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„Synthesis of isotopically labeled sodium 1-pyrroline-2-carboxylate - a potential proline precursor for selective protein labeling“

verfasst von / submitted by

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Abstract

Nuclear magnetic resonance (NMR) spectroscopy is a frequently used method for studying structures, dynamics and binding interactions of proteins. Sophisticated protein NMR experiments require the selective incorporation of ^{2}H, ^{13}C and ^{15}N isotopes into the amino acid sequence to either enhance or to deplete certain signals. For selective labeling experiments, a synthetic route for sodium 1-pyrroline-2-carboxylate, a potential metabolic precursor of *L*-proline, was established. This synthesis allows the introduction of ^{2}H-, ^{13}C- and ^{15}N-labels from cheap isotope sources in the molecule. Sodium 1-[5-^{13}C]-pyrroline-2-carboxylate was synthesized and tested in protein overexpression experiments in *E.coli*. Subsequent NMR experiments of the expressed protein (BRD4) did not reveal the incorporation of the precursor. Future experiments with proline auxotrophic *E.coli* strains will show the applicability of sodium 1-pyrroline-2-carboxylate as a selective proline precursor.

Table of Contents

1 Introduction

1.1 Protein NMR

Nuclear magnetic resonance (NMR) spectroscopy is one method for studying the structures, dynamics and ligand interactions of proteins. According to the protein databank[1] (PDB), 8.0% of the 137992 solved protein structures were elucidated by NMR (figure 1), the vast majority (124922, 90.5%) derive from electron density maps measured with x-ray crystallography and 1.5% of protein structures were measured with cryo-electron microscopy (cryo-EM). Despite the small number of protein structures solved by it, nuclear magnetic resonance is a very important tool to investigate biomolecules like proteins and has several advantages compared to static methods such as x-ray crystallography or cryo-electron microscopy.[2]

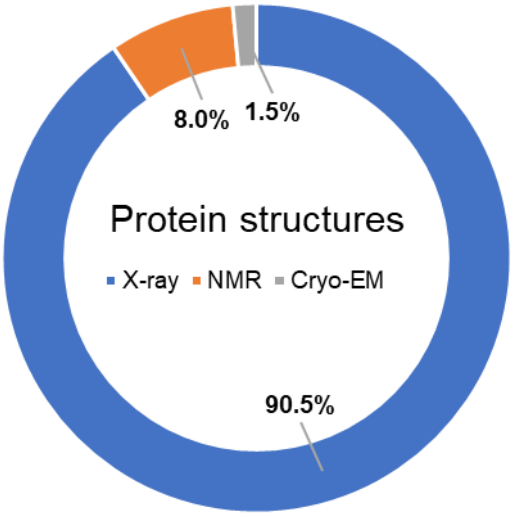


Figure 1: Methods used for protein structure elucidation.

Since protein NMR is usually done in solution, one can avoid the tedious task of growing well-ordered protein crystals and is also able to analyze samples at atomic or near atomic resolution which could not be crystallized. Protein NMR structures with reasonable resolution usually require the incorporation of NMR-active isotopes (I=1/2) of carbon (^{13}C) and nitrogen (^{15}N), because at their natural abundance of 1.1% for ^{13}C and 0.37% for ^{15}N they exhibit a poor relative sensitivity to ^{1}H of $1.8 \cdot 10^{-4}$ and $3.9 \cdot 10^{-6}$, respectively.[3] Isotope labeling strategies and techniques will be discussed in detail in an upcoming section. The information required for the calculation of three-dimensional structures is obtained from the recorded 2D-, 3D- or higher dimensional spectra. The first step is usually the assignment of each resonance in the spectrum to the specific nucleus in the amino acid chain. Hetero- and homonuclear multi-dimensional NMR-experiments with specific pulse sequences are used for backbone and side chain assignment.[4] The thereby obtained spectral data gives ac-

cess to torsion angle restraints via scalar coupling (J-coupling) and the Karplus equation which can be used together with chemical shifts to predict secondary structure elements (α-helices, β-sheets). Dipolar coupling between protons via the nuclear Overhauser effect (NOE) can give additional information of proximity of certain non-adjacent residues in space and leads to another set of structural restraints.[2] These distances and angles are then used to calculate a three-dimensional structure that fits the experimental data best. The main limitation of NMR-based structure determination is the size/molecular weight, the larger the protein gets the likelier it is that signals are overlapping and line-broadening occurs.[4]

Another application of nuclear magnetic resonance is the investigation of interactions and dynamics of proteins. Proteins are not static but rather in constant motion, different parts of the protein might be more flexible than others. To fully understand protein function, it is also necessary to take those fast or slow motions and conformational changes into account. The time scales on which NMR can be used for those observations range from picosecond to seconds.[5] Fast protein dynamics (ps to ns) can be determined by ^{15}N and $^{13}C'$ relaxation techniques to assess the backbone flexibility of proteins.[6] Slower processes (µs to ms) such as conformational changes can be monitored by changing chemical shifts of the involved nuclei. NMR can also be used to shed light on protein folding and unfolding processes at atomic resolution.[7] Conformational flexibility and changes are essential for proteins to fulfill their intrinsic tasks like molecular recognition, catalysis and signaling. Those tasks often involve interactions (ligand binding, protein-protein interactions) of two or more binding partners. NMR also provides tools for studying binding events. Ligand- or target-based titrations can be used to determine affinities in drug discovery. Chemical shift mapping can reveal the residues in the binding pocket of enzymes or receptors by looking at chemical shift perturbation of the nuclei involved. Protein-protein interactions (PPIs) usually cover a larger area than those of small molecules and proteins. Nevertheless, detection of PPIs can be done with NMR by chemical shift perturbation or analyzing intermolecular nuclear Overhauser effects.[4]

1.1.1 Isotope Labeling

Beside advances in hardware and new pulse sequences, isotope labeling techniques have had a large impact on the facilitation and sensitivity of protein NMR spectra over the recent decades. Since the proteins of interest are commonly produced in host organisms or extracts thereof, the growth medium or the remaining protein machinery is supplemented with isotopically labeled nutrients/building blocks.

The overall goal of isotope labeling is to either deplete or enrich signals from NMR-active nuclei with a spin of I=1/2 (^{1}H, ^{13}C, ^{15}N). Depletion of ^{1}H resonances is usually sought after in large proteins because of severe signal overlap and fast spin-spin relaxation. With the incorporation of deuterium (^{2}H, D) into the amino acid residues, it is possible to minimize these problems. Incorporation of ^{13}C and ^{15}N isotopes is necessary because their natural abundant isotopes are either NMR-inactive (^{12}C, I=0) or insensitive (^{14}N, I=1).[5]

1.1.1.1 Uniform Labeling

Supplementing the minimal growth medium of host organisms chosen for recombinant protein expression with certain isotopically labeled nutrients leads to uniform labeled proteins. This approach exploits the full metabolic pathway of a host organism, e.g. *E.coli*., and results in the incorporation of isotopes in all positions of the protein without any selectivity. Usually, the organism grows on a sole carbon and nitrogen source which will be metabolized. In case of carbon-13 labeling ^{13}C-glucose can be used, which is metabolized via glycolysis and thereby enters all amino acid biosynthetic pathways in *E.coli*.[8] An alternative for uniform ^{13}C labeling is ^{13}C-acetate.[9] $^{15}NH_4Cl$ is commonly used as a sole nitrogen source, which leads to ^{15}N incorporation into the protein via different transaminases during amino acid biosynthesis. Since full deuteration (perdeuteration) is normally not wanted because of the immediate loss of dipolar coupling and NOE information, random fractional deuteration (50-90%) is used to incorporate deuterium. This can be achieved by growing the cells in H_2O medium until a high cell density is reached, subsequently the cells are isolated and resuspended in D_2O minimal medium followed by induction of protein expression.[10,11]

1.1.1.2 Selective Labeling

Selective labeling allows the introduction of isotopes at defined positions in the amino acids of a protein. Specific labeling patterns in side chains are able to answer special questions regarding structure, dynamics and binding. Labeled amino acids or metabolic amino acid precursors can be used for this purpose. Isotopically labeled amino acids are usually employed in cell-free expression systems while metabolic amino acid precursors are supplemented to the minimal medium of cells and are biosynthetically converted to the corresponding amino acids.

It is absolutely necessary to know the metabolism of the chosen host organism when employing metabolic precursors. Picking the wrong precursor might lead to unwanted isotope scrambling. Some early metabolic precursors can create distinct isotope patterns of carbon throughout the protein. This can be accomplished by using [2-^{13}C]-glycerol as a sole carbon source and leads to the creation of alternating ^{13}C-^{12}C patterns.[10] Approaches to selectively protonate methyl groups of alanine, valine, leucine and isoleucine in an otherwise perdeuterated protein have also been reported. Protonated pyruvate as a sole carbon source and D_2O-containing growth medium lead primarily to CH_3-methyl groups. However, it should be noted that also CH_2D- and CHD_2-methyl groups arise.[12]

One class of metabolic precursors which has found broad applicability for amino acid selective labeling are α-ketoacids. They can be chemically synthesized from readily available isotope sources such as $^{13}CH_3I$, CD_3I, D_2O, ^{13}C-acetone etc., are supplemented to minimal growth media in relatively low amounts (10-100 mg/l) and can be considered as late metabolic precursors.[13] The corresponding α-ketoacids for valine, leucine, tyrosine, phenylalanine, histidine, methionine and tryptophan have been described with various isotope patterns and employed in selective isotope labeling of proteins.[14,15,16,17] Furthermore, α-ketobutyrate has been used as a selective isoleucine precursor.[18] α-Ketoacids are biosynthetically converted into the corresponding amino acids by transaminases. Simultaneously, ^{15}N can be introduced in the amino acid backbone by adding cheap $^{15}NH_4Cl$ to the growth medium. Other non-α-ketoacid precursors for tryptophan are indole and anthranilic acid.[19,20]

1.1.2 Protein Expression

1.1.2.1 Cell-free systems

Selective labeling can be achieved with isotopically labeled amino acids in cell-free protein expression systems. Cell-free systems usually consist of extracts of *E.coli* that still contain the transcriptional and translational machinery for protein production and DNA plasmids for the protein of interest. Since the metabolism is widely repressed, isotopically labeled amino acids can be used without isotope scrambling and are incorporated selectively via translation. Another advantage of cell-free systems is the possibility to produce and study toxic proteins that otherwise cannot be expressed in living cells. However, cell-free protein production is associated with high costs because of the labeled amino acids used, the elaborate preparation of the corresponding extracts and the low yield of the isotopically labeled protein.[11]

1.1.2.2 Cell-based methods

Cell-based methods exploit the metabolism of living cells to produce a protein of interest. The organism is transfected with a DNA-plasmid on which the overexpression vector is encoded and is grown in M9 minimal medium. Isotopically labeled nutrients or building blocks are supplemented to the medium which allows the incorporation of isotope patterns into the protein. Cell-based methods can be employed for uniform labeling as well as for selective labeling. As discussed above early metabolic precursors (^{13}C-glucose, ^{13}C-acetate, $^{15}NH_4Cl$) are used for uniformly labeled proteins while late metabolic precursors (isotopically labeled α-ketoacids etc.) are selectively converted to the corresponding amino acids via the host organism's metabolic pathways. The selection of an organism depends on the expressed protein. The most commonly used organism is *E.coli* because of its fast and robust growth rates in comparably cheap media, the yield of protein and ease of genetical manipulation. If more sophisticated posttranslational modifications of the protein are needed, eukaryotic yeast cells such as *Pichia pastoris* can be used.[10] Chinese hamster ovary (CHO)[21] and human embryonic kidney (HEK) cells[22] as mammalian cell lines and the fall armyworm *Spodoptera frugiperda* (Sf9) and *Drosophila* (S2) cells[23] as insect cell lines have also been reported for cell-based isotope labeling of proteins. However, mammalian and insect cells need more elaborate conditions to grow well in media and are usually associated with high costs and poor protein yields.[24,25]

1.2 Proline

1.2.1 Proline and its Biological Role

As one of the standard twenty proteinogenic amino acids, proline exhibits its uniqueness already by just looking at its chemical structure (figure 2). It is the only proteinogenic amino acid in which the backbone nitrogen is connected twice to the side chain. Firstly, via the α-carbon (C^{α}) like all other α-amino acids and secondly via the δ-carbon (C^{δ}) of the side chain forming a five-membered pyrrolidine ring.

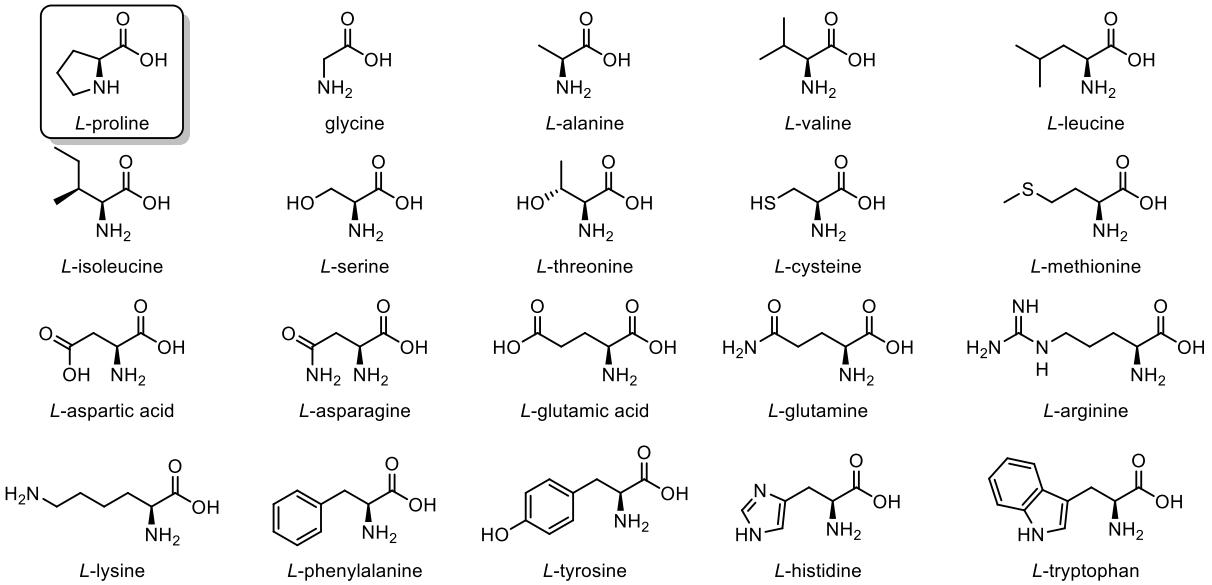


Figure 2: Chemical structures of the twenty proteinogenic amino acids.

This instance leads to several implications in the biological role of proline and hence in the places it is found throughout biomolecules. Since proline contains a secondary amine in its chemical structure, the resulting amides in peptide bonds with preceding residues are tertiary amides and not secondary amides as in X_{aa}-nonPro peptide bonds. Lacking an amide proton in the peptide bond, proline is only able to form one H-bond with its carbonyl oxygen as a H-bond acceptor. Protein secondary structures like α-helices and β-sheets are stabilized by a network of H-bonds. Additionally, the 5-membered pyrrolidine ring causes backbone torsion angle (Φ) restraints making the incorporation of proline especially in β-sheets but also to some extent in α-helices less likely.[26] Proline is therefore often found at the end of secondary structures in turns and loops and is considered as a structure breaker.

However, turns can also be considered as crucial elements for the three-dimensional structure of proteins and proline plays a key role in enabling and stabilizing them. CH-π interactions of proline and aromatic amino acids can contribute to the stabilization of turns. In turns proline is often preceded by histidine and is capable to form a CH-π hydrogen bond with the methylene group at the δ-position of the pyrrolidine ring. Such CH-π hydrogen bonds have also been found in Trp-Pro, Tyr-Pro-Tyr and Pro-Pro-Phe motifs throughout proteins.[27]

The most pivotal feature of proline is its ability to form cis-peptide bonds in proteins. X_{aa}-nonPro peptide bonds predominantly exists in the energetically favored trans conformation because the cis conformation would lead to a steric clash of the side chains (figure 3) which renders it energetically undesirable. However, X_{aa}-Pro peptide bonds exhibit lower energy differences between the trans and cis conformer because even in the trans conformer minor steric hindrance arises.

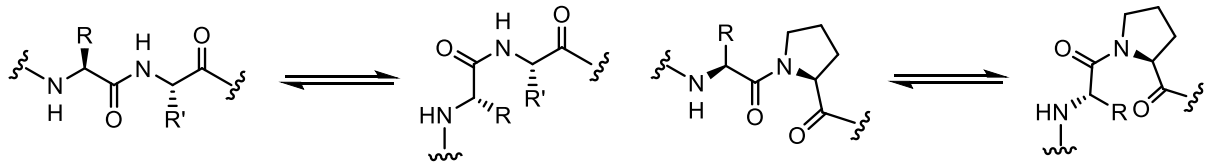


Figure 3: X_{aa}-nonPro and X_{aa}-Pro trans and cis peptide bonds.

While only 0.03% of non-proline peptide bonds in proteins are in cis conformation, up to 5% of X_{aa}-Pro peptide bonds in folded proteins are cis.[28] In an unfolded state numbers reaching from 5% to 80% of the cis X_{aa}-Pro conformer have been reported.[27,29] Proline cis-trans isomerization by prolyl peptidyl isomerases is a rate-limiting step in protein folding and therefore particularly interesting for understanding folding processes and protein dynamics.[30] In the 1970's it was found out that the ^{13}C NMR chemical shifts of C^{β} and C^{γ} in proline differ significantly when in cis or trans state.[31] This can be used to assess the population of each state. In proteins, this has been used for the first time by Dennis Torchia in 1984. He and his coworkers determined cis/trans population in unfolded chicken calvaria collagen by using selectively labeled proline at the γ-position.[32] On average the signal for $^{13}C^{\beta}$ is shifted downfield by 2.41 ppm in the cis conformation (31.75 ppm in trans vs 34.16 ppm in cis), the $^{13}C^{\gamma}$ signal on the other hand is shifted upfield by 2.74 ppm (27.26 ppm in trans vs

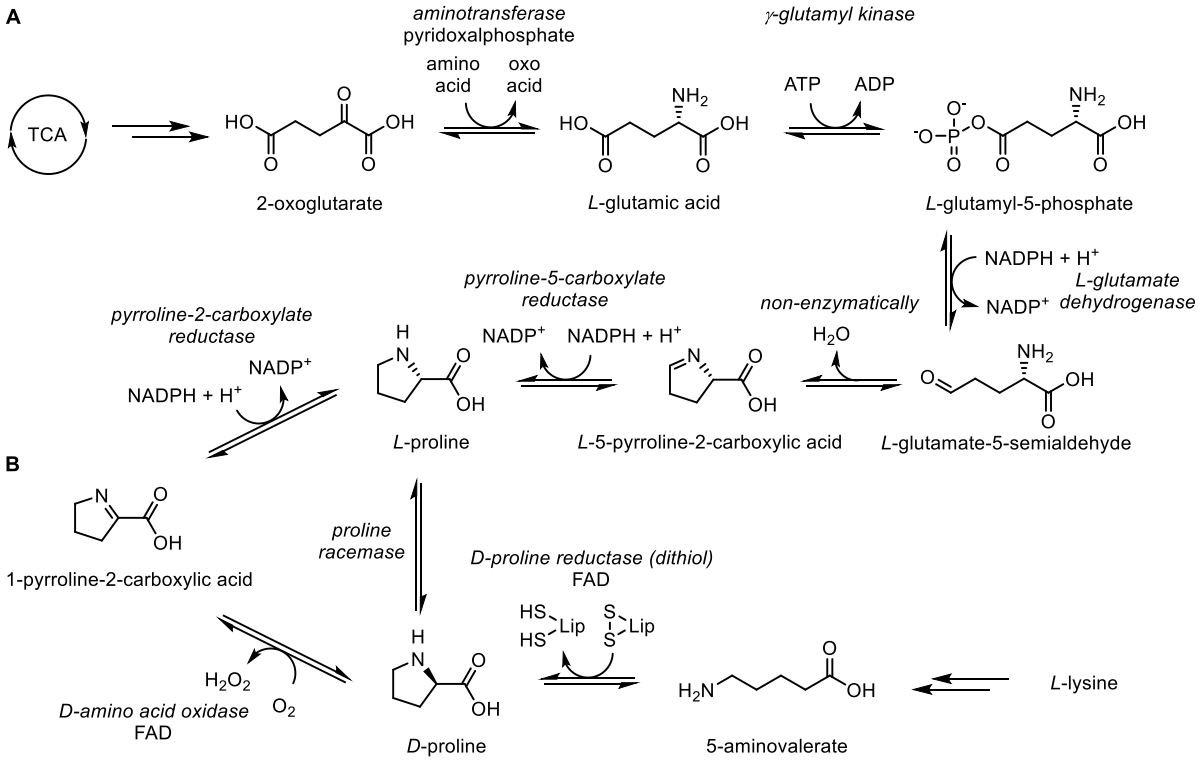
24.52 ppm in cis).[33] The change in chemical shift is caused by the different ring puckering. The pyrrolidine ring can either adopt a C^{γ}-endo or C^{γ}-exo conformation with respect to the carbonyl group sitting on C^{α}. The equilibrium between these two conformations is dependent on the cis/trans peptide bond. In case of the cis conformer the preferred ring conformation at C^{γ} is endo while with the trans conformer no preference between the endo and exo ring conformation could be detected.[34]

Another interesting feature of proline is its appearance in proline-rich motifs (PRMs) and proline-rich regions (PRRs). PRMs and PRRs play key roles in signal transduction in protein-protein interactions and are often found in intrinsically-disordered proteins (IDPs) or intrinsically-disordered regions (IDRs) of otherwise folded proteins. These types of proteins account for 30% of the human proteome and are particularly difficult to study because they only adopt ordered structures for a very short period of time during binding interactions.[35]

1.2.2 Proline Biosynthesis

The biosynthesis of proline is depicted in scheme 1. It starts with a product of the tri-

Scheme 1: Proline biosynthesis in eukaryotes and prokaryotes (**A**) and in mammalian tissue (**B**).



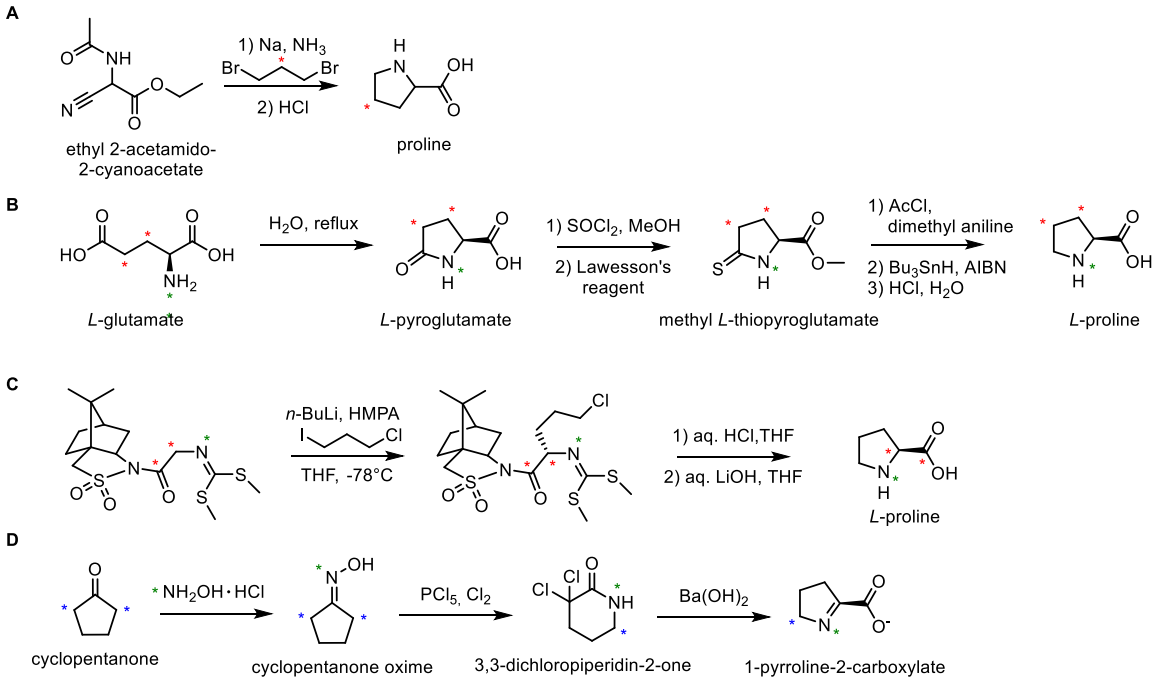
carboxylic acid cycle (TCA-, Krebs- or citrate acid cycle), 2-oxoglutarate which is converted to *L*-glutamate by transaminases and pyridoxal phosphate. *L*-Glutamate is then phosphorylated at the γ-position by the γ-glutamyl kinase under the consumption of ATP. *L*-Glutamate dehydrogenase can reduce *L*-glutamyl-5-phosphate to *L*-glutamate-5-semialdehyde which cyclizes non-enzymatically to *L*-1-pyrroline-5-carboxylate. The last step in the biosynthesis of *L*-proline is the reduction of *L*-1-pyrroline-5-carboxylate by the enzyme pyrroline-5-carboxylate reductase.[36] This pathway (scheme 1A) occurs in prokaryotes and eukaryotes, however, an additional pathway (scheme 1B) is available in mammalian tissue. 5-Amino-valerate, as a degradation product of *L*-lysine, can be converted to *D*-proline by a *D*-proline reductase (dithiol) and lipoic acid.[36] *D*-Proline can either be converted to *L*-proline by a proline racemase or oxidized by a *D*-amino acid oxidase to give 1-pyrroline-2-carboxylic acid. 1-Pyrroline-2-carboxylic acid or 1-pyrroline-2-carboxylate can then be reduced to *L*-proline by a pyrroline-2-carboxylate reductase.[37]

Interestingly, Wright and coworkers discovered that proline auxotrophic *E.coli* as a prokaryote can grow on 1-pyrroline-2-carboxylate as a proline source.[38] They supplemented the growth medium of *E.coli* strain X7026, lacking the two genes responsible for the conversion of *L*-glutamate to *L*-glutamyl-5-phosphate and then to *L*-glutamate-5-semialdehyde, with 1-pyrroline-2-carboxylate (80 mg/l). Only colonies supplemented with *L*-proline or 1-pyrroline-2-carboxylate grew and it was shown that 1-pyrroline-2-carboxylate was directly converted to *L*-proline without isomerization to the natural precursor, *L*-1-pyrroline-5-carboxylate.[39] This instance makes 1-pyrroline-2-carboxylate, next to *L*-1-pyrroline-5-carboxylate, an interesting target as a late metabolic proline precursor for selective isotope labeling of proteins. Wright and his coworkers concluded that 1-pyrroline-2-carboxylate should be considered a natural precursor also in *E.coli*. However, efforts finding the responsible enzyme mitigating the described conversion in *E.coli* were unsuccessful.[40,41]

1.2.3 Proline labeling approaches

A number of synthetic approaches are available for the incorporation of stable isotopes into proline. One racemic approach was used by Torchia and coworkers[32,42] (scheme 2A), who reacted ethyl 2-acetamido-2-cyanoacetate with 1,3-[2-^{13}C]dibromopropane under basic conditions. Deacetylation and hydrolyzation of the nitrile under acidic conditions followed by decarboxylation gave racemic [4-^{13}C]proline. An enantiopure synthesis of isotopically labeled proline from glutamate was reported by Cappon et al.[43] (scheme 2B). First, *L*-glutamate was refluxed to give *L*-pyroglutamate which is subjected to an esterification with thionyl chloride and methanol. In the following step the oxo-group was exchanged for a thiooxo-group with Lawesson's reagent. *N*-Acetylation followed by radical desulfurization with tributyl tin hydride/AIBN yielded methyl N-acetyl-*L*-prolinate. Deacetylation and hydrolysis of the ester gave *L*-[3,4-$^{13}C_2$]proline and *L*-[^{15}N]proline with a 60% yield based on *L*-glutamate and an *ee* of 97% after recrystallization.

Scheme 2: Synthesis of isotopically labeled proline and 1-pyrroline-2-carboxylate. * indicate ^{13}C-labels. * indicate ^{15}N-labels. * indicate ^{2}H/D-labels.



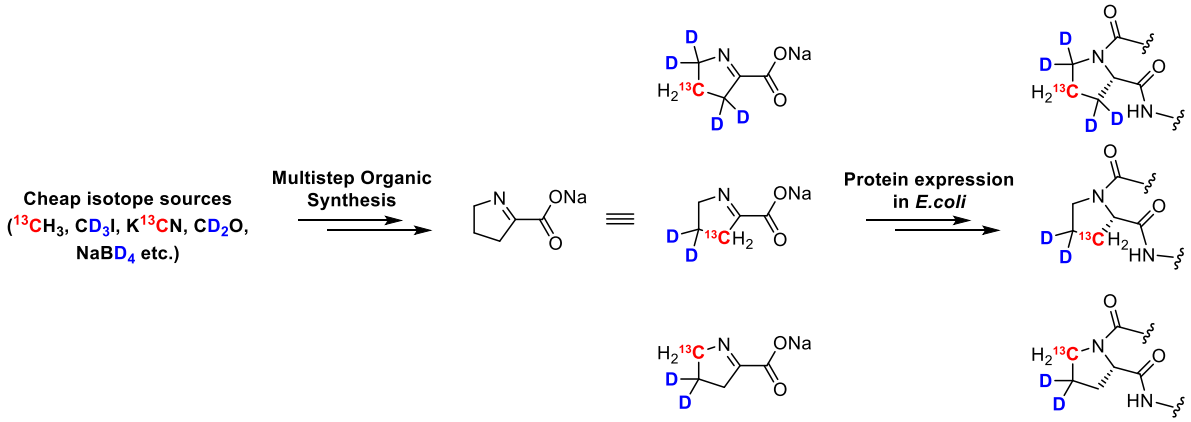
Lodwig et al.[44] exploited the chiral induction of Oppolzer's sultam[45] (scheme 2C), *bis*-(methylthio)methylidene *N*-protected glycine was connected to the sultam and alkylated with *n*-BuLi, HMPA and 3-chloroiodopropane stereoselectively. Removal of the

protection group with aq. HCl/THF and cleavage of the chiral inductor with aq. LiOH/THF gave *L*-proline with an *ee* of 97%. Isotopomers synthesized were [1,2-$^{13}C_2$,^{15}N]-*L*-proline and [^{15}N]-*L*-proline. Lewis et al. synthesized isotopically labeled 1-pyrroline-2-carboxylate starting from [2,2,5,5-$^{2}H_4$]cylcopentanone which was turned into the corresponding oxime (scheme 2D). Beckmann rearrangement with PCl_5, followed by chlorination of the α-position gave 3,3-dichloropiperidin-2-one which yielded 1-pyrroline-2-caboxylate after treatment with $Ba(OH)_2$. 1-[5,5-$^{2}H_2$]pyrroline-2-carboxylate was then used in their studies on metabolic pathways of proline auxotrophic *E.coli*.[39]

1.3 Aim of the Thesis

The aim of the thesis was to develop a late metabolic proline precursor for selective protein labeling. For this purpose, a modular synthesis of sodium 1-pyrroline-2-carboxylate should be developed which would allow the introduction of isotopic labels of ^{13}C, ^{15}N and ^{2}H at different positions in the molecule without changing the overall synthetic plan but rather only switching between isotopically labeled or unlabeled building blocks to access different isotopomers. A special focus should be drawn to the use of cheap isotope sources (scheme 3) such as $^{13}CH_3I$, CD_3I, $K^{13}CN$, $KC^{15}N$, CD_2O, $NaBD_4$ etc.. To avoid undesired scalar couplings and spin diffusion in future protein NMR experiments, the generation of isolated spin systems ($^{13}CH_2$ groups with adjacent CD_2 groups) should be taken into consideration. The ultimate goal of this work is to test ^{13}C-isotopomers of sodium 1-pyrroline-2-carboxylate in protein expression experiments in *E.coli* to assess if it is biosynthetically converted to proline and thereby its use as a proline precursor for selective protein labeling.

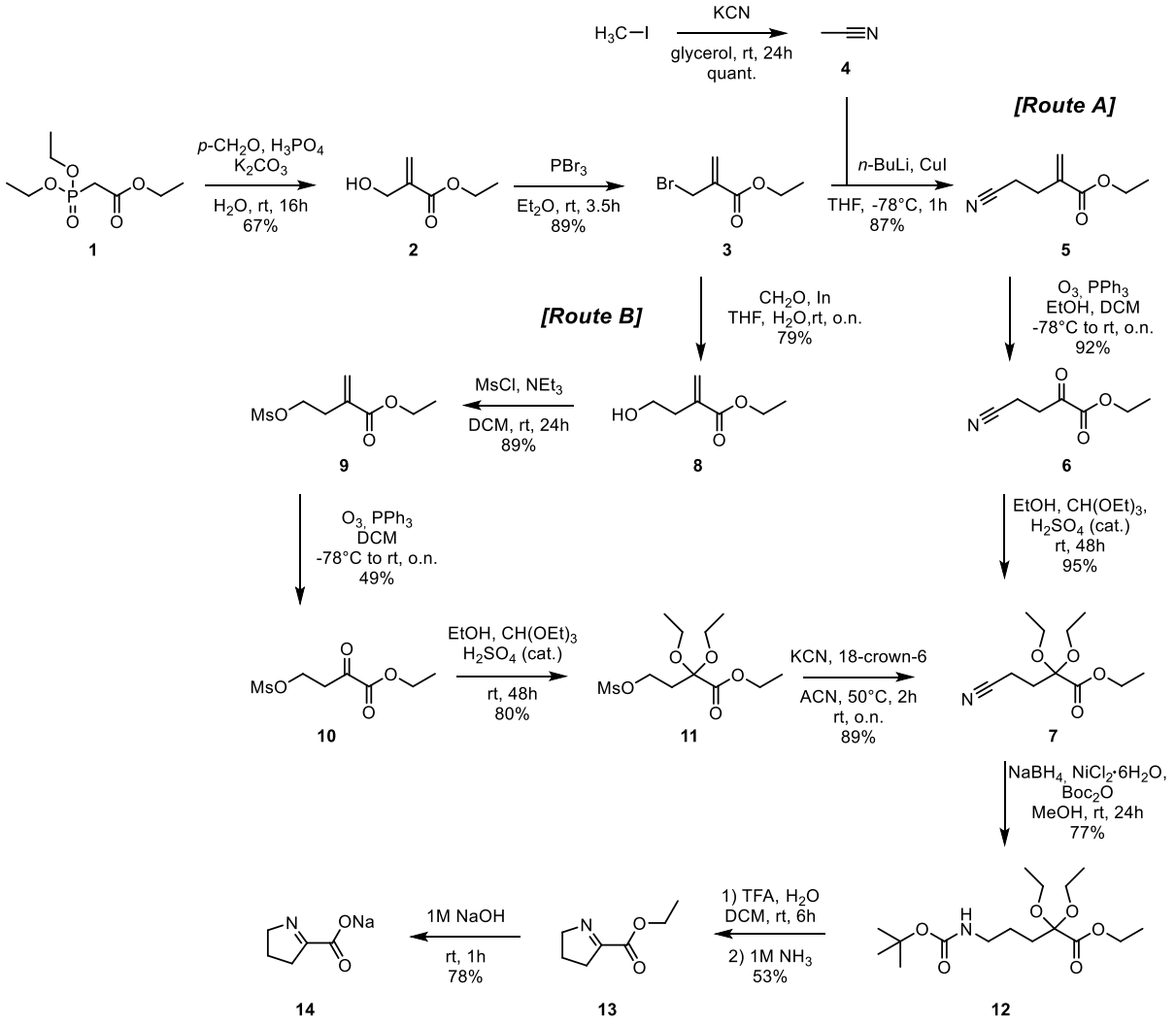
Scheme 3: Graphical description of the aim of this thesis.



