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MASTERARBEIT

Titel der Masterarbeit

New Concepts in Isotopic Labeling of Proteins

verfasst von

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Things never quite work as you expect them to;
this is one of the principal beauties of science.

The Prestige

ACKNOWLEDGMENT

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Furthermore, I would like to thank Prof. Walther Schmid for the opportunity to be part of his working group and, moreover, to deal with this interesting topic.

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Everything is theoretically impossible, until it is done. Therefore, I owe my deepest gratitude to my boyfriend for having the courage to make us possible.

At last I want to thank my family members, especially my parents and my sisters Pia and Lena, for encouraging and inspiring me to follow my dreams. Thank you for supporting me emotionally and financially throughout my life and my studies at university.

ABSTRACT

Isotope labeling is used to simplify complex NMR spectra of proteins by increasing the amount of structural information and facilitating signal assignment. Recently, we developed and tested various synthetic routes to selectively label small molecules; these are added as amino acid precursors to *E. coli* expression media of proteins. After successful ^{13}C incorporation of the 1-^{13}C-α-keto acid, we synthesized more elaborate side-chain labeled derivatives of indole as a precursor, featuring ^{13}C isotope labeling in the five-membered ring and an isolated ^{13}C-^{1}H spin system of the six-membered ring in an otherwise deuterated background.

Furthermore, we designed an economic and general reaction sequence applicable for several enantiopure amino acids such as Val, Phe, Leu, Tyr, Trp and potentially His; these could be used to enhance cell-free protein expression or solid phase synthesis.

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1. Introduction and Aims of the Master Thesis

Determination of protein structures is a useful target to analyze protein folding, dynamic properties and, furthermore, to shed light on their functions in organisms. The conformation of proteins provides information about cellular mechanisms and about their affinity to specific substrates. Therefore, diverse methods for three-dimensional protein determination are applicable; the most important methods include X-ray crystallography and specific NMR methods (NOESY, COSY, and TOCSY). X-ray crystallography enables determination of static high molecular weight proteins providing high accuracy. Complementary to this method, NMR spectroscopy has evolved into a new method to analyze proteins in aqueous solution and, consequently, protein folding and their dynamic properties providing three-dimensional information.[1] A major disadvantage of this method is the complexity of resulting NMR spectra of large proteins (>100 kDa); this complexity derives from signal overlapping and line broadening, which causes limited signal assignment and difficult interpretation of these spectra.[2] Thus, our research investigates new methods to simplify NMR spectra by eliminating unwanted signals in order to make signal assignment feasible.

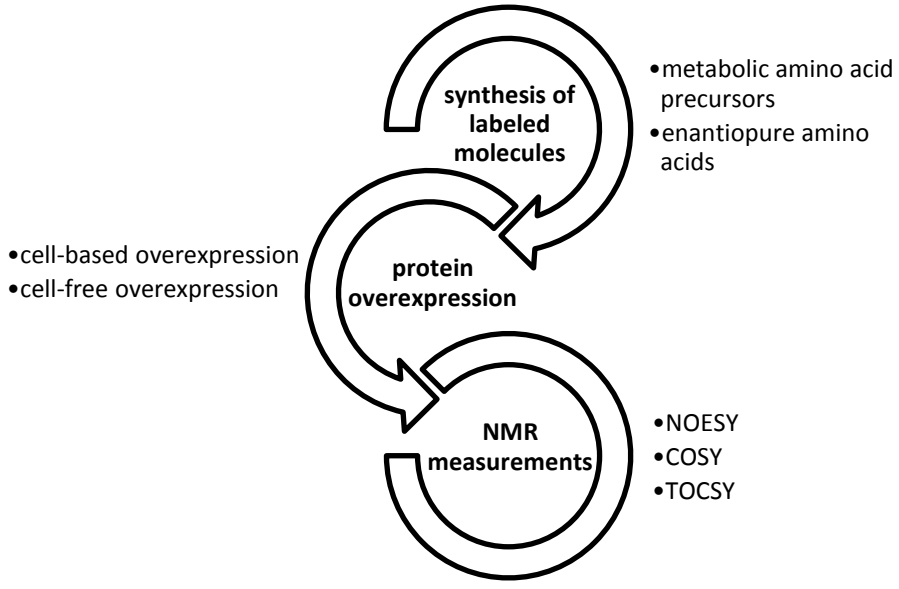


FIGURE 1: Three-step process of introducing isotopes into proteins and subsequent determination.

Protein labeling is the method of choice to eliminate superfluous signals and to increase the amount of structural information gained from NMR spectra. Therefore, small molecules with specific isotopic patterns (^{13}C, ^{2}H, ^{15}N) are synthesized; these are added to minimal growth media of protein overexpression host organisms (e.g. *E. coli*) and converted to amino acids *in vivo*. The resulting selective labeled target proteins can be investigated by certain NMR experiments (Figure 1).[1]

The intention of my master thesis was to develop economic novel routes to unprecedented ^{13}C and ^{2}H precursor compounds to be used in cell-based overexpression of selective labeled proteins. To this end, my master thesis was divided into two parts; on the one hand, we were interested in synthesizing labeled enantiopure amino acids and, on the other hand, we focused on different synthetic approaches to label Trp-precursors.

The synthetic route for enantiopure amino acids should be applicable for various amino acids (e.g. Val, Phe, Leu, Tyr, Trp and His). Starting from a cost-efficient substrate, glycine-1-^{13}C , selective isotope labeling is especially facilitated in the backbone positions of protein residues. These labeled enantiopure amino acids are relevant for cell-free protein overexpression as well as for solid-phase synthesis.

The second part dealt with the question, whether new metabolic Trp-precursors could be incorporated into small model proteins *via* cell-based protein overexpression in *E. coli*. Accordingly, indole-3-pyruvate, which is actually a degradation product in the metabolism of tryptophan, represents to be a convenient compound. Tryptophan synthase catalyzes formation as well as degradation of tryptophan into indole-3-pyruvate and, thus, this compound caught our attention.[3]

Moreover, indole serves as precursor in the tryptophan anabolism and catabolism; therefore, we focused on various selective ^{13}C and ^{2}H isotope labeling of indole, again starting from cost-efficient substrates.[4]

2. Theoretical Background

Since I worked on routes toward specific amino acids (Val, Phe, Leu, Tyr, Trp and His) I will address their metabolism. Especially the metabolism of tryptophan will explain our focus on specific metabolic compounds, which we have synthesized and used as precursors for protein labeling.

I will present various labeling strategies for these amino acids, which have already been reported in literature. Furthermore, this chapter comprises a detailed summary of protein overexpression and important NMR methods.

2.1. Metabolism of Specific Amino Acids: Val, Leu, Ile, Phe, Tyr, Trp and His

Protein biosynthesis in specific organisms always depends on the availability of all 20 amino acids.[5] Amino acids can be obtained from the environment, diet, degradation of existing proteins or provided by biosynthesis. In this chapter I will focus on the metabolic pathway of the amino acids Val, Leu, Ile, Phe, Tyr, Trp and His in the Gram-negative enteric bacterium *E. coli*.

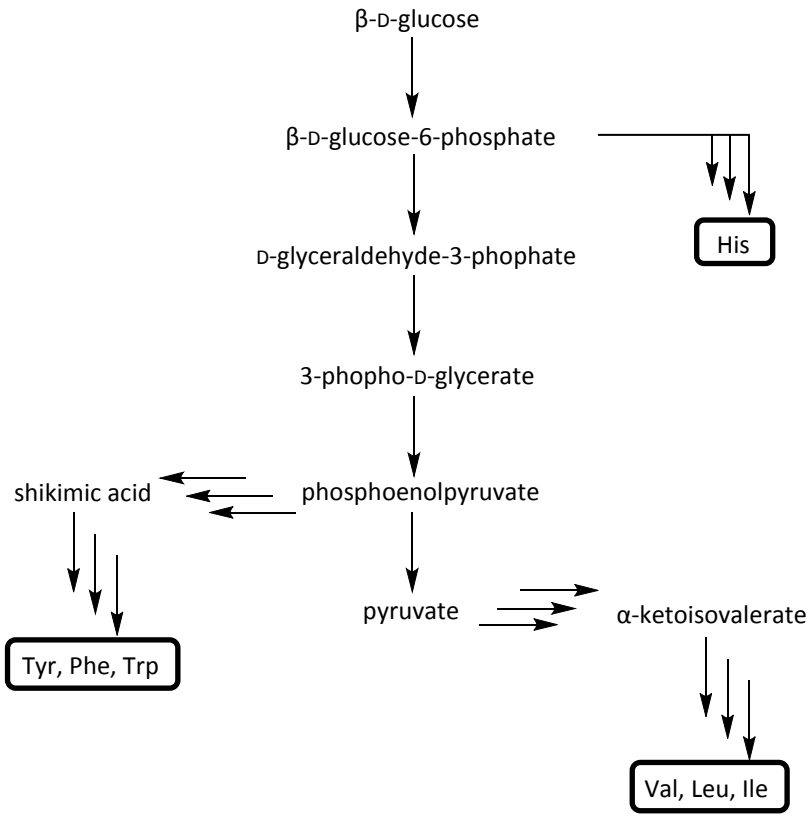


FIGURE 2: Overview of the metabolic pathways of Phe, Tyr, Trp, His, Ile, Val and Leu in *E. coli.*

Figure 2 (modified from lit.[3,6,7]) gives a brief overview of the biosynthesis of these amino acids. Phosphoenolpyruvate, intermediate of the glycolysis, is part of the shikimate pathway leading to aromatic amino acids Tyr, Phe and Trp, and, on the other hand, it can be converted into pyruvate, which is a precursor of the branched-chain amino acids Val, Leu and Ile. Histidine shows a slightly different pathway starting from β-D-glucose-6-phosphate.

2.1.1. Metabolism of Aromatic Amino Acids: Tyr, Phe, Trp and His

Phenylalanine, tyrosine and tryptophan are three proteinogenic aromatic amino acids, which are only accessible *via* one precursor, chorismate. Thereby, chorismate is obtained *via* chorismate biosynthesis, also known as shikimate pathway.[3]

Tyrosine is a polar amino acid. It plays a significant role in signal transduction processes and in posttranslational modifications as receiver of phosphate- or sulfate groups. Phosphate groups are transferred by protein kinases changing the protein's activity, while sulfation is catalyzed by sulfotransferases.[8]

The amino acid phenylalanine differs from tyrosine by the absence of the polar hydroxyl group and is classified as precursor for tyrosine.

Figure 3 (modified from lit.[3]) illustrates the metabolisms of L-tyrosine and L-phenylalanine showing interrelated pathways hardly differing from each other. First, both pathways convert chorismate to prephenate and in the third step, both produce the amino acid *via* transamination of a keto acid. The only objective difference is in the second reaction sequence; in the L-tyrosine pathway prephenate is converted into 4-hydroxyphenylpyruvate and in the L-phenylalanine pathway phenylpyruvate is generated.[3]

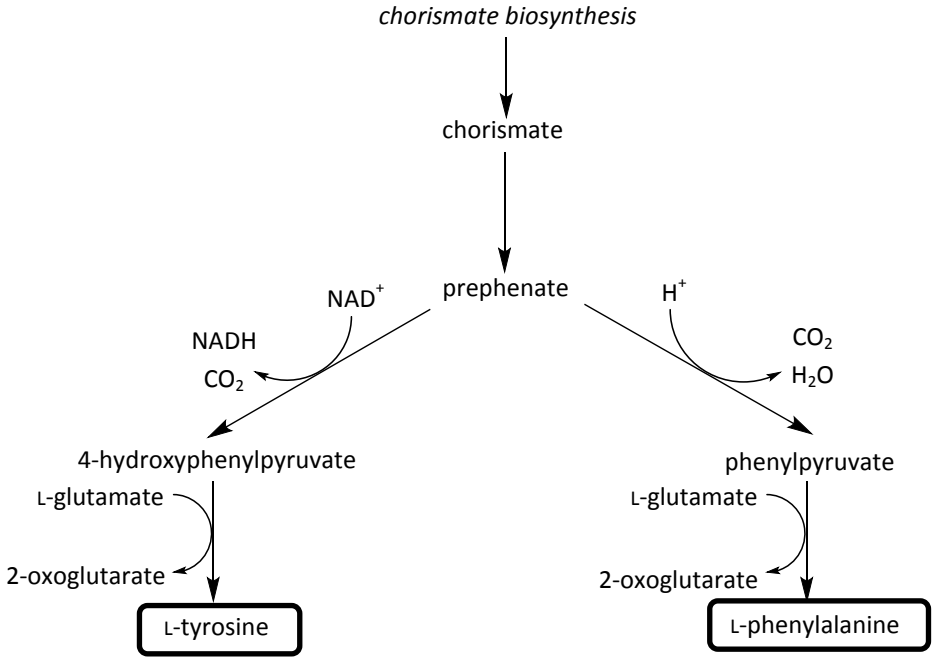


FIGURE 3: Biosynthesis of L-tyrosine and L-phenylalanine in *E. coli*.

Figure 4 (modified from lit.[3]) addresses the biosynthetic pathway of L-tryptophan in the Gram-negative enteric bacterium *E. coli*. Anthranilate synthase (EC 3.1.3.27) converts L-glutamine (provided *via* glutamine biosynthesis) and chorismate (furnished in the chorismate biosynthesis) to anthranilate. Anthranilate is further transformed to *N*-(5-phosphoribosyl)-anthranilate generating diphosphate as by-product and then to 1-(o-carboxylphenylamino)-1‘-deoxyribulose 5‘-phosphate. The next step provides ring opening and decarboxylation to form (1*S*, 2*R*)-1-C-(indole-3-yl)glycerol 3-phosphate. The last two steps comprise the function of tryptophan synthase (EC 4.2.1.20) catalyzing the conversion of 1-C-(indole-3-yl)glycerol 3-phosphate into indole and subsequent formation of L-tryptophan from indole and serine.[9]

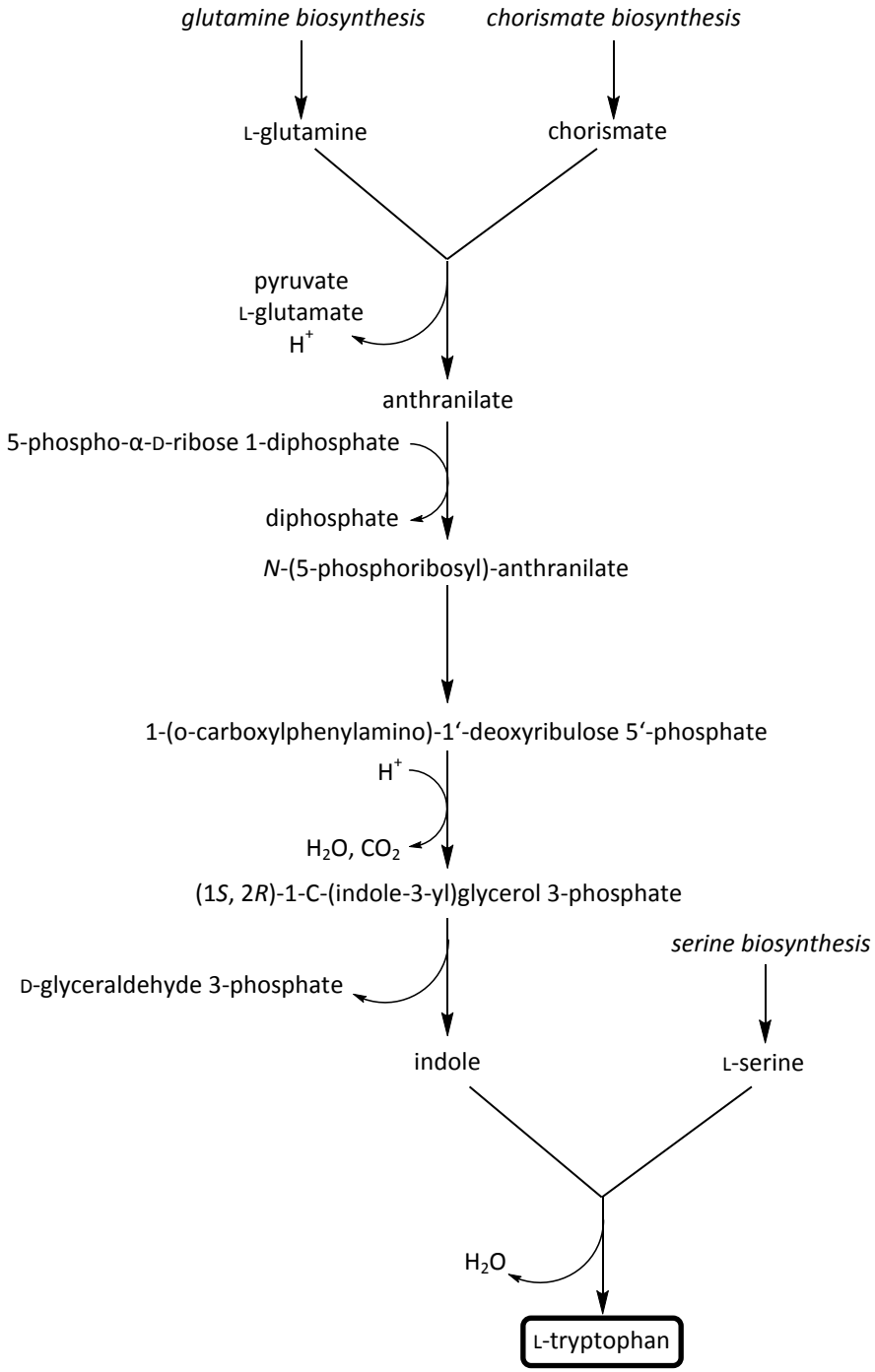


FIGURE 4: Biosynthetic pathway of L-tryptophan in *E. coli.*

Moreover, I want to outline the degradation of tryptophan; actually, this pathway provides interesting information concerning our thoughts on Trp-precursor synthesis. A more detailed assessment will be given in chapter 3.2.

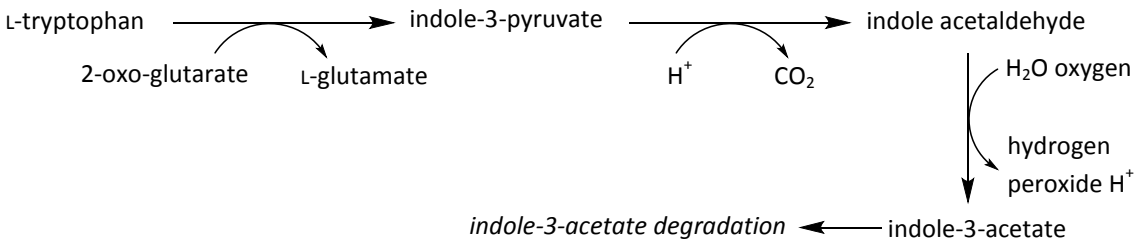


FIGURE 5: Tryptophan degradation *via* indole-3-pyruvate.

The enzyme of interest in the tryptophan degradation pathway, illustrated in Figure 5 (modified from lit.[3]), is Trp-transaminase (EC 2.6.1.27), which is present in most bacteria including *E. coli*. This enzyme operates close to equilibrium and is, therefore, interesting for our approach. Trp-transaminase catalyzes the degradation of L-tryptophan into indole-3-pyruvate as well as the conversion of indole-3-pyruvate into L-tryptophan. Thus, we synthesized labeled indole-3-pyruvate as precursor for protein overexpression in *E. coli* host organism realized *via* Trp-transaminase.

Histidine contains imidazole as a side-chain, which plays an important role for coordination of ligands. Moreover, histidine is very common in certain enzymes; it is part of the catalytic triade and acts as base. Thereby, it deprotonates the nucleophile (e.g. serine, cysteine) and forms hydrogen bonding to the corresponding acid moiety in the catalytic triade.[8]

Figure 6 (modified from lit.[3]) pictures the metabolic pathway of the amino acid histidine comprising several enzymatic steps. The ribose moiety undergoes a condensation reaction with ATP followed by a cleavage of the pyrophosphate group. The next step consists of the purine ring opening followed by formation of D-erythro-imidazole-glycerol-phosphate; thereby, amidotransfer is realised *via* glutamine. Dehydrogenation of this compound and transamination lead to L-histidinol-phosphate. Cleavage of the phosphate group finally forms L-histidinol. The last two steps comprise the conversion of L-histidinol to L-histidine *via* L-histidinealdehyde intermediate catalyzed by L-histidiniol dehydrogenase. This aldehyde intermediate is not released from the enzyme, but immediately converted to L-histidine.

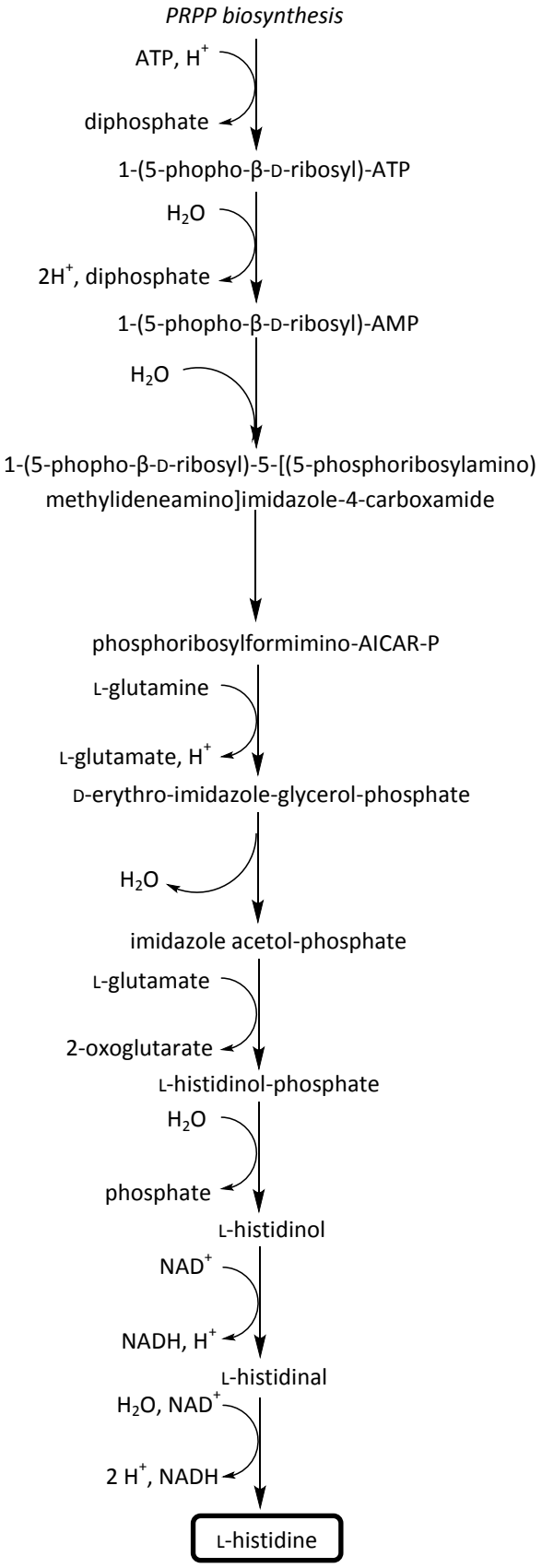


FIGURE 6: Biosynthesis of L-histidine in *E. coli*.

2.1.2. Metabolism of Aliphatic Amino Acids: Val, Leu and Ile

Glycolysis is part of the biosynthesis of the essential amino acids valine, leucine and isoleucine illustrated in Figure 2. Phosphoenolpyruvate acts as intermediate in the glycolysis and is converted into pyruvate. From here, Figure 7 (modified from lit.[3]) and Figure 8 (modified from lit.[3]) show the detailed biosynthesis of branched-chain amino acids valine, leucine and isoleucine.

The majority of enzymes are present in the anabolism of all three amino acids. Sharing many enzymes and components, it is difficult to use labeled precursors for protein synthesis, which selectively label one specific amino acid e.g. valine without any cross-labeling to leucine or isoleucine.[10] In which way cross-labeling can be avoided and which precursors support this will be explained in detail in chapter 2.3.1.

Figure 7 shows the biosynthesis of valine and leucine. In the first step acetolactate synthase (EC 4.1.3.18) catalyzes the formation of (*S*)-2-acetolactate deriving from two pyruvate moieties.[11] Thereby, an active acetaldehyde undergoes a condensation reaction with pyruvate. The next reaction step comprises a NADPH-dependent reduction forming a dihydroxy precursor. Dehydrogenation leads to the corresponding α-ketoisovalerate. At this point the metabolic pathways of valine and leucine separate. Transamination of α-ketoisovalerate finally leads to L-valine.[12]

Additionally, α-ketoisovalerate also serves as a substrate for the subsequent biosynthetic pathway of leucine. Chain-elongation is achieved by various enzymatic steps finally forming α-ketoisocaproate. This precursor is further converted into L-leucine *via* transaminase reaction.[12]

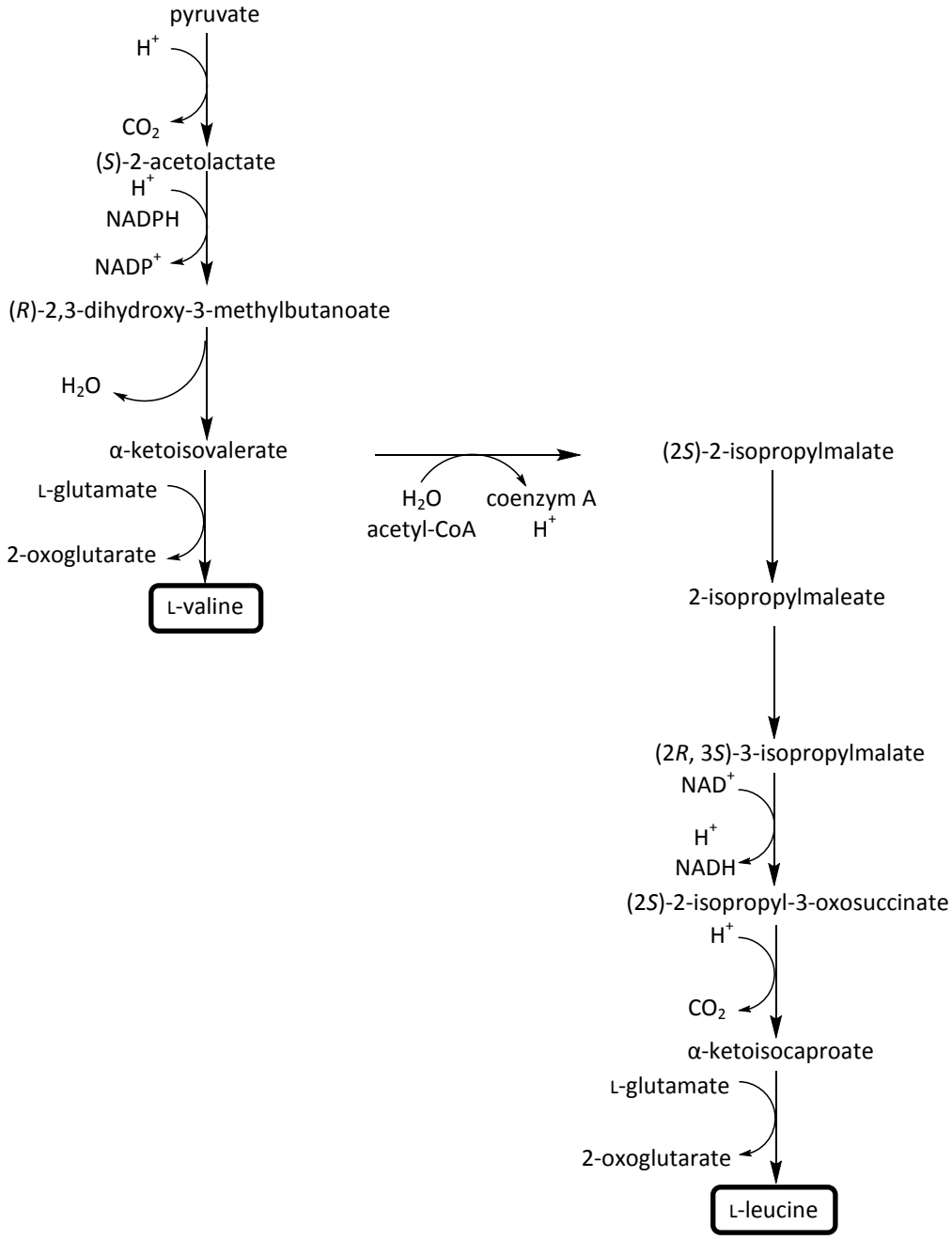


FIGURE 7: Metabolic pathway of L-valine and L-leucine in *E. coli*.

Isoleucine is part of the aspartate family; two of its six carbon atoms are provided by pyruvate and the four remaining ones by the L-threonine. Deamination of threonine provides α-ketobutyrate and subsequent chain-elongation is achieved by a pyruvate moiety forming (*S*)-2-aceto-2-hydroxybutanoate. After a skeleton rearrangement a NADPH-dependent reduction finally generates (*R*)-2,3-dihydroxy-3-methylpentanoate and following dehydration leads to α-keto-β-methylpyruvate. The last step provides a transamination reaction with L-glutamate finally forming L-isoleucine.[13]

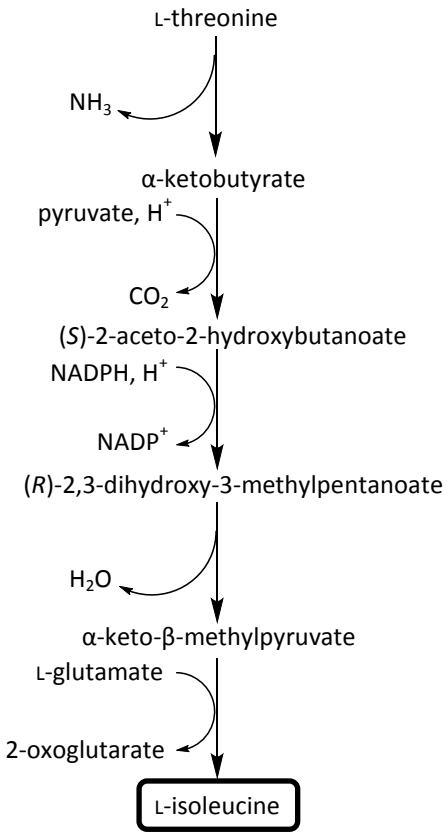


FIGURE 8: Biosynthesis of L-isoleucine in *E. coli*.

2.2. PROTEIN DETERMINATION

2.2.1. NMR METHODS

Two major methods, X-ray crystallography and NMR spectroscopy constitute 98.9% of analytical techniques used in protein structure determination. At present 89850 proteins (88.6%) are assigned *via* X-ray crystallography, while 10441 protein structures (10.3%) are identified by NMR methods.[14]

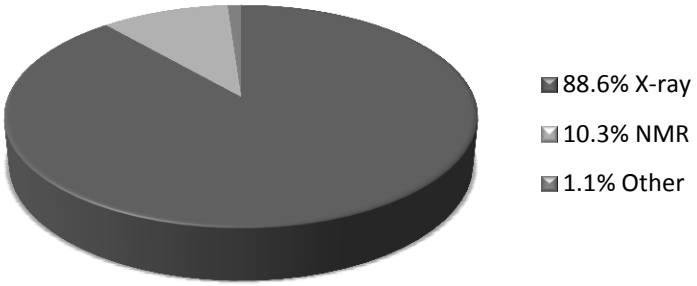


FIGURE 9: Experimental methods used for protein structure determination.

Protein structure determination started in the middle of the 20th century by Sir John Cowdery Kendrew and Max Perutz, who received the Nobel Prize in 1962 for studies on globular proteins *via* X-ray crystallography.[15] Since then, 89850 protein structures have been determined using this method.[14] This strategy requires stable proteins with a static structure; therefore, it is challenging to measure proteins inside membranes, which require specific solvents in isolation. According to this, X-ray crystallography enables determination of static proteins providing high accuracy and, moreover, structure identification of high molecular proteins.[16]

Complementary to this method, NMR spectroscopy has evolved into a new strategy to analyze proteins in aqueous solution. NMR experiments enable to determine the folding of proteins, molecular dynamic features and interactions of biological macromolecules. However, a drawback of this method is still the limitation of the size of proteins (~100 kDa).[2] This limitation arises from signal overlapping and line broadening, due to fast transversal relaxation and poor signal-to-noise ratios in NMR spectra.[17] At this point there are several methods to overcome these limitations and strategies to enhance NMR interpretation and determination of high molecular weight systems. Incorporation

of stable isotopes (e.g. ^{2}H, ^{13}C and ^{15}N) along with novel heteronuclear multidimensional NMR techniques simplify NMR spectra, alleviate interpretation and, moreover, improve sensitivity.[18]

At present there are several applicable NMR methods; therefore, it is important to distinguish between homonuclear and heteronuclear NMR strategies as well as between one-dimensional- and multidimensional NMR methods. Homonuclear coupling occurs between two NMR active nuclei of the same type (e.g. ^{1}H-^{1}H) *via* scalar spin-spin coupling (J-coupling). On the other hand, heteronuclear coupling is observed between two different NMR active nuclei (e.g. ^{1}H-^{15}N, ^{1}H-^{13}C).[19]

1D-NMR is usually restricted to measure small molecules since larger compounds or small proteins lead to a plethora of overlapping signals. The 2D-NMR technique is the method of choice to determine proteins providing two dimensional spectra. 2D-NMR spectroscopy is used for homonuclear as well as for heteronuclear measurements. One limitation of homonuclear NMR spectroscopy is caused by the overlap of peaks within a narrow range. The larger the protein, the more complex the NMR spectra. Therefore, various heteronuclear NMR methods (e.g. 3D-NMR, 4D-NMR) are applicable to overcome these limitations and to enhance spectra interpretation.[20]

Moreover, the phenomenon of NOE (Nuclear Overhauser Effect) is used to assess spin-spin distances in proteins. This effect arises through space and is therefore crucial for gaining three-dimensional information of protein structures. Spin coupling is usually observed through 2-3 chemical bonds; however, neighboring atoms in space (< 5 Å) can give NOE information. Fully protonated proteins lack the ability to achieve signal assignment caused by a multitude of NOE signals. Therefore, particularly deuterated proteins are synthesized to eliminate unnecessary signals and to decrease the complexity of NMR spectra.[21]

Various NMR methods, which are used for both homonuclear and heteronuclear techniques as well as for 2D- or multidimensional NMR methods, are listed below:

- COSY (Correlation Spectroscopy) observes couplings through 2-3 chemical bonds.
- TOCSY (Total Correlation Spectroscopy) comprises a suppression of transverse relaxation leading to improved spectral resolution and greater sensitivity for large molecules.

- NOESY (Nuclear Overhauser Effect Spectroscopy) correlates signals through space.[17]

Fully protonated molecules show ^{1}H-^{1}H dipolar spin relaxation, which leads to a loss of sensitivity and resolution. Therefore, selective exchange of ^{1}H protonated carbons against NMR-inactive ^{2}H (6.7-fold gyromagnetic ratio of ^{2}H to ^{1}H) results in elimination of many relaxation pathways, minimized spin-diffusion effects and, moreover, to simpler NMR spectra.[18] Using this method, signals, which are not relevant for determination, are extinguished, while important signals remain unchanged; this strategy makes NMR interpretation feasible.

On the other hand, perdeuteration of the whole molecule is of low analytical value, because ^{1}H coupling with heteronuclear atoms (NOE information) is immediately lost.[22] ^{13}C- and ^{15}N-isotops, which have low natural abundance, demonstrate low sensitivity due to their gyromagnetic ratio; therefore, they are incorporated into proteins to obtain appropriate signal intensities.[23] Both isotopes are important for heteronuclear measurements to study protein dynamics (e.g. ^{1}H-^{15}N, ^{1}H-^{13}C and ^{2}H-^{12}C couplings and spin interaction with the directly bonded heteroatom) and, moreover, for direct detections.[1,18]

2.2.2. Cell-Based Protein Overexpression

Cell-based protein overexpression describes the term of producing proteins *via* specific host organisms. *Escherichia coli* (*E. coli*), a Gram-negative bacterium, is the most frequently used organism, which can translate all 20 amino acids starting from simple carbon and nitrogen sources. The advantage of *E. coli* derives from the well-known metabolic processes as well as from using e.g. glucose, glycerol or acetate as carbon- and e.g. $^{15}NH_4Cl$ as nitrogen sources. Several metabolic pathways of amino acids have already been described in chapter 2.1. In fact, *E. coli* is unsuitable for protein expression, which require specific posttranslational modifications or for proteins having numerous disulfide bonds. Therefore, various yeast cells provide introduction of such parameters.[1] E. g. *Pichia pastoris* (*P. pastoris*) is used more frequently as host medium for proteins featuring disulfide bonds. In this host organism they are more likely formed than in *E. coli* systems.[1]

Using cell-based protein overexpression, it is possible to add amino acid precursors to the medium, which are translated to the corresponding amino acid. The

advantage of precursors includes the aspect of avoiding a costly synthesis of enantiopure amino acids. Precursors usually derive from a cheap isotopic source containing less synthetic steps than the synthesis of enantiopure amino acids.

2.2.3. Cell-Free Methods

In addition, cell-free protein overexpression is another useful approach for generating proteins. The cell-free strategy is a valuable method to produce proteins, which are toxic to the host organism, to avoid interconversion of amino acids and, moreover, to reduce cross-labeling of amino acids. In chapter 2.3.1 other routes to avoid cross-labeling will be discussed; thereby, specific precursors are used to facilitate independent valine and leucine labeling in cell-based protein overexpression.[10]

The method of cell-free overexpression is therefore an alternative possibility to avoid cross-labeling. Cell-free protein overexpression is carried out in e.g. *E. coli* extracts covering the protein synthesis system, but not the metabolic conversion, which exists in living host organisms. Moreover, another advantage is a high incorporation rate of labeled compounds.[1] Unfortunately, this method requires enantiopure labeled amino acids. The synthesis of enantiopure amino acids is more complex and it is carried out *via* longer and less economic reaction routes.

Cell-free overexpression is a useful target for the SAIL strategy, which will be discussed in detail in chapter 2.3.3.

2.3. LABELING STRATEGIES

At present isotope labeling of small molecules is the method of choice to introduce stable isotopic patterns into proteins in order to simplify complex NMR spectra. The complexity of NMR spectra is affected by signal overlapping and line broadening, due to fast transversal relaxation and poor signal-to-noise ratios.[17] Therefore, ^{2}H, ^{13}C and ^{15}N isotope labeling of proteins is frequently used to eliminate unwanted signals and to make interpretation of NMR spectra easier.[24]

Isotopic labeling can be categorized as:

- Uniform labeling: Isotopes are introduced into proteins without any selectivity. Each atom is replaced by an isotope, which is useful for a specific NMR experiment.[25]
    - ^{15}N can be incorporated *via E. coli* by adding $^{15}NH_4Cl$ as cost-efficient isotopic source to the minimal medium.
    - Uniform ^{13}C labeling can be achieved by using glucose, glycerol or acetate as ^{13}C source; however, this uniform ^{13}C labeling technique can lead to more complex spectra caused by ^{13}C-^{13}C couplings.
    - Random exchange of ^{1}H by ^{2}H was considered to be an appropriate strategy, because of extinction of all ^{1}H-^{1}H signal couplings and spin diffusion. Actually, it is not always an advantage, because coupling with heteronuclear atoms, which is useful for specific NMR experiments (NOE information), is immediately lost.
- Selective labeling: Isotopes are introduced at specific positions in the protein; this can be achieved by chemical synthesis of small labeled precursors or enantiopure amino acids, which are added to the *E. coli* host organism's growth medium used in cell lysates.

Choosing a suitable method of isotope labeling depends on the performed NMR experiments. Therefore, selective isotope labeling is the method of choice for NMR experiments, which include crucial NOE information. In order to extinguish spin diffusion by ^{1}H-atoms, uniform exchange of ^{1}H by ^{2}H is considered. Moreover, a combination of both labeling methods is realized nowadays; e.g. selectively ^{13}C labeled precursors are added to a host organism as well as $^{15}NH_4Cl$ as a nitrogen source. Consequently, proteins

display uniform ^{15}N labeling in the backbone and ^{13}C labeling patterns at selective positions deriving from specific precursors.

Synthesizing enantiopure amino acids with selective labeling patterns is one of many options to introduce isotopes into proteins. However, this approach is very useful, but it includes high costs of isotopic sources and long reaction sequences to finally separate the racemic amino acid. More recently, this method has gained importance as so-called SAIL strategy, which will be discussed in detail in chapter 2.3.3.

Another protein labeling strategy is to add achiral precursors of a specific amino acid to a host organism. This host organism transforms the precursor into the specific labeled amino acid in a target protein. This method of synthesizing precursors as building blocks of the amino acids is often generated more easily.

To decide and investigate which amino acid precursors are suitable for protein overexpression, it is essential consider their metabolic pathway in a specific host organism (e.g. *E. coli*). The metabolic processes present various amino acids precursors; some of them are finally synthesized using organic synthetic chemistry with specific isotopic patterns, added to minimal media and used as building blocks for labeled target proteins.

Chapter 2.1 discussed several metabolic pathways of the amino acids Val, Leu, Ile, Tyr, Phe, Trp and His. Most of them contain an α-keto acid displaying a keto group directly next to a carboxyl acid moiety (e.g. α-ketoisocaproate, α-ketobutyrate, α-ketoisovalerate, 4-hydroxyphenylpyruvate, phenylpyruvate and indole-3-pyruvate). Figure 10 (modified from lit.[7]) illustrates that transaminases can easily convert these compounds to the corresponding amino acids; the keto group finally carries an amine group and the carboxylate group becomes the carboxyl group of the amino acid (Figure 10). Thereby, the keto carbon serves as the α-carbon and the residual carbons as side-chain.[7]

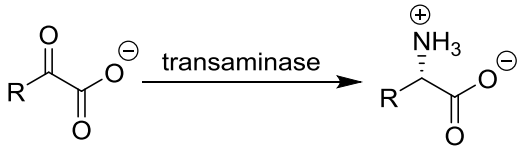


FIGURE 10: Conversion of an α-keto acid into an amino acid *via* transaminase.

In the following, I will present selective isotope labeling patterns in various amino acids and precursors, which have recently been published in literature.

2.3.1. Labeling of Branched-Chain Amino Acids

Amino acids with hydrophobic side-chains (Ala, Val, Leu, Ile) have become an increasingly important role in isotope labeling, because of their high abundance in proteins.[26] The signals of these amino acids are present in a narrow spectral range, leading to signal overlapping, restricted sensitivity and, therefore, to complex NMR spectra interpretation. Thus, in recent literature specific side-chain labeling has been reported to offer new opportunities to measure dynamic properties, protein motions and their folding.

Perdeuterated proteins lack in ^{1}H-^{1}H signals and, therefore, perdeuteration is unsuitable for NOE based NMR spectra (e.g. NOESY). In 1996 Kay et al realized selective protein labeling by adding protonated pyruvate as carbon source in a D_2O based media.[23] Thereby, they used advantages of perdeuteration while having a protonated pyruvate as carbon source. All carbons in α- and β position were almost uniformly deuterated, while the methyl groups of Ala, Val, Leu and Ile remained protonated. This method was devised to provide selective protonated methyl residues of the specific amino acids in a deuterated background; moreover, using this technique should offer new possibilities for NMR interpretation of larger proteins.[23] Unfortunately, the method led to proton deuterium exchange between pyruvate and D_2O and, therefore, other isotopic patterns of methyl groups were observed ($-CH_2D$, $-CHD_2$ and $-CD_3$) leading again to complex NMR spectra.[27]

Having these problems in mind, new strategies to selectively label proteins were developed. As already described, α-keto acids are useful metabolic components for protein overexpression acting as direct precursors for many amino acids.

α-Ketoisovalerate serves as precursor for both valine and leucine and α-ketobutyrate for isoleucine. In order to study high molecular weight proteins, signals of protons should be minimized in this narrow spectral range. This can be achieved by introducing $-^{13}CH_3$ and $-^{12}C^{2}H_3$ methyl groups into the aliphatic side-chains. Under these circumstances, branched-chain amino acids signal overlapping is highly decreased and sensitivity is improved.

Specific side-chain labeling was achieved starting from *tert*-butyl α-bromomethacrylate giving α-ketoisovaleric acid with $-^{12}C^{2}H_3$ and $-^{13}CH_3$ side-chain residue and α-ketobutyrate with $-^{13}CH_3$ residue shown in Figure 11 (modified from lit.[28]). Protein overexpression in *E. coli* stated that α-ketobutyrate and α-ketoisovalerate were transformed into the corresponding valine, leucine and isoleucine amino acids. This labeling strategy simplified NOESY spectra in the aliphatic region, because of reduced NOEs in the side-chain.[28]

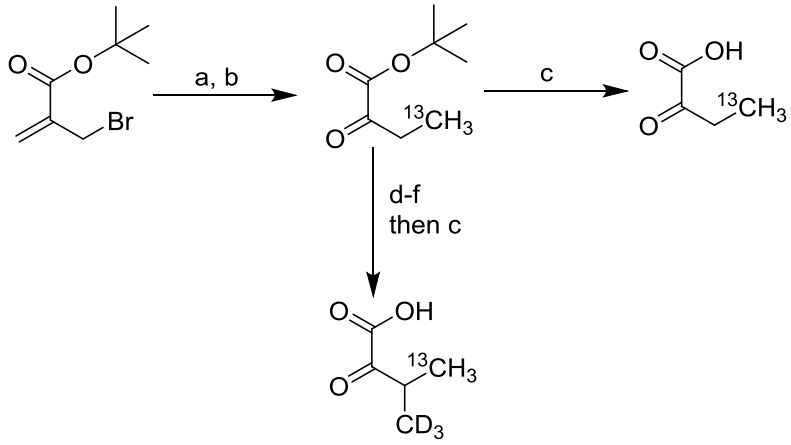


FIGURE 11: Synthetic pathway of labeled α-ketoisovaleric acid and labeled α-ketobutyric acid. Conditions: a) Mg, $^{13}CH_3I$, Et_2O, RT, 2 h b) O_3, CH_2Cl_2, -78 °C, PPh_3 c) HCl_g, CH_2Cl_2/Et_2O d) $H_2NN(CH_3)_2$, Et_2O, RT, 24 h e) LDA, CD_3I, THF, -78 °C, 3 h f) 1 N HCl, THF, Et_2O, RT, 1 h

Independent isoleucine labeling can be achieved by adding α-ketobuyrate or acetohydroxybutyrate to minimal media, while valine and leucine residues remain unlabeled.[29-31]

As it is known form literature, independent valine and leucine labeling can be achieved by adding unlabeled α-ketoisocaproate and labeled 2-ketoisovalerate to minimal growth medium. Thereby, leucine labeling is restricted, while labeled 2-ketoisovalerate is added as metabolic precursor for valine. Figure 12 (modified from lit.[10]) shows the synthetic sequence for labeling 2-ketoisovalerate and synthesizing unlabeled α-ketoisocaproate. Besides, labeled 2-ketoisocaproates can be used to selectively label leucine without any cross-labeling to valine; on the other hand, labeled 2-ketoisovalerate leads to simultaneously labeling of valine- and leucine-residues.[10]

Considering these novel strategies, selective labeling of aliphatic amino acid valine, leucine and isoleucine residues can be achieved.

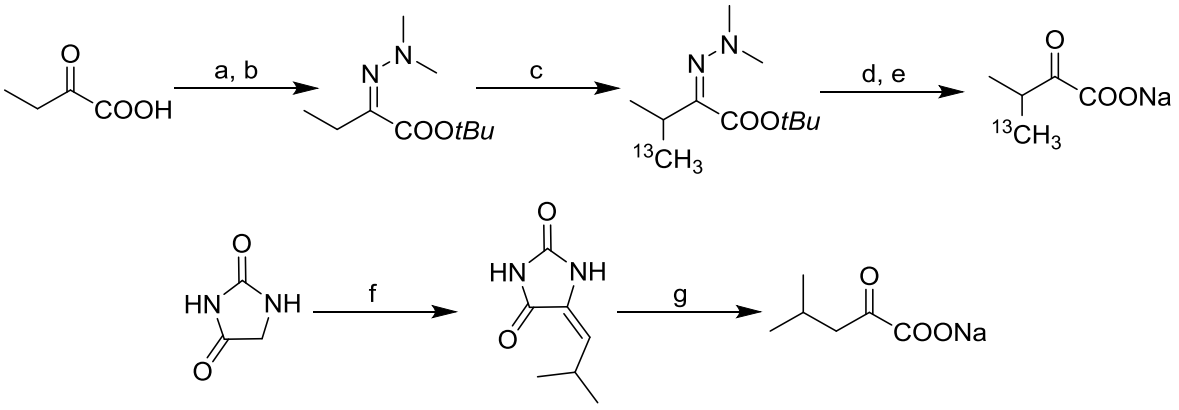


FIGURE 12: Synthetic route for labeled α-ketoisovalerate and unlabeled α-ketoisocaproate. Conditions: a) *t*BuOH, methanesulfonyl chloride, pyridine, THF, -5 °C – RT, 12 h b) *N,N*'-dimethylhydrazine, diethyl ether, 12 h c) lithium diisopropylamide, -78 °C, 1 h, then $^{13}CH_3I$, THF, 2h d) 1N HCl, THF, 3 h e) HCl_g, Et_2O/CH_2Cl_2 = 1:1, 0 °C – RT, then 1 M NaOH until pH = 8, lyophilize f) ethanolamine, isobutyraldehyde, H_2O, 110 °C, 9 h g) NaOH (20%), 100 °C, 5h

2.3.2. Labeling of Aromatic Amino Acids

Phenylalanine, tyrosine, histidine and tryptophan are aromatic amino acids. They are highly overrepresented in NMR spectra and, therefore, interpretation is quite difficult.[32] Moreover, their dynamic interactions with other side-chains as well as their rotation within proteins are a crucial aspect of structure determination and the folding of proteins. Due to these facts, selective labeling of aromatic side-chains can be used as a constructive tool for simplifying NMR spectra as well as for identifying their dynamic parameters.

Labeled aromatic α-keto acids (for Tyr, Phe) evolved to be appropriate amino acid precursors, which can be added to an *E. coli* host organisms. Synthesizing these selective labeled α-keto acids enables a simple and cost-efficient synthesis without introduction of a chiral center into the molecule. Selective labeling can be achieved in the backbone and in the side-chain of the precursors. ^{13}C labeling in the backbone is accomplished starting from glycine-1-^{13}C as isotopic source to form labeled hydantoin. Subsequent condensation of the hydantoin with benzaldehyde or 4-hydroxybenzaldehyde and hydrolysis give the corresponding α-keto acids. The precursors are obtained as salts *via* lyophilization and then incorporated into proteins *via E. coli* overexpression in the presence of $^{15}NH_4Cl$ and glucose nutrients. No cross-labeling was observed adding both labeled precursors into the host organism; this demonstrates independent labeling of tyrosine and phenylalanine residues.[33] More elaborate side-chain labeling can be achieved by introducing alternating ^{13}C-^{1}H and ^{12}C-^{2}H isotopic patterns in the side-chain

ring of these amino acids. This ring-labeling is achieved starting from labeled acetone-1,3-$^{13}C_2$ or acetone-2-^{13}C and $^{2}H_2O$. Isolated ^{13}C-^{1}H patterns in a deuterated background are a novel tool for precise signal assignment. Moreover, ^{13}C-^{13}C coupling is eliminated, because the aromatic residues include ^{13}C-^{12}C-^{13}C alternating patterns in the ring. The advantage of these alternating labeling strategies is discussed in detail in the next chapter.[1]

2.3.3. SAIL STRATEGY

The stereo-array isotope labeling (SAIL) method was invented to generate proteins featuring amino acids, which show stereo- and regio-specifically ^{2}H, ^{13}C and ^{15}N labeled positions.[18] Designing such amino acids includes the following rules for isotopic patterns:

- One of the two protons of a methylene group is exchanged with deuterium. Thereby, the remaining protons can be assigned more easily.
- In order to reduce spin diffusion, two protons of a methyl group are substituted with ^{2}H.
- In valine and leucine residues one methyl shows -$^{13}CHD_2$ and the other one -$^{12}CD_3$ isotopic patterns in order to facilitate a stereospecific assignment of one methyl group.
- Elimination of ^{13}C-^{13}C and ^{1}H-^{1}H couplings within the aromatic ring is completed by synthesizing alternating ^{1}H-^{13}C and ^{2}H-^{12}C labeling patterns in the aromatic residue.

This strategy of labeling amino acids simplifies NMR spectra by eliminating unnecessary NOEs. Furthermore, reduction of proton signals does not lead to loss of information, but increases both signal-noise ratios and signal assignment. Some of the amino acids, which have been synthesized, are now commercially available from SAIL Technologies (www.sail-technologies.com) and illustrated in Figure 13 (modified from lit.[1]).

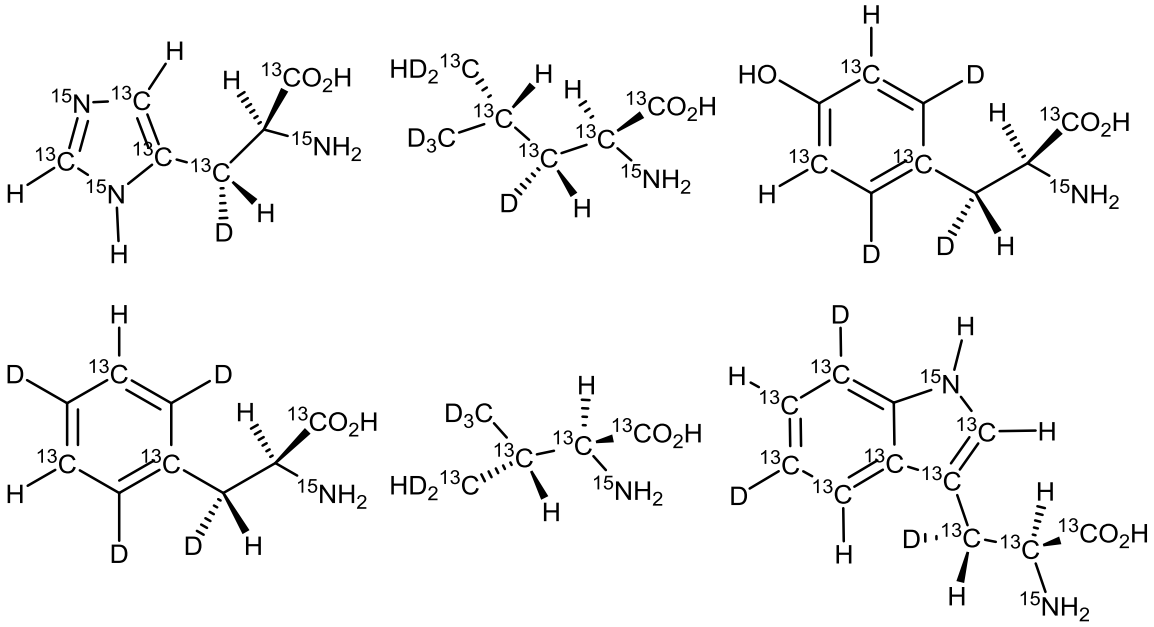


FIGURE 13: A small selection of various SAIL amino acids.

These costly labeled enantiopure amino acids are added to cell-free expression media (*E. coli* extracts) and incorporated into the target protein. This method usually provides a high incorporation rate without any cross-labeling; however, the incorporation rate varies for specific amino acids and target proteins. Therefore, it is crucial to optimize reaction conditions and find the right parameters to minimize the amount of required SAIL amino acids.

3. RESULTS AND DISCUSSION

In this chapter both the synthesis of enantiopure amino acids as well as the synthesis of Trp-precursors is discussed. Moreover, the last chapter gives a detailed summary of protein overexpression in *E. coli*.

3.1. SYNTHESIS OF ENANTIOPURE AMINO ACIDS

The general idea of designing a reaction route for isotopic labeled enantiopure amino acids includes several aspects:

- The linear reaction sequence must be as short as possible, including reaction steps, which provide good yields.
- The isotope patterns must be introduced from a cost-efficient substrate to enhance certain desired characteristics.
- The separation of enantiomers should be feasible, and, moreover, the yield of the required enantiomer should be clearly increased in contrast to the other enantiomer.
- The reaction sequence should be applicable for various amino acids.
- The synthetic approach should include reagents and solvents, which do not harm the environment, and, moreover, efficient use of substances is devised to minimize waste.

The reaction route, which is illustrated in Figure 14, includes a cheap labeled substrate, glycine, since ^{13}C permutations as well as ^{15}N labeling pattern of glycine are commercially available. Furthermore, the last reaction step provides an enzymatic reaction using acylase I in order to separate the enantiomers. The use of enzymes provides sustainability and, moreover, enzymes are biodegradable and, thus, particularly suitable for "green" reaction routes.[34] Acylase I catalyzes the hydrolysis of the L-amino acid, while the D-amino acid remains *N*-acetylated. The yield of labeled L-amino acid can be enhanced by racemization of the *N*-acetylated D-amino acid in the microwave oven to obtain again racemic *N*-acetylated amino acid, which can be used for the enzymatic reaction sequence. This reaction cycle is devised to be repeated. On the other hand, this synthetic route features the procurement of enantiopure D-amino acids *via* straightforward deacetylation of *N*-acetylated D-amino acids.

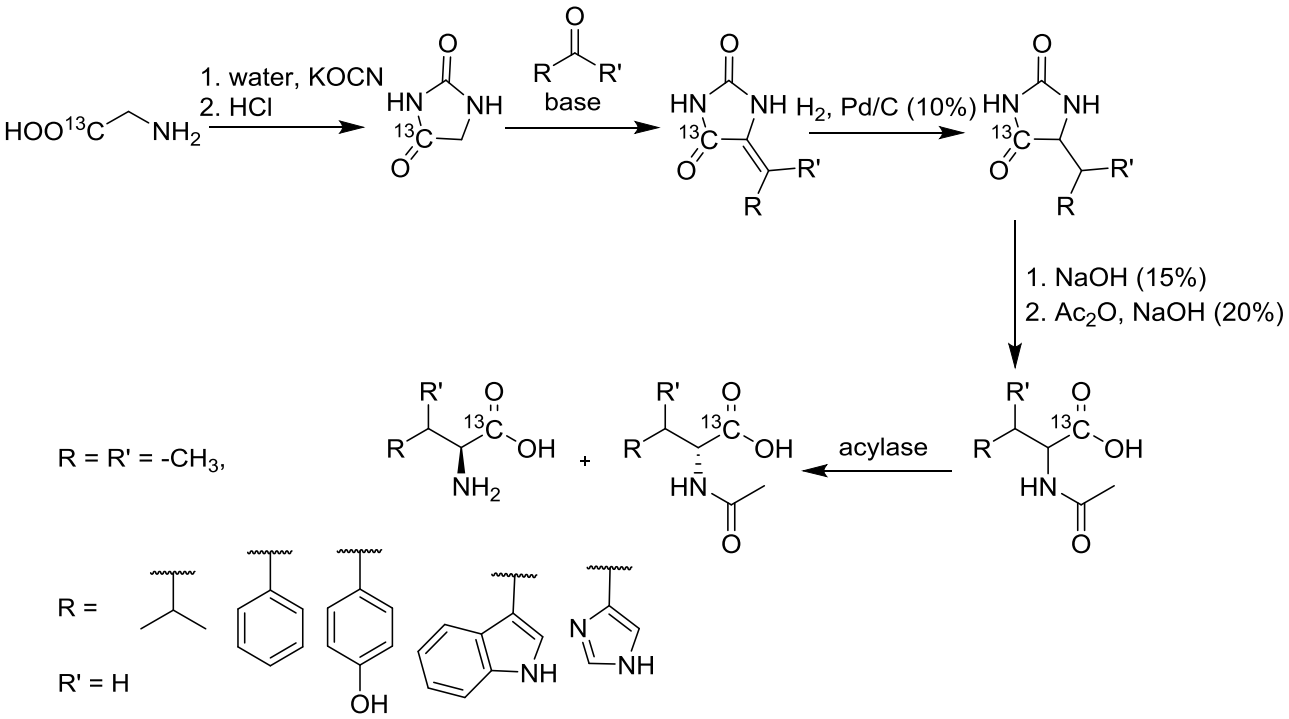


FIGURE 14: General synthetic route applicable for various amino acids.

