolyzed. However, because triazabutadienes are acid sensitive, the ester was hydrolyzed to the carboxylate salt rather than working it up to the acid, **162**.



Scheme 3.3 Synthesis of carboxylate triazabutadiene component

With the phosphine core, **136**, and carboxylate salt of the triazabutadiene, **162**, in hand, attempts were then made to couple the two components of the bifunctionalized reagent. However, making the ester link proved to be challenging. The use of (3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) for a traditional carbodiimide coupling of the two components resulted in the deprotection of the phosphine. Tertiary amines have been shown to deprotect borane protected phosphine species[92], it was therefore hypothesized that the tertiary amino group on EDC was the reason this was occurring. The use of N,N'-dicyclohexylcarbodiimide (DCC) and later N,N'-diisopropylcarbodiimide (DIC) was attempted.

Even though no deprotection was observed, these reactions yielded less than 5% of the bifunctionalized product, **163**(**Scheme 3.4.)**. Attempts to optimize these reactions further were not successful in increasing the yield. We believed this could be due to the sterics of the system.



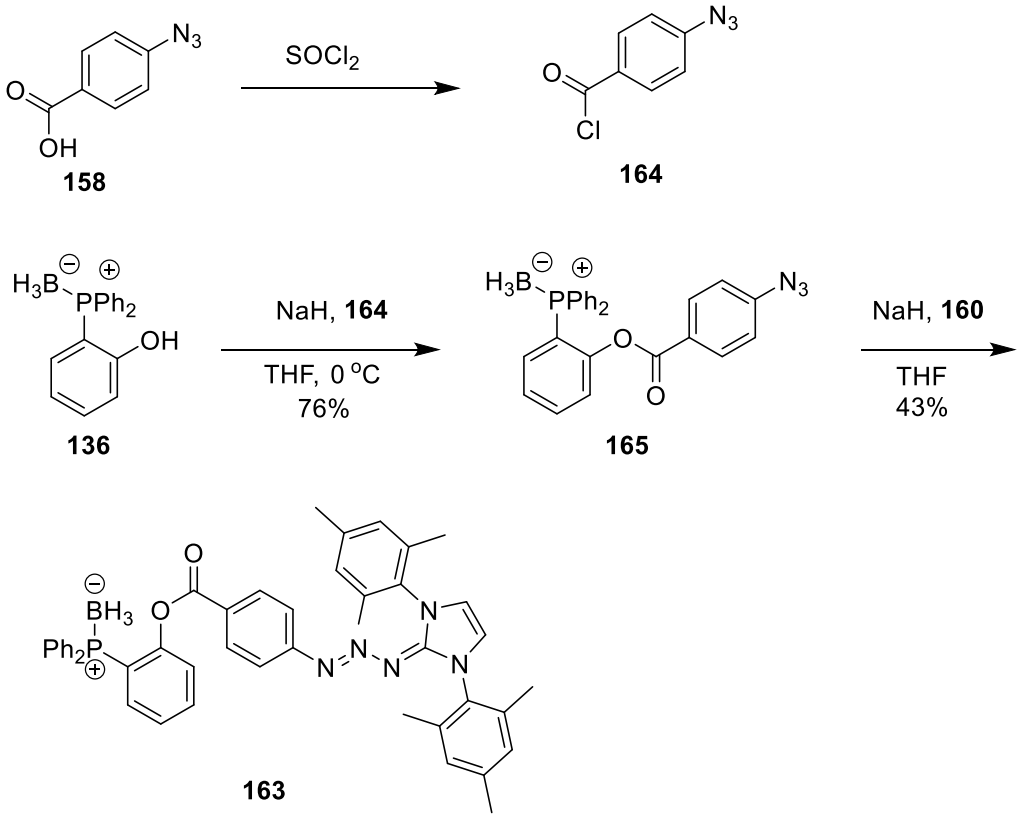
136 **162** **163**

Scheme 3.4 Attempts to couple components using carbodiimide chemistry

Knowing that benzoic esters of the traceless Bertozzi-Staudinger reagent have been previously synthesized a second approach was proposed. In this approach, the azidobenzene component of the triazabutadiene synthesis would be conjugated to the phosphine core in the first step. The triazabutadiene synthesis reaction would then be carried out between the azide that has been conjugated to the phosphine and an NHC salt to yield the bifunctionalized reagent (**Fig. 3.3**).

Initially the phosphine ester of 4-azidobenzoic acid was synthesized. A satisfactory yield of the ester, **165** (**Scheme 3.5**) was obtained from the reaction of the acid chloride of 4- azido benzoic acid, **164**, and the *in situ* generated phenolate of the phosphine core, **136**, The azide on

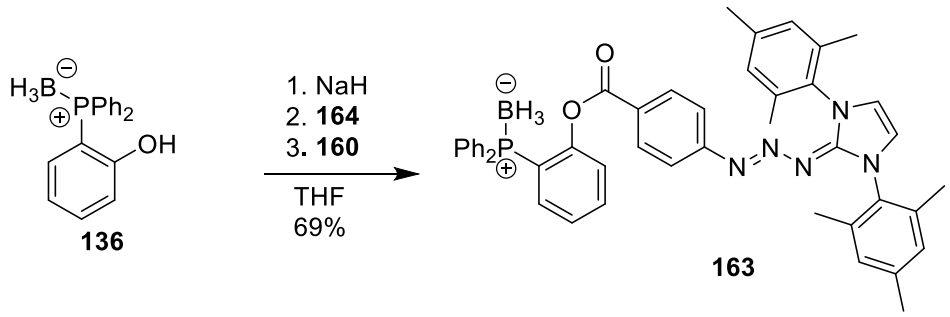
the phosphine was reacted with an in situ generated *N*-heterocyclic carbene in the next step. The known reaction of carbenes with boranes[93] was a cause for concern for the feasibility of this step. However, the bifunctionalized reagent was obtained in 43% yield, **163**.



Scheme 3.5 Two-step synthesis of bifunctionalized reagent

The observation of oxidized phosphine species during the purification process suggested that to increase the yield as well as for the sake of synthetic ease, the possibility of performing this reaction in one pot would be advantageous. Because both of the steps involved to make the

ester and triazabutadiene (**Scheme 3.5**) were performed under similar conditions, it was believed that this could be possible without significantly altering the procedures. An excess of NaH excess was used in the initial phase to generate the phenolate, to which was added the acid chloride, **164**(**Scheme 3.6**) to generate the ester intermediate. The NHC salt, **160**, was then added to the mixture after the intermediate ester had been formed. This resulted in the formation of the bifunctionalized reagent, **163**, in 69% yield, which was a greater than 2-fold increase over the previous two pot method.

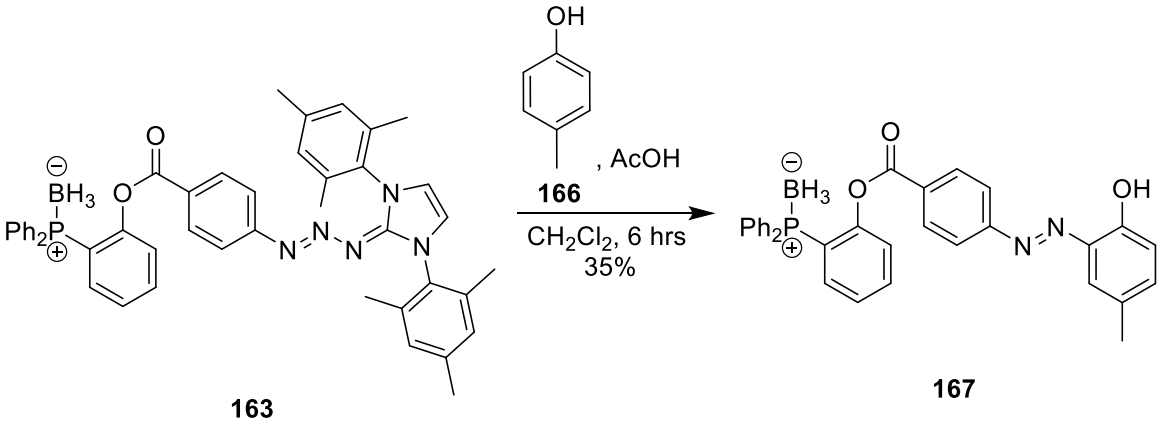


Scheme 3.6 A one pot synthesis of the bifunctionalized reagent

3.3 A bifunctionalized reagent for modifying traceless Bertozzi-Staudinger and triazabutadiene reagents

With the bifunctionalized reagent in hand, the orthogonality of the system and its synthetic utility was investigated to determine if this system could be used to modify or

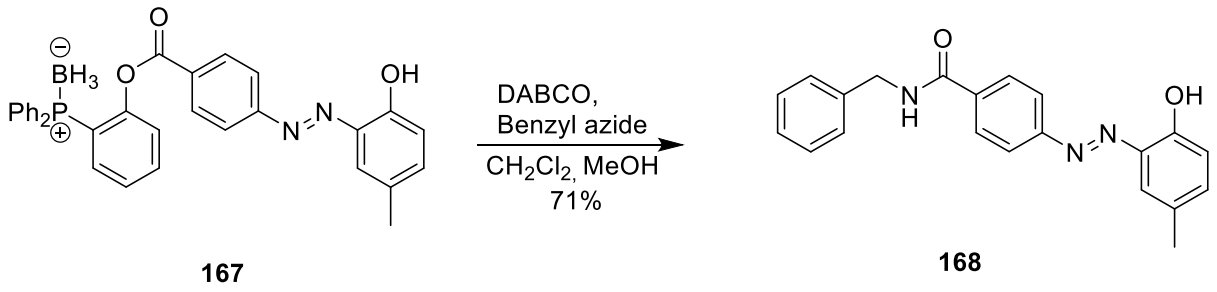
conjugate, under mild conditions, a cargo of interest to the traceless Bertozzi-Staudinger component of the system. The reagent was dissolved in dichloromethane with a model electron rich aromatic system, in this case *p*-cresol, **166**(**Scheme 3.7**). The solution was acidified by the addition of acetic acid and was allowed to stir at room temperature. This reaction lead to the formation of a traceless Bertozzi-Staudinger reagent functionalized with the *p*-cresol model cargo by an azo link, **167**. The phosphine system was stable under these mild conditions, and we envision that a wide variety of traceless Bertozzi-Staudinger reagents could be synthesized in this manner.



Scheme 3.7 A reaction to functionalize a traceless Bertozzi-Staudinger reagent

Next, we wanted to assess if the azo-modified traceless Bertozzi-Staudinger reagent from previous step was capable of conjugating with an azide containing moiety. To test this benzyl azide was synthesized following literature procedure.[94]

For phosphine reaction with organic azides to occur, deprotection of the phosphine is required. To avoid the isolation step of the oxidation prone free phosphine, a reaction condition was designed such that the deportection, using 1,4-diazabicyclo[2.2.2]octane (DABCO), was carried out in the presence of the benzyl azide yielding the amide linked benzyl azo system, **168**(**Scheme 3.8**).

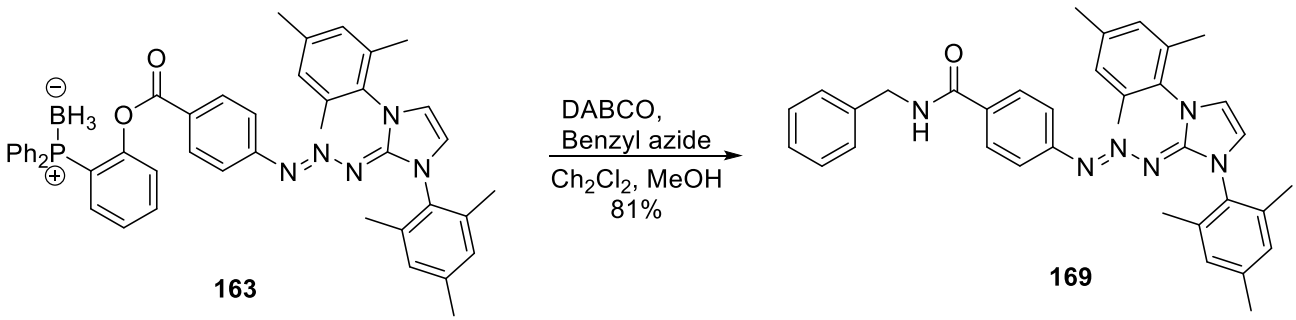


167 **168**

Scheme 3.8 Reaction of azo modified traceless Bertozzi-Staudinger reagent with benzyl-azide

After demonstrating that this system could be used to functionalize the traceless Bertozzi-Staudinger reagent, the possibility of using the bifunctionalized system to attach a label or cargo to the triazabutadiene system was then considered. The Staudinger ligation systems have been established to have exquisite selectivity for azides and that the reaction is highly functional group tolerant. To demonstrate this approach towards functionalizing a triazabutadiene system, benzyl azide and the bifunctionalized reagent, **163** (**Scheme 3.9**) were subjected to the same conditions previously used in the reaction the azo modified traceless Bertozzi-Staudinger product with

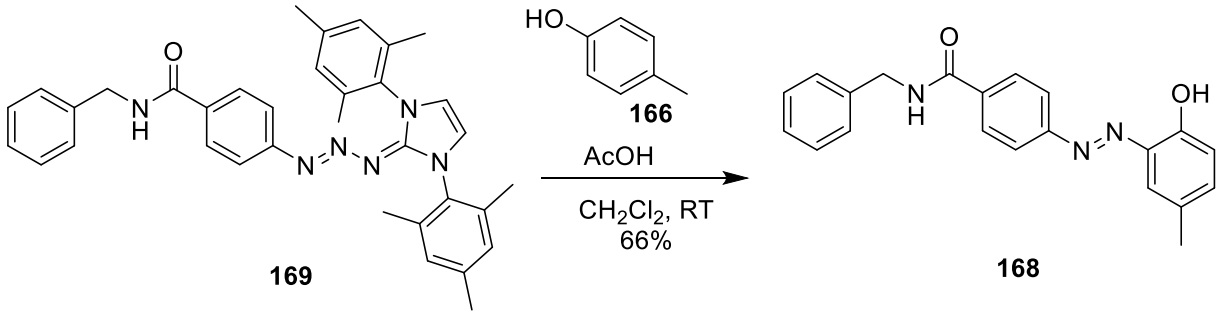
benzyl azide (**Scheme 3.8**). This reaction successfully yielded a benzyl functionalized triazabutadiene product, **169**(**Scheme 3.9**).



163 **169**

Scheme 3.9 A reaction to functionalize a triazabutadiene system

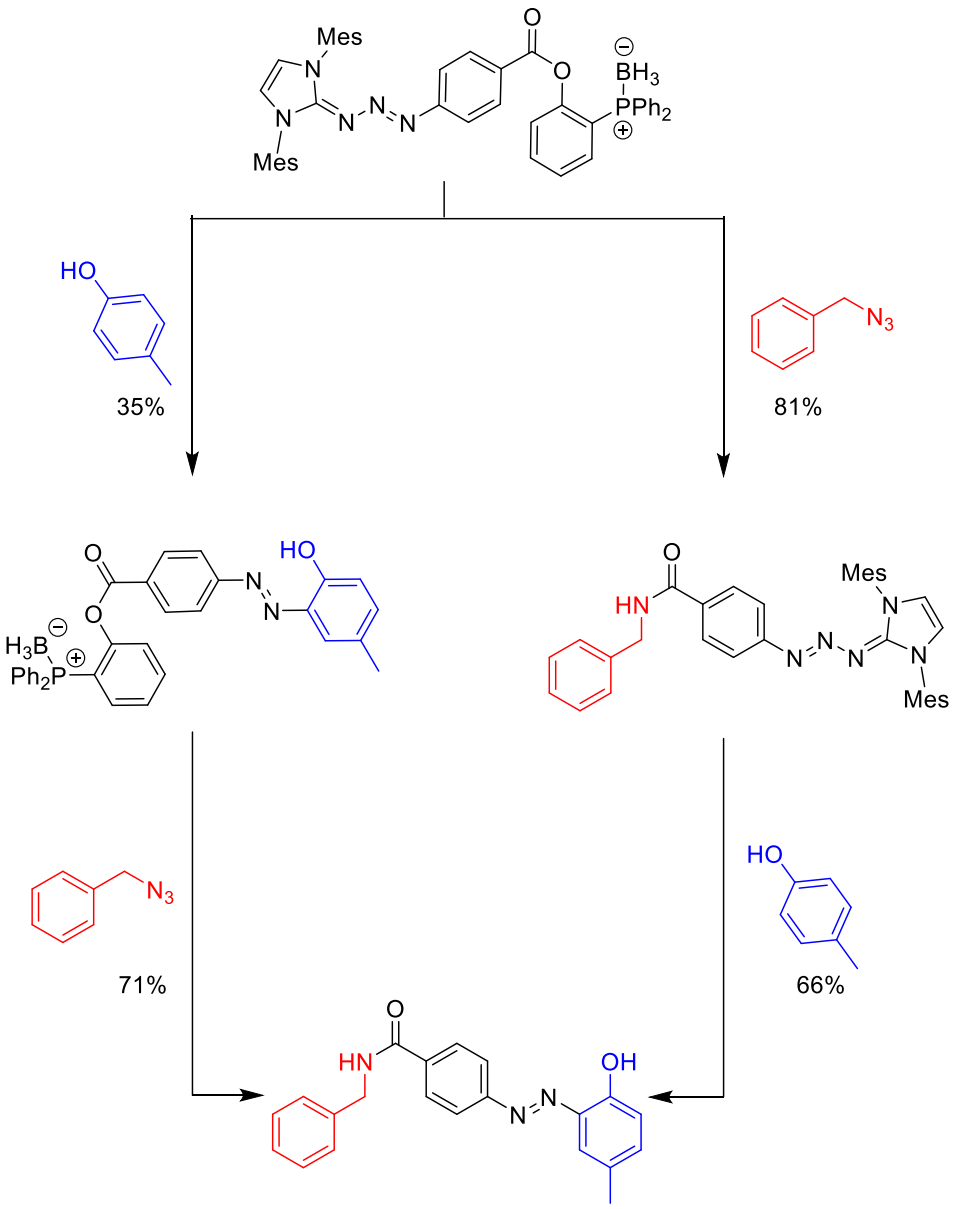
To establish whether the newly synthesized functionalized triazabutadiene can be used to label electron rich aromatic systems, it was reacted with *p*-cresol under similar conditions used for the reaction of the bifunctionalized reagent with *p*-cresol (**Scheme 3.7**). The expected benzyl labeled azo product was obtained from this reaction, **168** (**Scheme 3.10**).



169 **166** **168**

Scheme 3.10 Reaction of benzyl functionalized triazabutadiene with *p*-cresol

The bifunctionalized reagent proved to be modular and orthogonal allowing for modification of triazabutadienes and traceless Bertozzi-Staudinger reagents. The modified products also proved to react in the anticipated manner for the reagents. An overview of the reactions of the reagents is shown below (**Scheme. 3.11**).



Scheme 3.11 An overview of the reactions of the bifunctionalized traceless Bertozzi-Staudinger-triazabutadiene reagent

Chapter 4 : Future directions

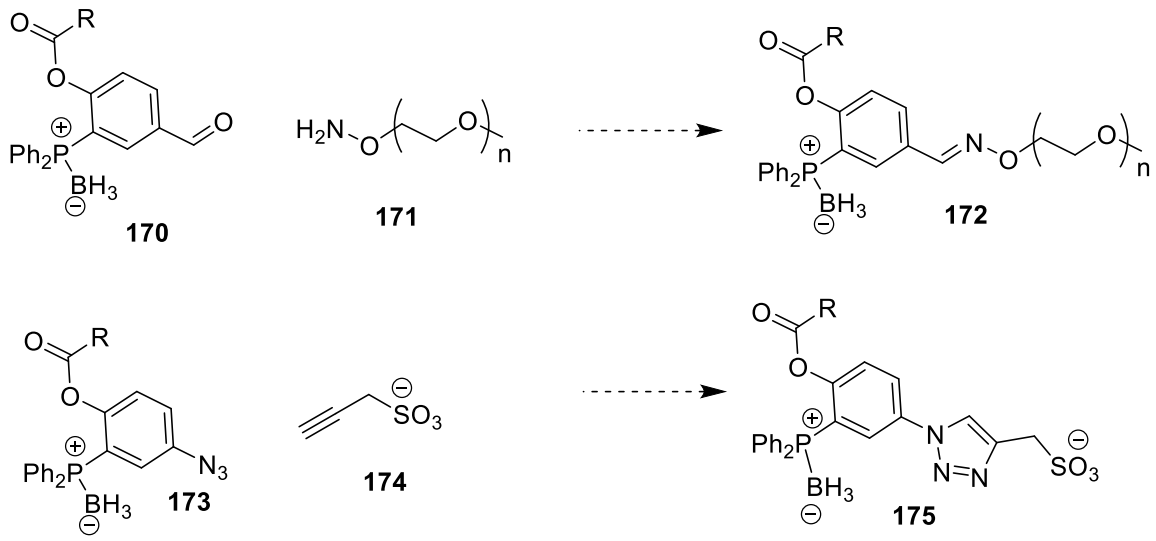
In this chapter, current work being conducted in our lab in regard to the projects discussed in this work and future directions will be discussed.

4.1 Modular traceless Bertozzi-Staudinger reagent

As described in chapter two of this work, the utility of the traceless Bertozzi-Staudinger reagents are limited by their low solubility in aqueous conditions. Current work that is being carried out in the lab aims to take advantage of the traceless Bertozzi-Staudinger reagents with modular handles that have been described in this work to address this limitations.

We envision that the aqueous solubility of these reagents can be increased by conjugation of a solubilizer to the traceless Bertozzi-Staudinger reagents using the modular handles. The proposed reagents with their synthetic scheme are shown below. In the case of the aldehyde modified reagent, **170** (**Scheme 4.1**) we propose a reaction with commercially available alpha effect nucleophile modified PEG solubilizer, **171**, will afford a PEG functionalized water-soluble traceless Bertozzi Staudinger reagent, **172**.

The azide modified reagent, **173**, can similarly be made water soluble by coupling to an alkyne modified solubilizer via copper catalyzed or strain promoted click reaction. A number of alkyne modified solubilizers are commercially available, in the proposed synthesis a commercially available sulfonate modified alkyne, **174**, is used to install a solubilizer group onto the traceless Bertozzi-Staudinger reagent.

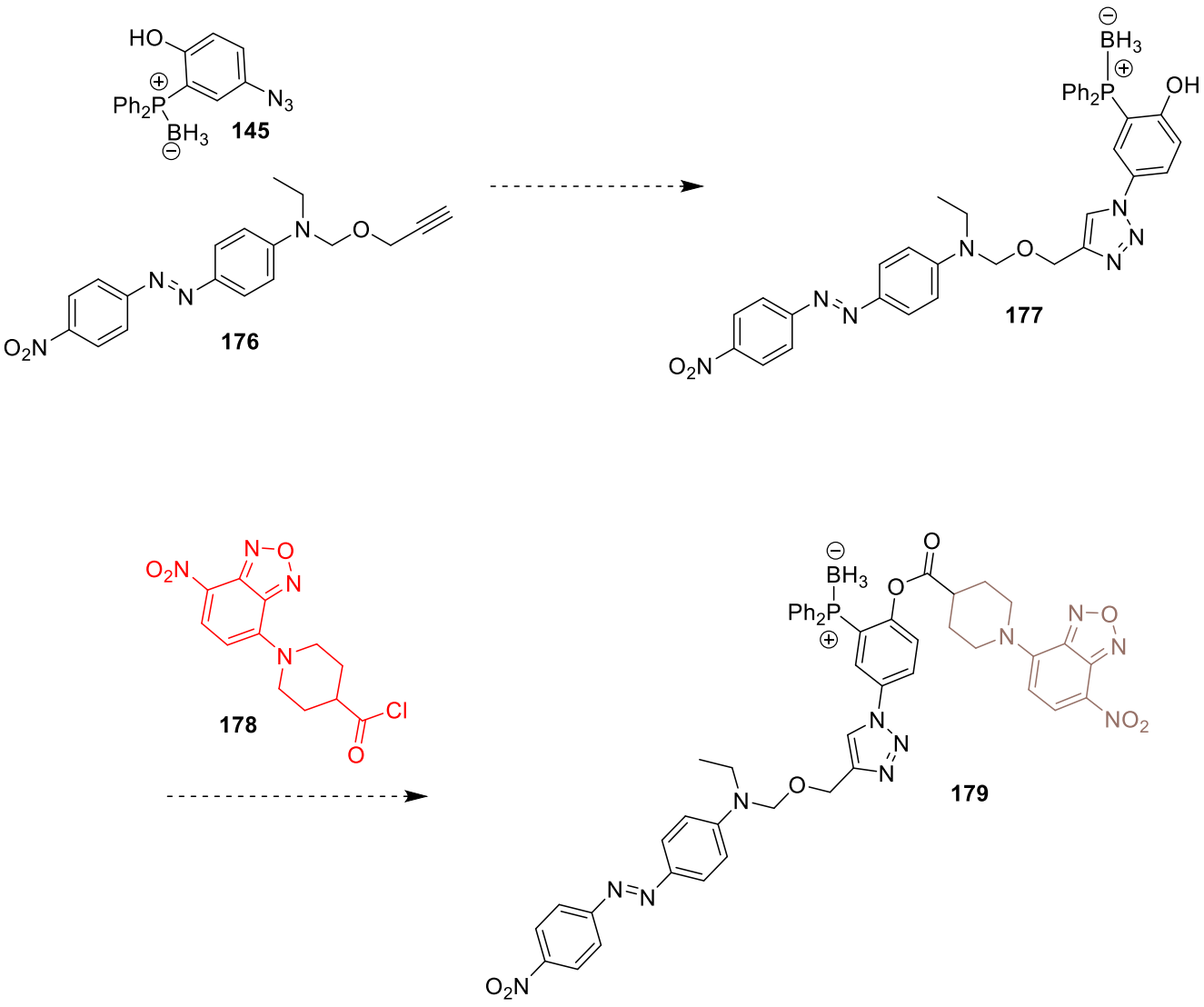


Scheme 4.1 Proposed schemes to install solubilizers onto modular traceless Bertozzi-Staudinger reagents

Another limitation we propose to address using this strategy is to design the first fluorogenic traceless Bertozzi-Staudinger reagent. As discussed in Chapter 2 of this work, fluorogenic versions of the Staudinger reagents have been synthesized, however background fluorescence and the challenge in the synthesis of these reagents has limited their application. We propose that the simplified synthesis developed in our lab and the modular nature of the handles that have been installed in these reagents by this strategy will offer an opportunity to design and synthesize a FRET based fluorogenic reagent that addresses these limitations.