2 Results & Discussion

The final synthesis of sodium 1-pyrroline-2-carboxylate (**14**) is summarized in scheme 4 and will be discussed in detail in this section.

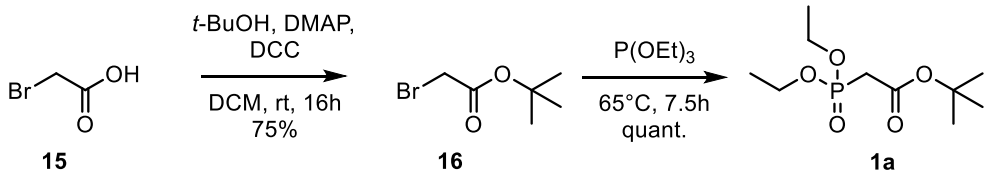
Scheme 4: Synthetic routes for sodium 1-pyrroline-2-carboxylate.



Initially, the project started with *tert*-butyl 2-(diethoxyphosphoryl)acetate (**1a**) which can be synthesized on a gram scale from bromoacetic acid (**15**, scheme 5) via an esterification with *tert*-butanol, *N,N'*-dicyclohexylcarbodiimide and dimethyl amino-pyridine followed by an Arbusow reaction with triethyl phosphite under neat conditions.[46] The *tert*-butyl ester was chosen because its bulky moiety renders it unreactive towards unwanted side reactions which might occur during the crucial reduction of a nitrile to the corresponding amine. Unfortunately, the *tert*-butyl ester proved to be

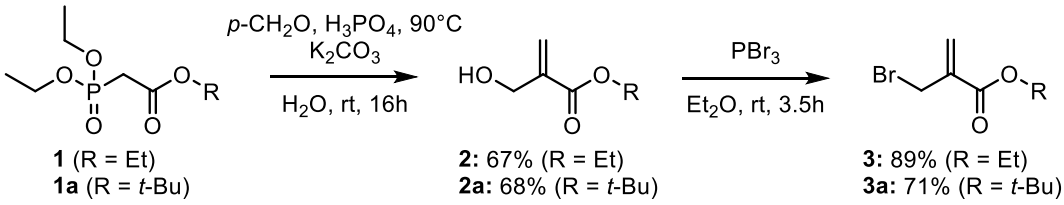
incompatible with the chosen protection group strategy in a later stage of the synthesis.

Scheme 5: Synthesis of *tert*-butyl 2-(diethoxyphophoryl)acetate from bromoacetic acid.



Phosphonoacetates **1** (commercially available) and **1a** were then subjected to a Horner-Wadsworth-Emmons reaction (scheme 6) with either a commercially available aqueous formaldehyde solution[47] or *para*-formaldehyde which has to be cracked by heating under acidic conditions. Vacuum distillation gave access to allylic alcohols **2** and **2a** in moderate yields. Bromination with phosphorus tribromide yielded in the corresponding bromomethyl acrylates which are the starting points for two different synthetic routes. Route A uses the electrophilic properties of **3** whereas in route B this compound is converted into a nucleophile by indium.

Scheme 6: Synthesis of bromomethylacrylates **3** and **3a**.

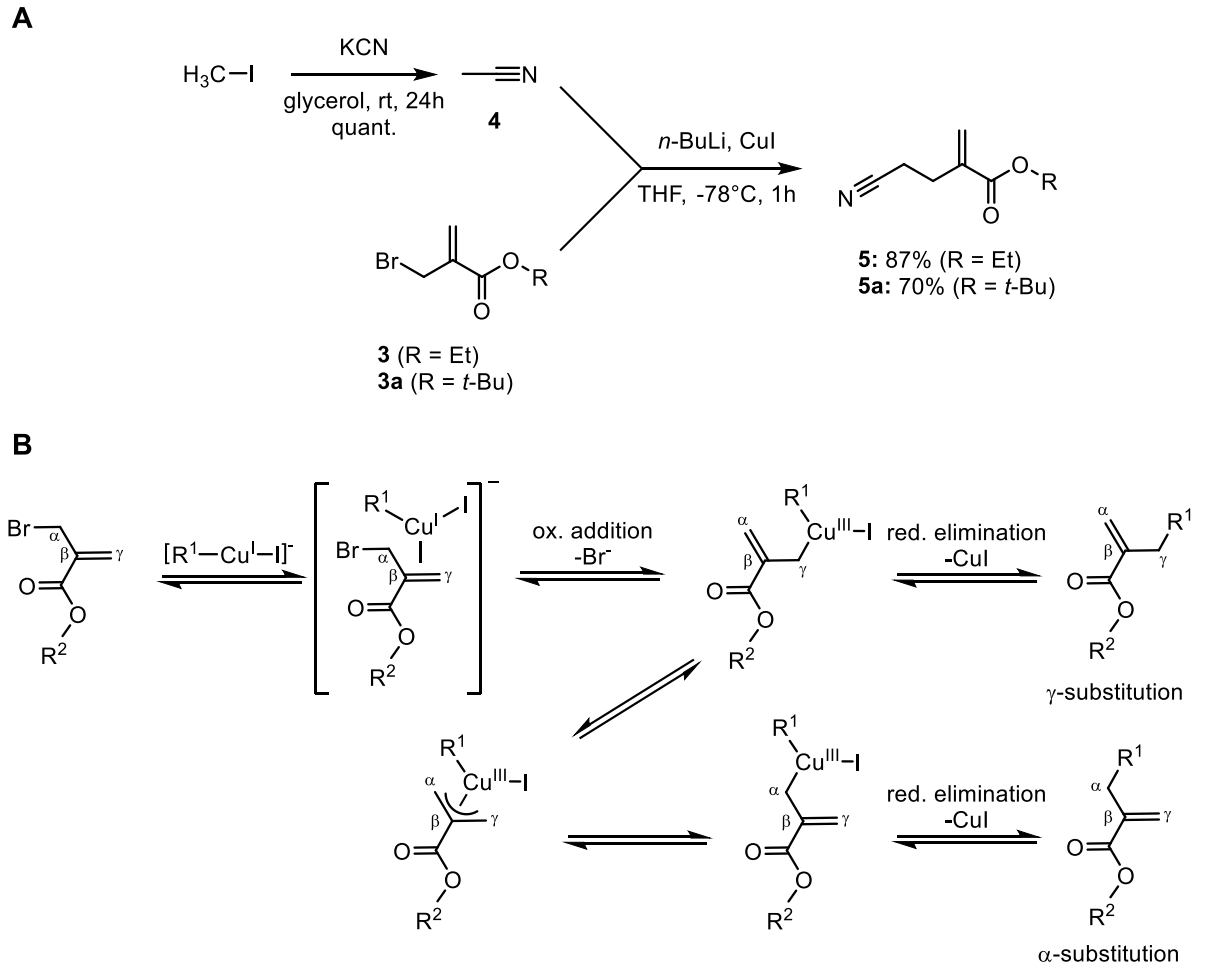


2.1 Route A

As mentioned above this route exploits the electrophilic properties of bromomethylacrylates **3** and **3a** in an allylic substitution reaction with acetonitrile, *n*-butyllithium and copper(I) iodide (scheme 7A). Acetonitrile was synthesized according to a protocol of Anthoni and Nielsen[48] from ordinary and cheap isotope sources (potassium cyanide and methyl iodide). The procedure was slightly modified, to avoid impurities of unreacted methyl iodide in the product, 1.1 equivalents of potassium cyanide (compared to methyl iodide) were used, instead of 1 equivalent in the original procedure. The chosen solvent for this reaction was glycerol which allowed acetonitrile to be distilled off from the reaction flask into a trap, cooled with liquid nitro-

gen, leaving glycerol with a boiling point of 290°C[49] behind. Since its simple and efficient synthesis (quantitative yield), acetonitrile is a good handle for the introduction of isotopic labels and for the generation of different isotopomers (^{15}N-label and ^{13}C-labels at position 1 or 2). Lithiation with *n*-butyllithium (2.5 M in hexanes) and treatment with copper(I) iodide renders acetonitrile active towards allylic substitution reactions with allylic bromides as reported by Corey and Kuwajima.[50] However, following their exact protocol gave poor results (yield <10%) which could be overcome by changing the addition sequence of the original procedure (solid copper(I) iodide was added to the lithiated acetonitrile).

Scheme 7: A: Synthesis of 2-(2-cyanoethyl)acrylates **5** and **5a** from 2-bromomethylacrylates **3** and **3a** and acetonitrile with *n*-BuLi and CuI. **B**: Reaction mechanism of Cu(I) mediated allylic substitution reactions.

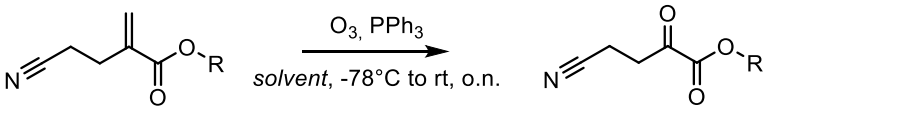


Best results were obtained by lithiating acetonitrile (1.5 equivalents) for 40 minutes in dry THF at -78°C followed by the dropwise addition of this mixture to a suspension of copper(I) iodide in THF at -78°C. The cuprate was then generated at -78°C for 15

min and at -25°C for 10 min. Bromomethylacrylates **3** and **3a** were then added to the red suspension at -78°C and stirred for 1 h yielding 87% of **5** and 70% of **5a**. The mechanism of copper(I) mediated allylic substitution reactions is shown in scheme 7B. After the oxidative addition of the cuprate, the copper(III)-γ-intermediate can undergo two reactions: either a reductive elimination takes place to yield the γ-substitution product or it isomerizes to the corresponding α-intermediate and finally gives the α-substitution product. Since the allylic system of **3** and **3a** possesses a terminal olefin and no additional substitution at the α-position next to the leaving group, this reaction yields the same product inconsiderate of the mechanism it underwent.

Ozonolysis of cyanoethylacrylates **5** and **5a** in dichloromethane at -78°C and reductive work-up with triphenylphosphine (dimethyl sulfide gave byproducts) yielded the corresponding 4-cyano-2-oxobutanoates **6** and **6a** (scheme 8).[51] Interestingly, under these conditions the yield of **6** was highly dependent on the scale of the reaction.

Scheme 8: Ozonolysis of 2-cyanoethylacrylates **5** and **5a** to 4-cyano-2-oxobutanoates **6** and **6a**.



5 (R = Et)
5a (R = *t*-Bu)

6: 51% w. DCM, 92% w. EtOH (R = Et)
6a: 99% w. DCM (R = *t*-Bu)

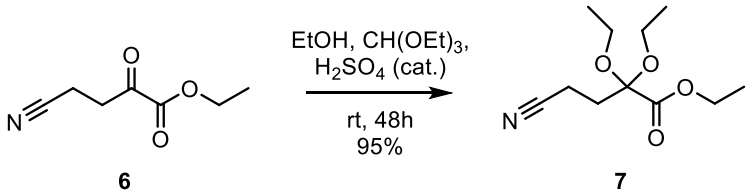
At a 50 mg (0.33 mmol) scale the reaction yielded 91% of **6**, while at a 266 mg (1.74 mmol) scale it only yielded 51% of **6**. The *tert*-butyl derivative **5a** on the other hand reacted smoothly to **6a** on a 250 mg (1.38 mmol) scale with a yield of 99%. The drop in yield of **6** could be avoided by using ethanol as a solvent during the ozonolysis followed by reductive work-up with triphenylphosphine and addition of dichloromethane to boost its solubility. Those optimized conditions yielded 92% of ethyl 4-cyano-2-oxobutanoate.

It was anticipated that the newly formed ketone should undergo acid catalyzed protection with either ethylene glycol or ethanol to give the corresponding ketals. Unfortunately, in the case of *tert*-butyl 4-cyano-2-oxobutanoate **6a** no conditions led to satisfying results. Both, ethylene glycol with 10 mol% *para*-toluenesulfonic acid and eth-

anol, triethyl orthoformate and catalytic amounts of concentrated sulfuric acid, gave unidentifiable product mixtures, presumably through transesterification which results in having two different alcohols in solution and subsequently in product mixtures. Because of this instance, work on the *tert*-butyl derivate was discontinued.

Fortunately, ethyl 4-cyano-2-oxo-butanoate could be easily protected with a procedure of Öhler and Schmidt[52] using ethanol, triethyl orthoformate to remove water from the equilibrium and catalytic amounts of concentrated sulfuric acid to give ethyl 4-cyano-2,2-diethoxybuanoate **7** (scheme 9) which is a shared intermediate of route A and B.

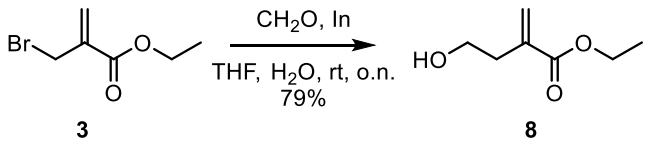
Scheme 9: Formation of diethyl ketal **7**.



2.2 Route B

The first reaction of route B is an indium-mediated allylation of formaldehyde with ethyl 2-bromomethylacrylate (**3**,scheme 10). Indium powder in Barbier-type reactions has the advantage of using water with organic solvents or pure water as a solvent, additionally indium reacts more readily and does not need activation (acid, heat or sonication) like other metals do such as zinc or tin. Indium-mediated allylations to aldehydes or ketones usually proceed under very mild conditions (room temperature) and give very good to excellent yields.[53,54]

Scheme 10: Indium-mediated allylation of formaldehyde with **3**.

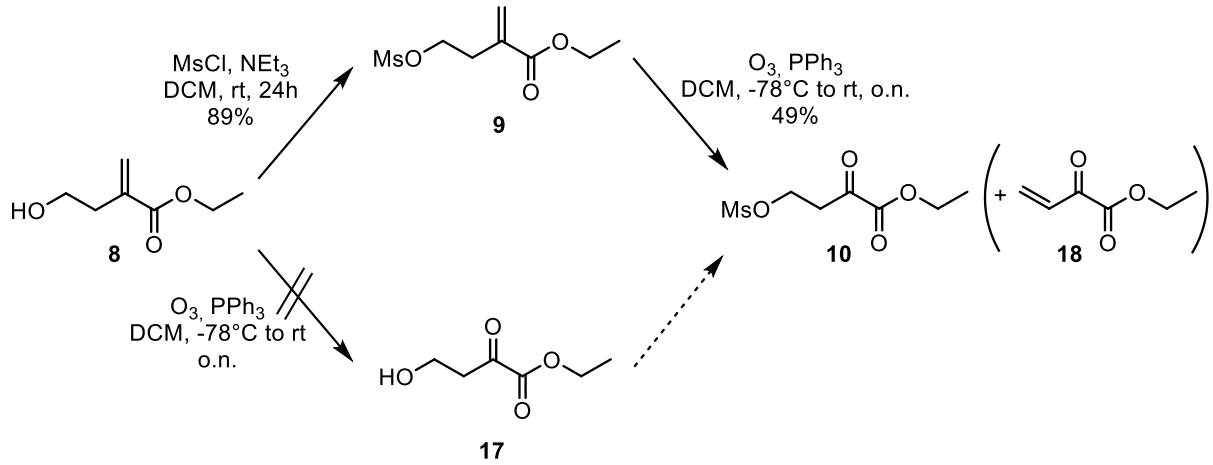


The reaction in this work followed a protocol by Nicponski[55] with a 13.4 M formaldehyde solution, indium powder and **3** in a 1:1.5 mixture of water and THF and yielded

79% of ethyl 2-(2-hydroxyethyl)acrylate after purification on silica gel. Although the crude product of the reaction appeared to be pure in the NMR spectrum, it was noticed that the yield of the subsequent mesylation of the alcohol dropped by 25% if crude **8** was used.

For a S_N2 reaction with potassium cyanide in a later stage of the synthesis, the hydroxyl group of **8** was converted into a leaving group by methanesulfonyl chloride and triethylamine in a very good yield of 89% (scheme 11).[17] Ethyl 2-(2-(methylsulfonyl)oxyethyl)acrylate was then subjected to ozonolysis in DCM to give ethyl 4-((methylsulfonyl)oxy)-2-oxobutanate **10** in a moderate yield of 49%. One problematic side reaction during the ozonolysis and even purification on silica gel was the elimination of the mesylate which leads to ethyl 2-oxobutenoate **18**. It was identified in the ^{1}H-NMR spectrum of **10** (figure 23) by its doublets of doublets at 6.91 ($^3J_{H,H}$ = 17.6 & 10.7 Hz), 6.56 ($^3J_{H,H}$ = 17.6 Hz, $^2J_{H,H}$ = 1.1 Hz) and 6.11 ($^3J_{H,H}$ = 10.7 Hz, $^2J_{H,H}$ = 1.1 Hz) ppm. Those signals correspond to the three protons on the double bond of **10** and are consistent with previously reported spectra.[56] Although this side reaction reduced the yield, the impurity was not troublesome in the next step.

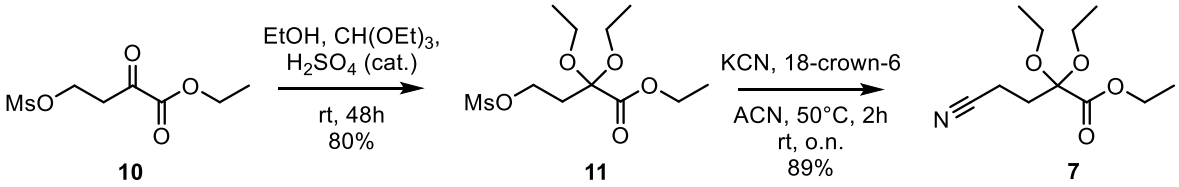
Scheme 11: Synthesis of ethyl 4-((methylsulfonyl)oxy)-2-oxobutanate **10** from ethyl 2-(2-hydroxyethyl)acrylate **8** via mesylation and subsequent ozonolysis of **9**.



Switching the reaction sequence from mesylation followed by ozonolysis to ozonolysis followed by mesylation did not prove to be useful, since the ozonolysis of **8** already lead to an unidentifiable product mixture.

The ketone in α-position to the ester was protected as the corresponding diethyl ketal with the same procedure[52] used in route A, yielding 80% of ethyl 2,2-diethoxy-4-((methylsulfonyl)oxy)butanoate **11** (scheme 12). This set the stage for nucleophilic substitution of the mesylate by potassium cyanide and thereby allowing the introduction of an isotopic label (^{13}C or ^{15}N) at a very late stage of the synthesis. The use of potassium cyanide as a nucleophile is hampered by its low solubility in common organic solvents, it is therefore necessary to use additives such as 18-crown-6 to boost its solubility and reactivity. Crown ethers have the ability to mask the positive charge of alkali metal ions by the partial negative charge of its oxygen atoms and thereby are able to solubilize inorganic salts in organic solvents and activating the negatively charged counterions. The ring size of 18-crown-6 fits the ionic radius of the K^+-ion best.[57,58]

Scheme 12: Ketal protection of **10** followed by a nucleophilic substitution reaction with KCN to form the common intermediate **7** of route A and B.

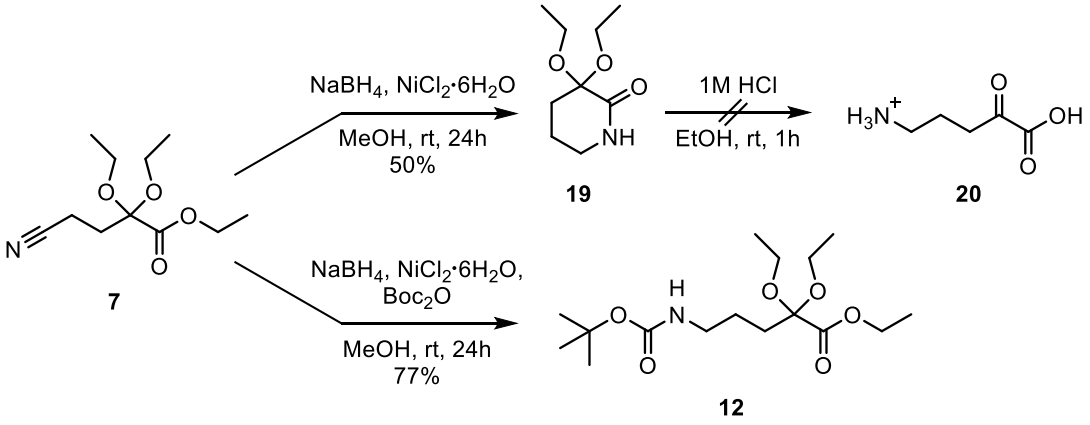


The nucleophilic substitution reaction with only 1.5 equivalents of potassium cyanide and 18-crown-6 was carried out in acetonitrile at 50°C for 2 hours and at room temperature overnight to yield 89% of ethyl 4-cyano-2,2-diethoxybutanoate which was used in the next step without further purification. Experiments with $K^{13}CN$ showed that the reaction can also be carried out with heating for 8.5 h without the necessity of overnight stirring.

Ethyl 4-cyano-2,2-diethoxybutanoate **7** is the shared intermediate of route A and B and the substrate for the reduction of the nitrile. A first attempt with Raney-Ni in a H-Cube® continuous flow hydrogenation reactor led to no conversion and the starting material was recovered even at 50 bar and 70°C. $LiAlH_4$, another commonly used reagent for the reduction of nitriles to amines, could not be applied since it would also reduce the ethyl ester in the molecule. Sodium borohydride alone is not able to reduce nitriles to amines but in combination with transition metal salts such as nickel(II)

chloride or cobalt(II) chloride in alcohols it can convert nitriles to the corresponding amines.[59] This transformation proceeds via at least two mechanisms. The in situ formed nickel or cobalt boride can act as a heterogeneous hydrogenation catalyst (hydrogen gas evolves from the addition of a hydride to a protic solvent). The second mechanism involves sodium borohydride itself, the nitrile gets activated by the boride allowing $NaBH_4$ to reduce the nitrile via a hydride reduction.[60] First attempts to reduce ethyl 4-cyano-2,2-diethoxybutanoate **7** with $NaBH_4$, $NiCl_2 \cdot 6H_2O$ in methanol did not yield the desired amine, but 50% of the δ-lactam **19** (scheme 13). As soon as the nitrile gets reduced to the amine the basic conditions of a hydride reduction cause the nitrogen to attack at the ester and form a six-membered ring. Reactions to open this ring under acidic conditions to give 5-amino-2-oxovalerate **20** were not successful.

Scheme 13: Nickel boride mediated reduction of nitrile **7** with and without trapping of the free amine by Boc_2O.



To circumvent the ring closure a protocol of Caddick et al.[61] was applied. They extended the nickel boride catalyzed reduction by an in situ Boc-protection. This method avoided the undesired δ-lactam **19** and yielded 77% of the desired Boc-protected amine **12**. Since both protecting groups on **12** are acid labile, several conditions (table 1) were investigated to cleave off the Boc-group as well as the ketal. However, neither different concentrations of aqueous HCl in different solvents nor *para*-toluenesulfonic acid did lead to any formation of ethyl 1-pyrroline-2-carboxylate.

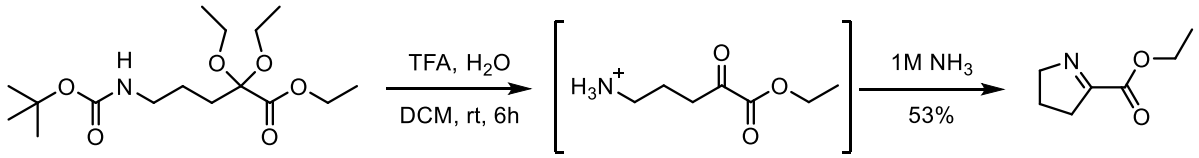
Table 1: Conditions investigated for the double deprotection and ring formation of **13**.

Entry	Conditions[a]	Solvent	Time [h]	Temperature	Yield of **13**
1	3M HCl	THF	2.5	rt	-
2	0.1M HCl	THF	2	rt	-
3	0.1M HCl	EtOH	2	rt	-
4	*p*-TsOH, H_2O	Acetone	16	rt	-
5	TFA, H_2O	DCM	6	rt	53%

[a]After the reaction time entries 1-4 were diluted with water and the pH was adjusted to 9 with 1M NH_3 followed by extraction with DCM. Entry 5 was extracted with H_2O followed by pH adjusting and extraction with DCM.

Only stirring with TFA and water in dichloromethane[62] for 6h at room temperature cleaved off the Boc-group and the ethoxy groups (scheme 14). After the reaction was judged complete by TLC, the mixture was extracted thrice with water to isolate protonated ethyl 5-amino-2-oxovalerate. The pH of the aqueous layer was then adjusted to 9 with 1M NH_3 which led to the formation of the 5-membered pyrroline ring of **13**. Ethyl 1-pyrroline-2-carboxylate was purified on silica gel with a 1:1 mixture of heptane/Et_2O (pentane should be used instead of heptane in future experiments because of its significantly lower boiling point).

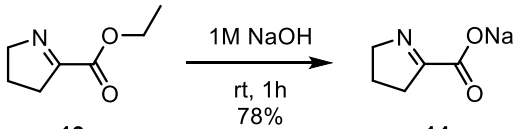
Scheme 14: Boc- and ketal-deprotection with TFA and water in DCM followed by ring formation under basic conditions.



12 **21** **13**

The last step in the synthesis of sodium 1-pyrroline-2-carboxylate is a simple saponification reaction with 1 equivalent of 1M NaOH that yielded 78% of sodium 1-pyrroline-2-carboxylate **14** after lyophilization (scheme 15). The NMR spectrum (figure 33) of **13** shows two triplets of triplets at 3.90 (tt, $^3J_{H,H}$ = 7.7 Hz, $^4J_{H,H}$ = 2.2 Hz, 2H) and 2.79 ppm (tt, $^3J_{H,H}$ = 8.2 Hz, $^4J_{H,H}$ = 2.1 Hz, 2H) and one quintet at 1.98 ppm (quint, $^3J_{H,H}$ = 7.9 Hz, 2H) which is consistent with previously reported NMR-data.[63]

Scheme 15: Saponification of **13** to yield sodium 1-pyrroline-2-carboxylate **14**.

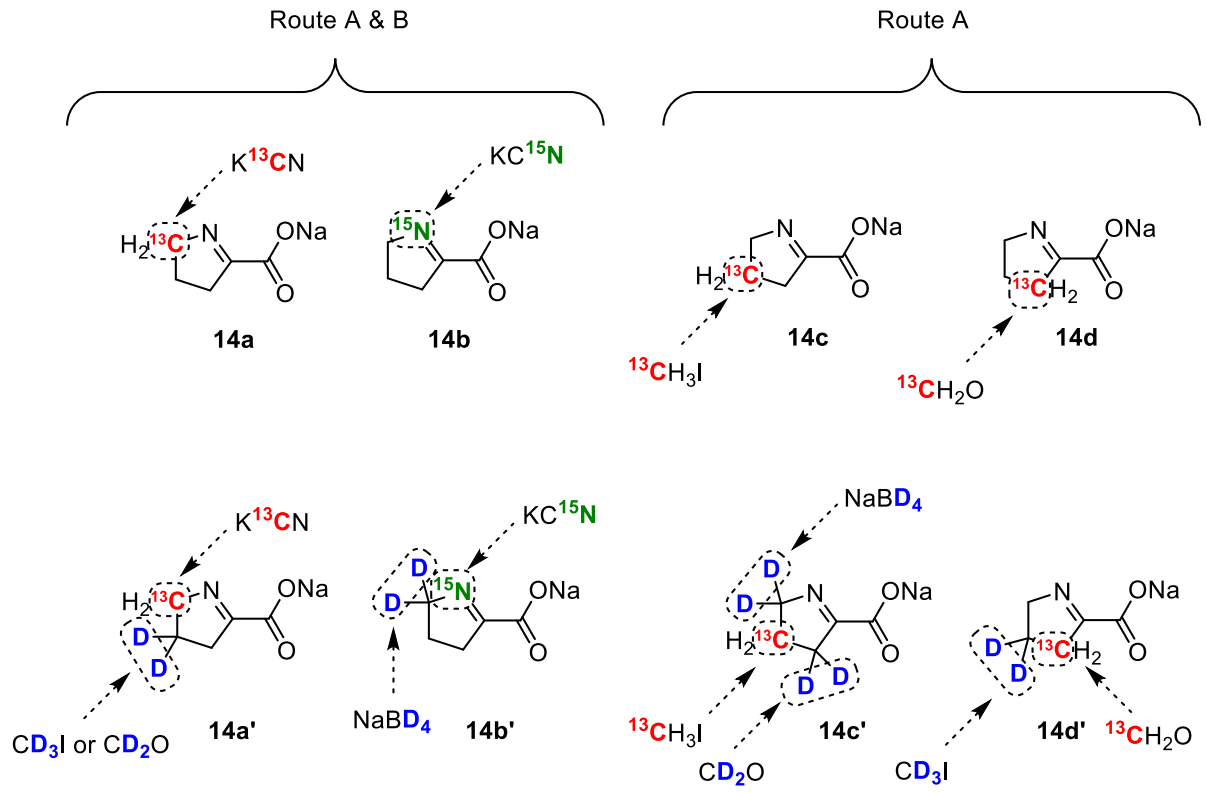


13 **14**

2.3 Accessible Isotopmers & Miscellaneous Experiments

The synthetic routes for sodium 1-pyrroline-2-carboxylate **14** (scheme 4) discussed in the previous section, in theory, allow the generation of various isotopomers of **14** (scheme 16). Isotopomers **14a** and **14b** can be synthesized via route A and B, either by using $K^{13}CN$ or $KC^{15}N$ in the synthesis of acetonitrile (route A) or in the late stage S_N2-reaction of **11** with $K^{13}CN$ or $KC^{15}N$ (route B). The corresponding compounds with isolated spin systems **14a'** and **14b'** are accessible just by switching to isotopically labeled versions of the reagents used in the designed synthesis. Deuteration at the γ-position (**14a'**) can be introduced either with CD_3I in the acetonitrile synthesis (route A) or by using CD_2O in the indium-mediated allylation of route B. Deuteration adjacent to the nitrogen label (**14b'**) is possible by using $NaBD_4$, MeOD and water-free nickel(II) chloride in the reduction of the corresponding nitrile.

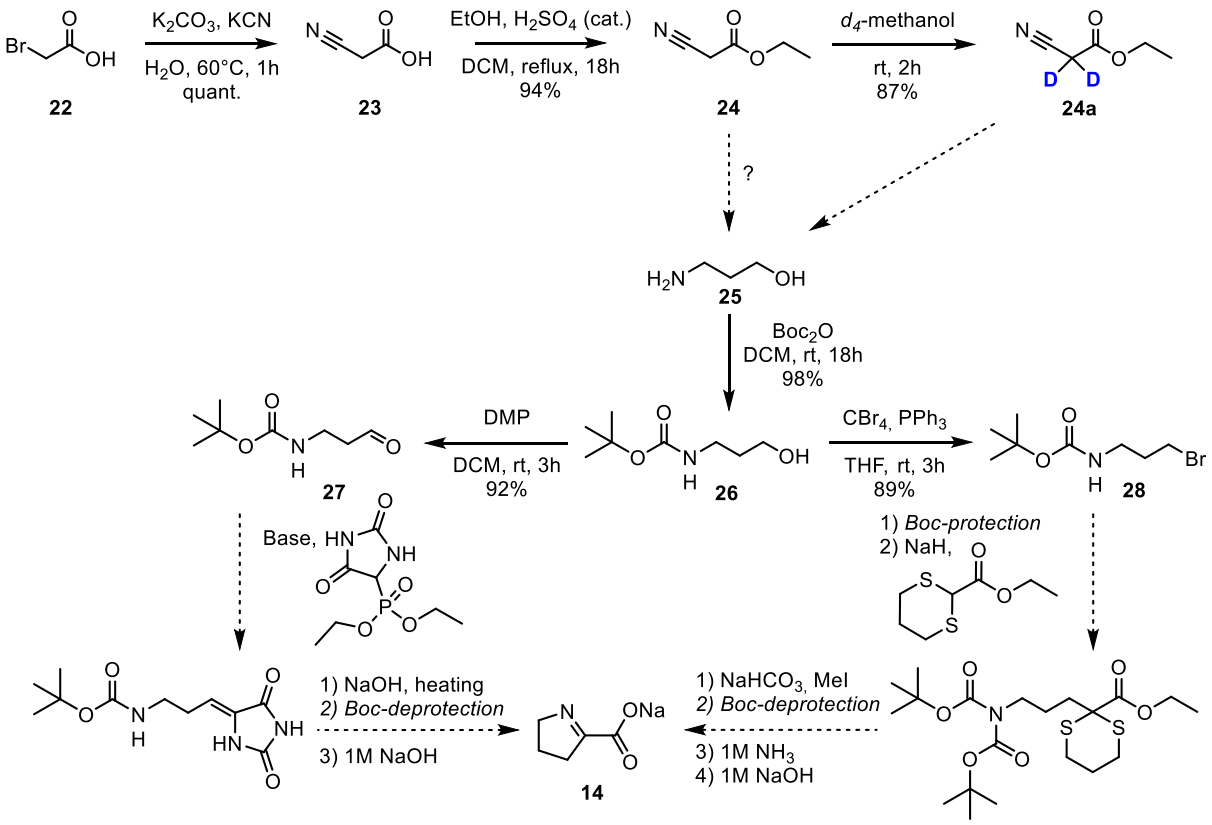
Scheme 16: Isotopomers of **14** accessible by route A and B (Scheme 4).



Route A also gives access to isotopomers **14c** and **14d**: **14c** may be synthesized by using $^{13}CH_3I$ in the acetonitrile synthesis. Adjacent deuteration for **14c'** can be achieved by $NaBD_4$, MeOD and water-free nickel(II) chloride in the reduction step at the δ-position and CD_2O in the Horner-Wadsworth-Emmons reaction. In theory, **14d**

and **14d'** can also be generated by using $^{13}CH_2O$ in the HWE-reaction to label the β-position and CD_3I in the acetonitrile synthesis to deuterate the γ-position. Since ^{13}C-formaldehyde is twice as expensive as [1-^{13}C]bromoacetic acid (58 €/mmol vs 27 €/mmol[64]) and is used in excess in the HWE-reaction, the development of a new route was started. The preliminary results are shown in scheme 17. The synthesis starts with a S_N2-reaction of KCN and bromoacetic acid under aqueous basic conditions[65] to give cyanoacetic acid **23** in a quantitative yield. Esterification with ethanol under acid catalysis in DCM[66] yielded ethyl cyanoacetate **24** which can be easily deuterated with *d*$_1$- or *d*$_4$-methanol.[67] Appropriate conditions for the reduction of **24** to 3-aminopropanol have yet to be found.

Scheme 17: Preliminary results of an alternative route for cost efficient ^{13}C β-position labeling of **14**.