In the past it was assumed that L-amino acids build up proteins exclusively; therefore, research focused on acquiring knowledge about L-amino acids, while D-amino acids were considered unusual having no biological functions. Over the last decades several peptides and antibiotics containing D-amino acids were discovered (e.g. gramicidin, octopine).[35] Therefore, Figure 14 represents a synthetic route for isotopically labeled L-amino acids as well as for D-amino acids.

The first step of this synthetic route provides the *Urech hydantoin synthesis* starting from glycine-1-^{13}C and potassium cyanate.[36] Hydantoin acid serves as intermediate and finally forms, after acidification, the ^{13}C labeled hydantoin. The hydantoin performs a condensation reaction with a ketone (acetone) or with aldehydes (isobutyraldehyde, indole-3-carboxaldehyde, benzaldehyde, 4-hydroxybenzaldehyde, imidazole-4-carbaldehyde). Selective hydrogenation of the previously formed double bond is achieved *via* a hydrogen balloon or an H-cube® continuous flow hydrogenation apparatus under Pd/C (10%) catalysis. The following steps comprise, on the one hand, the basic hydantoin ring opening to obtain the racemic amino acid, and, on the other hand, the acetylation of the amino group using acetylation reagent Ac_2O to acquire racemic *N*-acetylated amino acid. In the final step, a kinetic resolution *via* acylase I separating enantiopure L-amino acid from *N*-acetylated D-amino acid using ion-exchange

chromatography is performed. Thereby, acylase I catalyzes the enantioselective hydrolysis of *N*-acetylated L-amino acids.[37] Due to the wide variety of applications of acylase I, the enzyme accepts numerous *N*-acetylated amino acids in aqueous solution.

In order to increase the yield of L-amino acid, the *N*-acetylated D-amino acid is racemized in the microwave oven to achieve racemic *N*-acetylated amino acid, which can be used again for kinetic resolution.

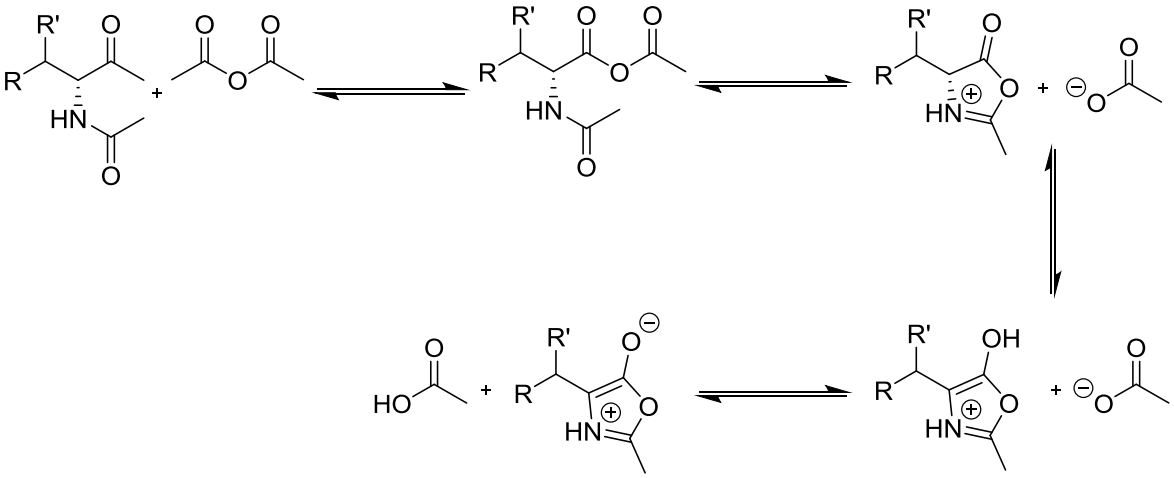


FIGURE 15: Posed mechanism for racemization of *N*-acetylated D-amino acids.

Figure 15 (modified from lit.[38]) poses the reaction mechanism of racemization including the formation of an asymmetric anhydride, a ring closure, keto-enol tautomerization as well as proton shift. The formation of an achiral oxazolium intermediate results in loss of chirality and, therefore, the reverse reactions form (*R* or *S*) enantiomers.[38]

This comprehensive reaction route, using low cost labeling compounds, is applicable for numerous amino acids including valine, phenylalanine, leucine, tyrosine, tryptophan, and some reaction sequences to form histidine.

In the following, I will describe in detail the syntheses of the above mentioned amino acids and I want to pinpoint all advantages and disadvantages of this reaction sequences. During my studies, I always performed all reactions unlabeled in the first place and after improving and optimizing reaction conditions and yields I started to introduce isotopic patterns.

3.1.1. SYNTHESIS OF LABELED L-VALINE

The first synthetic approach started form commercially available hydantoin in order to synthesize unlabeled L-valine. After optimizing all reaction steps and yields ^{13}C was introduced in the backbone of L-valine (Figure 16).

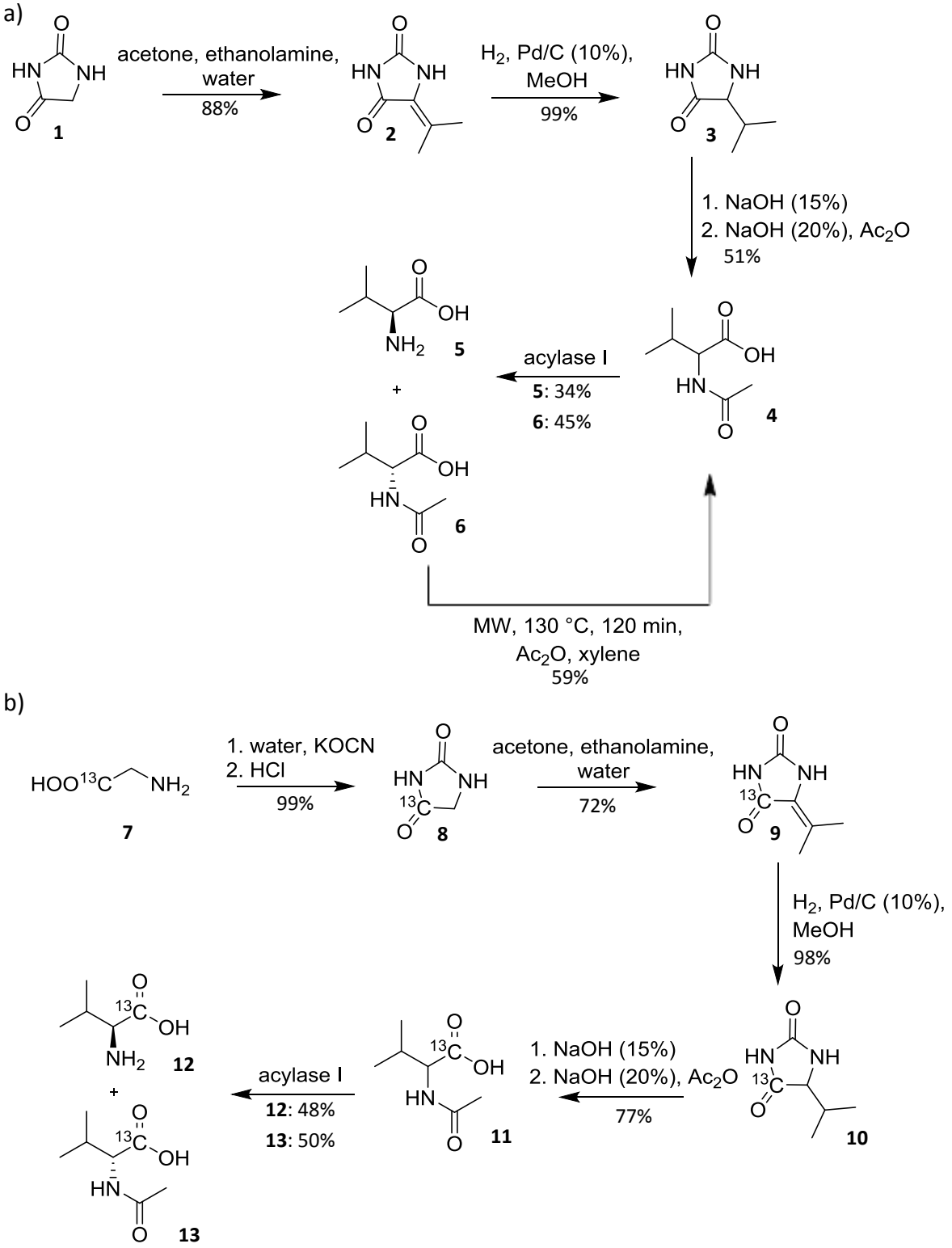


FIGURE 16: Synthesis of L-valine: a) unlabeled, b) ^{13}C labeled.

The first reaction step resulted in the formation of labeled hydantoin starting from glycine-1-^{13}C providing yields of 99%. This *Urech hydantoin synthesis* was carried out with potassium cyanate forming hydantoin acid and acidification of the reaction mixture led to an intramolecular attack of the amino group to the carbonyl group; subsequently, ring closure was observed forming hydantoin **8**.[36] Introducing acetone in a base catalyzed condensation reaction provided the formation of valine's side-chain.[39] Selective hydrogenation of the double bond was facilitated using either H-cube® or simple hydrogen balloon with Pd/C catalyst in methanol as solvent. The H-cube® provided yields up to 98%, while using a hydrogen balloon with a Pd/C (10%) catalyst comprised yields of not more than 96%. The following steps included basic hydantoin ring opening as well as acetylation of the amino group in order to accomplish compound **11**. Acylase I was used for kinetic resolution to separate the enantiomers; ion-exchange chromatography provided a successful isolation of labeled L-valine and *N*-acetylated D-valine.

Racemization was carried out with unlabeled *N*-acetylated D-valine giving pure compound **4**.[38]

Further determinations on the enantiomers were established by measuring the optical rotatory value comparing it to literature values, which will be discussed in the experimental section.

3.1.2. Synthesis of Labeled L-Phenylalanine

The second reaction pathway toward ^{13}C labeled L-phenylalanine was completed analogously to the previous synthetic route. Figure 17 illustrates all substrates and products used for the unlabeled synthesis as well as for the labeled pathway.

This reaction sequence hardly differs from valine, with one exception; the condensation of hydantoin and benzaldehyde under basic conditions using ethanolamine gave low yields.[40] Therefore, as reported in literature, different reaction conditions were tested to establish pure compound **19**.[41,42] Using benzaldehyde in combination with NH_4OAc and glacial acetic acid provided yields up to 74% of pure product.[43] Hydrogenation was carried out in a hydrogen balloon using Pd/C (10%) catalyst in methanol. This reaction step could straightforwardly be improved using the H-cube®, which provided yields up to almost 100%. Further reactions included the hydantoin ring opening under basic conditions, *N*-acetylation of the amino group as well as kinetic

resolution *via* acylase I. L-phenylalanine was further determined apart from NMR measurements by establishing the optical rotatory value.

Unlabeled *N*-acetylated D-phenylalanine was racemized using the microwave oven obtaining yields of 95%.

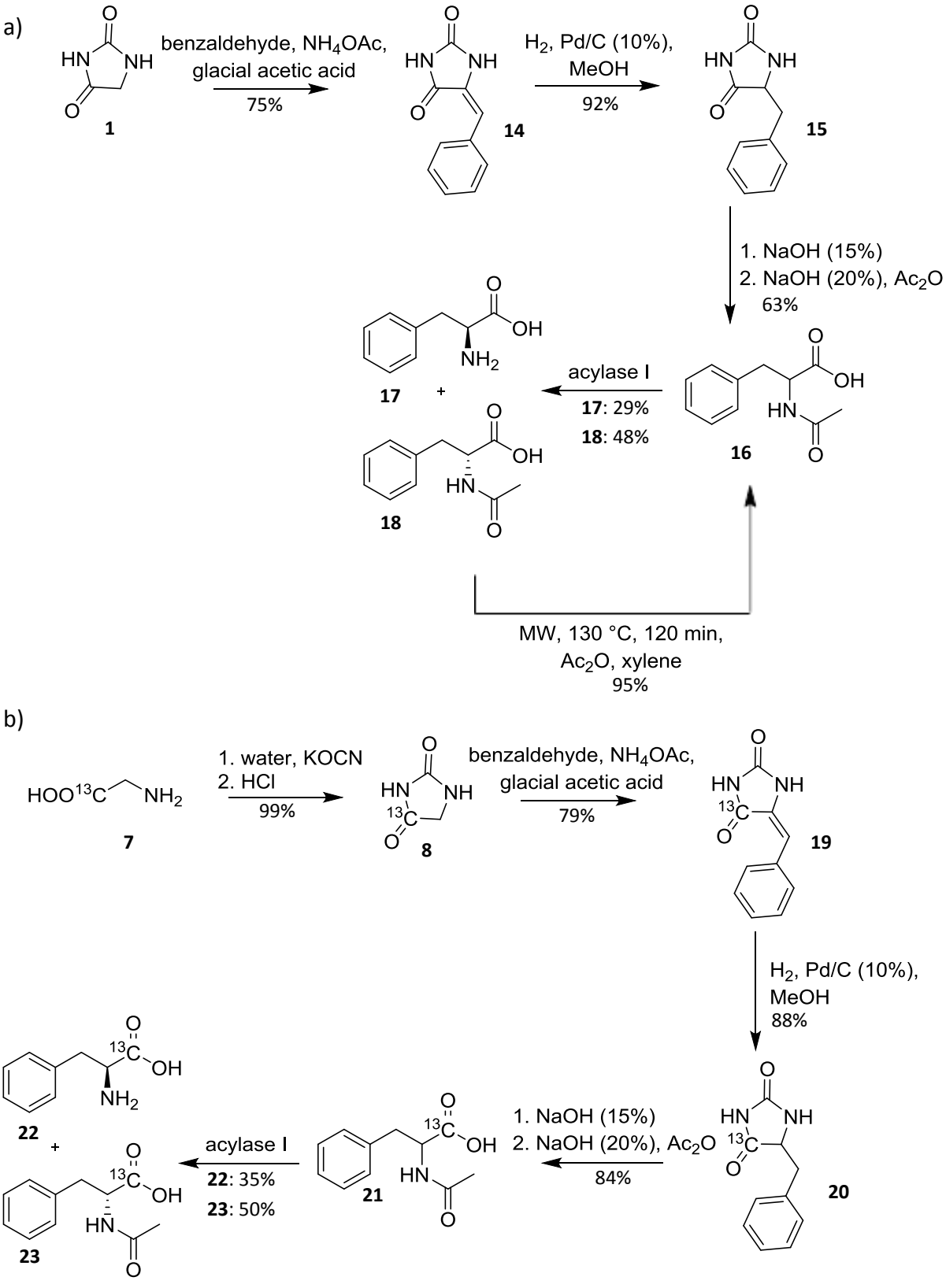


FIGURE 17: Synthesis of L-phenylalanine: a) unlabeled, b) ^{13}C labeled.

3.1.3. SYNTHESIS OF UNLABELED L-LEUCINE

The remaining amino acids, for which this reaction sequence was tested, were synthesized unlabeled; I have tried to enhance quality and efficiency of all reaction steps in order to introduce ^{13}C patterns in the future.

The same synthetic strategy was developed for L-Leucine (Figure 18). The first reaction step resulted in the formation of **24**, established by condensation of hydantoin with isobutyraldehyde under basic conditions using ethanolamine. Attempts to increase the yield were carried out using TEA.[44] Unfortunately I was not able to increase the yield of this condensation step and, therefore, it remains to be improved before introducing isotopes. The further reaction steps were completed as expected including acceptable yields finally leading to enantiopure L-leucine, which was further determined by establishing the optical rotatory value.

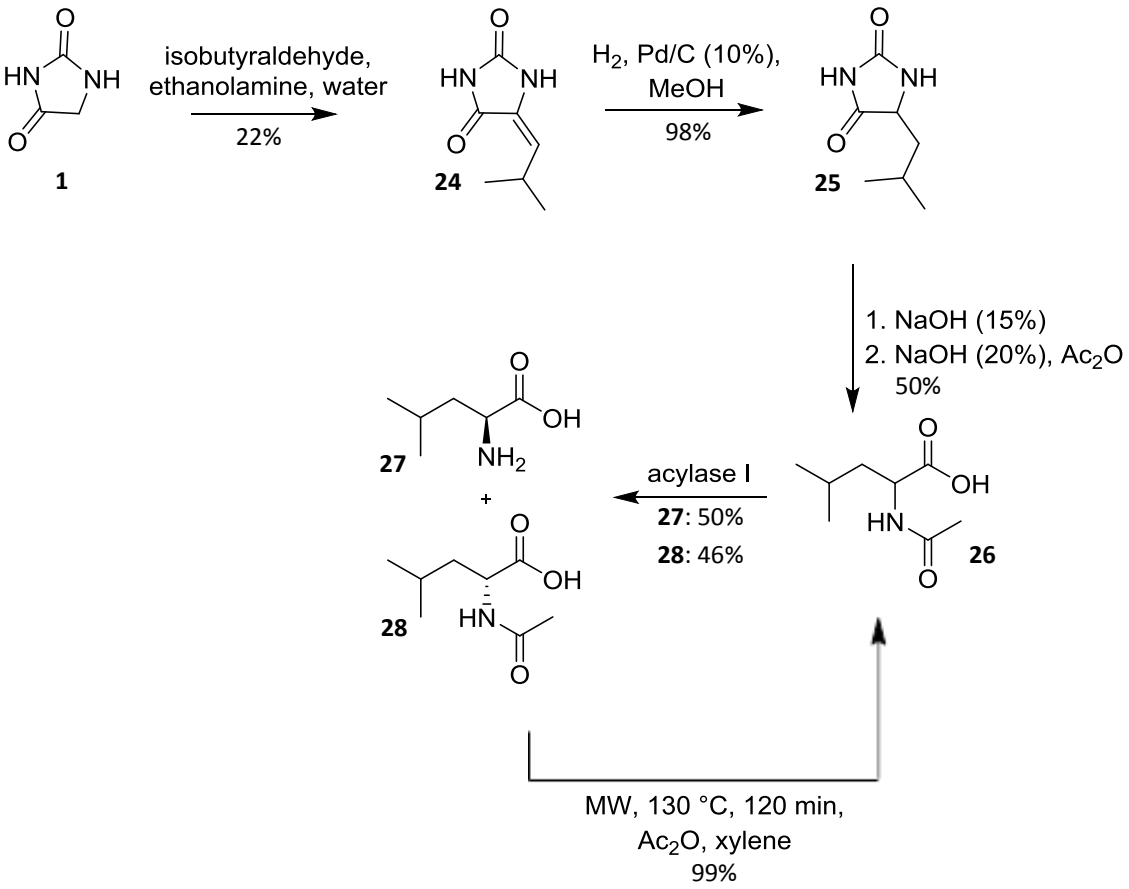


FIGURE 18: Synthesis of unlabeled L-leucine.

3.1.4. SYNTHESIS OF UNLABELED L-TYROSINE

L-tyrosine was the fourth amino acid for which this synthetic approach was desigend (Figure 19). The condensation step was carried out using 4-hydroxybenzaldehyde in piperidine to accomplish pure compound **29**.[45] Using ethanolamine instead of piperidine showed unsatisfactory yields as well as unwanted side products. This synthetic approach for tyrosine provided one challenging step, which was the acetylation of the amino group. The 4-hydroxy group of tyrosine's side-chain is in competition with the amino group. Thus, it was essential to use not more than one equivalent of the acetylation reagent in order to facilitate selective *N*-acetylation of the amino group.[46] Using excess of Ac_2O resulted in the formation of unwanted side products *via* diacetylation. The subsequent enzymatic reaction provided pure products of L-tyrosine and *N*-acetlyated D-tyrosine. Further determinations including the establishment of the optical rotatory value and the racemization step could not be finished so far.

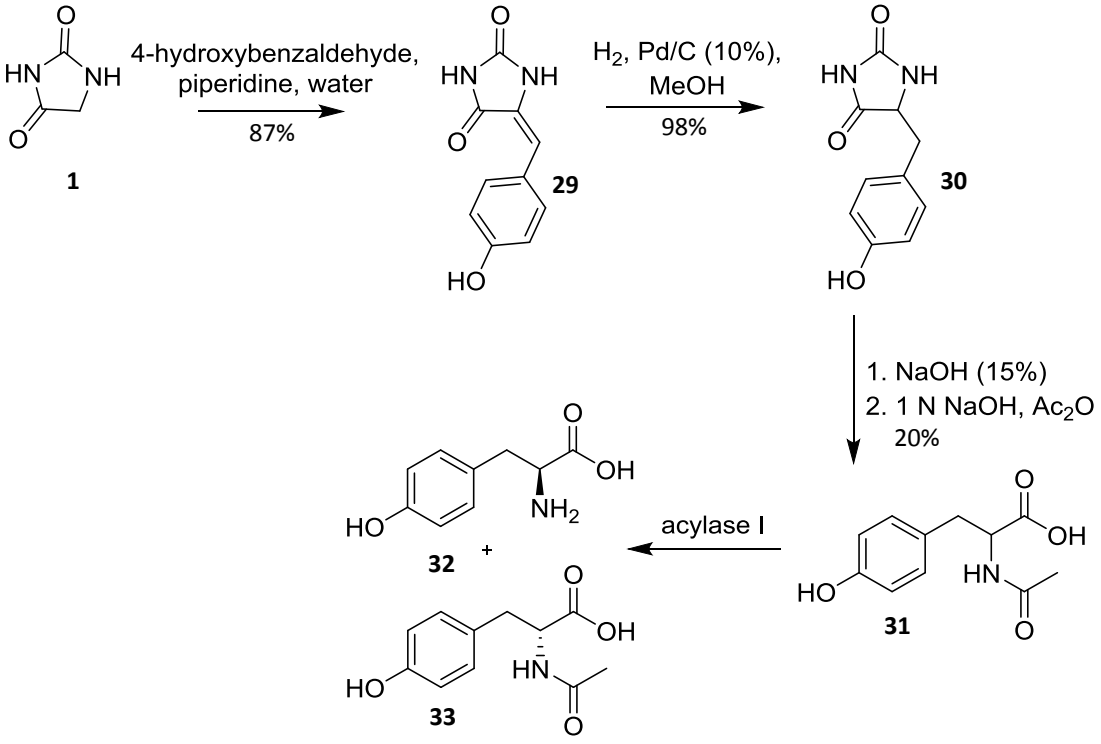


FIGURE 19: Synthesis of unlabeled L-tyrosine.

3.1.5. Synthesis of Unlabeled L-Tryptophan

The fifth reaction pathway consisted of the synthesis of L-tryptophan (Figure 20). This synthetic approach for enantiopure L-tryptophan provided acceptable yields in each reaction step, but so far the whole synthesis was just carried out using unlabeled substances. Pure L-tryptophan was obtained, but further investigations have not yet been carried out.

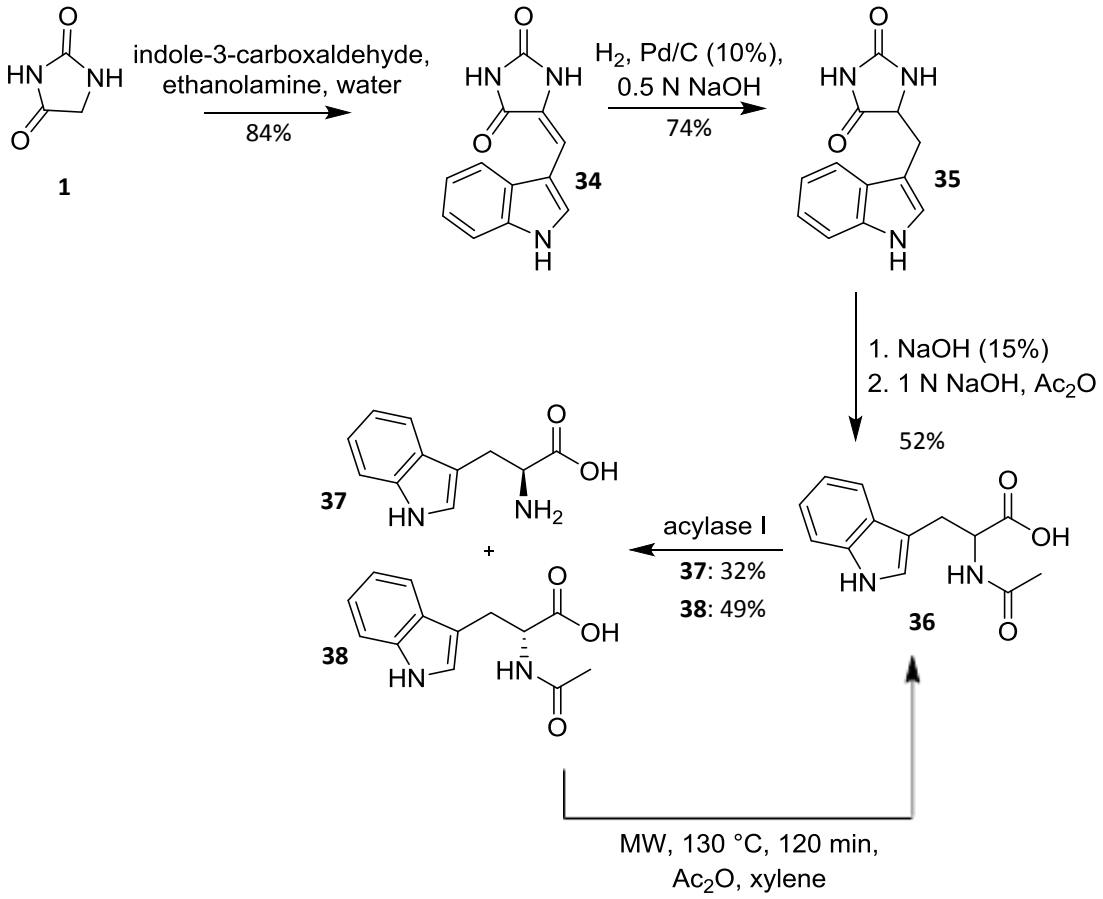


FIGURE 20: Synthesis of unlabeled L-tryptophan.

3.1.6. Synthesis of Unlabeled L-Histidine

The synthesis of unlabeled L-histidine turned out to be the most challenging synthesis (Figure 21). Imidazole-4-carbaldehyde could easily be introduced as side-chain of histidine using water as solvent in the condensation step under basic conditions. Moreover, hydrogenation of the double bond was carried out using the H-cube® and 0.5 N NaOH as solvent providing acceptable yields. Unfortunately, basic ring opening of the hydantoin included degradation of the side-chain of histidine. Therefore, different

reaction conditions have to be carried out in order to successfully synthesize compound **41**.

Using L-histidine as a substrate, acetylation of the amino group was successfully completed (Figure 21).[38]

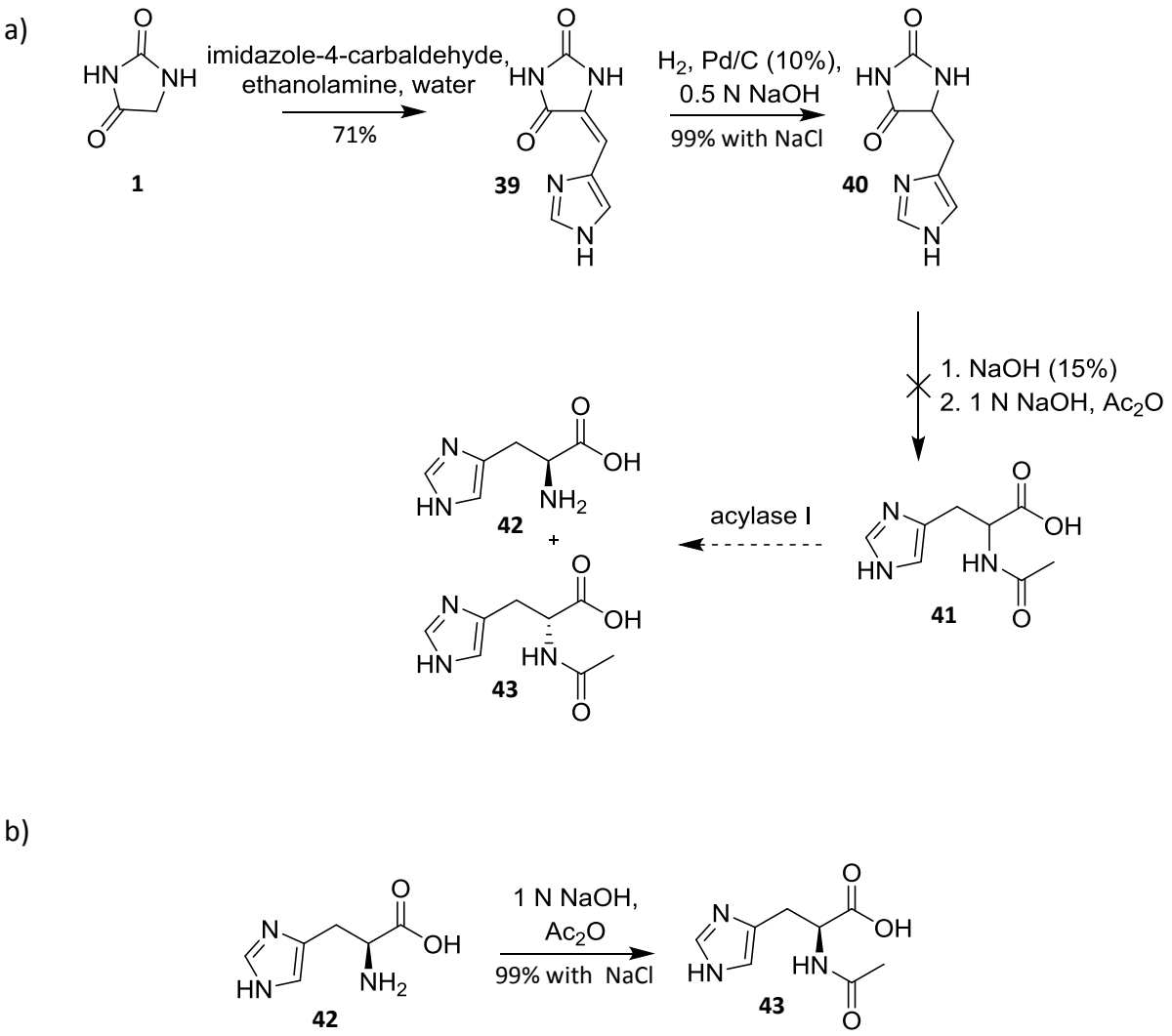


FIGURE 21: a) Synthetic route of unlabeled L-histidine. b) Acetylation of L-histidine.

3.2. TRP-PRECURSORS

The second part of my master thesis consisted of the synthesis of Trp-precursors, which are applied for cell-based protein overexpression.

Looking at the biosynthesis of tryptophan, as described in chapter 2.1.1, several potential precursors are eligible for being synthesized. Plants and microorganisms usually synthesize tryptophan from shikimic acid, transforming it into indole. Tryptophan synthase catalyzes the formation of tryptophan from indole and serine. Moreover, indole is a degradation product and, therefore, it is present in the anabolism as well as in the catabolism.[3]

Thus, this pathway pinpoints that indole could probably be used as precursor; therefore, the synthesis of labeled indole including various isotopic patterns is an interesting target compound to study. Moreover, it has briefly been reported in literature that indole serves as precursor for tryptophan.[4]

Having a look at the catabolism, indole-3-pyruvate is present as a degradation product. The degradation of tryptophan is catalyzed by Trp-transaminase, which works reversible. Therefore, indole-3-pyruvate could be used as precursor too. To this end, the idea was to synthesize two precursors with different isotopic labeling patterns: the indole-3-pyruvate and the indole in order to be overexpressed in *E. coli*.

3.2.1. SYNTHESIS OF INDOLE-3-PYRUVATE AS A TRP-PRECURSOR

A three step sequence was devised to provide ^{13}C labeled indole-3-pyruvate **45** in the backbone position (Figure 22). The first reaction step resulted in the formation of ^{13}C labeled hydantoin starting from labeled glycine performing the *Urech hydantoin synthesis*.[36] Introduction of the side-chain of the precursor was achieved by performing a condensation reaction with indole-3-carboxaldehyde. At this point the ring opening of the hydantoin under basic conditions was carried out to furnish amino acid precursor **45**. Numerous reaction conditions were carried out, due to the fact that literature reported inconsistent data. Indole-3-pyruvic acid's stability varies considerably with solvent and pH, caused by its oxidative character.[47] Decomposition of indole-3-pyruvic acid was observed under basic conditions in contact with atmospheric oxygen. Therefore, attempts to form the stable salt, indole-3-pyruvate, were made instead of instable indole-3-pyruvic acid. Caution is required performing this last reaction step; it is fundamental to bubble argon through the reaction mixture under basic conditions in order to prevent

decomposition of the keto acid. Thus, we had to try numerous reaction conditions to finally achieve a yield of 80%. In agreement with the literature we performed this reaction under exclusion of oxygen and under basic conditions. Moreover, literature reported to achieve pyruvic acid as a pale-brown solid, which could be filtered and another literature source stated that an oily residue to which water was added gave a colorless solid.[48,49] However, the combination of different reaction procedures from several literature sources and reasonable measures finally revealed the accurate reaction conditions to form indole-3-pyruvate.[50,51] Moreover, an extraction in the basic milieu was done while bubbling argon through the reaction mixture. After acidifying the solution, argon protection atmosphere was not further necessary. An extraction was carried out and subsequent lyophilization furnished **46**.

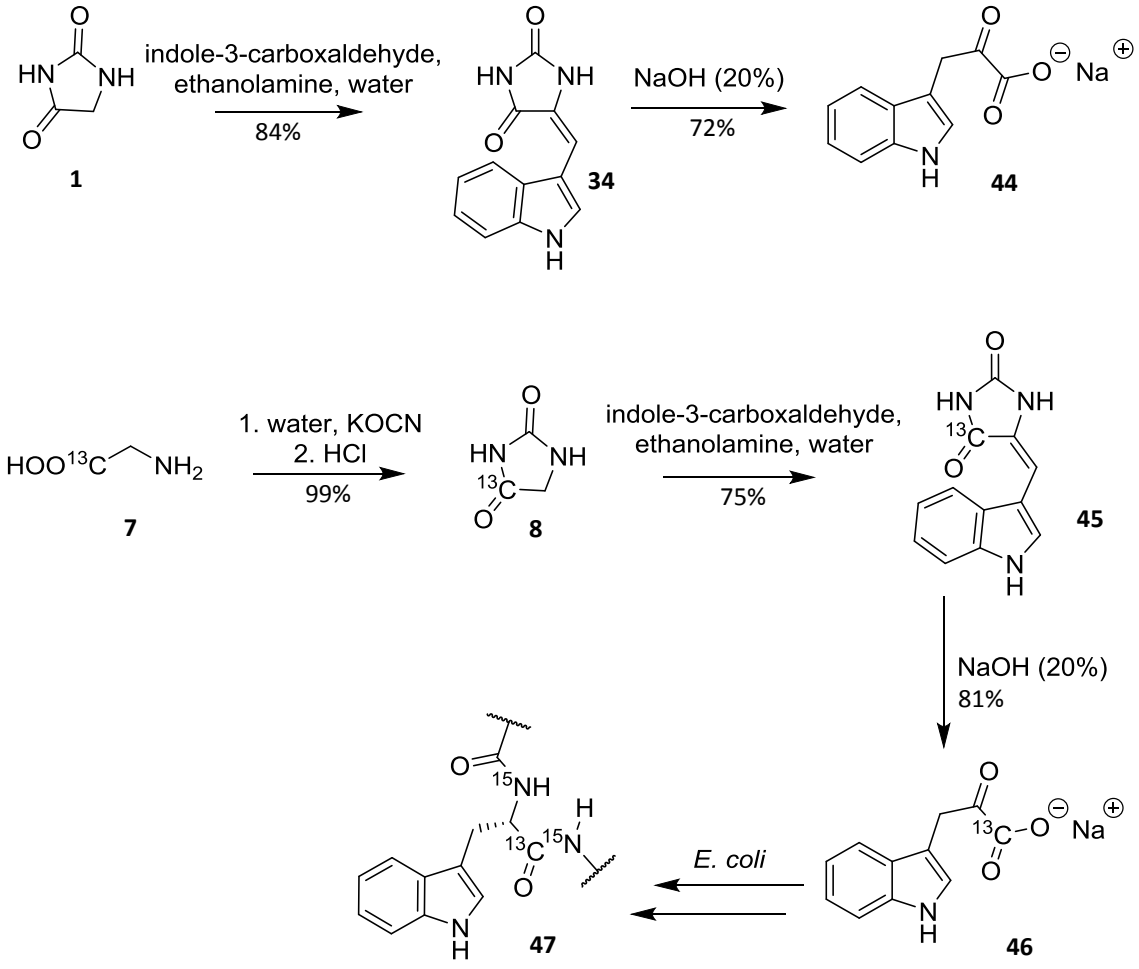


FIGURE 22: Synthesis of ^{13}C labeled indole-3-pyruvate and protein overexpression in *E. coli*.

The obtained ^{13}C labeled Trp-precursor **46** was incorporated into a small model protein GB1 (protein G B1 domain) in *E. coli* expression media in the group of Prof. Robert Konrat in the Department of Structural and Computational Biology, Max F. Perutz Laboratories, Dr. Bohr Gasse 9, 1030-Vienna (experiments performed by Tomas Sara).

Five 250 mL cultures of M9 minimal media were supplemented with $^{15}NH_4Cl$ as sole source of nitrogen and with the desired amount of ^{13}C labeled indole-3-pyruvate (10 mg/L, 20 mg/L, 50 mg/L, 100 mg/L, 200 mg/L). The protein concentration was measured *via* spectrophotometry and the tryptophan incorporation was measured by NMR HNCO.

Figure 23 addresses the relative intensity of the tryptophan peak signals and it reveals that saturation was already reached at about 50 mg/L.

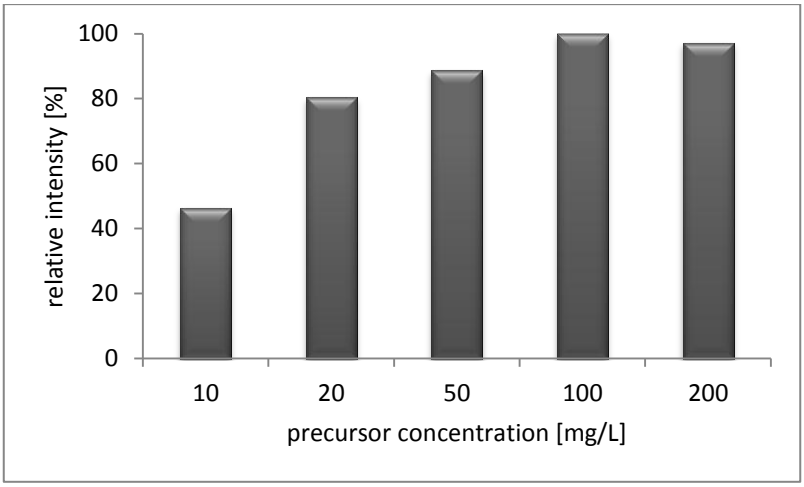


FIGURE 23: Relative intensities of the tryptophan peak signals.

Figure 24 illustrates an HNCO spectrum of GB1 protein, which was supplemented with 100 mg/L ^{13}C indole-3-pyruvate. This peak shows one carbon shift coming from a single carbon atom and, therefore, it shall be considered as evidence that indole-3-pyruvate was successfully incorporated into GB1 as the corresponding amino acid.

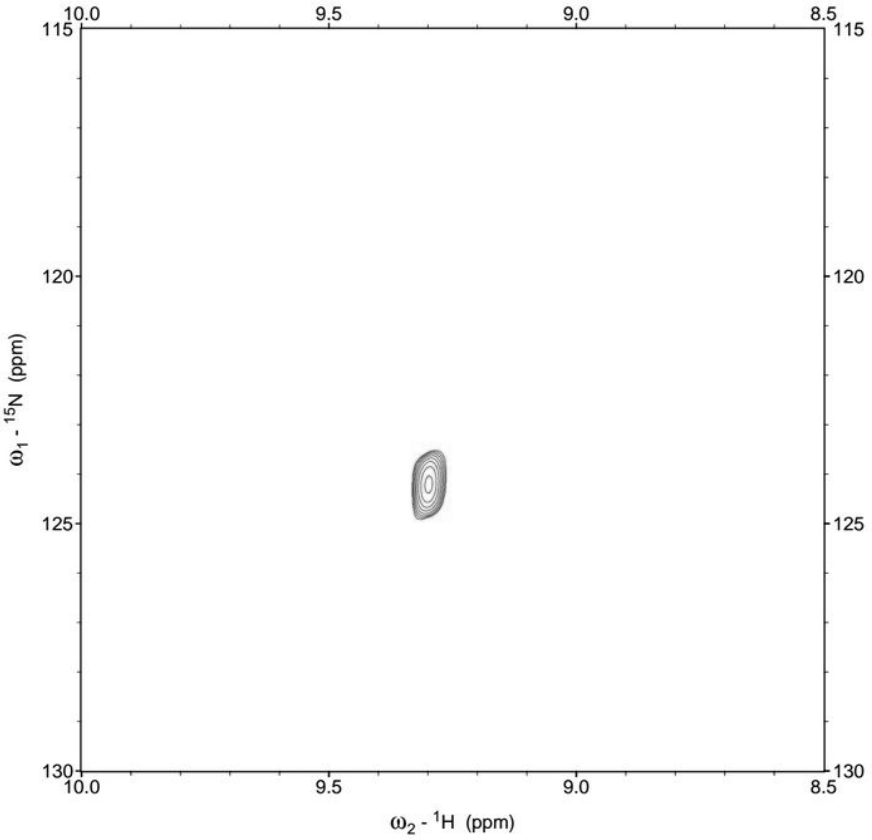


FIGURE 24: HNCO spectrum of GB1 protein supplemented with 100 mg/L ^{13}C indole-3-pyruvate. Sequence of GB1: MKHHHHHHPMKQYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGE**W**TYDDATKTFTVTE.

3.2.2. SYNTHESIS OF INDOLE AS A TRP-PRECURSOR

Besides, the second approach regarding Trp-precursors focused on the synthesis of indole. Indole as a precursor has been mentioned briefly in a patent. At that point we were interested whether indole is a suitable precursor for protein overexpression and, therefore, we designed a concise synthetic route to label indole in the five-membered ring with ^{13}C (Figure 28). First, all reactions were carried out unlabeled and after optimizing all reaction steps and yields ^{13}C was introduced. A *Vilsmeier-Haack reaction* was performed starting from indole and affording aldehyde **60**, which could be used as substrate to achieve indole-3-pyruvate with novel isotopic patterns.[52]

^{13}C was introduced by using $K^{13}CN$ as isotopic source in order to form hydroxyl acetonitrile in DCM. Literature recommended performing this reaction in diethyl ether for two days.[53] As in further reaction steps DCM is used as a solvent, we decided to run this reaction in DCM and, moreover, full conversion was achieved after one day. The next step included chlorination with thionylchloride to furnish compound **51**.[52] Chloroacetonitrile has a boiling point between 124-126 °C and hydroxyl acetonitrile about 100 °C.[54] Therefore, evaporation of the solvents was not possible without any product loss, due to their high volatility. Compound **51** was used to complete the *Houben-Hoesch reaction* with aniline hydrochloride.[52] Using commercially available chloroacetonitrile to perform this reaction, product **58** was formed having a yield of 77%. Pure product **52** was furnished with a yield of 48% over three steps. The last reaction step included a ring closure to obtain crude compound **53**.[52] Column chromatography finally gave pure indole **53**.

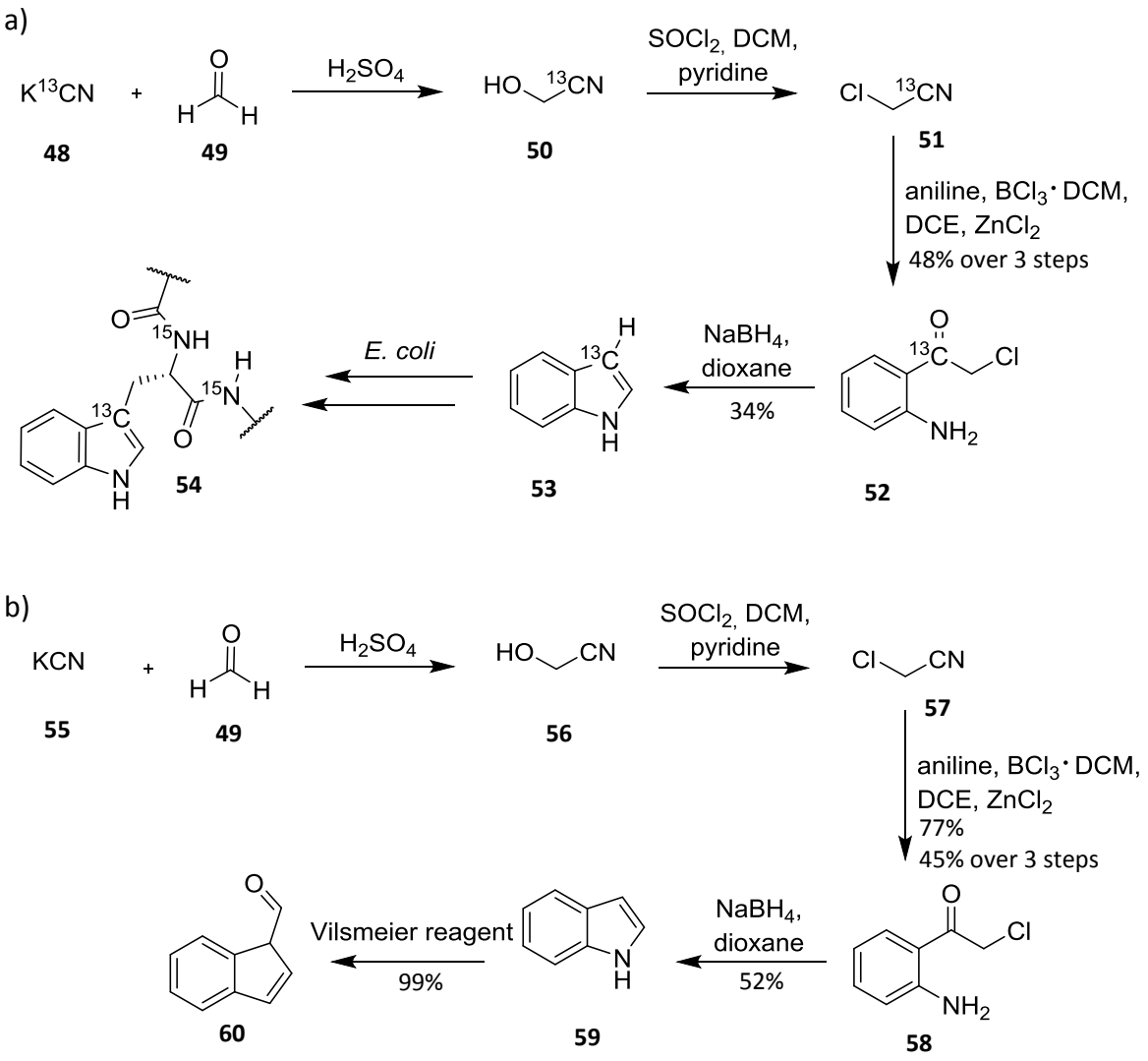


FIGURE 25: Synthesis of a) labeled indole and b) unlabeled indole.

Indole was further used as Trp-precursor for incorporation into protein GB1 in the group of Prof. Robert Konrat in the Department of Structural and Computational Biology, Max F. Perutz Laboratories, Dr. Bohr Gasse 9, 1030-Vienna (experiments performed by Tomas Sara).

Protein overexpression in *E. coli* indicated incorporation of ^{13}C into the protein. The HMQC data showed six peaks with different carbon shifts. The interpretation and further examination of these findings are currently an ongoing project (Figure 26).

Further studies about indole as tryptophan precursor are in progress as well as the synthesis of a more elaborate side-chain residue labeling with ^{13}C and deuterium for indole as well as for indole-3-pyruvate.

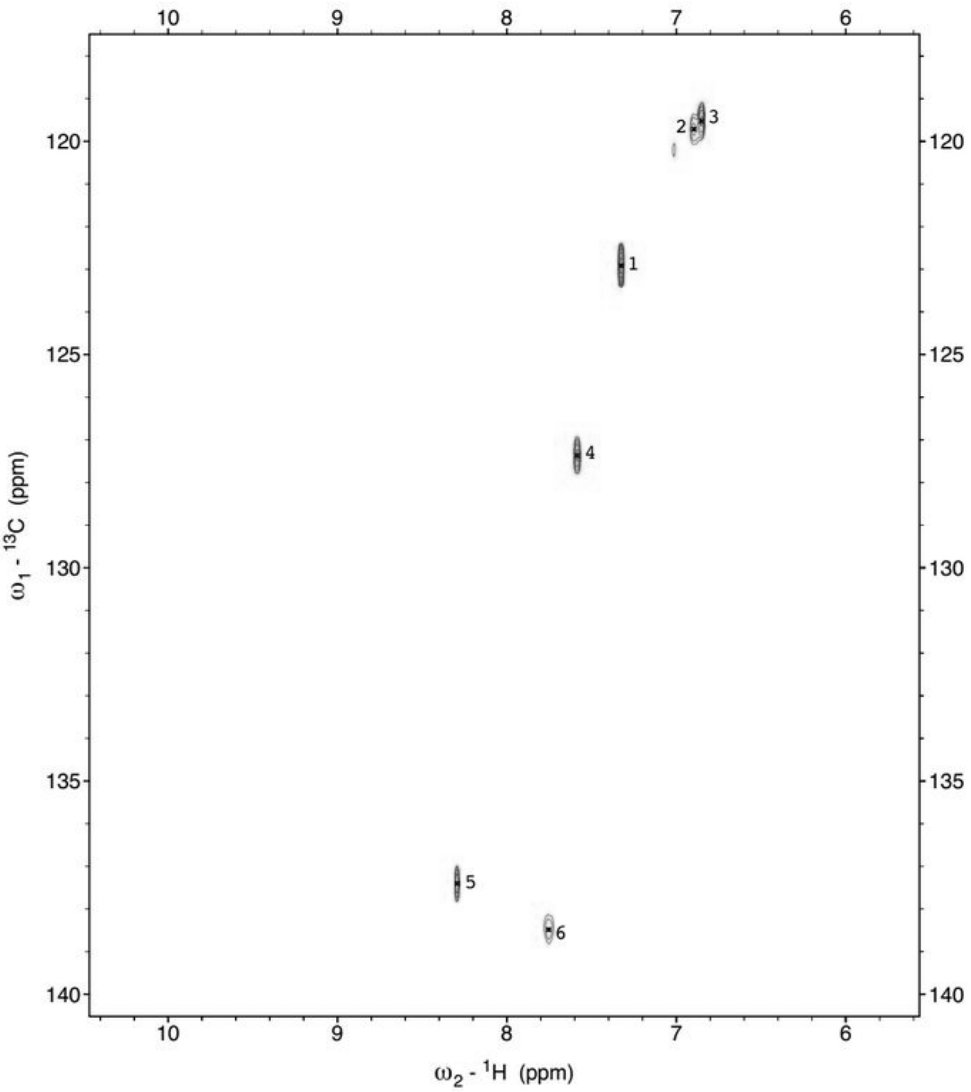


FIGURE 26: HMQC spectrum of GB1 protein supplemented with ^{13}C labeled indole.

Besides, a further synthesis of indole was carried out to estimate if the *Houben-Hoesch reaction* and the ring closure harm deuteration of aniline (Figure 27). Therefore, deuteration of anline was performed by Dr. Roman Lichtenecker in *para* and *ortho* position by treatment with D_2O in the microwave oven. Compound **63** was accomplished without any exchange of deuterium in *para* and *ortho* position of the amino group. Moreover, no loss of deuteration was observed after performing the ring closure to obtain product **64**. Thus, a more elaborate isotopic pattern was introduced into the tryptophan precursor.

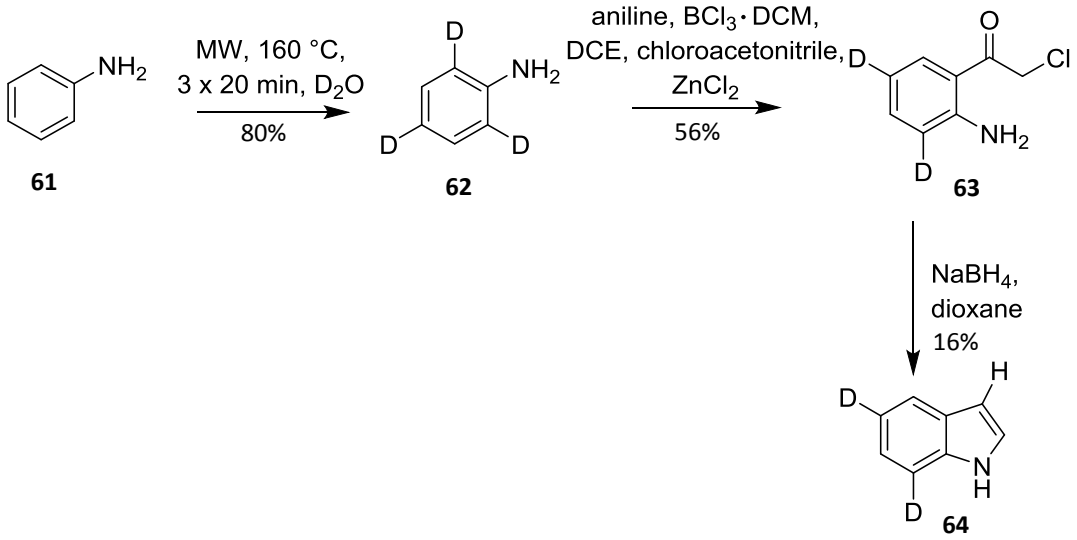


FIGURE 27: Synthesis of deuterated indole in the six-membered ring.

Figure 28 provides the synthetic approach for new labeling patterns in indole.[6] The first four reaction steps were carried out by Dr. Roman Lichtenecker.[55] ^{13}C labeling in the six-membered ring was achieved starting from acetone-2-^{13}C and nitromalon-aldehyde. *p*-Nitrophenol was reduced using an H-cube® apparatus for hydrogenation and further deuterated in *ortho* and *meta* position by using D_2O as solvent in the microwave oven. Deuteration was achieved due to two activating groups (-NH_2 and –OH), which enable hydrogen deuterium exchange in their *ortho* position. The hydroxyl group was cleaved by introducing a phenyl-tetrazolyl group in the first place, which was further cleaved using the H-cube® and Pd/C (10%) catalyst to furnish pure labeled aniline **69**. After successful synthesis of labeled aniline, reaction with chloroacetonitrile in presence of Lewis acids was performed to obtain pure compound **70**. The last reaction step comprised a ring closure and, moreover, a reduction by $NaBH_4$ giving pure compound **71**.

This new synthetic approach for a more elaborate labeling of the tryptophan precursor was successfully performed in order to incorporate it as indole or as indole-3-pyruvate into a model protein by Prof. Robert Konrat in the near future.

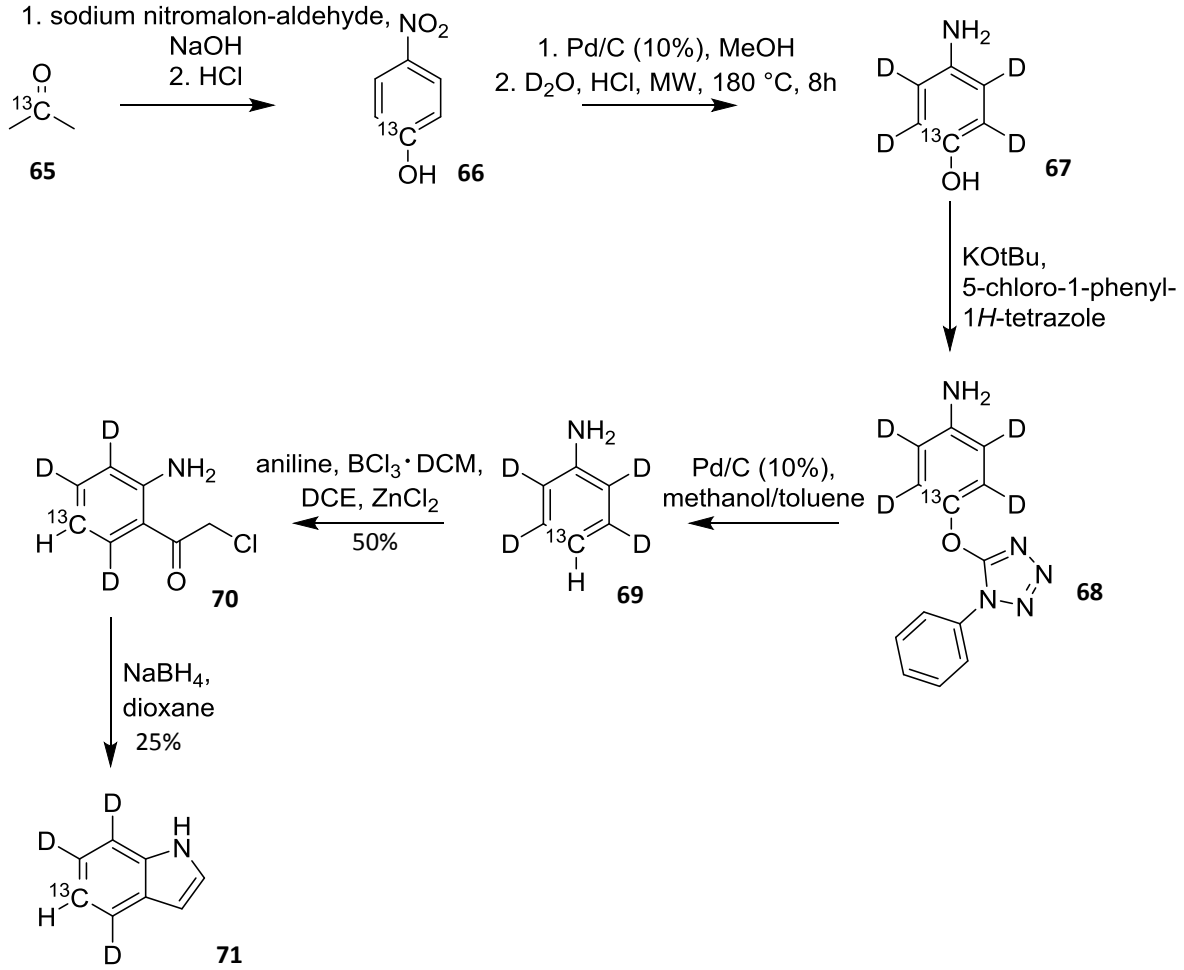


FIGURE 28: Synthesis of D and ^{13}C labeled indole precursor.

4. CONCLUSION AND OUTLOOK

4.1. CONCLUSION

At present there are two possibilities to determine the structure of proteins; on the one hand, there is the X-ray crystallography and, on the other hand, there is the NMR spectroscopy. X-ray crystallography enables the determination of the structure of static proteins, which provides high accuracy. Besides, NMR methods (NOESY, COSY, TOCSY) enable the possibility to analyze proteins in aqueous solution and, thus, the folding of proteins and their dynamic features. Large protein complexes (~100 kDa) generate signal overlapping and, thus, signal assignment is not feasible. Due to this, protein labeling is the method of choice eliminating unwanted signals, increasing the amount of structural information and, additionally, facilitating signal assignment. Therefore, small molecules with specific isotopic patterns (^{13}C, ^{2}H, ^{15}N) were synthesized, and further incorporated *via* host media (e.g. *E. coli*) into proteins in order to determine their structure *via* NMR methods. Small labeled molecules, which are further incorporated into proteins, can be e.g. metabolic precursors or amino acids.

Aim of the master thesis on hand was to develop and test a new synthetic approach for labeled enantiopure amino acids and to synthesize labeled Trp-precursors **46**, **53**, **71**.

