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Asymmetric Synthesis of Cα-methyl-γ- and δ-Amino Acids From a Common Synthon and Evaluation of Thionyl Chloride Assisted Peptide Esterifications

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ASYMMETRIC SYNTHESIS OF C^{α}-METHYL- γ- AND δ-AMINO ACIDS FROM A COMMON SYNTHON AND EVALUATION OF THIONYL CHLORIDE ASSISTED PEPTIDE ESTERIFICATIONS

by

Emily Rose Vogel

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
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December 2015

ABSTRACT

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C^{α}-methyl-γ- and δ-unnatural amino acids (UAAs) are important class of biomolecules used extensively as structural scaffolds, peptidomimetics, and in the development of pharmaceuticals. Due to steric congestion surrounding the quaternary center, asymmetric preparation of α,α-disubstituted UAAs are synthetically challenging. Herein, two methods for the synthesis of chiral synthons to prepare C^{α}-methyl-γ- and -δ-UAAs are reported. A crucial step in both strategies includes an enzymatic hydrolysis of prochiral malonic esters with pig liver esterase (PLE). The first method utilizes the Meyer Schuster rearrangement to prepare α,β-unsaturated diesters synthons, but the preparation of the precursor propargyl alcohol decomposes into uncontrollable retro-aldol products. Alternatively, synthons 3-(hydroxymethyl)-3-methylpyrrolidin-2-one and piperidin-2-one, are prepared via a phthalimide deprotection and stereoselective cyclization of the free amine toward an activated ester. This cyclization strategy allows for the preparation of both enantiomers through steric or electronic controls. Conversion of intermediate γ- or δ-lactams into mesylates, nucleophilic substitution, and ring opening led to the formation of $\gamma^{2,2}$ and $\delta^{2,2}$-serine, -azido, and -cysteine analogues. General application of the cyclization

strategy was found to be limited by sterics imposed by the neopentyl intermediate resulting in longer reaction times and inability to substitute some nucleophiles.

The esterification of oxidized glutathione with thionyl chloride and various alcohols was monitored for completeness using electrospray ionization mass spectrometry. Oxidized glutathione was found to be highly compatible with an excess of methanol and ethanol, but slow and incomplete with 2-propanol. The thionyl chloride esterification was applied to other small peptides to evaluate the limitations with various amino acid side chains. The results show incompatibilities with peptides containing both serine and cysteine but well tolerated with the remaining natural amino acids.

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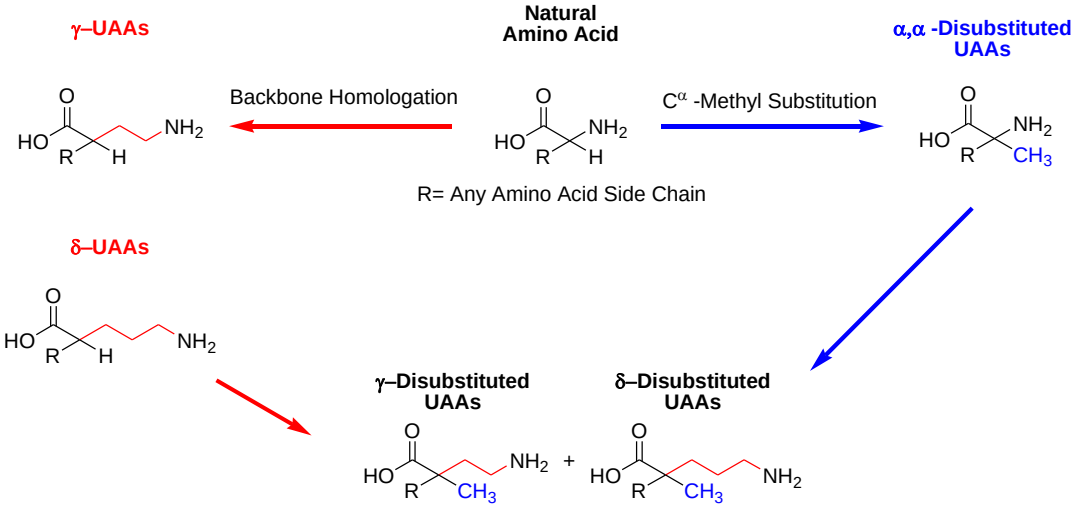
CHAPTER I

INTRODUCTION

The basic monomer unit of proteins and peptides are the 20 unique amino acids provided by nature. These naturally occurring amino acids all contain the same core backbone but structurally their side chains differ. The structural diversity of amino acid side chains are responsible for their unique chemical and physical properties. Over the last fifty years, chemically modified versions of amino acids have emerged with non-natural variations opening a new class of amino acids deemed unnatural amino acids (UAAs).[1-3]

The incorporation of UAAs into peptides/proteins has important implications such as stabilizing biological structures, resistances to enzymatic degradation, and improved biological functions. As a result, UAAs are promising research leads as peptidomimetics, foldamers, antibiotics, and pharmaceuticals.[4-8] UAAs are also valuable as asymmetric catalysts, structural scaffolds, and as reaction intermediates in synthesis of natural products.[1,9] γ- and δ-UAAs are an important class of UAAs gaining considerable attention over the last decade. These UAAs have methylene units inserted into the carbon backbone (Scheme 1).[10-12] In comparison to α-amino acids, the homologated γ/δ-UAA backbone allows these molecules to mimic dipeptides with increased flexibility to form secondary structures. Another promising class of UAAs are α,α-disubstituted UAAs with C^{α}-methyl substitution. The replacement of the quaternary hydrogen with a methyl group increases the in vivo half life, improve bioavailabilities, and restrict the conformational backbone freedom.[13] Marriage

between both the homologated and α,α-disubstituted backbone represents a novel class of UAAs worthy of synthetic exploration and value.



Scheme 1. Illustration of the classes of γ/δ-disubstituted UAAs studied**.**

Currently synthesis of disubstituted γ/δ-UAAs has not been thoroughly explored. Moreover, there are no reports of a method capable of preparing multiple derivatives of both enantiomers of γ- and δ-disubstituted UAAs. One of the goals set forth by The National Research Council is to, "develop methods that will enable synthesis of all important molecules in reasonable yields using compact synthetic schemes, so that no useful compound is inaccessible to practical synthesis."[14] The aim of this work is to make access to C^{α}-methyl-γ- and δ-UAAs easily accessible by developing chiral synthons to prepare these biomolecules. Additionally, The National Research Council also emphasized the importance of using enzymes to prepare molecular intermediates.[14] A crucial synthetic step in this work includes an enzymatic hydrolysis with Pig Liver Esterase (PLE) to prepare enantioenriched chiral half esters from prochiral malonic esters. Other UAAs synthesis often draw from scarce or expensive

chiral catalysts, chiral auxiliaries, or natural resources. In comparison, the PLE method is inexpensive and tunable to a specific enantiomer.[15] The goals of this research are:

- The development of γ/δ-UAAs common synthons from an enantioenriched PLE hydrolysis;
- Demonstrate the versatility of the common synthon by preparing multiple γ/δ-UAA analogues;
- The evaluation of thionyl chloride in the esterification of glutathione and other small peptides.

Applications and Uses of Unnatural Amino Acids

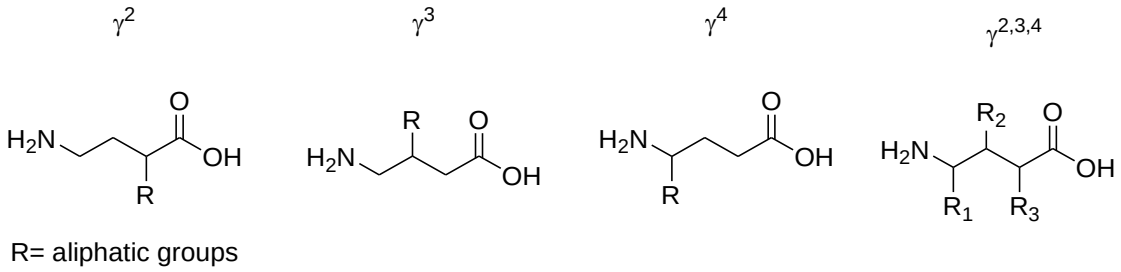
The past several decades α-UAAs have been used in peptidomimetics, foldamers, antibiotics, and as pharmaceuticals.[11,16,17] They have also been used as intermediates in the synthesis of chemical scaffolds, chiral auxiliaries, and as asymmetric organocatalysts.[1,9] Recently, interest in these molecules has expanded to include homologated γ/δ-UAAs. These homologues have greater flexibility and chemical diversity, due to the inserted methylene units, but still function as their α-counterparts.

UAAs as applied to the field of peptidomimetics

Peptidomimetics are used to mimic natural peptides in both structure and function while maintaining essential pharmacophore properties. As biomolecules, peptidomimetics have improved bioavailabilities,[18] improved receptor selectivities,[19] and are stable toward proteolysis.[20,21] These features make

peptidomimetics respectable candidates for drug discovery functioning as enzyme inhibitors.[22-24]

Homologated UAAs closely resemble dipeptides, therefore, γ/δ-UAAs are used as dipeptide peptidomimetics. The difference between the two molecules is one less peptide bond on the γ/δ-UAA peptidomimetic. This makes the γ/δ-UAA peptidomimetic less susceptible to enzyme hydrolysis improving their in vivo half lives. Frackenpohl et al. demonstrated the stability of aliphatic substituted γ^2, γ^3, γ^4, and $\gamma^{2,3,4}$ and found no degradation occurred after incubation with active enzymes pronase, proteinase K, penicillin amidase, and β-lactamase (Figure 1).[25] Unnatural γ-homologues were also used as somatostatin peptidomimetics. Somatostatin's natural 14-amino acid cyclic disulfide hormone sequence is mimicked with an open chain γ-analogue containing only three amide bonds.[26]



R= aliphatic groups

Figure 1. γ-UAAs studied by Frackenpohl et al. with active enzymes.

Foldamers composed of unnatural amino acids

Peptides and proteins containing well-defined conformational secondary and tertiary structures are responsible for many biological effects found in vivo. In order to function properly, peptides must maintain specific conformations either in solution or when bound to receptors. The major problem in designing peptides is they lack the stabilization necessary for the formation of secondary

structures. As a result, scientists interested in biologically active peptides explored the properties needed to stabilize particular conformations and foldamer research was born.[27] Early studies focused on creating foldamers strictly with α-UAAs, as it was generally assumed that the increased flexibility associated with UAA homologues without conformational restrictions in the backbone would lack the stabilization necessary to form secondary structures. This belief was discredited by both Seebach and Gellman who showed β-UAAs are capable of forming stable helices, turns, and sheets.[7,28-31]

Foldamer studies have since expanded to include γ-homologues. Hinterman et al. was the first to show hexamers composed of aliphatic mono-substituted γ-UAAs formed stable right handed helices in solution.[28] The stabilizing hydrogen bonds form between the carbonyl of *i* and the NH group of every (*i* +3) residue. Hanessian et al. later found only four amino acids residues are needed to stabilize a helix. The important findings of homologated peptides are summarized as follows:[30,32]

- γ-homologues fold into stabilized helices in as little as four amino acid residues in both methanol and pyridine solutions, or in solid state;
- The stability of the helices increases from α-, to β-, to γ-peptides, while the number of hydrogen bond decreases;
- The helicity reverses for each homologue; Right handed 3.6_{13}-α-, 3_{10}-α-, 2.6_{14}-γ-helicies and left handed for 3_{14}-β-helices;

- In each homologue, the direction of the macrodipole reverses pointing N- to C- in both α-, and γ-peptides and C- to N- in β-peptides.

γ-UAAs also form other secondary structures. Schreiber et al. reported α,β-unsaturated γ^4-UAAs (Figure 2, Type A) formed parallel sheets and α,β-unsaturated $\gamma^{2,4}$ (Figure 2, Type B) formed antiparallel sheets.[33] The conformational side chain orientations are also important factors dictating secondary structures stability. The *anti*-$\gamma^{2,4}$ (Figure 2, Type C) conformations stabilize structures where as *syn*-$\gamma^{2,4}$ (Figure 2, Type D) destabilized secondary structures.[34] Additionally, Hanessian showed that stereochemistry plays an important role in secondary structure determination by demonstrating (α-*S*)-cinnamyl γ-amino acid tetrapeptide adopts a helical structure, whereas, (α-*R*)-cinnamyl tetrapeptide forms a reverse turn in solution.[35] Other observed secondary structures included β-hairpins stabilized between a disulfide formed between two β-SH-γ-amino acids.[36] These examples demonstrate the importance of position in the substitution of the amino acid side chain and the need for asymmetric preparation of these biomolecules.

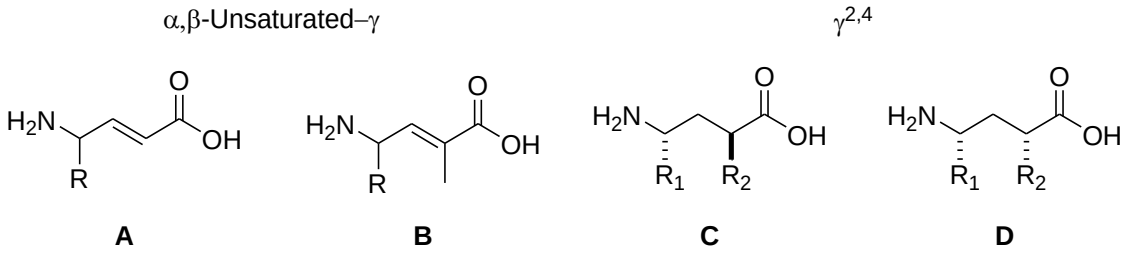


Figure 2. Types of γ-UAAs inducing or stabilizing conformational structures.

Since δ-UAAs represent a true isosteric replacement of dipeptides, δ-UAAs are often used as β-turns mimics.[10,37,38] Shankaramma et al. showed antiparallel β-sheets formed when δ-aminovaleric acid was inserted at the turn.[39] Gellman et al. demonstrated the importance of a rigidified backbone by introducing *trans* δ-UAA alkenes as a β-turn mimic (Figure 3).[40] Gardner et al. also showed $\delta^{2,5}$-UAAs induce β-turns.[37,38] All of these studies, including the mentioned γ-UAA studies, outline the importance of conformational restriction and/or specific substitution patterns along the UAA-backbone to induce stable turn mimetics.

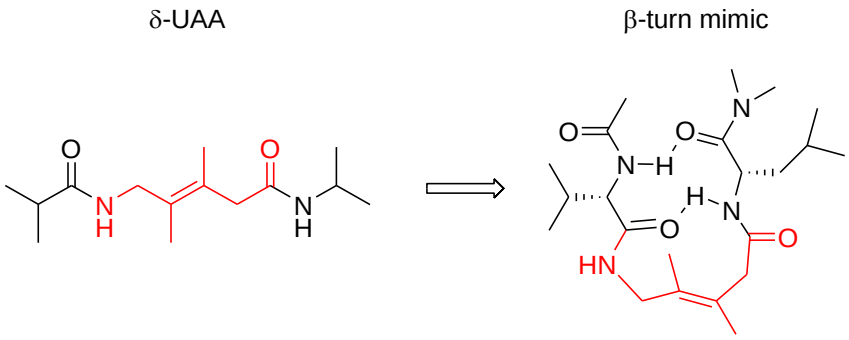


Figure 3. Gellman et al. β-turn mimetic using δ-unsaturated UAAs.

Pharmaceuticals and antibiotics composed of unnatural amino acids

The ability of UAAs to act as peptidomimetics and foldamers has led to the development of many pharmaceutical leads. Some of these are peptide-based drugs, whereas others are antifungal,[41,42] antibacterial,[43] and tumor reducing agents.[44] γ-UAAs as pharmaceuticals alone are studied extensively for their ability to mimic γ-aminobutyric acid (GABA), a central nervous system (CNS) neurotransmitter.[11,12] Currently, GABAergic drugs and prodrugs are used to treat Huntington's and Parkinson's disease, epilepsy, and aliments of psychiatric

disorders.[11,12] The flexibility and polarity of GABA's structure makes GABA incapable of crossing the blood brain barrier, therefore it is not administered orally (Figure 4). In order to gain entry, GABA analogues are designed with lipophilic and constrained moieties as seen in (*R*)-Baclofen, GABApentin, (*S*)-Vigabatrin, (*S*)-Pregabalin. The additional hydrocarbons and aromatic components make these drug candidates non-polar and more accessible to cross the blood brain barrier.[45,46]

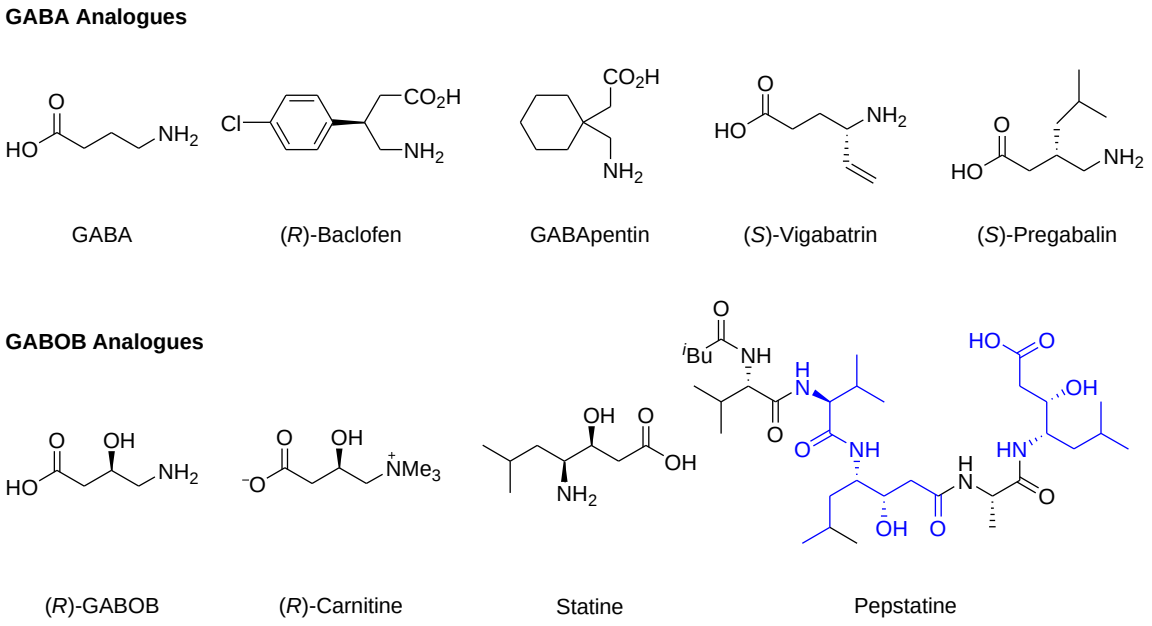


Figure 4. Current examples of γ-UAAs used as pharmaceuticals and inhibitors.

Another interesting class of γ–UAAs are 4-amino-3-hydroxy-butyric acid (GABOB) derivatives (Figure 4). GABOB is a naturally occurring marine microsclerodermin possessing both antitumor, antibacterial properties, and acts as an agonist to GABA. A N-methylated derivative, (*R*)-Carnitine, was developed to mimic GABOB. Similar in structure to GABOB is statine, a key component to natural hexapeptide pepstatine. Pepstatine is both an antibiotic and aspartic acid protease inhibitor. Aspartic acid proteases are responsible for the onset of aids,

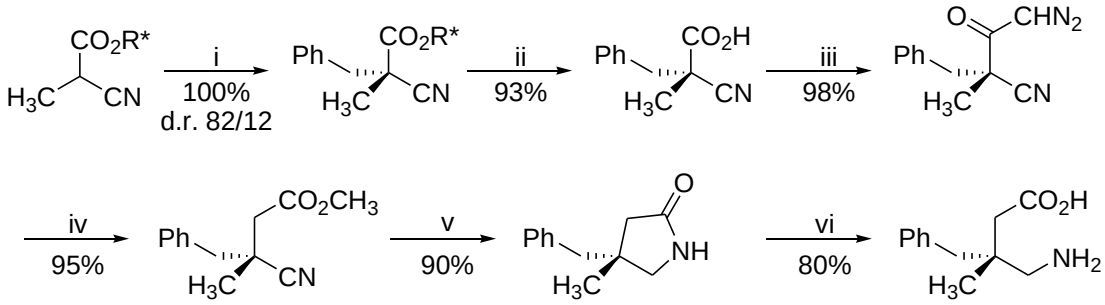
hypertension, malaria, and Alzheimer's disease.[11,47-49] Key to peptstatine's inhibitory effect is both the *syn* relationship between the hydroxy and amine groups of statine. If the *syn* relationship is missing pepstatine is not able to tightly bind and inhibit aspartic acid proteases.[49] Norstatine, cyclohexylstatine, and isostatine have emerged as peptide therapeutics derived from statines which include the hydroxy biological active component.[47]

Strategies for the Synthesis of γ/δ-Unnatural Amino Acids

The synthesis of γ/δ-UAAs is complex and highly customized. The amount of backbone substitution, degree of steric hindrance, and desired chirality must be considered when designing a synthesis. The synthesis γ/δ-UAAs has been reviewed extensively.[11,12,49] Herein, a few strategies to homologate the backbone are discussed. The asymmetric methods reported rely heavily on transition metal catalysts, chemoenzymatic routes, asymmetric catalysts, and/or draw from established chiral sources.[50] [48,51,52] Generally, these methods are specific to a particular amino acid side chain and require optimization of another method if different analogues are desired.

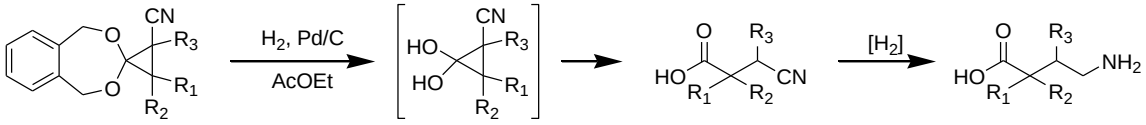
Due to the medicinal importance of GABA and GABOB analogues, considerable research is devoted to synthesizing γ-UAAs with β-substitutions. D. Aguirre et al. reported a stereoselective synthesis of (*R*)-γ-amino-β-benzyl-β-methylbutyric acid starting from chiral α-cyanoester (Scheme 2).[53] Chirality was established by a stereoselective α-alkylation of chiral 2-cyanoesters. The amino acid backbone was homologated after an Arndt-Eistert synthesis, and Wolff rearrangement of the diazoketone. Finally, the nitrile group reduced, cyclized,

and ring opened under acidic conditions to afford $\gamma^{3,3}$-amino acid. The Arndt-Eistert synthesis, Wolff rearrangement, and/or reduction of a nitrile is representative of one of the common approaches γ/δ-amino acids.[28] However, any steric hindrance prevents the formation of diazoketones limiting the Arndt-Eistert, Wolf rearrangements to uncongested substrates.[54]



Scheme 2. Aguirre et al. approach to synthesizing β,β-disubstituted γ-UAAs. Reagents and conditions: (i) K_2CO_3, BnBr, acetone; (ii) KOH, MeOH, D; (iii) $ClCO_2$, iBu, NMM,THF,-20 °C, then dry CH_2N_2 in ether; (iv) AgBzO, Et_3N, MeOH, THF; (v) H_2, Ni (Ra), NH_3/MeOH, 35 °C; (vi) HCl.

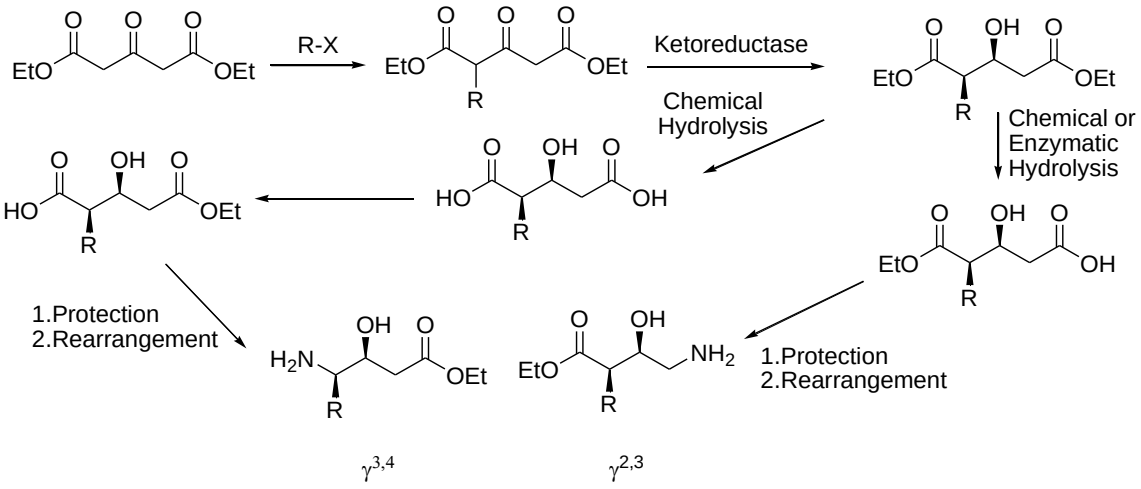
Adam et al. reported a similar asymmetric synthesis reducing a nitrile to create α-propyl-γ-serine.[55] Often, nitrile reductions are implemented to prepare disubstituted γ/δ-UAA analogues. Adam et al. used the nitrile group to prepare α-propyl-γ-serine and Doris et al. to prepare $\gamma^{2,2,3}$ substituted derivatives (Scheme 3).[55,56]



Scheme 3. Doris et al. synthesis of $\gamma^{2,3,3}$ substituted amino acids.

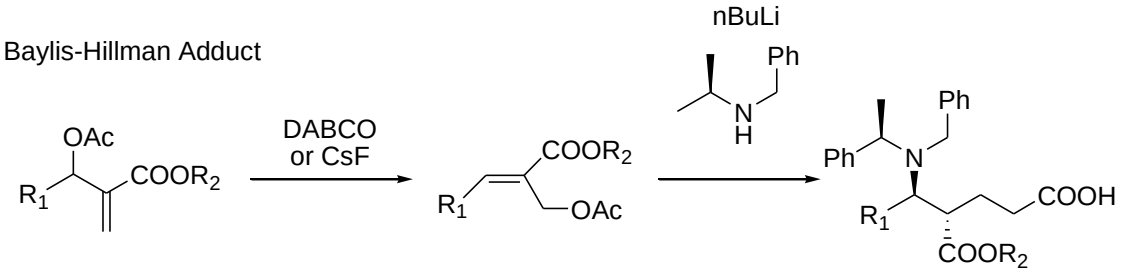
Other strategies to prepare γ/δ-UAA backbone include a Hoffman or Curtius rearrangements. Kambourakis et al. reported the low yielding chemo-enzymatic synthesis of $\gamma^{2,3}$ and $\gamma^{3,4}$ β-hydroxy analogues using ketoreductase

enzyme and a Hoffman/Curtius rearrangement (Scheme 4).[57] The additional chemical hydrolysis/esterification step prepared γ[3,4] derivatives, and an enzymatic hydrolysis produced the γ[2,3] derivatives.



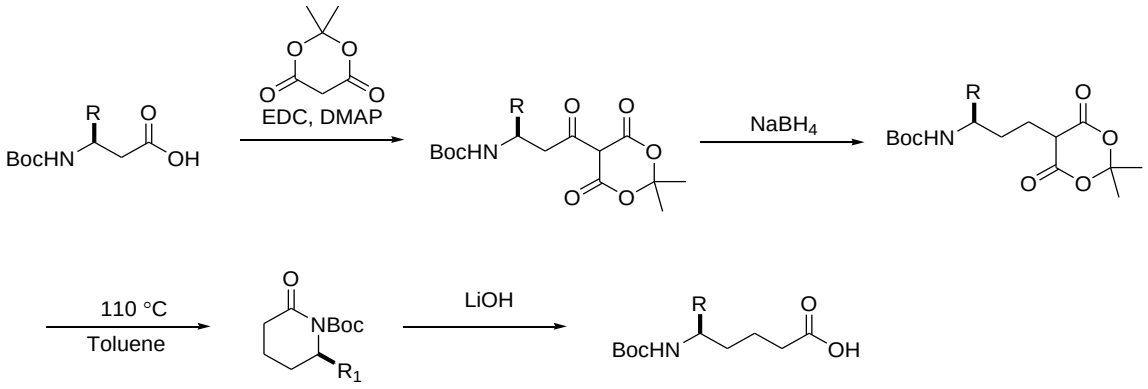
Scheme 4. Kambourakis enzymatic desymmetrization rearrangement to β-hydroxy γ-analogues.

Several methods use nitrooxazolines to prepare the carboxylic acids from oxazolines and amines from nitro groups.[58,59] Furanose sugars are stereoselective ring opened into both γ/δ-UAAs following a regiospecific reaction with periodate and azide reduction.[60] Garrido et al. described both a diastereoselective and enantioselective synthesis via an Ireland-Claisen rearrangement of a Baylis-Hilmann adducts and Michael addition of a chiral lithium amides to δ[3,4] analogues (Scheme 5).



Scheme 5. Garrido et al. method to prepare δ[3,4] amino acids.

Other strategies capable of preparing both γ/δ-UAAs have used Meldrum's acid as a scaffold (Scheme 6).[61,62] Finally, also common are Wittig olefination chemistries and nucleophilic aziridine ring openings.[28,63-67]



Scheme 6. Casimir et al. synthesis of δ^4 UAAs.

The number of synthetic methods to both γ/δ-UAAs are diverse. Many strategies involve a combination of either a ring opening, reduction of a nitrile/nitro group, and/or a rearrangement reaction. However, missing from all these strategies are methods capable of preparing a diverse array of α,α-disubstituted amino acids from a common synthon.

Influences of $C^{\alpha,\alpha}$-Disubstitution in Unnatural Amino Acids

The goal of biomimetic research is to design novel peptides with enhanced properties complementing natural peptides. One method to mimic peptides is to replace the chiral quaternary α-hydrogen with a methyl group. These substituted derivatives are called α-methyl amino acids. In comparison to a natural peptide, α–methyl amino acids restrict the conformational rotation of the backbone to add rigidity, increase metabolic stability, and hydrophobicity.[68,69] Initial studies containing α-methyl amino acids incorporated achiral 2-methylalanine analogues (Aib) into peptides. These studies found that Aib was a

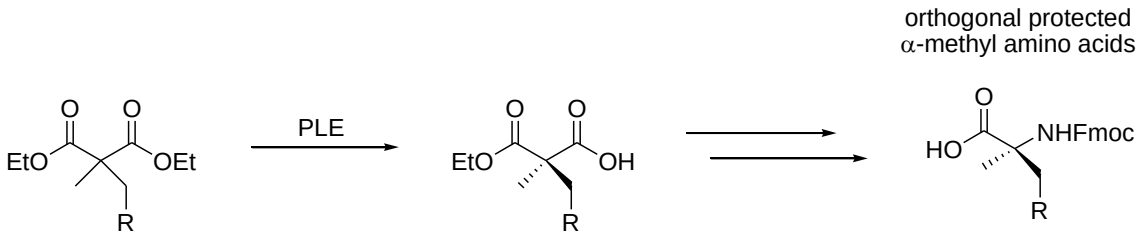
strong helix inducers, contained restricted rotation about the N-C^{α} (Φ) and C^{α}-C' (Ψ) bonds, and had limited Ramachandran torsion angles.[70,71] Overall the 3_{10}-helix is stabilized in a as few as eight residues, whereas the α-helix requires an average of 9-20 residues.[70] However, the missing stereogenic carbon of Aib results in a lack of helical screw sense, therefore the handiness is determined by the other amino acids in the peptide sequence.

The ability of Aib to stabilize secondary structures prompted other investigations into α-methyl amino acids. Altmann and Mutter[72] utilized (*R*)/(*S*) 2-methylaspartic acid incorporated into the *i*/(*i* +4) ends of a 16 amino acid residue peptide. They found chirality of the monomers plays a role in the helix inducing potential of α-methyl substituted amino acids. Other α-methyl amino acids capable of inducing helices or β-turns include derivatives of alanine, valine, leucine, and serine.[73]

In addition to inducing secondary structures, literature has shown α-methyl amino acids are effective enzyme inhibitors.[73] The methyl group increases receptor affinities by inducing binding conformations.[74] Examples of the inhibitor effects are documented with α-methyl-tyrosine, -tryptophan, and -aspartic acid.[73-75] As structural scaffolds α-methyl amino acids are used as intermediates in the synthesis of NK_1/NK_2 receptor antagonists,[76] tubulysin analogues,[77] and for acyl hydrolases fluorophore detection probes.[78] In summary, the ability of the disubstituted stereocenter to restrict the rotation of the backbone and as act as potent enzyme inhibitors make the synthesis of these biomolecules highly desirable.

Current synthetic methods

The synthesis of chiral α-methyl amino acids are considered a synthetic challenge. Direct methylation of amino acids result in a racemic mixtures and are not convenient approaches of establishing stereocenters. Two extensive reviews examining the asymmetric synthesis α,α-disubstituted α-amino acids have appeared.[69,73] In these reviews, there is a strong dependence on asymmetric catalysts and chiral auxiliaries. Generally, these methods are expensive and/or rely on established chiral pools of molecules not always available. Enzymes have recently emerged as an alternative to prepare chiral α-methyl amino acid analogues.[23,79,80] Masterson et al. reports the preparation of enantioenriched analogues of α-methyl cysteine, serine, lysine, and α-methyl-β-proline following an enzymatic hydrolysis with PLE (Scheme 7). The PLE method is also tunable for a particular enantiomer either by the addition of various co-solvents or using one of the six isolated PLE isoenzymes.[15,81] Since the PLE strategy is capable of resolving prochiral esters from inexpensive starting materials, and the method compliments the ideas set forth by The National Research Council the PLE method was used to prepare α,α-disubstituted γ- and δ-amino acid common synthons.



Scheme 7. Masterson et al. generic PLE strategy to orthogonal protected α-amino acids.

Project Overview

The proceeding sections outlined the benefits of UAAs as important biomolecules. Evident from the discussion is the emphasis placed of γ- and δ-UAAs, however, the synthesis of each analogue is expensive and time-consuming placing a bottleneck on the direct application of these biomolecules. Currently these amino acids are often prepared as a racemic mixture, draw from a chiral pool of molecules, or use expensive chiral catalysts. A review of current literature revealed there are no compact methods capable of preparing a variety of chiral γ- and δ-amino acid analogues. Additionally, conformational restricted α-methyl amino acids are important compounds because they induce secondary structures, improve bioavailabilities, and prevent enzymatic degradation. Therefore, the aim of this research is to develop an asymmetric synthesis with access to α,α-disubstituted γ- and δ-amino acids via a common synthon. This synthon should be easily obtained in a short compact synthesis, enantioenriched, and stable to wide ranging chemistries for further transformations to other amino acids.

Hypotheses

Hypothesis 1 postulates a γ-amino acid backbone can be prepared by utilizing the Meyer-Schuster rearrangement as a synthetic tool to form α,β-unsaturated diesters as a common intermediates in the synthesis of γ-unnatural amino acids. Chapter II focuses on the synthesis of the proposed intermediate following a chemoenzymatic PLE and Meyer-Schuster rearrangement strategy.

Hypothesis 2 postulates 3-(methylhydroxy)-3-methyl–γ/δ-lactams can be prepared via an intramolecular cyclization/reduction strategy and used as a common synthon in the synthesis enantioenriched $C^{\alpha,\alpha}$-disubstituted γ/δ-UAAs. Chapter III focuses on the chemistries associated with the stereoselective cyclization and functionalization of the lactam intermediates using substitution chemistry.

Hypothesis 3 postulates that esterification of small peptides with thionyl chloride can be used to completely esterify free carboxylic acids into methyl esters with limited side reactions and no purification. Chapter IV evolved during the synthesis of an unnatural glutathione peptide. Inefficient esterifications were observed following standard methods, and the discovery of thionyl chloride esterification proved to be highly efficient method not well documented. Therefore Chapter IV focuses on the compatibility of the esterification with various alcohols and other peptides.

CHAPTER II

APPLICATION OF THE MEYER SCHUSTER REARRANGEMENT TO PREPARE α,β-UNSATURATED DIESTERS AS A COMMON INTERMEDIATE IN γ-UNNANTURAL AMINO ACID SYNTHESIS

Hypothesis 1

A γ-amino acid backbone can be prepared by utilizing the Meyer-Schuster rearrangement as a synthetic tool to form α,β-unsaturated diesters as a common synthon in the synthesis of γ-unnatural amino acids.

Introduction

In the previous chapter, the properties, applications, and general synthetic strategies of UAAs were reviewed. Evident from this review is the need for homologated UAAs, but few general synthetic strategies are available. Moreover, none of these strategies are capable of preparing multiple analogues of C^{α}-methyl-γ-UAAs enantioselectively. This chapter focuses on meeting the challenges of The National Research Council in developing a compact synthesis to prepare biologically relevant C^{α}-methyl-γ-UAAs from a common synthon.

Designing a common synthon

The first step toward meeting The National Research Council's challenge was designing a synthon. The ideal candidate should be easily functionalized into both simple and complex analogues of γ-UAAs. In addition, self-imposed requirements dictated the compound be prepared asymmetrically through a PLE hydrolysis, be high yielding over a condensed multistep synthesis, and accessible to a general audience. Due to the variety of synthetic transformations

alkenes can undergo, intermediate **1,** an α,β-unsaturated diester, was proposed (Figure 5). This analogue contains the desired chiral quaternary center and is prepared through established PLE protocols.

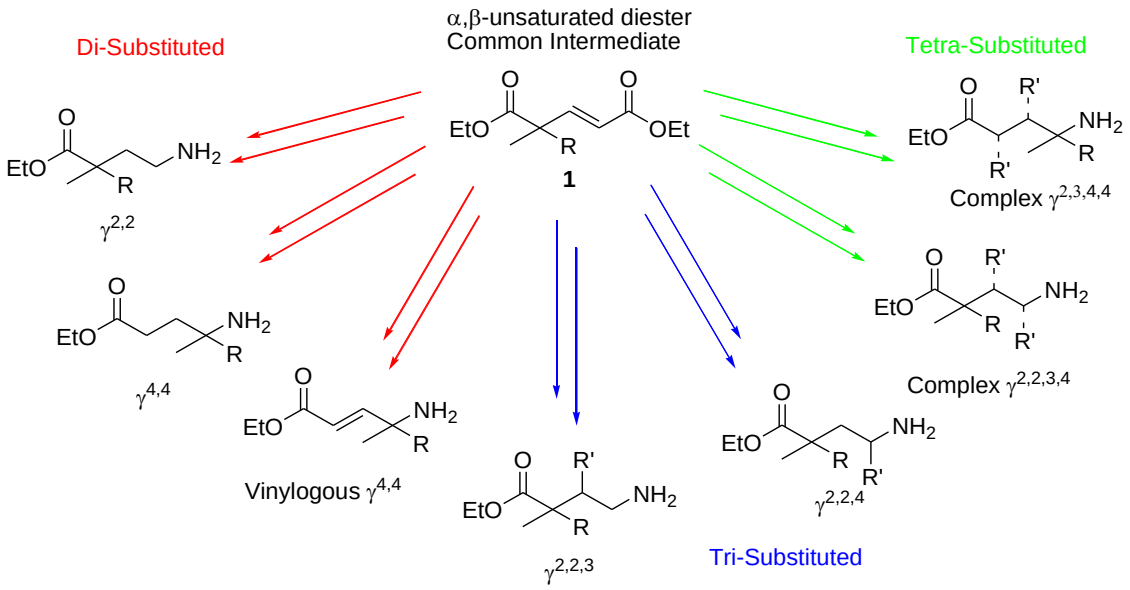


Figure 5. Proposed C$^{\alpha}$-methyl- α,β-unsaturated γ-UAA common intermediate.

The disadvantages of **1** as a synthon are the early commitment to specific side chains, and the need for a PLE active prochiral diester. However, those disadvantages are minor in comparison to the many higher ordered γ-derivatives capable of adding across the double bond. A generic overview of the synthetic transformations **1** can undergo are outlined in Figure 5. These syntheses included:

- The synthesis $\gamma^{2,2}$ and $\gamma^{4,4}$ analogues via hydrogenation;[82]
- The synthesis of vinylogous α,β-unsaturated $\gamma^{4,4}$-UAAs following a hydrolysis/Curtius reactions;
- The nucleophilic addition to the electron deficient β-carbon forming $\gamma^{2,2,3}$-UAAs with Michael additions[83-87] and organocopper Gilman's reagents;[88,89]

- The addition to the α-carbon producing $\gamma^{2,2,4}$-UAAs following a sequential silyl addition to the β-carbon, nucleophilic attack at the α-carbon, and desilylation;[90]
- The synthesis of $\gamma^{2,2,3,4}$- and $\gamma^{2,3,4,4}$-UAAs prepared via silyl chemistry and the addition of two equivalents of nucleophile;

Additionally, since, the designed intermediate are enantioenriched all additions follow a stereoinduced addition across the alkene. After addition, the compound is converted into an amino acids via standard hydrolysis/Curtius protocols. Use of the proposed synthon could access various enantioenriched α,α-disubstituted γ-UAAs in as little as 3 to 6 steps. The value of having a synthon with wide applicability is important to the scientific community and the synthesis of **1** is documented within this chapter.

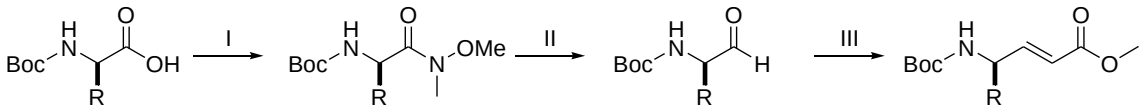
Other applications of the intermediate

The α,β-unsaturated diester synthon also has been used for other bio-relevant purposes such as the preparation of aminoglutethimide AG-1 analogues.[91,92] Additionally, conversion of **1** into an α,β-unsaturated amino esters are promising leads for the synthesis of natural products with antimalarial and anticancerous properties.[93-96] These amino ester are also used as inhibitors for serine and cysteine proteases.[87,97] For synthetic purpose α,β-unsaturated amino esters are used as substrates in Diels-Alder reactions,[63] epoxidations,[97] and 1,4-conjugate additions.[86,87] Due to the restricted backbone, these derivatives are also extremely desirable in foldamer research.[33,64,65] Schreiber et al. was the first to report the formation of parallel and anti-parallel sheet like

structures with vinylougous amino acids.[33] Grison et al. showed the ability to insert vinylogous glycine for GLY[3] of Leu-enkephalin.[65] In a later report, Grison found *Z*-vinylogous derivatives could form β-turn mimetics.[64] As research has shown proposed synthon **1** is highly a desirable compound with many potential biological applications.

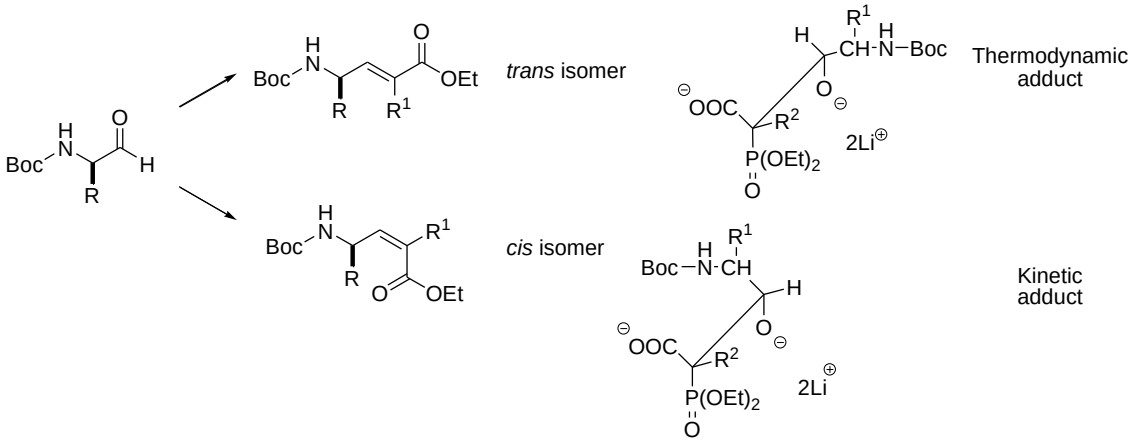
Synthesis of *γ-UAAs via olefination*

In order to synthesize α,β-unsaturated diesters, traditional olefination processes were considered. Typical olefinations require an aldehyde/ketone and a customized organophosphorus reagent. Classic examples of these processes include Wittig,[63,92,98] Horner-Wadsworth-Emmons (HWE), [99] Julia,[100] and Peterson methods.[101,102] The most utilized olefination for the preparation of α,β-unsaturated compounds are the adaptations of HWE protocols. In these reactions, phosphonate ylide intermediates are generated from alkali metals. Then the yliides undergo a stereoselective decomposition leading predominately *E*-isomers. This method was first employed in the synthesis of α,β-unsaturated γ-amino acids by Seebach's group (Scheme 8).[28] After struggling to make analogues via the Arndt-Eistert homologation Seebach opted to use a HWE synthesis. Their synthesis converted boc-protected amino acids into Weinreb amides, reduced the Weinreb amides into aldehydes using lithium aluminum hydride, and reacted the aldehydes with trimethyl phosphonoacetate over sodium hydride. This three step synthesis fostered a 22% combined yield of the α,β-unsaturated amino esters selective for the *E*-isomers. Finally, the vinylogous amino esters were reduced with palladium to the N-boc-protected γ-amino acids.



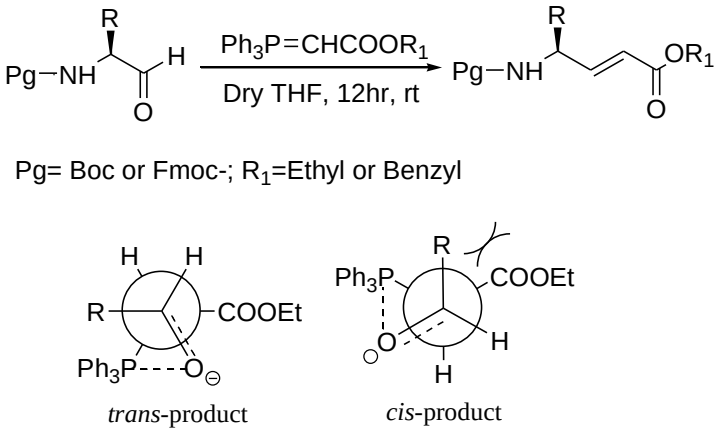
Scheme 8. Seebach HWE method to synthesizing vinylogous γ-amino acids. I. DCC, HOBt, Et_3N, MeONHMe,·H_2O. II. LAH III. NaH, $(PhO)_2P(O)CH_2CO_2Me$.

Grison et al. also used HWE reagents to prepare unsaturated-γ-UAAs (Scheme 9).[64,65] Unlike Seebach, Grison was capable of making both *E*- and *Z*-isomers by varying the alkali base used. The *E*-analogues were prepared using dilithiated dianions of 2-diethylphosphonpropanoic. Grison also showed the *Z*-olefin could be prepared by using a combination of potassium hydride/ethyl 2-bis(trifluoroethyl)-phosphonopropanoate or BuLi/ethyl 2-diethylphosphonopropanoate. Overall, the potassium hydride combination gave the highest *Z*-selectivity and was not affected by the size of R group or the nature of R^2 group. The selectivity was explained by the low temperature decomposition of the kinetic adduct and the reduced ability of potassium to form counter ion complexes. The lithiated base selectivity was only enhanced when R^1=F. While these methods provided an efficient way of making vinylogous γ-UAAs their methods were limited to monosubstituted substrates without bulky side chains.



Scheme 9. Grison et al. HWE method of preparing both E/ Z-γ- Vinylogous UAAs.

Gopi et al. established a similar method using Wittig reagents (Scheme 10). Like Seebach and Grison, Gopi reduced a Weinreb amide to form an amino aldehyde, and reacted it with the ylide formed by (triphenylphophoranylidiene)acetate. The reaction proceeds with a 100% *E*-selectivity to the N-boc protected α,β-unsaturated alanine amino ester in 93% yield. The same conditions prepared α-methylalanine in 75% yield. Only one variation, a Horner-Emmons reaction, emerged since Gopi's work to produce the same disubstituted compound,[99] but the advantage of Gopi's method is the compatibility with both boc- and fmoc-protected analogues.



Scheme 10. Gopi synthesis using traditional Wittig chemistry and their proposed transition states.

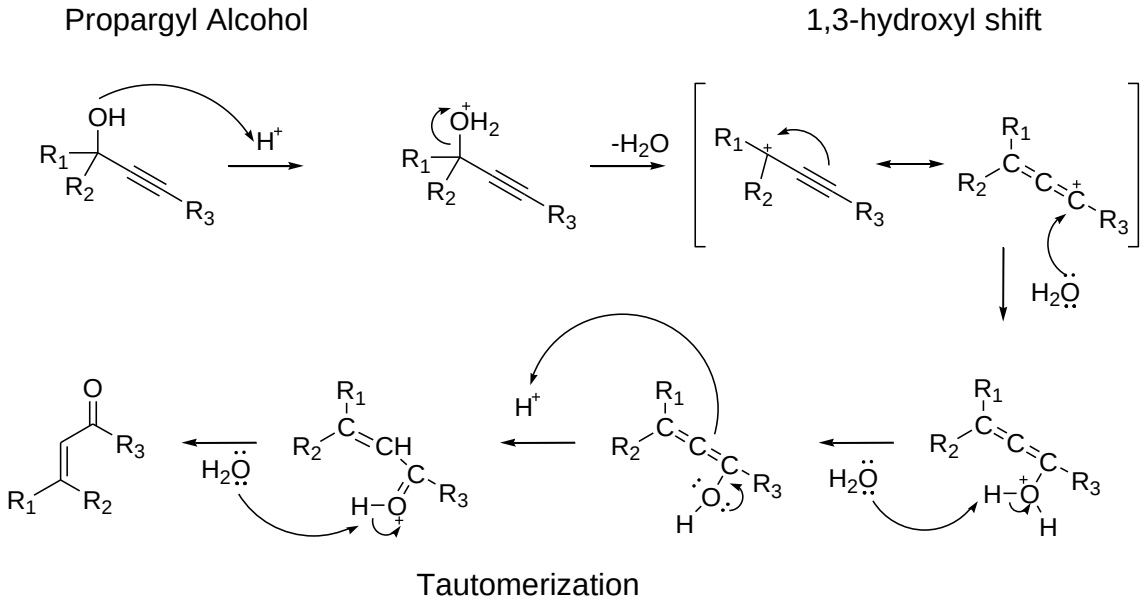
The strategies described were successful at making simple α-substituted UAAs but several limitations were encountered preparing disubstituted analogues. First, both the Horner and Witting reactions require expensive designer organophosphorus reagents. Typically, these reagents are synthesized individually and are bulky reagents. Moreover, mass is wasted and is not atom economical or affordable on a bulk scale. In addition, phosphorus reagents are notoriously hard to purify, sometimes requiring multiple columns to remove noxious byproducts completely. Finally, these olefination methods are limited to steric congestion surrounding the reacting ketones or aldehdyes. Those strategies preparing UAAs from vinylogous analogues resorted to using chiral catalysts[82] in order to reduce the double bond and avoided disubstituted α-carbons. Therefore, any chemistry used to prepare 1 must accommodate a congested chiral center.

