

**WAGING WARHEADS: INVESTIGATION OF ELECTROPHILIC WARHEADS FOR
TARGETED COVALENT INHIBITION OF TRANSGLUTAMINASE 2 AND BEYOND**

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Abstract

Covalent inhibition has re-emerged as a powerful therapeutic strategy, with attenuated electrophilic warheads enabling selective and sustained target engagement. Despite their central role, warheads are often treated primarily as elements for tuning electrophilicity, and their broader structural contributions to pharmacodynamic and pharmacokinetic behaviour remain underexplored.

This thesis investigates how warhead structure influences both reactivity and binding in covalent inhibitors, in the context of target engagement and metabolism. In Chapter 2, a library of electrophilic warheads was evaluated on a common scaffold targeting human tissue transglutaminase 2 (hTG2), demonstrating that warhead structure significantly impacts not only reactivity, but also binding affinity and isozyme selectivity across the transglutaminase family. In Chapter 3, a novel, fully non-peptidic internal alkynyl warhead scaffold was developed, revealing that sterically complex warheads can effectively inhibit hTG2 and providing a foundation for the development of new covalent inhibitor scaffolds. In Chapter 4, a library of warheads was evaluated as substrates for glutathione *S*-transferase (GST)-mediated metabolism using quantitative assays, demonstrating that metabolic susceptibility is a structurally tunable property that does not correlate with intrinsic reactivity. These results identify structural features that contribute to GST liability and provide strategies for modulating GST-mediated metabolism of covalent inhibitors.

Collectively, this work establishes a more integrated view of electrophilic warheads as tunable structural elements governing both reactivity and affinity-driven interactions and provides insight into how warhead design can be leveraged to optimize both target engagement and metabolic stability in covalent inhibitors.

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Dedication

*I dedicate this thesis to those who have fought—and continue to fight—for the opportunities that
allow me to pursue science.*

Epigraph

*“I would rather have questions that cannot be answered
than answers that cannot be questioned.”*

— attributed to Richard Feynman

Table of Contents

Examining Committee	ii
Abstract	iii
Acknowledgements	iv
Dedication	vi
Epigraph	vii
Chapter 1 – Introduction	1
1.1. Medicinal Chemistry	1
1.1.1. Ligand-Protein Interactions	2
1.2. Covalent Enzyme Inhibition	3
1.2.1. Targeted Covalent Inhibition.....	4
1.2.2. Electrophilic Warheads.....	6
1.2.3. Kinetic Evaluation of TCIs.....	10
1.2.4. Pharmacological Implications of Covalent Inhibition.....	12
1.3. Transglutaminases	13
1.3.1. Human Tissue Transglutaminase 2 (hTG2).....	14
1.3.2. Covalent Inhibition of hTG2	15
1.4. Glutathione S-Transferases (GSTs) and TCI Metabolism	18
1.4.1. Glutathione S-Transferase A1 (GSTA1)	19
1.4.2. Glutathione S-Transferase P1 (GSTP1).....	20
1.4.3. Electrophilic Warheads as Substrates for GSTs	21
1.5. Thesis Overview	23
1.6. Chapter 1 References	24
Chapter 2 – The War on hTG2: Warhead Optimization in Small-Molecule Human Tissue Transglutaminase Inhibitors	41
2.1. Preamble	41
2.2. Publication Information and Acknowledgements	42
2.3. Author Contributions	42
2.4. Correction to Published Version	42
2.5. Chapter 2 Published PDF	43
2.6. Chapter 2 Supporting Information	65
2.6.1. hTG2 Inhibition Kinetics Data	65
2.6.2. Isozyme Selectivity Data	70

2.6.3. Intrinsic Reactivity Data	74
2.6.4. LC-MS Data of Reaction Between 7f and GSH.....	75
2.6.5. HPLC Purity Analysis of Final Inhibitors	80
2.6.6. Synthesis of N-2-[4-[[1-Adamantanecarbonyl]-1-piperaziny]-2-oxoethyl]-2-methyl-propenamide (7r)	86
2.6.7. Crystal structure of 2-cyano-3-cyclopropylacrylic acid (I3)	86
2.6.8. NMR Spectra of Final Inhibitors and New Synthetic Intermediates	87
Chapter 3 – Discovery of an Internal Alkyne Warhead Scaffold for Irreversible hTG2 Inhibition	108
3.1. Preamble	108
3.2. Publication Information and Acknowledgements	109
3.3. Author Contributions	109
3.4. Correction to Published Version.....	109
3.5. Chapter 3 Published PDF	110
3.6. Chapter 3 Supporting Information	144
3.6.1. hTG2 Inhibition Kinetics Data	144
3.6.2. Isozyme Selectivity of 8R	155
3.6.3. Intrinsic Reactivity Data	156
3.6.4. HPLC Purity Traces of Final Compounds.....	158
3.6.5. NMR Spectra of Final Compounds and New Synthetic Intermediates	166
Chapter 4 – Don’t Lose Your (War)head: Structure-Activity Relationships of Covalent Warheads as Substrates for GST-Catalyzed GSH Conjugation	240
4.1. Preamble	240
4.2. Publication Information and Acknowledgements	241
4.3. Author Contributions	241
4.4. Chapter 4 Published PDF	242
4.5. Chapter 4 Supporting Information	260
4.5.1 Intrinsic Reactivity	260
4.5.1.1. Kinetic Data from Plate Reader and HPLC	260
4.5.2. Enzyme Kinetics.....	264
4.5.2.1. Direct Spectrophotometric Assay Kinetics and Standard Curves.....	267
4.5.2.2. Competitive Displacement and HPLC Rate Assay Kinetics and Standard Curves	276
4.5.3. Intrinsic Reactivity and Lipophilicity Correlation with Catalytic Efficiency	292

4.5.4. Molecular Modelling	294
4.5.5. Liver Cytosol Stability.....	295
4.5.5.1. Kinetic HPLC Data	298
4.5.6. LC-MS Traces of Select GSH Adducts	299
4.5.7. HPLC Purity Traces.....	302
4.5.8. NMR Spectra	310
Chapter 5 – Conclusions and Perspectives	337
5.1. Implications for Transglutaminase Inhibition	337
5.2. Expanding the Chemical Space of Alkyne Electrophiles.....	339
5.3. Tuning GST-Mediated Metabolism of Covalent Inhibitors	340
Additional Work Completed During Studies	342

List of Tables, Figures, and Schemes

Scheme 1.1. Mechanisms (A) and modes (B) of ligand-protein interactions.	3
Figure 1.1. Selected examples of clinically approved covalent drugs across diverse therapeutic areas.	6
Table 1.1. Chemistry for covalent protein modification.	8
Scheme 1.2. Kinetic scheme of irreversible enzyme inhibition.	10
Scheme 1.3. Catalytic cycle of transglutaminases.	13
Figure 1.2. Conformational regulation of hTG2.	15
Figure 1.3. Cartoon representation of targeted covalent inhibition of hTG2.	16
Figure 1.4. Representative inhibitors of hTG2.	17
Figure 1.5. Targeted covalent inhibitors are directly susceptible to phase II metabolism by GST-mediated GSH conjugation.	18
Figure 1.6. Crystal structures of (A) GSTA1 (PDB: 1PKW) and (B) GSTP1 (PDB: 6GSS).	20
Scheme 1.4. GSH conjugation to CDNB produces an increase in absorbance at 340 nm which is used to monitor the activity of GSTs.	22
Figure 2.1. Graphical abstract summarizing the work described in this chapter.	42
Figure 2.S1. Plots representing the disappearance of inhibitor and/or appearance of thiol-inhibitor adduct.	74
Figure 2.S2. Products of reaction of 7f with GSH with m/z values found by LC-MS.	75
Table 2.S1. The purity of the final inhibitors determined by HPLC.	80
Figure 3.1. Graphical summary of the work describing this chapter.	109
Figure 4.1. Graphical summary of the work describing this chapter.	241
Figure 4.S1. Intrinsic reactivity rate of Compound 21 decreases as pH increases.	260
Figure 4.S2. DMSO stability of (A) GSTA1 and (B) GSTP1 in CDNB activity assay.	264
Figure 4.S3. CDNB shows mixed non-competitive binding with compound 10 for GSTP1.	264
Figure 4.S4. Reporter substrate 2 shows competitive binding with compound 10 for (A) GSTA1 and (B) GSTP1.	265
Figure 4.S5. Spectral scan of compound 2 -GSH adduct (maximum difference between adduct and substrate Abs occurs at 380 nm) and compound 8 -GSH adduct (maximum difference between adduct and substrate Abs occurs at 420 nm).	266
Figure 4.S6. K_M^{app} of GSH at 0.25 mM reporter substrate 2 with (A) GSTA1 and (B) GSTP1.	266
Table 4.S1. Constants Expressed in Alternative Units	290
Figure 4.S7. Multiple linear regression analysis of $\log(k_{cat}/K_M^{app})^{GSTA1}$ against $\log(k_2^{GSH})$ and clogP	292
Figure 4.S8. Multiple linear regression analysis of $\log(k_{cat}/K_M^{app})^{GSTP1}$ against $\log(k_2^{GSH})$ and clogP	293
Figure 4.S9. Predicted model of compound 13 and GSH with (A) GSTA1 and (B) GSTP1.	294
Figure 4.S10. Predicted model of compound 6 and GSH with (A) GSTA1 and (B) GSTP1.	294
Figure 4.S11. Predicted model of osimertinib and GSH with (A) GSTA1 and (B) GSTP1.	294
Figure 4.S12. Inhibition of GSTA1 by ethacrynic acid (ETA) (A) Kinetic data (B) Time-dependent IC_{50} plots.	295
Figure 4.S13. Additional correlation analysis of liver cytosol stability.	296
Figure 4.S14. Example of liver cytosol stability time-course HPLC injections of compound 2	297
Figure 5.1. Potential optimization strategies for chapter 3 inhibitors.	339

List of Symbols and Abbreviations

Abs — Absorbance

AcOH — Acetic acid

ADME — Absorption, Distribution, Metabolism, Excretion

Ad — Adamantane

AL5 — Cbz-Glu(γ -*p*-nitrophenylester)Gly

BamHI — Restriction endonuclease from *Bacillus amyloliquefaciens* *H*

BL21 (DE3) — *E. coli* expression strain

Boc — tert-Butyloxycarbonyl

BOP — (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate

BTK — Bruton's tyrosine kinase

Cbz — Benzyloxycarbonyl

CDNB — 1-Chloro-2,4-dinitrobenzene

CYP450 — Cytochrome P450

DCC — N,N'-Dicyclohexylcarbodiimide

DCM — Dichloromethane

δ — Chemical shift

DIPEA — Diisopropylethylamine

DMAP — 4-Dimethylaminopyridine

DMF — Dimethylformamide

DMTA — Design–Make–Test–Analyze

DMP — Dess–Martin periodinane

DPPA — Diphenylphosphoryl azide

E. coli — Escherichia coli

EDCI — 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

EER — Enzymatic Efficiency Ratio

EGFR — Epidermal growth factor receptor

Et₂O — Diethyl ether

EtOAc — Ethyl acetate

EtOH — Ethanol

EWG — Electron-withdrawing group

FRET — Förster resonance energy transfer

GSH — Glutathione

GST — Glutathione S-transferase

GTP — Guanosine triphosphate

HATU — O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate

HBTU — N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate

HOBt — Hydroxybenzotriazole

HPLC — High-performance liquid chromatography

hTG2 — Human transglutaminase 2

IPTG — Isopropyl β -D-1-thiogalactopyranoside

J — Coupling constant

***k*₂^{GSH}** — Second-order intrinsic reactivity rate constant

***K*_a** — Acid dissociation constant

***k*_{cat}** — Enzymatic turnover number

***k*_{cat}/*K*_M** — Catalytic efficiency

***K*_D** — Binding constant

K_i — Reversible inhibition constant

K_I — Irreversible inhibition constant

k_{inact} — Rate constant for covalent inactivation

k_{inact}/K_I — Covalent inhibition efficiency

K_M — Substrate concentration at $\frac{1}{2} V_{max}$

k_{obs} — Observed rate constant

KRAS — Kirsten rat sarcoma viral oncogene homolog

LB — Lysogeny broth

LC-MS — Liquid chromatography–mass spectrometry

LG — Leaving group

MeCN — Acetonitrile

MeOH — Methanol

Na Asc — Sodium ascorbate

NEt₃ — Triethylamine

NMI — N-Methylimidazole

NMR — Nuclear magnetic resonance

PDB — Protein Data Bank

PD — Pharmacodynamics

PK — Pharmacokinetics

R.T. — Room temperature

SAR — Structure–Activity Relationships

T3P — Propanephosphonic acid anhydride

TCFH — N'-Tetramethylformamidinium hexafluorophosphate

TCI — Targeted covalent inhibitor

TCICA — Trichloroisocyanuric acid

TEMPO — 2,2,6,6-Tetramethylpiperidinyloxy

TFA — Trifluoroacetic acid

TGase — Transglutaminase

TLC — Thin-layer chromatography

***t*BuOH** — tert-Butanol

THF — Tetrahydrofuran

THP — Tetrahydropyranyl

v/v — Volume/volume

v_0 — Initial rate

$V_{\max}$ — Maximum enzymatic rate at saturation

w/v — Weight/volume

λ — Wavelength

XhoI — Restriction endonuclease from *Xanthomonas holcicola*

Chapter 1 – Introduction

1.1. Medicinal Chemistry

Medicinal chemistry is a multidisciplinary field that applies the principles of chemistry to the design, synthesis, and optimization of molecules capable of interacting with and modulating biomolecules (i.e., proteins, nucleic acids, lipids, and carbohydrates) for therapeutic or diagnostic purposes.^{1, 2} While the primary objective of the pharmaceutical *industry* is to develop clinically useful drugs, much of the foundational work that underpins modern drug discovery originates in academia,^{3, 4} where fundamental principles of molecular recognition, thermodynamics, and kinetics that inform the rational design of bioactive molecules are established. These efforts include target validation, characterization of ligand-biomolecule interactions, and molecular design and synthesis through iterative Design-Make-Test-Analyze (DMTA) cycles.⁵

The biological effects of bioactive molecules are often described within the framework of **pharmacodynamics (PD)** and **pharmacokinetics (PK)**.² PD describes the desired and undesired (side) effects that a molecule has on the body through its interactions with on- and off-target biomolecules. In contrast, PK describes how the body uses its biomolecules (e.g., enzymes and transporters) to process molecules through absorption, distribution, metabolism, and excretion (ADME). Both processes are impacted by various **physicochemical** drug properties such as solubility, lipophilicity, ionization, and molecular weight. Together, PD and PK determine the efficacy, safety, and overall clinical success of therapeutics, and optimization of both properties is crucial for drug development.

1.1.1. Ligand-Protein Interactions

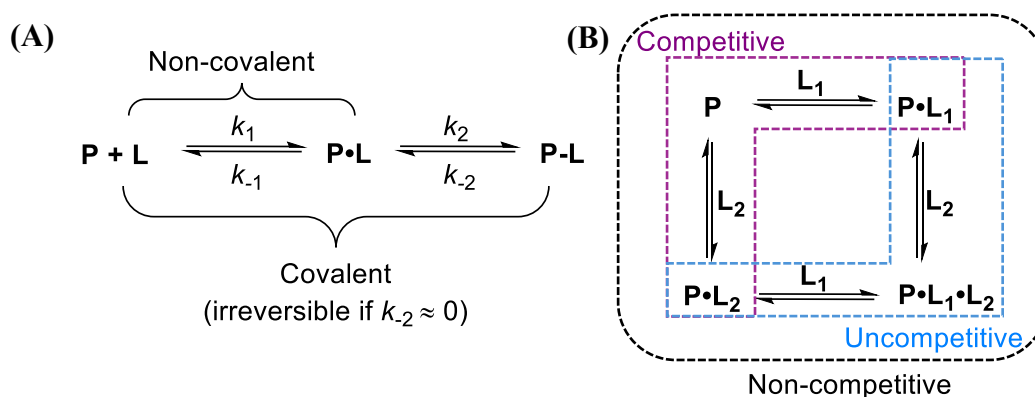
PD and PK processes arise from interactions between bioactive ligands and biomolecules. Understanding these interactions is therefore central to medicinal chemistry. In this context, proteins are particularly important, as they represent the most common pharmacological target class and mediate many processes involved in ADME.⁶⁻⁸

Ligands can interact with proteins through a **non-covalent** or **covalent mechanism** (**Scheme 1.1A**).⁴ Non-covalent interactions include hydrogen bonding, electrostatic interactions, van der Waals forces, and hydrophobic interactions. The resulting ligand-protein complex exists in equilibrium with the free protein and ligand in solution. Although non-covalent interactions are typically *reversible*, some ligands can exhibit very high affinity for their targets, resulting in tightly bound complexes with long residence times.⁹ In contrast, covalent ligands form covalent bonds with proteins, typically through the reaction of an electrophilic moiety on the ligand with a nucleophilic protein residue, which can be *irreversible* or *reversible* depending on the stability of the ligand-protein adduct.¹⁰

The classification of ligands as reversible or irreversible is typically based on *in vitro* characterization of ligand-protein interactions. However, effective reversibility of ligand binding *in vivo* depends on the physiological context of the target protein. If a ligand remains bound to a protein for the duration of its lifetime (i.e., until it is degraded), it is effectively irreversible, whereas ligands that dissociate prior to protein turnover are reversible.

When multiple ligands interact with the same protein, their *mode* of binding can be described based on how binding of one ligand influences binding of another.^{11, 12} In **competitive** interactions, two ligands compete for the same binding site on the free protein, and their binding is mutually exclusive. In **uncompetitive** interactions, binding of one ligand occurs to the protein-ligand

complex after the first ligand is bound. In **non-competitive** interactions, a ligand can bind to either the free protein or the protein-ligand complex with equal or differing affinity, representing a combination of competitive and uncompetitive binding modes. Although this terminology originates from classical enzyme inhibition,¹³ it broadly describes how multiple ligands, including substrates, inhibitors, and reporter molecules, used in biochemical assays interact with proteins relative to one another (**Scheme 1.1B**).



Scheme 1.1. Mechanisms (A) and modes (B) of ligand-protein interactions.

1.2. Covalent Enzyme Inhibition

Covalent ligand-protein interactions have historically been explored most extensively in the context of enzymology. Enzymes are catalysts primed for covalent modification; their catalytic mechanisms frequently involve nucleophilic residues positioned in well-defined active sites, making them particularly well suited for reacting with electrophilic ligands. As a result, the earliest covalent ligands were developed as probes for the study of enzyme function.¹⁴

Covalent probes or inhibitors are classified into three categories based on the mechanism by which covalent modification occurs.¹⁰ **Group-specific** reagents react with amino acid side chains chemoselectively, based on high intrinsic chemical reactivity, with minimal dependence on binding interactions, such as the cysteine alkylating agent iodoacetamide.¹⁵ **Mechanism-based** (suicide)

inhibitors are processed by the enzyme as substrate analogues to generate reactive intermediates that subsequently inactivate the enzyme, as exemplified by clavulanic acid, which irreversibly inhibits β -lactamases.¹⁴ **Affinity labels** combine a reactive electrophile with a recognition element that promotes covalent modification following initial non-covalent binding to the enzyme, such as substrate analogues bearing electrophilic groups that react with residues in the active site.

Interestingly, many of the first covalent drugs, such as aspirin (1890s) and penicillin (1940s), are affinity labels that were discovered through biological screening and used clinically before their covalent mechanisms of action were understood.¹⁶ Despite these early successes, initial attempts to intentionally design covalent inhibitors relied on highly reactive and non-selective electrophilic functional groups that produced widespread off-target effects. This led to hesitancy within the pharmaceutical industry, effectively stalling progress in this area for several decades.¹⁶

1.2.1. Targeted Covalent Inhibition

A resurgence of interest in covalent inhibitors emerged in the early 2000s as advances in structural biology, proteomics, and medicinal chemistry enabled rational, structure-based design of selective covalent ligands.¹⁶ High-resolution protein structures made it possible to identify nucleophilic residues positioned within or near ligand binding sites, whether catalytic or non-catalytic, while insights from physical organic chemistry led to the development of electrophilic functional groups with attenuated intrinsic reactivity.

Together, these developments enabled a strategy in which the ligand first binds the target through high-affinity *non-covalent* interactions, positioning a pendant electrophile in proximity to the nucleophilic residue where covalent bond formation can subsequently occur.¹⁷ In this *two-step mechanism* (**Scheme 1.1A**), reactivity is driven primarily by proximity and orientation effects within the target binding site rather than high intrinsic electrophile reactivity, which significantly

mitigates off-target reactivity. This strategy is now referred to as **Targeted Covalent Inhibition**, a modern medicinal-chemistry-optimized form of affinity labelling.

Clinical validation of this strategy was driven by three landmark targets: epidermal growth factor receptor (EGFR), Bruton's tyrosine kinase (BTK), and Kirsten rat sarcoma viral oncogene homolog (KRAS).¹⁸ Covalent inhibitors of EGFR and BTK demonstrated that selective targeting of non-catalytic cysteine residues within kinase active sites could produce highly potent and durable therapeutics, resulting in clinically approved drugs afatinib (2013) and ibrutinib (2013) for the treatment of lung and blood cancers, respectively.^{19, 20} More recently, covalent inhibitors of the KRAS G12C mutant demonstrated that covalent chemistry could be used to target proteins previously considered “undruggable” due to the absence of well-defined ligand-binding pockets, leading to the drug sotorasib (2021) for the treatment of KRAS mutant lung cancers.²¹ **Targeted covalent inhibitors (TCIs)** are now an established, safe, and effective therapeutic strategy for modulating challenging protein targets, and the field is currently experiencing rapid clinical expansion across diverse diseases (**Fig. 1.1**).¹⁸

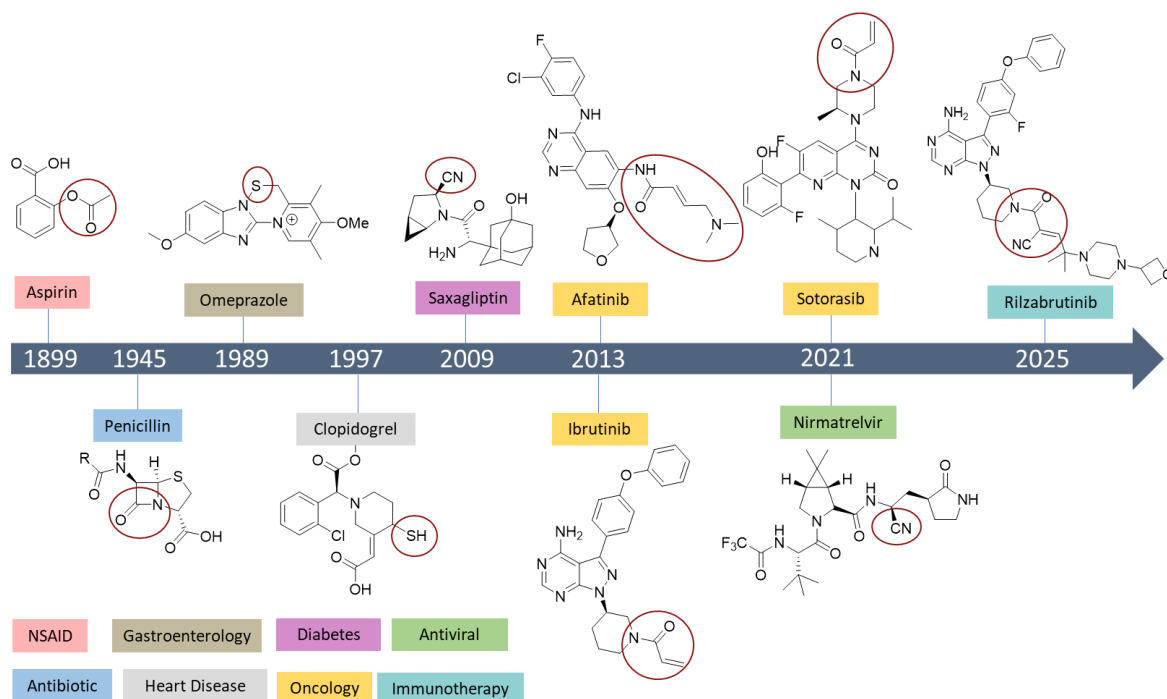


Figure 1.1. Selected examples of clinically approved covalent drugs across diverse therapeutic areas. Electrophilic groups are circled in red.

1.2.2. Electrophilic Warheads

The electrophilic functional group in a TCI that facilitates covalent reaction with a protein residue is commonly referred to as a **warhead**.¹⁶ The structure of the warhead dictates its **intrinsic reactivity**, chemoselectivity, and mechanism of reaction (i.e., reversible or irreversible) towards specific amino acids side chains, which are typically first studied *in vitro* using free amino acids, surrogate functional groups, or recombinant proteins.

Early covalent ligands frequently relied on highly reactive warheads capable of rapidly labelling protein residues, such as haloacetamides, epoxides, and activated esters. However, these electrophiles exhibited promiscuous reactivity that was unsuitable for *in vivo* drug development. As the field transitioned toward the affinity-driven paradigm of targeted covalent inhibition, a key objective became the identification of attenuated warheads, which possess sufficient reactivity to

undergo covalent reaction when appropriately positioned within a target binding site, while remaining largely unreactive towards off-target nucleophiles under physiological conditions. This attenuation was achieved through subtle electronic and steric modifications.²²⁻²⁵ For example, the use of α,β -unsaturated amides instead of their more reactive ketone counterparts,²² or chlorofluoroacetamides rather than chloroacetamides,²⁶ decreases electrophilicity through electronic effects. In addition, steric effects can reduce reactivity by hindering nucleophilic attack, as illustrated by the use of 2-butyramides in place of propionamides.²⁷

Beyond attenuation of electrophilicity, another major innovation in warhead design involved the development of reversible covalent warheads, such as α -cyanoacrylamides and α -ketoamides.²⁸⁻³⁰ These electrophiles exhibit relatively high reactivity but form thermodynamically unstable covalent adducts (i.e., electron-deficient Michael adducts or tetrahedral intermediates) that dissociate at rates that depend on the specific protein environment in which they are formed.

Currently, the landscape of warheads available for the design of TCIs is vast. The most prominent warheads, their mechanisms, and target amino acids are summarized in **Table 1.1**.^{25, 31} Among these, acrylamides and their substituted derivatives have emerged as the most widely used and clinically successful warheads to date.

Reaction	Representative Warheads	Target Residue(s)	Reversibility
1,4-Addition 		Cys	Irrev.
SN2-like 		Cys	Irrev.
SNAr/Addition-elimination 		Cys, Lys, Thr, His	Irrev.
1,2-Addition X = O, N, C		Cys, Lys, Thr, Glu	Irrev.
Acylation 		Cys, Lys, Ser, Thr	Rev.
Sulfur(VI)-LG exchange 		Arg	Rev.
Disulfide formation 		Lys, Tyr, Ser, Thr	Irrev.
Boronate complexation 		Lys, Tyr, Ser, Thr	Irrev.
		Cys	Rev.
		Ser, Thr	Rev.

Table 1.1. Chemistry for covalent protein modification. Reversibility of the chemistry refers to reversibility of *modification* not necessarily warhead regeneration.

Covalent inhibitors can, in principle, target a variety of nucleophilic amino acid side chains,²⁵ but the feasibility of targeting a given residue depends strongly on its physiological context. Several factors influence the reactivity of amino acids towards particular warheads, including their intrinsic nucleophilicity, the pK_a of the side chain and its resulting protonation state within the local protein environment, as well as their abundance and functional roles within proteins. For example, residues that participate in catalytic mechanisms and/or are located within well-defined binding pockets may be particularly susceptible to selective covalent modification due to local environmental effects that enhance nucleophilicity.

Cysteine is the most widely targeted amino acid in covalent drug discovery. Its thiolate side chain is highly nucleophilic and often participates in catalytic or regulatory mechanisms within proteins, while cysteine residues are relatively rare overall,³² which favours selectivity. Other nucleophilic residues, such as lysine, serine, threonine, and tyrosine are also commonly involved in catalysis and have been successfully targeted by TCIs. However, these residues are generally more challenging to target because their nucleophilicity is often reduced under physiological conditions due to protonation. More weakly nucleophilic residues, including histidine, aspartate/glutamate, arginine, and tryptophan present even greater challenges for covalent modification, and the development of strategies to selectively target these residues remains an active area of research. Another limitation when targeting residues other than cysteine is the difficulty of achieving irreversible covalent modification, as many electrophiles that react with these residues tend to form reversible adducts.²⁵

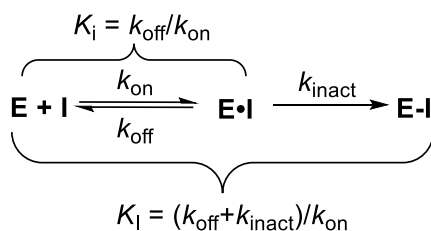
Importantly, the reactivity of electrophilic warheads toward protein residues is highly dependent on the specific protein environment in which the reaction occurs, which influences warhead positioning within the binding site.³³ Thus, understanding how different warheads behave

within the context of specific protein targets is an essential step of early medicinal chemistry campaigns for new targets.

1.2.3. Kinetic Evaluation of TCIs

Unlike non-covalent reversible inhibitors, whose potency can be described using a steady state equilibrium dissociation constant K_D or inhibition constant K_i ,¹³ TCIs require *time-dependent* kinetic analysis that accounts for both binding and the rate of covalent reaction.

For irreversible TCIs this is accomplished by measurement of the parameters K_I and k_{inact} (**Scheme 1.2**).^{34, 35} K_I is the apparent inhibition constant describing the steady-state balance between formation of the enzyme-inhibitor ($E \cdot I$) complex and its depletion through dissociation or covalent modification, and k_{inact} is the rate constant of covalent modification. Together these parameters describe the overall efficiency of covalent inhibition, which is expressed as k_{inact}/K_I . These parameters are analogous to Michaelis-Menten kinetic parameters K_M , k_{cat} , and k_{cat}/K_M .³⁶



Scheme 1.2. Kinetic scheme of irreversible enzyme inhibition.

Although K_I and k_{inact} are often interpreted as reflecting binding affinity and reactivity respectively, it is important to note that these parameters are not independent of each other. K_I is a collection of rate constants that includes k_{inact} (**Scheme 1.2**), such that the rate of covalent bond formation contributes directly to its observed value. For example, a highly reactive warhead may exhibit a large k_{inact} , but this can also lead to a higher K_I , reflecting reduced stability of the $E \cdot I$ complex. Therefore, while k_{inact}/K_I reflects the balance between these parameters, individual

characterization of k_{inact} and K_I provides mechanistic insight about whether binding affinity and/or reactivity require tuning.

In practice, warheads are often considered primarily as determinants of k_{inact} through their intrinsic reactivity. However, because inhibition proceeds through initial non-covalent binding the structural complementarity of the warhead can also influence true binding affinity (K_D) in the active site and have further confounding effects on K_I . This may be particularly significant for larger, structurally complex warheads, which contribute more substantially to overall ligand efficiency.⁴ As such, the role of the warhead as a structural determinant of binding affinity is often underappreciated in the design of TCIs.

Experimentally, k_{inact} and K_I are measured by monitoring time-dependent loss of enzyme activity or covalent protein modification directly.³⁷⁻³⁹ The development of experimental approaches and corresponding mathematical models for kinetic evaluation of TCIs is still an active area of research. Current approaches include continuous activity assays analyzed within the kinetic framework originally described by Kitz and Wilson,⁴⁰ discontinuous time-dependent IC_{50} methods described by Krippendorff and Keillor,⁴¹⁻⁴³ and RapidFire mass spectrometry for pseudo-continuous monitoring of covalent labelling.⁴⁴

For reversible covalent inhibitors, time-dependent inhibition typically arises when the rate of covalent bond dissociation is much slower than covalent bond formation, allowing the covalent adduct to accumulate during the assay. In such cases, an additional rate constant describing covalent bond dissociation must be considered, resulting in more complex kinetic models.^{38, 43} However, if covalent bond formation and dissociation both occur rapidly relative to the experimental timescale, the system rapidly reaches equilibrium and inhibition is effectively indistinguishable from non-covalent reversible inhibition.³⁸

1.2.4. Pharmacological Implications of Covalent Inhibition

A major advantage of covalent inhibitors is their ability to produce prolonged target engagement.^{45, 46} The initial rate of target engagement is determined by $k_{\text{inact}}/K_{\text{I}}$ as well as drug and protein concentrations. However, once substantial target engagement is achieved pharmacological activity becomes largely dependent on the rate of protein turnover rather than free drug concentration. Consequently, sustained efficacy may be maintained with relatively low drug exposure after initial target engagement, reducing systemic exposure and toxicity.^{47, 48}

Covalent inhibition can also enable targeting of proteins that are difficult to modulate with conventional reversible ligands. By exploiting nucleophilic residues located within shallow or transient binding pockets, covalent inhibitors can stabilize binding interactions that would otherwise be too weak to support effective reversible inhibition, enabling modulation of targets previously considered “undruggable”.⁴⁹

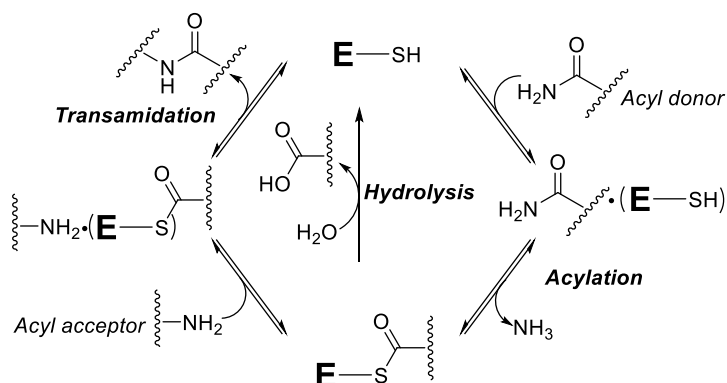
Reversible covalent inhibitors provide an additional layer of control by allowing prolonged residence times while avoiding permanent off-target modification. For example, structural modification of the α -cyanoacrylamide warhead has been shown to fine-tune the rates of covalent bond formation and dissociation with BTK, enabling prolonged on-target residence times while rapidly dissociating from off-target proteins.^{50, 51}

The choice between irreversible and reversible inhibition ultimately depends on the biological context of the target protein, particularly its rate of turnover and conformational dynamics. For rapidly resynthesized proteins, reversible inhibition may be advantageous to allow engagement of newly formed protein. Conversely, irreversible inhibition may be beneficial when sustained engagement of a specific conformational state is required for targets such as human tissue transglutaminase 2 (hTG2).

1.3. Transglutaminases

Transglutaminases (TGases) are a family of Ca^{2+} -dependent enzymes that catalyze the crosslinking of protein substrates by forming isopeptide bonds between the side chains of glutamine and lysine residues through an acyl transfer (transamidation) mechanism mediated by a catalytic cysteine residue (**Scheme 1.3**).⁵²⁻⁵⁵ This crosslinking activity plays an integral role in the stabilization of extracellular matrices and protein assemblies across diverse cell types.⁵⁶

The human TGase family comprises nine isozymes with distinct tissue distributions and physiological roles, including TGases 1-7 (TG1-TG7), factor XIII (FXIII), and erythrocyte membrane protein band 4.2 (EBP42).⁵⁷ TG1, TG3, and TG5 are primarily expressed in the epidermis, where they contribute to keratinocyte differentiation and skin barrier formation. FXIII functions in blood coagulation by stabilizing fibrin clots, while TG4 and TG6 exhibit more specialized roles in the prostate and central nervous system, respectively. EBP42 is a catalytically inactive transglutaminase homologue that serves a structural role in erythrocyte membranes.



Scheme 1.3. Catalytic cycle of transglutaminases. An amide acyl donor substrate (e.g., glutamine) forms a thioester intermediate with the active-site cysteine, which undergoes transamidation in the presence of an amine acyl acceptor (e.g., lysine) or hydrolysis to a carboxylic acid (e.g., glutamate) in the absence of an acyl acceptor. This figure is adapted from Keillor *et al.*⁵⁴

1.3.1. Human Tissue Transglutaminase 2 (hTG2)

Human tissue transglutaminase 2 (hTG2) is the most ubiquitously expressed and extensively studied member of the TGase family.⁵⁷ It is uniquely multifunctional: in addition to its transamidation activity, hTG2 functions as a GTP-binding protein (G-protein) and participates in many non-enzymatic processes, including protein-protein interactions, cell adhesion, and intracellular signalling.⁵⁸⁻⁶⁰ Through these diverse activities, hTG2 contributes to numerous physiological processes and has been implicated in a variety of pathological conditions.

The transamidation activity of hTG2 plays a central role in the stabilization of extracellular matrices through protein crosslinking,⁶¹ which is essential for normal tissue homeostasis but can become dysregulated in disease. In fibrotic disorders, excessive hTG2-mediated crosslinking contributes to tissue stiffening and impaired organ function.⁶²⁻⁶⁴ In celiac disease, hTG2-mediated hydrolysis of gluten peptides enhances their immunogenicity, driving an autoimmune response.⁶⁵⁻⁶⁷ hTG2 activity has also been implicated in neurodegenerative diseases, where aberrant crosslinking of proteins may contribute to protein aggregation.⁶⁸⁻⁷⁰ As a G-protein, hTG2 plays roles in cell survival, adhesion, and signalling, and contributes to cancer progression by promoting proliferation, metastasis, and drug resistance in a variety of cancers,⁷¹⁻⁷³ most notably breast,⁷⁴ ovarian,⁷⁵ and pancreatic.⁷⁶

The transamidase and G-protein activities of hTG2 are mutually exclusive, as they are dependent on distinct protein conformations (**Fig. 1.2**). hTG2 is composed of an N-terminal β -sandwich domain, a catalytic core domain containing the active-site cysteine, and two C-terminal β -barrel domains, which undergo large-scale conformational changes in response to cellular environment.⁷⁷ In the extracellular environment, where Ca^{2+} concentrations are high, Ca^{2+} binding stabilizes an extended 'open' conformation in which the transamidation active site is accessible

and the GTP-binding site is disrupted, enabling protein crosslinking while preventing G-protein activity.⁷⁸ In contrast, intracellular conditions, characterized by low Ca^{2+} concentrations and high GTP levels, favour a compact ‘closed’ conformation stabilized by GTP binding, in which the transamidation active site is occluded and crosslinking activity is suppressed.⁷⁹⁻⁸¹ Although intracellular and extracellular environments favour the closed and open conformations of hTG2, respectively, the enzyme exists in a dynamic conformational equilibrium, with minor populations of alternative states present under all conditions. Moreover, cellular stress or damage can lead to an influx of Ca^{2+} , which may perturb the intracellular equilibrium towards the open conformation.

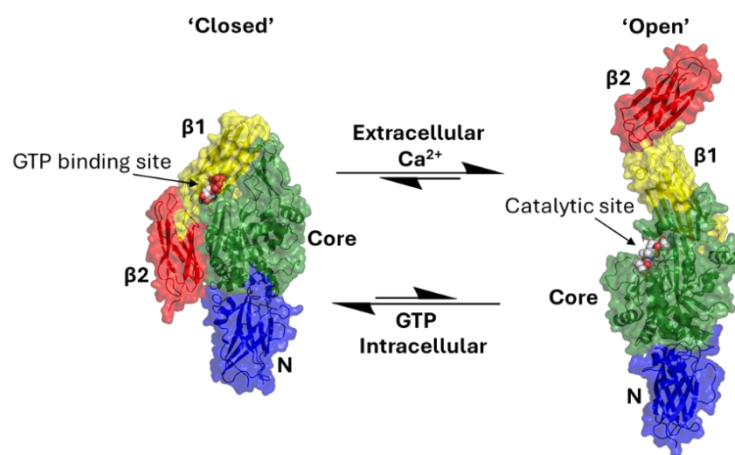


Figure 1.2. Conformational regulation of hTG2. PDB ‘Closed’: 4PYG,⁸¹ PDB ‘Open’: 2Q3Z.⁷⁷

1.3.2. Covalent Inhibition of hTG2

Given its multifunctionality and involvement in a wide range of pathologies, hTG2 is an attractive target for inhibition for both therapeutic and chemical biology purposes. hTG2 is particularly well-suited for covalent inhibition as it contains an active site cysteine (Cys277), is non-essential for animal survival and reproduction,⁵⁶ and displays moderate-slow turnover rates in some cancers.^{82, 83} Notably, its conformational dynamics enables a ‘two birds with one stone strategy’, whereby covalent modification of Cys277 in the catalytic site prevents it from adopting

its closed conformation. In this way not only is transamidase activity inhibited, but, through conformational locking, its G-protein activity is also abolished (**Fig. 1.3**).⁸⁴⁻⁸⁶

The advantages of this conformational locking are particularly relevant to intracellular inhibition of hTG2's G-protein activity. Direct inhibition of this function through competitive targeting of the nucleotide-binding site is challenging due to the high intracellular concentration of GTP and the widespread use of GTP by other proteins, which limits the selectivity of GTP analogues. In contrast, the transamidation active site recognizes distinct substrates, enabling structure- and substrate-based inhibitor design strategies. Although the open conformation represents a minor population under normal intracellular conditions, covalent trapping of this state effectively depletes it from the equilibrium, promoting continued population of the open conformation and enabling effective inhibition.^{87, 88}

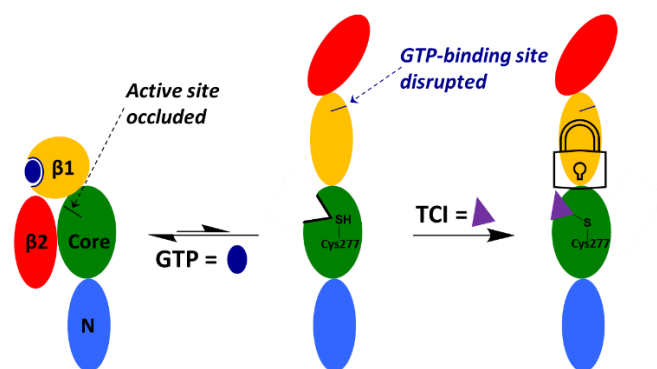


Figure 1.3. Cartoon representation of targeted covalent inhibition of hTG2. This figure is adapted from Akbar *et al.*⁸⁴

Historically, many hTG2 inhibitors have been based on peptidomimetic scaffolds designed to mimic protein substrate recognition sequences (**Fig 1.4**).^{85, 89, 90} These early scaffolds, and their application to the study of hTG2 structure and function were pioneered by the Keillor and Khosla groups,^{77, 91, 92} as well as Zedira GmbH.⁹³ Most notably, ZED1227 (Zedira) has progressed to phase II clinical trials for the treatment of celiac disease, targeting extracellular hTG2 in the intestinal

lumen.⁹⁴ While these peptidomimetic scaffolds have demonstrated high potency, selectivity, and early clinical success in targeting extracellular hTG2, they often suffer from poor physicochemical and pharmacokinetic properties, including high molecular weight, limited solubility, low cellular permeability and/or high efflux, and reduced metabolic stability, which limits their utility for intracellular applications.^{95, 96} In recent years, efforts in the Keillor lab,^{86, 97-99} as well as those of other researchers including Griffin,¹⁰⁰ Löser,¹⁰¹ and the CHDI foundation,¹⁰² have shifted toward smaller, non-peptidic scaffolds with more conventional drug-like properties for targeting intracellular hTG2 (**Fig. 1.4**).

A variety of electrophilic warheads have been employed in the design of hTG2 inhibitors, including epoxides, diazoketones, chloroacetamides, and most commonly Michael acceptors such as acrylamides and acrylates.⁸⁵ These warheads are typically appended to glutamine- or lysine-like side chains to facilitate substrate-like recognition of peptidomimetic scaffolds. Much of the warhead diversity explored to date has been in the context of these peptidomimetic architectures, rather than non-peptidic small-molecule scaffolds, where the contributions of warhead structure to binding affinity and covalent reactivity with hTG2 remain less well understood.

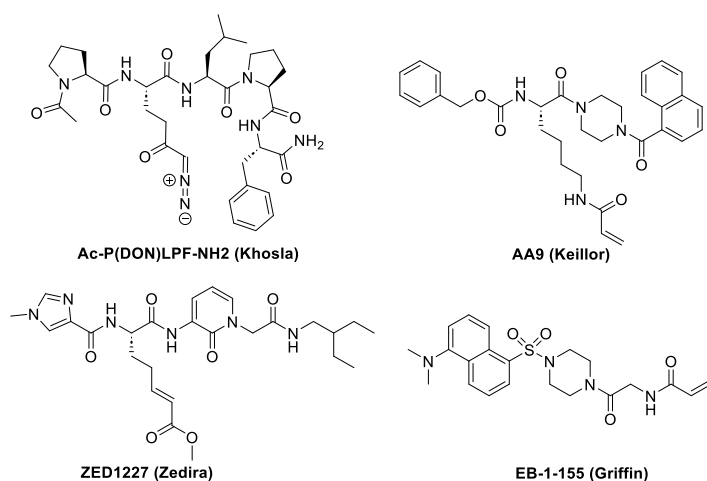


Figure 1.4. Representative inhibitors of hTG2.^{77, 84, 94, 100}

1.4. Glutathione S-Transferases (GSTs) and TCI Metabolism

Electrophilic warheads play a central role in the pharmacodynamics of TCIs, enabling effective target engagement. However, this functionality also introduces pharmacokinetic liabilities, most notably susceptibility to metabolic processes. Drugs are typically processed through two phases of metabolism: phase I reactions introduce or unmask functional groups to increase polarity and/or reactivity for phase II reactions in which additional functional groups are conjugated onto reactive moieties to facilitate further solubilization and clearance (**Fig. 1.5**).² In the context of TCIs, phase I is often mediated by cytochrome p450 (CYP450)-catalyzed oxidation, whereas phase II is dominated by glutathione (GSH) conjugation catalyzed by glutathione S-transferases (GSTs).¹⁷

By virtue of containing pre-installed electrophilic groups, TCIs can bypass the need for phase I activation and are inherently susceptible to direct phase II metabolism. This is particularly relevant for irreversible TCIs that target cysteine residues, such as in hTG2. GSH is an endogenous Cys-containing tripeptide present at high (mM) concentrations in cells and therefore represents a major competing nucleophile for Cys-targeting warheads that can not only react intrinsically (non-enzymatically), but whose reactivity is also enhanced *in vivo* by GSTs.^{8, 103}

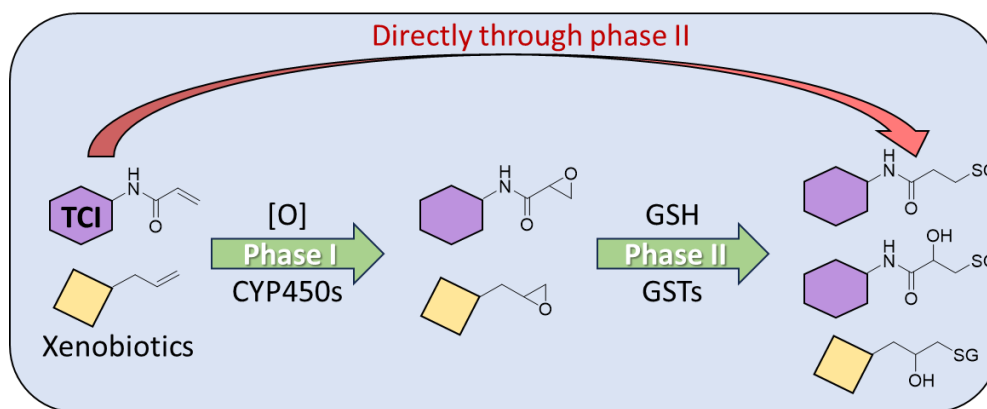


Figure 1.5. Targeted covalent inhibitors are directly susceptible to phase II metabolism by GST-mediated GSH conjugation.

GSTs are a superfamily of metabolic enzymes that play a central role in detoxification of xenobiotic compounds. GSTs are broadly categorized into several subfamilies based on their subcellular localization and sequence homology, including cytosolic, mitochondrial, and microsomal (membrane associated) isozymes.¹⁰⁴ Among these, the cytosolic GSTs, further subclassified into α , μ , π , θ , σ , ω , ζ variants,¹⁰⁵ are the most extensively studied and are primarily responsible for drug metabolism in key organs such as the liver and kidneys.

Structurally, cytosolic GSTs exist as homo- or heterodimers, with each monomer containing a highly conserved glutathione binding site (G-site) and a more variable hydrophobic binding site (H-site), capable of accommodating a wide range of substrates (**Fig. 1.6**).¹⁰⁶ In addition to these two sites facilitating proximity and orientation between the electrophile and GSH, the catalytic mechanism of GSTs involves stabilization of the GSH thiolate (GS^-) within the active site, effectively lowering its pK_a and increasing the concentration of the nucleophilic species.¹⁰⁷⁻¹⁰⁹ Under physiological conditions, intracellular GSH concentrations are typically at or above the K_M values for GSTs, such that the G-site is saturated and GSH is constitutively available for reaction as a co-substrate.¹⁰⁷ Together, these effects lead to an overall acceleration of GSH-conjugation.

1.4.1. Glutathione *S*-Transferase A1 (GSTA1)

Glutathione *S*-transferase A1 is a member of the α subclass of cytosolic GSTs and is the most highly expressed GST isoform in the liver.^{110, 111} In addition to its central role in hepatic detoxification, GSTA1 can be overexpressed as a resistance mechanism to cancer therapies.¹¹⁰ As a homodimeric Tyr-based GST, it uses a conserved Tyr residue (Tyr9) to stabilize the GSH thiolate through a hydrogen bonding interaction, thereby promoting nucleophilicity.¹¹² Structurally, GSTA1 possesses a relatively well-defined H-site (**Fig. 1.6A**), in part due to a C-terminal α -helix that contributes to a more enclosed binding pocket, which may influence substrate recognition.¹¹³

Owing to its central role in the primary metabolic organ, GSTA1 is widely studied as a model for systemic clearance pathways, particularly in the context of its broad substrate scope.

1.4.2. Glutathione *S*-Transferase P1 (GSTP1)

Glutathione *S*-transferase P1 is a member of the π subclass of cytosolic GSTs and is widely expressed in many extrahepatic tissues, such as the blood, kidneys, and lungs, with particularly high expression observed in many cancer types.¹¹⁴ GSTP1 is strongly implicated in resistance to drugs and has been extensively studied in the context of cancer biology. Like GSTA1, it functions as a homodimeric Tyr-based GST, using conserved Tyr7 during catalysis.¹¹² GSTP1 exhibits a more solvent-accessible H-site relative to GSTA1 (**Fig. 1.6B**), due to a disordered C-terminus, which may contribute to differences in substrate recognition and reactivity.¹¹⁵ In addition to its role in detoxification pathways throughout the body, GSTP1 has also been explored as a target for inhibition, as a strategy to overcome drug resistance.¹¹⁶

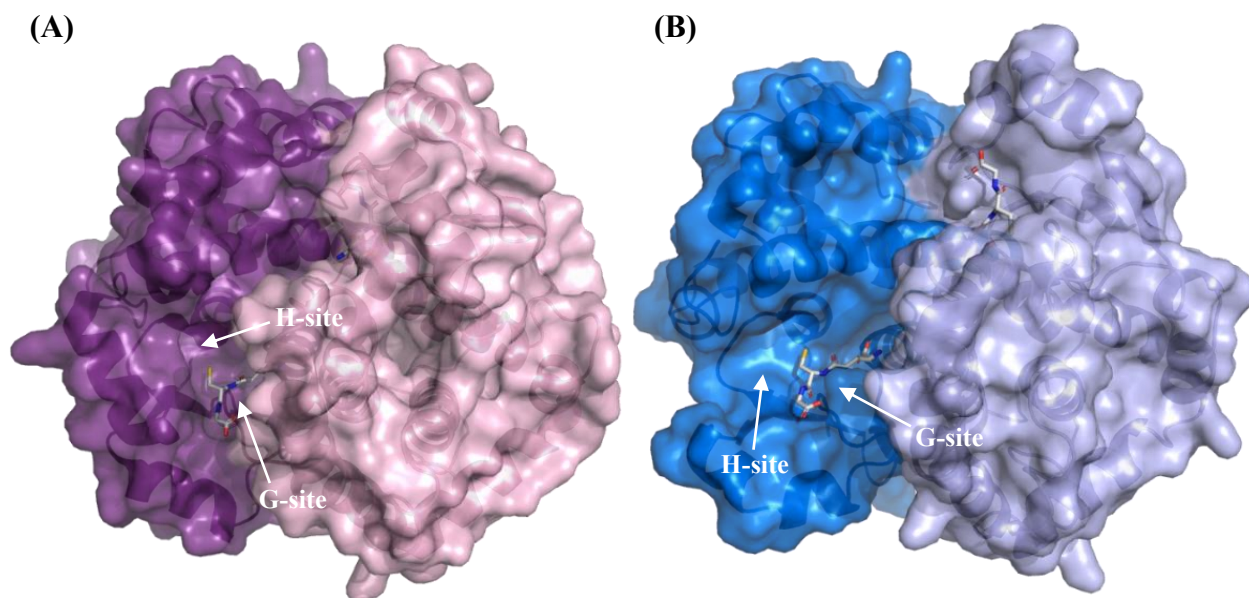


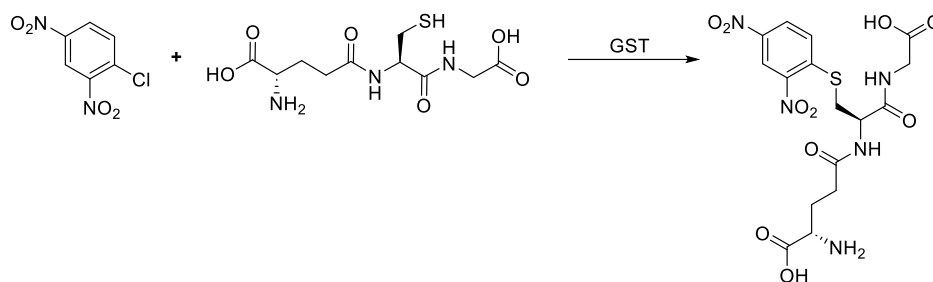
Figure 1.6. Crystal structures of (A) GSTA1 (PDB: 1PKW) and (B) GSTP1 (PDB: 6GSS).

1.4.3. Electrophilic Warheads as Substrates for GSTs

Together, GSTA1 and GSTP1 represent major pathways for the deactivation of covalent warheads. From a drug design perspective, selection of an electrophilic warhead should, in part, be guided by its susceptibility to these metabolic pathways. Reduced susceptibility is advantageous for maintaining free drug in circulation, whereas increased susceptibility, although often undesired, may be beneficial in cases where rapid clearance is required to mitigate toxicity.

The propensity of a warhead to undergo GSH conjugation is governed by two key factors: its intrinsic reactivity with free GSH and its efficiency as a substrate for GSTs. The former has been studied extensively for many warhead classes,²²⁻²⁴ as it can be readily characterized through reaction half-lives or rate constants with GSH in solution. In contrast, surprisingly little is known about how warheads behave as substrates for GSTs, particularly with respect to binding affinity and isozyme selectivity.

To date, much of the mechanistic understanding and substrate scope of GST-catalyzed reactions has been derived from studies of electrophiles that undergo nucleophilic aromatic substitution (S_NAr).¹¹⁷⁻¹²¹ These warheads are well suited for studying GSTs due to their relatively large, hydrophobic structures, which can sufficiently occupy the H-site even in the absence of a larger recognition scaffold, as well as their high intrinsic reactivity. Furthermore, GSH conjugation of S_NAr substrates typically produces a measurable increase in absorbance at convenient wavelengths (300-700 nm), enabling rapid spectrophotometric assays. Electrophiles of this class are widely used as model substrates for GST activity measurements, most notably in the canonical assay monitoring GSH conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) (**Scheme 1.4**).^{120, 122}



Scheme 1.4. GSH conjugation to CDNB produces an increase in absorbance at 340 nm which is used to monitor the activity of GSTs.

However, previous studies exploring structure-activity relationships of S_NAr electrophiles have primarily been conducted in the context of developing efficient assay substrates, particularly for GSTP1, rather than from a medicinal chemistry perspective aimed at minimizing susceptibility to GST-mediated metabolism. In addition, many of these studies predate the recent resurgence of targeted covalent drug development.

In contrast, other electrophilic warhead classes relevant to covalent drug design, such as attenuated Michael acceptors, have not been studied in a systematic manner as GST substrates. While isolated reports describe GST-mediated GSH conjugation of specific compounds, including drugs and natural products,¹²³⁻¹²⁵ these studies are generally limited to individual examples and do not establish generalizable structure-activity relationships. Consequently, current understanding of GST-mediated metabolism cannot be readily applied to the selection or optimization of electrophilic warheads in drug development.

1.5. Thesis Overview

The intrinsic reactivity of warheads is often used as a proxy to predict the reactivity of TCIs *in vivo*. However, intrinsic reactivity does not capture the complexity of protein mediated covalent reactions which depend not only on inherent electrophilicity, but also on interaction, proximity, and orientation effects within specific protein binding sites. We hypothesize that, beyond intrinsic reactivity alone, warheads may contribute to the PD and PK properties of covalent inhibition through affinity driven interactions with on and off-target proteins, and understanding these effects is crucial to the rational design of effective TCIs.

Thus, this thesis investigates how electrophilic warhead structure influences the PD and PK properties of TCIs, not only for hTG2 inhibition but also for general covalent drug design. Emphasis is placed on rigorous kinetic measurements to establish effects of warhead structure on target engagement, isozyme selectivity, intrinsic reactivity, and metabolic susceptibility.

Chapter 2 examines a library of warheads on a known small-molecule scaffold to elucidate their influence on binding affinity, reactivity, selectivity, and stability in the context of hTG2 inhibition. Chapter 3 explores the structure-activity relationships of a novel non-peptidic small-molecule scaffold incorporating a unique internal alkyne warhead for irreversible inhibition of hTG2. Finally, chapter 4 evaluates the GST-mediated metabolic susceptibility of a library of warheads commonly employed in covalent drug development.

While the new hTG2 inhibitors developed herein provide a foundation for future lead optimization efforts, the kinetic insights gained from this work demonstrate how warhead structure and reactivity govern both target engagement and off-target metabolic liabilities. Collectively, these findings position electrophilic warheads as key structural design elements rather than merely reactive functional groups, thereby informing the rational design of TCIs for hTG2 and beyond.

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Chapter 2 – The War on hTG2: Warhead Optimization in Small-Molecule Human Tissue Transglutaminase Inhibitors

2.1. Preamble

At the time this study was initiated, the Keillor lab was transitioning toward the development of smaller, more drug-like scaffolds for targeting hTG2 to improve pharmacokinetic properties. Although a variety of electrophilic warheads had previously been explored for hTG2 inhibition, these efforts were largely conducted with larger peptidomimetic scaffolds, where the relative contribution of the warhead to overall ligand efficiency is diminished. Moreover, the studies conducted by other groups in this field generally did not include detailed kinetic characterization, making it difficult to disentangle the respective contributions of warhead structure to binding affinity (K_I) and covalent reactivity (k_{inact}). As a result, the extent to which electrophilic warheads modulate inhibitor potency, particularly within smaller, more minimalistic scaffolds, remained unclear.

To address this gap, the work described in this chapter investigates a library of covalent inhibitors bearing diverse electrophilic warheads installed on a common small-molecule scaffold. Through systematic evaluation of inhibition kinetics and warhead stability, this study aims to elucidate how warhead structure influences both binding affinity and reactivity toward hTG2, as well as isozyme selectivity and susceptibility to metabolic deactivation.

2.2. Publication Information and Acknowledgements

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2.3. Author Contributions

L. K. M. – Conceptualization, synthesis, characterization, enzymatic assays, writing original draft

S. K. I. W. – Intrinsic reactivity measurements

H. R. I., L. N., and H. K. – Cellular pharmacokinetic measurements

J. W. K. – Conceptualization, supervision, funding, editing and reviewing

2.4. Correction to Published Version

On **page 52** of this thesis (**page 286** of the manuscript) in **Table 3** the k_2^{corr} value for compound **7c** should read 0.79 ± 0.09 and $(k_{\text{inact}}/K_1)/k_2^{\text{corr}}$ should read $\times 10^6$, making the enzymatic efficiencies on the order of millions rather than billions. This resulted from a mathematical error and has no impact on the rank order of inhibitors or conclusions of the manuscript.

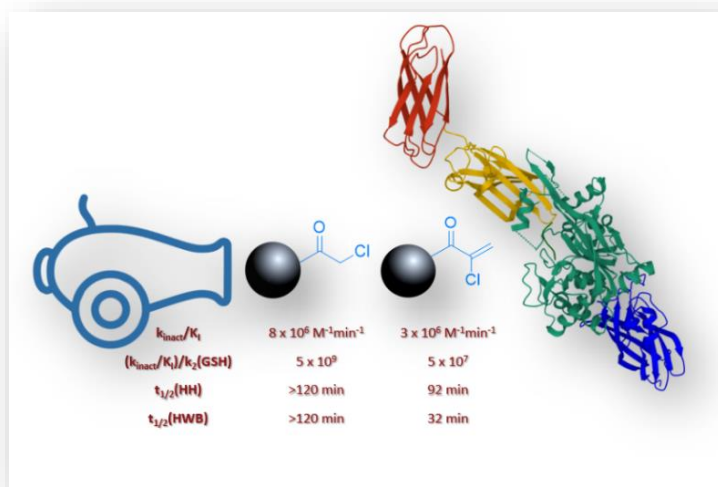


Figure 2.1. Graphical abstract summarizing the work described in this chapter.

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The war on hTG2: warhead optimization in small molecule human tissue transglutaminase inhibitors†

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Human tissue transglutaminase (hTG2) is a multifunctional enzyme with protein cross-linking and G-protein activity, both of which have been implicated in the progression of diseases such as fibrosis and cancer stem cell propagation when dysregulated, prompting the development of small molecule targeted covalent inhibitors (TCIs) possessing a crucial electrophilic 'warhead'. In recent years there have been significant advances in the library of warheads available for the design of TCIs; however, the exploration of warhead functionality in hTG2 inhibitors has remained relatively stagnant. Herein, we describe a structure-activity relationship study entailing rational design and synthesis for systematic variation of the warhead on a previously reported small molecule inhibitor scaffold, and rigorous kinetic evaluation of inhibitory efficiency, selectivity, and pharmacokinetic stability. This study reveals a strong influence on the kinetic parameters k_{inact} and K_i with even subtle variation in warhead structure, suggesting that the warhead plays a significant role in not only reactivity, but also binding affinity, which consequently extends to isozyme selectivity. Warhead structure also influences *in vivo* stability, which we model by measuring intrinsic reactivity with glutathione, as well as stability in hepatocytes and in whole blood, giving insight into degradation pathways and relative therapeutic potential of different functional groups. This work provides fundamental structural and reactivity information highlighting the importance of strategic warhead design for the development of potent hTG2 inhibitors.

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Introduction

Human tissue transglutaminase (hTG2) is one of eight enzymes comprising the transglutaminase (TGase) family that are characterized by their ability to cross-link proteins in the extracellular matrix. Using a catalytic cysteine residue, these enzymes form $N^{\epsilon}(\gamma\text{-glutaminy})\text{lysine}$ bonds *via* an acyl transfer (transamidation) mechanism between acyl donor and acyl acceptor substrates. This transamidase site is also capable of isopeptidic cleavage and hydrolysis.^{1–4} Among this family, hTG2 is uniquely multifunctional, in that it can also bind and hydrolyze guanosine triphosphate (GTP) in its function as a G-protein in cell signalling pathways.^{1,5,6} These two biological activities are mutually exclusive as they are dependent on the enzyme's ability to adopt remarkably

distinct conformations associated with each.^{6,7} In the extracellular environment, high Ca^{2+} concentrations favour a linear 'open' conformation, stabilized by the binding of these ions, in which the transamidase site is formed and the GTP binding site is distorted, through which G-protein function is lost. Intracellularly however, lower Ca^{2+} concentrations favour a compact 'closed' conformation in which the enzyme folds to expose a GTP binding site, while abolishing the transamidase site and cross-linking activity.^{6–9}

Dysregulation of both enzymatic functions has been implicated in the progression of a wide range of diseases such as fibrosis,^{10–12} cancer,^{13–15} and celiac disease,^{16,17} all while hTG2 knockout mice appear developmentally and reproductively viable.¹⁸ Thus, hTG2 serves as an attractive target for inhibition and has been extensively studied for decades with promising results in many therapeutic avenues, such as the recent progression of a peptidomimetic inhibitor to phase II clinical trials for the treatment of celiac disease.¹⁷

Considering the mutual exclusivity of the two binding sites of hTG2, we, and many others, have developed libraries of irreversible, targeted covalent inhibitors (TCIs) that

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selectively bind to the transamidase site of hTG2, locking the enzyme in an open conformation and thereby abolishing both G-protein and transamidase activity.^{19–22} These inhibitors vary in structure from small molecule to larger peptidomimetic scaffolds; however, a critical feature they all share is an electrophilic ‘warhead’ that forms a covalent bond with the catalytic cysteine residue. This warhead must be designed to fit in the enzyme’s active site in the correct conformation, and be electrophilic enough to react with the cysteine, while also possessing enough stability to survive cellular conditions and prevent off-target reactions.²³ Over the years we and others have investigated many different warheads for their suitability based on these criteria, including acrylamides, dihydroisoxazoles, thiadiazoles, and epoxides, but often on different scaffolds (Fig. 1), making it difficult to assess the influence of the warhead alone.^{24–30} In addition, many studies outside of our lab rely predominantly on IC₅₀ values to assess inhibitor efficiency, leaving the distinct parameters of k_{inact} and K_{i} unaccounted for, along with a full characterization of the impact that a warhead may have on reactivity and binding affinity, respectively.

So far, the acrylamide group appears to provide the best balance between reactivity and stability and is the most commonly used warhead for hTG2 inhibition.¹⁹ Since these initial studies, significant advances have been made in the development of warheads available for the design of TCIs.^{23,31,32} Recognizing the lack of a current and systematic structure–activity relationship study of warheads for hTG2 inhibition, in this work we used a previously reported, potent, adamantyl-piperazine-glycine (Ad-Pip-Gly) scaffold (Fig. 1),^{22,28} to incorporate and screen a variety of different warheads. These inhibitors were rigorously characterized with respect to their enzymatic efficiency, intrinsic reactivity, pharmacokinetic stability and isozyme selectivity, revealing interesting trends relevant to the design of inhibitors targeting hTG2, as well as other TGases.

Results and discussion

Design

For this type of systematic study in which the most reactive group of the inhibitor is varied, it was important to select a scaffold that is not only efficient for inhibition, but also synthetically accessible and enables late-stage diversification. Recently we confirmed that the Ad-Pip-Gly scaffold originally published by Badarau *et al.* confers excellent affinity and efficiency to inhibitors.^{22,28} This small molecule scaffold is also very convenient to synthesize as it allows the warhead to be incorporated in the very last step (Scheme 1), as opposed to other peptidomimetic scaffolds we have developed,^{19–21} making it ideal for this study.

Our subsequent selection of warheads (Scheme 2) was guided by a collection of works characterizing warheads for intrinsic reactivity and/or enzymatic inhibition, some of which have never been investigated for hTG2 inhibition in any scaffold that we know of. Results from previous hTG2 warhead studies,^{24–30} coupled with our molecular modelling of small molecule inhibitors,²² led us to believe that relatively small warheads were more suitable for inhibition as they could be better accommodated by the narrow ‘tunnel’ leading from the acyl-donor substrate binding site, down to the catalytic cysteine residue (Cys277) in the enzyme’s active site (Fig. 2).⁷ Hence, we took size and electrophilicity into consideration to see how expected trends in sterics and electronics would correlate with inhibition parameters k_{inact} and K_{i} .

We have previously shown that current hTG2 crystal structures are not completely appropriate for predicting binding affinity for small molecule inhibitors.²² Our attempts at docking molecules with different warheads using our established methods did not reveal any significant trends or correlation to kinetic parameters, so we did not use molecular modelling to refine our choice of warheads, apart

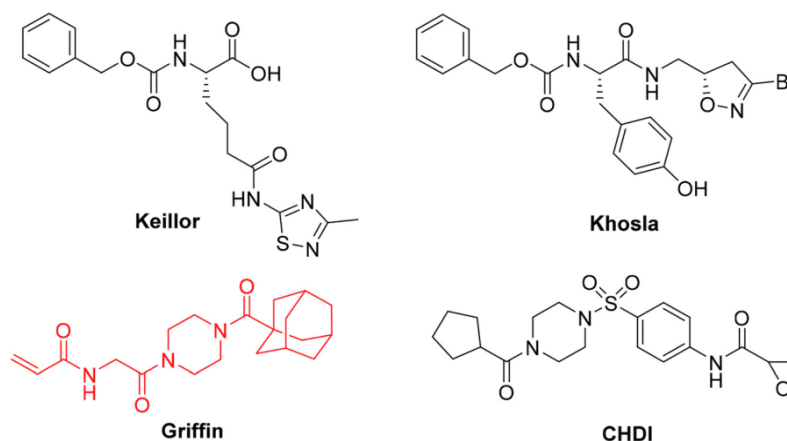
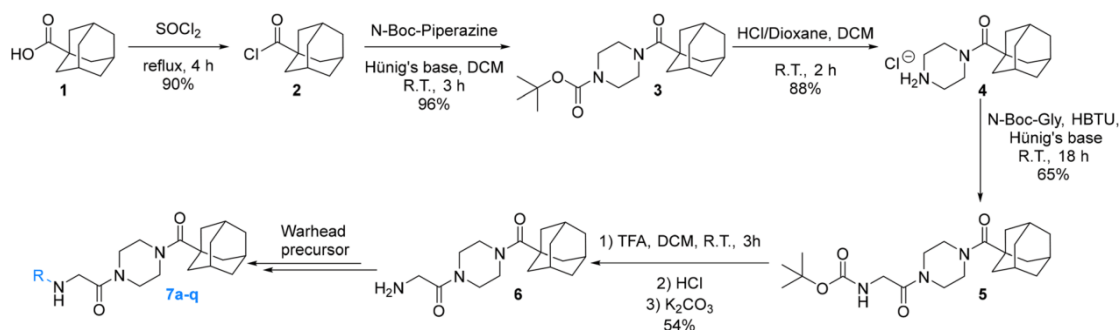
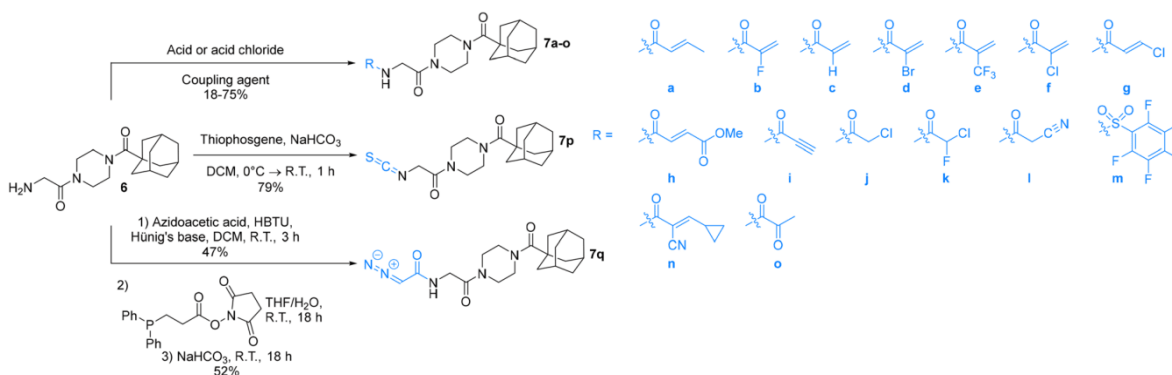


Fig. 1 Representative small molecule hTG2 inhibitors with different warheads.^{25,28–30} The Ad-Pip-Gly scaffold used in this study is shown in red.



Scheme 1 Synthesis of Ad-Pip-Gly scaffold enabling late-stage warhead diversification.



Scheme 2 Synthesis of final inhibitors with varying warheads.

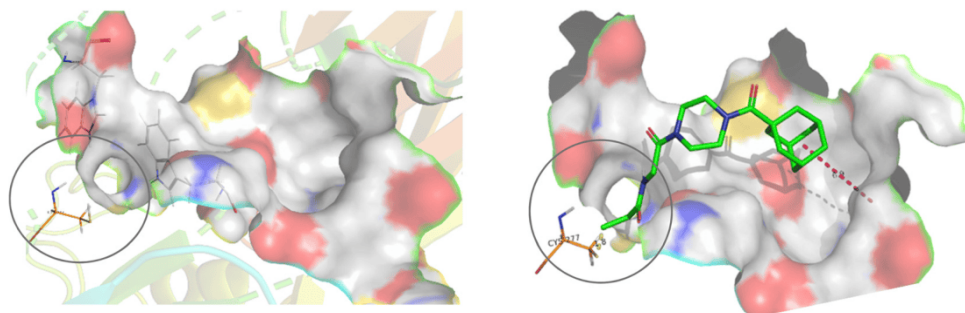


Fig. 2 Active site of hTG2 (PDB: 2Q3Z) with narrow 'tunnel' leading to Cys277, shown with and without inhibitor.

from considering the general structure of the active site as a guide.

An obvious place to start was an acrylamide series with various substituents at the α and β positions (**7a–h**) to investigate the effect of increasing steric demand and/or electrophilicity on this commonly used warhead. Of particular interest were certain 'mediator' warheads that are known for their intrinsic stability,³³ such as α -fluoroacrylamide (**7b**) or for their improved selectivity, such as sterically hindered fumarates (**7h**).³⁴ We also included propiolamide (**7i**) as our previous studies suggest that alkyne-based inhibitors are quite potent for hTG2

inhibition.^{35,36} Chloroacetamide (**7j**) has been previously reported by Badarau *et al.*,²⁸ but not characterized fully with respect to individual kinetic parameters. While chloroacetamides are excellent electrophiles,³⁷ they are believed to be too reactive to survive cellular conditions, for this reason we investigated a less reactive counterpart, chlorofluoroacetamide (**7k**), which has been shown to be less electrophilic than chloroacetamides, possibly due to the strong electron-withdrawing effect of the fluorine inducing a larger partial positive charge on the α -carbon atom and thereby shortening/strengthening the C–Cl bond.³⁸

We also studied some other warheads commonly employed in the design of TCIs, including cyanoacetamide (**7l**) and isothiocyanate (**7p**), the latter of which is present in an anticancer agent that can be used to inhibit TG2.^{23,39} Pentafluorobenzenesulfonamide (**7m**), bearing a warhead that reacts with thiols by S_NAr but has never been studied with TG2,⁴⁰ and diazoacetamide (**7q**),⁴¹ an amide version of the 6-diazo-5-oxo-L-norleucine (DON) warhead that was used in inhibitor-hTG2 crystallization studies,⁷ were also studied herein.

Finally, this Ad-Pip-Gly scaffold, traditionally used for irreversible inhibitors, was also used to investigate potentially reversible covalent inhibition with α -cyanoacrylamide (**7n**) and α -ketoamide (**7o**), warheads known to react reversibly with thiols,^{42,43} the latter of which has even previously been explored specifically for hTG2 inhibition.⁴⁴

With this series of inhibitors, we aimed to rigorously investigate a broad range of warheads with potential to confer potency, while still exploring specific trends that would provide fundamental information, rather than screening a large number of warheads at random.

Synthesis

The synthesis of the Ad-Pip-Gly scaffold follows previously reported routes developed by our lab and Badarau *et al.* (Scheme 1).^{22,28} For this study it was necessary to stockpile pure **6** from which late-stage diversification takes place for maximum synthetic efficiency. It is noteworthy that the final deprotection step to produce amine precursor **6** works optimally with TFA followed by extraction of the free base, as reported by Badarau *et al.*,²⁸ rather than with HCl/dioxane. Typically, we have observed much cleaner deprotection products with HCl/dioxane; however, in this specific case HCl/dioxane deprotection led to very poor yields due to the formation of unidentified side products. With TFA we were able to scale this reaction up to several grams, which proved to be necessary, since some of the warhead attachments were found to be difficult and gave poor yields, requiring larger amounts of starting material.

Inhibitors **7a–o** were synthesized by coupling amine **6** to either the carboxylic acid or the acid chloride of each warhead precursor (see Materials and methods) (Scheme 2). All of these precursors are commercially available; however, we opted to synthesize α -fluoroacrylic acid (**12**) and α -cyano- β -cyclopropylacrylic acid (**13**) by literature routes (see Materials and methods). Since the stereochemical configuration of **13** cannot be established by NMR, and is described rather ambiguously in previous methods,^{45,46} we confirmed the *E*-configuration of this compound by X-ray crystallography, as shown in the ESI.†

While performing these seemingly straightforward amide couplings for inhibitors **7a–o**, we found that certain acrylic acids did not react well with certain amide coupling agents. More specifically, many of them were sensitive to pre-activation, gave low yields, or simply did not react at all.

Hence the use of a diverse array of coupling agents, rather than one general synthetic method, was necessary for the different warheads. Ultimately, we did not focus on synthetic optimization and did not perform extensive screening on coupling conditions but did obtain acceptable yields for all reactions.

Inhibitor **7p** was synthesized easily by reacting amine **6** with thiophosgene, while inhibitor **7q** was obtained by first synthesizing the azidoacetamide (**15**) by an amide coupling with azidoacetic acid (**14**), followed by a ‘deimidogenation’ reaction using a phosphine reagent developed by Myers and Raines (Scheme 2).⁴⁷

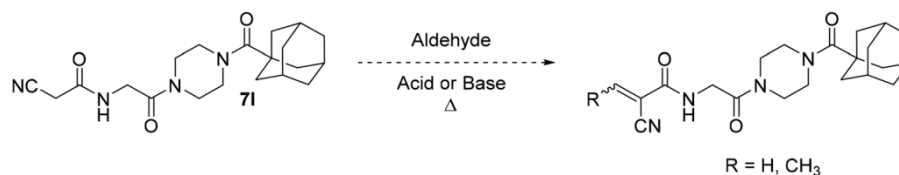
It is worth noting that originally, we wanted to synthesize less sterically hindered α -cyanoacrylamides from inhibitor **7l** by a Knoevenagel condensation with either formaldehyde or acetaldehyde (Scheme 3); however, we were not able to successfully isolate these compounds under a variety of attempted conditions as they would immediately degrade upon contact with water during work up, suggesting they would not have been stable to subsequent assay conditions. These types of compounds are known to be very reactive,³¹ and it is possible that without a relatively bulky alkyl group at the β -position, they are too unstable to be used as viable warheads. However, the α -cyano- β -cyclopropylacrylamide warhead has been previously reported several times and was relatively easy to synthesize, so we decided to incorporate that instead.^{45,46,48}

We also synthesized an inhibitor with an α -methylacrylamide warhead (see ESI,† compound **7r**), in order to serve as a good comparison to its isostere α -chloroacrylamide (**7f**) and its isomer β -methylacrylamide (**7a**). However, we found that its solubility in polar solvents such as DMSO, DMF, ACN, and MeOH was surprisingly poor, and therefore this analogue could not be analyzed for purity or evaluated in our kinetic assay.

When necessary, inhibitors could be purified easily by flash chromatography generally up to >95% purity, typically using MeOH/EtOAc as the mobile phase for optimal separation, taking care to dry load the crude products as they had poor solubility in this solvent system. In some cases, further washes with water and/or pentane were necessary to remove residual by-products from coupling agents and contaminant grease. Overall, the synthesis of these inhibitors followed very standard conditions after some necessary troubleshooting, and the majority of issues were posed by acrylic acid precursors rather than the Ad-Pip-Gly scaffold.

Kinetic evaluation

All inhibitors were then kinetically evaluated for their inhibitory action against recombinant hTG2 using our previously developed continuous kinetic assay (Table 1).^{19,49} hTG2-mediated hydrolysis of substrate AL5 was analyzed by monitoring the release of chromogenic product *p*-nitrophenolate. Incubation with irreversible inhibitors resulted in time-dependent inactivation of the enzyme. Under



Scheme 3 Attempted synthesis of some α -cyanoacrylamides.

Kitz and Wilson conditions,⁵⁰ each inhibitor was tested at varying concentrations and the corresponding curves of absorbance *versus* time were fitted to a mono-exponential association equation to give observed first order rate constants of inhibition (k_{obs}) (Fig. 3a). These rate constants were then plotted against inhibitor concentrations corrected for the presence of the competitive substrate, and then fitted to a hyperbolic equation representative of saturation kinetics, giving inhibition parameters k_{inact} and K_{I} (Fig. 3b).^{50–52} We also performed a simple linear regression of the low inhibitor concentration data points, from which the ratio of $k_{\text{inact}}/K_{\text{I}}$ could be obtained directly with less relative error and compared to the ratio calculated using the individually obtained parameters.⁵¹

Starting with the acrylamide series, a rough correlation can be seen between expected electrophilicity of the β carbon, where the thiolate attacks *via* Michael addition, and k_{inact} values ranging between $0.51\text{--}7.28\text{ min}^{-1}$ (Table 1, **7a–f**). As the electronegativity of the substituent at the α -position increases, the π -bond between the α and β carbon becomes more polarized, making the β carbon more electrophilic. As a result, the k_{inact} value (the first order rate constant of covalent bond formation within the enzyme-inhibitor complex) also increases. The apparent exception to this is α -fluoroacrylamide (**7b**), whose intrinsic stability (*i.e.*, lower reactivity) has been well studied and is thought to be a result of the mesomeric electron donating ability of fluorine, which compensates at least partially for its electron withdrawing inductive effects.⁵³ This warhead gives a k_{inact} of 1.17 min^{-1} , compared to a value of 2.64 min^{-1} for acrylamide **7c**. Beyond this general trend in the k_{inact} values, the construction of Hammett and Charton plots for substituents at the alpha position of inhibitors **7b–f** did not provide any correlation (not shown).

More interesting and perplexing results are found in the corresponding K_{I} values. For inhibitor **7a**, a large decrease is observed in the binding affinity compared to parent compound **7c**, with K_{I} values of 61.8 *versus* $3.98\text{ }\mu\text{M}$ respectively. We believe this is due to the increased steric demand at the β position, resulting in the warhead not being able to fit properly in the active site ‘tunnel’. Surprisingly, inhibitors **7b** and **7e**, both containing fluorine, also display K_{I} values an order of magnitude higher than **7c**, while other halo-acrylamide inhibitors **7d** and **7f** have relatively unchanged binding affinity despite posing increased steric demand at the α -position. These results suggest that the α -position seems to tolerate bulkier substituents more readily

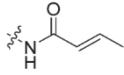
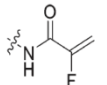
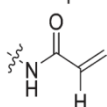
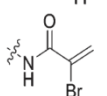
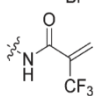
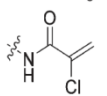
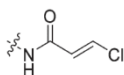
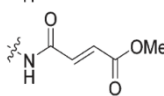
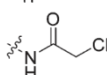
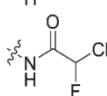
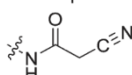
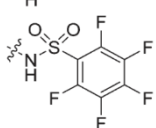
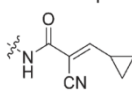
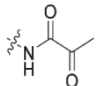
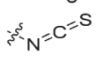
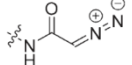
than the β -position, further supported by the fact that **7g** and **7h** show no significant inhibition. However, the binding affinity cannot be explained by steric factors alone. It seems that fluorine-based warheads are not well tolerated in the enzyme’s active site. We believe that this may be due to some fluorine mediated polar interaction or halogen–hydrogen bonding that disorients the inhibitor. Although there is currently no crystallographic evidence to support this hypothesis, we have previously discovered that fluorines on the N-terminus of our peptidomimetic inhibitors actually increase binding affinity.²¹ It is possible that the fluorine on the warheads studied herein directs these inhibitors to the same substrate binding site interaction as the peptidomimetic inhibitors, instead of down the active site ‘tunnel’.

Due to the increase in k_{inact} values of **7d** and **7f**, while maintaining modest K_{I} values, these inhibitors show an order of magnitude greater overall inhibitory efficiency, with $k_{\text{inact}}/K_{\text{I}}$ values of $2\,412\,000\text{ M}^{-1}\text{ min}^{-1}$ and $3\,134\,000\text{ M}^{-1}\text{ min}^{-1}$ respectively, compared to **7c** ($k_{\text{inact}}/K_{\text{I}} = 662\,000\text{ M}^{-1}\text{ min}^{-1}$), which is on par with chloroacetamide **7j** and quite remarkable.

Propiolamide **7i** gives a k_{inact} value (6.83 min^{-1}) that is much higher than that of **7c**. This is consistent with intrinsic reactivity studies performed by Flanagan *et al.* which show that this warhead is more reactive towards thiol addition than both acrylamides and chloroacetamides, presumably due to the sp hybridization state of this warhead which stabilizes negative charge more effectively.³² In fact, this is also reminiscent of our previous studies on alkyne inhibitors, which showed similarly high k_{inact} values.³⁶ Regardless, the low binding affinity of this inhibitor, possibly due to the inappropriate orientation of this linear warhead, makes it a relatively poor inhibitor.

Outside of the acrylamide and propiolamide warheads, k_{inact} does not strictly correlate to the electrophilicity of a warhead, indicating that it is likely influenced by both intrinsic reactivity and orientation relative to the thiolate in the active site. Chloroacetamides are known to be highly reactive, and within this series of compounds **7j** displays the best overall efficiency ($k_{\text{inact}}/K_{\text{I}} = 8\,047\,000\text{ M}^{-1}\text{ min}^{-1}$). However, this is largely due to a relatively high binding affinity rather than high reactivity, as shown by the relative K_{I} and k_{inact} values, respectively. Intrinsically it is known that chloroacetamides are more reactive than acrylamides,³² so it is interesting that the k_{inact} values do not correlate with this intrinsic reactivity, as the k_{inact} value (1.23 min^{-1}) for **7j** is

Table 1 Inhibition parameters measured for inhibitors **7a–q**. n.d.: no inhibition detected up to 100 μM

Compound	Warhead	k_{inact} (min^{-1})	K_{I} or K_{I} (μM) ^a	$k_{\text{inact}}/K_{\text{I}}$ ($\times 10^3 \text{ M}^{-1} \text{ min}^{-1}$)	$k_{\text{inact}}/K_{\text{I}}$ ($\times 10^3 \text{ M}^{-1} \text{ min}^{-1}$) (linear regression)
7a		0.51 ± 0.05	61.8 ± 10.2	8.3 ± 1.6	6.7 ± 0.7
7b		1.17 ± 0.12	38.4 ± 6.2	30.4 ± 5.9	28.5 ± 1.9
7c		2.64 ± 0.39	3.98 ± 0.79	662 ± 164	580 ± 40
7d		4.69 ± 0.65	1.95 ± 0.32	2412 ± 522	2320 ± 81
7e		5.22 ± 0.96	30.6 ± 6.9	171 ± 50	209 ± 13
7f		7.28 ± 1.41	2.32 ± 0.52	3134 ± 921	3141 ± 143
7g		n.d. ^b	n.d.	n.d.	n.d.
7h		n.d.	n.d.	n.d.	n.d.
7i		6.83 ± 1.75	70.1 ± 25.0	97.5 ± 42.8	87.7 ± 4.5
7j		1.23 ± 0.30	0.15 ± 0.09	8047 ± 4887	4545 ± 289
7k		1.57 ± 0.29	88.5 ± 21.5	17.8 ± 5.4	16.0 ± 1.3
7l		n.d.	n.d.	n.d.	n.d.
7m		n.d. ^c	n.d.	n.d.	n.d.
7n		—	n.d.	—	—
7o		—	154 ± 60	—	—
7p		0.23 ± 0.03	8.83 ± 2.41	26.3 ± 8.1	17.7 ± 2.4
7q		2.82 ± 0.49	26.3 ± 6.5	107 ± 53	95.0 ± 2.4

^a K_{I} for irreversible inhibitors and K_{I} for reversible inhibitors (**7n** and **7o**). ^b No inhibition detected up to 50 μM . ^c No inhibition detected up to 10 μM . Note: a threshold inhibitor concentration at which no inhibition was observed was chosen depending on solubility constraints

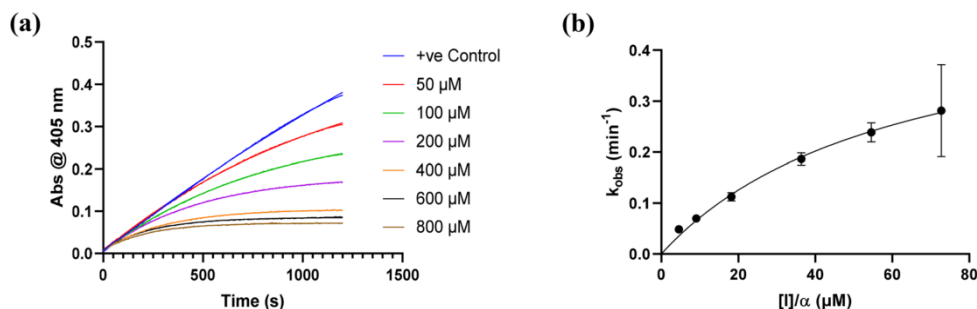


Fig. 3 Kinetic curves obtained during hTG2 inhibition with representative inhibitor **7a**. (a) k_{obs} for the loss of activity and (b) hyperbolic fitting to saturation kinetics model (see Materials and methods and ESI†).

smaller than that of **7c**. Furthermore, chlorofluoroacetamide **7k** displays an approximately equivalent k_{inact} value to **7j**, despite generally being less reactive. Note that the addition of a fluorine results in the increase of K_i by two orders of magnitude (0.15 vs. 88.5 μM) consistent with the observation noted above, that fluorine may be detrimental to binding affinity.

Inhibitors **7l** and **7m** showed no significant inhibition. We believe this is due to the nitrile of the cyanoacetamide not being electrophilic enough to react efficiently. This observation is also aligned with previous hTG2 inhibition studies in which inhibitors with any type of nitrile warhead showed no detectable inhibition.³⁰ It also comes as no surprise that the pentafluorobenzenesulfonamide warhead is inadequate likely due to its large size and perhaps abundance of fluorine atoms.

With isothiocyanate inhibitor **7p**, we again observed a low k_{inact} value (0.23 min^{-1}) for a notoriously reactive warhead, but in this case **7p** displayed a relatively high K_i as well as 8.83 μM . Nonetheless, this is consistent with recently published inhibition parameters for sulforaphane, a naturally-occurring anticancer agent with an isothiocyanate warhead, with TG2.³⁹ Sulforaphane gives values of $k_{\text{inact}} = 0.27 \pm 0.07 \text{ min}^{-1}$ and $K_i = 366 \pm 157 \mu\text{M}$; comparing these values with those of **7p**, it is evident that k_{inact} is approximately the same while the binding affinity has been improved as a result of the Ad-Pip-Gly scaffold. It is likely that, in part, the binding affinity is still low due to the inhibitor being too small in length to properly allow the warhead to reach into the active site ‘tunnel’.

The last irreversible warhead we tested was diazoacetamide **7q**. We expected this warhead to show attenuated reactivity relative to its keto version, due to the additional resonance donating abilities of nitrogen which may impact the conjugation and reactivity of the diazo-carbonyl system, but we also observed a decrease in binding affinity. Evidently, any linear elongation of the warhead leads to a dramatic decrease in binding affinity. This is consistent with our previous observation that elongation of the glycine linker leads to a decrease in affinity.²² This phenomenon may also be responsible for the fact that the highly reactive reversible warhead of inhibitor **7n** displayed no detectable inhibition.

We did have moderate success with reversible α -ketoamide **7o**, for which we were able to extract a K_i value (referred to expressly as K_i instead of K_i to distinguish between reversible and irreversible inhibition). As expected, this inhibitor displayed time-independent inhibition of hTG2 consistent with rapid binding, competitive, reversible inhibition. We performed the same kinetic experiment described for irreversible inhibition at different inhibitor concentrations and obtained initial rates at each from the absorbance *versus* time plot (Fig. 4a). This experiment was then repeated at a different substrate concentration and the data from the two experiments were used to construct a Dixon plot, from which the K_i was extrapolated by the point of intersection of the two resulting lines (Fig. 4b). We also performed a simple test to validate that this was in fact reversible inhibition by running the assay for an extended period of time and allowing the substrate to be completely consumed in both the uninhibited and inhibited reaction. If inhibition is reversible, both reactions should plateau at the same final absorbance value corresponding to the same initial concentration of substrate, albeit at different times. This is exactly what we observed, as shown in the ESI†. Although the binding affinity for this inhibitor is poor ($K_i = 154 \mu\text{M}$), it does represent one of the first fully characterized covalent reversible inhibitors of hTG2, demonstrating that it is a viable avenue to explore for hTG2 inhibition.

Taken together, this thorough kinetic evaluation has revealed some interesting trends in the reactivity and affinity of different warheads for hTG2 and highlights how subtle changes in warhead structure can dramatically influence inhibitory efficiency. It appears acrylamides are still the best suited class of warheads for targeting hTG2, and we have identified two analogues that confer potency far greater than that of an unsubstituted acrylamide group and have never been reported for hTG2 inhibition elsewhere.

Isozyme selectivity

We also tested our top four inhibitors against other therapeutically relevant TGases, in order to assess the relative

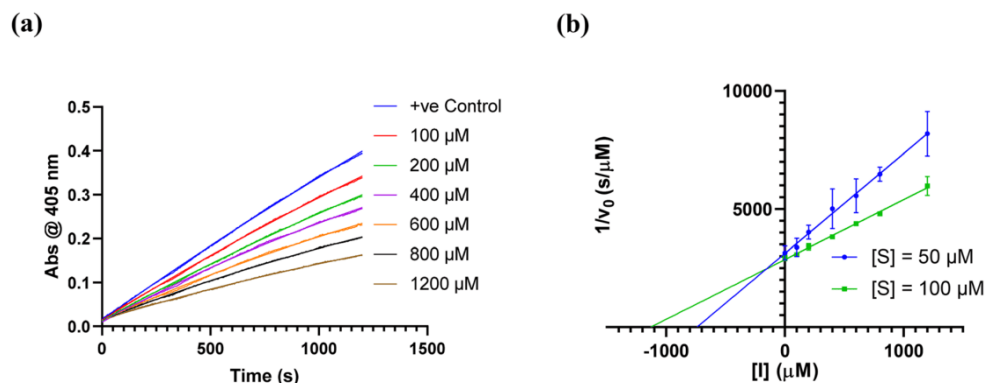


Fig. 4 Kinetic analysis of inhibitor **7o**. (a) Representative time-independent rate decrease at $[S] = 100 \mu\text{M}$. Average initial rates (v_0 for the uninhibited reaction and for the inhibited reactions) were determined over the first 10% of conversion of the uninhibited reaction. (b) Dixon plot used to determine K_i . See ESI† and Materials and methods for details.

selectivity imparted by the warhead (Table 2). The assay described above for hTG2 inhibition was also used to monitor hTG1 and hTG6 inhibition,¹⁹ whereas for hTG3a and hFXIIIa, a commercially available continuous fluorescence assay that uses a peptidic FRET-quenched substrate was used.^{54,55} In each case, we chose a corrected inhibitor concentration ($[I]/\alpha$) that gave a k_{obs} value of approximately 0.5 min^{-1} with hTG2, and then adjusted inhibitor concentrations to give an identical $[I]/\alpha$ value for each enzyme/substrate pair, in order to permit comparison between isozymes. In this fashion, k_{obs} values were obtained whenever time-dependent inhibition was observed. Interestingly, some isoforms displayed time-independent inhibition (*i.e.*, instantaneous and constant decrease in rate), in which case a percent inhibition ($v_{\text{inhibited}}/v_{\text{uninhibited}} \times 100\%$) was measured instead. We recognize that % inhibition values cannot be directly compared to k_{obs} values to assess selectivity for hTG2; however, they may be used to compare selectivity between different warheads (see ESI†).

Previously reported inhibitor **7c** (Fig. 5) shows roughly 20-fold selectivity for hTG2, over hTG1 and hTG6, consistent with the findings of Badarau *et al.*²⁸ However, in our assay

this inhibitor is far less selective for hTG2 relative to hTG3a and hFXIIIa than reported in the original study, to the point where it appears to be a rather efficient inhibitor of these isozymes. We believe this may be due to the different assays used to measure inhibition. Indirect, coupled-enzyme assays are prone to initial lag periods that may lead to underrepresented inhibition,²² which may be further exacerbated by the intrinsically slow reactivity of hTG3a and hFXIIIa. The sensitive continuous fluorescence assay used herein may allow for more accurate measurement of inhibition of hTG3a and hFXIIIa.

While some isozymes seem to have lower affinity for the scaffold, it is interesting to note that within the same scaffold, certain isozymes show selectivity for different warheads. All of these inhibitors target hTG1 in a similar manner and show approximately the same selectivity for hTG2 over this isozyme, whereas the selectivity is more diverse with other isozymes. No inhibitor, other than **7c**, inhibits hTG6. Similarly, hTG3a is only inhibited reversibly by previously reported inhibitors **7c** and **7j**, but not inhibited by our new inhibitors **7d** and **7f**. hFXIIIa is inhibited irreversibly by **7c** and **7j**, reversibly by **7f**, and not inhibited

Table 2 Selectivity of top inhibitors against TGase isoforms. n.d.: no inhibition detected

Compound	Warhead	$[I]/\alpha$	k_{obs} TG2 (min^{-1})	k_{obs} TG1 (min^{-1})	k_{obs} TG6 (min^{-1})	% inhibition TG3a	hFXIIIa
7c		0.91	0.500 ± 0.016	0.023 ± 0.003	$0.020 \pm 9.00 \times 10^{-4}$	59.1 ± 0.7	$k_{\text{obs}} = 0.051 \pm 0.001 \text{ min}^{-1}$
7d		0.23	0.497 ± 0.017	0.018 ± 0.002	n.d.	n.d.	n.d.
7f		0.18	0.509 ± 0.002	$0.022 \pm 9.00 \times 10^{-5}$	n.d.	n.d.	% inhibition = 76.0 ± 3.3
7j		0.14	0.537 ± 0.019	0.018 ± 0.003	n.d.	41.5 ± 0.9	$k_{\text{obs}} = 0.013 \pm 0.002 \text{ min}^{-1}$

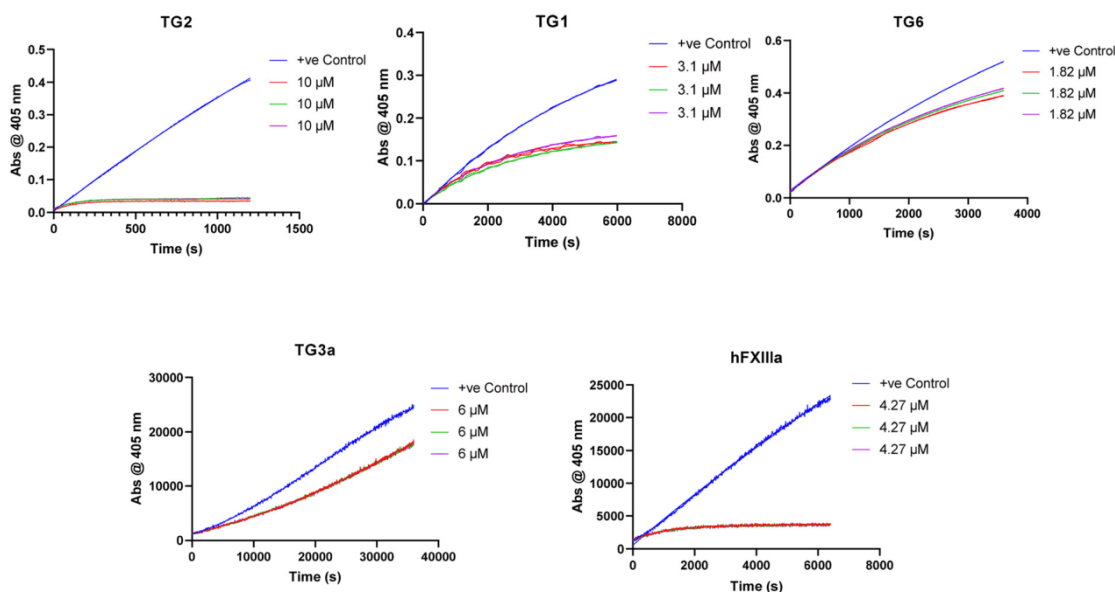


Fig. 5 Isozyme selectivity data for inhibitor 7c.

by **7d**. It is possible that the α -chloroacrylamide exhibits reversible covalent inhibition with hFXIIIa due to increased acidity of the α -hydrogen as a result of the electron withdrawing presence of the chlorine. More explicitly, there may be an amino acid residue on hFXIIIa near this hydrogen that is basic enough to facilitate the reverse E1cB reaction. A related change in inhibition mechanism has been reported for the inhibition of rhodesain, a cysteine protease; while a vinylsulfone inhibitor was irreversible, its α -fluoro analogue was found to be slowly reversible.⁵⁶ On the other hand, for inhibitors that show reversible inhibition with other TGases but not with hTG2, it is also possible that their warheads are simply not oriented in the isozyme's active site in an appropriate manner to react, and therefore they act as reversible non-covalent inhibitors. The reversibility observed in this work is clearly related to a function of the isozyme, and not to the intrinsic reactivity of the warhead.

Importantly, these results clearly demonstrate that increasing warhead reactivity does not necessarily have a negative impact on selectivity. To this point, our new haloacrylamide inhibitors show the most selectivity overall among the top inhibitors, suggesting that it is possible to tune both reactivity and isozyme selectivity through strategic warhead design.

Intrinsic reactivity

The same top inhibitors, excluding **7d** due to its poor aqueous solubility, were then evaluated further for their intrinsic reactivity, offering insight into the efficiency of the enzymatic reaction relative to reaction with a thiol in solution by simple comparison of rate constants (Table 3). We chose

GSH as a representative thiol in order to also provide a rough idea of the expected reactivity of a given inhibitor with the millimolar concentration of GSH found intracellularly and therefore the potential for off-target reactions.⁵⁷

Each inhibitor was incubated with GSH under pseudo-first order conditions at room temperature and at a pH that allowed sufficient reactivity. The formation of an inhibitor-GSH adduct and the corresponding disappearance of inhibitor were both monitored simultaneously using HPLC analysis. The resulting concentration *versus* time data were used to measure pseudo-first order rate constants which were then converted to intrinsic second order rate constants (k_2^{corr}) by taking into account the pH dependent concentration of excess thiolate in solution (see ESI†).

The kinetic parameter k_{inact}/K_I demonstrates overall efficiency of inhibition against hTG2. Intrinsically this is the second order rate constant of the reaction between the enzyme and the inhibitor, accounting for not only the rate of the covalent reaction (k_{inact}) but also binding affinity (K_I) and orientation/proximity effects that the enzyme's active site provides. This parameter is most relevant to compare to the second order rate constant of reaction with GSH (k_2^{corr}), as opposed to k_{inact} which is a first order rate constant. By calculating the ratio between the two second order rate constants we arrived at a parameter ($(k_{\text{inact}}/K_I)/k_2^{\text{corr}}$) that quantifies enzymatic efficiency relative to reaction with GSH in solution. All of the inhibitors demonstrate the extreme acceleration an enzymatic reaction provides over a typical chemical reaction in solution, with enzymatic efficiency ratios of at least 10 million. In fact, reactions with GSH were generally observed to be very slow at a pH of 7.4 and an increased pH of 8.0 was required to drive the reaction. With

Table 3 Comparison of warhead reactivity with hTG2 and GSH

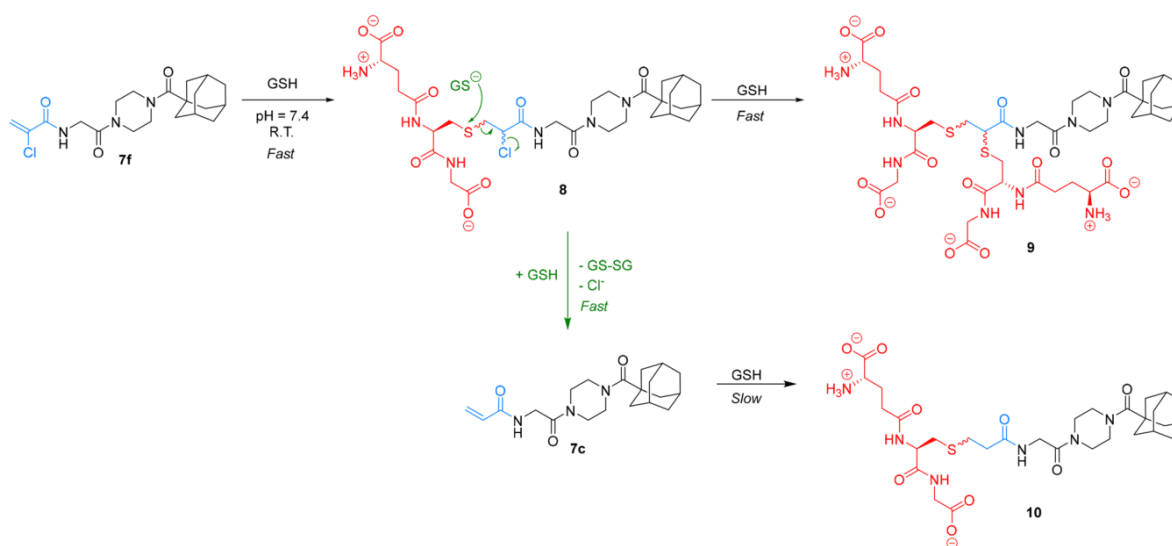
Compound	Warhead	$k_{\text{inact}}/K_{\text{I}}$ ($\times 10^3 \text{ M}^{-1} \text{ min}^{-1}$)	k_2^{corr} ($\times 10^{-3} \text{ M}^{-1} \text{ min}^{-1}$)	$(k_{\text{inact}}/K_{\text{I}})/k_2^{\text{corr}}$ ($\times 10^9$)
7c		662 ± 164	0.079 ± 0.009	0.843 ± 0.231
7f		3134 ± 921	59.387 ± 1.610	0.053 ± 0.016
7j		8047 ± 4887	1.480 ± 0.191	5.437 ± 3.376

acrylamide inhibitor (7c) we measured an enzymatic efficiency ratio of just under a billion ($0.849 \pm 0.232 \times 10^9$) as well as the slowest reaction with GSH, as expected based on the inherent stability of the acrylamide group compared to the other two warheads. The α -chloroacetamide (7j) displays reactivity with GSH that is two orders of magnitude faster than that of 7c, highlighting the intrinsic electrophilic superiority of α -halocarbonyls compared to the acrylamide group. This compound reacts roughly 5 billion times faster with hTG2 than with GSH.

We were particularly interested in comparing the reactivity profiles of chloroacetamide 7j and chloroacrylamide 7f, as it was not clear to us which electrophile is intrinsically more reactive. When we reacted 7f with GSH at a pH of 8.0 we observed such rapid consumption of the warhead that it was difficult to characterize, and the pH had to be adjusted back down to 7.4. Interestingly, we also noticed the formation of multiple products, instead of just one GS-inhibitor adduct. Upon careful inspection, we noted that while one product was formed at the beginning of the reaction, presumably

the GS-inhibitor mono-addition product, over time this adduct reacted further to produce other products, even after the original inhibitor had been entirely consumed (Scheme 4). The initial product was also eventually consumed in this second reaction. We hypothesized that the mono-addition product (8) can react further with another molecule of GSH through nucleophilic displacement of the chloride on the α -carbon to form a di-substituted product (9). Both mono and di-substitution would produce diastereomeric products which may be separated by HPLC, leading to the formation of multiple broad peaks on the chromatogram. Another possibility is the attack of a second equivalent of GSH on the sulfur of the initial adduct, leading to formation of glutathione disulfide and displacement of chloride in an elimination reaction that would lead to the formation of acrylamide 7c. This acrylamide would itself react slowly with another equivalent of GSH at this pH to form adduct 10.

We characterized these products using LC-MS analysis and were able to monitor and confirm the disappearance of inhibitor 7f, the formation and subsequent disappearance of

**Scheme 4** Side products of the reaction between GSH and 7f.

mono-substituted adduct **8**, the corresponding formation of double-substituted adduct **9** and formation of inhibitor **7c**, confirming our hypothesis (see ESI†). However, there were also other side products that we could not identify based on the mass to charge ratio. Although we could not analyze α -bromoacrylamide inhibitor **7d** in this assay, it is reasonable to assume that it could behave in a similar fashion, especially considering that bromide is a better leaving group than chloride.

Ultimately, by simply monitoring the disappearance of inhibitor **7f** by our original HPLC method, we determined that this warhead reacts an order of magnitude faster than the chloroacetamide. Owing to this relatively high and multifaceted reactivity with GSH, its reaction with hTG2 is only $0.053 \pm 0.016 \times 10^9$ times more efficient, which is the lowest efficiency ratio of the top warheads. However, it is particularly interesting to note that inhibitor **7f** can react with GSH, in a metabolically relevant reaction, to form another potent and more stable inhibitor **7c**.

Pharmacokinetic stability

The conjugation of electrophilic agents with GSH during phase 2 metabolism is most often mediated by one or more isozymes of glutathione *S*-transferase (GST).⁵¹ Taking this into account is necessary to assess the stability and suitability of a drug from a biochemical perspective. Therefore, we studied the stability of the same top three representative inhibitors in hepatocytes and whole blood, as *in vitro* models of *in vivo* conjugation mediated by the highly relevant α and π isozymes of human GST, respectively.^{58,59}

Inhibitors were incubated in human hepatocytes and human whole blood at 37 °C over a 120-minute period. Aliquots were taken over time and the amount of inhibitor remaining was determined by LC-MS/MS. In cases where decay was observed over this time frame, the half-life of each inhibitor was determined to model their relative stabilities (Table 4). In hepatocytes, acrylamide inhibitor **7c** and chloroacetamide **7j** show excellent stability with half-lives >120 min, whereas α -chloroacrylamide **7f** is notably less stable, with a half-life of 92 min. In human whole blood we

observed no degradation of inhibitors **7c** and **7j** over 120 minutes; however, **7f** showed a lower half-life of about 32 min, similar to that observed in hepatocytes. Overall, these relative stabilities are consistent with our in-solution inhibitor-GSH intrinsic reactivity studies, underlining the instability of the α -chloroacrylamide warhead compared to the chloroacetamide, and the superiority of the acrylamide warhead from an *in vivo* viability perspective. Although the degradation of inhibitors in these media cannot be directly linked only to GST activity, as there are many other proteins and compounds that may contribute, it is important to consider that while a given scaffold may help to lower affinity for GST, the warhead can also be optimized for low reactivity within the active site of GST, contributing to the overall *in vivo* stability of an inhibitor.

Conclusions

Rigorous kinetic evaluation of small molecule hTG2 inhibitors has revealed a strong influence on inhibitory parameters with even subtle variation in warhead structure, highlighting that it is equally important to optimize the warhead as it is the inhibitor scaffold. While the latter is clearly important for initial non-covalent binding and orientation of the warhead, the warhead itself may also contribute to binding affinity and the efficiency of the subsequent reaction. We have demonstrated the opportunity for tuning (isozyme) selectivity with varying warhead structure and identified two new inhibitors with excellent overall inhibitory efficiency and modest selectivity.

In addition, the pharmacokinetic stability of various functional groups must also be considered early in drug development. Warhead optimization provides an additional avenue to improve *in vivo* stability, particularly if reactivity with GST isoforms is taken into consideration. Here we confirm that the acrylamide warhead is still superior in terms of off-target stability and is most suitable for therapeutic inhibitor design. Other warheads we have incorporated can be applied to study hTG2 in a more fundamental manner, such as in the development of chemical probes, and provide interesting new insights for tuning reactivity, selectivity, and stability of hTG2 inhibitors.

Materials and methods

Synthesis

General remarks. All reagents and solvents were purchased from commercial sources and used without further purification. Anhydrous solvents were obtained using a Phoenix Solvent Dispensing System (JC Meyer Solvent Systems) with neutral alumina packed columns. Thin layer chromatography was performed using EMD aluminum-backed silica 60 F254-coated plates and were visualized using either UV-light (254 nm), KMnO₄, Hanessian's stain, or

Table 4 *In vitro* half-lives of top inhibitors in human hepatocytes (HH) and human whole blood (HWB)

Compound	Warhead	HH $t_{1/2}$ (min)	HWB $t_{1/2}$ (min)
7c		>120	>120
7f		92 ± 16	32 ± 4
7j		>120	>120

ninhydrin stain. Column chromatography was carried out using standard flash technique with silica (Siliaflash-P60, 230-400 mesh Silicycle) under compressed air pressure. ^1H NMR spectra were obtained at 300 MHz or 400 MHz, ^{13}C NMR spectra were obtained at 75 or 100 MHz, and ^{19}F NMR spectra were obtained at 283 MHz on Bruker instruments. NMR chemical shifts (δ) are reported in ppm and are calibrated against residual solvent signals of CDCl_3 , $\text{DMSO}-d_6$, or CD_3OD . Spectra were processed using MNOVA 14.1.2 (Mestrelab Research S.L., Santiago de Compostela, Spain). Fourier transform was conducted using a linear phase shift group delay, an exponential-fit 1 Hz apodization, and a zero-filling linear prediction of 65 536 points. An automatic phase correction was applied and manually refined to provide a flatter baseline. A baseline correction, employing a Whittaker smoothing function with a 2.60 Hz filter and a smoothing factor of 16 384 was employed on the spectrum between -2.00 and 15.00 ppm. Integration was measured using the manual integration tool and referenced to a relevant aliphatic proton. Multiplet analysis was conducted using the algorithm as implemented in the software, confirmed by manual analysis. High resolution mass spectrometry (HRMS) was performed using a quadrupole time-of-flight (QTOF) analyzer and electrospray ionization (ESI).

Crystallographic measurements on **13** were performed using a Bruker AXS SMART single-crystal diffractometer equipped with an APEX II CCD detector, using graphite-monochromated $\text{Mo K}\alpha$ radiation ($\lambda = 0.71073 \text{ \AA}$) from a sealed tube source. The crystal chosen was mounted on the tip of a $200 \text{ }\mu\text{m}$ MicroLoop using Parabar 10 312 oil, and the data collection was performed at room temperature. Raw data collection and processing were performed with the Bruker APEX II software package. A multi-scan absorption correction was applied (SADABS). The crystal structure was solved *via* direct methods and refined using a full-matrix least-squares method on F^2 using the SHELXTL program suite and the Olex2 user interface. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms bonded to carbon were included at geometrically idealized positions [riding model] and were not refined. The isotropic thermal parameters of these hydrogen atoms were fixed at $1.2 U_{\text{eq}}$ of the parent carbon atom. The hydrogen atom bonded to oxygen in the carboxylic acid moiety was located in the Fourier difference map, allowing isotopic refinement with the O–H distance restrained to $0.86 (0.01) \text{ \AA}$. A relatively large crystal (with dimensions $0.268 \times 0.447 \times 0.461 \text{ mm}^3$) was purposely chosen to compensate for the somewhat weak diffraction, caused by the lack of atoms heavier than oxygen in the compound. This compound crystallized in the monoclinic space group $P2_1/c$. The extended structure of this crystal comprises hydrogen-bonded dimers of the compound *via* the carboxylic acid moiety. Short contacts ($2.5351(22) \text{ \AA}$) are also present between H4 (alkene proton) and N1 (nitrile N).

N-(*tert*-Butoxycarbonyl)glycine (**11**)

To a stirring solution of NaOH (1.60 g, 39.96 mmol, 1.5 eq) in 1:1 H_2O /dioxane (50 mL) was added glycine (2.00 g, 26.64 mmol, 1.0 eq). The solution was cooled to 0°C and stirred for 15 min. Di-*tert*-butyl dicarbonate (8.72 g, 39.96 mmol, 1.5 eq) was dissolved in dioxane (10 mL) and added dropwise to the solution. The reaction mixture was allowed to warm to R.T. and stir for 16 h until the reaction was confirmed to be complete by TLC analysis (10% MeOH in DCM, $R_f = 0.46$). The reaction mixture was concentrated *in vacuo* and the residue was redissolved in H_2O (50 mL). The aqueous phase was washed with Et_2O ($3 \times 50 \text{ mL}$) and then acidified with 12 M HCl to pH 2. The aqueous phase was then extracted with EtOAc ($3 \times 50 \text{ mL}$). The organic phase was washed with brine (25 mL), dried with MgSO_4 , and concentrated *in vacuo* to give a clear oil which dried into a crystalline white solid overnight (4.26 g, 91%). ^1H NMR (400 MHz, CDCl_3) δ 3.97–3.89 (m, 2H, rotamers), 1.44 (s, 9H). ^{13}C NMR (100 MHz, CDCl_3) δ 175.0/174.1 (1C, rotamers), 157.4/156.1 (1C, rotamers), 81.9/80.6 (1C, rotamers), 43.5/42.3 (1C, rotamers), 28.4. Characterization data are consistent with previously reported values.²²

Adamantanecarbonyl chloride (**2**)

1-Adamantanecarboxylic acid (1.8 g, 10 mmol) and thionyl chloride (5 mL) were stirred at reflux under an inert atmosphere with a bubbler attached to monitor gas evolution. After 4 h the reaction was complete as confirmed by ^{13}C NMR analysis (shift in carbonyl carbon resonance). The reaction was evaporated to dryness under reduced pressure to give the product as a white crystalline solid (1.77 g, 90%). ^1H NMR (300 MHz, CDCl_3) δ 2.14–2.11 (m, 3H), 2.03–2.02 (m, 6H), 1.82–1.69 (m, 6H). ^{13}C NMR (75 MHz, CDCl_3) δ 180.0, 51.2, 39.1 (3C), 36.2 (3C), 28.0 (3C). Characterization data are consistent with previously reported values.²²

N-(Adamantanecarbonyl)-*N*-(*tert*-butoxycarbonyl)-piperazine (**3**)

To a stirring solution of *N*-Boc-piperazine (0.446 g, 2.396 mmol, 1.0 eq) and Hünig's base (0.835 mL, 4.792 mmol, 2.0 eq) in dry DCM (10 mL) at 0°C was added **2** (0.500 g, 2.516 mmol, 1.05 eq) portion wise. The ice bath was removed, and the reaction was stirred at R.T. for 3 h when it was confirmed to be complete by TLC analysis (1:1 hexanes/ EtOAc , $R_f = 0.67$). The reaction mixture was diluted with DCM and washed with 5% AcOH ($3 \times 20 \text{ mL}$) and brine (25 mL). The organic phase was dried over MgSO_4 and concentrated *in vacuo* to give the product as a pale-yellow solid (0.797 g, 96%). ^1H NMR (400 MHz, CDCl_3) δ 3.66–3.63 (t, $J = 5.6 \text{ Hz}$, 4H), 3.42–3.38 (t, $J = 5.6 \text{ Hz}$, 4H), 2.05–2.00 (m, 3H), 1.98–1.97 (m, 6H), 1.76–1.68 (m, 6H), 1.46 (s, 9H). ^{13}C NMR (100 MHz, CDCl_3) δ 176.1, 154.8, 80.3, 45.2, 41.8, 39.2, 36.7, 28.6, 28.5.

Characterization data are consistent with previously reported values.²²

***N*-(Adamantanecarbonyl)piperazine hydrochloride (4)**

To a stirring solution of **3** (0.797 g, 2.287 mmol, 1.0 eq) in DCM (5 mL) was added 5 mL of HCl (4 M in dioxane). A precipitate formed as the reaction progressed and the disappearance of starting material was monitored by TLC (1:1 hexanes/EtOAc), completely disappearing in 2 h. The reaction mixture was concentrated *in vacuo* and the resulting solid was resuspended in DCM (7 mL) and Et₂O (7 mL), filtered, and washed with Et₂O (3 × 5 mL) to give the product as a white solid (0.575 g, 88%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.33 (br s, 2H), 3.82–3.78 (t, *J* = 5.1 Hz, 4H), 3.05–3.01 (t, *J* = 5.1 Hz, 4H), 2.00–1.93 (m, 3H), 1.88–1.80 (m, 6H), 1.74–1.58 (m, 6H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 174.8, 43.0, 41.7, 41.0, 38.3, 36.0, 27.9. Characterization data are consistent with previously reported values.²²

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl] carbamic acid *tert*-butyl ester (5)**

Compound **1** (0.244 g, 1.389 mmol, 1.1 eq), HBTU (0.765 g, 2.022 mmol, 1.6 eq), and Hünig's base (0.9 mL, 5.052 mmol, 4.0 eq) were stirred in DCM (20 mL) at R.T. After 1 h, **4** (0.360 g, 1.263 mmol, 1.0 eq) was added. The reaction was left to stir overnight (~18 h). After confirmed to be complete by TLC (10% MeOH/DCM, *R*_f = 0.71) the reaction was concentrated *in vacuo* and the residue was redissolved in EtOAc (50 mL). The organic phase was washed with 5% AcOH (3 × 25 mL), brine (25 mL), NaHCO₃ (50 mL), and brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a pale-yellow solid, which was washed with Et₂O to give a white solid (0.330 g, 65%). ¹H NMR (400 MHz, CDCl₃) δ 5.47 (br s, 1H), 3.97–3.96 (d, *J* = 4.0 Hz, 1H), 3.71–3.68 (m, 4H), 3.63–3.59 (m, 2H), 3.39–3.39 (t, *J* = 5.2 Hz, 2H), 2.06–2.04 (m, 3H), 1.98–1.97 (m, 6H), 1.77–1.68 (m, 6H), 1.44 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 176.2, 167.4, 155.9, 79.9, 45.4, 44.8, 44.6, 42.4, 42.3, 41.9, 39.1, 36.7, 28.5, 28.4. Characterization data are consistent with previously reported values.²²

1-[4-(Adamantane-1-carbonyl)piperazin-1-yl]-2-amino-ethanone (6)

Compound **5** (0.203 g, 0.5 mmol, 1.0 eq) was dissolved in DCM (7.0 mL) and treated with TFA (0.31 mL, 4.0 mmol, 8.0 eq) until the starting material was completely consumed by TLC (10% MeOH/DCM) in 3 h. The reaction mixture was diluted with DCM (15 mL) and washed with 0.5 M HCl (3 × 25 mL). The pH of the combined aqueous layer was adjusted to 9–10 using sat. K₂CO₃ and then extracted using DCM (3 × 35 mL). The combined organic layer was dried over MgSO₄ and concentrated *in vacuo* to give a white solid (0.0827 g, 54%) (10% MeOH/DCM, *R*_f = 0.27). ¹H NMR (300 MHz, CDCl₃) δ 3.75–3.59 (m, 6H), 3.49 (apparent s, 2H), 3.39–3.34

(m, 2H), 2.05 (apparent s, 3H), 1.99–1.98 (m, 6H), 1.79–1.67 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.3, 171.6, 45.7, 44.8, 44.5, 43.7, 42.3, 41.9, 39.2, 36.7, 28.5. Characterization data are consistent with previously reported values.²⁸

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]-3-methyl-propenamide (7a)**

Crotonic acid (0.032 g, 0.363, 1.0 eq), Hünig's base (0.15 mL, 0.871 mmol, 2.4 eq), EDCI-HCl (0.084 g, 0.436 mmol, 1.2 eq), and HOBT-H₂O (0.067 g, 0.436 mmol, 1.2 eq) were stirred in anhydrous DCM (4 mL) under an atmosphere of nitrogen at R.T. **6** (0.100 g, 0.327 mmol, 0.9 eq) was then added as a solution in DCM (1 mL). The reaction was stirred for 18 h at R.T. when TLC analysis confirmed the reaction as complete (7.5% MeOH/EtOAc, *R*_f = 0.51). The reaction mixture was diluted with DCM (7 mL) and washed with 5% AcOH (3 × 7 mL), brine (7 mL), sat. NaHCO₃ (2 × 10 mL), and brine again (10 mL). The organic phase was then dried over MgSO₄ and concentrated *in vacuo* to give a white solid which was purified by silica gel column chromatography (7.5% MeOH/EtOAc, *R*_f = 0.51, dry loading using silica) to yield the product as a white chalk-like solid (0.0632 g, 52%). ¹H NMR (300 MHz, CDCl₃) δ 6.93–6.81 (dq, *J* = 15.3, 6.9 Hz, 1H), 6.52 (br s, 1H), 5.92–5.86 (dq, *J* = 15.3, 1.8 Hz, 1H), 4.14–4.12 (d, *J* = 4.2 Hz, 2H), 3.73–3.70 (m, 4H), 3.65–3.62 (m, 2H), 3.44–3.41 (m, 2H), 2.06 (apparent s, 3H), 1.99–1.98 (m, 6H), 1.88–1.85 (dd, *J* = 6.9, 1.8 Hz, 3H), 1.78–1.68 (m, 6H, overlapping with H₂O signal). ¹³C NMR (75 MHz, CDCl₃) δ 176.2, 167.1, 166.0, 140.7, 124.7, 45.3, 44.9, 44.7, 42.4, 41.9, 41.4, 39.2, 36.7, 28.5, 17.9. HRMS (ESI) calc'd for C₂₁H₃₁N₃O₃Na [MNa]⁺: 396.2263, found: 393.2260.

2-Fluoroacrylic acid (I2)

2-Fluoroacrylic acid methyl ester (0.500 g, 4.80 mmol) was dissolved in EtOH/H₂O (8.7:1.3 mL, 10 mL). NaOH (2 M) was then added dropwise until pH 11 was reached, and the resulting mixture was stirred for 30 min, after which the solution was concentrated *in vacuo* to yield the sodium salt as a white solid. The salt was suspended in Et₂O (20 mL) and HCl (6 M) was added dropwise until the solid dissolved. The layers were separated, and the aqueous layer was extracted with Et₂O (2 × 10 mL). The combined organic layer was dried over MgSO₄ and evaporated *in vacuo* to yield the desired acid as a white solid (0.306 g, 71%). ¹H NMR (CDCl₃, 300 MHz) δ 11.01 (br s, 1H), 5.93–5.77 (dd, *J*_{HF} = 42.9, *J*_{HH} = 3.3 Hz, 1H), 5.52–5.47 (dd, *J*_{HF} = 12.6, *J*_{HH} = 3.3 Hz, 1H). ¹³C NMR (CDCl₃, 75 MHz) δ 165.6 (d, *J*_{CF} = 36.9 Hz), 152.6 (d, *J*_{CF} = 259.2 Hz), 105.4 (d, *J*_{CF} = 14.6 Hz). ¹⁹F NMR (CDCl₃, 283 MHz) δ -118.3. Characterization data are consistent with previously reported values.⁶⁰

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]-2-fluoro-propenamide (7b)**

2-Fluoroacrylic acid (0.062 g, 0.684 mmol, 1.1 eq), HBTU (0.378 g, 0.995 mmol, 1.6 eq), and Hünig's base (0.433 mL,

2.49 mmol, 4.0 eq) were stirred in anhydrous DCM (5 mL) at 0 °C for 30 min. **6** (0.190 g, 0.622 mmol, 1.0 eq) was then added dropwise as a solution in DCM (2 mL) at R.T. and the reaction was stirred for 5 h at R.T. at which point it was confirmed complete by TLC analysis (2% MeOH/EtOAc, R_f = 0.60). The reaction was concentrated *in vacuo* and the residue was redissolved in EtOAc (25 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (15 mL), NaHCO₃ (15 mL), and brine again (15 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give an orange solid, which was washed with Et₂O/hexanes (10 mL: 10 mL) and then further purified by silica gel column chromatography (2% MeOH/EtOAc, dry loading using celite) to give the product as a pale-yellow solid (0.055 g, 24%). ¹H NMR (300 MHz, CDCl₃) δ 7.35 (br s, 1H), 5.76–5.60 (dd, J_{HF} = 46.8, J_{HH} = 3.3 Hz, 1H), 5.52–5.47 (dd, J_{HF} = 14.7, J_{HH} = 3.3 Hz, 1H), 4.15–4.14 (d, J = 4.2 Hz, 2H), 3.74–3.71 (m, 4H), 3.66–3.63 (m, 2H), 3.44–3.41 (m, 2H), 2.06 (apparent s, 3H), 1.99 (apparent s, 6H), 1.78–1.68 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.3, 166.1, 159.7 (d, J_{CF} = 31.2 Hz), 156.1 (d, J_{CF} = 268.9 Hz), 99.3 (d, J_{CF} = 14.6 Hz), 45.4, 44.8, 44.7, 42.4, 41.9, 41.2, 39.2, 36.7, 28.5. ¹⁹F NMR (CDCl₃, 283 MHz) δ –120.5. HRMS (ESI) calc'd for C₂₀H₂₈FN₃O₃Na [MNa]⁺: 400.2012, found: 400.2010.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]-2-propenamide (7c)**

Compound **6** (0.085 g, 0.278 mmol, 1.0 eq), and NEt₃ (0.2 mL, 1.390 mmol, 5.0 eq) were stirred in anhydrous DCM (5.0 mL) under an atmosphere of nitrogen at 0 °C. Acryloyl chloride (0.025 mL, 0.306 mmol, 1.1 eq) was then added dropwise. The reaction was allowed to warm to R. T. over 3 h while monitoring product formation by TLC (10% MeOH/DCM, R_f = 0.59). The reaction mixture was concentrated *in vacuo* and the residue was redissolved in EtOAc (100 mL) and washed with sat. NaHCO₃ (3 × 50 mL) and brine (50 mL). The organic phase was then dried over MgSO₄ and concentrated *in vacuo*. The resulting solid was purified by silica gel column chromatography (4% MeOH/EtOAc, R_f = 0.37, dry loading using celite) and then washed with pentane (3 × 5 mL) to give the product as a white solid (0.073 g, 73%). ¹H NMR (300 MHz, CDCl₃) δ 6.75 (br s, 1H), 6.35–6.20 (dd, J = 1.3, 17.0 Hz, 1H), 6.17–6.14 (dd, J = 10.1, 17.0 Hz, 1H), 5.70–5.66 (dd, J = 1.1, 10.1 Hz, 1H), 4.16–4.15 (d, J = 4.1 Hz, 1H), 3.72–3.70 (m, 4H), 3.65–3.62 (m, 2H), 3.45–3.42 (m, 2H), 2.05–2.04 (m, 3H), 1.98–1.97 (m, 6H), 1.77–1.67 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.2, 166.9, 165.5, 130.4, 127.1, 45.3, 44.9, 44.7, 42.4, 41.9, 41.4, 39.2, 36.7, 28.5. HRMS (ESI) calc'd for C₂₀H₂₉N₃O₃Na [MNa]⁺: 382.2107, found: 382.2101.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]-2-bromo-propenamide (7d)**

To a stirring solution of bromoacrylic acid (0.055 g, 0.360 mmol, 1.1 eq) and **6** (0.1 g, 0.327 mmol, 1.0 eq) in anhydrous

DCM (2 mL) under an atmosphere of nitrogen at 0 °C was added a solution of DCC (0.075 g, 0.360 mmol, 1.1 eq) and DMAP (0.001 g, 2 mol%) in anhydrous DCM (2 mL). The ice bath was then removed, and the reaction was stirred for 3 h. The mixture was then filtered through celite and the filtrate was diluted with EtOAc (50 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (25 mL), sat. NaHCO₃ (25 mL), and brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a white solid, which was purified by silica gel column chromatography (100% EtOAc, R_f = 0.55, dry loading using celite) (0.048 g, 34%). ¹H NMR (300 MHz, CDCl₃) δ 7.73 (br s, 1H), 7.00 (d, J = 1.5 Hz, 1H), 6.06 (d, J = 1.5 Hz, 1H), 4.12 (d, J = 4.2 Hz, 2H), 3.73–3.72 (m, 4H), 3.67–3.65 (m, 2H), 3.44–3.41 (m, 2H), 2.06 (m, 3H), 1.99–1.98 (m, 6H), 1.78–1.69 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.3, 166.2, 161.2, 127.8, 122.5, 45.4, 44.8, 44.7, 42.4, 42.4, 41.9, 39.2, 36.7, 28.5. HRMS (ESI) calc'd for C₂₀H₂₈BrN₃O₃Na [MNa]⁺: 460.1212, found: 460.1210.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]-2-trifluoromethyl-propenamide (7e)**

Compound **6** (0.100 g, 0.327 mmol, 1.0 eq) and Hünig's base (0.23 mL, 1.310 mmol, 4.0 eq) were dissolved in anhydrous DCM (3 mL) under an atmosphere of nitrogen. The solution was cooled to 0 °C and BOP (0.160 g, 0.360 mmol, 1.1 eq) and 2-trifluoromethylacrylic acid (0.051 g, 0.360 mmol, 1.1 eq) were added. The reaction was stirred for 1 h (note: running the reaction longer than this led to product degradation) and then poured into EtOAc (50 mL). The organic phase was washed with H₂O (50 mL) and brine (50 mL) and then dried over MgSO₄ and concentrated *in vacuo* to give a yellow oil. The crude material was purified by silica gel column chromatography (5% MeOH/EtOAc, R_f = 0.56, dry loading using celite) to give a white solid (0.025 g, 18%). ¹H NMR (300 MHz, CDCl₃) δ 7.21 (br s, 1H), 6.56 (quint, J_{HH} = 1.5 Hz, J_{HF} = 1.5 Hz, 1H), 5.52–5.47 (quint, J_{HH} = 1.5 Hz, J_{HF} = 1.5 Hz, 1H), 4.17 (d, J = 3.9 Hz, 2H), 3.75–3.71 (m, 4H), 3.67–3.63 (m, 2H), 3.44–3.41 (m, 2H), 2.06 (apparent s, 3H), 1.99–1.98 (m, 6H), 1.78–1.68 (m, 6H). ¹³C NMR (75 MHz, CD₃OD) δ 177.2, 167.9, 163.7, 134.8 (q, J_{CF} = 30.5 Hz), 127.5 (q, J_{CF} = 5.3 Hz), 122.4 (q, J_{CF} = 270.7 Hz), 54.0, 45.3, 45.0, 42.5, 42.1, 41.1, 39.2, 36.7, 28.1. ¹⁹F NMR (CDCl₃, 283 MHz) δ –64.1. HRMS (ESI) calc'd for C₂₁H₈F₃N₃O₃Na [MNa]⁺: 450.1980, found: 450.1972.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]-2-chloro-propenamide (7f)**

α-Chloroacrylic acid (0.073 g, 0.687 mmol, 2.1 eq) was dissolved in anhydrous DCM (2 mL) under an atmosphere of nitrogen. DCC (0.071 g, 0.343 mmol, 1.05 eq) in anhydrous DCM (1 mL) was added slowly and the reaction was stirred at R. T. for 1 h. The solution was then filtered to isolate the symmetric anhydride and the filtrate was added to a solution of **6** (0.1 g, 0.327 mmol, 1.0 eq) and NEt₃ (0.091 mL, 0.654 mmol, 2.0 eq) in DCM (5 mL) under nitrogen and stirred for 6 h. The

reaction was then diluted with EtOAc (50 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (25 mL), sat. NaHCO₃ (25 mL), and brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a white solid, which was purified by silica gel column chromatography (2% MeOH/EtOAc, *R_f* = 0.56, dry loading using celite) (0.080 g, 60%). ¹H NMR (300 MHz, CDCl₃) δ 7.71 (br s, 1H), 6.59 (d, *J* = 1.2 Hz, 1H), 5.82 (d, *J* = 1.2 Hz, 1H), 4.14 (d, *J* = 4.5 Hz, 2H), 3.73–3.64 (m, 6H), 3.45–3.41 (m, 6H), 2.06–2.04 (m, 3H), 1.99–1.98 (m, 6H), 1.78–1.69 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.2, 166.2, 161.1, 132.2, 123.0, 45.4, 44.8, 44.7, 42.5, 42.1, 41.9, 39.2, 36.7, 28.5. HRMS (ESI) calc'd for C₂₀H₂₈ClN₃O₃Na [MNa]⁺: 416.1717, found: 416.1706.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]-3-chloro-propenamide (7g)**

To a stirring solution of *trans*-3-chloroacrylic acid (0.075 g, 0.360 mmol, 1.1 eq) and **6** (0.1 g, 0.327 mmol, 1.0 eq) in anhydrous DCM (2 mL) under an atmosphere of nitrogen at 0 °C was added a solution of DCC (0.075 g, 0.360 mmol, 1.1 eq) and DMAP (0.001 g, 2 mol%) in anhydrous DCM (2 mL). The ice bath was then removed, and the reaction was stirred for 3 h. The mixture was then filtered through celite and the filtrate was diluted with EtOAc (50 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (25 mL), sat. NaHCO₃ (25 mL), and brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a white solid, which was purified by silica gel column chromatography (2% MeOH/EtOAc, *R_f* = 0.39, dry loading using celite) (0.050 g, 39%). ¹H NMR (300 MHz, CDCl₃) δ 7.35 (d, *J* = 12.6 Hz, 1H), 6.69 (br s, 1H), 6.36 (d, *J* = 12.6 Hz, 1H), 4.17 (d, *J* = 4.2 Hz, 2H), 3.78–3.74 (m, 4H), 3.70–3.66 (m, 2H), 3.47–3.44 (m, 2H), 2.10 (m, 3H), 2.03–2.02 (m, 6H), 1.82–1.72 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.1, 166.4, 163.1, 134.6, 126.2, 45.2, 44.7, 44.6, 42.3, 41.8, 41.3, 39.1, 36.5, 28.4. HRMS (ESI) calc'd for C₂₀H₂₈ClN₃O₃Na [MNa]⁺: 416.1685, found: 416.1695.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]-3-methylester-propenamide (7h)**

To a stirring solution of methyl fumarate (0.047 g, 0.360 mmol, 1.1 eq) and **6** (0.1 g, 0.327 mmol, 1.0 eq) in anhydrous DCM (2 mL) under an atmosphere of nitrogen at 0 °C was added a solution of DCC (0.075 g, 0.360 mmol, 1.1 eq) and DMAP (0.001 g, 2 mol%) in anhydrous DCM (2 mL). The ice bath was then removed, and the reaction was stirred for 3 h. The mixture was then filtered through celite, and the filtrate was diluted with EtOAc (50 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (25 mL), sat. NaHCO₃ (25 mL), and brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a white solid, which was purified by silica gel column chromatography (2% MeOH/EtOAc, *R_f* = 0.44, dry loading using celite) (0.067 g, 49%). ¹H NMR (300 MHz, CDCl₃) δ 7.02–6.98 (m, 2H, NH overlapping with olefin proton), 6.85 (d, *J* = 11.4 Hz, 1H), 4.17 (d, *J* = 3.0 Hz, 2H), 3.80 (s, 3H),

3.74–3.72 (m, 4H), 3.67–3.64 (m, 2H), 3.44–3.42 (m, 2H), 2.06–2.04 (m, 3H), 1.99–1.98 (m, 6H), 1.77–1.69 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.3, 166.4, 166.0, 163.6, 135.9, 130.6, 52.4, 45.3, 44.8, 44.7, 42.5, 41.9, 41.7, 39.2, 36.7, 28.5. HRMS (ESI) calc'd for C₂₂H₃₁N₃O₅Na [MNa]⁺: 440.2161, found: 440.2150.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl] propiolamide (7i)**

To a stirring solution of propiolic acid (0.023 mL, 0.360 mmol, 1.1 eq) and **6** (0.1 g, 0.327 mmol, 1.0 eq) in anhydrous DCM (2 mL) under an atmosphere of nitrogen at 0 °C was added a solution of DCC (0.075 g, 0.360 mmol, 1.1 eq) and DMAP (0.001 g, 2 mol%) in anhydrous DCM (2 mL). The ice bath was then removed, and the reaction was stirred for 3 h. The mixture was then filtered through celite and the filtrate was diluted with EtOAc (50 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (25 mL), sat. NaHCO₃ (25 mL), and brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a white solid, which was purified by silica gel column chromatography (2% MeOH/EtOAc, *R_f* = 0.59, dry loading using celite) (0.062 g, 53%). ¹H NMR (300 MHz, CDCl₃) δ 7.03 (br s, 1H), 4.12 (d, *J* = 3.9 Hz, 2H), 3.75–3.63 (m, 6H), 3.40–3.38 (m, 2H), 2.84 (s, 1H), 2.06–2.04 (m, 3H), 1.99–1.98 (m, 6H), 1.78–1.68 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.3, 165.8, 152.0, 76.9, 74.1, 45.3, 44.8, 44.7, 42.5, 41.9, 41.6, 39.2, 36.7, 28.5. HRMS (ESI) calc'd for C₂₀H₂₇N₃O₃Na [MNa]⁺: 380.1950, found: 380.1953.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl] chloroacetamide (7j)**

Under an atmosphere of nitrogen at –78 °C, chloroacetyl chloride (0.024 mL, 0.297 mmol, 1.1 eq.) was slowly added to a solution of **6** (0.082 g, 0.270 mmol, 1.0 eq.) and NEt₃ (0.190 mL, 1.350 mmol, 5.0 eq.) in anhydrous DCM (3 mL). The reaction mixture was stirred at –78 °C for 3 h, diluted with DCM (7 mL) and washed with 0.2 M HCl (3 × 10 mL). The organic layer was dried over MgSO₄, and the solvent was evaporated *in vacuo* to give the product as an off-white solid (0.0777 g, 75%). ¹H NMR (300 MHz, CDCl₃) δ 7.61 (apparent s, 1H), 4.11–4.10 (d, *J* = 4.2 Hz, 2H), 4.08 (s, 2H), 3.75–3.70 (m, 4H), 3.66–3.63 (m, 2H), 3.43–3.40 (m, 2H), 2.06 (apparent s, 3H), 1.99–1.98 (m, 6H), 1.78–1.68 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.3, 166.3, 166.2, 45.4, 44.8, 44.7, 42.5, 42.4, 41.9, 41.6, 39.2, 36.7, 28.5. HRMS (ESI) calc'd for C₁₉H₂₈ClN₃O₃Na [MNa]⁺: 404.1717, found: 404.1745.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl] chlorofluoroacetamide (7k)**

To a stirring solution of sodium chlorofluoroacetate (0.066 g, 0.491 mmol, 1.5 eq) and **6** (0.1 g, 0.327 mmol, 1.0 eq) in anhydrous DCM:DMF (3.0:0.5 mL) under an atmosphere of nitrogen at 0 °C was added T3P (50% by weight in EtOAc)

(0.3 mL, 0.491 mmol, 1.5 eq) and Hünig's base (0.12 mL, 0.654 mmol, 2.0 eq). The reaction was stirred at R.T. for 3.5 h and then diluted with EtOAc (50 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (25 mL), sat. NaHCO₃ (25 mL), and brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a white solid which was then washed with pentane (0.040 g, 31%). ¹H NMR (300 MHz, CDCl₃) δ 7.47 (br s, 1H), 6.33 (d, *J*_{HF} = 50.4 Hz, 1H), 4.13 (d, *J* = 4.2 Hz, 2H), 3.76–3.71 (m, 4H), 3.67–3.64 (m, 2H), 3.43–3.40 (m, 2H), 2.06–2.04 (m, 3H), 1.99–1.98 (m, 6H), 1.78–1.68 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.3, 165.7, 164.1 (d, *J*_{CF} = 24.4 Hz), 93.9 (d, *J*_{CF} = 255.2 Hz), 45.4, 44.7, 44.7, 42.5, 41.9, 41.4, 39.2, 36.7, 28.5. ¹⁹F NMR (CDCl₃, 283 MHz) δ -144.3. HRMS (ESI) calc'd for C₁₉H₂₇ClFN₃O₃Na [MNa]⁺: 422.1623, found: 422.1623.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]cyanoacetamide (7l)**

Cyanoacetic acid (0.031 g, 0.360 mmol, 1.1 eq), EDCI-HCl (0.075 g, 0.392 mmol, 1.2 eq), HOBT-H₂O (0.060 g, 0.392 mmol, 1.2 eq), and Hünig's base (0.14 mL, 0.785 mmol, 2.4 eq) were stirred in dry DCM (1 mL) at R.T. for 30 min. Compound **6** (0.100 g, 0.327 mmol, 1.0 eq) was then added as a solution in DCM (1 mL). The reaction was left to stir for 24 h, after which point it was diluted with DCM (15 mL) and washed with H₂O (2 × 15 mL), followed by brine (15 mL). The organic layer was dried over MgSO₄ and concentrated *in vacuo* to give a yellow solid which was purified by silica gel column chromatography (7.5% MeOH/EtOAc, *R*_f = 0.45, dry loading using celite) to give a white solid (0.022 g, 18%). ¹H NMR (300 MHz, CDCl₃) δ 7.35 (br s, 1H), 4.11 (d, *J* = 4.1 Hz, 1H), 3.73–3.70 (m, 4H), 3.64–3.60 (m, 2H), 3.46 (s, 2H), 3.42–3.39 (m, 2H), 2.05 (apparent s, 3H), 1.97–1.96 (m, 6H), 1.77–1.67 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.2, 166.2, 161.4, 114.4, 45.2, 44.7, 44.7, 42.4, 41.9, 41.9, 39.2, 36.6, 28.5, 25.9. HRMS (ESI) calc'd for C₂₀H₂₃N₄O₃Na [MNa]⁺: 395.2059, found: 395.2088.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]pentafluorobenzenesulfonamide (7m)**

To a stirring solution of **6** (0.1 g, 0.327 mmol, 1.0 eq) in anhydrous DCM (2 mL) under an atmosphere of nitrogen at 0 °C. Was added Hünig's base (0.12 mL, 0.654 mmol, 2.0 eq) and pentafluorobenzenesulfonyl chloride (0.055 mL, 0.360 mmol, 1.1 eq). The reaction was warmed to R.T. and stirred for 2 h and then diluted with EtOAc (30 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (25 mL), sat. NaHCO₃ (25 mL), and brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a white solid which was purified by silica gel column chromatography (30% EtOAc/hexanes, *R*_f = 0.43, dry loading using celite) (0.157 g, 90%). ¹H NMR (300 MHz, CDCl₃) δ 4.95 (s, 2H), 3.79–3.73 (m, 4H), 3.59–3.58 (m, 2H), 3.47–3.44 (m, 2H), 2.06–2.04 (m, 3H), 2.00–1.99 (m, 6H), 1.79–1.69 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.3, 163.9, 147.4–147.2 (m),

143.9–143.7 (m), 139.2–139.5 (m), 136.5–136.1 (m), 115.8, 52.1, 45.4, 45.4, 44.6, 42.9, 41.9, 39.2, 36.6, 28.5. ¹⁹F NMR (CDCl₃, 283 MHz) δ -132.8 (br s), -141.8–142.1 (m), -158.2–158.4 (m). HRMS (ESI) calc'd for C₁₉H₂₅F₅N₃O₆S [M-H]⁻: 534.1486, found: 534.1457.

2-Cyano-3-cyclopropylacrylic acid (I3)

To a suspension of cyanoacetic acid (0.85 g, 10 mmol, 1.0 eq) in toluene (10 mL) in a sealed tube was added cyclopropanecarboxaldehyde (0.75 mL, 10 mmol, 1.0 eq) and NH₄OAc (0.039 g, 0.5 mmol, 0.05 eq). The reaction was stirred at 110 °C for 2 h and then cooled down to R.T. The resulting precipitate was filtered, washed with hexanes, and dried *in vacuo* to yield a yellow crystalline solid (1.04 g, 73%) which was used without further purification. ¹H NMR (400 MHz, CDCl₃) δ 7.11 (d, *J* = 11.6 Hz, 1H), 1.95–1.86 (m, 1H), 1.27–1.22 (m, 2H), 1.04–1.00 (m, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 169.0, 163.2, 116.0, 106.5, 25.3, 16.4, 11.5. Characterization data are consistent with previously reported values.⁴⁵ *E* configuration confirmed by X-ray crystallography (see ESI†).

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]-2-cyano-3-cyclopropylpropenamide (7n)**

To a stirring solution of **I3** (0.054 g, 0.392 mmol, 1.2 eq) and **6** (0.1 g, 0.327 mmol, 1.0 eq) in anhydrous DCM (2 mL) under an atmosphere of nitrogen at 0 °C was added a solution of DCC (0.075 g, 0.360 mmol, 1.1 eq) and DMAP (0.001 g, 2 mol%) in anhydrous DCM (2 mL). The ice bath was then removed, and the reaction was stirred for 3 h. The mixture was then filtered through celite and the filtrate was diluted with EtOAc (50 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (25 mL), sat. NaHCO₃ (25 mL), and brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a white solid, which was purified by preparative thin layer chromatography (0–2% MeOH/DCM, *R*_f = 0.43, dry loading using celite) (0.083 g, 57%). ¹H NMR (300 MHz, CDCl₃) δ 7.17 (br s, 1H), 7.00 (d, *J* = 11.4 Hz, 1H), 4.14 (d, *J* = 4.2 Hz, 2H), 3.75–3.63 (m, 6H), 3.43–3.40 (m, 2H), 2.07–2.02 (m, 4H, CH of cyclopropyl overlapping with adamantane protons), 1.98–1.97 (m, 6H), 1.77–1.73 (m, 6H), 1.32–1.24 (m, 2H), 0.96–0.91 (m, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 176.2, 166.1 (2C), 160.1, 115.5, 106.6, 45.5, 44.7, 44.7, 42.5, 41.9, 41.8, 39.2, 36.7, 28.5 (4C), 16.0, 11.3. HRMS (ESI) calc'd for C₂₄H₃₂N₄O₃Na [MNa]⁺: 447.2362, found: 447.2353.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl]-2-oxo-propenamide (7o)**

Pyruvic acid (0.027 mL, 0.392 mmol, 1.2 eq) was added to a flame-dried 2-neck round bottom flask, flushed with nitrogen, and dissolved in anhydrous DCM (1 mL). Anhydrous DMF (catalytic, 2 drops) was added, and the mixture was cooled to 0 °C. A bubbler was attached to the

flask and oxalyl chloride (2 M in DCM) (0.2 mL, 0.392 mmol, 1.2 eq) was added dropwise, and the reaction was stirred at R.T. for 3 h. A solution of **6** (0.1 g, 0.327 mmol, 1.0 eq) and NEt₃ (0.070 mL, 0.491 mmol, 1.5 eq) in DCM (1 mL) was then added to the reaction mixture at 0 °C. White fumes immediately formed along with a precipitate. An additional 2 mL of DCM was then added to the reaction mixture to enable uniform stirring. The reaction was left to stir at R.T. for 15 h, after which it was quenched with 1 M HCl (5 mL). The layers were separated, and the organic layer was washed with brine (5 mL), sat. NaHCO₃ (5 mL), and brine (5 mL) again. The organic layer was dried over MgSO₄ and concentrated *in vacuo*. The crude material was purified by silica gel column chromatography using 0–5% MeOH/EtOAc gradient elution (100% EtOAc, *R*_f = 0.36, dry loading using celite) to give a white solid (0.059 g, 48%). ¹H NMR (300 MHz, CDCl₃) δ 7.82 (br s, 1H), 4.07 (s, 2H), 3.73–3.63 (m, 6H), 3.42 (apparent s, 2H), 2.45 (s, 3H), 2.03–2.02 (m, 3H), 1.97–1.96 (m, 6H), 1.76–1.66 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 195.8, 176.3, 165.9, 160.3, 45.4, 44.7, 44.7, 42.4, 41.9, 41.0, 39.1, 36.6, 28.4, 24.6. HRMS (ESI) calc'd for C₂₀H₂₉N₃O₄Na [MNa]⁺: 398.2056, found: 398.2071.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl] isothiocyanate (7p)**

To a solution of **6** (0.1 g, 0.327 mmol, 1.0 eq) in DCM (2 mL) was added sat. NaHCO₃ (2 mL). The biphasic mixture was stirred vigorously and cooled to 0 °C before adding thiophosgene (0.028 mL, 0.360 mmol, 1.1 eq) dropwise. The reaction was stirred at 0 °C for 10 min then at R.T. for 1 h, at which point the reaction was complete by TLC analysis (10% MeOH/DCM, *R*_f = 0.77). The layers were separated, and the aqueous phase was extracted with DCM (3 × 5 mL). The combined organic layer was dried over MgSO₄ and concentrated *in vacuo* to yield yellow oil which was triturated with hexanes to give a pale-yellow solid (0.09 g, 79%). ¹H NMR (300 MHz, CDCl₃) δ 4.31 (s, 2H), 3.75–3.69 (m, 4H), 3.65–3.61 (m, 2H), 3.35–3.32 (m, 2H), 2.05–2.02 (m, 3H), 1.97–1.96 (m, 6H), 1.76–1.67 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.1, 163.6, 139.6, 47.6, 45.4, 45.2, 44.5, 42.8, 41.8, 39.1, 36.5, 28.4. HRMS (ESI) calc'd for C₁₈H₂₅N₃O₂SNa [MNa]⁺: 370.1565, found: 370.1558.

Azidoacetic acid (14)

To a solution of bromoacetic acid (2 g, 14.40 mmol, 1.0 eq) in H₂O (5 mL) was added a solution of NaN₃ (1.84 g, 28.80 mmol, 2.0 eq) in H₂O (5 mL). The reaction was stirred for 24 h at room temperature, followed by dropwise addition of 12 M HCl until pH = 1 was reached. The aqueous mixture was extracted using Et₂O (3 × 20 mL) and the combined organic later was dried over MgSO₄ and concentrated *in vacuo* to obtain azidoacetic acid as a pale-yellow oil which was used without further purification (1.42 g, 98%). ¹H NMR (300 MHz, CDCl₃) δ 8.29 (br s, 1H), 3.95 (s, 1H). ¹³C NMR (75 MHz, CDCl₃) δ

173.5, 50.1. Characterization data are consistent with previously reported values.⁶¹

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl] azidoacetamide (15)**

Azidoacetic acid (**14**) (0.1 g, 0.98 mmol, 1.5 eq), HBTU (0.397 g, 1.05 mmol, 1.6 eq), Hünig's base (0.35 mL, 1.97 mmol, 3.0 eq), and **6** (0.2 g, 0.655 mmol, 1.0 eq) were stirred in anhydrous DCM (5 mL) at R.T. for 3 h at which point the reaction was confirmed complete by TLC analysis. The reaction was concentrated *in vacuo* and the residue was redissolved in EtOAc (50 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (15 mL), sat. NaHCO₃ (15 mL), and brine again (15 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a brown solid, which was purified by silica gel column chromatography (5% MeOH/EtOAc, *R*_f = 0.43, dry loading using celite) and then washing with water to give a white solid (0.12 g, 47%). ¹H NMR (300 MHz, CDCl₃) δ 7.29 (br s, 1H), 4.10 (d, *J* = 3.9 Hz, 2H), 4.02 (s, 2H), 3.73–3.70 (m, 4H), 3.65–3.63 (m, 2H), 3.43–3.39 (m, 2H), 2.06–2.04 (m, 3H), 1.99–1.98 (m, 6H), 1.78–1.68 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.3, 166.9, 166.3, 52.7, 45.4, 44.8, 44.7, 42.4, 41.9, 41.2, 39.2, 36.7, 28.5. HRMS (ESI) calc'd for C₁₉H₂₈N₆O₃Na [MNa]⁺: 411.2107, found: 411.2121.

***N*-2-[4-[[1-Adamantanecarbonyl]-1-piperazinyl]-2-oxoethyl] diazoacetamide (7q)**

15 (0.05 g, 0.129 mmol, 1.0 eq) was dissolved in a solution of THF/H₂O (2 mL/0.3 mL) at R.T. and sparged with nitrogen for 5 minutes. *N*-Succinimidyl 3-(diphenylphosphino)propionate (0.048 g, 0.135, 1.05 eq) was then added and the reaction was stirred for 18 h. A solution of sat. NaHCO₃ (2 mL) was added, and the reaction was stirred for another 18 h, after which the reaction mixture was diluted with brine (15 mL) and extracted with EtOAc (3 × 15 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a yellow solid, which was purified by silica gel column chromatography (5% MeOH/EtOAc, *R*_f = 0.27, dry loading using celite) to give a yellow solid (0.025 g, 52%) ¹H NMR (300 MHz, CDCl₃) δ 6.31 (br s, 1H), 4.87 (s, 1H), 4.13 (d, *J* = 4.2 Hz, 2H), 3.73–3.69 (m, 4H), 3.63–3.60 (m, 2H), 3.47–3.40 (m, 2H), 2.05–2.04 (m, 3H), 1.98–1.97 (m, 6H), 1.77–1.67 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.2, 167.2, 165.6, 47.6, 45.2, 44.9, 44.7, 42.4, 41.9, 41.7, 39.2, 36.7, 28.5. HRMS (ESI) calc'd for C₁₉H₂₇N₅O₃Na [MNa]⁺: 396.2012, found: 396.1992.

TG2 inhibition assay

Recombinant TG2 was expressed from *E. coli* and purified as described previously.¹⁹ TG2 activity was determined according to a previously published colorimetric activity assay using the chromogenic substrate Cbz-Glu(γ-*p*-nitrophenylester)Gly (AL5).^{19,46} In order to determine irreversible inhibition parameters for each inhibitor, enzymatic assays were run under Kitz and Wilson

conditions,⁵⁰ in the presence of 100 μM AL5 substrate, in triplicate. A large excess of substrate is used to ensure that any curvature in the slope is not attributed to depletion of the substrate, but rather to time-dependent inhibition. Buffered solutions of 50 mM of 3-(4-morpholino)propanesulfonic acid (MOPS) (pH 6.9), 7.5 mM CaCl_2 , 100 μM AL5, and various concentrations of inhibitor (from 0.25 to 1200 μM , depending on the inhibitor) were prepared in a 96-well polystyrene microplate with a final volume of 200 μL at 25 $^\circ\text{C}$. AL5 and inhibitor stocks were prepared in DMSO ensuring that the final concentration of this co-solvent did not exceed 5% v/v, with the exception of inhibitor **7d**, for which a stock solution was prepared in DMF (due to poor solubility in DMSO) and the final volume of DMF was less than 1% v/v. If necessary, working stocks of each inhibitor were diluted with water to maintain less than 5% v/v DMSO. To initiate the enzymatic reaction, 5 mU mL^{-1} (0.25 μM) TG2, or water for the blank, was added to the well and the formation of the hydrolysis product, *p*-nitrophenolate, was followed at 405 nm for 20 min (a period of time over which the positive control is linear and not being impacted by substrate depletion) using a BioTek Synergy 4 plate reader. Background AL5 hydrolysis was corrected for by subtracting the blank from each reaction. Observed first order rate constants of inactivation (k_{obs}) were obtained by fitting the inhibition data sets with non-linear regression to mono-exponential association eqn (1) using GraphPad Prism software.

$$\text{Abs}_t = \text{Abs}_{\text{max}}(1 - e^{-k_{\text{obs}}t}) \quad (1)$$

The rate constants measured at different inhibitor concentrations were then fitted by non-linear regression to a saturation kinetics model, using eqn (2).⁵¹

$$k_{\text{obs}} = \frac{k_{\text{inact}}[I]}{[I] + K_I \left(1 + \frac{[S]}{K_M}\right)} \quad (2)$$

In order to correct for the competition with the assay substrate, AL5, the inhibitor concentrations were divided by α , which equals $1 + [S]/K_M$, where $K_M = 10 \mu\text{M}$. Inhibition parameters, k_{inact} and K_I , were then extrapolated from the fitting.

Simple linear regression was performed on the k_{obs} values measured at the lowest concentrations of each inhibitor for validation of k_{inact}/K_I ratios.

For inhibitor **7o**, the assay was performed in a similar manner as described above at AL5 concentrations of 100 μM and 50 μM . Initial rates of the uninhibited reaction (positive control) and the inhibited reactions (v_0) were determined over the first 10% of conversion of the positive control. The reciprocals of the average initial rate ($1/v_0$) were plotted against inhibitor concentration at each AL5 concentration to construct a Dixon plot according to eqn (3) from which the K_i was determined by the point of intersection of the two resulting lines.⁶²

$$\frac{1}{v_0} = \frac{K_M}{V_{\text{max}}[S]K_i}[I] + \frac{[S] + K_M}{V_{\text{max}}[S]} \quad (3)$$

Isozyme selectivity

First, for each inhibitor, a single corrected inhibitor concentration ($[I]/\alpha$) was chosen, which would result in the complete inactivation of TG2 in under 5 min. Then for each other isozyme, the concentration of inhibitor was adjusted according to the value of α determined by the concentration and K_M value of the substrate of each isozyme assay. In this way the effective inhibitor concentration ($[I]/\alpha$) remained constant for each isozyme.

The activities of TG1 and TG6 were measured by a colorimetric assay using the substrate AL5. Assays were performed as previously described for TG2 under Kitz & Wilson conditions established for each transglutaminase isoform by varying the concentration of substrate to 112 μM and 436 μM of AL5 for TG1 and TG6, respectively. The reaction was initiated with the addition of enzyme, 0.10 μM TG1 or 0.32 μM TG6. Formation of the hydrolysis product, *p*-nitrophenolate, was followed at 405 nm for 100 min for TG1 or 60 min for TG6.¹⁹

The isopeptidase activities of activated TG3a and hFXIIIa (purchased from Zedira) were measured by a fluorescence-based assay based on the use of the peptidic FRET-quenched probe A101 (Zedira). The final assay mixture comprised 50 mM Tris (pH 7.0), 10 mM CaCl_2 , 100 mM NaCl, 2.8 mM TCEP, 50 μM A101 and 14 mM H-Gly-OMe. The reaction was monitored at 25 $^\circ\text{C}$ using a BioTek Synergy 4 plate reader (Ex/Em: 318/413 nm). Enzymatic inhibition assays were run under Kitz and Wilson conditions, which were established for TG3a and FXIIIa at a substrate (A101) concentration of 50 μM using enzyme concentrations of 0.17 μM and 0.11 μM for TG3a and FXIIIa, respectively.^{19,54,55} Experiments were completed at least in triplicate.

In cases where irreversible inhibition (time-dependent curvature) was clearly observed, a k_{obs} value was obtained as described above. If the inhibition resembled a reversible mode (instantaneous decrease in rate, linear product increase), a percent inhibition ($\% \text{ inhibition} = v_{\text{uninhibited}}/v_{\text{inhibited}} \times 100\%$) was determined.

Intrinsic reactivity

HPLC traces were collected by Gilson-Mandel GXP271 high performance liquid chromatography (HPLC) with UV detection at 214 and 254 nm (Phenomenex Luna, 150 mm $\times$ 4.6 mm, 30 min, 1.5 mL min^{-1} flow rate, 20–95% $\text{CH}_3\text{-CN}$ with 0.1% TFA in H_2O with 0.1% TFA, 20 min method).

Three inhibitors from the series were selected in order to study the intrinsic reaction rates with a 30-fold excess of

GSH. In 2-mL HPLC vials fitted with a pre-slit screw cap, 150 μL of 0.1 M GSH stock solution in the corresponding aqueous buffer was diluted to a final concentration of 15 mM in 830 μL aq buffer. The reactions were initiated by addition of 20 μL of 25 mM warhead stock solution in DMSO to a final concentration of 0.5 mM with 2% v/v DMSO as co-solvent. Once initiated, the HPLC vials were inverted, placed on the autosampler tray and an initial aliquot was taken immediately. The vials were incubated at 22 $^{\circ}\text{C}$ with aliquots taken at pre-determined time points over the course of at least 5–6 half lives. Both the disappearance of inhibitor and appearance of adduct were monitored for inhibitors **7c** and **7j**. Only the disappearance of inhibitor **7f** was monitored due to further reaction of the thiol adduct and formation of side products. Experiments were completed in duplicate for each inhibitor.

The increasing area under the curve (AUC) of the adduct peaks of the HPLC chromatograms were fitted to a mono-exponential association model with the constraint that $Y_0 = 0$ (eqn (4)).

$$\text{AUC} = \text{AUC}_{\text{max}}(1 - e^{-k_{\text{obs}}t}) \quad (4)$$

The decreasing AUC of the inhibitor peaks of the HPLC chromatograms were fitted to a mono-exponential decay model, with the constraint that the lower plateau = 0 (eqn (5)).

$$\text{AUC} = \text{AUC}_0(e^{-k_{\text{obs}}t}) \quad (5)$$

Both of these methods were used to obtain replicate pseudo-first order k_{obs} values. Second order rates constants were then calculated by dividing these pseudo-first order rate constants by the concentration of excess thiol (eqn (6)).

$$k_2^{\text{calc}} = \frac{k_{\text{obs}}}{[\text{thiol}]} \quad (6)$$

The final, corrected second order rate constants were then calculated by dividing by the fraction of thiolate at the reaction pH, using the corresponding pK_a value (8.7) of the thiol (eqn (7)).

$$k_2^{\text{corr}} = \frac{k_2^{\text{calc}}}{\left(\frac{K_a}{[\text{H}^+] + K_a}\right)} \quad (7)$$

To identify side products formed in the reaction between **7f** and GSH, the reaction was initiated as described above and monitored by liquid chromatography-mass spectrometry on a Shimadzu 2020 LCMS with UV detection at 214 nm (Agilent Eclipse Plus C18, 3.5 μm , 4.6 $\times$ 150 mm, 1.0 mL min^{-1} , 5–95% CH_3CN with 0.05% formic acid in H_2O with 0.05% formic acid, 15 min method) at room temperature at 0, 3, 6, and 24 h intervals.

Pharmacokinetic stability

Hepatocyte incubations. Pooled cryopreserved human hepatocytes from 20 donors were purchased from BioIVT

(NY, US). They were thawed according to supplier's recommendation. Following thawing, cell viability was performed with trypan blue exclusion and each cell preparation showed a viability higher than 95%. The hepatocytes were resuspended in INVITROGRO KHB medium (BioIVT) with a cell density of $\sim 1.3 \times 10^6$ cells per mL. A total of 700 μL incubation media with $\sim 450\,000$ cells per well containing 1 μM test compound in a 24-well plate, were incubated in a humidified CO_2 incubator for up to 2 h, in duplicate. Aliquots of 75 μL were collected at 0, 5, 15, 30, 60, 120 min to determine metabolic half-life. The samples were deproteinized with 2.5 volumes of acetonitrile containing 0.1% formic acid and 0.5 μM glyburide (as an internal standard), and the supernatant obtained by centrifugation (2500 rpm, 10 min) were analyzed by LC-MS/MS. Midazolam and 7-ethoxycoumarin were utilized as positive controls for the assay.

Whole blood incubations. Gender pooled human whole blood containing $\text{K}_3\text{-EDTA}$ as an anti-coagulant was purchased from BioIVT. The incubation was carried out with 1 μM or 5 μM test compound by adding 2 μL of test compound (either 100 or 500 μM sub-stock solutions) to 198 μL whole blood. Aliquots of 20 μL of the incubation were removed at 0, 15, 30, 60 and 120 min and quenched with 180 μL acetonitrile containing 0.1% formic acid and 0.5 μM glyburide (as an internal standard). The supernatant of the samples obtained by centrifugation (2500 rpm, 10 min) were analyzed by LC-MS/MS. Propantheline bromide was used as a positive control for the assay.

Analytical method. All samples were analyzed by using a Sciex triple quadrupole LC-MS/MS (positive electrospray ionization) equipped with an Agilent 1100 HPLC. A Kinetic reverse-phase C_{18} column (100 $\times$ 4.6 mm) from Phenomenex was used for method development to monitor for compound selectivity, sensitivity and linearity. The percent of unchanged parent compound was calculated by taking the ratio of peak area (compound) over peak area (internal standard) and compared to the initial concentration ($T = 0$ min). An exponential decay regression was performed, and the half-life calculated using the equation: $y = Ae^{-kt}$, where A = ratio of compound at $T = 0$ min and k = first order rate constant of elimination.

List of abbreviations

DCM	Dichloromethane
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
AcOH	Acetic acid
HBTU	<i>N,N,N',N'</i> -Tetramethyl- <i>O</i> -(1 <i>H</i> -benzotriazol-1-yl)uronium hexafluorophosphate
MeOH	Methanol
TFA	Trifluoroacetic acid
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
HObt	Hydroxybenzotriazole
R.T.	Room temperature

EtOH	Ethanol
DCC	<i>N,N'</i> -Dicyclohexylcarbodiimide
DMAP	4-Dimethylaminopyridine
BOP	(Benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate
NEt ₃	Triethylamine
DMF	Dimethylformamide
T3P	Propanephosphonic acid anhydride

Author contributions

Conceptualization: J. W. K. and L. M.; funding acquisition: J. W. K.; supervision: J. W. K.; investigation and methodology: L. M. (synthesis, characterization, and enzymatic assays), S. K. I. W. (intrinsic reactivity), H. R. I., L. N., and H. K. (pharmacokinetic measurements); writing – original draft: L. M., writing – reviewing and editing: all authors.

Conflicts of interest

There are no conflicts of interest to declare.