The synthetic route for enantiopure amino acids was designed to be applicable for various amino acids (e.g. Val, Phe, Leu, Tyr, Trp and His). Starting from a cost-efficient substrate, glycine-1-^{13}C, facilitated the formation of hydantoin **8**, which enabled in a following condensation step the introduction of specific side-chains for various amino acids. This eight step sequence, starting from glycine-1-^{13}C, provided a successful synthesis of pure L-amino acids as well as *N*-acetylated D-amino acids. These labeled enantiopure amino acids are devised to be used for cell-free overexpression and, moreover, for solid-phase synthesis.

The second part of my master thesis dealt with the synthesis of Trp-precursors. First, indole-3-pyruvate was synthesized starting from glycine-1-^{13}C, which was successfully incorporated into a model protein. The second tryptophan precursor, labeled indole, showed ^{13}C labeling in the five-membered ring starting from cheap K^{13}CN. After protein overexpression in *E. coli*, we synthesized a more elaborate side-chain labeled

derivative of indole as a precursor, featuring an isolated ^{13}C-^{1}H spin system in an otherwise deuterated background of the six-membered ring. Labeled indole could further be converted into indole-3-carboxaldehyde *via Vilsmeier-Haack reaction* serving as labeled side-chain for indole-3-pyruvate as Trp-precursor.

4.2. OUTLOOK

The synthetic sequence for enantiopure amino acids was devised for six different amino acids (Val, Phe, Leu, Tyr, Trp and His). During my master thesis I was able to produce labeled enantiopure L-Val and L-Phe with acceptable yields. Moreover, L-Trp was generated in satisfying yields and will be synthesized with isotopic labeling patterns in the future. The synthesis of enantiopure leucine and tyrosine both included one challenging reaction step, respectively. First, the condensation reaction of the hydantoin with the aldehyde to form the side-chain of the amino acid leucine and, second, the acetylation of tyrosine have to be improved before introducing isotopes. Furthermore, the objective to synthesize enantiopure L-histidine must be achieved. Thereby, the problem of the ring opening of the hydantoin must be solved. As this reaction has always been performed at basic reaction conditions using NaOH (15%), the influence of different pH values leaves room for further investigations to perform this reaction successfully. Of course, there are still other options like using different bases, but in my opinion the employment of less basic conditions would be most promising.

If all reaction conditions and yields are optimized, other isotopes can be introduced into the amino acids and, of course, more elaborate side-chain labeling can be achieved. On the one hand, ^{15}N can be introduced starting from labeled glycine-^{15}N and, on the other hand, various isotopic patterns in aldehydes and in acetone can be used for labeled side-chains. Figure 29 illustrates that acetone-1,3-^{13}C, which can be purchased by Sigma Aldrich, is used as substrate to achieve side-chain labeling of valine. Precursors of labeled 4-hydroxybenzaldehyde, benzaldehyde and isobutyraldehyde have already been synthesized in our laboratory and could be used for more elaborate side-chain labeling of tyrosine, phenylalanine and leucine. Selective side-chain labeling of tryptophan at different positions is in progress.

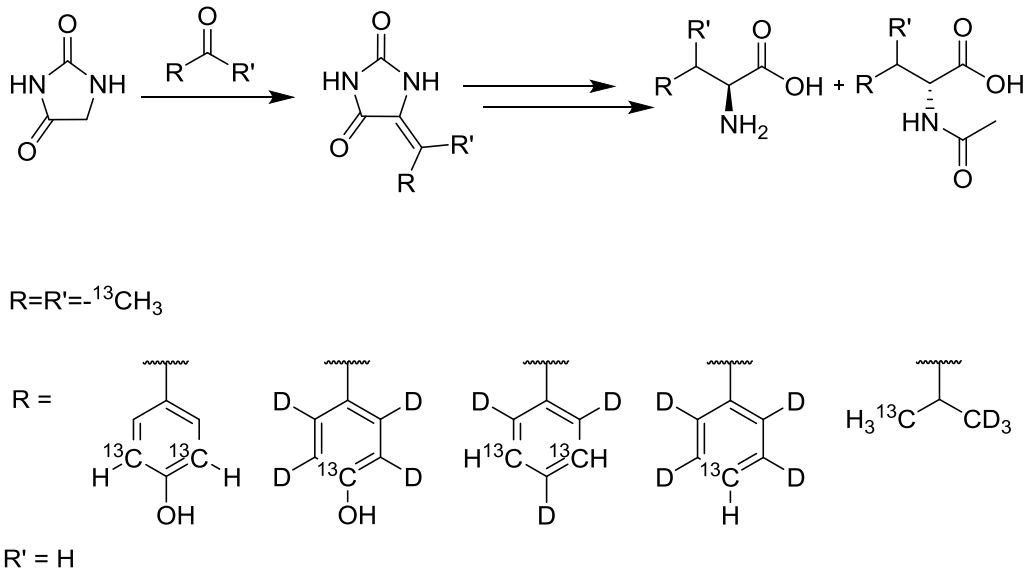


FIGURE 29: Novel labeling strategies for Val, Tyr, Phe and Leu.

^{13}C labeled indole-3-pyruvate was synthesized as a tryptophan precursor and successfully incorporated into a model protein. Moreover, isotopic patterns in the side-chain of tryptophan are realized by the synthesis of selectively labeled indole. Thereby, indole-3-carboxaldehyde is achieved *via* a *Vilsmeier-Haack reaction* performed on indole. This labeled aldehyde can be attached to the hydantoin and a subsequent basic ring opening results in the formation of a new labeled indole-3-pyruvate. Therefore, several selective labeling methods are in progress to have, on the one hand, indole- and, on the other hand, indole-3-pyruvate as tryptophan precursor. These novel labeling strategies for tryptophan precursors will further be tested for incorporation in various model proteins.

5. Experimental Section

5.1. General Comments

All reactions were stirred magnetically, oxygen- and moisture sensitive reactions were carried out under an argon atmosphere and yields refer to pure compounds unless stated otherwise. Used solvents and chemicals were purified and dried according to common procedures. Dry solvents were stored over 4 Ångström molecular sieves under argon atmosphere and deionized water was purified *via* Milli-Q* Reference A+ Systems.

Acylase I from porcine kidney [EC 3.5.1.14] was purchased from Sigma-Aldrich with an activity of 541 units / mg (CAS: 9012-37-7) as lyophilized powder.

The reactions were monitored *via* thin layer chromatography (TLC) on silica gel 60 with fluorescent indicator UV_{254} by MACHEREY-NAGEL GmbH & Co. KG. Detection was carried out using an ultraviolet lamp (254 nm) and by application of specific reagents: Mo-$Ce(SO_4)_2$ complex solution (48 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ and 2 g $Ce(SO_4)_2$ in 1 L 10% H_2SO_4), bromocresol purple (BCP) or ninhydrine solution (3 g ninhydrine in 100 mL butanol and 3 mL of acetic acid) with subsequent branding by hot-gun. Flash column chromatography was performed on silica gel 60 (0.040-0.063 mm) from Merck and filtration was carried out using Celite® S from Sigma-Aldrich. Ion-exchange chromatography was performed using DOWEX® 50W x 4, 200-400 mesh.

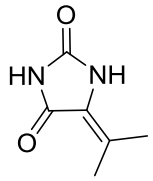
Hydrogenation was carried out *via* ThalesNano H-Cube® and ThalesNano CatCart®-cartridges and optical rotatory power was measured *via* Perkin Elmer Polarimeter 341. Deuteration and racemization were performed using initiator microwave EU from Biotage®. Freeze-drying was done with liquid nitrogen and subsequent lyophilization with freeze dryer alpha 1-4 LD_{plus} from CHRIST®.

^{1}H-NMR and ^{13}C-NMR spectroscopic data were recorded on a Bruker AVANCE-DRX 400 or DRX 600 MHz spectrometer. NMR spectra were measured in different solvents and analyzed with specific values: 2.50 ppm (6d-DMSO), 4.79 ppm (D_2O), 7.26 ppm ($CDCl_3$). Chemical shifts (δ) are given in ppm (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, q = quartet, m = multiplet, b = broadened signal) and reported relative to the residual solvent peaks. Coupling constants (J) are given in Hertz (Hz).

All mass spectra were carried out on a Finnigan MAT 900 mass spectrometer *via* electron impact ionization.

5.2. Synthetic Protocols of Enantiopure Amino Acids

5-(Propane-2-ylidene)hydantoin (2)



Molecular weight:	140.14 g/mol
Formula:	$C_6H_8N_2O_2$
Appearance:	white solid
Yield:	88%

To a stirred solution of hydantoin (500 mg, 5.0 mmol) in 3 mL water at 70 °C was added ethanolamine (0.3 mL, 5.0 mmol) and acetone (2.2 mL, 29.9 mmol). The reaction mixture was stirred at 70 °C overnight and then cooled to room temperature. The mixture was treated with 1 N HCl until pH = 3 and the white precipitate was separated from the mixture *via* filtration giving pure **2** as a white solid (616 mg, 4.4 mmol, 88%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

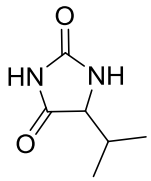
δ 1.80 (s, 3H, C**H$_3$**), 2.12 (s, 3H, C**H$_3$**), 9.75 (s, 1H, CON**H**CO), 10.79 (s, 1H, CON**H**C).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 18.4 (**C**H$_3$), 21.1 (**C**H$_3$), 124.3 (**C**CH$_3$), 125.4 (NH**C**CO), 154.4 (NH**C**ONH), 165.0 (NH**C**OC).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_6H_8N_2NaO_2$ 163.0484; found 163.0481.

5-Isopropylhydantoin (3)



Molecular weight:	142.16 g/mol
Formula:	$C_6H_{10}N_2O_2$
Appearance:	white solid
Yield:	99%

Compound **2** (400 mg, 2.9 mmol) was dissolved in 75 mL methanol and hydrogenated using the H-cube® with a Pd/C catalyst (10%) cartridge. The following reaction conditions were set: full H_2 and a flow rate of 1 mL/min. The hydrogenation cycle was repeated until NMR spectra showed full conversion of the starting material. Methanol was evaporated under reduced pressure affording pure **3** as a white solid (402 mg, 2.8 mmol, 99%).

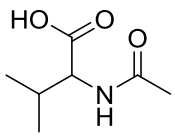
Alternatively, catalyst 10% Pd/C (75 mg, 5 mol%) was evacuated in a round bottom flask and exposed to H_2. 5-(propan-2-ylidene)hydantoin (200 mg, 1.4 mmol) was dissolved in 40 mL methanol and added to the catalyst under hydrogen atmosphere. The reaction mixture was stirred overnight at room temperature. The palladium catalyst was filtered off and the solvent was removed *in vacuo* giving pure **3** as a white solid (195 mg, 1.4 mmol, 96%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 0.78 (d, ^3J = 6.81 Hz, 3H, C**H$_3$**), 0.92 (d, ^3J = 6.97 Hz, 3H, C**H$_3$**), 1.93-2.01 (m, 1H, C**H**CH$_3$), 3.87 (dd, ^3J = 1.18, 3.58 Hz, 1H, NHC**H**), 7.87 (s, 1H, CON**H**CO), 10.44 (bs, 1H, CON**H**CH).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 16.3 (**C**H$_3$), 19.0 (**C**H$_3$), 30.0 (**C**HCH$_3$), 63.2 (NH**C**H), 158.4 (NH**C**ONH), 176.0 (NH**C**OCH).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_6H_{10}N_2NaO_2$ 165.0640; found 165.0635.

_N_-Acetylvaline (4)



Molecular weight:	159.19 g/mol
Formula:	$C_7H_{13}NO_3$
Appearance:	white solid
Yield:	51%

Compound **3** (157 mg, 1.1 mmol) in 5 mL NaOH (15%) was stirred for five hours at 130 °C. The reaction mixture was cooled and the pH was adjusted to 2 with 6 N HCl resulting in precipitation of a white solid. The solution was neutralized by addition of 1 N NaOH and then Ac_2O (270 µL, 2.9 mmol) and NaOH (20%) (270 µL, 8.2 mmol) were added simultaneously. The addition of Ac_2O (270 µL, 2.9 mmol) and NaOH (20%) (270 µL, 8.2 mmol) was repeated after half an hour and the reaction mixture was stirred overnight. Subsequently, the pH was adjusted to 1 with 1 N HCl and the reaction mixture was diluted with EtOAc. The aqueous layer was extracted with EtOAc (4 x 20 mL), the combined organic extracts were dried over $MgSO_4$, filtered and evaporated *in vacuo* to dryness. The oily residue was dissolved in EtOAc and PE was added slowly until a white precipitate was observed. Further precipitation was performed at 0 °C and the precipitate was filtered off obtaining pure **4** as a white solid (90 mg, 0.6 mmol, 51%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 0.86 (d, ^3J = 6.80 Hz, 3H, CHC**H$_3$**), 0.86 (d, ^3J = 6.87 Hz, 3H, CHC**H$_3$**), 1.85 (s, 3H, COC**H$_3$**), 1.96-2.05 (m, 1H, C**H**CH$_3$), 4.11 (dd, ^3J = 5.80, 8.51 Hz, 1H, NHC**H**), 7.94 (d, ^3J = 8.64 Hz, 1H, N**H**), 12.51 (bs, 1H, COO**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 18.5 (CH**C**H$_3$), 19.6 (CH**C**H$_3$), 22.8 (CO**C**H$_3$), 30.2 (**C**HCH$_3$), 57.7(NH**C**H), 169.9 (NH**C**O), 173.6 (**C**OOH).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_7H_{13}NNaO_3$ 182.0793; found 182.0780.

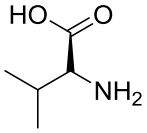
<u>Racemization:</u>

N-Acetyl-D-valine **6** (100 mg, 0.6 mmol) was dissolved in 440 µL xylene and Ac_2O (8 µL, 0.1 mmol) was added. The mixture was heated to 130 °C in the microwave oven for two hours. The reaction was quenched with water in order to hydrolyze Ac_2O. The solvents were evaporated under reduced pressure and the oily residue was dissolved in

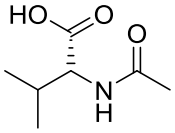
EtOAc. PE was added slowly until formation of a white precipitate, which was further precipitated at 0 °C. The solid was filtered off giving pure racemic compound **4** as white solid (59 mg, 0.4 mmol, 59%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 0.84 (d, ^{3}J = 6.83 Hz, 6H, CHC**H$_3$**), 1.85 (s, 3H, COC**H$_3$**), 1.94-2.04 (m, 1H, C**H**CH$_3$), 4.09 (dd, ^{3}J = 5.71, 8.50 Hz, 1H, NHC**H**), 7.94 (d, ^{3}J = 8.44 Hz, 1H, N**H**).

L-Valine (5) and *N*-acetyl-D-valine (6)



Molecular weight:	117.15 g/mol
Formula:	$C_5H_{11}NO_2$
Appearance:	white solid
Yield:	34%



Molecular weight:	159.19 g/mol
Formula:	$C_7H_{13}NO_3$
Appearance:	white solid
Yield:	45%

Racemic *N*-acetylvaline **4** (100 mg, 0.6 mmol) was dissolved in 10 mL water and the pH was adjusted to 7.8 by addition of 0.5 M KOH. Acylase I (3.2 mg) was added to the mixture and then it was stirred for 18.5 hours at room temperature. The mixture was loaded onto an ion-exchange resin (equilibrated with 1 N HCl and brought to neutral with water). *N*-Acetyl-D-valine was eluted with water and L-valine with 1 N NH_3. The fractions of the corresponding product were combined and concentrated under reduced pressure. The oily residue of compound **6** was dissolved in EtOAc and slowly treated with PE, forming a white precipitate. This solid was further precipitated at 0 °C and filtered off to give pure **6** as white solid (45 mg, 0.3 mmol, 45**%**). L-Valine **5** was afforded as a white solid (25 mg, 0.2 mmol, 34%).

^{1}H-NMR (400.13 MHz; D_2O, NaOD) δ [ppm] of compound **5**:
δ 0.89 (d, ^{3}J = 6.87 Hz, 3H, C**H$_3$**), 0.97 (d, ^{3}J = 6.92 Hz, 3H, C**H$_3$**), 1.90-1.98 (m, 1H, C**H**CH$_3$), 3.07 (d, ^{3}J = 5.34 Hz, 1H, NH_2C**H**).

^{13}C-NMR (150.90 MHz; D_2O, NaOD) δ [ppm] of compound **5**:
δ 16.6 (**C**H$_3$), 19.0 (**C**H$_3$), 31.7 (**C**HCH$_3$), 61.8 (NH_2**C**H).

HRMS (ESI$^+$) of compound **5** *m/z* = [M + H]$^+$ calculated for $C_5H_{12}NO_2$ 118.0869; found 118.0861.

$\boldsymbol{\alpha}\ \boldsymbol{\frac{20}{D}}$ = -6 (0.15 g/100mL, EtOH) of compound **5**.

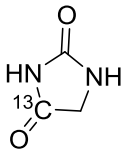
^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm] of compound **6**:
δ 0.85 (d, ^3J = 6.79 Hz, 3H, CHC**H$_3$**), 0.86 (d, ^3J = 6.89 Hz, 3H, CHC**H$_3$**), 1.86 (s, 3H, COC**H$_3$**), 1.96-2.07 (m, 1H, C**H**CH$_3$), 4.10 (dd, ^3J = 5.90, 8.56 Hz, 1H, NHC**H**), 7.94 (d, ^3J = 8.57 Hz, 1H, N**H**), 12.49 (bs, 1H, COO**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm] of compound **6**:
δ 18.5 (CH**C**H$_3$), 19.6 (CH**C**H$_3$), 22.8 (CO**C**H$_3$), 30.3 (**C**HCH$_3$), 57.8 (NH**C**H), 173.6 (NH**C**O), 174.6 (**C**OOH).

HRMS (ESI$^+$) of compound **6** *m/z* = [M + Na]$^+$ calculated for $C_7H_{13}NNaO_3$ 182.0793; found 182.0782.

$\boldsymbol{\alpha}\ \boldsymbol{\frac{20}{D}}$ = -5.0 (0.55 g/100mL, EtOH) of compound **6**.

[1-^{13}C] Hydantoin (8)



Molecular weight:	101.07 g/mol
Formula:	$C_2{}^{13}CH_4N_2O_2$
Appearance:	white solid
Yield:	99%

A mixture of glycine-1-^{13}C (500 mg, 6.6 mmol) and KOCN (800 mg, 25.7 mmol) in 4 mL water was heated for three hours at 100 °C. The solution was then stirred at 0 °C, 1.6 mL conc. HCl were slowly added and the mixture was again heated to 120 °C for four hours. The solvent was evaporated under reduced pressure until a white solid precipitated. Further precipitation was achieved at 0 °C; the precipitate was filtered off obtaining pure hydantoin **8** as a white solid (658 mg, 6.5 mmol, 99%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

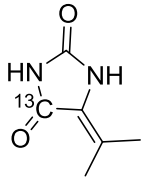
δ 3.83 (dd, $^2J_{C,H}$ = 5.90 Hz, ^3J = 1.04 Hz, 1H, C**H$_2$**), 7.68 (d, $^2J_{C,H}$ = 6.13 Hz, 1H, CON**H**CO), 10.59 (s, 1H, CON**H**CH$_2$).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 47.4 (**C**H$_2$), 158.7 (NH**C**ONH), 174.3 (NH**C**OCH$_2$).

HRMS (ESI$^+$) *m/z* = [M + H]$^+$ calculated for $C_2{}^{13}CH_5N_2O_2$ 102.0385; found 102.0381.

[1-^{13}C] 5-(Propane-2-ylidene)hydantoin (9)



Molecular weight:	141.13 g/mol
Formula:	$C_5{}^{13}CH_8N_2O_2$
Appearance:	white solid
Yield:	72%

Labeled hydantoin **8** (300 mg, 3.0 mmol) was dissolved in 2 mL water, heated to 70 °C and the solution was then treated with ethanolamine (200 µL, 3.3 mmol) and acetone (1.3 mL, 17.7 mmol). The reaction mixture was stirred overnight at 70 °C and was then cooled. The solution was brought to pH = 3 by addition of 1 N HCl resulting in precipitation of a white solid, which was separated by filtration, giving pure **9** as a white solid (302 mg, 2.1 mmol, 72%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

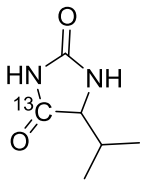
δ 1.77 (s, 3H, C**H$_3$**), 2.10 (s, 3H, C**H$_3$**), 9.66 (d, $^{2}J_{C,H}$ = 7.07 Hz, 1H, N**H**CO), 10.74 (s, 1H, N**H**C).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 21.1 (2 x **C**H$_3$), 124.4 (**C**CH$_3$), 154.4 (NH**C**ONH), 165.0 (NH**C**OC).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_5{}^{13}CH_8N_2NaO_2$ 164.0517; found 164.0508.

[1-^{13}C] 5-Isopropylhydantoin (10)



Molecular weight:	143.15 g/mol
Formula:	$C_5{}^{13}CH_{10}N_2O_2$
Appearance:	beige crystalls
Yield:	98%

Compound **9** (185 mg, 1.3 mmol) was dissolved in 40 mL methanol and hydrogenated *via* H-cube® using Pd/C catalyst (10%) cartridge. The reaction conditions included: full H_2 and a flow rate of 1.0 mL/min. The solvent was reduced *in vacuo* giving pure **10** as a beige solid (184 mg, 1.3 mmol, 98%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

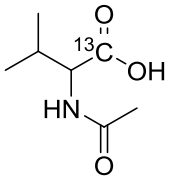
δ 0.78 (d, ^3J = 6.62 Hz, 3H, C**H$_3$**), 0.92 (d, ^3J = 7.02 Hz, 3H, C**H$_3$**), 1.92-2.01 (m, 1H, C**H**CH$_3$), 3.86-3.89 (m, 1H, NHC**H**), 7.83 (d, $^2J_{C,H}$ = 7.52 Hz, 1H, CON**H**CO), 10.54 (s, 1H, CON**H**CH).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 19.0 (2 x **C**H$_3$), 30.0 (**C**HCH$_3$), 63.4 (NH**C**H), 158.2 (NH**C**ONH), 175.9 (NH**C**OCH).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_5{}^{13}CH_{10}N_2NaO_2$ 166.0674; found 166.0666.

[1-^{13}C] *N*-Acetylvaline (11)



Molecular weight:	160.18 g/mol
Formula:	$C_6{}^{13}CH_{13}NO_3$
Appearance:	white solid
Yield:	77%

Hydantoin **10** (160 mg, 1.1 mmol) was treated with 5 mL NaOH (15%) and heated to 130 °C. After stirring for five hours at this temperature, the mixture was cooled to room temperature and the pH was adjusted to 2 with 6 N HCl. The reaction mixture was neutralized by addition of 1 N NaOH and then Ac_2O (270 µL, 2.9 mmol) and NaOH (20%) (270 µL, 8.2 mmol) were added simultaneously. The addition of Ac_2O (270 µL, 2.9 mmol) and NaOH (20%) (270 µL, 8.2 mmol) was repeated after half an hour and the reaction mixture was stirred overnight at room temperature. The pH was adjusted to 1 by addition of 1 N HCl and the reaction mixture was diluted with EtOAc. The aqueous phase was extracted with EtOAc (4 x 20 mL); the combined organic extracts were dried over $MgSO_4$, filtered and evaporated under reduced pressure. The obtained oily residue was dissolved in EtOAc and PE was added slowly until precipitation was observed. This solid was further precipitated at 0 °C and separated by filtration obtaining pure **11** as a white solid (138 mg, 0.9 mmol, 77%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

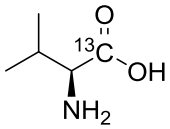
δ 0.87 (d, ^3J = 6.82 Hz, 6H, CHC**H$_3$**), 1.86 (s, 3H, COC**H$_3$**), 1.96-2.06 (m, 1H, C**H**CH$_3$), 4.09-4.15 (m, 1H, NHC**H**), 7.95 (d, ^3J = 8.49 Hz, 1H, N**H**), 12.48 (s, 1H, COO**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

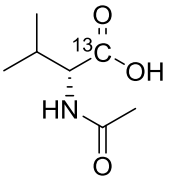
δ 18.5 (CH**C**H$_3$), 19.6 (CH**C**H$_3$), 22.8 (CO**C**H$_3$), 30.2 (**C**HCH$_3$), 170.0 (NH**C**O), 173.6 (**C**OOH).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_6{}^{13}CH_{13}NNaO_3$ 183.0827; found 183.0822.

L-[1-^{13}C] Valine (12) and D-[1-^{13}C] *N*-acetylvaline (13)



Molecular weight:	118.14 g/mol
Formula:	$C_4{}^{13}CH_{11}NO_2$
Appearance:	white solid
Yield:	48%



Molecular weight:	160.18 g/mol
Formula:	$C_6{}^{13}CH_{13}NO_3$
Appearance:	white solid
Yield:	50%

The solution of compound **11** (150 mg, 0.9 mmol) in 15 mL water was adjusted to pH = 7.8 by addition of 0.5 M KOH. The mixture was treated with acylase I (4.8 mg) and stirred for 18.5 hours at room temperature. The solution was applied to ion-exchange chromatography column (equilibrated with 1 N HCl and brought to neutral with water). D-[1-^{13}C] *N*-Acetylvaline was eluted with water and L-[1-^{13}C] valine with 1 N NH_3. The fractions with the corresponding product were combined and evaporated under reduced pressure. The oily compound **13** was dissolved in EtOAc and PE was added slowly, resulting in a white precipitate. This solid was further precipitated at 0 °C and separated *via* filtration giving pure **13** as a white solid (75 mg, 0.5 mmol, 50**%**). The fractions containing L-[1-^{13}C] valine were combined and evaporated under reduced pressure affording **12** as pure white solid (53 mg, 0.4 mmol, 48%).

^{1}H-NMR (400.13 MHz; D_2O, NaOD) δ [ppm] of compound **12**:
δ 0.87 (d, ^3J = 6.80 Hz, 3H, C**H$_3$**), 0.94 (d, ^3J = 6.80 Hz, 3H, C**H$_3$**), 1.88-1.96 (m, 1H, C**H**CH_3), 3.03-3.05 (m, 1H, NH_2C**H**).

^{13}C-NMR (100.61 MHz; D_2O, NaOD) δ [ppm] of compound **12**:
δ 16.7 (**C**H_3), 19.0 (**C**H_3), 31.8 (**C**HCH_3), 61.6 (NH_2**C**H), 183.1 (**C**OOH).

HRMS (ESI$^+$) of compound **12** *m/z* = $[M + H]^+$ calculated for $C_4{}^{13}CH_{12}NO_2$ 119.0902; found 119.0895.

$\boldsymbol{\alpha}_{\boldsymbol{D}}^{\boldsymbol{20}}$ = -12.3 (0.41 g/100mL, EtOH) of compound **12**.

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm] of compound **13**:
δ 0.86-0.88 (m, 6H, CHC**H$_3$**), 1.86 (s, 3H, COC**H$_3$**), 1.96-2.05 (m, 1H, C**H**CH_3), 4.09-4.14 (m, 1H, NHC**H**), 7.95 (d, ^3J = 8.33 Hz, 1H, N**H**CH), 12.49 (s, 1H, COO**H**).

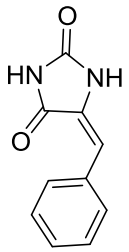
^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm] of compound **13**:

δ 18.1 (CH**C**H_3), 19.6 (CH**C**H_3), 22.8 (CO**C**H_3), 30.8 (**C**HCH$_3$), 174.6 (**C**OOH).

HRMS (ESI$^+$) of compound **13** *m/z* = [M + Na]$^+$ calculated for $C_6{}^{13}CH_{13}NNaO_3$ 183.0827; found 183.0820.

$\boldsymbol{\alpha}\ \boldsymbol{\frac{20}{D}}$ = -2.4 (0.94 g/100mL, EtOH) of compound **13**.

5-(Benzylidene)hydantoin (14)



Molecular weight:	188.19 g/mol
Formula:	$C_{10}H_8N_2O_2$
Appearance:	yellow solid
Yield:	75%

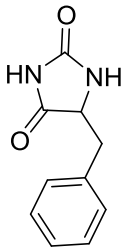
Hydantoin **1** (300 mg, 3.0 mmol) was treated with anhydrous NH_4OAc (231 mg, 3.0 mmol), benzaldehyde (300 µL, 3.0 mmol) and glacial acetic acid (700 µL, 12.2 mmol). The reaction mixture was connected to a drying tube filled with $CaCl_2$ and stirred overnight at 120 °C. After cooling the mixture to room temperature, cold water was added. The resulting yellow precipitate was filtered off and washed with cold water giving pure **14** as yellow solid (421 mg, 2.2 mmol, 75%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 6.39 (s, 1H, C**H**), 7.31 (t, ^3J = 7.40 Hz, 1H, *p*-C**H**$_{\text{arom.}}$), 7.38 (t, ^3J = 7.43 Hz, 2H, *m*- C**H**$_{\text{arom.}}$), 7.59 (d, ^3J = 7.39 Hz, 2H, *o*-C**H**$_{\text{arom.}}$), 10.51, 11.24 (2 x bs, 2 x 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 108.7 (**C**H), 128.5 (**C**CH), 128.8 (*p*-**C**$_{\text{arom.}}$H), 129.2 (*o*-**C**$_{\text{arom.}}$H), 129.8 (*m*-**C**$_{\text{arom.}}$H), 133.4 (**C**$_{\text{q arom.}}$), 156.2 (NH**C**ONH), 166.0 (NH**C**OC).

HRMS (ESI$^+$) *m/z* = $[M + Na]^+$ calculated for $C_{10}H_8N_2NaO_2$ 211.0484; found 211.0482.

5-Benzylhydantoin (15)



Molecular weight:	190.20 g/mol
Formula:	$C_{10}H_{10}N_2O_2$
Appearance:	white solid
Yield:	92%

A solution of compound **14** (300 mg, 1.6 mmol) in 50 mL methanol was hydrogenated *via* the H-cube® using Pd/C catalyst (10%) cartridge under following reaction conditions: full H_2 and a flow rate of 0.5 mL/min. The hydrogenation cycle was repeated until full conversion of the starting material was accomplished. The solvent was removed under reduced pressure giving pure **15** as a white solid (279 mg, 1.5 mmol, 92%).

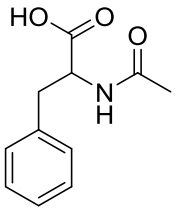
Alternatively, 10% Pd/C catalyst (57 mg, 5 mol%) was evacuated in a round bottom flask and purged with H_2. Compound **14** (200 mg, 1.1 mmol) was dissolved in 30 mL methanol and added to the catalyst under hydrogen atmosphere. The reaction mixture was stirred overnight at room temperature. The palladium catalyst was filtered off and methanol was removed *in vacuo* affording pure **3** as white solid (163 mg, 0.9 mmol, 81%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 2.92 (t, ^3J = 4.94 Hz, 2H, C**H$_2$**), 4.30-4.33 (m, 1H, C**H**), 7.16-7.30 (m, 5H, C**H$_{arom.}$**), 7.88 (s, 1H, CON**H**CO), 10.39 (s, 1H, CON**H**CH).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 36.9 (**C**H$_2$), 58.9 (**C**H), 127.1 (*p*-**C$_{arom.}$**H), 128.5 (*o*-**C$_{arom.}$**H), 130.2 (*m*-**C$_{arom.}$**H), 136.1 (**C$_{q\ arom.}$**), 157.6 (NH**C**ONH), 175.7 (NH**C**OCH).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_{10}H_{10}N_2NaO_2$ 213.0640; found 213.0637.

_N_-Acetylphenylalanine (16)



Molecular weight:	207.23 g/mol
Formula:	$C_{11}H_{13}NO_3$
Appearance:	white solid
Yield:	63%

Substrate **15** (260 mg, 1.4 mmol) in 8 mL NaOH (15%) was stirred for five hours at 130 °C. The mixture was cooled to room temperature and the pH was adjusted to 2 with 6 N HCl resulting in the formation of a white precipitate. The solution was neutralized by treatment with 1 N NaOH and then Ac_2O (320 µL, 3.4 mmol) and NaOH (20%) (320 µL, 9.8 mmol) were added simultaneously. The addition of Ac_2O (320 µL, 3.4 mmol) and NaOH (20%) (320 µL, 9.8 mmol) was repeated after half an hour and the reaction mixture was stirred overnight at room temperature. After acidification (pH = 1) with 1 N HCl, the reaction mixture was diluted with EtOAc. The aqueous phase was extracted with EtOAc (4 x 20 mL); the combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The oily residue was dissolved in EtOAc and PE was added slowly resulting in precipitation to give a white solid. The solid was further precipitated at 0 °C and then filtered off giving pure **16** as a white solid (178 mg, 0.8 mmol, 63%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 1.76 (s, 3H, C**H$_3$**), 2.83 (dd, ^2J = 13.79 Hz, ^3J = 9.55 Hz, 1H, CH**H**), 3.04 (dd, ^2J = 13.81 Hz, ^3J = 4.97 Hz, 1H, C**H**H), 4.37-4.43 (m, 1H, C**H**), 7.18-7.29 (m, 5H, $C_{arom.}$**H**), 8.14 (d, ^3J = 8.18 Hz, 1H, N**H**CO), 12.57 (bs, 1H, COO**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 22.8 (**C**H$_3$), 37.3 (**C**H$_2$), 53.9 (**C**H), 126.9 (*p*-**C$_{arom.}$**H), 128.6 (*o*-**C$_{arom.}$**H), 129.5 (*m*-**C$_{arom.}$**H), 138.2 (**C$_{q\ arom.}$**), 169.7 (NH**C**O), 173.6 (**C**OOH).

HRMS (ESI$^+$) *m/z* = [M]$^+$ calculated for $C_{11}H_{13}NNaO_3$ 230.0793; found 230.0788.

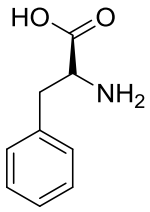
<u>Racemization:</u>

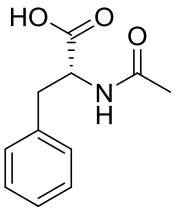
N-acetyl-D-phenylalanine **18** (100 mg, 0.5 mmol) was dissolved in 320 µL xylene and Ac_2O (5 µL, 0.1 mmol) was added. The reaction mixture was heated to 130 °C in the microwave oven for two hours. The mixture was quenched with water in order to

hydrolyze Ac_2O. The solvents were concentrated under reduced pressure; the oily residue was dissolved in EtOAc and PE was added slowly until yellow precipitate was formed. The residue was further precipitated at 0 °C and filtered off giving pure racemic compound **16** as yellow solid (95 mg, 0.5 mmol, 95%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 1.76 (s, 3H, C**H$_3$**), 2.81 (dd, ^2J = 13.74 Hz, ^3J = 9.57 Hz, 1H, CH**H**), 3.02 (dd, ^2J = 13.68 Hz, ^3J = 4.94 Hz, 1H, C**H**H), 4.35-4.41 (m, 1H, C**H**), 7.18-7.28 (m, 5H, C**H$_{arom.}$**), 8.13 (d, ^3J = 8.09 Hz, 1H, N**H**CO), 12.59 (bs, 1H, COO**H**).

L-Phenylalanine (17) and *N*-acetyl-D-phenylalanine (18)





Molecular weight:	165.19 g/mol	Molecular weight:	207.23 g/mol
Formula:	$C_9H_{11}NO_2$	Formula:	$C_{11}H_{13}NO_3$
Appearance:	white solid	Appearance:	white solid
Yield:	29%	Yield:	48%

Compound **11** (200 mg, 1.0 mmol) was dissolved in 17 mL water and the pH was adjusted to 7.8 with 0.5 M KOH. Acylase I (5.2 mg) was added and the reaction mixture was stirred for 39 hours at room temperature. The mixture was loaded onto an ion-exchange resin (equilibrated with 1 N HCl and then neutralized with water). *N*-Acetyl-D-phenylalanine was eluted with water; the corresponding fractions were combined and evaporated under reduced pressure. The oily compound **18** was dissolved in EtOAc and treated with PE until a white precipitate was formed. This solid was further precipitated at 0 °C and separated *via* filtration giving pure **18** as a white solid (96 mg, 0.5 mmol, 48**%**). L-Phenylalanine was eluted with 1 N NH_3; the fractions containing the corresponding product were combined and the solvent was concentrated under reduced pressure giving pure L-phenylalanine **17** as a white solid (46 mg, 0.3 mmol, 29%).

^{1}H-NMR (400.13 MHz; D_2O, NaOD) δ [ppm] of compound **17**:

δ 2.87 (dd, ^{2}J = 13.46 Hz, ^{3}J = 7.21 Hz, 1H, CH**H**), 3.01 (dd, ^{2}J = 13.46 Hz, ^{3}J = 5.76 Hz, 1H, C**H**H), 3.53 (dd, ^{3}J = 5.71, 7.11 Hz, 1H, C**H**), 7.29-7.42 (m, 5H, C$\mathbf{H}_{\mathbf{arom.}}$).

^{13}C-NMR (100.61 MHz; D_2O, NaOD) δ [ppm] of compound **17**:

δ 36.3 (**C**H_2), 127.8 (*p*-$\mathbf{C}_{\mathbf{arom.}}$H), 129.2 (*o*-$\mathbf{C}_{\mathbf{arom.}}$H), 129.4 (*m*-$\mathbf{C}_{\mathbf{arom.}}$H).

HRMS (ESI$^+$) of compound **17** *m/z* = $[M + Na]^+$ calculated for $C_9H_{11}NNaO_2$ 188.0688; found 188.0679.

$\boldsymbol{\alpha}_{\boldsymbol{D}}^{\mathbf{20}}$ = -20.0 (0.10 g/100mL, water) of compound **17**.

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm] of compound **18**:

δ 1.77 (s, 3H, C$\mathbf{H_3}$), 2.83 (dd, ^{2}J = 13.88 Hz, ^{3}J = 9.52 Hz, 1H, CH**H**), 3.04 (dd, ^{2}J = 13.88 Hz, ^{3}J = 5.00 Hz, 1H, C**H**H), 4.37-4.42 (m, 1H, C**H**), 7.20-7.29 (m, 5H, C$\mathbf{H}_{\mathbf{arom.}}$), 8.15 (d, ^{3}J = 8.15 Hz, 1H, N**H**CO), 12.63 (bs, 1H, COO**H**).

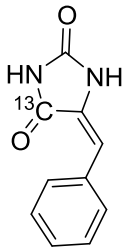
^{13}C-NMR (150.90 MHz; 6d-DMSO) δ [ppm] of compound **18**:

δ 22.3 (**C**H_3), 36.7 (**C**H_2), 53.5 (**C**H), 126.4 (*p*-$\mathbf{C}_{\mathbf{arom.}}$H), 128.2 (*o*-$\mathbf{C}_{\mathbf{arom.}}$H), 129.0 (*m*-$\mathbf{C}_{\mathbf{arom.}}$H), 137.7 ($\mathbf{C}_{\mathbf{q\ arom.}}$), 169.2 (NH**C**O), 173.2 (**C**OOH).

HRMS (ESI$^+$) of compound **18** *m/z* = $[M + Na]^+$ calculated for $C_{11}H_{13}NNaO_3$ 230.0793; found 230.0788.

$\boldsymbol{\alpha}_{\boldsymbol{D}}^{\mathbf{20}}$ = -21.9 (1.02 g/100mL, EtOH) of compound **18**.

[1-^{13}C] 5-(Benzylidene)hydantoin (19)



Molecular weight:	189.18 g/mol
Formula:	$C_9{}^{13}CH_8N_2O_2$
Appearance:	yellow solid
Yield:	79%

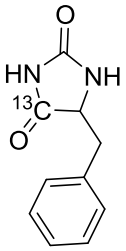
Labeled compound **8** (300 mg, 3.0 mmol) was transferred into a round bottom flask and anhydrous NH_4OAc (231 mg, 3.0 mmol), benzaldehyde (300 µL, 3.0 mmol) and glacial acetic acid (700 µL, 12.2 mmol) were added. The resulting mixture was connected to a drying tube filled with $CaCl_2$ and stirred overnight at 120 °C. After cooling the mixture to room temperature, cold water was added. The resulting orange precipitate was filtered off and washed with cold water. The precipitate was recrystallized from EtOH affording **19** as a pure yellow solid (444 mg, 2.4 mmol, 79%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 6.39 (d, $^3J_{C,H}$ = 6.08 Hz, 1H, C**H**), 7.31 (t, ^3J = 7.33 Hz, 1H, *p*-C**H**$_{\text{arom.}}$), 7.38 (dd, ^3J = 7.33, 7.52 Hz, 2H, *m*-C**H**$_{\text{arom.}}$), 7.60 (d, ^3J = 7.52 Hz, 2H, *o*-C**H**$_{\text{arom.}}$), 10.51, 11.20 (2 x bs, 2 x 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 108.7 (**C**H), 128.8 (*p*-**C**$_{\text{arom.}}$H), 129.2 (*o*-**C**$_{\text{arom.}}$H), 129.8 (*m*-**C**$_{\text{arom.}}$H), 133.5 (**C**$_{\text{q arom.}}$), 156.1 (NH**C**ONH), 166.0 (NH**C**OC).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_9{}^{13}CH_8N_2NaO_2$ 212.0517; found 212.0510.

[1-^{13}C] 5-Benzylhydantoin (20)



Molecular weight:	191.19 g/mol
Formula:	$C_9{}^{13}CH_{10}N_2O_2$
Appearance:	white solid
Yield:	88%

10% Pd/C catalyst (36 mg, 2 mol%) was evacuated in a round bottom flask and purged with H_2. Compound **19** (300 mg, 1.6 mmol) was dissolved in 30 mL methanol and added to the catalyst under hydrogen atmosphere. The mixture was stirred overnight at room temperature. The catalyst was filtered off and the solvent was reduced *in vacuo* giving pure **20** as white solid (267 mg, 1.4 mmol, 88%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

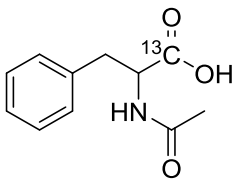
δ 2.87-2.98 (m, 2H, C**H$_2$**), 4.29-4.33 (m, 1H, C**H**), 7.17-7.29 (m, 5H, C**H$_{arom.}$**), 7.85 (d, $^2J_{C,H}$ = 7.82 Hz, 1H, CON**H**CO), 10.41 (bs, 1H, CON**H**CH).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 36.9 (**C**H$_2$), 58.6 (**C**H), 127.1 (*p*-**C$_{arom.}$**H), 128.5 (*o*-**C$_{arom.}$**H), 130.2 (*m*-**C$_{arom.}$**H), 136.2 (**C$_{q\ arom.}$**), 157.7 (NH**C**ONH), 175.8 (NH**C**OCH).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_9{}^{13}CH_{10}N_2NaO_2$ 214.0674; found 214.0665.

[1-^{13}C] *N*-Acetylphenylalanine (21)



Molecular weight:	208.22 g/mol
Formula:	$C_{10}{}^{13}CH_{13}NO_3$
Appearance:	white solid
Yield:	84%

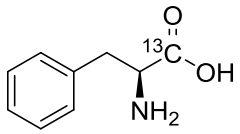
Compound **20** (290 mg, 1.5 mmol) was dissolved in 10 mL NaOH (15%), heated to 130 °C and stirred for five hours. The reaction mixture was cooled to room temperature and the pH was set to 2 by addition of 6 N HCl resulting in the formation of a white precipitate. The solution was neutralized by treatment with 1 N NaOH and then Ac_2O (370 µL, 3.9 mmol) and NaOH (20%) (370 µL, 11.3 mmol) were added simultaneously. The addition of Ac_2O (370 µL, 3.9 mmol) and NaOH (20%) (370 µL, 11.3 mmol) was repeated after half an hour and the reaction mixture was stirred overnight at room temperature. The pH was adjusted to 1 with 1 N HCl and the mixture was diluted with EtOAc. The aqueous phase was extracted with EtOAc (4 x 20 mL); the combined organic extracts were dried over $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The oily residue was dissolved in EtOAc and PE was added slowly resulting in precipitation of a white solid. The solid was further precipitated at 0 °C and filtered off affording pure **21** as a white solid (265 mg, 1.3 mmol, 84%).

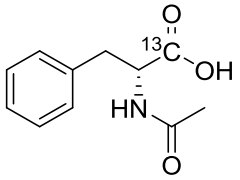
^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 1.76 (s, 3H, C**H$_3$**), 2.77-2.85 (m, 1H, CH**H**), 2.94-3.04 (m, 1H, C**H**H), 4.35-4.41 (m, 1H, C**H**), 7.15-7.28 (m, 5H, C**H$_{arom.}$**), 8.14 (d, ^3J = 8.01 Hz, 1H, N**H**CO), 12.65 (bs, 1H, COO**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 22.8 (**C**H$_3$), 37.3 (**C**H$_2$), 126.9 (*p*-**C$_{arom.}$**H), 128.6 (*o*-**C$_{arom.}$**H), 129.5 (*m*-**C$_{arom.}$**H), 138.2 (**C$_{q\ arom.}$**), 169.7 (NH**C**O), 173.6 (**C**OOH).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_{10}{}^{13}CH_{13}NNaO_3$ 231.0827; found 231.0818.

[1-^{13}C] L-Phenylalanine (22) and [1-^{13}C] *N*-acetyl-D-phenylalanine (23)





Molecular weight:	166.18 g/mol	Molecular weight:	208.22 g/mol
Formula:	$C_8{}^{13}CH_{11}NO_2$	Formula:	$C_{10}{}^{13}CH_{13}NO_3$
Appearance:	white solid	Appearance:	white solid
Yield:	35%	Yield:	50%

Substrate **21** (200 mg, 1.0 mmol) was dissolved in 17 mL water and 0.5 M KOH was added until pH = 7.8. The reaction mixture was treated with acylase I (5.2 mg) and stirred for 39 hours at room temperature. The resin for ion-exchange column chromatography (equilibrated with 1 N HCl and brought to neutral with water) was charged with the mixture. [1-^{13}C] *N*-acetyl-D-phenylalanine was eluted with water; all fractions containing this product were combined and concentrated under reduced pressure. The oily residue was dissolved in EtOAc and PE was added slowly until a white precipitate was formed. This solid was further precipitated at 0 °C and filtered off giving pure **23** as a white solid (99 mg, 0.5 mmol, 50**%**). [1-^{13}C] L-phenylalanine was eluted with 1 N NH_3; the fractions containing the corresponding product were combined and the solvent was removed under reduced pressure giving L-phenylalanine **22** as a pure white solid (56 mg, 0.3 mmol, 35%).

^{1}H-NMR (400.13 MHz; D_2O, NaOD) δ [ppm] of compound **22**:
δ 2.83-2.89 (m, 1H, CH**H**), 2.97-3.03 (m, 1H, C**H**H), 3.49-3.53 (m, 1H, C**H**), 7.29-7.41 (m, 5H, C**H**$_{\mathbf{arom.}}$).

^{13}C-NMR (100.61 MHz; D_2O, NaOD) δ [ppm] of compound **22**:
δ 126.6 (*p*-**C**$_{\mathbf{arom.}}$H), 128.6 (*o*-**C**$_{\mathbf{arom.}}$H), 129.5 (*m*-**C**$_{\mathbf{arom.}}$H), 182.5 (**C**OOH).

HRMS (ESI$^-$) of compound **22** *m/z* = $[M - H]^-$ calculated for $C_8{}^{13}CH_{10}NO_2$ 165.0744; found 165.0746.

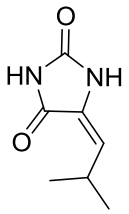
^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm] of compound **23**:
δ 1.77 (s, 3H, C**H**$_{\mathbf{3}}$), 2.77-2.85 (m, 1H, CH**H**), 2.94-3.04 (m, 1H, C**H**H), 4.27-4.41 (m, 1H, C**H**), 7.15-7.28 (m, 5H, C**H**$_{\mathbf{arom.}}$), 8.16 (d, ^3J = 8.13 Hz, 1H, N**H**CO), 12.70 (bs, 1H, COO**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm] of compound **23**:

δ 22.8 (**C**H_3), 37.3 (**C**H_2), 126.9 (*p*-$\mathbf{C_{arom.}}$H), 128.6 (*o*-$\mathbf{C_{arom.}}$H),129.5 (*m*-$\mathbf{C_{arom.}}$H), 138.2 ($\mathbf{C_{q\ arom.}}$), 169.7 (NH**C**O), 173.6 (**C**OOH).

HRMS (ESI$^-$) of compound **23** *m/z* = [M - H]$^-$ calculated for $C_{10}{}^{13}CH_{12}NO_3$ 207.0850; found 207.0859.

5-(2-Methylpropylidene)hydantoin (24)



Molecular weight:	154.17 g/mol
Formula:	$C_7H_{10}N_2O_2$
Appearance:	white solid
Yield:	22%

Hydantoin (3000 mg, 30.0 mmol) in 18 mL water was treated with ethanolamine (1.8 mL, 30.1 mmol) and isobutyraldehyde (4.1 mL, 44.9 mmol). The reaction mixture was stirred overnight at 110 °C. After cooling the yellow mixture to room temperature, precipitation of a white solid was observed. The pH was adjusted to 3 by addition of 1 N HCl. The white precipitate was filtered off and recrystallized from PE:EtOAc = 1:1 affording **24** as a pure white solid (1031 mg, 6.7 mmol, 22%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

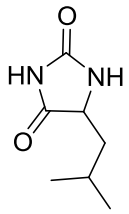
δ 1.00 (d, ^{3}J = 6.63 Hz, 6H, C**H$_3$**), 2.60-2.66 (m, 1H, C**H**CH$_3$), 5.36 (d, ^{3}J = 10.29 Hz, 1H, CC**H**), 10.14, 10.83 (2 x bs, 2 x 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 22.6 (**C**H$_3$), 26.2 (**C**HCH$_3$), 118.8 (C**C**H), 129.1 (NH**C**), 155.3 (NH**C**ONH), 165.2 (NH**C**OC).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_7H_{10}N_2NaO_2$ 177.0640; found 177.0636.

5-Isobutylhydantoin (25)



Molecular weight:	156.19 g/mol
Formula:	$C_7H_{12}N_2O_2$
Appearance:	white solid
Yield:	98%

Compound **24** (500 mg, 3.2 mmol) was dissolved in 30 mL methanol and hydrogenated *via* the H-cube® using a Pd/C catalyst (10%) cartridge under the following reaction conditions: full H_2 and a flow rate of 0.5 mL/min. Methanol was evaporated under reduced pressure affording **25** as a pure white solid (497 mg, 3.2 mmol, 98%).

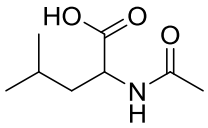
Alternatively, 10% Pd/C catalyst (70 mg, 5 mol%) was transferred into a round bottom flask, evacuated and exposed to H_2. Compound **24** (200 mg, 1.3 mmol) was dissolved in 30 mL methanol and added to the palladium catalyst under hydrogen atmosphere. The mixture was stirred overnight at room temperature. The catalyst was filtered off and methanol was evaporated under reduced pressure giving **25** as a pure white solid (167 mg, 1.1 mmol, 82%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 0.88 (dd, ^3J = 6.34, 6.47 Hz, 6H, C**H$_3$**), 1.34-1.52 (m, 2H, C**H$_2$**), 1.71-1.82 (m, 1H, C**H**CH$_3$), 3.97-4.01 (m, 1H, NHC**H**), 7.99, 10.52 (2 x s, 2 x 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 21.9 (**C**H$_3$), 23.6 (**C**H$_3$), 24.6 (**C**HCH$_3$), 41.3 (**C**H$_2$), 56.6 (NH**C**H).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_7H_{12}N_2NaO_2$ 179.0797; found 179.0792.

_N_-Acetylleucine (26)



Molecular weight:	173.21 g/mol
Formula:	$C_8H_{15}NO_3$
Appearance:	white solid
Yield:	50%

Compound **25** (100 mg, 0.6 mmol) was dissolved in 3 mL NaOH (15%) and stirred for five hours at 130 °C. The reaction mixture was cooled to room temperature and the pH was set to 2 by addition of 6 N HCl resulting in precipitation of a bright pink solid. The solution was neutralized by treatment with 0.5 N NaOH and then Ac_2O (200 µL, 2.1 mmol) and NaOH (20%) (200 µL, 6.1 mmol) were added simultaneously. The addition of Ac_2O (200 µL, 2.1 mmol) and NaOH (20%) (200 µL, 6.1 mmol) was repeated after half an hour and the mixture was stirred overnight at room temperature. After acidification (pH = 1) by addition of 1 N HCl, the reaction mixture was diluted with EtOAc. The aqueous phase was extracted with EtOAc (3 x 20 mL); the combined organic extracts were dried over $MgSO_4$, filtered and reduced *in vacuo*. The oily residue was dissolved in EtOAc and PE was added slowly resulting in the formation of a white precipitate. The solid was further precipitated at 0 °C and filtered off giving pure **26** as a white solid (55 mg, 0.3 mmol, 50%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 0.82 (d, ^3J = 6.51 Hz, 3H, CHC**H$_3$**), 0.87 (d, ^3J = 6.59 Hz, 3H, CHC**H$_3$**), 1.43-1.51 (m, 2H, C**H$_2$**), 1.56-1.66 (m, 1H, C**H**CH$_3$), 1.81 (s, 3H, COC**H$_3$**), 4.15-4.21 (m, 1H, NHC**H**), 8.06 (d, ^3J = 7.97 Hz, 1H, N**H**CH), 12.39 (s, 1H, COO**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 21.8 (CH**C**H$_3$), 22.8 (CH**C**H$_3$), 23.3 (CO**C**H$_3$), 24.8 (**C**HCH$_3$), 50.7 (NH**C**H), 169.7 (**C**OOH), 174.7 (**C**OCH$_3$).

HRMS (ESI$^-$) *m/z* = [M - H]$^-$ calculated for $C_8H_{14}NO_3$ 172.0973; found 172.0975.

<u>Racemization:</u>

N-Acetyl-D-leucine **28** (100 mg, 0.6 mmol) in 440 µL xylene was treated with Ac_2O (8 µL, 0.1 mmol). The mixture was heated to 130 °C in the microwave oven for two hours. The reaction was quenched with water to hydrolyze Ac_2O. The solvents were evaporated under reduced pressure; the oily residue was dissolved in EtOAc and PE was added slowly until precipitation of a white solid was observed. The residue was further precipitated at

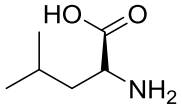
0 °C and filtered off giving pure racemic compound **26** as a white solid (99 mg, 0.6 mmol, 99%).

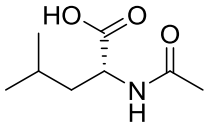
^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 0.82 (d, ^{3}J = 6.51 Hz, 3H, CHC**H$_3$**), 0.87 (d, ^{3}J = 6.61 Hz, 3H, CHC**H$_3$**), 1.42-1.51 (m, 2H, C**H$_2$**), 1.55-1.64 (m, 1H, C**H**CH$_3$), 1.81 (s, 3H, COC**H$_3$**), 4.12-4.20 (m, 1H, NHC**H**), 8.09 (d, ^{3}J = 7.95 Hz, 1H, N**H**CH), 12.51 (bs, 1H, COO**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 21.8 (CH**C**H$_3$), 22.8 (CH**C**H$_3$), 23.3 (CO**C**H$_3$), 24.8 (**C**HCH$_3$), 50.7 (NH**C**H), 169.7 (**C**OOH), 174.7 (**C**OCH$_3$).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_8H_{15}NNaO_3$ 196.0950; found 196.0945.

L-Leucine (27) and *N*-acetyl-D-leucine (28)





Molecular weight:	131.18 g/mol	Molecular weight:	173.21 g/mol
Formula:	$C_6H_{13}NO_2$	Formula:	$C_8H_{15}NO_3$
Appearance:	white solid	Appearance:	white solid
Yield:	50%	Yield:	46%

The pH of compound **26** (97 mg, 0.6 mmol) in 10 mL water was adjusted to 7.8 by addition of 0.5 M KOH. Acylase I (1.2 mg) was added and the mixture was stirred for 24 hours at room temperature. The reaction mixture was loaded onto an ion-exchange resin (equilibrated with 1 N HCl and then neutralized with water). *N*-Acetyl-D-leucine was eluted with water; the corresponding fractions were combined and evaporated under reduced pressure. The oily compound **28** was dissolved in EtOAc and PE was added until a white precipitate was formed. Further precipitation was achieved at 0 °C and the solid was separated *via* filtration giving pure **28** as a white solid (45 mg, 0.3 mmol, 53**%**). L-phenylalanine was eluted with 1 N NH_3; the fractions containing the corresponding product were combined and the solvent was removed under reduced pressure giving pure L-leucine **27** as a white solid (37 mg, 0.3 mmol, 50%).

^{1}H-NMR (400.13 MHz; D_2O, NaOD) δ [ppm] of compound **27**:

δ 0.94 (dd, ^{3}J = 6.61, 6.68 Hz, 6H, C**H$_3$**), 1.37-1.54 (m, 2H, C**H$_2$**), 1.64-1.74 (m, 1H, C**H**CH_3), 3.29 (dd, ^{3}J = 6.54, 7.61 Hz, 1H, NH_2C**H**).

^{13}C-NMR (100.61 MHz; D_2O, NaOD) δ [ppm] of compound **27**:

δ 21.4 (**C**H_3), 22.4 (**C**H_3), 24.4 (**C**HCH_3), 44.3 (**C**H_2), 54.7 (NH_2**C**H).

HRMS (ESI$^+$) of compound **27** *m/z* = $[M + H]^+$ calculated for $C_6H_{14}NO_2$ 132.1025; found 132.1019.

$\boldsymbol{\alpha}_{\boldsymbol{D}}^{\boldsymbol{20}}$ = -12.4 (0.11 g/100mL, water) of compound **27**.

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm] of compound **28**:

δ 0.84 (d, ^{3}J = 6.48 Hz, 3H, CHC**H$_3$**), 0.89 (d, ^{3}J = 6.67 Hz, 3H, CHC**H$_3$**), 1.44-1.52 (m, 2H, C**H$_2$**), 1.57-1.67 (m, 1H, C**H**CH_3), 1.82 (s, 3H, COC**H**$_3$), 4.16-4.22 (m, 1H, NHC**H**), 8.05 (d, ^{3}J = 7.97 Hz, 1H, N**H**CH), 12.46 (s, 1H, COO**H**).

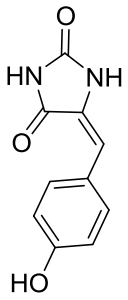
^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm] of compound **28**:

δ 21.8 (CH**C**H_3), 22.8 (CH**C**H_3), 23.3 (CO**C**H_3), 24.8 (**C**HCH_3), 50.7 (NH**C**H), 169.7 (**C**OOH), 174.7 (**C**OCH_3).

HRMS (ESI$^+$) of compound **28** *m/z* = $[M + Na]^+$ calculated for $C_8H_{15}NNaO_3$ 196.0950; found 196.0939.

$\boldsymbol{\alpha}_{\boldsymbol{D}}^{\boldsymbol{20}}$ = 18.1 (0.23 g/100mL, EtOH) of compound **28**.