All of the mentioned strategies involve the conversion of an amino aldehyde to the vinylogous amino ester. After a literature review it is obvious very few strategies are capable of synthesizing C $^{\alpha,\alpha}$- disubstituted-unsaturated

diesters.[92,103-108] Some of these methods used traditional Witting[92] and HWE[109] methods, while others used non-traditional olefination chemistries. The Meyer Schuster rearrangement was considered as an alternative strategy to avoid the challenges associated the synthesis of α,α-disubstituted with traditional olefination chemistries.

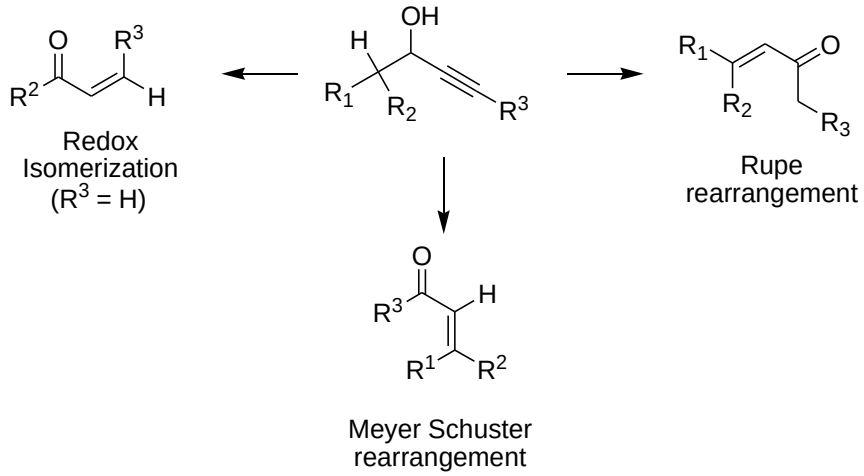
Meyer Schuster rearrangement

Recently, the Meyer Schuster rearrangement gained significant attention as chemists continue to develop greener alternative chemistries.[110] The Meyer Schuster rearrangement is not limited by sterically hindered ketones/aldehydes. The only requirement to promote the reaction is a propargyl alcohol and a catalytic amount of Brønsted acid. Once activated, the propargyl alcohol undergoes a 1,3-hydroxyl shift and tautomerization to an α,β-unsaturated ketone (Scheme 11).[111,112]



Scheme 11. Mechanism of the Meyer Schuster rearrangement.

The Meyer Schuster reaction is not without limitations, and the biggest challenge is selectively promoting the desired rearrangement over competing pathways (Scheme 12).[113-116] The Rupe rearrangement, a 1,2-hydroxy shift, is the major competitor due to a lower energy transition state. However, the Rupe pathway only occurs if a β-hydrogen is located adjacent to the propargyl alcohol.[115,116] There is also potential for an internal redox process with a simultaneous oxidation of the alcohol and reduction of the C≡C bond. Early on limitations of using the Meyer Schuster rearrangement was the harsh refluxing acidic reaction conditions needed to effect transformation and lack of regioselectives. Today, these limitations are overcome using new transition-metal catalysts.



Scheme 12. Possible competing reactions of Meyer Shuster rearrangement.

Original Meyer Schuster catalysts included oxides and oxo-complexes centered on activating hydroxyl groups.[116] One of the first successful transition metal catalysts used were vanadium metal complexes.[117] Still, lack of stereocontrol and high reaction temperatures offset the low to moderate reaction

yields. In 2006, Engle and Dudley screened late transition-metal soft Lewis acids and found 5 mol % gold (III) chloride catalysts could promote the desired reaction.[118] The high affinity for gold to coordinate acetylenic π-bonds enabled the rearrangement to proceed under milder reaction conditions (Figure 6). This mode of activation differed from traditional Brønsted main group elements and early transition-metal Lewis acids which bound preferentially to harder Lewis basic sites.

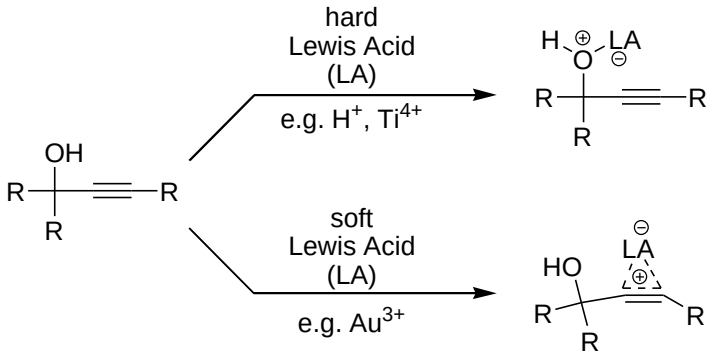


Figure 6. Hard/soft Lewis acid activation of Meyer Schuster rearrangements.

Dudley's group continued to optimize reaction conditions for increased stereoselectivities under milder reaction conditions using cheaper catalysts.[116,119,120] Dudley's results are summarized below:

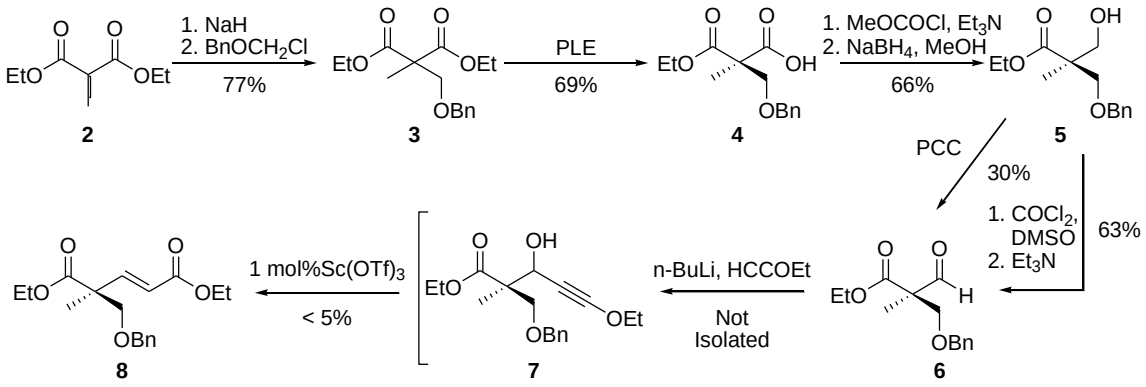
- AuCl/$AgSbF_6$ affects the E/Z selectivity;
- 5.0 equivalents of ethanol solvent promotes 1,3-hydroxyl migration;
- A solvent mixture of THF-CH_2Cl_2 (1:1) was *E*-selective;
- 1 mol% catalyst loading of either copper(II) triflate, indium(III) chloride, or scandium(III) triflate produces higher yields.

These observations allowed them to synthesize several α,β-unsaturated ester in under a hour at room temperature. Yields were greater than 70% with an E/Z ratio ranging from 40:60 to 100:0.

Dudley's Meyer Schuster protocol inspired the synthesis of **1** as a key step in the synthesis of the common synthon. By using the Meyer Schuster rearrangement reaction organophosphorus reagents are avoided, the chemistry is atom economical, and steric problems are eliminated. Moreover, since the precursor propargyl alcohol contains no β-hydrogen the favorable Rupe rearrangement is not a potential pathway. The results of the adaption of the Meyer Schuster method are documented below.

Results and Discussion

The preparation of the common synthon was optimized using (*S*)-α[2,2]-serine analogues, because previous research in the Masterson Research Group showed high enantioselectivities of this substrate toward PLE.[23,121] The PLE method has become a staple in the Masterson Research Group as it allows for easy access to UAAs containing α-methyl substitution from readily available and cheap diethyl methylmalonate. In addition, the tunability of PLE with serine analogue 3 (Scheme 13), is influenced by the careful choice of co-solvent.[122] γ-serine analogues contain the important alcohol functionality and represent potential pharmaceutical leads as GABOB and statine derivatives which are the most widely synthesized class of γ-UAAs.[12]



Scheme 13. Synthesis of α,β-unsaturated diester common intermediate.

Optimization of the common synthon

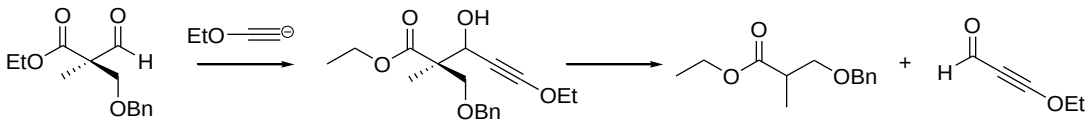
The synthesis of (*S*)-diethyl 2-((benzyloxy)methyl)-2-methylmalonate was carried out following documented procedures (Scheme 13).[23,121] An enolate formed over sodium hydride was added to diethyl methylmalonate, **2**, and refluxed with benzyl chloromethyl ether to **3**. The prochiral diester was hydrolyzed with PLE in 0.1 M phosphate buffer at pH 7.4. The progress of the reaction was monitored on an automatic titrator set to deliver 1.0 M NaOH as the acid formed. These studies were conducted without co-solvents but previous reports had shown the addition of 10% isopropyl alcohol increases the enantioselectivity up to 97% *ee* at the expense of lower reaction yields.[122] After the addition of one mole equivalent of base, the reaction was complete (approximately 24 h), and half ester **4** was isolated (69% yield, 70% *ee*).

In order to form the needed precursor propargyl alcohol, carboxylic acid **4** was converted into an aldehyde following a reduction/oxidation sequence. First, primary alcohol **5** was prepared by converting **4** into a mix anhydride with methyl chloroformate in the presence of triethylamine.[123] The slow addition of sodium borohydride was followed by the addition of one equivalent of methanol over 2

hours. The mild reduction selectively reduced the activated anhydride to **5** leaving the ethyl ester intact. Intermediate **5** was oxidized using pyridinium chlorochromate (PCC), but required multiple columns, long reaction times, and resulted in low yields (30% overall yield), of a toxic byproducts. A Swern oxidation was picked as an alternative method based on the overall quicker reaction times and reduced toxicity. The Swern Oxidation proceeded via an in situ generation of dimethylchlorosulphonium ion from the reaction of dimethyl sulfoxide and oxalyl chloride. To the solution was added **5** at -78 °C forming a sulfur ylide which decomposed into aldehdye **6** (63% yield after column purification).

In a two-step reaction, the aldehyde was converted to the propargyl alcohol **7**. First, N-butyllithium was added to ethyl ethnyl ether at -78 °C to form the nucleophilic acetylide anion. Then, via syringe, the acetylide anion was added to a chilled solution of 6 at -60 °C, warmed to room temperature, stirred for 3 hours, and quenched with ammonium chloride. However, after workup **7** was not isolated and no indication of the rearranged Meyer Schuster product **8** was present. Instead, crude ^{1}H-NMR showed a mixture of starting material and a side product. After a preparative TLC, ethyl 3-(benzyloxy)-2-methylpropanoate, **9** was isolated. The observance of **9** indicates the propargyl alcohol formed, but prior to isolation decomposed into products **9** and **10** (Scheme 14). Aldol reactions produce beta-hydroxy ketones from the addition of an enolate to an aldehyde. Since the propargyl alcohol contained the structural scaffold of an aldol product, a beta-hydroxy ester moiety, the reverse aldol reaction was occuring. This

means the bond between the α-carbon and β-hydroxy carbon cleaved to form both **9** and **10**. However, compound **10** was never isolated likely due to the volatility and/or reactivity of the substrate. These results prompted further investigation into optimizing the conditions to minimize decomposition.



7 **8** **9** **10**

Scheme 14. The decomposition of the propargyl alcohol into the retro aldol products.

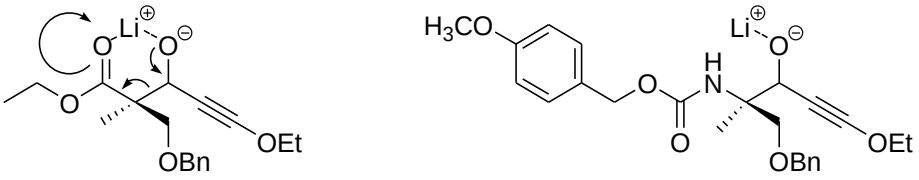
Propargyl alcohol isolation studies

In attempts to isolate the desired propargyl alcohol, a series of reaction conditions were varied. Previously, the acetylide anion was drawn into a dry syringe and added directly to the solution. This technique may have quenched some anion in the process. In addition, the internal reaction temperature was not monitored, and the solution could have warmed significantly during the addition. These experimental techniques were addressed by using a cannula under an inert atmosphere of nitrogen gas to deliver the acetylide to the aldehyde while maintaining the solution temperature at -78 °C ± 5 °C. After stirring at room temperature for 3 hours the reaction was quenched. Unfortunately, these adjustments had no influence in the observed product, and the reaction and quenching temperature were varied. In a second attempt, the reaction stirred for 30 minutes at -78 °C ± 5 °C and quenched with saturated ammonium chloride after acetylide addition. This time considerable amounts of starting material was isolated. The reaction was setup again and allowed to react longer (3 hours)

before quenching. Initially the mass corresponding to **7** was present in the ESI MS, but re-examination of the isolated fraction showed the sample had completely decomposed into the thermodynamically more stable retro aldol product. It was concluded that the propargyl alcohol was too thermodynamically unstable to be isolated in significant quantities and will decompose into aldol products upon storage.

In a final attempt, without isolating the intermediate propargyl alcohol, the serine diester was made. Without characterization of the propargyl alcohol, the reaction was taken immediately to the Meyer Schuster rearrangement following Dudley's reported scandium (III) triflate catalyzed method. After 3 hours of stirring at room temperature the reaction was worked up.[120] Surprisingly, this modification resulted in the formation of the desired α,β-unsaturated diester **8**, but yields still remained low. After column purification, a 3:1 mixture of (*E*):(*Z*) isomer was isolated (less than 5% overall yield). At this point it was concluded further optimization with the serine diester would not yield significant amounts of the desired α,β-unsaturated diester. Since these results suggested the Retro-aldol product was more stable than the propargyl alcohol, a new analogue was devised limiting the ability of the diester to act as good leaving group.

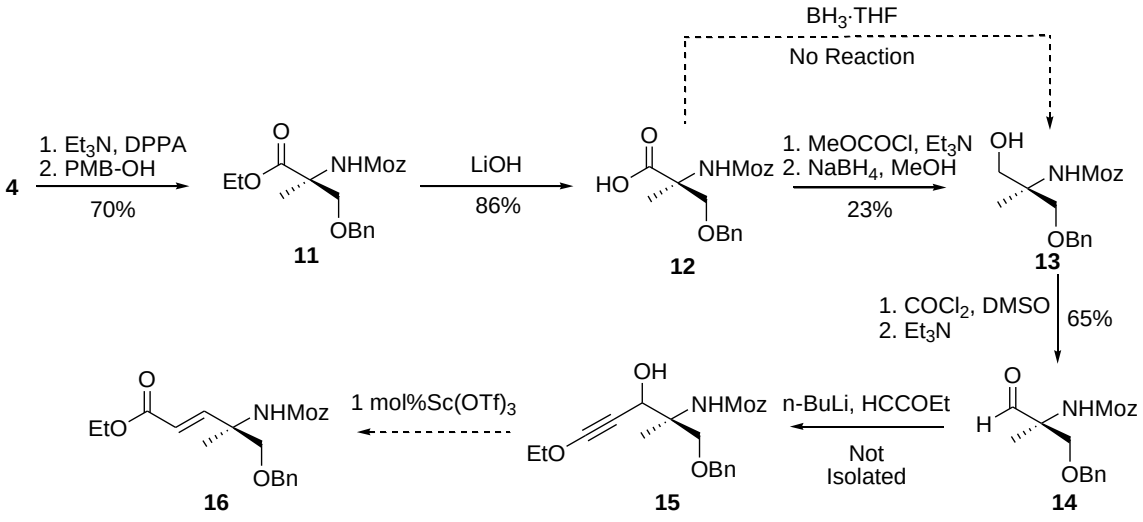
Optimization of the vinylogous amino ester



Electron Withdrawing Electron Donating

Figure 7. Proposed propargyl alcohol intermediates studied to control the retro-aldol decomposition.

Previously, the propargyl intermediate, **7**, contained an ethyl ester. It was hypothesized this ethyl ester activated the compound toward the rearrangement and if replaced with an electron donating group decomposition could be avoided (Figure 7). Additionally, the lithium salts formed during the acetylide addition were capable of coordinating the β-hydroxy alcohol and carbonyl facilitating the rearrangement. Since the end goal is to make amino acids, in the long run, converting the ester into nitrogen seemed beneficial (Scheme 15). The amino group would not act as a good leaving group, due to nitrogen's donation of electron density, therefore avoiding the retro aldol reaction.[124]



Scheme 15. Synthesis of the α,β-unsaturated amide.

The modified procedure starts with half ester **4** formed from the PLE hydrolysis and was reacted under Curtius protocol. After formation of the acyl azide the intermediate was quenched with 4-methoxybenzyl alcohol to form the moz protected carbamate **11** in good overall yield (86%). The ester was hydrolyzed to **12**, and reduced and oxidized to **14**. Unlike the reduction of β-hydroxy ester **5**, the synthesis of the amino ester **13** was challenging. Reduction under mixed anhydride conditions resulted in low yields of **13**, while both reductions via dimethylsulfide-borane and borane-tetrahydrofuran complex gave no reduced product. At this point, the low-yielding mixed anhydride protocol was followed and oxidation to aldehyde **14** was completed under Swern conditions (70% yield after purification).

The optimized procedures used previously to form the propargyl alcohol were followed to prepare **15**. Due to the presence of an acidic amide proton, an added equivalent of acetylide acted as a sacrificial base in the reaction. One acetylide served as a nucleophile adding to the carbonyl of the aldehyde and the other deprotonated the amide hydrogen. Thus, the acetylide anion was added, reacted, and quenched at -78 °C. Immediately after workup, the crude material was taken on to the Meyer Schuster rearrangement. After a 3-hour reaction time with 1 mol% scandium (III) triflate catalyst no evidence of **16** was observed. At this point the inability to control the competing side reaction forced the abandonment of the Meyer Schuster rearrangement as a tool to form the common synthon.

Future Directions

At the time of these experiments, there was no literature precedence of similar amino substrates. Since then, a few example have emerged. One example showed that propargyl amines, instead of alcohols, are capable of promoting the Meyer Schuster rearrangement.[124] Another example demonstrated an intramolecular cyclization of β- and γ-amino-ynones into a hemi-aminal propargylic intermediates rearranging into to vinylogous pyrrolidine analogs.[125] Key to this reaction was the addition of methanesulfonic acid with varying amounts of co-solvent methanol. These two examples also were only successful when the amine was tertiary. If future investigations into the Meyer Schuster rearrangements are conducted then conversion of the amino group into tertiary amines should be considered. This means only one equivalent of the acetylide anion is needed and less chance of competing side reactions with the excess of alkynyl reagents. Another consideration would be trapping the formed propargyl alcohol. Zanoni et al. showed the ability to trap the alcohol as a propargylic acetate. These Meyer Schuster rearrangement reactions were mediated with expensive gold.[126] In theory, the ability to trap, isolate the intermediate alcohol, and still show reactivity toward the Meyer Schuster is important even at the expenses of an additional step to remove the trapping agent.

Conclusions

Due to uncontrollable formation of reverse aldol products during the synthesis of the propargyl alcohol, the Meyer Schuster rearrangement is not a

viable method of preparing γ-UAAs. Attempts at eliminating the side reaction via reaction conditions and electronic effects were unsuccessful. Evidence of the desired product came only when the Meyer Schuster reaction was performed consecutively allowing no time for isolation of the propargyl alcohol. This was confirmed by a crude ^{1}H-NMR where the α,β-unsaturated diester was isolated along with residual starting material (less than 5% yield). Based on these results it appears as if the Meyer Schuster rearrangement is capable of occurring but the rate of degradation to the reverse aldol product is significantly faster and product more thermodynamically stable. These results forced consideration of an alternative synthon discussed in Chapter III.

Experimental

General Experimental

All reagents were used as received from commercial vendors, unless otherwise noted. Tetrahydrofuran, methylene chloride, 1,2-dichloroethane, diethyl ether, N,N-dimethylformamide, and triethylamine were obtained from a solvent system dried over a column of activated alumina. Solutions of N-butylithium were titrated prior to use using the Gilman protocol.[127] Crude PLE was purchased directly from Sigma and used as received. All enzymatic hydrolyses were performed on a 798 MPT Titrino or a Radiometer Analytical TIM 854 Automatic Titrator. High Pressure hydrogenation was conducted on a Parr Pressure Reaction Apparatus. Flash chromatography was performed using P-60 silica gel. TLC analysis was conducted on Aluminum backed 200 μm Silica XG TLC Plates. The TLC plates were visualized using UV light, phosphomolybdic

acid, bromocresol green, ninhydrin, or potassium permanganate stains. High Resolution Mass Spectra were obtained from Old Dominion University and analyzed on a Bruker 12 Tesla APEX –Qe FTICR-MS with and Apollo II ion source. NMR spectra were acquired with a Bruker Avanced III 400 MHz or Varian Mercury 300 MHz spectrometer and referenced to TMS or other residual solvent protons. IR spectra were acquired with a Thermo-Nicolet Nexus 470-FT-IR using a diamond anvil ATR accessory. Optical rotation measurements were recorded with a 1 dm cell at ambient temperature on a Rudolph Research Autopol III autopolarimeter or a Rudolph Research Autopol IV Automatic Polarimeter. Melting points were determined using a Thomas Hoover capillary melting point apparatus.

*Diethyl 2-((benzyloxy)methyl)-2-methylmalonate (**3**):*

The title compound was synthesized according to documented literature procedure and confirmed with the characterization data.[23,122]

*(R)-2-(ethoxycarbonyl)-3-(benzyloxy)-2-methylpropanoic acid (**4**):*

The title compound was synthesized according to reported literature procedure, and isolated material was confirmed with reported characterization data.[23,122]

*(S)-ethyl 3-(benzyloxy)-2-(hydroxymethyl)-2-methylpropanoate (**5**):*

In a three-neck round bottom flask under N_2 atmosphere 4 (48.3 mmol, 12.8 g) was dissolved into 200 mL of dry THF. The solution was cooled to -15 °C and triethylamine (53.2 mmol, 7.4 mL) was slowly added. After 15 min, methyl chloroformate (50.7 mmol, 3.92 mL) added, and stirred for an additional 15 min.

The white solids formed were removed over a sintered glass filter, filtrate placed back into the reaction flask, and cooled to -10 °C. In one portion $NaBH_4$ (144 mmol, 5.48 g) was added at 0 °C followed by the slow addition of methanol (31.8 mL) via a syringe pump over 1.5 h. After the addition, the solution was kept at 0 °C for 2 h, and stirred at rt for 12 h. The reaction was quenched with 10% HCl over an ice bath, and concentrated under reduced pressure to remove the THF. The solution was extracted with Et_2O (3×100 mL), organic layers combined, and extracted with saturated $NaHCO_3$ (3×50 mL), 10% HCl (3×25 mL), and a saturated brine solution (3×25 mL). The final organic layer was dried over $MgSO_4$, filtered, and was concentrated under reduced pressure. A short column eluting with 40:60 EtOAc:Hexanes led to 7.11 g of 5 (28.1 mmol, 58% yield) as a pure oil. R_f =0.17 (30:70 Et_2O:Hexanes). The isolated material was confirmed with reported characterization data.[123,128]

*(R)-ethyl 3-(benzyloxy)-2-formyl-2-methylpropanoate (**6**):*

A flask containing oxalyl chloride (8.72 mmol, 0.75 mL) was dissolved into 25 mL of CH_2Cl_2. The solution was cooled on a 2-propanol bath to -60 °C using an immersion chiller. DMSO (15.9 mmol, 1.13 mL) was added to the solution and stirred for 15 min. To the flask dissolved into 10 mL of CH_2Cl_2 was added 5 (15.9 mmol, 2.00 g) and stirred for 15 min. Then triethylamine (36.0 mmol, 5.0 mL) was added and warmed to rt over an hour. The reaction was quenched with 50 mL of H_2O and the aqueous layer extracted with CH_2Cl_2 (5×25 mL). The organic layers were combined and extracted with H_2O (3×10 mL), a saturated brine solution (1×25 mL), dried over $MgSO_4$, filtered, and concentrated under

reduced pressure. A silica gel column eluting with 30:70 Et_2O:Hexanes solution, R_f =0.53 (30:70 Et_2O:Hexanes), resulted in 6 (1.25 g, 5.00 mmol, 42% yield). The compound was stored under nitrogen at -4 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 9.82 (s, 1H), 7.37 – 7.24 (m, 5H), 4.52 (s, 2H), 4.22 (q, *J* = 7.1 Hz, 2H), 3.86 (d, *J* = 9.0 Hz, 1H), 3.69 (d, *J* = 9.0 Hz, 1H), 1.33 (s, 3H), 1.26 (t, *J* = 7.1 Hz, 3H).

*(S)-diethyl 4-((benzyloxy)methyl)-4-methylpent-2-enedioate (**8**):*

In a 3-neck round bottom flask under N_2 atmosphere ethyl ethynyl ether (1.76 mmol, 0.41 mL) was dissolved into 5.0 mL of dry THF and cooled to -78 °C on a 2-propanol bath with an immersion chiller. An acetylide anion was formed upon the addition of 1.39 M n-butyllithium (1.09 mL) via syringe, warmed to 0 °C for 90 min, and chilled back down to -78 °C. In a separate flask 6 (1.60 mmol, 0.40 g) was cooled to -78 °C. The acetylide solution was added via cannula, stirred 4 h, and quenched with 1.0 mL of saturated ammonium chloride. The crude material was dissolved into Et_2O, extracted with H_2O (3×5 mL), saturated $NaHCO_3$ (3×5 mL), and a saturated brine solution (1×5 mL). The organic layers were combined, dried over $MgSO_4$, filtered off, and concentrated under reduced pressure. The crude material was used immediately without further purification.

In an open flask, crude 7 and 7.8 mg of $Sc(OTf)_3$ (1 mol%) catalyst was suspended into a solution of 4:1 CH_2Cl_2:EtOH. The solution stirred 3 h and concentrated under vacuum. The crude material was purified on a silica gel column eluting with 30:70 Et_2O:Hexanes. Compound 8 elutes as a mixture with starting material (10 mg of mixture isolated, less than 5% yield). R_f = 0.30 (30:70 Et_2O:Hexanes). ^{1}H-NMR results indicate the formation of both *E/Z* isomers along

with unreacted starting material. Based on the ratios of the alkene protons the (*E*/*Z*)-ratio is 3:1. Additional purification was not attempted and ^{1}H-NMR values are reported as a mixture of isomers. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.23 (m, 5H), 7.10 (d, *J* = 16.1 Hz, 1H), 6.27 (d, *J* = 12.1 Hz, 1H), 5.90 (d, *J* = 16.1 Hz, 1H), 5.88 (d, *J* = 12.1 Hz, 1H), 4.52 (d, *J* = 3.7 Hz, 2H), 4.26 – 4.15 (m, 4H), 3.70 (d, *J* = 8.6 Hz, 1H), 3.55 (d, *J* = 8.7 Hz, 1H), 1.48 (s, 3H), 1.20 (t, *J* = 7.1 Hz, 6H). LRMS (ESI-MS) *m/z*: $[M+Na]^+$ calcd for $C_{18}H_{24}O_5Na$ 343.2; Found 343.3.

*ethyl 3-(benzyloxy)-2methylpropanoate (**9**):*

The title compound was isolated as a product from the synthesis of 8. The ^{1}H-NMR spectra confers with the reported literature data.[129]

*4-methoxybenzyl (S)-2-(ethoxycarbonyl)-1-(benzyloxy)propan-2-ylcarbamate (**11**):*

The title compound was synthesized according to reported literature procedure, and isolated material confirmed with reported characterization data.[23]

*Serine, [[(94-methoxyphenyl]methoxy]carbonyl]-2-methyl-O-(phenylmethyl) (**12**):*

In a flask containing **11** (2.49 mmol, 1.0 g) dissolved into 40:60 EtOH:H_2O (v/v) solution LiOH (9.96 mmol, 0.238 g) was added. The reaction stirred open to the atmosphere at rt for 36 h. The EtOH layer was concentrated and crude material extracted with Et_2O (3×50 mL). The combined organic layers were set aside. The aqueous layer was acidified to a pH=2.0 with 4.0 N HCl, and extracted with Et_2O (3×50 mL). All of the organic layers were combined, dried over $MgSO_4$, and concentrated under reduce pressure. The crude material was purified on a gradient silica gel column eluting with a mobile phase of 30:70 EtOAc:Hexanes (R_f =0.07). Pure **12** was isolated after two columns (0.800g,

2.14 mmol, 86% yield) and crystallized after several weeks in storage. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.61 (s, 1H), 7.37 – 7.21 (m, 8H), 6.87 (d, *J* = 8.7 Hz, 2H), 5.74 (s, 2H), 5.02 (s, 2H), 4.52 (s, 2H), 3.79 (s, 3H), 1.58 (s, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 159.67, 155.78, 137.28, 129.97, 128.50, 128.28, 127.95, 127.74, 113.98, 73.61, 72.56, 66.77, 59.96, 55.30, 20.36. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ calcd for $C_{20}H_{23}NO_6Na$ 396.1; found 396.0.

*4-methoxybenzyl (R)-3-(benzyloxy)-1-hydroxy-2-methylpropan-2-ylcarbamate (**13**):*

In a flask under N_2 atmosphere was dissolved of **12** (5.6 mmol, 2.09 g) into 20 mL of dry THF. The solution was cooled to -15 °C and triethylamine (11.2 mmol, 1.56 mL) was added. After 15 min of stirring, methyl chloroformate (5.88 mmol, 0.45 mL) was added, and stirred for 15 min. A white solid formed was removed over a sintered glass filter. The filtrate was placed back into the flask, and cooled to -10 °C. In a single portion $NaBH_4$ (16.8 mmol, 0.635g) was added. A syringe pump was set to deliver 3.70 mL of methanol over 1.5 h at 0 °C. After the addition, the solution was kept at 0 °C for 2 h and rt for 12 h. The reaction was quenched with 10% HCl over an ice bath, and THF was concentrated under reduced pressure. The remaining aqueous portion was extracted with Et_2O (3×20 mL), organic layers combined, and extracted with saturated $NaHCO_3$ (3×20 mL), a 10% HCl (3×20 mL), and a saturated brine solution (3×20 mL). The final organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. After a short column eluting with 30:70 EtOAc:Hexanes pure **13** (1.32 mmol, 0.474 g, 23% yield) was isolated as an oil. R_f = 0.33 (30:70

EtOAc:Hexanes) IR (cm^{-1}) = 3325 br, 2961, 2455 br, 2068, 1642. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.37 – 7.26 (m, 7H), 6.88 (d, *J* = 8.7 Hz, 2H), 5.39 (s, 1H), 4.99 (s, 2H), 4.50 (s, 2H), 3.80 (s, 3H), 3.71 (d, *J* = 6.9 Hz, 1H), 3.61 (d, *J* = 9.2 Hz, 1H), 3.60 (d, *J* = 10.5 Hz, 2H), 3.50 (d, *J* = 9.2 Hz, 1H), 1.26 (s, 3H).^{13}C-NMR ($CDCl_3$, 100 MHz): δ 159.61, 156.13, 137.63, 129.93, 128.50, 127.89, 127.65, 113.96, 74.47, 73.63, 68.44, 66.40, 56.53, 55.29, 19.78.

*Synthesis of 4-methoxybenzyl (S)-1-(benzyloxy)-2-formylpropan-2-ylcarbamate (***14***):*

In a flask under inert N_2 atmosphere at -65 °C, oxalyl chloride (1.38 mmol, 0.108 mL) was added to 5.0 mL of CH_2Cl_2 followed by DMSO (2.77 mmol, 0.19 mL) and stirred for 25 min. A solution of **13** (1.12 mmol, 0.449 g) dissolved in 5.0 mL of CH_2Cl_2 was added, stirred for 15 min. before triethylamine (3.15 mmol, 0.436 mL) was added. The reaction warmed to -40 °C, stirred for an additional 90 min., warmed to rt, and quenched with 10 mL of H_2O. The aqueous layer was extracted with CH_2Cl_2 (3×15 mL), and organic layers were combined, extracted with 10% HCl solution (5×15 mL), and rinsed with a saturated brine solution (3×15 mL). The resulting organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure isolating **14** (0.322g, 0.90 mmol, 65% yield) as a pure liquid. The compound was stored under nitrogen at 4 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ. 9.49 (s, 1H), 7.31 (dddd, *J* = 21.5, 19.3, 8.9, 4.2 Hz, 8H), 6.88 (d, *J* = 8.7 Hz, 2H), 5.62 (s, 1H), 5.02 (s, 2H), 4.47 (d, *J* = 3.3 Hz, 2H), 3.80 (s, 3H), 3.67 (s, 2H), 1.40 (s, 3H).^{13}C-NMR ($CDCl_3$, 100 MHz): δ 199.9, 159.7,

155.5, 137.3, 130.0, 128.5, 128.3, 128.0, 127.7, 114.0, 73.6, 71.3, 66.7, 63.1, 55.3, 17.4.

CHAPTER III

THE PREPARATION OF C^{α}-METHYL-γ- AND δ-AMINO ACID ANALOGUES VIA 3-(METHYLHYDROXY)-3-METHYL-γ/δ-LACTAM SYNTHONS

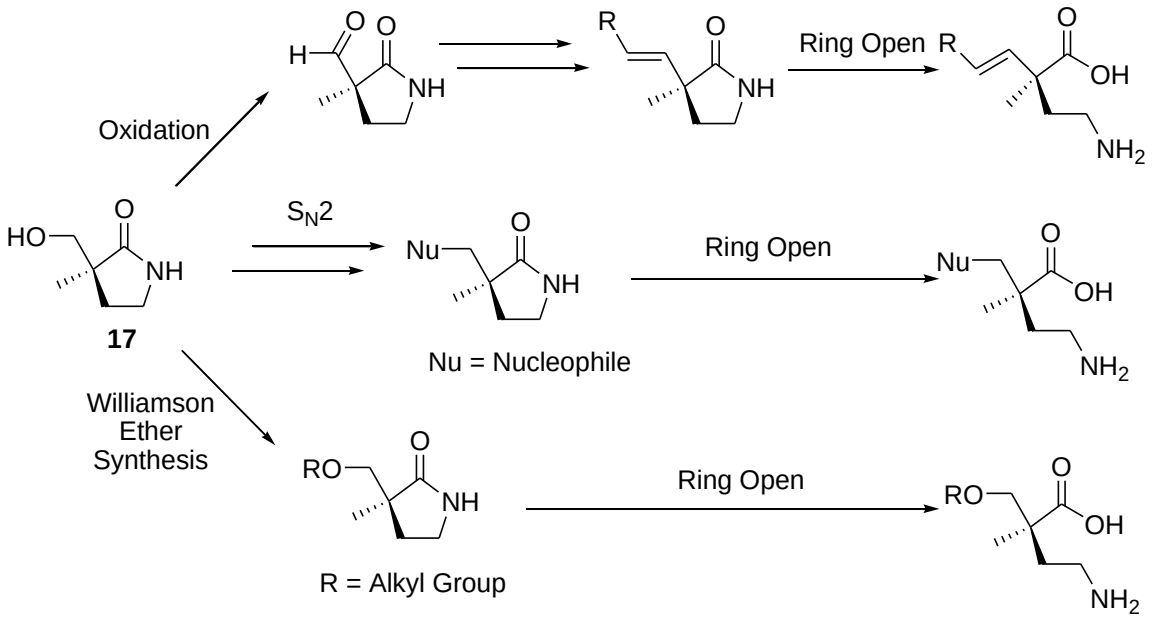
Hypothesis 2

3-(methylhydroxy)-3-methyl–γ/δ-lactams can be prepared via an intramolecular cyclization/reduction strategy and used as a synthon in the synthesis enantioenriched $C^{\alpha,\alpha}$-disubstituted γ/δ-UAAs.

Introduction

After limited success in optimizing the Meyer Schuster rearrangement, a new intermediate containing a core pyrrolidin-2-one was designed. Unlike the Meyer Schuster synthesis, this method establishes the γ-backbone before functionalizing the side chain. This would avoid problems associated with late stage homologation and prevent unwanted side reactions observed previously. Like the Meyer Schuster synthon, the new synthon also follows the same self-imposed characteristics discussed in chapter II. Since a cyclization strategy to prepare ethyl 3-methyl-2-oxopyrrolidine-3-carboxylic acid had already been established,[130] γ-lactam-alcohol **17** was picked as the new synthon. As a synthon, the reactivity of the alcohol group would serve as a site for further functionalization (Scheme 16). In comparison to the initially-proposed α,β-unsaturated intermediate, the γ-lactam intermediate has the advantage of being functionalized into many types of $C^{\alpha,\alpha}$-disubstituted γ-UAAs. The previous α,β-unsaturated diester synthon was limited to PLE compatible substrates. In

addition, the cyclization strategy is also capable of preparing both enantiomers of γ-UAAs through steric and stereoelectronic control of the cyclization.



Scheme 16. Proposed γ-lactam intermediate for the synthesis of α,α-γ-UAAs.

As an intermediate **17** can be functionalized by:

- Oxidation of **17** to the aldehyde, nucleophilic substitution, dehydration, and ring opening into amino acids with unsaturated side chains.
- The use of S_N2 chemistries by converting **17** into a good leaving group, nucleophilic substitution, and ring opening to a variety of functional groups.
- The alcohol group of **17** can be converted into other ethers following a Williamson ether synthesis and ring opening.

Moreover, the same synthetic sequence can be applied to the synthesis of δ-UAAs expanding the methodology to another important class of biomolecules. The following introduction will focus strictly on the applications and general synthetic strategies of preparing lactams. In addition, a short overview of lactam

functional group transformations and lactam ring opening strategies are briefly discussed.

Introduction

Lactams as synthetic intermediates

Both γ- and δ-lactams occur frequently in nature and the synthesis of lactams provides access to many biologically relevant compounds. These include the synthesis of inhibitors of lactacystin,[131,132] hepatitus C,[133] type II diabetes,[134] and various cancer inhibitors.[135-138] In addition, lactams are used as intermediates to prepare photo switchable ligands in inonotropic glutamate receptors.[139,140] Synthesis of 3,3-disubstituted pyrrolidones/piperidone derivatives (Figure 8) are documented to a lesser extent but have been used in natural products synthesis and as organocatalysis.[141] Disubstituted lactams have served as enzymes inhibitors, peptidomimetics of nipecotic acid and β-proline, and as potent GABA analogues.[141-144] The above examples demonstrate the potential use of optically pure $C^{\alpha,\alpha}$-disubstituted γ/δ lactams as synthetic intermediates of biomolecules with medicinally important functions. The synthesis of intermediates with multiple purposes keeps with the ideas established by The National Research Council and is valuable to the scientific community.

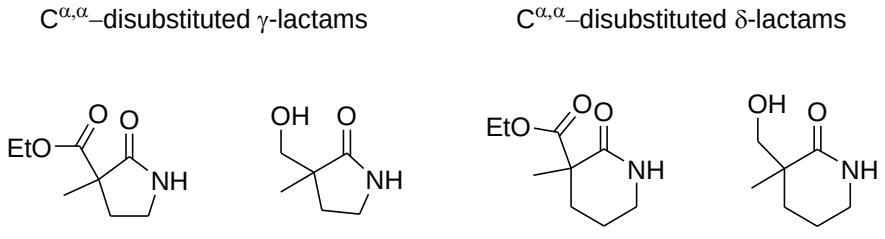
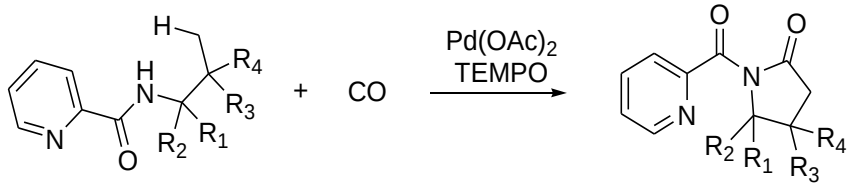


Figure 8. The class of α,α-disubstituted γ/δ-lactams.

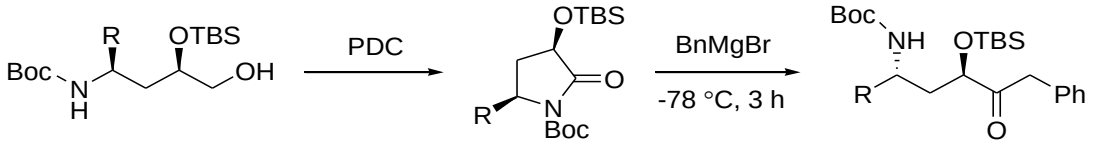
Current state of synthesis of γ/δ-lactams

The synthesis of lactams varies widely and includes both asymmetric and non-asymmetric variants of intramolecular and intermolecular cyclizations. Recently, interest in palladium catalyzed directed C(sp^3)-H bond carbonylation with carbon monoxide has been reported.[145,146] The most current example (Scheme 17) was documented by Wang et al. in the synthesis of 2-pyrrolidones with oxidant 2,2,6,6-tetramethylpiperidinyloxy (TEMPO).[146] While not an asymmetric strategy, the racemic synthesis was used to prepare pregblain, a GABA analogue. Other intramolecular cyclization strategies include palladium catalyzed allylations,[147] atom transfer radical cyclizations,[148] ruthenium catalyzed metathesis and Mannich cascade reactions,[149] and reductive cyclizations.[150]



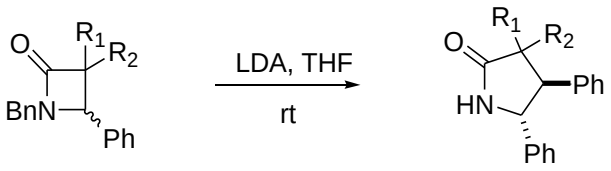
Scheme 17. Wang et al. transition metal catalyzed carbonylation with carbon monoxide synthesis of 2-pyrrolidones.

Zhou recently published an efficient method for the preparation of 3-hydrozyl-5-substituted 2-pyrrolidones following an oxidative cyclization(Scheme 18).[151]



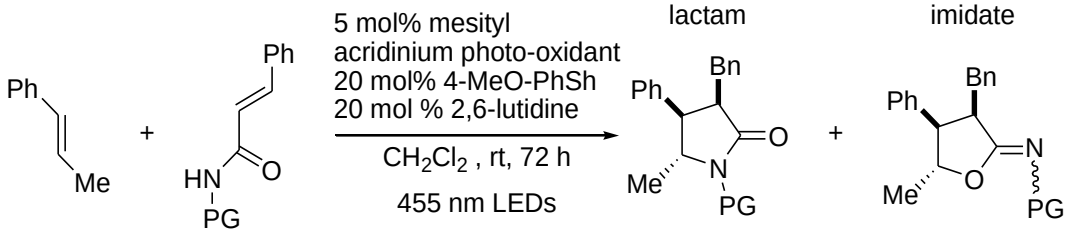
Scheme 18. Zhou oxidation/cyclization and Grignard ring opening.

Park et al. showed γ-lactam rings could be expanded following a β-lactam cleavage and cyclization strategy (Scheme 19).[152]



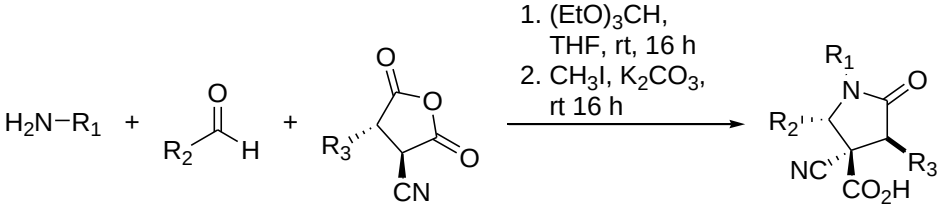
Scheme 19. Park et al. β-lactams ring expansion into γ-lactams.

Asymmetric intermolecular examples also appear. Gesmundo et al. reported a photo-oxidant radical reaction between alkenes and conjugated carbamates (Scheme 20).[153] Their research showed electron deficient amide protecting group selectively produces substituted lactams over imidates.



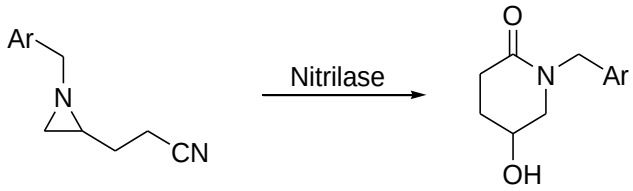
Scheme 20. Gesmundo et al. polar radical lactam formation.

Tan et al. reported a strategy to prepare substituted pyrrolidones following a one-pot generation of an imines and cyclization with chiral disubstituted anhydrides (Scheme 21).[154,155] Younai et al. also used imines and anhydride cyclization to prepare sulfur pyrrolidone analogues.[156]



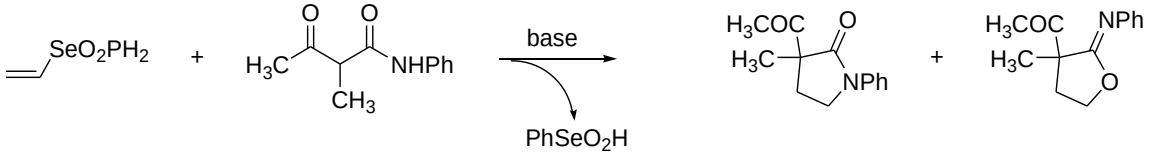
Scheme 21. Tan et al. Imines formation and cyclization strategy.

Many strategies also exist to synthesize δ-lactams and are similar to those to prepare γ-lactams. Synthetic preparation of δ-lactams include intramolecular cyclization with Lewis acid catalysts,[157,158] palladium-catalyzed hydroamidations,[159] ring expansion with N-heterocyclic carbine organocatalysts,[160] and addition of β-ketoamides to alkenes.[161] Vervisch et al. recently reported both chemical and enzymatic synthesis of δ-lactams using nitrilase (Scheme 22).[162]



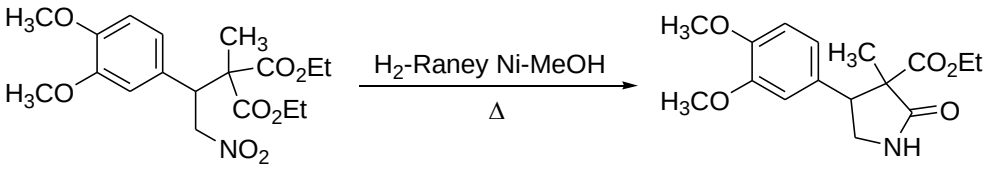
Scheme 22. Vervisch et al. enzymatic synthesis of δ-lactams.

The synthesis of α,α-disubstituted lactams are documented to a lesser extent. The most recent example comes from Sternativo et al. following a Michael addition and cyclization with vinyl selenone; however, the reaction produces both lactams and cyclic imidates as products and is not enantioselective (Scheme 23).[163]



Scheme 23. Sternativo et al. Michael addition/cyclization strategy.

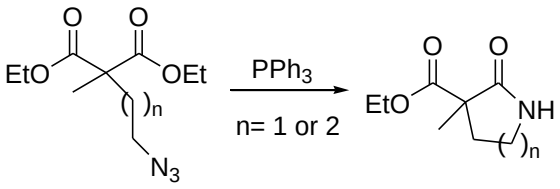
Marivet et al. showed the internal reductive cyclization of a malonic diester and nitro group to prepare phosphodiesterase inhibitors (Scheme 24).[150]



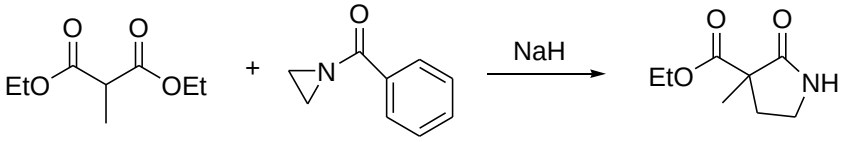
Scheme 24. Marivet et al. reductive cyclization strategy.

There are three synthesis documented to make ethyl, 3-methyl-2-oxopyrrolidine-3-carboxylate, the proposed ethyl ester intermediate (Scheme 25)[130,164,165] Khouhki et al. prepared the pyrrolidone as a racemic mixture following the reduction of a γ-azido ester and cyclization. Budny et al. showed diethylmethyl malonate enolates could react with aziridines to form the lactam. Banerjee et al. is the only example of an asymmetric synthesis following a stereoselective cyclization of optical enriched amino diesters. They showed cyclization was selectively controlled by steric and stereoelectronic effects.[130] The cyclization strategy proposed by Banerjee represents a novel access to α,α-disubstituted lactams and the strategy was used to prepare common synthon **17**.

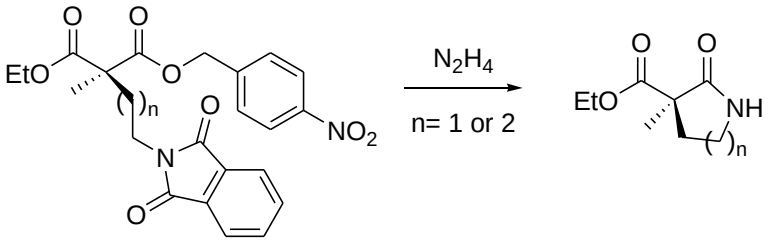
Khouki et al. method



Budny et al. method



Banerjee et al. method

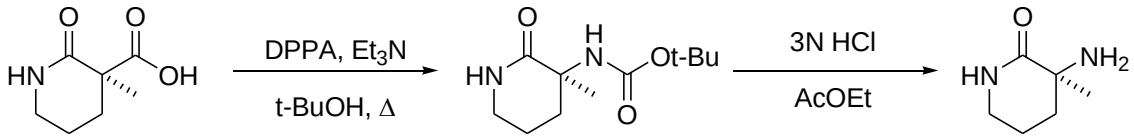


Scheme 25. Reported literature methods to prepare ethyl, 3-methyl-2-oxopyrrolidine-3-carboxylate.