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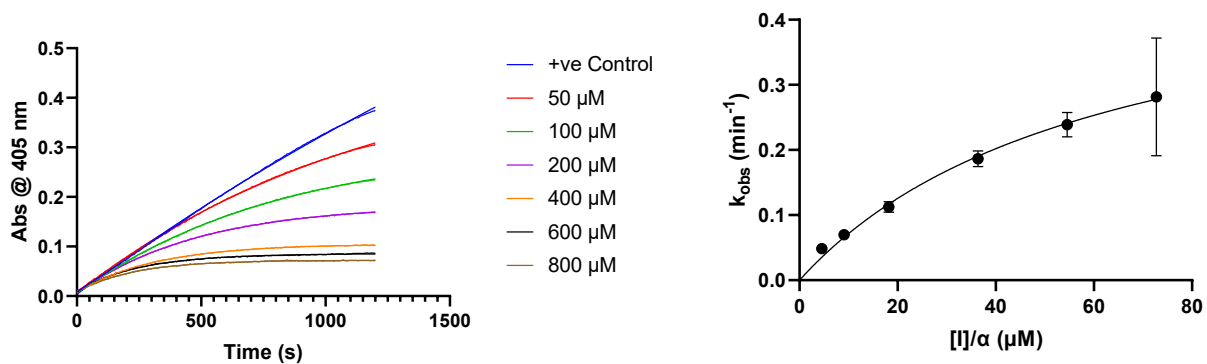
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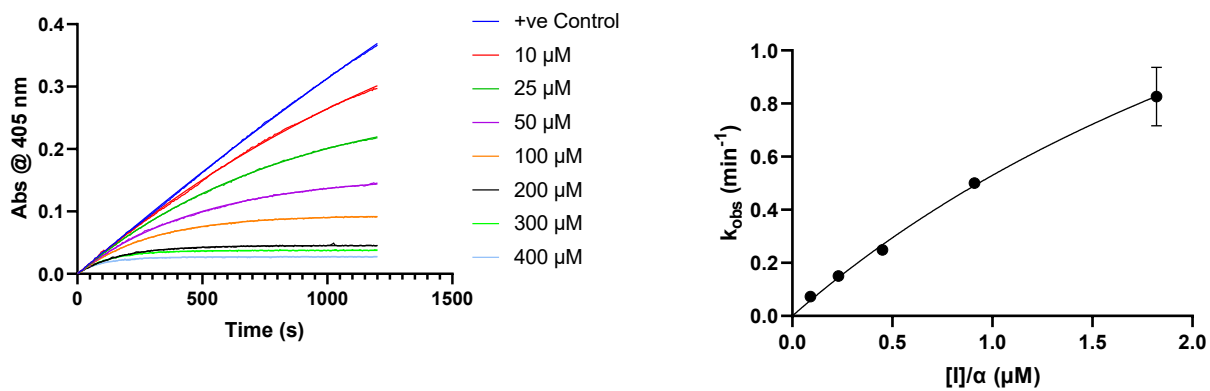
2.6. Chapter 2 Supporting Information

2.6.1. hTG2 Inhibition Kinetics Data

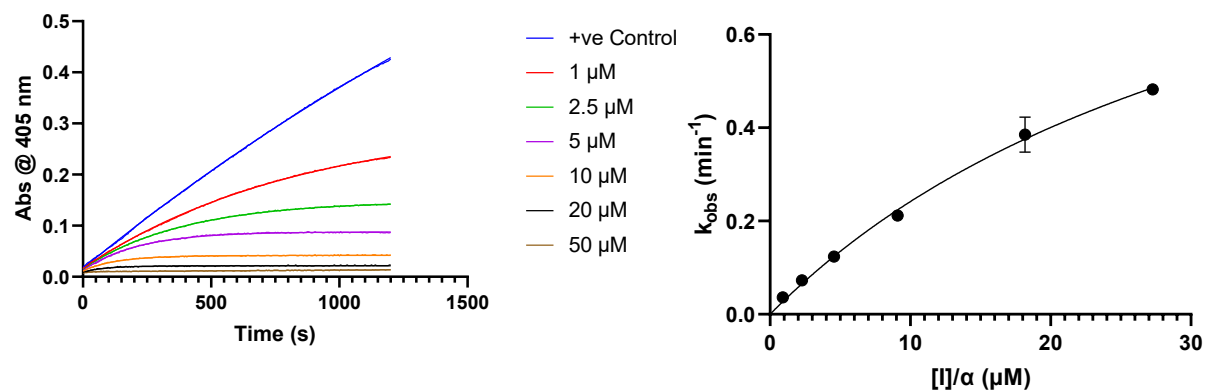
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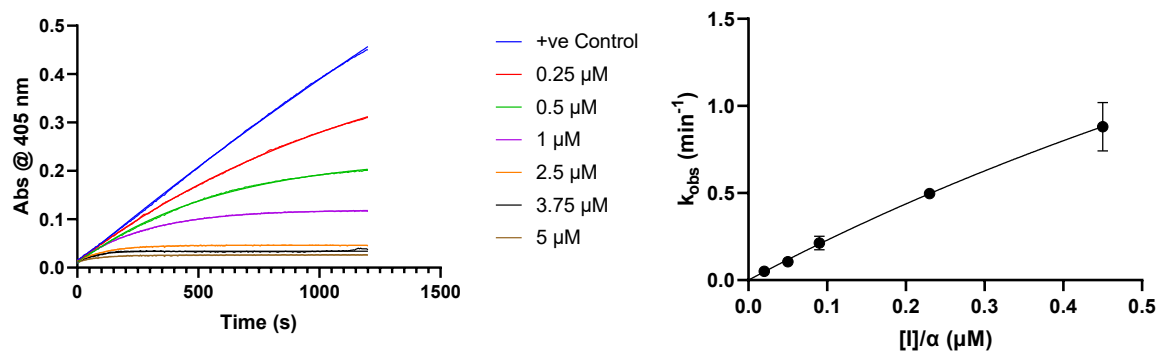
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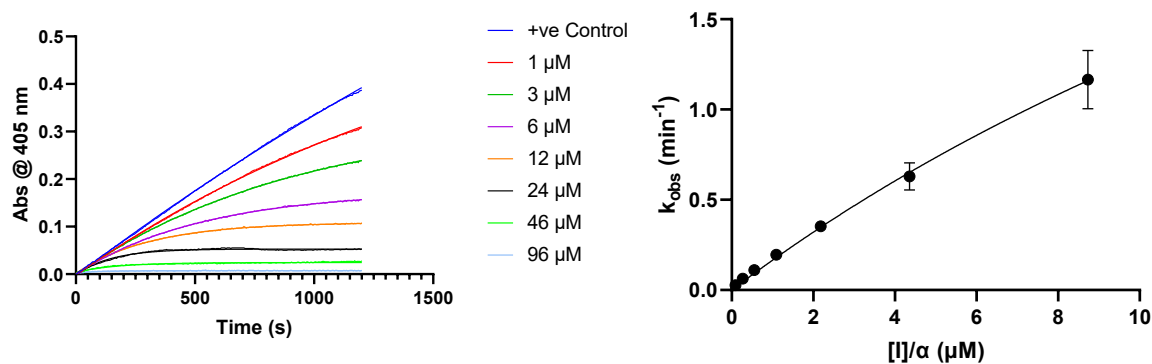
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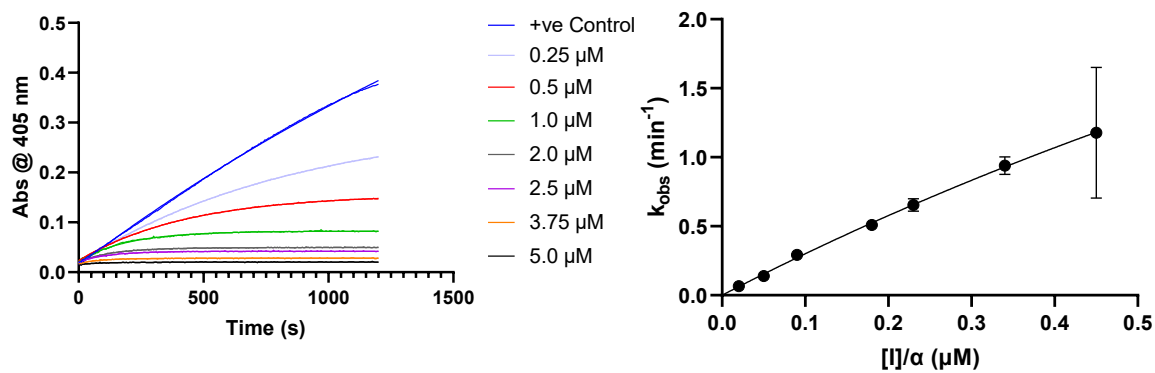
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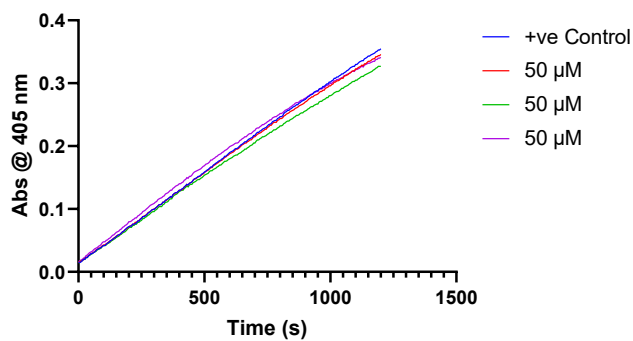
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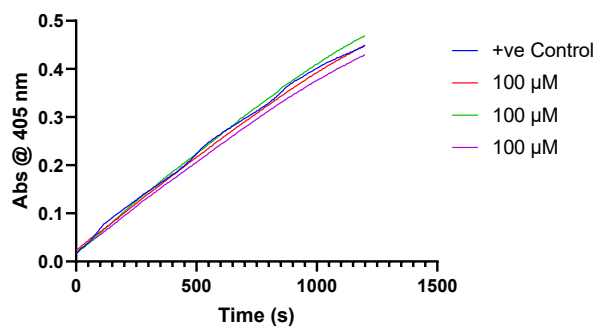
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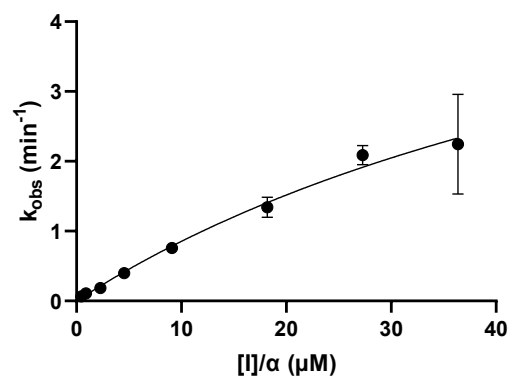
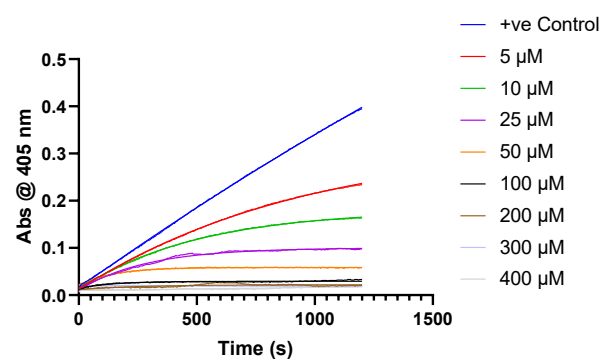
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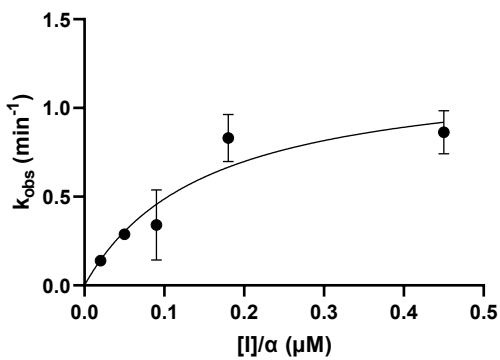
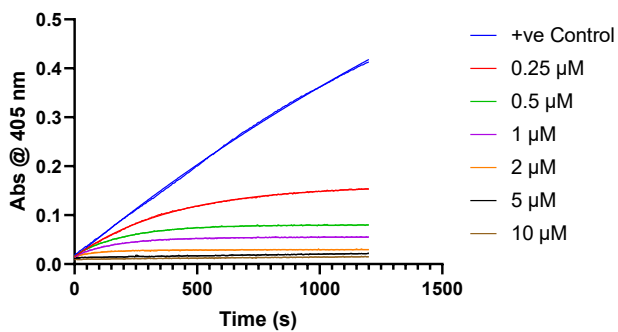
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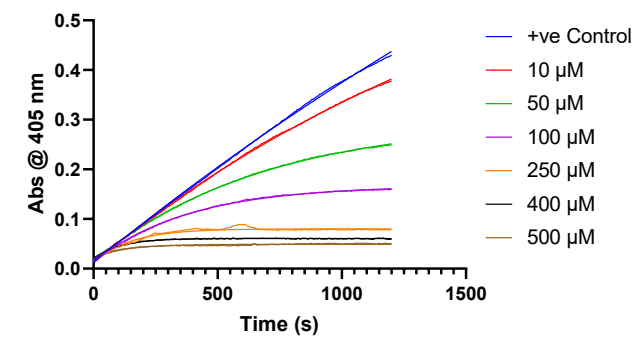
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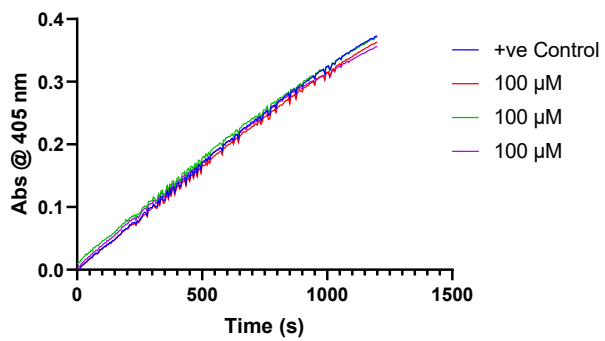
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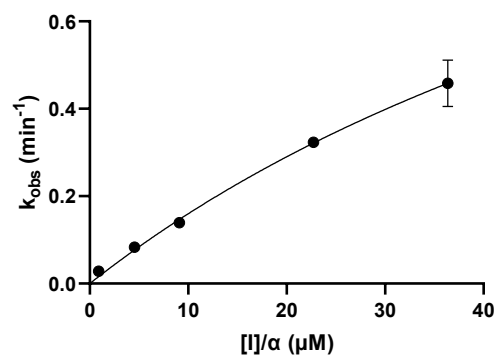
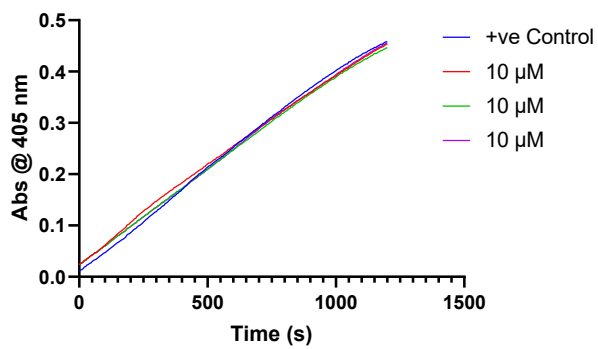
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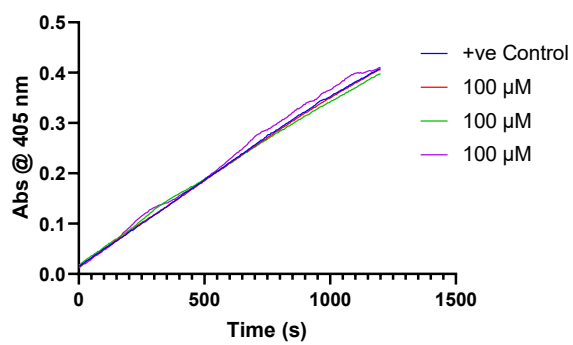
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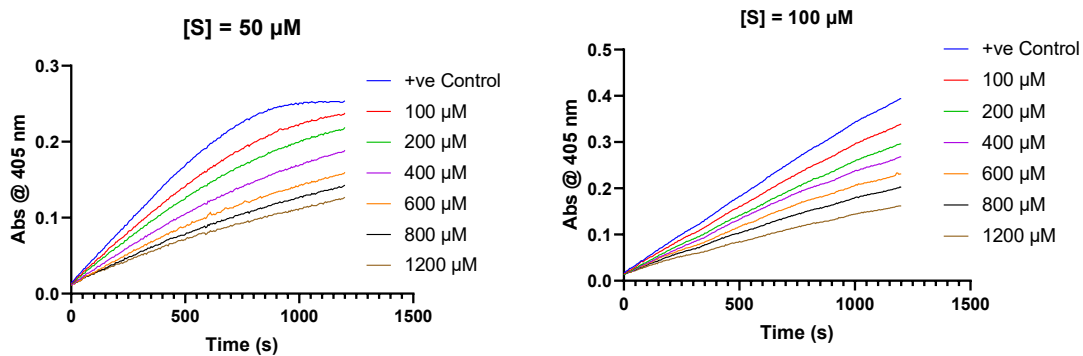
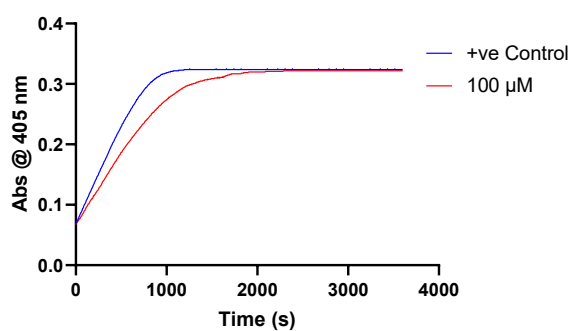
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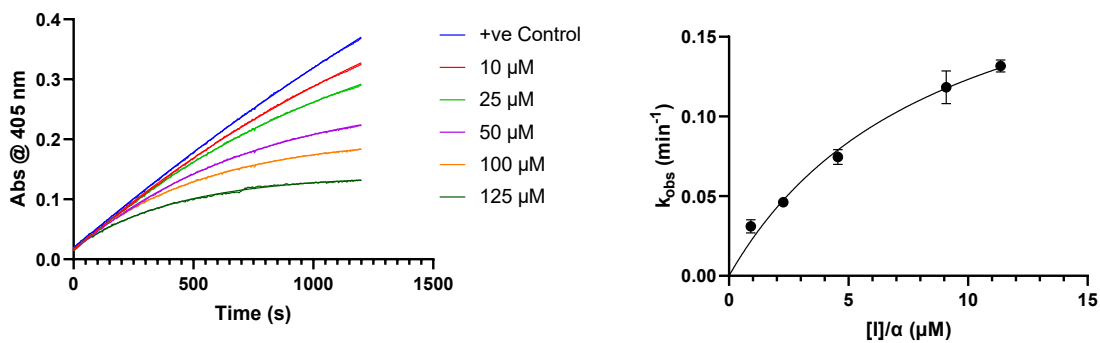
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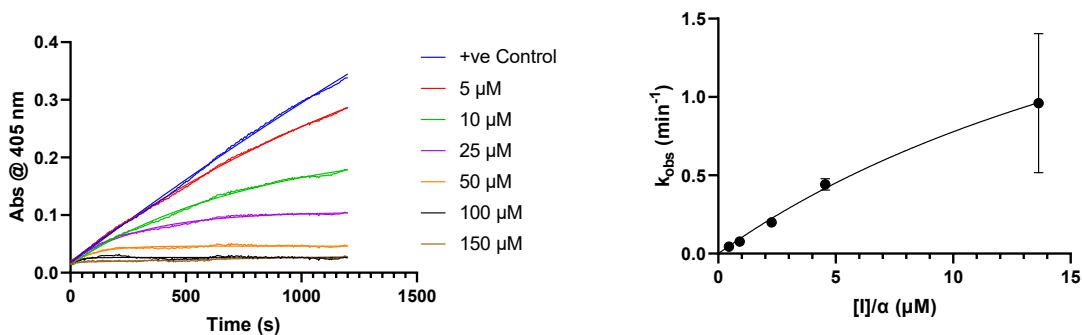
7o

Reversibility Check ($[S] = 50 \mu\text{M}$)

7p

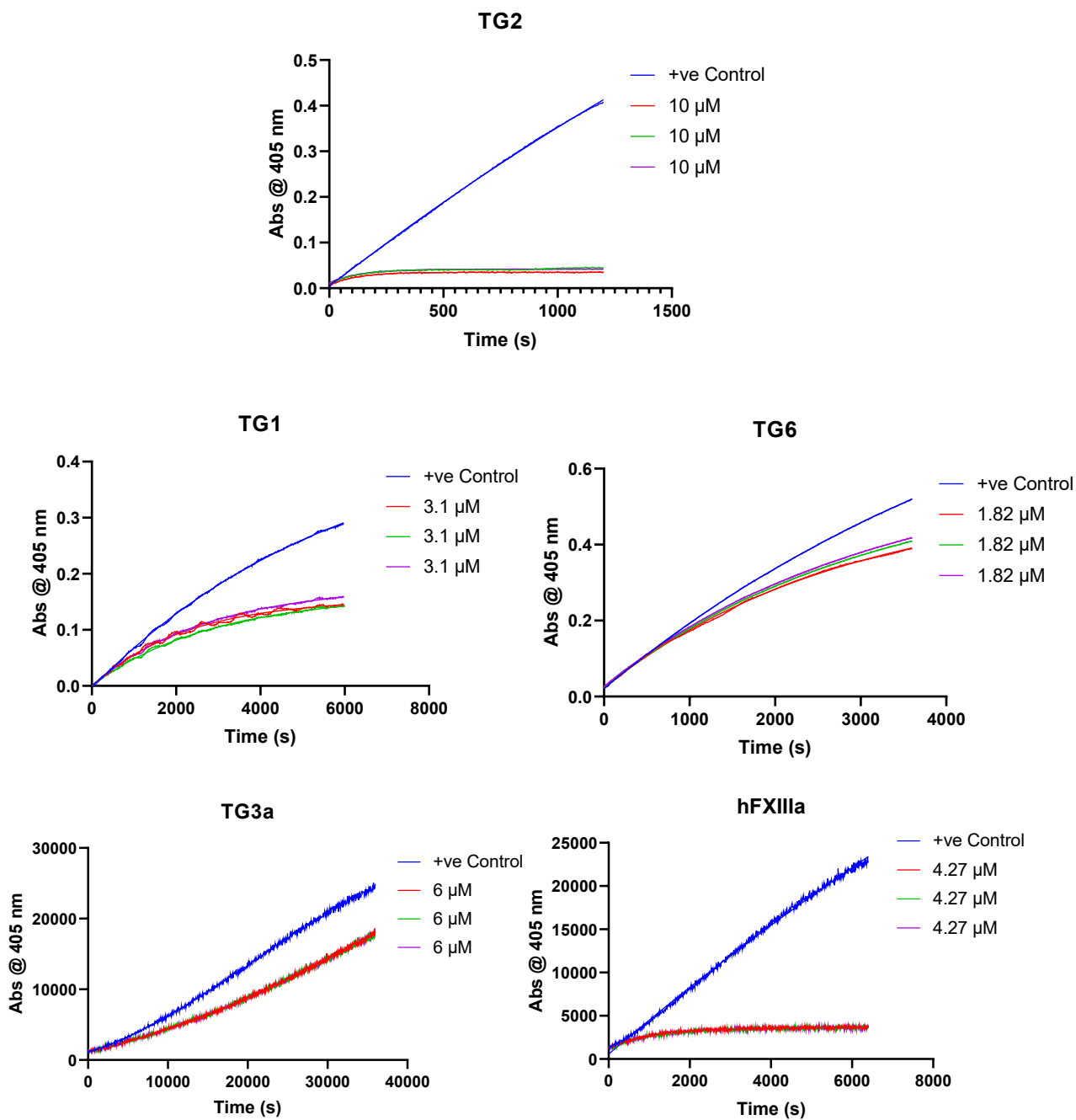


7q

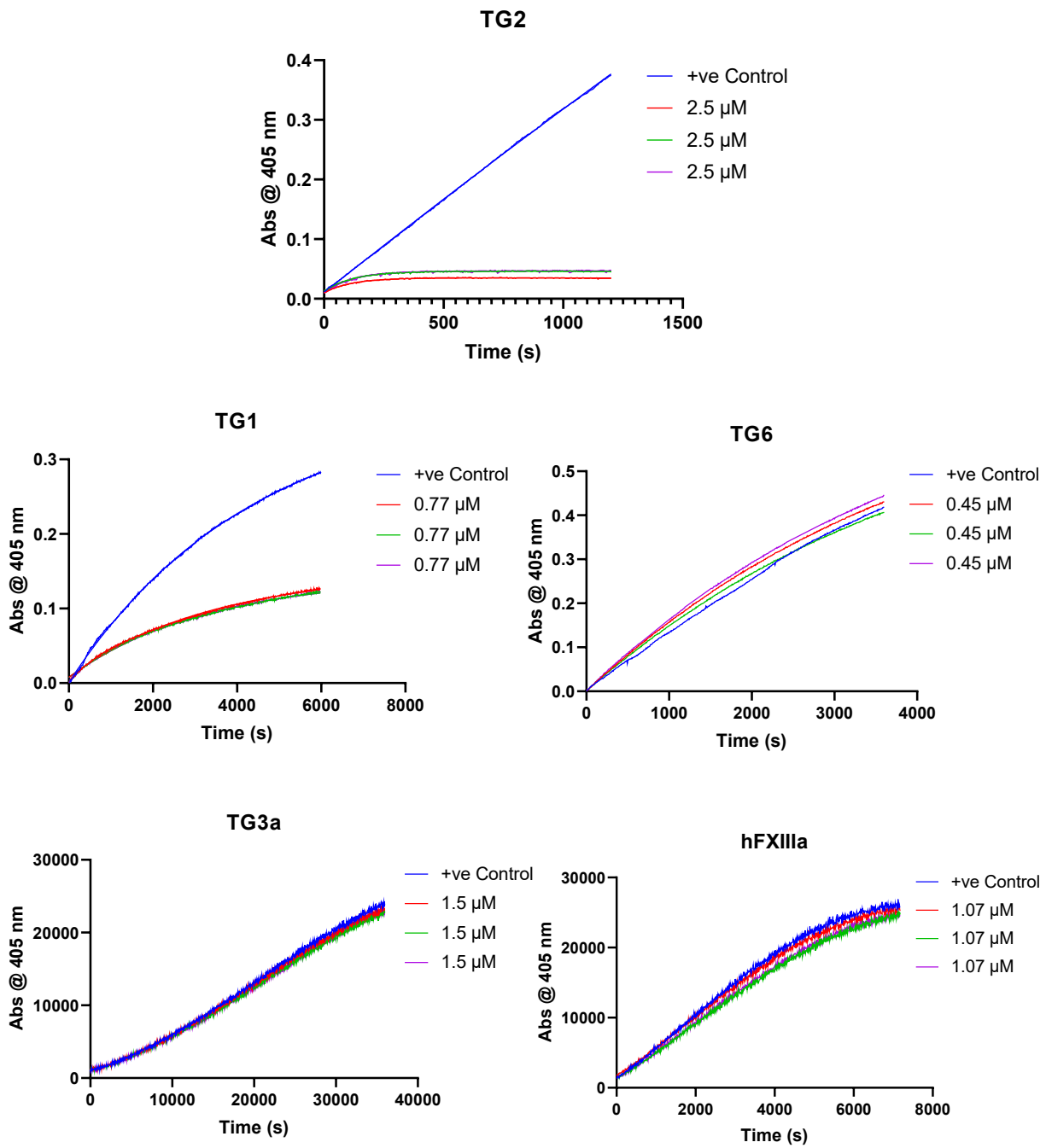


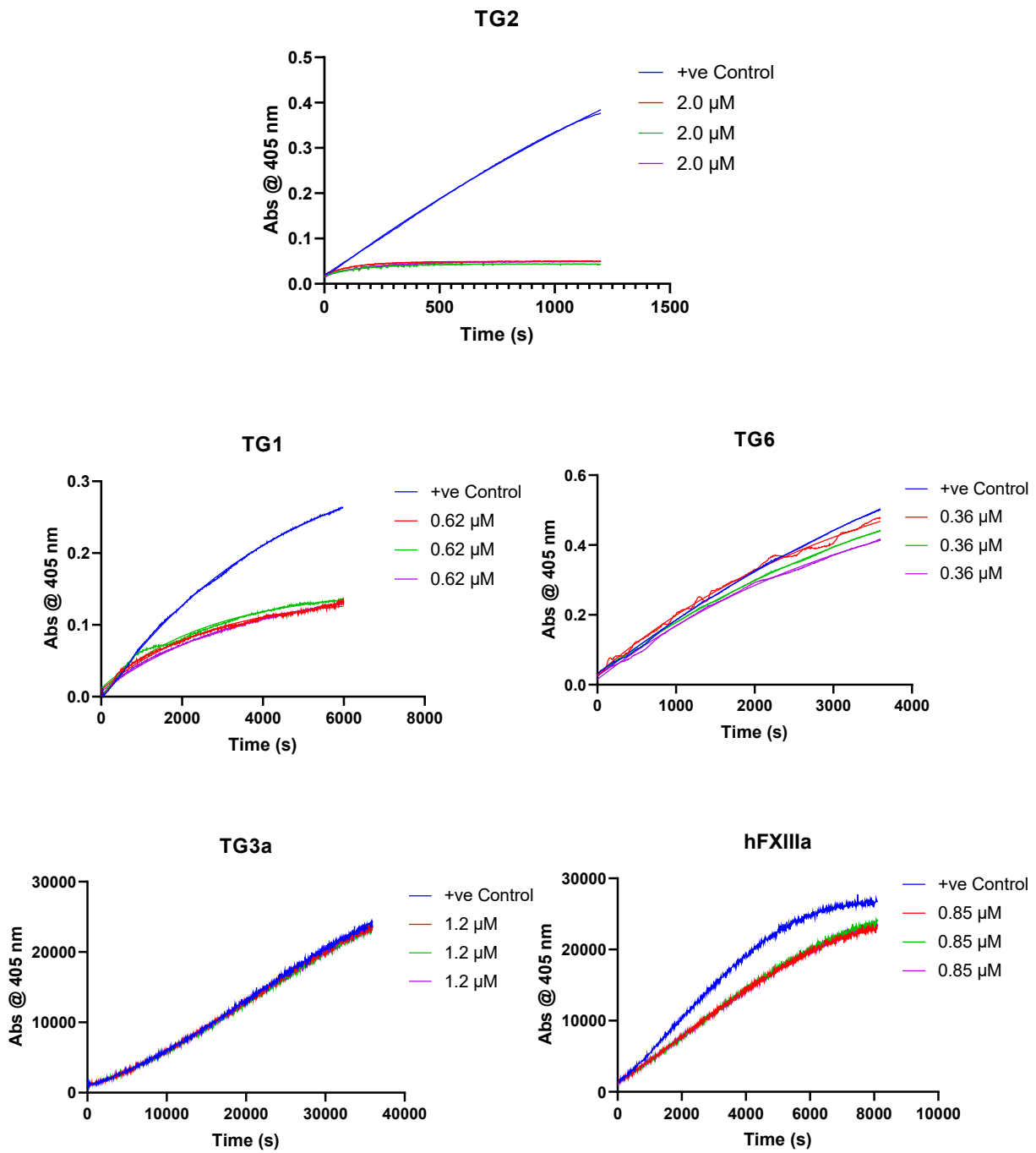
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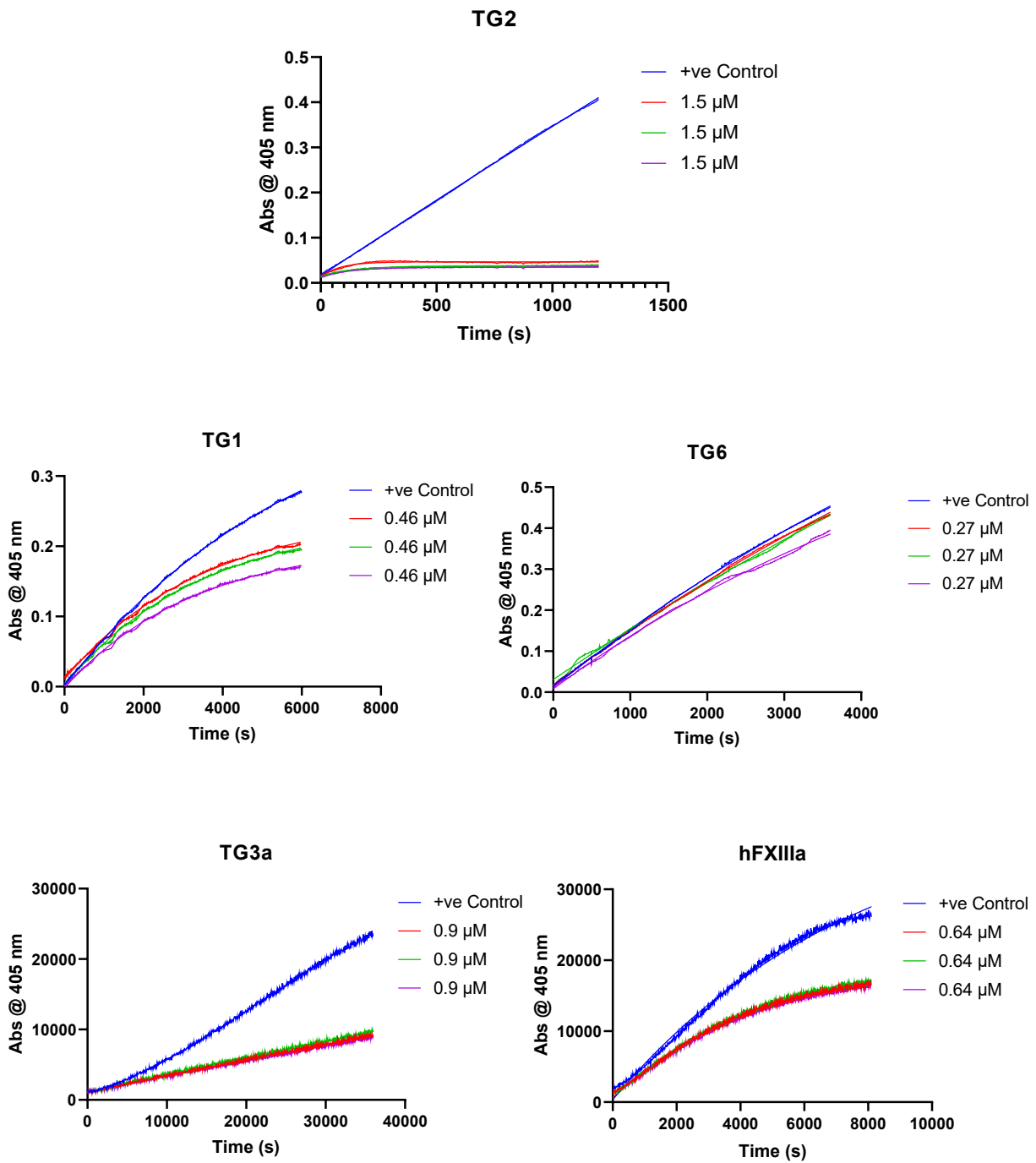
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7d







2.6.3. Intrinsic Reactivity Data

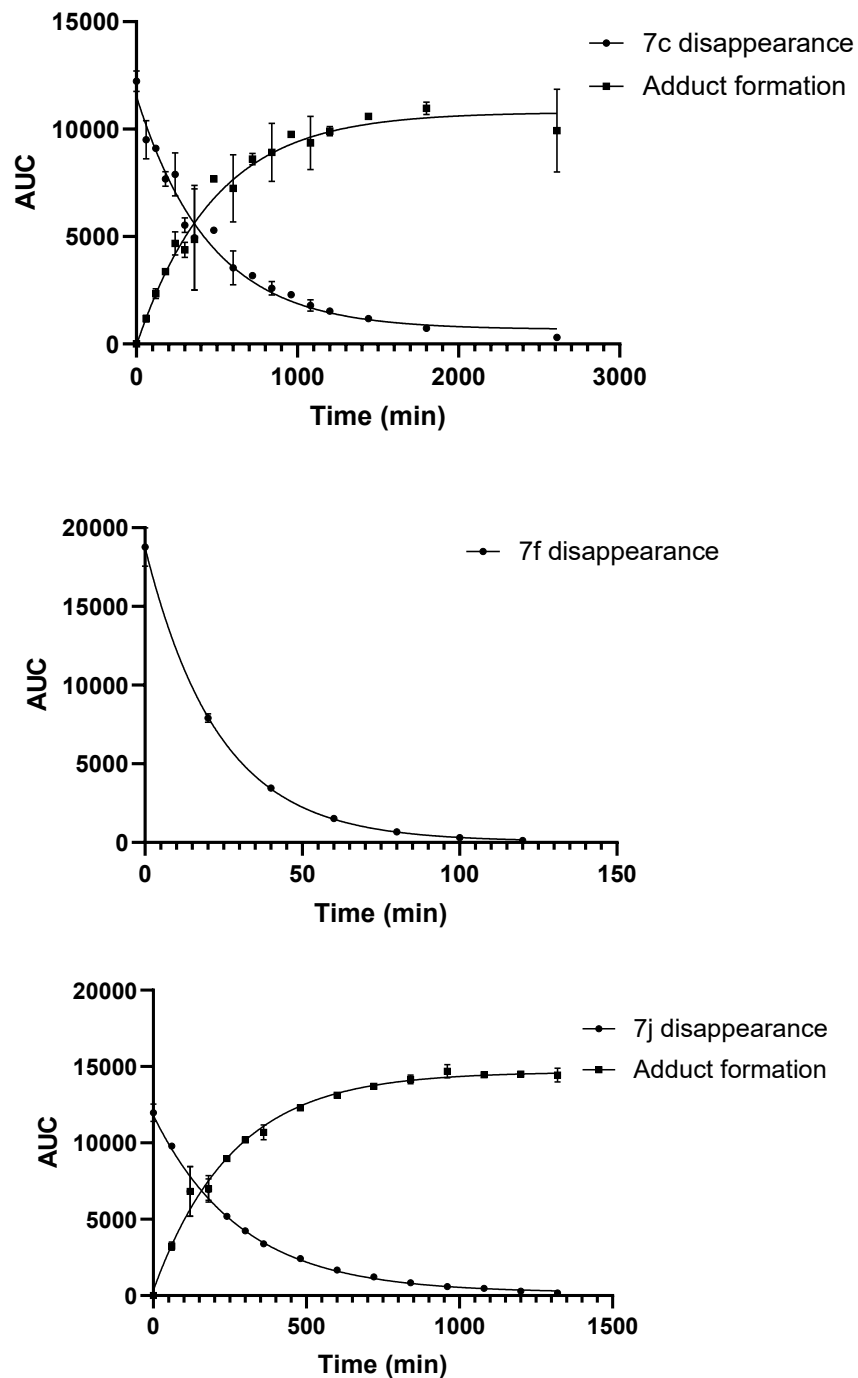


Figure 2.S1. Plots representing the disappearance of inhibitor and/or appearance of thiol-inhibitor adduct. The area under the curve (AUC) were plotted over time and fitted to a mono-exponential equation to give pseudo-first order reaction rates (k_{obs}), (a) **7c** + GSH, (b) **7f** + GSH, (c) **7j** + GSH.

2.6.4. LC-MS Data of Reaction Between 7f and GSH

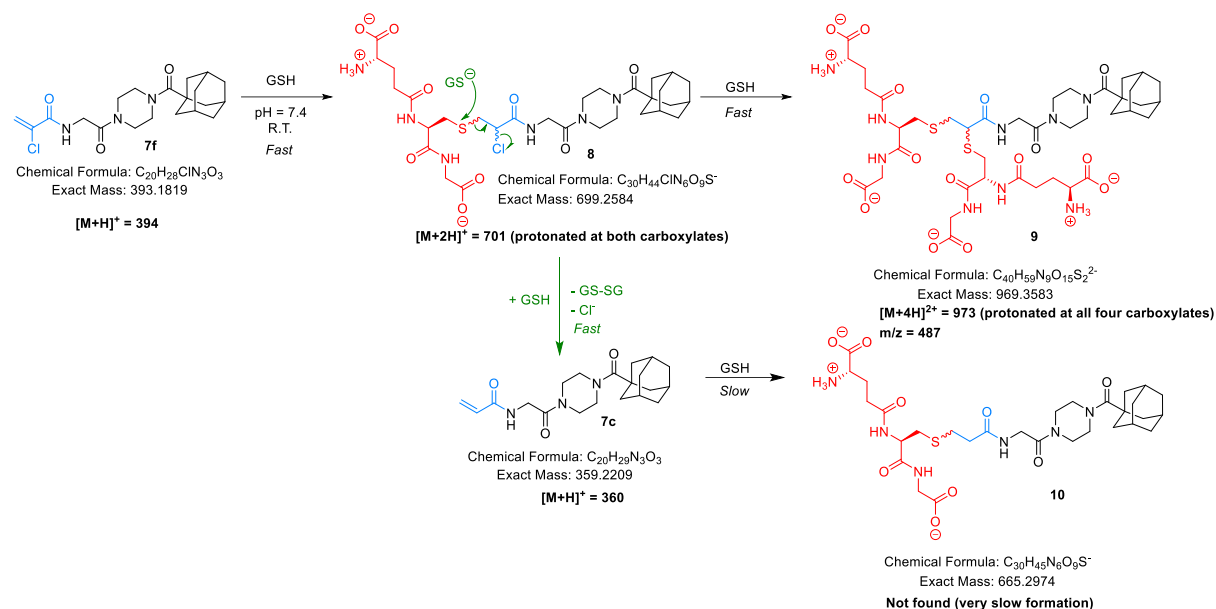
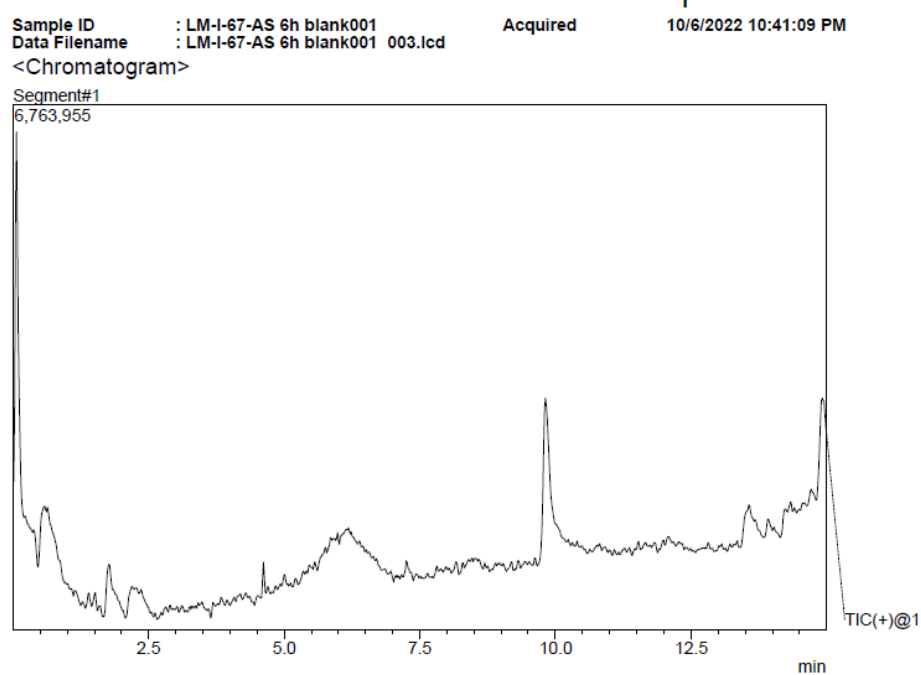


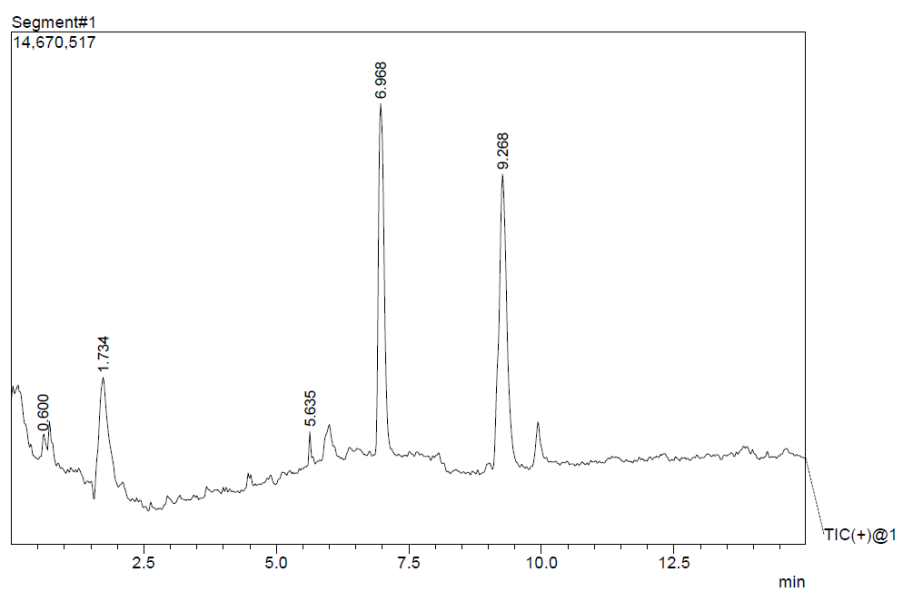
Figure 2.S2. Products of reaction of **7f** with GSH with m/z values found by LC-MS.

Chromatograms:

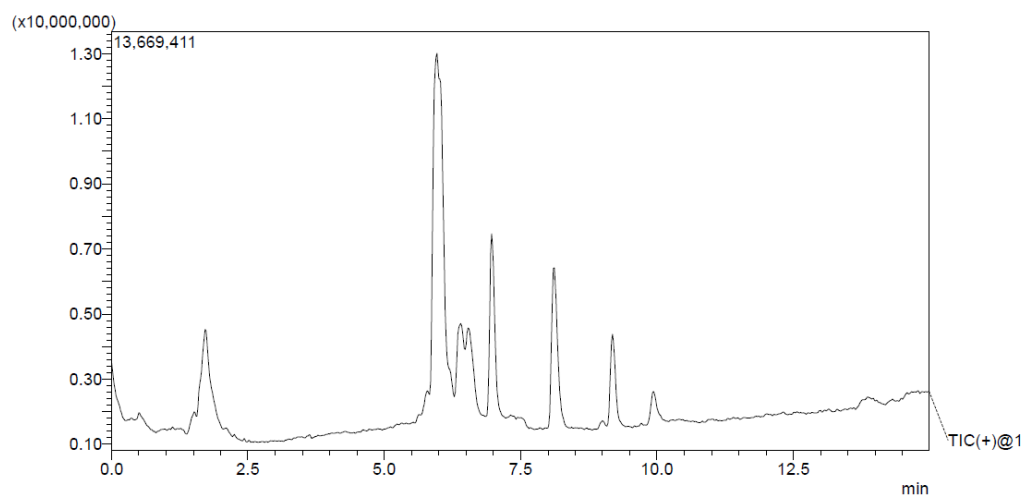
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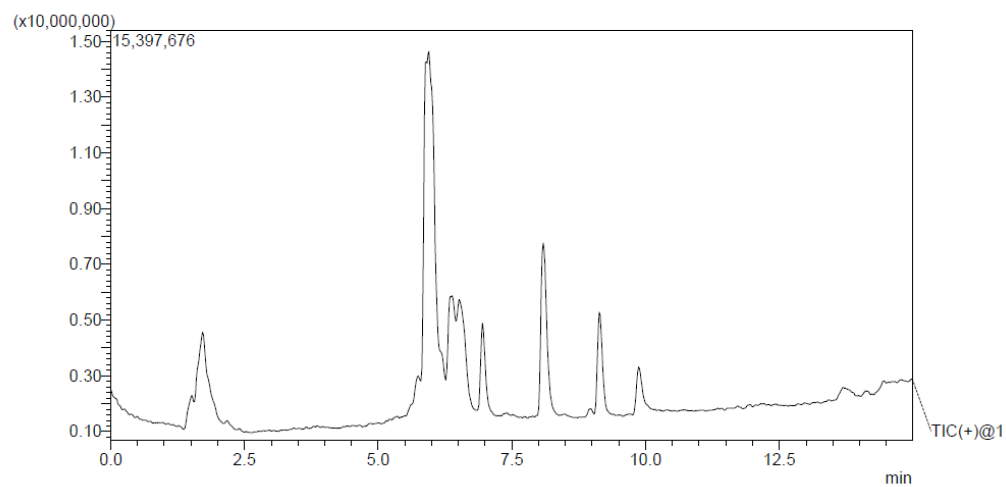
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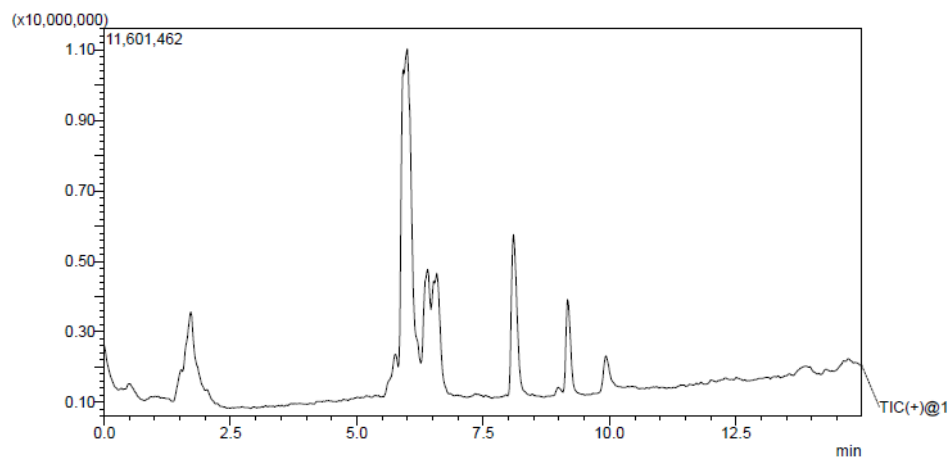
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(T = 6h)



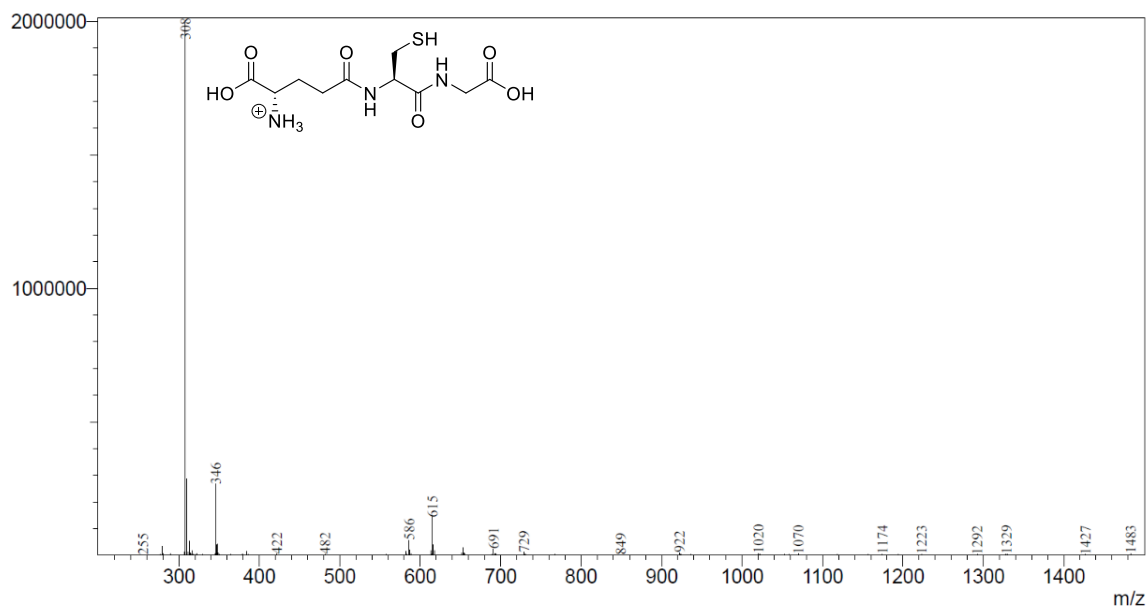
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Mass Spectra of identifiable compounds (Compound, Retention Time):

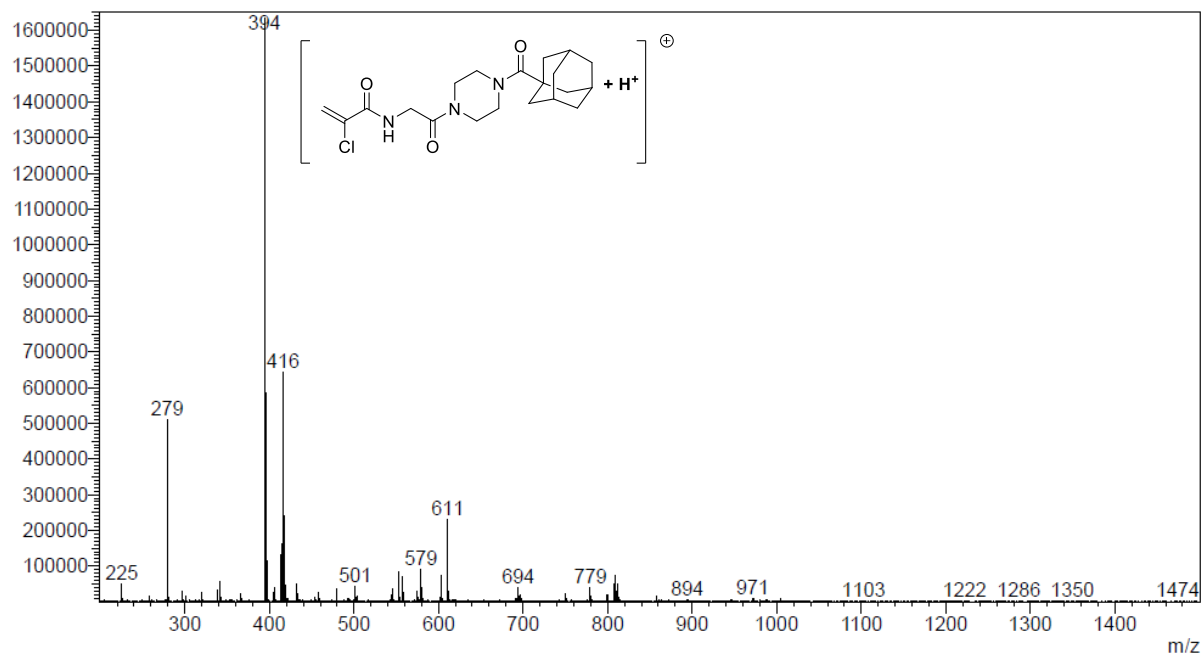
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BG Mode:None Segment 1 - Event 1



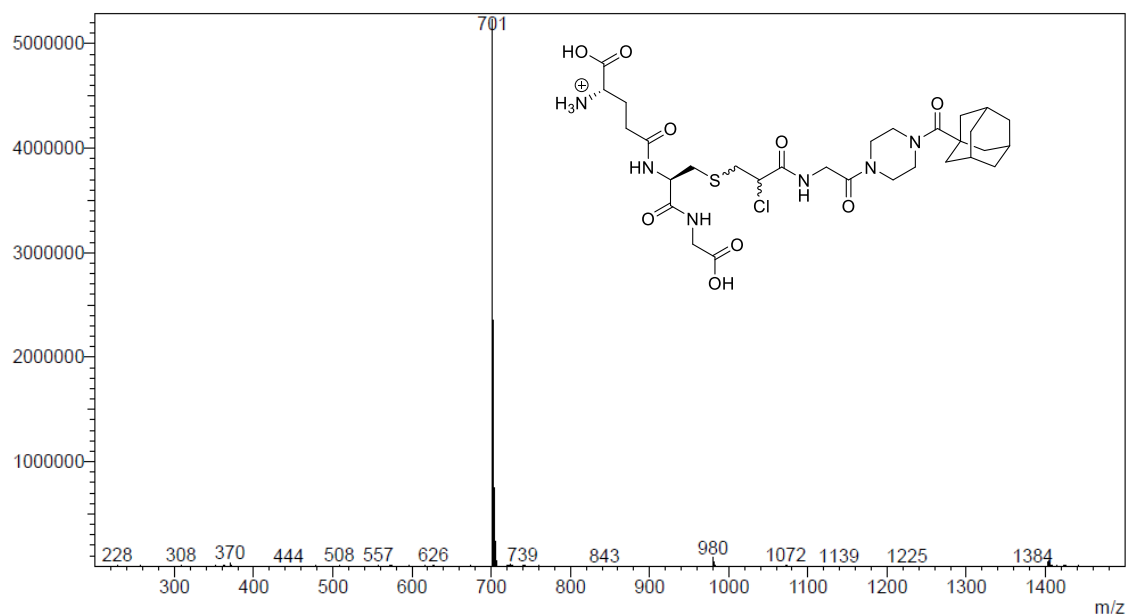
(7f, 9.333 min)

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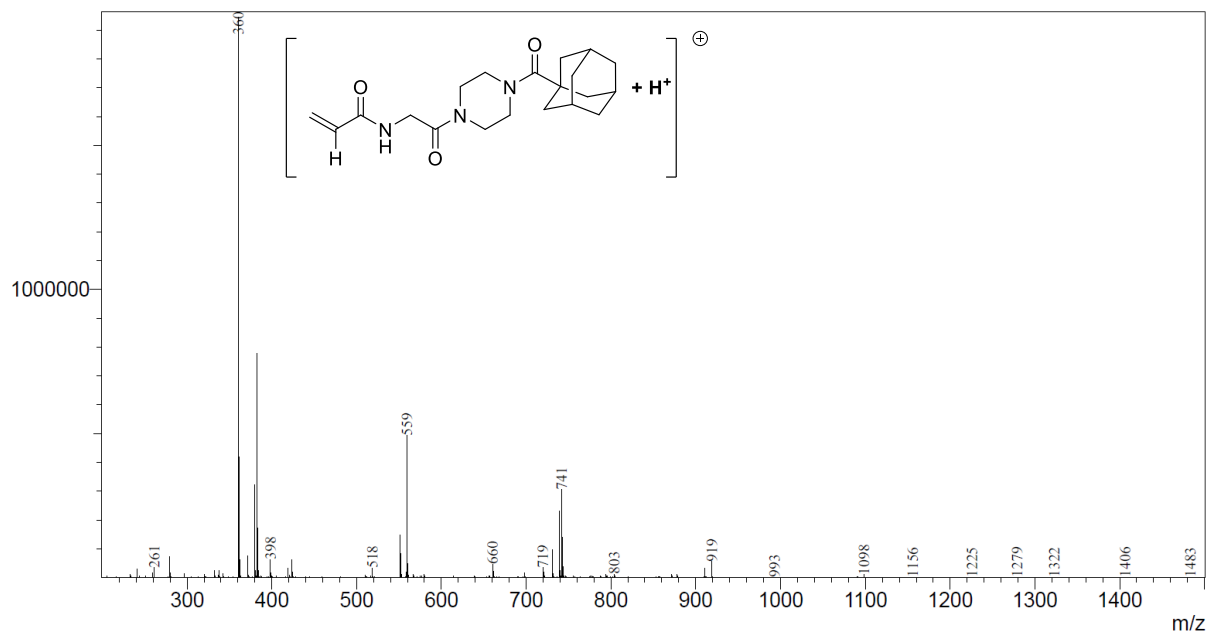
(8, 7.350 min)

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Spectrum Mode:Averaged 6.950-6.983(418-420)
BG Mode:Calc Polarity:Positive Segment 1 - Event 1



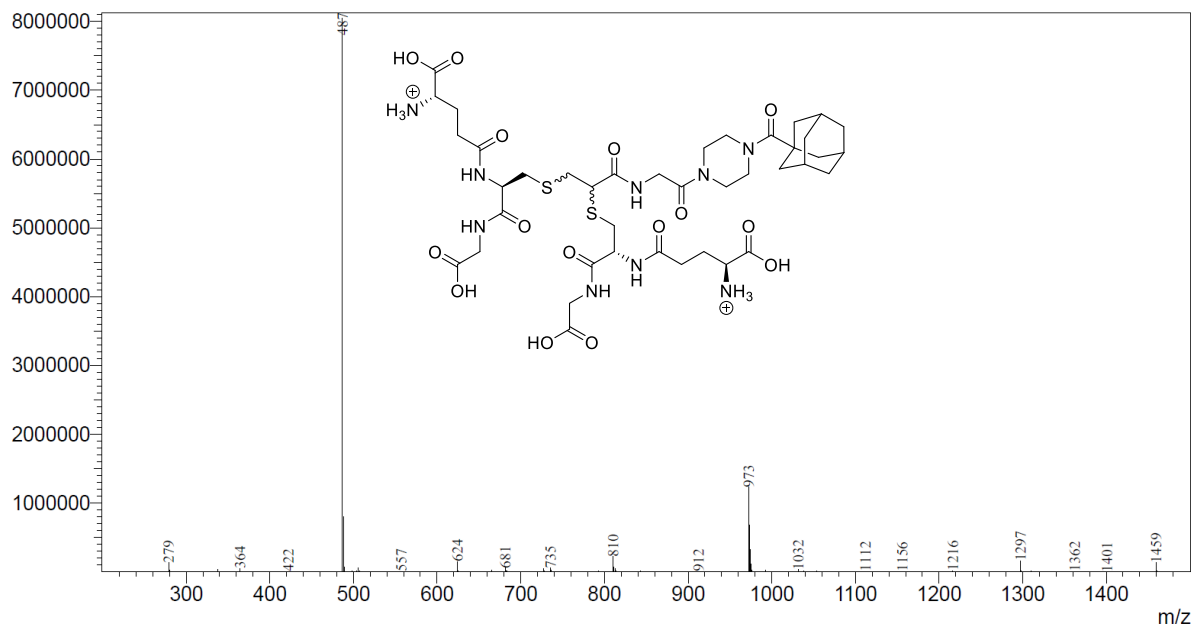
(7c, 8.083 min)

Line# 1 R.Time:8.083(Scan#:486)
MassPeaks:1344
RawMode:Single 8.083(486) BasePeak:360(1944438)
BG Mode:None Segment 1 - Event 1



(9, 5.933 min)

Line# 1 R.Time:5.933(Scan#:357)
MassPeaks:1365
RawMode:Single 5.933(357) BasePeak:487(8044516)
BG Mode:None Segment 1 - Event 1

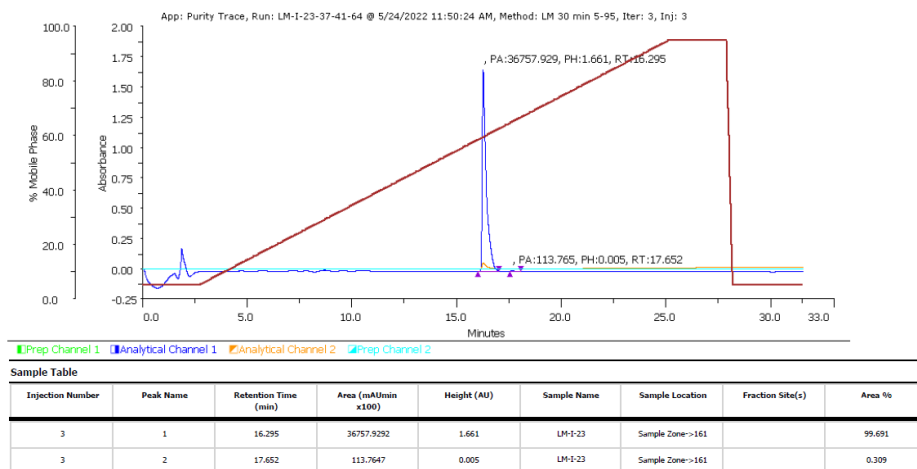


2.6.5. HPLC Purity Analysis of Final Inhibitors

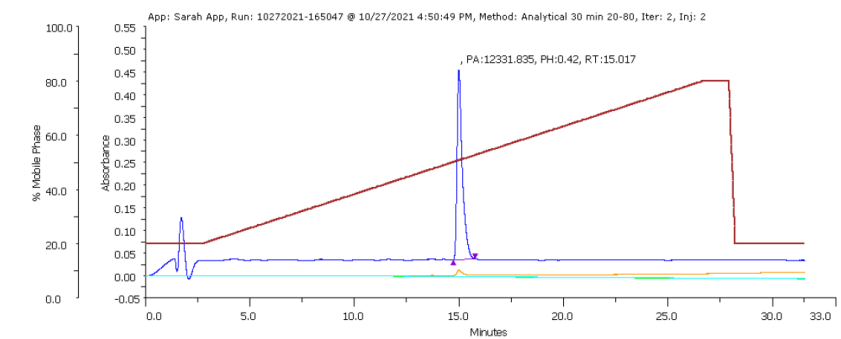
Inhibitor	Retention Time (min)	Purity (%)
7a	16.295	99.691
7b*	15.017	100
7c*	12.520	96.285
7d	18.468	99.670
7e	18.838	93.586
7f	17.996	99.741
7g	17.374	99.824
7h	16.823	100
7i	16.075	100
7j*	14.270	98.501
7k	17.477	90.763
7l	15.803	99.702
7m	27.028	100
7n	18.817	97.148
7o	16.550	98.985
7p	20.905	100
7q	14.142	91.349

Table 2.S1. The purity of the final inhibitors determined by HPLC. A Gilson-Mandel GXP271 high performance liquid chromatography (HPLC) with UV detection at 214 and 254 nm was used (Phenomenex Luna, 150 mm × 4.6 mm, 30 min, 1.5 mL/min flow rate, 5-95% 0.1% TFA in MeCN/0.1% TFA in H₂O unless otherwise indicated). *20-80% 0.1% TFA in MeCN/0.1% TFA in H₂O.

7a

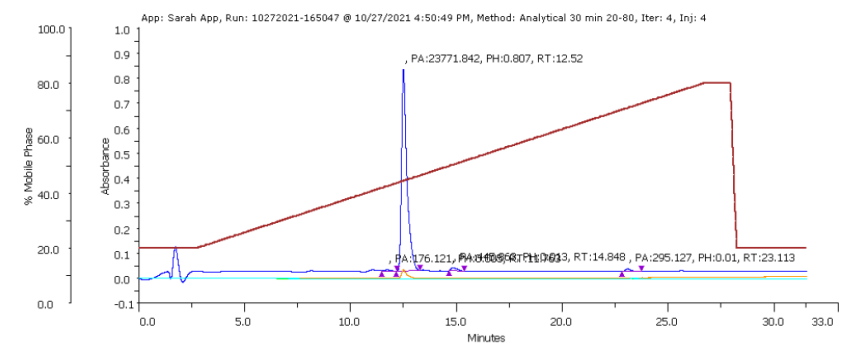


7b



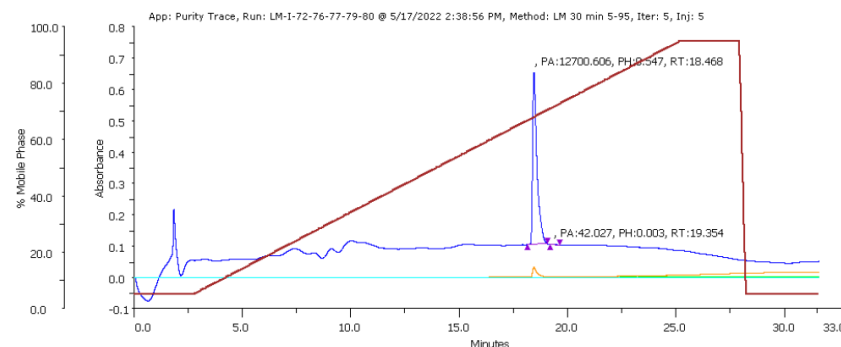
Sample Table								
Injection Number	Peak Name	Retention Time (min)	Area (mAUmin x100)	Height (AU)	Sample Name	Sample Location	Fraction Site(s)	Area %
2	1	15.017	12331.8346	0.42	LM-1-17	Sample Zone->152		100

7c



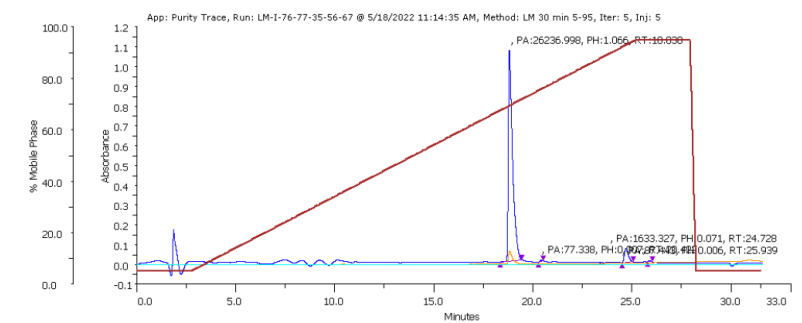
Sample Table								
Injection Number	Peak Name	Retention Time (min)	Area (mAUmin x100)	Height (AU)	Sample Name	Sample Location	Fraction Site(s)	Area %
4	1	12.52	23771.8418	0.807	LM-1-10	Sample Zone->154		96.285
4	2	14.848	445.863	0.013	LM-1-10	Sample Zone->154		1.806
4	3	23.113	295.1269	0.01	LM-1-10	Sample Zone->154		1.195
4	4	11.763	176.1212	0.005	LM-1-10	Sample Zone->154		0.713

7d



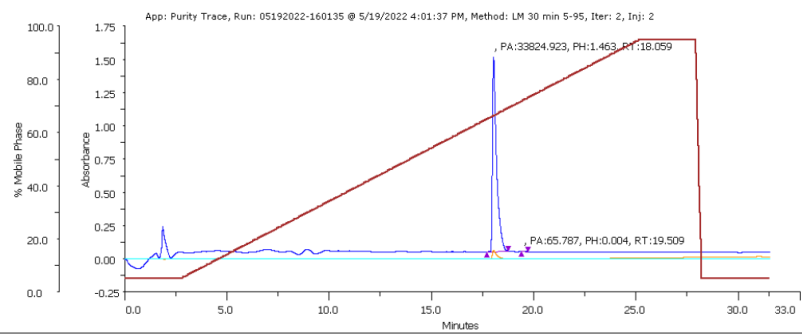
Sample Table								
Injection Number	Peak Name	Retention Time (min)	Area (mAUmin x100)	Height (AU)	Sample Name	Sample Location	Fraction Site(s)	Area %
5	1	18.468	12700.6061	0.547	LM-1-79	Sample Zone->159		99.67
5	2	19.354	42.0266	0.003	LM-1-79	Sample Zone->159		0.33

7e



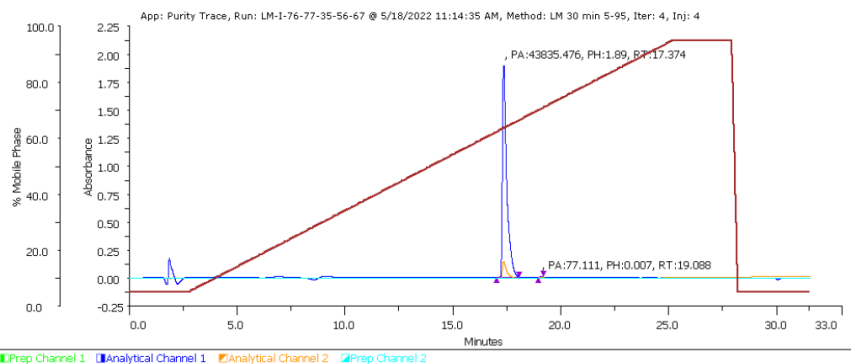
Injection Number	Peak Name	Retention Time (min)	Area (mAUmin x100)	Height (AU)	Sample Name	Sample Location	Fraction Site(s)	Area %
5	1	18.838	26236.9977	1.066	LM-I-35	Sample Zone->163		93.586
5	2	20.422	77.3385	0.007	LM-I-35	Sample Zone->163		0.276
5	3	24.728	1633.3269	0.071	LM-I-35	Sample Zone->163		5.826
5	4	25.939	87.443	0.006	LM-I-35	Sample Zone->163		0.312

7f



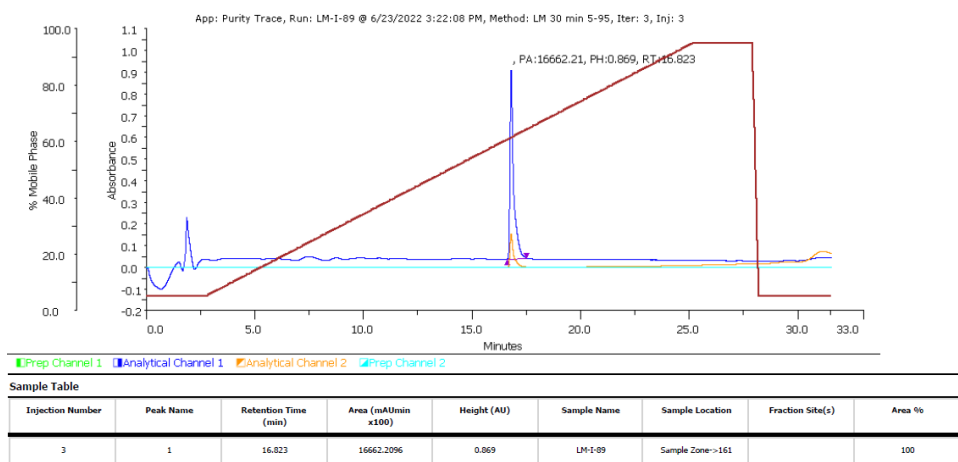
Injection Number	Peak Name	Retention Time (min)	Area (mAUmin x100)	Height (AU)	Sample Name	Sample Location	Fraction Site(s)	Area %
2	1	18.059	33824.9231	1.463	LM-I-67	Sample Zone->161		99.806
2	2	19.509	65.7872	0.004	LM-I-67	Sample Zone->161		0.194

7g

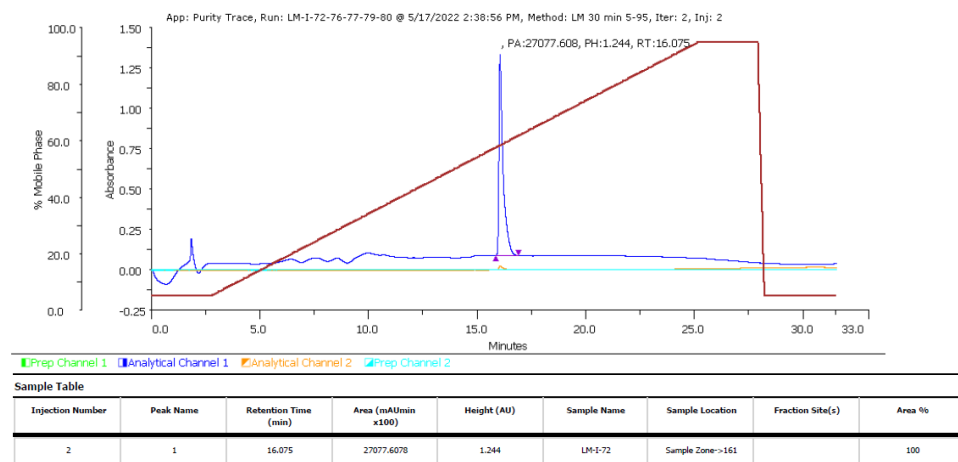


Injection Number	Peak Name	Retention Time (min)	Area (mAUmin x100)	Height (AU)	Sample Name	Sample Location	Fraction Site(s)	Area %
4	1	17.374	43835.4756	1.89	LM-I-77	Sample Zone->162		99.824
4	2	19.088	77.111	0.007	LM-I-77	Sample Zone->162		0.176

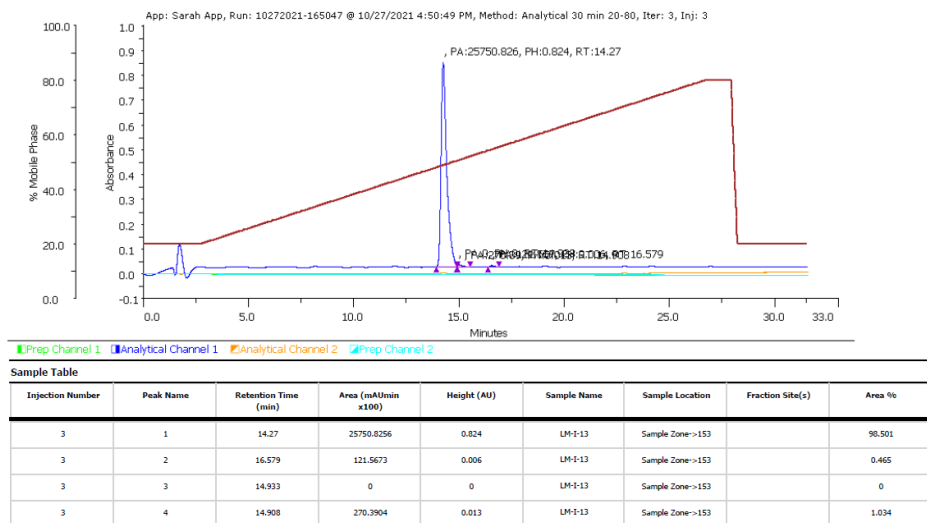
7h



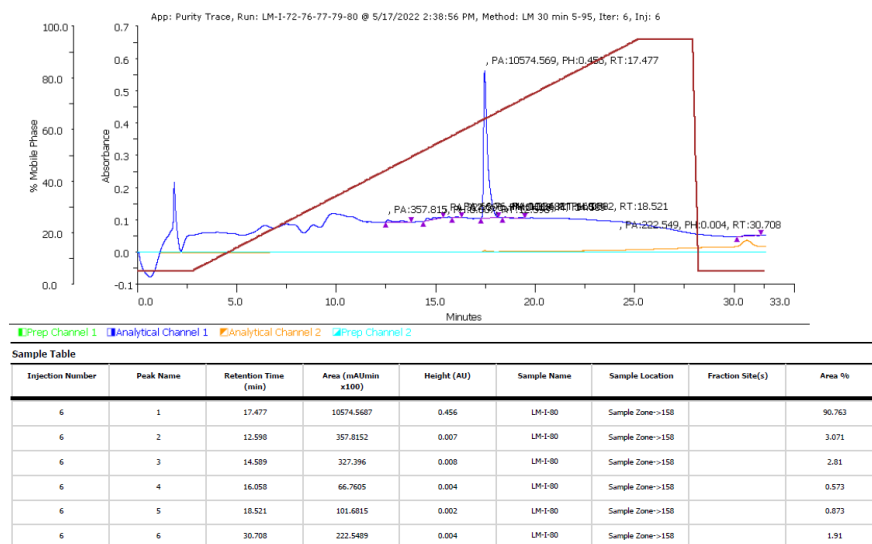
7i



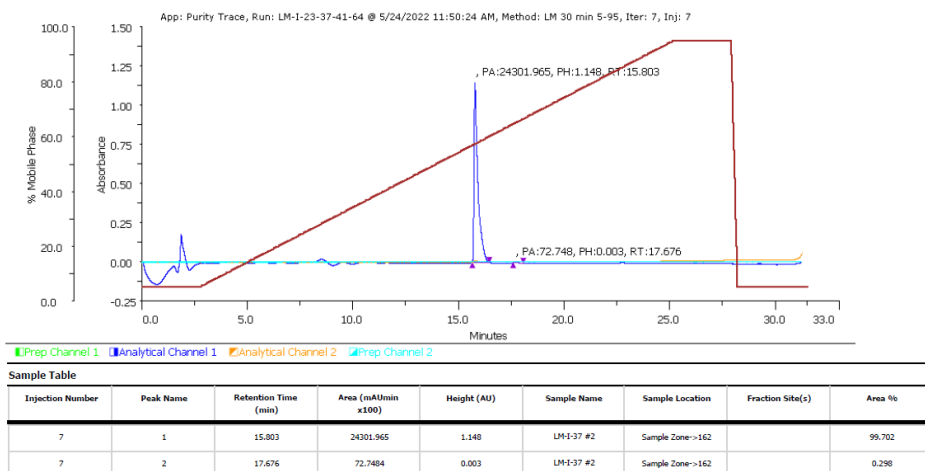
7j



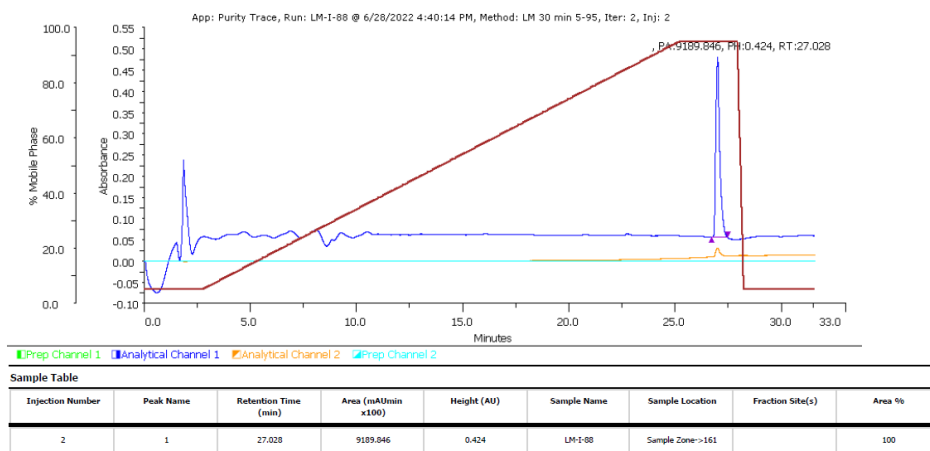
7k



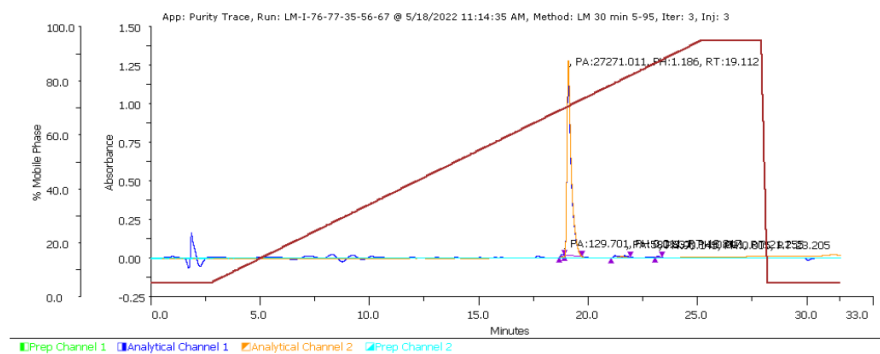
7l



7m



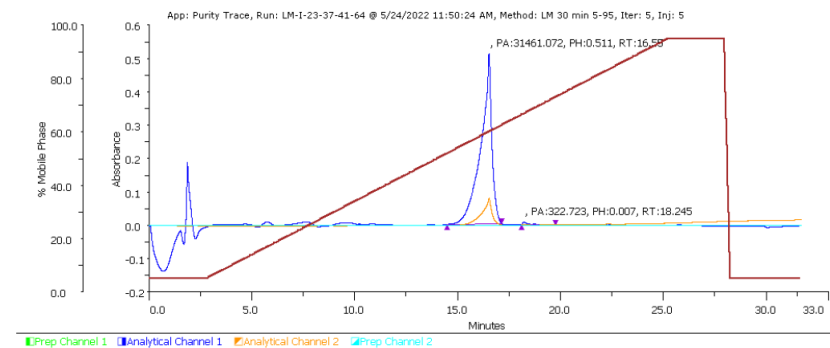
7n



Sample Table

Injection Number	Peak Name	Retention Time (min)	Area (mAUmin x100)	Height (AU)	Sample Name	Sample Location	Fraction Site(s)	Area %
3	1	18.817	129.7009	0.011	LM-1-76	Sample Zone->161		0.462
3	2	19.112	27271.0106	1.186	LM-1-76	Sample Zone->161		97.148
3	3	21.355	580.8367	0.011	LM-1-76	Sample Zone->161		2.069
3	4	23.205	90.1447	0.005	LM-1-76	Sample Zone->161		0.321

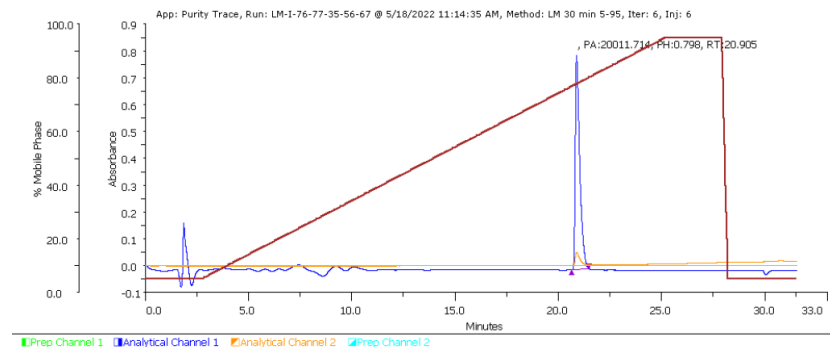
7o



Sample Table

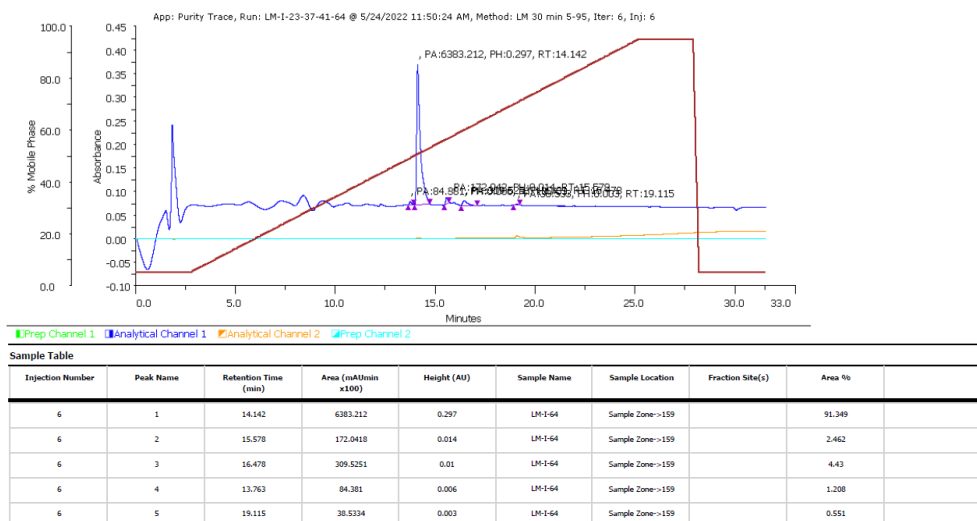
Injection Number	Peak Name	Retention Time (min)	Area (mAUmin x100)	Height (AU)	Sample Name	Sample Location	Fraction Site(s)	Area %
5	1	16.55	31461.072	0.511	LM-1-41	Sample Zone->163		98.985
5	2	18.245	322.7227	0.007	LM-1-41	Sample Zone->163		1.015

7p



Sample Table

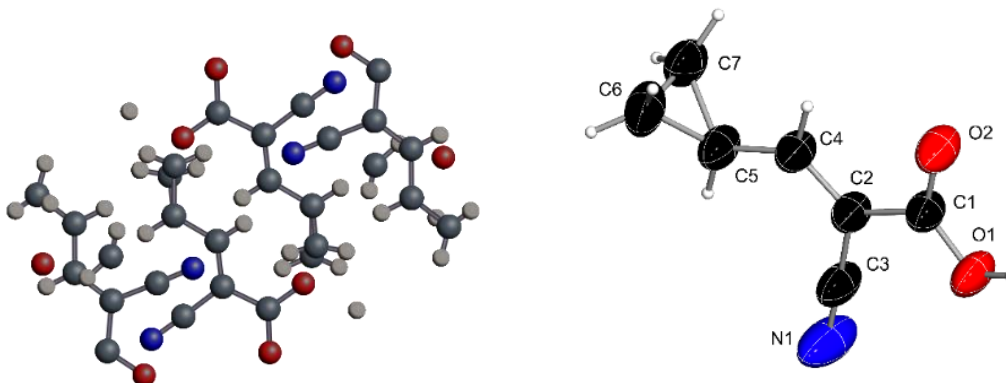
Injection Number	Peak Name	Retention Time (min)	Area (mAUmin x100)	Height (AU)	Sample Name	Sample Location	Fraction Site(s)	Area %
6	1	20.905	20011.7141	0.798	LM-1-56	Sample Zone->159		100



2.6.6. Synthesis of N-2-[4-[[1-Adamantanecarbonyl]-1-piperaziny]-2-oxoethyl]-2-methylpropenamide (7r)

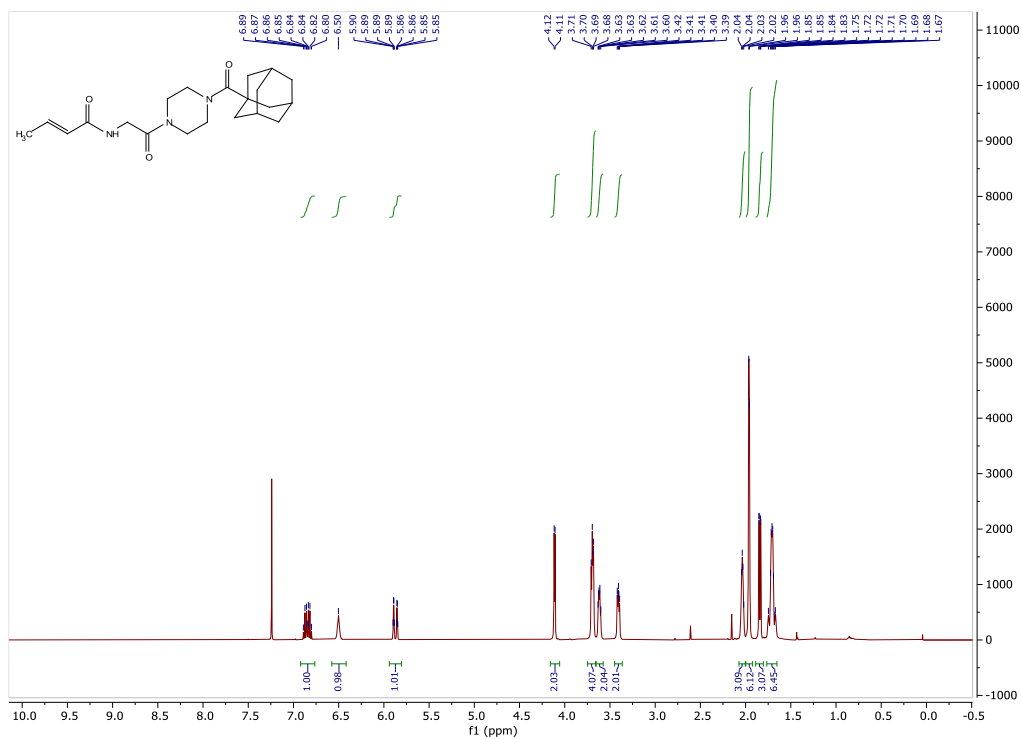
Methacrylic acid (0.031 mL, 0.361 mmol, 1.1 eq), HBTU (0.2 g, 0.524 mmol, 1.6 eq), Hünig's base (0.17 mL, 0.98 mmol, 3.0 eq), and **6** (0.1 g, 0.327 mmol, 1.0 eq) were stirred in anhydrous DCM (5 mL) at R.T. for 16 h, at which point the reaction was confirmed complete by TLC analysis. The reaction was concentrated *in vacuo* and the residue was redissolved in EtOAc (50 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (25 mL), NaHCO₃ (25 mL), and brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a light orange solid, which was purified by silica gel column chromatography (5% MeOH/EtOAc, dry load using celite, R_f = 0.41) and then washing with pentane to give a white solid (0.028 g, 23%). ¹H NMR (300 MHz, CDCl₃) δ 5.78-5.77 (m, 1H), 5.37-5.36 (m, 1H), 4.07 (s, 2H), 3.71-3.61 (m, 4H), 3.60-3.57 (m, 2H), 3.44-3.40 (m, 2H), 2.02-2.00 (m, 3H), 1.96-1.93 (m, 9H), 1.74-1.64 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.2, 168.2, 167.0, 139.2, 120.6, 45.3, 44.9, 44.7, 42.3, 41.9, 41.5, 39.2, 36.6, 28.5, 18.6. HRMS (ESI) calc'd for C₂₁H₃₁N₃O₃Na [MNa]⁺: 396.2263, found: 396.2243.

2.6.7. Crystal structure of 2-cyano-3-cyclopropylacrylic acid (I3)

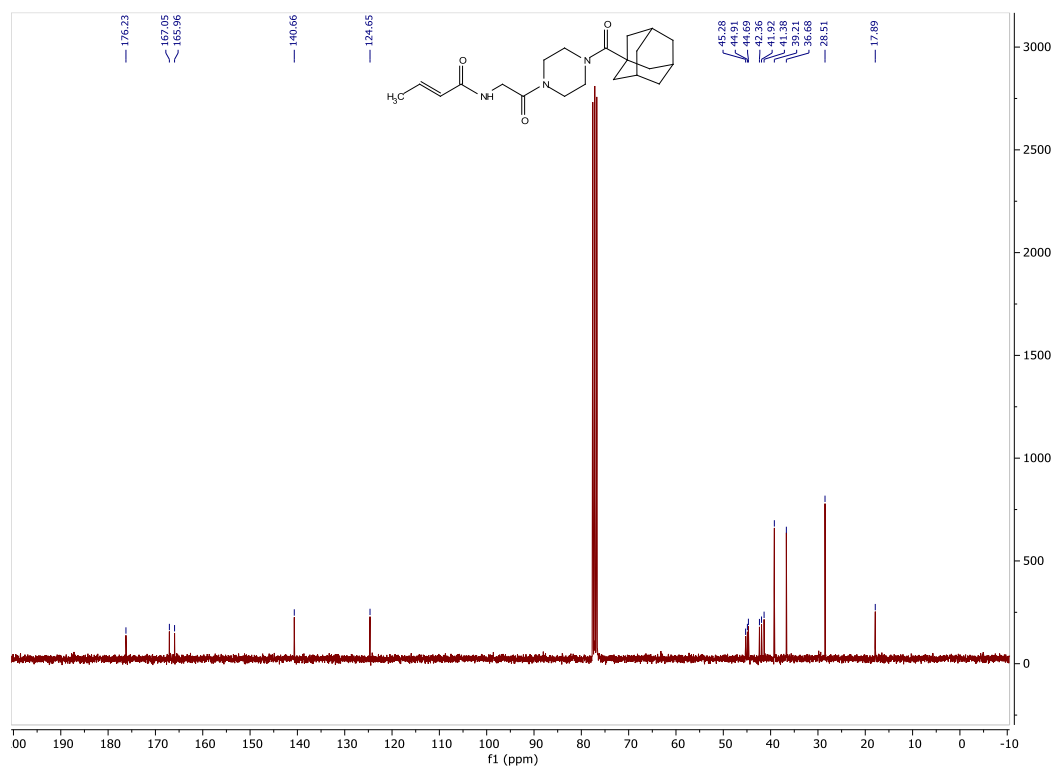


2.6.8. NMR Spectra of Final Inhibitors and New Synthetic Intermediates

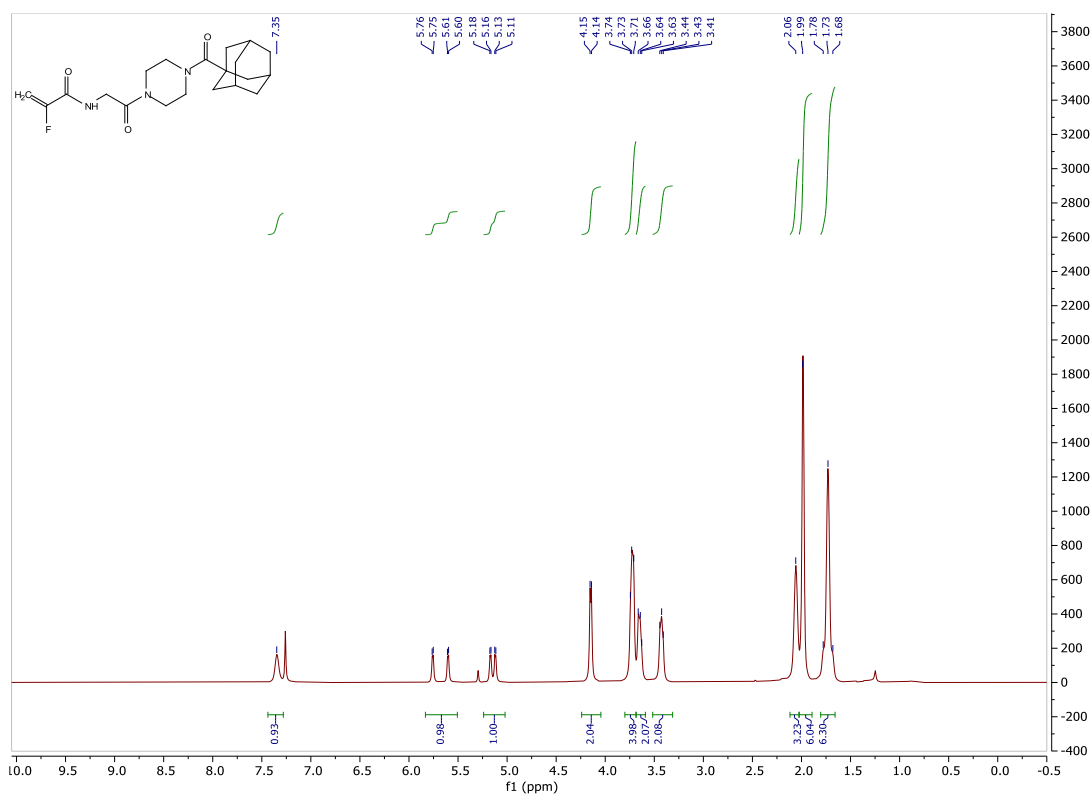
^1H NMR (300 MHz, CDCl_3) Spectra of Compound **7a**



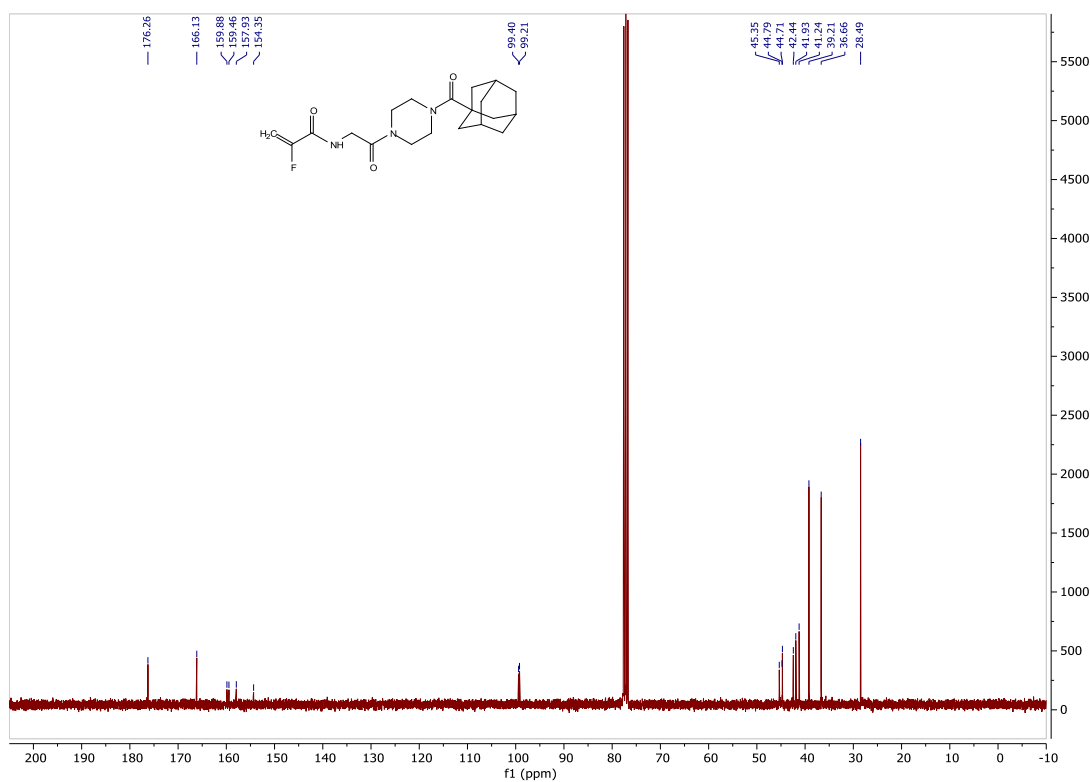
^{13}C NMR (75 MHz, CDCl_3) Spectra of Compound **7a**



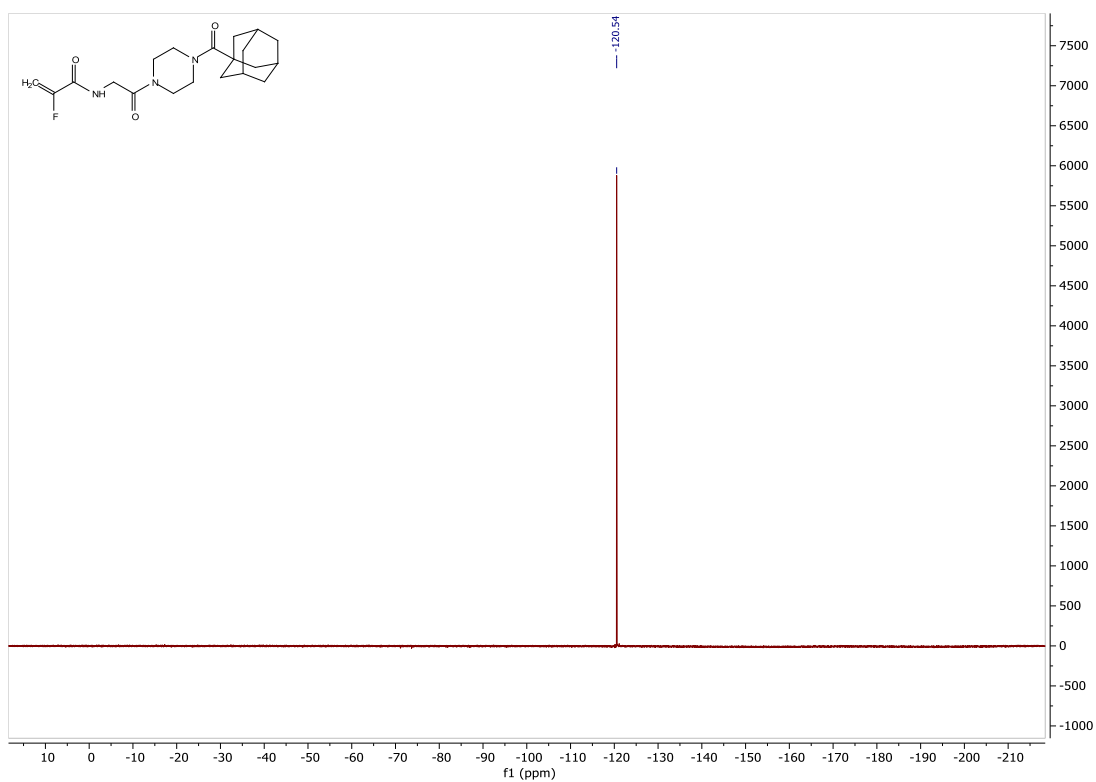
¹H NMR (300 MHz, CDCl₃) Spectra of Compound **7b**



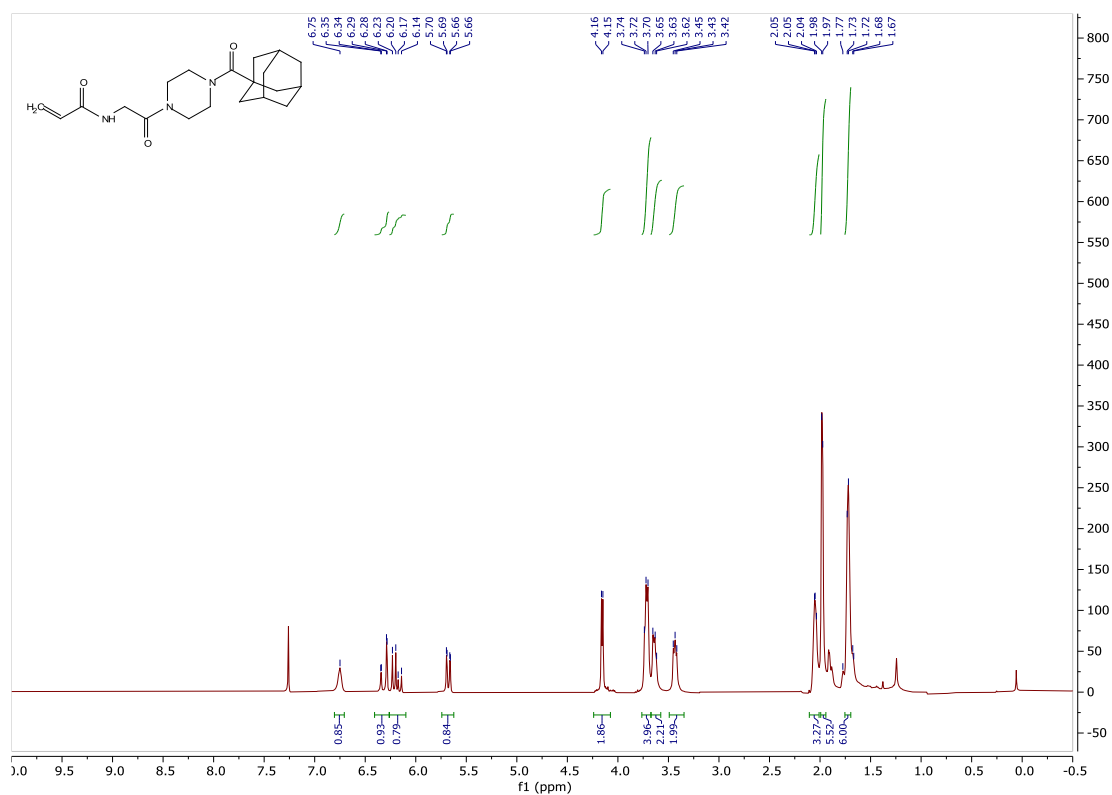
¹³C NMR (75 MHz, CDCl₃) Spectra of Compound **7b**



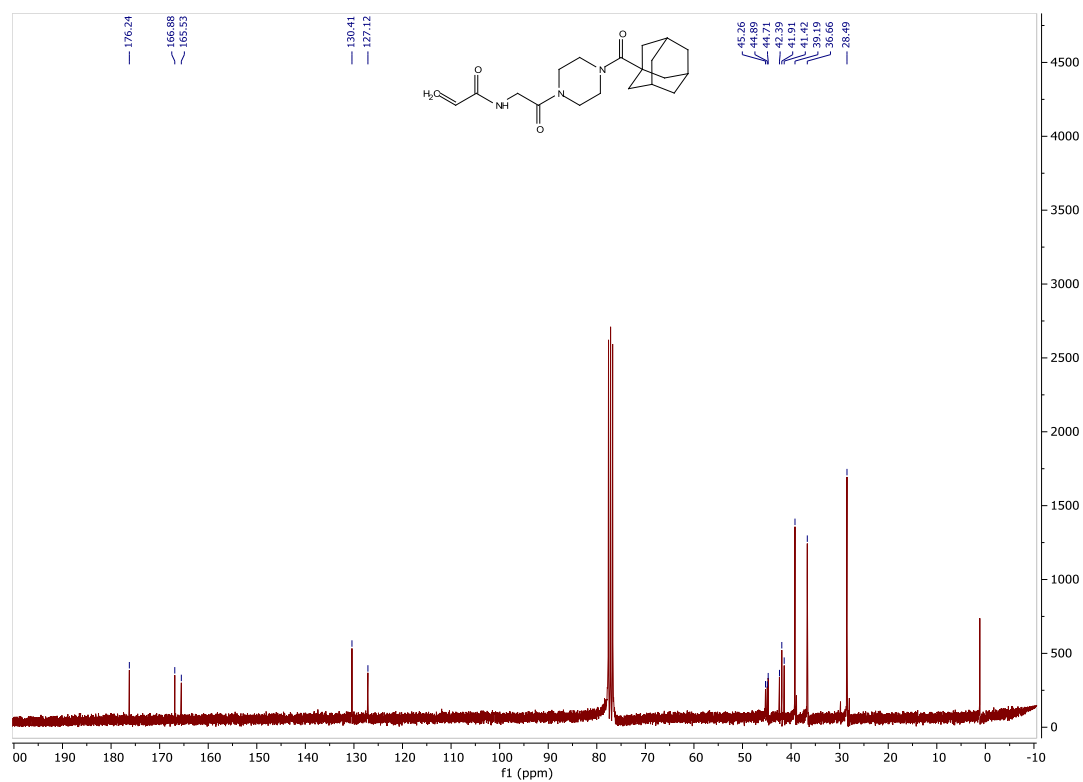
^{19}F NMR (283 MHz, CDCl_3) Spectra of Compound **7b**



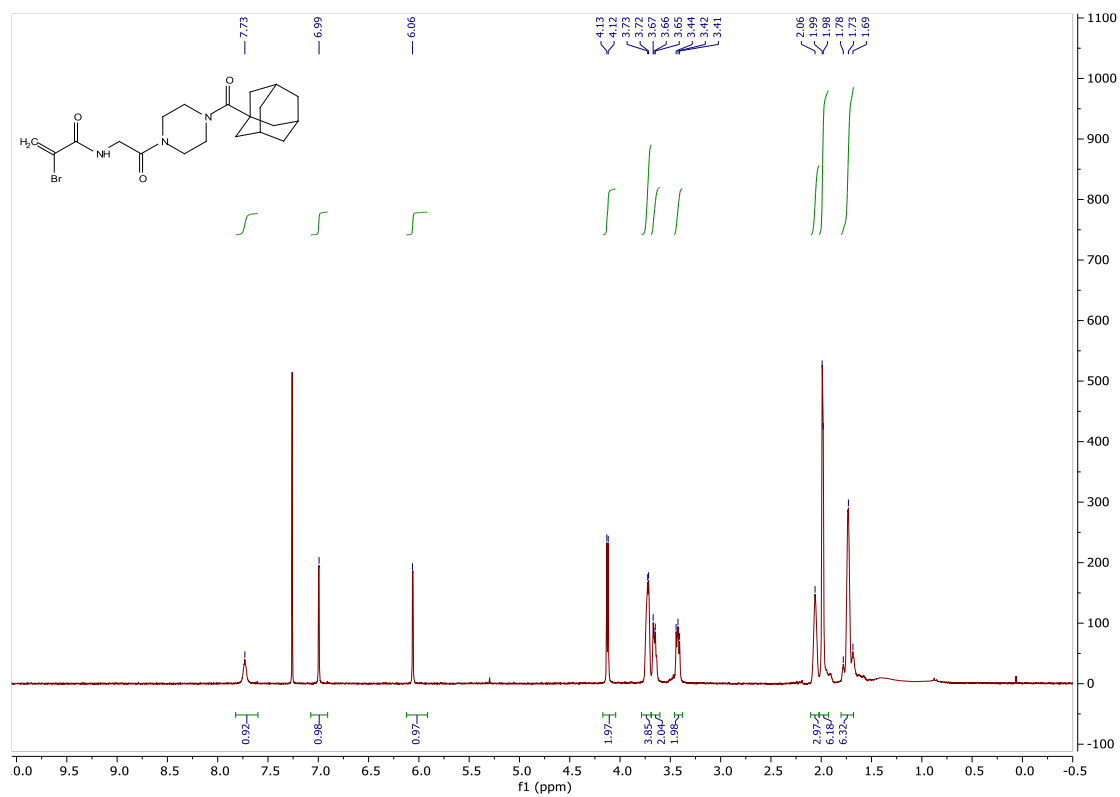
^1H NMR (300 MHz, CDCl_3) Spectra of Compound **7c**



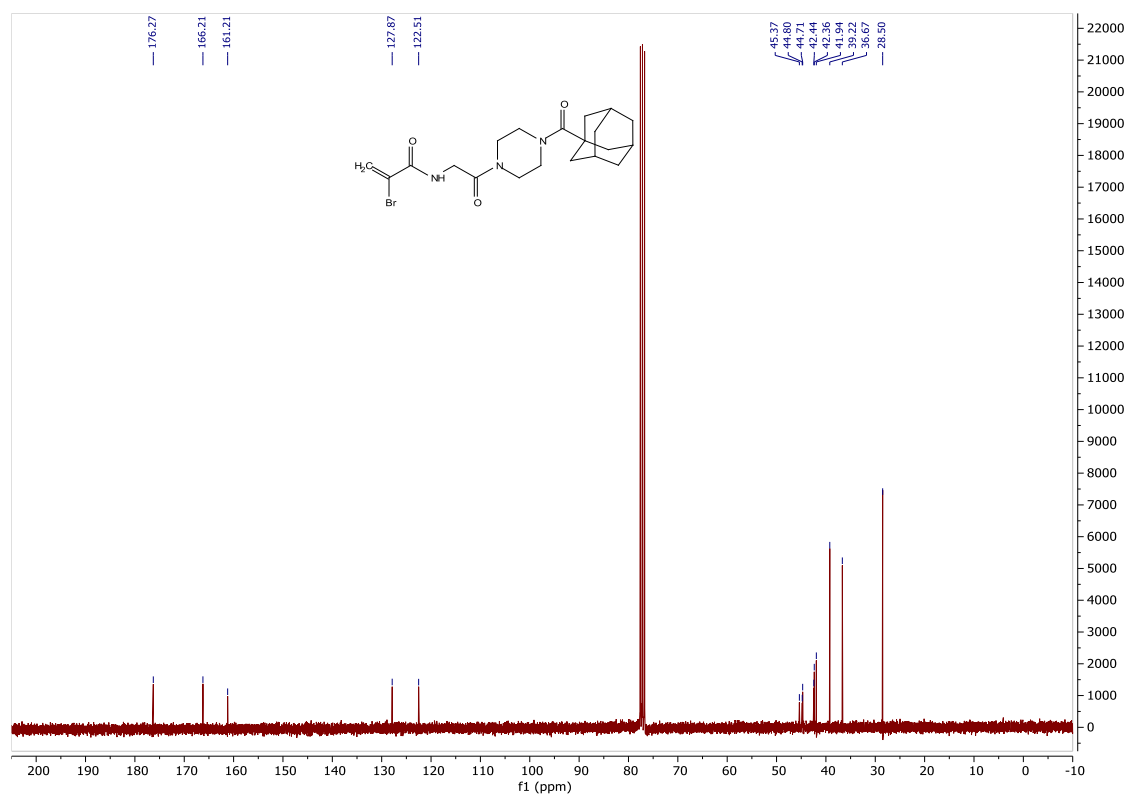
^{13}C NMR (75 MHz, CDCl_3) Spectra of Compound **7c**



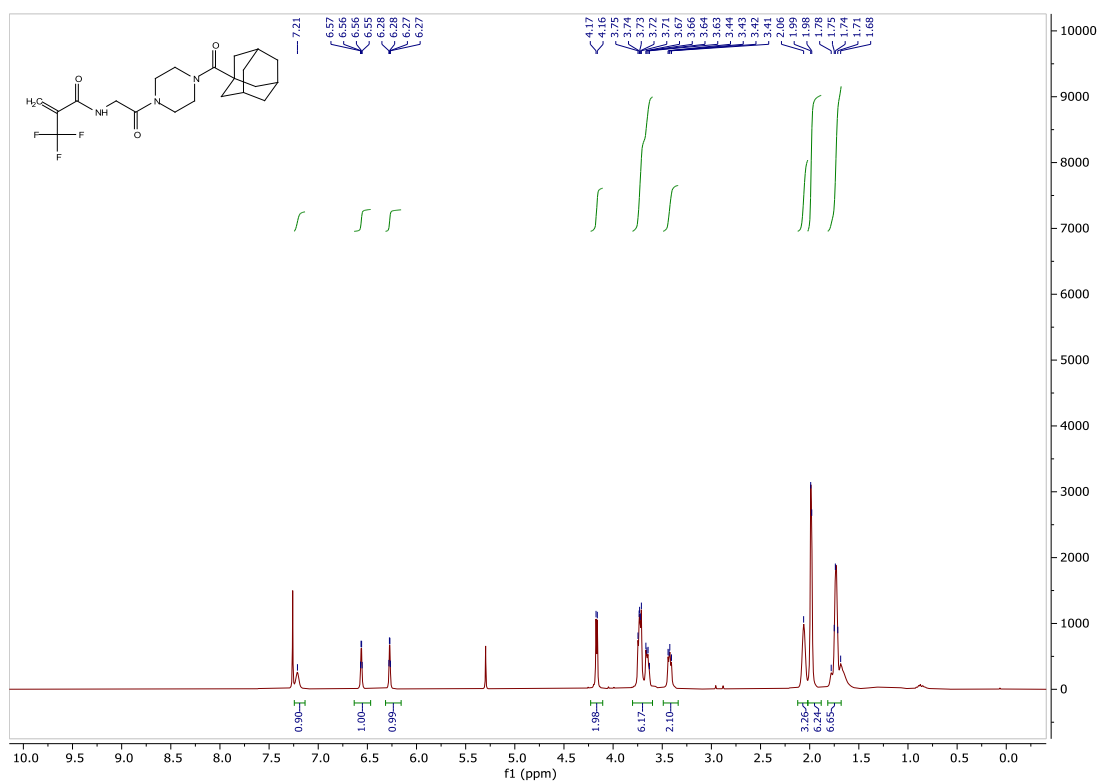
^1H NMR (300 MHz, CDCl_3) Spectra of Compound **7d**



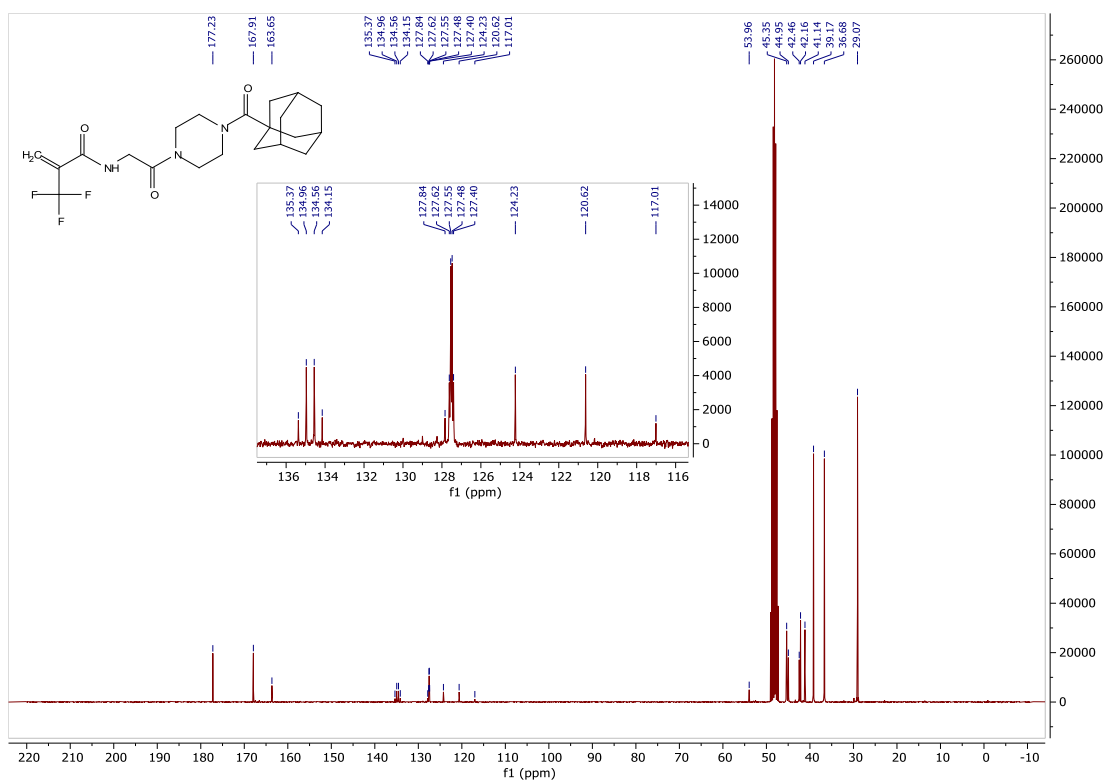
^{13}C NMR (75 MHz, CDCl_3) Spectra of Compound **7d**



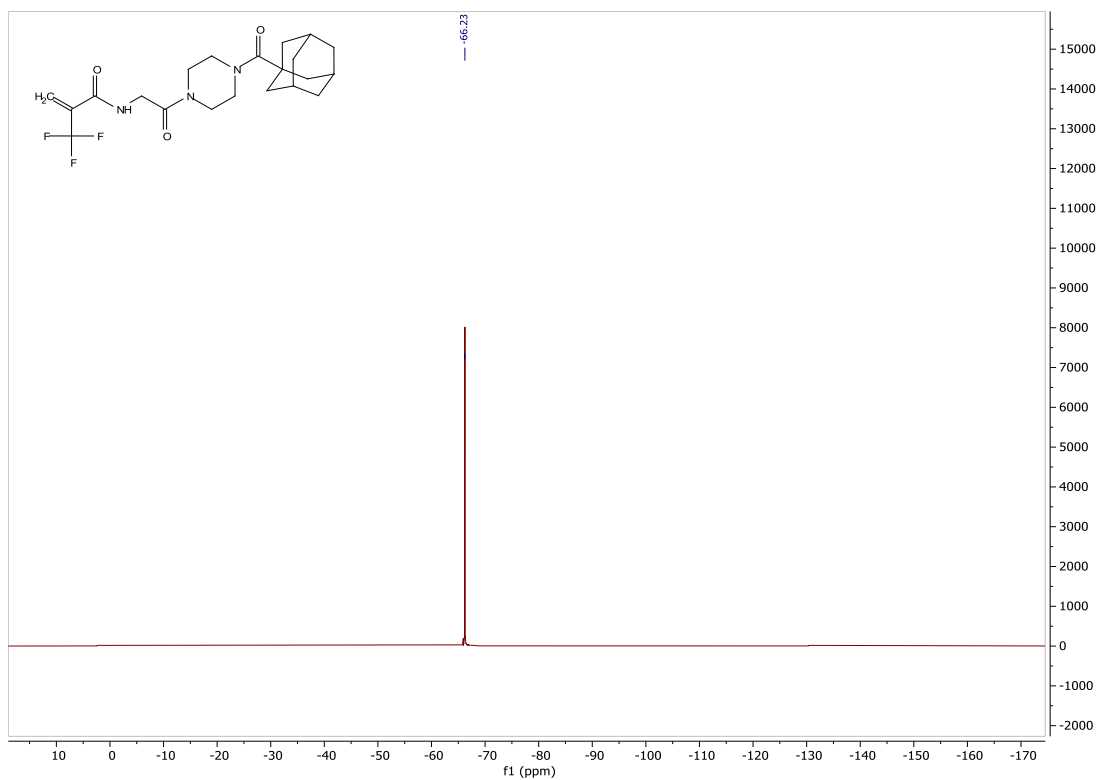
^1H NMR (300 MHz, CDCl_3) Spectra of Compound **7e**



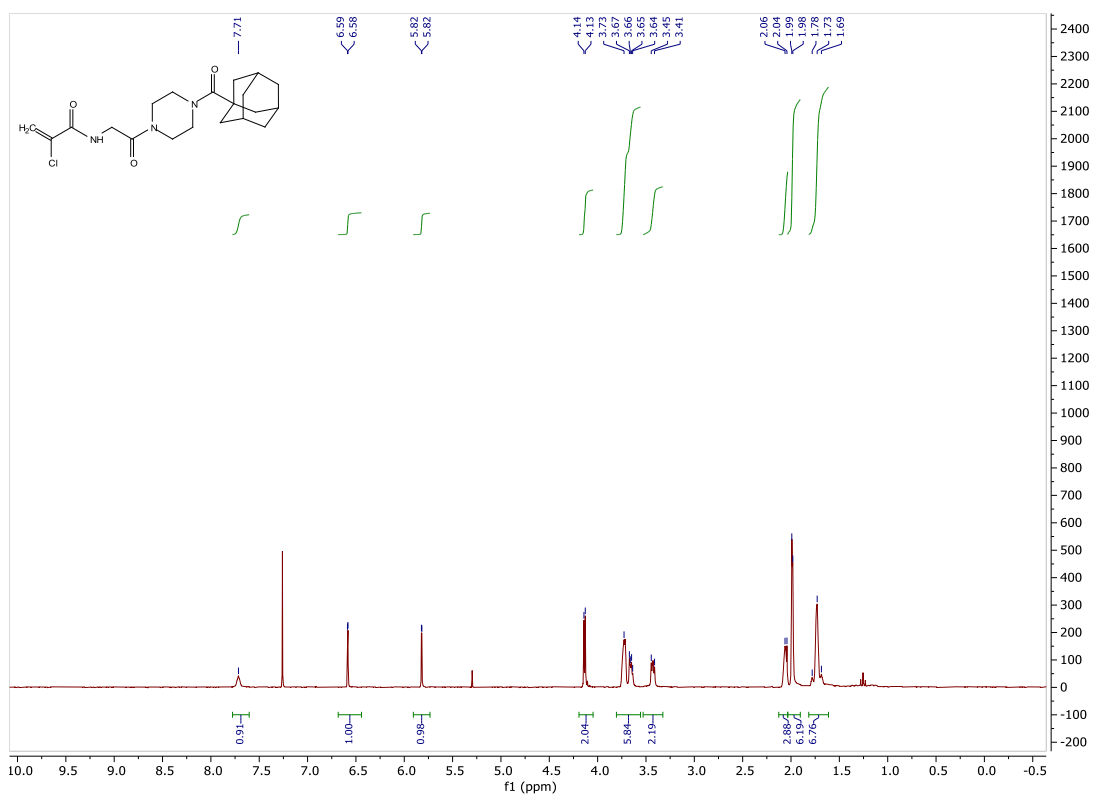
¹³C NMR (75 MHz, CD₃OD) Spectra of Compound **7e**



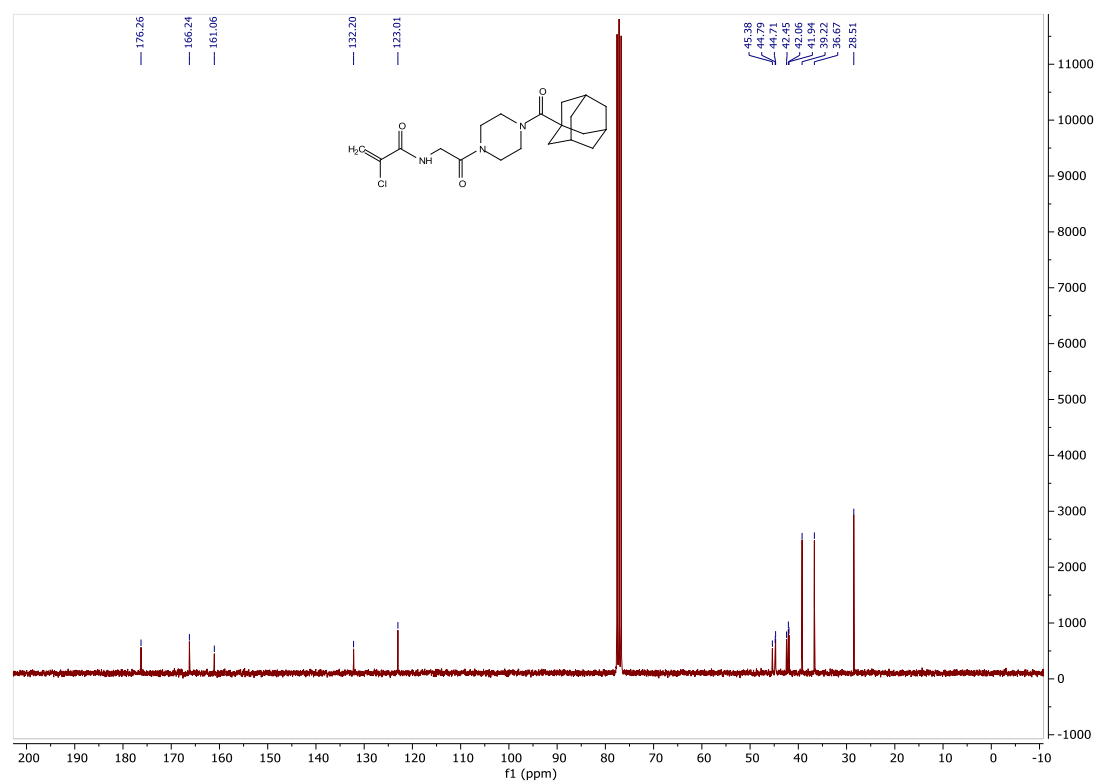
¹⁹F NMR (283 MHz, CDCl₃) Spectra of Compound **7e**



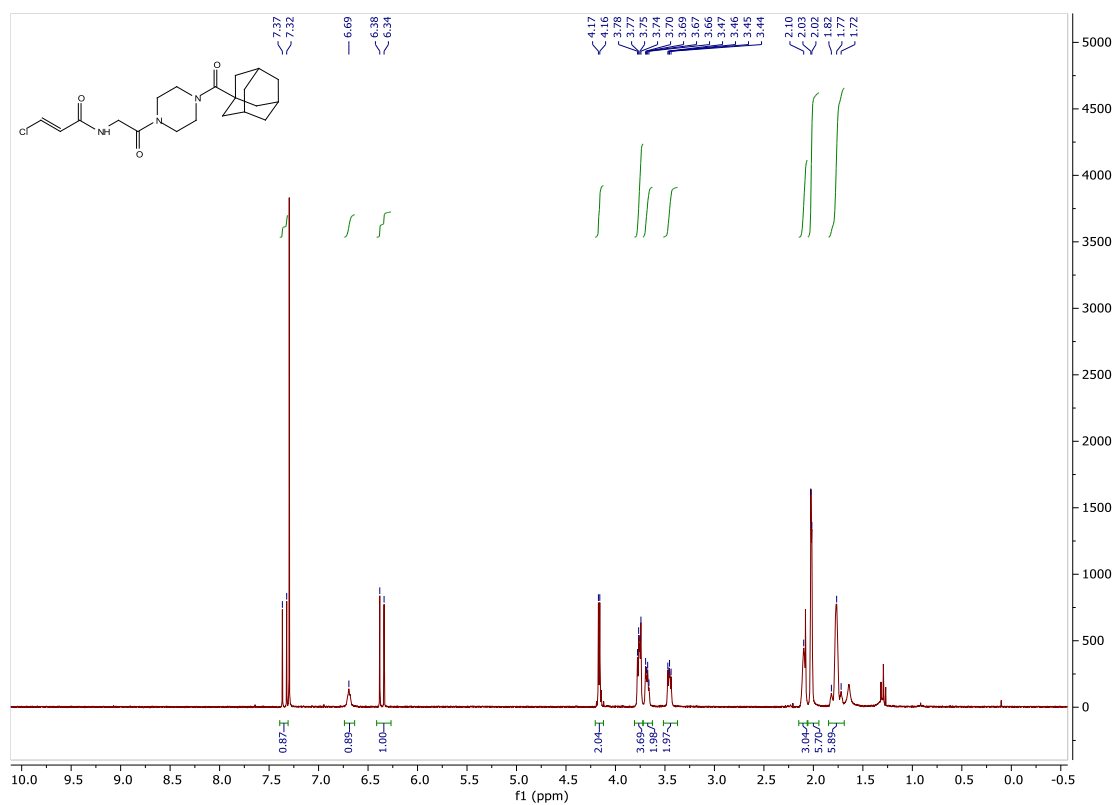
^1H NMR (300 MHz, CDCl_3) Spectra of Compound **7f**



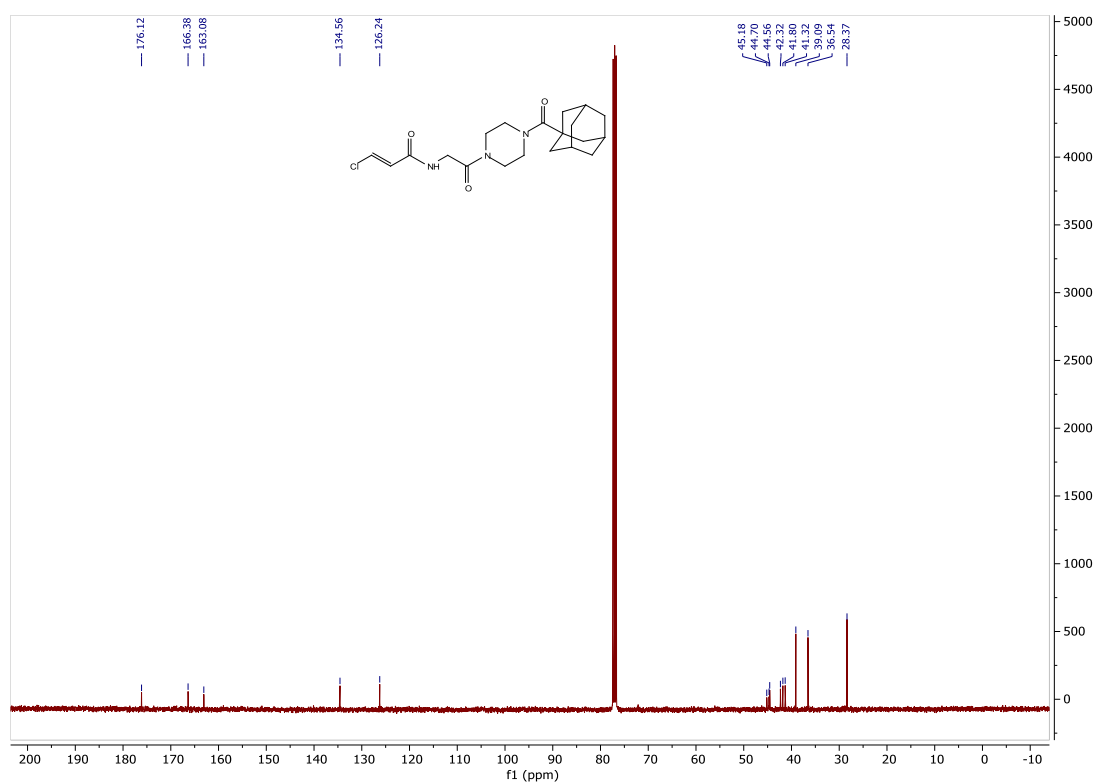
^{13}C NMR (75 MHz, CDCl_3) Spectra of Compound **7f**



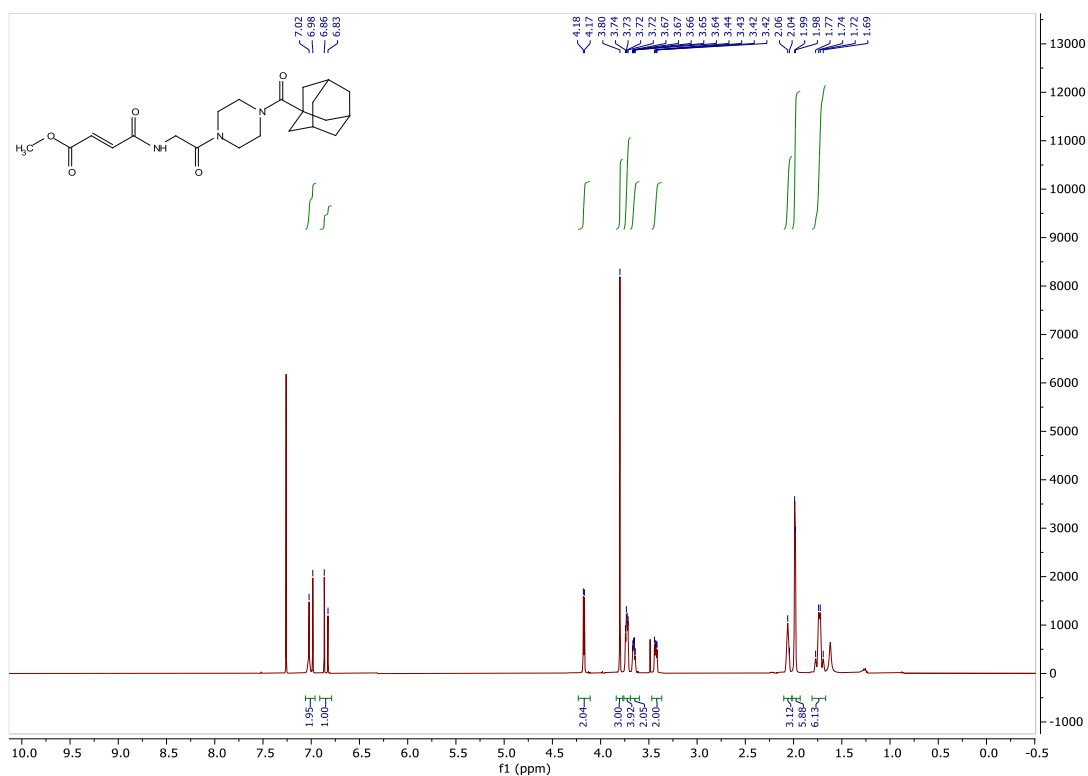
^1H NMR (300 MHz, CDCl_3) Spectra of Compound **7g**



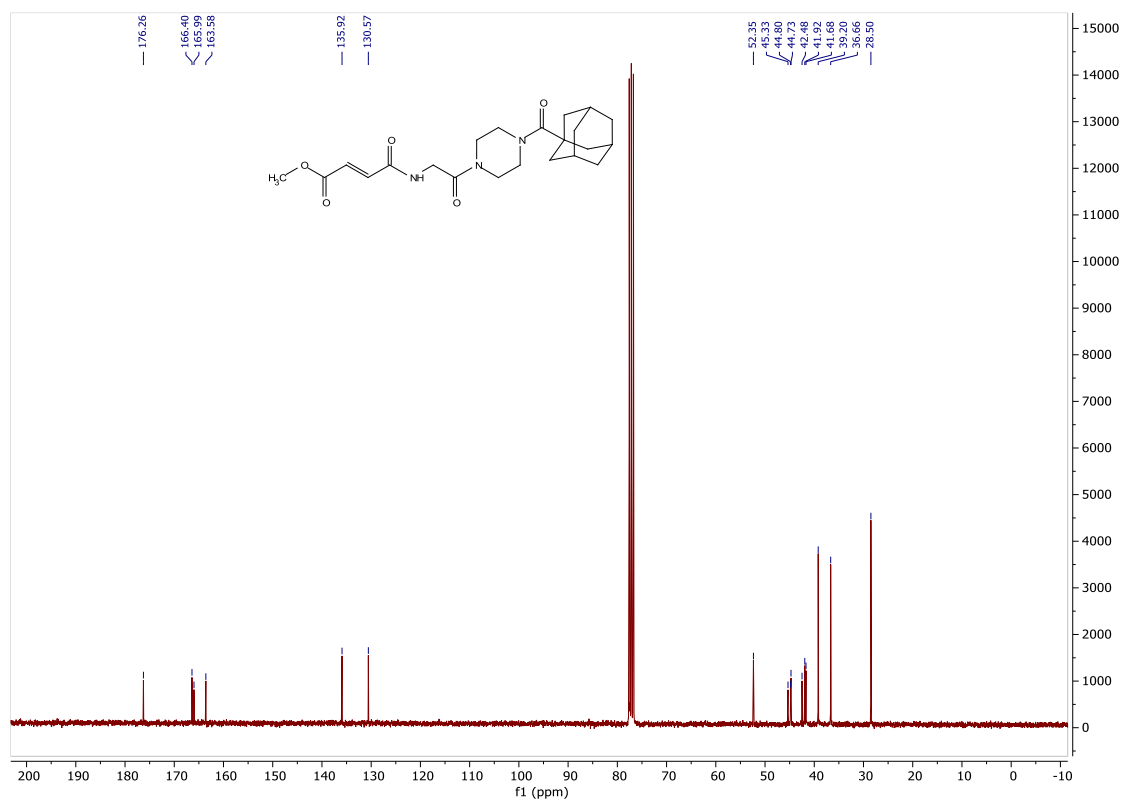
^{13}C NMR (75 MHz, CDCl_3) Spectra of Compound **7g**



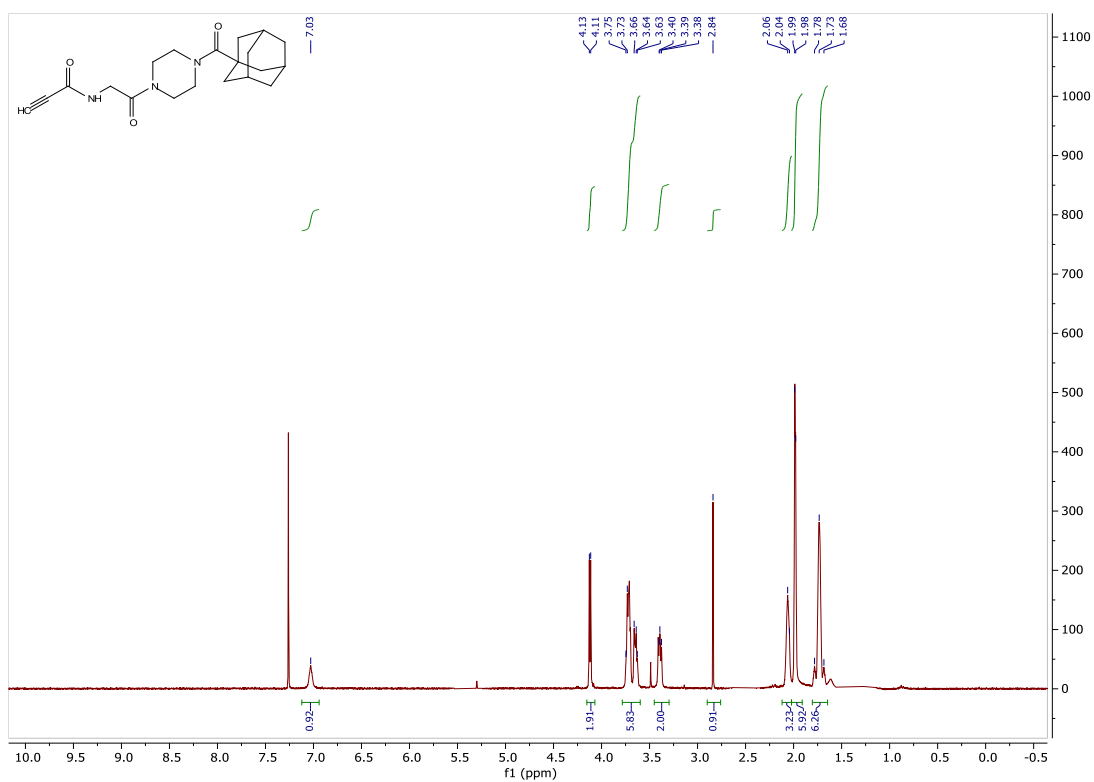
¹H NMR (300 MHz, CDCl₃) Spectra of Compound **7h**



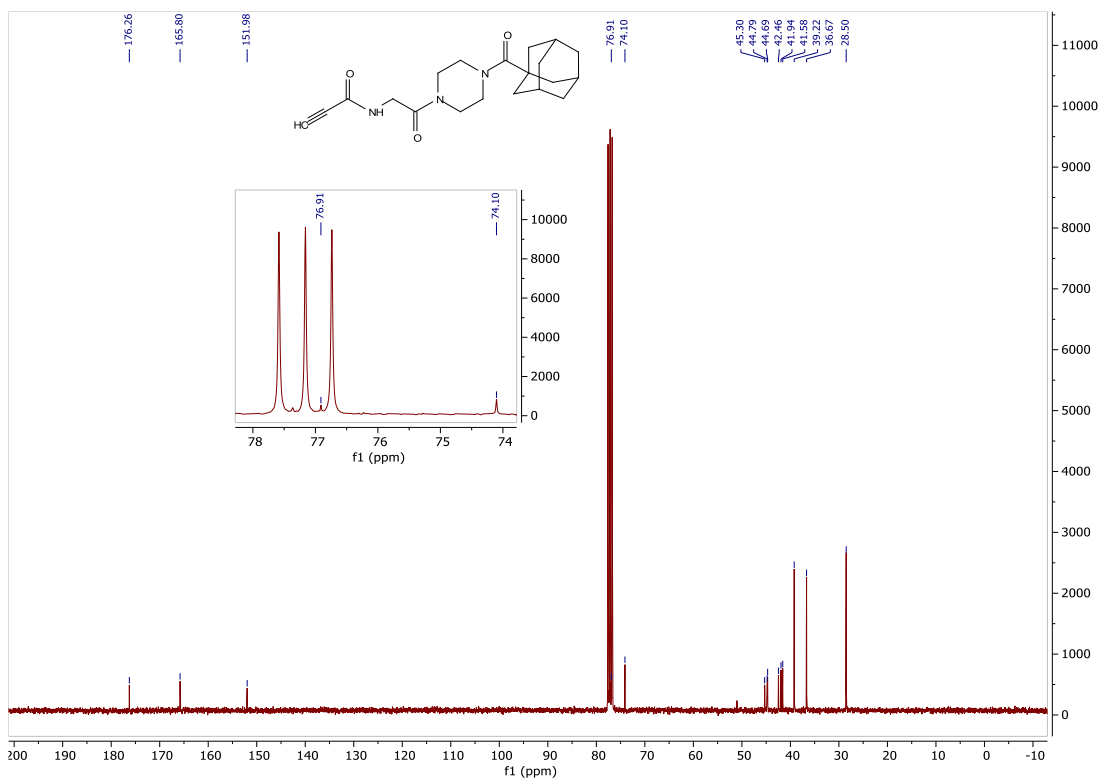
¹³C NMR (75 MHz, CDCl₃) Spectra of Compound **7h**



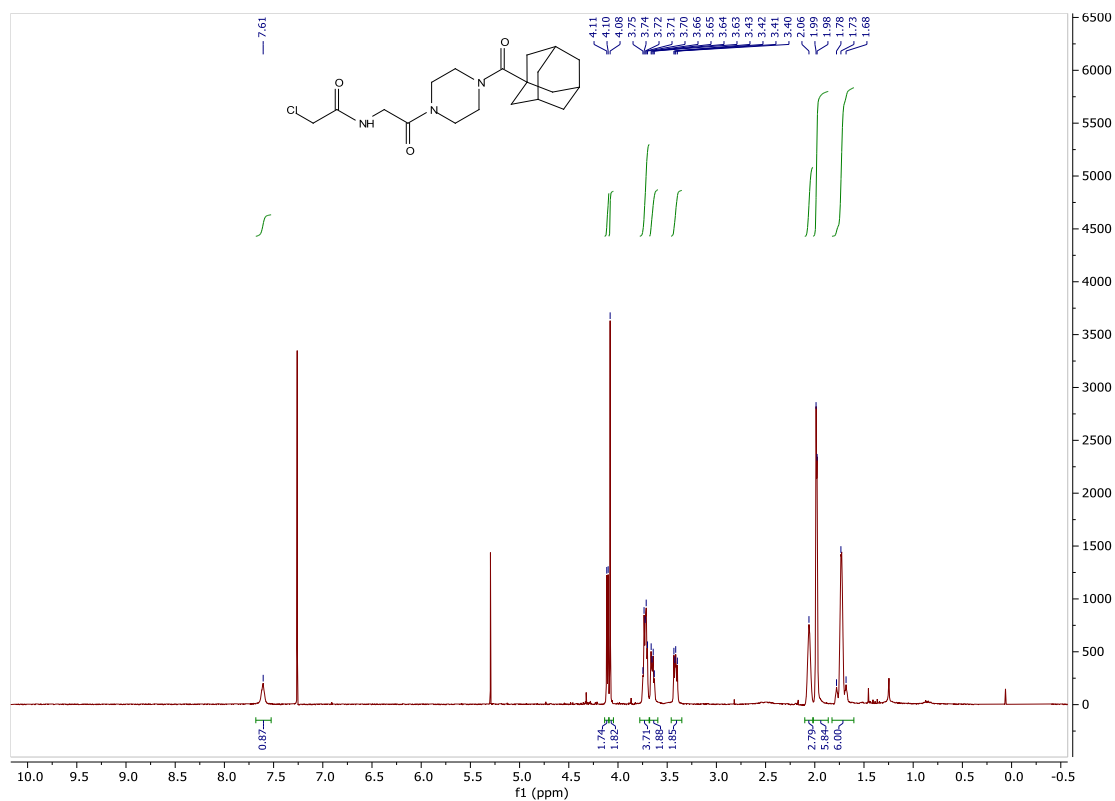
¹H NMR (300 MHz, CDCl₃) Spectra of Compound **7i**



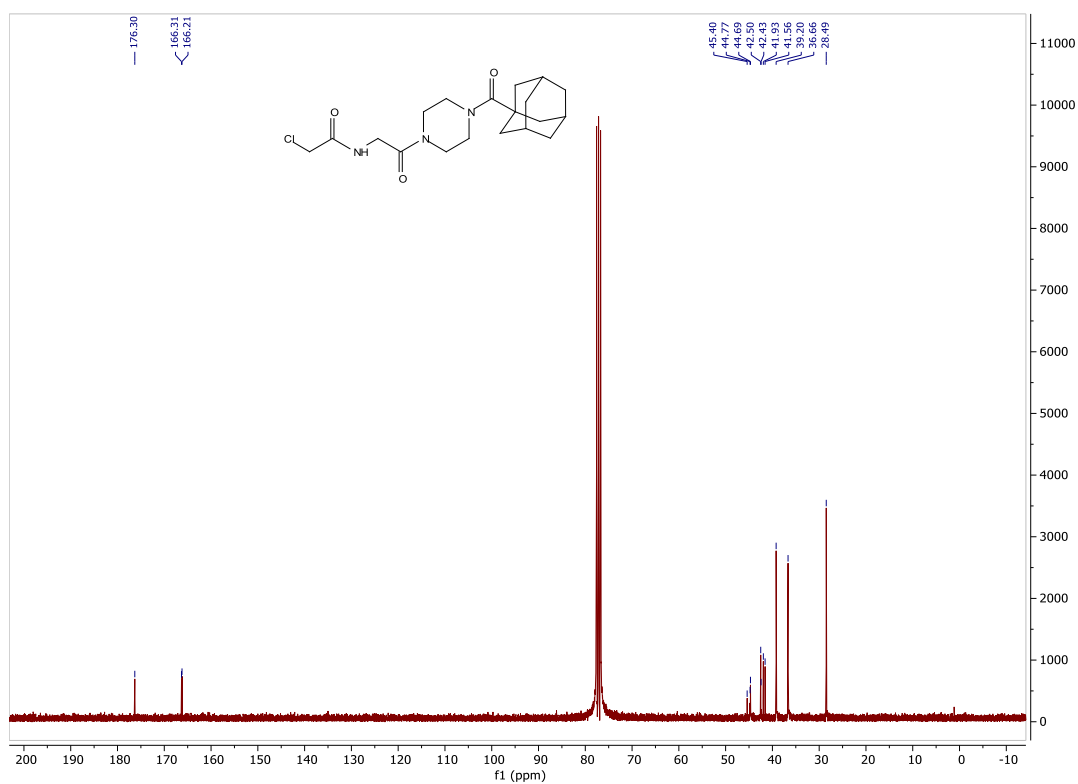
¹³C NMR (75 MHz, CDCl₃) Spectra of Compound **7i**



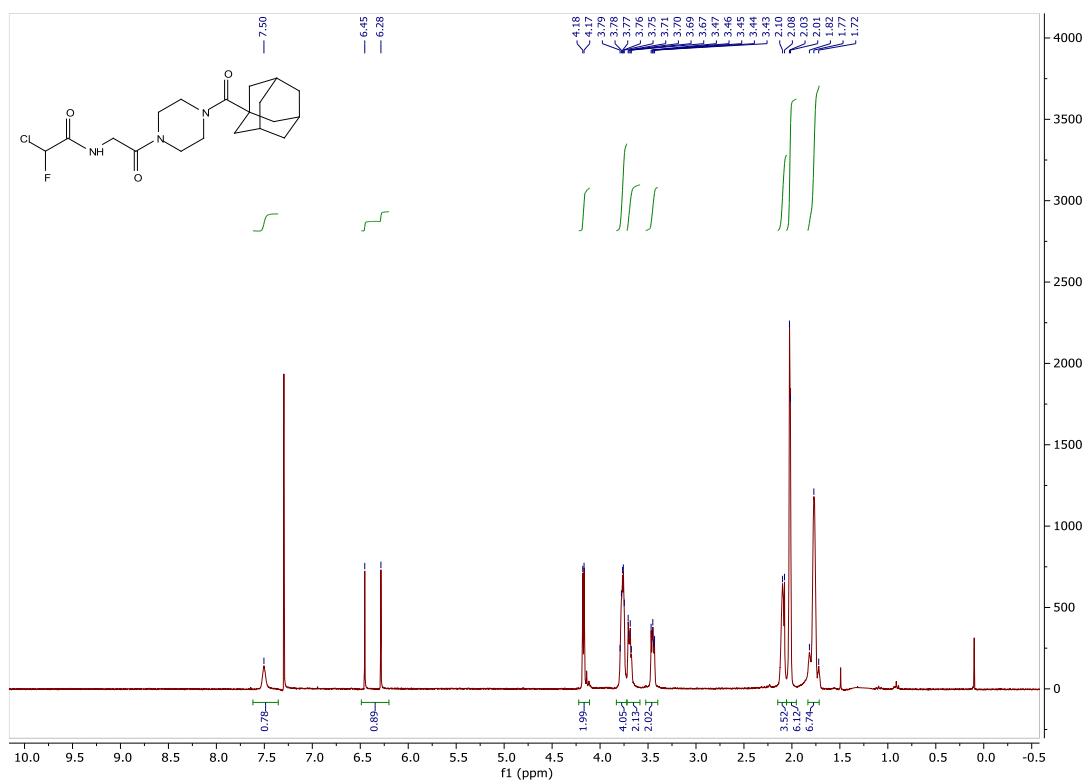
¹H NMR (300 MHz, CDCl₃) Spectra of Compound **7j**



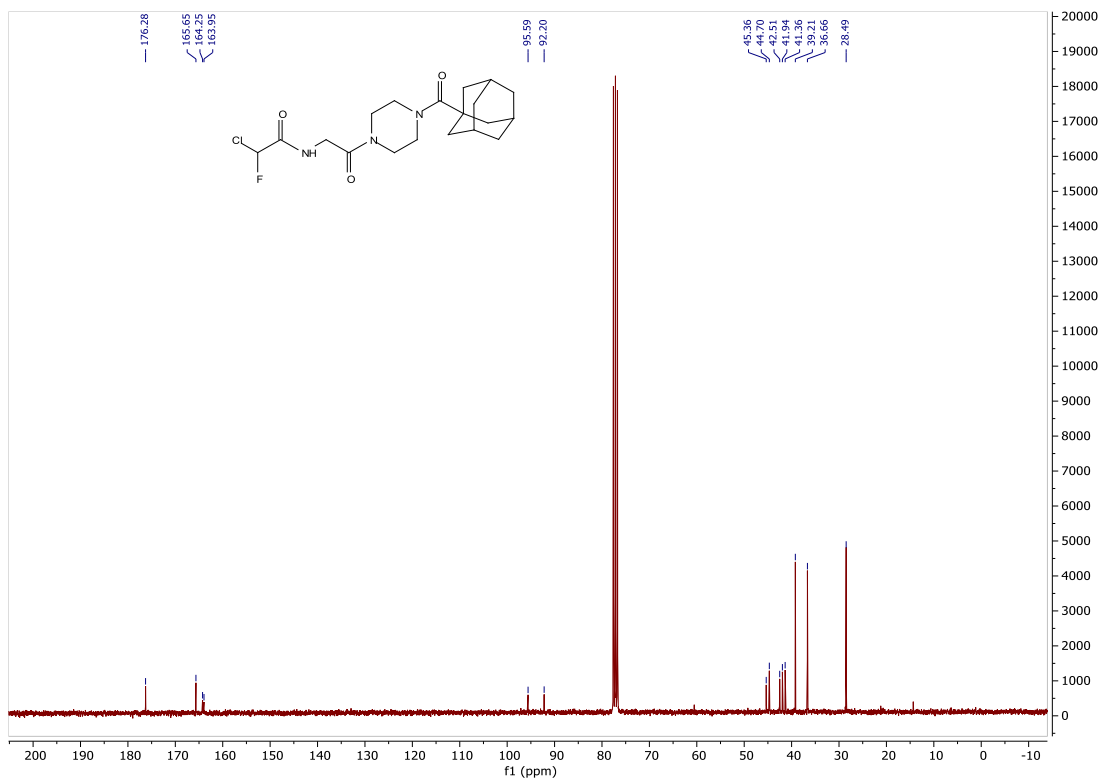
¹³C NMR (75 MHz, CDCl₃) Spectra of Compound **7j**



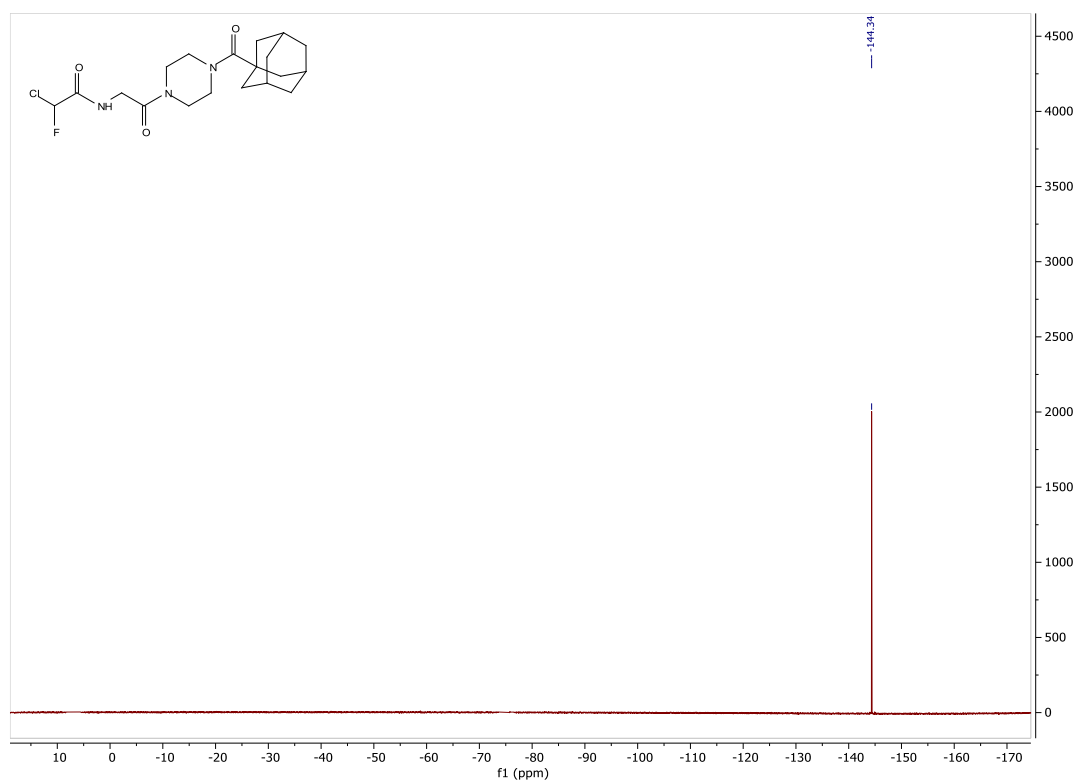
¹H NMR (300 MHz, CDCl₃) Spectra of Compound **7k**



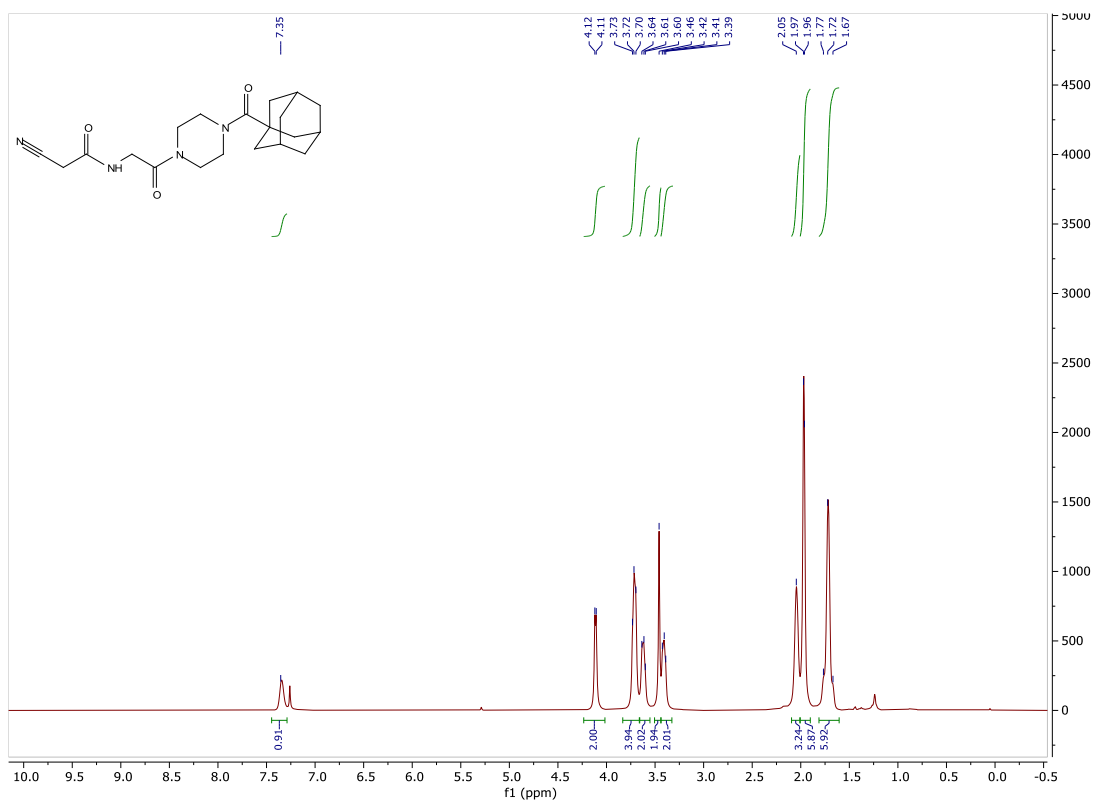
¹³C NMR (75 MHz, CDCl₃) Spectra of Compound **7k**



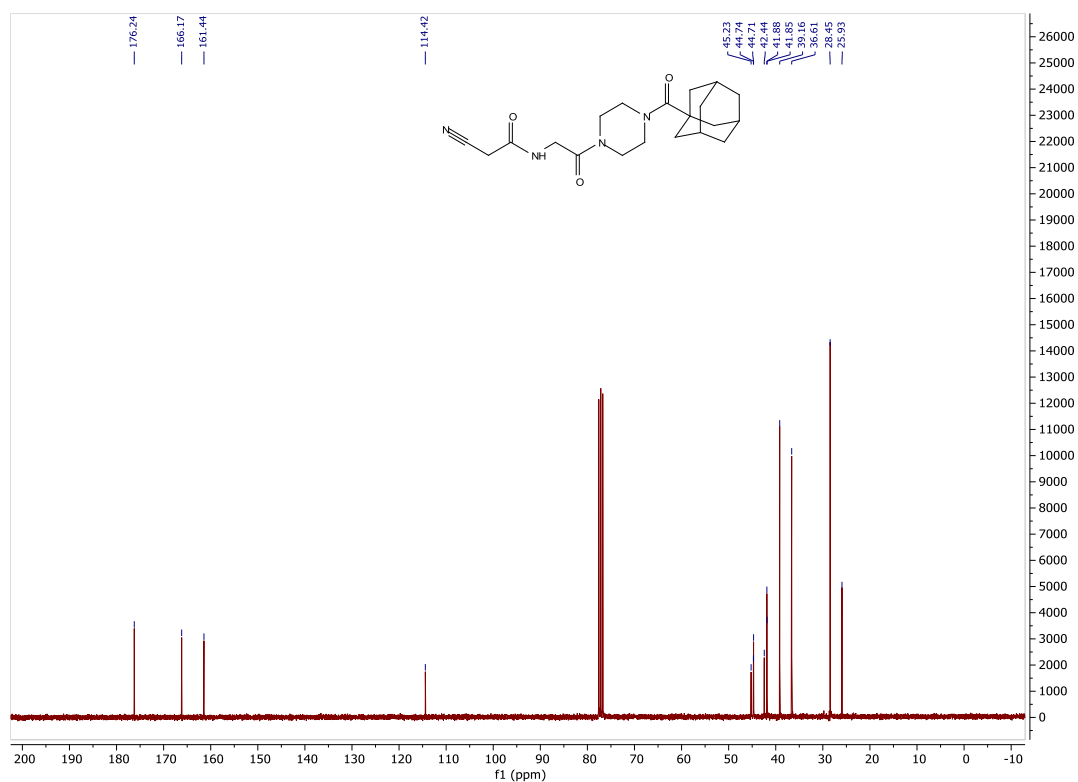
^{19}F NMR (283 MHz, CDCl_3) Spectra of Compound **7k**



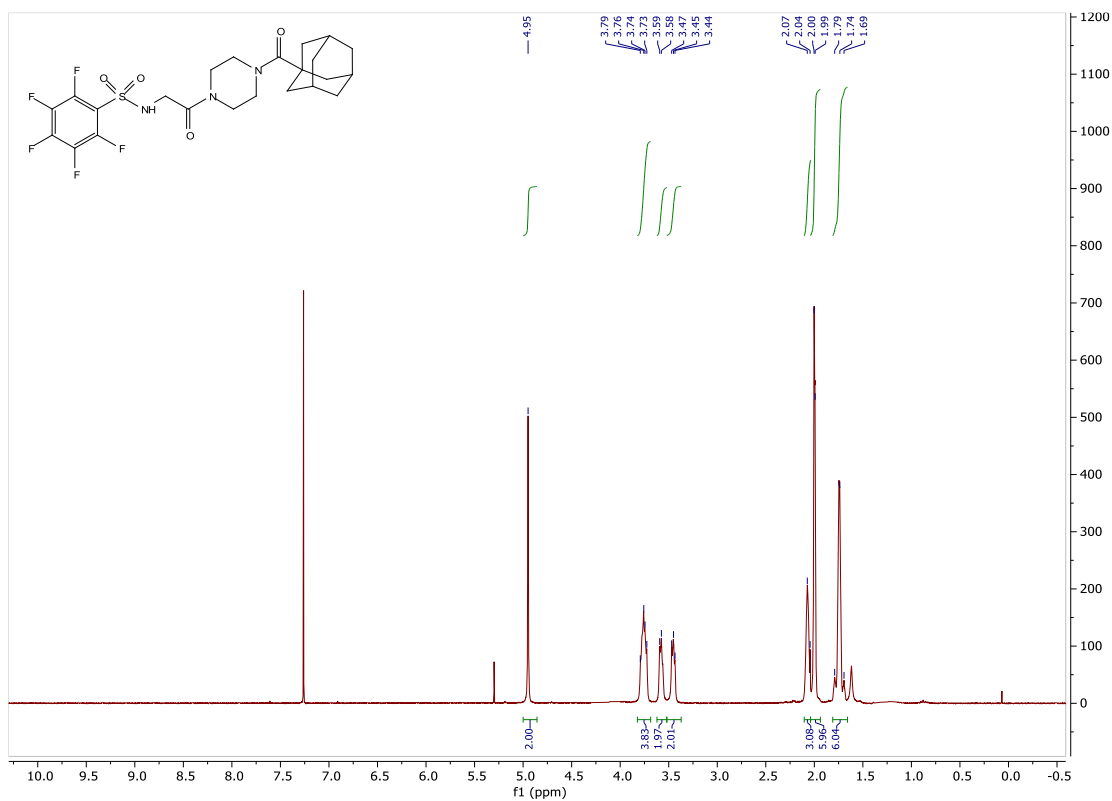
^1H NMR (300 MHz, CDCl_3) Spectra of Compound **7l**



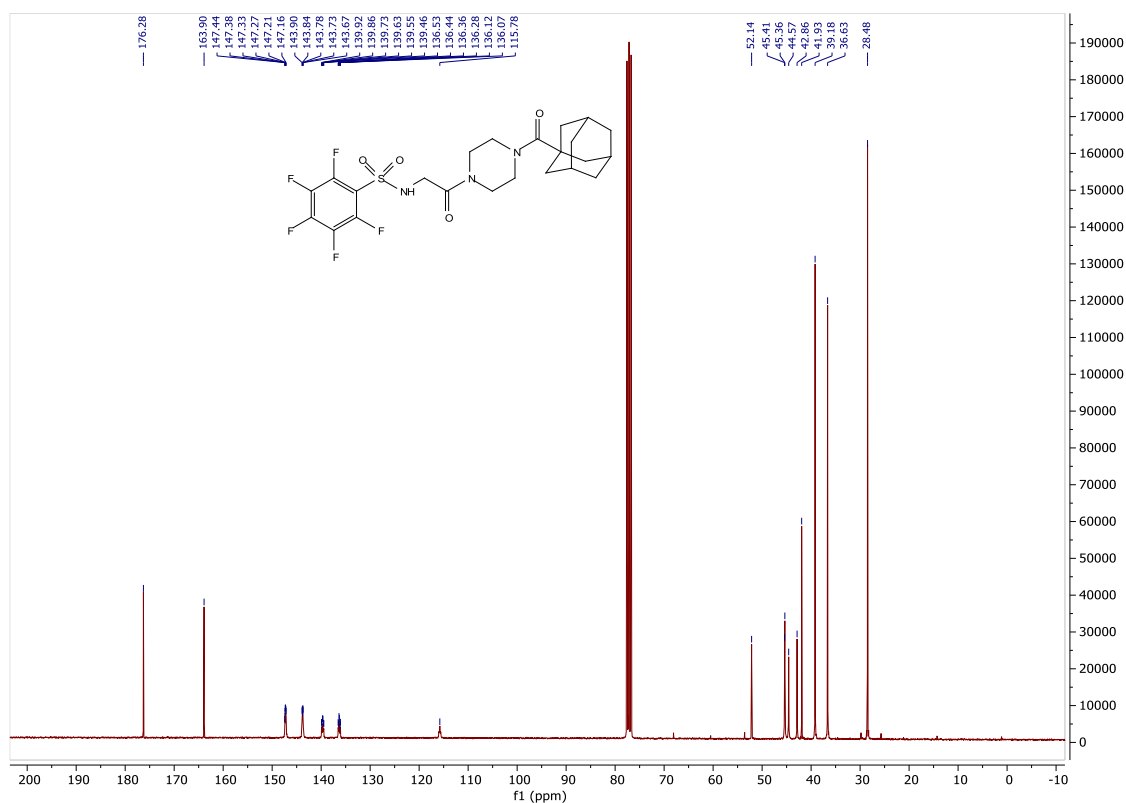
^{13}C NMR (75 MHz, CDCl_3) Spectra of Compound **7l**



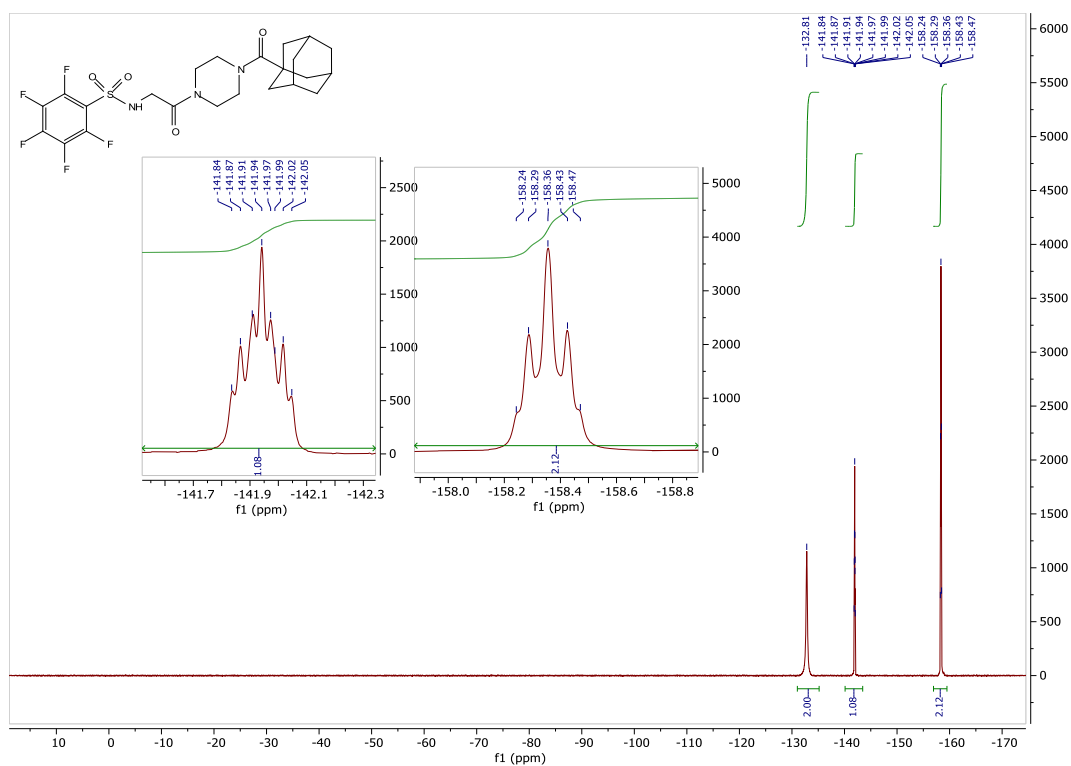
^1H NMR (300 MHz, CDCl_3) Spectra of Compound **7m**



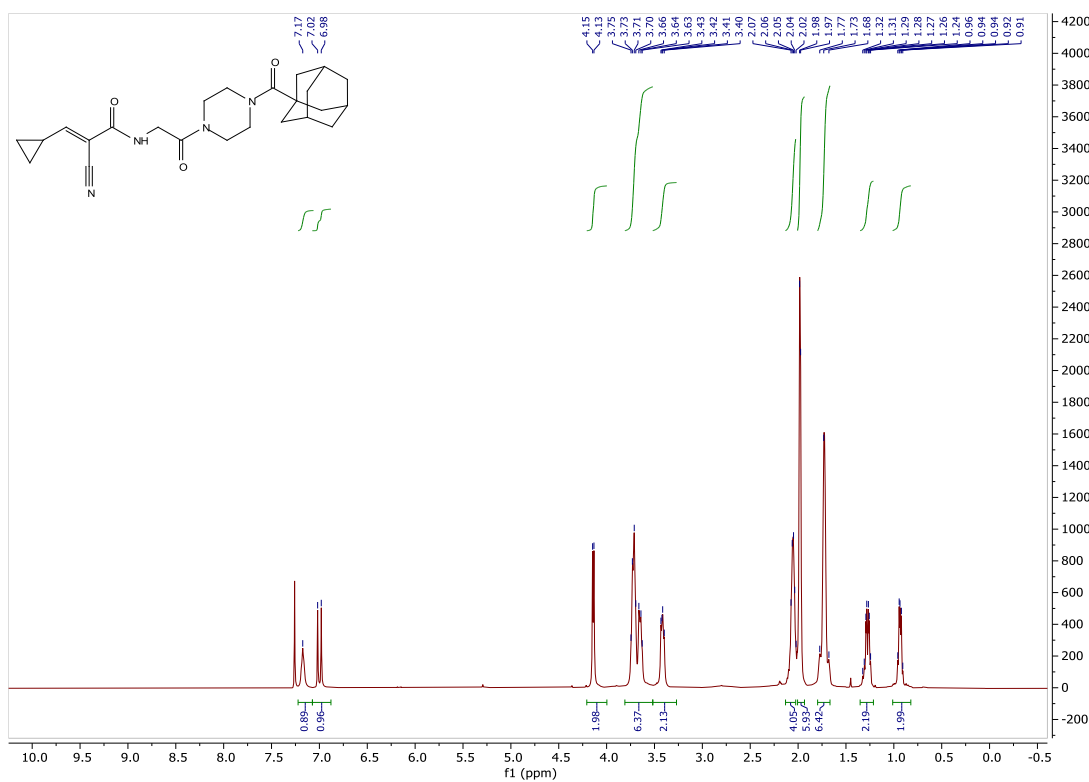
^{13}C NMR (75 MHz, CDCl_3) Spectra of Compound **7m**



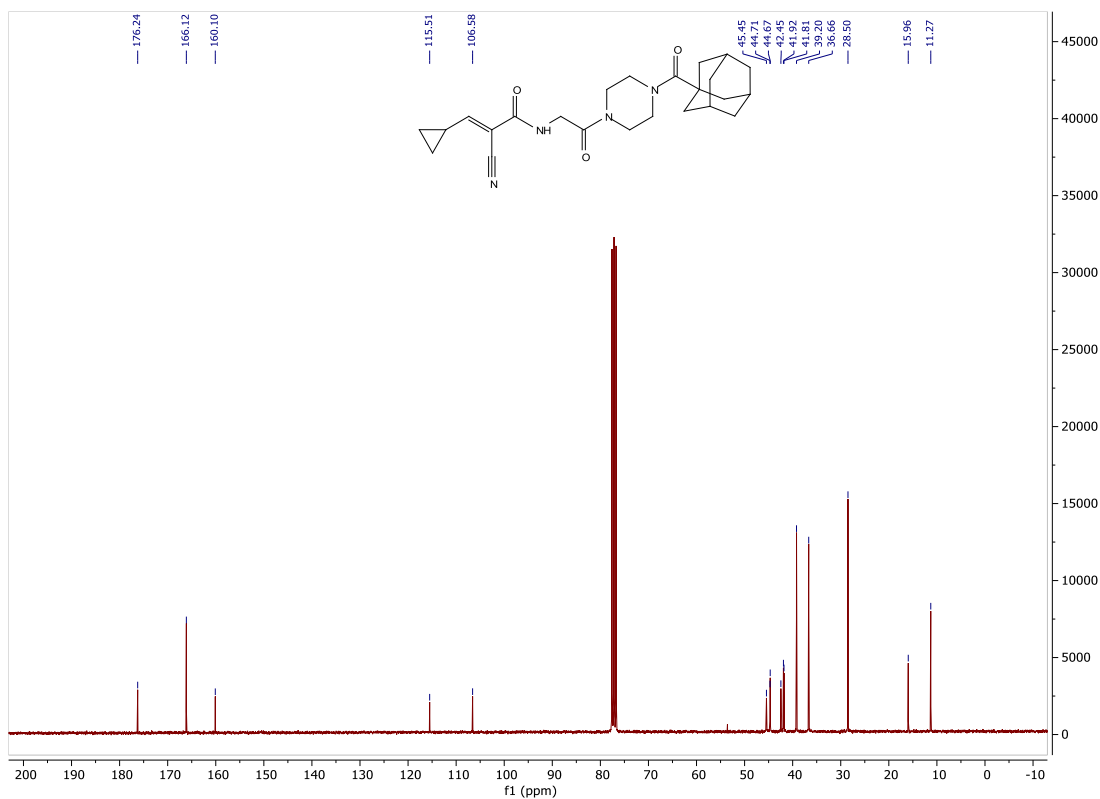
^{19}F NMR (283 MHz, CDCl_3) Spectra of Compound **7m**



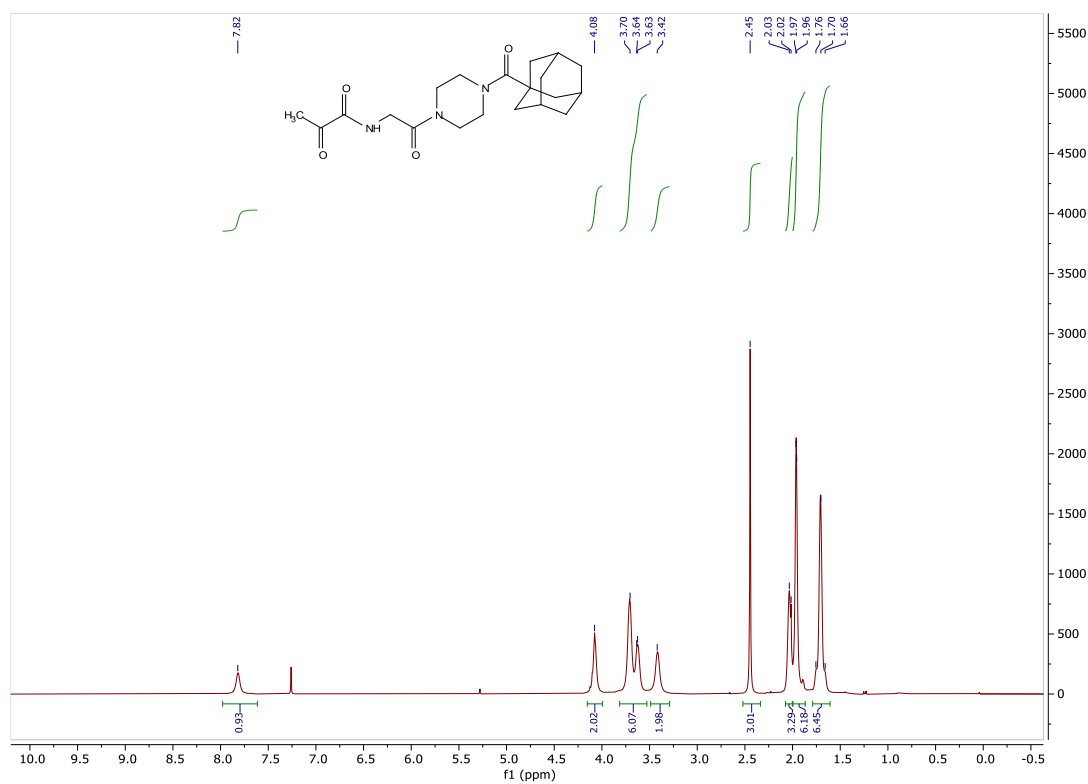
^1H NMR (300 MHz, CDCl_3) Spectra of Compound **7n**



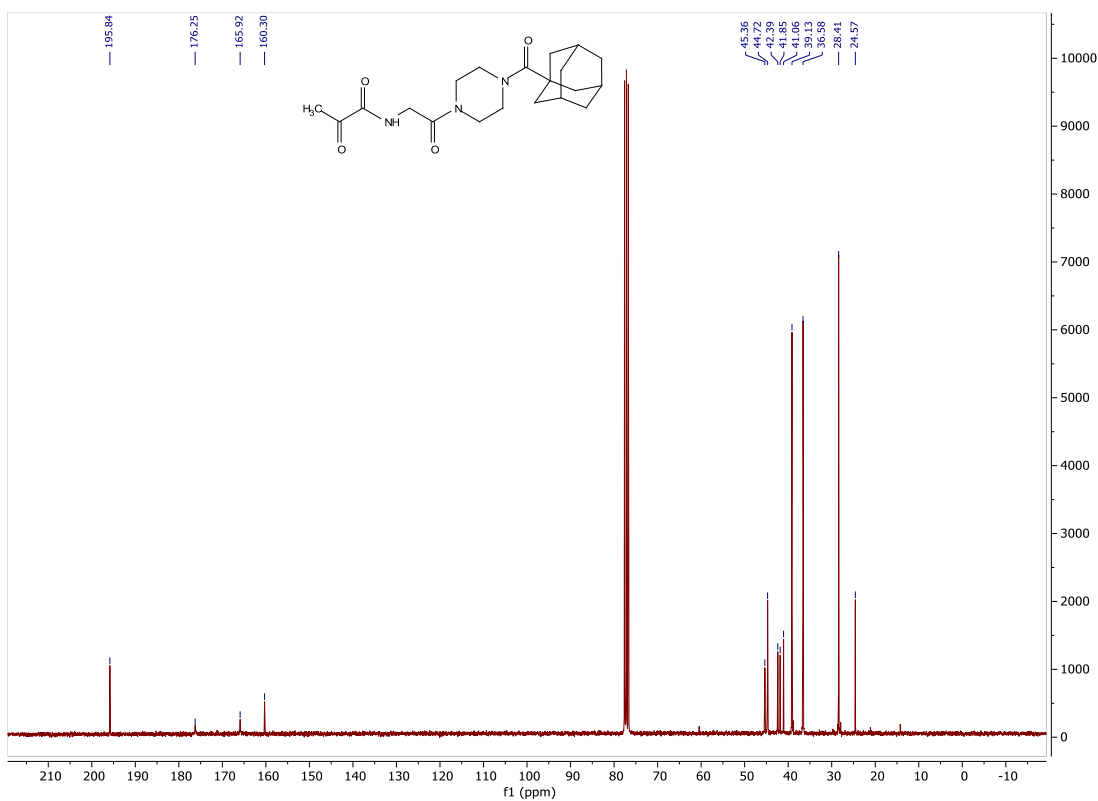
^{13}C NMR (75 MHz, CDCl_3) Spectra of Compound **7n**



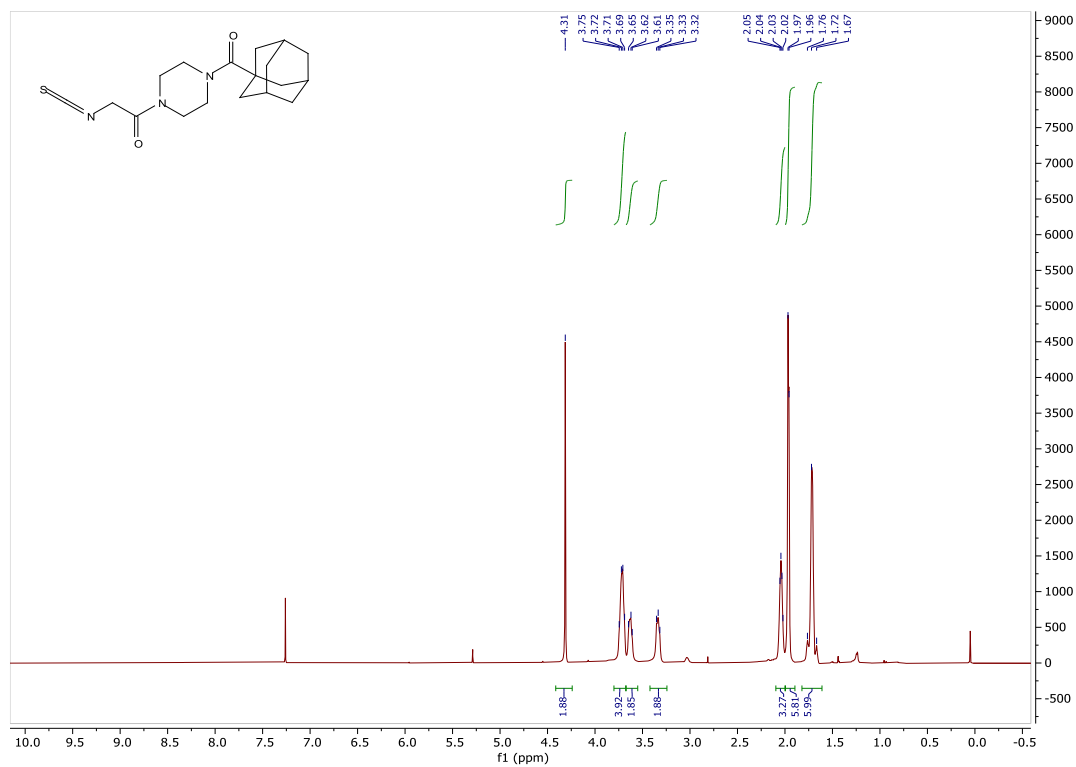
¹H NMR (300 MHz, CDCl₃) Spectra of Compound **7o**



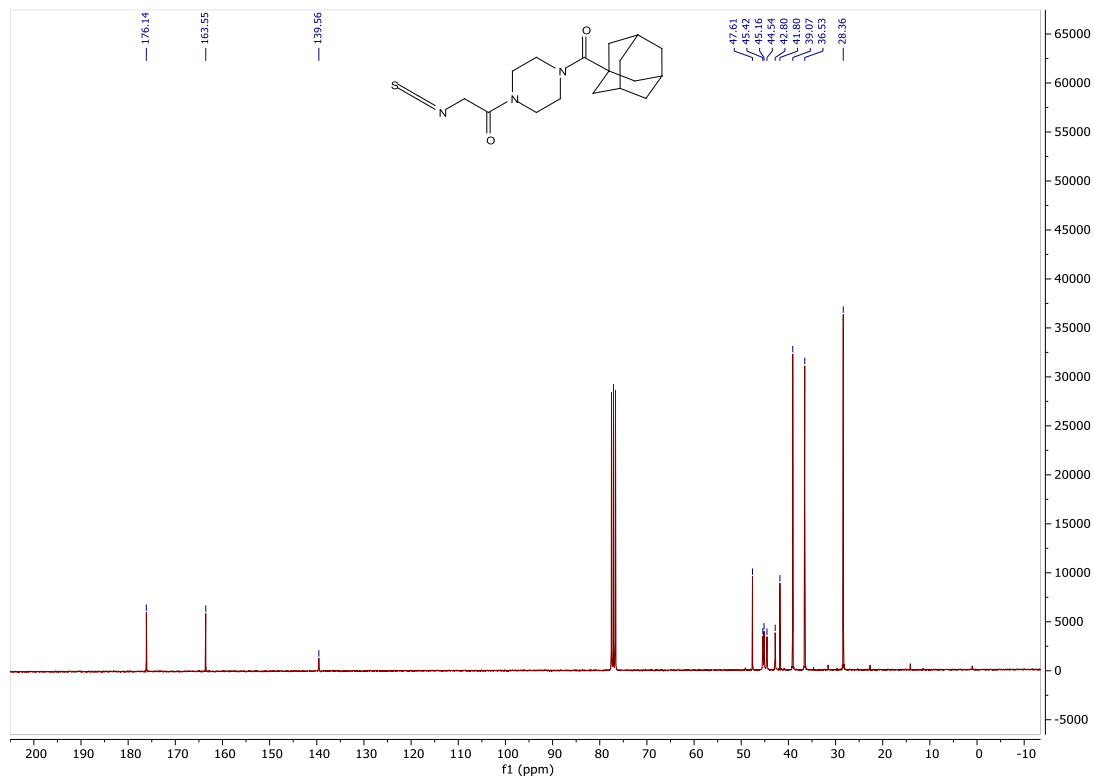
¹³C NMR (75 MHz, CDCl₃) Spectra of Compound **7o**



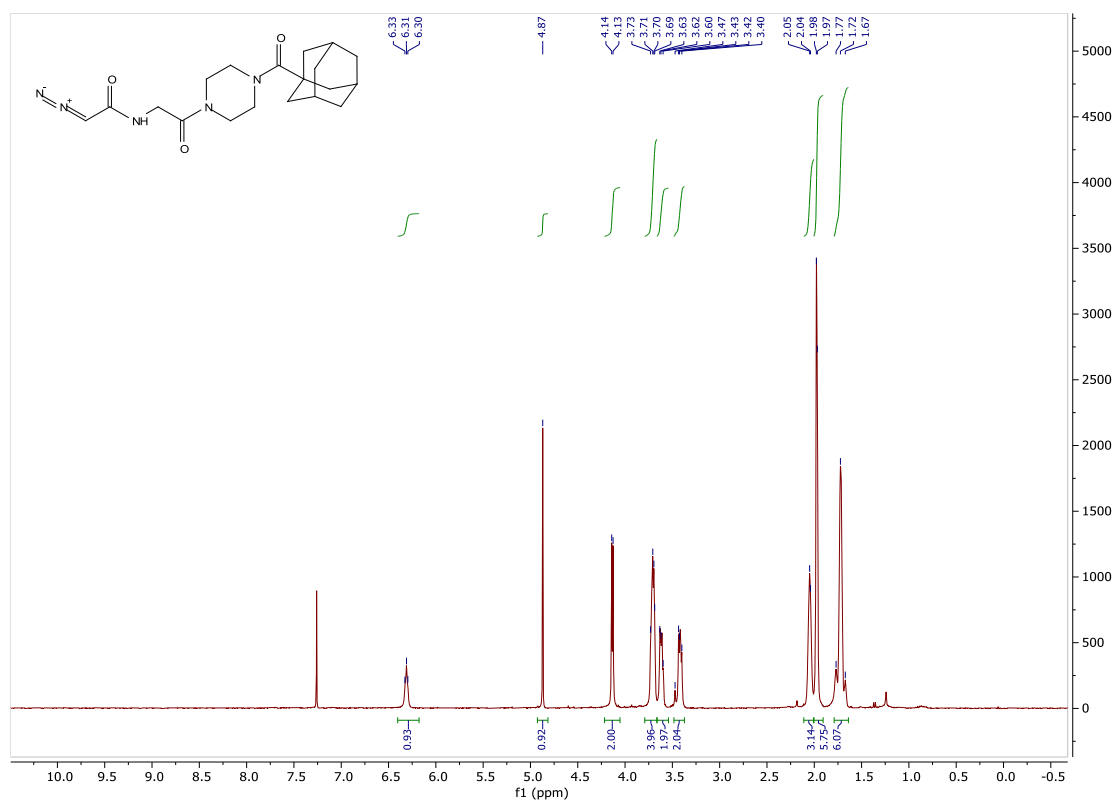
¹H NMR (300 MHz, CDCl₃) Spectra of Compound **7p**



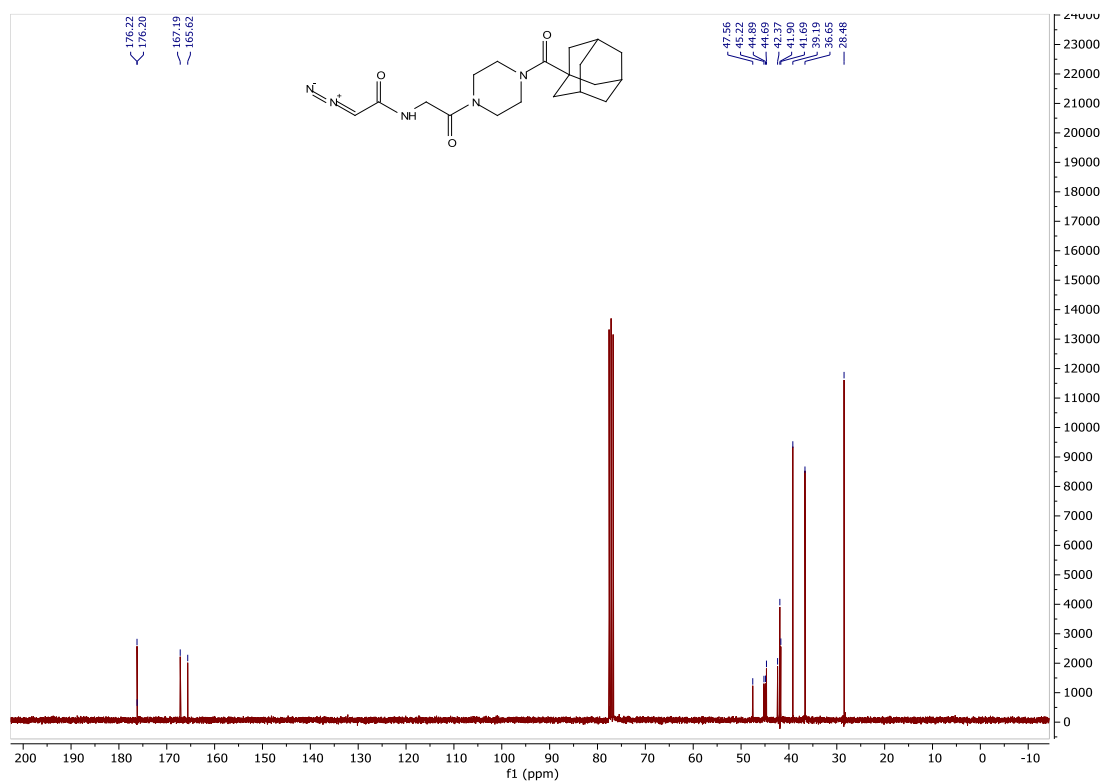
¹³C NMR (75 MHz, CDCl₃) Spectra of Compound **7p**



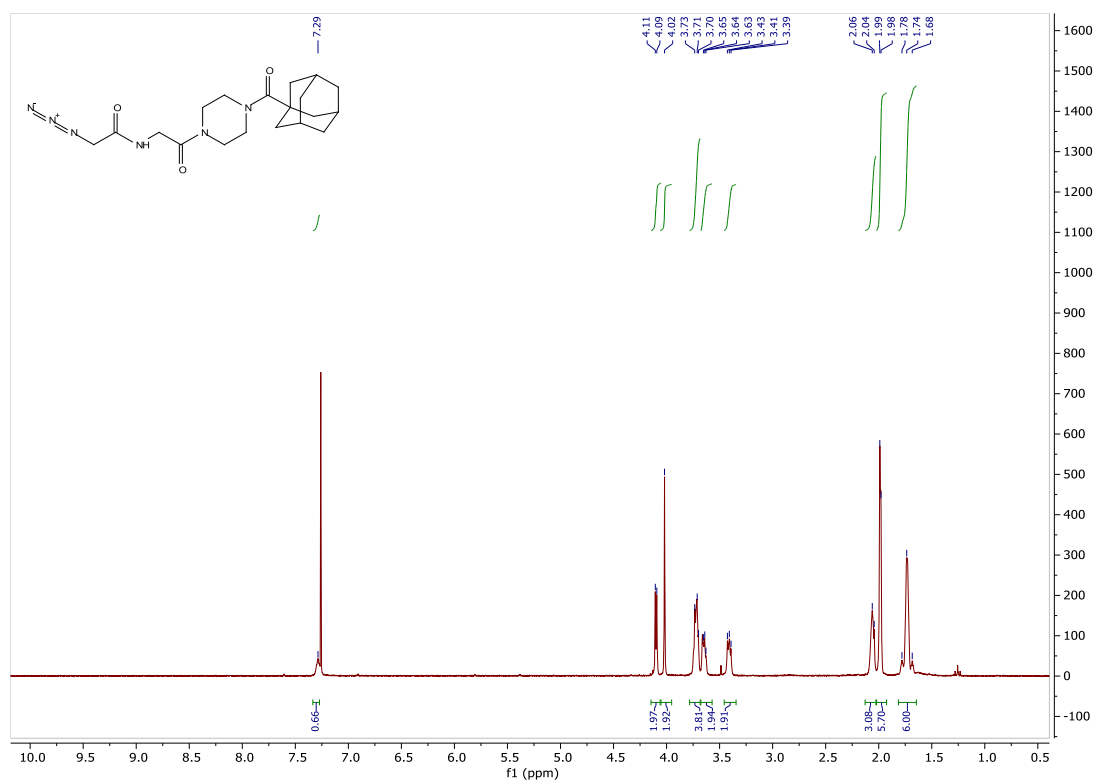
¹H NMR (300 MHz, CDCl₃) Spectra of Compound **7q**



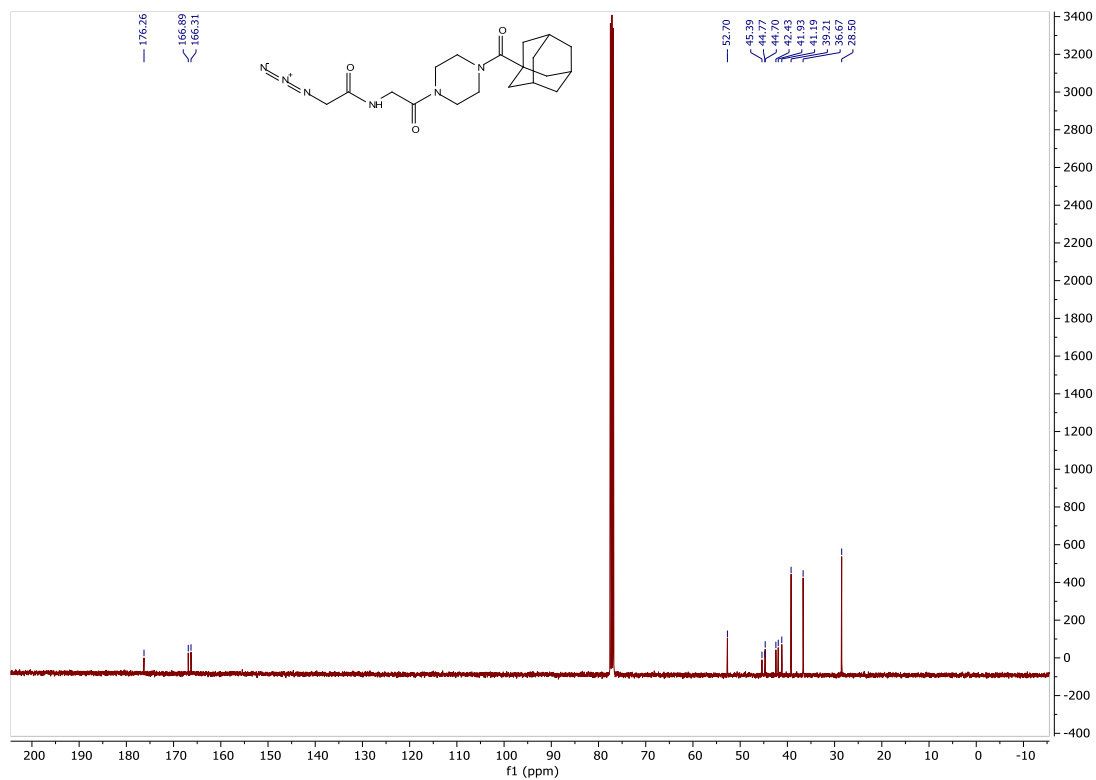
¹³C NMR (75 MHz, CDCl₃) Spectra of Compound **7q**



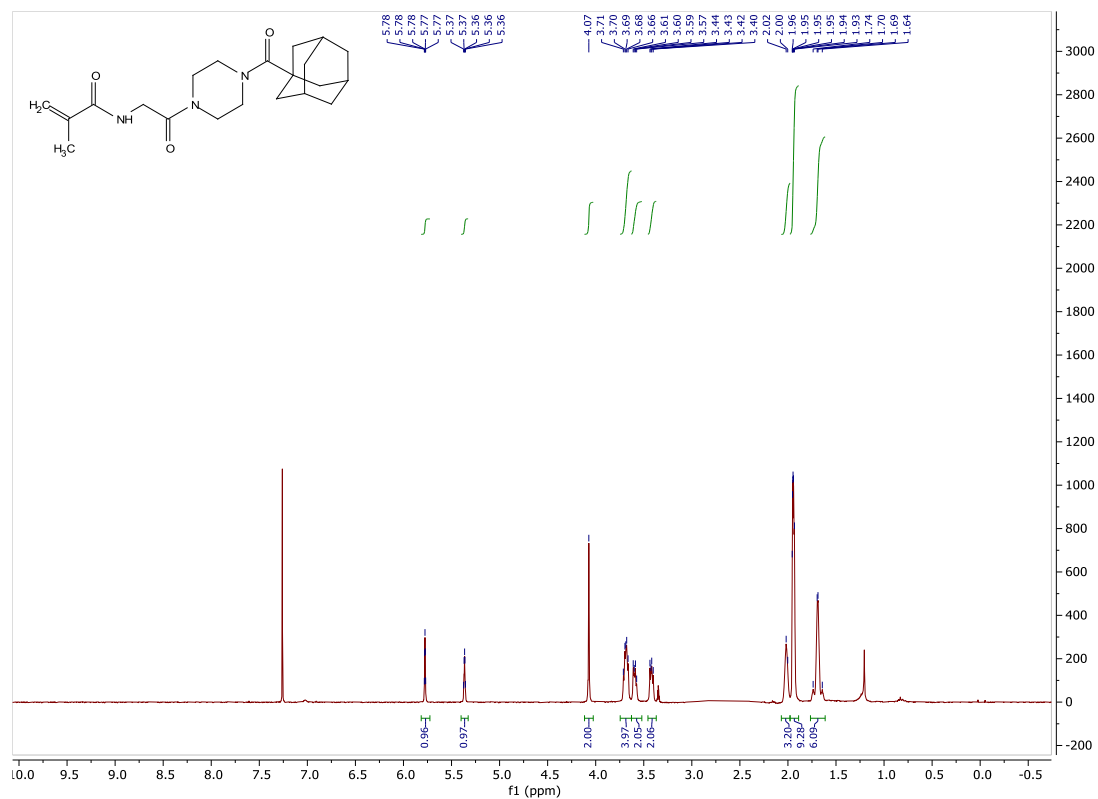
¹H NMR (300 MHz, CDCl₃) Spectra of Compound **I5**



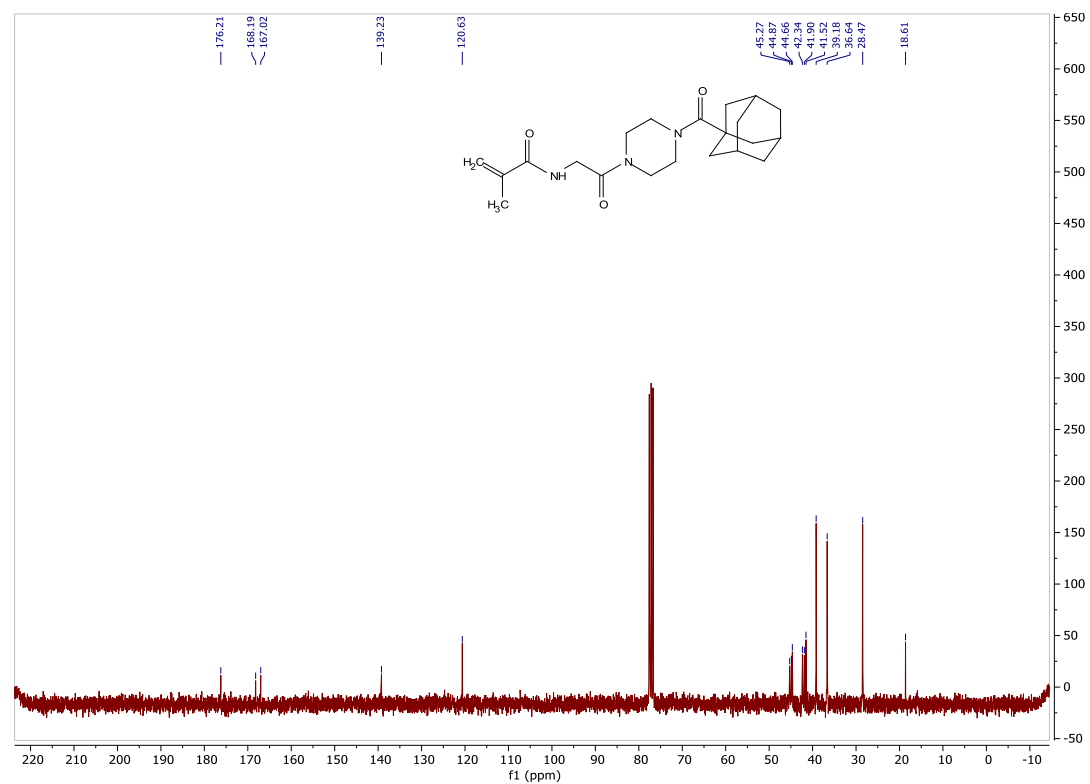
¹³C NMR (75 MHz, CDCl₃) Spectra of Compound **I5**



¹H NMR (300 MHz, CDCl₃) Spectra of Compound **7r**



¹³C NMR (75 MHz, CDCl₃) Spectra of Compound **7r**



Chapter 3 – Discovery of an Internal Alkyne Warhead Scaffold for Irreversible hTG2 Inhibition

3.1. Preamble

Consistent with prior literature on hTG2 inhibition, the results presented in Chapter 2 further support the preference for relatively small electrophilic warheads, even within smaller scaffolds. This trend is generally attributed to steric hindrance within the hTG2 active site, where the narrow ‘tunnel’ leading to Cys277 limits the accommodation of sterically bulky electrophiles.