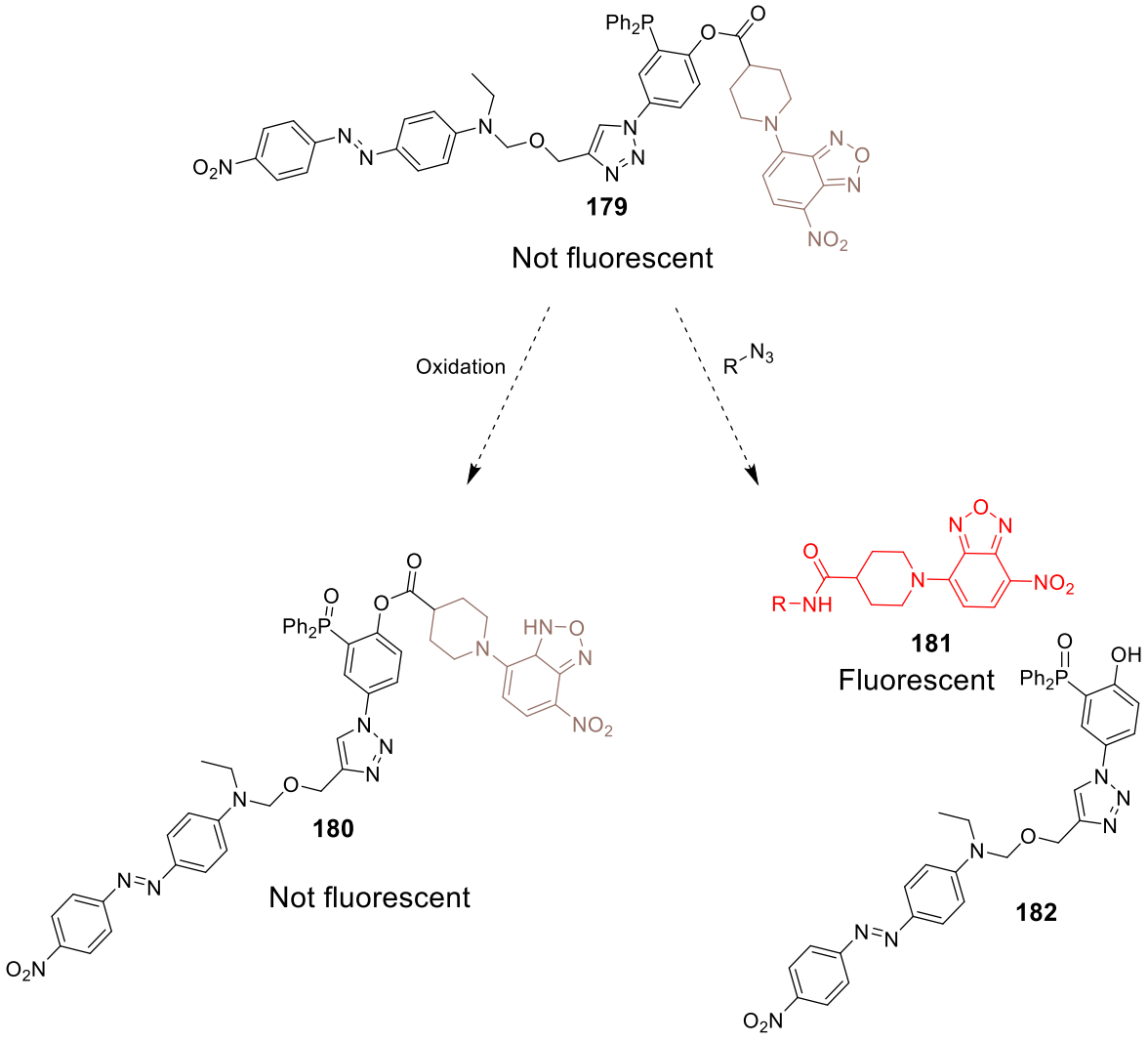
In our proposed scheme for the synthesis of the fluorogenic system, **179** (**Scheme 4.2**), an alkyne modified disperse red quencher, **176**, is installed onto the reagent by utilizing the azide handle. After which, the 4-nitro-2,1,3-benzoxadiazole (NBD) fluorophore, **178**, label can be

installed using acid chloride chemistry. The proximity of the fluorophore and quencher should result in reduction of fluorescent signal.



Scheme 4.2 Proposed scheme for the synthesis of a fluorogenic traceless Bertozzi-Staudinger reagent

The reaction of the proposed fluorogenic reagent with an organic azide would result in loss of proximity of the fluorophore and the quencher resulting in the recovery of fluorescence of the label, **181** (**Scheme 4.3**). This reagent addresses the background fluorescence limitation associated with the coumarin based fluorogenic Bertozzi-Staudinger ligation reagent previously reported and discussed in Chapter 2 of this work, as the oxidation of the phosphine prior to reaction with an azide, **180**, would not result in the loss of proximity of the fluorophore and quencher there by maintaining the quenching effect of the unreacted reagent.



Scheme 4.3 Reaction of the fluorogenic system with organic azide and molecular oxygen

4.2 A bifunctionalized traceless Bertozzi-Staudinger-triazabutadiene reagent

As described in Chapter 3 of this work, the bifunctionalized Bertozzi-Staudinger-triazabutadiene reagent developed in our lab allows for the modular functionalization of the traceless Bertozzi-Staudinger reagent as well as for the triazabutadiene systems. Current work in our lab aims to take advantage of this capability to synthesize a variety of the functionalized systems with the aim of utilizing them in biological studies.

Proposed modifications are designed with the label that is desired to be delivered to in vitro and in vivo samples that are currently being investigated in our lab. The mild conditions required for this functionalization allows us the capability to incorporate a wide variety of labels. Currently, work is being carried out to functionalize these reagents with fluorescent labels, **183** & **184** (**Figure 4.1**), and purification handles, **185** & **186**. Some of the proposed products are shown below.

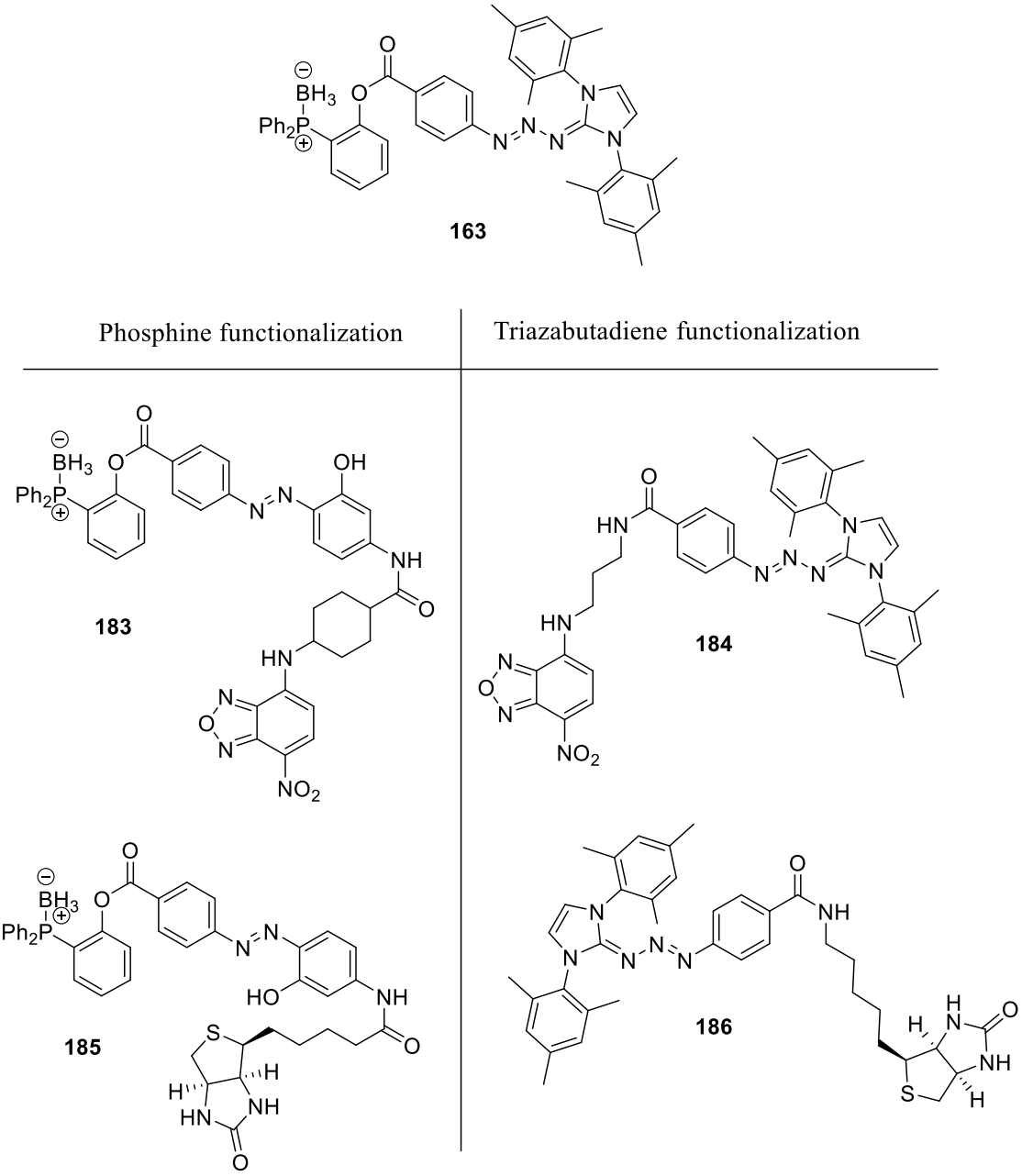
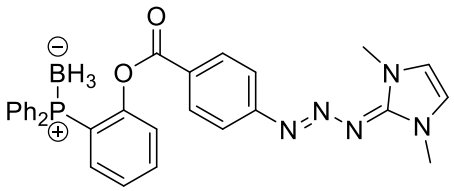


Figure 4.1 Proposed products from functionalization reaction of the bifunctionalized reagent: **184**, Fluorophore cargo functionalized traceless Bertozzi-Staudinger reagent; **185**, Fluorophore cargo functionalized triazabutadiene; **190**, Biotin functionalized traceless Bertozzi-Staudinger reagent for purification and pull down; **191**, Biotin functionalized triazabutadiene reagent for purification and pull down

Another proposed application for this reagent is to deliver a triazabutadiene at azide modified locations of biological samples. Understanding protein interactions between host and viral proteins is a major area of research in the Jewett lab. As part of this work, strategies to incorporate azide functionalities onto viral proteins have been developed. The azide functionalities have been used to fluorescently label viral surfaces. An ability to deliver a crosslinking capability to the viral surface without having to design a new crosslinker incorporation strategy but rather utilizing the azide functionalities would be advantageous. This strategy could also be utilized by the wider chemical biology community to perform similar modifications. However, the pH required to activate the triazabutadiene moiety of the current designed reagent might not be appropriate for some studies. With this in mind, a reagent that can be activated at higher pH is being designed, **187** (**Figure 4.2**).



187

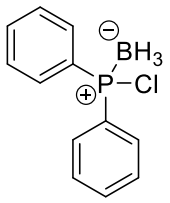
Figure 4.2 Proposed bifunctionalized system for activation at higher pH

Chapter 5 : Experimental Section

5.1. Experimental conditions and characterizations

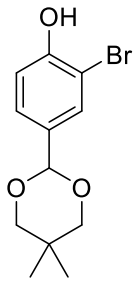
All reactions were performed under an argon atmosphere, with the flasks oven dried and cooled in a vacuum oven prior to use. *n*-BuLi, chlorodiphenyl phosphine, 3-bromo-4-hydroxybenzaldehyde, 2,2-dimethyl-1,3-propanediol, *p*-toluenesulfonic acid, $SnCl_2 \cdot 5H_2O$, 2-bromo-4-nitrophenol, 2-bromophenol, (2-hydroxyphenyl)diphenylphosphine, sodium hydride and the solvents were commercially obtained and used as received. ^{1}H, ^{13}C and ^{31}P NMR data were acquired on Bruker DRX-400 and referenced with residual solvent peaks; $CDCl_3$ 7.26 ppm and 77.0 ppm; $(CD_3)_2SO$ 2.50 ppm and 39.5 ppm; D_2O 4.80 ppm. Coupling constants are expressed in hertz and abbreviations for multiplicities given as s = singlet, d = doublet, t = triplet, dd = doublet of doublets, ddd = doublet of doublets of doublets, td = triplet of doublets, and m = multiplet where applicable. High resolution mass spectral analysis was performed on a Bruker ICR-ESI. Low-res GC/MS was run on a Shimadzu GCMS QP2010s.

5.1.1. Chlorodiphenylphosphineborane (134)



To a solution of Chlorodiphenylphosphine (234 mg, 1.0 mmol, 1.0 equiv.) in dry THF (5 ml), was added borane tetrahydrofuran complex 1 M solution (3 ml, 3.0 mmol, 3.0 equiv.) and allowed to stir at that temperature for under argon 3 hours, at 0 °C. The resulting solution was then concentrated and used in the next reaction without further purification.

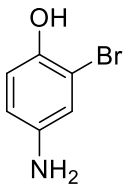
5.1.2. 2-bromo-4-(5,5-dimethyl-1,3-dioxan-2-yl)phenol (139)



To a solution of 3-bromo-4-hydroxybenzaldehyde (400 mg, 2.0 mmol, 1 equiv.) in toluene (10 ml) was added 2,2-dimethyl-1,3-propanediol (208 mg, 2.0 mmol, 1 equiv.) and *p*-toluenesulfonic acid (17 mg, 0.1 mmol, 0.05 equiv.) and refluxed overnight in a Dean-Stark apparatus. Solution was cooled to room temperature and washed with 10% $NaHCO_3$ solution (3 X 10 ml), H_2O (1 X

10ml) and brine (1 X 10 ml). Organic layer was dried with $MgSO_4$, filtered and concentrated. Residue was purified by flash chromatography (hexane: ethyl acetate). The desired compound was obtained as a white solid. (406 mg, 71%). R_f 0.33 (hexane/ethyl acetate, 7:3). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.33 (s, 1H), 7.48 (dd, J = 2.1, 0.5 Hz, 1H), 7.22 (ddd, J = 8.4, 2.1, 0.5 Hz, 1H), 6.92 (d, J = 8.3 Hz, 1H), 5.30 (s, 1H), 3.71 – 3.49 (m, 4H), 1.16 (s, 3H), 0.74 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 154.22, 131.20, 130.58, 126.69, 115.76, 108.57, 99.83, 76.46, 29.72, 22.71, 21.36. HRMS calculated for $C_{12}H_{15}BrO_3$. [M-H]- 285.01208, measured 285.01368.

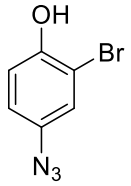
5.1.3. 4-amino-2-bromophenol (142)



To a mixture of 2-bromo-4-nitrophenol (1.09 g, 5 mmol, 1 equiv.) and $SnCl_2 \cdot 5H_2O$ (5.25 g, 15 mmol, 3 equiv.) was added 20 ml of absolute ethanol was added and heated at 70 °C for 2 hours. Solution allowed to cool to room temperature and was poured into an ice slurry resulting in the formation of a precipitate. Saturated solution of sodium bicarbonate was added until pH was neutral. To the suspension was added ethyl acetate (50ml) and filtered. The organic layer was separated, washed with brine, dried over $MgSO_4$ and filtered. Solution was concentrated to give the desired product as a light yellow solid (0.683 g, 73%). NMR in chloroform matched reported spectrum.[95] ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (s, 1H), 6.71 (d, J = 2.6 Hz, 1H), 6.65 (d, J =

8.5 Hz, 1H), 6.41 (dd, J = 8.5, 2.7 Hz, 1H). HRMS calculated for C_6H_6BrNO. [M-H]- 187.97055, measured 187.97087.

5.1.4. 4-azido-2-bromophenol (143)



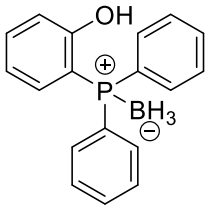
A suspension of 4-amino-2-bromo-phenol (335 mg, 1.8 mmol) in water (2.5 mL), was cooled to -5 ^{o}C and concentrated hydrochloric acid (0.5 mL) added slowly. A solution of sodium nitrite (130 mg, 1.9 mmol, 0.1M), in water was added dropwise while stirring. The resulting solution was stirred for 30 min at -5 ^{o}C. The reaction mixture was poured into a solution of sodium azide (130 mg, 2 mmol, 0.12M) in water and ice (5 g). The solution was extracted with diethyl ether, washed with brine and dried over $MgSO_4$. Solution was concentrated to yield the desired product. (315 mg, 82 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.30 (s, 1H), 7.22 (m, 1H), 6.99 – 6.94 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 152.18, 131.41, 123.67, 120.02, 117.63, 110.40.

5.2 General procedure for the synthesis of phosphine

To a solution of 2-bromophenol (2 mmol, 1 equiv.) in 5 ml THF was added NaH (1.2 equiv.) in THF at 0 °C and stirred at this temperature for 2 hours. The reaction was then cooled to -78 °C

and previously synthesized chlorodiphenylphosphine borane (1 equiv.) in 2 ml THF was added drop wise over 15 minutes. The reaction was allowed to stir at this temperature for 2 hours and an additional 2 hours at room temperature. . After which the reaction was cooled to -78 °C and *n*-BuLi (1 equiv.) was added dropwise and the reaction stirred for an additional 2 hours at this temperature. The reaction was then quenched by the addition of water. To the solution was added 10 ml ethyl acetate and washed with water (1 X 10) and brine (1 X 10). Organic layer was dried over MgSO4, filtered and concentrated. The residue was subjected to flash chromatography (hexane: ethyl acetate) to yield the desired product.

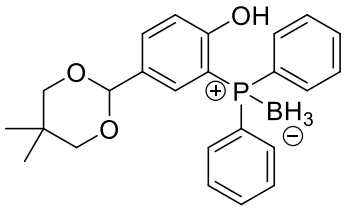
5.2.1 (2-Hydroxyphenyl)diphenylphosphine borane (136)



Following the general procedure, 2-bromophenol (344 mg, 2 mmol, 1 equiv.) in 5 ml THF was added NaH (1.2 equiv.) and chlorodiphenylphosphine borane (1 equiv.) were reacted followed by the addition of *n*-BuLi (1 equiv.) yielding a white solid. (380 mg, 65%). R_f 0.25 (hexane/ethyl acetate, 4:1). NMR matched reported spectrum.[96] ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.61 – 7.53 (m, 6H), 7.52 – 7.42 (m, 5H), 7.03 (m, 1H), 6.99 – 6.89 (m, 2H). ^{13}C NMR (101 MHz, CDCl3) δ 160.72, 160.62, 134.55, 134.52, 134.15, 134.13, 133.18, 133.08, 131.76, 131.73,

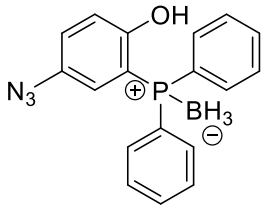
129.16, 129.05, 128.41, 127.79, 120.83, 120.75, 118.67, 118.60, 112.10, 111.52, 77.16. ^{31}P NMR (162 MHz, $CDCl_3$) δ 12.86.

5.2.2. 4-(5,5-dimethyl-1,3-dioxan-2-yl)- (2-Hydroxyphenyl)diphenylphosphine Borane (140)



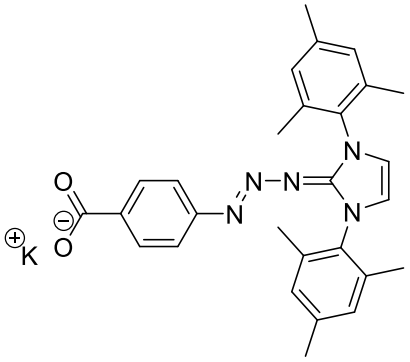
Following the general procedure, 2-bromo-4-(5,5-dimethyl-1,3-dioxan-2-yl)phenol (**139**) (572 mg, 2 mmol, 1 equiv.) in 5 ml THF was added NaH (1.2 equiv.) and chlorodiphenylphosphine borane (1 equiv.) were reacted followed by the addition of n-BuLi (1 equiv.) yielding a white solid. (464 mg, 57%). R_f 0.28 (hexane/ethyl acetate, 4:1). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.61 (dddd, *J* = 8.5, 2.2, 0.9, 0.4 Hz, 1H), 7.59 – 7.50 (m, 6H), 7.48 – 7.41 (m, 4H), 7.08 – 7.03 (m, 1H), 7.01 (dd, *J* = 8.5, 5.1 Hz, 1H), 3.66 (dt, *J* = 11.3, 1.4 Hz, 2H), 3.56 – 3.51 (m, 2H), 1.18 (s, 3jH), 0.75 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.92, 133.13, 133.03, 132.65, 132.08, 131.58, 131.56, 128.99, 128.88, 128.07, 127.46, 118.47, 101.03, 30.08, 22.82, 21.81. ^{31}P NMR (162 MHz, $CDCl_3$) δ 12.84. HRMS calculated for $C_{24}H_{28}BO_3P$. [M-H]- 407.19461, measured 407.19597.

5.2.3. 4-azido-(2-Hydroxyphenyl)diphenylphosphine borane (144)



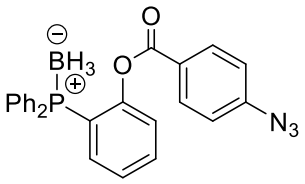
Following the general procedure, 4-azido-2-bromophenol (**143**) (426 mg, 2 mmol, 1 equiv.) in 5 ml THF was added NaH (1.2 equiv.) and chlorodiphenylphosphine borane (1 equiv.) were reacted followed by the addition of n-BuLi (1 equiv.) yielding a white solid. (367 mg, 43%). R_f 0.25 (hexane/ethyl acetate, 4:1). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.73 – 7.65 (m, 5H), 7.54 – 7.43 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.60, 133.01, 132.91, 132.46, 131.87, 129.15, 129.04, 127.53, 126.92, 124.42, 124.16, 119.99. ^{31}P NMR (162 MHz, $CDCl_3$) δ 15.67.