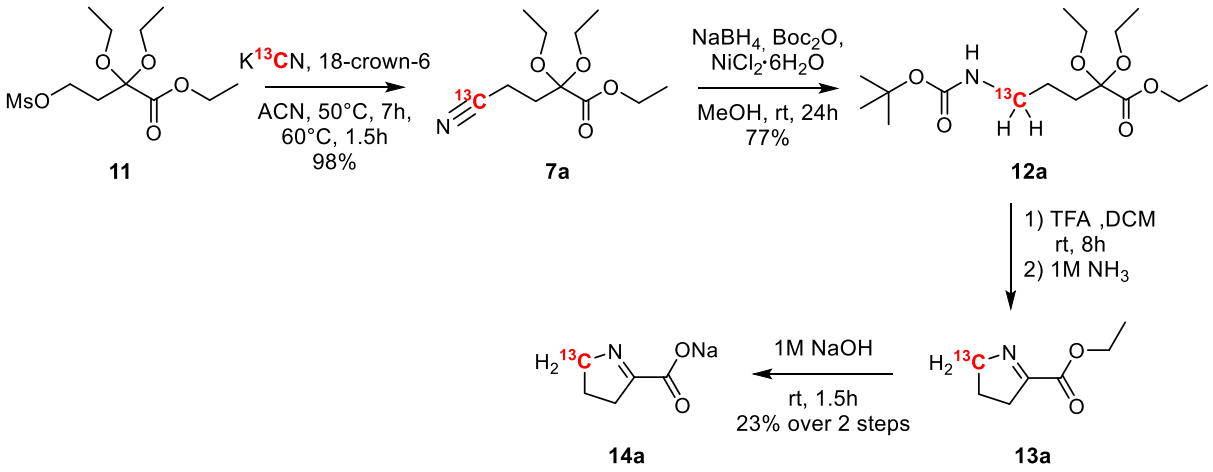
Boc-protection[68] of the amino group followed by oxidation of the alcohol to the corresponding aldehyde with DMP[69] gave **27** in a very good yield. In future experiments one could try to synthesize the corresponding hydantoin in a Horner-Wadsworth-Emmons reaction.[70,71] The hydantoin could then be converted under basic conditions and heating to the α-keto acid.[14] Boc-deprotection followed by ring closure and neu-

tralization of the acid would give sodium 1-pyrroline-2-carboxylate **14**. Alternatively, alcohol **26** can be converted into bromide **28** via an Appel-reaction[67], double Boc-protection followed by nucleophilic replacement of the bromide with the Umpolung-reagent ethyl 1,3-dithiane-2-carboxylate should give the corresponding dithiane. Removal of the dithiane may be achieved by $NaHCO_3$ and MeI.[72] Boc-deprotection followed by ring closure with aqueous ammonia and saponification with sodium hydroxide should give access to **14**.

2.4 Synthesis of sodium 1-[5-^{13}C]pyrroline-2-carboxylate & Protein Labeling

To assess the applicability of isotopically labeled sodium 1-pyrroline-2-carboxylate as a proline precursor for selective protein labeling, isotopomer **14a** was synthesized (scheme 18). With mesylate **11** in hand, route B provides the fastest way for the introduction of a ^{13}C-label. K^{13}CN was used as an isotope source and the same reaction sequence was applied as in the unlabeled synthesis: The S_N2-reaction with K^{13}CN gave nitrile **7a** followed by the reduction to yield Boc-protected amine **12a**. Finally, double-deprotection, cyclization and saponification gave 77 mg of sodium 1-[5-^{13}C]pyrroline-2-carboxylate **14a**.

Scheme 18: Synthesis of sodium 1-[5-^{13}C]pyrroline-2-carboxylate.



In collaboration with Dr. Gerald Platzer and Prof. Robert Konrat compound **14a** was tested as a late metabolic proline precursor in the expression of bromodomain-containing protein 4 (BRD4) in *E.coli* strain BL21(DE3). BRD4 binds to acetylated lysines in histones and is an interesting target for cancer therapy.[19,73] It is not particu-

larly interesting regarding the conformation of its 12 prolines but it is a stable expression system and was therefore chosen to test the incorporation of **14a** into the protein. BRD4 was expressed in M9 minimal medium supplemented with 10 and 100 mg/l of sodium 1-[5-^{13}C]pyrroline-2-carboxylate. Unfortunately, consecutive ^{1}H-^{13}C HSQC NMR experiments with the expressed protein showed no incorporation of **14a** into the amino acid sequence of BRD4. This is probably due to the fact that *E.coli* produces proline biosynthetically from 1-pyrroline-5-carboxylate and only under proline auxotrophy switches to 1-pyrroline-2-carboxylate.[38-41] Since proline auxotrophic *E.coli* strains are available by manipulating their genetic code with biomolecular tools[74] follow up experiments in *E.coli*, unable to synthesize proline itself, will show the applicability of isotopically labeled sodium 1-pyrroline-2-carboxylate in selective protein labeling.

3 Summary & Outlook

For selective protein labeling experiments, a simple and robust synthetic route for sodium 1-pyrroline-2-carboxylate was developed. The synthesis allows the introduction of ^{13}C-labels at the β- and γ-position whose chemical shifts are commonly used for the assessment of cis and trans conformations in X_{aa}-Pro peptide bonds. The γ-position can be labeled with cheap $^{13}CH_3I$ and the adjacent methylene groups can be deuterated with CD_2O (C^{β}) and $NaBD_4$ (C^{δ}) to generate an isolated spin system. Labeling of the β-position is possible with $^{13}CH_2O$ and deuteration at the neighboring γ-position can be achieved with CD_3I. Furthermore, the synthesis also allows the introduction of a ^{15}N-label with $KC^{15}N$ and a ^{13}C-label with $K^{13}CN$ at the δ-position of **14**.

Sodium 1-[5-^{13}C]pyrroline-2-carboxylate (**14a**) was synthesized to assess its applicability in proline selective isotope labeling in proteins. Unfortunately, protein overexpression experiments in *E.coli* strain BL21(DE3) did not show any incorporation into the corresponding protein residues of BRD4. Future experiments with proline auxotrophic strains will show the applicability of isotopically labeled sodium 1-pyrroline-2-carboxylate in selective protein labeling.

Nevertheless, the described synthesis may also be expanded to access novel isotopomers of *L*-proline for cell-free protein labeling or solid-phase peptide synthesis. This might be achieved by chemical[75] or enzymatical[76] means.

4 Experimental Section

4.1 General remarks

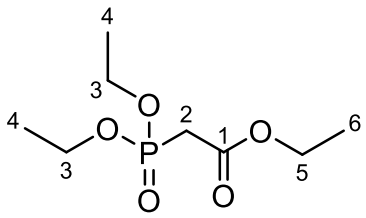
All chemicals were purchased from commercial vendors and were used without further purification unless otherwise stated. Water was deionized prior to use. Reactions under argon atmosphere were carried out in oven-dried flasks which were subjected to three flame-drying and argon backfilling cycles prior to use.

Reaction control was done on precoated TLC sheets ALUGRAM® Xtra SIL G/UV_{254}. TLC-spots were visualized with UV-light, $KMnO_4$-solution (3 g $KMnO_4$ and 20 g K_2CO_3 dissolved in 300 ml H_2O and 5 ml 5% aq. NaOH), cerium ammoniummolybdate solution (48 g $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ and 2.43 g $Ce(SO_4)_2\cdot 4H_2O$ dissolved in 1 l 10% aq. H_2SO_4) or ninhydrin solution (1 g ninhydrin dissolved in 10 ml acetic acid and 100 ml 1-butanol) followed by heating with a heat gun. For column chromatography silica 60 (0.04-0.063 mm) was used.

NMR spectra were recorded on a Bruker AV III 400 MHz spectrometer at 400.27 (^{1}H), 100.65 (^{13}C), 162.03 (^{31}P) MHz. The residual solvent signals were used as internal reference (7.26 (^{1}H) & 77.16 (^{13}C) ppm for $CDCl_3$, 4.79 (^{1}H) ppm for D_2O, 2.50 (^{1}H) & 39.52 (^{13}C) ppm for *d*$_6$-DMSO) for the chemical shifts of ^{1}H and ^{13}C signals. Mass spectra were measured on a Bruker maXis UHR-TOF spectrometer or on a Thermo Scientific LTQ Orbitrap Velos spectrometer with electrospray ionization.

4.2 Synthesis of Sodium 1-pyrroline-2-carboxylate

4.2.1 Triethyl phophonoacetate (1)

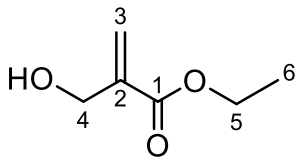


$C_8H_{17}O_5P$
224.19 g/mol

Triethyl phosphite (11.144 g, 67.1 mmol, 1.02 eq.) was added to ethyl bromoacetate (10.981 g, 65.8 mmol, 1 eq) and the mixture was refluxed at 65°C for 7 h. Upon completion of the reaction bromoethane and remaining triethyl phosphite were removed under reduced pressure (bath temperature 60°C). The reaction yielded 14.742 g triethyl phosphonoacetate as a colourless liquid (100%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.21-4.11 (m, 6H, H^3 & H^5), 2.94 (d, $^{2}J_{H,P}$ = 21.6 Hz, 2H, H^2), 1.33 (t, $^{3}J_{H,H}$ = 7.5 Hz, 6H, H^4), 1.27 (t, $^{3}J_{H,H}$ = 7.1 Hz, 3H, H^6) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 166.0 (C^1), 62.8 (d, $^{2}J_{C,P}$ = 6.3 Hz, C^3), 61.7 (C^5), 34.6 (d, $^{1}J_{C,P}$ = 134.3 Hz, C^2), 16.5 (d, $^{3}J_{C,P}$ = 6.2 Hz, C^4), 14.2 (C^6) ppm.
^{31}P NMR (162 MHz, $CDCl_3$): δ 19.74 ppm.
HRMS (ESI$^-$) *m/z*: 223.0375 $[M-H]^-$, calculated for $C_8H_{16}O_5P^-$: 223.0741.

4.2.2 Ethyl 2-(hydroxymethyl)acrylate (2)

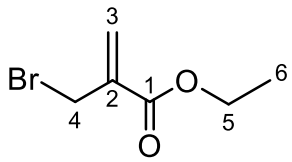


$C_6H_{10}O_3$
130.14 g/mol

Paraformaldehyde (7.193 g, 239.5 mmol, 4 eq.) was suspended in 60 ml H_2O in a three necked flask equipped with a reflux condenser. 0.3 ml 1 M H_3PO_4 was added and the mixture was stirred at 90°C until the white suspension became a clear solution (2 h). The formaldehyde solution was then cooled to room temperature before triethyl phophonoacetate (13.425 g, 59.9 mmol, 1 eq.) was added. The reflux condenser was exchanged for a dropping funnel charged with a sat. aq. solution of K_2CO_3 (13.241 g, 95.8 mmol, 1.6 eq.). The K_2CO_3-solution was added slowly to maintain a reaction temperature below 40°C. After the addition the reaction mixture was stirred at room temperature for 16 h. 75 ml Et_2O was added and the layers were separated. The aqueous layer was extracted thrice with Et_2O and the combined organic layers were washed with H_2O and brine. The organic layer was then dried over $MgSO_4$ and filtered. The solvent was evaporated and the crude product was purified via vacuum distillation (bp: 95-100°C at 11 torr, to avoid polymerization during the distillation, a spatula tip of hydroquinone was added). The reaction yielded 5.252 g of ethyl 2-(hydroxymethyl)acrylate as a colourless liquid (67%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.25 (s, 1H, H^3), 5.82 (s, 1H, H^3), 4.33 (d, $^3J_{H,H}$ = 6.4 Hz, 2H, H^4), 4.25 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^5), 2.26 (t, $^3J_{H,H}$ = 6.5 Hz, 1H, OH), 1.32 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^6) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 166.5 (C^1), 139.7 (C^2), 125.7 (C^3), 62.8 (C^5), 61.0 (C^4), 14.3 (C^6) ppm.
HRMS (ESI$^+$) *m/z*: 153.0520 $[M+Na]^+$, calculated for $C_6H_{10}O_3Na^+$: 153.0522.

4.2.2.1 Ethyl 2-(bromomethyl)acrylate (3)



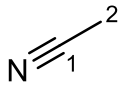
$C_6H_9BrO_2$
193.04 g/mol

Ethyl 2-(hydroxymethyl)acrylate (5.252 g, 40.4 mmol, 1 eq.) was dissolved in 40 ml abs. Et_2O under an argon atmosphere and cooled to -20°C. PBr_3 (5.899 g, 2.070 ml, 21.8 mmol, 0.54 eq.) was added dropwise to the solution. The reaction mixture was then warmed to room temperature and stirred for 3.5 h. The reaction was slowly quenched with 20 ml H_2O at -20°C. After warming to room temperature, the layers were separated and the aqueous layer was extracted thrice with heptane. The combined organic layers were washed with H_2O and brine, dried over $MgSO_4$ and filtered. The solvent was evaporated and the crude product was purified with silica gel chromatography (hept/EtOAc 19:1, $KMnO_4$-stain) to give 5.926 g of ethyl 2-(bromomethyl)acrylate as a colourless liquid (89%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.33 (s, 1H, H^3), 5.94 (s, 1H, H^3), 4.27 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^5), 4.18 (s, 2H, H^4), 1.33 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^5) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 165.0 (C^1), 137.8 (C^2), 129.0 (C^3), 61.5 (C^5), 29.5 (C^4), 14.3 (C^6) ppm.
HRMS (ESI$^+$) *m/z*: 214.9677 $[M+Na]^+$, calculated for $C_6H_9BrO_2Na^+$: 214.9678.

4.2.3 Route A

4.2.3.1 Acetonitrile (4)

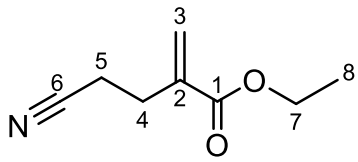


C_2H_3N
41.05 g/mol

Freshly powdered KCN (440 mg, 6.8 mmol, 1.1 eq.) was suspended in 0.4 ml glycerol (dried under high vacuum) in a 5 ml round bottom flask. Methyl iodide (872 mg, 0.38 ml, 6.1 mmol, 1 eq.) was added and the flask was sealed with a lubricated stopper which was secured by a metal clip. The reaction mixture was stirred at 40°C for 1 h, cooled to room temperature and stirred for 24 h. Acetonitrile was then directly distilled from the reaction flask into a liquid N_2 cold trap under reduced pressure. To obtain a high yield the flask was warmed to 30°C and the distillation was allowed to proceed for 1 h. The reaction yielded 252 mg of acetonitrile (100%).

^{1}H NMR (400 MHz, $CDCl_3$: δ 1.93 (s, 3H, H^2) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 116.5 (C^1), 1.6 (C^2) ppm.

4.2.3.2 Ethyl 2-(2-cyanoethyl)acrylate (5)



$C_8H_{11}NO_2$
153.18 g/mol

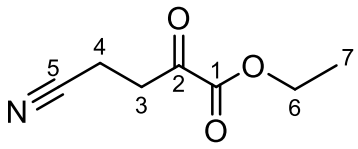
Acetonitrile (478 mg, 11.7 mmol, 1.5 eq.) was dissolved in 24 ml abs. THF under an argon atmosphere and cooled to -78°C. *n*-butyllithium (2.5 M in hexanes, 4.660 ml, 11.7 mmol, 1.5 eq.) was added dropwise to the solution. The reaction mixture was then stirred at -78°C for 40 min before the pale pink mixture was added to a suspension of CuI (2.249 g, 11.8 mmol, 1.52 eq) in 20 ml abs. THF at -78°C under an argon atmosphere. After the dropwise addition the mixture was stirred for 15 min at -78°C and for 10 min at -25°C before it was cooled to -78°C again. Ethyl 2-(bromomethyl)acrylate (1.500 g, 7.8 mmol, 1 eq.), dissolved in 5 ml abs. THF, was slowly added to the red-brownish reaction mixture and it was stirred at -78°C for 1 h. The reaction was slowly quenched with 10 ml sat. aq. NH_4Cl-solution and warmed to room temperature. The layers were separated and the aqueous layer was extracted thrice with EtOAc. The combined organic layers were vigorously washed with sat. aq. NH_4Cl-solution three times followed by washing with H_2O and brine. The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated and the crude product was purified with silica gel chromatography (hept/EtOAc 4:1, $KMnO_4$-stain) to give 1.038 g of ethyl 2-(2-cyanoethyl)acrylate as a colourless oil (87%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.36 (s, 1H, H^3), 5.78 (s, 1H, H^3), 4.26 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^7), 2.69-2.60 (m, 4H, H^4 & H^5), 1.34 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^8) ppm.

^{13}C NMR (101 MHz, $CDCl_3$): δ 166.1 (C^1), 136.9 (C^2), 128.2 (C^3), 119.0 (C^6), 61.2 (C^7), 28.54 (C^4), 17.06 (C^5), 14.29 (C^8) ppm.

HRMS (ESI$^+$) *m/z*: 176.0683 $[M+Na]^+$, calculated for $C_8H_{11}NO_2Na^+$: 176.0682.

4.2.3.3 Ethyl 4-cyano-2-oxobutanoate (6)

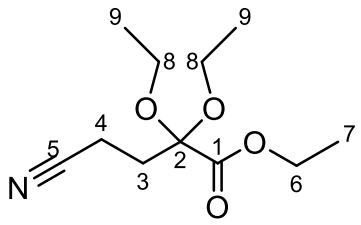


$C_7H_9NO_3$
155.15 g/mol

Ethyl 2-(2-cyanoethyl)acrylate (304 mg, 2 mmol, 1 eq.) was dissolved in 20 ml abs. EtOH and cooled to -78°C. Ozone was bubbled through the solution until the clear solution became murky (7 min). Dry air was then bubbled through the solution until it became clear again. PPh_3 (573 mg, 2.2 mmol, 1.1 eq.) was added to the reaction followed by 20 ml abs. DCM to improve the solubility of PPh_3 in the mixture. The reaction mixture was then allowed to warm to room temperature overnight. The solvent was evaporated and the crude product was purified with silica gel chromatography (EtOAc, $KMnO_4$-stain) to give 292 mg of ethyl 4-cyano-2-oxobutanoate as a colourless oil which solidifies at 4°C (92%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.35 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^6), 3.26 (t, $^3J_{H,H}$ = 7.1 Hz, 2H, H^3), 2.65 (t, $^3J_{H,H}$ = 7.1 Hz, 2H, H^4), 1.38 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^7) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 190.3 (C^2), 159.9 (C^1), 118.20 (C^5), 63.2 (C^6), 35.3 (C^3), 14.1 (C^7), 11.2 (C^4) ppm.
HRMS (ESI$^+$) *m/z*: 178.0472 $[M+Na]^+$, calculated for $C_7H_9NO_3Na^+$: 178.0475.

4.2.3.4 Ethyl 4-cyano-2,2-diethoxybutanoate (7)



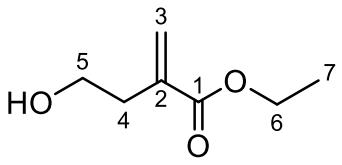
$C_{11}H_{19}NO_4$
229.28 g/mol

Ethyl 4-cyano-2-oxobutanoate (721 mg, 4.7 mmol, 1 eq.) was dissolved in 925 µl abs. EtOH in a microwave vial. Triethyl orthoformate (1.377 g, 1.548 ml, 9.3 mmol, 2 eq.) was added and the vial was purged with argon and sealed. Concentrated H_2SO_4 (18.4 mg, 10 µl, 0.2 mmol, 0.04 eq.) was added and the reaction mixture was stirred at room temperature for 2 days. Volatile reaction components were removed under reduced pressure to give 1.009 g of ethyl 4-cyano-2,2-diethoxybutanoate as a colourless oil which was used without further purification (95%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.28 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^6), 3.60–3.45 (m, 4H, H^8), 2.39–2.35 (m, 2H, H^3), 2.25–2.21 (m, 2H, H^4), 1.33 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^7), 1.23 (t, $^3J_{H,H}$ = 7.1 Hz, 6H, H^9) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 168.4 (C^1), 119.1 (C^5), 100.1 (C^2), 62.0 (C^6), 58.5 (C^8), 30.5 (C^3), 15.2 (C^9), 14.28 (C^7), 12.0 (C^4) ppm.
HRMS (ESI$^+$) *m/z*: 252.1204 $[M+Na]^+$, calculated for $C_{11}H_{19}NO_4Na^+$: 252.1206.

4.2.4 Route B

4.2.4.1 Ethyl 2-(2-hydroxyethyl)acrylate (8)

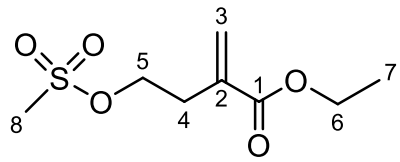


$C_7H_{12}O_3$
144.17 g/mol

Ethyl 2-(bromomethyl)acrylate (1.685 g, 8.7 mmol, 1 eq.) was suspended in 8.7 ml H_2O to give a 1 M suspension. 13.1 ml THF was added under vigorous stirring followed by formaldehyde (13.3 M, 980 µl, 13.1 mmol, 1.5 eq.) and indium powder (1.103 g, 9.6 mmol, 1.1 eq.). The reaction mixture was then stirred for 24h at room temperature. 15 ml 1 M HCl was slowly added and stirring was continued until the white suspension turned into a clear solution. 20 ml EtOAc was added and the layers were separated. The aqueous layer was extracted thrice with EtOAc and the combined organic layers were washed with H_2O and brine followed by drying over $MgSO_4$ and filtering. The solvent was evaporated and the crude product was purified with silica gel chromatography (hept/EtOAc 3:1, $KMnO_4$-stain) to give 998 mg of ethyl 2-(2-hydroxyethyl)acrylate as a colourless liquid (79%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.25 (s, 1H, H^3), 5.66 (s, 1H, H^3), 4.23 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^6), 3.77 (t, $^3J_{H,H}$ = 6.2 Hz, 2H, H^5), 2.59 (t, $^3J_{H,H}$ = 6.1 Hz, 2H, H^4), 1.82 (bs, 1H, OH), 1.31 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^7) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 167.6 (C^1), 137.9 (C^2), 127.2 (C^3), 61.9 (C^6), 61.1 (C^5), 35.8 (C^4), 14.3 (C^7) ppm.
HRMS (ESI$^+$) *m/z*: 167.0678 $[M+Na]^+$, calculated for $C_7H_{12}O_3Na^+$: 167.0679.

4.2.4.2 Ethyl 2-(2-(methylsulfonyl)oxyethyl)acrylate (9)

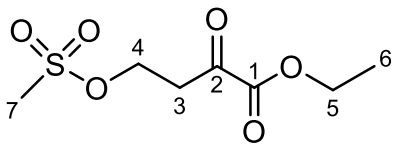


$C_8H_{14}O_5S$
222.26 g/mol

Ethyl 2-(2-hydroxyethyl)acrylate (200mg, 1.4 mmol, 1 eq.) was dissolved in 2.8 ml abs. DCM under an argon atmosphere and cooled to 0°C. NEt_3 (421 mg, 577 µl, 4.2 mmol, 4 eq.) was added slowly followed by the dropwise addition of MsCl (238 mg, 160 µl, 2.1 mmol, 2 eq.) The reaction mixture was then warmed to room temperature and stirred for 24h. 2 ml DCM and 4 ml H_2O were added and the layers were separated. The aqueous layer was extracted thrice with DCM. The combined organic layers were washed with H_2O and brine, dried over $MgSO_4$ and filtered. The solvent was evaporated and the crude product was purified with silica gel chromatography (hept/EtOAc 2:1, $KMnO_4$-stain) to give 266 mg of ethyl 2-(2-(methylsulfonyl)oxyethyl)acrylate as a colourless oil (89%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.31 (s, 1H, H^3), 5.73 (s, 1H, H^3), 4.37 (t, $^3J_{H,H}$ = 6.5 Hz, 2H, H^5), 4.23 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^6), 2.99 (s, 3H, H^8), 2.76 (t, $^3J_{H,H}$ = 6.5 Hz, 2H, H^4), 1.31 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^7) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 166.4 (C^1), 135.4 (C^2), 128.6 (C^3), 68.1 (C^5), 61.2 (C^6), 37.6 (C^8), 32.41 (C^4), 14.3 (C^7) ppm.
HRMS (ESI$^+$) *m/z*: 245.0444 $[M+Na]^+$, calculated for $C_8H_{14}O_5SNa^+$: 245.0454.

4.2.4.3 Ethyl 4-((methylsulfonyl)oxy)-2-oxobutanate (10)

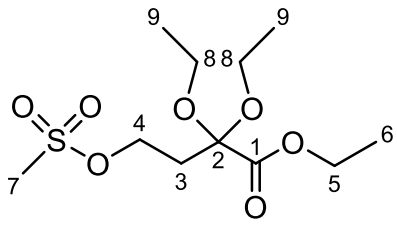


$C_7H_{12}O_6S$
224.23 g/mol

2-(2-(methylsulfonyl)oxyethyl)acrylate (250 mg, 1.1 mmol, 1 eq.) was dissolved in 11 ml abs. DCM and cooled to -78°C. Ozone was bubbled through the solution until it turned blue (6 min). Dry air was then bubbled through the solution until it turned colourless again. PPh_3 (325 mg, 1.2 mmol, 1.1 eq.) was added and the reaction mixture was allowed to warm to room temperature overnight. The solvent was evaporated and the crude product was purified with silica gel chromatography (EtOAc, $KMnO_4$-stain) to give 122 mg of ethyl 4-((methylsulfonyl)oxy)-2-oxobutanate as a colourless oil (49%) with minor impurities of the elimination product (yield was corrected by NMR).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.53 (t, $^3J_{H,H}$ = 5.8 Hz, 2H, H^4), 4.35 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^5), 3.31 (t, $^3J_{H,H}$ = 5.8 Hz, 2H, H^3), 3.06 (s, 3H, H^7), 1.39 (t, $^3J_{H,H}$ = 7.2 Hz, 3H, H^6) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 190.5 (C^2), 160.1 (C^1), 63.5 (C^4), 63.2 (C^5), 38.7 (C^3), 37.5 (C^7), 14.1 (C^6) ppm.
HRMS (ESI$^+$) *m/z*: 247.0247 $[M+Na]^+$, calculated for $C_7H_{12}O_6SNa^+$: 247.0247.

4.2.4.4 Ethyl 2,2-diethoxy-4-((methylsulfonyl)oxy)butanoate (11)

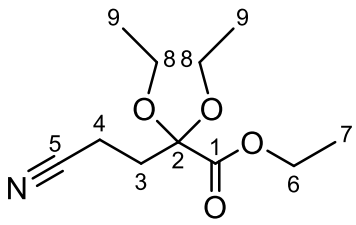


$C_{11}H_{22}O_7S$
298.35 g/mol

Ethyl 4-((methylsulfonyl)oxy)-2-oxobutanate (680 mg, 3 mmol, 1 eq.) was dissolved in 600 µl abs. EtOH in a microwave vial. Triethyl orthoformate (899 mg, 1.010 ml, 6.1 mmol, 2 eq.) was added and the vial was purged with argon and sealed. Concentrated H_2SO_4 (18.4 mg, 10 µl, 0.2 mmol, 0.07 eq.) was added and the reaction mixture was stirred at room temperature for 2 days. Volatile reaction components were removed under reduced pressure and the crude product was purified with silica gel chromatography (hept/EtOAc 3:1, CAM-stain) to give 720 mg of ethyl 2,2-diethoxy-4-((methylsulfonyl)oxy)butanoate as a white solid (80%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.26 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^5), 4.24 (t, $^3J_{H,H}$ = 6.7 Hz, 2H, H^4), 3.60-3.46 (m, 4H, H^8), 2.99 (s, 3H, H^7), 2.36 (t, $^3J_{H,H}$ = 6.7 Hz, 2H, H^3), 1.33 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^6), 1.24 (t, $^3J_{H,H}$ = 7.1 Hz, 6H, H^9) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 168.9 (C^1), 99.6 (C^2), 65.8 (C^4), 62.0 (C^5), 58.3 (C^8), 37.2 (C^7), 34.2 (C^3), 15.3 (C^9), 14.3 (C^6) ppm.
HRMS (ESI$^+$) *m/z*: 321.0970 $[M+Na]^+$, calculated for $C_{11}H_{22}O_7SNa^+$: 321.0978.

4.2.4.5 Ethyl 4-cyano-2,2-diethoxybutanoate (7)

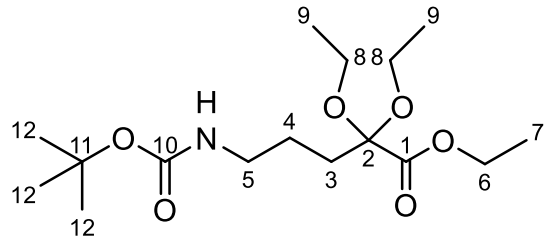


$C_{11}H_{19}NO_4$
229.28 g/mol

Ethyl 2,2-diethoxy-4-(mesyloxy)butanoate (25 mg, 0.08 mmol, 1 eq.), KCN (8 mg, 0.13 mmol, 1.5 eq.) and 18-crown-6 (33 mg, 0.13 mmol, 1.5 eq.) were dissolved in 170 µl abs. ACN in a sealed microwave vial under argon atmosphere. The reaction mixture was stirred for 2 h at room temperature before another 170 µl abs. ACN was added. Stirring was continued for 2h at 50 °C and at room temperature overnight. 500 µl EtOAc and 500 µl H_2O were added and the layers were separated. The aqueous layer was extracted thrice with EtOAc and the combined organic layers were washed with H_2O and brine, dried over $MgSO_4$ and filtered. The solvent was evaporated to give 17 mg of ethyl 4-cyano-2,2-diethoxybutanoate as a yellow oil which was used without further purification (89%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.27 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^6), 3.59-3.45 (m, 4H, H^8), 2.39-2.35 (m, 2H, H^3), 2.25-2.21 (m, 2H, H^4), 1.33 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^7), 1.23 (t, $^3J_{H,H}$ = 7.1 Hz, 6H, H^9) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 168.4 (C^1), 119.1 (C^5), 100.1 (C^2), 62.0 (C^6), 58.5 (C^8), 30.5 (C^3), 15.2 (C^9), 14.3 (C^7), 12.0 (C^4) ppm.
HRMS (ESI$^+$) *m/z*: 252.1217 $[M+Na]^+$, calculated for $C_{11}H_{19}NO_4Na^+$: 252.1206.

4.2.5 Ethyl 5-((*tert*-butoxycarbonyl)amino)-2,2-diethoxypentanoate (12)

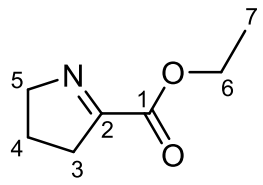


$C_{16}H_{31}NO_6$
333.43 g/mol

Ethyl 4-cyano-2,2-diethoxybutanoate (150 mg, 0.65 mmol, 1 eq.) was dissolved in 6.5 ml abs. MeOH under an argon atmosphere. $NiCl_2 \cdot 6H_2O$ (39 mg, 0.16 mmol, 0.25 eq.) and Boc_2O (286 mg, 301 µl, 1.3 mmol, 2 eq.) were added to the solution. The reaction mixture was then cooled to 0°C and $NaBH_4$ (173 mg, 4.58 mmol, 7 eq.) was added in small portions over 30 min. The black reaction mixture was warmed to room temperature und stirred for 24 h followed by the addition of diethylenetriamine (169 mg, 177 µl 1.64 mmol, 2.5 eq.) and 177 µl H_2O. The reaction mixture was then stirred until it turned into a purple solution. Volatile reaction components were removed under reduced pressure and the crude product was purified with silica gel chromatography (hept/EtOAc 3:1, CAM-stain) to give 167 mg of ethyl 5-((*tert*-butoxycarbonyl)amino)-2,2-diethoxypentanoate as a colourless liquid (77%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.53 (bs, 1H, NH), 4.25 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^6), 3.58-3.42 (m, 4H, H^8), 3.12-3.07 (m, 2H, H^5), 1.91-1.87 (m, 2H, H^3), 1.43 (s, 11H, H^4 & H^{12}), 1.31 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^7), 1.22 (t, $^3J_{H,H}$ = 7.1 Hz, 6H, H^9) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 169.4 (C^1), 156.0 (C^{10}), 101.8 (C^2), 79.3 (C^{11}), 61.5 (C^6), 57.9 (C^8), 40.4 (C^5), 32.0 (C^3), 28.5 (C^{12}), 24.1 (C^4), 15.3 (C^9), 14.4 (C^7) ppm.
HRMS (ESI$^+$) *m/z*: 356.2042 $[M+Na]^+$, calculated for $C_{16}H_{31}NO_6Na^+$: 356.2044.

4.2.6 Ethyl 1-pyrroline-2-carboxylate (13)

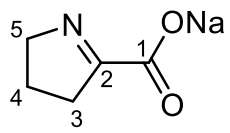


$C_7H_{11}NO_2$
141.17 g/mol

5-((*tert*-butoxycarbonyl)amino)-2,2-diethoxypentanoate (171 mg, 0.5 mmol, 1 eq.) was dissolved in 1 ml DCM and H_2O (21 mg, 21 µl, 1.19 mmol, 6.5 eq.) was added. The reaction mixture was cooled to -15°C before TFA (439 mg, 294 µl, 3.9 mmol, 7.5 eq) was added dropwise. The reaction mixture was stirred for 5 h at room temperature when another equivalent of TFA (59 mg, 39 µl) was added. After 1 h, TLC indicated completion of the reaction and it was cooled to 0°C. 1 ml H_2O was added and the reaction mixture was warmed to room temperature. The layers were separated and the organic layer was extracted thrice with H_2O. The pH of the combined aqueous layers was adjusted to 9 with 1 M aq. NH_3 followed by extraction with DCM three times. The combined organic layers of this extraction were dried over $MgSO_4$ and filtered. The solvent was evaporated and the crude product was purified with silica gel chromatography (hept/Et_2O 1:1, CAM-stain) to give 38 mg of ethyl 1-pyrroline-2-carboxylate as a colourless liquid (53%).
Note: Instead of heptane it is better to use pentane during silica gel chromatography.

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.34 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^6), 4.11 (tt, $^3J_{H,H}$ = 7.5 Hz, $^4J_{H,H}$ = 2.5 Hz, 2H, H^5), 2.83 (tt, $^3J_{H,H}$ = 8.2 Hz, $^4J_{H,H}$ = 2.5 Hz, 2H, H^3), 1.98 (quint, $^3J_{H,H}$ = 8.0 Hz, 2H, H^4), 1.37 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^7) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 168.7 (C^2), 163.0 (C^1), 62.7 (C^5), 61.9 (C^6), 35.5 (C^3), 22.3 (C^4), 14.2 (C^7) ppm.
HRMS (ESI$^+$) *m/z*: 142.0867 $[M+H]^+$, calculated for $C_7H_{12}NO_2^+$: 142.0863,
164.0688 $[M+Na]^+$, calculated for $C_7H_{11}NO_2Na^+$: 164.0682.

4.2.7 Sodium 1-pyrroline-2-carboxylate (14)



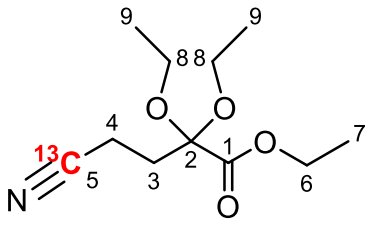
$C_5H_6NNaO_2$
135.10 g/mol

Ethyl 1-pyrroline-2-carboxylate (24 mg, 0.17 mmol, 1 eq.) was cooled to 0°C. 1 M aq. NaOH (170 µl, 0.17 mmol, 1 eq.) was added dropwise under stirring. The reaction mixture was stirred until judged complete by TLC (1 h, CAM-stain). Overnight lyophilization yielded 18 mg of sodium 1-pyrroline-2-carboxylate as a white solid (78%).