Strategies to Functionalize Lactams

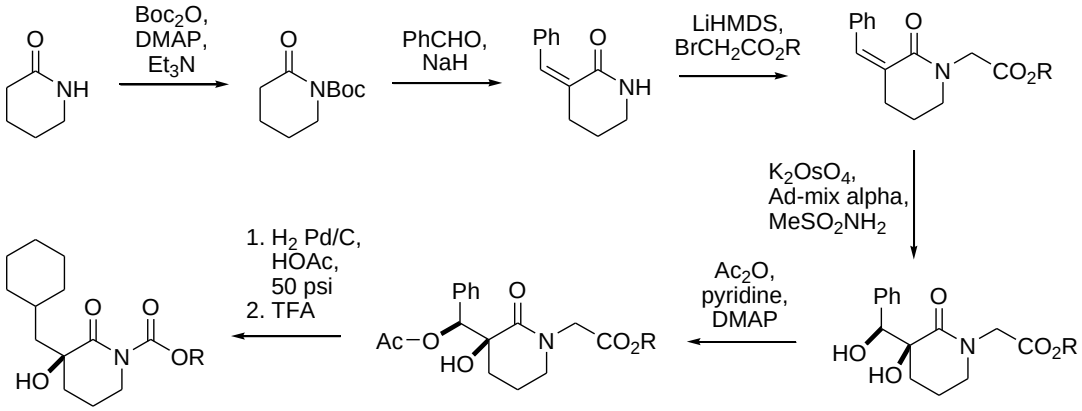
Skeletal backbone substitutions of lactams are generally established prior to or during lactam formation. However, addition to the lactam framework is possible through a handful of functional group transformations. The lactam scaffold frequently used for derivatization is an α,β-unsaturated lactam, because they undergo nucleophilic additions at the 4 position, electrophilic addition at the 3 position, and cycloaddition reactions.[166] The problem with α,β-unsaturated lactams is their inability to make the desired $C^{\alpha,\alpha}$-disubstituted γ/δ-lactam. Another method reported by Oguri et al. followed a Curtius rearrangement of a

carboxylic acid and deprotection of carbamate to form amino lactams. The α,α-disubstituted lactams were later used as natural product scaffolds (Scheme 26).[141]



Scheme 26. Oguri et al. method of functionalizing lactams.

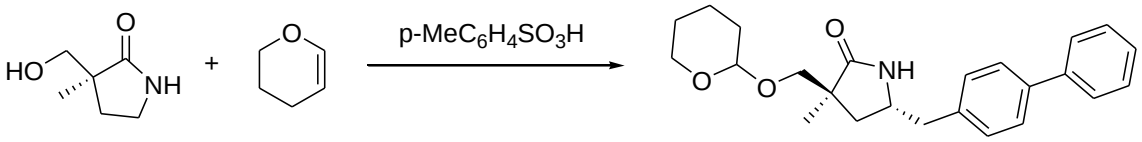
Minami et al. reported an asymmetric synthesis of α,α-hydroxy δ-lactam via a dihydroxylation to serve as D-Phe-L-Pro surrogates (Scheme 27).[167] While not a direct example of functionalizing α,α-disubstituted lactams, chiral disubstituted lactams were formed in the process. Moreover, addition across the double bond represents a standard method for adding chemical complexities.



Scheme 27. Minami et al. strategy to prepare α,α-disubstituted δ-lactams.

Recently Fleury reported the synthesis of a neprilysin inhibitor via an addition of dihydropyran to an alcohol (Scheme 28).[136,137] Tetrahydropyranyl ethers are general used as acid labile protecting groups of alcohols. Fleury's example represents one of the few instances where addition occurs adjacent to a sterically congested stereocenter. The lack of literature precedence to

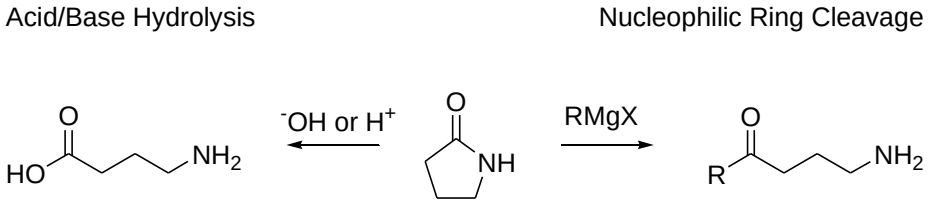
functionalize α,α-disubstituted lactams makes the development of synthon **17** worthy of exploration.



Scheme 28. Fleury et al. neprilysin inhibitor intermediate.

Ring opening of lactams

Ring opening of lactams is a common practice previously used in the preparation of UAAs,[168-172] GABA,[173,174] Daptomycin,[175] and Tubulysin analogues.[77] Lactam ring opening is also used in the controlled released drug delivery of polyvinylpyrolidone-drug conjugates.[176] There are two different strategies commonly used to open lactams depending on the desired product. These strategies are acid/base hydrolysis,[177,178] and/or nucleophilic ring cleavage (Scheme 29).[170,179-181] Early studies showed that hydrolysis of unprotected lactams undergo ring opening under harsh acidic/basic reflux. Overtime it was found that activation of the amide with electron deficient carbamates would allow for milder reaction conditions.[182] Nucleophilic ring cleavage is also a possible pathway.



Scheme 29. Ring opening strategies of lactams.

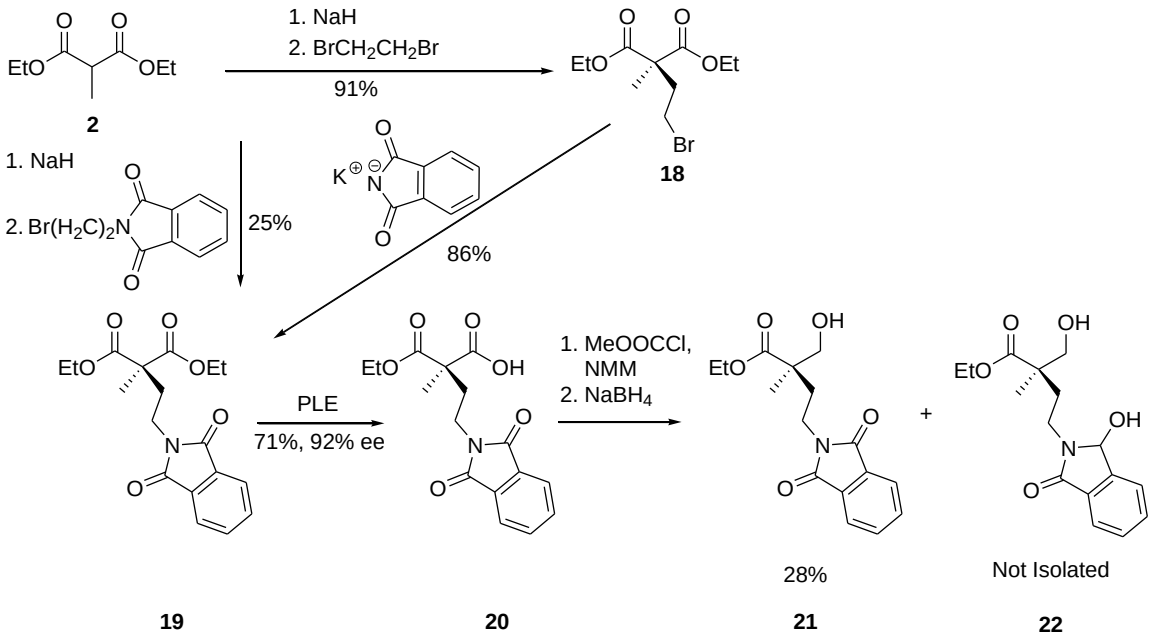
The above discussion outlined the use of lactams in the synthesis of natural products. Additionally, ring opening of lactams into biomolecules is well

documented and a common method to prepare both γ/δ-UAAs. However, a large gap remains in the ability to quickly convert chiral disubstituted lactams into other useful functionalized derivatives conveniently.

Results and Discussion

Synthesis of γ-backbone following a 2-carbon phthalimide strategy

A different strategy was envisioned prior to preparing **17** as a common synthon. Both strategies used diethyl 2-methyl-2-(2-(1,3-dioxoisoindolin-2-yl)ethyl)malonate,**19**, as a basis to establish the γ-backbone but lactam cyclization was not originally part of the plan (Scheme 30). First, an enolate was formed with diethyl methyl malonate, and reacted with 1,2-dibromoethane to form **18** after distillation (91% yield). Diester **18** was heated to 90 °C with potassium phthalimide using Gabriel's conditions and **19** isolated in good yield (86% after recrystallization). Alternatively, **19** could be prepared in a one-step synthesis directly from diethyl methyl malonate and 2-(2-bromoethyl)isoindoline-1,3-dione. However, reaction yields were low (25% yield) due to an elimination occurring on the side chain. Under these conditions, a two-step Gabriel's reaction was more efficient (79% overall yield). The prochiral diester was hydrolyzed with PLE and **20** was isolated (71% yield and 92% *ee*).[130]



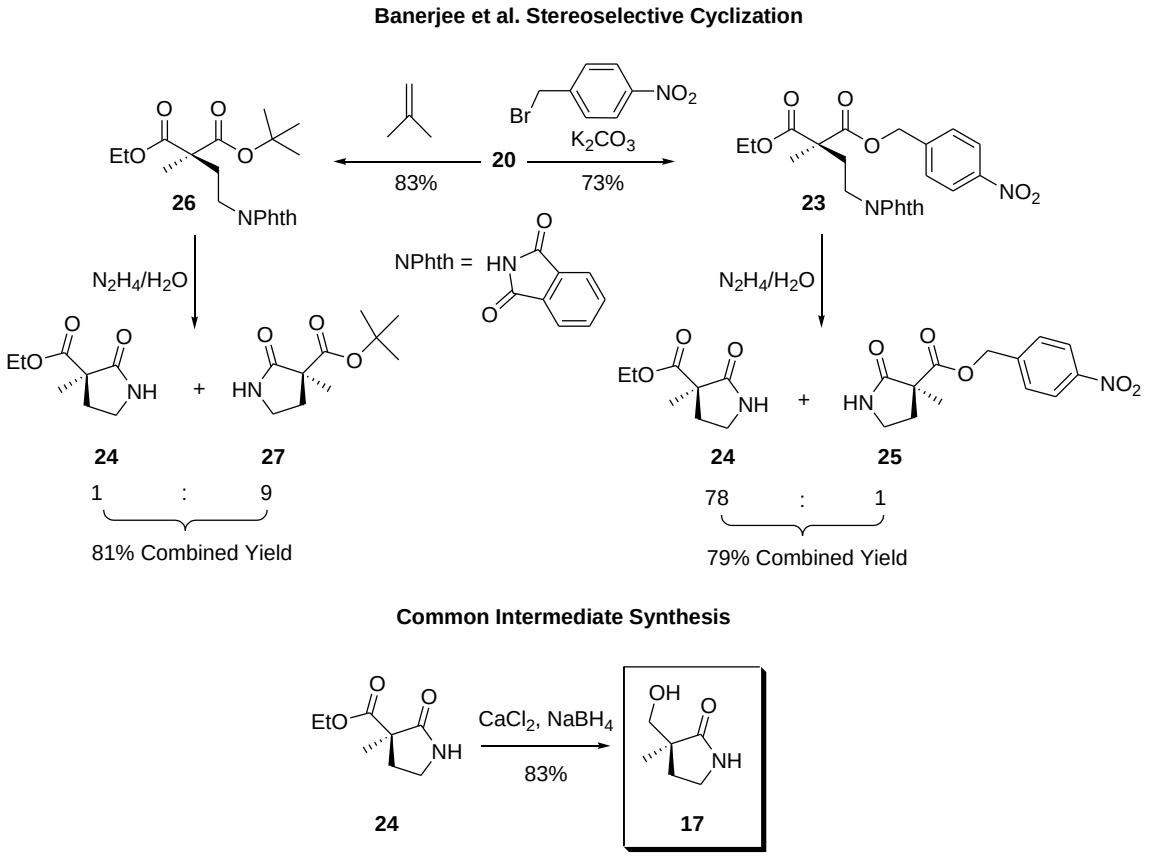
Scheme 30. Synthesis of γ-UAAs from (*R*)-2(ethoxycarbonyl)-2-methyl-4-(1,3-dioxoisooindolin-2-yl)butanoic acid.

The reduction of **21** was a challenge because all attempts led to over reduced phthalimide **22**. Initially, methyl chloroformate and triethylamine were used to form the mixed anhydride and reduced with sodium borohydride. However, only 10% of product was isolated and over reduced **22** dominated the reaction. The reduction was repeated varying reaction conditions by controlling the temperature, isolating the triethylammonium salts formed prior to reduction, and changing the number of equivalents of sodium borohydride used. None of these parameters increased product yields. Upon literature review, a patent was found using a similar mixed anhydride protocol to successful in reduce (*S*)-3-((benzyloxy)carbonyl)-2-(1,3-dioxoisoindolin-2-yl)proppanoic acid.[183] This procedure was intriguing because it required a less nucleophilic base N-methylmorpholine (NMM), a solution of sodium borohydride in water, and a reaction time of less than 5-minutes. These conditions were replicated with **20**

and increased the overall yield to 28%. Unfortunately, the side product **22** was still present, and minimizing reaction times to 2-minutes failed to produce the desired compound.

Synthesis of common intermediate 3-(hydroxymethyl)-3-methyl-pyrrolidone *via a stereoselective cyclization strategy*

Previously, the Masterson Research Group reported the stereoselective synthesis of γ-lactams from PLE resolved carboxylic acid **20** with an electron withdrawing ester (Scheme 31).[130] The activated ester was cyclized following deprotection of phthalimide and nucleophilic attack of the free amine toward the more electrophilic ester carbonyl. Hammett plot studies indicated substrate **23** containing a *p*-nitrobenzyl ester provided the best cyclized selectivity. After cyclization ethyl ester **24** was isolated over the *p*-nitrobenzyl ester **25** in a 78:1 ratio. This strategy is used as the foundation to prepare common intermediate **17**. The opposite amino acid enantiomer could be prepared by switching the electron withdrawing ester to a sterically hindered *tert*-buty ester. This time stereoselective cyclization to the least hindered ester carbonyl was observed.[130] In order to prepare *tert*-butyl ester **26** isobutylene is added to **20** under acid catalyzed conditions. After phthalimide deprotection, the amine attacks the carbonyl of the ethyl ester forming *tert*-butyl ester **27** over ethyl ester **24** in a 9:1 ratio. Since the ability to form both enantiomers had already been established, the synthesis using the bulky *tert*-butyl group was not carried out. Synthesis of intermediate **17** was reduced to **24** following an in situ generation of calcium borohydride. After purification **17** was isolated in 83% yield.

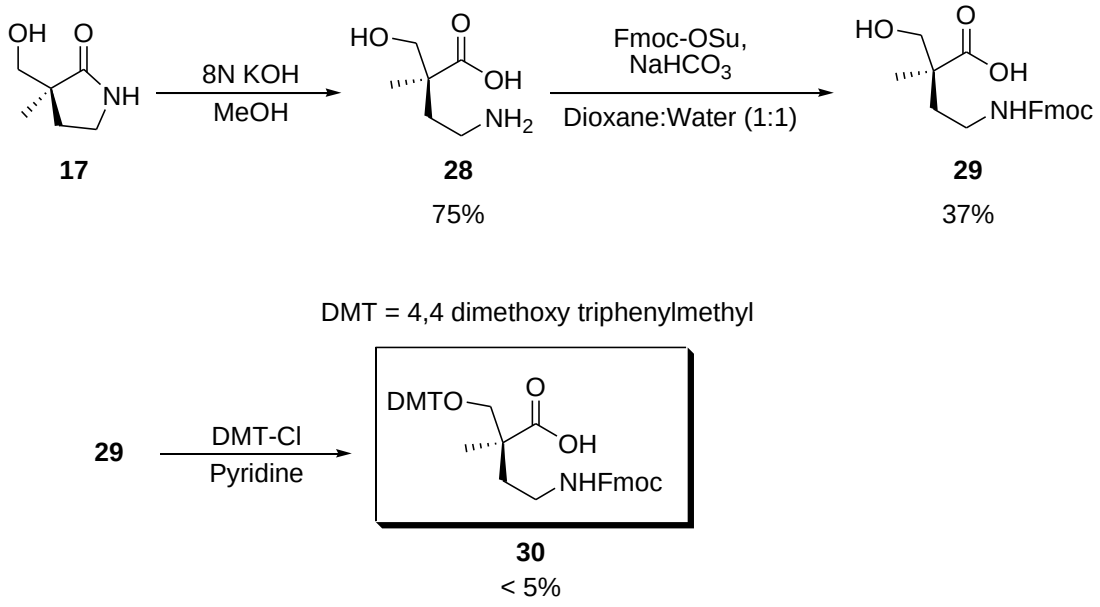


Scheme 31. Cyclization strategy outline by Banerjee et al. and reduction to the lactam alcohol common synthon.

Synthesis of Fmoc/DMT-γ-serine analogue

A three-step synthesis from **17** was designed to prepare a serine analogue (Scheme 32). A solution of 8.0 N KOH ring opened the γ-lactam alcohol to **28** via a hydrolysis. The harsh basic conditions and the polarity of the newly formed amino acid made isolating the compound from water and potassium salts difficult. In order to make the synthesis more appealing to peptide chemists the amino group of **28** was protected with 9-fluorenylmethyloxycarbonyl (Fmoc. After the addition of Fmoc-Succinimide, **29** was formed (37% isolated yield) and protected with 4,4 dimethoxy triphenymethyl chloride to **30.** However, the compound deprotected while purifying **30** on a C18

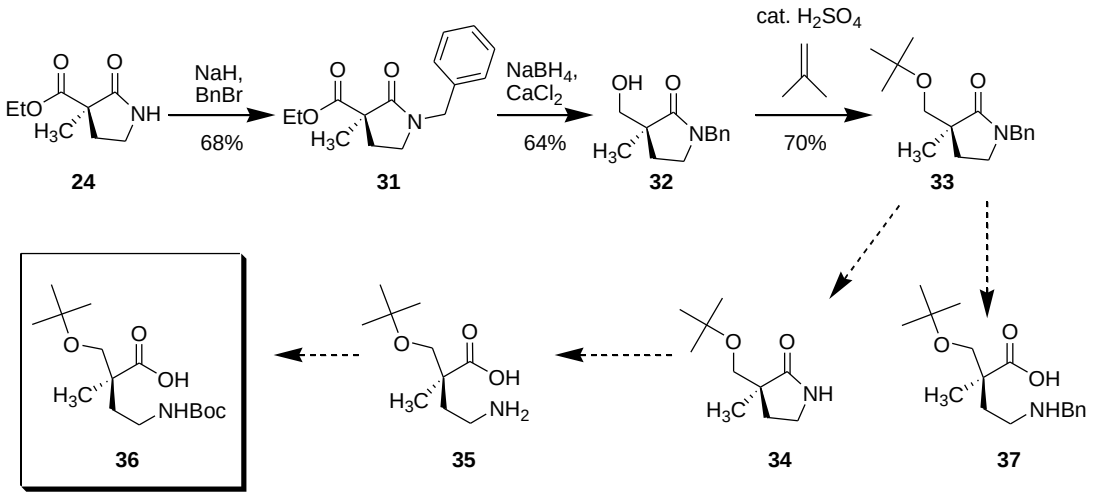
HPLC column, and less than 5% of compound was isolated. While this method was capable of producing the desired analogue, the end product was too unstable and the overall synthesis-inefficient (<1% combined reaction yields). These results prompted an alternative strategy in which isolation of intermediate products was easier and stable toward Solid phase peptide synthesis (SPPS) conditions.



Scheme 32. Fmoc protected synthesis of γ-serine.

Attempted synthesis of boc/t-butyl protected of γ-serine via benzylation strategy

Since ring opening to unprotected **28** was inconvenient substrate to manipulate, a lactam/alcohol protection strategy was designed. Previously, Banerjee et al. observed increased organic solubility, easier purifications, and higher reaction yields by inserting a benzyl group onto the amide of α-methyl-β-proline.[130] This benzyl protection strategy was applied **24** in the synthesis of **36** (Scheme 33).



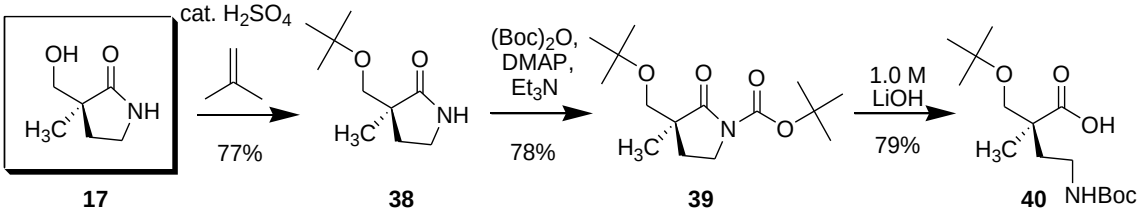
Scheme 33. Benzyl protection strategy for the synthesis of Boc/*t*-butyl γ-serine.

Starting from **24**, benzyl bromide was added under basic conditions to protect the amide to **31** and reduced to **32** with calcium borohydride. Isobutylene was used to *tert*-butyl protect the alcohol to **33**. At this point the anticipated strategy was to deprotect the benzyl group to **34**, ring open to the free amine **35** , and N-boc protect to **36**. However, the removal of the benzyl group under standard palladium on carbon reduction was not sufficient. Classic Bouveault Blanc reduction with sodium metal also was unsuccessful. Initially, these results were surprising but according to literature amide N-debenzylation of lactams are notorious for withstanding reduction conditions.[184] Successful N-debenzylations only occurred with limited successes under extreme conditions using Birch reductions (Na/NH_3),[185] napthalene catalyzed lithiations,[186] or carbanion-mediated oxidative deprotections with butyllithium.[187] Since these methods were beyond the labs capabilities and are inconvenient, the reduction was deferred until after ring opening. However, a hydrolysis to **37** in refluxing 8.0 N KOH did not produce the desired compound. These results provided insight into the

electronic factors dictating the ring opening of the lactam. Since the benzyl group acts by donating electron density into the lactam, the carbonyl is deactivated toward hydrolysis. In order to activate the system, a protecting group capable of withdrawing electron density away from the amide nitrogen was needed to increase the electrophilicity of the carbonyl. This would make the carbonyl carbon electrophilic increasing the rate of lactam hydrolysis. This new strategy was applied and results are described below.

Synthesis of N-boc/tert-butyl protected γ-serine analogue

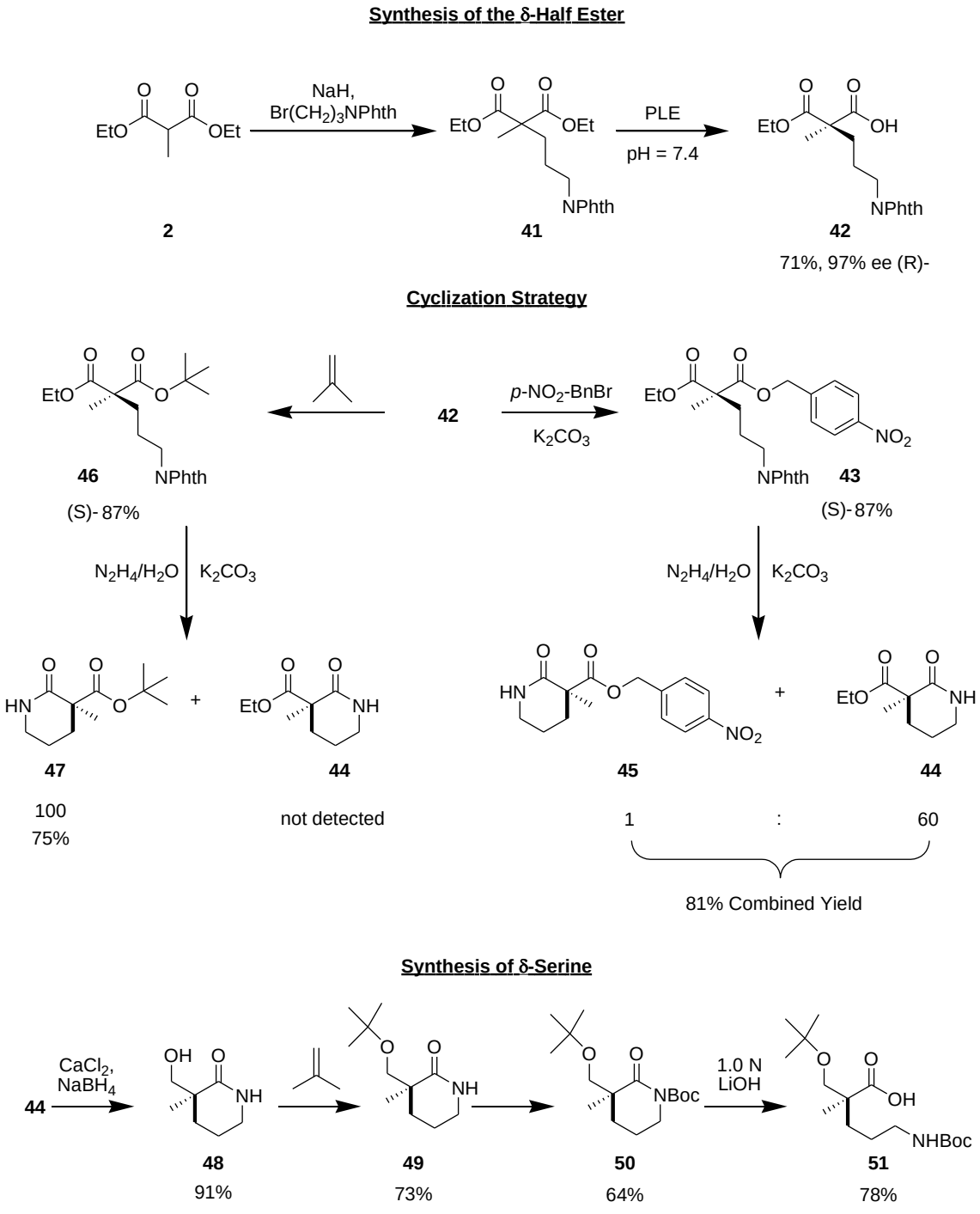
The synthesis of N-boc protected lactam was developed after noting the N-benzyl group deactivated the system (Scheme 34). In order to prepare the γ-serine analogue, common intermediate **17** was *tert*-butyl protected with a catalytic amount of sulfuric acid and condensed isobutylene. This formed protected alcohol **38** in good yield (77 % isolated yield) which was reacted with di-*tert*-butyl dicarbonate to N-boc protected **39**. The activated lactam was hydrolyzed using 1.0 M LiOH at room temperature where after a short workup pure **40** was isolated. Over three steps the (*S*)-α-methyl-α-*tert*-butyl serine γ-UAA was prepared in a 47% yield. Since the analogue was also prepared from the stereoselective PLE cyclized the opposite enantiomer could be synthesized.



Scheme 34. Synthesis of α-methyl-γ-serine using the N-boc/*tert*-butyl strategy**.**

Synthesis of N-boc/t-butyl protected δ-serine analogue

Shortly after the discovery of the cyclization strategy the same strategy was applied to diester **41**. Addition of the phthalimide side chain to diethyl methyl malonate formed **41**, and was PLE hydrolyzed to **42** (97% ee, *R*) isolating the half ester in 71% yield. To the enantiomerically enriched chiral half ester *p*-nitrobenzyl bromide was added under basic K_2CO_3 conditions to form the activated ester **43** in 87% yield. After phthalimide deprotection, the free amine formed, attacked the electronic deficient carbonyl, and cyclized to **44** selectively over the *p*-nitrobenzyl ester **45** in a 60:1 ratio. However, unlike the γ- lactam, the formation of the δ-lactam required the removal of the formed phthalhydrazide and the addition K_2CO_3 to assist in the cyclization process. Once again, the opposite enantiomer can be prepared by converting to *tert*-butyl ester **46** and cyclizing to **47** as the only isolated product.

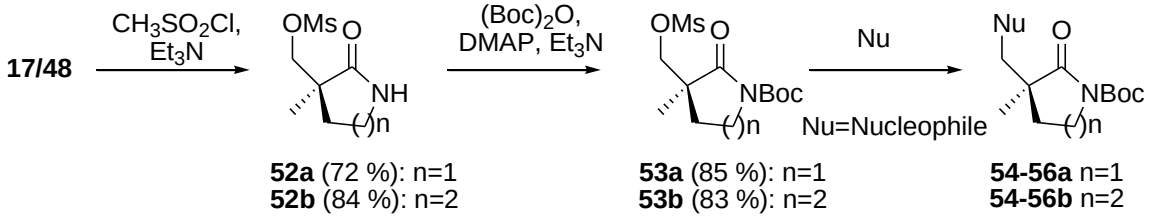


Scheme 35. Synthesis of α-methyl-δ-serine.

Synthesis of N-boc γ/δ-unnatural amino acids via S_N2 substitution

At this point, the same series of reactions used to prepare the γ-serine amino acid were applied to the synthesis of the δ-serine analogue. The common

synthon was prepared by the reduction of the lactam to **48** (91% yield), and then protected to *tert*-butyl ether **49**. This compound was boc-protected to **50**, and ring opened to **51** with LiOH isolating δ-serine in 78% yield. The three step reaction from intermediate **48** proceeded in a modest 36% overall yield.



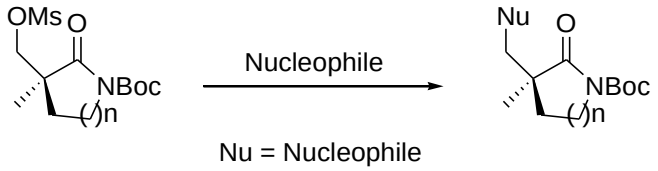
Scheme 36. Synthesis of γ/δ-UAAs using S_N2 chemistry.

In order to make common synthons **17**/**48** versatile, substitution (S_N2) chemistries were applied (Scheme 36). First, the alcohol groups of **17**/**48** were converted into good leaving groups with treatment of methanesulfonyl chloride to **52**, and the amides were N-boc-protected to **53** in good yields. At this point **53a** and **53b** were used to explore S_N2 chemistries with small nucleophiles (Table 1). Traditionally, S_N2 chemistry requires primary substrates with unhindered access to the carbon's anti-bonding molecular orbitals adjacent to the leaving group. Any steric congestion near the site of substitution limits the rate of reaction, but does not necessarily prevent substitution. Since **53a**/**53b** are neopentyl, they were not expected to behave as typical primary substrates. Instead, the quaternary centers would limit the nucleophiles approach and reaction rates were expected to be slow. Therefore, S_N2 studies on substrates **53a**/**53b** were explored using small, linear, and strong nucleophiles to minimize steric interactions.

Recently, amino acids incorporating azides and alkynes as side chains have been used to tether peptides together to form stabilized secondary structures.[188,189] Therefore, the first nucelophiles tested were azides and acetylides which undergo “click” chemistry together to form 1,2,3-triazoles and and staple peptides together. In a sealed tube, **53a/53b** was added to sodium azide (2 equiv.) and monitored by both TLC, and ^{1}H-NMR. The reaction was complete in 5-days. After purification, **54a** was isolated in 68% yield and **54b** was isolated in 46% yield. Due to the higher yields observed with the γ-lactam, it was used optimize all other nucleophilic substitution conditions.

Table 1

Small linear nucleophilic substitution of the γ/δ-mesylate intermediates.



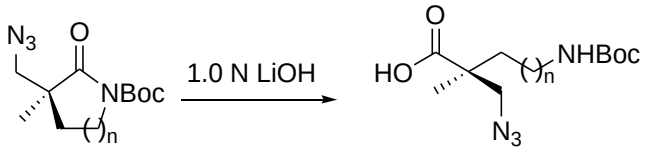
53a n=1
53b n=2

54-56a n=1
54-56b n=2

Entry	Reactant	Nu	Product	Time (h)	Nucleophile (mol equiv.)	Solvent	Yields (%)
1	**53a**	-N_3	**54a**	120	2	DMF	68
2	**53b**	-N_3	**54b**	120	2	DMF	46
3	**53a**	-CCH	**55a**	72	2.5	DMSO	ND
4	**53a**	-CN	**56a**	120	1.1	DMSO	ND
5	**53a**	-CN	**56a**	120	1.1	DMF	ND
6	**53a**	-CN	**56a**	120	4	DMF	ND

A solution of lithium acetylide, ethylenediamine complex was used to synthesize the alkynyl derivative **55a**. After 72 hours **55a** was not detected. Since the acetylide anion is both a good nucleophile and base these results were unexpected. The inability to form **55a** was attributed to the ethylenediamine complex added to prevent polymerization. Unfortunately, the in situ generation of acetylide required costly equipment not available to our lab and potassium cyanide was considered as an alternative nucleophile.

A few bimolecular applications examples had shown the "clickable" nature of azides with nitriles to form tetrazoles.[188,190] Thus, substitution of a nitrile group could serve as both a small linear nucleophile and peptide staple. However, reaction of potassium cyanide with **53a** produced no observable detection of **56a**. A series of reactions optimizations were attempted, but change of solvent (Entry 5), additional mole equivalents of nucleophile (Entry 6), and increased temperature produced the same results. Despite the lack of functionalization with small linear nucleophiles the substituted azide was successfully ring opened (Scheme 37) to amino acids **57** under mild basic conditions.



53a n=1
53b n=2

57a n=1 (68%)
57b n=2 (88%)

Scheme 37. Synthesis of γ- and δ-azido UAAs.

In order to rationalize the observed results, x-ray quality crystals of **53a** and **53b** were grown and sent off to Dr. Douglas Powell. The returned CIF files were analyzed in Spartan '08 molecular modeling program (Figure 9 and Figure

10). Evident from the x-ray crystals was a sterically hindered carbon adjacent to the methylsulfonate leaving group. Since steric hindrance was already considered as one of the factors limiting the reaction rate, an energy profile modeling the associated energies was calculated as it was rotated about a 360° dihedral angle. Each data point reflects the calculated lowest energy conformation and associated 3D gas phase model of the molecule at the particular dihedral angle. The energy of these models was plotted against the dihedral angle (degree of rotation) for each of the calculated models. The lowest and highest energy 3D models were extracted and used as a qualitative assessment to compare the relative hindrance toward an approaching nucleophile. Evident in the lowest energy models of both the γ- and δ-lactams was the obstructed approach to the nucleophile. Interestingly, the highest energy models, in which steric hindrance is minimized, make these conformation better suited for nucleophilic substitution. Since both the higher energy conformations exhibit unhindered access to the anti-bonding molecular orbital's nucleophilic substitutions is not completely excluded. Instead, the substitution reactions may require increased energy or longer reaction times.

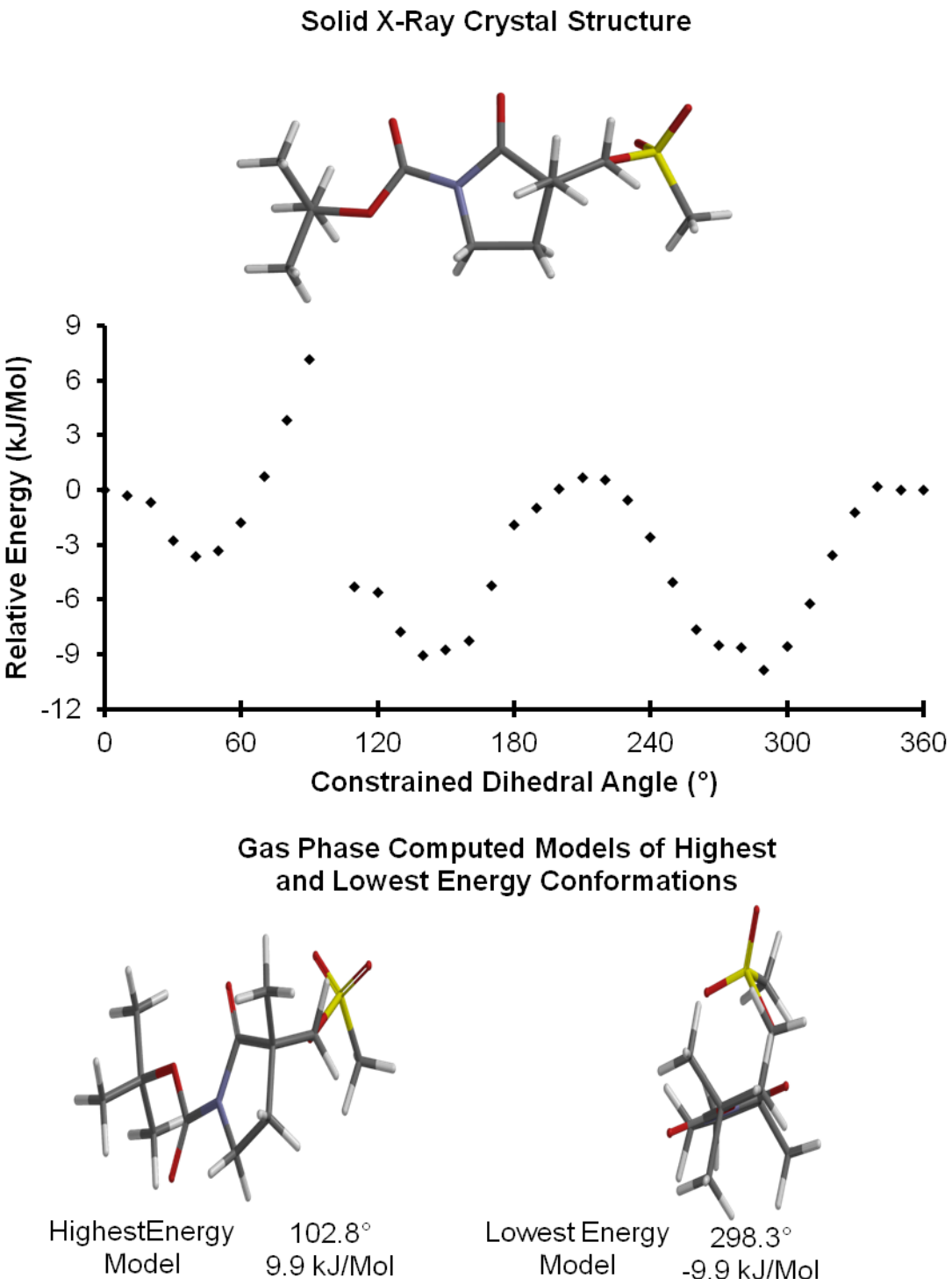


Figure 9. Conformational energies associated with the geometry optimized computed models of the constrained dihedral angles of **53a**.

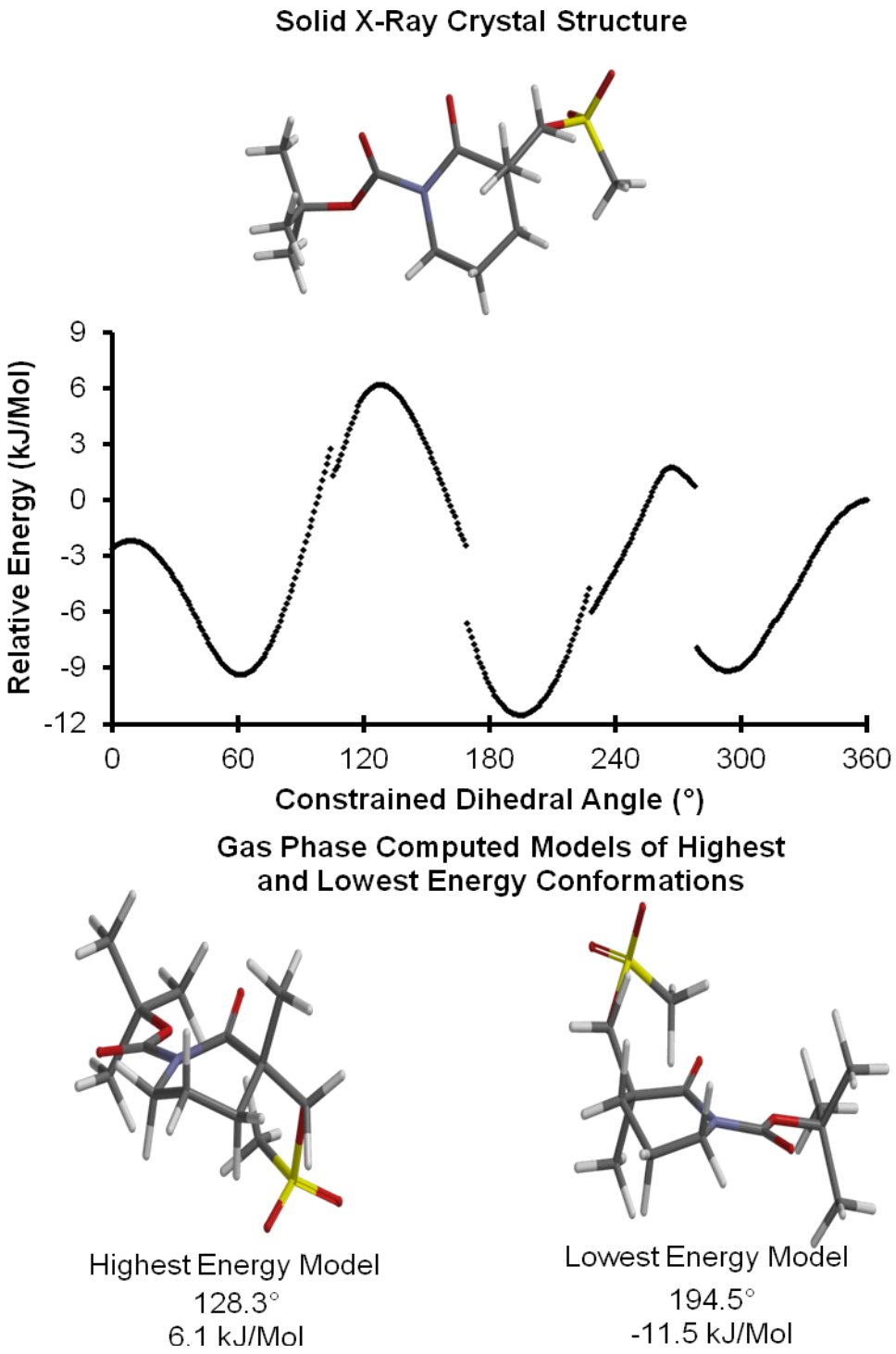
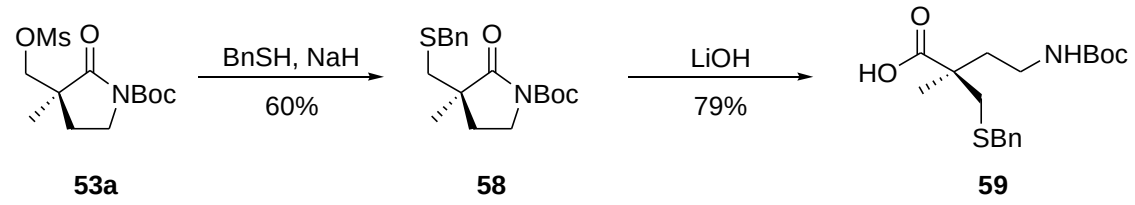


Figure 10. Conformational energies associated with the geometry optimized computed models of the constrained dihedral angles of **53b**.

As a final challenge to the mesylate intermediate, a cysteine analogue was proposed (Scheme 38). In contrast to the small linear nucleophiles studied,

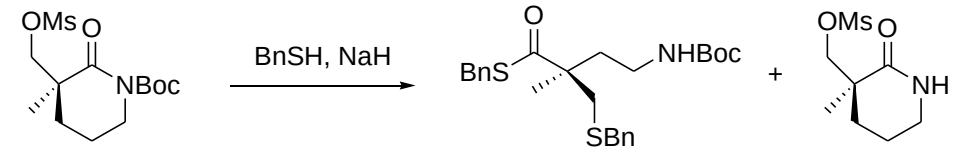
cytsteine's sulfur atom is bigger, polarizable, and a weak base. The γ-cysteine derivative was synthesized by adding sodium hydride to benzyl mercaptan and reacting the sulfur anion with mesylate intermediate **53a**. Surprisingly, all starting material disappeared within 6 hours and **58a** isolated after purification. The thiol lactam was ring opened under mild 1.0 N LiOH to provide **59** in 79% yield. The same procedure was repeated with the δ-lactam, but the result was not the same. Instead, excess benzyl mercaptan displaced the mesylate and attacked the lactam carbonyl forming a ring opened thioester **60** as a minor product**.** After purification, N-boc deprotected lactam **61** was isolated as the major component. Overall, the reaction was low yielding (37%). The thioester initially was hydrolyzed with K_2CO_3; however, it was not basic enough to promote the reaction, therefore solid KOH was added to the solution. This promoted the hydrolysis to **62** where it was isolated in a 65% yield.

Synthesis of γ-cysteine

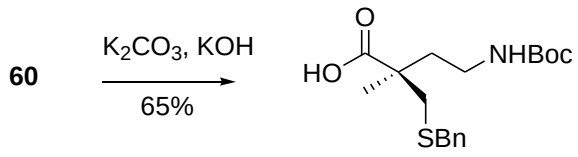


53a **58** **59**

Synthesis of δ-cysteine



53b **60** 37% **61** 47%



62

Scheme 38. Synthesis of γ/δ-cysteine UAAs.

Summary of the common synthon to prepare γ/δ-UAAs

The synthesis of intermediates **17**/**48** were easily prepared from the stereoselective cyclization and reduction strategy. The three-step process to synthesize γ/δ-serine-UAAs and the four step synthesis to synthesize γ/δ-azido-, and γ/δ-cysteine UAAs were isolated in reasonable overall yields (Figure 11). The highest overall yields were found during the synthesis of the γ/δ-serine analogues. The three-step synthesis benefited from being two atom away from the quaternary center and the unnecessary need to form the mesylate intermediate. Both serine analogues were isolated as (*S*)-enantiomers where γ-serine was isolated in 54% and δ-serine in 36% overall yields. The additional

step and increased time needed to prepare the azido analogues led to diminished yields but isolations of both γ/δ-azido(*S*)-enantiomers occurred in 28% overall yields. At this time, it is still unclear as to why the cysteine analogues proceeded through different pathways. Regardless both pathways led to the (*R*)-enantiomers of the desired cysteine compounds where γ-cysteine was made in 29% and δ-cysteine was prepared in 15% overall yields. All of the synthesized α-methyl γ/δ-amino acids are novel and having been prepared from PLE are enantiomerically enriched. Finally, the stereoselective cyclization to the lactams esters makes this process enantiodivergent.

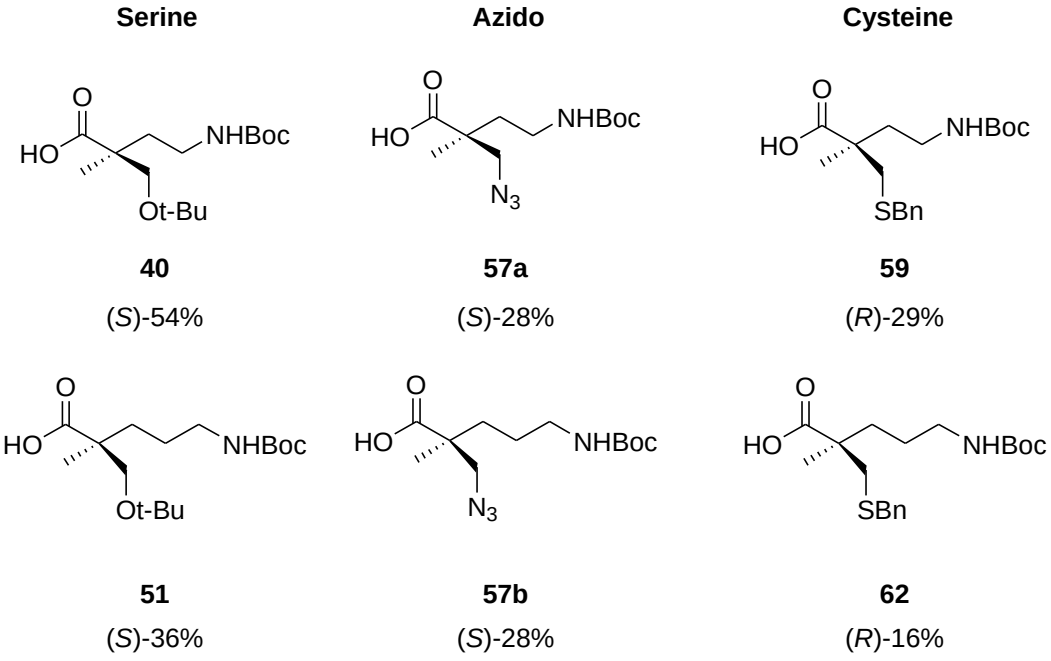


Figure 11. Overall yields from the common synthon to prepare γ/δ-serine, -azido, and -cysteine UAAs.

Future Directions

There is still considerable work needed to optimize the lactams for use as a common synthon. Alternative pathways to functionalize the lactams via the Williamson ether synthesis and conversion to an aldehyde have been attempted,

but isolation of either product has not been successful. Repeated attempts and alternative methods are needed before eliminating either of these pathways.

Improvements to the S_N2 substitution chemistry

Additional work is still needed to elucidate all factors governing the S_N2 chemistry with the mesylate lactams. Further exploration using nucleophiles of different sizes and strengths will be considered. The long reaction times for the linear nucleophiles and short reaction times with the thiol nucleophile contradict expected results. Moreover, carbon nucleophiles, such as the acetylides and cyanides, were completely unreactive whereas heteroatom nucleophiles of the azides and thiols worked well. Thus a series of heteroatomic and carbon nucleophiles containing both bulky and linear components would help establish the factors governing the nucleophilic substitution.

The difference in reactivates between the γ- and δ-cysteine is also puzzling. Both mesylate intermediates appear to be equally hindered from the conformation modeling studies. Repeated attempts to replicate the nucleophilic attack at the carbonyl will be performed. In addition, a comparison of the reactivates observed with different leaving groups, such as a series of sulfonate esters (mesylate, tosylates, triflates), would also be beneficial.