5.3.1 Potassium (E)-4-((1,3-dimesityl-1,3-dihydro-2H-imidazol-2-ylidene)triaz-1-en-1-yl)benzoate (162)



To a solution of the methyl ester compound (**164**) (480 mg, 1 mmol) in 5.0 mL methanolic potassium hydroxide. This mixture was heat to reflux and upon conversion to the potassium salt (5h, reaction followed by TLC, more potassium hydroxide added as required), the solvent was removed by rotavap to complete dryness. The residue left was picked up in DCM and filtered (Eastman filter paper) to get rid of excess KOH, giving the final product as a yellow solid. (249 mg, 49 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.48 – 7.45 (d, J = 8.5 Hz, 2H), 7.15 (s, 2H), 7.08 (m, 4H), 6.22- 6.18 (d, J = 8.5 Hz, 1H), 2.35 (s, 6H), 2.08 (s, 12H). ^{13}C NMR (101 MHz, DMSO) δ 168.63, 151.26, 150.77, 138.70, 137.95, 134.57, 134.12, 129.01, 128.85, 119.05, 117.60, 39.52, 20.69, 17.44.

5.3.2. 2-(diphenylphosphanyl)phenyl 4-azidobenzoate borane (165)

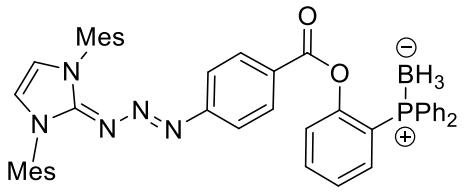


To 4-azidobenzoic acid (325 mg, 1 mmol, 1.2 equiv.) was added $SOCl_2$ and the solution was refluxed for 2 hours. The solution was concentrated and used in the next step.

To (2-Hydroxyphenyl)diphenylphosphine Borane (240 mg, 0.83 mmol, 1 equiv.) in THF (10ml) was added NaH (24 mg, 1 mmol, 1.2 equiv.) at 0 °C and stirred at this temperature for 30

minutes. The azide chloride that was previously synthesized was dissolved in minimal amount of THF and added dropwise to the solution. Reaction was allowed to gradually warm to room temperature overnight. Reaction was quenched by addition of water. Ethyl acetate (10 ml) was added to the solution and the organic layer washed with water (1 X 10), Brine (1 X 10) and dried under $MgSO_4$. The solution was filtered and concentrated and the residue was purified by sila gel column chromatography (hexane: ethyl acetate). Desired product was obtained as a light yellow solid. (270 mg, 76 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.70 – 7.57 (m, 7H), 7.46 – 7.40 (m, 2H), 7.36 (m, 5H), 7.31 – 7.20 (m, 2H), 6.95 – 6.87 (m, 2H). 13C NMR (101 MHz, CDCl3) δ 163.07, 152.56, 145.40, 134.83, 134.76, 133.28, 133.18, 132.87, 132.25, 131.41, 128.95, 128.85, 128.43, 127.85, 126.23, 126.14, 125.00, 124.28, 118.63, 77.16. ^{31}P NMR (162 MHz, $CDCl_3$) δ 18.99. HRMS calculated for $C_{25}H_{21}BN_3O_2P$. [M-H]- 440.11585, measured 440.11706.

5.4. 2-(diphenylphosphanyl)phenyl (E)-4-((1,3-dimesityl-1,3-dihydro-2H-imidazol-2-ylidene)triaz-1-en-1-yl)benzoate borane (163)



Method 1

To a solution of 2-(diphenylphosphanyl)phenyl 4-azidobenzoate borane, (**168**), (50 mg, 0.11 mmol, 1 equiv.) and Dimesityl NHC Salt, (**163**), (40 mg, 0.11 mmol, 1 equiv.) in THF was added NaH (1.5 equiv.) in one portion and allowed to stir for 6 hours under argon. Ethyl acetate (10 ml) was added and the organic layer washed with brine (1 X 10 ml). Organic layer was dried over $MgSO_4$ and concentrated. Residue was purified by sila gel column chromatography with hexane-ethyl acetate eluent to yield the desired product. (35 mg, 43 %) R_f 0.35 (hexane/ethyl acetate, 7:3). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.67 – 7.65 (m, 1H), 7.65 – 7.62 (m, 1H), 7.62 – 7.60 (m, 1H), 7.38 – 7.32 (m, 4H), 7.32 – 7.26 (m, 8H), 7.02 – 7.00 (m, 4H), 6.65 (s, 2H), 6.44 – 6.40 (d, J = 8.7 Hz, 0H), 2.38 (s, , 6H), 2.17 (s, 12H). ^{13}C NMR (101 MHz, CDCl3) δ 164.34, 153.28, 139.57, 135.33, 134.21, 133.50, 133.08, 131.58, 131.55, 131.11, 129.86, 129.19, 129.09, 128.83, 128.25, 126.24, 126.14, 124.83, 124.78, 124.68, 122.87, 122.31, 120.91, 117.86, 77.16, 21.65, 18.40. ^{31}P NMR (162 MHz, Chloroform-*d*) δ 18.74. HRMS calculated for $C_{24}H_{28}BO_3P$. [M-H]- 742.34845, measured 742.34834.

Method 2

To 4-azidobenzoic acid (325 mg, 1 mmol, 1.2 equiv.) was added $SOCl_2$ and the solution was refluxed for 2 hours. The solution was concentrated and used in the next step.

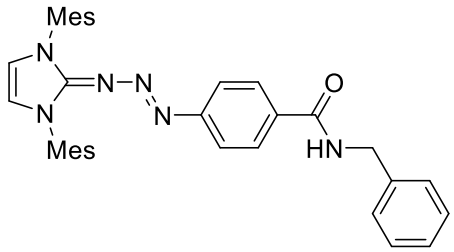
To (2-Hydroxyphenyl)diphenylphosphine Borane (240 mg, 0.83 mmol, 1 equiv.) in THF (10ml) was added NaH, (3.3 mmol, 4 equiv.) at 0 °C and stirred at this temperature for 30 minutes under argon. The azide chloride that was previously synthesized was dissolved in minimal amount of THF and added dropwise to the solution. Reaction was allowed to gradually warm to room temperature over 8 hrs. To the solution was added Dimesityl NHC Salt, (**163**), (340 mg,1 mmol,

1.2 equiv.) in one portion. Reaction was allowed to stir over night. . Ethyl acetate (10 ml) was added and the organic layer washed with brine (1 X 10 ml). Organic layer was dried over $MgSO_4$ and concentrated. Residue was purified by sila gel column chromatography with hexane-ethyl acetate eluent to yield the desired product. (424 mg, 69 %). R_f 0.35 (hexane/ethyl acetate, 7:3).

5.5. General condition for the reaction of benzyl azide with phosphine borane

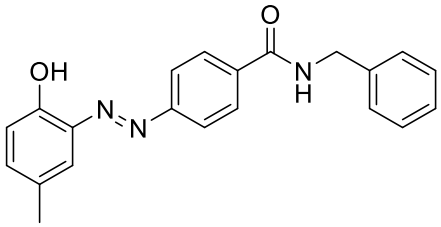
To a solution of phosphine borane (1 equiv.) and azide (1.5 equiv.) in CH_2Cl_2 was added 1,4-diazabicyclo[2.2.2]octane (DABCO) (1.3 equiv.) and solution allowed to stir overnight. To the solution was then added MeOH and stirred for an additional one hour. Ethyl acetate (10 ml) was added and the organic layer washed with brine (1 X 10 ml). Organic layer was dried over MgSO4 and concentrated. Residue was purified by sila gel column chromatography

5.5.1. (E)-N-benzyl-4-((1,3-dimesityl-1,3-dihydro-2H-imidazol-2-ylidene)triaz-1-en-1-yl)benzamide (169)



Following the general procedure, the bifunctionalized reagent (**163**) (100 mg, 0.13 mmol, 1 equiv.) and benzyl azide (1.5 equiv.) in CH_2Cl_2 (5 mL) was added 1,4-diazabicyclo[2.2.2]octane (DABCO) (1.3 equiv.) yielded product as a yellow solid (59 mg, 81%)**.** R_f 0.4 (hexane/ethyl acetate, 6:4). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.85 (t, J = 6.0 Hz, 1H), 7.60 – 7.49 (m, 2H), 7.34 – 7.26 (m, 4H), 7.09 (p, J = 0.6 Hz, 4H), 7.09 (p, J = 0.6 Hz, 1H), 6.41 – 6.29 (m, 1H), 4.44 (d, J = 6.0 Hz, 2H), 2.36 (t, J = 0.7 Hz, 6H), 2.08 (d, J = 0.6 Hz, 12H). ^{13}C NMR (101 MHz, DMSO) δ 138.66, 134.94, 129.37, 128.69, 127.96, 127.55, 127.11, 120.36, 118.55, 21.14, 17.84. HRMS calculated for $C_{24}H_{28}BO_3P$. [M-H]- 557.30234, measured 557.30605.

5.5.2. (E)-N-benzyl-4-((2-hydroxy-5-methylphenyl)diazenyl)benzamide (168)

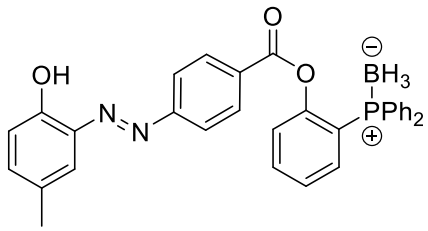


Following the general procedure, 2-(diphenylphosphanyl)phenyl (E)-4-((2-hydroxy-5-methylphenyl)diazenyl)benzoate borane (**167**) (30 mg, 0.06 mmol, 1 equiv.) and benzyl azide (1.5 equiv.) in CH_2Cl_2 (3 mL) was added 1,4-diazabicyclo[2.2.2]octane (DABCO) (1.3 equiv.) yielded product as a brown solid (14 mg, 71%)**.** R_f 0.3 (hexane/ethyl acetate, 1:1). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (s, 1H), 9.19 (t, *J* = 6.0 Hz, 1H), 8.23 – 7.93 (m, 4H), 7.56 (dd, *J* = 2.2, 1.0 Hz, 1H), 7.35 – 7.32 (m, 4H), 7.29 – 7.21 (m, 2H), 6.98 (d, *J* = 8.4 Hz, 1H), 4.51 (d, *J* = 6.0 Hz, 1H), 2.29 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 166.14, 153.49, 153.35, 139.84, 138.67, 136.39, 135.61, 129.26, 128.94, 128.80, 127.71, 127.31, 122.89, 122.51, 118.61, 43.20, 20.36.

5.6. General condition for reaction of triazabutadiene with *p*-cresol

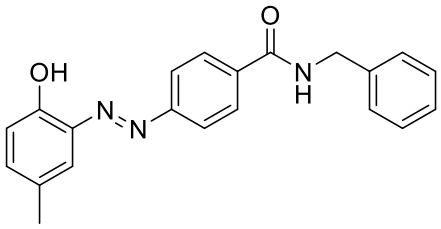
To a solution of the triazabutadiene (1 equiv.) and *p*-cresol (3 equiv.) in CH_2Cl_2 was added acetic acid (10 equiv.). The solution was stirred at room temperature for six hours. The organic layer was washed several times with water, brine and was dried over $MgSO_4$ and concentrated. Residue was purified by sila gel column chromatography (hexane : ethyl acetate).

5.6.1 2-(diphenylphosphanyl)phenyl (E)-4-((2-hydroxy-5-methylphenyl)diazenyl)benzoate borane (167)



To a solution of the triazabutadiene (200 mg, 0.27 mmol, 1 equiv.) and *p*-cresol (87 mg, 0.81 mmol, 3 equiv.) in CH_2Cl_2 was added acetic acid (10 equiv.). The solution was stirred at room temperature for six hours. The organic layer was washed several times with water, brine and was dried over $MgSO_4$ and concentrated. Residue was purified by sila gel column chromatography (hexane : ethyl acetate). (50 mg, 35%). Rf 0.3 (hexane/ethyl acetate, 1:1). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (s, 1H), 8.14 – 8.08 (m, 2H), 8.04 – 7.99 (m, 2H), 7.62 – 7.54 (m, 6H), 7.51 – 7.42 (m, 9H), 7.23 (ddt, *J* = 8.4, 2.3, 0.6 Hz, 1H), 6.95 (d, *J* = 8.3 Hz, 1H), 2.32 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 150.98, 136.19, 134.42, 134.38, 133.99, 133.60, 133.04, 132.95, 132.70, 132.06, 131.96, 131.60, 131.57, 129.81, 129.01, 128.90, 128.67, 128.31, 127.70, 122.38, 120.67, 120.59, 118.52, 118.46, 118.22, 20.30. ^{31}P NMR (162 MHz, $CDCl_3$) δ 18.91. HRMS calculated for (oxidized under MS conditions) $C_{32}H_{25}N_2O_4P$. [M-H]- 555.14441, measured 555.14342.

5.6.2. (E)-N-benzyl-4-((2-hydroxy-5-methylphenyl)diazenyl)benzamide (168)



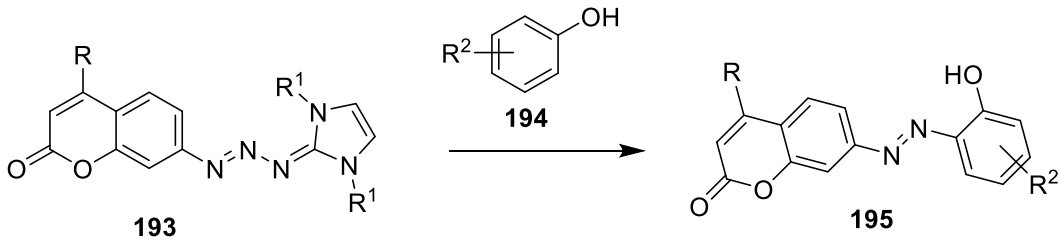
To a solution of the triazabutadiene (20 mg, 0.036 mmol, 1 equiv.) and *p*-cresol (12 mg, 0.11 mmol, 3 equiv.) in CH_2Cl_2 was added acetic acid (10 equiv.). The solution was stirred at room temperature for six hours. The organic layer was washed several times with water, brine and was dried over $MgSO_4$ and concentrated. Residue was purified by sila gel column chromatography (hexane : ethyl acetate). (10.4 mg, 81%). R_f 0.3 (hexane/ethyl acetate, 1:1). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (s, 1H), 9.19 (t, *J* = 6.0 Hz, 1H), 8.23 – 7.93 (m, 4H), 7.56 (dd, *J* = 2.2, 1.0 Hz, 1H), 7.35 – 7.32 (m, 4H), 7.29 – 7.21 (m, 2H), 6.98 (d, *J* = 8.4 Hz, 1H), 4.51 (d, *J* = 6.0 Hz, 1H), 2.29 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 166.14, 153.49, 153.35, 139.84, 138.67, 136.39, 135.61, 129.26, 128.94, 128.80, 127.71, 127.31, 122.89, 122.51, 118.61, 43.20, 20.36.

Appendix A: Work done in collaboration

A1. Collaboration with Jewett group members

A) A fluorogenic coumarin based triazabutadiene reagent (Collaboration with Mehrdad Shadmehr)

In this project fluorogenic triazabutadiene compounds **193**, were synthesized. Photophysical characterization of the reagents and their reaction products, **195**, with model compounds were carried out.



Scheme Appendix.1 Reaction of coumarin based fluorogenic triazabutadiene with model compounds

The compounds that were characterized with obtained data are shown below (**Fig. Appendix 1**).

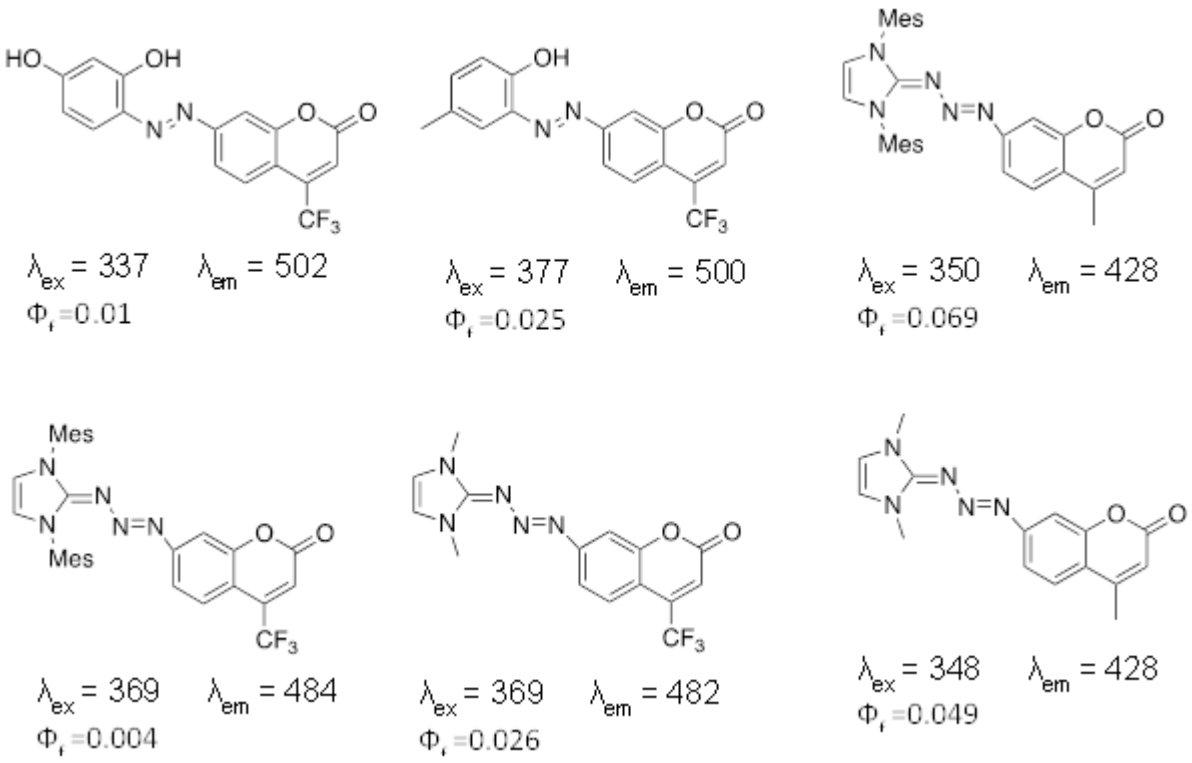


Figure Appendix 1 Spectral data for fluorogenic triazabutadiene reagent and conjugation products

In this project I participated in the design of the reagents and their photophysical characterization. I also contributed in obtaining the absorption and emission spectra as well as the quantum yield and molar absorptivity of the compounds. Absorption spectra were measured with an Agilent 8453 UV−vis spectrophotometer. Fluorescence spectra were measured with a QuantaMaster 400 Steady State Spectrofluorometer and PTI fluorescence spectrophotometer. Quantum yield was calculated using coumarin 151 and rhodamine in ethanol as reference.

B) Azocoumarin fluorophore with increased fluorescence upon photo irradiation

(Collaboration with Mehrdad Shadmehr)

An azocoumarin fluorophore demonstrated an increase in fluorescence upon irradiation at 360 nm. Some loss of fluorescence was observed upon storage after irradiation. Current working theory is that this is due to the isomerization of the azo system leading to increased electron donation into the coumarin moiety. Work is currently being carried out to have a greater understanding of the system.

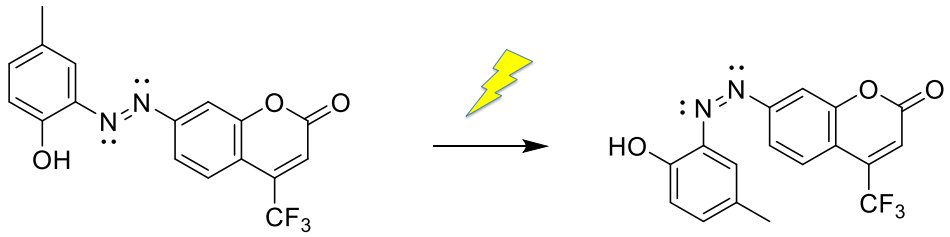


Figure Appendix 2 Proposed mechanism for increased fluorescence of azocoumarin fluorophore

Below is shown the increased the emission spectrum observed for this pre and post irradiation (**Fig. appendix 3**)

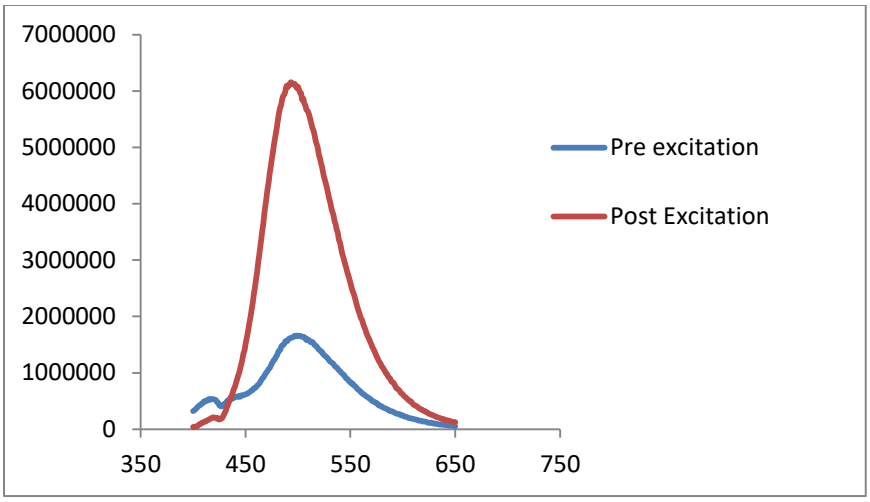


Figure Appendix 3 Emission spectrum from irradiation experiment of azocoumarin dye

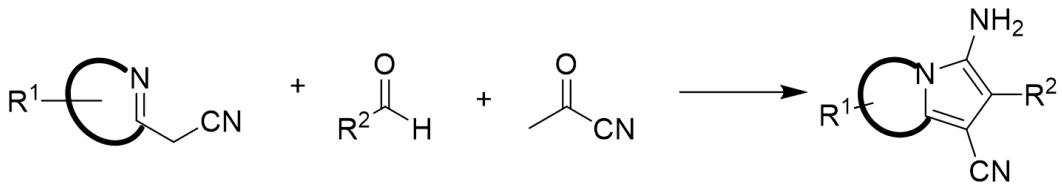
In this project my contribution was obtaining the emission spectra azocoumarin pre and post irradiation. Fluorescence spectra were measured with a QuantaMaster 400 Steady State Spectrofluorometer.

A2. Collaboration with other research groups

A) Synthesis of fluorescent heterocycles via a Knoevenagel/[4+1]-cycloaddition cascade using acetyl cyanide[97] (Collaboration with the Hulme group, University of Arizona)

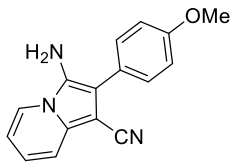
In this work a one-pot three-component reaction that affords fluorescent indolizines, benzo[d]pyrrolo[2,1-b]thiazoles, and pyrrolo[1,2-a]pyrazines was designed. The methodology involves the formation of a heterocyclic 1-aza-1,3-diene derived from a Knoevenagel

condensation between an aldehyde and 2-methyl-ene-cyano aza-heterocycles, followed by [4 + 1] cycloaddition of acetyl cyanide behaving as a non-classical isocyanide replacement.

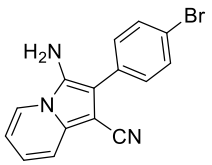


Scheme Appendix 2 Tandem Knoevenagel [4 + 1] cycloaddition reaction

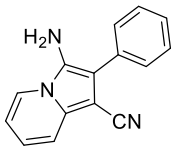
The methodology allowed for the formation of at least three different types of heterocycles: indolizines), benzo[d]pyrrolo[2,1-b]thiazoles , and pyrrolo[1,2-a]pyrazines . Most of the synthesized scaffolds exhibited fluorescence. The structure and fluorescence data for the synthesized molecules is shown below (**Fig. Appendix 4**).