However, earlier work in the Keillor lab revealed an observation that challenges this trend. An internal keto-alkyne warhead-containing inhibitor, **KA22b**, originally reported by PhD student Kim Apperley, exhibited strong inhibition of hTG2 despite incorporating a more sterically demanding electrophile. Although initially characterized as a reversible inhibitor, a subsequent correction noted in the final stages of that work suggested that **KA22b** instead operates through an irreversible mechanism.

KA22b is one of very few potent and fully non-peptidic irreversible hTG2 inhibitors. Since most hTG2 inhibitors are peptidomimetic in nature, often leading to poor drug-like properties particularly for intracellular hTG2 targeting, this scaffold presents unique opportunities for drug development. Furthermore, TCI architecture with a warhead positioned in the middle of the scaffold is rare in the field of covalent inhibition, which may offer improved selectivity and expanded chemical/reactivity space for the design of TCIs.

Motivated by this finding, the work described in this chapter investigates the structure-activity relationships of this scaffold as a novel class of irreversible hTG2 inhibitors, with emphasis placed on improvement of drug-like properties and fine-tuning of reactivity of this unique warhead class.

3.2. Publication Information and Acknowledgements

This chapter has been directly reproduced from the following reference: Mader, L. K.; Maunick N.; Borean, J. E.; Keillor, J. W., *RSC Medicinal Chemistry* **2025**, 16 (12), 6228-6261 (<https://doi.org/10.1039/D5MD00777A>) with permission from the Royal Society of Chemistry.

3.3. Author Contributions

L. K. M. – Conceptualization, synthesis, characterization, inhibition assays, intrinsic reactivity assays, GTP binding assay, molecular modelling, writing original draft.

N. M. – Synthesis, characterization, and inhibition kinetics of compounds **36**, **38**, and **40**.

J. E. B. – Synthesis, characterization, and inhibition kinetics of compounds **8R** and **18**.

J. W. K. – Conceptualization, supervision, funding, editing and reviewing.

3.4. Correction to Published Version

On **page 122** of this thesis (page 6240 of the manuscript) in **Table 5** the rest of the structure of compound **13** is a 1-(4-cyanobenzyl)triazolyl group (i.e., the structure given in **Table 2** on page 118 of this thesis, and page 6236 of the manuscript, is the correct structure).

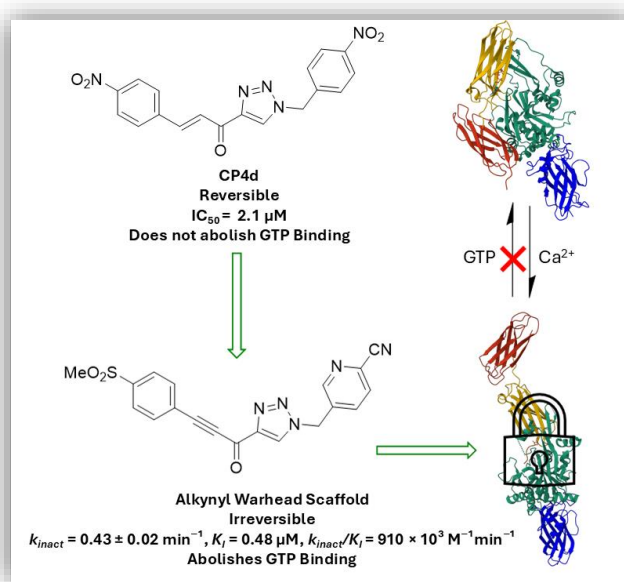


Figure 3.1. Graphical summary of the work describing this chapter.

RESEARCH ARTICLE

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Discovery of an internal alkyne warhead scaffold for irreversible hTG2 inhibition

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Jessica E. Borean  and Jeffrey W. Keillor *

Human tissue transglutaminase (hTG2) is a multifunctional enzyme with both protein cross-linking and G-protein activity. Dysregulation of these functions has been implicated in diseases such as celiac disease and cancer, prompting the development of hTG2 inhibitors, many of which act covalently via a pendant electrophilic warhead. Most small molecule hTG2 inhibitors to date feature terminal, sterically minimal warheads, based on the assumption that bulkier electrophiles impair binding and reactivity. Here, we report structure–activity relationships (SAR) of a novel internal alkynyl warhead scaffold for irreversible inhibition of hTG2. This series includes one of the most potent non-peptidic hTG2 inhibitors reported to date. We demonstrate that this scaffold not only inhibits transamidase activity but also abolishes GTP binding, while exhibiting excellent isozyme selectivity. In addition, we investigate the tunability and stability of this warhead, providing insights into its broader applicability. Through detailed kinetic analysis, this study establishes a new scaffold for irreversible hTG2 inhibition and expands the design principles for covalent warheads beyond traditional terminal systems.

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Introduction

Human tissue transglutaminase (hTG2) is one of nine enzymes in the transglutaminase (TGase) family, characterized by their ability to catalyze protein cross-linking in the extracellular matrix via a transamidation reaction involving a catalytic cysteine (Cys277).^{1,2} Uniquely, hTG2 is multifunctional: in addition to transamidation, it can bind guanosine triphosphate (GTP) and act as a G-protein in intracellular signalling. These two functions are mutually exclusive; each is associated with a distinct conformational state.^{1,3,4} In the extracellular space, high calcium concentrations favour an extended “open” conformation that exposes the transamidase active site but abolishes the GTP binding pocket. Conversely, under intracellular conditions, low calcium concentrations favour a compact “closed” conformation, allowing the GTP binding site to form and occluding the transamidase site.^{5–8} Dysregulation of either function has been linked to pathologies including celiac diseases,⁹ fibrosis,¹⁰ and cancer,^{11,12} making hTG2 an attractive therapeutic target.

The conformationally-based exclusivity of the two functions enables a unique targeted covalent inhibition (TCI) strategy, whereby irreversible covalent modification of Cys277 in the transamidase site locks hTG2 in its open

conformation, simultaneously blocking both of its functions.¹³ Several small molecule and peptidomimetic TCI scaffolds have been developed (Fig. 1),^{14–19} but peptidic inhibitors often suffer from poor pharmacokinetics, prompting increased interest in non-peptidic scaffolds.¹⁴

A key determinant of covalent inhibition is the nature of the electrophilic warhead. In a recent study, we evaluated multiple warheads on a scaffold (**EB-2-16**) originally reported by Badarau *et al.*^{17,20} We found that effective Cys277 engagement generally required “terminal” warheads (*i.e.*, minimally hindered electrophiles capable of accessing the active site tunnel). This is consistent with most reported irreversible hTG2 inhibitors, which typically feature a simple acrylamide warhead (Fig. 1). However, our group also previously identified **CP4d**,¹⁹ a structurally distinct inhibitor bearing a bulky, internal electrophilic warhead. Despite the presence of this warhead, **CP4d** acted reversibly and failed to react irreversibly with Cys277 or to lock hTG2 into the open state,^{11,21} and was not pursued further.

Here, we revisit **CP4d** with a library of alkynyl analogues, which interestingly, function as potent irreversible inhibitors that abolish GTP binding and exhibit high isozyme selectivity. These compounds feature an internal keto-alkyne-phenyl warhead, with tunable reactivity properties (k_{inact} parameter). Our findings challenge the prevailing view that hTG2 covalent inhibitors require terminal, sterically minimal warheads, and provide a novel scaffold for future structure-based inhibitor design.

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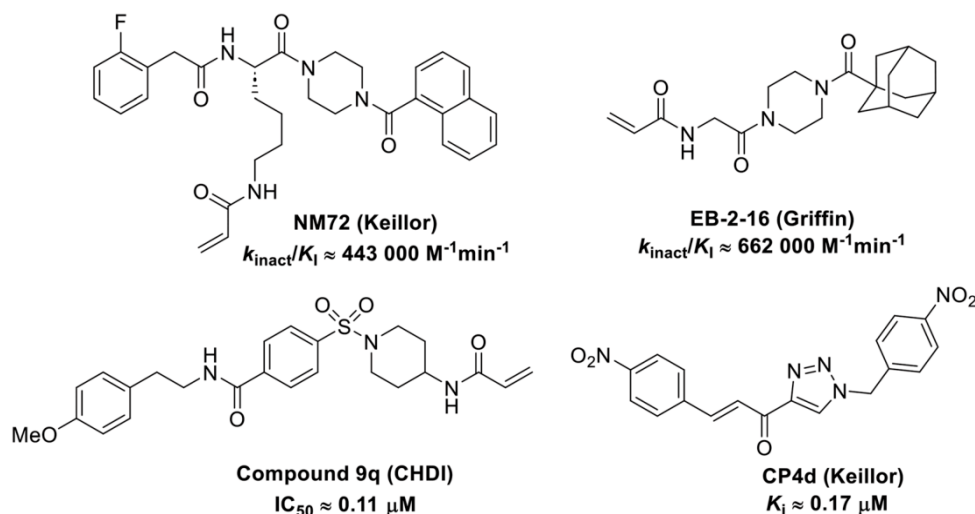


Fig. 1 Representative small molecule hTG2 inhibitors.^{15,17–19}

Results and discussion

Discovery of alkynyl scaffold

CP4d represents a peculiar class of hTG2 inhibitors. It bears an obvious electrophilic group and is a competitive inhibitor with the acyl-donor substrate of our standard activity assay (as a surrogate for transamidase activity), but does not irreversibly modify Cys277 or lock hTG2 in an open conformation.^{11,19,21} Given the structure of its warhead, **CP4d** would be expected to react covalently, even if only through reversible covalent modification, due to the strong electron withdrawing nitrophenyl group.²² Reactivity studies with **CP4d** and glutathione in solution showed rapid and irreversible GSH adduct formation in a matter of minutes, highlighting the high intrinsic reactivity of the warhead.²³ These findings suggest that the compound may bind in the active site but not in a conformation that allows reaction with Cys277, leading to reversible competitive binding.

Prior efforts in our group probed the reactivity of this scaffold with alkyne (**KA22b**) and bis-triazole derivatives.²⁴ At the time it was assumed that these analogues were also reversible inhibitors, as kinetic analysis was performed using

initial rate data over a very short period of time. However, an independent dialysis experiment later showed that no hTG2 activity was recovered after incubating with **KA22b** and dialyzing the inhibitor into buffer.²³ Given the fact that the apparent IC_{50} and K_I values of this inhibitor were in the micromolar range, we ruled out “tight” binding as a cause of this result. This led us to our current hypothesis that the alkyne scaffold was in fact capable of irreversible inhibition and its evaluation and subsequent SAR analysis required rigorous irreversible inhibition kinetic analysis.

We evaluated **KA22b** (also referred to as **8A** in this work) following our standard AL5 assay protocol, carried out under Kitz and Wilson conditions, and indeed observed classic time-dependent inhibition as expected for an irreversible inhibitor.^{25–27} The observed rate constants of inhibition (k_{obs}) acquired from fitting of curves of absorbance vs. time (Fig. 2A) were plotted against inhibitor concentration corrected for the presence of a competitive substrate. The resulting hyperbolic curve (Fig. 2B) was fitted to a saturation kinetics model to obtain $k_{\text{inact}} = 0.90 \pm 0.09\text{ min}^{-1}$ and $K_I = 1.80 \pm 0.24\text{ }\mu\text{M}$ with an overall inhibitor efficiency of $k_{\text{inact}}/K_I = 499 \pm 0.85 \times 10^3\text{ M}^{-1}\text{ min}^{-1}$. This represents a remarkably

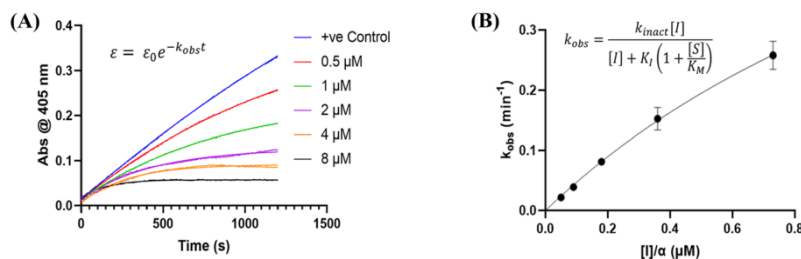


Fig. 2 Representative hTG2 inhibition kinetic curves with inhibitor **8A**. (A) Rate constants for the loss of activity (k_{obs}) and (B) their hyperbolic fitting to saturation kinetic model (see Material and methods and SI).

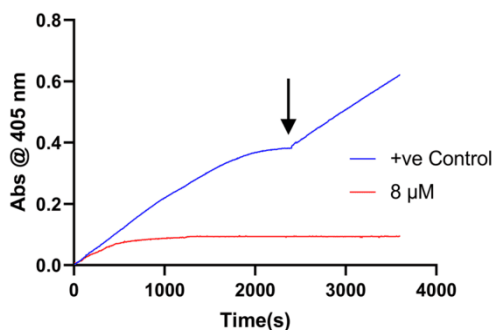


Fig. 3 Substrate spike test showing irreversible nature of **8A** inhibition.

efficient inhibitor to identify early in a campaign, on par with the lead scaffolds shown in Fig. 1.

We also demonstrated the irreversibility of inhibition by **8A** by performing a substrate spike test as previously described,²⁵ in which the assay is allowed to run until a plateau is reached in the uninhibited and inhibited reaction (Fig. 3). This plateau results from substrate depletion in the former, but complete enzyme inactivation in the latter. More substrate is then added (resulting in a concentration that is equivalent to its initial concentration in the assay), effectively diluting the reactions with substrate solution (Fig. 3, arrow). The activity of both reactions is then monitored again. The inhibition is confirmed to be irreversible as no activity is regained, whereas the activity of the uninhibited control returns to its initial activity, as observed in the first phase of the assay. The uninhibited control is crucial here to ensure that lack of activity is not due to enzyme degradation. Further validation of the irreversibility of this scaffold is provided by its ability to completely abolish GTP binding, unlike **CP4d** (see *Inhibition of GTP binding* below).

Design and synthesis

Confirmation of **8A** as a potent irreversible hTG2 inhibitor prompted us to design and synthesize various derivatives to give better understanding of the pharmacophore of this scaffold and a potential binding mode, as well as reactivity of the warhead. **8A**, while potent, poses several challenges. It is a very symmetrical, flat molecule which imparts poor solubility, and it also contains nitro groups which have mutagenic/toxicity potential.²⁸ Moreover, the activated keto-alkyne warhead is very intrinsically reactive and has poor stability in GSH reactivity assays (see *Intrinsic reactivity* below).²³ Therefore, prominent goals were to increase solubility and replace the aryl nitro groups, while tuning the warhead for stability.

The synthesis of most analogues follows our previously reported procedure (Scheme 1).^{23,24} Aryl iodides **1A–H** were either commercially available or synthesized according to known procedures (see *Materials and methods*). THP-protected propargyl alcohol (**2B**) was synthesized as previously described and azides **7A–J** were

synthesized by standard literature procedures. In the case of the pentafluorobenzene analogue (**8K**), additional protection and deprotection steps were required as side products from an S_NAr reaction were observed when the free alcohol was used in the first Sonogashira reaction. Compound **12**, bearing only a hydrogen at the terminal end of the alkyne, was also synthesized by a slightly modified route (Scheme 2), since working with a terminally unsubstituted alkyne (*i.e.*, without a phenyl ring) would likely lead to highly reactive and volatile intermediates, making the original route challenging. Here, TEMPO/TCICA was used as the oxidant, since DMP resulted in poor yields.

In the first series of compounds (**8A–I**), we altered the 1-position of the 1,4-substituted triazole with a variety of alkyl, cyclic/heterocyclic, and substituted phenyl moieties. When a group that replaced the nitro group while retaining potency and improving solubility was identified (see *Kinetic evaluation* below), that substituent was retained in the second series of compounds (**8J–O**, **12**) to allow kinetic analysis without solubility as a barrier. **8J–O**, and **12** probe the stereo electronic effects on the terminal phenyl ring attached to the alkyne warhead. Based on the results from the first two series (see *Kinetic evaluation* below), the elements that increased potency, solubility, and replaced nitro groups were combined to form compounds **8P–R**.

Next, we made a series of derivatives (**13**, **18**, **23A–E**) to probe and attempt to improve the stability (*i.e.*, lower intrinsic reactivity) of the alkynyl warhead while retaining sufficient reactivity and binding affinity to inhibit hTG2. Compound **13**, synthesized *via* a Luche reduction (Scheme 3), lowers the electrophilicity of the warhead by removing electron withdrawing affects from the adjacent carbonyl.

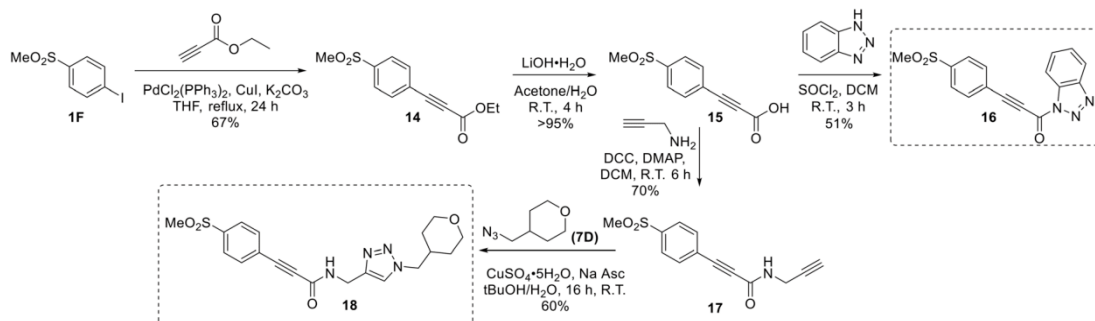
We also synthesized propiolamide derivatives **16**, **18**, **23A–E** (Schemes 4 and 5), as the electron donation of the amine to the carbonyl is known to attenuate the intrinsic reactivity of α,β -unsaturated carbonyls.²⁹ Some additional modifications were also made to the terminal substituent of the alkyne within the propiolamide series, to probe the effects of sterics and electronics, at the end of the alkyne, on intrinsic reactivity (see *Intrinsic reactivity* below).

Compound **16** was synthesized in similar fashion to a previously described protocol,²⁴ and compound **18** was obtained by an amide coupling of intermediate **15** with propargyl amine, followed by a copper-catalysed click reaction.

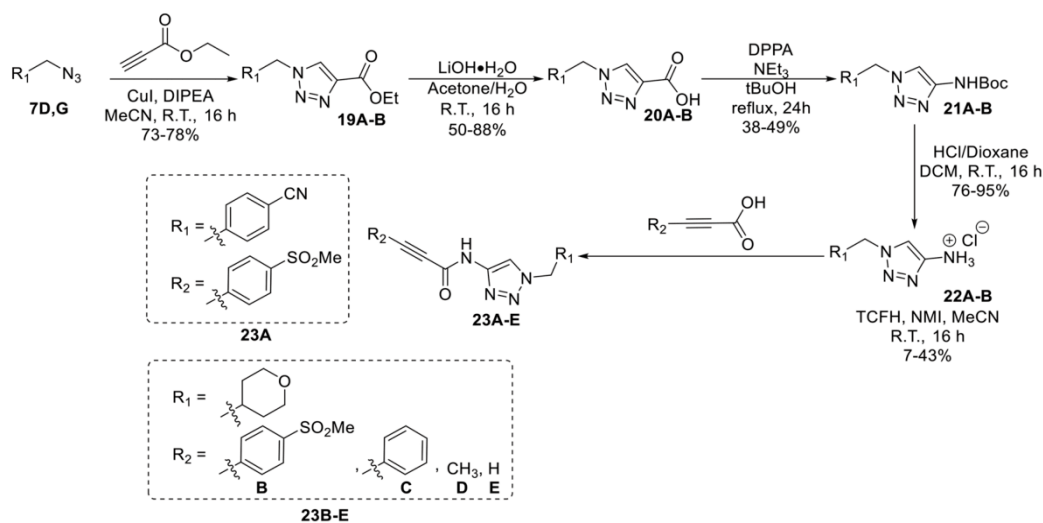
Synthesis of compounds **23A–E** features a click reaction with ethyl propiolate and subsequent hydrolysis to produce intermediate **20**. This intermediate was then subjected to a Curtius rearrangement and deprotection to install a free amine at the 4-position of the triazole (**22**). This intermediate was then coupled with various synthesized or commercially available propiolic acids using TCFH/NMI, which is known to facilitate challenging amide couplings with electron deficient amines.³⁰

Given the stability that a terminal methyl group is known to provide to alkynone warheads (see *Intrinsic reactivity*),





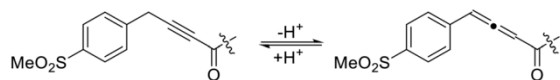
Scheme 4 Synthesis of inhibitors **16** and **18**.



Scheme 5 Synthesis of compounds **23A-E**.

attempts were made to synthesize an analogue with a methylene unit between the alkyne and the phenyl group, to disrupt any conjugation that may contribute to increased electrophilicity of the warhead. However, attempts to synthesize this type of compound yielded rearrangement to an allene in mildly basic aqueous conditions (Scheme 6, data not shown), so this was not pursued further.

We also found it peculiar that hTG2 could not tolerate steric bulk around the warhead of a scaffold like **EB-2-16**, but apparently could do so for **8A**, calling into question whether different small molecule scaffolds bind at all similarly to hTG2. No crystal structures are known of hTG2 in complex with a non-peptidic small molecule inhibitor; however, a structure obtained with Ac-P(DON)



Scheme 6 Rearrangement from alkyne to allene.

LPF-NH₂ (PDB: 2Q3Z) is widely used for structure-based design,⁶ which features three distinct pockets: an A-site, the active site tunnel, and a hydrophobic D-site. This crystallographic structure features a distinct active site 'tunnel', in which it appears the warhead must fit to access Cys277. This was used to rationalize the need for 'terminal' warheads in **EB-2-16**.¹⁷ However, in another structure of hTG2 (PDB: 3S3S), obtained with an analogue of **ZED013**,³¹ which does feature a small but internal warhead, the upper wall of this tunnel is absent in the crystal structure, suggesting that it is dynamic in nature. Perhaps surpassing a certain threshold of steric bulk forces the tunnel open, to accommodate a larger warhead. Interestingly, molecular docking of these inhibitors in the 2Q3Z vs. 3S3S structures produced significantly different poses. While **EB-2-16** could be modelled in 2Q3Z (Fig. 4A), **8A** could not, as none of the resulting poses placed the warhead in the tunnel near Cys277 (data not shown). Docking of **8A** in 3S3S, however, resulted in a plausible binding mode shown in Fig. 4B. Given its more rigid and

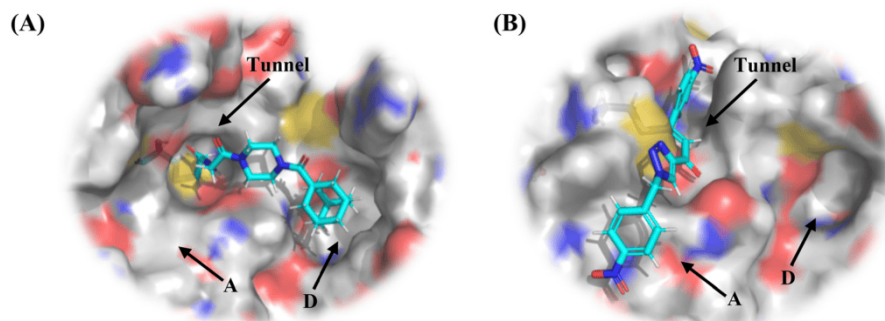
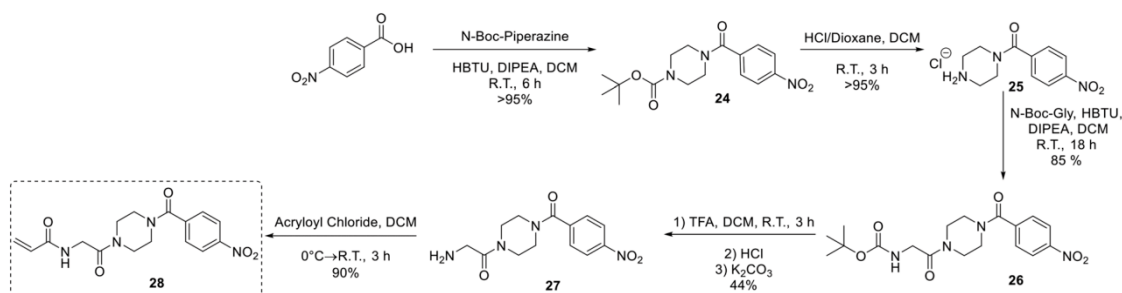


Fig. 4 Molecular modelling of (A) **EB-2-16** in crystal structure PDB: 2Q3Z and (B) **8A** in crystal structure PDB: 3S3S. Tunnel, D-site, and A-site are shown by arrows (see Materials and methods).



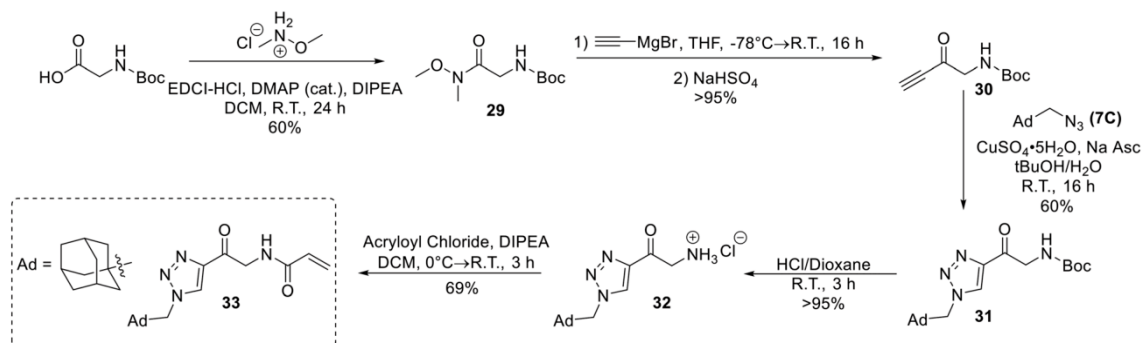
Scheme 7 Synthesis of inhibitor **28**.

linear structure, it is unlikely that **8A** could both occupy the D-site and wrap around the adjacent groove to access Cys277. However, binding in the A-site would allow the warhead direct access to Cys277 without compromising its extended conformation.

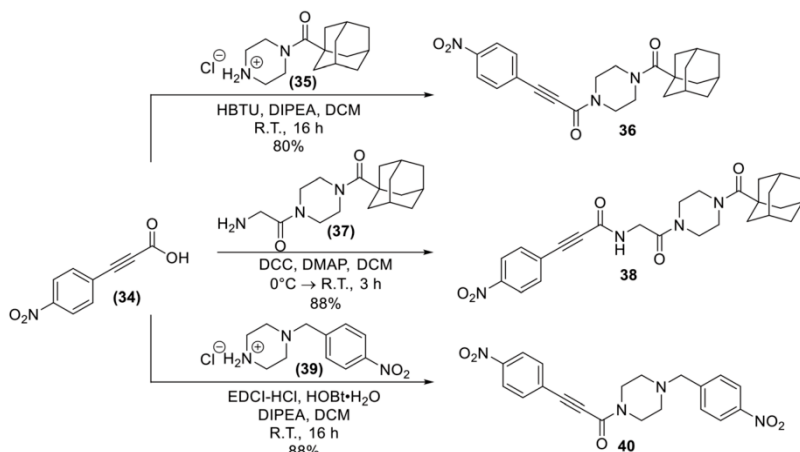
To investigate these differences experimentally, we designed a series of ‘hybrid’ compounds (**28**, **33**, **36**, **38**, **40**) that combined elements of **EB-2-16** with elements of **8A**. If these two inhibitors bind similarly to TG2, some degree of cooperativity between their structural features would be expected, whereas the absence of cooperativity would suggest they adopt different binding poses.

An analogue of **EB-2-16** bearing a *para*-nitrophenyl group in the place of the adamantane group (**28**) was synthesized using our previously established protocol (Scheme 7),²⁰ beginning with *para*-nitrobenzoic acid.

Next, an analogue replacing the piperazine group of **EB-2-16** with a triazole was synthesized (**33**). This was achieved by accessing an alkynone derivative of Boc-glycine (**30**) *via* a Weinreb amide and subsequent Grignard reaction with ethynylmagnesium bromide. **30** was then clicked to 1-(azidomethyl)adamantane (**7C**), followed by deprotection of the amine and coupling to acryloyl chloride to obtain inhibitor **33** (Scheme 8).



Scheme 8 Synthesis of inhibitor **33**.



Scheme 9 Synthesis of inhibitors **36**, **38**, and **40**.

Lastly, analogues that combined the warhead of **8A** with the scaffold of **EB-2-16**, with and without the glycine spacer were synthesized (**36** and **38**), as absence of the linker more closely resembles the size of **8A**, but including the glycine linker is more consistent with the original **EB-2-16** scaffold. We also included a compound with a *para*-nitrobenzyl replacing the adamantane carbonyl moiety (**40**), to resemble the rotatable bond and overall compound symmetry found in **8A**. These compounds were obtained through amide couplings of intermediates we have previously synthesized (Scheme 9).²⁰

Structure–activity relationships

All compounds were tested as described above for **8A**. Whenever possible, individual k_{inact} and K_{I} parameters were determined using nonlinear regression. However, in cases where inhibitors showed poor solubility and/or poor inhibition, such that they could not be tested at concentrations high enough to produce a saturation plot, double reciprocal fitting was used to estimate these parameters. However, it should be noted that this double reciprocal approach often produces high errors in the fitting of individual rate and inhibition constants.²⁵ In these cases, the ratio of $k_{\text{inact}}/K_{\text{I}}$ is taken to be the more reliable measure of potency. All results were interpreted relative to benchmark values for **8A** ($k_{\text{inact}} = 0.90 \pm 0.09 \text{ min}^{-1}$, $K_{\text{I}} = 1.80 \pm 0.24 \text{ }\mu\text{M}$, $k_{\text{inact}}/K_{\text{I}} = 499 \pm 0.85 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$) and **EB-2-16** ($k_{\text{inact}} = 2.64 \pm 0.39 \text{ min}^{-1}$, $K_{\text{I}} = 3.98 \pm 0.79 \text{ }\mu\text{M}$, $k_{\text{inact}}/K_{\text{I}} = 662 \pm 164 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$).²⁰

1-substituted triazole variants

We first started by altering the substituent at the 1-position of the triazole ring, with the goal of replacing the nitro group on the phenyl rings and increasing solubility, while retaining potency. We kept any substituents on the phenyl ring in the *para* position, as it was previously observed in **CP4d** and **8A**

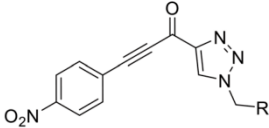
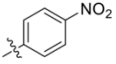
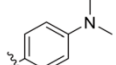
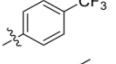
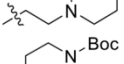
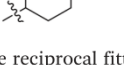
derivatives that *ortho* and *meta* substituents led to a decrease in potency.^{19,23}

In general, we found that a variety of groups ranging from simple alkyl chains to more complex aromatic and aliphatic heterocycles could be tolerated at this position, while maintaining overall inhibitor efficiencies of $k_{\text{inact}}/K_{\text{I}} \sim 300\text{--}500 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$ (Table 1). K_{I} values in this series of compounds were around 1–2 μM and k_{inact} ranged from about 0.5–1 min^{-1} , diverging very little from parent compound **8A**. However, it should be noted that moieties with a hydrogen bond acceptor showed slightly increased inhibition efficiency.

The most efficient compounds from this series were the tetrahydropyranyl derivative (**8D**) and the *para*-cyanobenzyl derivative (**8G**). The latter showed roughly 2-fold increase in potency compared to parent compound **8A**, owing largely to an increase in binding affinity (decrease in K_{I}), suggesting that the presence of a cyano group as a hydrogen bond acceptor in this position was superior to a nitro (**8A**), amine (**8B**), ether (**8D/8H**), or ester (**8I**). The least efficient compound in this series was the *para*-trifluoromethyl derivative (**8E**). Fluorine is known to have variable effects on binding affinity with hTG2 depending on the scaffold. In the case of **NM72** (Fig. 1) it provided a notable increase in binding affinity for this peptidomimetic scaffold.¹⁵ However, in the **EB-2-16** scaffold (Fig. 1), the addition of a fluorine, particularly near the warhead, resulted in a significant loss of binding affinity.²⁰ Here, it seems to not drastically reduce affinity but is not particularly desirable to include either. Note that **8C** is not necessarily a poor inhibitor, but it displayed very poor solubility and could not be sufficiently tested. However, it was synthesized to serve as an interesting comparison to the ‘hybrid’ compound series discussed below.

Although **8G** displayed the highest overall efficiency, for the majority of the subsequent series of compounds we chose to move forward with a tetrahydropyranyl group (**8D**) at the

Table 1 Kinetic parameters of triazole variants

				
Inh.	R	k_{inact} (min^{-1})	K_{I} (μM)	$k_{\text{inact}}/K_{\text{I}}$ ($\times 10^3 \text{ M}^{-1} \text{ min}^{-1}$)
8A		0.90 ± 0.09	1.80 ± 0.24	499 ± 85
8B^a		0.54 ± 0.38	1.03 ± 0.74	536 ± 27
8C^b		n.d.	n.d.	n.d.
8D		1.24 ± 0.19	2.30 ± 0.56	538 ± 156
8E^a		0.62 ± 0.29	1.75 ± 0.39	353 ± 136
8F^a		0.89 ± 0.12	2.43 ± 0.33	368 ± 3
8G		0.77 ± 0.07	0.96 ± 0.13	805 ± 128
8H		1.21 ± 0.14	2.84 ± 0.46	425 ± 84
8I^a		0.20 ± 0.02	0.40 ± 0.03	511 ± 15

^a Determined by double reciprocal fitting. ^b No inhibition detected up to solubility limit of compound ($1 \mu\text{M}$).

4-position as it provided higher solubility and allowed us to test compounds at higher concentrations.

Warhead variants

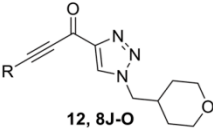
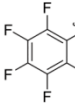
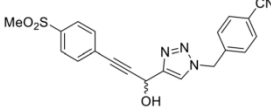
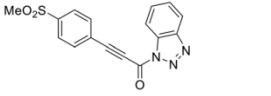
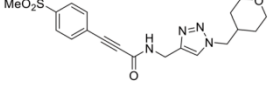
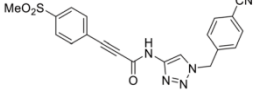
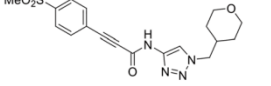
Next, we probed the effects of modifications to the terminal alkynyl substituent of the warhead on reactivity and binding affinity with hTG2 (Table 2). Surprisingly, completely removing the phenyl ring (**12**) or removing just its *para* substituent (**8J**) resulted in equally poor binding affinity. While both modifications also reduced reactivity k_{inact} , retaining the phenyl ring resulted in 3-fold higher reactivity than having no phenyl ring at all. This suggests that the phenyl ring is not providing potency by way of binding affinity, but its conjugation to the alkyne is increasing the reactivity of the warhead. This is corroborated by the intrinsic reactivity of the warheads with glutathione (see *Intrinsic reactivity*).

Preliminary results previously showed that electron donating groups on this portion of the scaffold resulted in poor potency.²³ Thus, we opted to keep electron withdrawing groups on the phenyl ring and investigate how the strength of the electron withdrawing group

affects reactivity. Starting with a perfluorobenzene (**8K**), it seems that inductively withdrawing groups do not increase k_{inact} at all, and fluorine at this position significantly decreases binding affinity, similar to what was observed with an α -fluoroacrylamide warhead on the **EB-2-16** scaffold.²⁰ Interestingly, while introducing and increasing the strength of a conjugated electron withdrawing group does increase k_{inact} , this effect is not as strong as expected (*e.g.*, **8L** vs. **8M**), and k_{inact} seems to plateau around a value of 1 min^{-1} , regardless of electron withdrawing group strength beyond a cyano group. However, again it seems that the presence of an electron withdrawing group containing a hydrogen bond acceptor, specifically one that contains a double-bonded oxygen, is required for optimal binding affinity (*e.g.*, **8L** and **8N**).

Previously we showed that a propiolamide warhead on the **EB-2-16** scaffold had a k_{inact} value of $6.83 \pm 1.75 \text{ min}^{-1}$.²⁰ Compounds **8A–O** are not propiolamides, as their keto-alkyne moiety is directly attached to the triazole ring, so there is very little attenuation of electrophilicity due to resonance from this side of the molecule. What is peculiar here, is that despite the high intrinsic reactivity of these

Table 2 Kinetic parameters of warhead variants

 12, 8J-O				
Inh.	R/Structure	k_{inact} (min ⁻¹)	K_{I} or K_{I} (μM)	$k_{\text{inact}}/K_{\text{I}}$ (×10 ³ M ⁻¹ min ⁻¹)
12	H	0.12 ± 0.05	19.4 ± 9.1	6.30 ± 1.00
8J		0.36 ± 0.02	23.9 ± 4.1	15.0 ± 3.0
8K		0.25 ± 0.02	7.30 ± 0.80	33.8 ± 4.3
8L		0.36 ± 0.05	1.36 ± 0.36	267 ± 81
8M		1.04 ± 0.18	2.99 ± 0.77	349 ± 109
8N		0.98 ± 0.14	2.05 ± 0.48	479 ± 131
8O		0.90 ± 0.13	3.19 ± 0.68	282 ± 74
13^a		0.06 ± 0.01	18.9 ± 2.8	3.33 ± 0.54
16^b		—	0.19 ± 0.02	—
18^c		n.d.	n.d.	n.d.
23A^a		0.59 ± 0.13	11.4 ± 2.9	52.0 ± 2.0
23B		0.48 ± 0.06	21.5 ± 4.3	22.0 ± 4.0

^a Determined by double reciprocal fitting. ^b Reversible inhibitor. ^c No inhibition detected up to solubility limit of compound (100 μM).

compounds (see *Intrinsic reactivity*), their k_{inact} values are relatively low. Given the generally higher binding affinity that this scaffold shows compared to **EB-2-16**, it seems that it is able to bind tightly to hTG2, but this bound conformation does not optimally position the warhead relative to Cys277 for reaction, resulting in lower k_{inact} values. This could be due to improper positioning of these sterically hindered warheads in the active site tunnel, but it

could also suggest a binding mode entirely different from the one proposed in (Fig. 4), perhaps accessing a pocket that has not yet been discovered crystallographically, since all crystal structures have been obtained with peptidomimetic inhibitors. The best inhibitor of this series was the methylsulfonyl derivative **8N**, which allowed us to retain excellent inhibitor efficiency while replacing the second nitro group.

We also evaluated a variety of compounds with attenuated warhead reactivity. Inhibitor **13** features an internal propargyl alcohol rather than a keto-alkyne, in which the alkyne is much less activated (less electron deficient). Surprisingly, this inhibitor did still display time-dependent irreversible inhibition with hTG2, albeit with a very low k_{inact} value of $0.06 \pm 0.01 \text{ min}^{-1}$ and lower binding affinity. Next, we investigated a variety of propiolamide derivatives, starting with a compound (**16**) inspired from a benzotriazole-cinnamoyl adduct (**CP15n**), from which **CP4d** was first discovered.¹⁹ Interestingly, this compound was a very potent but reversible inhibitor with a K_i of 190 nM (see SI). This suggests that having the triazole group and phenyl groups closer to the warhead in a fused ring structure impacted binding affinity very positively, but disrupted the positioning of the warhead such that it could no longer react with Cys277 at all. Strangely, it seems that minor structural changes can have significant impact on the exact binding site/pose of these compounds, without perturbing the overall binding affinity.

Other, more classic propiolamide derivatives **18** and **23A–B** showed substantially reduced potency, largely due to a decrease in binding affinity, suggesting that more distance between the triazole and warhead leads to poorer binding. With the 2-atom spacer between the triazole and the warhead in compound **18**, no significant inhibition was detected. However, with the 1-atom spacer in **23A–B**, appreciable binding affinity had returned. The only difference between **23A** and **23B** is the substituent at the 1-position of the triazole. The *para*-cyanobenzyl derivative showed roughly 2-fold higher potency than the

tetrahydropyranyl derivative. This is consistent the previous findings with compounds **8D** and **8G** (Table 1), confirming that there were no unusual cooperativity effects taking place when altering substituents at the 1 and 4-positions of the triazole together.

EB-2-16 and 8A hybrids

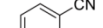
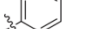
Compounds bearing elements of both the **EB-2-16** and **8A** scaffold were evaluated to determine if any cooperativity existed between their binding modes (Table 3). Inhibitor **28** features a *para*-nitrophenyl group in the place of the adamantane of **EB-2-16**. This derivative showed ~50-fold reduction in binding affinity compared to **EB-2-16**. This suggests that the substituent at the 1-position of the triazole in **8A** is not similar in its binding pose/interactions as the hydrophobic unit of **EB-2-16**.

Inhibitor **33** contains a triazole ‘spacer’ unit in between the hydrophobic moiety and glycine linker/warhead component, instead of a piperazine. It should be noted, however, that the hydrophobic moiety does not contain a carbonyl linkage to the triazole like it does to piperazine in the parent compound. This is due to the fact that a click reaction with an acyl azide precursor would be needed to retain the carbonyl, and because this is synthetically impractical, we carried forward with a methylene linkage instead. Inhibitor **33** showed ~4-fold decrease in binding affinity, but it was still a relatively efficient inhibitor. It is unclear whether the reduction in binding affinity came solely from replacing the triazole with the piperazine and/or removing the carbonyl linkage. However, given the fact that compound **40**, in which the triazole of **8A** was replaced with a

Table 3 Kinetic parameters of **EB-2-16** and **8A** hybrids

Inh.	Structure	k_{inact} (min^{-1})	K_i (μM)	k_{inact}/K_i ($\times 10^3 \text{ M}^{-1} \text{ min}^{-1}$)
28		2.17 ± 0.45	181 ± 54	12.0 ± 4.3
33		2.24 ± 0.56	16.9 ± 5.5	133 ± 54
36^a		0.40 ± 0.22	17.7 ± 12.5	22.7 ± 3.8
38^b		n.d.	n.d.	n.d.
40^b		n.d.	n.d.	n.d.

^a Determined by double reciprocal fitting. ^b No inhibition detected up to solubility limit of compound (50 μM).

Inh.	R1	R2	k_{inact} (min^{-1})	K_1 (μM)	k_{inact}/K_1 ($\times 10^3 \text{ M}^{-1} \text{ min}^{-1}$)
8P	SO ₂ Me		0.42 ± 0.01	0.52 ± 0.03	802 ± 50
8Q	SO ₂ NMe ₂		0.14 ± 0.01	0.22 ± 0.03	604 ± 82
8R	SO ₂ Me		0.43 ± 0.02	0.48 ± 0.05	910 ± 113

Compounds **36** and **38** contain the warhead of **8A**, but with the rest of the **EB-2-16** scaffold, without and with the glycine linker respectively. Again, these compounds display significantly reduced potency compared to the parent scaffolds. However, it is interesting to note that the smaller size/length of **36**, which is more similar to the size of **8A**, shows greater potency than **38**.

warhead could be replaced with a methylsulfone and that a *para*-cyanobenzyl group at the 1-position of the triazole increased binding affinity. Therefore, we combined these elements together to give inhibitor **8P**. The synergy of these two changes pushed the inactivation constant (K_I) down into the nanomolar range, while retaining a modest k_{inact} (Table 4). We also synthesized and evaluated a derivative with a dimethylsulfonamide instead of a methylsulfone (**8Q**); however, this compound showed relatively poor solubility in our kinetic assay, and its overall inhibition efficiency was also lower. To achieve solubilizing effects similar to those of the tetrahydropyranyl moiety of **8D**, we replaced the simple benzene ring with a pyridine in inhibitor **8R**. This compound displayed the highest inhibitor efficiency among all compounds ($k_{\text{inact}} = 0.43 \pm 0.02 \text{ min}^{-1}$, $K_I = 0.48 \pm 0.05 \text{ }\mu\text{M}$, $k_{\text{inact}}/K_I = 910 \pm 113 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$), representing one of the most potent non-peptidic small molecule hTG2 inhibitors to date.

Optimization of inhibitor potency

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sulfonylphenyl group is engaged in pi-pi stacking with Trp332 and hydrogen bonding with Thr360, the triazole forms a hydrogen bond with Asn333, and the cyanopyridine group is positioned to engage in a pi-pi stacking interaction with Phe334 and a hydrogen bond with Gln169 *via* the nitrile group. The increased affinity observed with **8P** and **8R** compared to **8A** may be due to the better positioning of the nitrile group, relative to the nitro group, to act as a hydrogen bond acceptor.

Isozyme selectivity

Since hTG2 is part of a family of TGases it is always important to establish the selectivity of new scaffolds against a panel of other, therapeutically relevant TGases, namely TG1, TG6, TG3a, and FXIIIa. The AL5 assay described above for hTG2 inhibition was also used to monitor hTG1 and hTG6 inhibition,¹⁶ whereas for hTG3a and hFXIIIa, a commercially available continuous fluorescence assay that uses a peptidic FRET-quenched substrate was used.^{32,33} In each case the same effective inhibitor concentration ($[I]/\alpha$, where $\alpha = (1 + ([S]/K_M))$, to account for competition with substrate) was used for each enzyme/substrate pair in the assays, in order to permit comparison between isozymes. A single k_{obs} value was determined, in triplicate, for each isozyme with inhibitor **8R** (see SI).¹⁶ As shown in Fig. 6 and the SI (page S12), **8R** does not show significant inhibition of any of the other isozymes. In fact, this scaffold shows better selectivity than **EB-2-16**,²⁰ and our other previously reported peptidomimetic scaffolds,¹⁴ making **8R** one of the most selective non-peptidic hTG2 inhibitor scaffolds reported to date.

Inhibition of GTP binding

One of the key advantages of covalent modification of Cys277 of hTG2 is that it locks hTG2 in its open conformation and thereby abolishes its ability to bind GTP. We have recently shown that molecules as small as acrylamide itself completely lock hTG2 in its open conformation, even if they have poor binding affinity.³⁴ Therefore, it appears that any compound covalently

modifying Cys277 will abolish GTP binding. Compound **8R** was evaluated for its impact on GTP binding using our previously reported assay.³⁴ The enzyme was incubated with and without inhibitor and then subjected to dialysis to wash out any unbound inhibitor. Both samples were then mixed with a nonhydrolyzable GTP analogue (GTP γ S FL BODIPY) whose fluorescence increases when bound to hTG2. As shown in Fig. 7, enzyme incubated with inhibitor showed negligible GTP binding compared to the uninhibited control. This provides further validation that the alkynyl scaffold is in fact irreversible and likely reacting with Cys277.

Intrinsic reactivity of alkynyl warheads

Although none of the warheads with attenuated reactivity were able to react/bind as efficiently with hTG2 as **8A**, this type of internal warhead is underexplored in the field of TCIs. Therefore, we further investigated the intrinsic reactivity of different alkynyl warhead analogues by measuring the corrected second order rate constants (k_2^{corr}) of their reaction with glutathione (GSH), in solution, under pseudo-first order conditions, at room temperature (Table 5). Reactions were carried out at pH 7.4 (phosphate buffer) or pH 10.4 (CAPS buffer) depending on how reactive the compound was and if rate acceleration was needed by increasing the pH to observe the reaction over a reasonable time frame. Corrected second order rate constants (*i.e.*, corrected for pH and accounting for $[GS^-]$ in solution) provide a much more robust picture of reactivity than half-lives or pseudo-first order rate constants alone, as they are not dependent on assay conditions and allow direct comparison between literature values.³⁵

We started with compound **8N** as a representative of the potent alkynyl hTG2 inhibition scaffold reported herein. Unfortunately, this compound showed rapid reaction with GSH and was highly unstable ($t_{1/2} \leq 10$ min at pH 7.4). This is likely due to the fact there are two strong electron withdrawing groups attached directly to the alkyne (*para*-methylsulfone phenyl and ketone) making it a highly activated electrophile.

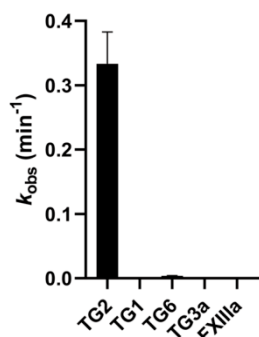


Fig. 6 Isozyme selectivity of **8R** at $[I]/\alpha = 0.73 \mu\text{M}$ with each isozyme.

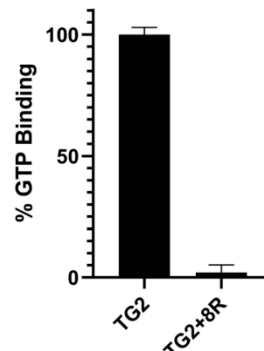
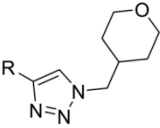
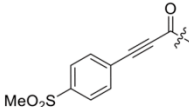
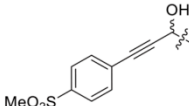
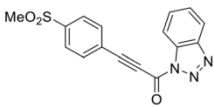
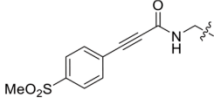
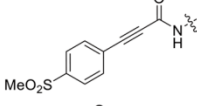
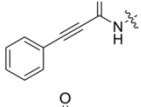
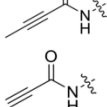
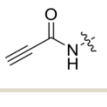


Fig. 7 Inhibition of GTP binding by **8R** at $[I] = 0.92 \mu\text{M}$.

Table 5 Intrinsic reactivity of representative alkynyl warheads and their enzymatic efficiency ratios

 8N, 13, 18, 23B-E			
Cpd.	R/Structure	k_2^{corr} ($\text{M}^{-1} \text{min}^{-1}$)	$\sim(k_{\text{inact}}/K_1)/k_2^{\text{corr}}$
8N		>100	—
13		0.12 ± 0.01	28 000
16		>100	—
18		4.85 ± 0.19	—
23B		36.7 ± 1.0	600
23C		11.2 ± 0.31	—
23D		0.51 ± 0.08	—
23E		69.5 ± 4.81	—

Compound **16**, a potent reversible inhibitor, also showed rapid consumption and instability in this assay. Due to its poor solubility, its reactivity with GSH was measured by ^1H NMR in $\text{DMSO}-d_6$ (see SI, page S13). Even in non-aqueous conditions, its half-life was under 10 minutes. With this compound, it is plausible that along with the alkyne, the amide bond could be cleaved by glutathione (or in solution during kinetic assays) due to the fact that benzotriazole is a good leaving group. For this reason, we also tested benzotriazole itself and intermediate **15** (Scheme 4) in our inhibition assay to see if inhibition actually resulted from one of these individual precursors rather than the compound itself due to hydrolysis. However, none of these precursors showed any inhibition of hTG2 on their own (see SI, page S9), suggesting that the inhibition observed in our assay was not due to degradation products. The evaluation of **8N** and **16** provided half-lives that were less than 10 min, allowing us to set a limit of their

k_2^{corr} values as being $>100 \text{ M}^{-1} \text{min}^{-1}$ (see *Materials and methods* for related equations).

The alkyne in compound **13** is not conjugated to a carbonyl, which explains why it is the least intrinsically reactive compound in this series with a k_2^{corr} of $0.12 \pm 0.01 \text{ M}^{-1} \text{min}^{-1}$. However, the conjugated methanesulfonylbenzene ring still provides significant activation, such that this compound is only about 4–8 fold less reactive than a standard *N*-alkyl acrylamide warhead, which typically have k_2^{corr} values of $\sim 0.4\text{--}0.8 \text{ M}^{-1} \text{min}^{-1}$ at room temperature,^{20,29,36} suggesting that it is still a viable warhead, especially if it offers greater binding affinity with specific targets.

We also tested a variety of propiolamide derivatives with different terminal alkyne substituents. Compound **18**, featuring an *N*-alkyl amide linkage showed a $k_2^{\text{corr}} = 4.85 \pm 0.19 \text{ M}^{-1} \text{min}^{-1}$, whereas an analogue with an *N*-triazole

amide linkage (**23B**) has a $k_2^{\text{corr}} = 36.7 \pm 1.0 \text{ M}^{-1} \text{ min}^{-1}$. This is likely because the electron donating capability of the nitrogen is stronger in the *N*-alkyl linkage, whereas the *N*-triazole linkage splits the electron donation through resonance between the adjacent carbonyl and the triazole, making the warhead overall about 10-fold more reactive. This is very similar to the difference in reactivity between *N*-alkyl and *N*-phenyl acrylamides.^{35,36}

Next, we explored the effect of the methylsulfone group on the terminal phenyl ring. Removing this electron withdrawing group in compound **23C** decreased the $k_2^{\text{corr}} \sim 4$ -fold to $11.2 \pm 0.08 \text{ M}^{-1} \text{ min}^{-1}$ compared to **23B**, revealing the significant contribution of this electron withdrawing group to warhead reactivity. Removing the phenyl group and replacing it with a methyl group (**23D**) resulted in a significant increase in stability, with a $k_2^{\text{corr}} = 0.52 \pm 0.08 \text{ M}^{-1} \text{ min}^{-1}$. This is an interesting result, as it seems that the steric hindrance conferred by the methyl group far outweighs any steric hindrance effects imparted by the phenyl group, and that the electronic effects of the phenyl group dominate. It is likely that, when directly attached to the alkyne, the planar phenyl ring is orthogonal to the anti-bonding pi-orbitals of the α,β -unsaturated carbonyl system. However, a tetrahedral methyl group is free to rotate and partially block attack from the incoming nucleophile. The stabilizing effects of the methyl group are also seen in compound **23E**, where replacing the methyl group with a hydrogen (*i.e.*, effectively removing the terminal substituent) resulted in significant loss of stability (~ 140 -fold) with a $k_2^{\text{corr}} = 69.5 \pm 5.81 \text{ M}^{-1} \text{ min}^{-1}$.

The $k_{\text{inact}}/K_{\text{I}}$ ratio represents the second order rate constant for reaction between an inhibitor and enzyme, taking into account both binding affinity and reactivity. On the other hand, k_2^{corr} represents the second order rate constant for the reaction of inhibitor with glutathione thiolate (GS^-). We divided ($k_{\text{inact}}/K_{\text{I}}$) by k_2^{corr} for every inhibitor for which these values were measured, to provide an 'enzymatic efficiency ratio' (EER), as we have previously described.²⁰ This ratio gives information about how well the enzyme is able to facilitate thiol addition to the inhibitor, compared to GSH in solution. A higher ratio indicates higher expected on-target reactivity and selectivity. Interestingly, compound **13**, which is a less efficient inhibitor than **23B**, shows an EER of $\sim 28\,000$ (Table 5), roughly 50-fold higher than the EER obtained with **23B** (~ 600), meaning the enzyme is much more selective in facilitating the reaction with the intrinsically low reactivity warhead, likely due to binding affinity and/or conformational effects. This is generally true and consistent with what we found in our previous warhead screening study on **EB-2-16**.²⁰ However, for good lead candidates this ratio should be on the order of millions,²⁰ which is far above the EER values of our current series.

These results point to some routes that may be explored to improve the stability and efficiency of these inhibitors. While it seems that propiolamide derivatives show significant decrease in binding affinity, and that the pharmacophore is very sensitive to changes at the 4-position of the triazole, attempts can be made to further improve the binding affinity of

analogues like **13** and **23A/B**, as they show modest inhibition efficiency and stability. Additionally, although attempts to synthesize a propargyl derivative of our scaffold (Scheme 6) by introducing a methylene unit between the alkyne and the phenyl ring were unsuccessful, it is likely that this type of modification, in principle, will increase stability similarly to a terminal methyl group. However, this type of modification will likely require more than one methylene unit to prevent allene formation, as well as more complex synthetic routes.

Conclusions

In this study, we revisited our previously reported reversible hTG2 inhibitor scaffold **CP4d** and discovered a unique and highly potent internal alkynyl scaffold (**8A**) that irreversibly inhibits hTG2. Comprehensive kinetic SAR analysis revealed how modifications to the triazole 1-substituent, warhead reactivity, and incorporation of elements from the **EB-2-16** scaffold affect both binding affinity (K_{I}) and reactivity (k_{inact}). These insights guided the design of inhibitor **8R**, in which potentially toxic nitrophenyl groups were replaced with methylsulfonyl-phenyl and cyano-pyridinyl substituents, while improving solubility and inhibitor efficiency substantially. With a k_{inact} of $0.43 \pm 0.02 \text{ min}^{-1}$, a K_{I} of $0.48 \pm 0.05 \text{ }\mu\text{M}$, and a $k_{\text{inact}}/K_{\text{I}}$ of $910 \pm 113 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$, **8R** ranks among the most effective non-peptidic hTG2 inhibitors reported to date. It also demonstrates excellent isozyme selectivity and fully abolishes GTP binding.

Hybrid derivatives combining the scaffolds of **8A** and **EB-2-16** showed no cooperative inhibition, suggesting distinct binding poses and underscoring the need for structural elucidation of non-peptidic hTG2 inhibitors *via* crystallography or cryo-EM to enhance rational structure-based design. Investigation into the intrinsic reactivity of various internal alkynyl warheads highlighted the stabilizing effect of terminal methyl groups and the significant influence of extended conjugation and electron-withdrawing groups on reactivity. Although the most potent inhibitors have limited stability, promising strategies such as propiolamide and propargyl derivatives have been identified for future optimization.

Overall, this work establishes a novel internal alkynyl warhead scaffold for irreversible hTG2 inhibition that not only provides a valuable chemical biology tool but also expands the scope of covalent warheads beyond traditional terminal electrophiles, with potential applications extending beyond hTG2.

Materials and methods

Synthesis

General remarks. All reagents and solvents were purchased from commercial sources and used without further purification. Anhydrous solvents were obtained using a Phoenix Solvent Dispensing System (JC Meyer Solvent Systems) with neutral alumina-packed columns. Thin layer chromatography was performed using EMD aluminum-backed silica 60 F254-coated plates and were visualized using either UV-light (254 nm),

KMnO₄, Hanessian's stain, or ninhydrin stain. Column chromatography was carried out using standard flash technique with silica (Siliaflash-P60, 230–400 mesh Silicycle) under compressed air pressure. ¹H-NMR spectra were obtained at 300, 400, 500, or 600 MHz, ¹³C-NMR spectra were obtained at 75, 100, 125, or 150 MHz, and ¹⁹F-NMR spectra were obtained at 283 MHz on Bruker instruments. NMR chemical shifts (δ) are reported in ppm and are calibrated against residual solvent signals of CDCl₃, DMSO-*d*₆, or CD₃OD. Spectra were processed using MNOVA 14.1.2 (Mestrelab Research S.L., Santiago de Compostela, Spain). Fourier transform was conducted using a linear phase shift group delay, an exponential-fit 1 Hz apodization, and a zero-filling linear prediction of 65 536 points. An automatic phase correction was applied and manually refined to provide a flatter baseline. A baseline correction, employing a Whittaker smoothing function with a 2.60 Hz filter and a smoothing factor of 16384 was employed on the spectrum between –2.00 and 15.00 ppm. Integration was measured using the manual integration tool and referenced to a relevant proton. Multiplet analysis was conducted using the algorithm as implemented in the software, confirmed by manual analysis. High resolution mass spectrometry (HRMS) was performed using a quadrupole time-of-flight (QTOF) or magnetic sector analyzer and electrospray ionization (ESI) or electron impact (EI), respectively. All measured *m/z* values were within 3 mmu of the calculated value (generally < 10 ppm discrepancy) unless otherwise noted. High performance liquid chromatography (HPLC) purity traces were collected on a Gilson-Mandel GXP271 with UV detection at 214 and 254 nm (Phenomenex Luna, 150 mm × 4.6 mm, 30 min, 1.5 mL min^{–1} flow rate, 5–95% or 20–80%, CH₃CN with 0.1% TFA in H₂O with 0.1% TFA, 30 min method).

1-Iodo-4-(methylsulfonyl)benzene (1F)

To a solution of 4-(methylsulfonyl)aniline (2.00 g, 11.68 mmol, 1.0 eq.) in H₂O (10 mL) and conc. HCl (2.5 mL) at 0 °C was added a solution of NaNO₂ (0.88 g, 12.84 mmol, 1.1 eq.) in H₂O (2 mL) dropwise. The reaction was stirred for 1 h and then a solution of KI (2.12 g, 12.84 mmol, 1.2 eq.) in H₂O (2 mL) was added dropwise at 0 °C. The reaction was allowed to warm to R. T. and then refluxed for 1 h. The reaction mixture was then cooled to 0 °C prior to addition of sat. NaHSO₃ (100 mL). The aqueous solution was extracted with Et₂O (3 × 50 mL) and then washed with brine (100 mL). The organic layer was then dried over MgSO₄ and concentrated *in vacuo* to give a yellow solid which was purified by flash column chromatography (30% EtOAc/Hex *R*_f = 0.47) to give a white powder (1.05 g, 35%). ¹H NMR (500 MHz, CDCl₃) δ 7.99 (d, *J* = 8.6 Hz, 2H), 7.70 (d, = 8.6 Hz, 2H), 3.09 (s, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 140.4, 138.8, 128.9, 101.7, 44.6. Characterization data are consistent with previously reported values.³⁷

1-Bromo-4-((trifluoromethyl)sulfonyl)benzene (1G)

To a solution of 4-bromophenyl trifluoromethyl sulfide (0.30 mL, 1.94 mmol, 1.0 eq.) in DCM (20 mL) at 0 °C was added

*m*CPBA (1.67 g, 9.70 mmol, 5.0 eq.) portion wise. The reaction mixture was allowed to warm to R.T while stirring overnight. The reaction mixture was then diluted with DCM (100 mL) and washed with sat. NaHCO₃ (2 × 100 mL) and then brine (100 mL) followed by drying over MgSO₄ and concentrating *in vacuo*. The residue was purified by flash column chromatography (5% EtOAc/Hex, *R*_f = 0.45, dry loading using celite) to give a white solid (0.50 g, 89%). ¹H NMR (300 MHz, CDCl₃) δ 7.90 (d, *J* = 9.0 Hz, 2H), 7.83 (d, *J* = 9.0 Hz, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 119.8 (q, *J*_{C,F} = 324.3 Hz), 130.4, 132.3, 133.0, 133.4. ¹⁹F NMR (282 MHz, CDCl₃) δ –78.25. Characterization data are consistent with previously reported values.³⁸

2-(Prop-2-yn-1-yloxy)tetrahydro-2H-pyran (2B)

To a solution of propargyl alcohol (1.10 mL, 17.84 mmol, 1.0 eq.) in DCM (15 mL) were added dihydropyran (2.50 mL, 26.76 mmol, 1.5 eq.) and 4 drops of 12.5 M HCl. The reaction mixture was stirred vigorously and R.T. for 24 h, after which it was quenched with sat. NaHCO₃ (50 mL). The organic layer was separated, and the aqueous layer was further extracted using DCM (3 × 50 mL). The combined organic layer was washed with brine (150 mL) and dried over MgSO₄ before being concentrated *in vacuo* to give a colourless oil (2.14 g, 86%). ¹H NMR (300 MHz, CDCl₃) δ 4.83 (t, *J* = 3.0 Hz, 1H), 4.27 (dd, *J* = 15.6, 2.4 Hz, 2H), 3.90–3.74 (m, 1H), 3.60–3.47 (m, 1H), 2.42 (t, *J* = 2.4 Hz, 1H), 1.92–1.44 (m, 6H, overlapping with H₂O). ¹³C NMR (75 MHz, CDCl₃) δ 96.8, 79.7, 73.9, 61.9, 53.9, 30.2, 25.3, 19.0. Characterization data are consistent with previously reported values.³⁹

General procedure 1 (GP1): Sonogashira reaction²⁴

In a flame-dried round-bottom flask filled with N₂ were added *para* substituted aryl iodide or aryl bromide (**1A–H**) (1.0 eq., commercially available unless indicated otherwise), PdCl₂(PPh₃)₂ (2 mol%) and CuI (2 mol%). The solids were dissolved in anhydrous MeCN (~1 M) followed by the addition of NEt₃ (4.0 eq.). The reaction mixture was stirred for 10 min at R.T. followed by dropwise addition of propargyl alcohol (**2A**) or THP-protected propargyl alcohol (**2B**) (1.2 eq.). The reaction was heated to reflux and stirred for 2–24 h. The reaction was concentrated *in vacuo* and then diluted with H₂O (100 mL) and EtOAc (100 mL) and filtered through celite. The layers of the filtrate were separated, and the aqueous layer was extracted with EtOAc (2 × 100 mL). The combined organic layer was washed with sat. NH₄OH (100 mL) and then water (100 mL) followed by drying over MgSO₄ and concentrating *in vacuo*. The residue was purified by flash column chromatography as described below.

3-(4-Nitrophenyl)prop-2-yn-1-ol (3A)

Synthesized according to GP1 using 4-iodonitrobenzene (**1A**) (1.24 g, 5.00 mmol) and purified by flash column chromatography (50% EtOAc/Hexanes, *R*_f = 0.61, dry loading using celite) to give an orange solid (0.74 g, 84%). ¹H NMR

(300 MHz, CDCl₃) δ 8.19 (d, J = 8.7 Hz, 2H), 7.58 (d, J = 8.7 Hz, 2H), 4.54 (s, 2H), 1.70 (br s, overlapping with H₂O, 1H). ¹³C NMR (75 MHz, CDCl₃) δ 147.3, 132.4, 129.4, 123.6, 92.4, 83.8, 51.5. Characterization data are consistent with previously reported values.²⁴

3-Phenylprop-2-yn-1-ol (3B)

Synthesized according to **GP1** using 4-iodobenzene (**1B**) (1.00 g, 4.90 mmol) and purified by flash column chromatography (30% EtOAc/Hex, R_f = 0.39, dry loading using celite) to give a yellow solid (0.68 g, >95%). ¹H NMR (400 MHz, CDCl₃) δ 7.46–7.43 (m, 2H), 7.35–7.31 (m, 3H), 4.52 (s, 2H), 1.72 (br d, J = 2.0 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 131.7, 128.5, 128.3, 122.5, 87.7, 85.7, 51.6. Characterization data are consistent with previously reported values.²³

2-((3-(Perfluorophenyl)prop-2-yn-1-yl)oxy)tetrahydro-2H-pyran (3C1)

Synthesized according to **GP1** using iodopentafluorobenzene (**1C**) (1.00 mL, 7.50 mmol), **2B** (0.87 g, 6.45 mmol), and purified by flash column chromatography (10% EtOAc/Hex, R_f = 0.53, dry loading using celite) to give a colourless oil (1.50 g, 65%). ¹H NMR (300 MHz, CDCl₃) δ 4.88 (t, J = 3.1 Hz, 1H), 4.54 (s, 2H), 4.01–3.77 (m, 1H), 3.61–3.54 (m, 1H), 1.97–1.41 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 148.4–148.3 (m), 146.8–146.6 (m), 142.6–142.4 (m), 140.9–140.7 (m), 138.5–138.3 (m), 136.9–136.6 (m), 99.7–99.5 (m), 98.2, 97.1, 69.8, 62.1, 54.5, 30.2, 25.3, 18.9. ¹⁹F NMR (282 MHz, CDCl₃) δ –135.79–135.92 (m, 2F), –152.17–152.34 (m, 1F), –161.66–161.86 (m, 2F). HRMS (EI) calc'd for C₁₄H₁₁F₅O₂ [M]⁺: 306.0679, found: 306.0702.

3-(Perfluorophenyl)prop-2-yn-1-ol (3C2)

To a solution of **3C1** (1.40 g, 4.90 mmol, 1.0 eq.) in MeOH (15 mL) was added *p*TsOH·H₂O (0.10 g, 0.49 mmol, 0.1 eq.). The reaction was stirred for 1 h at R.T. at which point it was confirmed complete by TLC analysis. The reaction was concentrated *in vacuo*, and the residue was purified by flash column chromatography (10% EtOAc/Hex, R_f = 0.16, dry loading using celite) to give a white crystalline solid (0.92 g, 85%). ¹H NMR (300 MHz, CDCl₃) δ 4.60 (s, 2H), 1.93 (br s, 1H). ¹³C NMR (75 MHz, CDCl₃) δ 148.4–148.2 (m), 146.7–146.5 (m), 142.7–142.5 (m), 140.9–140.7 (m), 138.5–138.3 (m), 136.9–136.6 (m), 99.8, 99.5–99.2 (m), 69.7, 51.5. ¹⁹F NMR (282 MHz, CDCl₃) δ –135.90–136.03 (m, 2F), –151.82–151.98 (m, 1F), –161.46–161.66 (m, 2F). HRMS (EI) calc'd for C₉H₃F₅O [M]⁺: 222.0104, found: 222.0091.

1-(4-(3-Hydroxyprop-1-yn-1-yl)phenyl)ethan-1-one (3D)

Synthesized according to **GP1** using 4-iodoacetophenone (**1D**) (1.00 g, 4.06 mmol) and purified by flash column chromatography (10% EtOAc/DCM, R_f = 0.43, dry loading using celite) to give a yellow solid (0.65 g, 92%). ¹H NMR

(300 MHz, CDCl₃) δ 7.92 (d, J = 8.5 Hz, 2H), 7.53 (d, J = 8.5 Hz, 2H), 4.54 (s, 2H), 2.61 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 197.3, 136.5, 131.8, 128.2, 127.4, 90.4, 84.9, 51.6, 26.6. Characterization data are consistent with previously reported values.²³

4-(3-Hydroxyprop-1-yn-1-yl)benzonitrile (3E)

Synthesized according to **GP1** using 4-iodobenzonitrile (**1E**) (1.00 g, 4.37 mmol) and purified by flash column chromatography (50% EtOAc/Hex, R_f = 0.63, dry loading using celite) to give a yellow solid (0.60 g, 87%). ¹H NMR (400 MHz, CDCl₃) δ 7.59 (d, J = 8.4 Hz, 2H), 7.50 (d, J = 8.4 Hz, 2H), 4.51 (s, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 132.1, 132.0, 127.5, 118.3, 111.8, 91.7, 83.9, 51.4. Characterization data are consistent with previously reported values.⁴⁰

3-(4-(Methylsulfonyl)phenyl)prop-2-yn-1-ol (3F)

Synthesized according to **GP1** using **1F** (0.60 g, 2.12 mmol) and purified by flash column chromatography (20% EtOAc/Hex, R_f = 0.37, dry loading using celite) to give an off-white solid (0.35 g, 80%). ¹H NMR (300 MHz, CDCl₃) δ 7.90 (d, J = 8.6 Hz, 2H), 7.61 (d, J = 8.6 Hz, 2H), 4.53 (s, 2H), 3.06 (s, 3H), 1.76 (br s, 1H). ¹³C NMR (75 MHz, CDCl₃) δ 44.6, 51.5, 83.9, 91.9, 127.5, 128.8, 132.6, 140.0. Characterization data are consistent with previously reported values.⁴¹

3-((Trifluoromethyl)sulfonyl)phenyl)prop-2-yn-1-ol (3G)

Synthesized according to **GP1** using **1G** (0.50 g, 1.73 mmol) and purified by flash column chromatography (5% EtOAc/DCM, R_f = 0.47, dry loading using celite) to give a brown oil (0.34 g, 73%). ¹H NMR (600 MHz, CDCl₃) δ 8.00 (d, J = 8.6 Hz, 2H), 7.70 (d, J = 8.6 Hz, 2H), 4.57 (s, 2H), 1.98 (br s, 1H). ¹³C NMR (150 MHz, CDCl₃) δ 132.7, 131.5, 130.7, 130.4, 119.7 (q, $J_{C,F}$ = 324.3 Hz), 93.7, 83.5, 51.5. ¹⁹F NMR (282 MHz, CDCl₃) δ –78.25. HRMS (EI) calc'd for C₁₀H₆F₃O₃S [M – H]⁺: 262.9990, found: 262.9950 (note: 5 mmu (15 ppm) discrepancy).

4-(3-Hydroxyprop-1-yn-1-yl)-*N,N*-dimethylbenzenesulfonamide (3H)

Synthesized according to **GP1** using 4-bromo-*N,N*-dimethylbenzenesulfonamide (**1H**) (1.00 g, 3.79 mmol) and purified by flash column chromatography (70% EtOAc/Hex, R_f = 0.41, dry loading using celite) to give a pale-yellow solid (0.77 g, 84%). ¹H NMR (600 MHz, CDCl₃) δ 7.72 (d, J = 8.6 Hz, 2H), 7.57 (d, J = 8.6 Hz, 2H), 2.71 (s, 6H). ¹³C NMR (150 MHz, CDCl₃) δ 135.3, 132.2, 127.8, 127.5, 91.0, 84.2, 51.7, 38.0. HRMS (EI) calc'd for C₁₁H₁₃NO₃S [M]⁺: 239.0616, found: 239.0636.

General procedure 2 (GP2): DMP oxidation²³

To a solution of alcohol (**3A-H** and **5A-H**) (1.0 eq.) in DCM (~0.15 M) at 0 °C was added DMP (1.2 eq.) portion wise. The ice bath was then removed, and the reaction was stirred at

R.T. for 3 h. The reaction was concentrated *in vacuo* and diluted with EtOAc (100 mL). Sat. Na₂S₂O₃ and NaHCO₃ (150 mL:150 mL) were added to the reaction mixture and the solution was stirred vigorously until the precipitate dissolved. The layers were separated, and the aqueous layer was extracted with EtOAc (2 × 100 mL). The organic layer was washed with sat. NaHCO₃ (100 mL), brine (200 mL), and then dried over MgSO₄. After concentrating *in vacuo*, the residue was purified by flash column chromatography or carried forward without further purification as described below.

3-(4-Nitrophenyl)propionaldehyde (4A)

Synthesized according to GP2 using 3A (0.74 g, 4.15 mmol) and purified by flash column chromatography (30% EtOAc/Hexanes, *R*_f = 0.57, dry loading using celite) to give a yellow solid (0.67 g, 92%). ¹H NMR (300 MHz, CDCl₃) δ 9.46 (s, 1H), 8.28 (d, *J* = 9.0 Hz, 2H), 7.78 (d, *J* = 9.0 Hz, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 176.1, 184.8, 133.9, 126.0, 123.9, 90.8, 90.6. Characterization data are consistent with previously reported values.²³

3-Phenylpropionaldehyde (4B)

Synthesized according to GP2 using 3B (0.63 g, 4.70 mmol) and the resulting solid was carried forward without further purification (5% EtOAc/DCM, *R*_f = 0.71) (0.60 g, >95%). ¹H NMR (400 MHz, CDCl₃) δ 9.44 (s, 1H), 7.64–7.61 (m, 2H), 7.53–7.48 (m, 1H), 7.44–7.40 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 176.8, 133.3, 131.3, 128.7, 119.4, 95.1, 88.4. Characterization data are consistent with previously reported values.²³

3-(Perfluorophenyl)propionaldehyde (4C)

Synthesized according to modified procedure GP2 using 3C2 (0.30 g, 1.40 mmol). NaHCO₃ (0.47 g, 4.60 mmol, 4.0 eq.) was added to the reaction to neutralize the AcOH in the reaction mixture. The resulting white powder (0.28 g, >95%) was carried forward without further purification (10% EtOAc/Hex, *R*_f = 0.41). ¹H NMR (300 MHz, CDCl₃) δ 9.41 (s, 1H). ¹³C NMR (75 MHz, CDCl₃) δ 175.2, 149.9–149.7 (m), 146.5–146.3 (m), 145.6–145.2 (m), 142.1–141.7 (m), 139.4–139.2 (m), 136.3–136.0 (m), 97.3–97.7 (m, 2C overlapping), 60.7. ¹⁹F NMR (282 MHz, CDCl₃) δ -132.68–132.82 (m, 2F), -146.49–146.65 (m, 1F), -160.17–160.41 (m, 2F). HRMS (EI) calc'd for C₉HF₅O [M]⁺: 219.9948, found: 219.9937.

3-(4-Acetylphenyl)propionaldehyde (4D)

Synthesized according to GP2 using 3D (0.65 g, 3.73 mmol) and purified by flash column chromatography (10% EtOAc/DCM, *R*_f = 0.67, dry loading using celite) to give a yellow solid (0.60 g, 93%). ¹H NMR (300 MHz, CDCl₃) δ 9.46 (s, 1H), 7.99 (d, *J* = 8.7 Hz, 2H), 7.71 (d, *J* = 8.7 Hz, 2H), 2.64 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 196.9, 176.4, 138.5, 133.3, 128.4, 123.9, 92.9, 89.8, 26.7.

Characterization data are consistent with previously reported values.²³

4-(3-Oxoprop-1-yn-1-yl)benzonitrile (4E)

Synthesized according to GP2 using 3E (0.60 g, 3.75 mmol) and the resulting yellow solid (0.59 g, >95%) was carried forward without further purification (50% EtOAc/Hex, *R*_f = 0.56). ¹H NMR (300 MHz, CDCl₃) δ 9.43 (s, 1H), 7.71–7.66 (m, 4H). ¹³C NMR (75 MHz, CDCl₃) δ 176.2, 133.5, 132.4, 124.2, 117.8, 114.6, 91.3, 90.3. Characterization data are consistent with previously reported values.⁴²

3-(4-(Methylsulfonyl)phenyl)propionaldehyde (4F)

Synthesized according to GP2 using 3F (0.35 g, 1.68 mmol) and purified by flash column chromatography (5% EtOAc/DCM, *R*_f = 0.55, dry loading using celite) to give an off-white solid (0.31 g, 91%). ¹H NMR (300 MHz, CDCl₃) δ 9.46 (s, 1H), 8.00 (d, *J* = 8.7 Hz, 2H), 7.79 (d, *J* = 8.7 Hz, 2H), 3.08 (3H, s). ¹³C NMR (75 MHz, CDCl₃) δ 176.2, 142.5, 133.8, 127.7, 125.1, 91.2, 89.2, 44.4. HRMS (EI) calc'd for C₁₀H₈O₃S [M]⁺: 208.0194, found: 208.0181.

3-(4-((Trifluoromethyl)sulfonyl)phenyl)propionaldehyde (4G)

Synthesized according to GP2 using 3G (0.30 g, 0.76 mmol) and the resulting orange oil (0.30 g, >95%) was carried forward without further purification (5% EtOAc/DCM = 0.91). ¹H NMR (300 MHz, CDCl₃) δ 9.47 (s, 1H), 8.09 (d, *J* = 8.3 Hz, 2H), 7.87 (d, *J* = 8.3 Hz, 2H). ¹³C NMR (600 MHz, CDCl₃) δ 175.9, 133.9, 133.2, 130.9, 128.1, 119.7 (q, *J*_{C,F} = 324.3 Hz), 91.0, 89.9. ¹⁹F NMR (282 MHz, CDCl₃) δ -77.89. HRMS (EI) calc'd for C₁₀H₅F₃O₃S [M]⁺: 261.9911, found: 261.9912.

N,N-Dimethyl-4-(3-oxoprop-1-yn-1-yl)benzenesulfonamide (4H)

Synthesized according to GP2 using 3H (0.75 g, 3.12 mmol) and the resulting yellow solid (0.73 g, >95%) was carried forward without further purification (70% EtOAc/Hex, *R*_f = 0.67). ¹H NMR (600 MHz, CDCl₃) δ 9.47 (s, 1H), 7.84 (d, *J* = 8.0 Hz, 2H), 7.78 (d, *J* = 8.0 Hz, 2H), 2.77 (s, 6H). ¹³C NMR (150 MHz, CDCl₃) δ 176.3, 138.0, 133.5, 127.9, 123.9, 91.8, 89.8, 37.9. HRMS (EI) calc'd for C₁₁H₁₁NO₃S [M]⁺: 237.0460, found: 237.0473.

General procedure 3 (GP3): Grignard reaction with ethynyl magnesium bromide²⁴

In a flamed dried flask under N₂, 4A-H (1.0 eq.) was dissolved in anhydrous THF (~0.1 M) and the solution was cooled to -78 °C. Ethynylmagnesium bromide (2.0 eq.) was then added dropwise. The reaction was stirred at this temperature for 3 h and then quenched with H₂O (10 mL) and sat. NH₄Cl (5 mL), and then extracted with EtOAc (3 × 50 mL). The organic layer was dried over MgSO₄ and concentrated *in vacuo*. The resulting solid was purified by flash column chromatography as described below.

1-(4-Nitrophenyl)penta-1,4-diyn-3-ol (5A)

Synthesized according to **GP3** using **4A** (0.20 g, 1.14 mmol) and purified by flash column chromatography (30% EtOAc/hexanes, R_f = 0.27, dry loading using celite) to give a yellow solid (0.12 g, 54%). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 8.21 (d, J = 8.9 Hz, 2H), 7.62 (d, J = 8.9 Hz, 2H), 5.38 (dd, J = 7.5, 2.4 Hz, 1H), 2.67 (d, J = 2.4 Hz, 1H), 2.42 (br d, J = 7.5 Hz, 1H). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 147.6, 132.7, 128.5, 123.6, 90.3, 82.5, 80.0, 73.6, 52.4. Characterization data are consistent with previously reported values.²⁴

1-Phenylpenta-1,4-diyn-3-ol (5B)

Synthesized according to **GP3** using **4B** (0.24 g, 1.84 mmol) and purified by flash column chromatography (30% EtOAc/Hexanes, R_f = 0.51, dry loading using celite) to give a yellow oil (0.25 g, 87%). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.50–7.47 (m, 2H), 7.38–7.31 (m, 3H), 5.37 (d, J = 2.4 Hz, 1H), 2.63 (d, J = 2.4 Hz, 1H), 2.44 (br s, 1H). $^{13}\text{C NMR}$ (100 MHz, CDCl_3) δ 131.8, 128.9, 128.3, 121.7, 85.4, 84.7, 80.8, 72.9, 52.5. Characterization data are consistent with previously reported values.²³

1-(Perfluorophenyl)penta-1,4-diyn-3-ol (5C)

Synthesized according to **GP3** using **4C** (0.20 g, 0.90 mmol) and purified by flash column chromatography (10% EtOAc/hexanes, R_f = 0.21, dry loading using celite) to give a pale-yellow powder (0.12 g, 56%). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 5.40 (dd, J = 8.0, 2.3 Hz, 1H), 2.66 (d, J = 2.3 Hz, 1H), 2.53 (d, J = 8.0 Hz, 1H). $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 148.6–148.5 (m), 146.9–146.8 (m), 143.2–142.9 (m), 141.5–141.3 (m), 138.7–138.5 (m), 137.0–136.8 (m), 99.01–98.8 (m), 97.8, 79.6, 69.0, 52.6. $^{19}\text{F NMR}$ (282 MHz, CDCl_3) δ -135.15–-135.29 (m, 2F), -150.69–-150.86 (m, 1F), -161.12–-161.32 (m, 2F). **HRMS (EI)** calc'd for $\text{C}_{11}\text{H}_3\text{F}_5\text{O}$ [M^+]: 246.0104, found: 246.0111.

1-(4-(3-Hydroxypenta-1,4-diyn-1-yl)phenyl)ethan-1-one (5D)

Synthesized according to **GP3** using **4D** (0.20 g, 1.16 mmol) and purified by flash column chromatography (30% EtOAc/hexanes, R_f = 0.27, dry loading using celite) to give a yellow oil (0.20 g, 86%). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.92 (d, J = 8.5 Hz, 2H), 7.53 (d, J = 8.5 Hz, 2H), 4.54 (s, 2H), 2.61 (s, 3H). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 197.3, 136.5, 131.8, 128.2, 127.4, 90.4, 84.9, 51.6, 26.6. Characterization data are consistent with previously reported values.²³

4-(3-Hydroxypenta-1,4-diyn-1-yl)benzonitrile (5E)

Synthesized according to **GP3** using **4E** (0.20 g, 1.30 mmol) and purified by flash column chromatography (30% EtOAc/hexanes, R_f = 0.31, dry loading using celite) to give a yellow solid (0.14 g, 61%). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 1H NMR (300 MHz, CDCl_3) δ 7.62 (d, J = 8.8 Hz, 2H), 7.55 (d, J = 8.8 Hz, 2H), 5.37 (dd, J = 7.3, 2.3 Hz, 1H), 2.65 (d, J = 2.3 Hz, 1H), 2.44 (br d, J = 7.3 Hz, 1H). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ

132.3, 132.0, 126.6, 118.2, 112.4, 89.5, 82.8, 80.1, 73.5, 52.4. **HRMS (EI)** calc'd for $\text{C}_{12}\text{H}_7\text{NO}$ [M^+]: 181.0528, found: 181.0516.

1-(4-(Methylsulfonyl)phenyl)penta-1,4-diyn-3-ol (5F)

Synthesized according to **GP3** using **4F** (0.20 g, 0.96 mmol) and purified by flash column chromatography (15% EtOAc/DCM, R_f = 0.41, dry loading using celite) to give a yellow solid (0.15 g, 66%). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.89 (d, J = 8.7 Hz, 2H), 7.62 (d, J = 8.7 Hz, 2H), 5.37 (d, J = 2.3 Hz, 1H), 3.06 (s, 3H), 2.65 (d, J = 2.3 Hz, 1H). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 140.3, 132.6, 127.6, 127.4, 89.4, 82.6, 80.2, 73.5, 52.4, 44.4. **HRMS (EI)** calc'd for $\text{C}_{12}\text{H}_{10}\text{O}_3\text{S}$ [M^+]: 234.0351, found: 234.0325.

1-(4-((Trifluoromethyl)sulfonyl)phenyl)penta-1,4-diyn-3-ol (5G)

Synthesized according to **GP3** using **4G** (0.30 g, 1.14 mmol) and purified by flash column chromatography (8% EtOAc/DCM, R_f = 0.65, dry loading using celite) to give a dark orange oil (0.18 g, 54%). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 8.01 (d, J = 8.7 Hz, 2H), 7.73 (d, J = 8.7 Hz, 2H), 5.39 (d, J = 2.3 Hz, 1H), 2.66 (d, J = 2.3 Hz, 1H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 133.0, 131.0, 130.7, 130.6, 119.7 (q, $J_{\text{C,F}}$ = 324.3 Hz), 91.4, 82.2, 79.9, 73.8, 52.4. $^{19}\text{F NMR}$ (282 MHz, CDCl_3) δ -78.16. **HRMS (EI)** calc'd for $\text{C}_{12}\text{H}_7\text{F}_3\text{O}_3\text{S}$ [M^+]: 288.0068, found: 288.0055.

4-(3-Hydroxypenta-1,4-diyn-1-yl)-*N,N*-dimethylbenzenesulfonamide (5H)

Synthesized according to **GP3** using **4H** (0.30 g, 1.25 mmol) and the resulting yellow solid (0.27 g, 75%) was carried forward without further purification (70% EtOAc/Hex, R_f = 0.49). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 7.76 (d, J = 8.7 Hz, 2H), 7.64 (d, J = 8.7 Hz, 2H), 5.39 (dd, J = 7.6, 2.3 Hz, 1H), 2.73 (s, 6H), 2.67 (d, J = 2.3 Hz, 1H), 2.50 (d, J = 7.6 Hz, 1H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 135.5, 132.4, 127.9, 127.7, 88.9, 82.9, 80.3, 73.5, 52.5, 37.9. **HRMS (EI)** calc'd for $\text{C}_{13}\text{H}_{13}\text{NO}_3\text{S}$ [M^+]: 263.0616, found: 263.0639.

1-(4-Nitrophenyl)penta-1,4-diyn-3-one (6A)

Synthesized according to **GP2** using **5A** (0.12 g, 0.60 mmol) and purified by flash column chromatography (30% EtOAc/hexanes, R_f = 0.50, dry loading using celite) to give a yellow solid (0.11 g, 88%). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 8.28 (d, J = 8.9 Hz, 2H), 7.79 (d, J = 8.9 Hz, 2H), 3.46 (s, 1H). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 159.4, 148.9, 134.0, 125.7, 123.8, 91.1, 88.0, 81.8, 80.3. Characterization data are consistent with previously reported values.²⁴

1-Phenylpenta-1,4-diyn-3-one (6B)

Synthesized according to **GP2** using **5B** (0.25 g, 1.60 mmol) and the resulting solid (0.24 g, >95%) was carried forward without further purification (30% EtOAc/hexanes, R_f = 0.77). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.65–7.63 (m, 2H), 7.54–7.49 (m, 1H), 3.80 (s, 1H). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 160.1, 133.5,

131.5, 128.7, 119.1, 92.5, 89.0, 82.2, 79.0. Characterization data are consistent with previously reported values.²³

1-(Perfluorophenyl)penta-1,4-diyn-3-one (6C)

Synthesized according to modified **GP2** using **5C** (0.12 g, 0.49 mmol) and the resulting white powder (0.11 g, >95%) was carried forward without further purification (10% EtOAc/Hex, R_f = 0.53). **¹H NMR** (600 MHz, CDCl₃) δ 3.49 (s, 1H). **¹³C NMR** (150 MHz, CDCl₃) δ 158.7, 149.2–149.1 (m), 147.5–147.4 (m), 144.9–144.6 (m), 143.1–142.9 (m), 138.7–138.5 (m), 137.0–136.8 (m), 97.6, 97.0–96.7 (m), 81.5, 81.1, 74.5. **¹⁹F NMR** (282 MHz, CDCl₃) δ –131.87–132.00 (m, 2F), –145.45–145.62 (m, 1F), –159.49–159.76 (m, 2F). **HRMS (EI)** calc'd for C₁₁HF₅O [M]⁺: 243.9948, found: 243.9922.

1-(4-Acetylphenyl)penta-1,4-diyn-3-one (6D)

Synthesized according to **GP2** using **5D** (0.20 g, 1.0 mmol) and purified by flash column chromatography (30% EtOAc/hexanes, R_f = 0.43, dry loading using celite) to give a grey solid (0.18 g, 92%). **¹H NMR** (300 MHz, CDCl₃) δ 7.99 (d, J = 8.7 Hz, 2H), 7.73 (d, J = 8.7 Hz, 2H), 3.43 (s, 1H), 2.64 (s, 3H). **¹³C NMR** (75 MHz, CDCl₃) δ 196.9, 159.8, 138.6, 133.5, 128.4, 123.6, 90.4, 90.2, 82.0, 79.7, 26.7. Characterization data are consistent with previously reported values.²³

4-(3-Oxopenta-1,4-diyn-1-yl)benzonitrile (6E)

Synthesized according to **GP2** using **5E** (0.14 g, 0.77 mmol) and purified by flash column chromatography (30% EtOAc/Hexanes, R_f = 0.48, dry loading using celite) to give a yellow solid (0.12 g, 88%). **¹H NMR** (300 MHz, CDCl₃) δ 7.71 (s, 4H), 3.44 (s, 1H). **¹³C NMR** (75 MHz, CDCl₃) δ 159.7, 133.7, 132.5, 124.0, 117.8, 114.9, 90.9, 88.7, 81.9, 80.4. **HRMS (EI)** calc'd for C₁₂H₅NO [M]⁺: 179.0371, found: 179.0362.

1-(4-(Methylsulfonyl)phenyl)penta-1,4-diyn-3-one (6F)

Synthesized according to **GP2** using **5F** (0.14 g, 0.60 mmol) and the resulting yellow solid (0.14 g, >95%) was used without further purification (15% EtOAc/DCM, R_f = 0.69). **¹H NMR** (600 MHz, CDCl₃) δ 8.00 (d, J = 8.6 Hz, 2H), 7.81 (d, J = 8.7 Hz, 2H), 3.45 (s, 1H), 3.06 (s, 3H). **¹³C NMR** (150 MHz, CDCl₃) δ 159.7, 142.7, 134.1, 127.8, 124.9, 90.5, 88.6, 82.0, 80.3, 77.4, 77.2, 76.9, 44.5. **HRMS (EI)** calc'd for C₁₂H₈O₃S [M]⁺: 232.0194, found: 232.0187.

1-(4-((Trifluoromethyl)sulfonyl)phenyl)penta-1,4-diyn-3-one (6G)

Synthesized according to **GP2** using **5G** (0.18 g, 0.62 mmol) the resulting oil was triturated with hexanes to give a beige solid (0.14 g, 80%) which was used without further purification (30% EtOAc/DCM, R_f = 0.62). **¹H NMR** (300 MHz, CDCl₃) δ 8.09 (d, J = 8.3 Hz, 2H), 7.89 (d, J = 8.3 Hz, 2H), 3.49 (s, 1H). **¹³C NMR** (150 MHz, CDCl₃) δ 159.3, 134.0, 133.3,

130.9, 127.8, 119.7 (q, $J_{C,F}$ = 324.3 Hz), 91.4, 87.2, 81.7, 80.7. **¹⁹F NMR** (282 MHz, CDCl₃) δ –77.88. **HRMS (EI)** calc'd for C₁₂H₅F₃O₃S [M]⁺: 285.9911, found: 284.9909.

N,N-Dimethyl-4-(3-oxopenta-1,4-diyn-1-yl)benzenesulfonamide (6H)

Synthesized according to **GP2** using **5H** (0.27 g, 1.03 mmol, 1.0 eq.) and the resulting yellow solid (0.26 g, >95%) was used without further purification (70% EtOAc/Hex, R_f = 0.75). **¹H NMR** (600 MHz, CDCl₃) δ 7.81–7.77 (m, 4H), 3.45 (s, 1H), 2.74 (s, 6H). **¹³C NMR** (150 MHz, CDCl₃) δ 159.7, 138.2, 133.7, 127.9, 123.6, 90.3, 89.1, 81.9, 80.1, 37.9. **HRMS (EI)** calc'd for C₁₃H₁₁NO₃S [M]⁺: 261.0460, found: 261.0442.

General procedure 4: synthesis of azides

To a stirring solution of alkyl bromides or chlorides (1.0 eq., commercially available unless indicated otherwise) in DMF (~0.5 M) was added NaN₃ (2.0 eq.). The reaction was stirred at 60 °C for 16 h, after which it was concentrated *in vacuo*, diluted with water (20 mL), and then extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with brine (25 mL), dried over MgSO₄, and concentrated *in vacuo* to give azides (**7A**, **E–J**) which were used without further purification.

1-(Azidomethyl)-4-nitrobenzene (7A)

Synthesized according to **GP4** using 4-nitrobenzyl bromide (2.16 g, 10.00 mmol) to give a yellow oil (1.43 g, 80%). **¹H NMR** (300 MHz, CDCl₃) δ 8.24 (d, J = 6.6 Hz, 2H), 7.50 (d, J = 6.6 Hz, 2H), 4.50 (s, 2H). **¹³C NMR** (75 MHz, CDCl₃) δ 142.4, 137.8, 124.8, 120.6, 55.8. Characterization data are consistent with previously reported values.²⁴

4-(Dimethylamino)phenyl)methanol (7B1)

To a solution of 4-(dimethylamino)benzaldehyde (0.50 g, 3.35 mmol, 1.0 eq.) in EtOH (20 mL) at R.T. was added NaBH₄ (0.14 g, 3.69 mmol, 1.1 eq.) portion wise. The reaction was stirred at R.T. for 5 h, concentrated *in vacuo* and then quenched with H₂O (25 mL). The aqueous phase was extracted with EtOAc (3 × 25 mL) and the combined organic layer was washed with brine (50 mL), dried over MgSO₄ and concentrated *in vacuo* to give a clear oil (0.48 g, 95%). **¹H NMR** (300 MHz, CDCl₃) δ 7.24 (d, J = 8.7 Hz, 2H), 6.73 (d, J = 8.7 Hz, 2H), 4.56 (s, 2H), 2.95 (s, 6H). Characterization data are consistent with previously reported values.⁴³

4-(Azidomethyl)-*N,N*-dimethylaniline (7B2)

To a solution of **7B1** (0.43 g, 2.81 mmol, 1.0 eq.) in anhydrous THF (15 mL) at 0 °C were added diphenylphosphoryl azide (0.91 mL, 4.22 mmol, 1.5 eq.) and DBU (0.63 mL, 4.22 mmol, 1.5 eq.) dropwise. The reaction was warmed to R.T. and stirred for 16 h. The reaction mixture was quenched with 1 M HCl (30 mL) and extracted with EtOAc (25 mL). The aqueous layer was then basified with 1 M NaOH to pH ≈ 8 and extracted with EtOAc (3 × 15 mL). The

combined organic layer was washed with brine (50 mL), dried over MgSO_4 and concentrated *in vacuo* to give a yellow oil which was purified by flash column chromatography (10% EtOAc/hexanes, R_f = 0.52, dry loading using celite) to give a clear colourless oil (0.23 g, 46%). ^1H NMR (300 MHz, CDCl_3) δ 7.19 (d, J = 8.7 Hz, 2H), 6.72 (d, J = 8.7 Hz, 2H), 4.23 (s, 2H), 2.97 (s, 6H). ^{13}C NMR (75 MHz, CDCl_3) δ 150.5, 129.6, 122.7, 112.4, 54.7, 40.5. Characterization data are consistent with previously reported values.⁴⁴

(Adamantan-1-yl)methyl methanesulfonate (7C1)

To a solution of 1-adamantanemethanol (1.00 g, 6.00 mmol, 1.0 eq.) in DCM (25 mL) was added NEt_3 (2.10 mL, 15.00 mmol, 2.5 eq.) and the mixture was cooled to 0 °C. Then methanesulfonyl chloride (0.60 mL, 7.20 mmol, 1.2 eq.) was added dropwise and the reaction mixture was allowed to warm to R.T. and stirred for 4 h. The reaction was quenched with sat. NaHCO_3 (25 mL) and extracted with EtOAc (3 $\times$ 30 mL). The combined organic layer was washed with sat. NH_4Cl (50 mL), brine (50 mL), and then dried over MgSO_4 and concentrated *in vacuo* to give a pale-yellow solid which was used without further purification (1.43 g, >95%). ^1H NMR (300 MHz, CDCl_3) δ 3.78 (s, 2H), 2.99 (2, 3H), 2.02 (apparent s, 3H), 1.76–1.63 (m, 6H), 1.57 (m, 6H). ^{13}C NMR (75 MHz, CDCl_3) δ 79.5, 38.9, 37.2, 36.9, 33.6, 28.0. Characterization data are consistent with previously reported values.⁴⁵

1-(Azidomethyl)adamantane (7C2)

To a solution of 7C1 in DMF (10 mL) was added NaN_3 (1.95 g, 30.00 mmol, 10.0 eq.) and the reaction was stirred at 120 °C for 48 h. The reaction mixture was quenched with H_2O (20 mL) and extracted with 1:4 EtOAc/hexanes (3 $\times$ 25 mL). The combined organic layer was washed with brine (25 mL), dried over MgSO_4 and concentrated *in vacuo* to give a pale-yellow oil which was used without further purification (0.47 g, 82%). ^1H NMR (300 MHz, CDCl_3) δ 2.95 (s, 2H), 2.01–1.98 (m, 3H), 1.76–1.59 (m, 6H), 1.53–1.52 (m, 6H). ^{13}C NMR (75 MHz, CDCl_3) δ 64.3, 40.0, 36.8, 34.8, 28.2. Characterization data are consistent with previously reported values.⁴⁵

4-(Azidomethyl)tetrahydro-2H-pyran (7D)

To a solution of tetrahydropyran-4-methanol (0.35 mL, 3.00 mmol, 1.0 eq.) in DCM (15 mL) was added NEt_3 (1.00 mL, 7.50 mmol, 2.5 eq.) and the mixture was cooled to 0 °C. Then methanesulfonyl chloride (0.30 mL, 3.60 mmol, 1.2 eq.) was added dropwise and the reaction mixture was allowed to warm to R.T. and stirred for 4 h. The reaction was quenched with sat. NaHCO_3 (15 mL) and extracted with DCM (2 $\times$ 10 mL). The combined organic layer was washed with brine (25 mL) and then dried over MgSO_4 and concentrated *in vacuo* to give a pale-yellow oil. The oil was dissolved in DMF (10 mL) and to this solution was added NaN_3 (0.98 g, 15.00 mmol, 5.0 eq.) and the reaction was stirred at 80 °C for 16 h. The reaction mixture was quenched with H_2O (20

mL) and extracted with Et_2O (3 $\times$ 10 mL). The combined organic layer was washed with brine (25 mL), dried over MgSO_4 and concentrated *in vacuo* to give a pale-yellow oil which was used without further purification (0.30 g, 72%). ^1H NMR (300 MHz, CDCl_3) δ 4.00–3.94 (m, 2H), 3.37 (td, J = 11.7, 2.1 Hz, 2H), 3.17 (d, J = 6.9 Hz, 2H), 1.83–1.72 (m, 1H), 1.68–1.60 (m, 2H), 1.41–1.24 (m, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ 67.6, 57.3, 35.5, 30.5. Characterization data are consistent with previously reported values.⁴⁶

1-(Azidomethyl)-4-(trifluoromethyl)benzene (7E)

Synthesized according to GP4 using 4-trifluoromethylbenzyl bromide (0.50 g, 2.10 mmol) give a clear colourless liquid (0.27 g, 65%). ^1H NMR (300 MHz, CDCl_3) δ 7.66 (d, J = 7.8 Hz, 2H), 7.45 (d, J = 7.8 Hz, 2H), 4.43 (s, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ 139.4, 130.5 (q, J_{CF} = 32.3 Hz), 128.3, 125.8 (q, J_{CF} = 1.8 Hz), 124.0 (q, J_{CF} = 270.3 Hz), 54.1. ^{19}F NMR (283 MHz, CDCl_3) δ –62.6. Characterization data are consistent with previously reported values.⁴⁷

1-Azidohexane (7F)

Synthesized according to GP4 using 1-bromohexane (0.42 mL, 3.00 mmol) to give a clear colourless liquid (0.32 g, 84%). ^1H NMR (300 MHz, CDCl_3) δ 3.25 (t, J = 6.9 Hz, 2H), 1.66–1.51 (m, 2H), 1.45–1.20 (m, 6H), 0.89 (t, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 51.6, 31.5, 28.9, 26.5, 22.6, 14.1. Characterization data are consistent with previously reported values.⁴⁸

4-(Azidomethyl)benzonitrile (7G)

Synthesized according to GP4 using 4-cyanobenzyl bromide (0.50 g, 2.50 mmol) to give a clear colourless liquid (0.33 g, 83%). ^1H NMR (300 MHz, CDCl_3) δ 7.66 (d, J = 8.0 Hz, 2H), 7.43 (d, J = 8.0 Hz, 2H), 4.47 (s, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ 140.8, 132.6, 128.5, 118.4, 112.1, 53.7. Characterization data are consistent with previously reported values.⁴⁹

4-(2-Azidoethyl)morpholine (7H)

Synthesized according to GP4 using 4-(2-Chloroethyl)morpholine (0.18 mL, 1.30 mmol) to give a colourless oil (0.20 g, 96%). ^1H NMR (400 MHz, CDCl_3) 3.72 (t, J = 4.4 Hz, 4H), 3.35 (t, J = 6.0 Hz, 2H), 2.59 (t, J = 6.0 Hz, 2H), 2.59 (t, J = 4.4 Hz, 4H). δ ^{13}C NMR (100 MHz, CDCl_3) δ 67.0, 57.7, 53.7, 48.0. Characterization data are consistent with previously reported values.⁵⁰

tert-Butyl 4-(azidomethyl)piperidine-1-carboxylate (7I)

Synthesized according to GP4 using tert-butyl 4-(bromomethyl)piperidine-1-carboxylate (0.50 g, 1.80 mmol) to give a colourless oil (0.41 g, 94%). ^1H NMR (400 MHz, CDCl_3) 4.11 (d, J = 12.8 Hz, 2H), 3.17 (d, J = 6.0 Hz, 2H), 2.67 (td, J = 13.2, 2.8 Hz, 2H), 1.73–1.63 (m, 3H), 1.44 (s, 9H), 1.20–1.09 (m, 2H). δ ^{13}C NMR (100 MHz, CDCl_3) δ 154.9, 79.6,

57.1, 43.6, 36.4, 29.7, 28.6. Characterization data are consistent with previously reported values.⁵¹

5-(Azidomethyl)picolinonitrile (7J)

Synthesized according to **GP4** using 2-cyano-5-bromomethylpyridine (0.40 g, 2.00 mmol) to give a brown solid (0.26 g, 82%). ¹H NMR (600 MHz, CDCl₃) δ 8.70 (s, 1H), 7.84 (dd, *J* = 8.3, 1.8 Hz, 1H), 7.75 (d, *J* = 8.0 Hz, 1H), 4.55 (s, 2H). ¹³C NMR (150 MHz, CDCl₃) 150.3, 136.2, 135.3, 133.6, 128.4, 116.9, 51.5. Characterization data are consistent with previously reported values.⁵²

General procedure 4 (GP5): CuAAC reaction with CuI and DIPEA²³

To a solution of **6A–H** (1.0 eq.), **7A–J** (1.0 eq.), and CuI (1.0 eq.) in anhydrous MeCN (~0.1 M relative to **6**) under N₂ at R. T. was added DIPEA (1.0 eq.) dropwise and the reaction was stirred for 16 h. The reaction mixture was then diluted with DCM (20 mL) and washed with 1 M HCl (2 × 15 mL), brine (20 mL), sat. NH₄OH (4 × 15 mL, until aqueous phase was no longer blue), and brine (20 mL) again. The organic layer was then dried over MgSO₄ and concentrated *in vacuo* to give a residue which was purified by flash column chromatography as described below.

1-(1-(4-Nitrobenzyl)-1H-1,2,3-triazol-4-yl)-3-(4-nitrophenyl)prop-2-yn-1-one (8A)

Synthesized according to **GP5** using **6A** (0.10 g, 0.50 mmol) and **7A** (0.09 g, 0.50 mmol) and purified by flash column chromatography (1–5% EtOAc/DCM, in 2% EtOAc/DCM *R_f* = 0.23, dry loading using celite) to give a yellow solid (0.10 g, 54%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.29 (s, 1H), 8.36 (d, *J* = 9.0 Hz, 2H), 8.26 (d, *J* = 8.7 Hz, 2H), 8.04 (d, *J* = 9.0 Hz, 2H), 7.59 (d, *J* = 8.7 Hz, 2H), 5.91 (s, 2H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 168.2, 148.6, 147.4, 146.9, 142.6, 134.3, 130.9, 129.2, 125.4, 124.1, 124.0, 89.5, 88.7, 52.4. HRMS (EI) calc'd for C₁₈H₁₁N₅O₅ [M]⁺: 377.0760, found: 377.0750. Characterization data are consistent with previously reported values.²³

1-(1-(4-(Dimethylamino)benzyl)-1H-1,2,3-triazol-4-yl)-3-(4-nitrophenyl)prop-2-yn-1-one (8B)

Synthesized according to **GP5** using **6A** (0.09 g, 0.45 mmol) and **7B** (0.08 g, 0.45 mmol) and purified by flash column chromatography (1–5% EtOAc/DCM, in 2% EtOAc/DCM *R_f* = 0.27, dry loading using celite) to give an orange solid (0.12 g, 71%). ¹H NMR (300 MHz, CDCl₃) δ 8.27 (d, *J* = 9.0 Hz, 2H), 8.00 (s, 1H), 7.86 (d, *J* = 9.0 Hz, 2H), 7.21 (d, *J* = 8.7 Hz, 2H), 6.71 (d, *J* = 8.7 Hz, 2H), 5.91 (s, 2H), 2.98 (s, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 170.0, 161.2, 148.7, 148.0, 134.1, 130.0, 126.8, 126.7, 123.9, 120.1, 112.7, 90.1, 89.9, 54.7, 40.5. HRMS (EI) calc'd for C₂₀H₁₇N₅O₃ [M]⁺: 377.1331, found: 375.1324.

1-(1-((Adamantan-1-yl)methyl)-1H-1,2,3-triazol-4-yl)-3-(4-nitrophenyl)prop-2-yn-1-one (8C)

Synthesized according to **GP5** using **6A** (0.08 g, 0.40 mmol) and **7C** (0.08 g, 0.40 mmol) and purified by flash column chromatography (2% EtOAc/DCM, *R_f* = 0.43, dry loading using celite) to give a pale-yellow solid (0.10 g, 67%). ¹H NMR (300 MHz, CDCl₃) δ 8.28 (d, *J* = 9.0 Hz, 2H), 8.10 (s, 1H), 7.89 (d, *J* = 9.0 Hz, 2H), 4.12 (s, 2H), 2.03 (apparent s, 3H, 1.75–1.58 (m, 6H), 1.53–1.52 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 170.1, 148.8, 147.6, 134.2, 128.0, 126.8, 123.9, 90.1 (2 alkyne C), 62.8, 40.3, 36.5, 34.4, 28.1. HRMS (ESI) calc'd for C₂₂H₂₂N₄O₃Na [M + Na]⁺: 413.1589, found: 413.1590.

3-(4-Nitrophenyl)-1-(1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)prop-2-yn-1-one (8D)

Synthesized according to **GP5** using **6A** (0.08 g, 0.40 mmol) and **7D** (0.05 g, 0.40 mmol) and purified using preparative thin layer chromatography by eluting 3 times (1% MeOH/DCM, *R_f* = 0.19) to give a pale-yellow solid (0.03 g, 19%).⁵³ ¹H NMR (300 MHz, CDCl₃) δ 8.27 (d, *J* = 9.0 Hz, 2H), 8.16 (s, 1H), 7.85 (d, *J* = 9.0 Hz, 2H), 4.33 (d, *J* = 7.2 Hz, 2H), 4.00–3.96 (m, 2H), 3.35 (td, *J* = 11.4, 2.1 Hz, 2H), 2.29–2.11 (m, 1H), 1.54–1.34 (m, 4H). ¹³C NMR (75 MHz, CDCl₃) δ 169.8, 148.7, 147.8, 134.1, 127.3, 126.6, 123.8, 90.1, 89.8, 67.1, 56.3, 36.2, 30.2. HRMS (EI) calc'd for C₁₇H₁₆N₄O₄ [M]⁺: 340.1172, found: 340.1149.

3-(4-Nitrophenyl)-1-(1-(4-(trifluoromethyl)benzyl)-1H-1,2,3-triazol-4-yl)prop-2-yn-1-one (8E)

Synthesized according to **GP5** using **6A** (0.08 g, 0.40 mmol) and **7E** (0.08 g, 0.40 mmol) and purified using flash chromatography (1% EtOAc/DCM, *R_f* = 0.21, dry loading using celite) to give a beige solid (0.11 g, 71%). ¹H NMR (300 MHz, CDCl₃) δ 8.27 (d, *J* = 9.0 Hz, 2H), 8.17 (s, 1H), 7.85 (d, *J* = 9.0 Hz, 2H), 7.67 (d, *J* = 8.1 Hz, 2H), 7.43 (d, *J* = 8.1 Hz, 2H), 5.69 (s, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 169.7, 148.8, 148.5, 137.2, 134.2, 131.7 (q, *J_{CF}* = 32.8 Hz), 128.6, 127.0, 126.6, 126.5 (q, *J_{CF}* = 3.7 Hz), 123.9, 123.7 (q, *J_{CF}* = 293.0 Hz), 90.4, 89.8, 54.1. ¹⁹F NMR (282 MHz, CDCl₃) δ –62.8. HRMS (EI) calc'd for C₁₉H₁₁F₃N₄O₃ [M]⁺: 400.0783, found: 400.0784.

1-(1-Hexyl-1H-1,2,3-triazol-4-yl)-3-(4-nitrophenyl)prop-2-yn-1-one (8F)

Synthesized according to **GP5** using **6A** (0.08 g, 0.40 mmol) and **7F** (0.05 g, 0.40 mmol) and purified using flash chromatography (0 → 3% EtOAc/DCM, 3% EtOAc/DCM *R_f* = 0.53, dry loading using celite) to give a pale-yellow solid (0.08 g, 61%). ¹H NMR (300 MHz, CDCl₃) δ 8.31 (d, *J* = 9.0 Hz, 2H), 8.22 (s, 1H), 7.91 (d, *J* = 9.0 Hz, 2H), 4.49 (t, *J* = 7.2 Hz, 2H), 2.04–1.95 (m, 2H), 1.43–1.29 (m, 6H), 0.92 (t, *J* = 7.2 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 169.9, 148.6, 147.9, 134.1, 126.7, 123.8, 90.0, 89.8, 51.0, 31.1, 30.1, 26.0, 22.4, 13.9. HRMS (EI) calc'd for C₁₇H₁₈N₄O₃ [M]⁺: 326.1379, found: 326.1357.

4-((4-(3-(4-Nitrophenyl)propioloyl)-1H-1,2,3-triazol-1-yl)methyl)benzonitrile (8G)

Synthesized according to GP5 using 6A (0.06 g, 0.32 mmol) and 7G (0.05 g, 0.32 mmol) and purified by flash column chromatography (5% EtOAc/DCM, R_f = 0.29, dry loading using celite) to give a pale-yellow solid (0.09 g, 77%). ^1H NMR (300 MHz, DMSO- d_6) δ 9.24 (s, 1H), 8.31 (d, J = 9.0 Hz, 2H), 8.01 (d, J = 9.0 Hz, 2H), 7.84 (d, J = 8.4 Hz, 2H), 7.49 (d, J = 8.4 Hz, 2H), 5.82 (s, 2H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 168.2, 148.6, 146.9, 140.7, 134.3, 132.8, 130.8, 128.8, 125.4, 118.5, 111.2, 89.5, 88.7, 52.7. HRMS (ESI) calc'd for $\text{C}_{38}\text{H}_{22}\text{N}_{10}\text{O}_6$ [$2\text{M} + \text{Na}$] $^+$: 737.1645, found: 737.1648.

1-(1-(2-Morpholinoethyl)-1H-1,2,3-triazol-4-yl)-3-(4-nitrophenyl)prop-2-yn-1-one (8H)

Synthesized according to GP5 using 6A (0.08 g, 0.40 mmol) and 7H (0.06 g, 0.40 mmol), and purified by flash column chromatography (2% MeOH/EtOAc, R_f = 0.44, dry loading using celite) to give a pale-yellow solid (0.08 g, 56%). ^1H NMR (400 MHz, CDCl_3) δ 8.38 (s, 1H), 8.27 (d, J = 9.0 Hz, 2H), 7.87 (d, J = 9.0 Hz, 2H), 4.56 (t, J = 6.0 Hz, 2H), 3.70 (t, J = 4.8 Hz, 2H), 2.87 (t, J = 6.0 Hz, 2H), 2.52 (t, J = 4.8 Hz, 4H). ^{13}C NMR (100 MHz, CDCl_3) δ 170.0, 148.8, 148.0, 134.1, 127.7, 126.8, 123.9, 90.1, 90.0, 66.9, 57.6, 53.5, 47.7. HRMS (ESI) calc'd for $\text{C}_{17}\text{H}_{18}\text{N}_5\text{O}_4$ [$\text{M} + \text{H}$] $^+$: 356.1359, found: 356.1320.

tert-Butyl 4-((4-(3-(4-nitrophenyl)propioloyl)-1H-1,2,3-triazol-1-yl)methyl)piperidine-1-carboxylate (8I)

Synthesized according to GP5 using 6A (0.10 g, 0.50 mmol) and 7I (0.12 g, 0.50 mmol), and purified by flash column chromatography (10–30% EtOAc/DCM, R_f (30% EtOAc/DCM) = 0.72, dry loading using celite) to give a yellow solid (0.15 g, 68%). ^1H NMR (400 MHz, CDCl_3) δ 8.26 (d, J = 8.8 Hz, 2H), 8.15 (s, 1H), 7.85 (d, J = 8.8 Hz, 2H), 4.32 (d, J = 7.2 Hz, 2H), 4.15–4.07 (m, 2H), 2.67 (t, J = 12.8 Hz, 2H), 2.18–2.06 (m, 1H), 1.63–1.56 (m, 2H), 1.43 (s, 9H), 1.27–1.17 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 169.8, 154.6, 148.7, 147.9, 134.1, 127.3, 126.6, 123.8, 90.2, 89.8, 79.8, 56.1, 43.2, 37.3, 29.4, 28.4. (ESI) calc'd for $\text{C}_{22}\text{H}_{25}\text{N}_5\text{O}_5\text{Na}$ [$\text{M} + \text{Na}$] $^+$: 462.1753, found: 462.1755.

3-Phenyl-1-(1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)prop-2-yn-1-one (8J)

Synthesized according to GP5 using 6B (0.06 g, 0.39 mmol) and 7D (0.06 g, 0.39 mmol), and purified by flash column chromatography (10% EtOAc/DCM, R_f = 0.26, dry loading using celite) to give a pale-yellow solid (0.07 g, 62%). ^1H NMR (300 MHz, CDCl_3) δ 8.15 (s, 1H), 7.75–7.71 (m, 2H), 7.51–7.39 (m, 2H), 4.33 (d, J = 7.2 Hz, 2H), 4.02–3.97 (m, 2H), 3.37 (td, J = 11.3, 3.7 Hz, 2H), 2.29–2.15 (m, 1H), 1.58–1.33 (m, 4H). ^{13}C NMR (75 MHz, CDCl_3) δ 170.2, 148.2, 133.5, 131.1, 128.6, 127.2, 119.9, 94.2, 87.1, 77.2, 77.0, 76.8, 67.1, 56.2, 36.2, 30.2. HRMS (EI) calc'd for $\text{C}_{17}\text{H}_{17}\text{N}_3\text{O}_2$ [M] $^+$: 295.1321, found: 295.1341.

3-(Perfluorophenyl)-1-(1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)prop-2-yn-1-one (8K)

Synthesized according to GP5 using 6C (0.08 g, 0.33 mmol), and 7D (0.05 g, 0.33 mmol), and purified by preparative thin layer chromatography (5% MeOH/DCM, R_f = 0.20, developed 3 $\times$) to give a pale-yellow solid (0.08 g, 63%). ^1H NMR (600 MHz, CDCl_3) δ 8.18 (s, 1H), 4.34 (d, J = 7.3 Hz, 2H), 4.03–3.97 (m, 2H), 3.37 (td, J = 11.7, 2. Hz, 2H), 2.31–2.15 (m, 1H), 1.57–1.35 (m, 4H). ^{13}C NMR (150 MHz, CDCl_3) δ 168.6, 149.1–149.0 (m), 147.5, 147.4–147.3 (m), 144.5–144.3 (m), 142.7–142.6 (m), 138.7–138.5 (m), 137.0–136.8 (m), 127.7, 97.6–97.4 (m), 96.4, 76.0, 67.1, 56.3, 36.2, 30.2. ^{19}F NMR (282 MHz, CDCl_3) δ -132.09–-132.23 (m, 2F), -146.59–-146.77 (m, 1F), -159.88–-160.04 (m, 2F). HRMS (EI) calc'd for $\text{C}_{17}\text{H}_{12}\text{F}_5\text{N}_3\text{O}_2$ [M] $^+$: 385.0850, found: 385.0839.

3-(4-Acetylphenyl)-1-(1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)prop-2-yn-1-one (8L)

Synthesized according to GP5 using 6D (0.08 g, 0.41 mmol) and 7D (0.06 g, 0.41 mmol), and purified by flash column chromatography (20–40% EtOAc/DCM, in 20% EtOAc/DCM R_f = 0.26, dry loading using celite) to give a pale-yellow solid (0.05 g, 36%). ^1H NMR (400 MHz, CDCl_3) δ 8.16 (s, 1H), 8.00 (d, J = 8.4 Hz, 2H), 7.81 (d, J = 8.4 Hz, 2H), 4.34 (d, J = 6.0 Hz, 2H), 3.41–3.33 (m, 2H), 3.37 (td, J = 11.7, 2.1 Hz, 2H), 2.64 (s, 3H), 2.29–2.17 (m, 1H), 1.56–1.36 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3) δ 197.1, 170.0, 148.0, 138.2, 133.4, 128.3, 127.1, 124.4, 92.1, 88.8, 67.1, 56.2, 36.2, 30.1, 26.7. HRMS (EI) calc'd for $\text{C}_{19}\text{H}_{19}\text{N}_3\text{O}_3$ [M] $^+$: 337.1426, found: 337.1429.

4-(3-Oxo-3-(1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)prop-1-yn-1-yl)benzonitrile (8M)

Synthesized according to GP5 using 6E (0.07 g, 0.39 mmol) and 7D (0.06 g, 0.39 mmol), and purified by flash column chromatography (20% EtOAc/DCM R_f = 0.29, dry loading using celite) to give a pale-yellow solid (0.05 g, 38%). ^1H NMR (600 MHz, CDCl_3) δ 8.16 (s, 1H), 7.81 (d, J = 8.7 Hz, 2H), 7.71 (d, J = 8.7 Hz, 2H), 4.34 (d, J = 7.2 Hz, 2H), 4.00 (m, 2H), 3.37 (td, J = 11.8, 2.3 Hz, 2H), 2.23 (m, 1H), 1.57–1.49 (m, 2H), 1.50–1.36 (m, 2H). ^{13}C NMR (150 MHz, CDCl_3) δ 169.9, 148.0, 133.7, 132.3, 127.3, 124.8, 118.0, 114.3, 90.7, 89.4, 77.3, 77.1, 76.9, 67.2, 56.4, 36.3, 30.3. HRMS (EI) calc'd for $\text{C}_{18}\text{H}_{16}\text{N}_4\text{O}_2$ [M] $^+$: 320.1273, found: 320.1240.

3-(4-(Methylsulfonyl)phenyl)-1-(1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)prop-2-yn-1-one (8N)

Synthesized according to GP5 using 6F (0.08 g, 0.34 mmol) and 7D (0.05 g, 0.34 mmol), and purified by flash column chromatography (40% EtOAc/DCM R_f = 0.25, dry loading using celite) to give a pale-yellow solid (0.07 g, 55%). ^1H NMR (300 MHz, CDCl_3) δ 8.21 (s, 1H), 8.03 (d, J = 8.7 Hz, 2H), 7.93 (d, J = 8.7 Hz, 2H), 4.38 (d, J = 7.2 Hz, 2H), 4.03 (m, 2H), 3.40 (td, J = 11.7, 2.6 Hz, 2H), 3.12 (s, 3H), 2.26 (m, 1H), 1.59–1.39

(m, 4H). ¹³C NMR (75 MHz, CDCl₃) δ 169.9, 147.9, 142.2, 133.9, 127.6, 127.3, 125.7, 90.6, 89.0, 67.1, 56.3, 44.4, 36.2, 30.1. HRMS (EI) calc'd for C₁₈H₁₉N₃O₄S [M]⁺: 373.1096, found: 373.1095.

1-((Tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)-3-((trifluoromethyl)sulfonyl)phenyl)prop-2-yn-1-one (8O)

Synthesized according to GP5 using 6G (0.08 g, 0.28 mmol) and 7D (0.04 g, 0.28 mmol), and purified by flash column chromatography (15% EtOAc/DCM *R_f* = 0.51, dry loading using celite) to give a beige solid (0.04 g, 33%). ¹H NMR (600 MHz, CDCl₃) δ 8.18 (s, 1H), 8.09 (d, *J* = 8.8 Hz, 2H), 7.97 (d, *J* = 8.8 Hz, 2H), 4.35 (d, *J* = 7.2 Hz, 2H), 4.00 (m, 2H), 3.37 (td, *J* = 11.6, 2.5 Hz, 2H), 2.32–2.11 (m, 1H), 1.59–1.27 (m, 4H). ¹³C NMR (150 MHz, CDCl₃) δ 169.6, 147.7, 134.1, 132.8, 130.8, 128.7, 127.3, 119.7 (q, *J*_{C,F} = 324.3 Hz), 90.3, 89.3, 67.1, 56.3, 36.2, 30.1. ¹⁹F NMR (282 MHz, CDCl₃) δ -77.95. HRMS (EI) calc'd for C₁₈H₁₆F₃N₃O₄S [M]⁺: 427.0814, found: 427.0787.

4-((4-(3-(4-(Methylsulfonyl)phenyl)propioloyl)-1H-1,2,3-triazol-1-yl)methyl)benzonitrile (8P)

Synthesized according to GP5 using 6F (0.06 g, 0.25 mmol) and 7G (0.04 g, 0.25 mmol), and purified by flash column chromatography (20% EtOAc/DCM *R_f* = 0.4, dry loading using celite) to give a white powder (0.07 g, 95%). ¹H NMR (300 MHz, CDCl₃) δ 8.19 (s, 1H), 8.02 (d, *J* = 8.2 Hz, 2H), 7.92 (d, *J* = 8.2 Hz, 2H), 7.74 (d, *J* = 8.0 Hz, 2H), 7.43 (d, *J* = 8.0 Hz, 2H), 5.71 (s, 2H), 3.11 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 169.5, 148.5, 142.2, 138.4, 133.9, 133.1, 128.6, 127.6, 126.8, 125.5, 117.8, 113.4, 90.8, 88.8, 77.2, 77.0, 76.8, 53.9, 44.3. HRMS (EI) calc'd for C₂₀H₁₄N₄O₃S [M]⁺: 390.0787, found: 390.0778.

4-(3-(1-(4-Cyanobenzyl)-1H-1,2,3-triazol-4-yl)-3-oxoprop-1-yn-1-yl)-N,N-dimethylbenzenesulfonamide (8Q)

Synthesized according to GP5 using 6H (0.12 g, 0.44 mmol) and 7G (0.07 g, 0.44 mmol), and purified by flash column chromatography (15% EtOAc/DCM *R_f* = 0.53, dry loading using celite) to give a white powder (0.13 g, 72%). ¹H NMR (300 MHz, CDCl₃) δ 8.20 (s, 1H), 7.88 (d, *J* = 8.7 Hz, 2H), 7.83 (d, *J* = 8.7 Hz, 2H), 7.73 (d, *J* = 8.6 Hz, 2H), 7.43 (d, *J* = 8.6 Hz, 2H), 5.71 (s, 2H), 2.76 (s, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 169.6, 148.5, 138.5, 137.7, 133.7, 133.1, 128.6, 127.7, 126.9, 124.3, 117.8, 113.4, 91.3, 88.6, 53.8, 37.8. HRMS (EI) calc'd for C₂₁H₁₇N₅O₃S [M]⁺: 419.1052, found: 419.1056.

5-((4-(3-(4-(Methylsulfonyl)phenyl)propioloyl)-1H-1,2,3-triazol-1-yl)methyl)picolinonitrile (8R)

Synthesized according to GP5 using 6G (0.06 g, 0.25 mmol) and 7J (0.04 g, 0.25 mmol), and purified by flash column chromatography (50% EtOAc/DCM *R_f* = 0.40, dry loading using celite) to give an off-white powder (0.05 g, 47%). ¹H NMR (600 MHz, DMSO-*d*₆) δ 9.25 (s, 1H), 8.83 (s, 1H), 8.09–87.99 (m, 6H), 5.91 (s, 2H), 3.30 (s, 3H). ¹³C NMR (150 MHz, DMSO-*d*₆) 168.3, 150.9, 146.9, 142.8,

137.6, 135.3, 133.8, 132.4, 130.8, 129.1, 127.6, 123.9, 117.3, 89.3, 88.5, 50.4, 43.1. HRMS (ESI) calc'd for C₁₉H₁₃N₅O₃Na [M + Na]⁺: 414.0637, found: 414.0649.

(1-((Tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl) methanol (9)

To a solution of propargyl alcohol (0.11 mL, 1.82 mmol, 1.0 eq.) and 7D (0.26 g, 1.82 mmol, 1.0 eq.) in 6 mL of *t*BuOH/H₂O (2:1) were added CuSO₄·5H₂O (0.02 g, 0.09 mmol, 5 mol%) and sodium ascorbate (0.04 g, 0.18 mmol, 10 mol%). The mixture was stirred vigorously for 16 h at R.T. and then diluted with H₂O (50 mL) and extracted with EtOAc (3 × 25 mL). The organic layer was washed with brine (50 mL), dried over MgSO₄, and concentrated *in vacuo* to give a pale yellow oil (0.17 g, 50%) which was used without further purification (100% EtOAc, *R_f* = 0.13). ¹H NMR (600 MHz, CDCl₃) δ 7.54 (s, 1H), 4.24 (d, *J* = 7.2 Hz, 2H), 4.08–3.90 (m, 2H), 3.36 (td, *J* = 11.8, 2.1 Hz, 2H), 2.17 (m, 1H), 1.65–1.46 (m, 2H), 1.40 (m, 2H). ¹³C NMR (150 MHz, CDCl₃) δ 147.8, 122.3, 67.2, 56.3, 55.8, 36.2, 30.2. HRMS (EI) calc'd for C₉H₁₅N₃O₂ [M]⁺: 197.1164, found: 197.1171.

1-((Tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazole-4-carbaldehyde (10)

To a solution of 9 (0.17 g, 0.91 mmol, 1.0 eq.) in acetone (10 mL) at 0 °C were added trichloroisocyanuric acid (0.19 g, 0.83 mmol, 1.05 eq.) and TEMPO (0.001 g, 0.008 mmol, 0.01 eq.). The reaction was allowed to gradually warm to R.T. over 3 h at which point TLC analysis confirmed completion (100% EtOAc, *R_f* = 0.68). The reaction was concentrated *in vacuo*, redissolved in DCM (50 mL) and washed with sat. NaHCO₃ (25 mL), 1 M HCl (25 mL), and then brine (50 mL). The organic layer was dried over MgSO₄ and concentrated *in vacuo* to give a yellow solid (0.16 g, >95%) which was used without further purification. ¹H NMR (600 MHz, CDCl₃) δ 10.16 (s, 1H), 8.10 (s, 2H), 4.34 (d, *J* = 7.2 Hz, 2H), 4.09–3.93 (m, 2H), 3.56–3.20 (td, *J* = 11.8, 2.1 Hz, 2H), 2.23 (m, 1H), 1.65–1.45 (m, 2H), 1.43 (m, 2H). ¹³C NMR (150 MHz, CDCl₃) δ 185.1, 147.7, 125.5, 67.1, 56.2, 36.1, 30.1. HRMS (EI) calc'd for C₉H₁₃N₃O₂ [M]⁺: 195.1008, found: 195.1011.

1-((1-((Tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)prop-2-yn-1-ol (11)

In a flamed dried flask under N₂, 10 (0.10 g, 0.51 mmol, 1.0 eq.) was dissolved in anhydrous THF (8 mL) and the solution was cooled to -78 °C. Ethynylmagnesium bromide (0.5 M in THF) (2.00 mL, 1.02 mmol, 2.0 eq.) was then added dropwise. The reaction was stirred for 4 h, then quenched with H₂O (10 mL) and sat. NH₄Cl (5 mL), and extracted with EtOAc (3 × 50 mL). The organic layer was dried over MgSO₄ and concentrated *in vacuo*. The resulting solid was purified by flash column chromatography (50% EtOAc/DCM, *R_f* = 0.29, dry loading using celite) to give a yellow oil (0.08 g, 75%). ¹H NMR (600 MHz, CDCl₃) δ 7.56 (s, 1H), 5.64 (d, *J* = 3.0 Hz, 1H), 4.18 (d, *J* = 7.2 Hz, 2H), 3.99–3.83 (m, 2H), 3.29 (td, *J* =

11.8, 2.2 Hz, 2H), 2.56 (d, J = 2.3 Hz, 1H), 2.11 (m, 1H), 1.53–1.40 (m, 2H), 1.34 (m, 2H). ^{13}C NMR (150 MHz, CDCl_3) δ 146.8, 121.1, 81.0, 72.9, 66.1, 55.9, 54.9, 35.2, 29.2, 29.2. HRMS (EI) calc'd for $\text{C}_{11}\text{H}_{15}\text{N}_3\text{O}_2$ $[\text{M}]^+$: 221.1164, found: 221.1159.

1-(1-((Tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)prop-2-yn-1-one (12)

To a solution of **11** (0.08 g, 0.36 mmol, 1.0 eq.) in acetone (5 mL) at 0 °C were added trichloroisocyanuric acid (0.09 g, 0.38 mmol, 1.05 eq.) and TEMPO (0.002 g, 0.004 mmol, 0.01 eq.). The reaction was allowed to gradually warm to R.T. over 3 h. The reaction was concentrated *in vacuo*, redissolved in DCM (10 mL) and washed with sat. NaHCO_3 (10 mL), 1 M HCl (10 mL), and then brine (20 mL). The organic layer was dried over MgSO_4 and concentrated *in vacuo* and purified by flash column chromatography (30% EtOAc/DCM, R_f = 0.62) to give a white solid (0.06 g, 75%). ^1H NMR (600 MHz, CDCl_3) δ 8.15 (s, 1H), 4.34 (d, J = 7.2 Hz, 2H), 4.03–3.93 (m, 2H), 3.53 (s, 1H), 3.38 (td, J = 11.9, 2.1 Hz, 2H), 2.23 (m, 1H), 1.55 (m, 2H), 1.44 (m, 2H). ^{13}C NMR (150 MHz, CDCl_3) δ 169.3, 147.5, 127.4, 81.4, 80.2, 67.1, 56.2, 36.1, 30.1. HRMS (EI) calc'd for $\text{C}_{11}\text{H}_{13}\text{N}_3\text{O}_2$ $[\text{M}]^+$: 219.1008, found: 219.1008.

4-((4-(1-Hydroxy-3-(4-(methylsulfonyl)phenyl)prop-2-yn-1-yl)-1H-1,2,3-triazol-1-yl)methyl)benzonitrile (13)

To a solution of **8P** (0.03 g, 0.06 mmol, 1.0 eq.) in MeOH (2 mL) at R.T. was added $\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$ (0.05 g, 0.13, 2.0 eq.) and the mixture was stirred for 10 min before the addition of NaBH_4 (0.005 g, 0.128 mmol, 2.0 eq.) and stirring for another 1 h. The reaction mixture was then concentrated *in vacuo*, diluted with EtOAc (20 mL) and washed with 1 M HCl (2 $\times$ 10 mL) and brine (20 mL). The organic layer was then dried over MgSO_4 and concentrated *in vacuo* to give a yellow solid which was purified by preparative thin layer chromatography (2.5% MeOH/DCM R_f = 0.13, developed 3 $\times$) to give a white powder (0.02 g, 60%, mixture of enantiomers).⁵³ ^1H NMR (500 MHz, CDCl_3) δ 7.93 (d, J = 8.7 Hz, 2H), 7.77–7.68 (m, 3H), 7.66 (d, J = 8.7 Hz, 2H), 7.42 (d, J = 8.4 Hz, 2H), 5.99 (s, 1H), 5.67 (s, 2H), 3.20 (s, 1H), 3.10 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 148.4, 140.4, 139.3, 133.0, 132.6, 128.5, 127.8, 127.4, 121.7, 118.0, 113.0, 90.8, 84.0, 57.7, 53.6, 44.4. HRMS (ESI) calc'd for $\text{C}_{20}\text{H}_{16}\text{N}_4\text{O}_3\text{SNa}$ $[\text{M} + \text{Na}]^+$: 415.0837, found: 415.0841.

Ethyl 3-(4-(methylsulfonyl)phenyl)propionate (14)

In a flame dried round bottom flask filled with N_2 were added **1G** (0.50 g, 1.77 mmol, 1.0 eq.), $\text{PdCl}_2(\text{PPh}_3)_2$ (0.03 g, 0.04 mmol, 2 mol%), CuI (0.01, 0.07 mmol, 4 mol%), and K_2CO_3 (0.50 g, 3.54 mmol, 2.0 eq.). The solids were dissolved in 5 mL of anhydrous THF followed by the addition of ethyl propionate (0.72 mL, 7.08 mmol, 4.0 eq.). The reaction was heated to reflux and stirred for 24 h. The reaction was concentrated *in vacuo* and then diluted with H_2O (100 mL) and EtOAc (50 mL) and filtered through celite. The layers of

the filtrate were separated, and the aqueous layer was extracted with EtOAc (2 $\times$ 50 mL). The combined organic layer was washed brine (100 mL) followed by drying over MgSO_4 and concentrating *in vacuo* to give a brown solid. The residue was purified by flash column chromatography (40% EtOAc/Hex, R_f = 0.41, dry loading using celite) to give a brown solid (0.35 g, 77%). ^1H NMR (600 MHz, CDCl_3) δ 7.96 (d, J = 8.7 Hz, 2H), 7.76 (d, J = 8.7 Hz, 2H), 4.32 (q, J = 7.2 Hz, 2H), 3.07 (s, 3H), 1.37 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, CDCl_3) δ 153.3, 141.9, 133.5, 127.6, 125.4, 83.3, 83.0, 62.5, 44.3, 14.0. HRMS (EI) calc'd for $\text{C}_{12}\text{H}_{12}\text{O}_4\text{S}$ $[\text{M}]^+$: 252.0456, found: 252.0435.

3-(4-(Methylsulfonyl)phenyl)propionic acid (15)

To a solution of **14** (0.33 g, 1.31 mmol, 1.0 eq.) in acetone/ H_2O (5:1 mL) was added $\text{LiOH} \cdot \text{H}_2\text{O}$ (0.22 g, 5.24 mmol, 4.0 eq.). The reaction was stirred for 4 h, concentrated *in vacuo*, and then diluted with sat. Na_2CO_3 (20 mL). The aqueous layer was washed with DCM (3 $\times$ 10 mL), acidified to pH $\approx$ 1, and extracted with EtOAc ((3 $\times$ 20 mL). The combined organic layer was washed brine (50 mL) followed by drying over MgSO_4 and concentrating *in vacuo* to give a brown solid that was carried forward without further purification (40% EtOAc/Hex, R_f = 0) (0.22 g, >95%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.01 (d, J = 8.7 Hz, 2H), 7.90 (d, J = 8.7 Hz, 2H), 3.29 (s, 3H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 153.9, 142.2, 133.3, 127.4, 124.1, 83.9, 82.1, 43.1. HRMS (EI) calc'd for $\text{C}_{10}\text{H}_8\text{O}_4\text{S}$ $[\text{M}]^+$: 224.0143, found: 224.0118.

1-(1H-Benzo[d][1,2,3]triazol-1-yl)-3-(4-(methylsulfonyl)phenyl)prop-2-yn-1-one (16)

To a solution of benzotriazole (0.21 g, 1.80 mmol, 4.0 eq.) in anhydrous DCM (3 mL) was added SOCl_2 (0.03 mL, 0.45 mmol, 1.0 eq.) and the mixture was stirred for 30 min at R.T. followed by the addition of **15** (0.10 g, 0.45 mmol, 1.0 eq.). The reaction mixture was stirred for 3 h and then diluted with EtOAc (20 mL), filtered over celite, and washed with 2 M NaOH (3 $\times$ 10 mL). The organic layer was concentrated *in vacuo* to give a yellow solid which was washed with Et $_2\text{O}$ to give a white powder (0.04 g, 51%) (50% EtOAc/Hex R_f = 0.51). ^1H NMR (400 MHz, CDCl_3) δ 8.31 (d, J = 8.2 Hz, 1H), 8.18 (d, J = 8.2 Hz, 1H), 8.06 (d, J = 8.7 Hz, 2H), 7.98 (d, J = 8.7 Hz, 2H), 7.73 (t, J = 8.2 Hz, 1H), 7.58 (t, J = 8.2 Hz, 1H), 3.11 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 149.9, 146.5, 142.9, 134.3, 131.0, 130.9, 127.9, 127.1, 124.8, 120.7, 114.3, 92.5, 83.4, 44.5. HRMS (ESI) calc'd for $\text{C}_{16}\text{H}_{11}\text{N}_5\text{O}_3\text{S}$ $[\text{M} + \text{Na}]^+$: 348.0419, found: 348.0389.

3-(4-(Methylsulfonyl)phenyl)-N-(prop-2-yn-1-yl)propionamide (17)

To a solution of **15** (0.10 g, 0.45 mmol, 1.1 eq.) and propargyl amine (0.03 mL, 0.41 mmol, 1.0 eq.) in anhydrous DCM (3 mL) under N_2 was added a solution of DCC (0.09 g, 0.25 mol, 1.1 eq.) and DMAP (0.001 g, 0.008 mmol, 2 mol%) in anhydrous DCM (2 mL). The reaction was stirred at R.T. for

3 h and then diluted EtOAc (20 mL) and filtered over celite. The organic layer was washed with 5% AcOH (3 × 10 mL), brine (20 mL), sat. NaHCO₃ (3 × 10 mL), and then brine (20 mL) again. The combined organic layer was dried over MgSO₄ and concentrated *in vacuo* to give a brown solid that was purified by flash column chromatography (50% EtOAc/Hex, *R_f* = 0.18) to give a pale-yellow solid (0.05 g, 42%). ¹H NMR (500 MHz, CDCl₃ + CD₃OD) δ 7.93 (d, *J* = 6.5 Hz, 2H), 7.72 (d, *J* = 6.5 Hz, 2H), 4.14–4.00 (m, 1H), 3.47–3.37 (m, 2H), 3.13 (s, 4H), 1.69–1.66 (m, 1H). ¹³C NMR (125 MHz, CDCl₃ + CD₃OD) δ 152.5, 141.3, 133.2, 127.5, 125.9, 85.2, 82.7, 78.3, 71.9, 44.2, 33.7. HRMS (ESI) calc'd for C₁₃H₁₁O₃S [M]⁺: 261.0460, found: 261.0466.

3-(4-(Methylsulfonyl)phenyl)-N-((1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl)propionamide (18)

To a solution of 17 (0.05 g, 0.19 mmol, 1.0 eq.) and 7D (0.03 g, 0.19 mmol, 1.0 eq.) in *t*BuOH/H₂O (2:2 mL) were added CuSO₄·5H₂O (0.003 g, 0.009 mmol, 5 mol%) and sodium ascorbate (0.004 g, 0.019 mmol, 10 mol%). The reaction mixture was stirred vigorously for 16 h and was then concentrated *in vacuo* to give a yellow solid, which was purified by flash column chromatography (2% MeOH/EtOAc *R_f* = 0.23, dry loading using celite) to give a white powder (0.04 g, 51%). ¹H NMR (600 MHz, CDCl₃) δ 7.98 (d, *J* = 8.6 Hz, 2H), 7.74 (d, *J* = 8.6 Hz, 2H), 7.64 (s, 1H), 6.97 (s, 1H), 4.67 (d, *J* = 5.7 Hz, 2H), 4.28 (d, *J* = 7.1 Hz, 2H), 4.02 (dd, *J* = 11.4, 2.9 Hz, 2H), 3.40 (td, *J* = 11.8, 2.3 Hz, 2H), 3.11 (s, 3H), 2.26–2.16 (m, 1H), 1.58–1.54 (m, 2H), 1.49–1.39 (m, 2H). ¹³C NMR (150 MHz, CDCl₃) δ 152.6, 143.4, 141.6, 133.3, 127.6, 125.8, 122.9, 85.4, 82.7, 67.1, 55.9, 44.3, 36.2, 35.3, 30.3. HRMS (ESI) calc'd for C₁₉H₂₂N₄O₄SNa [M + Na]⁺: 425.1259, found: 425.1241.

Ethyl 1-(4-cyanobenzyl)-1H-1,2,3-triazole-4-carboxylate (19A)

To a solution of 7G (0.20 g, 1.26 mmol, 1.0 eq.), ethyl propionate (0.13 mL, 1.26 mmol, 1.0 eq.), and CuI (0.24 g, 1.26 mmol, 1.0 eq.) in anhydrous MeCN (10 mL) under N₂ at R.T. was added DIPEA (0.22 mL, 1.26 mmol, 1.0 eq) dropwise and the reaction was stirred for 16 h. The reaction mixture was then diluted with DCM (20 mL) and washed with 1 M HCl (2 × 15 mL), brine (20 mL), sat. NH₄OH (4 × 15 mL, until the aqueous phase was no longer blue), and brine (20 mL) again. The organic layer was then dried over MgSO₄ and concentrated *in vacuo* to give a brown solid that was purified by flash column chromatography (10% EtOAc/DCM *R_f* = 0.47, dry loading using celite) to give the title compound as white fluffy needles (0.35 g, 78%). ¹H NMR (500 MHz, CDCl₃) δ 8.05 (s, 1H), 7.69 (d, *J* = 8.4 Hz, 2H), 7.37 (d, *J* = 8.4 Hz, 2H), 5.65 (s, 2H), 4.41 (q, *J* = 7.1 Hz, 2H), 1.39 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 160.6, 141.2, 139.0, 133.2, 128.7, 127.6, 118.1, 113.4, 77.4, 77.2, 76.9, 61.6, 53.8, 14.4. HRMS (ESI) calc'd for C₁₃H₁₂N₄O₂Na [M + Na]⁺: 279.0858, found: 279.0828.

1-(4-Cyanobenzyl)-1H-1,2,3-triazole-4-carboxylic acid (20A)

To a solution of 19A (0.10 g, 0.40 mmol, 1.0 eq.) in acetone/H₂O (5:1 mL) was added LiOH·H₂O (0.07 g, 1.60 mmol, 4.0 eq.). The reaction was stirred for 4 h, concentrated *in vacuo*, and then diluted with sat. Na₂CO₃ (10 mL). The aqueous layer was washed with DCM (3 × 10 mL), acidified to pH ≈ 1, and extracted with EtOAc (3 × 20 mL). The combined organic layer was washed brine (20 mL) followed by drying over MgSO₄ and concentrating *in vacuo* to give a white powder that was carried forward without further purification (50% EtOAc/Hex, *R_f* = 0) (0.08 g, 88%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.82 (s, 1H), 7.86 (d, *J* = 8.7 Hz, 2H), 7.49 (d, *J* = 8.7 Hz, 2H), 5.77 (s, 2H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 162.0, 141.4, 140.5, 129.9, 129.3, 119.0, 111.5, 52.9. HRMS (ESI) calc'd for C₁₁H₈N₄O₂Na [M + Na]⁺: 251.0545, found: 251.0557.

tert-Butyl 1-(4-cyanobenzyl)-1H-1,2,3-triazol-4-yl)carbamate (21A)

To a suspension of 20A (0.83 g, 3.65 mmol, 1.0 eq.) in *t*BuOH (20 mL) were added NEt₃ (0.63 mL, 4.40 mmol, 1.2 eq.) and DPPA (0.95 mL, 4.40 mmol, 1.2 eq.). The reaction was stirred for 24 h at 90 °C and then concentrated *in vacuo*. The crude residue was purified by flash column chromatography (70% EtOAc/Hex *R_f* = 0.71, dry loading using celite) to give a white powder (0.41 g, 38%). ¹H NMR (500 MHz, CDCl₃) δ 7.60 (s, 1H), 7.56 (d, *J* = 8.3 Hz, 2H), 7.25 (d, *J* = 8.3 Hz, 2H), 7.19 (s, 1H), 5.41 (s, 2H), 1.39 (s, 9H). ¹³C NMR (125 MHz, CDCl₃) δ 152.1, 145.0, 139.7, 132.9, 128.5, 118.2, 112.9, 111.6, 81.5, 53.9, 28.2. HRMS (ESI) calc'd for C₁₅H₁₇N₅O₂Na [M + Na]⁺: 322.1280, found: 322.1304.

4-((4-Amino-1H-1,2,3-triazol-1-yl)methyl)benzonitrile hydrochloride (22A)

A solution of 21A (0.20 g, 0.67 mmol) in DCM:4 M HCl in Dioxanes (2 mL:2 mL) was stirred at R.T. for 16 h. The reaction was concentrated, and the residue was washed with Et₂O to give a yellow solid (0.13 g, >95%) which was used without further purification. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.27 (s, 1H), 7.88 (d, *J* = 8.3 Hz, 2H), 7.50 (d, *J* = 8.3 Hz, 2H), 5.75 (s, 2H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 141.0, 139.7, 132.8, 128.8, 118.5, 117.3, 111.1, 52.8. HRMS (ESI) calc'd for C₁₀H₁₀N₅ [M]⁺: 200.0936, found: 200.0925.

Ethyl 1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazole-4-carboxylate (19B)

To a solution of 7D (0.40 g, 2.83 mmol, 1.0 eq.), ethyl propionate (0.30 mL, 2.83 mmol, 1.0 eq.), and CuI (0.54 g, 2.83 mmol, 1.0 eq.) in anhydrous MeCN (10 mL) under N₂ at R.T. was added DIPEA (0.50 mL, 2.83 mmol, 1.0 eq.) dropwise and the reaction was stirred for 16 h. The reaction mixture was then diluted with DCM (50 mL) and washed with 1 M HCl (2 × 25 mL), brine (25 mL), sat. NH₄OH (4 × 25 mL, until aqueous phase was no longer blue), and brine (25 mL) again. The organic layer was then dried over MgSO₄ and

concentrated *in vacuo* to give an orange solid which was washed with Et₂O to give the title compound as pale-orange powder (0.50 g, 73%) that was carried forward without further purification. ¹H NMR (500 MHz, CDCl₃) δ 8.05 (s, 1H), 4.42 (q, *J* = 7.1 Hz, 2H), 4.29 (d, *J* = 7.1 Hz, 2H), 4.04–3.90 (m, 2H), 3.35 (td, *J* = 11.7, 2.4 Hz, 2H), 2.19 (m, 1H), 1.61–1.14 (m, 7H). ¹³C NMR (125 MHz, CDCl₃) δ 160.9, 140.4, 127.9, 67.3, 61.5, 56.2, 36.3, 30.3, 14.5. HRMS (ESI) calc'd for C₁₁H₁₇N₃O₃Na [M + Na]⁺: 262.1184, found: 262.1168.

1-((Tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazole-4-carboxylic acid (20B)

To a solution of **19B** (0.50 g, 2.09 mmol, 1.0 eq.) in acetone/H₂O (20:5 mL) was added LiOH·H₂O (0.35 g, 8.36 mmol, 4.0 eq.). The reaction was stirred for 4 h, concentrated *in vacuo*, and then diluted with sat. Na₂CO₃ (25 mL). The aqueous layer was washed with DCM (3 × 25 mL), acidified to pH ≈ 1, and extracted with EtOAc (3 × 25 mL). The combined organic layer was washed with brine (25 mL), dried over MgSO₄, and concentrated *in vacuo* to give a white powder that was carried forward without further purification (50% EtOAc/Hex, *R*_f = 0) (0.21 g, 50%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.68 (s, 1H), 4.32 (d, *J* = 7.2 Hz, 2H), 3.82 (dd, *J* = 11.5, 2.6 Hz, 1H), 3.24 (td, *J* = 11.5, 2.6 Hz, 2H), 2.10 (m, 1H), 1.62–0.96 (m, 4H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 162.2, 140.0, 129.8, 66.8, 55.2, 35.8, 30.1. HRMS (ESI) calc'd for C₉H₁₃N₃O₃Na [M + Na]⁺: 234.0837, found: 234.0855.

tert-Butyl 1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl]carbamate (21B)

To a suspension of **20B** (0.90 g, 4.26 mmol, 1.0 eq.) in tBuOH (20 mL) were added NEt₃ (0.90 mL, 6.39 mmol, 1.2 eq.) and DPPA (1.40 mL, 6.39 mmol, 1.2 eq.). The reaction was stirred for 24 h at 90 °C and then concentrated *in vacuo*. The crude residue was purified by flash column chromatography (80% EtOAc/Hex *R*_f = 0.48, dry loading using celite) to give a white powder (0.59 g, 49%). ¹H NMR (600 MHz, CDCl₃) δ 7.71 (s, 1H), 7.15 (s, 1H), 4.17 (d, *J* = 7.2 Hz, 2H), 3.97 (apparent dd, *J* = 9.4, 4.6 Hz, 2H), 3.36 (td, *J* = 11.8, 2.2 Hz, 2H), 2.16 (m, 2H), 1.57–1.44 (m, 11H), 1.40 (m, 2H). ¹³C NMR (150 MHz, CDCl₃) δ 152.2, 144.2, 112.0, 81.3, 67.3, 56.3, 36.2, 30.3, 28.3. HRMS (ESI) calc'd for C₁₃H₂₂N₄O₃Na [M + Na]⁺: 305.1590, found: 305.1595.

1-((Tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-amine hydrochloride (22B)

A solution of **21B** (0.25 g, 0.89 mmol) in DCM:4 M HCl in dioxanes (3 mL:3 mL) was stirred at R.T. for 16 h. The reaction was concentrated, and the residue was washed with Et₂O to give a white powder (0.20 g, >95%) which was used without further purification. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.11 (s, 1H), 4.30 (d, *J* = 7.1 Hz, 2H), 3.91–3.74 (m, 2H), 3.26 (td, *J* = 11.5, 2.5 Hz, 2H), 2.09 (m, 1H), 1.44–1.37 (m, 2H),

1.34–1.11 (m, 2H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 139.8, 117.5, 66.8, 55.7, 35.9, 30.1. HRMS (ESI) calc'd for C₈H₁₅N₄ [M]⁺: 183.1246, found: 183.1248.

General procedure 6 (GP6): amide coupling with TCFH and NMI³⁰

To a solution of propiolic acids (1.0 eq., commercially available unless indicated otherwise), **22A** or **22B** (1.3 eq.), and NMI (3.5 eq.) in anhydrous MeCN (~0.1 M relative to acid) at R.T. was added TCFH (1.2 eq.). The reaction was stirred at R.T. for 3 h and then diluted with EtOAc (20 mL). The organic layer was washed with 5% AcOH (3 × 10 mL), brine (20 mL), sat. NaHCO₃ (3 × 10 mL), and brine again (20 mL). The organic layer was then dried over MgSO₄ and concentrated *in vacuo*. The crude residue was purified by flash column chromatography as described below.

N-(1-(4-Cyanobenzyl)-1H-1,2,3-triazol-4-yl)-3-(4-(methylsulfonyl)phenyl)propiolamide (23A)

Synthesized according to GP6 using **15** (0.08 g, 0.39 mmol) and **22A** (0.12 g, 0.50 mmol), and purified by flash column chromatography (40% EtOAc/Hex *R*_f = 0.40, dry loading using celite) to give a white powder (0.06 g, 36%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.08 (s, 1H), 8.39 (s, 1H), 8.03 (d, *J* = 8.5 Hz, 2H), 7.91 (d, *J* = 8.5 Hz, 2H), 7.86 (d, *J* = 8.4 Hz, 2H), 7.49 (d, *J* = 8.4 Hz, 2H), 5.72 (s, 2H), 3.28 (s, 3H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 148.8, 142.6, 142.1, 141.9, 141.4, 133.3, 142.8, 128.8, 127.5, 124.6, 118.6, 115.3, 110.9, 85.4, 83.4, 52.5, 43.2. HRMS (ESI) calc'd for C₂₀H₁₅N₅O₃Na [M + Na]⁺: 428.0793, found: 428.0811.

3-(4-(Methylsulfonyl)phenyl)-N-(1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)propiolamide (23B)

Synthesized according to GP6 using **15** (0.16 g, 0.70 mmol) and **22B** (0.20 g, 0.91 mmol), and the resulting residue was suspended in MeOH (10 mL) and DCM (1 mL) and then filtered to give the title compound as a pale-yellow solid which was used without further purification (0.02 g, 7%) (50% EtOAc/Hex *R*_f = 0.59). ¹H NMR (600 MHz, CDCl₃) δ 11.98 (s, 1H), 8.21 (s, 1H), 8.00 (d, *J* = 8.7 Hz, 2H), 7.87 (d, *J* = 8.7 Hz, 1H), 4.24 (d, *J* = 7.1 Hz, 2H), 3.79 (apparent d, *J* = 11.5 Hz, 3H), 3.25–3.17 (m, 5H), 2.10–2.02 (m, 1H), 1.53–1.12 (m, 4H). ¹³C NMR (150 MHz, CDCl₃) δ 149.2, 142.7, 142.6, 133.7, 128.0, 125.1, 115.8, 86.0, 83.8, 66.9, 55.4, 55.4, 43.7, 36.0, 30.2. HRMS (ESI) calc'd for C₁₈H₂₀N₄O₄Na [M + Na]⁺: 411.1103, found: 411.1108.

3-Phenyl-N-(1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)propiolamide (23C)

Synthesized according to GP6 using phenylpropionic acid (0.07 g, 0.46 mmol) and **22B** (0.12 g, 0.55 mmol), and the resulting residue was suspended in MeOH (5 mL) and DCM (0.5 mL) and then filtered to give the title compound as a pale-yellow solid which was used without further purification

(0.03 g, 21%) (50% EtOAc/Hex R_f = 0.65). ^1H NMR (600 MHz, DMSO- d_6) δ 8.21 (s, 1H), 7.80–7.52 (m, 2H), 7.60–7.23 (m, 3H), 4.27 (d, J = 7.1 Hz, 2H), 3.83 (apparent dd, J = 12.0, 4.1 Hz, 2H), 3.25 (td, J = 11.7, 2.3 Hz, 2H), 2.09 (ddd, J = 11.3, 7.4, 4.0 Hz, 1H), 1.49–1.05 (m, 4H). ^{13}C NMR (150 MHz, CDCl_3) δ 149.7, 142.9, 132.9, 131.2, 129.5, 120.0, 115.6, 85.8, 83.8, 66.9, 55.4, 36.0, 30.2. HRMS (ESI) calc'd for $\text{C}_{17}\text{H}_{18}\text{N}_4\text{O}_2\text{Na}$ [$\text{M} + \text{Na}$] $^+$: 333.1327, found: 333.1337.

***N*-(1-((Tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)but-2-ynamide (23D)**

Synthesized according to GP6 using 2-butyric acid (0.02 g, 0.27 mmol) and 22B (0.07 g, 0.35 mmol), and purified by flash column chromatography (50% EtOAc/Hex R_f = 0.54, dry loading using celite) to give a white powder (0.02 g, 31%). ^1H NMR (300 MHz, CDCl_3) δ 8.70 (s, 1H), 7.97 (s, 1H), 4.20 (d, J = 7.2 Hz, 2H), 3.98 (apparent dd, J = 12.2, 4.3 Hz, 2H), 3.36 (td, J = 11.7, 2.4 Hz, 2H), 2.16 (ddd, J = 11.4, 7.9, 4.0 Hz, 1H), 1.53–1.30 (m, 4H). ^{13}C NMR (150 MHz, CDCl_3) δ 149.8, 142.8, 114.3, 86.4, 74.4, 67.4, 56.5, 36.3, 30.4, 4.0. HRMS (ESI) calc'd for $\text{C}_{12}\text{H}_{16}\text{N}_4\text{O}_2\text{Na}$ [$\text{M} + \text{Na}$] $^+$: 271.1171, found: 271.1192.

***N*-(1-((Tetrahydro-2H-pyran-4-yl)methyl)-1H-1,2,3-triazol-4-yl)propionamide (23E)**

Synthesized according to GP6 using propionic acid (0.02 mL, 0.30 mmol) and 22B (0.08 g, 0.37 mmol), and purified by flash column chromatography (50% EtOAc/Hex R_f = 0.51, dry loading using celite) to give a pale-yellow solid (0.03 g, 43%). ^1H NMR (600 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$) δ 7.95 (s, 1H), 4.15 (d, J = 7.2 Hz, 3H), 3.97–3.82 (m, 2H), 3.33–3.29 (m, 2H, overlapping with residual methanol), 3.07 (s, 1H), 2.09 (ddd, J = 11.1, 7.4, 3.8 Hz, 1H), 1.49–1.41 (m, 2H), 1.41–1.27 (m, 2H). ^{13}C NMR (150 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$) δ 149.1, 142.5, 114.8, 76.4, 75.5, 75.5, 67.1, 56.1, 35.9, 30.0. HRMS (ESI) calc'd for $\text{C}_{11}\text{H}_{14}\text{N}_4\text{O}_2\text{Na}$ [$\text{M} + \text{Na}$] $^+$: 257.1014, found: 257.1034.

***tert*-Butyl 4-(4-nitrobenzoyl)piperazine-1-carboxylate (24)**

4-Nitrobenzoic acid (0.49 g, 2.95 mmol, 1.1 eq.), HBTU (1.32 g, 3.48 mmol, 1.3 eq.), and DIPEA (1.40 mL, 8.04 mmol, 3.0 eq.) were stirred in DCM (25 mL) at R.T. for 15 min, after which *N*-Boc-piperazine (0.50 g, 2.68 mmol, 1.0 eq.) was added. The reaction was stirred for 3 h and then diluted with EtOAc (100 mL). The organic layer was washed with 5% AcOH (3 $\times$ 25 mL), brine (50 mL), sat. NaHCO_3 (50 mL), and brine (50 mL) again, and then dried over MgSO_4 and concentrated *in vacuo* to give a yellow solid (0.90 g, >95%), which was used without further purification. ^1H NMR (300 MHz, CDCl_3) δ 8.29 (d, J = 8.9 Hz, 2H), 7.57 (d, J = 8.9 Hz, 2H), 3.76–3.35 (m, 8H), 1.46 (s, 9H). ^{13}C NMR (75 MHz, CDCl_3) δ 167.3, 153.8, 147.8, 142.0, 128.3, 123.7, 79.2, 46.7 (2C), 41.4 (2C), 28.0. Characterization data are consistent with previously reported values.⁵⁴

(4-Nitrophenyl)(piperazin-1-yl)methanone hydrochloride (25)

To a solution of 24 (0.50 g, 1.50 mmol, 1.0 eq.) in DCM (5 mL) was added 4 M HCl/dioxane (5 mL) and the reaction was stirred for 3 h at R.T. Upon completion, the reaction was concentrated *in vacuo* and the resulting solid was washed with DCM (3 $\times$ 10 mL) to give a white solid (0.37 g, 93%). ^1H NMR (300 MHz, DMSO- d_6) δ 9.41 (br s, 2H), 8.31 (d, J = 8.9 Hz, 2H), 7.75 (d, J = 8.9 Hz, 2H), 3.85 (apparent br s, 2H), 3.50 (apparent br s, 2H), 3.18–3.12 (m, 4H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 168.2, 148.5, 142.2, 128.2, 124.1, 49.0, 46.6, 46.0, 43.5. Characterization data are consistent with previously reported values.⁵⁴

***tert*-Butyl (2-(4-(4-nitrobenzoyl)piperazin-1-yl)-2-oxoethyl) carbamate (26)**

N-Boc-glycine (0.25 g, 1.43 mmol, 1.1 eq.), HBTU (0.64 g, 1.69 mmol, 1.3 eq.), and DIPEA (0.90 mL, 5.20 mmol, 3.0 eq.) were stirred in DCM (15 mL) at R.T. for 15 min after which 25 (0.37 g, 1.30 mmol, 1.0 eq.) was added. The reaction was stirred for 3 h and then diluted with EtOAc (100 mL). The organic layer was washed with 5% AcOH (3 $\times$ 25 mL), brine (50 mL), sat. NaHCO_3 (50 mL), and brine (50 mL) again, and then dried over MgSO_4 and concentrated *in vacuo*. The residue was washed with hexanes (3 $\times$ 25 mL) to give a yellow solid (0.44 g, 85%), which was used without further purification. ^1H NMR (300 MHz, CDCl_3) δ 8.31 (d, J = 8.9 Hz, 2H), 7.59 (d, J = 8.9 Hz, 2H), 5.43 (br s, 1H), 3.99 (br s, 2H), 3.81–3.41 (m, 8H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, CDCl_3) δ 168.4, 167.5, 155.9, 148.8, 141.1, 128.3, 124.2, 80.1, 47.3, 44.3, 42.3 (3C), 28.5. HRMS (ESI) calc'd for $\text{C}_{18}\text{H}_{24}\text{N}_4\text{O}_6\text{Na}$ [$\text{M} + \text{Na}$] $^+$: 415.1576, found: 415.1594.

2-Amino-1-(4-(4-nitrobenzoyl)piperazin-1-yl)ethan-1-one (27)

26 (0.38 g, 0.97 mmol, 1.0 eq.) was dissolved in DCM (5.0 mL) and treated with TFA (1.00 mL, 5.90 mmol, 6.0 eq.) until the starting material was completely consumed by TLC (10% MeOH/DCM) in 3 h. The reaction mixture was diluted with DCM (15 mL) and washed with 0.5 M HCl (3 $\times$ 25 mL). The pH of the combined aqueous layer was adjusted to 9–10 using sat. K_2CO_3 and then extracted using DCM (3 $\times$ 35 mL). The combined organic layer was dried over MgSO_4 and concentrated *in vacuo* to give a white solid (0.12 g, 44%) (10% MeOH/DCM, R_f = 0.27). ^1H NMR (300 MHz, DMSO- d_6) δ 8.29 (d, J = 8.9 Hz, 2H), 7.70 (d, J = 8.9 Hz, 2H), 3.61–3.27 (m, 19H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 171.5, 167.3, 147.9, 142.0, 128.4, 123.8, 46.7, 43.3, 42.6 41.4. HRMS (EI) calc'd for $\text{C}_{13}\text{H}_{16}\text{N}_4\text{O}_4$ [M] $^+$: 292.1172, found: 292.1175.