^{1}H NMR (400 MHz, D_2O): δ 3.90 (tt, $^3J_{H,H}$ = 7.7 Hz, $^4J_{H,H}$ = 2.2 Hz, 2H, H^5), 2.79 (tt, $^3J_{H,H}$ = 8.2 Hz, $^4J_{H,H}$ = 2.1 Hz, 2H, H^3), 1.98 (quint, $^3J_{H,H}$ = 7.9 Hz, 2H, H^4) ppm.
^{13}C NMR (101 MHz, D_2O): δ 175.7 (C^2), 171.9 (C^1), 60.2 (C^5), 35.7 (C^3), 21.8 (C^4) ppm.
HRMS (ESI$^+$) *m/z*: 158.0195 $[M+Na]^+$, calculated for $C_5H_6NO_2Na_2^+$: 158.0188.

4.3 Synthesis of Sodium 1-[5-^{13}C]pyrroline-2-carboxylate

4.3.1 Ethyl 4-[^{13}C]cyano-2,2-diethoxybutanoate (7a)

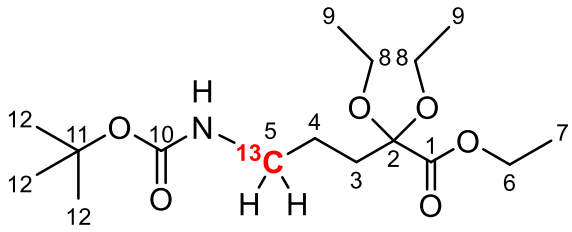


$C_{10}{}^{13}CH_{19}NO_4$
230.27 g/mol

Ethyl 2,2-diethoxy-4-((methylsulfonyl)oxy)butanoate (1.026 g, 3.44 mmol, 1 eq.), K^{13}CN (341 mg, 5.18 mmol, 1.5 eq.) and 18-crown-6 (1.364 mg, 5.18 mmol, 1.5 eq.) were dissolved in 10 ml abs. ACN in a sealed microwave vial under argon atmosphere. The reaction mixture was stirred for 7 h at 50°C before raising the temperature to 60°C for 0.5 h. To drive the reaction to completion another 0.15 equivalents of K^{13}CN (34 mg, 0.51 mmol) and 18-crown-6 (136 mg, 0.51 mmol), dissolved in 1 ml abs. ACN, were added to the reaction mixture. Stirring was continued for 1 h. After the reaction cooled to room temperature, 5 ml H_2O was added and the layers were separated. The aqueous layer was extracted thrice with EtOAc and the combined organic layers were washed with H_2O and brine, dried over $MgSO_4$ and filtered. The solvent was evaporated to give 773 mg of ethyl 4-[^{13}C]cyano-2,2-diethoxybutanoate as a yellow oil which was used without further purification (98%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.27 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^6), 3.59-3.45 (m, 4H, H^8), 2.40-2.34 (m, 2H, H^3), 2.26-2.21 (m, 2H, H^4), 1.33 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^7), 1.23 (t, $^3J_{H,H}$ = 7.1 Hz, 6H, H^9) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 119.2 (C^5) ppm.
HRMS (ESI$^+$) *m/z*: 253.1241 [M+Na]$^+$, calculated for $C_{10}{}^{13}CH_{19}NO_4Na^+$: 253.1240.

4.3.2 Ethyl 5-((*tert*-butoxycarbonyl)amino)-2,2-diethoxy[5-^{13}C]pentanoate (12a)



$C_{15}{}^{13}CH_{31}NO_6$
334.42 g/mol

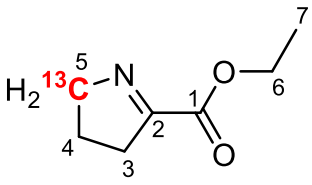
Ethyl 4-[^{13}C]cyano-2,2-diethoxybutanoate (770 mg, 3.3 mmol, 1 eq.) was dissolved in 33 ml abs. MeOH under an argon atmosphere. $NiCl_2 \cdot 6H_2O$ (199 mg, 0.8 mmol, 0.25 eq.) and Boc_2O (1.460 g, 1.536 ml, 6.7 mmol, 2 eq.) were added to the solution. The reaction mixture was then cooled to 0°C and $NaBH_4$ (886 mg, 23.4 mmol, 7 eq.) was added in small portions over 30 min. The black reaction mixture was warmed to room temperature und stirred for 24 h followed by the addition of diethylenetriamine (863 mg, 908 µl, 8.4 mmol, 2.5 eq.) and 908 µl H_2O. The reaction mixture was then stirred for 1 h before another 908 µl of diethylenetriamine and 908 µl of H_2O were added. Stirring was continued until the reaction mixture turned into a purple solution (1 h). Volatile reaction components were removed under reduced pressure and the crude product was purified with silica gel chromatography (hept/EtOAc 3:1, CAM-stain) to give 856 mg of ethyl 5-((*tert*-butoxycarbonyl)amino)-2,2-diethoxy-[5-^{13}C]pentanoate as a colourless oil (77%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.53 (bs, 1H, NH), 4.25 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^6), 3.58-3.42 (m, 4H, C H^8), 3.29-3.24 (m, 1H, H^5), 2.94-2.89 (m, 1H, H^5), 1.92-1.86 (m, 2H, H^3), 1.43 (s, 11H, H^4 & H^{12}), 1.31 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^7), 1.22 (t, $^3J_{H,H}$ = 7.1 Hz, 6H, H^9) ppm.

^{13}C NMR (101 MHz, $CDCl_3$): δ 40.5 (C^5) ppm.

HRMS (ESI$^+$) *m/z*: 357.2082 [M+Na]$^+$, calculated for $C_{15}{}^{13}CH_{31}NO_6Na^+$: 357.2077.

4.3.3 Ethyl 1-[5-^{13}C]pyrroline-2-carboxylate (13a)



$C_6{}^{13}CH_{11}NO_2$
142.16 g/mol

5-((*tert*-butoxycarbonyl)amino)-2,2-diethoxy-[5-^{13}C]-pentanoate (824 mg, 2.5 mmol, 1 eq.) was dissolved in 5 ml abs. DCM and H_2O (288 mg, 288 µl, 16 mmol, 6.5 eq.) was added. The reaction mixture was cooled to -15°C before TFA (2.107 g, 1.414 ml, 18.5 mmol, 7.5 eq) was added dropwise. The reaction mixture was stirred for 6 h at room temperature when another equivalent of TFA (281 mg, 189 µl) was added. After 2 h, TLC indicated completion of the reaction and it was cooled to 0°C. 5 ml H_2O was added and the reaction mixture was warmed to room temperature. The layers were separated and the organic layer was extracted thrice with H_2O. The pH of the combined aqueous layers was adjusted to 9 with 1 M aq. NH_3 followed by extraction with DCM three times. The combined organic layers of this extraction were dried over $MgSO_4$ and filtered. The solvent was evaporated and the crude product was purified with silica gel chromatography (hept/Et_2O 1:1, CAM-stain) to give 112 mg of ethyl 1-[5-^{13}C]pyrroline-2-carboxylate as a colourless liquid with traces of heptane.

Note: Instead of heptane it is better to use pentane during silica gel chromatography.

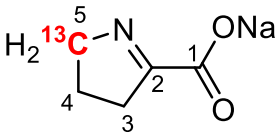
^{1}H NMR (400 MHz, $CDCl_3$): δ 4.34 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^6), 4.04 (dtt, $^1J_{H,C}$ = 140.9 Hz, $^3J_{H,H}$ = 7.5 Hz, $^4J_{H,H}$ = 2.5 Hz, 2H, H^5), 2.86-2.80 (m, 2H, H^3), 1.98 (dquint, $^3J_{H,H}$ = 7.9 Hz, $^2J_{H,C}$ = 2.9 Hz, 2H, H^4), 1.37 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H^7) ppm.

^{13}C NMR (101 MHz, $CDCl_3$): δ 62.7 (C^5) ppm.

HRMS (ESI$^+$) *m/z*: 143.0894 $[M+H]^+$, calculated for $C_6{}^{13}CH_{12}NO_2{}^+$: 143.0896.

165.0714 $[M+Na]^+$, calculated for $C_6{}^{13}CH_{11}NO_2Na^+$: 165.0716.

4.3.4 Sodium 1-[5-^{13}C]pyrroline-2-carboxylate (14a)



$C_4{}^{13}CH_6NNaO_2$
136.09 g/mol

1-[5-^{13}C]pyrroline-2-carboxylate (112 mg, 0.79 mmol, 1 eq.) was cooled to 0°C. 1 M aq. NaOH (788 µl, 0.79 mmol, 1 eq.) was added dropwise under stirring. The reaction mixture was stirred until judged complete by TLC (1.5 h, CAM-stain). Overnight lyophilization yielded 77 mg of sodium 1-[5-^{13}C]pyrroline-2-carboxylate as a white solid (23% over two steps).

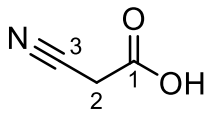
^{1}H NMR (400 MHz, D_2O): δ 3.90 (dtt, $^1J_{H,C}$ = 140.7 Hz, $^3J_{H,H}$ = 7.7 Hz, $^4J_{H,H}$ = 2.3 Hz, 2H, H^5), 2.83-2.77 (m, 2H, H^3), 1.99 (dquint, $^3J_{H,H}$ = 7.9 Hz, $^2J_{H,C}$ = 2.9 Hz, 2H, H^4) ppm.

^{13}C NMR (101 MHz, D_2O): δ 60.2 (C^5) ppm.

HRMS (ESI$^+$) *m/z*: 159.0220 $[M+Na]^+$, calculated for $C_4{}^{13}CH_6NO_2Na_2{}^+$: 159.0222.

4.4 Miscellaneous experiments

4.4.1 Cyanoacetic acid (23)

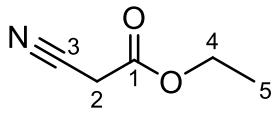


$C_3H_3NO_2$
85.06 g/mol

Bromoacetic acid (8.440 g, 60.7 mmol, 1 eq.) was dissolved in 18 ml H_2O and carefully neutralized under stirring with K_2CO_3 (4.617 g, 33.4 mmol, 0.55 eq.) until pH 9 was reached. KCN (4.252 g, 65.3 mmol, 1.075 eq.), dissolved in 15 ml H_2O, was added dropwise and the reaction mixture was stirred for 15 min at room temperature and for 1h at 60°C. After the reaction was cooled to 10°C, 6 M HCl (11.03 ml, 66.2 mmol, 1.09 eq.) was slowly added. The aqueous mixture was then placed in a liquid-liquid extractor and extracted with Et_2O for 72 h. The organic layer was dried over $MgSO_4$, filtered and the solvent was evaporated. The reaction yielded 5.167 g of cyanoacetic acid as a white solid which was used without further purification (100%).

^{1}H NMR (400 MHz, *d*$_6$-DMSO): δ 13.40 (bs, 1H, COOH), 3.86 (s, 2H, H^2) ppm.
^{13}C NMR (101 MHz, *d*$_6$-DMSO): δ 165.7 (C^1), 115.6 (C^3), 24.6 (C^2) ppm.

4.4.2 Ethyl cyanoacetate (24)

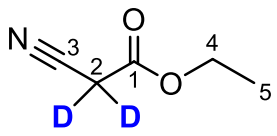


$C_5H_7NO_2$
113.12 g/mol

Cyanoacetic acid (2.191 g, 25.8 mmol, 1 eq.) was dissolved in 95 ml DCM. Ethanol (5.933 g, 7.51 ml, 128.8 mmol, 5 eq.) and concentrated H_2SO_4 (75 mg, 41 µl, 0.8 mmol, 0.03 eq.) were added and the mixture was refluxed for 18 h with a reversed Dean-Stark apparatus to trap the liberated H_2O. After the reaction cooled down to room temperature 65 ml 10% aq. $NaHCO_3$-solution was added and the layers were separated. The aqueous layer was extracted twice with DCM. The combined organic layers were dried over $MgSO_4$, filtered and the solvent was evaporated. The reaction yielded 2.752 g ethyl cyanoacetate as a colourless liquid which was used without further purification (94%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.28 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H^4), 3.44 (s, 2H, H^2), 1.33 (t, $^3J_{H,H}$ = 7.2 Hz, 3H, H^5) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 163.0 (C^1), 113.2 (C^3), 63.1 (C^4), 24.9 (C^2), 14.1 (C^5) ppm.

4.4.3 Ethyl [2-$^{2}H_2$]cyanoacetate (24a)



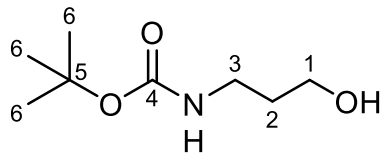
$C_5H_5D_2NO_2$
115.13 g/mol

Ethyl cyanoacetate (25 mg, 0.22 mmol, 1 eq.) was dissolved in 400 µl [$^{2}H_4$]methanol and stirred for 2 h at room temperature. The solvent was evaporated to give 22 mg of ethyl [2-$^{2}H_2$]cyanoacetate as a colourless liquid (87% yield, 79% deuteration).

Note: Instead of [$^{2}H_4$]methanol one can also use [*O*-^{2}H]methanol. To elevate the deuteration level one can simply run the reaction again with fresh [*O*-^{2}H]methanol.

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.27 (q, $^{3}J_{H,H}$ = 7.2 Hz, 2H, H^4), 3.44-3.42 (m, 0.21 H, H^2), 1.32 (t, $^{3}J_{H,H}$ = 7.2 Hz, 3H, H^5) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 163.0 (C^1), 113.2 (C^3), 63.1 (C^4), 24.9-24.5 (m, C^2), 14.1 (C^5) ppm.

4.4.4 *tert*-Butyl (3-hydroxypropyl)carbamate (26)

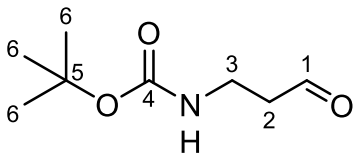


$C_8H_{17}NO_3$
175.23 g/mol

3-aminopropanol (9.83 g, 10 ml, 131 mmol, 1 eq.) was dissolved in 100 ml abs. DCM under an argon atmosphere. Boc_2O (31.42 g, 33 ml, 144 mmol, 1 eq.), dissolved in 70 ml abs. DCM, was added over 30 min via a dropping funnel. The argon balloon was exchanged with a gas bubbler to allow the forming gas to escape and the reaction was stirred at room temperature for 18 h. The reaction mixture was diluted with 200 ml Et_2O and washed with sat. aq. $NaHCO_3$-solution and brine. The organic layer was dried over $MgSO_4$, filtered and the solvent was evaporated. The crude product was purified with silica gel chromatography (hept/EtOAc 1:1, $KMnO_4$-stain) to give 22.474 g of *tert*-butyl (3-hydroxypropyl)carbamate as a colourless oil (98%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.73 (bs, 1H, NH), 3.66 (t, 2H, H^1), 3.28 (t, 2H, H^3), 2.54 (bs, 1H, OH), 1.66 (quint, 2H, H^2), 1.45 (s, 9H, H^6) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 79.8 (C^5), 59.5 (C^1), 37.1 (C^3), 33.1 (C^2), 28.5 (C^6) ppm.
HRMS (ESI$^+$) *m/z*: 198.1102 $[M+Na]^+$, calculated for $C_8H_{17}NO_3Na^+$: 198.1101.

4.4.5 *tert*-Butyl (3-oxopropyl)carbamate (27)

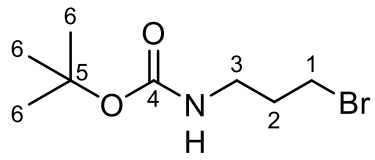


$C_8H_{15}NO_3$
173.21 g/mol

tert-Butyl (3-hydroxypropyl)carbamate (1.520 g, 8.7 mmol 1 eq.) was dissolved in 45 ml abs. DCM under an argon atmosphere. Dess-Martin periodinane (4.450 g, 10.5 mmol, 1.2 eq.) was added and the reaction mixture was stirred at room temperature for 3 h. The reaction was diluted with 70 ml Et_2O and washed with 20% aq. $Na_2S_2O_3$-solution, followed by sat. aq. $NaHCO_3$-solution. The combined aqueous layers were extracted twice with Et_2O and the combined organic layers were washed with 20% aq. $Na_2S_2O_3$-solution, sat. aq. $NaHCO_3$-solution and brine. The organic layer was dried over $MgSO_4$, filtered and the solvent evaporated to give 1.380 g of *tert*-butyl (3-oxopropyl)carbamate as a colourless oil which was used without further purification (92%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.81 (s, 1H, H^1), 4.88 (bs, 1H, NH), 3.42 (q, $^3J_{H,H}$ = 6.0 Hz, 2H, H^3), 2.70 (t, $^3J_{H,H}$ = 5.8 Hz, 2H, H^2), 1.43 (s, 9H, H^6) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): δ 201.5 (C^1), 156.0 (C^4), 79.7 (C^5), 44.5 (C^2), 34.2 (C^3), 28.5 (C^6) ppm.
HRMS (ESI$^+$) *m/z*: 174.1125 $[M+H]^+$, calculated for $C_8H_{16}NO_3^+$: 174.1125.

4.4.6 *tert*-Butyl (3-bromopropyl)carbamate (28)



$C_8H_{16}BrNO_2$
238.13 g/mol

tert-Butyl (3-hydroxypropyl)carbamate (2.000 g, 11.4 mmol, 1 eq.) and triphenylphosphine (5.980 g, 22.8 mmol, 2 eq.) was dissolved in 115 ml abs. THF under argon atmosphere. CBr_4 (7.570 g, 22.8 mmol, 2 eq.) was slowly added to the mixture whereupon the colourless solution turned yellow. After stirring for 3 h at room temperature, the solvent was evaporated. The crude product was purified with silica gel chromatography (hept/EtOAc 5:1, ninhydrin-stain) to give 2.420 g of *tert*-butyl (3-bromopropyl)carbamate as a colourless oil (89%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.65 (bs, 1H, NH), 3.43 (t, $^3J_{H,H}$ = 6.5 Hz, 2H, H^1), 3.27 (q, $^3J_{H,H}$ = 6.4 Hz, 2H, H^3), 2.04 (quint, $^3J_{H,H}$ = 6.5 Hz, 2H, H^2), 1.44 (s, 9H, H^6) ppm.
^{13}C NMR (101 MHz, $CDCl_3$): 156.1 (C^4), 79.6 (C^5), 39.1 (C^3), 32.9 (C^2), 30.9 (C^1), 28.5 (C^6) ppm.
HRMS (ESI$^+$) *m/z*: 260.0256 $[M+Na]^+$, calculated for $C_8H_{16}BrNO_2Na^+$: 260.0257.

4.5 Protein Expression

Bromodomain-containing protein 4 (BRD4) was expressed in *E.coli* BL21(DE3). The BRD4 plasmid contained a *N*-terminal TEV-cleavable His6-tag to facilitate purification, a lac-operon and a kanamycin resistance to select for transformants. Overnight cultures were grown in 10 ml LB-medium containing kanamycin (1 mM). 4x1 L LB medium was inoculated with overnight cultures and the cells were agitated at 37°C until an OD_{200} 0.7 was reached. The mixture was centrifuged at 4000 rpm and the resulting pellet was suspended in 1 l M9 minimal medium supplemented with sodium 1-[5-^{13}C]pyrroline-2-carboxylate (10 or 100 mg/l, dissolved in H_2O at a concentration of 100 mg/ml). The cells were agitated for 40 min at 37°C before induction with 0.5 mM IPTG. After 18 h of expression at 18°C cells were harvested by centrifugation at 4000 rpm. 5 µl protease inhibitor was added to the resulting pellet and it was resuspended in 40 ml buffer A (50 mM TRIS, 500 mM NaCl, pH 7.5) and 20 mM imidazole. Lysis was achieved by sonication for 3x2 min at 0°C. The lysate was then centrifuged for 20 min at 18000 rpm and 0°C. The supernatant was loaded onto a Ni^{2+}-column (HisTrap HP, 5 mL, GE Healthcare) which was washed with buffer A + 20 mM imidazole. The protein was eluted with buffer A + 500 mM imidazole and identical fractions were pooled and concentrated to 1 ml with 3 kDa Amicon vials. The concentrate was diluted to 10 ml with buffer A and β-mercapto ethanol (1 mM) and 1 mg TEV-protease were added. The TEV-cut was run at 4°C overnight. The TEV-cut sample was loaded onto a Ni^{2+}-column for a final purification step and the flow through was collected (buffer A was used as eluent). Samples containing BRD4 were pooled and concentrated. NMR samples were prepared in 10 mM sodium phosphate, 100 mM NaCl, 1 mM dithiothreitol, pH 7.5.

5 Appendix

5.1 NMR Spectra

5.1.1 Triethyl phosphonoacetate (1)

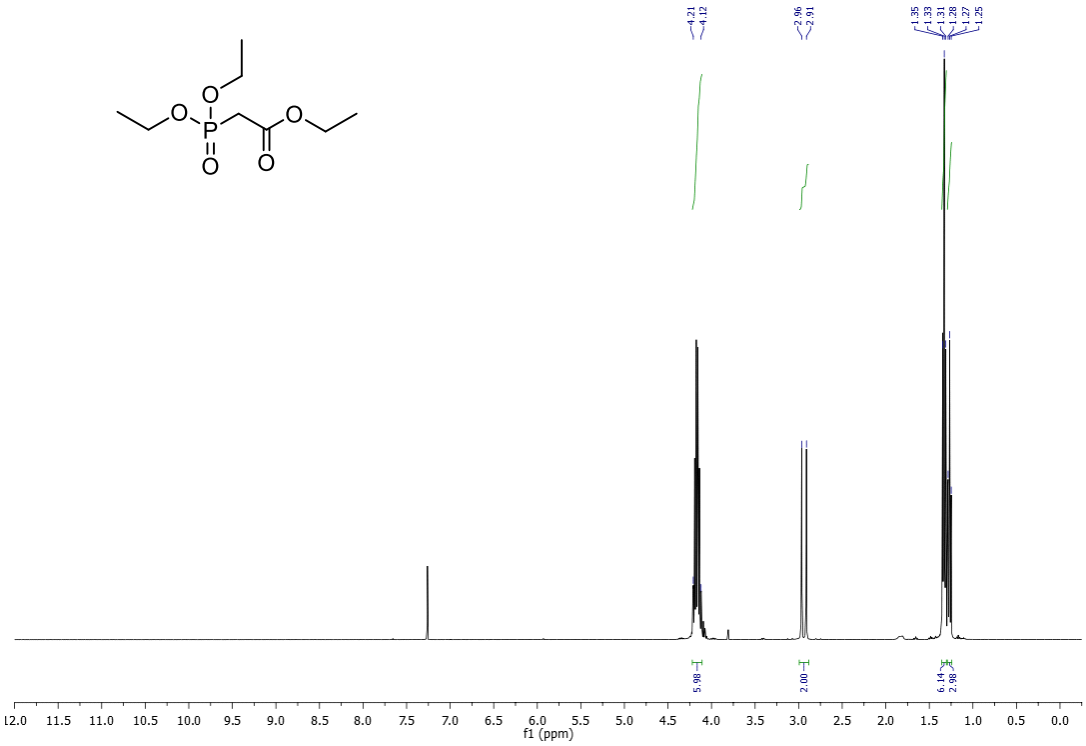


Figure 4: ^{1}H-NMR (400 MHz, $CDCl_3$) of triethyl phosphonoacetate.

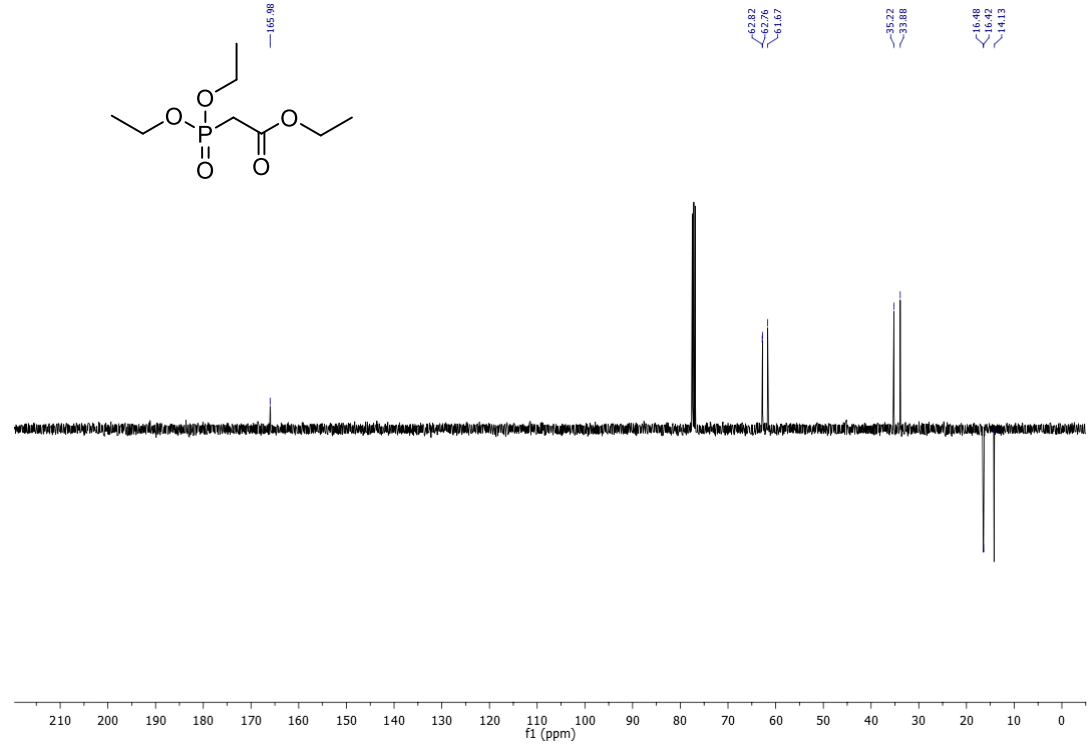


Figure 5: ^{13}C-NMR (101 MHz, $CDCl_3$) of triethyl phosphonoacetate.

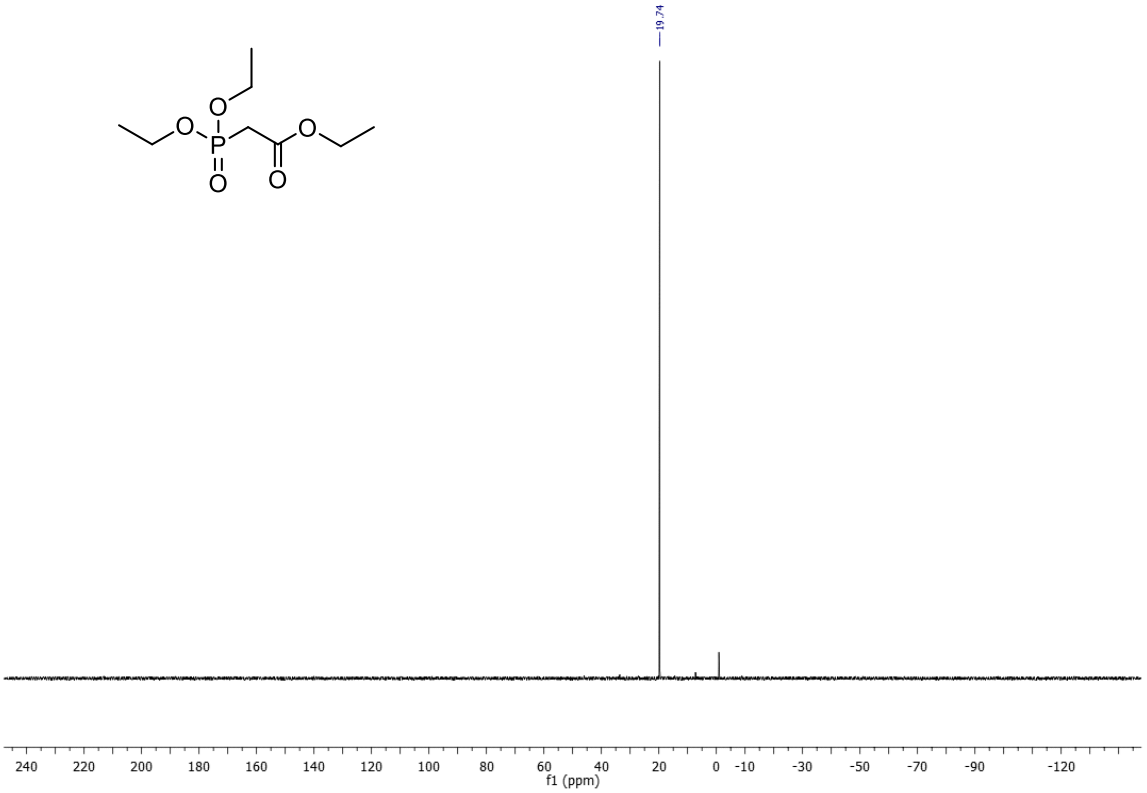


Figure 6: ^{31}P-NMR (162 MHz, $CDCl_3$) of triethyl phosphonoacetate.

5.1.2 Ethyl 2-(hydroxymethyl)acrylate (2)

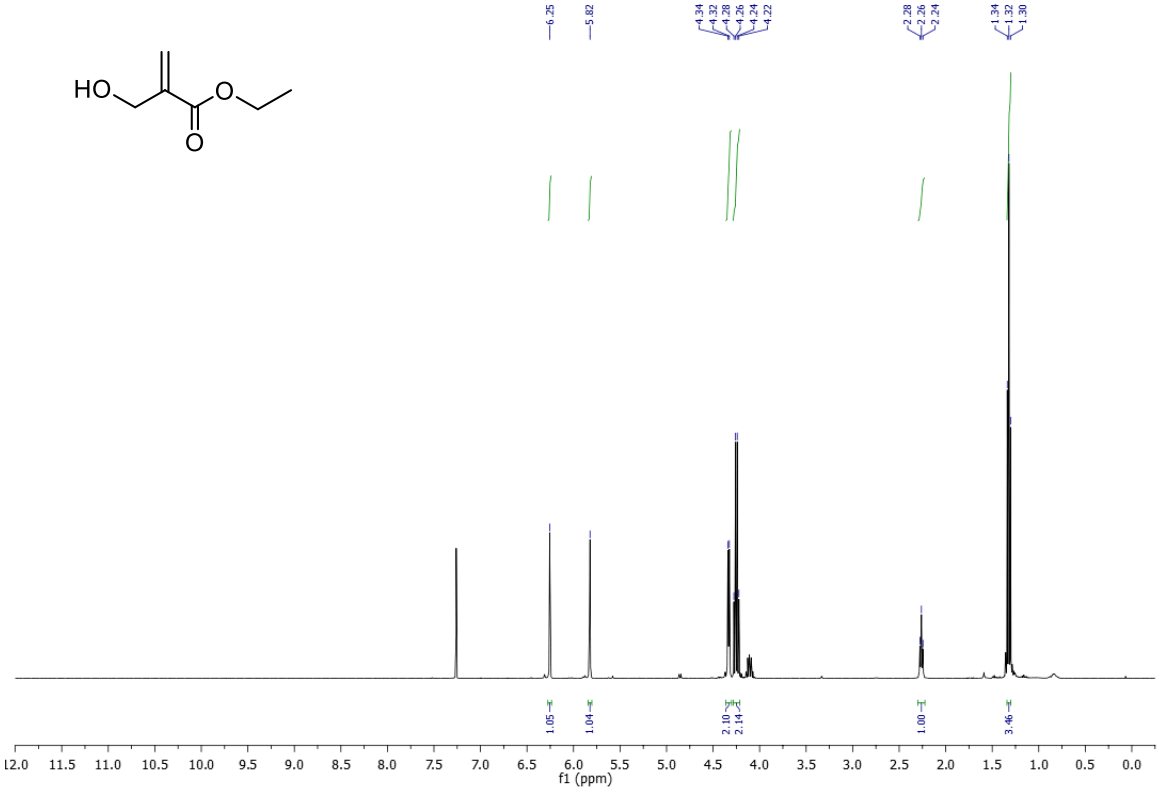


Figure 7: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 2-(hydroxymethyl)acrylate.

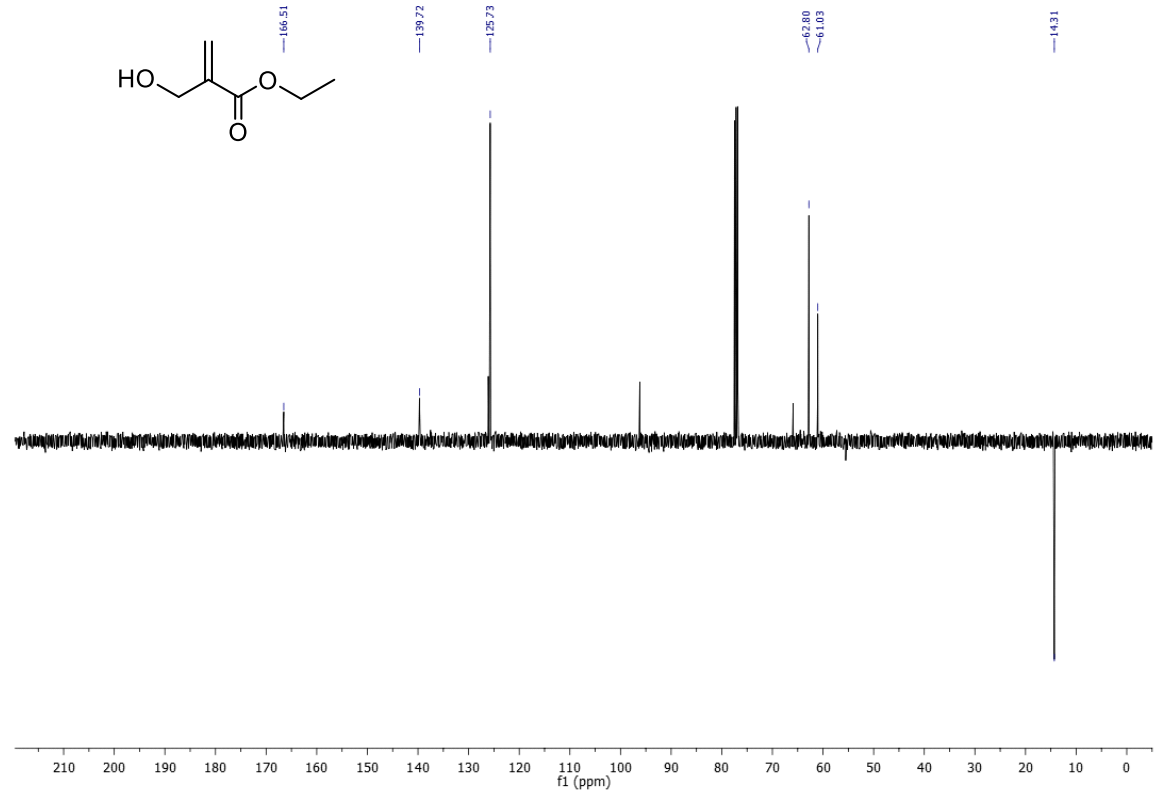


Figure 8: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 2-(hydroxymethyl)acrylate.