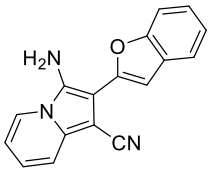
λ_{ex} = 292, 393 nm
λ_{em} = 497 nm
Φ_f = 0.03



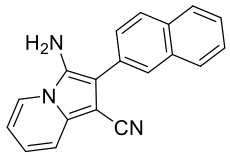
λ_{ex} = 282, 398 nm
λ_{em} = 493 nm
Φ_f = 0.01



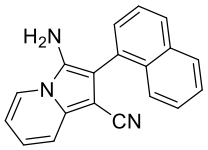
λex = 287, 396 nm
λem = 496 nm
Φ_f = 0.02



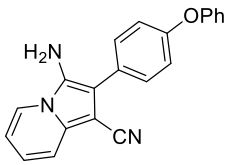
λ_{ex} = 294, 332, 373 nm
λ_{em} = 476 nm
Φ_f = 0.06



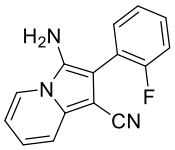
λ_{ex} = 292, 398 nm
λ_{em} = 505 nm
Φ_f = 0.02



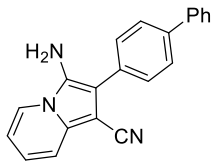
λ_{ex} = 297, 397 nm
λ_{em} = 512 nm
Φ_f = 0.05



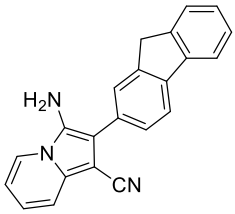
λ_{ex} = 300, 400 nm
λ_{em} = 494 nm
Φ_f = 0.05



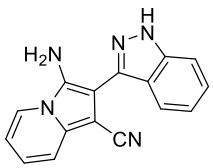
λ_{ex} = 280, 323, 383 nm
λ_{em} = 493 nm
Φ_f = 0.02



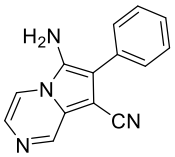
λ_{ex} = 292, 398 nm
λ_{em} = 496 nm
Φ_f = 0.02



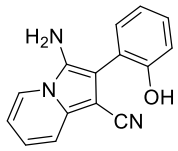
λ_{ex} = 300, 398 nm
λ_{em} = 490 nm
Φ_f = 0.10



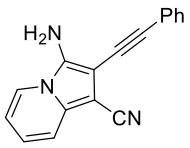
λ_{ex} = 299, 399 nm
λ_{em} = 493 nm
Φ_f = 0.07



λ_{ex} = 295, 399 nm
λ_{em} = 494 nm
Φ_f = 0.22



λ_{ex} = 291, 392 nm
λ_{em} = 494 nm
Φ_f = 0.03



λ_{ex} = 304, 348 nm
λ_{em} = 404 nm
Φ_f = 0.24

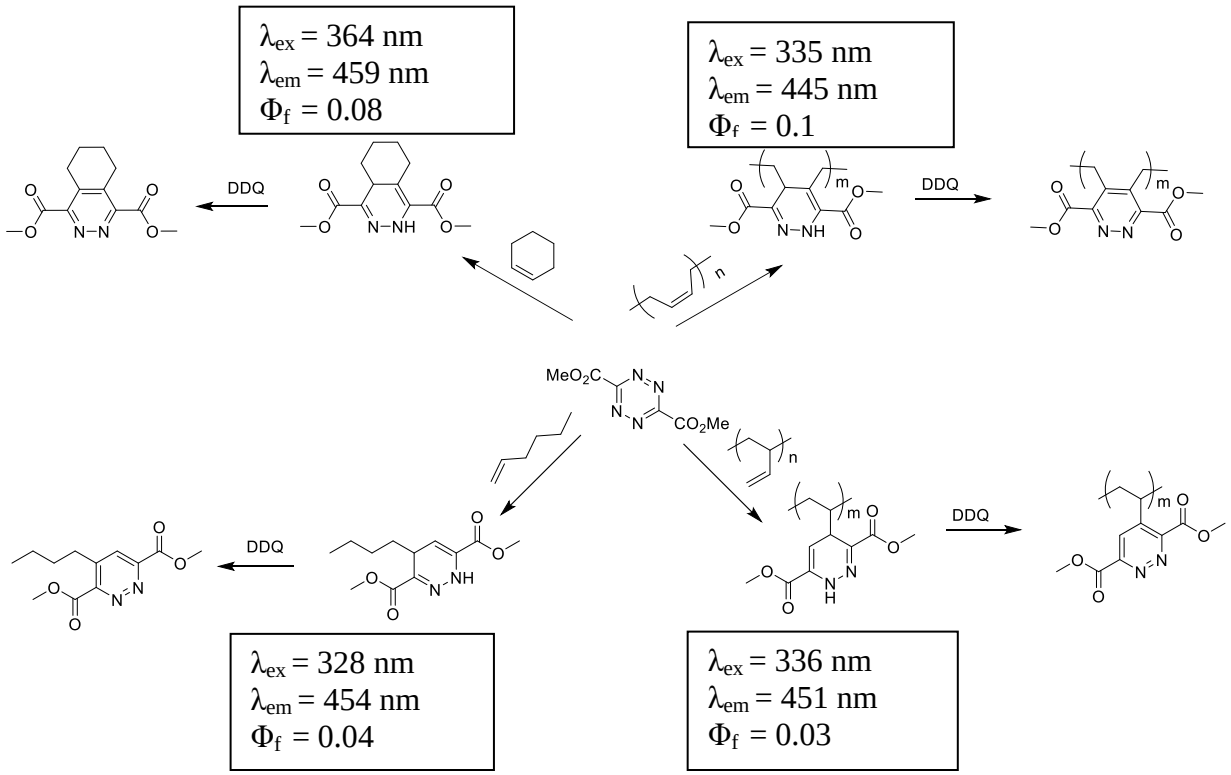
Figure Appendix 4 Fluorescence data of compounds synthesized by novel Knoevenagel/[4 + 1]-cycloaddition cascade with acetyl cyanide

In this project my contribution was obtaining the quantum yield and molar absorptivity of the compounds. Absorption spectra were measured with an Agilent 8453 UV−vis

spectrophotometer. Fluorescence spectra were measured with a QuantaMaster 400 Steady State Spectrofluorometer and PTI fluorescence spectrophotometer. Quantum yield was calculated using coumarin 151 and rhodamine in ethanol as reference.

B) Fluorescent anti-oxidant macromolecules through click modification of polybutadiene with dimethyl 1,2,4,5-tetrazine-3,6-dicarboxylate (Collaboration with the Loy group, University of Arizona)

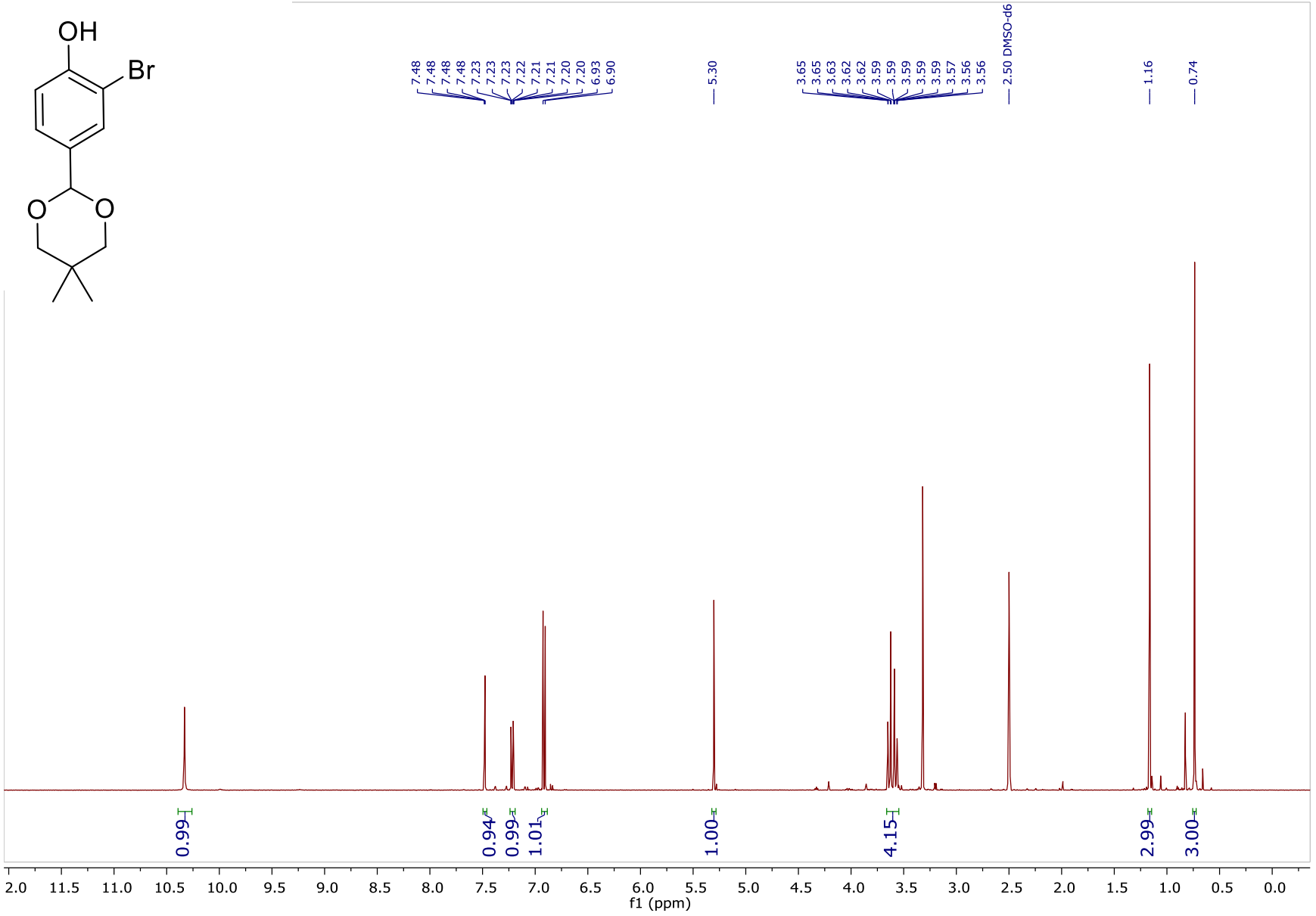
In this work, a simple click modification of polybutadienes (PDB) with dimethyl 1,2,4,5-tetrazine-3,6-dicarboxylate (DMTD) that modifies the polymer chains, replacing PBD C=C repeat units with 1,4-dihydropyridazine-3,6-dicarboxylate and transforms PBD into macromolecular antioxidants is described. Poly(dimethyl 1,4-dihydropyridazine-3,6-dicarboxylates) fluoresce blue light upon long wave UV excitation, but lose fluorescence from electron transfer quenching upon oxidation providing a visual method to monitor extents of oxidation for the materials.

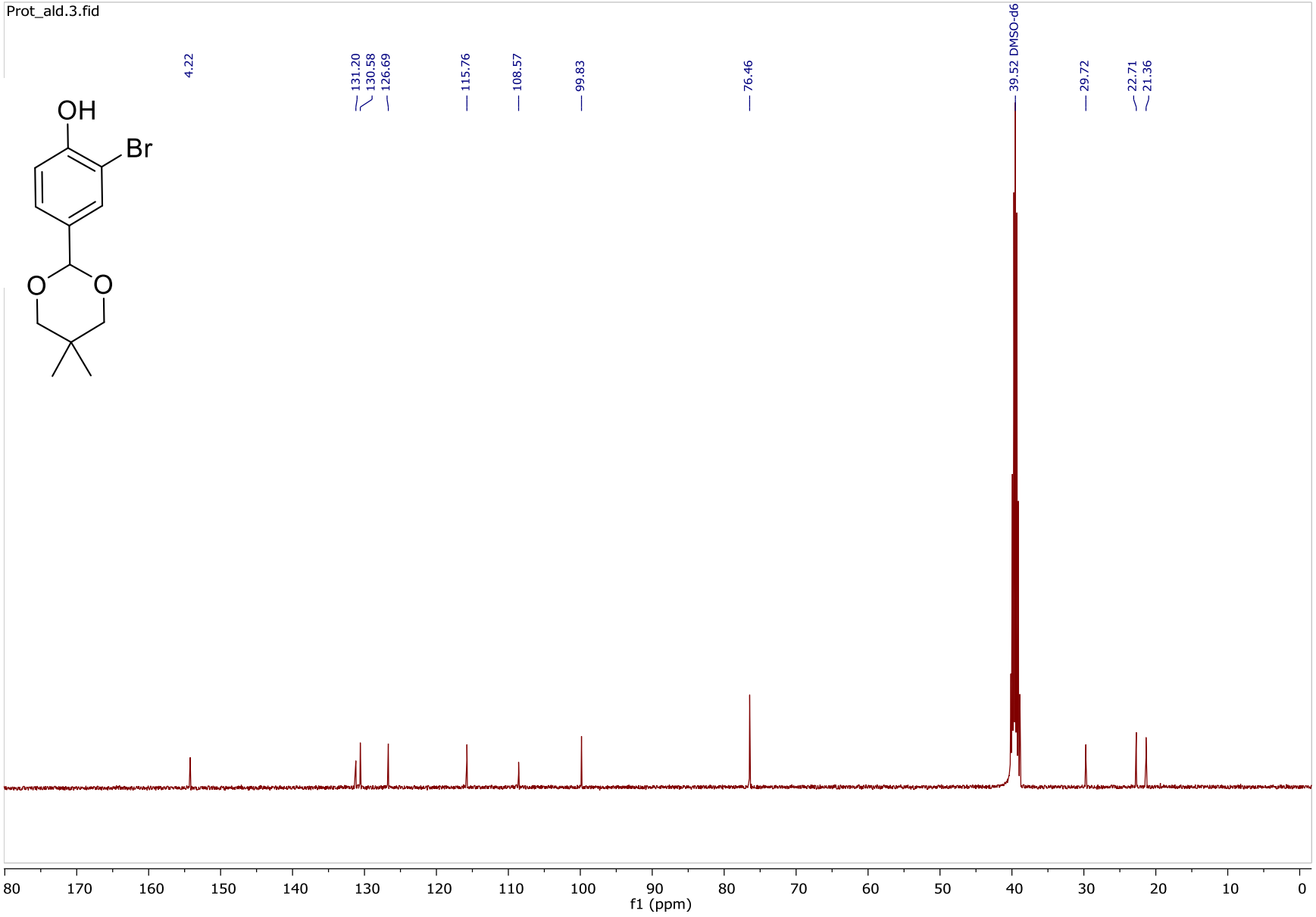


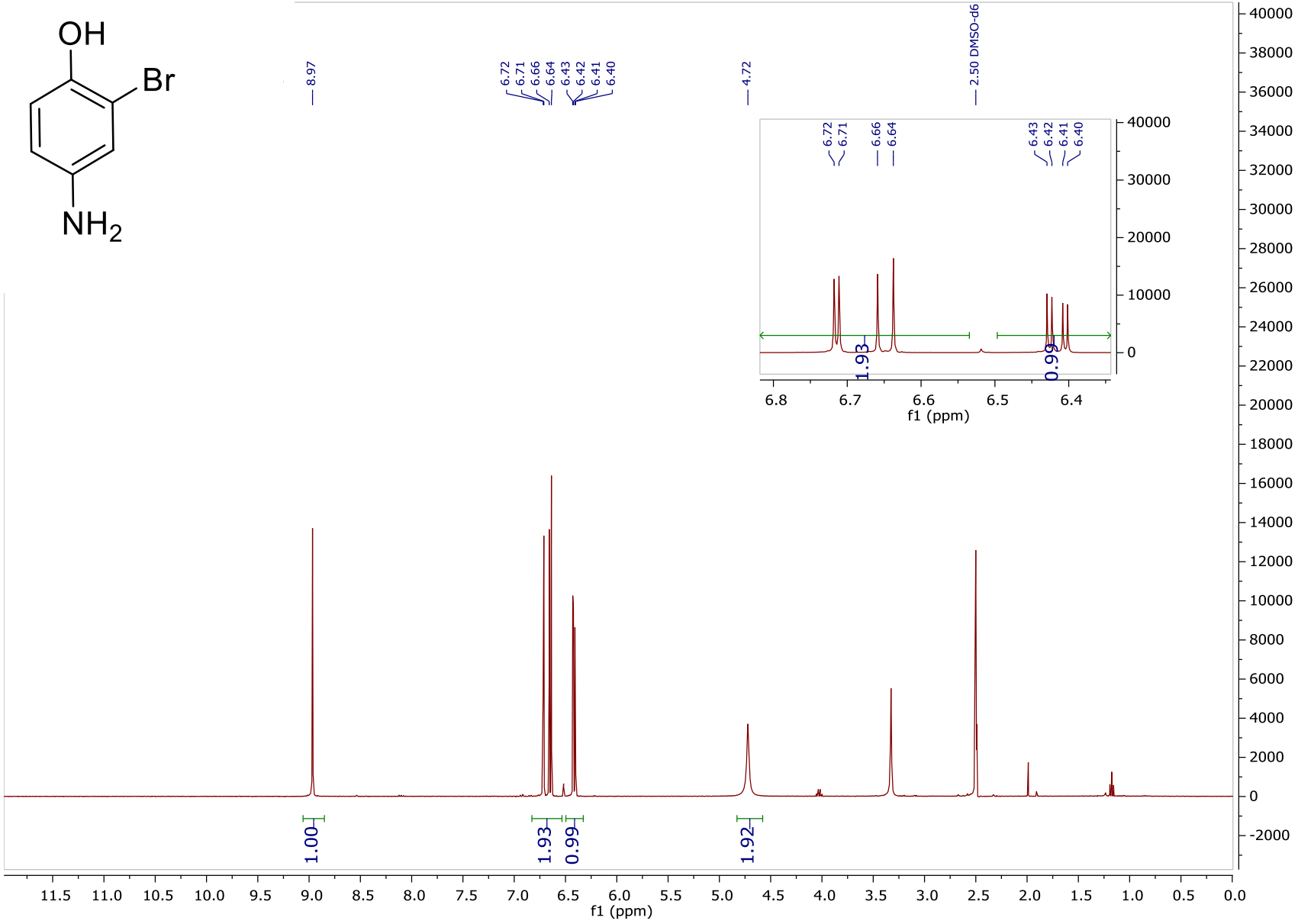
Scheme Appendix.3 Reaction scheme of modification of diene with dimethyl 1,2,4,5-tetrazine-3,6-dicarboxylate and fluorescence data

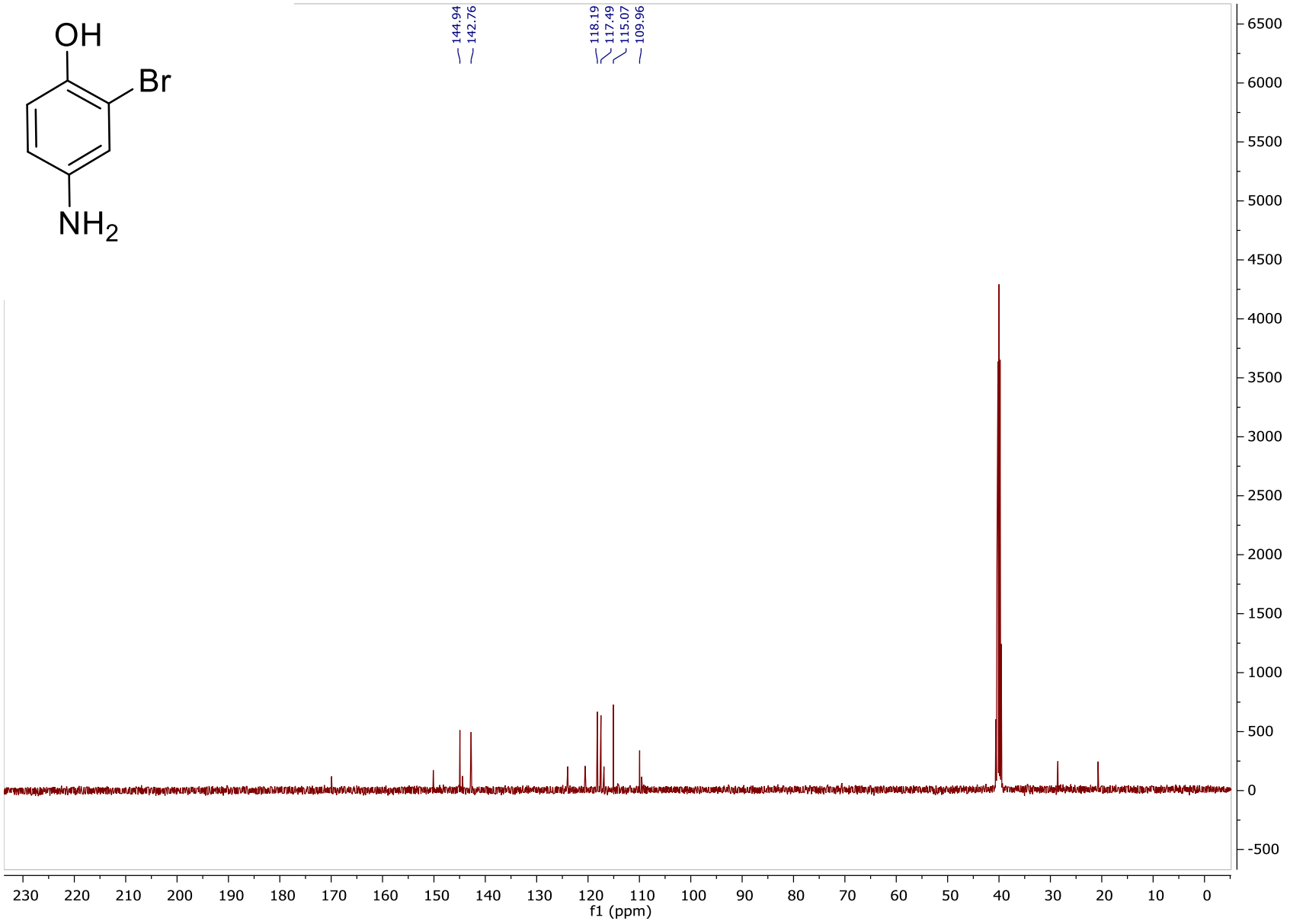
In this project my contribution was obtaining the quantum yield of the fluorescent products. Absorption spectra were measured with an Agilent 8453 UV−vis spectrophotometer. Fluorescence spectra were measured with a QuantaMaster 400 Steady State Spectrofluorometer and PTI fluorescence spectrophotometer. Quantum yield was calculated using coumarin 151 and rhodamine in ethanol as reference.