***N*-(2-(4-(4-Nitrobenzoyl)piperazin-1-yl)-2-oxoethyl)acrylamide (28)**

27 (0.12 g, 0.35 mmol, 1.0 eq.) and DIPEA (0.20 mL, 1.10 mmol, 3.0 eq.) were dissolved in anhydrous DCM (5.0 mL) and cooled to 0 $^\circ\text{C}$. To this solution was added acryloyl chloride (0.0630 mL, 0.39 mmol, 1.1 eq.) and the reaction was stirred

under nitrogen for 3 h. The reaction was concentrated and purified by flash column chromatography (5% MeOH/EtOAc, R_f = 0.33) to give a white solid (0.12 g, 90%). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 8.31 (d, J = 8.9 Hz, 2H), 7.59 (d, J = 8.9 Hz, 2H), 6.67 (br s, 1H), 6.35–6.29 (m, 2H), 5.69 (dd, J = 9.9, 1.8 Hz, 1H), 4.18 (s, 2H), 3.81–3.45 (m, 8H). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 168.4, 167.0, 165.5, 148.8, 141.0, 130.3, 128.3, 127.3, 124.2, 47.2, 44.4, 42.2 (2C), 41.4. **HRMS (ESI)** calc'd for $\text{C}_{16}\text{H}_{18}\text{N}_4\text{O}_5\text{Na}$ [$\text{M} + \text{Na}$] $^+$: 369.1175, found: 369.1171.

tert-Butyl (2-(methoxy(methyl)amino)-2-oxoethyl)carbamate (29)

To a solution of *N*-Boc-Gly (2.00 g, 11.42 mmol, 1.0 eq.), *N*,*O*-dimethylhydroxylamine hydrochloride (1.23 g, 12.56, 1.1 eq.), and DIPEA (8.00 mL, 45.68 mmol, 4.0 eq.) in DCM (50 mL) at 0 °C were added EDCI-HCl (2.41 g, 12.56 mmol, 1.1 eq.) and DMAP (0.14 g, 1.14 mmol, 0.1 eq.). The reaction was allowed to warm to R.T. and stirred for 24 h. The reaction was diluted with EtOAc (150 mL) and washed with 1 M HCl (50 mL), brine (50 mL), sat. NaHCO_3 (2 $\times$ 50 mL), and brine again (50 mL). The organic layer was then dried over MgSO_4 and concentrated *in vacuo* to give the product as a white solid (1.50 g, 60%). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 5.26 (br s, 1H), 4.08–4.09 (d, J = 5.1 Hz, 2H), 3.71 (s, 3H), 3.20 (s, 3H), 1.45 (s, 9H). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 170.3, 156.0, 79.6, 61.5, 41.7, 32.4, 28.3. Characterization data are consistent with previously reported values.⁵⁵

tert-Butyl (2-oxobut-3-yn-1-yl)carbamate (30)

In a flame-dried round-bottom flask flushed with nitrogen, **29** (0.97 g, 4.60 mmol, 1.0 eq.) was dissolved in anhydrous THF (80 mL) and the solution was cooled to –78 °C. Ethynyl magnesium bromide (0.5 M in THF) (37.00 mL, 18.40 mmol, 4.0 eq.) was then added dropwise and the reaction was allowed to warm to R.T. over 16 h. The reaction was quenched with cold 1 M NaHSO_4 (50 mL) and concentrated to removed majority of the THF. The aqueous layer was then extracted with EtOAc (3 $\times$ 100 mL). The combined organic layer was washed with sat. NaHCO_3 (100 mL) and brine (100 mL), dried over MgSO_4 , and concentrated *in vacuo* to give an orange oil (0.83 g, >95%) which was used without further purification. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 5.16 (br s, 1H), 4.21–4.19 (d, J = 5.1 Hz, 2H), 3.38 (s, 1H), 1.48 (s, 9H). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 183.1, 155.6, 81.7, 80.3, 79.7, 52.2, 28.3. Characterization data are consistent with previously reported values.⁵⁵

tert-Butyl (2-(1-((adamantan-1-yl)methyl)-1H-1,2,3-triazol-4-yl)-2-oxoethyl)carbamate (31)

To a solution of **30** (0.50 g, 2.73 mmol, 1.0 eq.) and **7C** (0.52 g, 2.73 mmol, 1.0 eq.) in 1:1 *t*BuOH/ H_2O (14 mL) were added freshly prepared aqueous solutions of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (50 mg mL^{-1}) (0.70 mL, 0.14 mmol, 0.05 eq.) and 1 M sodium ascorbate (0.55 mL, 0.55 mmol, 0.2 eq.). The reaction mixture was stirred vigorously overnight, at which point the reaction was still in complete by TLC. Another 0.2 mL of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

solution and 0.1 mL of sodium ascorbate solution were added, and the reaction was stirred for another 3 h, after which the reaction mixture was diluted with water (25 mL) and extracted with EtOAc (3 $\times$ 20 mL). The combined organic layer was dried over MgSO_4 and concentrated *in vacuo* to give a yellow oil which was purified by flash column chromatography (dry loading with celite, 40% EtOAc/hexanes, R_f = 0.38) to give a white foam (0.46 g, 40%). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 8.01 (s, 1H), 5.32 (br s, 1H), 4.76–4.74 (d, J = 5.1 Hz, 2H), 4.07 (s, 2H), 2.00 (apparent s, 3H), 1.72–1.55 (m, 6H), 1.48 (apparent s, 6H), 1.46 (s, 9H). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 190.0, 155.9, 145.4, 127.0, 79.9, 62.6, 48.3, 40.2, 36.5, 34.3, 28.5, 28.1. **HRMS (ESI)** calc'd for $\text{C}_{16}\text{H}_{18}\text{N}_4\text{O}_5\text{Na}$ [$\text{M} + \text{Na}$] $^+$: 397.2216, found: 397.2203.

1-(1-((Adamantan-1-yl)methyl)-1H-1,2,3-triazol-4-yl)-2-aminoethan-1-one hydrochloride (32)

To a solution of **31** (0.24 g, 0.64 mmol, 1.0 eq.) in DCM (5 mL) was added 5 mL of HCl (4 M in dioxane). The reaction was stirred for 4 h at room temperature and then concentrated *in vacuo* to give an off-white solid which was washed with DCM (3 $\times$ 20 mL) and used without further purification (0.18 g, 89%). $^1\text{H NMR}$ (300 MHz, CD_3OD) δ 8.65 (s, 1H), 4.58 (s, 2H), 4.19 (s, 2H), 1.97 (apparent s, 3H), 1.73–1.61 (m, 6H), 1.55–1.54 (m, 6H). $^{13}\text{C NMR}$ (75 MHz, CD_3OD) δ 185.6, 128.5, 126.9, 61.7, 42.0, 39.8, 36.2, 33.8, 28.2. **HRMS (ESI)** calc'd for $\text{C}_{15}\text{H}_{23}\text{N}_4\text{O}$ [M] $^+$: 275.1872, found: 275.1890.

***N*-(2-(1-((Adamantan-1-yl)methyl)-1H-1,2,3-triazol-4-yl)-2-oxoethyl)acrylamide (33)**

A solution of **32** (0.15 g, 0.48 mmol, 1.0 eq.) and DIPEA (0.25 mL, 1.44 mmol, 3.0 eq.) in anhydrous DCM (7 mL) was stirred at 0 °C until complete dissolution of the amine. Acryloyl chloride (0.04 mL, 0.53 mmol, 1.1 eq.) was then added dropwise and the reaction was allowed to stir at R.T. for 3 h and then concentrated *in vacuo*. The crude product was purified by flash column chromatography (dry loading using celite, 100% EtOAc, R_f = 0.58) to give a white solid (0.11 g, 69%). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 8.05 (s, 1H), 6.61 (apparent s, 1H), 6.36–6.18 (m, 2H), 5.68 (dd, J = 9.6, 1.5 Hz, 1H), 4.94 (d, J = 4.8 Hz, 1H), 4.07 (s, 2H), 1.99 (apparent s, 3H), 1.71–1.54 (m, 6H), 1.49 (apparent s, 6H). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 189.3, 165.6, 145.2, 130.5, 127.2, 127.1, 62.6, 47.3, 40.2, 36.5, 34.3, 28.1. **HRMS (EI)** calc'd for $\text{C}_{18}\text{H}_{24}\text{N}_4\text{O}_2$ [M] $^+$: 328.1899, found: 328.1874.

1-(4-(Adamantane-1-carbonyl)piperazin-1-yl)-3-(4-nitrophenyl)prop-2-yn-1-one (36)

To a stirring solution of **35**, which was synthesized as previously described,²⁰ (0.19 g, 0.58 mmol, 1.1 eq.) and **34**, which was synthesized as previously described,²⁴ (0.10 g, 0.52 mmol, 1.0 eq.) in anhydrous DCM (10 mL) was added HBTU (0.32 g, 0.84 mmol, 1.6 eq.) and Hünig's base (0.36 mL, 2.09 mmol, 4.0 eq.). The reaction mixture was stirred overnight

at R.T, under N₂. The reaction was confirmed complete by TLC (10% MeOH/DCM). The reaction mixture was concentrated *in vacuo* and the solid was resuspended in EtOAc (30 mL). The organic phase was washed with 5% AcOH (3 × 25 mL), brine (25 mL), NaHCO₃ (25 mL) and then brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a yellow solid, which was purified by silica gel column chromatography (10% MeOH/DCM, *R_f* = 0.74, dry loading using celite), giving a pale-yellow solid (0.18 g, 80%). ¹H NMR (300 MHz, CDCl₃) δ 8.30–8.20 (d, *J* = 9.0 Hz, 2H), 7.77–7.67 (d, *J* = 9.0 Hz, 2H), 3.84–3.64 (m, 8H), 2.14–2.04 (m, 3H), 2.04–1.90 (m, 6H), 1.86–1.65 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.2, 152.3, 148.3, 133.2, 126.9, 123.8, 88.5, 84.5, 47.3, 45.3, 42.1, 39.2, 36.5, 28.4. HRMS (ESI) calc'd for C₂₄H₂₇N₃O₄Na [M + Na]⁺: 444.1894, found: 444.1899.

N-(2-(4-(Adamantane-1-carbonyl)piperazin-1-yl)-2-oxoethyl)-3-(4-nitrophenyl)propiolamide (38)

Compound 37, which was synthesized as previously described,²⁰ (0.10 g, 0.33 mmol, 1.0 eq.) and 34 (0.07 g, 0.36 mmol, 1.1 eq.) were stirred in anhydrous DCM (2 mL) under N₂ at 0 °C. A solution of DCC (0.07 g, 0.36 mmol, 1.1 eq.) and DMAP (0.001 g, 0.008 mmol, 2 mol%) in anhydrous DCM (2 mL) was added, the ice bath was removed and the reaction was stirred for 3 h. The mixture was filtered through celite and the filtrate was diluted with EtOAc (50 mL). The organic phase was washed with 5% AcOH (3 × 15 mL), brine (25 mL), NaHCO₃ (25 mL), and brine again (25 mL). The organic phase was dried over MgSO₄ and concentrated *in vacuo* to give a yellow solid, which was purified by silica gel column chromatography (8% MeOH/DCM, *R_f* = 0.42, dry loading using celite) (0.14 g, 88%). ¹H NMR (300 MHz, CDCl₃) δ 8.33–8.25 (d, *J* = 9.0 Hz, 2H), 7.78–7.72 (d, *J* = 9.0 Hz, 2H), 4.27–4.19 (d, *J* = 3.0 Hz, 2H), 3.87–3.66 (m, 6H), 3.57–3.39 (m, 2H), 2.15–2.08 (m, 3H), 2.06–2.00 (m, 6H), 1.86–1.67 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.12, 165.7, 152.2, 148.3, 133.4, 126.7, 86.2, 82.5, 45.2, 44.6, 41.8, 41.6, 39.1, 36.5, 28.4. HRMS (ESI) calc'd for C₂₆H₃₀N₄O₅Na [M + Na]⁺: 501.2126, found: 501.2114.

1-(4-(4-Nitrobenzyl)piperazin-1-yl)-3-(4-nitrophenyl)prop-2-yn-1-one (40)

39, which was synthesized as previously described,⁵⁶ (0.05 g, 0.25 mmol, 1.1 eq.), 34 (0.06 g, 0.23 mmol, 1.0 eq.), EDCI-HCl (0.05 g, 0.27 mmol, 1.2 eq.) and HOBT-H₂O (0.04 g, 0.27 mmol, 1.2 eq.) were stirred in dry DCM (2 mL) under N₂. After 24 h, the reaction mixture was diluted with DCM (15 mL) and washed with H₂O (2 × 15 mL), followed by brine (15 mL). The organic layer was then dried over MgSO₄ and concentrated *in vacuo*, to give a white solid, which was purified by silica gel column chromatography, giving a white solid (5% DCM/EtOAc, *R_f* = 0.52, dry loading using celite) (0.02 g, 20%). ¹H NMR (300 MHz, CDCl₃) δ 8.28–8.15 (m, 4H), 7.74–7.64 (d, *J* = 9 Hz, 2H), 7.62–7.43

(d, *J* = 9 Hz, 2H), 3.95–3.79 (m, 2H), 3.79–3.69 (m, 2H), 3.69–3.54 (s, 2H), 2.70–2.37 (m, 4H). ¹³C NMR (75 MHz, CDCl₃) δ 152.1, 148.2, 147.4, 145.5, 133.1, 129.5, 127.0, 123.8, 88.1, 84.8, 61.8, 52.4, 47.0, 41.6. HRMS (ESI) calc'd for C₂₀H₁₈N₄O₅ [M + H]⁺: 395.1357, found: 395.1387.

TG2 inhibition assay

Recombinant TG2 was expressed from *E. coli* and purified as described previously.⁵⁷ TG2 activity was determined according to a previously published colorimetric activity assay using the chromogenic substrate Cbz-Glu(γ-pnitrophenylester)Gly (AL5).⁵⁸ In order to determine irreversible inhibition parameters for each inhibitor, enzymatic assays were run under Kitz and Wilson conditions,^{25,26} in the presence of 100 μM AL5 substrate, in triplicate. A large excess of substrate was used to ensure that any curvature in the slope is not attributed to depletion of the substrate, but rather to time-dependent inhibition. Buffered solutions of 50 mM of 3-(4-morpholino)propanesulfonic acid (MOPS) (pH 6.9), 7.5 mM CaCl₂, 100 μM AL5, and various concentrations of inhibitor (from 0.25 to 1200 μM, depending on the inhibitor) were prepared in a 96-well polystyrene microplate with a final volume of 200 μL at 25 °C. AL5 and inhibitor stocks were prepared in DMSO ensuring that the final concentration of this co-solvent did not exceed 10% v/v. To initiate the enzymatic reaction, 5 mU mL⁻¹ (0.25 μM) TG2, or water for the blank, was added to the well and the formation of the hydrolysis product, *p*-nitrophenolate, was followed at 405 nm for 20 min (a period of time over which the positive control is linear and not being impacted by substrate depletion) using a BioTek Synergy 4 plate reader. Background AL5 hydrolysis was corrected for by subtracting the blank from each reaction. Observed first order rate constants of inactivation (*k_{obs}*) were obtained by fitting the inhibition data sets by non-linear regression to mono-exponential association eqn (1) using GraphPad Prism software.

$$\text{Abs}_t = \text{Abs}_{\text{max}}(1 - e^{-k_{\text{obs}}t}) \quad (1)$$

The rate constants measured at different inhibitor concentrations were then fitted by non-linear regression to a saturation kinetics model, using eqn (2).

$$k_{\text{obs}} = \frac{k_{\text{inact}}[I]}{[I] + K_I \left(1 + \frac{[S]}{K_M}\right)} \quad (2)$$

In order to correct for the competition with the assay substrate, AL5, the inhibitor concentrations were divided by α, which equals (1 + ([S]/K_M)), where K_M = 10 μM. Inhibition parameters, *k_{inact}* and *K_I*, were then extrapolated from the fitting.

In cases where a saturation plot could not be generated due to solubility constraints and only a few inhibitor

concentrations tested, a double-reciprocal plot of (eqn (2)) was made to estimate k_{inact} and K_i according to eqn (3).

$$\frac{1}{k_{\text{obs}}} = \frac{K_i}{k_{\text{inact}}} \cdot \frac{\alpha}{[I]} + \frac{1}{k_{\text{inact}}} \quad (3)$$

Simple linear regression was performed on the k_{obs} values measured at the lowest concentrations of each inhibitor for validation of k_{inact}/K_i ratios.

For reversible inhibitor **16**, initial rates of inhibition (v_i) were measured at various inhibitor concentrations instead of k_{obs} values. These rates were then normalized with respect to the uninhibited control (% v_i/v_0). These percent relative rate values were plotted against the inhibitor concentration on a logarithmic scale to generate a IC_{50} curve. An IC_{50} value was determined using four-parameter fitting eqn (4) in GraphPad Prism 9.

$$Y = \frac{\text{Top} - \text{Bottom}}{1 + \left(\frac{\text{IC}_{50}}{[I]}\right)^{\text{HillSlope}}} + \text{Bottom} \quad (4)$$

An estimated K_i value was then calculated using the Cheng–Prusoff eqn (5)⁵⁹

$$K_i = \frac{\text{IC}_{50}}{1 + \frac{[S]}{K_M}} \quad (5)$$

Isozyme selectivity

First, for each inhibitor, a single corrected inhibitor concentration ($[I]/\alpha$) was chosen, which would result in the complete inactivation of TG2 in under 5 min. Then for each isozyeme, the concentration of inhibitor was adjusted according to the value of α determined by the concentration and K_M value of the substrate of each isozyeme assay. In this way the effective inhibitor concentration ($[I]/\alpha$) remained constant for each isozyeme.²⁰

The activities of TG1 and TG6 were measured by a colorimetric assay using the substrate AL5. Assays were performed as previously described for TG2 under Kitz & Wilson conditions established for each transglutaminase isoform by varying the concentration of substrate to 112 μM and 436 μM of AL5 for TG1 and TG6, respectively. The reaction was initiated with the addition of enzyme, 0.10 μM TG1 or 0.32 μM TG6. Formation of the hydrolysis product, *p*-nitrophenolate, was followed at 405 nm for 100 min for TG1 or 60 min for TG6.¹⁶

The isopeptidase activities of activated TG3a and hFXIIIa (purchased from Zedira) were measured by a fluorescence-based assay based on the use of the peptidic FRET-quenched probe A101 (Zedira).^{16,32} The final assay mixture comprised 50 mM TRIS (pH 7.0), 10 mM CaCl_2 , 100 mM NaCl, 2.8 mM TCEP, 50 μM A101 and 14 mM H-Gly-OMe. The reaction was monitored at 25 °C using a BioTek Synergy 4 plate reader (Ex/Em: 318/413 nm). Enzymatic inhibition assays were run under Kitz and Wilson conditions, which were established for TG3a and FXIIIa at a substrate (A101) concentration of 50 μM using enzyme concentrations of 0.17 μM and 0.11 μM for

TG3a and FXIIIa, respectively. Experiments were completed at least in triplicate. A k_{obs} value was obtained for each isozyeme as described above.

GTP binding assay

GTP binding experiments were performed following a previously described protocol.³⁴ hTG2 (20 μg) was incubated at 25 °C for 30 min with or without an irreversible inhibitor (each at a concentration of $2 \times K_i$) with 15 mM CaCl_2 in 100 mM MOPS (pH = 6.91). The buffer was then exchanged *via* dialysis to 100 mM MOPS (pH = 7.0), 1 mM EGTA, and 5 mM MgCl_2 to remove calcium using a 14-kDa molecular weight cut-off membrane cuvette (purchased from Millipore-Sigma, Oakville, ON, Canada). The fluorescent, nonhydrolyzable GTP analogue BODIPY GTP- γ -S (purchased from Invitrogen, Waltham, MA, USA), whose fluorescence increases when bound to protein, was then added at a final concentration of 0.5 μM . Fluorescence was then measured on a microplate reader after 10 min of incubation (Ex/Em: 490/520 nm).

Intrinsic reactivity

HPLC traces were collected by Gilson-Mandel GXP271 high performance liquid chromatography (HPLC) with UV detection at 214 and 254 nm (Phenomenex Luna, 150 mm $\times$ 4.6 mm, 30 min, 1.5 mL min⁻¹ flow rate, 5–95% CH_3CN with 0.1% TFA in H_2O with 0.1% TFA, 30 min method).

Intrinsic reactions were monitored with a 10-fold excess of GSH for compounds **8N**, **18**, **23B**, **23C**, **23E** and a 100-fold excess for compounds **13** and **23D**, depending on the stability of the warhead. In 2 mL HPLC vials fitted with a pre-slit screw cap, 1250 μL of aqueous buffer was added (pH = 7.4, phosphate buffer for **8N**, **16**, **18**, **23B**, **23C**, **23E** and pH = 10.4 CAPS buffer for **13** and **23D**, buffer stability over 24 h was confirmed in all cases). Then, 150 μL of a 25- or 250-mM stock solution of GSH in the same buffer was added. The reactions were initiated by the addition of 150 μL of 2.5-mM warhead stock solution in DMSO to a final concentration of 0.25 mM with 10% v/v DMSO as co-solvent. The final concentration of GSH was either 2.5 or 25 mM. Once initiated, the HPLC vials were inverted, placed on the autosampler tray and an initial aliquot was taken immediately. The vials were incubated at 22 °C with aliquots taken at pre-determined time points over the course of at least 5–6 half lives. The disappearance or decrease in the area under the curve (AUC) of the chromatogram of each compound was measured in triplicate.

The decreasing AUC of the inhibitor peaks of the HPLC chromatograms were fitted to a mono-exponential decay model, with the constraint that the lower plateau = 0 (eqn (6)).

$$\text{AUC} = \text{AUC}_0(e^{-k_{\text{obs}}t}) \quad (6)$$

Second order rates constants were then calculated by dividing these pseudo-first order rate

constants by the concentration of excess thiol (eqn (7)).

$$k_2^{\text{calc}} = \frac{k_{\text{obs}}}{[\text{thiol}]} \quad (7)$$

The final, corrected second order rate constants were then calculated by dividing by the fraction of thiolate at the reaction pH, using the corresponding $\text{p}K_a$ value (8.7) of the thiol (eqn (8)).³⁵

$$k_2^{\text{corr}} = \frac{k_2^{\text{calc}}}{\left(\frac{K_a}{[\text{H}^+] + K_a}\right)} \quad (8)$$

For compound **16** (12 mM), due to limited aqueous solubility, a stability half-life ($t_{1/2}$) with 80 mM GSH was determined by ^1H NMR in DMSO- d_6 .²³

Molecular docking in molecular operating software (MOE)

Two crystals were selected as a target receptor enzyme, and were imported from their PDB files, namely 2Q3Z and 3S3S. The structure was prepared using the preparation tool from MOE. First, water molecules, salts and ions were removed from the structure. All hydrogen atoms were then added (electrostatics: $1/r^2$, dielectric: 2, solvent: 80, van der Waals: 12–6) and the protein structures were finally verified, and corrected manually, for any problems or warnings such as chain breaks, termini missing or unreasonable charges. Ligands were drawn in ChemDraw and imported to MOE; partial charges were calculated using a MMFF94x forcefield and the system was eventually minimized following a $0.0001 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$ gradient. After solvation, minimization was repeated.

The “compute” tool from MOE was used to perform docking analysis of each ligand by a *non-covalent* approach. Ligand placement was achieved using the Triangle Matcher protocol (London dG) to produce 30 poses. In addition, a Rigid Receptor refinement protocol was performed (GBVI/WSA dG) and a total of 5 to 15 final poses were obtained. Finally, the top pose (lowest S-score) was chosen and using the builder tool from MOE, the covalent bond between residue CYS277 and the warhead of the bound inhibitors were manually created, prior to minimization of the system ($0.001 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$).

Author contributions

Conceptualization: J. W. K. and L. K. M.; funding acquisition: J. W. K.; supervision: J. W. K.; investigation and methodology: L. K. M., N. M., J. E. B.; writing – original draft: L. K. M., writing – reviewing and editing: all authors.

Conflicts of interest

There are no conflicts of interest to declare.

List of abbreviations

AcOH	Acetic acid
Ad	Adamantane
Boc	<i>tert</i> -Butyloxycarbonyl
Cys	Cysteine
DCC	<i>N,N'</i> -Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMP	Dess–Martin Periodinane
DPPA	Diphenylphosphoryl azide
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
Et ₂ O	Diethyl ether
EtOAc	Ethyl acetate
EtOH	Ethanol
HBTU	<i>N,N,N',N'</i> -Tetramethyl- <i>O</i> -(1 <i>H</i> -benzotriazol-1-yl)uronium hexafluorophosphate
HOBt	Hydroxybenzotriazole
MeCN	Acetonitrile
MeOH	Methanol
Na Asc	Sodium Ascorbate
NEt ₃	Triethylamine
NMI	<i>N</i> -Methyl imidazole
R.T.	Room temperature
<i>t</i> BuOH	<i>tert</i> -butanol
TCFH	<i>N'</i> -tetramethylformamidine hexafluorophosphate
TCICA	Trichloroisocyanuric acid
TEMPO	2,2,6,6-Tetramethylpiperidinyloxy
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
THP	Tetrahydropyranyl

Data availability

The data supporting this article (kinetic graphs, HPLC traces, and NMR spectra) have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: <https://doi.org/10.1039/d5md00777a>.

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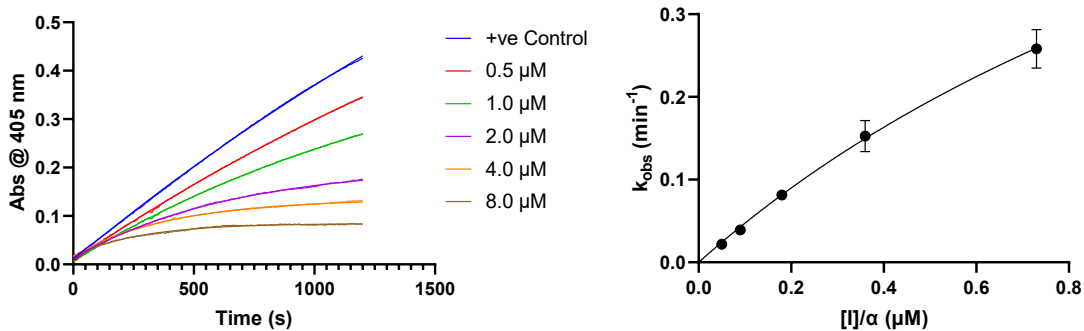
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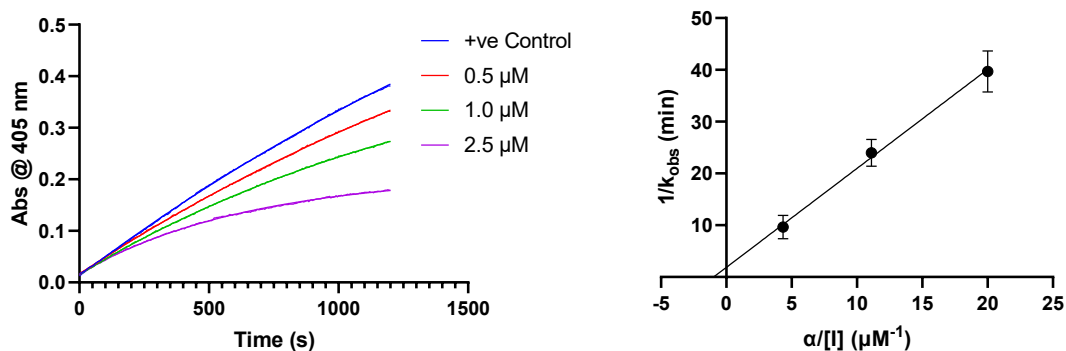
3.6. Chapter 3 Supporting Information

3.6.1. hTG2 Inhibition Kinetics Data

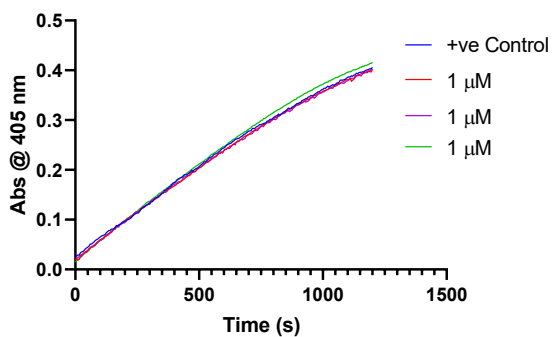
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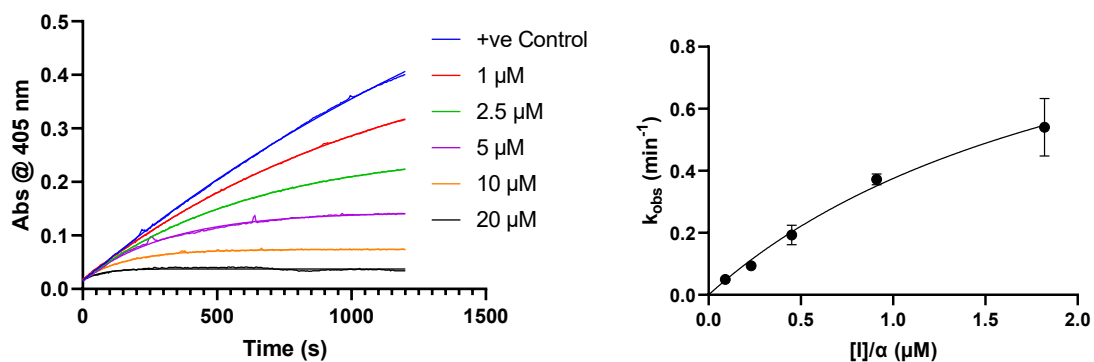
8B



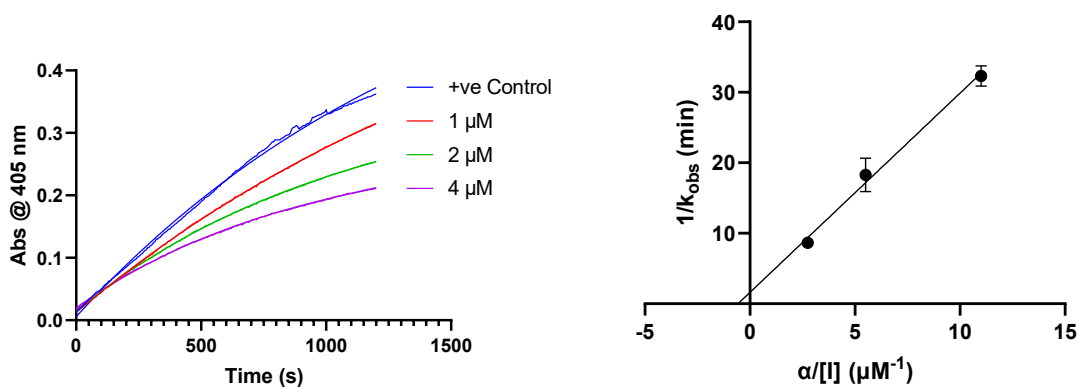
8C



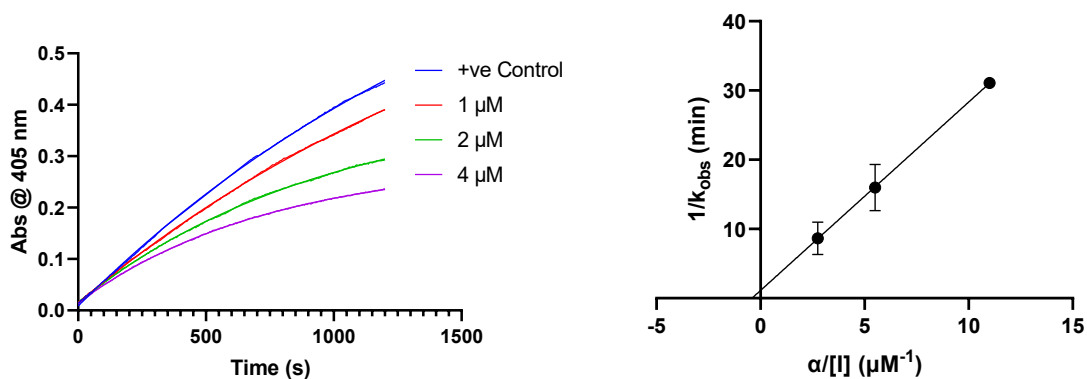
8D



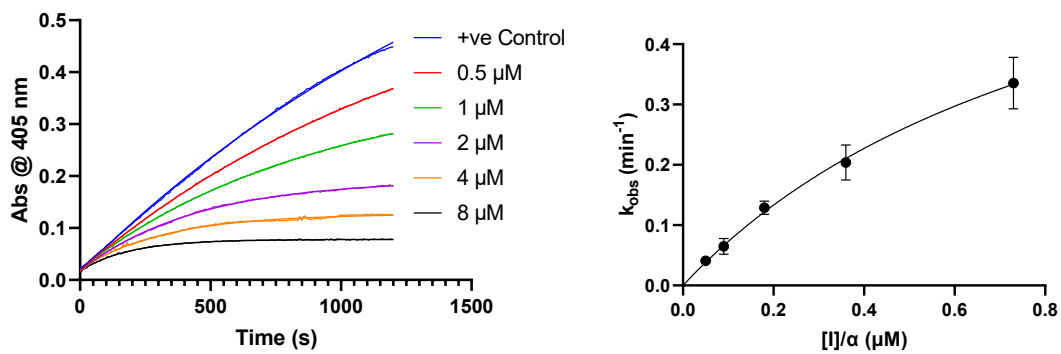
8E



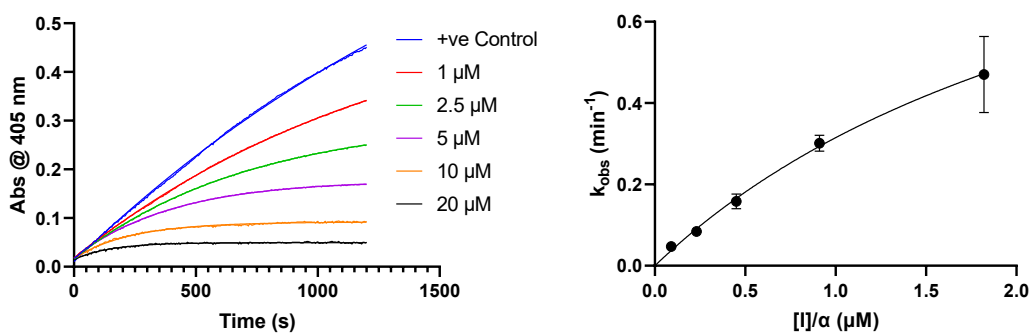
8F



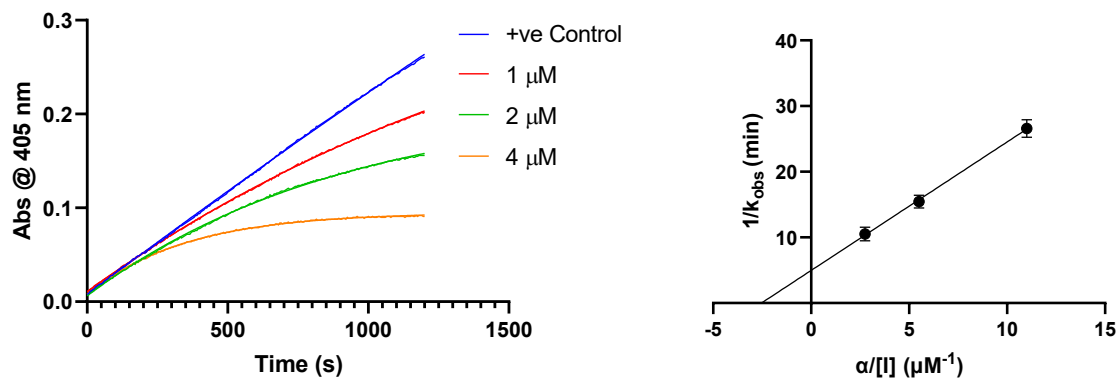
8G



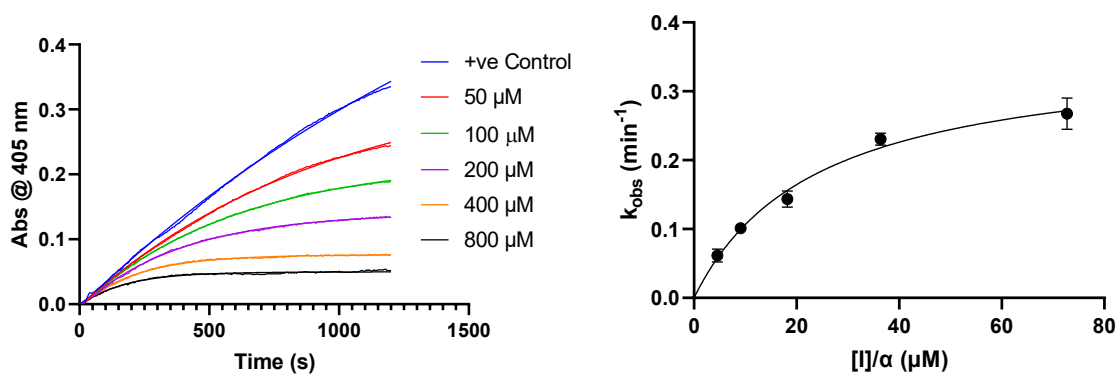
8H



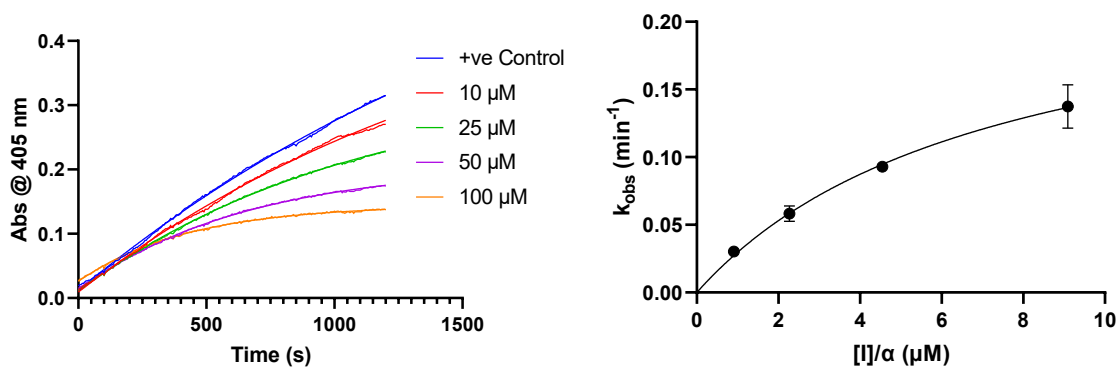
8I



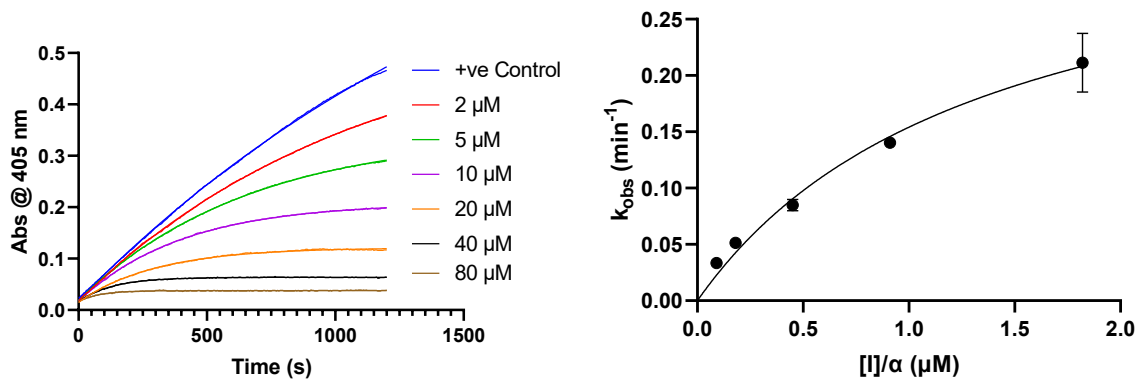
8J



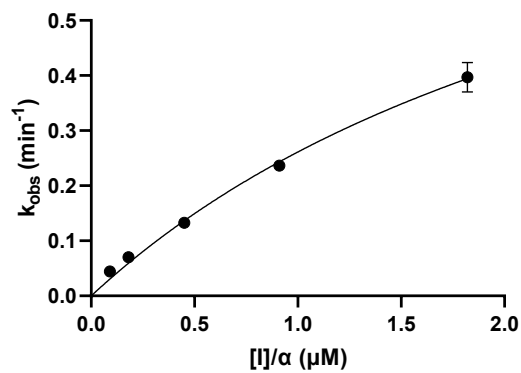
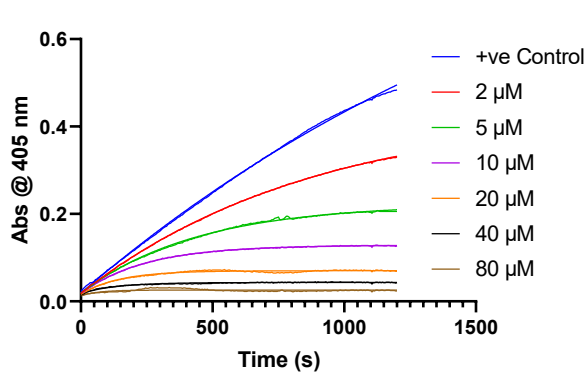
8K



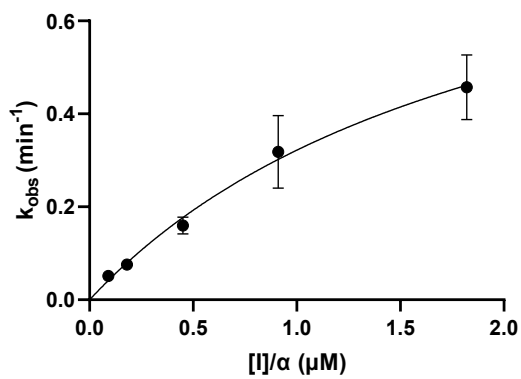
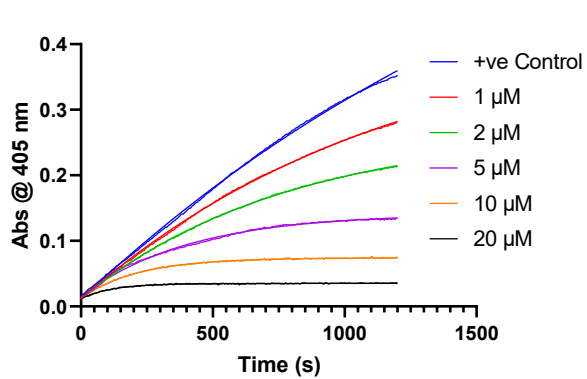
8L



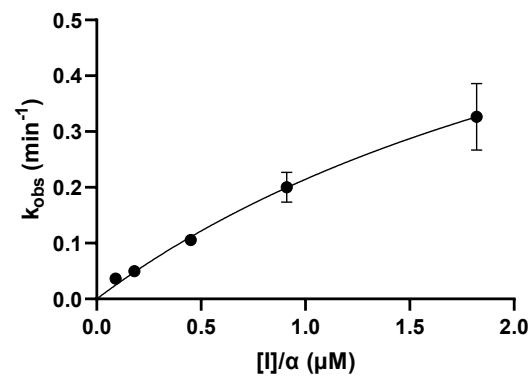
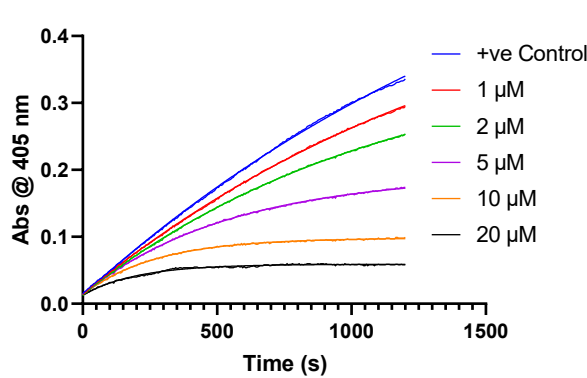
8M



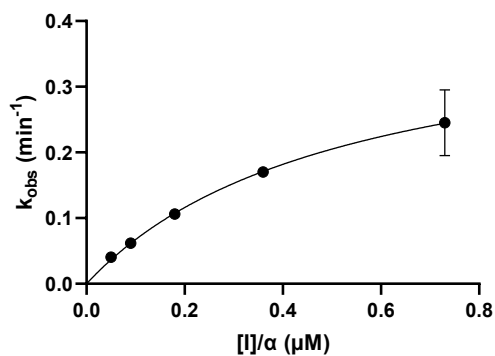
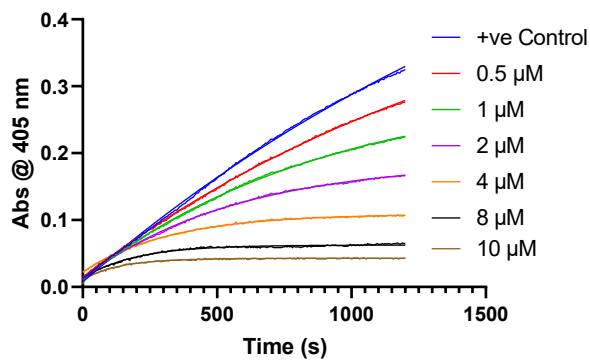
8N



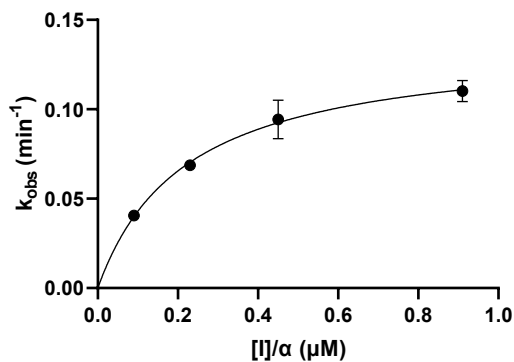
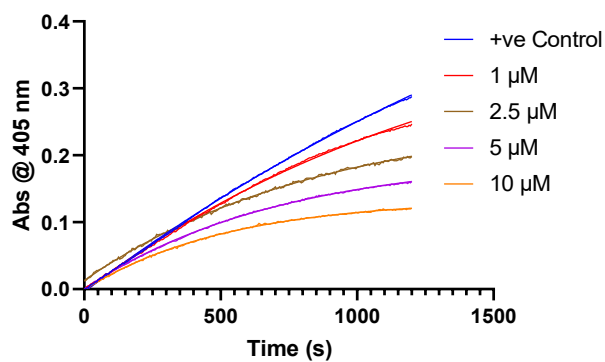
8O



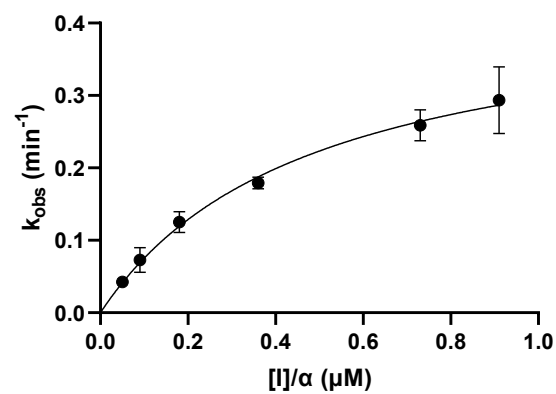
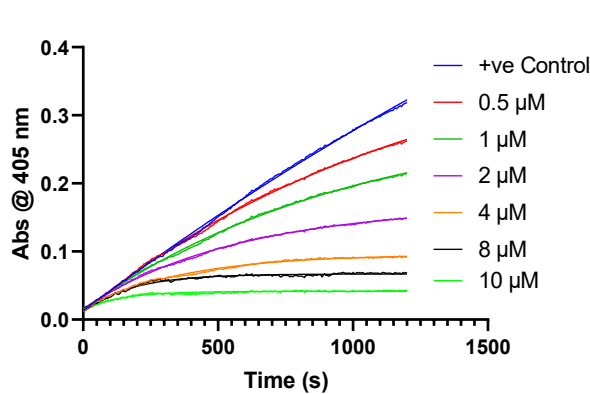
8P



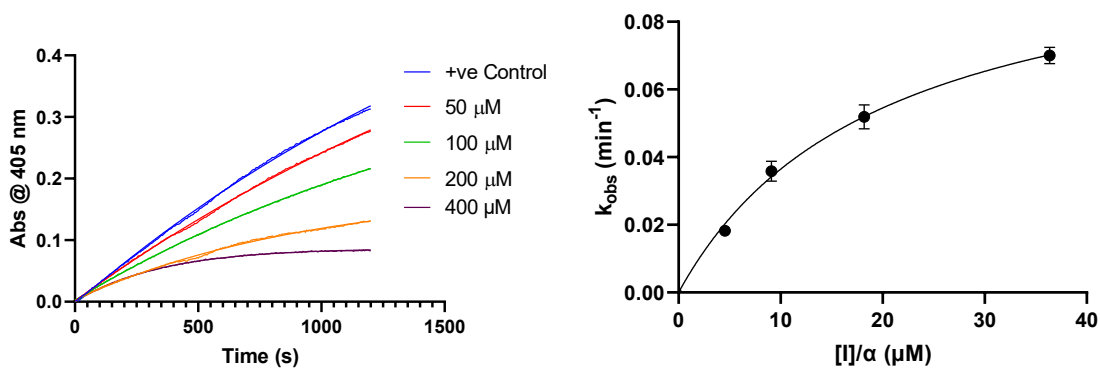
8Q



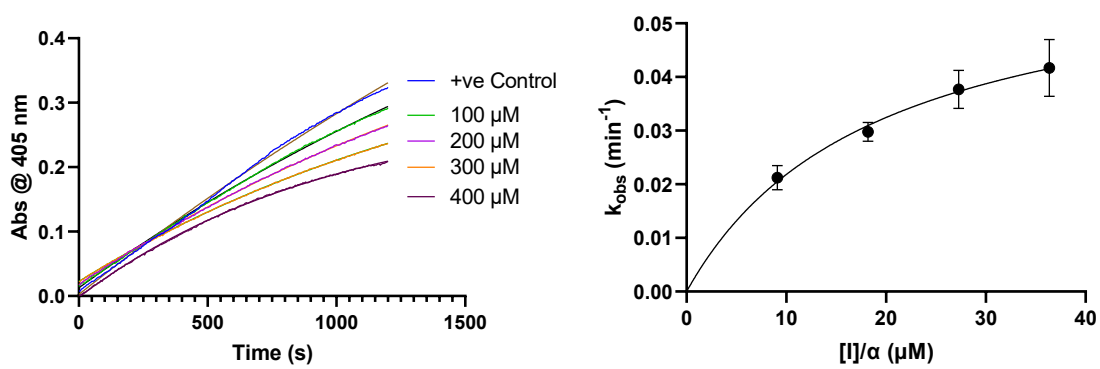
8R

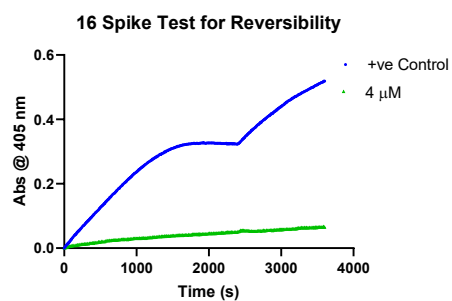
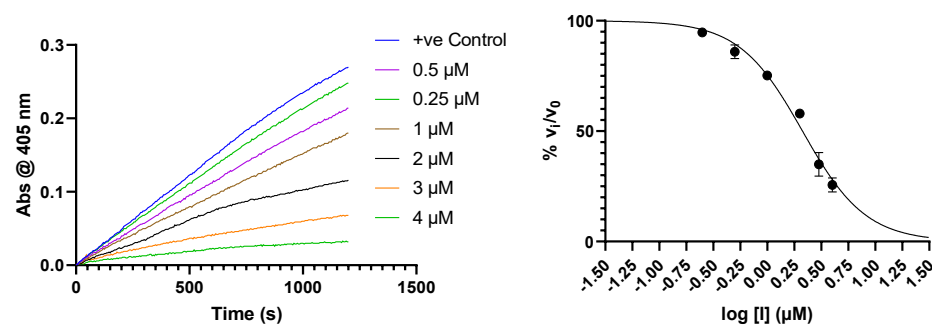


12

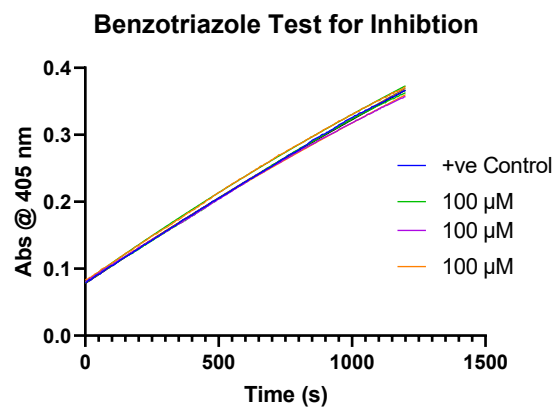
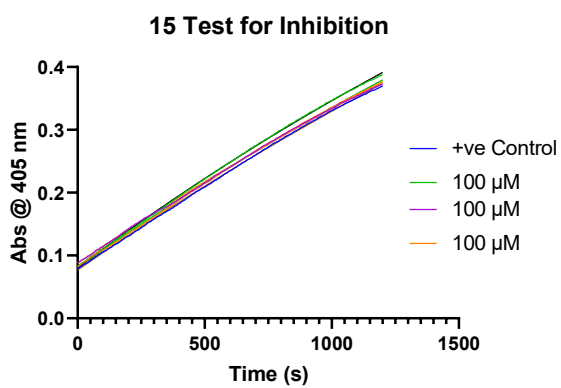


13

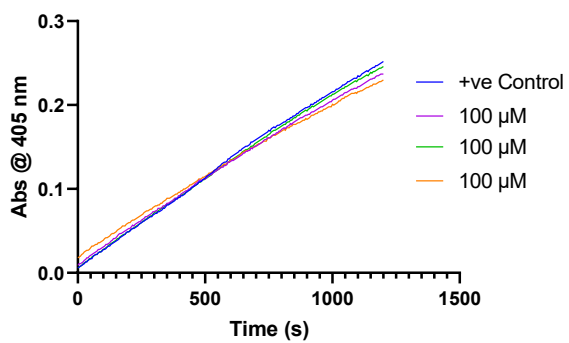




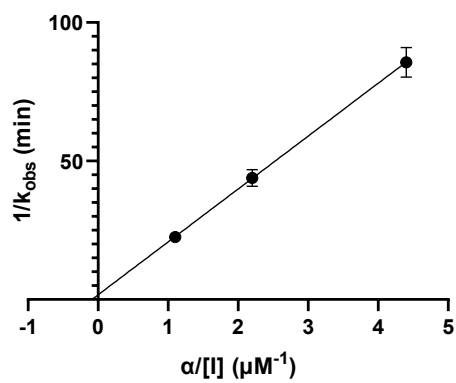
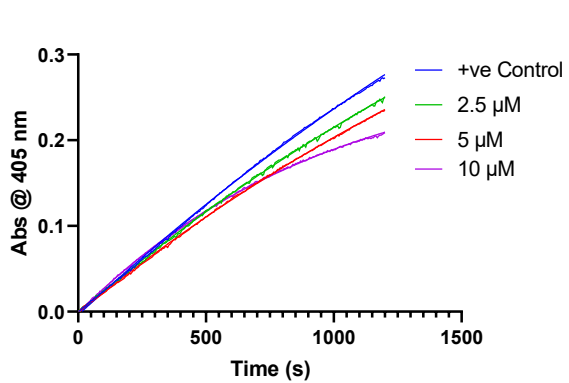
15 and Benzotriazole (To verify inhibitor 16)



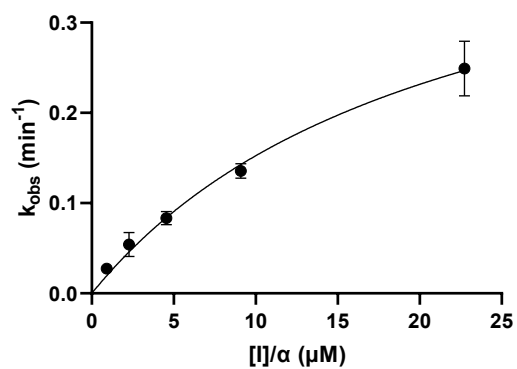
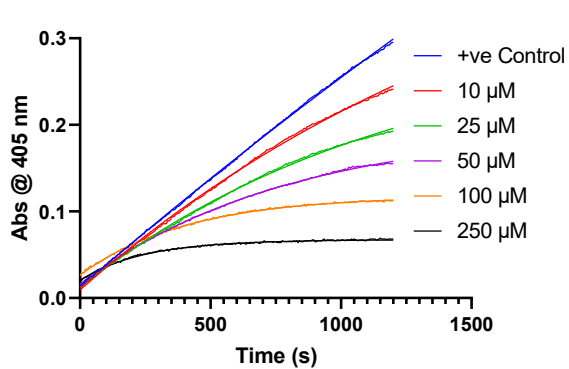
18



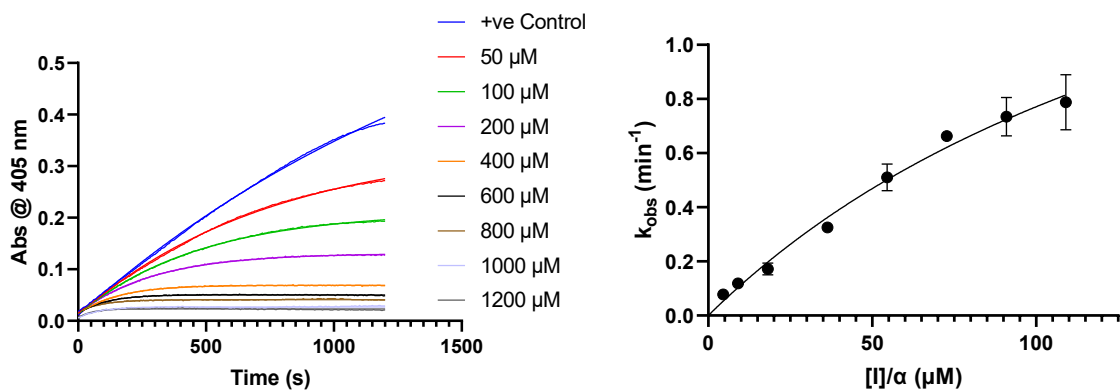
23A



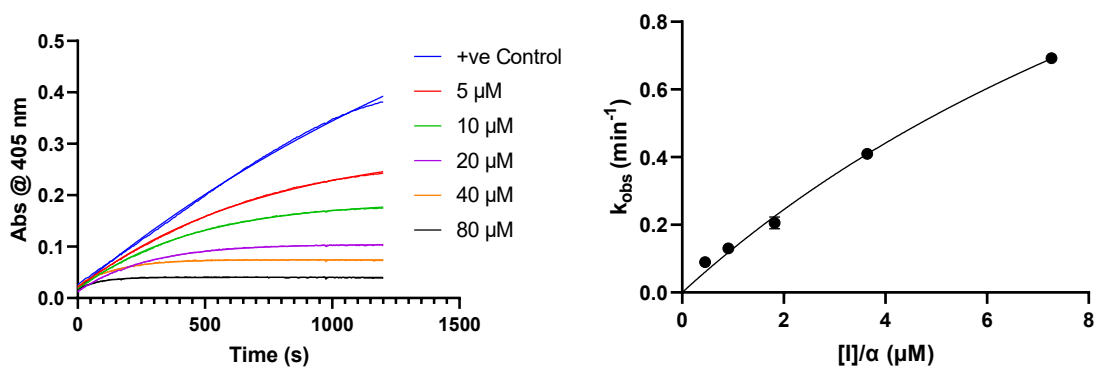
23B



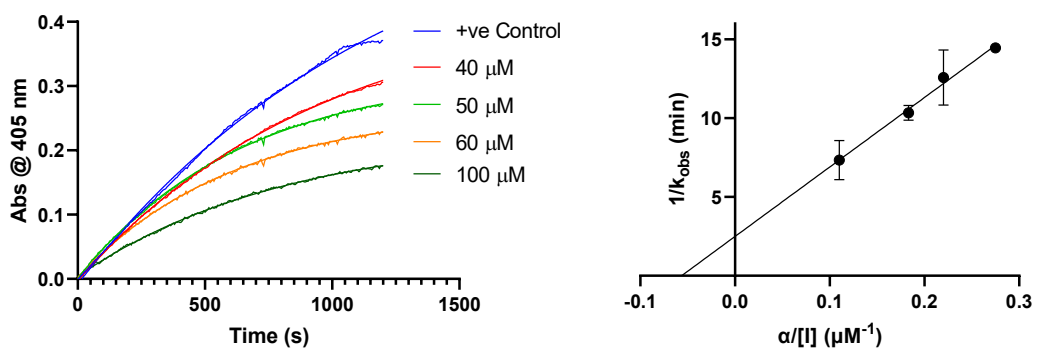
28



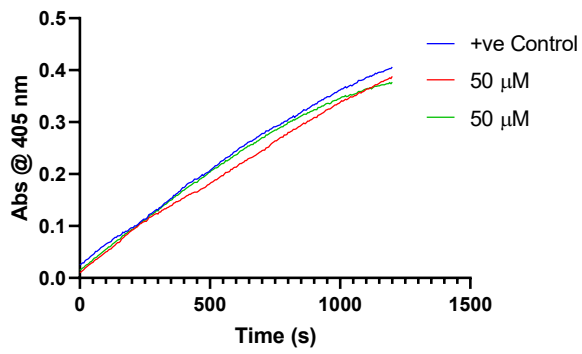
33



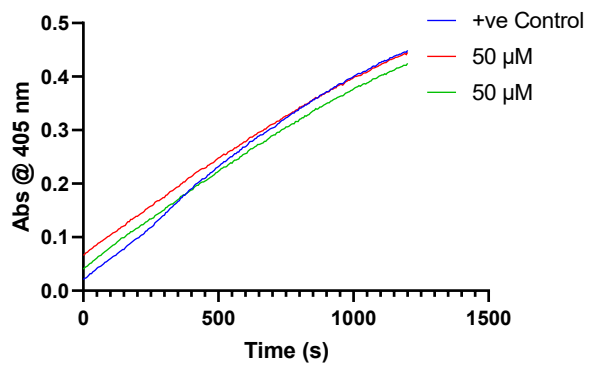
36



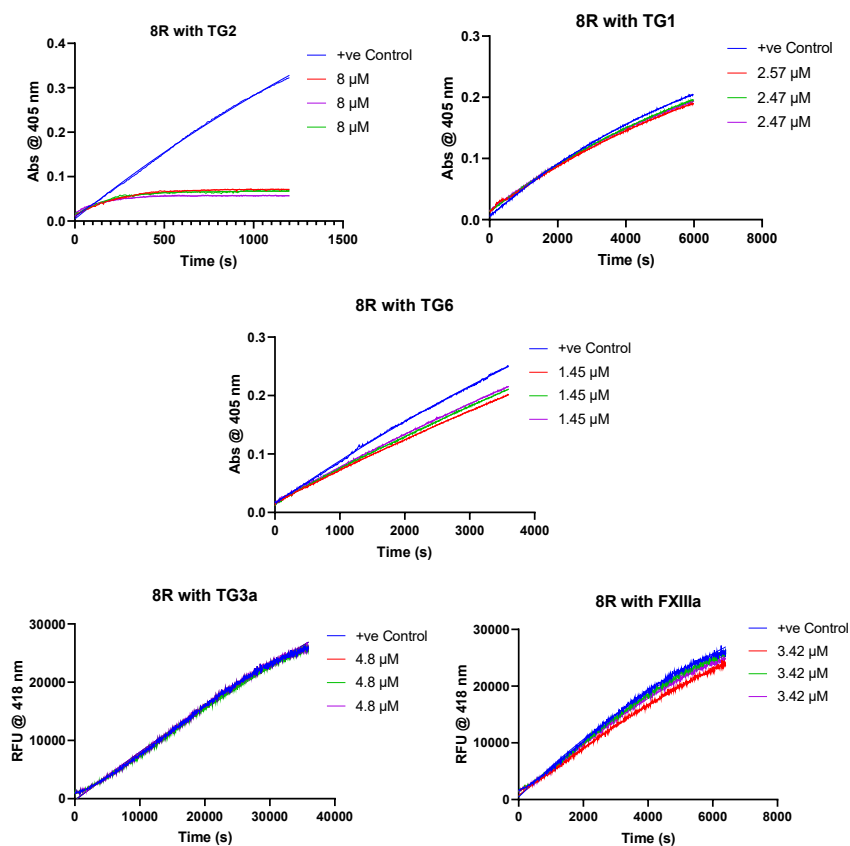
38



40



3.6.2. Isozyme Selectivity of 8R

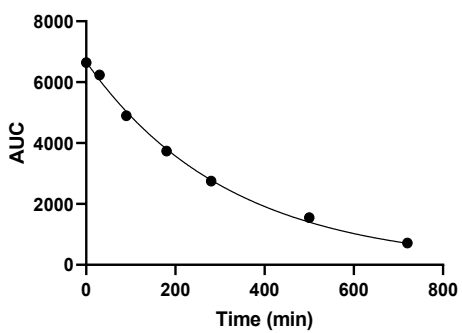


$[8R]/\alpha = 0.73 \mu\text{M}$	<i>TG2</i>	<i>TG1</i>	<i>TG6</i>	<i>TG3</i>	<i>FXIIIa</i>
$k_{\text{obs}} (\text{min}^{-1})$	0.3332 ± 0.0497	n.d.	0.0040 ± 0.0001	n.d.	n.d.

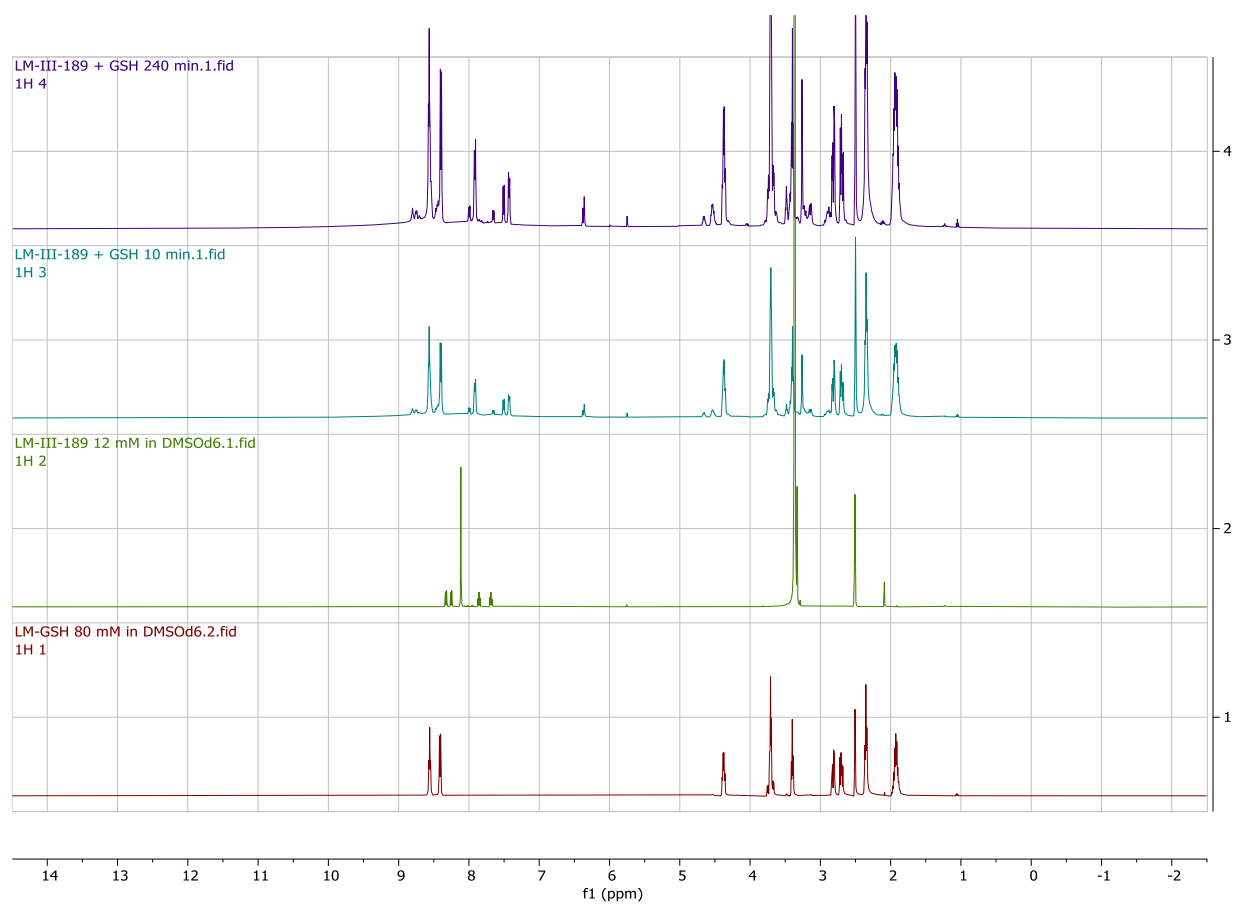
n.d. : Not determined. Unable to measure a rate constant distinct from the no-inhibitor control.

3.6.3. Intrinsic Reactivity Data

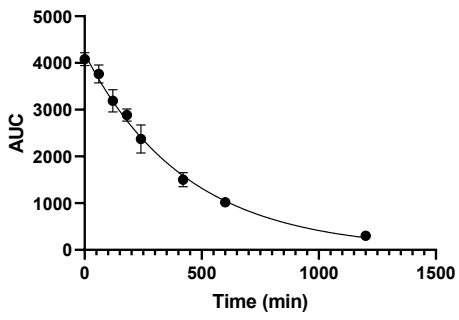
13



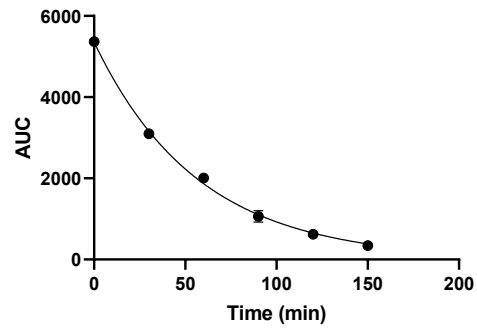
16



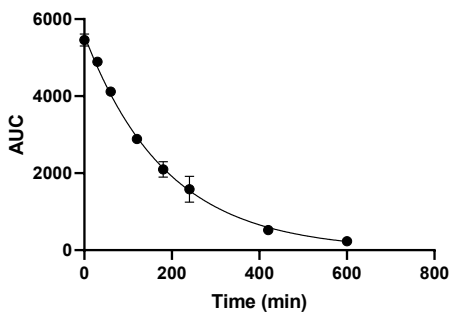
18



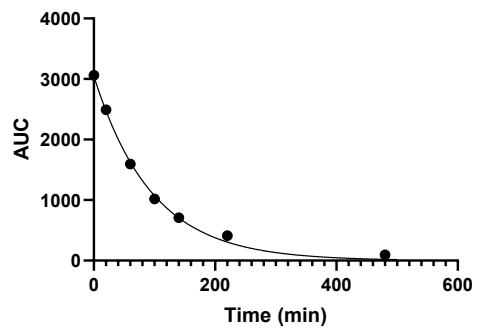
23B



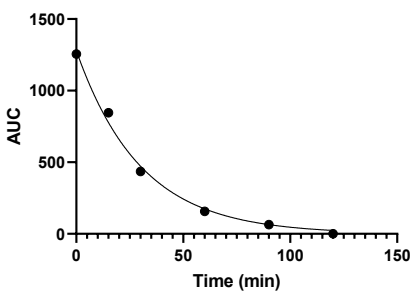
23C



23D

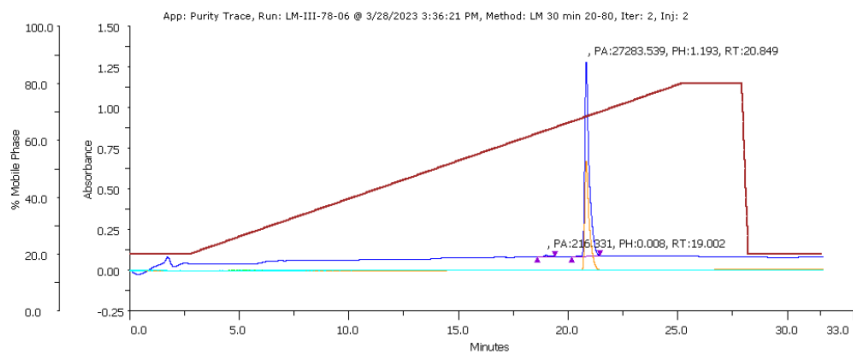


23E

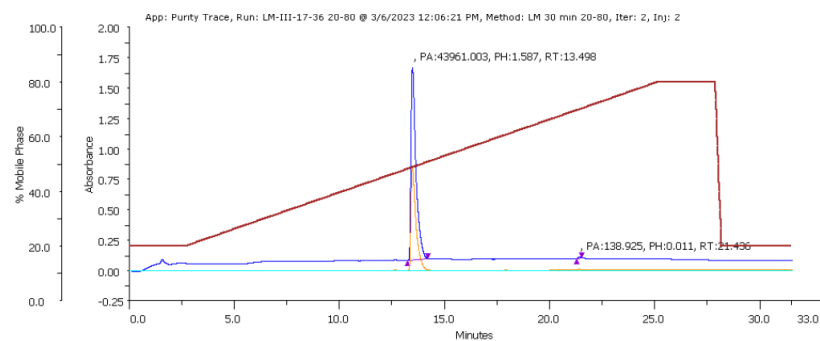


3.6.4. HPLC Purity Traces of Final Compounds

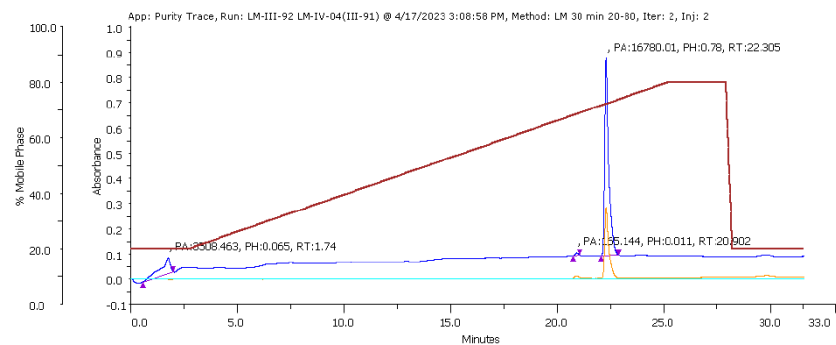
8A (99%)



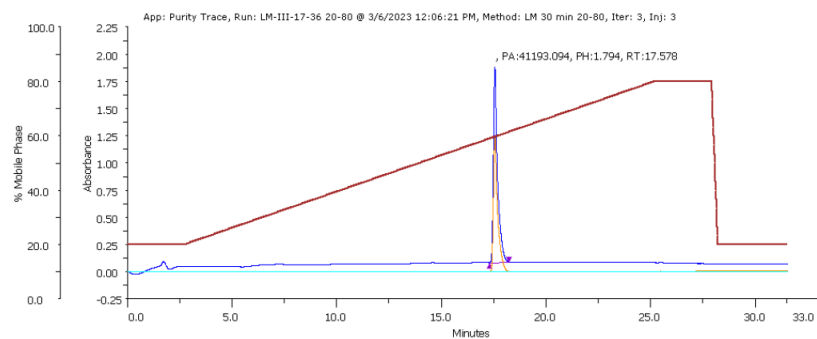
8B (99%)



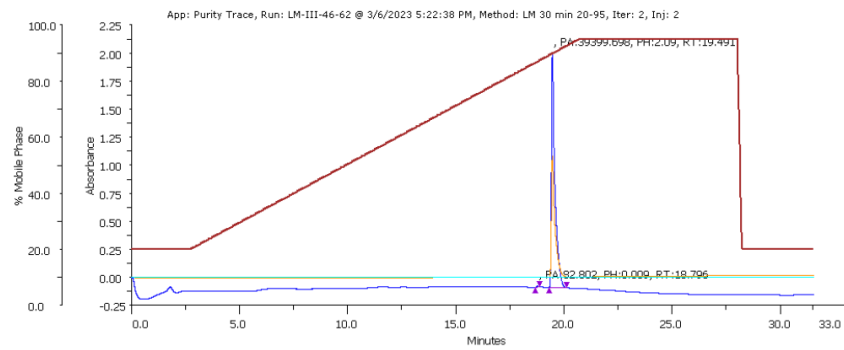
8C (98%)



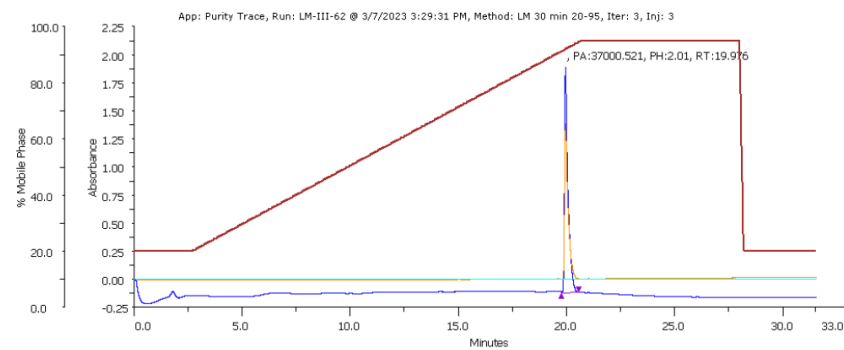
8D (100%)



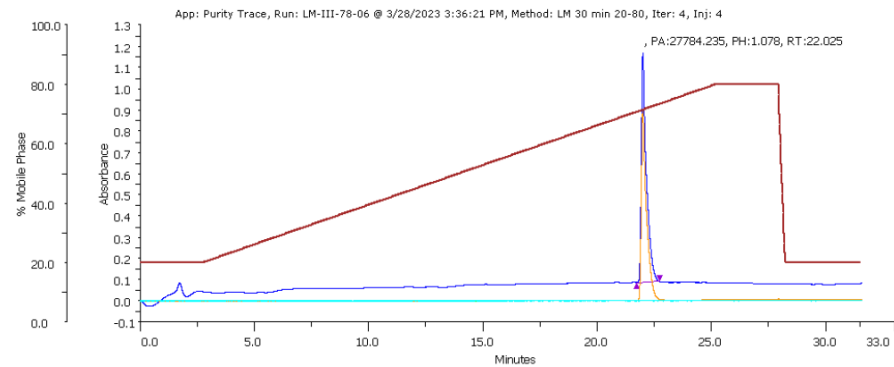
8E (99%)



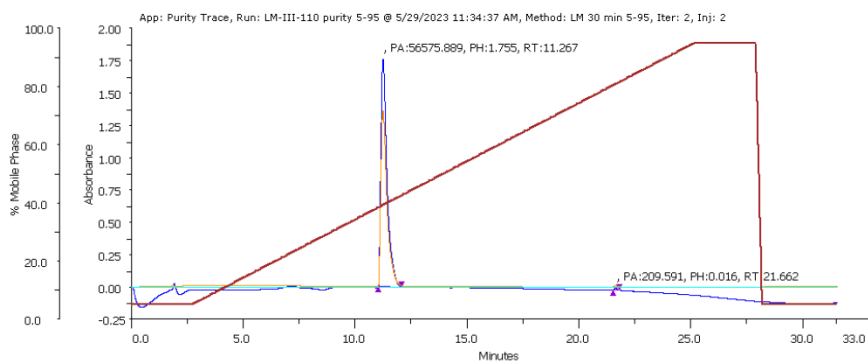
8F (100%)



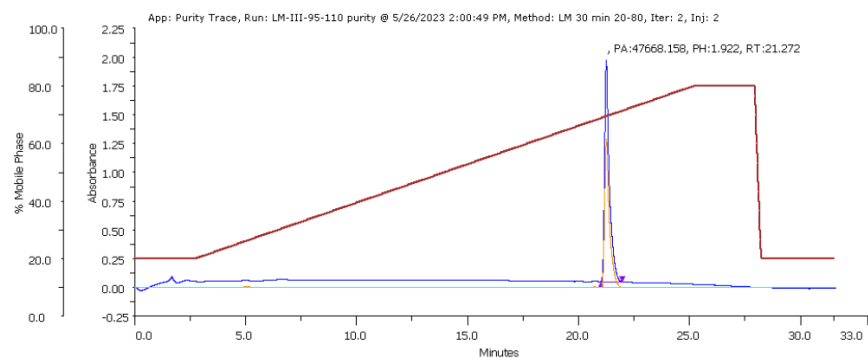
8G (100%)



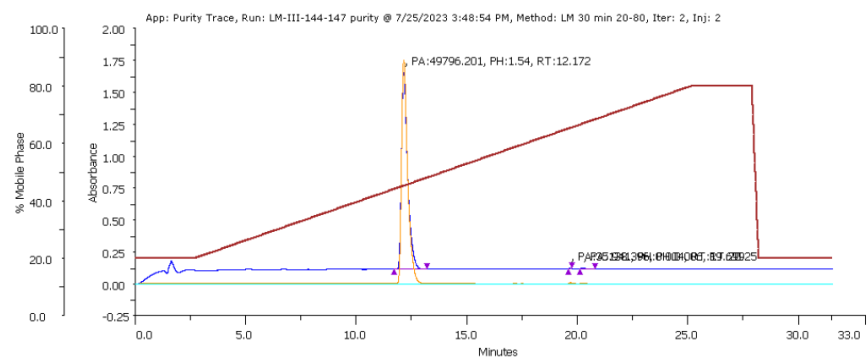
8H (99%)



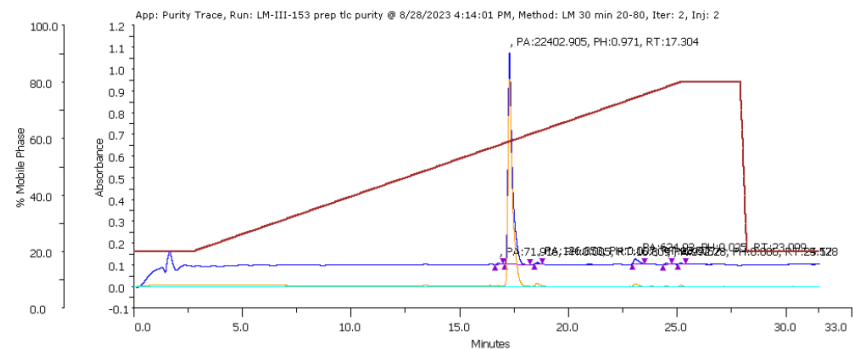
8I (100%)



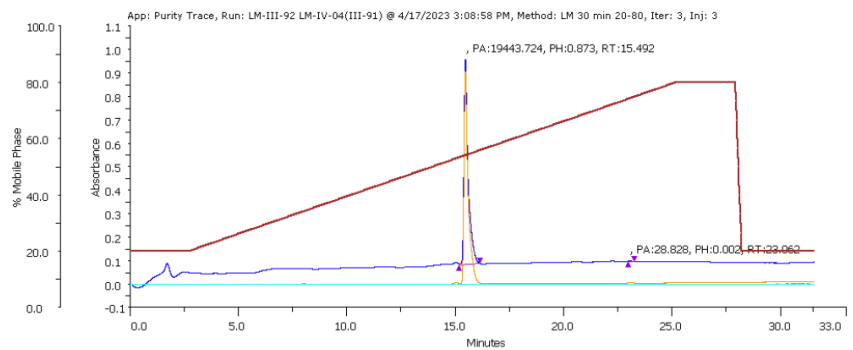
8J (99%)



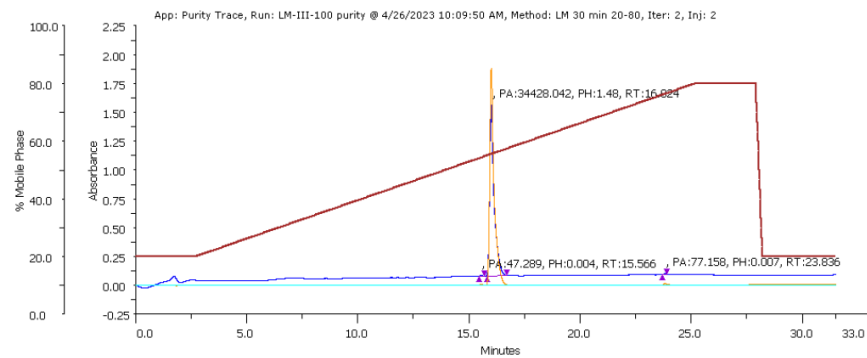
8K (96%)



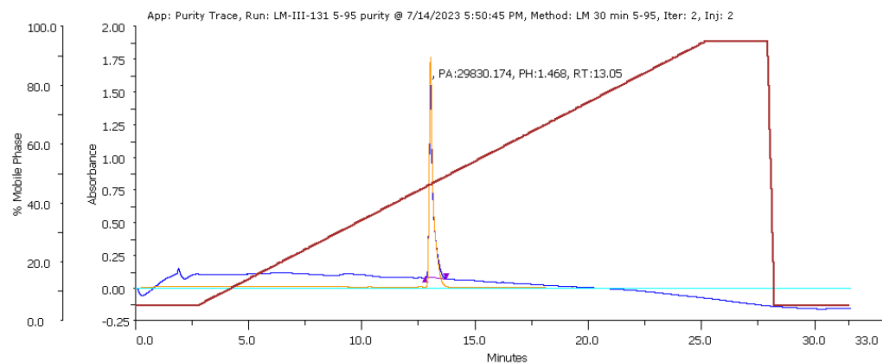
8L (99%)



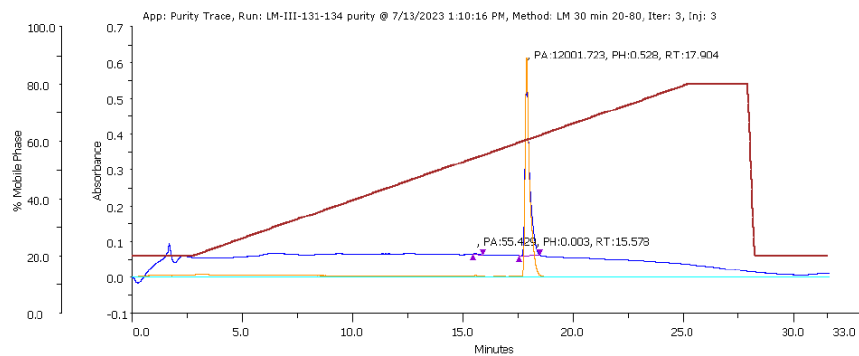
8M (99%)



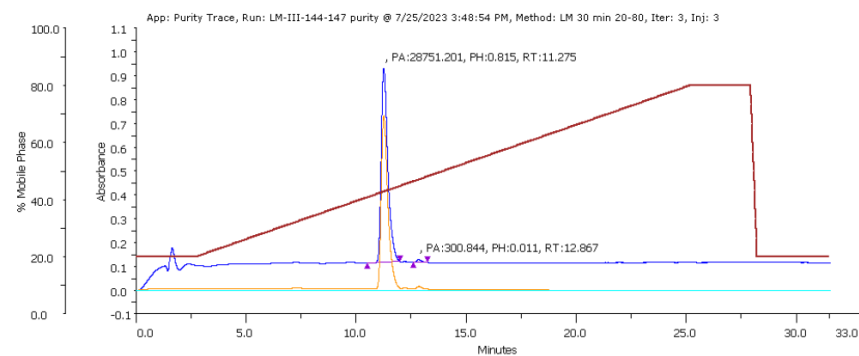
8N (100%)



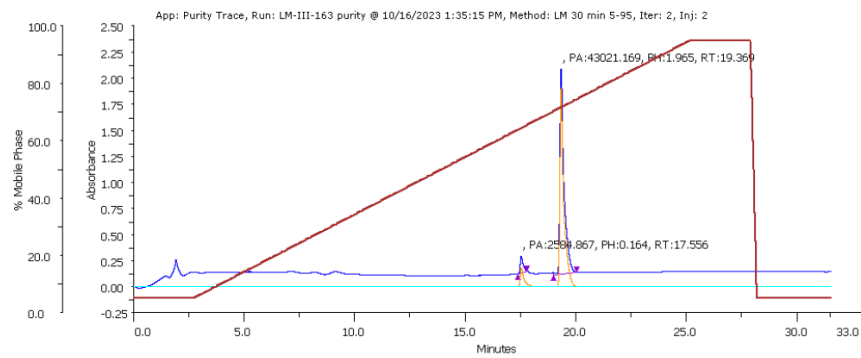
8O (99%)



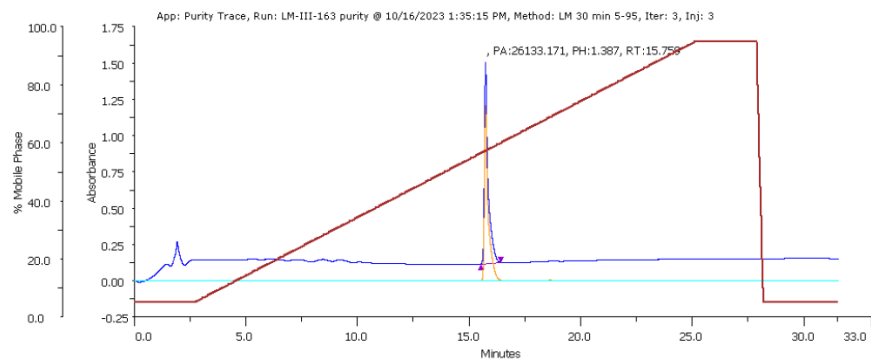
8P (98%)



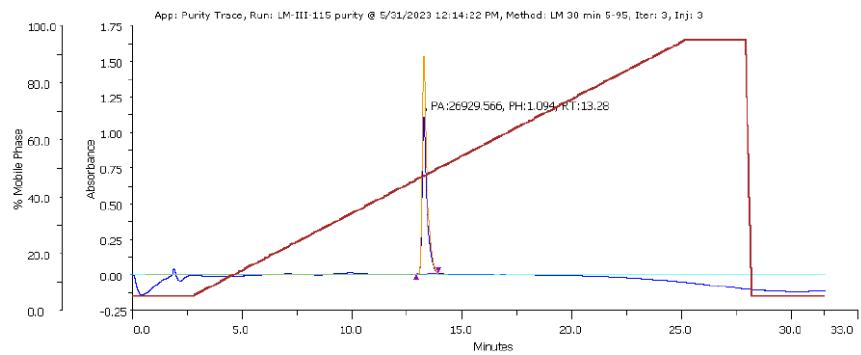
8Q (95%)



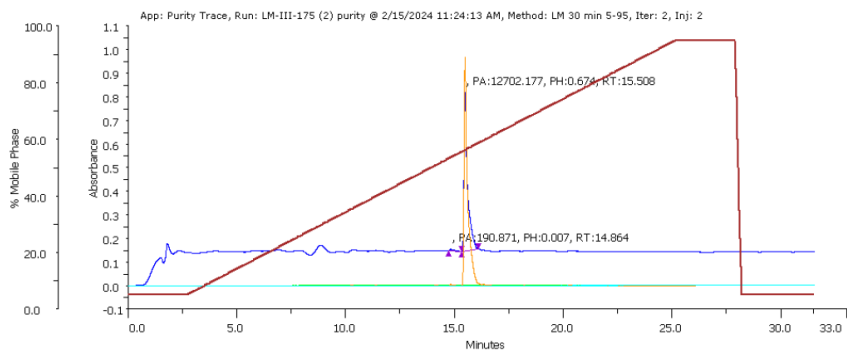
8R (100%)



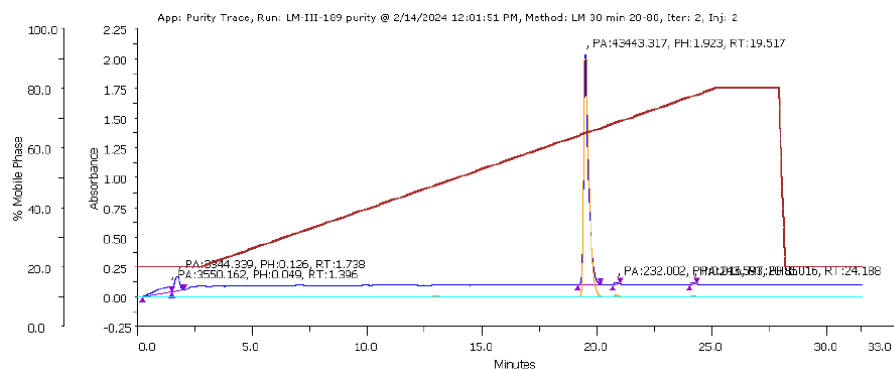
12 (100%)



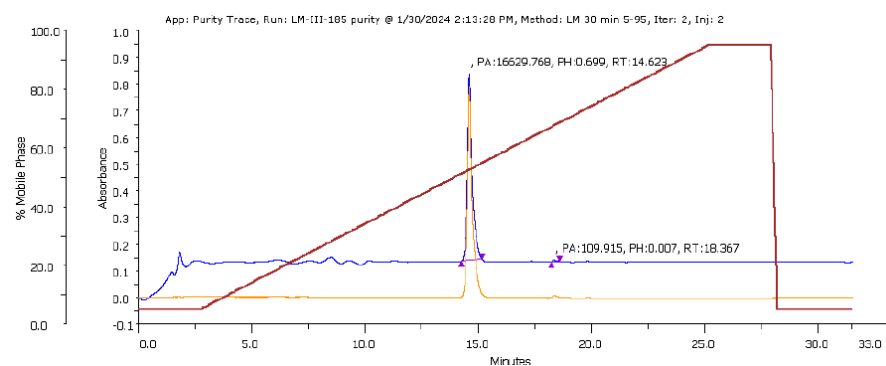
13 (98%)



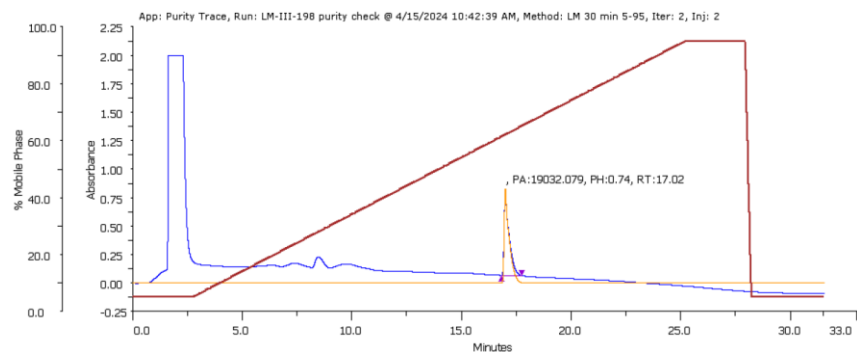
16 (99%)



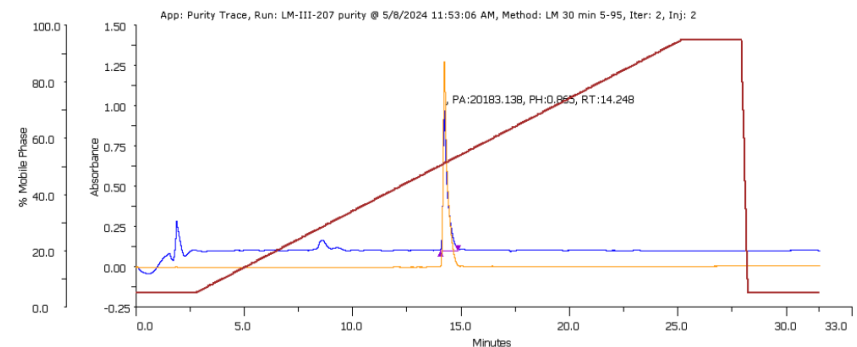
18 (99%)



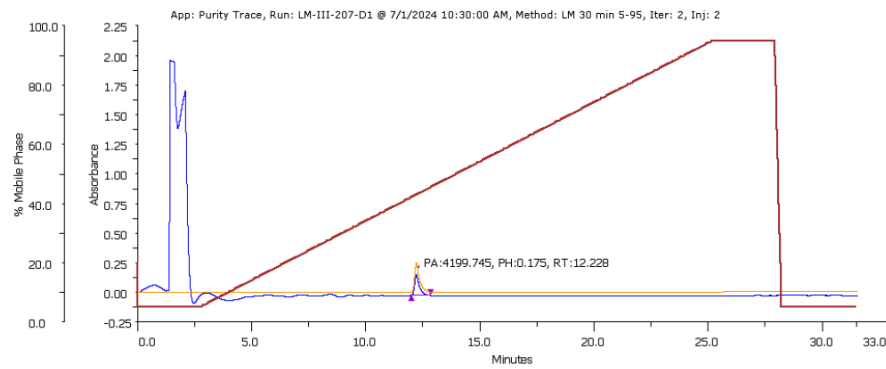
23A (100%)



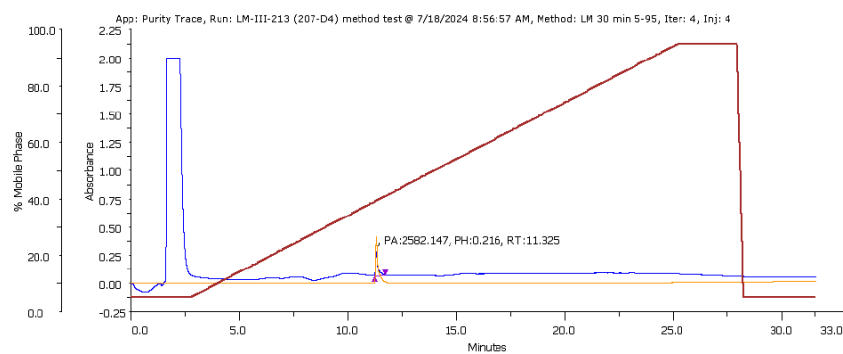
23B (100%)



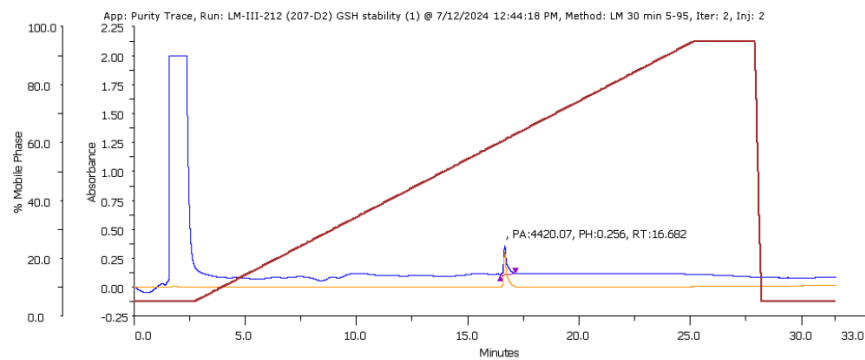
23C (100%)



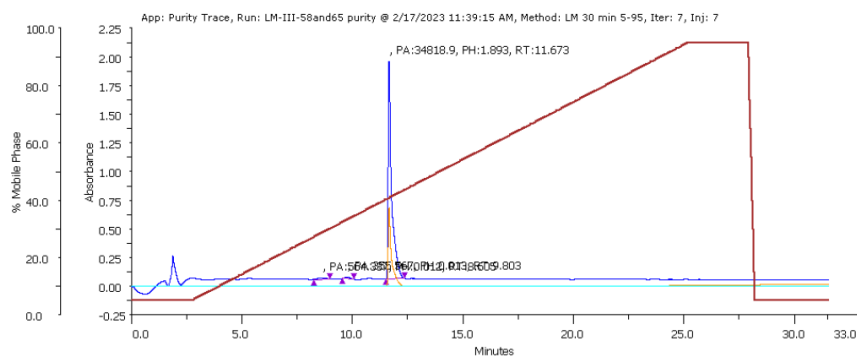
23D (100%)



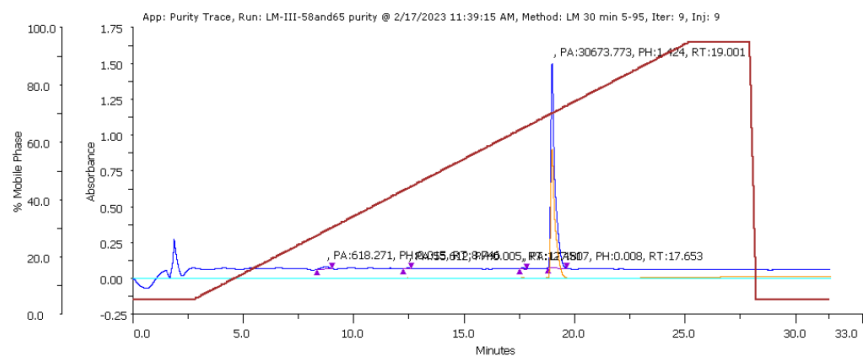
23E (100%)



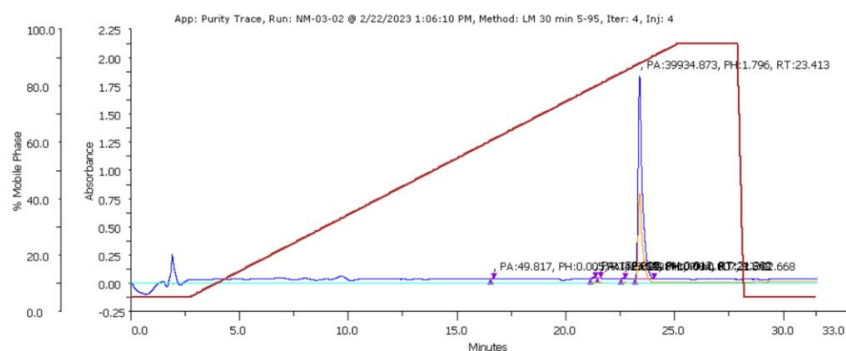
28 (97%)



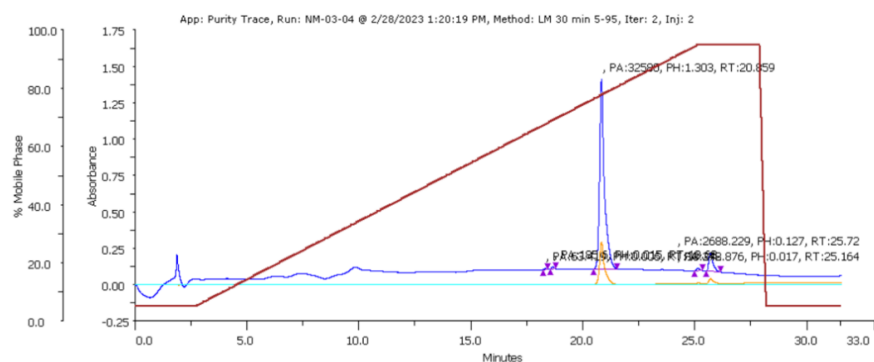
33 (97%)



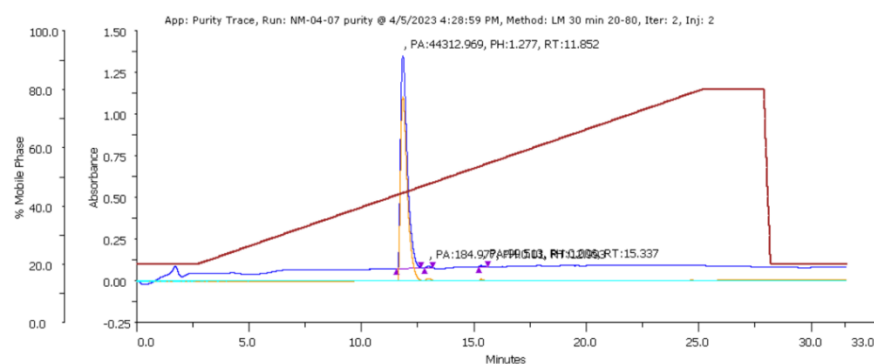
36 (99%)



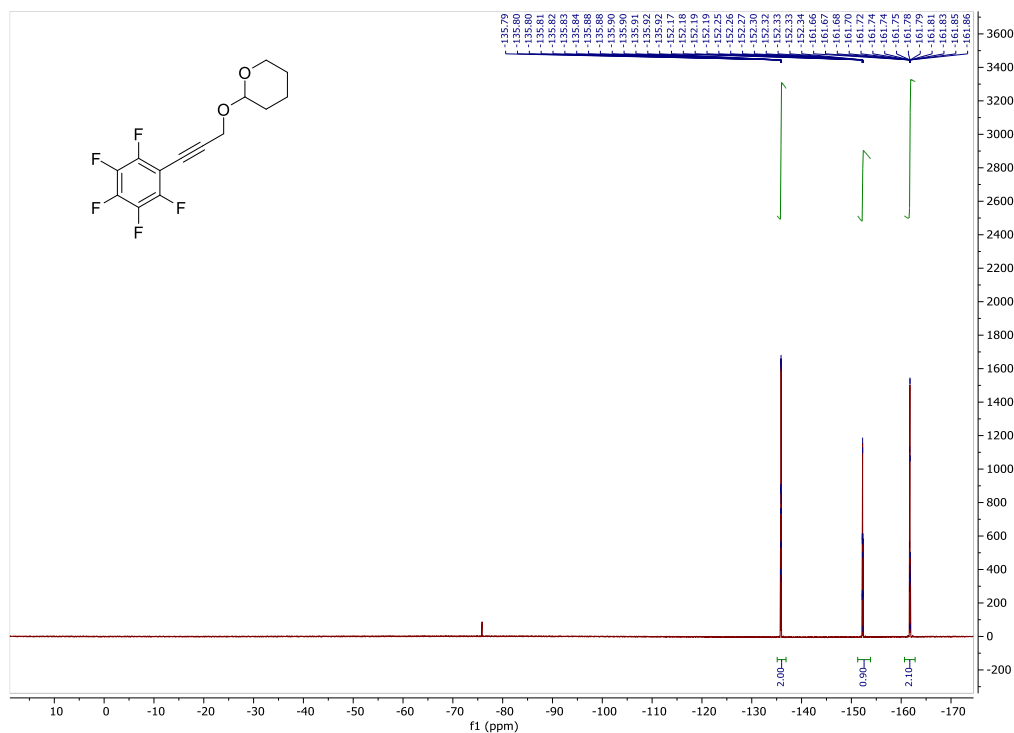
38 (91%)



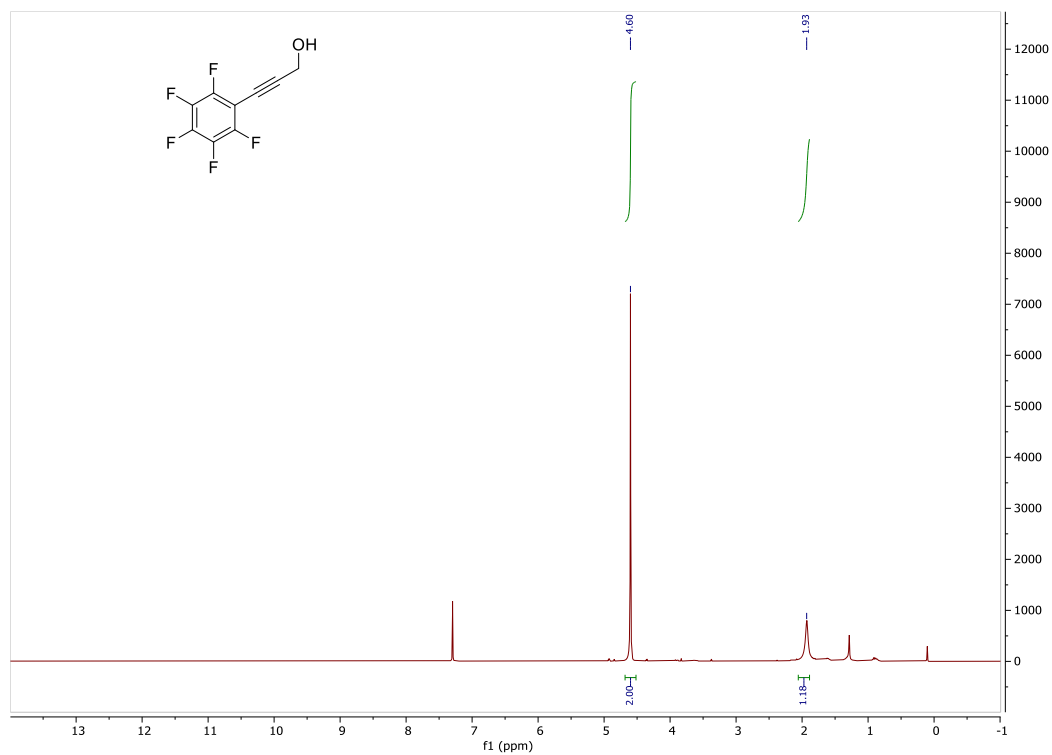
40 (99%)



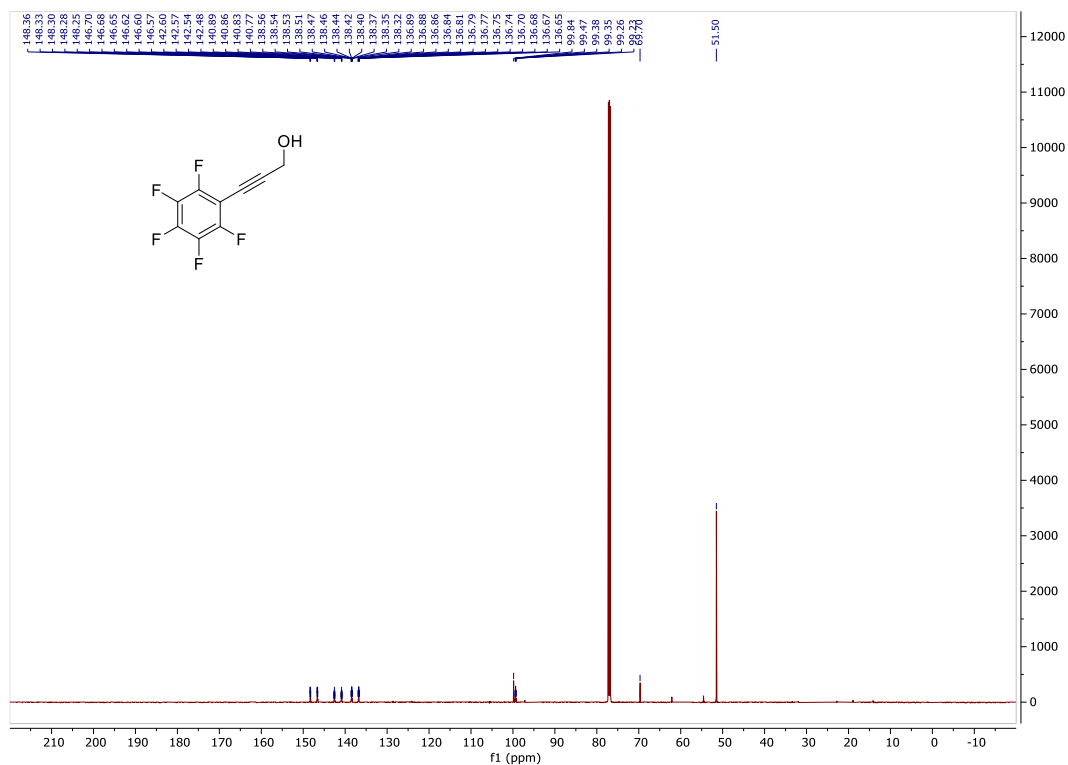
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **3C1**



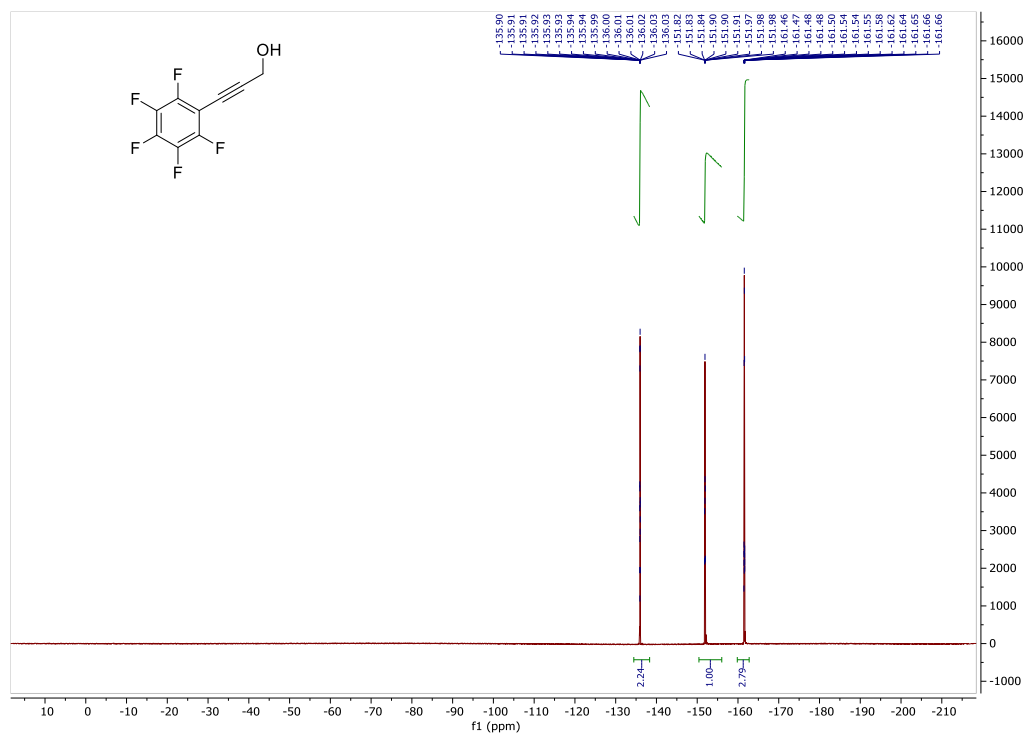
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **3C2**



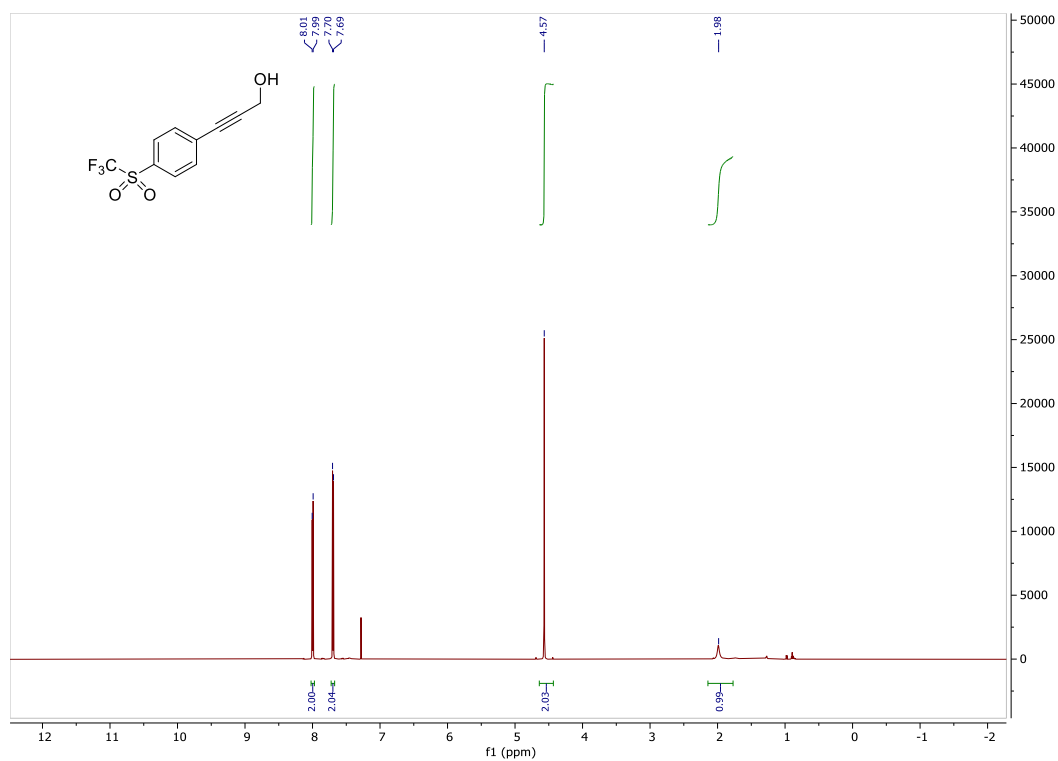
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **3C2**



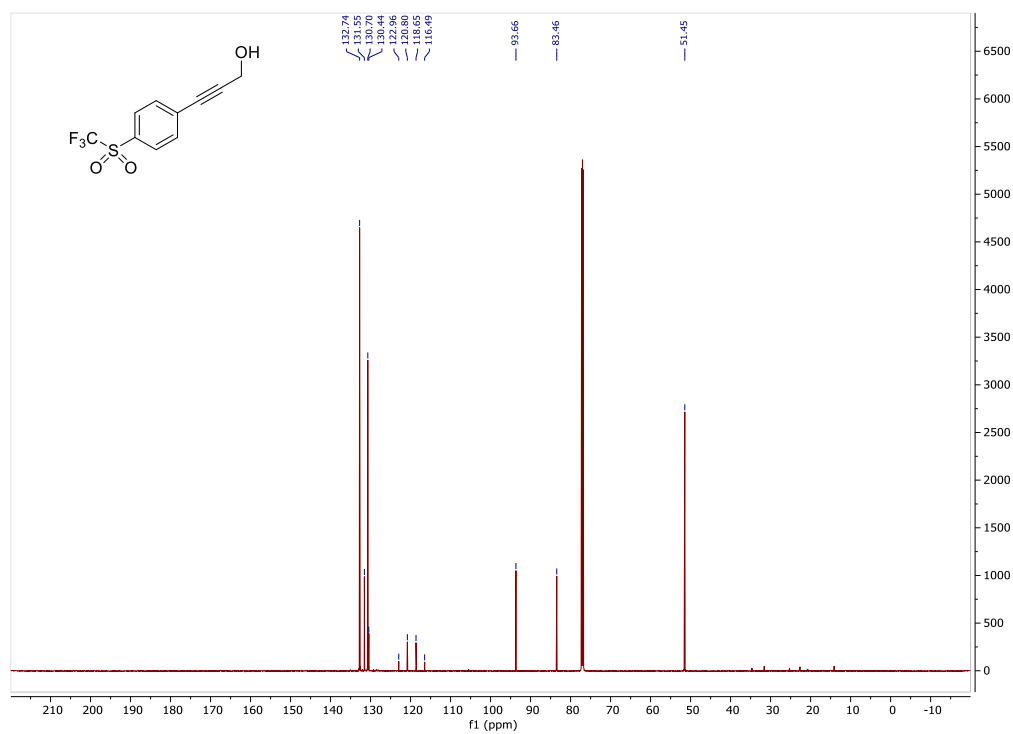
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **3C2**



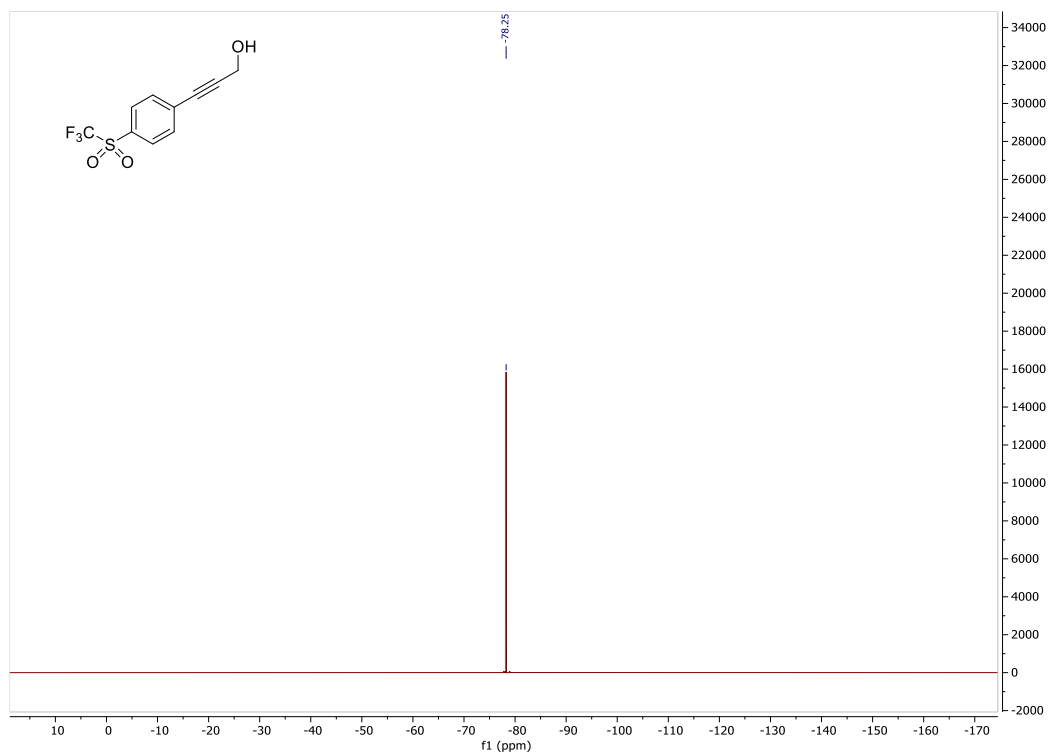
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **3G**



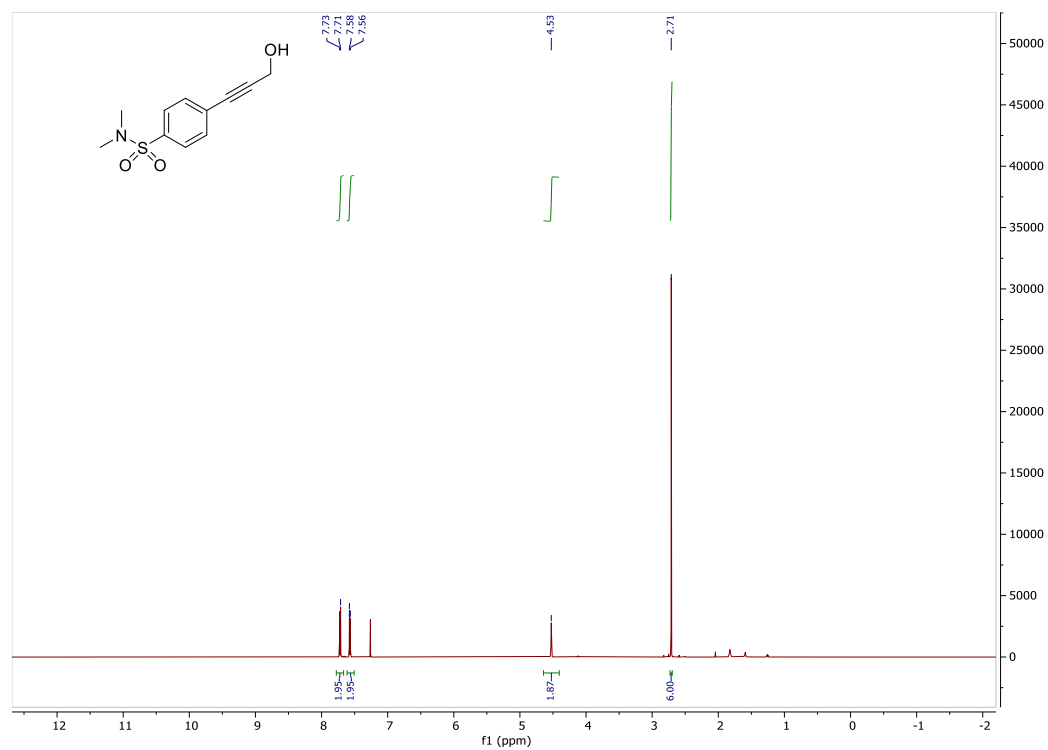
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **3G**



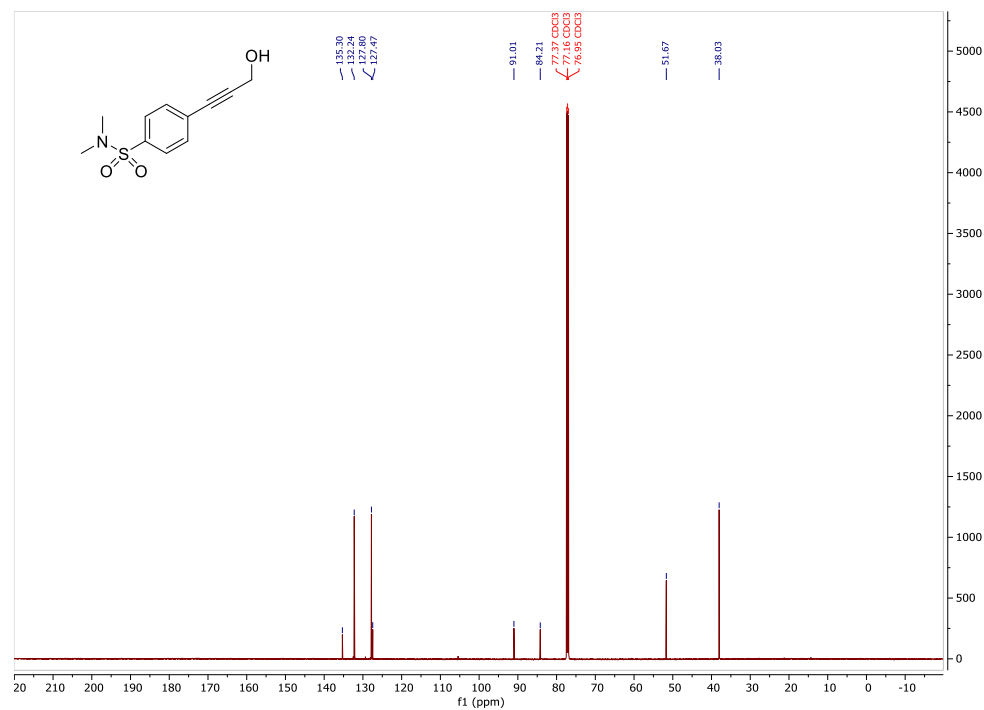
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **3G**



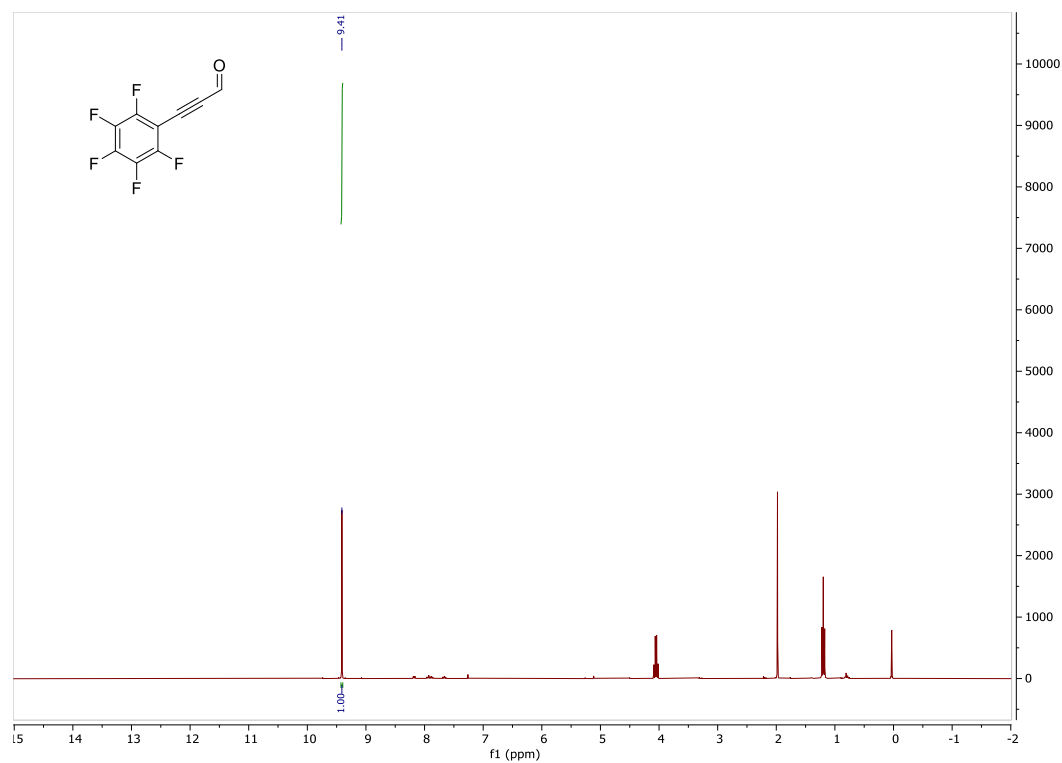
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **3H**



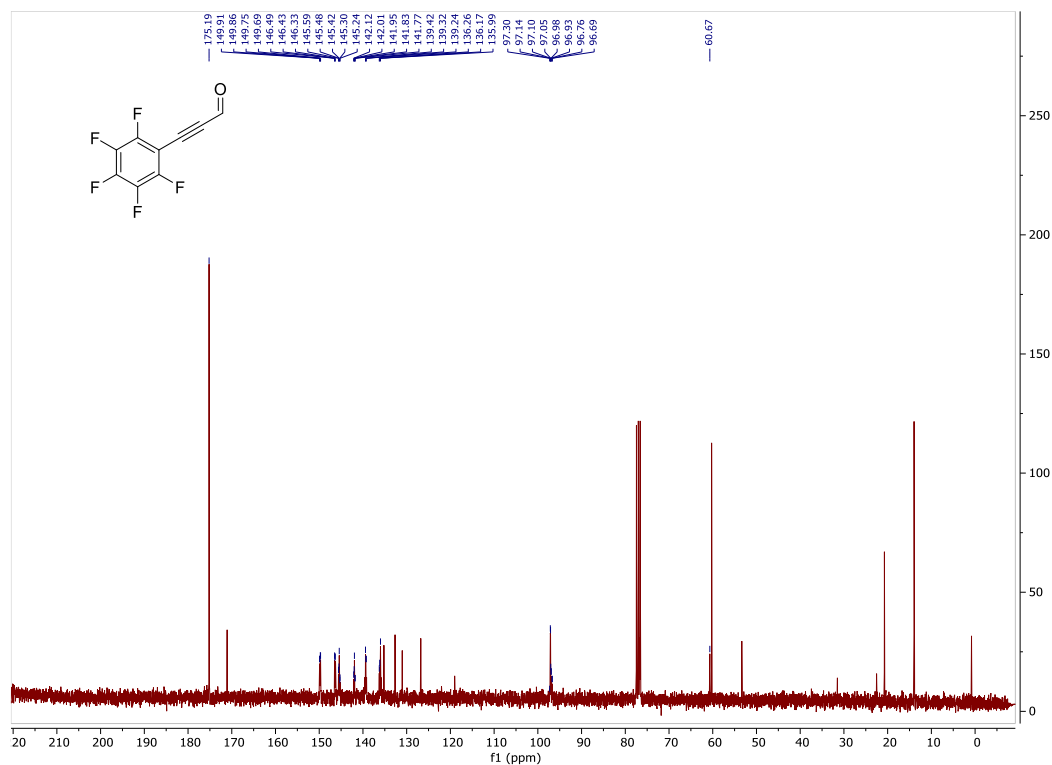
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **3H**



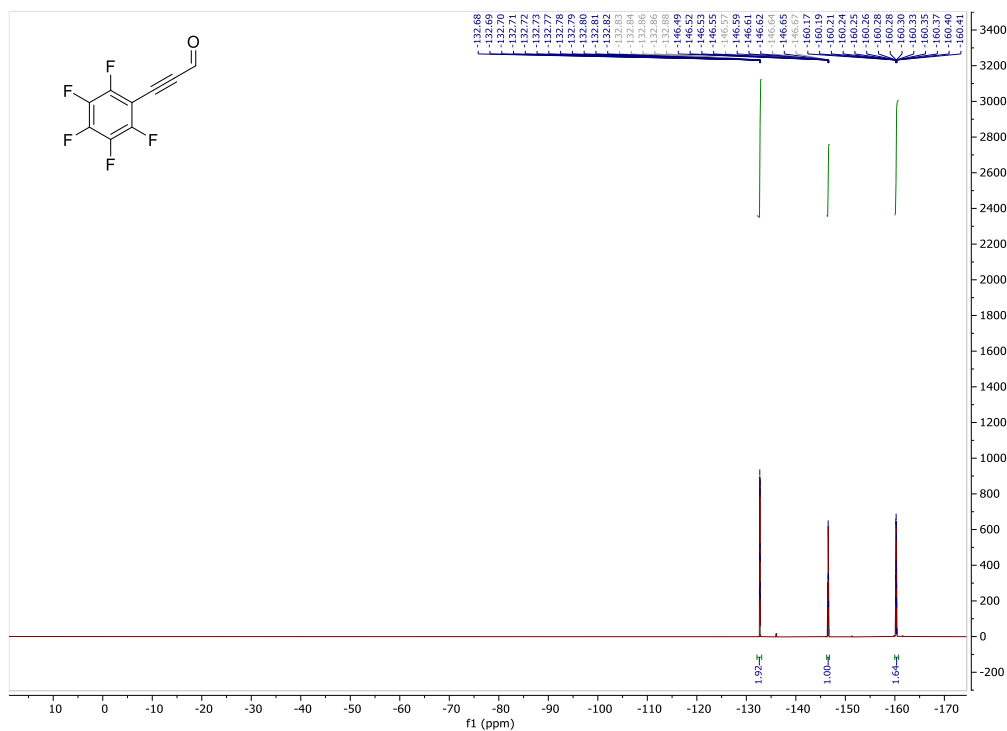
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **4C**



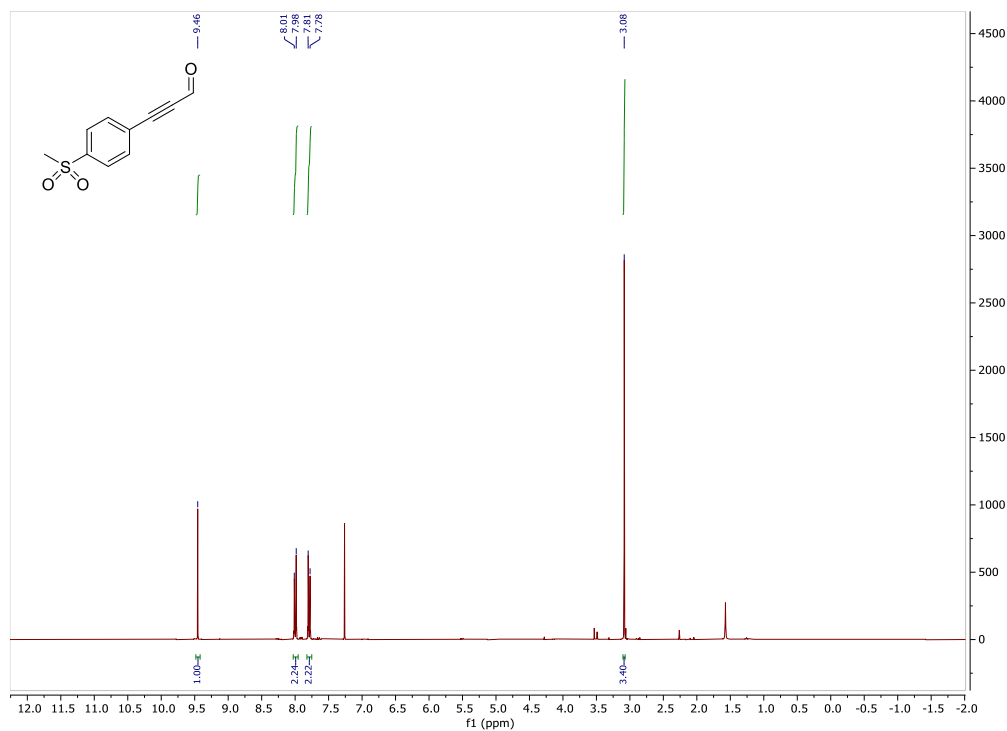
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **4C**



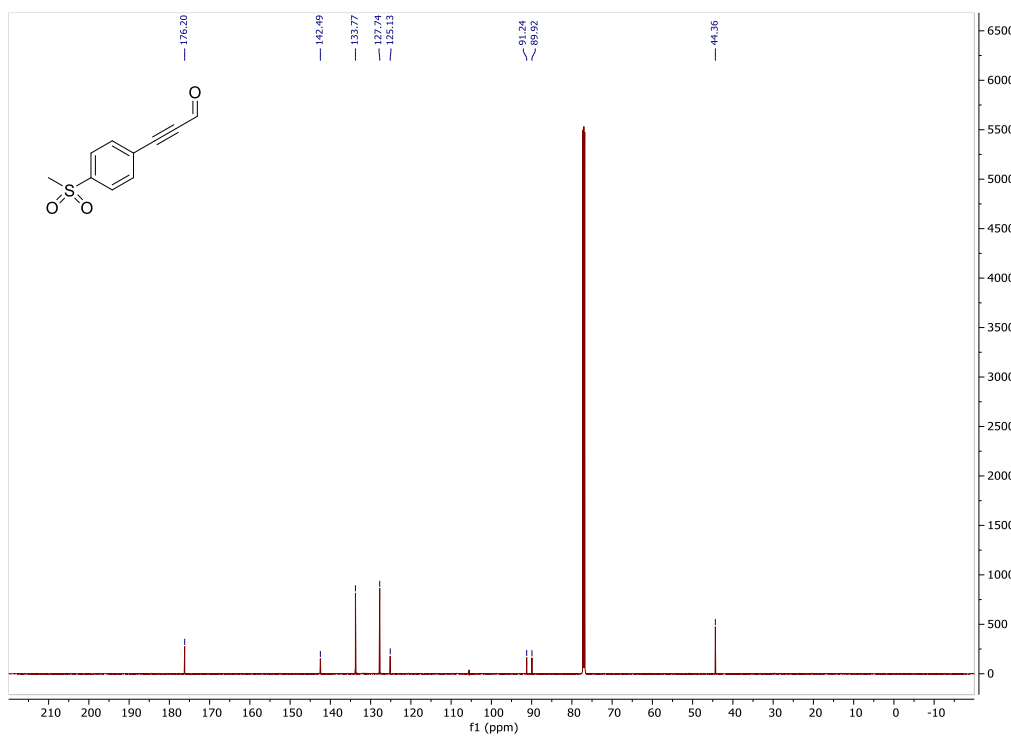
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **4C**



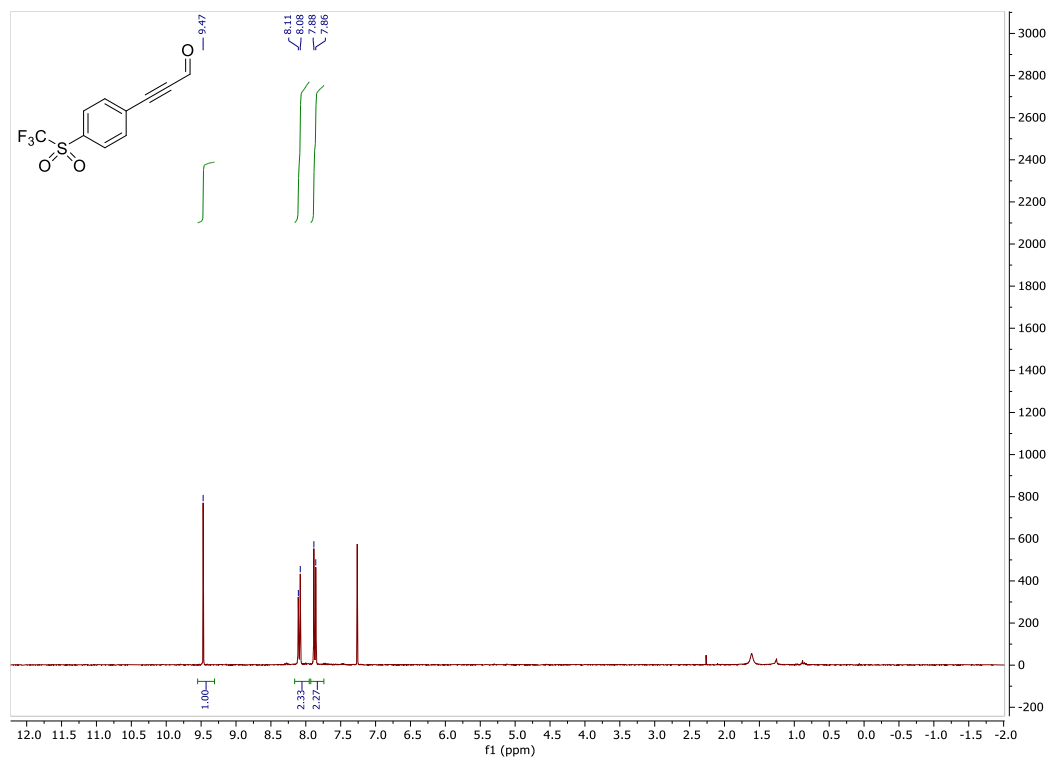
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **4F**



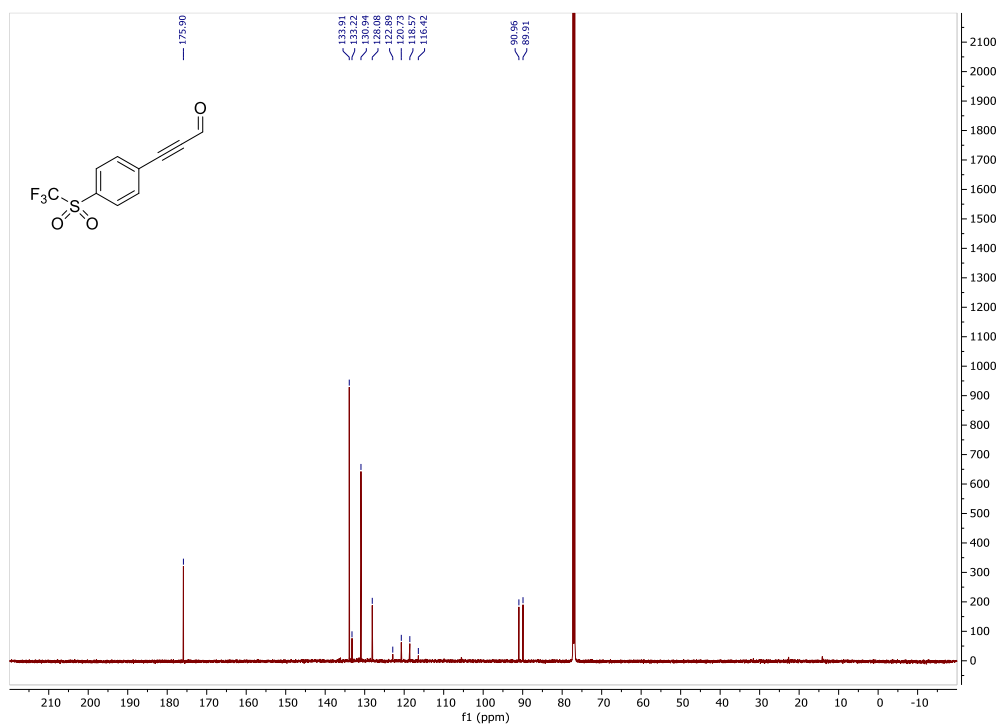
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **4F**



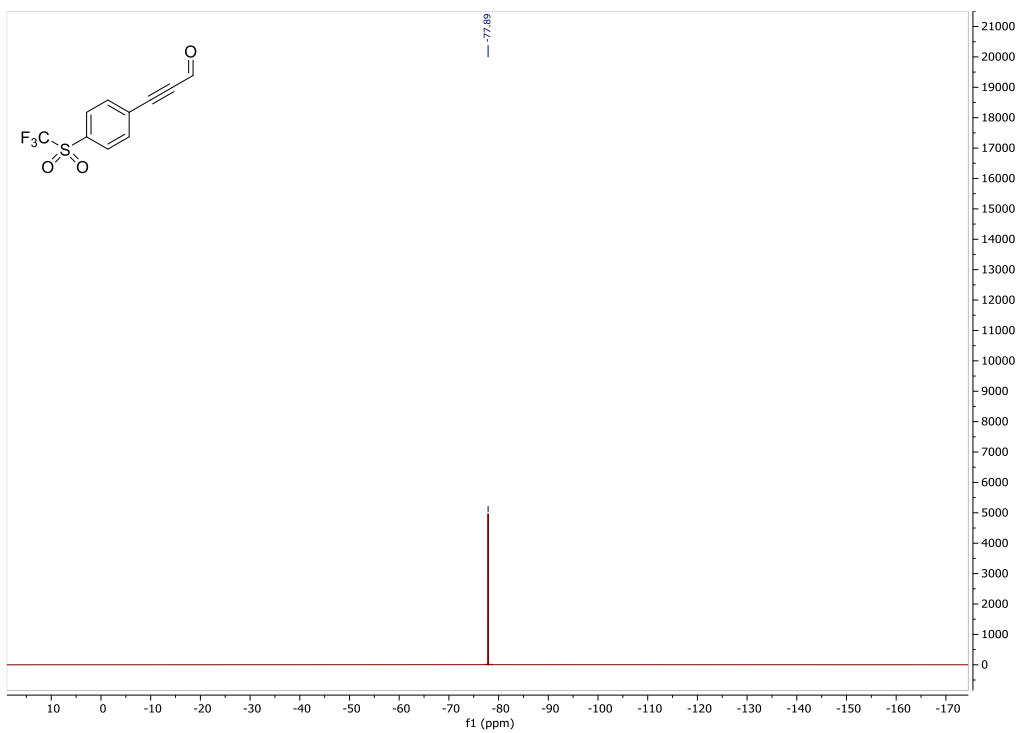
¹H NMR (300 MHz, CDCl₃) Spectrum of Compound **4G**



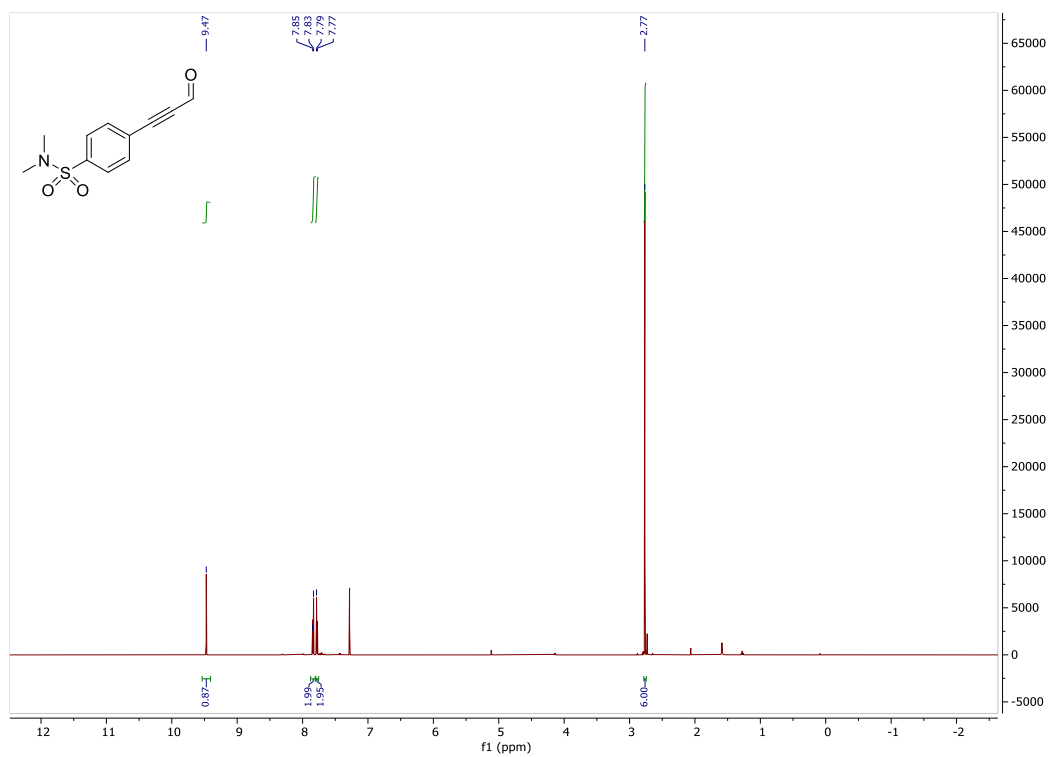
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **4G**



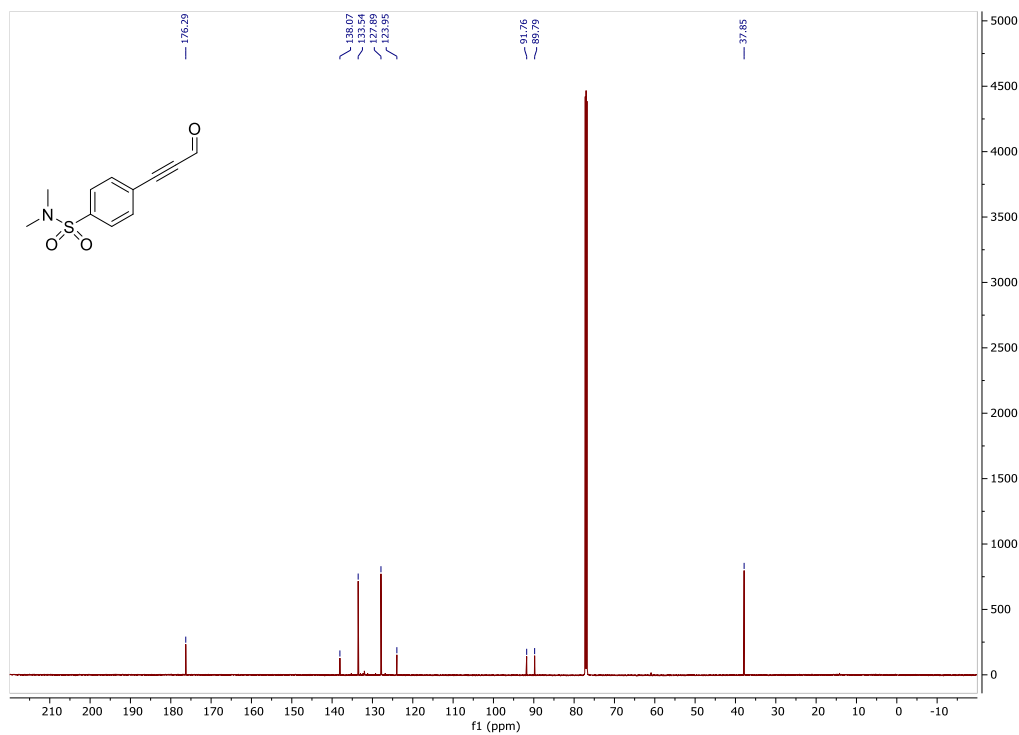
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **4G**



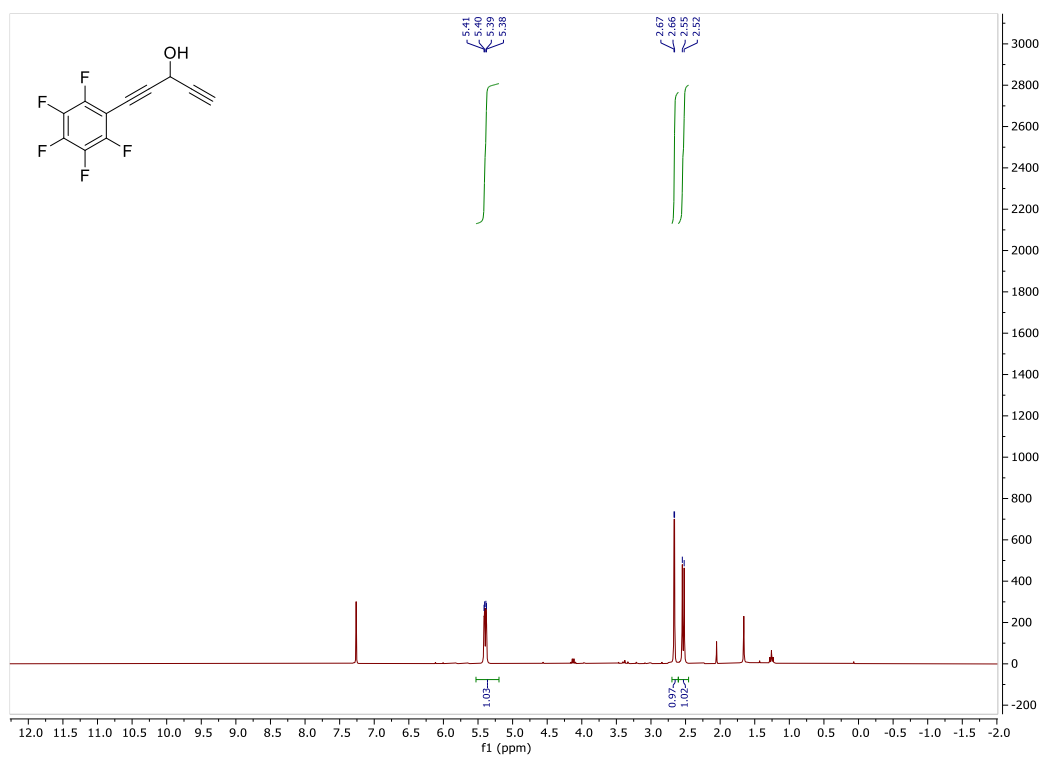
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **4H**



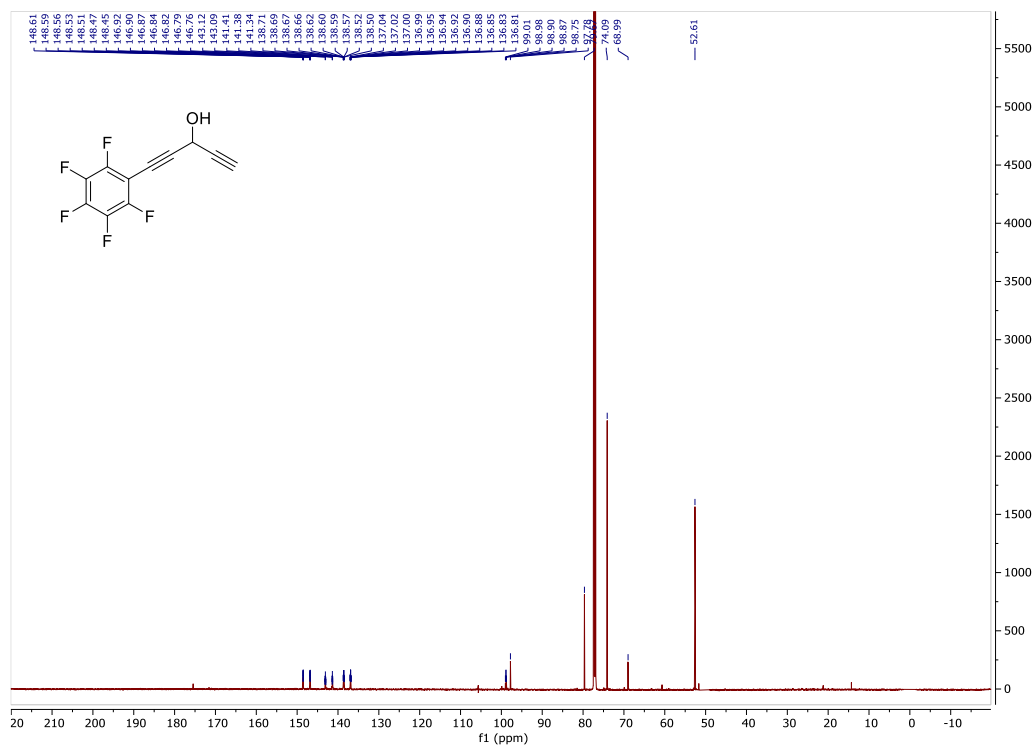
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **4H**



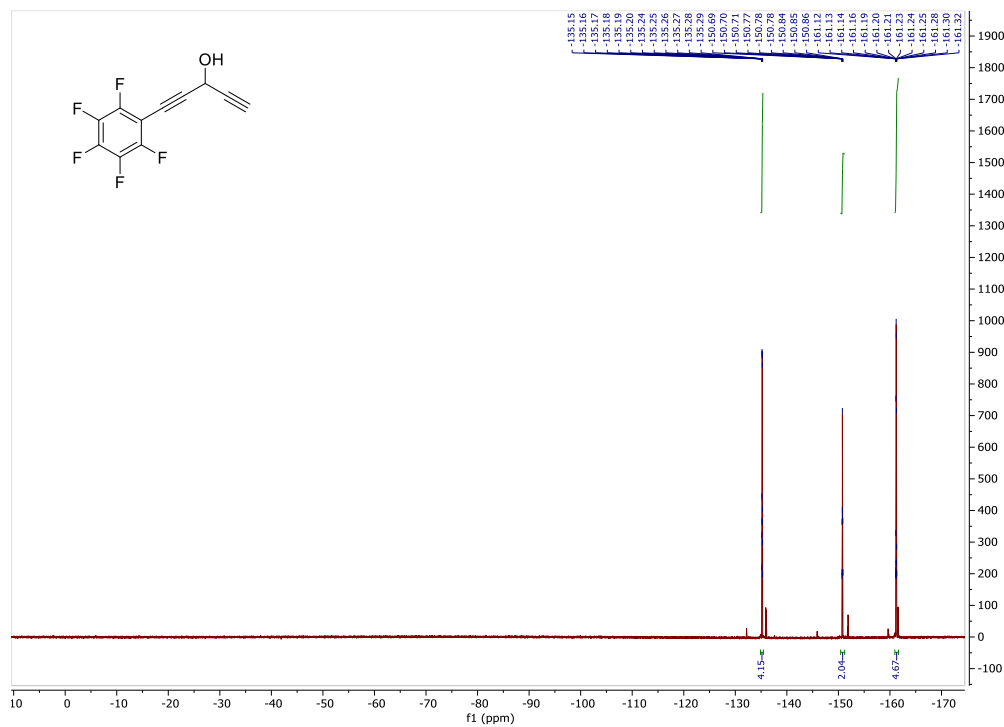
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **5C**



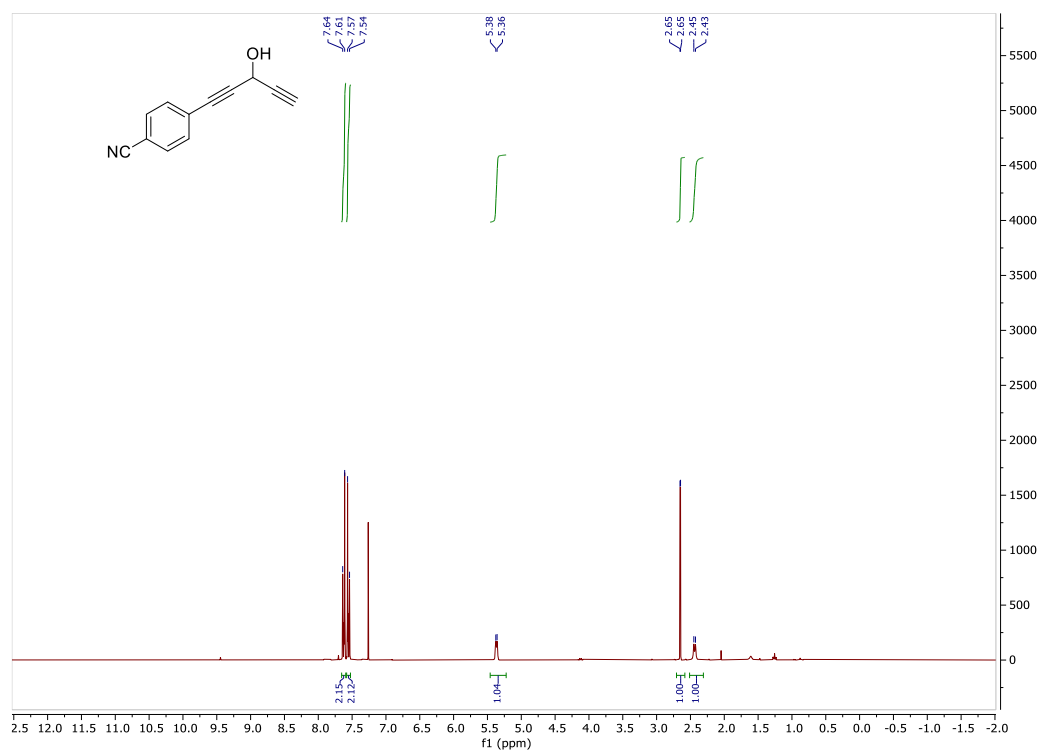
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **5C**



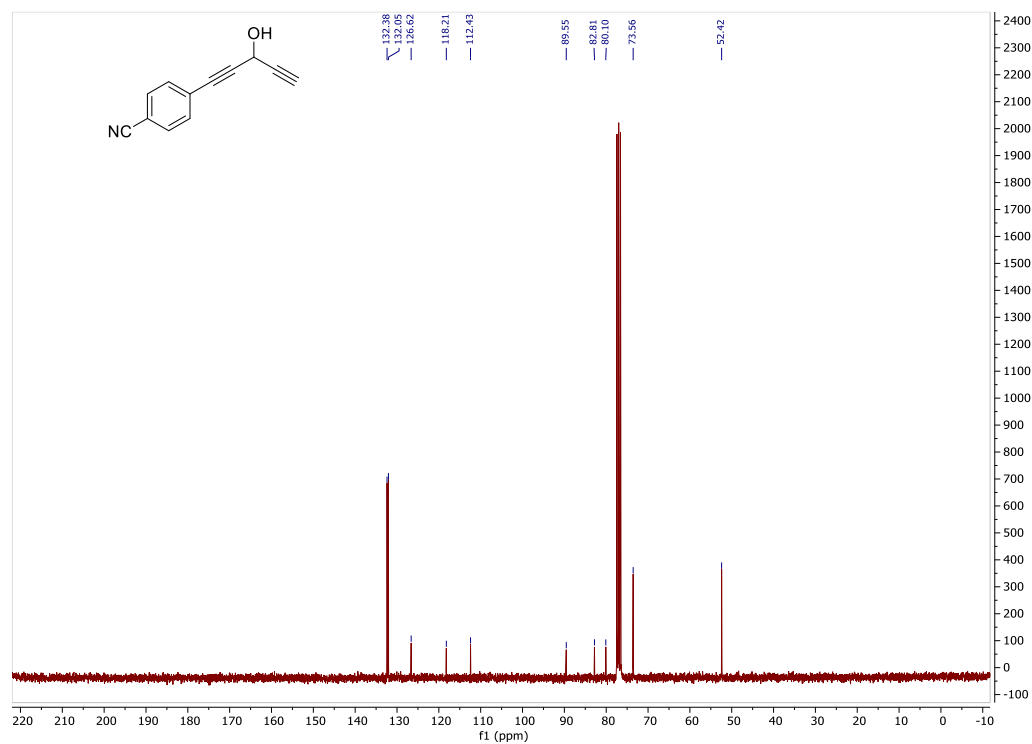
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **5C**



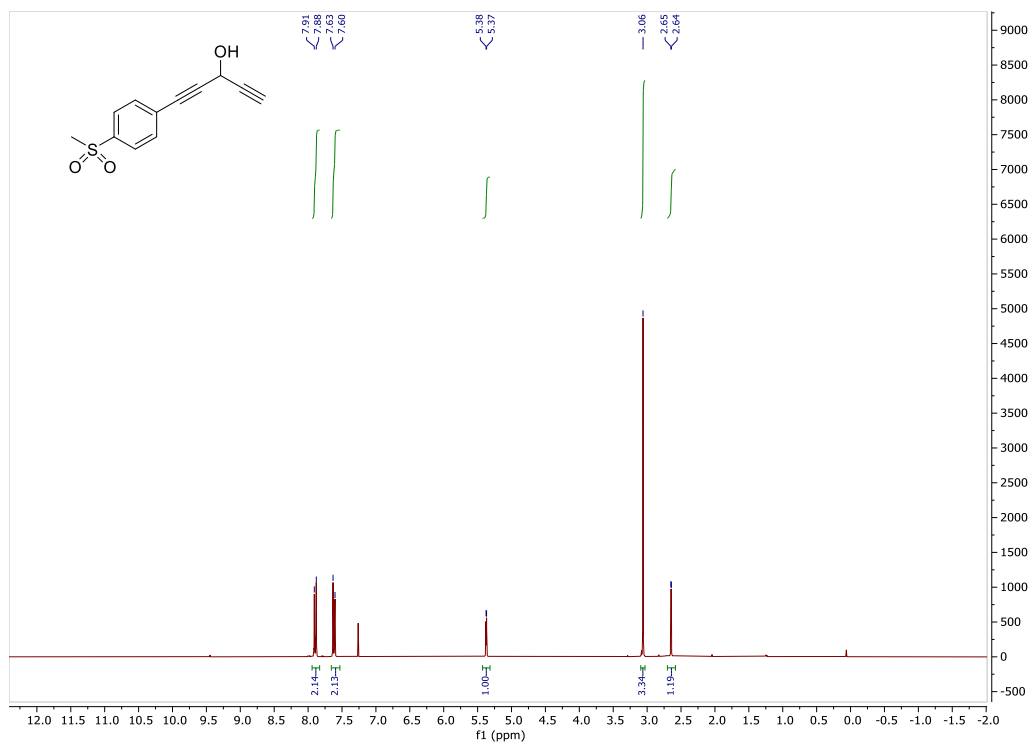
¹H NMR (300 MHz, CDCl₃) Spectrum of Compound **5E**



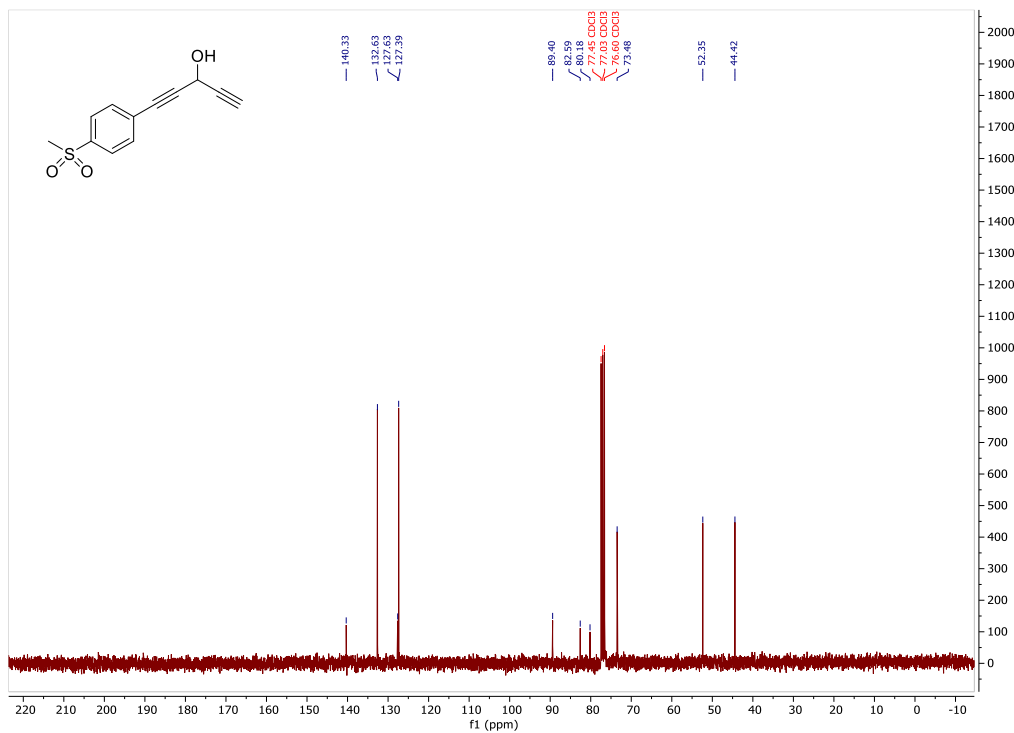
¹³C NMR (75 MHz, CDCl₃) Spectrum of Compound **5E**



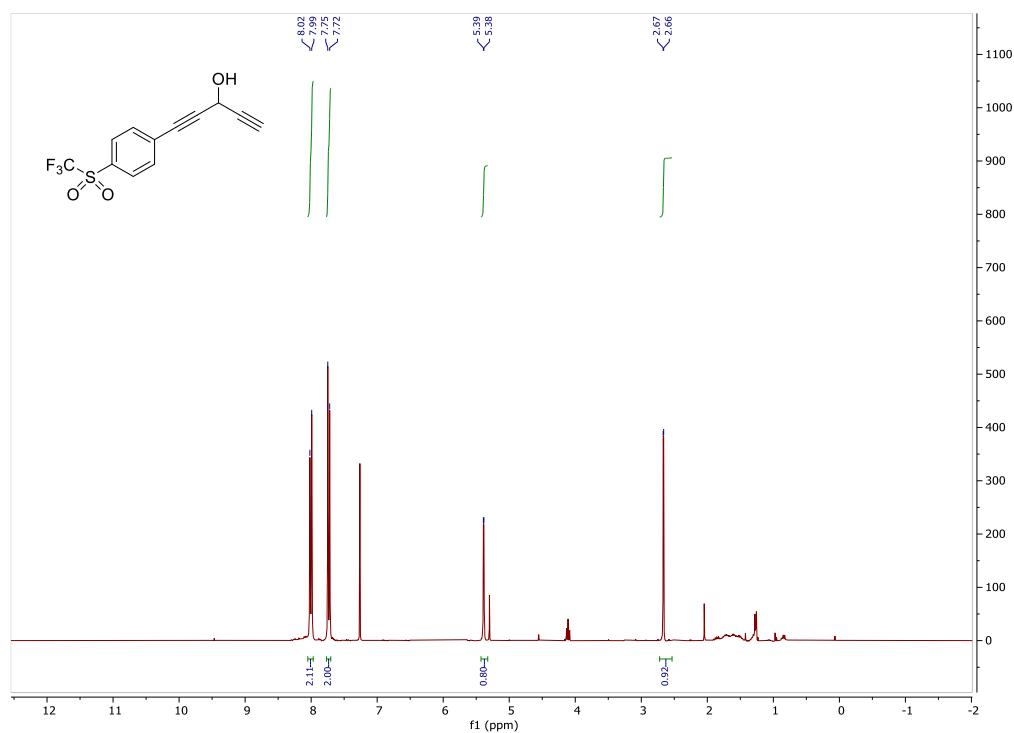
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **5F**



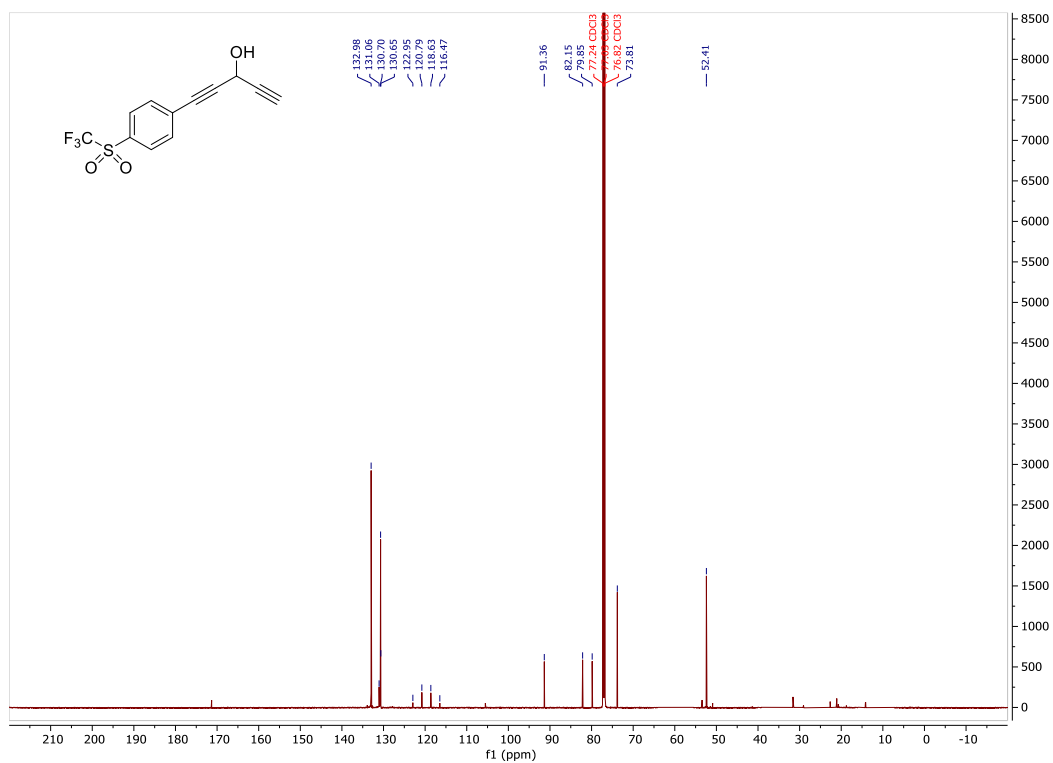
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **5F**



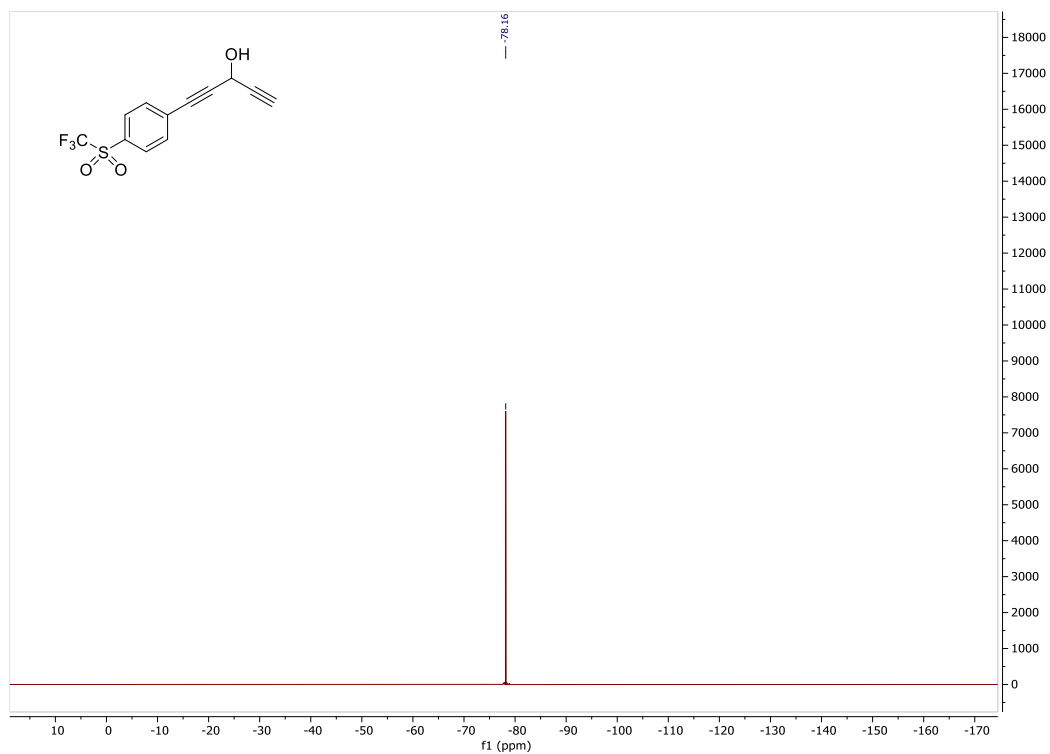
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **5G**



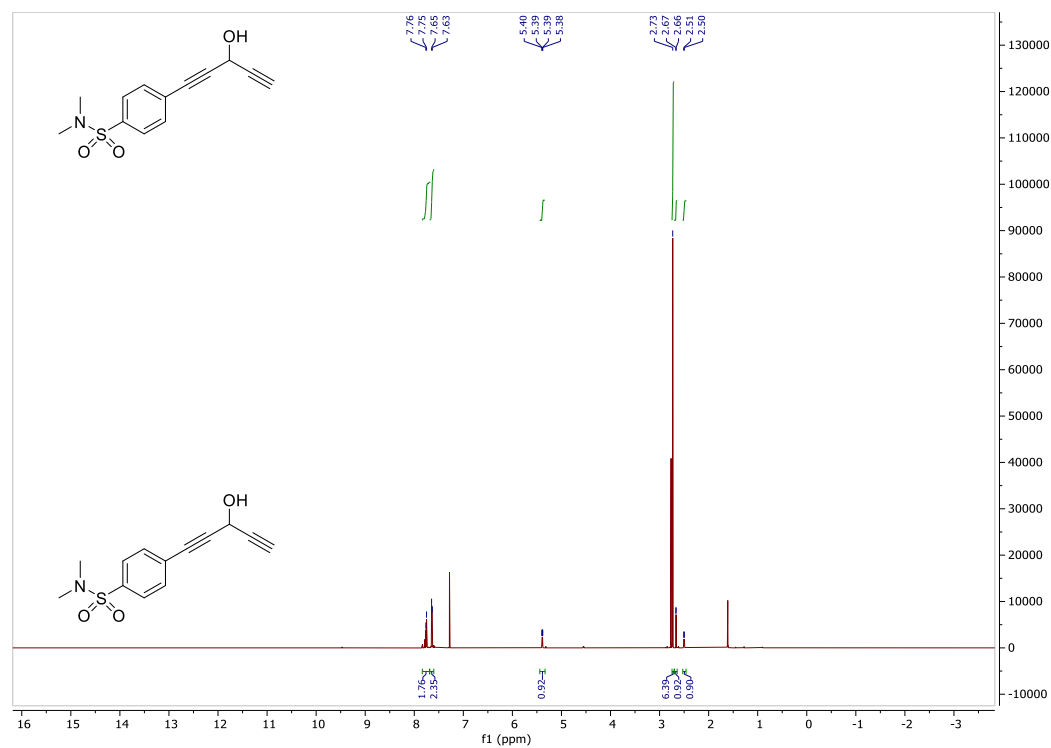
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **5G**



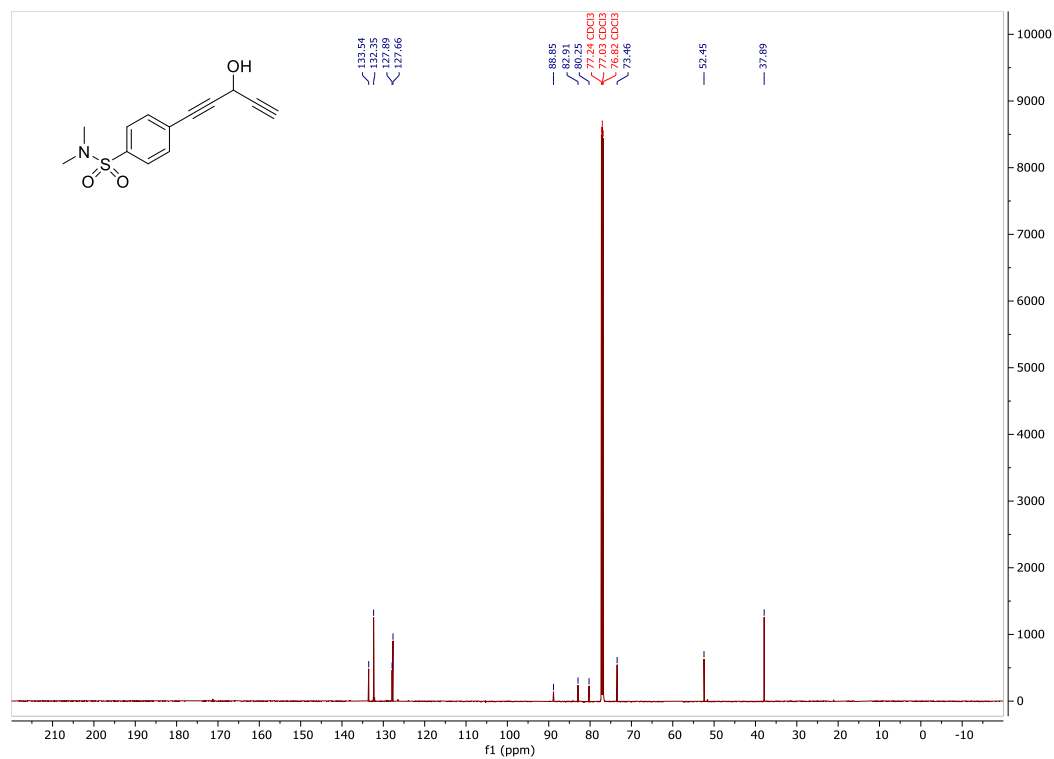
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **5G**