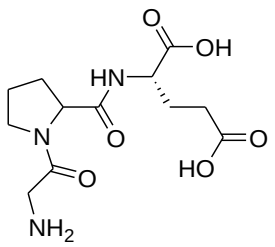
Potential applications and uses for the synthesized γ/δ-UAAs analogues

All of the synthesized γ/δ-UAAs are valuable analogues which could easily be studied as peptidomimetic, pharmaceutical, or foldamers. The cysteine analogues could be used to form disulfide bonds stabilizing β-hairpins. The

serine analogues could be studied as potential GABA, GABOB, and statine derived analogues.

The azides ability to undergo functionalization into triazoles and tetrazoles makes amino acids containing azido functional groups valuable synthetic intermediates. Recently Hung et al. reported the synthesis of two tripeptide peptidomimetics of insulin like growth factor I with amino acid sequence GPE.[190] The synthesized tripeptides replaced one of the carboxylic acids of glutamic acid with a tetrazole moiety. The result was a tetrazole substituted γ-glutatmic acid derivative at both α- and γ-positions (Figure 12). Since the tetrazole moiety serves as a carboxylic acid surrogate and improves metabolic stability, it would be interesting to see if C^{α}-methyl-α-azido-γ- and δ-analogues have the same structural activity relationship.

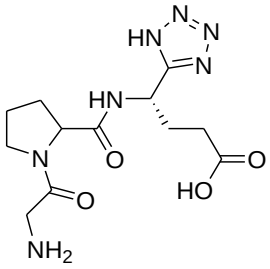
Insulin-like growth factor I



Glycyl-L-prolyl-L-glutamic acid

GPE

α-tetrazole



γ-tetrazole

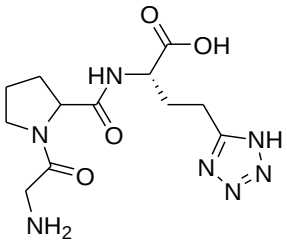


Figure 12. Insulin like growth factor I peptide mimics synthesized by Hung et al.

Conclusions

Initial studies conducted prior to cyclization of the γ-backbone resulted in an uncontrollable reduction of phthalimide. These results prompted the investigation into the phthalimide deprotection/cyclization strategy where γ- and

δ-lactams are formed stereoselectively and reduced to 3-(methylhydroxy)-3-methyl-γ/δ-lactam intermediates. The inconvenience of ring opening an unprotected γ-lactam led to the development two different lactam protection strategies. First, N-benzyl lactams were utilized, but difficulties removing the benzyl group led to the implementation of a N-boc protecting strategy. N-boc protection of the lactams proved to be an essential to ring opening the synthons into protected serine, azido, and cysteine γ/δ-UAAs. The limitation imposed by the sterically hindered neopentyl nature of the mesylate substrates severely limits the general application toward S_N2 chemistries. Future work will focus on exploring other methods of functionalizing the synthons and incorporating the synthesized amino acids into relevant biomolecules.

Experimental

*(S)-3-(hydroxymethyl)-3-methyl pyrrolidin-2-one (**17**):*

In a flask, **24** (2.92 mmol, 0.500 g) was dissolved into 10 mL of MeOH followed by $CaCl_2$ (3.5 mmol, 0.388 g). The solution was placed on an ice bath at 0 °C, under N_2 atmosphere while solid $NaBH_4$ (3.5 mmol, 0.132 g) was added in 3 equal portions over 30 min. The reaction stirred at 0 °C for 2 h, warmed to rt for 12 h, and quenched with 6 M HCl until a pH=2.0. The formed solids were filtered over a Buchner funnel and discarded. The filtrate was concentrated, dissolved into MeOH, and triturated with CH_2Cl_2 until no solids fell out of solution. The solids were isolated and the solution concentrated. The crude material was purified on a silica gel column eluted with 5:95 MeOH:CH_2Cl_2 (R_f = 0.33,10:90 MeOH:CH_2Cl_2). After purification, 0.312 g of **17** was isolated as a solid (2.42

mmol, 83%). $[\alpha]_D^{22}$ = - 5.8 (c =1, MeOH). mp = 122 ˚C. IR (cm^{-1}) = 3325 br, 3235, 3962, 2862, 1648. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 6.26 (s, 1H), 3.68 (dd, *J* = 10.9, 7.2 Hz, 1H), 3.54 (dd, *J* = 10.9, 4.6 Hz, 1H), 3.37 (ddd, *J* = 5.3, 4.0, 1.9 Hz, 2H), 2.93 (s, 1H), 2.24 (dt, *J* = 12.7, 8.1 Hz, 1H), 1.85 (ddd, *J* = 12.4, 6.9, 4.6 Hz, 1H), 1.19 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 182.21, 67.85, 44.59, 39.13, 31.20, 19.53. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_6H_{11}NO_2Na$ 152.1; Found 152.1.

*Diethyl 2-(2-bromoethyl)-2-methylmalonate (**18**):*

The title compound was prepared according to reported literature procedures, and confirmed with characterization data.[130,191]

*Diethyl 2-methyl-2-(2(1,3-dioxoisoindolin-2-yl)ethyl)malonate (**19**):*

The title compound was first synthesized according to reported literature procedures.[80] A more efficient method was discovered shortly after, and all isolated material was confirmed with reported characterization data.[130]

*(R)-2-(ethoxycarbonyl)-2-methyl-4-(1,3-dioxoisoindolin-2-yl)butanoic acid (**20**):*

The title compound was prepared according to reported literature procedures, and confirmed with characterized data.[80,130]

*(R)-ethyl-2-(hydroxymethyl)-2-methyl-4-(1,3-dioxoisoindolin-2-yl)butanoate (**21**):*

In a 3-neck round bottom flask under N_2 atmosphere **20** (1.56 mmol, 0.500 g) was dissolved into 7.7 mL of dry THF. The solution was cooled to -15 °C, N-methylmorpholine (1.56 mmol, 1.72 mL) added, followed by methyl chloroformate (1.56 mmol, 0.121 mL). After 5 min of stirring $NaBH_4$ (4.68 mmol, 0.177 g) dissolved in 0.71 mL of water was added via syringe, stirred for 2.5 min, and

quenched with 8.0 mL of MeOH. The crude material was suspended into CH_2Cl_2, filtered over a pasture pipette packed with glass wool, and concentrated. A column packed with 4:96 MeOH:CH_2Cl_2 eluted 0.138 g of **21** (0.451mmol, 28% yield) as pure oil. R_f = 0.54 (4:96 MeOH:CH_2Cl_2) $[\alpha]_D^{23}$ = -0.82 (c =2, CH_2Cl_2). IR (cm^{-1}) = 3499 br, 2979, 2940, 1770, 1701. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.87 (m, 2H), 7.74 (m, 2H), 4.22 (m, 2H), 3.82 (m, 4H), 2.45 (t, *J* = 6.8 Hz, 1H), 2.06 (m, 2H), 1.31 (t, *J* = 7.1 Hz, 3H), 1.27 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 176.0, 168.1, 133.9, 132.1, 123.2, 67.9, 61.1, 46.5, 34.0, 33.9, 19.8, 14.1. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{16}H_{19}NO_5Na$ 328.1; Found 328.1.

*(S)-1-(4-nitrobenzyl) 3-ethyl 2-methyl-2-(2-(1,3-dioxoisoindolin-2-yl)ethyl)malonate (**23**):*

The title compound was prepared according to reported literature procedures, and confirmed with characterization data.[130]

*(R)-ethyl 3-methyl-2-oxopyrrolidine-3-carboxylate (**24**):*

The title compound was prepared according to reported literature procedures, and confirmed with characterization data.[130]

*(S)-4-nitrobenzyl 3-methyl-2-oxopyrrolidine-3-carboxylate (**25**):*

The title compound was prepared according to reported literature procedures, and confirmed with characterization data.[130]

*(S)-1-tert-butyl 3-ethyl 2-methyl-2-(2-(1,3-dioxoisoindolin-2-yl)ethyl)malonate (**26**):*

The title compound can be synthesized according to reported literature procedure.[130]

*(S)-tert-butyl 3-methyl-2-oxopyrrolidine-3-carboxylate (**27**):*

The title compound can be synthesized according to reported literature procedure.[130]

*(S)-4-amino-2-(hydroxymethyl)-2-methylbutanoic acid (**28**):*

In a flask containing **17** (2.42 mmol, 312 mg) dissolved into 8 mL of MeOH was added 15 mL of 8 N KOH, and brought to a reflux. After 16h the reaction was acidified to pH 7.0 with 4N HCl. The salts were filtered off and the filtrate concentrated under reduced pressure. The crude material was triturated with 2:1 MeOH:CH_2Cl_2 solution, washed with pentanes, and left under vacuum for 24 h with concentrated H_2SO_4. After drying 0.270 g of **28** (1.83 mmol, 75%) was isolated. R_f = 0.01 (10:90 MeOH:CH_2Cl_2). $[\alpha]_D^{23}$ = +10.8 (c =1, MeOH). IR (cm^{-1}) = 3287 br, 2929, 2872, 1664. ^{1}H-NMR (MeOD, 400 MHz): COOH, NH, & OH peaks not present δ 3.51 (q, *J* = 10.7 Hz, 2H), 2.67 (t, *J* = 8.0 Hz, 2H), 1.79 – 1.69 (m, 1H), 1.68 – 1.58 (m, 1H), 1.10 (s, 3H). ^{13}C-NMR (MeOD, 100 MHz): 183.25, 68.46, 46.64, 39.88, 37.60, 20.27. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_6H_{11}NO_3Na$ 168.1; Found 168.1.

*(R)-4-[[(9H-fluoren-9-ylmethoxy)carbonyl]amino]-2-(hydroxymethyl)-2-methylbutanoic acid (**29**):*

In a flask 0.240 g of **28** (1.63 mmol) and 0.257 g of $NaHCO_3$ (3.0 mmol) was dissolved into 6.0 mL of H_2O at 0 °C. The pH adjusted to 7 by the dropwise addition of 0.100 M HCl. A solution of Fmoc-OSu (2.12mmol, 0.716 g) dissolved in 6.0 mL of dioxane was dripped into the flask. After the addition, the reaction was warmed to rt, stirred for 20 h, and diluted with 10 mL of H_2O. The crude

material was acidified to pH=3.0 with 1M $NaHSO_4$, and extracted with CH_2Cl_2 (3×15 mL). The organic layer combined, extracted with H_2O (10×15 mL), dried with 1M NaH_2SO_4, filtered, and concentrated. A gradient column (0:100 MeOH:CH_2Cl_2 to 10:90 MeOH:CH_2Cl_2) eluted **29** (209 mg, 0.596 mmol, 37%). R_f = 0.36 (10:90 MeOH:CH_2Cl_2). $[\alpha]_D^{23}$ = +2.58 (c =1, MeOH). IR (cm^{-1}) = 3326 br, 2942, 1693. ^{1}H-NMR (MeOD, 400 MHz): δ 7.80 (d, *J* = 7.5 Hz, 2H), 7.65 (d, *J* = 7.4 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.32 (t, *J* = 7.4 Hz, 2H), 4.33 (d, *J* = 6.9 Hz, 2H), 4.20 (t, *J* = 6.9 Hz, 1H), 3.66 (d, *J* = 11.3 Hz, 2H), 3.36 (s, 2H), 3.18 (dd, *J* = 15.2, 9.0 Hz, 2H), 1.90 – 1.78 (m, 1H), 1.72 – 1.62 (m, 1H), 1.20 (s, 3H). δ ^{13}C-NMR (MeOD, 100 MHz): δ 178.37, 157.32, 143.94, 141.18, 127.35, 126.73, 124.77, 119.50, 67.45, 66.29, 48.46, 47.08, 36.64, 34.91, 18.44. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{21}H_{23}NO_5Na$ 392.2; Found 392.1.

*2-[bis(4-methyoxyphenyl)phenylmethoxy]-4-[[9H-fluoren-9-ylmethoxy]carbonyl]amino]-2-methylbutanoic acid (**30**):*

In a flask containing a solution of **29** (0.23 mmol, 88 mg) dissolved into 2.0 mL of pyridine freshly distilled was added 4,4'-Dimethoxytrityl chloride (0.30 mmol, 105 mg) and stirred for 16 h. The product was purified on Rigel 5 µm C18 10x250mm HPLC column, but was not isolated (product deprotect on column). HPLC conditions used: Flow rate of 3 mL/min, mobile phase programmed to pump 60:40 H_2O:MeCN to 0:100 H_2O:MeCN in 20 minutes. A 1.0 mL sample loop was installed and 500 µL injected. The UV detector was set to 254 nm and zeroed prior to injection. A 5.0 mg/mL solution of crude material was dissolved

into a solution of 60:40 H_2O:MeCN and filtered through a Whatman13mm 0.2 µm PP filter. Product elutes at R_f = 16.3 m.

*(R)-ethyl 1-benzyl-3-methyl-2-oxopyrrolidine-3-carboxylate (**31**):*

The title compound was prepared according to reported literature procedures, and confirmed with reported characterization data.[130]

*(S)-1-benzyl-3-(hydroxymethyl)-3-methylpyrrolidin-2-one (**32**):*

In a dry flask under N_2 atmosphere, a solution of **31** (1.11 mmol, 0.290g) was dissolved into 2.22 mL of MeOH followed by $CaCl_2$ (1.11 mmol, 0.123 g). The temperature was adjusted to 0 °C and $NaBH_4$ (2.22 mmol, 0.084 g) added. The reaction was warmed to rt, stirred for 16h, and quenched with 3.0 N citric acid (pH~3.5). The MeOH was removed and solution extracted with CH_2Cl_2 (4×5 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated to a viscous oil. The crude material was purified on silica gel column eluting with a mobile phase of 20:80 EtOAc:Hexanes resulting in 0.157 g of **32** (0.715 mmol, 64%). R_f = 0.22 (20:80 EtOAc:Hexanes). $[\alpha]_D^{21}$ = +3.5 (c =1, CH_2Cl_2). IR (cm^{-1}) = 3290 br, 2913,2871, 1656. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.32 (dt, *J* = 14.4, 7.7 Hz, 3H), 7.24 – 7.19 (m, 2H), 4.45 (q, *J* = 14.7 Hz, 2H), 3.70 (dd, *J* = 10.8, 7.6 Hz, 1H), 3.56 (dd, *J* = 10.8, 4.5 Hz, 1H), 3.26 – 3.15 (m, 2H), 2.80 (dd, *J* = 7.5, 4.6 Hz, 1H), 2.07 (ddd, *J* = 12.7, 7.6, 3.8 Hz, 1H), 1.72 (ddd, *J* = 12.7, 7.6, 3.8 Hz, 1H), 1.21 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 178.45, 136.31, 128.76, 127.96, 127.63, 68.22, 46.66, 45.59, 43.67, 28.74, 19.71. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{13}H_{17}NO_2Na$ 242.1152; Found 242.1153.

*(S)-3-(tert-butoxymethyl)-1-benzyl-3-methylpyrrolidin-2-one (**33**):*

In a sealed tube **32** (2.71 mmol, 0.746 g) 0.224 mL of concentrated H_2SO_4 was dissolved into 11.2 mL of CH_2Cl_2 and placed on an ice bath. In a separate container isobutylene (23.5 mmol, 2.25 mL) was condensed at -15 °C, poured into the sealed tube, capped, and warmed to rt. After 12 h the sealed tube was opened, stirred 2 h, and concentrated. The crude material was purified on a silica gel column eluting with 20:80 EtOAc:Hexanes (R_f = 0.13) isolating 0.622 g of **33** (2.4 mmol, 70%) as a pure oil. $[\alpha]_D^{21}$ = +19.3 (c =1, CH_2Cl_2). IR (cm^{-1}) = 2970, 2927, 2869, 1682. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.34 – 7.21 (m, 5H), 4.47 (dd, *J* = 168.5, 14.9 Hz, 2H), 3.54 (d, *J* = 8.2 Hz, 1H), 3.26 – 3.19 (m, 1H), 3.17 (d, *J* = 8.2 Hz, 1H), 3.08 (td, *J* = 9.1, 4.4 Hz, 1H), 2.21 (ddd, *J* = 12.9, 8.7, 4.4 Hz, 1H), 1.74 (ddd, *J* = 12.6, 9.0, 6.4 Hz, 1H), 1.14 (s, 12H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 177.91, 136.71, 128.46, 127.95, 127.23, 72.59, 67.66, 46.83, 45.27, 44.21, 29.54, 27.45, 20.95. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{17}H_{25}NO_2Na$ 298.1778; Found 298.1778.

*(S)-3-(tert-butoxymethyl)-3-methylpyrrolidin-2-one (**38**):*

In a sealed tube containing 20 mL of a solution of CH_2Cl_2:$CHCl_3$ 1:1 v/v was dissolved **17** (2.81 mmol, 0.364 g) and chilled to -10 °C. A catalytic amount of H_2SO_4 (2.04 mmol, 0.109 mL) and 2.5 mL of condensed isobutylene (26.1 mmol) was added, and stirred at rt. After 12 h the reaction was opened to the atmosphere, stirred for 1 h, and concentrated. The crude material was suspended into 20 mL of Et_2O and extracted with 1% KOH solution (3×10 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated

to 0.3115 g of pure **38** (1.68 mmol, 60% yield). R_f = 0.15 (45:55 EtOAc:Hexanes). $[\alpha]_D^{21}$ = -6.48 (c =1, $CHCl_3$). IR (cm^{-1}) = 3249, 2967, 2931, 2900, 2869, 1650. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 5.62 (s, 1H), 3.47 (d, *J* = 8.4 Hz, 1H), 3.37 – 3.29 (m, 1H), 3.28 – 3.21 (m, 1H), 3.18 (d, *J* = 8.4 Hz, 1H), 2.34 (ddd, *J* = 12.9, 8.3, 4.8 Hz, 1H), 1.86 (ddd, *J* = 12.7, 8.6, 6.2 Hz, 1H), 1.15 (s, 9H), 1.12 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz):δ 181.48, 72.60, 67.03, 44.07, 39.31, 32.07, 27.45, 20.45. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{10}H_{19}NO_2Na$ 208.1308; Found 208.1310.

*Synthesis of (S)-tert-butyl 3-(tert-butoxymethyl)-3-methyl-2-oxopyrrolidine-1-carboxylate (***39***):*

A flask containing **38** (1.62 mmol, 0.300 g) was dissolved into 8.0 mL of CH_2Cl_2 and cooled to 0 °C under N_2 atmosphere. To the solution was added DMAP (0.162 mmol, 19 mg), triethylamine (4.86 mmol, 0.677 mL), and di-*tert*-butyl dicarbonate (2.43 mmol, 0.558 mL). The reaction warmed to rt, stirred 12h and concentrated. The crude material was purified on a gradient column eluting with 15:85 EtOAc:Hexanes increasing the polarity to 20:80 EtOAc:Hexanes (R_f =0.20, 20:80 EtOAc:Hexanes). After purification, 0.328 g of **39** was isolated as a solid (1.27 mmol, 78 %). $[\alpha]_D^{23}$ = -24.9 (c =1, CH_2Cl_2). mp = 60 °C. IR (cm^{-1}) = 2975, 2933, 2873, 1768,1691. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 3.69 (ddd, *J* = 10.4, 8.5, 7.0 Hz, 1H), 3.60 (ddd, *J* = 10.4, 8.8, 4.9 Hz, 1H), 3.49 (d, *J* = 8.4 Hz, 1H), 3.19 (d, *J* = 8.4 Hz, 1H), 2.20 (ddd, *J* = 13.2, 8.5, 4.9 Hz, 1H), 1.71 (ddd, *J* = 8.8, 6.4, 1.9 Hz, 1H), 1.53 (s, 9H), 1.13 (s, 12H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 177.79, 150.57, 82.45, 72.84, 67.43, 47.06, 43.84, 28.59, 28.08, 27.37, 20.53.

HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{15}H_{27}NO_4Na$ 308.1832; Found 308.1837.

*(S)-4-[[(1,1-dimethylethoxy)carbonyl]amino]-2-(1,1-dimethylethoxy)-2-methyl-butanoic acid (**40**):*

In a flask at rt was dissolved **39** (4.97 mmol, 1.42 g of) into a 24.8 mL of THF followed by the addition of 3 equivalents of a 1.0 N LiOH solution (14.9 mmol). The solution stirred 6 h, concentrated, and acidified to pH=4.0 with 10% acetic acid solution. The crude material was extracted with Et_2O (4×20 mL), dried over $MgSO_4$, filtered, and concentrated under high vacuum pressure. The crude material was purified on a silica gel column eluted with 30:70 EtOAc:Hexanes (R_f =0.02, 55:45 EtOAc:Hexanes). After purification 1.346 g of **40** (3.87 mmol, 78% yield) was isolated. $[\alpha]_D^{24}$ = +5.70 (c =1, CH_2Cl_2). IR (cm^{-1}) = 3332 br, 2973, 2932, 2874, 1697. ^{1}H-NMR ($CDCl_3$, 400 MHz): COOH peak not present δ 4.90 (s, 1H), 3.42 (s, 2H), 3.29 – 3.10 (m, 2H), 1.83 (d, *J* = 4.9 Hz, 2H), 1.41 (d, *J* = 21.7 Hz, 9H), 1.22 (s, *J* = 12.7 Hz, 9H), 1.21 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 178.64, 155.95, 79.16, 74.63, 66.18, 45.24, 36.61, 35.57, 28.43, 27.28, 20.65. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{15}H_{29}NO_5Na$326.1938; Found 326.1939.

*diethyl 2-methyl-2-(3-(1,3-dioxoisoindolin-2-yl)propyl)malonate(**41**):*

The title compound was prepared according to reported literature procedures, and confirmed with reported characterization data.[191]

*(R)-2-(ethoxycarbonyl)-2-methyl-5-(1,3-dioxoisoindolin-2-yl)pentanoic acid (**42**):*

The title compound was prepared according to reported literature procedures, and confirmed with reported characterization data.[191]

*(S)-1-(4-nitrobenzyl) 3-ethyl 2-methyl-2-(3-(1,3-dioxoisoindolin-2-yl)propyl)malonate (**43**):*

The title compound can be synthesized according to reported literature procedure.[191]

(R)-ethyl 3-methyl-2-oxopiperidine-3-carboxylate (**44**):

The title compound can be synthesized according to reported literature procedure.[191]

*(S)-(hydroxymethyl)-3-methylpiperidin-2-one (**48**):*

In a flask containing 7.0 mL of MeOH was dissolved **44** (3.24 mmol, 0.600 g) followed by $CaCl_2$. (3.88 mmol, 0.431 g). The solution was placed on an ice bath at 0 °C under N_2 atmosphere and solid $NaBH_4$ (6.48 mmol, 1.08 g) added in 3 equal portions over 30 min. The reaction stirred at 0 °C for 2 h, warmed to rt, and quenched after 12 h with 6 M HCl until a pH=2.0. The solid formed was filtered off over a Büchner funnel, and discarded. The filtrate was concentrated, suspended into MeOH, and triturated with CH_2Cl_2, and concentrated again once no more solid fell out of solution. The crude material was purified on a silica gel column and eluted in a 5% MeOH in CH_2Cl_2 (R_f = 0.42, 10:90 MeOH:Hexanes). Pure **48** (2.93 mmol, 91%, 0.424 g) was isolated as a solid. $[\alpha]_D^{22}$ = - 47.0 (c =1, CH_2Cl_2). mp = 79 °C. IR (cm^{-1}) = 3313Br, 3251, 2969, 2945, 2898, 2869, 1639. ^{1}H-NMR (MeOD, 400 MHz): OH peak not present δ 3.67 (d, *J* = 10.6 Hz, 1H),

3.28 – 3.19 (m, 2H), 3.18 – 3.12 (m, 2H), 2.02 – 1.87 (m, 1H), 1.79 – 1.68 (m, 2H), 1.50 – 1.37 (m, 1H), 1.02 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 178.41, 69.88, 42.39, 42.18, 30.79, 22.12, 18.93. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_7H_{13}NO_2Na$ 166.1; Found 166.1.

*(S)-3-(tert-butoxymethyl)-3-methylpiperidin-2-one (**49**):*

In a sealed tube on an ice bath containing **48** (2.96 mmol, 0.424 g), 0.127 mL of concentrated H_2SO_4 dissolved into 15 mL of CH_2Cl_2 was added 1.25 mL of isobutylene (13.0 mmol) at -10 °C. The tube was capped, warmed to rt, and stirred. After 12 h the cap was removed and stirred for 1 h. The solution was concentrated, suspended into Et_2O, and extracted with 2% KOH solution. The organic layer was dried over $MgSO_4$, filtered, and concentrated isolating 0.431 g of **49** (2.17 mmol, 73%) as a pure solid. R_f = 0.08 (20:80 EtOAc:Hexanes) $[\alpha]_D^{22}$ = - 15.7 (c =1, CH_2Cl_2). mp = 77 °C. IR (cm^{-1}) = 3281, 3187, 3063, 2971, 2863, 1650. ^{1}H-NMR (MeOD, 400 MHz): NH group not present δ 3.53 (d, *J* = 8.1 Hz, 1H), 3.13 (dd, *J* = 7.3, 4.9 Hz, 2H), 3.00 (d, *J* = 8.1 Hz, 1H), 2.07 – 1.98 (m, 1H), 1.77 (dddd, *J* = 8.2, 4.9, 3.5, 1.5 Hz, 1H), 1.72 – 1.61 (m, 1H), 1.42 (ddd, *J* = 13.2, 6.7, 3.4 Hz, 1H), 1.06 (s, 9H), 1.01 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 176.58, 72.45, 68.09, 42.91, 42.63, 31.13, 27.49, 22.88, 19.78. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{11}H_{21}NO_2Na$ 222.1465; Found 222.1466.

*(S)-tert-butyl 3-(tert-butoxymethyl)-3-methyl-2-oxopiperidine-1-carboxylate (**50**):*

A flask containing **49** (2.17 mmol, 0.4325 g), DMAP (0.217 mmol, 26 mg), triethylamine (6.51 mmol, 0.907 mL), 0.710 g of di-*tert*-butyl dicarbonate (3.25 mmol, 0.907 mL) was dissolved into 12 mL of CH_2Cl_2 at 0 °C under a N_2

atmosphere. The reaction was warmed to rt, stirred 12 h, and concentrated. The crude material was purified on a 20:80 EtOAc:Hexanes silica gel column isolating 0.279 g of **50** (1.40 mmol, 64%) as a pure solid. R_f =0.44, 20:80 EtOAc:Hexanes. $[\alpha]_D^{23}$ = -49.8 (c =1, CH_2Cl_2). mp = 60 °C. IR (cm^{-1}) = 2972, 2871, 1769, 1708. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 3.68 (dddd, *J* = 12.0, 5.9, 4.8, 1.4 Hz, 1H), 3.60 (d, *J* = 8.1 Hz, 1H), 3.54 (ddd, *J* = 12.4, 9.3, 4.4 Hz, 1H), 3.17 (d, *J* = 8.1 Hz, 1H), 2.13 – 2.02 (m, 1H), 1.93 – 1.85 (m, 1H), 1.81 – 1.70 (m, 1H), 1.57 – 1.52 (m, 1H), 1.51 (s, 9H), 1.18 (s, 3H), 1.13 (s, 9H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 176.27, 153.49, 82.32, 72.59, 68.77, 47.50, 46.03, 31.56, 28.06, 27.44, 23.23, 20.10. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{16}H_{29}NO_4Na$ 322.1988; Found 322.1990.

*5-[[1,1-dimethylethoxy]carbonyl]amino -2-[2(1,1-dimethylethoxy)methyl]- 2-methyl-pentanoic acid (**51**):*

In a flask, **39** (1.1 mmol, 0.279 g) was dissolved into a 5.5 mL of THF followed by the addition of 1.0 N LiOH (3.3 mmol. 3.35 mL) at rt. After 6 h the THF layer was removed, remaining aqueous layer acidified to a pH of 4.0 with 4 N HCl, and extracted with Et_2O (4×20 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under high vacuum pressure, resulting in 0.272 g of **51** (0.858 mmol, 78%). R_f =0.00, (20:80 EtOAc:Hexanes). $[\alpha]_D^{23}$ = +2.62 (c =1, $CHCl_3$). IR (cm^{-1}) = 3336 br, 2973, 2932, 2872, 1700. ^{1}H-NMR ($CDCl_3$, 400 MHz): COOH not present δ 4.60 (s, 1H), 3.39 (q, *J* = 8.7 Hz, 2H), 3.11 (d, *J* = 5.6 Hz 2H), 1.74 – 1.61 (m, 1H), 1.61 – 1.40 (m, 3H), 1.44 (s, 9H), 1.23 (s, 9H), 1.17 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz):δ 178.14, 155.99,

79.15, 74.63, 66.26, 45.93, 40.77, 32.98, 28.42, 27.29, 24.85, 20.10. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{16}H_{31}NO_5Na$ 340.2094; Found340.2095.

((S)-3-methyl-2-oxopyrrolidin-3-yl)methyl methanesulfonate (***52a***):

In a flask was dissolved **17** (3.00 mmol, 0.387 g) and triethylamine (6.00 mmol, 0.83 mL) into 6.0 mL of dry CH_2Cl_2. The solution was cooled to 0 °C, methanesulfonyl chloride (3.3 mmol, 0.255 mL) added, and warmed to rt. After 2 h the reaction was extracted with 2.0 M HCl (3×3.0 mL), water (3×3.0 mL), and saturated sodium bicarbonate (3×3.0 mL). After each extraction, the aqueous layer was back extracted with CH_2Cl_2. All organic layers were combined, washed with saturated brine, and dried over $MgSO_4$. The crude material was concentrated, and recrystallized from cold ether isolating 0.450 g of **52a** as a white solid (2.17 mmol, 72%). R_f = 0.38 (9% MeOH/EtOAc) $[\alpha]_D^{24}$ = - 3.63 (c =1, $CHCl_3$). mp = 79 °C. IR (cm^{-1}) = 3255 br, 3018, 2973, 2936, 1689, 1654. ^{1}H -NMR (400 MHz, $CDCl_3$) NH not observed δ 6.17 (s, 1H), 4.29 (d, *J* = 9.7 Hz, 1H), 4.10 (d, *J* = 9.7 Hz, 1H), 3.43 – 3.33 (m, 2H), 3.03 (d, *J* = 4.7 Hz, 3H), 2.43 (dt, *J* = 13.1, 7.8 Hz, 1H), 2.01 – 1.91 (m, 1H), 1.21 (d, *J* = 3.8 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 178.66, 73.26, 43.65, 38.63, 37.06, 30.48, 19.60. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_7H_{13}NO_4SNa$ 230.0457; Found 230.0458.

*((S)-3-methyl-2-oxopiperidin-3-yl)methyl methanesulfonate (**52b**):*

In a flask was dissolved **48** (4.36 mmol, 0.625 g) and triethylamine (8.73 mmol, 1.21 mL) into 10 mL of dry CH_2Cl_2. The reaction was placed onto an ice bath at 0 ^{o}C and methanesulfonyl chloride (4.36 mmol, 0.337mL) was added over 5 min. and warmed to rt for 2 h. The solution was extracted with 2.0 M HCl

(3×3.0 mL), water (3×3.0 mL), and saturated sodium bicarbonate (3×3.0 mL). After each extraction, the aqueous layer was back extracted with CH_2Cl_2. All organic layers were combined, washed with saturated brine, and dried over $MgSO_4$. The resulting material was concentrated, and recrystallized from cold ether where 0.802 g of ***52b*** (3.6 mmol, 83%) was isolated as a white solid. R_f = 0.26 (9% MeOH/EtOAc). $[\alpha]_D^{22}$ = - 26.5 (c =1, $CHCl_3$). mp = 93°C. IR (cm^{-1}) = 3186 br, 3070, 2958, 2938, 2873, 1643. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 5.83 (s, 1H), 4.54 (d, *J* = 9.2 Hz, 1H), 3.96 (d, *J* = 9.2 Hz, 1H), 3.34 (ddd, *J* = 8.6, 5.8, 2.4 Hz, 2H), 3.02 (s, 3H), 2.13 (ddd, *J* = 13.6, 10.8, 5.0 Hz, 1H), 1.98 – 1.83 (m, 2H), 1.69 – 1.63 (m, 1H), 1.25 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz):173.9, 75.4, 42.7, 36.9, 30.1, 22.1, 19.1. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_8H_{15}NO_4SNa$ 244.0; Found 244.1.

*((S)-1-(tert-butoxycarbonyl)-3-methyl-2-oxopyrrolidin-3-yl)methyl methanesulfonate (**53a**):*

In a round bottom flask, **52a** (1.9 mmol, 0.400 g) was dissolved into 15.0 mL of anhydrous CH_2Cl_2 under a N_2 atmosphere. To the solution was sequentially added Et_3N (6.25 mmol, 0.871 mL), DMAP (0.19 mmol, 23.3 mg), 0.654 g of di-*tert*-butyl-dicarbonate (2.85 mmol). The reaction stirred for 20 h at rt, concentrated, and purified on a column packed in 25:75 EtOAc:Hexanes. After purification 0.4980 g of **53a** (1.62 mmol, 85.0%) was isolated as a pure white solid. R_f =0.08 (25:75 EtOAc:Hexanes) $[\alpha]_D^{22}$ = -10.00 (c =1, CH_2Cl_2). mp =106°C. IR (cm^{-1}) = 2979, 2935, 1766, 1690. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 4.32 (d, *J* = 9.8 Hz, 1H), 4.10 (d, *J* = 9.8 Hz, 1H), 3.84 – 3.74 (m, 1H), 3.66 (dt, *J* =

10.9, 8.1 Hz, 1H), 3.03 (d, *J* = 0.5 Hz, 3H), 2.31 (dt, *J* = 13.0, 8.8 Hz, 1H), 1.82 (ddd, *J* = 13.0, 7.8, 3.4 Hz, 1H), 1.54 (s, 9H), 1.22 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz):δ 175.06, 149.87, 83.42, 72.63, 46.38, 42.85, 37.12, 28.01, 26.81, 19.66. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{12}H_{21}NO_6SNa$ 330.0981; Found 330.0980.

((S)-1-(tert-butoxycarbonyl)-3-methyl-2-oxopiperidin-3-yl)methyl methanesulfonate (***53b***):

In a flask was dissolved **52b** (0.927 mmol, 205 mg) into 15.0 mL of anhydrous CH_2Cl_2 under a N_2 atmosphere, and sequentially was added Et_3N (1.85 mmol, 0.257 mL), DMAP (9.2 µmmol, 11.3 mg), di-*tert*-butyl-dicarbonate (1.85 mmol, 0.425 g). The solution stirred 20 h at rt, concentrated, and purified on a gradient column eluting 20:80 EtOAc:Hexanes to 35:65 EtOAc:Hexanes. After purification 0.226 g of **53b** (0.704 mmol, 75.0%) was isolated as a pure white solid. R_f =0.65 (5:95 MeOH:CH_2Cl_2). $[\alpha]_D^{22}$ = -24.5 (c =1, CH_3Cl). mp = 59 °C. IR (cm^{-1}) = 2954, 1753, 1668. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.54 (d, *J* = 9.3 Hz, 1H), 3.97 (d, *J* = 9.3 Hz, 1H), 3.77 (dtd, *J* = 12.6, 4.6, 1.6 Hz, 1H), 3.60 – 3.48 (m, 1H), 3.02 (s, 3H), 2.19 – 2.10 (m, 1H), 1.96 – 1.84 (m, 2H), 1.70 – 1.62 (m, 1H), 1.52 (s, 9H), 1.28 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.61, 152.68, 83.26, 75.52, 47.37, 45.05, 36.95, 30.53, 27.98, 22.67, 19.43. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{13}H_{23}NO_6SNa$ 344.1; Found 344.1.

*(S)-tert-butyl 3-(azidomethyl)-3-methyl-2-oxopyrrolidine-1-carboxylate (**54a**):*

In a sealed tube was dissolved **53a** (1.00 mmol, 0.307 g) and sodium azide (6.00 mmol, 0.395 g) into 2 mL of DMF. The tube was capped, stirred at

90 °C for 5 days, and extracted with CH_2Cl_2 (3×7 mL). The organic layers were combined, extracted with H_2O (5×5 mL), dried over $MgSO_4$, filtered, and concentrated. The crude material was purified on a silica gel column eluting in 20:80EtOAc:Hexanes (R_f =0.28) to yield 0.186 g of pure **54a** (0.732 mmol, 73%). $[\alpha]_D^{22}$ = +8.99 (c =1, CH_3Cl). mp = 66 °C. IR (cm^{-1}) = 2972, 2928, 2095, 1766, 1689. ^1H NMR (400 MHz, $CDCl_3$) δ 3.77 (ddd, *J* = 11.0, 9.0, 4.1 Hz, 1H), 3.71 – 3.59 (m, 2H), 3.34 (d, *J* = 12.1 Hz, 1H), 2.17 (ddd, *J* = 13.0, 8.9, 7.8 Hz, 1H), 1.78 (ddd, *J* = 12.9, 8.0, 4.1 Hz, 1H), 1.55 (s, 9H), 1.22 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.89, 150.18, 83.20, 57.16, 46.92, 42.90, 28.01, 27.97, 20.96. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{11}H_{18}N_4O_3Na$ 277.1271; Found 277.1270.

*(S)-tert-butyl 3-(azidomethyl)-3-methyl-2-oxopiperidine-1-carboxylate (**54b**):*

In a sealed tube 0.200 g of **53b** (0.786 mmol) and 0.121 g of sodium azide (1.86 mmol) was dissolved into 3 mL of DMF. The reaction was capped and stirred at 90 °C. After 7 days, the reaction was extracted with CH_2Cl_2 (3×7 mL). The organic layers were combined and extracted with H_2O (5×5 mL), dried over $MgSO_4$, filtered, and concentrated. The crude material was purified on a silica gel column eluting in 40:60EtOAc:Hexanes (R_f =0.59) to yield 0.100 g of pure **54b** (0.372 mmol, 60%). $[\alpha]_D^{21}$ = -1.7 (c =1, CH_3Cl). IR (cm^{-1}) = 2977, 2935, 2872, 2100, 1766, 1712. ^1H NMR (400 MHz, $CDCl_3$) δ 3.81 (d, *J* = 11.8 Hz, 1H), 3.75 (dtd, *J* = 12.5, 4.7, 1.6 Hz, 1H), 3.53 (ddd, *J* = 12.5, 8.4, 6.1 Hz, 1H), 3.21 (d, *J* = 11.8 Hz, 1H), 2.05 – 1.96 (m, 1H), 1.92 – 1.83 (m, 2H), 1.64 – 1.56 (m, 1H), 1.52 (s, 9H), 1.25 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.66, 153.21, 83.02,

59.51, 47.35, 45.72, 31.44, 27.99, 23.93, 19.59. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{12}H_{20}N_4O_3Na$ 291.1, observed = 291.1.

*(S)-2-(azidomethyl)-4-[[(1,1-dimethylethoxy)carbonyl]amino]-2-methyl-butanoic acid (**57a**):*

In a flask containing **54a** (0.786 mmol, 0.200) dissolved into 4 mL of THF was added 2.36 ml of 1.0 M LiOH. The reaction stirred 5 h, quenched with 1.0 M HCl until the pH=3.0, and extracted with Et_2O (3×15 mL). The organic layers were combined and extracted with saturated $NaHCO_3$ (3×5 mL), and saturated brine (3×5 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure to yield 71.8 mg of **57a** (0.534 mmol, 68%). R_f = 0.13 (30:70 EtOAc:Hexanes). $[\alpha]_D^{21} =$ -6.10 (c = 2.3, $CHCl_3$). IR (cm^{-1}) = 3320 br,2977, 2932, 2101, 1700. ^{1}H-NMR (MeOD, 400 MHz): COOH and NH peaks not present δ 3.47 (d, *J* = 12.1 Hz, 1H), 3.31 (d, *J* = 12.1 Hz, 1H), 2.97 (dt, *J* = 9.2, 5.7 Hz, 2H), 1.69 (ddd, *J* = 15.7, 9.3, 6.5 Hz, 1H), 1.58 (ddd, *J* = 13.5, 9.2, 6.6 Hz, 1H), 1.33 (s, 9H), 1.11 (s, 3H). ^{13}C-NMR (MeOD, 100 MHz): δ ^{13}C NMR (101 MHz, MeOD) δ 178.37, 158.29, 80.00, 59.35, 46.66, 37.36, 28.76, 20.81. HRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{11}H_{20}N_4O_3Na$ 295.1376; Found 295.1374.

*(S)-2-(azidomethyl)-5-[[[(1,1-dimethylethoxy)carbonyl]amino]propyl]-2-methyl-pentanoic acid (**57b**)*

A solution of **54b** (0.343 mmol, 92 mg) dissolved into 2 mL of THF was added to 1.02 ml of 1.0 M LiOH. The reaction stirred for 5 h, quenched with 1.0 M HCl until the pH=2.0, and extracted with CH_2Cl_2 (5×5 mL). The organic layers were combined and extracted with water (3×5 mL), brine (3×5 mL), dried over

anhydrous $MgSO_4$, and concentrated under reduced pressure to yield 87 mg of **57b** as a clear gum (0.30 mmol, 88%). R_f = 0.22 (40:60 EtOAc:Hexanes) $[\alpha]_D^{23}$ = -4.1 (c =1, MeOH). IR (cm^{-1}) = 3329, 2976, 2933, 2100, 1700, 1653. ^{1}H-NMR (MeOD, 400 MHz): COOH and NH peaks not present δ 3.57 (d, *J* = 12.1 Hz, 1H), 3.38 (d, *J* = 12.1 Hz, 1H), 3.02 (t, *J* = 6.6 Hz, 2H), 1.70 – 1.57 (m, 1H), 1.56 – 1.47 (m, 2H), 1.45 – 1.40 (m, 1H), 1.44 (s, 9H), 1.19 (s, 3H). ^{13}C-NMR (MeOD, 100MHz): δ 178.86, 158.54, 79.89, 59.40, 47.69, 41.56, 34.95, 28.77, 25.99, 20.83. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{12}H_{22}N_4O_3Na$ 309.1; Found 309.1.

*(R)-3-((benzylthio)methyl)-3-methylpiperidin-2-one (**58a**)*

A flask containing NaH (0.586 mmol, 14.0 mg) was washed with pentane under inert atmosphere, and replaced with 0.5 mL of DMF on an ice bath. To the solution benzyl mercaptan (0.586mmol, 68 µL) was added slowly and stirred 5 min. A solution of **53a** (0.325 mmol, 100 mg) dissolved in 0.5 mL of DMF and added to the reaction over 10 min. After the addition the reaction was stirred at rt for 12 h, quenched with 0.4 mL of 4 N HCl, and extracted with Et_2O (5×5 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under vacuum. The crude material was purified on a silica gel column eluting with 20:80 EtOAc:Hexanes isolating 65 mg of **58a** (0.190 mmol, 57%). R_f = 0.21 (20:80 EtOAc:Hexanes) IR (cm^{-1}) = 2974, 2928, 1779, 1743, 1712. ^{1}H-NMR (COD, 400 MHz): δ 7.34 – 7.28 (m, 4H), 7.26 – 7.21 (m, 1H), 3.74 (q, *J* = 13.3 Hz, 2H), 3.69 – 3.53 (m, 2H), 2.65 (dd, *J* = 34.3, 13.1 Hz, 2H), 2.08 (ddd, *J* = 12.9, 8.6, 7.4 Hz, 1H), 1.73 (ddd, *J* = 12.8, 7.9, 4.7 Hz, 1H), 1.53 (s, 9H), 1.21 (s, 3H).

^{13}C-NMR (MeOD, 100 MHz): δ 177.18, 150.35, 138.16, 129.01, 128.52, 127.12, 82.95, 47.03, 43.00, 39.04, 37.78, 29.48, 28.04, 22.73. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{18}H_{25}NO_3SNa$ 358.1; Found 358.1.

(R)-4-[[[(1,1-dimethylethoxy)carbonyl]amino]propyl]-2-methyl-2-[[(phenylmethyl]thio]methyl]-butanoic acid (**59**):

In a flask containing 2.7 mL of THF was added **58a** (0.177 mmol, 59 mg) followed by 1.0 M LiOH (1.02 mL). After 6 h the solution was concentrated and acidified to pH=3.0 with 4N HCl. The aqueous layer was extracted with Et_2O (5×5 mL) and extracts washed with saturated brine (3×5 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure to yield 49.7 mg of **59** as a gum (0.140 mmol, 79%). R_f = 0.06 (5:95 MeOH:CH_2Cl_2) ^{1}H-NMR (MeOD, 400 MHz): COOH and NH peaks not observed δ 7.31 – 7.06 (m, 5H), 3.64 (s, 2H), 3.00 – 2.80 (m, 2H), 2.59 (dd, *J* = 59.0, 12.9 Hz, 2H), 1.72 (ddd, *J* = 13.8, 10.1, 6.0 Hz, 1H), 1.57 (ddd, *J* = 13.4, 10.0, 5.8 Hz, 1H), 1.32 (s, 9H), 1.09 (s, 3H). ^{13}C-NMR (MeOD, 75 MHz): δ 158.27, 140.06, 130.09, 129.41, 127.95, 79.92, 47.03, 41.56, 39.13, 38.65, 37.79, 37.72, 28.79, 22.13. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{18}H_{27}NO_4SLi$ 360.1; Found 360.1.

*tert-butyl (S)-4-((benzylthio)carbonyl)-5-(benzylthio)-4-methylpentrylcarbamate (**60**):*

In a dry flask under a N_2 atmosophere NaH (0.896 mmol, 35.8 mg) was washed with pentane, and replaced with 1.0 mL of DMF on an ice bath at 0 °C. To the solution benzyl mercaptan (0.896 mmol, 0.10 mL) was added and stirred

for 5 min. A solution of **53b** (0.497 mmol, 0.160 g) was dissolved into 1.0 mL of DMF and added over 10 min. The reaction was stirred for 12 h at rt, quenched with 0.8 mL of 4 N HCl, and extracted with Et_2O (5×5 mL). The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under vacuum. This crude material was purified on a silica gel column eluting with 20:80 EtOAc:Hexanes isolating **60** as an oil (0.184 mmol, 37.0%). R_f = 0.30 (20:80 EtOAc:Hexanes) $[\alpha]_D^{23}$ = -11.2 (c =1, $CHCl_3$). IR (cm^{-1}) = 3359, 2973, 1671 br. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.33 – 7.20 (m, 10H), 4.40 (s, 1H), 4.10 (s, 2H), 3.64 (s, 2H), 2.99 (dd, *J* = 12.3, 6.0 Hz, 2H), 2.68 (dd, *J* = 65.4, 12.8 Hz, 2H), 1.72 – 1.62 (m, 1H), 1.56 – 1.47 (m, 1H), 1.44 (s, 9H), 1.35 – 1.18 (m, 2H), 1.26 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 204.01, 155.88, 138.18, 137.47, 128.97, 128.92, 128.58, 128.48, 127.25, 127.07, 79.13, 53.72, 40.44, 37.96, 35.95, 33.24, 28.43, 24.78, 21.59. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{26}H_{35}NO_3S_2Na$ 496.1; Found 496.1.

*(R)-3-((benzylthio)methyl)-3-methylpiperidin-2-one (**61**):*

The title compound was isolated (59 mg, 0.236 mmol, 47%) as a product in the synthesis of **60**. R_f = 0.02 (20:80 EtOAc:Hexanes). $[\alpha]_D^{23}$ = -52.5 (c =1, $CHCl_3$). IR (cm^{-1}) = 3199, 3060, 2933, 1653. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.45 – 7.15 (m, 5H), 5.89 (s, 1H), 3.76 (q, *J* = 13.2 Hz, 2H), 3.39 – 3.16 (m, 2H), 2.96 (d, *J* = 12.8 Hz, 1H), 2.54 (d, *J* = 12.8 Hz, 1H), 2.10 – 1.97 (m, 1H), 1.88 – 1.72 (m, 2H), 1.61 (dd, *J* = 31.5, 6.3 Hz, 1H), 1.60 – 1.50 (m, 1H), 1.25 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 176.29, 138.66, 128.98, 128.44, 126.93, 42.90, 42.83,

41.60, 37.95, 32.16, 25.61, 19.44. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{14}H_{19}NOSNa$ 272.1; Found 272.2.

*(R)-5-[[[(1,1-dimethylethoxy)carbonyl]amino]propyl]-2-methyl-2-[[(phenylmethyl]thio]methyl]-pentanoic acid (**62**):*

In a flask **60** (0.1667 mmol, 79 mg) was dissolved into a solution of 4:1 THF:water, and K_2CO_3 (1.447mmo, 200 mgl). The reaction stirred at rt under a N_2 atmosphere for 4 h. The pH was adjusted with KOH (0.71 mmol, 45 mg) to bring the pH=11. After 24 h the reaction was concentrated, acidified to pH=3 with 4 N HCl, and extracted with Et_2O (5×12 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure to yield 40 mg of **62** as a light brown gum (0.1088 mmol, 65% yield). R_f = 0.04 (20:80 EtOAc:Hexanes). $[\alpha]_D^{24}$ = -0.144 (c =1, $CHCl_3$). IR (cm^{-1}) =3326, 2974, 2928, 1697 br. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ. 7.36 – 7.16 (m, 5H), 5.89 (s, 1H), 4.55 (s, 1H), 3.72 (s, 2H), 3.05 (s, 2H), 2.68 (dd, *J* = 54.0, 12.8 Hz, 2H), 1.65 (td, *J* = 12.6, 4.9 Hz, 1H), 1.57 – 1.48 (m, 1H), 1.42 (s, 9H), 1.40 – 1.30 (m, 2H), 1.21 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 181.15, 156.00, 138.26, 128.96, 128.50, 127.07, 79.26, 46.72, 40.66, 39.85, 37.94, 35.14, 28.42, 25.14, 21.61. LRMS (ESI-MS) *m/z*: $[M+Na]^+$ Calcd for $C_{19}H_{29}NO_4SNa$ 390.1; Found 390.1.

CHAPTER IV

ESI-MS STUDIES ON THE ESTERIFICATION OF GLUTATHIONE AND OTHER SMALL PEPTIDES WITH THIONYL CHLORIDE

Hypothesis 3

The esterification of small peptides with thionyl chloride can be used to completely esterify free carboxylic acids into methyl esters with limited side reactions and no purification.

