

From Probes to Cell Surface Labelling: Towards the Development of New Chemical Biology Compounds and Methods

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“Diversity of opinion about a work of art shows the work is new, complex, and vital. When critics disagree, the artist is in accord with himself. We can forgive a man for making a useful thing as long as he does not admire it. The only excuse for making a useless thing is if one admires it intensely.

All art is quite useless.”

-Oscar Wilde

“Every good scientist is half B.F. Skinner and half P.T. Barnum.”

-W. Seymour Skinner

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Abstract

Chemical biology encompasses the study and manipulation of biological system using chemistry, often by virtue of small molecules or unnatural amino acids. Much insight has been gained into the mechanisms of biological processes with regards to protein structure and function, metabolic processes and changes between healthy and diseased states. As an ever expanding field, developing new tools to interact with and impact biological systems is an extremely valuable goal.

Herein, work is described towards the synthesis of a small library of heterocyclic-containing small molecules and the mechanistic details regarding the interesting and unexpected chemical compounds that arose; an alternative set of non-toxic copper catalyzed azide-alkyne click conditions for *in vivo* metabolic labelling; and the synthesis of an unnatural amino acid for further chemical modification via [3+2] cycloadditions with nitrones upon incorporation into a peptide of interest. Altogether, these projects strive to supplement pre-existing methodology for the synthesis of small molecule libraries and tools for metabolic labelling, and thus provide further small molecules for understanding biological systems.

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List of Abbreviations

aaRS	aminoacyl tRNA synthetase
BARAC	biarylazacyclooctynone
BTES	2-(4-((bis((1-tert-butyl-1 <i>H</i> -1,2,3-triazol-4-yl)methyl)amino)methyl)-1 <i>H</i> -1,2,3-triazol-1-yl)ethanesulfonic acid
CARS	coherent anti-Stokes Raman scattering
CuAAC	copper catalyzed azide-alkyne cycloaddition
DBU	1,8-diazabicycloundec-7-ene
DCM	dichloromethane
DIFO	difluorinated cyclooctyne
DMAD	dimethyl acetylenedicarboxylate
DMF	dimethyl formamide
DOS	diversity oriented synthesis
ESI	electrospray ionization
Et ₃ N	triethylamine
FITC	fluorescein isothiocyanate
GC-MS	gas chromatography mass spectroscopy
HCV	hepatitis C virus
HPLC	high pressure liquid chromatography
HTS	high throughput screen
Huh 7.5	human hepatoma cells
KHMDS	potassium hexamethyldisilazane
KOtBu	potassium <i>tert</i> -butoxide
LC-MS	liquid chromatography mass spectrometry
LUMO	lowest unoccupied molecular orbital
MCR	multi-component reaction
MP	melting point

MTT	metabolism of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHC	<i>N</i> -heterocyclic carbene
NHS	<i>N</i> -hydroxysuccinimide
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
OBOC	One bead one compound
PBS	phosphate buffer saline
R _f	retention factor
RT	room temperature
SAR	structure activity relationship
SBP	sulfonated bathophenanthroline
SPAAC	strain promoted azide-alkyne cycloaddition
TAMRA	tetra amino methyl rhodamine azide
TBTA	tris((1-benzyl-1 <i>H</i> -1,2,3-triazol-4-yl)methyl)amine
THF	tetrahydrofuran
TLC	thin layer chromatography
tRNA	transfer ribonucleic acid
UAA	unnatural amino acid

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1.1 Introduction to Chemical Biology

The research performed within this thesis encompasses two different topics from the field chemical biology. That is to say, the work herein has primarily been performed with the aim of studying or manipulating biological systems and processes using small organic compounds. The different chapters involve different applications of chemical biology which will be discussed in brief in this introductory chapter and are: chemical library generation and metabolic labelling using small molecules and unnatural amino acids (UAAs). In the first section, topics such as the purpose of generating large chemical libraries through synthetic or biological methods will be discussed, the different means of doing so, and some classical and recent relevant discoveries made through this process. The second section will look at metabolic labelling and subsequent bioorthogonal reactions that can provide a better understanding of metabolic processes as well as the limitations of such reactions. The strategies behind incorporating UAAs into a biological system will be outlined as well as their limitations, while also briefly discussing several previously reported UAAs and their purpose.

1.2 Chemical Libraries in Chemical Biology

1.2.1 The Need for Chemical Libraries

Nature is an extremely complex system driven by chemical reactions that ultimately necessitate transformations requisite for life. The diversity of natural products that abound in nature is seemingly without end. These natural products have found much use in pharmaceutical and industrial applications. However, it is very rare that these natural products are optimized for the task for which humans wish to exploit them, and are thus often further modified by chemists (for example, through SAR). There is a poor overlap between pharmaceutical drugs and naturally occurring products, in part due to the requirements for solubility, selectivity, membrane permeability and pharmacokinetics.

To obtain novel compounds of potential interest from a biological perspective, chemists often turn to the development of synthetic libraries to uncover compounds that can perturb a gene product and deliver a specific phenotype (a process known as chemical genetics)[1]. Ideally, these libraries can deliver a wide variety of structurally varied compounds in relatively little time when compared to target directed synthesis: the synthesis of compounds with a target in mind, usually complemented with exhaustive structural studies of said target, thus leading to a more focused series of compounds[2]. The unfortunate reality, however, is that often these libraries suffer from laborious manipulations leading for the most part, to compounds without much interest. The scope of chemical space (the descriptor space encompassing organic carbon based molecules)[3] that can be occupied by different plausible molecules has been documented to range between 10^{30} and 10^{200} [4]. However, studies suggest that while these enormous numbers comprise the total number of possible permutations, only roughly 10^9 are likely to be of interest based on the relatively small sample of possibilities exhibited in nature[5].

To minimize the investment of both time and resources towards uncovering compounds with little to no interest, researchers often turn to nature for their starting point. The majority of combinatorial chemistry begins with a molecular scaffold or privileged structure [6] found in nature: the idea being that these natural products have been shaped over time with the evolution of nature, and thus pose to occupy the most relevant chemical space to be potentially active as chemical probes or as pharmaceuticals. From a biological perspective, genetics can often be useful in providing some clarity in terms of what natural products will be synthesized in a given organism. Often, there are genetic “trackers” that are conserved greatly from one organism to another that equip said organism for the synthesis of specific natural products. An ever increasing documentation and understanding of genomes provides researchers with a better understanding of which natural products will occur where, which can alleviate tedious isolation and characterization work. Being able to deduce this helps to locate new natural products that can serve as scaffolds or inspiration of scaffolds for further diversification via combinatorial chemistry. [7]

1.2.2 Methods for Obtaining Chemical Libraries

A combinatorial approach to synthesizing a library of compounds is most easily represented by peptide synthesis. The strategy involves the anchoring of an amino acid to a resin, followed by a series of deprotection and coupling steps, eventually leading to a peptide chain that could be macrocyclized by various methods or simply cleaved from the resin as a linear peptide and was first presented by Furka *et al.* [8] This method could be modified through split pool synthesis [9] where various beads with different amino acids anchored to them are introduced into a vessel containing a set of reagents, then transferred to another vessel with a separate set of reagents and so on. Thus, differential sequences could be obtained readily to obtain a diverse set of compounds. The capacity for this process to be automated and the lack of purification steps (simple washing of excess reagents between each step was sufficient) also added to the upside of this methodology. A convergent approach allows groups to perform combinatorial chemistry on two separate components and finally link them together in the final transformation to generate a series of novel compounds. This strategy is limited in the chemistry that can be performed, and care must be taken in the choice of protecting groups to lead to the “one bead one-compound” (OBOC)[10] concept. As well, this kind of approach is limited by the length of the peptide, as efficiency seems to decrease with increasing oligopeptide size. Also because of OBOC, the extent of molecular complexity obtained is directly proportional to the number of beads used. Additionally, a pre-mix alternative to the mix and split method described above is able to provide an equivalent amount of diversity by first creating a pool of premixed amino acids that will serve as common “tags” on each bead. After a series of couplings, the beads are split and subjected to treatment similar to the mix and split methodology. This route circumvents the OBOC “issue”, but also the differences on each bead are conserved from bead to bead. Changes in concentrations of different amino acids during the pre mix stage can compensate for varying kinetic rates of reaction to ensure the additions are consistent from bead to bead and minimizes bias. [11]

Combining MALDI-TOF-MS analysis data with peptide library screens can permit researchers to focus their library synthesis. They can determine common residues or conserved sequences present in all active compounds (be they active as inhibitors of enzymes

such as kinases [12] or proteases [13]; as ligands [14]; or as antigens [15], to name a few examples) from the library and reiterate these findings in subsequent syntheses.

However, where this strategy suffers is in the limitations of the chemistry that can be applied to the system. While simple coupling and deprotection steps are tolerable, more complex chemical reactions are not possible, essentially limiting this type of methodology to peptide coupling. This in itself was a problem within the realm of library synthesis, since peptides represented a relatively small portion of naturally occurring active compounds when being contrasted to small molecules. Additionally, the lack of analytical data obtained throughout the process makes it a kind of black box chemistry process. Nevertheless, several important molecules have arisen from this strategy and will be mentioned in the subsequent section.

Variability in pendant chemical motifs, chemical composition, stereochemistry and skeletal makeup [16] chemical libraries is achieved through a subset of combinatorial chemistry known as diversity oriented synthesis (DOS), [17] where a set of large complex molecules is synthesized and modified, and can be said as being “natural product-like”. Potentially, both the largest advantage and hurdle presented by DOS is the goal of achieving backbone diversity from a common scaffold. It has been noted that scaffold diversity within a library is likely the most important factor in obtaining a large number of hits, but is also the most challenging feat to attain. [18]

Alternatively, multi-component reactions (MCR) and tandem/domino reactions also permit the synthesis of a large series of compounds, often in one pot. Some examples that have been applied to the synthesis of chemical libraries include the Hantzsch, Biginelli, Passerini and Ugi multi-component reactions [19]. Using these chemical reactions to synthesize molecular scaffolds with orthogonal points for introducing diversity has been an effective strategy in the synthesis of natural product-like libraries[20].

From a non-synthetic perspective, there are interesting alternatives, such as phage-display and combinatorial biosynthesis. In the former, a virulent fusion bacteriophage infects cultured cells. Bacteriophages have the capacity to use the host machinery to replicate their DNA, and fusion bacteriophages can accept large segments of DNA within their own

genome and be tailored to synthesize large linear or cyclic libraries of novel peptides based on this sequence [21]. This strategy is limited by the fact that only the 20 naturally occurring amino acids can be incorporated, and the transformation efficiency. However, a large number of phages (and thus, peptides) can be relatively easily prepared, and the size of the gene products is not limited as in the case of synthetic peptide preparation. The latter process involves providing natural products as substrates to biological (for example, microbial) systems and allowing them to modify them chemically. This process works well for certain chemical transformations such as glycosylation, redox, sulfation, methylation, isoprenylation, halogenations, and acetylation[22-24]. This process only works with certain natural substrates, but can be often streamlined with synthetic chemistry to provide further modifications upon isolation. Additionally, DNA-encoded chemical libraries are a newer alternative that allows researchers to perform iterative split and mix cycles of oligonucleotide replication which results in libraries of evolved sequences leading to a diverse compilation of gene products coded by specific gene tags of interest. [25]

The outcomes of chemical libraries is often unpredictable, but there are enough success stories that merit further research to uncover novel chemical scaffolds and methodology with the hope of uncovering potentially useful compounds, particularly from a therapeutic perspective.

1.2.3 Outcomes of Chemical Libraries

Of recent note, combinatorial chemistry has led to the discovery of several pharmaceutically relevant compounds. These include, but are not limited to, those summarized in figure 1-1. They may have been obtained from a peptide synthesis approach, an MCR approach, or a DOS approach and represent a wide array of therapeutic targets that may have been identified through high throughput screening (HTS), reverse or forward chemical genetics. Often, hits are determined through simple binding assays through immobilization on a column treated with a target of choice, but other screens are also possible, and include cell-line specific cytotoxicity assays, microscopy for the tracking of a fluorescent protein, or observation of some altered cellular process. The assay used depends on the target of interest and also the method used to prepare the samples. Follow up studies are typically performed to confirm the mechanism of action and assure that a false positive

has not been revealed. Examples provided represent hits with higher citation numbers or those discovered more recently. As the list of libraries generated through combinatorial chemistry is quite large and encompasses a wide array of natural product and alkaloid inspiration, an exhaustive list is not presented. [26]

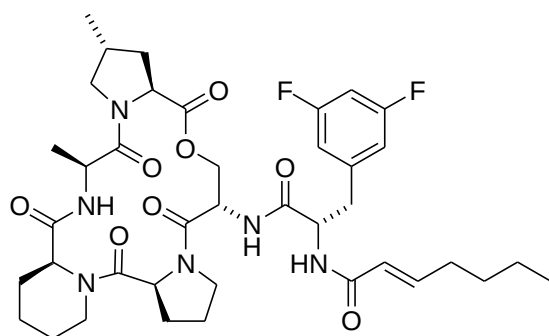
ADEP 4 [27] was discovered through the synthesis of a library of Enopeptin analogues via Joullié-Ugi MCR and was found to be a potent antibacterial agent. Robotnikin [28] was identified via a scaffold with three points of diversity and closed using Grubbs' ring closing olefin metathesis and found to be a potent inhibitor of the sonic hedgehog regulatory pathway, implicated in various cancers and physiological diseases such as cyclopia [29]. The Bcl-2 inhibitor depicted was produced from a library of bi- and tricyclic containing alkaloids encompassing roughly 15,000 compounds [30]. Finally, the 3-benzazepine scaffold provides a molecular framework for introducing three points of diversity to lead to compounds structurally related to naturally occurring alkaloids such as aphanorphine and cephalotaxine [31]. LLP2A arose from a peptidomimetic library coupled with highly stringent screening strategies to identify this compound which selectively (pM) targets $\alpha 4\beta 1$ of T- and B-lymphoma xenografts, and due to the presence of unnatural and D amino acids in its backbone, was less susceptible to proteolysis. Galanthamine, a potent acetylcholinesterase inhibitor, was modified through DOS, varying four different positions with various chemical groups to generate a library of 2,527 molecules. Using a cell-based phenotypic assay monitoring protein trafficking, one compound, secramine, was found to exhibit inhibitory properties of this pathway by affecting export from the golgi apparatus. DOS thus provided a scaffold for modification which led to the synthesis of a compound with unanticipated properties when compared to its parent compound that served as the scaffold.[32] A library of diverse quinoline based compounds were synthesized in a DOS manner, and several hits (one included above) showed selective and potent cytotoxicity towards various cancer tumour cell lines. [33]

While these libraries can be synthesized with a specific target in mind, they can also be synthesized and screened at random in a high throughput manner. A chemical library being screened against a typical phenotype, such as cell death, is a process known as forward chemical genetics. In a screen of over 70,000 compounds Erastin was identified as producing

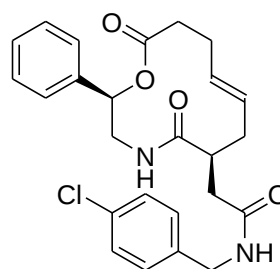
this phenotype in several tumor cell lines. Further studies were performed to determine the mechanism of action was not merely due to toxicity, and the current investigations suggest that iron and mitochondrial respiratory activity is affected[34].

While this list is by no means exhaustive, it does provide insight into the molecular complexity that can be achieved through the power of combinatorial chemistry not easily attainable through traditional target oriented synthesis.

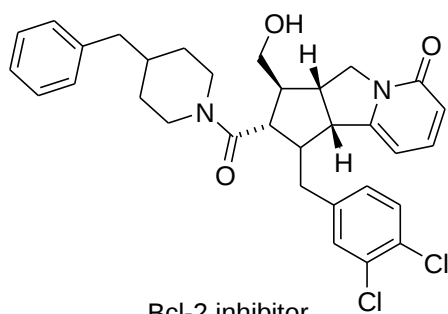
Identification of lead compounds can occur from a pre-existing knowledge of the activity of the desired target or via a phenotypic or genotypic screen. Irrespective of the knowledge possessed when beginning, the generation of large, diverse or focused libraries allows researchers to tap a vast array of compounds for potential advancement.



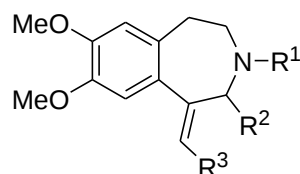
ADEP4



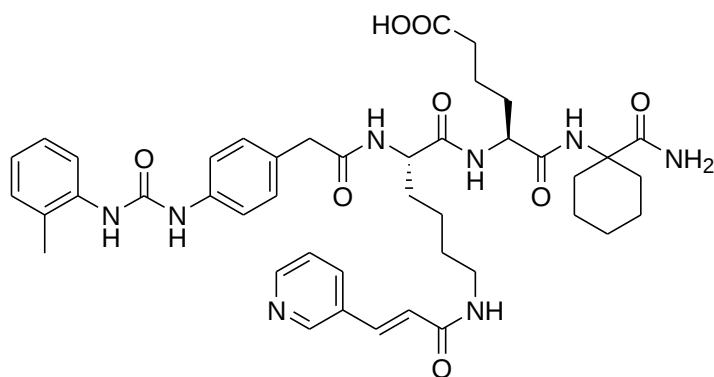
Robotnikin



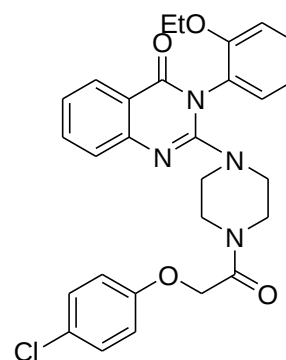
Bcl-2 inhibitor



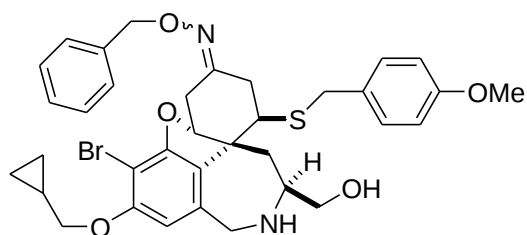
3-benzazepine
framework



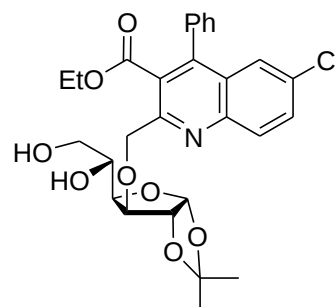
LLP2A



Erastin



Secramine



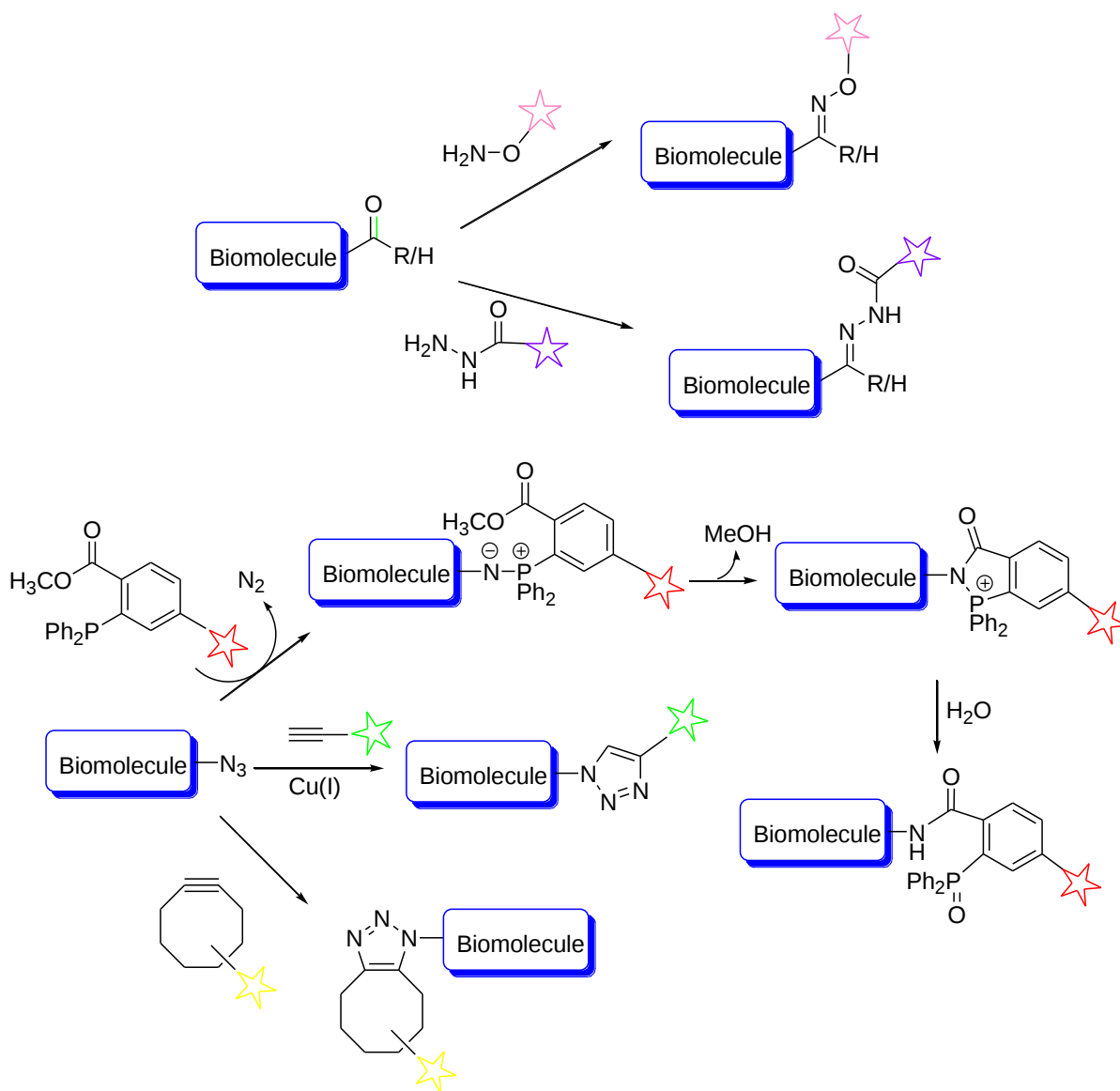
Quinoline analogue

Figure 1-1 Representative structures of small molecules synthesized and identified through chemical library syntheses.

1.3 Metabolic Labelling and Live Cell Imaging Using Small Molecules

The labelling and subsequent visualization of certain metabolites has been achieved in a variety of biological systems ranging from bacterium to mice. Most commonly, a sugar that has been chemical modified with a bioorthogonal (that is, one which does not perturb the biological environment) moiety is incorporated into the diet of the species including *Caenorhabditis elegans*[35], mice[36], developing zebrafish[37] and *Sacchromyces cerevisiae*[38] to be placed under surveillance. Once the compound has been metabolized by the species, further modification can be achieved by virtue of chemical transformations which fall under the umbrella of “click chemistry”: rapid, selective and mild means of obtaining irreversible covalent modification [39] for further study. This strategy introduces tolerable changes to the metabolite prior to its incorporation, thus minimizing disparity between metabolism of the natural and modified substrate.

Several traditional organic chemistry reactions fit these criteria, and have thus been applied for metabolic labelling. Incorporation of an aldehyde or ketone moiety (which are typically absent from cell surfaces) onto a protein using biotin ligase or aldehyde tags [40,41] allows for the introduction of a chemical handle capable of selective modification. Indeed, this has been performed on cell surface proteoglycans using either aminoxy or hydrazide probes [42,43] as depicted in scheme 1-1 to give the resultant biotinylated oxime or hydrazone linkages respectively.



Scheme 1-1. A series of bioorthogonal reactions following labelling of a biomolecule with either an aldehyde/ketone or an azide. Presented are condensation reactions leading to oxime (pink star) or hydrazone (purple star) linkages, as well as the Staudinger ligation (red star), copper mediated Huisgen [3+2] cycloaddition (green star) and strain promoted azide alkyne cycloaddition (yellow star).

The Staudinger reaction (originally a reaction between azides and triphenylphosphines, [44] has been applied to ligate a biomolecule containing an orthogonal azide and a triphenyl phosphine probe containing both a reporter tag and an ortho methyl ester. As depicted in Scheme 1-1 this allows the formation of an aza-ylide which subsequently cyclises to remove methanol. Reaction with water oxidizes the phosphine and produces a very stable amide linkage between the reporter tag and the biomolecule of interest. As with the previous case,

this strategy requires the incorporation of a bioorthogonal chemical group onto the biomolecule of interest.

Quite possibly the most applied of the click reactions is the 1,3-dipolar cycloaddition of azides with alkynes, traditionally known as the Huisgen cycloaddition. It has received the most notoriety due to its tolerance of aqueous conditions, the stability of its aromatic 1,2,3-triazole product, and the bioorthogonality of the azide and alkyne moieties. The application of these reactions has been limited for the most part by their inability to be applied *in vivo* due to the cytotoxicity issues related to using a copper catalyst to employ the reaction under physiological conditions. This reaction is depicted in scheme 1-1 in its traditional, copper mediated sense (green star).

Development for the Huisgen [3+2] cycloaddition has thus been spent on deriving potentially less toxic alternatives, specifically chemical moieties that do not require copper to react, as will be described below. The inherent toxicity issues have thus been attributed to copper, or its ligated form *in situ*. One such approach employed the use of an electron deficient oxanorbornadiene which first reacts with the azide partner to give the triazole product and a furan byproduct through a retro Diels-Alder reaction, however the toxicity issue of this furan by-product however has limited its biological application[45].

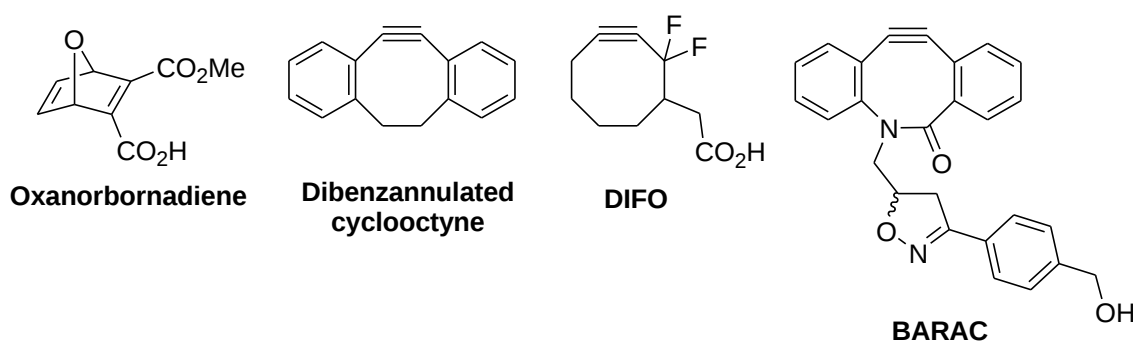


Figure 1-2 Representative structures of varying strategies over time for performing copper free click chemistry.

Using internal ring strain within the alkyne moiety has provided an alternative route for labelling known as strain-promoted azide alkyne cycloadditions (SPAAC). The energy (18 kcal/mol for the simple cyclooctyne[46] released upon reaction with an azide has allowed this reaction to occur readily in the absence of a copper catalyst, permitting their application

in *in vivo* studies. Much effort has been put into the development of cyclooctynes with increased ring strain to improve reaction kinetics, including the introduction of flanking benzene rings[47], through introduction of alpha electron withdrawing groups and in effect lowering the lowest unoccupied molecular orbital (LUMO) of the alkyne as in difluorinated cyclooctyne (DIFO) [48], and through increased sp² hybridization through the inclusion of an amide within the ring as in biarylazacyclooctynone [49].

One of the most well adopted means of labelling is through the metabolic incorporation of azide labelled sugars. This strategy has been applied to many living systems. *Caenorhabditis elegans* treated with azide bound galactose derivatives were subsequently labelled with a fluorophore conjugated DIFO to identify membrane bound glycans which showed colocalization with the physiological Mucin components [35]. Cell surface sialic acid derivatives (the metabolic product of mannose) containing azides were also successfully labelled using SPAAC in live mouse tissue isolates [50]. The diffusion properties and mobility of hippocampal neurons was performed using DIFO-biotin and a streptavidin linked quantum dot using single particle tracking[51].Also, *in vivo* imaging in zebrafish embryos [52] has also been achieved with this azido sugar as well as azido sugar nucleotides has provided insight into the localization of cell surface glycans during the early stages of development. This strategy has even obtained tissue specificity through the use of a protease substrate which is then cleaved by prostate specific antigen to target labelling or therapy of cancer cells[49].

Despite the aforementioned advancements in SPAAC and instances of application, the relative kinetics do not match those found in the copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction ($k = 10^{-1}\text{M}^{-1}\text{s}^{-1}$ for DIFO/dibenzannulated cyclooctyne). In contrast, the CuAAC reaction can have a k_{obs} ranging from this (no ligand) to $30\text{M}^{-1}\text{s}^{-1}$ when using rate-accelerating ligands[53]. Additionally, the large size of the alkyne limits to some extent the application of this approach. As well, its stability is diminished and can thus lead to decomposition or potentially non-specific reactivity. Finally, the synthetic routes to the various cyclooctynes are far from trivial, and often involve numerous steps with a low overall yield (i.e. 3 steps and 24% overall for dibenzannulated cyclooctyne, 12 steps and 1% for DIFO, and 6 steps and 18% yield for biarylazacyclooctynone (BARAC)). Without

compromising cellular viability or stability of starting materials, it would be ideal to obtain comparable kinetics and convenient reagent preparation, similar to those found in the CuAAC. Researchers have recently uncovered (through compound library screens) water soluble bis(tert-butyltriazoly) ligand (BTTEs)[54] that stabilizes reduced copper in biological systems in an attempt to achieve this feat, which suggests a desire for non-toxic variants of the CuAAC for metabolic labelling.

1.4 Metabolic Labelling Using UAAs

1.4.1 Chemical Diversity through UAAs

Another means of chemically tagging cellular components is through the metabolic incorporation of UAAs. Twenty naturally occurring amino acids make up the vast majority of proteins found in nature. While these amino acids convey much of the functionality required for life, there are some processes that require the use of cellular cofactors or post-translational modifications to occur.

The incorporation of unnatural functional groups into biological systems provides a means of accomplishing selective, orthogonal chemical transformations via, for example, the Staudinger ligation[44] or [3+2] azide alkyne Huisgen cycloaddition[55]. Reactive partners for these types of transformations can be incorporated either *in vitro* or through metabolic labeling. The latter can be accomplished using modified metabolites such as sugars, as described above and the subject of chapter three, or by use of UAAs, which will be the subject of chapter four.

Limited only by the ingenuity of the researcher, UAAs can provide a wide range of functionality to a peptide chain, permitting researchers to manipulate the biological system of interest as necessary. Functionalities previously incorporated include those capable of cleaving the peptide backbone upon activation [56]; that can be introduced to modify the hydrophobic region of a particular peptide with unique steric properties[57]; those which have bioorthogonal partners for click chemistry (i.e. an alkyne [58]; those capable of crosslinking with other peptides [59]; those which are fluorescent [60,61]; those which

provide structural information for ion channels; ones with unique spectral properties for monitoring changes in protein folding.

As well as introducing UAAs, modified naturally occurring amino acids can also be incorporated by similar strategies to those which will be described in section 1.4.2. These include protected or caged amino acids which are typically spatially and temporally controlled through the use of photocages [62,63], and only become activated or uncaged upon excitation from a specific wavelength of light; this strategy has been applied for protecting serine, allowing for the spatial and temporal control of phosphorylation of a target transcription factor Pho4[62]. Likewise, natural amino acids often undergo post-translational modifications to attenuate or modify their functionality, including the transfer of sulphates[64], and methylation[65] of tyrosine, and with the UAA analogues of these modifications they can be incorporated at the whim of the researcher. Thus, UAAs provide a selective means of manipulating, observing, monitoring and understanding proteins, their function, their localization, and their interactions with other biological molecules.

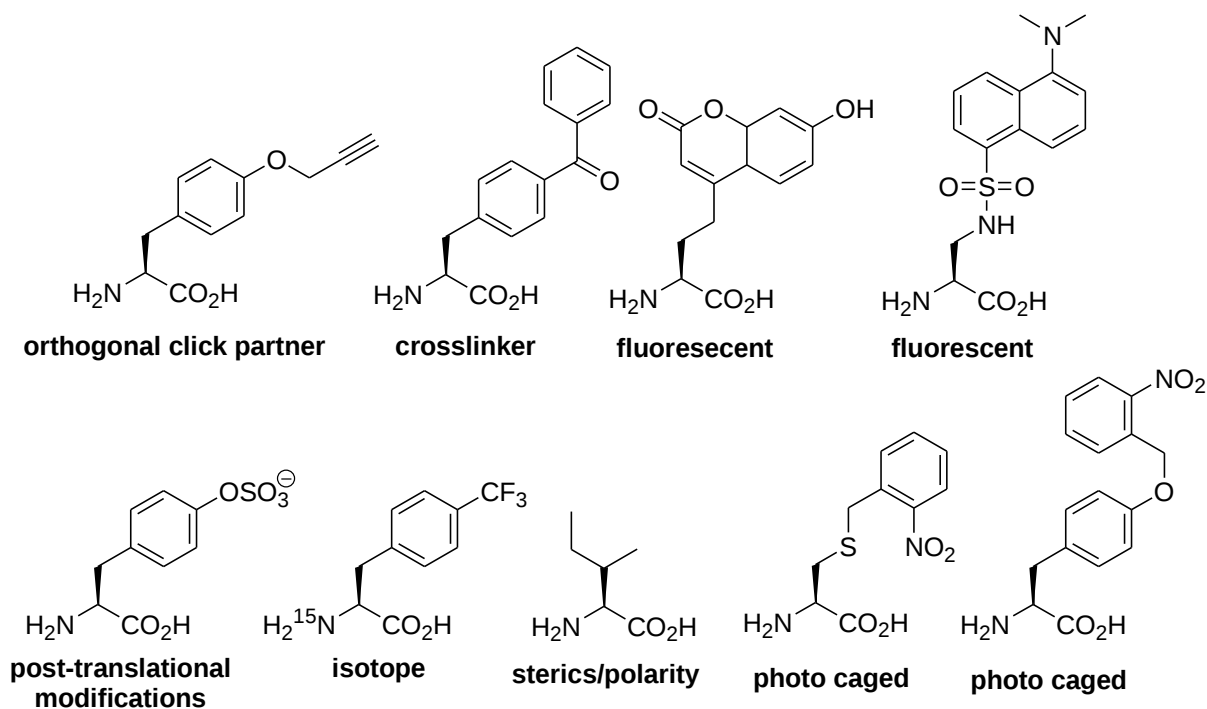


Figure 1-3 Various amino acids exemplifying the kind of chemical diversity that can be incorporated *in vivo* for various applications discussed above.

1.4.2 Incorporating UAAs

The breadth of chemistry [66-68] provided by UAAs makes them a powerful tool in the study of biological systems. Their introduction into peptides has been achieved by several means, the most widely used being through the translational machinery as a natural amino acid would. Three (TAG, TAA, and TGA: known as amber, ochre and opal respectively) of the total 64 permutations of three unit codon sequences are unassociated with any natural amino acid, and have thus been utilized by researchers as a programmed codon for the introduction of UAAs. Through rigorous two step selection processes, a UAA can be partnered with a tRNA and an aminoacyl tRNA synthetase(aaRS), the two necessary components for introduction via translation. The capacity to introduce a UAA *in vivo* by such a method is limited by the UAA's, tRNA's, and the aaRS' orthogonality within the system and with respect to their endogenous counterparts. Obviously, stability of the UAA, toxicity, local concentration and localization are all factors that must be taken into account as well.

The selection process by which these components are derived was first described in *Escherchia coli*[69]. In eukaryotes, a similar system was derived by co-transformation of a *Gal4* transcriptional activator protein transcript with an *E. coli* derived tRNA which codes tyrosine and targets the amber codon and a library of active site mutant TyrRS also derived from *E. coli* into *S. cerevisiae* rendered auxotrophic for histidine. The activator protein *Gal4* activates transcription of two genes, *HIS3* and *URA3*[70], and its (*GAL4*) transcript contains two amber stop codons. The yeast's synthesis of histidine in the absence of any exogenous source is performed by the product of *HIS3*. Only cells supplemented with UAAs that fit in the active site of the mutant aaRS are successfully translated to provide the activator for the *HIS3* gene and the downstream effect is synthesis of histidine and survival of the yeast. However, this does not rule out the possibility that some natural amino acid was actually incorporated into the translation product. To determine this, a negative selection process is performed with all surviving mutants, where control of *URA3* is the deciding factor (a gene whose product converts 5-fluorootic acid to toxic 5-fluorouracil) [71] is performed. Yeast are treated with 5-fluorootic acid and in the absence of the UAA, only surviving mutants are considered to have contained an aaRS selective for the UAA, while those that died were

likely coding for a natural amino acid. This selection process has been used for the incorporation of dozens of UAAs with high fidelity into *S. cerevisiae* and subsequently used these tRNA/aaRS pairs in higher ordered mammals.

Alternative methods have also been explored, including rendering the target host auxotrophic in a specific amino acid for which the unnatural variant is structurally similar. This method suffers from its inability to scrutinize between different sites within a peptide, and thus is only capable of an all or nothing effect. Additionally, all proteins containing the analogous amino acid will be affected, so results must be. Finally, synthetic chemistry on both solid phase and in solution have been used to introduce UAAs, however these have limitations in terms of protein length. This can be overcome through modern techniques such as native chemical ligation, which allows the splicing of two individual peptides [72], but is limited by the requirement of a conserved sequence capable of carrying out the ligation. Similar to drawbacks regarding the solid phase synthesis of peptide libraries, this strategy also requires careful attention to the protecting group strategies to avoid individual functional groups from cross-reacting, and characterization is only possible at the final stage.

Apart from the aforementioned limitations, imagination and interest drive forward the type of UAAs that researchers design and use as an investigative tool for their specific needs and research interests.

1.5 Outline of Work

Chapter two discusses work leading to the novel methodology towards the synthesis of unique, nitrogenous, bicyclic compounds. The application of these kinds of compounds in biological studies is irrefutable, however the synthesis of unique molecular scaffolds is a virtually limitless field and herein we report the thought process towards a novel methodology, the discoveries, and the mechanistic experiments and explanations for the observations made.

Chapter three discusses copper toxicity in CuAAC with respect to the ligand bound to the active complex. L-Histidine, a naturally occurring amino acid bearing an imidazole functionality, is chosen to perform this reaction in live cells, and serve as a less toxic tool for chemical biologists. This is demonstrated through the metabolic incorporation of a

mannosamine analogue bearing a terminal alkyne and subsequent labelling using CuAAC, thus expanding the scope of cellular imaging strategies by circumventing the synthesis of more complex reactive partners as mentioned in section 1.3

Finally, chapter four looks at unnatural amino acids as tools for chemical biology. Modifying peptides site-specifically allows researchers to enact control over systems and has found widespread application in the realm of chemical biology. This chapter looks at synthetic measures taken to synthesize two UAAs, one containing an α,β -unsaturated ketone, the other mimicking a charged L-histidine, relating to the compounds discussed in chapters one and two.

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Chapter 2: Novel intramolecular reactions and rearrangements involving *N*-heterocyclic carbenes derived from azolium salts

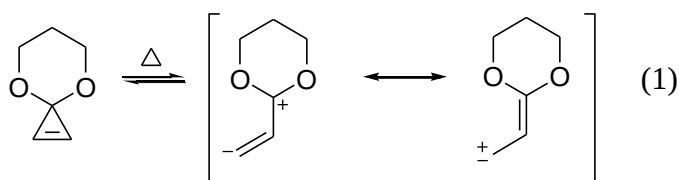
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2.1 Introduction

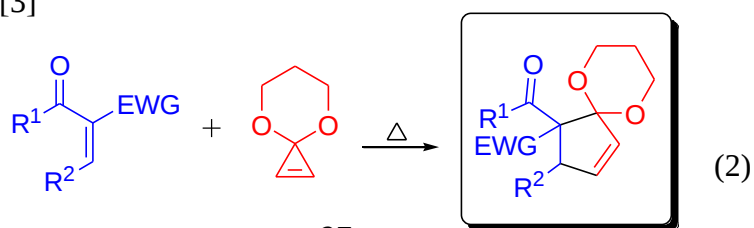
During this chapter, the discussion of a methodology towards a small library of polycyclic nitrogen bearing heterocycles through reaction between an intermediate similar to the vinyl ketone observed for the thermal ring opening of cyclopropenone ketals and different molecular traps. Unexpected rearrangement products were observed, and attention was turned towards understanding the reaction pathways and operative mechanisms.

2.1.1 Boger's Cyclopropenone Ketal

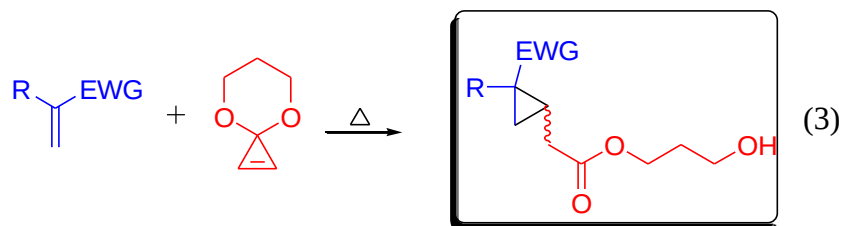
In an attempt to expand the synthetic toolbox, an unprecedented thermally promoted three carbon 1,3-dipole was extensively studied in terms of its formation, reactivity and application. Work performed primarily by Boger and Brotherton in the mid 1980's led to the discovery of a thermally reactive synthon that was applied to a broad family of substrates - which will be further described - ultimately delivering a variety of different products.[1] This research followed the observations and work of Albert and Butler[2] on cyclopropenone ketals like the canonical compound depicted in Equation 1. It was determined that this particular structure could serve as a synthon for vinyl carbenes as very useful and reactive synthetic tools. The reactive intermediates have been best described as a hybrid of 1,3- and 1,1- dipoles, that is, a nucleophilic, singlet carbene.



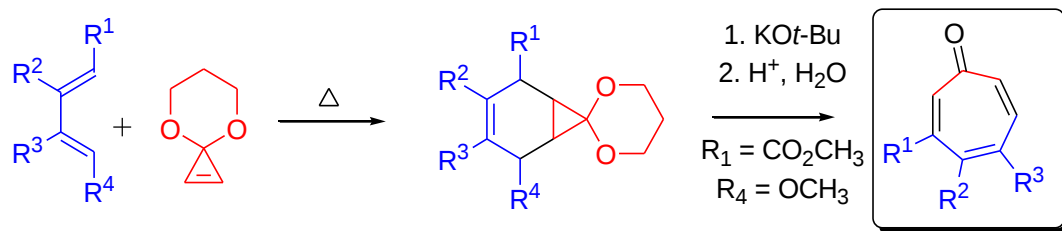
A summary of the synthetic application of this synthon is provided in the following Equations and schemes. Specifically, the cyclopropenone ketal can be thermally reacted with electron deficient olefins to yield the corresponding unsaturated cyclopentane via a formal [3+2] cycloaddition.[3]



Their work was then extended to using the *in situ* generated 1,1-dipole of the cyclopropenone ketal functional group in cycloadditions with less electron deficient olefins to give the corresponding cyclopropanes.[4]

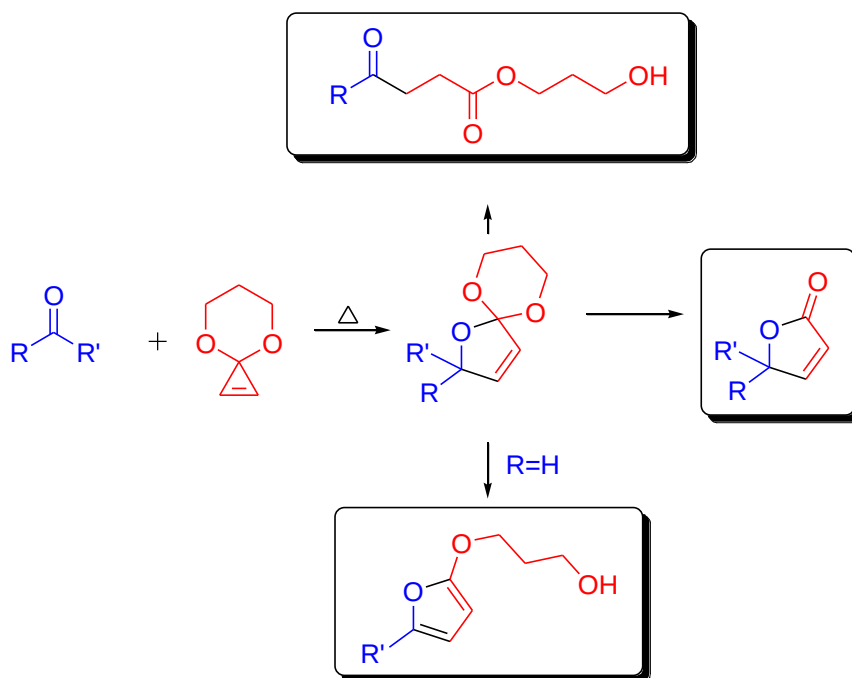


The strain in the cyclopropenone ring allowed extension of this functional group to Diels-Alder type [4+2] cycloadditions as well as inverse electron demand Diels-Alder cycloadditions with a variety of dienes as described in Scheme 2-1. The scope of the reaction was tested and showed yields ranging from 33% to 80% for the normal Diels-Alder and 0-96% for the inverse electron demand reaction. The *exo* adduct predominated in both series of reactions with the exception of cyclopentadiene which yielded a 1:1 mixture of stereoisomers. This approach could also lead to the synthesis of tropones via acid catalyzed hydrolysis of the unsaturated tricyclic intermediate.[5]



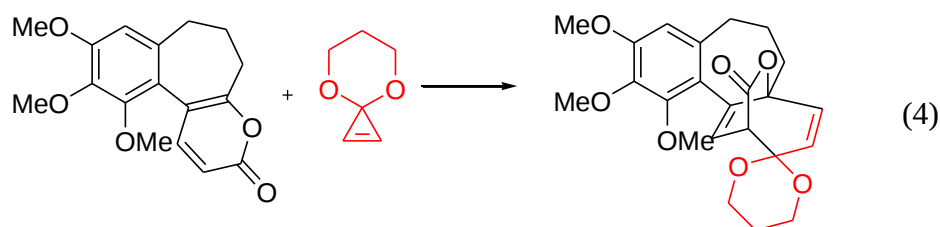
Scheme 2-1 Synthesis of inverse electron demand Diels-Alder cycloadducts and tropones using cyclopropenone ketal methodology.

The application of the cyclopropenone ketal was further extended to its reaction with aldehydes and ketones to give butenolide ortho esters via [3+2] cycloaddition as depicted in Scheme 2-2. These could further be modified to provide various functionalities such as γ -keto esters, γ -butenolides or furans depending on the nature of the substrate and the workup procedure.[6] Particular merit lay in the lability of the ketal protecting group, permitting its removal or modification to give the corresponding products.

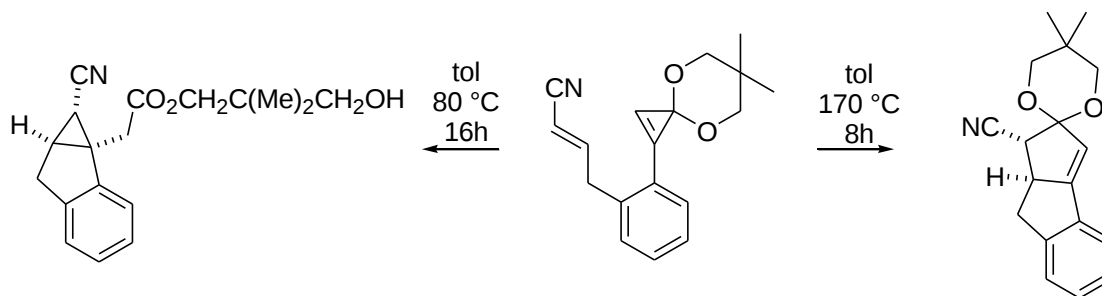


Scheme 2-2 Reactivity of cyclopropenone ketal with aldehydes and ketones.

The synthetic application of the aforementioned cyclopropenone ketal [3+2] chemistry was applied towards the formal total synthesis of the mitotic inhibiting natural product colchicines[1,7] by reaction with Eschenmoser's α -pyrone to give the subsequent bridged intermediate.[8]



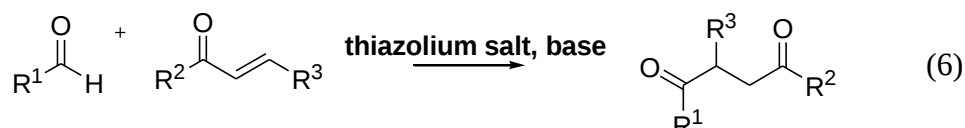
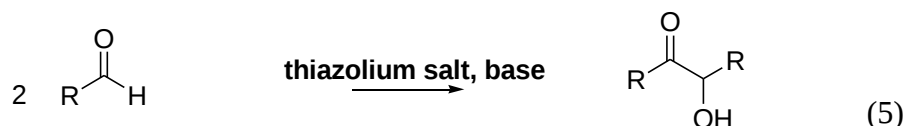
Recent studies have demonstrated that this type of reactivity can be attained in an intramolecular fashion, as well as in the previously demonstrated intermolecular manners (Scheme 2-3). While the reactivity at different temperatures and with different substrates from the intramolecular variant led to the formation of unexpected products, be they tricyclic or tetracyclic, both arising from a common intermediate, the reaction showed tolerance to varying tether lengths and substitution patterns[9],[10].



Scheme 2-3 Intramolecular variant of the cyclopropenone ketal chemistry leading to two different products.

2.1.2 N-Heterocyclic Carbenes

NHCs have received especially widespread attention in the field of organic chemistry since their first isolation as both an air stable liquid (dicarbene) [11] and solid.[12] Their unique characteristics have been employed towards many synthetic aims, perhaps most importantly in their ability to generate Umpolung synthons. Namely, the reaction of NHCs, and their isoelectronic thiazolium counterparts, with aldehydes has been used in countless syntheses, employing the concept of reverse polarity to generate either α -keto alcohols through the benzoin condensation[13] and 1,4-diketones via the Stetter reaction between aldehydes and α,β -unsaturated ketones[14], as shown in Equations 5 and 6 respectively.



These chemical reactions have been performed in aqueous media.[15] The reaction has also been able to achieve enantioselectivity, using chiral thiazolium salts as catalysts.[16] This type of reactivity largely mimics the types of transformations prevalent in biological systems performed by the cofactor thiamine.[17] Benzoin Oxy-Cope to give cyclopentenones [18], and tandem reduction/hydroacylation of ketones[19] and have all been achieved using NHCs as the organocatalyst.

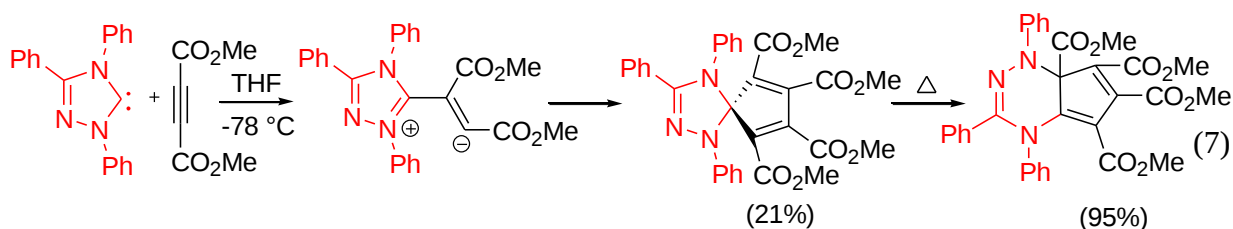
The application of NHCs has also been greatly advantageous to inorganic chemists as they have served as stabilizing ligands for a variety of transition metal centers, namely: ruthenium, platinum, palladium, rhodium, nickel, silver and gold. In many instances, these ligands have substituted pre-existing complexes involving phosphines.[20] Transformations including but not limited to hydrogenation[21], hydrosilylation[22,23], cycloadditions & rearrangements [24], aryl amination[25], Suzuki-Miyaura cross-coupling [26], α -arylation of ketones [27] and tandem reactions of the aforementioned transformations have all been achieved by NHC ligated metal catalysts or reagents. 1,3-bis(2,4,6-Trimethylphenyl)imidazolidine was employed in the second generation Grubbs' catalyst, a catalyst that has served as the elementary catalyst for performing carbocyclizations[28,29] via olefin metathesis.

The applicability of NHCs in the various fields of chemistry has been due to their unique properties. Unlike more classical types of carbenes, NHCs do not exhibit a similar type of chemical reactivity i.e. CH, OH, and NH insertion chemistry. It is for these reasons that NHCs have been such widely used tools. However, their versatility need not be, and has not been, limited to the aforementioned applications. Despite limited literature precedents, NHCs have been employed as stoichiometric reagents to be transformed into novel interesting compounds.

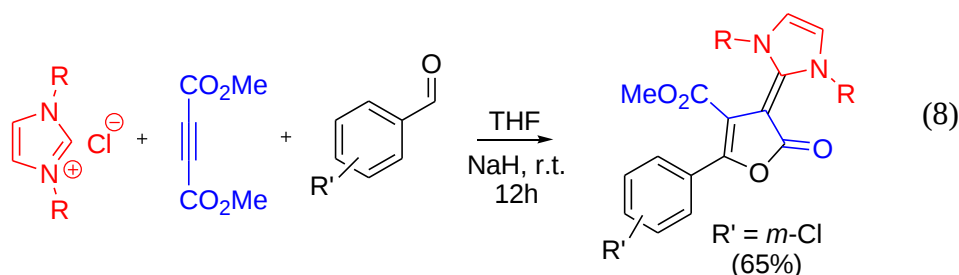
2.1.3 N-Heterocyclic Carbenes as Reagents

N-heterocyclic carbene application in organic synthesis has not been limited to that of catalysis. The study of N-heterocyclic carbenes as reactive partners with different substrates including maleimides[30], alkenes[31], and isocyanates [32]. Interestingly, several instances have been reported whereby NHC's have reacted with alkynes to deliver a reactive intermediate similar to that of the Boger intermediate.

As described in Equation 7, reaction of a triazolylidene with dimethyl acetylenedicarboxylate (DMAD) leads to an intermediate which resembles the 1,3-singlet carbene of the cyclopropenone ketal. Upon formation, this intermediate can further react with another equivalent of DMAD to give the subsequent spiro product, and finally, the thermally favourable bicyclic product [33].



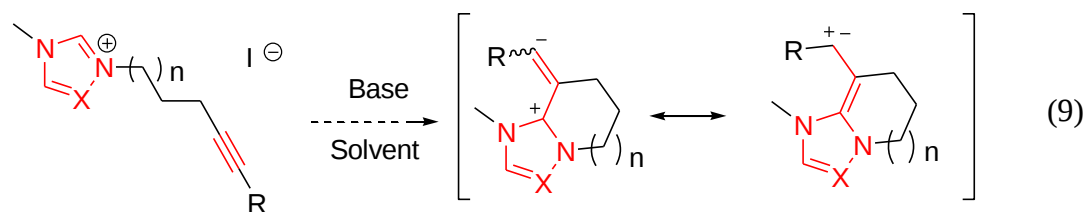
Further investigation into these was reported by introducing a third component into the reaction mixture. Novel, polycyclic compounds were able to be generated in one pot from the imidazolium and imidazolinium salts upon treatment with base, an electron deficient alkyne such as DMAD and various substituted aldehydes as depicted in Equation 8[34].



Thus, we came to the idea of combining the findings in Equations 7 and 8 with the research performed on the cyclopropenone ketal with the hope that a similar type of chemistry could be achieved with imidazolium and triazolium salts to ultimately deliver a wide array of novel products.

2.1.4 N-Heterocyclic Carbenes as Reagents via a Boger Intermediate

Taking the versatility of such precedence into consideration, we focused on studying an analogous system involving nitrogen containing heterocycles due to their importance and abundance in the search for medicinally or biologically active compounds.[35-37] Presumably if a reactive intermediate (similar to the Boger intermediates in Equation 1) could be obtained, a diverse array of novel molecules could be synthesized upon further reaction with a variety of substrates. Such a system was proposed to be accessible via the base-promoted intramolecular cyclization of alkyne-tethered azolium salts via an *N*-heterocyclic carbene intermediate.



It was thought that through this strategy, a small library of novel polycyclic compounds could be obtained by combining the reactive vinyl singlet carbene intermediates with a variety of compounds similar to those used in the work performed by Boger. A literature search unveiled that such structures are virtually absent from the canon of pre-existing chemicals, though the reactive intermediate scaffold bears resemblance to a class of naturally occurring cyclic structures which have not as of yet been exploited in the synthesis of medicinal compounds[38] (Figure 2-1). These compounds all share a tetrasubstituted carbon center that connects three of the four rings, as well as containing either an imidazole or oxazole derived ring and are depicted in Figure 2-1.