5.1.3 Ethyl 2-(bromomethyl)acrylate (3)

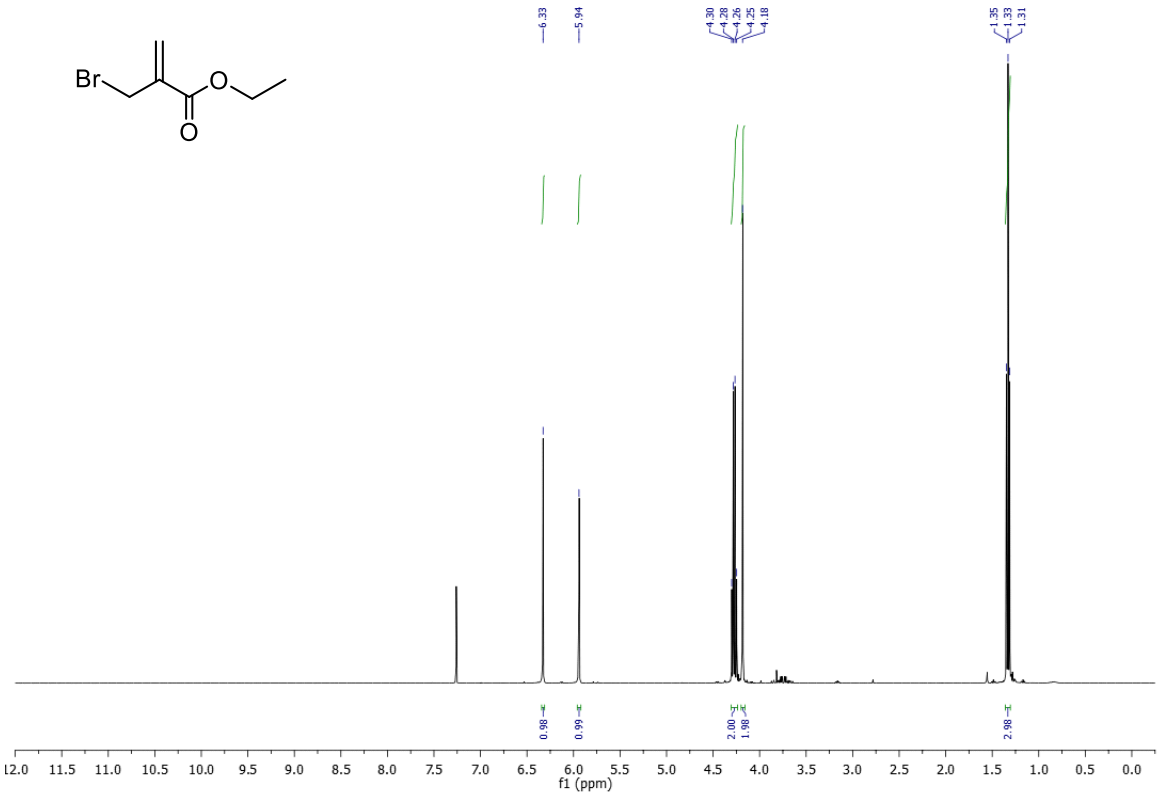


Figure 9: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 2-(bromomethyl)acrylate.

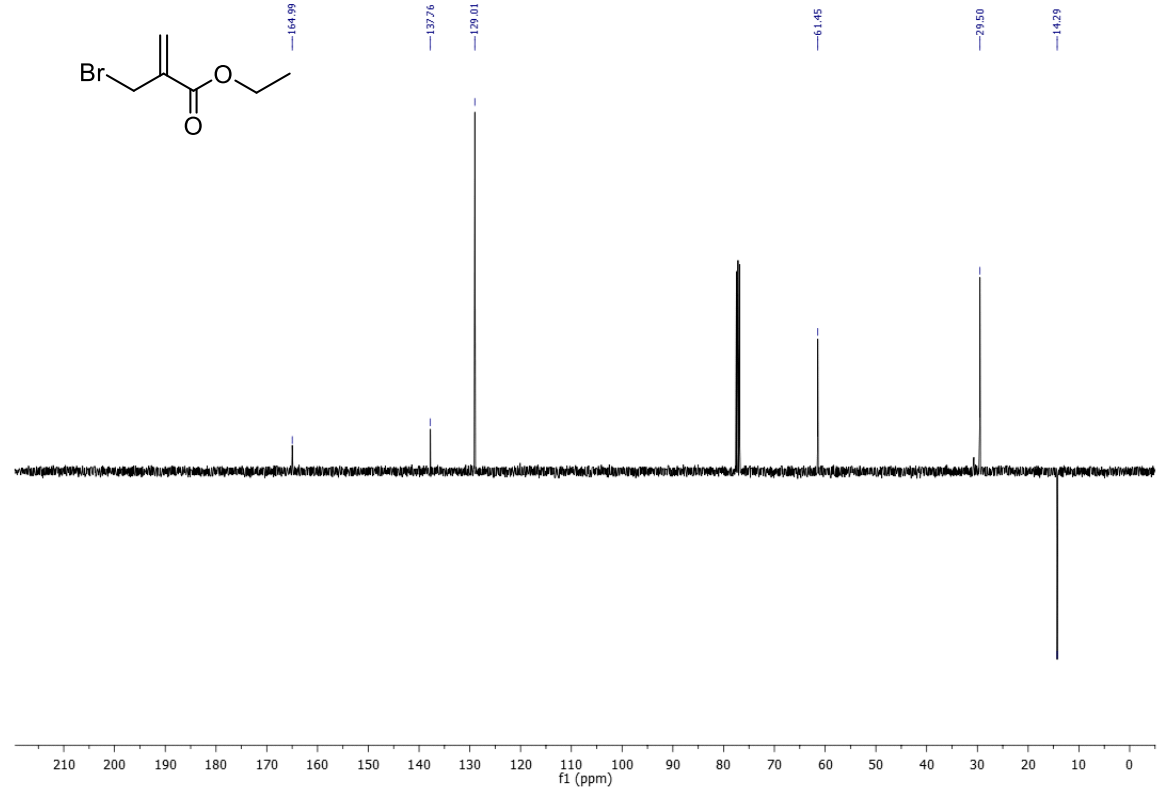


Figure 10: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 2-(bromomethyl)acrylate.

5.1.4 Acetonitrile (4)

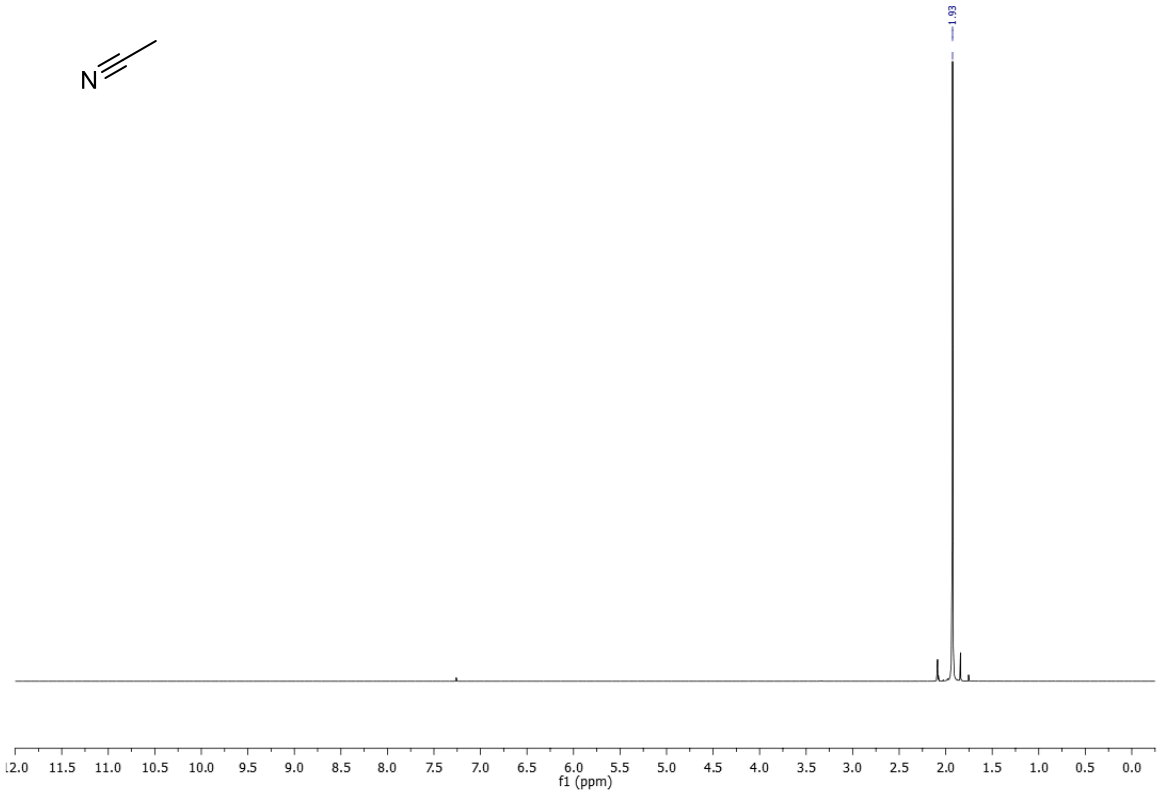


Figure 11: ^{1}H-NMR (400 MHz, $CDCl_3$) of acetonitrile.

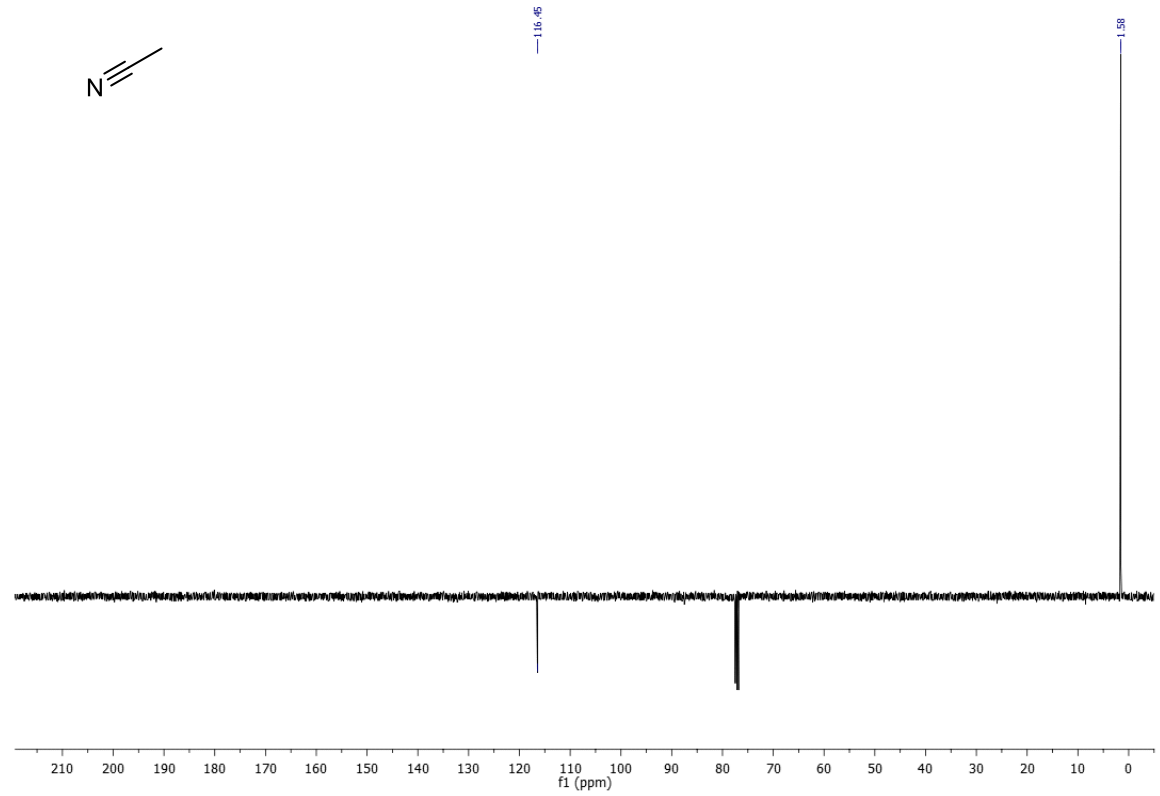


Figure 12: ^{13}C-NMR (101 MHz, $CDCl_3$) of acetonitrile.

5.1.5 Ethyl 2-(2-cyanoethyl)acrylate (5)

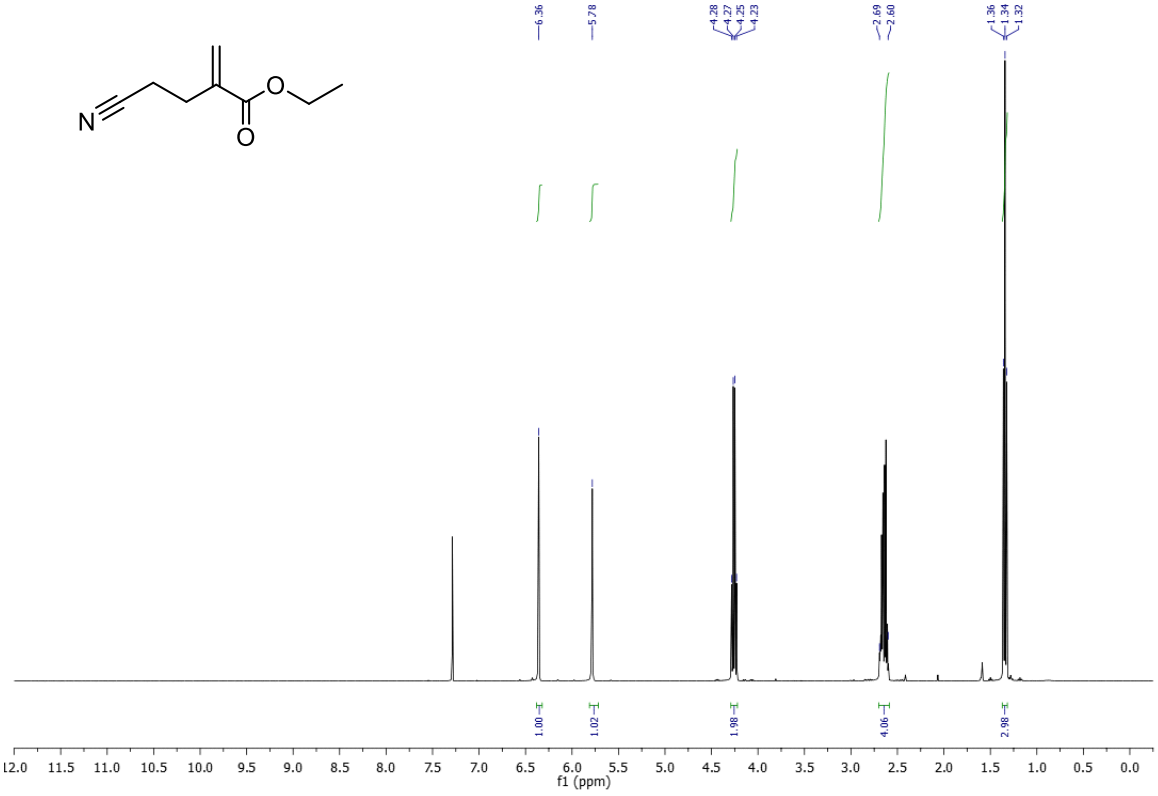


Figure 13: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 2-(2-cyanoethyl)acrylate.

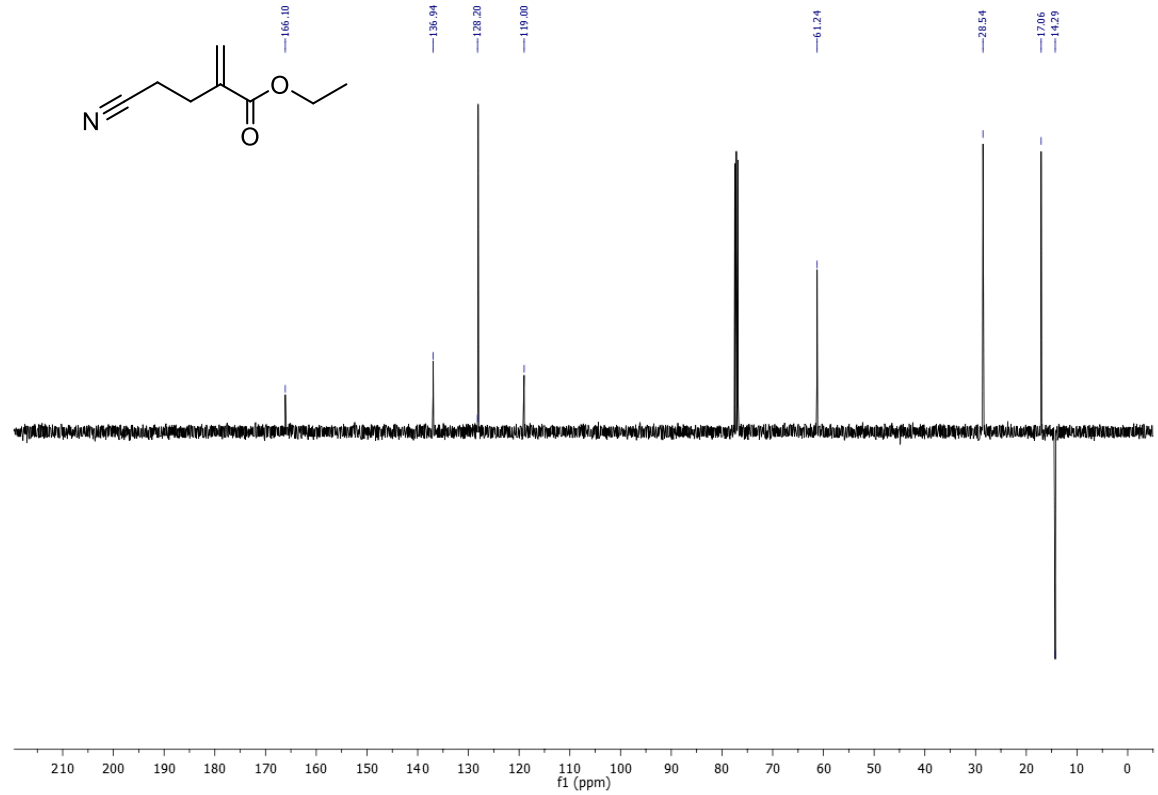


Figure 14: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 2-(2-cyanoethyl)acrylate.

5.1.6 Ethyl 4-cyano-2-oxobutanoate (6)

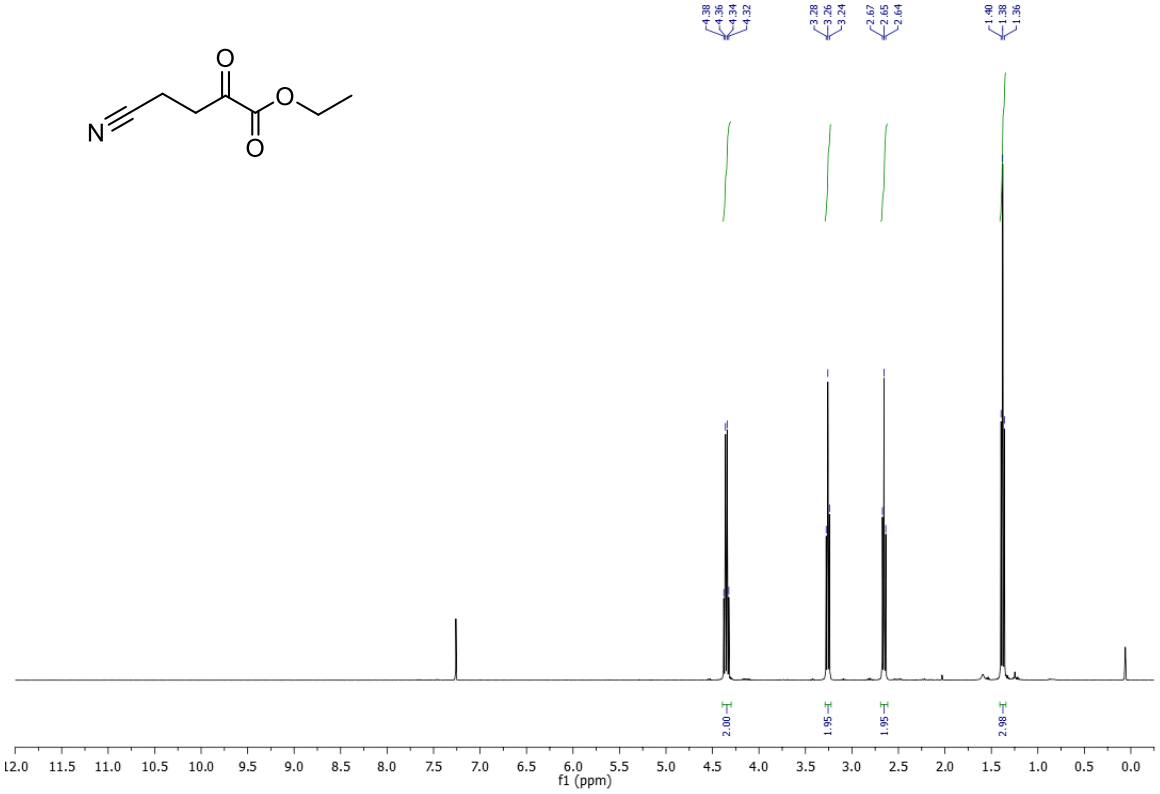


Figure 15: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 4-cyano-2-oxobutanoate.

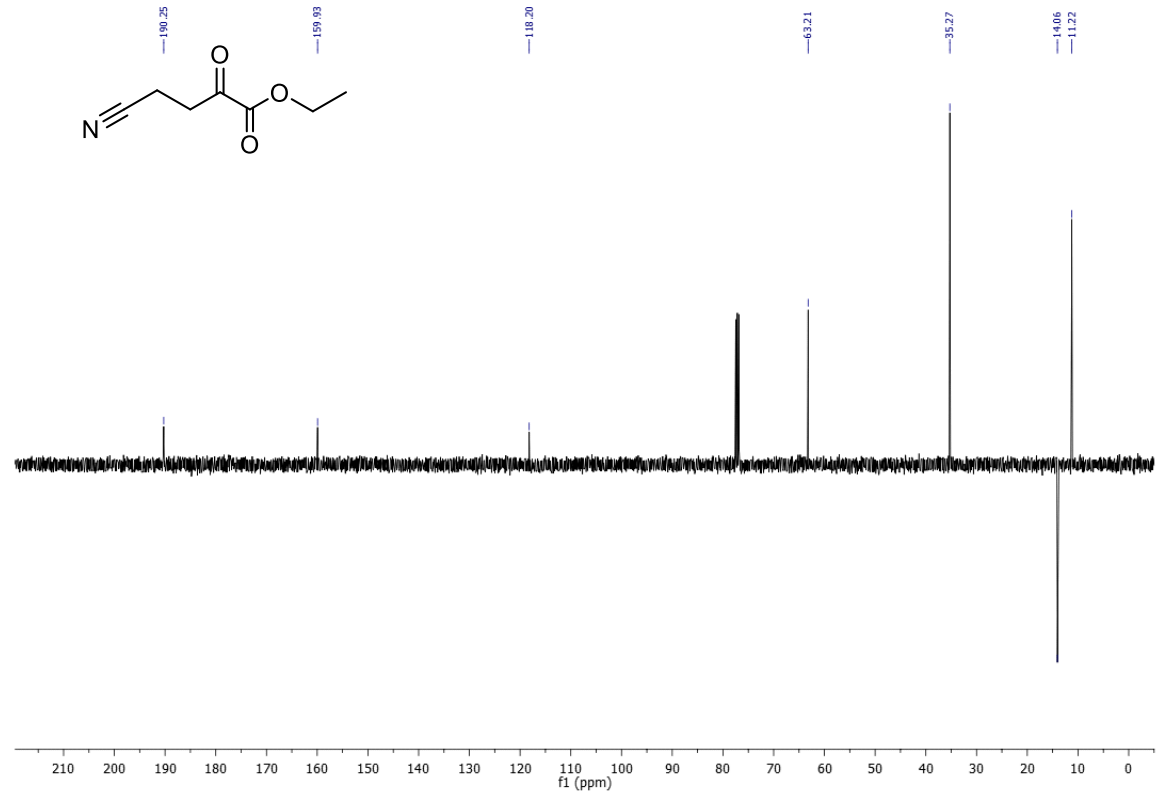


Figure 16: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 4-cyano-2-oxobutanoate.

5.1.7 Ethyl 4-cyano-2,2-diethoxybutanoate (7)

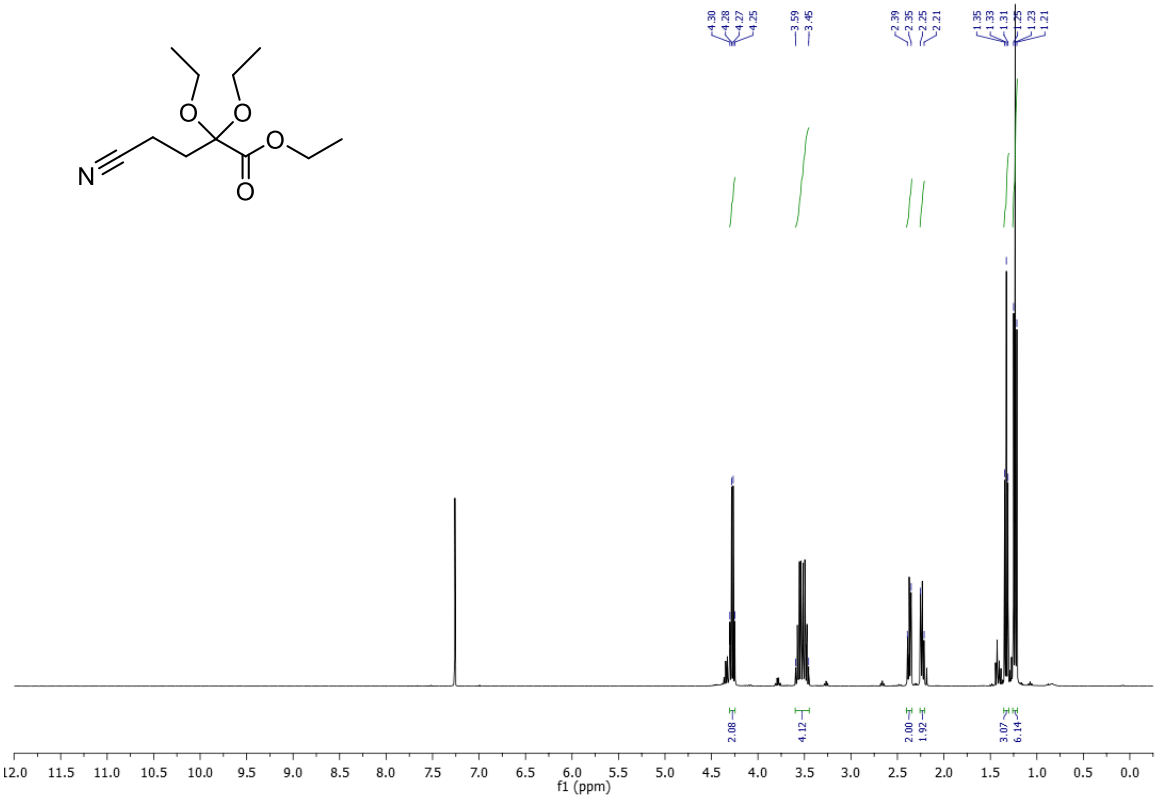


Figure 17: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 4-cyano-2,2-diethoxybutanoate.

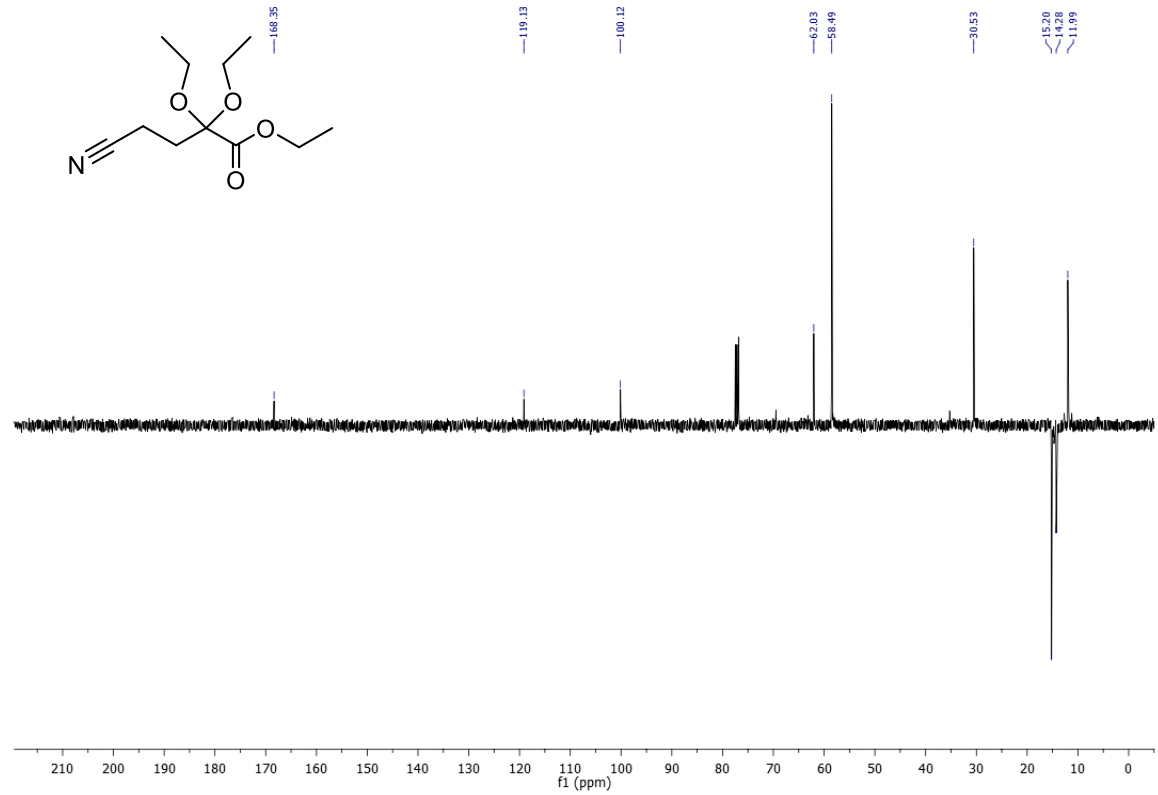


Figure 18: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 4-cyano-2,2-diethoxybutanoate.

5.1.8 Ethyl 2-(2-hydroxyethyl)acrylate (8)

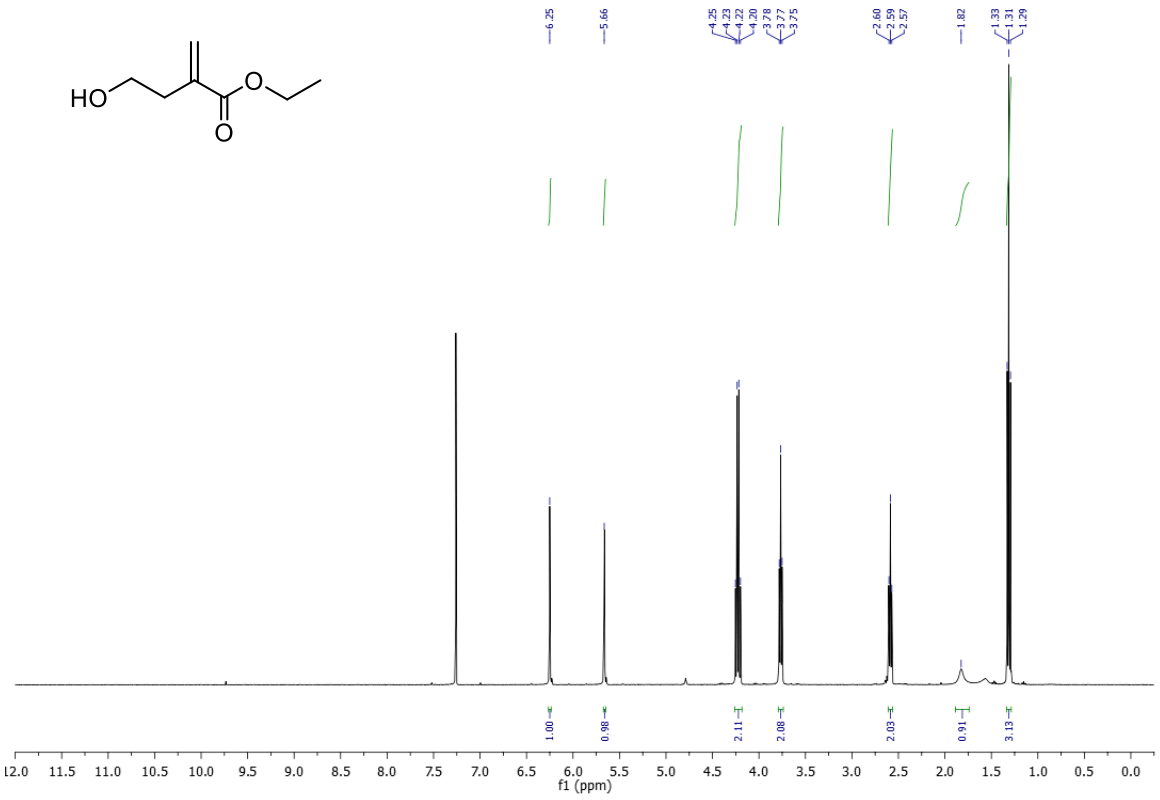


Figure 19: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 2-(2-hydroxyethyl)acrylate.

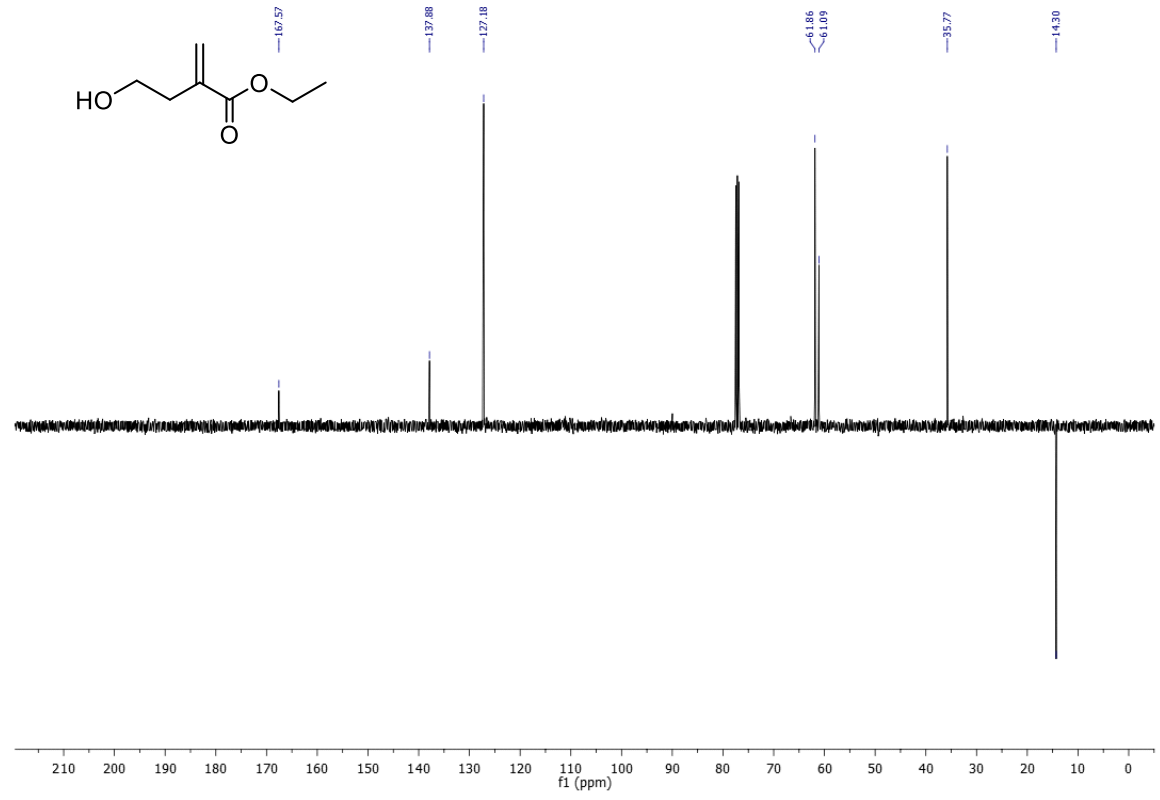


Figure 20: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 2-(2-hydroxyethyl)acrylate.