Introduction

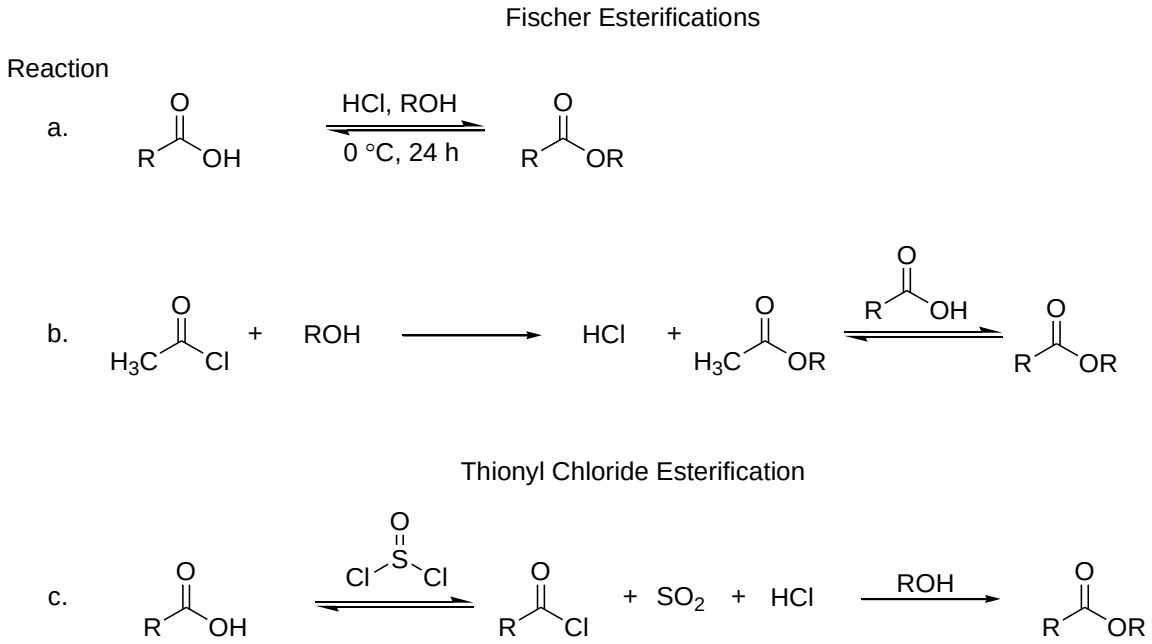
Esterification of peptides play an important role in peptide detection, isolation, and synthesis of peptidomimetics. A variety of methods exists to complete the transformation of carboxylic acids into their respective esters.[192-194] Classically, methanolic HCl has been the standard in C-terminal peptide methylation, but those reactions are plagued with competing side reactions, long reaction times, and incomplete conversion to products. Several esterification methods including a thionyl chloride assisted esterification are described. In addition, the significance as it pertains to mass spectrometry and the synthesis of an unnatural glutathione analogue are discussed.

Overview of peptide esterification methods

Esterification of carboxylic acids is an important tool to synthetic and peptide chemists. The conversion of an acid into an ester makes isolation and chromatographic separations easier due to increased organic solubility, lower boiling point, and increased volatility. Peptides are diverse in their chemical composition, therefore modifications, are synthetically challenging. Side

reactions such as amino acid side chain modification, peptide bond hydrolysis, and oxidation/reduction reactions are often observed. Therefore, the chemical composition must be considered when designing a synthesis surrounding peptides.

Earlier esterifications followed standard Fischer Esterification methods. These esterifications require a saturated solution of peptides, coat of HCl gas, and an excess of alcohol to drive the equilibrium to completion (Scheme 39, reaction a).[195] The disadvantages of this method are incompletely formed products, long reaction times at 0 °C, and the formation of multiple side products. In addition, the side reactions are destructive toward tryptophan. Other studies found the Fischer esterification incompatible with amino acids arginine, histidine, and tyrosine.[195] Today, these methods are used for the synthesis of organic compounds, but use of Fischer esterification in peptide has been replaced with modern methods.



Scheme 39. Various peptide esterification methods.

The second method frequently utilized is the generation of HCl via the alcoholysis of acetyl chloride. The foundation of this esterification was developed by Fraenkel-Cohrat and Olcott.[192] Together they outlined a mild protocol using 0.05 to 0.2 N aqueous HCl or acetyl chloride to catalyze the esterification of various proteins(Scheme 39, reaction b).[192] They determined only catalytic amounts of HCl were needed to esterify proteins, and found smaller quantities of HCl eliminated most side reactions. Additionally, they proposed acetyl chloride was reacting with the dry alcohol to liberate HCl to catalyze the reaction. Modernized methods have replaced the acetyl chloride method with methanolic HCl. However, commercially available methanolic HCl is still prepared via generation of HCl in the presence of acetyl chloride and methanol.

Fraenkel-Cohrat and Olcott also investigated the esterification with different alcohol solvents. Methanol esterification was the quickest and most

complete. After 6 days, other higher ordered primary alcohols (ethanol, n-propanol) were capable of esterifying proteins but not completely. Secondary alcohol 2-propanol also was tested but inefficient (less than 5% of isolated product). Since Fraenkel-Cohrat and Olcott's publication new adaptations of the in situ generation of HCl method have emerged.[196-198]

Thionyl chloride esterification is an alternative esterification used less frequently (Scheme 39, reaction c). Instead of the acid catalyzed Fischer esterifications, thionyl chloride converts the carboxylic acid into a reactive acyl chloride and the chloride leaves once displaced with the methanol solvent. Unlike the Fisher esterifications, equilibrium of the thionyl chloride esterification is non-reversible due to the formation of easily removed gaseous byproducts. A study using thionyl chloride to esterify amino acids with *n*-propanol determined temperature was more important than the molar concentration of thionyl chloride.[199] Frequently, experiments with thionyl chloride call for solvent reflux but these conditions lead to the formation of pyrrolidones with amino acids and hydrolysis of peptide bonds.[200] This method has been adapted in organic synthesis including the esterification of gelatin,[201] palmitic acid,[202] and aromatic carboxylic acids.[203] Generally, due to harsh acidic conditions, thionyl chloride is considered peptide incompatible with acid labile protecting groups and potential for oxidative transformations. However, thionyl chloride is used in peptide coupling of amino acids employing an Fmoc-protection strategy so the use of thionyl chloride should not be completely ruled out as an esterification reagent.

Applications of peptide esterification

C-terminal esterification has played a central role in the detection of amino acids and peptides. In mass spectrometry, esterification is used to chromatographically resolve peptides, elucidate and quantify proteins in complex mixtures, and enhance the detection of the secondary ion yields of hydrophilic peptides.[196] Esterification is also crucial to immobilized metal affinity chromatography (IMAC) of unphosphorylated peptides.[204] By derivatizing the free carboxylic acids to esters the negative charge is removed which increasing the specificity for enriching phosphorylated peptides.[205] As a tool for elucidating peptide structure, methyl esterification has been used to profile crustacean neuropeptidome.[206,207] However, during elucidation chemical artifacts of modified peptides were misidentified as occurring naturally.[208] These peptides were linked back to the methanolic HCl sample preparation. Therefore, having a method to quickly convert peptides into esterified products is a valuable tool for mass spectrometry detection.

The use of thionyl chloride as an esterification reagent in mass spectrometry has been well documented. Some of these application include LC-MS pharmacokinetic studies,[209] fatty acid GC-MS, and IMAC phosphoproteomics.[210,211] Unfortunately, the esterification protocol for many of these compounds called for a 2 hour reflux, where others are conducted at ambient temperature. Due to the delicate nature of peptides these conditions are prohibitive for peptide analysis and modified decomposed peptides are expected. Craig and Fischer at the Salk Institute are the only ones who have published a

short case study to compare the compatibility of methanolic HCl to thionyl chloride.[212] However, their study was limited to two peptides and the application of thionyl chloride to small molecule peptides was not explored.

Esterification of glutathione analogues

Interest in thionyl chloride esterification developed out of a synthetic need to prepare a mixed disulfide analogue of oxidized glutathione (GSSG, **63**, Figure 13). Currently, new anti-malarial treatments are considered a global health priority,[213,214] and glutathione analogues represent a novel class of molecules with potential therapeutic leads.[215,216] One of the first lines of defense against malarial parasites is oxidative stress which is regulated by glutathione reductase (GR). Important for cellular health, GR maintains the balance between GSSG to reduced glutathione (GSH, **64**, Figure 13) by catalyzing the reduction of GSSG to give GSH. Significant research has gone into designing potent GR inhibitors, because Inhibition of GR decreases the amount of invasion by malarial parasites.[217-219]

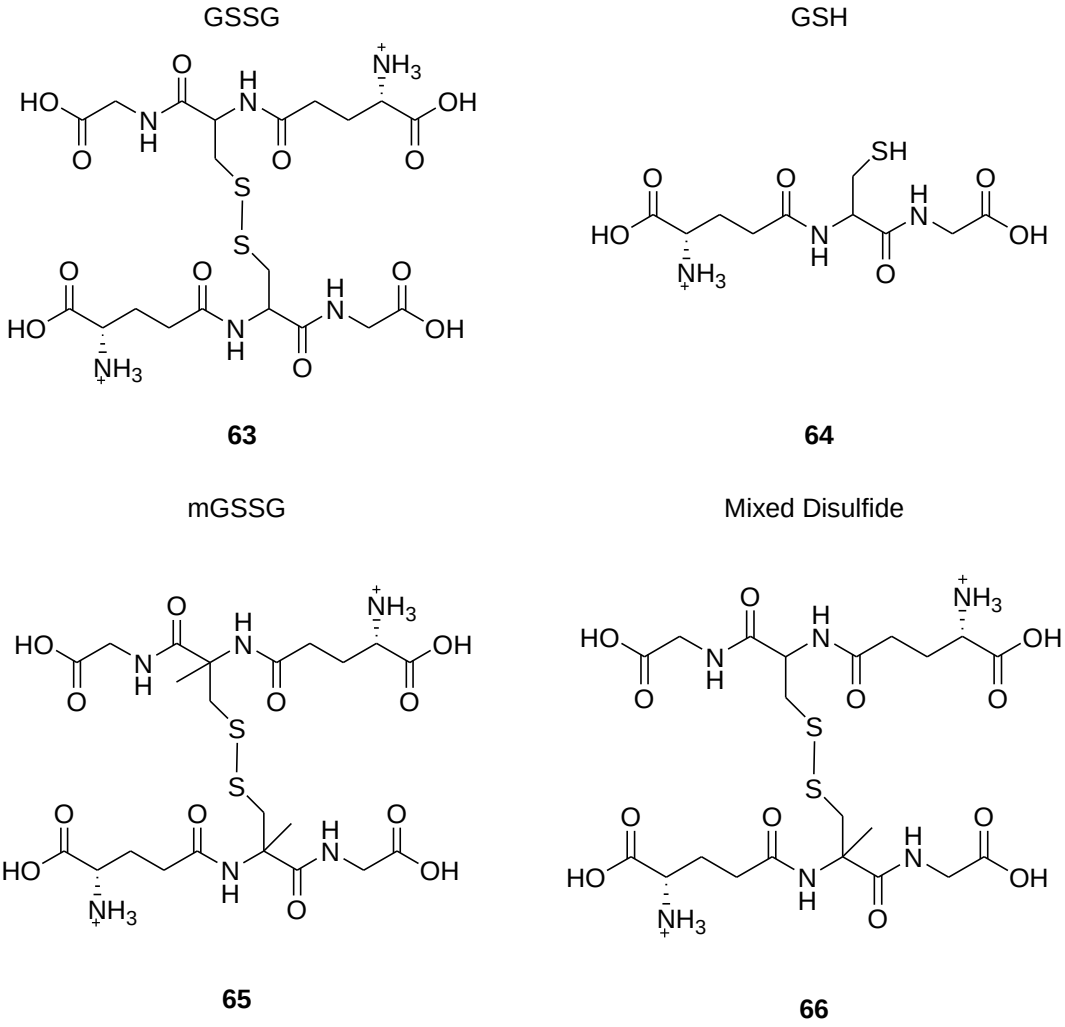
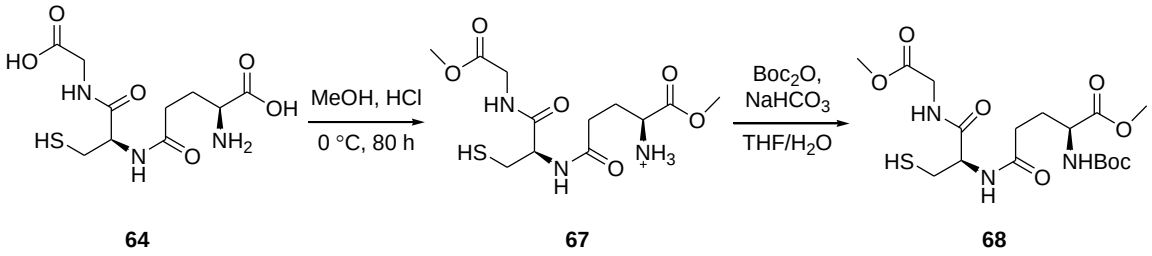


Figure 13. Glutathione and glutathione analogues.

Kedrowski et al. synthesized L-γ-glutamyl-2-methyl-L-cysteinyl-glycine disulphide, (mGSSG,**65**), to study how enzymes catalyze reactions of disulfides in peptides.[79,220,221] They found the mGSSG analogue consisting of two methylated cysteine residues,**65,** bond to the enzyme's active site and was a mild competitive inhibitor of GR; however the distorted disulfide bond was attributed for GR inability to reduce mGSSG. Therefore, Kedrowski et al. proposed to replace one cysteine on GSSG with an unnatural cysteine analogue, **66**, in hopes the dihedral angle of C-S-S-C would be less distorted and fit the

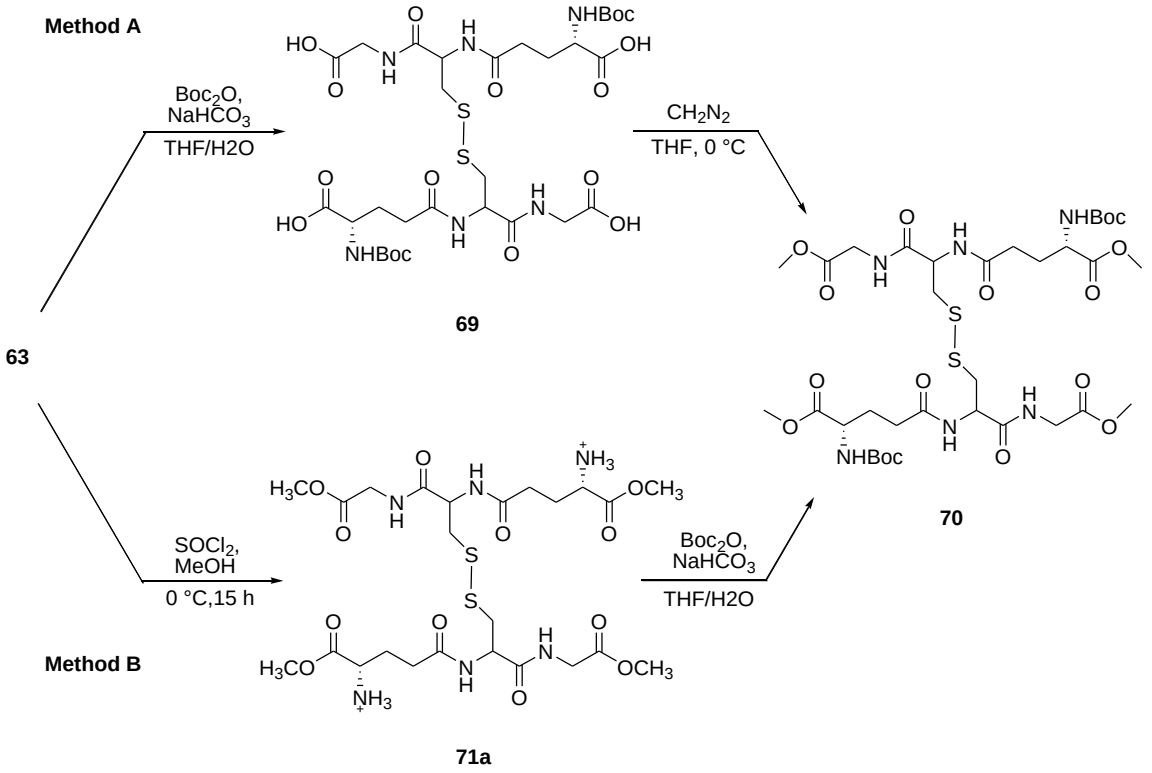
primary binding spot of the enzyme to inhibit GR. The challenge of synthesizing **66** was taken on by Masterson and Jackson.[222]



64 **67** **68**

Scheme 40. Fischer esterification and N-boc protection of GSH.

Esterified GSH, **67** (Scheme 40), was needed as an intermediate during the process of preparing, **66**. The ester functions as a convenient protecting group for carboxylic acids and was required for the coupling between natural and unnatural cysteine. Initially, a reported two-step synthesis was attempted to prepare **68**.[223] Unfortunately, under an atmosphere of HCl at 0 °C over 80 hours, multiple side products were observed making purification difficult.[224,225] Additionally, the material was extremely hygroscopic and low yielding. A repeated attempt at the esterification showed no improvement and an alternative approach was followed.



Scheme 41. Two synthetic pathways proposed to prepare the natural cysteine component of for the mixed disulfide bond of GSSG. Method (a) N-Boc protection and diazomethane esterification of GSSG. Method (b) Thionyl chloride methyl esterification and N-boc protection of GSSG.

GSSG, is significantly cheaper than GSH, and lacks the reactive sulfhydryl functional group responsible for the side reactions observed previously. Therefore, two methods for the synthesis of the natural component of the mixed disulfide bond were devised (Scheme 41). First, following Method A, GSSG was N-boc protected to **69**, and then esterified using diazomethane to give **70**. However, diazomethane is toxic, potentially explosive, and required specialized glassware not readily available. An alternative approach (Method B) was to convert the carboxylic acids into acyl chlorides with thionyl chloride and react it with an excess of methanol to form **71a**, and N-boc protect to **70**. Surprisingly, thionyl chloride esterification was highly efficient and **71a** was isolated without

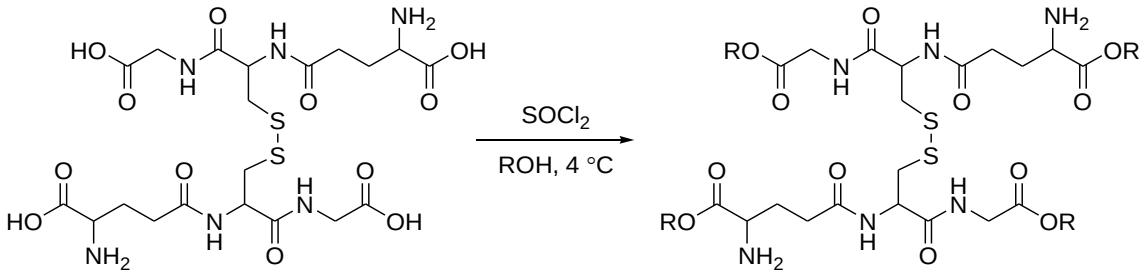
purification. In comparison to the commercial cost of the tetra ester ($1290 per 100 mg), the quantitative recovery of **71a** using the thionyl chloride made the commercial price seem inflated. The thionyl chloride esterification economic value alone is worthy of recognition.

A literature review reveals that only a few references documented the synthesis of methyl esterified GSSG. Many followed the inefficient methanol HCl esterification and column purification process.[223,225,226] Only one research group used the the modified HCl and acetyl chloride esterification route.[227] Another reference reported using TMS-Cl in methanol.[228,229] Su et al. was the only reference documenting a thionyl chloride methyl esterification.[230] After these experiments were completed, a similar method using oxalyl chloride emerged.[231] Esterified GSSG analogues have been used in photoactivated GSH/glutathione transferase tags,[228,229] protein refolding reagents,[232-234] screening of Wnt signaling inhibitors/activators,[235] GSH pseudopeptide analogues,[236,237] and the examination of metallothionein GSSG/GSH complexes.[227] The broad applications of GSSG analogues are clearly demonstrated and having methods such as the thionyl chloride esterification available is of value to the extended scientific community.

Results and Discussion

As evident from the literature review, interest in esterified GSSG analogues is widespread. During the process of preparing a GSSG tetra ester the thionyl chloride method was highly efficient. However, investigation into the exact time needed for complete esterification was missing. As a result, a study

was setup to monitor the relative GSSG rate of esterification using electrospray ionization mass spectrometry (ESI-MS). Additionally, since methyl esters are not the only esters of synthetic value, the compatibility of different alcohol solvents were explored (Scheme 42). Finally, in order to evaluate the compatibility with amino acids, the thionyl chloride esterification was applied to other peptides.



63

71a, R= -CH_3
71b, R= -CH_2CH_3
71c, R= -$CH(CH_3)_2$

Scheme 42. Thionyl chloride GSSG MS studies with methanol, ethanol, and isopropyl alcohol.

Study of thionyl chloride esterification on GSSG with various alcohols

In order to monitor the esterification, a soft ionization method via ESI-MS was developed to quantify the esterified compounds (Figure 14). All reactions were conducted at 4 °C on 250 mg of GSSG and reacted with a 3.44 M thionyl chloride solution in anhydrous methanol. At various time points 1.0 mL samples were drawn from the reaction, concentrated, and suspended into 1.0 mL of a 1% acetic acid solution of 1:1 methanol:water v/v. The reaction progress was quantified by comparing the relative signal intensity of the tetra esters to the sum of all esterified and unreacted GSSG detected. Graphs comparing the percent of GSSG tetra ester against time was plotted (Figure 15). Once the percentage of tetra ester plateaued experimental data collection was stopped and the reaction

deemed complete. All alcohol esterification reactions were repeated in triplicate. In all GSSG samples, the observed masses correlated directly to the $[M+H]^+$ esterified products. No evidence of dimers or side products were observed.

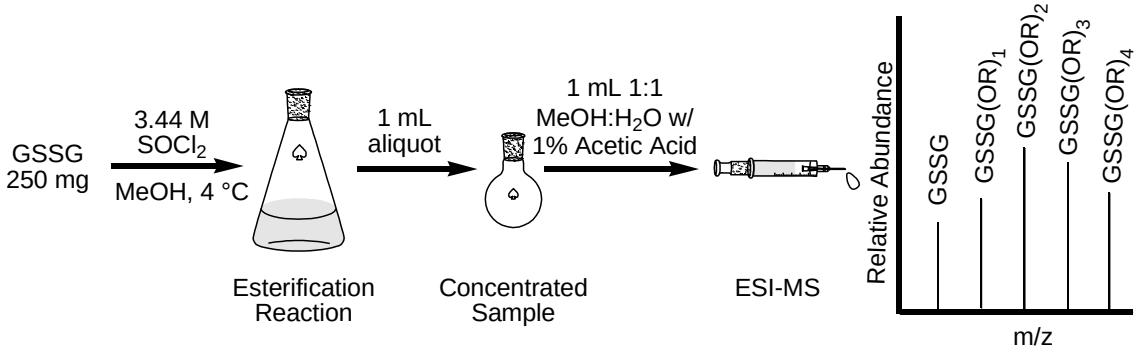


Figure 14. Illustration of the ESI-MS method used in the thionyl chloride esterification studies of GSSG.

The esterification of GSSG with methanol went to 82% completion in 16 hours before plateauing. After 24 hours, a ^{1}H-NMR was recorded, and revealed the sample to be completely esterified. Anhydrous ethanol was also capable of esterifying GSSG to **71b**, but the reaction progress was significantly slower (144 h). The isopropyl alcohol esterification differs significantly from the methanol and ethanol studies where conversion to **71c** (144 h) went only a 14% completion along with the appearance of several side products. Other secondary and tertiary alcohols were not studied, given the incompatibility of isopropyl alcohol.

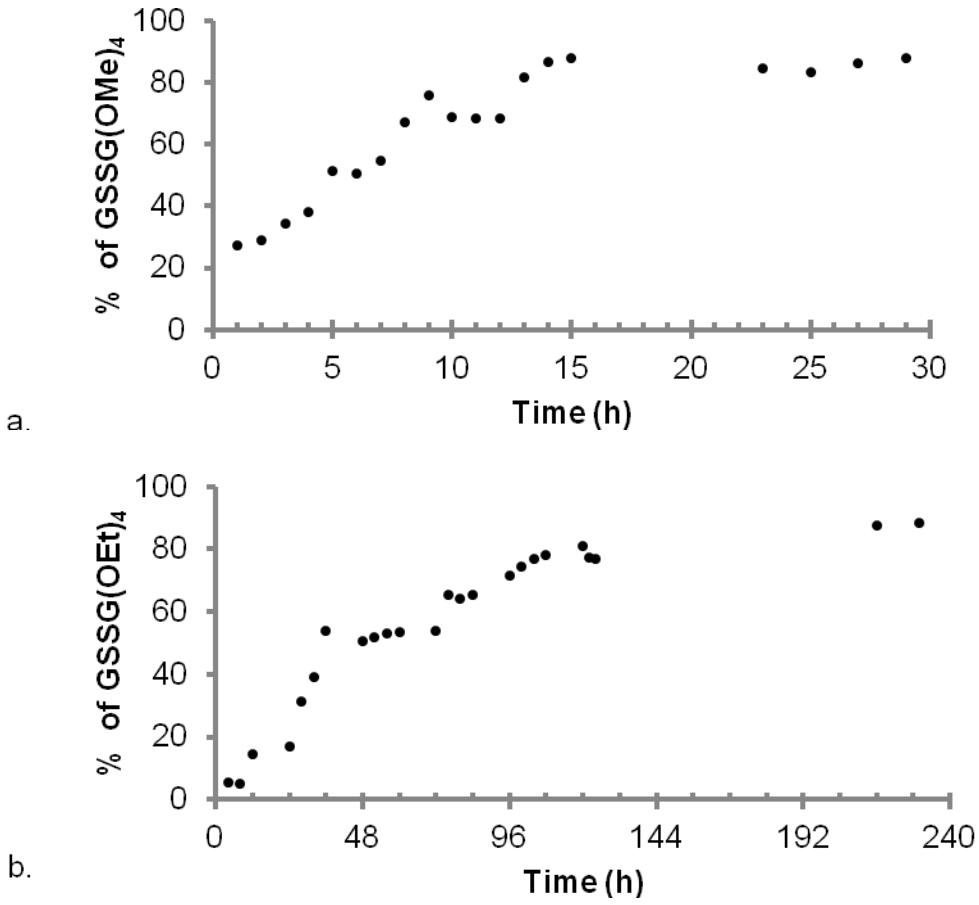


Figure 15. Plot of the observed esterification rate of GSSG with methanol and ethanol as determined by ESI-MS. Graph (a) is the esterification with thionyl chloride and methanol, and graph (b) is the thionyl chloride esterification with ethanol.

Studies of thionyl chloride methanol esterification with small peptides

In order to extend the application of the thionyl chloride method a series of methanol esterifications were conducted on other peptides (Table 2). All peptides were commercially obtained and of similar molecular weight to GSSG. All amino acids were represented to examine the general compatibility as a tool for peptide analysis. These reactions were scaled down for economical purposes and reaction progress was not monitored. Instead, 1.0 mg of peptide

was dissolved into a 3.44 M thionyl chloride methanol solution, concentrated after 24-hour incubation at 4 °C, and analyzed on the ESI-MS.

Table 2

Thionyl chloride peptide esterification study with methanol

Entry[a]	Peptide	Esterified Peptide	Peptide Sequence	Number of COOH	Estimated % Conversion[c]
1	L-glutathione oxidized	**71a**	2ECG[b]	4	100
2	L-glutathione reduced	**67**	ECG	2	26
3	Fibronectin fragment	**72**	GRADSPK	2	23
4	Bradykinin (1-7)	**73**	RPPGFSP	1	63
5	Necrofibrin, rat	**74**	WTVPTA	1	64
6	[D-Ala2,D-Met5]-Enkephalin	**75**	YAGFM	1	90
7	Angiotensin II, human	**76**	DRVYIHPF	2	100
8	Thymopentin (TP-5)	**77**	RKDVY	2	100
9	Neurotensin (9-13)	**78**	RPYIL	1	100
10	[Ile3]-Pressinoic acid	**79**	CYIQNC[b]	1	94

Notes (a) Reaction conditions of the peptides were run using 3.44 M thionyl chloride in methanol at 4 °C for 24 h. (b) Peptide contains a disulfide bridge between the two cysteine residues. (c) Estimate based on the relative abundance of product(s) compared to total relative abundance of all fragments.

In many peptides (Table 2), the reaction proceeded smoothly (Entries 1 & 6-10) with near complete conversion to esterified product. Other peptides (Entries 2-5) were not as tolerant to the reaction conditions. Common to both Entry 3 and Entry 4 was a fragment with 18 AMU addition from the expected esterified mass and a signature 3:1 chlorine isotopic ratio. Based on this evidence it was concluded that both peptides were esterified but serine residues were chemically modified to an alkyl chloride (Figure 16).[238-240] Since thionyl chloride is a chlorinating reagent, these results were not surprising. Still, the esterified mass containing the unaltered serine was present indicating the potential for reaction optimization. Moreover, the estimated conversion is significantly different between Entry 3 and Entry 4. In the case of the fibronectin fragment, chlorinated compound **80** was the major component but in bradykinin **81** was the minor product. It is true these results could be the difference in ionization of the peptides. However, in comparison to bradykinin, the spectra of fibronectin contained many other unidentified fragments suggesting location of the serine in sequence may be a contributing factor. On both peptides, proline is attached to the C-terminus of serine but differ in their N-terminus attachments. The fibronectin fragment contains two basic (lysine and arginine) and one acid (aspartic acid) amino acid in its sequence. Apart from serine, bradykinin only has arginine at the N-terminus and overall is more hydrophobic than fibronectin making bradykinin less prone to reactivity. Unfortunately, additional studies of the serine residues were not explored and are left for future studies. Other amino acids containing alcohol side chains, threonine and tyrosine, were also

analyzed. Interestingly, both underwent smooth conversion to the ester with no evidence of the alkyl chloride.

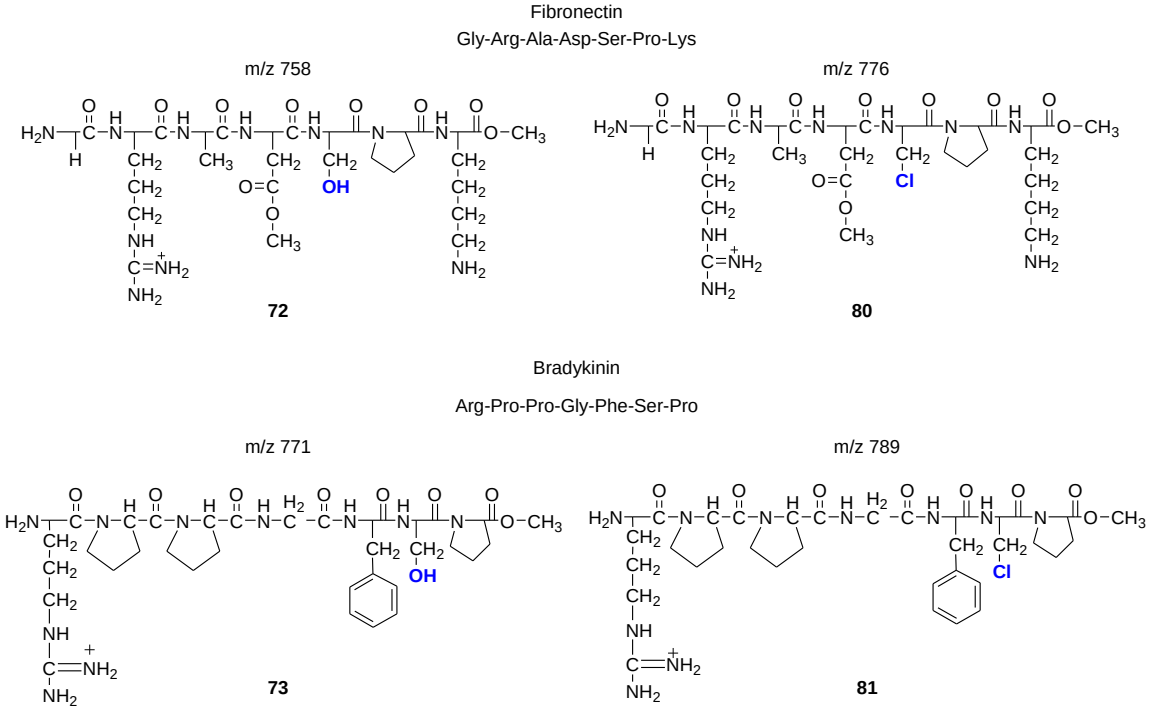


Figure 16. Observed modifications of fibronectin and bradykinin by thionyl chloride.

Another peptide demonstrating incomplete formation of the tetra ester was necrofibrin (Entry 5). The expected mono ester was observed in 64% with two impurities. These impurities were traced back to masses present on the supplied manufactures certificate of analysis and not an impurity arising from thionyl chloride esterification. The only other incompatibility arose from the free sulfhydryl of cysteine on GSH (Entry 2) where minor amounts of esterified GSH were observed. In addition, the reaction contained significant amounts of oxidized GSSG and other unidentified species. However, methionine of Enkephalin and the disulfide of [Ile3]-Pressinoic acid and GSSG were cleanly converted to their respective esters. Additionally, aspartic acid and glutamic acid

were both esterified without side reactions. All other proteinogenic amino acids were tolerant with thionyl chloride reaction conditions.

Future Directions

After completing this short study many questions have evolved. Literature suggests higher temperatures could be used to increase the reaction rate with higher ordered alcohols.[199] If GSSG is capable of withstanding increased temperatures what are those limits? If higher temperatures are possible then higher order alcohols could be studied and shorter reaction times would be expected. Also, a complete investigation of reaction rates would include varying the proportions of each reactants. Since this study strictly focused on replicating the initially discovered 3.44 M thionyl chloride concentration it was held constant. Based on other research[212] this thionyl chloride concentration seems excessive, but yet effective. How will the increase/decrease of thionyl chloride concentration affect the observed rate?

The small peptide esterifications were not repeated in triplicate; therefore, good analytical practices dictates these studies should be repeated. First, a scale up, isolation, and complete characterization of the esterified peptides would eliminate any experimental error in these studies. Additionally, physically isolating the peptides will eliminate any discrepancy in the observed side products. If these results agree with the recorded mass data then further analysis is unnecessary. However, it would be interesting to see if alkyl chloride formation could be controlled on the peptides containing serine. The different proportion of chlorinated serine suggests that control of the chlorination may be

possible. It is possible that the location of the serine residue in the peptide sequence may govern the extent of the formed alkyl chloride. Finally, other cysteine containing peptides should be considered. Since the free sulfhydril can readily undergo oxidation, the addition of β-mercaptoethanol could prevent the formation of the oxidized disulfide.

Conclusions

In conclusion, GSSG methyl esters were esterified with thionyl chloride and methanol in high yields requiring no purification. GSSG ethyl esters also were prepared with thionyl chloride but required longer reaction times at the expense of increased reaction times. These conditions were replicated on a 1.0 mmol scale with small peptides comparable in size to GSSG and found to be highly compatible. The only observed limitations occurred in cases where serine and cysteine were present in the peptide sequence. All other amino acids were tolerant of the reactions conditions.

Experimental

General experimental

NMR spectra were acquired on a Bruker 400 MHz NMR in proton-decoupled mode. Residual solvent signal for MeOD was used as an internal standard in the NMR experiments. ESI MS was carried out on a ThermoFisher LXQ ESI-Ion trap mass spectrometer using Optima LCMS grade methanol and water from Fisher Scientific. Methanol was distilled from calcium hydride, and 2-propanol distilled from sodium. Prior to use, absolute ethanol was stored over molecular sieves for 24h. L-oxidized glutathione and reduced glutathione were

purchased directly from Sigma-Aldrich. American Peptide Company supplied the peptides and peptide fragments and were used as received. Each peptide and peptide fragment came furnished with Certificate of Analysis (COA) and some contained trace impurities. Trace impurities are noted in the MS tables in the appendix.

LXQ instrument conditions

The LXQ was tuned with a 1.0 mg/mL sample of GSSG dissolved into 1% acetic acid 1:1 MeOH:H_2O (v/v) solution. The sample was ionized at 320 °C and tuned to detect $[M+H]^+$ of GSSG (*m/z* = 612). After a steady signal was obtained, the file was saved and used as a tune file for all experiments.

General thionyl chloride esterification procedures for alcohol studies

In a 125 mL Erlenmeyer flask, L-oxidized glutathione (0.408 mmol, 0.250 g) was added to 50 mL of alcohol, capped with a rubber septum, and cooled to 0 °C. Thionyl chloride (34.4 mmol, 2.5 mL) was added slowly; the flask swirled, and placed into a refrigerator at 4 °C. The reaction was monitored by taking 1.0 mL reaction aliquots, concentrating the samples under reduced pressure at 35 °C, and suspending the crude material1.0 mL of a 1% acetic acid 1:1 MeOH:H_2O (v/v) solution. All samples were analyzed on the ESI-MS using the GSSG LXQ optimized tune file.

*Oxidized glutathione methyl ester (**71a**):*

The above general experimental conditions were followed for the synthesis of **71a.** Recorded characterization data matched ^{1}H-NMR and ^{13}C-NMR reported by literature.[229] ^{1}H-NMR (400MHz, CD_3OD) δ 4.67 (m, 2H), 4.05

(m, 2H), 3.88 (s, 4H), 3.76 (s, 6H), 3.62 (s, 6H), 3.21 (s, 4H), 2.93-2.81 (m, 2H), 2.49 (m, 4H), 2.12 (m, 4H). ^{13}C-NMR (100MHz, CD_3OD) δ 174.3, 173.0, 171.6, 170.7, 54.0, 53.9, 53.7, 52.8, 42.0, 41.4, 32.3, 27.0. LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{24}H_{44}N_6O_{12}S_2$ 669.2; Found 669.2.

*Oxidized glutathione ethyl ester (**71b**):*

The above general experimental conditions were followed for the synthesis of **71b.** Recorded characterization data matched ^{1}H-NMR and ^{13}C-NMR reported by literature.[235] ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 4.78 (dd, J = 9.6, 4.6 Hz, 2H), 4.33 (q, J = 7.1 Hz, 4H), 4.19 (q, J = 7.1 Hz, 4H),4.13(m, 2H), 3.97 (d, J = 2.2 Hz, 4H), 3.28 (dd, J = 14.0, 4.6 Hz, 2H), 2.99 (dd, J = 13.9, 9.7 Hz, 2H), 2.61 (t, J = 7.1 Hz, 4H), 2.23 (m, J = 21.7, 14.6, 7.5 Hz, 4H), 1.35 (t, J = 7.1 Hz, 6H), 1.28 (t, J = 7.2 Hz, 6H). ^{13}C-NMR(100MHz, CD_3OD) δ 174.4, 173.0, 171.1, 170.2, 63.4, 62.4, 53.9, 53.7, 42.1, 41.3, 32.6, 27.0, 14.5, 14.43. LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{28}H_{44}N_6O_{12}S_2$725.3; Found, 725.3.

*Oxidized glutathione isopropyl ester (**71c**):*

The above general experimental conditions were followed for the synthesis of **71c.** LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{32}H_{57}N_6O_{12}S_2$ 781.4; Found 781.3.

*Reduced glutathione methyl ester (**67**):*

The general experimental conditions were followed for the synthesis of **67.** LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{12}H_{22}N_3O_6S$ 336.1; Found 336.1.

General thionyl chloride esterification procedures for peptide studies:

The evaluation of the small molecule peptides were scaled down and all performed in methanol. Only 1.0 mg of peptide was used in each of the studies, and was incubated at 4 °C for 24 h. For every carboxylic acid present in the peptide, 756 equivalents of MeOH and 105 equivalents of thionyl chloride were used to give an overall 3.44 M solution of thionyl chloride in methanol. At the end of the incubation the crude material was concentrated, dissolved into 1% acetic acid 1:1 MeOH:H_2O solution, and analyzed on the ESI-MS.

*Fibronectin methyl ester (**72**):*

A vial containing Fibronectin (1 µmol, 1.0 mg) was dissolved into anhydrous MeOH (2.075 mmol, 83.9 µL) and placed on an ice bath at 0 °C. Thionyl chloride (0.289 mmol, 21.0 µL) was added slowly, the vial swirled, and placed into a refrigerator at 4 °C. After 24 h the sample was concentrated, dissolved into 1.0 ml of 1% acetic acid 1:1 MeOH:H_2O (v/v), and analyzed on the ESI-MS. LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{31}H_{56}N_{11}O_{11}$ 758.4; Found 758.4.

*Bradykinin methyl ester (**73**):*

A vial containing Bradykinin (1 µmol, 1.0 mg) was dissolved into 40.5 µL of anhydrous methanol (1.00 mmol, 40.5 µL) and placed on an ice bath at 0 °C. Thionyl chloride (0.14 mmol, 10.1 µL) was added slowly, the vial swirled, and placed into the refrigerator at 4 °C. After 24 h the sample was concentrated under reduced pressure, suspended into 1.0 ml of 1% acetic acid 1:1 MeOH:H_2O (v/v), and analyzed on the ESI-MS. LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{36}H_{55}N_{10}O_9$ 771.41; Found771.4.

*necrofibrin, rat methyl ester (**74**):*

A vial containing necrofibrin, rat (1 µmol, 1.0 mg) was dissolved into anhydrous methanol (1.12 mmol, 45.5 µL) and placed on an ice bath at 0 °C. Thionyl chloride (0.156 mmol, 11.4 µL) was added slowly, swirled, and placed into a refrigerator at 4 °C. After 24 h, the sample was concentrated under reduced pressure, suspended into 1.0 ml of 1% acetic acid 1:1 MeOH:H_2O (v/v), and analyzed on the ESI-MS. LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{33}H_{50}N_7O_9$ 688.37; Found 688.2.

*[DAla2,DMet5] Enkephalin methyl ester (**75**):*

A vial containing [DAla2,DMet5] Enkephalin (2 µmol, 1.0 mg) dissolved into anhydrous methanol (1.29 mmol, 52.1 µL) and placed on an ice bath at 0 °C. Thionyl chloride (0.18 mmol, 13.0 µL) was slowly added, swirled, and placed into a refrigerator at 4 °C. After 24 h the sample was concentrated under reduced pressure, dissolved into 1.0 ml of 1% acetic acid in a 1:1 solution of methanol:water, and analyzed on the ESI-MS. LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{29}H_{40}N_5O_7S$ 602.3; Found 602.2.

*angiotensin II, human methyl ester (**76**):*

A vial containing angiotensin II, human (1 µmol, 1.0 mg) was dissolved into anhydrous methanol (1.45 mmol, 58.6 µL) and placed on an ice bath at 0 °C. Thionyl chloride (0.20 mmol, 14.6 µL) was slowly added, swirled, and placed into a refrigerator at 4 °C. After 24 h, the sample was concentrated under reduced pressure, dissolved into 1.0 ml of 1% acetic acid 1:1 MeOH:H_2O (v/v), and

analyzed on the ESI-MS. LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{52}H_{76}N_{13}O_{12}$ 1074.57; Found 1074.6.

Thymopentin (TP-5) methyl ester (**77**):

A vial containing Thymopentin (TP-5) (1 µmol, 1.0 mg) was dissolved into anhydrous methanol (2.22 mmol, 90.1 µL) and placed on an ice bath at 0 °C. Thionyl chloride (0.289 mmol, 22.5 µL) was slowly added, swirled, and placed into the fridge at 4 °C. After 24 h the sample was concentrated under reduced pressure, dissolved into 1.0 ml of 1% acetic acid 1:1 MeOH:H_2O (v/v), and analyzed on the ESI-MS. LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{32}H_{54}N_9O_9$ 708.4; Found 708.4. Masses present in starting material from COA: 341(14%), 680 (100%), 1358(8%).

*Neurotensin methyl ester (**78**):*

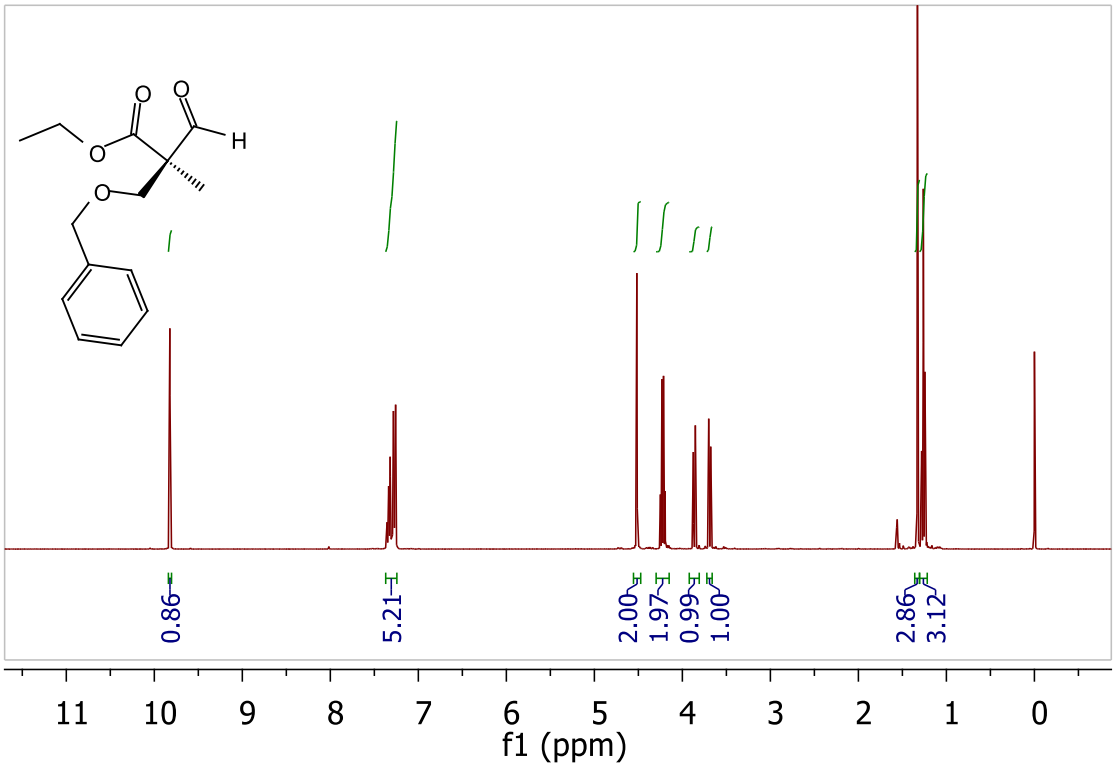
A vial containing Neurotensin (9-13) (1 µmol, 1.0 mg) was dissolved into anhydrous methanol (2.22 mmol, 90.1 µL) and placed on an ice bath at 0 °C. Thionyl chloride (0.289 mmol, 22.5 µL) was slowly added, swirled, and placed into the fridge at 4 °C. After 24 h, the sample was concentrated under reduced pressure, dissolved into 1.0 ml of 1% acetic acid 1:1 MeOH:H_2O (v/v), and analyzed on the ESI MS. LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{32}H_{54}N_9O_9$ 675.4; Found675.4.

*[Ile3] Pressinoic Acid methyl ester (**79**):*

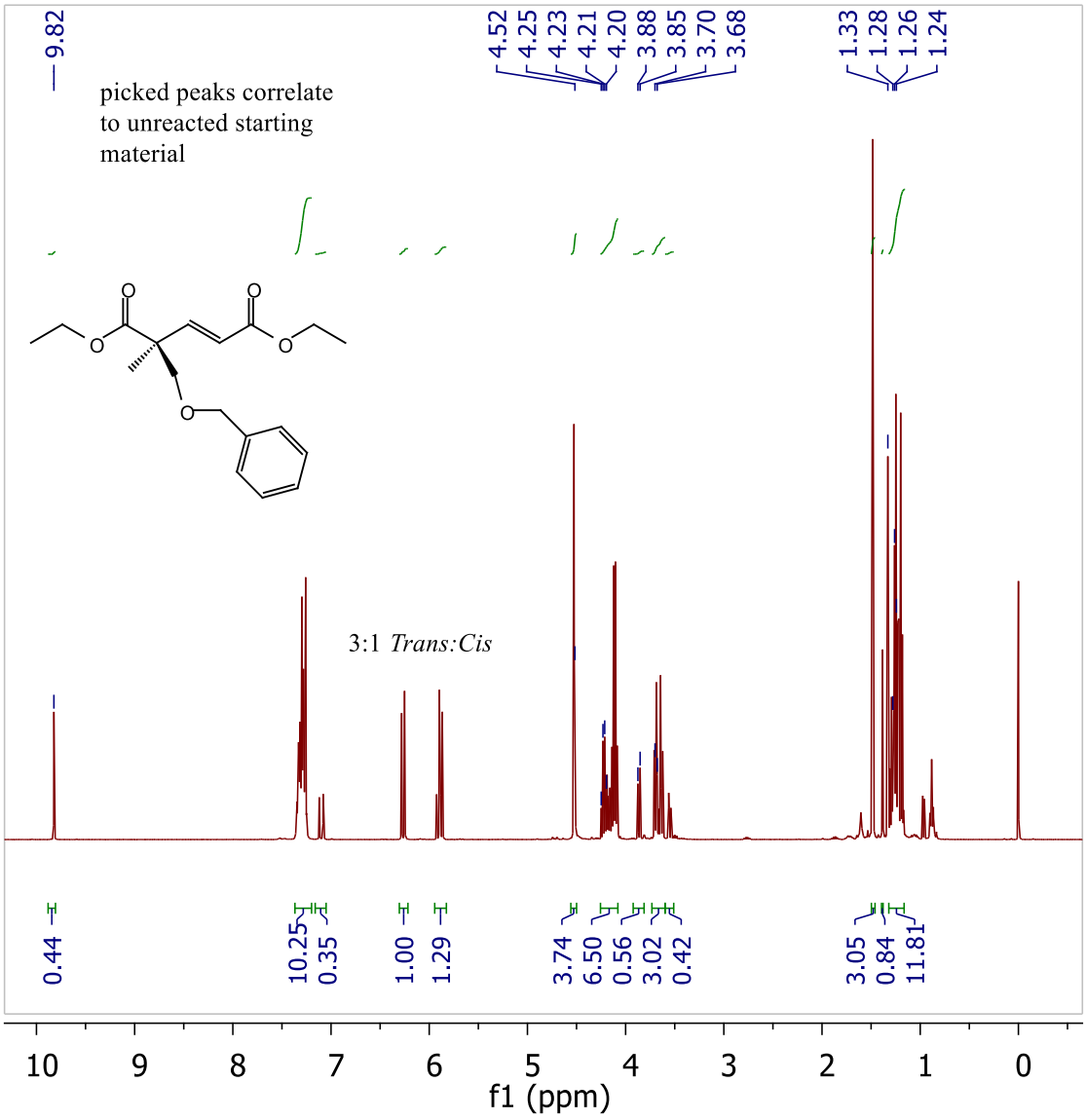
A vial containing [Ile3] Pressinoic Acid (1 µmol, 1.0 mg) was dissolved into anhydrous methanol (1.02 mmol, 41.3 µL) and placed on an ice bath at 0 °C. Thionyl chloride (0.14 mmol, 10.3 µL) was slowly added, swirled, and placed into

a refrigerator at 4 °C. After 24 h the sample was concentrated under reduced pressure, dissolved into 1.0 ml of 1% acetic acid 1:1 MeOH:H_2O (v/v), and analyzed on the ESI-MS. LRMS (ESI-MS) *m/z*: $[M+H]^+$ Calcd for $C_{31}H_{47}N_8O_{10}S_2$ 755.2; Found 755.2.