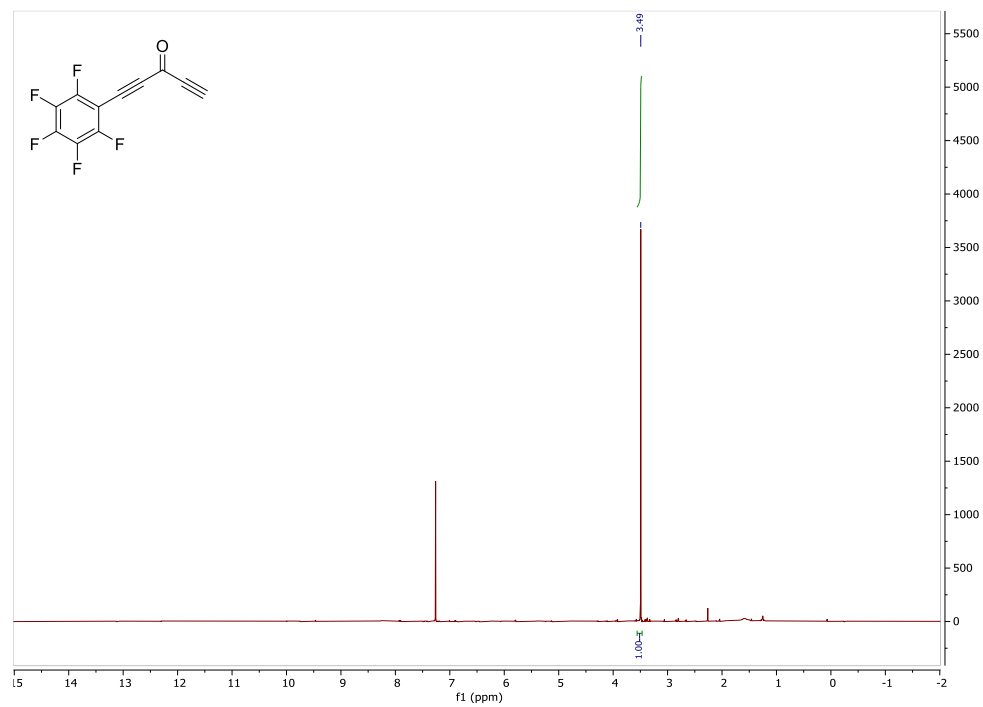
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **5H**



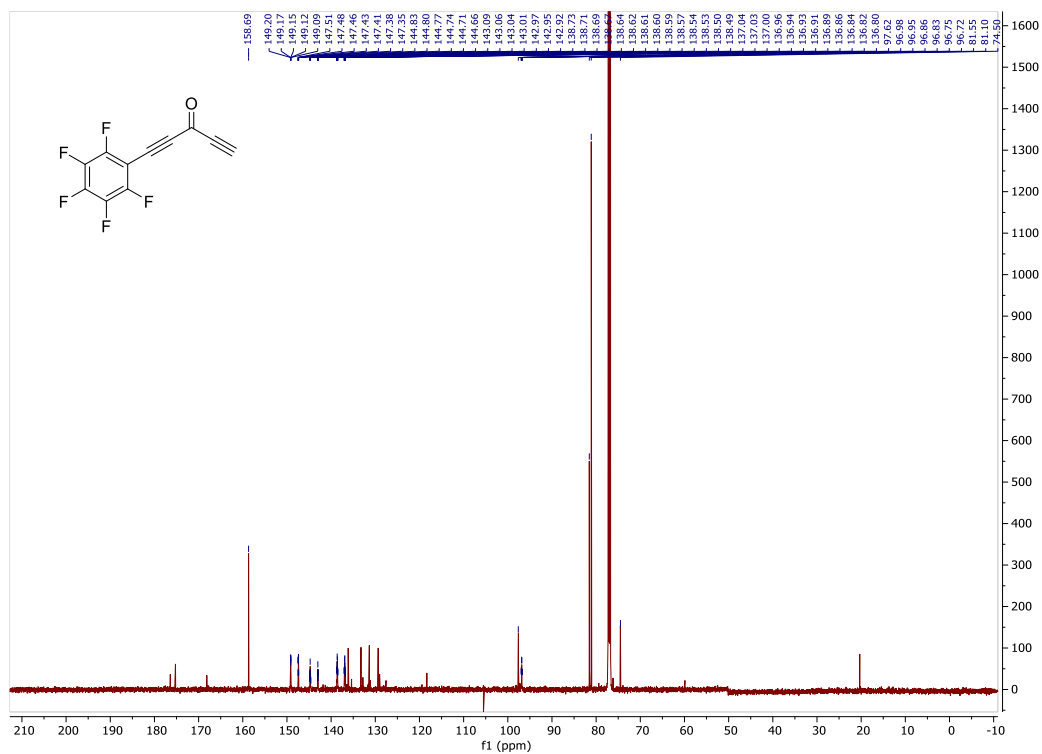
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **5H**



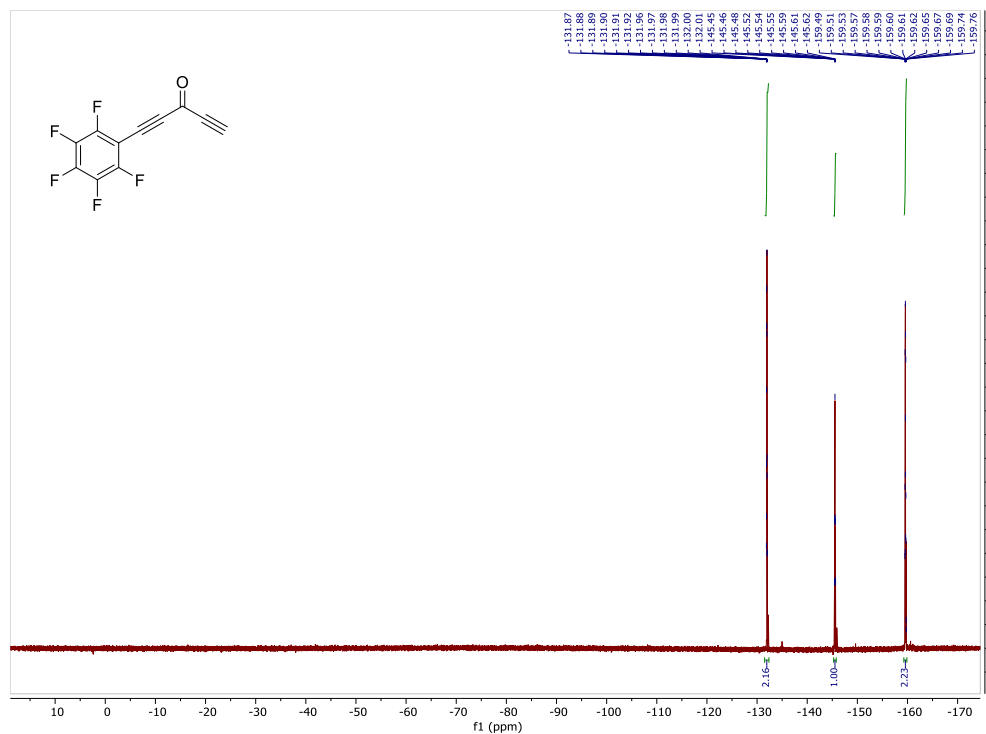
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **6C**



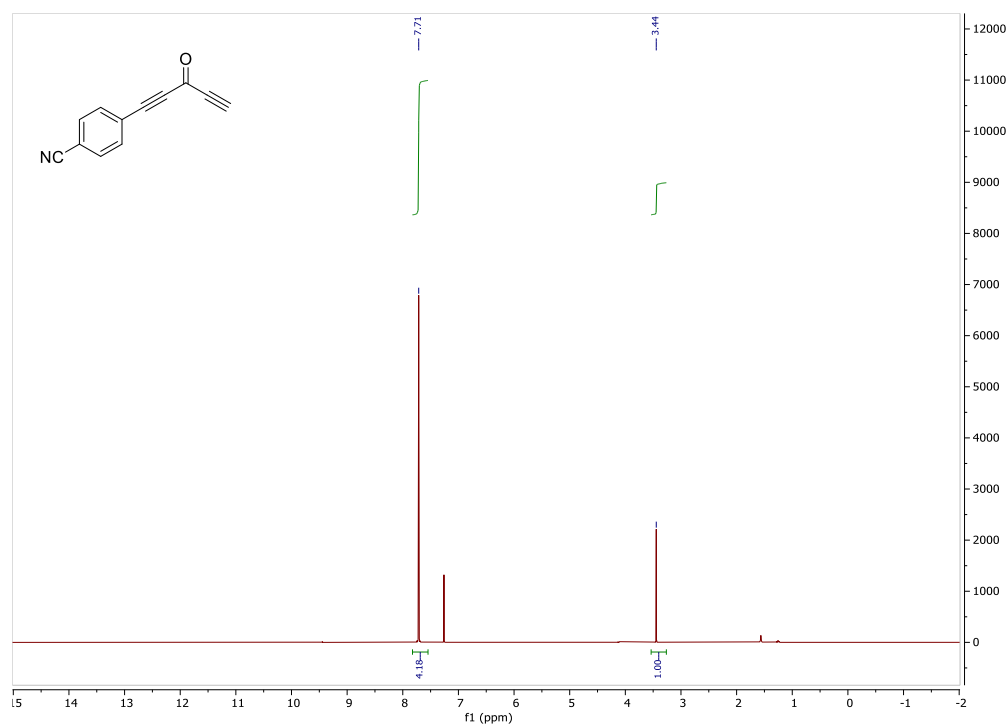
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **6C**



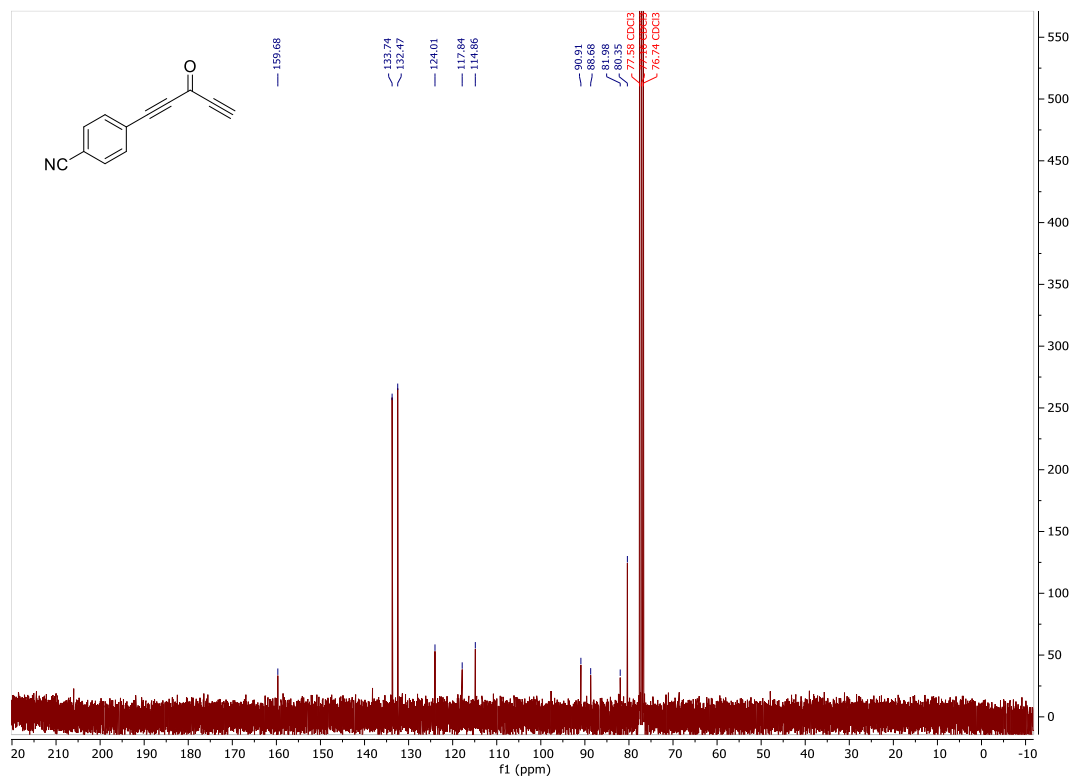
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **6C**



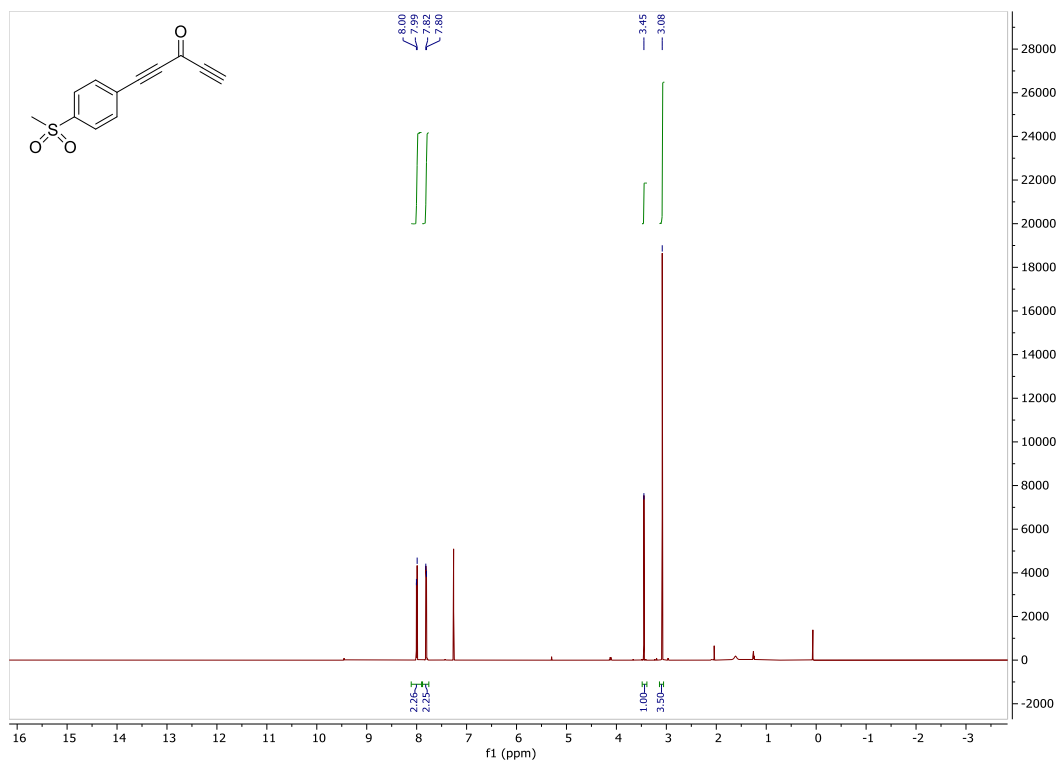
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **6E**



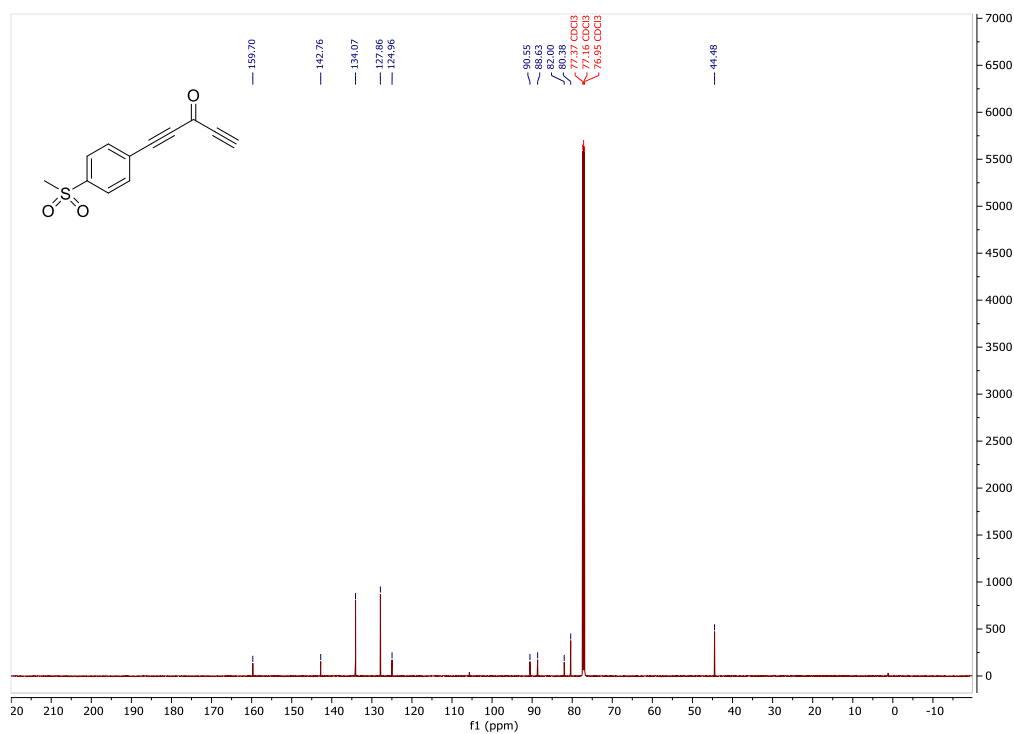
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **6E**



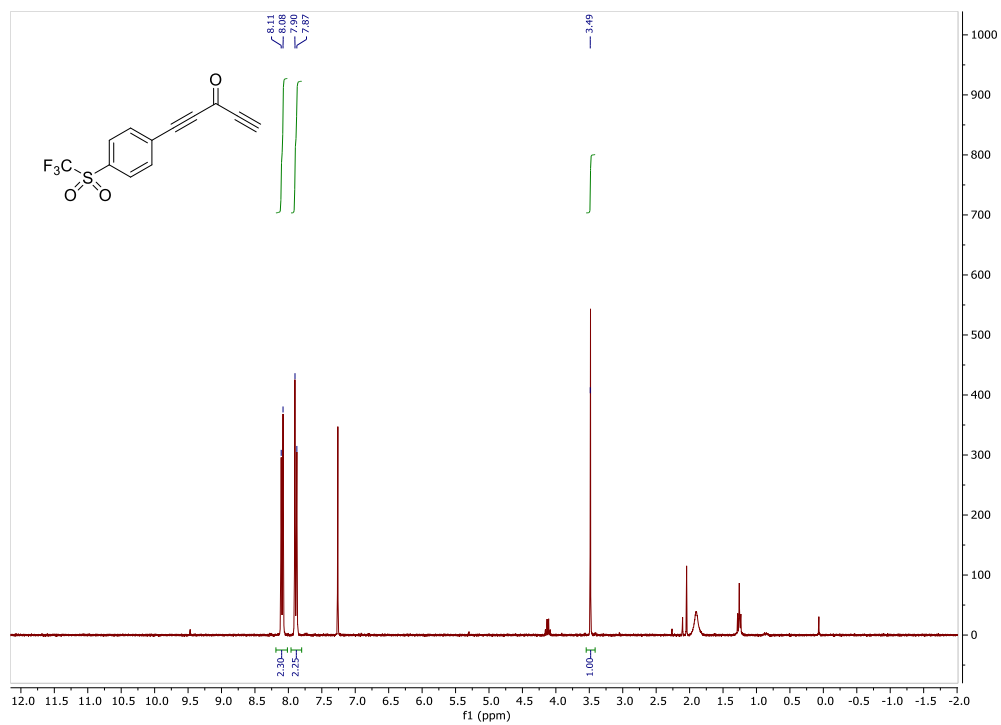
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **6F**



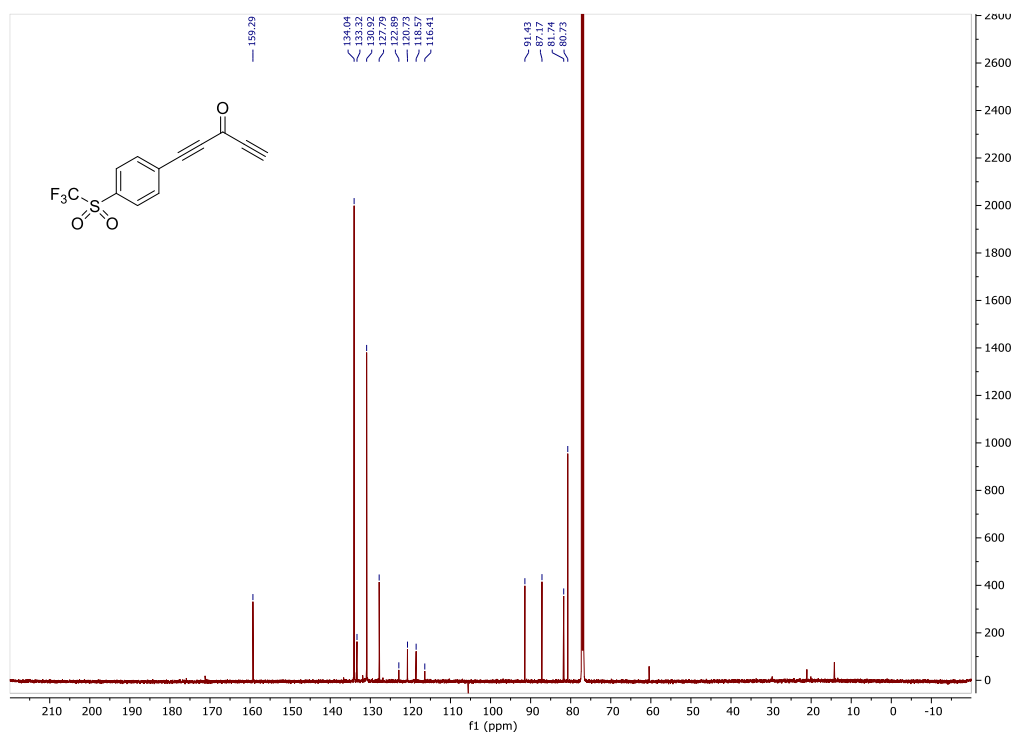
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **6F**



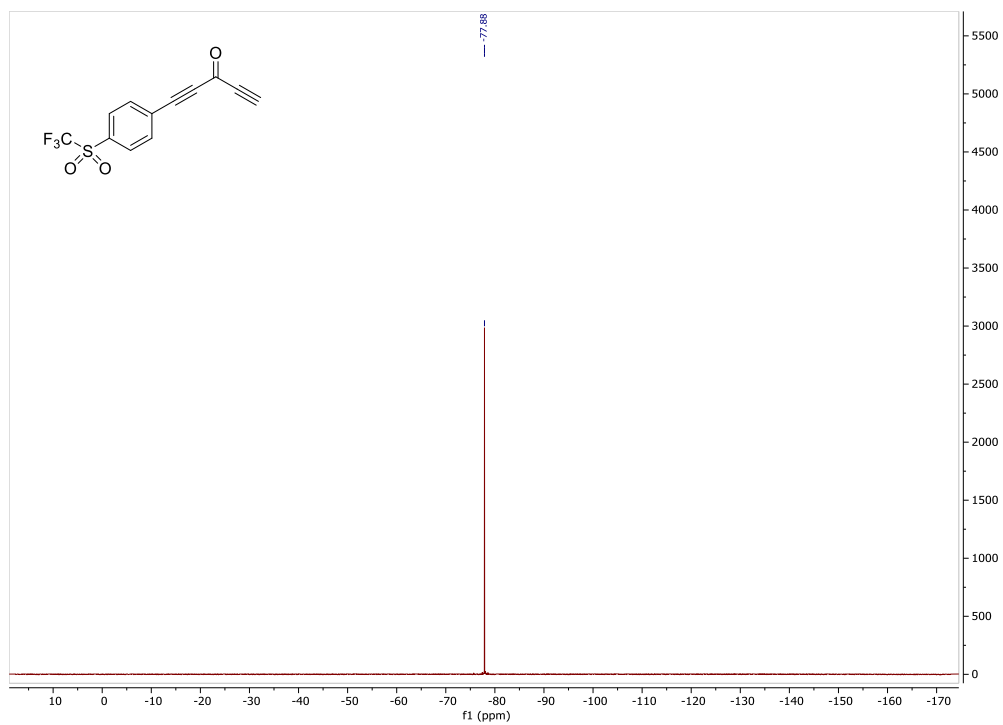
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **6G**



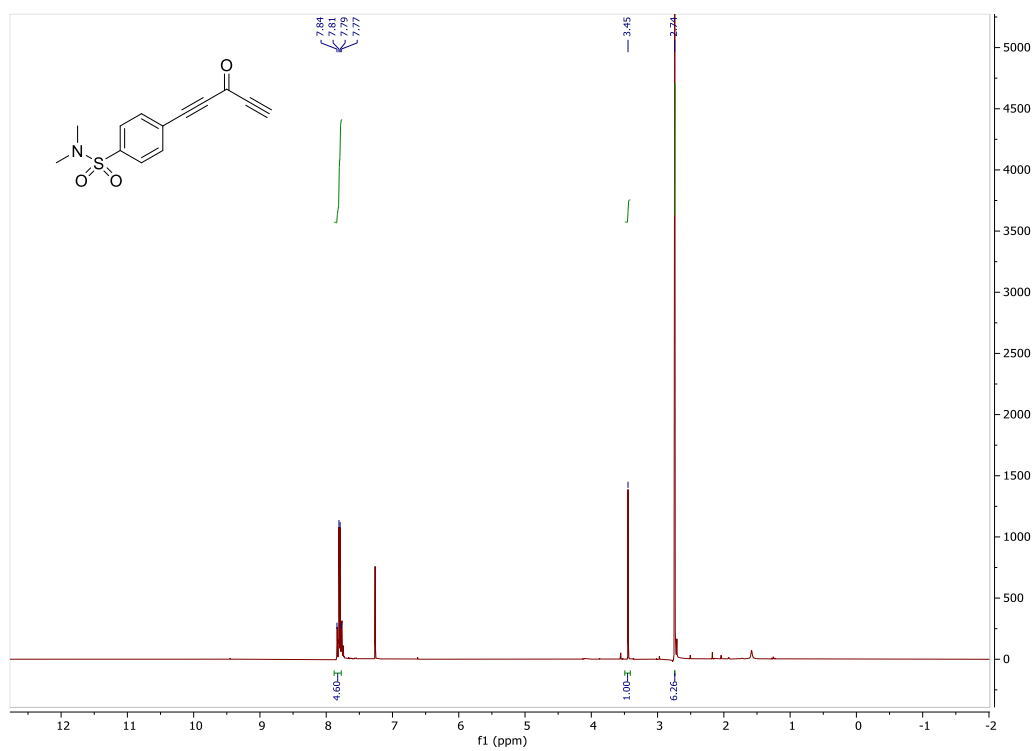
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **6G**



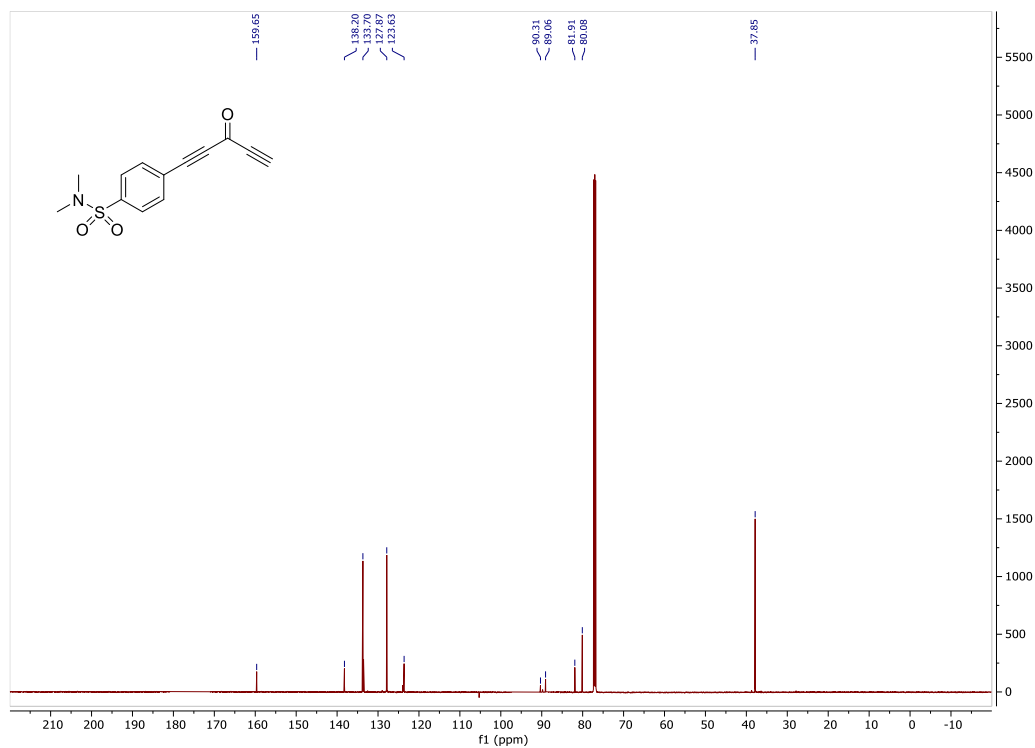
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **6G**



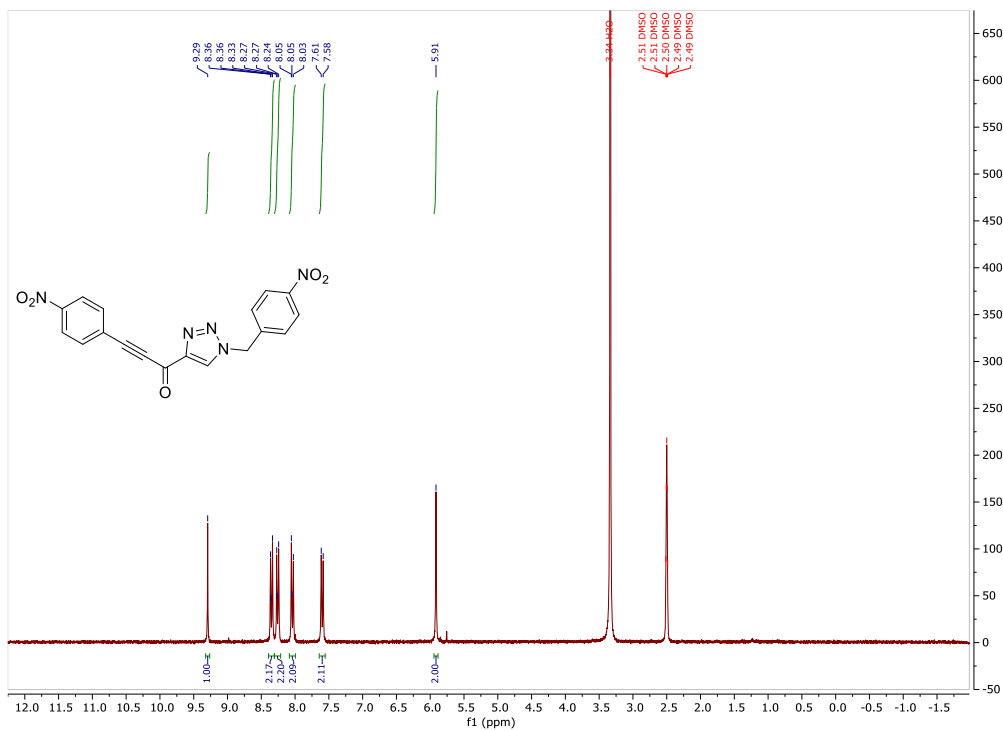
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **6H**



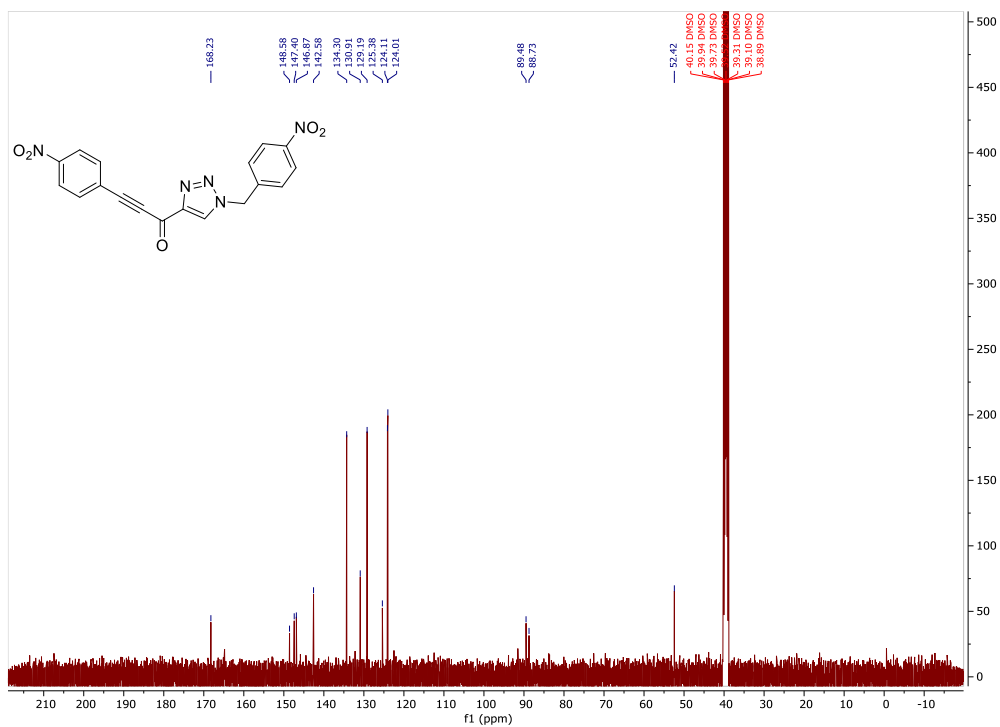
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **6H**



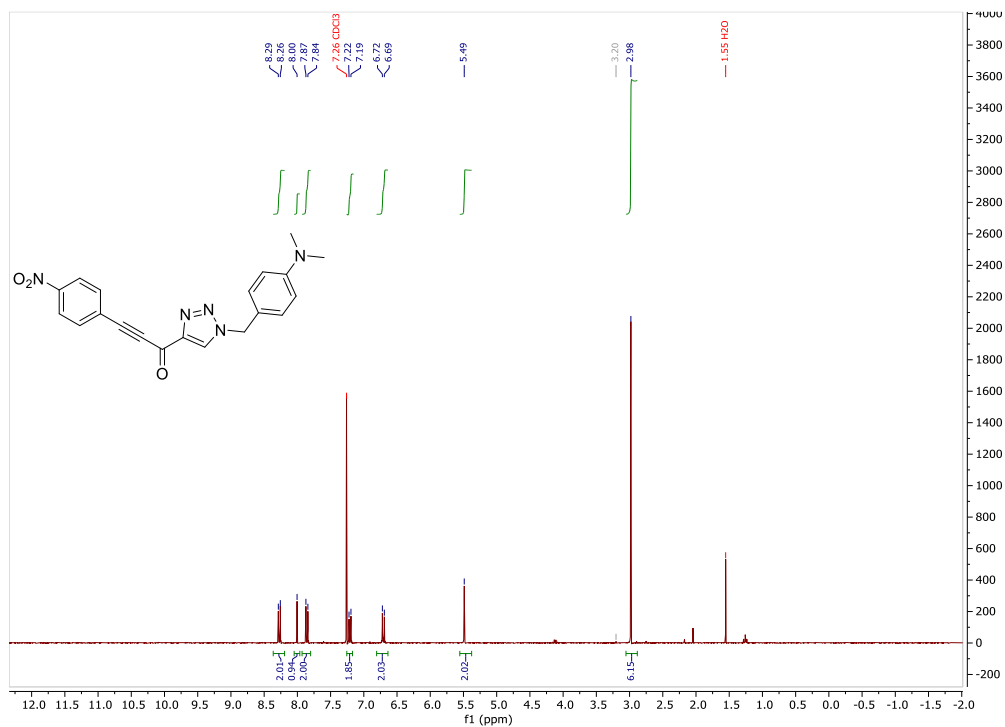
^1H NMR (300 MHz, $\text{DMSO}-d_6$) Spectrum of Compound **8A**



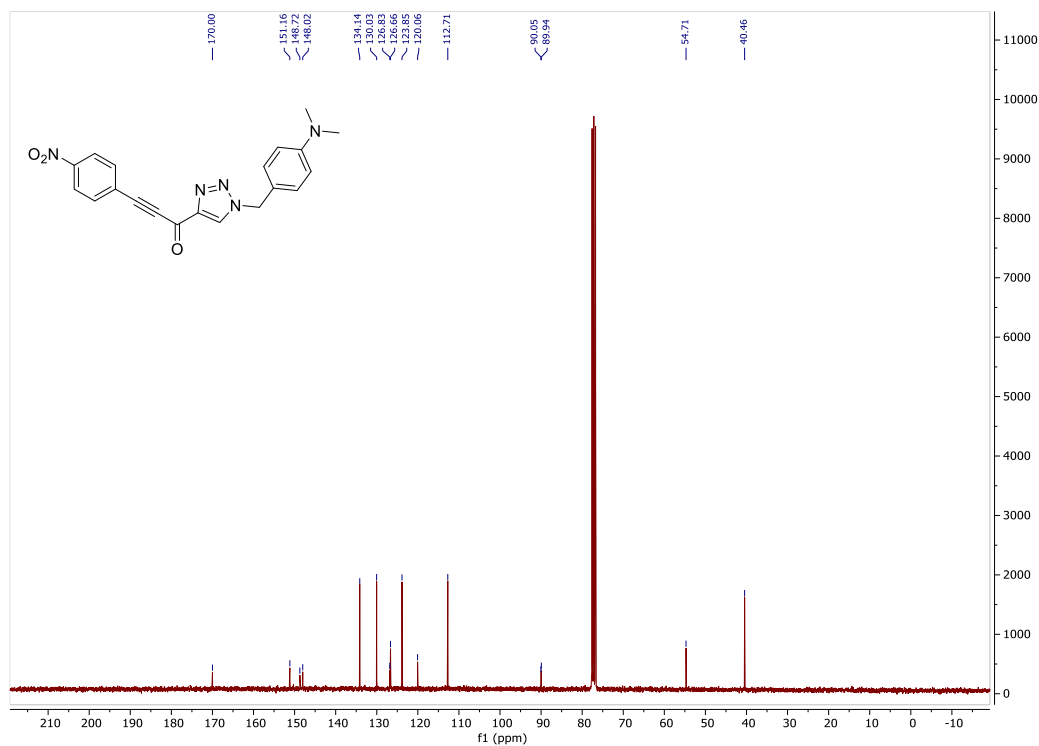
¹³C NMR (75 MHz, DMSO-*d*₆) Spectrum of Compound **8A**



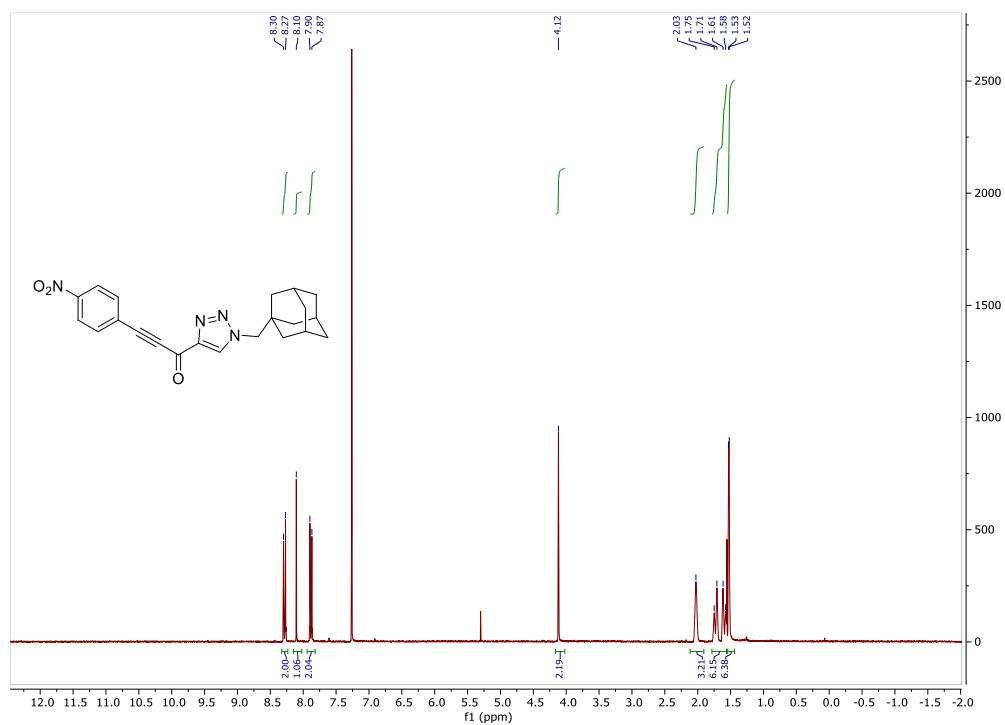
¹H NMR (300 MHz, CDCl₃) Spectrum of Compound **8B**



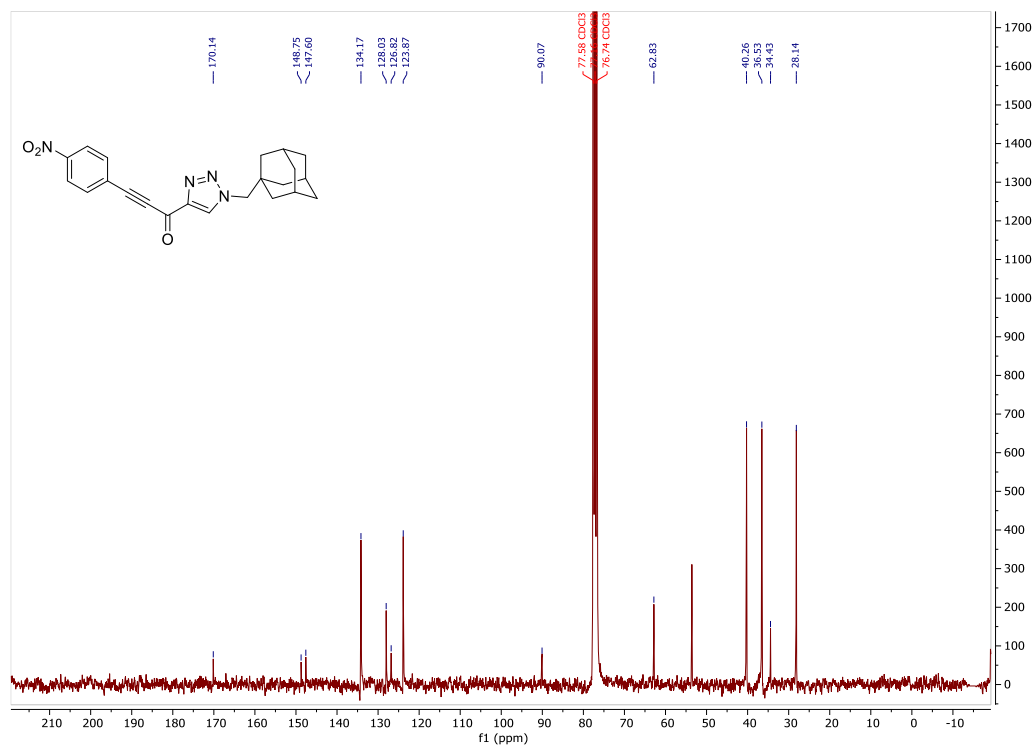
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **8B**



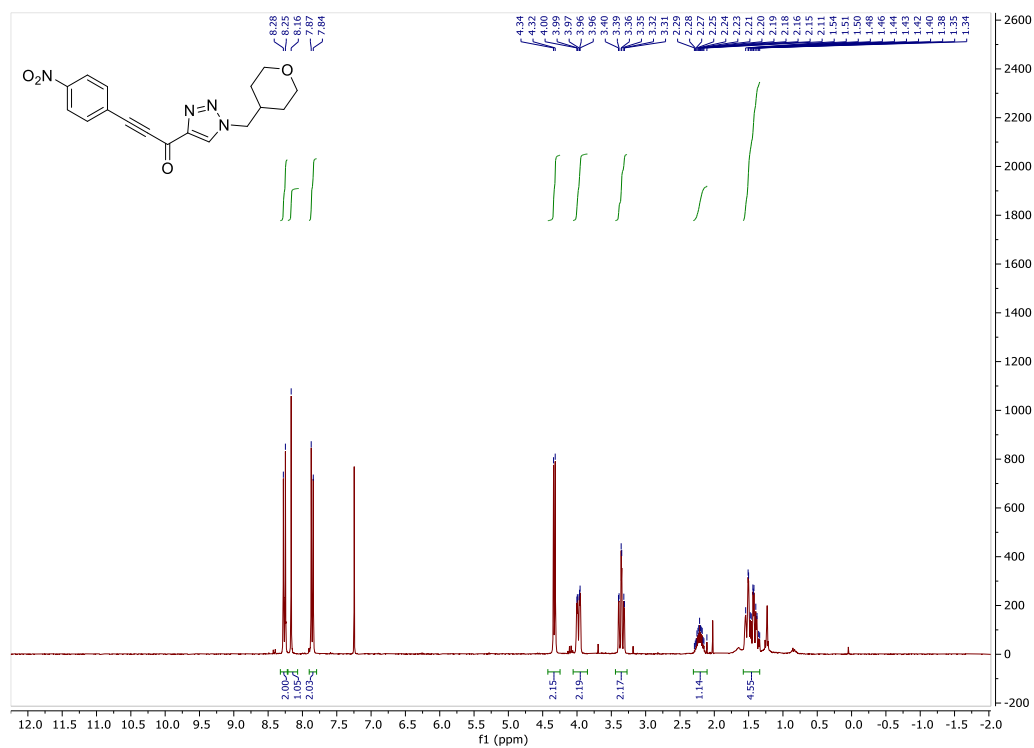
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **8C**



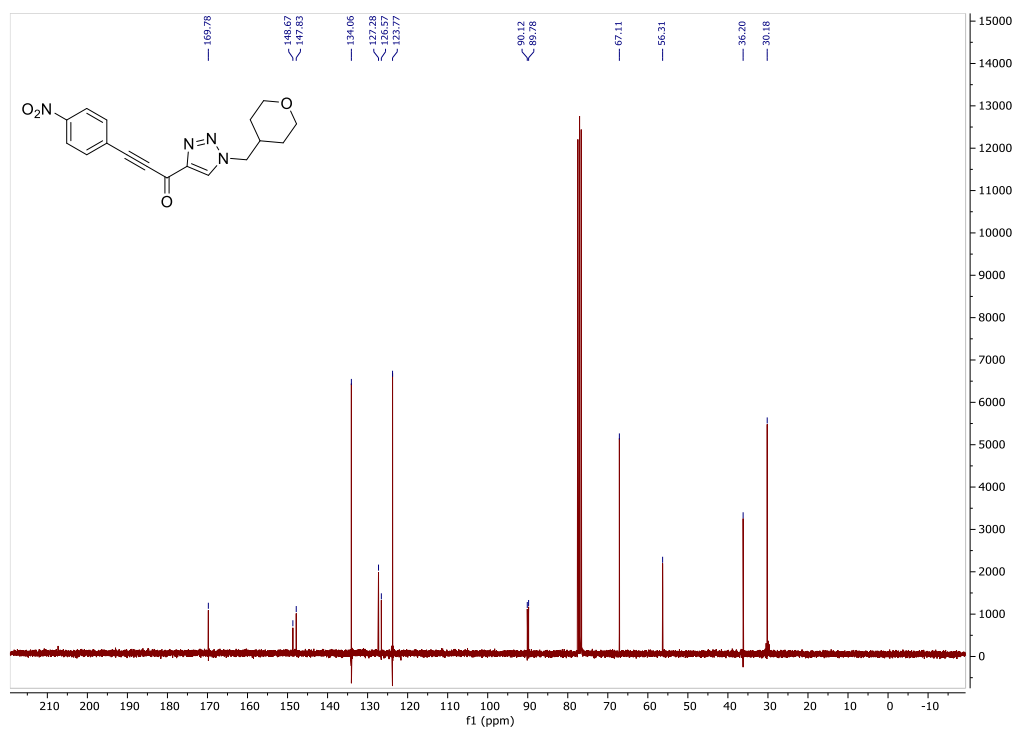
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **8C**



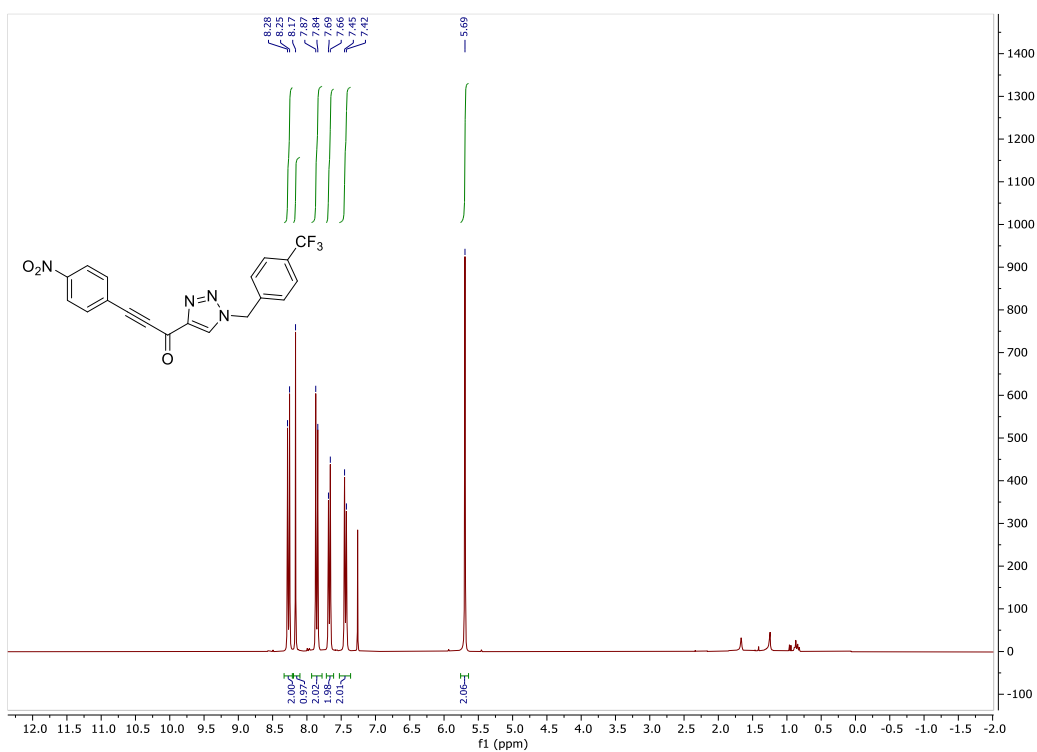
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **8D**



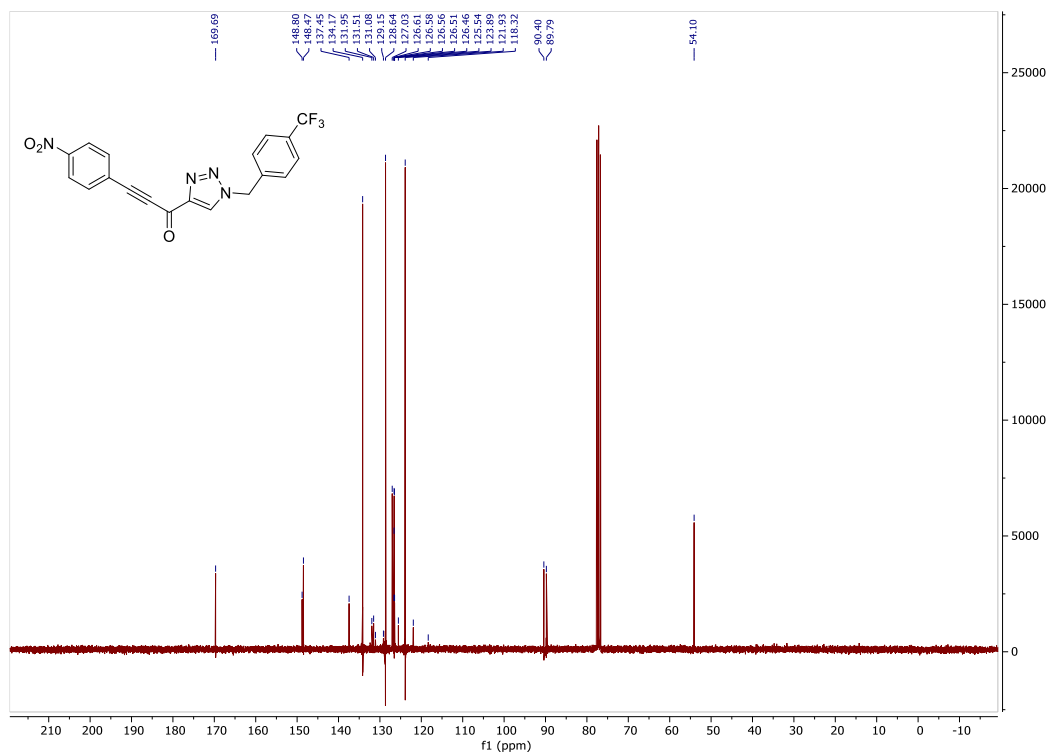
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **8D**



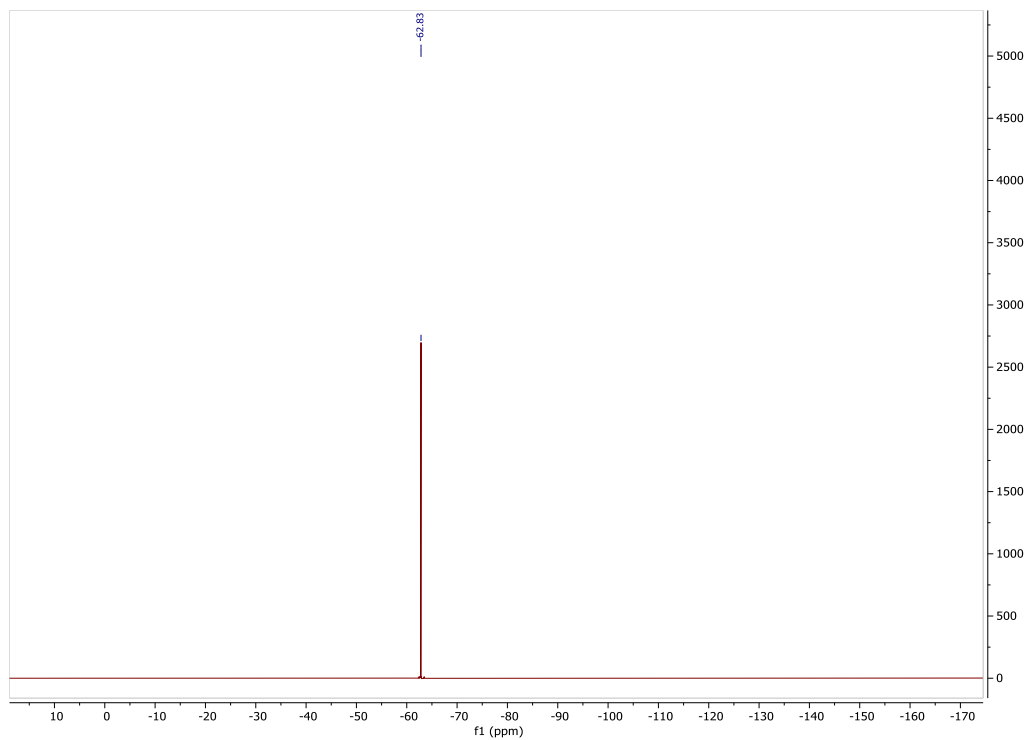
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **8E**



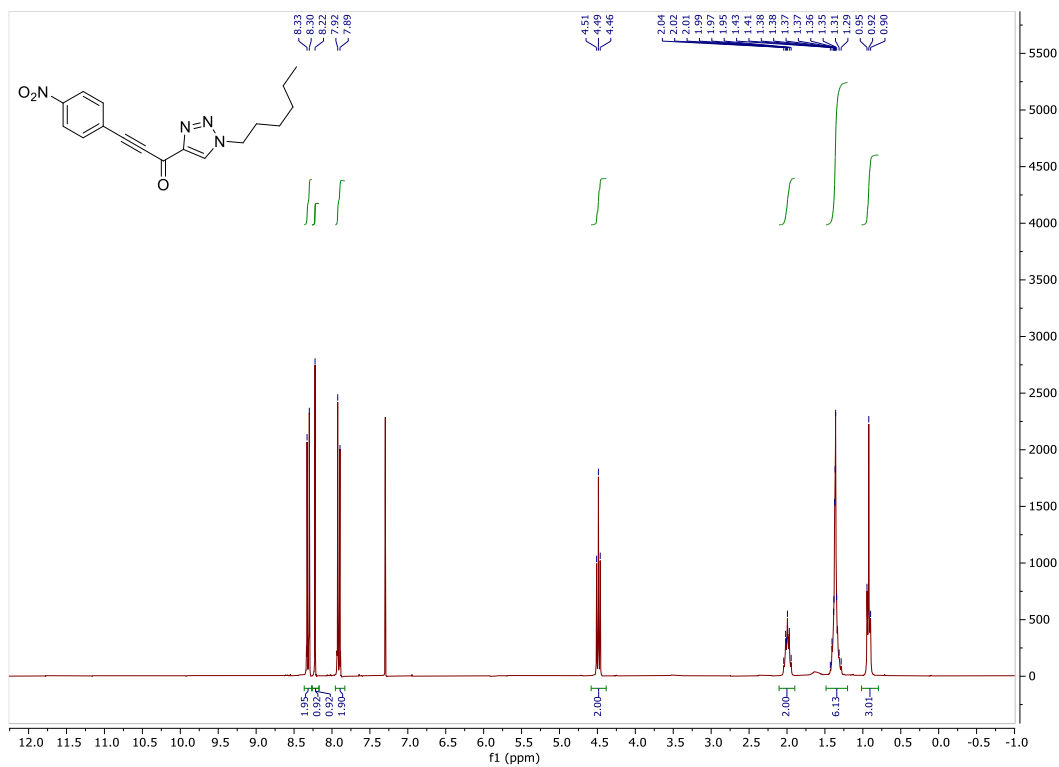
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **8E**



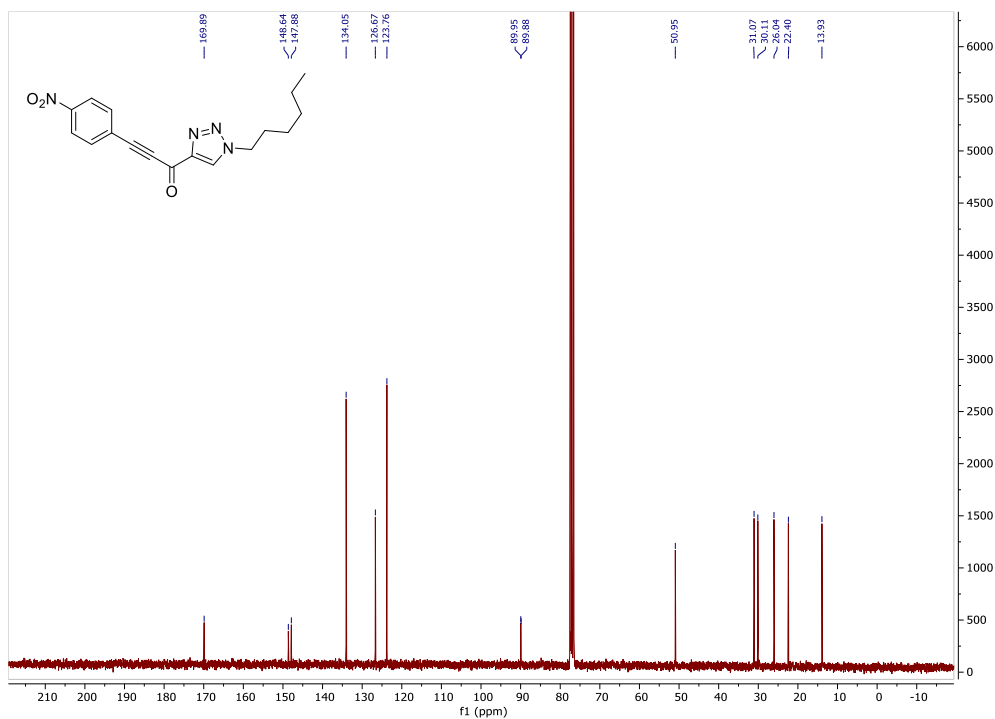
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **8E**



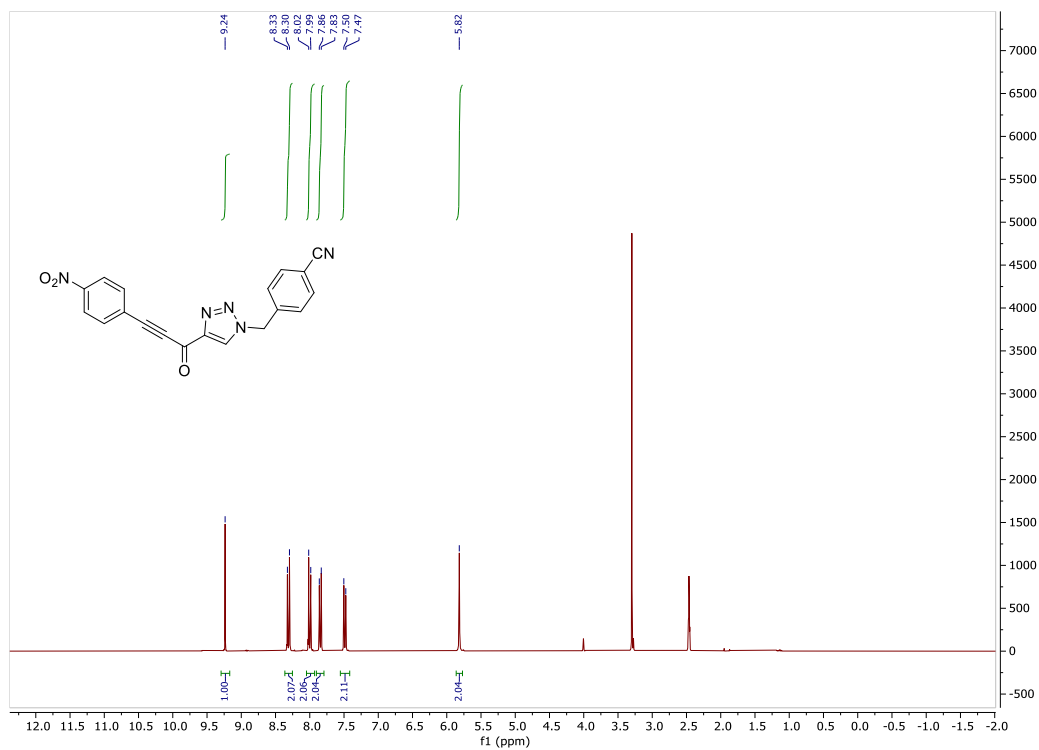
¹H NMR (300 MHz, CDCl₃) Spectrum of Compound **8F**



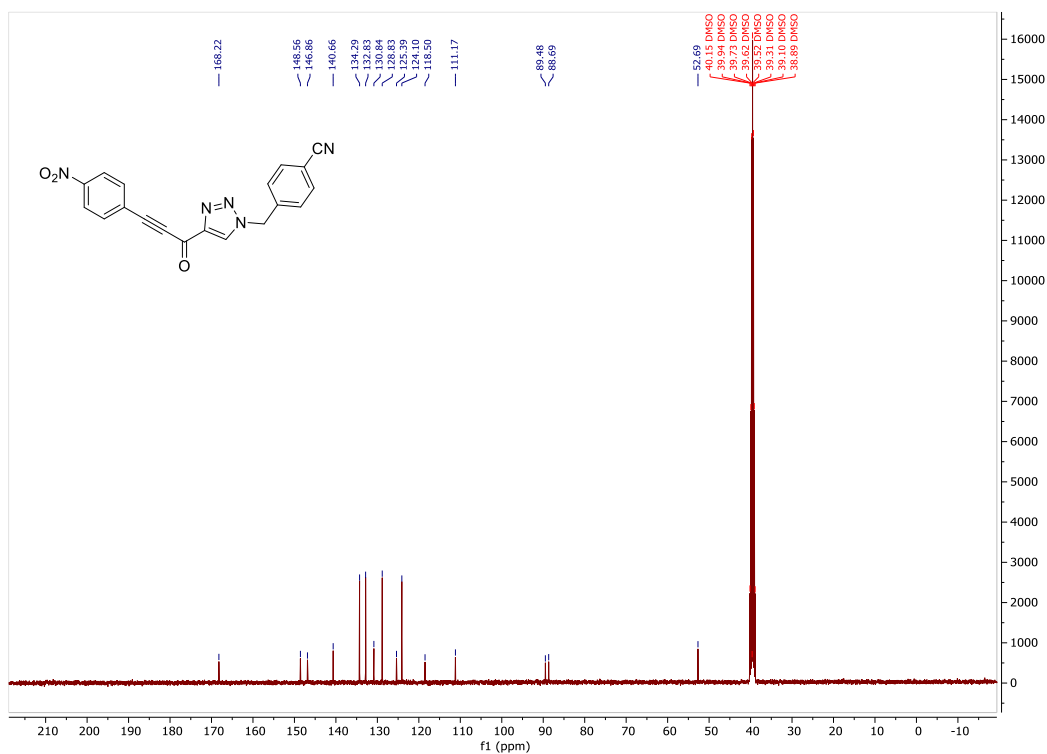
¹³C NMR (75 MHz, CDCl₃) Spectrum of Compound **8F**



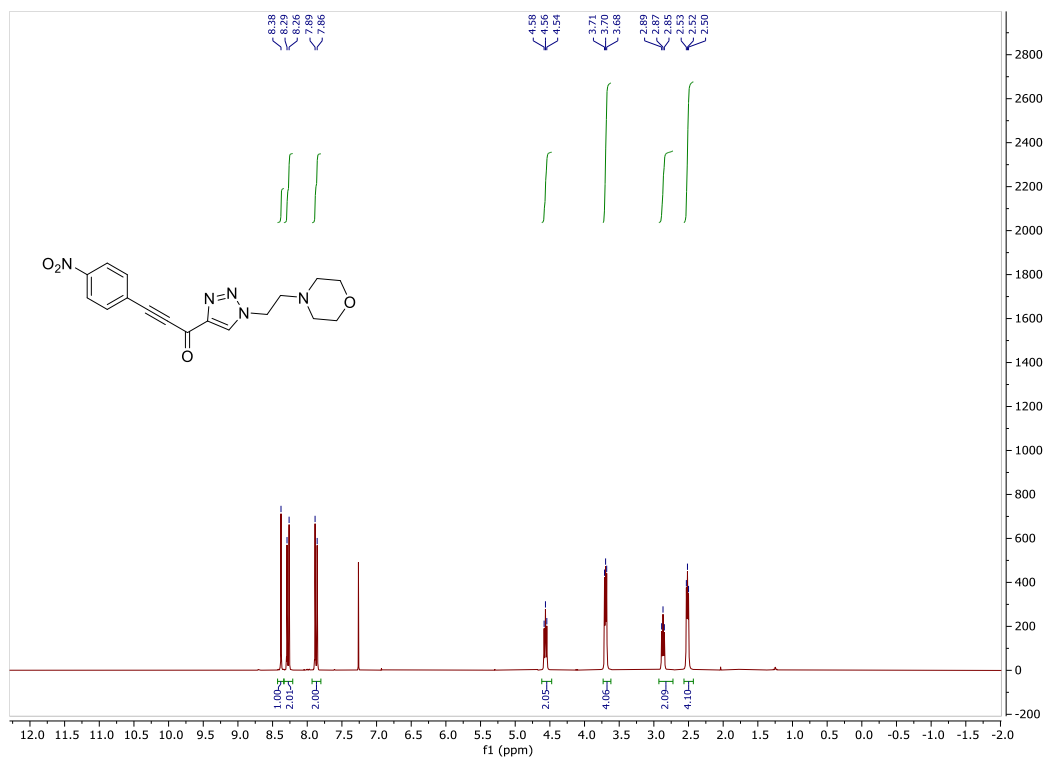
^1H NMR (300 MHz, $\text{DMSO-}d_6$) Spectrum of Compound **8G**



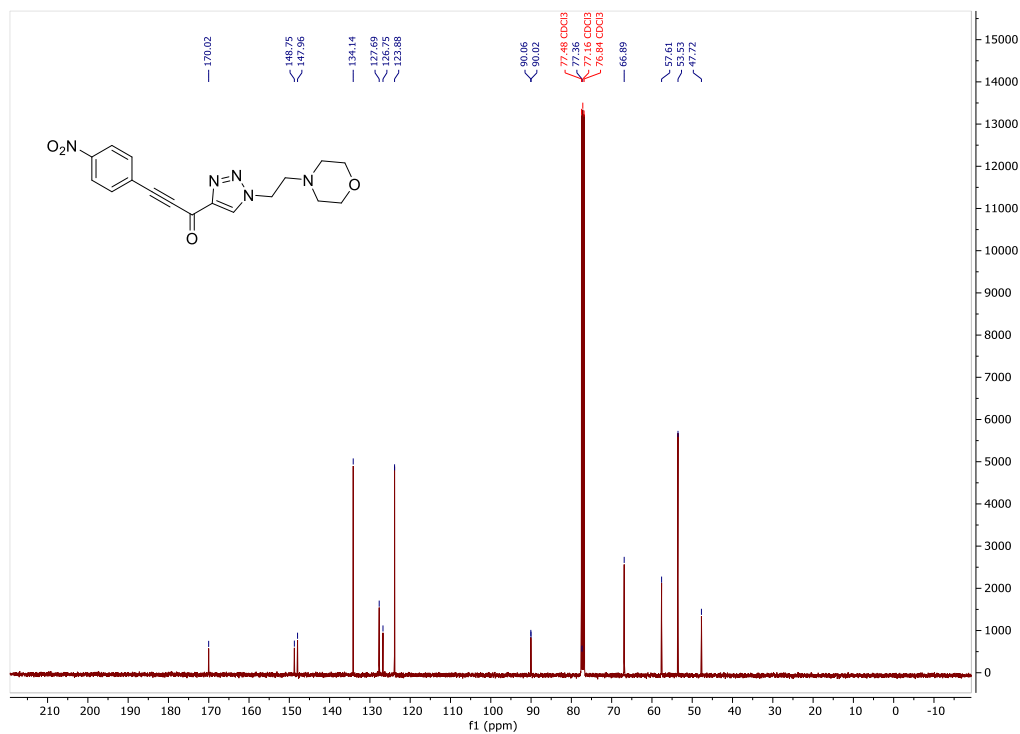
^{13}C NMR (75 MHz, $\text{DMSO-}d_6$) Spectrum of Compound **8G**



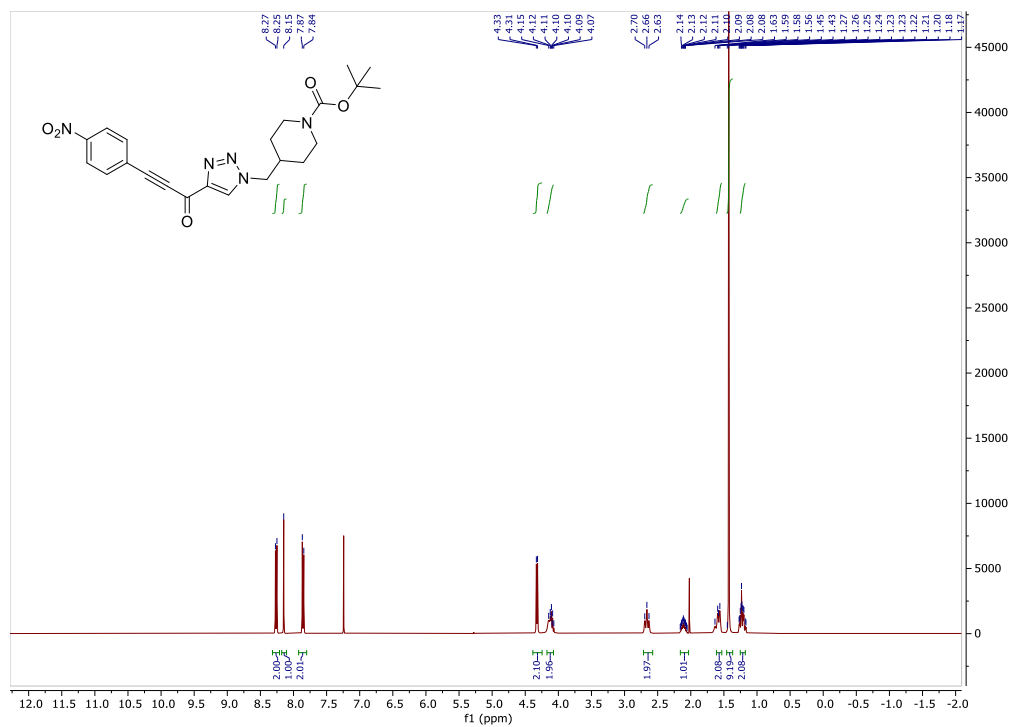
¹H NMR (400 MHz, CDCl₃) Spectrum of Compound **8H**



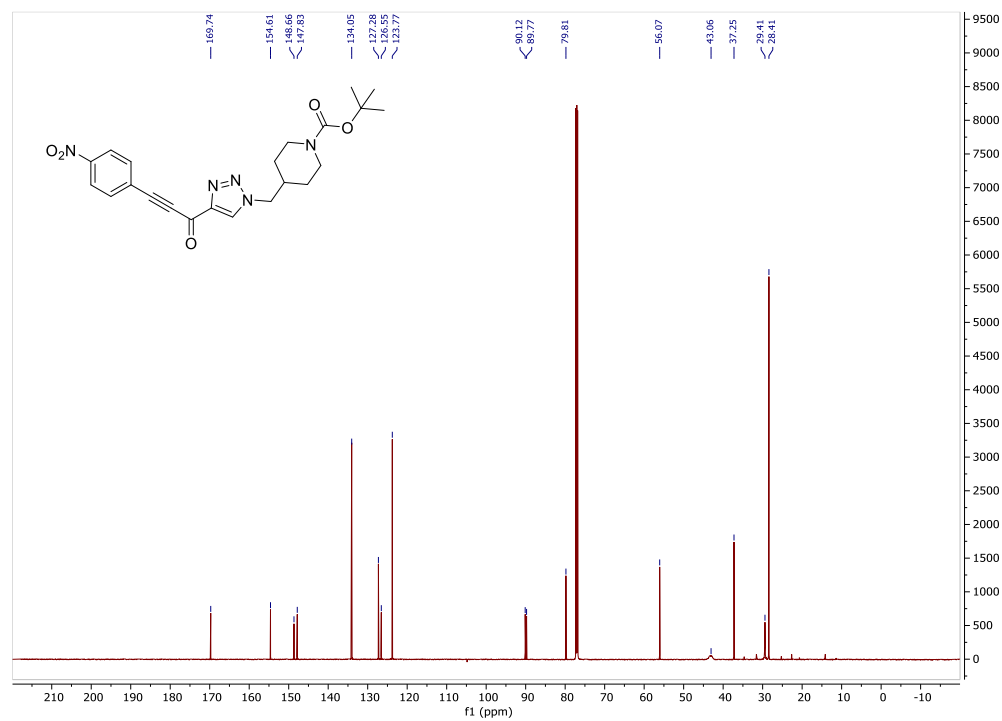
¹³C NMR (100 MHz, CDCl₃) Spectrum of Compound **8H**



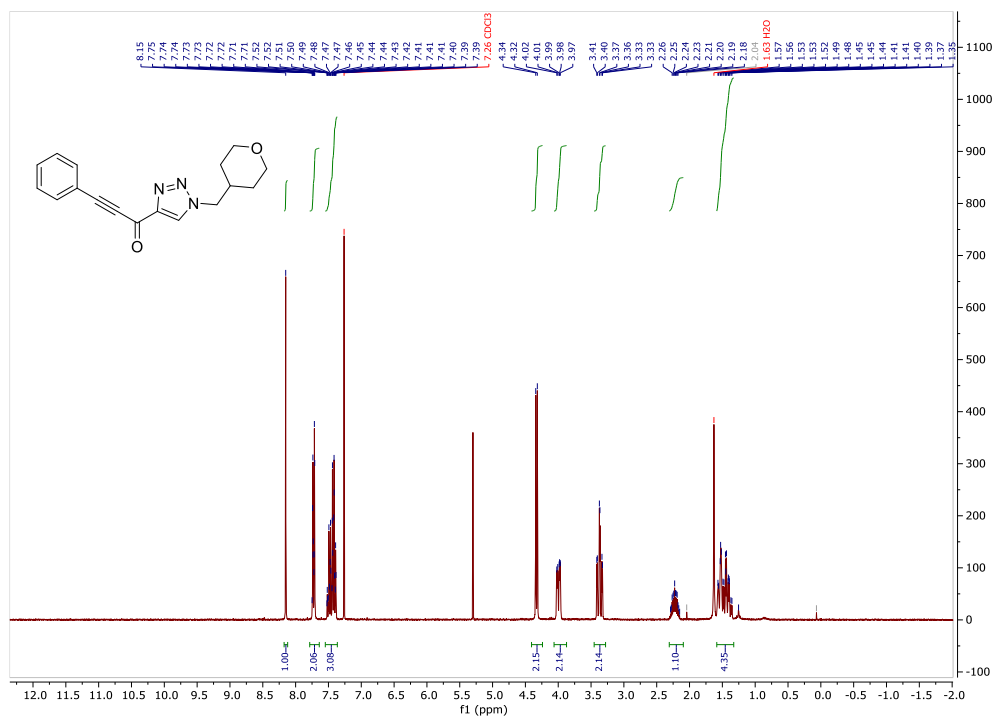
¹H NMR (400 MHz, CDCl₃) Spectrum of Compound **8I**



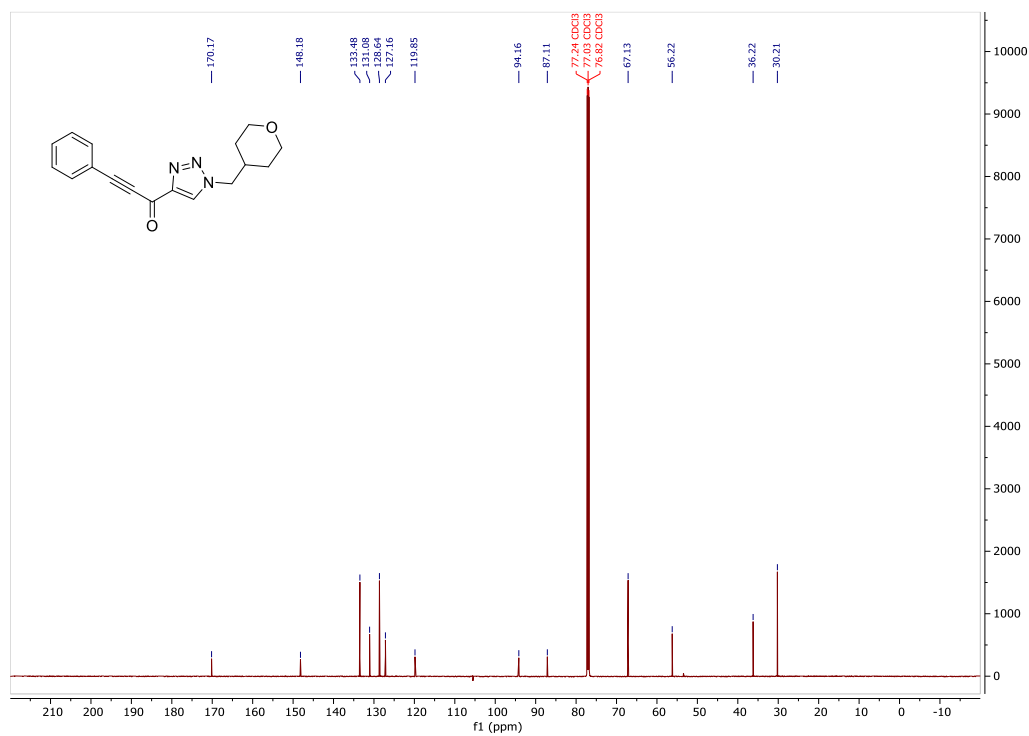
¹³C NMR (100 MHz, CDCl₃) Spectrum of Compound **8I**



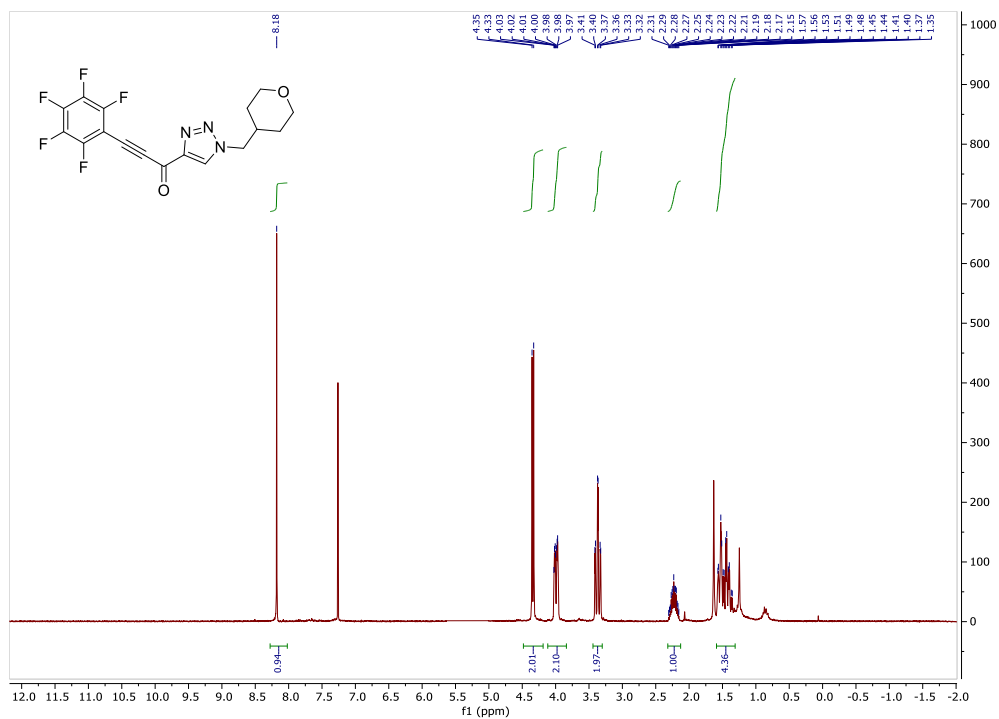
¹H NMR (400 MHz, CDCl₃) Spectrum of Compound **8J**



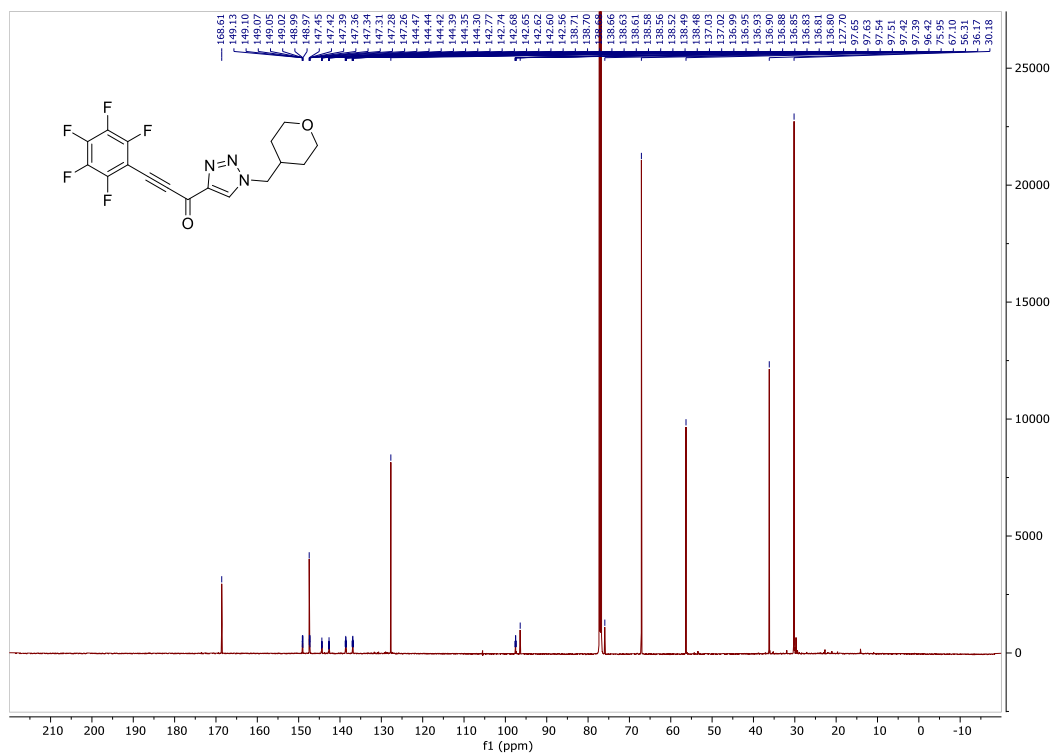
¹³C NMR (100 MHz, CDCl₃) Spectrum of Compound **8J**



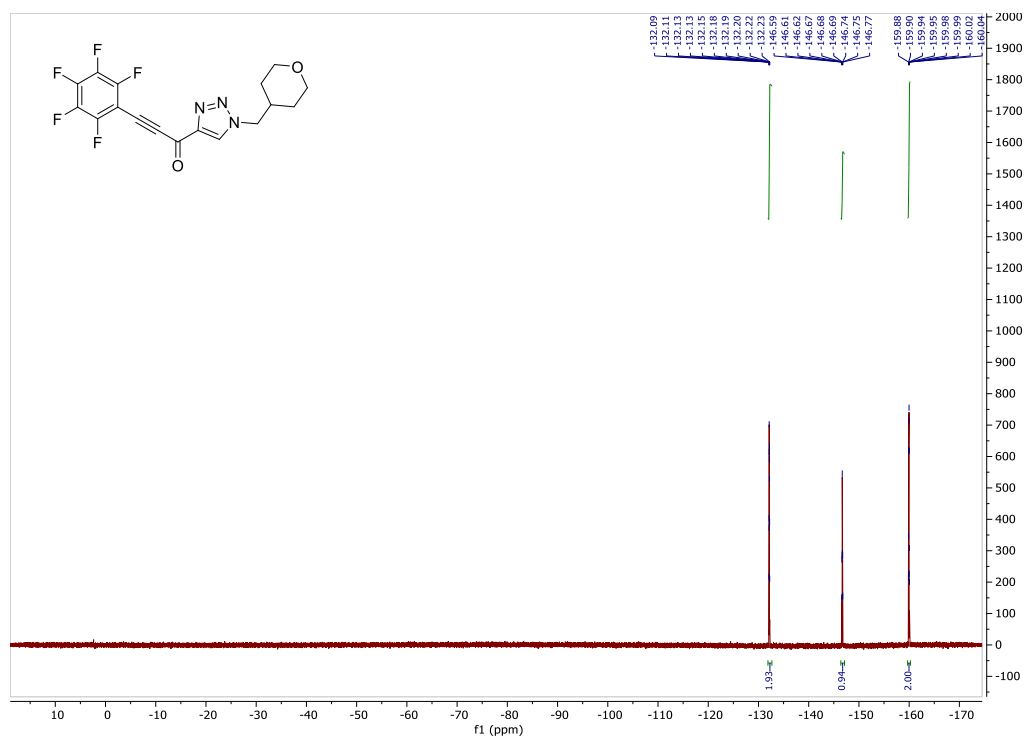
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **8K**



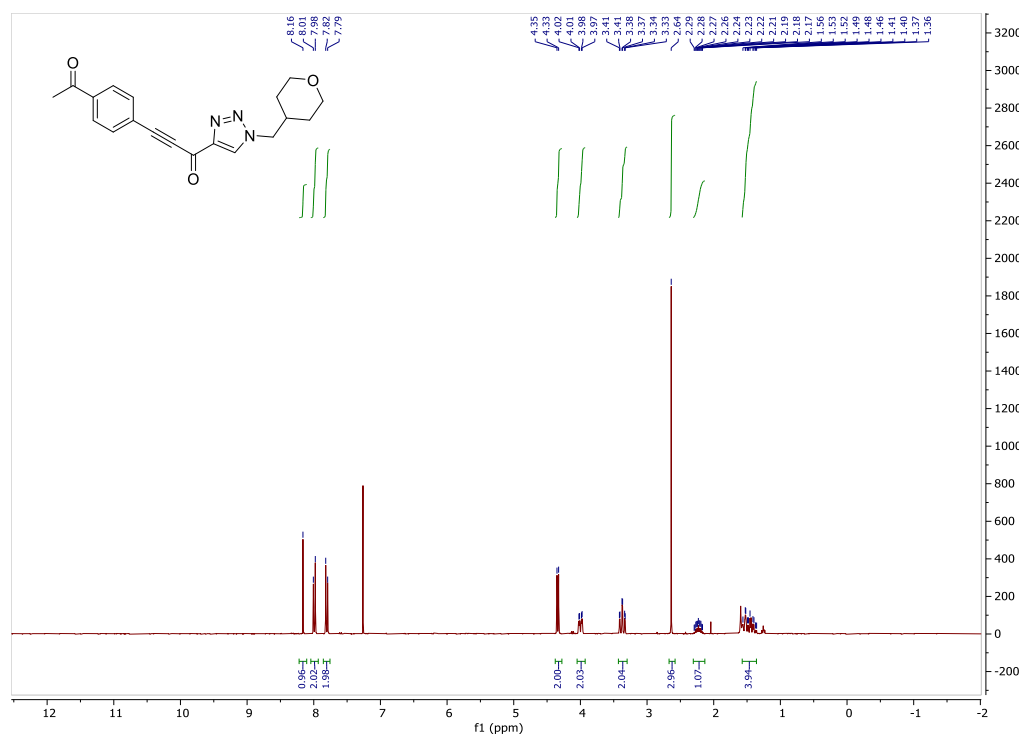
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **8K**



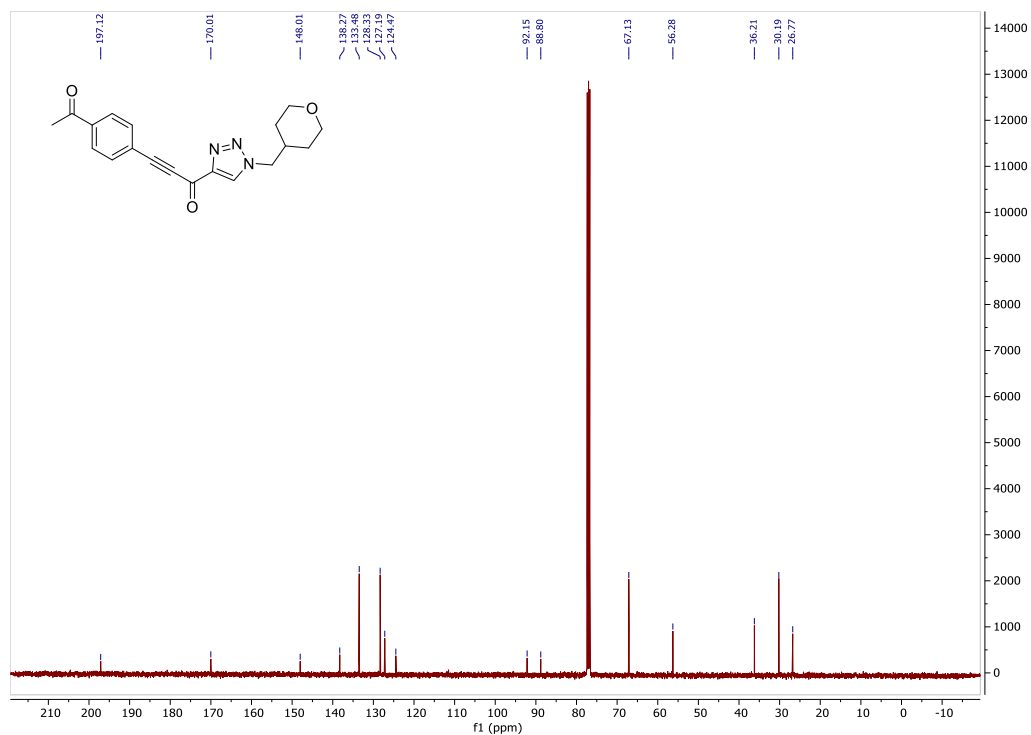
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **8K**



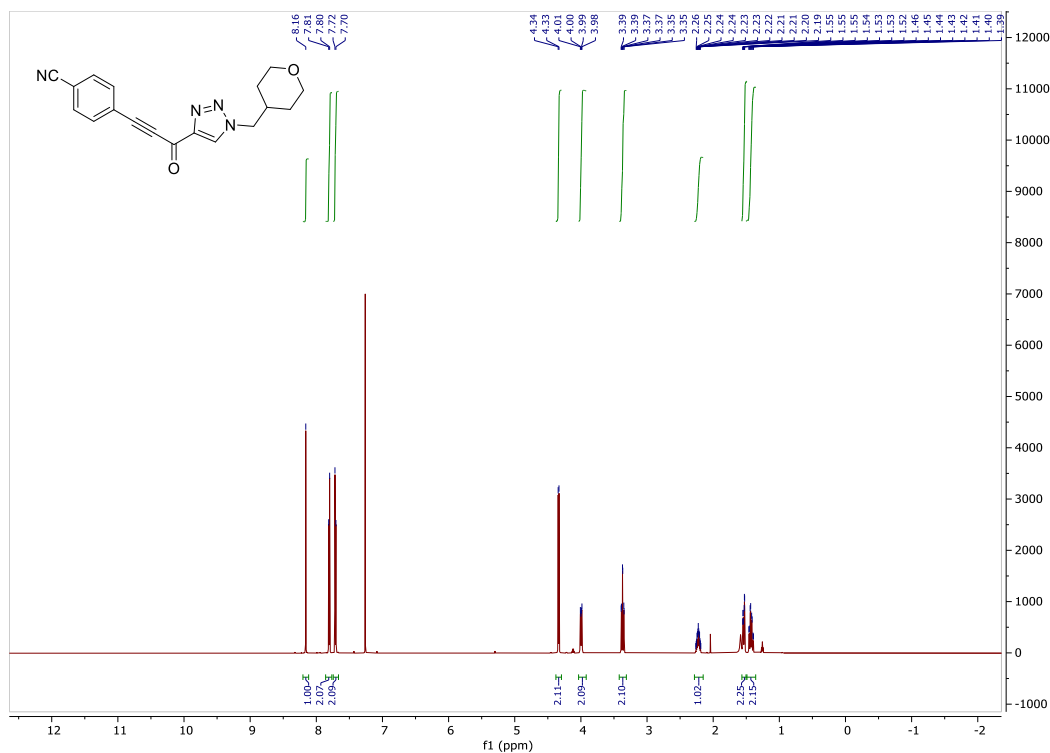
^1H NMR (400 MHz, CDCl_3) Spectrum of Compound **8L**



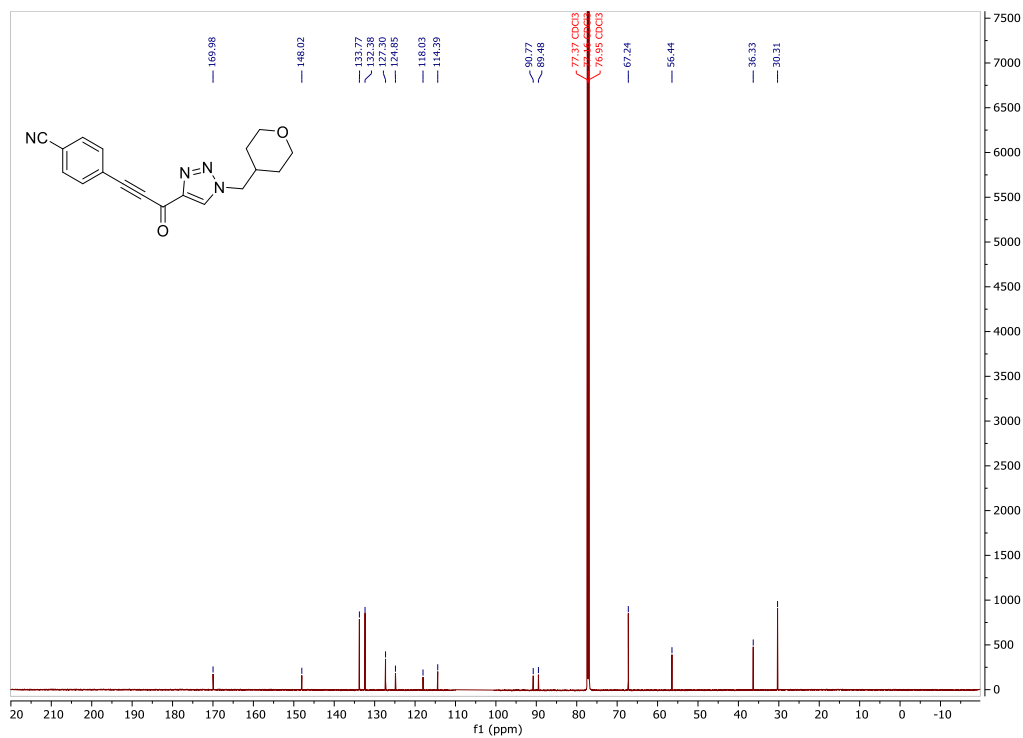
^{13}C NMR (100 MHz, CDCl_3) Spectrum of Compound **8L**



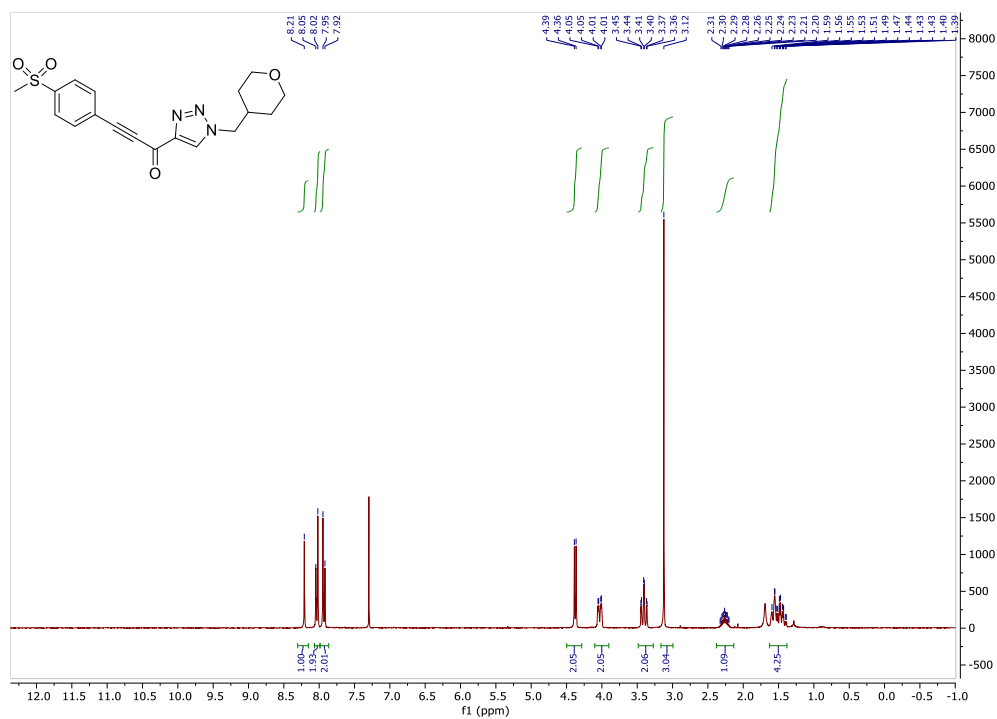
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **8M**



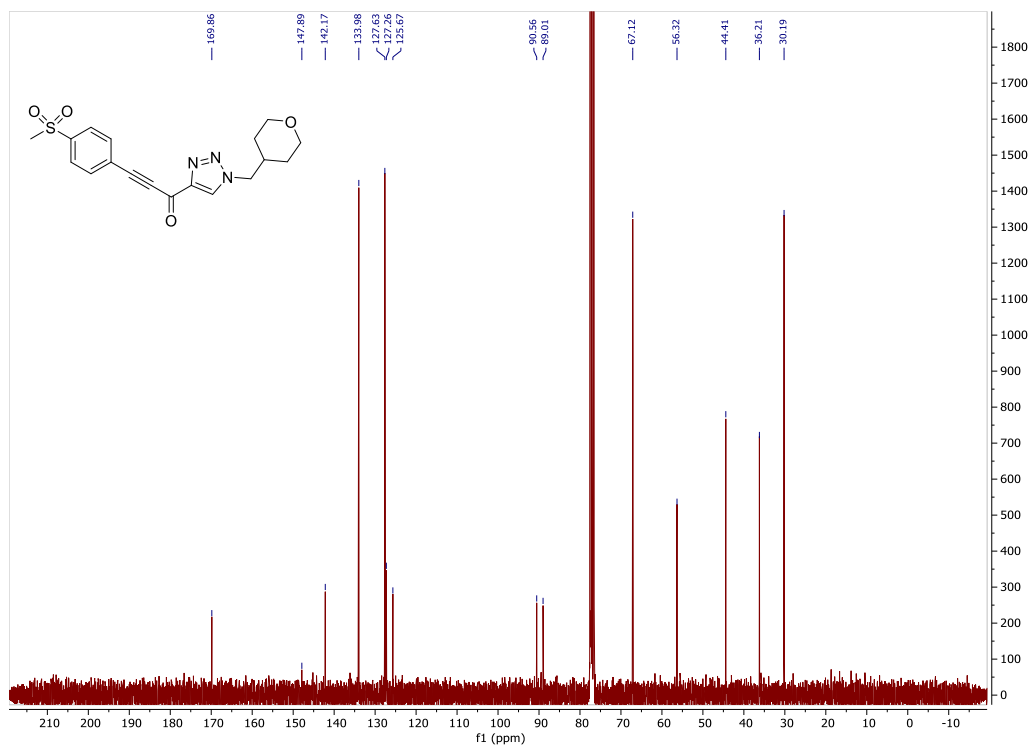
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **8M**



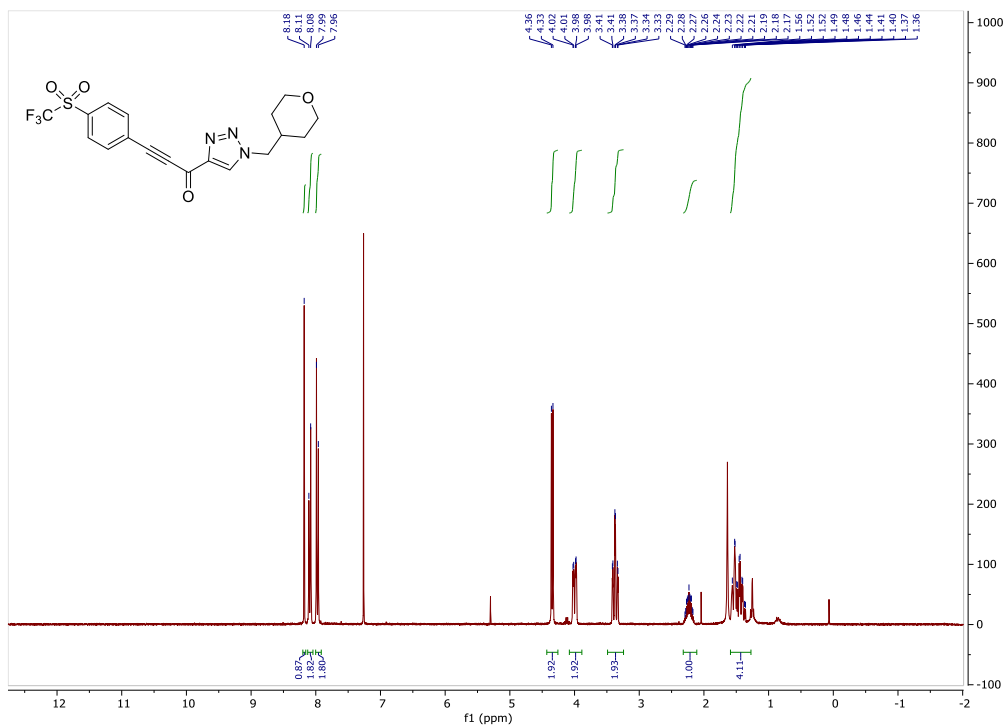
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **8N**



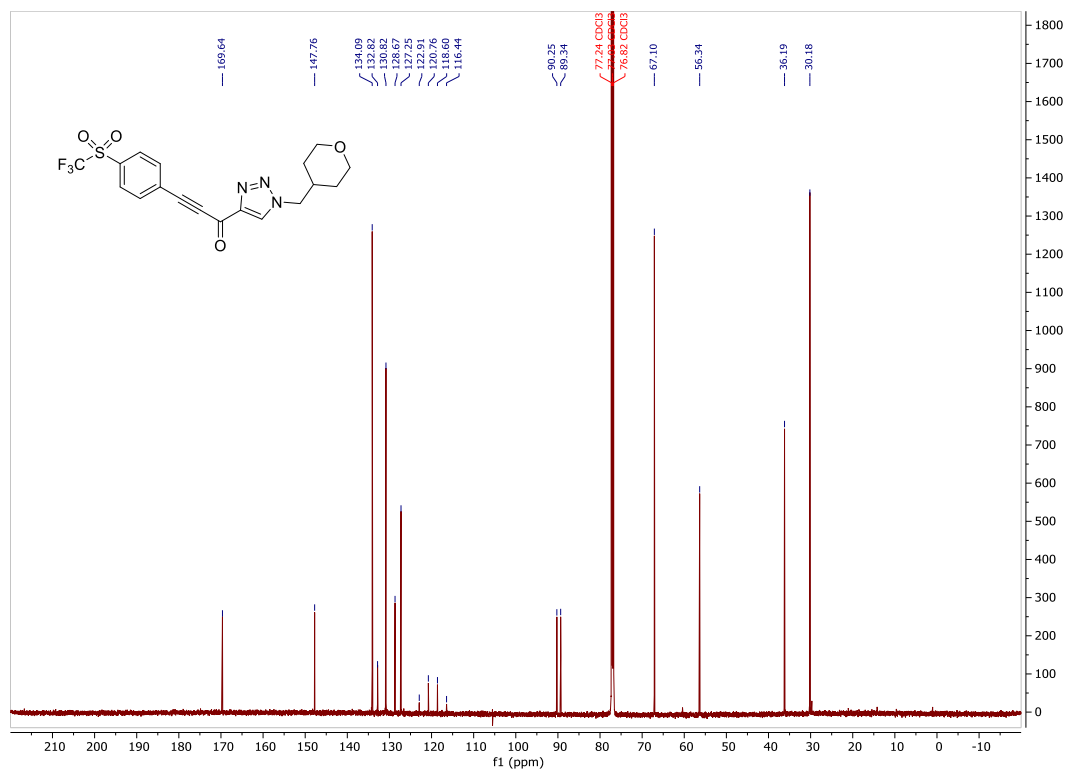
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **8N**



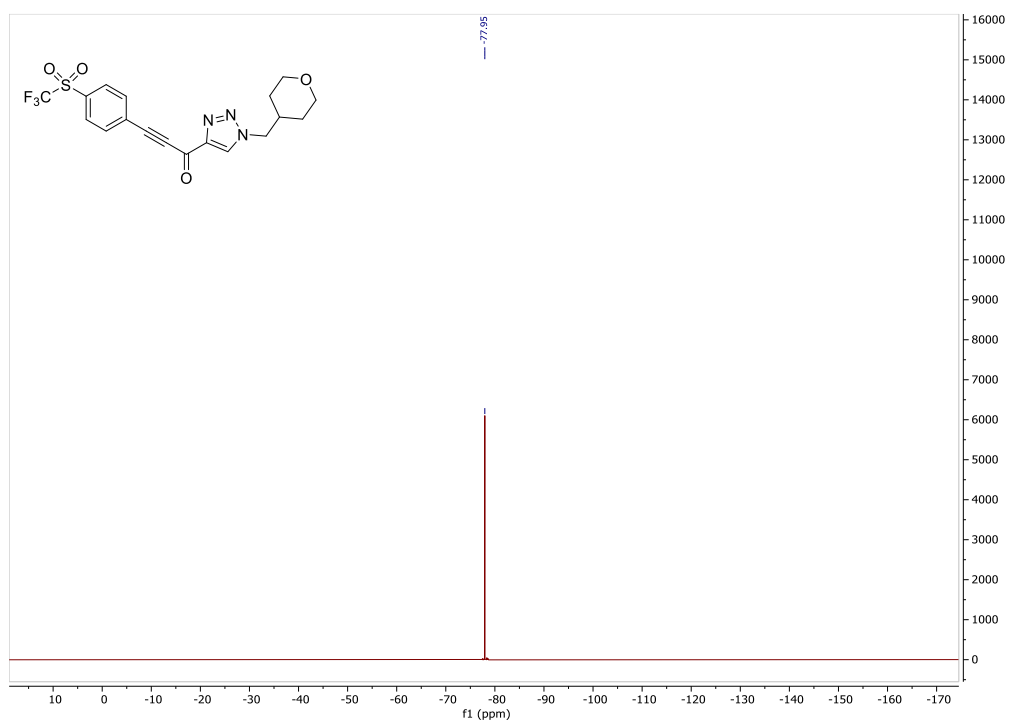
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **8O**



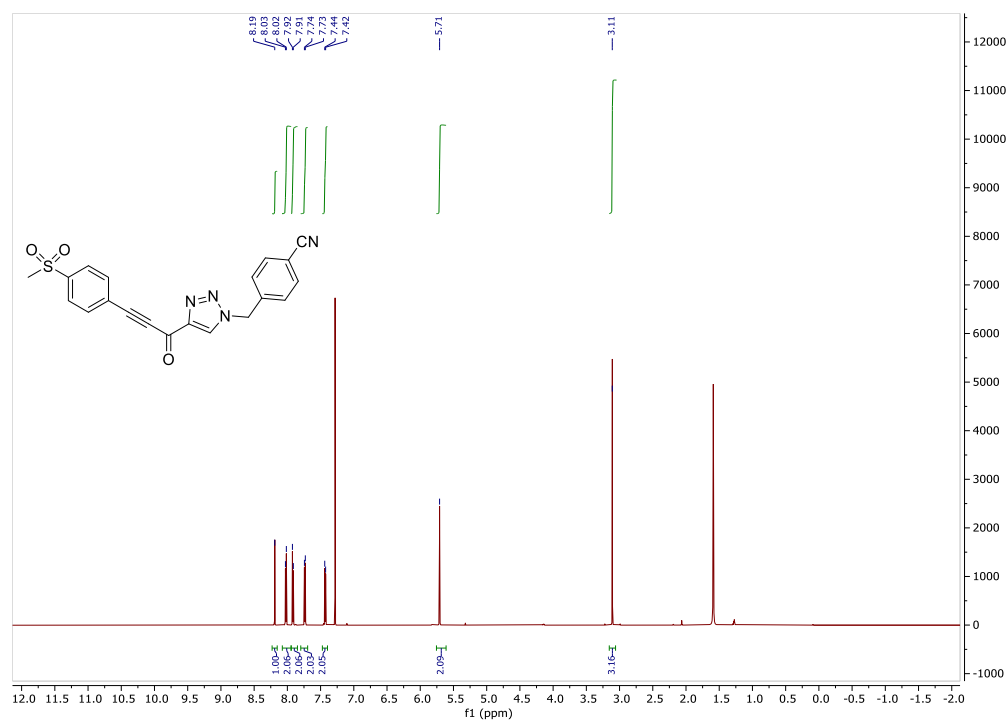
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **80**



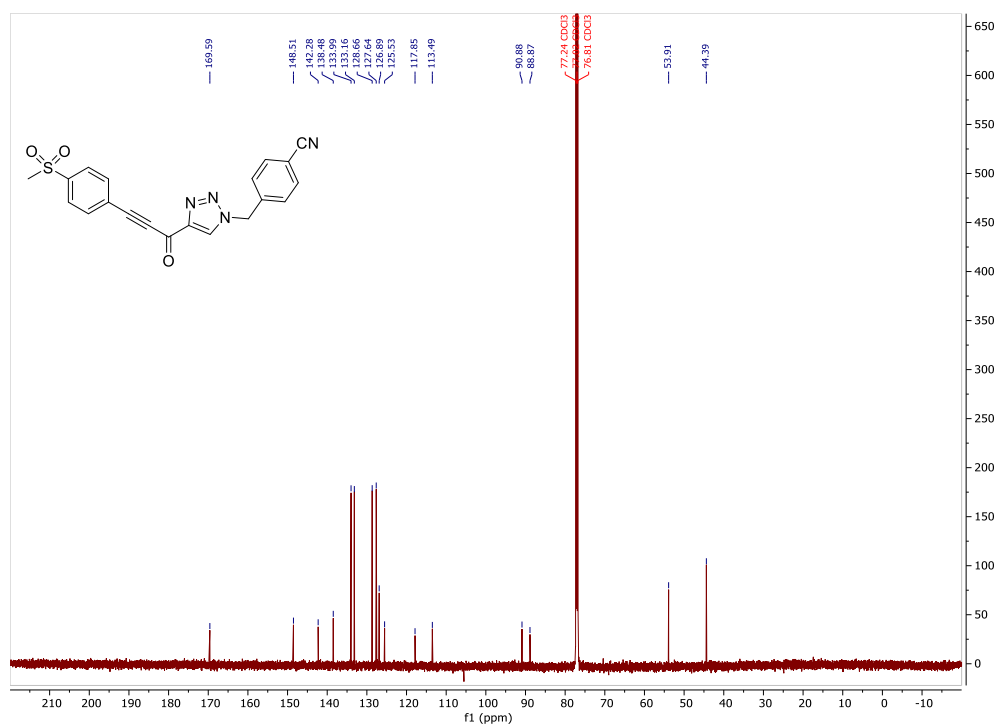
^{19}F NMR (282 MHz, CDCl_3) Spectrum of Compound **80**



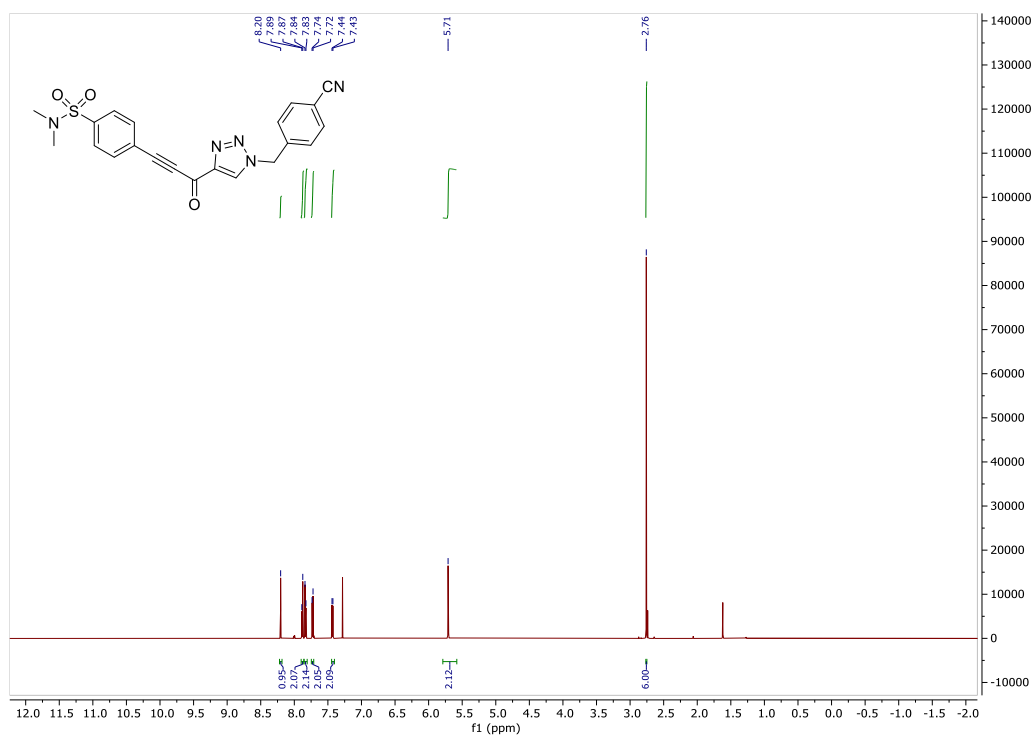
¹H NMR (300 MHz, CDCl₃) Spectrum of Compound **8P**



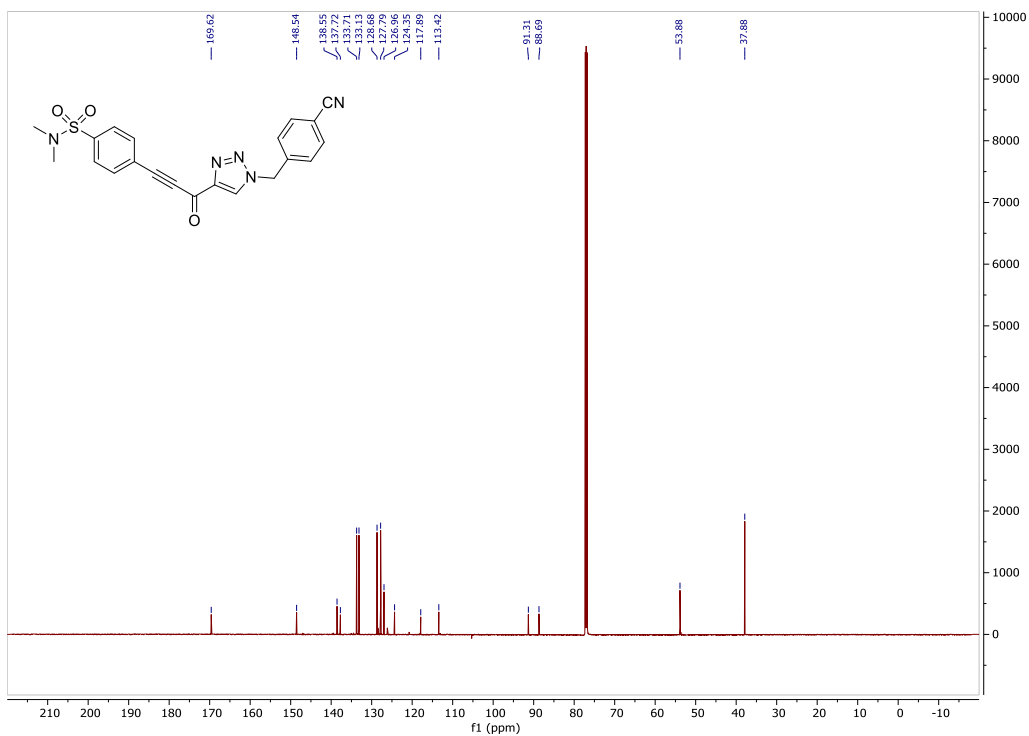
¹³C NMR (75 MHz, CDCl₃) Spectrum of Compound **8P**



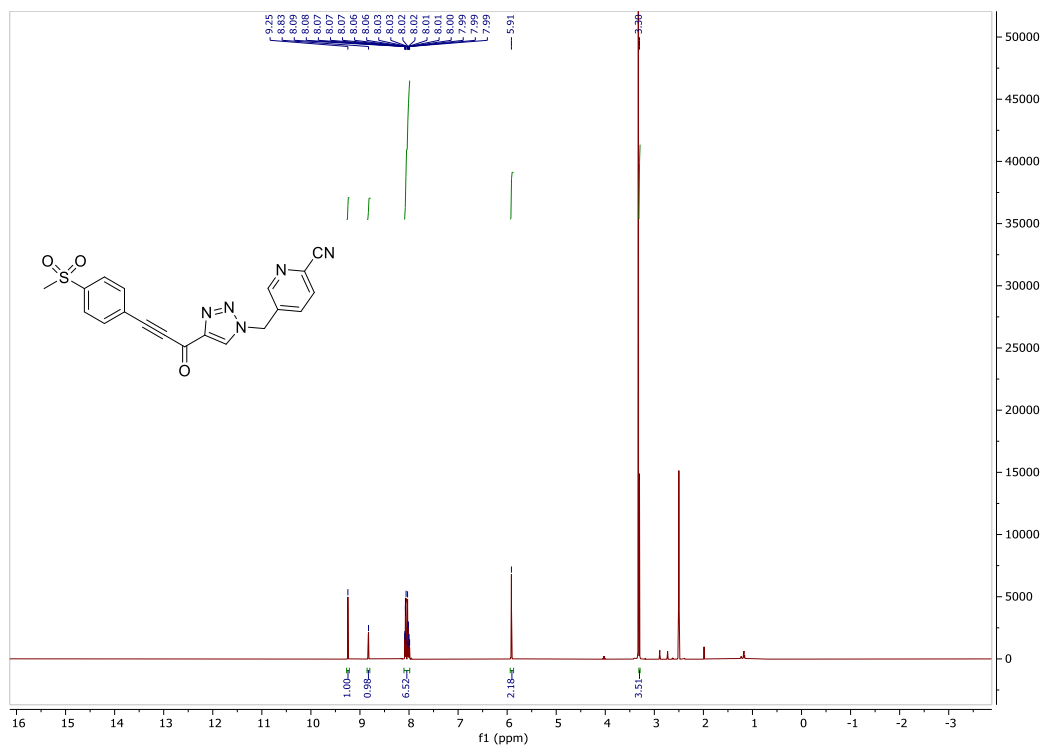
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **8Q**



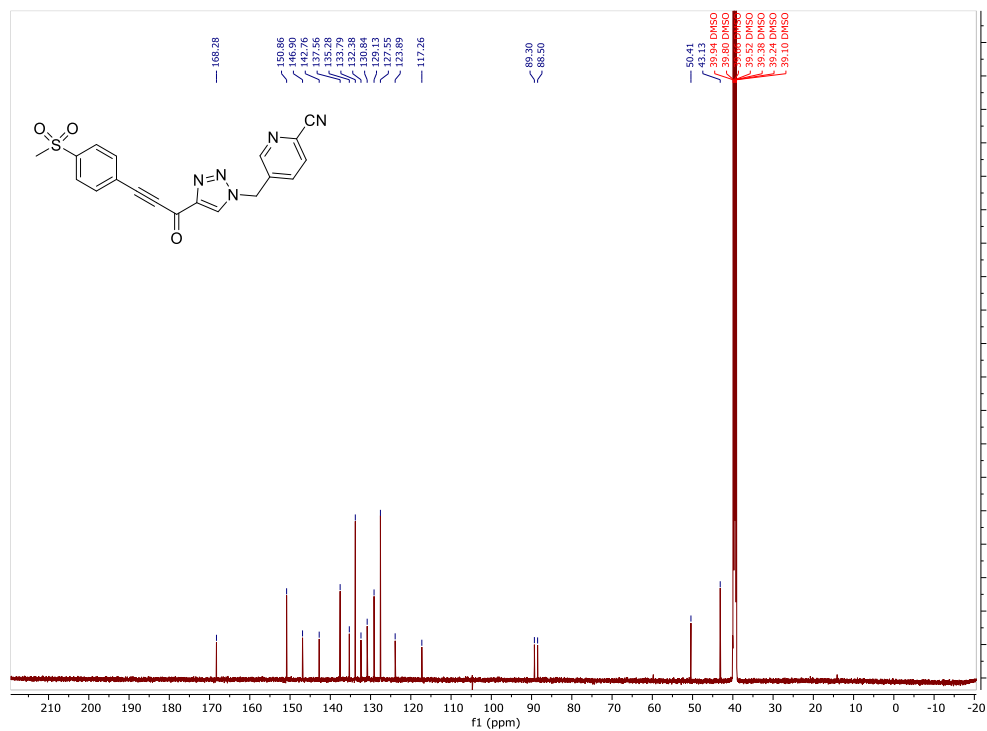
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **8Q**



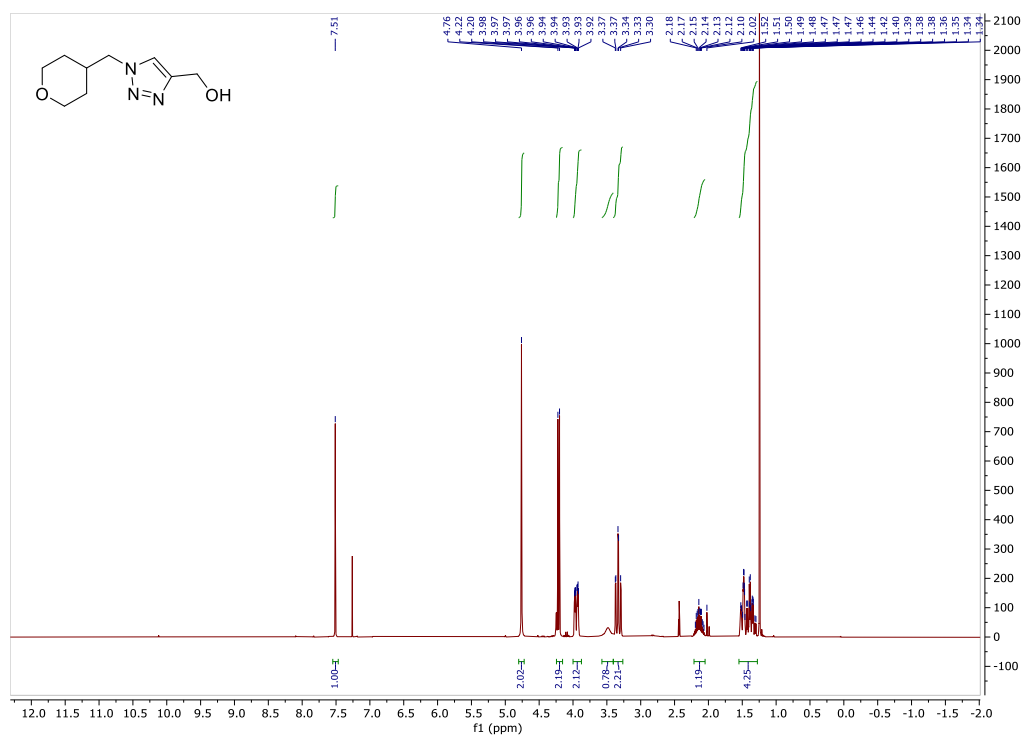
^1H NMR (600 MHz, $\text{DMSO}-d_6$) Spectrum of Compound **8R**



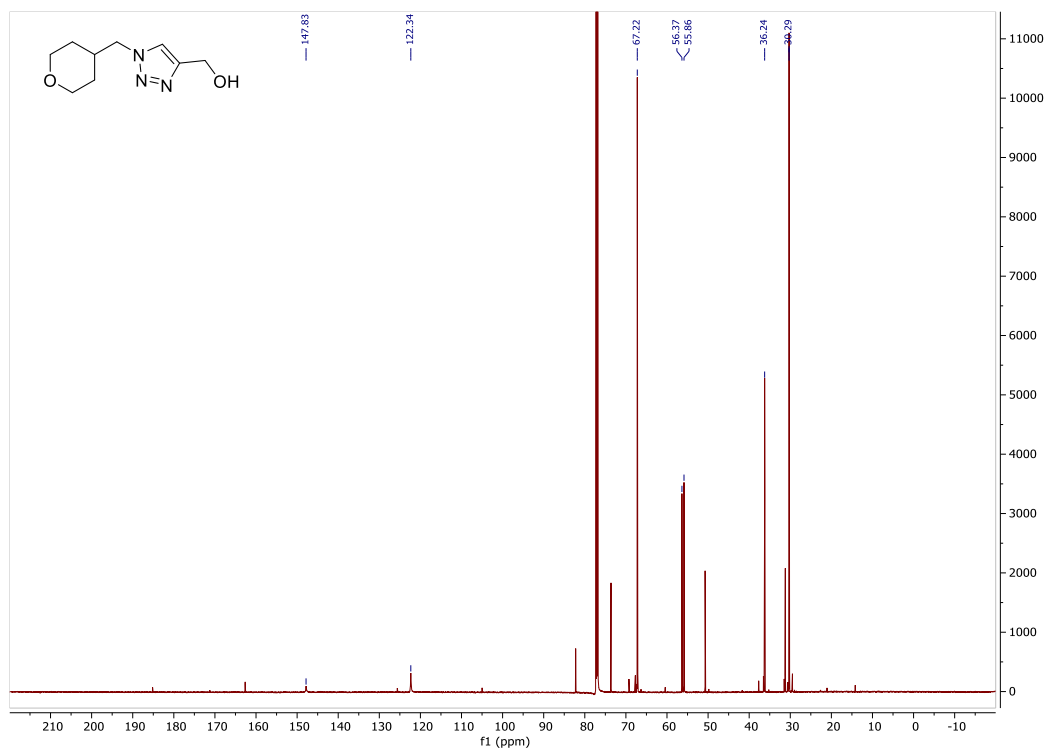
^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) Spectrum of Compound **8R**



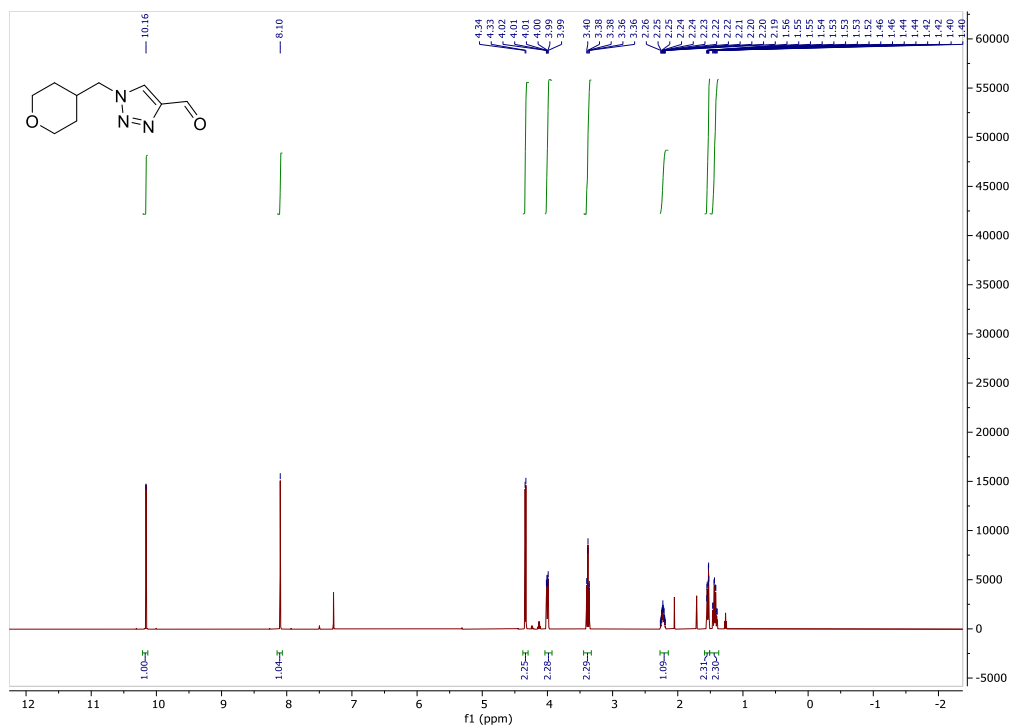
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **9**



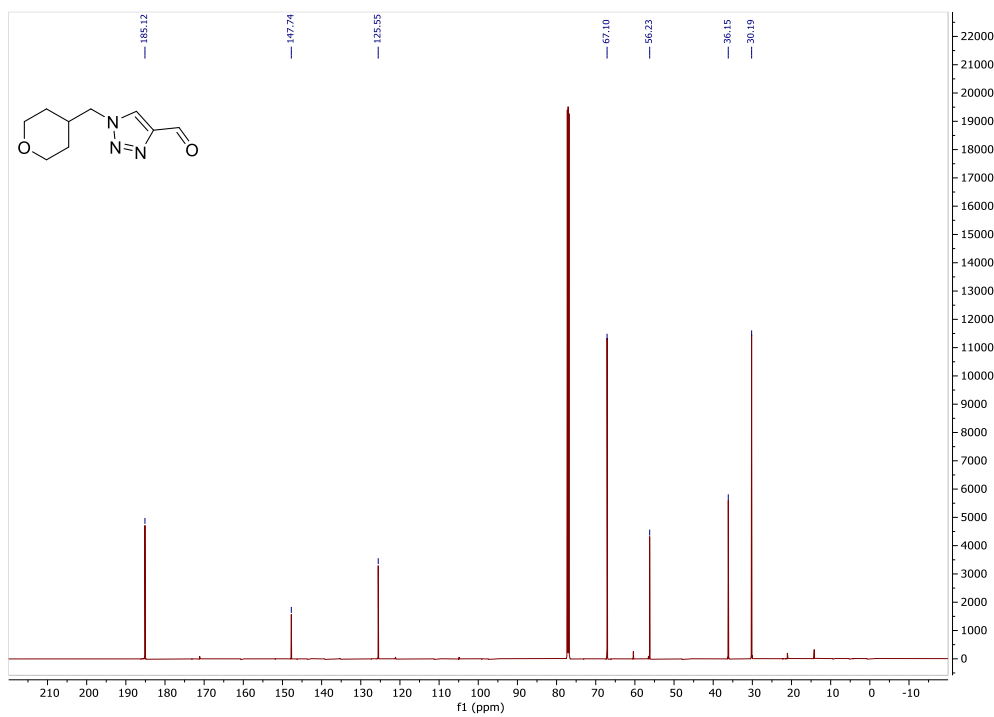
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **9**



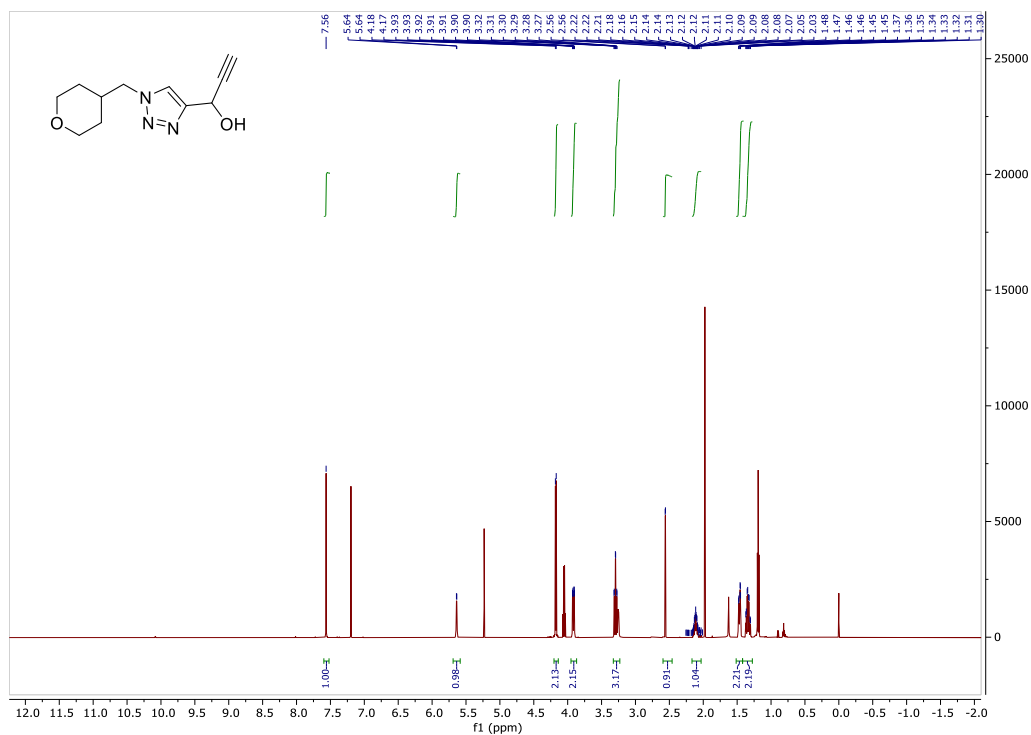
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **10**



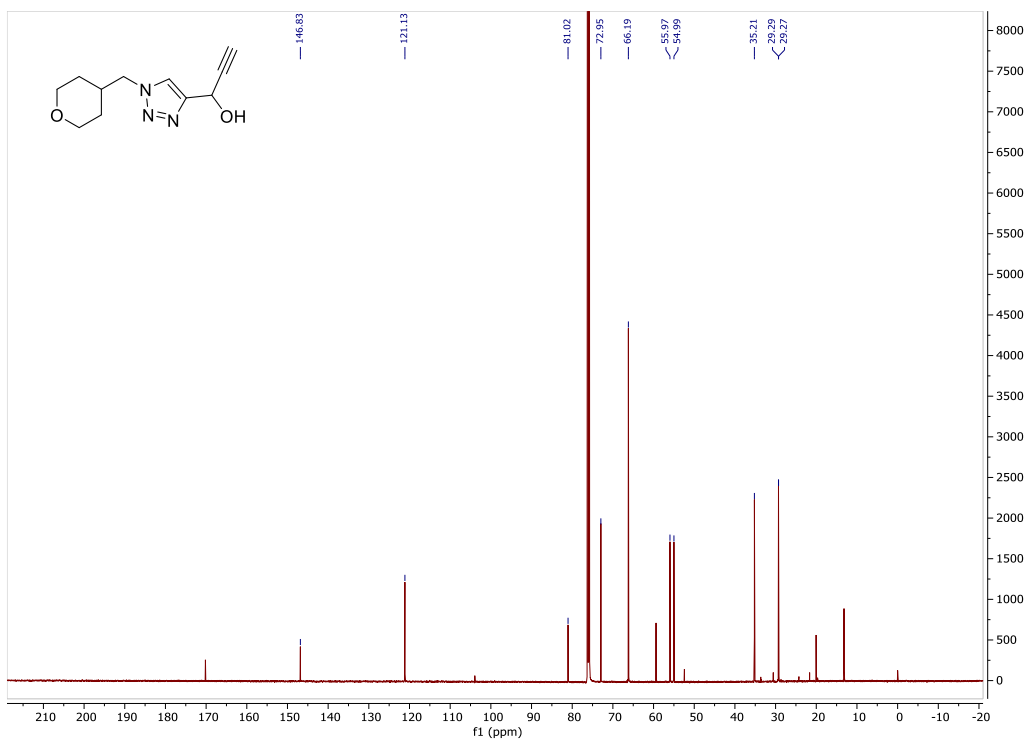
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **10**



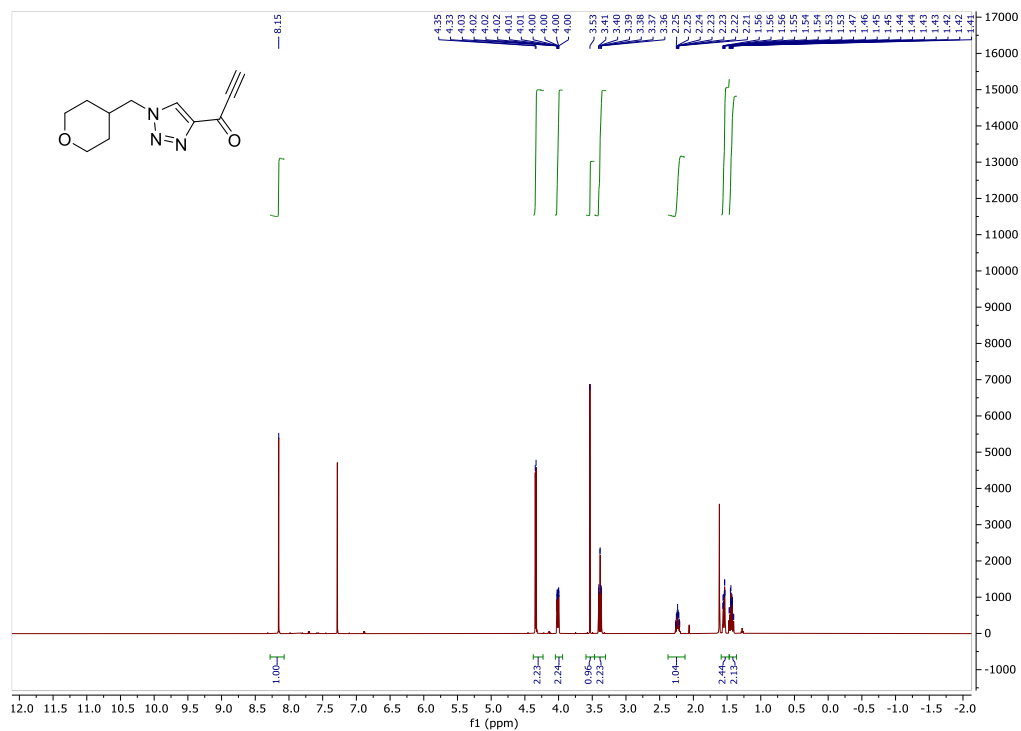
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **11**



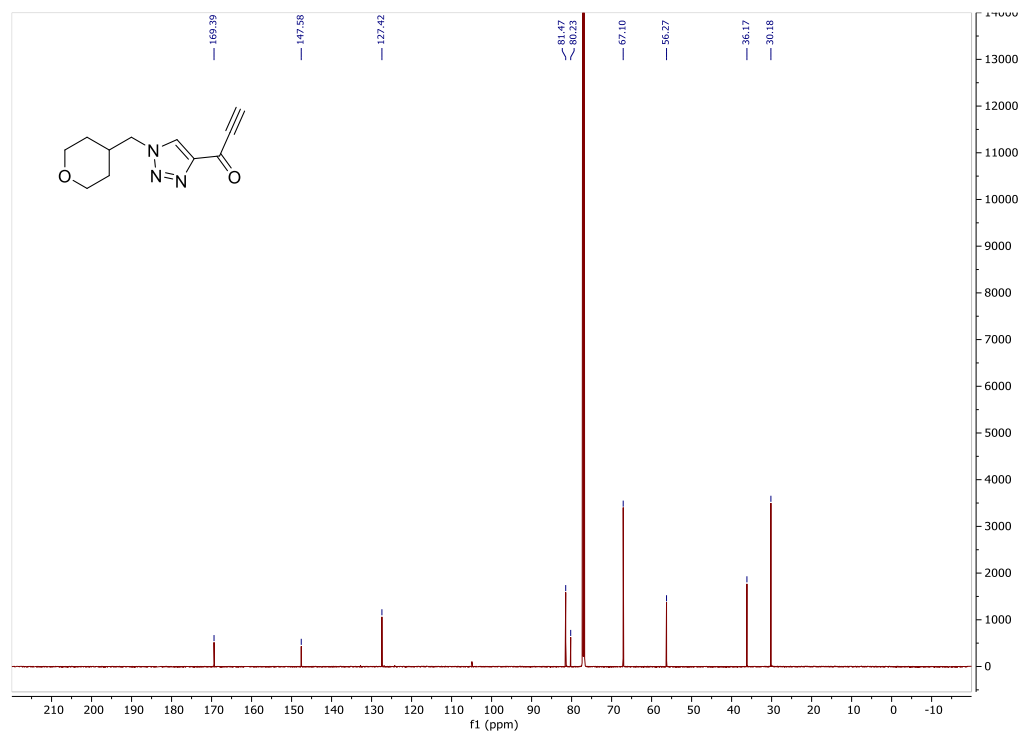
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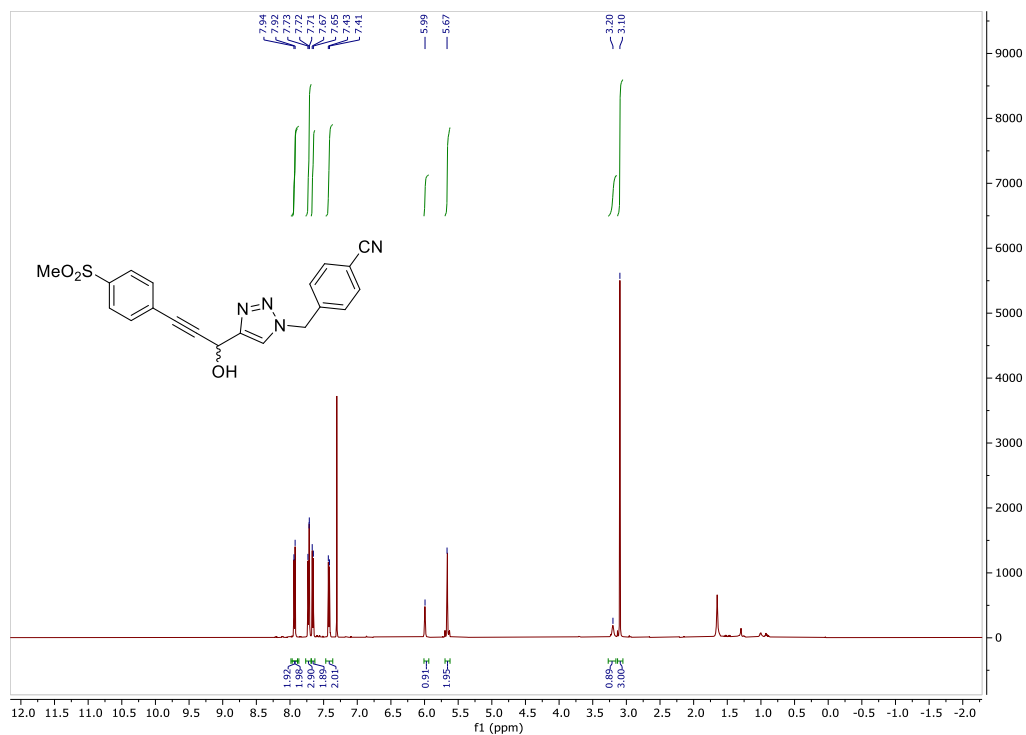
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **12**



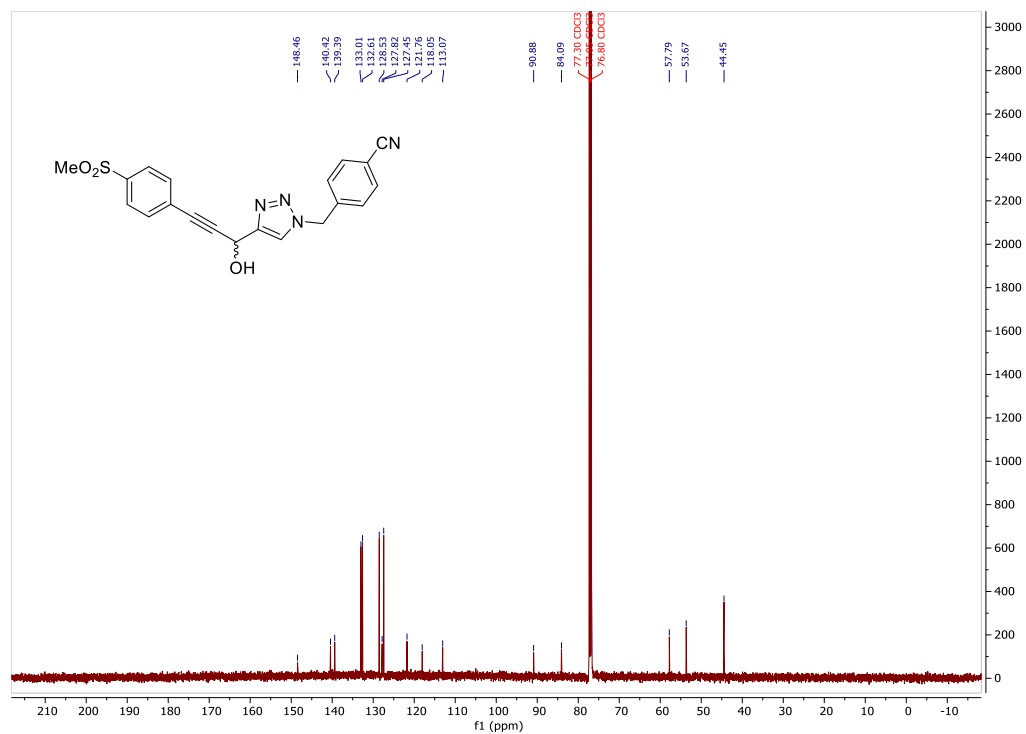
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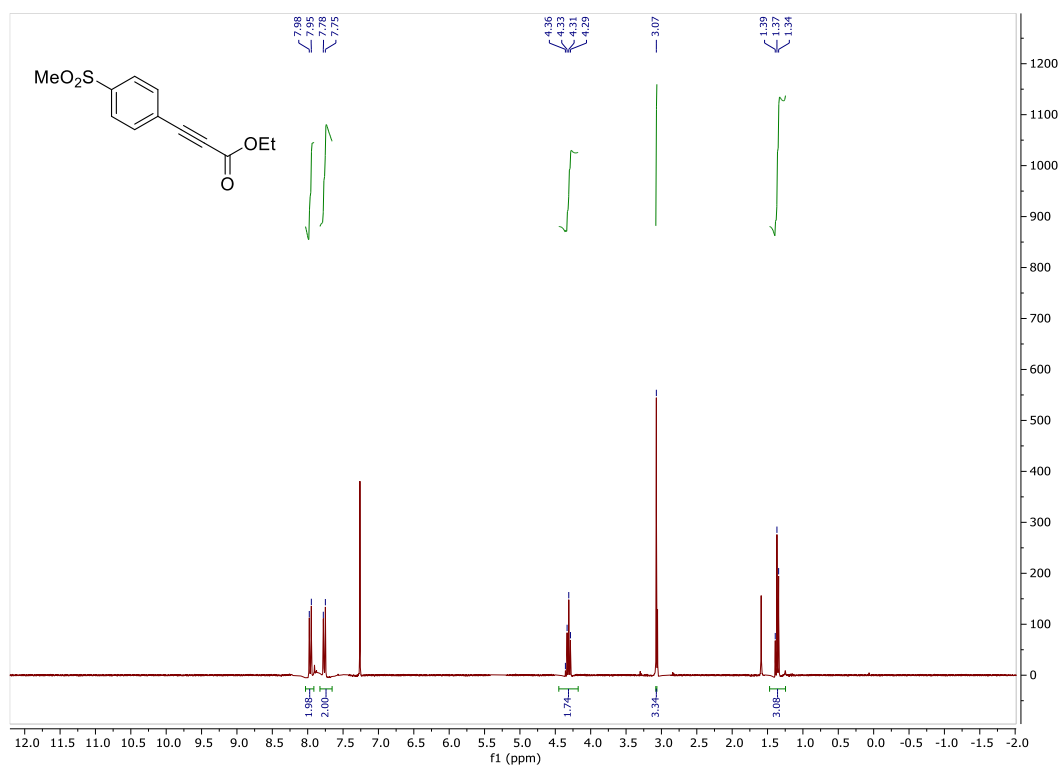
¹H NMR (500 MHz, CDCl₃) Spectrum of Compound **13**



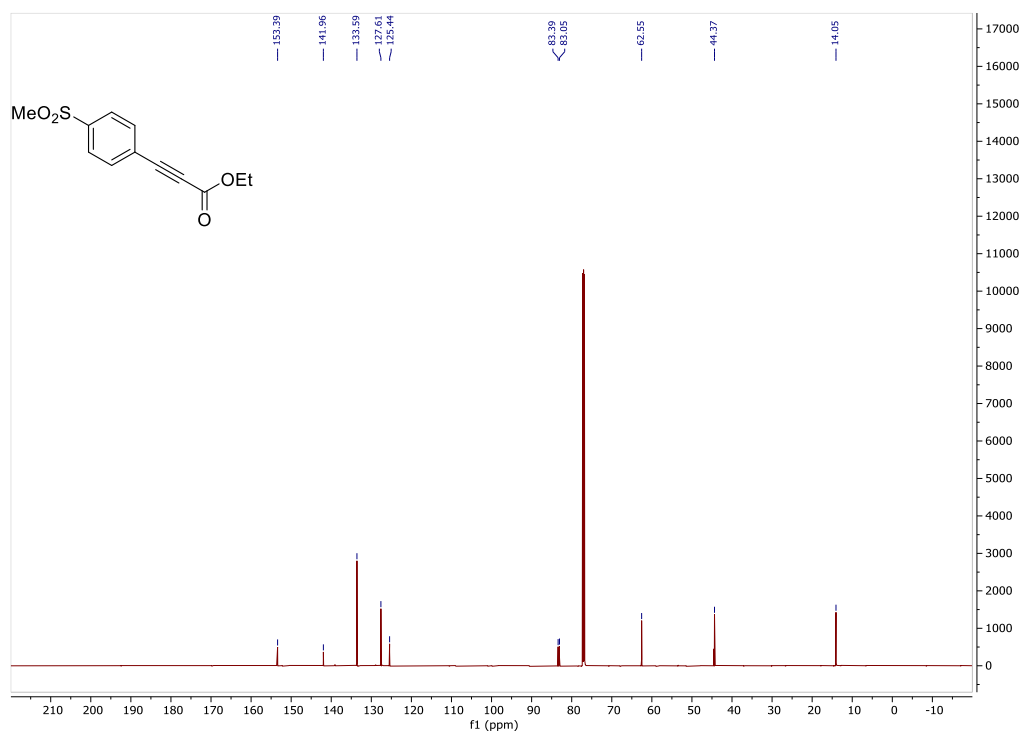
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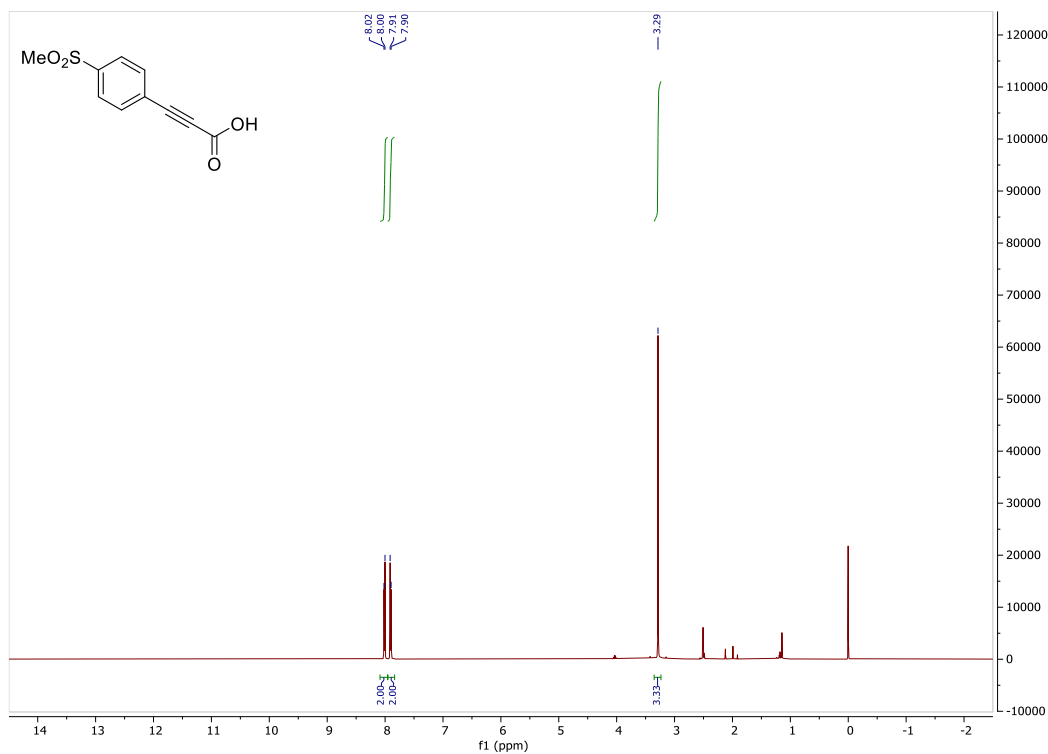
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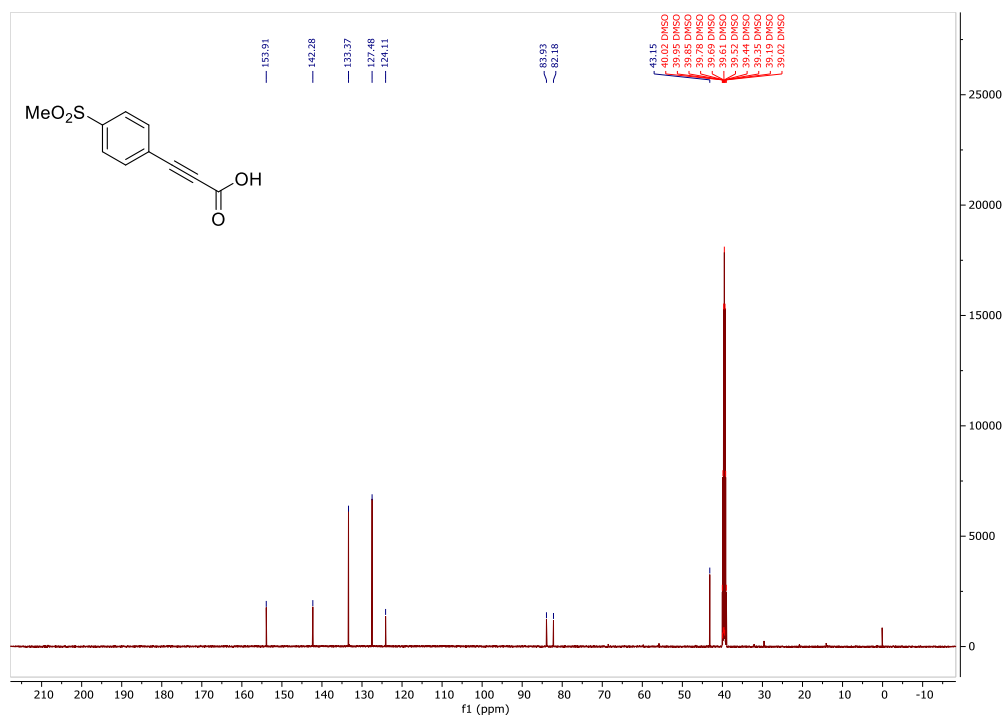
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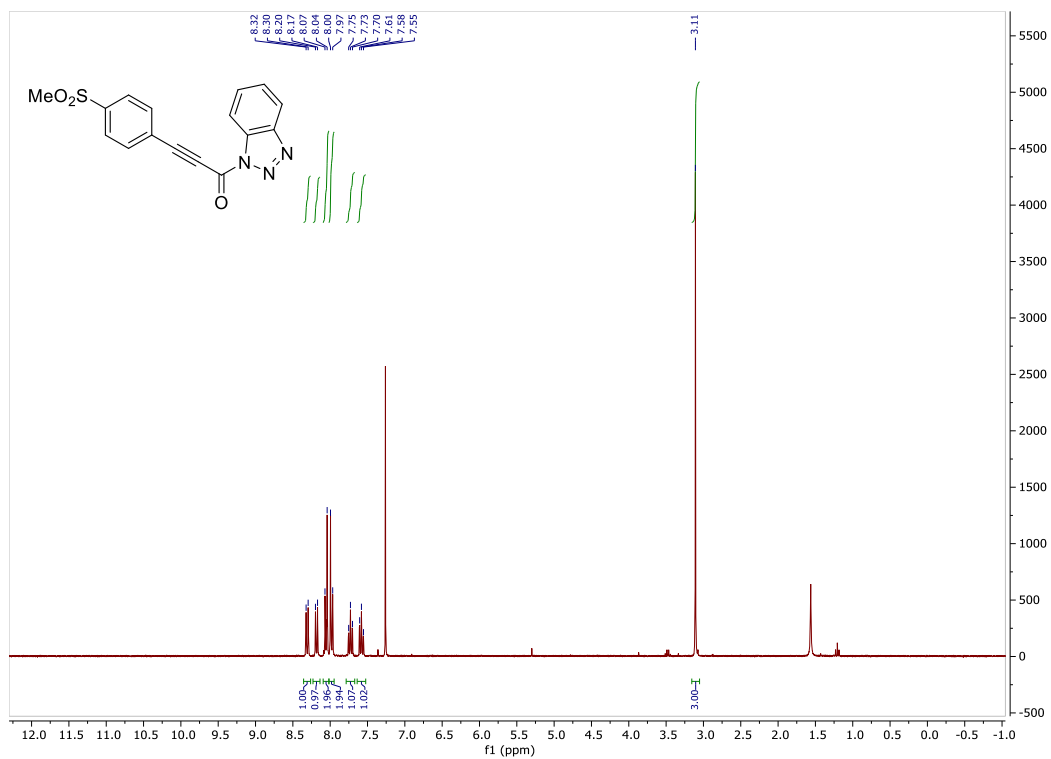
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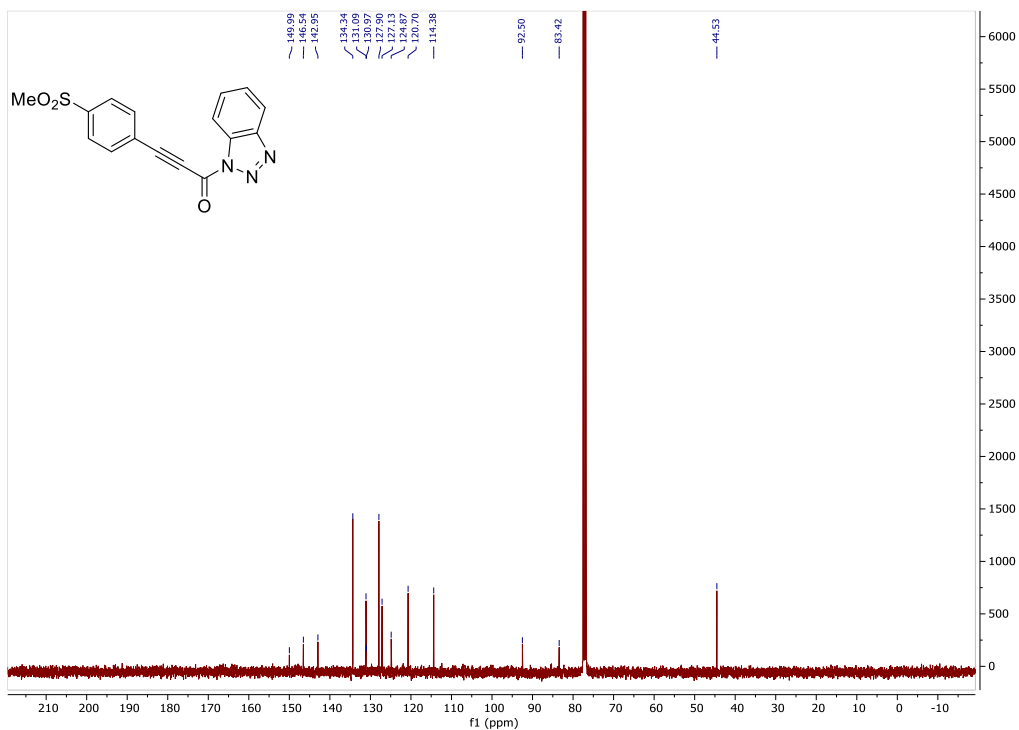
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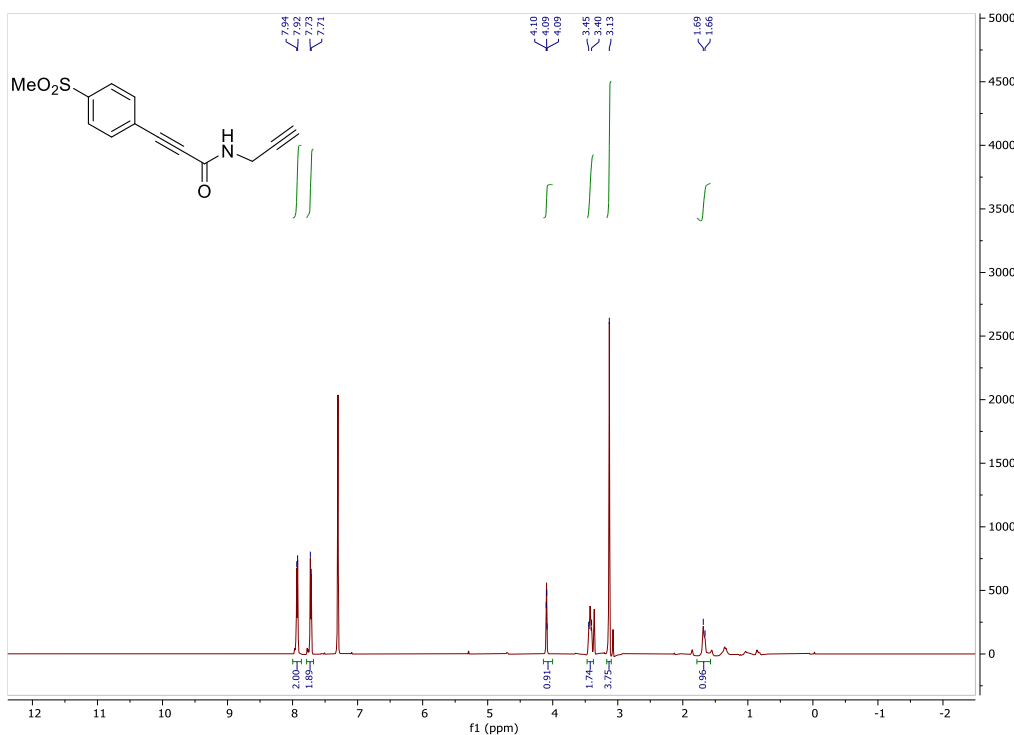
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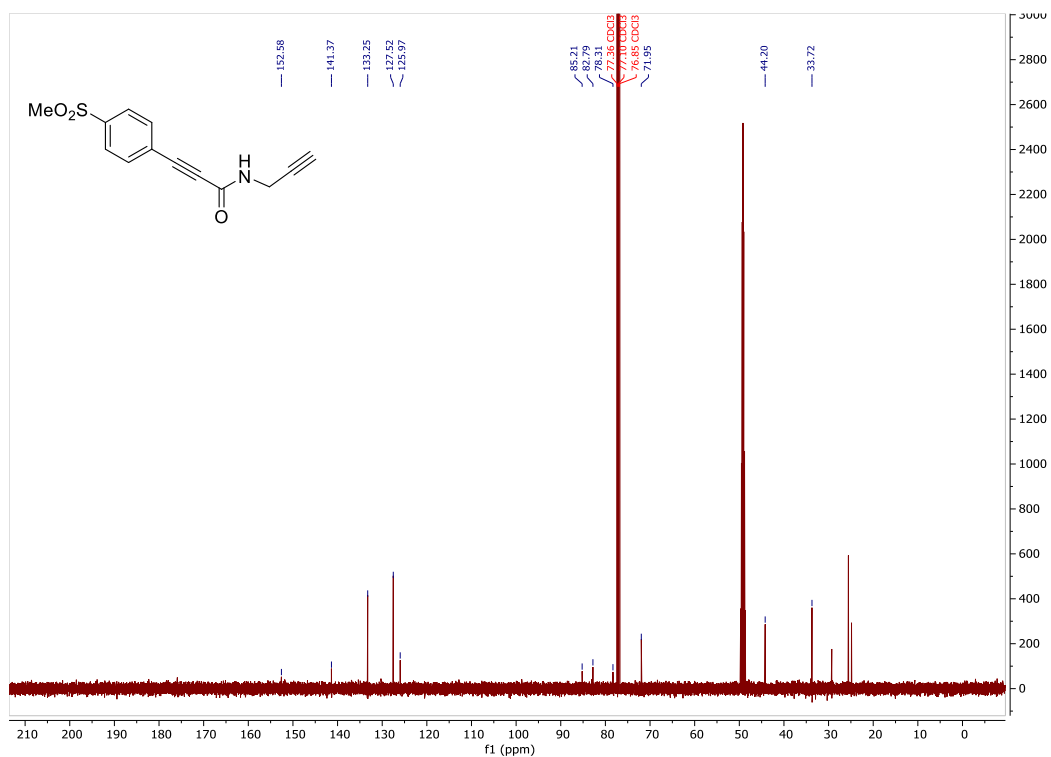
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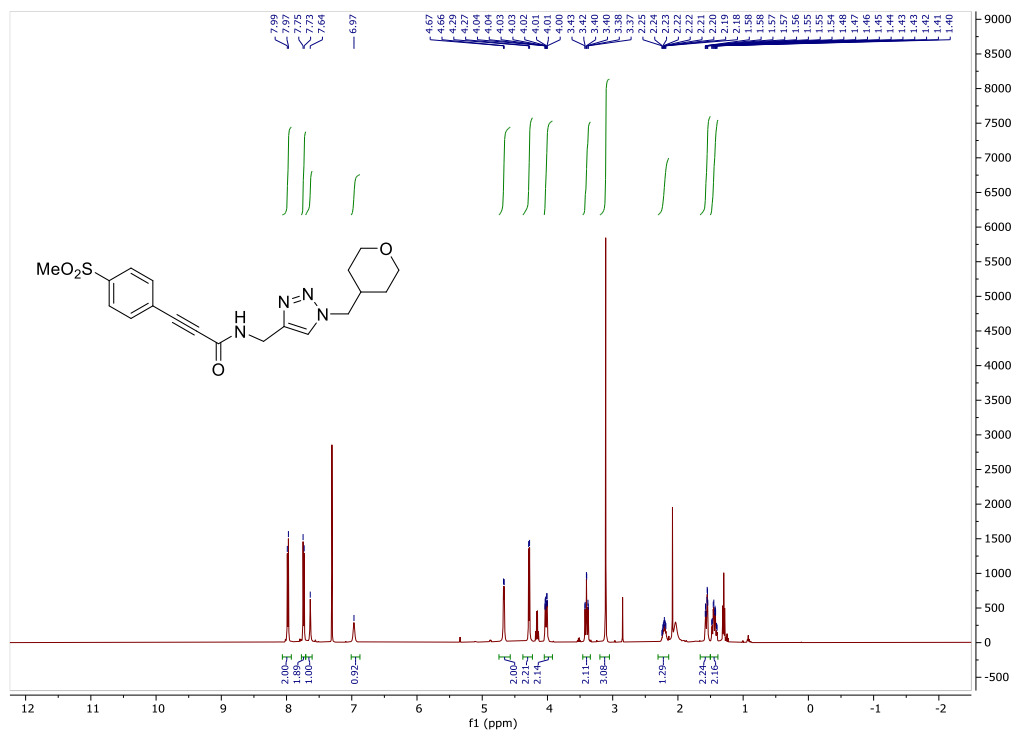
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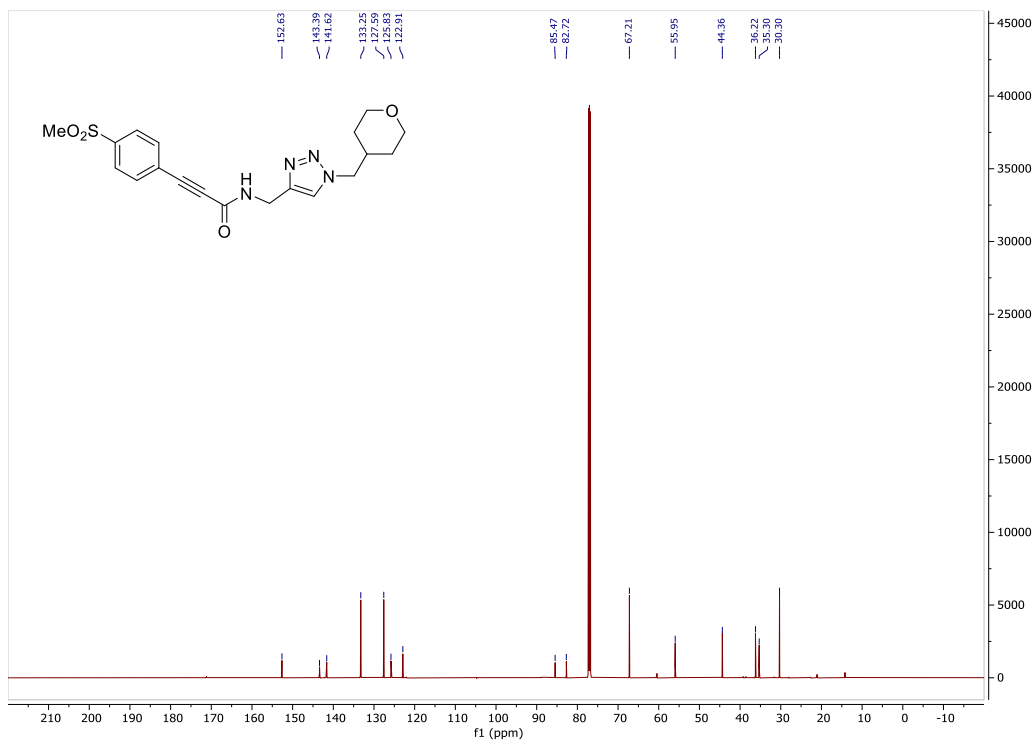
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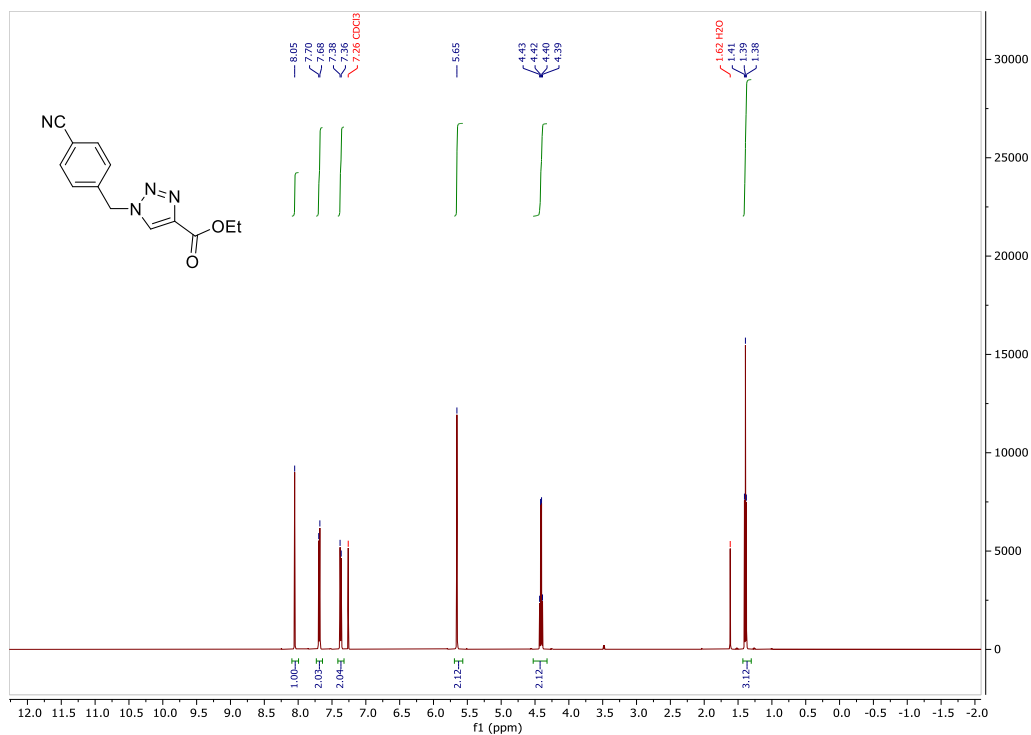
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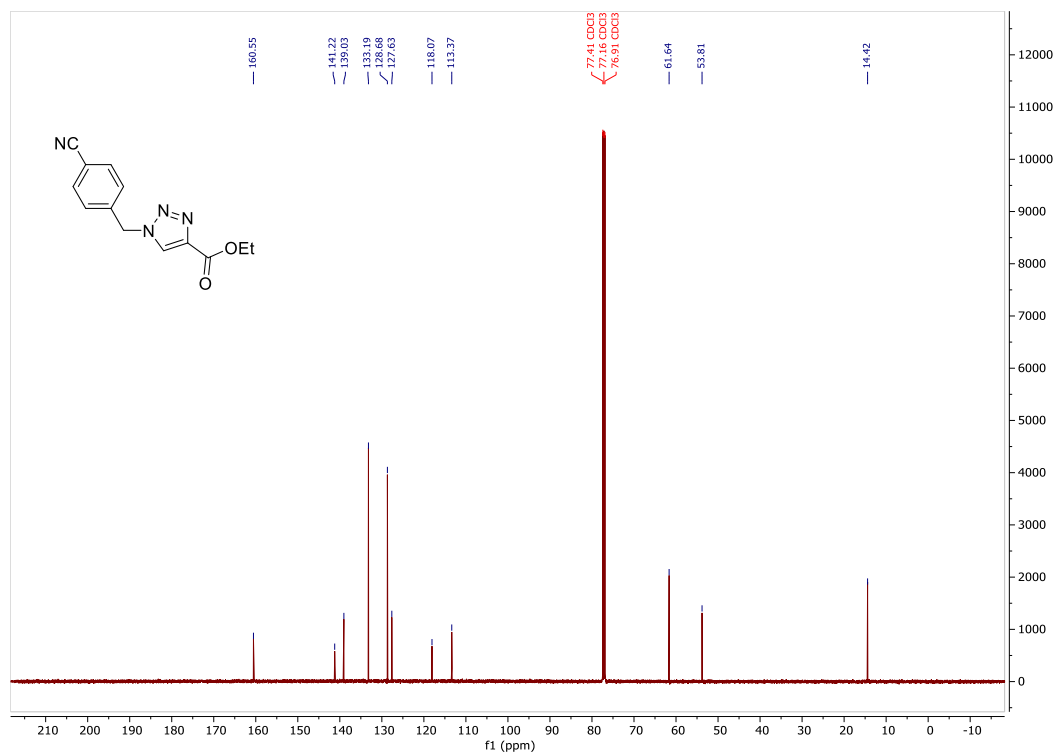
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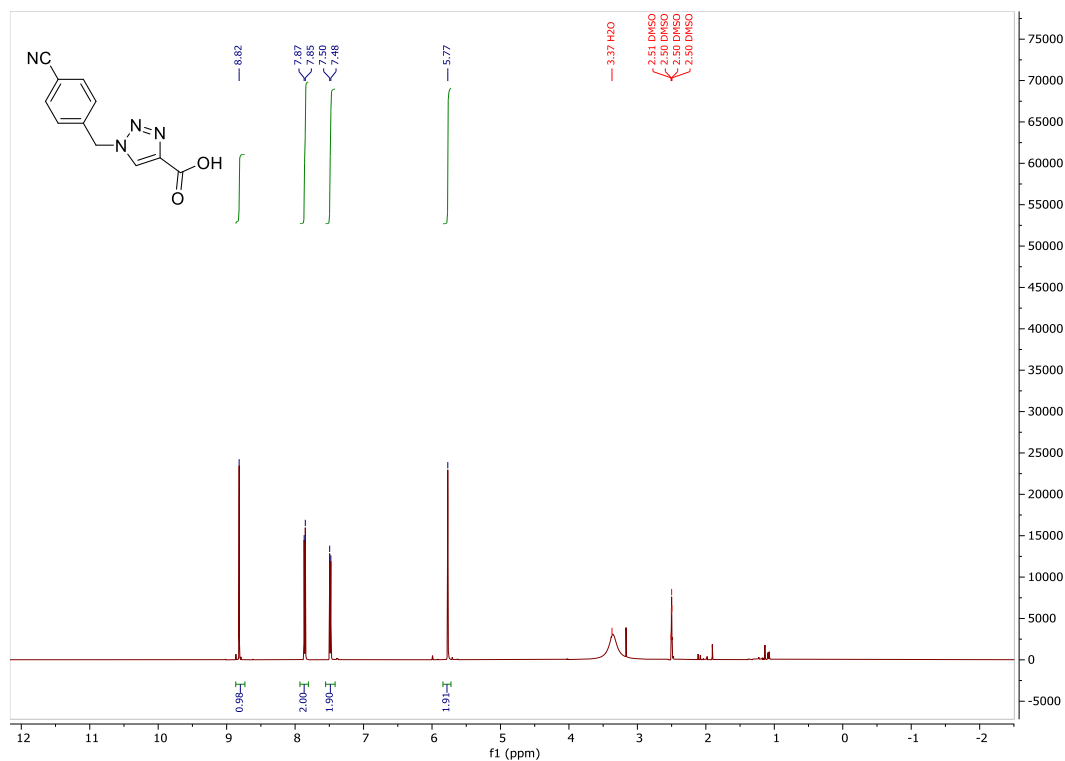
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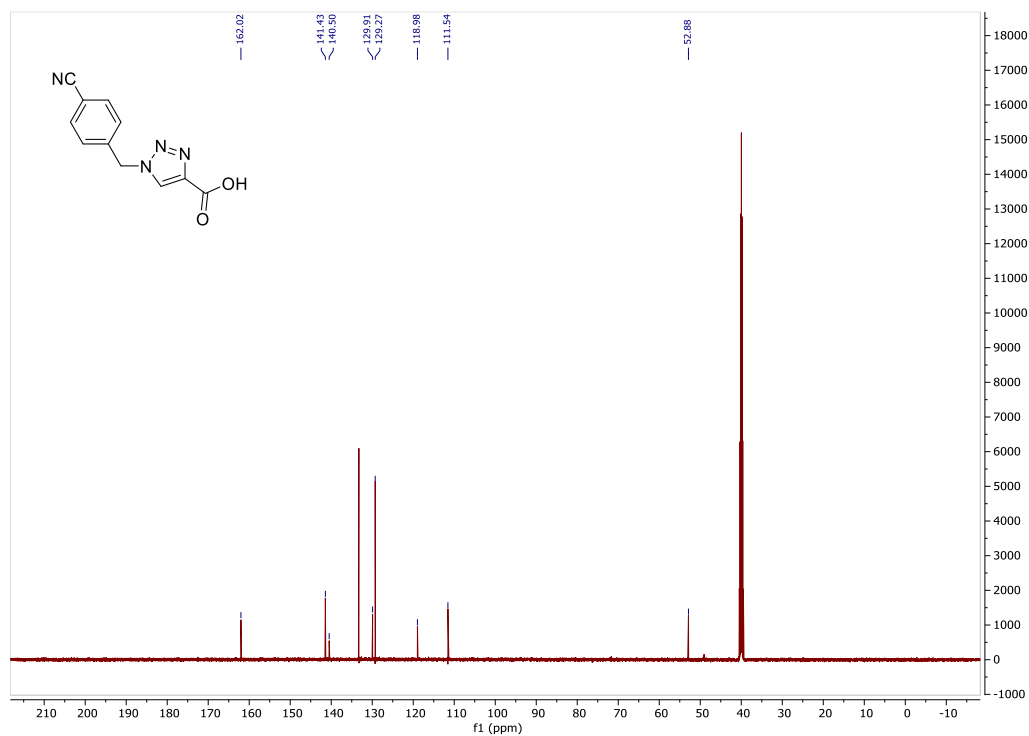
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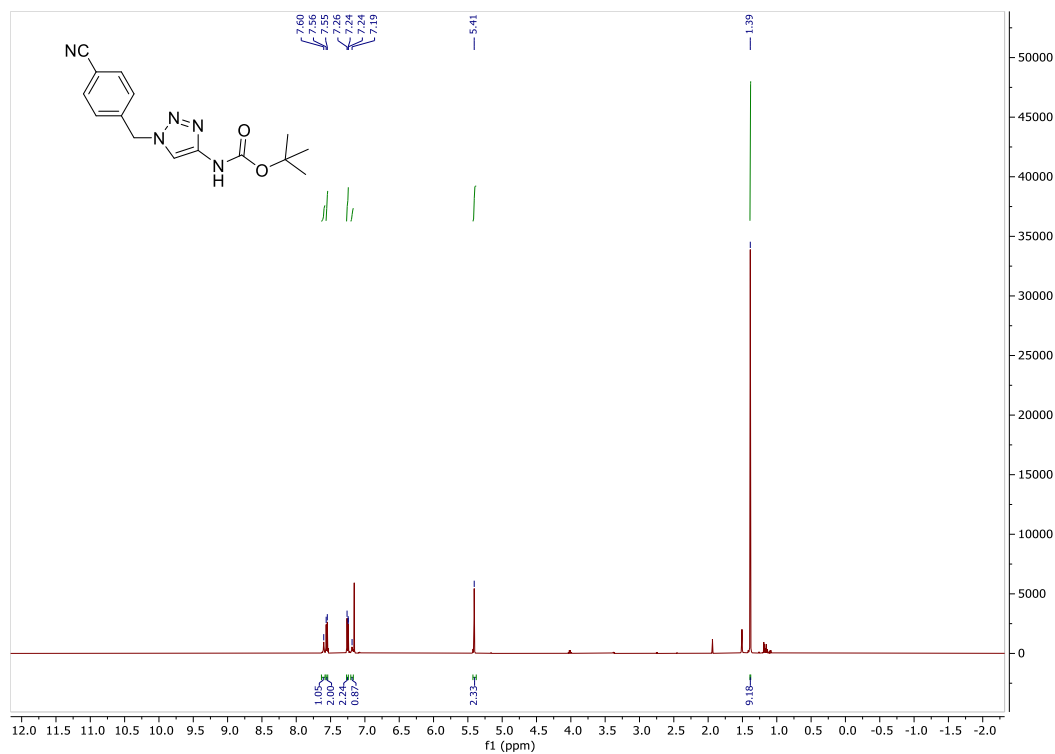
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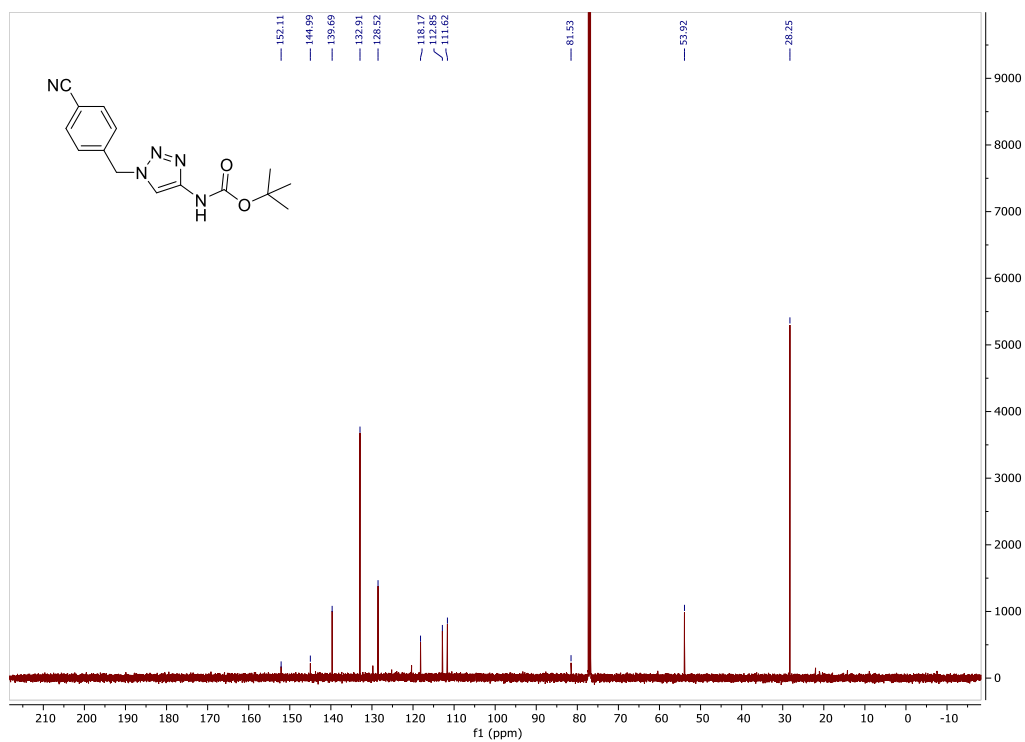
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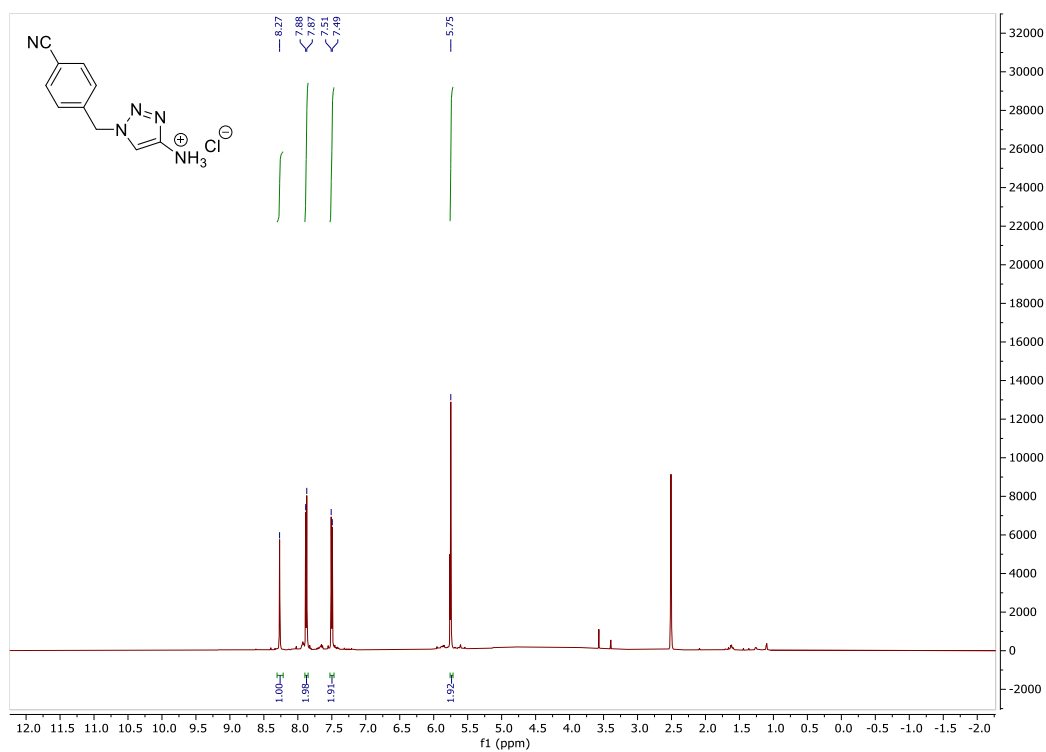
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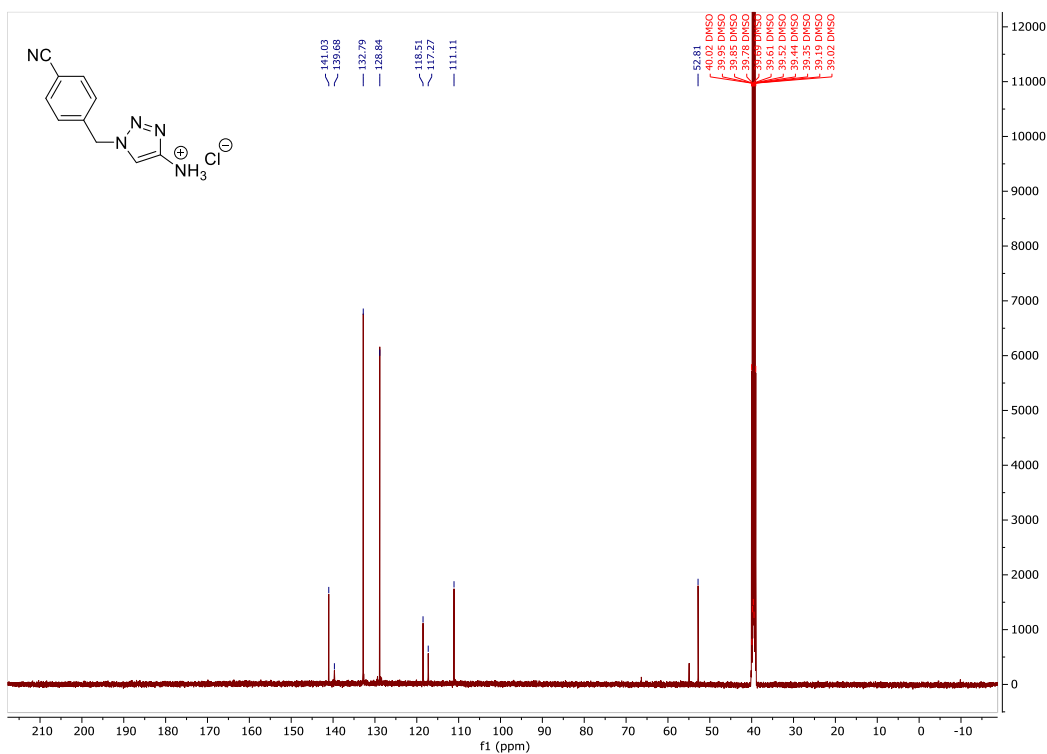
¹³C NMR (125 MHz, CDCl₃) Spectrum of Compound **21A**