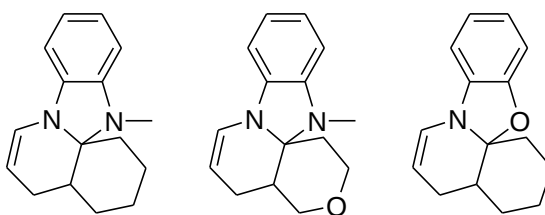


Figure 2-1 Privileged natural product substructures largely absent from drug trade collections.

While many methods exist to modify different azoles[39-42], we chose what would likely be the simplest route to the azolium salts. The precursors could be obtained in as few as three steps [43-45], to give the iodide salts which in some cases bore physical resemblance to ionic liquid properties [46]. Likewise, while a strategy for the intramolecular reaction involving carbenes and alkenes in a nickel mediated method[47], no attempts at a base-promoted intramolecular reaction has been reported to our knowledge. The following section is a discussion of the synthesis of the various salts, as well as the preliminary observations and results of these compounds under basic conditions with and without reactive partners.

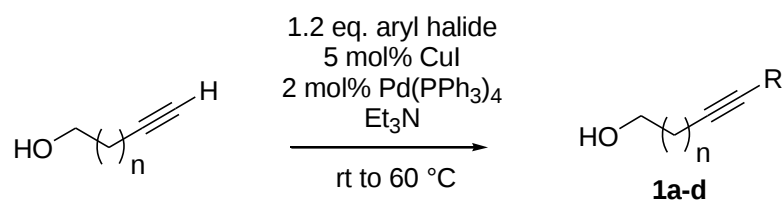
2.2 Results & Discussion

2.2.1 Synthesis of Azolium Salts

A diverse array of salts comprising of various tethered lengths and electronic properties were synthesized for the purposes of generating a small library of compounds with privileged scaffolds. Phenyl, *p*-nitro, C₆H₄-*p*-methyl, C₆H₄-*m*-methyl, methyl ester, and alkynes with no substitution were attained in very few synthetic steps. While overall yields were better via substitution occurring on the alkyne prior to substitution onto the azole ring (Scheme 2-4, step i), a more general and applicable route was achieved by first adding the alkyne to the azole and following this with substitution or coupling of the alkyne (Scheme 2-4, step v).

The first step for the phenyl substituted compounds was to perform the Sonogashira coupling reaction [48] which proceeded fairly well for both the 5 and 6 carbon alkynes (Table 2-1)

Table 2-1 Yields for alkyne and iodobenzene cross coupling reaction

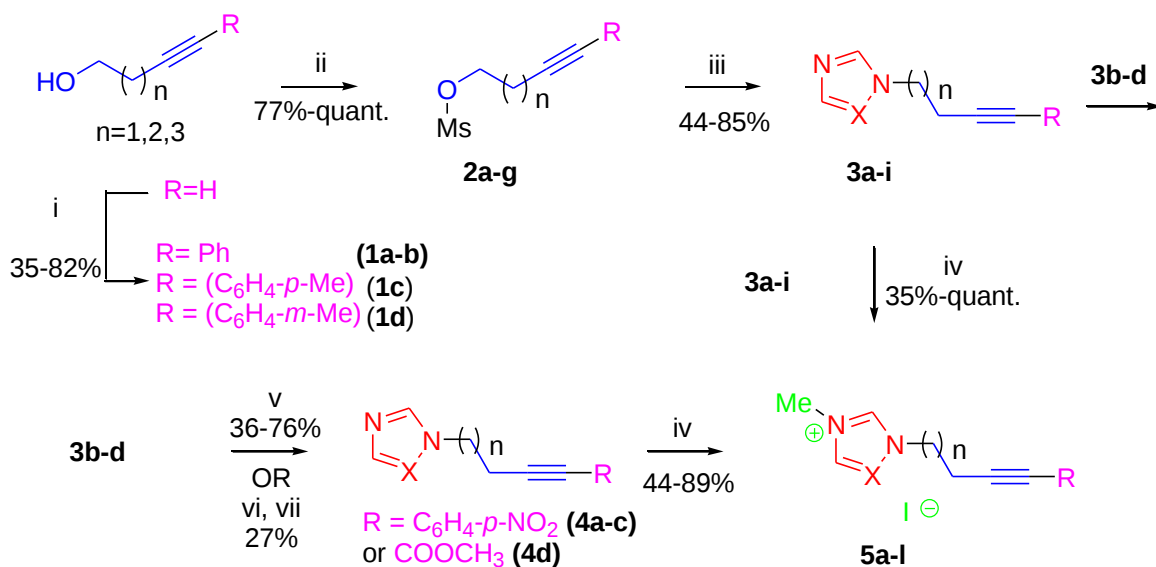


1.2 eq. aryl halide
 5 mol% CuI
 2 mol% Pd(PPh₃)₄
 Et₃N
 rt to 60 °C

1a-d

Entry	n	R	Product	Yield (%)
1	2	Ph	1 a	82
2	3	Ph	1 b	71
3	2	C ₆ H ₄ - <i>p</i> -Me	1 c	63
4	2	C ₆ H ₄ - <i>m</i> -Me	1 d	35

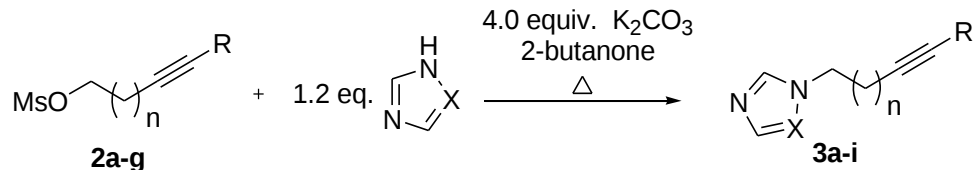
Following this, mesylation of the alcohols on both the unsubstituted and substituted alkynes were performed. This procedure provided products that were directly applied in the substitution step without the need for any further purification.



Scheme 2-4 Summary of chemical transformations leading to the synthesis of salts **5a-5l**:
 i) Aryl halide, CuI, Pd(PPh₃)₄, Et₃N, rt to 60 °C; ii) 1.5 equiv. MsCl, 2.0 equiv. Et₃N, DCM, 0 °C to RT; iii) imidazole, K₂CO₃, 2-butanone, reflux; iv) MeI, EtOAc, sealed, 60 °C; v) 4-iodonitrobenzene, CuI, PdCl₂(PPh₃)₂, Et₃N, RT; vi) n-BuLi, -78 °C, THF; vii) Methyl chloroformate, 0 °C, THF

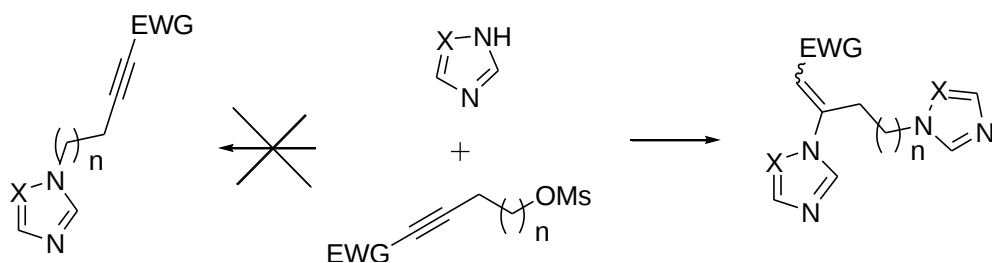
Table 2-2 Yields for the various mesylation reactions

$ \text{HO-CH}_2\text{-(CH}_2\text{)}_n\text{-C}\equiv\text{C-R} \xrightarrow[\text{0 } ^\circ\text{C to rt}]{\substack{\text{1.5 equiv. MsCl} \\ \text{2.0 equiv. Et}_3\text{N} \\ \text{DCM}}} \text{MsO-CH}_2\text{-(CH}_2\text{)}_n\text{-C}\equiv\text{C-R} \quad \textbf{2a-g} $					
Entry	n	R	Product	Yield (%)	
1	1	H	2 a	89	
2	2	H	2 b	93	
3	3	H	2 c	77	
4	2	Ph	2 d	81	
5	3	Ph	2 e	>97	
6	2	C ₆ H ₄ - <i>p</i> -Me	2 f	>97	
7	2	C ₆ H ₄ - <i>m</i> -Me	2 g	>97	

Table 2-3 Yields for the various substitution reactions

Entry	Mesylate	X	Product	Yield (%)
1	n = 1, R = H (2 a)	CH	3 a	44
2	n = 2, R = H (2 b)	CH	3 b	53
3	n = 3, R = H (2 c)	CH	3 c	85
4	n = 2, R = H (2 b)	N	3 d	62
5	n = 2, R = Ph (2 d)	CH	3 e	58
6	n = 3, R = Ph (2 e)	CH	3 f	73
7	n = 2, R = Ph (2 d)	N	3 g	54
8	n = 2, R = C ₆ H ₄ - <i>p</i> -Me (2 f)	CH	3 h	68
9	n = 2, R = C ₆ H ₄ - <i>m</i> -Me (2 g)	CH	3 i	63

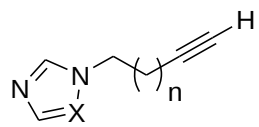
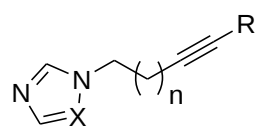
The substitution reaction did not tolerate electron deficient alkynes (Scheme 2-5), for such compounds (para nitro and methyl ester) functionalization was performed from the prepared unsubstituted azole alkynes rather than being done prior to substitution. As an added bonus, parent azoles **3a** through **3d** could potentially lead to a variety of different compounds, thus bulk preparation of these was performed. When the nucleophile used was a triazole, two possible constitutional isomers formed. The chemoselectivity was roughly 9:1 (**3d** and **3g**) with the N-1 substitution being preferred.

**Scheme 2-5** Competing reaction for alkynes bearing electron withdrawing groups

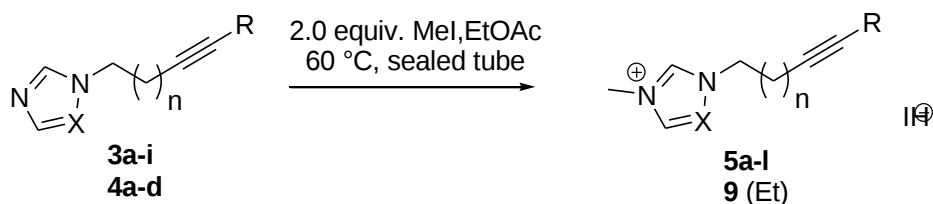
Sonogashira coupling with the para nitro iodobenzene provided the corresponding precursors **4a** through **4c**, while base promoted substitution of the methyl chloroformate provided the ester derivative **4d**. This was rather unfortunate given that we predicted electron

poor alkynes to be the most likely to lead to a desired intermediate. Chromatographic resolution of the intermediates **4a** and **4b** were especially difficult to purify from the excess imidazole in the reaction mixture and were thus subjected to the subsequent methylation step without further purification.

Table 2-4 Yields for the various cross coupling or substitution reactions

 3b-d 1.2 equiv. iodobenzene 3 mol% CuI, 3 mol% Pd(PPh₃)₄ Et₃N, rt -OR- 1) 1.2 equiv. n-BuLi -78 °C, THF 2) 1.2 equiv. Methyl chloroformate, 0 °C, THF  4a-d				
Entry	Alkyne	R	Product	Yield (%)
1	n = 2, X = CH (3 b)	C ₆ H ₄ - <i>p</i> -NO ₂	4 a	Nd
2	n = 3, X = CH (3 c)	C ₆ H ₄ - <i>p</i> -NO ₂	4 b	36
3	n = 2, X = N (3 d)	C ₆ H ₄ - <i>p</i> -NO ₂	4 c	76
4	n = 2, X = N (3 d)	COOMe	4 d	27

Upon obtaining the various tethered azole alkynes, a simple methylation procedure^[45] was chosen to furnish the iodide salts. The final methylation of the precursors (to give the iodide salts **5a** through **5k**, Table 2-5) proceeded in a sealed tube under an atmosphere of argon rather rapidly. Several of the substrates formed immediately, appearing as a biphasic mixture of organic (ethyl acetate, excess iodomethane) and ionic liquid (lower phase). In some instances (i.e. **5e**) the final product would crystallize out of solution upon washing with cold ethyl acetate, permitting a rather easy purification by filtration. In other instances, column chromatography was necessary to obtain analytically pure final product. No counterion exchanges were performed. Overall yields for the syntheses of these imidazolium and triazolium salts ranged from seven to 52% over three or four steps.

Table 2-5 Isolated yields for the methylation step

Entry	Substrate	Yield (%)	Product	Overall Yield ^a (%)
1	n = 1, R = H, X = CH (3 a)	53	5 a	22
2	n = 2, R = H, X = CH (3 b)	96	5 b	47
3	n = 3, R = H, X = CH (3 c)	56	5 c	37
4	n = 2, R = H, X = N (3 d)	83	5 d	47
5	n = 2, R = Ph, X = CH (3 e)	80	5 e	30
6	n = 3, R = Ph, X = CH (3 f)	>97	5 f	52
7	n = 2, R = Ph, X = N (3 g)	84	5 g	30
8	n = 2, R = C ₆ H ₄ - <i>p</i> -Me, X = CH (3 h)	65	5 h	28
9	n = 2, R = C ₆ H ₄ - <i>p</i> -NO ₂ , X = CH (4 a)	Nd	5 i	Nd
10	n = 3, R = C ₆ H ₄ - <i>p</i> -NO ₂ , X = CH (4 b)	89	5 j	21
11	n = 2, R = C ₆ H ₄ - <i>p</i> -NO ₂ , X = N, (4 c)	84	5 k	36
12	n = 2, R = COOMe, X = N (4 d)	44	5 l	7
13 ^b	n = 2, R = C ₆ H ₄ - <i>m</i> -Me, X = CH (3 i)	35	9	8

a – Calculated from yields obtained for steps listed in tables 2-1 to 2-4.

b – Ethyl iodide used in place of methyl iodide.

2.2.2 Solvent & Base Screens

A very general solvent and base screen was performed upon synthesis of the salts. Using **5e** and **5g** as test salts (rather trivially selected because of their abundance) a series of bases and solvents representing different characteristic properties were screened and the crude samples were analyzed by ¹H NMR 16 hours after treatment.

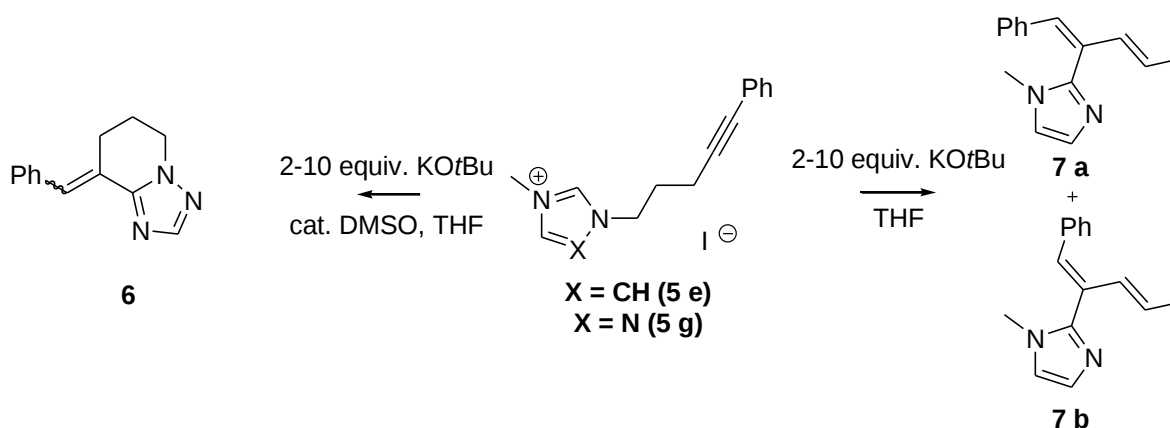
Upon screening, the least complex mixtures were chosen for further investigation; these included DMF with DBU or KO^tBu and THF or DCM with KO^tBu. Furthermore, NMR temperature studies showed the deprotonation occurring rapidly at room temperature with the appearance of several products. At 0 °C deprotonation occurred readily, while at lower temperatures the deprotonation did not seem to occur.

As mentioned, the intended purpose of the *N*-heterocyclic salts was ultimately to provide a reactive intermediate that could act as a scaffold for further derivatization to

generate novel polycyclic heteroatom bearing compounds. Initially, a solvent and base screen was done on **5e** and **5g** due to their relative ease of synthesis, their being solids at room temperature and the presence of a group capable of stabilizing any charge buildup during cyclization. KHMDS, DBU, KOtBu, NaH, Cs₂CO₃ and NaOAc were screened as bases in DCM, THF, DMF and isopropanol representing various kinds of solvents and bases that are commonly used for NHC chemistry. The crude mixtures were washed with water and extracted into chloroform and visualized by NMR. All of the NMR spectra were qualitatively assessed for the simplest system to choose for further reactivity. Unfortunately, no spectra obtained provided anything but a complex mixture of various products that were unidentifiable. Qualitatively, the DMF reactions were the cleanest in terms of solubility. KOtBu in THF was chosen as a point of further investigation on the other salts, as there were alkene peaks with the cleanest spectra obtained, suggesting to us that cyclization had occurred. In none of the reactions was the intact starting material deconvoluted from the spectrum.

2.2.3 Dienes & Demethylated Products

Diene rearrangement products and demethylated cyclised products were formed respectively from the imidazolium and triazolium 5 carbon tethered phenyl iodide salts **5e** and **5g** using KOtBu in excess in THF, with a catalytic amount of DMSO in the latter case as described in Scheme 2-6.



Scheme 2-6 Formation of products using conditions screened on azolium salts with five carbon tethers and phenyl substituted alkynes. In the presence of catalytic DMSO, the triazolium precursor **5g** yields the cyclised alkyne **6** and the imidazolium precursor **5e** yields the stereoisomeric dienes **7a** and **7b**.

The visualization of characteristic alkene peaks found in these products was what led to further investigation into the character of these products and the possible mechanism for their formation. It is worth noting that the formation of this kind of product was not achieved using other salts of equal tether length under identical conditions.

2.2.3.1 Discovery

In the presence of an excess of hindered base such as potassium *tert*-butoxide, the formation of a polar compound was observed as the major product. Isolation and characterization of this product confirmed it as the (*Z,E*) diene diastereoisomer **7a**, additionally the (*E,E*) diastereomer **7b** was produced, but with a slightly lower isolated yield.

These unexpected observations were analyzed using mass spectrometry which showed the correct molecular ion signal of 225.1 for both and by proton NMR. This substitution pattern of heteroaromatics on dienes is absent from any literature precedent.

In the presence of a catalytic amount of DMSO, with an excess of KO*t*Bu as a base and in THF, cyclic compound **6** (Scheme 2-6).

2.2.3.2 Diene Characterization & Reactivity

Absolute stereochemistry of the double bonds of the diene side products was established using NOE. The presence of an NOE signal for the hydrogens as depicted in Figure 2-2 largely supported the proposed structures. Additionally, while most of the shifts were similar in both spectra, the *N*-methyl resonance was more upfield for **7a** than **7b** (0.4 ppm) suggesting a larger anisotropy contribution from the phenyl ring when it was on the same side of the double bond as the imidazole ring.

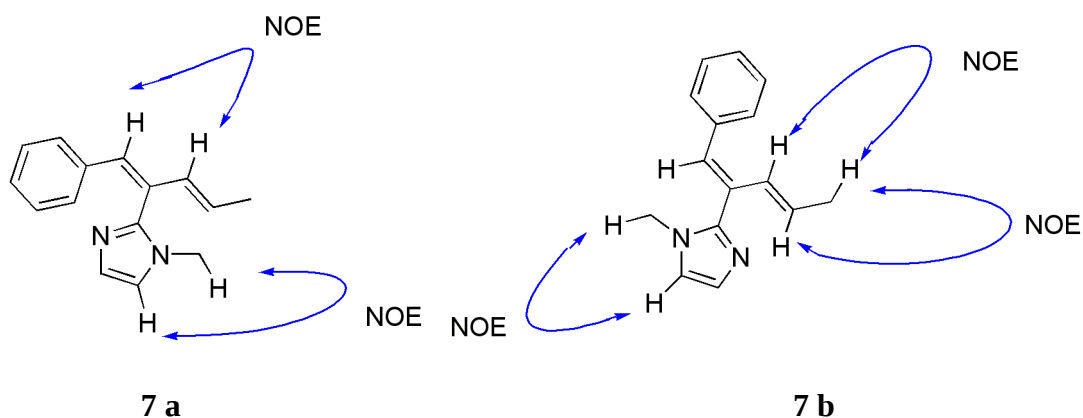
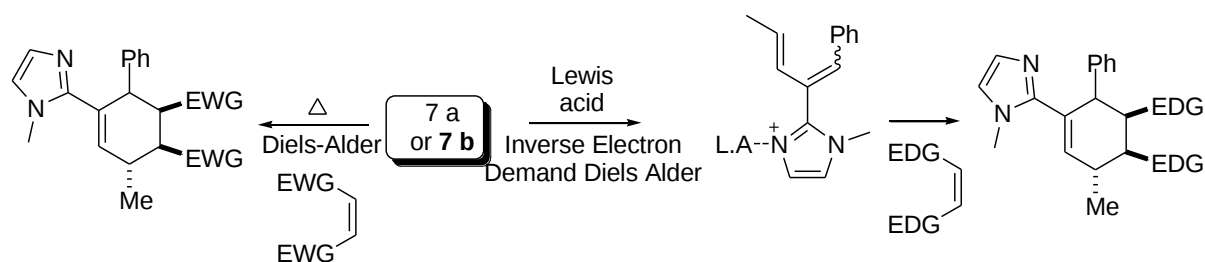


Figure 2-2 Important NOE resonances for determination of region and stereochemistry of protons in diene molecules **7b** and **7a**.

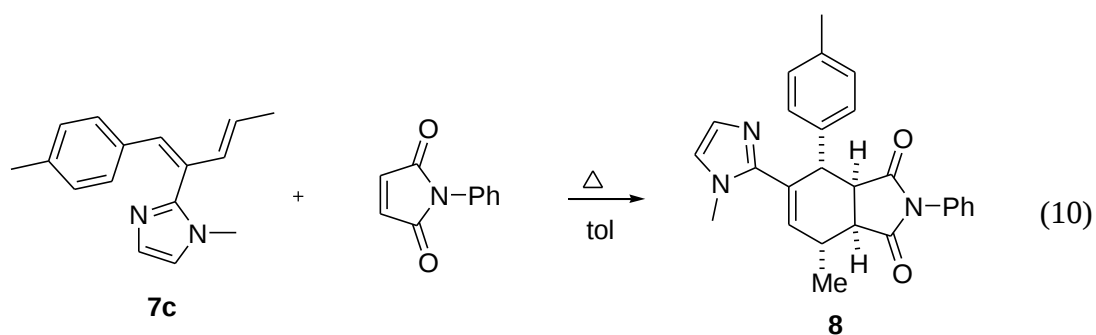
The dienes themselves were thought to be structurally interesting, particularly due to the imidazole moiety that was thought could potentially serve as a Lewis base. Thus, Lewis acid catalyzed inverse electron demand Diels-Alder reactions were thought to be possible, though not within the scope of this work. It is conceivable that this diene could act as both a thermally active diene for Diels-Alder reactions, while also being an inverse electron demand diene under Lewis acid conditions as described in Scheme 2-7. To our knowledge, this kind of tuneable diene has not been explored with imidazole structures and is currently being further investigated.



Scheme 2-7 Proposed tuneable reactivity of dienes using heat or Lewis acids.

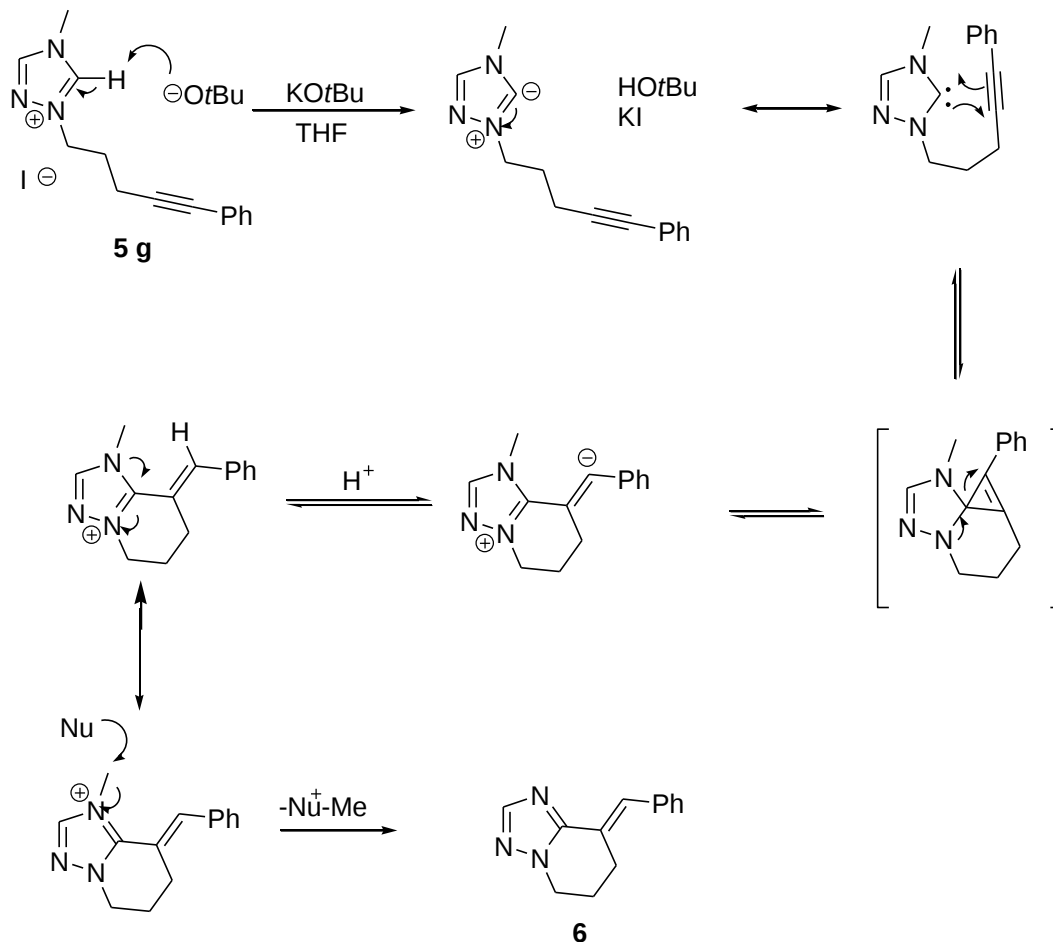
It was believed that further evidence towards establishing such structures would be attainable using a Diels-Alder reaction. Reaction with maleic anhydride proved to be ineffective even at higher temperatures. The *E,E* diene obtained from reaction of compound **5h** under the standard diene formation conditions (**7c**) was subsequently reacted with *N*-Phenyl maleimide to undergo a thermally promoted Diels-Alder reaction, and yielded the thermodynamically favoured *exo* cycloadduct **8** in moderate yield, as depicted in Equation

10. These results further confirm the structure of the diene products, as well as the relative geometry of the double bonds in the less polar diene. The observed NOESY spectrum was consistent with the NOE signals established in the diene precursor. This data suggests these dienes can be further modified with dienophiles to generate unique imidazole linked cycloadducts.



2.2.3.3 Mechanistic Proposals

The proposed mechanism for the formation of the demethylated triazole bicyclic product **6** that was isolated is shown in Scheme 2-8.



Scheme 2-8 Proposed demethylation mechanism to give product **6**, Nu= DMSO/H₂O.

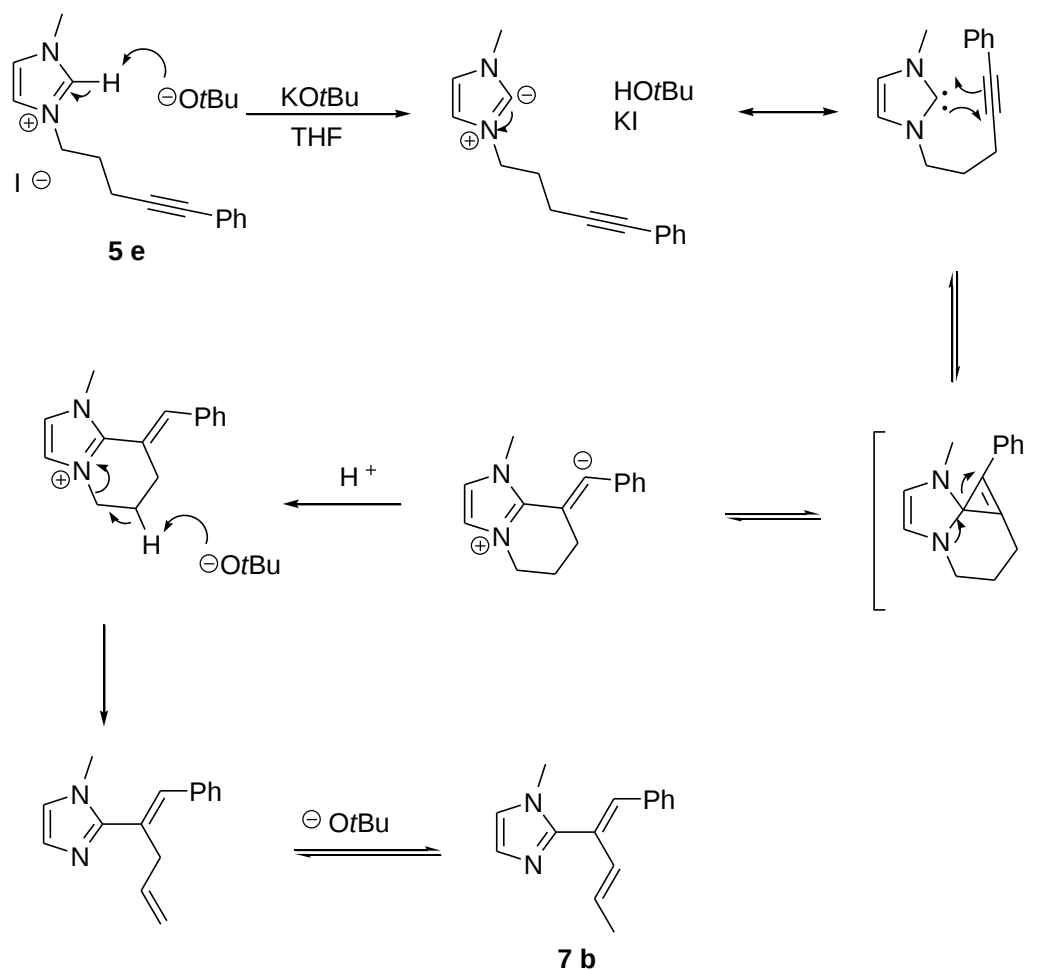
The proposed mechanism involves the deprotonation of the imidazolium salt to give the reactive NHC intermediate. This intermediate leads to a high energy intermediate that ring opens to give the vinyl carbene that is then demethylated and protonated upon workup with water. This mechanism resembles the proposed mechanism for the diene formation, however we propose that due to the presence of an additional nitrogen within the heterocyclic framework there exists a strong resonance character towards stationing the positive charge on the methylated nitrogen. The electronegative nitrogen destabilizes the positive charge on

the tethered alpha nitrogen. As a result, there exists a very unstable pseudo 1,3-allyllyl system.

This was believed to deliver to the methyl group some electrophilic character to alleviate this strain within the fixed bicyclic system. The small yield of observed demethylated product was accredited to the steric bulk of the butoxide anion and thus the requirement for water or DMSO to perform the demethylation. Proposed means for shutting down this pathway include the synthesis of alternatively substituted salts such as with mesityl or tert-butyl salts. While the former is a very common and stabilizing substituent on NHCs, the latter is difficult to achieve synthetically without resorting to harsh conditions. However, these considerations might lead to future studies on the N- substituent's influence on this pathway.

A proposed mechanism for the formation of **7b** is described in Scheme 2-9 and involves the deprotonation of imidazolium salt, followed by carbene insertion into the alkyne unsaturation to give a proposed reactive intermediate analogous to the Boger intermediate that spontaneously ring opens to give the 1,3-dipole. In the presence of excess hindered base, the E2 elimination product accessed by a deprotonation at the beta position of the positive center is then believed to be observed followed by isomerisation to give the internal alkene is then believed to occur in a base promoted manner. This mechanism was believed to operate only in the presence of an excess of hindered base, and other pathways were believed to be accessible by varying the base chosen and its stoichiometric value. Presumably the ring opening of the reactive intermediate could also be altered to give the seven membered ring and resultant 1,3-dipole, and this ring opening was believed to be heavily influenced by the favoured entropy of six membered ring cyclization as well as the stereoelectronic effects of the alkynyl R group.

Of course, the stereochemistry of the double bond bearing the phenyl group was observed to be a mixture of *Z* and *E* configurations. This is supported by the argument of the 1,3-dipole intermediate, which possesses a vinyl carbene resonance structure that allows free rotation of the R substituent. This mechanism proposal supports the initial hypothesis that these compounds could provide a reactive Boger like intermediate for further modification.

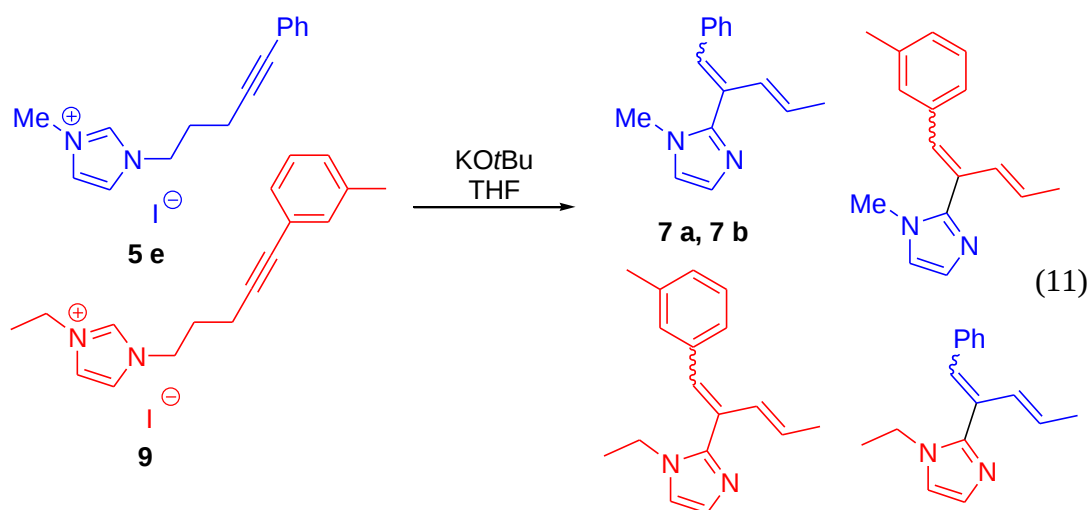


Scheme 2-9 Proposed mechanism for the formation of the imidazole-linked diene **7b**.

It was proposed that this elimination pathway could be shut down altogether by replacing the hydrogens with methyl groups in the imidazolium salt precursor. Thus, not only could this mechanism be tested, also presumably the reactive intermediate could be more likely to yield desired products by eliminating one of the unexpected pathways. The proposed mechanisms and hypothesized reactivity suggest a reactive intermediate would form upon ring formation and that this intermediate would possess the character of either a 1,3-dipole, or a vinyl carbene. Despite efforts, this intermediate could not be observed directly.

2.2.4 Crossover Experiment

A better understanding of the reaction mechanism was required for us to attempt and direct the outcome from these unexpected products to the originally desired Boger-like intermediate. An equal mixture of two salts **5e** and **9** in one flask under the same conditions that prompted the formation of the dienes **7a** and **7b** in Scheme 2-9 were used to determine if there would be any crossover products, thus serving as a proof towards the nature of the mechanism leading to the diene formation. The salts chosen represent different two different tether groups, as shown in the Equation below.



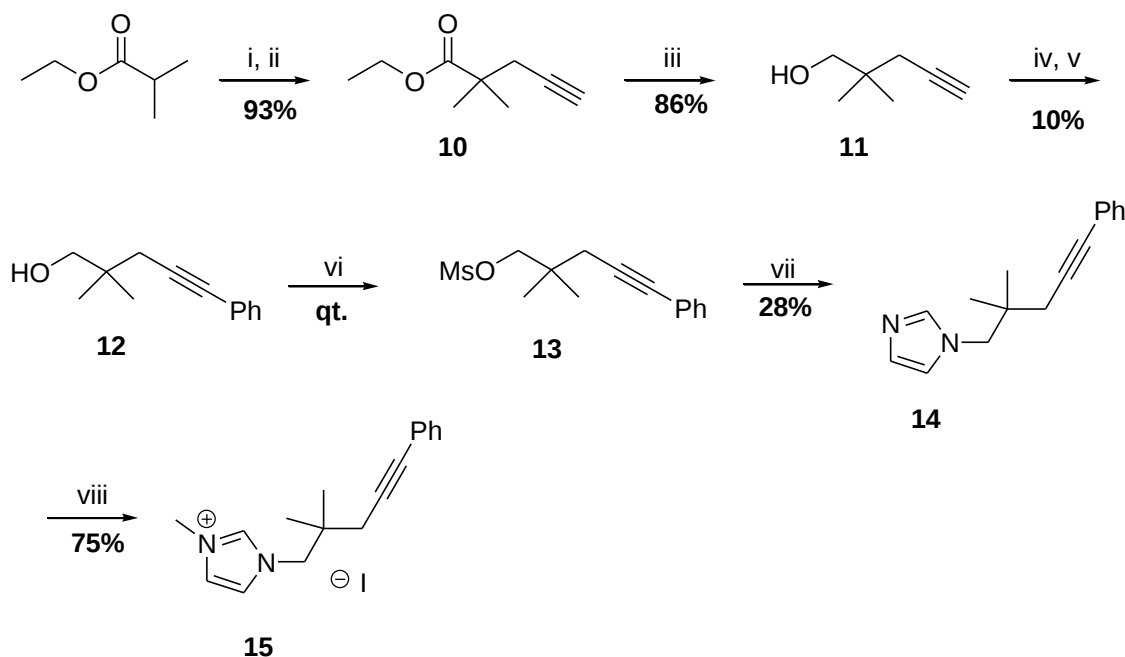
One could not rule out the possibility that the dienes were being formed in an intermolecular fashion, rather than through an intramolecular cyclization followed by elimination. This crossover experiment was performed to serve as negative evidence for the support of the latter mechanism. Should the transformation occur through an intramolecular cyclization then only the solid blue and red structures should be observed, while in the case of an intermolecular reaction of the nucleophilic carbene with the alkyne of another molecule one would expect a mixture of the four constitutional isomers presented in Equation 11. The only observed products were the solid blue diene stereoisomers, suggesting an intramolecular mechanism such as the one presented in Scheme 2-10 (or some variant) was operative.

The intent of the crossover experiment was to determine whether or not the formation of the diene was occurring in an inter- or intramolecular fashion. Should the latter be the

case, it would suggest that the formation of the desired reactive vinyl singlet carbene intermediate was occurring, followed by elimination and subsequent isomerisation of the double bond. Should the former be the case, then one would expect a more complex mechanism. The lack of any cis double bonds on the alkene bearing the methyl group suggests that the isomerisation subsequent to elimination is occurring in an antiperiplanar fashion under thermodynamic control. Likewise, the presence of two stereoisomers with regards to the other double bond would suggest an intermediate that has at least some free rotation, as would be the case in one resonance structure of the singlet vinyl carbene. While encouraged by the lack of crossover products in the crossover experiment, it was nonetheless discouraging to discover that only oxidation and hydrolyzed products were obtained from these reactions, as well as the diene product, even with subsequent addition of reactive trapping agents.

2.2.5 Gem-dimethyl Salts

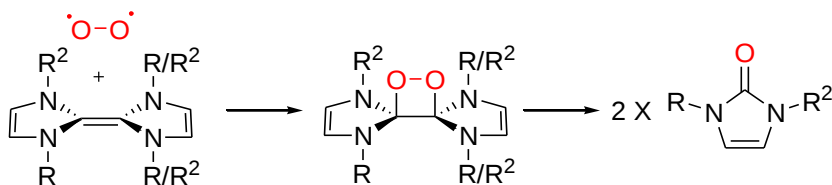
As a better understanding of the elimination pathway was gained through crossover experiments, suggesting a mechanism similar to that depicted in Scheme 2-9 was operative, the inability to trap any reactive intermediates (either through trapping intermolecularly or intramolecularly via a benzylic CH insertion) prompted further investigation into the pathway. Gem-dimethyl salts were synthesized via the synthetic pathway depicted in Scheme 2-10 to test the proposed mechanisms for the formation of the diene by-products **7a-c**. It was believed that following an intramolecular cyclization, the resultant elimination and isomerisation lead to the formation of the dienes, and should this be the case, such a mechanism could be attenuated by substituting the appropriate hydrogens with methyl groups. This would aid in the trapping of the reactive intermediates by essentially shutting down the diene pathway. The synthesis was carried out over six steps and is described in Scheme 2-10.



Scheme 2-10 Synthetic route towards phenyl substituted gem-dimethyl salt **15**: i)LDA, -78 °C to 0 °C, THF; ii)Propargyl bromide, -78 °C; iii)LiAlH₄, Et₂O, 0 °C; iv)Iodobenzene, Pd(PPh₃)₂Cl₂, CuI, Et₃N, rt 30 min; v) 60 °C, 18h; vi)MsCl, Et₃N, DCM, 0 °C to rt; vii)Imidazole, K₂CO₃, 2-butanone, reflux; viii)MeI, EtOAc, 60°C, sealed tube.

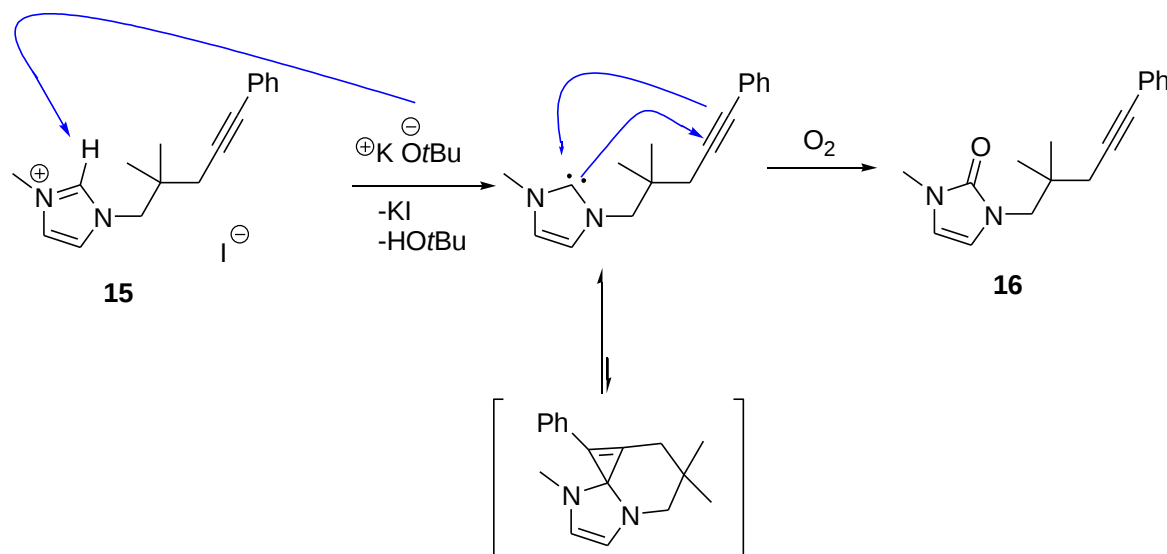
The synthesis of the gem-dimethyl salt involved first the alkylation of ethyl isopropanoate with propargyl bromide. Following this, lithium aluminum hydride reduction yielded the terminal alcohol. The following steps were similar to those performed to obtain the other phenyl substituted salts; Sonogashira coupling of the aryl halide to the alkyne, mesylation, substitution and finally methylation to provide the iodide salt.

Treating the gem-dimethyl salt **15** with an excess of KO^tBu in THF failed to yield any diene product, as expected. This supported the proposed mechanism for diene formation as presented in Scheme 2-9. What was isolated was a large amount of the urea byproduct, suggesting that the carbene, without a reactive partner and inability to eliminate, lay in favour of the formation of the oxidized product. It has been previously been proposed that this kind of reactivity occurs from a Wanzlick intermediate in the case of bridged imidazolylidenes reacting with triplet oxygen as described in Scheme 2-11[49]. Alternatively, a similar product can be proposed through reaction with excess water, potentially from the KO^tBu.



Scheme 2-11 Proposed source of urea derivatives arising from a Wanzlick intermediate reacting with dioxxygen.

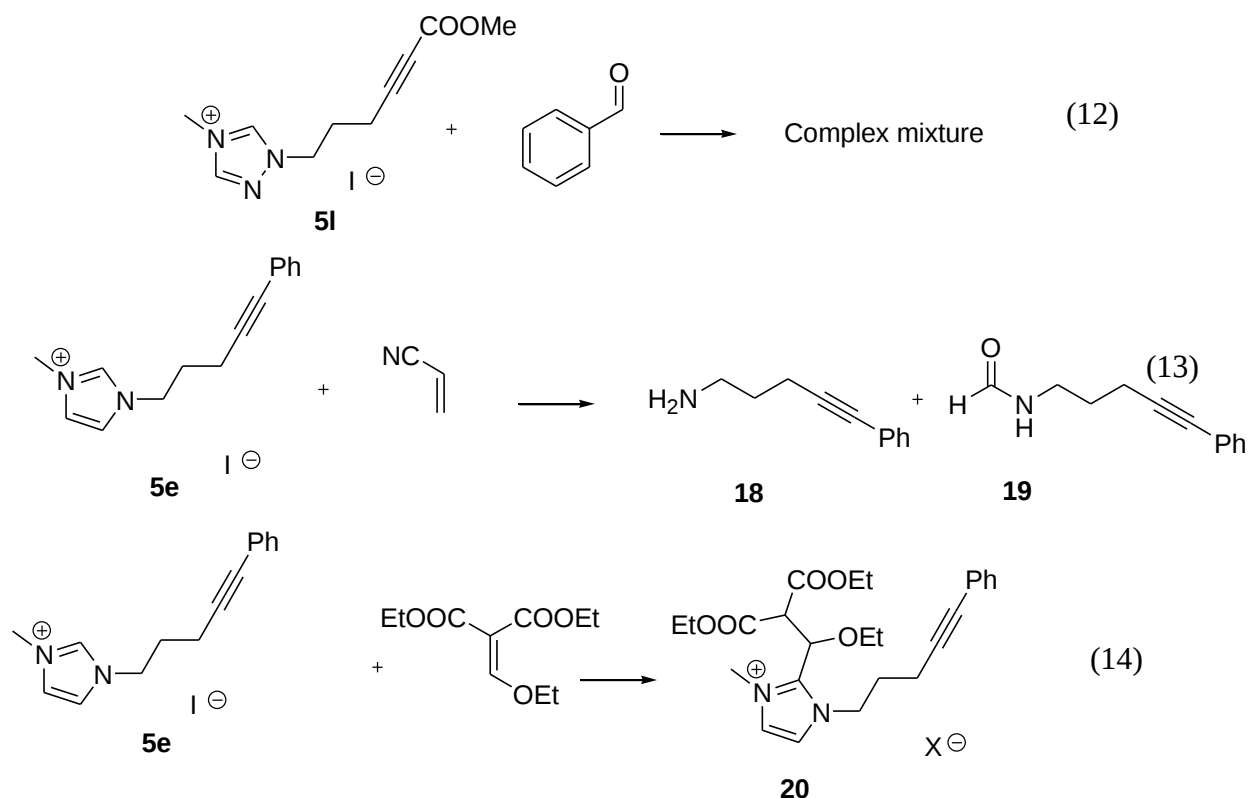
One would expect that any intramolecular cyclization occurring in the saturated analogue would be greatly enhanced in the methylated compound, by virtue of the Thorpe-Ingold effect. Thus it can be surmised that, while cyclization was likely occurring, this cyclization is likely in equilibrium with the uncyclized starting material and that the cyclized product is a high energy intermediate. Over time, the uncyclized carbene reacts with trace triplet oxygen to give the irreversible formation of the urea product **16** as presented in Scheme 2-12. These findings suggest a high energy intermediate is formed but its lifetime is limited by either reversion to the uncyclized NHC or by demethylation (Scheme 2-8) or elimination (Scheme 2-9) where possible. Similar results were seen when reacting the unsubstituted salt **5f** over longer periods of time to give the analogous oxidation product **17**.



Scheme 2-12 Proposed mechanism explaining observed results for gem-dimethyl reaction

2.2.6 Further Cyclization Attempts

Attempts to cyclise products were performed using acrylonitrile, benzaldehyde and diethyl (ethoxymethylene) malonate. Temperature, length of time elapsed before adding the reactive partner, and salt were tested. Various different products were obtained, though none of the potential cyclised products were observed under any conditions. The most notable results are summarized below in the following three Equations.

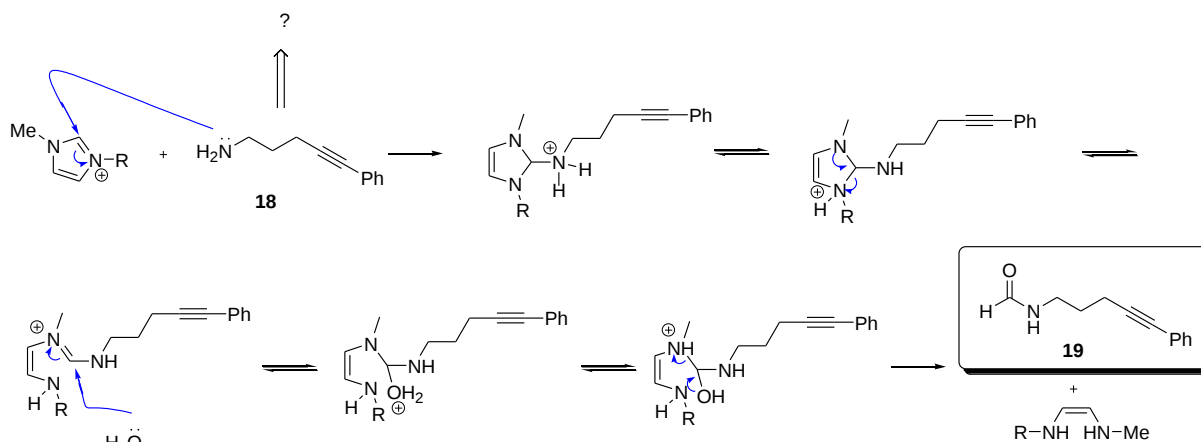


The testing of salts **5e**, **5f**, and the analogous gem-dimethyl five carbon tethered salt **15** under consistent conditions (excess of K^tBu in THF with heat) was performed. What was observed was a common low molecular weight and extremely polar compound was being formed, first observed with the addition of acrylonitrile as a capture agent for the reactive intermediate in all cases. For **5f**, an amine compound **18** was flushed out of the column using 100% methanol on silica gel to give the corresponding free amine as the major product. Mass spec analysis of the other reactions involving **5e** and **15** showed the formation

of compounds of similar purity and mass units differing by one and two saturated carbons respectively.

To elucidate the origins of such a product, several control reactions were performed using **5e**. The omission of either heat or base was performed, and both yielded the formation of the amine by-product, suggesting that its formation is dependent primarily on the salt and the olefin. Presumably, such a compound is acting more as a nucleophile than as a trap, and as a result of this, a less nucleophilic trapping agent was sought out to avoid the formation of such displacement product. Encouragingly, the mass for a product involving the salt and the trap was present in the case of gem-dimethyl salt **15** with acrylonitrile, however not present for any of the hydrogen saturated salts. It was our hope that if a proper trap could be found, this reaction could be driven to give the desired compound as the exclusive product. Disparagingly, it was also determined that using column chromatography with or without base pre-treatment would not be possible without sustaining decomposition to these products.

Aldehydes used as a trap were unsuccessful as well, even when using the more electron deficient imidazolium or triazolium salts (**5h-5k**). The general observation was that reactivity of these salts generally leads to a Breslow type of intermediate, rather than a Boger intermediate. Results were further confirmed when using diethyl (ethoxymethylene) malonate, and seeing compound **20** only, with the alkyne remaining intact. It was also apparent that introducing heat was not favouring desired reactivity, rather decomposition and the formation of the unwanted products. Thus, it was believed that while deprotonation might be kinetically favoured, the cyclization was not occurring in a timely fashion. Overnight stirring prior to quenching with a trap was thus attempted again failing to yield any desired cyclic products.



Scheme 2-13 Proposed source of formamide byproduct **19**[50]

Scheme 2-20 describes a potential mechanism based on literature precedent that explains the potential source of the formamide product **19** (also observed for other salts). It involves the nucleophilic substitution of the amine product **18** leading to decomposition of the five membered ring and eventual hydrolysis of the iminium ion. However, the absence of any unsaturated diamine suggests this mechanism is not correct. It also necessitates the formation of **18** which also presumably arises from decomposition of the imidazolium core ring structure for which there is no literature precedent to the best of our knowledge.

2.3 Summary & Future Work

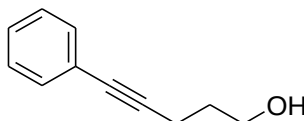
We have attempted to synthesize a library of small molecule polycyclic compounds containing nitrogen bearing heterocycles through a carbene mediated intramolecular cyclization of azolium salts followed by reaction with an electrophilic partner. We have synthesized various alkyne tethered salts of varying tether lengths and of varying electronic properties. We have screened these salts against a variety of solvent/base conditions and further investigated one of these systems that provided the cleanest reaction. Of particular interest, the five carbon tethered salts containing a phenyl group were extensively studied because of the products that arose. Triazolium salts led to the isolation of a demethylated cyclic product, while imidazolium salts led to a diene product. Various undesired products were also observed including oxidation, amine and formamide products. The possible mechanisms leading to these various products have been proposed and tested by various

experiments including the shut-down of elimination through site-specific introduction of methyl groups, and crossover experiments. Spectroscopic and experimental confirmation of the diene by-product was performed. Results obtained from reactions with exogenous electrophiles suggest this strategy is not viable for the majority of these salts for obtaining the originally predicted products. Alternative applications of these salts as reducing agents have been briefly outlined.

Work left to be done on this project includes exploring the application of the dienes in both normal and inverse electron demand Diels-Alder reactions as well as finding other applications for the imidazolium and triazolium salts produced. As mentioned in the introduction, these can often be applied as ionic liquids for a diverse range of applications ranging from use in synthesis[51] to use as recyclable solvents[52]. Additionally, it has recently been shown that alkyl azolium salts can serve as reducing agents *in vivo* and in cases they are often less toxic and more effective than glutathione[53]. This could be a potential application for making use of these salts.