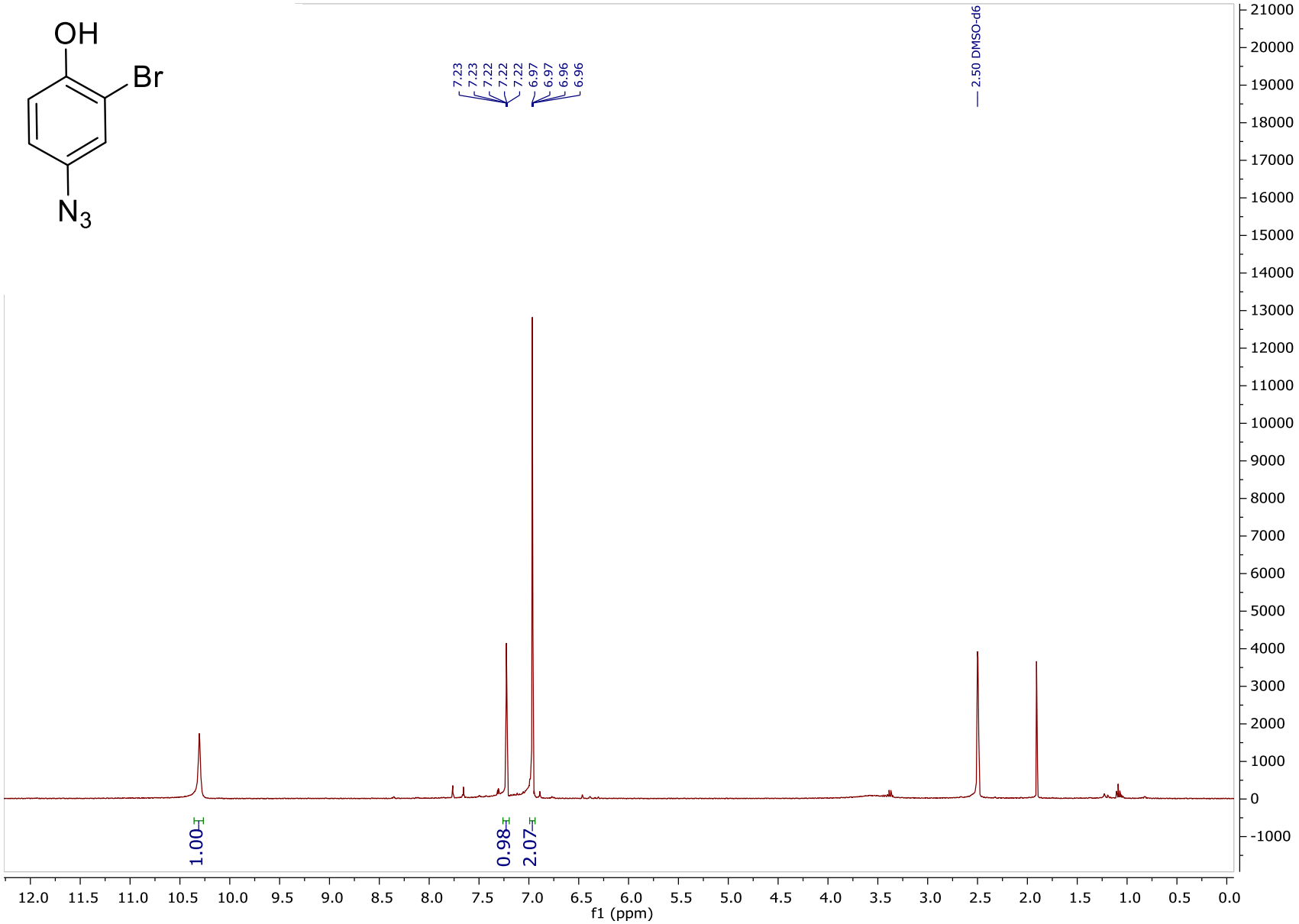
Appendix B: Spectral data

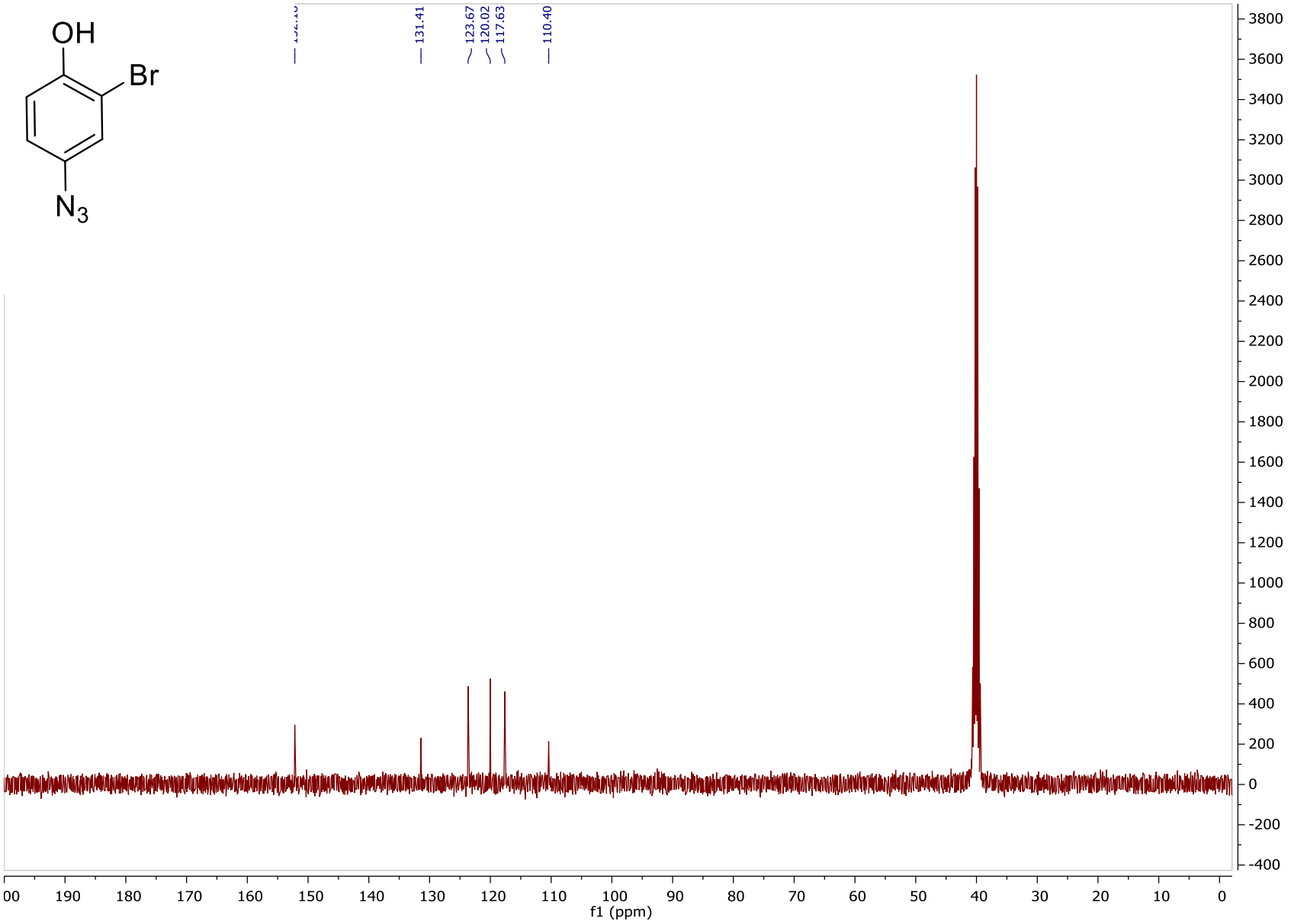

OH
Br
O
O
7.48
7.48
7.48
7.48
7.23
7.23
7.23
7.22
7.21
7.21
7.20
7.20
6.93
6.90
5.30
3.65
3.65
3.63
3.62
3.62
3.59
3.59
3.59
3.59
3.59
3.57
3.56
3.56
2.50 DMSO-d6
1.16
0.74
0.99
0.94
0.99
1.01
1.00
4.15
2.99
3.00
2.0
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

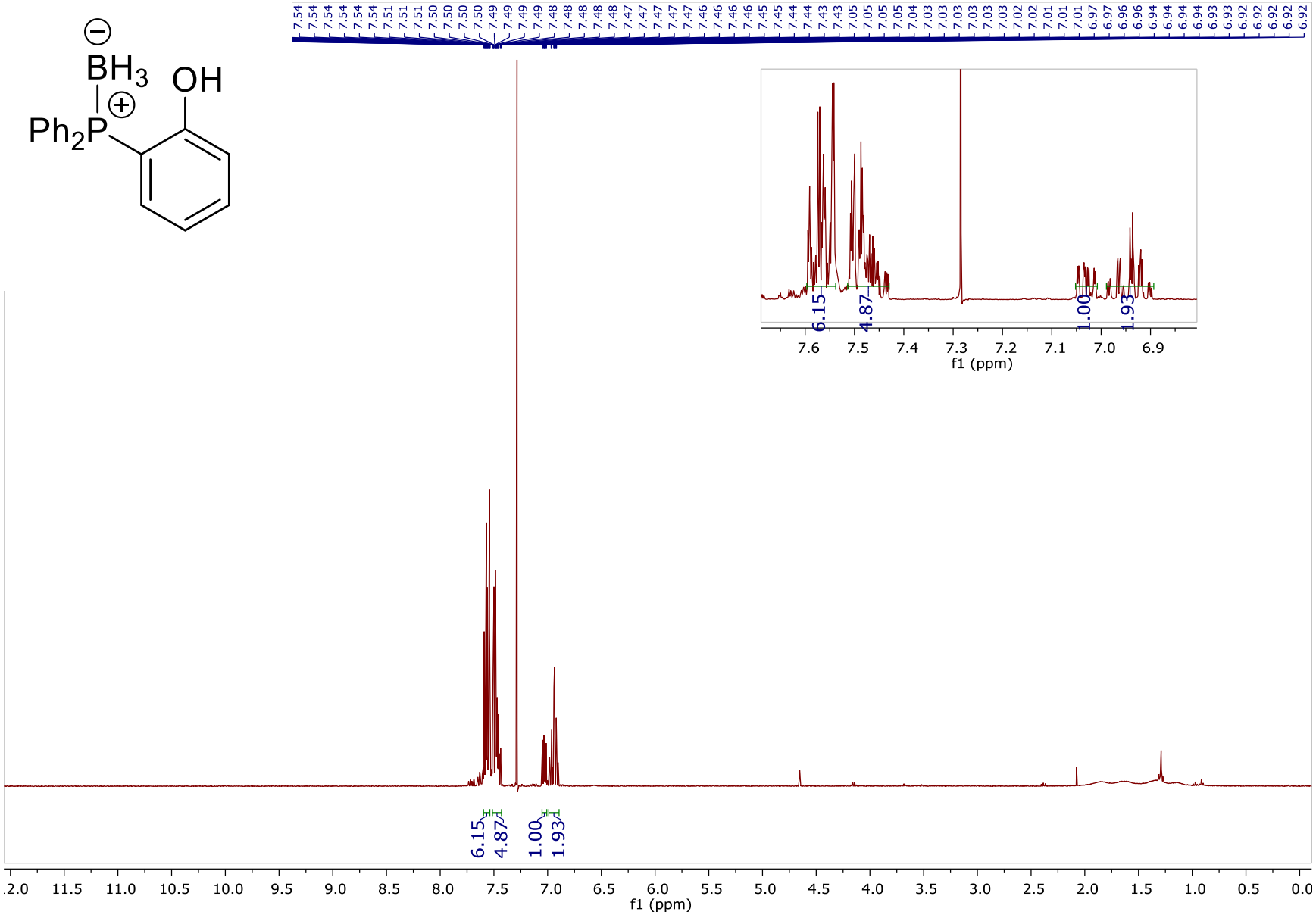

Prot_ald.3.fid
4.22
131.20
130.58
126.69
115.76
108.57
99.83
76.46
39.52 DMSO-d6
29.72
22.71
21.36
OH
Br
O
O
80
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

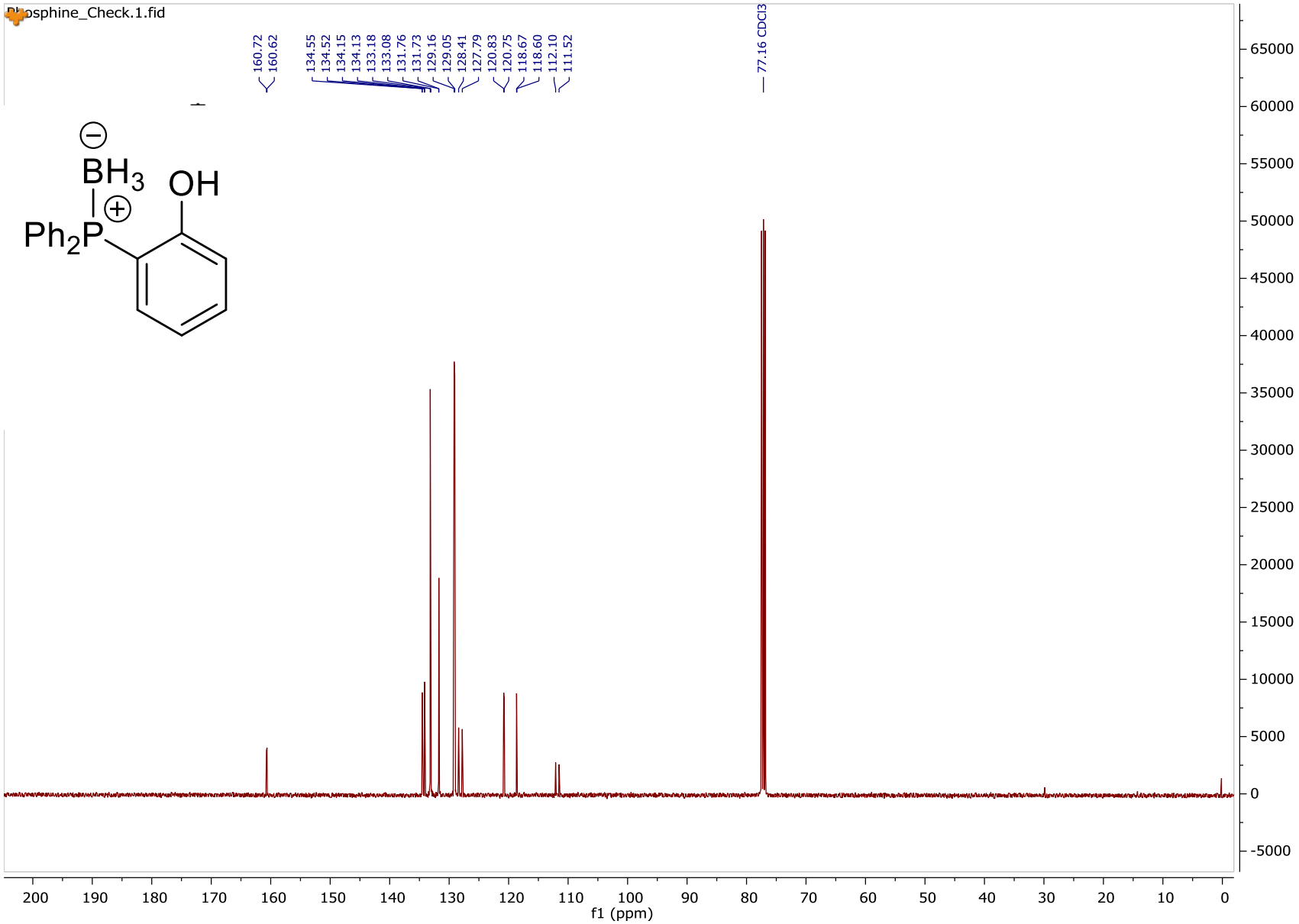

OH
Br
NH2
8.97
6.72
6.71
6.66
6.64
6.43
6.42
6.41
6.40
4.72
2.50 DMSO-d6
1.00
1.93
0.99
1.92
f1 (ppm)


OH
Br
NH2
144.94
142.76
118.19
117.49
115.07
109.96
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


OH
Br
N3
7.23
7.23
7.22
7.22
7.22
6.97
6.97
6.96
6.96
2.50 DMSO-d6
1.00
0.98
2.07
f1 (ppm)


OH
Br
N3
131.41
123.67
120.02
117.63
110.40
f1 (ppm)


BH3
OH
Ph2P
6.15
4.87
1.00
1.93
f1 (ppm)


Phosphine_Check.1.fid
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160.62
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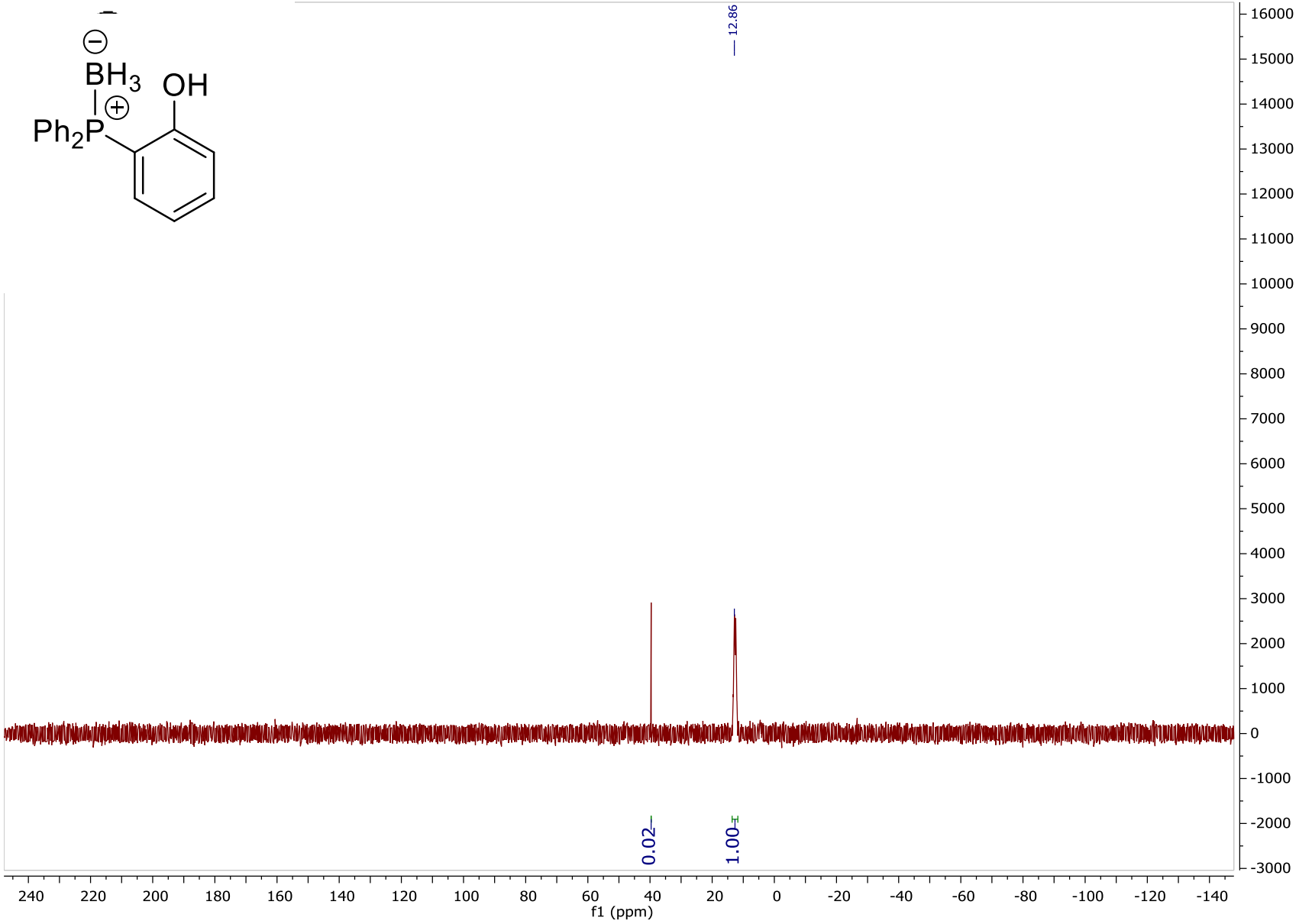
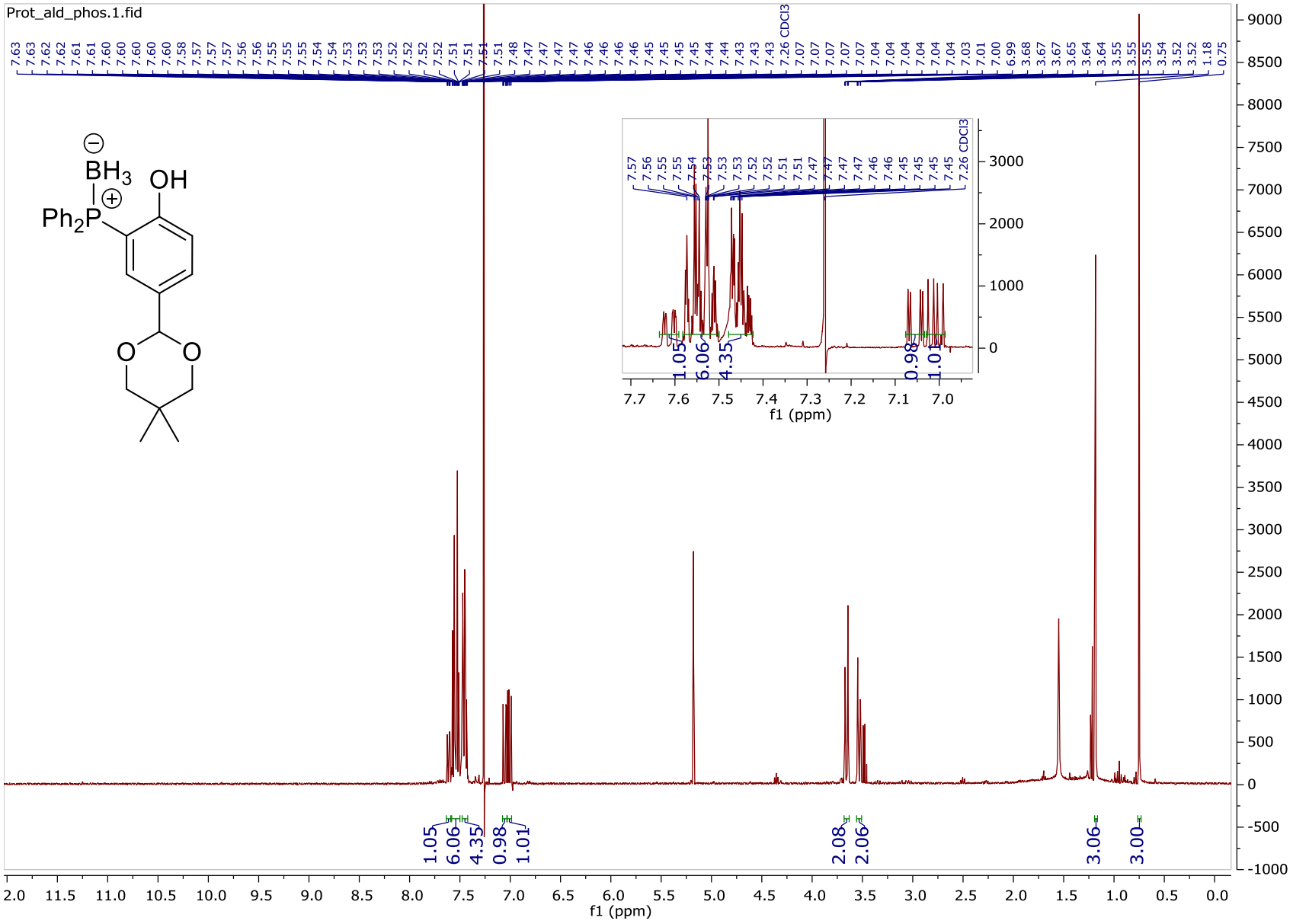
134.13
133.18
133.08
131.76
131.73
129.16
129.05
128.41
127.79
120.83
120.75
118.67
118.60
112.10
111.52
77.16 CDCl3
BH3
OH
Ph2P
65000
60000
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
-5000
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


BH3
OH
Ph2P
12.86
0.02
1.00
f1 (ppm)