5.1.9 Ethyl 2-(2-(methylsulfonyl)oxyethyl)acrylate (9)

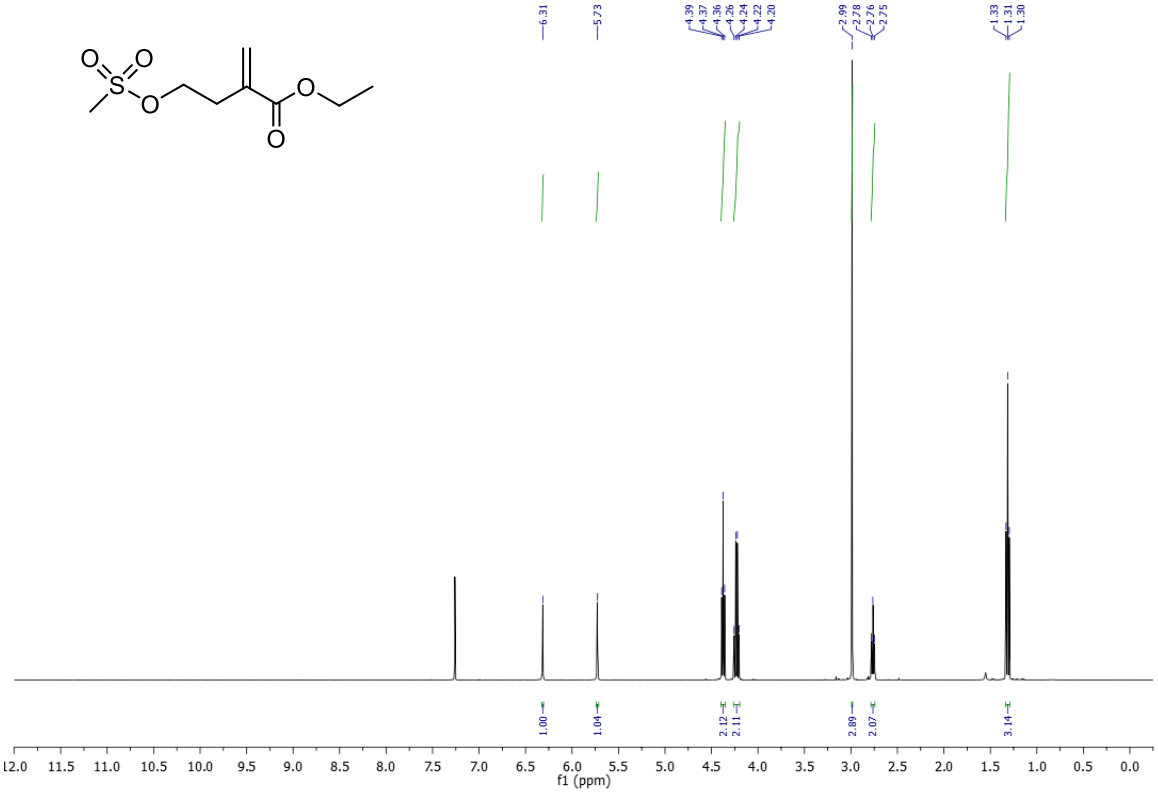


Figure 21: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 2-(2-(methylsulfonyl)oxyethyl)acrylate.

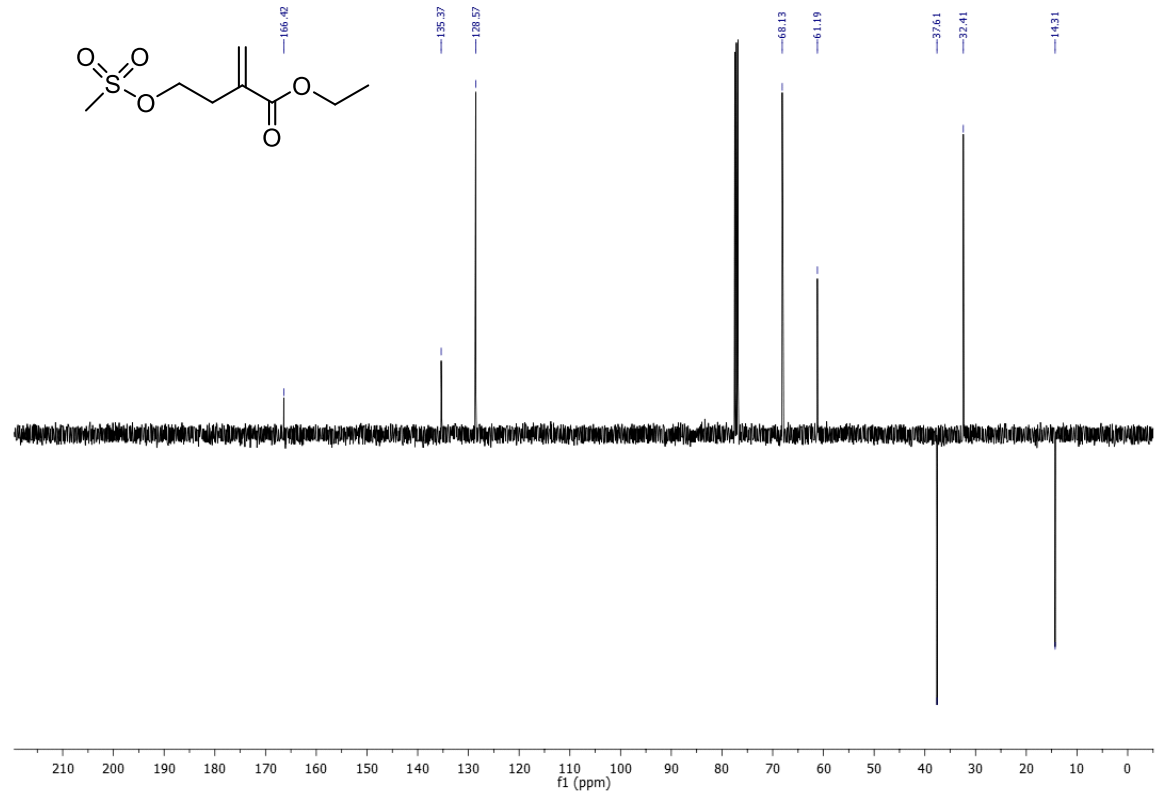


Figure 22: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 2-(2-(methylsulfonyl)oxyethyl)acrylate.

5.1.10 Ethyl 4-((methylsulfonyl)oxy)-2-oxobutanoate (10)

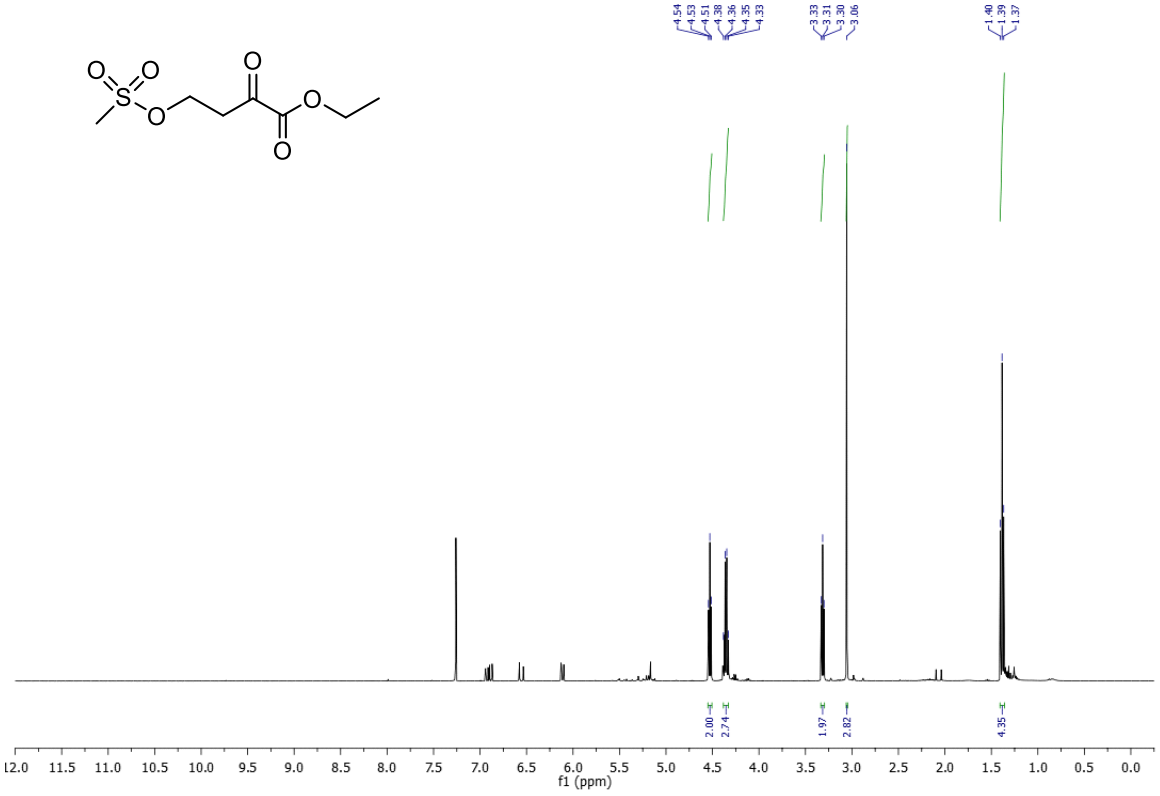


Figure 23: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 4-((methylsulfonyl)oxy)-2-oxobutanoate.

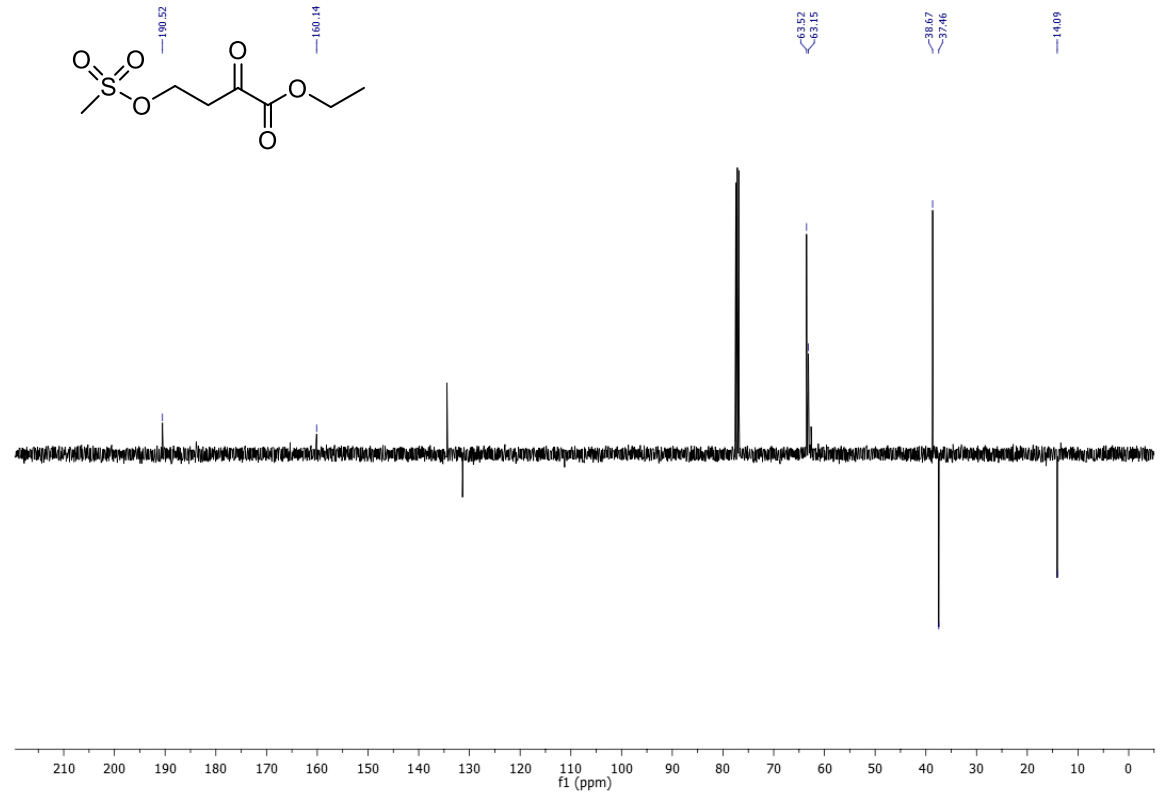


Figure 24: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 4-((methylsulfonyl)oxy)-2-oxobutanoate.

5.1.11 Ethyl 2,2-diethoxy-4-((methylsulfonyl)oxy)butanoate (11)

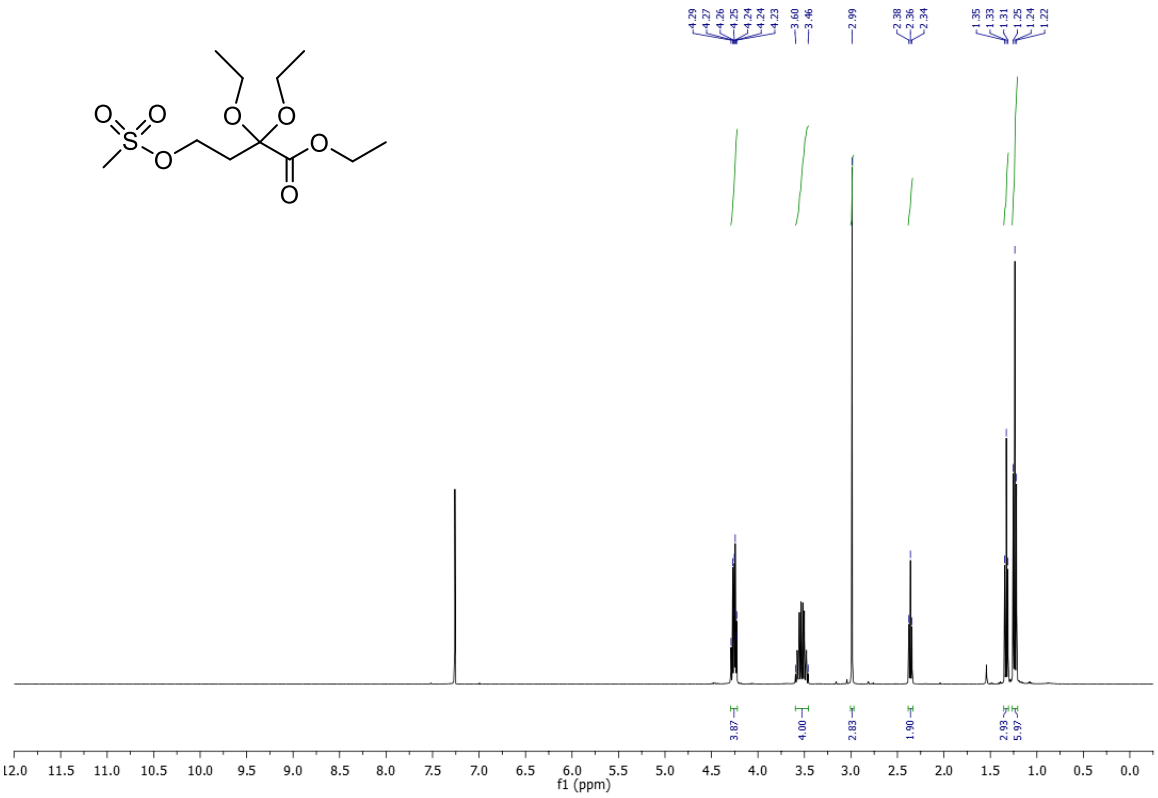


Figure 25: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 2,2-diethoxy-4-((methylsulfonyl)oxy)butanoate.

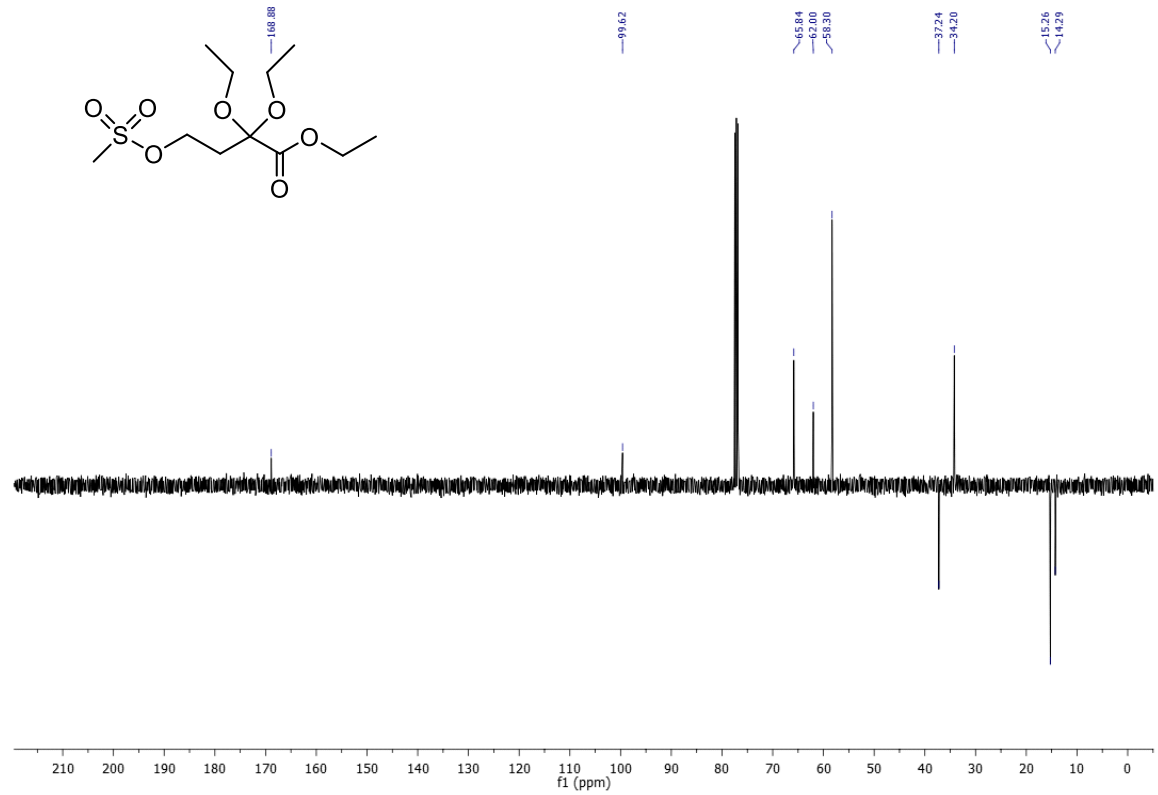


Figure 26: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 2,2-diethoxy-4-((methylsulfonyl)oxy)butanoate.

5.1.12 Ethyl 4-cyano-2,2-diethoxybutanoate (7)

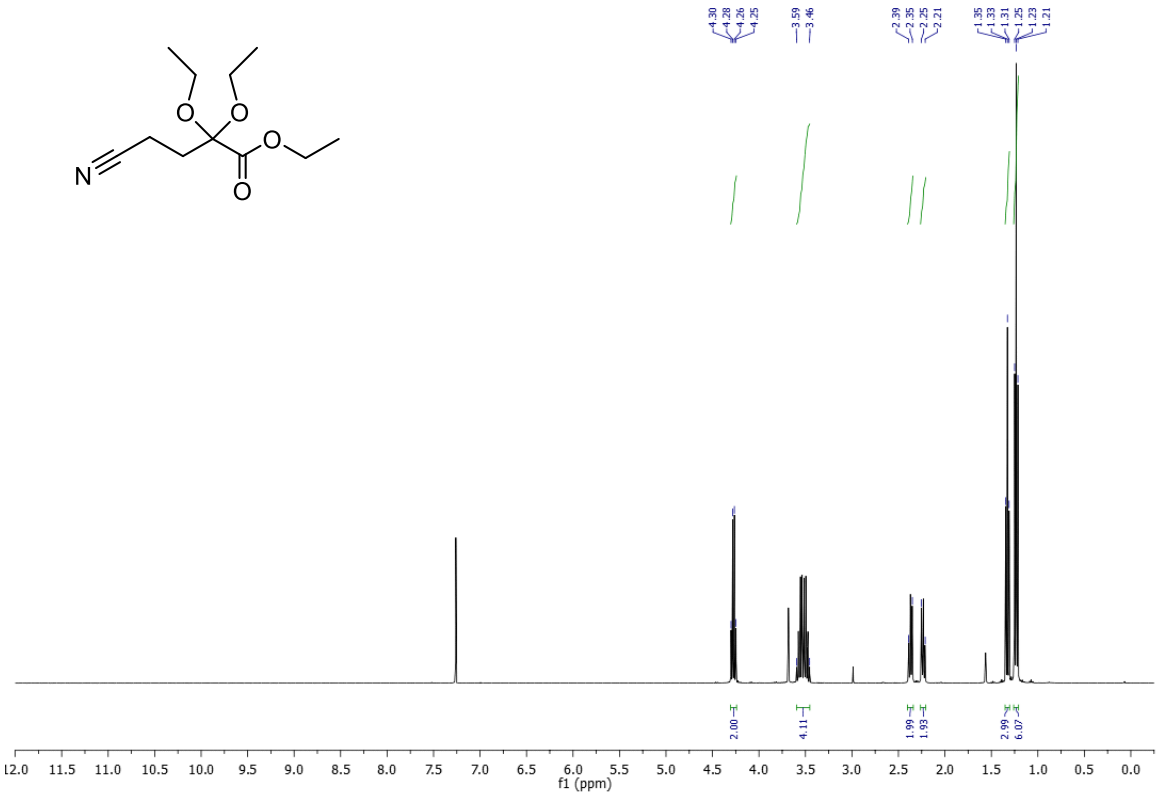


Figure 27: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 4-cyano-2,2-diethoxybutanoate.

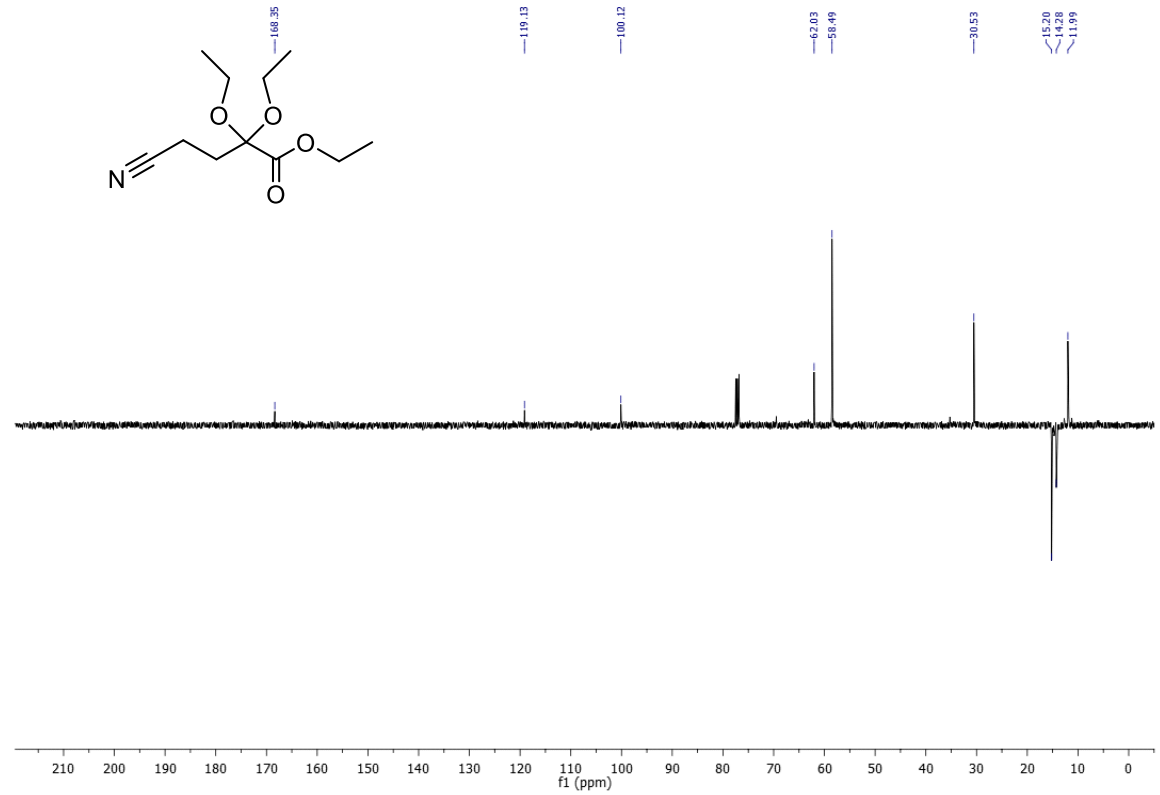


Figure 28: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 4-cyano-2,2-diethoxybutanoate.

5.1.13 Ethyl 5-((tert-butoxycarbonyl)amino)-2,2-diethoxypentanoate (12)

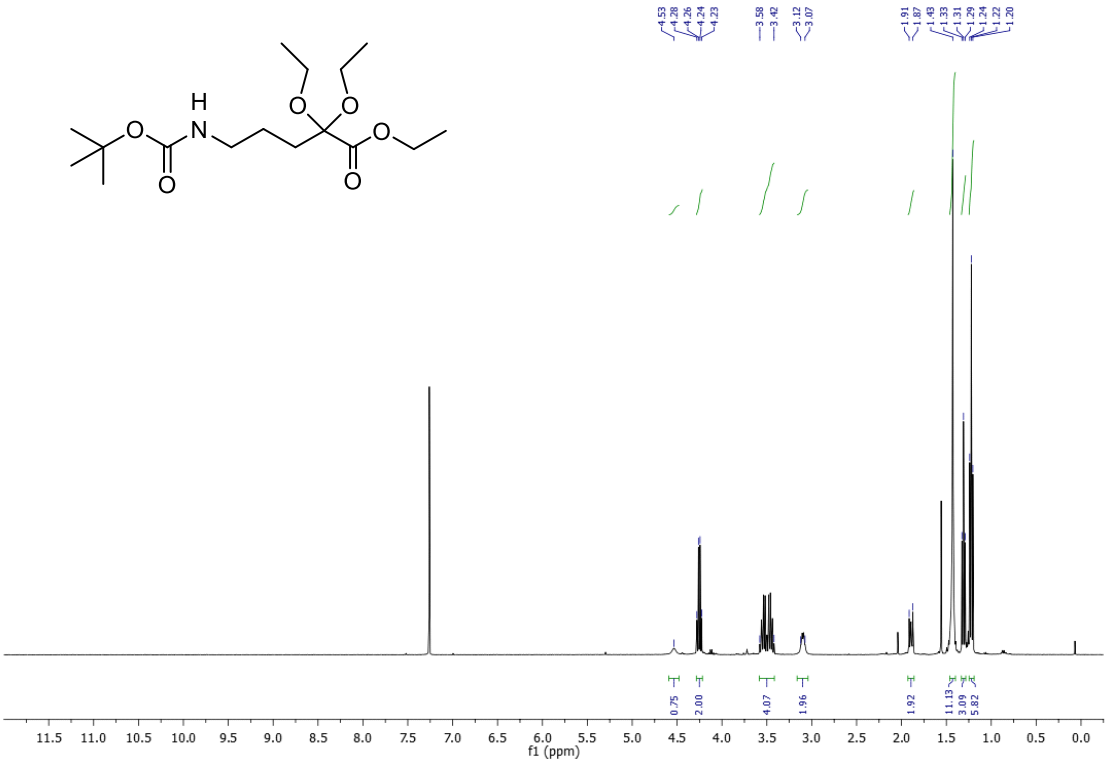


Figure 29: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 5-((tert-butoxycarbonyl)amino)-2,2-diethoxypentanoate.

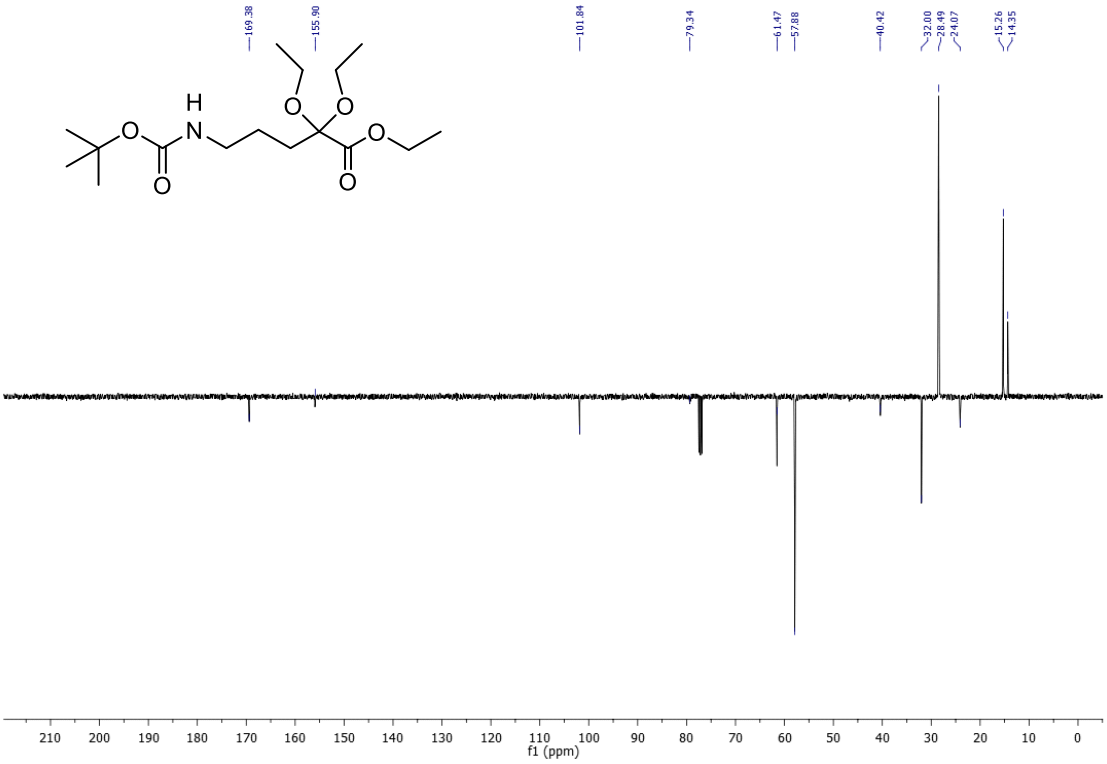


Figure 30: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 5-((tert-butoxycarbonyl)amino)-2,2-diethoxy pentanoate.

5.1.14 Ethyl 1-pyrroline-2-carboxylate (13)

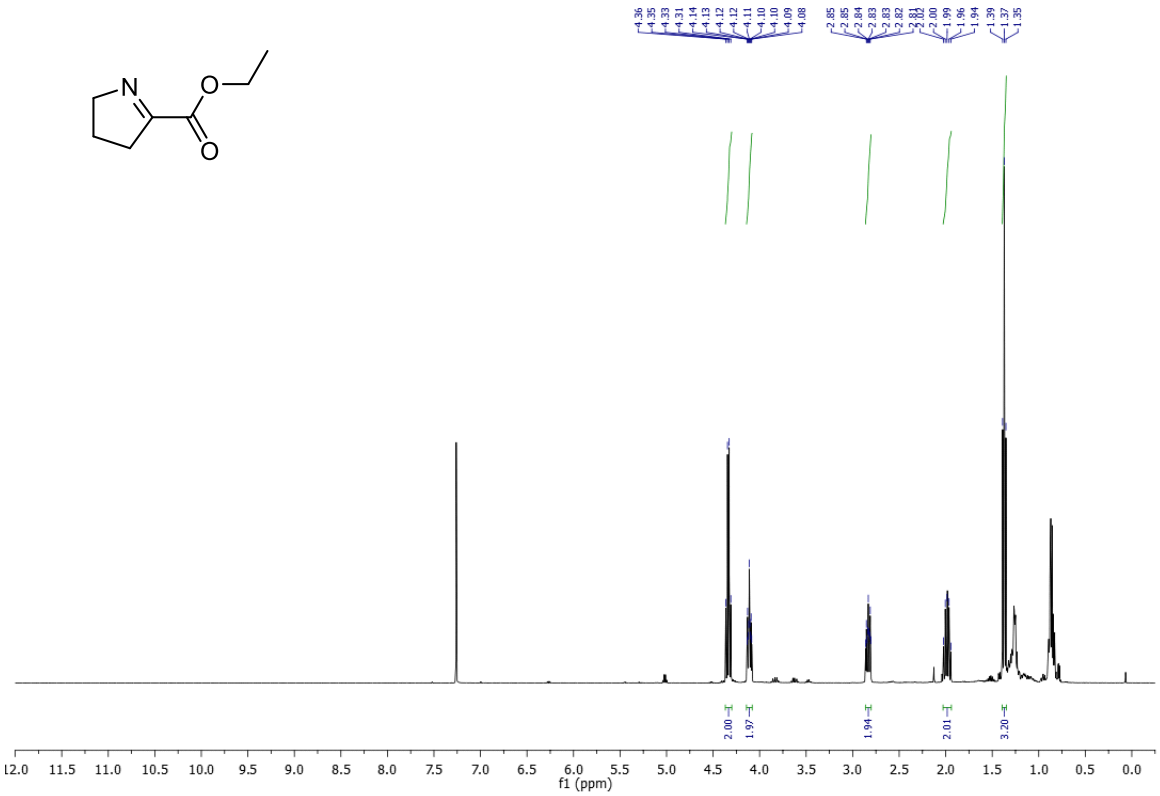


Figure 31: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 1-pyrroline-2-carboxylate.

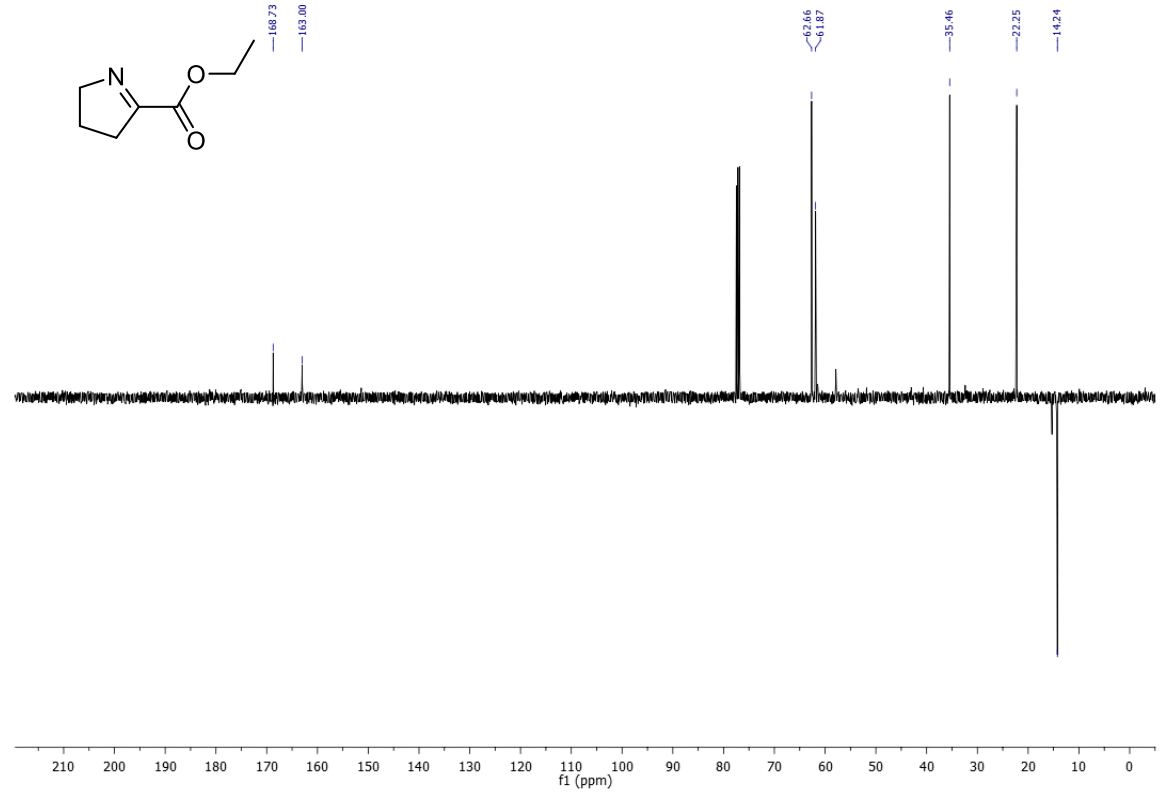


Figure 32: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 1-pyrroline-2-carboxylate.