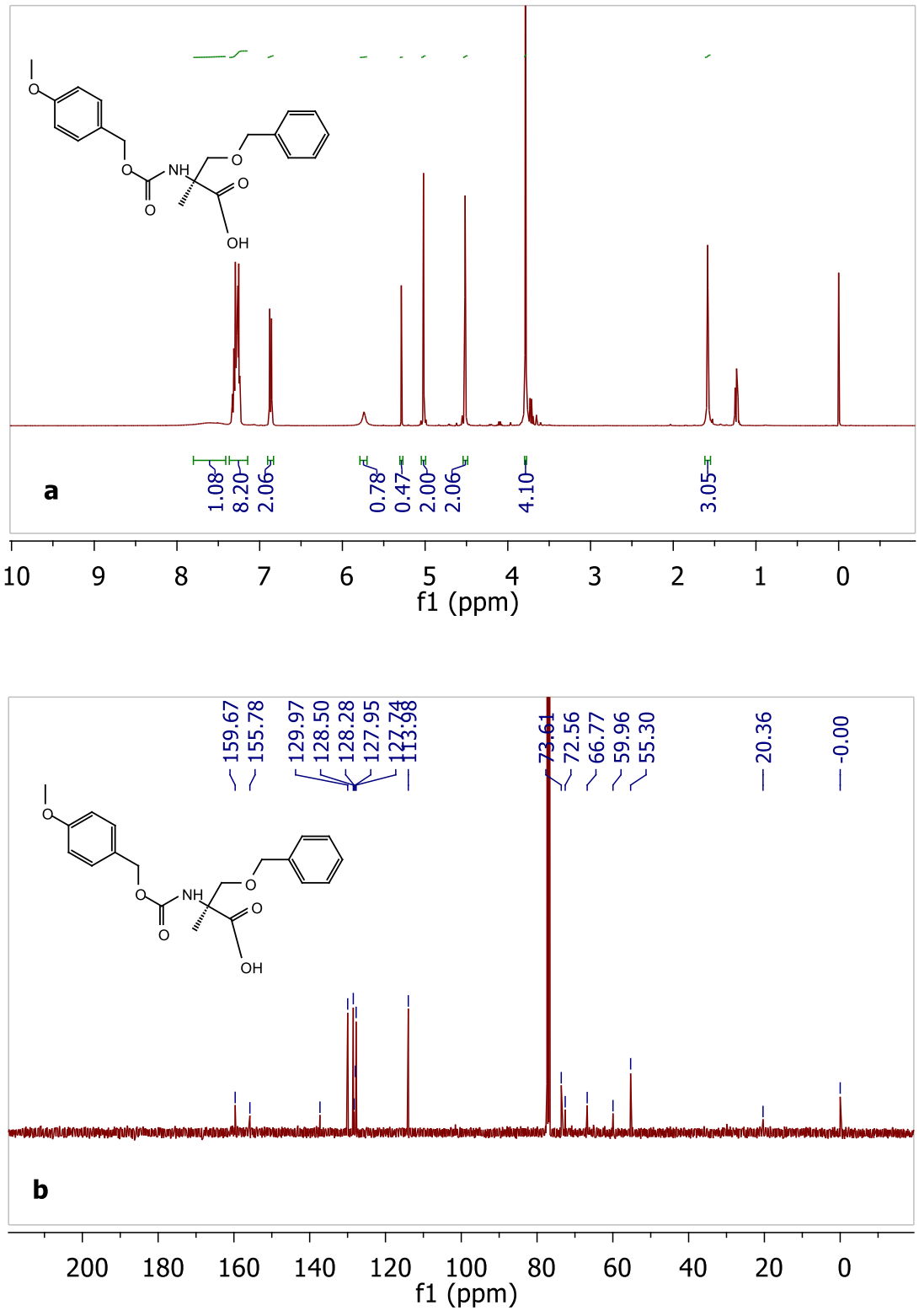
APPENDIX



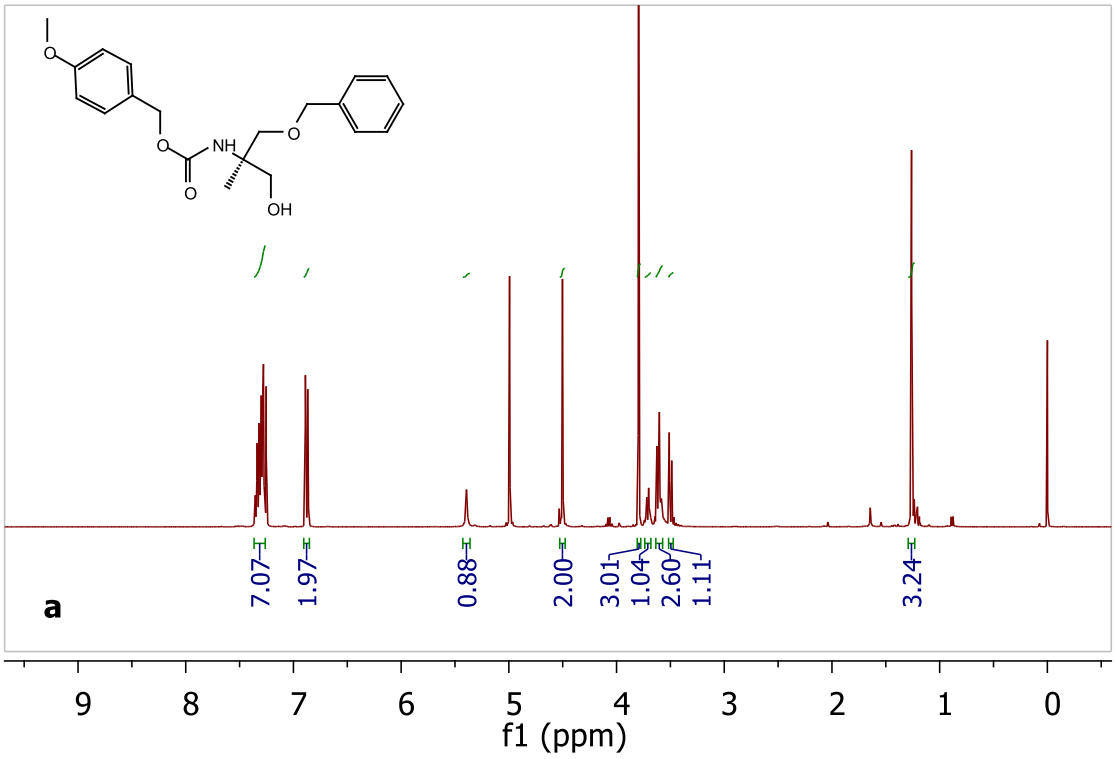
A. 1 ^{1}H-NMR of **6**.

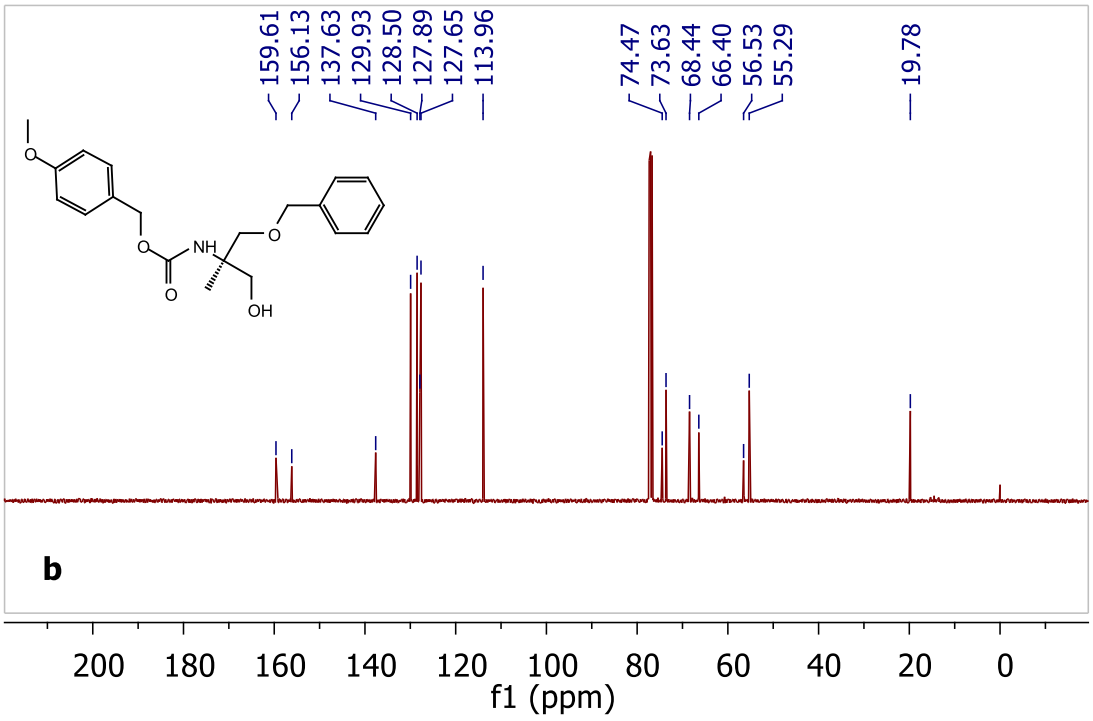


A. 2 Crude ^{1}H-NMR mixture of *cis/trans* isomers of **8** and unreacted **6**.

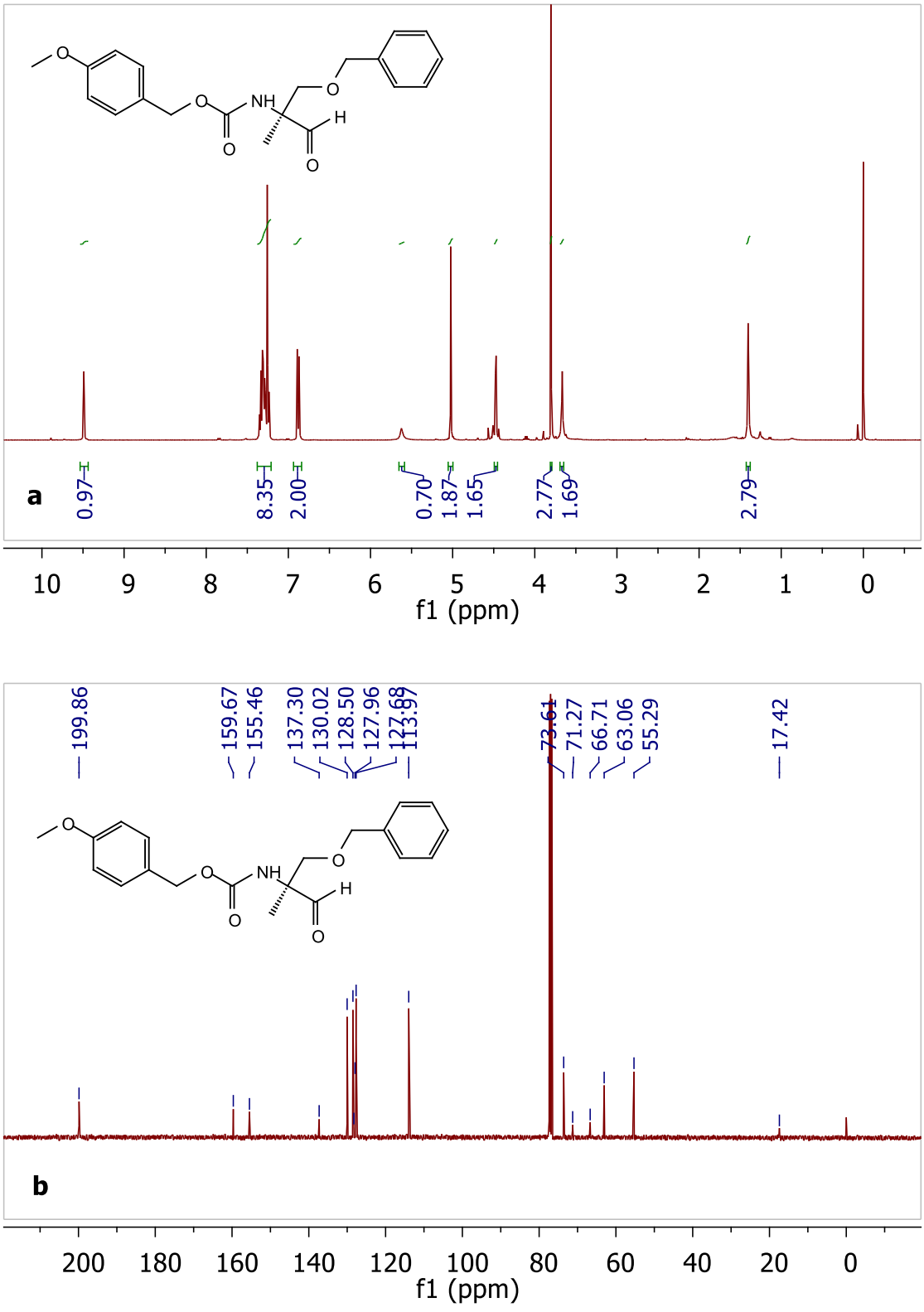


A. 3 a) ^{1}H-NMR of **12**; b) ^{13}C-NMR of **12**.

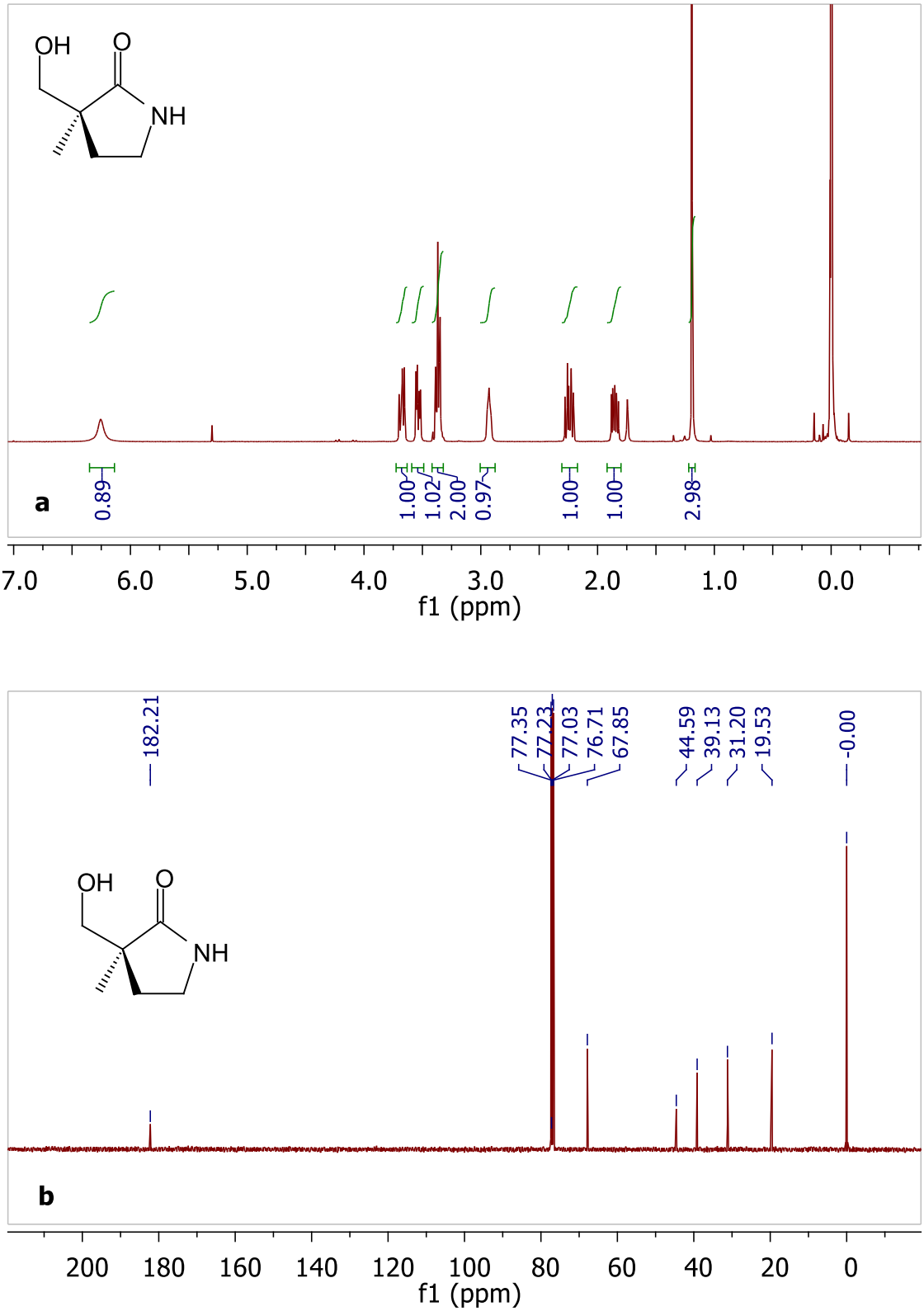




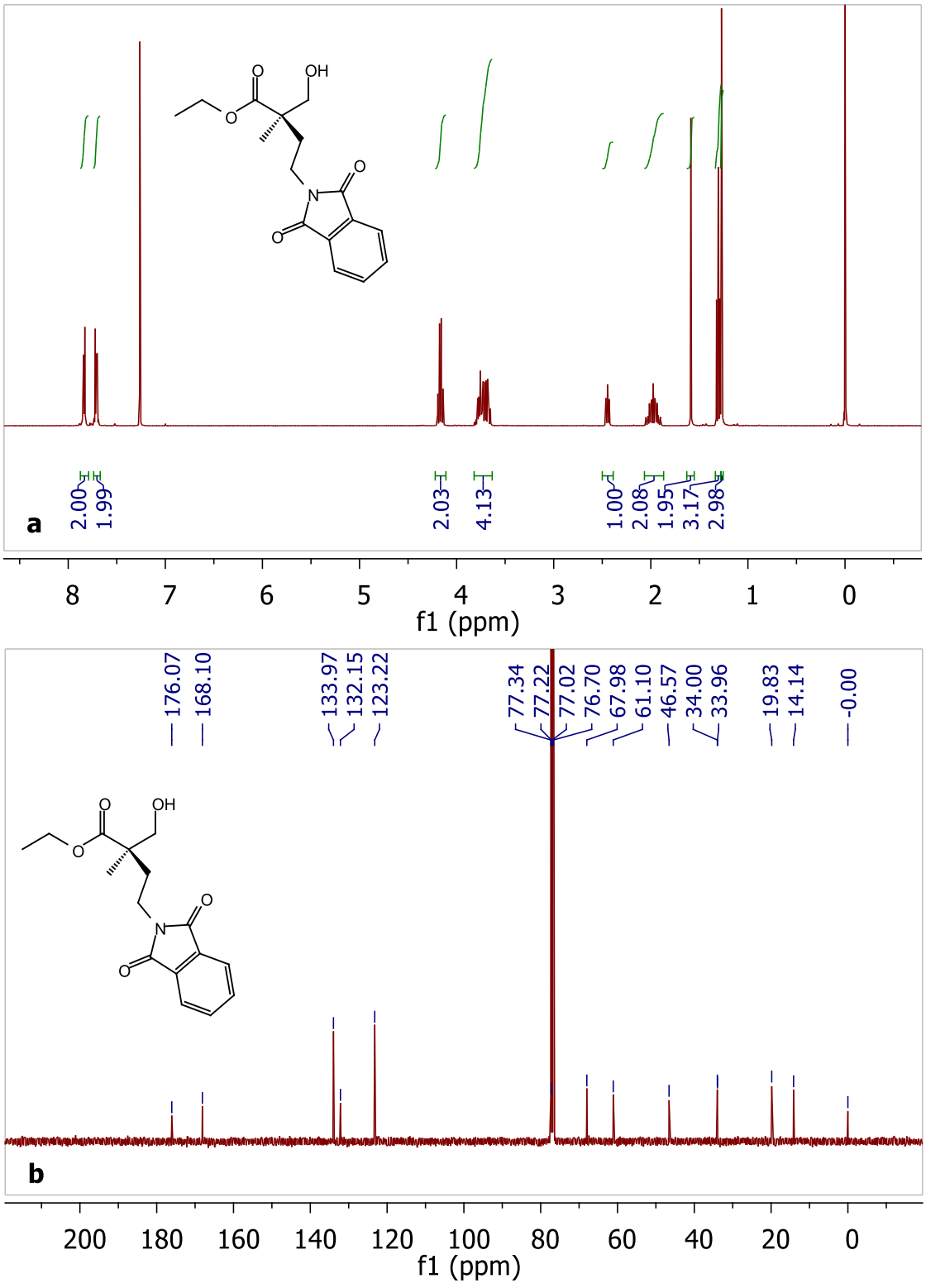
A. 4 a) ^{1}H-NMR of **13**; b) ^{13}C-NMR of **13.**



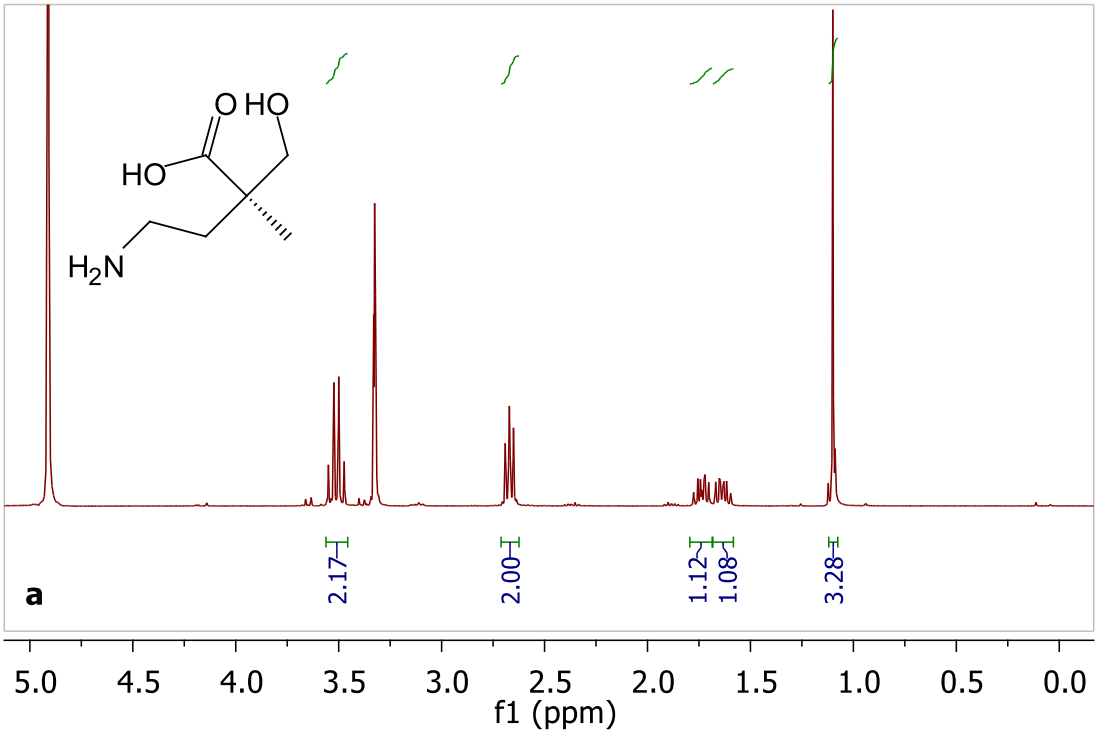
A. 5 a) ^{1}H-NMR of **14**; b) ^{13}C-NMR of **14**.

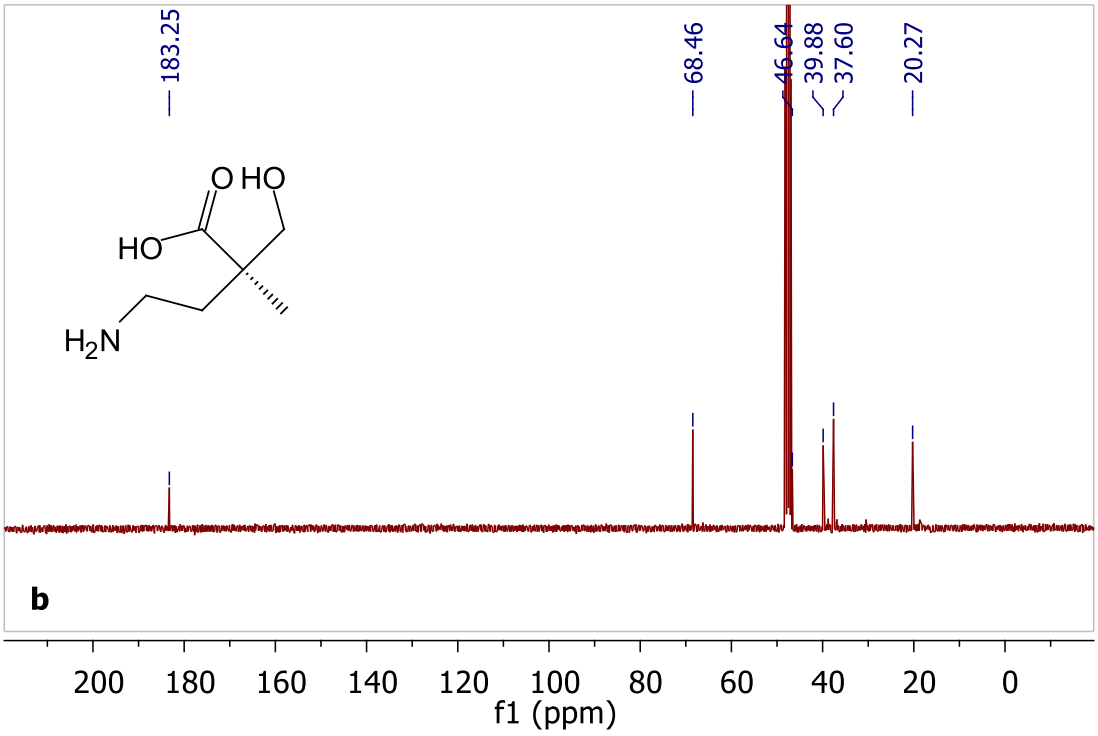


A. 6 a) ^{1}H-NMR of **17**; b) ^{13}C-NMR of **17**.

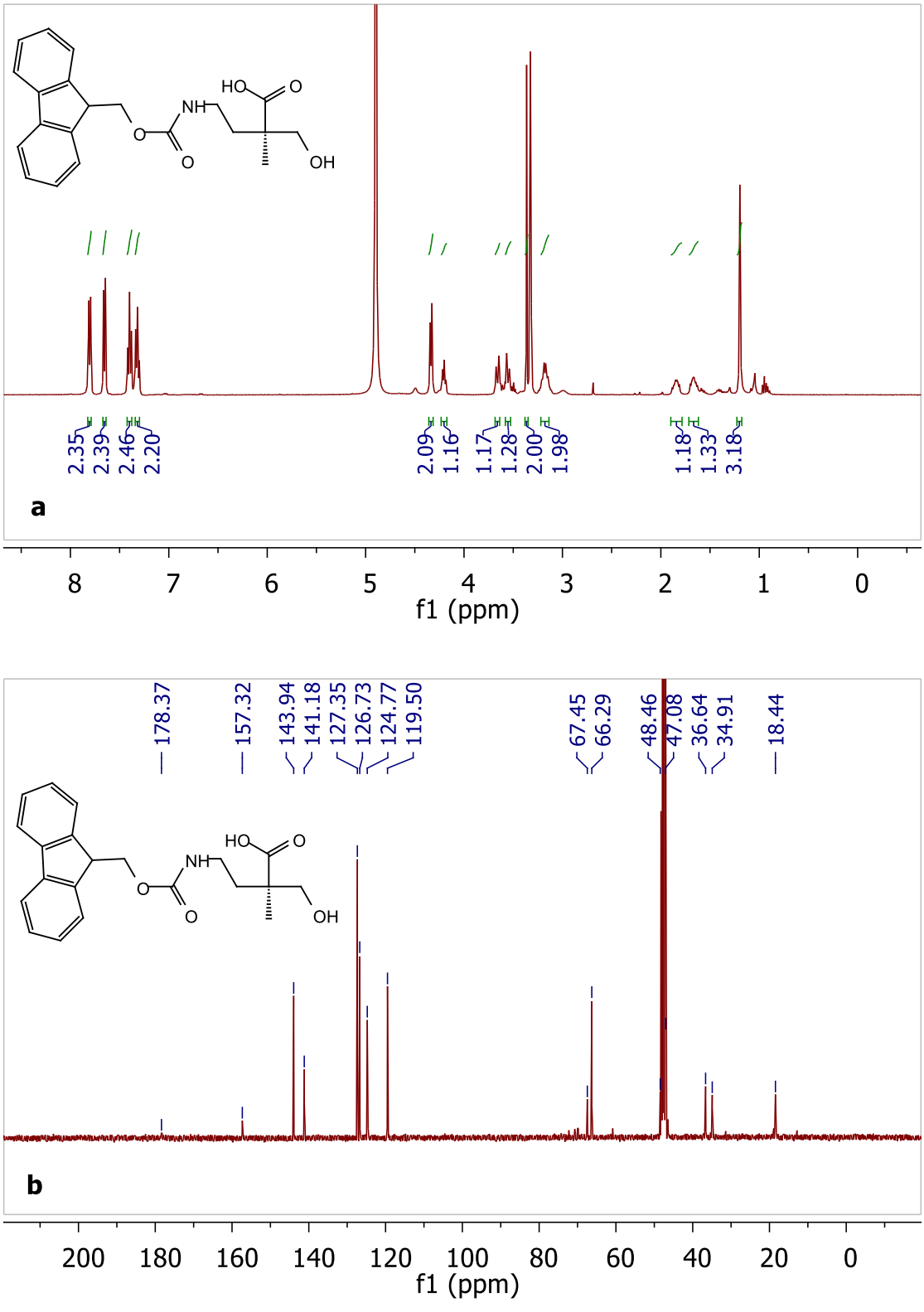


A. 7 a) ^{1}H-NMR of **21**; b) ^{13}C-NMR of **21**.

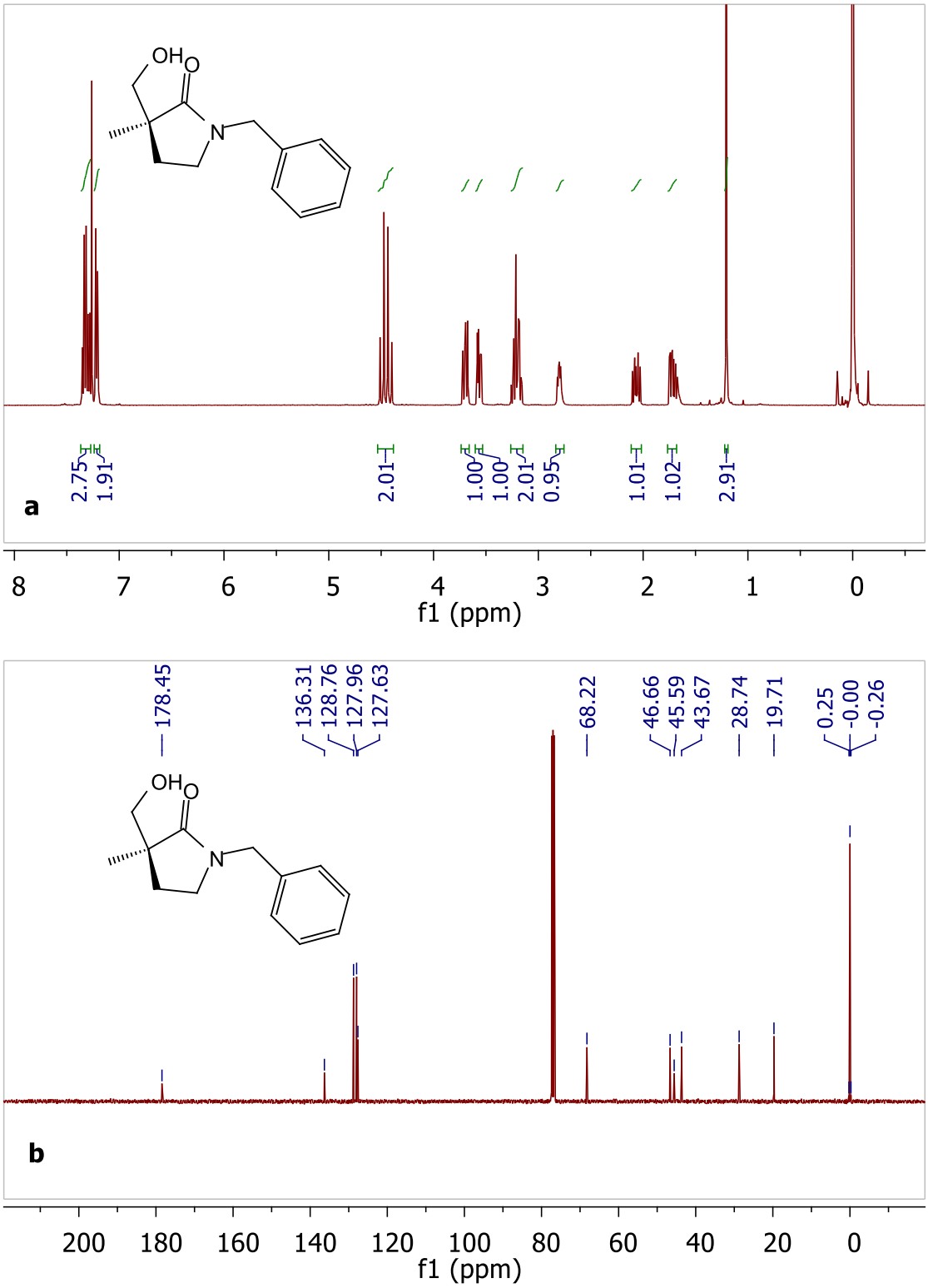




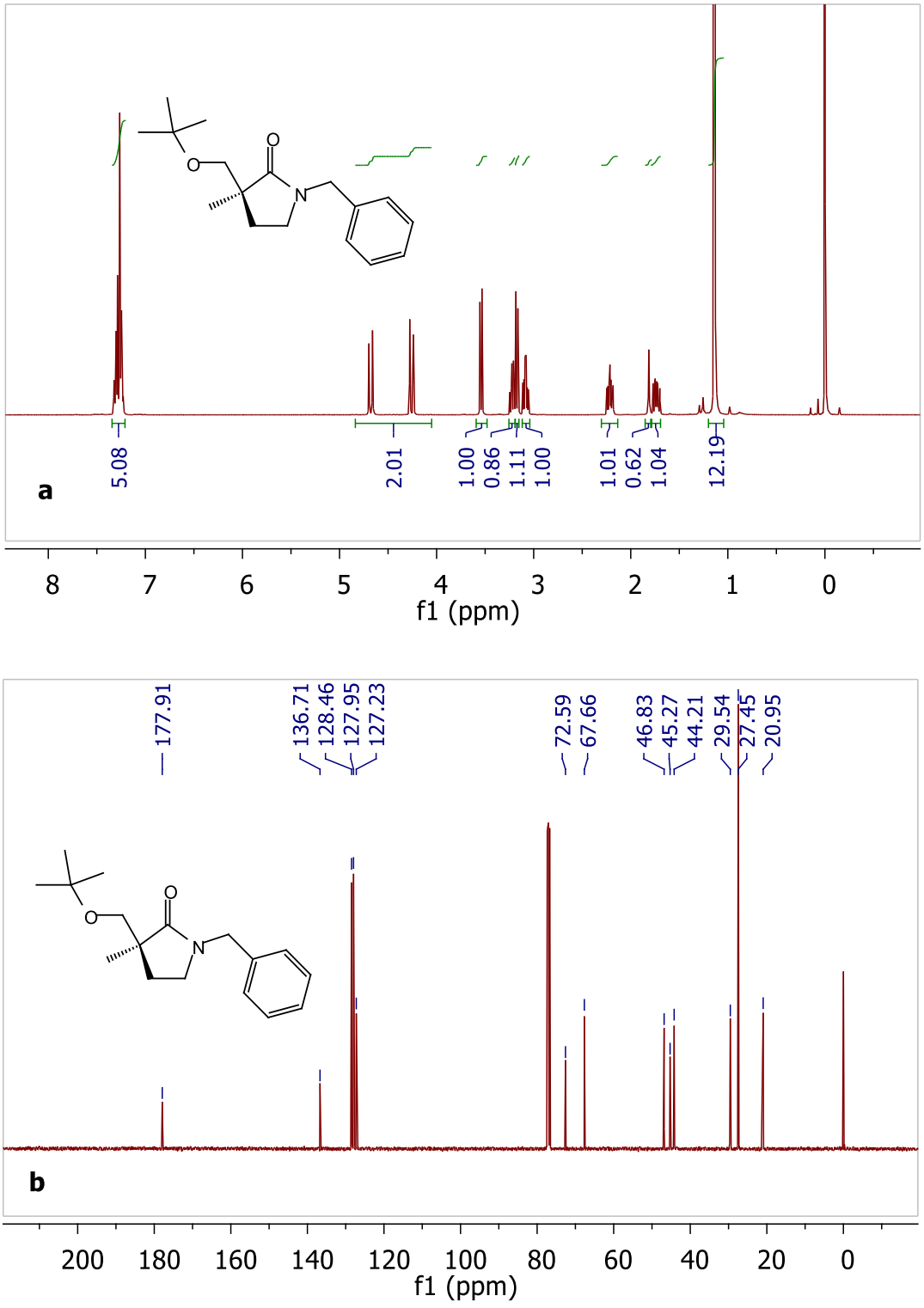
A. 8 a) ^{1}H-NMR of **28**; b) ^{13}C-NMR of **28**.



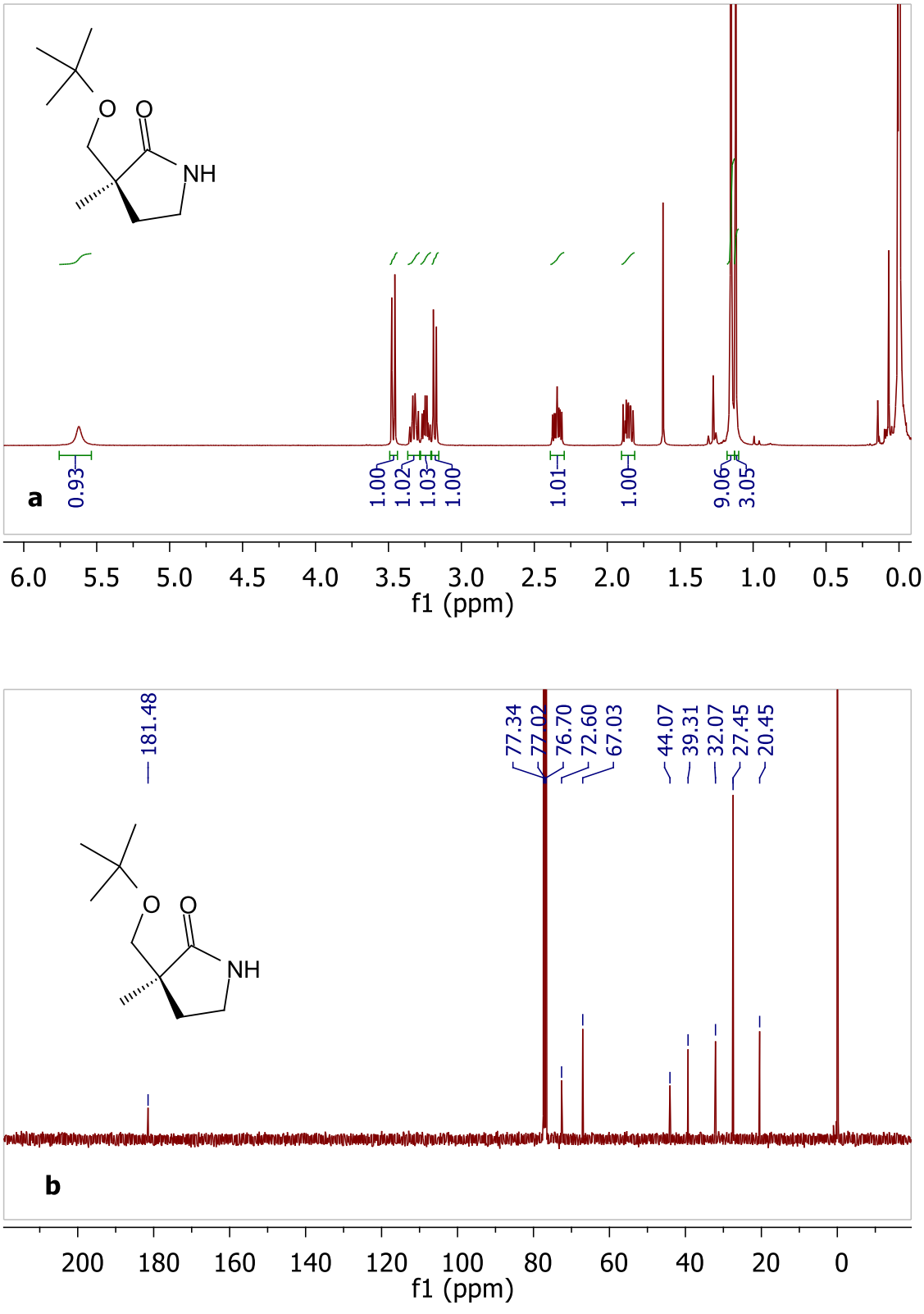
A. 9 a) ^{1}H-NMR of **29**; b) ^{13}C-NMR of **29**.



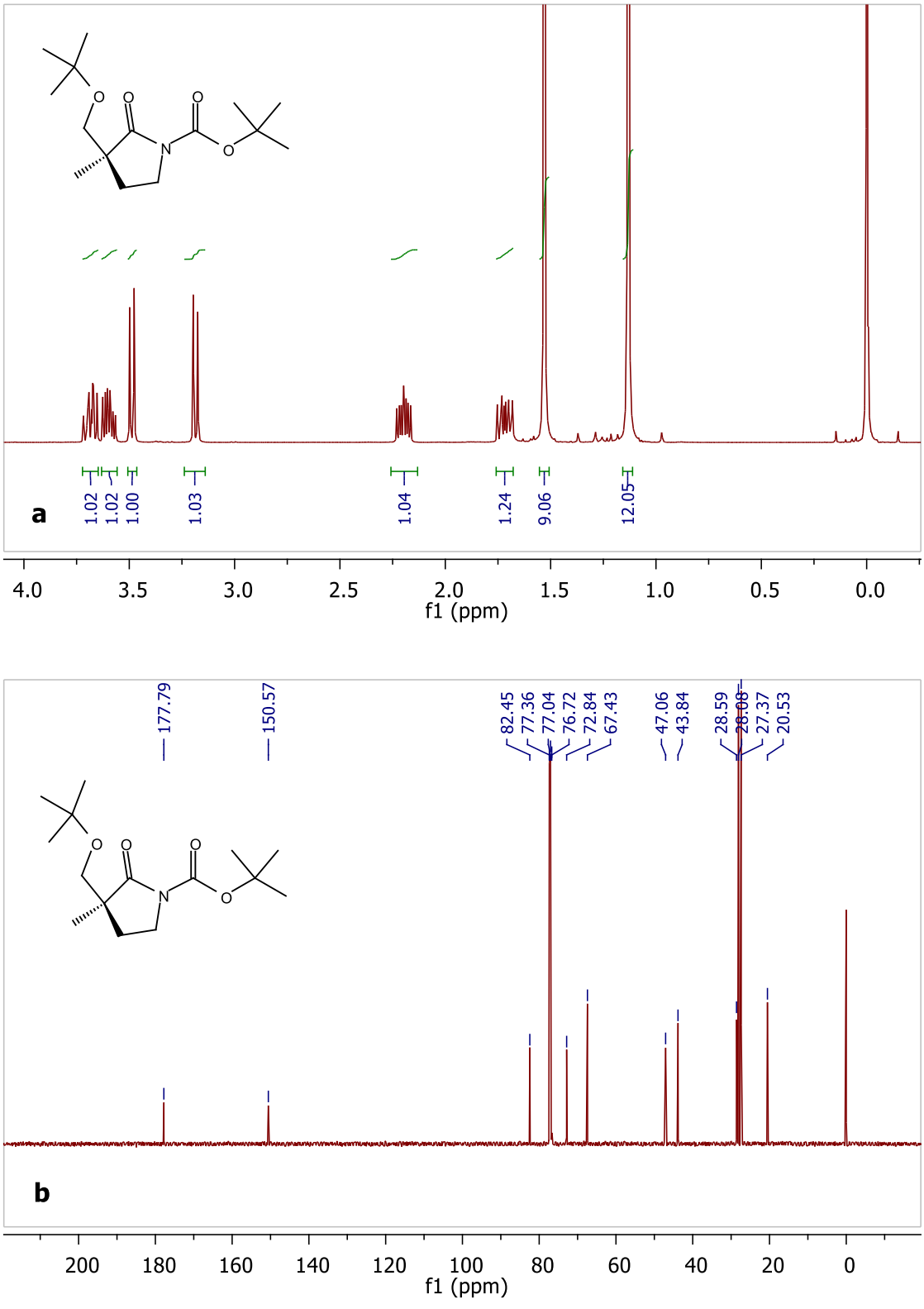
A. 10 a) ^{1}H-NMR of **32**; b) ^{13}C-NMR of **32**.



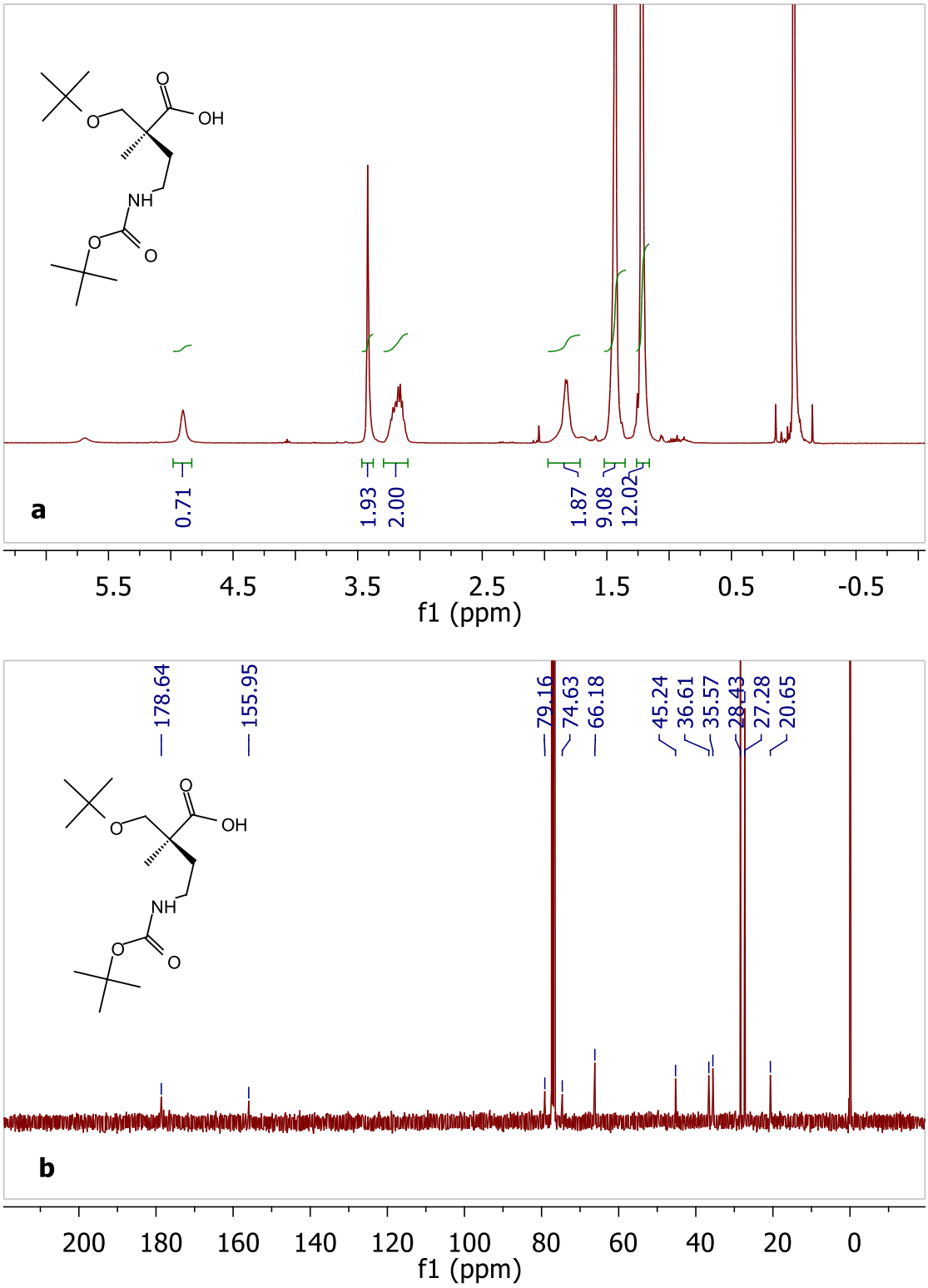
A. 11 a) ^{1}H-NMR of **33**; b) ^{13}C-NMR of **33**.