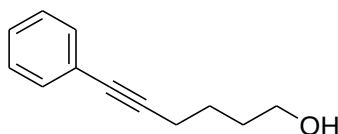
2.4 Synthetic Experimental Methods

5-Phenyl-pent-4-yn-1-ol (1a)



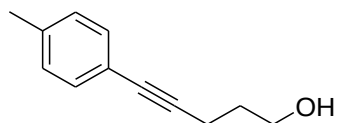
To a stirred solution of copper iodide (214 mg, 1.12 mmol, 5 mol%), and Tetrakis(triphenylphosphine)Palladium (452 mg, 0.38 mmol, 2 mol%) in triethylamine (40 mL) was added iodobenzene (2.8 mL, 25.0 mmol, 1.16 equiv.). The reaction was stirred at room temperature for 30 minutes. 4-Pentyn-1-ol (2.0 mL, 21.5 mmol, 1.0 equiv.) was then added and the mixture was stirred at 60 °C for 18 hours. The resulting solution was diluted with ether, filtered over celite, washed with ether, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (0-20% EtOAc/Hexanes, R_f = 0.31) provide the product as a yellow oil (2.83 g, 82%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.87 (quintet, J = 6.8 Hz, 2H), 2.54 (t, J = 6.8 Hz, 2H), 3.83 (t, J = 6.4 Hz, 2H), 7.27-7.29 (m, 3H), 7.37-7.40 (m, 2H). NMR matched literature [54]

6-Phenyl-hex-5-yn-1-ol (1b)



To a stirred solution of copper iodide (485 mg, 2.25 mmol, 5 mol%) and Tetrakis(triphenylphosphine)Palladium (1.2g, 1.02 mmol, 2 mol %), in triethylamine (75 mL) was added iodobenzene (6.25 mL, 56.0 mmol, 1.1 equiv.) and the mixture was stirred at room temperature for 30 minutes. Following this, 5-hexyn-1-ol (5.0 g, 50.9 mmol, 1.0 equiv.) was added and the reaction was stirred at 60 °C for 18 h under argon. The resultant mixture was then cooled to room temperature, diluted with ether (50 mL) and filtered over celite. The solid was washed with a subsequent 50 mL of ether, and the filtrate was concentrated *in vacuo*. The crude material was purified using column chromatography (0-20% EtOAc/Hexanes) to give the product as a yellow oil (6.33 g, 71%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.48 (s, 1H), 1.65-1.79 (m, 4H), 2.45 (t, $J = 6.8$ Hz, 2H), 3.70 (t, $J = 6.3$ Hz, 2H), 7.25-7.28 (m, 3H), 7.37-7.40 (m, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 18.9, 25.4, 30.1, 46.6, 81.4, 88.8, 123.5, 127.7, 128.2, 131.4. ESI-MS [$\text{C}_{12}\text{H}_{14}\text{O}$] + 1 = 175.1. Matched Literature [55]

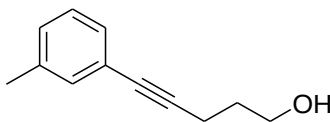
5-*p*-Tolylpent-4-yn-1-ol



To a stirred solution of copper iodide (214 mg, 1.12 mmol, 5 mol%) and Tetrakis(triphenylphosphine)Palladium (452 mg, 0.380 mmol, 2 mol %), in triethylamine (40 mL) was added *p*-tolyl iodobenzene (5.45 g, 25.0 mmol, 1.2 equiv.) and the mixture was stirred at room temperature for 30 minutes. Following this, 4-Pentyn-1-ol (2.0 mL, 21.5 mmol, 1.0 equiv.) was added and the reaction was stirred at 60 °C for 18 h under argon. The resultant mixture was then cooled to room temperature, diluted with ether (50 mL) and filtered over celite. The solid was washed with a subsequent 50 mL of ether, and the filtrate was concentrated *in vacuo*. The crude material was purified using column chromatography (20% EtOAc, $R_f = 0.25$) to give the product as a yellow oil (2.30 g, 62%). ^1H NMR δ_{H} (400

MHz, CDCl₃) 1.55 (br, 1H), 1.86 (quintet, $J = 6.4$ Hz, 2H), 2.33 (s, 3H), 2.53 (t, $J = 6.9$ Hz, 2H), 3.83 (br, 2H), 7.09 (d, $J = 8.0$ Hz, 2H), 7.28 (d, $J = 8.0$ Hz, 2H). ¹³C NMR δ_C (100 MHz, CDCl₃) 16.0, 21.4, 31.4, 61.9, 81.2, 88.4, 120.6, 128.9, 131.4, 137.7. [C₁₂H₁₄O] + 1 = 175.1. Matched literature [56]

5-*m*-Tolylpent-4-yn-1-ol



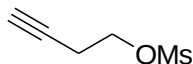
To a stirred solution of copper iodide (214 mg, 1.12 mmol, 5 mol%) and Tetrakis(triphenylphosphine)Palladium (452 mg, 0.380 mmol, 2 mol %), in triethylamine (40 mL) was added *p*-tolyl iodobenzene (3.20 mL, 25.0 mmol, 1.2 equiv.) and the mixture was stirred at room temperature for 30 minutes. Following this, 4-Pentyn-1-ol (2.0 mL, 21.5 mmol, 1.0 equiv.) was added and the reaction was stirred at 60 °C for 18 h under argon. The resultant mixture was then cooled to room temperature, diluted with ether (50 mL) and filtered over celite. The solid was washed with a subsequent 50 mL of ether, and the filtrate was concentrated *in vacuo*. The crude material was purified using column chromatography (25% EtOAc, $R_f = 0.19$) to give the product as an orange oil (1.32 g, 35%). ¹H NMR δ_H (400 MHz, CDCl₃) 1.58 (br, 1H), 1.86 (quintet, $J = 6.8$ Hz, 2H), 2.31, (s, 3H), 2.54 (t, $J = 6.9$ Hz, 2H), 3.82 (br, 2H), 7.08-7.15 (m, 1H), 7.17-7.22 (m, 3H). ¹³C NMR δ_C (100 MHz, CDCl₃) 15.9, 21.2, 31.4, 61.9, 81.3, 88.9, 123.4, 128.1, 128.6, 132.1, 137.8. [C₁₂H₁₄O] + 1 = 175.1

General Mesylation Procedure

Compounds 2a through 2g

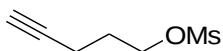
Methanesulfonyl chloride (1.5 equiv.) was added dropwise to a solution of the appropriate alkynol (1.0 equiv.) and triethylamine (2.0 equiv.) in an appropriate volume of anhydrous dichloromethane at 0 °C under argon. After 3 hours, water (100 mL) was added. The organic layer was separated, and washed with water (4 X 100 mL) and dried over Na₂SO₄, filtered, and concentrated *in vacuo*. The crude oil product was carried onto the next step without further purification

Methanesulfonyl but-3-ynyl ester (2a)



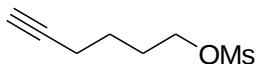
Methanesulfonyl chloride (18.1 g, 158 mmol), 3-Butyn-1-ol (8.00 mL, 106 mmol), triethylamine (29.4 mL, 211 mmol) and DCM (125 mL) were reacted according to the general mesylation procedure to give a yellow oil (13.8 g, 89%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.06 (t, $J = 2.6$ Hz, 1H), 2.64 (td, $J = 6.7, 2.6$ Hz, 2H), 3.04 (s, 3H), 4.28 (t, $J = 6.7$ Hz, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 19.6, 37.6, 67.1, 70.9, 78.6. NMR matched literature. [57]

Methanesulfonyl pent-4-ynyl ester (2b)



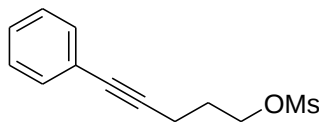
Methanesulfonyl chloride (7.27 g, 63.5 mmol, 1.2 equiv.*), 4-Pentyn-1-ol (5.20 mL, 52.9 mmol), triethylamine (8.84 mL, 63.4 mmol) and DCM (80 mL) were reacted according to the general mesylation procedure to give a yellow oil (7.23 g, 92%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.97 (quintet, $J = 6.4$ Hz, 2H), 2.01 (t, $J = 2.8$ Hz, 1H), 2.36 (td, $J = 6.8, 2.4$ Hz, 2H), 3.03 (s, 3H), 4.36 (t, $J = 6.0$ Hz, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 14.6, 27.7, 37.2, 68.2, 69.7, 82.0. NMR matched literature. [57]

Methanesulfonyl hex-5-ynyl ester (2c)



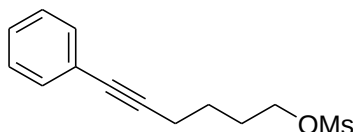
Methanesulfonyl chloride (17.1 g, 150 mmol), 5-Hexyn-1-ol (10.8 mL, 100 mmol), triethylamine (27.9 mL, 200 mmol) and DCM (200 mL) were reacted according to the general mesylation procedure to give a yellow oil (13.6 g, 77%). NMR matched literature. [57]

Methanesulfonic acid 5-phenyl-pent-4-ynyl ester (2d)



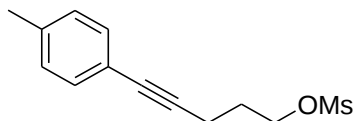
Methanesulfonyl chloride (1.93 mL, 25.0 mmol), **1a** (2.67 g, 16.7 mmol), triethylamine (4.60 mL, 33.3 mmol) and DCM (30 mL) were reacted according to the general mesylation procedure to give a yellow oil (3.19 g, 81%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.07 (quintet, $J = 6.4$ Hz, 2H), 2.62 (t, $J = 6.8$ Hz, 2H), 3.06 (s, 3H), 4.44 (t, $J = 6.4$ Hz, 2H), 7.31-7.33 (m, 3H), 7.40-7.43 (m, 2H).

Methanesulfonic acid 6-phenyl-hex-5-ynyl ester (2e)



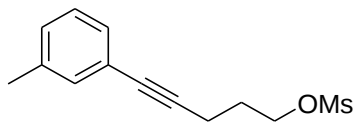
Methanesulfonyl chloride (4.54 g, 39.8 mmol), **1b** (6.33 g, 36.2 mmol), triethylamine (5.56 mL, 39.8 mmol) and DCM (100 mL) were reacted according to the general mesylation procedure to give a yellow oil (9.10 g, >97%) NMR matched literature. [58]

Methane sulfonic acid 5-*p*-tolyl-pent-4-ynyl ester (2f)



Methanesulfonyl chloride (1.00 mL, 12.9 mmol), **1c** (1.50 g, 8.60 mmol), triethylamine (2.40 mL, 17.2 mmol) and DCM (20 mL) were reacted according to the general mesylation procedure to give a yellow oil (2.4 g, qt). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.03 (quintet, $J = 6.4$ Hz, 2H), 2.33 (s, 3H), 2.57 (t, $J = 6.8$ Hz, 2H), 3.03 (s, 3H), 4.41 (t, $J = 6.1$ Hz, 2H), 7.10 (d, $J = 7.9$ Hz, 2H), 7.28 (d, $J = 7.9$ Hz, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 15.6, 21.3, 28.0, 37.2, 68.6, 82.0, 86.7, 120.2, 129.0, 131.3, 137.9.

Methane sulfonic acid 5-*m*-tolyl-pent-4-ynyl ester (2g)



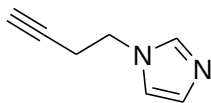
Methanesulfonyl chloride (0.213 mL, 2.76 mmol), **1d** (320 mg, 1.8 mmol), triethylamine (0.500 mL, 3.6 mmol) and DCM (5 mL) were reacted according to the general mesylation procedure to give an orange oil (451 mg, >97%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.04 (quintet, $J = 6.6$ Hz, 2H), 2.32 (s, 3H), 2.85 (t, $J = 6.8$ Hz, 2H), 3.04 (s, 3H), 4.42 (t, $J = 6.1$ Hz, 2H), 7.09-7.11 (m, 1H), 7.16-7.21 (m, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 15.7, 21.2, 28.1, 37.3, 68.5, 87.1, 123.1, 128.2, 128.6, 128.8, 132.1, 137.9.

General Substitution Procedure

Compounds **3a** through **3i**

To a round bottom flask equipped with a water cooled condenser containing a solution of either imidazole or 1-*H*-[1,2,4]-Triazole (1.2 equiv.) and potassium carbonate (4.0 equiv.) in an appropriate volume of 2-butanone was added the corresponding mesylate (1.0 equiv.) and the reaction was heated to reflux and stirred for 18 hours. The reaction was cooled to room temperature and quenched with water (200 mL) and the aqueous phase was extracted with DCM (3 X 100 mL). The combined organic extracts were dried over Na_2SO_4 , filtered, and concentrated *in vacuo*. The crude products were purified using flash column chromatography.

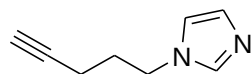
1-But-3-ynyl-1*H*-imidazole (**3a**)



Imidazole (7.62 g, 112 mmol), potassium carbonate (51.6 g, 373 mmol), and **2a** (13.8 g, 93.2 mmol) in 2-butanone (200 mL) were reacted according to the general substitution procedure. Purification by flash column chromatography (0-2.5% MeOH/DCM) provided the product as a yellow oil (4.92 g, 44%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.07 (t, $J = 2.6$ Hz, 1H), 2.64 (td, $J = 6.7, 2.6$ Hz, 2H), 4.10 (t, $J = 6.7$ Hz, 2H), 6.98 (s, 1H), 7.07 (s, 1H), 7.54

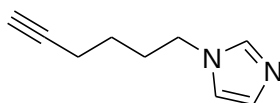
(s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 21.7, 45.8, 71.8, 80.2, 119.5, 130.4, 138.0. ESI-MS $[\text{C}_7\text{H}_8\text{N}_2] = 121.0$.

1-Pent-4-ynyl-1H-imidazole (3b)



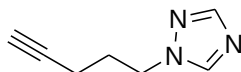
Imidazole (9.57 g, 141 mmol), potassium carbonate (65.0 g, 469 mmol) and **2b** (13.8 g, 93.2 mmol) in 2-butanone (200 mL) were reacted according to the general substitution procedure. Purification by flash column chromatography (2.5% MeOH/DCM) provided the product as a yellow oil (8.30 g, 53%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.93 (quintet, $J = 6.7$ Hz, 2H), 2.02 (t, $J = 2.6$ Hz, 1H), 2.14 (td, $J = 6.7, 2.6$ Hz, 2H), 4.07 (t, $J = 6.7$ Hz, 2H), 6.89 (s, 1H), 7.03 (s, 1H), 7.46 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 15.4, 29.5, 45.3, 70.4, 82.5, 119.4, 130.3, 138.0. ESI-MS $[\text{C}_8\text{H}_{10}\text{N}_2] + 1 = 149.1$.

1-Hex-5-ynyl-1H-imidazole (3c)



Imidazole (6.31 g, 92.8 mmol), potassium carbonate (42.8 g, 309 mmol) and **2c** (13.6 g, 77.3 mmol) in 2-butanone (200 mL) were reacted according to the general substitution procedure. Purification by flash column chromatography (2.5% MeOH/DCM) provided the product as a yellow solid (9.68 g, 85%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.49 (quintet, $J = 7.0$ Hz, 2H), 1.88 (quintet, $J = 7.2$ Hz, 2H), 1.94 (t, $J = 2.6$ Hz, 1H), 2.19 (td, $J = 6.9, 2.6$ Hz, 2H), 3.93 (t, $J = 7.1$ Hz, 2H), 6.88 (s, 1H), 7.02 (s, 1H), 7.43 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 17.9, 25.2, 29.9, 46.5, 69.1, 83.3, 118.7, 129.5, 137.0. ESI-MS $2[\text{C}_9\text{H}_{12}\text{N}_2] + 1 = 297.3$.

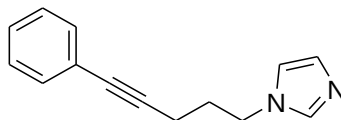
1-Pent-4-ynyl-1H-[1,2,4]triazole (3d)



1.H-[1,2,4]-Triazole (1.66 g, 24.0 mmol), potassium carbonate (11.1 g, 80.0 mmol) and **2b** (3.24 g, 20.0 mmol) in 2-butanone (60 mL) were reacted according to the general substitution procedure. Purification by flash column chromatography (2.5% MeOH/DCM)

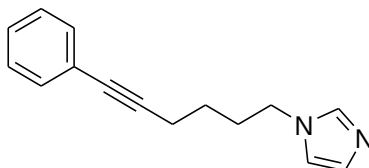
provided the product (1.66g, 62%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.03-2.18 (m, 5H), 4.32 (t, $J = 6.4$ Hz, 2H), 7.93 (s, 1H), 8.08 (s, 1H). ESI – MS $[\text{C}_7\text{H}_9\text{N}_3] + 1 = 136.0$.

1-(5-Phenylpent-4-ynyl)-1H-imidazole (3e)



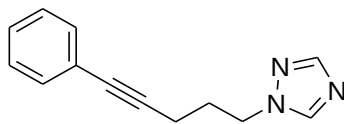
Imidazole (1.09 g, 16.0 mmol), potassium carbonate (7.36 g, 53.2 mmol) and **2d** (3.19 g, 13.3 mmol) in 2-butanone (20 mL) were reacted according to the general substitution procedure. Purification by flash column chromatography (0-5% MeOH/DCM) provided the product as a yellow syrup (1.60 g, 58%). $R_f = 0.55$ (5% MeOH/DCM). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.05 (quintet, $J = 6.8$ Hz, 2H), 2.41 (t, $J = 6.8$ Hz, 2H), 4.16 (t, $J = 6.4$ Hz, 2H), 6.97 (s, 1H), 7.09 (s, 1H), 7.30-7.31 (m, 3H), 7.40-7.42 (m, 2H), 7.56 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 16.4, 29.7, 45.8, 82.3, 87.4, 118.9, 123.4, 128.0, 128.2, 129.4, 131.5, 137.2. ESI-MS $[\text{C}_{14}\text{H}_{14}\text{N}_2] + 1 = 211.1$.

1-(6-Phenylhex-5-ynyl)-1H-imidazole (3f)



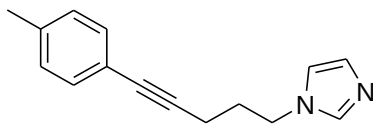
Imidazole (2.94 g, 43.3 mmol), potassium carbonate (20.0 g, 144 mmol) and **2e** (9.10 g, 36.1 mmol) in 2-butanone (75 mL) were reacted according to the general substitution procedure. Purification by flash column chromatography (0-2.5% MeOH/DCM) provided the product as a dark yellow oil (5.90 g, 73%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.44-1.62 (m, 2H), 1.93-2.00 (m, 2H), 2.44 (t, $J = 6.9$ Hz, 2H), 3.99 (t, $J = 7.1$ Hz, 2H), 6.9 (br, 1H), 7.08 (br, 1H), 7.26-7.28 (m, 3H), 7.36-7.38 (m, 2H), 7.49 (br, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 18.9, 25.4, 30.1, 46.5, 81.4, 88.8, 123.5, 126.5, 127.7, 128.2, 128.6, 131.4. ESI-MS $[\text{C}_{15}\text{H}_{16}\text{N}_2] + 1 = 225.2$; $(2[\text{C}_{15}\text{H}_{16}\text{N}_2]) + 1 = 449.4$.

1-(5-Phenylpent-4-ynyl)-1*H*-triazole (3g)



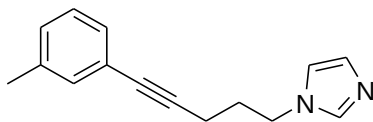
1*H*-[1,2,4]-Triazole (264 mg, 3.80 mmol), potassium carbonate (1.76 g, 12.7 mmol) and **2d** (768 mg, 3.2 mmol) in 2-butanone (10 mL) were reacted according to the general substitution procedure using smaller workup volumes. Purification by flash column chromatography (5% MeOH/DCM) provided the product as a yellow oil (360 mg, 54%). *R*_f = 0.43 (5% MeOH/DCM). ¹H NMR δ_{H} (400 MHz, CDCl₃) 2.18 (quintet, *J* = 6.8 Hz, 2H), 2.42 (t, *J* = 6.4 Hz, 2H), 4.39 (t, *J* = 6.4 Hz, 2H), 7.29-7.31 (m, 3H), 7.39-7.42 (m, 2H), 7.97 (s, 1H), 8.14 (s, 1H). ¹³C NMR δ_{C} (100 MHz, CDCl₃) 16.4, 28.3, 48.1, 82.3, 87.3, 123.3, 128.0, 128.3, 131.6, 143.4, 152.1. ESI-MS [C₁₃H₁₃N₃] + 1 = 212.1

1-(5-*p*-tolylpent-4-ynyl)-1*H*-imidazole (3h)



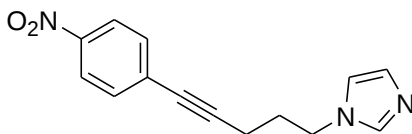
Imidazole (700 mg, 10.3 mmol), potassium carbonate (4.13 g, 34.4 mmol) and **2f** (2.16 g, 8.60 mmol) in 2-butanone (20 mL) were reacted according to the general substitution procedure. Purification by flash column chromatography (0-5% MeOH/EtOAc) provided the product as a yellow oil (1.3g, 68%). ¹H NMR δ_{H} (400 MHz, CDCl₃) 2.03 (quintet, *J* = 6.7 Hz, 2H), 2.34 (s, 3H), 2.39 (t, *J* = 6.7 Hz, 2H), 4.14 (t, *J* = 6.7 Hz, 2H), 6.95 (s, 1H), 7.07 (s, 1H), 7.11 (d, *J* = 7.9 Hz, 3w3w2H), 7.30 (d, *J* = 7.9 Hz, 2H), 7.52 (s, 1H). ¹³C NMR δ_{C} (100 MHz, CDCl₃) 16.4, 21.4, 29.8, 45.4, 82.3, 86.7, 118.8, 120.2, 129.0, 129.6, 131.4, 137.3, 138.0. ESI-MS [C₁₅H₁₆N₂] + 1 = 225.3

1-(5-*m*-tolylpent-4-ynyl)-1*H*-imidazole (3i)



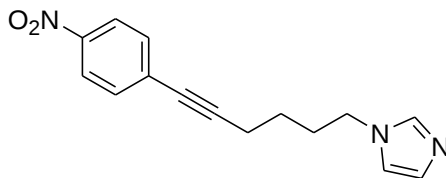
Imidazole (142 mg, 2.10 mmol), potassium carbonate (837 mg, 6.96 mmol) and **2g** (440 mg, 1.74 mmol) in 2-butanone (15 mL) were reacted according to the general substitution procedure. Purification by flash column chromatography (1:4 Hex/DCM, *R_f* = 0.44) provided the product as a yellow oil (152 mg, 68%). ¹H NMR δ_{H} (400 MHz, CDCl₃) 2.03 (quintet, *J* = 6.7 Hz, 2H), 2.32 (s, 3H), 2.39 (t, *J* = 6.7 Hz, 2H), 4.14 (t, *J* = 6.7 Hz, 2H), 6.98 (s, 1H), 7.08-7.17 (m, 2H), 7.18-7.23 (m, 3H), 7.54 (s, 1H). ¹³C NMR δ_{C} (100 MHz, CDCl₃) 16.3, 21.1, 29.7, 45.4, 82.4, 87.0, 123.0, 128.2, 128.5, 128.8, 132.1, 137.9.

1-[5-(4-Nitro-phenyl)-pent-4-ynyl]-1*H*-imidazole (4a)



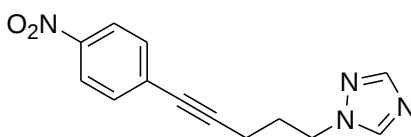
To a solution of 4-nitroiodobenzene (3.09 g, 12.4 mmol, 1.0 equiv.) in triethylamine (35 mL) were added subsequently PdCl₂(PPh₃)₂ (260 mg, 0.37 mmol, 3 mol%), copper iodide (70.8 mg, 0.37 mmol, 3 mol %), and **3b** (2.00 g, 14.9 mmol, 1.2 equiv.). The solution was stirred for 16 hours at room temperature. The mixture was treated with saturated ammonium chloride (200 mL) and the resultant mixture was extracted with DCM (3 X 150 mL), dried over sodium sulphate, filtered, and concentrated *in vacuo*. Purification was achieved via column chromatography (40% DCM/hex to 2.5% MeOH/DCM) to give the product as a crude inseparable mixture with the starting material. The compound was used without further purification in the methylation step. ESI-MS [C₁₄H₁₃N₃O₂] + 1 = 256.2

1-[6-(4-Nitro-phenyl)-hex-5-ynyl]-1H-imidazole (4b)



To a solution of 4-iodonitrobenzene (1.01 g, 4.00 mmol, 1.2 equiv.) in triethylamine (10 mL) were added subsequently $\text{PdCl}_2(\text{PPh}_3)_2$ (70.2 mg, 0.10 mmol, 3 mol%), copper iodide (19.2 mg, 0.10 mmol, 3 mol%), and **3c** (500 mg, 3.37 mmol, 1.0 equiv.) at room temperature. Stirring was continued at room temperature for 16 hours. The mixture was quenched with saturated NH_4Cl , extracted with DCM (3 X 20 mL), dried over Na_2SO_4 , filtered, and concentrated *in vacuo*. The crude solid was purified by first adsorbing to silica gel and then performing column chromatography (DCM to 10% MeOH/DCM) to give the product (R_f = 0.14 in 5%MeOH/DCM) as a yellow oil that was inseparable from unreacted starting material, this was used without further purification (330 mg, 36 % estimated by NMR conversion). ESI-MS $[\text{C}_{15}\text{H}_{15}\text{N}_3\text{O}_2] + 1 = 270.2$

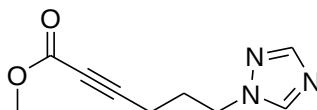
1-[5-(4-Nitro-phenyl)-pent-4-ynyl]-1H-[1,2,4]triazole (4c)



To a solution of 4-nitroiodobenzene (2.56 g, 10.3 mmol, 1.0 equiv.) in triethylamine (35 mL) were added subsequently $\text{PdCl}_2(\text{PPh}_3)_2$ (217 mg, 0.31 mmol, 3 mol%), copper iodide (58.5 mg, 0.31 mmol, 3 mol%), and **3d** (1.66 g, 12.3 mmol, 1.2 equiv.) at 25 °C. Stirring was continued for 16 hours. The reaction mixture was treated with saturated ammonium chloride (200 mL) and the resultant mixture was extracted with DCM (3X 150 mL). The combined organic extracts were dried over sodium sulphate, filtered, and concentrated *in vacuo*. The crude product was purified using column chromatography (40% EtOAc/Hexanes > 10% MeOH/EtOAc) to give the product (1.99 g, 76.3%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.21 (quintet, J = 6.4 Hz, 2H), 2.48 (t, J = 6.8 Hz, 2H), 4.36 (t, J = 6.8 Hz, 2H), 7.51 (d, J = 8.8 Hz, 2H), 7.96 (s, 1H), 8.11 (s, 1H), 8.15 (d, J = 8.8 Hz, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3)

16.6, 28.1, 48.1, 80.5, 93.4, 104.6, 123.5, 130.2, 132.3, 143.2, 152.2. ESI-MS [$C_{13}H_{12}N_4O_2$] + 1 = 257.2.

6-[1,2,4]Triazol-1-yl-hex-2-ynoic acid methyl ester (4d)



3b (1.00 g, 7.40 mmol, 1.0 equiv.) was dissolved as a 0.27 M solution in dry THF (27.4 mL) and cooled to -78 °C. Dropwise addition of n-BuLi (3.55 mL, 8.88 mmol, 1.2 equiv.) was followed by stirring for 20 minutes. The solution was transferred to an ice bath (0 °C) and methyl chloroformate (688 μ L, 8.88 mmol, 1.2 equiv.) was added dropwise followed by stirring at 0 °C for 30 minutes. The reaction was quenched with saturated NH_4Cl , and DCM and water were added as the solution was transferred to a separatory funnel and the organic layer was washed with water (2X), brine (1X), dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified using column chromatography to give the final compound as a yellow oil (390 mg, 27.2 %). 1H NMR δ_H (400 MHz, $CDCl_3$) 2.18 (quintet, J = 6.5 Hz, 2H), 2.35 (t, J = 6.8 Hz, 2H), 3.78 (s, 3H), 4.32 (t, J = 6.7 Hz, 2H), 7.96 (s, 1H), 8.11 (s, 1H). ^{13}C NMR δ_C (100 MHz, $CDCl_3$) 15.6, 27.2, 48.0, 53.0, 74.2, 86.6, 143.4, 152.2, 153.8. ESI-MS [$C_9H_{11}N_3O_2$] + 1 = 193.1.

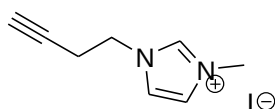
General Methylation Procedure to Obtain Azolium Iodide Salts

Compounds 5a through 5l

To a tube containing the corresponding alkynyl-tethered triazole or imidiazole (1.0 equiv.) and a small volume of ethyl acetate was added iodomethane (2.0 equiv.) (or iodoethane for compound **9**) and the tube was purged with argon for two minutes. The tube was then sealed and stirred at 60 °C overnight. The organic supernatant was removed and the resultant ionic liquid was purified by one of the following two methods: **A**) washing with ethyl acetate (3 X 5 mL) then dissolving in chloroform, transferring to a round bottom flask, and concentrating *in vacuo* to give the product as an ionic liquid – OR – **B**) precipitating by treatment with ethyl acetate and then isolating by suction filtration and washing the solid with 5 mL of cold ethyl acetate. Further purification may have been performed using flash

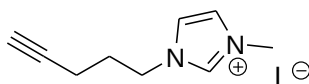
column chromatography or recrystallization as necessary. Where appropriate, the melting point is given for the recrystallized compound.

3-But-3-ynyl-1-methyl-3*H*-imidazol-1-ium iodide (5a)



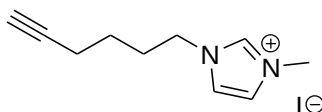
3a (1.00 g, 8.30 mmol) and iodomethane (1.03 mL, 16.6 mmol) in ethyl acetate (2 mL) were reacted as described in the general methylation procedure and purified using method B to give a peach coloured solid (1.14 g, 53%) ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.18 (t, $J = 2.6$ Hz, 1H), 2.91 (td, $J = 6.3, 2.6$ Hz, 2H), 4.11 (s, 3H), 4.56 (t, $J = 6.3$ Hz, 2H), 7.48 (s, 1H), 7.66 (s, 1H), 10.04 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 21.0, 37.4, 48.9, 73.5, 79.2, 123.5, 123.8, 138.1. ESI-MS $[\text{C}_8\text{H}_{11}\text{N}_2\text{I} - \text{I}]^+ = 135.0$.

1-Methyl-3-pent-4-ynyl-2,3-dihydro-1*H*-imidazol-1-ium iodide (5b)



3b (593 mg, 4.42 mmol) and iodomethane (548 μL , 8.84 mmol) in ethyl acetate (2.2 mL) were reacted as described in the general methylation procedure and purified using method B to give an orange coloured solid (1.18g, 96%). $R_f = 0.33$ (10% MeOH/DCM). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.10 (t, $J = 2.6$ Hz, 1H), 2.20 (quintet, $J = 6.8$ Hz, 2H), 2.35 (td, $J = 6.5, 2.4$ Hz, 2H), 4.11 (s, 3H), 4.52 (t, $J = 7.0$ Hz, 2H), 7.42-7.44 (m, 2H), 10.10 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 15.5, 28.6, 37.4, 49.0, 71.5, 81.8, 123.1, 124.1, 138.1. ESI-MS $[\text{C}_9\text{H}_{13}\text{N}_2\text{I} - \text{I}]^+ = 149.1$.

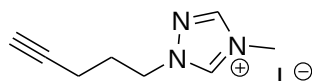
3-Hex-5-ynyl-1-Methyl-2,3-dihydro-1*H*-imidazol-1-ium iodide (5c)



3c (0.500 g, 3.37 mmol) and iodomethane (0.420 mL, 6.75 mmol) in ethyl acetate (1.7 mL) were reacted as described in the general methylation procedure and purified using method A

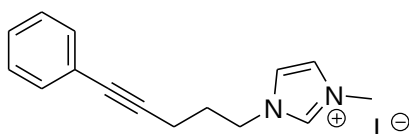
then the crude product was purified using flash column chromatography (10-15% MeOH/DCM) to obtain the final product as a yellow oil (550 mg, 56%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.35 (quintet, $J = 7.0$ Hz, 2H), 1.81-1.88 (m, 3H), 2.04 (td, $J = 6.9, 2.6$ Hz, 2H), 3.88 (s, 3H), 4.20 (t, $J = 7.3$ Hz, 2H), 7.47 (s, 1H), 7.48 (s, 1H), 9.61 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 17.1, 24.0, 28.4, 36.4, 48.7, 69.1, 82.5, 121.8, 123.1, 135.4. ESI-MS $[\text{C}_9\text{H}_{13}\text{N}_2\text{I} - \text{I}]^+ = 163.0$

4-Methyl-2-pent-4-ynyl-3,4-dihydro-2H-[1,2,4]triazol-1-ium iodide (5d)



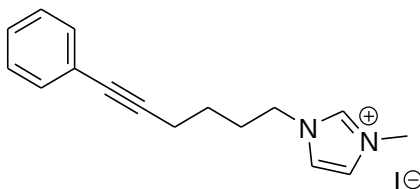
3d (0.760 g, 5.62 mmol) and iodomethane (708 μL , 11.2 mmol) in ethyl acetate (2.8 mL) were reacted as described in the general methylation procedure and purified using method B then the crude product was purified using flash column chromatography (5-15% MeOH/DCM) to obtain the final product as a yellow solid (1.30g, 83%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.0 (t, $J = 2.4$ Hz, 1H), 2.16 (quintet, $J = 6.4$ Hz, 2H), 2.30 (td, $J = 6.8, 2.4$ Hz, 2H), 4.14 (s, 3H), 4.55 (t, $J = 7.2$ Hz, 2H), 8.69 (s, 1H), 10.92 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 15.8, 27.2, 36.1, 52.0, 71.3, 81.9, 144.2, 145.3. ESI-MS $[\text{C}_8\text{H}_{12}\text{N}_3\text{I} - \text{I}]^+ = 164.0$

1-Methyl-1-(5-phenylpent-4-ynyl)-3H-imidazol-1-ium iodide (5e)



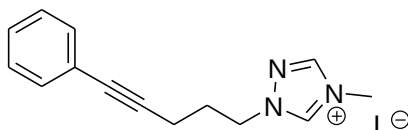
3e (0.390 g, 1.90 mmol) and iodomethane (0.230 mL, 3.70 mmol) in ethyl acetate (1.0 mL) were reacted as described in the general methylation procedure and purified using method B (530 mg, 80%). ^1H NMR δ_{H} (400 MHz, CD_3Cl_3) 2.27 (quintet, $J = 6.7$ Hz, 2H), 2.59 (t, $J = 6.5$ Hz, 2H), 4.01 (s, 3H), 4.56 (t, $J = 6.9$ Hz, 2H), 7.29-7.31 (m, 4H), 7.36-7.41 (m, 3H), 10.19 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CD_3Cl_3) 16.5, 28.7, 37.0, 49.2, 82.6, 86.7, 122.4, 122.9, 123.2, 128.2, 128.4, 131.5, 137.5. ESI-MS $[\text{C}_{15}\text{H}_{17}\text{N}_2\text{I} - \text{I}]^+ = 225.2$. mp 111 -113 $^{\circ}\text{C}$ (recrystallized from EtOAc/MeOH).

1-Methyl-3-(6-phenyl-hex-5-ynyl)-3H-imidazol-1-ium iodide (5f)



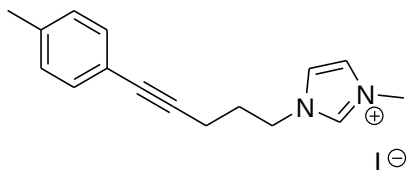
3f (5.90 g, 26.3 mmol) and iodomethane (3.27 mL, 52.6 mmol) in ethyl acetate (5 mL) were reacted as described in the general methylation procedure and purified using method A to obtain the final product as a dark yellow syrup (9.50g, qt). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.60-1.67 (m, 2H), 2.05-2.13 (m, 2H), 2.47 (t, $J = 6.9$ Hz, 2H), 4.04 (s, 3H), 4.38 (t, $J = 7.3$ Hz, 2H), 7.22-7.25 (m, 3H), 7.33-7.36 (m, 2H), 7.49 (s, 1H), 7.50 (s, 1H), 9.95 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 18.8, 29.2, 36.9, 49.6, 81.4, 88.7, 122.2, 123.3, 123.6, 127.7, 128.2, 131.4, 136.6. ESI-MS $[\text{C}_{16}\text{H}_{19}\text{N}_2\text{I} - \text{I}]^+ = 239.2$.

4-Methyl-1-(5-phenylpent-4-ynyl)-1H-[1,2,4]triazol-4-ium iodide (5g)



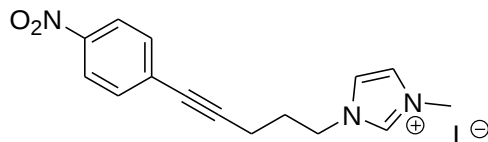
3g (0.360 g, 1.71 mmol) and iodomethane (213 μL , 3.42 mmol) in ethyl acetate (1.0 mL) were reacted as described in the general methylation procedure and purified using method B to give a white salt (506 mg, 84%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.37 (t, $J = 6.7$ Hz, 2H), 2.66 (t, $J = 6.5$ Hz, 2H), 4.04 (s, 3H), 4.73 (t, $J = 6.8$ Hz, 2H), 7.29-7.31 (m, 3H), 7.37-7.40 (m, 2H), 8.32 (s, 1H), 11.50 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 16.8, 27.3, 35.4, 52.2, 82.2, 86.9, 122.9, 128.1, 128.4, 131.6, 143.5, 144.0. ESI-MS $[\text{C}_{14}\text{H}_{16}\text{N}_3\text{I} - \text{I}]^+ = 226.2$. mp 122-124 $^{\circ}\text{C}$ (recrystallized from EtOAc/MeOH).

1-Methyl-3-(5-*p*-tolylpent-4-ynyl)-3*H*-imidazol-1-ium iodide (5h)



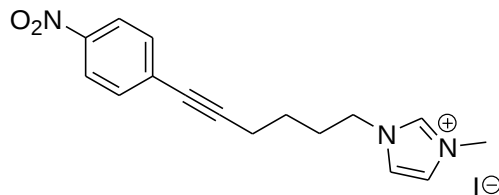
3h (450 mg, 2.0 mmol) and iodomethane (249 μ L, 4.0 mmol) in ethyl acetate (1.5 mL) were reacted as described in the general methylation procedure and purified using method B to give the product as a yellow solid (475 mg, 65%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.24 (quintet, $J = 6.8$ Hz, 2H), 2.32 (s, 3H), 2.56 (t, $J = 6.6$ Hz, 2H), 4.00 (s, 3H), 4.54 (t, $J = 6.9$ Hz, 2H), 7.09 (d, $J = 7.9$ Hz, 2H), 7.26 (d, $J = 7.9$ Hz, 2H), 7.39 (s, 1H), 7.46 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 16.5, 21.4, 28.7, 37.0, 49.1, 82.6, 86.0, 119.8, 122.5, 123.4, 129.1, 131.4, 137.2, 138.2. ESI-MS $[\text{C}_{16}\text{H}_{19}\text{N}_2\text{I} - \text{I}]^+ = 239.3$.

3-Methyl-1-[5-(4-nitro-phenyl)-pent-4-ynyl]-2,3-dihydro-1*H*-imidazol-1-ium iodide (5h)



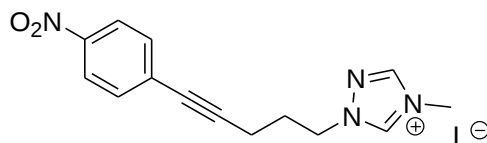
Crude **4a** (840 mg) and iodomethane (409 μ L, 6.58 mmol) were reacted as described in the general methylation procedure and purified using method B to provide the product as a dark solid (1.72g, no yield determined because of impure starting materials). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.31 (quintet, $J = 6.9$ Hz, 2H), 2.65 (t, $J = 6.7$ Hz, 2H), 4.06 (s, 3H), 4.56 (t, $J = 7.2$ Hz, 2H), 7.40 (s, 1H), 7.48 (s, 1H), 7.57 (d, $J = 8.9$ Hz, 2H), 8.14 (d, $J = 8.9$ Hz, 2H), 10.04 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 16.7, 28.6, 37.1, 49.0, 81.0, 92.7, 122.3, 123.5, 123.6, 129.9, 132.5, 137.3, 146.9. ESI-MS $[\text{C}_{15}\text{H}_{16}\text{N}_3\text{O}_2\text{I} - \text{I}]^+ = 270.1$

1-Methyl-3-[6-(4-nitro-phenyl)-hex-5-ynyl]-2,3-dihydro-1H-imidazol-1-ium iodide (5i)



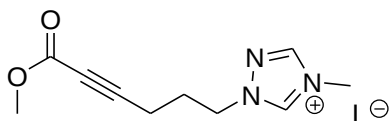
4b (0.330 g, 1.22 mmol) and iodomethane (152 μ L, 2.45 mmol) in ethyl acetate (1.0 mL) were reacted as described in the general methylation procedure and purified using method B then further purified using HPLC (10-90% MeCN/H₂O over 10 minutes with 0.1% TFA, retention time = 5.5 minutes to 6.5 minutes) to give the final product as a green ionic liquid (450 mg, 89%). ¹H NMR δ_{H} (400 MHz, CDCl₃) 1.64 (quintet, J = 6.8 Hz, 2H), 2.04 (quintet, J = 6.9 Hz, 2H), 2.50 (t, J = 6.7 Hz, 2H), 3.94 (s, 3H), 4.27 (t, J = 6.9 Hz, 2H), 7.34 (s, 1H), 7.37 (s, 1H), 7.47 (d, J = 8.7 Hz, 2H), 8.09 (d, J = 8.6 Hz, 2H), 9.34 (s, 1H). ¹³C NMR δ_{C} (100 MHz, CDCl₃) 18.9, 24.8, 29.3, 36.4, 49.8, 80.5, 95.2, 122.8, 124.1, 124.4, 131.4, 133.1, 137.8, 147.5. ESI-MS [$\text{C}_{16}\text{H}_{18}\text{N}_3\text{O}_2\text{I} - \text{I}$]⁺ = 284.1.

4-Methyl-1-[5-(4-nitro-phenyl)-pent-4-ynyl]-1H-[1,2,4]triazol-4-ium iodide (5j)



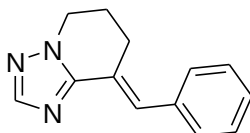
4c (1.28 g, 5.00 mmol) and iodomethane (622 μ L, 10.0 mmol) in ethyl acetate (2.5 mL) were reacted as described in the general methylation procedure and purified using method B to give the product as a yellow powder (1.67g, 84%). ¹H NMR δ_{H} (400 MHz, CDCl₃) 2.42 (tt, J = 6.8, 6.8, 2H), 2.71 (t, J = 6.8 Hz, 2H), 4.20 (s, 3H), 4.75 (t, J = 6.8 Hz, 2H), 7.59, (d, J = 8.8 Hz, 2H), 8.17 (d, J = 8.8 Hz, 2H), 8.42 (s, 1H), 11.55 (s, 1H). ¹³C NMR (100 MHz, CDCl₃) 16.9, 27.2, 35.6, 52.0, 80.9, 92.7, 123.6, 126.7, 129.9, 132.6, 143.8, 147.0 ESI-MS [$\text{C}_{14}\text{H}_{15}\text{N}_4\text{O}_2\text{I} - \text{I}$]⁺ = 271.2.

1-(5-Methoxycarbonyl-pent-4-ynyl)-4-methyl-4,5-dihydro-1H-[1,2,4]triazol-4-ium iodide (5k)



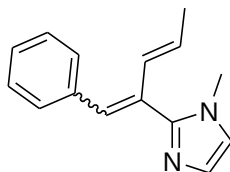
4d (0.380 g, 1.97 mmol) and iodomethane (244 μ L, 3.93 mmol) in ethyl acetate (1.9 mL) were reacted as described in the general methylation procedure and purified using method A then the crude product was further purified using flash column chromatography (15% MeOH/DCM) to obtain the final product as an off white solid (290 mg, 44%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 2.33 (t, J = 6.7 Hz, 2H), 2.65 (t, J = 6.7 Hz, 2H), 3.74 (s, 3H), 4.20 (s, 3H), 4.71 (t, J = 6.6 Hz, 3H), 8.82 (s, 1H), 10.84 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 16.3, 26.2, 35.7, 52.3, 53.1, 74.2, 87.0, 144.6, 144.9, 153.8. ESI-MS $[\text{C}_{10}\text{H}_{14}\text{N}_3\text{O}_2\text{I} - \text{I}]^+ = 208.1$.

8-Benzylidene-5,6,7,8-tetrahydro-[1,2,4]triazolo[1,5-a]pyridine (6)



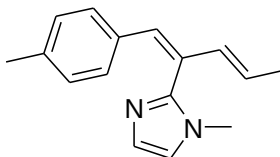
To a flame dried flask **5g** (0.200g, 0.560 mmol, 1.0 equiv.) was added and dissolved in dry THF (5 mL). To this solution was added potassium *tert*-butoxide (693 mg, 5.00 mmol, 10 equiv.) and the mixture was stirred overnight. The resultant solution was quenched with the addition of water (10 mL) and extracted into EtOAc (3 X 10 mL). The organic extracts were combined, dried over sodium sulphate, filtered and concentrated *in vacuo*. The product was isolated and purified using column chromatography (20-100% EtOAc/Hexanes) (R_f = 0.45 1:1 EtOAc/Hexanes). (15 mg, 13%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.99 (t, J = 7.2 Hz, 2H), 2.86, (ddd, J = 7.6, 7.6, 2.0 Hz, 2H), 3.69 (t, J = 7.2 Hz, 2H), 6.31 (s, 1H), 7.20-7.36 (m, 5H), 8.88 (s, 1H). (400 MHz, CDCl_3) 23.0, 37.6, 39.2, 105.0, 127.2, 128.0, 128.4, 128.8, 129.4, 133.9, 161.3. ESI-MS $[\text{C}_{13}\text{H}_{13}\text{N}_3] + 1 = 212.1$.

2-(1-Benzylidene-but-2-enyl)-1-Methyl-1H-imidazole (7a & 7b)



5e (0.100 g, 0.280 mmol, 1.0 equiv.) was dissolved in THF (1 mL). An excess of potassium *tert*-butoxide (342 mg, 2.80 mmol, 10 equiv.) was added and the reaction stirred overnight. The mixture was transferred to a separatory funnel and water (10 mL) was added. The mixture was extracted with dichloromethane (3 X 10 mL), dried over sodium sulphate, filtered, concentrated *in vacuo*. The resultant crude product was purified using column chromatography with a mobile phase gradient of 20-100 % EtOAc/Hexanes to give the resultant compound as an amorphous red product and yellow oil respectively. [Isomer **7a** (*Z,E*) = Rf 0.30, 1:1 hexanes/EtOAc] (17.1 mg, 27%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.79 (dd, J = 6.8, 1.0 Hz, 3H), 3.17 (s, 3H), 5.47 (dq, J = 15.3, 6.8 Hz, 1H), 6.42 (dd, J = 15.3, 1.0 Hz, 1H), 6.69 (dd, J = 8, 1.6 Hz, 2H), 6.74 (s, 1H), 6.89 (d, J = 1.0 Hz, 1H), 7.14-7.17 (m, 3H), 7.18 (d, J = 1.0 Hz, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 18.3, 32.5, 120.4, 127.4, 128.2, 128.4, 128.6, 129.7, 130.3, 133.1, 133.7, 136.2, 144.5. ESI-MS [$\text{C}_{15}\text{H}_{16}\text{N}_2$] + 1 = 225.3. [isomer **7b**(*E,E*), Rf = 0.15, 1:1 hexanes/EtOAc] (12.0 mg, 19%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.80 (dd, J = 6.8, 1.1 Hz, 3H), 3.57 (s, 3H), 5.53 (dq, J = 15.5, 6.8 Hz, 1H), 6.59 (s, 1H), 6.72 (dq, J = 15.6, 1.1 Hz, 1H), 6.91 (d, J = 1.0 Hz, 1H), 7.08 (d, J = 1.0 Hz, 1H), 7.27-7.41 (m, 5H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 18.6, 33.7, 120.9, 127.4, 127.7, 128.0, 128.3, 129.4, 130.4, 132.2, 132.5, 136.5, 148.3. ESI-MS [$\text{C}_{15}\text{H}_{16}\text{N}_2$] + 1 = 225.3.

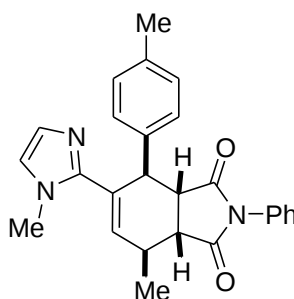
1-Methyl-2-[1-(4-methyl benzylidene)but-2-enyl]-1H-imidazole (7c)



5h (183 mg, 0.500 mmol, 1.0 equiv.) was suspended in THF (1 mL) and KO*t*Bu (168 mg, 1.50 mmol, 3.0 equiv.) was added portionwise and the reaction was stirred under argon for 2 hours. The mixture was quenched with water (5 mL) and extracted into EtOAc (3 X 5 mL).

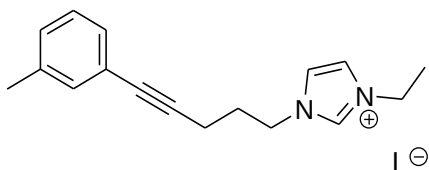
The organic extracts were combined, dried over sodium sulphate, filtered and concentrated *in vacuo*. The crude mixture was purified using column chromatography (50-100% EtOAc in hexanes) to give the (Z,E) stereoisomer (confirmed through NOESY of starting material and Diels-Alder cycloadduct **9**) as a yellow oil (30 mg, 25 %). (EtOAc, R_f = 0.48). ¹H NMR δ_H (400 MHz, CDCl₃) 1.78 (dd, *J* = 6.8, 1.0 Hz, 1H), 2.25 (s, 3H), 3.17 (s, 3H), 5.43 (dq, *J* = 15.3, 6.8 Hz, 1H), 6.40 (dq, *J* = 15.3, 1.0 Hz, 1H), 6.58 (d, *J* = 8.2 Hz, 2H), 6.70 (s, 1H), 6.88 (d, *J* = 0.9 Hz, 1H), 6.96 (d, *J* = 8.2 Hz, 2H), 7.17 (d, *J* = 1.0 Hz, 1H). ¹³C NMR δ_C (100 MHz, CDCl₃) 18.3, 21.1, 32.5, 120.3, 128.1, 128.6, 129.1, 129.2, 129.4, 133.2, 133.4, 133.8, 137.3, 144.7. ESI-MS [C₁₆H₁₈N₂] + 1 = 239.1.

7-Methyl-5-(1-methyl-1*H*-imidazol-2-yl)-2-phenyl-4*p*-tolyl-3a,4,7,7a-tetrahydroisindole-1,3-dione (**8)**



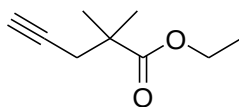
7c (7.5 mg, 0.060 mmol, 1.1 equiv.) and *N*-Phenyl maleimide (5.0 mg, 0.057 mmol, 1.0 equiv.) were mixed in a flask in toluene (0.5 mL) and heated to 110 °C overnight. The crude reaction mixture was concentrated *in vacuo* and purified using column chromatography (1:1 Hex/EtOAc, R_f = 0.20) to give the product as a brown solid (7 mg, 59%). [Exo cycloadduct] ¹H NMR δ_H (400 MHz, CDCl₃) 1.80 (d, *J* = 7.6 Hz, 3H), 2.24 (s, 3H), 3.12-3.20 (m, 1H), 3.38 (dd, *J* = 8.4, 8.3 Hz, 1H), 3.52 (s, 3H), 3.68 (t, *J* = 8.0 Hz, 1H), 4.74 (d, *J* = 7.5 Hz, 1H), 6.11 (d, *J* = 3.6 Hz, 1H), 6.45-6.49 (m, 2H), 6.70 (d, *J* = 0.8 Hz, 1H), 6.94 (d, *J* = 0.8 Hz, 1H), 7.01 (d, *J* = 8.0 Hz, 2H), 7.08 (d, *J* = 8.0 Hz, 2H), 7.23-7.26 (m, 3H). ¹³C NMR δ_C (100 MHz, CDCl₃) 18.1, 20.9, 29.6, 34.0, 40.2, 42.9, 45.8, 121.7, 126.3, 127.6, 128.2, 128.6, 129.2, 129.6, 131.3, 133.9, 135.0, 137.4, 146.6, 175.5, 175.9. ESI-MS [C₂₆H₂₅N₃O₂] + 1 = 412.2

1-Ethyl-3-(5-m-tolyl-pent-4-ynyl)-3H-imidazol-1-ium iodide (9)



3i (35.0 mg, 0.150 mmol) and iodoethane (25.0 μ L, 0.300 mmol) in ethyl acetate (0.5 mL) were reacted as described in the general methylation procedure and purified using method B and the resultant yellow powder was used without further purification (20 mg, 38%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.57 (t, $J = 7.4$ Hz, 3H), 2.27 (quintet, $J = 6.7$ Hz, 2H), 2.31 (s, 3H), 2.57 (t, $J = 6.6$ Hz, 2H), 4.34 (q, $J = 7.4$ Hz, 2H), 4.58 (t, $J = 6.9$ Hz, 2H), 7.10-7.20 (m, 4H), 7.33-7.34 (m, 1H), 7.41-7.42 (m, 1H), 10.29 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 15.3, 16.5, 21.2, 28.8, 45.5, 49.1, 82.8, 86.4, 121.4, 122.4, 122.7, 128.2, 128.6, 129.0, 132.1, 136.8, 138.1. ESI-MS $[\text{C}_{17}\text{H}_{21}\text{N}_2\text{I} - \text{I}]^+ = 253.2$.

2,2-Dimethyl-pent-4-ynoic acid ethyl ester (10)



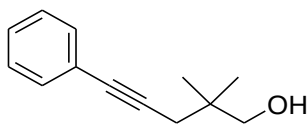
To a solution of 2.0 M LDA (13.5 mL, 26.9 mmol, 1.04 equiv.) in dry THF at -78°C was added ethyl isobutyrate (3.00 g, 25.8 mmol, 1.0 equiv.) and the reaction was stirred at 0°C for 10 minutes. It was again cooled to -78°C and propargyl bromide (80% in toluene, 3.16 mL, 28.2 mmol, 1.1 equiv.) was added. The mixture was warmed to room temperature and stirred for 1 hour. The reaction was quenched with saturated aqueous NH_4Cl , diluted with DCM and the organic phase was washed with water. The collected organic extracts were dried with Na_2SO_4 , filtered, and concentrated. The resultant crude brown oil was purified using column chromatography (15% EtOAc/Hexanes, $R_f = 0.7$ in 10 % EtOAc/Hexanes) to give the product as a volatile, yellow oil (3.71 g, 93 %). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.25 (t, $J = 7.2$ Hz, 3H), 1.26 (s, 6H), 1.99 (t, $J = 2.7$ Hz, 1H), 2.42 (d, $J = 2.7$ Hz, 2H), 4.13 (q, $J = 7.2$ Hz, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 14.2, 24.6, 29.7, 42.2, 61.0, 70.7, 81.5, 177.6. ESI-MS $[\text{C}_9\text{H}_{14}\text{O}_2] + 1 = 155.2$.

2,2-Dimethyl-pent-4-yn-1-ol (11)



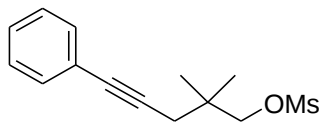
To a suspension of lithium aluminum hydride (1.00 g, 26.4 mmol, 1.1 equiv.) in ether (50 mL) was added **10** (3.70 g, 24.0 mmol, 1.0 equiv.) as a solution in ether (10 mL) under an atmosphere of argon at 0 °C. A solution of 10% (w/v) aqueous KOH was added dropwise at 0 °C. The mixture was warmed to room temperature and stirred for 1 hour and became a milky solution. The mixture was filtered over a pad of celite and the filtrate was concentrated *in vacuo*. The residue was purified using column chromatography (20 % EtOAc/Hexanes) to give the product as a pale yellow oil (2.31g, 86 %). $R_f = 0.14$ (10% EtOAc/Hex). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.23 (s, 6H), 1.70-1.75 (br, 1H), 1.99 (t, $J = 1.4$ Hz, 1H), 2.15 (d, $J = 2.5$ Hz, 2H), 3.42 (s, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 23.8, 28.4, 35.6, 70.6, 71.2, 82.6. ESI-MS $[\text{C}_7\text{H}_{12}\text{O}] + 1 = 113.2$.

2,2-Dimethyl-5-phenyl-pent-4-yn-1-ol (12)



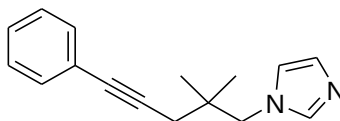
To a stirring solution of copper iodide (85.0 mg, 0.450 mmol, 5 mol%) and Tetrakis(triphenylphosphine)Palladium (190 mg, 0.18 mmol, 2 mol%), in triethylamine (10 mL) was added iodobenzene (1.54 mL, 9.81 mmol, 1.1 equiv.). The reaction was stirred at room temperature for 30 minutes. **11** (1.0 g, 8.97 mmol, 1.0 equiv.) was added and the reaction was stirred at 60 °C for 18 hours. The mixture was cooled to room temperature and diluted with ether. It was then filtered over celite and the filtrate was concentrated *in vacuo*. The crude reaction mixture was purified using column chromatography and a gradient of 5-10% EtOAc/Hexanes to give the product as a dark yellow oil (150 mg, 10%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.04 (s, 6H), 1.59 (br), 2.38 (s, 2H), 3.49 (d, $J = 5.4$ Hz, 2H), 7.27-7.30 (m, 3H), 7.39-7.41 (m, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 24.0, 29.5, 36.2, 71.5, 83.1, 88.2, 124.5, 128.4, 129.0, 132.3. . ESI-MS $[\text{C}_{13}\text{H}_{16}\text{O}] + 1 = 189.1$.

Methanesulfonic acid 2,2-dimethyl-5-phenyl-pent-4-ynyl ester (13)



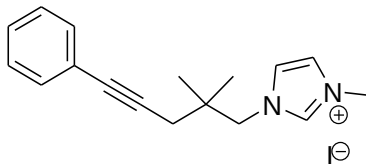
Methanesulfonyl chloride (0.090 mL, 1.20 mmol), **12** (145 mg, 0.770 mmol), triethylamine (214 μ L, 1.50 mmol) and DCM (20 mL) were reacted according to the general mesylation procedure to give an orange oil (204 mg, qt). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.12 (s, 6H), 2.44 (s, 2H), 3.04 (s, 3H), 4.08 (s, 2H), 7.28-7.31 (m, 3H), 7.38-7.41 (m, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 23.9, 29.3, 34.9, 37.0, 76.4, 83.2, 86.2, 123.4, 127.9, 128.3, 131.5. ESI-MS $[\text{C}_{14}\text{H}_{18}\text{SO}_3] + 1 = 266.4$.