5.1.15 Sodium 1-pyrroline-2-carboxylate (14)

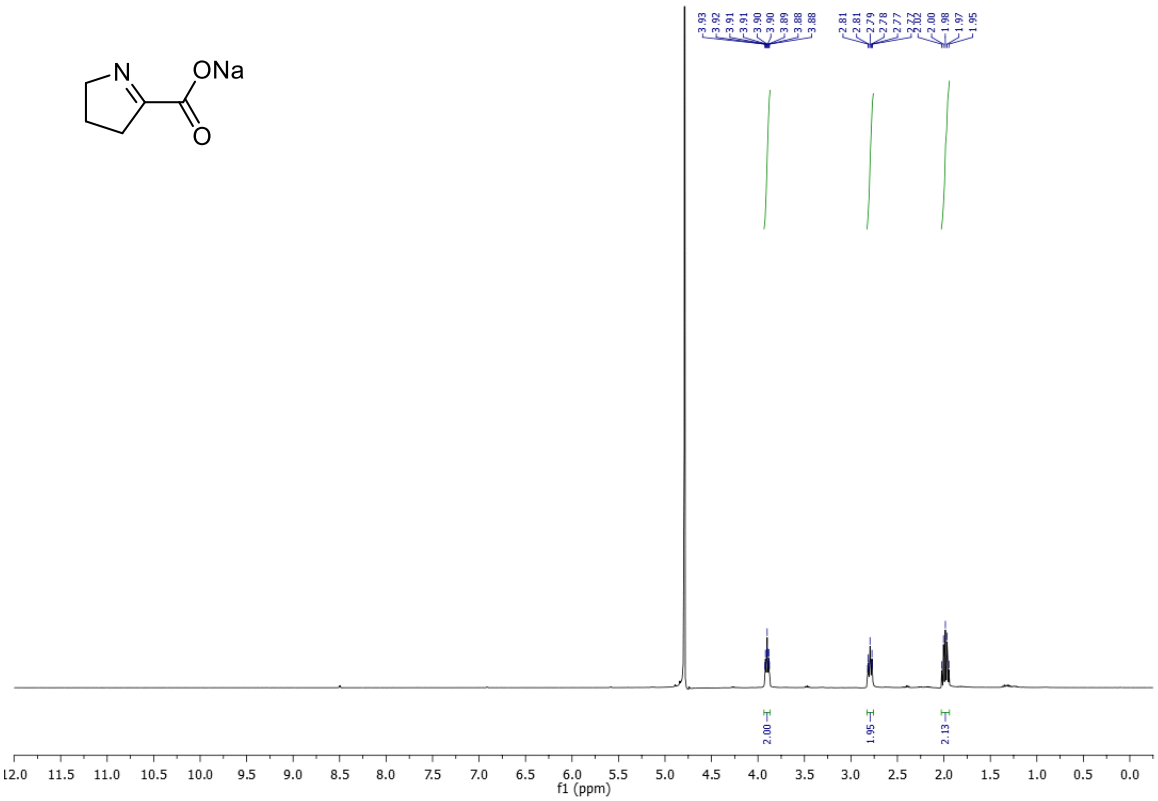


Figure 33: ^{1}H-NMR (400 MHz, D_2O) of sodium 1-pyrroline-2-carboxylate.

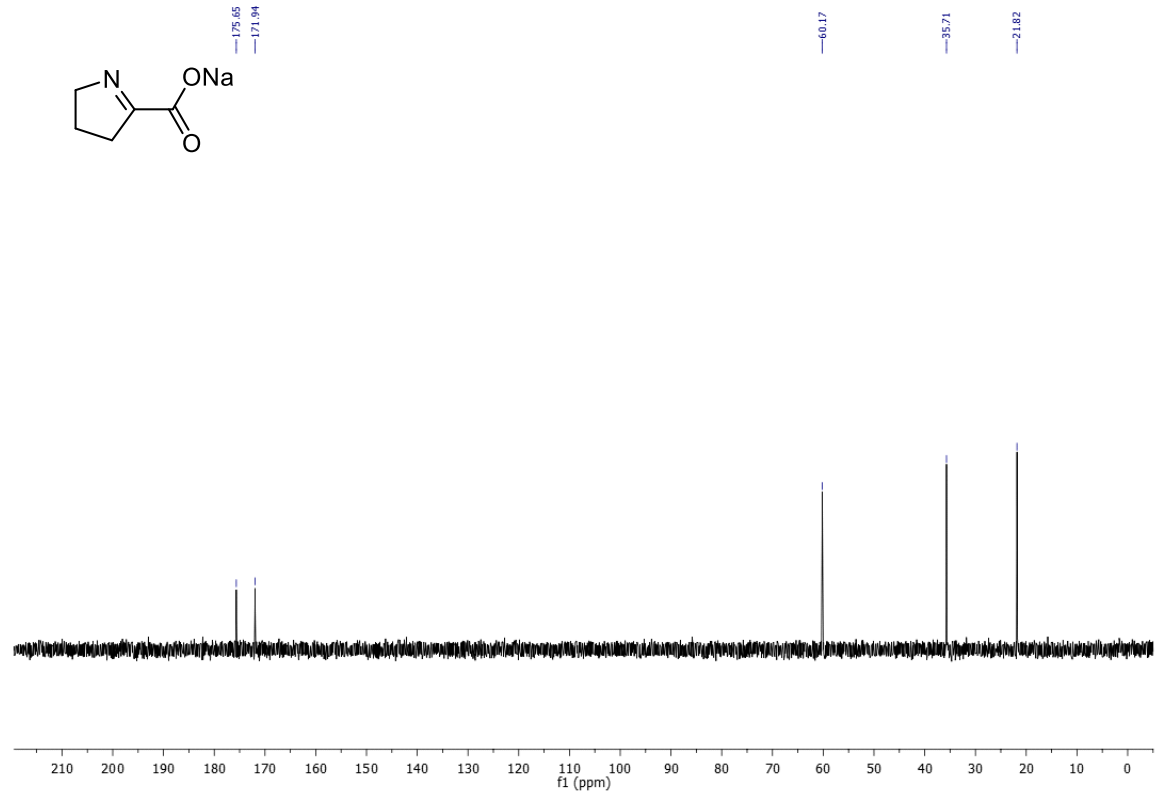


Figure 34: ^{13}C-NMR (101 MHz, D_2O) of sodium 1-pyrroline-2-carboxylate.

5.1.16 Ethyl 4-[13C]cyano-2,2-diethoxybutanoate (7a)

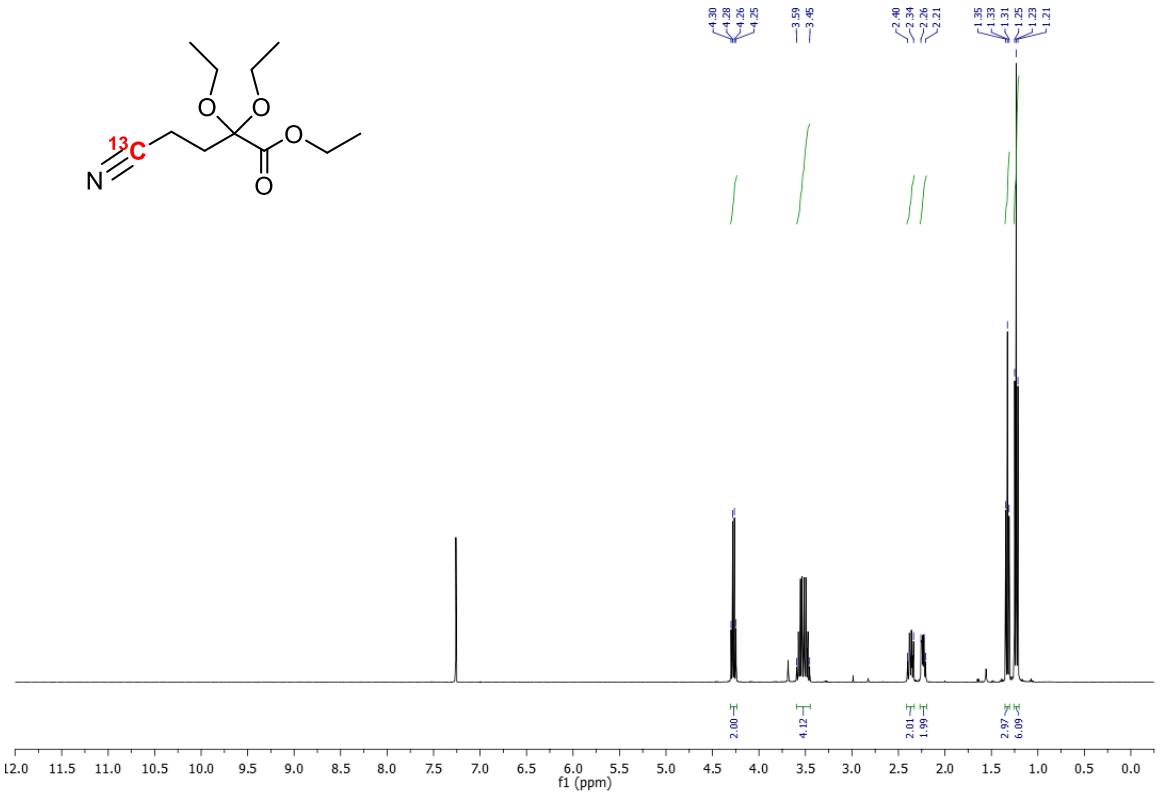


Figure 35: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 4-[^{13}C]cyano-2,2-diethoxybutanoate.

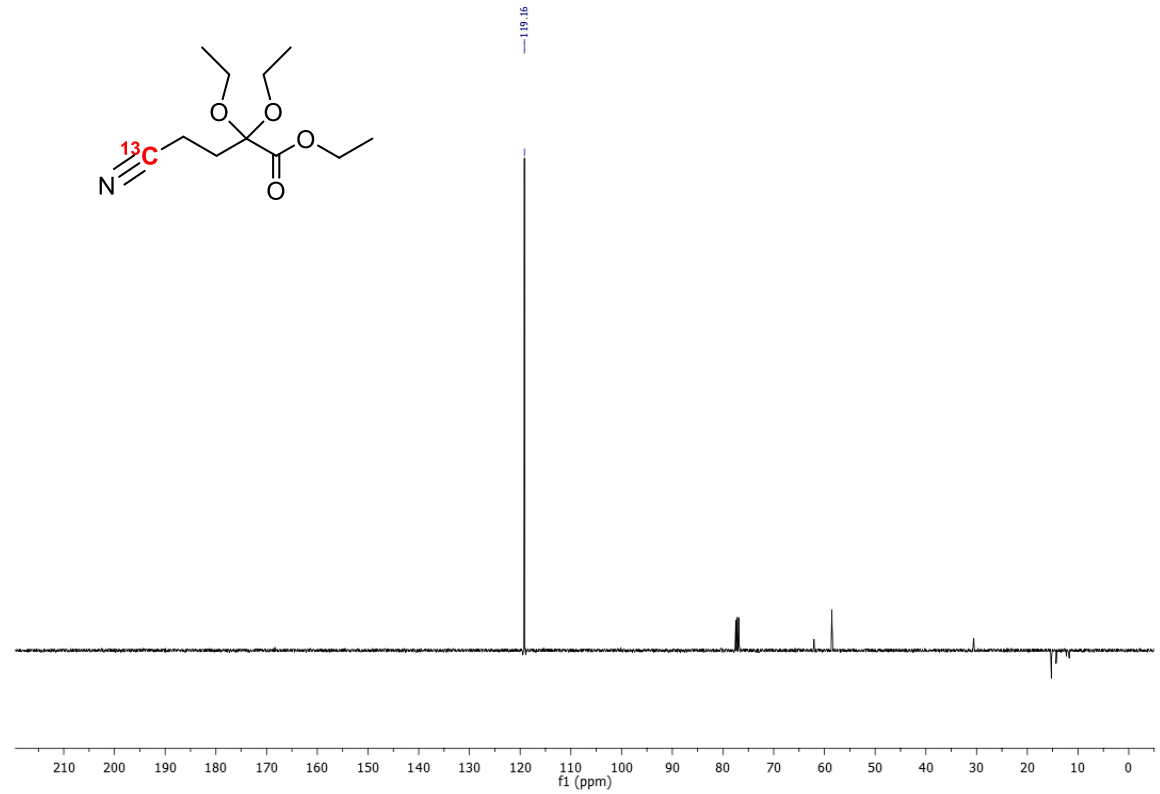


Figure 36: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 4-[^{13}C]cyano-2,2-diethoxybutanoate.

5.1.17 Ethyl 5-((tert-butoxycarbonyl)amino)-2,2-diethoxy-[5-^{13}C]pentanoate (12a)

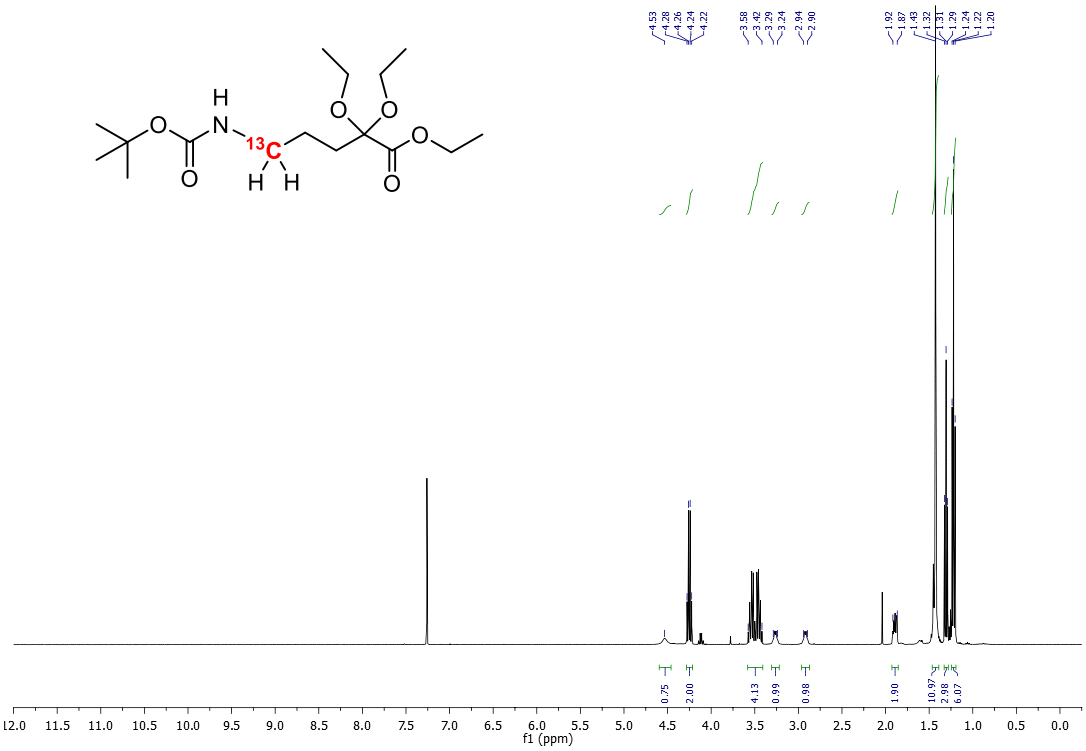


Figure 37: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 5-((*tert*-butoxycarbonyl)amino)-2,2-diethoxy [5-^{13}C]pentanoate.

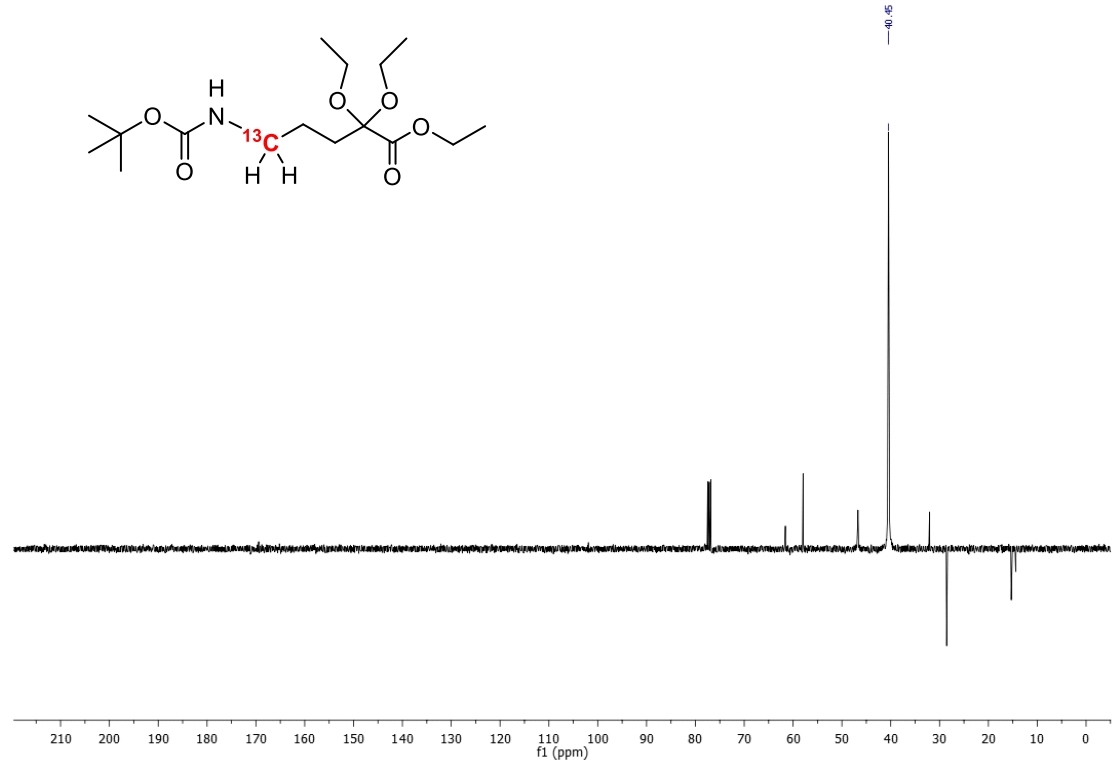


Figure 38: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 5-((*tert*-butoxycarbonyl)amino)-2,2-diethoxy [5-^{13}C]pentanoate.

5.1.18 Ethyl 1-[5-^{13}C]pyrroline-2-carboxylate (13a)

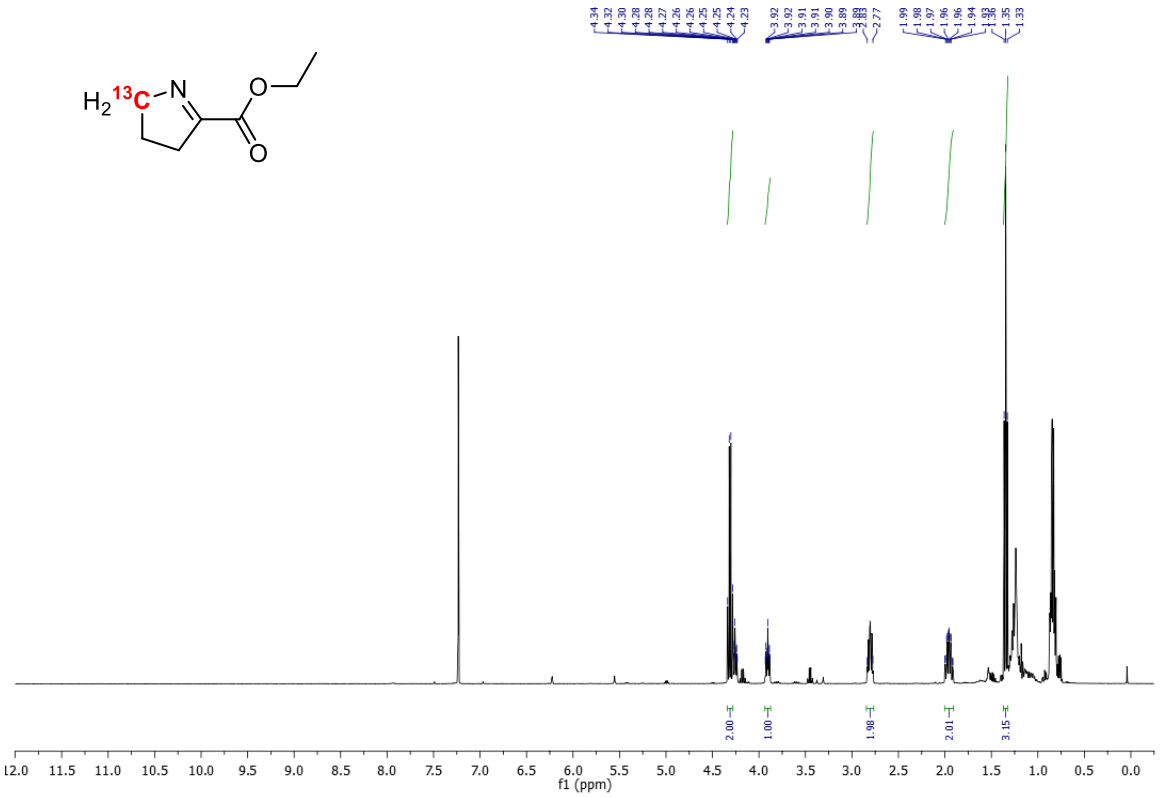


Figure 39: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl 1-[5-^{13}C]pyrroline-2-carboxylate.

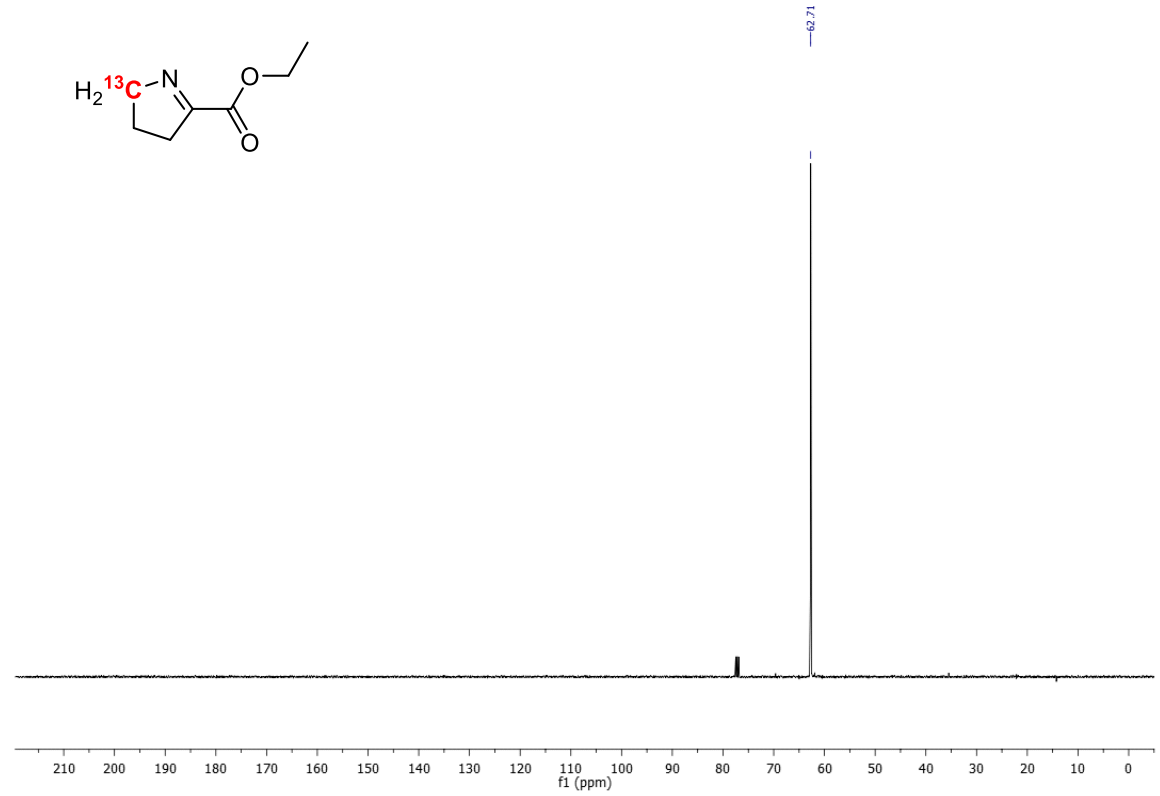


Figure 40: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl 1-[5-^{13}C]pyrroline-2-carboxylate.

5.1.19 Sodium 1-[5-^{13}C]pyrroline-2-carboxylate (14a)

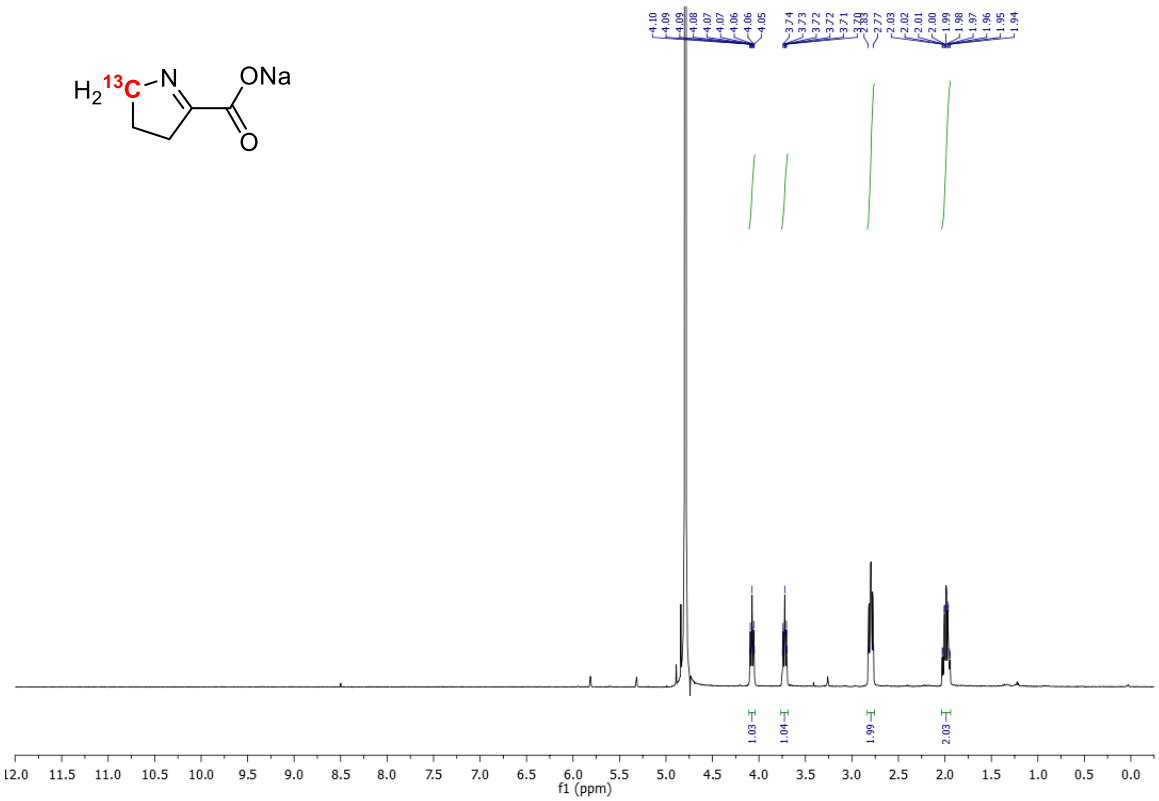


Figure 41: ^{1}H-NMR (400 MHz, D_2O) of sodium 1-[5-^{13}C]pyrroline-2-carboxylate.

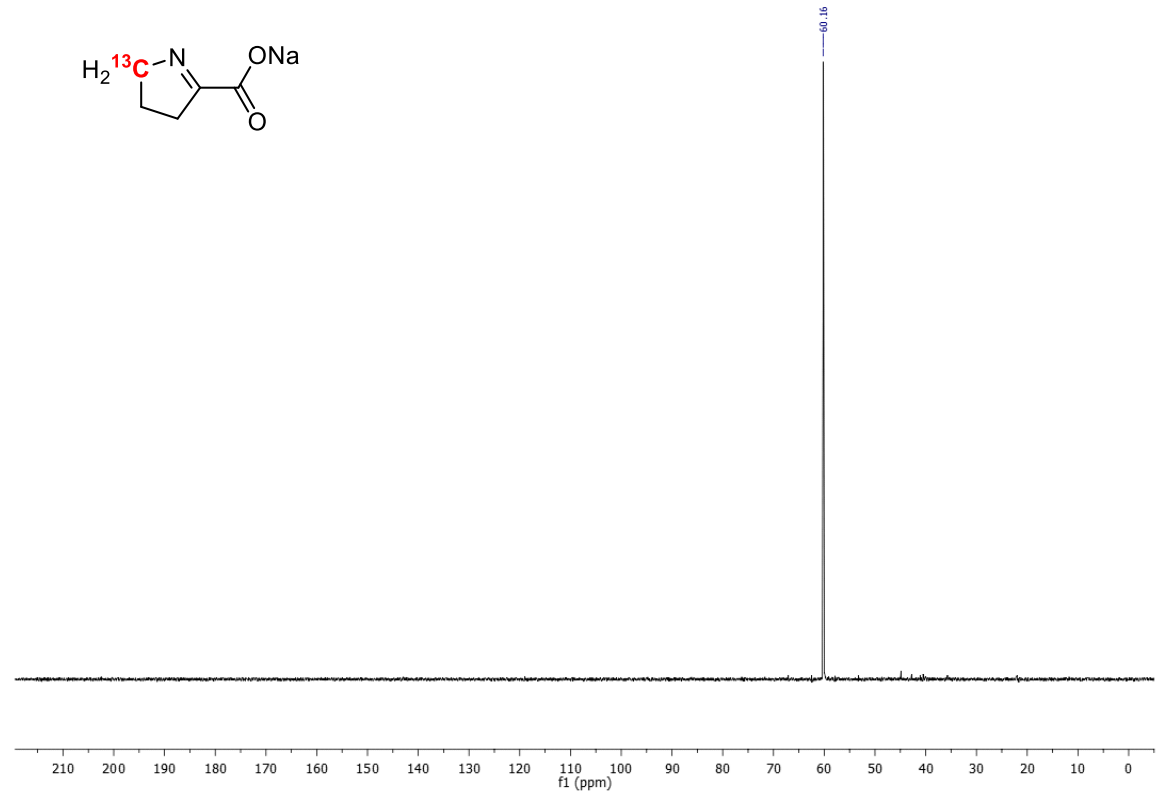


Figure 42: ^{13}C-NMR (101 MHz, D_2O) of sodium 1-[5-^{13}C]pyrroline-2-carboxylate.

5.1.20 Cyanoacetic acid (23)

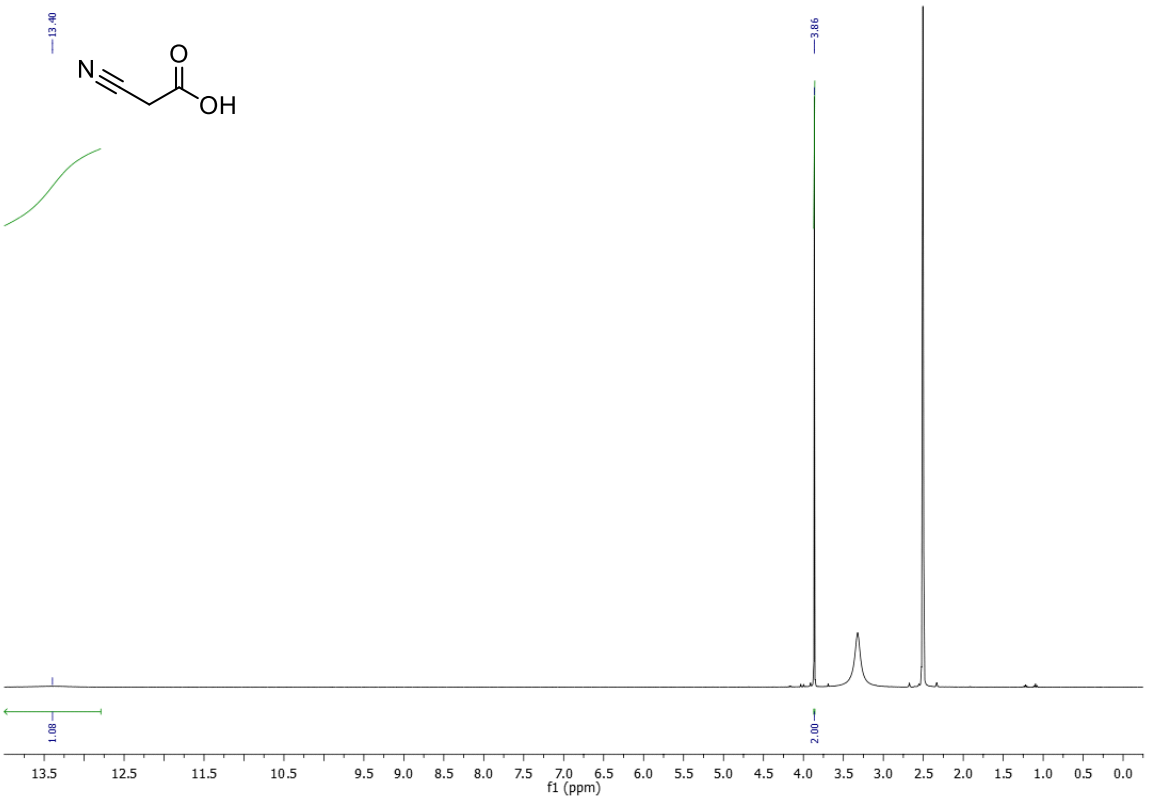


Figure 43: ^{1}H-NMR (400 MHz, d_6-DMSO) of cyanoacetic acid.