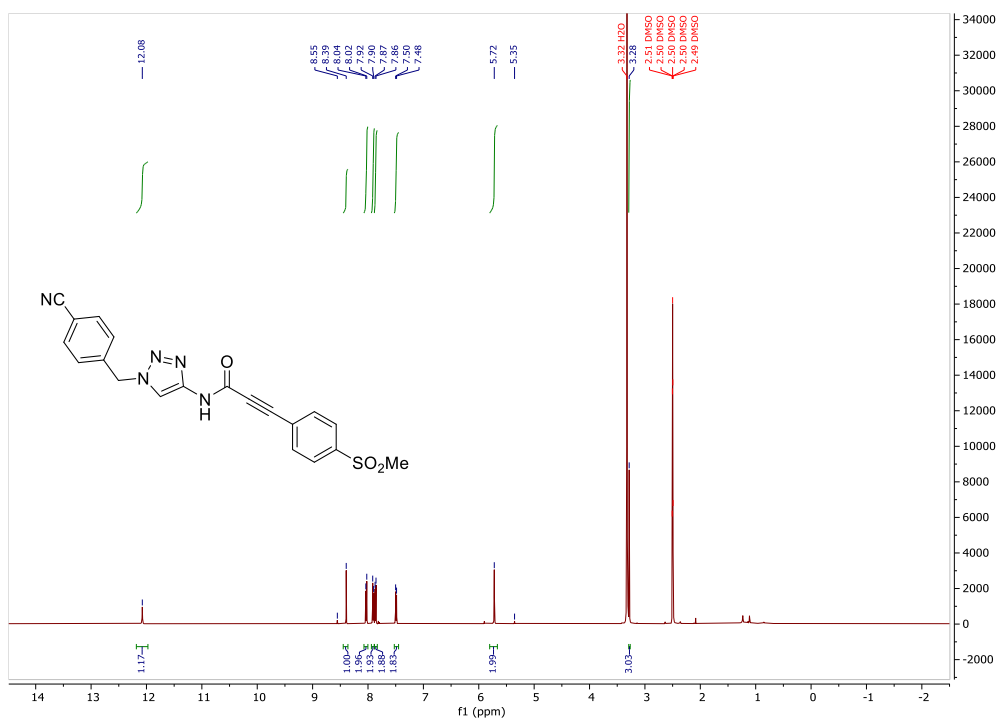
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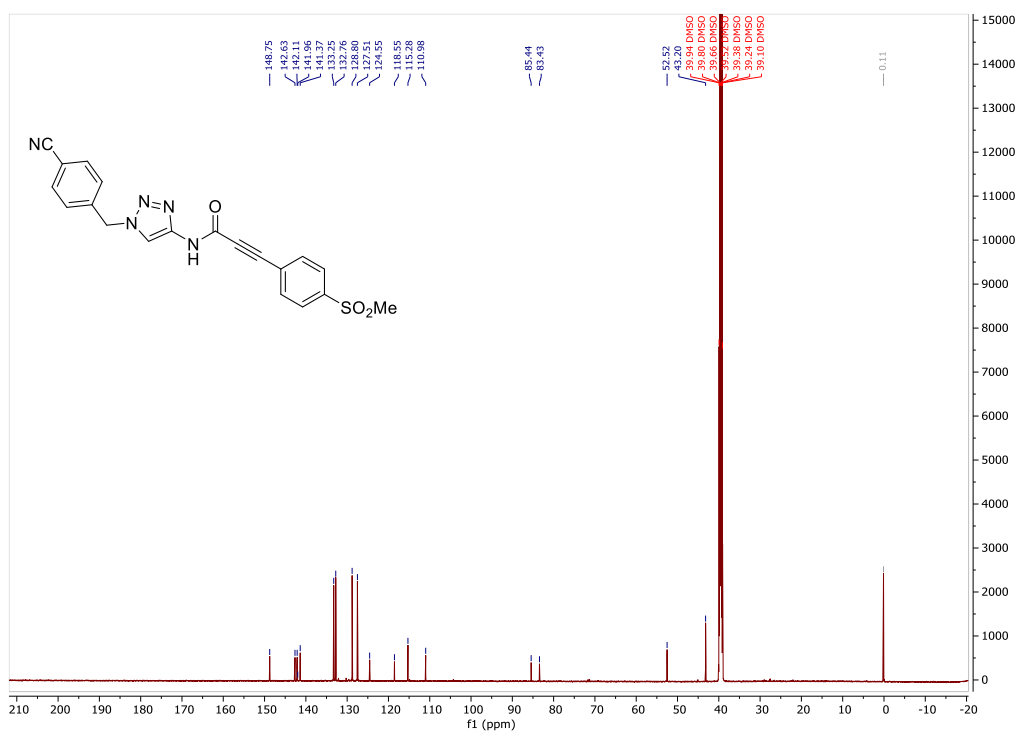
¹³C NMR (125 MHz, DMSO-*d*₆) Spectrum of Compound **22A**



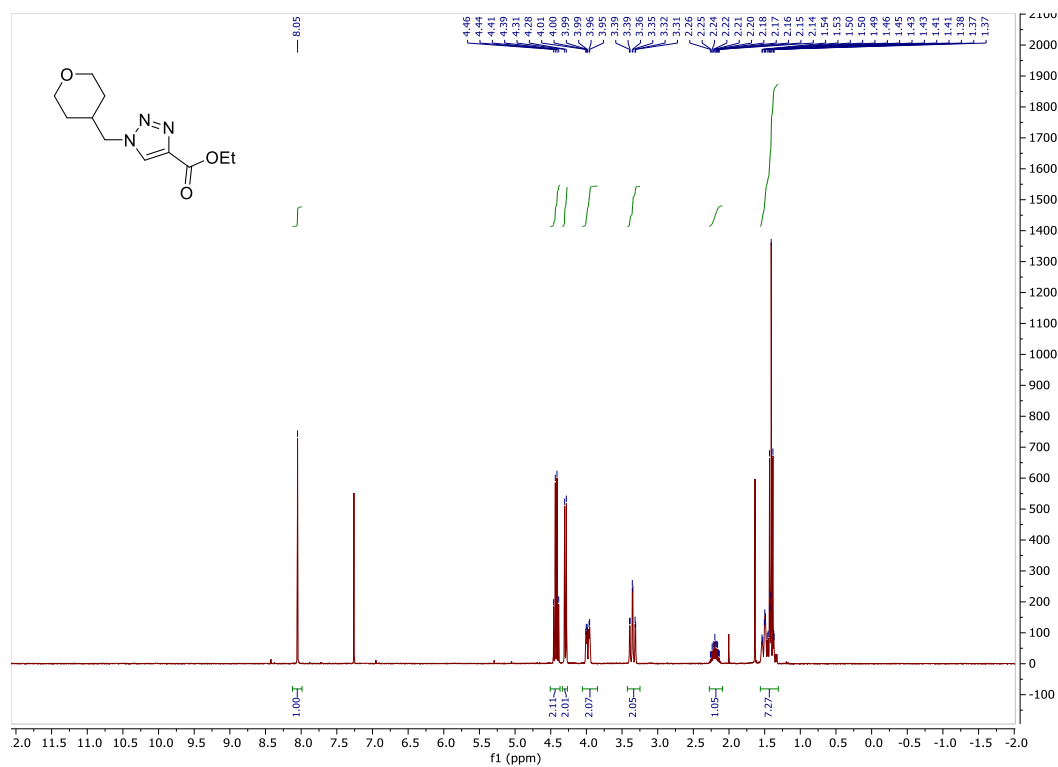
^1H NMR (500 MHz, $\text{DMSO}-d_6$) Spectrum of Compound **23A**



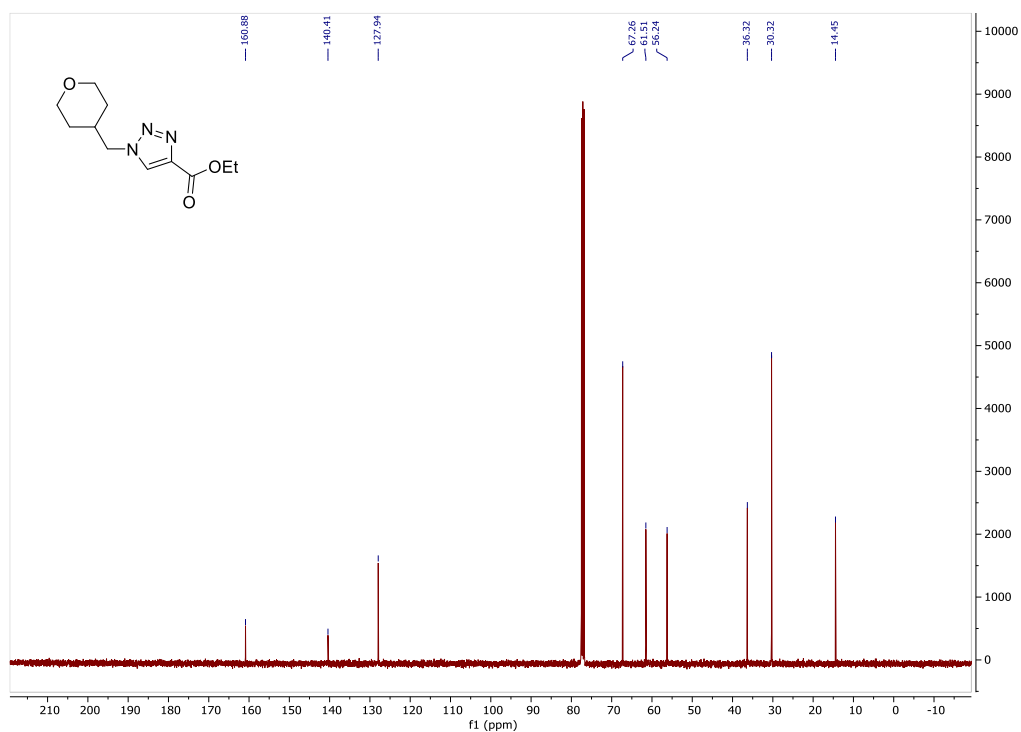
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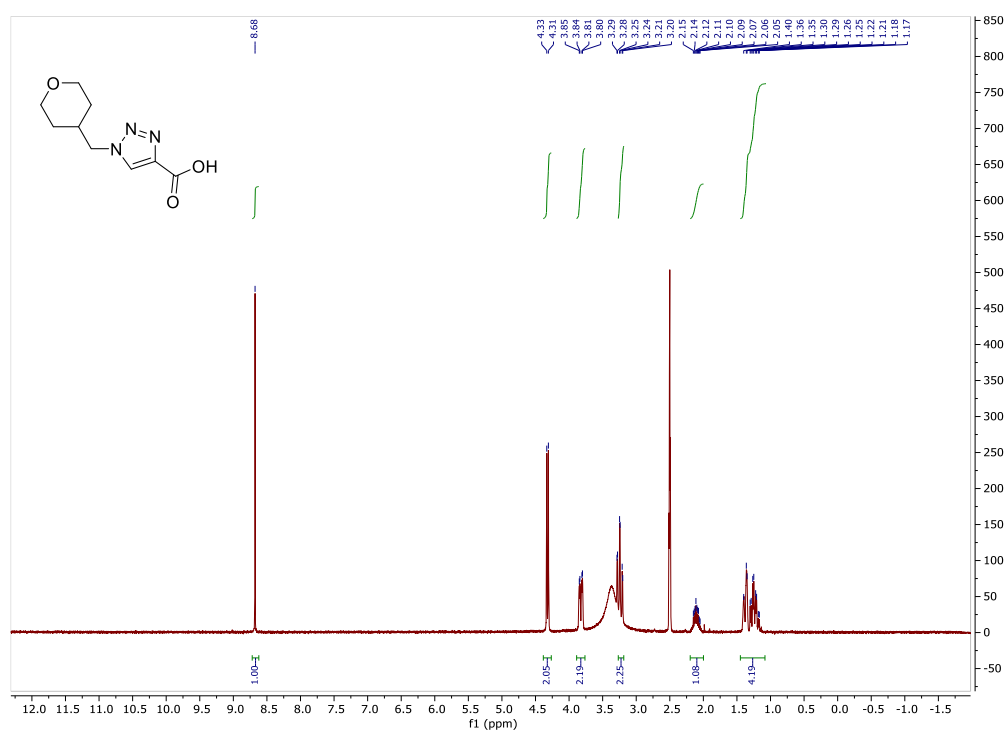
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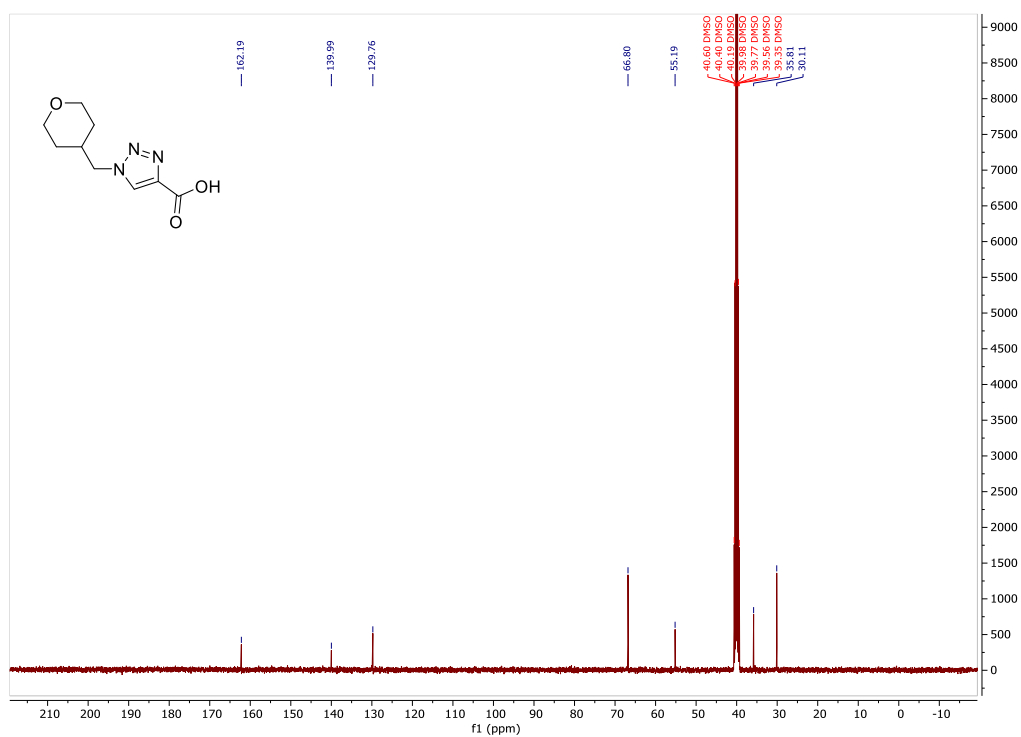
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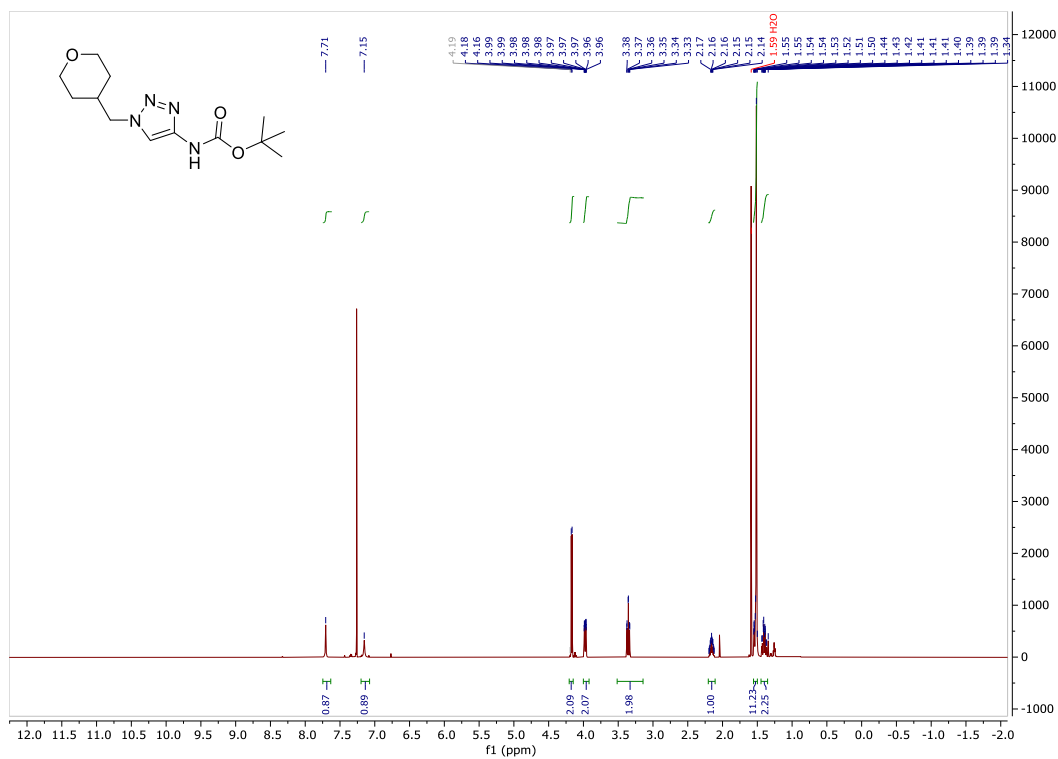
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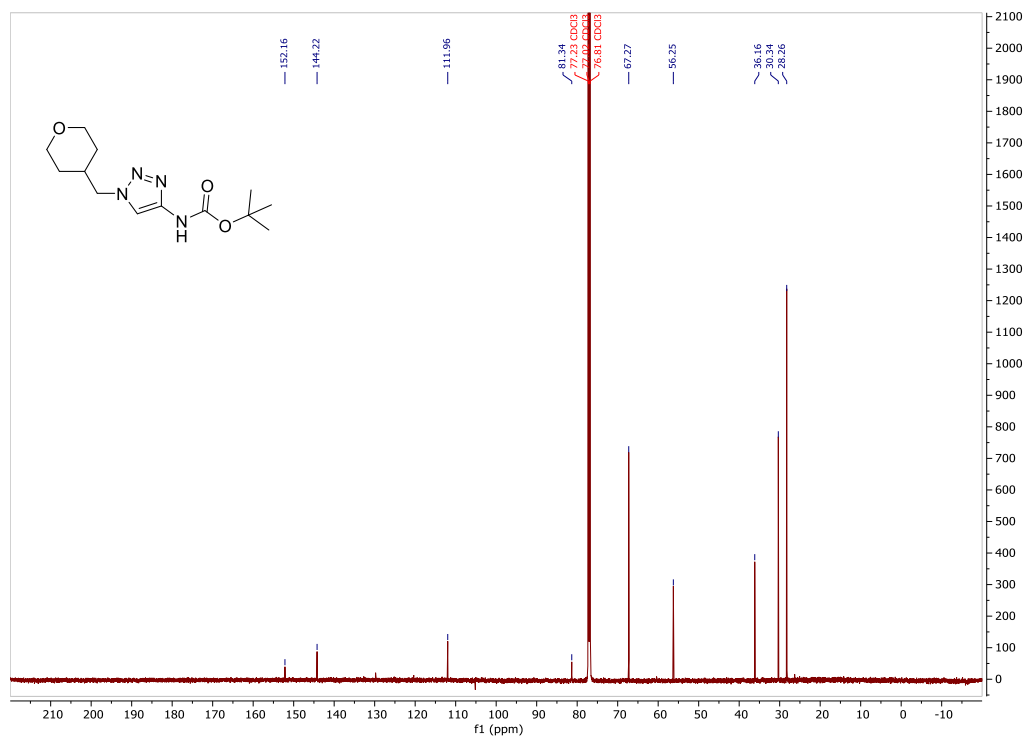
^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) Spectrum of Compound **20B**



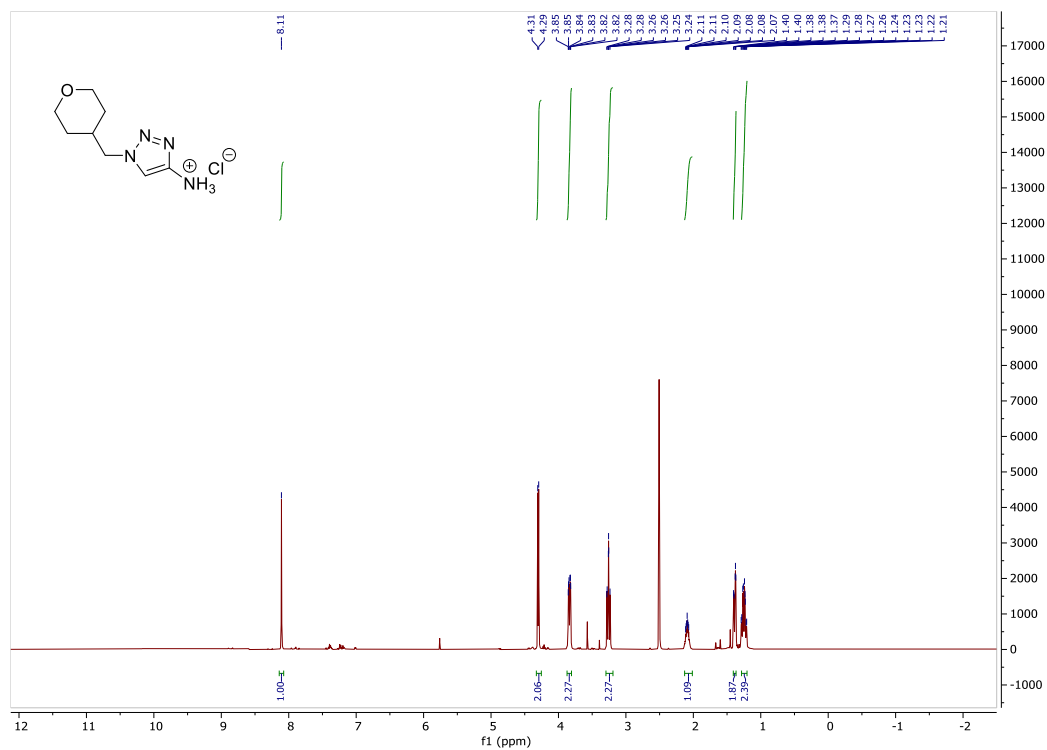
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **21B**



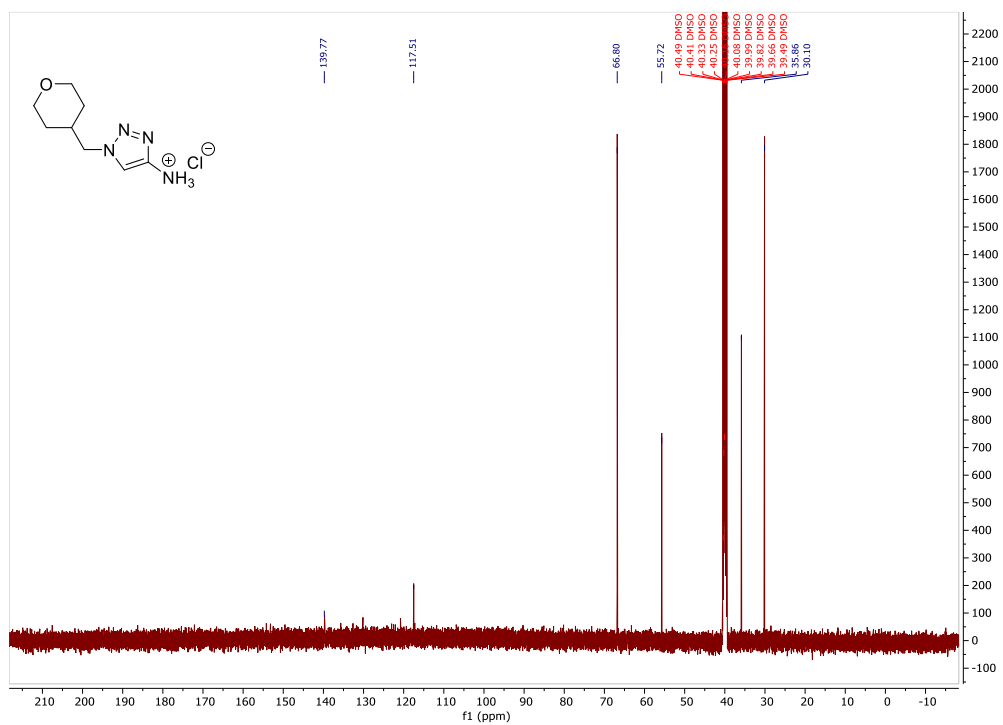
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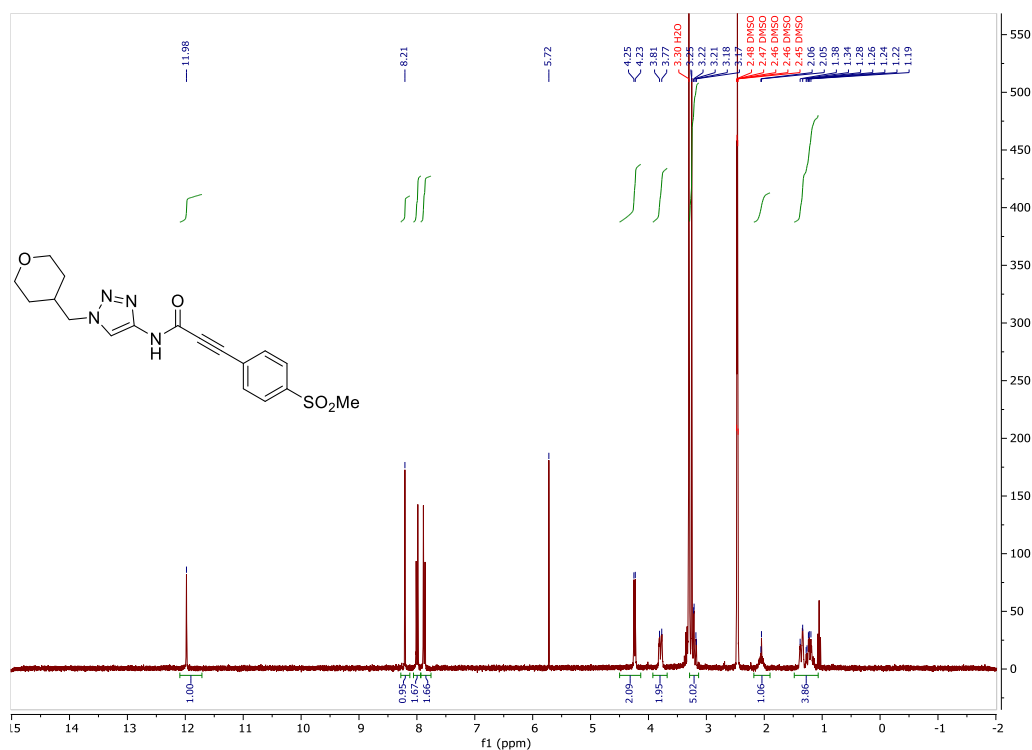
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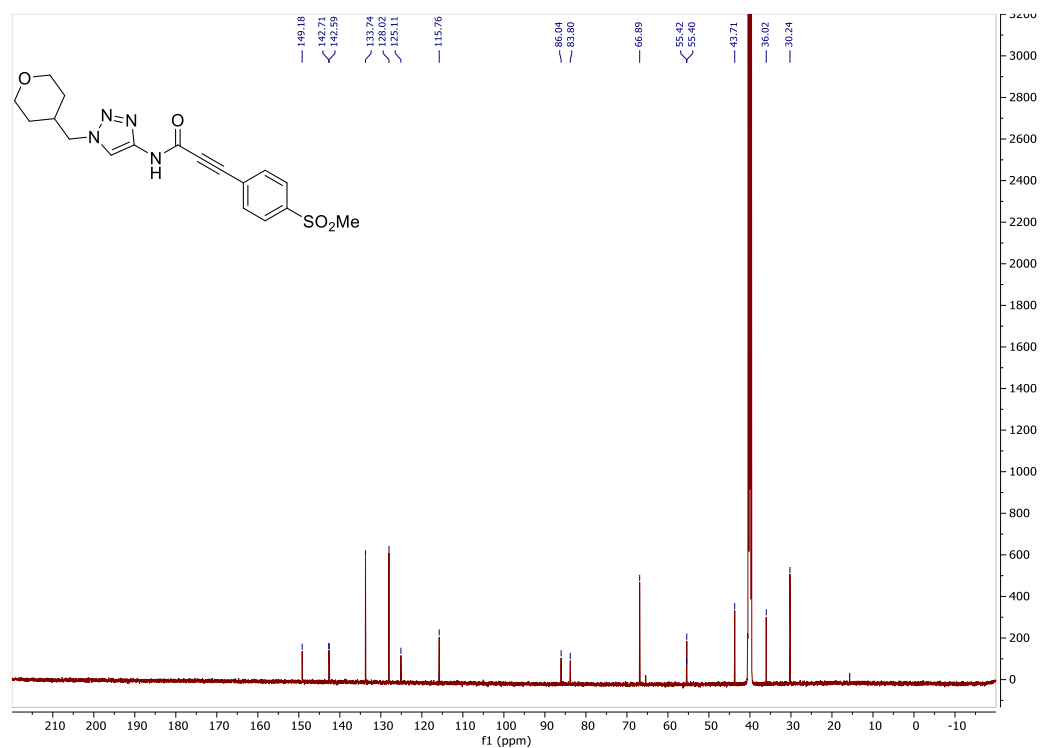
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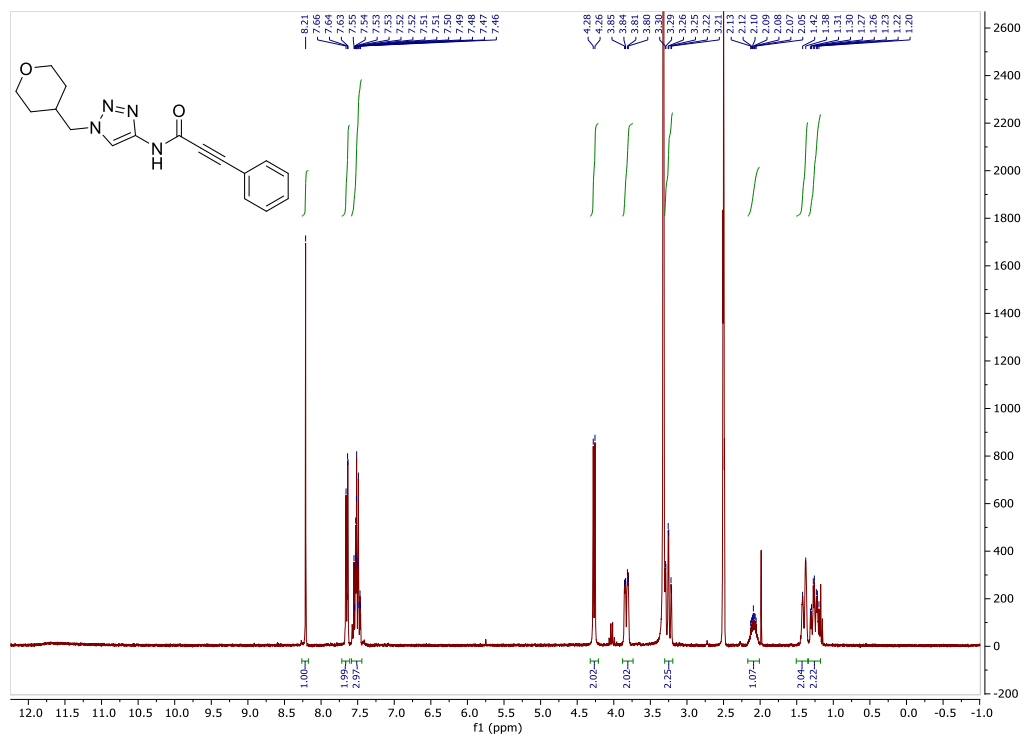
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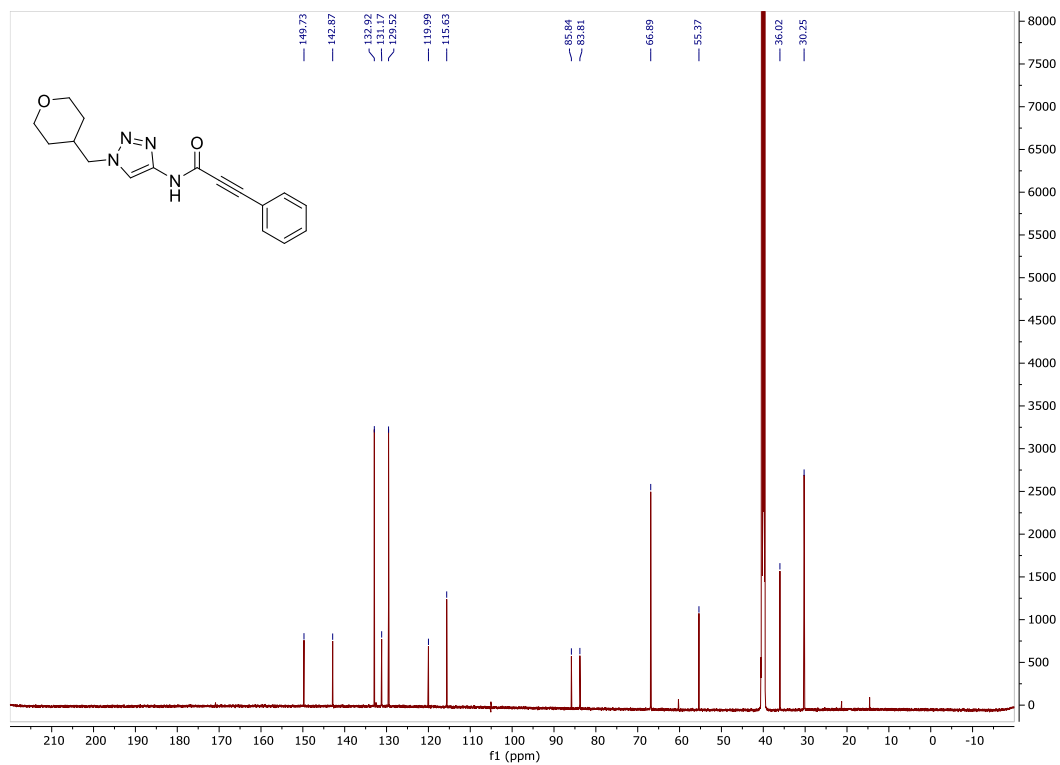
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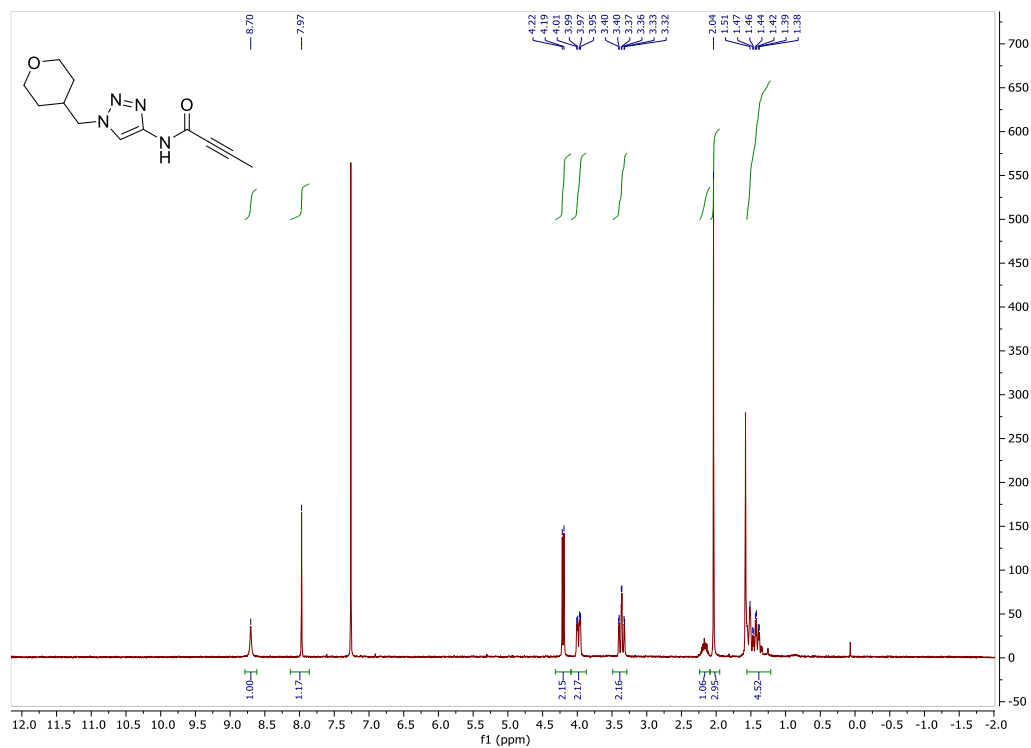
^1H NMR (600 MHz, $\text{DMSO}-d_6$) Spectrum of Compound **23C**



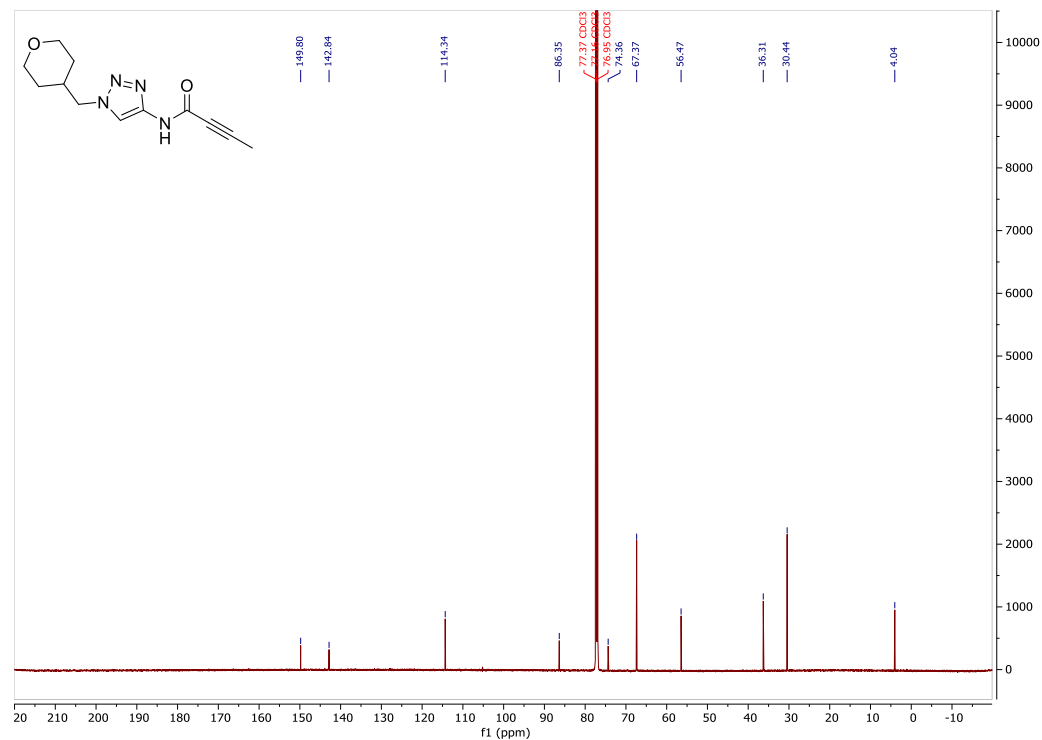
^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) Spectrum of Compound **23C**



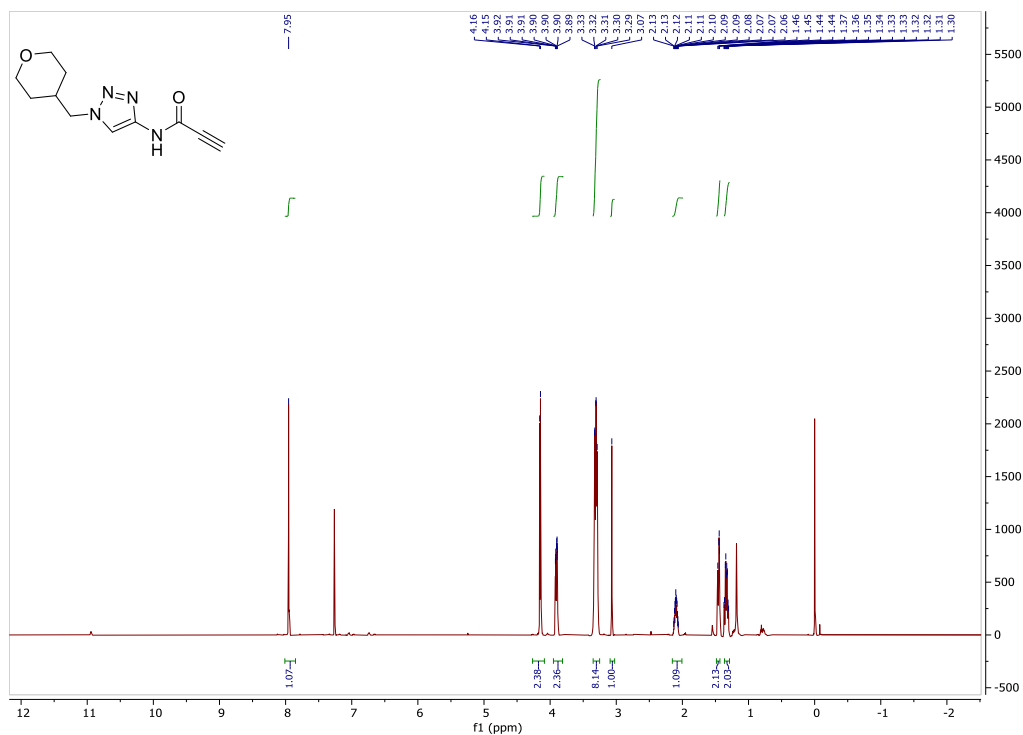
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **23D**



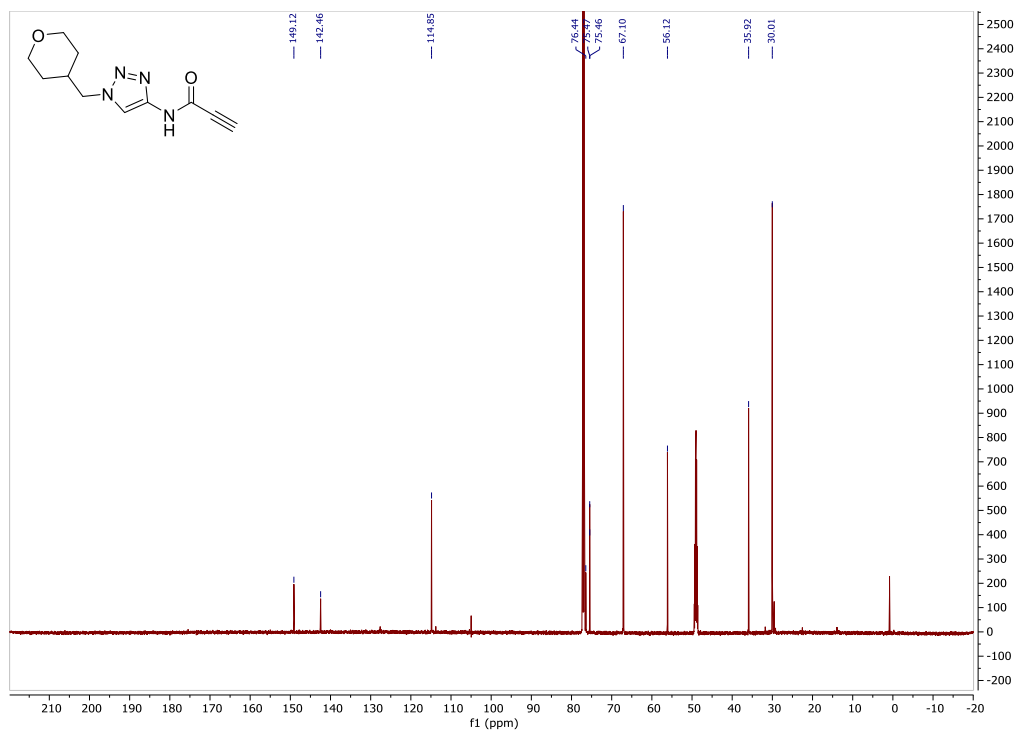
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **23D**



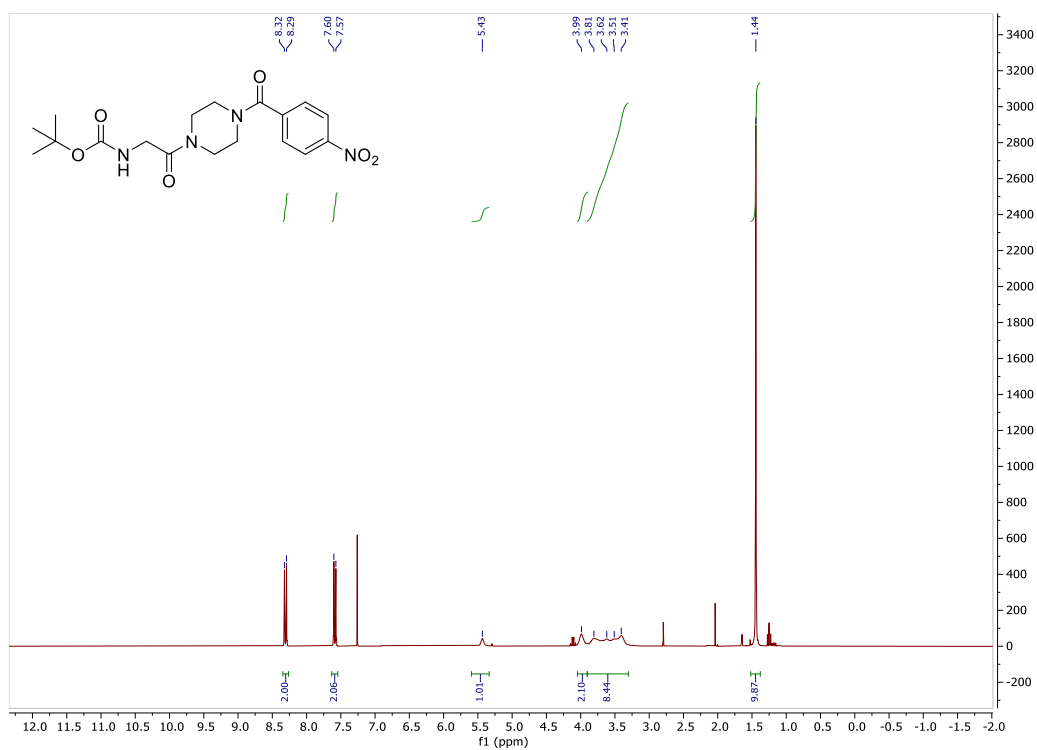
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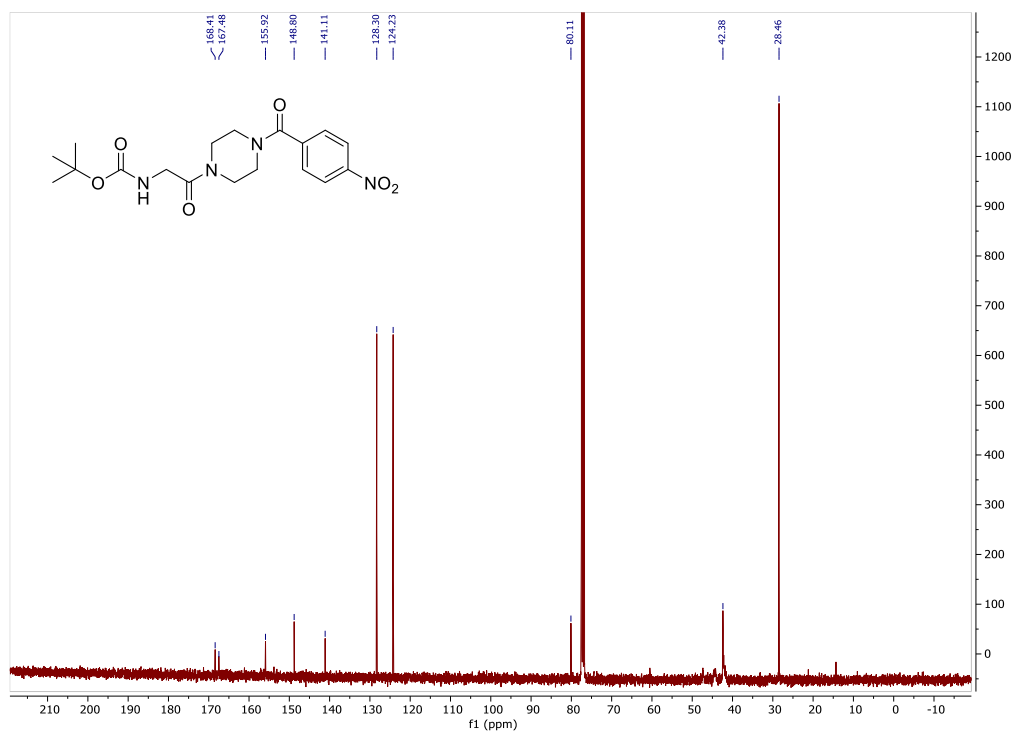
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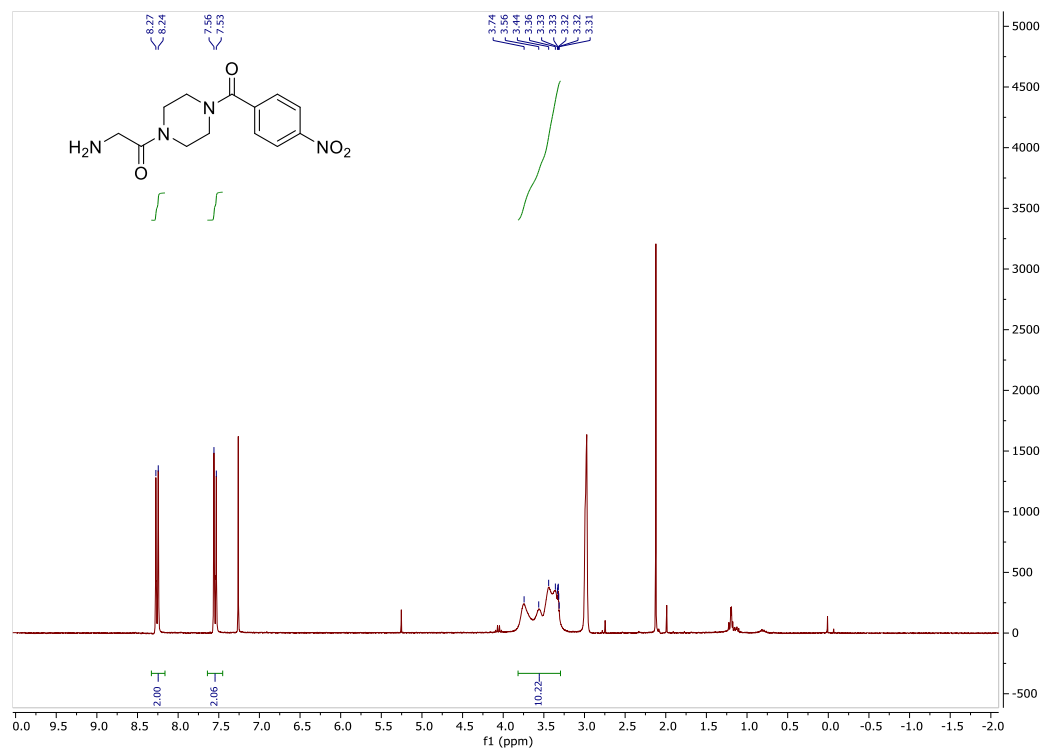
¹H NMR (300 MHz, CDCl₃) Spectrum of Compound **26**



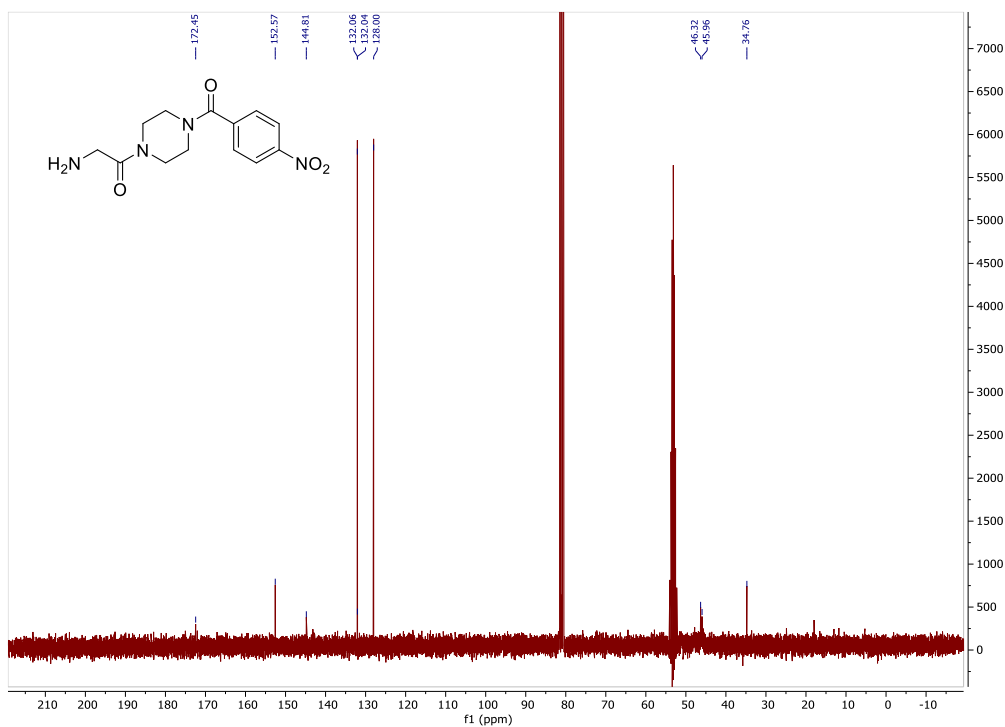
¹³C NMR (75 MHz, CDCl₃) Spectrum of Compound **26**



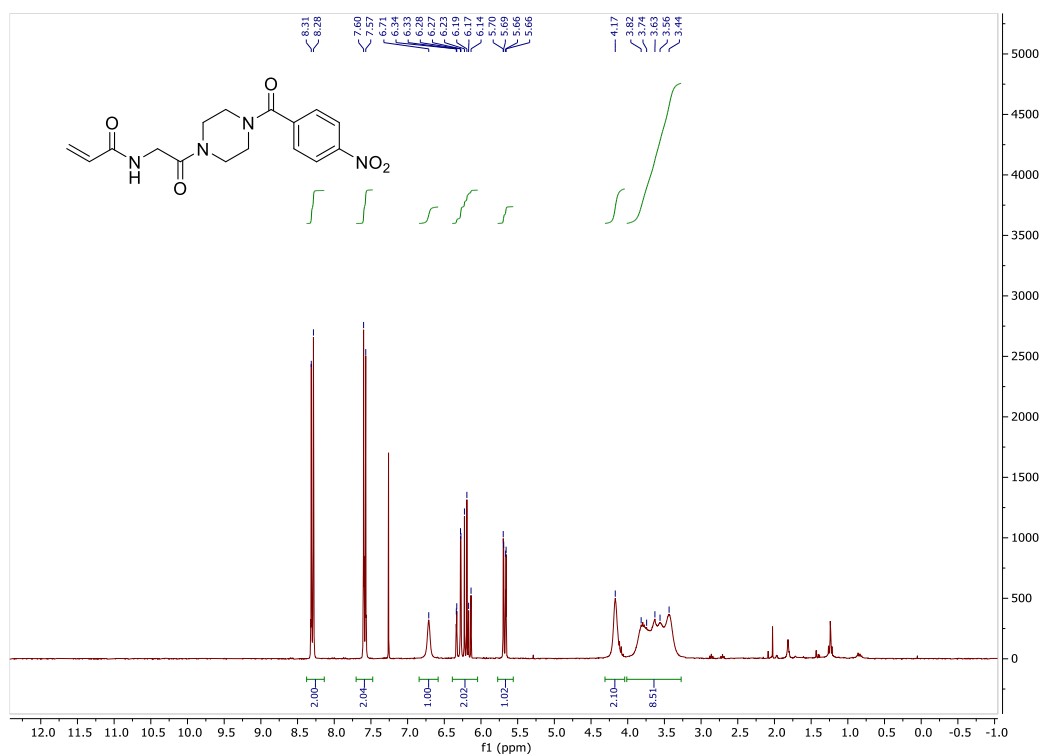
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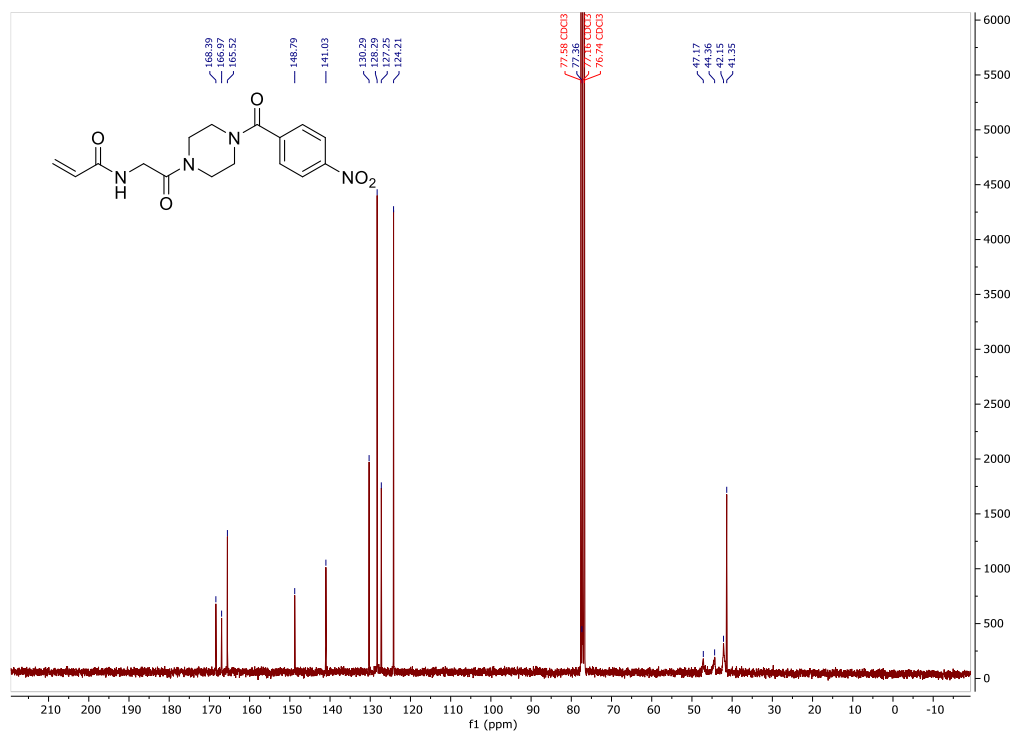
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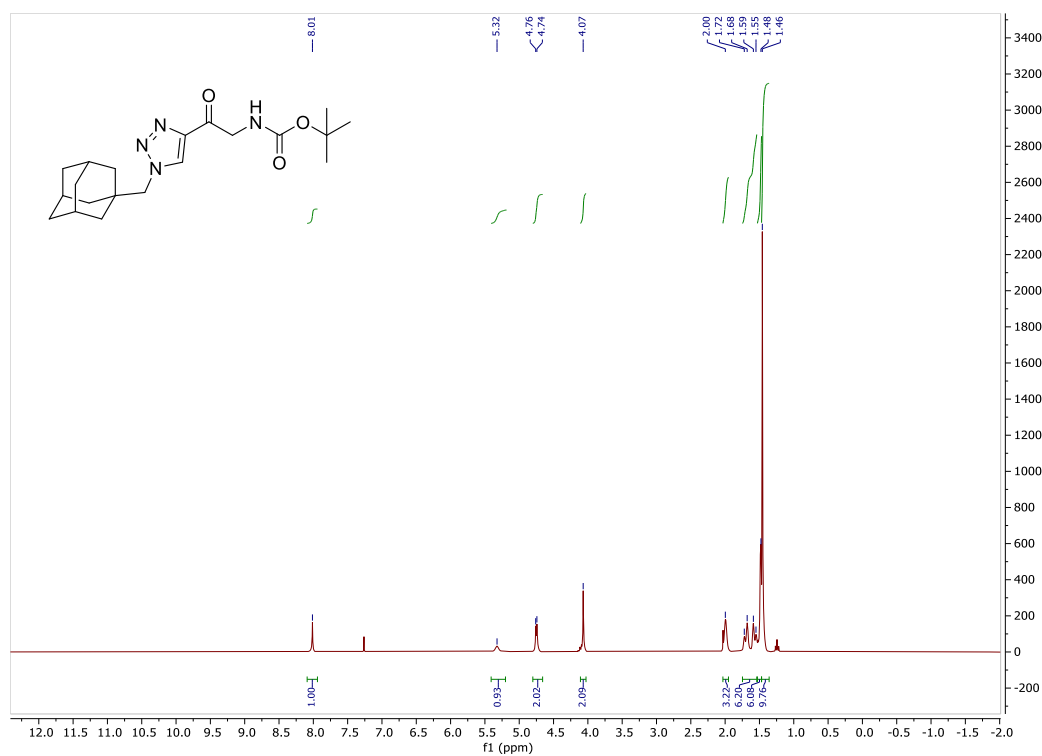
¹H NMR (300 MHz, CDCl₃) Spectrum of Compound **28**



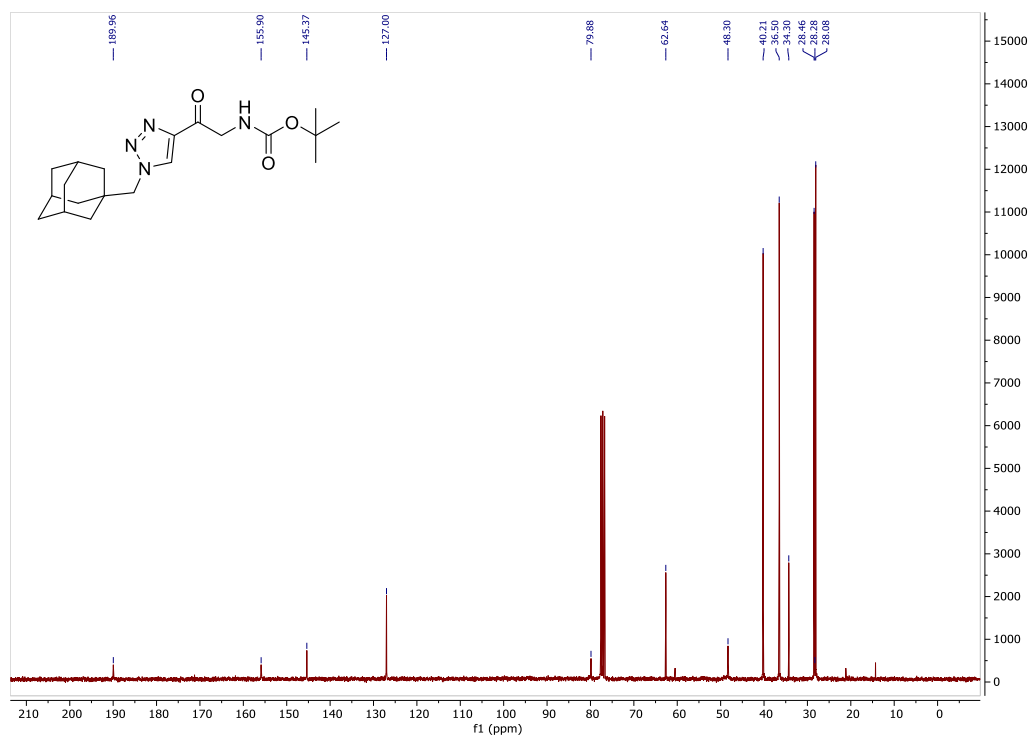
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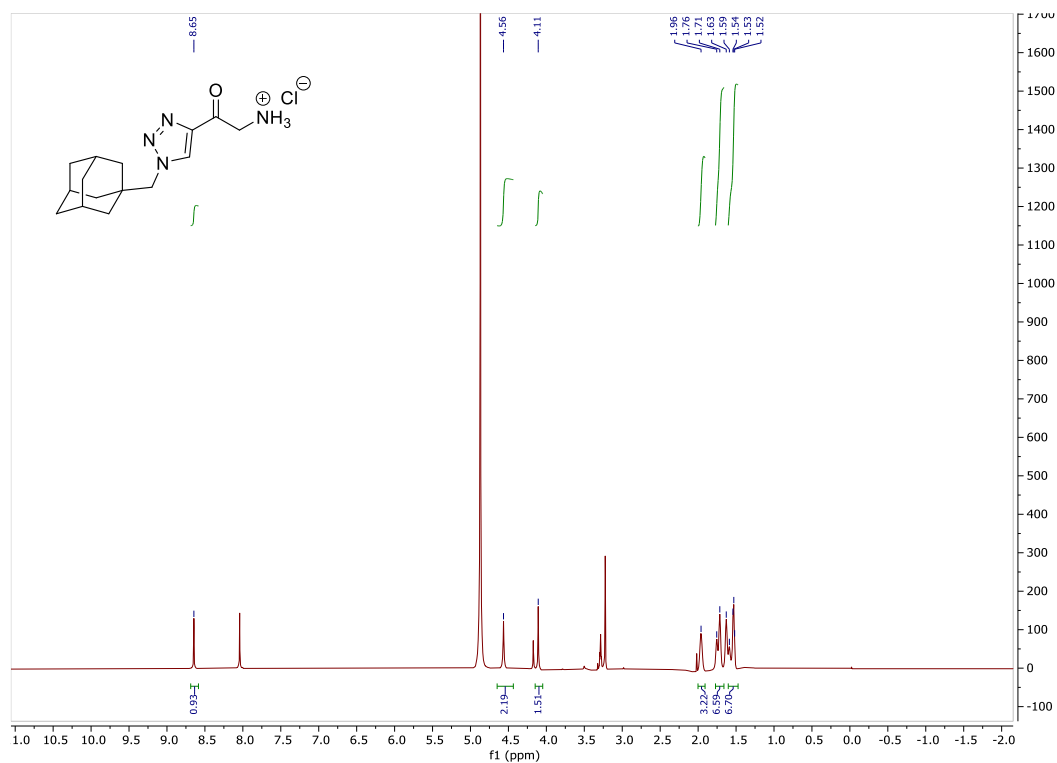
¹H NMR (300 MHz, CDCl₃) Spectrum of Compound **31**



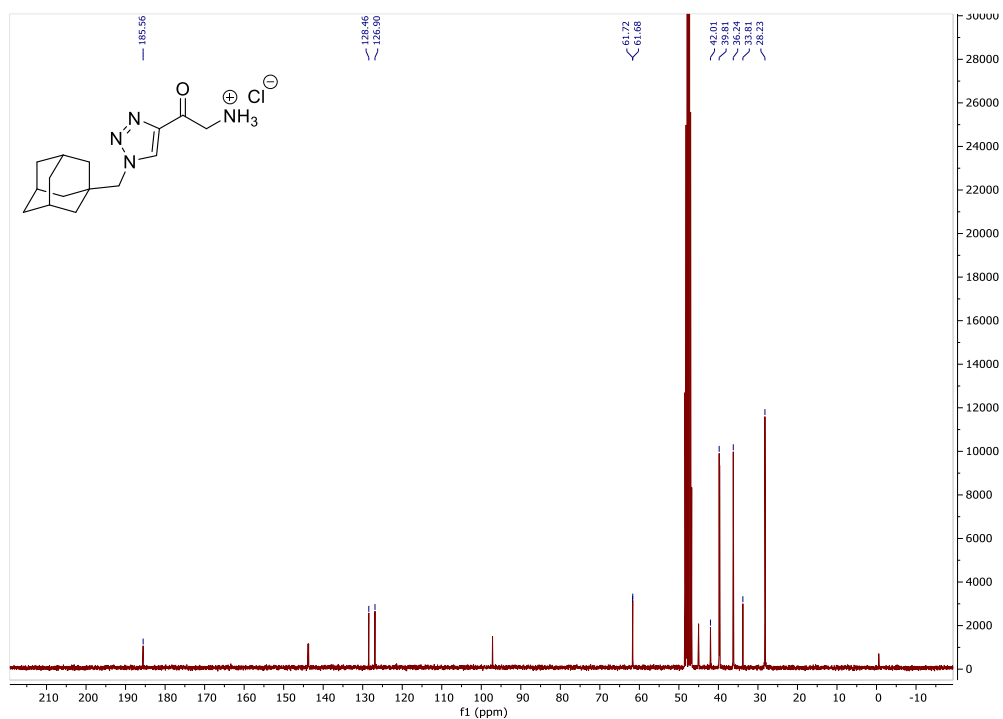
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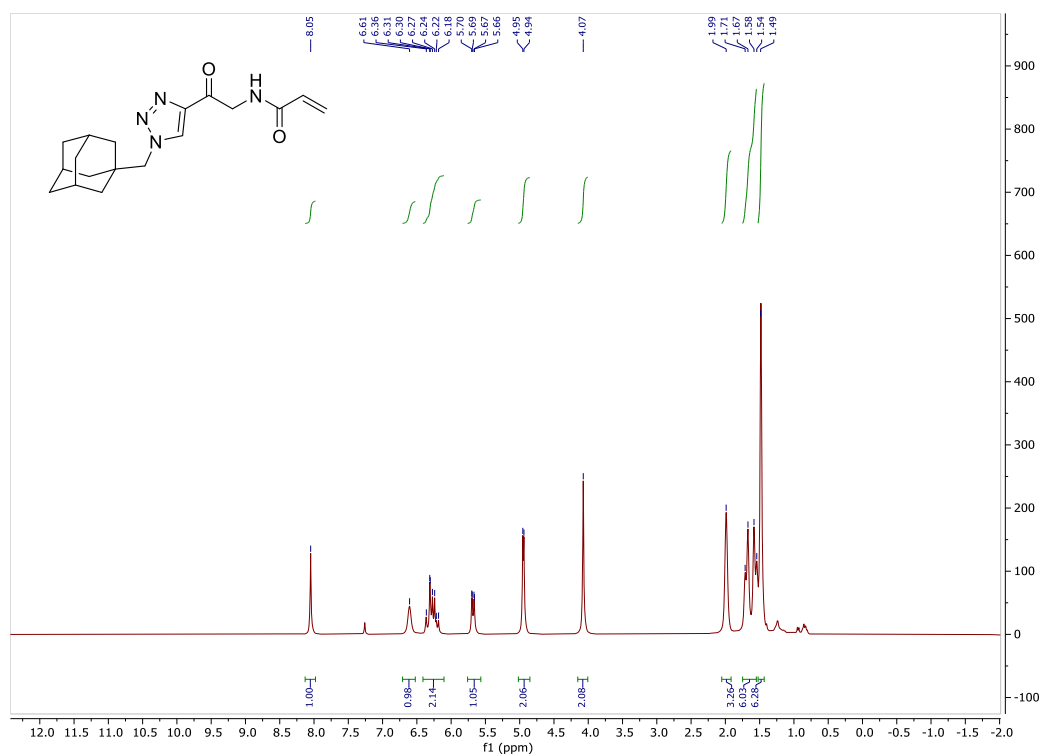
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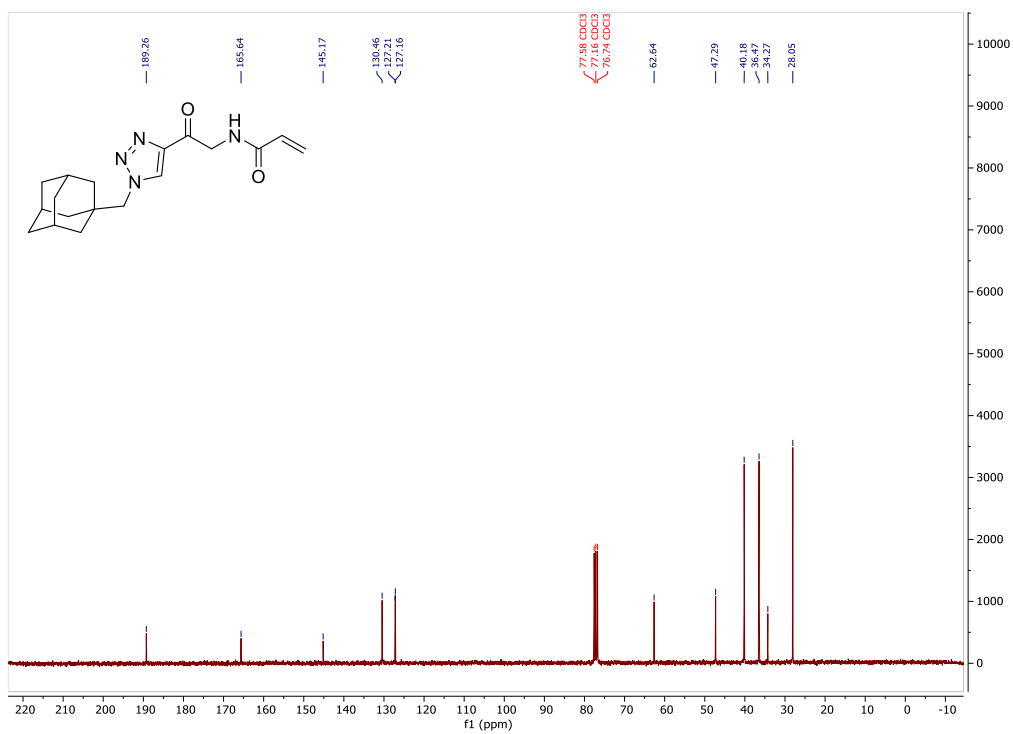
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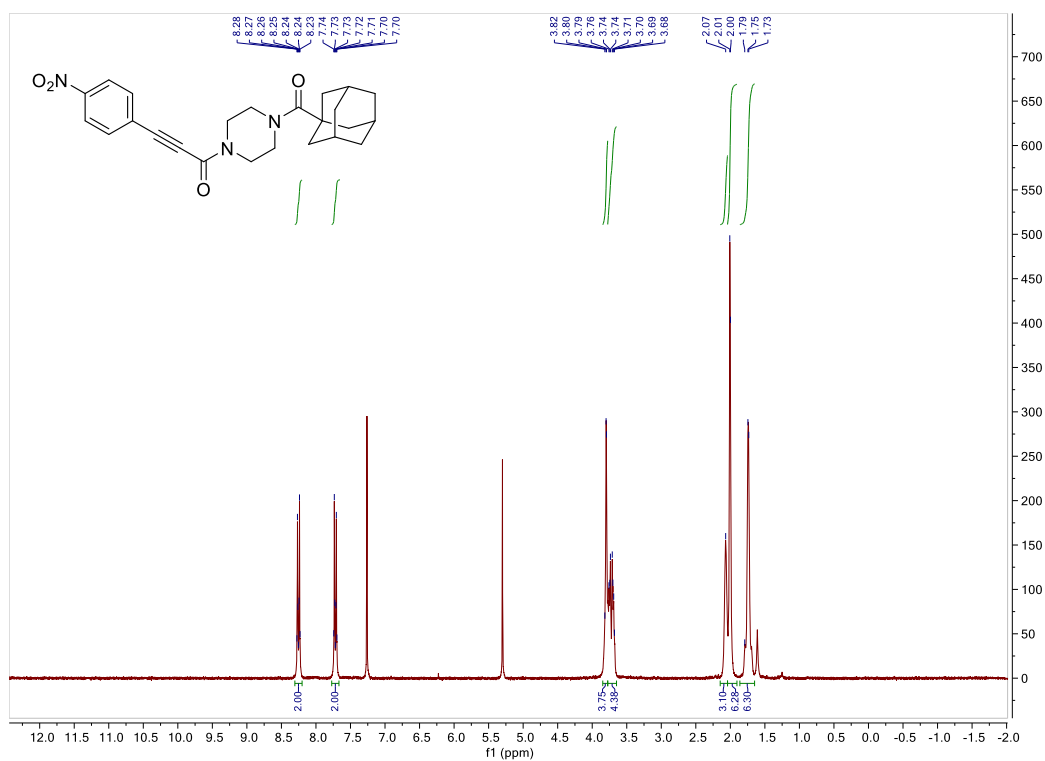
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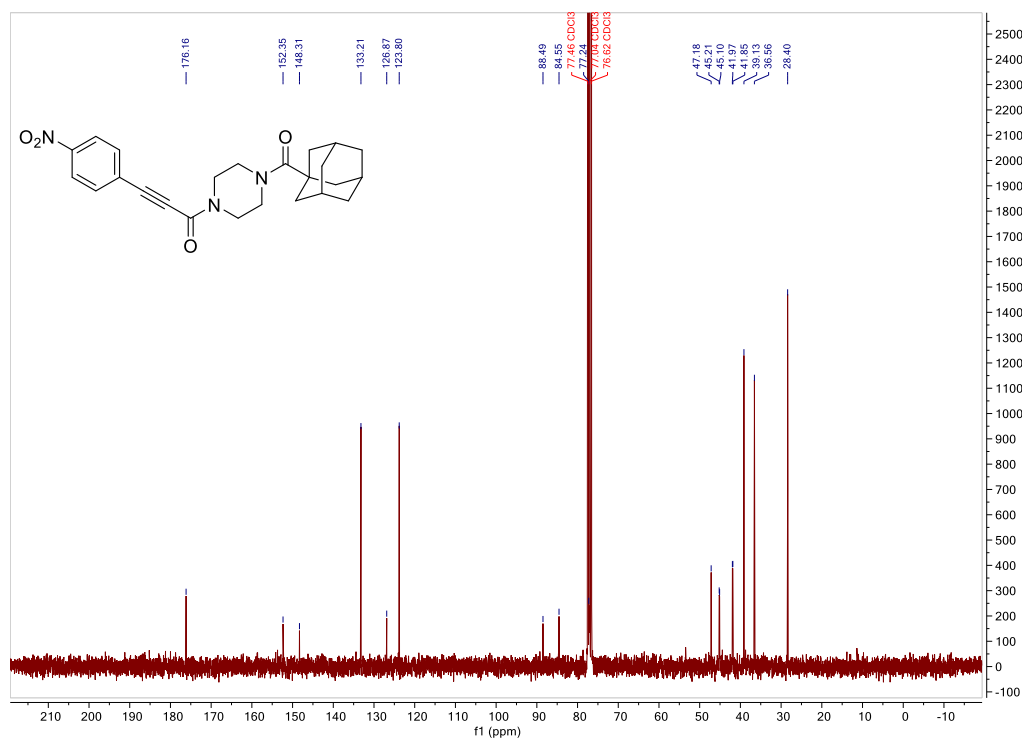
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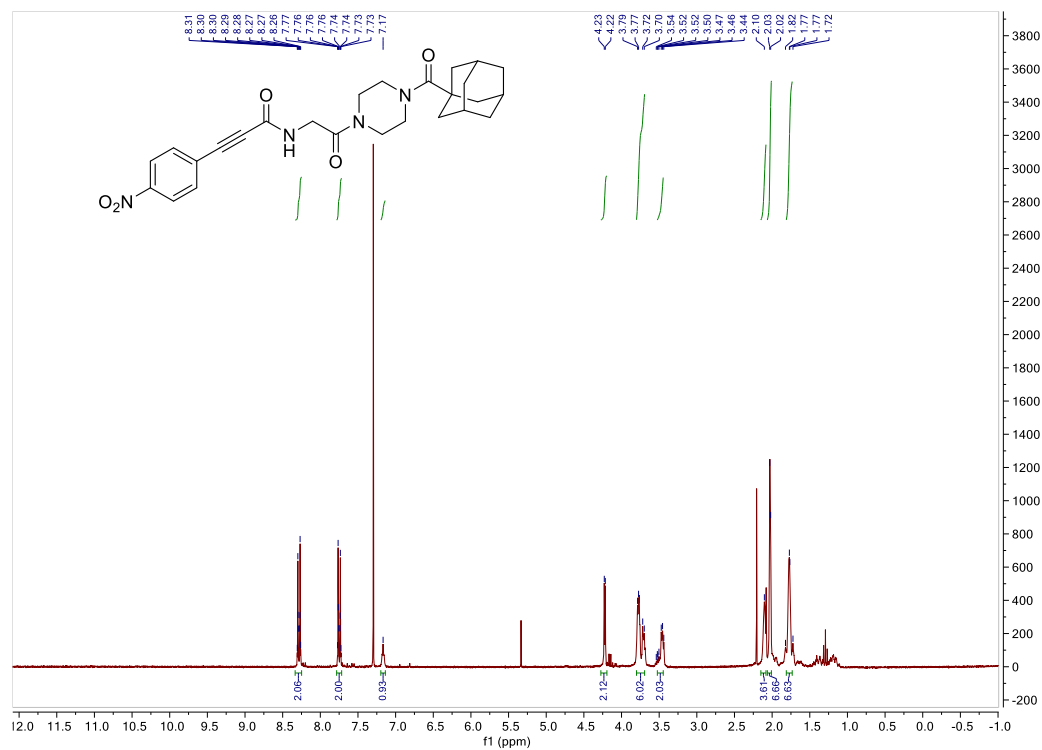
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **36**



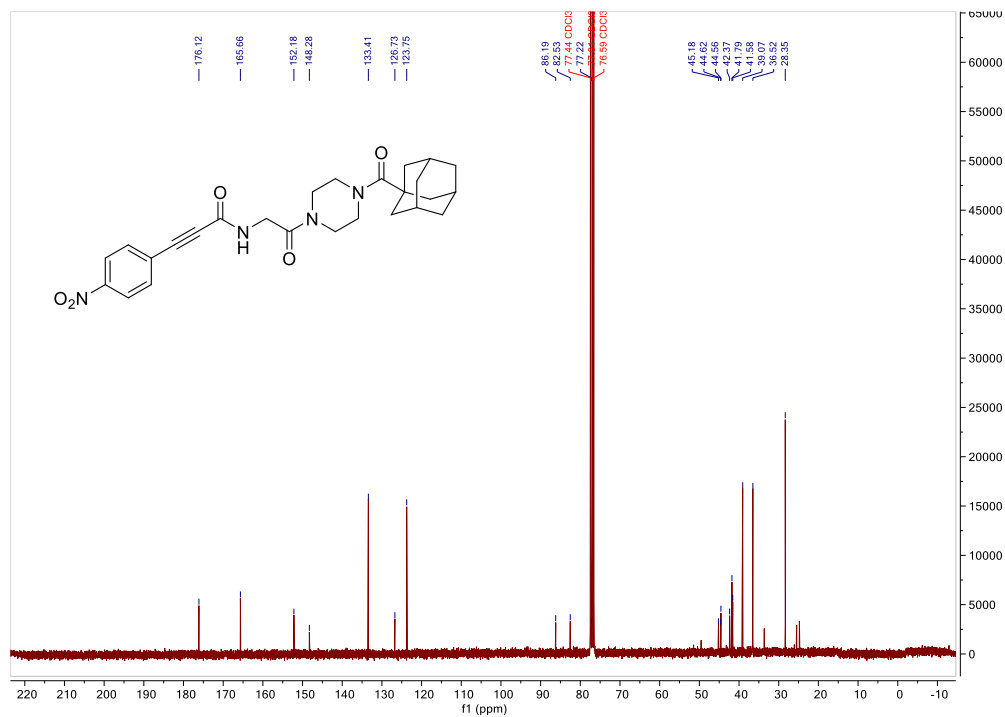
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **36**



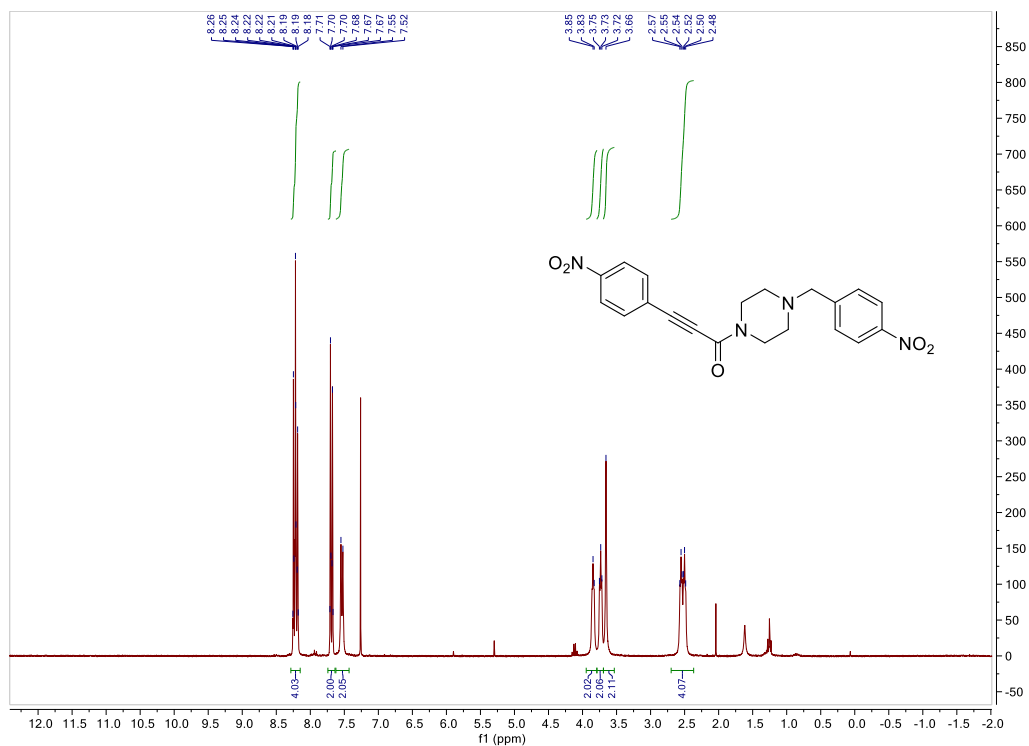
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **38**



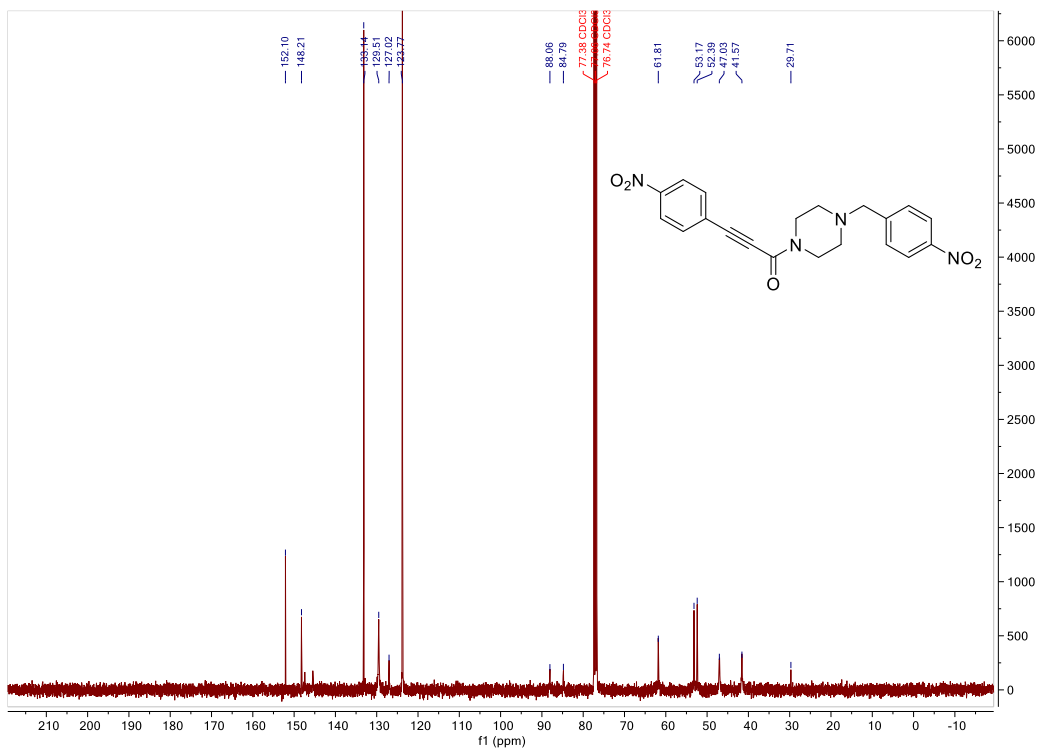
^{13}C NMR (75 MHz, CDCl_3) Spectrum of Compound **38**



¹H NMR (300 MHz, CDCl₃) Spectrum of Compound **40**



¹³C NMR (75 MHz, CDCl₃) Spectrum of Compound **40**



Chapter 4 – Don't Lose Your (War)head: Structure-Activity Relationships of Covalent Warheads as Substrates for GST-Catalyzed GSH Conjugation

4.1. Preamble

Chapters 2 and 3 explore the role of electrophilic warheads in covalent inhibition of hTG2, with a particular focus on target engagement (pharmacodynamics). The metabolic liability (pharmacokinetics) of the warheads was mostly characterized through intrinsic reactivity with GSH, as a proxy for phase II GSH-conjugation, which is currently standard in the field.

However, such measurements do not account for the enzymatic contribution of glutathione *S*-transferases (GSTs), which play a central role in catalyzing GSH conjugation *in vivo*. Furthermore, because the scope of substrates studied with GSTs is quite limited, the extent to which intrinsic reactivity reflects GST-mediated metabolism remains unclear.

To address this, the work described in this chapter investigates a library of electrophilic warheads as substrates for GSTs. By systematically evaluating structure-activity relationships across commonly used warhead classes, this study aims to characterize how warhead structure influences GST-catalyzed reactivity and to identify features that may enable tuning of metabolic stability. In contrast to the target-specific focus of the preceding chapters, this work adopts a perspective on electrophile design relevant to covalent medicinal chemistry more broadly.

4.2. Publication Information and Acknowledgements

This chapter has been directly reproduced from the following reference: Mader, L. K.; Borean, J. E.; Keillor, J. W. *Journal of Medicinal Chemistry*, **2026**, 69 (9), 11574-11591 (<https://doi.org/10.1021/acs.jmedchem.6c00826>) with permission from the American Chemical Society.

4.3. Author Contributions

L. K. M. – Conceptualization, synthesis, characterization, enzymatic assays, intrinsic reactivity assays, hepatic stability assays, molecular modelling, writing original draft.

J. E. B. – Synthesis and characterization of compounds **4**, **7**, and **8**. Enzymatic and intrinsic reactivity assays of compound **8**.

J. W. K. – Conceptualization, supervision, funding, editing and reviewing.

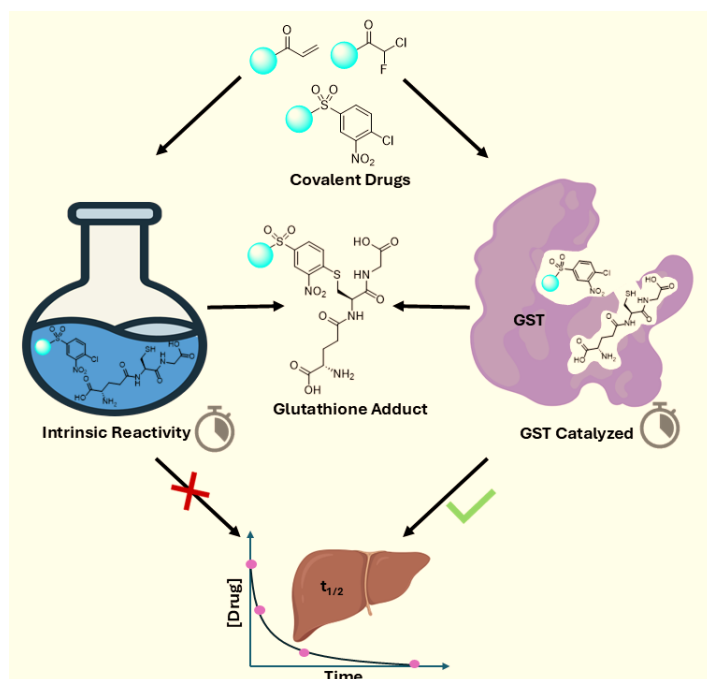


Figure 4.1. Graphical summary of the work describing this chapter.

Don't Lose Your (War)head: Structure–Activity Relationships of Covalent Warheads as Substrates for GST-Catalyzed Glutathione Conjugation

Lavleen K. Mader, Jessica E. Borean, and Jeffrey W. Keillor*

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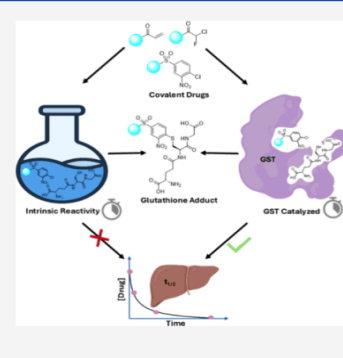
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ABSTRACT: The resurgence of targeted covalent inhibitors (TCIs) has expanded the diversity of electrophilic warheads used in drug discovery. TCIs must balance efficient target engagement with resistance to rapid metabolic clearance. In drug development campaigns, intrinsic reactivity toward glutathione (GSH) is commonly used to estimate metabolic liability; however, *in vivo* GSH conjugation is primarily catalyzed by glutathione S-transferases (GSTs), a phase II metabolic pathway that is not captured by intrinsic reactivity measurements. Here, we establish a quantitative assay to determine GST k_{cat} and K_{M} values across a panel of structurally diverse warheads. We show that their intrinsic reactivities correlate poorly with GST-catalyzed conjugation rates, which are instead governed by warhead- and scaffold-dependent enzyme–substrate interactions. In contrast, GST $k_{\text{cat}}/K_{\text{M}}$ values correlate closely with compound half-lives in human liver cytosol. Together, these findings establish GST susceptibility as a structurally tunable determinant of metabolic GSH conjugation and provide new principles for the optimization of TCIs.



INTRODUCTION

Targeted covalent inhibition has become an established modality in medicinal chemistry,^{1,2} enabling high potency and durable target engagement through the formation of a covalent bond between an electrophilic warhead and a nucleophilic protein residue, most commonly cysteine.^{3,4} Advances in electrophile design have expanded the range of warheads for TCI development, enabling fine-tuning of reactivity, binding affinity/selectivity, and reversibility.^{3,5} As a result, warhead selection has evolved into a deliberate drug design variable, with direct implications for efficacy and safety.

The successful application of covalent drugs, however, requires careful balancing of on-target covalent engagement with off-target reactivity and metabolic stability *in vivo*. Given that most warheads are either designed for, or exhibit significant reactivity with cysteine thiols,⁴ reaction with glutathione (GSH), which is present in millimolar concentrations in most cells, is a major metabolic clearance pathway for compounds bearing these warheads. As such, reactivity with GSH is frequently invoked as a key liability during early drug discovery,⁶ and intrinsic reactivity with GSH (k_2^{GSH}) is widely used as a proxy for metabolic susceptibility, with high reactivity often interpreted as an indicator of poor stability.^{3,7} However, *in vivo* GSH conjugation is predominantly mediated by glutathione S-transferases (GSTs), a family of phase II metabolic enzymes that catalyze the addition of GSH to electrophilic substrates, thereby enhancing solubility and clearance.⁸ While intrinsic (nonenzymatic) GSH reactivity informs inherent electrophilicity, it does not capture the

enzyme-catalyzed process that governs stability in biological systems (Figure 1).

The rate of enzymatic modification is dictated not only by the intrinsic reactivity of substrates but also by binding affinity and substrate orientation. GSTs are described as broadly tolerant enzymes, attributed to a large hydrophobic H-site adjacent to their highly conserved GSH-binding G-site.⁹ Consequently, GST turnover is often assumed to be governed primarily by electrophile reactivity rather than substrate-specific recognition. Despite this assumption, systematic, scaffold-matched comparisons of how distinct electrophilic warheads are processed by individual GST isoforms, particularly in terms of catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$), remain limited. As a result, the relationship between intrinsic reactivity and GST-catalyzed conjugation remains poorly defined.

Here, we report structure–activity relationships (SAR) of a focused warhead library to directly define and compare intrinsic and GST-catalyzed GSH reactivity on a common scaffold. Given their prominent roles in drug metabolism, we examine two clinically relevant isoforms of GST, namely GSTA1, the major hepatic isoform, and GSTP1, a widely expressed extrahepatic isoform.^{10–12} Using intrinsic reactivity,

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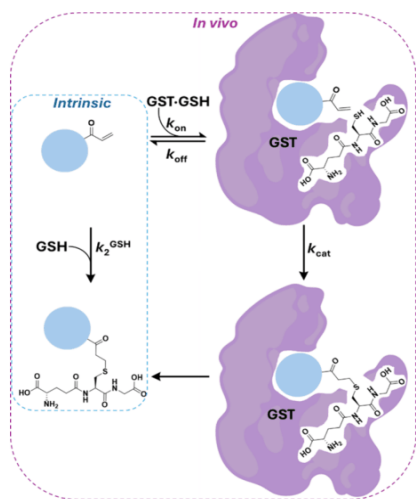


Figure 1. Glutathione conjugation of electrophilic compounds (e.g., acryloyl group) through intrinsic reactivity in solution (blue box) and biologically relevant GST catalysis (purple box).

enzyme kinetic parameters, and stability in human liver cytosol, we quantitatively define how warhead structure influences GST-mediated metabolism. Together, this work establishes a framework for understanding and predicting the metabolic stability of electrophilic warheads commonly employed in drug development.

RESULTS AND DISCUSSION

Substrate Design and Synthesis

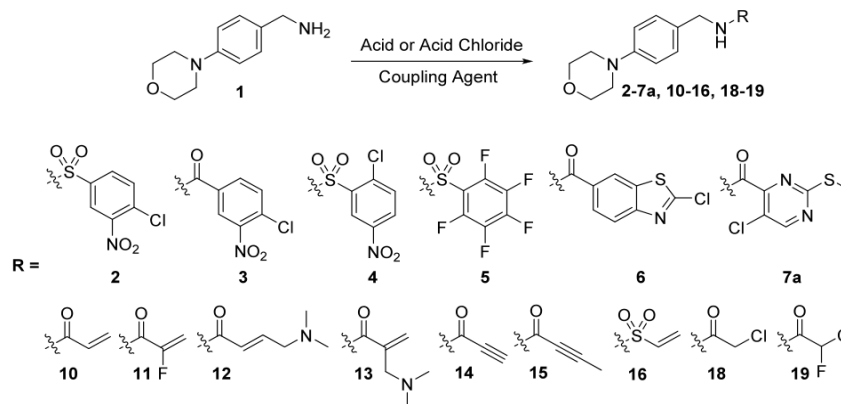
To isolate warhead-dependent effects while minimizing scaffold contributions, we employed a compact benzyl-morpholine core (**1**), which was held constant throughout the study. This drug-like scaffold provides balanced polarity and hydrophobic character while enabling late-stage installation of diverse electrophiles.¹³ Warheads were selected to represent diverse mechanistic and structural features commonly used in current covalent medicinal chemistry.³

Warheads capable of nucleophilic aromatic substitution (S_NAr) are of increasing interest in covalent drug design, due to their tunable electronic properties and potential for productive binding interactions.^{3,14–17} Notably, S_NAr reactions are efficiently catalyzed by GSTs, as exemplified by the canonical 1-chloro-2,4-dinitrobenzene (CDNB) assay for GST activity.¹⁸ We therefore prepared a series of S_NAr warheads (**2–9**) that differ in aromatic framework, substitution pattern, and electrophile orientation, to evaluate how these features influence GST-mediated conjugation. These compounds were prepared primarily through late-stage amide coupling or direct aromatic substitution (Schemes 1, 2, 3, and 4).

Michael acceptors, particularly α,β -unsaturated amides, represent the most widely used warhead class in covalent medicinal chemistry.³ FDA-approved covalent drugs such as osimertinib, afatinib, and acalabrutinib, contain acrylamide- or butynamide-based electrophiles that balance modest intrinsic reactivity with productive target engagement.^{1,19,20} Major metabolic pathways for these warheads include GSH-conjugation through Michael addition, or in the case of acrylamides, oxidation to epoxides followed by hydrolysis or GSH addition. Although GSH-adducts have been observed as metabolites of these drugs,²¹ the structural features governing susceptibility to GST-catalyzed conjugation remain poorly understood. Accordingly, a series of α,β -unsaturated amides and a vinyl sulfonamide (**10–16**) were prepared to examine how substitution pattern and electronic tuning influence GST turnover (Schemes 1 and 3). We also included a newly emerging Michael acceptor, vinylpyridine (**17**),²² whose reactivity is even further attenuated than acrylamides, while potentially offering additional target binding interactions through its heteroaromatic ring (Scheme 3 and 5).

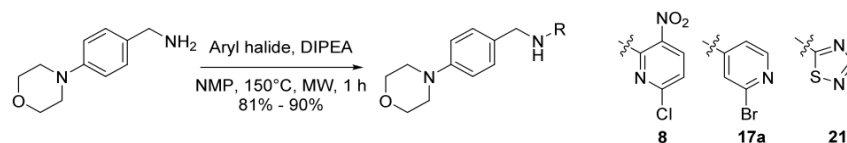
Electrophiles that participate in S_N2 -like reactions, such as chloroacetamide (**18**),²³ which serves as a benchmark for highly reactive thiol targeting electrophiles frequently used in chemical biology,⁴ were also evaluated. More recently developed attenuated analogues such as chlorofluoroacetamide (CFA) (**19**) and bicyclobutane (BCB) (**20**), which reduce intrinsic reactivity while introducing structural features that may influence binding interactions,^{24,25} were also included to probe how reactivity attenuation and structural changes impact GST-mediated conjugation (Schemes 1 and 6).

Scheme 1. Synthesis of Compounds **2–7a**, **10–16**, **18–19** by Amide Coupling^a

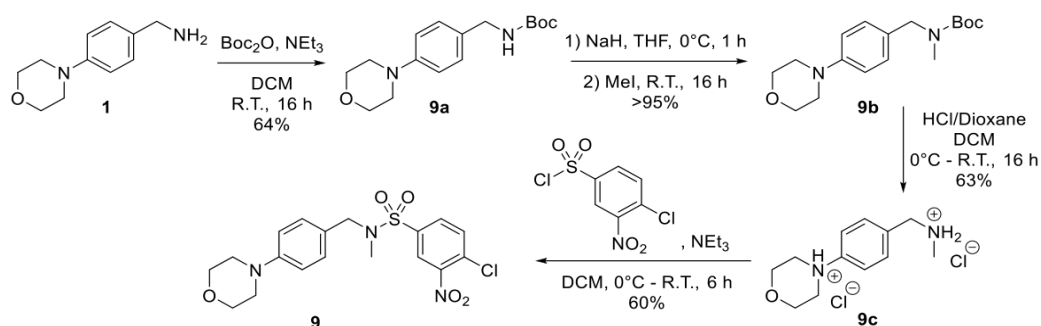


^aSee Experimental Section for details.

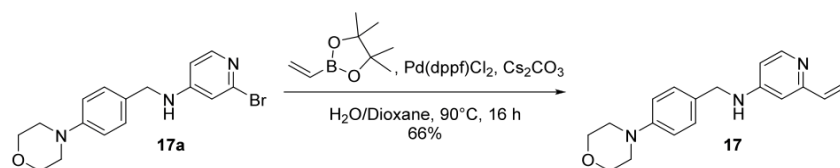
Scheme 2. Synthesis of Compound 7

Scheme 3. Synthesis of Compounds 8, 17a, 21 by S_NAr ^a^aSee Experimental Section for details.

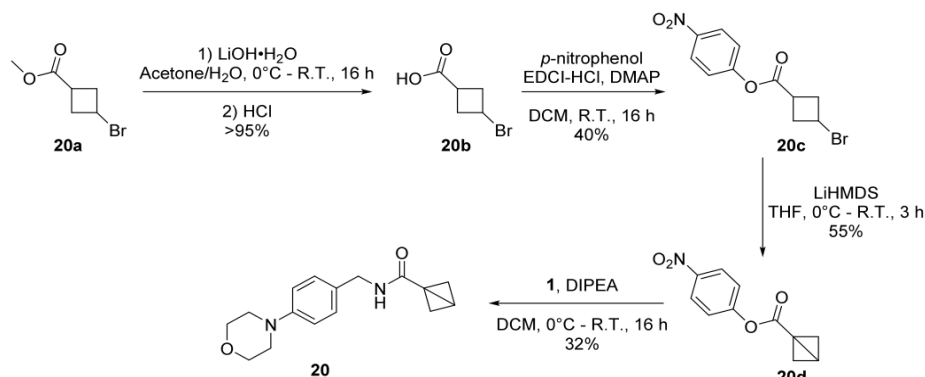
Scheme 4. Synthesis of Compound 9



Scheme 5. Synthesis of Compound 17



Scheme 6. Synthesis of Compound 20



Finally, less conventional heterocyclic electrophiles featuring distinct covalent addition mechanisms, including thiadiazole (21) and 3-bromo-4,5-dihydroisoxazole (22),^{26–28} were included, to assess mechanistic divergence from canonical

thiolate addition (Schemes 3 and 7). The 1,3-dipolar cycloaddition in Scheme 7 proceeded regioselectively, affording a single detectable isoxazoline regioisomer, consistent with established precedent for nitrile oxide additions to

Scheme 7. Synthesis of Compound 22

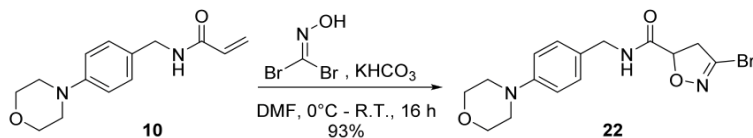


Table 1. Second Order Rate Constants for the Nonenzymatic Reaction between Warhead Substrates 2-22 and GSH

Cpd.	Warhead	k_2^{GSH} ($\text{M}^{-1} \text{min}^{-1}$)	Cpd.	Warhead	k_2^{GSH} ($\text{M}^{-1} \text{min}^{-1}$)
2		38.5 ± 0.2	12 ^a		0.379 ± 0.020
3		30.2 ± 0.2	13 ^a		1.80 ± 0.29
4		14.5 ± 0.3	14		72.7 ± 4.6
5		82.0 ± 0.5	15		0.439 ± 0.017
6		7.92 ± 0.01	16		30.7 ± 0.9
7		6670 ± 25	17		0.382 ± 0.017
8		53.3 ± 0.9	18		6.95 ± 0.08
9		38.2 ± 0.1	19		0.0792 ± 0.0025
10		0.787 ± 0.040	20		0.0380 ± 0.0020
11		0.104 ± 0.003	21 ^a		66.8 ± 0.6
			22		3.68 ± 0.14

^aCompeting pH effects were observed; reported values were measured at pH 7.4. See Table S1 for values expressed in $\text{M}^{-1}\text{s}^{-1}$.

electron-deficient alkenes.²⁹ Collectively, this library spans a wide range of electrophilic warheads used broadly in covalent drug discovery, enabling systematic evaluation of how intrinsic reactivity, warhead structure, and reaction mechanism impact GST-catalyzed GSH conjugation.

Intrinsic Reactivity

Intrinsic reactivity of warheads toward GSH has been previously reported,^{30–32} often as half-lives ($t_{1/2}$) or apparent pseudo-first order rate constants (k_{obs}). These values depend strongly on assay conditions, including the concentration of GSH and pH (i.e., concentration of the nucleophilic thiolate (GS^-) in solution), and are therefore not directly comparable

across studies. To enable internally consistent comparison across our library, intrinsic reactivity was determined as absolute second-order rate constants (k_2^{GSH}) under standardized conditions with explicit correction for pH.¹⁹ Reactions were performed under pseudo-first order conditions with excess GSH and k_{obs} values were converted to k_2^{GSH} by accounting for the fraction of thiolate in solution. Reaction progress was monitored spectrophotometrically for $\text{S}_{\text{N}}\text{Ar}$ and thiadiazole substrates and by HPLC for all other electrophiles. Representative LC-MS traces of warhead-GSH adducts are shown in the Supporting Information.

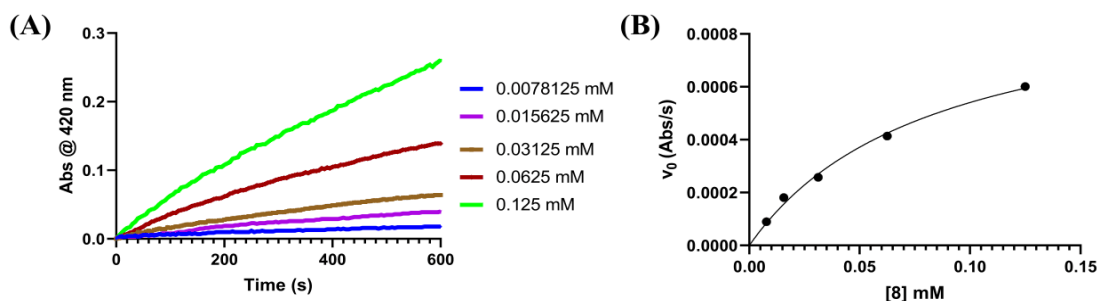


Figure 2. Kinetic evaluation of representative compound **8** with GSTA1 using direct spectrophotometric monitoring. (A) Product formation vs time curves from which initial rates (v_0) were determined. (B) Michaelis–Menten plot used to measure k_{cat} and K_M values. $k_{\text{cat}} = 31.1 \pm 1.6 \text{ min}^{-1}$, $K_M = 0.0833 \pm 0.0090 \text{ mM}$, $k_{\text{cat}}/K_M = (373 \pm 44) \times 10^3 \text{ M}^{-1}\text{min}^{-1}$. Assays were conducted using 0.05 mU/mL ($\approx 186 \text{ nM}$) GSTA1, at 25°C in phosphate buffer (pH 6.5).

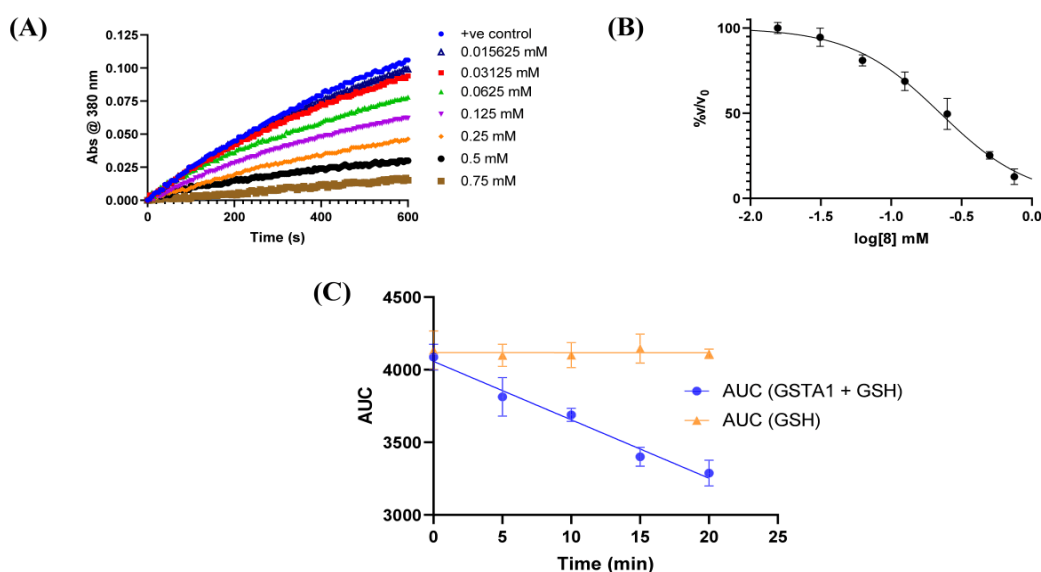


Figure 3. Kinetic evaluation of representative compound **8** with GSTA1 by competitive displacement of reporter substrate **2** and initial rate analysis by HPLC. (A) The rate of product formation of reporter substrate **2** decreases in the presence of competitive substrate **8**. (B) EC_{50} plot used to determine K_M . (C) Initial rate of substrate **8** consumption used to calculate k_{cat} . $k_{\text{cat}} = 27.9 \pm 2.6 \text{ min}^{-1}$, $K_M = 0.0920 \pm 0.0031 \text{ mM}$, $k_{\text{cat}}/K_M = (303 \pm 91) \times 10^3 \text{ M}^{-1}\text{min}^{-1}$. Assays were conducted using 0.25 mM of reporter substrate **2**, 0.05 mU/mL ($\approx 186 \text{ nM}$) GSTA1 in competitive displacement, 0.025 mU/mL ($\approx 93 \text{ nM}$) GSTA1 in HPLC rate determination, at 25°C in phosphate buffer (pH 6.5).

The resulting k_2^{GSH} values of our panel of 21 warheads span approximately 5 orders of magnitude (Table 1). The slowest reactivity was exhibited by attenuated warheads such as CFA (**19**) and BCB (**20**), whereas the fastest rates were obtained for certain $\text{S}_\text{N}\text{Ar}$ warheads (**5** and **7**), unsubstituted propiolamide (**14**), and thiadiazole (**21**). These data highlight the broad range of reactivity accessible across contemporary warheads, while also illustrating that mechanistically distinct warhead classes can have similar reactivity (e.g., **5** and **14**).

Overall, intrinsic reactivity trends were consistent with established steric and electronic effects.^{30,31} However, the dimethylaminomethyl (DMAM)-substituted acrylamides (**12** and **13**) deviated from simple steric expectations. Although α - or β -substitution typically attenuates acrylamide reactivity, neither analogue showed substantially reduced reactivity relative to the parent acrylamide (**10**), and compound **13** was modestly more reactive. This behavior is consistent with prior reports suggesting that protonation of the dimethylamino

substituent can stabilize the transition state of thiolate addition through electrostatic effects, partially offsetting steric hindrance.^{31,33} Interestingly, the thiadiazole warhead (**21**) also exhibited distinct pH-dependent behavior, displaying decreased reaction rates at higher pH (Figure S1). This inverse relationship suggests that thiolate concentration alone does not dictate reactivity and is consistent with a protonation-dependent step preceding thiolate addition. Although modest pH adjustments were used where necessary to enable practical measurement of slow-reacting substrates (accompanied by the requisite correction for thiolate concentration), k_2^{GSH} values for **12**, **13**, and **21** were determined under standardized biologically relevant conditions (pH 7.4) to ensure consistent comparison across the library. These intrinsic rate constants provide a quantitative benchmark of nonenzymatic electrophile reactivity for subsequent comparison with GST-catalyzed conjugation kinetics.

GSTA1 and GSTP1 Kinetics

Assay Design. Warhead substrates that exhibited a measurable absorbance change upon GSH conjugation (2–9, 21) were monitored directly by spectrophotometry under GST-catalyzed conditions. Initial rates were determined and used to generate Michaelis–Menten plots, from which k_{cat} and K_M were determined (Figure 2).³⁴

For electrophiles that did not produce a measurable spectral change upon GSH conjugation (10–20, 22) direct multi-concentration kinetic analysis by HPLC would have been prohibitively time intensive. We therefore determined K_M values through competitive displacement, relating the EC_{50} of displacement of the reporter substrate to K_M using the Cheng–Prusoff equation (Figure 3A,B).³⁵

CDNB was initially evaluated as the reporter substrate for this application; however, under our assay conditions it did not display purely competitive behavior with respect to our warhead substrates (Figure S3), consistent with previous reports.³⁶ This behavior likely reflects sparse occupation of the GST H-site by the small CDNB scaffold, permitting simultaneous binding of additional substrates.

To enable competitive kinetic analysis, compound 2 was instead employed as a scaffold-matched reporter substrate. Incorporation of the same drug-like core used throughout our library increased H-site occupancy and resulted in strict competitive behavior (Figure S4). K_M values were then determined from competitive displacement experiments, and k_{cat} values were subsequently calculated from initial rates measured at a single substrate concentration at or above K_M , using HPLC-based quantification (Figure 3C). As GST catalysis proceeds via a bisubstrate mechanism, all kinetic parameters reported represent apparent values determined at saturating GSH concentration (2.5 mM) (Figure S6).³⁷

Notably, both GSTA1 and GSTP1 retained full catalytic activity in the presence of elevated DMSO concentrations up to 20% (Figure S2), enabling the evaluation of compounds with limited aqueous solubility. In the competitive displacement assay, the concentration of the reporter substrate can be reduced, to allow testing of inhibitors at lower concentrations. This flexibility is particularly important, given the relatively high apparent K_M values of electrophilic GST substrates, which may otherwise require assay concentrations that exceed aqueous solubility limits.

To validate consistency between assay formats, compound 8 was evaluated with GSTA1 using both direct Michaelis–Menten kinetics and competitive displacement with HPLC quantification (Figures 2, 3 and S5). The resulting catalytic efficiencies are in close agreement within approximately 1.2-fold, supporting consistency between the two assay formats.

Warhead Structure–Activity Relationships. Across the warhead panel, catalytic efficiencies (k_{cat}/K_M) spanned 7 orders of magnitude (i.e., from 10^0 to $10^7 \text{ M}^{-1}\text{min}^{-1}$). Dissection of k_{cat} and K_M values (Table 2) revealed several SAR trends that were consistent across both GSTA1 and GSTP1 isozymes.

$S_N\text{Ar}$ warheads (2–9) were generally processed most efficiently, exhibiting high k_{cat} values and low K_M values, relative to other warhead classes. This preference reflects strong mechanistic compatibility of $S_N\text{Ar}$ with GST-catalyzed reactivity and that hydrophobic aryl electrophiles are particularly well accommodated within the H-site.³⁸ Importantly, these results suggest that this warhead class is especially susceptible to GST-mediated metabolism, both hepatically and extrahepatically. Interestingly, this preference also extends to

Table 2. Catalytic Parameters of Warhead Substrates 2–22 with GSTA1 and GSTP1^c

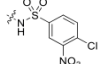
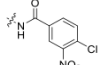
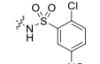
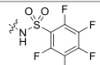
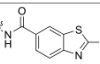
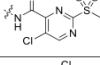
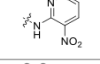
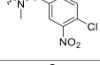
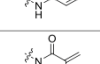
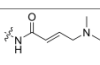
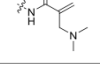
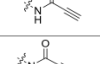
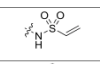
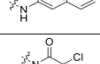
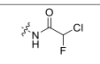
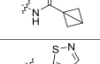
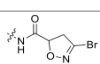
Cpd.	Warhead	$k_{\text{cat}}^{\text{app}}$ (min^{-1})		K_M^{app} (mM)		$k_{\text{cat}}/K_M^{\text{app}}$ ($\times 10^3 \text{ M}^{-1} \text{ min}^{-1}$)	
		GSTA1	GSTP1	GSTA1	GSTP1	GSTA1	GSTP1
2		89.3 ± 5.1	101 ± 18	0.176 ± 0.023	0.780 ± 0.280	506 ± 71	130 ± 5
3		38.7 ± 1.9	22.2 ± 1.2 ^a	0.731 ± 0.041	1.98 ± 0.22 ^a	53.0 ± 3.9	11.3 ± 0.7 ^a
4		16.9 ± 1.7	N.D.	0.451 ± 0.070	N.D.	37.4 ± 6.9	N.D.
5		167 ± 5	175 ± 17	0.0140 ± 0.0022	0.184 ± 0.022	11900 ± 1900	954 ± 61
6 ^a		15.3 ± 6.7	19.9 ± 7.7	5.47 ± 1.70	1.78 ± 0.45	2.79 ± 0.15	11.2 ± 0.3
7		N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
8		31.1 ± 1.6	31.4 ± 5.4	0.0833 ± 0.0090	0.167 ± 0.048	373 ± 44	188 ± 12
9		53.3 ± 3.0	41.2 ± 3.9	0.0434 ± 0.0049	0.102 ± 0.020	1230 ± 155	405 ± 85
10		3.12 ± 0.41	3.29 ± 0.32	1.84 ± 0.07	4.51 ± 0.14	1.69 ± 0.23	0.729 ± 0.074
11		4.08 ± 0.25	3.12 ± 0.54	0.310 ± 0.070	1.52 ± 0.19	13.2 ± 3.1	2.05 ± 0.44
12		0.963 ± 0.120	0.343 ± 0.073	15.2 ± 0.7	46.2 ± 5.5	0.0633 ± 0.0080	0.00743 ± 0.00100
13		5.42 ± 0.17	6.13 ± 0.39	7.23 ± 0.15	9.21 ± 0.27	0.751 ± 0.058	0.666 ± 0.047
14		16.6 ± 1.7	18.1 ± 1.0	2.15 ± 0.03	5.15 ± 0.24	7.75 ± 0.78	3.51 ± 0.24
15		0.850 ± 0.050	N.D.	4.26 ± 0.84	>10 ^b	0.200 ± 0.044	N.D.
16		11.2 ± 0.3	9.78 ± 0.75	0.570 ± 0.060	2.85 ± 0.22	19.7 ± 3.1	3.32 ± 0.37
17		4.71 ± 0.21	5.59 ± 0.15	0.0793 ± 0.0089	0.190 ± 0.006	67.3 ± 8.2	29.4 ± 2.1
18		3.19 ± 0.77	3.06 ± 0.11	1.28 ± 0.09	3.69 ± 0.65	2.49 ± 0.75	0.83 ± 0.15
19		3.72 ± 0.34	2.89 ± 0.23	0.550 ± 0.050	1.90 ± 0.31	6.76 ± 0.86	1.52 ± 0.27
20		1.62 ± 0.05	1.36 ± 0.07	2.41 ± 0.07	5.27 ± 0.19	0.675 ± 0.023	0.257 ± 0.016
21		N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
22		12.1 ± 0.2	14.8 ± 0.2	0.183 ± 0.025	0.360 ± 0.040	67.4 ± 7.6	41.1 ± 4.6

Table 2. continued

^aDue to solubility constraints, saturation could not be observed in Michaelis–Menten plots, so Lineweaver–Burk plots were used to estimate k_{cat} and K_{M} . ^bExact value could not be determined due to solubility constraints (See Supporting Information page S25). N.D.: Not determined. See Table S1 for values expressed in s^{-1} , μM , and $\text{M}^{-1}\text{s}^{-1}$. ^cCompounds 2–9 were evaluated using direct spectrophotometry. Compounds 10–22 were evaluated using competitive displacement and HPLC rate analysis.

dihydrobromoisoxazole (22) which features a similar addition–elimination mechanism, but with a nonaromatic heterocyclic ring structure. However, higher GST-mediated reactivity is generally observed for $\text{S}_{\text{N}}\text{Ar}$ warheads bearing *para*-positioned leaving groups (LG), and a lower susceptibility for those in which the leaving group is positioned *meta*- or *ortho*- to the scaffold (e.g., 2 vs 4 and 8), suggesting that LG positioning is key for mitigating the metabolic susceptibility of this warhead class.

Across all mechanistic classes, substitution pattern strongly influenced substrate engagement. Warheads bearing substitutions flanking the electrophilic center (e.g., 12 and 15) displayed the highest K_{M} and lowest k_{cat} values with both isozymes. Even distal substitutions such as α -DMAM (13) resulted in increased K_{M} values as compared to unsubstituted acrylamide 10. This suggests that steric hindrance can impede productive binding and reaction with GSTs, independently of intrinsic reactivity. This effect may be relevant to covalent drugs such as afatinib (β -DMAM acrylamide) and acalabrutinib (2-butynamide), which display improved metabolic stability compared to acrylamide-containing drugs such as ibrutinib.^{21,39}

Electronic modification also modulated GST susceptibility. Incorporation of a sulfonamide linkage generally improved binding affinity and reactivity 2- to 3-fold compared to amide

linkages (e.g., 2 vs 3 and 10 vs 16), possibly due to additional hydrogen bonding or more productive warhead geometry/positioning. Fluorination on the warhead similarly tended to improve binding affinity ~ 3 -fold across matched pairs (e.g., 10 vs 11 and 18 vs 19), likely due to increased hydrophobicity. This resulted in comparable or increased susceptibility to GSTs compared to nonfluorinated warheads, despite the decreased intrinsic reactivity conferred by fluorine substitution. These combined effects underlie the exceptionally high $k_{\text{cat}}/K_{\text{M}}$ ($\sim 10^6 \text{ M}^{-1}\text{min}^{-1}$) of pentafluorobenzenesulfonamide (5) with both isozymes, suggestive of high metabolic susceptibility.

Scaffold-level modification adjacent to the warhead also proved consequential to GST catalysis. *N*-Methylation on substrate 9 did not influence intrinsic reactivity (Table 1) compared to its nonmethylated analogue (2) yet resulted in a 4- and 8-fold lower K_{M} value with GSTA1 and GSTP1, respectively. Although methylation adjacent to the warhead has previously been proposed to decrease GST engagement,⁴⁰ our results indicate that its impact is highly scaffold- and/or warhead-dependent.

Two warheads, methylsulfonylpyrimidine (7) and thiadiazole (21), displayed no measurable reaction with either GST in our assay. For compound 7, intrinsic reactivity with GSH was notably higher (Table 1) than all other warheads, suggesting that rapid solution-phase conjugation with excess GSH can outcompete enzyme-mediated catalysis beyond a threshold of intrinsic reactivity. Conversely, 21 displayed intrinsic reactivity similar to $\text{S}_{\text{N}}\text{Ar}$ warheads for which GST catalysis was readily measurable. Given its distinct protonation-dependent reaction mechanism, these results suggest mechanistic incompatibility with the canonical thiolate addition chemistry that GSTs are known to mediate. Thus, thiadiazole represents a unique case in which high intrinsic reactivity does not translate to enhanced GST-mediated metabolism.

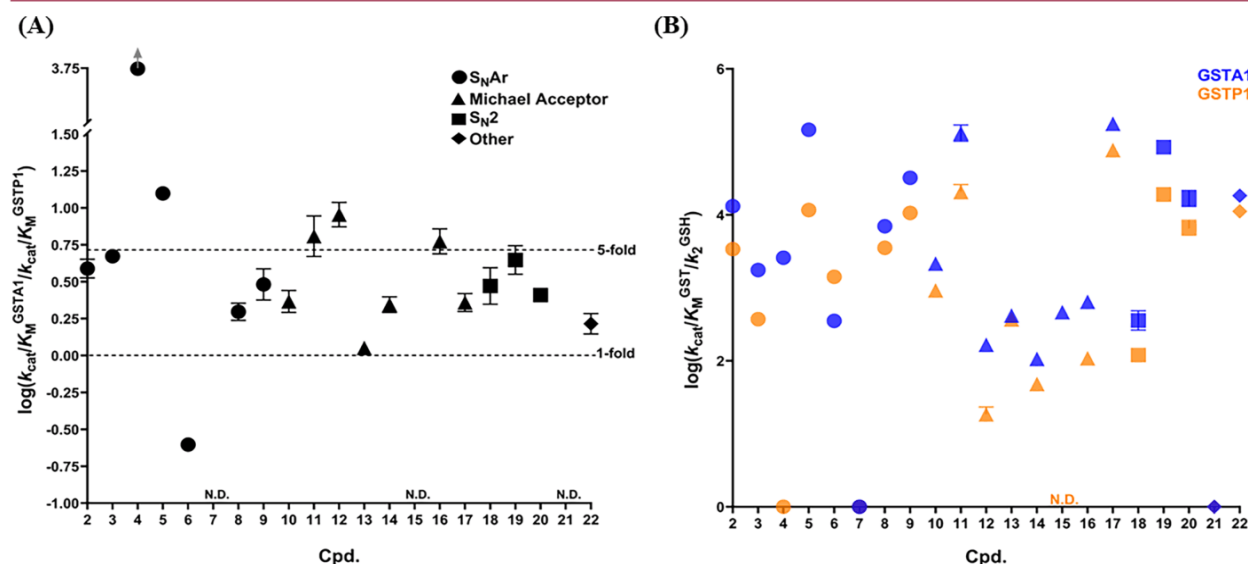


Figure 4. Comparisons of second order rate constants. (A) Ratios of catalytic efficiencies ($k_{\text{cat}}/K_{\text{M}}$) with GSTA1 and GSTP1, reflecting isozyme selectivity. 1-Fold and 5-fold selectivity is marked by dotted lines. (B) Ratios of catalytic efficiency with GSTA1 (blue) and GSTP1 (orange) to intrinsic reactivity (k_2^{GSH}) indicating fold acceleration provided by enzyme for GSH addition to warhead. Classes of warheads are differentiated by symbols (circle = $\text{S}_{\text{N}}\text{Ar}$, triangle = Michael acceptor, square = $\text{S}_{\text{N}}2$, diamond = other). N.D.: not determined.

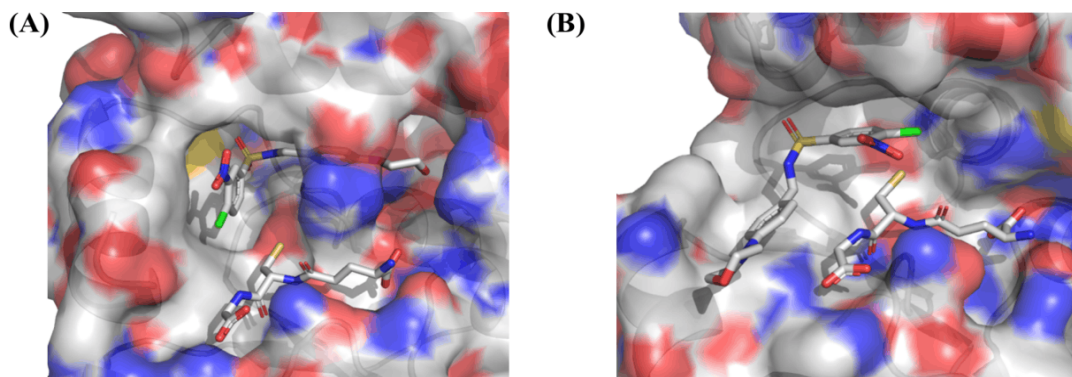


Figure 5. Molecular model of the predicted binding pose of compound 2 and GSH with (A) GSTA1 and (B) GSTP1.

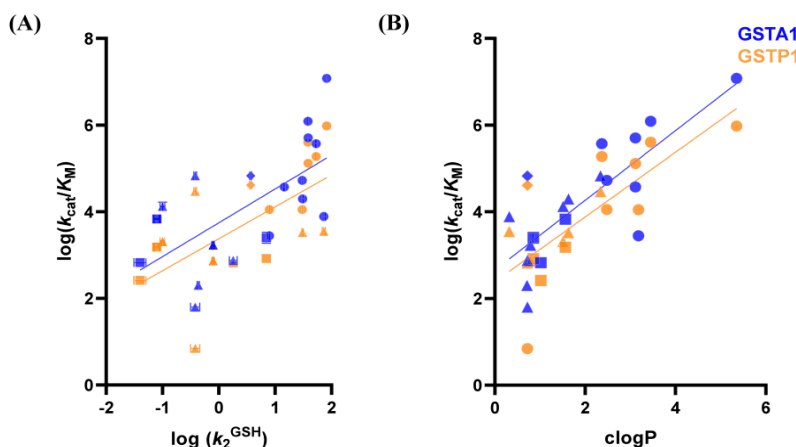


Figure 6. Correlation of catalytic efficiency ($\log(k_{\text{cat}}/K_{\text{M}})$) with (A) intrinsic reactivity ($\log(k_2^{\text{GSH}})$) and (B) lipophilicity (clogP) for GSTA1 and GSTP1. Linear regression (least-squares) analysis yielded the following relationships: (A) GSTA1: $Y = 0.78X + 3.74$ ($R^2 = 0.40$; Spearman $\rho = 0.65$, $p < 0.05$). GSTP1: $Y = 0.73X + 3.37$ ($R^2 = 0.41$; Spearman $\rho = 0.71$, $p < 0.05$). (B) GSTA1: $Y = 0.80X + 2.66$ ($R^2 = 0.62$; Spearman $\rho = 0.69$, $p < 0.05$). GSTP1: $Y = 0.75X + 2.39$ ($R^2 = 0.57$; Spearman $\rho = 0.69$, $p < 0.05$). Compounds for which $k_{\text{cat}}/K_{\text{M}}$ could not be determined (4, 7, 15 (for GSTP1), and 21) were excluded from correlation analyses. Data for GSTA1 and GSTP1 are shown in blue and orange, respectively. Classes of warheads are differentiated by symbols (circle = $\text{S}_{\text{N}}\text{Ar}$, triangle = Michael acceptor, square = $\text{S}_{\text{N}}2$, diamond = other).

Across the library, isozyme selectivity was generally modest, with most substrates displaying a 2- to 4-fold preference for GSTA1 over GSTP1 (Figure 4A). Selectivity was driven by lower K_{M} values rather than higher k_{cat} values, consistent with stronger scaffold engagement within the more defined H-site of GSTA1, shaped by its C-terminal α -helix, relative to the more solvent exposed H-site of GSTP1 (Figure 5).⁴¹

However, several warheads showed ≥ 5 -fold selectivity for GSTA1. These include fluorinated warheads 5, 11, and 19, suggesting that fluorination not only increases binding affinity for both enzymes, but also increases propensity for hepatic metabolism. Compound 12 (β -DMAM acrylamide) displayed nearly 10-fold selectivity for GSTA1, indicating that even though β -substitution of α,β -unsaturated amides reduces binding affinity to both isozymes, hepatic metabolism through GSTA1 may be the major GSH conjugation pathway for these warheads. In contrast to its matched pair 12, compound 13 (α -DMAM acrylamide) showed no preference for either isozyme. Given that the substrate scaffold tends to favor GSTA1, the α -DMAM substitution decreases productive binding and reaction with GSTA1, which may be due to steric hindrance between

the α -substituent and the H-site in GSTA1, forcing the scaffold into a more solvent-exposed pose (Figure S9).

Remarkably, compound 4, whose $\text{S}_{\text{N}}\text{Ar}$ leaving group is positioned *ortho* to the core scaffold, displayed moderate turnover with GSTA1, but no measurable reaction with GSTP1. For this compound, a $k_{\text{cat}}/K_{\text{M}}$ value equal to the experimentally defined lower detection limit ($\sim 7 \text{ M}^{-1} \text{ min}^{-1}$) was assigned to GSTP1, to allow visualization of the highest preference for GSTA1 in the series in Figure 4A. Although molecular modeling did not fully rationalize this divergence, the result indicates that subtle geometric perturbation can abolish turnover in one isozyme while preserving activity in another, with implications for spatially selective metabolism.

In contrast, bicyclic $\text{S}_{\text{N}}\text{Ar}$ substrate 6 was the only compound favoring GSTP1 over GSTA1 (~ 4 -fold). Interestingly, modeling of 6 in GSTA1 shows the warhead buried deep into the H-site, distant from the GSH thiol (Figure S10), consistent with an unproductive binding mode due to a rigid warhead structure. Together, compounds 4 and 6 demonstrate that modest structural variation within even the most GST-compatible warhead class can decrease metabolic susceptibility.

The isoform-dependent behavior of enzyme-mediated GSH-conjugation parallels CYP450 isozyme oxidation selectivity, where distinct isoforms exhibit differential substrate selectivity and metabolic profiles.⁴² Similarly, GSTA1 and GSTP1 display distinct preferences for electrophilic substrates, reinforcing that GSH conjugation is not a uniform detoxification pathway but an isoform-dependent process.

Comparison of catalytic efficiency (k_{cat}/K_M) with intrinsic reactivity (k_2^{GSH}) defined an enzymatic efficiency ratio (EER) for each warhead (Figure 4B),⁴³ which represents the magnitude of acceleration the enzyme provides relative to solution-phase chemistry. For most substrates, GSTs enhanced GSH addition by approximately 4 orders of magnitude. Although substantial, this acceleration is modest relative to substrate-optimized enzymes,⁴⁴ consistent with the broad substrate tolerance of detoxification enzymes. However, in some cases, lower EER values reflected high intrinsic reactivity rather than weak enzymatic turnover (e.g., 14 and 16).

Next, we explored the extent to which catalytic efficiency (k_{cat}/K_M) correlated with key physicochemical properties such as intrinsic reactivity (k_2^{GSH}) (Figure 6A) and calculated lipophilicity ($\log P$) as a proxy for hydrophobicity (Figure 6B). Catalytic efficiency generally increases with both intrinsic reactivity and lipophilicity, although neither parameter alone fully accounts for the observed variability across the panel. A positive correlation was observed between $\log(k_{\text{cat}}/K_M)$ and $\log(k_2^{\text{GSH}})$ for both isozymes, indicating that more intrinsically reactive electrophiles generally exhibit higher catalytic efficiency. However, intrinsic reactivity accounted for only ~40% of the variance, suggesting that electrophilicity alone is insufficient to predict GST turnover. Lipophilicity ($\log P$) displayed a stronger relationship with catalytic efficiency, explaining ~60% of the variance for both GSTA1 and GSTP1. This relationship tracks with vinylpyridine (17) being the best substrate for GSTA1 and GSTP1 within the Michael acceptor class, despite having the lowest intrinsic reactivity, reflecting improved engagement within the hydrophobic H-site owing to its pyridine ring.

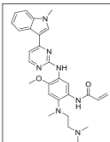
To evaluate whether intrinsic reactivity and lipophilicity contribute independently to catalytic efficiency, multiple linear regression analysis was performed (Figures S7 and S8). The combined model yielded $R^2 \approx 0.7$ for both enzymes. Both lipophilicity and intrinsic reactivity retained positive coefficients and remained statistically significant ($p < 0.05$), indicating that each parameter accounts for variance not captured by the other. These results suggest that, within the measurable catalytic regime, GST-mediated turnover reflects an interplay between electrophilicity and lipophilicity, with lipophilicity emerging as the stronger predictor in this data set.

Compounds for which k_{cat}/K_M could not be reliably determined, due to either undetectable enzymatic turnover or solubility limitations, were excluded from regression analyses. Notably, substrates (4, 7, 21) that showed no measurable enzymatic reaction despite possessing k_2^{GSH} and $\log P$ values comparable to reactive warheads, together with the substantial scatter observed within the measurable data set, indicate that intrinsic electrophilicity and lipophilicity do not fully account for GST-mediated turnover. These findings suggest that productive catalysis, in part, requires appropriate substrate orientation and complementarity within the H-site and effective transition-state stabilization, features not captured by conventional physicochemical descriptors. Thus, the linear models define trends within the productive catalytic regime but

do not capture structural features that can shift substrates outside this regime entirely.

GST-Mediated Conjugation of the Covalent EGFR Inhibitor Osimertinib. To assess whether the trends observed with model substrates extend to a complex drug scaffold, we evaluated the FDA-approved covalent EGFR inhibitor osimertinib,⁴⁵ which contains an *N*-phenylacrylamide warhead, which is more intrinsically reactive than *N*-alkyl acrylamides (Table 3).^{19,30,46} Osimertinib was measurably

Table 3. Kinetic Parameters of Osimertinib

	$k_{\text{cat}}^{\text{app}}$ (min ⁻¹)		K_M^{app} (mM)		$k_{\text{cat}}/K_M^{\text{app}}$ ($\times 10^3 \text{ M}^{-1} \text{ min}^{-1}$)		k_2^{GSH} ($\text{M}^{-1} \text{ min}^{-1}$)
	GSTA1	GSTP1	GSTA1	GSTP1	GSTA1	GSTP1	
	12.3 ± 0.2	7.10 ± 0.24	5.14 ± 0.09	18.7 ± 0.5	2.41 ± 0.14	0.380 ± 0.020	27.8 ± 1.3

turned over by both isozymes. However, relative to the *N*-alkyl acrylamide analogue on the morpholinobenzylamine scaffold (10), osimertinib is a comparatively poor substrate, primarily due to its high K_M values. Catalytic efficiency favored GSTA1 (~7-fold), with GSTA1 providing ~100-fold acceleration relative to intrinsic GSH reactivity, whereas GSTP1 provided only ~15-fold acceleration.

Despite possessing both high intrinsic reactivity and lipophilicity ($\log P = 4.32$), features that correlated with increased GST turnover within the congeneric warhead series (Figure 6), osimertinib deviated from these trends. Molecular modeling (Figure S11) suggests that substitutions proximal to the warhead restrict productive binding orientations, leaving much of the scaffold solvent exposed within both isozymes. These results reinforce the notion that scaffold context, not electrophilicity or lipophilicity alone, governs susceptibility for GST-mediated conjugation.

Consistent with this low GST turnover, osimertinib is cleared predominantly via CYP-oxidation, with GSH conjugation reported as a secondary metabolic pathway, particularly in hepatocytes.²¹ The observed preference for GSTA1 over GSTP1 aligns with reports implicating hepatic GSTA1 in resistance phenotypes,⁴⁷ supporting isoform-dependent metabolism of this drug. Interestingly, deuteration of the β -hydrogens on the acrylamide of osimertinib has been shown to decrease CYP-oxidation,⁴⁸ indicating that isotope effects related to warheads can impact metabolism and, although beyond the scope of this work, should be evaluated in the context of GSTs as well.

From a pharmacokinetic perspective, quantification of both GST-catalyzed and intrinsic GSH reactivity provides insight into the kinetic competition between drug-target engagement and detoxification pathways. For osimertinib, the value of k_{inact}/K_I with EGFR is $\sim 7.62 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$,⁴⁹ which exceeds intrinsic GSH reactivity by approximately 5 orders of magnitude and surpasses GSTA1 and GSTP1 catalytic efficiencies by roughly 3 and 4 orders of magnitude, respectively. This substantial kinetic separation favors target modification over metabolic modification under typical conditions. However, the balance between these processes would also depend on tissue-specific enzyme abundance and GSH concentration, particularly in environments characterized by elevated GST expression.

Stability in Human Liver Cytosol

The *in vivo* stability of drugs is often modeled through surrogate assays in either whole blood or hepatocytes, representing extrahepatic metabolism (e.g., due to GSTP1) and hepatic metabolism (e.g., due to GSTA1 and CYP450) respectively.⁷ However, in both systems multiple competing processes can contribute to overall stability. In whole blood, metabolic outcomes reflect not only intracellular enzymatic activity (e.g., erythrocyte-localized GSTP1), but also compound partitioning between plasma and cellular components, as well as potential nonenzymatic reactivity. Similarly, hepatocytes integrate cytosolic conjugation and microsomal oxidation pathways, making it difficult to isolate the specific contribution of GST-catalyzed metabolism.

To determine whether trends observed with recombinant GST translate to a more physiologically relevant system, representative compounds spanning the full dynamic range of GSTA1 catalytic efficiencies (including high, intermediate, and minimal turnover cases) were evaluated for stability in pooled human liver cytosol. Hepatic cytosolic metabolism is dominated by GST activity, with GSTA1 representing the most abundant isoform,^{7,12} minimizing contributions from other metabolic pathways and enabling direct comparison with recombinant $k_{\text{cat}}/K_{\text{M}}^{\text{GSTA1}}$ values.

A strong correlation ($R^2 = 0.9$) was observed between recombinant $k_{\text{cat}}/K_{\text{M}}^{\text{GSTA1}}$ and cytosolic GSH conjugation rates (expressed as $1/t_{1/2}$), with higher catalytic efficiency corresponding to lower stability (Figure 7). In contrast,

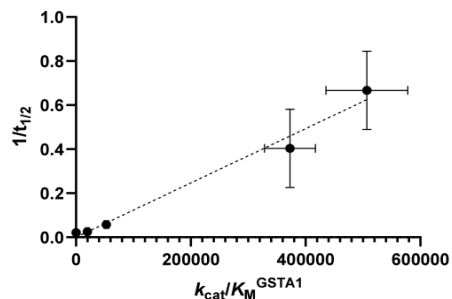


Figure 7. Correlation ($R^2 = 0.9$) of stability in human liver cytosol ($1/t_{1/2}$) with GSTA1 catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}^{\text{GSTA1}}$) for compounds 2, 3, 8, 16, 21.

intrinsic reactivity (k_2^{GSH}) showed no meaningful relationship with cytosolic turnover, and $\log P$ values displayed only a moderate correlation ($R^2 = 0.6$) (Figure S13). Although absolute rates differed from purified enzyme measurements, the preservation of substrate rank order clearly indicates that recombinant GST kinetics capture susceptibility to hepatic GSH conjugation more effectively than intrinsic reactivity and hydrophobicity alone.

To confirm that cytosolic clearance reflected GST catalysis rather than nonspecific thiol chemistry, incubations were repeated in the presence of ethacrynic acid, a pan-GST inhibitor (Figure S12).⁵⁰ Under these inhibition conditions, substrate half-lives were no longer well described by $k_{\text{cat}}/K_{\text{M}}^{\text{GSTA1}}$ and instead aligned with k_2^{GSH} (Figure S13), consistent with suppression of GSTA1 activity. Furthermore, the magnitude of enzymatic acceleration, defined as the ratio of half-lives in the absence versus the presence of inhibitor, correlated with $k_{\text{cat}}/K_{\text{M}}^{\text{GSTA1}}/k_2^{\text{GSH}}$ (i.e., EER) (Figure S13).

Together, these results demonstrate that GST catalytic efficiency quantitatively predicts the enzyme-driven component of hepatic cytosolic clearance, highlighting the relevance of measuring GST reactivity, as a metric of metabolic stability, in drug development campaigns.

Implications for Warhead Design

While increased metabolic stability is most often desired in drug development, there are cases in which rapid metabolism and clearance may be required, to mitigate off-target effects.⁵¹ Either way, the SAR trends discussed above suggest several warhead design principles for tuning (i.e., either decreasing or increasing) susceptibility to GST-mediated metabolism during covalent drug development (Figure 8). $S_{\text{N}}\text{Ar}$ electrophiles were

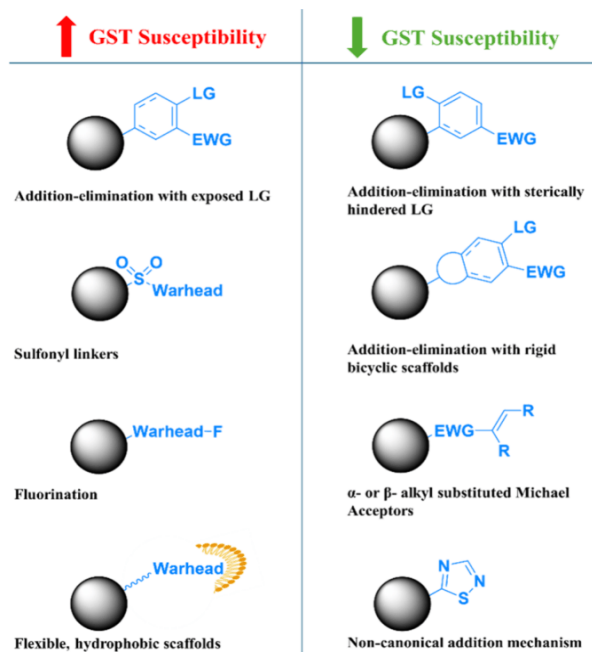


Figure 8. Structural features influencing susceptibility of electrophilic warheads to GST-catalyzed GSH conjugation. LG = leaving group, EWG = electron withdrawing group.

consistently among the most efficient GST substrates, indicating that aryl electrophiles undergoing addition–elimination mechanisms are particularly compatible with GST-catalyzed glutathione conjugation. However, structural modifications within this class can significantly alter enzyme susceptibility. For example, *ortho*-positioning of the leaving group substantially reduced GSTP1 turnover, while incorporation of a rigid bicyclic motif decreased catalytic efficiency with GSTA1, demonstrating that relatively subtle geometric perturbations can disrupt productive substrate engagement within the enzyme H-site.

Within the Michael acceptor and $S_{\text{N}}2$ classes, moderate GST susceptibility was observed, which may partly explain their broad application. However, substitution patterns surrounding the electrophilic center strongly influenced catalytic efficiency. β -Substitution of α,β -unsaturated amides consistently decreased turnover with both isozymes, likely through steric effects that impair productive binding, whereas α -substitution selectively reduced GSTA1 engagement. In contrast, the use of

a vinyl pyridine, fluorination and sulfonamide linkages on warheads generally enhanced GST-mediated conjugation through increased binding affinity. Notably, certain highly reactive electrophiles (e.g., thiadiazole) exhibited negligible GST turnover despite substantial intrinsic reactivity, suggesting that warheads operating outside canonical thiolate addition mechanisms may evade GST-mediated detoxification.

These observations highlight important considerations for covalent warhead design. New electrophiles are often selected or optimized primarily to reduce intrinsic chemical reactivity. However, reduced solution reactivity does not necessarily translate to reduced metabolic susceptibility, if the same modifications enhance binding within the GST H-site (e.g., α -fluorination of acetamides and acrylamides). While extremely high intrinsic reactivity is obviously unfavorable, moderate attenuation of electrophilicity may not improve metabolic stability, if GST engagement is simultaneously strengthened. Our findings therefore suggest that evaluating GST susceptibility alongside intrinsic reactivity, during early covalent warhead optimization, is crucial. The assays described herein provide a practical framework for integrating such screening into drug discovery campaigns.

CONCLUSIONS

This work presents the first systematic SAR study of a diverse library of electrophilic warheads as substrates for hepatic (GSTA1) and extrahepatic (GSTP1) glutathione S-transferases, enabling quantitative assessment of their GST-mediated metabolic liability. Characterization of individual k_{cat} and K_{M} parameters was enabled by a competitive displacement assay, whose broader applicability was demonstrated through the profiling of the covalent EGFR inhibitor osimertinib. While increased intrinsic electrophilicity and hydrophobicity were generally associated with enhanced GST-mediated conjugation, these parameters were not solely predictive. Instead, local scaffold features surrounding the electrophilic center, governing productive binding and orientation within the H-site, substantially influenced catalytic efficiency. Importantly, $k_{\text{cat}}/K_{\text{M}}$ values measured for recombinant GSTA1 strongly correlated with GSH conjugation rates in pooled human liver cytosol, whereas intrinsic reactivity and lipophilicity showed less meaningful relationships.

Collectively, these findings establish GST catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) as a more informative predictor of hepatic GSH conjugation than intrinsic reactivity and hydrophobicity alone. Most importantly, they also demonstrate that GST susceptibility is structurally tunable. By integrating intrinsic reactivity, enzyme kinetics, isozyme selectivity, and hepatic validation, this work provides a practical, quantitative framework to guide warhead selection and modification strategies aimed at modulating GST-mediated clearance in covalent drug discovery.

EXPERIMENTAL SECTION

Synthesis

General Remarks. All reagents and solvents were purchased from commercial sources and used without further purification. Osimertinib (Catalog No. HY-15772) and ethacrynic acid (Catalog No. HY-B1640) were purchased from MedChemExpress. Anhydrous solvents were obtained using a Phoenix Solvent Dispensing System (JC Meyer Solvent Systems) with neutral alumina-packed columns. Thin layer chromatography was performed using EMD aluminum-backed silica 60 F254-coated plates and were visualized using either UV-light (254

nm), KMnO_4 , Hanessian's stain, or ninhydrin stain. Column chromatography was carried out using standard flash technique with silica (Siliaflash-P60, 230–400 mesh Silicycle) under compressed air pressure. ^1H NMR spectra were obtained at 300, 400, 500, or 600 MHz, ^{13}C NMR spectra were obtained at 75, 100, 125, or 150 MHz, and ^{19}F -NMR spectra were obtained at 283 MHz on Bruker instruments. NMR chemical shifts (δ) are reported in ppm and are calibrated against residual solvent signals of CDCl_3 , $\text{DMSO}-d_6$, or CD_3OD . Spectra were processed using MNOVA 14.1.2 (Mestrelab Research S.L., Santiago de Compostela, Spain). High resolution mass spectrometry (HRMS) was performed using a quadrupole time-of-flight (QTOF) or magnetic sector analyzer and electrospray ionization (ESI) or electron impact (EI), respectively. All found m/z values were within 3 mmu of the calculated value (generally <10 ppm discrepancy) unless otherwise noted. HPLC traces were collected on a Gilson-Mandel GXP271 with UV detection at 214 and 254 nm (Phenomenex Luna, 150 mm $\times$ 4.6 mm, 30 min, 1.5 mL/min flow rate, 5–55% (20 min method) or 5–95% (20 or 30 min method), MeCN with 0.1% TFA in H_2O with 0.1% TFA. All final compounds were $>95\%$ pure by HPLC analysis. LCMS traces were collected on a Shimadzu with UV detection at 254 nm (Agilent Eclipse Plus C18, 3.5 μm , 4.6 mm $\times$ 150 mm, 1.0 mL/min, 5–95% MeCN with 0.05% formic acid in H_2O with 0.05% formic acid, 20 min method). No unexpected, new, or significant hazards are associated with this work.

4-Chloro-N-(4-morpholinobenzyl)-3-nitrobenzenesulfonamide (2). To a solution of 4-(morpholinophenyl)methanamine (**1**) (0.20 g, 1.04 mmol, 1.0 equiv) and NEt_3 (0.30 mL, 2.10 mmol, 2.0 equiv) in anhydrous DCM (10 mL) at 0°C under a nitrogen atmosphere, was added 4-chloro-3-nitrobenzenesulfonyl chloride (0.30 g, 1.20 mmol, 1.1 equiv). The reaction was allowed to gradually warm to R.T. over 3 h and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3 $\times$ 25 mL) and brine (50 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (10% EtOAc/DCM, R_f = 0.41, dry loaded using Celite) to give the title compound as a yellow powder (0.27 g, 60%). ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 8.43 (br s, 1H), 8.15 (d, J = 1.9 Hz, 1H), 7.87 – 7.43 (m, 2H), 6.94 (d, J = 8.7 Hz, 2H), 6.67 (d, J = 8.7 Hz, 2H), 3.98 (s, 2H), 3.69 – 3.66 (m, 4H), 2.99 – 2.96 (m, 4H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 150.7, 147.2, 141.9, 133.2, 131.7, 129.3, 127.2, 124.5, 114.9, 66.5, 48.7, 46.4. HRMS (ESI) calc'd for $\text{C}_{17}\text{H}_{18}\text{ClN}_3\text{O}_5\text{SNa}$ [$\text{M} + \text{Na}$] $^+$: 434.0553, found: 434.0558.

4-Chloro-N-(4-morpholinobenzyl)-3-nitrobenzamide (3). To a solution of **1** (0.20 g, 1.04 mmol, 1.0 equiv) and 4-chloro-3-nitrobenzoic acid (0.20 g, 1.20 mmol, 1.1 equiv) in anhydrous DCM (6 mL) at 0°C under a nitrogen atmosphere, was added a solution of DCC (0.30 g, 1.20 mmol, 1.1 equiv) and DMAP (~1 mg, cat.) in anhydrous DCM (4 mL) dropwise. The reaction was allowed to gradually warm to R.T. over 3 h and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3 $\times$ 25 mL) and brine (50 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (50% EtOAc/DCM, R_f = 0.56, dry loaded using Celite) to give the title compound as a pale-yellow powder (0.32 g, 78%). ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 9.31 (t, J = 5.8 Hz, 1H), 8.58 (d, J = 2.1 Hz, 1H), 8.21 (dd, J = 8.4, 2.1 Hz, 1H), 7.95 (d, J = 8.4 Hz, 1H), 7.25 (d, J = 8.6 Hz, 2H), 6.95 (d, J = 8.6 Hz, 2H), 4.44 (d, J = 5.8 Hz, 2H), 3.77 – 3.76 (m, 4H), 3.11 – 3.09 (m, 4H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ 163.0, 150.2, 147.3, 134.4, 132.4, 131.9, 129.5, 128.4, 127.8, 124.4, 115.0, 66.0, 48.6, 42.4. HRMS (ESI) calc'd for $\text{C}_{18}\text{H}_{19}\text{ClN}_3\text{O}_4$ [$\text{M} + \text{H}$] $^+$: 376.1053, found: 376.1036.

2-Chloro-N-(morpholinobenzyl)-5-nitrobenzenesulfonamide (4). To a solution of 2-chloro-5-nitrobenzenesulfonyl chloride (0.20 g, 0.78 mmol, 1.0 equiv) and **1** (0.20 g, 1.04 mmol, 1.3 equiv) in anhydrous DCM (10 mL) in a flame-dried flask under a nitrogen atmosphere at 0°C was added triethylamine (0.30 mL, 2.20 mmol, 2.1 equiv). The reaction mixture was allowed to gradually warm to room temperature and stirred overnight. The reaction was diluted with DCM (20 mL) and washed with brine (2 $\times$ 15 mL), before

being dried over MgSO_4 , filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (25% EtOAc/hex, $R_f = 0.38$, dry loaded using Celite) to obtain the title compound as a light-yellow powder (0.20 g, 63%). $^1\text{H NMR}$ (600 MHz, $\text{DMSO}-d_6$) δ 8.67 (t, $J = 5.9$ Hz, 1H), 8.27 (dd, $J = 8.7, 2.8$ Hz, 1H), 8.23 (d, $J = 2.8$ Hz, 1H), 7.78 (d, $J = 8.7$ Hz, 1H), 6.95 (d, $J = 8.7$ Hz, 2H), 6.61 – 6.58 (m, 2H), 4.07 (d, $J = 5.9$ Hz, 2H), 3.69 – 3.67 (m, 4H), 2.94 – 2.92 (m, 4H). $^{13}\text{C NMR}$ (150 MHz, $\text{DMSO}-d_6$) δ 155.2, 150.8, 145.3, 142.4, 138.2, 134.2, 133.0, 132.8, 131.5, 130.4, 130.3, 119.4, 71.2, 53.3, 51.2. **HRMS** (ESI) calc'd for $\text{C}_{17}\text{H}_{18}\text{N}_3\text{O}_5\text{SClNa}$ [$\text{M} + \text{Na}$] $^+$: 434.0553, found: 434.0573.

2,3,4,5,6-Pentafluoro-N-(4-morpholinobenzyl)-benzenesulfonamide (5). To a solution of **1** (0.10 g, 0.52 mmol, 1.0 equiv) and DIPEA (0.20 mL, 1.04 mmol, 2.0 equiv) in anhydrous DCM (10 mL) at 0 °C under a nitrogen atmosphere, was added pentafluorobenzenesulfonyl chloride (0.08 mL, 0.52 mmol, 1.0 equiv) dropwise. The reaction was stirred for 1 h at 0 °C and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3 $\times$ 25 mL) and brine (50 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (50% EtOAc/Hex, $R_f = 0.53$, dry loaded using Celite) to give the title compound as a white powder (0.15 g, 70%). $^1\text{H NMR}$ (600 MHz, $\text{DMSO}-d_6$) δ 9.16 (t, $J = 6.3$ Hz, 1H), 7.07 (d, $J = 8.7$ Hz, 2H), 6.79 (d, $J = 8.7$ Hz, 2H), 4.18 (d, $J = 6.3$ Hz, 2H), 3.76 – 3.75 (m, 4H), 3.05 – 3.04 (m, 4H). $^{13}\text{C NMR}$ (150 MHz, $\text{DMSO}-d_6$) δ 150.8, 144.7, 143.7, 143.0, 142.0, 138.4–138.2 (9 apparent m), 136.7–136.5 (apparent m), 129.3, 126.5, 117.8–117.6 (apparent m), 114.6, 66.5, 48.6, 46.4. $^{19}\text{F NMR}$ (283 MHz, $\text{DMSO}-d_6$) δ -137.20, -149.13, -160.77. **HRMS** (ESI) calc'd for $\text{C}_{17}\text{H}_{16}\text{F}_5\text{N}_2\text{O}_3\text{S}$ [$\text{M} + \text{H}$] $^+$: 423.0796, found: 423.0785.

2-Chloro-N-(4-morpholinobenzyl)benzo[d]thiazole-6-carboxamide (6). To a solution of **1** (0.20 g, 1.04 mmol, 1.0 equiv) and 2-chloro-1,3-benzothiazole-6-carboxylic acid (0.22 g, 1.04 mmol, 1.0 equiv) in anhydrous MeCN (10 mL) at R.T. under a nitrogen atmosphere, were added TCFH (0.35 g, 1.25 mmol, 1.2 equiv) and NMI (0.29 mL, 3.64 mmol, 3.5 equiv). The reaction was stirred for 16 h at which point a precipitate had formed. The precipitate was collected by vacuum filtration and washed with H_2O (3 $\times$ 10 mL) and Hexanes (3 $\times$ 10 mL) to give the title compound as a yellow solid (0.26 g, 63%) which was used without further purification. $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 8.30 (s, 1H), 7.96 (d, $J = 8.5$ Hz, 1H), 7.81 (d, $J = 8.5$ Hz, 1H), 7.29 (d, $J = 8.6$ Hz, 2H), 6.91 (d, $J = 8.6$ Hz, 2H), 6.35 (t, $J = 5.4$ Hz, 1H), 4.59 (d, $J = 5.4$ Hz, 2H), 3.87 – 3.85 (m, 4H), 3.16 – 3.15 (m, 4H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 166.1, 155.9, 152.9, 151.0, 136.5, 132.0, 129.2, 128.9, 125.0, 122.8, 120.9, 115.9, 66.8, 49.2, 44.0. **HRMS** (ESI) calc'd for $\text{C}_{19}\text{H}_{19}\text{ClN}_3\text{O}_3\text{S}$ [$\text{M} + \text{H}$] $^+$: 388.0881, found: 388.0887.

5-Chloro-2-(methylthio)-N-(4-morpholinobenzyl)pyrimidine-4-carboxamide (7a). To a solution of 5-chloro-2-methylthiopyrimidine-6-carboxylic acid (0.21 g, 1.04 mmol, 1.0 equiv) in anhydrous THF (5 mL) in a flame-dried flask under a nitrogen atmosphere, was added HATU (0.60 g, 1.58 mmol, 1.5 equiv) and DIPEA (0.74 mL, 4.16 mmol, 4.0 equiv). The reaction was stirred for 15 min before **1** (0.20 g, 1.04 mmol, 1.0 equiv) was added and stirred at R.T. overnight. The reaction was diluted with water (25 mL) and extracted with DCM (3 $\times$ 25 mL). The organic phase was washed with brine (2 $\times$ 25 mL), before being dried over MgSO_4 , filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (75% EtOAc/hex, $R_f = 0.78$, dry loaded using Celite) to give the title compound as a pale yellow solid (0.21 g, 57%). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 8.64 (s, 1H), 7.83 (br s, 1H), 7.27 (d, $J = 8.7$ Hz, 4H), 6.90 (d, $J = 8.7$ Hz, 2H), 4.55 (d, $J = 6.0$ Hz, 2H), 3.87 – 3.85 (m, 4H), 3.16 – 3.15 (m, 4H), 2.55 (s, 3H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 169.9, 161.4, 160.4, 151.9, 150.9, 129.1, 128.7, 125.0, 115.8, 66.9, 49.25, 43.1, 14.6. **HRMS** (ESI) calc'd for $\text{C}_{17}\text{H}_{20}\text{ClN}_4\text{O}_2\text{S}$ [$\text{M} + \text{H}$] $^+$: 379.0990, found: 379.1012.

5-Chloro-2-(methylsulfonyl)-N-(4-morpholinobenzyl)pyrimidine-4-carboxamide (7). To a solution of **7a** (0.05 g, 0.13 mmol, 1.0 equiv) in methanol (3 mL) was added a solution of Oxone (0.12 g, 0.40 mmol, 3.0 equiv) in H_2O (3 mL). The reaction was stirred

overnight before being concentrated *in vacuo*. The crude solid was resuspended in methanol (20 mL) and filtered. The filtrate was collected, concentrated *in vacuo* and purified by prep-TLC (50% MeOH/EtOAc, $R_f = 0.16$) to obtain the title compound as an off-white solid (0.02 g, 46%). $^1\text{H NMR}$ (600 MHz, Methanol- d_4) δ 9.15 (s, 1H), 7.99 (d, $J = 9.2$ Hz, 2H), 7.74 (d, $J = 9.2$ Hz, 2H), 4.01 (s, 2H), 4.40 – 4.33 (m, 4H), 4.12 – 4.10 (m, 2H), 3.97 – 3.95 (m, 2H), 3.02 (s, 3H). $^{13}\text{C NMR}$ (150 MHz, Methanol- d_4) δ 171.4, 164.1, 162.7, 157.0, 149.1, 144.1, 131.7, 130.8, 121.5, 66.9, 62.5, 43.4, 40.4. **HRMS** (ESI) calc'd for $\text{C}_{17}\text{H}_{20}\text{ClN}_4\text{O}_4\text{S}$ [$\text{M} + \text{H}$] $^+$: 411.0888, found: 411.0898.

6-Chloro-N-(4-morpholinobenzyl)-3-nitropyridin-2-amine (8). A solution of **1** (0.20 g, 1.04 mmol, 1.05 equiv), 2,6-dichloro-3-nitropyridine (0.20 g, 1.00 mmol, 1.0 equiv), DIPEA (0.50 mL, 3.00 mmol, 3.0 equiv), and NMP (2 mL) was prepared in a microwave vial and stirred at 150 °C in a microwave reactor for 1 h. The reaction mixture was diluted with EtOAc (50 mL), washed with brine (3 $\times$ 25 mL), and the organic layer was dried over MgSO_4 , filtered and concentrated *in vacuo*. The resulting solid was purified by flash column chromatography (30% EtOAc/Hexanes, $R_f = 0.32$, dry loaded using Celite) to obtain the title compound as a yellow powder (0.21 g, 62%). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 8.53 (s, 1H), 8.35 (d, $J = 8.6$ Hz, 1H), 7.30 (d, $J = 8.7$ Hz, 2H), 6.91 (d, $J = 8.7$ Hz, 2H), 6.63 (d, $J = 8.6$ Hz, 1H), 4.70 (d, $J = 5.4$ Hz, 2H), 3.88 – 3.85 (m, 4H), 3.18 – 3.15 (m, 4H). $^{13}\text{C NMR}$ (100 MHz, CDCl_3) δ 156.8, 151.9, 150.9, 137.7, 129.2, 128.7, 126.7, 115.9, 112.1, 66.9, 49.3, 45.1. **HRMS** (ESI) calc'd for $\text{C}_{16}\text{H}_{17}\text{N}_4\text{O}_3\text{ClNa}$ [$\text{M} + \text{Na}$] $^+$: 371.0887, found 371.0895.

tert-Butyl (4-morpholinobenzyl)carbamate (9a). To a solution of **1** (0.25 g, 1.30 mmol, 1.2 equiv) and NEt_3 (0.36 mL, 2.60 mmol, 2.0 equiv) in DCM (10 mL) was added Boc_2O (0.35 g, 1.60 mmol, 1.2 equiv). The reaction was stirred for ~16 h and then concentrated *in vacuo*. The residue was purified by flash column chromatography (10% EtOAc/Hexanes, $R_f = 0.44$) to give the title compound white solid (0.24 g, 64%). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 7.22 (d, $J = 8.1$ Hz, 2H), 6.89 (d, $J = 8.1$ Hz, 2H), 4.79 (br s, 1H), 4.25 (d, $J = 5.8$ Hz, 2H), 3.88 – 3.87 (m, 4H), 3.16 – 3.15 (m, 4H), 1.48 (s, 9H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 155.8, 150.6, 130.3, 128.6, 115.8, 79.3, 66.9, 49.4, 44.2, 28.4. **HRMS** (ESI) calc'd for $\text{C}_{16}\text{H}_{25}\text{N}_3\text{O}_3$ [$\text{M} + \text{H}$] $^+$: 293.1860, found 293.1851.

tert-Butyl methyl(4-morpholinobenzyl)carbamate (9b). In a flame-dried round-bottom flask under an atmosphere of nitrogen, NaH (60% dispersion in mineral oil) (0.06 mg, 1.58 mmol, 2.0 equiv) was suspended in anhydrous THF (2.5 mL) at 0 °C. A solution of **9a** (0.23 g, 0.79 mmol, 1.0 equiv) in anhydrous THF (2.5 mL) was then added dropwise and the reaction was stirred at 0 °C for 1 h. MeI (0.06 mL, 0.95 mmol, 1.2 equiv) was then added and the reaction was allowed to warm to R.T. overnight. The reaction was then quenched with H_2O (10 mL) and extracted with EtOAc (3 $\times$ 25 mL). The combined organic layer was dried over MgSO_4 , filtered, and concentrated *in vacuo* to give the title compound as a white powder (0.24 g, > 95%) which was used without further purification. $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 7.15 (apparent br s, 2H), 6.87 (d, $J = 8.2$ Hz, 2H), 4.34 (br s, 2H), 3.87 – 3.85 (m, 4H), 3.15 – 3.14 (m, 4H), 2.80–2.75 (m, -NCH₃ rotamers, 3H), 1.48 (s, 9H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 155.8, 150.5, 129.5, 128.4, 115.7, 79.5, 66.9, 49.4, 33.6, 29.7, 28.5. **HRMS** (ESI) calc'd for $\text{C}_{17}\text{H}_{27}\text{N}_2\text{O}_3$ [$\text{M} + \text{H}$] $^+$: 307.2016, found 307.2017.