5-(4-Hydroxybenzylidene)hydantoin (29)



Molecular weight:	204.19 g/mol
Formula:	$C_{10}H_8N_2O_3$
Appearance:	bright yellow solid
Yield:	87%

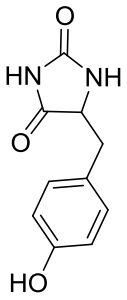
Hydantoin (140 mg, 1.4 mmol) was treated with 4-hydroxybenzaldehyde (171 mg, 1.4 mmol) and piperidine (460 µL, 4.7 mmol). The reaction mixture was connected to a drying tube filled with $CaCl_2$ and stirred for one hour at 130 °C. 5 mL hot water (60 °C) were added and then 0.5 mL conc. HCl to the brown oily reaction mixture, resulting in precipitation of a bright yellow solid. The precipitate was separated *via* filtration and washed with water affording pure **29** as a bright yellow solid (249 mg, 1.2 mmol, 87%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 6.34 (s, 1H, C**H**), 6.79 (d, ^3J = 8.67 Hz, 2H, *m*-C$\mathbf{H}_{\mathbf{arom.}}$), 7.48 (d, ^3J = 8.64 Hz, 2H, *o*- C$\mathbf{H}_{\mathbf{arom.}}$), 9.83, 10.32, 11.07 (3 x bs, 3 x 1H, O**H**, 2 x N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 109.8 (**C**H), 116.2 (*m*-$\mathbf{C}_{\mathbf{arom.}}$H), 124.3 ($\mathbf{C}_{\mathbf{q\ arom.}}$C), 125.8 (NH**C**), 131.7 (*o*-$\mathbf{C}_{\mathbf{arom.}}$H), 156.1 (NH**C**ONH), 158.5 ($\mathbf{C}_{\mathbf{q\ arom}}$OH), 166.1 (NH**C**OC).

HRMS (ESI$^+$) *m/z* = $[M + Na]^+$ calculated for $C_{10}H_8N_2NaO_3$ 227.0433; found 227.0420.

5-(4-Hydroxybenzyl)hydantoin (30)



Molecular weight:	206.20 g/mol
Formula:	$C_{10}H_{10}N_2O_3$
Appearance:	white solid
Yield:	98%

Compound **29** (500 mg, 2.5 mmol) was dissolved in 200 mL methanol and hydrogenated *via* the H-cube® using a Pd/C catalyst (10%) cartridge. The following reaction conditions were set: full H_2 and a flow rate of 0.5 mL/min. The hydrogenation cycle was repeated until NMR spectra showed full conversion of the starting material. Methanol was removed under reduced pressure affording pure **30** as a white solid (495 mg, 2.4 mmol, 98%).

Alternatively, catalyst 10% Pd/C (10 mg, 2 mol%) was evacuated in a round bottom flask and exposed to H_2. Compound **29** (100 mg, 0.5 mmol) was dissolved in 50 mL methanol and added to the catalyst under hydrogen atmosphere. The reaction mixture was stirred overnight at room temperature. The palladium catalyst was filtered off and methanol was removed under reduced pressure giving pure **30** as a white solid (88 mg, 0.4 mmol, 87%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

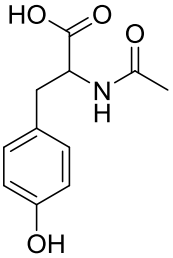
δ 2.81 (d, ^3J = 4.86 Hz, 2H, C**H$_2$**), 4.21-4.24 (m, 1H, C**H**), 6.64 (d, ^3J = 8.53 Hz, 2H, *m*-C**H$_{arom.}$**), 6.96 (d, ^3J = 8. 47 Hz, 2H, *o*-C**H$_{arom.}$**), 7.85, 9.22, 10.39 (3 x s, 3 x 1H, O**H,** 2 x N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 36.1 (**C**H$_2$), 59.1 (**C**H), 115.3 (*m*-**C$_{arom.}$**H), 131.1 (*o*-**C$_{arom.}$**H), 156.6 (NH**C**ONH).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_{10}H_{10}N_2NaO_3$ 229.0589; found 229.0584.

***N*-Acetyltyrosine (31)**



Molecular weight:	223.23 g/mol
Formula:	$C_{11}H_{13}NO_4$
Appearance:	light brown oil
Yield:	20%

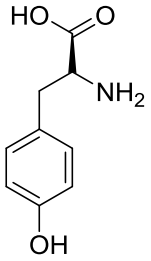
Substrate **30** (200 mg, 1.0 mmol) in 8 mL NaOH (15%) was stirred for five hours at 130 °C. The reaction mixture was cooled to room temperature and the pH was adjusted to 2 by addition of 6 N HCl resulting in precipitation of a white solid. The solution was neutralized with 1 N NaOH and treated with Ac_2O (82 µL, 0.9 mmol). The pH was adjusted to 10 by treatment with 1 N NaOH and the reaction mixture was stirred for four hours at 130 °C. After acidification (pH = 1-2) by addition of 1 N HCl, the solution was concentrated under reduced pressure giving a white solid. This solid was dissolved in acetone and filtrated. Acetone was removed under reduced pressure giving an oily residue, which was dissolved in EtOAc and washed with water. The organic layer was dried over $MgSO_4$, filtered and evaporated *in vacuo* giving **31** as a light brown oil (43 mg, 0.2 mmol, 20%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 1.76 (s, 3H, C**H$_3$**), 2.69 (dd, ^2J = 13.84 Hz, ^3J = 9.43 Hz, 1H, CH**H**), 2.88 (dd, ^2J = 13.84 Hz, ^3J = 4.97 Hz, 1H, C**H**H), 4.26-4.32 (m, 1H, C**H**), 6.66 (d, ^3J = 8.46 Hz, 2H, *m*-C**H$_{arom.}$**), 6.97 (d, ^3J = 8.46 Hz, 2H, *o*-C**H$_{arom.}$**), 8.06 (d, ^3J = 8.18 Hz, 1H, N**H**), 9.17 (s, 1H, CO**H**), 12.60 (bs, 1H, COO**H**).

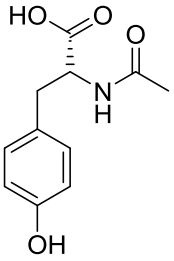
^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 22.8 (**C**H$_3$), 36.5 (**C**H$_2$), 54.3 (**C**H), 115.4 (*m*-**C$_{arom.}$**H), 128.2 (**C$_{q\ arom.}$**C), 130.4 (*o*-**C$_{arom.}$**H), 156.3 (**C$_{q\ arom.}$**OH), 169.6 (NH**C**O), 173.7 (**C**OOH).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_{11}H_{13}NNaO_4$ 246.0743; found 246.0736.

L-Tyrosine (32) and *N*-acetyl-D-tyrosine (33)



Molecular weight:	181.19 g/mol
Formula:	$C_9H_{11}NO_3$
Appearance:	white solid



Molecular weight:	223.23 g/mol
Formula:	$C_{11}H_{13}NO_4$
Appearance:	oily residue

Racemic *N*-acetyltyrosine **31** (50 mg, 0.2 mmol) in 5 mL water was treated with 0.5 M KOH until pH = 7.8 and acylase I (1 mg) was added. The reaction mixture was stirred for 18 hours at room temperature. The mixture was loaded onto an ion-exchange resin (equilibrated with 1 N HCl and neutralized with water). *N*-Acetyl-D-tyrosine was eluted with water and L-tyrosine with 1 N NH_3. All fractions containing compound **33** were combined and evaporated under reduced pressure giving an oily residue of pure compound **33**. The fractions containing the corresponding product **32** were combined and the solvent was concentrated *in vacuo* affording pure **32** as a white solid.

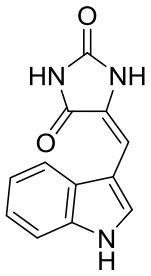
^{1}H-NMR (400.13 MHz; D_2O, NaOD) δ [ppm] of compound **32**:
δ 1.87 (d, ^3J = 10.41 Hz, 2H, C**H$_2$**), 2.16 (s, 1H, C**H**), 6.50 (d, ^3J = 7.81 Hz, 2H, *m*-C**H$_{arom.}$**), 6.93 (d, ^3J = 7.62 Hz, 2H, *o*-C**H$_{arom.}$**).

^{13}C-NMR (100.61 MHz; D_2O, NaOD) δ [ppm] of compound **32**:
δ 118.6 (*m*-**C$_{arom.}$**H).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm] of compound **33**:
δ 1.74 (s, 3H, C**H$_3$**), 2.70 (dd, ^2J = 13.89 Hz, ^3J = 9.41 Hz, 1H, CH**H**), 2.90 (dd, ^2J = 13.89 Hz, ^3J = 4.94 Hz, 1H, C**H**H), 4.27-4.32 (m, 1H, C**H**), 6.64 (d, ^3J = 8.37 Hz, 2H, *m*- C**H$_{arom.}$**), 6.97 (d, ^3J = 8.28 Hz, 2H, *o*-C**H$_{arom.}$**), 8.09 (d, ^3J = 8.03 Hz, 1H, N**H**), 9.24 (s, 1H, CO**H**), 12.47 (bs, 1H, COO**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm] of compound **33**:
δ 22.8 (**C**H$_3$), 36.5 (**C**H$_2$), 54.3 (**C**H), 115.5 (*m*-**C$_{arom.}$**H), 128.2 (**C$_{q\ arom.}$**C), 130.4 (*o*-**C$_{arom.}$**H), 156.4 (**C$_{q\ arom.}$**OH), 169.7 (NH**C**O), 173.7 (**C**OOH).

5-((1*H*-Indole-3-yl)methylene)hydantoin (34)



Molecular weight:	227.22 g/mol
Formula:	$C_{12}H_9N_3O_2$
Appearance:	yellow solid
Yield:	84%

Hydantoin (200 mg, 2.0 mmol) was dissolved in 2 mL water at 70 °C and the solution was neutralized with $NaHCO_3$. Ethanolamine (200 µL, 3.3 mmol) was added; the reaction mixture was heated to 90 °C and treated with indole-3-carboxaldehyde (348 mg, 2.4 mmol) dissolved in 25 mL ethanol. The mixture was stirred overnight at 120 °C forming a yellow precipitate. The solution was cooled to room temperature; the precipitate was filtered off and washed with EtOH:water = 1:5 affording **34** as a yellow solid (380 mg, 1.7 mmol, 84%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

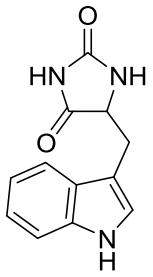
δ 6.77 (s, 1H, C**H**), 7.12-7.22 (m, 2H, C$\mathbf{H}_{\mathbf{arom.6,7}}$), 7.46 (d, ^3J = 7.99 Hz, 1H, C$\mathbf{H}_{\mathbf{arom.8}}$), 7.80 (d, ^3J = 7.73 Hz, 1H, C$\mathbf{H}_{\mathbf{arom.5}}$), 8.16 (d, ^3J = 2.35 Hz, 1H, C$\mathbf{H}_{\mathbf{arom.2}}$), 10.11, 11.01, 11.83 (3 x s, 3 x 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 108.8 ($\mathbf{C}_{\mathbf{q\ arom.3}}$), 112.3 ($\mathbf{C}_{\mathbf{arom.8}}$H), 118.5 ($\mathbf{C}_{\mathbf{arom.5}}$H), 120.6 ($\mathbf{C}_{\mathbf{arom.6}}$H), 122.8 ($\mathbf{C}_{\mathbf{arom.7}}$H), 127.2 ($\mathbf{C}_{\mathbf{arom.2}}$H), 127.4 (NH**C**), 136.3 ($\mathbf{C}_{\mathbf{q\ arom.9}}$).

HRMS (ESI$^+$) *m/z* = $[M + Na]^+$ calculated for $C_{12}H_9N_3NaO_2$ 250.0593; found 250.0579.

5-((1*H*-Indole-3-yl)methyl)hydantoin (35)



Molecular weight:	229.24 g/mol
Formula:	$C_{12}H_{11}N_3O_2$
Appearance:	white solid
Yield:	74%

Compound **34** (119 mg, 0.5 mmol) was dissolved in 90 mL 0.5 N NaOH and hydrogenated *via* the H-cube® using a Pd/C catalyst (10%) cartridge. The following reaction conditions were set: full H_2 and a flow rate of 0.5 mL/min. The pH was adjusted to 7 with 6 N HCl resulting in precipitation of a white solid. The precipitate was separated by filtration giving pure **35** as a white solid (89 mg, 0.4 mmol, 74%).

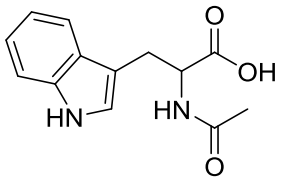
Alternatively, catalyst 10% Pd/C (19 mg, 2 mol%) was evacuated in a round bottom flask and exposed to H_2. Compound **34** (200 mg, 0.9 mmol) was dissolved in 70 mL 0.5 N NaOH and added to the catalyst under hydrogen atmosphere. The reaction mixture was stirred overnight at room temperature. After filtration of the palladium catalyst, the pH of the solution was neutralized with 1 N HCl resulting in precipitation of a white solid. The precipitate was filtered off affording pure **35** as a white solid (143 mg, 0.6 mmol, 71%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 3.05 (d, ^3J = 4.88 Hz, 2H, C**H$_2$**), 4.29 (dt, ^3J = 1.04; 4.86 Hz, 1H, C**H**), 6.93-7.06 (m, 2H, C**H$_{arom.6,7}$**), 7.12 (d, ^3J = 2.36 Hz, 1H, C**H$_{arom.2}$**), 7.33 (d, ^3J = 8.05 Hz, 1H, C**H$_{arom.8}$**), 7.53 (d, ^3J = 7.93 Hz, 1H, C**H$_{arom.5}$**), 7.86, 10.32, 10.87 (3 x s, 3 x 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 27.0 (**C**H$_2$), 58.8 (**C**H), 108.5 (**C$_{q\ arom.3}$**), 111.7 (**C$_{arom.8}$**H), 118.8 (**C$_{arom.5}$**H), 119.1 (**C$_{arom.6}$**H), 121.3 (**C$_{arom.7}$**H), 124.6 (**C$_{arom.2}$**H), 128.0 (**C$_{q\ arom.4}$**), 136.4 (**C$_{q\ arom.9}$**), 157.9 (NH**C**ONH), 176.2 (NH**C**OCH$_2$).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_{12}H_{11}N_3NaO_2$ 252.0749; found 252.0742.

***N*-Acetyltryptophan (36)**



Molecular weight:	246.27 g/mol
Formula:	$C_{13}H_{14}N_2O_3$
Appearance:	bright yellow solid
Yield:	52%

Compound **35** (97 mg, 0.4 mmol) in 3 mL NaOH (15%) was heated to 130 °C and stirred for five hours. The reaction mixture was cooled and the pH was adjusted to 2 with 6 N HCl resulting in precipitation of a white solid. The solution was neutralized by addition of 1 N NaOH and then Ac_2O (100 µL, 1.1 mmol) and NaOH (20%) (100 µL, 3.1 mmol) were added simultaneously. The addition of Ac_2O (100 µL, 1.1 mmol) and NaOH (20%) (100 µL, 3.1 mmol) was repeated after half an hour and the reaction mixture was stirred overnight. On the next day the pH was adjusted to 1 with 1 N HCl and the reaction mixture was diluted with EtOAc. The aqueous layer was extracted with EtOAc (4 x 20 mL); the combined organic extracts were dried over $MgSO_4$, filtered and evaporated *in vacuo* to dryness. The oily residue was dissolved in EtOAc and PE was added slowly until precipitation of a bright yellow solid was observed. Further precipitation was performed at 0 °C and filtered off obtaining pure **36** as a bright yellow solid (54 mg, 0.2 mmol, 52%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 1.80 (s, 3H, C**H$_3$**), 2.99 (dd, ^2J = 14.66 Hz, ^3J = 8.65 Hz, 1H, CH**H**), 3.16 (dd, ^2J = 14.66 Hz, ^3J = 4.99 Hz, 1H, C**H**H), 4.43-4.49 (m, 1H, C**H**), 6.96-7.08 (m, 2H, C**H$_{arom.6,7}$**), 7.14 (d, ^3J = 2.29 Hz, 1H, C**H$_{arom.2}$**), 7.34 (d, ^3J = 8.05 Hz, 1H, C**H$_{arom.8}$**), 7.53 (d, ^3J = 7.90 Hz, 1H, C**H$_{arom.5}$**), 8.09 (d, ^3J = 7.92 Hz, N**H**CO), 10.83 (s, 1H, N**H**C$_{arom.}$), 12.51 (bs, 1H, COO**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 22.9 (**C**H$_3$), 27.6 (**C**H$_2$), 53.4 (**C**H), 110.5 (**C$_{q\ arom.3}$**), 111.8 (**C$_{arom.8}$**H), 118.6 (**C$_{arom.5}$**H), 118.9 (**C$_{arom.6}$**H), 121.4 (**C$_{arom.7}$**H), 124.0 (**C$_{arom.2}$**H), 127.7 (**C$_{q\ arom.4}$**), 136.6 (**C$_{q\ arom.9}$**), 169.7 (NH**C**O), 174.0 (**C**OOH).

HRMS (ESI$^+$) *m/z* = [M + Na]$^+$ calculated for $C_{13}H_{14}N_2NaO_3$ 269.0902; found 269.0887.

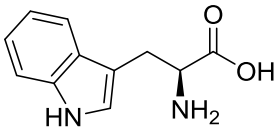
<u>Racemization:</u>

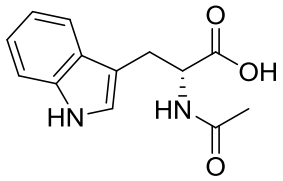
N-Acetyl-D-tryptophan **38** (17 mg, 0.1 mmol) was dissolved in 500 µL xylene and Ac_2O (1 µL, 0.01 mmol) was added. The mixture was heated to 130 °C in the microwave

oven for two hours. The reaction was quenched with water in order to hydrolyze Ac_2O. The solvents were evaporated under reduced pressure and the oily residue was dissolved in EtOAc. PE was added slowly until formation of an orange precipitate, which was further precipitated at 0 °C. The solution was decanted affording racemic compound **36** as an orange solid.

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 1.76 (s, 3H, C**H$_3$**), 2.95 (dd, ^2J = 14.45 Hz, ^3J = 8.53 Hz, 1H, CH**H**), 3.13 (dd, ^2J = 14.54 Hz, ^3J = 5.01 Hz, 1H, C**H**H), 4.40-4.45 (m, 1H, C**H**), 6.94-7.06 (m, 2H, C**H$_{arom.6,7}$**), 7.11 (d, ^3J = 1.97 Hz, 1H, C**H$_{arom.2}$**), 7.31 (d, ^3J = 8.09 Hz, 1H, C**H$_{arom.8}$**), 7.51 (d, ^3J = 7.78 Hz, 1H, C**H$_{arom.5}$**), 8.08 (d, ^3J = 7.68 Hz, N**H**CO), 10.82 (s, 1H, N**H**C$_{arom.}$).

L-Tryptophan (37) and *N*-acetyl-D-tryptophan (38)





Molecular weight:	204.23 g/mol
Formula:	$C_{11}H_{12}N_2O_2$
Appearance:	brown solid
Yield:	32%

Molecular weight:	246.27 g/mol
Formula:	$C_{13}H_{14}N_2O_3$
Appearance:	white solid
Yield:	49%

Racemic *N*-acetyltryptophan **36** (93 mg, 0.4 mmol) was dissolved in 10 mL water and pH was adjusted to 7.8 by addition of 0.5 M KOH. Acylase I (0.8 mg) was added to the mixture and then it was stirred for 23.5 hours at room temperature. The mixture was loaded onto an ion-exchange resin (equilibrated with 1 N HCl and neutralized with water). *N*-Acetyl-D-tryptophan was eluted with water and L-tryptophan with 1 N NH_3. All fractions of the corresponding product were combined and evaporated under reduced pressure. The oily residue of compound **38** was dissolved in EtOAc and slowly treated with PE, resulting in precipitation of a white solid. This precipitate was further precipitated at 0 °C and filtrated to give pure **38** as a white solid (46 mg, 0.2 mmol, 49**%**). The fractions containing L-tryptophan were combined and concentrated *in vacuo* affording pure **37** as a white solid (25 mg, 0.1 mmol, 32%).

^{1}H-NMR (400.13 MHz; D_2O, NaOD) δ [ppm] of compound **37**:

δ 3.17 (dd, ^{2}J = 14.50 Hz, ^{3}J = 7.04 Hz, 1H, CH**H**), 3.34 (dd, ^{2}J = 14.61 Hz, ^{3}J = 4.94 Hz, 1H, C**H**H), 4.37-4.41 (m, 1H, C**H**), 7.19-7.30 (m, 3H, C**H**$_{\mathbf{arom.2,6,7}}$), 7.54 (d, ^{3}J = 8.05 Hz, 1H, C**H**$_{\mathbf{arom.8}}$), 7.75 (d, ^{3}J = 7.81 Hz, 1H, C**H**$_{\mathbf{arom.5}}$).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm] of compound **38**:

δ 1.79 (s, 3H, C**H**$_{\mathbf{3}}$), 2.97 (dd, ^{2}J = 14.51 Hz, ^{3}J = 8.49 Hz, 1H, CH**H**), 3.15 (dd, ^{2}J = 14.59 Hz, ^{3}J = 4.94 Hz, 1H, C**H**H), 4.40-4.46 (m, 1H, C**H**), 6.95-7.07 (m, 2H, C**H**$_{\mathbf{arom.6,7}}$), 7.12 (d, ^{3}J = 2.08 Hz, 1H, C**H**$_{\mathbf{arom.2}}$), 7.32 (d, ^{3}J = 8.05 Hz, 1H, C**H**$_{\mathbf{arom.8}}$), 7.53 (d, ^{3}J = 7.85 Hz, 1H, C**H**$_{\mathbf{arom.5}}$), 8.06 (d, ^{3}J = 7.69 Hz, N**H**CO), 10.82 (s, 1H, N**H**$C_{arom.}$).

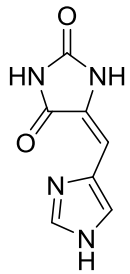
^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm] of compound **38**:

δ 22.9 (**C**H_3), 27.7 (**C**H_2), 53.6 (**C**H), 110.6 (**C**$_{\mathbf{q\ arom.3}}$), 111.8 (**C**$_{\mathbf{arom.8}}$H), 118.7 (**C**$_{\mathbf{arom.5}}$H), 118.8 (**C**$_{\mathbf{arom.6}}$H), 121.3 (**C**$_{\mathbf{arom.7}}$H), 123.9 (**C**$_{\mathbf{arom.2}}$H), 127.8 (**C**$_{\mathbf{q\ arom.4}}$), 136.5 (**C**$_{\mathbf{q\ arom.9}}$), 169.5 (NH**C**O), 174.0 (**C**OOH).

HRMS (ESI^{+}) of compound **38** *m/z* = $[M + Na]^{+}$ calculated for $C_8H_{15}NNaO_3$ 269.0902; found 269.0886.

$\boldsymbol{\alpha}\ \mathbf{^{20}_{D}}$ = 19.4 (0.46 g/100mL, MeOH) of compound **38**.

5-((1*H*-Imidazole-4-yl)methylene)hydantoin (39)



Molecular weight:	178.15 g/mol
Formula:	$C_7H_6N_4O_2$
Appearance:	beige solid
Yield:	71%

Hydantoin (500 mg, 5.0 mmol) was dissolved in 2 mL water at 70 °C and treated with ethanolamine (200 µL, 3.3 mmol) and 4-imidazolecarboxaldehyde (527 mg, 5.5 mmol) dissolved in 3 mL ethanol and 3 mL water. The mixture was stirred overnight at 90 °C forming a beige precipitate. The solution was cooled to room temperature and the precipitate was filtered off affording **39** as a beige solid (632 mg, 3.6 mmol, 71%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

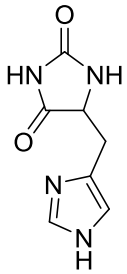
δ 6.44 (s, 1H, C**H**), 7.62 (s, 1H, C$\mathbf{H}_{\mathbf{arom.5}}$), 7.89 (s, 1H, C$\mathbf{H}_{\mathbf{arom.2}}$), 9.96, 11.97, 12.51 (3 x s, 3 x 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 102.2 (**C**H), 154.4 (NH**C**ONH).

HRMS (ESI^{+}) *m/z* = $[M + H]^{+}$ calculated for $C_7H_7N_4O_2$ 179.0570; found 179.0565.

5-((1*H*-Imidazole-4-yl)methyl)hydantoin (40)



Molecular weight:	180.17 g/mol
Formula:	$C_7H_8N_4O_2$
Appearance:	beige solid
Yield:	99% (product and NaCl)

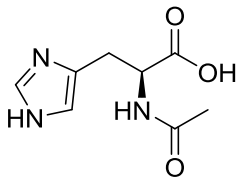
Compound **39** (200 mg, 1.1 mmol) was dissolved in 30 mL 0.5 N NaOH and hydrogenated *via* the H-cube® using a Pd/C catalyst (10%) cartridge. The following reaction conditions were set: full H_2 and a flow rate of 0.5 mL/min. The pH was adjusted to 6-7 with 1 N HCl and the solvent was evaporated under reduced pressure affording product **40** as a white solid with NaCl (200 mg, 1.1 mmol, 99%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 2.90 (dd, ^2J = 15.20 Hz, ^3J = 7.69 Hz, 1H, CH**H**), 3.10 (dd, ^2J = 15.29 Hz, ^3J = 4.81 Hz, 1H, C**H**H), 4.37-4.41 (m, 1H, C**H**), 7.37 (s, 1H, C$\mathbf{H}_{\mathbf{arom.5}}$), 8.00 (s, 1H, C$\mathbf{H}_{\mathbf{arom.2}}$), 8.98 (d, ^2J = 1.32 Hz, 1H, CON**H**CH), 10.66, 14.70 (2 x s, 2 x 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 57.2 (**C**H), 174.9 (NH**C**OCH).

HRMS (ESI$^+$) *m/z* = $[M + H]^+$ calculated for $C_7H_9N_4O_2$ 181.0726; found 181.0716.

_N_-Acetyl-L-histidine (43)



Molecular weight:	197.19 g/mol
Formula:	$C_8H_{11}N_3O_3$
Appearance:	white solid
Yield:	99% (product with NaCl)

Compound **42** (100 mg, 0.7 mmol) in 7 mL water was set to pH = 9-10 by addition of 1 N NaOH and was treated with Ac_2O (200 µL, 2.1 mmol) at 0 °C. The pH was again adjusted to 10 with NaOH (15%) and the reaction mixture was stirred for four hours at room temperature. The solution was acidified (pH = 2) with 1 N HCl and reduced *in vacuo* affording a white solid. This solid was dissolved in methanol and filtered off. The filtrate was evaporated under reduced pressure to give product **43** as a white solid with NaCl (126 mg, 0.6 mmol, 99%).

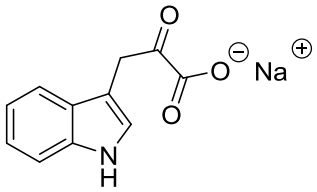
^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 1.83 (s, 3H, C**H$_3$**), 2.93 (dd, ^2J = 14.98 Hz, ^3J = 9.04 Hz, 1H, CH**H**), 3.06 (dd, ^2J = 15.07 Hz, ^3J = 4.93 Hz, 1H, C**H**H), 4.44-4.50 (m, 1H, C**H**), 7.15 (s, 1H, C**H$_{arom.5}$**), 8.30 (d, ^3J = 7.78 Hz, 1H, CON**H**), 8.44 (s, 1H, C**H$_{arom.2}$**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 22.9 (**C**H$_3$), 27.7 (**C**H$_2$), 52.2 (**C**H), 117.2 (**C$_{arom.5}$**H), 131.5 (**C$_{q\ arom.4}$**), 169.8 (NH**C**O), 173.0 (**C**OOH).

HRMS (ESI$^+$) *m/z* = [M + H]$^+$ calculated for $C_8H_{12}N_3O_3$ 198.0879; found 198.0868.

5.3. SYNTHETIC PROTOCOLS OF TRP-PRECURSORS

Indole-3-pyruvate (44)



Molecular weight:	225.18 g/mol
Formula:	$C_{11}H_8NNaO_3$
Appearance:	bright-orange powder
Yield:	73%

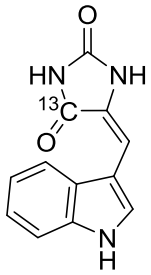
Substrate **34** (100 mg, 0.4 mmol) was transferred into a three necked flask and purged with argon. 6 mL NaOH (20%) were added and the reaction mixture was stirred at 110 °C for four hours exposed to continuous argon flow, which was purged through the reaction mixture. The yellow solution was cooled to room temperature, diluted with Et_2O and the aqueous phase was extracted with Et_2O (2 x 10 mL) under continuous argon flow. The organic layer was removed with a syringe and cannula and the pH of the aqueous phase was adjusted to 2 by addition of 6 N HCl. After acidification, the argon protection was removed and the mixture was diluted with Et_2O. The aqueous phase was extracted with Et_2O (4 x 20 mL); the organic extracts were combined, dried over $MgSO_4$, filtered and evaporated under reduced pressure. The brown residue was redissolved in water and the pH was adjusted to 6-7 by careful addition of 0.5 N NaOH. Freezing with liquid nitrogen and subsequent lyophilization afforded pure **44** as a bright-orange powder (71 mg, 0.3 mmol, 72%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

δ 3.86 (s, 2H, C**H$_2$**), 6.91-7.05 (m, 2H, C**H$_{arom.6,7}$**), 7.13 (d, ^3J = 2.28 Hz, 1H, C**H$_{arom.2}$**), 7.30-7.32 (m, 1H, C**H$_{arom.8}$**), 7.41 (d, ^3J = 7.79 Hz, 1H, C**H$_{arom.5}$**), 10.79 (s, 1H, N**H**).

HRMS (ESI$^-$) *m/z* = [M - Na]$^-$ calculated for $C_{11}H_8NO_3^-$ 202.0510; found 202.0515.

[1-^{13}C] 5-((1*H*-Indole-3-yl)methylene)hydantoin (45)



Molecular weight:	228.22 g/mol
Formula:	$C_{11}{}^{13}CH_9N_3O_2$
Appearance:	yellow solid
Yield:	75%

Labeled hydantoin **8** (400 mg, 4.0 mmol) was dissolved in 4 mL water at 70 °C and the solution was neutralized with $NaHCO_3$. Ethanolamine (400 µL, 6.7 mmol) was added, the reaction mixture was heated to 90 °C and treated with indole-3-carboxaldehyde (690 mg, 4.8 mmol) dissolved in 50 mL ethanol. The mixture was stirred overnight at 120 °C resulting in the formation of a yellow solid. After cooling the solution to room temperature, the precipitate was filtered off affording **45** as a yellow solid (677 mg, 3.0 mmol, 75%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

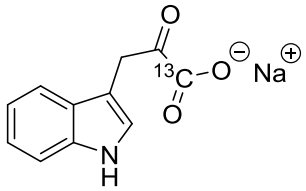
δ 6.75 (d, $^3J_{C,H}$ = 5.67 Hz, 1H, C**H**), 7.11-7.21 (m, 2H, C**H**$_{\mathbf{arom.6,7}}$), 7.42 (d, ^3J = 8.13 Hz, 1H, C**H**$_{\mathbf{arom.8}}$), 7.76 (d, ^3J = 7.88 Hz, 1H, C**H**$_{\mathbf{arom.5}}$), 8.15 (d, ^3J = 2.44 Hz, 1H, C**H**$_{\mathbf{arom.2}}$), 10.12, 11.03, 11.81 (3 x s, 3 x 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 108.8 (**C**$_{\mathbf{q\ arom.3}}$), 112.3 (**C**$_{\mathbf{arom.8}}$H), 118.5 (**C**$_{\mathbf{arom.5}}$H), 120.6 (**C**$_{\mathbf{arom.6}}$H), 122.8 (**C**$_{\mathbf{arom.7}}$H), 123.7 (**C**H), 127.2 (**C**$_{\mathbf{arom.2}}$H), 127.4 (NH**C**), 136.3 (**C**$_{\mathbf{q\ arom.9}}$), 155.8 (NH**C**ONH), 165.8 (NH**C**OC).

HRMS (ESI$^+$) *m/z* = [M + H]$^+$ calculated for $C_{11}{}^{13}CH_{10}N_3O_2$ 229.0807; found 229.0793.

[1-^{13}C] Indole-3-pyruvate (46)



Molecular weight:	226.17 g/mol
Formula:	$C_{10}{}^{13}CH_8NNaO_3$
Appearance:	bright-orange powder
Yield:	81%

Substrate **45** (100 mg, 0.4 mmol) was purged with argon in a three necked flask. 6 mL NaOH (20%) were added and the mixture was stirred under a continuous argon flow, which was purged through the reaction mixture, for four hours at 110 °C. The yellow solution was cooled to room temperature, diluted with Et_2O and the aqueous phase was extracted with Et_2O (2 x 10 mL) under an argon atmosphere. The organic layer was removed with a syringe and a cannula and the pH of the aqueous phase was adjusted to 2 by addition of 6 N HCl. After acidification, the argon protection was removed and the mixture was diluted with Et_2O. The aqueous phase was extracted with Et_2O (4 x 20 mL); the organic extracts were combined, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The brown residue was redissolved in water and the pH was adjusted to 6-7 by careful addition of 0.5 N NaOH. Freezing with liquid nitrogen and subsequent lyophilization afforded pure **46** as a bright-orange powder (80 mg, 0.4 mmol, 81%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

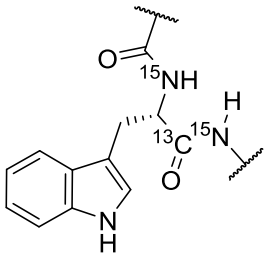
δ 3.82 (s, C**H$_2$**), 6.89-7.06 (m, 2H, C**H$_{arom.6,7}$**), 7.12 (d, *J* = 2.37 Hz, 1H, C**H$_{arom.2}$**), 7.28-7.32 (m, 1H, C**H$_{arom.8}$**), 7.40 (d, ^3J = 7.76 Hz, 1H, C**H$_{arom.5}$**), 10.76 (s, 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 124.7 (**C$_{arom.2}$**H), 167.6 (**C**OONa).

HRMS (ESI$^-$) *m/z* = [M - Na]$^-$ calculated for $C_{10}{}^{13}CH_8NO_3{}^-$ 203.0543; found 203.0545.

GB1 overexpression in *E. coli* (47)

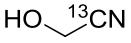


Five 250 ml cultures of M9 minimal media, supplemented with 1 g/L $^{15}NHCl_4$ as the sole source of nitrogen, were inoculated with 5 mL LB medium overnight culture of *E. coli* BL21(DE3) transformed with plasmid coding for his-tagged GB1 (EMBL) and incubated at 37 °C to OD_{600} ~ 0.4. Each culture was supplemented with desired amount of ^{13}C labeled tryptophan precursor and incubated another hour; by then the OD_{600} reached a value of approximately 0.8. The cultures were induced with 0.8 mM IPTG and incubated at 30 °C overnight.

Cultures were harvested by centrifugation at 5000 g for 10 minutes at 4 °C and the cells were resuspended in 25 mL of buffer A (10 mM Bis-Tris, pH = 7.0, 30 mM imidazole). The cells were lysed by sonication on ice and the lysate was clarified by centrifugation at 50000 g for one hour at 4 °C. Cell lysates were applied on a HisTrap FF 1 mL column (GE Healthcare) previously equilibrated with five column volumes of buffer A. Unbound proteins were washed with five column volumes of buffer A followed by ten column volumes of buffer B (10 mM Bis-Tris, pH = 7.0, 50 mM imidazole, 2 M NaCl) and further five column volumes of buffer A. The retained GB1 was eluted isocratically with 4 mL of buffer C (10 mM Bis-Tris, pH = 7.0, 300 mM imidazole, 1 mM EDTA). The samples were concentrated by centrifugation on a membrane to approximately 300 µL and the buffer was exchanged to buffer D (10 mM Bis-Tris, pH = 7.0, 1 mM EDTA); the samples were further concentrated to approximately 250 µL.

The protein concentration was measured by spectrophotometry and for all samples the concentration of GB1 was adjusted to 1.5 mM. 360 µL samples were supplemented with 10 % D_2O and the tryptophan incorporation was measured by NMR HNCO experiment at 500 MHz field strength.

[1-^{13}C] 2-Hydroxyacetonitrile (50)



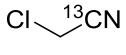
Molecular weight:	58.04 g/mol
Formula:	$C^{13}CH_3NO$
Appearance:	dissolved in DCM

A stirred solution of $K^{13}CN$ (1000 mg, 15.1 mmol) in 5 mL water was cooled to 0 °C and treated with paraformaldehyde (466 mg, 15.5 mmol). The pH of the reaction mixture was adjusted to 2.5 by addition of conc. H_2SO_4 while stirring for half an hour at 0 °C. The reaction mixture was extracted with 150 mL DCM in a liquid-liquid extraction apparatus for 28 hours at 70 °C. The clear organic solution was carefully concentrated under reduced pressure to approximately 50 mL to give a pure product **50** dissolved in DCM.

^{1}H-NMR (400.13 MHz; $CDCl_3$) δ [ppm]:
δ 4.38 (dd, $^2J_{C,H}$ = 6.38 Hz, ^3J = 7.11 Hz, 2H, C**H$_2$**).

^{13}C-NMR (100.61 MHz; $CDCl_3$) δ [ppm]:
δ 53.5 (**C**H$_2$), 117.5 (**C**N).

[1-^{13}C] 2-Chloroacetonitrile (51)



Molecular weight:	76.47 g/mol
Formula:	$C^{13}CH_2ClN$
Appearance:	dissolved in DCM

A solution of compound **50** (878 mg, 15.1 mmol) in approximately 50 mL DCM was treated with pyridine (4.0 mL, 49.6 mmol) and thionyl chloride (2.5 mL, 34.7 mmol), which was added slowly over 45 minutes at 0 °C. The reaction mixture was stirred for one hour at 0 °C and for three hours at room temperature. 5 mL saturated aq. NaCl were added to the mixture and the aqueous layer was removed. The organic phase was extracted with 5 mL saturated aq. NaCl and dried over $MgSO_4$ affording a pure product **51** dissolved in DCM.

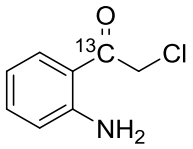
^{1}H-NMR (400.13 MHz; $CDCl_3$) δ [ppm]:

δ 4.13 (d, $^2J_{C,H}$ = 7.84 Hz, 2H, C**H$_2$**).

^{13}C-NMR (100.61 MHz; $CDCl_3$) δ [ppm]:

δ 53.4 (**C**H$_2$), 114.4 (**C**N).

[1-^{13}C] 1-(2-Aminophenyl)-2-chloroethanone (52)



Molecular weight:	170.60 g/mol
Formula:	$C_7{}^{13}CH_8ClNO$
Appearance:	greenish yellow crystals
Yield:	48% over three steps

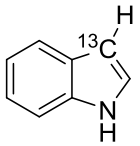
Aniline hydrochloride (2000 mg, 15.4 mmol) was dissolved in 40 mL anhydrous DCE under an argon atmosphere. The solution was treated drop-wise with 1 M boron trichloride in DCM (14.6 mL, 14.6 mmol) and stirred at room temperature until complete dissolution. Chloroacetonitrile **51** (1157 mg, 15.1 mmol) in DCM was added slowly to the reaction mixture followed by $ZnCl_2$ (2400 mg, 17.6 mmol). The reaction mixture was stirred overnight at 75 °C forming an orange precipitate. After cooling to room temperature, 80 mL 1 N HCl were added and the mixture was stirred for two hours at 75 °C to guarantee ketamine hydrolysis. The reaction mixture was cooled to room temperature and diluted with DCM. The aqueous layer was extracted with DCM (5 x 10 mL); the organic extracts were combined, dried over $MgSO_4$, filtered and evaporated under reduced pressure giving pure **52** as greenish yellow crystals (1239 mg, 7.3 mmol, 48%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 4.99 (d, $^2J_{C,H}$ = 3.57 Hz, 2H, C**H$_2$**), 6.50-6.54 (m, 1H, *m*-C**H$_{arom.5}$**), 6.76-6.78 (m, 1H, *m*-C**H$_{arom.3}$**), 7.21-7.28 (m, 3H, *p*-C**H$_{arom.4}$**, N**H$_2$**), 7.66-7.70 (m, 1H, *o*-C**H$_{arom.6}$**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 47.8 (**C**H$_2$), 114.9 (*m*-**C$_{arom.3}$**H), 117.6 (*m*-**C$_{arom.5}$**H), 131.7 (*o*-**C$_{arom.6}$**H), 135.3 (*p*-**C$_{arom.4}$**H), 152.1 (*o*-**C$_{q\ arom.2}$**), 192.9 (**C**O).

HRMS (ESI$^+$) *m/z* = [M + H]$^+$ calculated for $C_7{}^{13}CH_9ClNO$ 171.0407; found 171.0404.

[3-^{13}C] Indole (53)



Molecular weight:	118.14 g/mol
Formula:	$C_7{}^{13}CH_7N$
Appearance:	white solid
Yield:	34%

Substrate **52** (555 mg, 3.3 mmol) in 20 mL dioxane was treated with $NaBH_4$ (134 mg, 3.5 mmol). The reaction mixture was stirred overnight at 110 °C forming a pale precipitate. After cooling to room temperature, the solvent was removed under reduced pressure and the solid residue was dissolved in DCM and water. The aqueous layer was extracted with DCM (5 x 20 mL); the organic extracts were combined, dried over $MgSO_4$, filtered and concentrated *in vacuo* affording a crude brownish oily product. Silica gel flash column chromatography (DCM) gave pure **53** as a white solid (131 mg, 1.1 mmol, 34%).

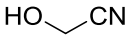
R_f 0.80 (DCM).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 6.17, 6.60 (2 x s, 1H, C**H**$_{\mathbf{arom.3}}$), 6.93-7.07 (m, 2H, C**H**$_{\mathbf{arom.6,7}}$), 7.28-7.32 (m, 1H, C**H**$_{\mathbf{arom.2}}$), 7.37 (d, ^3J = 8.17 Hz, 1H, C**H**$_{\mathbf{arom.8}}$), 7.49-7.52 (m, 1H, C**H**$_{\mathbf{arom.5}}$), 11.04 (s, 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 101.4 (**C**$_{\mathbf{arom.3}}$H), 111.8 (**C**$_{\mathbf{arom.8}}$H), 119.2 (**C**$_{\mathbf{arom.7}}$H), 120.4 (**C**$_{\mathbf{arom.5}}$H), 121.3 (**C**$_{\mathbf{arom.6}}$H), 125.6 (**C**$_{\mathbf{arom.2}}$H).

HRMS (ESI$^+$) *m/z* = $[M + H]^+$ calculated for $C_7{}^{13}CH_8N$ 119.0691; found 119.0688.

2-Hydroxyacetonitrile (56)



Molecular weight:	57.05 g/mol
Formula:	C_2H_3NO
Appearance:	dissolved in DCM

KCN (1000 mg, 15.4 mmol) in 5 mL water was stirred at 0 °C and treated with paraformaldehyde (466 mg, 15.5 mmol). The pH of the mixture was adjusted to 2.5 by treatment with conc. H_2SO_4 while stirring for half an hour at 0 °C. The reaction mixture was extracted with 150 mL DCM in a liquid-liquid extraction apparatus for 23.5 hours at 70 °C. The clear organic solution was carefully evaporated to approximately 25 mL under reduced pressure to give a pure product **56** dissolved in DCM.

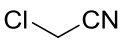
^{1}H-NMR (400.13 MHz; $CDCl_3$) δ [ppm]:

δ 4.36 (d, ^{3}J = 7.13 Hz, 2H, C**H$_2$**).

^{13}C-NMR (100.61 MHz; $CDCl_3$) δ [ppm]:

δ 53.4 (**C**H_2).

2-Chloroacetonitrile (57)



Molecular weight:	75.50 g/mol
Formula:	C_2H_2ClN
Appearance:	dissolved in DCM

A solution of compound **56** (876 mg, 15.4 mmol) in approximately 50 mL DCM was treated with pyridine (4.0 mL, 49.6 mmol) and thionyl chloride (2.5 mL, 34.7 mmol), which was added slowly over 45 minutes at 0 °C. The reaction mixture was stirred for one hour at 0 °C and for three hours at room temperature. 5 mL saturated aq. NaCl were added to the reaction mixture and then the aqueous layer was removed. The organic phase was extracted with 5 mL saturated aq. NaCl and dried over $MgSO_4$ affording a pure product **57** dissolved in DCM.

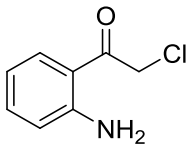
^{1}H-NMR (400.13 MHz; $CDCl_3$) δ [ppm]:

δ 4.09 (s, 2H, C**H$_2$**).

^{13}C-NMR (100.61 MHz; $CDCl_3$) δ [ppm]:

δ 24.6 (**C**H$_2$).

1-(2-Aminophenyl)-2-chloroethanone (58)



Molecular weight:	169.61 g/mol
Formula:	C_8H_8ClNO
Appearance:	brownish yellow crystals
Yield:	45% over three steps

Aniline hydrochloride (2000 mg, 15.4 mmol) was dissolved in 40 mL anhydrous DCE under an argon atmosphere. The solution was treated drop-wise with 1 M boron trichloride in DCM (14.6 mL, 14.6 mmol) and stirred at room temperature until complete dissolution. Chloroacetonitrile **57** (1159 mg, 15.4 mmol) in DCM was added slowly to the reaction mixture followed by $ZnCl_2$ (2400 mg, 17.6 mmol). The reaction mixture was stirred overnight at 75 °C forming an orange precipitate. After cooling to room temperature, 80 mL 1 N HCl were added and the mixture was stirred for two hours at 75 °C to guarantee ketamine hydrolysis. The reaction mixture was cooled to room temperature and was diluted with DCM. The aqueous layer was extracted with DCM (5 x 10 mL); the organic extracts were combined, dried over $MgSO_4$, filtered and evaporated under reduced pressure giving pure **58** as brownish yellow crystals (1172 mg, 6.9 mmol, 45%).

Alternatively, aniline hydrochloride (207 mg, 1.6 mmol) in 10 mL anhydrous DCE was treated drop-wise with 1 M boron trichloride in DCM (5.2 mL, 5.2 mmol) and stirred at room temperature. Chloroacetonitrile (350 µL, 5.5 mmol) and $ZnCl_2$ (870 mg, 6.4 mmol) were added. The reaction mixture was stirred overnight at 75 °C forming an orange precipitate. After cooling, 10 mL 1 N HCl were added and the mixture was stirred for two hours at 75 °C. The reaction mixture was cooled and diluted with DCM. The aqueous layer was extracted with DCM (5 x 10 mL); the organic extracts were combined, dried over $MgSO_4$, filtered and concentrated *in vacuo* giving pure **58** as yellow crystals (209 mg, 1.2 mmol, 77%).

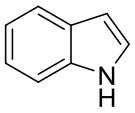
^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 4.98 (s, 2H, C**H$_2$**), 6.52-6.56 (m, 1H, *m*-C**H$_{arom.5}$**), 6.81 (dd, ^3J = 8.45 Hz, ^4J = 0.83 Hz, 1H, *m*-C**H$_{arom.3}$**), 7.23-7.30 (m, 3H, *p*-C**H$_{arom.4}$**, N**H$_2$**), 7.70 (dd, ^3J = 8.23 Hz, ^4J = 1.43 Hz, 1H, *o*-C**H$_{arom.6}$**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 48.0 (**C**H_2), 114.9 (*m*-$\mathbf{C_{arom.3}}$H), 117.5 (*m*-$\mathbf{C_{arom.5}}$H), 131.7 (*o*-$\mathbf{C_{arom.6}}$H), 135.3 (*p*-$\mathbf{C_{arom.4}}$H), 152.1 (*o*-$\mathbf{C_{q\ arom.2}}$).

HRMS (ESI$^+$) *m/z* = [M + H]$^+$ calculated for C_8H_9ClNO 170.0373; found 170.0365.

Indole (59)



Molecular weight:	117.15 g/mol
Formula:	C_8H_7N
Appearance:	white solid
Yield:	52%

Substrate **58** (570 mg, 3.4 mmol) in 20 mL dioxane was treated with $NaBH_4$ (138 mg, 3.7 mmol). The reaction mixture was stirred overnight at 110 °C forming a pale precipitate. After cooling to room temperature, the solvent was removed under reduced pressure and the solid residue was dissolved in DCM and water. The aqueous layer was extracted with DCM (5 x 20 mL); the organic extracts were combined, dried over $MgSO_4$, filtered and concentrated *in vacuo* affording crude brownish oil as product. Silica gel chromatography (DCM) gave pure **59** as a white solid (205 mg, 1.8 mmol, 52%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

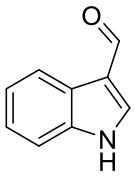
δ 6.40-6.42 (m, 1H, C$\mathbf{H_{arom.3}}$), 6.95-7.08 (m, 2H, C$\mathbf{H_{arom.6,7}}$), 7.31 (t, ^3J = 2.77 Hz, 1H, C$\mathbf{H_{arom.2}}$), 7.37-7.39 (m, 1H, C$\mathbf{H_{arom.8}}$), 7.51-7.54 (m, 1H, C$\mathbf{H_{arom.5}}$), 11.05 (s, 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 101.4 ($\mathbf{C_{arom.3}}$H), 111.8 ($\mathbf{C_{arom.8}}$H), 119.2 ($\mathbf{C_{arom.7}}$H), 120.4 ($\mathbf{C_{arom.5}}$H), 121.3 ($\mathbf{C_{arom.6}}$H), 125.6 ($\mathbf{C_{arom.2}}$H), 128.1 ($\mathbf{C_{q\ arom.4}}$), 136.3 ($\mathbf{C_{q\ arom.9}}$).

HRMS (ESI$^+$) *m/z* = [M + H]$^+$ calculated for $C_7{}^{13}CH_8N$ 118.0657; found 118.0653.

Indole-3-carboxaldehyde (60)



Molecular weight:	145.16 g/mol
Formula:	C_9H_7NO
Appearance:	red crystals
Yield:	99%

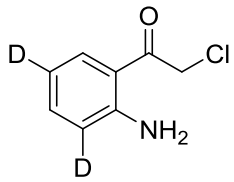
Indole **59** (159 mg, 1.4 mmol) was dissolved in 470 µL DMF. The Vilsmeier-Haack reagent was prepared in a separate flask containing 800 µL DMF, which was cooled to 0 °C and treated with phosphorus oxychloride (235 µL, 2.5 mmol). This Vilsmeier-Haack reagent was added slowly to the solution of indole in DMF and stirred for one hour at 35 °C forming a brown precipitate. 1.2 mL water were added in order to dissolve the solids resulting in a clear reddish solution. Subsequently, 700 µL 4.8 N NaOH were added slowly to the reaction mixture followed by the addition of 1650 µL 4.8 N NaOH in one portion. This reaction mixture was heated for 20 minutes to reflux at 160 °C resulting in precipitation. After cooling to room temperature, the reaction mixture was extracted with EtOAc (5 x 15 mL). The organic extracts were combined, dried over $MgSO_4$, filtered and concentrated under reduced pressure giving pure **60** as red crystals (195 mg, 1.3 mmol, 99%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 7.18-7.26 (m, 2H, C**H**$_{\mathbf{arom.6,7}}$), 7.49 (d, ^3J = 7.85 Hz, 1H, C**H**$_{\mathbf{arom.8}}$), 8.08 (d, ^3J = 7.41 Hz, 1H, C**H**$_{\mathbf{arom.5}}$), 8.26 (d, ^3J = 3.12 Hz, 1H, C**H**$_{\mathbf{arom.2}}$), 9.92 (s, 1H, C**H**O), 12.12 (s, 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:
δ 112.9 (**C**$_{\mathbf{arom.8}}$H), 118.6 (**C**$_{\mathbf{q\ arom.3}}$), 121.3 (**C**$_{\mathbf{arom.6}}$H), 122.6 (**C**$_{\mathbf{arom.7}}$H), 123.9 (**C**$_{\mathbf{arom.8}}$H), 124.6 (**C**$_{\mathbf{q\ arom.4}}$), 137.5 (**C**$_{\mathbf{q\ arom.9}}$), 138.9 (**C**$_{\mathbf{arom.2}}$H), 185.4 (**C**HO).

HRMS (ESI$^+$) *m/z* = $[M + H]^+$ calculated for C_9H_8NO 146.0607; found 146.0603.

1-(2-Aminophenyl-3,5-dideuterio)-2-chloroethanone (63)



Molecular weight:	171.62 g/mol
Formula:	$C_8H_6D_2ClNO$
Appearance:	brown crystals
Yield:	56%

2,4,6-Trideuterioaniline (200 mg, 2.1 mmol) was dissolved in 10 mL anhydrous DCE under an argon atmosphere. The solution was treated drop-wise with 1 M boron trichloride in DCM (5.2 mL, 5.2 mmol) and stirred at room temperature until complete dissolution. Chloroacetonitrile (350 µL, 5.5 mmol) was added slowly to the reaction mixture followed by $ZnCl_2$ (870 mg, 6.4 mmol). The reaction mixture was stirred overnight at 75 °C forming an orange precipitate. After cooling to room temperature, 10 mL 1 N HCl were added and the mixture was stirred for two hours at 75 °C to guarantee ketamine hydrolysis. The reaction mixture was cooled to room temperature and was diluted with DCM. The aqueous layer was extracted with DCM (5 x 10 mL); the organic extracts were combined, dried over $MgSO_4$, filtered and evaporated under reduced pressure giving pure **63** as brown crystals (200 mg, 1.2 mmol, 56%).

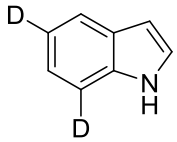
^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

δ 4.98 (s, 2H, C**H$_2$**), 7.06-7.28 (bs and s, 3H, *p*-C**H$_{arom.4}$**, N**H$_2$**), 7.68 (s, 1H, *o*-C**H$_{arom.6}$**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 48.0 (**C**H$_2$), 131.6 (*o*-**C$_{arom.6}$**H), 135.1 (*p*-**C$_{arom.4}$**H), 152.1 (**C$_{q\ arom.2}$**), 192.9 (**C**O).

6,8-Dideuterioindole (64)



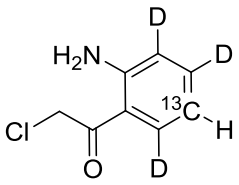
Molecular weight:	119.16 g/mol
Formula:	$C_8H_5D_2N$
Appearance:	white solid
Yield:	16%

Substrate **63** (185 mg, 1.1 mmol) in 7 mL dioxane was treated with $NaBH_4$ (50 mg, 1.3 mmol). The reaction mixture was stirred overnight at 110 °C forming a pale precipitate. After cooling to room temperature, the solvent was evaporated under reduced pressure and the solid residue was dissolved in DCM and water. The aqueous layer was extracted with DCM (5 x 20 mL); the organic extracts were combined, dried over $MgSO_4$, filtered and concentrated *in vacuo* affording crude brown oil as a product. Silica gel chromatography (DCM) gave pure **64** as a white solid (21 mg, 0.2 mmol, 16%).

R_f 0.80 (DCM).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:
δ 6.39-6.40 (m, 1H, C**H**$_{\text{arom.3}}$), 7.07 (s, 1H, C**H**$_{\text{arom.7}}$), 7.31 (t, *J* = 2.76 Hz, 1H, C**H**$_{\text{arom.2}}$), 7.52 (s, 1H, C**H**$_{\text{arom.5}}$), 11.02 (s, 1H, N**H**).

1-(2-Aminophenyl-5-^{13}C-3,4,6-trideuterio)-2-chloroethanone (70)



Molecular weight:	173.62 g/mol
Formula:	$C_7{}^{13}CH_5D_3ClNO$
Appearance:	brown crystals
Yield:	50%

4-^{13}C-2,3,5,6-Tetradeuterioaniline (100 mg, 1.0 mmol) was dissolved in 5 mL anhydrous DCE under an argon atmosphere. The solution was treated drop-wise with 1 M boron trichloride in DCM (2.6 mL, 2.6 mmol) and stirred at room temperature. Chloroacetonitrile (175 µL, 2.8 mmol) and $ZnCl_2$ (435 mg, 3.2 mmol) were added to the reaction mixture consecutively. The reaction mixture was stirred overnight at 75 °C forming an orange precipitate. After cooling to room temperature, 5 mL 1 N HCl were added and the mixture was stirred for two hours at 75 °C to guarantee ketamine hydrolysis. The reaction mixture was cooled to room temperature and was diluted with DCM. The aqueous layer was extracted with DCM (5 x 10 mL); the organic extracts were combined, dried over $MgSO_4$, filtered and evaporated under reduced pressure giving pure **70** as brown crystals (89 mg, 0.5 mmol, 50%).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

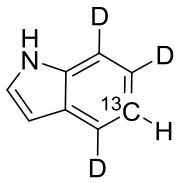
δ 5.02 (s, 2H, C**H$_2$**), 6.35, 6.76 (2 x s, 1H, *m*-C**H$_{arom.5}$**), 7.27 (bs, 2H, N**H$_2$**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

δ 114.7 (*m*-**C$_{arom.5}$**H).

HRMS (ESI$^+$) *m/z* = [M + H]$^+$ calculated for $C_7{}^{13}CH_8N$ 174.0595; found 174.0588.

6-^{13}C-5,7,8-Trideuterioindole (71)



Molecular weight:	121.16 g/mol
Formula:	$C_7{}^{13}CH_4D_3N$
Appearance:	yellow solid
Yield:	25%

Substrate **70** (80 mg, 0.5 mmol) in 5 mL dioxane was treated with $NaBH_4$ (106 mg, 2.8 mmol). The reaction mixture was stirred overnight at 110 °C forming a pale precipitate. After cooling to room temperature, the solvent was evaporated under reduced pressure and the solid residue was dissolved in DCM and water. The aqueous layer was extracted with DCM (5 x 20 mL); the organic extracts were combined, dried over $MgSO_4$, filtered and concentrated *in vacuo* affording crude brown oil as product. Silica gel chromatography (DCM) gave pure **71** as a yellow solid (14 mg, 0.1 mmol, 25%).

R_f 0.82(DCM).

^{1}H-NMR (400.13 MHz; 6d-DMSO) δ [ppm]:

δ 6.40-6.41 (m, 1H, C**H**$_{\mathbf{arom.3}}$), 6.77, 7.17 (2 x s, 1H, C**H**$_{\mathbf{arom.6}}$), 7.31-7.32 (m, 1H, C**H**$_{\mathbf{arom.2}}$), 11.07 (s, 1H, N**H**).

^{13}C-NMR (100.61 MHz; 6d-DMSO) δ [ppm]:

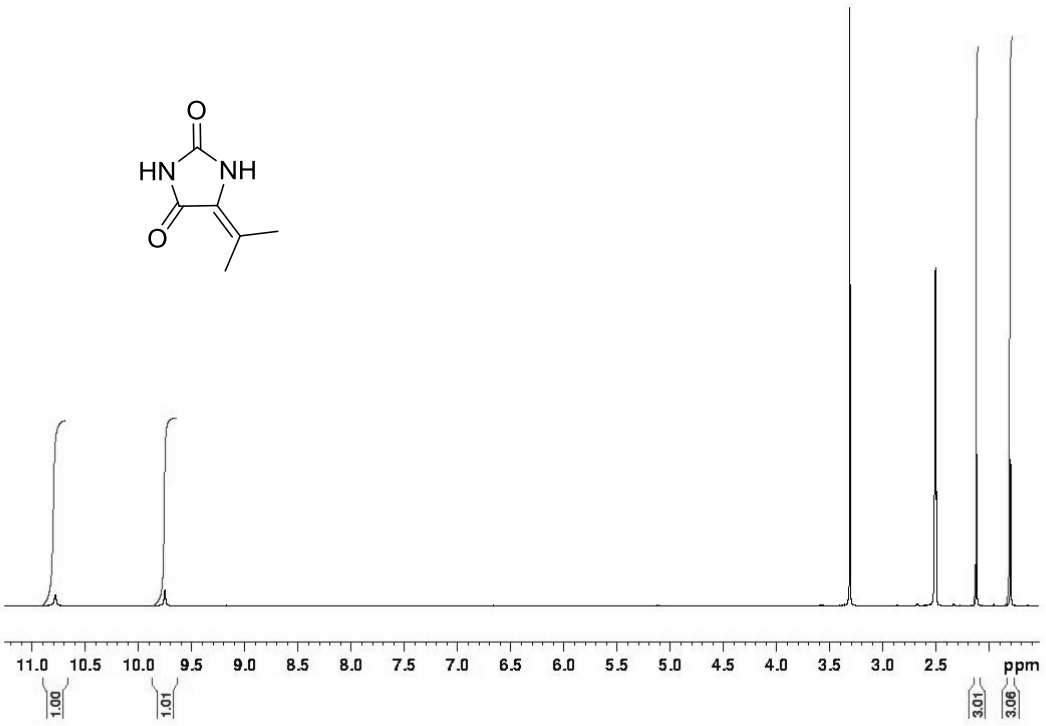
δ 101.4 (**C**$_{\mathbf{arom.3}}$H), 115.3 (**C**$_{\mathbf{arom.8}}$D), 119.0 (**C**$_{\mathbf{arom.7}}$D), 122.4 (**C**$_{\mathbf{arom.6}}$H), 125.6 (**C**$_{\mathbf{arom.2}}$H).