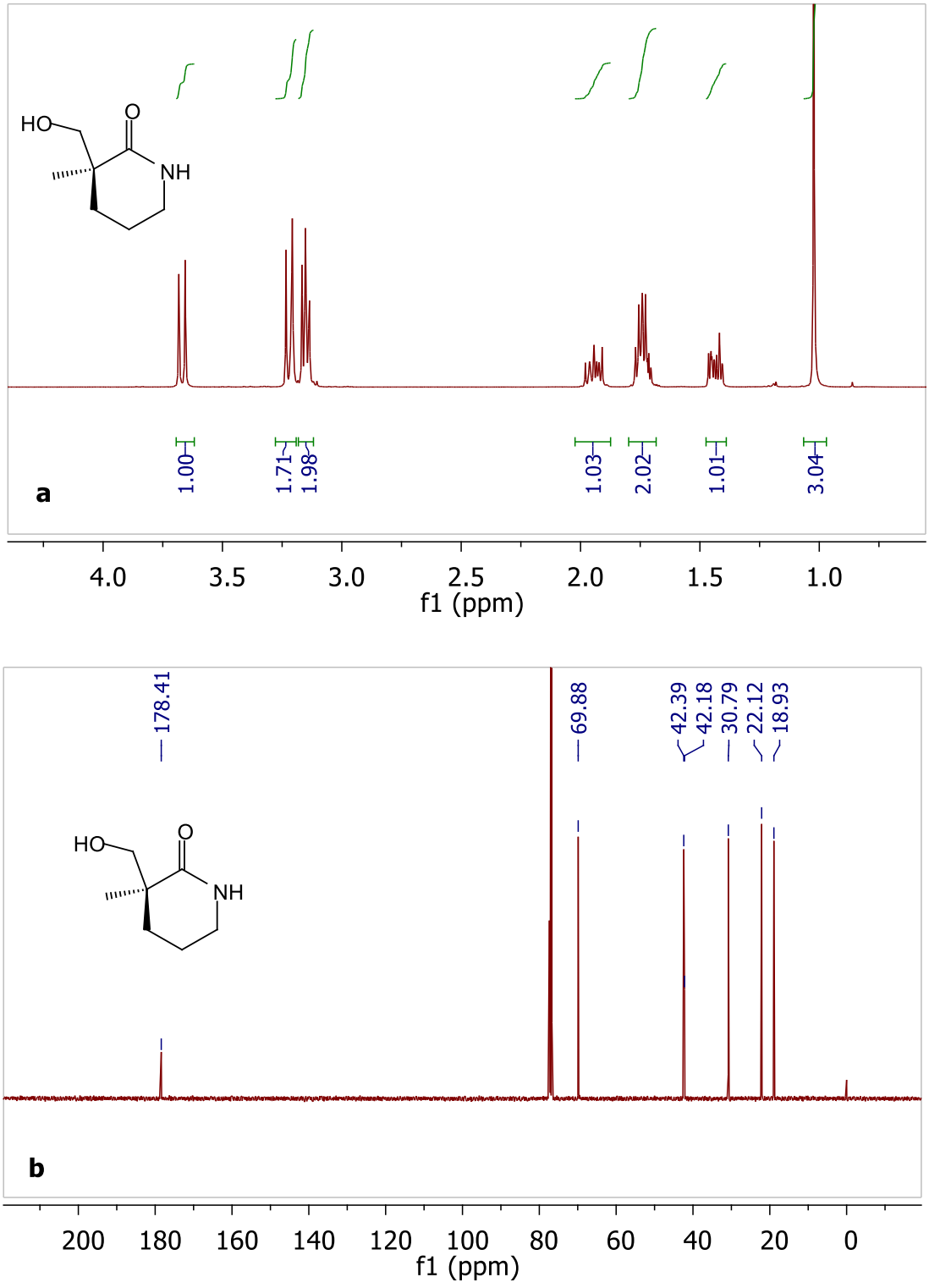
A. 12 a) ^{1}H-NMR of **38**; b) ^{13}C-NMR of **38**.



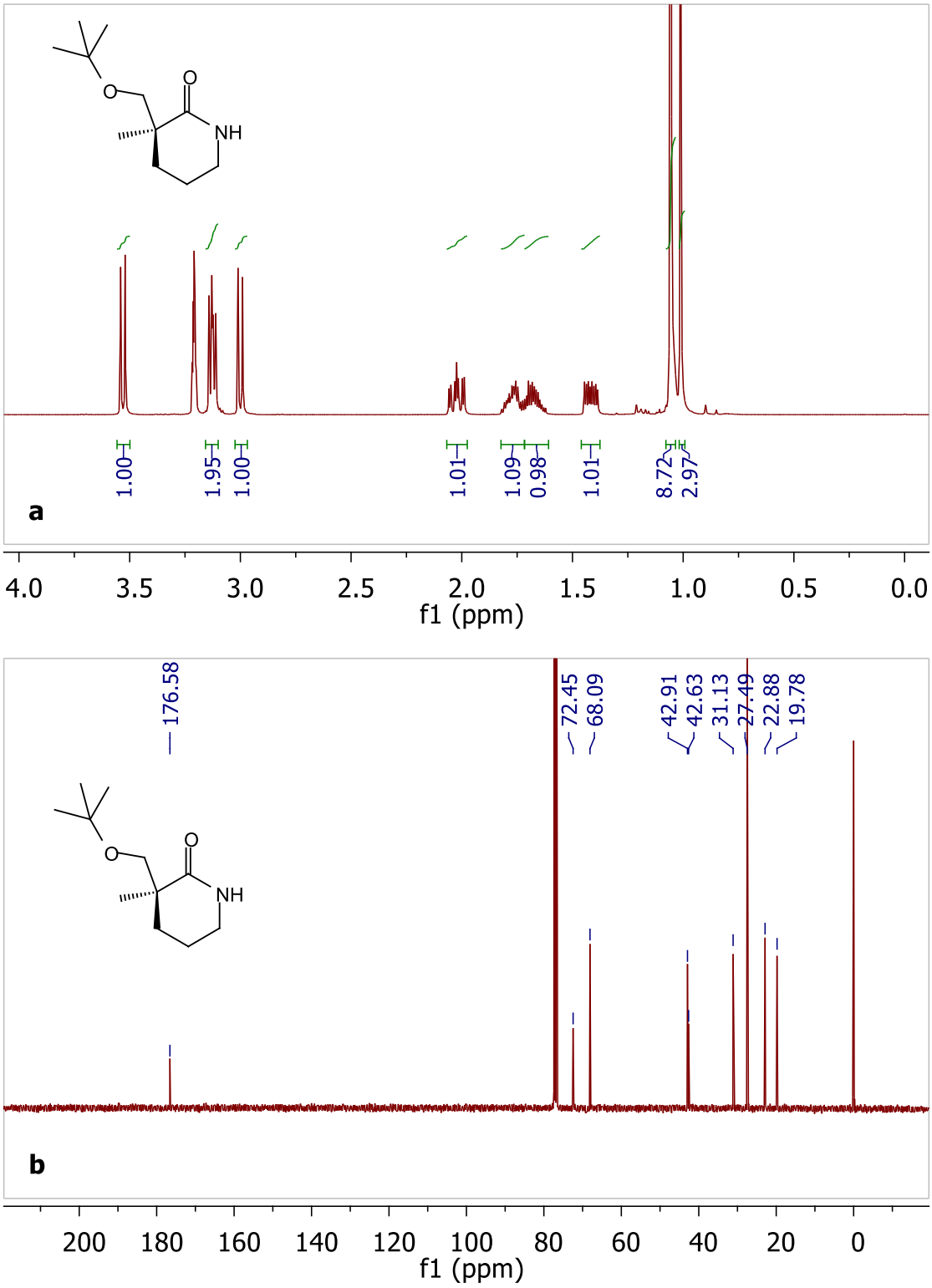
A. 13 a) ^{1}H-NMR of **39**; b) ^{13}C-NMR of **39**.



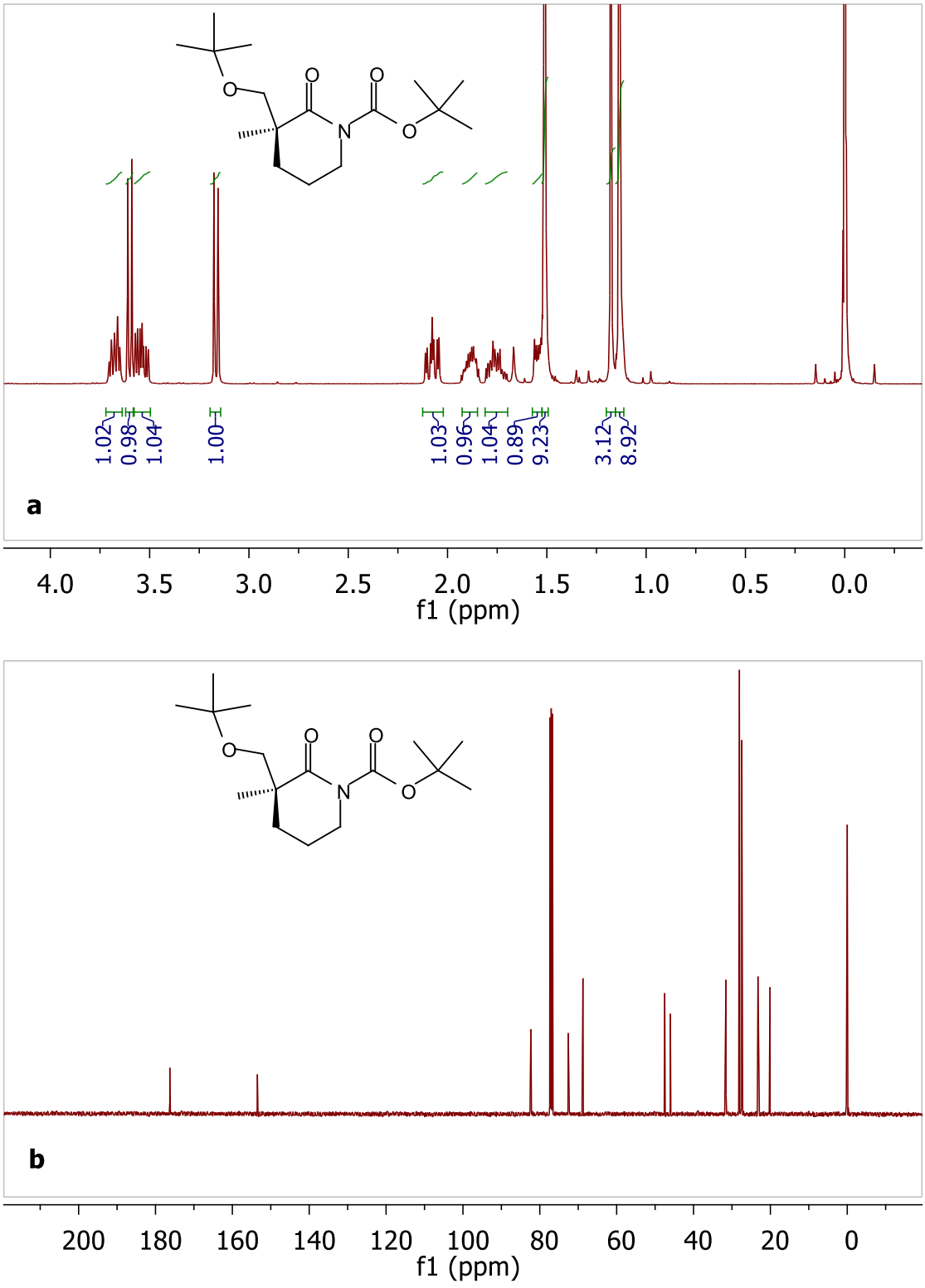
A. 14 a) ^{1}H-NMR of **40**; b) ^{13}C-NMR of **40**.



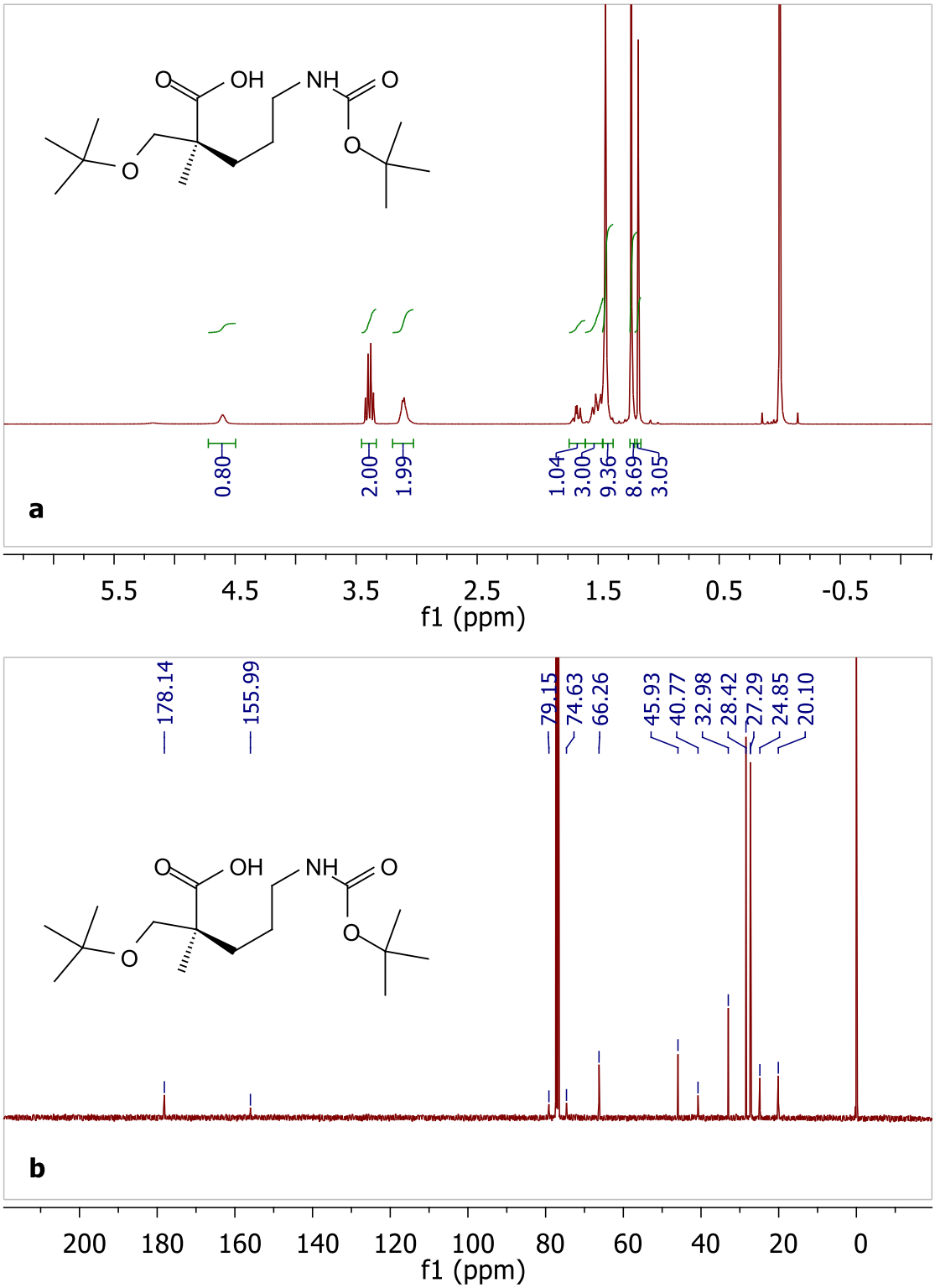
A. 15 ^{1}H-NMR of **48**; b) ^{13}C-NMR of **48**.



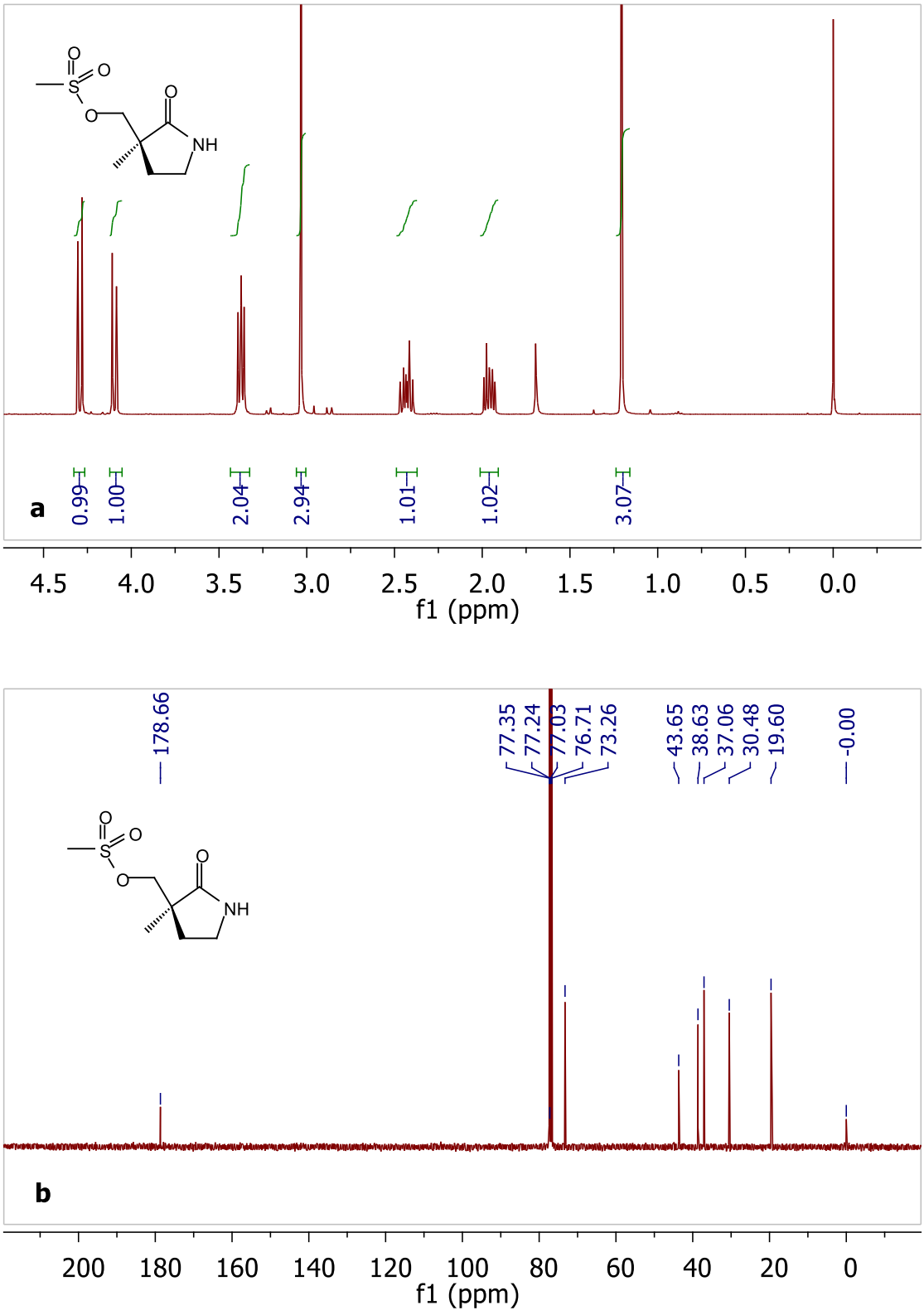
A. 16 a) ^{1}H-NMR of **49**; b) ^{13}C-NMR of **49**.



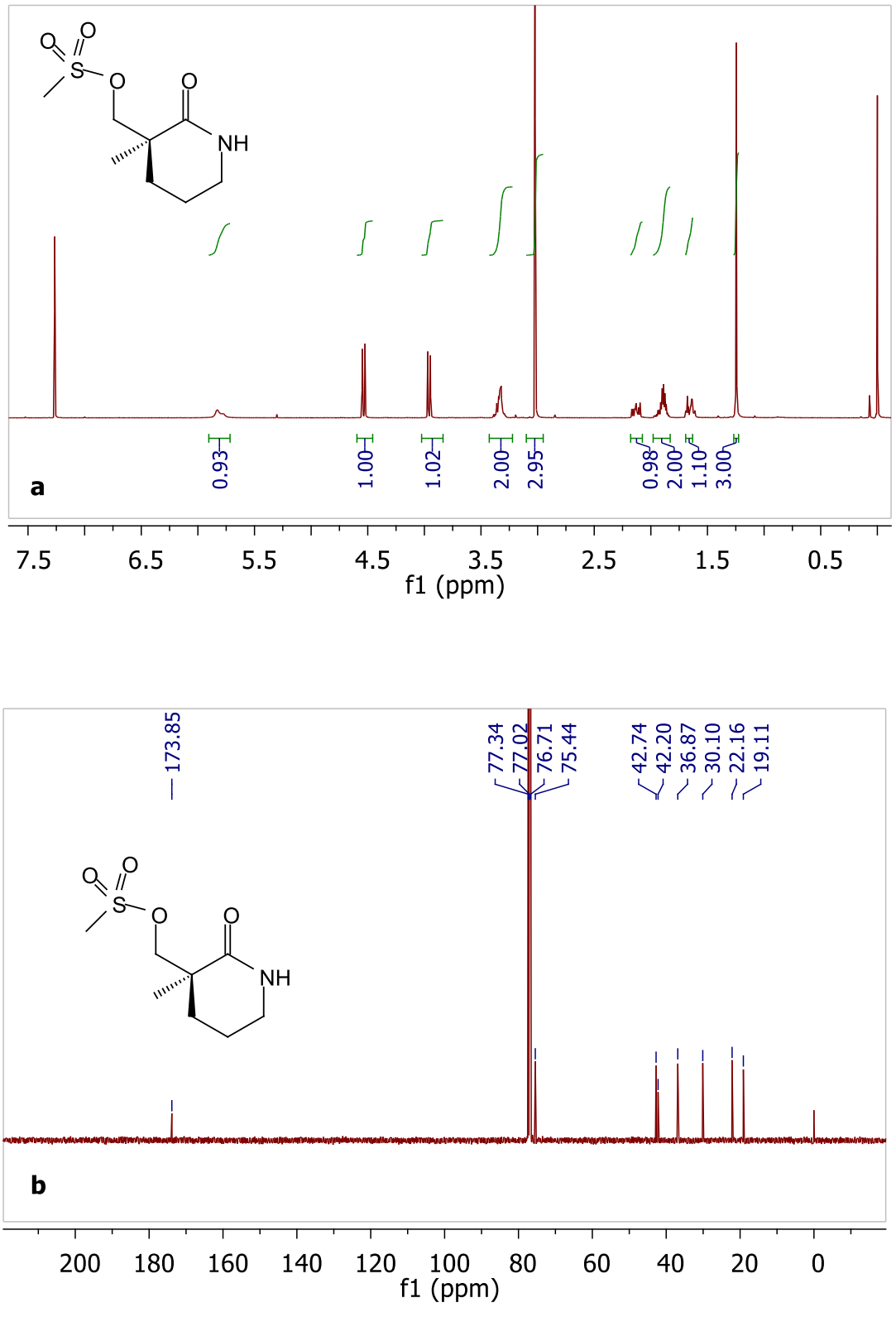
A. 17 a) ^{1}H-NMR of **50**; b) ^{13}C-NMR of **50**.



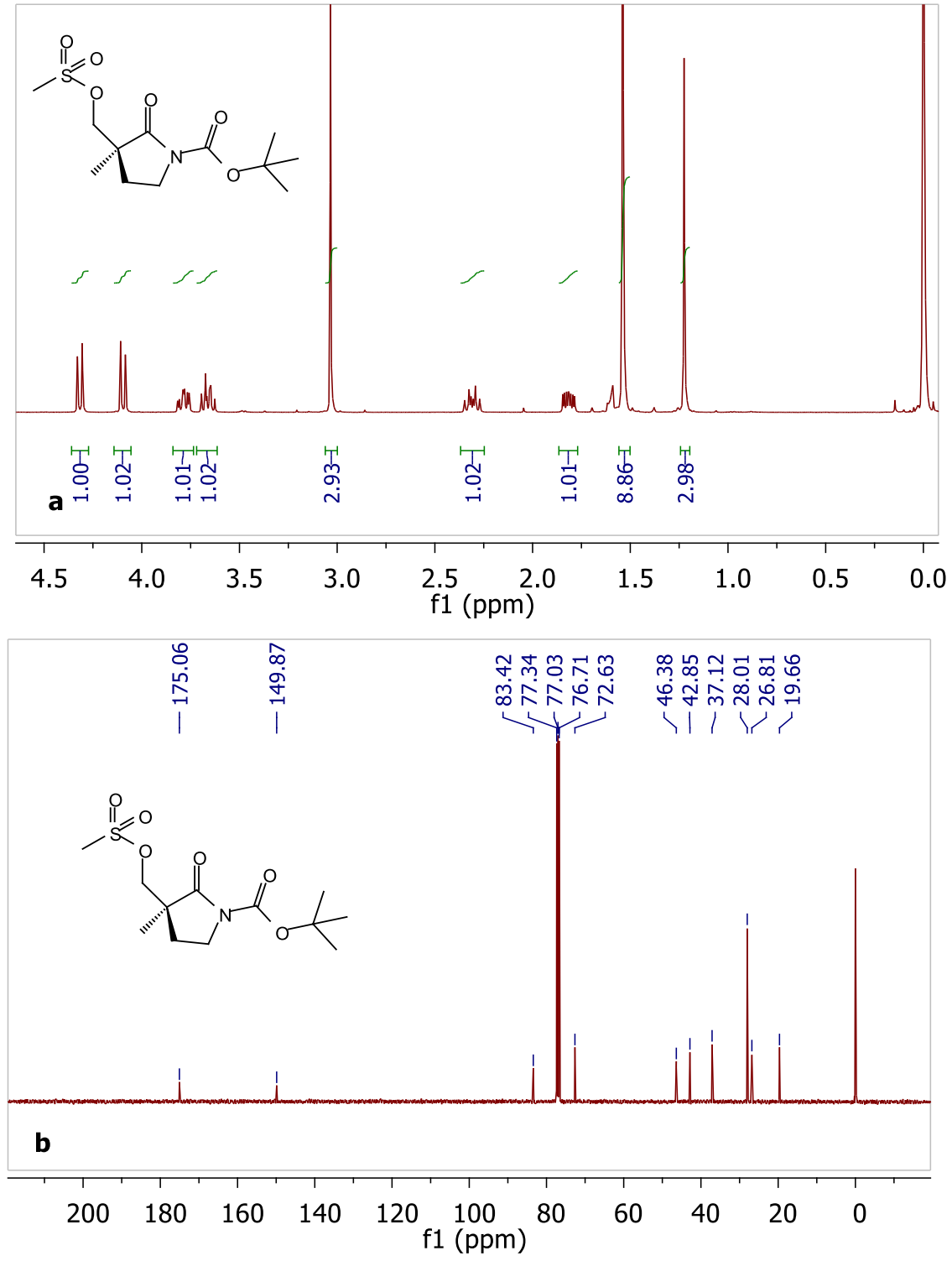
A. 18 a) ^{1}H-NMR of **51**; b) ^{13}C-NMR of **51**.



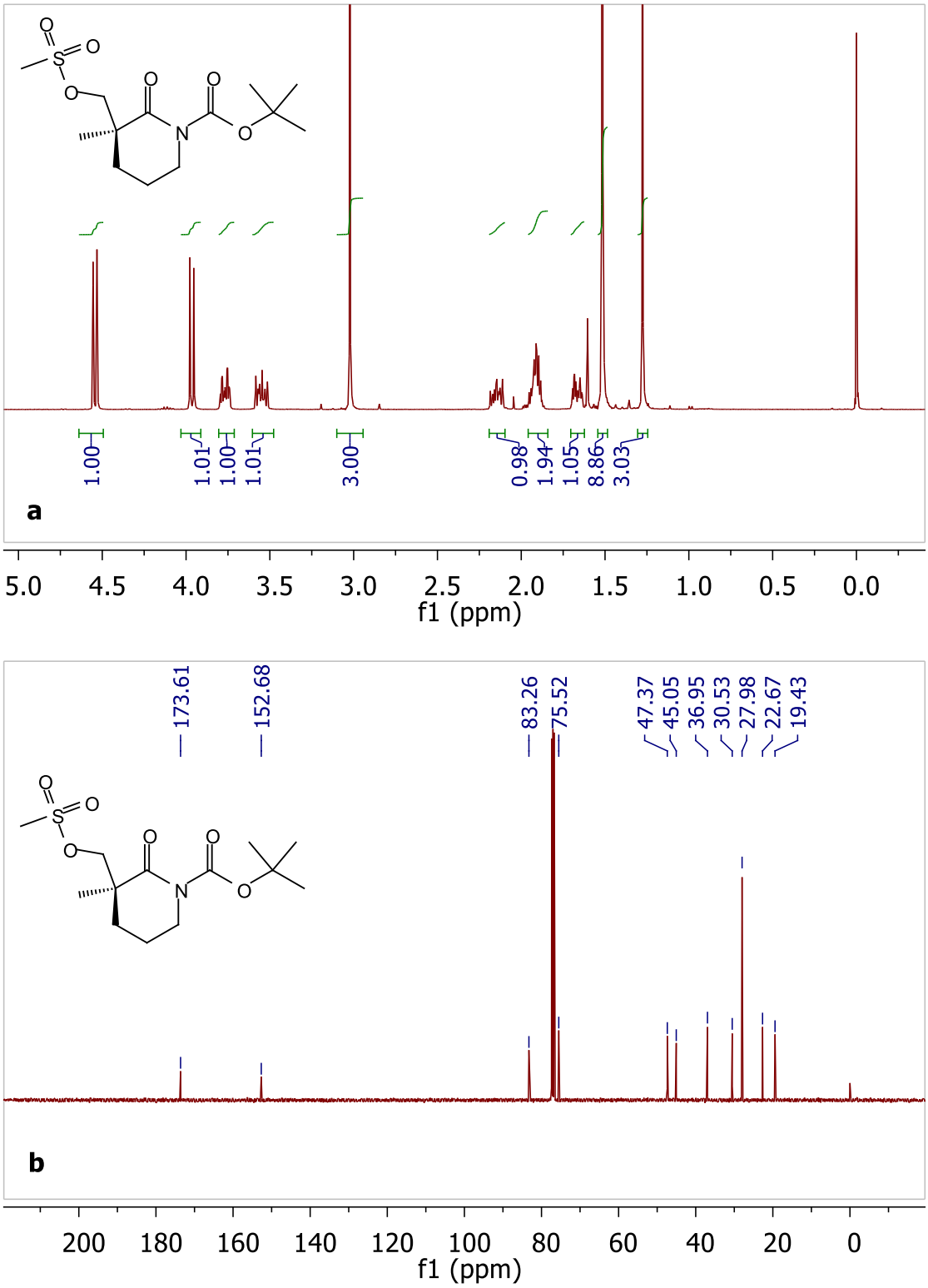
A. 19 a) ^{1}H-NMR of **52a**; b) ^{13}C-NMR of **52a**.



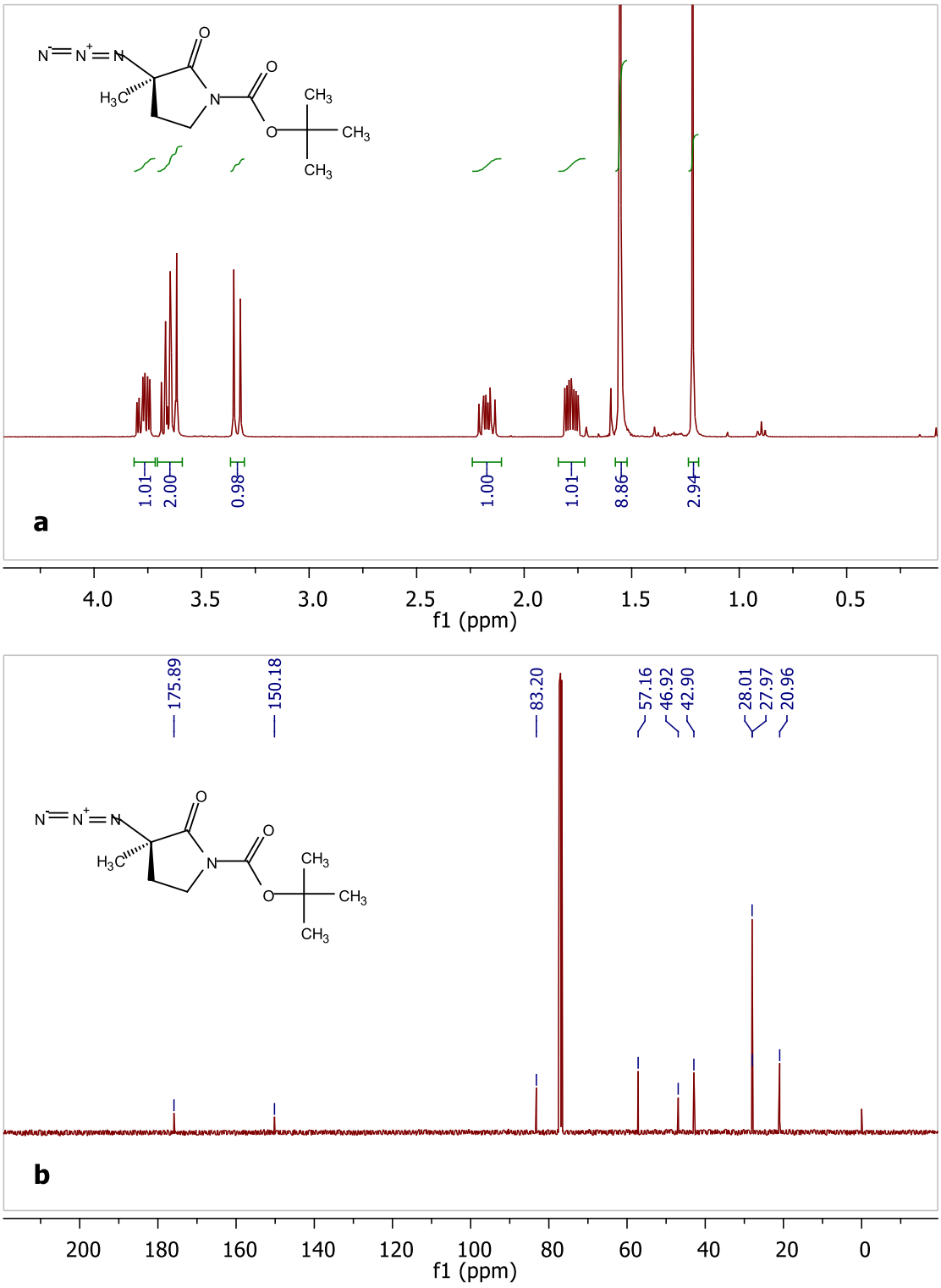
A. 20 a) ^{1}H-NMR of **52b**; b) ^{13}C-NMR of **52b**.



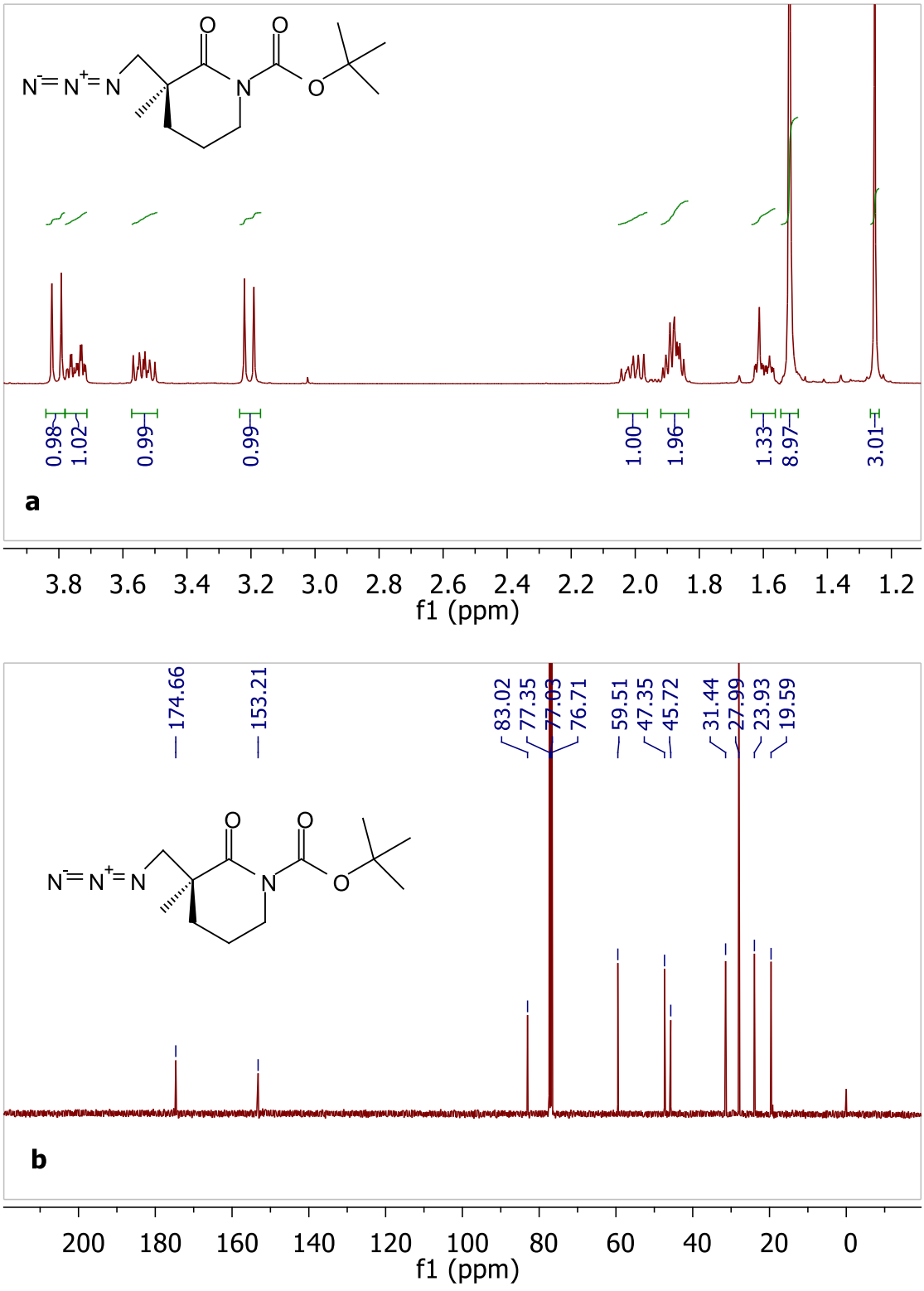
A. 21 a) ^{1}H-NMR of **53a**; b) ^{13}C-NMR of **53a**.



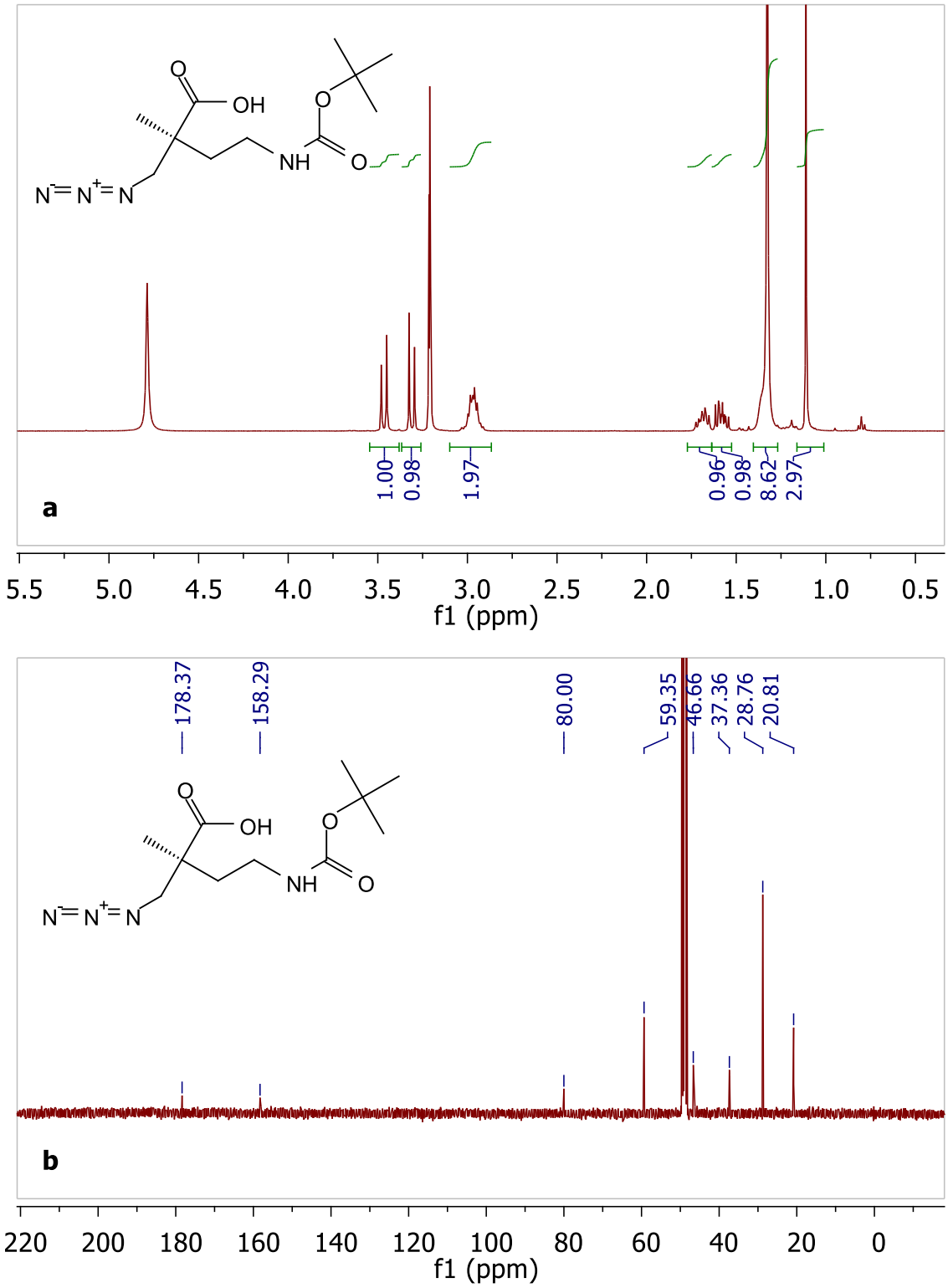
A. 22 a) ^{1}H-NMR of **53b**; b) ^{13}C-NMR of **53b**.



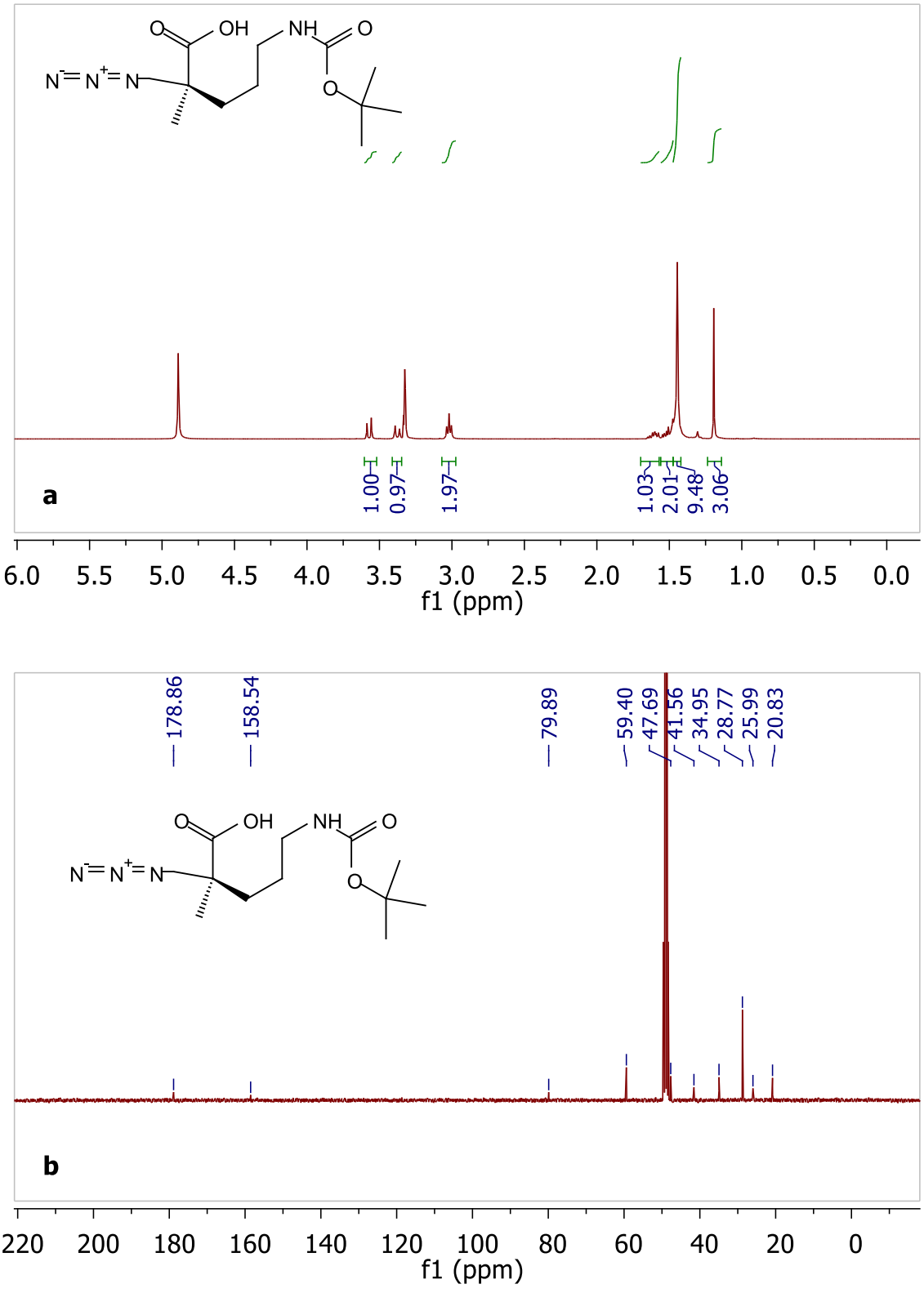
A. 23 a) ^{1}H-NMR of **54a**; b) ^{13}C-NMR of **54a**.



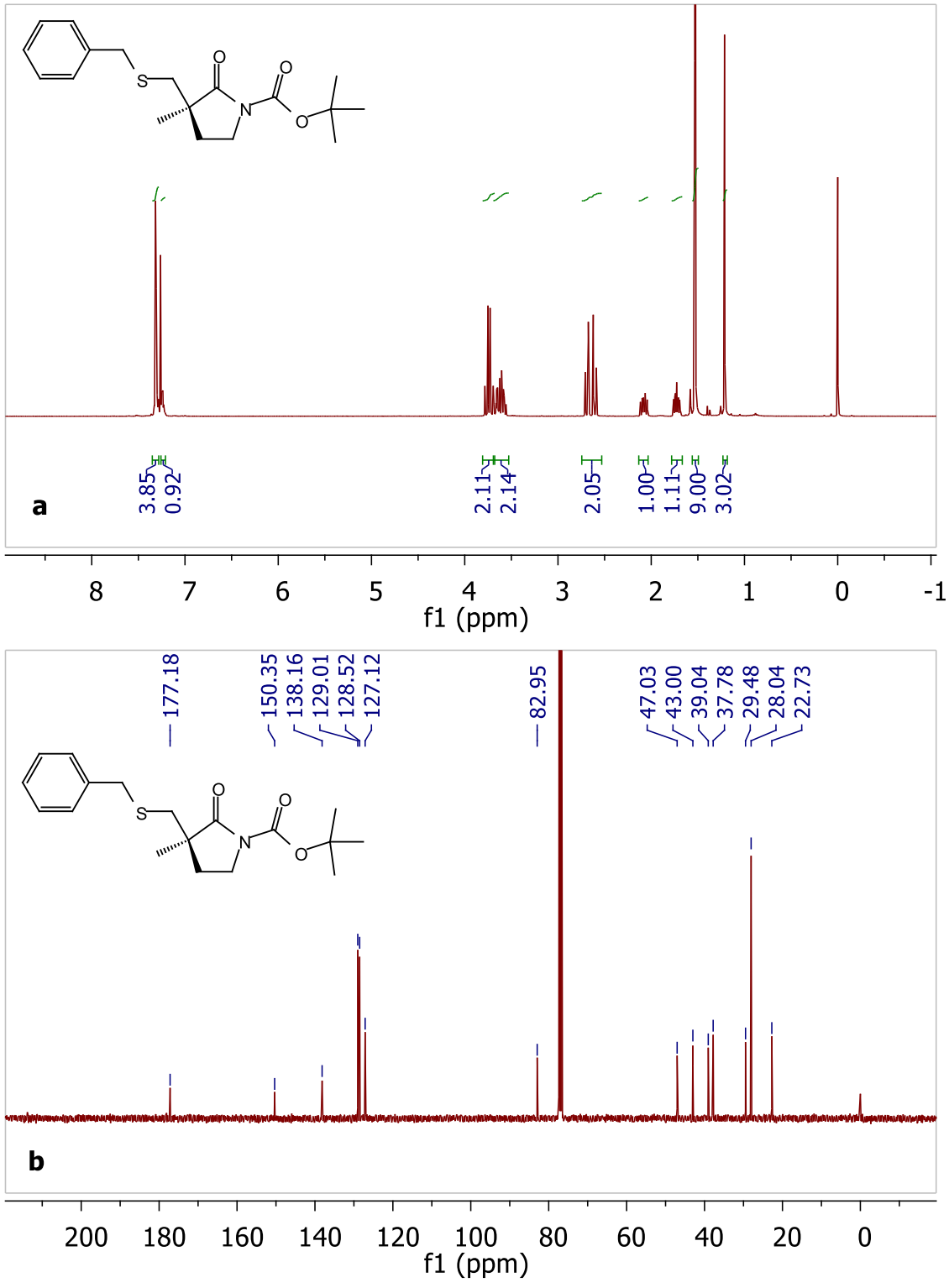
A. 24 a) ^{1}H-NMR of **54b**; b) ^{13}C-NMR of **54b**.