1-(2,2-Dimethyl-5-phenyl-pent-4-ynyl)-1H-imidazole (14)



Imidazole (62.0 mg, 0.900 mmol), potassium carbonate (415 mg, 3.00 mmol) and **13** (0.200 g, 0.750 mmol) in 2-butanone (5 mL) were reacted according to the general substitution procedure. Purification by flash column chromatography (50.0 mg, 28%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.07 (s, 6H), 2.27 (s, 2H), 3.92 (s, 2H), 6.99 (s, 1H), 7.05 (s, 1H), 7.29-7.32 (m, 3H), 7.43-7.46 (m, 2H), 7.53 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 25.4 (CH_3), 30.2 (CH_2), 35.8 (C), 55.8 (CH_2), 83.8 (C), 86.4 (C), 120.5 (CH), 123.3 (C), 128.0 (CH), 128.1 (CH), 128.6 (CH), 128.8 (CH), 138.2 (CH). . ESI-MS $[\text{C}_{16}\text{H}_{18}\text{N}_2] + 1 = 238.1$.

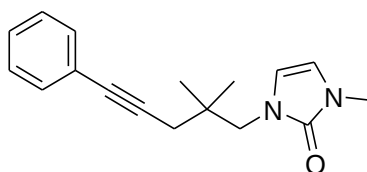
3-(2,2-Dimethyl-5-phenyl-pent-4-ynyl)-1-Methyl-3H-imidazol-1-ium iodide (15)



14 (50 mg, 0.21 mmol) and iodomethane (26 μ L, 0.42 mmol) in ethyl acetate (mL) were reacted as described in the general methylation procedure and purified using method B then

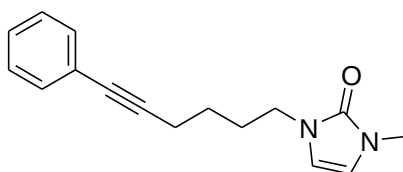
the crude product was purified using flash column chromatography (MeOH/DCM) to obtain the final product as a yellow solid. Column conditions, dark yellow ionic liquid (60 mg, 75%) ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.19 (s, 6H), 2.40 (s, 2H), 4.08 (s, 3H), 4.36 (s, 2H), 7.31-7.34 (m, 4H), 7.39-7.42 (m, 3H), 10.17 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 25.3 (CH₃), 30.3 (CH₂), 35.7 (C), 37.1 (CH₃), 58.3 (CH₂), 84.6 (C), 85.3 (C), 122.6 (C), 123.0 (CH), 123.2 (CH), 126.6 (CH), 128.4 (CH), 128.5 (CH), 128.8 (CH). ESI-MS [$\text{C}_{17}\text{H}_{21}\text{N}_2\text{I} - \text{I}$] $^+ = 252.3$.

1-(2,2-Dimethyl-5-phenyl-pent-4-ynyl)-3-methyl-1,3-dihydro-imidazol-2-one (16)



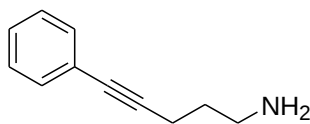
Standard procedure for the formation of the dienes using **15** (0.020 g, 0.050 mmol) led to the minimal formation of this decomposition by-product as a yellow oil (8.0 mg, 50%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.09 (s, 6H), 2.36 (s, 2H), 3.25 (s, 3H), 3.59 (s, 2H), 6.15 (d, $J = 2.9$ Hz, 1H), 6.36 (d, $J = 2.9$ Hz, 2H), 7.28-7.30 (m, 3H), 7.40-7.43 (m, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 25.3, 30.5, 30.6, 36.7, 52.2, 83.2, 87.3, 110.9, 111.6, 123.7, 127.8, 128.2, 131.5, 153.9.

Methyl-3-(6-phenyl-hex-5-ynyl)-1,3-dihydro-imidazol-2-one (17)



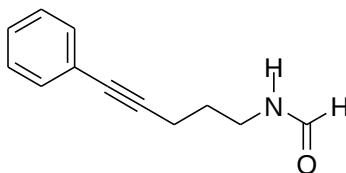
Standard reaction conditions for cyclization using **5f** (37 mg, 0.10 mmol). Oxidized product was obtained as a fine solid (0.010 g, 39%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.59-1.66 (m, 2H), 1.71-1.88 (m, 2H), 2.45 (t, $J = 7.0$ Hz, 2H), 3.25 (s, 3H), 3.66 (t, $J = 7.0$ Hz, 2H), 6.17 (d, $J = 13.8$ Hz, 1H), 6.18 (d, $J = 13.8$ Hz, 1H), 7.27-7.29 (m, 3H), 7.37-7.39 (m, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 19.0, 25.6, 28.7, 30.3, 40.0, 81.0, 89.5, 109.9, 111.3, 123.8, 127.6, 128.2, 131.5, 153.3. ESI=MS [$\text{C}_{16}\text{H}_{18}\text{N}_2\text{O}$] + 1 = 255.2.

5-Phenyl-pent-4-ynylamine (18)



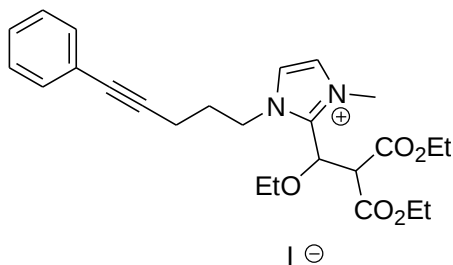
Formed using 2.0 equiv. of KOtBu and 1.0 equiv. of acrylonitrile added at 0°C degrees and heated to 70 °C with **5e**. Isolated using column chromatography and a gradient of 10-100% MeOH/DCM. ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.74 (quintet, $J = 6.9$ Hz, 2H), 2.17 (s, 2H)*, 2.48 (t, $J = 7.0$ Hz, 2H), 2.87 (t, $J = 6.8$ Hz, 2H), 7.27 (m, 3H), 7.38 (m, 2H). ESI-MS $[\text{C}_{11}\text{H}_{13}\text{N}] + 1 = 160.1$.

N-(5-Phenyl-pent-4-ynyl)-formamide (19)



Standard procedure for the formation of the dienes from **5a** followed by aqueous acid workup (5 mL of 1M HCl) and then neutralization with addition of base (5 mL of 1M KOH) and extraction with ether led to the minimal formation of this decomposition by-product as a yellow oil (8 mg). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.86 (quintet, $J = 6.9$ Hz, 2H), 2.50 (t, $J = 6.9$ Hz, 2H), 3.50 (q, $J = 6.6$ Hz, 2H), 5.72 (br, 0.5H??), 7.28-7.30 (m, 3H), 7.37-7.40 (m, 2H), 8.20 (s, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 17.0, 28.2, 37.5, 81.6, 88.6, 123.5, 127.9, 128.3, 131.5, 161.2. ESI-MS $[\text{C}_{12}\text{H}_{13}\text{NO}] + 1 = 188.2$.

2-(1-Ethoxy-2,2-bis-ethoxycarbonyl-ethyl)-1-Methyl-3-(5-phenyl-pent-4-ynyl)-3H-imidazol-1-ium iodide (20)



5e (0.500 mmol) was stirred at 0 °C with KO^tBu (1.00 mmol) for twenty minutes. Diethyl (ethoxymethylene) malonate (0.500 mmol) was then added and the reaction left to stir at room temperature for one hour. The crude reaction mixture was filtered and concentrated *in vacuo*, then purified using column chromatography (0-2% MeOH/DCM) to give the product as a yellow oil. ¹H NMR δ_H (400 MHz, CDCl₃) 1.16 (t, *J* = 7.0 Hz, 3H), 1.18 (t, *J* = 7.1 Hz, 3H), 1.23 (t, *J* = 7.1 Hz, 3H), 2.21-2.30 (m, 2H), 2.59-2.71 (m, 2H), 3.55-3.64 (m, 2H), 4.06-4.13 (m, 2H), 4.11 (d, *J* = 10.5 Hz, 1H), 4.14 (s, 3H), 4.21-4.28 (m, 2H), 4.56-4.76 (m, 2H), 5.36 (d, *J* = 10.0 Hz, 1H), 7.27-7.31 (m, 3H), 7.38-7.41 (m, 2H), 8.09-8.12 (m, 2H). ¹³C NMR δ_C (100 MHz, CDCl₃) 13.8 (CH₃) 13.9 (CH₃) 14.8 (CH₃) 16.5 (CH₂) 29.2 (CH₂) 37.1 (CH₃) 47.9 (CH₂) 55.9 (CH) 62.7 (CH₂) 63.1 (CH₂) 68.4 (CH₂) 69.9 (CH) 82.8 (C) 86.9 (C) 123.0 (C) 123.5 (CH) 125.9 (CH) 128.1 (CH) 128.2 (CH) 131.6 (CH) 141.2 (C) 165.2 (C) 165.3 (C). ESI-MS [C₂₅H₃₃N₂O₅I – I] + = 441.3.

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Chapter 3: Histidine as a non-toxic ligand for click chemistry

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3.1 Introduction

Copper catalyzed azide alkyne cycloaddition (CuAAC) chemistry has proved to be a powerful chemical biology tool since its first concurrent but independent application by Sharpless[1] and Meldal [2]. It has a broad set of applications within, for example, the domains of proteomics[3] and metabolomics[4]. The copper is often introduced as Cu(II) and reduced *in situ* to generate the active Cu(I) species, which is often caged by ligands that maintain its oxidation state. While copper toxicity is often cited as being a limiting factor for these studies in the literature, to date no study on the adverse effects of copper in these types of transformations has been performed.

Preliminary work [5] by our group on copper uptake and its effect on cellular metabolism was done using coherent anti-Stokes Raman scattering (CARS) microscopy: a tool that can tune into CH₂ stretches which are abundant in lipids making it an excellent tool for monitoring lipid metabolism in a relatively non-invasive manner[6-8]. Copper toxicity, uptake and effects on lipid metabolism were found to be directly affected by the source of copper and the length of exposure. Despite the metabolic flux exhibited by the cells, we wanted to know whether or not these effects were severe enough to limit the application of these findings. To expand on the observation that copper toxicity appeared to decrease when bound to histidine, a naturally occurring ligand for copper, we sought to apply this to a methodology for CuAAC on live cells. Since human systemic copper levels are regulated primarily by the liver, a hepatoma cell line was chosen to demonstrate the effects of copper uptake on cell viability.

It was our hypothesis that the toxicity of copper in the cells would be largely dependent on its complexation character, and that by introducing non-toxic ligands, the toxicity of this transformation could be largely reduced. It has been well reported that the kinetics of the CuAAC reaction are largely ligand dependent, and to date the polydentate ligand tris((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)amine (TBTA)[9] has been one of the most widely used ligands due to its favourable kinetics. We believed the naturally occurring amino acid L-histidine would be sufficient to allow the CuAAC reaction to occur, and would serve as a less toxic ligand, compared to EDTA, TBTA, sulfonated bathophenanthroline (SBP), and free copper. We demonstrate this methodology both *in situ* and *in vivo* using

human hepatoma 7.5 (Huh 7.5) cells as an alternative approach to SPAAC for cell surface labelling in live cells.

3.2 Results

3.2.1 Toxicity Assay & Test Reactions

To determine the relative values of toxicity for various copper-ligand complexes, a metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) was performed, the results for which are outlined in Table 3.1 This work and the subsequent CARS imaging were performed by Dr. David Kennedy, a research assistant from our group, and is presented herein to provide a more complete understanding of the overall project results.

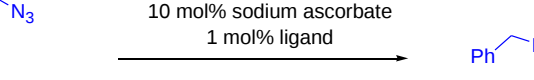
Table 3-1 Toxicity, increase in lipid content as determined by CARS, and metal uptake of copper complexes in Huh7.5 cells.

Ligand	IC₅₀ (μM ± 5%)	Metal Uptake after 24 hrs (ng/10⁶ cells ± 20)
mock	-	-
none ^a	100	120
histidine	>1000	195
EDTA	150	185
TBTA	15	170
SBP	1	250

a- These results are for CuSO₄ with no-exogenous ligands. In each subsequent experiment, CuSO₄ was used as the copper source to which the ligands were added. CuCl₂ was also tested and yielded similar results both for ligand free and for ligated samples.

Additional work performed within our group demonstrated that under reaction conditions involving catalytic loading of copper and sodium ascorbate in 2:1 DMSO/H₂O illustrated that histidine can serve as a ligand in the CuAAC reaction between benzyl azide and phenyl acetylene with yields comparable to the widely accepted TBTA ligand. Additionally, in the absence of any ligand the yield suffered over 24 hours. This work was performed by Craig S. McKay, a Ph.D. candidate from our group, and is presented to provide a more complete understanding of the results of the project.

Table 3-2 Effect of ligand on copper (I) catalyzed azide-alkyne cycloadditions ^a



1 mol% CuSO₄
10 mol% sodium ascorbate
1 mol% ligand

2:1 DMSO/H₂O, 24 hrs

Ligand	Yield % ^b
none	23
histidine (1)	90
EDTA	18
TBTA	93
SBP	88

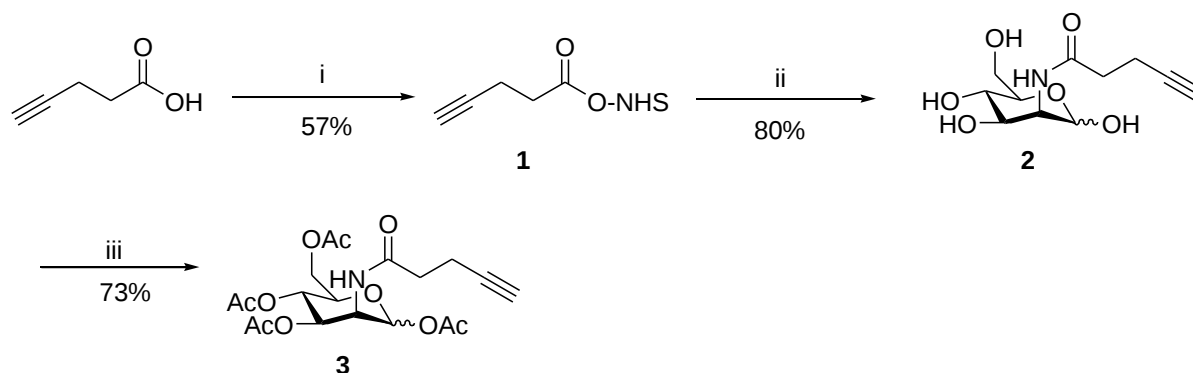
a - Reactions were performed in scintillation vials with no effort to exclude oxygen either than capping with screw cap.

b - Yields were determined by gas chromatography.

While these results imply that CuAAC with histidine as the ligand is a valid methodology, we sought to test whether or not we could achieve live cell images employing this system. Thus, we set out to determine whether or not Cu(his)₂ could be applied as an alternative to SPAAC for biological labelling studies using azide labelled sugars in Huh 7.5 cells.

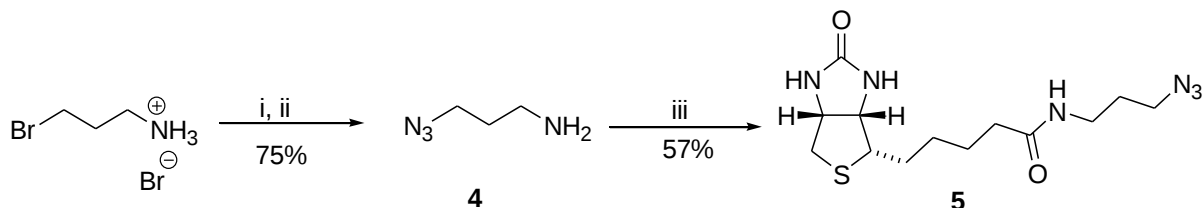
3.2.2 Synthesis of Reagents for Live Cell Imaging

The synthesis of sugar probe **3** has been previously reported elsewhere and was replicated with minor changes to pre-existing methodology [4,10,11] (see experimental p.p. 97-99 for details). The synthetic route was three overall steps from commercially available starting materials and had an overall yield of 33%. It is described in Scheme 3-1.



Scheme 3-1 Synthetic route towards sugar probe **3**: i)NHS, DCC, dioxane/EtOAc; ii)Et₃N, D-mannosamine•HCl; iii)Ac₂O, pyridine.

Likewise, the synthesis of biotin azide **5** has been previously reported in the literature [12] [13] [14] (see experimental for details p.p. 99-100) and was performed (Scheme 3-2) from commercially available starting materials over two steps in 43% overall yield.

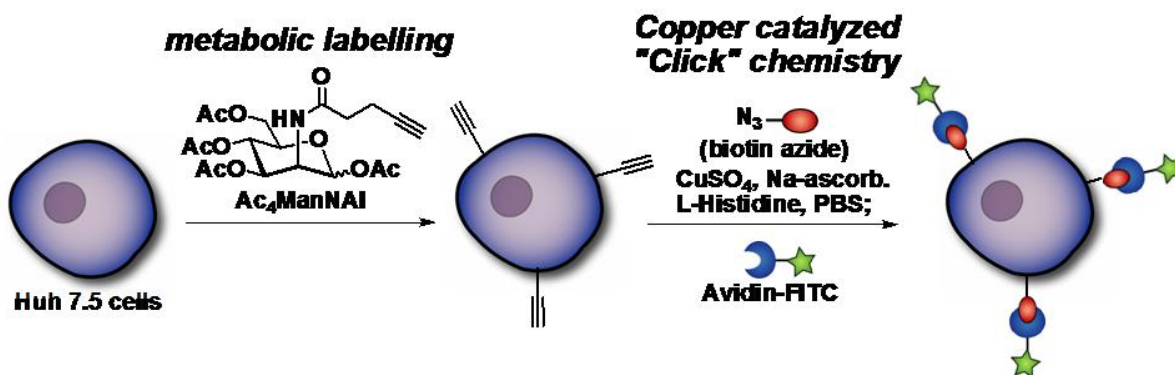


Scheme 3-2 Synthesis of (+)biotin azide **5**: i) NaN_3 , H_2O , 18h; ii) KOH , $0\text{ }^\circ\text{C}$, Et_2O ; iii) (+)-biotin-NHS, Et_3N .

TBTA was synthesized in a one step procedure previously reported [9] and tetra amino methyl rhodamine azide (TAMRA) was synthesized from pre-existing literature [15] by combining the rhodamine fluorophore to **7** and used as a mixture of isomers.

3.2.3 Biological Sample Trials

Live cells were imaged post-treatment without being fixed. The strategy is described in Scheme 3-3 and successful trials are depicted below from trials II and VI in Figure 3-1. Live cells were treated according to the conditions summarized in Table 3-3 over various trials.



Scheme 3-3 Strategy applied towards the metabolic labelling of Huh 7.5 cells. In a two step labelling process, cells are first treated with Ac_4ManNAI 3 and incubated for three days. Cells were then subjected to standard click chemistry conditions with histidine as a ligand, using biotin (red oval) azide 5 to tag cell surface glycoproteins expressing the metabolized sugar. Finally, treatment with streptavidin-FITC (blue half moon/green star respectively) permitted the labelling of the tagged cells and visualization using fluorescence and confocal microscopy. Image produced by Craig McKay and used with permission.

Table 3-3 Conditions for cell surface labelling experiment from trial to trial. All samples were seeded on borosilicate Lab-Tek chambers (VWR, Mississauga, ON) with or without collagen, then treated with the sugar **3** (50 μ M) and left to metabolize this for three days before treatment with click reagents ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (50 μ M), Na(L)-ascorbate (100 μ M), L-his (100 μ M)) as well as director two step introduction of a fluorophore. Cells were imaged and the number of cells was also assessed qualitatively.

<i>Trial</i>	<i>Fluorophore</i>	<i>Collagen</i>	<i>Fluorescence</i>	<i>Cell Viability</i> ^a
I	Cell count 7 (50 μ M) PBS washes	No	Non-specific labelling, high background	Moderate
II	5 (50 μ M), Streptavidin- FITC (1 μ g/mL), PBS washes	No	Cell surface	Low
III	5 (50 μ M), Streptavidin- FITC (1 μ g/mL). Media with serum ^b washes	No	None	Low
IV	5 (50 μ M), Streptavidin- FITC (1 μ g/mL), PBS/media ^c washes	Yes	None	High
V	5 (50 μ M), Streptavidin- FITC (1 μ g/mL) PBS washes	Yes	None	Low
VI	5 (50 μ M), Streptavidin- FITC (1 μ g/mL) PBS/media ^c washes	No	Cell surface	High

a – Cell viability was qualitatively compared from sample to sample

b – Cells were washed with media containing 10% serum

c – Cells were washed with PBS prior to fluorophore treatment, then washed once with PBS, once with media and imaged in media

3.2.4 Biological Sample Imaging

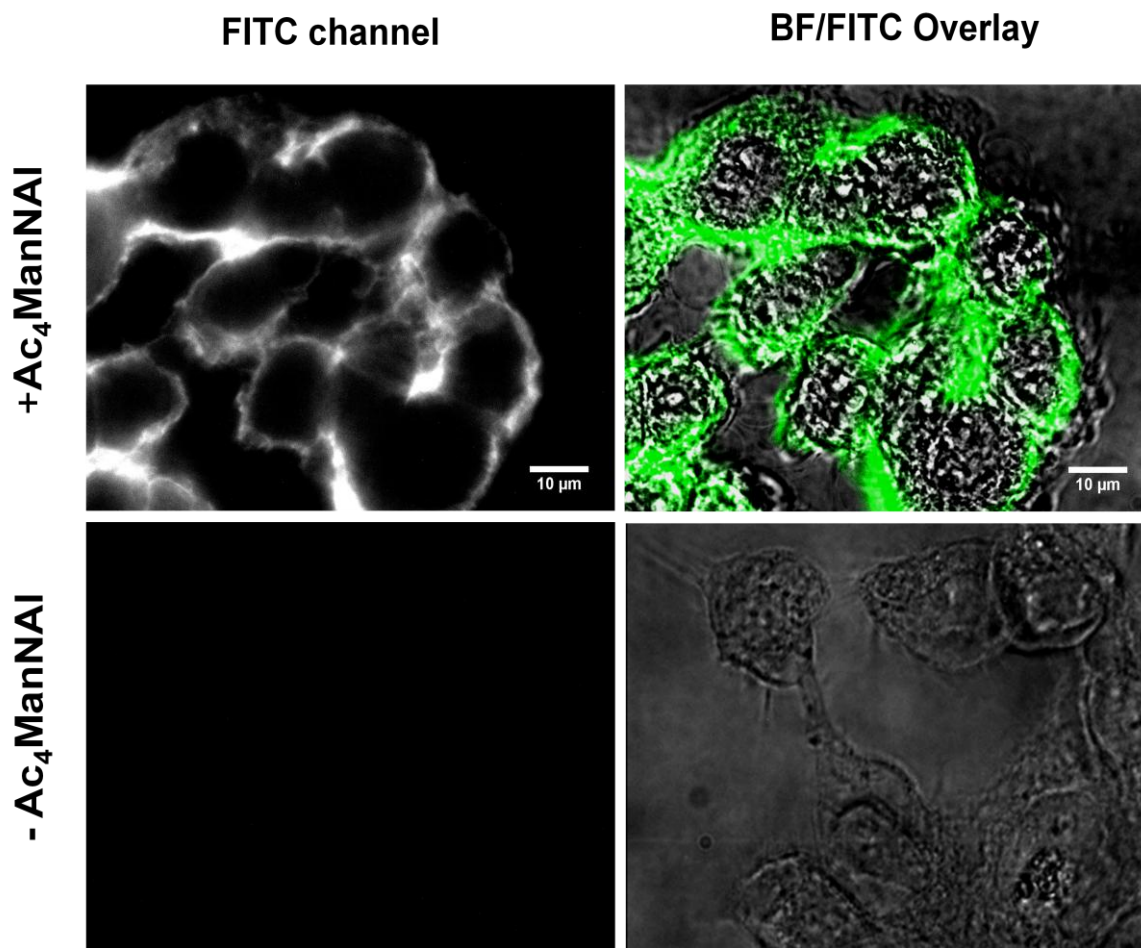


Figure 3-1 FITC and overlay of FITC and broad field channel images with false colouring (green) of live cell copper mediated click chemistry using histidine as a ligand in presence or absence of alkyne sugar **3**. Trial II depicted (see Table 3-3)

In addition to cells treated as described in Scheme 3-3 (images in Figure 3-1), controls were also performed during each trial. TBTA (100 μ M) in place of histidine was performed as a comparative sample. No streptavidin-FITC was also performed as a negative control for the establishment of background and autofluorescence.

3.3 Discussion

3.3.1 Toxicity Assay & Test Reactions

MTT assays measure a cell's ability to metabolize the exogenous metabolite to formazan. Both the precursor and metabolized compound have different characteristic absorbances by UV/Vis and thus a colorimetric ratio can be determined which can be extrapolated to living versus dead cells. The results obtained in Table 3-1 demonstrate that a lower IC₅₀ (inhibitory concentration for 50% of cells) value was obtained for the cells treated with the more traditional complexes (EDTA, TBTA) over those treated with l-histidine, promoting further investigation into the viability of this strategy.

Additionally, the *in vitro* click chemistry reactions (Table 3-2) performed by reacting benzyl azide and phenyl acetylene to provide the resultant triazole suggest that the efficacy of using L-histidine as a ligand in the CuAAC reaction is not hampered when compared to using the more traditional ligands, and was better at generating product when compared to the ligand-free conditions. However, these reactions were not performed under standard solvent conditions, and thus might not provide an accurate picture to *in vivo* results. These conditions were chosen to ensure complete solubility of the reagents. It was proposed that the best way to test the effectiveness of the copper-histidine complex would be to use an *in vivo* metabolic labelling strategy.

3.3.2 Synthesis

To perform a metabolic labelling experiment, a biological probe was needed to label the cells. The choice of probe was made based upon pre-existing literature, and was achieved in comparable yields with minor adjustments made to purification techniques (references in section 3.2.2, discussed in experimental). This sugar probe was chosen based on the fact that it was incorporated via the sialic acid metabolic pathway, one that is conserved in the cell

line we had readily available. Also, it was chosen because of its relative ease of synthesis relative to alternative sugar probes. Biotin azide **5**, the TBTA ligand **6**, and rhodamine azide **7** were also all synthesized using the pre-existing literature procedures referenced in Chapter 3.2.2.

3.3.3 Cell Treatments and Imaging

The Huh 7.5 cell line is a second generation line derived from human hepatoma cells, and are used in our lab as a primary cell line for the study of HCV. These cells possess a sialic acid pathway and were chosen for both their convenience and relevance to our research group. Cells were grown to (near confluency) and seeded on CARS slides at a concentration of 6.7×10^5 cells/mL and incubated overnight prior to treatment with the modified sugar, or no treatment for mock cells. To obtain a high level of incorporation of the unnatural mannosamine **3**, cells were incubated for three days as previously reported by other groups. At this time, cells were treated and either fixed using a formaldehyde solution or visualized directly after treatment.

The images obtained initially using rhodamine as the labelling agent provided poor contrast from sample to sample (images not included). The inefficiency in washes even when employing an excessive number of washes was the source of this error. It has been established previously in our group that rhodamine can be difficult to remove without employing conditions that would be too harsh for our purposes (i.e. KOH/DMSO mixture). Thus, it was decided that an alternate two-step approach might facilitate better contrast resolution.

Instead of directly labelling the cells with a fluorophore conjugated to an azide, the implementation of a biotin linked to an azide would provide more specific labelling. It has been well established in the literature that the conjugate fluorophore/streptavidin unit is readily removed with PBS buffer washes. Likewise, the biotin streptavidin interaction is one of the strongest non covalent interactions known in nature ($K_d \approx 10^{15}$ [16]) thus it was unlikely that any false negative results would be observed. Cells that were visualized post fixation provided good images and suggested the click chemistry in the presence of copper

histidine was successful, however to test our hypothesis it was necessary to image the cells while still living.

Treatment under conditions that were used for the fixed samples was a fairly long process and it was not shocking to observe that upon completion, most of the cells had washed off. While it was encouraging to discover that the labelling worked selectively in the appropriate samples, the issue with cells lifting provided poor resolution for some of the negative controls and thus a replicate experiment was required. To determine whether or not this was a consequence of manipulation post treatment or whether or not it was due to toxicity was determined by examining mock samples. Even in the untreated samples cells appeared to be lifting and it was deemed a consequence of the strategy rather than the treatments.

Using rat tail collagen IV prior to the seeding of cells (trials IV and V) was considered as a potential solution for improving the cells' adherence to the surface. During treatment, it was observed that the cell count was indeed larger in the samples on surfaces pre-treated with collagen, however, no differential fluorescence was observed between samples treated with all the necessary reagents and the negative control samples (no **3**, or no streptavidin-FITC). It was believed that the collagen was preventing uptake of the sugar, and indeed, it has been previously reported that collagen can disrupt certain cell receptor function [17]. An alternative suggestion would be that the collagen is preventing cell surface peptidoglycans from interacting with the click reagents, since Dommerhalt *et al.* have recently demonstrated that cell imaging under SPAAC is achievable on a collagen surface [18], albeit not with Huh 7.5 cells so the results are not necessarily translational. Regardless, it was decided that lower cell count would have to be a sacrifice inherent to the success of the experiment. Again, it is important to note that low cell count is due to the experimental conditions, and not the toxicity of the reagents.

A modified preparation procedure was developed where washes were performed using media containing serum rather than PBS buffer. However, these results gave no labelling in any cell samples. It was hypothesized that the concentration of albumin present in the serum was potentially sequestering any copper and out-competing the Cu-his₂ complex for available catalyst and thus no click reaction was possible. Therefore, we decided to perform

all washes with media to satisfy the cells in their environment. However, we also decided to perform the actual click reaction in PBS buffer. This was only a ten minute treatment therefore it was believed that the cells could tolerate such conditions.

Cells washed with pre-warmed media (serum free) for all steps subsequent the click reaction step (secondary biotin/streptavidin labelling) provided images similar in appearance to those found with only PBS washes. An interesting observation was made over the course of the imaging experiment: over time (>1h) cells that initially showed phenotypic cell surface imaging changed in appearance. Eventually they showed internal fluorescence above background levels, while no longer exhibiting characteristic surface labelling. This was an intriguing enough observation with regards to internalization of cell surface glycoproteins that it prompted a time based study to confirm these results were due to some cellular mechanism and not merely photo-bleaching. This research is currently ongoing within our group and is not the focus of this chapter.

Trial II provided the images found in Figure 3-1 and displays characteristic halo effect around the cells in the samples with all the reagents. In the absence of **3** there is no such observable fluorescence. This suggests that the click chemistry is working on the cell surface specifically with the cells that have metabolized and incorporated **3**.

Intriguingly, control samples treated with TBTA **6** as a ligand, however, were giving a similar effect. It was postulated that while histidine might be a non-toxic ligand during the CuAAC reaction, it was not binding as tightly as the multi-dentate **6** which is characteristic for its ability to stabilize the Cu(I) active oxidation state. Therefore, it was proposed that addition of more reducing agent (a non-toxic reagent) could circumvent this problem and maintain a steady level of Cu(I) in the wells. This hypothesis showed to be accurate on fixed cells was also tested successfully on live cells.

Additional optimization by Dr. David Kennedy and Dana Danielson during the preparation of this work has shown that additional FITC was required to obtain replicable results that supported the preliminary ones found herein including longer incubation times of 20 minutes for the click reaction, a 100-fold excess of sodium ascorbate relative to copper and a four-fold increase in the concentration of labelling dye. Sodium ascorbate in such high

concentrations was shown to be non-toxic to the cells, though further optimization is necessary.

3.4 Summary & Future Directions

L-histidine was successfully employed in live cell imaging using CuAAC on Huh 7.5 cells that were incubated with an alkyne sugar **3** that has been previously reported to be metabolically incorporated onto cellular surfaces. Combining these results with previous observations including reduced cellular toxicity, it has been shown that L-histidine can serve as a safer ligand, with comparable efficiency to traditional choices. This strategy could be particularly amenable to living model organisms (e.g. mice; murine models).

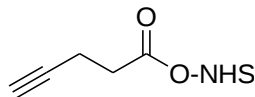
It is worth noting that during the finalization of this work, an article was published detailing a similar approach to reducing cellular copper toxicity[19]. A TBTA analogue, bis (*tert*-butyltriazoly) ligand, was discovered through a screen and used due to its optimal solubility and reactivity. The group demonstrated successful click reactions on developing zebrafish embryo surfaces in a similar metabolic labelling fashion. It is our opinion that this work further supports the desire for ligands that permit CuAAC in a biological milieu.

Application of this methodology on live cells for the study of lipid metabolism during HCV infection and viral life cycle in combination with Coherent Anti-Stokes Raman Scattering (CARS) microscopy is currently under investigation.

3.5 Experimental

3.5.1 Synthesis of Compounds for Imaging

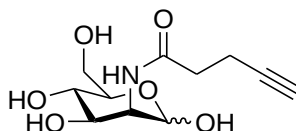
N-succinimidyl-4-pentynoate (**1**) [4][11]



To a stirred solution of 4-pentynoic acid (500 mg, 5.1 mmol, 1.0 equiv.) and *N*-hydroxysuccinimide (590 mg, 5.1 mmol, 1.0 equiv.) in a 1:1 solution of dioxane/ethyl acetate (60 mL) at 0 °C was added *N,N'*-dicyclohexylcarbodiimide (1.05 g, 5.1 mmol, 1.0

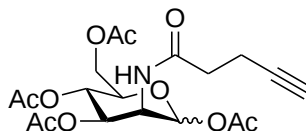
equiv.) in one portion. The resulting mixture was stirred at room temperature for five hours. The DCU formed was filtered off and the filtrate was concentrated *in vacuo*. The residue was dissolved in ethyl acetate (300 mL) and the solution was washed with 5% NaHCO₃ (2 X 75 mL) and brine (75 mL). The organic layer was separated, dried over sodium sulphate, filtered, and concentrated *in vacuo*. Recrystallization from DCM/pentanes provided the compound as white crystals (570 mg, 57%). ¹H NMR δ_H (400 MHz, CdCl₃) 2.05 (t, *J* = 2.6 Hz, 1H), 2.61 (dt, *J* = 7.0 Hz, 2.6 Hz, 2H), 2.84 (s, 4H), 2.88 (t, *J* = 7.0 Hz, 2H). ¹³C NMR δ_C (100 MHz, CdCl₃) 14.1, 25.6, 30.3, 70.0, 80.8, 167.0, 168.9.

N-4-Pentynoylmannosamine (mixture of α/β anomers) (2) [11][20]



A mixture of d-mannosamine hydrochloride (276 mg, 1.28 mmol, 1.0 equiv.), **1** (250 mg, 1.28 mmol, 1.0 equiv.) and triethylamine (535 μ L, 3.84 mmol, 3.0 equiv.) in DMF (15 mL) was stirred at room temperature overnight. The mixture was concentrated *in vacuo* and the residue was purified using flash column chromatography (8:1 CHCl₃/MeOH) to give **2** as a slightly impure* white solid (242 mg, 80%). ¹H NMR δ_H (400 MHz, D₂O) 2.37 (t, *J* = 2.3 Hz, 1H), 2.47-2.62 (m, 6H), 3.38-3.43 (m, 0.4 H), 3.52 (t, *J* = 9.8 Hz, 0.4H), 3.62 (t, *J* = 9.6 Hz, 1H), 3.77-3.90 (m, 4.4H), 4.05 (dd, *J* = 9.8, 4.7 Hz, 1H), 4.35 (dd, *J* = 4.6, 1.4 Hz, 1H), 4.48 (dd, *J* = 4.4, 1.4 Hz, 0.4H), 5.03 (d, *J* = 1.6 Hz, 0.4H), 5.12 (d, *J* = 1.4 Hz, 1H). ¹³C NMR δ_C (100 MHz, D₂O) 14.3, 14.4, 34.1, 34.3, 53.2, 54.0, 60.4, 66.5, 66.8, 68.8, 69.9, 70.1, 72.0, 76.3, 83.5, 83.9, 92.9, 93.2, 175.4, 176.1.

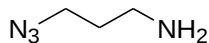
1,3,4,6-Tetra-O-Acetyl-N-4-Pentynoylmannosamine (mixture of α/β anomers) (3) [11][20]



A mixture of **2** (242 mg, 1.03 mmol, 1.0equiv.) and acetic anhydride (467 μ L, 4.91 mmol, 1.2 equiv.) in pyridine (6 mL) was stirred at room temperature overnight. The reaction

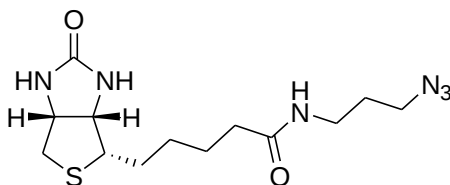
mixture was concentrated *in vacuo* and the residue was dissolved in DCM (10 mL) and washed with water (10 mL). The organic layer was dried over sodium sulphate, filtered, and concentrated *in vacuo*. The resultant residue was purified by flash column chromatography (gradient of 1:4 EtOAc/Hexanes to 1:1 EtOAc/Hexanes) to give **3** as white crystals (319 mg, 73%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.65 (s, 8H), 2.00 (s, 3H), 2.01 (s, 3H), 2.06 (s, 6H), 2.10 (s, 6H), 2.11 (s, 3H), 2.18 (s, 3H), 2.45-2.58 (m, 8H), 3.80 (ddd, $J = 9.8$ Hz, 5.1 Hz, 2.5 Hz, 1H) 4.02-4.11 (m, 3H) 4.26-4.31 (m, 2H), 4.67 (ddd, $J = 9.2$, 4.4, 1.8 Hz, 1H), 4.80 (ddd, $J = 9.1$, 4.0, 1.7 Hz, 1H) 5.03 (dd, $J = 9.9$, 4.0 Hz, 1H), 5.14-5.24 (m, 2H), 5.32 (dd, $J = 10.2$, 4.4 Hz, 1H), 5.86 (d, $J = 1.7$ Hz, 1H), 6.04 (d, $J = 1.7$ Hz, 1H), 6.08 (d, $J = 9.2$ Hz, 1H), 6.12 (d, $J = 9.0$ Hz, 1H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 15.0, 15.1, 20.6, 20.7, 20.7, 20.7, 20.8, 20.9, 35.4, 25.5, 49.3, 49.5, 61.8, 61.9, 65.2, 65.4, 68.8, 70.1, 70.1, 70.5, 71.3, 73.5, 82.5, 82.6, 90.6, 91.7, 168.1, 168.4, 169.3, 170.1, 170.2, 170.5, 170.5, 171.2, 171.7.

3-aminopropyl-1.azide (**4**) [12,14]



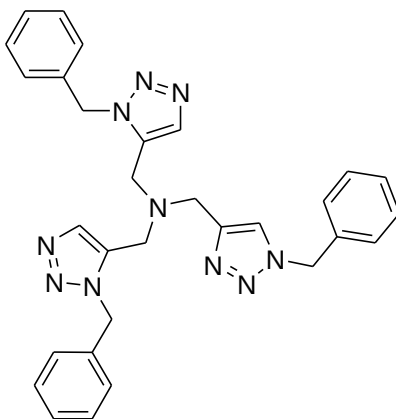
To a solution of 3-bromopropylamine hydrobromide (3.2 g, 14.6 mmol, 1.0 equiv.) in water (10 mL) was slowly added sodium azide (3.2 g, 49.2 mmol, 3.4 equiv.) as a solution in water (15 mL). The resulting solution was allowed to reflux gently overnight. After cooling, two thirds of the water was removed *in vacuo* and the residue was dissolved in diethyl ether (50 mL). The biphasic mixture was cooled to 0 °C and KOH (4.0 g) was added slowly. The resultant phases were separated, and the aqueous phase was extracted with diethyl ether (2 X 30 mL), dried over sodium sulphate, filtered and concentrated. The crude, slightly yellow oil was stored under argon at -20 °C and used without further purification (1.10 g, 75%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.24 (bs, 2H), 1.68 (quintet, $J = 6.8$ Hz, 2H), 2.75 (t, $J = 6.8$ Hz, 2H), 3.33 (t, $J = 6.6$ Hz, 2H). ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 32.3, 39.2, 49.0.

Biotin azide (5) [14] [13]



To a solution of (+)-biotin-*N*-hydroxysuccinimide ester (34 mg, 0.1 mmol, 1.0 equiv.) and triethylamine (28 μ L, 0.2 mmol, 2.0 equiv.) was added **3.9** (14.7 μ L, 0.15 mmol, 1.5 equiv.) and the reaction was left to stir at room temperature under argon overnight. The reaction was filtered and the filtrate was concentrated to dryness. The residue was purified using flash column chromatography (10% MeOH/EtOAc) to give the product as a white solid (19.2 mg, 57%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.47 (q, J = 7.6 Hz, 2H), 1.68-1.74 (m, 2H), 1.76-1.81 (m, 2H), 2.18-2.26 (m, 2H), 2.93 (dd, J = 12.9, 4.8 Hz, 1H), 3.16-3.2 (m, 2H), 3.32-3.39 (m, 2H), 4.34 (dd, J = 6.9, 4.4 Hz, 1H), 4.53 (dd, J = 7.2, 4.4 Hz, 1H), 5.04 (s, 1H), 5.63 (s, 1H), 5.90 (s, 1H) ^{13}C NMR δ_{C} (100 MHz, CDCl_3) 25.4, 27.9, 28.0, 28.7, 35.9, 37.0, 40.5, 49.3, 55.2, 60.3, 61.9, 164.2, 172.8. ESI-MS [$\text{C}_{13}\text{H}_{22}\text{N}_6\text{O}_2\text{S}$] + 1 = 327.1

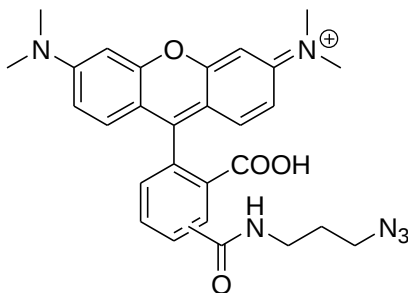
Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (6) [9]



Tripropargylamine (3.0 g; 23 mmol) in acetonitrile (40 mL) was treated sequentially with benzyl azide (13.6g, 100 mmol), 2,6-lutidine (2.43g, 23 mmol), and $\text{u}(\text{MeCN})_4\text{PF}_6$ (1.3 mol %). Upon addition of the copper salt, the reaction warmed and was cooled in an ice bath. After the mixture was stirred at room temperature for 3 days, a white crystalline solid precipitated from the reaction. Filtration and washing with cold acetonitrile afforded fine

light pink powder (7.6g, 63%). ^1H NMR δ_{H} (400 MHz, CdCl_3) 3.70 (s, 6 H), 5.49 (s, 6 H), 7.26 (m, 6 H), 7.34 (m, 9 H), 7.67 (s, 3H). ^{13}C NMR (100 MHz, CdCl_3) 47.1, 54.2, 123.8, 128.0, 128.8, 129.1, 134.7. ESI-MS $[\text{C}_{27}\text{H}_{30}\text{N}_{10}] + 1 = 531.0$

Rhodamine azide [15] (mixture of regioisomers, performed by Craig McKay) (**7**)



Carboxytetramethylrhodamine (TAMRA) as a mixture of regioisomers, prepared from commercially available starting materials. (43.0 mg, 0.1 mmol, 1.0 equiv.) was dissolved in DMF (5 mL) then TSTU (33.1 mg, 0.11 mmol, 1.1 equiv.) and triethylamine (195 μL , 1.4 equiv., 15 mmol) were added and the solution was stirred for 2 hours at room temperature. **4** (30 μL , 0.2 mmol, 3.0 equiv.) was then added and the reaction was stirred for 24 hours at room temperature. The solution was concentrated *in vacuo* and the residue purified using flash column chromatography (0-20% MeOH/DCM) to give the product as a dark red solid. ^1H as reported in literature.

3.5.2 Cell Culture & Fluorescence Microscopy (TRIAL II) [11,20][10]

Hepato cellular carcinoma (Huh-7.5) cells were seeded 6.7×10^5 cells/mL borosilicate Lab-Tek chambers (VWR, Mississauga, ON) 2 mL of LB media and incubated with or without **3** (50 μM) for three days. The cells were washed with PBS (3X) and treated with the following reagents for 10 minutes at 37 $^\circ\text{C}$ in 2 mL of PBS: sodium ascorbate (100 μM), $\text{CuSO}_4 \cdot \text{H}_2\text{O}$ (50 μM), L-histidine (100 μM), and **5** (50 μM). The cells were washed with PBS (3X) and blocked with 1% BSA for 20 minutes at r.t. and then stained with FITC-streptavidin (1 $\mu\text{g/mL}$ in PBS). The cells were incubated in the dark for 30 minutes at r.t. then washed with PBS (2X), media (1X) and then imaged live-cell in 1 mL of phenol-red free DMEM. Imaging was done with an Olympus 1X81 spinning-disk confocal microscope equipped with a FITC filter (Semrock, Excitation: 465-499nm, Emission: 516-556 nm) and a

Photometrics (Coolsnap ES) camera using 60x magnification. Images were taken of samples and controls (no **3**) using both bright-field and the FITC channel (10 second exposure). Image processing was done using ImageJ software, using the Colour Merge plugin to apply pseudocolour to FITC channel images and merging with bright-field images. The same pixel-intensity ranges were applied and displayed for all images taken.

3.6 References

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Chapter 4: Optimizing the synthesis of an unnatural tyrosine analogue

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4.1 Introduction

Unnatural amino acids (UAAs) expand the breadth of chemical transformations that can be accomplished by proteins. As discussed in Chapter 1.4.1, they are capable of changing the physical properties of the protein into which they are incorporated. In reality, the limit of versatility provided by UAAs is truly set by the researcher's ingenuity and imagination, thus making them a most valuable tool for chemical biologists and their study of biological systems.

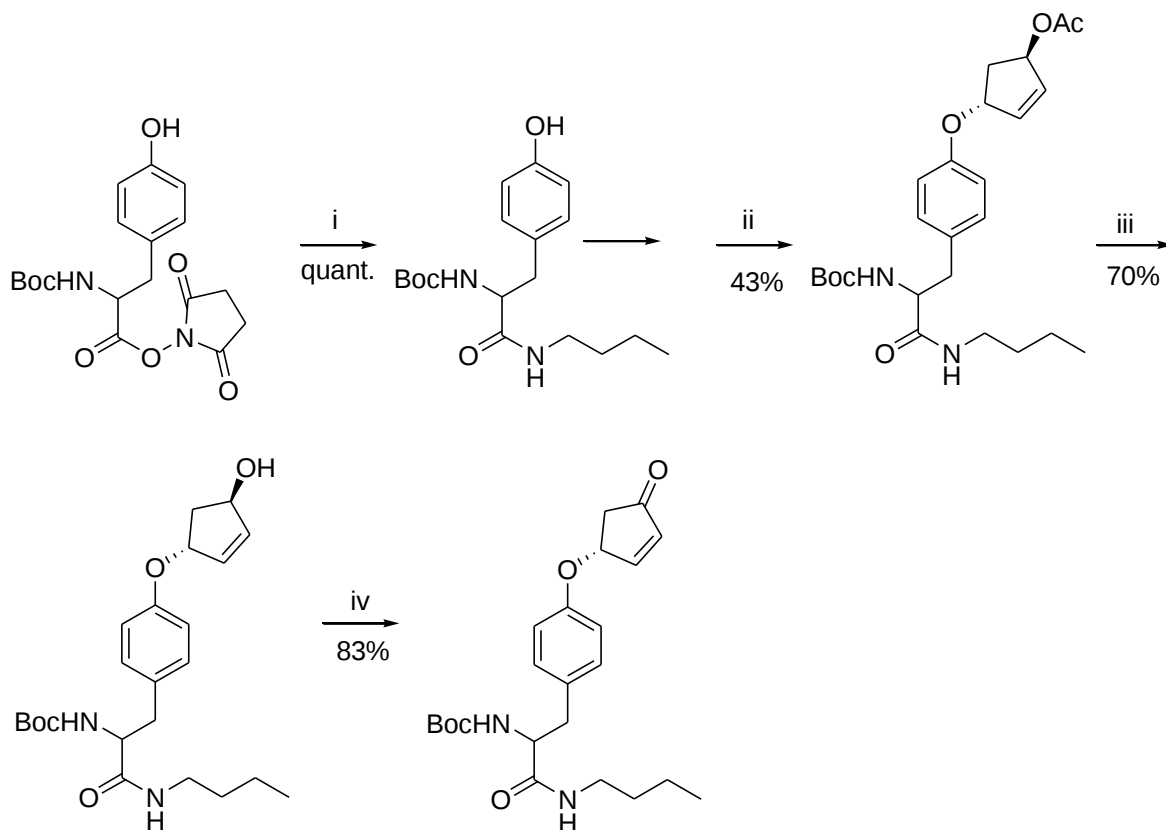
Also, as mentioned in Chapter 1.4.2, incorporation of these UAAs into biological systems has been achieved within several organisms with high fidelity. The different methods for incorporation, including auxotrophic bacteria and the site specific incorporation using amber stop codons and engineered aaRS and tRNA pairs have both been well established and show tolerance to a wide variety of chemical structures, allowing researchers to design diverse series of UAAs.

Our lab sought to design and synthesize a UAA to take advantage of the aforementioned benefits of research using UAAs. We designed a tyrosine analogue with an α,β -unsaturated ketone moiety within a five membered ring. Computational studies performed by Gianni Lorello suggested that this particular α,β -unsaturated ketone was optimal for reaction with nitrones, a functional group that our group has performed a large degree of research on in terms of application within aqueous media, while being less reactive towards nucleophilic addition of thiols. Thus it was believed that we could incorporate this UAA into a growing peptide by either of the two methods above, for further modification through a [3+2] nitrone-alkene cycloaddition, which could allow for site specific incorporation of fluorophores, biotin, or other chemical functional groups.

While the synthesis of this UAA was carried out previously by Dr. Bojana Rakić, issues regarding its classification as a UAA were brought to attention, and this chapter discusses an alternate route towards the synthesis of the UAA for the eventual goal of incorporation into, and study of, proteins.

4.1.1 Unnatural Tyrosine Analogue

Our lab has successfully synthesized an UAA tyrosine analogue containing a 5-membered α,β -unsaturated ketone moiety.[1] This chiral analogue was synthesized in 26% overall yield over five steps as the butyl amide (Scheme 4-1).



Scheme 4-1 First synthetic pathway towards NHBoc protected UAA tyrosine analogue with butyl amide chain: i) butylamine, reflux, DCM, 1h; ii) DIAD, PPH₃, (1S,4R)-cis-4-acetoxy-2-cyclopenten-1-ol, THF, 24h; iii) LiOH, THF, 24h; iv) PDC, DCM, 7h.

Upon synthesis of the UAA analogue, further studies were performed to better evaluate the reactivity of this particular tool towards [3+2] cycloadditions in aqueous media prior, as a model system for physiological conditions.

4.1.2 Reactivity

The cyclopentenone functional group was installed to serve as a reactive partner with nitrones: 1,3-dipoles studied extensively in biological media within the research group. A 1,3-dipolar cycloaddition between the two would lead to the formation of a bicyclic system.

This particular unsaturated ketone was chosen because of its presumed relative inertness towards conjugate addition relative to other possible α,β -unsaturated ketones.

A model reaction between cyclopentenone and nitrones generated *in situ* was conducted to determine the tolerance of such a reaction. *N*-Hydroxylamine was chosen rather ambiguously, and aldehydes (R = -N(Me)₂, -OH, -OMe, -Me, -Cl, -CN, or -NO₂) of varying electronic properties were chosen for a competition reaction with benzaldehyde to derive stereoelectronic information using pseudo first order reaction conditions (i.e. cyclopentenone as limiting reagent in the rate limiting step, benzaldehyde and one of the several substituted para benzaldehyde in equal amounts and in excess) and reactions were monitored by LCMS. Upon formation of the nitrone intermediate, reaction under physiological conditions with cyclopent-2-enone would yield a ratio of products based on the reactivity of each nitrone, and this ratio is summarized in Figure 4-1 as a Hammett plot. The ρ value of -0.94 was to be expected for a concerted but unsymmetrical reaction favouring electron rich aldehydes. Explanation for the outlier p-hydroxy benzaldehyde reaction was with regards to the equilibrium existing between the alcohol and its conjugate base, which would be unable to enter the hydrophobic micelle core, thus unable to partake in the reaction. Multiple trials for R = Cl were done with different pre-incubation times (triangle = 10 minutes, circles = 2 hours).

Moreover, this suggested that the unnatural amino acid could also serve, under physiological conditions, as a reactive partner with nitrones to provide chemically modified unnatural peptides. This could result in a diverse number of amino acids, and serve as a means of introducing fluorophores or other bioorthogonal reagents post-incorporation. The reaction was replicated with the UAA and benzaldehyde as shown in Scheme 4-2.

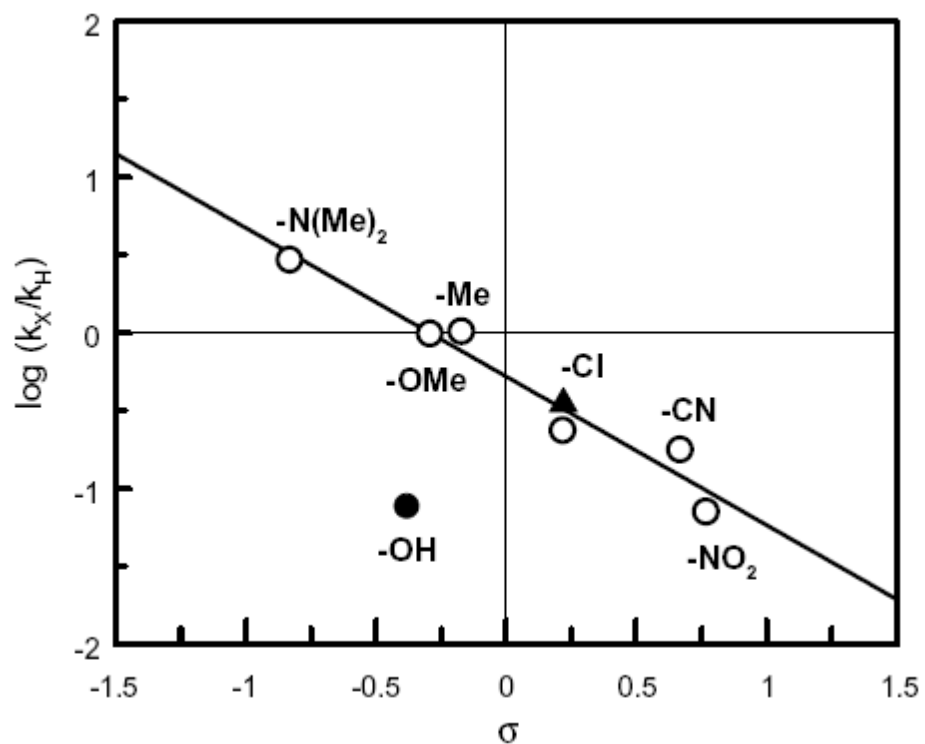
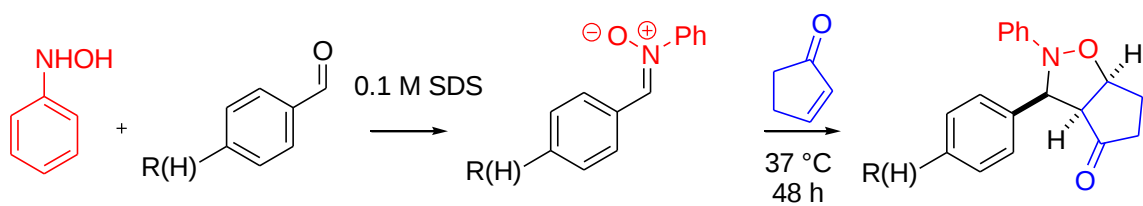
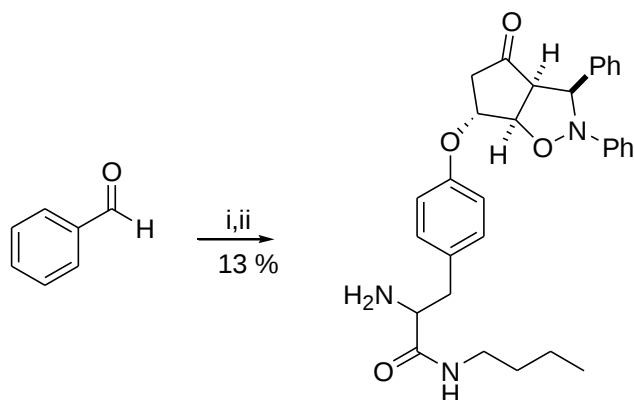


Figure 4-1 Hammett plot for the competitive model study of the UAA functionality involving cyclopentenone and a variety of nitrones in a one pot reaction. The ρ value was found to be -0.94.



Scheme 4-2 Reaction of UAA tyrosine analogue with benzaldehyde in a [3+2] cycloaddition reaction: i)PhNHOH, 0.1 M SDS, sonicate, rt, 2h; 2)cyclopent-2-enone, 37°C, 48h.