HRMS (ESI$^+$) *m/z* = $[M + H]^+$ calculated for $C_7{}^{13}CH_5D_3N$ 122.0879; found 122.0868.

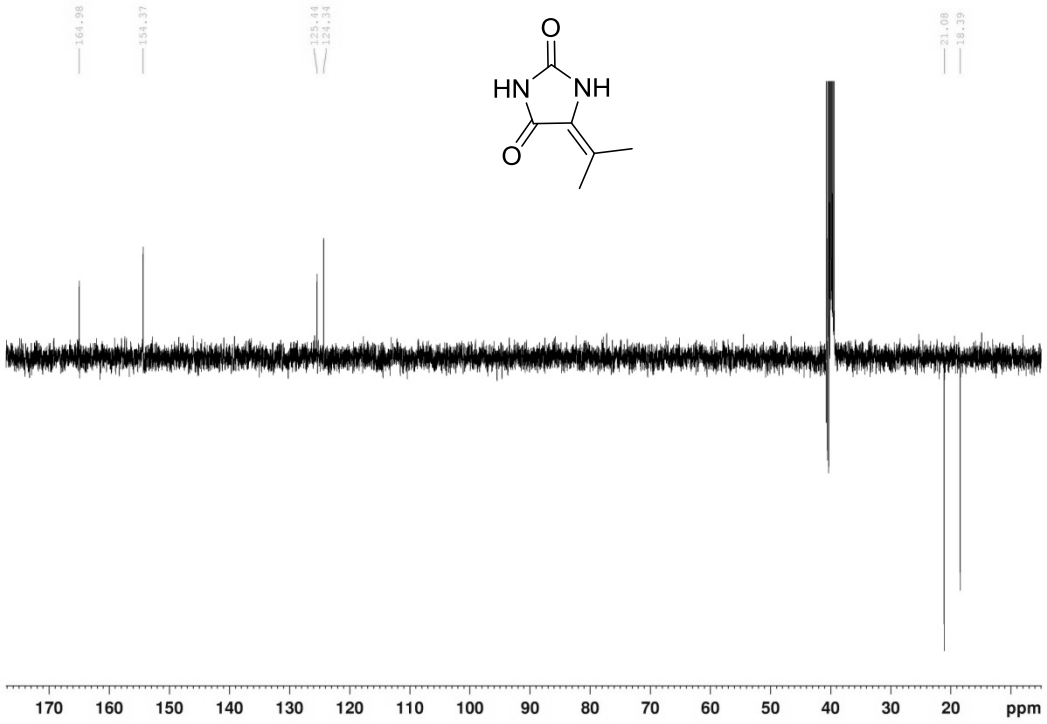
6. APPENDIX

6.1. SPECTRA

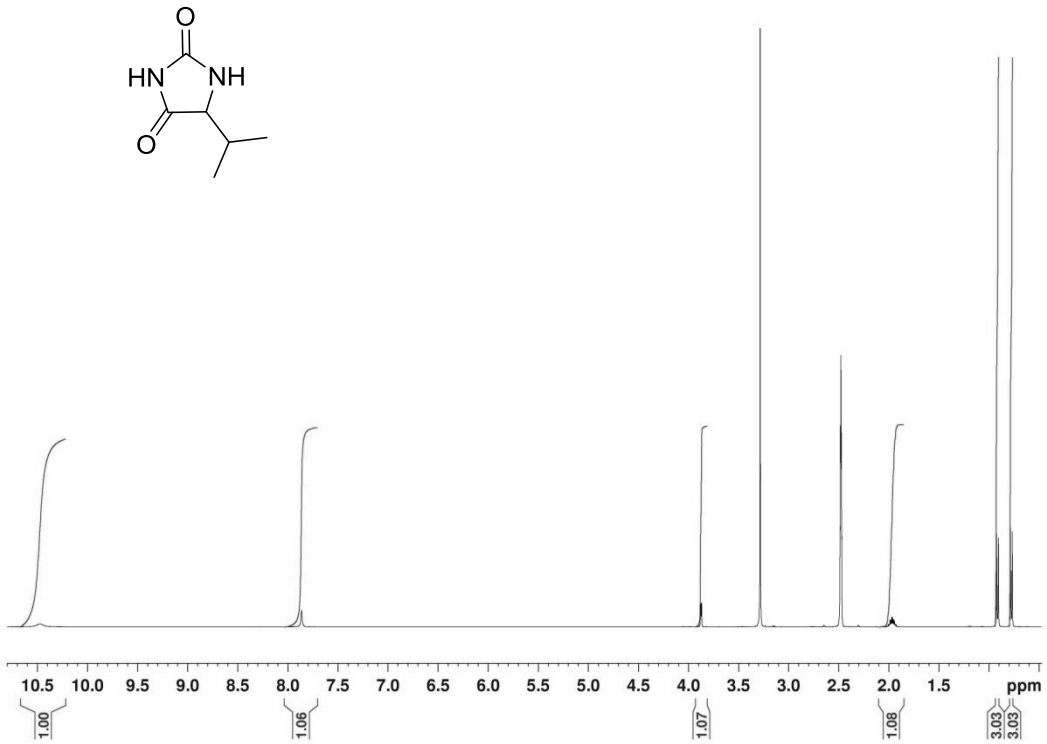
5-(Propane-2-ylidene)hydantoin **2** (^{1}H; 400.13 MHz; 6d-DMSO)



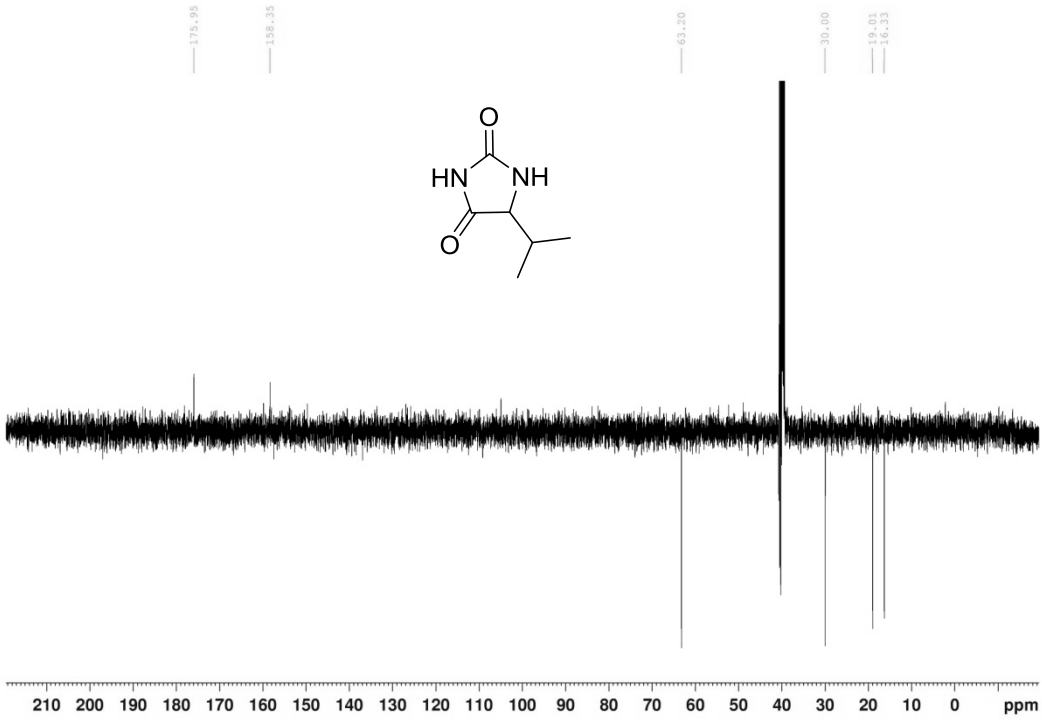
5-(Propane-2-ylidene)hydantoin **2** (^{13}C; 100.61 MHz; 6d-DMSO)



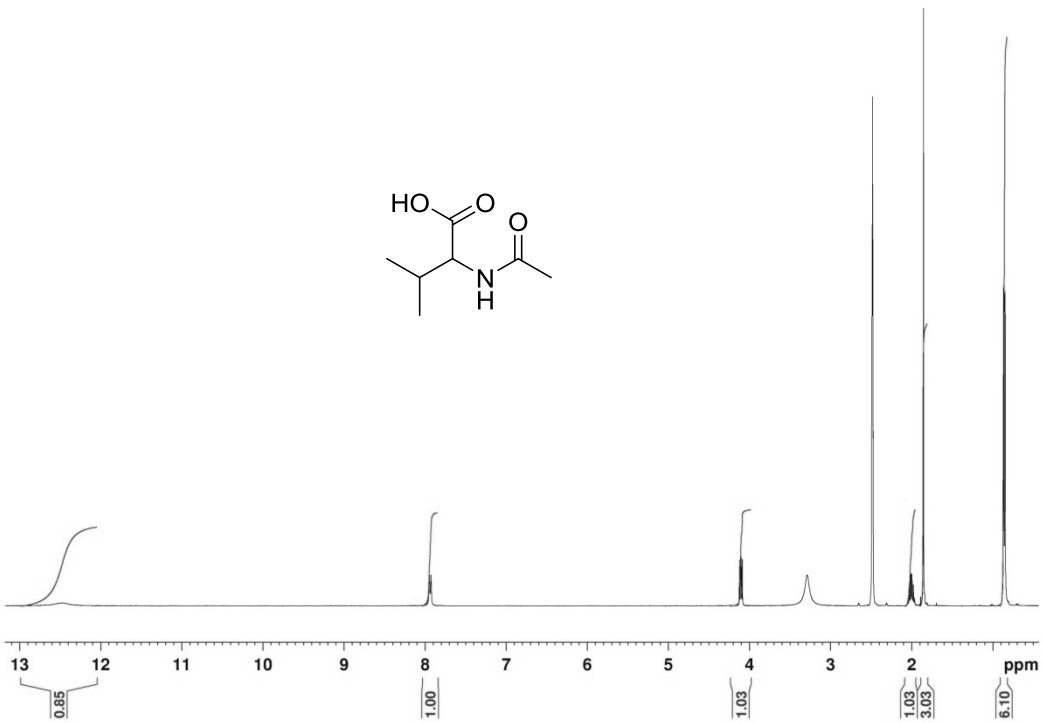
5-Isopropylhydantoin **3** (^{1}H; 400.13 MHz; 6d-DMSO)



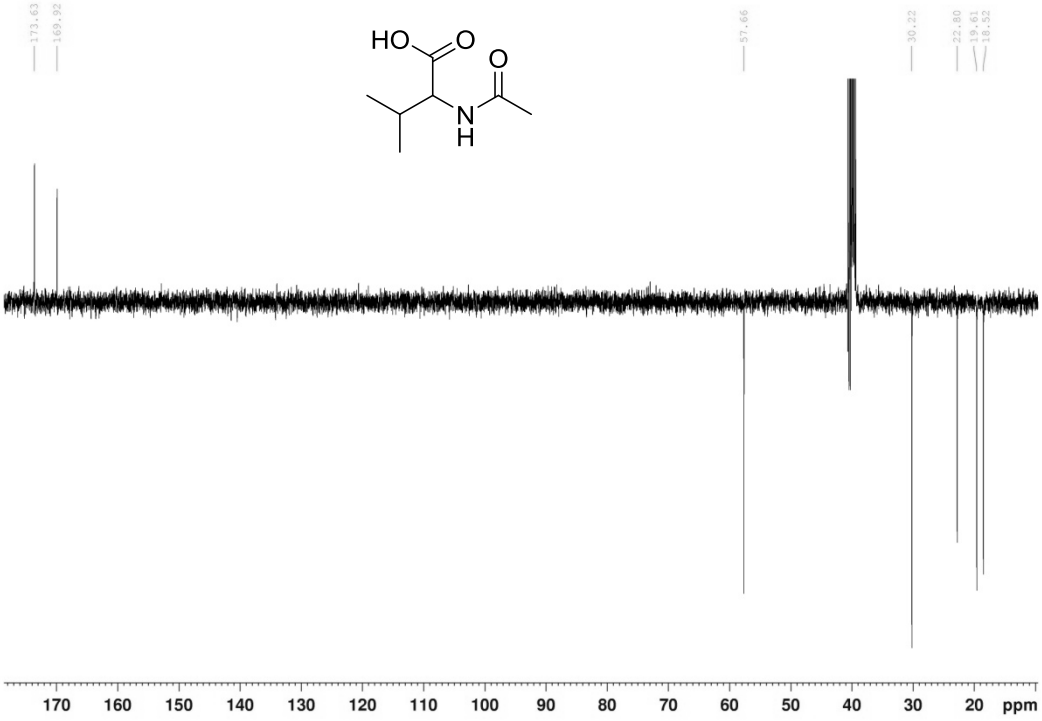
5-Isopropylhydantoin **3** (^{13}C; 100.61 MHz; 6d-DMSO)



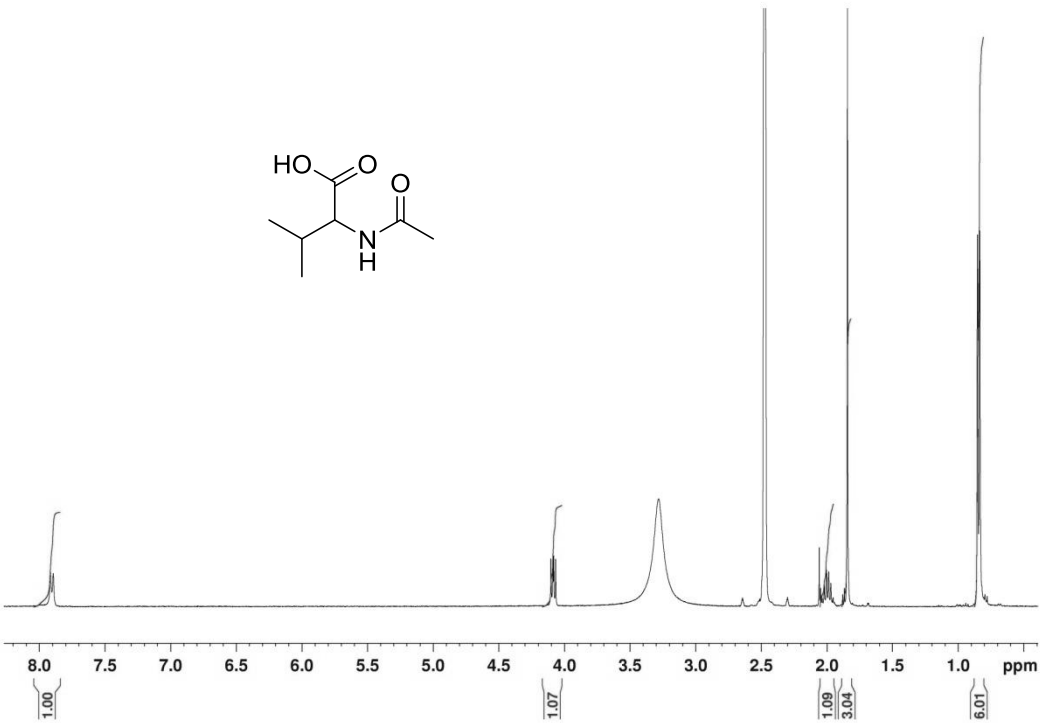
N-Acetylvaline **4** (^{1}H; 400.13 MHz; 6d-DMSO)



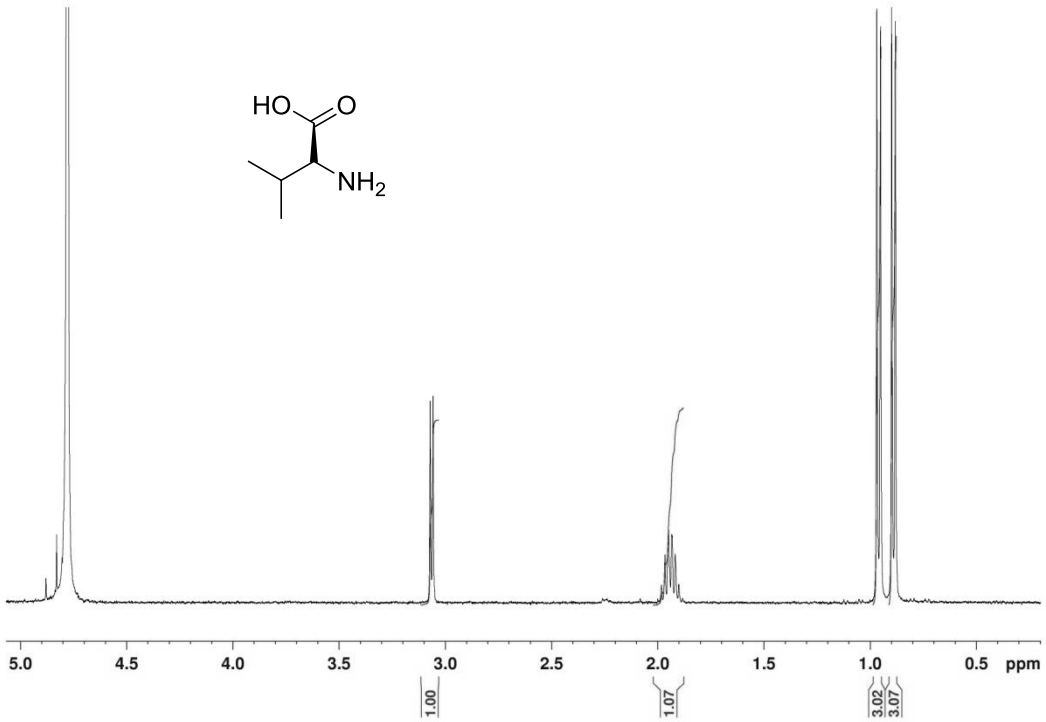
N-Acetylvaline **4** (^{13}C; 100.61 MHz; 6d-DMSO)



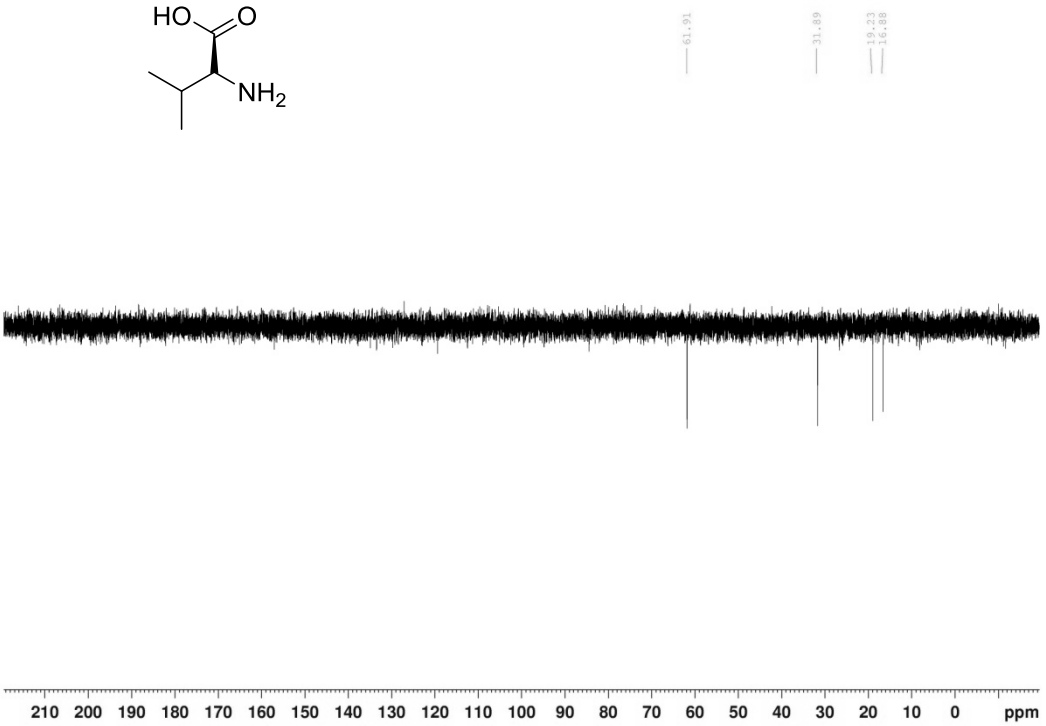
N-Acetylvaline **4** (^{1}H; 400.13 MHz; 6d-DMSO) after racemization



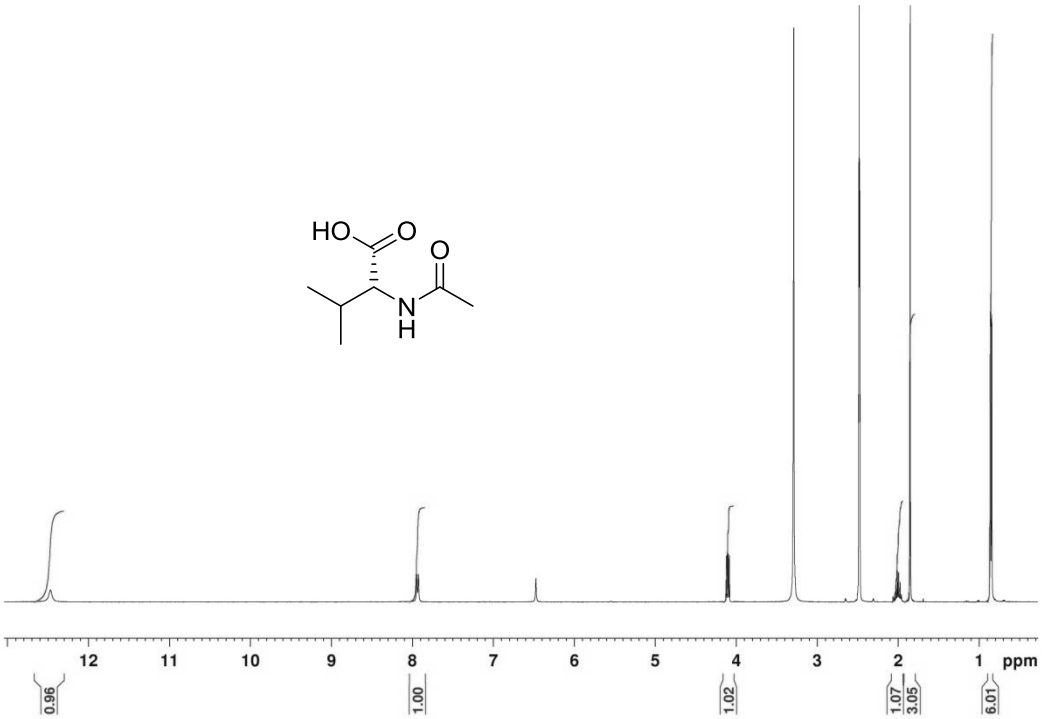
L-Valine **5** (^{1}H; 400.13 MHz; D_2O, NaOD)



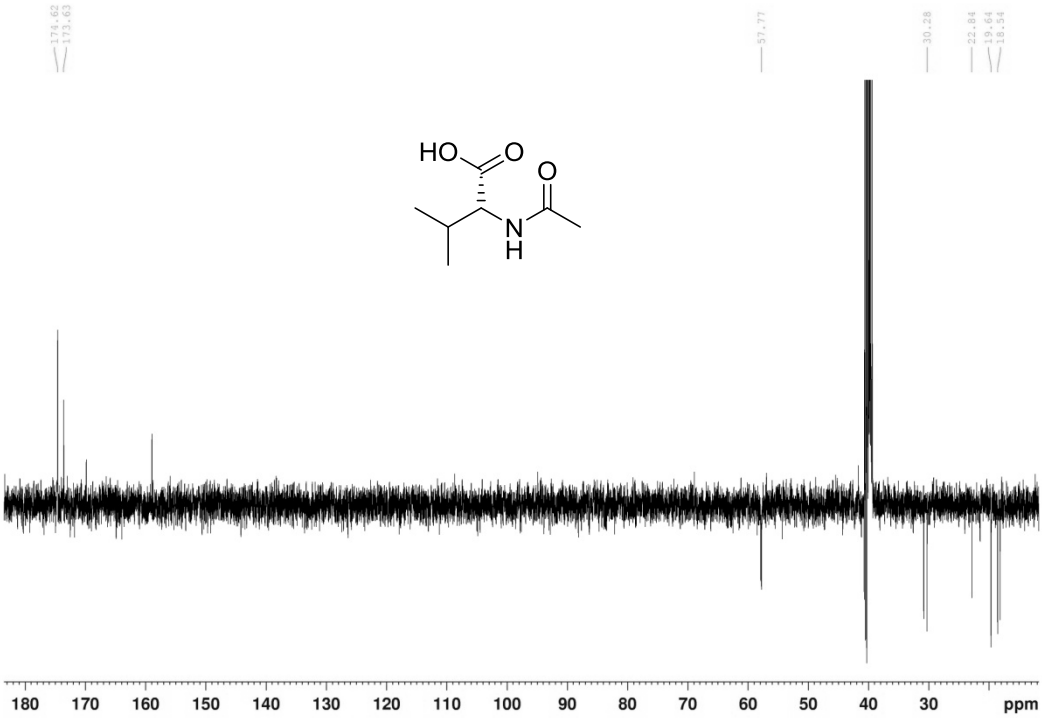
L-Valine **5** (^{13}C; 150.90 MHz; D_2O, NaOD)



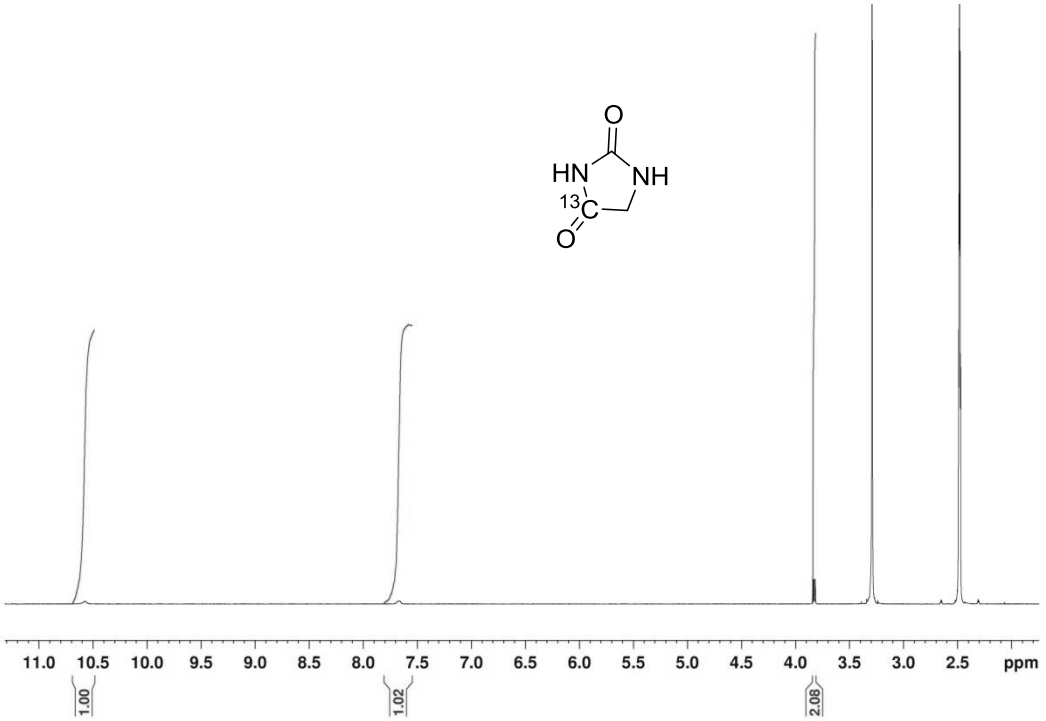
N-Acetyl D-valine **6** (^{1}H; 400.13 MHz; 6d-DMSO)



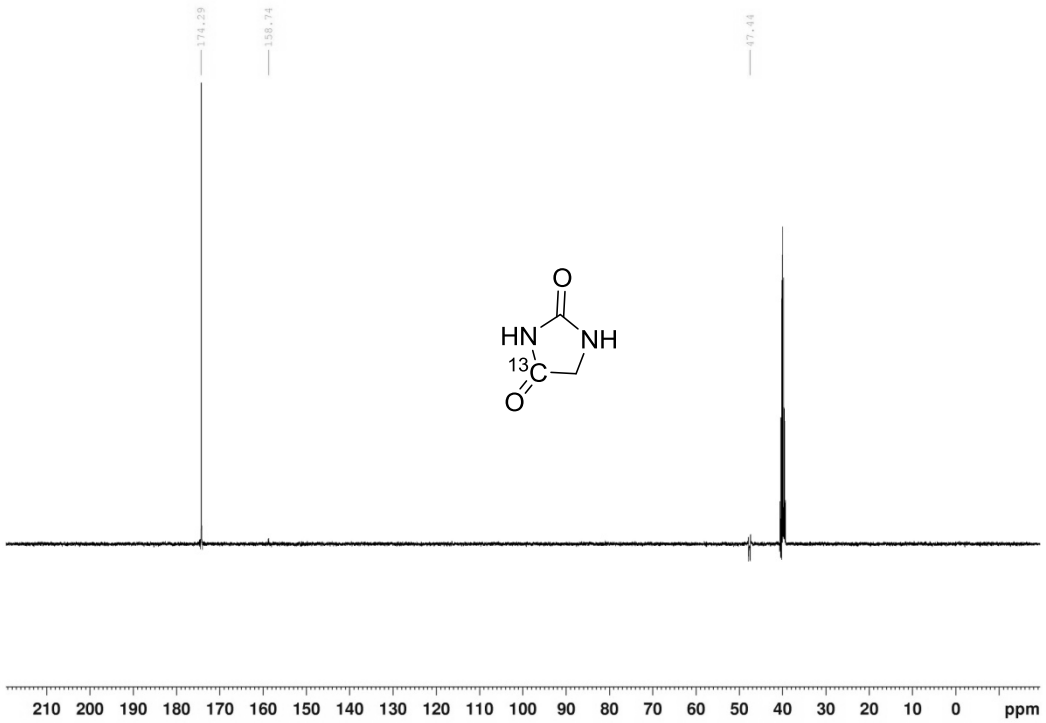
N-Acetyl D-valine **6** (^{13}C; 100.61 MHz; 6d-DMSO)



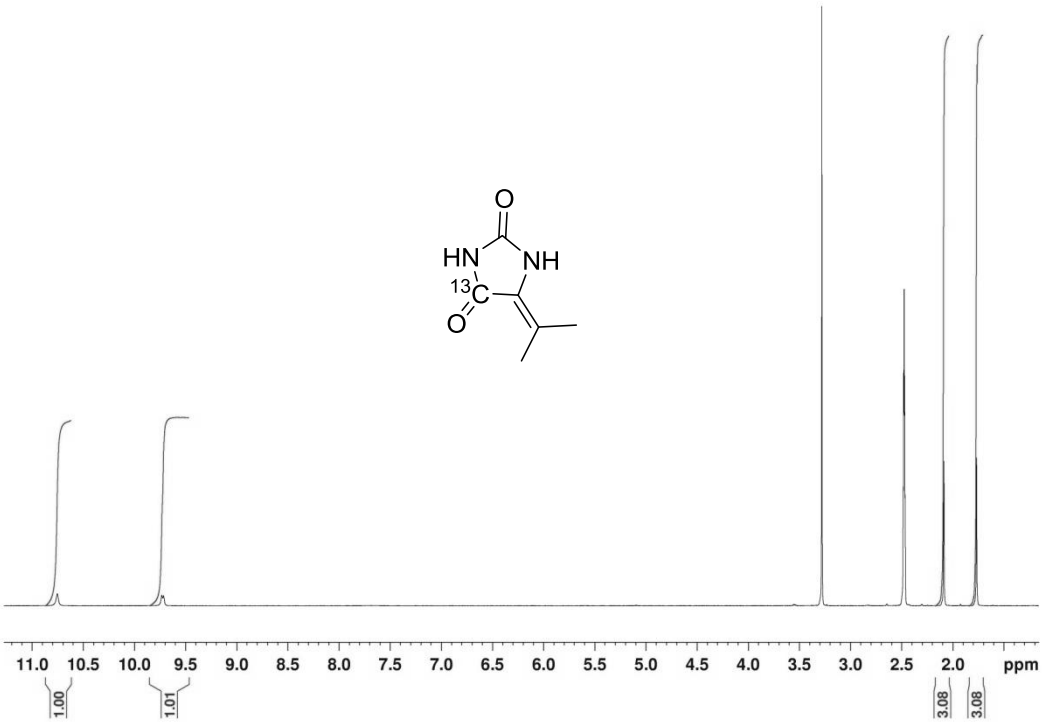
[1-^{13}C] Hydantoin **8** (^{1}H; 400.13 MHz; 6d-DMSO)



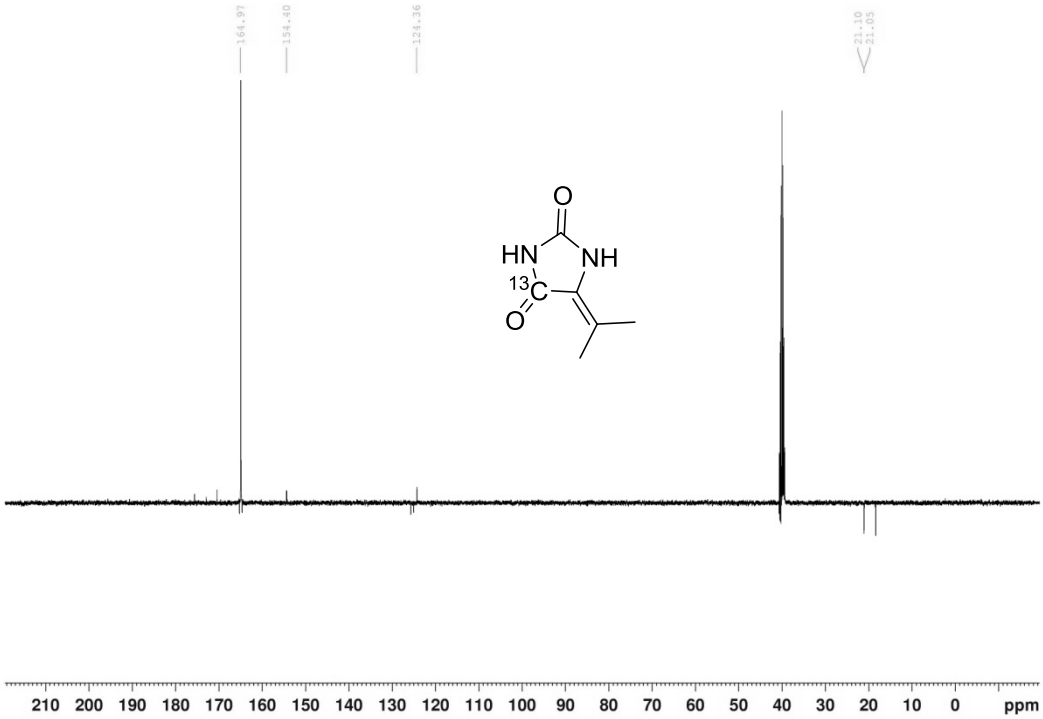
[1-^{13}C] Hydantoin **8** (^{13}C; 100.61 MHz; 6d-DMSO)



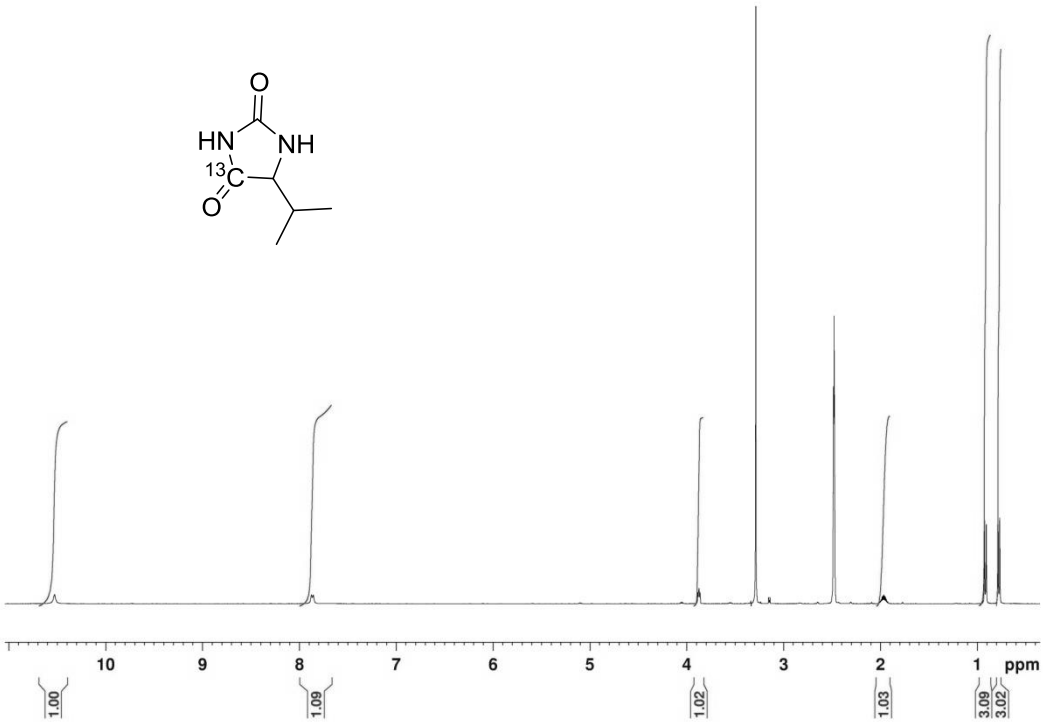
[1-^{13}C] 5-(Propane-2-ylidene)hydantoin **9** (^{1}H; 400.13 MHz; 6d-DMSO)



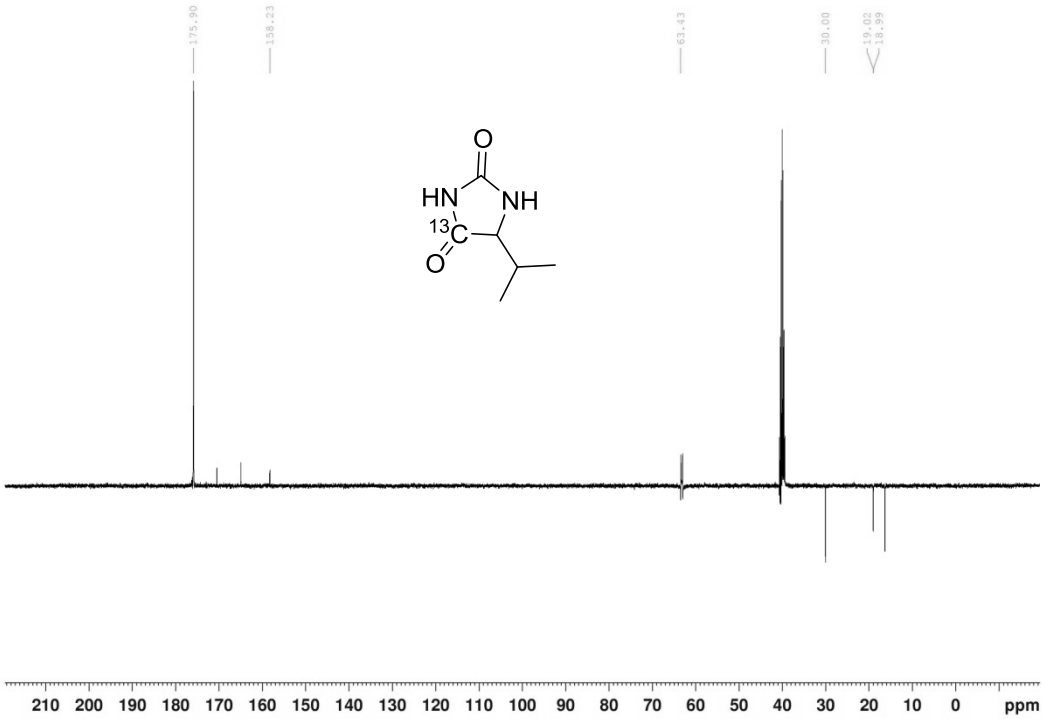
[1-^{13}C] 5-(Propane-2-ylidene)hydantoin **9** (^{13}C; 100.61 MHz; 6d-DMSO)



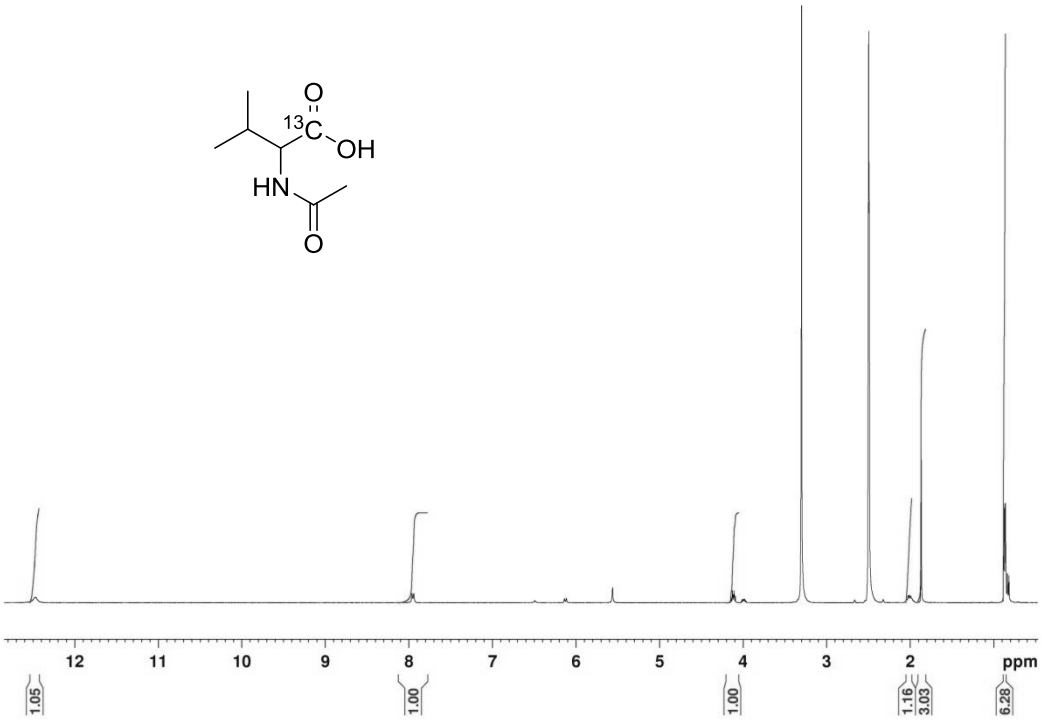
[1-^{13}C] 5-Isopropylhydantoin **10** (^{1}H; 400.13 MHz; 6d-DMSO)



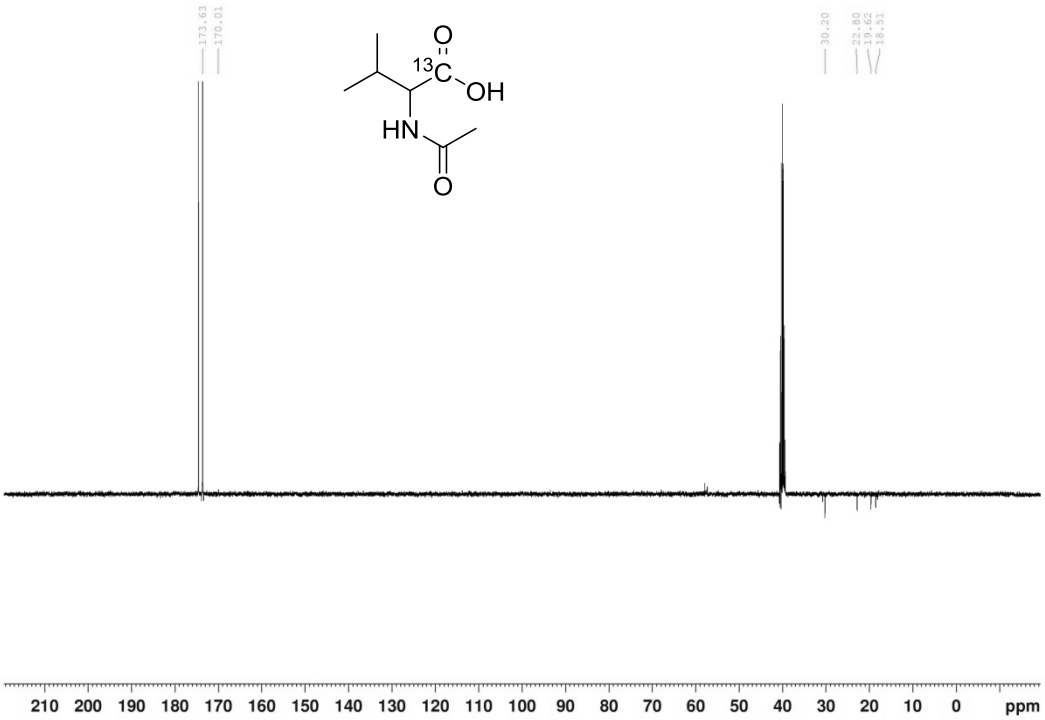
[1-^{13}C] 5-Isopropylhydantoin **10** (^{13}C; 100.61 MHz; 6d-DMSO)



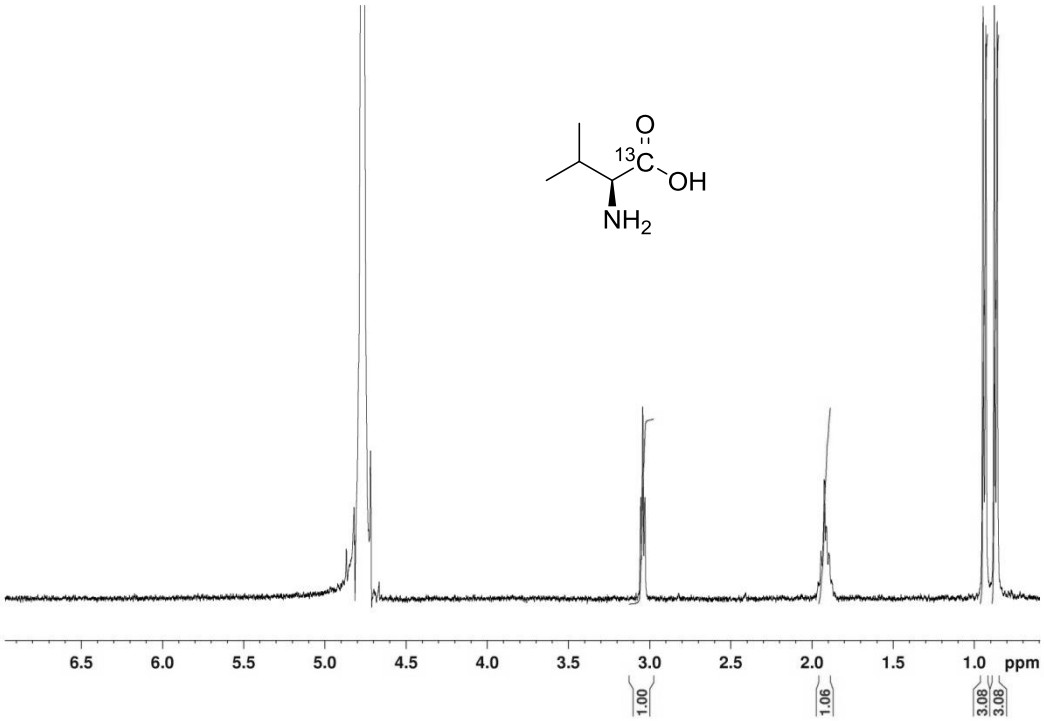
[1-^{13}C] *N*-Acetylvaline **11** (^{1}H; 400.13 MHz; 6d-DMSO)



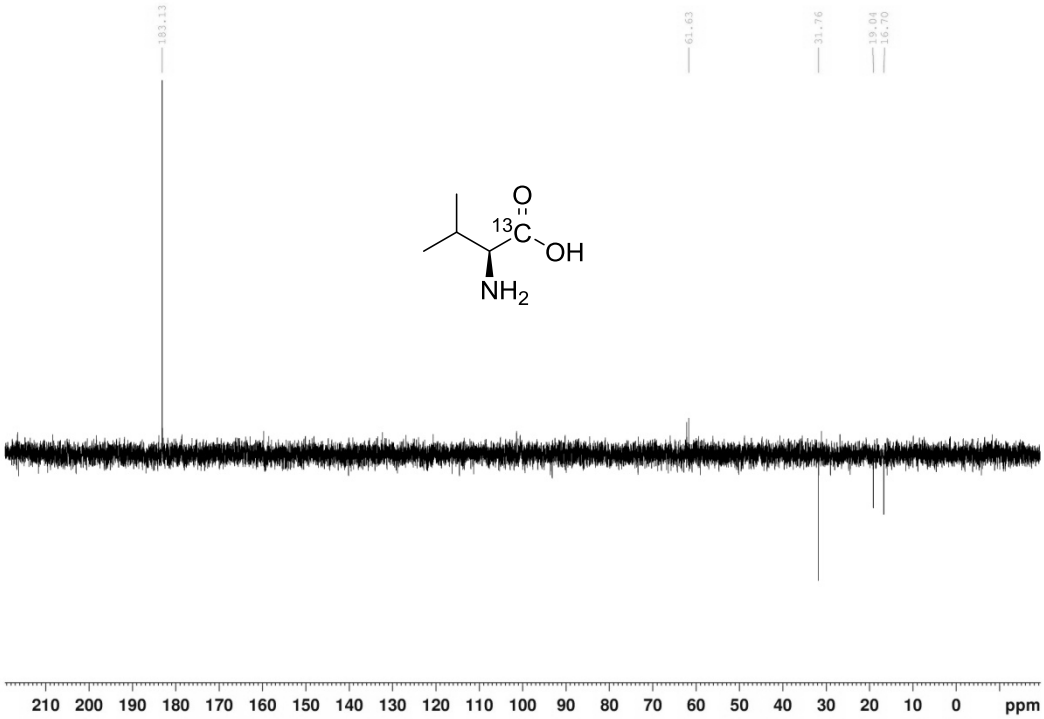
[1-^{13}C] *N*-Acetylvaline **11** (^{13}C; 100.61 MHz; 6d-DMSO)



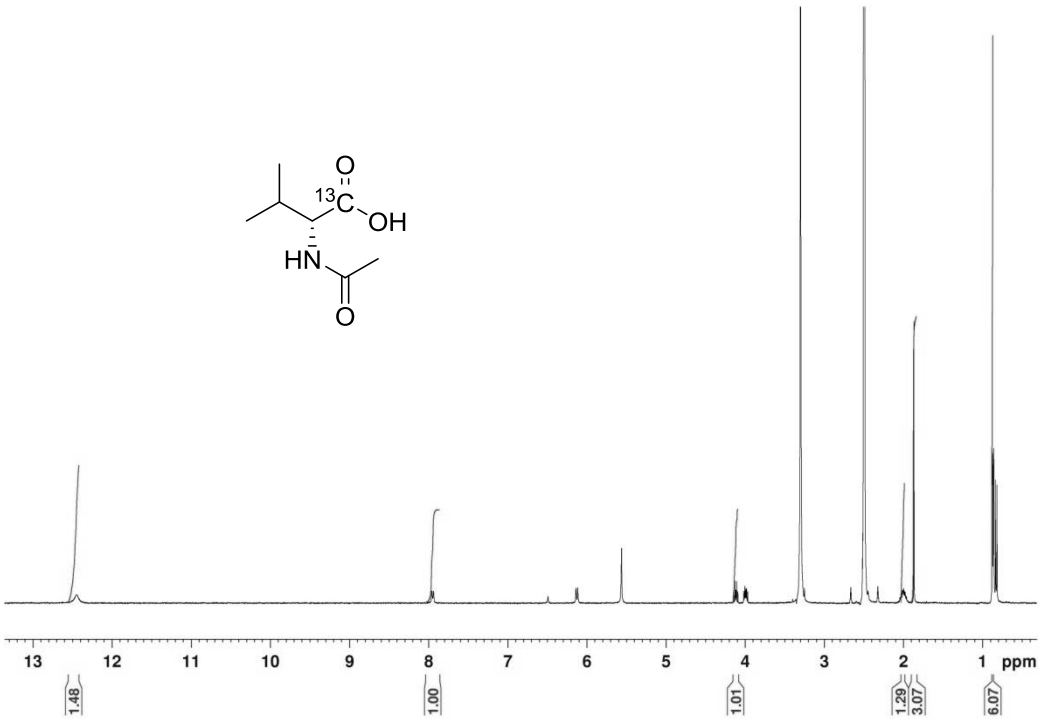
L-[1-^{13}C] Valine **12** (^{1}H; 400.13 MHz; D_2O, NaOD)



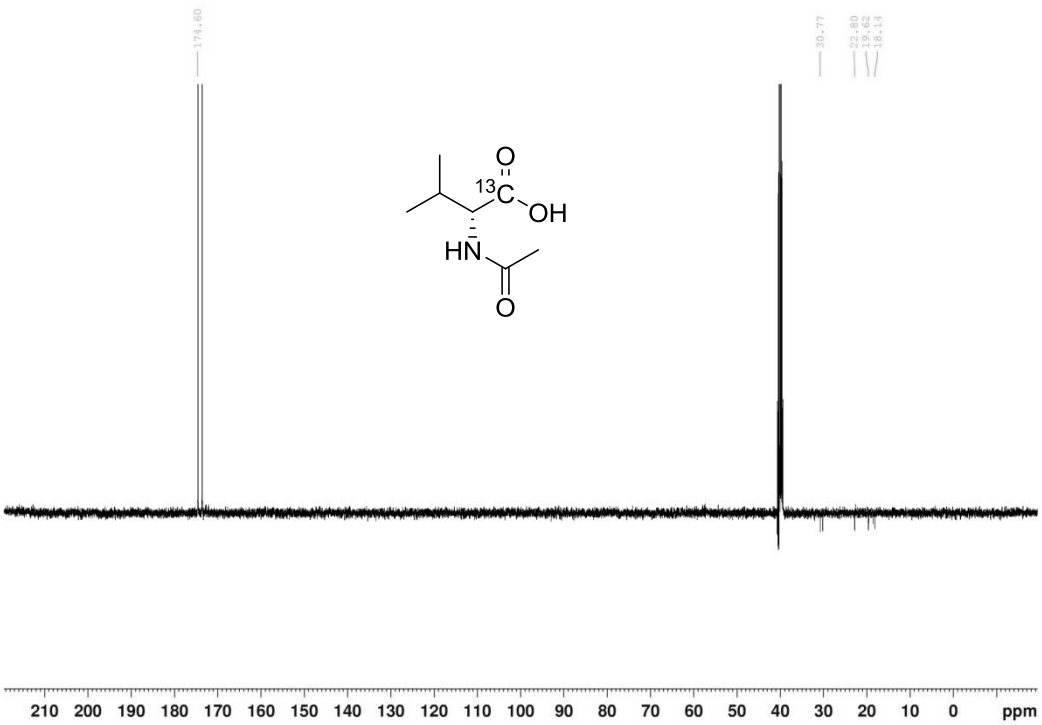
L-[1-^{13}C] Valine **12** (^{13}C; 100.61 MHz; D_2O, NaOD)



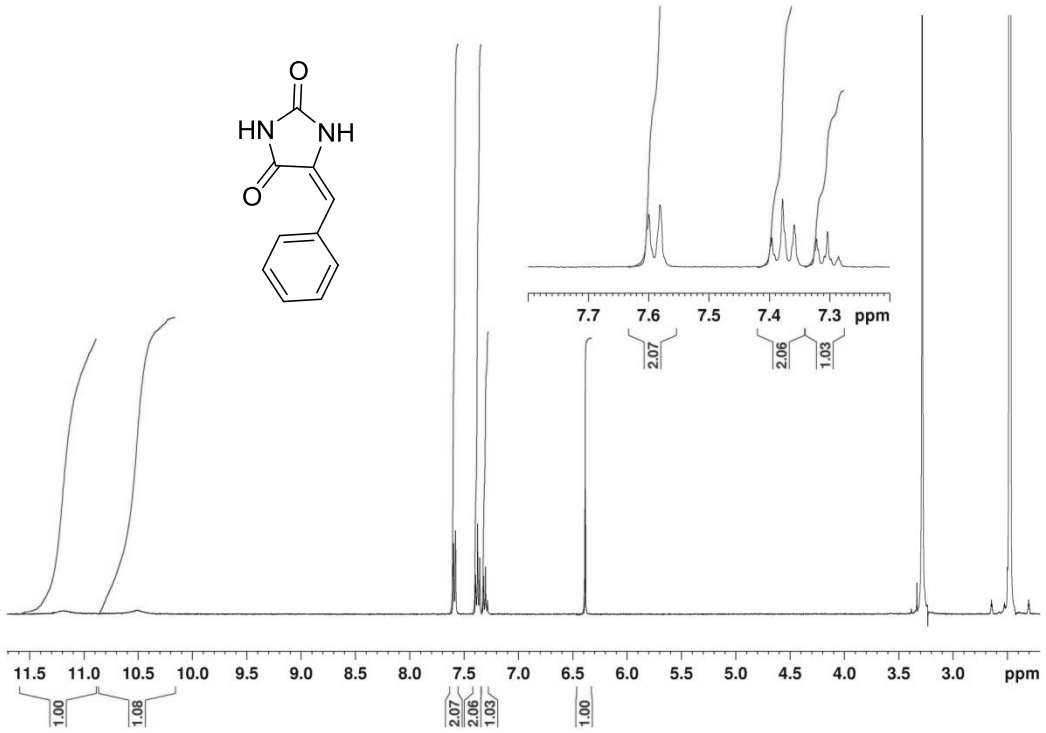
D-[1-^{13}C] *N*-Acetylvaline **13** (^{1}H; 400.13 MHz; 6d-DMSO)



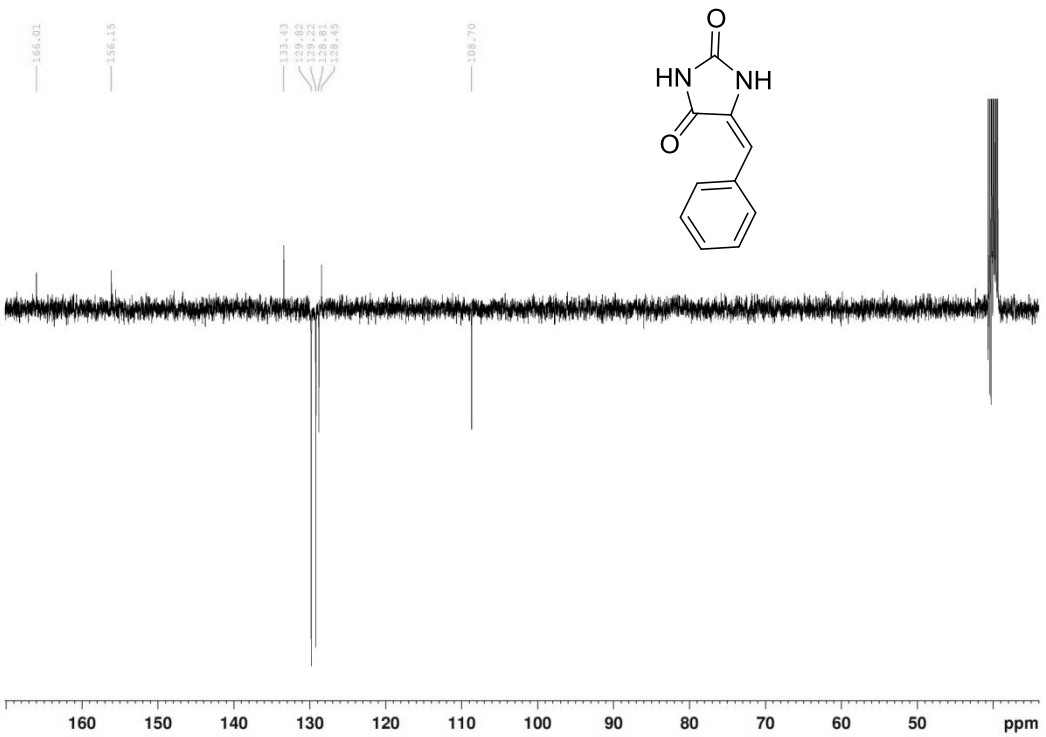
D-[1-^{13}C] *N*-Acetylvaline **13** (^{13}C; 100.61 MHz; 6d-DMSO)