Prot_ald_phos.1.fid
BH3
OH
Ph2P
O
O
f1 (ppm)
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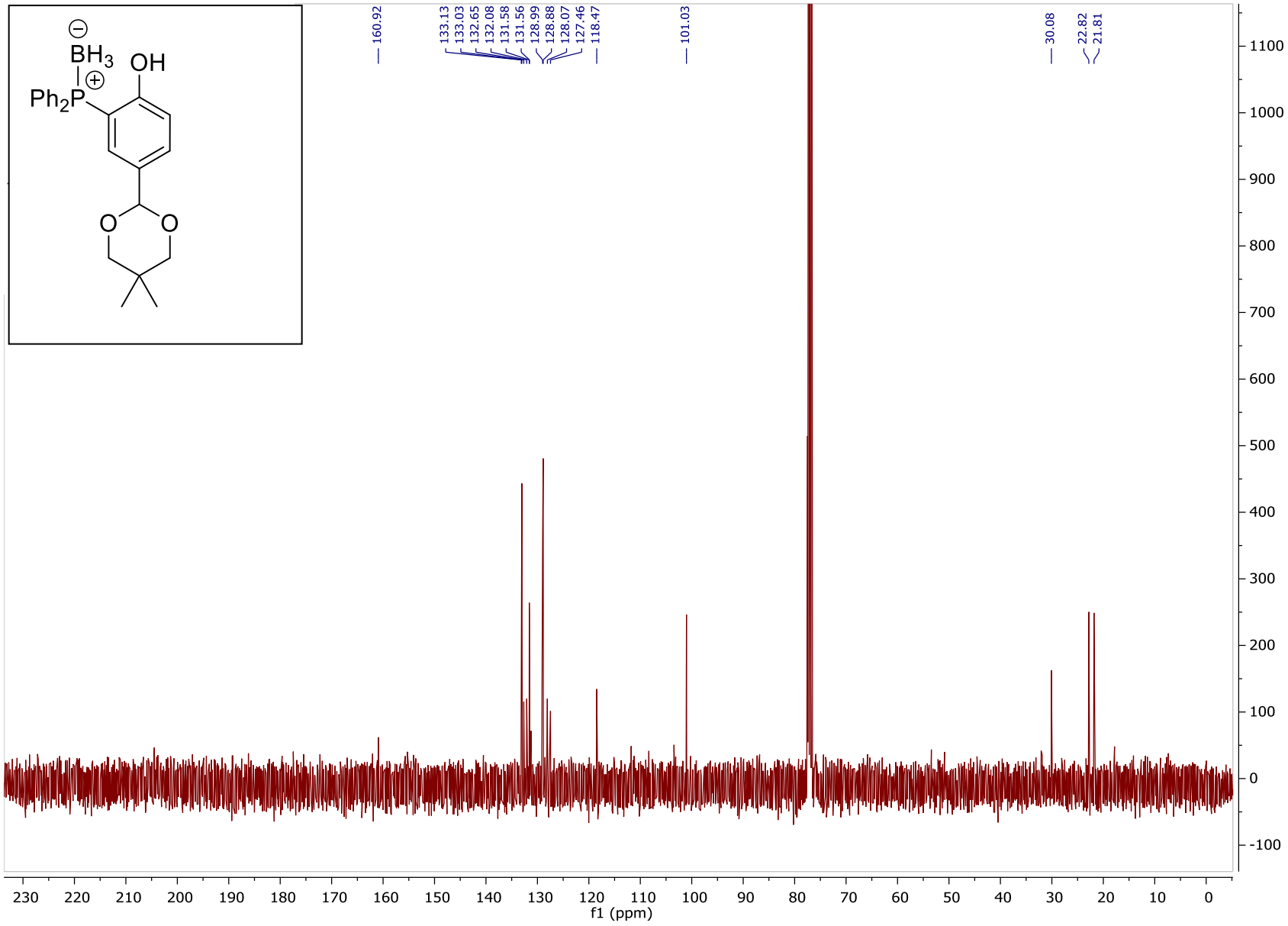
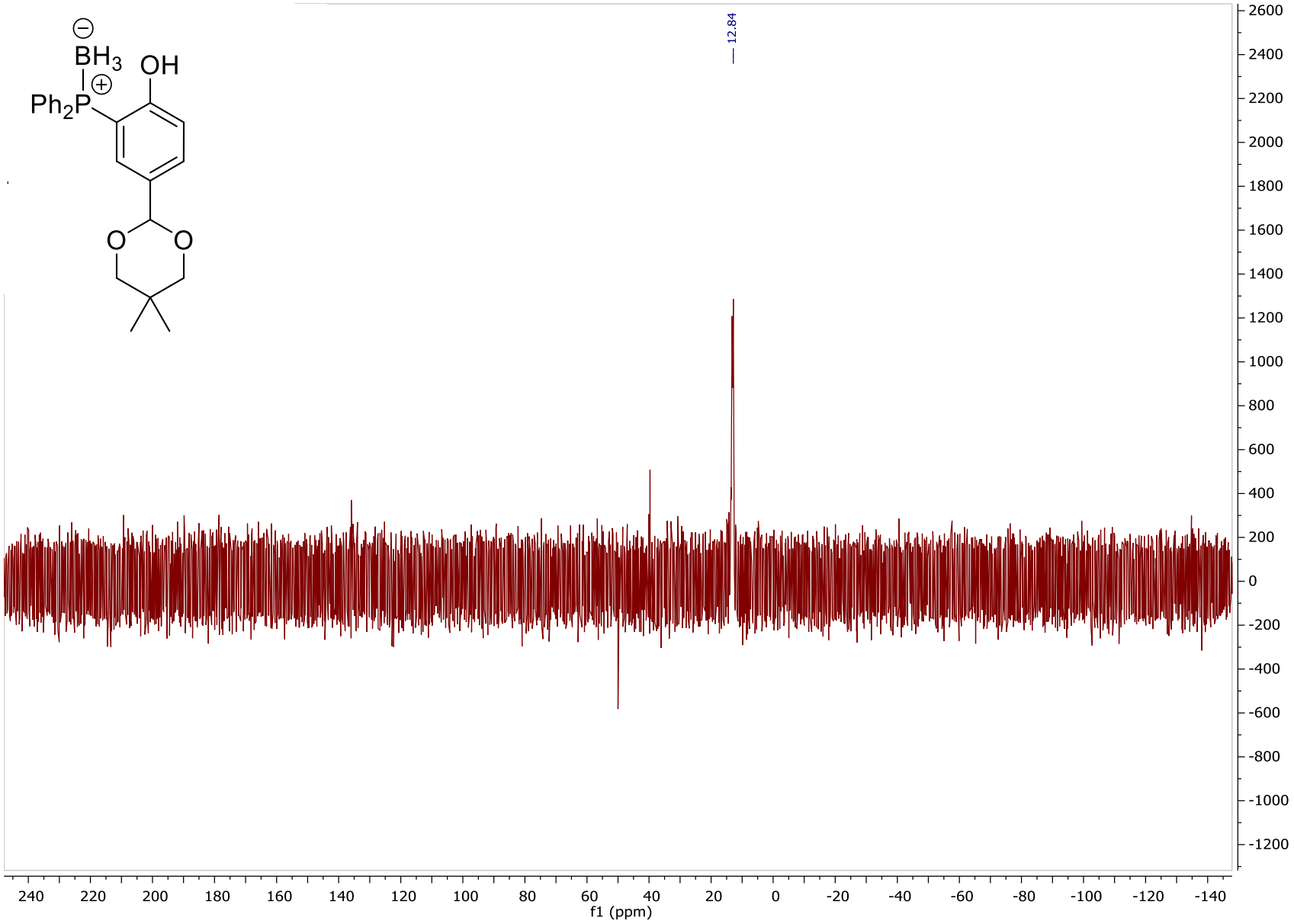
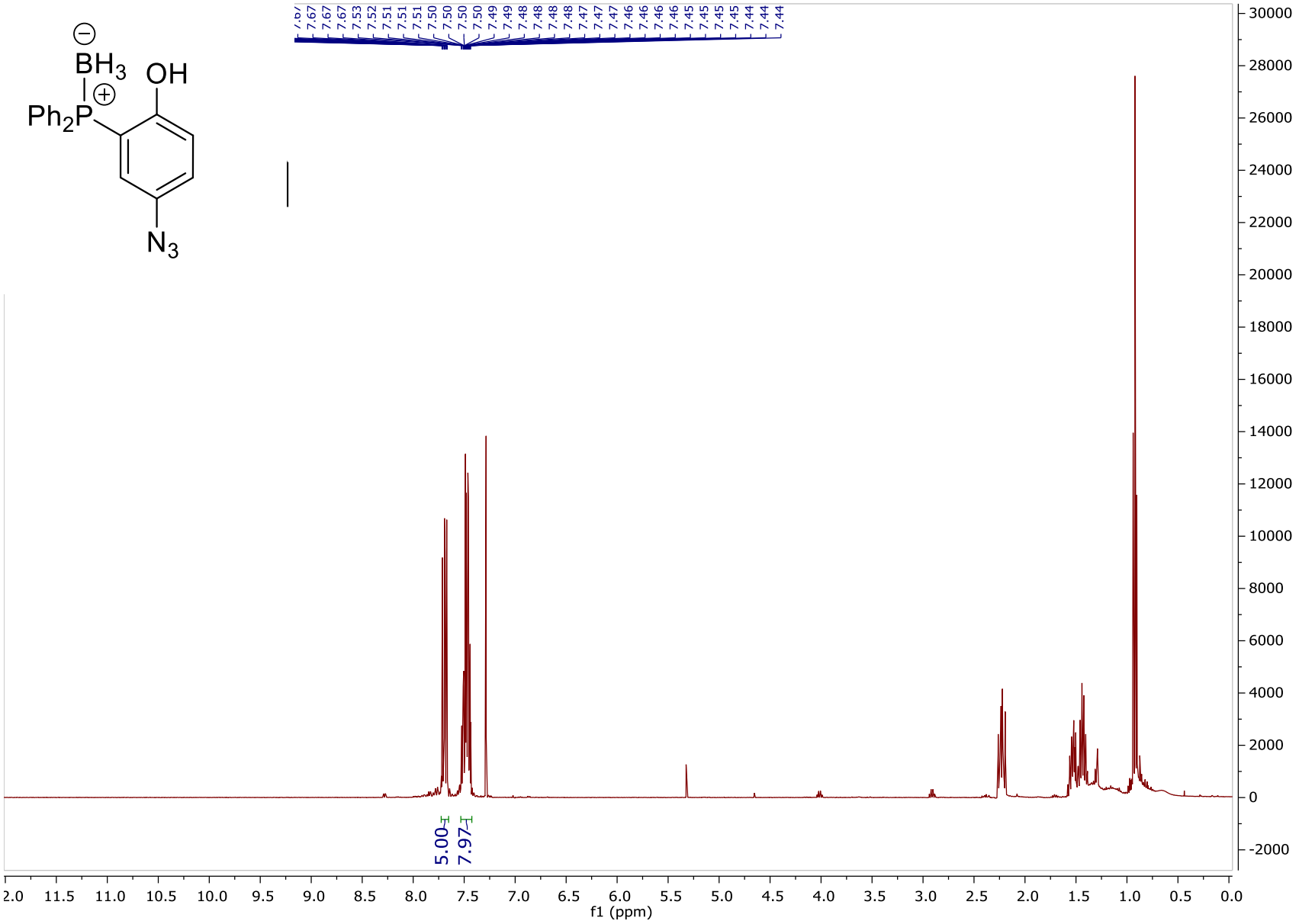
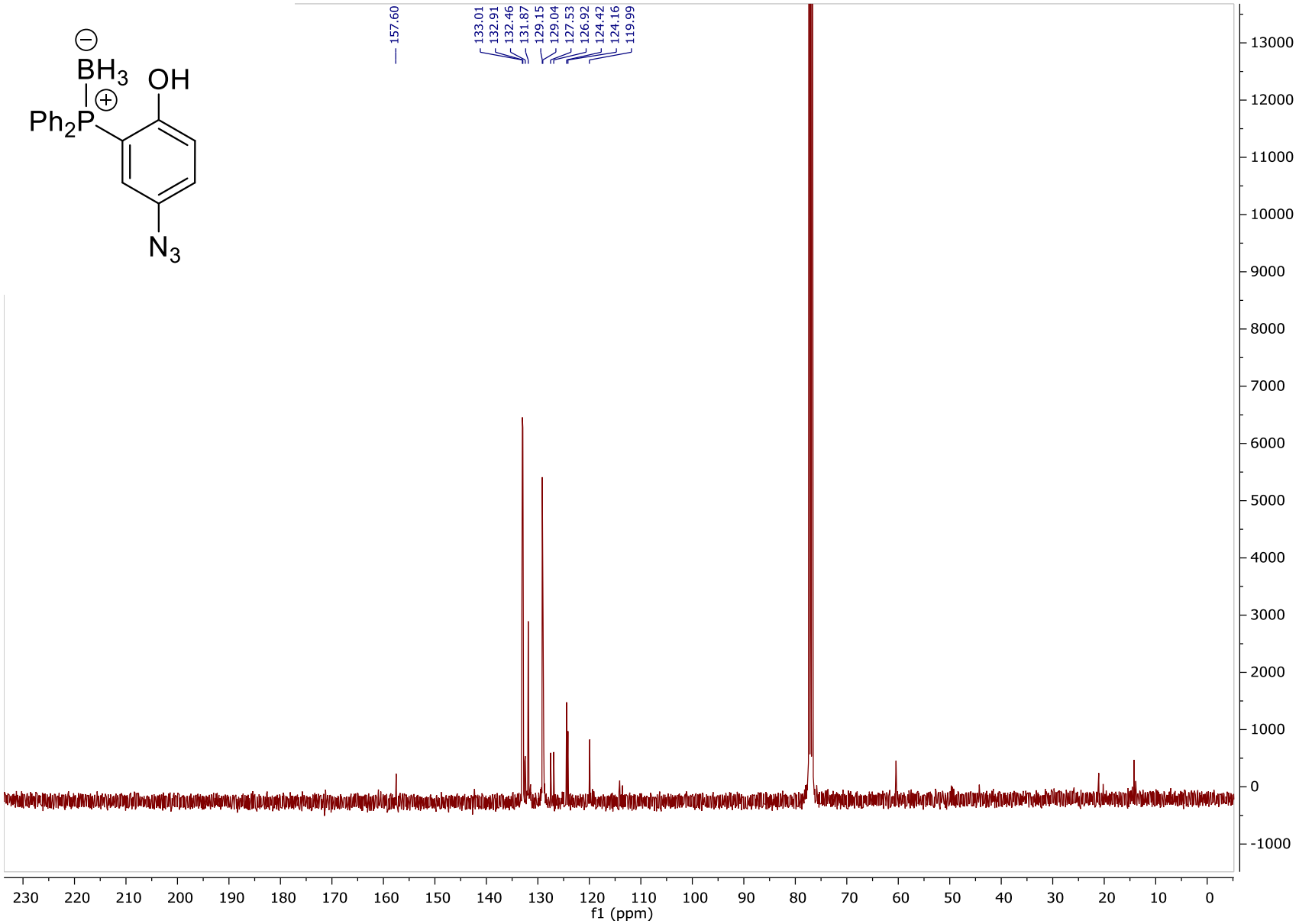
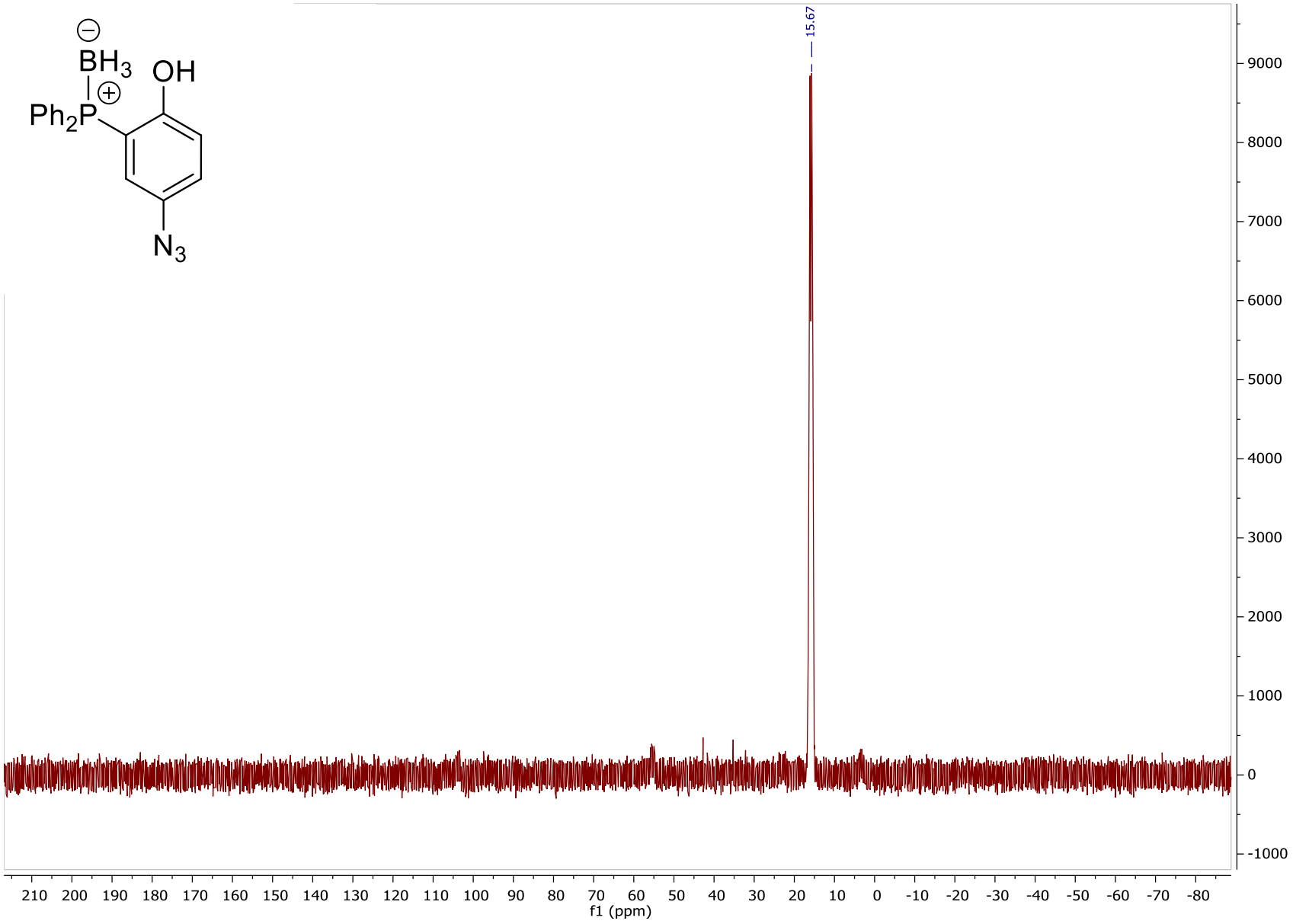
6.06
4.35
0.98
1.01
2.08
2.06
3.06
3.00

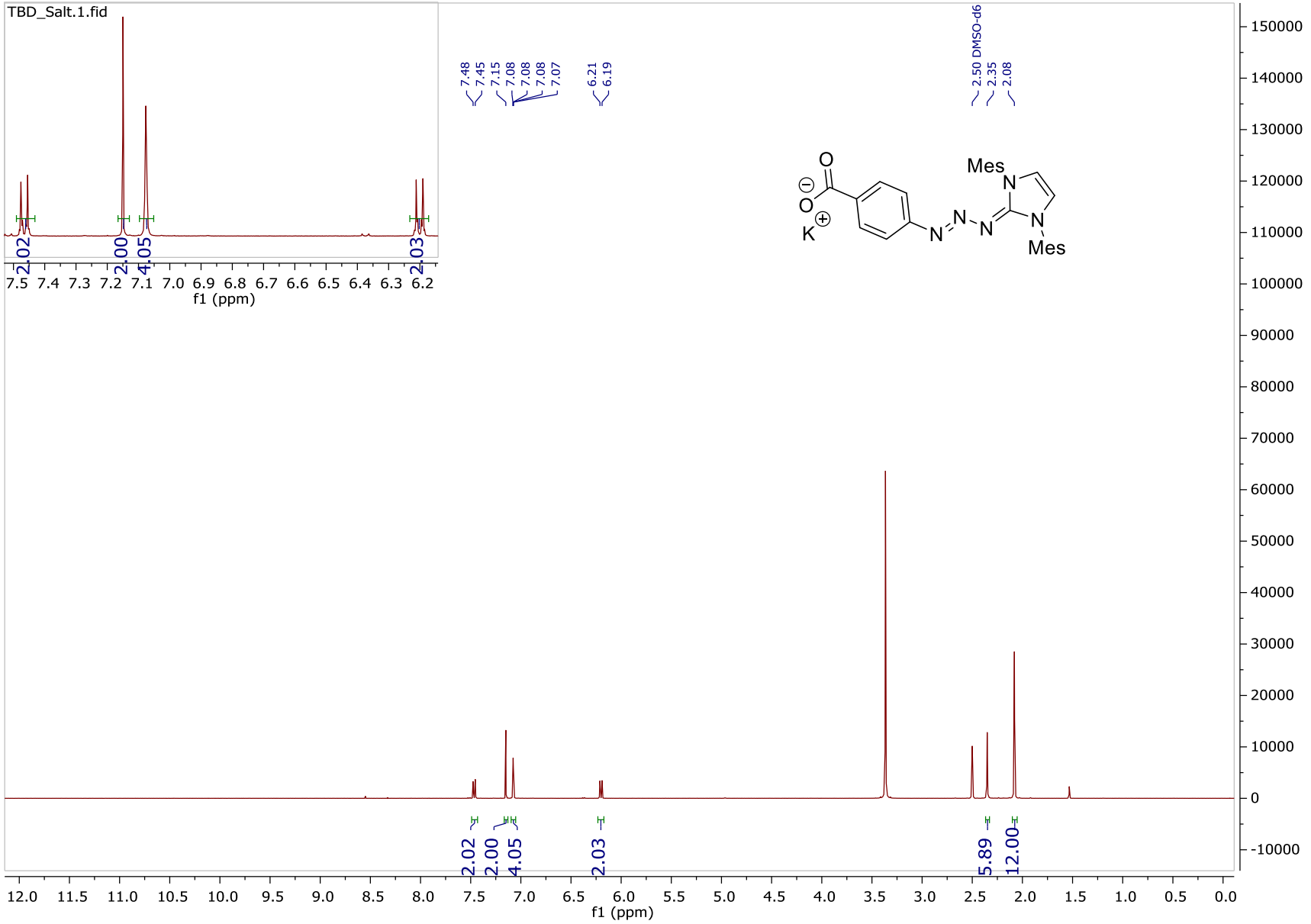

BH3
OH
Ph2P
O
O
160.92
133.13
133.03
132.65
132.08
131.58
131.56
128.99
128.88
128.07
127.46
118.47
101.03
30.08
22.82
21.81
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

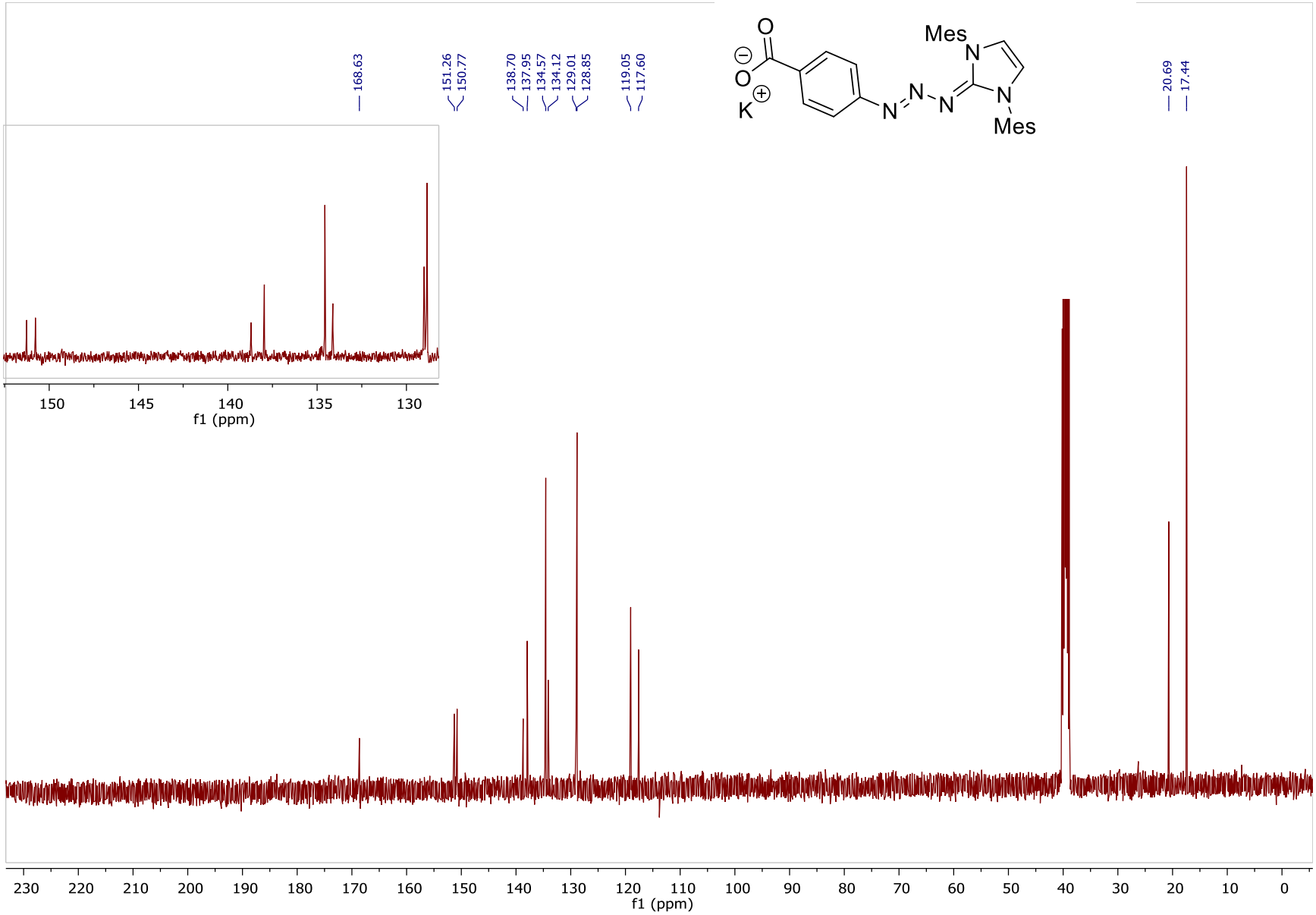

BH3
OH
Ph2P
O
O
12.84
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
-400
-600
-800
-1000
-1200
240
220
200
180
160
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
f1 (ppm)