N-Methyl-1-(4-morpholinophenyl)methanaminium dihydrochloride (9c). To a solution of **9b** (0.24 g, 0.79 mmol, 1.0 equiv) in DCM (2 mL) at 0 °C was added a solution of 4 M HCl in dioxane (2 mL). The reaction was allowed to warm to R.T. overnight at which point a precipitate had formed, which was collected by vacuum filtration, and washed with Et_2O (3 $\times$ 25 mL) to give the title compound as a white powder (0.14 g, 63%) which was carried forward without further purification. $^1\text{H NMR}$ (600 MHz, $\text{DMSO}-d_6$) δ 9.39 (br s, 1H), 7.52 (d, $J = 8.2$ Hz, 2H), 7.25 (d, $J = 8.2$ Hz, 2H), 4.05 (t, $J = 5.8$ Hz, 2H), 3.88 – 3.86 (m, 4H), 3.29 – 3.28 (m, 4H), 2.55 (br s, 2H), 2.51 (t, $J = 5.4$ Hz, 3H). $^{13}\text{C NMR}$ (150 MHz, $\text{DMSO}-d_6$) δ 149.8, 131.6, 125.1, 116.8, 65.9, 51.0, 49.9, 32.1. Characterization data are consistent with previously reported values.⁵³

4-Chloro-N-methyl-N-(4-morpholinobenzyl)-3-nitrobenzenesulfonamide (9). To a solution of **9c** (0.13 g, 0.47 mmol, 1.0 equiv) and NEt_3 (0.26 mL, 1.88 mmol, 4.0 equiv) in anhydrous DCM (5 mL) under an atmosphere of nitrogen at 0 °C, was added 4-chloro-3-nitrobenzenesulfonyl chloride (0.13 g, 0.52 mmol, 1.1 equiv). The reaction was allowed to gradually warm to R.T. over 6 h and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3 $\times$ 25 mL) and brine (50 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (10% EtOAc/DCM, R_f = 0.72, dry loaded using Celite) to give the title compound as a white powder (0.27 g, 60%). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 8.25 (d, J = 2.1 Hz, 2H), 7.94 (dd, J = 8.4, 2.1 Hz, 2H), 7.74 (d, J = 8.4 Hz, 2H), 7.20 (d, J = 8.7 Hz, 2H), 6.88 (d, J = 8.7 Hz, 2H), 4.18 (s, 2H), 3.89 – 3.87 (m, 4H), 3.19 – 3.17 (m, 4H), 2.71 (s, 3H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 151.2, 147.9, 138.4, 132.8, 131.4, 131.2, 129.6, 125.3, 124.4, 115.5, 66.8, 53.7, 48.9, 34.3. **HRMS** (ESI) calc'd for $\text{C}_{18}\text{H}_{21}\text{ClN}_3\text{O}_3\text{S}$ [$\text{M} + \text{H}$] $^+$: 426.0885, found 426.0858.

N-(4-Morpholinobenzyl)acrylamide (10). To a solution of **1** (0.50 g, 2.60 mmol, 1.0 equiv) and DIPEA (0.95 mL, 5.20 mmol, 2.0 equiv) in anhydrous DCM (15 mL) at 0 °C under a nitrogen atmosphere, was added acryloyl chloride (0.26 mL, 3.12 mmol, 1.2 equiv) dropwise. The reaction was allowed to warm to R.T. over 3 h and then diluted with EtOAc (100 mL). The organic layer was washed with sat. NaHCO_3 (3 $\times$ 50 mL) and brine (50 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (70% EtOAc/Hex, R_f = 0.46, dry loaded using Celite) to give the title compound as a white powder (0.56 g, 88%). $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 7.22 (d, J = 8.6 Hz, 2H), 6.88 (d, J = 8.6 Hz, 2H), 6.31 (d, J = 17.0 Hz, 1H), 6.08 (dd, J = 17.0, 10.3 Hz, 1H), 5.73 (s, 1H), 5.66 (d, J = 10.3 Hz, 1H), 4.44 (d, J = 5.6 Hz, 2H), 3.87 – 3.85 (m, 4H), 3.15 – 3.13 (m, 4H). $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 165.3, 150.9, 130.8, 129.3, 129.1, 126.7, 115.9, 66.9, 49.3, 43.3. **HRMS** (ESI) calc'd for $\text{C}_{14}\text{H}_{18}\text{N}_2\text{O}_2\text{Na}$ [$\text{M} + \text{Na}$] $^+$: 269.1266, found: 269.1265.

2-Fluoro-N-(4-morpholinobenzyl)acrylamide (11). To a solution of **1** (0.20 g, 1.04 mmol, 1.0 equiv) and 2-fluoroacrylic acid (0.13 g, 1.15 mmol, 1.1 equiv) in anhydrous DCM (6 mL) at R.T. under a nitrogen atmosphere, was added a solution of DCC (0.24 g, 1.15 mmol, 1.1 equiv) and DMAP ($\approx$ 1 mg, cat.) in anhydrous DCM (4 mL) dropwise. The reaction was stirred for 3 h and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3 $\times$ 25 mL) and brine (25 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (20% EtOAc/DCM, R_f = 0.44, dry loaded using Celite) to give the title compound as a yellow powder (0.20 g, 73%). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 7.24 (d, J = 8.7 Hz, 2H), 6.90 (d, J = 8.7 Hz, 2H), 6.49 (br s, 1H), 5.74 (dd, J_{HF} = 47.4 Hz, J_{HH} = 3.2 Hz, 1H), 5.15 (dd, J_{HF} = 15.3 Hz, J_{HH} = 3.2 Hz, 1H), 4.47 (d, J = 5.7 Hz, 2H), 3.89 – 3.87 (m, 4H), 3.18 – 3.16 (m, 4H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 159.3 (d, J_{CF} = 30.8 Hz, 1C), 156.3 (d, J_{CF} = 270.1 Hz, 1C), 151.0, 129.1, 128.4, 115.8, 99.0 (d, J_{CF} = 14.9 Hz, 1C), 66.8, 49.2, 43.0. $^{19}\text{F NMR}$ (283 MHz, CDCl_3) δ –121.18. **HRMS** (ESI) calc'd for $\text{C}_{14}\text{H}_{18}\text{FN}_2\text{O}_2$ [$\text{M} + \text{H}$] $^+$: 265.1347, found: 265.1357.

(E)-4-(Dimethylamino)-N-(4-morpholinobenzyl)but-2-enamide (12). To a solution of **1** (0.20 g, 1.04 mmol, 1.0 equiv) and 3-((dimethylamino)methyl) acrylic acid (0.21 g, 1.25 mmol, 1.2 equiv) in anhydrous MeCN (10 mL) at R.T. under a nitrogen atmosphere, were added HATU (0.60 g, 1.56 mmol, 1.5 equiv) and DIPEA (0.73 mL, 4.16 mmol, 4.0 equiv). The reaction was stirred for $\sim$ 16 h and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3 $\times$ 25 mL) and brine (25 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (9% MeOH/30% DCM/60% EtOAc/1% NEt_3 , R_f = 0.13, dry loaded using Celite) to give the title compound as a white powder (0.15 g, 47%). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 7.20 (d, J = 8.1 Hz, 2H), 6.86 – 6.80 (m, 3H), 5.95 (d, J = 15.3 Hz, 1H), 5.85 (br s, 1H), 4.41 (d, J = 5.8 Hz, 2H), 3.85 – 3.83 (m, 4H), 3.13 – 3.11 (m, 4H), 3.06 – 3.03 (m, 2H), 2.24

(s, 6H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 164.1, 149.7, 139.7, 128.4, 128.0, 124.6, 114.8, 65.8, 59.2, 48.2, 44.3, 42.1. **HRMS** (ESI) calc'd for $\text{C}_{17}\text{H}_{26}\text{N}_3\text{O}_2$ [$\text{M} + \text{H}$] $^+$: 304.2020, found: 304.2010.

2-((Dimethylamino)methyl)-N-(4-morpholinobenzyl)acrylamide (13). To a solution of **1** (0.20 g, 1.04 mmol, 1.0 equiv) and 2-((dimethylamino)methyl) acrylic acid (0.14 g, 1.04 mmol, 1.0 equiv) in anhydrous MeCN (10 mL) at R.T. under a nitrogen atmosphere, were added TCFH (0.35 g, 1.25 mmol, 1.2 equiv) and NMI (0.29 mL, 3.64 mmol, 3.5 equiv). The reaction was stirred for $\sim$ 16 h and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3 $\times$ 25 mL) and brine (25 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography (20% DCM/79% EtOAc/1% NEt_3 , R_f = 0.11, dry loaded using Celite) to give the title compound as an oil which was triturated with Hexanes (25 mL) to give a white powder (0.09 g, 30%). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 9.44 (br s, 1H), 7.19 (d, J = 8.8 Hz, 1H), 6.87 (d, J = 8.8 Hz, 1H), 6.25 (d, J = 2.0 Hz, 1H), 5.41 – 5.40 (m, 1H), 4.44 (d, J = 5.6 Hz, 2H), 3.87 – 3.84 (m, 4H), 3.15 – 2.12 (m, 6H), 2.18 (s, 6H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 166.9, 150.3, 138.5, 130.3, 128.2, 125.3, 115.8, 66.9, 62.9, 49.4, 44.4, 42.4. **HRMS** (ESI) calc'd for $\text{C}_{17}\text{H}_{26}\text{N}_3\text{O}_2$ [$\text{M} + \text{H}$] $^+$: 304.2020, found: 304.2026.

N-(4-Morpholinobenzyl)propiolamide (14). To a solution of **1** (0.20 g, 1.04 mmol, 1.0 equiv) and propiolic acid (0.07 mL, 1.15 mmol, 1.1 equiv) in anhydrous DCM (6 mL) at R.T. under a nitrogen atmosphere, was added a solution of DCC (0.24 g, 1.15 mmol, 1.1 equiv) and DMAP ($\sim$ 1 mg, cat.) in anhydrous DCM (4 mL) dropwise. The reaction was stirred for 6 h and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3 $\times$ 25 mL) and brine (25 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (80% EtOAc/Hex, R_f = 0.53, dry loaded using Celite) to give the title compound as a white powder (0.16 g, 64%). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 7.14 (d, J = 8.7 Hz, 2H), 6.82 (d, J = 8.7 Hz, 2H), 4.34 (d, J = 5.7 Hz, 2H), 3.80 – 3.78 (m, 4H), 3.09 – 3.07 (m, 4H), 2.71 (s, 1H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 151.8, 151.1, 129.2, 128.4, 128.1, 115.8, 73.3, 66.8, 49.2, 43.5. **HRMS** (ESI) calc'd for $\text{C}_{14}\text{H}_{17}\text{N}_2\text{O}_2$ [$\text{M} + \text{H}$] $^+$: 245.1285, found: 245.1281.

N-(4-Morpholinobenzyl)but-2-ynamide (15). To a solution of **1** (0.20 g, 1.04 mmol, 1.0 equiv) and 2-butyric acid (0.10 g, 1.15 mmol, 1.1 equiv) in anhydrous DCM (6 mL) at R.T. under a nitrogen atmosphere, was added a solution of DCC (0.24 g, 1.15 mmol, 1.1 equiv) and DMAP ($\sim$ 1 mg, cat.) in anhydrous DCM (4 mL) dropwise. The reaction was stirred for 6 h and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3 $\times$ 25 mL) and brine (25 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (100% EtOAc, R_f = 0.61, dry loaded using Celite) to give the title compound as a white powder (0.19 g, 71%). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 7.20 (d, J = 8.5 Hz, 1H), 6.87 (d, J = 8.5 Hz, 1H), 5.91 (br s, 1H), 4.38 (d, J = 5.7 Hz, 1H), 3.86 – 3.85 (m, 4H), 3.15 – 3.13 (m, 4H), 1.92 (s, 1H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ 153.2, 150.9, 129.1, 128.6, 115.8, 83.5, 74.8, 66.9, 49.2, 43.3, 3.6. **HRMS** (ESI) calc'd for $\text{C}_{15}\text{H}_{18}\text{N}_2\text{O}_2$ [$\text{M} + \text{H}$] $^+$: 259.1441, found: 259.1451.

N-(4-Morpholinobenzyl)ethanesulfonamide (16). To a solution of **1** (0.20 g, 1.04 mmol, 1.0 equiv) and 2-bromoethanesulfonyl chloride (0.22 g, 1.04 mmol, 1.0 equiv) in anhydrous DCM (15 mL) at 0 °C under a nitrogen atmosphere, was added NEt_3 (0.30 mL, 2.08 mmol, 2.0 equiv). The reaction was gradually allowed to warm to R.T. over 3 h and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3 $\times$ 25 mL) and brine (25 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (50% EtOAc/Hexanes, R_f = 0.53, dry loaded using Celite) to give the title compound as a yellow powder (0.14 g, 47%). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ 7.21 (d, J = 8.3 Hz, 2H), 6.88 (d, J = 8.3 Hz, 2H), 6.48 (dd, J = 16.6, 9.9 Hz, 1H), 6.27 (d, J = 16.6 Hz, 1H), 5.93 (d, J = 9.9 Hz, 1H), 4.40 (br s, 1H), 4.14 (d, J = 6.0 Hz, 2H), 3.87 – 3.85 (m, 4H),

3.16 – 3.14 (m, 4H). ^{13}C NMR (150 MHz, CDCl_3) δ 151.1, 136.1, 129.1, 127.4, 126.7, 115.7, 66.8, 49.1, 46.6. HRMS (ESI) calc'd for $\text{C}_{13}\text{H}_{19}\text{N}_2\text{O}_3\text{S}$ $[\text{M} + \text{H}]^+$: 283.1111, found: 283.1125.

2-Bromo-N-(4-morpholinobenzyl)pyridin-4-amine (17a). Compound **1** (0.25 g, 1.30 mmol, 1.2 equiv), 2-bromo-4-fluoropyridine (0.12 mL, 1.08 mmol, 1.0 equiv), DIPEA (0.45 mL, 2.40 mmol, 2.2 equiv), and NMP (2 mL) were added to a microwave vial and stirred at 150 °C in a microwave reactor for 1 h. The reaction was poured into cold H_2O (20 mL), and the resulting precipitate was collected by vacuum filtration and washed with cold Et_2O (3×10 mL) to give the title compound as a beige powder which was used without further purification (0.30 g, 81%). ^1H NMR (300 MHz, CDCl_3 + Methanol- d_4) δ 7.79 (d, J = 5.9 Hz, 1H), 7.17 (d, J = 8.6 Hz, 2H), 6.86 (d, J = 8.6 Hz, 2H), 6.60 (d, J = 2.2 Hz, 1H), 6.37 (dd, J = 5.9, 2.2 Hz, 1H), 5.12 (br s, 1H), 4.20 (d, J = 3.7 Hz, 2H), 3.84 – 3.81 (m, 4H), 3.13 – 3.09 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3 + Methanol- d_4) δ 155.1, 150.8, 149.1, 142.4, 128.3, 118.3, 115.9, 110.0, 107.5, 66.8, 49.2 (overlapping with Methanol- d_4), 46.2. HRMS (ESI) calc'd for $\text{C}_{16}\text{H}_{19}\text{BrN}_3\text{O}$ $[\text{M} + \text{H}]^+$: 348.0706, found: 348.0711.

N-(4-Morpholinobenzyl)-2-vinylpyridin-4-amine (17). Compound **17a** (0.30 g, 0.87 mmol, 1.0 equiv), vinylboronic acid pinacol ester (0.23 mL, 1.32 mmol, 1.5 equiv), $\text{Pd}(\text{dppf})\text{Cl}_2$ (0.06 g, 0.09 mmol, 0.1 equiv), and Cs_2CO_3 (0.84 g, 2.61 mmol, 3.0 equiv) were combined in a pressure tube under a stream of nitrogen and suspended in Dioxane/ H_2O (3:1 mL). The reaction was stirred 90 °C for ~16 h and then concentrated *in vacuo*. The resulting residue was purified by flash column chromatography (79% EtOAc/20% Hexanes, 1% NEt_3 , R_f = 0.34, dry loaded using Celite) to give the title compound as an orange powder (0.17 g, 66%). ^1H NMR (600 MHz, DMSO- d_6) δ 8.01 (d, J = 5.6 Hz, 1H), 7.25 (d, J = 8.6 Hz, 2H), 7.02 (t, J = 5.6 Hz, 1H), 6.95 (d, J = 8.6 Hz, 2H), 6.65 – 6.60 (m, 2H), 6.44 (dd, J = 5.6, 2.3 Hz, 1H), 6.09 (dd, J = 17.4, 2.0 Hz, 1H), 5.33 (dd, J = 10.7, 2.0 Hz, 1H), 4.26 (d, J = 5.9 Hz, 2H), 3.77 – 3.75 (m, 4H), 3.11 – 3.09 (m, 4H). ^{13}C NMR (150 MHz, DMSO- d_6) δ 155.1, 154.7, 150.6, 149.6, 138.3, 130.0, 128.6, 117.0, 115.6, 107.0, 105.8, 66.6, 49.1, 45.3. HRMS (ESI) calc'd for $\text{C}_{18}\text{H}_{22}\text{N}_3\text{O}$ $[\text{M} + \text{H}]^+$: 296.1757, found: 296.1760.

2-Chloro-N-(4-morpholinobenzyl)acetamide (18). To a solution of **1** (0.20 g, 1.04 mmol, 1.0 equiv) and NEt_3 (0.56 mL, 4.16 mmol, 4.0 equiv) in anhydrous DCM (10 mL) at 0 °C under an atmosphere of nitrogen, was added chloroacetyl chloride (0.09 mL, 1.14 mmol, 1.1 equiv). The reaction was stirred at 0 °C for 3 h and then quenched with sat. NaHCO_3 (25 mL) and extracted with EtOAc (3×25 mL). The combined organic layer was dried over MgSO_4 , filtered, and concentrated *in vacuo*. The resulting solid was purified by flash column chromatography (75% EtOAc/Hexanes, R_f = 0.53, dry loaded using Celite) to give the title compound as a white powder (0.23 g, 82%). ^1H NMR (600 MHz, CDCl_3) δ 7.23 (d, J = 8.8 Hz, 2H), 6.91 (d, J = 8.8 Hz, 2H), 6.78 (br s, 1H), 4.43 (d, J = 5.7 Hz, 2H), 4.11 (s, 1H), 3.89 – 3.87 (m, 4H), 3.18 – 3.17 (m, 4H). ^{13}C NMR (150 MHz, CDCl_3) δ 165.6, 150.9, 129.0, 128.4, 115.8, 66.8, 49.2, 43.4, 42.6. HRMS (ESI) calc'd for $\text{C}_{13}\text{H}_{18}\text{ClN}_2\text{O}_2$ $[\text{M} + \text{H}]^+$: 269.1051, found: 269.1062.

2-Chloro-2-fluoro-N-(4-morpholinobenzyl)acetamide (19). To a solution of **1** (0.20 g, 1.04 mmol, 1.0 equiv) and chlorofluoroacetate (0.21 g, 1.56 mmol, 1.5 equiv) in anhydrous DMF (5 mL) at R.T. under a nitrogen atmosphere, were added a solution of T3P (0.96 mL, 1.56 mmol, 1.5 equiv) and DIPEA (0.37 mL, 2.08 mmol, 2.0 equiv). The reaction was stirred for 24 h and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3×25 mL) and brine (25 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (80% EtOAc/Hexanes, R_f = 0.77, dry loaded using Celite) to give the title compound as a white powder (0.19 g, 63%). ^1H NMR (600 MHz, CDCl_3) δ 7.21 (d, J = 8.1 Hz, 2H), 6.90 (d, J = 8.1 Hz, 2H), 6.50 (br s, 1H), 6.32 (d, J_{HF} = 59.4 Hz, 1H), 4.48 – 4.41 (m, 2H), 3.86 – 3.85 (m, 4H), 3.17 – 3.15 (m, 4H). ^{13}C NMR (150 MHz, CDCl_3) δ 163.8 (d, J_{CF} = 21.8 Hz, 1C), 151.1, 129.1, 115.8, 94.6 (d, J_{CF} = 256.2 Hz, 1C), 66.8, 49.1, 43.2 (2C). ^{19}F NMR (283

MHz, CDCl_3) δ –143.82. HRMS (ESI) calc'd for $\text{C}_{13}\text{H}_{17}\text{ClFN}_2\text{O}_2$ $[\text{M} + \text{H}]^+$: 287.0957, found: 287.1970.

3-Bromocyclobutane-1-carboxylic acid (20b). To a solution of methyl 3-bromocyclobutane-1-carboxylate (**20a**) (0.50 g, 2.60 mmol, 1.0 equiv) in acetone: H_2O (15 mL:5 mL) at 0 °C was added $\text{LiOH} \cdot \text{H}_2\text{O}$ (0.44 g, 10.40 mmol, 4.0 equiv). The reaction was allowed to gradually warm to R.T. overnight and then concentrated *in vacuo*. The residue was dissolved in H_2O (50 mL) and acidified to pH $\approx$ 2 using conc. HCl. The aqueous phase was extracted using EtOAc (3×50 mL). The combined organic layer was dried over MgSO_4 , filtered, and concentrated *in vacuo* to give the title compound as a colorless oil (0.47 g, > 95%) which was carried forward without further purification. ^1H NMR (300 MHz, CDCl_3) δ 4.74 – 4.64 (m, 1H), 3.49 – 3.39 (m, 1H), 3.03 – 2.94 (m, 2H), 2.81 – 2.70 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.7, 40.3, 36.7, 36.2. Characterization data are consistent with previously reported values.⁵⁴

4-Nitrophenyl 3-bromocyclobutane-1-carboxylate (20c). To a solution of **20b** (0.47 g, 2.60 mmol, 1.0 equiv) and EDCI-HCl (0.65 g, 3.40 mmol, 1.3 equiv) in anhydrous DCM (10 mL) under an atmosphere of nitrogen were added *p*-nitrophenol (0.72 g, 5.2 mmol, 2.0 equiv) and DMAP (0.07 g, 0.52 mmol, 0.2 equiv). The reaction was stirred for ~16 h and then diluted with a solution of sat. NaHCO_3 (25 mL) and extracted with EtOAc (3×25 mL). The combined organic layers were washed with brine (3×25 mL), dried over MgSO_4 , filtered, and concentrated *in vacuo*. The resulting solid was purified by flash column chromatography (0–10% EtOAc/Hexanes, R_f = 0.30 in 10% EtOAc/Hexanes, dry loaded using Celite) to give the title compound as a pale-yellow oil (0.31 g, 40%). ^1H NMR (300 MHz, CDCl_3) δ 8.28 (d, J = 9.3 Hz, 1H), 7.29 (d, J = 9.3 Hz, 1H), 4.75 – 4.66 (m, 1H), 3.75 – 3.65 (m, 1H), 3.13 – 3.04 (m, 2H), 2.89 – 2.79 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 172.3, 155.3, 145.6, 125.4, 122.4, 40.1, 37.5, 36.2. Characterization data are consistent with previously reported values.⁵⁴

4-Nitrophenyl bicyclo[1.1.0]butane-1-carboxylate (20d). To a solution of **20c** (0.30 g, 1.00 mmol, 1.0 equiv) in anhydrous THF (10 mL) at 0 °C under an atmosphere of nitrogen was added LiHMDS (1.30 mL, 1.30 mmol, 1.3 equiv). The reaction was allowed to warm to R.T. over 3 h and then quenched with sat. NH_4Cl (25 mL) and extracted with EtOAc (3×25 mL). The combined organic phase was dried over MgSO_4 , filtered, and concentrated *in vacuo*. The resulting oil was purified by flash column chromatography (25% EtOAc/Hexanes, R_f = 0.44, dry loaded using Celite) to give the title compound as a white solid (0.16 g, 55%). ^1H NMR (400 MHz, CDCl_3) δ 8.26 (d, J = 9.1 Hz, 2H), 7.29 (d, J = 9.1 Hz, 2H), 2.54 (dt, J = 3.3, 1.2 Hz, 2H), 2.45 (p, J = 3.3 Hz, 1H), 1.38 (dt, J = 3.3, 1.2 Hz, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 170.7, 155.6, 145.1, 125.1, 122.3, 36.5, 19.4, 9.3. Characterization data are consistent with previously reported values.⁵⁴

N-(4-Morpholinobenzyl)bicyclo[1.1.0]butane-1-carboxamide (20). To a solution of **1** (0.14 g, 0.70 mmol, 1.0 equiv) and **20d** (0.16 g, 0.70 mmol, 1.0 equiv) in DCM (10 mL) at 0 °C was added DIPEA (0.25 mL, 1.40 mmol, 2.0 equiv). The reaction was stirred ~16 h and then diluted with EtOAc (50 mL). The organic layer was washed with sat. NaHCO_3 (3×25 mL) and brine (25 mL), before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography (75% EtOAc/Hexanes R_f = 0.28, dry loaded using Celite) to give the title compound as white powder (0.06 g, 32%). ^1H NMR (600 MHz, CDCl_3) δ 7.21 (d, J = 8.5 Hz, 2H), 6.88 (d, J = 8.5 Hz, 2H), 5.78 (br s, 1H), 4.39 (d, J = 5.6 Hz, 2H), 3.87 – 3.85 (m, 4H), 3.15 – 3.14 (m, 4H), 2.26 – 2.25 (m, 2H), 2.00 – 1.98 (m, 1H), 1.03 – 1.03 (m, 2H). ^{13}C NMR (150 MHz, CDCl_3) δ 171.5, 150.9, 129.8, 129.1, 115.9, 67.0, 49.4, 43.5, 34.1, 14.1, 11.1. HRMS (ESI) calc'd for $\text{C}_{16}\text{H}_{21}\text{N}_2\text{O}_2$ $[\text{M} + \text{H}]^+$: 273.1603, found: 273.1604.

N-(4-Morpholinobenzyl)-1,2,4-thiadiazol-5-amine (21). Compound **1** (0.25 g, 1.30 mmol, 1.2 equiv), 5-chloro-1,2,4-thiadiazole (0.09 mL, 1.08 mmol, 1.0 equiv), DIPEA (0.45 mL, 2.40 mmol, 2.2 equiv), and NMP (2 mL) were added to a microwave vial and stirred at 150 °C in a microwave reactor for 1 h. The reaction was poured into H_2O (20 mL) and extracted with EtOAc (3×25 mL). The

combined organic layer was dried over MgSO_4 and concentrated *in vacuo* to give a brown solid which was tritiated with DCM (5 mL) and Hexanes (5 mL) and then filtered to give the title compound as an orange solid (0.24 g, 83%) which was used without further purification. ^1H NMR (300 MHz, CDCl_3) δ 7.76 (s, 1H), 7.28 (d, J = 8.7 Hz, 2H, overlapping with CDCl_3), 7.04 (br s, 1H), 6.90 (d, J = 8.7 Hz, 2H), 4.36 (d, J = 5.1 Hz, 2H), 3.88 – 3.84 (m, 4H), 3.18 – 3.15 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3) δ 184.4, 160.0, 151.3, 129.0, 126.7, 115.7, 66.8, 50.2, 49.0. HRMS (ESI) calc'd for $\text{C}_{13}\text{H}_{17}\text{N}_4\text{O}_5$ $[\text{M} + \text{H}]^+$: 277.1118, found: 277.1129.

3-Bromo-N-(4-morpholinobenzyl)-4,5-dihydroisoxazole-5-carboxamide (22). To a solution of **10** (0.10 g, 0.41 mmol, 1.0 equiv) in DMF (4 mL) at 0 °C were added dibromoformaldoxime (0.13 g, 0.62 mmol, 1.5 equiv) and KHCO_3 (0.13 g, 1.23 mmol, 3.0 equiv). The reaction was stirred allowed to warm to R.T. overnight and then diluted with H_2O (50 mL). The aqueous layer was extracted with EtOAc (3 $\times$ 25 mL) before being dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (50% EtOAc/Hexanes, R_f = 0.21, dry loaded using Celite) to give the title compound as a white powder (0.14 g, 93%). ^1H NMR (600 MHz, CDCl_3) δ 7.18 (d, J = 8.7 Hz, 2H), 6.88 (m, 3H (2 aryl H + NH overlapping)), 5.06 (dd, J = 11.2, 6.3, 1H), 4.46 (d, J = 14.5, 6.0 Hz, 1H), 4.29 (dd, J = 14.5, 5.3, 1H), 3.87 – 3.85 (m, 4H), 3.61 – 3.52 (m, 2H), 3.16 – 3.14 (m, 4H). ^{13}C NMR (150 MHz, CDCl_3) δ 169.1, 150.9, 138.7, 128.9, 128.7, 115.8, 79.2, 66.8, 49.2, 45.7, 42.9. HRMS (ESI) calc'd for $\text{C}_{15}\text{H}_{19}\text{BrN}_3\text{O}_3$ $[\text{M} + \text{H}]^+$: 368.0610, found: 368.0638.

Intrinsic Reactivity

Reactions with GSH were monitored at R.T. with ≥ 10 -fold excess of GSH for all compounds in either 0.1 M phosphate buffer pH = 6.5, 7.4, 8.0, or 0.5 M 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer pH = 10.4, with 10% v/v DMSO. GSH concentration and pH were adjusted to ensure reaction completion within a time frame of <24 h, except for compounds **12**, **13**, and **21** for which pH was held at 7.4. See [Supporting Information](#) for exact concentrations. The 24-h buffer stability of all compounds was confirmed prior to evaluation.

Compounds **2**–**9**, **21** were evaluated by monitoring adduct formation by an increase in absorbance at the appropriate wavelength (see [Direct Spectrophotometric Assay](#) below) on a BioTek Synergy 4 plate reader. Compounds **10**–**20**, **22** were evaluated by monitoring disappearance of substrate by HPLC.

The Abs vs time plots were fitted to a monoexponential association model (eq 1).

$$\text{Abs} = \text{Abs}_{\text{max}}(1 - e^{-k_{\text{obs}}t}) \quad (1)$$

The decreasing AUC of the substrate peaks of the HPLC chromatograms were fitted to a monoexponential decay model, with the constraint that the lower plateau = 0 (eq 2).

$$\text{AUC} = \text{AUC}_0(e^{-k_{\text{obs}}t}) \quad (2)$$

Both of these methods were used to obtain replicate pseudo-first order k_{obs} values. Second order rates constants were then calculated by dividing these pseudo-first order rate constants by the concentration of excess thiol (eq 3).

$$k_2^{\text{calc}} = \frac{k_{\text{obs}}}{[\text{thiol}]} \quad (3)$$

The final, corrected second order rate constants were then calculated by dividing by the fraction of thiolate at the reaction pH, using the corresponding pK_a value (8.7) of the GSH thiol (eq 4).¹⁹

$$k_2^{\text{GSH}} = \frac{k_2^{\text{calc}}}{\left(\frac{K_a}{[\text{H}^+] + K_a}\right)} \quad (4)$$

6X-His-Tagged hGSTP1 and hGSTA1 Expression

An expression plasmid for 6X-His-hGSTP1–1 and 6X-His-hGSTA1–1 was obtained using GenScript Biotech Inc. cloning services. Clone ID: OHu18057 hGSTP1–1 and OHu17474 hGSTA1–1 were cloned into pET-15b between *Xho*I/*Bam*HI restriction sites, in frame with the hexahistidine tag with codon optimization for *E. coli*. Each commercial lyophilized plasmid (10 μg) was diluted with water to a concentration of 100 ng/ μL and 5 μL of stock plasmid solution was used to transform BL21 (DE3) cells. Transformants were then selected on ampicillin (100 $\mu\text{g}/\text{mL}$) containing LB agar plates. A single colony was inoculated into 5 mL of LB medium containing ampicillin (100 $\mu\text{g}/\text{mL}$) and incubated overnight at 37 °C, shaking at 250 rpm. The small culture was then added to 500 mL of the same medium and large-scale expression and purification was completed for each isozyme as previously reported,⁵⁵ with the exception that homogenization (AVESTIN) was used for cell lysis and a His 60 Ni Superflow resin (Takara) was used for purification with elution using 150 mM imidazole buffer (pH 8.0) (10 column volumes per fraction). The purity of the fractions was assessed using SDS-PAGE and fractions containing pure protein were concentrated using a 10-kDa Amicon tube (Sigma-Aldrich) and the buffer was exchanged with 3 $\times$ 10 volumes of storage buffer (0.1 M potassium phosphate, pH 6.5 containing 1 mM EDTA and 1 mM DTT), and the protein was ultimately collected in approximately 1.0 mL of this buffer. Protein concentration was determined according to a Bradford assay, 3.1 mg/mL for GSTP1, and 13.3 mg/mL for GSTA1. Specific activity was measured using a previously reported CDNB activity assay with 1 mM CDNB and 2.5 mM GSH,⁵⁵ 148 U/mL for GSTP1, 143 U/mL for GSTA1. Protein aliquots were flash-frozen and stored at –80 °C until use. Both enzymes were stable on ice >16 h and at R.T. > 4 h, and their activity was unaffected by DMSO concentrations up to 20% v/v ([Figure S2](#)).

Direct Spectrophotometric Assay

Assays were conducted in 0.1 M potassium phosphate buffer (pH 6.5) at R.T. in a polystyrene 96-well microplate with a final reaction volume of 200 μL . Reactions were performed using 2.5 mM of reduced GSH ($>30 \times K_M$ relative to model substrate **2**) ([Figure S6](#)) and various ranges of substrate concentrations (0.007–0.75 mM) up to their solubility limits. Substrate stock solutions were prepared in DMSO such that the final DMSO concentration in the reaction was maintained at 10% v/v. A final concentration of 250 mU/mL GSTP1 (≈ 200 nM) and 50 mU/mL (≈ 186 nM) GSTA1 was used unless otherwise specified in the [Supporting Information](#). All reactions were initiated by addition of GSH and the change in absorbance was monitored using a Biotek Synergy 4 plate reader at the optimal wavelength for each substrate. The optimal wavelength was determined by performing a spectral scan of a blank reaction (substrate in buffer) and control reaction (with enzyme and GSH) after a 20 min incubation at R.T. The absorption spectrum of both reactions was measured at 10 nm wavelength intervals from 300 to 700 nm. The wavelength that displayed the largest increase in absorbance between the enzymatic and blank reaction was selected for all subsequent measurements.

Reactions were monitored up to 10 min, and initial rates (v_0) at each substrate concentration were calculated from the slope of Abs vs time plots and subtracting the background (nonenzymatic) GSH reactivity at each substrate concentration. All reactions were performed in triplicate. A Michaelis–Menten plot was constructed by plotting v_0 against $[\text{S}]$ and fitted to the Michaelis–Menten equation (eq 5) using GraphPad Prism 10 software to obtain the apparent K_M and V_{max} values.³⁴

$$v_0 = \frac{V_{\text{max}}^{\text{app}}[\text{S}]}{K_M^{\text{app}} + [\text{S}]} \quad (5)$$

For substrates that could not be tested at sufficiently high concentrations (**5** and **6**) to observe saturation, due to solubility limits, Lineweaver–Burk analysis was used instead by performing linear regression according to eq 6. The values for apparent K_M and

$V_{\max}$ were extrapolated from the x- and y-intercepts of the resulting line, which are equal to $-1/K_M^{\text{app}}$ and $1/V_{\max}^{\text{app}}$, respectively.⁵⁶

$$\frac{1}{v_0} = \frac{K_M^{\text{app}}}{V_{\max}^{\text{app}}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}^{\text{app}}} \quad (6)$$

$V_{\max}^{\text{app}}$ values in Abs/s were converted to M/min using a standard curve of the substrate-GSH adduct. Briefly, three reactions were prepared using the highest concentration of substrate tested in the initial kinetic assay and monitored at the appropriate wavelength until the absorbance plateaued, indicating reaction completion. Each reaction was then serially diluted and the absorbance was measured, along with a blank containing the corresponding concentration of enzyme, GSH and buffer. Absorbances were corrected by subtracting the blank and plotted against adduct concentration to yield the standard curve. The $V_{\max}^{\text{app}}$ was divided by the slope of the standard curve and subsequently converted into a $k_{\text{cat}}^{\text{app}}$ value by dividing by the concentration of enzyme used in the kinetic assay. The overall enzymatic efficiency was then calculated using as the ratio of (k_{cat}/K_M)^{app}.

Competitive Displacement and HPLC Rate Assay

Competitive displacement assays were performed using a final concentration of 0.25 mM reporter substrate (S_R) **2** and 2.5 mM GSH for both isozymes, and 250 mU/mL GSTP1 and 50 mU/mL GSTA1, unless otherwise specified in the Supporting Information. Test substrates (S_T) were evaluated at various ranges of concentrations up to their solubility limits, spanning 0–100 mM, depending on the substrate. Test substrate, reporter substrate, and enzyme were combined in buffer (pH 6.5) prior to initiation of the reaction by addition of GSH. DMSO was maintained at 10% v/v final concentration. Initial rates were determined at each test substrate concentration as described above, in triplicate. The initial rates were normalized with respect to the rate observed with reporter substrate only (% v_0) and plotted against the log of test substrate concentration to determine an EC_{50} value according to eq 7.

$$\% \frac{v_0}{v_0} = \frac{\text{top} - \text{bottom}}{1 + \left(\frac{EC_{50}}{[S_T]} \right)^{\text{hill slope}}} + \text{bottom} \quad (7)$$

A K_M^{app} value for the test substrate was then calculated using the Cheng-Prusoff eq 8.³⁵

$$K_{M,S_T}^{\text{app}} = \frac{EC_{50}}{1 + \frac{[S_R]}{K_{M,S_R}^{\text{app}}}} \quad (8)$$

A v_0 in AUC/min at a single test substrate concentration ($2-10 \times K_M^{\text{app}}$, depending on solubility) was measured by incubating the test substrate with GSH and enzyme in buffer at the same concentrations as the competitive displacement assay, unless otherwise specified in the Supporting Information. Reactions were quenched at specific time points by dilution with cold MeCN (variable amount depending on [substrate] used) and TCA (2% v/v) prior to centrifugation at 14,000 rpm for 10 min. For exact concentrations and dilution volumes, see Supporting Information. The supernatant was collected and analyzed by HPLC. The reaction was monitored by disappearance of the substrate peak over time. Initial rates (v_0) were then used to calculate $V_{\max}^{\text{app}}$ using eq 5. A standard curve of AUC vs test substrate concentration was prepared and used to convert AUC/min to M/min units. $k_{\text{cat}}^{\text{app}}$ was determined by dividing $V_{\max}^{\text{app}}$ by the concentration of enzyme used in the assay. The overall enzymatic efficiency was then calculated using as the ratio of (k_{cat}/K_M)^{app}.

Liver Cytosol Stability

Pooled human liver cytosol from 10 donors was purchased from ThermoFisher Scientific (Catalog No. HMCYPL, 20 mg/mL total protein concentration). Stability assays were performed by incubating 100 μ M of test substrate with 1 mg/mL liver cytosol and 2.5 mM GSH in 0.1 M phosphate buffer, pH 7.4, at 37 °C with 2% v/v DMSO. Reactions were quenched at specific time points by dilution

with cold MeCN (48% v/v) and TCA (2% v/v) prior to centrifugation at 14,000 rpm for 10 min. The supernatant was collected and analyzed by HPLC. The decreasing AUC of the test substrate peaks were fitted to eq 2 using GraphPad Prism to determine k_{obs} and $t_{1/2} = \ln(2)/k_{\text{obs}}$. Ethacrynic acid-inhibited stability assays were performed in a similar manner, except reactions were incubated with 100 μ M of the inhibitor ($\sim 25 \times IC_{50}$) (Figure S12) for 15 min prior to addition of the test substrate, to ensure GSTA1 activity was completely suppressed. Note that this preincubation is required, since ethacrynic acid exhibits time-dependent product inhibition.

Chai-1 Structure Prediction

Co-structure prediction of GST-GSH-substrate was performed using the Chai-1 Discovery Web server.⁵⁷ Enzyme sequences obtained from UniProt and SMILES codes for compounds were used. Structure prediction was run using templates with multiple sequence alignment, no restraints were specified. The top ranked prediction, which placed the electrophilic center on the substrate within ~ 3 Å of the GSH thiol, was selected and rendered on PyMOL.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.6c00826>.

Supplementary figures; kinetic plate reader and HPLC data, Michaelis–Menten and Lineweaver–Burk plots, EC_{50} plots, standard curves, molecular models, compound purity; NMR spectra of new compounds, HPLC traces of the final compounds, LC-MS traces of representative GSH adducts (PDF)

Molecular formula strings; SMILES codes, k_{cat}/K_M values, $t_{1/2}$ in liver cytosol, $\log P$ values (CSV)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CDNB	1-chloro-2,4-dinitrobenzene
DIPEA	diisopropylethylamine
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EER	Enzymatic Efficiency Ratio
EGFR	Epidermal Growth Factor Receptor
Et ₂ O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
EWG	Electron withdrawing group
GSH	glutathione
HATU	O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
k_2^{GSH}	second order intrinsic reactivity rate constant
k_{cat}	enzymatic turnover number
K_M	substrate concentration at $1/2 V_{max}$
k_{cat}/K_M	catalytic efficiency
LG	Leaving group
MeCN	acetonitrile
MeOH	methanol
NEt ₃	triethylamine
NMI	N-methyl imidazole
TCFH	N'-tetramethylformamidium hexafluorophosphate
TCI	Targeted Covalent Inhibitor
T3P	propylphosphonic anhydride
SAR	Structure–Activity Relationships
v_0	initial rate
V_{max}	maximum enzymatic rate at saturation

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4.5. Chapter 4 Supporting Information

4.5.1 Intrinsic Reactivity

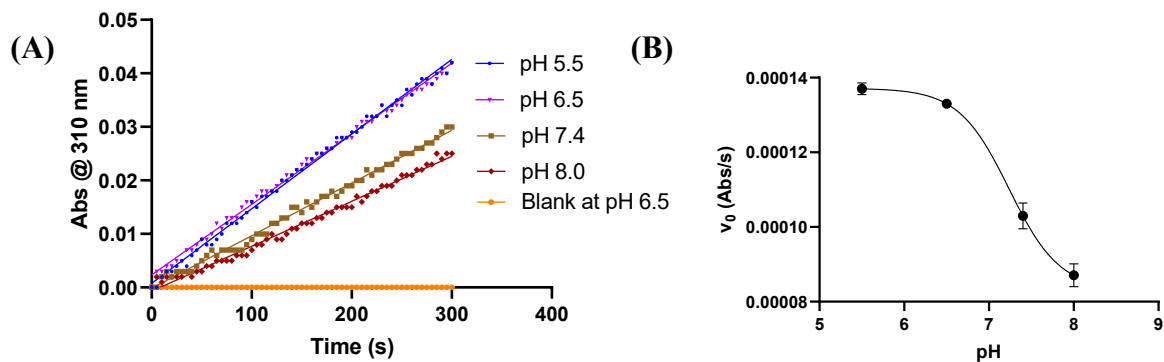
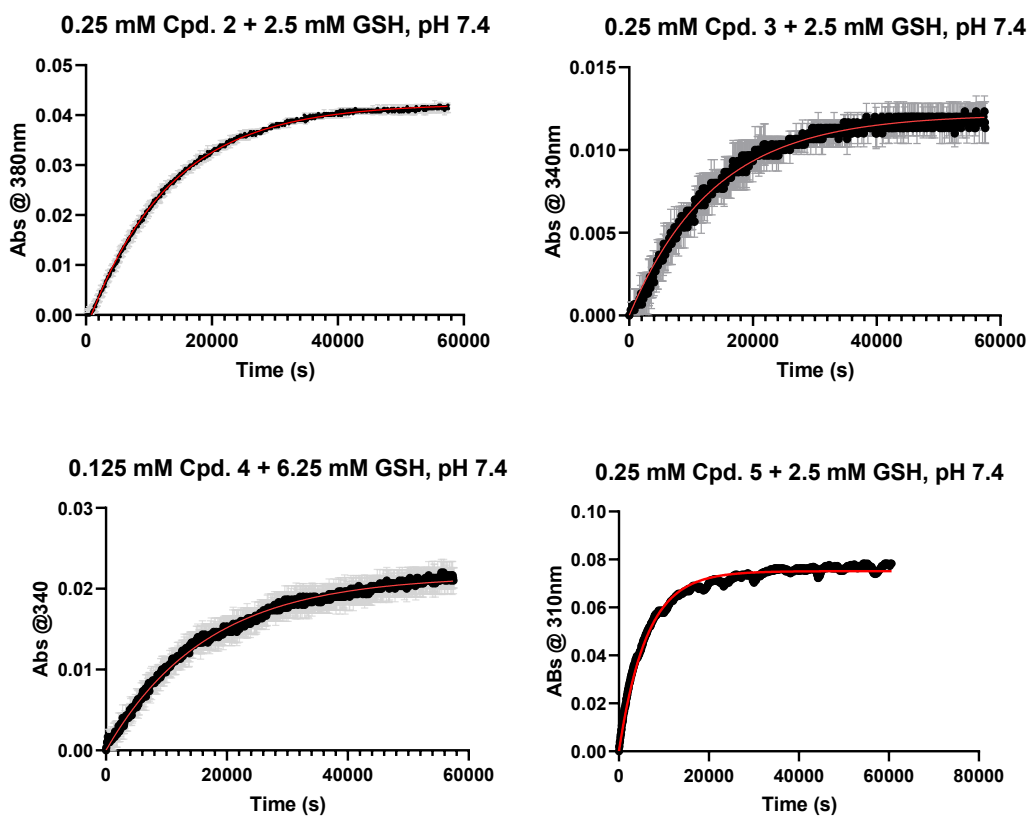
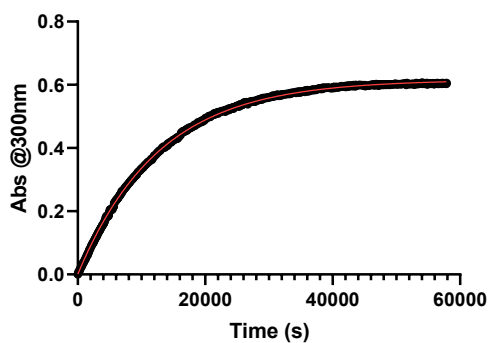


Figure 4.S1. Intrinsic reactivity rate of Compound **21** decreases as pH increases. (A) Initial rate data. (B) Rate vs. pH profile.

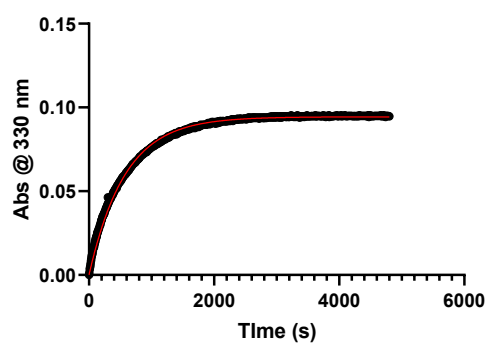
4.5.1.1. Kinetic Data from Plate Reader and HPLC



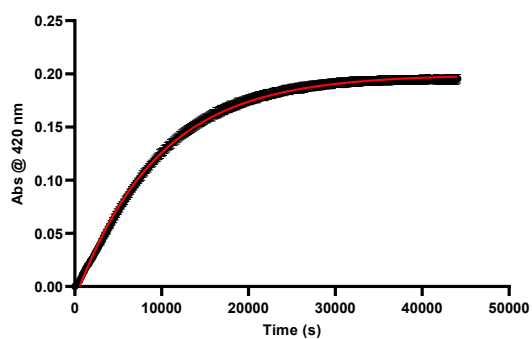
0.25 mM Cpd. 6 + 12.5 mM GSH, pH 7.4



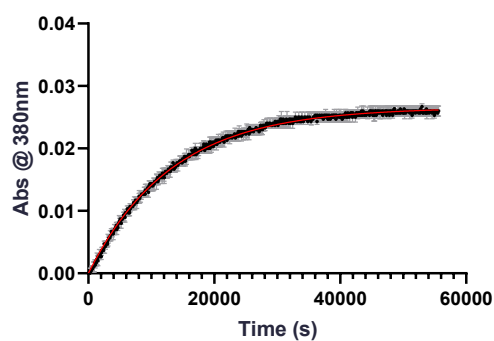
0.25 mM Cpd. 7 + 2.5 mM GSH, pH 6.5



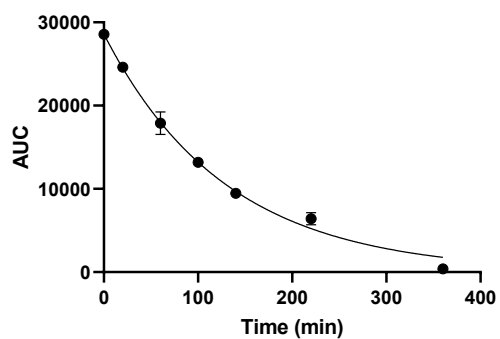
0.25 mM Cpd. 8 + 2.5 mM GSH, pH 7.4



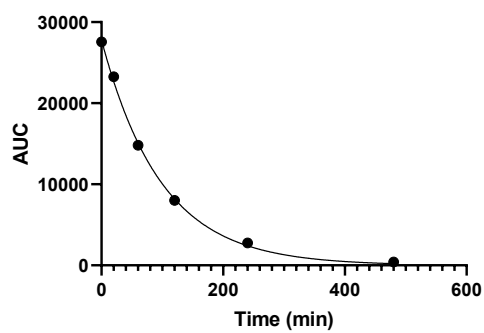
0.25 mM Cpd. 9 + 2.5 mM GSH, pH 7.4

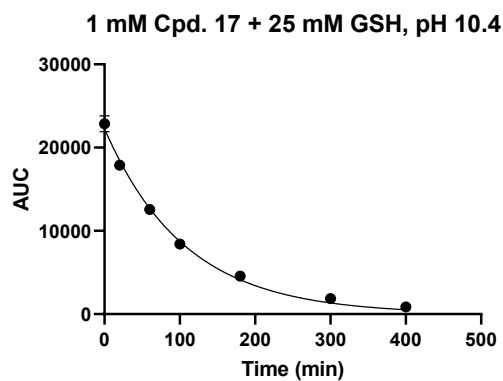
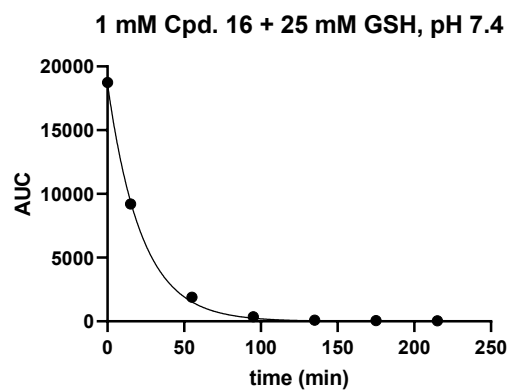
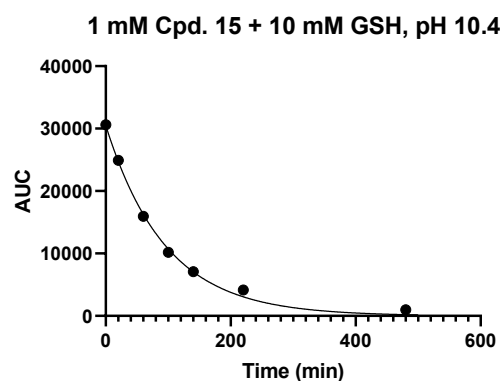
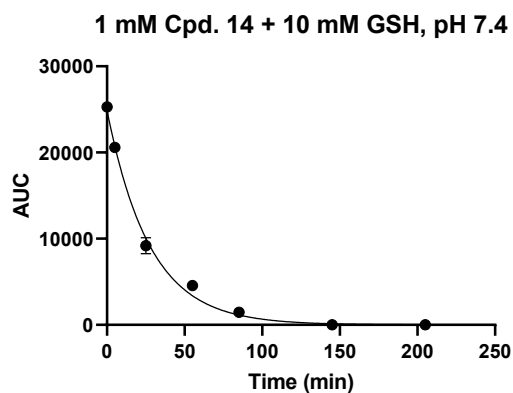
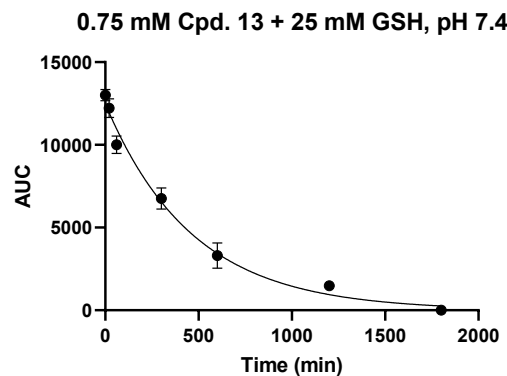
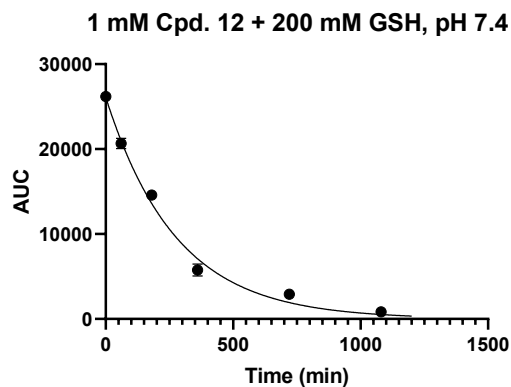


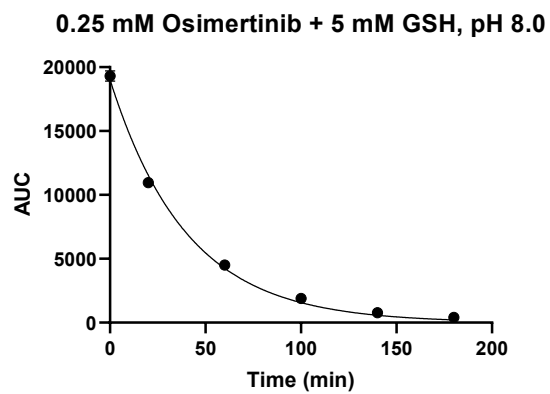
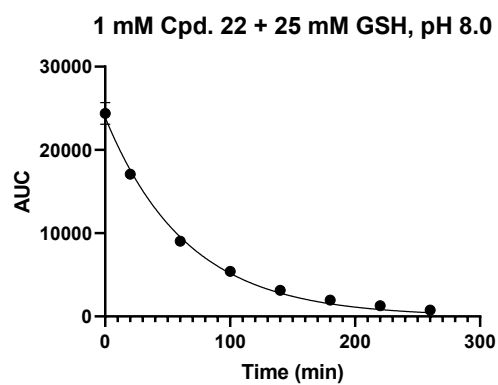
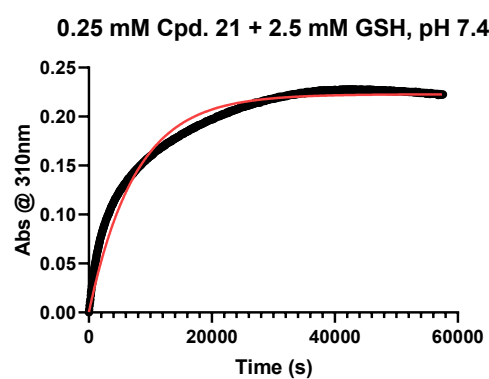
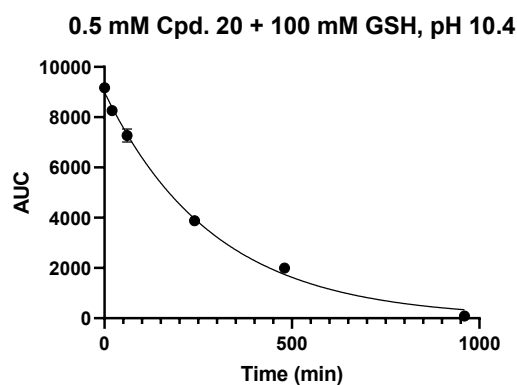
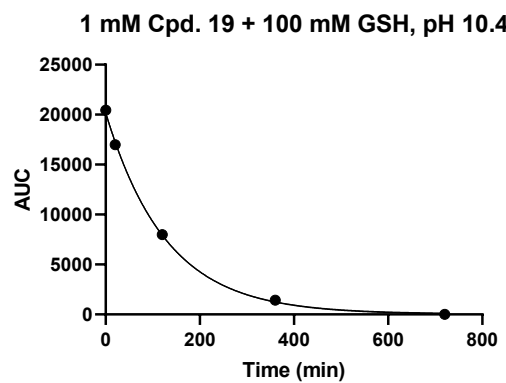
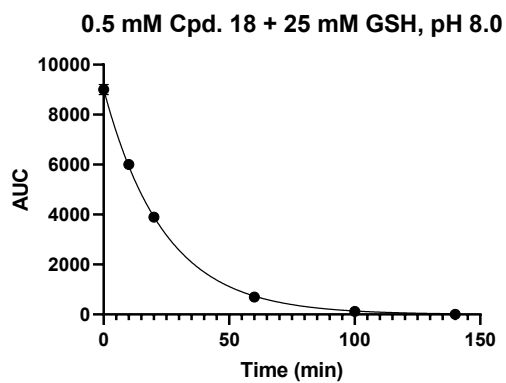
1 mM Cpd. 10 + 10 mM GSH, pH 10.4



1 mM Cpd. 11 + 100 mM GSH, pH 10.4







4.5.2. Enzyme Kinetics

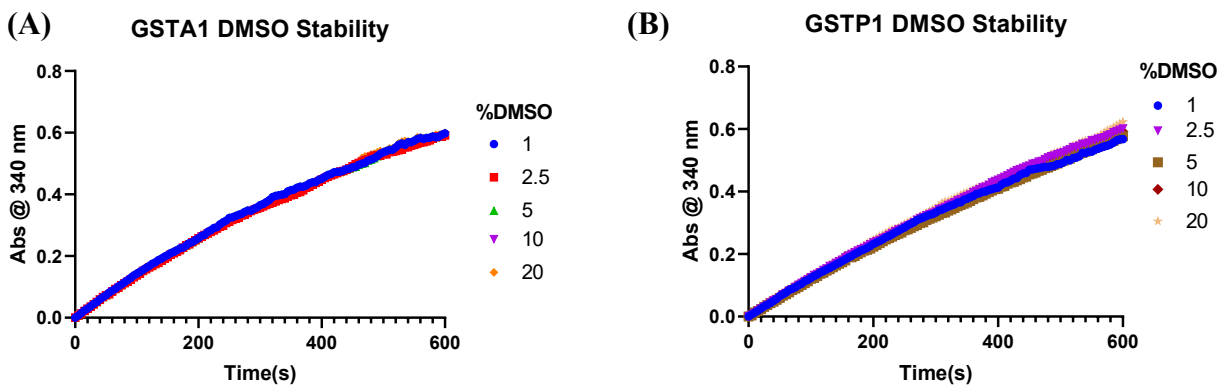


Figure 4.S2. DMSO stability of (A) GSTA1 and (B) GSTP1 in CDNB activity assay.

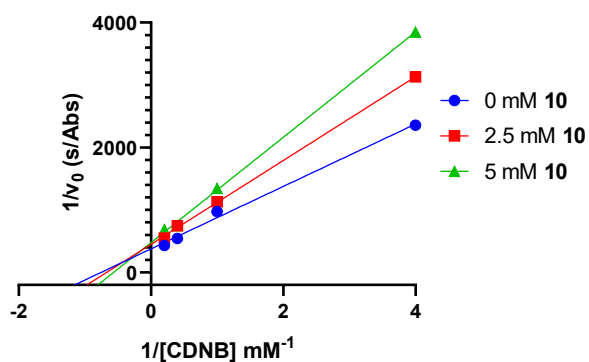


Figure 4.S3. CDNB shows mixed non-competitive binding with compound **10** for GSTP1. This is evident by the negative-x, negative-y quadrant intersection of the Lineweaver-Burk plots.

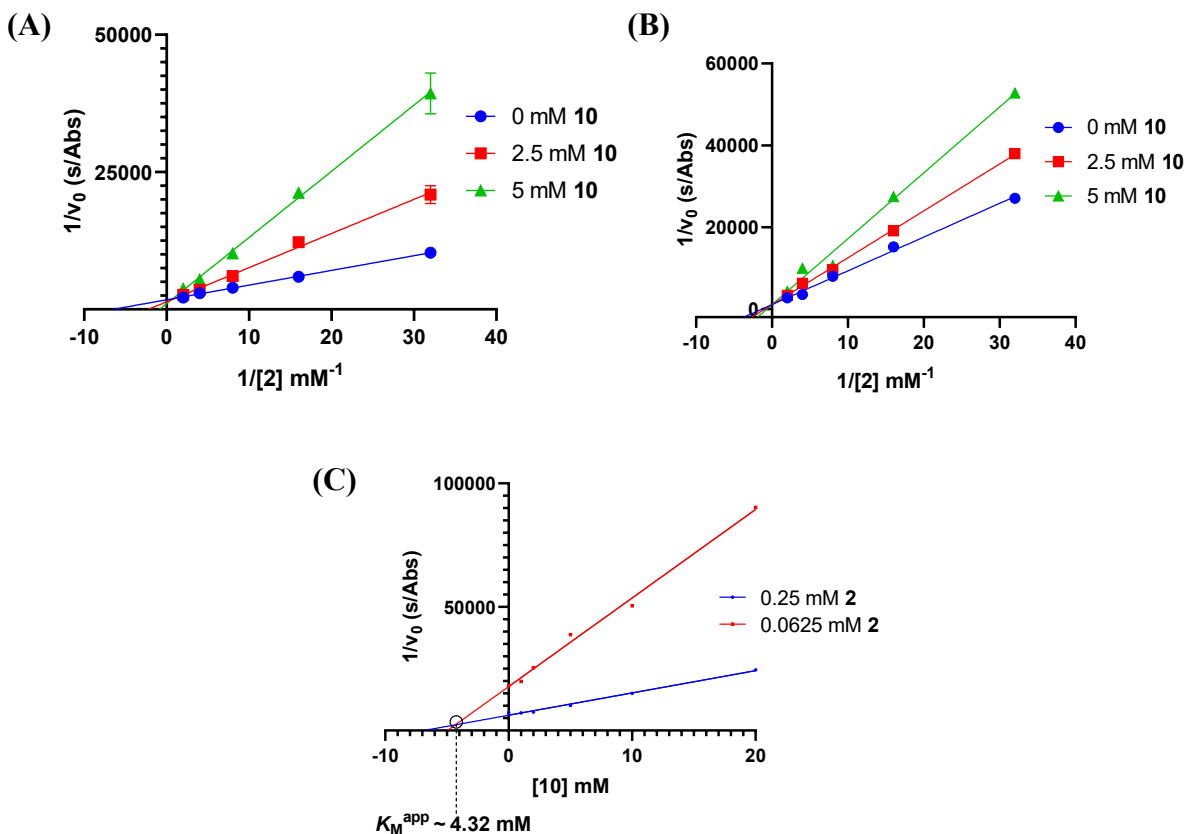


Figure 4.S4. Reporter substrate **2** shows competitive binding with compound **10** for (A) GSTA1 and (B) GSTP1. This is evident by the y-intercept intersection of the Lineweaver-Burk plots. (C) Dixon plot is consistent with competitive behaviour and yields a K_M^{app} for GSTP1 consistent with value obtained by Cheng-Prusoff estimation.

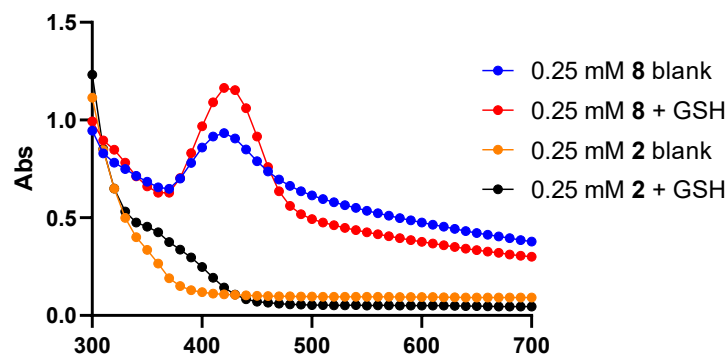


Figure 4.S5. Spectral scan of compound **2**-GSH adduct (maximum difference between adduct and substrate Abs occurs at 380 nm) and compound **8**-GSH adduct (maximum difference between adduct and substrate Abs occurs at 420 nm). This difference in absorbance spectra allowed compound **8** to be evaluated in the competitive displacement assay with reporter substrate **2** without spectral interference.

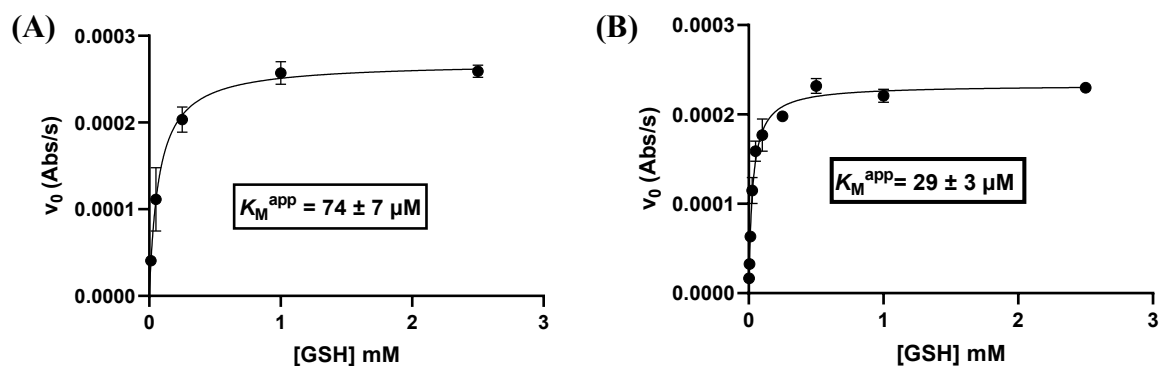
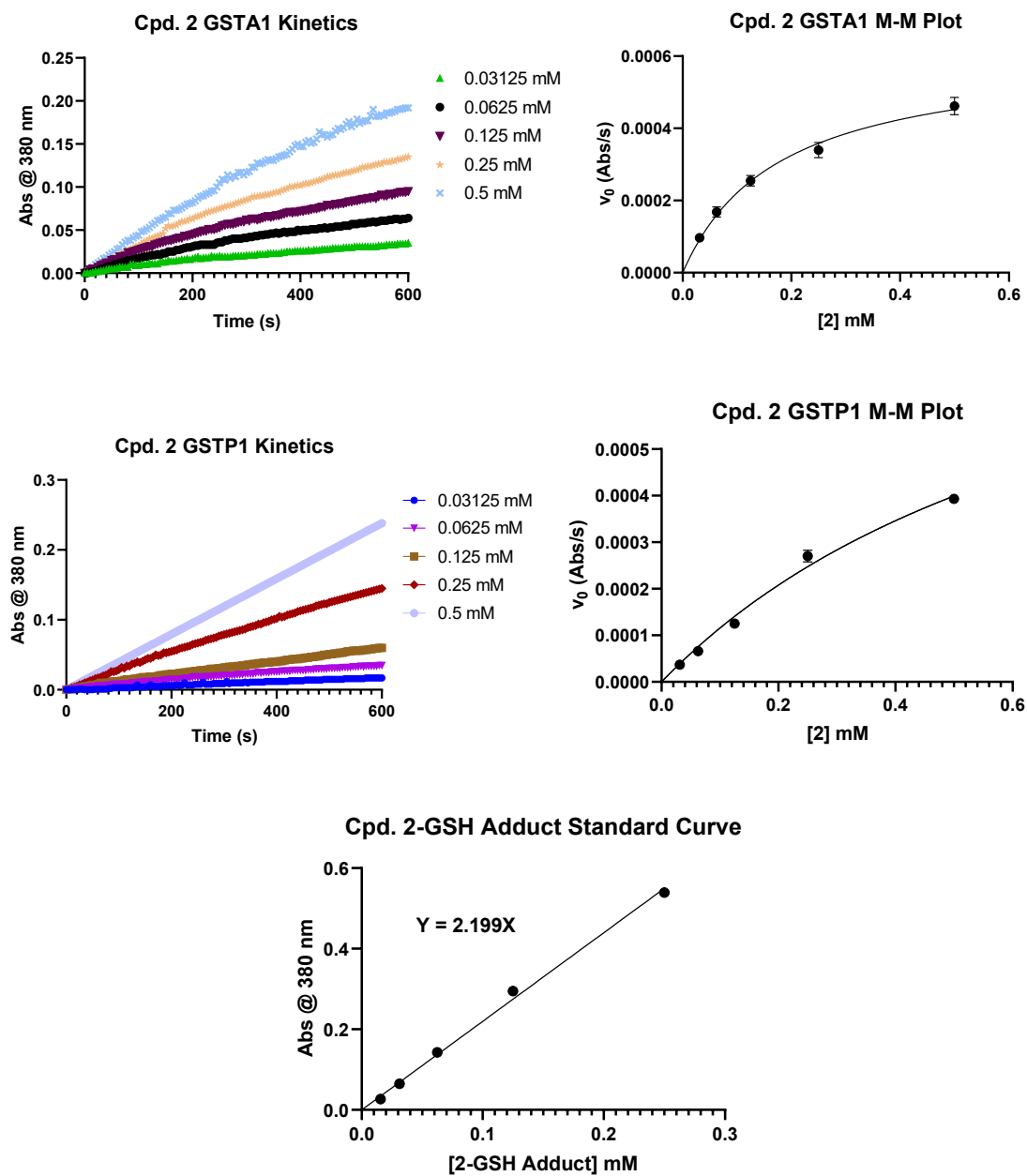


Figure 4.S6. K_M^{app} of GSH at 0.25 mM reporter substrate **2** with (A) GSTA1 and (B) GSTP1.

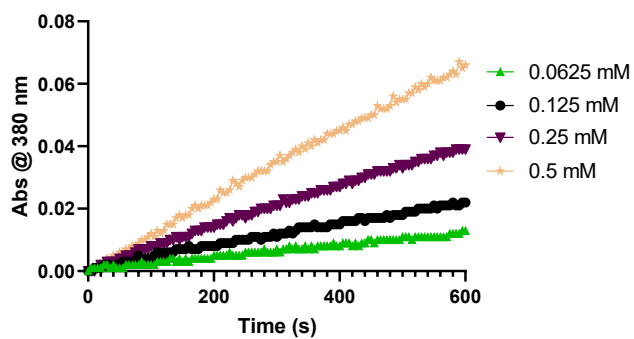
4.5.2.1. Direct Spectrophotometric Assay Kinetics and Standard Curves

Compound 2

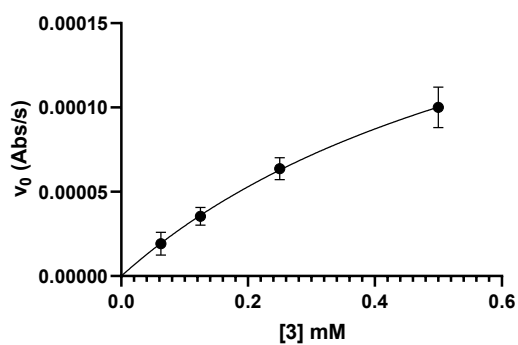


Compound 3

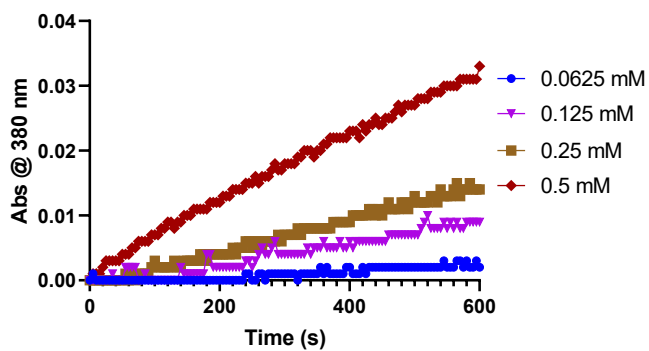
Cpd. 3 GSTA1 Kinetics



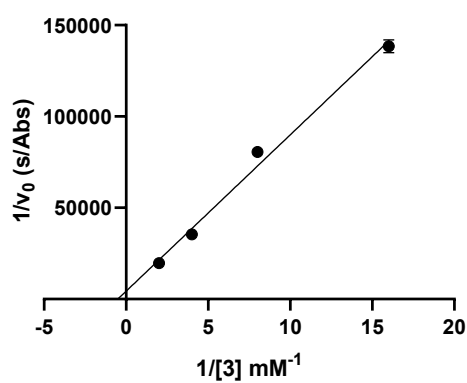
Cpd. 3 GSTA1 M-M Plot



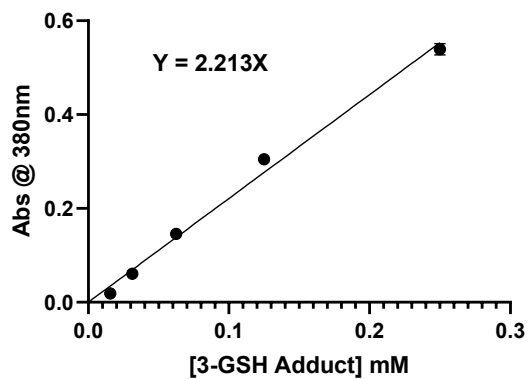
Cpd. 3 GSTP1 Kinetics



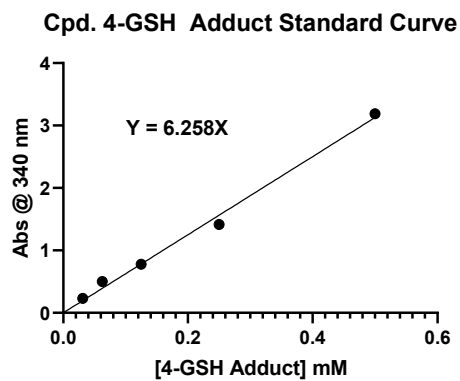
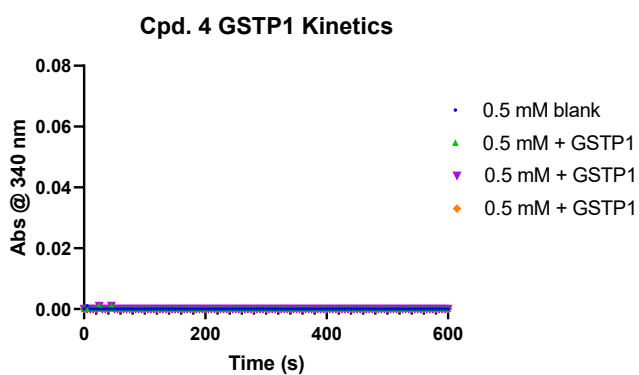
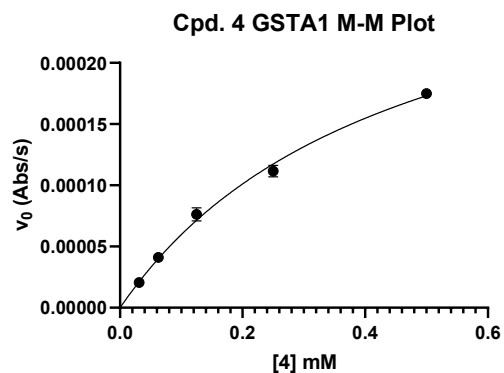
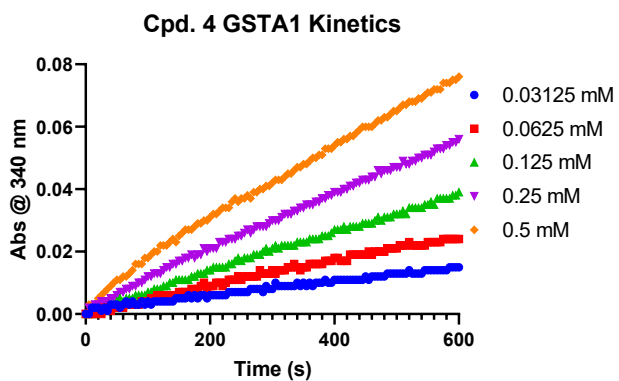
Cpd. 3 GSTP1 LWB Plot



Cpd. 3-GSH Adduct Standard Curve

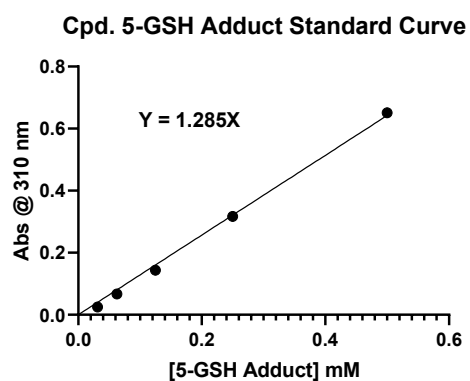
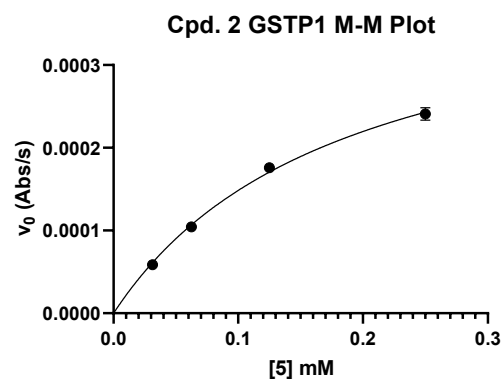
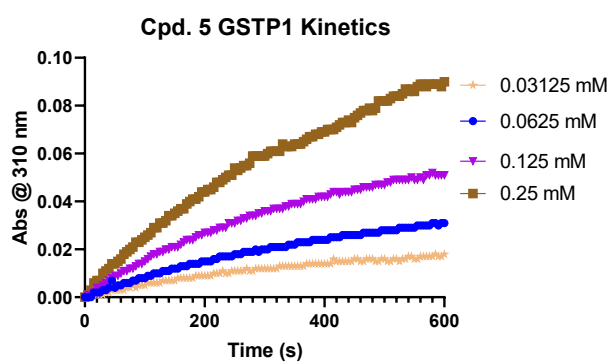
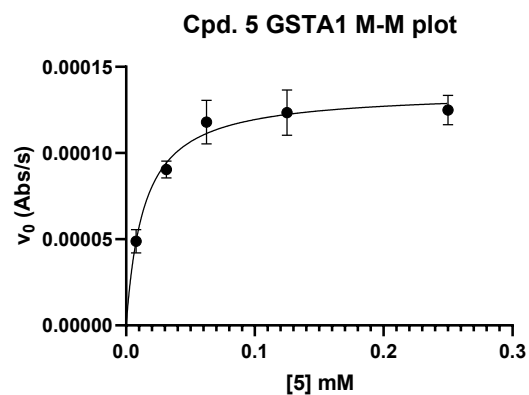
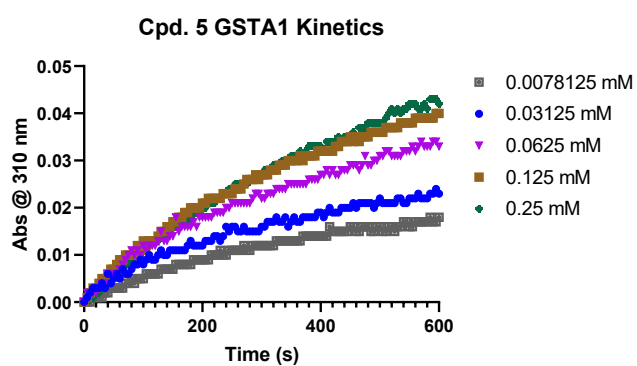


Compound 4



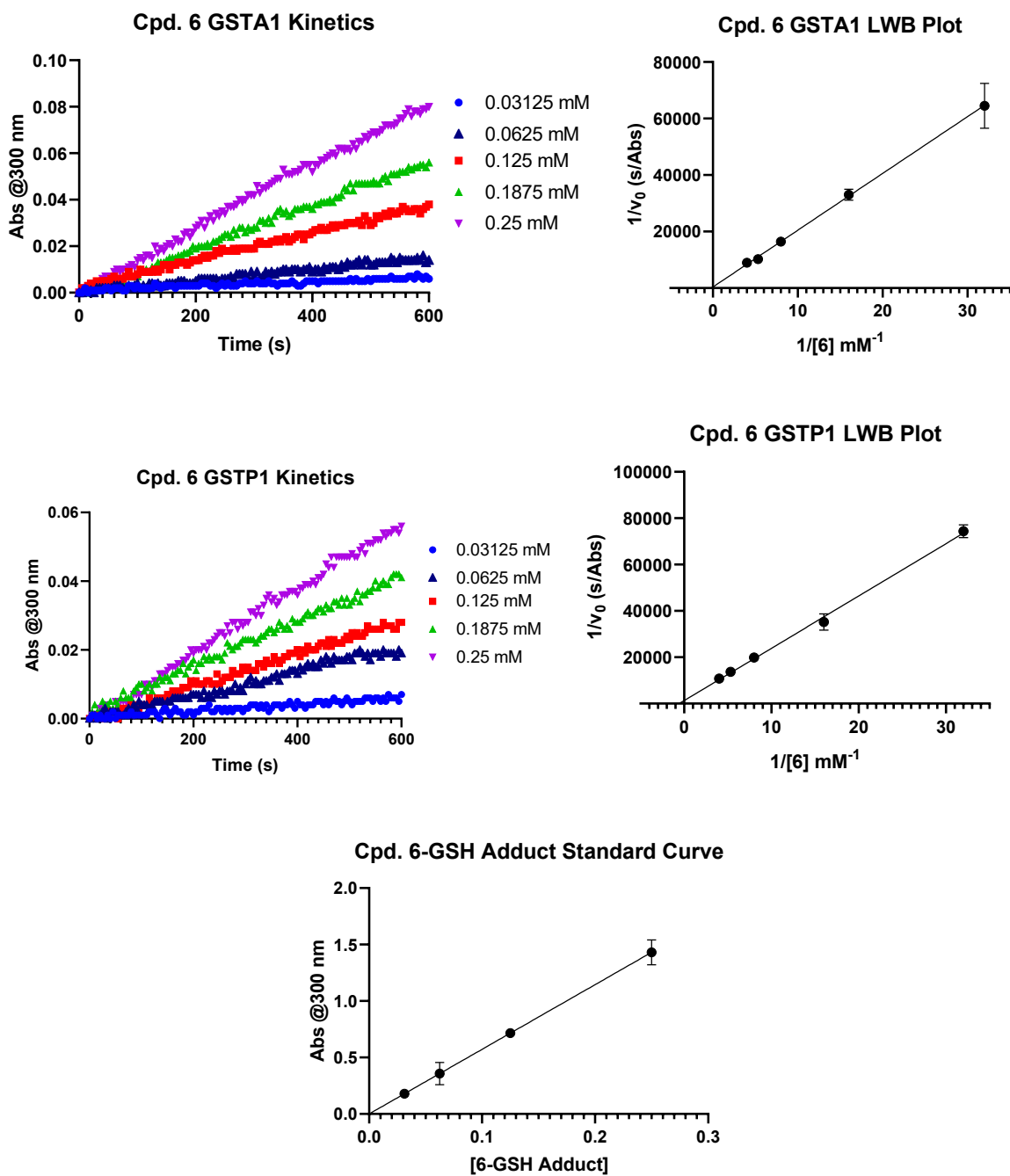
*Note: Cpd. 4 was tested with GSTP1 using 1000 mU/mL ($\approx$ 1000 nM) of enzyme.

Compound 5



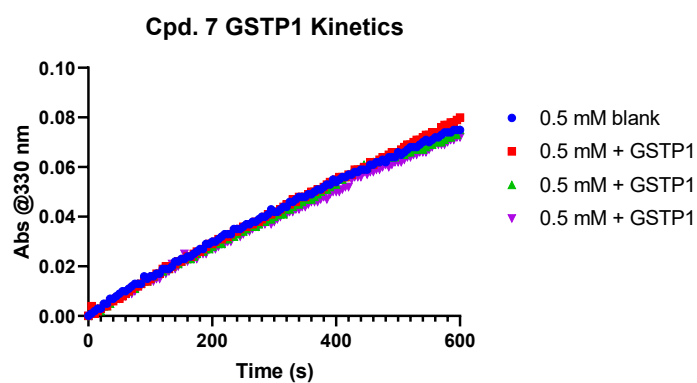
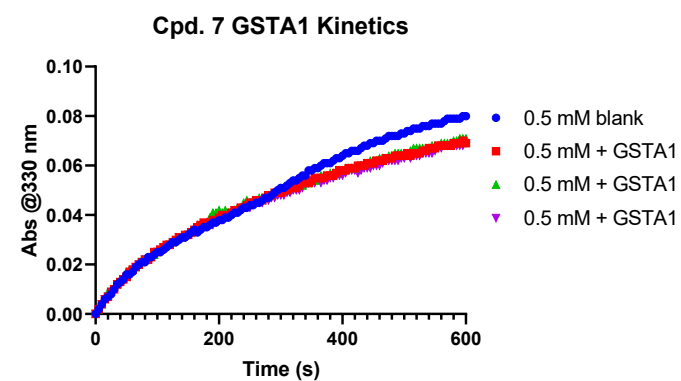
*Note: 10 mU/mL (≈ 37 nM) GSTA1 and 100 mU/mL (≈ 100 nM) GSTP1 were used.

Compound 6



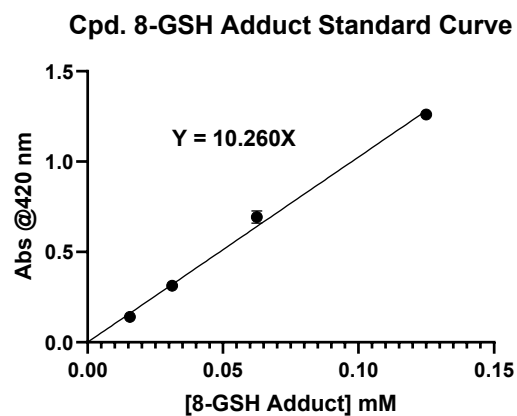
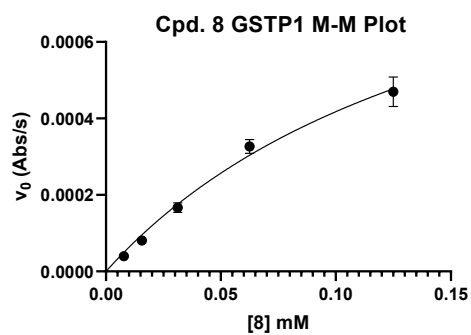
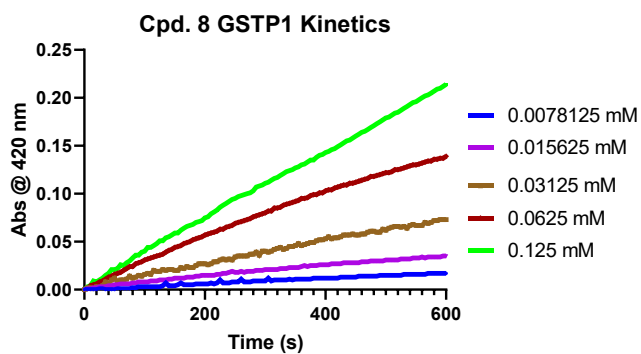
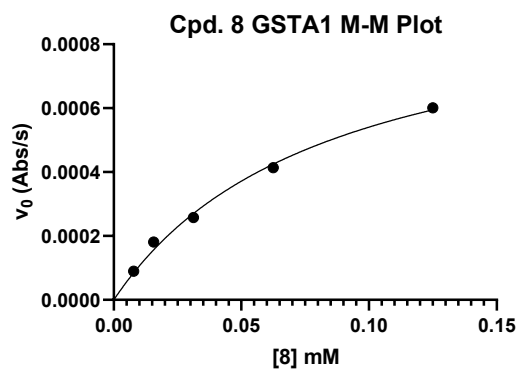
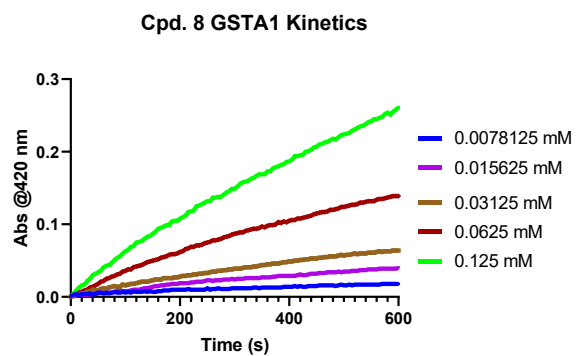
*Note 500 mU/mL (≈ 1864 nM) of GSTA1 and 500 mU/mL (≈ 500 nM) of GSTP1 were used.

Compound 7

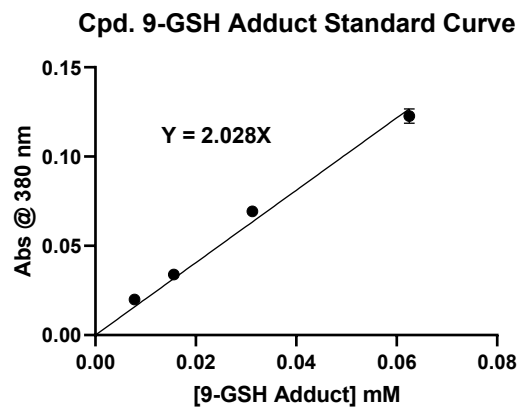
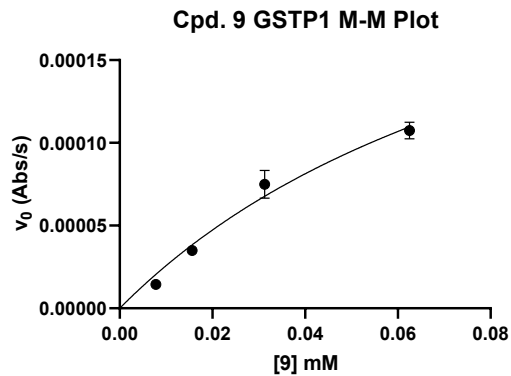
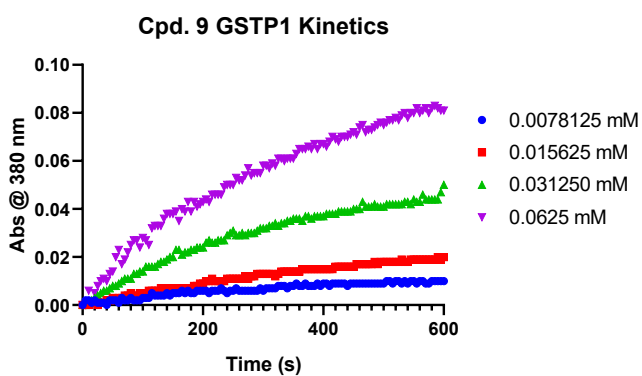
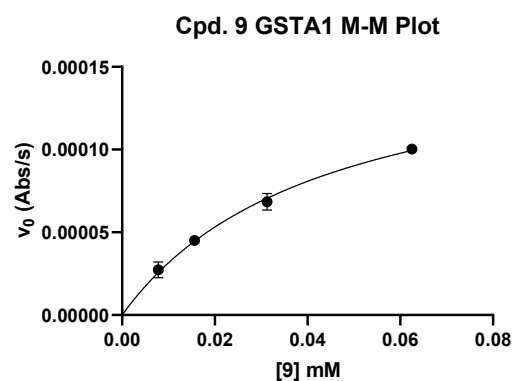
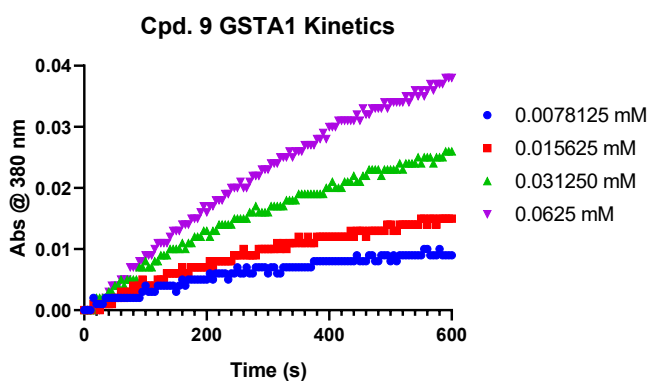


**Note: 500 mU/mL (≈ 1864 nM) of GSTA1 and 1000 mU/mL (≈ 1000 nM) GSTP1 were used.*

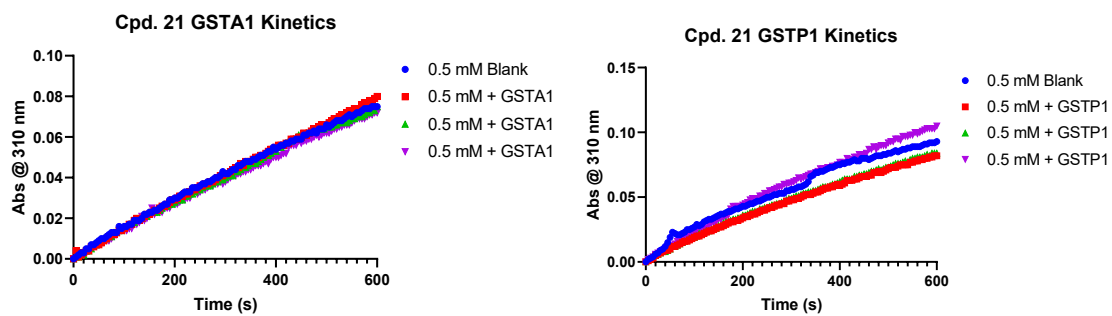
Compound 8



Compound 9



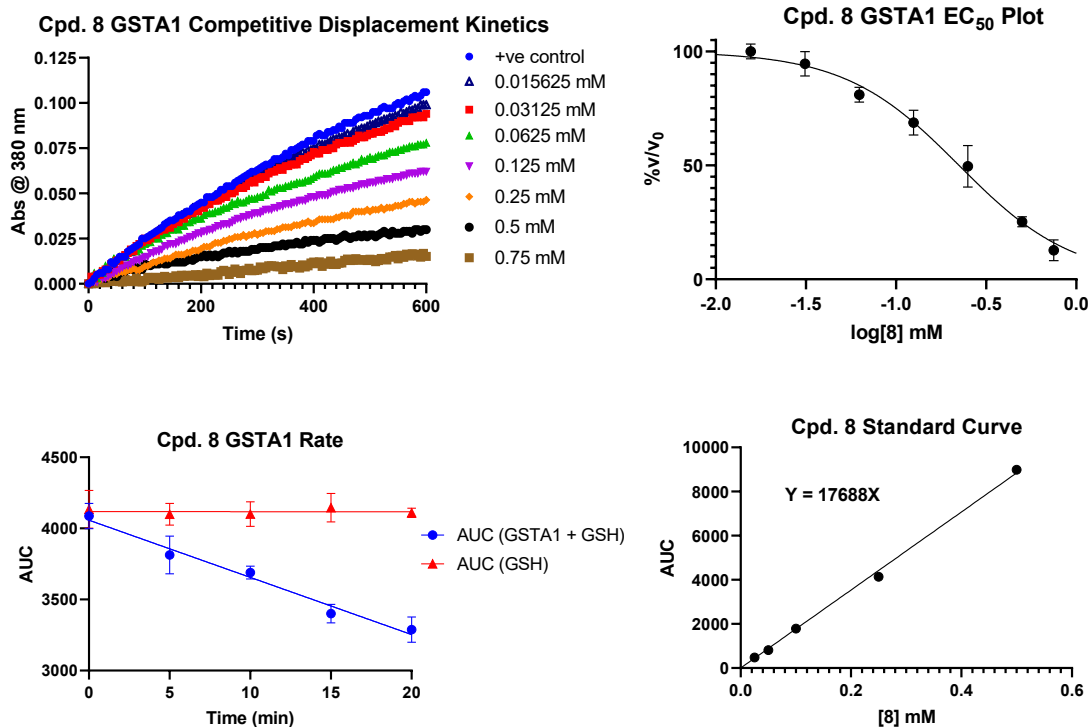
Compound 21



**Note: 500 mU/mL (≈ 1864 nM) of GSTA1 and 1000 mU/mL (≈ 1000 nM) GSTP1 were used.*

4.5.2.2. Competitive Displacement and HPLC Rate Assay Kinetics and Standard Curves

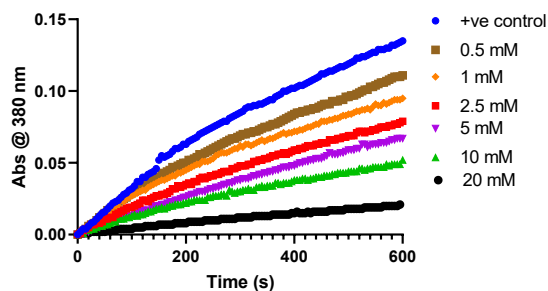
Compound 8



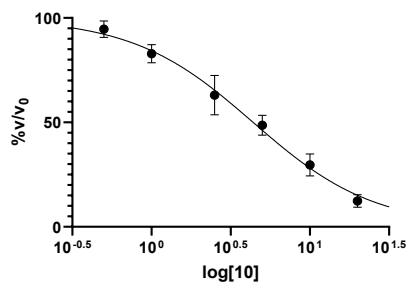
*Note 25 mU/mL ($\approx$ 93 nM) GSTA1 was used, 0.75 mM Cpd. 8 was used for HPLC rate determination, and the reaction was diluted 3-fold prior to analysis.

Compound 10

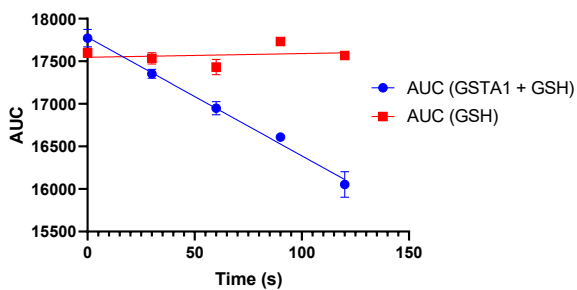
Cpd. 10 GSTA1 Competitive Displacement Kinetics



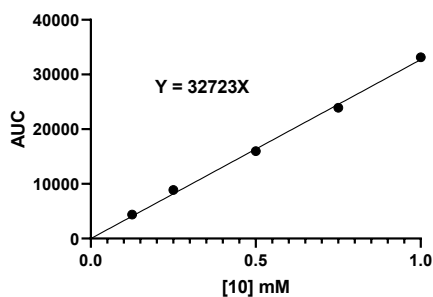
Cpd. 10 GSTA1 EC₅₀ Plot



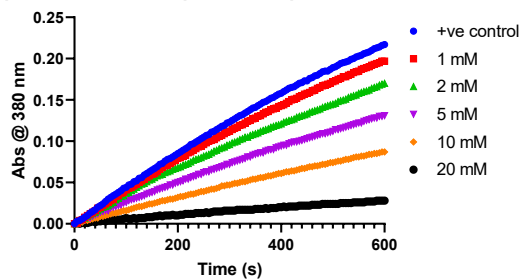
Cpd. 10 GSTA1 Rate



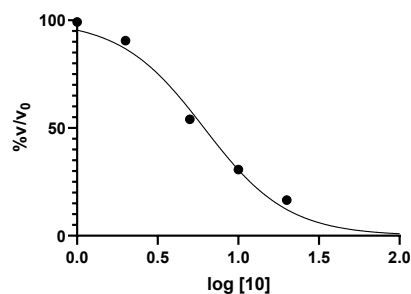
Cpd. 10 Standard Curve



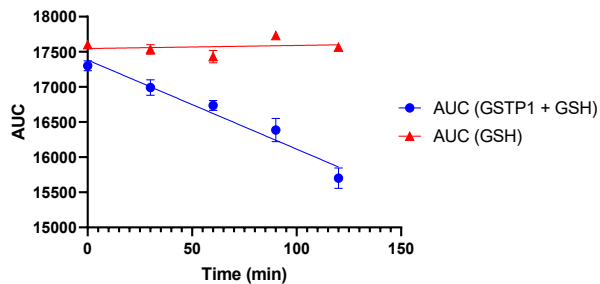
Cpd. 10 GSTP1 Competitive Displacement Kinetics



Cpd. 10 GSTP1 EC₅₀ Plot



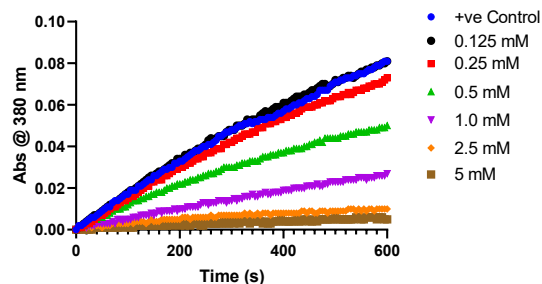
Cpd. 10 GSTP1 Rate



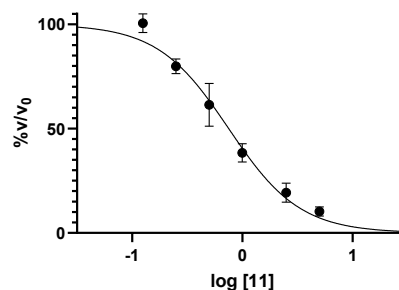
**Note: 5 mM of Cpd. 10 was used in HPLC rate determination, and the reaction was diluted 10-fold prior to analysis.*

Compound 11

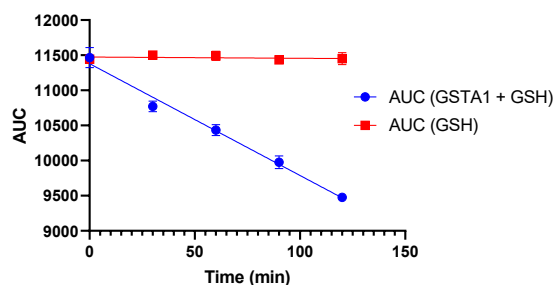
Cpd. 11 GSTA1 Competitive Displacement Kinetics



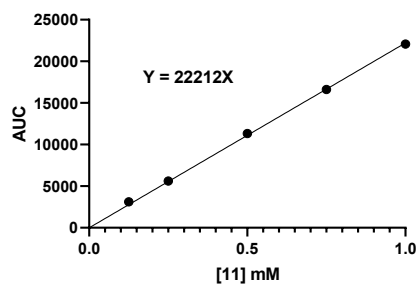
Cpd. 11 GSTA1 EC₅₀ Plot



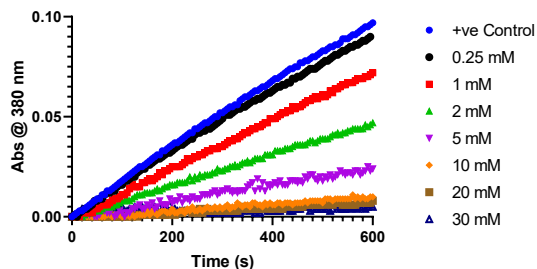
Cpd. 11 GSTA1 Rate



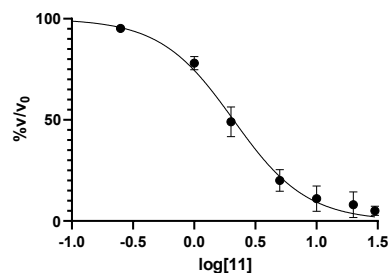
Cpd. 11 Standard Curve



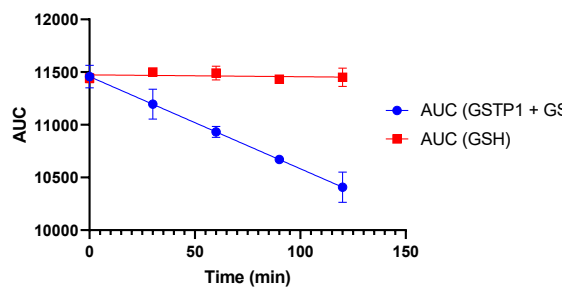
Cpd. 11 GSTP1 Competitive Displacement Kinetics



Cpd. 11 GSTP1 EC₅₀ Plot



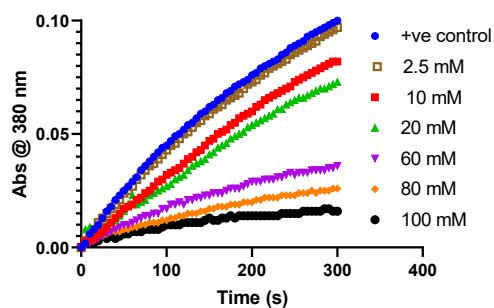
Cpd. 11 GSTP1 Rate



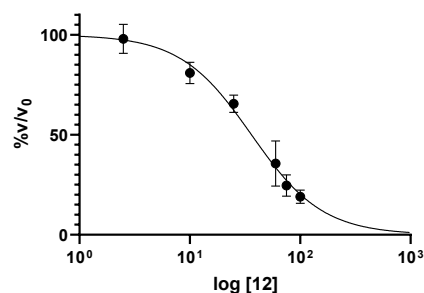
**Note: 5 mM of Cpd. 11 was used in HPLC rate determination, and the reaction was diluted 10-fold prior to analysis.*

Compound 12

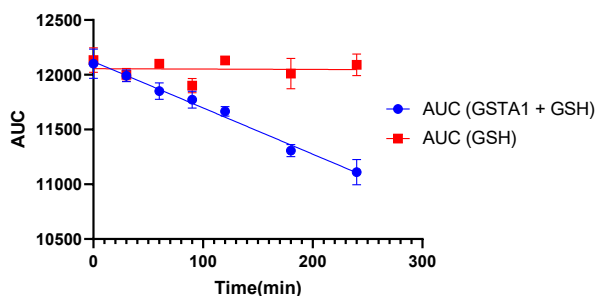
Cpd. 12 GSTA1 Competitive Displacement Kinetics



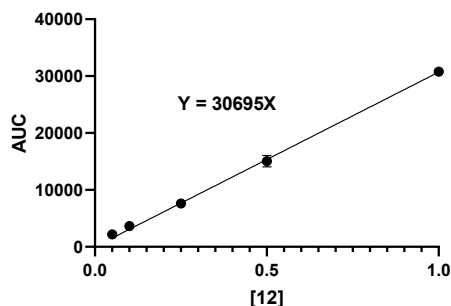
Cpd. 12 GSTA1 EC₅₀ Plot



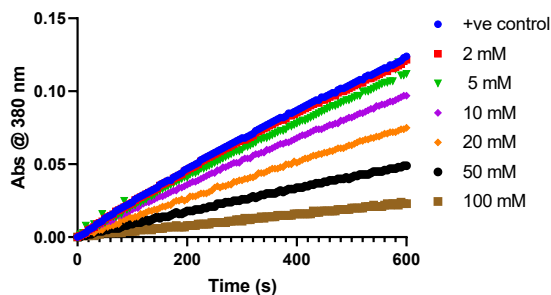
Cpd. 12 GSTA1 Rate



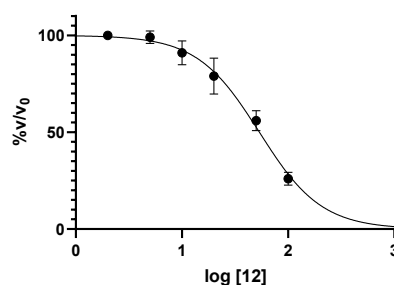
Cpd.12 Standard Curve



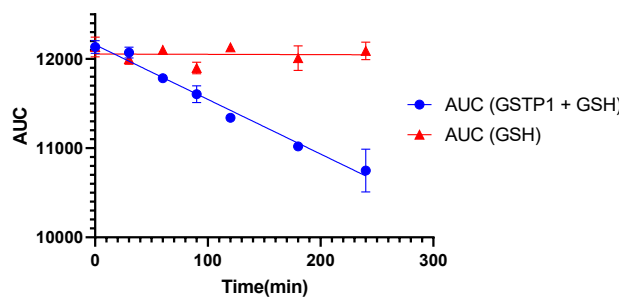
Cpd. 12 GSTP1 Competitive Displacement Kinetics



Cpd. 12 GSTP1 EC₅₀ Plot



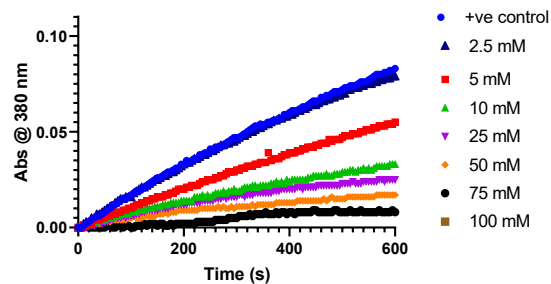
Cpd. 12 GSTP1 Rate



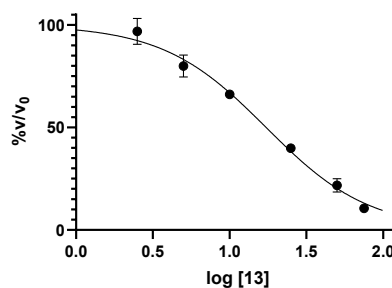
**Note: 0.125 mM reporter substrate was used in competitive displacement assay. 1000 mU/mL ($\approx$ 1000 nM) of GSTP1 was used in the HPLC assay. 50 mM of Cpd. 12 was used in each HPLC assay, and the reaction was diluted 100-fold prior to analysis.*

Compound 13

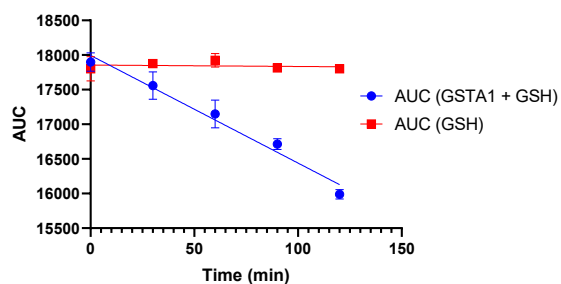
Cpd. 13 GSTA1 Competitive Displacement Kinetics



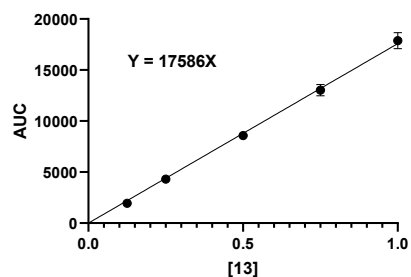
Cpd. 13 GSTA1 EC₅₀ Plot



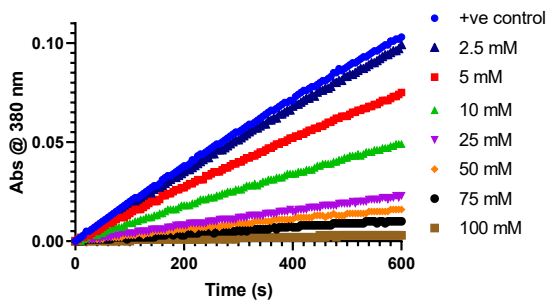
Cpd. 13 GSTA1 Rate



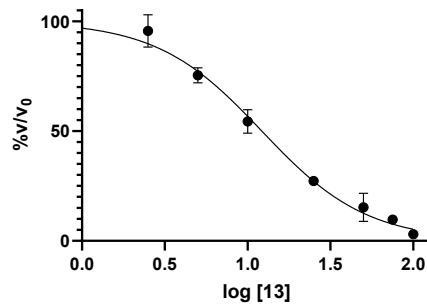
Cpd. 13 Standard Curve



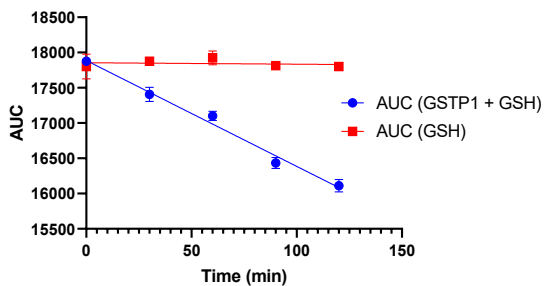
Cpd. 13 GSTP1 Competitive Displacement Kinetics



Cpd. 13 GSTP1 EC₅₀ Plot



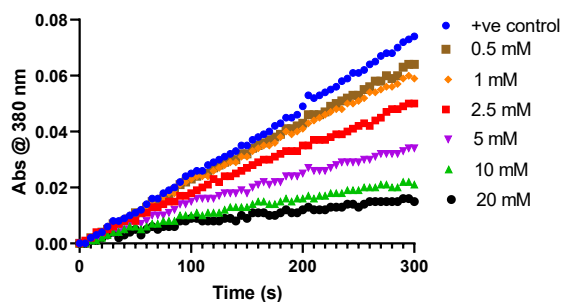
Cpd. 13 GSTP1 Rate



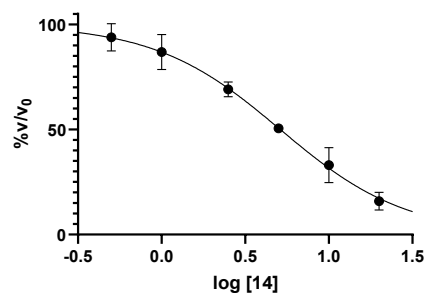
**Note: 50 mM of Cpd. 13 was used in each HPLC assay, and the reaction was diluted 50-fold prior to analysis.*

Compound 14

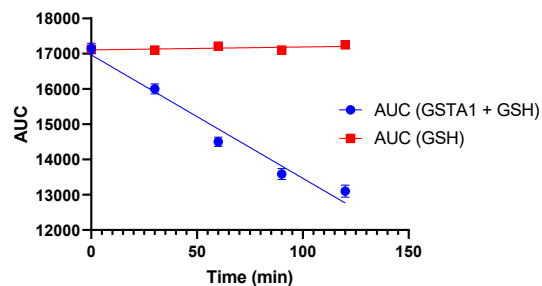
Cpd. 14 GSTA1 Competitive Displacement Kinetics



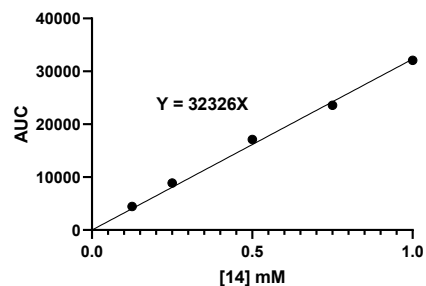
Cpd. 14 GSTA1 EC₅₀ Plot



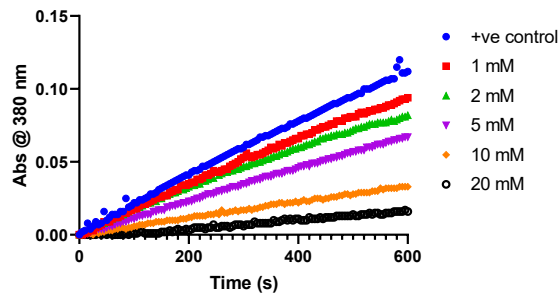
Cpd. 14 GSTA1 Rate



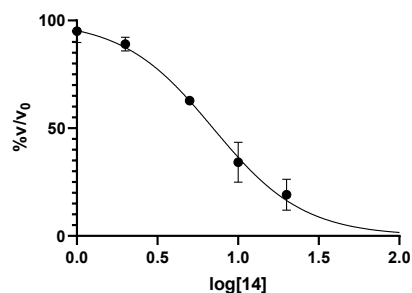
Cpd. 14 Standard Curve



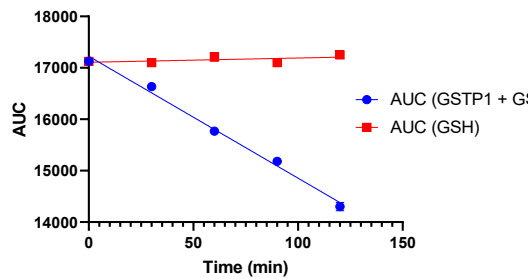
Cpd. 14 GSTP1 Competitive Displacement Kinetics



Cpd. 14 GSTP1 EC₅₀ Plot

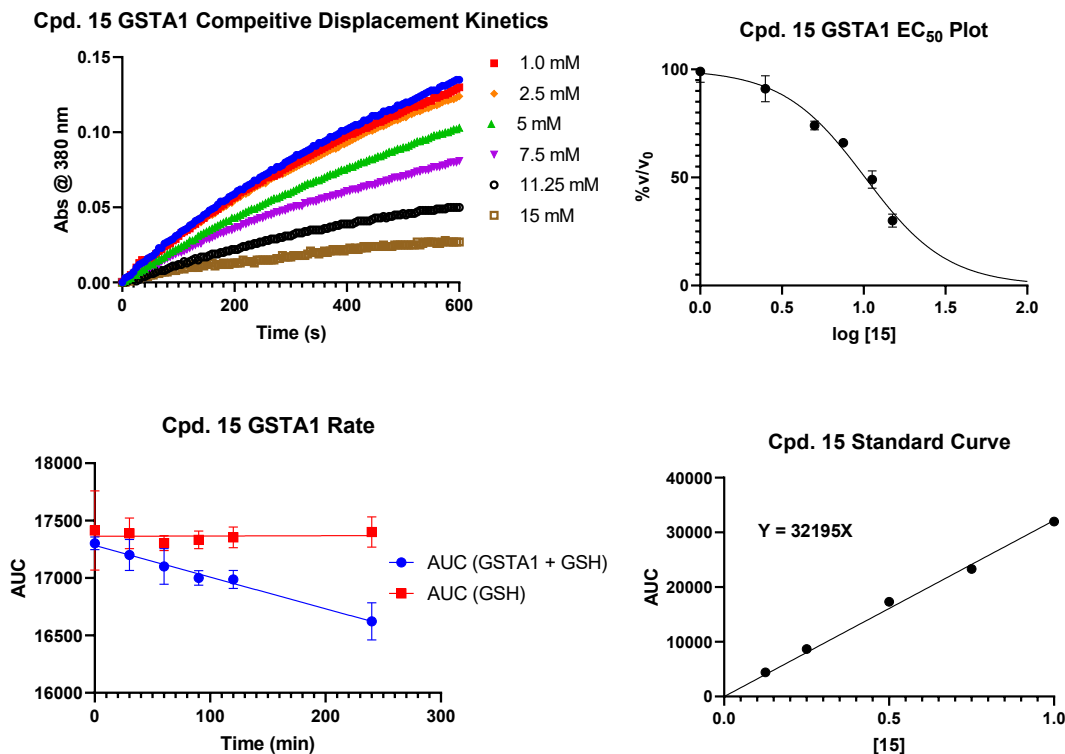


Cpd. 14 GSTP1 Rate

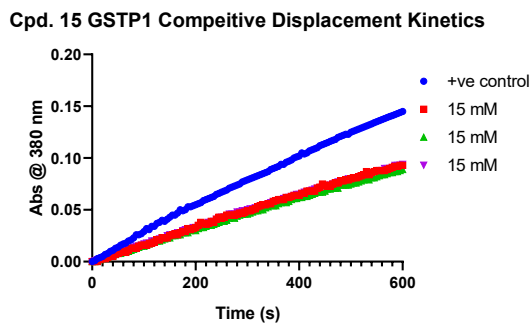


**Note: 5 mM of Cpd. 14 was used in each HPLC assay, and the reaction was diluted 10-fold prior to analysis. 25 mU/mL ($\approx$ 93 nM) of GSTA1 and 100 mU/mL ($\approx$ 100 nM) of GSTP1 were used in the HPLC assay.*

Compound 15



*Note: 5 mM of Cpd. 15 was used in each HPLC assay, and the reaction was diluted 10-fold prior to analysis.

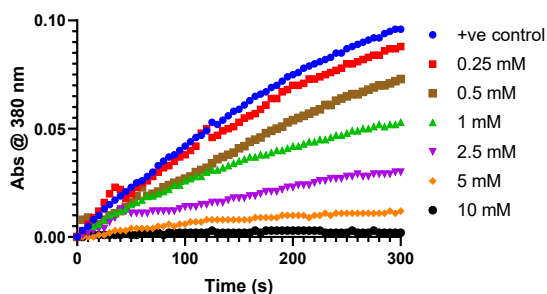


*With GSTP1 and 0.125 mM reporter substrate, EC₅₀ > 15 mM. If EC₅₀ is set to 15 mM the Cheng-

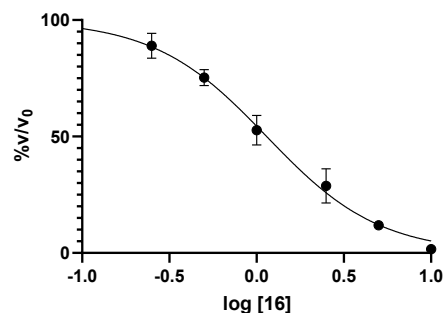
Prusoff equation: $K_{M,S_T}^{app} = \frac{EC_{50}}{1 + \frac{[S_R]}{K_{M,S_R}^{app}}}$ gives a K_M^{app} of 12 mM, therefore we state that $K_M^{app} > 10$ mM.

Compound 16

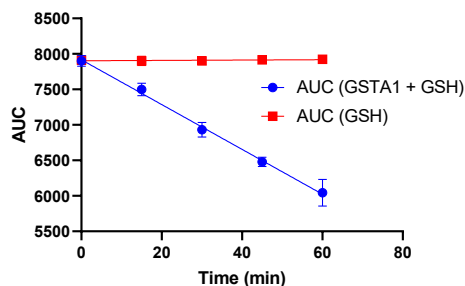
Cpd. 16 GSTA1 Competitive Displacement Kinetics



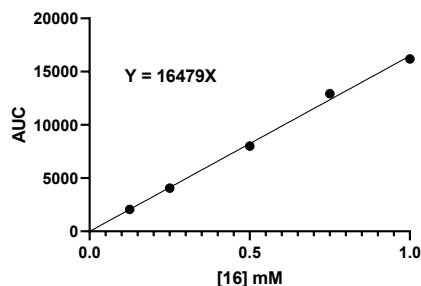
Cpd. 16 GSTA1 EC₅₀ Plot



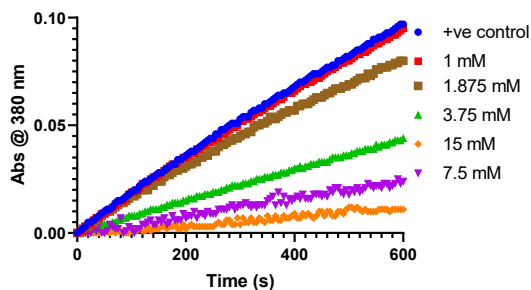
Cpd. 16 GSTA1 Rate



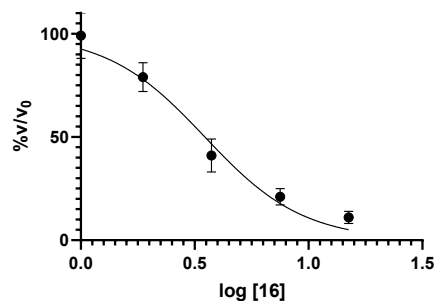
Cpd. 16 Standard Curve



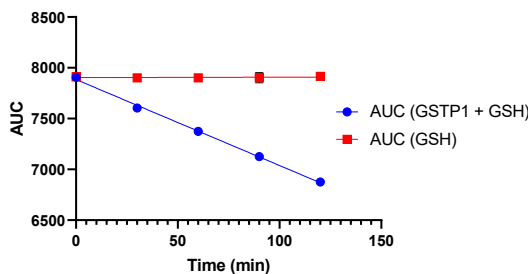
Cpd. 16 GSTP1 Competitive Displacement Kinetics



Cpd. 16 GSTP1 EC₅₀ Plot



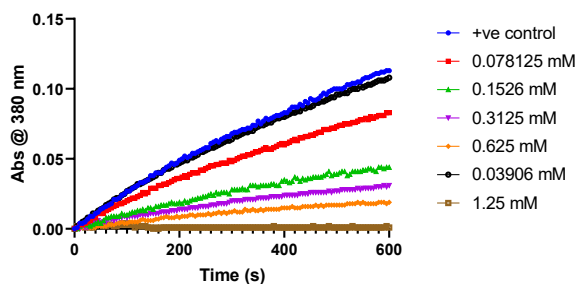
Cpd. 16 GSTP1 Rate



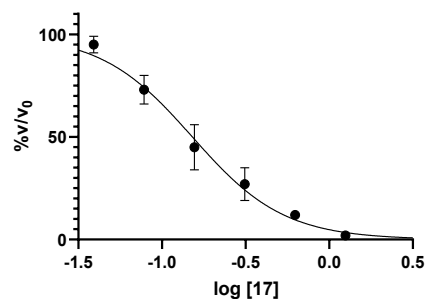
**Note: 5 mM of Cpd. 16 was used in each HPLC assay, and the reaction was diluted 10-fold prior to analysis. 100 mU/mL ($\approx$ 100 nM) of GSTP1 was used in the HPLC assay.*

Compound 17

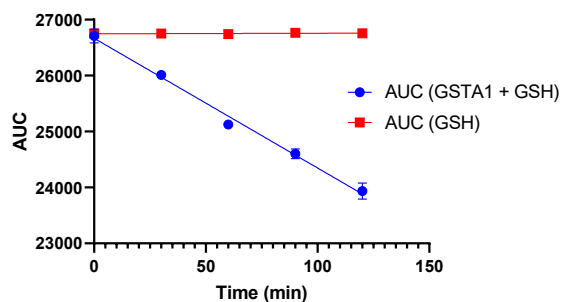
Cpd. 17 GSTA1 Competitive Displacement Kinetics



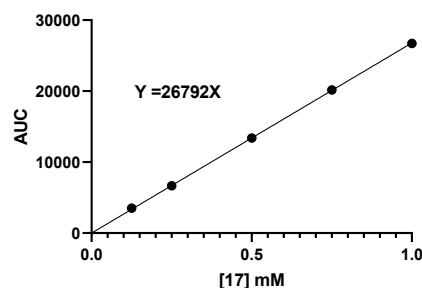
Cpd. 17 GSTA1 EC₅₀ Plot



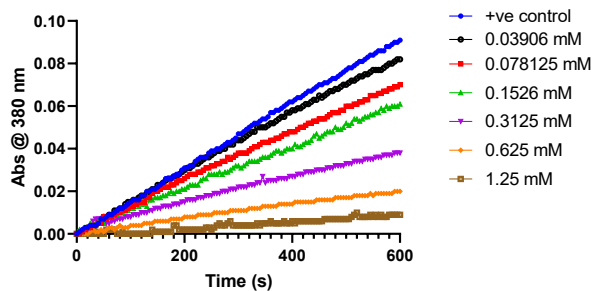
Cpd. 17 GSTA1 Rate



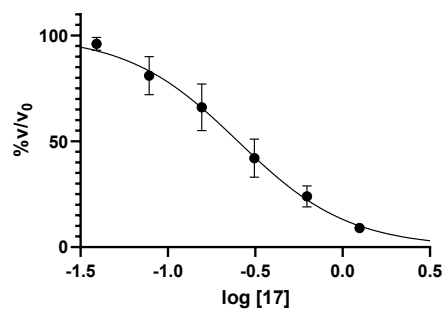
Cpd. 17 Standard Curve



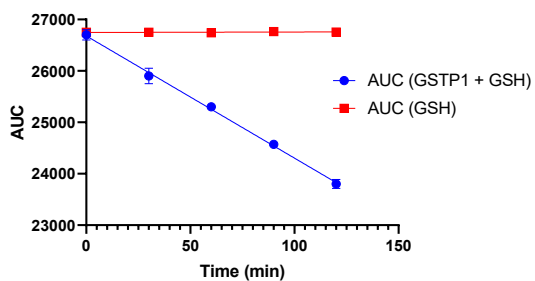
Cpd. 17 GSTP1 Competitive Displacement Kinetics



Cpd. 17 GSTP1 EC₅₀ Plot



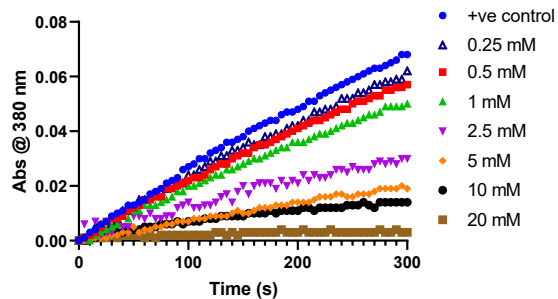
Cpd. 17 GSTP1 Rate



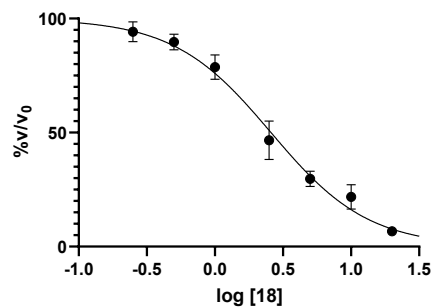
**Note: 5 mM of Cpd. 17 was used in each HPLC assay, and the reaction was diluted 5-fold prior to analysis.*

Compound 18

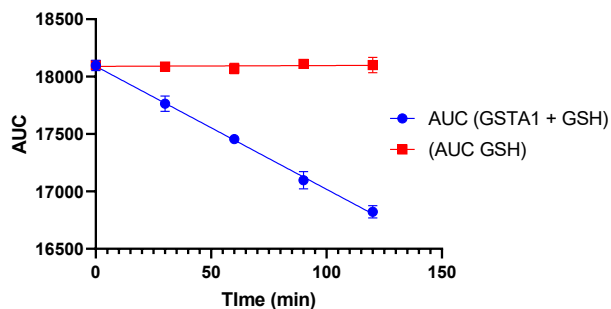
Cpd. 18 GSTA1 Competitive Displacement Kinetics



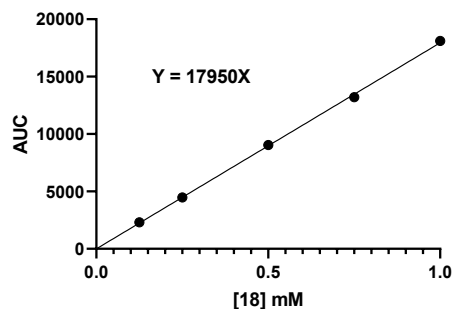
Cpd. 18 GSTA1 EC₅₀ Plot



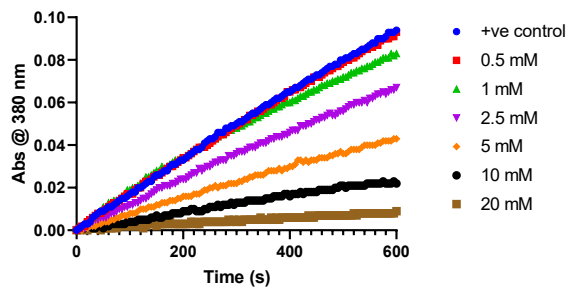
Cpd. 18 GSTA1 Rate



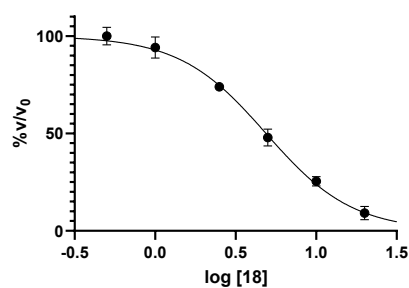
Standard Curve



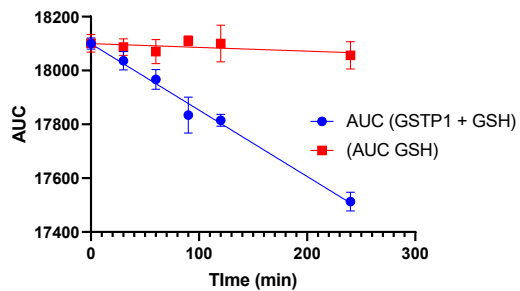
Cpd. 18 GSTP1 Competitive Displacement Kinetics



Cpd. 18 GSTP1 EC₅₀ Plot



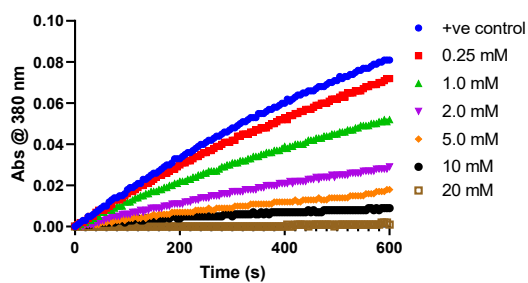
Cpd. 18 GSTP1 Rate



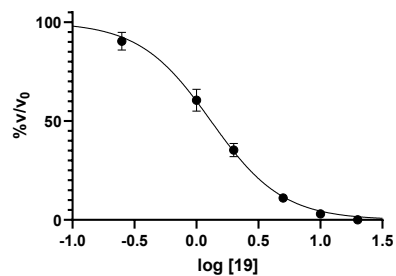
**Note: 5 mM of Cpd. 18 was used with GSTA1 and 15 mM with GSTP1 in the HPLC assay, and the reaction was diluted 5-fold and 15-fold respectively prior to analysis.*

Compound 19

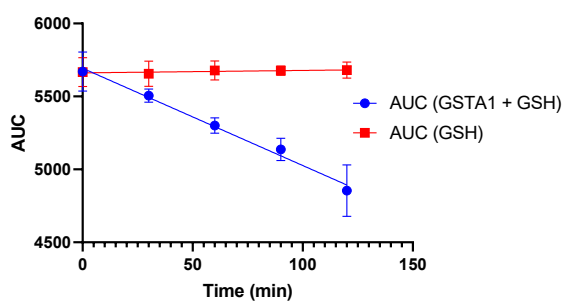
Cpd. 19 GSTA1 Competitive Displacement Kinetics



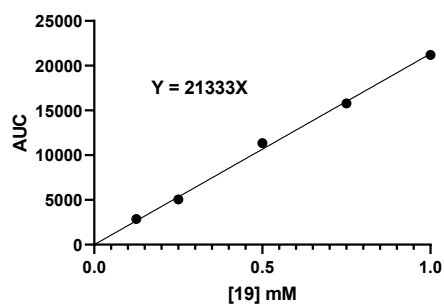
Cpd. 19 GSTA1 EC₅₀ Plot



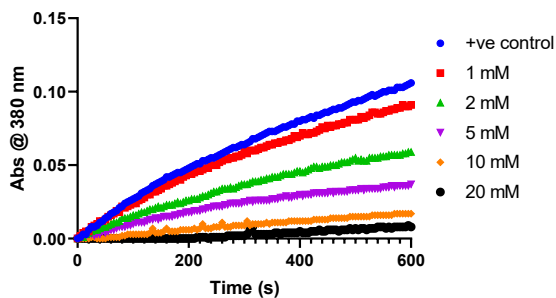
Cpd. 19 GSTA1 Rate



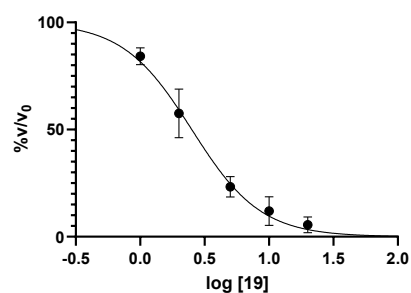
Cpd. 19 Standard Curve



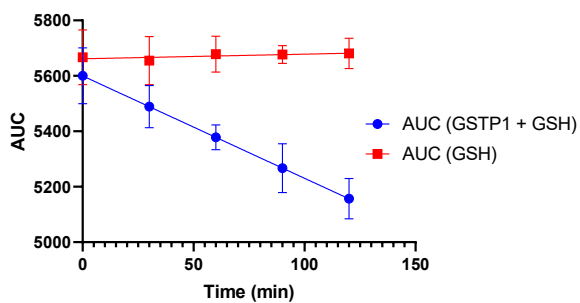
Cpd. 19 GSTP1 Competitive Displacement Kinetics



Cpd. 19 GSTP1 EC₅₀ Plot



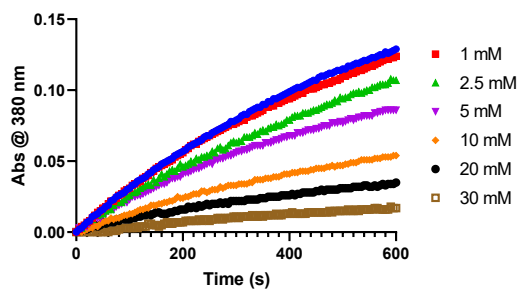
Cpd. 19 GSTP1 Rate



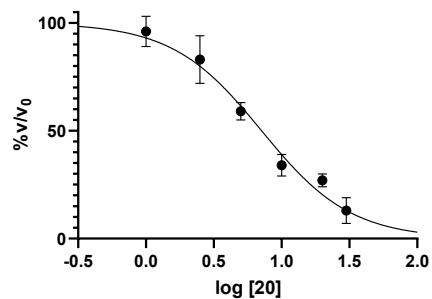
**Note: 5 mM of Cpd. 19 was used in the HPLC assay, and the reaction was diluted 20-fold prior to analysis.*

Compound 20

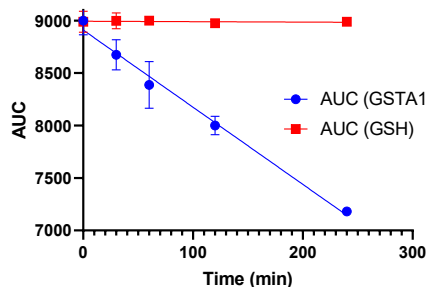
Cpd. 20 GSTA1 Competitive Displacement Kinetics



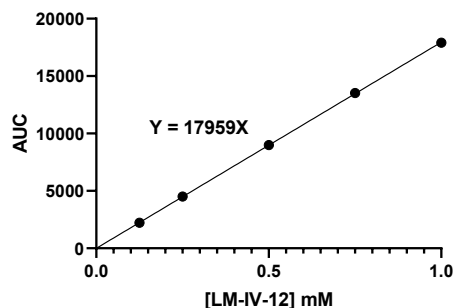
Cpd. 20 GSTA1 EC₅₀ Plot



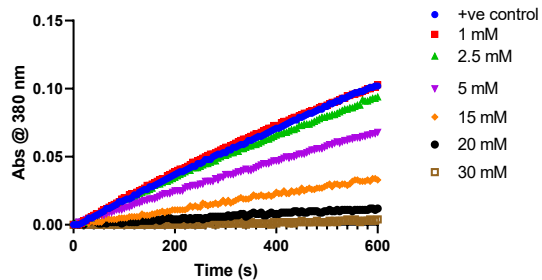
Cpd. 20 GSTA1 Rate



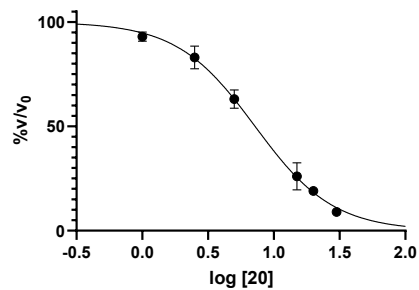
Cpd. 20 Standard Curve



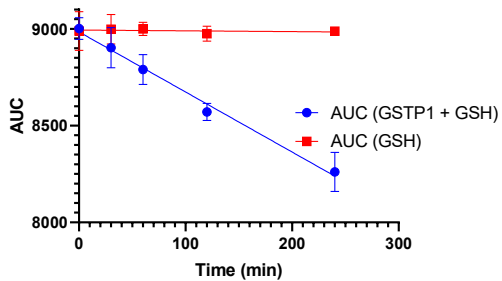
Cpd. 20 GSTP1 Competitive Displacement Kinetics



Cpd. 20 GSTP1 EC₅₀ Plot



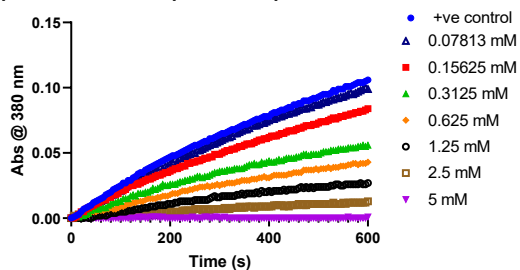
Cpd. 20 GSTP1 Rate



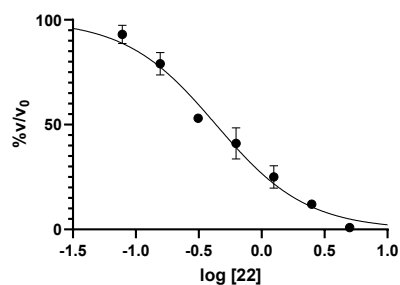
**Note: 10 mM of Cpd. 20 in the HPLC assay, and the reaction was diluted 20-fold prior to analysis.*

Compound 22

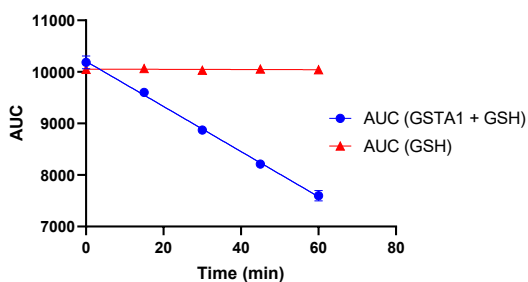
Cpd. 22 GSTA1 Competitive Displacement Kinetics



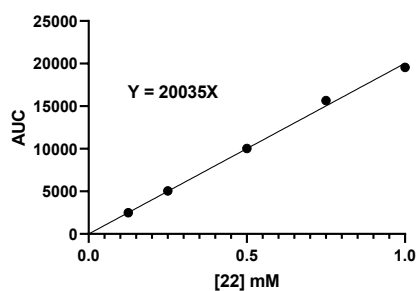
Cpd. 22 GSTA1 EC₅₀ Plot



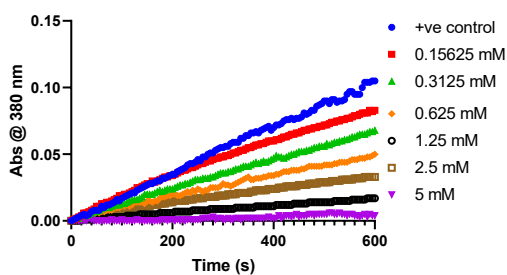
Cpd. 22 GSTA1 Rate



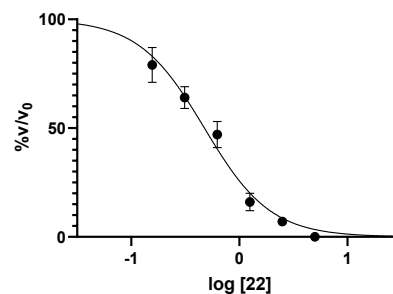
Cpd. 22 Standard Curve



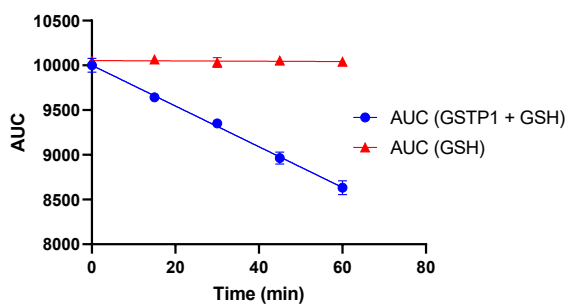
Cpd. 22 GSTP1 Competitive Displacement Kinetics



Cpd. 22 GSTP1 EC₅₀ Plot



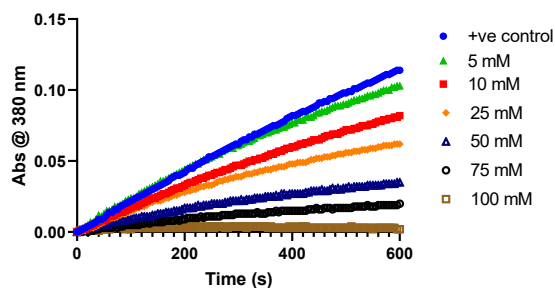
Cpd. 22 GSTP1 Rate



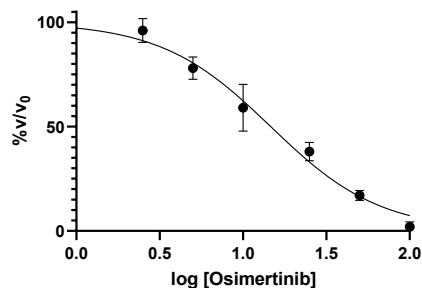
**Note: 5 mM of Cpd. 22 and 100 mU/mL (≈ 93 nM) of GSTP1 were used in the HPLC assay, and the reaction was diluted 10-fold prior to analysis.*

Osimertinib

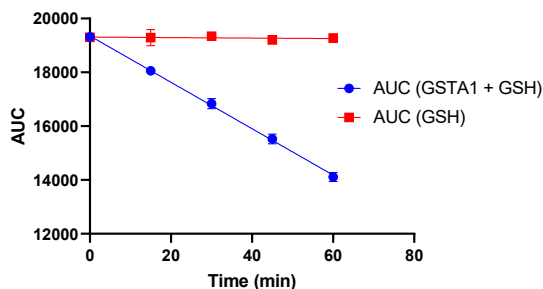
Osimertinib GSTA1 Competitive Displacement Kinetics



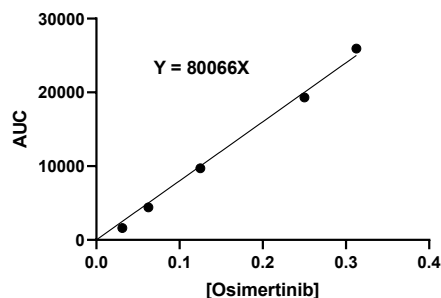
Osimertinib GSTA1 EC₅₀ Plot



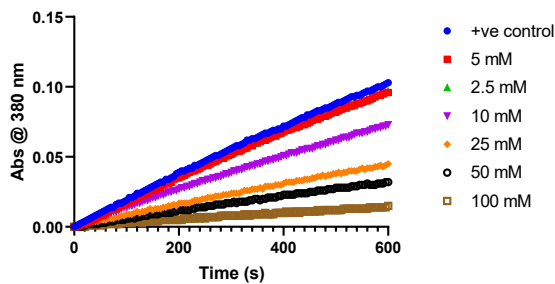
Osimertinib GSTA1 Rate



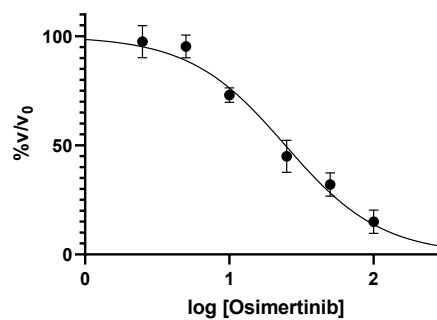
Osimertinib Standard Curve



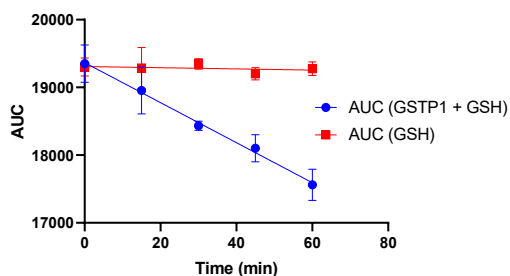
Osimertinib GSTP1 Competitive Displacement Kinetics



Osimertinib GSTP1 EC₅₀ Plot

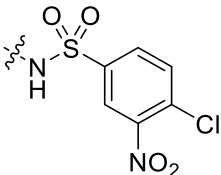
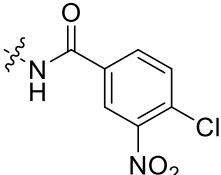
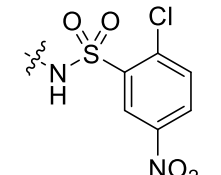
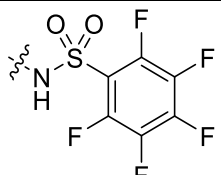
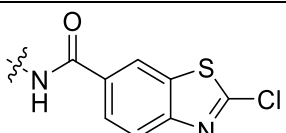
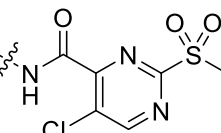
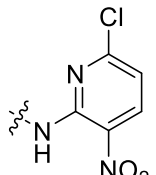
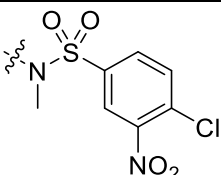


Osimertinib GSTP1 Rate



**Note: 10 mM and 50 mM of osimertinib was used with 50 mU/mL (≈ 186 nM) GSTA1 and 100 mU/mL (≈ 93 nM) of GSTP1 in the HPLC assay, respectively. The reaction was diluted 40- and 200-fold prior to analysis, respectively.*

Table 4.S1. Constants Expressed in Alternative Units

Cpd	Warhead	k_2^{GSH} ($M^{-1}s^{-1}$)	K_M^{app} (μM)		k_{cat}^{app} (s^{-1})		k_{cat}/K_M^{app} ($\times 10^3 M^{-1}s^{-1}$)	
			GSTA1	GSTP1	GSTA1	GSTP1	GSTA1	GSTP1
2		0.642 ± 0.003	177 ± 23	780 ± 280	1.49 ± 0.09	1.68 ± 0.30	8.43 ± 1.20	2.17 ± 0.08
3		0.503 ± 0.004	731 ± 41	1980 ± 220	0.645 ± 0.032	0.37 ± 0.02	0.883 ± 0.065	0.188 ± 0.012
4		0.242 ± 0.006	451 ± 70	N.D.	0.282 ± 0.028	N.D.	0.623 ± 0.120	N.D.
5		1.37 ± 0.01	14.0 ± 2.2	184 ± 22	2.78 ± 0.08	2.92 ± 0.28	198 ± 32	15.9 ± 1.0
6		0.132 ± 0.001	5470 ± 1800	1780 ± 450	0.255 ± 0.110	0.332 ± 0.130	0.0465 ± 0.0025	0.187 ± 0.005
7		111 ± 1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
8		0.888 ± 0.016	83.3 ± 9.0	167 ± 48	0.518 ± 0.027	0.523 ± 0.090	6.22 ± 0.73	3.13 ± 0.20
9		0.637 ± 0.002	43.4 ± 5.0	102 ± 20	0.888 ± 0.050	0.687 ± 0.065	20.5 ± 2.6	6.75 ± 1.4

10		0.0131 ± 0.0007	1840 ± 70	4510 ± 140	0.0520 ± 0.0068	0.0548 ± 0.0053	0.0282 ± 0.0038	0.0121 ± 0.0012
11		0.00173 ± 0.00006	310 ± 70	1520 ± 190	0.0680 ± 0.0042	0.052 ± 0.009	0.220 ± 0.050	0.0342 ± 0.0073
12		0.00632 ± 0.00033	15200 ± 700	46200 ± 5500	0.0161 ± 0.0020	0.00572 ± 0.00120	0.00105 ± 0.00013	0.000124 ± 0.000017
13		0.0300 ± 0.0048	7230 ± 150	9210 ± 270	0.0903 ± 0.0028	0.102 ± 0.007	0.0125 ± 0.0010	0.0111 ± 0.0008
14		1.21 ± 0.08	2150 ± 32	5150 ± 240	0.277 ± 0.028	0.302 ± 0.016	0.129 ± 0.013	0.0585 ± 0.0040
15		0.00732 ± 0.00028	4260 ± 840	>10000	0.0142 ± 0.0008	N.D.	0.00333 ± 0.00073	N.D.
16		0.512 ± 0.015	570 ± 60	2850 ± 220	0.187 ± 0.005	0.163 ± 0.013	0.328 ± 0.052	0.0553 ± 0.0062
17		0.00637 ± 0.00028	79.3 ± 8.9	190 ± 5.8	0.0785 ± 0.0035	0.0932 ± 0.0025	1.12 ± 0.14	0.49 ± 0.04
18		0.116 ± 0.001	1280 ± 94	3690 ± 650	0.0532 ± 0.0130	0.051 ± 0.002	0.0415 ± 0.013	0.0138 ± 0.0025
19		0.00132 ± 0.00004	550 ± 51	1900 ± 310	0.0620 ± 0.0057	0.0482 ± 0.0038	0.113 ± 0.014	0.0253 ± 0.0045
20		0.000633 ± 0.000030	2410 ± 70	5270 ± 190	0.0270 ± 0.0009	0.0227 ± 0.0011	0.0113 ± 0.0004	0.00428 ± 0.00027
21		1.11 ± 0.01	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
22		0.0613 ± 0.0023	183 ± 25	360 ± 40	0.202 ± 0.004	0.247 ± 0.003	1.12 ± 0.13	0.685 ± 0.077

4.5.3. Intrinsic Reactivity and Lipophilicity Correlation with Catalytic Efficiency

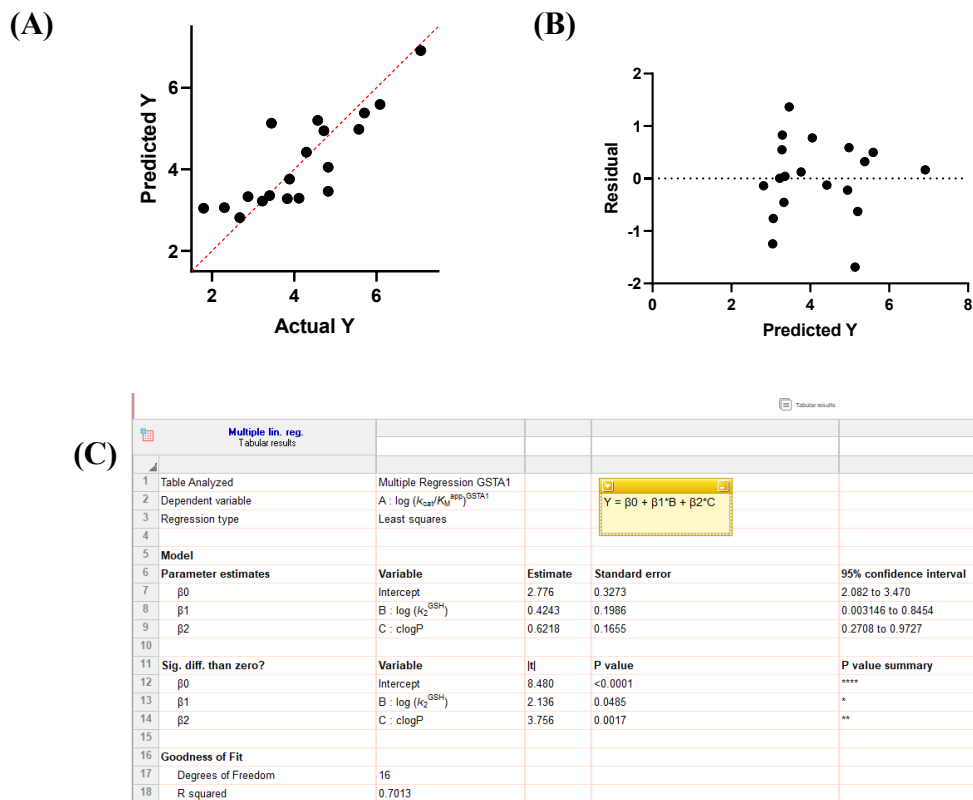


Figure 4.S7. Multiple linear regression analysis of $\log(k_{cat}/K_M^{app})^{GSTA1}$ against $\log(k_2^{GSH})$ and clogP. (A) Predicted vs. actual Y value plot. (B) Residual plot. (C) Summary of fitted parameters.

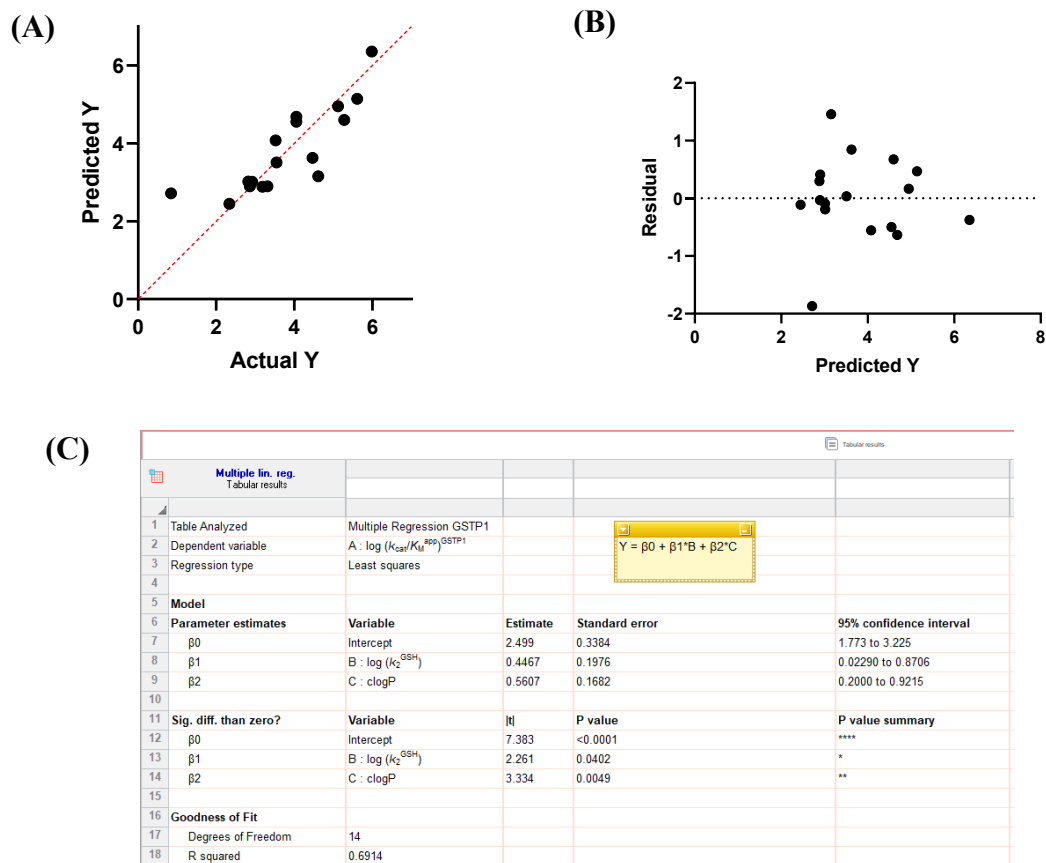


Figure 4.S8. Multiple linear regression analysis of $\log(k_{cat}/K_M^{app})^{GSTP1}$ against $\log(k_2^{GSH})$ and clogP . (A) Predicted vs. actual Y value plot. (B) Residual plot. (C) Summary of fitted parameters.

4.5.4. Molecular Modelling

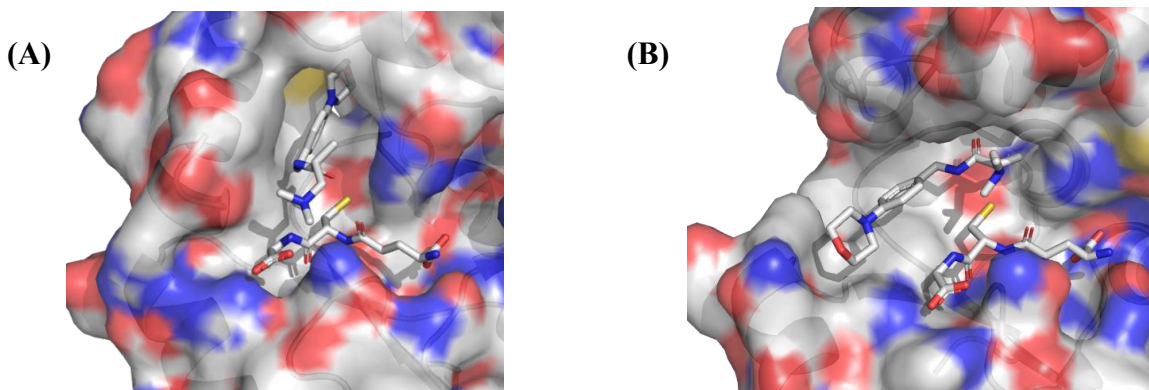


Figure 4.S9. Predicted model of compound 13 and GSH with (A) GSTA1 and (B) GSTP1.

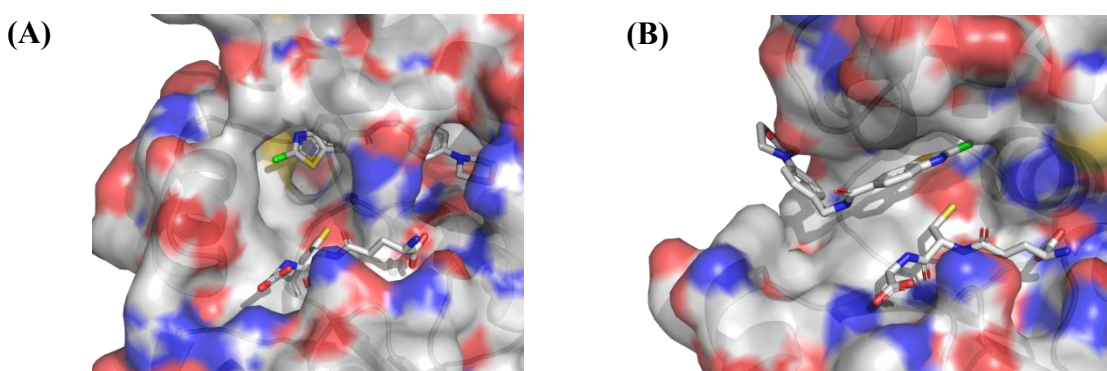


Figure 4.S10. Predicted model of compound 6 and GSH with (A) GSTA1 and (B) GSTP1.

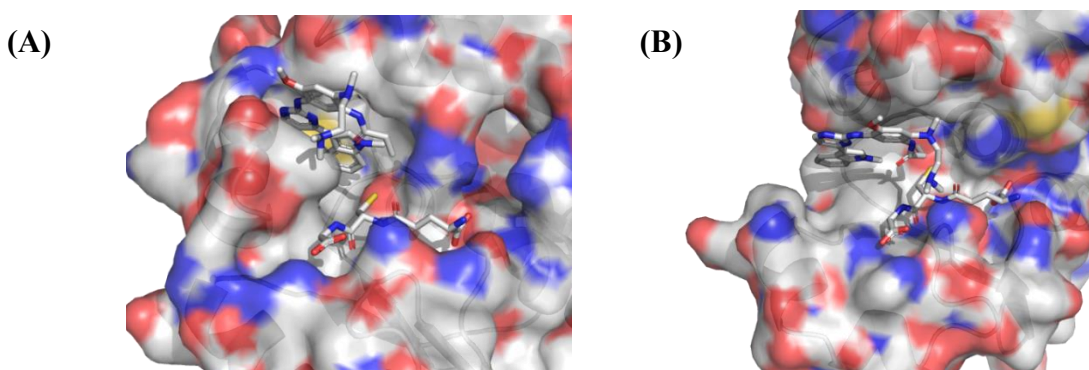


Figure 4.S11. Predicted model of osimertinib and GSH with (A) GSTA1 and (B) GSTP1.

4.5.5. Liver Cytosol Stability

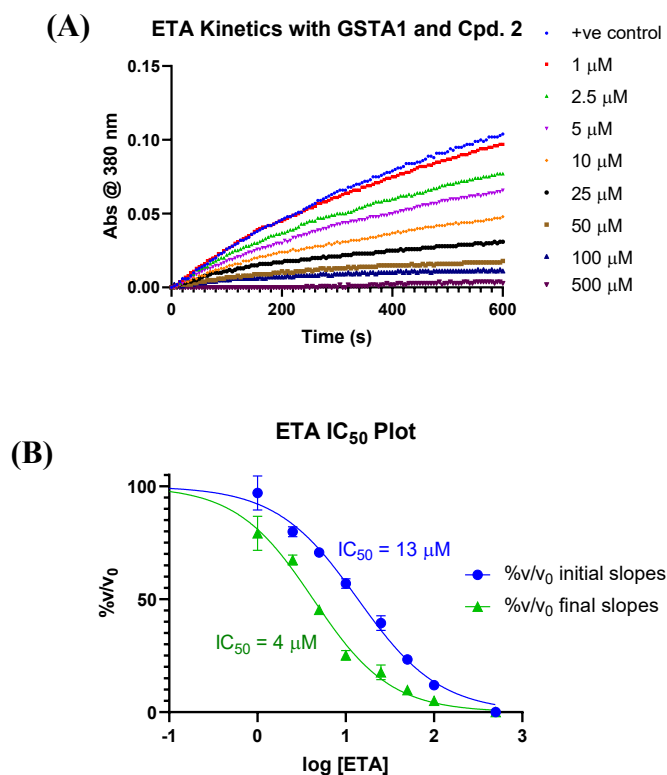


Figure 4.S12. Inhibition of GSTA1 by ethacrynic acid (ETA) **(A)** Kinetic data **(B)** Time-dependent IC_{50} plots.

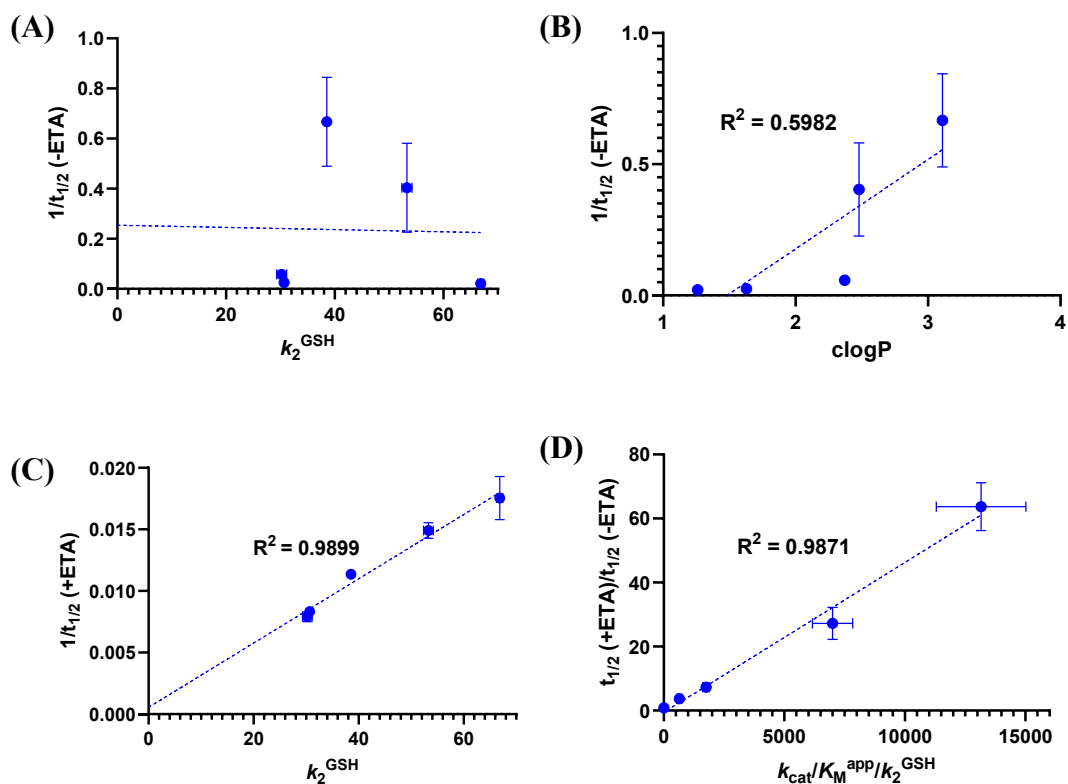


Figure 4.S13. Additional correlation analysis of liver cytosol stability. **(A)** Stability without GST inhibitor ETA does not correlate with intrinsic reactivity (k_2^{GSH}). **(B)** Stability without GST inhibitor shows moderate correlation with $clogP$. **(C)** Stability in the presence of ETA shows excellent correlation with k_2^{GSH} . **(D)** Ratio of stability with ETA to without ETA shows excellent correlation with the enzymatic efficiency ratio (EER) ($(k_{cat}/K_M^{app})/k_2^{GSH}$).

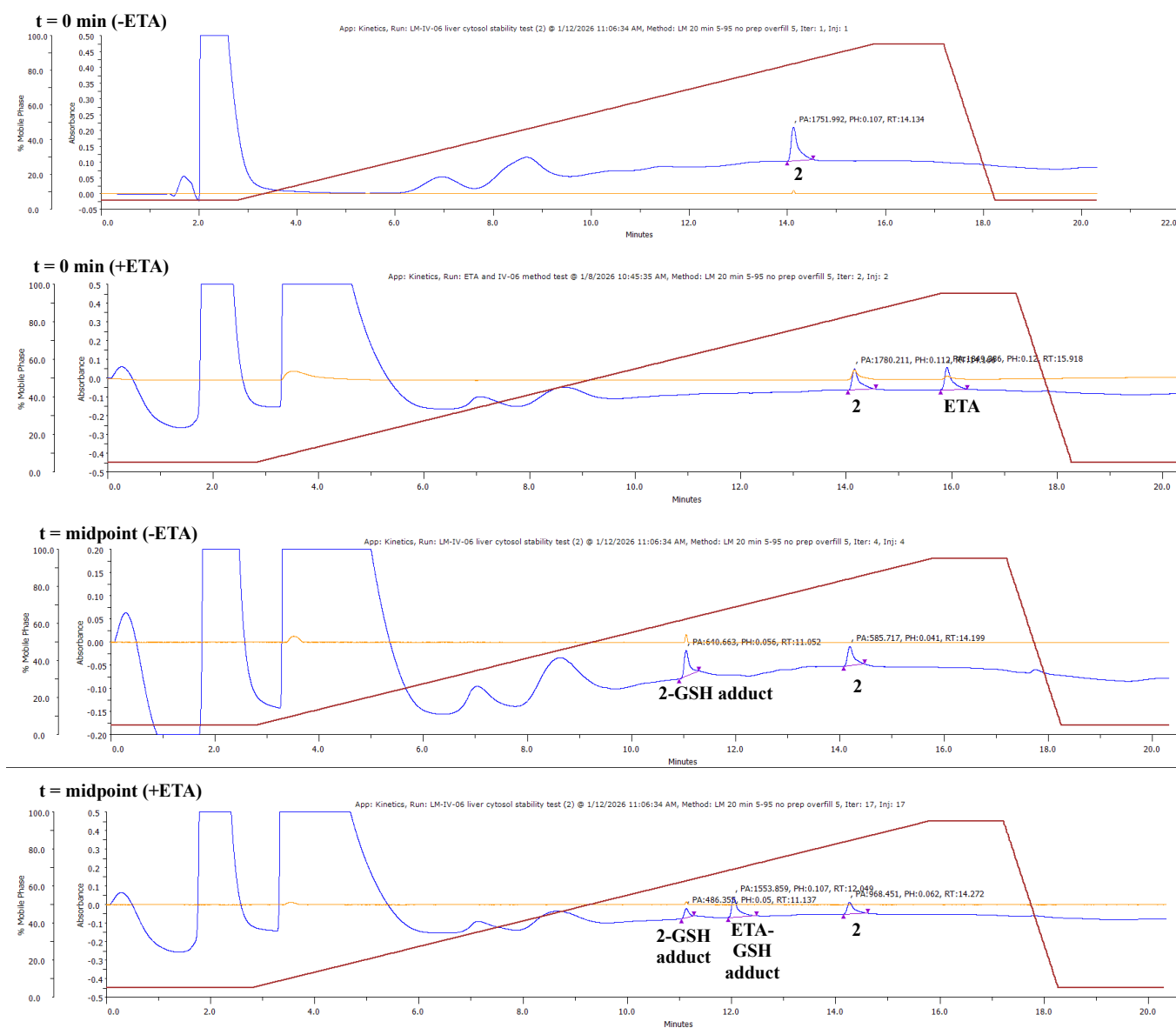
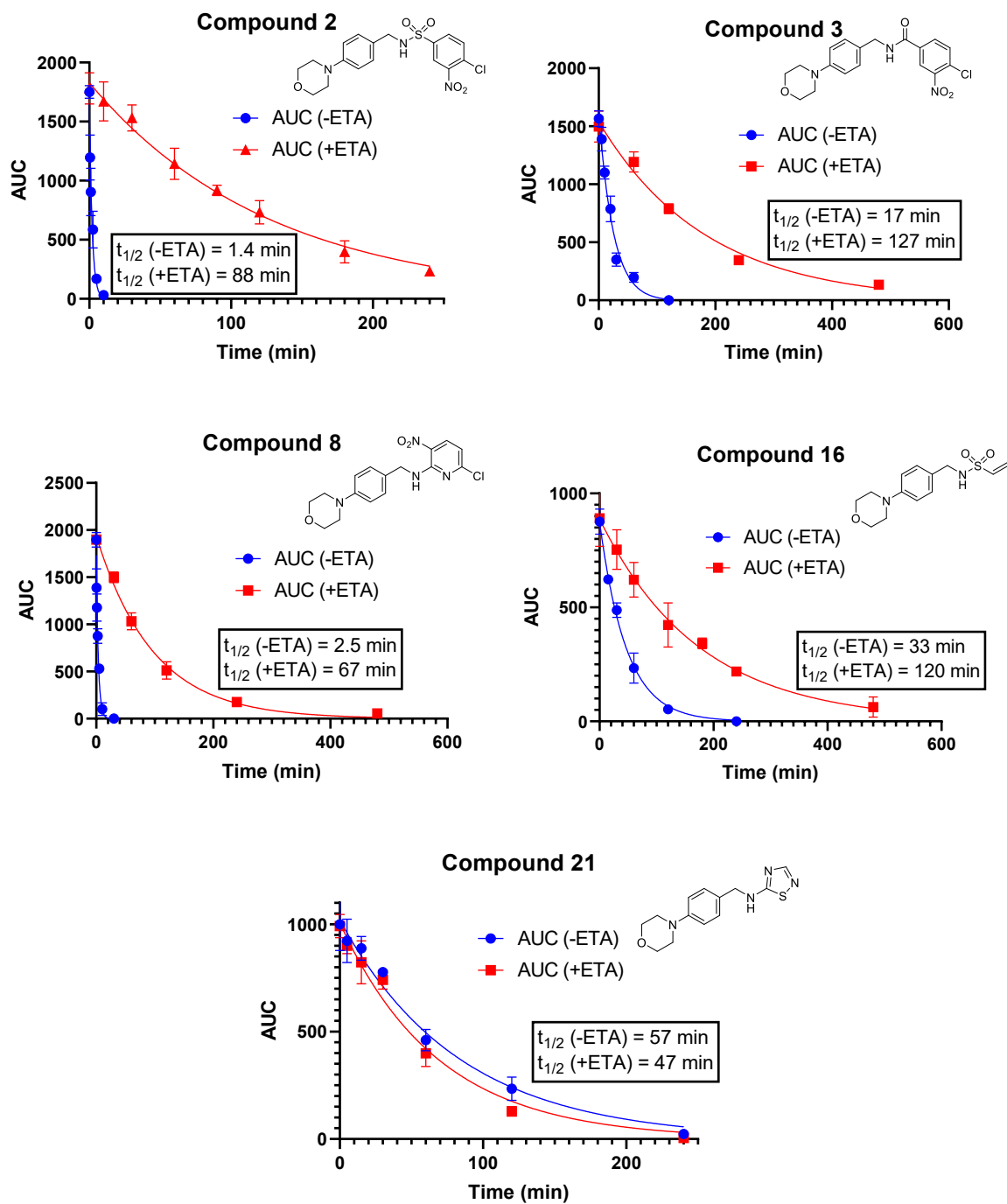


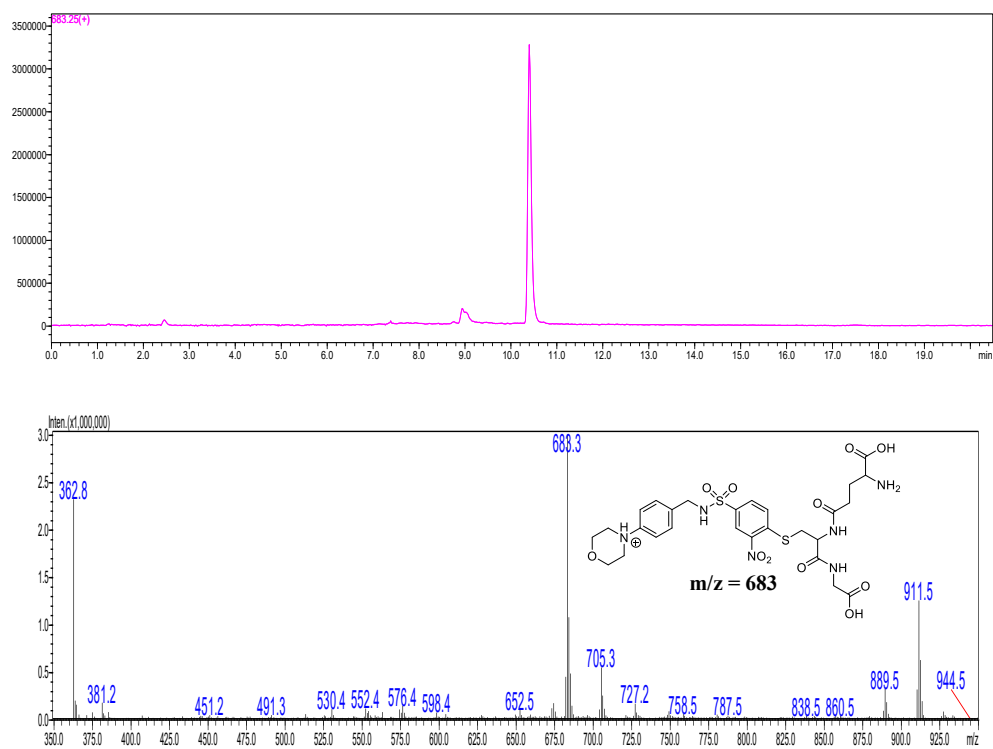
Figure 4.S14. Example of liver cytosol stability time-course HPLC injections of compound 2.