4.1.3 Issues

However, criticisms that the tyrosine analogue was not in fact a canonical amino acid were justifiably presented. The butyl group, which was employed to mimic the micellar environment presented in the literature as being requisite for improved kinetics of the 1,3-dipolar cycloaddition[2] was not easily converted into the acid to yield the zwitterionic free UAA. Therefore, a slightly modified synthetic route was sought and each step optimized, with the goal of synthesizing the UAA as a canonical zwitterion for further research towards the application of the UAA and the aqueous nitron methodology to biological systems. The results of the former endeavours are presented in this chapter.

4.2 Results

4.2.1 Trypsin Digest

The simplest route towards the free UAA would be the direct hydrolysis of the amide bond to yield the acid. Chymotrypsin was chosen as the hydrolytic enzyme,[3] however, only non-specific cleavage was observed, and no mass for the purified UAA could be seen.

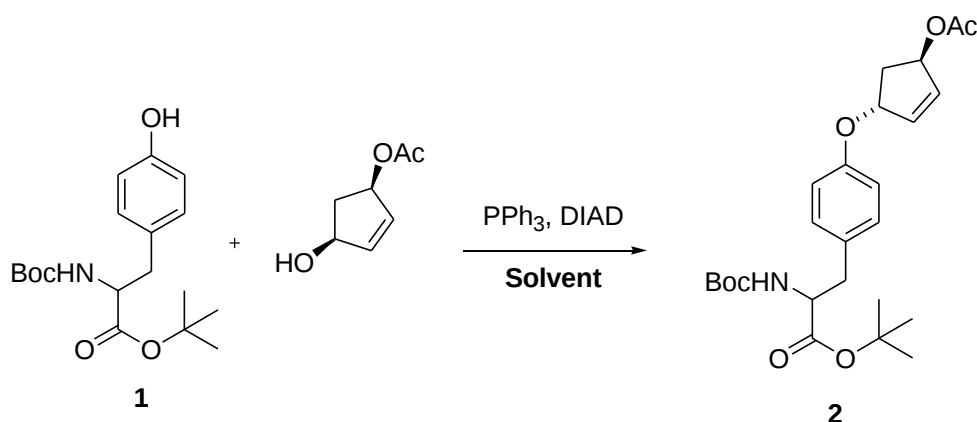
4.2.2 Alternate Routes & Optimization of Individual Steps

A simple protection strategy change was employed to synthesize the UAA rather than attempt to remove the butylamine following the original synthesis strategy. The slight

optimization of each step is presented in Tables 4-1 through 4-4 and the overall route is summarized in Scheme 4-3.

Attempts to convert the butylamine into the free carboxylic acid using a chymotrypsin enzyme proved unsuccessful. Only non-specific degradation (hydrolysis of the ester linkage) or no deprotection whatsoever was able to be observed by mass spectrometry. Therefore, it was believed that an alternative route to the final free amino acid would be more effective. Based on pre-existing literature for the protection of tyrosine[4], an alternative protection step was first sought out. This step proceeded excellently to give compound **1** in 97% yield. It was presumed that this *t*-boc protected amide and *t*-butyl ester could both be removed in the final stage via an acid treatment.

Table 4-1 Optimization of Mitsunobu step

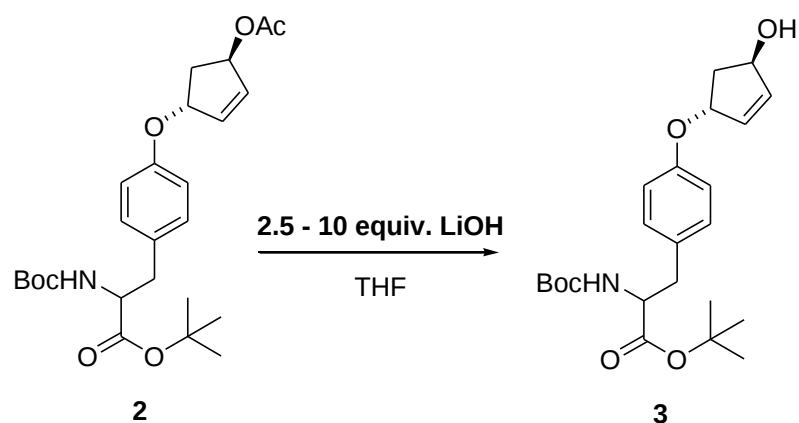


Entry	Solvent	Yield
1	DCM	67%
2	THF	52%

For the Mitsunobu[5] reaction, DCM and THF (Table 4-1, entries 1 and 2) were both tested as polar aprotic solvents on the new substrate. The solvent that provided the best yield was DCM (67%) of the ether product **2** which was a marked improvement over the original 43% yield, however part of this might be due to the substrate being modified, as all the solvents showed an improvement over the original method. Longer reaction times did not

lead to enhanced yields, in fact, by TLC the reaction showed no further progress after three hours in either solvent.

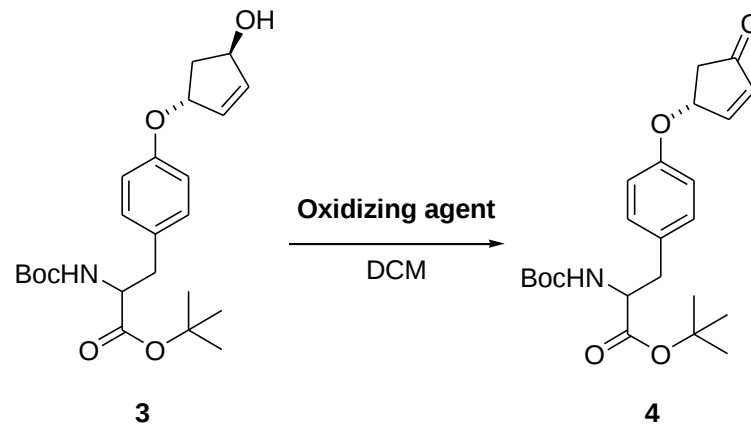
Table 4-2 Optimization of LiOH hydrolysis step



Entry	Equivalents of LiOH	Time for conversion by TLC (h)	Isolated Yield (%)
1	10	1.5	97
2	5	2.5	97
3	3	23	87
4	2.5	>26	82

The hydrolysis reaction to give the alcohol **3** was optimized to a lower concentration of lithium hydroxide. While 10 equiv. was used originally, concentrations as low as 5 equiv. could achieve similar results in similar time span (Table 4-2, entry 2). Lower concentrations (3 equiv.) required a longer conversion time of 23 hours and lower still required >26h for complete conversion (Table 4-2, entries 3 & 4 respectively). Yields also suffered. Notably, it was found that by performing the reaction in a mixture of THF/MeOH greatly improved the rate of the reaction when compared to the original conditions of using a simple aqueous LiOH solution.[6]

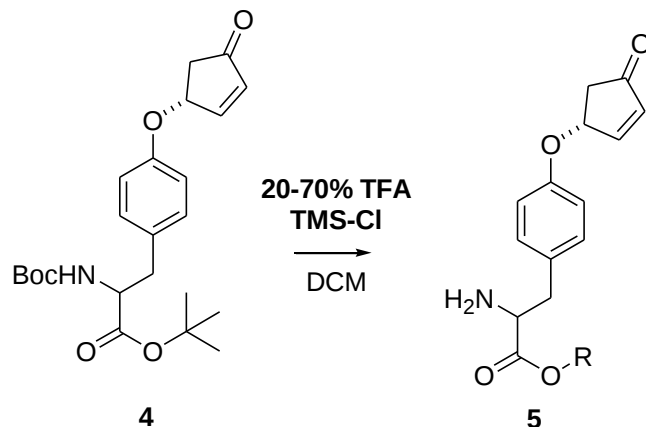
Table 4-3 Optimization of oxidation step



3 **4**

Entry	[O]	Time (h)	Yield (%)
1	2 equiv. DMP	20	36
2	2 equiv. PDC	9.5	93
3	1.1 equiv. PDC	16	60
4	1.5 equiv. PDC	16	76

Subsequently, the oxidation reaction was attempted with both PCC[7] and Dess-Martin periodinate[8] and the results are summarized in Table 4-3. Neither of these provided better yields than PDC (entry 2) which is especially effective at oxidizing allylic alcohols[9] to the corresponding α,β -unsaturated ketones. PCC (data not shown) demonstrated comparable yields to PDC, however PDC was found to be a more practical reagent to use. Lower loading of PDC (entries 3 and 4) showed incomplete conversion even after 48h, suggesting that at least a two-fold excess of the oxidizing agent was required for complete oxidation of **3** to **4** to occur.

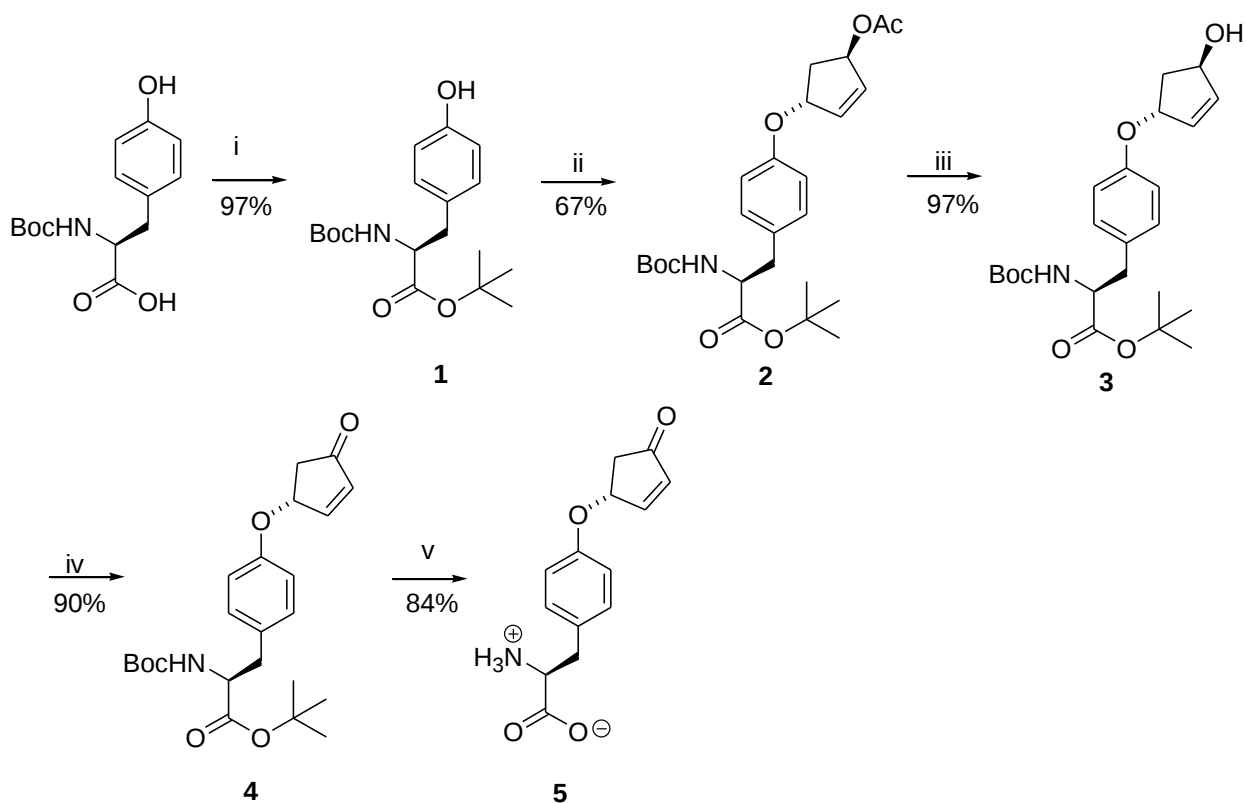
Table 4-4 Optimization of deprotection step

Entry	% TFA	Time (h)	Cation Scavenger	R (Major)
1	20	2	No	<i>tert</i> -butyl
2	50	12	No	H
3	50→70	4	Yes	H

The preceding results obtained during optimization are summarized in Scheme 4-3.

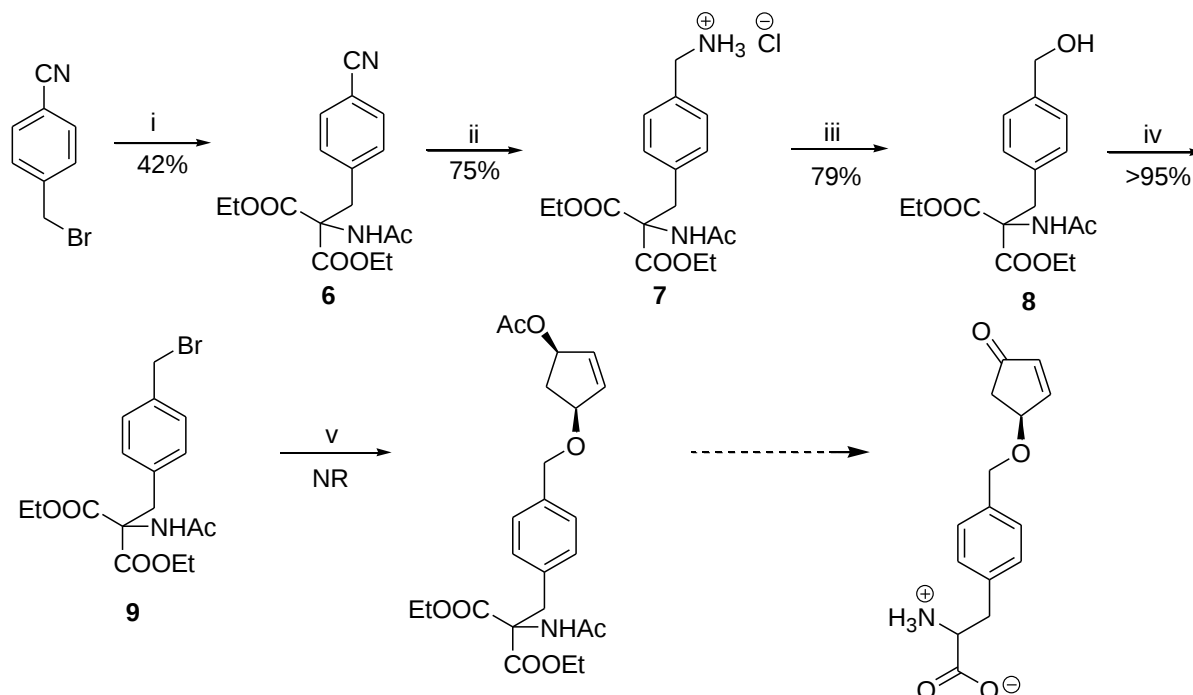
Finally, concurrent removal of the two protecting groups was believed to be possible using a TFA/CH₂Cl₂ treatment[10]. The deprotection step proved to be quite sensitive to the acidic conditions required for complete removal of the protecting groups to give the free UAA **5**. As described in Table 4-4, at lower concentrations of acid, the *t*-butyl group was not removed, while at higher concentrations of acid(>50%) hydrolysis of the aromatic ether linkage to give tyrosine was observed. Addition of a proton scavenger (TMS-Cl) while maintaining a 1:1 v/v mixture of TFA/DCM overnight at room temperature provided the final UAA in appreciable yield. However, more simplistic workup was achievable when the proton scavenger was omitted from the reaction.

Thus the overall yield of the modified reaction Scheme was achieved in 48% overall yield and is summarized in Scheme 4-3. Scale up to give around 100 mg of compound is required to generate a mutant tRNA/aaRS pair for the UAA. The synthetic procedure uses commercially available starting materials and is limited only by the cost of (1*S*,4*R*)-*cis*-4-Acetoxy-2-cyclopenten-1-ol, a compound obtained by kinetic resolution.



Scheme 4-3 Summary of optimized synthetic manipulations towards UAA **5**: i)(Boc)₂O, H₂O, rt, 2h; ii) DIAD, PPH₃, (1*S*,4*R*)-*cis*-4-Acetoxy-2-cyclopenten-1-ol, DCM, 24h; iii) 5 equiv. LiOH, THF/MeOH (1:6), 2.5 h; iv) 2 equiv. PDC, 9.5h, DCM; v) TFA/DCM (1:1 v/v), cat. TMS-Cl, 25h.

The acid stability of the compound due to the aromatic ether linkage was somewhat of a concern for the application of this UAA, so a route towards an analogous UAA that would be more acid stable was concurrently sought and is described in Scheme 4-4.



Scheme 4-4 Summary of alternative route towards diastereomeric mixture of acid stable UAA analogues. . i) Diethyl acetamidomalonate, NaOEt, EtOH, reflux, 36 h . ii) H₂ (1.0 atm.), Pd/c, HCl (conc.), EtOH. iii) NaNO₂, H₂O. iv) PBr₃, DCM. NaH, (1*S*,4*R*)-*cis*-4-acetoxy-2-cyclopenten-1-ol, THF, MeOH.

While the UAA **5** proved to be stable to acidic conditions harsher than those found in a biological system (1:1 TFA/DCM v/v) a concurrent synthesis was sought that would lead to a more acid stable UAA variant (Scheme 4-4). Beginning with *p*-cyanobenzyl bromide, substitution of the amino acid backbone precursor was achieved in 42% yield via a base promoted substitution. Following this, reduction of the nitrile group in 75% yield to the chloride salt and oxidation to the benzyl alcohol (79%), and bromine substitution (97%) were achieved. Attempts towards the Williamson ether synthesis described in Scheme 4-4 (step v) were performed yet failed to yield any desired product, presumably due to the self reactivity with the alkoxy and the ester within (1*S*,4*R*)-*cis*-4-acetoxy-2-cyclopenten-1-ol. Following this, a similar route to that described earlier could be envisioned followed by acid catalyzed hydrolysis of the amide bond, saponification of the esters and then decarboxylation to yield the free amino acid. The synthesis of these precursors is well established in the literature. An alternative route using silver(I) oxide[11] was considered, as this method has been used

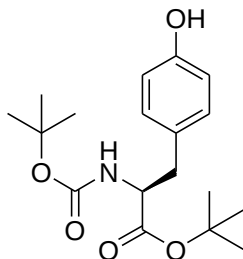
to couple acetyl bearing alcohols to benzyl bromide. However, there failed to be any reaction between **9** and the (1*S*,4*R*)-*cis*-4-acetoxy-2-cyclopenten-1-ol.

4.3 Summary & Future Direction

The tyrosine analogue UAA previously reported has been synthesized by an alternate route, and each step has been optimized to give the optimal yield. The final product contains an α,β -unsaturated ketone moiety that we have shown can react with nitrones to give the [3+2] cycloadducts. Unlike our previous methodology, this route permits the synthesis of the UAA as its free zwitterions, thus permitting its incorporation into biological systems through the development of mutant, orthogonal tRNA/aaRS pairs. A route towards an analogous compound with an extra carbon linker between the cyclopenten-1-one and phenyl functional groups has also been worked towards.

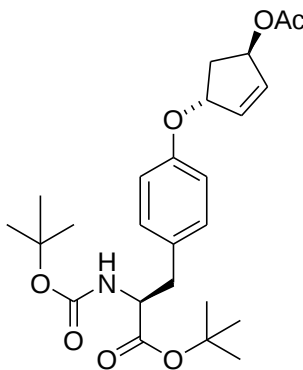
4.4 Experimental Synthetic Methodology

2-*tert*-Butoxycarbonylamino-3-(4-hydroxy-phenyl)-propionic acid *tert*-butyl ester (1)



To a stirring suspension of L-(O-*t*Bu)-tyrosine (593 mg, 2.5 mmol, 1.0 equiv.) in degassed water, Boc anhydride (600 mg, 2.75 mmol, 1.1 equiv.) was added. The reaction was stirred for 2 hours at room temperature. The crude reaction mixture was extracted with EtOAc (3X 10 mL), and the organic extracts were combined, dried over sodium sulphate, filtered, and concentrated *in vacuo*. The crude product was purified using column chromatography (3:7 EtOAc/Hex, *R_f* = 0.32) to give the product as a clear gel (816 mg, 97%). ¹H NMR δ_{H} (400 MHz, CDCl_3) 1.41 (s, 9H), 1.42 (s, 9H), 1.63 (s, 1H), 2.91-3.01 (m, 2H), 4.39 (dd, *J* = 13.7, 6.1 Hz, 2H), 5.01 (d, *J* = 8.0 Hz, 1H), 5.82 (s, 1H), 6.72 (d, *J* = 8.1 Hz, 2H), 7.00 (d, *J* = 8.1 Hz, 2H). ¹³C NMR δ_{C} (100 MHz, CDCl_3) 27.9, 28.3, 37.7, 55.0, 79.8, 82.1, 115.2, 128.0, 130.6, 154.8, 155.2, 171.2. ESI-MS [$\text{C}_{18}\text{H}_{26}\text{NO}_5$] + 1 = 338.4.

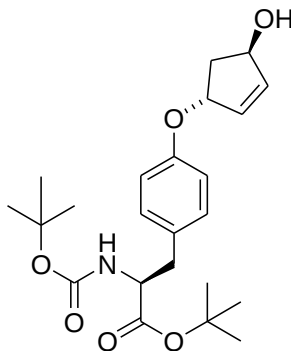
3-[4-(4-acetoxy-cyclopent-2-enyloxy)-phenyl]-2-*tert*-butoxycarbonylamino-propionic acid *tert*-butyl ester (2)



To a dry round bottomed flask, **1** (564 mg, 1.67 mmol, 1.0 equiv.), (1*S*,4*R*)-*cis*-4-Acetoxy-2-cyclopenten-1-ol (285 mg, 2.0 mmol, 1.2 equiv.) and PPh_3 (876 mg, 3.34 mol, 2.0 equiv.) were added and dissolved in dry DCM (10 mL). DIAD (657 μL , 3.34 mmol, 2.0 equiv.) was

added dropwise with stirring. The reaction was left to for three hours. The reaction was quenched with water (10 mL) and the organic layer was separated. The aqueous layer was extracted with DCM (3X 10 mL) and the combined organic extracts were dried over magnesium sulphate, filtered, and concentrated *in vacuo*. The resultant crude mixture was purified using column chromatography (3:1 Hex/EtOAc) (R_f = 0.44 5:4 Hex/EtOAc) to give the product as a solid white powder (527 mg, 67%). ^1H NMR δ_{H} (400 MHz, CdCl_3) 1.41 (s, 9H), 1.42 (s, 9H), 2.05 (s, 3H), 2.26-2.38 (m, 2H), 2.94-3.03 (m, 2H), 4.40 (dd, J = 13.7, 6.1 Hz, 1H), 4.97 (d, J = 8.0, 1H), 5.44-5.47 (m, 1H), 5.83-5.85 (m, 1H), 6.16 (ddd, J = 5.6, 2.0, 0.9 Hz, 1H) 6.25 (ddd, J = 5.6, 2.0, 0.9 Hz, 1H), 6.80 (d, J = 8.6 Hz, 2H), 7.07 (d, J = 8.6 Hz, 2H). ^{13}C NMR δ_{C} (100 MHz, CdCl_3) 21.1, 27.9, 28.3, 37.6, 37.9, 54.9, 78.6, 81.0, 81.9, 115.2, 128.8, 130.6, 134.7, 134.8, 136.2, 136.6, 155.1, 156.8, 170.8. ESI-MS $[\text{C}_{25}\text{H}_{34}\text{NO}_7] + 1 = 462.3$.

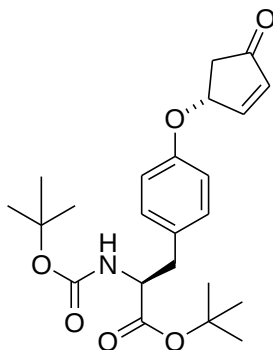
2-*tert*-Butoxycarbonylamino-3-[4-(4-hydroxy-cyclopent-2-enyloxy)-phenyl]-propionic acid *tert*-butyl ester (3)



LiOH (178 mg, 7.5 mmol, 5.0 equiv.) was dissolved in water (3 mL) and added to a stirring solution of **2** (688 mg, 1.5 mmol, 1.0 equiv.) in 3 mL of THF/MeOH (1:6). The reaction was stirred for 4 hours. Water (5 mL) and EtOAc (5 mL) were added, and the phases were separated. The aqueous phase was extracted with EtOAc (2X 5 mL) and the combined organic extracts were dried over magnesium sulphate. The crude product was purified using column chromatography (8:7 EtOAc/Hex) (R_f = 0.15, 2:1 Hexanes/EtOAc) to provide the product as a solid (534 mg, 97%). ^1H NMR δ_{H} (400 MHz, CdCl_3) 1.41 (s, 9H), 1.42 (s, 9H), 1.76 (br, 1H), 2.17 (ddd, J = 14.6, 6.7, 3.1 Hz, 1H), 2.32 (ddd, J = 14.6, 6.9, 2.9 Hz, 1H), 2.94-3.03 (m, 2H), 4.39 (dd, J = 13.7, 5.9 Hz, 1H), 5.11 (d, J = 2.9 Hz, 1H), 5.45-5.47 (m, 1H), 6.13-6.17 (m, 2H), 6.80 (d, J = 8.6 Hz, 2H), 7.07 (d, J = 8.6 Hz, 2H). ^{13}C NMR δ_{C} (100

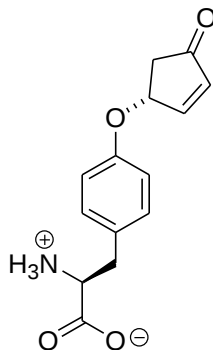
MHz, CdCl_3) 27.9, 28.3, 37.6, 41.1, 54.9, 76.1, 79.7, 81.4, 81.9, 115.2, 128.6, 130.6, 133.8, 139.0, 155.1, 156.9. ESI-MS $2[\text{C}_{23}\text{H}_{32}\text{NO}_6] + 1 = 840.0$

2-*tert*-Butoxycarbonylamino-3-[4-(4-oxo-cyclopent-2-enyloxy)-phenyl]-propionic acid *tert*-butyl ester (4)



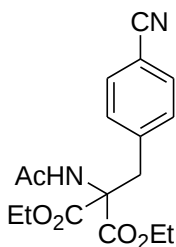
3 (376 mg, 0.90 mmol, 1.0 equiv.) was dissolved in a small round bottom flask containing dry DCM (5 mL). PDC (680 mg, 1.8 mmol, 2.0 equiv.) was added with stirring and the reaction was capped and stirred for 9.5 hours. The mixture was diluted with Et_2O and filtered over a pad of celite. The filtrate was washed with water (2X 5 mL) and dried over magnesium sulphate, filtered, and concentrated *in vacuo*. The crude product was purified by column chromatography (1:2 EtOAc/Hex , $R_f = 0.29$) to give the product as a yellow oil (339 mg, 90%). ^1H NMR δ_{H} (400 MHz, CdCl_3) 1.41 (s, 9H), 1.42 (s, 9H), 2.44 (dd, $J_{\text{AB}} = 18.4$ Hz, $J_{\text{BX}} = 2.0$ Hz, 1H) 2.88 (dd, $J_{\text{AB}} = 18.4$ Hz, $J_{\text{AX}} = 5.9$ Hz, 1H), 2.95-3.06 (m, 2H), 4.41 (dd, $J = 13.6, 6.0$ Hz, 1H), 4.98 (d, 7.8 Hz, 1H), 5.42-5.44 (m, 1H), 6.36 (dd, $J = 5.7, 1.2$ Hz, 1H), 6.84 (d, $J = 8.6$ Hz, 2H), 7.11 (d, $J = 8.6$ Hz, 2H), 7.69 (dd, $J = 5.7, 2.3$ Hz, 1H). ^{13}C NMR δ_{C} (100 MHz, CdCl_3) 27.9, 28.3, 37.7, 41.8, 54.9, 75.1, 79.7, 82.0, 115.2, 129.7, 130.8, 136.5, 155.0, 156.2, 159.6, 170.9, 205.1. ESI-MS $[\text{C}_{23}\text{H}_{31}\text{NO}_6] + 1 = 418.3$.

2-Amino-3-[4-(4-oxo-cyclopent-2-enyloxy)-phenyl]-propionic acid (5)



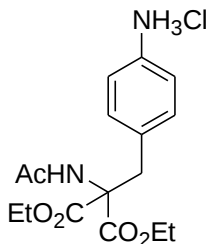
4 (15 mg, 36 μ mol) was dissolved in a 1:1 v/v mixture of TFA and DCM (total volume of 250 μ L) and stirred at room temperature for 12 hours. The mixture was concentrated to half volume then an equal volume of DCM was added and the sample concentrated to dryness to obtain the product as a white powder (9.4 mg, quant.). ^1H NMR δ_{H} (400 MHz, d_6 DMSO) 2.18-2.22 (m, 1H), 2.82-3.09 (m, 4H), 5.54-5.58 (m, 1H), 6.41 (d, $J = 5.5$, 1H), 6.94 (d, $J = 8.1$ Hz, 2H), 7.20 ($J = 8.1$ Hz, 2H), 7.87 (dd, $J = 5.5$, 2.0 Hz, 1H), ^{13}C NMR δ_{C} (100 MHz, CD_3OD) 30.6, 37.3, 42.7, 76.5, 79.4, 116.9, 129.9, 131.8, 136.9, 158.3, 162.1, 207.8. ESI-MS $[\text{C}_{14}\text{H}_{15}\text{NO}_4] + 1 = 262.2$

2-Acetylamino-2-(4-cyano-benzyl)-malonic acid diethyl ester (6) [12]



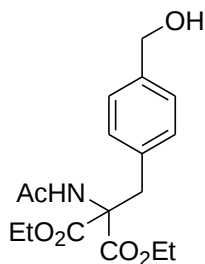
A solution of sodium (632 mg, 28 mmol, 1.0 equiv.), diethyl acetamidomalonate (5.43g, 25 mmol, 1.0 equiv.) and p-cyanobenzyl bromide (4.90 g, 25 mmol, 1.0 equiv.) in 50 mL of dry ethanol was refluxed for 36 hours. After cooling and addition of water (100 mL) the crystalline material was collected by filtration and washed twice with 15 mL of cold water (3.5g, 42%). ^1H NMR δ_{H} (400 MHz, CDCl_3) 1.29 (t, $J = 7.2$ Hz, 6H), 2.03 (s, 3H), 3.73 (s, 2H), 4.26 (q, 7.2 Hz, 4H), 6.52 (br, 1H), 7.12 (d, $J = 8.2$ Hz, 2H), 7.55 (d, $J = 8.2$ Hz, 2H). ESI-MS $[\text{C}_{17}\text{H}_{20}\text{N}_2\text{O}_5] + 1 = 333.3$.

4-(2-Acetylamino-2,2-bis-ethoxycarbonyl-ethyl)-benzyl-ammonium chloride (7)[12]



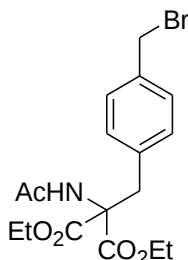
6 (3.25g, 9.78 mmol) was hydrogenated at atmospheric pressure and room temperature for 22 hours in ethanol (50 mL) and concentrated HCl (4.5 mL) with Palladium (10%) on activated carbon as a catalyst (652 mg). After filtration over celite, the solution was concentrated to dryness. Water (100 mL) was added to the residue and unreacted material was removed by filtration. The filtrate was again concentrated *in vacuo* to obtain the product as a yellow viscous gel (2.73 g, 75%). ^1H NMR δ_{H} (400 MHz, DMSO- d_6 Cl $_3$) 1.18 (t, J = 7.2 Hz, 6H), 1.95 (s, 3H), 3.45 (s, 2H), 3.97 (s, 2H), 4.15 (q, 2H), 7.01 (d, J = 8.2 Hz, 2H), 7.38 (d, J = 8.2 Hz, 2H), 7.98 (s, 1H), 8.34 (br, 3H). ESI-MS $[\text{C}_{16}\text{H}_{24}\text{N}_2\text{O}_5\text{Cl} - \text{Cl}]^+ = 324.3$.

2-Acetylamino-2-(4-hydroxymethyl-benzyl)-malonic acid diethyl ester (8)[12]



A solution of **7** (2.50 g, 6.71 mmol, 1.0 equiv.) NaNO $_2$ (648 mg, 9.40 mmol, 1.4 equiv.) and water (125 mL) was heated for 2h at 100 °C, cooled and extracted with EtOAc. The extract was washed with 1 M HCl (20 mL), water (20 mL), 5% NaHCO $_3$ (20 mL), water (20 mL) and brine (20 mL), dried over sodium sulphate, filtered and concentrated *in vacuo* to give the product as yellow needles (1.78 g, 79%). ^1H NMR δ_{H} (400 MHz, CdCl $_3$) 1.29 (t, J = 7.2 Hz, 6H), 1.70 (br, 1H), 2.03 (s, 3H), 3.65 (s, 2H), 4.26 (q, 7.2 Hz, 4H), 4.67 (s, 2H), 6.52 (br, 1H), 7.00 (d, J = 8.2 Hz, 2H), 7.27 (d, J = 8.2 Hz, 2H). ESI-MS $[\text{C}_{16}\text{H}_{23}\text{NO}_6] + 1 = 337.2$.

2-Acetylamino-2-(4-bromomethyl-benzyl)-malonic acid diethyl ester (9) [13]



To a dry flask containing **8** (1.60 g, 4.56 mmol, 1.0 equiv.) in dry DCM (10 mL) was added dropwise phosphorous tribromide (859 μ L, 9.0 mmol, 2.0 equiv.) and the reaction was stirred for 24 hours. Water (20 mL) was added slowly, and the organic layer was separated and washed again with 20 mL of water. The product was used without further purification (peach solid, 1.81 g, qt). ^1H NMR δ_{H} (400 MHz, CdCl_3) 1.29 (t, $J = 7.2$ Hz, 6H), 2.03 (s, 3H), 3.64 (s, 2H), 4.27 (q, 7.2 Hz, 4H), 4.46 (s, 2H), 6.53 (br, 1H), 6.98 (d, $J = 8.0$ Hz, 2H), 7.28 (d, $J = 8.0$ Hz, 2H). ^{13}C NMR δ_{C} (100 MHz, CdCl_3) 14.0, 23.1, 33.1, 37.5, 62.7, 67.1, 129.0, 130.3, 135.7, 136.7, 167.4, 169.0. ESI-MS $[\text{C}_{17}\text{H}_{22}\text{BrNO}_5] + 1 = 402.1$.

4.4 References

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Appendix A:

Instrumentation and Chemicals

Chemicals

All compounds were synthesized from commercially available sources purchased through Sigma-Aldrich unless otherwise indicated, and used without further purification. All deuterated NMR solvents were purchased from Cambridge Isotope Laboratories.

Nuclear Magnetic Resonance Spectroscopy (NMR).

Characterization of novel compounds was done using NMR spectroscopy. All experiments were carried out on a Bruker DRX-400 using a frequency of 400.13 MHz for ^1H and 100.61 MHz for ^{13}C . Spectra were recorded using a broad band direct detection probe. All data were processed using Bruker TOPSPIN 2.1 software.

High Pressure Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (HPLC-MS/MS).

A WATERS system consisting of a WATERS 996 Photodiode Array Detector, an Alliance HT – WATERS 2795 Separations Module, a WATERS Micromass ZQ 2000 unit equipped with a pneumatically-assisted electrospray ionization source was used for HPLC-MS analysis. Samples were run on a WATERS Sunfire C18 (100 mm x 2.10 mm x 3.5 μm) column. Standard conditions used, unless otherwise stated, were a gradient of 10-95% acetonitrile/0.1% formic acid in H_2O /0.1% formic acid over 15 minutes with a flow rate of 0.2 mL/min. Eluent was directed first to the diode array detector and then to the mass spectrometer. The source temperature was set at 80 $^\circ\text{C}$, desolvation gas temperature was set at 200 $^\circ\text{C}$, and an electrospray capillary was set at 3.5 kV with a cone voltage set at 10 V. For MS analysis, the sample simply bypassed the column and was injected directly into the mass spectrometer. Data were collected in single ion recording mode and processed using Masslynx software.

High Pressure Liquid Chromatography (HPLC) - Optimization

HPLC was used for the purification of several compounds (as described within the respective experimental procedures) where silica gel flash column chromatography proved to be ineffective at obtaining analytically pure samples. An Agilent system consisting of a quaternary pump, an Agilent 1100 degasser, an auto injector and a UV/Vis detector set at 254 nm was used for the optimization of methods. All samples were run using a SunFire C18 4.6x100mm, 3.5µm column and data were processed using ChemStation software.

High Pressure Liquid Chromatography (HPLC) – Preparative

Purification was performed using a WATERS Delta Prep 4000 with 996 PDA detector and a SunFire C18 19x100mm, 5µm column. Specific gradient details are listed with compounds purified by this method.

Melting Point Apparatus

Melting points for various solid compounds were obtained using a Fisher-Johns melting point apparatus and size 18, number 2 thickness Fisher Scientific glass circle cover slips.

Solvent Purification System

DMF, DCM, and THF solvents were dried using a PureSolv system.

Confocal Microscope

Imaging was done with an Olympus 1X81 spinning-disk confocal microscope equipped with a FITC filter (Semrock, Excitation: 465-499nm, Emission: 516-556 nm) and a Photometrics (Coolsnap ES) camera using 60x magnification. Images were taken using both bright-field and the FITC channel (10 second exposure). Image processing was done using ImageJ software, using the Colour Merge plugin to apply pseudocolour to FITC channel images and merging with bright-field images. The same pixel-intensity ranges were applied and displayed for all images taken.

GC-MS

Analysis was performed using an Agilent 6970 GC/5975 MSD column - HP-5, 30m x 0.25mm x 0.25um. Helium carrier gas was used with a constant flow of 1.5 mL/min. An autosampler was used to inject 1 µL sample in DCM. The injector temperature was 250 °C and split injection mode was used with split ratio 1:50. The oven temperature program was: initial temp. 100 °C kept for 1 minute then temperature ramp of 10 °C / min to 280 °C. The MSD detector was working in scan mode with a mass range of 40 – 550 mass units.

Appendix B: ^1H and ^{13}C NMR Spectra for Novel Compounds

Chapter	Compound	Page	Chapter	Compound	Page
2	2 d	129	4	1	167
2	2 f	130	4	2	168
2	2 g	131	4	3	169
2	3 a	132	4	4	170
2	3 b	133	4	5	171
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2	3 d	135			
2	3 e	136			
2	3 f	137			
2	3 g	138			
2	3 h	139			
2	3 i	140			
2	4 c	141			
2	4 d	142			
2	5 a	143			
2	5 b	144			
2	5 c	145			
2	5 d	146			
2	5 e	147			
2	5 f	148			
2	5 g	149			
2	5 h	150			
2	5 i	151			
2	5 j	152			
2	5 k	153			
2	5 l	154			
2	6	155			
2	7 a	156			
2	7 b	157			
2	7 c	158			
2	8	159-160			
2	9	161			
2	13	162			
2	15	163			
2	17	164			
2	19	165			
2	20	166			

Chemical structure: CC1=CC=C(C=C1)C#CCCCO

¹H NMR spectrum (400 MHz, CDCl₃) data:

Chemical Shift (ppm)	Integration
7.08 - 7.26	0.94, 2.88
3.81 - 3.85	1.92
2.31	3.00
1.56 - 1.98	1.97, 0.98, 1.94, 1.92

Mass spectrum data (m/z vs. Relative Intensity):

m/z	Relative Intensity
122.0	100.0
105.0	10.0
91.0	5.0
77.0	3.0
65.0	2.0
51.0	1.0

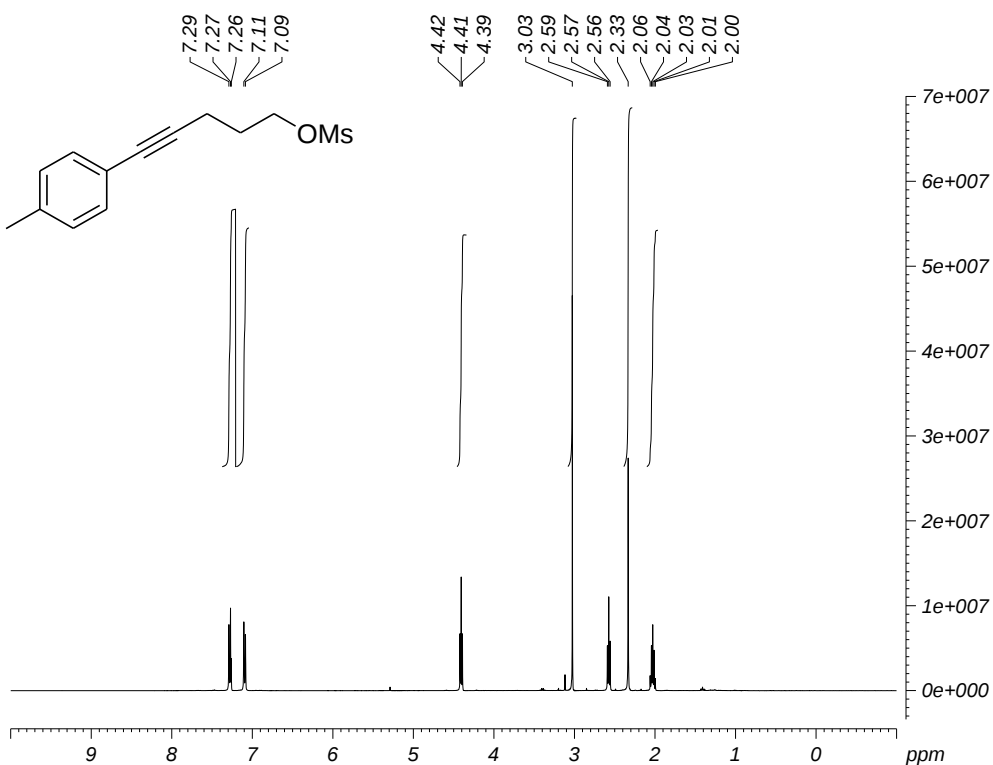
137.85
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128.10
123.45
88.87
81.26
77.32
77.20
77.00
76.68
61.85
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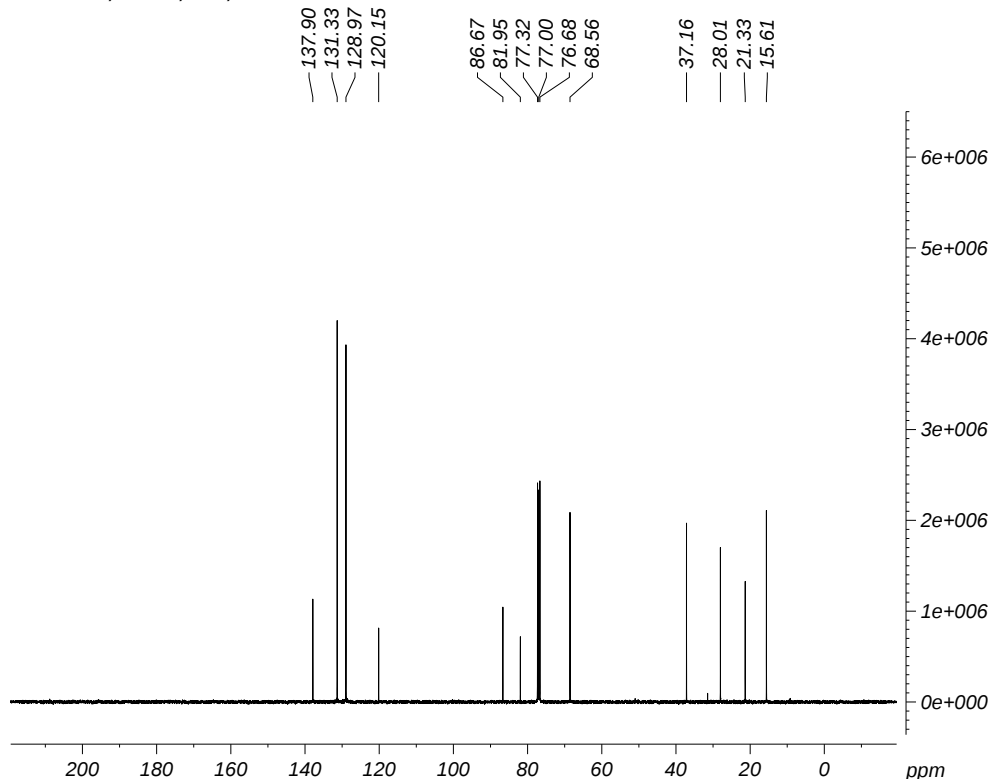


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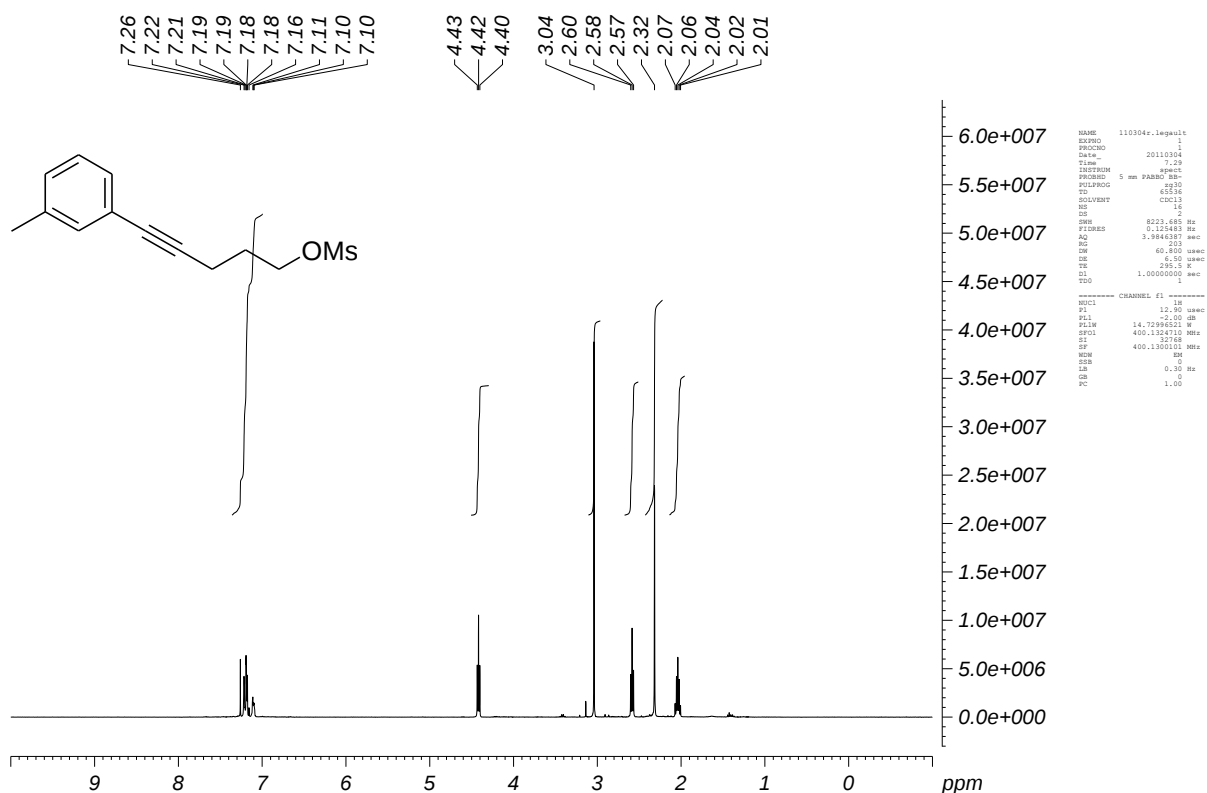


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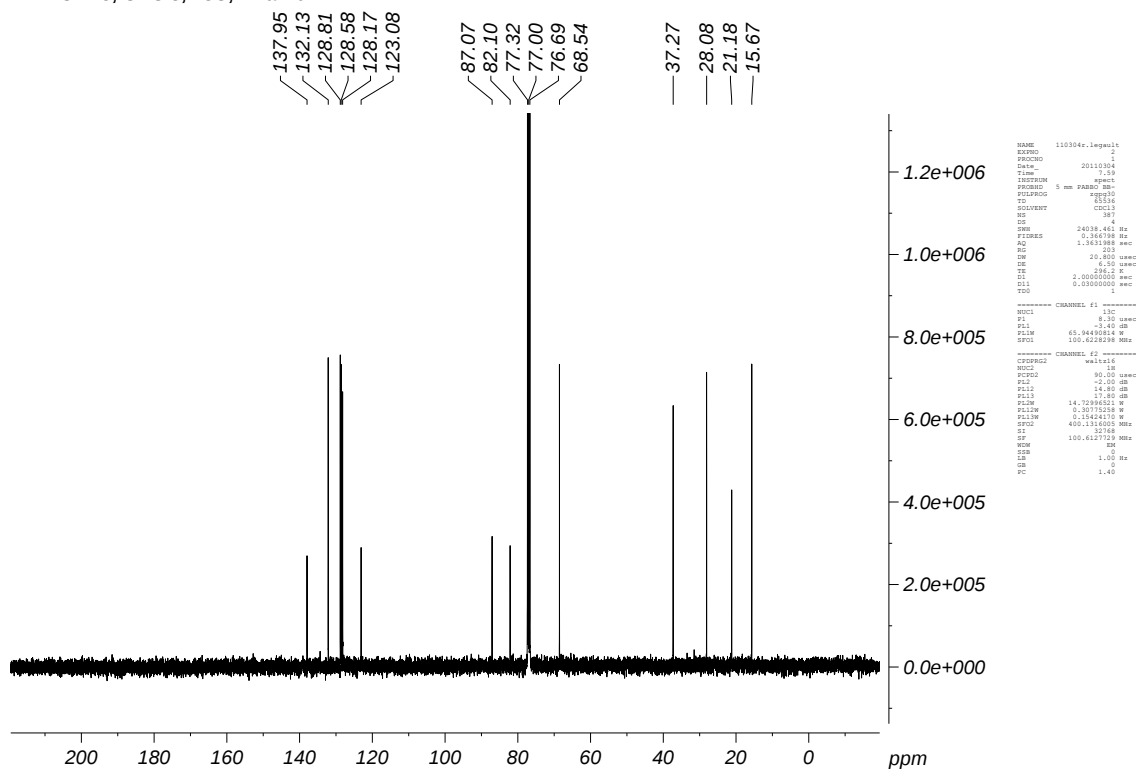
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PL 0.00 dB
PL1W 65.9449814 W
SFO1 100.6264598 MHz
----- CHANNEL f2 -----
CPDPRG2 waltz16
NUC2 1H
P2 90.00 usec
PL2 0.00 dB
PL1 14.80 dB
PL1W 17.80 dB
SFO2 400.1324710 MHz
SFO1W 0.3075258 W
PL1W 0.14424170 W
SFO2 400.1316005 MHz
SI 32768
SF 100.6127794 MHz
WDW EM
GB 1.00 Hz
LB 0.00 Hz
GB 1.40
PC 1.40

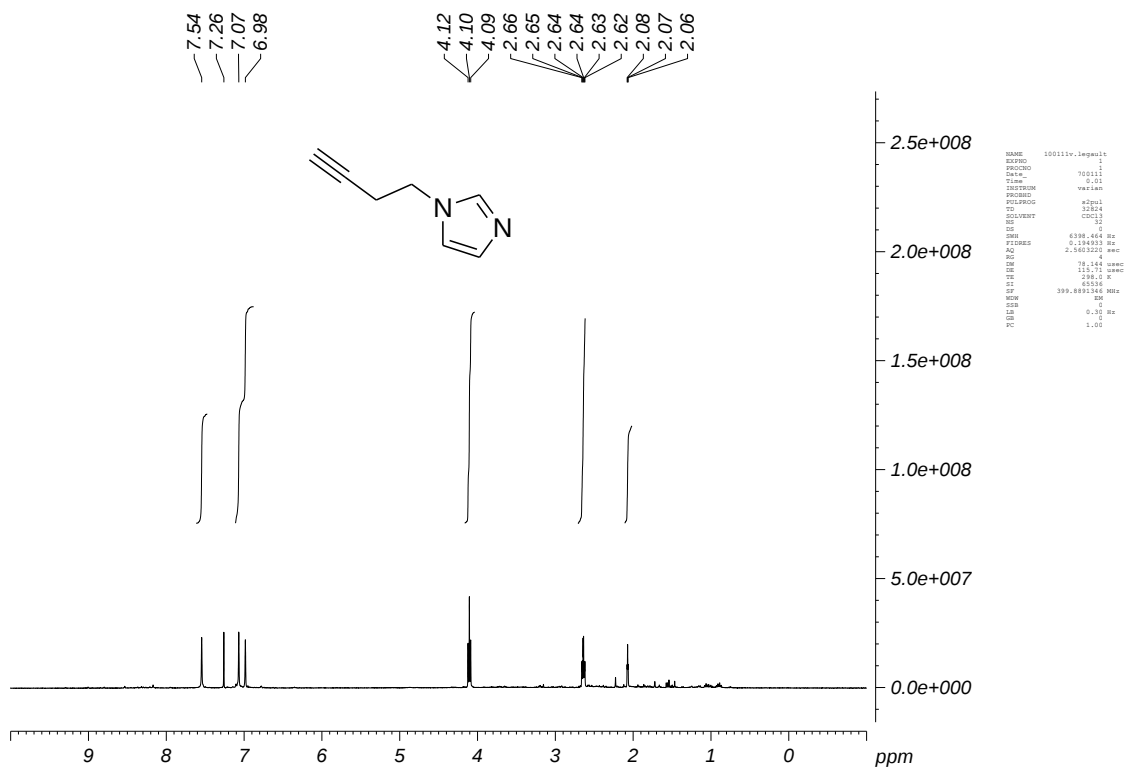
```

MLC 175, CDCl₃, 1H, 4Mar2011



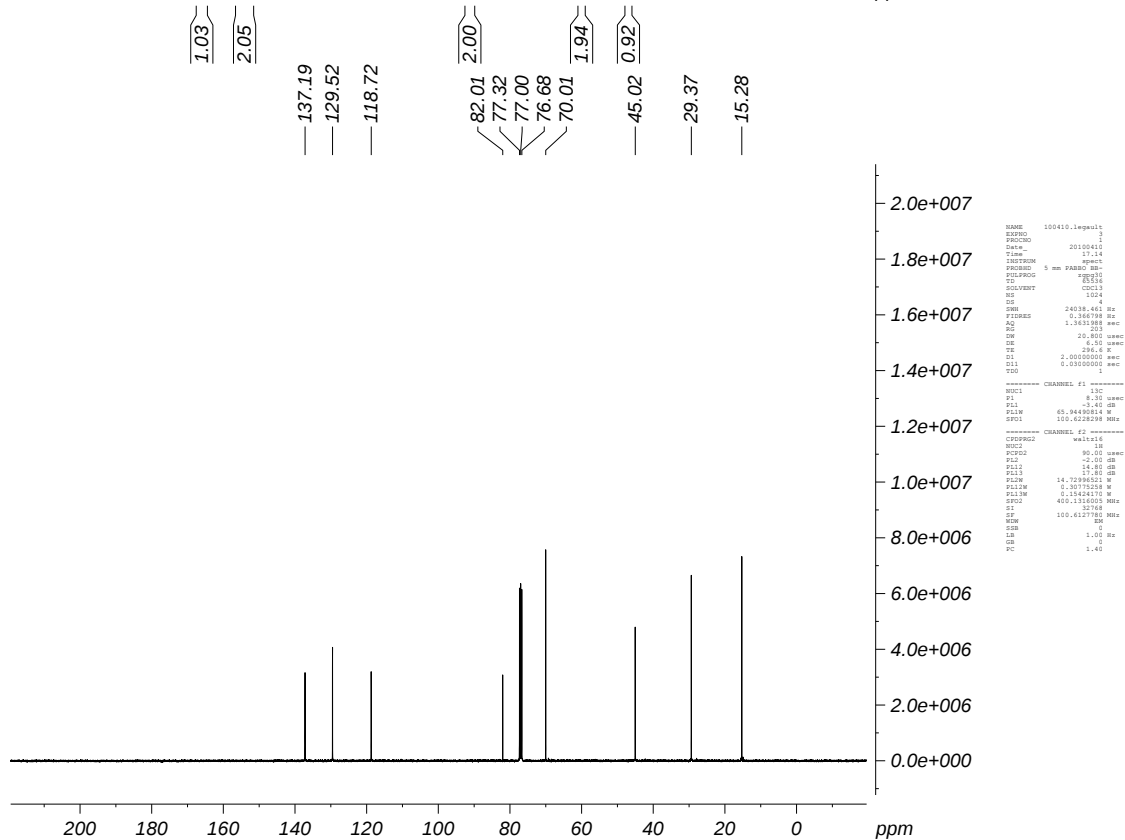
MLC 175, CDCl₃, 13C, 4Mar2011