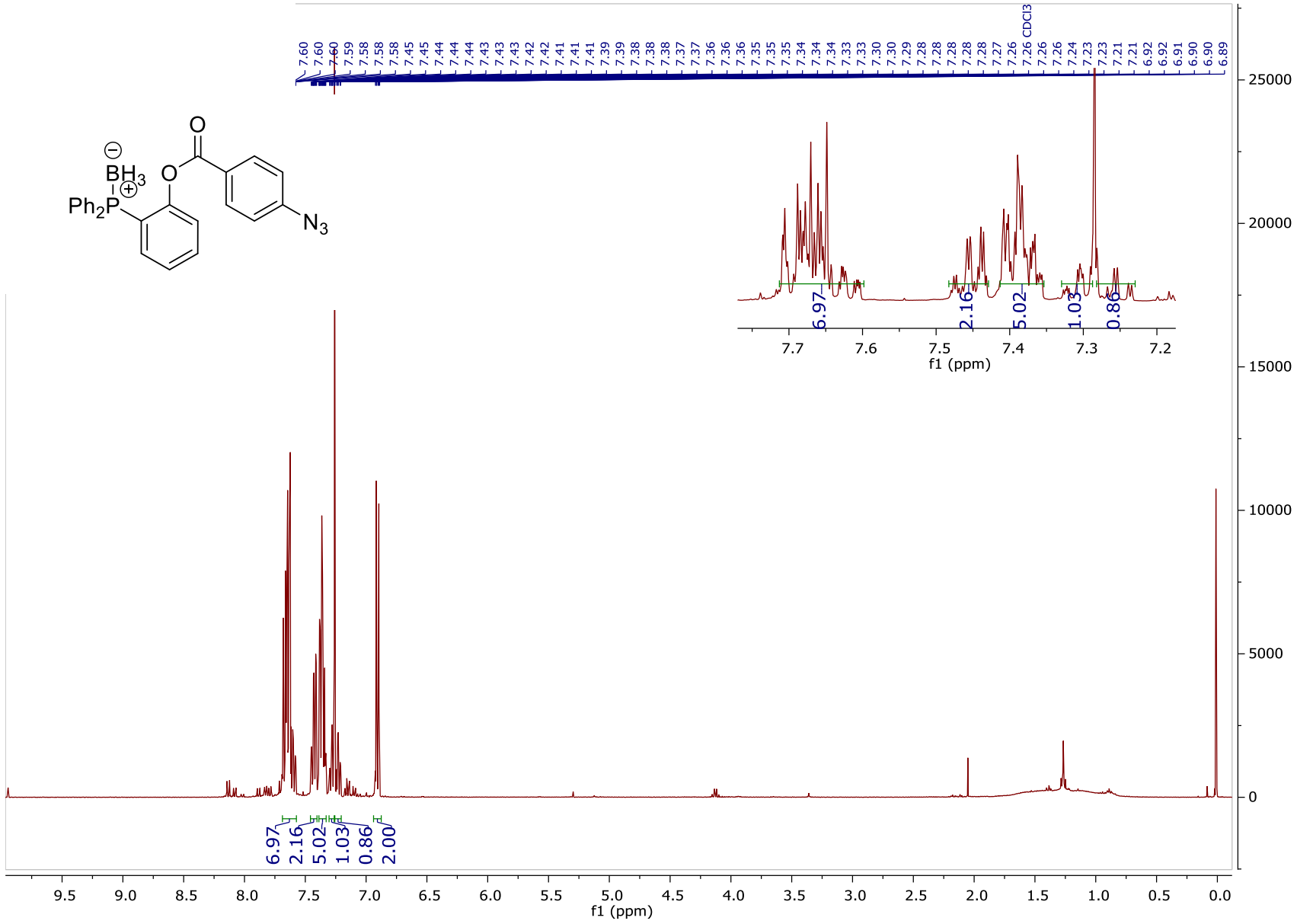

BH3
OH
Ph2P
N3
7.67 7.67 7.67 7.53 7.52 7.51 7.51 7.51 7.50 7.50 7.50 7.49 7.49 7.48 7.48 7.48 7.48 7.47 7.47 7.47 7.46 7.46 7.46 7.45 7.45 7.45 7.44 7.44 7.44
5.00
7.97
f1 (ppm)

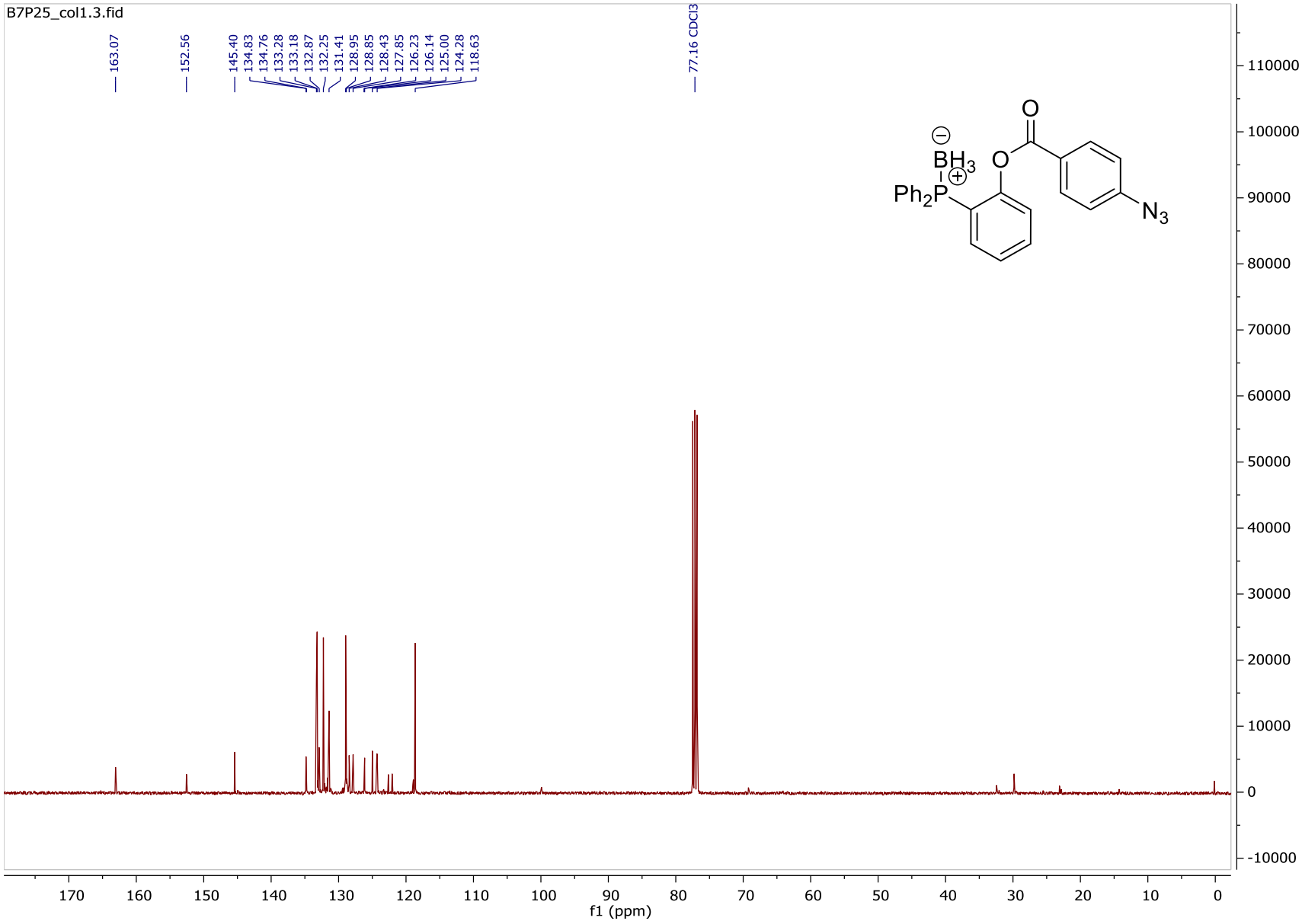

BH3
OH
Ph2P
N3
157.60
133.01
132.91
132.46
131.87
129.15
129.04
127.53
126.92
124.42
124.16
119.99
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

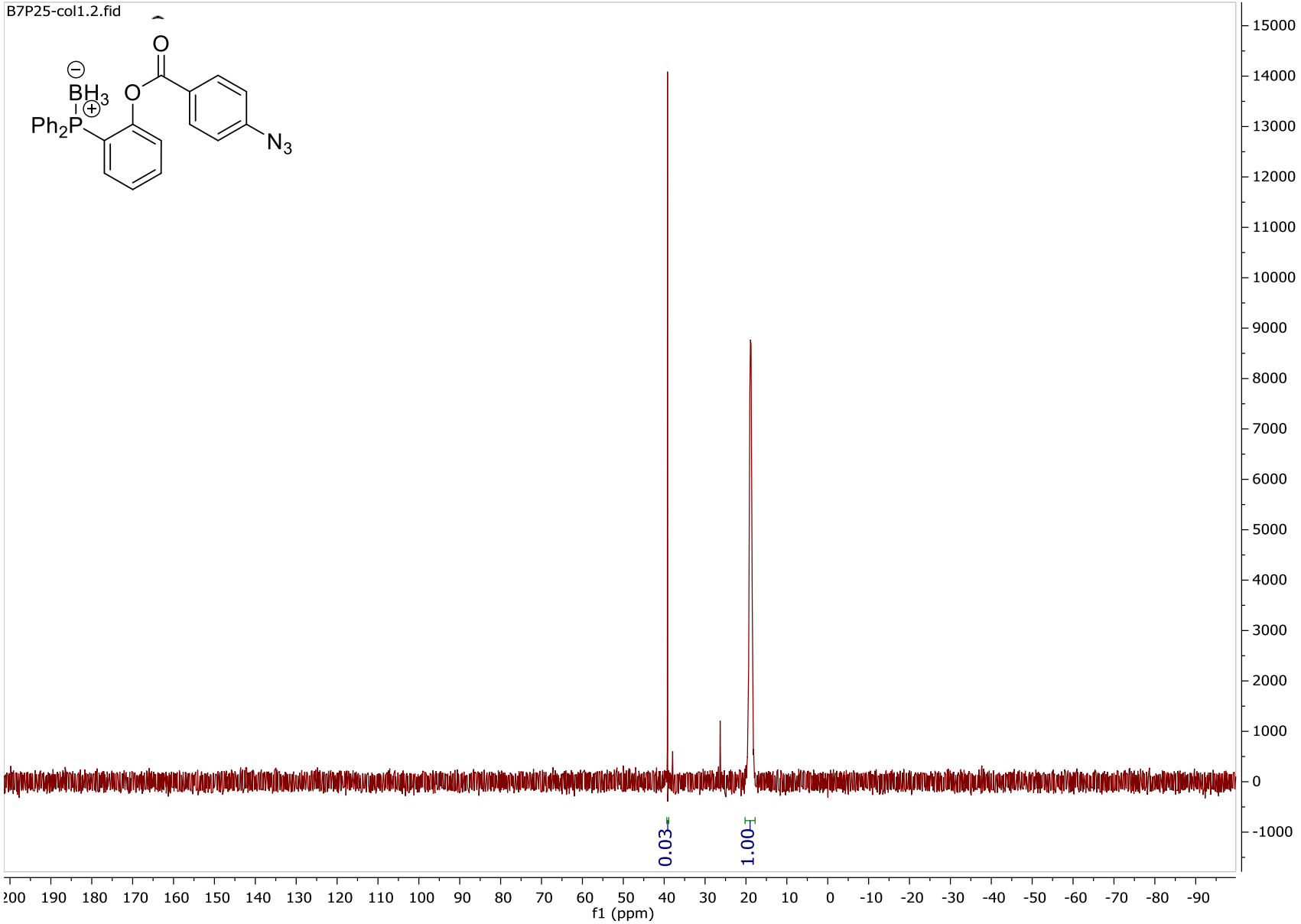

15.67
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30 -40 -50 -60 -70 -80
f1 (ppm)

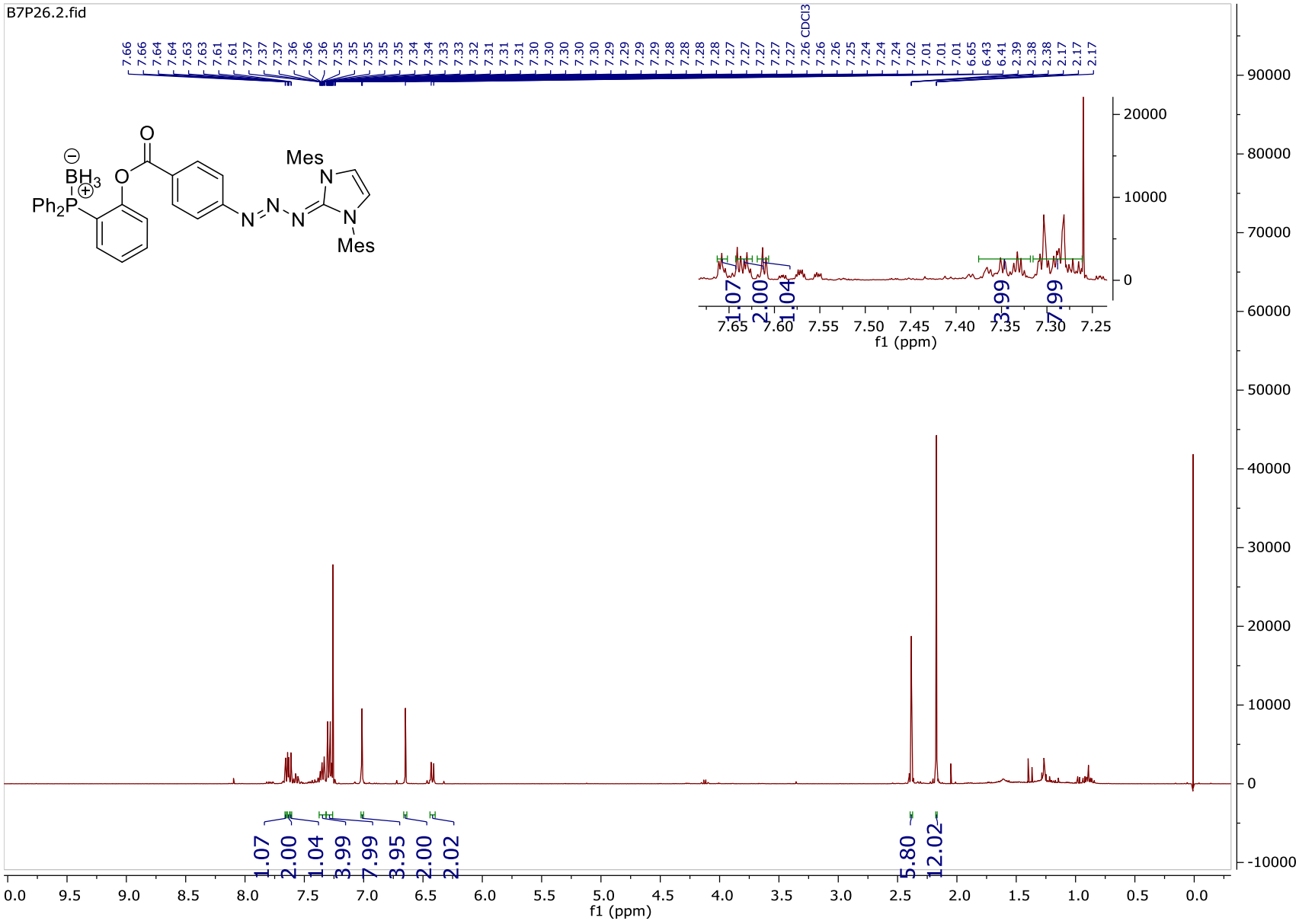

TBD_Salt.1.fid
2.02
2.00
4.05
2.03
7.5 7.4 7.3 7.2 7.1 7.0 6.9 6.8 6.7 6.6 6.5 6.4 6.3 6.2
f1 (ppm)
7.48
7.45
7.15
7.08
7.08
7.08
7.07
6.21
6.19
2.50 DMSO-d6
2.35
2.08
O
O
K
N
N
N
Mes
N
Mes
2.02
2.00
4.05
2.03
5.89
12.00
12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)
150000
140000
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
-10000

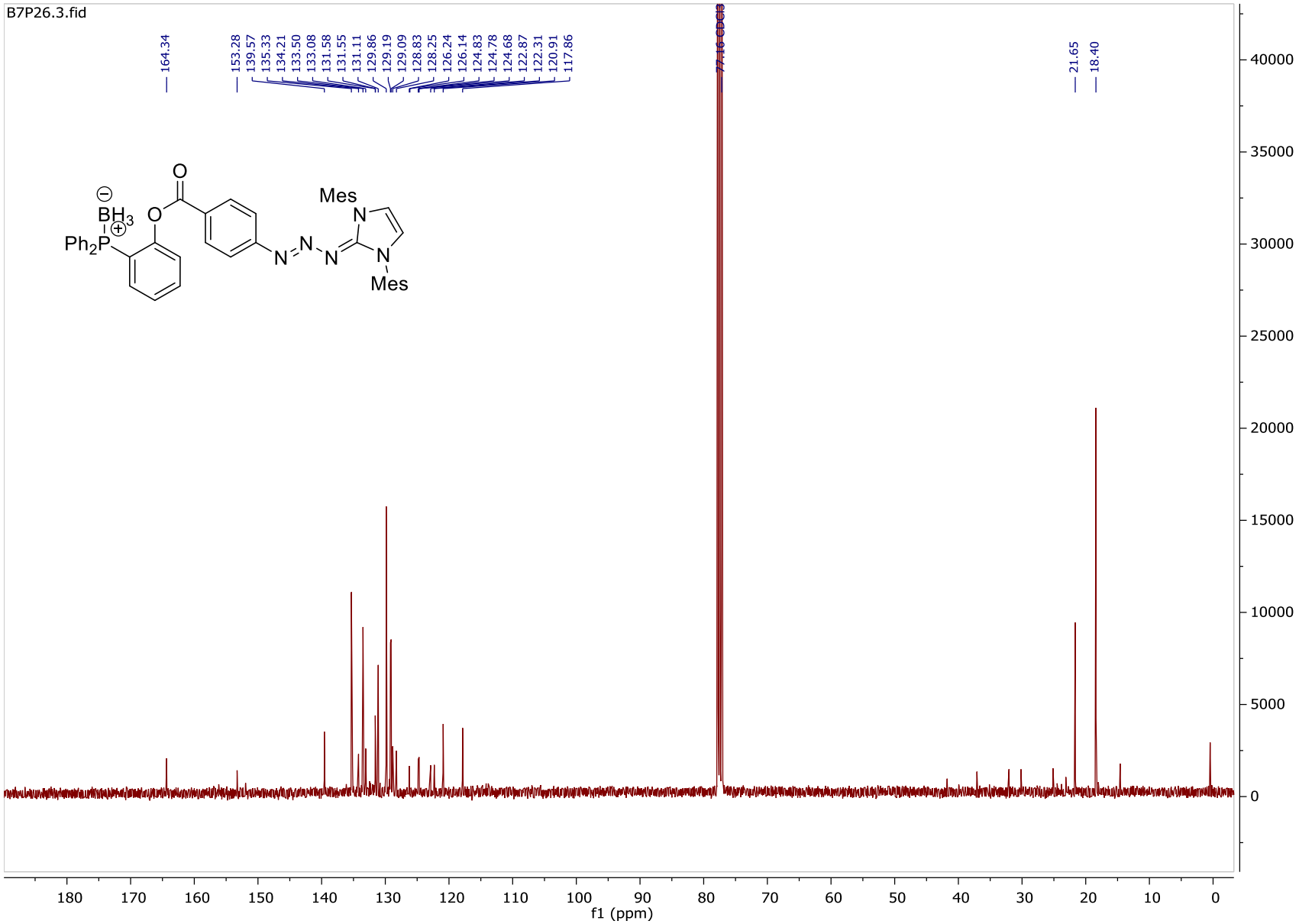

168.63
151.26
150.77
138.70
137.95
134.57
134.12
129.01
128.85
119.05
117.60
20.69
17.44
Mes
Mes
f1 (ppm)
f1 (ppm)

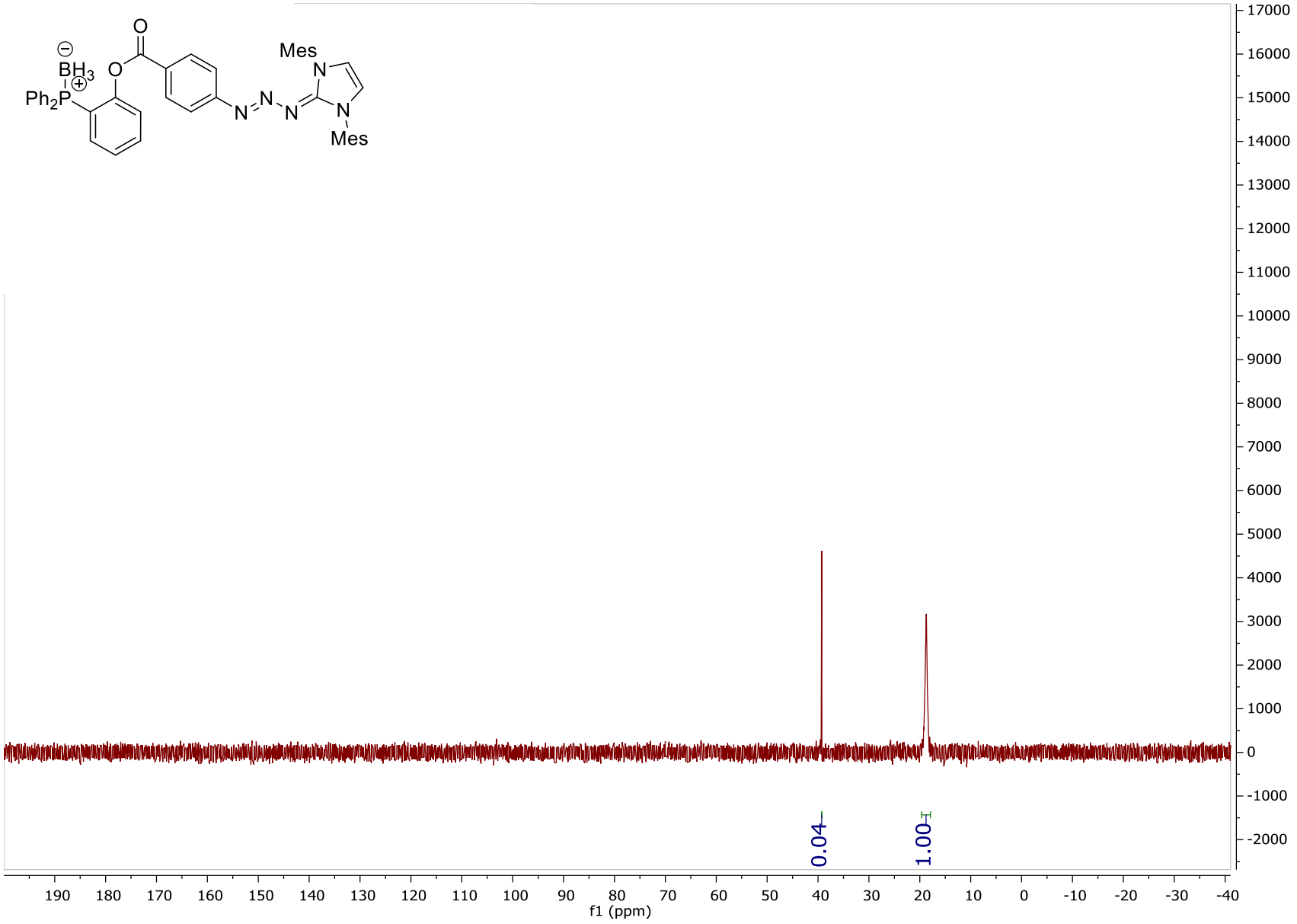

7.60 7.60 7.60 7.59 7.58 7.58 7.58 7.45 7.45 7.44 7.44 7.44 7.43 7.43 7.43 7.42 7.42 7.41 7.41 7.41 7.39 7.39 7.38 7.38 7.38 7.37 7.36 7.36 7.36 7.35 7.35 7.35 7.34 7.34 7.34 7.33 7.33 7.30 7.30 7.29 7.28 7.28 7.28 7.28 7.28 7.27 7.26 7.26 CDCl3 7.26 7.24 7.23 7.23 7.21 7.21 6.92 6.92 6.91 6.90 6.90 6.89
O
O
BH3
Ph2P
N3
6.97
2.16
5.02
1.03
0.86
7.7
7.6
7.5
7.4
7.3
7.2
f1 (ppm)
25000
20000
15000
10000
5000
0
6.97
2.16
5.02
1.03
0.86
2.00
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