4.5.5.1. Kinetic HPLC Data

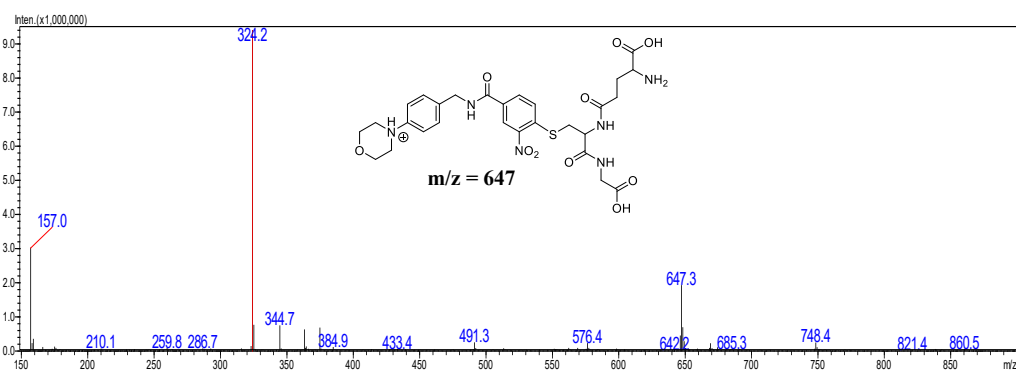
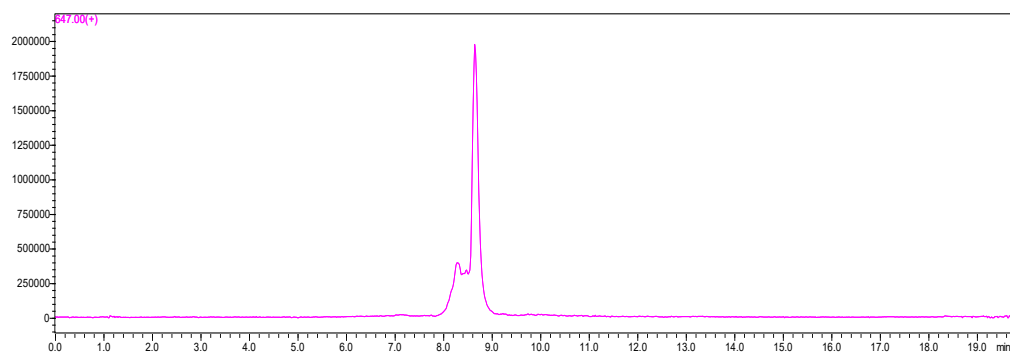


4.5.6. LC-MS Traces of Select GSH Adducts

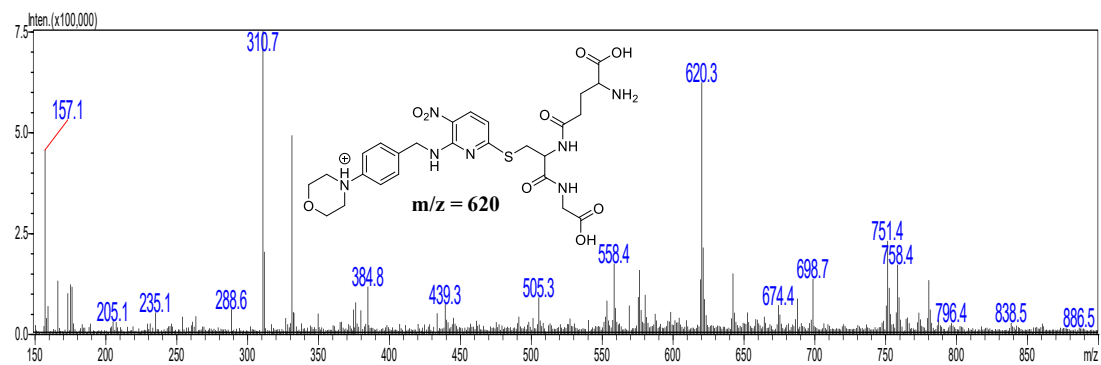
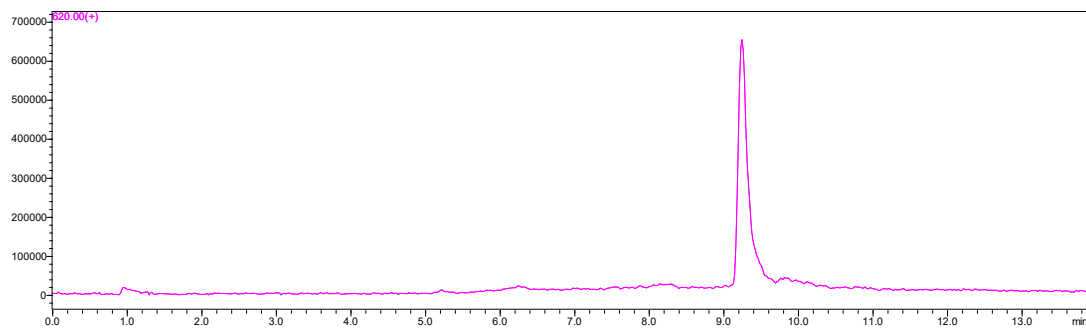
Compound 2:



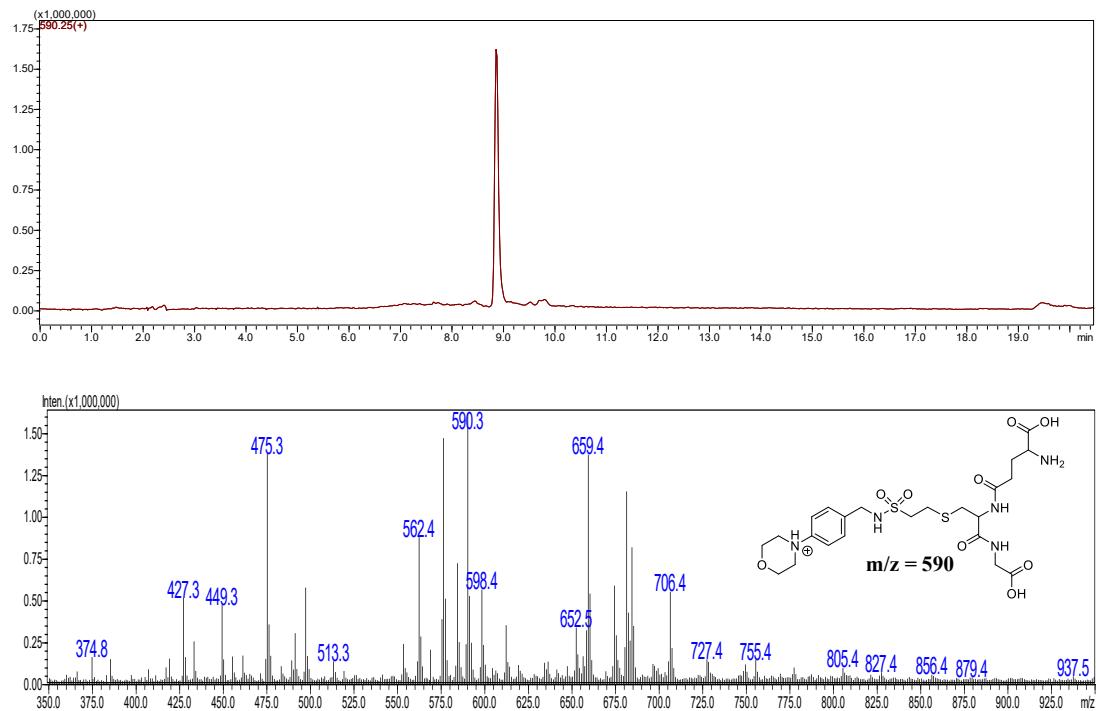
Compound 3:



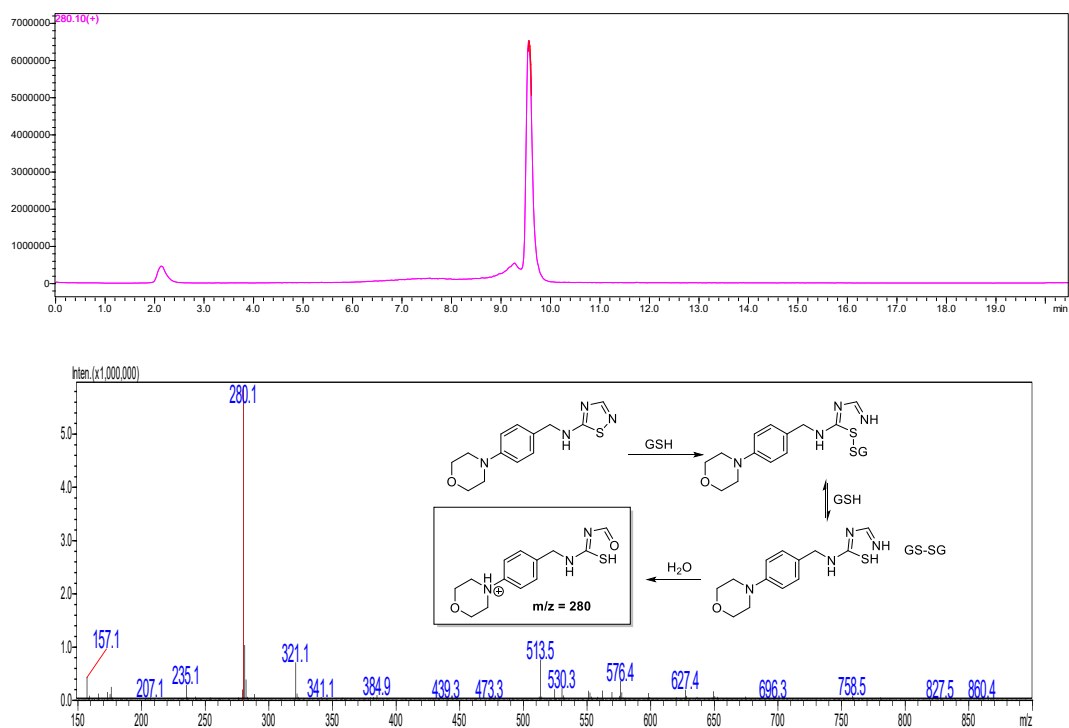
Compound 8:



Compound 16:

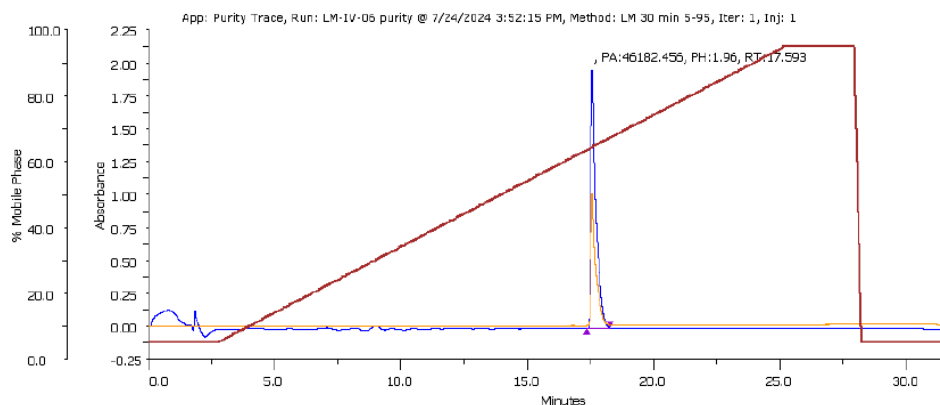


Compound 21:

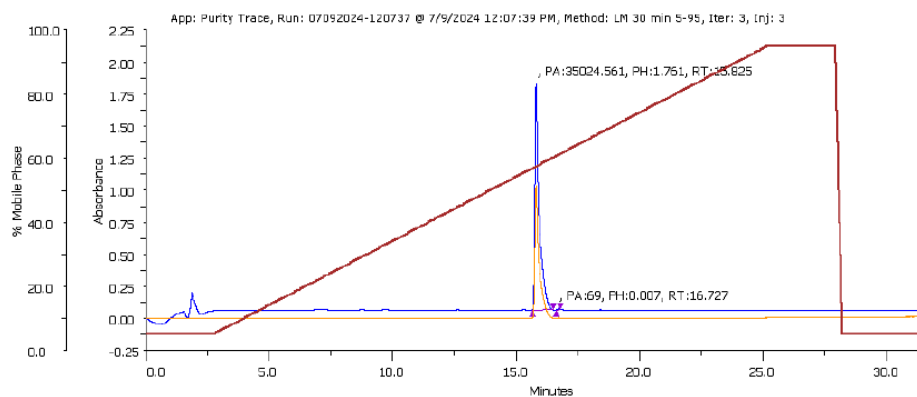


4.5.7. HPLC Purity Traces

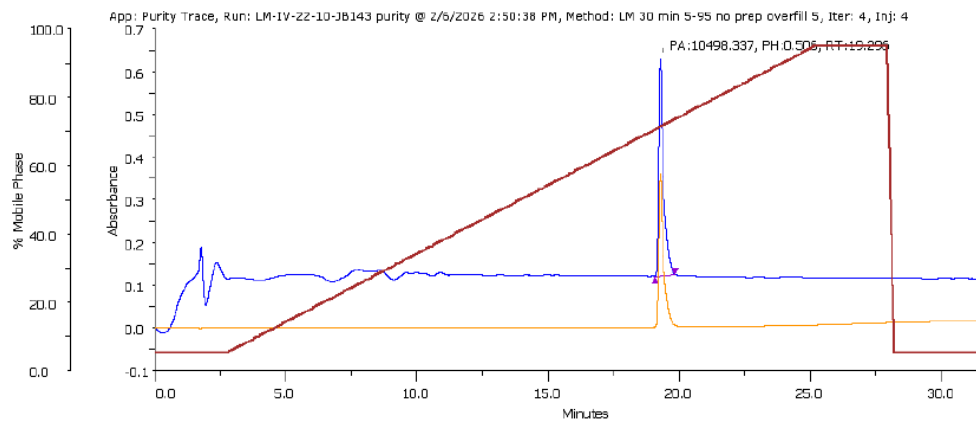
Compound 2 (100%)



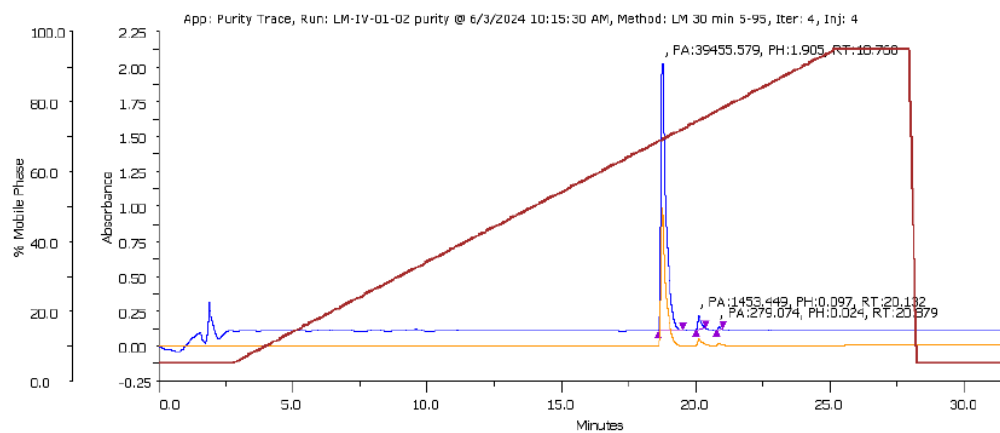
Compound 3 (99%)



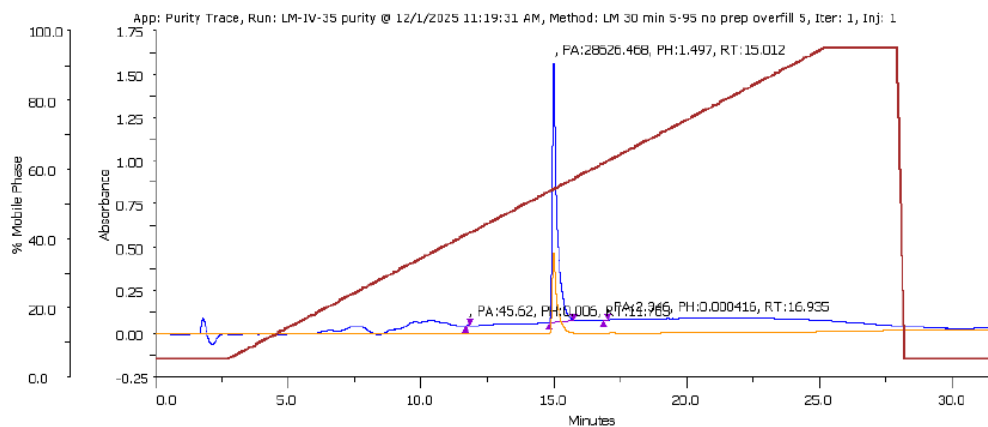
Compound 4 (100%)



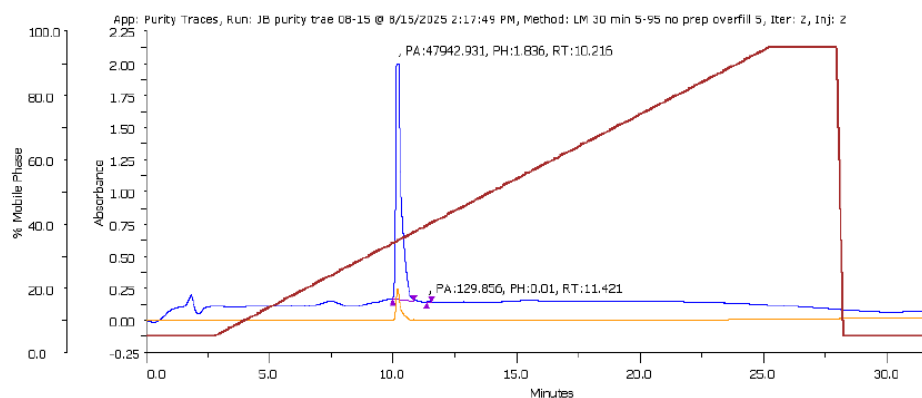
Compound 5 (96%)



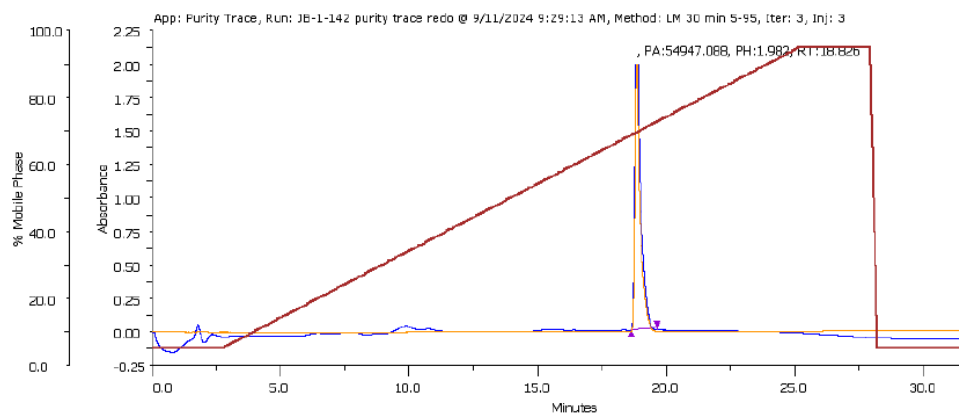
Compound 6 (99%)



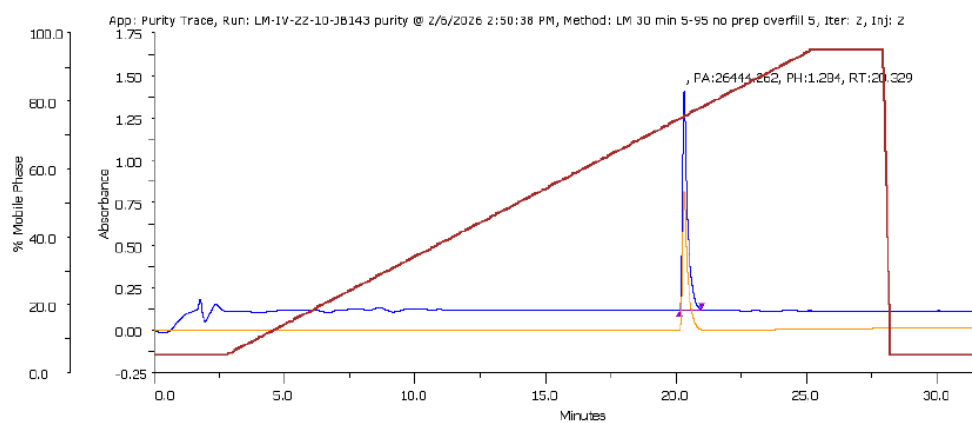
Compound 7 (99%)



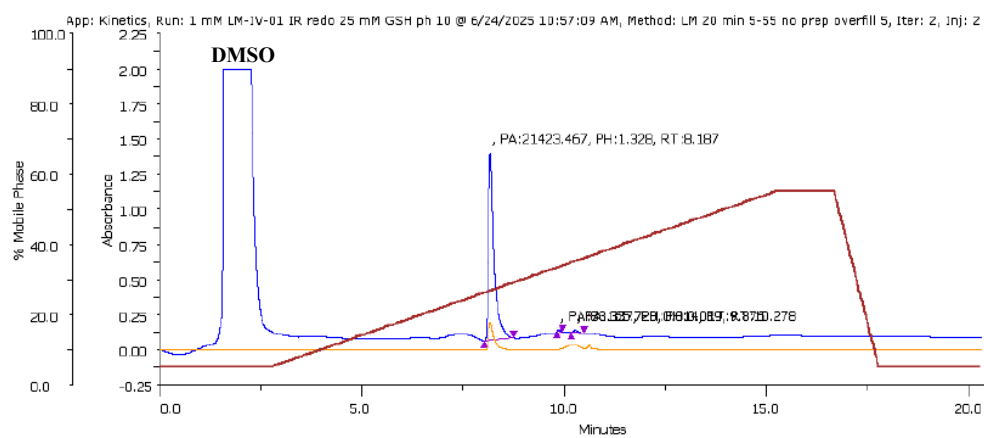
Compound 8 (100%)



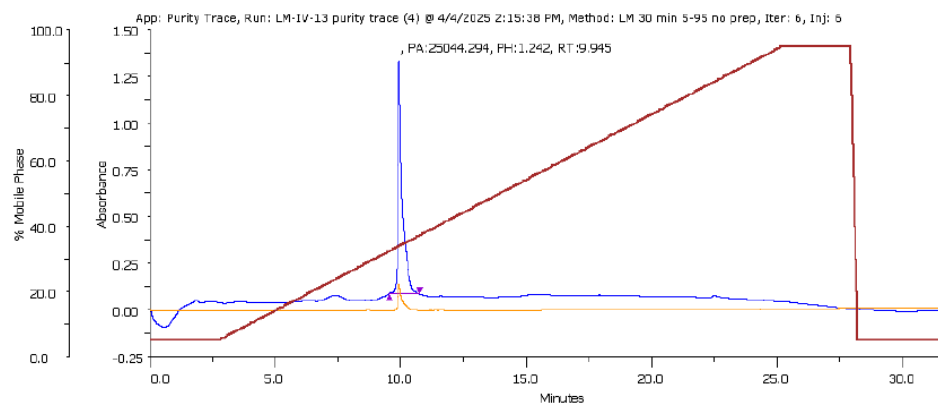
Compound 9 (100%)



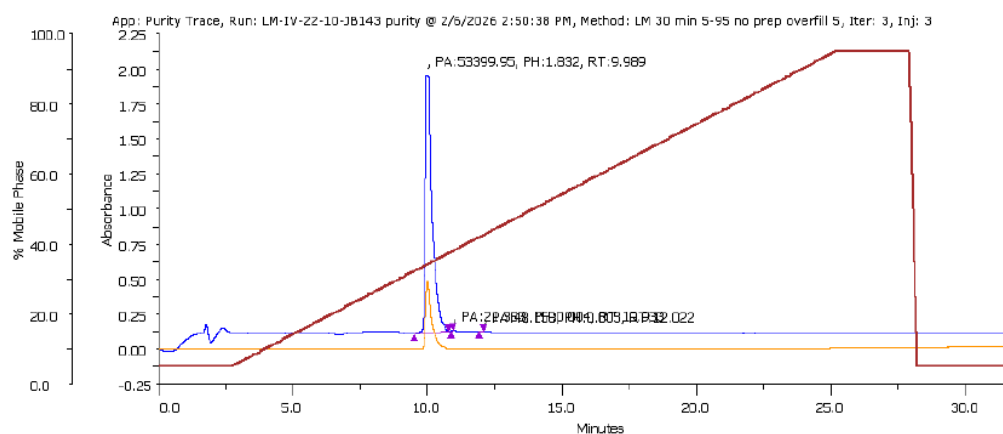
Compound 10 (98%)



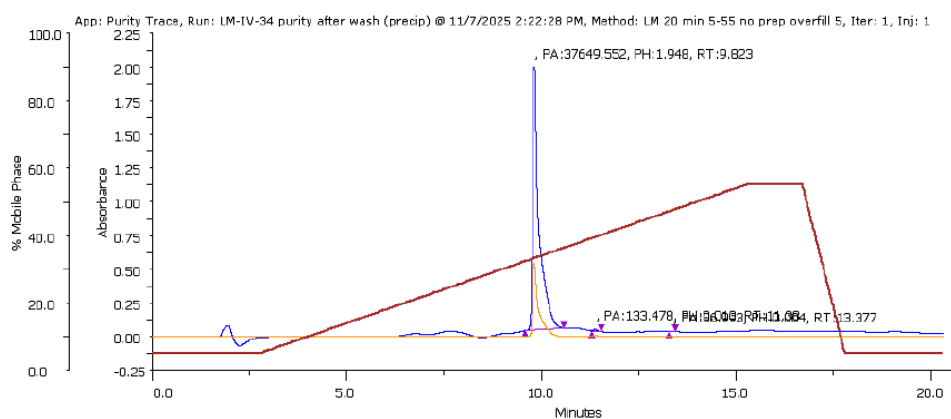
Compound 11 (100%)



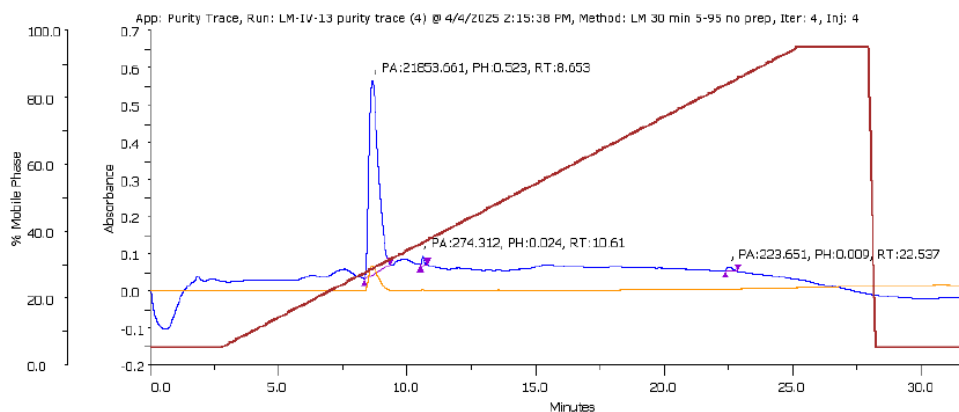
Compound 12 (99%)



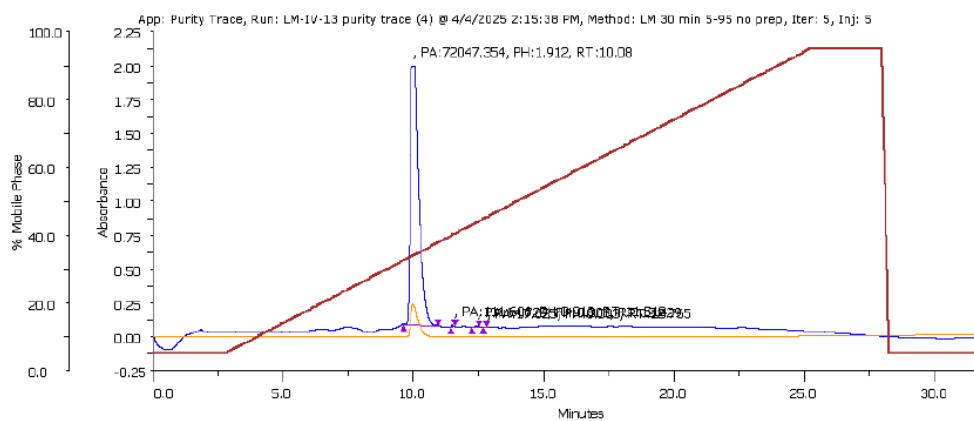
Compound 13 (99%)



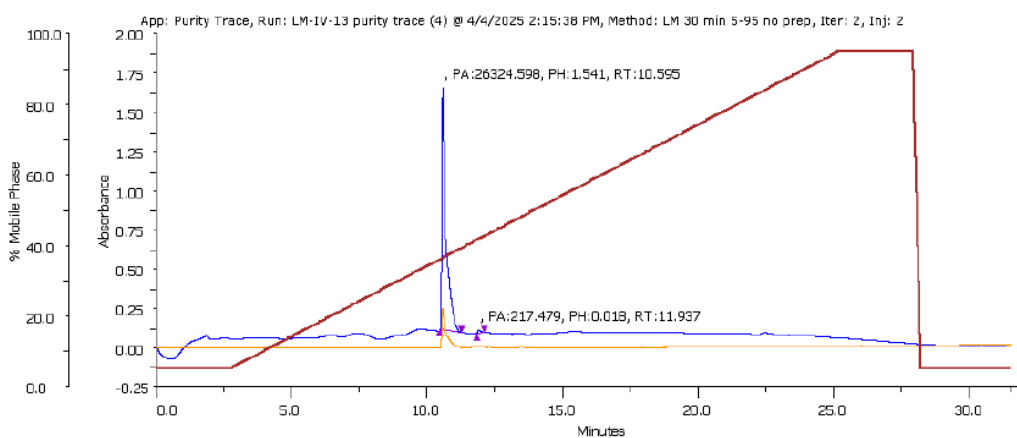
Compound 14 (97%)



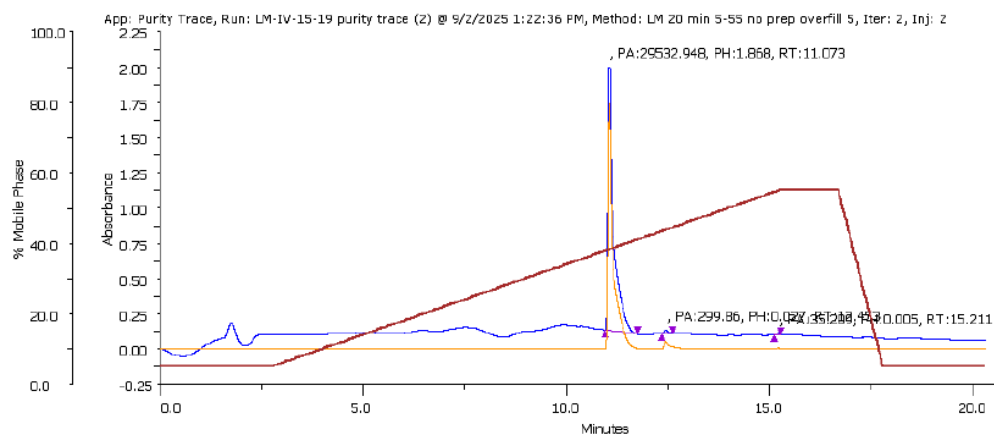
Compound 15 (99%)



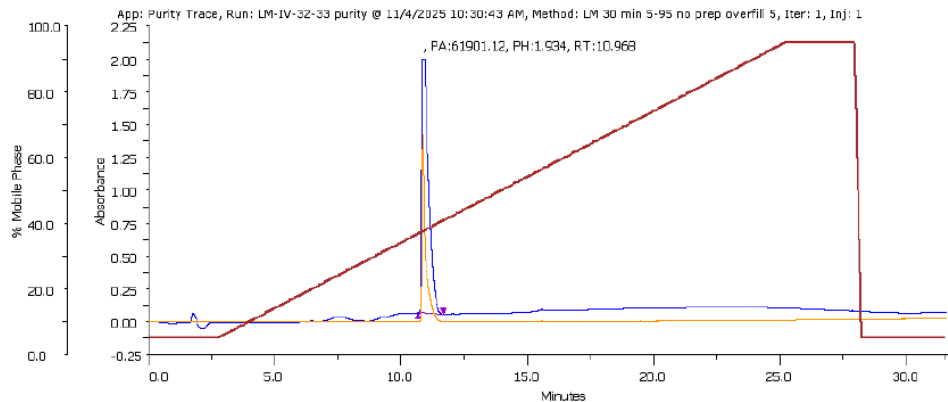
Compound 16 (99%)



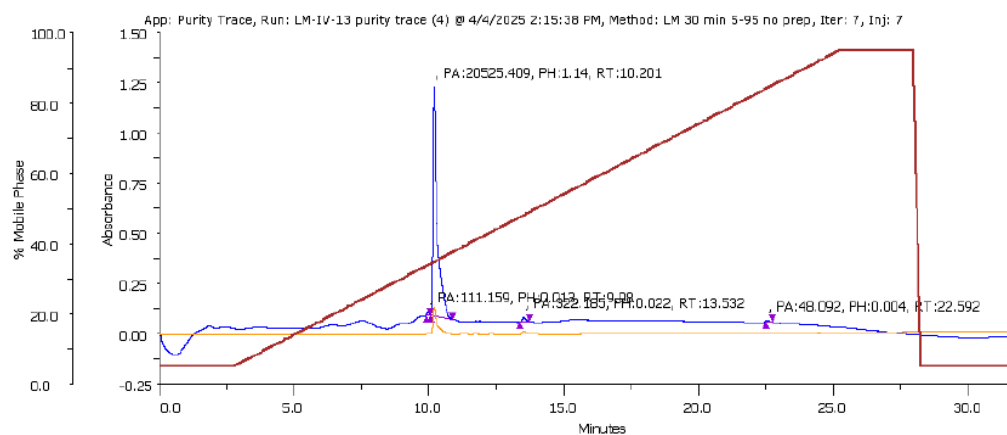
Compound 17 (98%)



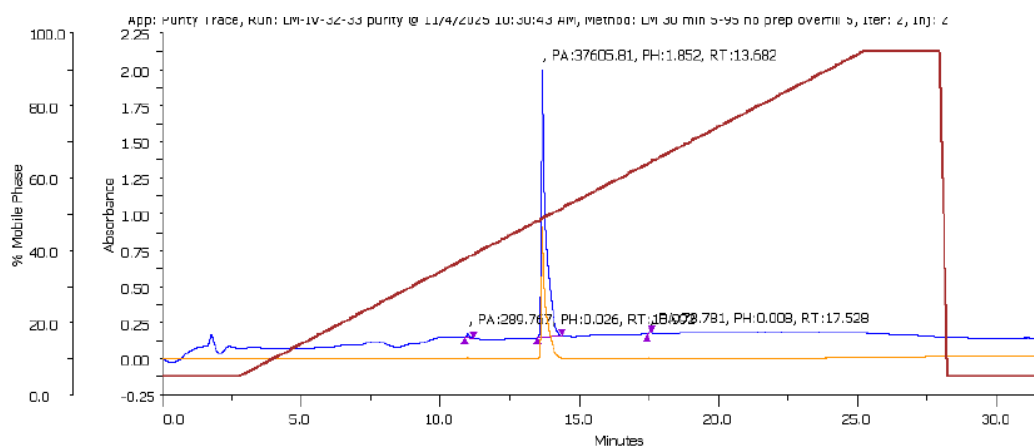
Compound 18 (100%)



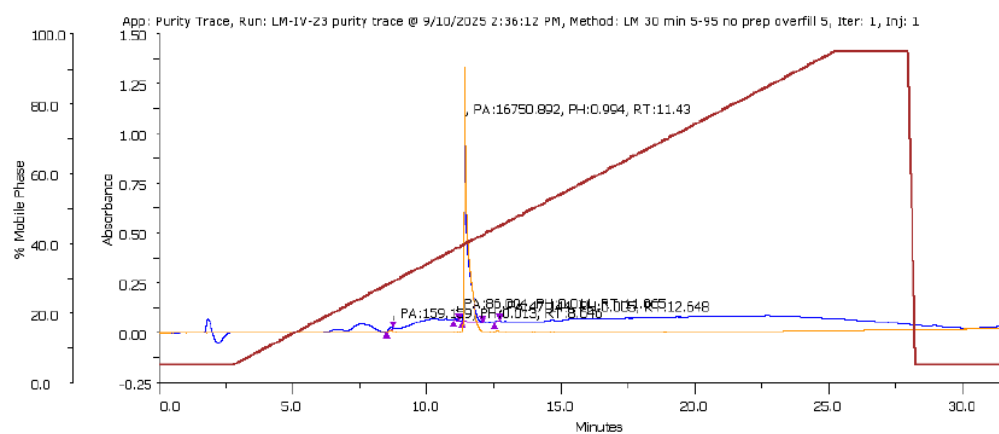
Compound 19 (97%)



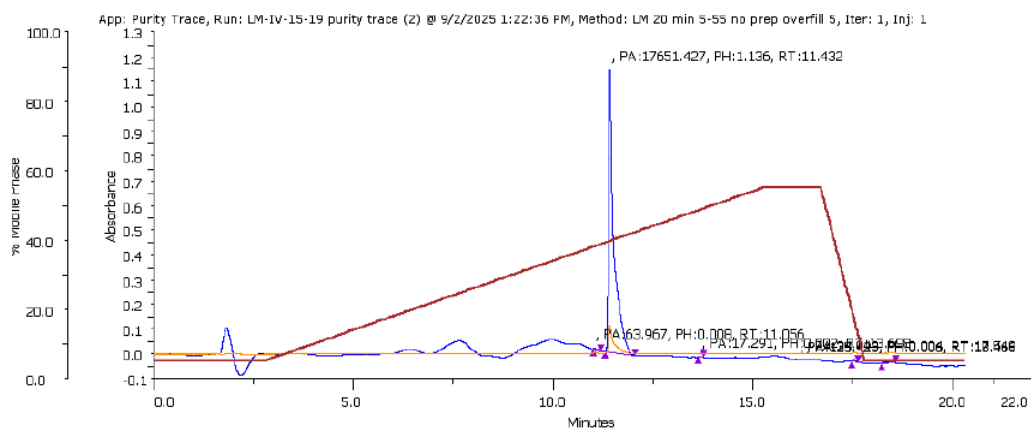
Compound 20 (99%)



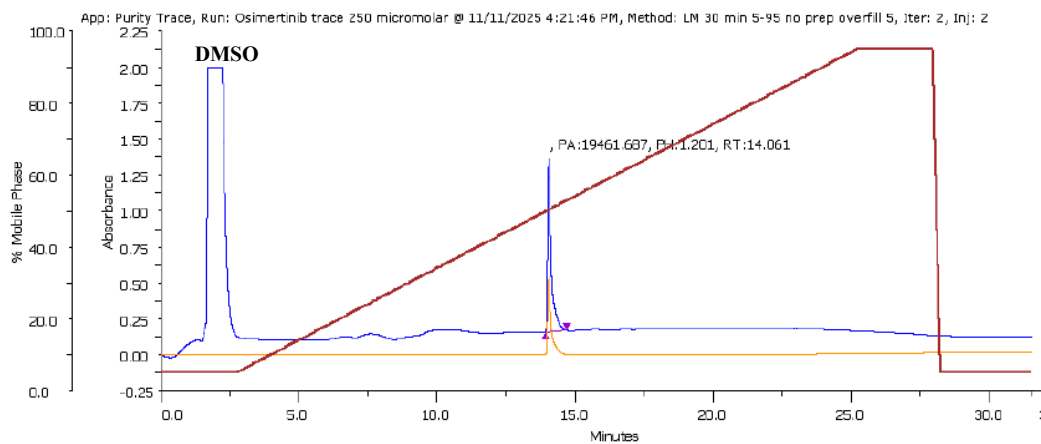
Compound 21 (98%)



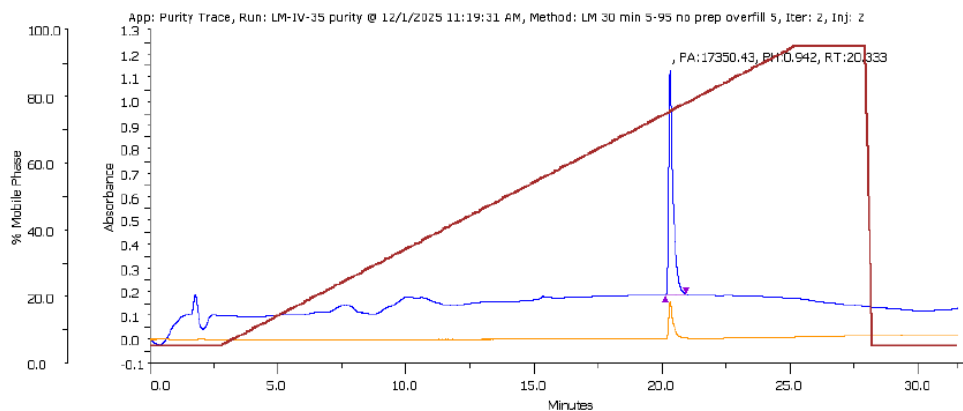
Compound 22 (98%)



Osimertinib (100%)

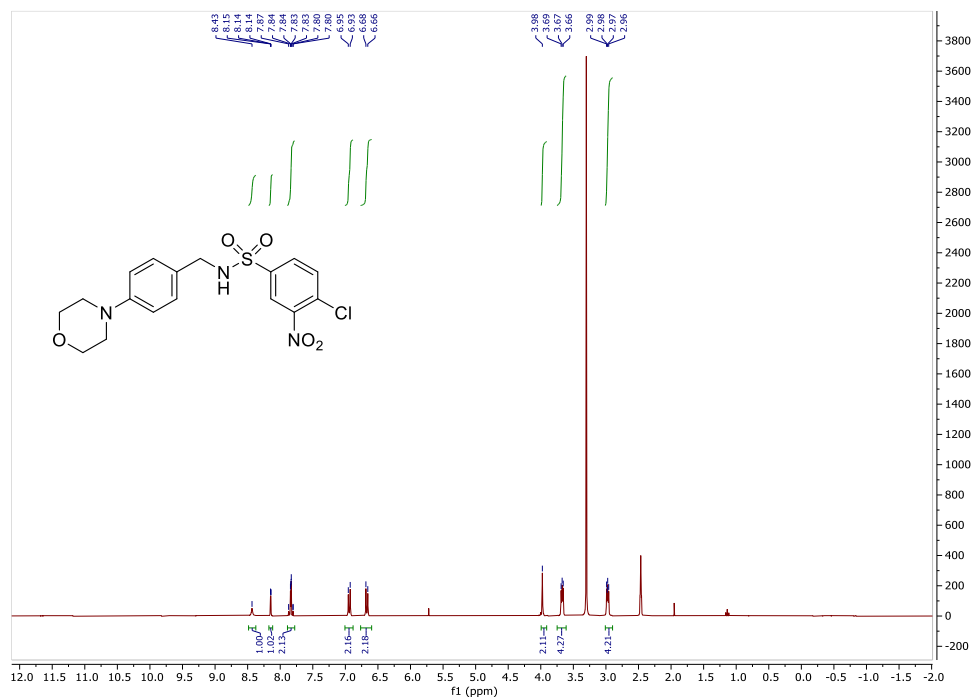


Ethacrynic acid (100%)

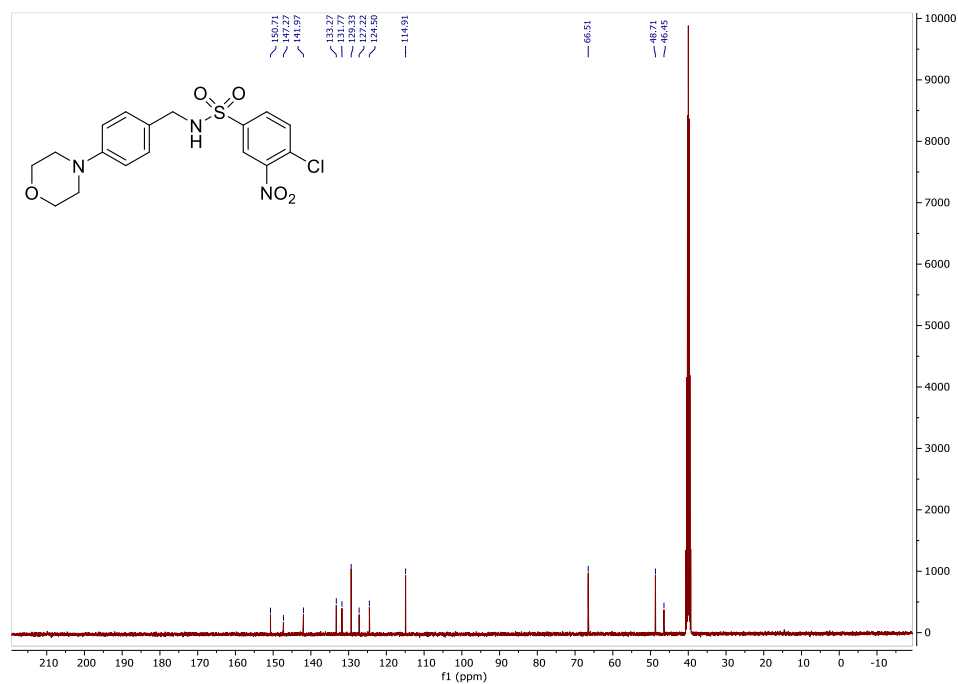


4.5.8. NMR Spectra

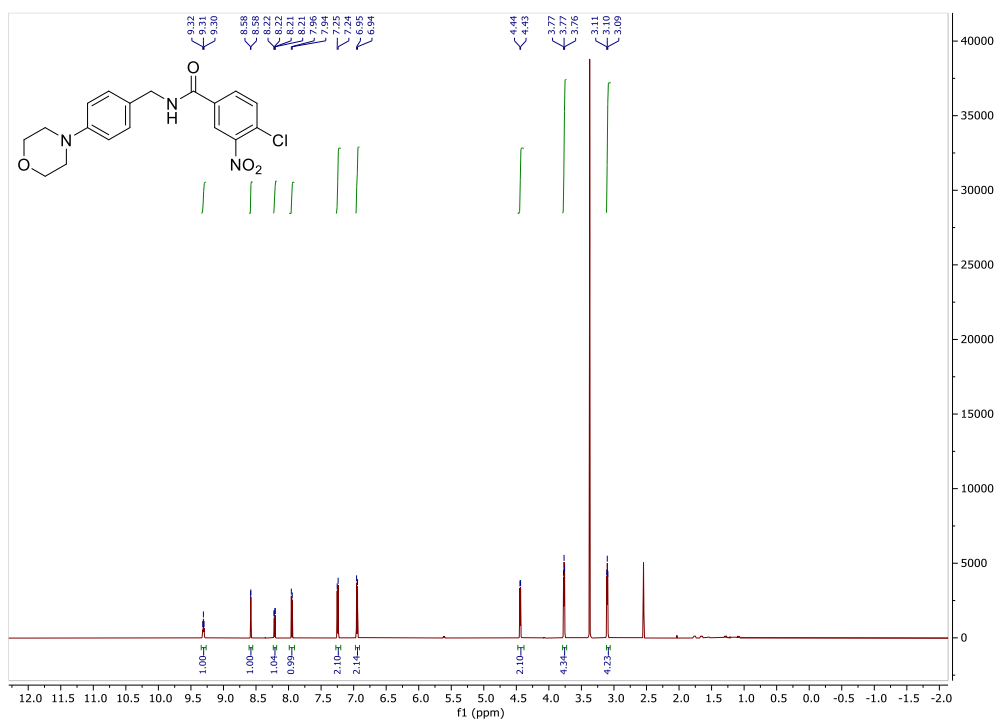
^1H NMR (300 MHz, $\text{DMSO}-d_6$) Spectrum of Compound 2



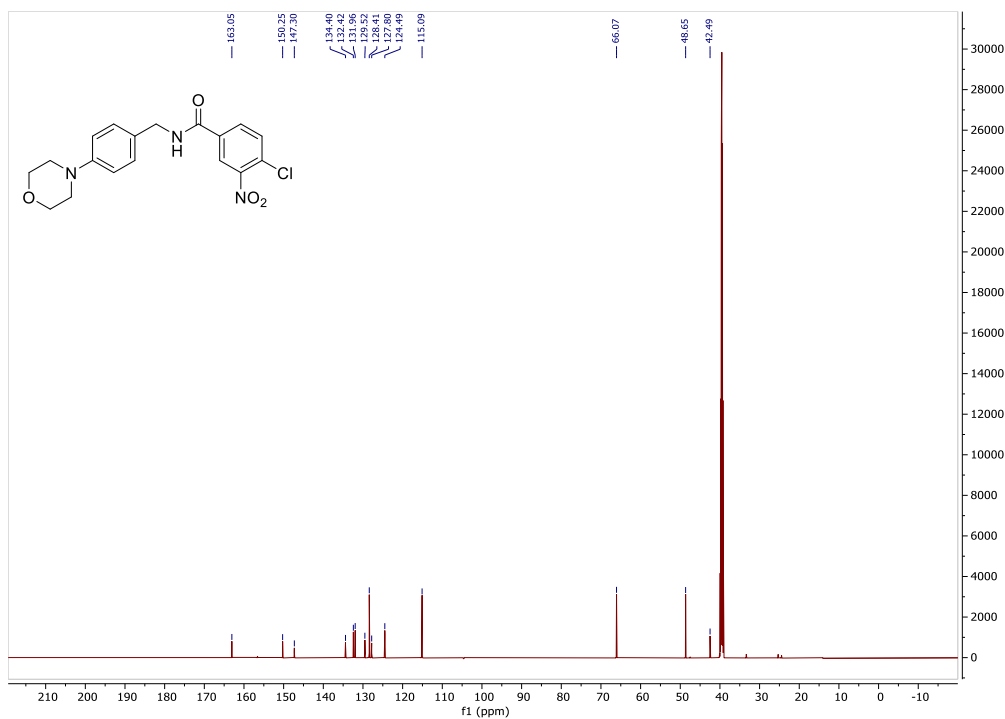
^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) Spectrum of Compound 2



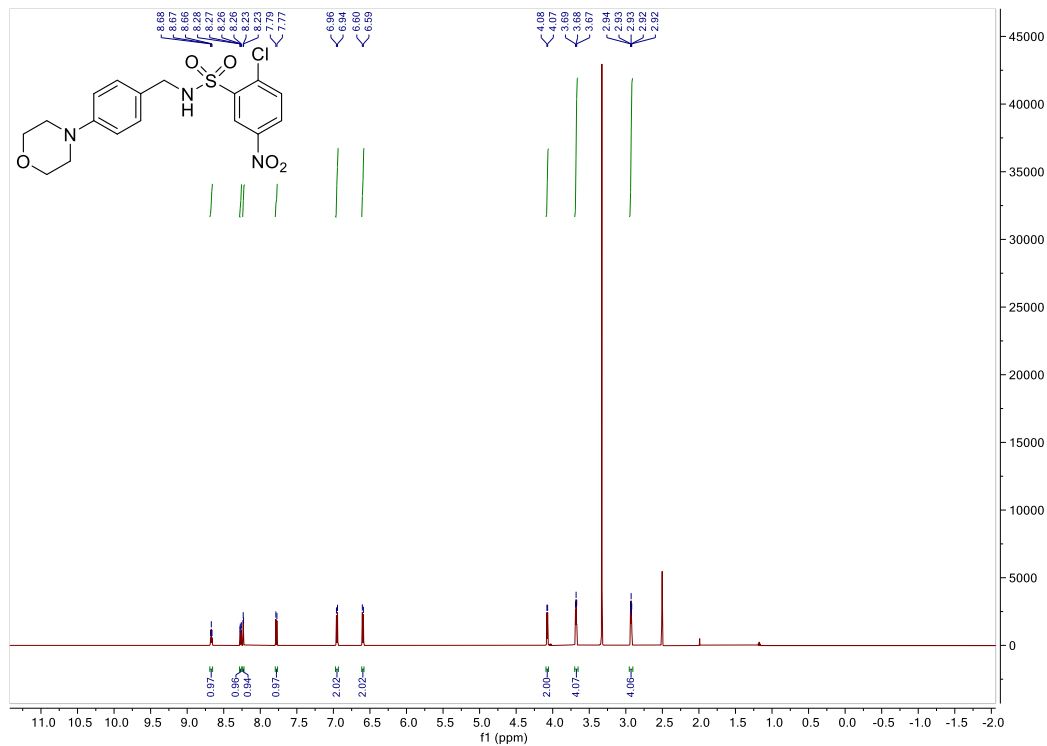
¹H NMR (600 MHz, DMSO-*d*₆) Spectrum of Compound **3**



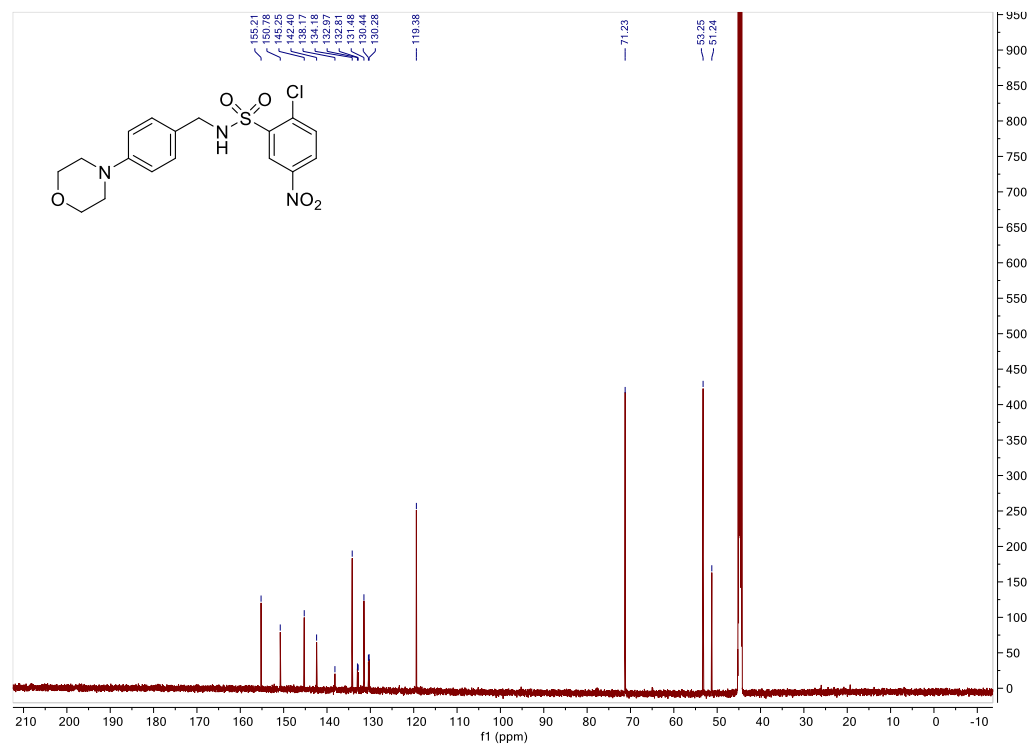
¹³C NMR (150 MHz, DMSO-*d*₆) Spectrum of Compound **3**



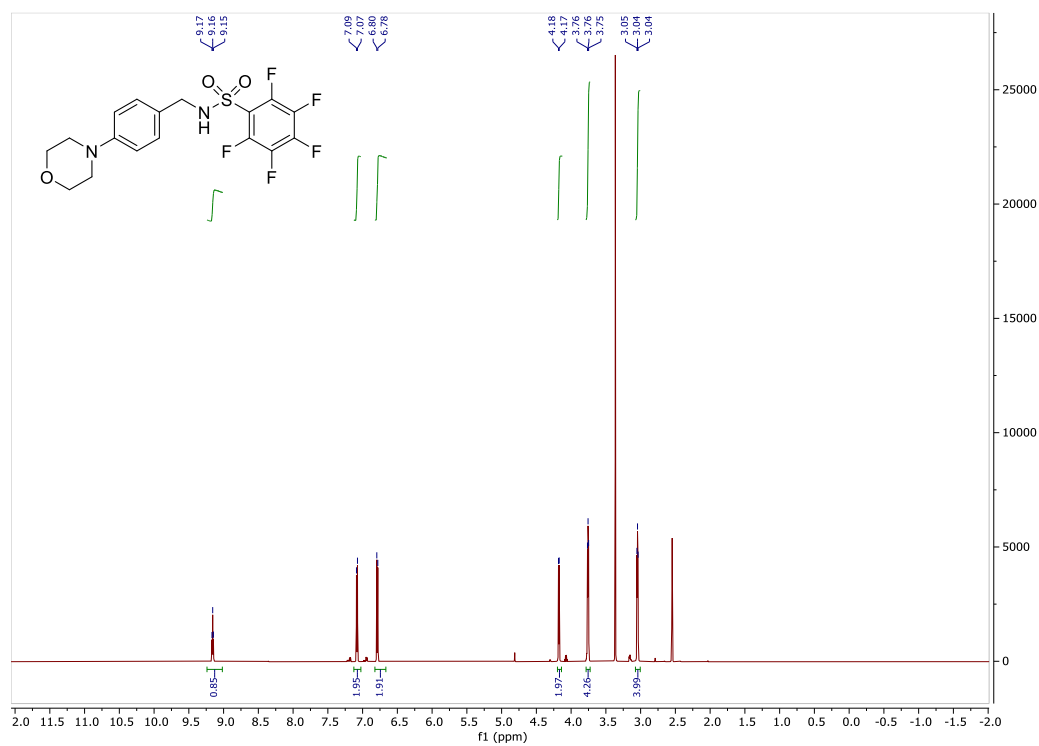
¹H NMR (600 MHz, DMSO-*d*₆) Spectrum of Compound **4**



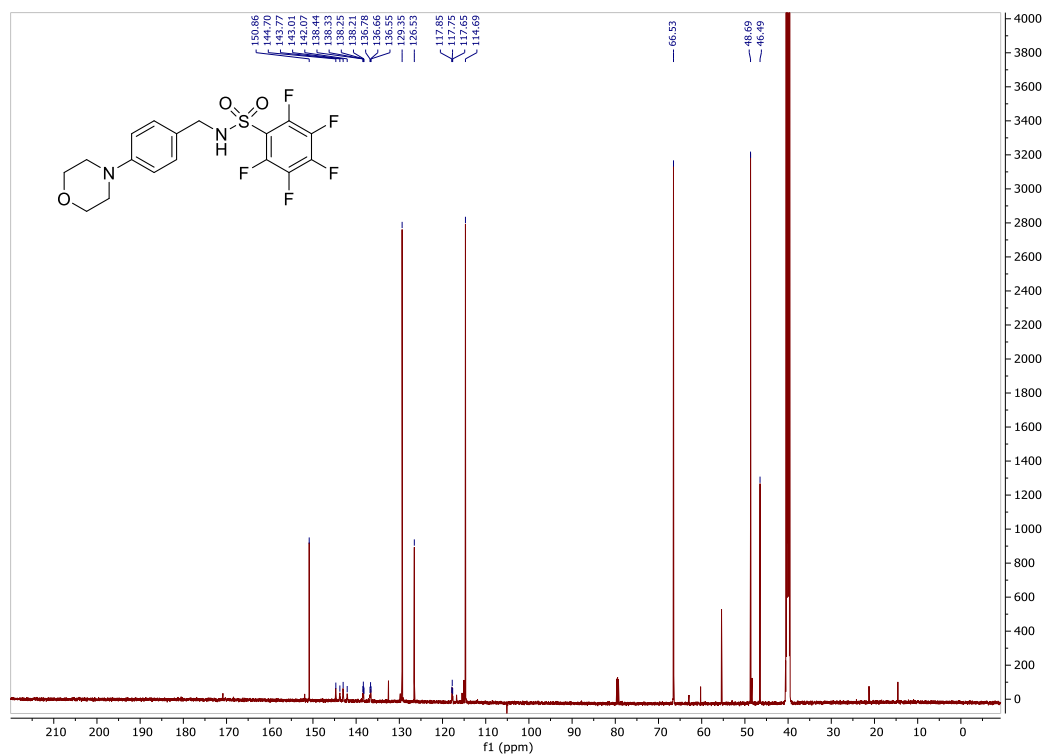
¹³C NMR (150 MHz, DMSO-*d*₆) Spectrum of Compound **4**



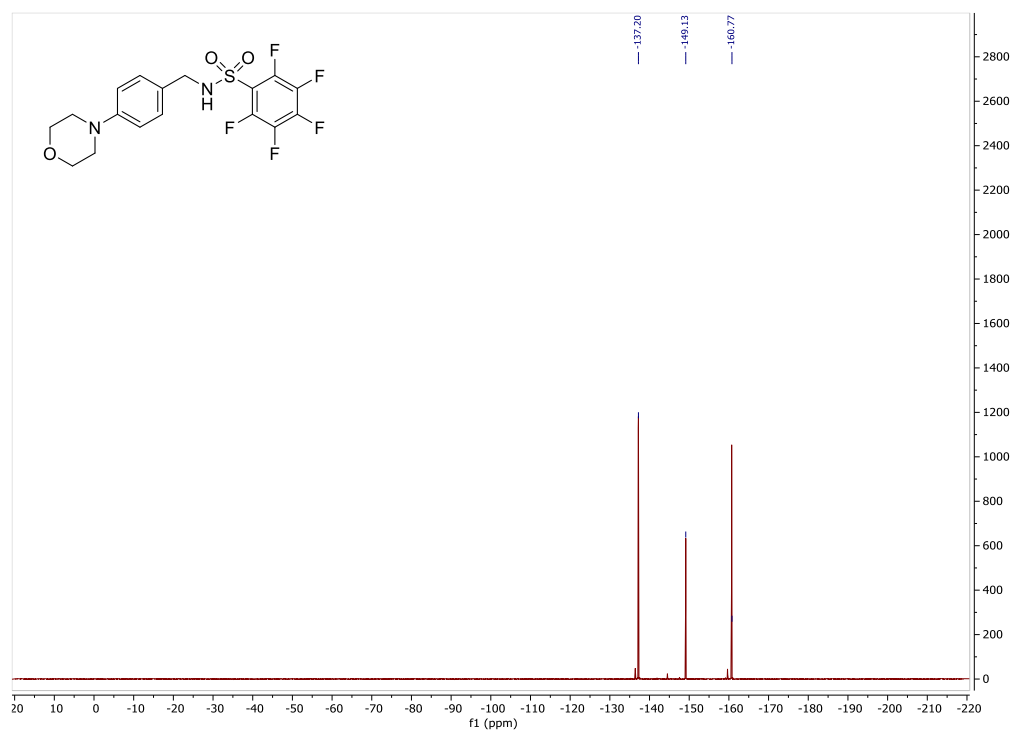
¹H NMR (600 MHz, DMSO-*d*₆) Spectrum of Compound **5**



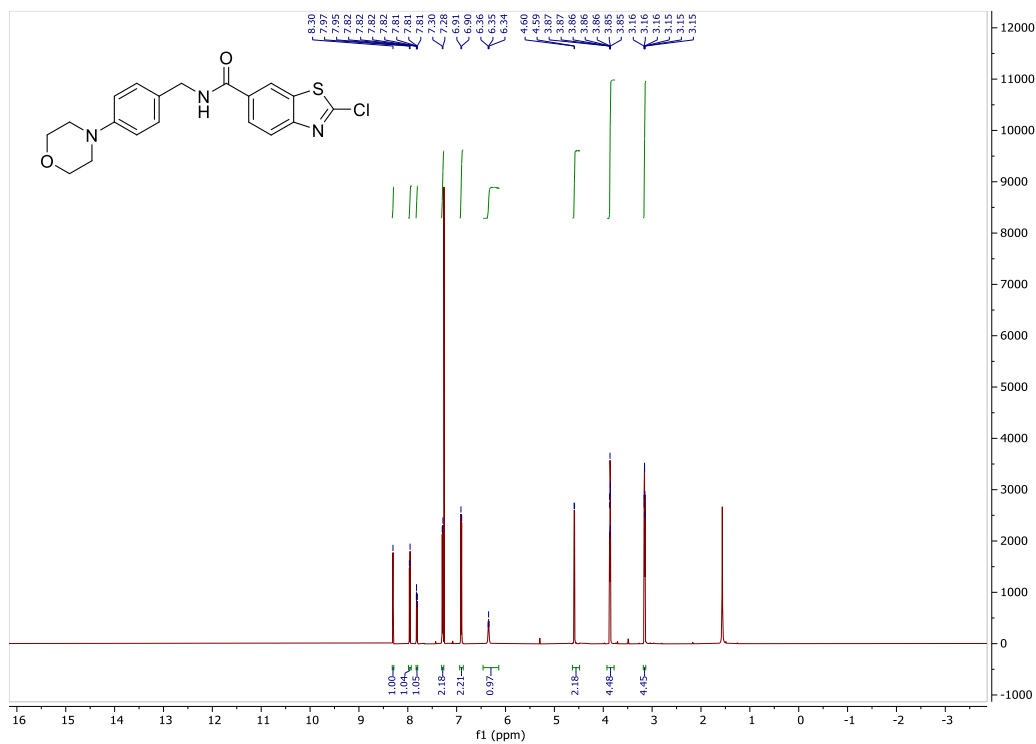
¹³C NMR (150 MHz, DMSO-*d*₆) Spectrum of Compound **5**



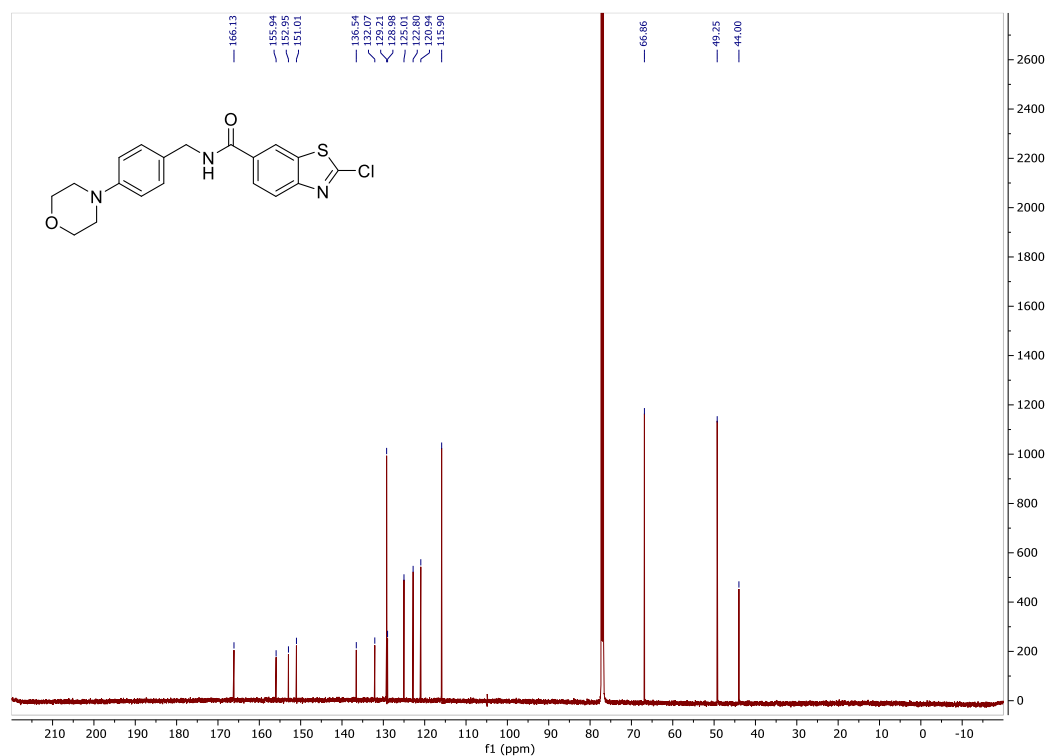
^{19}F NMR (283 MHz, $\text{DMSO-}d_6$) Spectrum of Compound **5**



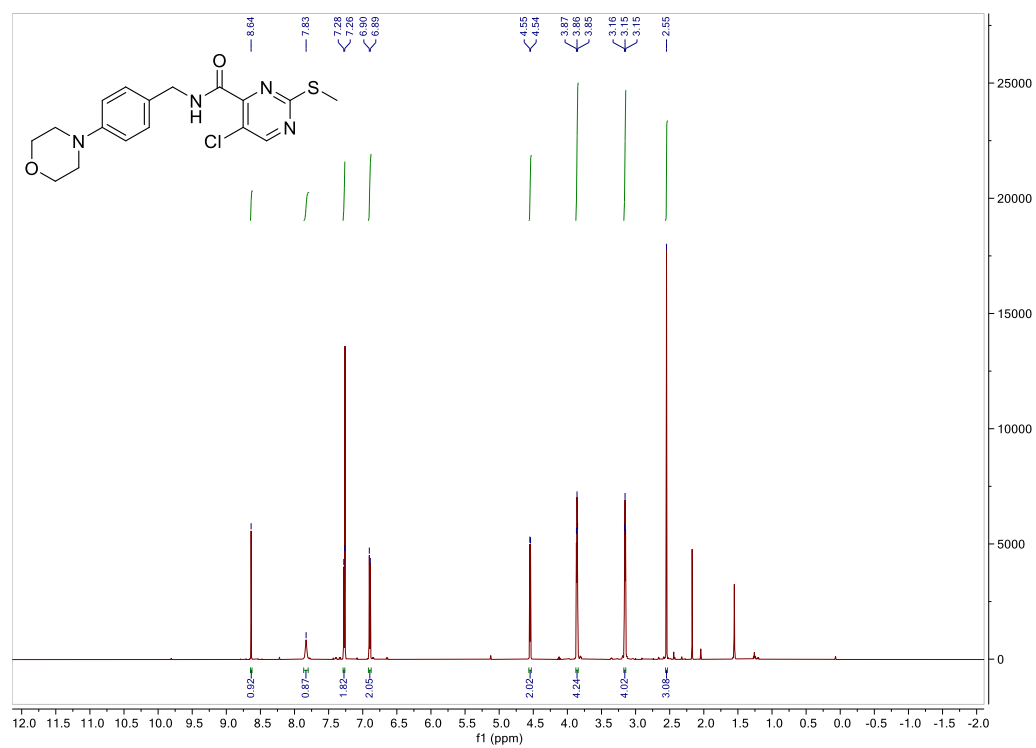
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **6**



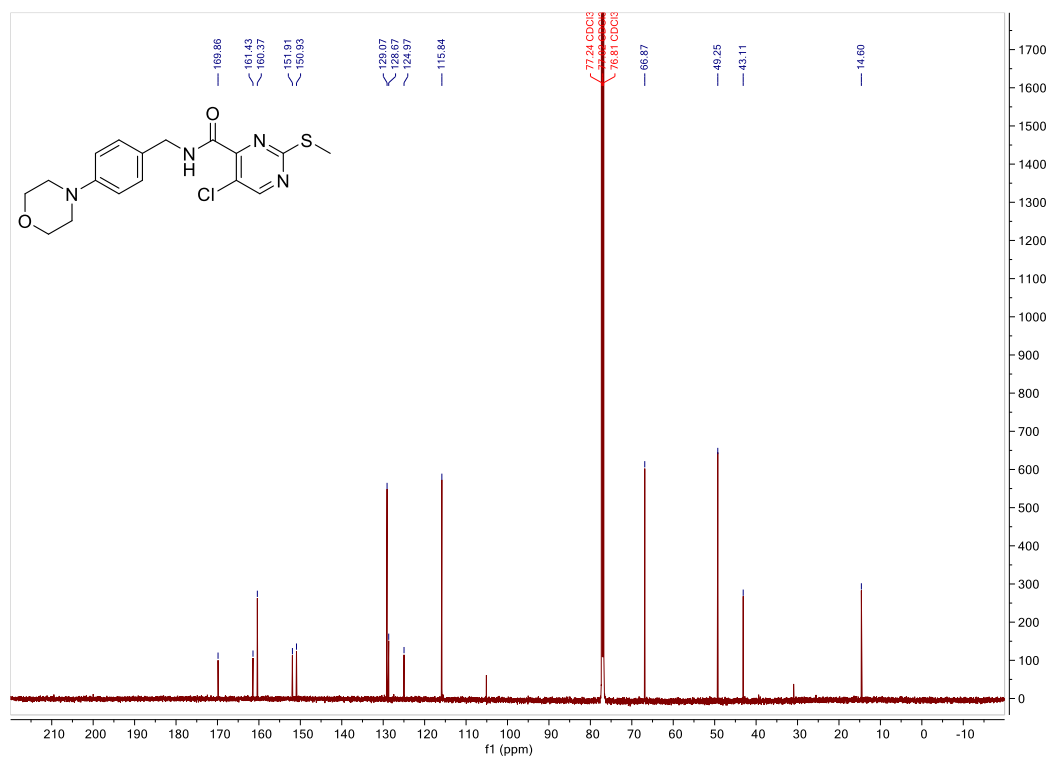
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **6**



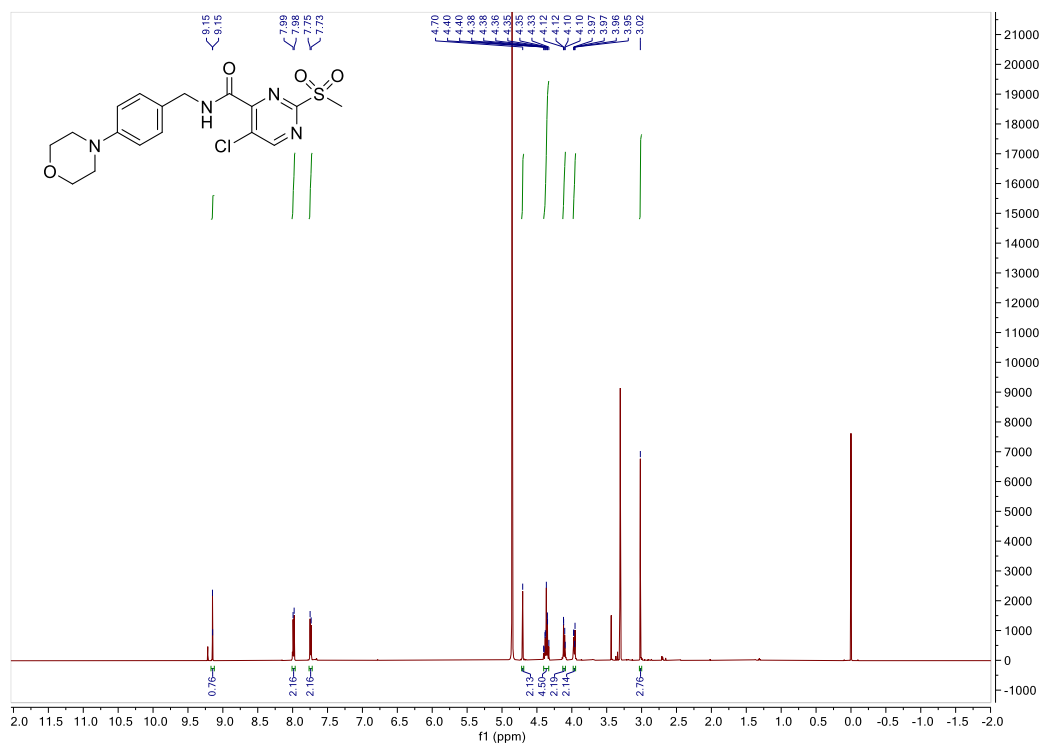
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **7a**



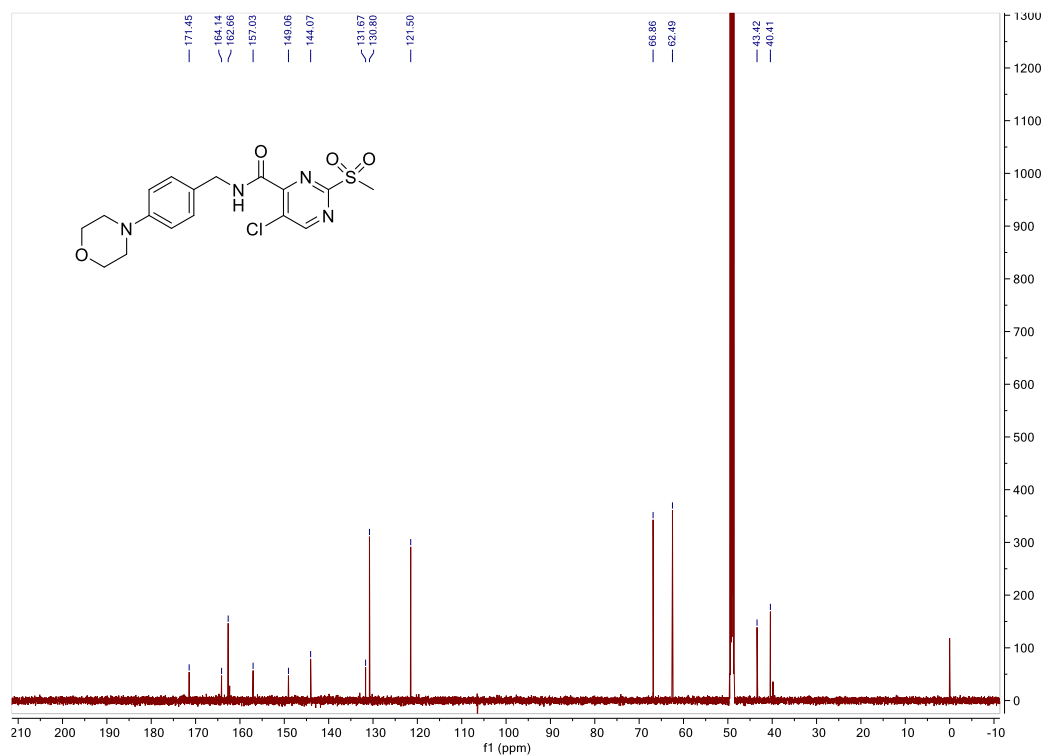
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **7a**



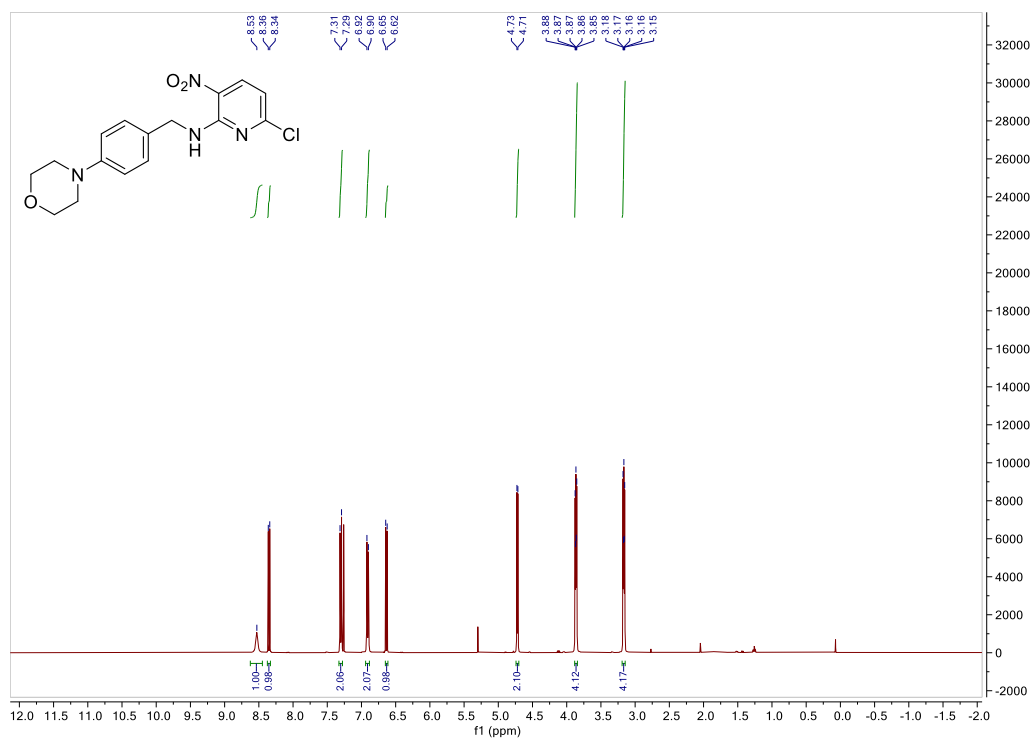
^1H NMR (600 MHz, $\text{Methanol-}d_4$) Spectrum of Compound **7**



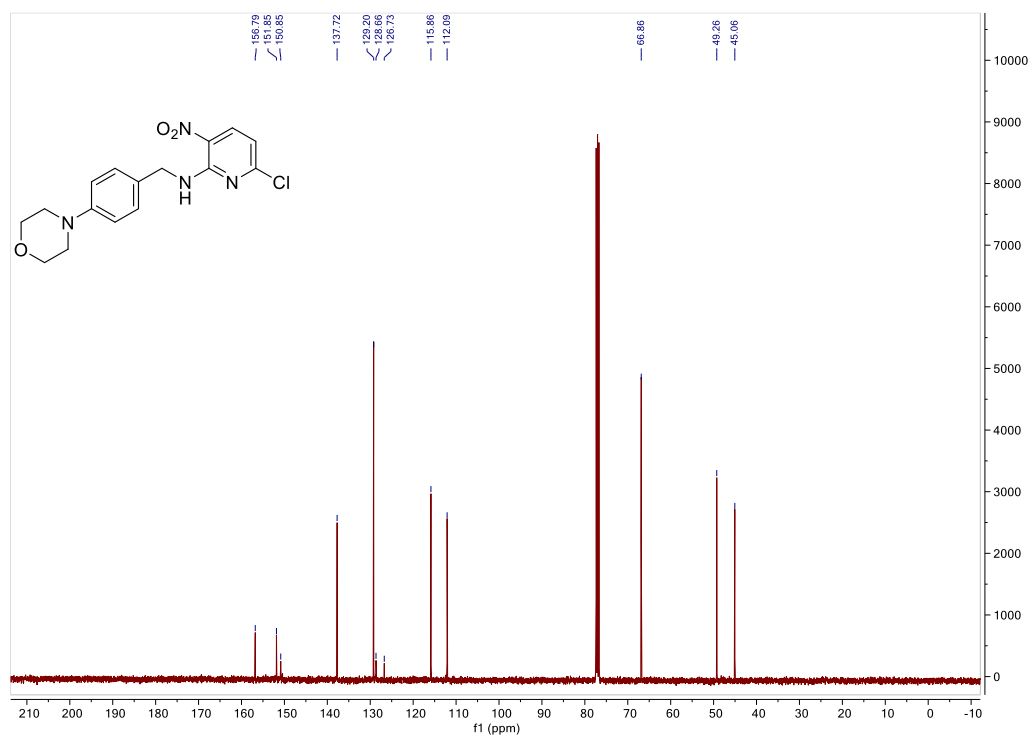
^{13}C NMR (150 MHz, Methanol- d_4) Spectrum of Compound **7**



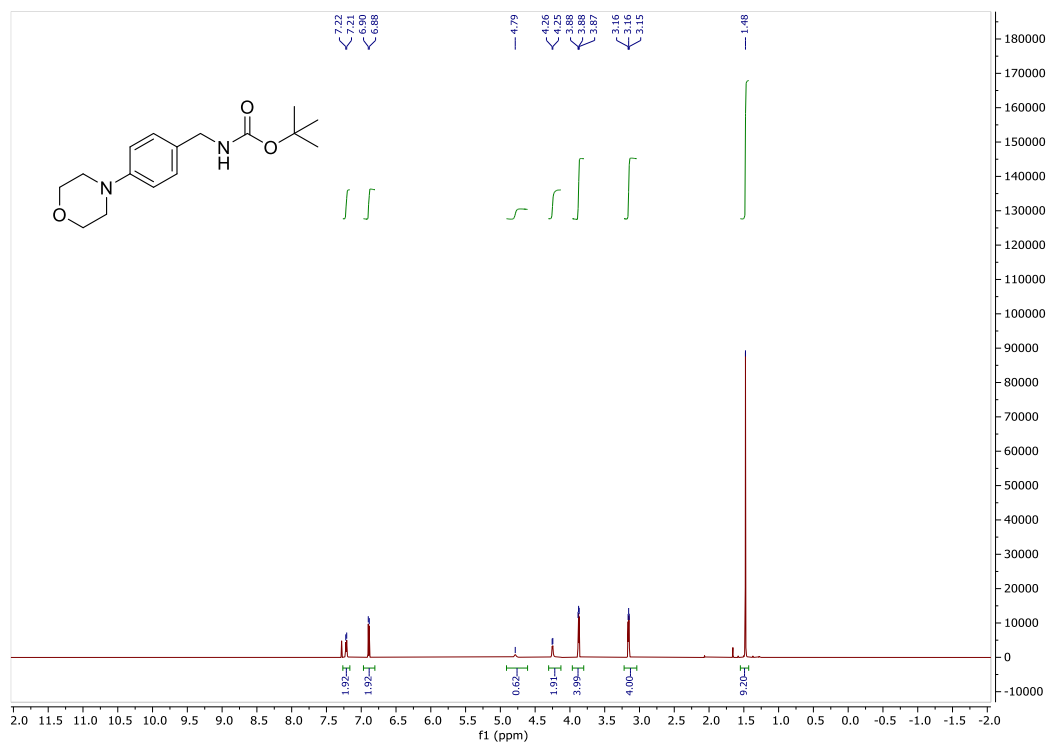
^1H NMR (400 MHz, CDCl_3) Spectrum of Compound **8**



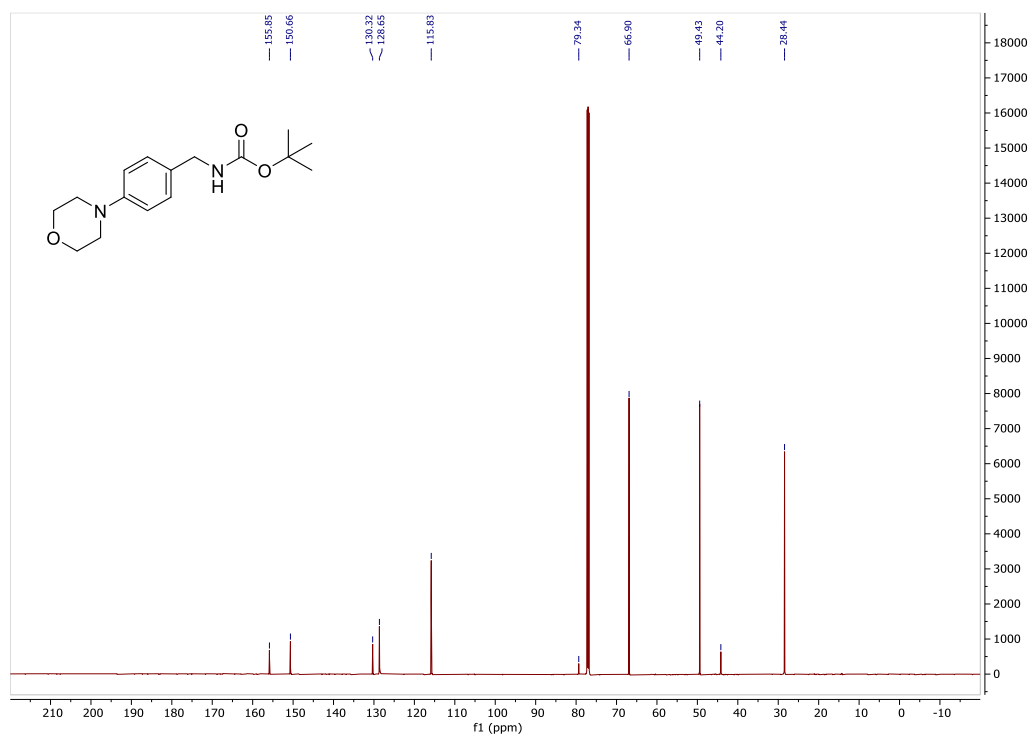
^{13}C NMR (100 MHz, CDCl_3) Spectrum of Compound **8**



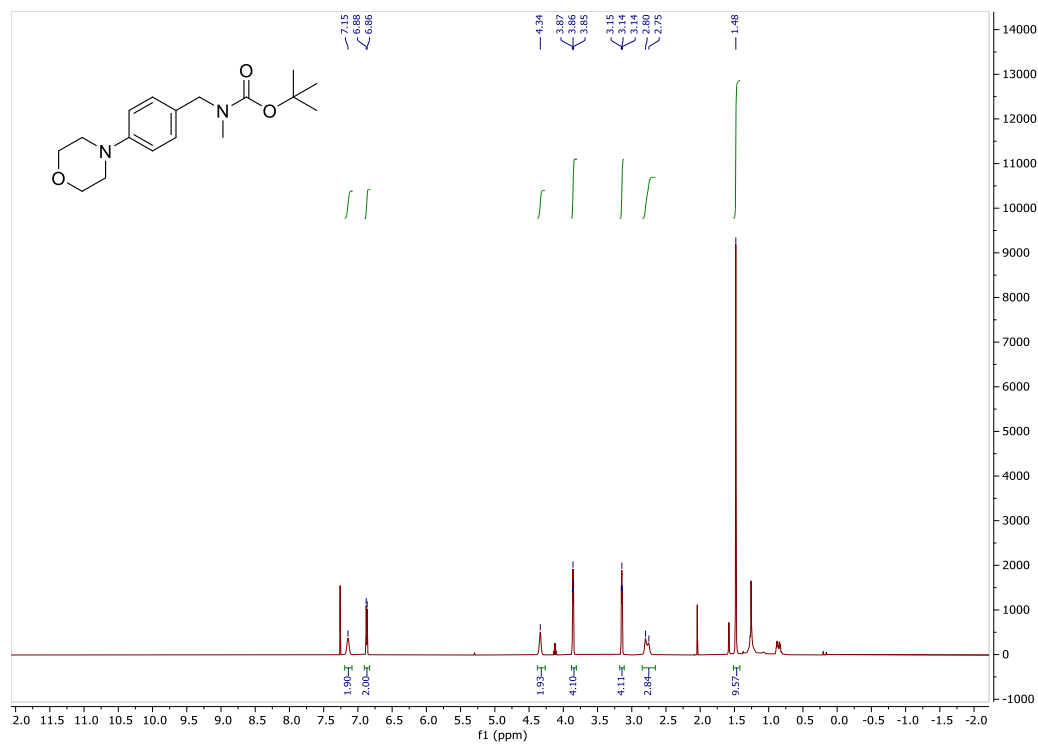
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **9a**



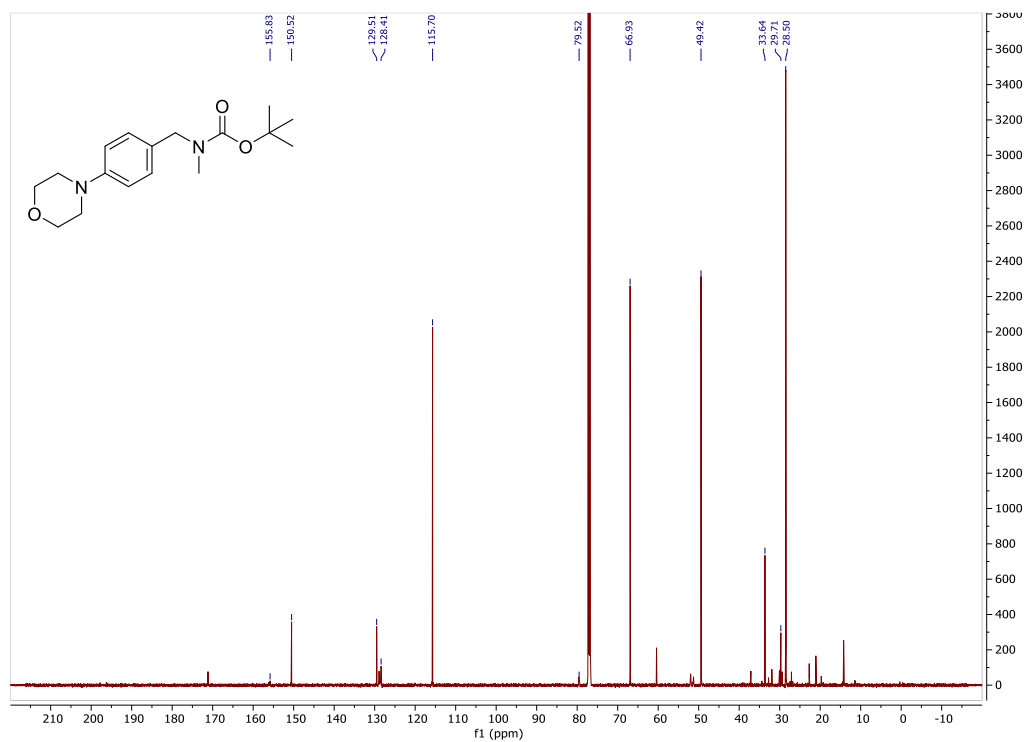
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **9a**



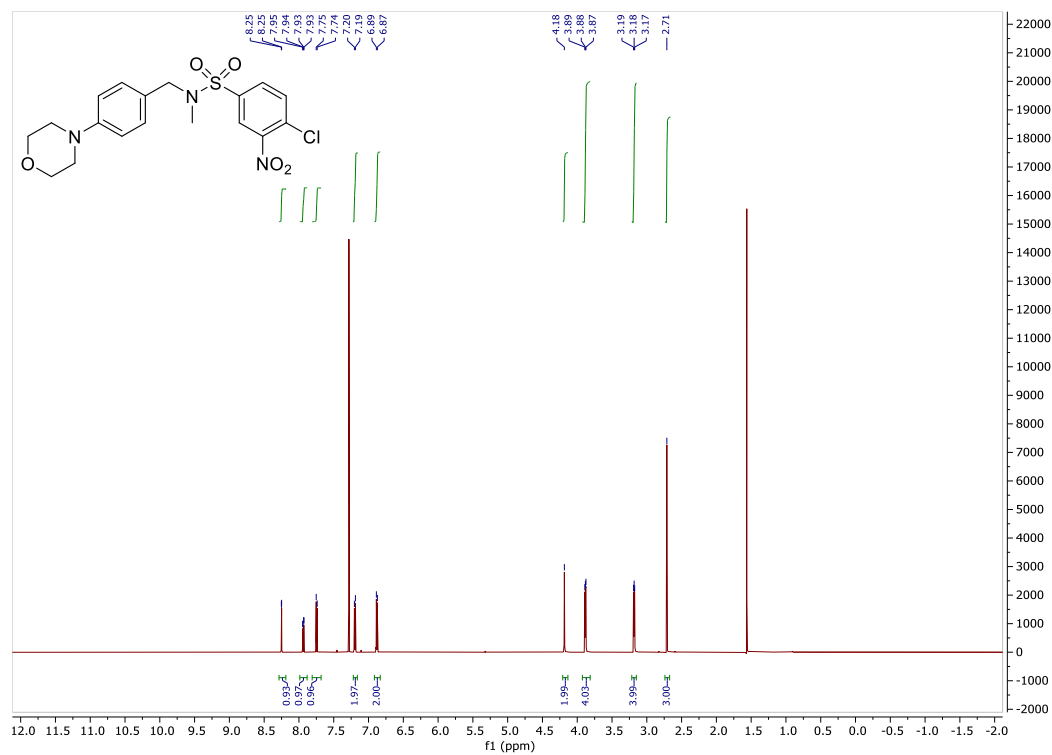
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **9b**



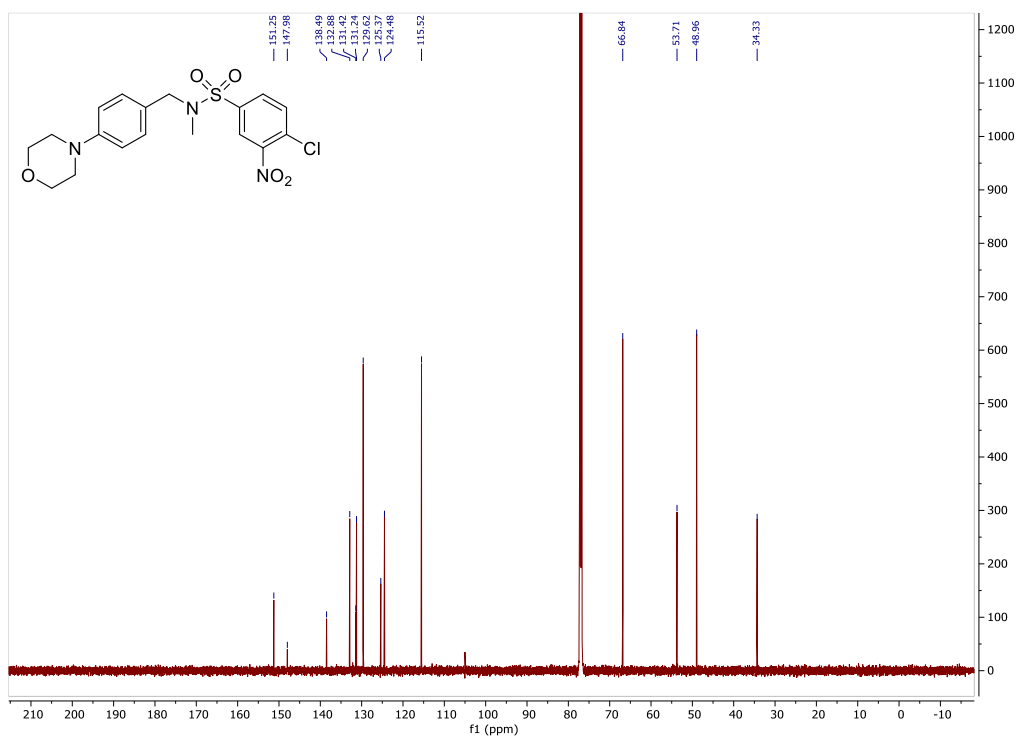
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **9b**



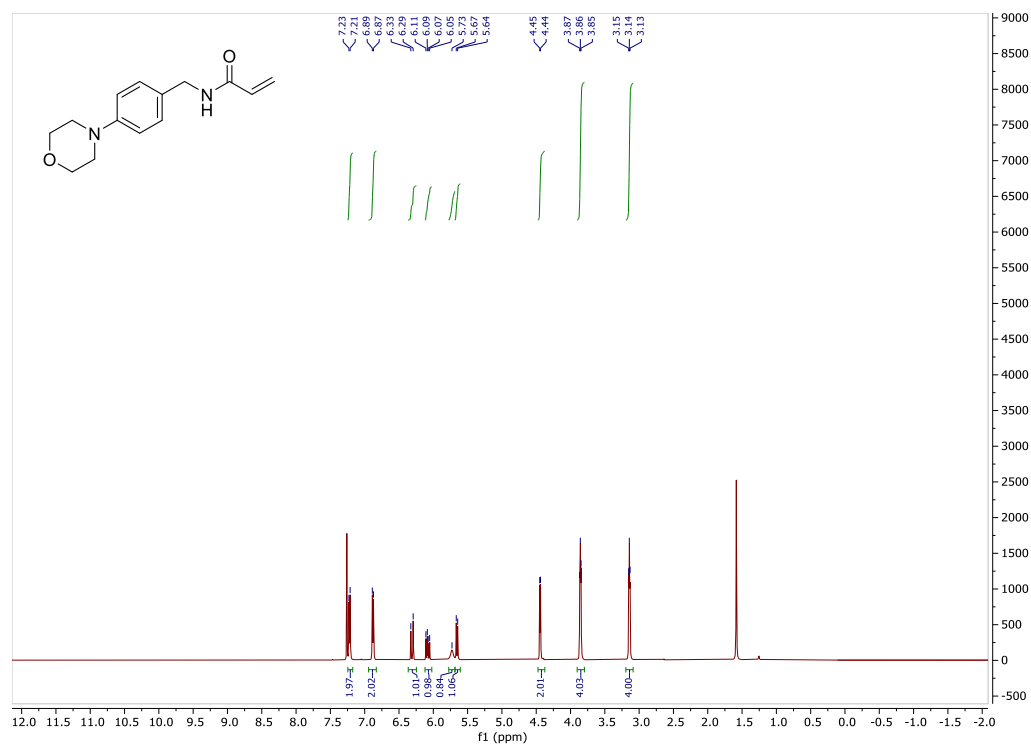
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **9**



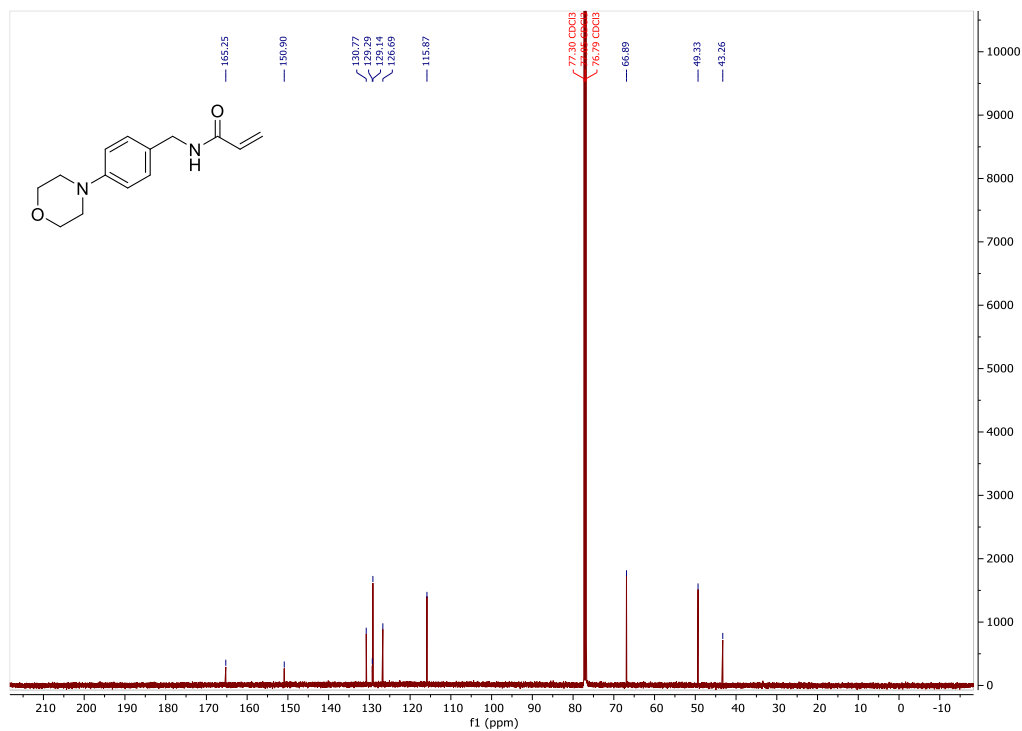
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **9**



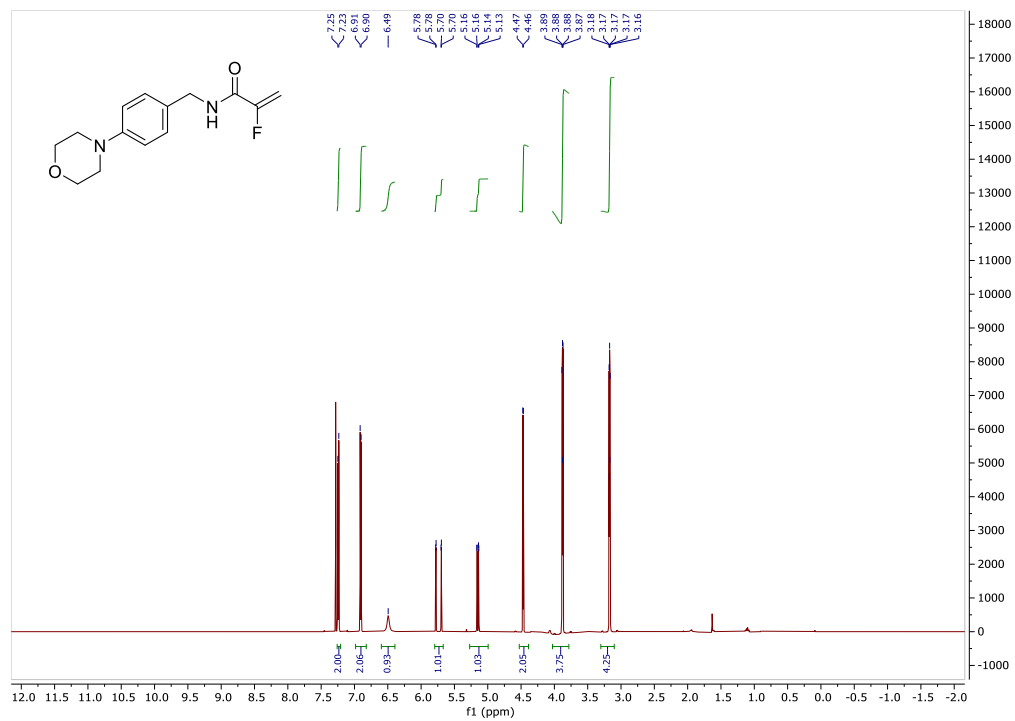
¹H NMR (500 MHz, CDCl₃) Spectrum of Compound **10**



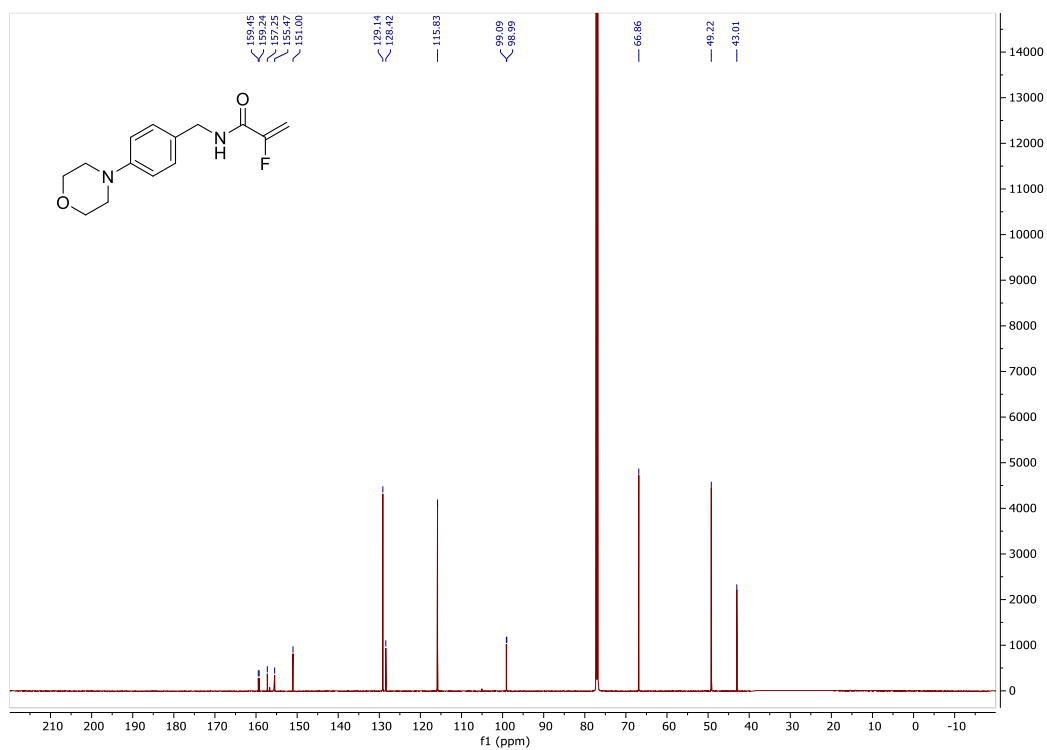
¹³C NMR (125 MHz, CDCl₃) Spectrum of Compound **10**



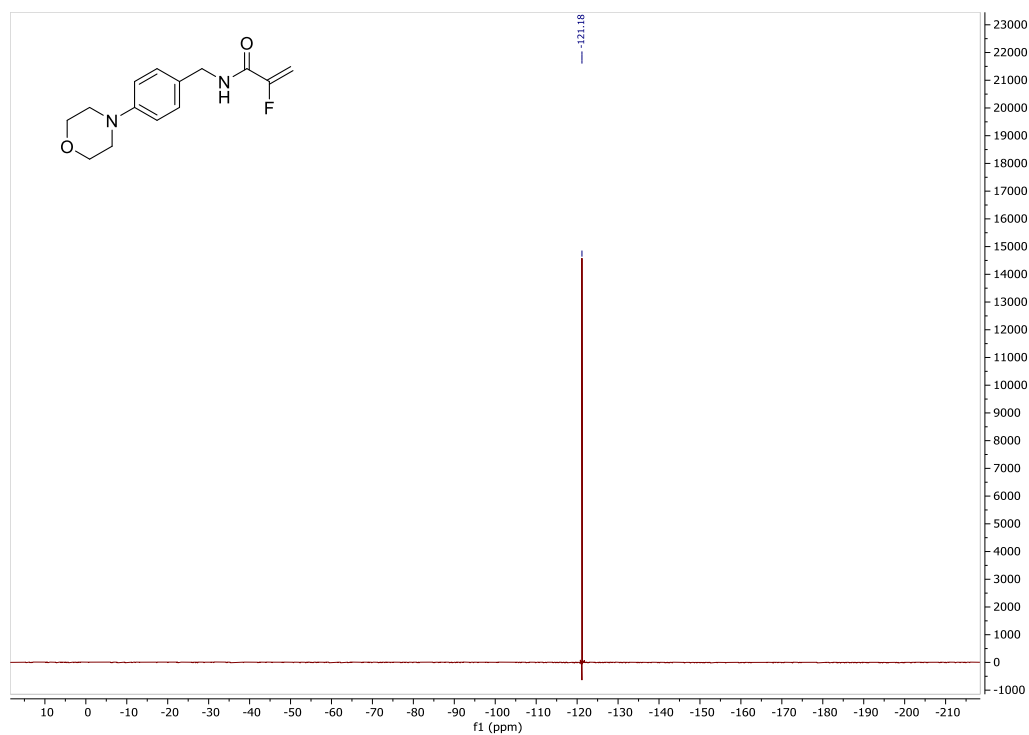
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **11**



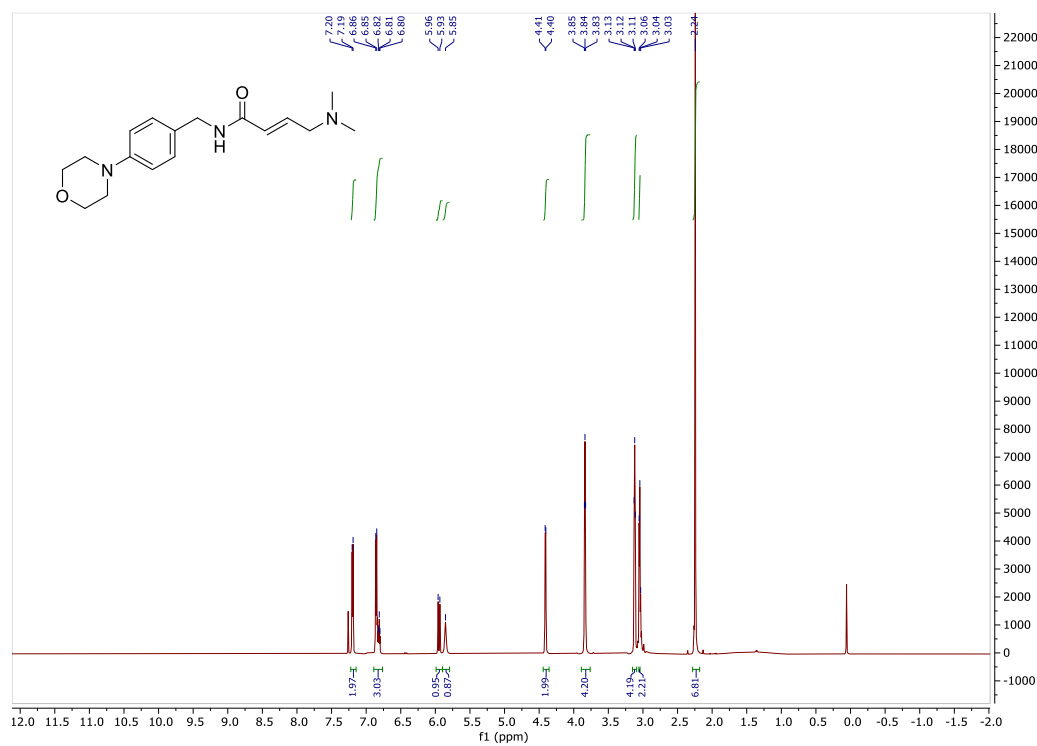
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **11**



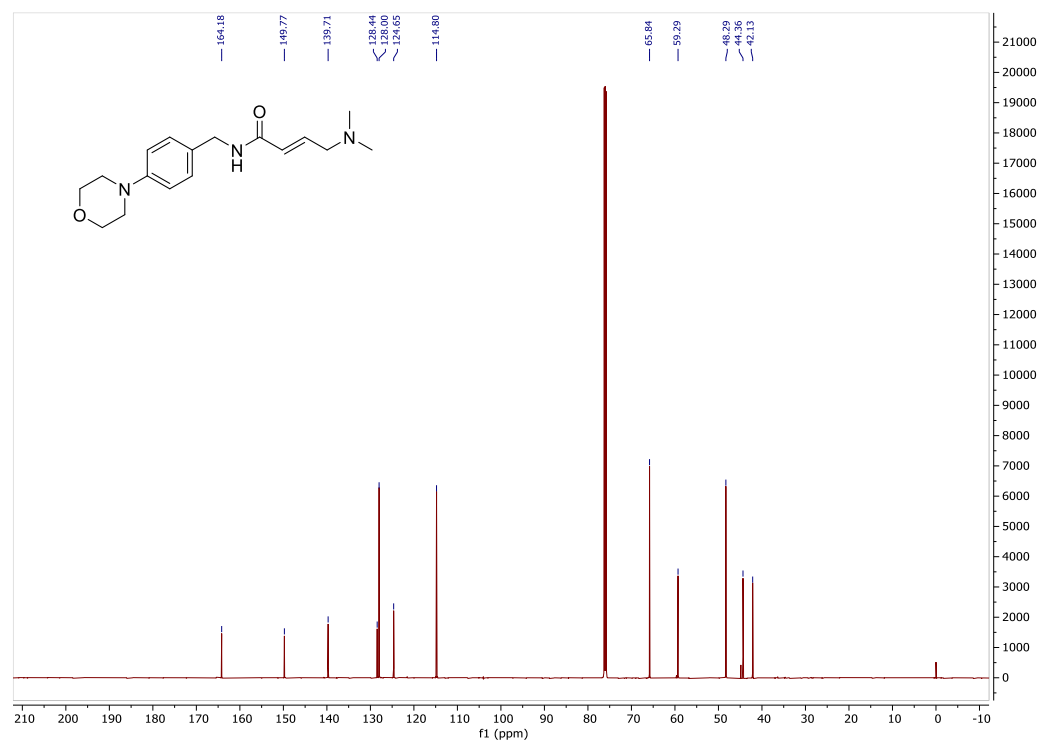
^{19}F NMR (283 MHz, CDCl_3) Spectrum of Compound **11**



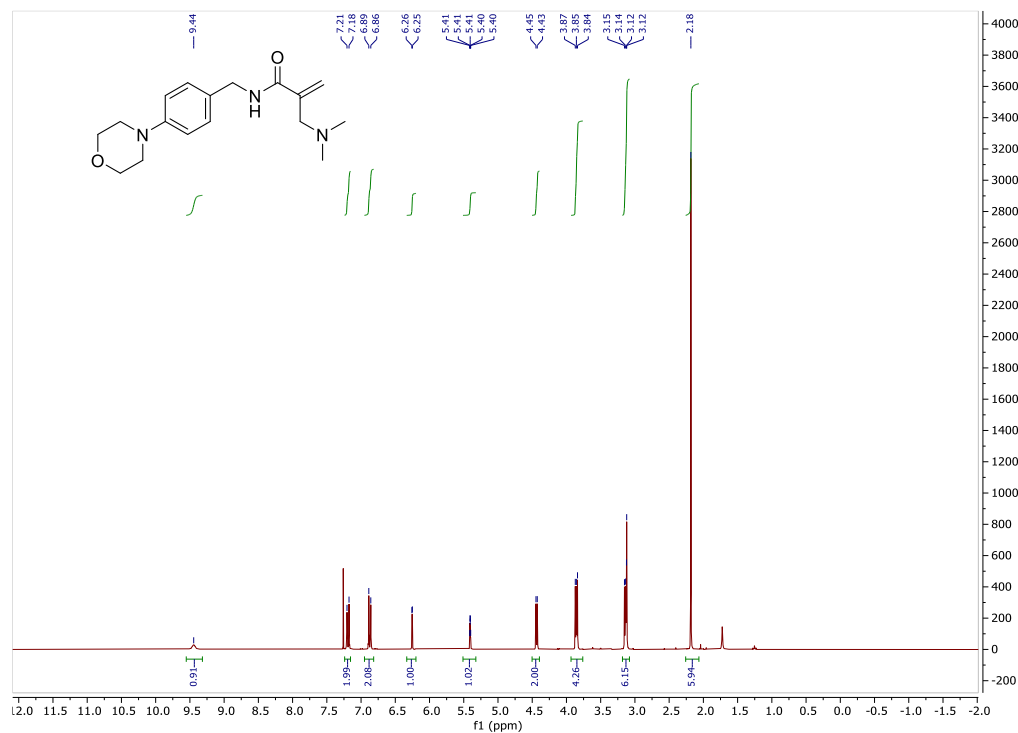
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **12**



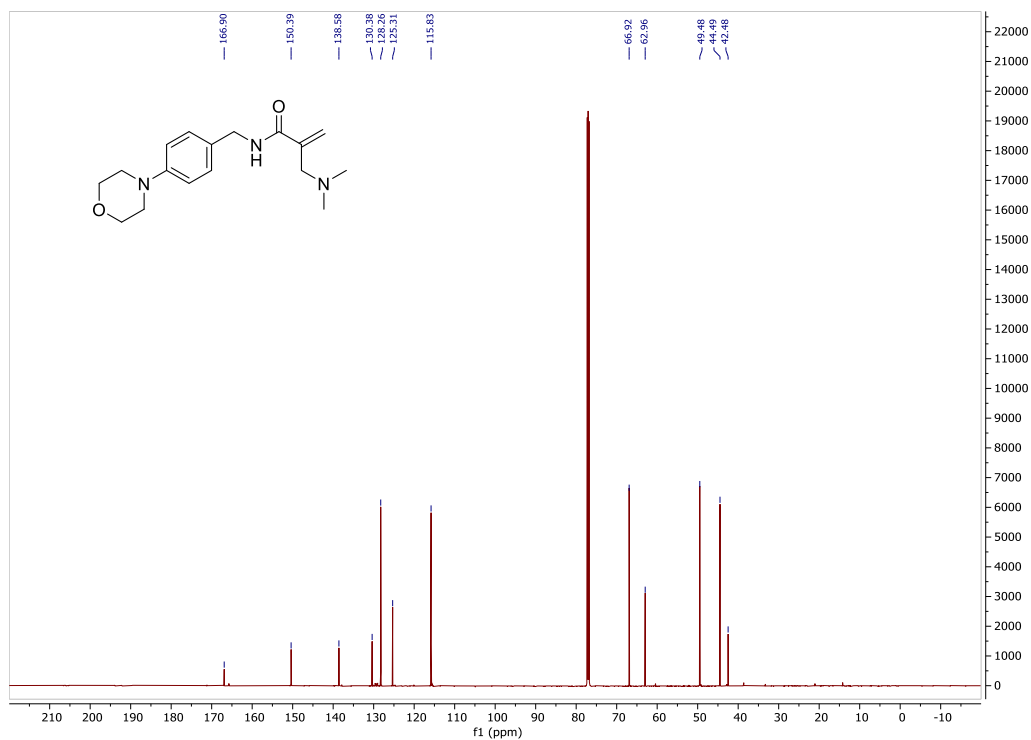
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **10**



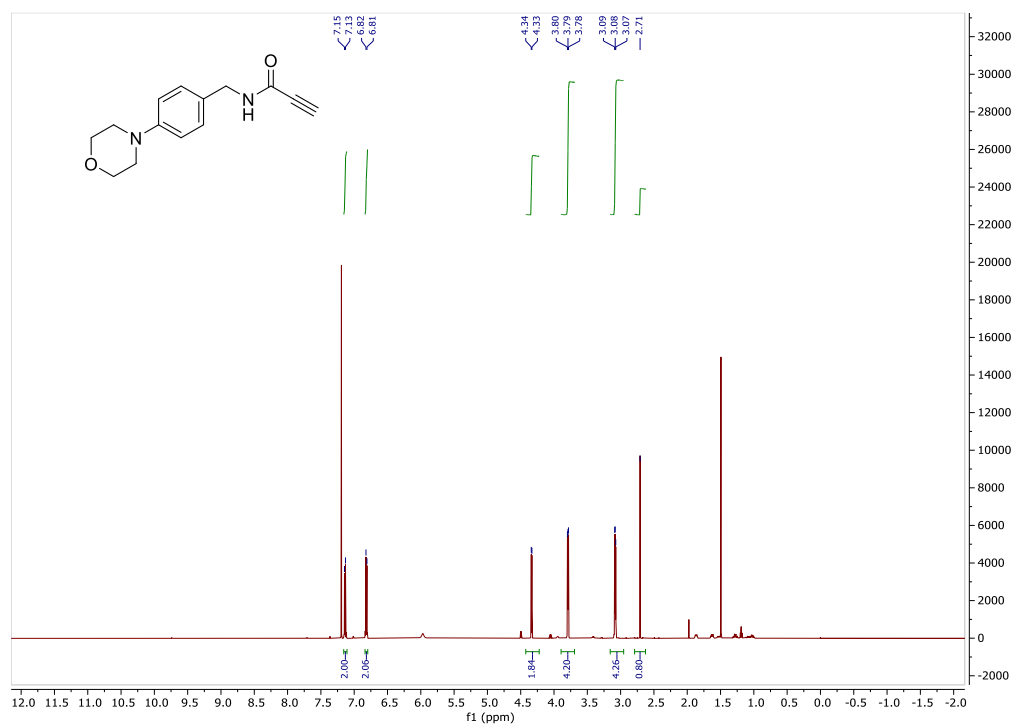
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **13**



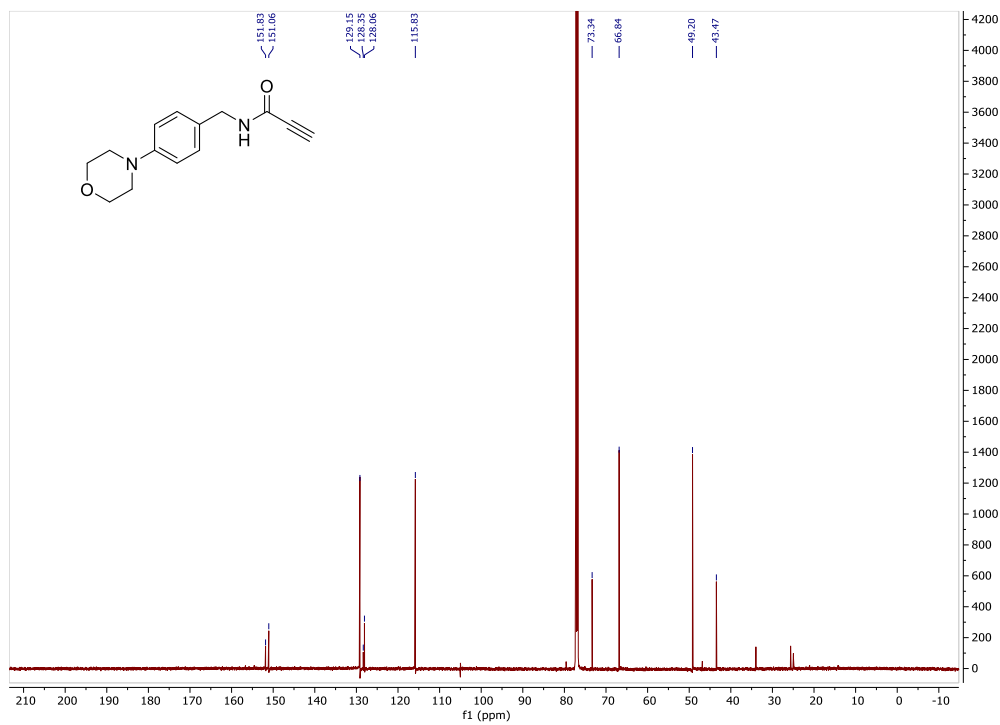
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **13**



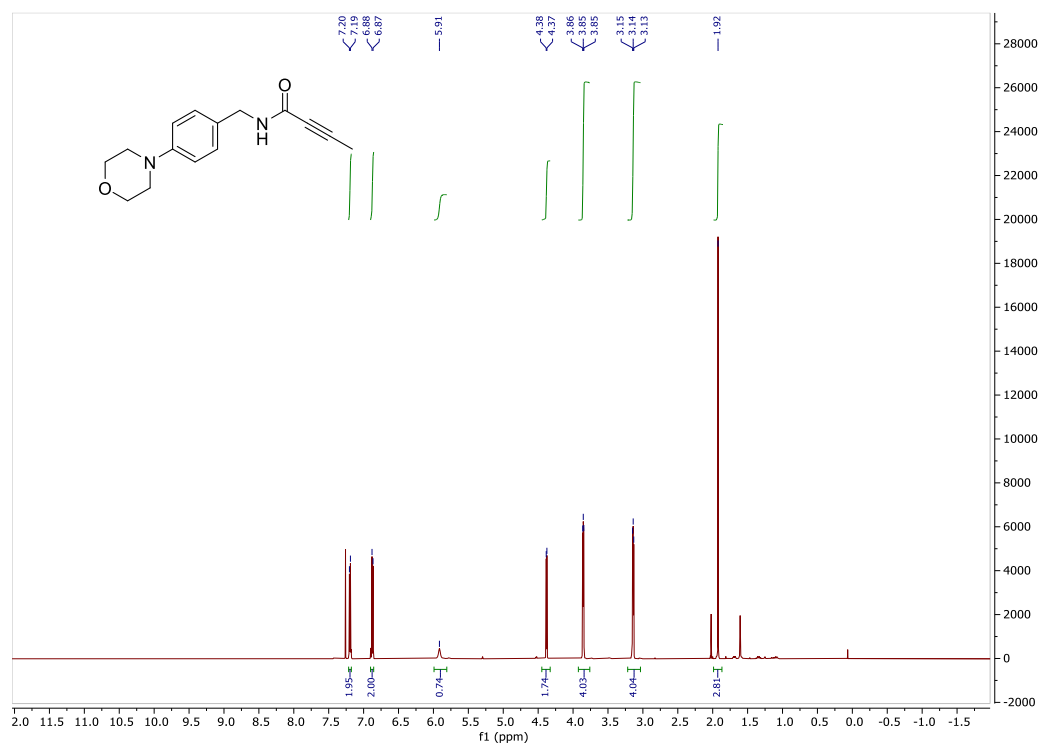
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **14**



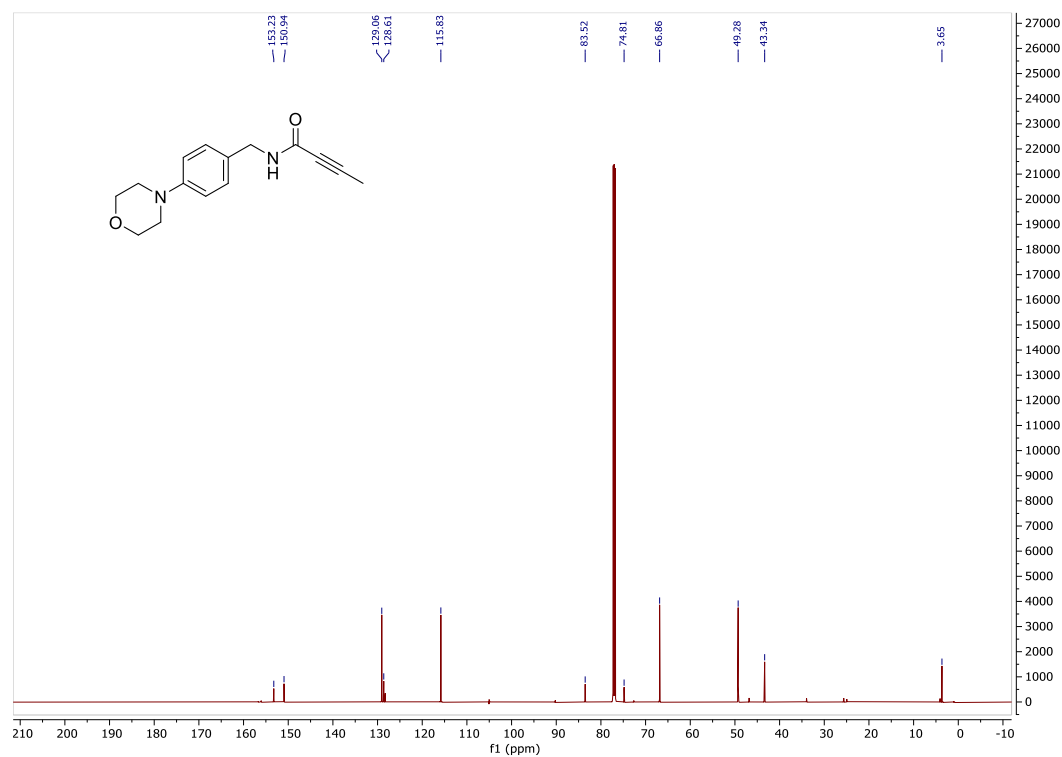
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **14**



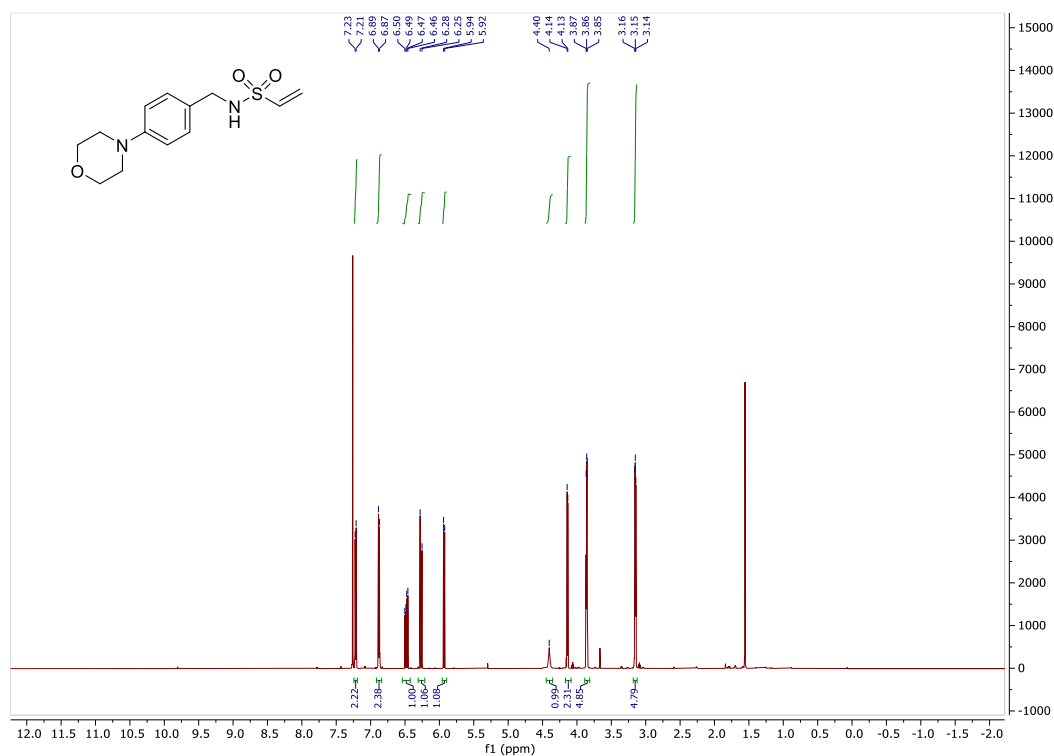
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **15**



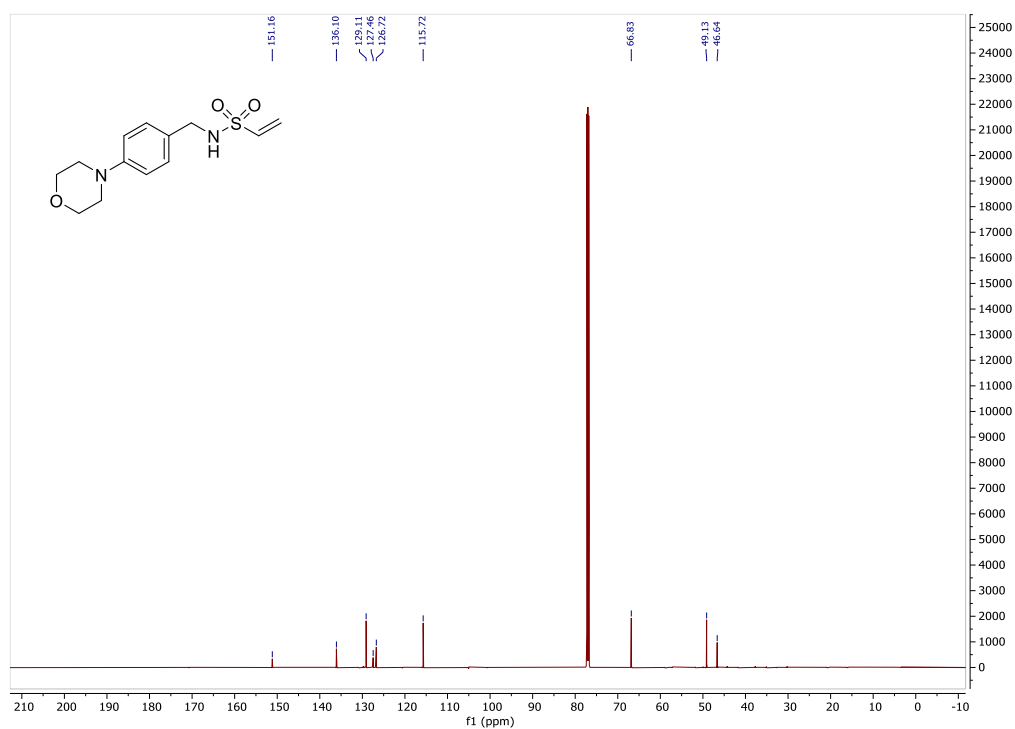
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **15**



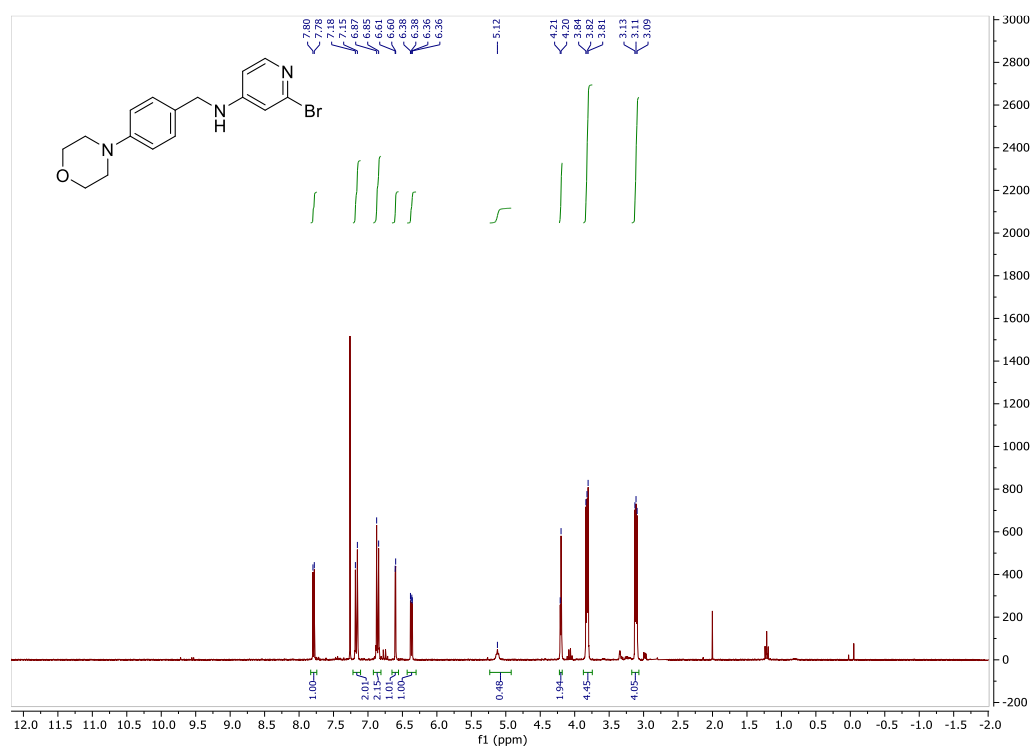
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **16**



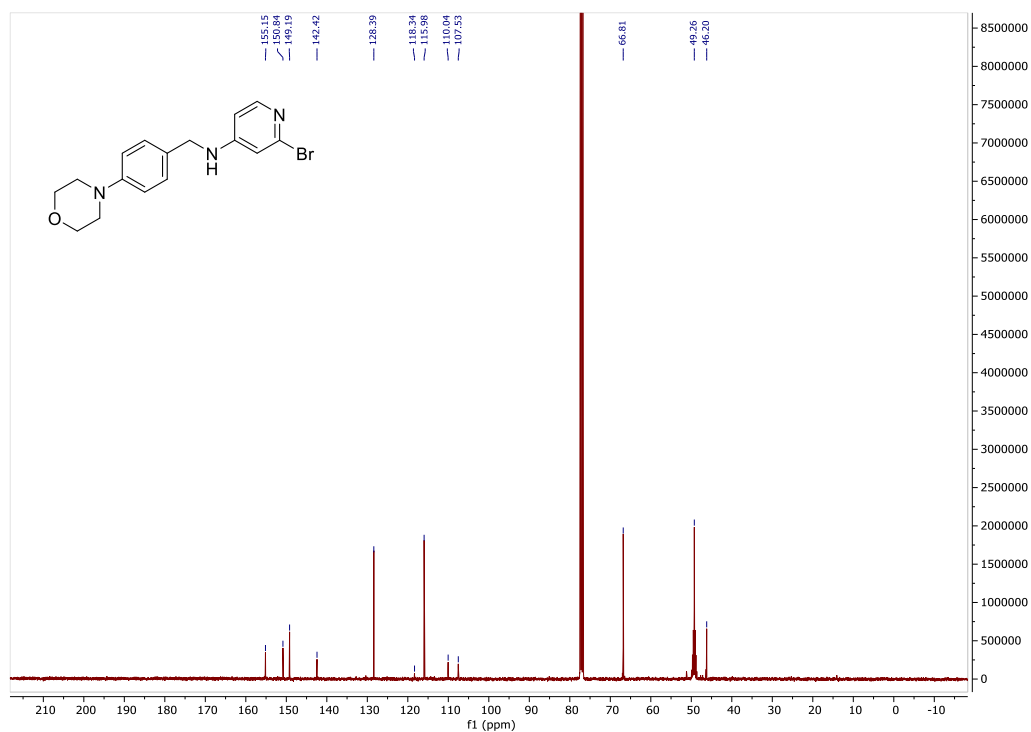
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **16**



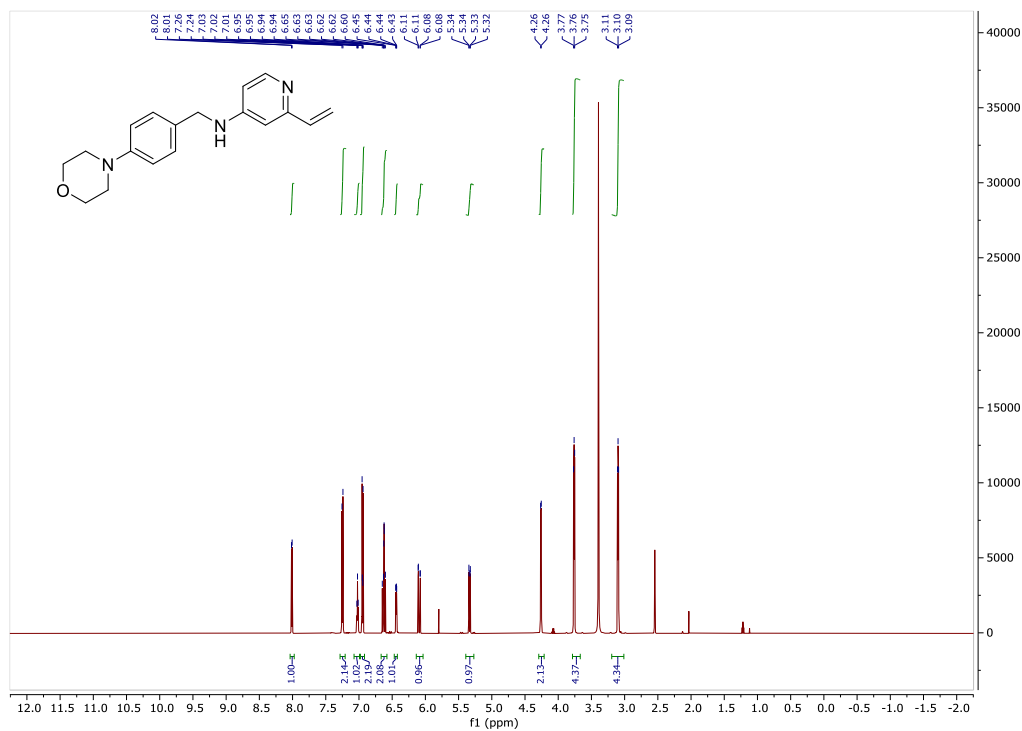
^1H NMR (300 MHz, CDCl_3 + $\text{Methanol-}d_4$) Spectrum of Compound **17a**



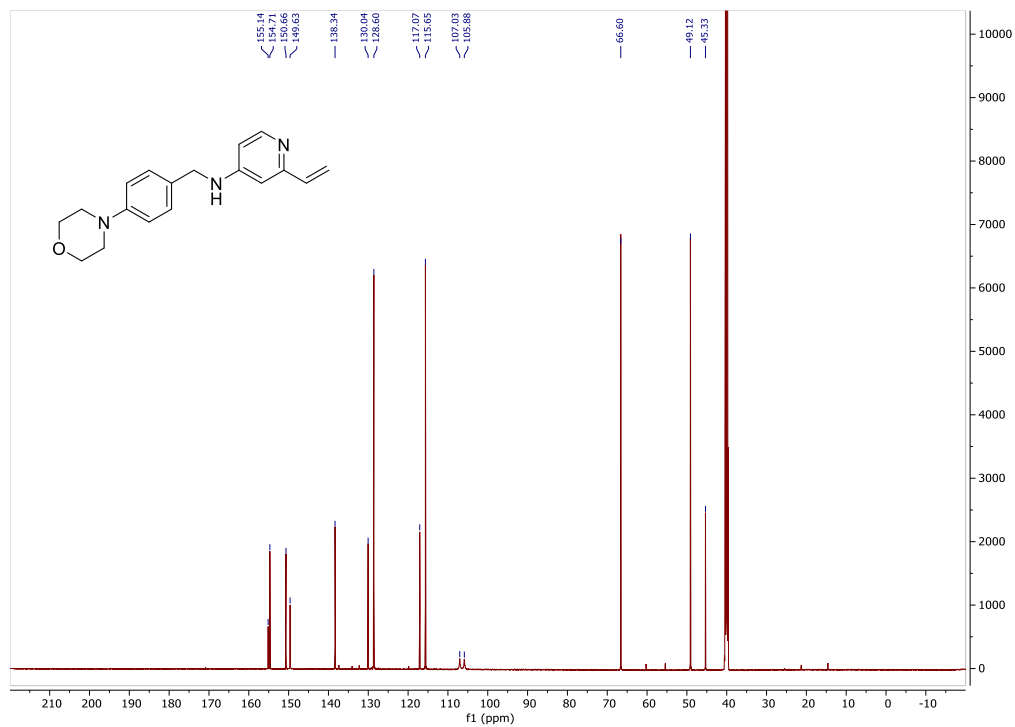
^{13}C NMR (100 MHz, CDCl_3 + $\text{Methanol-}d_4$) Spectrum of Compound **17a**



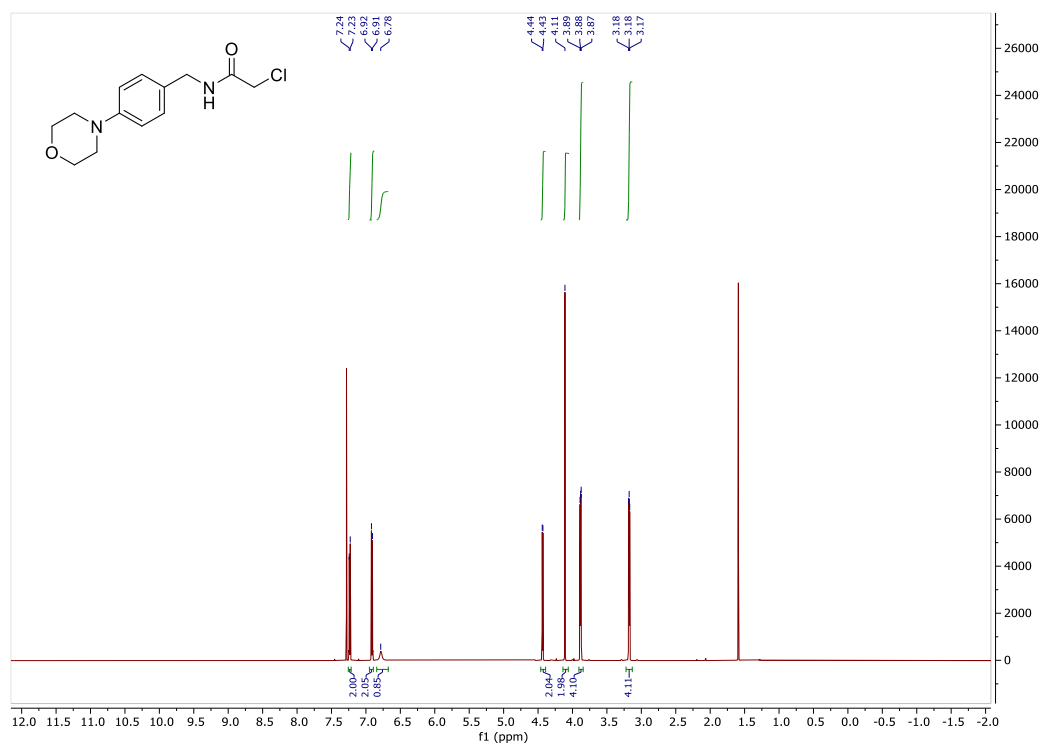
¹H NMR (600 MHz, DMSO-*d*₆) Spectrum of Compound **17**



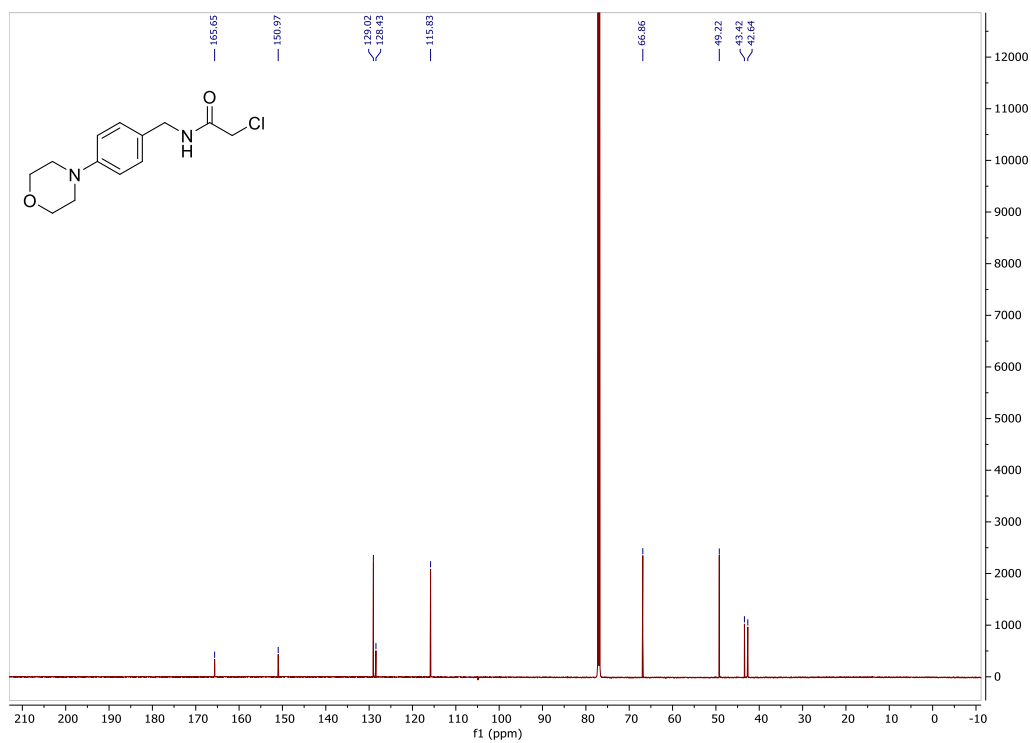
¹³C NMR (150 MHz, DMSO-*d*₆) Spectrum of Compound **17**



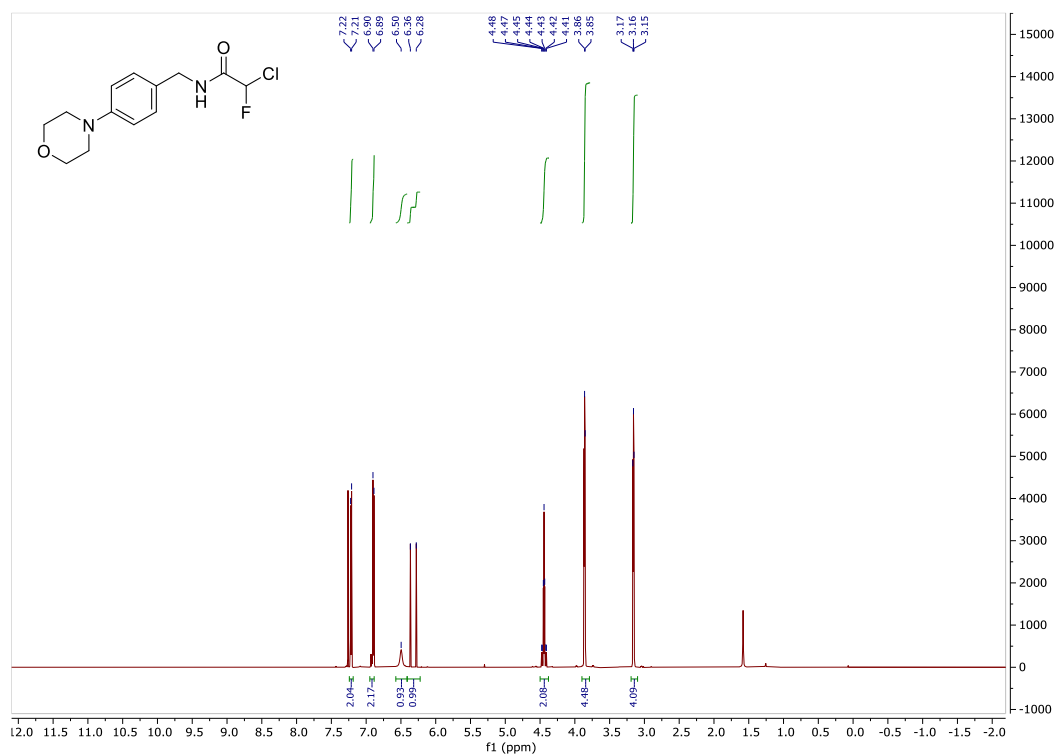
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **18**



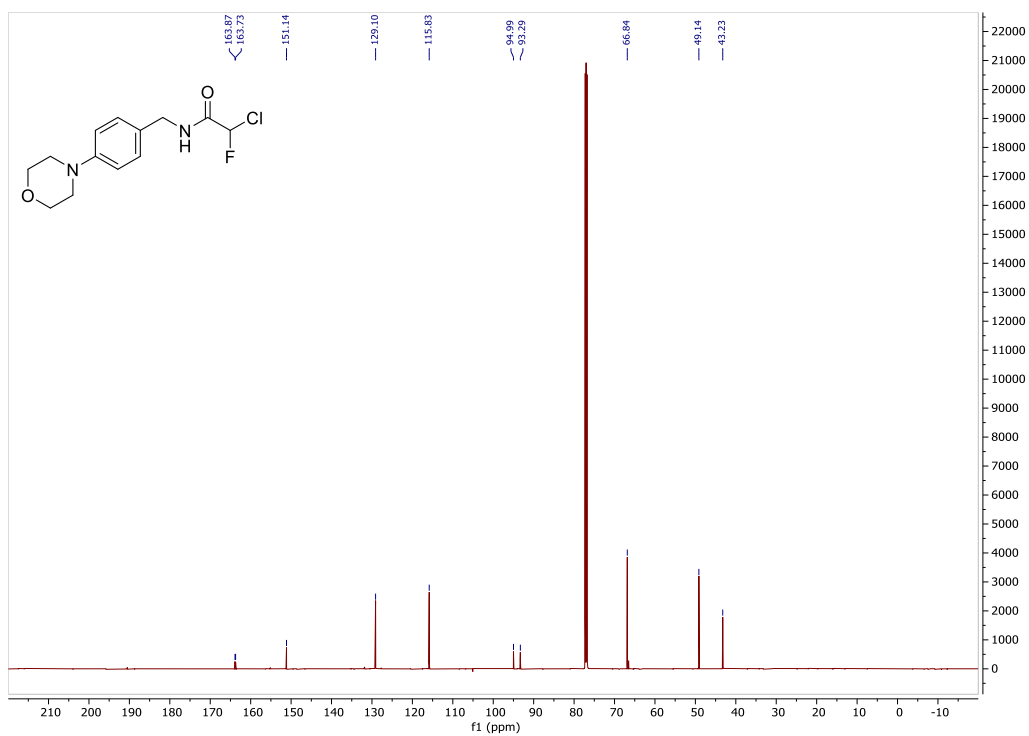
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **18**



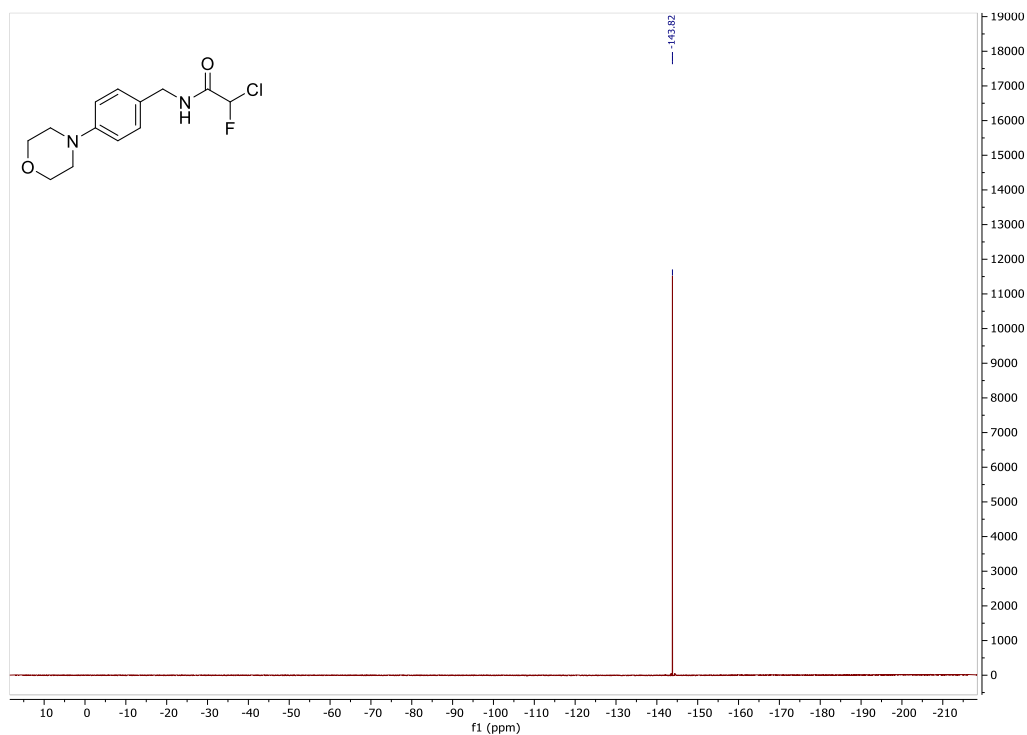
¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **19**



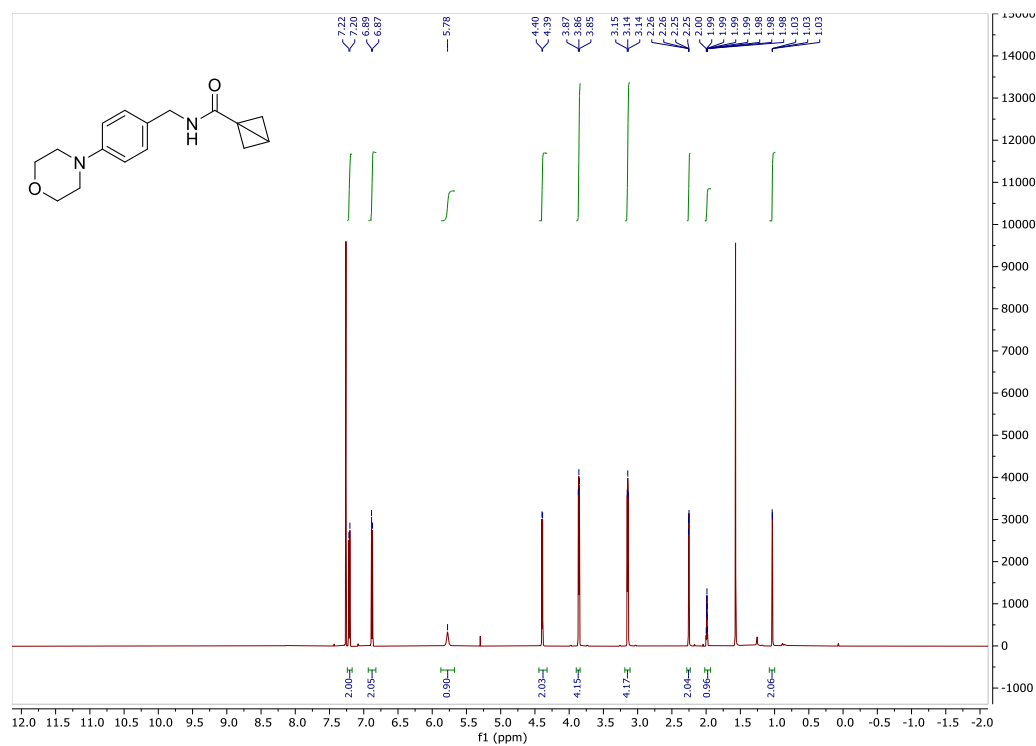
¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **19**



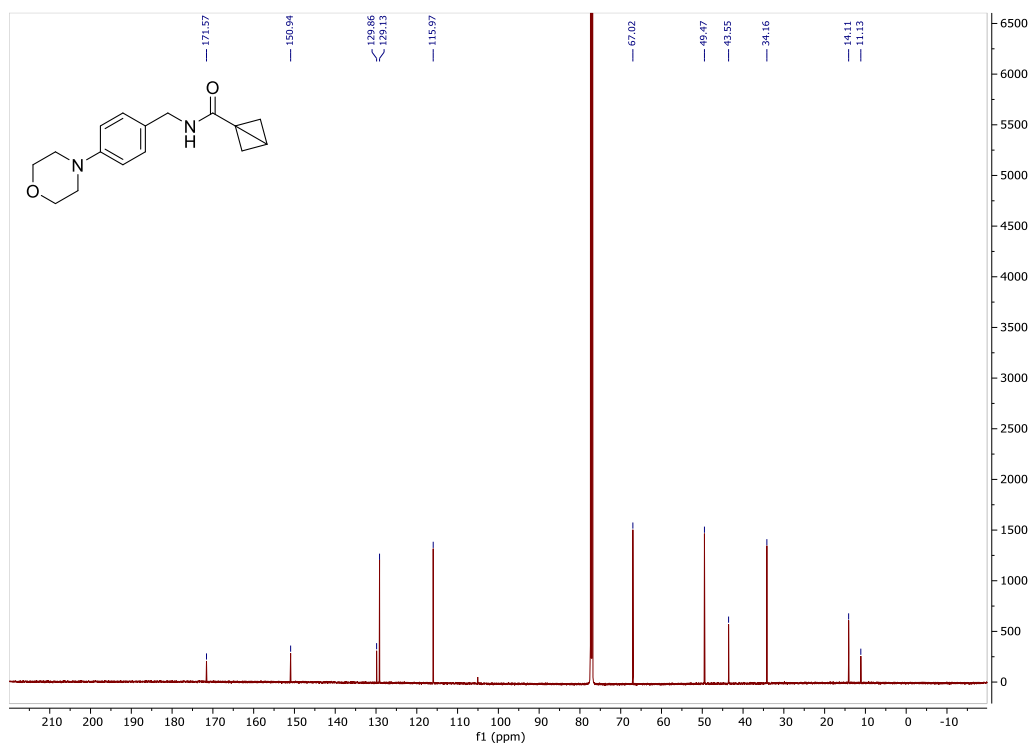
^{19}F NMR (283 MHz, CDCl_3) Spectrum of Compound **19**



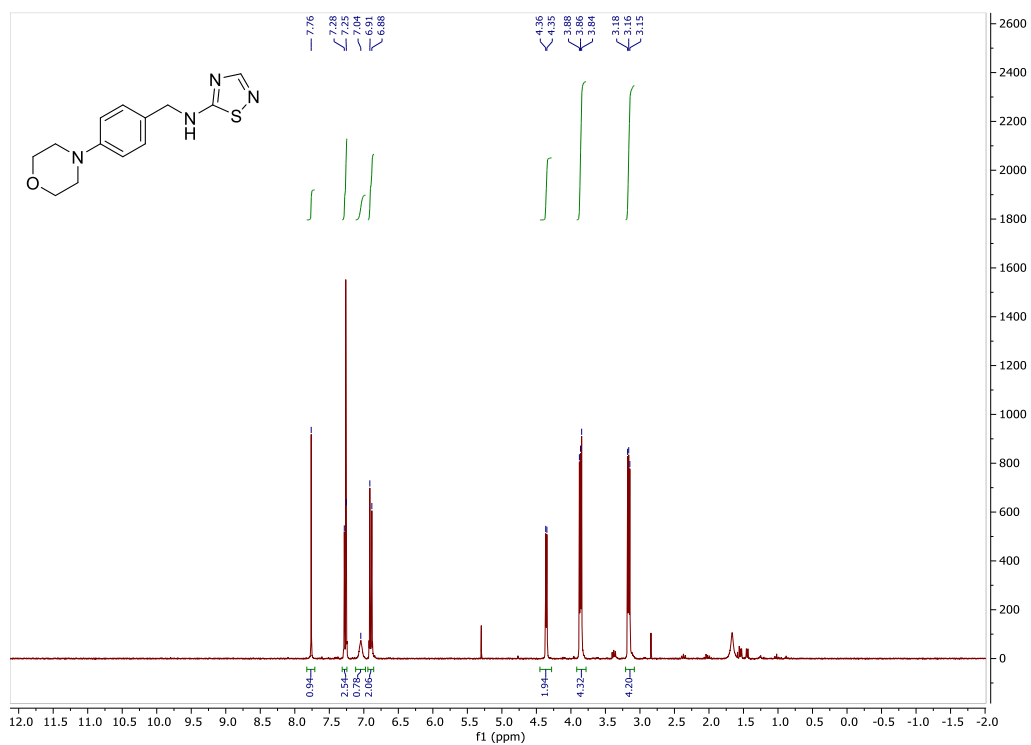
^1H NMR (600 MHz, CDCl_3) Spectrum of Compound **20**



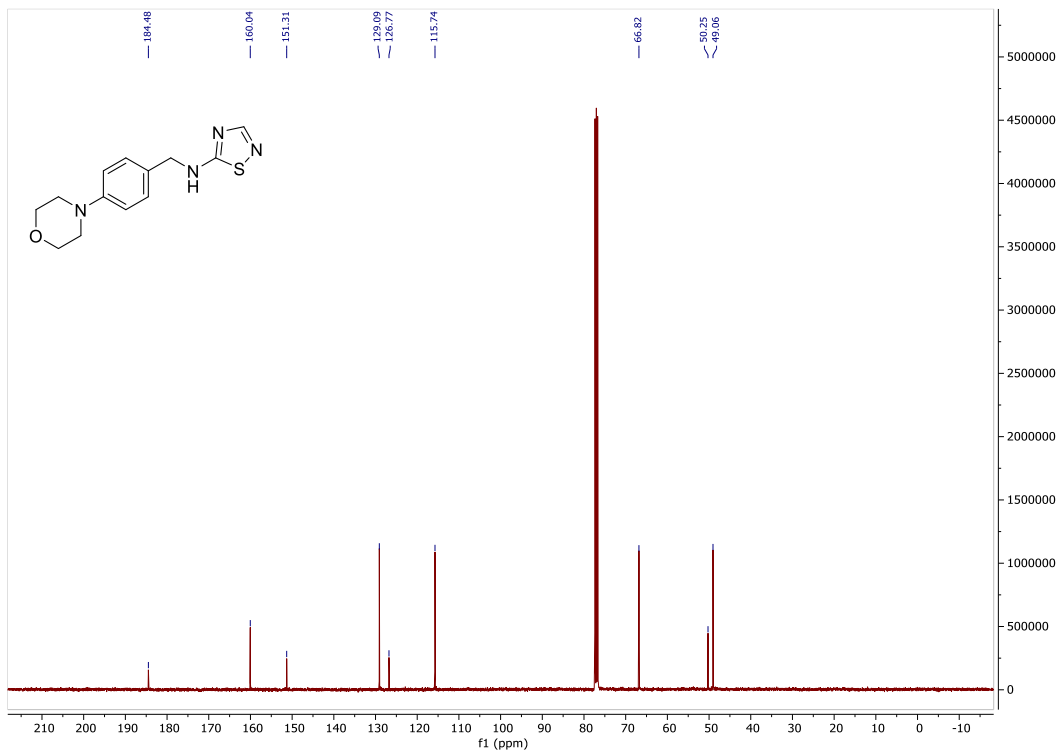
^{13}C NMR (150 MHz, CDCl_3) Spectrum of Compound **20**



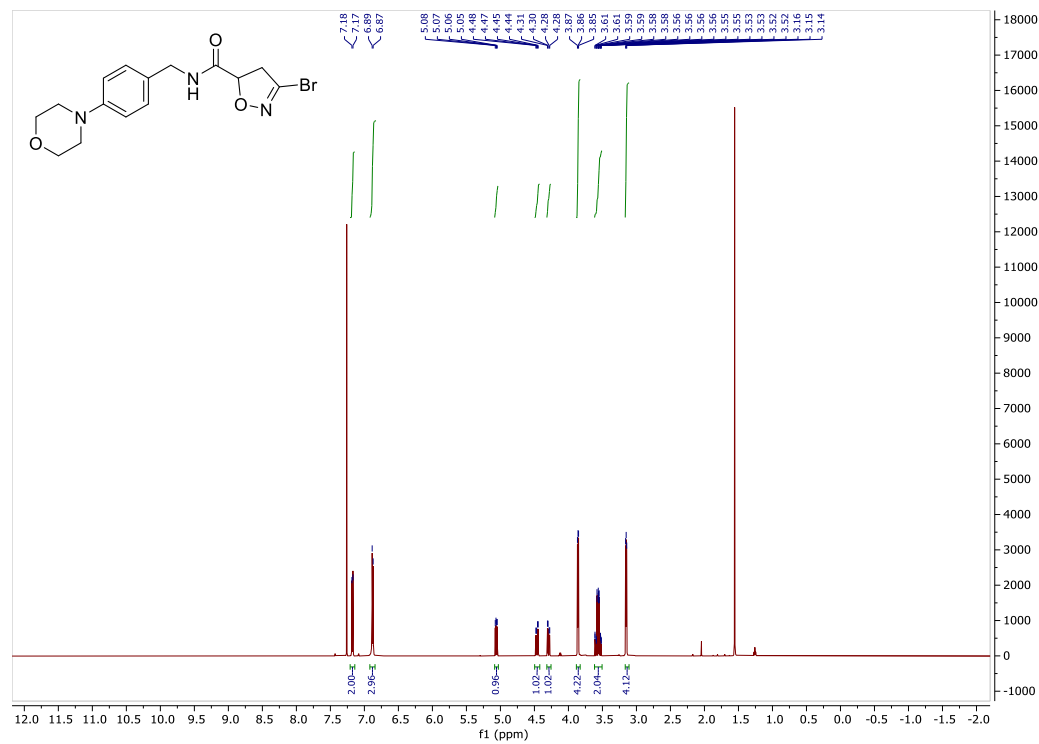
^1H NMR (300 MHz, CDCl_3) Spectrum of Compound **21**



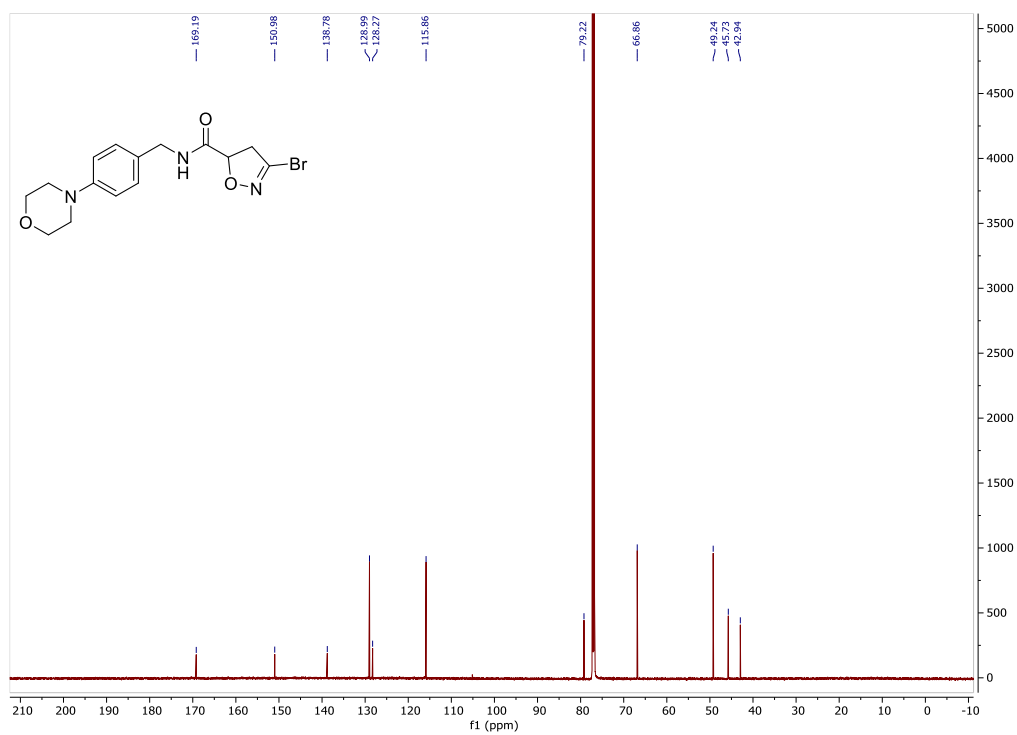
¹³C NMR (100 MHz, CDCl₃) Spectrum of Compound **21**



¹H NMR (600 MHz, CDCl₃) Spectrum of Compound **22**



¹³C NMR (150 MHz, CDCl₃) Spectrum of Compound **22**



Chapter 5 – Conclusions and Perspectives

5.1. Implications for Transglutaminase Inhibition

The results of Chapters 2 and 3 highlight that electrophilic warheads play a central and multifaceted role in governing target engagement not only with hTG2, but across the transglutaminase family. Notably, in Chapter 2, the relationship between intrinsic reactivity and selectivity was found to be non-intuitive, with more reactive warheads generally exhibiting greater selectivity than attenuated acrylamide analogues, which displayed broader inhibition profiles. This trend suggests that subtle differences in warhead substitution may significantly impact positioning within the active site and influence whether inhibitors adopt reactive or non-productive binding orientations. In this context, warhead structure contributes not only to reactivity, but also to binding affinity, highlighting that overall inhibitory potency arises from a balance between these parameters. The observed activity of the **EB-2-16** scaffold against related enzymes, particularly TG3 and FXIII, further highlights the potential to exploit warhead variation on this scaffold as a starting point for the development of inhibitors targeting other TGases of therapeutic interest.

Comparison of the warhead library in Chapter 2 with the internal alkyne scaffold described in Chapter 3 demonstrates that optimal warhead design is highly dependent on the molecular context in which it is embedded. While the results of Chapter 2 suggested that sterically minimal electrophiles are required to access the narrow active site tunnel of hTG2, the internal alkyne scaffold challenges this assumption by demonstrating that sterically complex electrophiles can still effectively engage Cys277. These findings suggest that the active site may be more flexible than previously assumed, and that different scaffolds may access distinct binding modes or regions within the enzyme, consistent with the lack of synergy between the **EB-2-16** and alkynyl scaffolds. Consequently, warheads cannot be treated as modular elements that can be transferred between

scaffolds without consideration of structural context but must instead be optimized in conjunction with the overall scaffold.

Although the internal alkyne scaffold does not yet achieve an optimal balance between potency and stability, it represents one of the most potent fully non-peptidic scaffolds reported for hTG2 inhibition and provides a strong foundation for further development. The structure-activity relationships identified in this work suggest several potential strategies for optimization (**Fig. 5.1A**). Greater diversity at the 1-position of the triazole may be explored to further improve potency and solubility, as a broad range of substituents are tolerated at this position and may offset the decrease in potency observed with more attenuated amide warhead analogues. The warhead may be further attenuated by steric hindrance introduced by an ortho-methyl substituent. Additionally, installation of two or more methylene units between the alkyne and terminal phenyl group may attenuate reactivity while mitigating rearrangement to an allene, although this approach may present synthetic challenges.

Notably, the highest potency inhibitor in chapter 3 (**8R**) bears structural resemblance to features of glutamine-mimetic acrylate-based inhibitors developed by Zedira (i.e., **ZED1227**), particularly given that triazoles are known bioisosteres of amides. This suggests that a hybrid approach between our alkyne scaffold and the glutamine-like linkage to the warhead may lead to potent inhibitors (**Fig. 5.1B**).

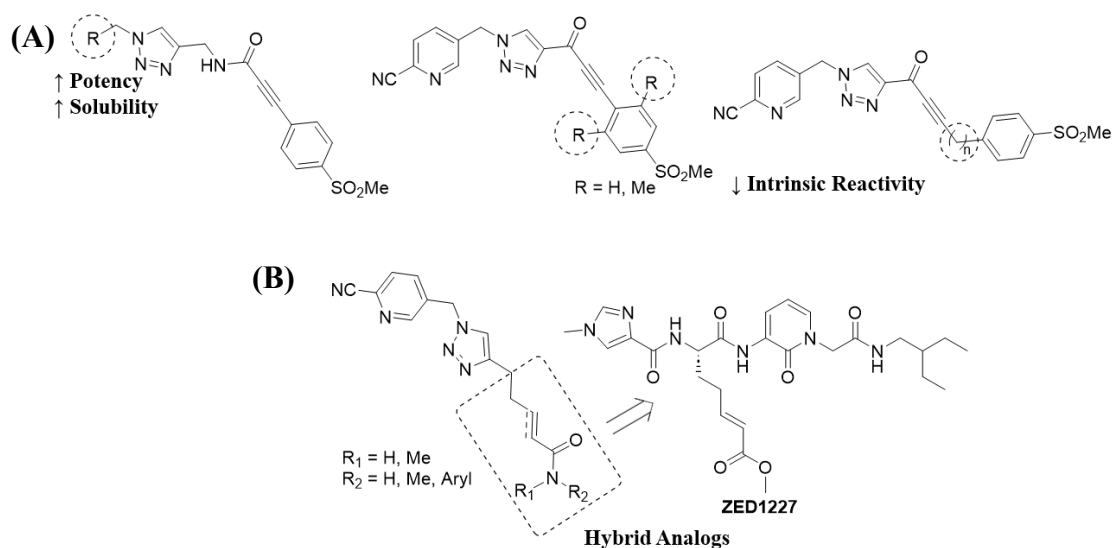


Figure 5.1. Potential optimization strategies for chapter 3 inhibitors. **(A)** Modifications on original alkynyl scaffold. **(B)** Hybrid approach with glutamine-mimetic warhead linker.

Taken together, these findings support a more integrated view of electrophilic warheads as key determinants of covalent inhibitor behaviour within the transglutaminase family. Rather than serving as passive reactive handles, warheads can influence binding affinity, selectivity, and inhibition mechanism in a highly context-dependent manner. These results challenge common assumptions in covalent inhibitor design, particularly the notion that increased electrophilicity necessarily leads to reduced selectivity or that sterically minimal warheads are required for effective inhibition. Recognizing and leveraging this complexity will be essential for the rational design of selective and effective covalent inhibitors targeting TGases.

5.2. Expanding the Chemical Space of Alkyne Electrophiles

Internal warheads represent an underexplored area of electrophile design in covalent inhibition. In contrast to conventional warheads, which are typically appended as terminal functional groups, the internal alkynyl scaffold described in Chapter 3 integrates the electrophile within the core of the molecular framework. This structural feature represents a distinct design

strategy in which the warhead is not a modular component, but rather a fundamental element of the scaffold that dictates the geometry and orientation of the entire recognition system.

A notable feature of this warhead is the degree of tunability that can be achieved through substitution on the terminal aryl group, which directly modulates the electronic properties and reactivity of the alkyne. This provides a means of modulating covalent reactivity within a largely conserved steric framework, enabling systematic variation of electrophilicity without substantial changes to molecular size or geometry. Further exploration of substituent effects, including variation in electron-donating or electron-withdrawing character and positional effects relative to the alkyne, may provide strategies for tuning reactivity. Incorporation of heteroaryl systems such as pyridine or imidazole may offer additional means of modulating electronic properties. A more comprehensive study of structure-intrinsic reactivity relationships for this warhead, for example through correlation with Hammett parameters, could provide a foundation for more predictive tuning of electrophilicity and clarify the relative contributions of steric and electronic effects.

While further work is required to establish the applicability of this warhead to enzymes beyond hTG2, this work provides a foundation for expanding the chemical space of covalent inhibitor design through more complex and integrated electrophile architectures.

5.3. Tuning GST-Mediated Metabolism of Covalent Inhibitors

Chapter 4 demonstrates that a paradigm shift is needed in how susceptibility to GSH conjugation is evaluated during early-stage warhead design. Standard measurements of intrinsic reactivity with GSH do not predictively reflect GST-mediated metabolism, which instead depends on substrate binding affinity and orientation within the GST H-site, reminiscent of the results of chapter 2 and 3 with respect to warhead effects on target engagement. This shows that GST-

mediated metabolism is a structurally tunable parameter rather than one inextricably correlated with electrophilicity.

The quantitative assays developed herein provide a general framework for evaluating GST susceptibility that can be readily integrated into early-stage medicinal chemistry campaigns. While the library of warheads examined in this study represents only an initial exploration, these results highlight the potential for further systematic structure-activity relationship studies to define GST substrate scope and identify structural features, not only in warheads but also in other scaffold elements, associated with metabolic liability. In contrast to phase I oxidative metabolism, where predictive tools for CYP-oxidation sites/substrates are well established, no frameworks currently exist for forecasting GST-mediated conjugation beyond general recognition of electrophiles. As GST-focused optimization strategies become more widely adopted in covalent drug design, the accumulation of experimental datasets may enable the development of predictive models to inform how both warhead and scaffold structure may contribute to GST susceptibility.

Across chapters 2-4 it is clear that electrophilic warheads play an essential structural, not solely electrophilic, role in governing both pharmacodynamic and pharmacokinetic behaviour in covalent inhibitor design. While a wide range of electrophilic functionalities are known, covalent drug development has largely centred around a small number of well-characterized warheads that provide a reliable balance between reactivity and selectivity. A shift toward viewing warheads as tunable structural features, capable of influencing binding, selectivity, and metabolism, may enable more deliberate and diverse approaches to covalent inhibitor design. As these perspectives continue to evolve, a more integrated understanding of how warhead structure contributes to both pharmacodynamics and pharmacokinetics is expected to guide the development of next-generation covalent therapeutics across a broader range of targets.

Additional Work Completed During Studies

To present this thesis in the most cohesive manner, only major projects were included as discrete chapters. Other publications resulting from my PhD studies are listed below for interest of the reader.

Development of new kinetic modelling methods (EPIC-Fit) for time-dependent IC₅₀ evaluations of covalent inhibitors:

Mader, L. K.; Keillor, J. W., Fitting of k_{inact} and K_I Values from Endpoint Pre-incubation IC₅₀ Data. *ACS Medicinal Chemistry Letters* **2024**, *15* (5), 731-738.
<https://doi.org/10.1021/acsmedchemlett.4c00054>

Mader, L. K.; Keillor, J. W., Methods for kinetic evaluation of reversible covalent inhibitors from time-dependent IC₅₀ data. *RSC Medicinal Chemistry* **2025**, *16*, 2517-2531.
<https://doi.org/10.1039/D5MD00050E>

A review on assays and kinetic models for evaluating covalent inhibitors:

Mader, L. K.; Borean, J. E.; Keillor, J. W., A practical guide for the assay-dependent characterisation of irreversible inhibitors. *RSC Medicinal Chemistry* **2025**, *16*, 63-76.
<https://doi.org/10.1039/D4MD00707G>

Contributions to a new synthetic methodology for accessing tetrahydroisoquinolines:

Lewin, H. M.; **Mader, L. K.**; Edupuganti, V. V. S. R.; Hawkins, B. C.; Gamble, A. B.,
Synthesis of Heterocyclic Scaffolds from Nitrobenzyl Alcohols Using Reduction-Generated
Azaquinone Methides and Cyclization. *European Journal of Organic Chemistry* **2026**,
e202600014. <https://doi.org/10.1002/ejoc.202600014>