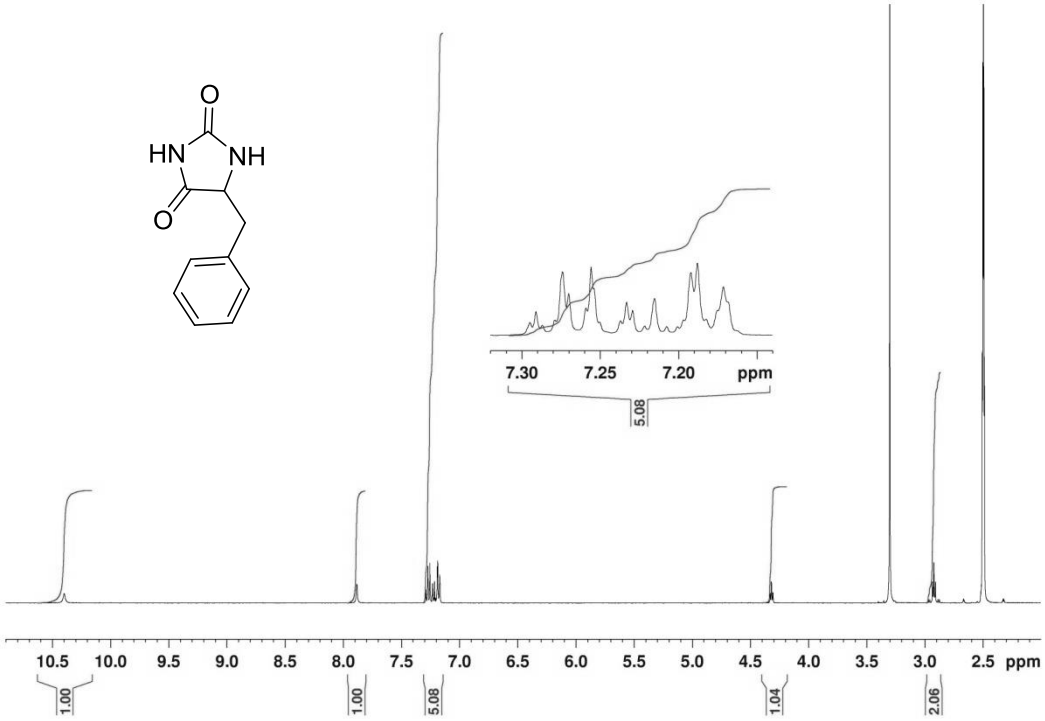
5-(Benzylidene)hydantoin **14** (^{1}H; 400.13 MHz; 6d-DMSO)



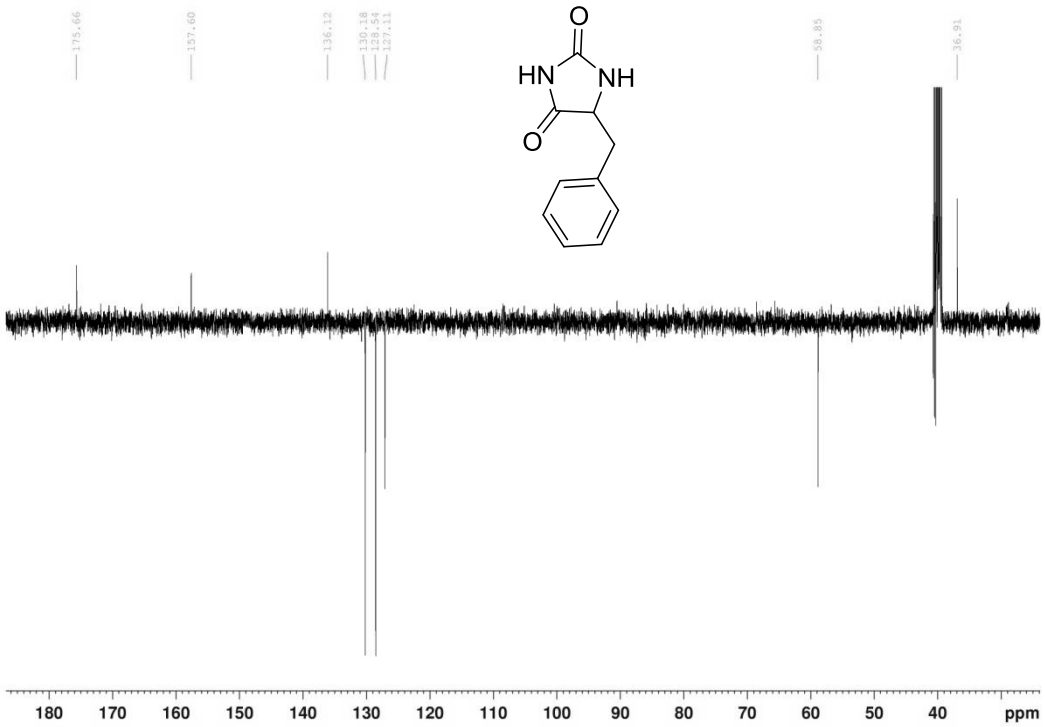
5-(Benzylidene)hydantoin **14** (^{13}C; 100.61 MHz; 6d-DMSO)



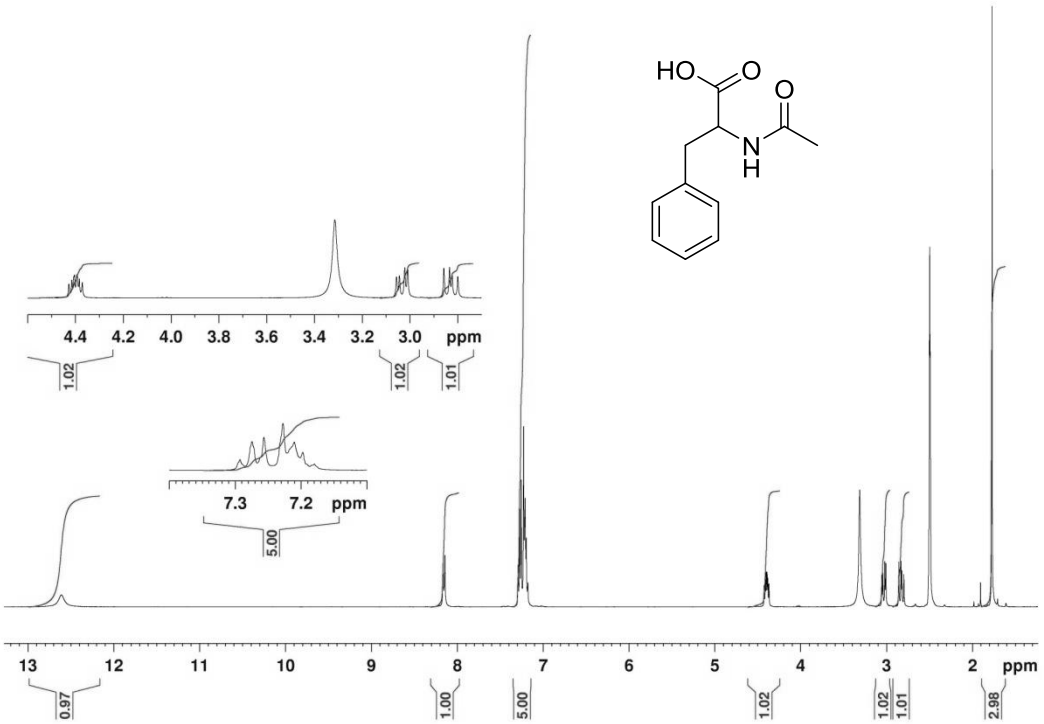
5-Benzylhydantoin **15** (^{1}H; 400.13 MHz; 6d-DMSO)



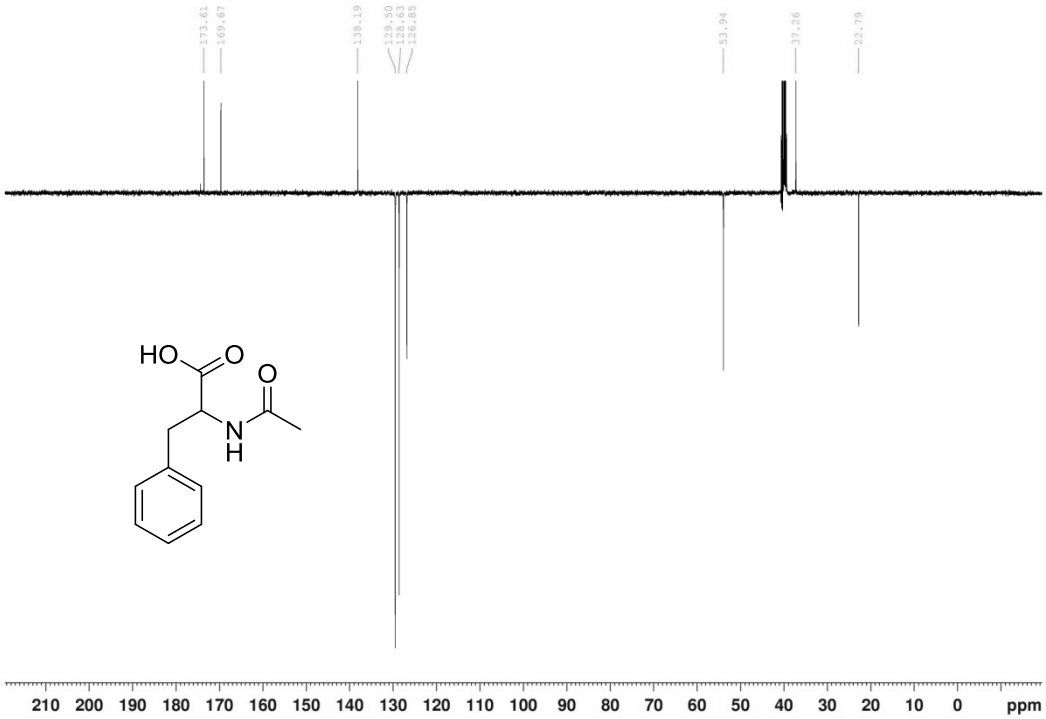
5-Benzylhydantoin **15** (^{13}C; 100.61 MHz; 6d-DMSO)



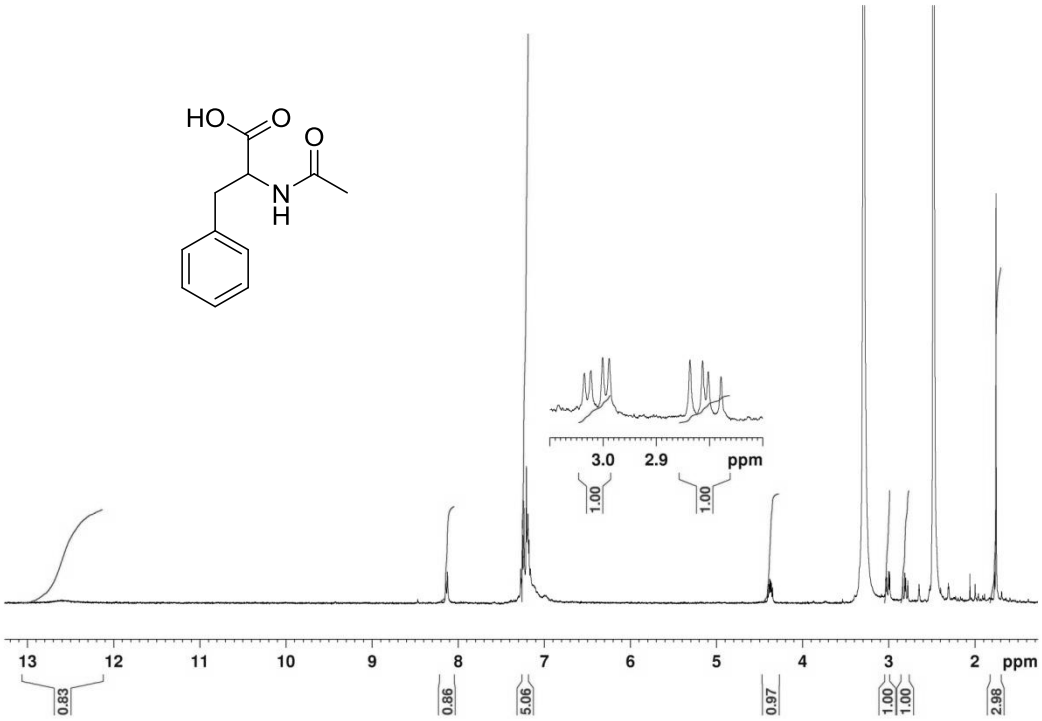
N-Acetylphenylalanine **16** (^{1}H; 400.13 MHz; 6d-DMSO)



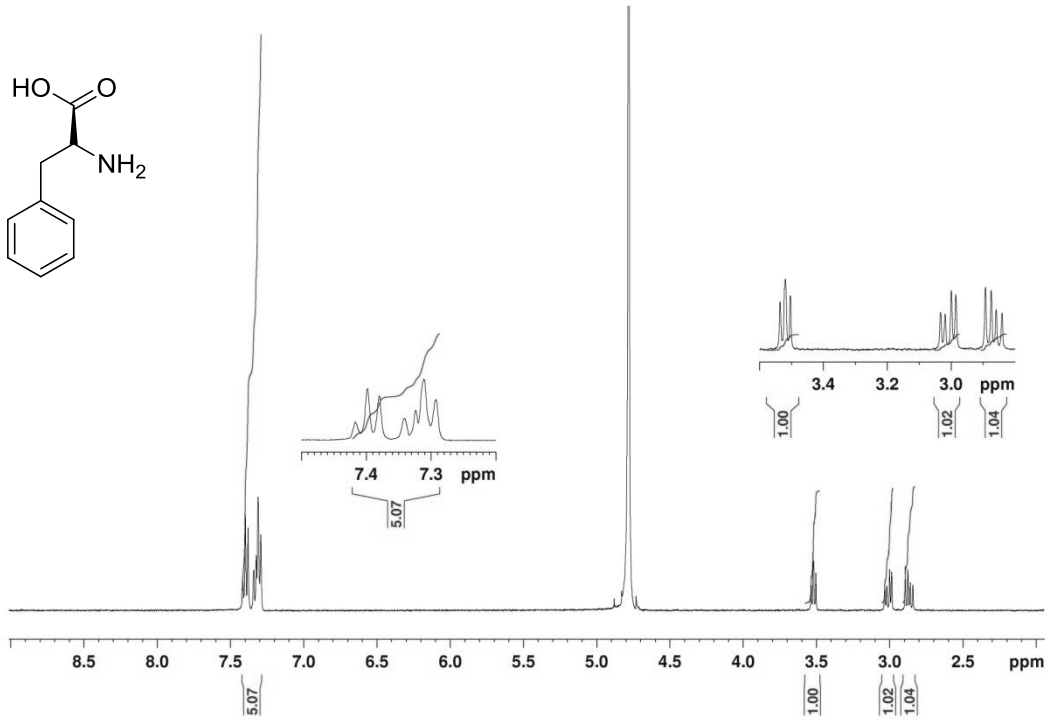
N-Acetylphenylalanine **16** (^{13}C; 100.61 MHz; 6d-DMSO)



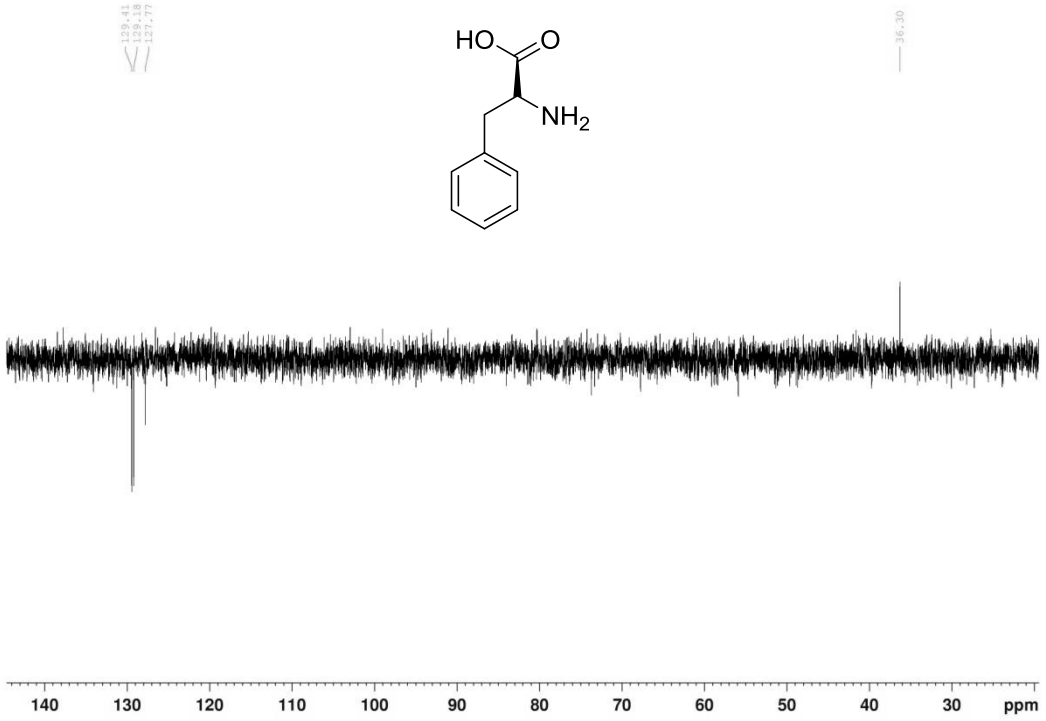
N-Acetylphenylalanine **16** (^{1}H; 400.13 MHz; 6d-DMSO) after racemization



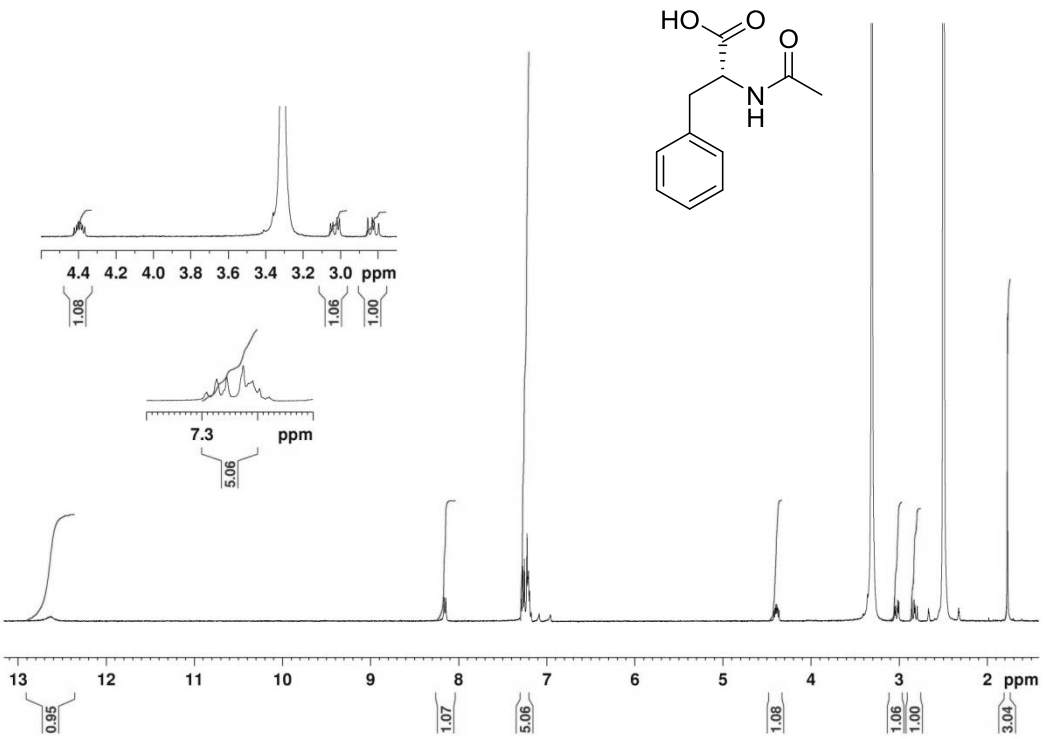
L-Phenylalanine **17** (^{1}H; 400.13 MHz; D_2O, NaOD)



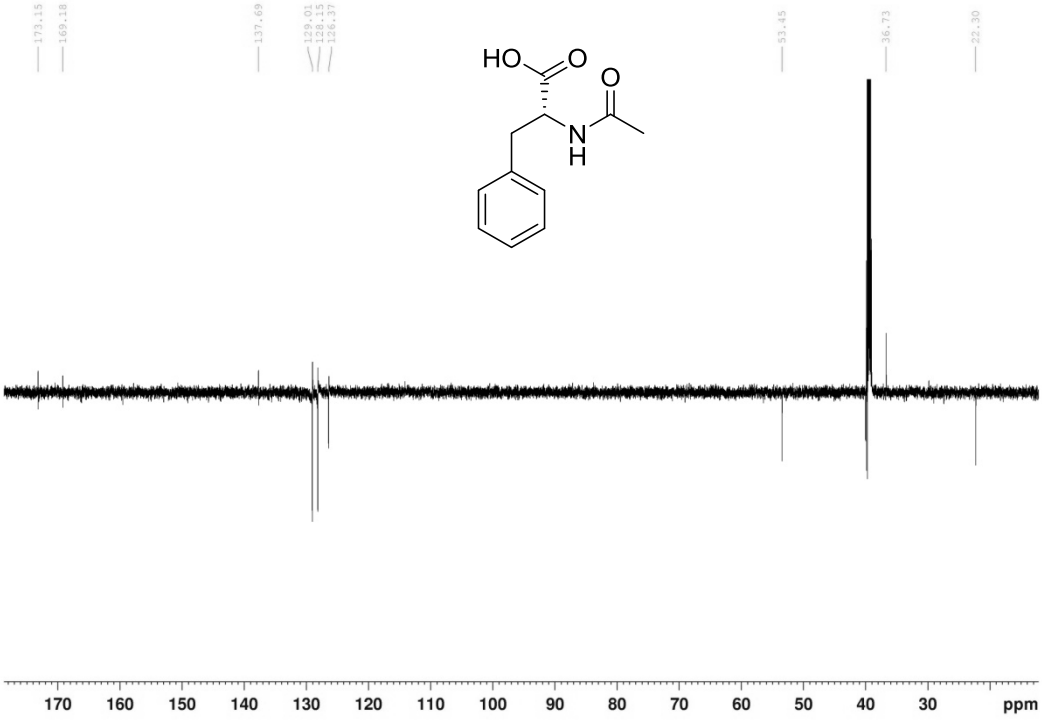
L-Phenylalanine **17** (^{13}C; 100.61 MHz; D_2O, NaOD)



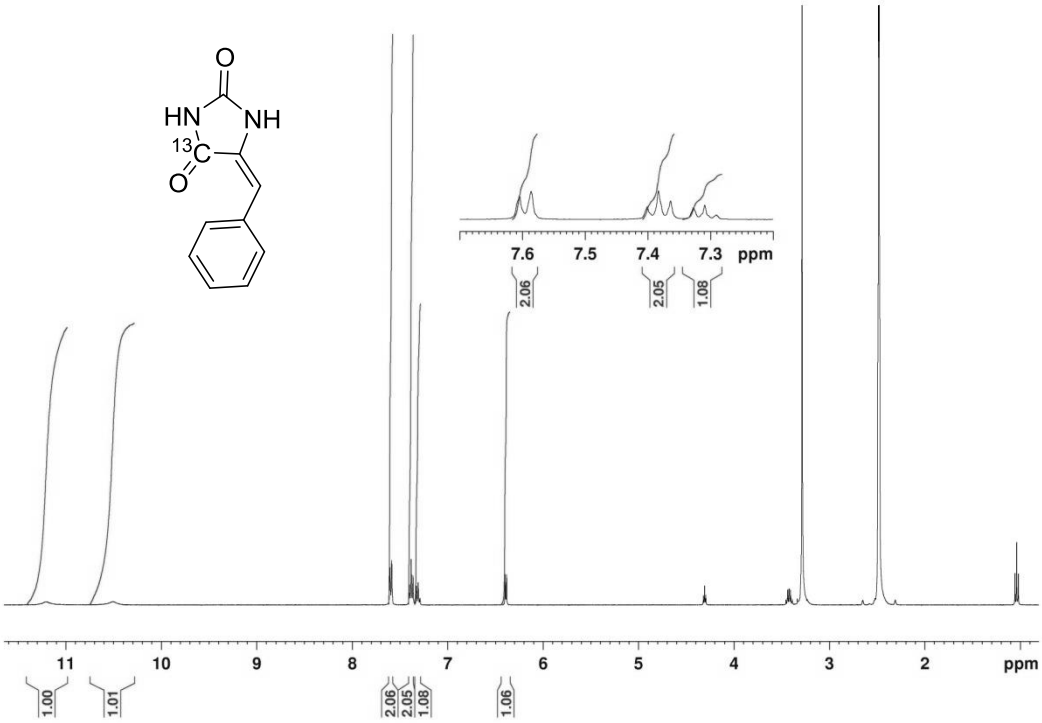
N-Acetyl D-phenylalanine **18** (^{1}H; 400.13 MHz; 6d-DMSO)



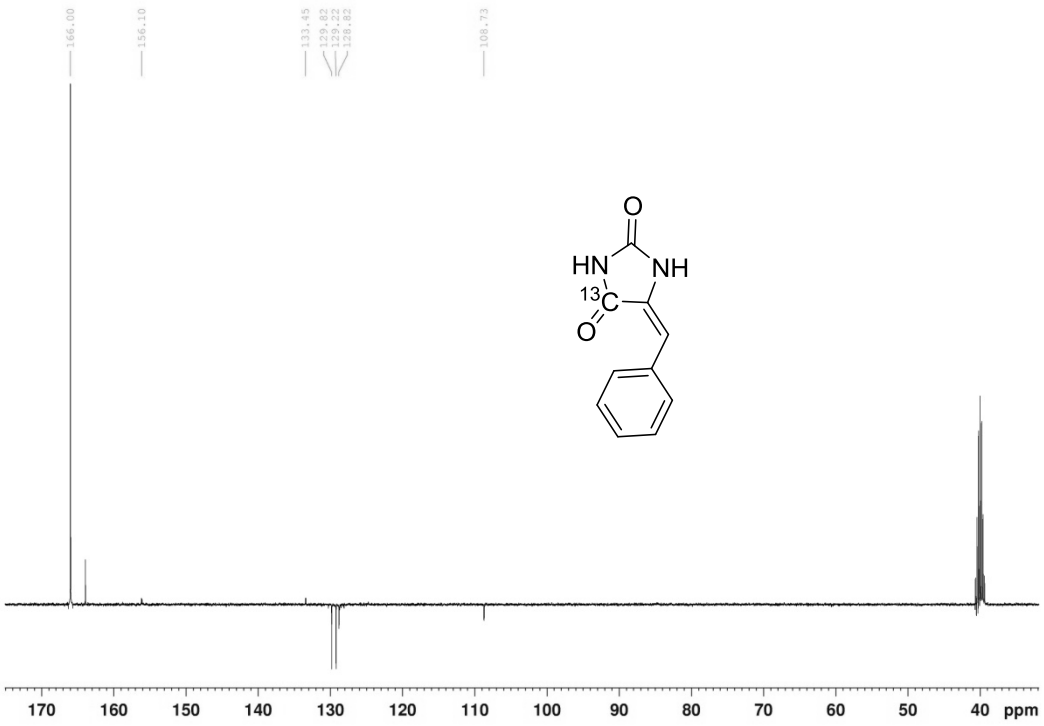
N-Acetyl-D-phenylalanine **18** (^{13}C; 150.90 MHz; 6d-DMSO)



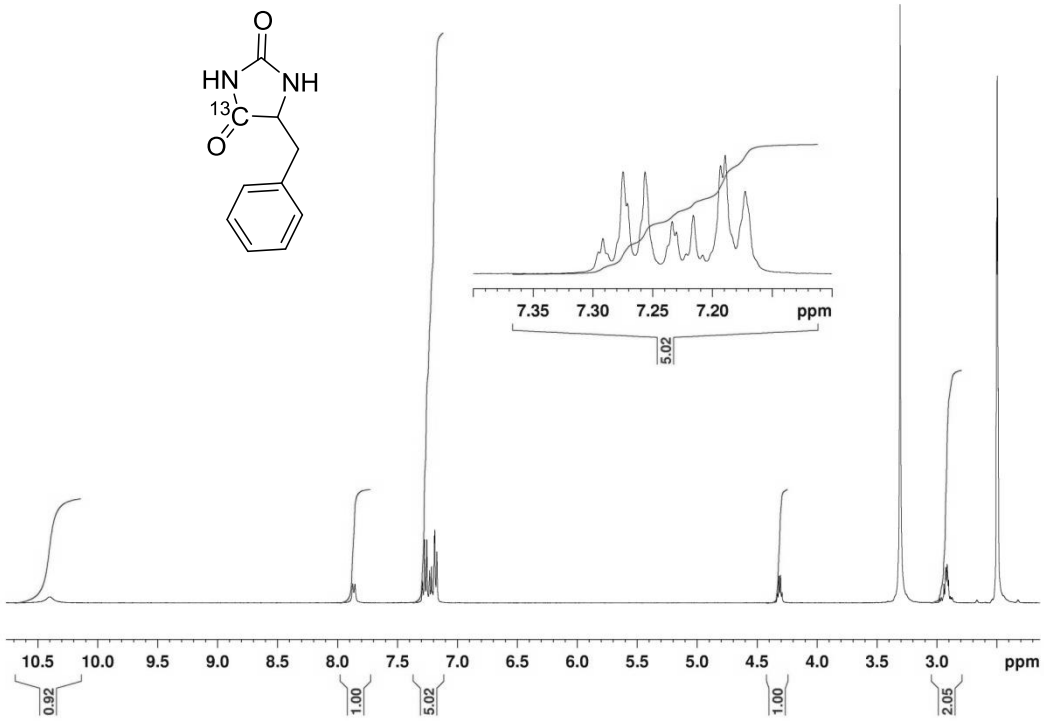
[1-^{13}C] 5-(Benzylidene)hydantoin **19** (^{13}C; 100.61 MHz; 6d-DMSO)



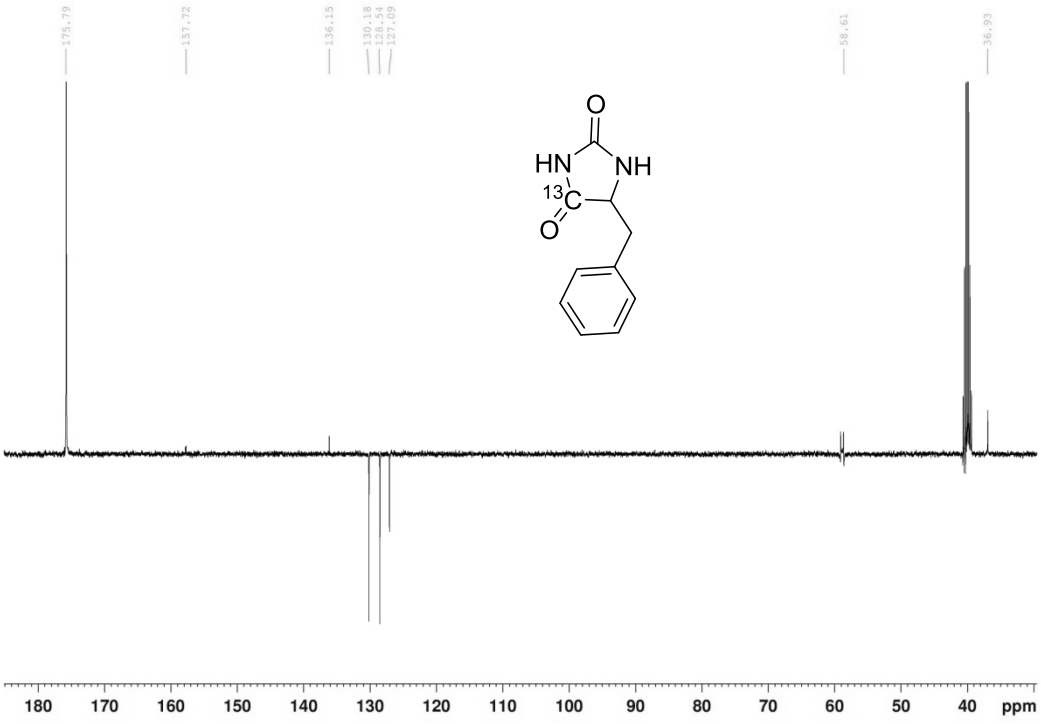
[1-^{13}C] 5-(Benzylidene)hydantoin **19** (^{13}C; 100.61 MHz; 6d-DMSO)



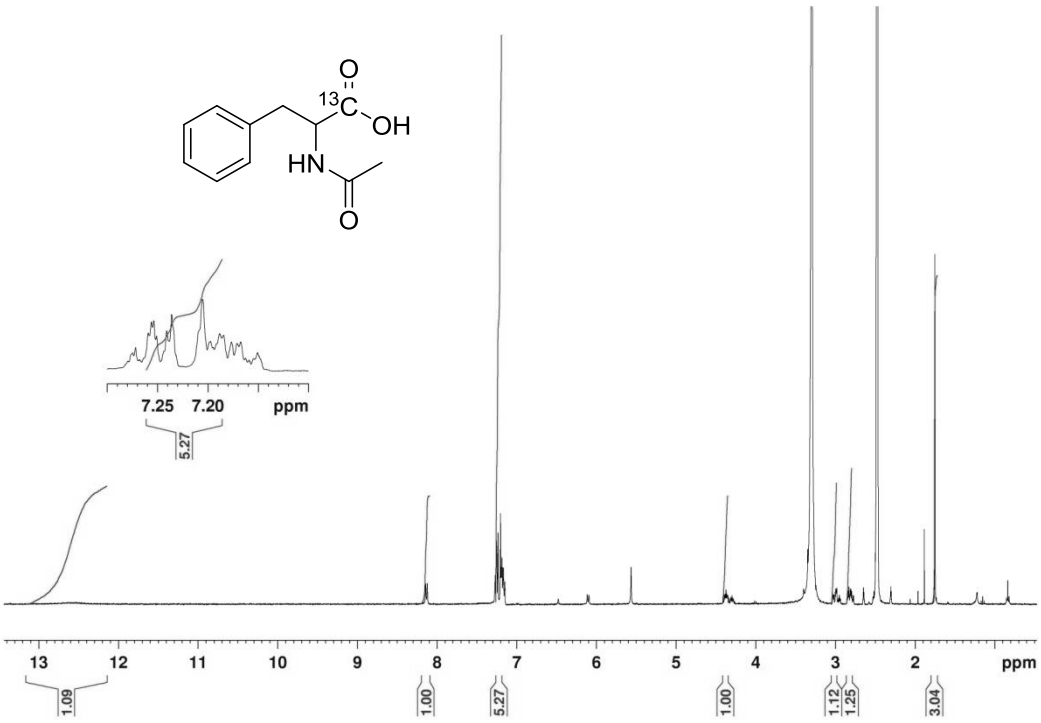
[1-^{13}C] 5-Benzylhydantoin **20** (^{1}H; 400.13 MHz; 6d-DMSO)



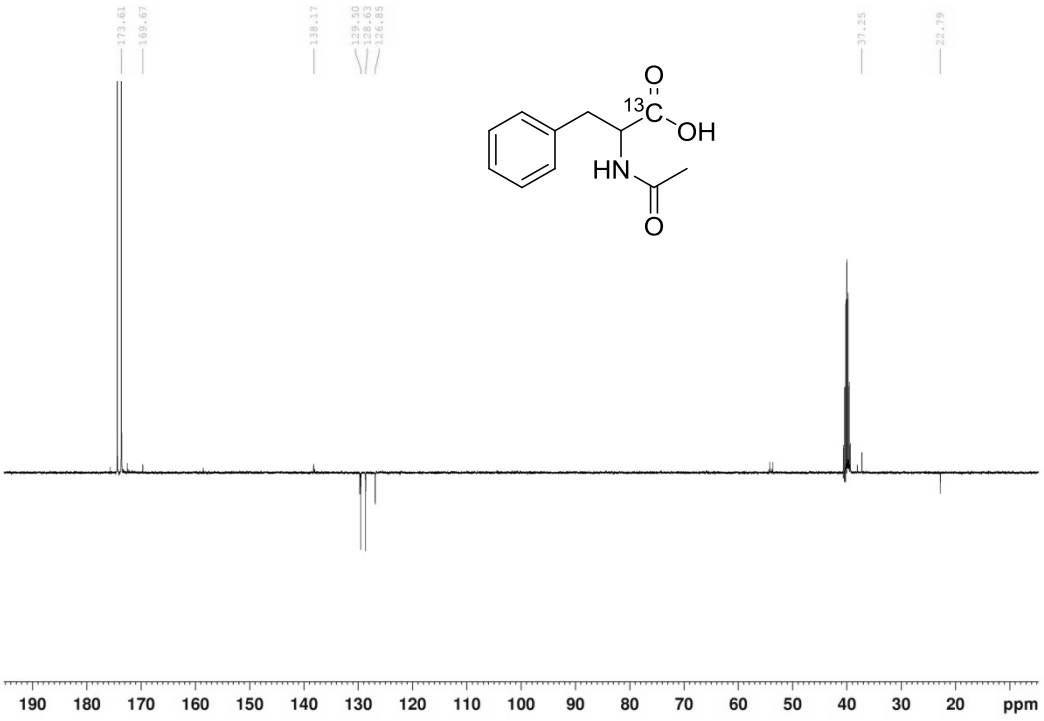
[1-^{13}C] 5-Benzylhydantoin **20** (^{13}C; 100.61 MHz; 6d-DMSO)



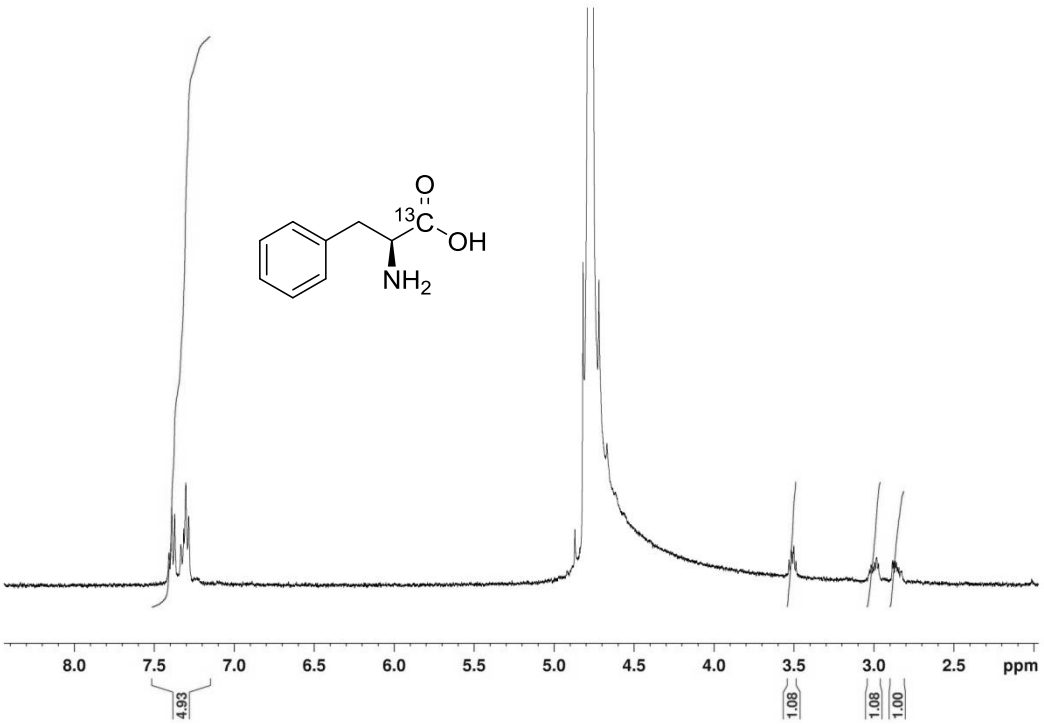
[1-^{13}C] *N*-Acetylphenylalanine **21** (^{1}H; 400.13 MHz; 6d-DMSO)



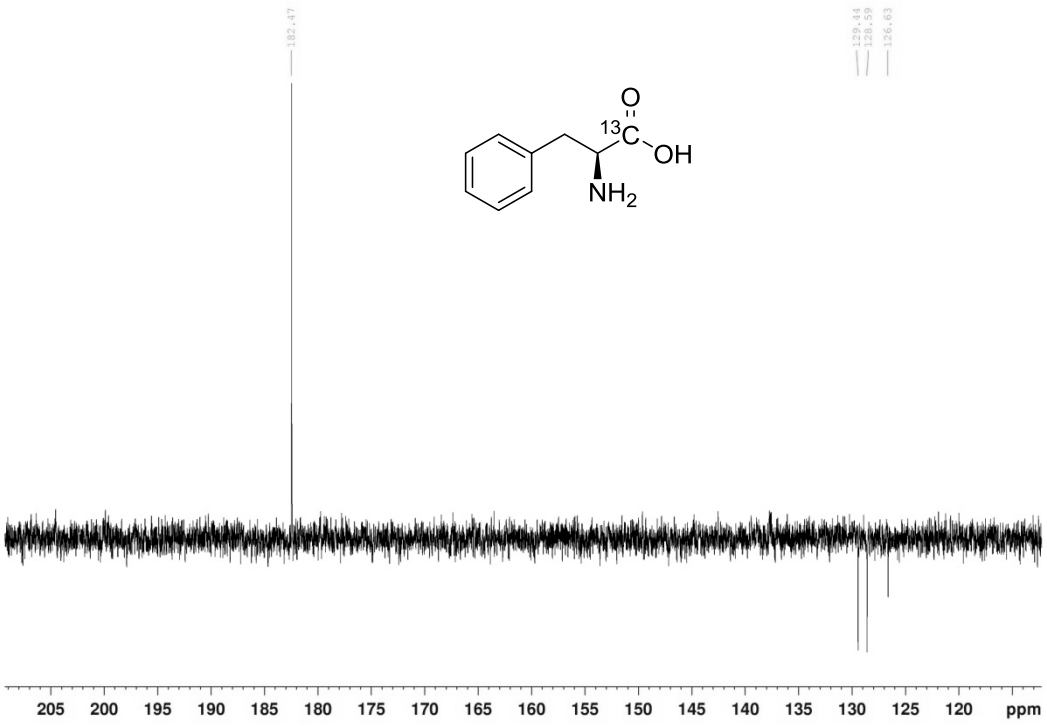
[1-^{13}C] *N*-Acetylphenylalanine **21** (^{13}C; 100.61 MHz; 6d-DMSO)



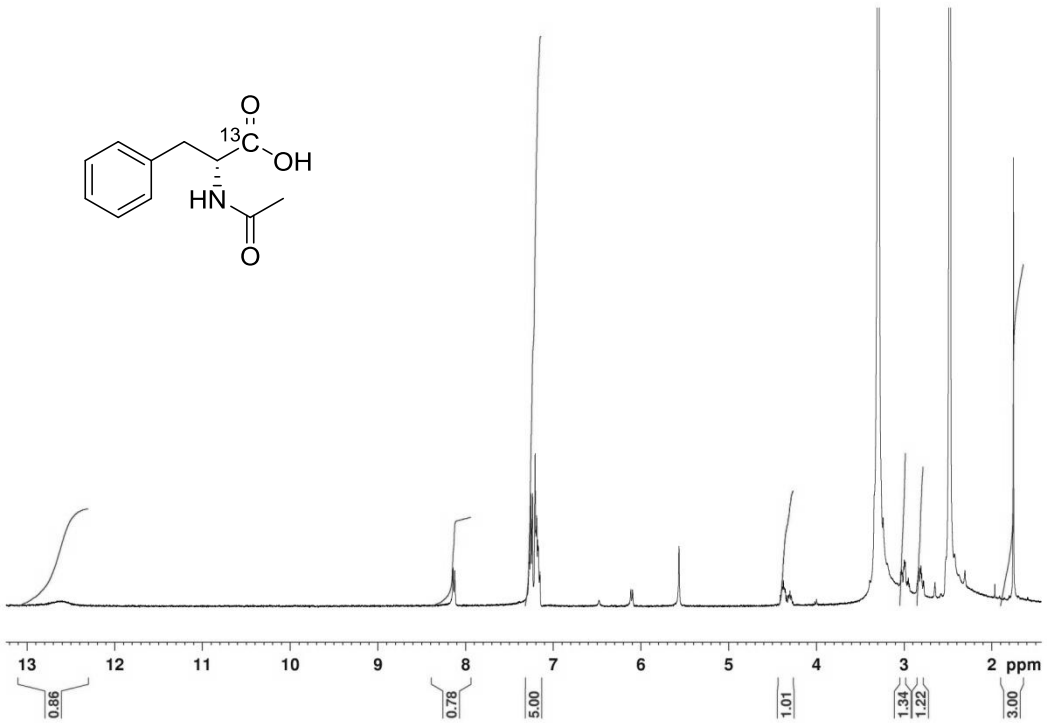
[1-^{13}C] L-Phenylalanine **22** (^{1}H; 400.13 MHz; D_2O, NaOD)



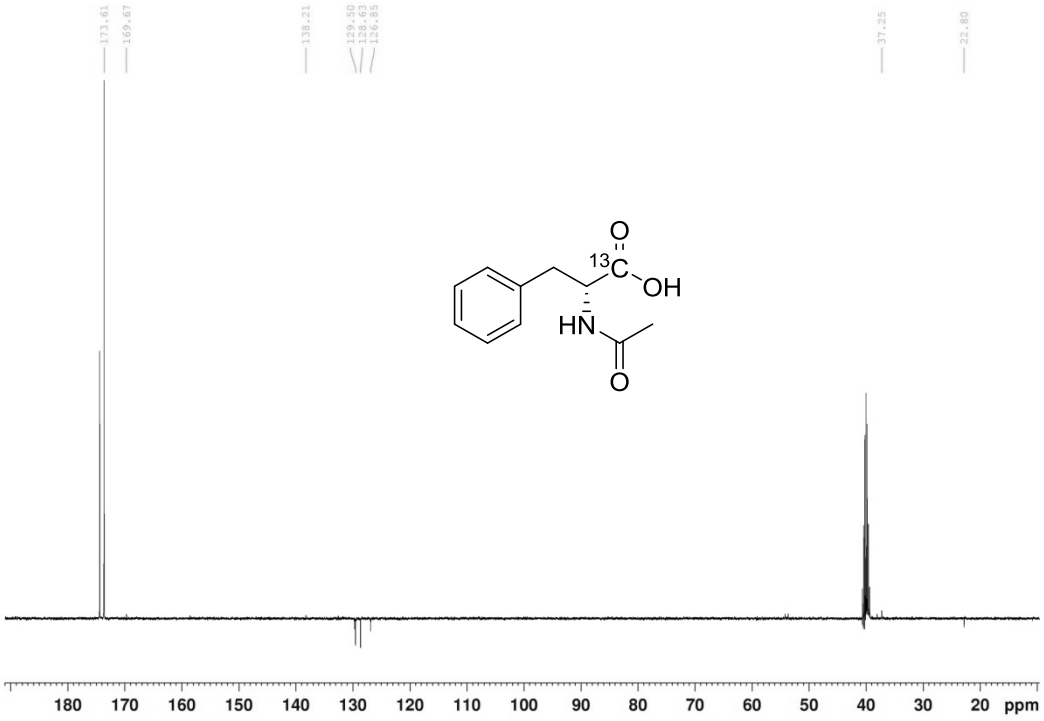
[1-^{13}C] L-Phenylalanine **22** (^{13}C; 100.61 MHz; D_2O, NaOD)



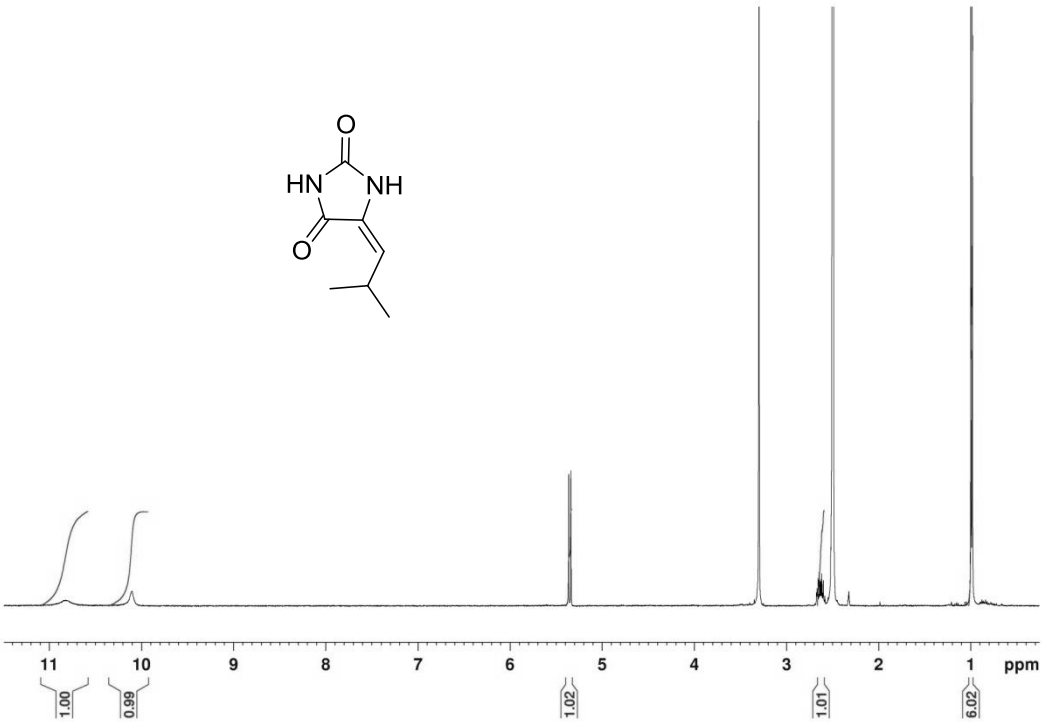
[1-^{13}C] *N*-Acetyl-D-phenylalanine **23** (^{1}H; 400.13 MHz; 6d-DMSO)



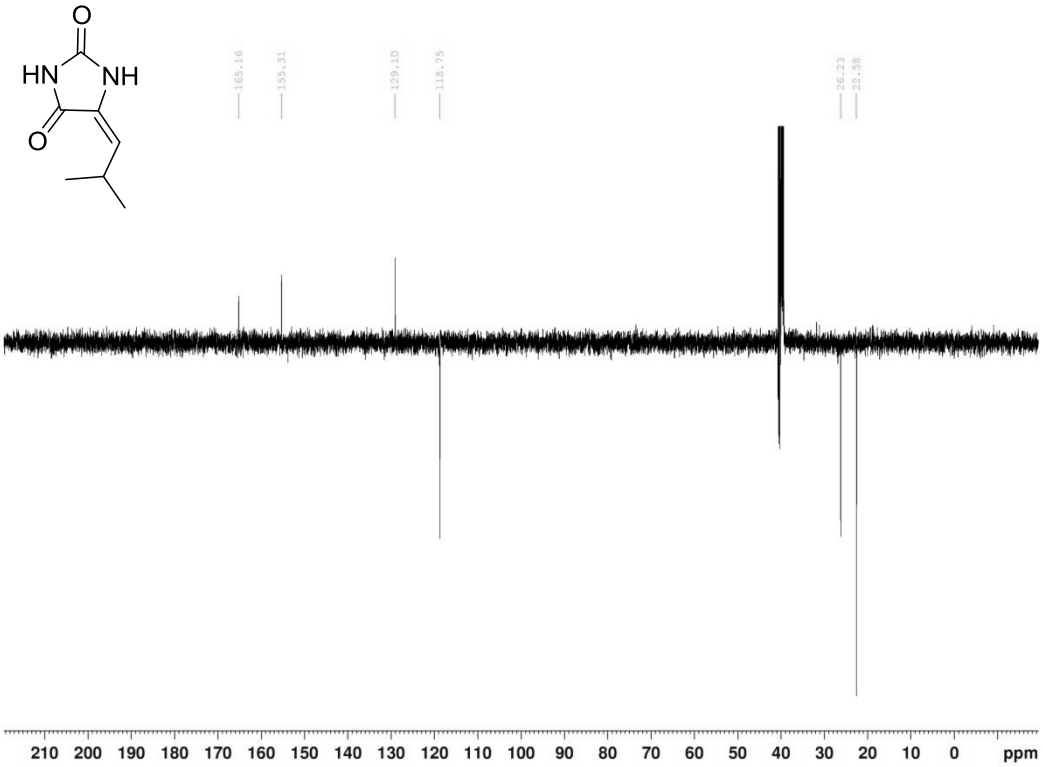
[1-^{13}C] *N*-Acetyl-D-phenylalanine **23** (^{13}C; 100.61 MHz; 6d-DMSO)



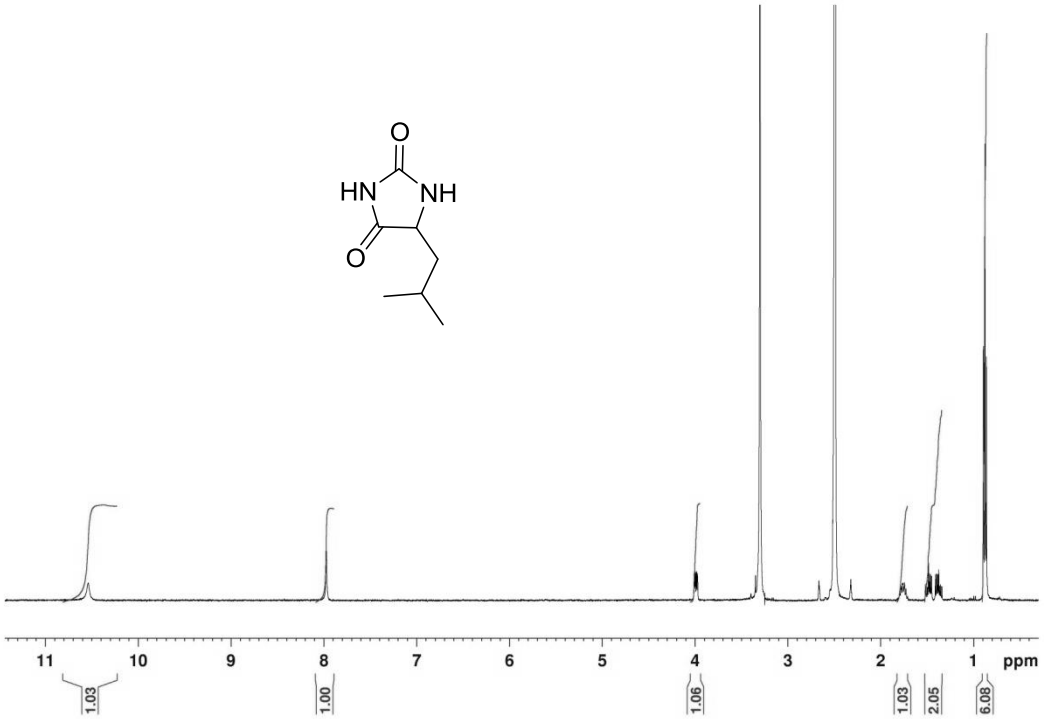
5-(2-Methylpropylidene)hydantoin **24** (^{1}H; 400.13 MHz; 6d-DMSO)



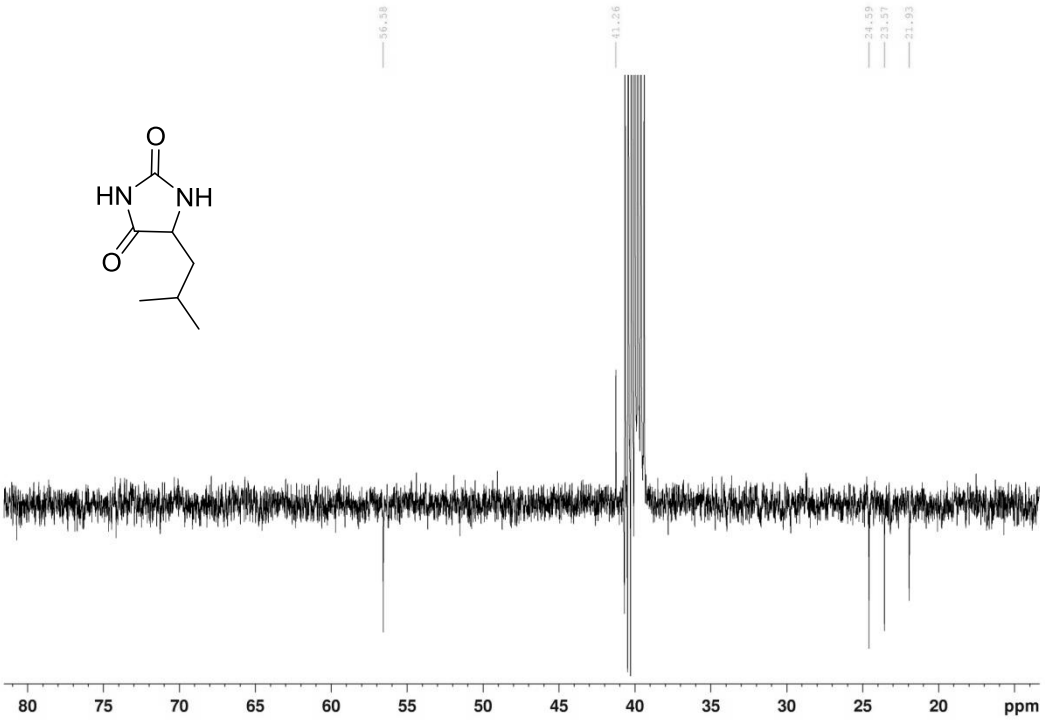
5-(2-Methylpropylidene)hydantoin **24** (^{13}C; 100.61 MHz; 6d-DMSO)



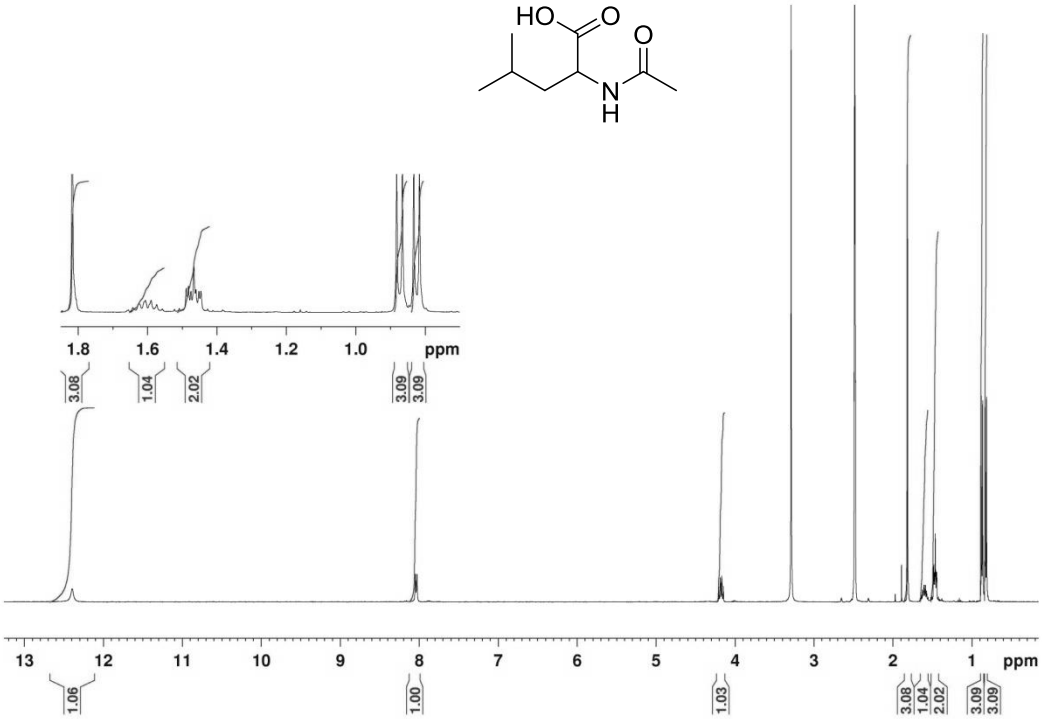
5-Isobutylhydantoin **25** (^{1}H; 400.13 MHz; 6d-DMSO)