```

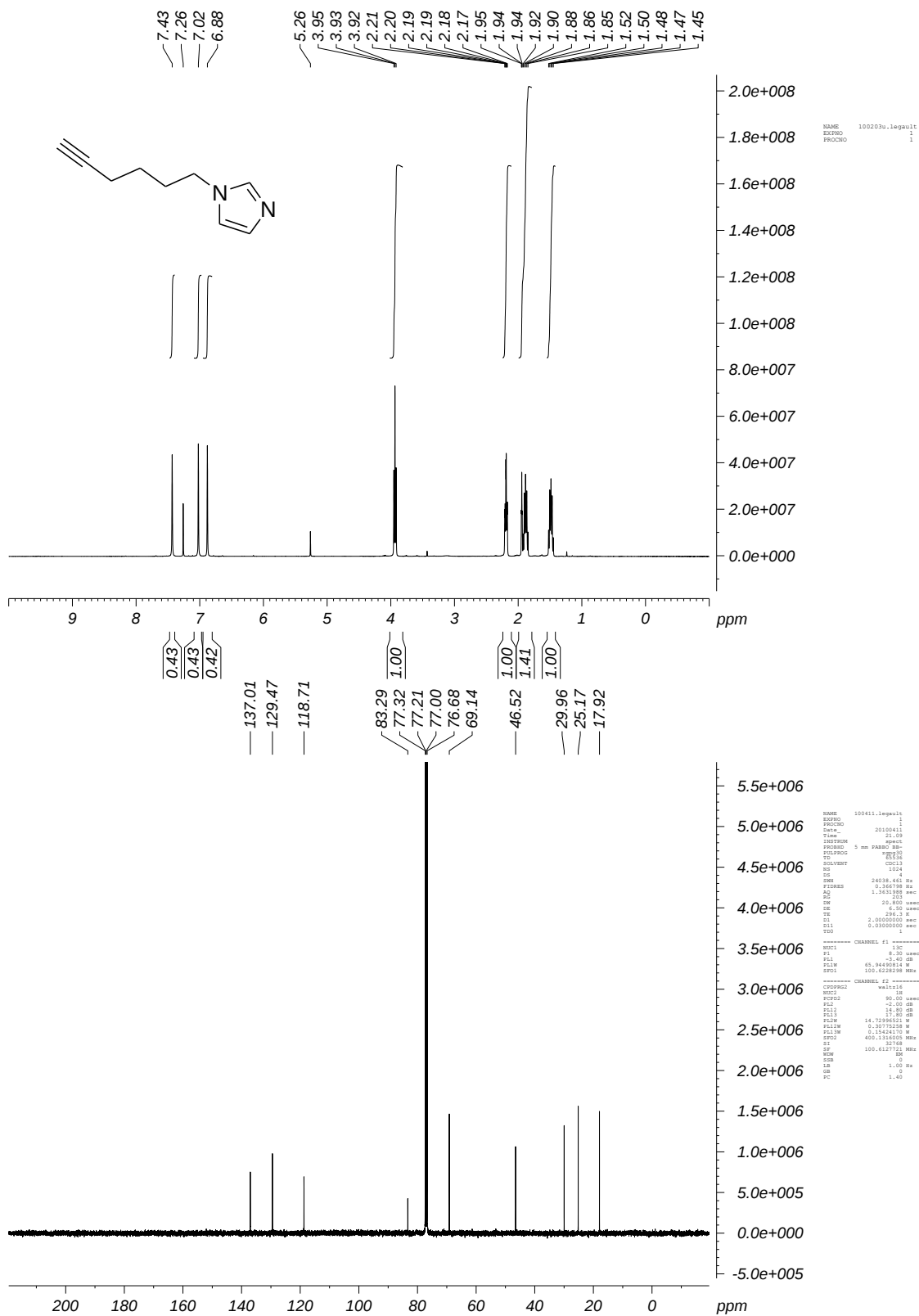
NAME      10011iv.1exp01
EXPNO     1
PROCNO    1
Date_     20110411
Time      17.14
INSTRUM    spect
PROBHD     5 mm
PULPROG    zgpg30
TD         65536
SOLVENT    CDCl3
NS         1024
DS         4
SWH         24038.461 Hz
FIDRES     0.184333 Hz
AQ         2.5603215 sec
RG          327.5
SI          327.5
SF          399.8891346 MHz
WDW         EM
SS          0
LB          0.30 Hz
GB          0
PC          1.00
  
```



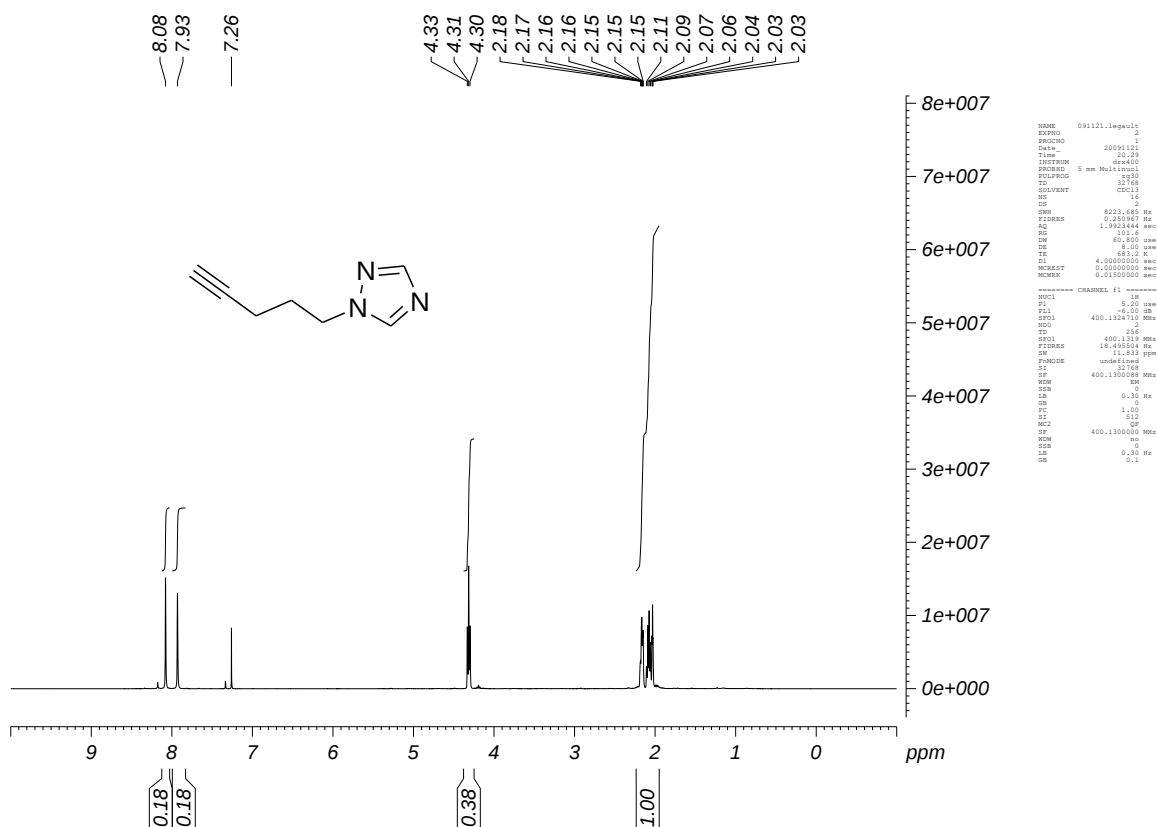
```

NAME      100410.1exp01
EXPNO     1
PROCNO    1
Date_     20100410
Time      17.14
INSTRUM    spect
PROBHD     5 mm
PULPROG    zgpg30
TD         65536
SOLVENT    CDCl3
NS         1024
DS         4
SWH         24038.461 Hz
FIDRES     0.184333 Hz
AQ         2.5603215 sec
RG          327.5
SI          327.5
SF          399.8891346 MHz
WDW         EM
SS          0
LB          0.30 Hz
GB          0
PC          1.00
  
```



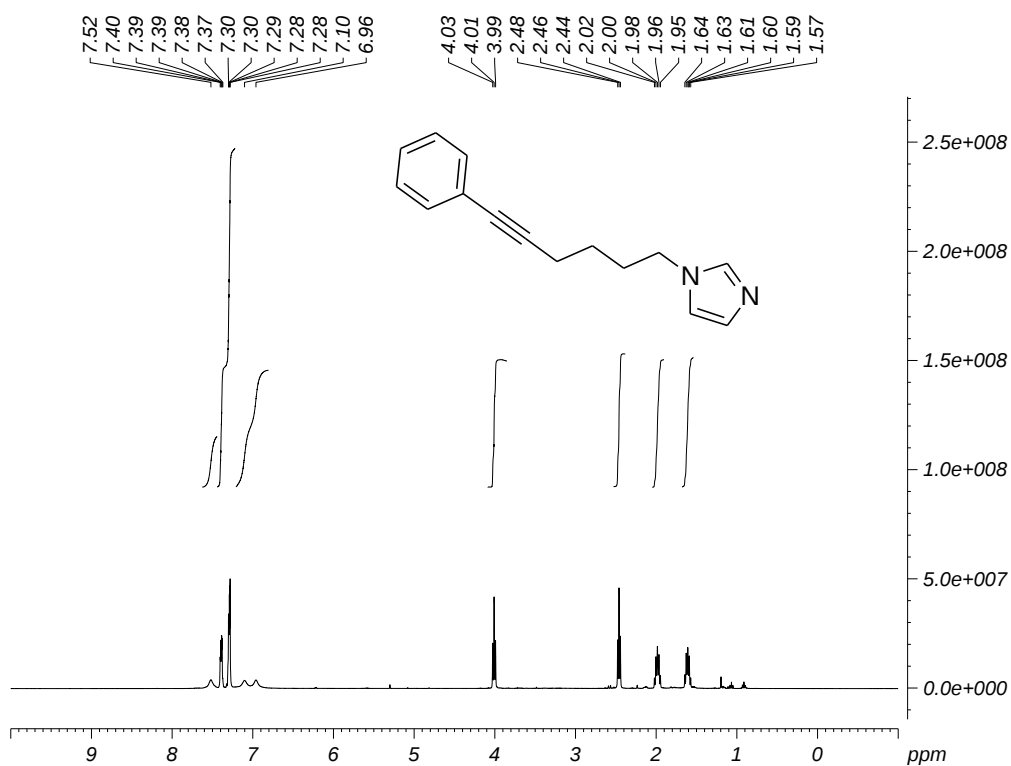


mlb125 cdcl3



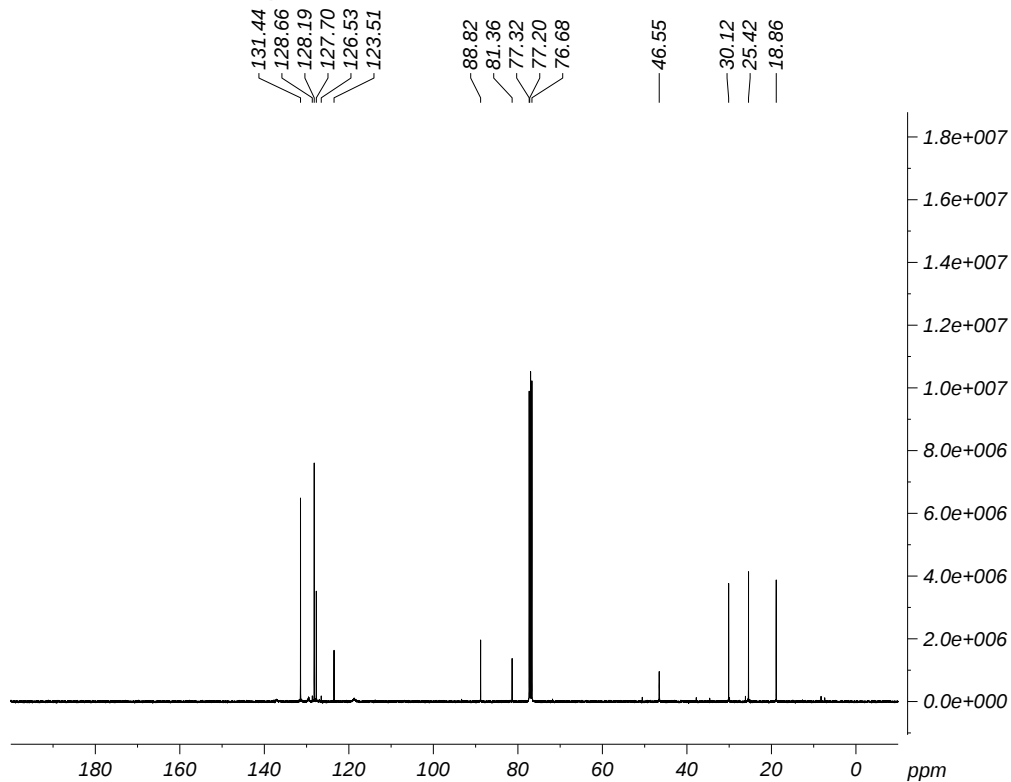


MLB171, CDCl₃, 1H, 11Jan2010

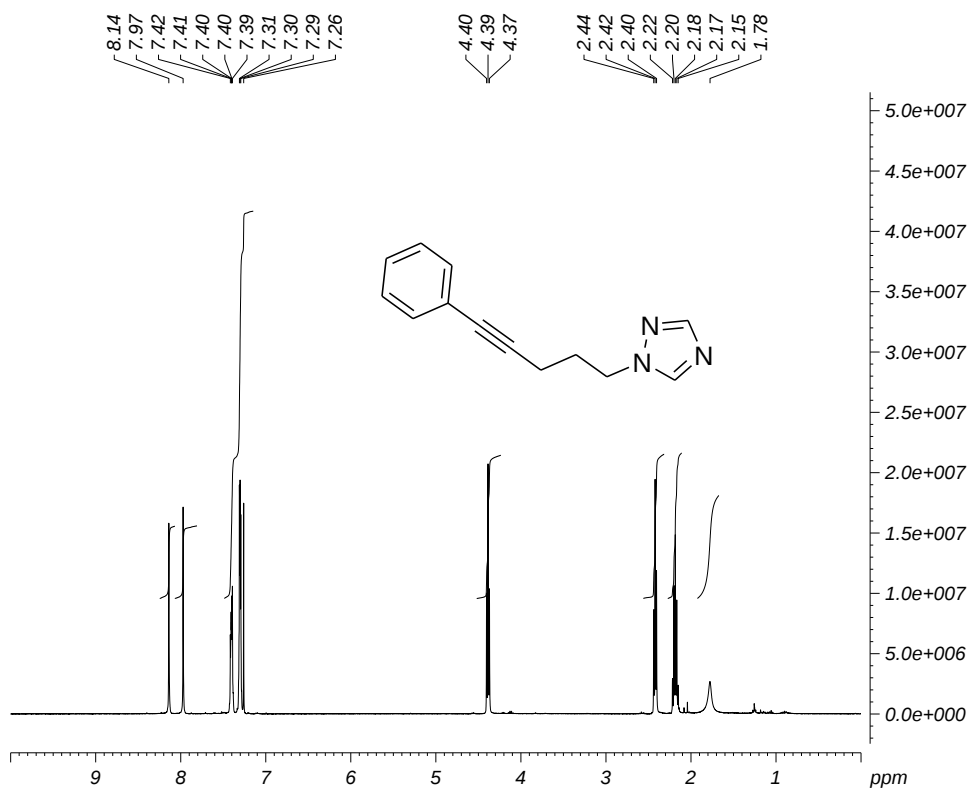


```
NAME 100111a.1segul1
EXPNO 1
PROCNO 1
Date_ 700111
Time 0.21
INSTRUM varian
PULPROG zgpg30
TE 300.2
SOLVENT CDCl3
NS 2
DS 0
SWH 6398.464 Hz
FIDRES 0.194012 Hz
AQ 2.5603220 sec
RG 32
DR 78.144 usec
DE 115.71 usec
TE 298.0 K
AQ 65536
SF 399.892368 MHz
WDW EM
GB 0
LB 0.30 Hz
GB 0
PC 1.00
```

mlb171, CDCl₃, 13C, 10Apr2010



```
NAME 100410.1segul1
EXPNO 1
PROCNO 1
Date_ 20100410
Time 14.42
INSTRUM spect
PULPROG 5 mm PABBO BBO
TE 300.2
SOLVENT CDCl3
NS 1024
DS 0
SWH 24038.462 Hz
FIDRES 0.346798 Hz
AQ 1.363398 sec
RG 320
DR 20.800 usec
DE 6.50 usec
TE 298.0 K
AQ 2.0000000 sec
SF 100.622394 MHz
WDW EM
GB 0
LB 1.00 Hz
GB 0
PC 1.40
```



```

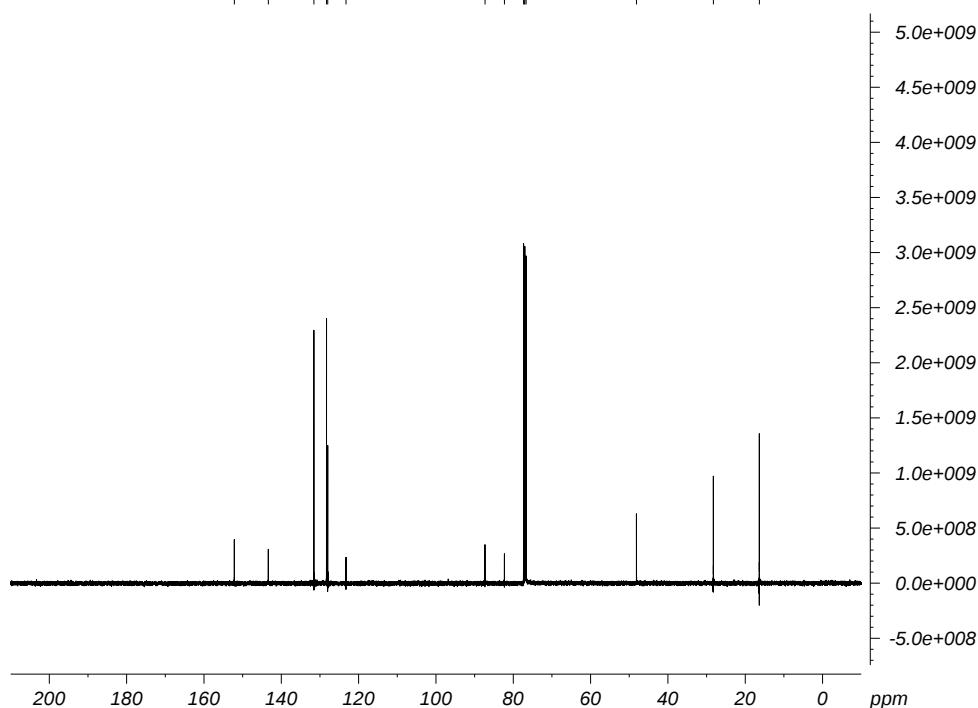
NAME      090910.1apex011
EXPNO     1
PROCNO    1
Date_     20090911
Time      17.08
INSTRUM   spect
PROBHD    5 mm Multispec1
PULPROG   zgpg30
TD         65536
SOLVENT   CDCl3
NS         2048
DS         4
SWH         8223.680 Hz
F2RES     0.200000 Hz
AQ         1.9921444 sec
RG         655.360
DE         8.00 umsec
TE         300.2 K
D1         4.0000000 sec
d11        0.0000000 sec
NCHSTRT   0.0000000 sec
NCHXPR    0.0100000 sec

```

```

===== CHANNEL f1 =====
NUC1       13C
P1         9.00 umsec
PL1        -1.00 dB
SFO1       400.1314000 MHz
SF         400.1314000 MHz
WDW         EM
SSB         0
GB          0
SR          0.70 Hz
GB          0
PC          1.00

```



```

NAME      090912.1apex011
EXPNO     1
PROCNO    1
Date_     20090912
Time      17.08
INSTRUM   spect
PROBHD    5 mm Multispec1
PULPROG   zgpg30
TD         65536
SOLVENT   CDCl3
NS         2048
DS         4
SWH         24154.580 Hz
F2RES     0.137140 Hz
AQ         0.6183476 sec
RG         5782.4
DE         20.700 umsec
TE         300.2 K
D1         0.3200000 sec
d11        0.0000000 sec
NCHSTRT   0.0000000 sec
NCHXPR    0.0100000 sec

```

```

===== CHANNEL f1 =====
NUC1       13C
P1         9.00 umsec
PL1        -1.00 dB
SFO1       100.6213293 MHz

```

```

===== CHANNEL f2 =====
CPDPRG2   waltz16
NUC2       13C
PCPD2      80.00 umsec
PL2        -1.00 dB
PL12       16.00 dB
SFO2       400.1314000 MHz
SF         400.1314000 MHz
WDW         EM
SSB         0
GB          0
SR          0.70 Hz
GB          0
PC          1.00

```

Chemical structure: Cc1ccc(cc1)C#CCCN2C=CN=C2

¹H NMR spectrum (400 MHz, CDCl₃) data:

Chemical Shift (ppm)	Integration
7.52, 7.31, 7.29, 7.26, 7.12, 7.10, 7.07, 6.95	0.96, 2.48, 2.90, 0.93
4.16, 4.14, 4.12	2.00
2.41, 2.39, 2.37, 2.34, 2.06, 2.04, 2.04, 2.03, 2.01, 1.99	4.87, 2.16

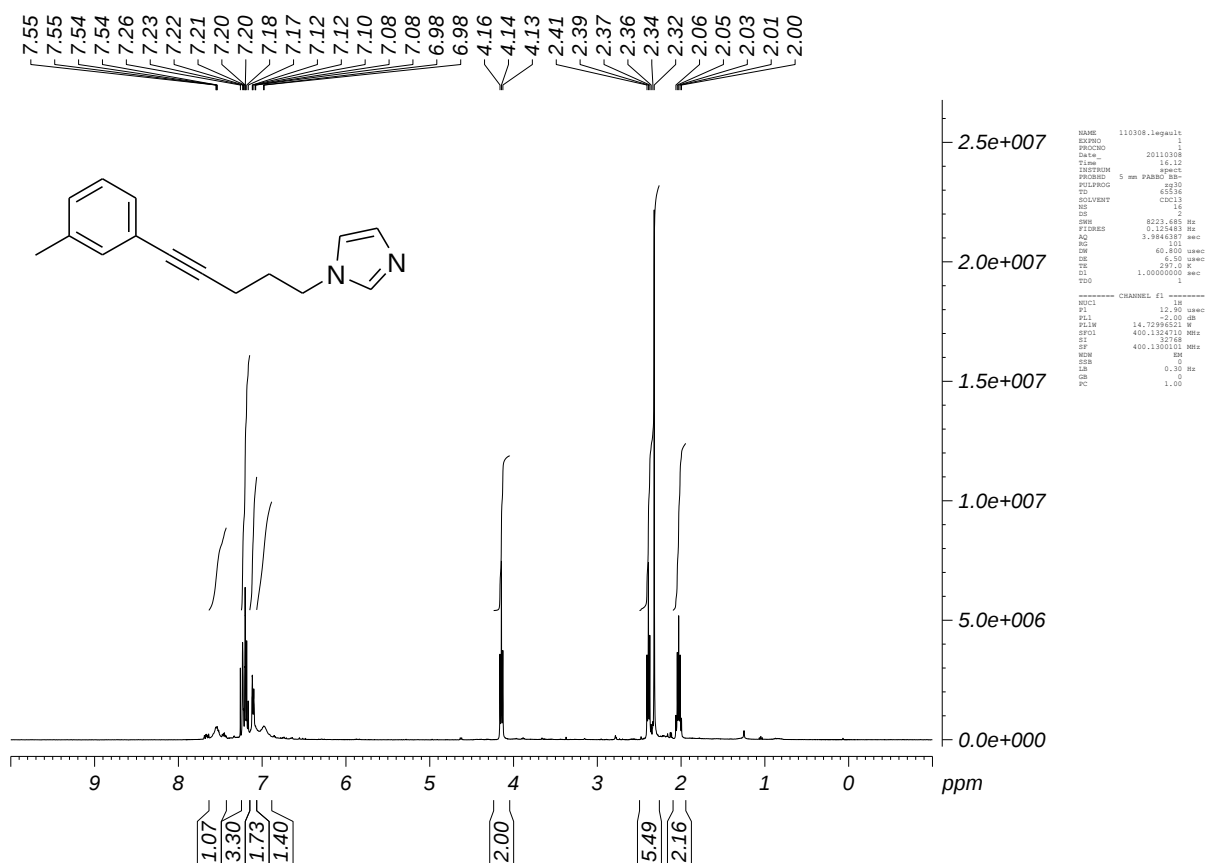
137.99
137.30
131.38
129.60
129.02
120.18
118.81
86.67
82.27
77.31
77.00
76.68
45.35
29.75
21.38
16.35

1.8e+007
1.6e+007
1.4e+007
1.2e+007
1.0e+007
8.0e+006
6.0e+006
4.0e+006
2.0e+006
0.0e+000

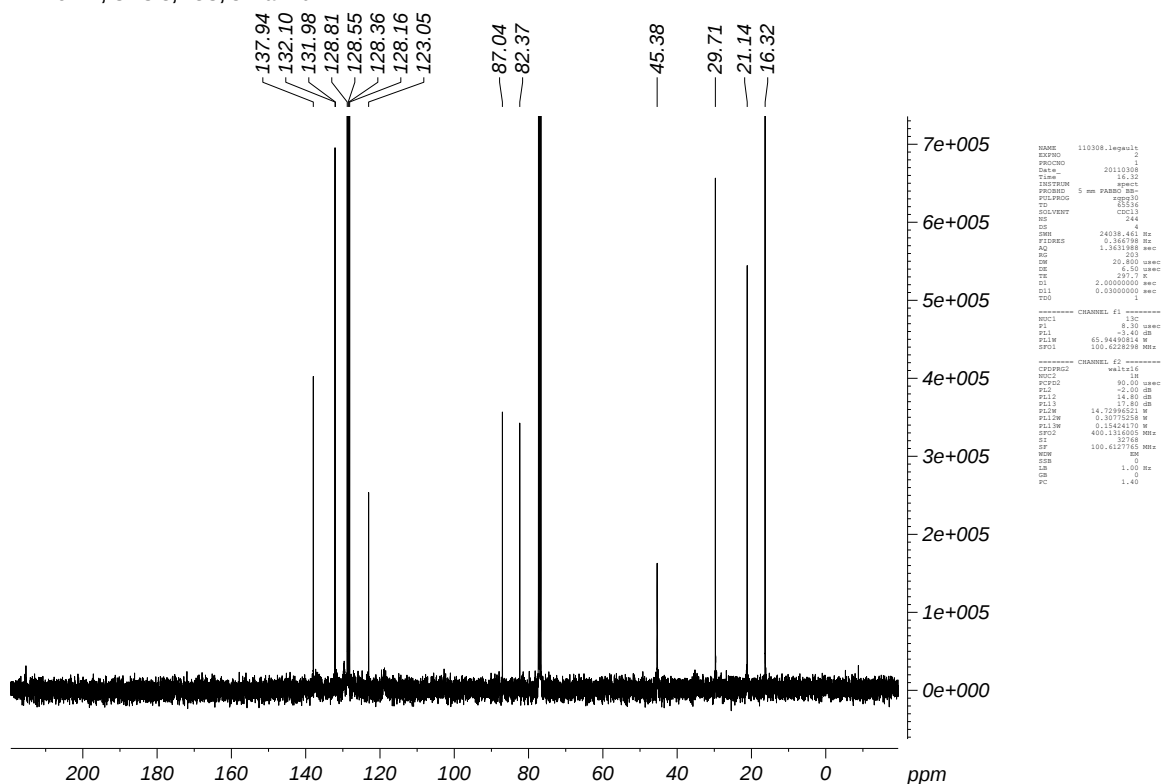
200 180 160 140 120 100 80 60 40 20 0 ppm

NAME 110120.Legend1	
EXPNO	3
PROCNO	1
Date_	20110120
Time	18.16
INSTRUM	nmr4h
PROBHD	5 mm QNP1H
PULPROG	zgpg30
TD	65536
CYC1	1024
SOLVENT	CDCl3
NS	1024
DS	4
SWH	24038.461 Hz
F2HRES	0.164758 Hz
AQ	1.3631988 sec
RG	320
WE	20.800 usec
DE	6.50 usec
TE	300.2 K
D1	2.00000000 sec
d11	0.13000000 sec
TD0	1
----- CHANNEL f1 -----	
NUC1	13C
PC1	~1.40 GH
PL1W	65.94492614 W
SFO1	100.6228298 MHz
----- CHANNEL f2 -----	
CPDPRG2	waltz16
NUC2	1H
PCPD2	90.00 usec
PL2	-2.00 GH
PL12	14.80 GH
PL13	17.80 GH
PL1W	14.72998521 W
PL1W	0.10779158 W
PL1W	0.1164170 W
SFO2	400.116055 MHz
DE	12.00 usec
DE	100.6127751 MHz
NS2	0
DS2	1.00 Hz
LA	1.00 Hz
PC	1.40

mlc177, CDCl₃, 1H, 3 mar 2011



mlc177, CDCl₃, 13C, 8 mar 2011



Chemical structure: CC1=CC=C(C#CCCN2C=NC=N2)C=C1[N+](=O)[O-]

¹H NMR spectrum (ppm) showing peaks and integration values:

Chemical Shift (ppm)	Integration
8.17, 8.16, 8.15, 8.14, 8.11	~4.0
7.96, 7.53, 7.50, 7.26	~3.0
4.38, 4.36, 4.34	~1.0
2.50, 2.48, 2.47, 2.25, 2.23, 2.21, 2.20, 2.18	~3.0

[illegible]

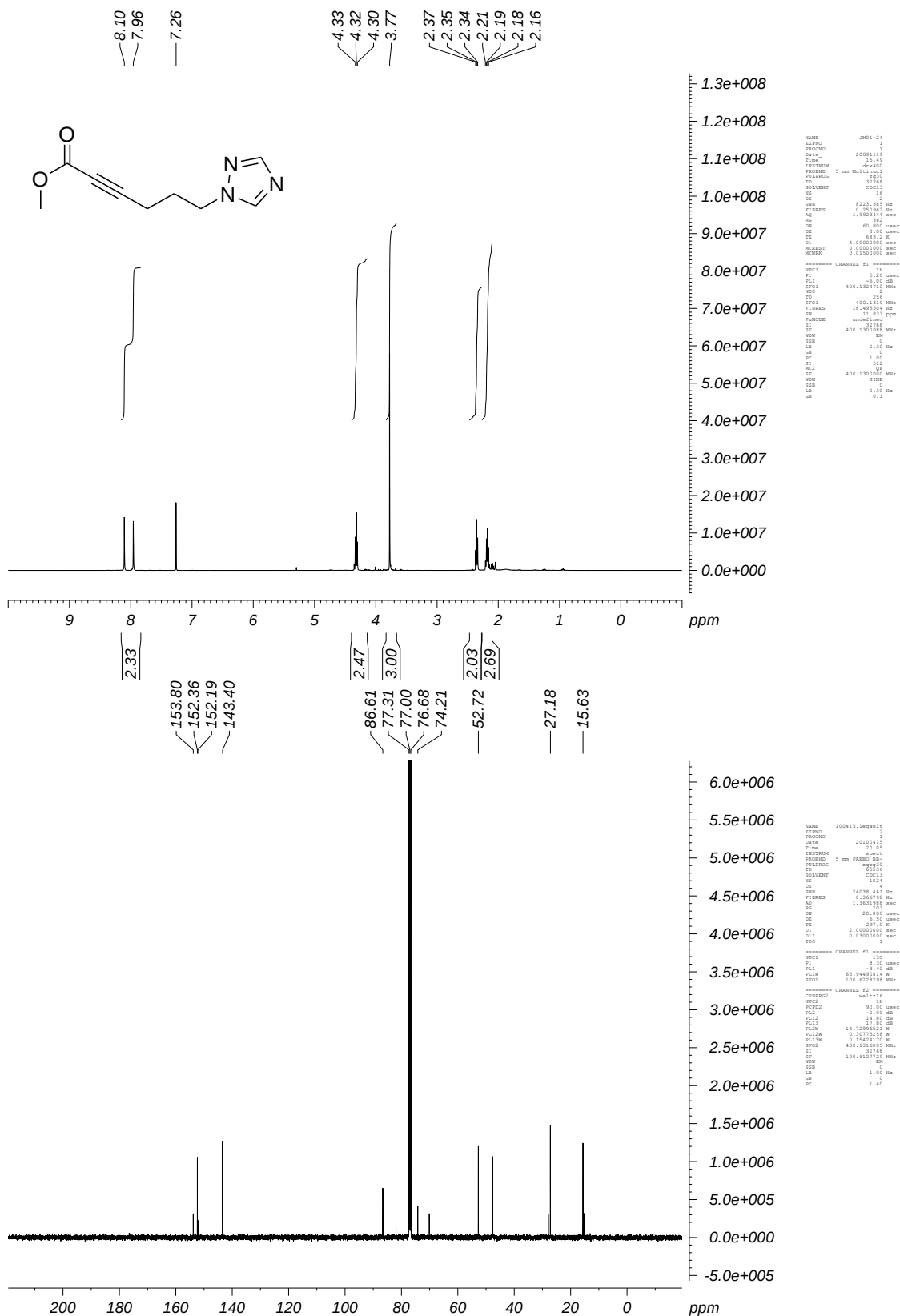
152.26
143.36
132.32
130.24
123.53
104.55
93.42
80.53
77.32
77.00
76.68
48.13
28.08
16.62

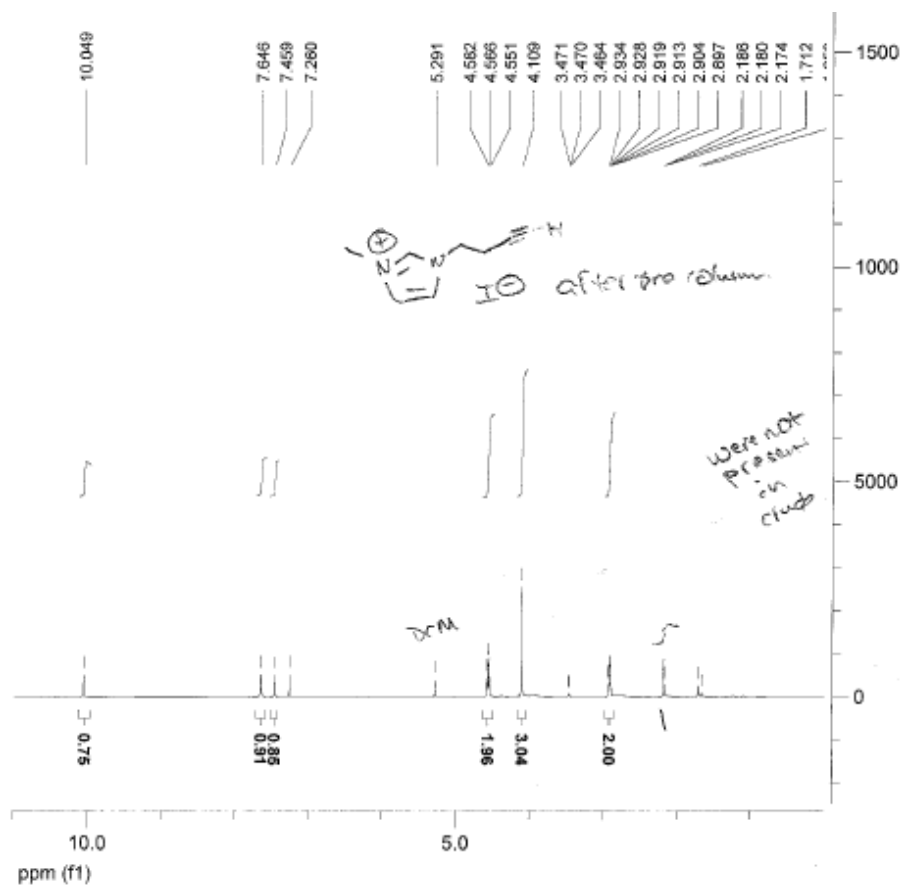
3.0e+008
2.5e+008
2.0e+008
1.5e+008
1.0e+008
5.0e+007
0.0e+000
-5.0e+007

200 180 160 140 120 100 80 60 40 20 0 ppm

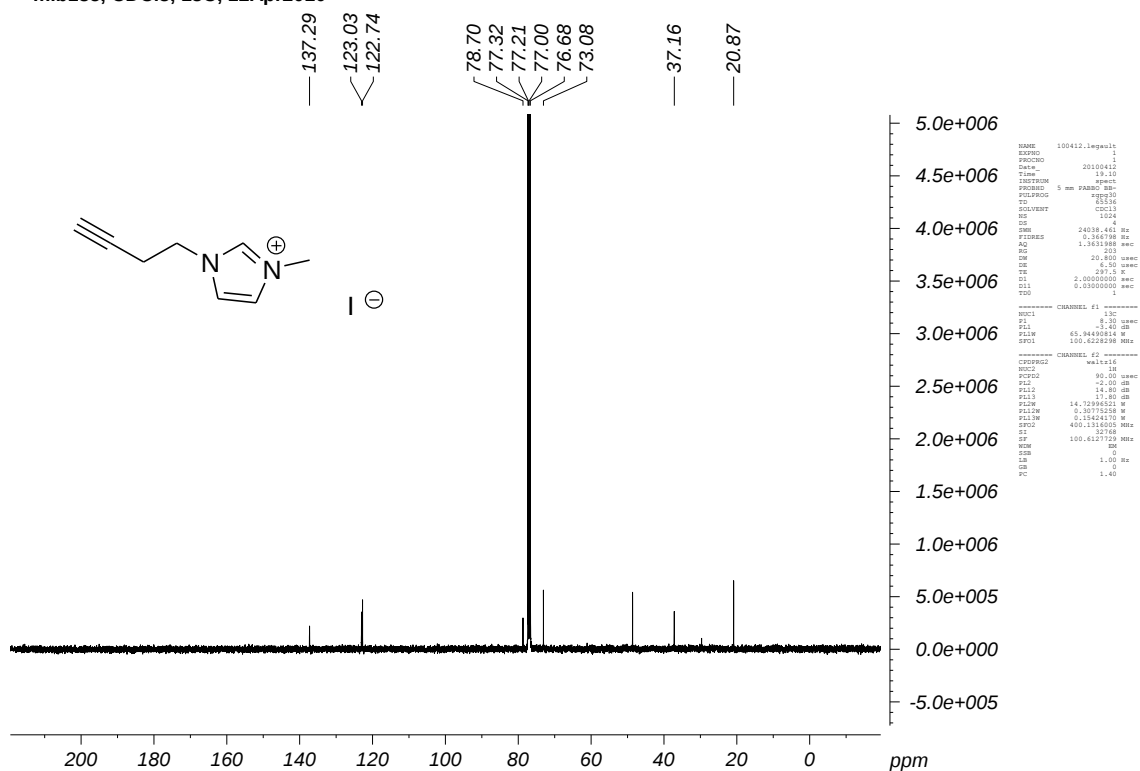
3.0e+008
2.5e+008
2.0e+008
1.5e+008
1.0e+008
5.0e+007
0.0e+000
-5.0e+007

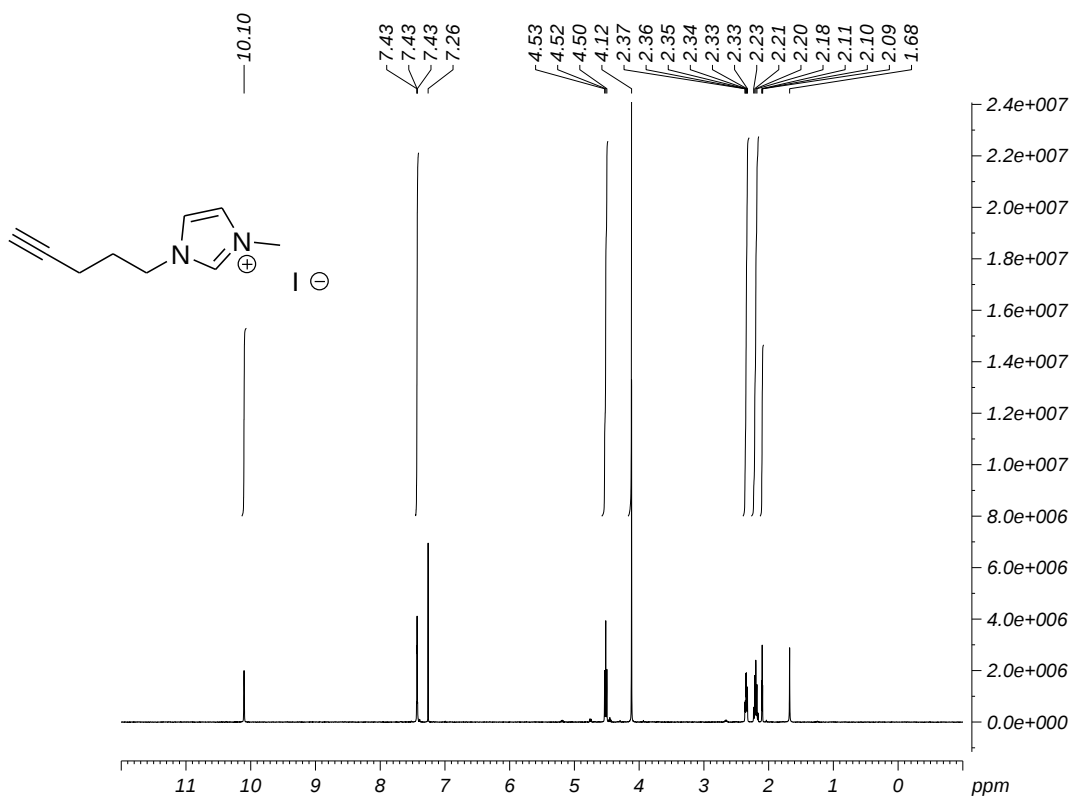
```
NAME          091122.legalc
EXPNO         1
PROCNO        1
PROCDATE      20091122
Time          16:10
INSTRUM       spect
PROBHD        5 mm Multinuc1
PULPROG       zgpg30
SOLVENT       CDCl3
SS            5000
SI            0
GB            0
SFO1          24154.150 Hz
FIDRES        0.371740 Hz
AQ            0.6783474 sec
RG            64
DM            20.700 usec
DE            0.00 usec
TE            283.2 K
G1            0.32000000 sec
G2            0.03000000 sec
MPCNST        0.0000000 sec
MORPHY        0.0000000 sec
----- CHANNEL f1 -----
NUC1           13C
F1             7.20 MHz
SFO1           100.622393 Hz
----- CHANNEL f2 -----
CPDPRG2       wait4a
SI            32
PC12          80.00 usec
F2            +3.31 dB
FIDRES        16.820 Hz
SFO2          400.115355 MHz
SI            32
DM            37.768
SFO1          100.6127394 MHz
SS            0
GB            0
LQ            0.00 Hz
SGB           0
SI            0
```





mlb185, CDCl₃, 13C, 12Apr2010

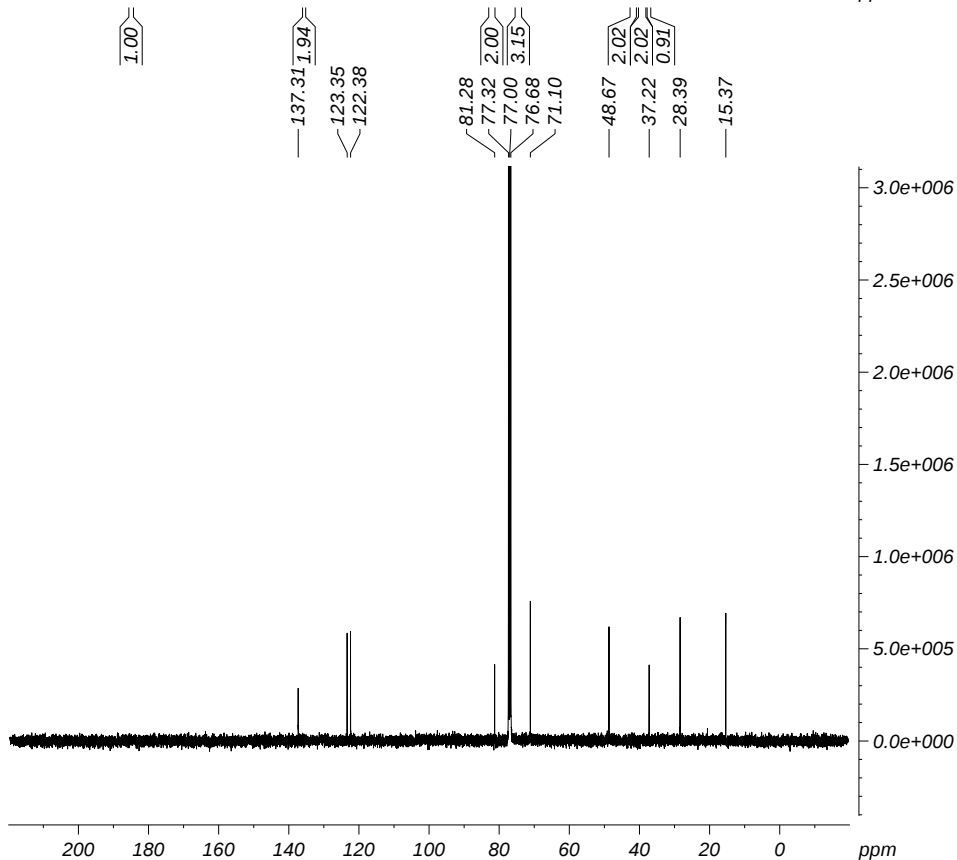




```

NAME 100427.1apex15
EXPNO 3
PROCNO 1
Date_ 20100427
Time 18.01
INSTRUM spect
PROBHD 5 mm QNP1H1
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 1634
DS 4
SWH 8223.662 Hz
FIDRES 0.123483 Hz
AQ 0.1864327 sec
RG 320
AQ 60.800 usec
DE 6.20 usec
TE 296.1 K
D1 1.00000000 sec
D11 1
===== CHANNEL f1 =====
NUC1 1H
P1 12.00 usec
PL1 -2.00 dB
PL12 14.72948015 W
SFO1 400.1304710 MHz
SF 400.1304710 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 1.00
PC 1.00

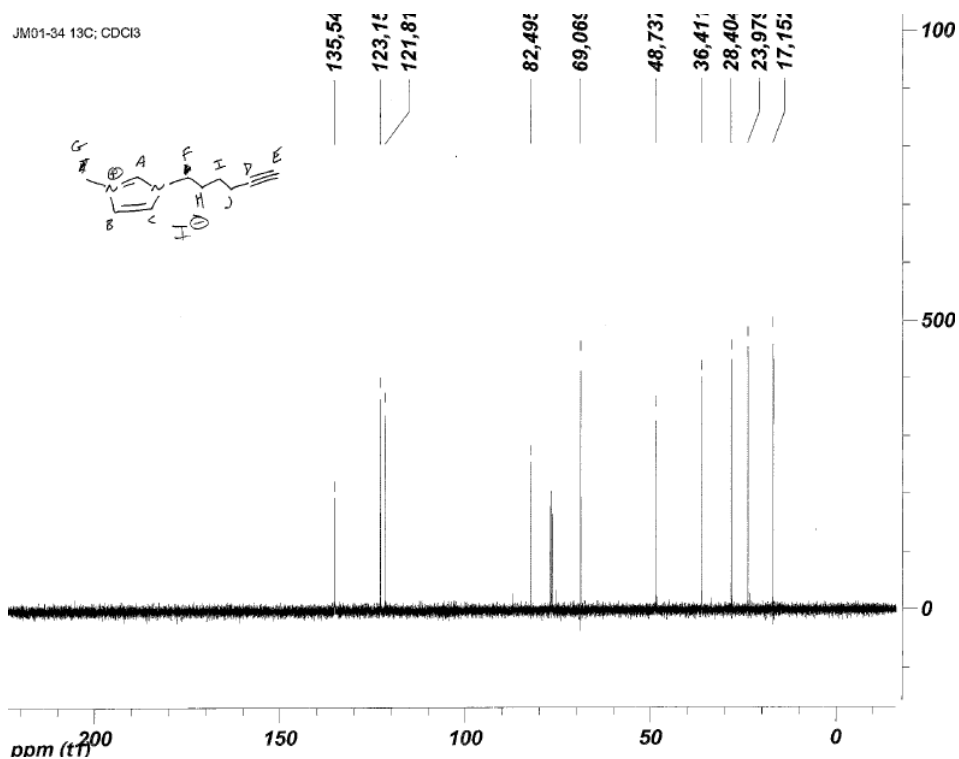
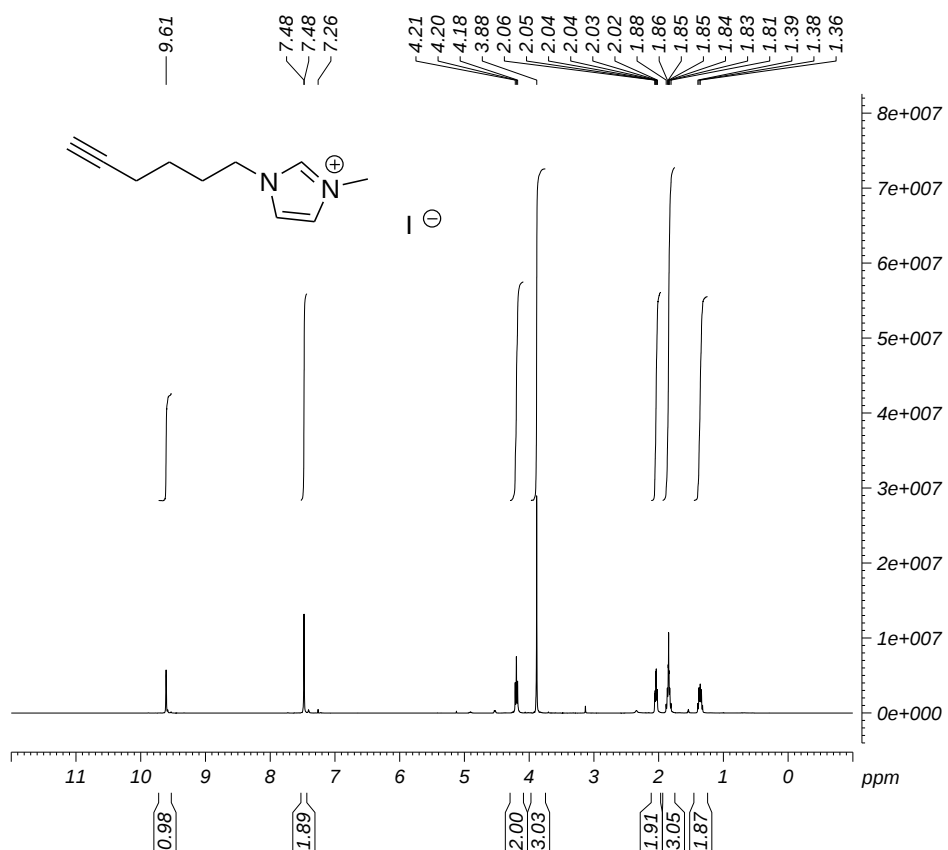
```

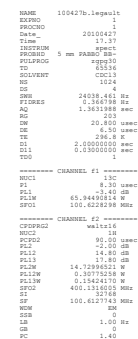
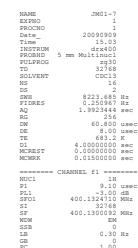


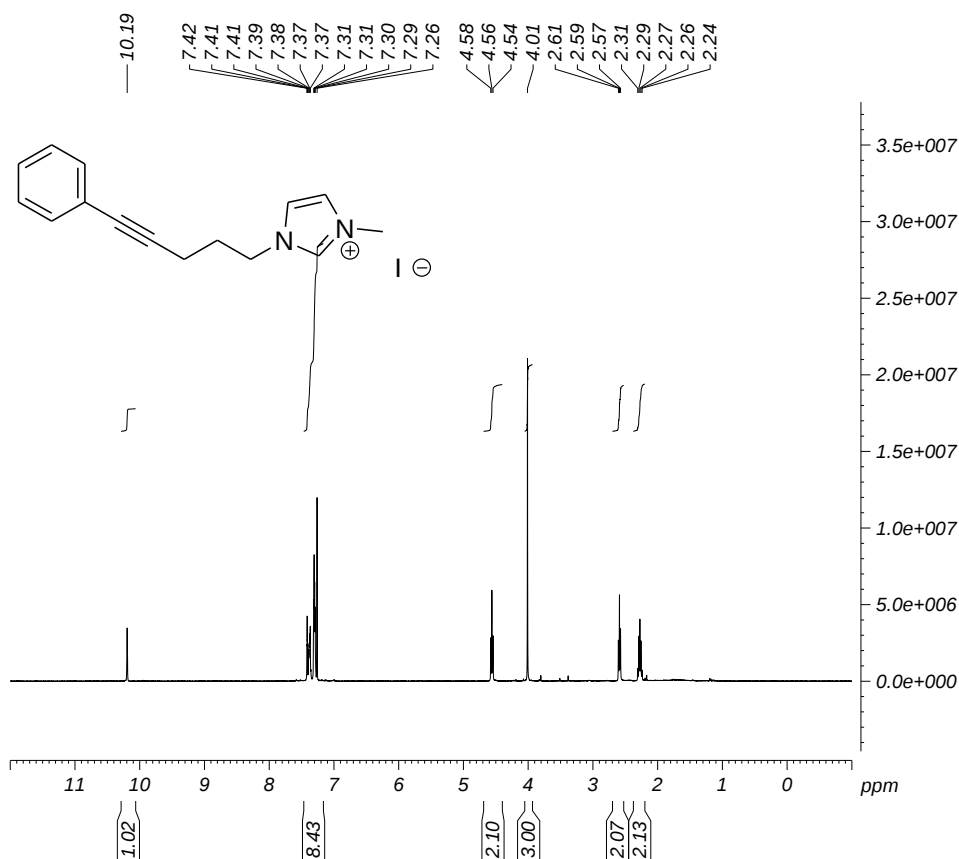
```

NAME 100427.1apex15
EXPNO 3
PROCNO 1
Date_ 20100427
Time 18.01
INSTRUM spect
PROBHD 5 mm QNP1H1
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 1634
DS 4
SWH 24038.441 Hz
FIDRES 0.266778 Hz
AQ 0.1864327 sec
RG 320
AQ 60.800 usec
DE 6.20 usec
TE 296.1 K
D1 1.00000000 sec
D11 1
===== CHANNEL f1 =====
NUC1 13C
P1 8.00 usec
PL1 -3.40 dB
PL12 101.6228238 W
SFO1 101.6228238 MHz
===== CHANNEL f2 =====
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 1634
DS 4
SWH 80.00 usec
FIDRES 0.123483 Hz
AQ 0.1864327 sec
RG 320
AQ 60.800 usec
DE 6.20 usec
TE 296.1 K
D1 1.00000000 sec
D11 1
===== CHANNEL f2 =====
NUC1 13C
P1 8.00 usec
PL1 -3.40 dB
PL12 101.6228238 W
SFO1 101.6228238 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 1.00
PC 1.00

```



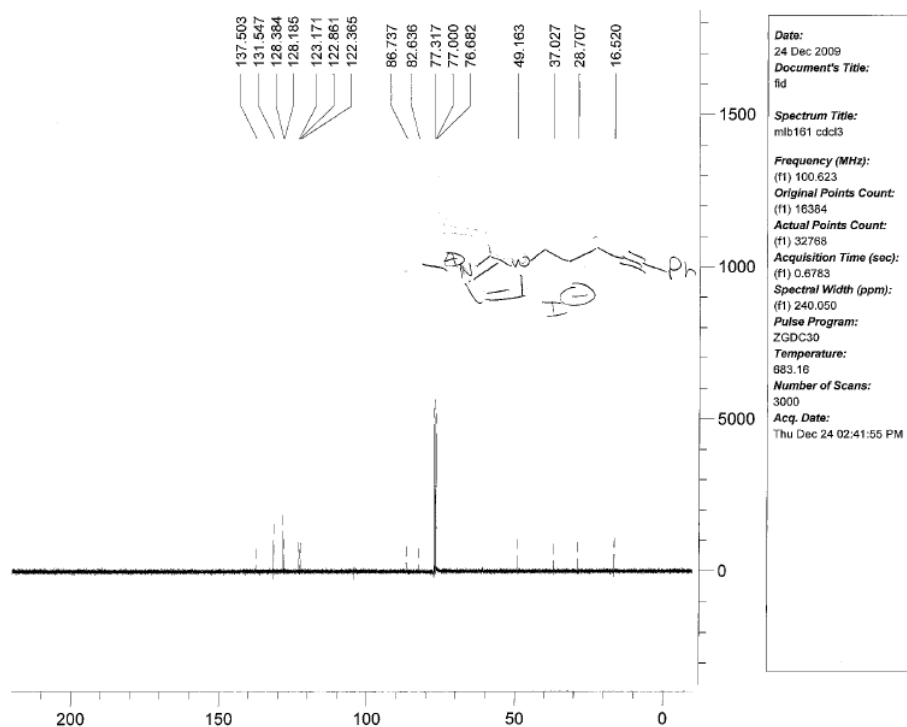




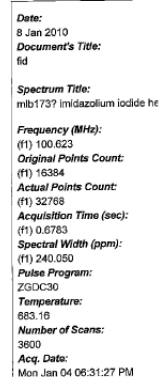
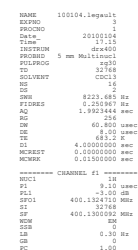
```