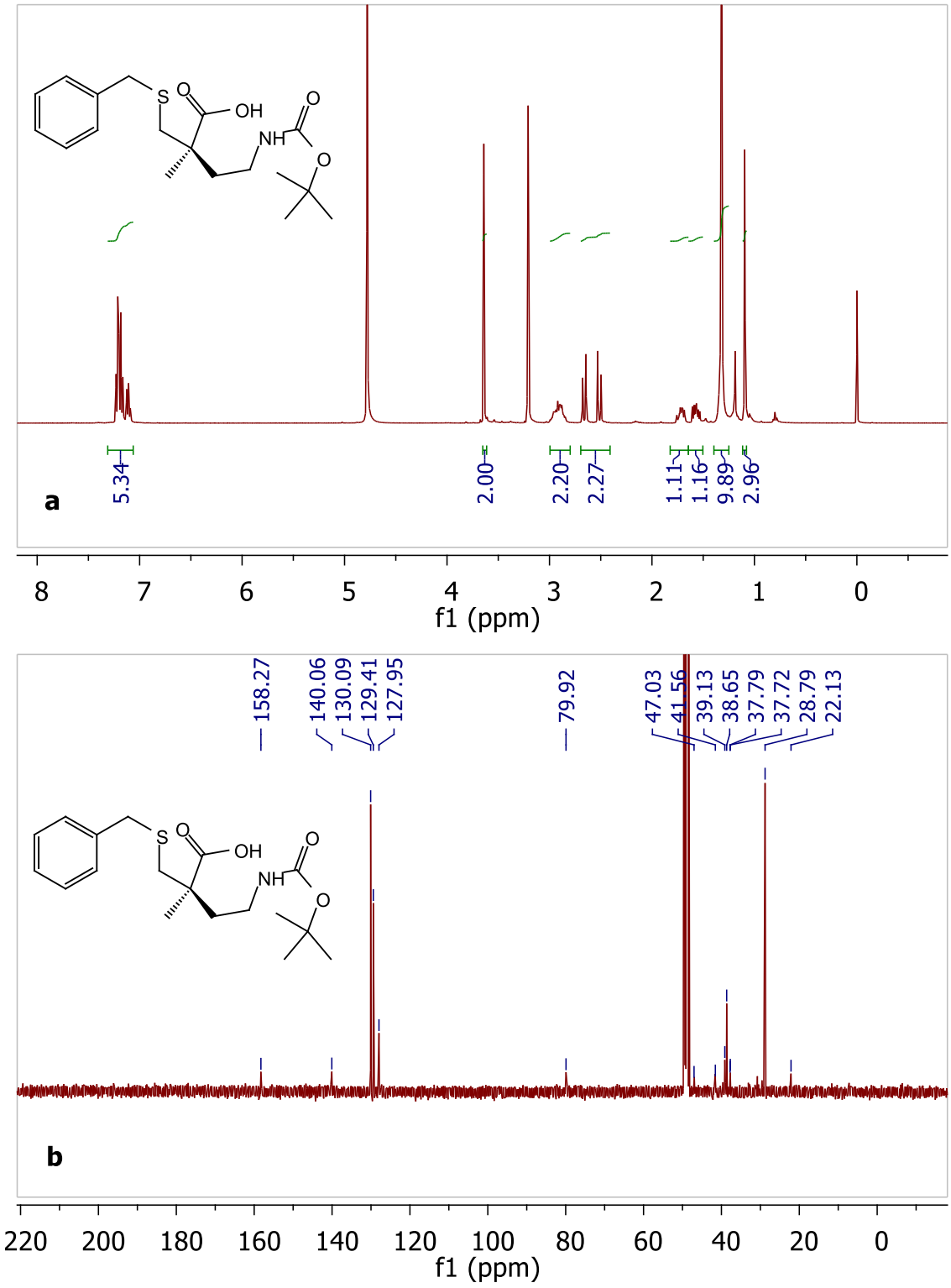
A. 25 a) ^{1}H-NMR of **57a**; b) ^{13}C-NMR of **57a**.



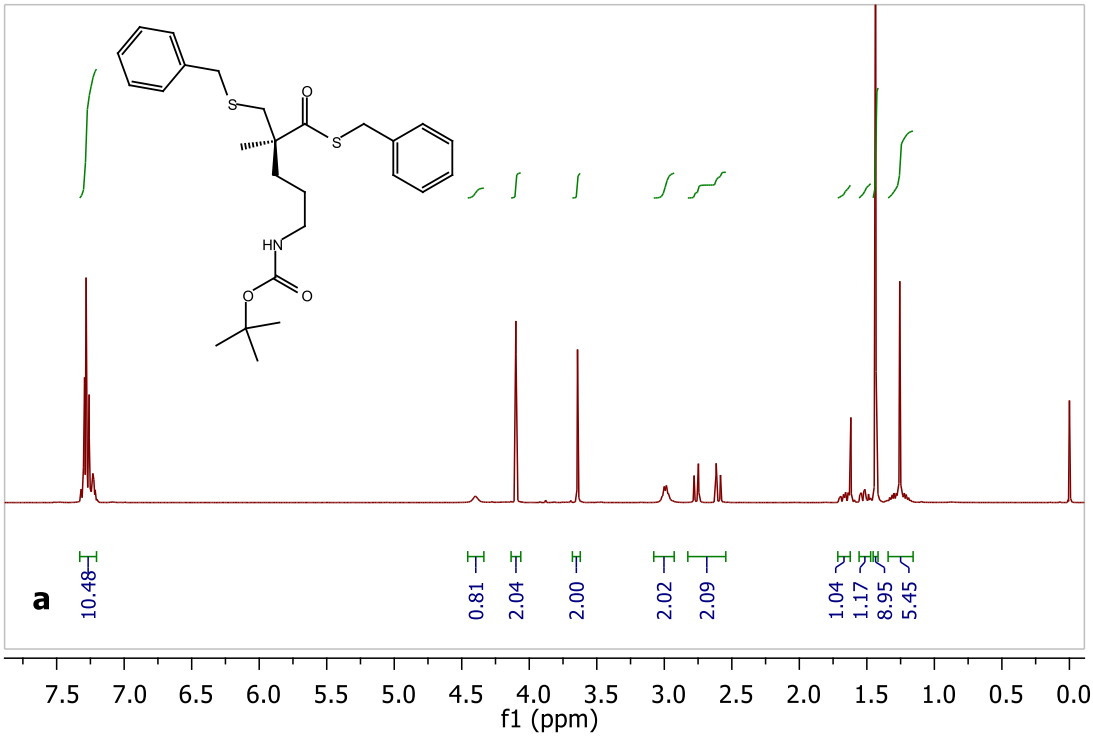
A. 26 a) ^{1}H-NMR of **57b**; b) ^{13}C-NMR of **57b**.

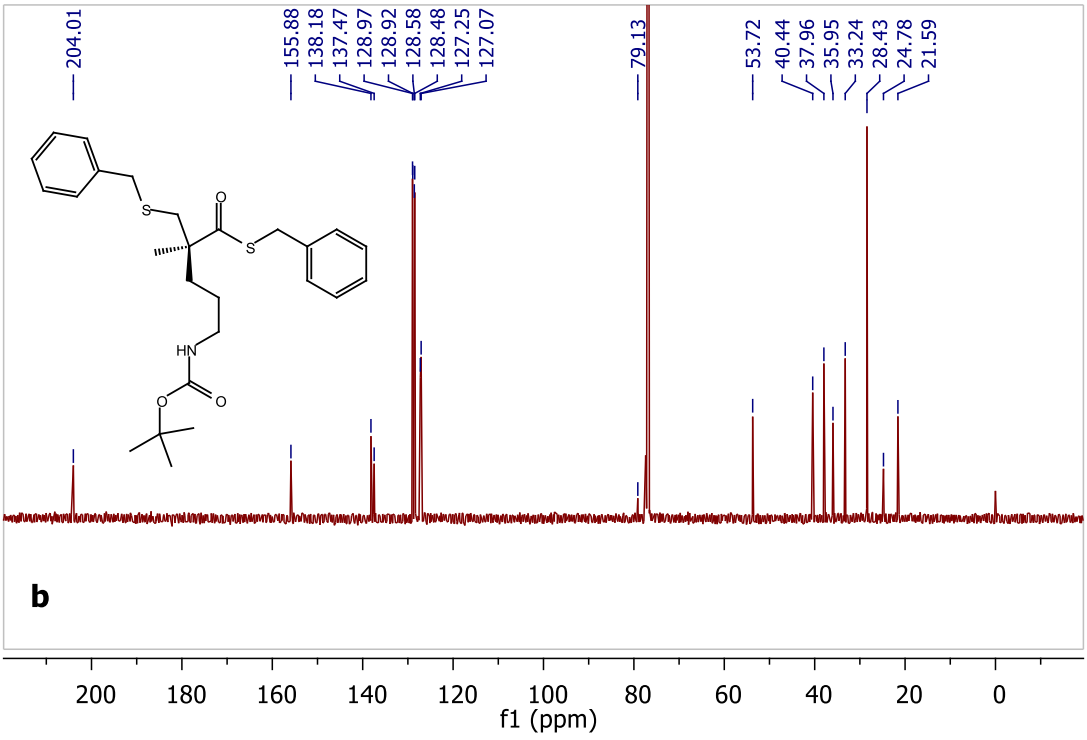


A. 27 a) ^{1}H-NMR of **58a**; b) ^{13}C-NMR of **58a**.

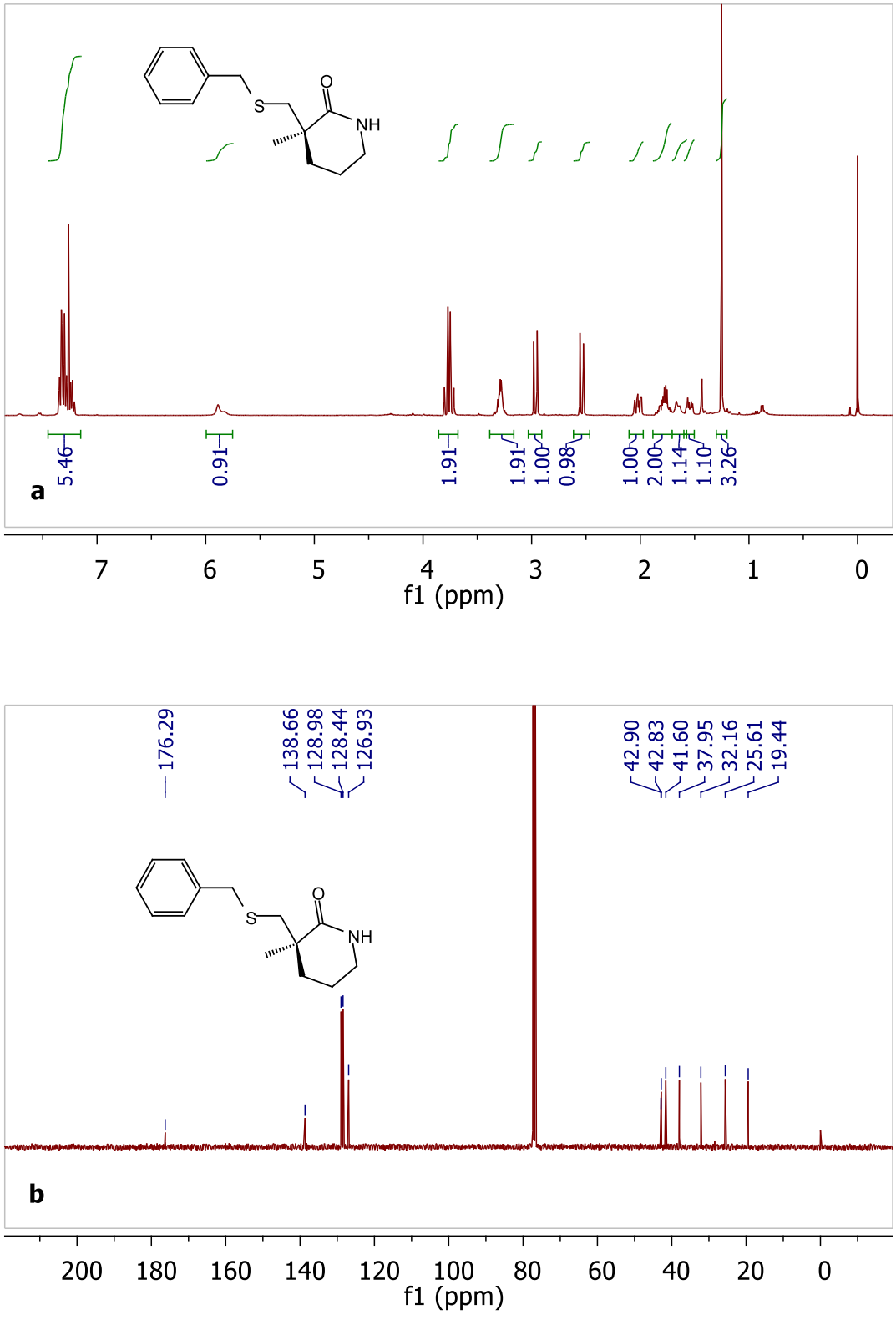


A. 28 ^{1}H-NMR of **59**; b) ^{13}C-NMR of **59**.

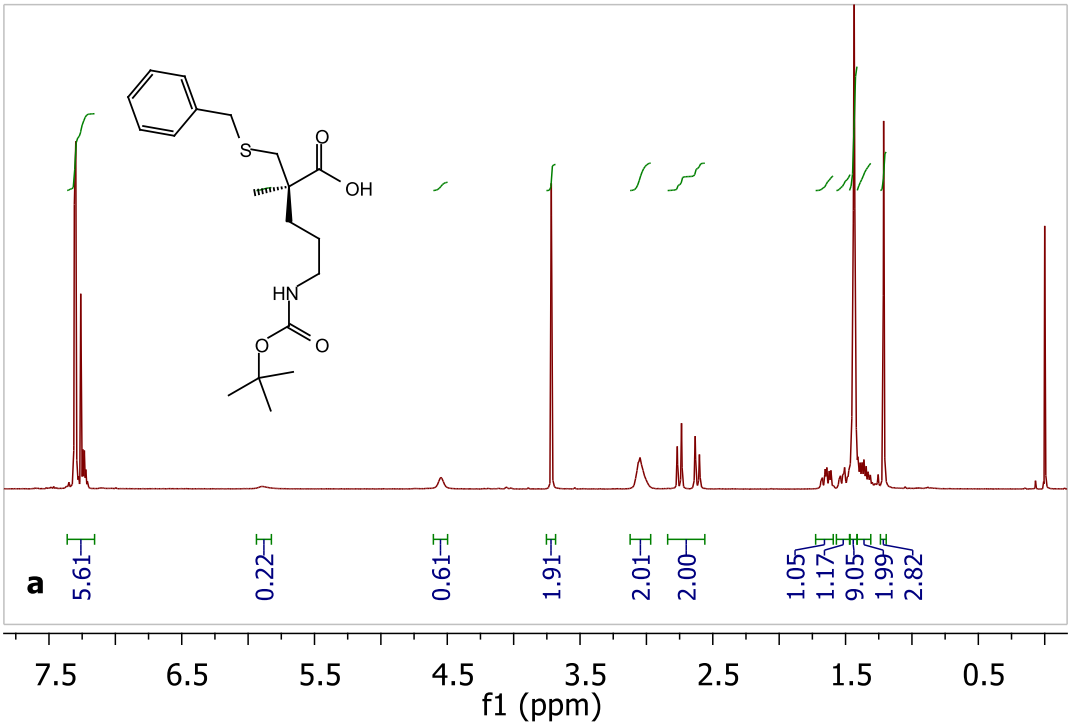


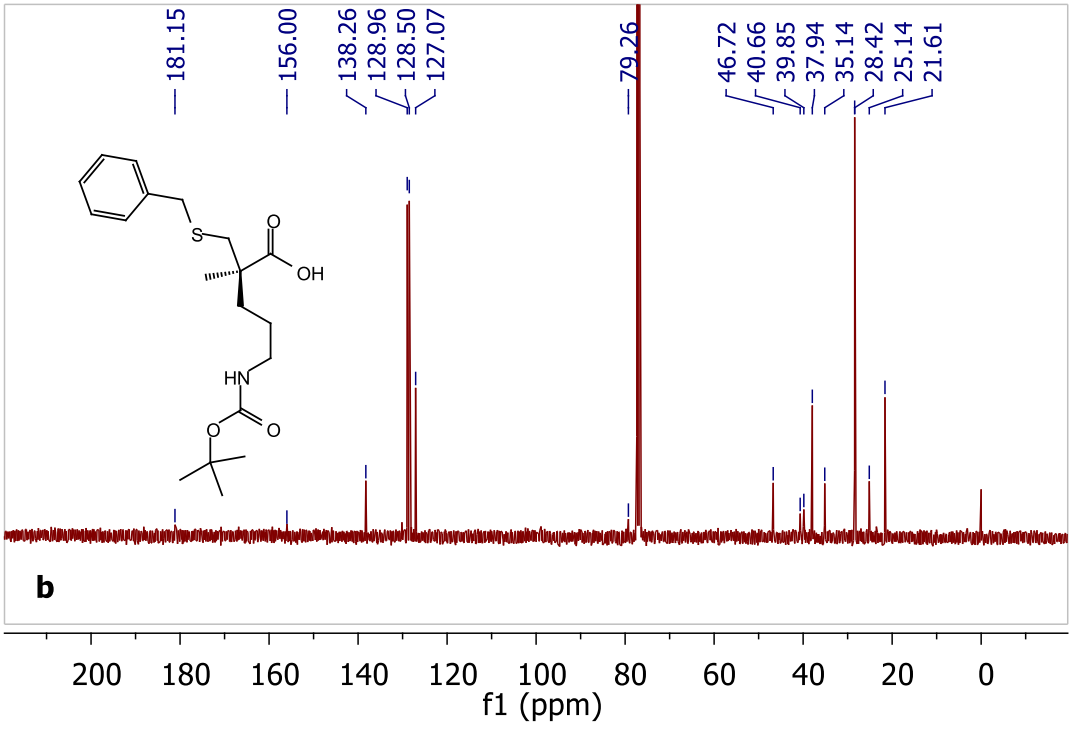


A. 29 a) ^{1}H-NMR of **60**; b) ^{13}C-NMR of **60**.

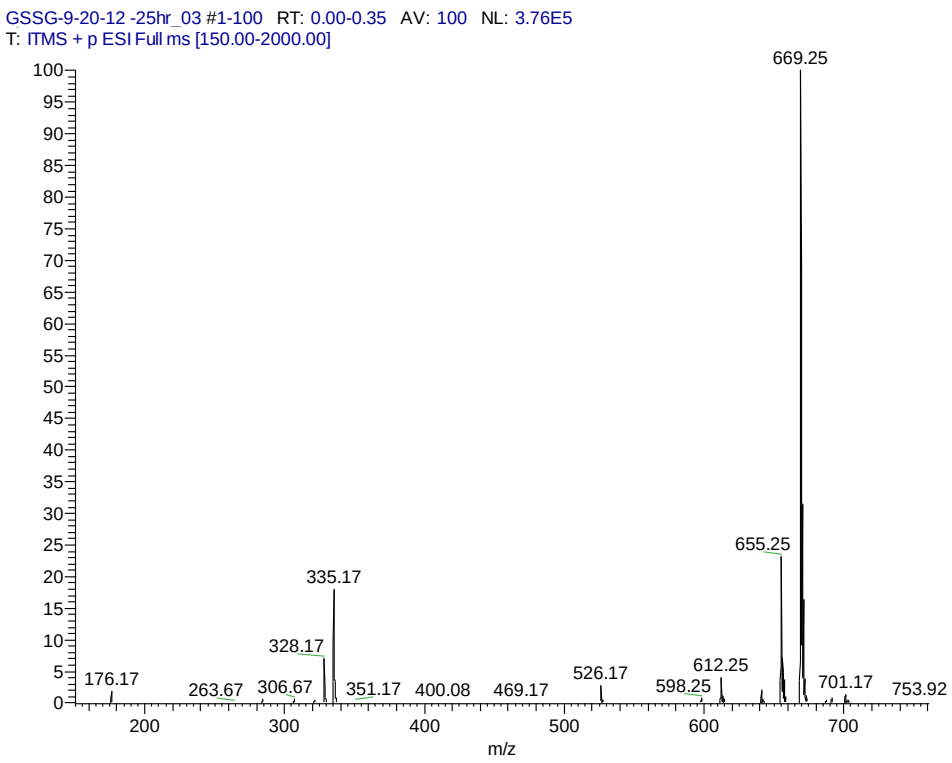


A. 30 a) ^{1}H-NMR of **61**; b) ^{13}C-NMR of **61**.





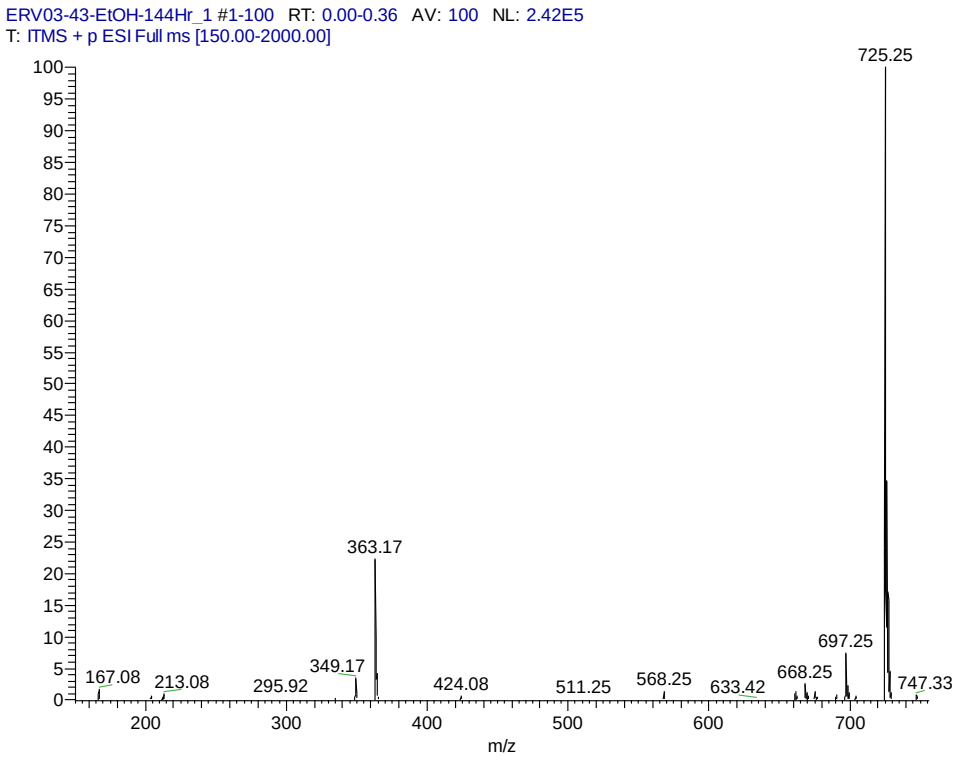
A. 31 a) ^{1}H-NMR of **62**; b) ^{13}C-NMR of **62**.



GSSG thionyl chloride esterification with methanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comments	Number of Esters
669.25	100.00%	$[M+H]^+$	4
655.25	24.50%	$[M+H]^+$	3
335.17	18.32%	$[M+2H]^{2+}$	4
328.17	7.56%	$[M+2H]^{2+}$	3

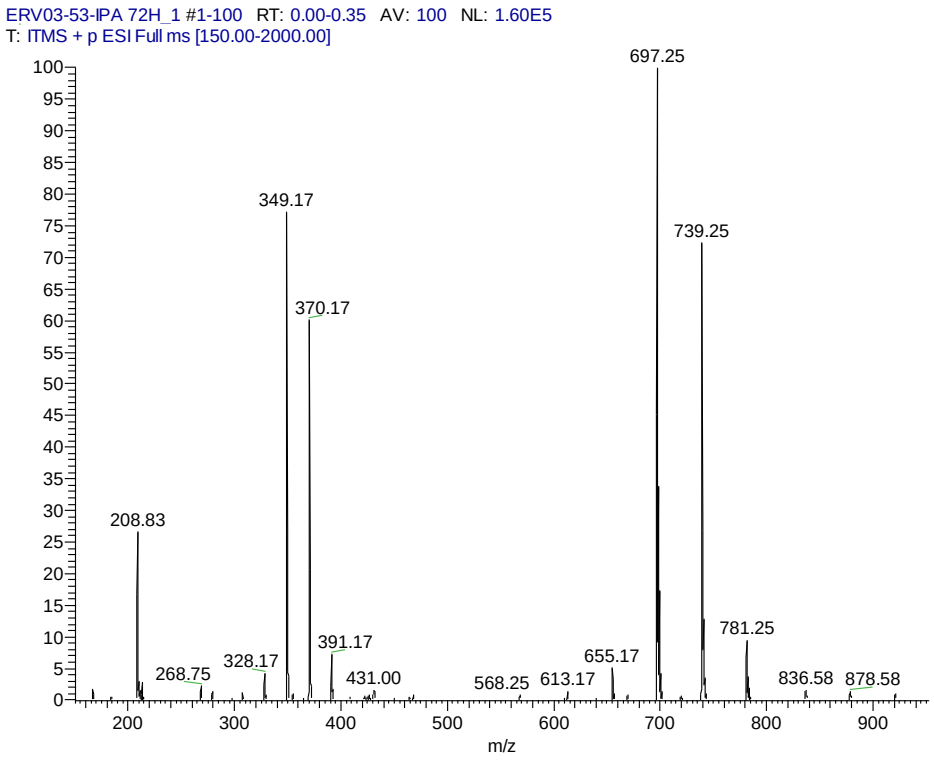
A. 32 ESI MS and mass list of **71a**.



GSSG thionyl chloride esterification with ethanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comments	Number of Esters
725.25	100%	$[M+H]^+$	4
363.17	24.00%	$[M+2H]^{2+}$	4
697.25	7.05%	$[M+H]^+$	3

A. 33 ESI MS and mass list of **71b**.

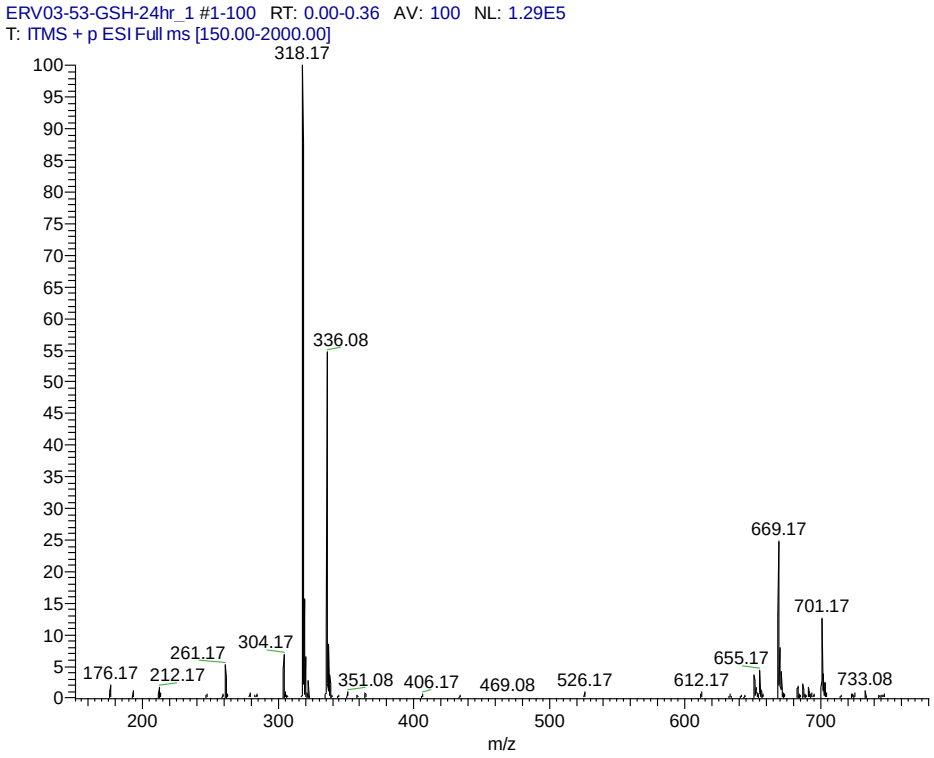


GSSG thionyl chloride esterification with 2-propanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comments	Number of Esters
697.25	100.00%	$[M+H]^+$	2
349.17	78.04%	$[M+2H]^{2+}$	2
739.25	74.30%	$[M+H]^+$	3
370.17	62.30%	$[M+2H]^{2+}$	3
208.83	27.00%	UK	UK
781.25	9.00%	$[M+H]^+$	4
391.17	7.50%	$[M+H]^{2+}$	4

UK=unknown

A. 34 ESI MS and mass list of **71c**.

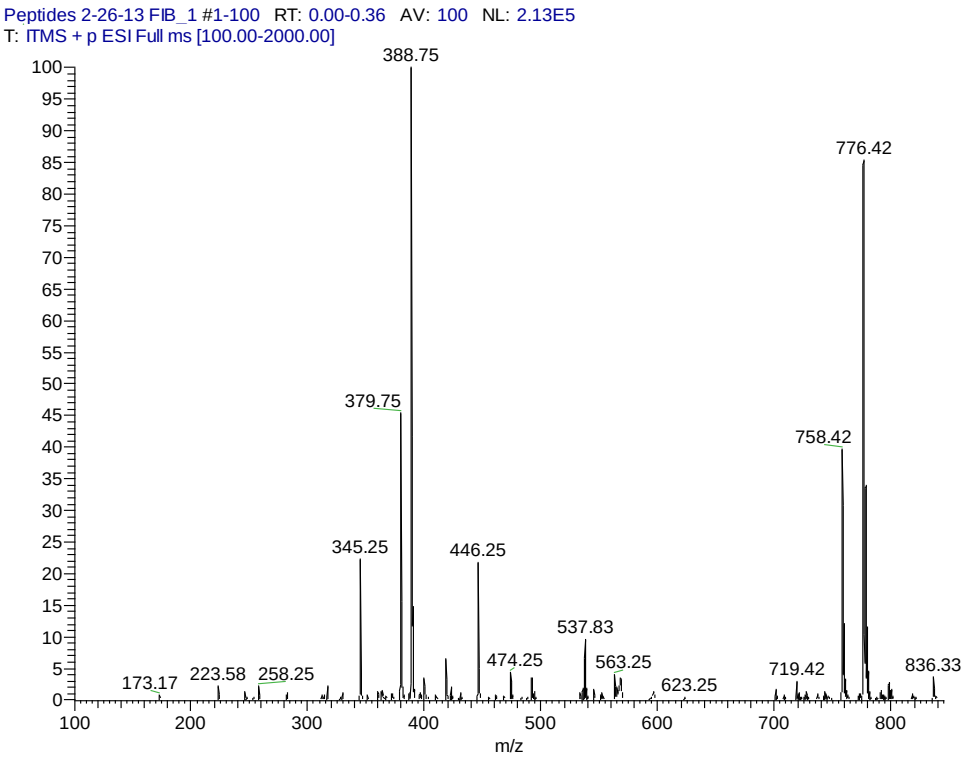


GSH thionyl chloride esterification with 2-propanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comments	Number of Esters
318.17	100%	UK	UK
336.08	56.70%	[M +H]+	2
669.17a	26.40%	[M +H]+	4
701.17	13.25%	UK	UK
304.17	8.30%	UK	UK
655.164[a]	5.80%	[M +H]+	3
261.17	5.50%	UK	UK

UK=unknown. (a) M=$[C_{24}H_{44}N_6O_{12}S_2]$+, Oxidized Glutathione.

A. 35 ESI MS and mass list of **67**.

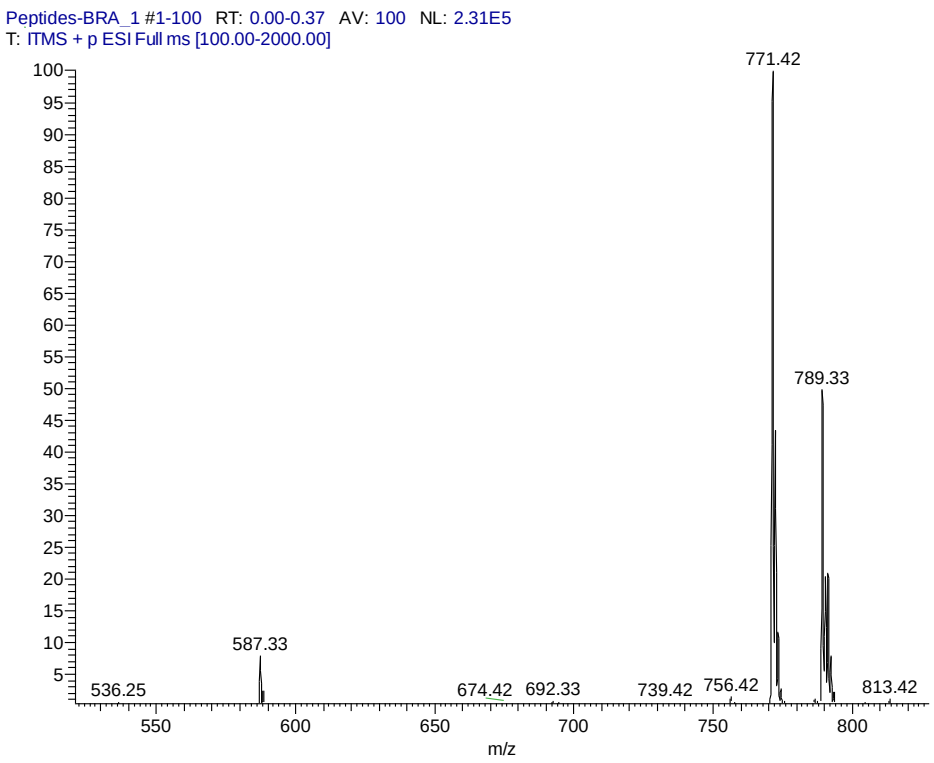


Fibronectin fragment thionyl chloride esterification with methanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comments
388.75[a]	100.00%	$[M+2H]^{2+}$
776.42[a]	85.40%	$[M+H]^{+}$, ^{35}Cl
758.42	39.66%	$[M+H]^{+}$
778.365[a]	33.97%	$[M+H]^{+}$, ^{37}Cl
379.75	25.28%	$[M+2H]^{2+}$
345.25	22.36	UK
446.25	20.94	UK
537.83	9.43%	UK

UK=unknown. (a) M= $C_{31}H_{55}ClN_{11}O_{10}$.

A. 36 ESI MS and mass list of **72**.

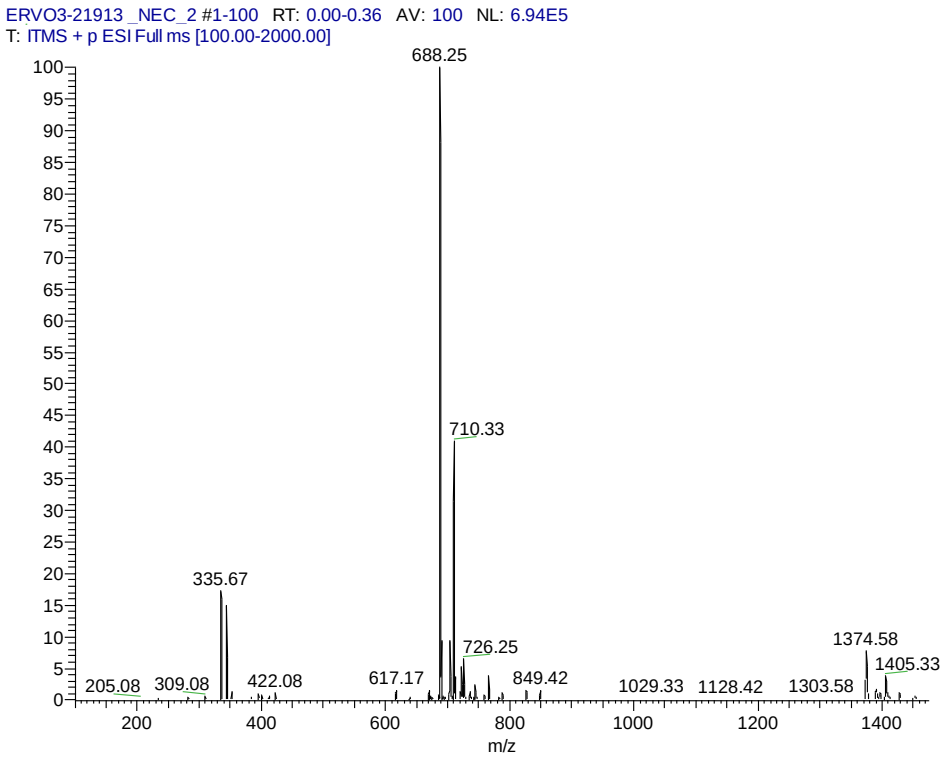


Bradykinin thionyl chloride esterification with methanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comment
771.42	100.00%	$[M+H]^+$
789.33[a]	49.97%	$[M+H]^+$, ^{35}Cl
791.34[a]	21.34%	$[M+H]^+$, ^{37}Cl
587.33	7.26%	UK

UK=unknown. (a) M= $C_{36}H_{54}ClN_{10}O_8$.

A. 37 ESI MS and mass list of **73**.



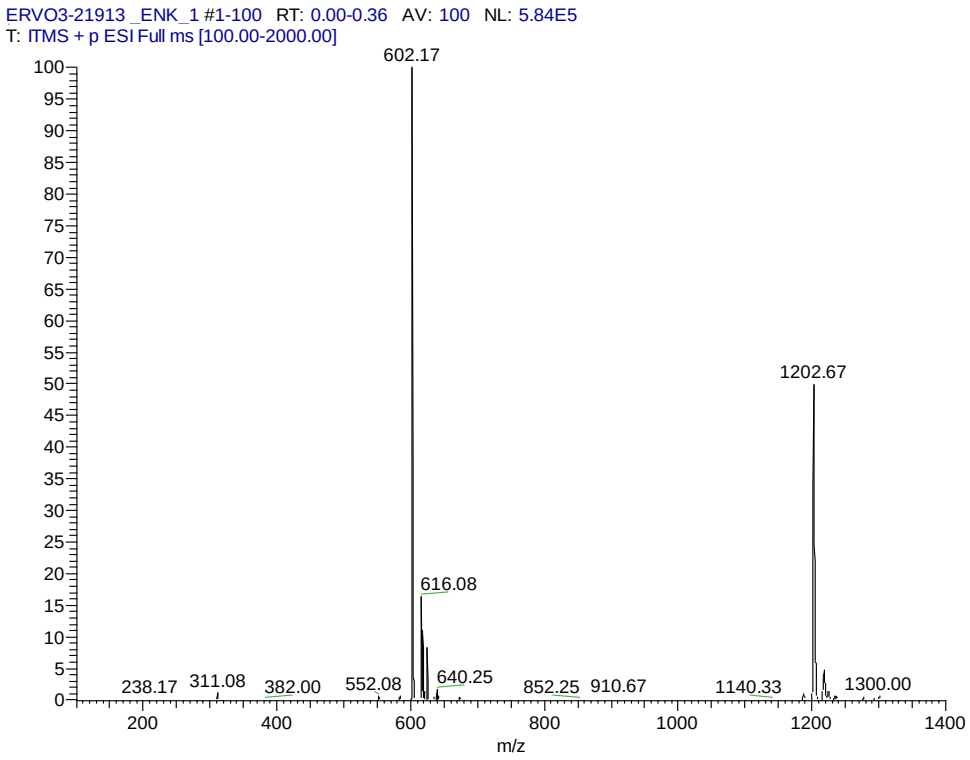
Necrofibrin, rat thionyl chloride esterification with methanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comments[a]
688.25	100.00%	$[M+H]^+$
710.33[b]	37.07%	$[M+H]^+$
335.678[b]	18.46%	$[M+2H]^{2+}$
344.51	16.51%	$[M+2H]^{2+}$
1374.58[c]	9.4%	$[M+H]^+$

(a) Impurities present in starting peptide m/z (relative abundance): 674 (55%), 696 (100%), 1346 (19%), 1368 (10%).

(b) Impurity from starting material undergoing methyl esterification *m/z*=696 (c) Impurity from starting material undergoing two methyl esterifications *m/z*=1346

A. 38 ESI MS and mass list of **74**.

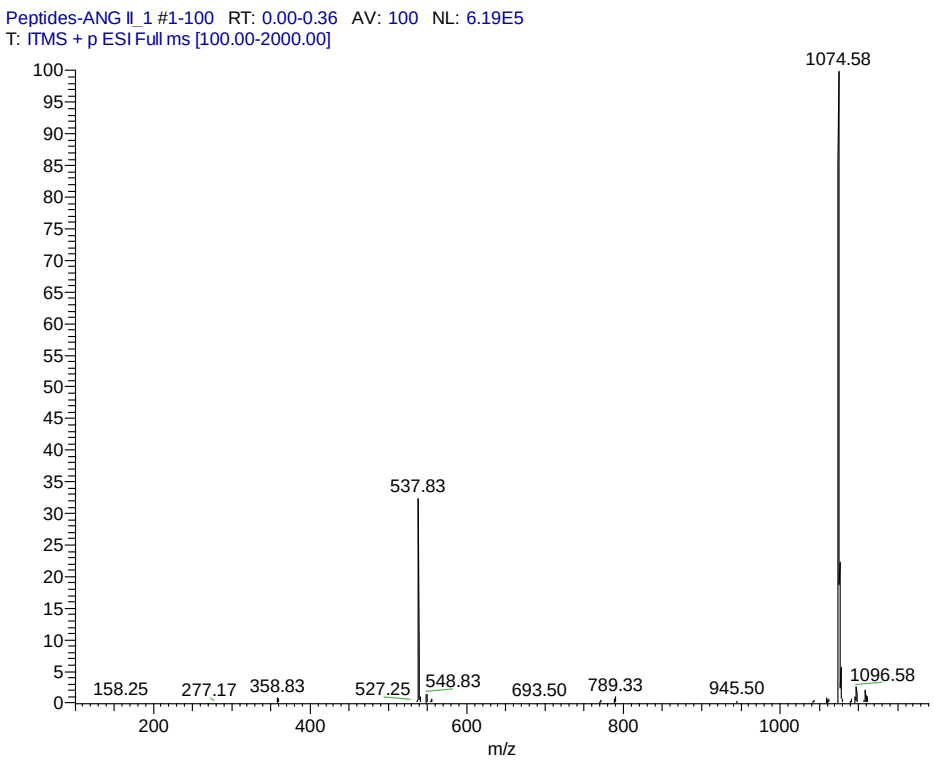


Enkephalin thionyl chloride esterification with methanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comments
602.17	100%	$[M+H]^+$
1202.67	54.00%	$[2M+H]^+$
616.08	16.54%	UK

UK=unknown. (a) Impurities present in starting peptide m/z (relative abundance): 411 (8%), 587 (100%), 610 (23%), 1174 (42%).

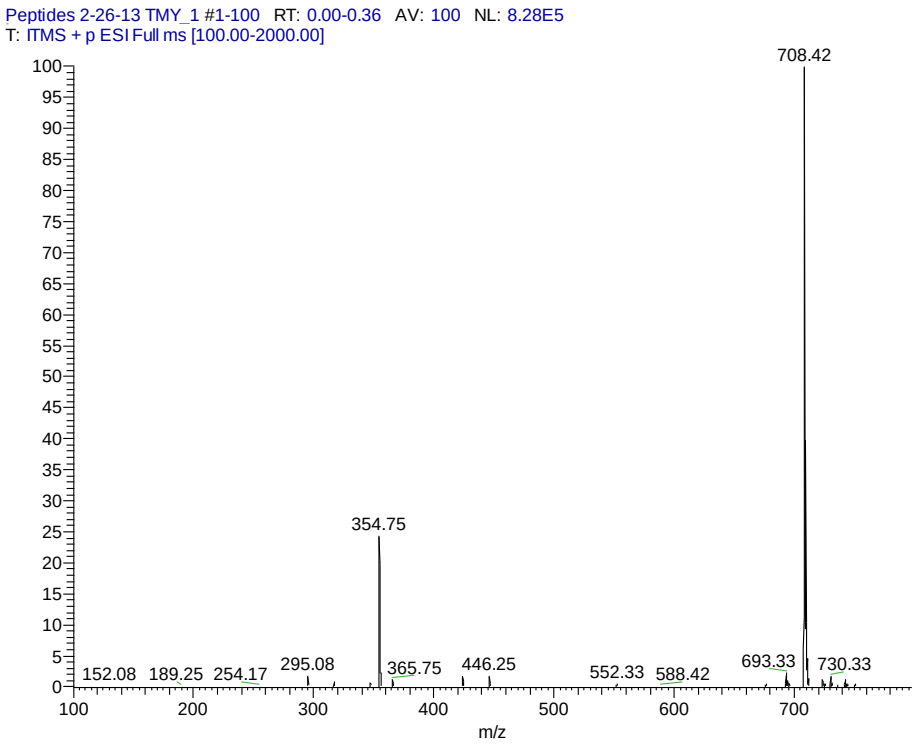
A. 39 ESI MS and mass list of **75**.



Angiotensin II, human thionyl chloride esterification with methanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comments
1074.58	100.00%	$[M+H]^{+}$
537.83	35.34%	$[M+2H]^{2+}$

A. 40 ESI MS and mass list of **76**.

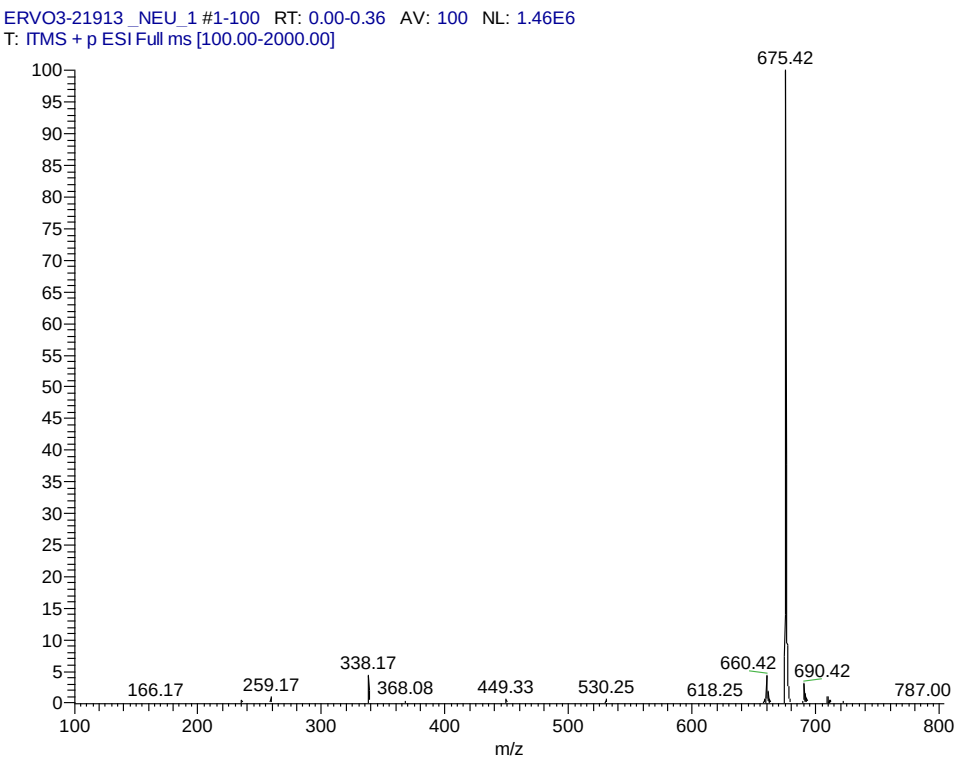


Thymopentin (TP-5) thionyl chloride esterification with methanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comments
708.42	100.00%	$[M+H]^+$
354.76	25.40%	$[M+2H]^{2+}$

(a) Impurities present in starting peptide m/z (relative abundance): 341(14%), 680 (100%), 1358(8%).

A. 41 ESI MS and mass list of **77**.

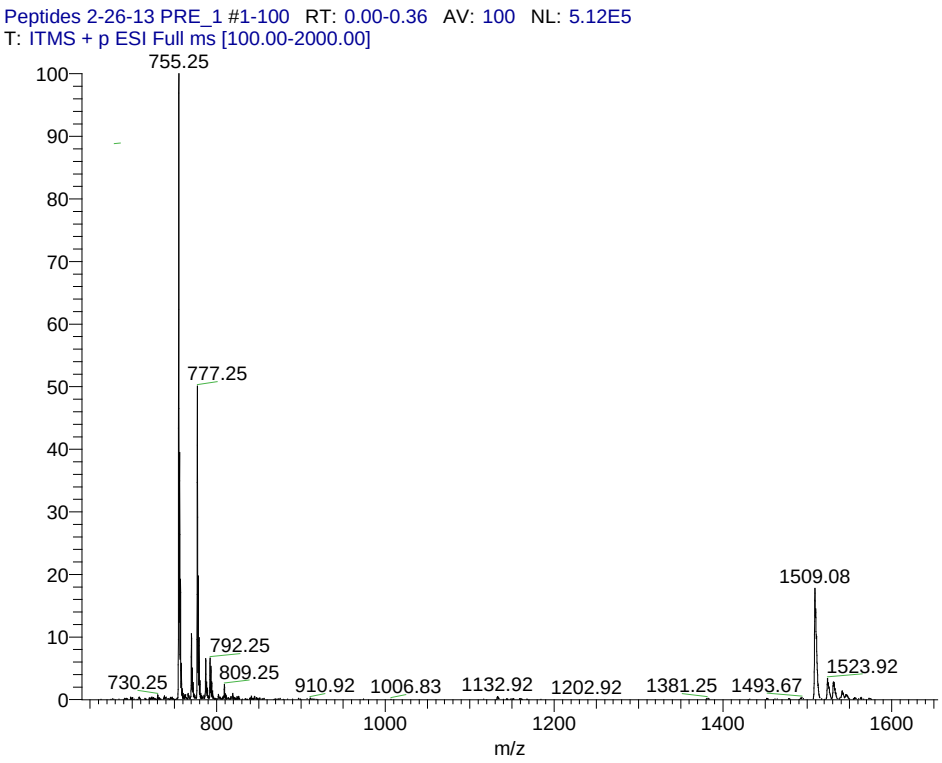


Neurotensin (9-13) thionyl chloride esterification with methanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comment
675.42	100.00%	$[M+H]^+$

(a) Impurities present in starting peptide m/z (relative abundance): 661 (100%), 1320 (56%), 1322 (40%).

A. 42 ESI MS and mass list of **78**.



[Ille3]-pressinoic acid thionyl chloride esterification with methanol

Observed Mass (*m/z*)	Relative Abundance (%)	Comments
755.25	100.00%	$[M+H]^+$
777.25	50.00%	$[M+Na]^+$
1509.08	24.55%	$[2M+H]^+$
770.212	10.54%	UK

UK=unknown. (a) Impurities present in starting peptide m/z (relative abundance): 726 (10%), 742 (100%), 764 (21%), 781 (16%), 1483 (10%), 1522 (20%).

A. 43 ESI MS and mass list of **79**.

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