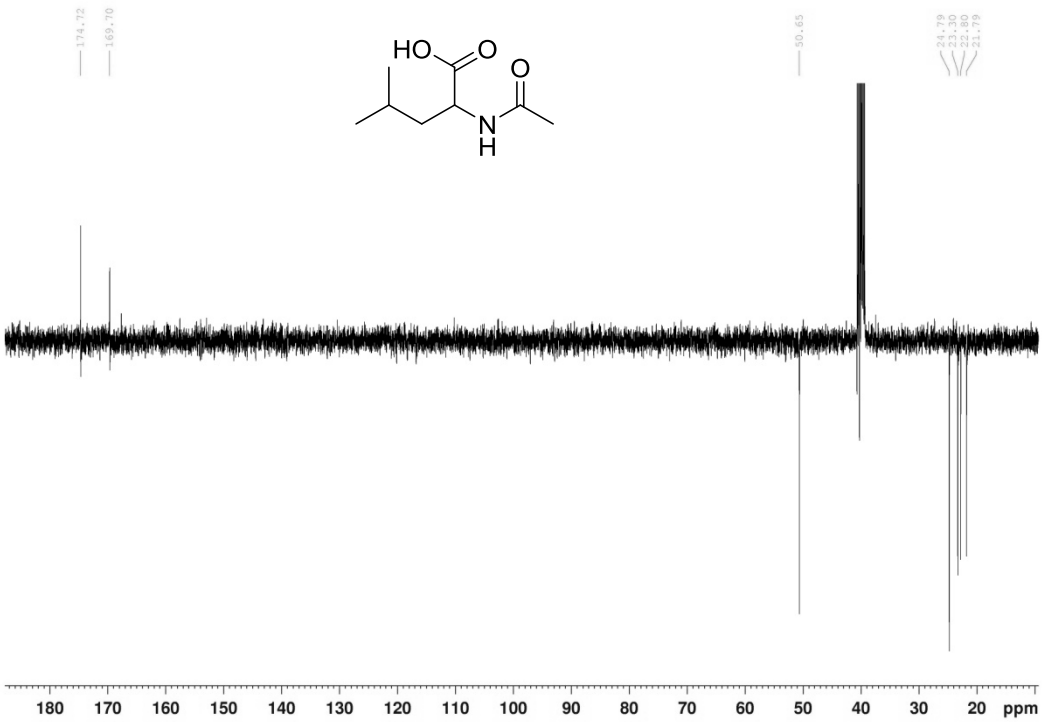
5-Isobutylhydantoin **25** (^{13}C; 100.61 MHz; 6d-DMSO)



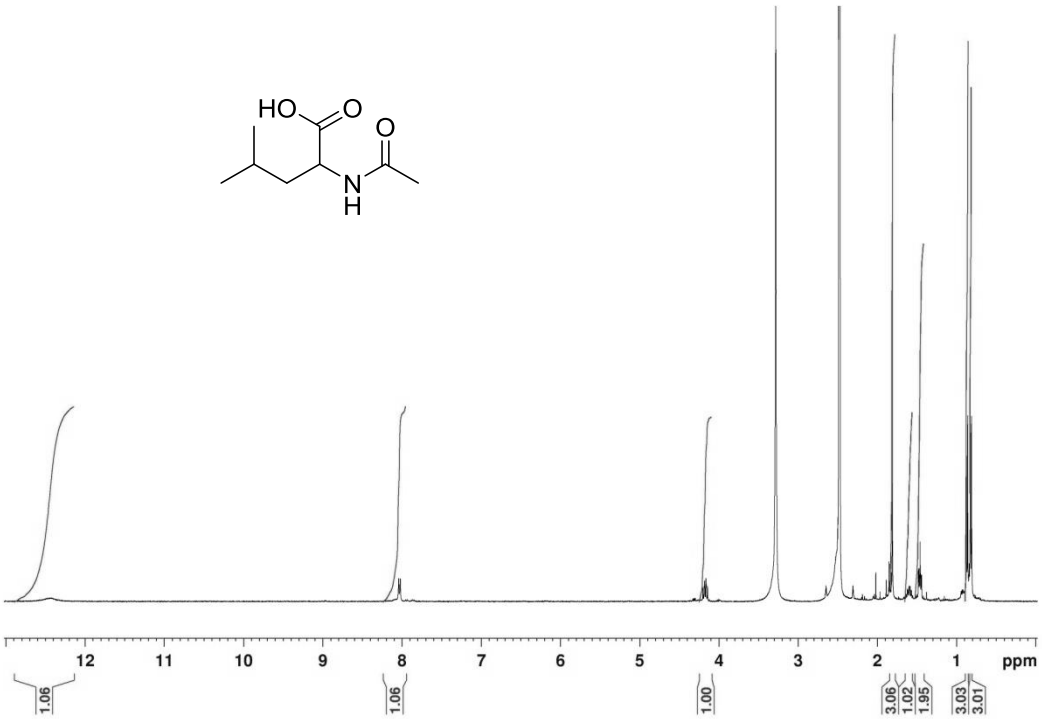
N-Acetylleucine **26** (^{1}H; 400.13 MHz; 6d-DMSO)



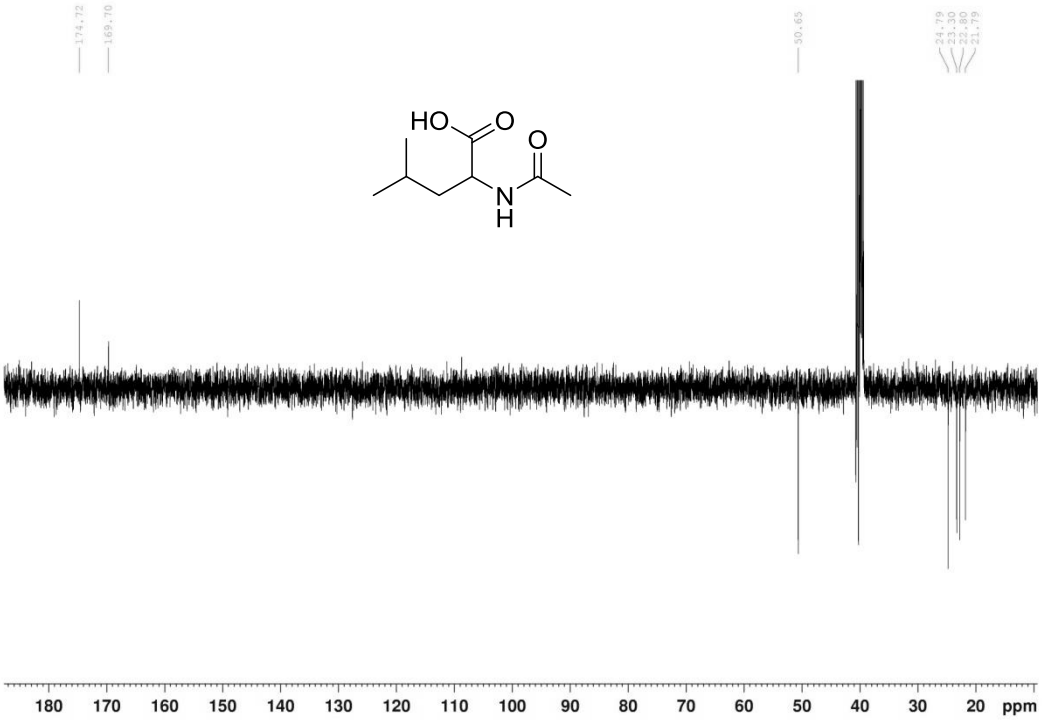
N-Acetylleucine **26** (^{13}C; 100.61 MHz; 6d-DMSO)



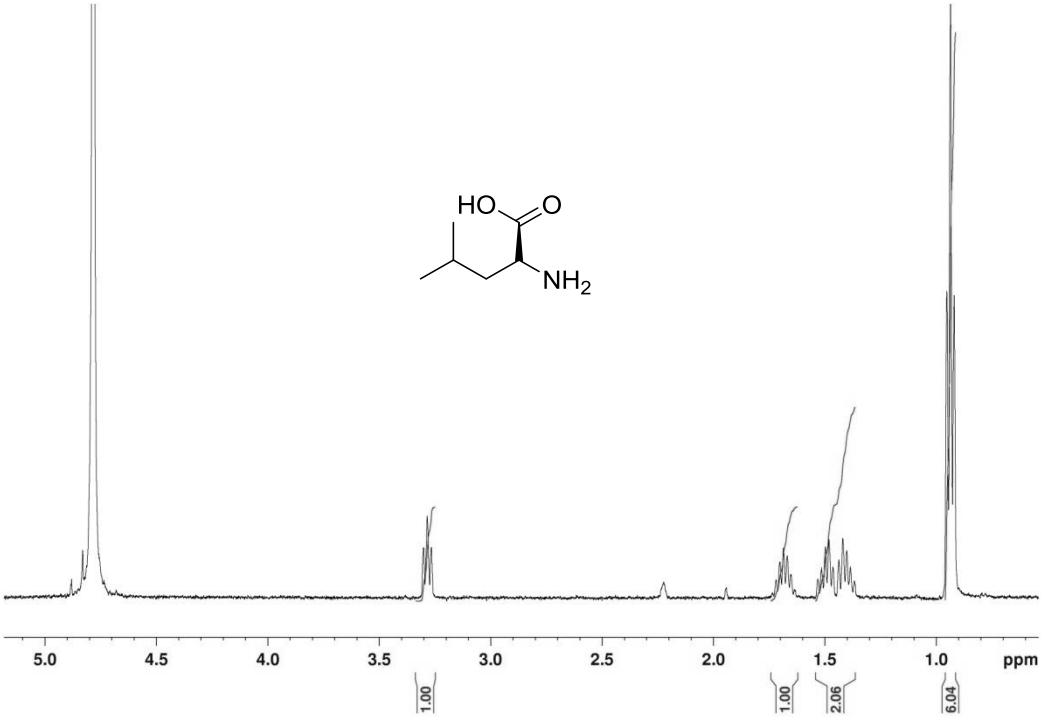
N-Acetylleucine **26** (^{1}H; 400.13 MHz; 6d-DMSO) after racemization



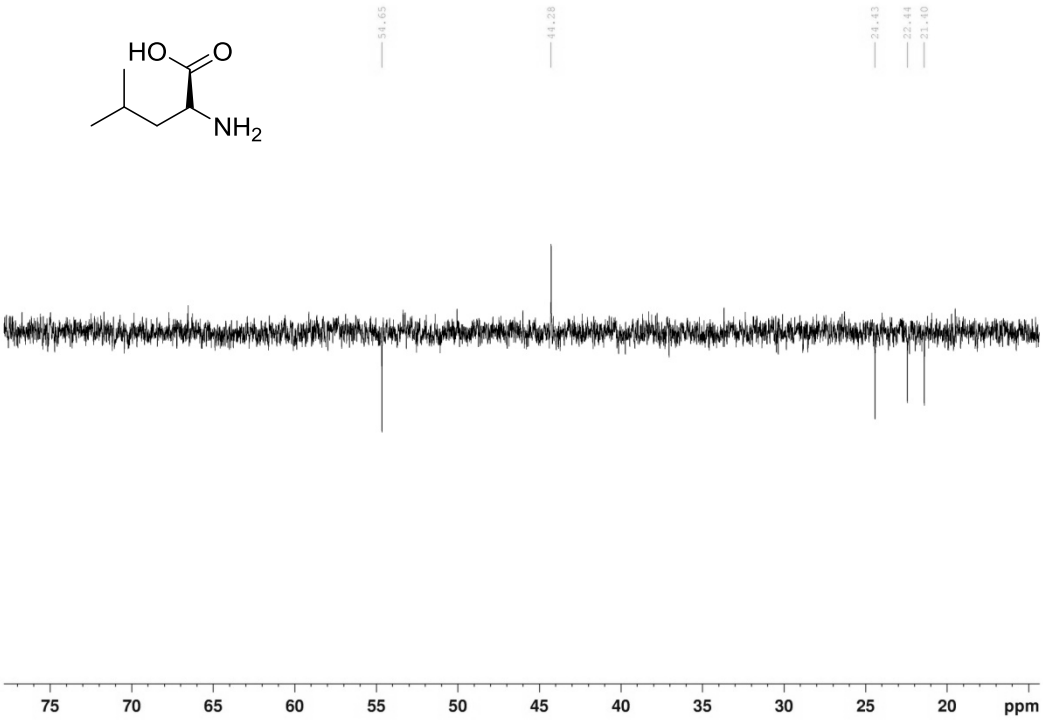
N-Acetylleucine **26** (^{13}C; 100.61 MHz; 6d-DMSO) after racemization



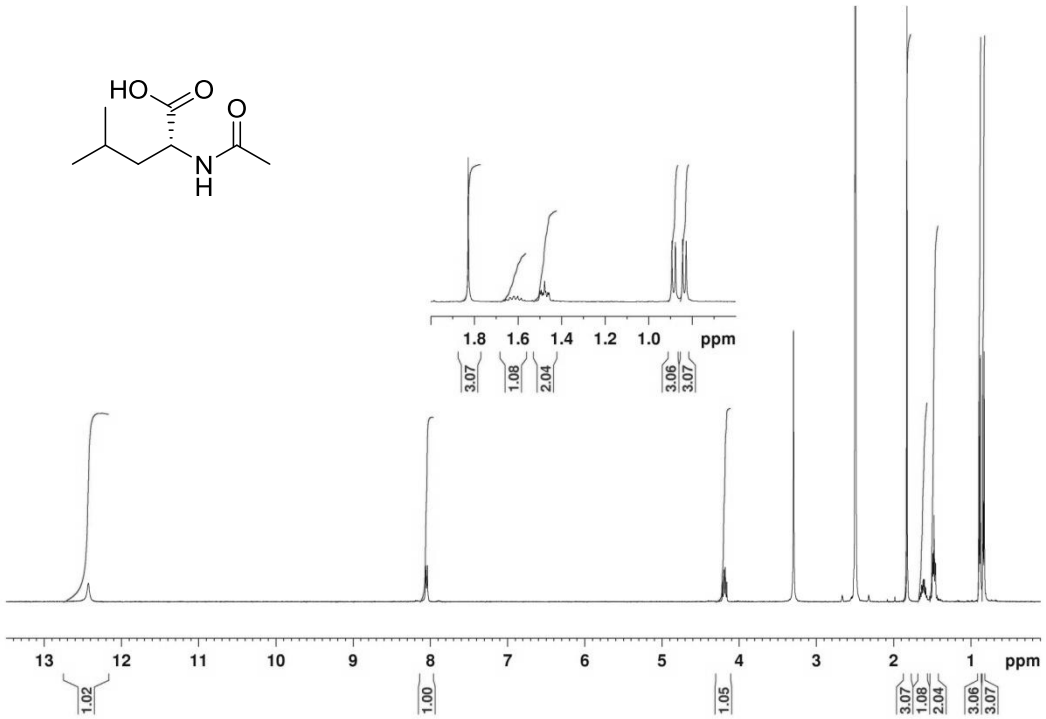
L-Leucine **27** (^{1}H; 400.13 MHz; D_2O, NaOD)



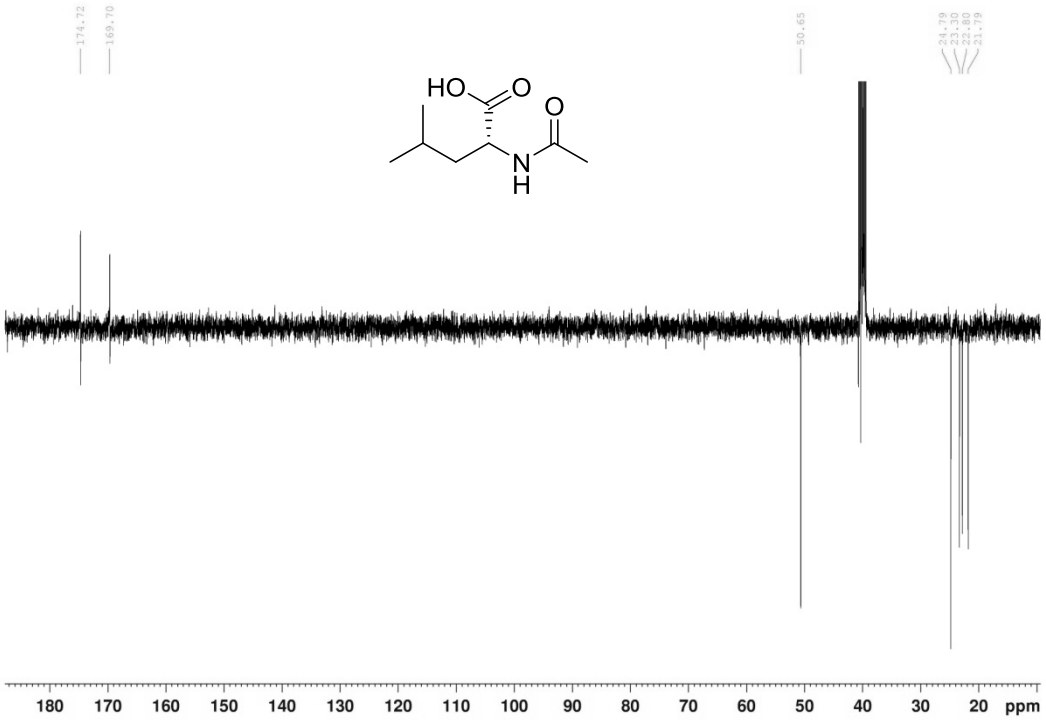
L-Leucine **27** (^{13}C; 100.61 MHz; D_2O, NaOD)



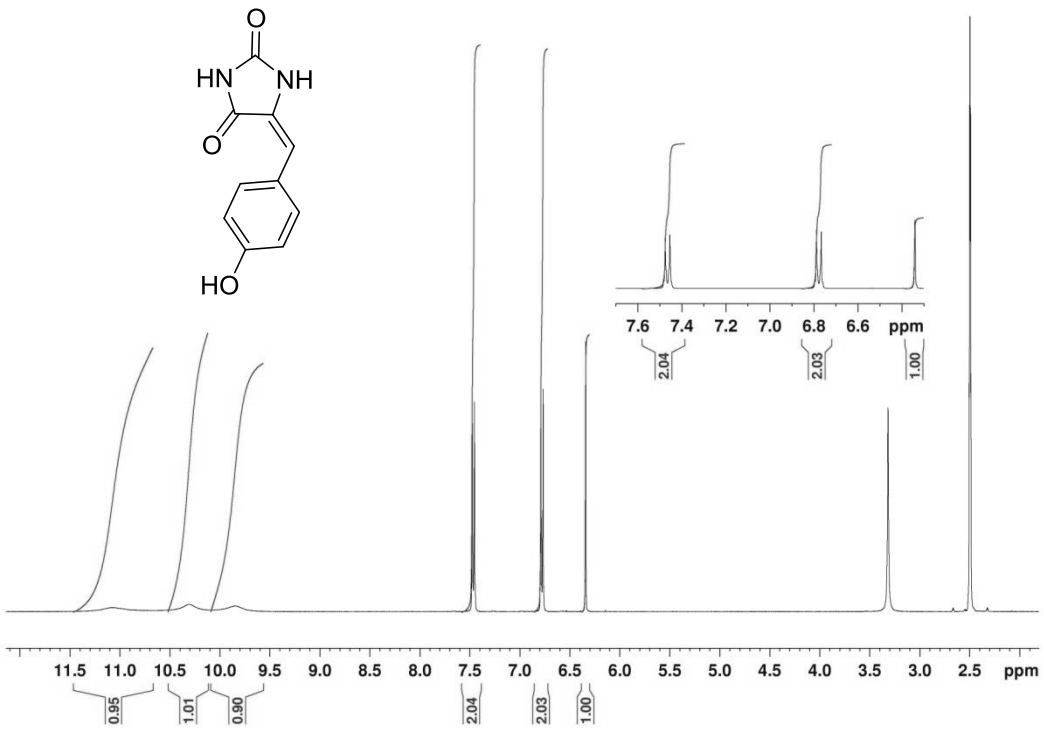
N-Acetyl-D-leucine **28** (^{1}H; 400.13 MHz; 6d-DMSO)



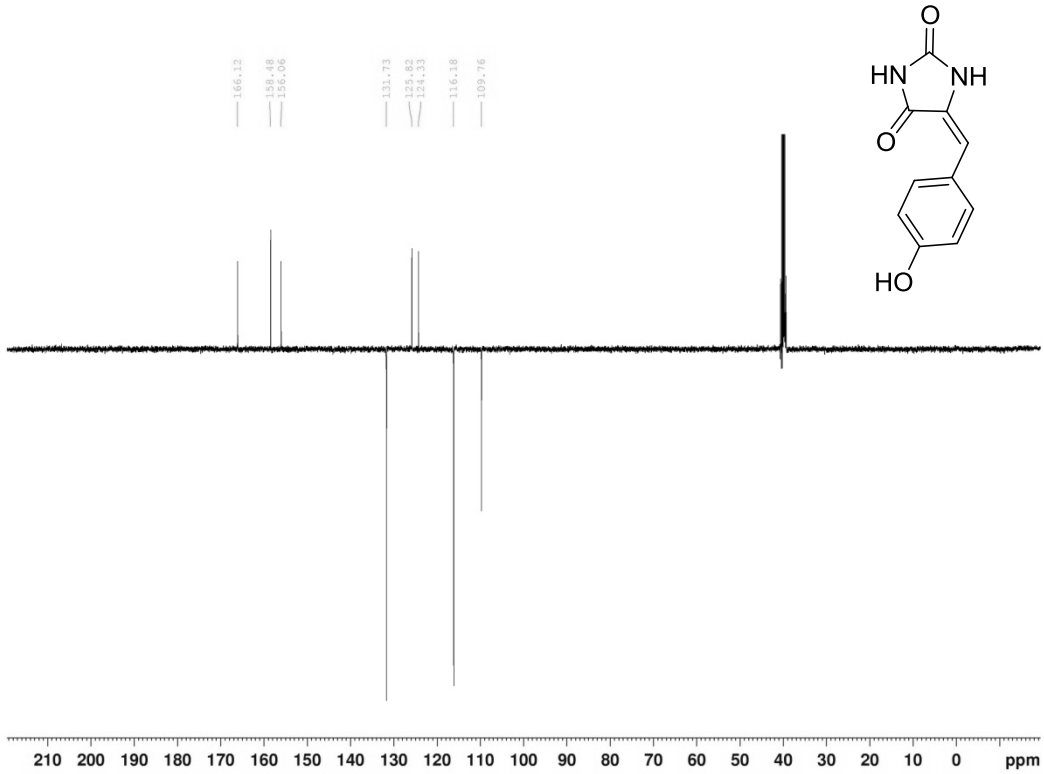
N-Acetyl-D-leucine **28** (^{13}C; 100.61 MHz; 6d-DMSO)



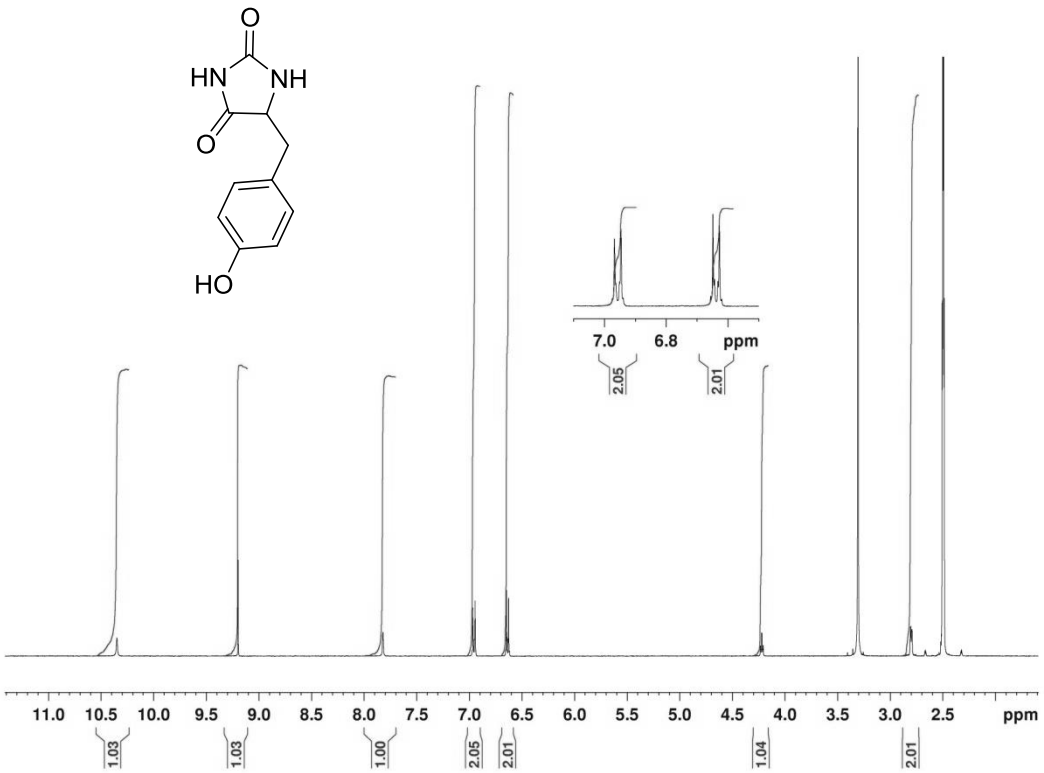
5-(4-Hydroxybenzylidene)hydantoin **29** (^{1}H; 400.13 MHz; 6d-DMSO)



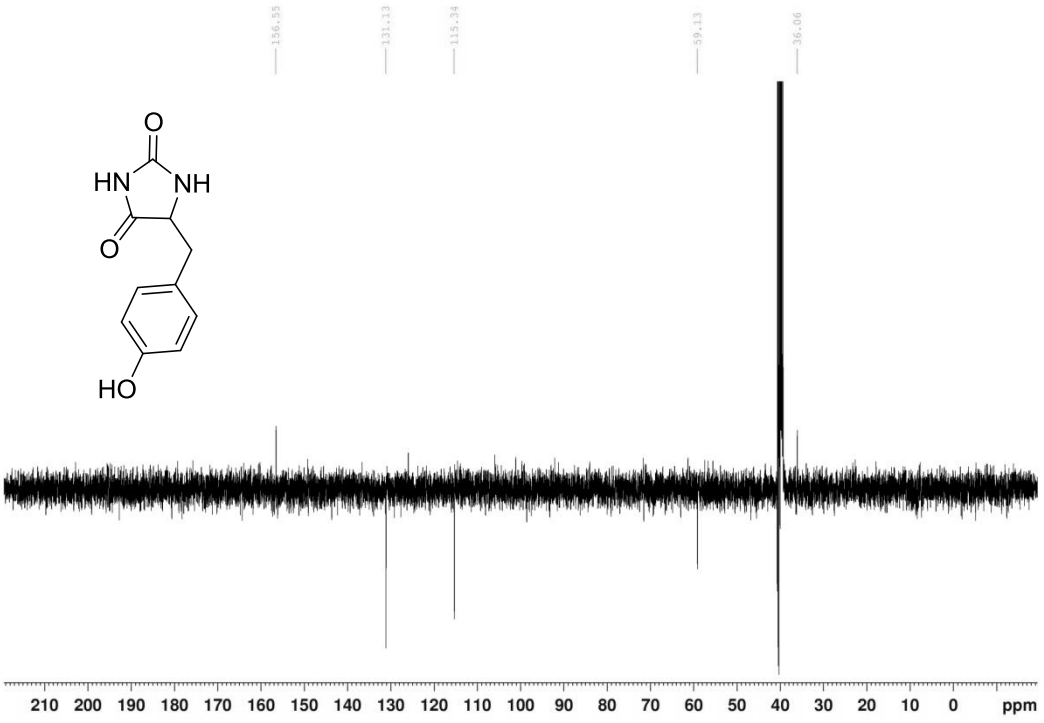
5-(4-Hydroxybenzylidene)hydantoin **29** (^{13}C; 100.61 MHz; 6d-DMSO)



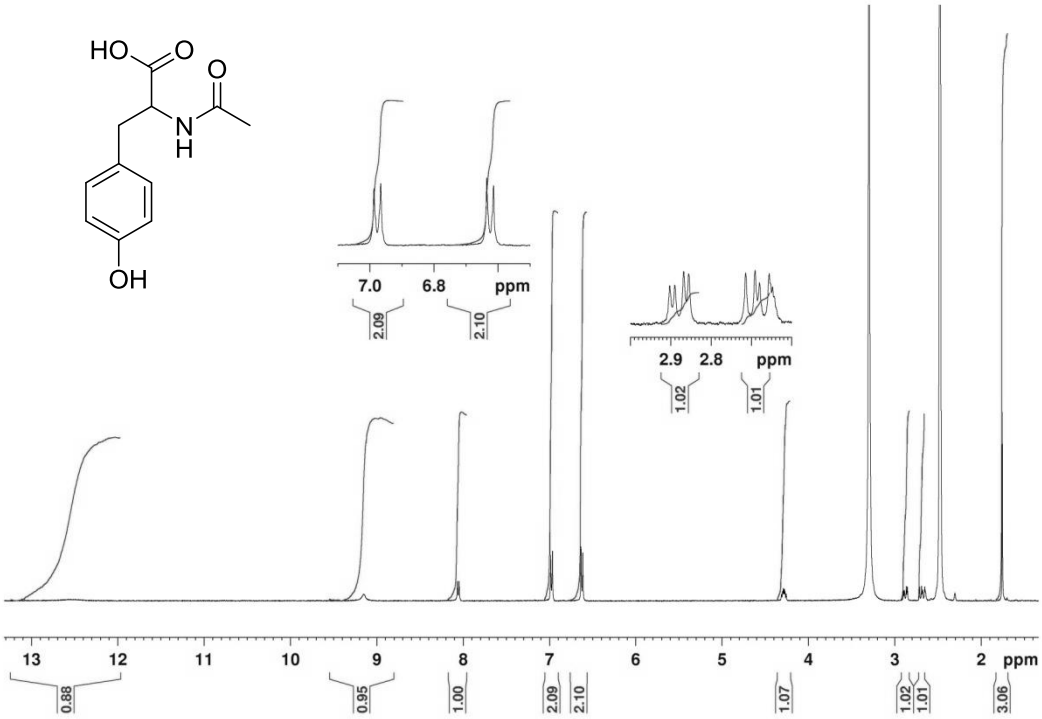
5-(4-Hydroxybenzyl)hydantoin **30** (^{1}H; 400.13 MHz; 6d-DMSO)



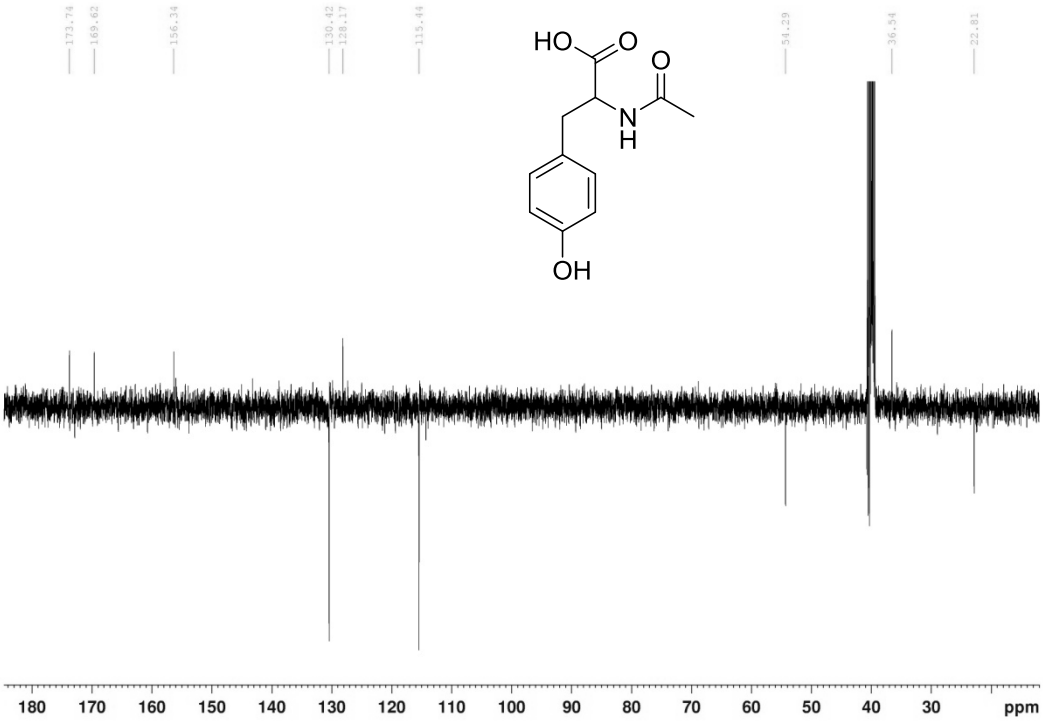
5-(4-Hydroxybenzyl)hydantoin **30** (^{13}C; 100.61 MHz; 6d-DMSO)



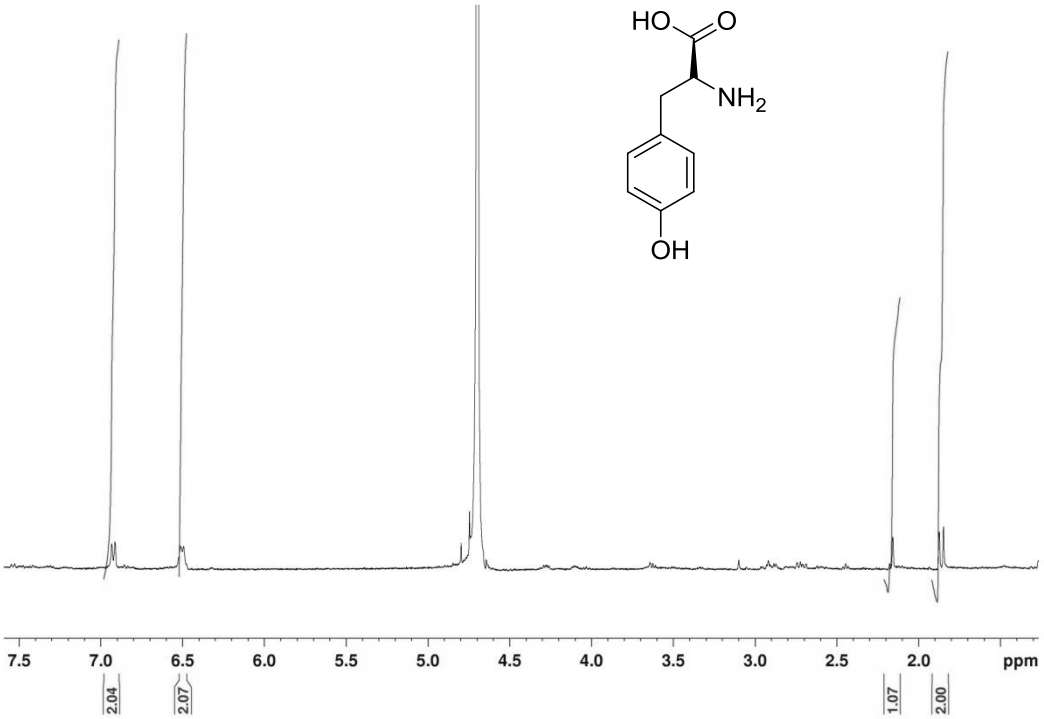
N-Acetyltyrosine **31** (^{1}H; 400.13 MHz; 6d-DMSO)



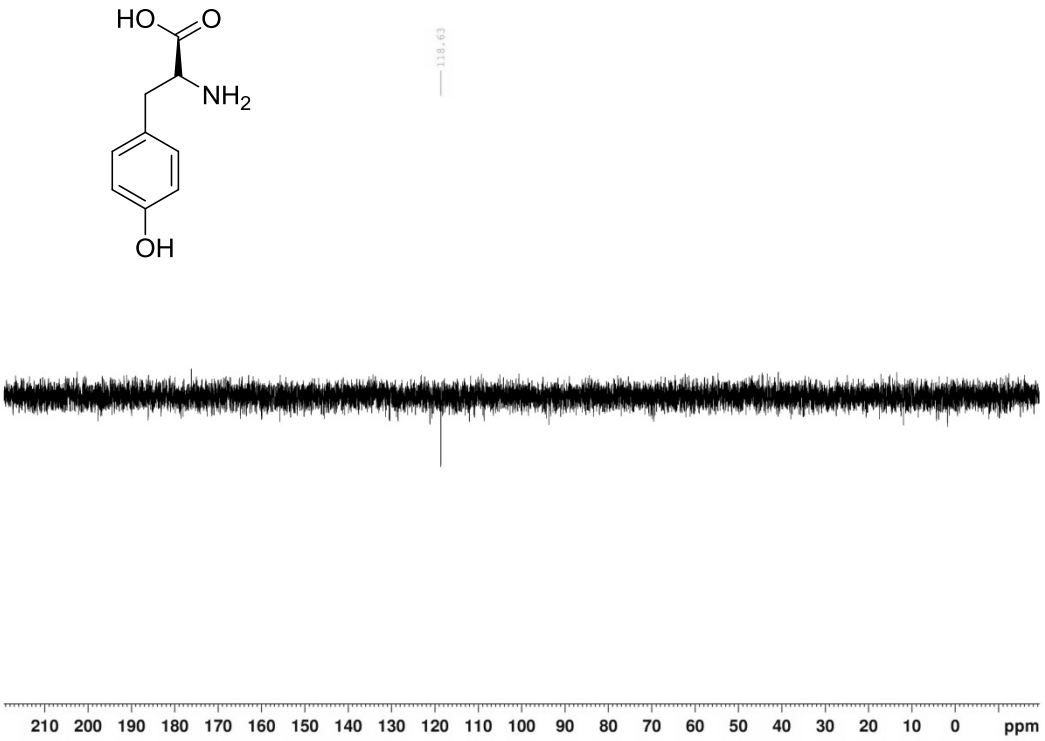
N-Acetyltyrosine **31** (^{13}C; 100.61 MHz; 6d-DMSO)



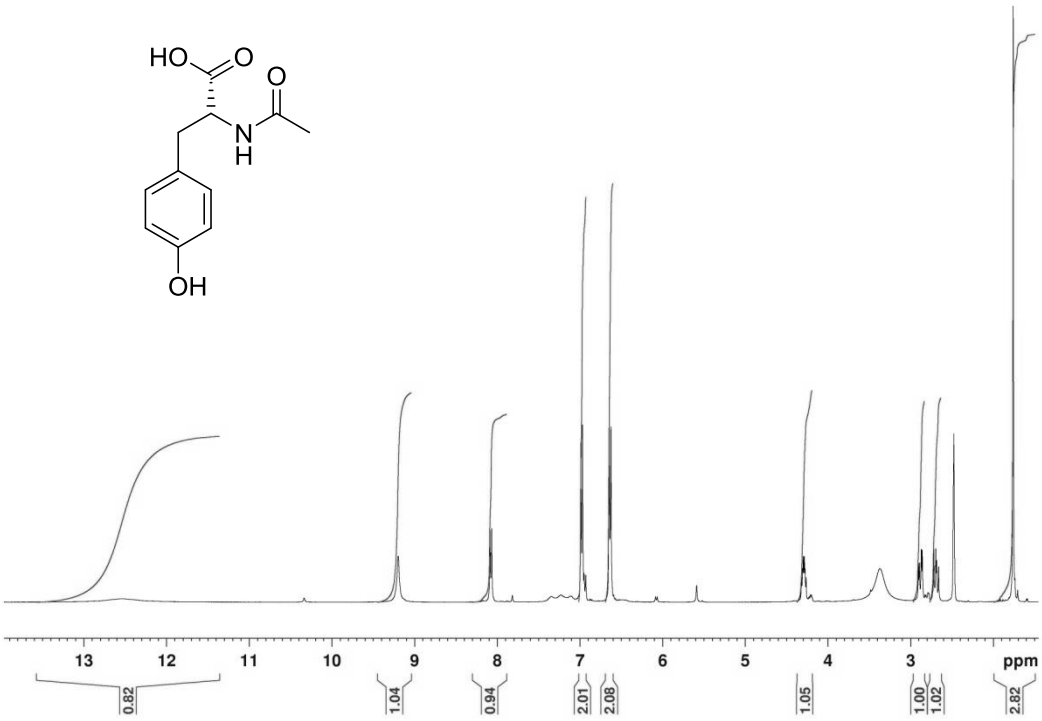
L-Tyrosine **32** (^{1}H; 400.13 MHz; D_2O, NaOD)



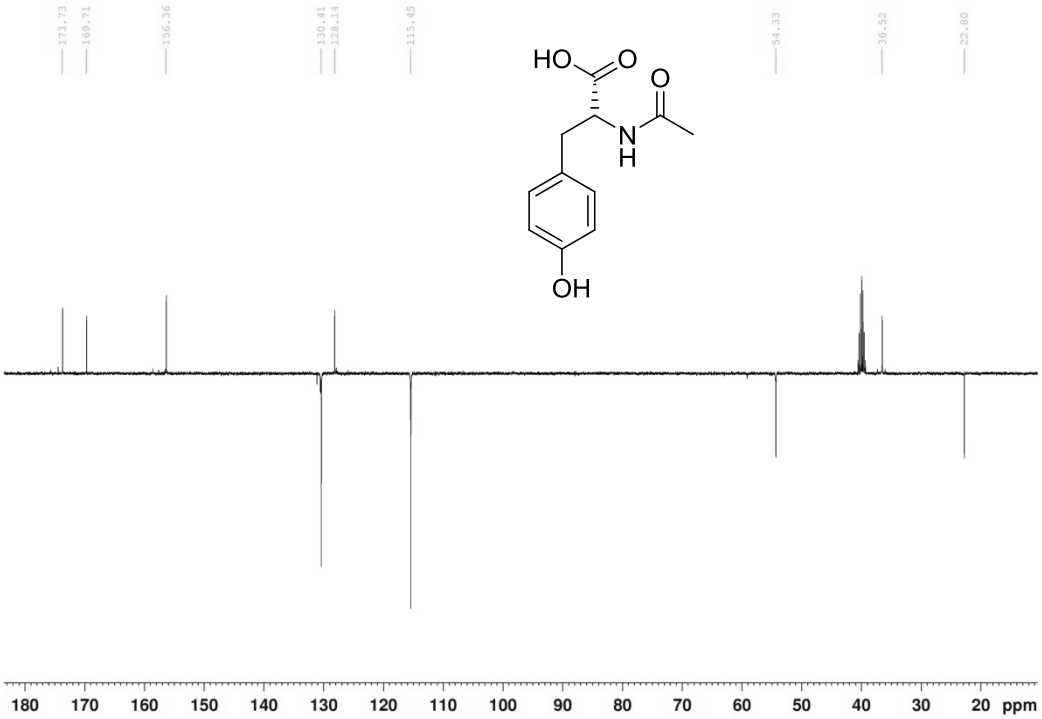
L-Tyrosine **32** (^{13}C; 100.61 MHz; D_2O, NaOD)



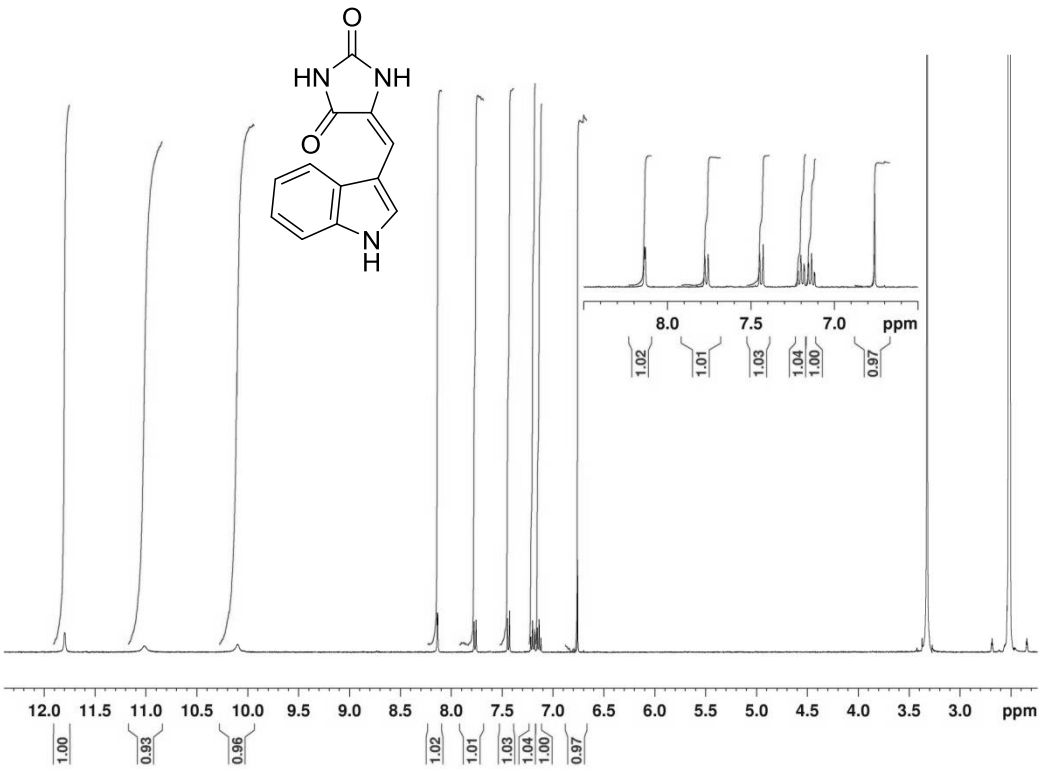
N-Acetyl-D-tyrosine **33** (^{1}H; 400.13 MHz; 6d-DMSO)



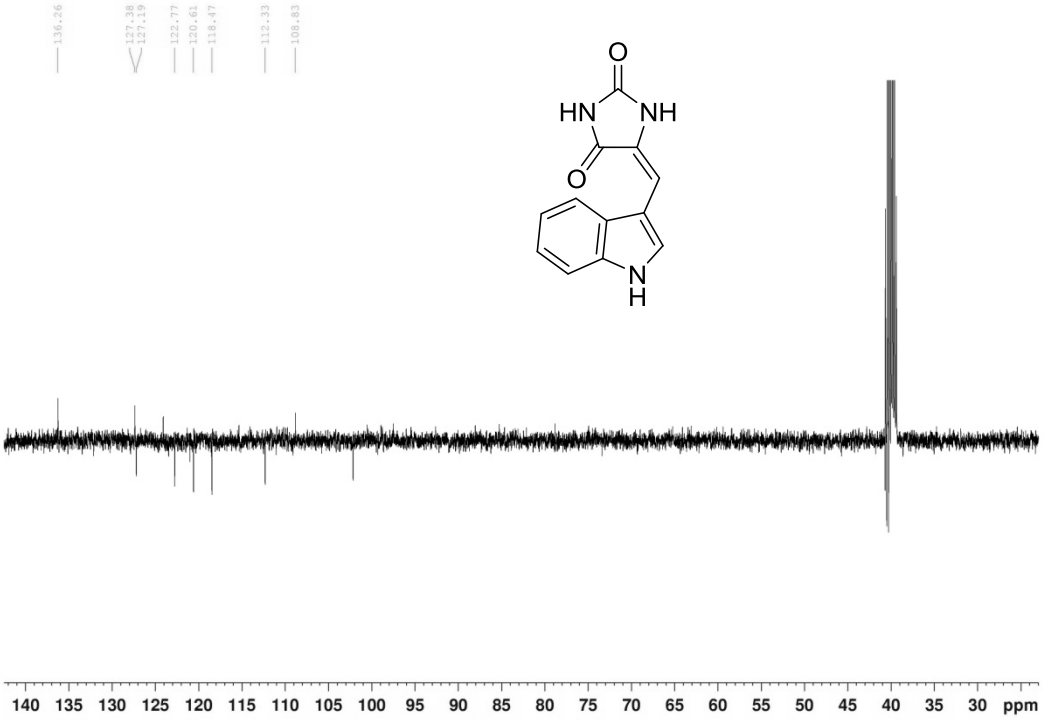
N-Acetyl-D-tyrosine **33** (^{13}C; 100.61 MHz; 6d-DMSO)



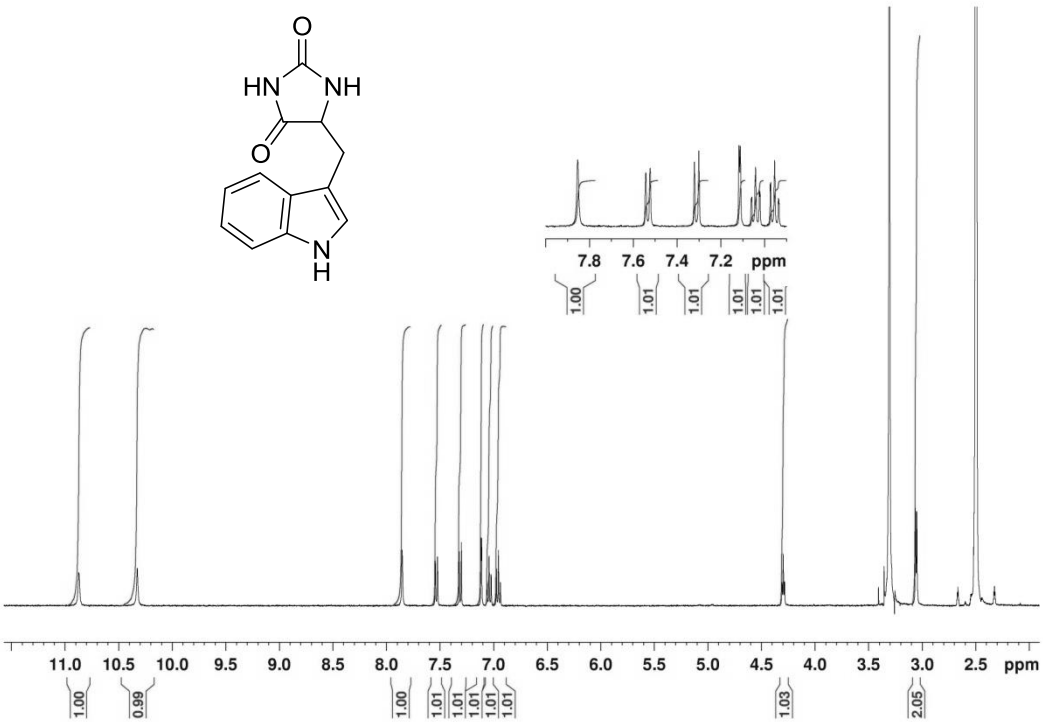
5-((1*H*-Indole-3-yl)methylene)hydantoin **34** (^{1}H; 400.13 MHz; 6d-DMSO)



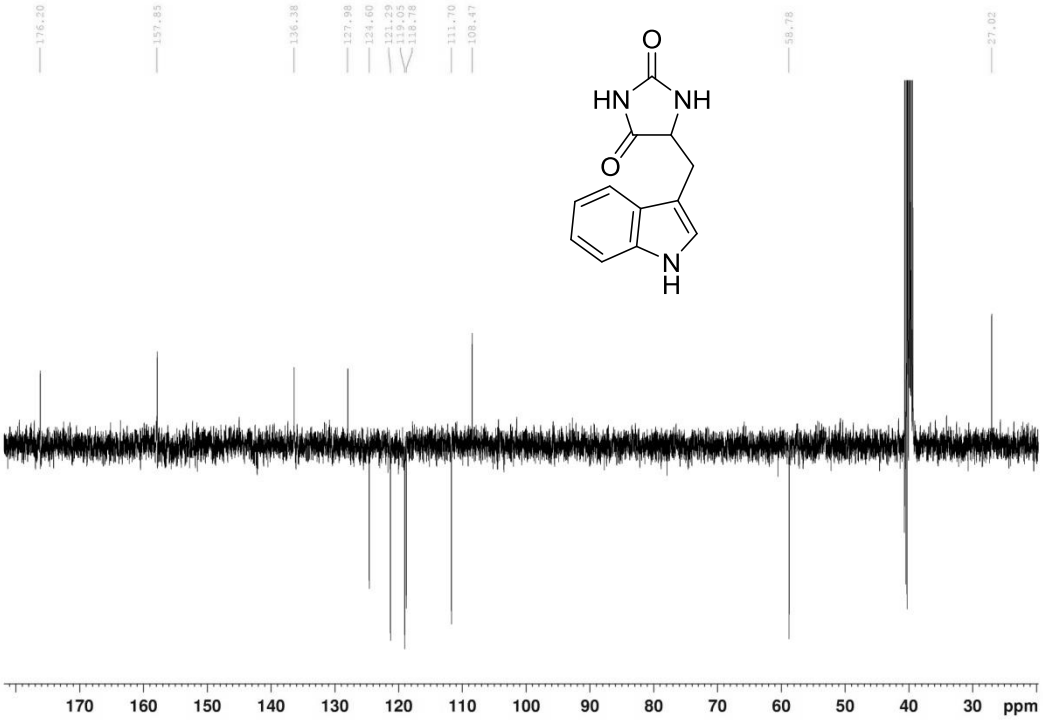
5-((1*H*-Indole-3-yl)methylene)hydantoin **34** (^{13}C; 100.61 MHz; 6d-DMSO)



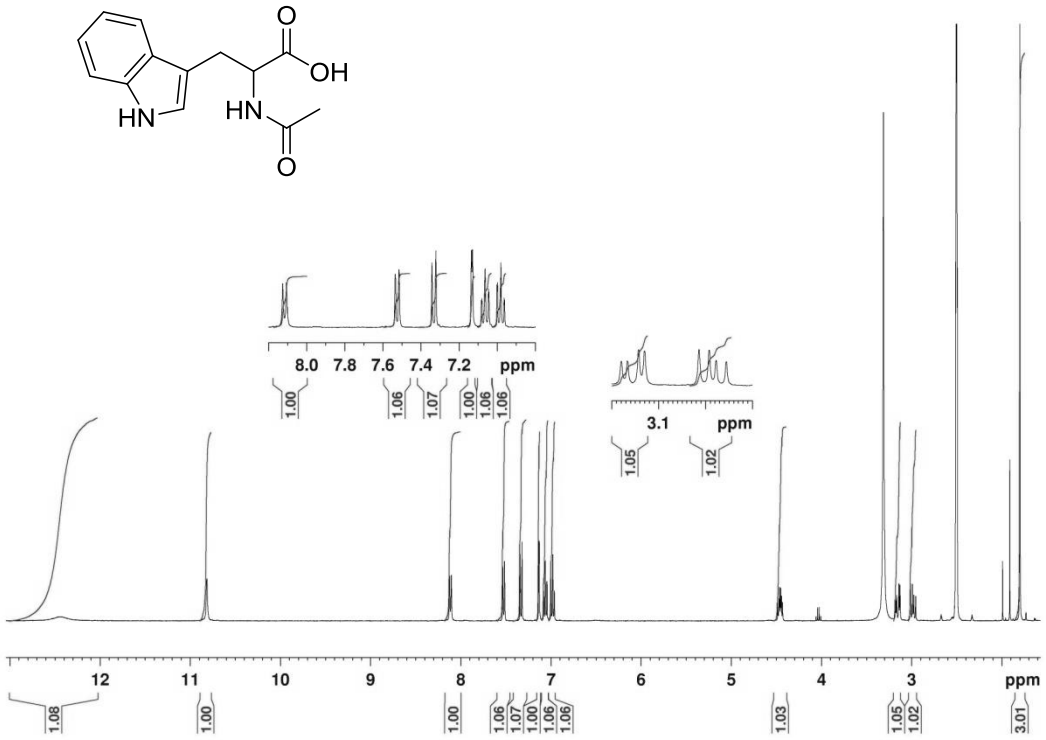
5-((1*H*-Indole-3-yl)methyl)hydantoin **35** (^{1}H; 400.13 MHz; 6d-DMSO)



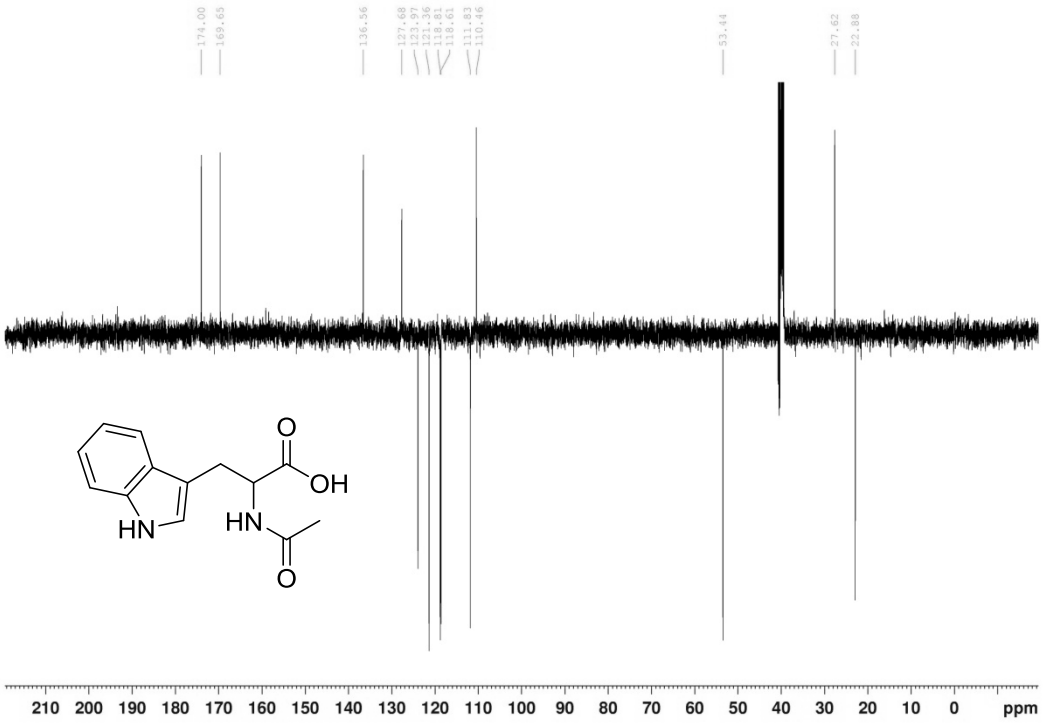
5-((1*H*-Indole-3-yl)methyl)hydantoin **35** (^{13}C; 100.61 MHz; 6d-DMSO)