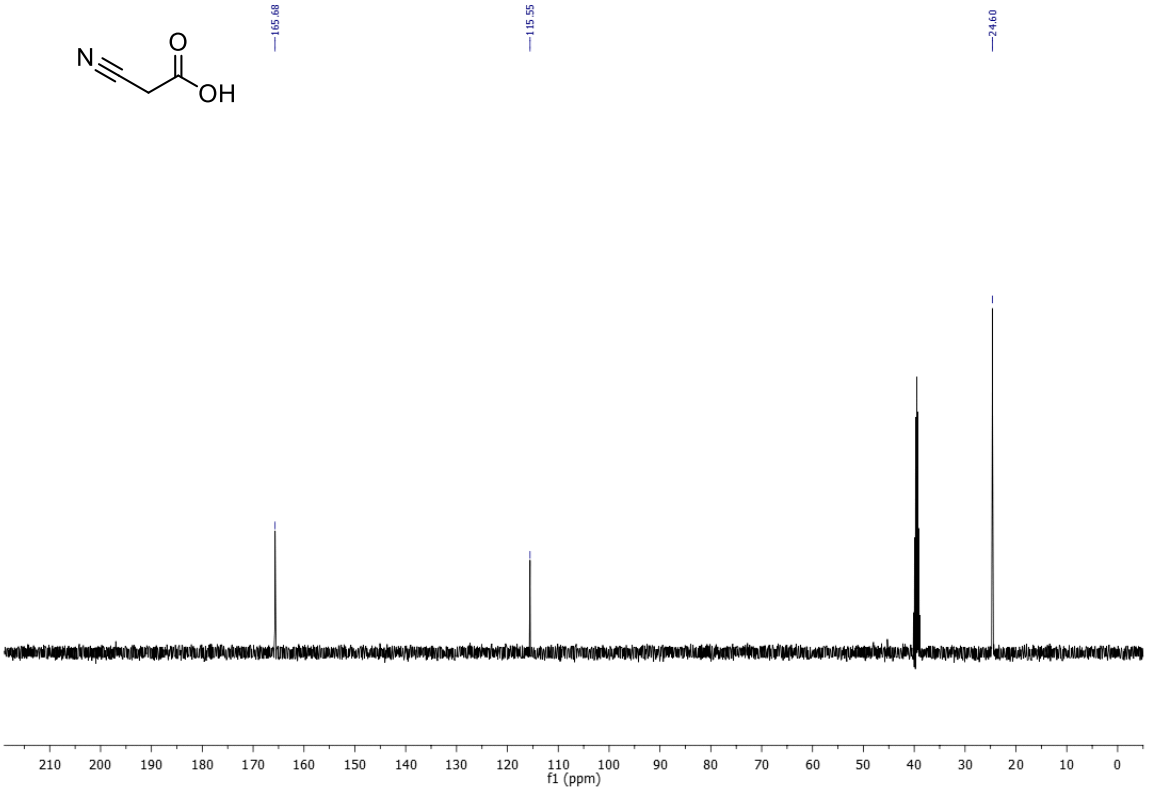


Figure 44: ^{13}C-NMR (101 MHz, d_6-DMSO) of cyanoacetic acid.

5.1.21 Ethyl cyanoacetate (24)

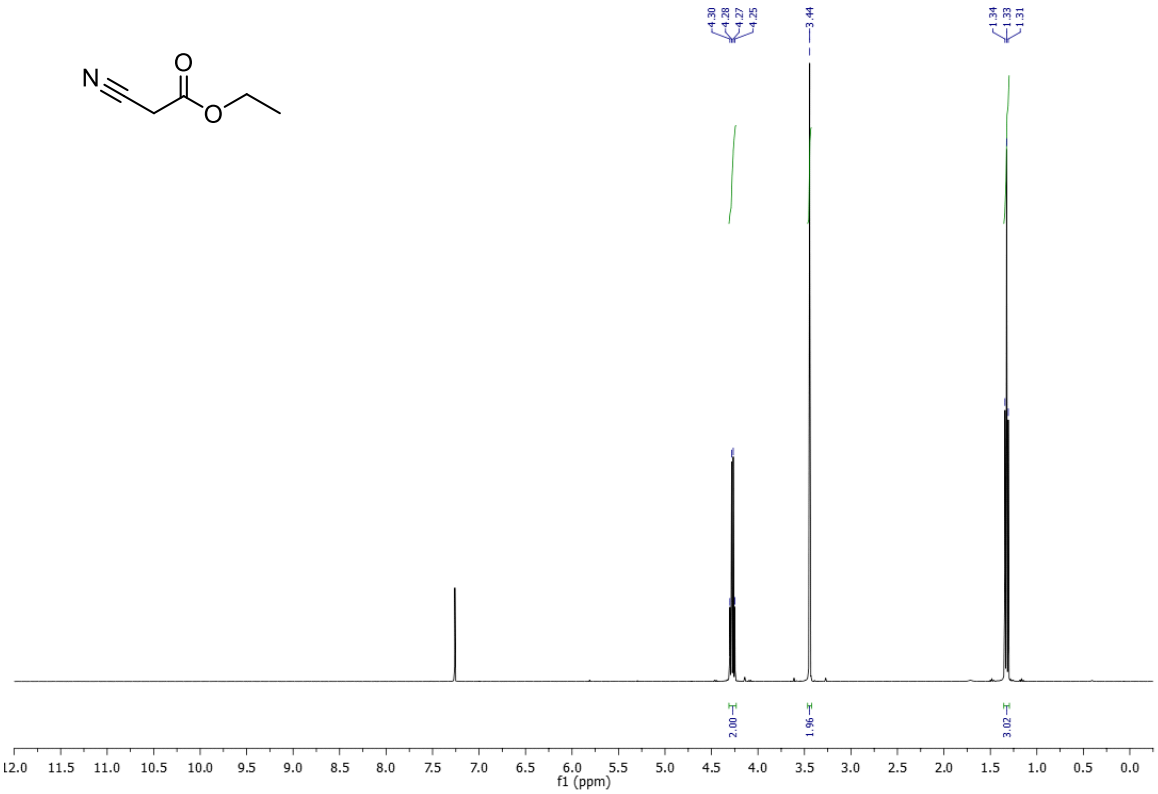


Figure 45: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl cyanoacetate.

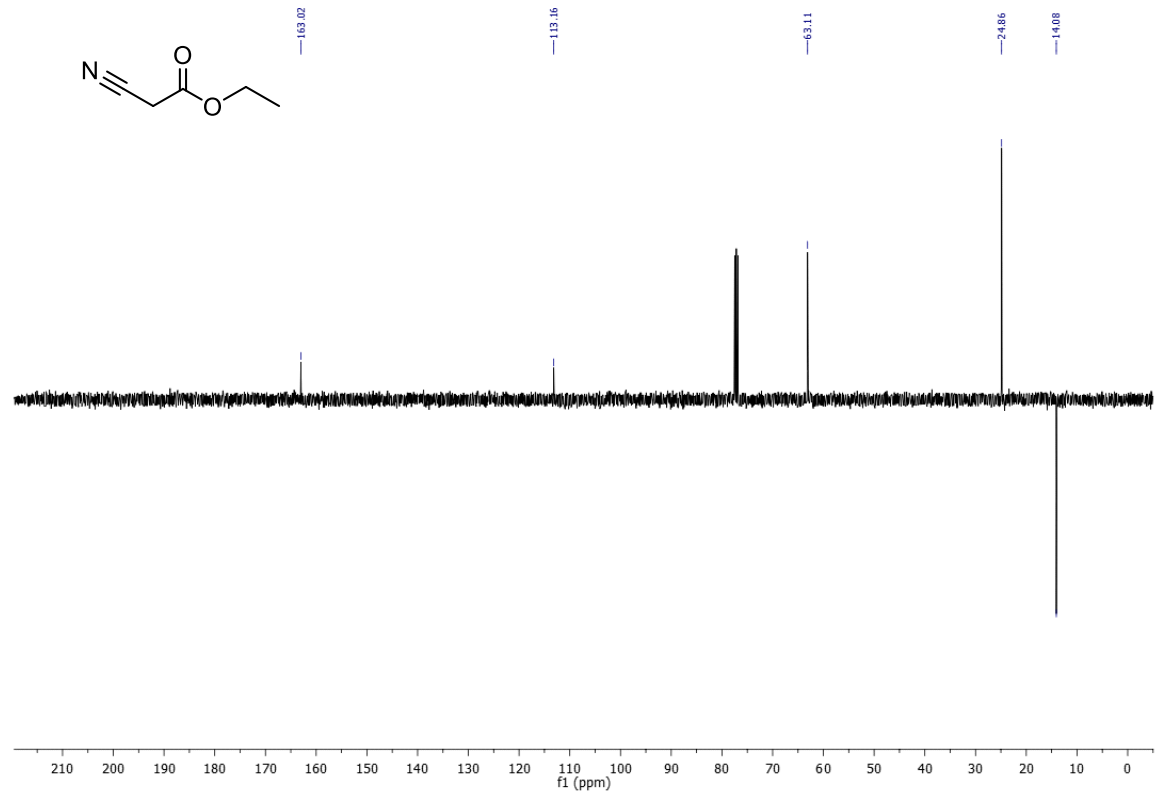


Figure 46: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl cyanoacetate.

5.1.22 Ethyl [2-$^{2}H_2$]cyanoacetate (24a)

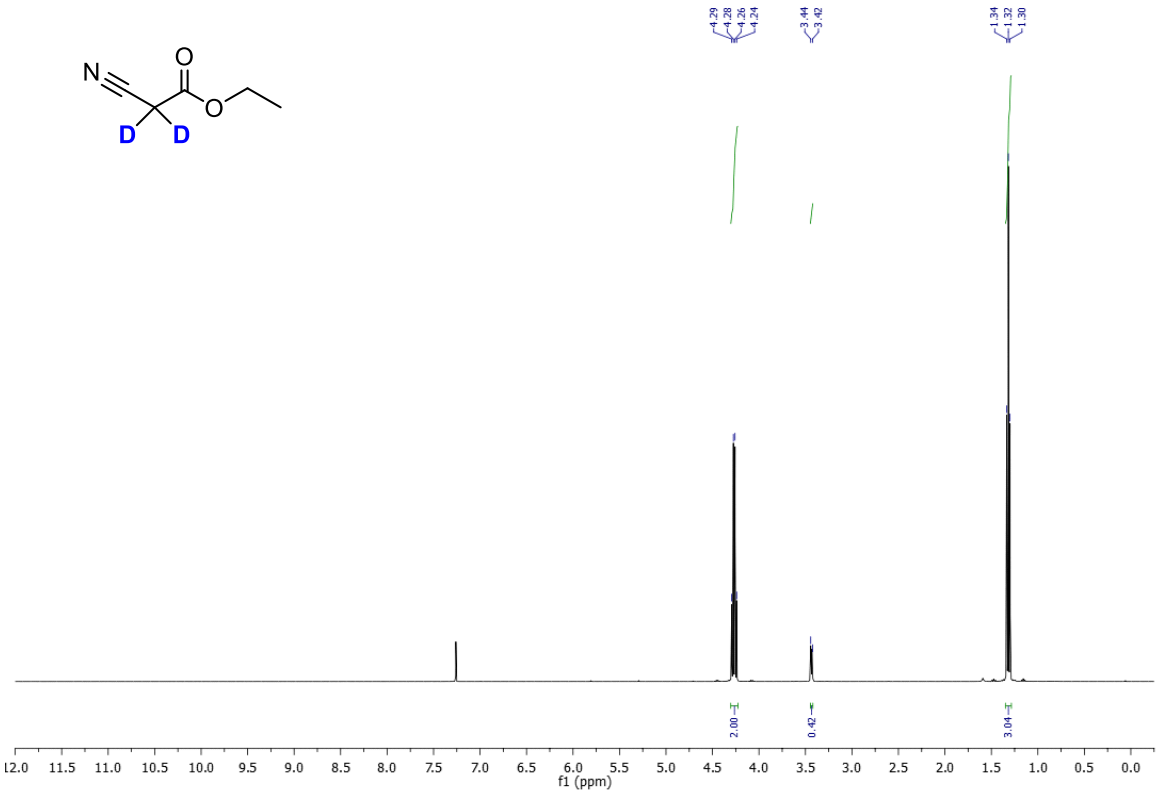


Figure 47: ^{1}H-NMR (400 MHz, $CDCl_3$) of ethyl [2-$^{2}H_2$]cyanoacetate.

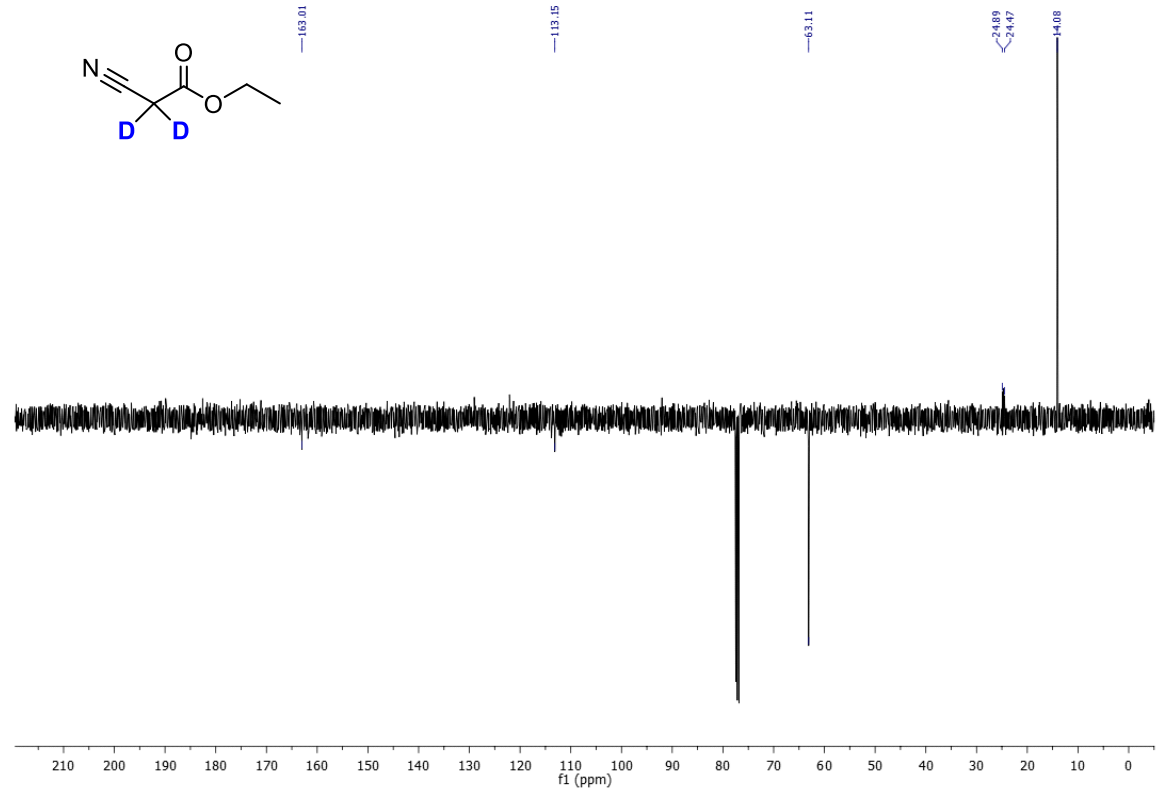


Figure 48: ^{13}C-NMR (101 MHz, $CDCl_3$) of ethyl [2-$^{2}H_2$]cyanoacetate.

5.1.23 *tert*-Butyl (3-hydroxypropyl)carbamate (26)

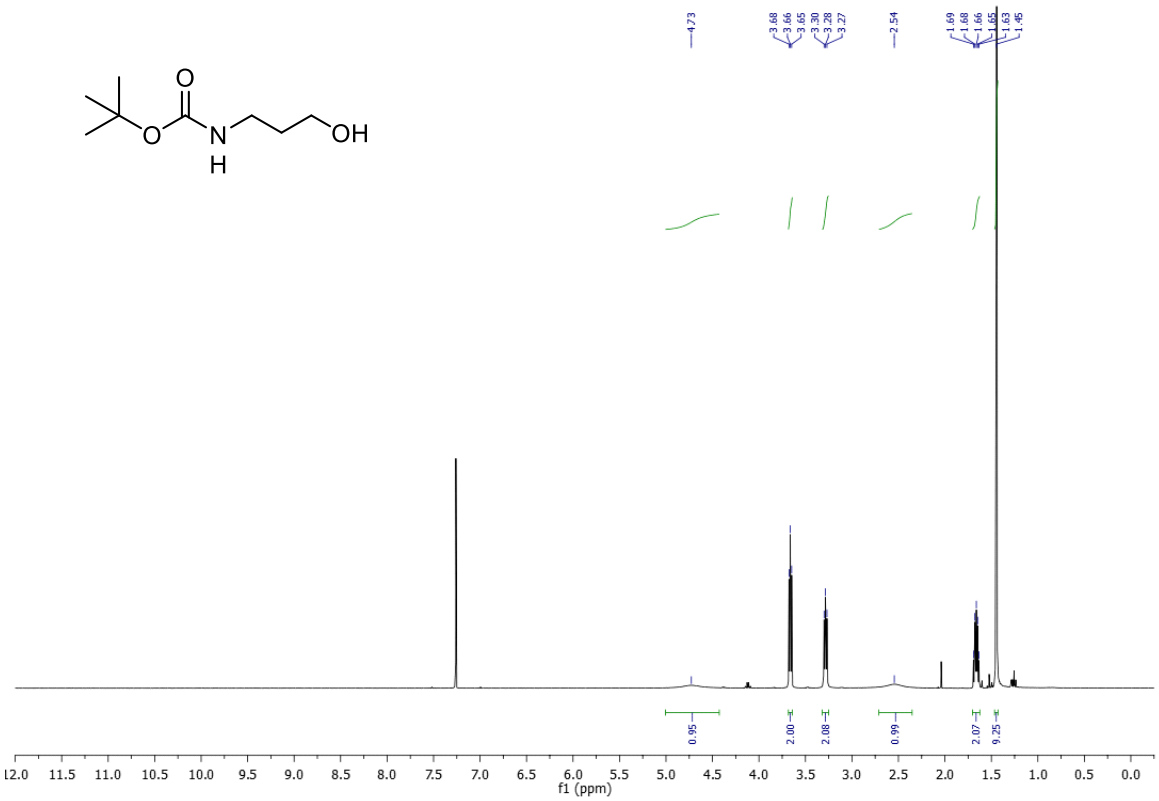


Figure 49: ^{1}H-NMR (400 MHz, $CDCl_3$) of *tert*-butyl (3-hydroxypropyl)carbamate.

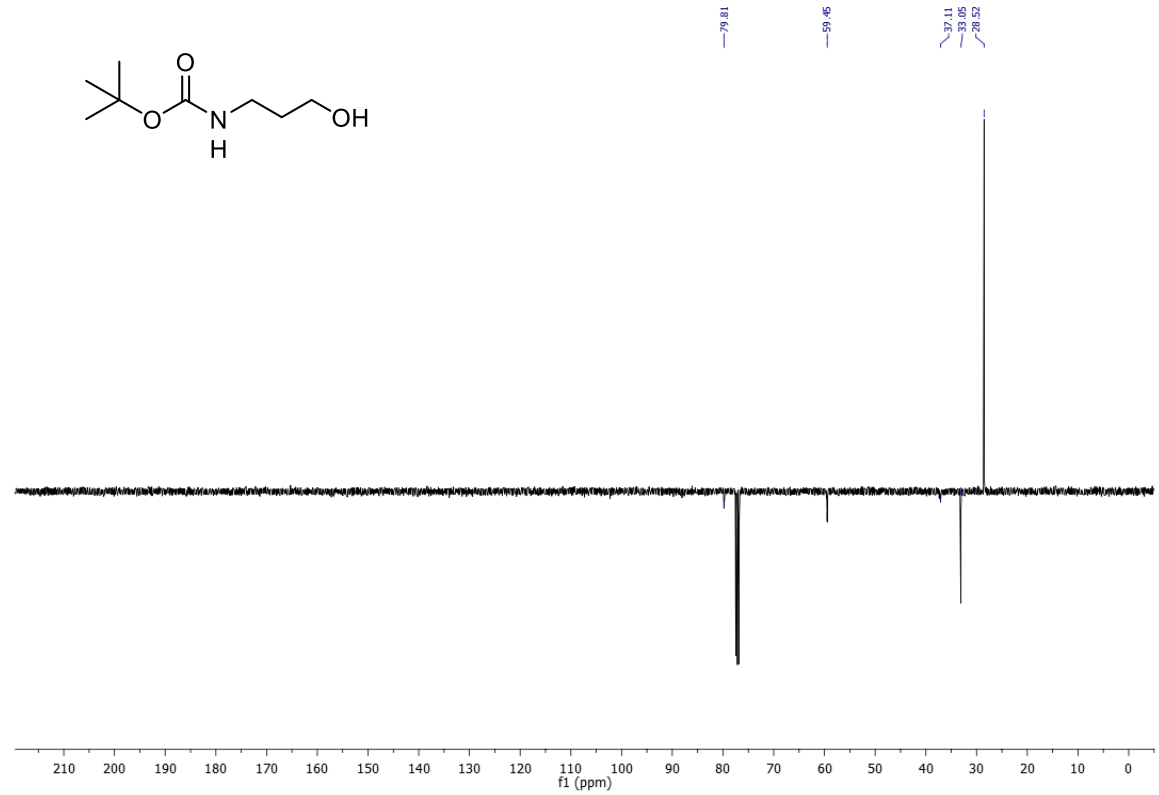


Figure 50: ^{13}C-NMR (101 MHz, $CDCl_3$) of *tert*-butyl (3-hydroxypropyl)carbamate.

5.1.24 *tert*-Butyl (3-oxopropyl)carbamate (27)

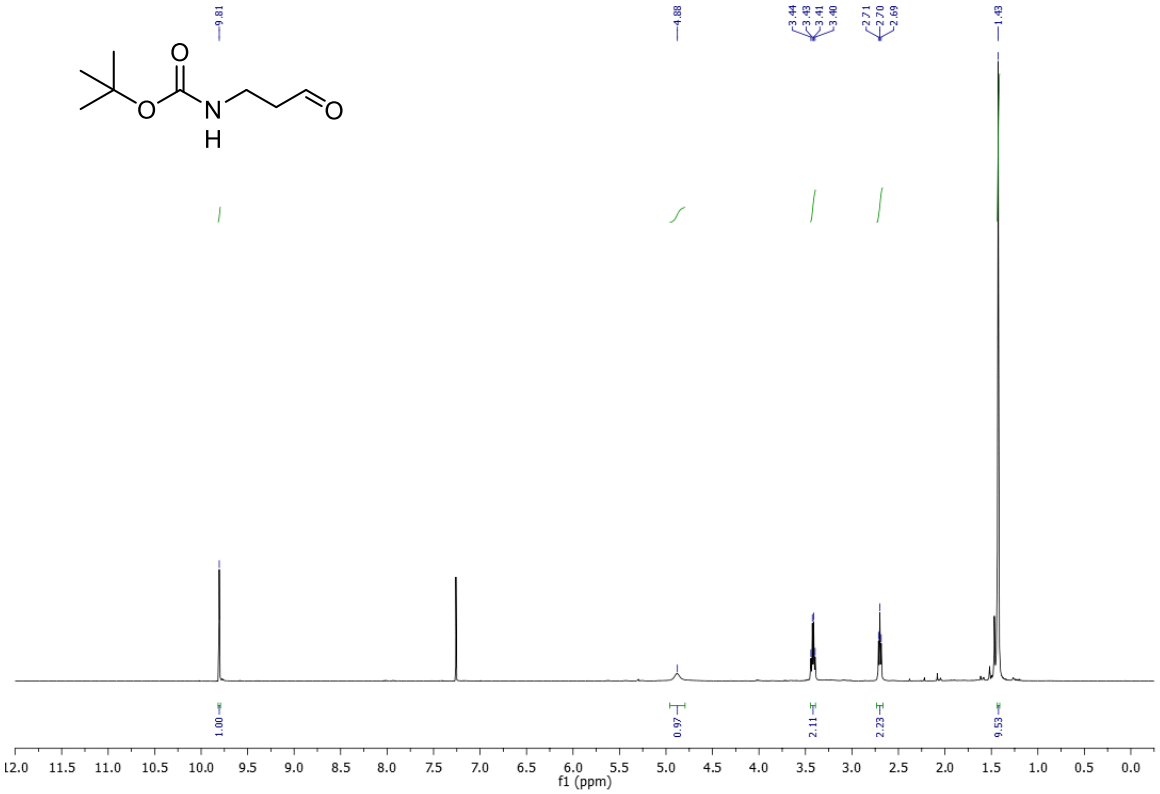


Figure 51: ^{1}H-NMR (400 MHz, $CDCl_3$) of *tert*-butyl (3-oxopropyl)carbamate.

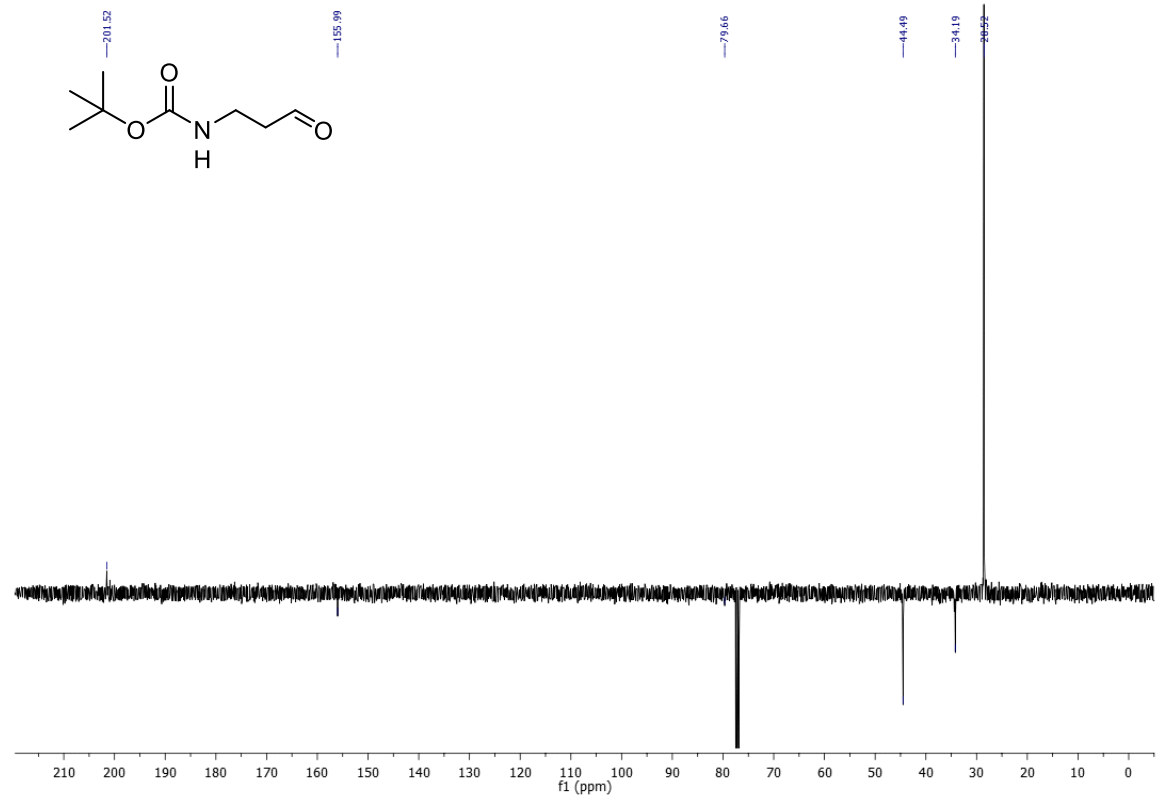


Figure 52: ^{13}C-NMR (101 MHz, $CDCl_3$) of *tert*-butyl (3-oxopropyl)carbamate.

5.1.25 *tert*-Butyl (3-bromopropyl)carbamate (28)

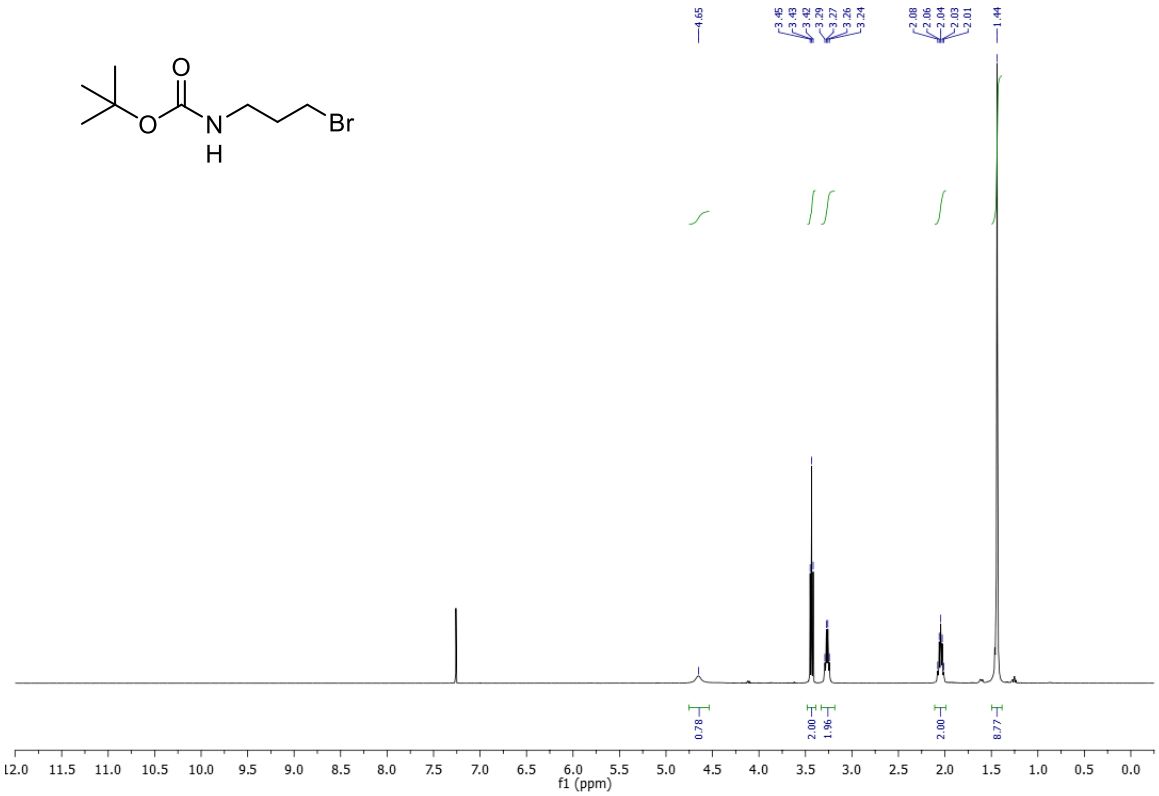


Figure 53: ^{1}H-NMR (400 MHz, $CDCl_3$) of *tert*-butyl (3-bromopropyl)carbamate.

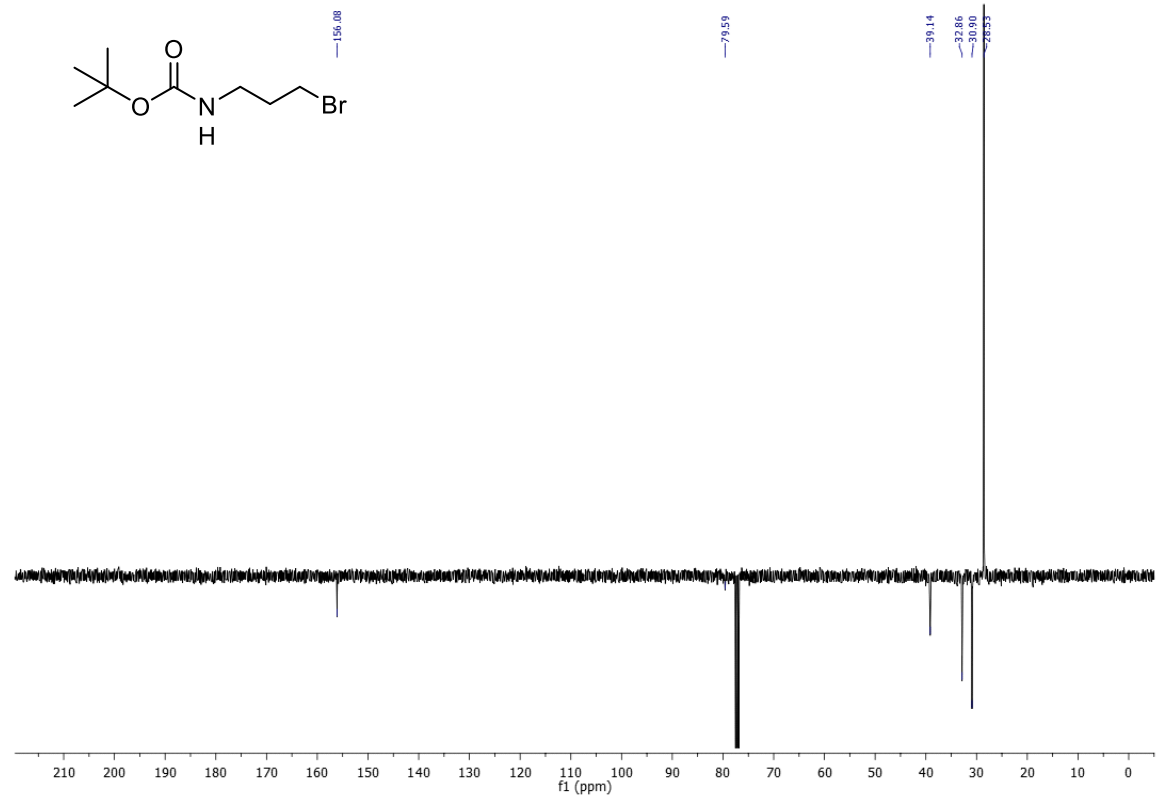


Figure 54: ^{13}C-NMR (101 MHz, $CDCl_3$) of *tert*-butyl (3-bromopropyl)carbamate.

5.2 Deutsche Zusammenfassung

Kernspinmagnetresonanzspektroskopie (NMR) ist eine häufig eingesetzte Methode um die Strukturen, Dynamiken und Wechselwirkungen von Proteinen zu untersuchen. Moderne Protein-NMR-Experimente benötigen den selektiven Einbau der Isotope ^{2}H, ^{13}C und ^{15}N in die Aminosäuresequenz, um entweder gewisse Signal zu verstärken oder zu verringern. Für selektive Isotopenmarkierungsexperimente wurde eine Syntheseroute für Natrium-1-pyrrolin-2-carboxylat, eine potentielle metabolische Vorstufe von *L*-Prolin, entwickelt. Diese Synthese ermöglicht den Einbau von ^{2}H-, ^{13}C- und ^{15}N-Markierungen im Molekül, ausgehend von leicht erhältlichen und günstigen Isotopenquellen. Natrium-1-[5-^{13}C]pyrrolin-2-carboxylat wurde synthetisiert und in Proteinüberexpressionen in *E.coli* getestet. Darauffolgende NMR-Experimente des exprimierten Proteins (BRD4) zeigten keinen Einbau der Vorstufe. Zukünftige Experimente mit Prolin-auxotrophen *E.coli*-Stämmen werden die Anwendbarkeit von Natrium-1-pyrrolin-2-carboxylat als selektive Prolinvorstufe zeigen.

5.3 Abbreviations and acronyms

ADP	adenosine diphosphate
ACN	acetonitrile
ATP	adenosine triphosphate
Cryo-EM	cryo electron microscopy
d	doublet
dtt	doublet of triplets of triplets
DCC	*N,N'*-dicyclohexylcarbodiimide
DCM	dichloromethane
DMAP	4-dimethylaminopyridine
DMP	dess–martin periodinane
E.coli	*Escherichia coli*
ee	enantiomeric excess
ESI-MS	electrospray mass spectrometry
FAD	flavin adenine dinucleotide
IDPs	intrinsically disordered proteins
IDRs	instrinsically disordered regions
NADP	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
PDB	protein databank
PPIs	protein-protein interactions
PRMs	proline-rich motifs
PRRs	proline-rich regions
q	quartet
s	singlet
t	triplet
tt	triplet of triplets
TCA cycle	tricarboxylic acid cycle
TFA	trifluoroacetic acid
THF	tetrahydrofuran
X_{aa}	any amino acid

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