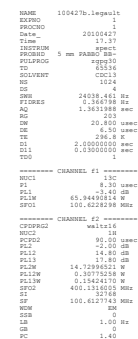
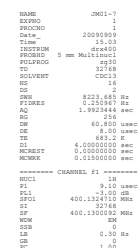
NAME 091224.lapex15
EXPNO 1
PROCNO 1
Date_ 20091224
Time 13:50
INSTRUM dr4000
PROBHD 5 mm Multinuc1
PULPROG zgpg30
TD 32768
SOLVENT cdcl3
AQ 12.768
RG 32
DS 1.0
FIDRES 0.223465 Hz
AQ 1.395244 sec
PC 512
DE 60.800 usec
TE 8.00 usec
TD 683.2 K
DE 4.00000000 sec
MCREST 0.00000000 sec
MCHRG 0.01000000 sec
===== CHANNEL f1 =====
NUC1 13
P1 9.10 usec
PL 0.00 dB
SFO1 400.1324710 MHz
SI 32768
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

```

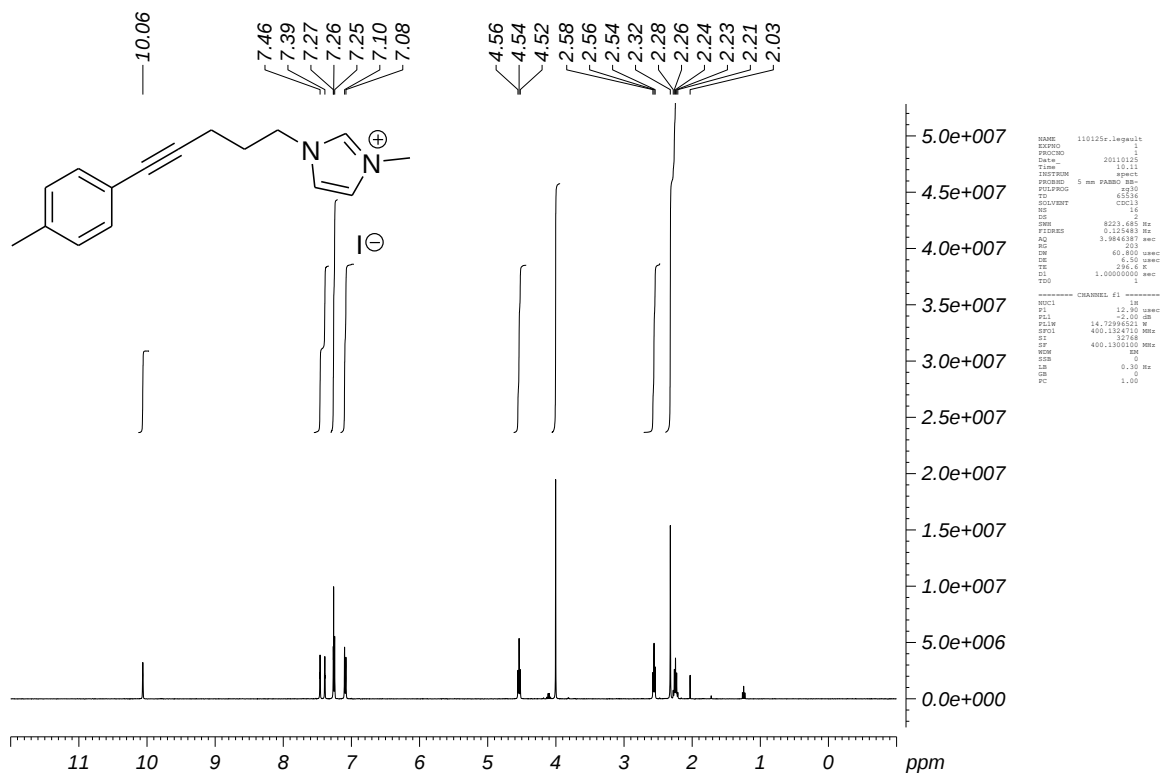


Date: 24 Dec 2009
Document's Title: fid
Spectrum Title: mlb161 cdcl3
Frequency (MHz): (f1) 100.623
Original Points Count: (f1) 16384
Actual Points Count: (f1) 32768
Acquisition Time (sec): (f1) 0.6783
Spectral Width (ppm): (f1) 240.050
Pulse Program: ZGDC30
Temperature: 083.16
Number of Scans: 3000
Acq. Date: Thu Dec 24 02:41:55 PM

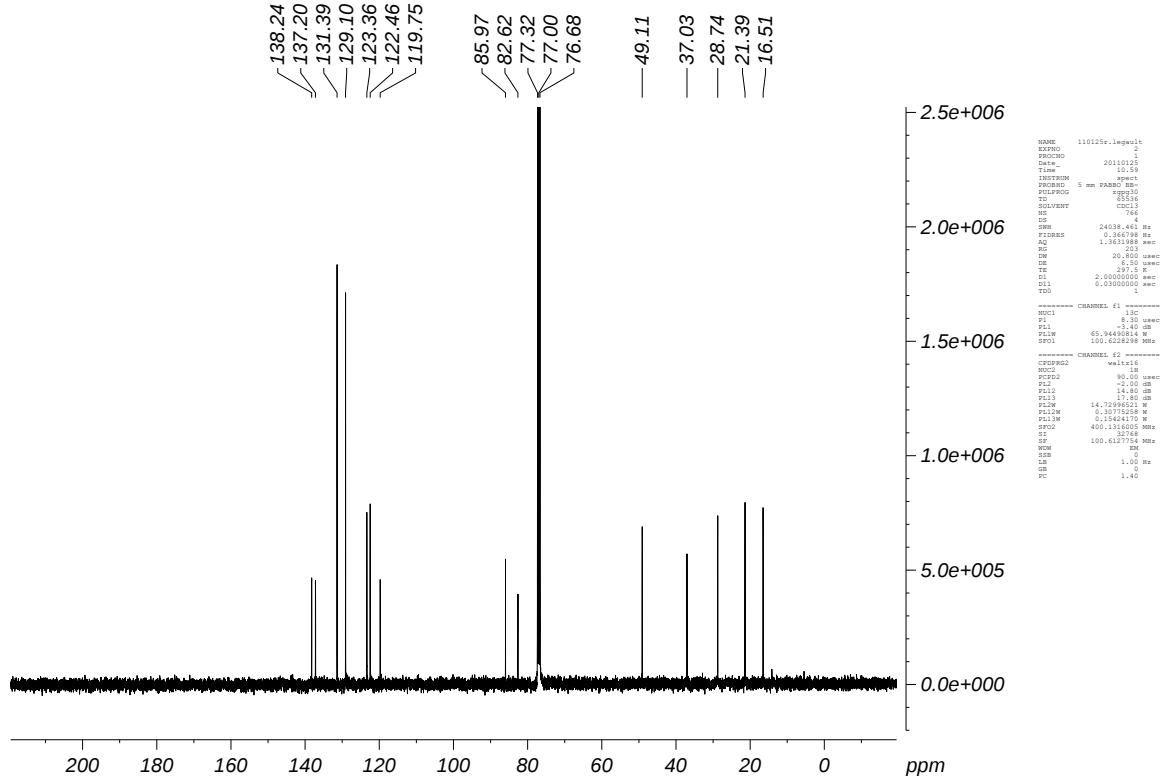


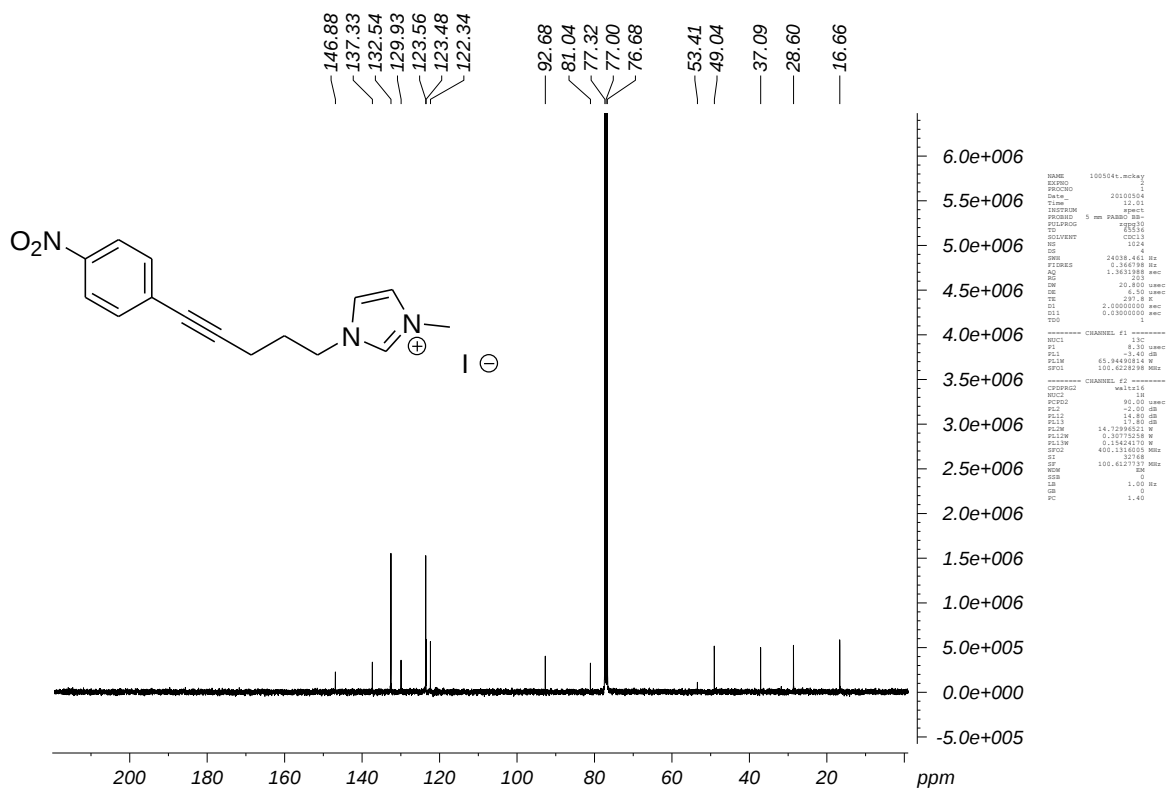


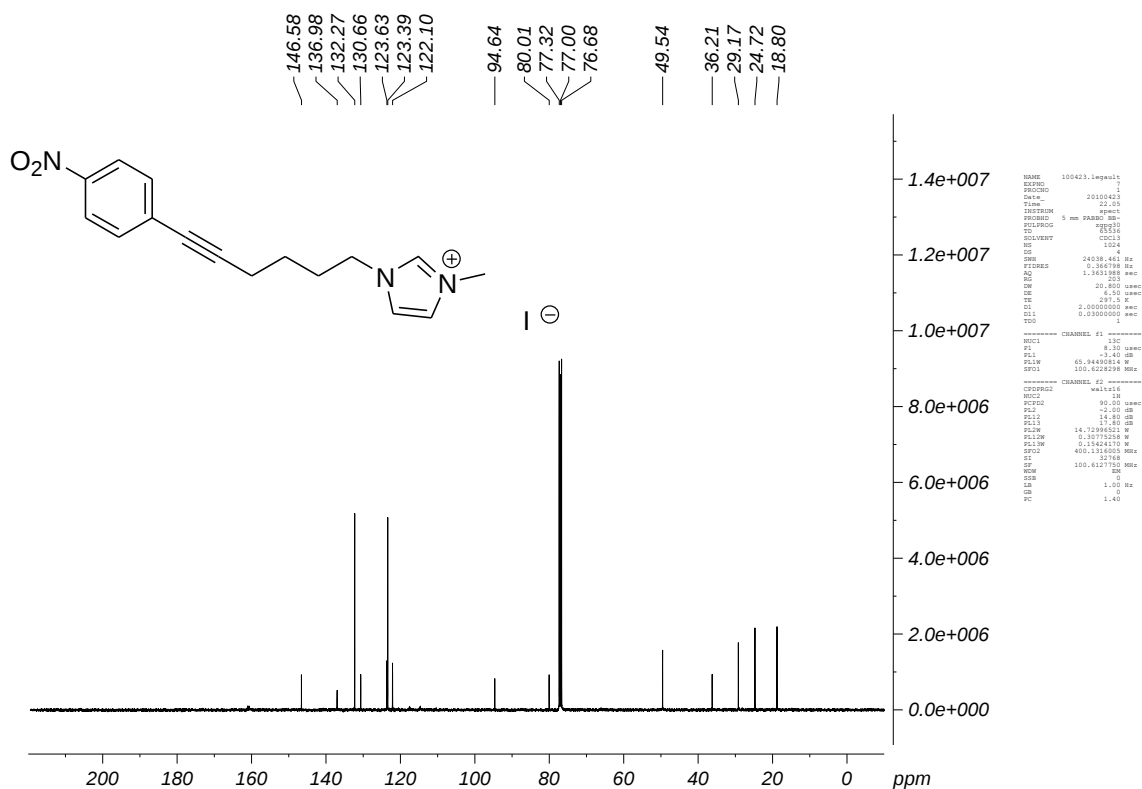
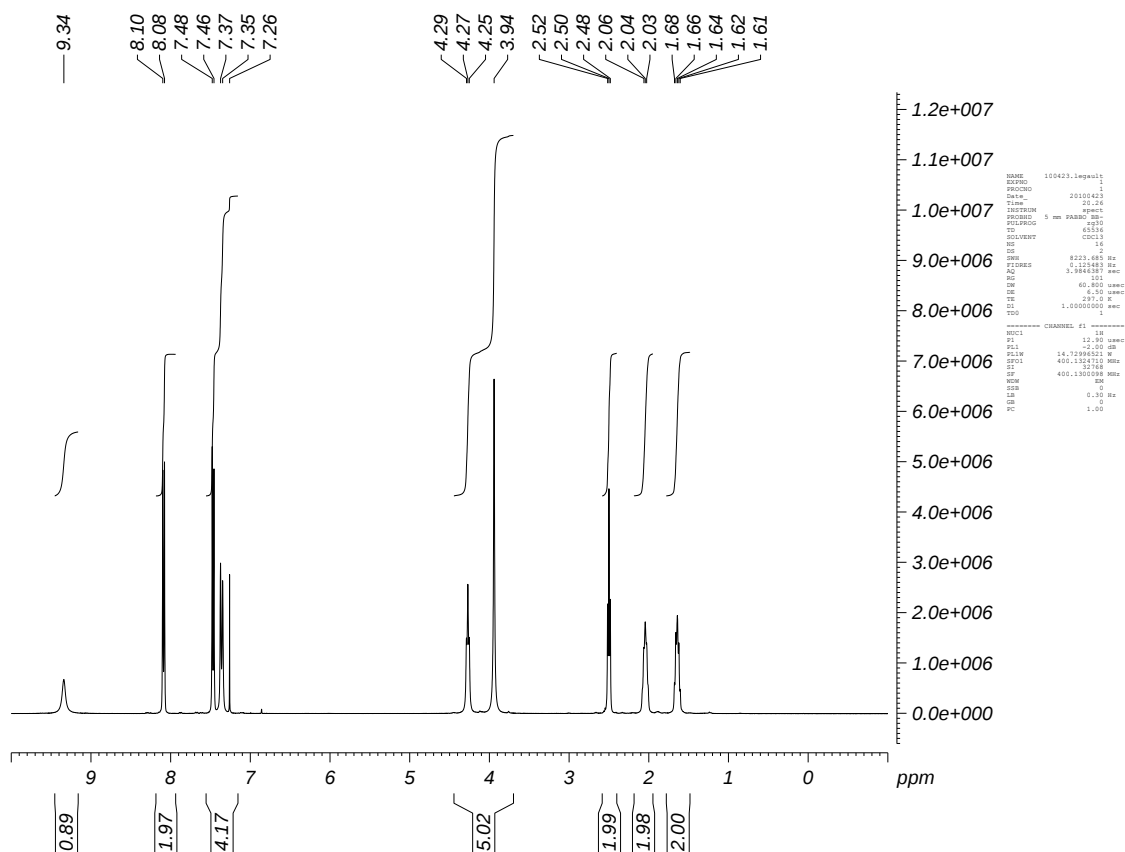
MLC149, CDCl₃, 1H, 25Jan2011

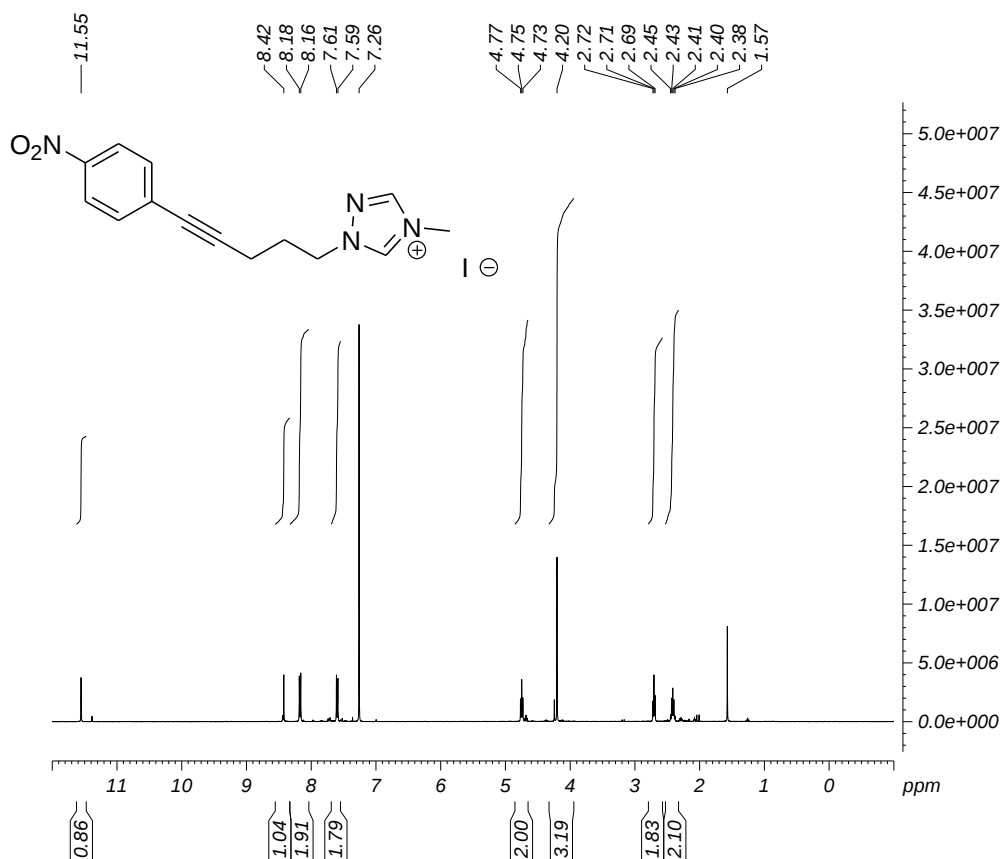


MLC149, CDCl₃, 13C, 25Jan2011







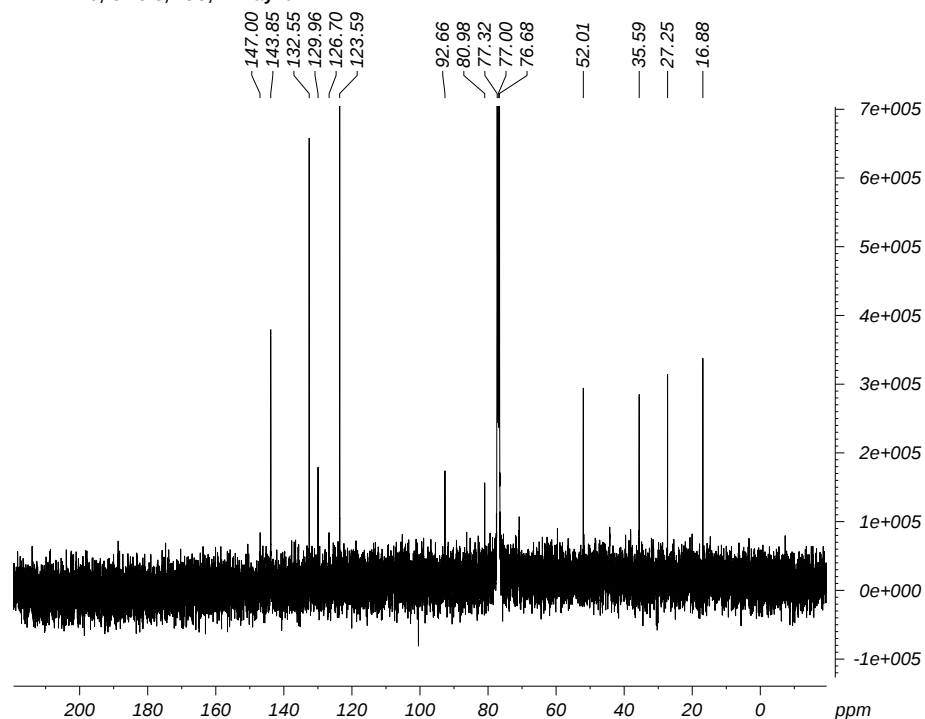


```

NAME 091123.lapex11
EXPNO 1
PROCNO 1
Date_ 20091123
Time 21:25
INSTRUM spect
PULPROG zgpg30
PROBHD 5 mm Maltinnoc1
NUC1 13C
SOLVENT CDCl3
NS 2768
DS 4
SWH 8223.660 Hz
FIDRES 1.225964 Hz
AQ 1.1602444 sec
RG 724.2
WDW 60.800 usec
SS 8.20 usec
TE 300.2 K
D1 4.0000000 sec
d11 0.0000000 sec
MCHST 2
RG 0.1100000 sec
===== CHANNEL f1 =====
NUC1 13C
P1 18.00 usec
PL1 0.00 dB
SFO1 400.1324710 MHz
WDW 60.800 usec
SS 8.20 usec
TE 300.2 K
D1 4.0000000 sec
d11 0.0000000 sec
MCHST 2
RG 0.1100000 sec
===== CHANNEL f2 =====
NUC2 1H
P2 12.00 usec
PL2 0.00 dB
SFO2 400.1324710 MHz
WDW 60.800 usec
SS 8.20 usec
TE 300.2 K
D2 0.0000000 sec
d21 0.0000000 sec
MCHST 2
RG 0.1100000 sec
===== CHANNEL f3 =====
NUC3 1H
P3 12.00 usec
PL3 0.00 dB
SFO3 400.1324710 MHz
WDW 60.800 usec
SS 8.20 usec
TE 300.2 K
D3 0.0000000 sec
d31 0.0000000 sec
MCHST 2
RG 0.1100000 sec

```

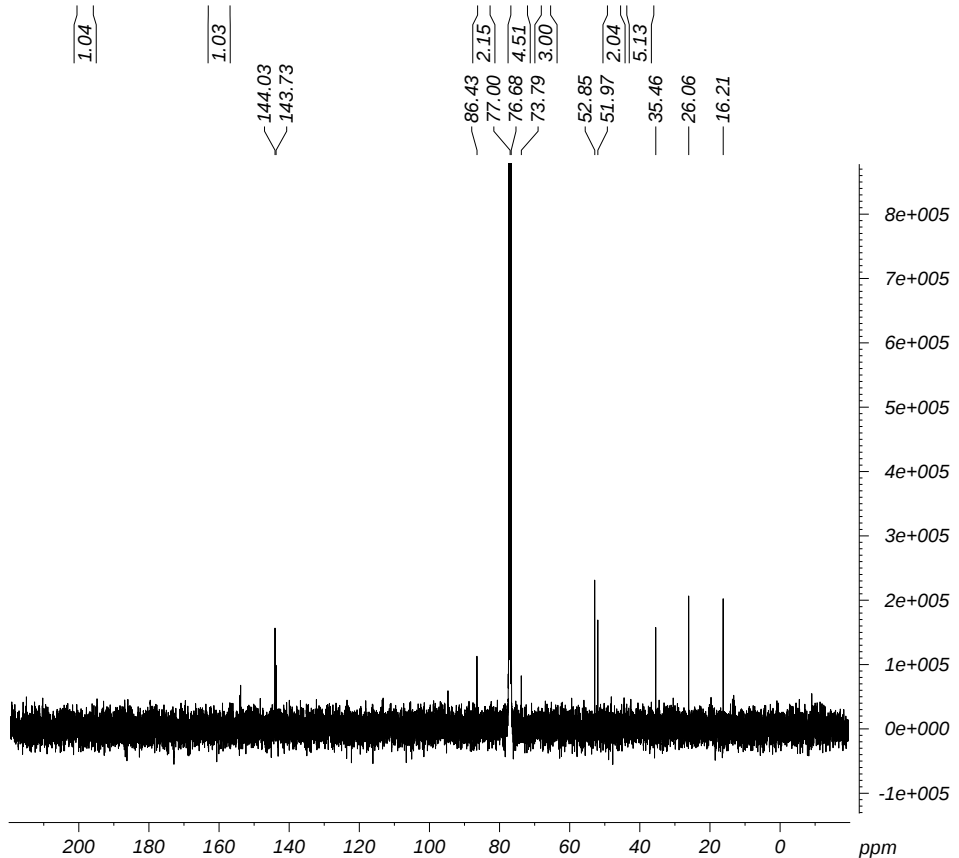
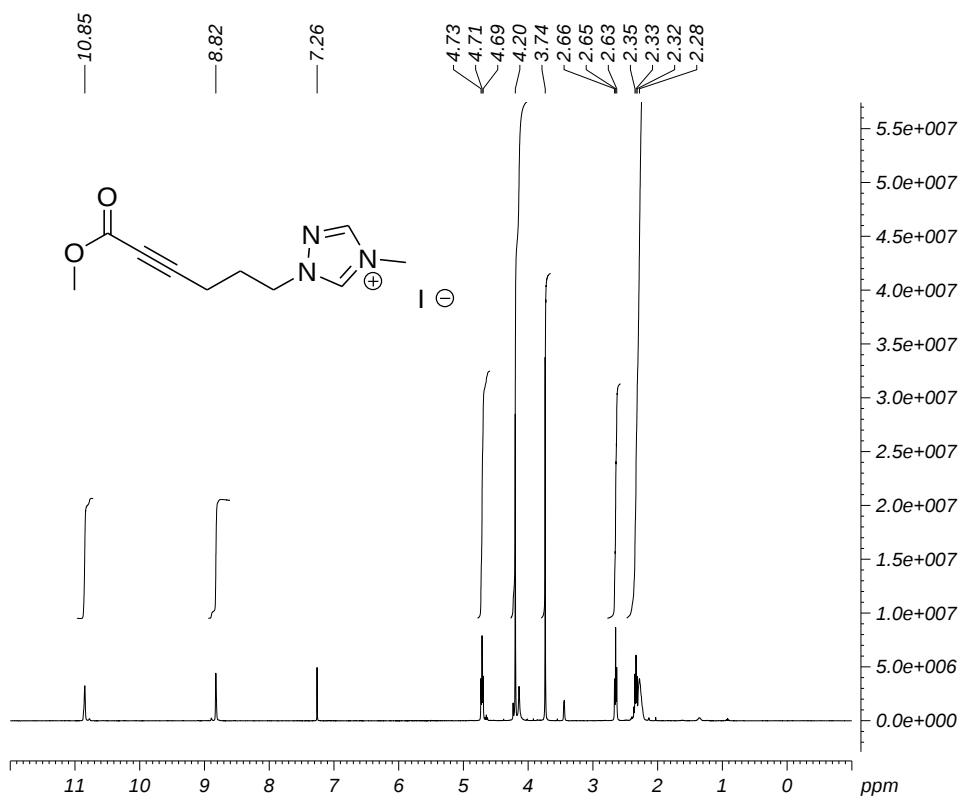
MLB129, CDCl3, 13C, 11May2011



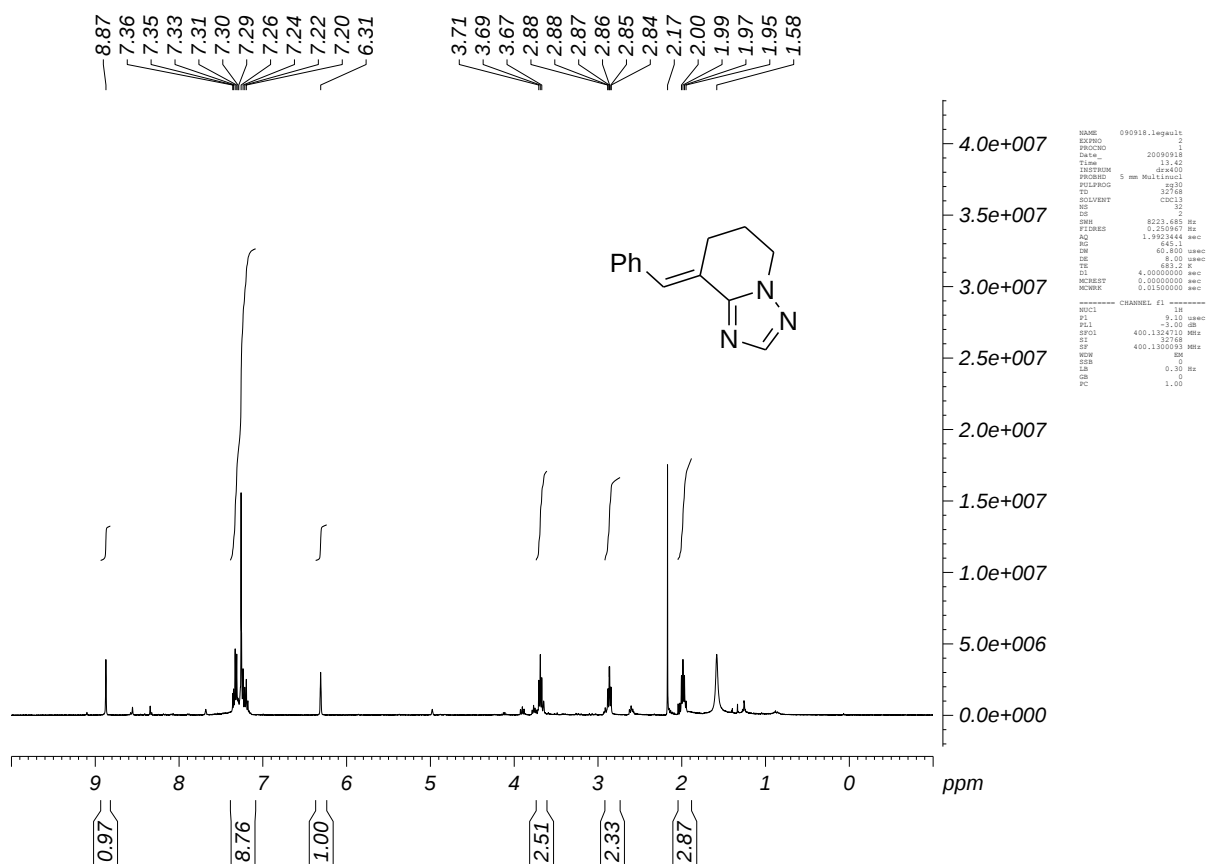
```

NAME 110511r.lapex11
EXPNO 1
PROCNO 1
Date_ 20110511
Time 17:37
INSTRUM spect
PULPROG zgpg30
PROBHD 5 mm QNP1H1
NUC1 13C
SOLVENT CDCl3
NS 6334
DS 4
SWH 24038.460 Hz
FIDRES 0.346768 Hz
AQ 1.3831000 sec
RG 277.8
WDW 60.800 usec
SS 8.20 usec
TE 300.2 K
D1 2.0000000 sec
d11 0.0000000 sec
MCHST 2
===== CHANNEL f1 =====
NUC1 13C
P1 18.00 usec
PL1 0.00 dB
SFO1 100.6261260 MHz
===== CHANNEL f2 =====
NUC2 1H
P2 12.00 usec
PL2 0.00 dB
SFO2 400.1324710 MHz
WDW 60.800 usec
SS 8.20 usec
TE 300.2 K
D2 0.0000000 sec
d21 0.0000000 sec
MCHST 2
RG 0.1100000 sec
===== CHANNEL f3 =====
NUC3 1H
P3 12.00 usec
PL3 0.00 dB
SFO3 400.1324710 MHz
WDW 60.800 usec
SS 8.20 usec
TE 300.2 K
D3 0.0000000 sec
d31 0.0000000 sec
MCHST 2
RG 0.1100000 sec

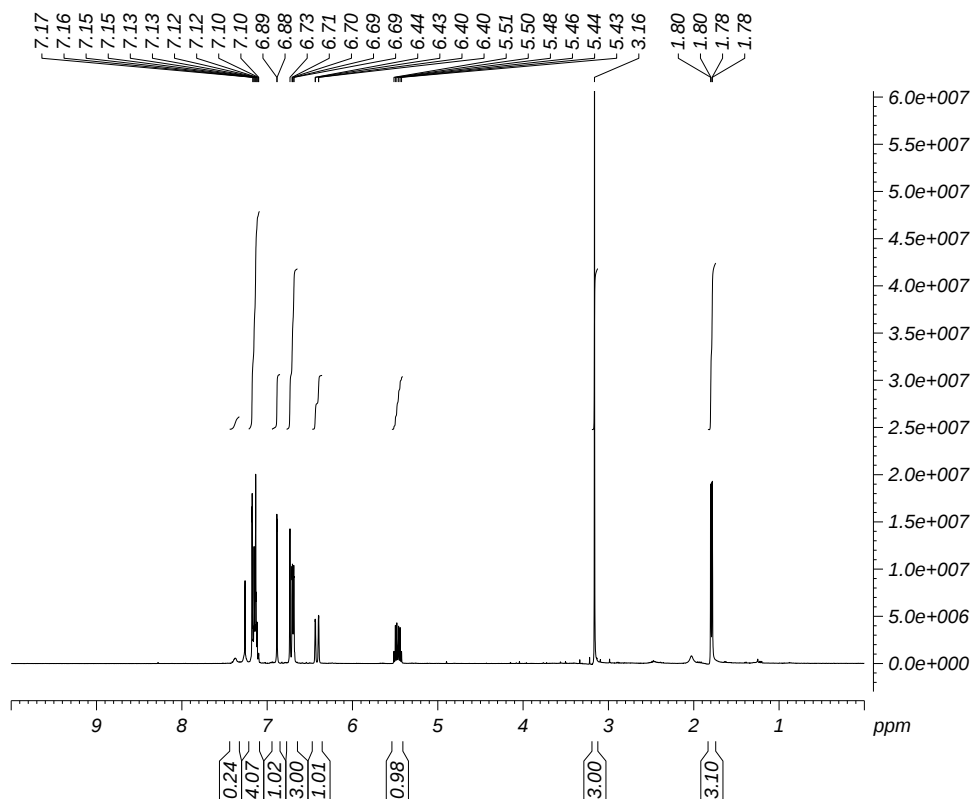
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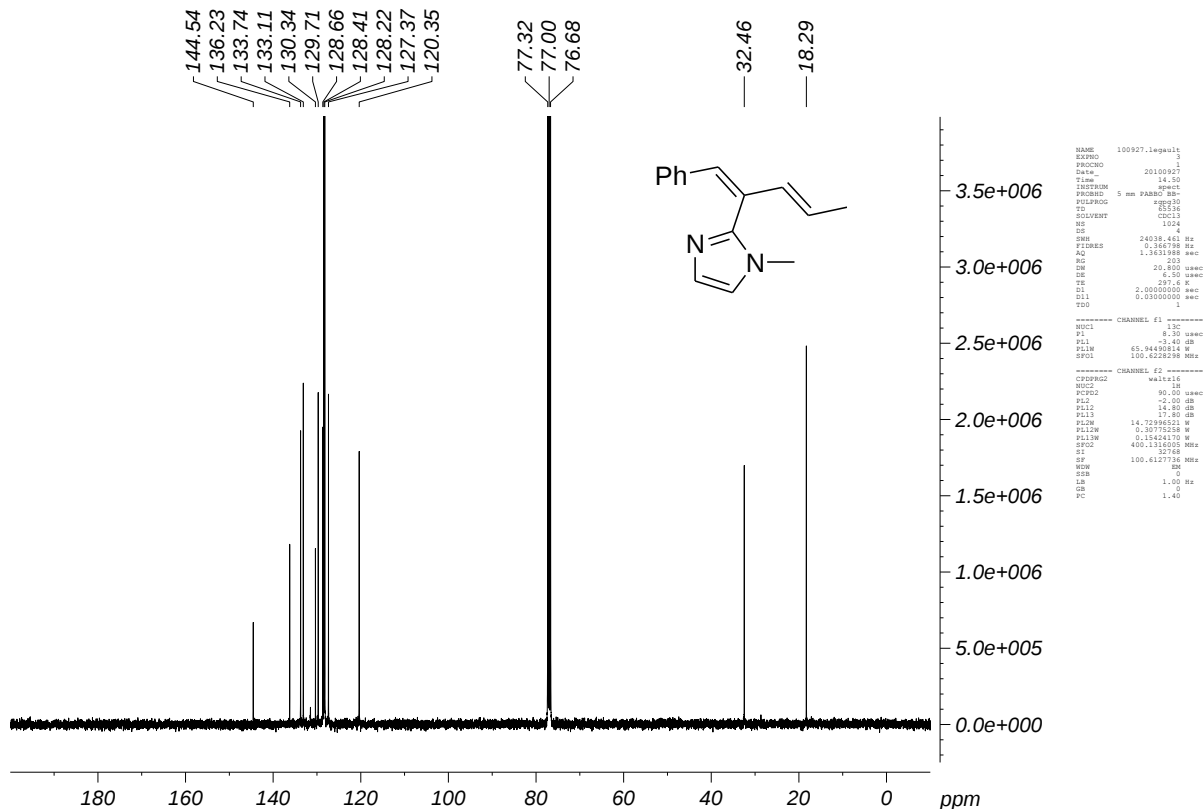
mlb87 b cdc13



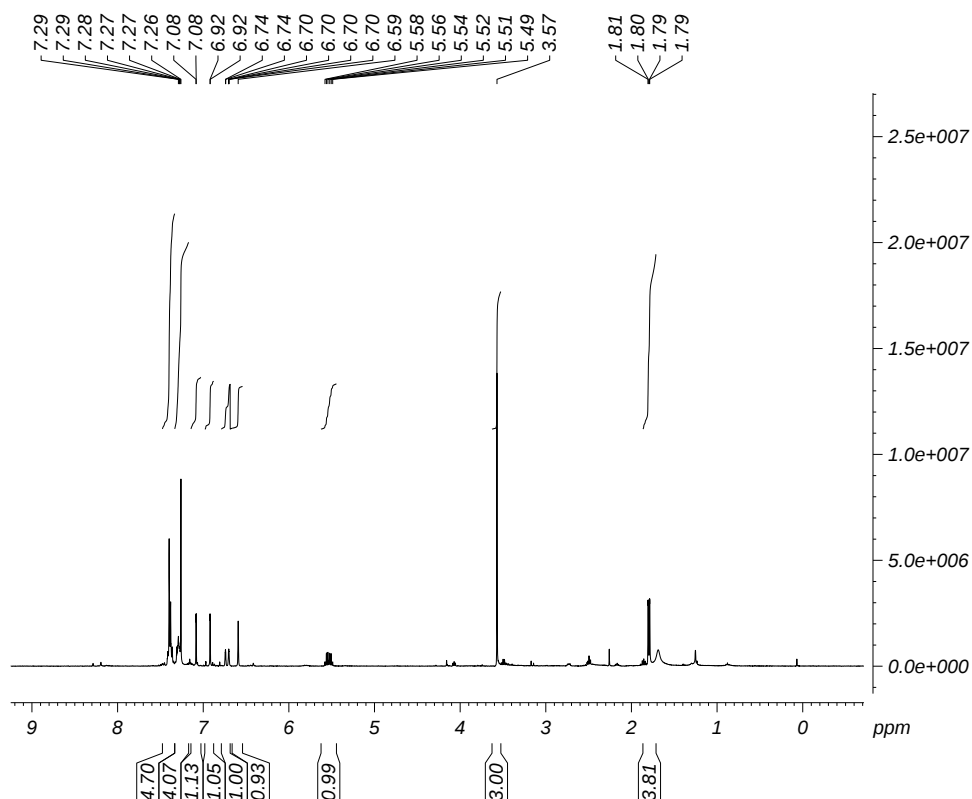
mlc129-4-8, CDCl₃, 1H, 27 SEP 2010



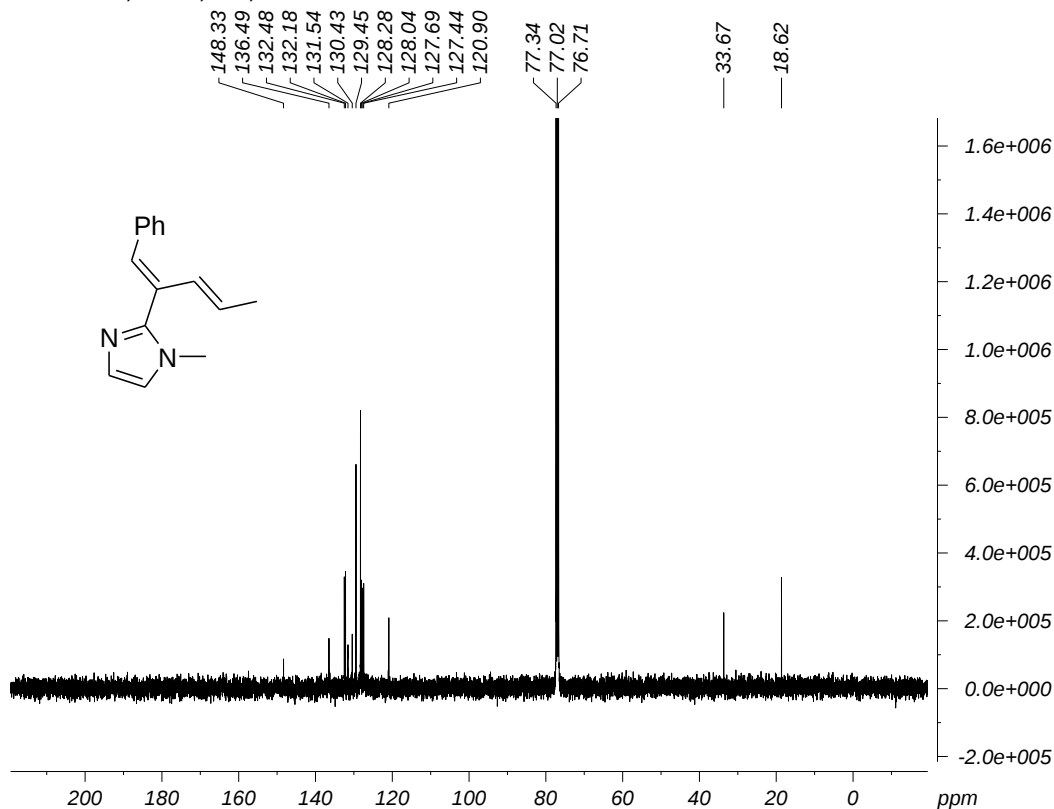
mlc129-4-8, CDCl₃, 13C, 27 SEP 2010



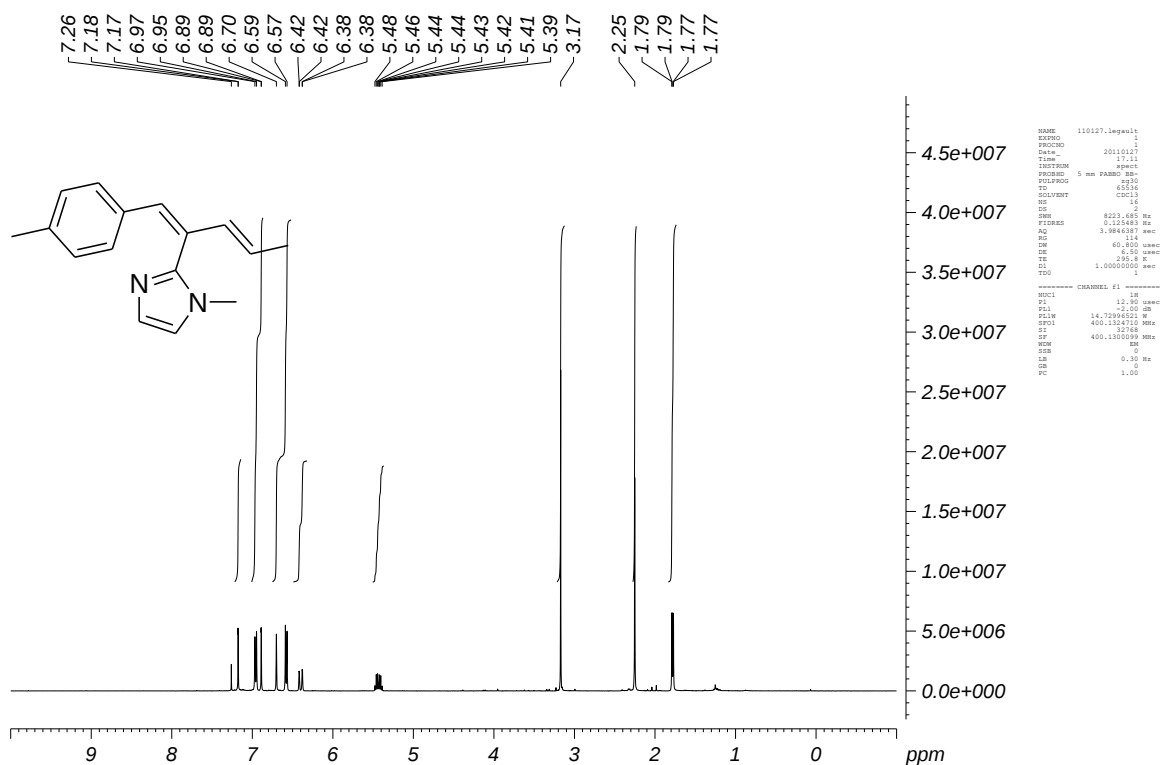
mlc129-11-24, CDCl₃, 1H, 27 SEP 2010



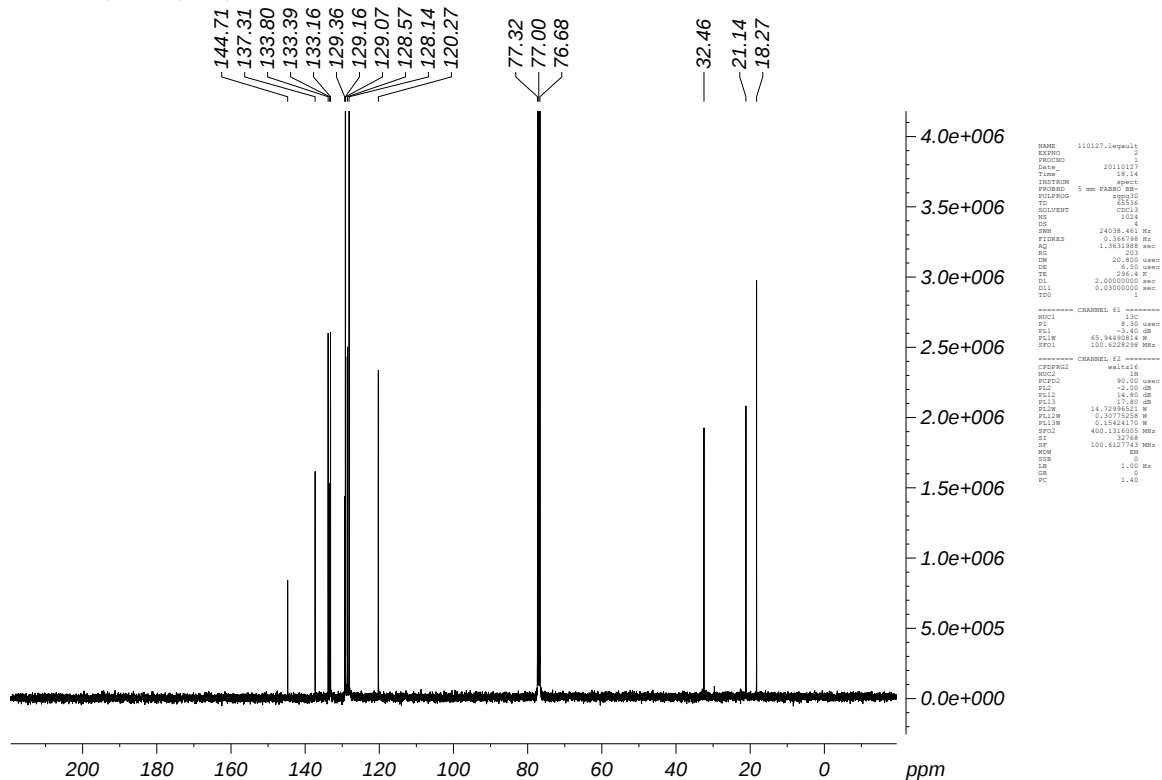
mlc129-11-24, CDCl₃, 13C, 27 SEP 2010



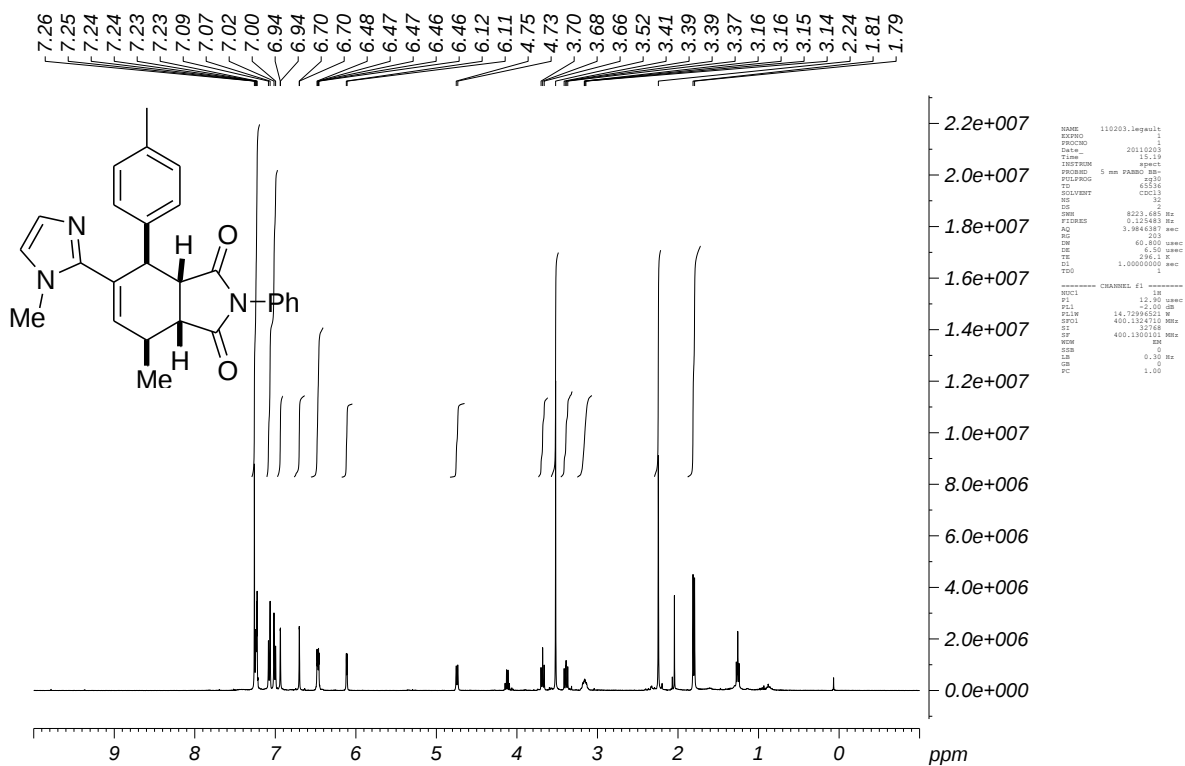
mlc149-1, CDCl₃, 1H, 27 Jan 2011



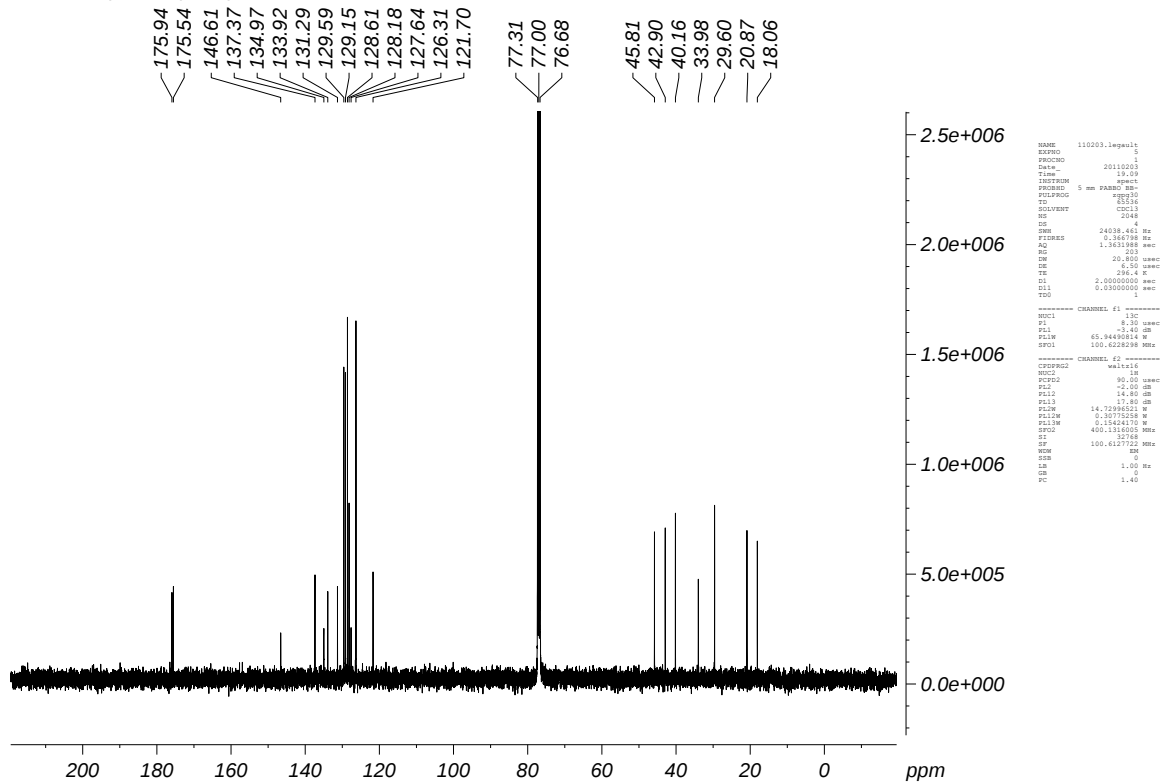
mlc149-1, CDCl₃, 13C, 27 Jan 2011

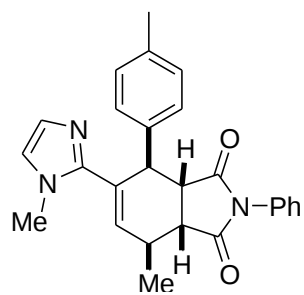
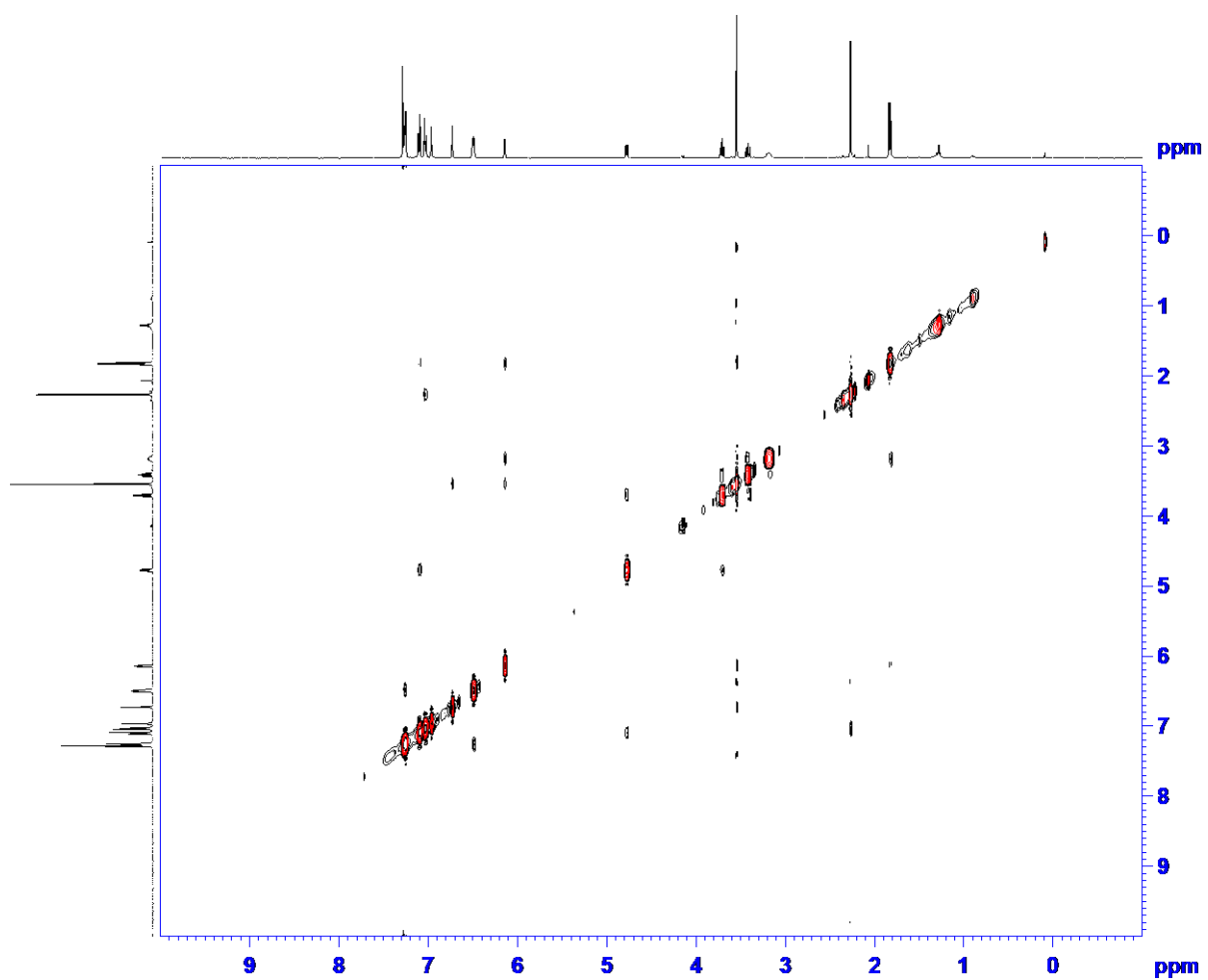


mlc151, CDCl3, 1H, 3Feb2011

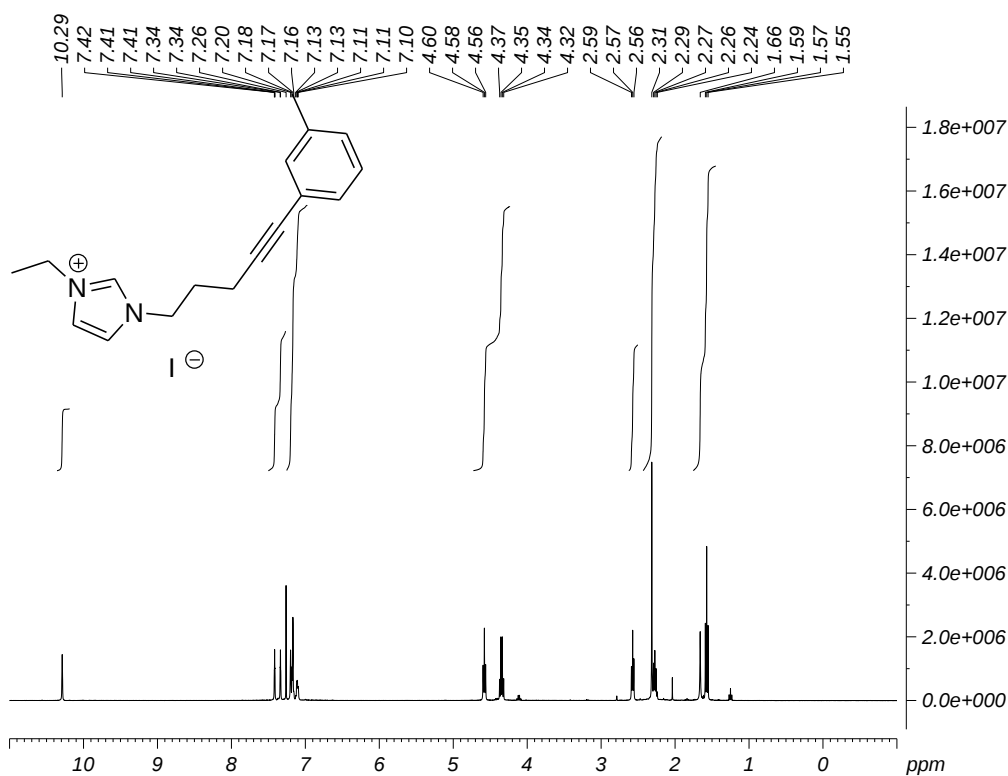


mlc151, CDCl3, 13C, 3Feb2011





MLD3, CDCl3, 1H, 11Apr2011

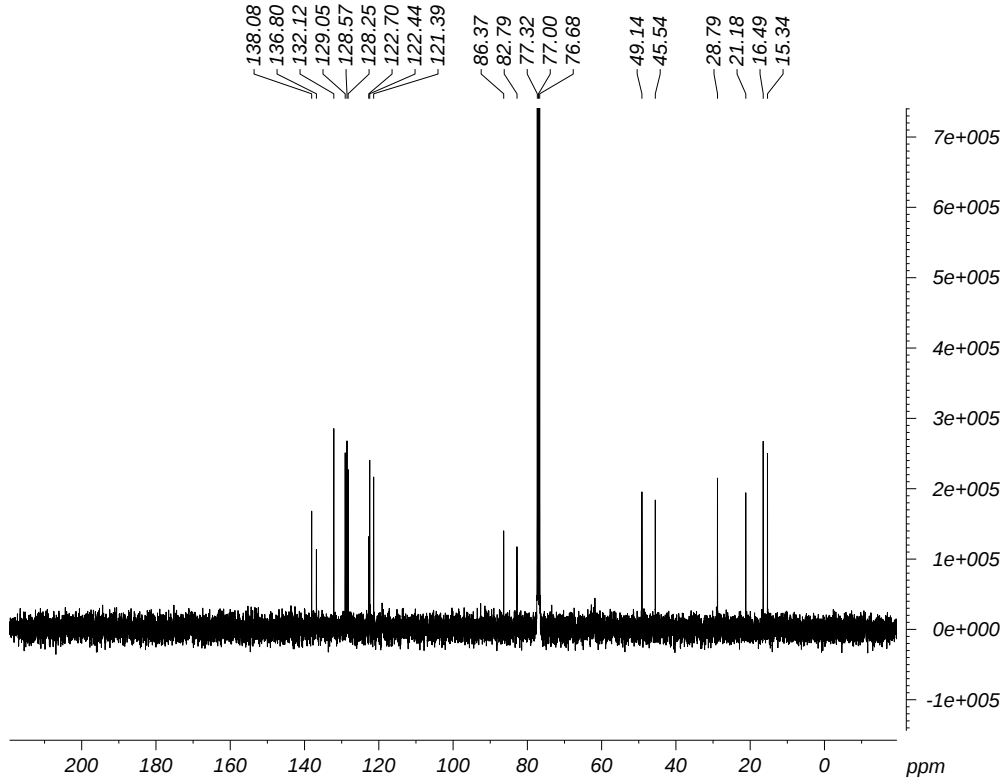


```

NAME 110411.1segant
EXPNO 2
PROCNO 1
Date_ 20110411
Time 15.15
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 16
DS 8223.685 Hz
FIDRES 0.12648 Hz
AQ 3.3846387 sec
RG 203
SW 60.800 usec
DE 6.50 usec
TE 297.6 K
D1 1.00000000 sec
TDS 1
===== CHANNEL f1 =====
NUC1 13C
P1 12.00 usec
PL1 -2.00 dB
PL12 14.72946215 W
SFO1 400.1324710 MHz
SI 32768
SF 400.1300113 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