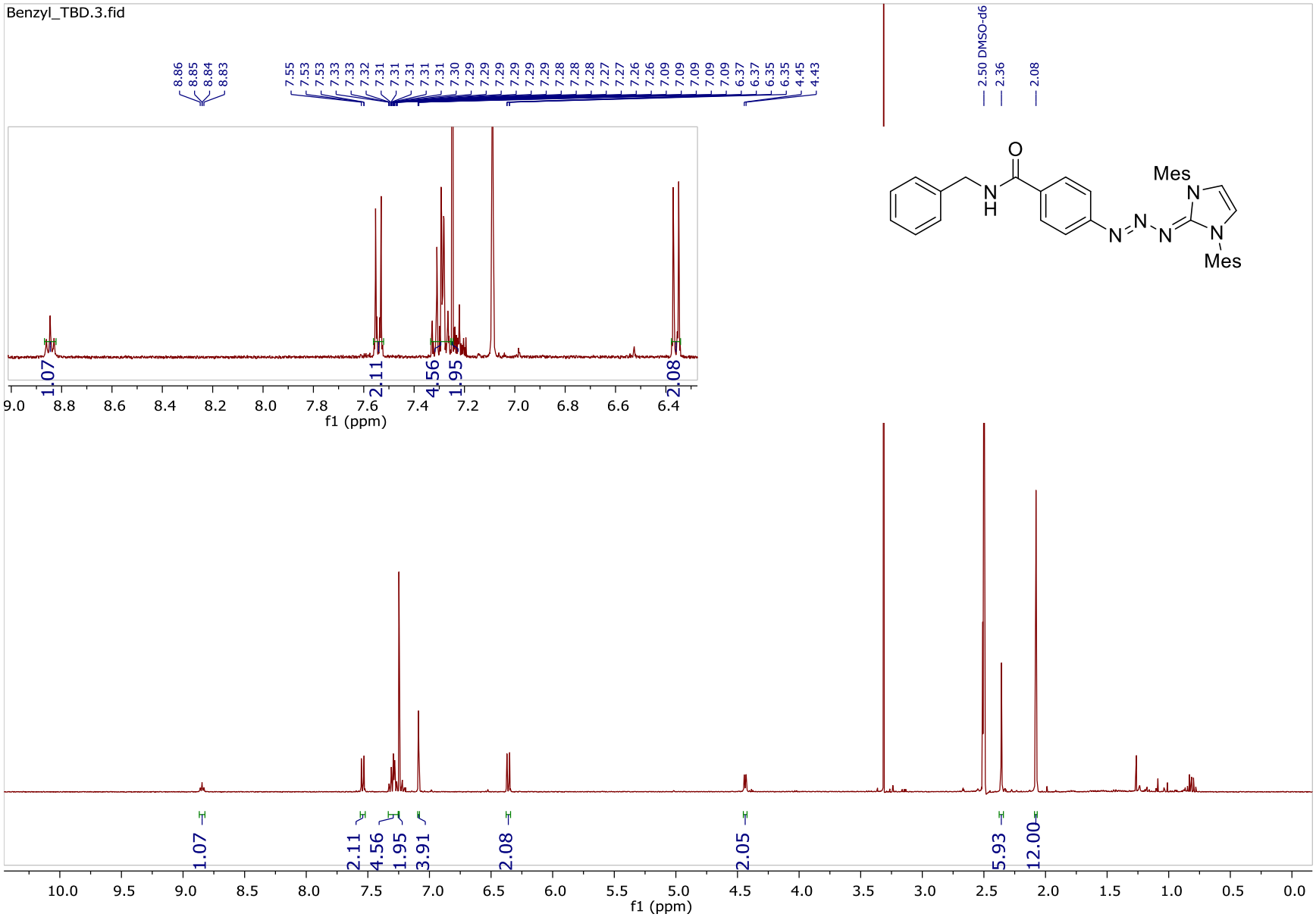

B7P25_col1.3.fid
163.07
152.56
145.40
134.83
134.76
133.28
133.18
132.87
132.25
131.41
128.95
128.85
128.43
127.85
126.23
126.14
125.00
124.28
118.63
77.16 CDCl3
O
BH3
O
Ph2P
N3
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
-10000
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

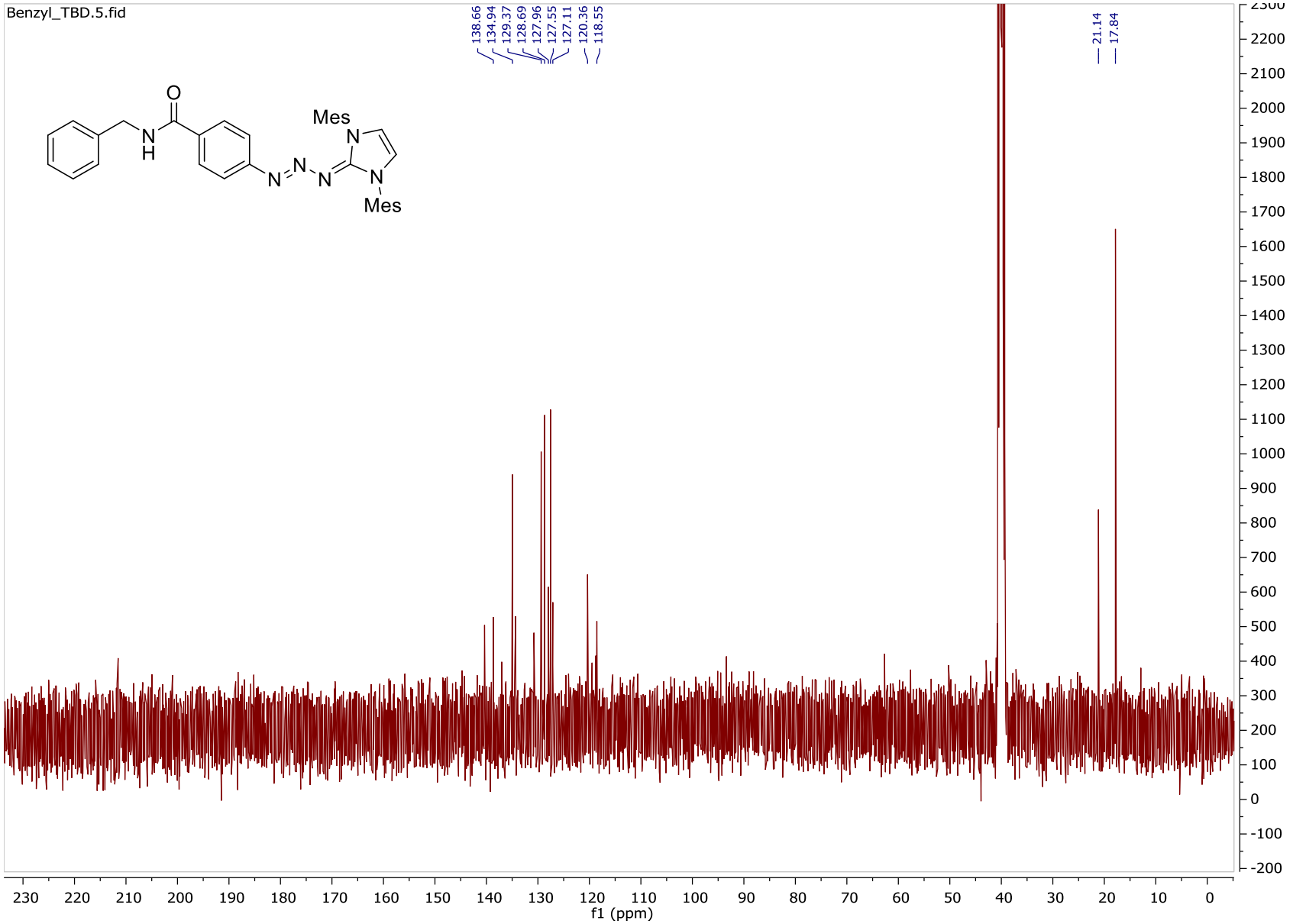

B7P25-col1.2.fid
O
BH3
O
Ph2P
N3
0.03
1.00
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90
f1 (ppm)

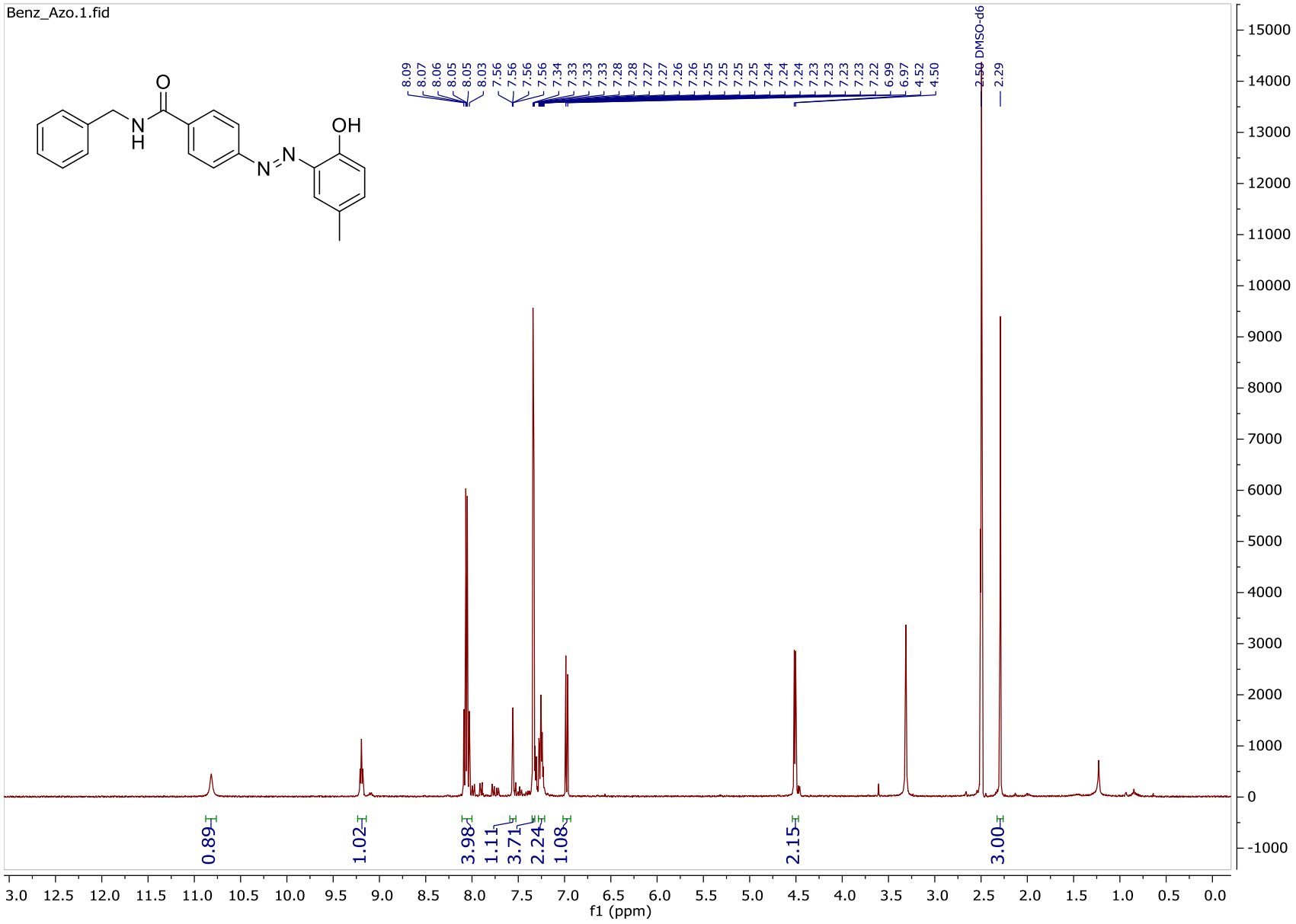

B7P26.2.fid
CDCl3
Mes
Mes
f1 (ppm)
f1 (ppm)

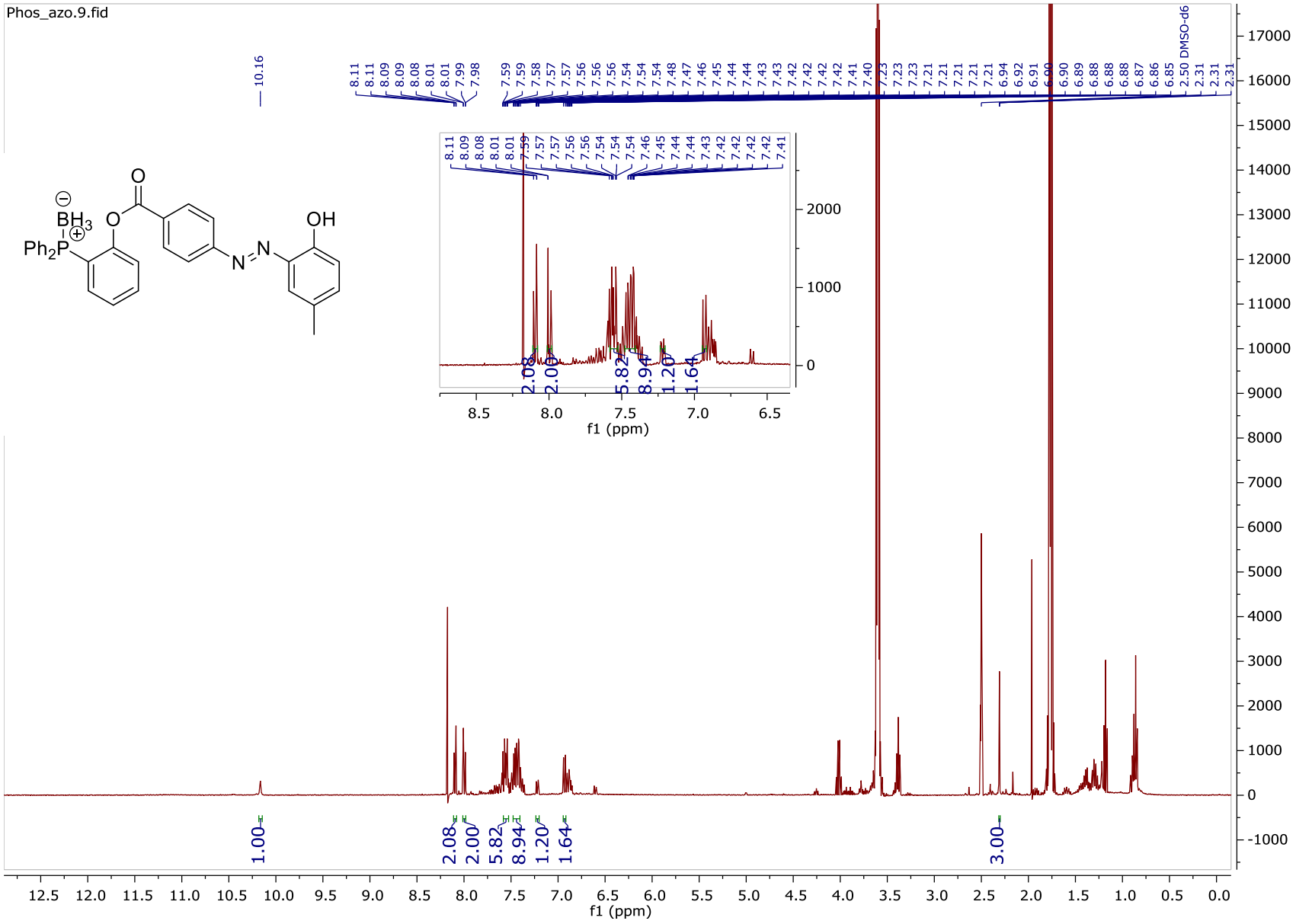

B7P26.3.fid
164.34
153.28
139.57
135.33
134.21
133.50
133.08
131.58
131.55
131.11
129.86
129.19
129.09
128.83
128.25
126.24
126.14
124.83
124.78
124.68
122.87
122.31
120.91
117.86
77.16 CDCl3
21.65
18.40
Mes
Mes
f1 (ppm)

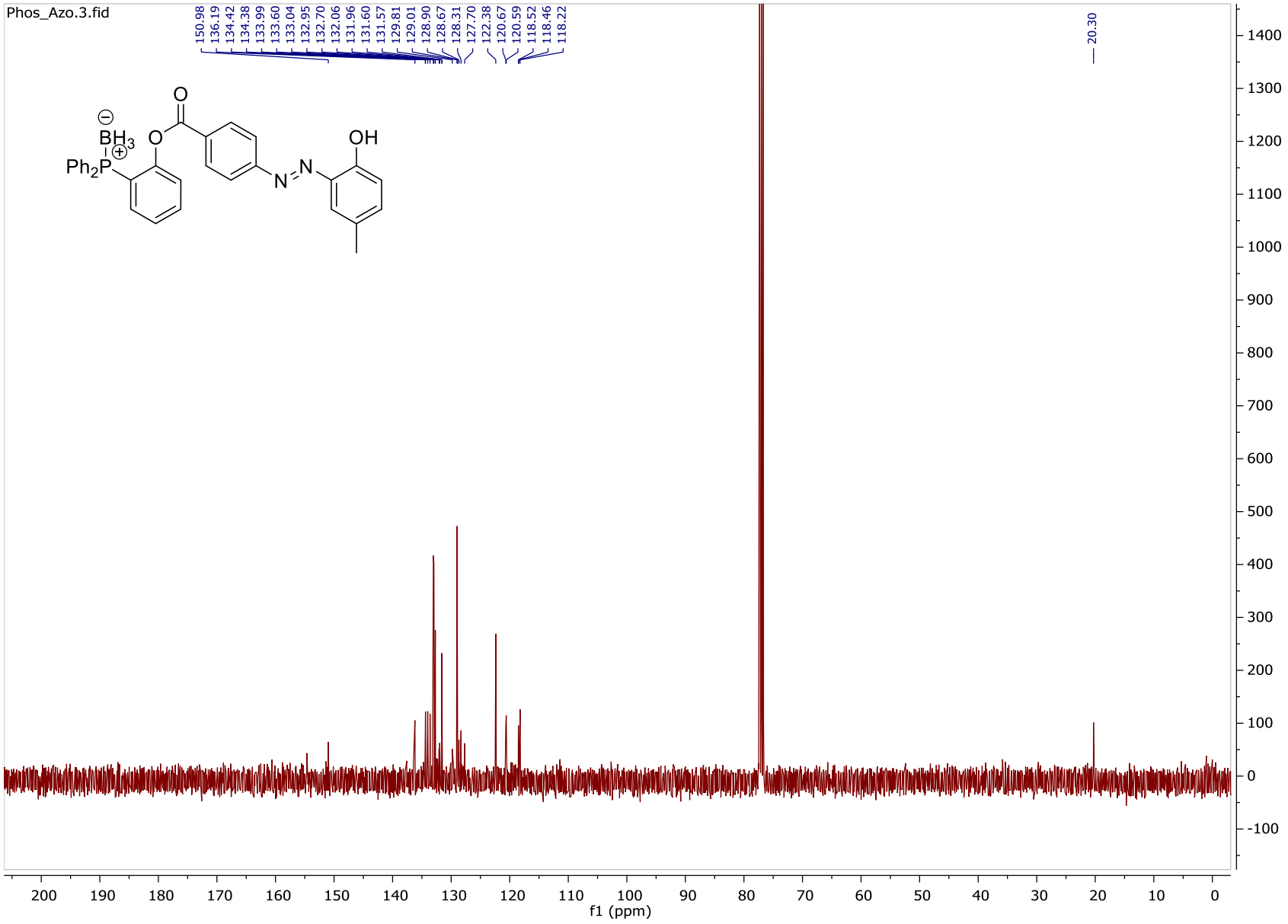

Mes
N
N
N
N
Mes
O
O
BH3
Ph2P
0.04
1.00
f1 (ppm)

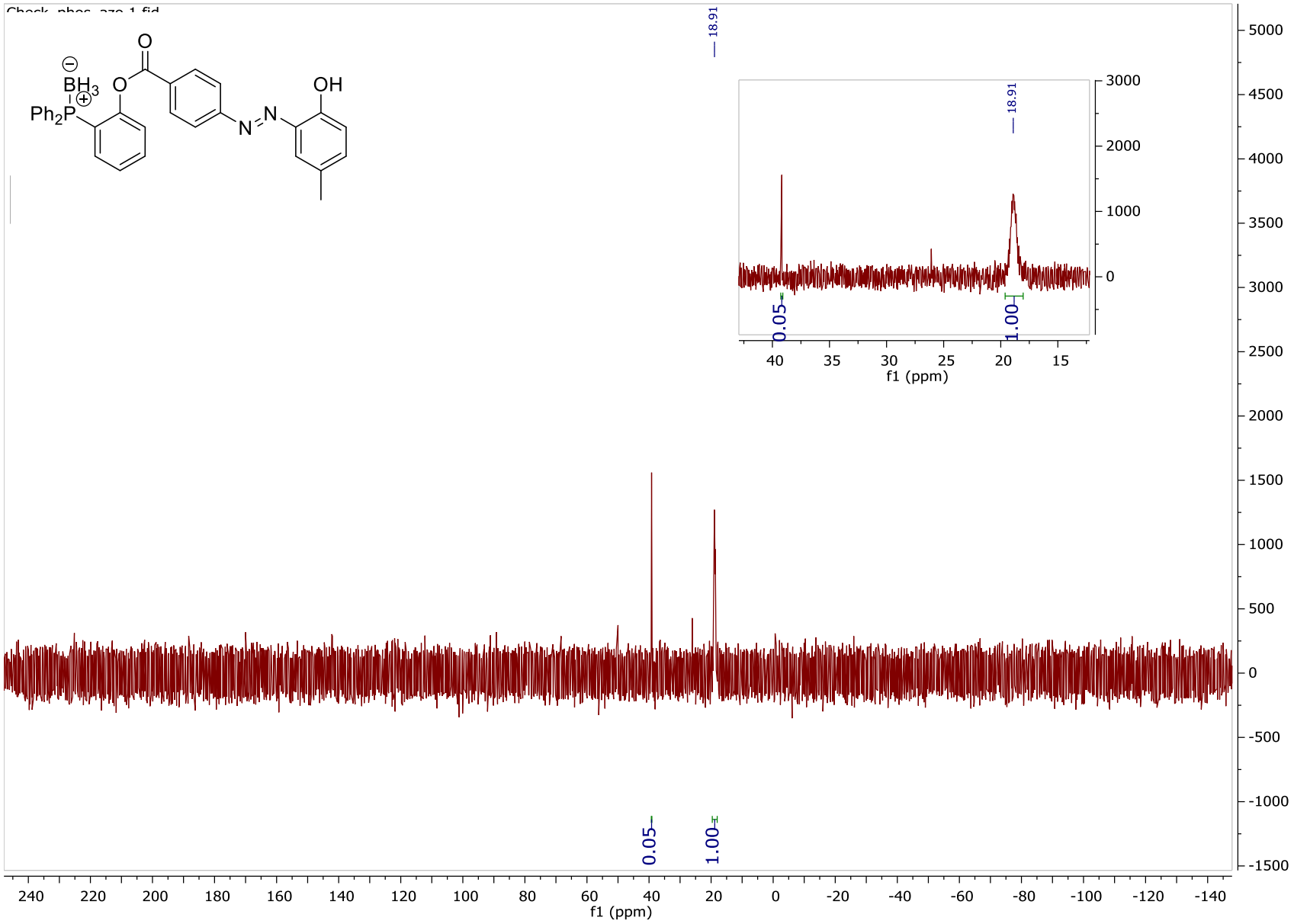

Benzyl_TBD.3.fid
8.86 8.85 8.84 8.83
7.55 7.53 7.53 7.33 7.33 7.32 7.31 7.31 7.31 7.31 7.30 7.29 7.29 7.29 7.29 7.29 7.28 7.28 7.28 7.27 7.27 7.26 7.26 7.09 7.09 7.09 7.09 6.37 6.35 6.35 4.45 4.43
2.50 DMSO-d6
2.36
2.08
O
N
H
N
N
N
Mes
N
N
Mes
1.07
2.11
4.56
1.95
2.08
f1 (ppm)
1.07
2.11
4.56
1.95
3.91
2.08
2.05
5.93
12.00
f1 (ppm)


Benzyl_TBD.5.fid
138.66
134.94
129.37
128.69
127.96
127.55
127.11
120.36
118.55
21.14
17.84
Mes
Mes
f1 (ppm)


Benz_Azo.1.fid
O
N
H
N
N
OH
8.09
8.07
8.06
8.05
8.05
8.03
7.56
7.56
7.56
7.56
7.34
7.33
7.33
7.33
7.28
7.28
7.27
7.27
7.26
7.26
7.25
7.25
7.25
7.25
7.24
7.24
7.24
7.23
7.23
7.23
7.23
7.22
6.99
6.97
4.52
4.50
2.50 DMSO-d6
2.29
0.89
1.02
3.98
1.11
3.71
2.24
1.08
2.15
3.00
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
3.0
12.5
12.0
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


Phos_azo.9.fid
DMSO-d6
f1 (ppm)
1.00
2.08
2.00
5.82
8.94
1.20
1.64
3.00


Phos_Azo.3.fid
150.98
136.19
134.42
134.38
133.99
133.60
133.04
132.95
132.70
132.06
131.96
131.60
131.57
129.81
129.01
128.90
128.67
128.31
127.70
122.38
120.67
120.59
118.52
118.46
118.22
20.30
O
BH3
O
Ph2P
N
N
OH
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


18.91
0.05
1.00
f1 (ppm)
18.91
0.05
1.00
f1 (ppm)

References

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