```

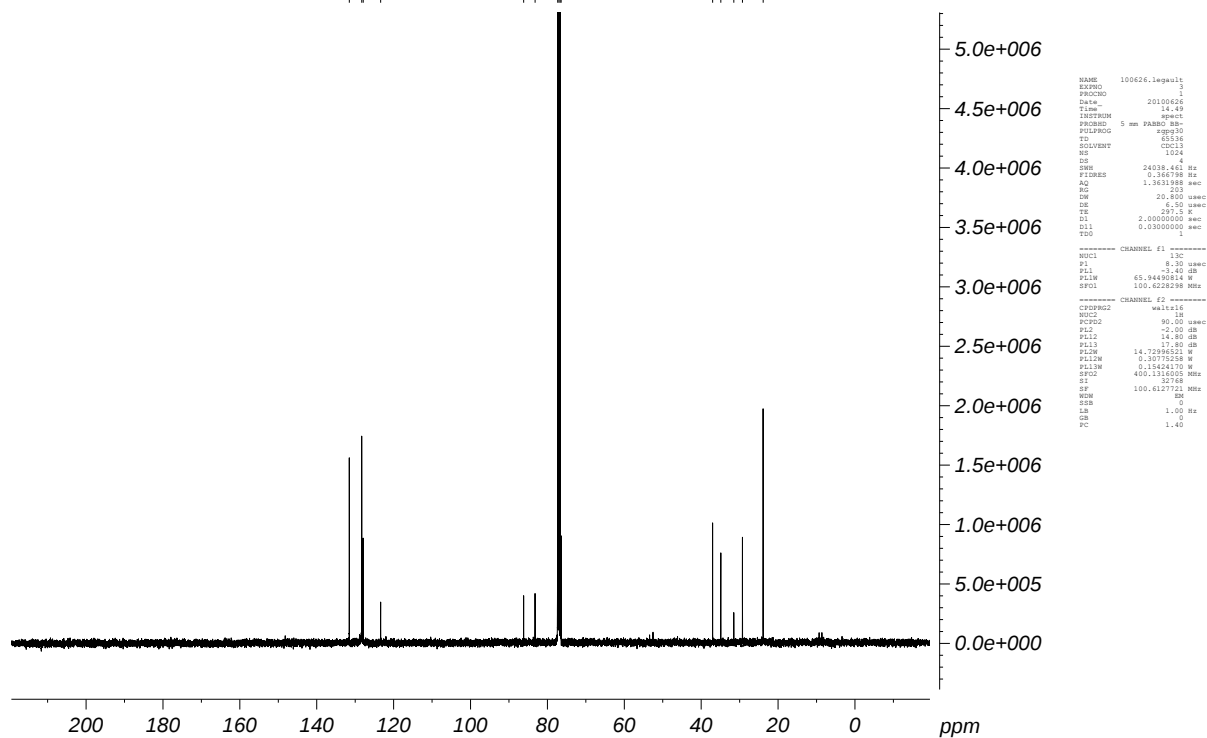
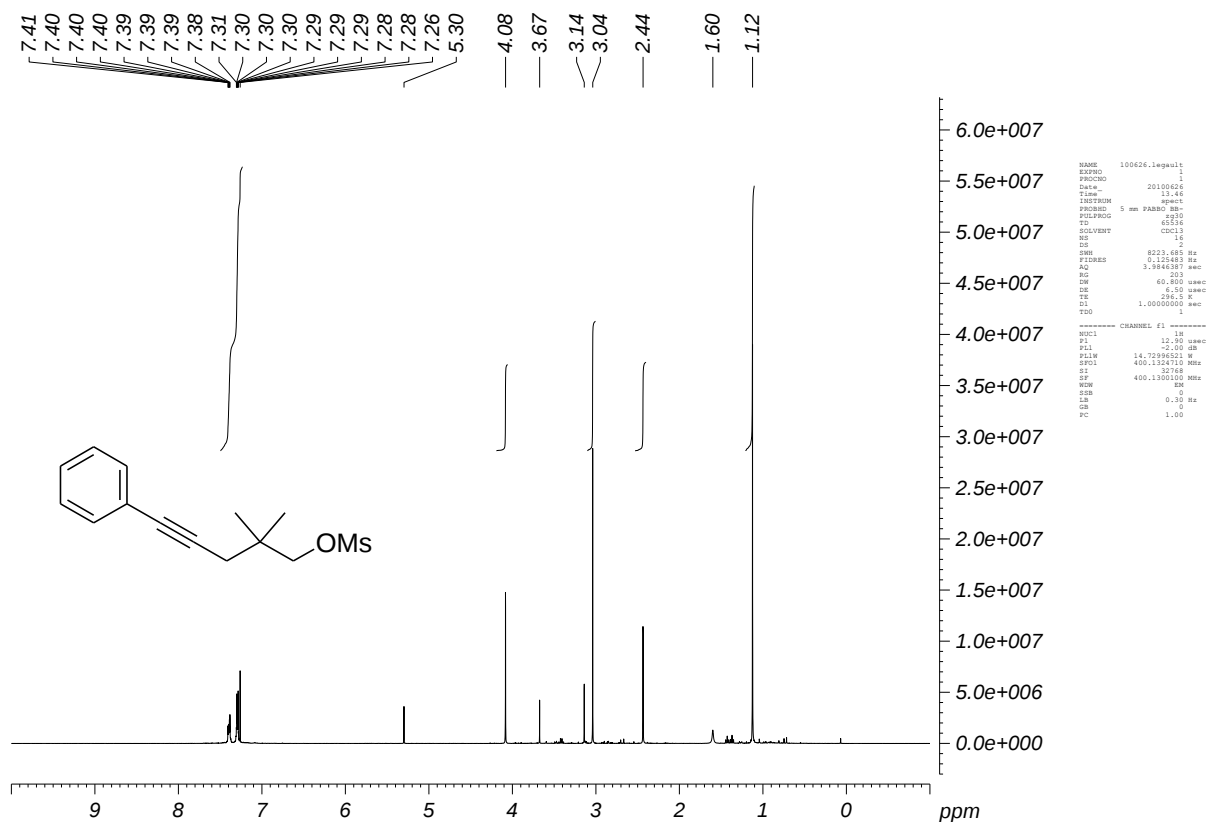
MLD3, CDCl3, 1H, 11Apr2011

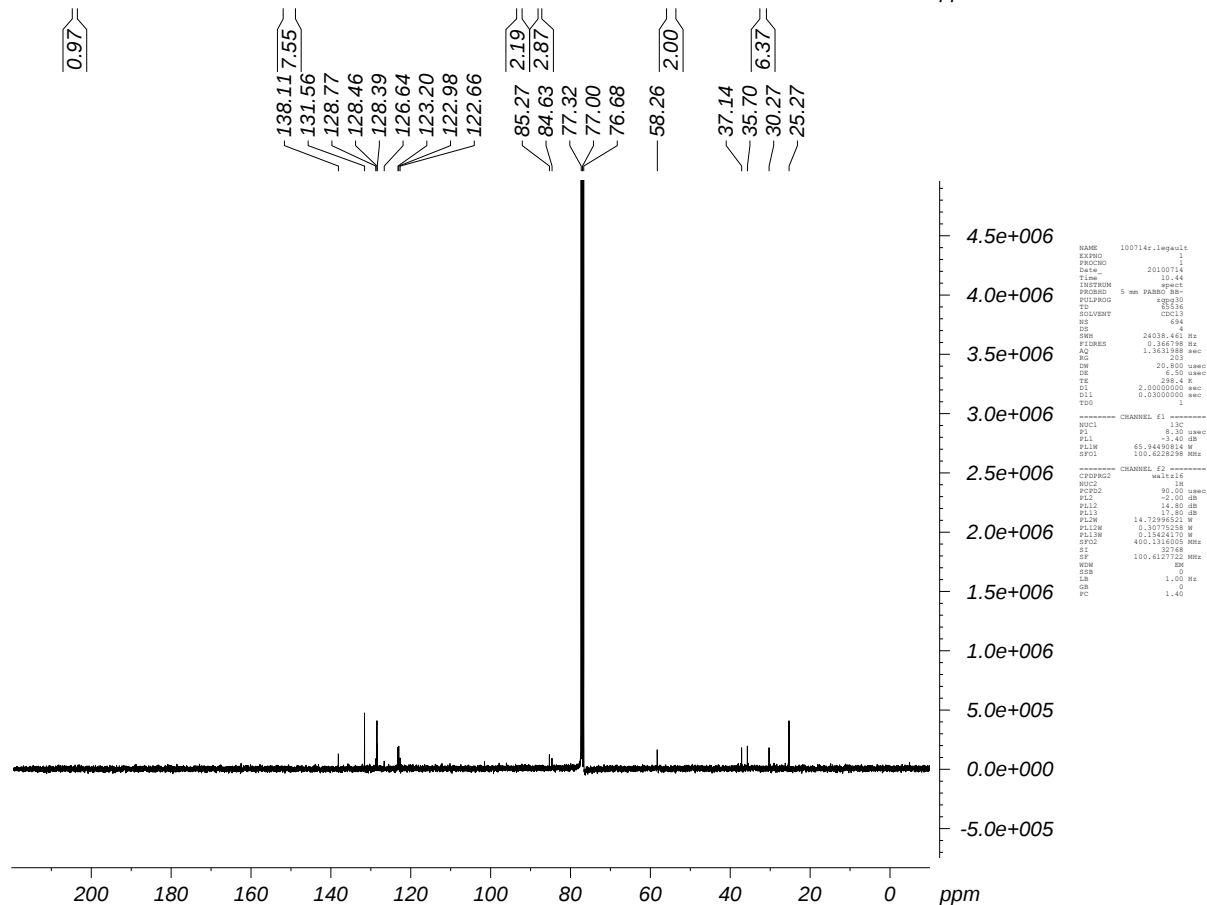
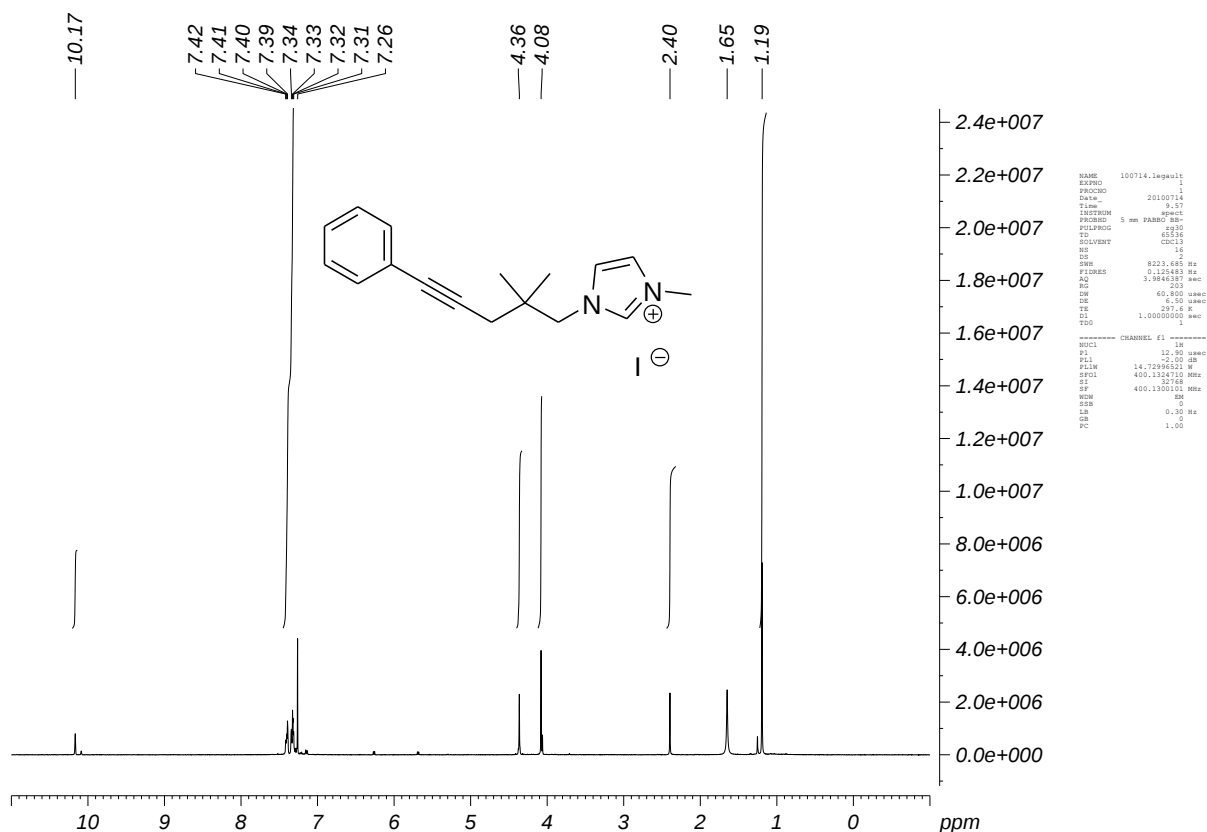


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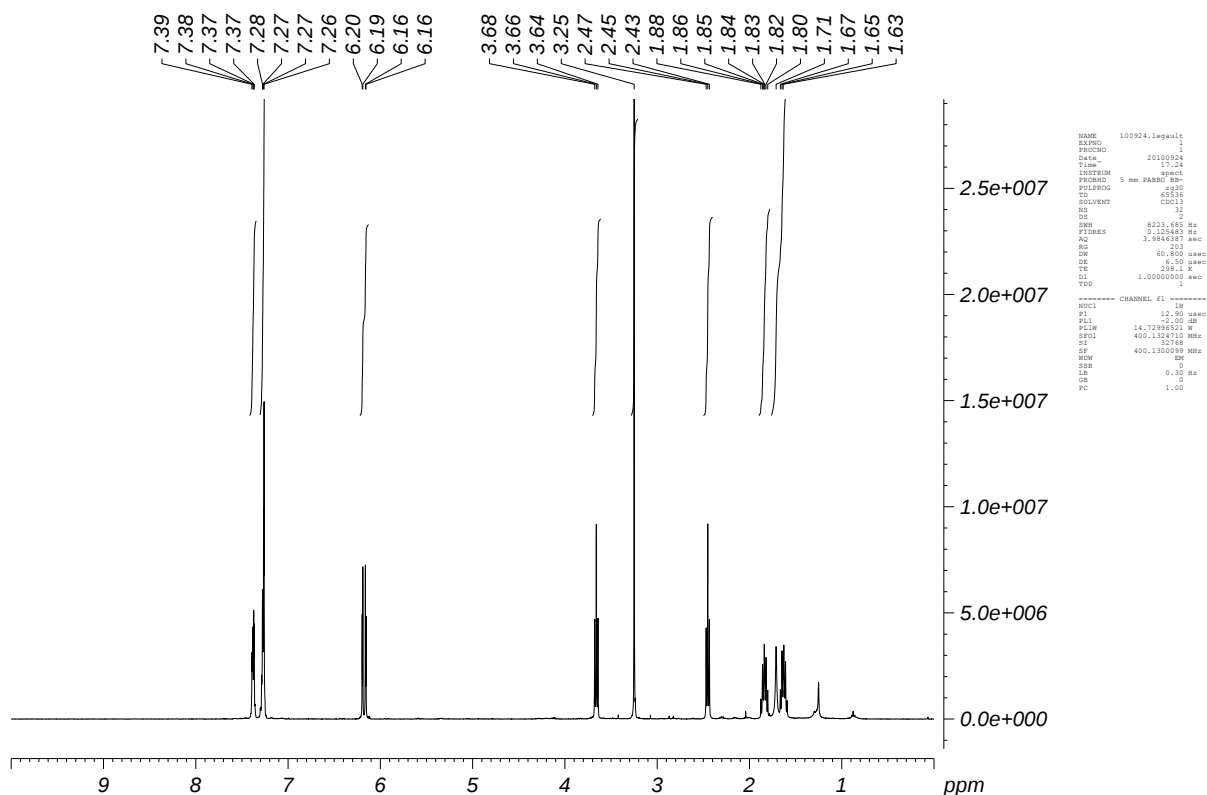
NAME 110411.1segant
EXPNO 2
PROCNO 1
Date_ 20110411
Time 16.04
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 16
DS 24038.463 Hz
FIDRES 0.366798 Hz
AQ 1.361198 sec
RG 203
SW 26.800 usec
DE 6.50 usec
TE 298.3 K
D1 2.00000000 sec
D11 0.03000000 sec
TDS 1
===== CHANNEL f1 =====
NUC1 13C
P1 8.30 usec
PL1 -2.00 dB
PL12 65.84480814 W
SFO1 100.6282624 MHz
===== CHANNEL f2 =====
CPDPRG2 waltz16
NUC2 1H
PCPD2 90.00 usec
PL2 -2.00 dB
PL13 14.00 dB
PL14 17.00 dB
PL15 14.72946215 W
PL16 0.30775258 W
PL17 0.18454170 W
SFO2 400.1316003 MHz
SI 32768
SF 100.6127729 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

```

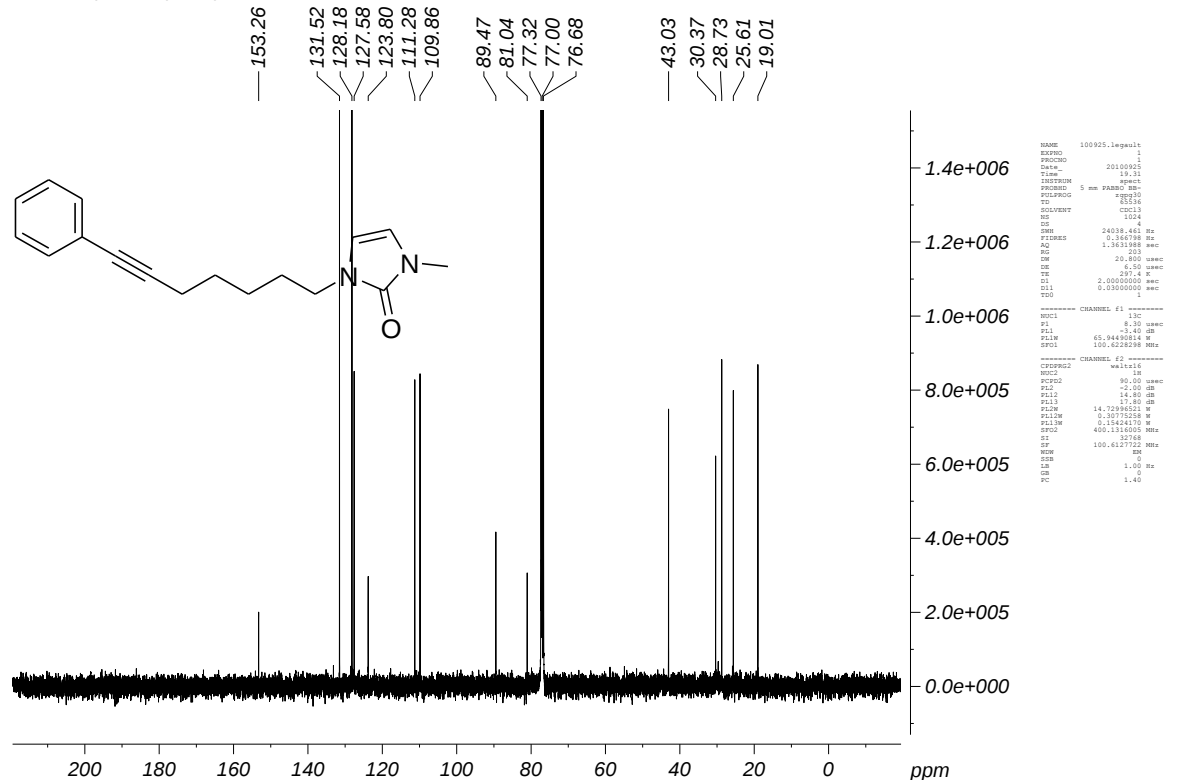




mlc115, CDCl3, 1H, 24 SEP 2010



mlc115, CDCl3, 13C, 25 SEP 2010



Chemical structure: O=CCNC#CCCC#Cc1ccccc1

¹H NMR spectrum (400 MHz, CDCl₃) data:

Chemical Shift (ppm)	Integration
9.70	1.00
7.30-7.40	1.00
2.10-2.20	1.00

Peak list (ppm): 9.70, 7.40, 7.39, 7.39, 7.39, 7.38, 7.37, 7.30, 7.29, 7.29, 7.28, 7.26, 3.58, 3.52, 3.50, 3.49, 3.47, 3.45, 3.43, 2.52, 2.50, 2.48, 1.89, 1.88, 1.86, 1.84, 1.83, 1.82, 1.81, 1.80.

161.22 131.54 128.29 127.86 123.52 88.57 81.64 77.34 77.02 76.70 37.51 28.22 17.04

1.2e+006
1.0e+006
8.0e+005
6.0e+005
4.0e+005
2.0e+005
0.0e+000
-2.0e+005

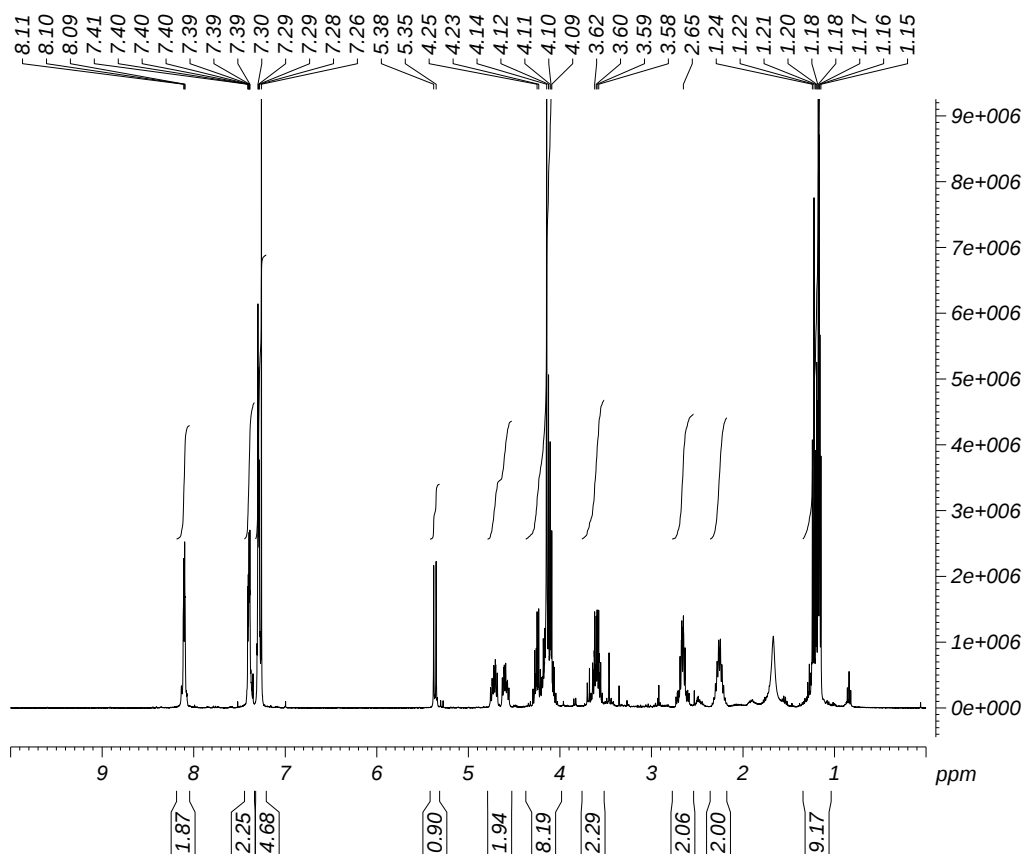
180 160 140 120 100 80 60 40 20 0 ppm

NAME 100928.1expn1
EXPNO 2
PROCNO 1
Date_ 20100928
Time 17.46
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 1024
DS 4
SWH 24039.461 Hz
F2PRESS 0.360798 Hz
AQ 1.361188 sec
RG 203
WDW 20.600 usec
SSB 0.50 usec
LB 297.2 Hz
GB 2.00000000 sec
CU1 0.00000000 sec
TD0 1

===== CHANNEL f1 =====
NUC1 13C
P1 8.30 usec
PL1 -1.40 dB
PL1W 65.96490814 W
SFO1 100.6228299 MHz

===== CHANNEL f2 =====
CPDPRG2 waltz16
NUC2 1H
PCPD2 90.00 usec
PL2 -2.00 dB
PL12 14.80 dB
PL13 21.80 dB
PL1W 14.7099821 W
PL12W 0.10775258 W
PL13W 0.10424170 W
SFO2 400.1316005 MHz
SI 32768
SF 100.6127490 MHz
WDW 0
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

mlc113, CDCl₃, 1H, 13Aug2010

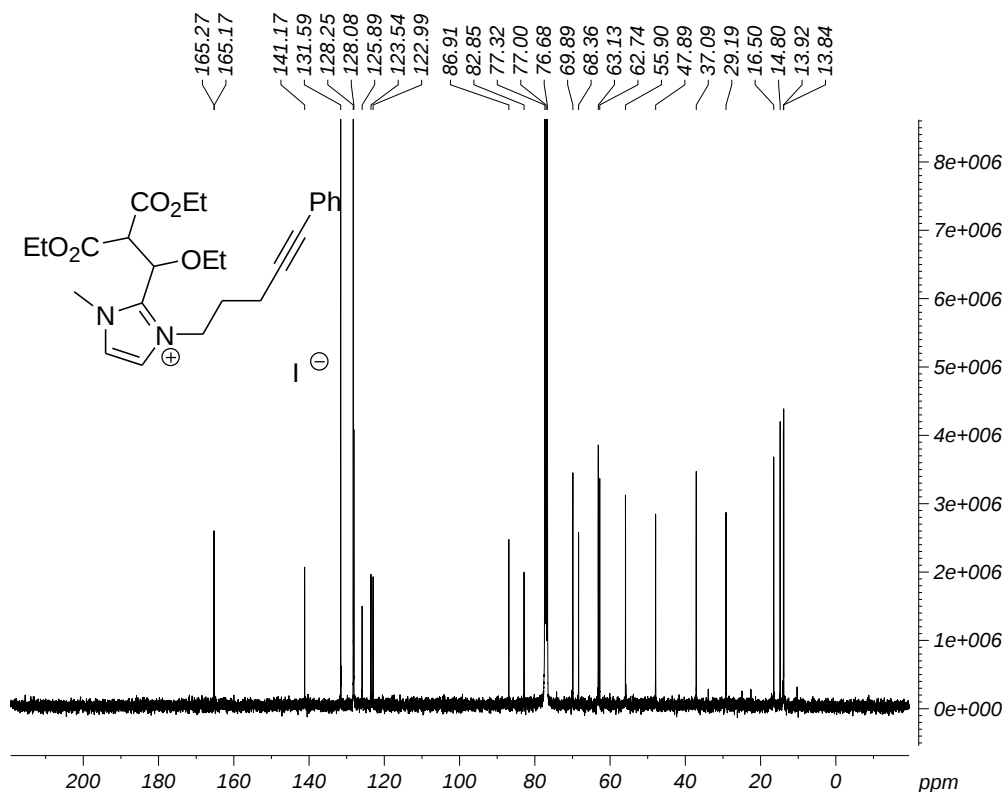


```

NAME 100813.1egault
EXPNO 1
PROCNO 1
Date_ 20100813
Time 15:10
INSTRUM spect
PROBHD 5 mm PABBO WB
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 32
DS 2
SWH 8223.685 Hz
FIDRES 0.125883 Hz
AQ 3.9846387 sec
RG 320
DM 60.800 usec
DE 6.50 usec
TE 298.2 K
D1 1.00000000 sec
TDO
===== CHANNEL f1 =====
NUC1 13C
P1 12.90 usec
PL1 -2.00 dB
PL12 14.72996521 W
SFO1 400.1324710 MHz
SI 32768
SF 400.1300101 MHz
WDW EM
SSB 0
GB 0.30 Hz
PC 1.00

```

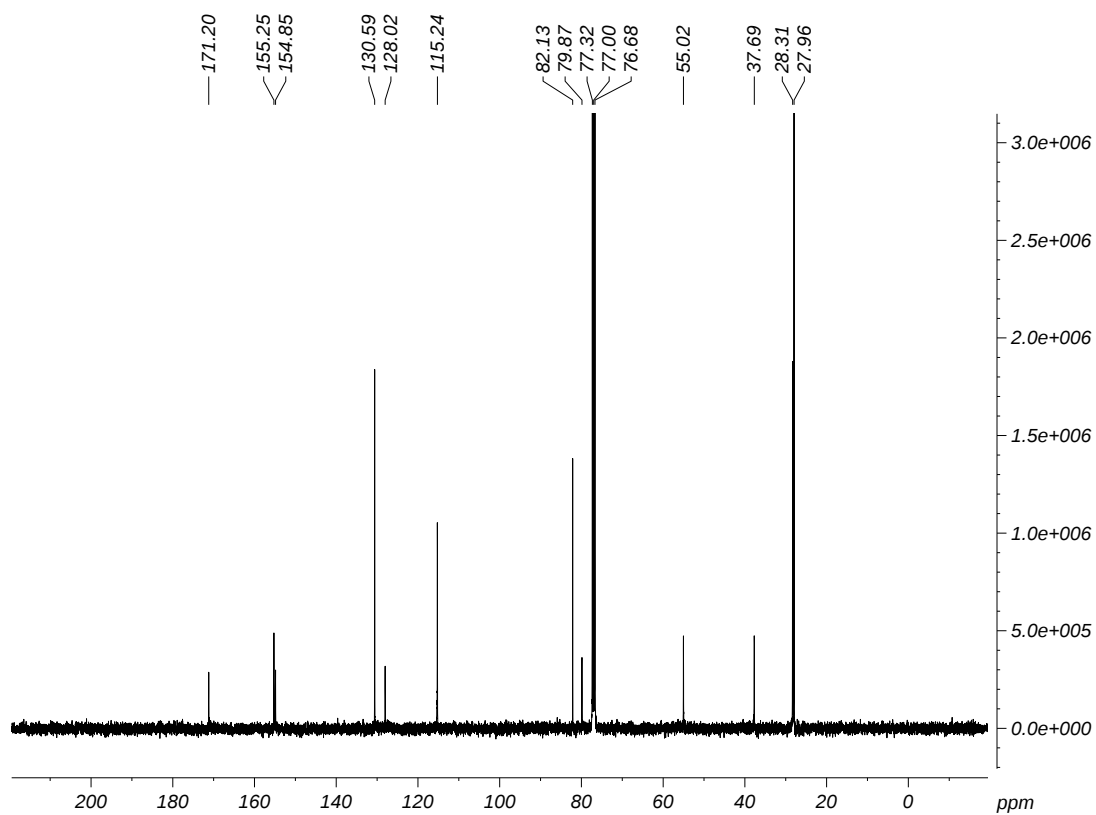
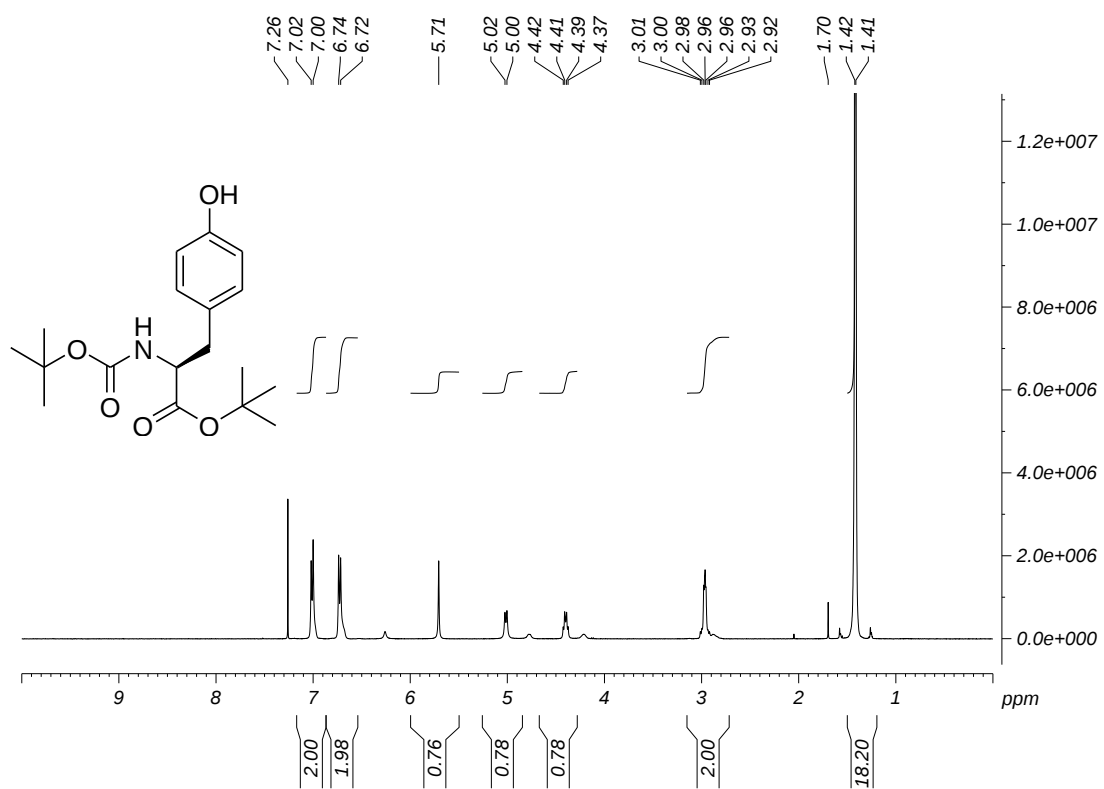
mlc113, CDCl₃, 13C, 14Aug2010



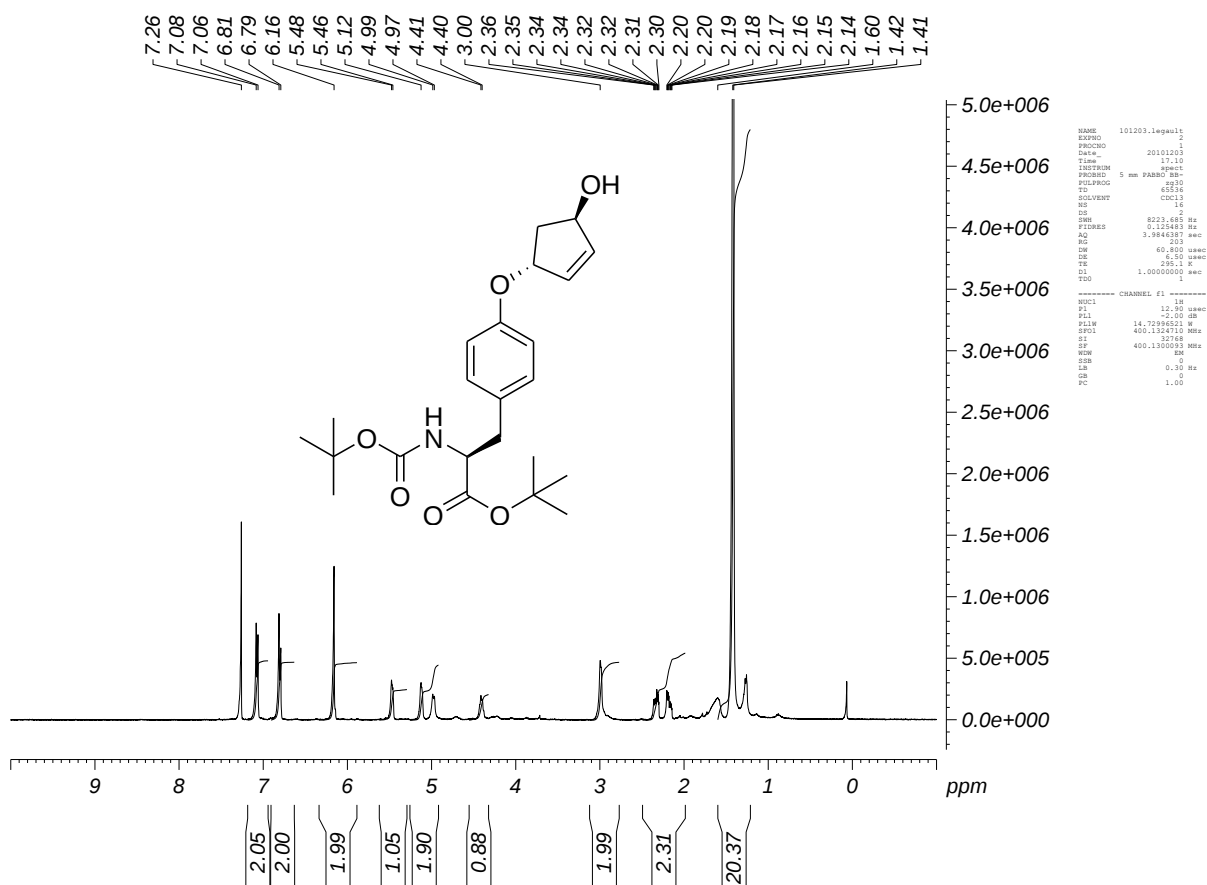
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NAME 100813.1egault
EXPNO 1
PROCNO 1
Date_ 20100814
Time 21:10
INSTRUM spect
PROBHD 5 mm PABBO WB
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 10000
DS 2
SWH 24039.460 Hz
FIDRES 0.136798 Hz
AQ 1.3633988 sec
RG 320
DM 20.800 usec
DE 6.50 usec
TE 298.2 K
D1 2.00000000 sec
D11 0.03000000 sec
TDO
===== CHANNEL f1 =====
NUC1 13C
P1 16.35 usec
PL1 -2.40 dB
PL12 69.84498814 W
SFO1 100.6228299 MHz
===== CHANNEL f2 =====
CPDPRG2 waltz16
NUC2 15N
P2 90.00 usec
PL2 -2.00 dB
PL12 14.80 dB
PL13 17.60 dB
PL14 14.72996521 W
PL15 0.1077528 W
PL16 0.1044470 W
SFO2 400.1316005 MHz
SI 32768
SF 100.6127728 MHz
WDW EM
SSB 0
GB 1.00 Hz
PC 1.40

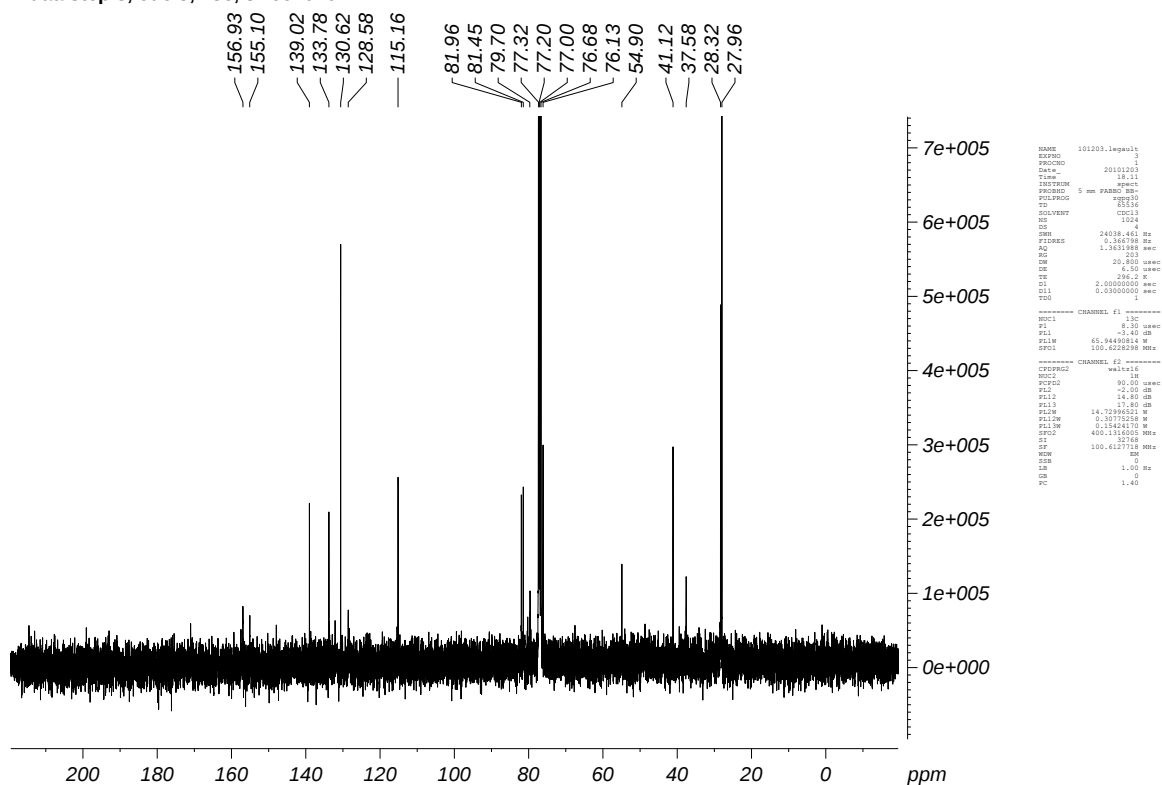
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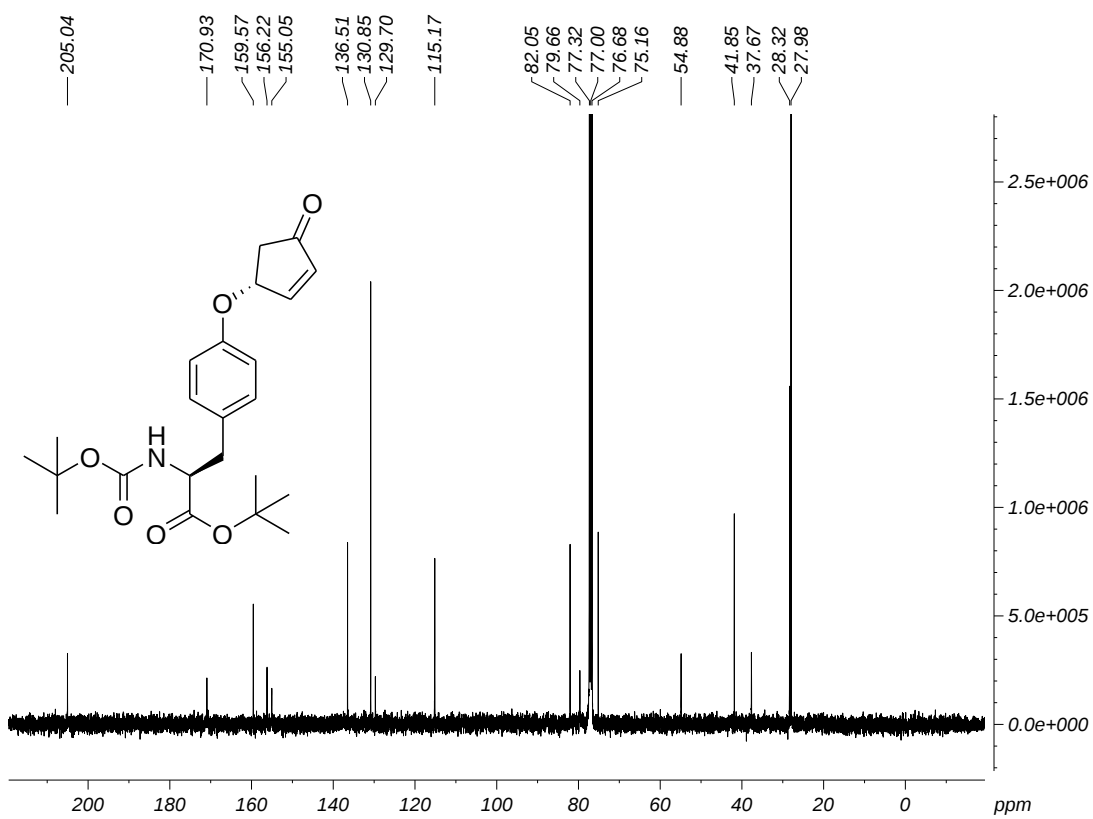
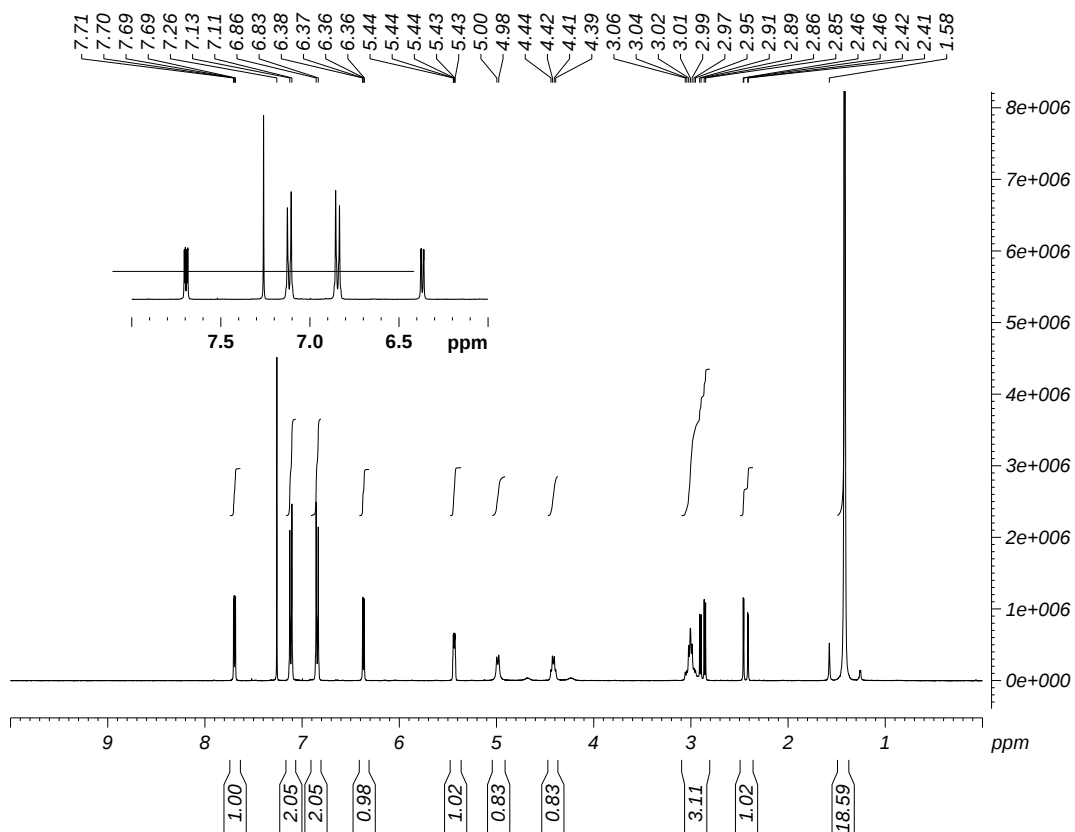


uaa step 3, cdcl3, 1H, 3Dec2010

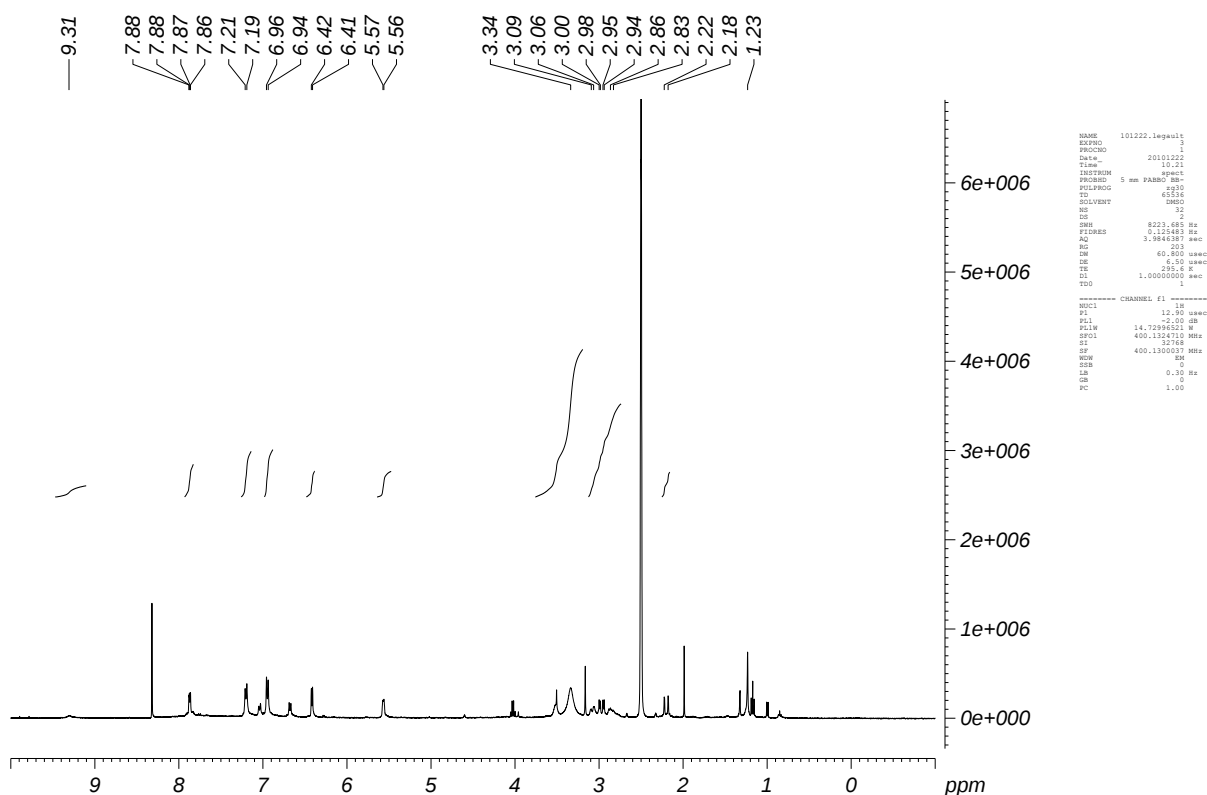


uaa step 3, cdcl3, 13C, 3Dec2010





uaa, Dmso, 1h, 22dec2010



UAA, CD3OD, 13C, 6Dec2010