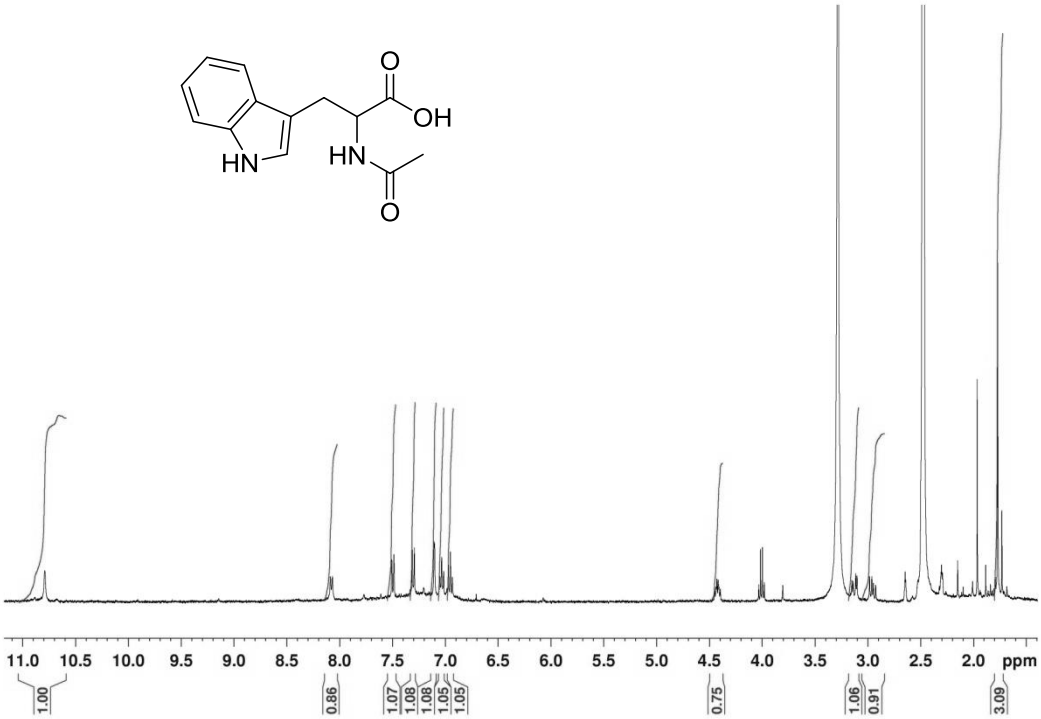
N-Acetyltryptophan **36** (^{1}H; 400.13 MHz; 6d-DMSO)



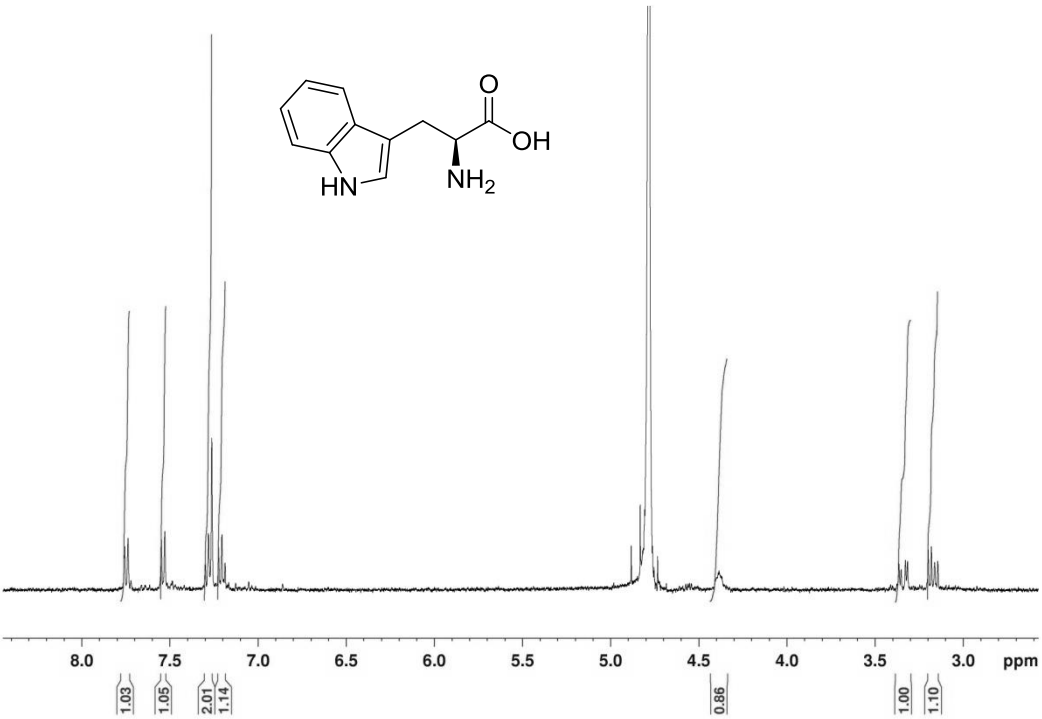
N-Acetyltryptophan **36** (^{13}C; 100.61 MHz; 6d-DMSO)



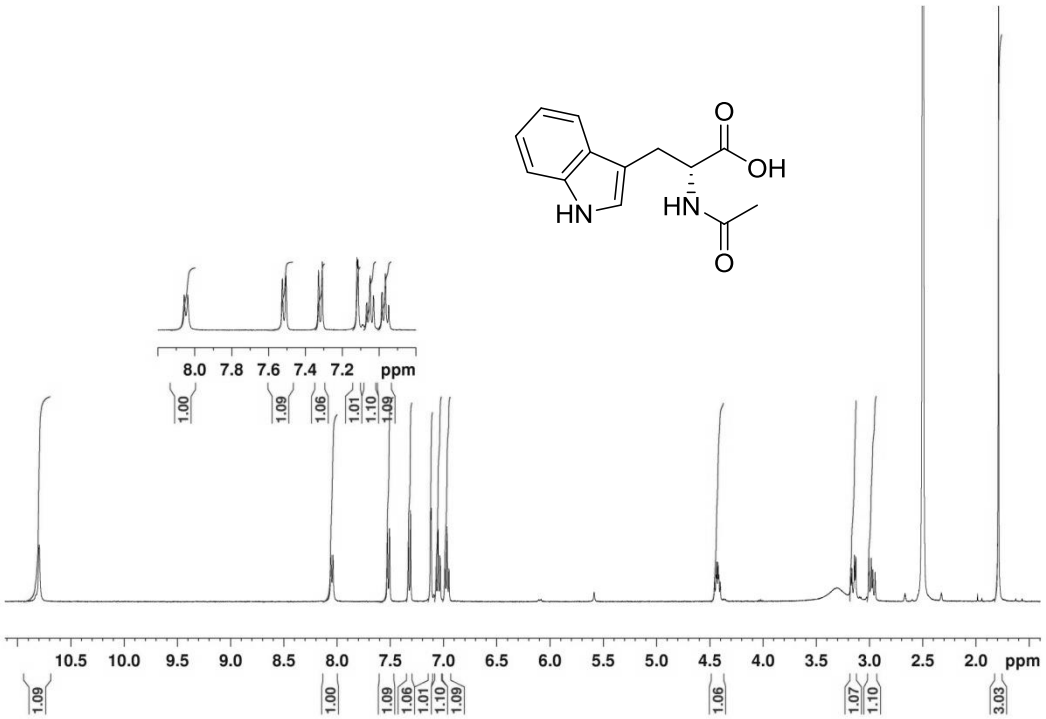
N-Acetyltryptophan **36** (^{1}H; 400.13 MHz; 6d-DMSO) after racemization



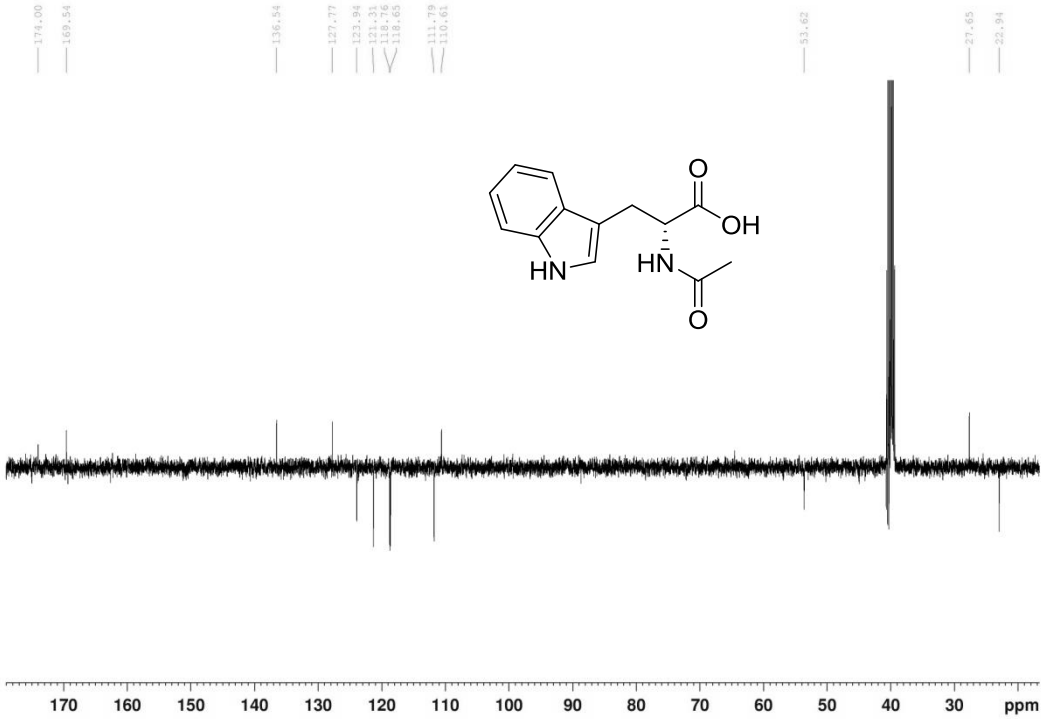
L-Tryptophan **37** (^{1}H; 400.13 MHz; D_2O, NaOD)



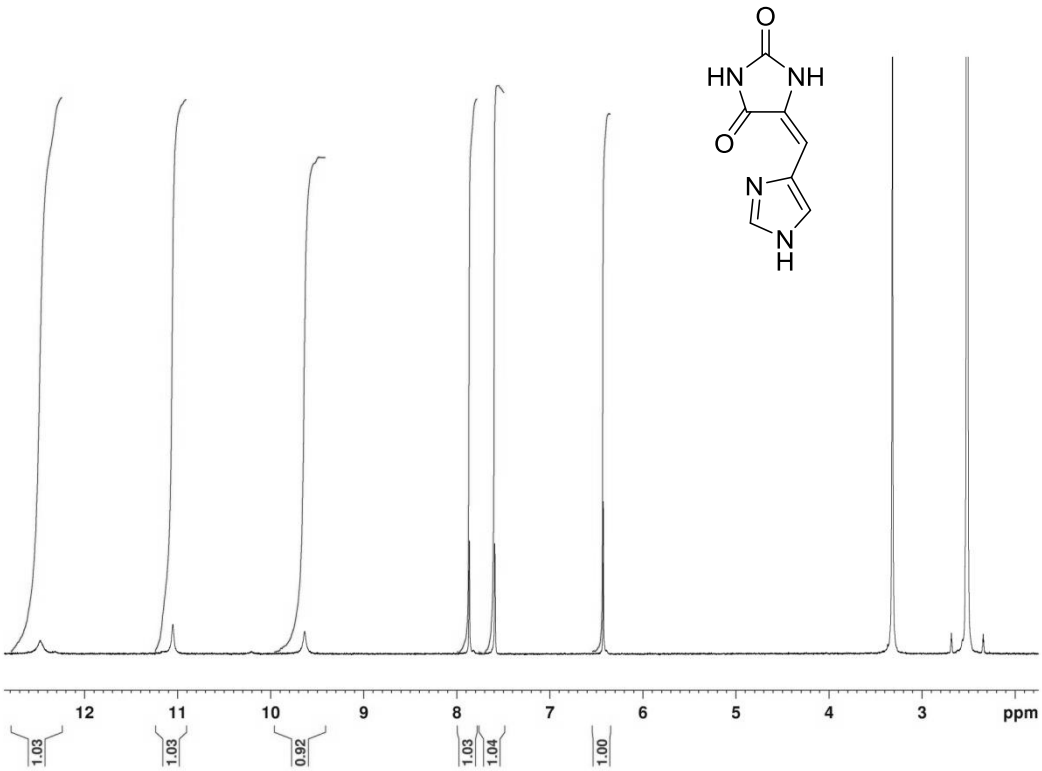
N-Acetyl-D-tryptophan **38** (^{1}H; 400.13 MHz; 6d-DMSO)



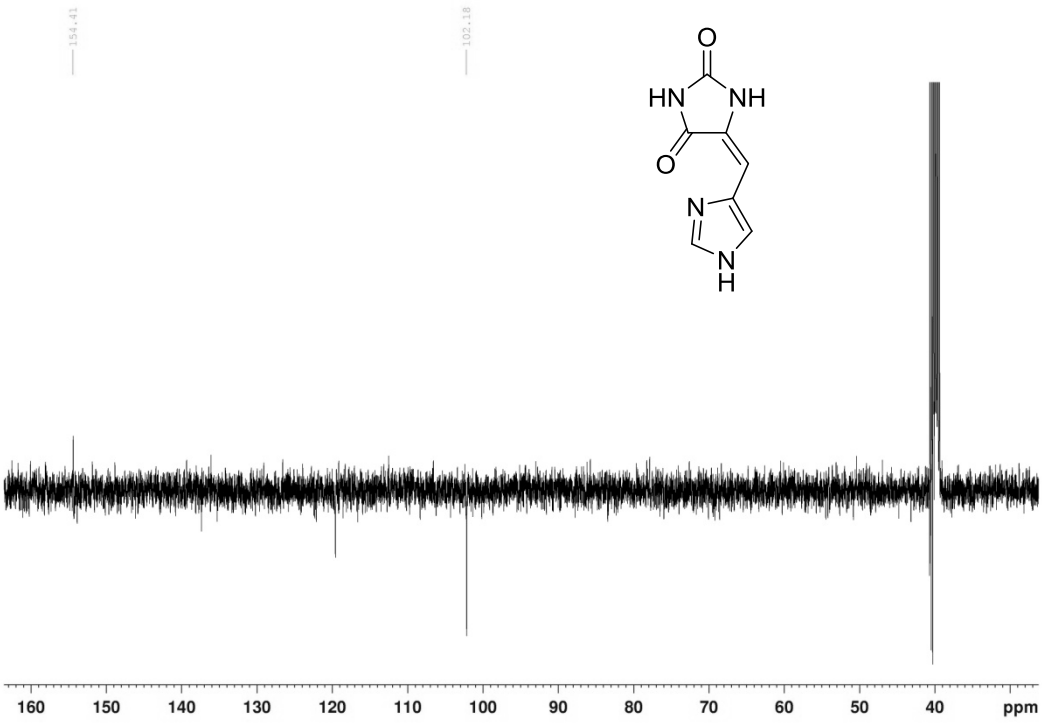
N-Acetyl-D-tryptophan **38** (^{13}C; 100.61 MHz; 6d-DMSO)



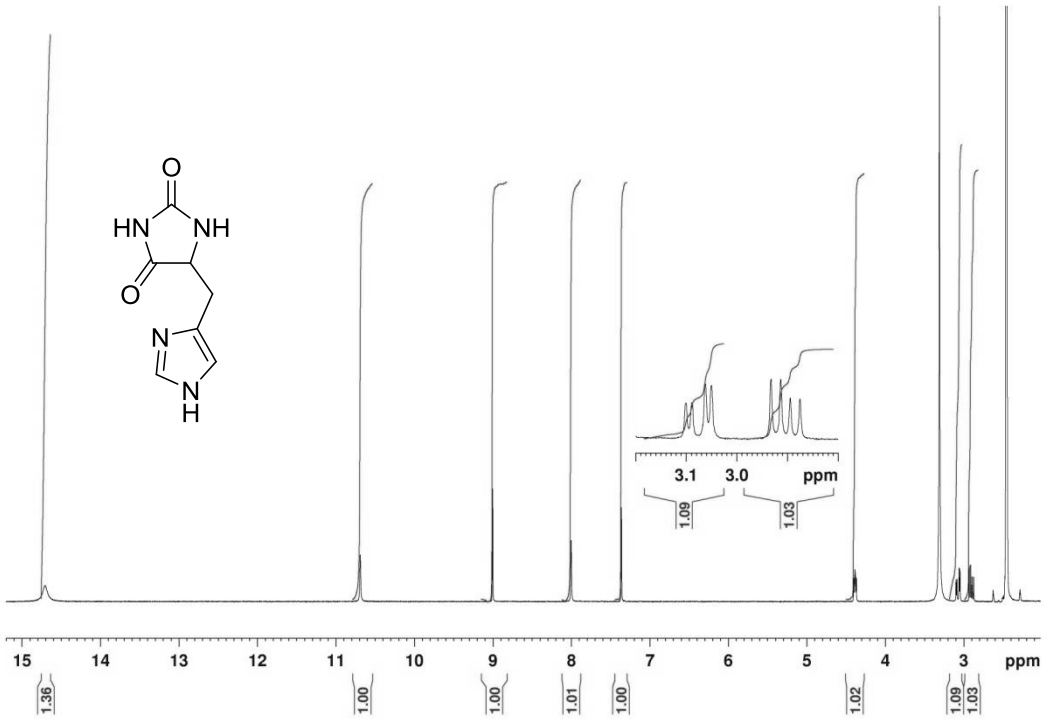
5-((1*H*-Imidazole-4-yl)methylene)hydantoin **39** (^{1}H; 400.13 MHz; 6d-DMSO)



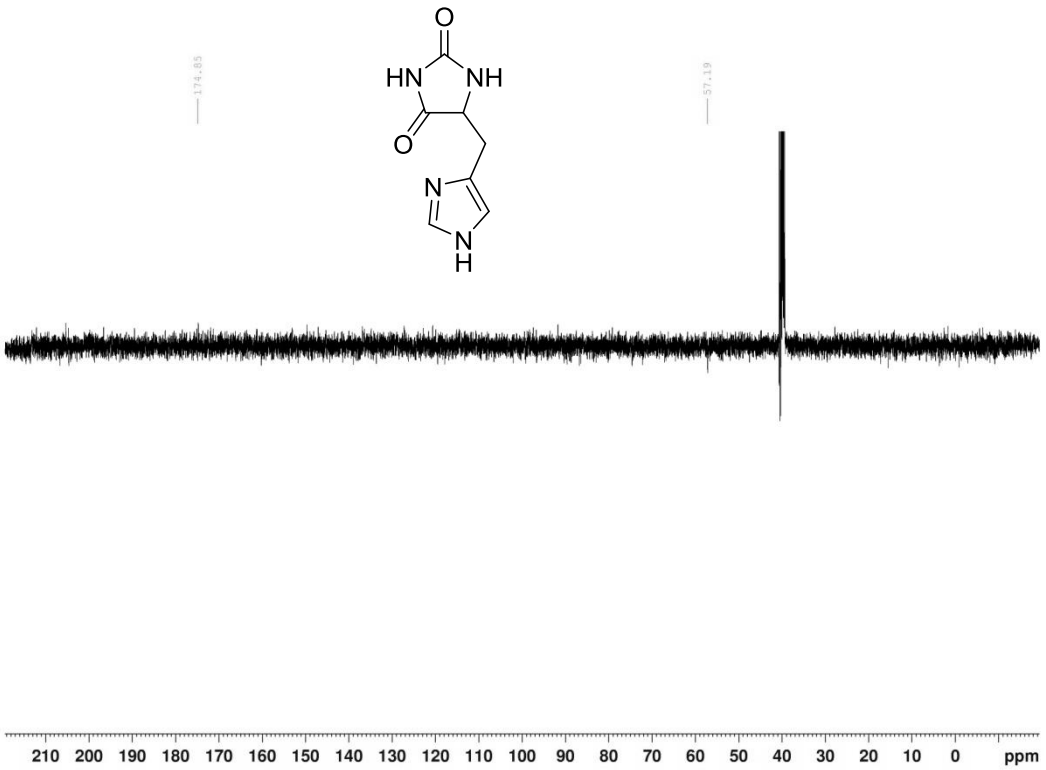
5-((1*H*-Imidazole-4-yl)methylene)hydantoin **39** (^{13}C; 100.61 MHz; 6d-DMSO)



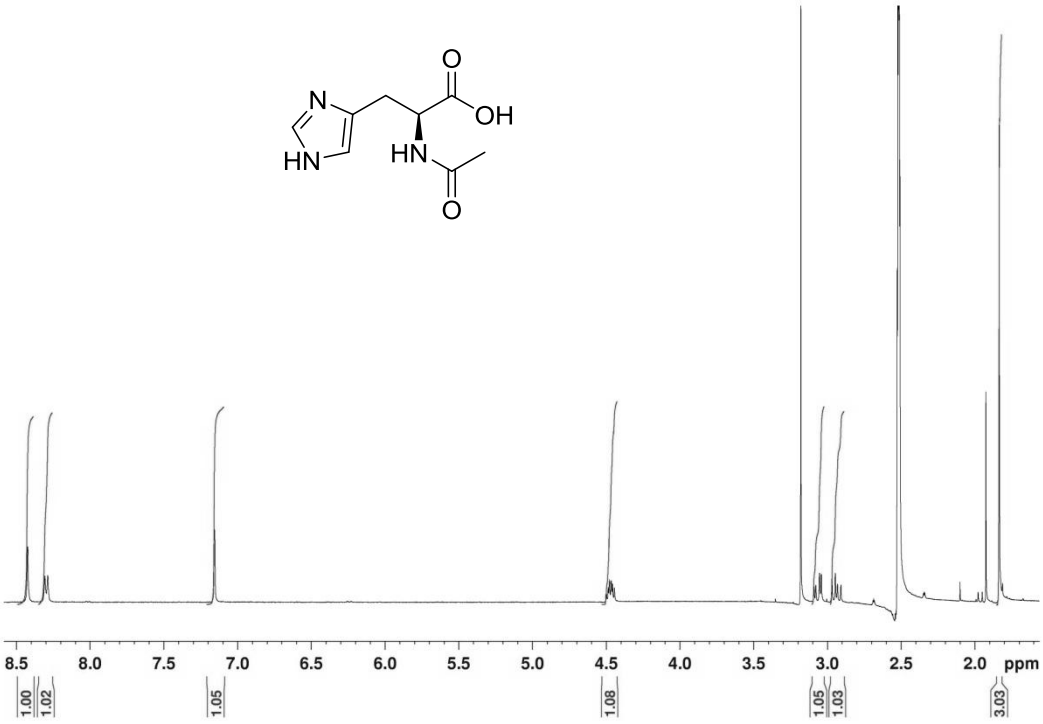
5-((1*H*-Imidazole-4-yl)methyl)hydantoin **40** (^{1}H; 400.13 MHz; 6d-DMSO)



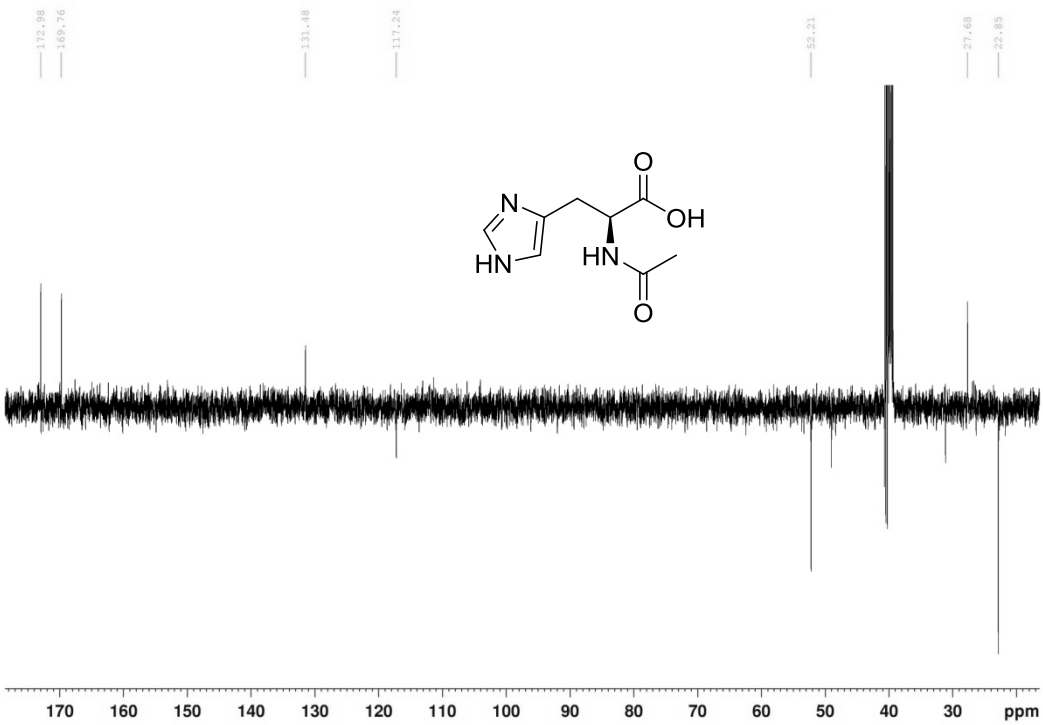
5-((1*H*-Imidazole-4-yl)methyl)hydantoin **40** (^{13}C; 100.61 MHz; 6d-DMSO)



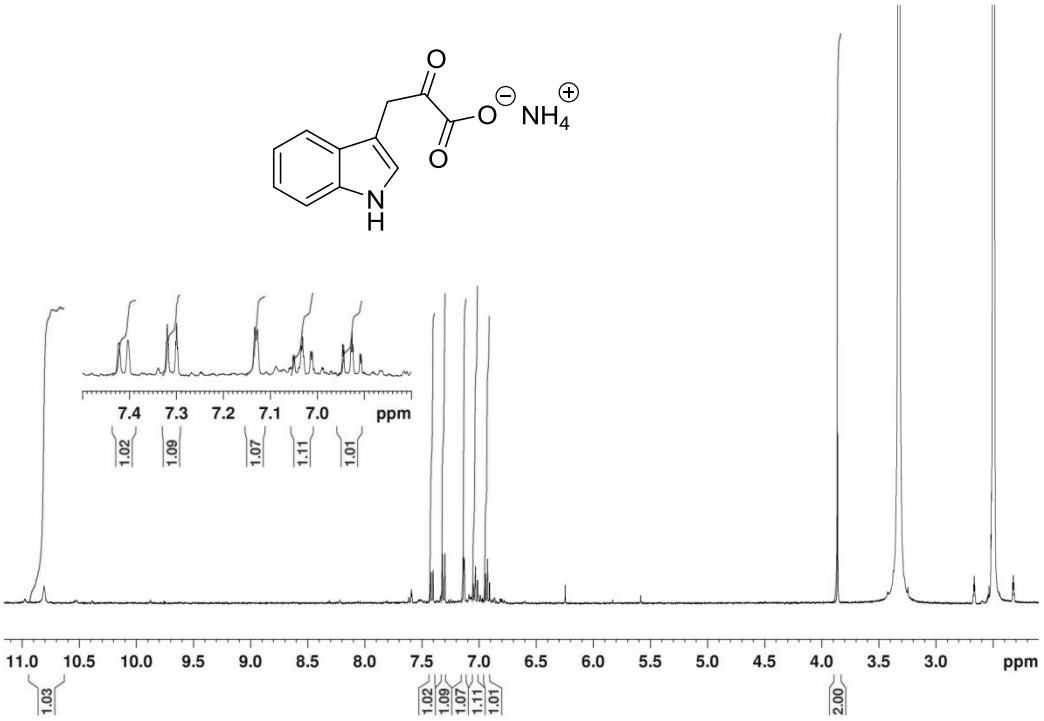
N-Acetyl-L-histidine **43** (^{1}H; 400.13 MHz; 6d-DMSO)



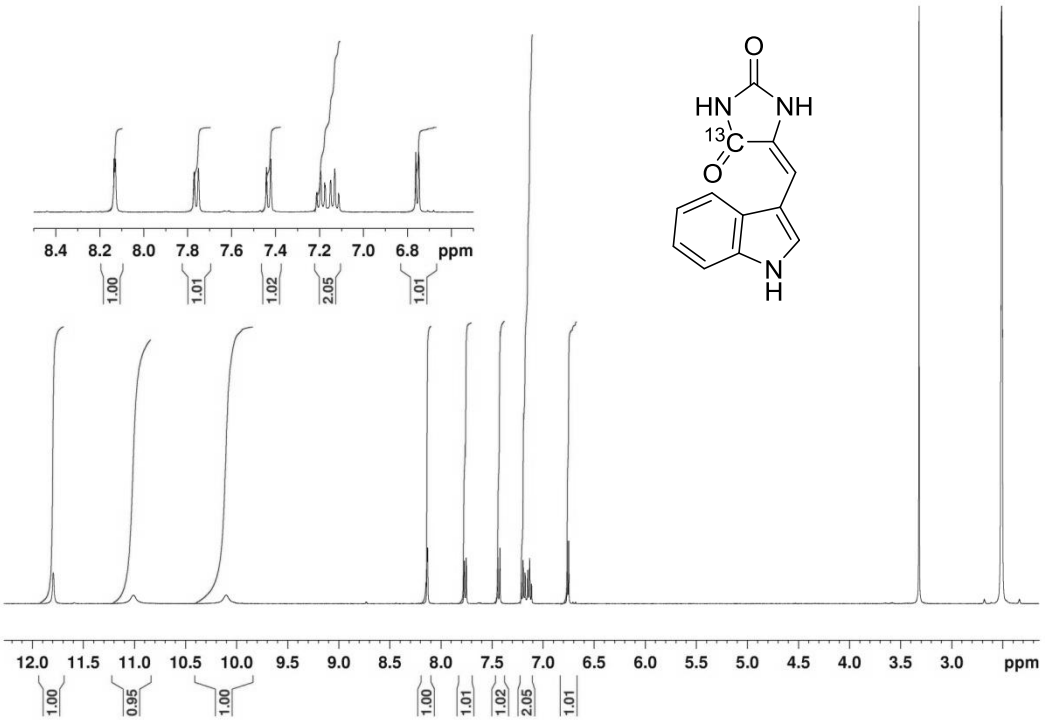
N-Acetyl-L-histidine **43** (^{13}C; 100.61 MHz; 6d-DMSO)



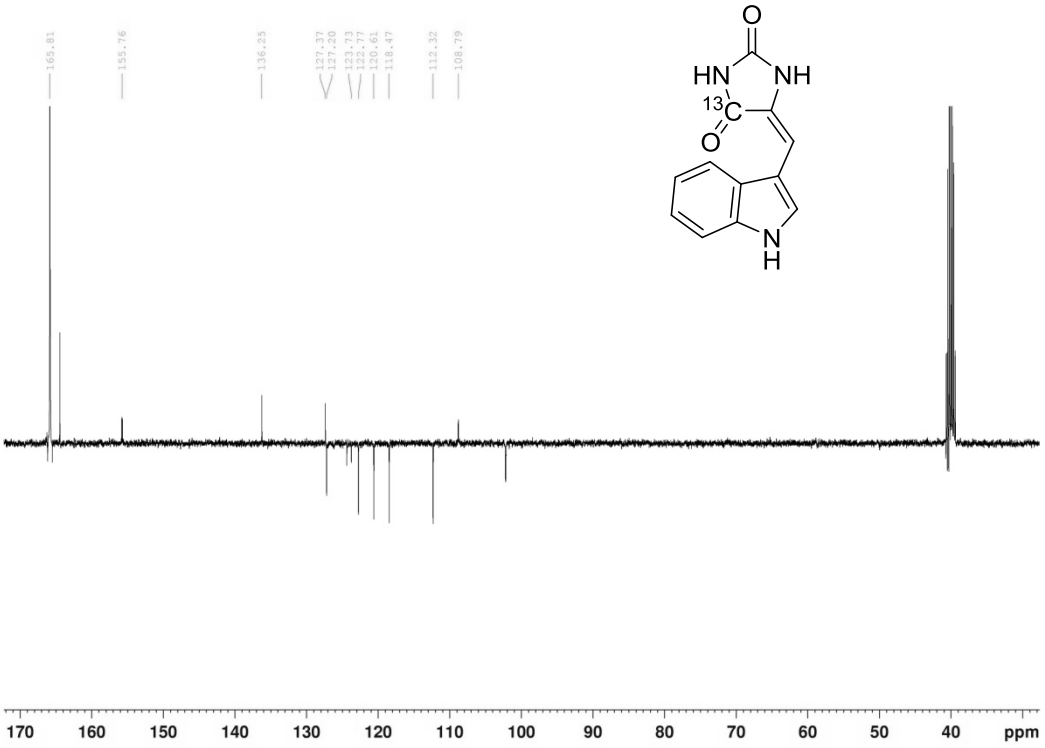
Indole-3-pyruvate **44** (^{1}H; 400.13 MHz; 6d-DMSO)



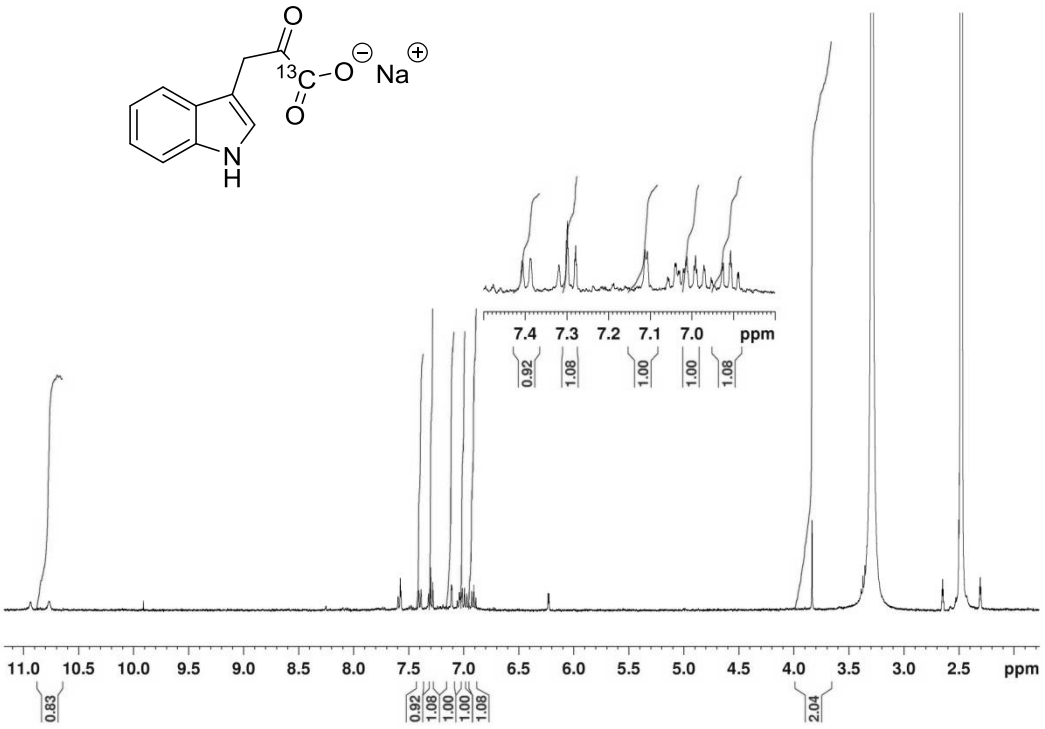
[1-^{13}C] 5-((1*H*-Indole-3-yl)methylene)hydantoin **45**



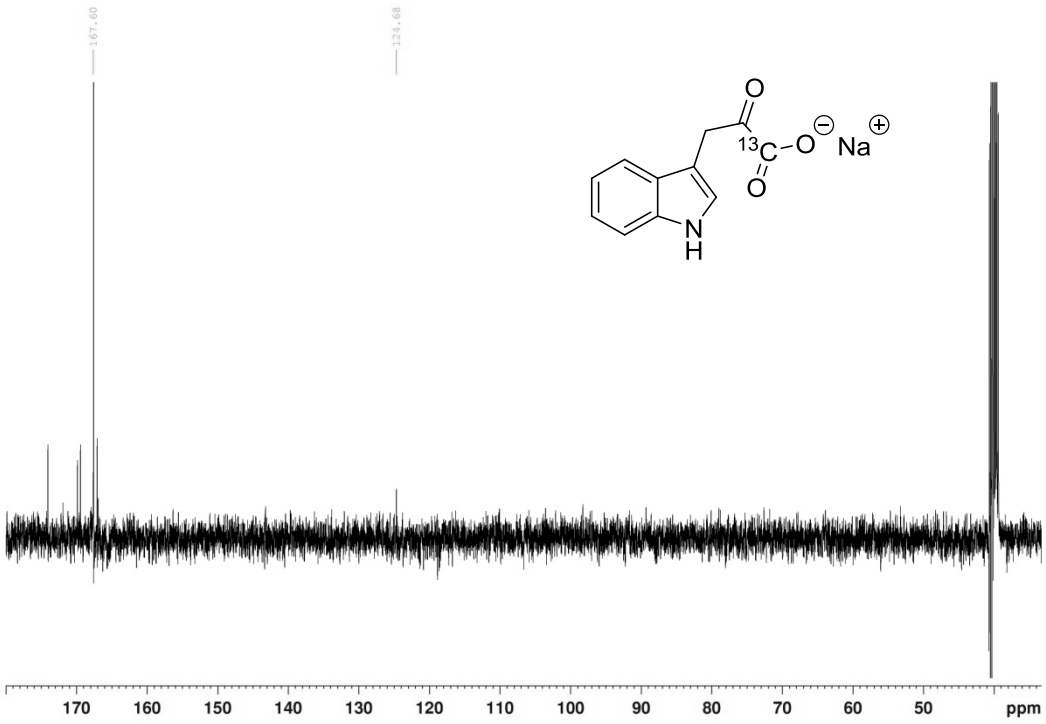
[1-^{13}C] 5-((1*H*-Indole-3-yl)methylene)hydantoin **45** (^{13}C; 100.61 MHz; 6d-DMSO)



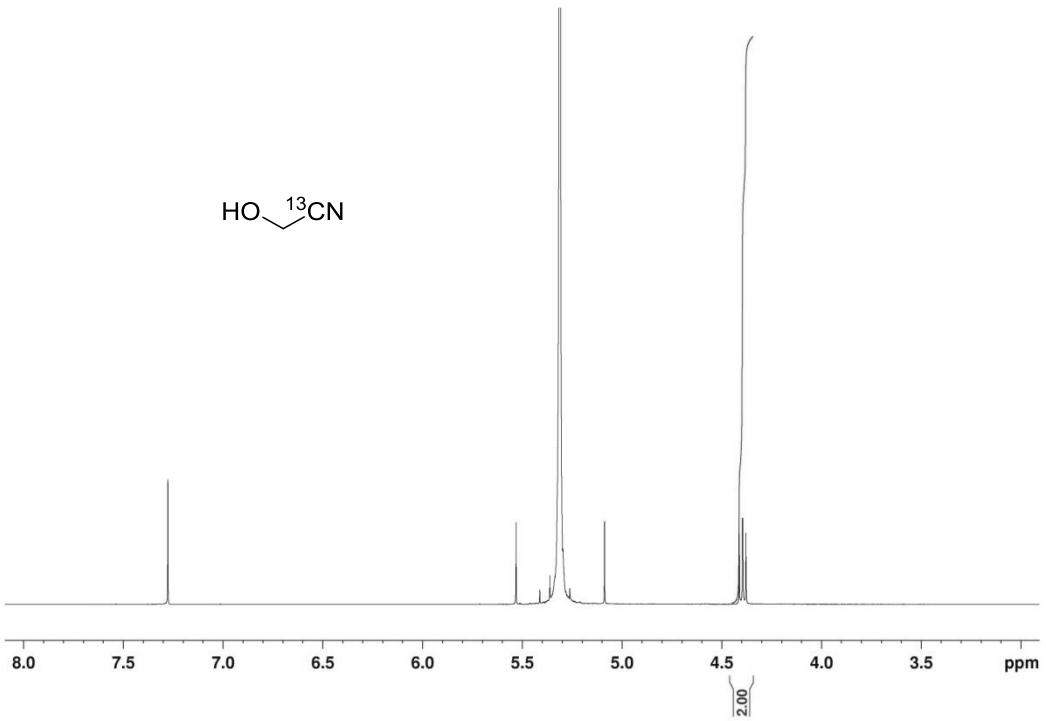
[1-^{13}C] Indole-3-pyruvate **46** (^{1}H; 400.13 MHz; 6d-DMSO)



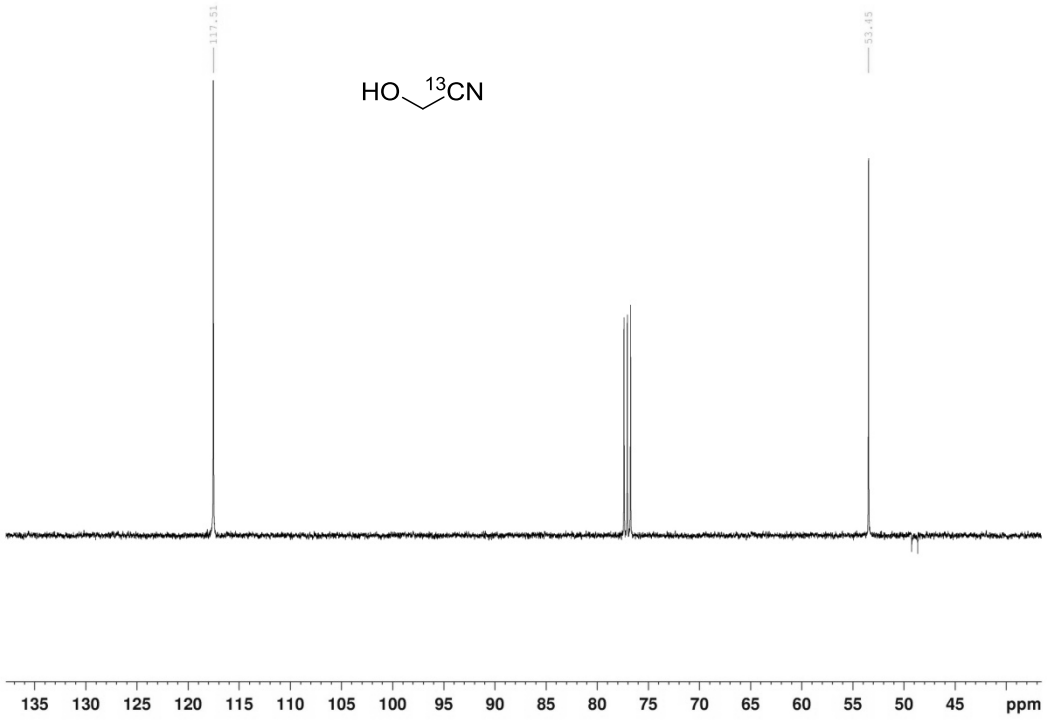
[1-^{13}C] Indole-3-pyruvate **46** (^{13}C; 100.61 MHz; 6d-DMSO)



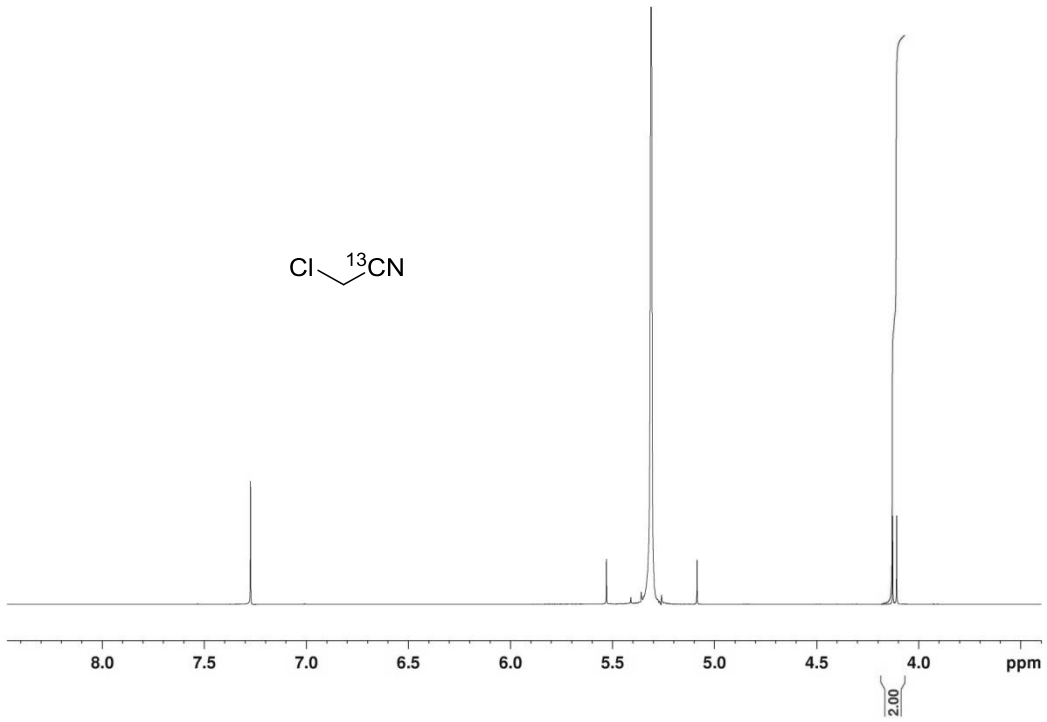
[1-^{13}C] 2-Hydroxyacetonitrile **50** (^{1}H; 400.13 MHz; $CDCl_3$)



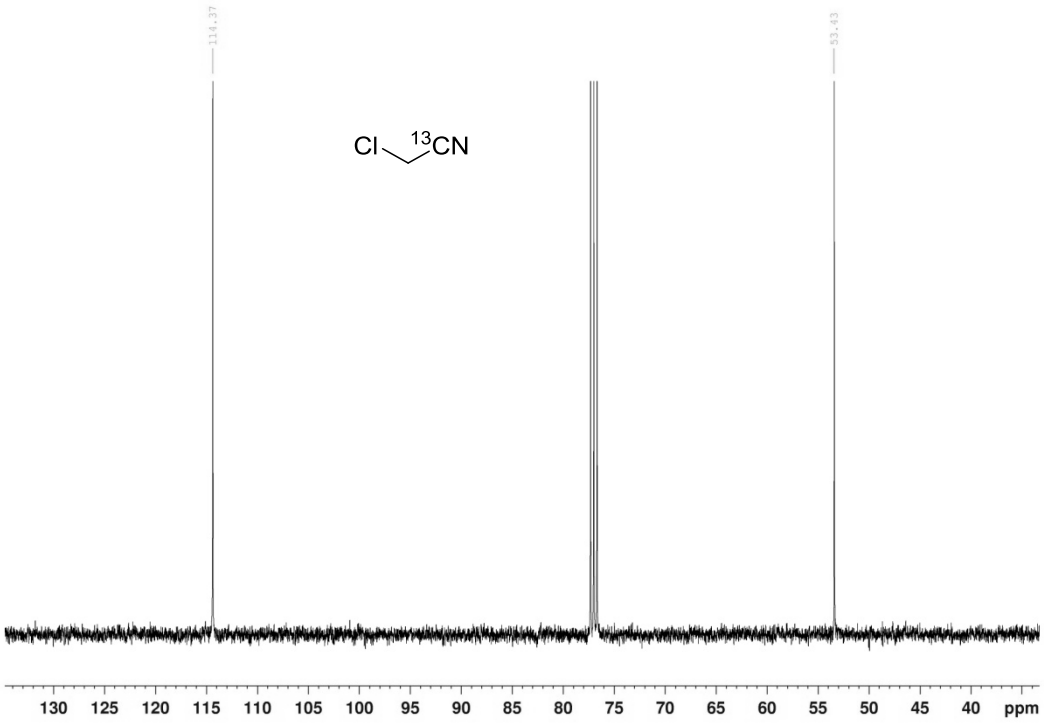
[1-^{13}C] 2-Hydroxyacetonitrile **50** (^{13}C; 100.61 MHz; $CDCl_3$)



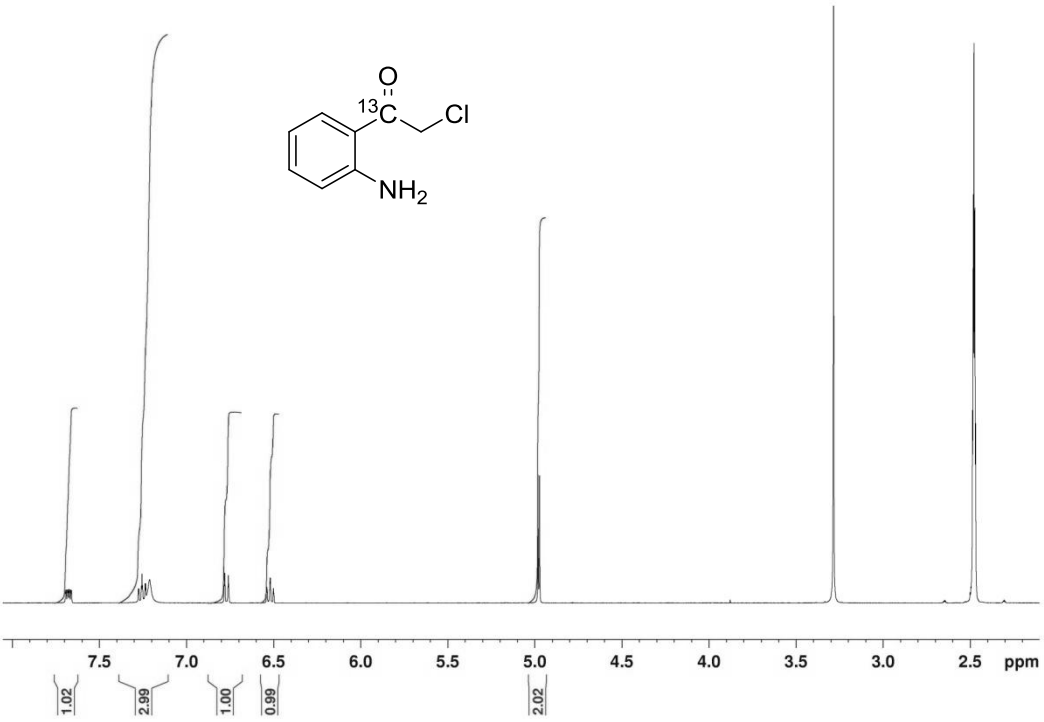
[1-^{13}C] 2-Chloroacetonitrile **51** (^{1}H; 400.13 MHz; $CDCl_3$)



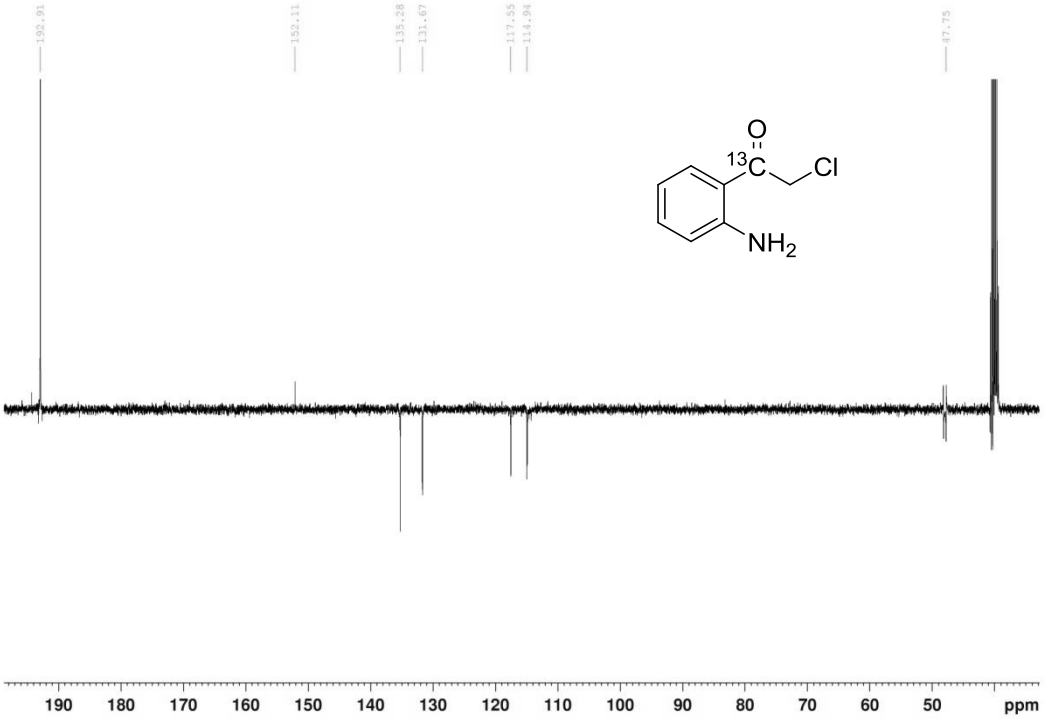
[1-^{13}C] 2-Chloroacetonitrile **51** (^{13}C; 100.61 MHz; $CDCl_3$)



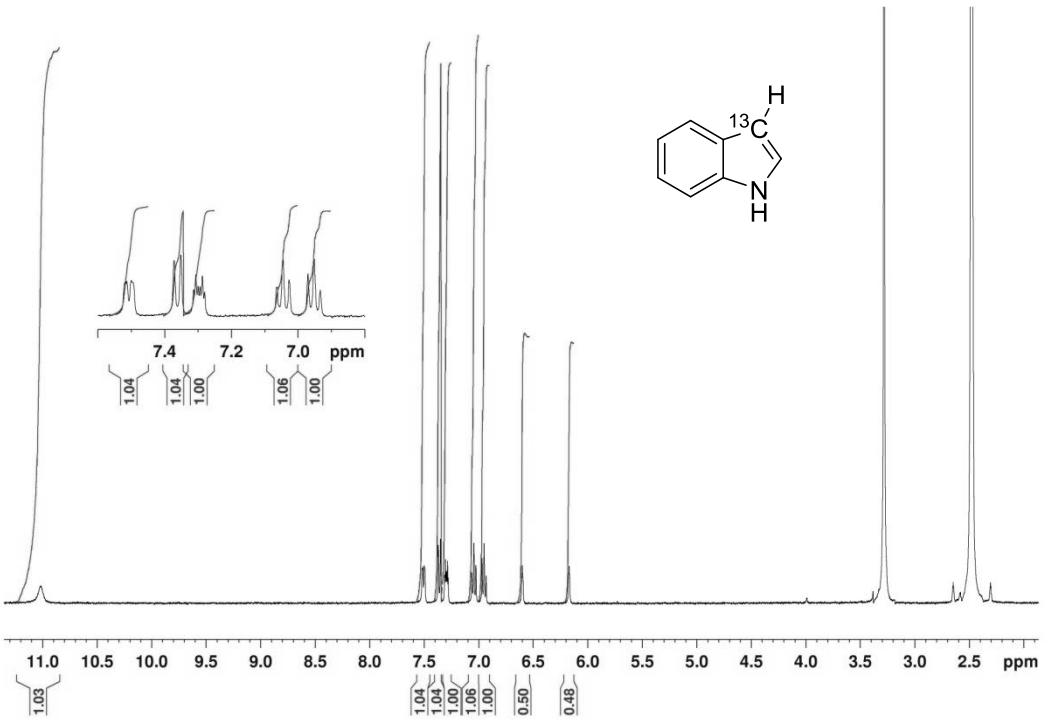
[1-^{13}C] 1-(2-Aminophenyl)-2-chloroethanone **52** (^{1}H; 400.13 MHz; 6d-DMSO)



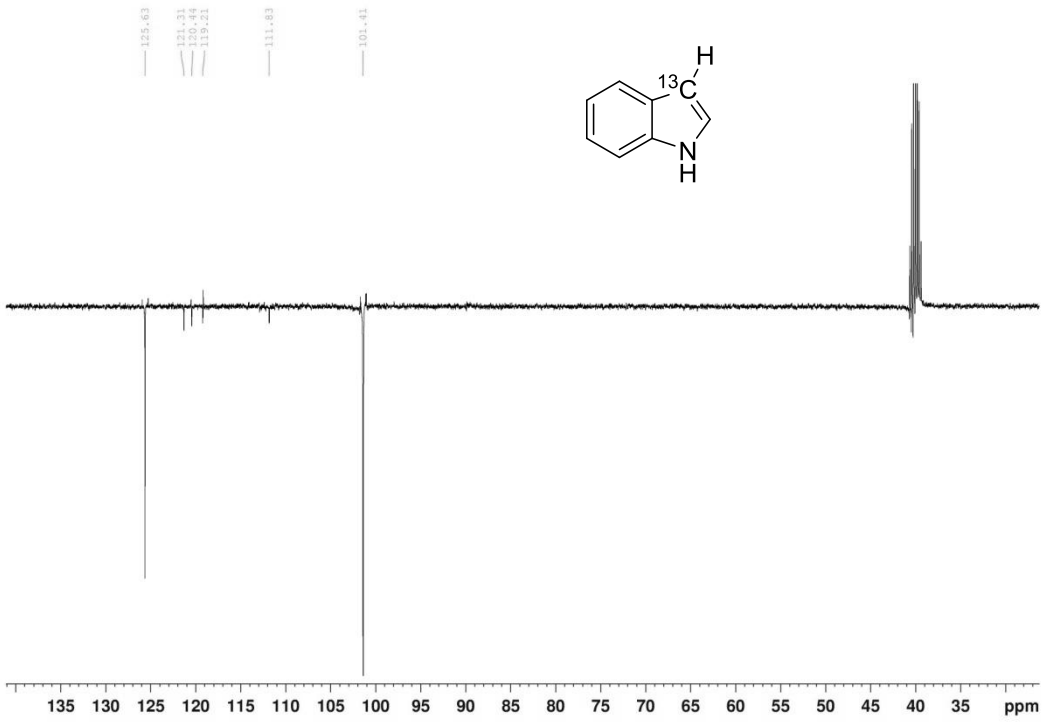
[1-^{13}C] 1-(2-Aminophenyl)-2-chloroethanone **52** (^{13}C; 100.61 MHz; 6d-DMSO)



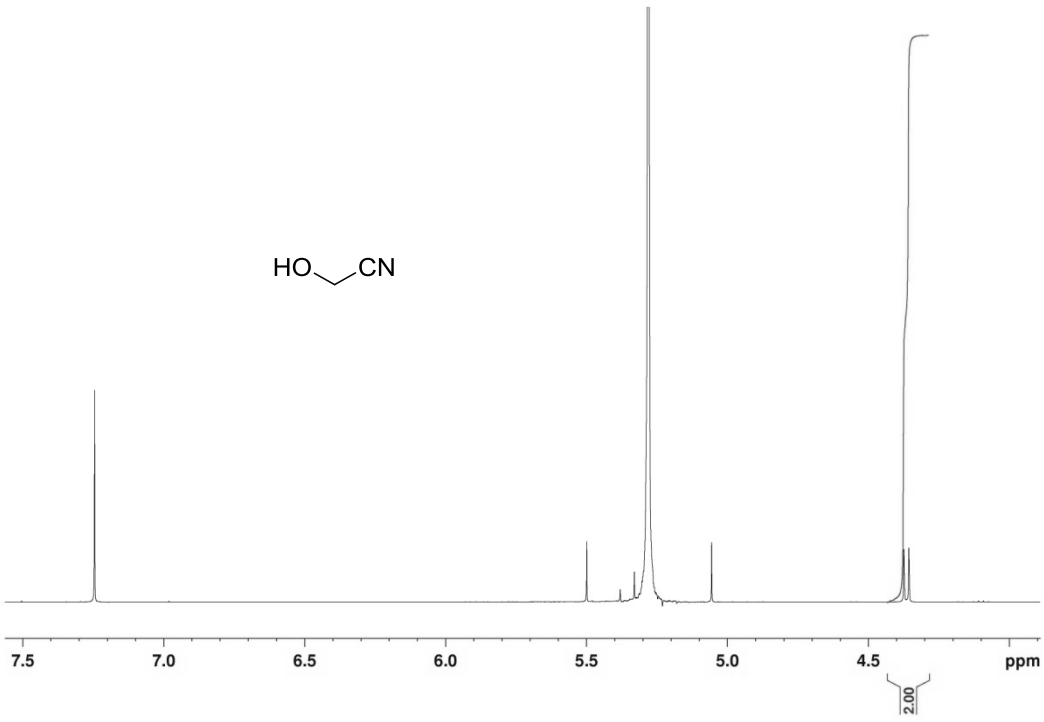
[3-^{13}C] Indole **53** (^{1}H; 400.13 MHz; 6d-DMSO)



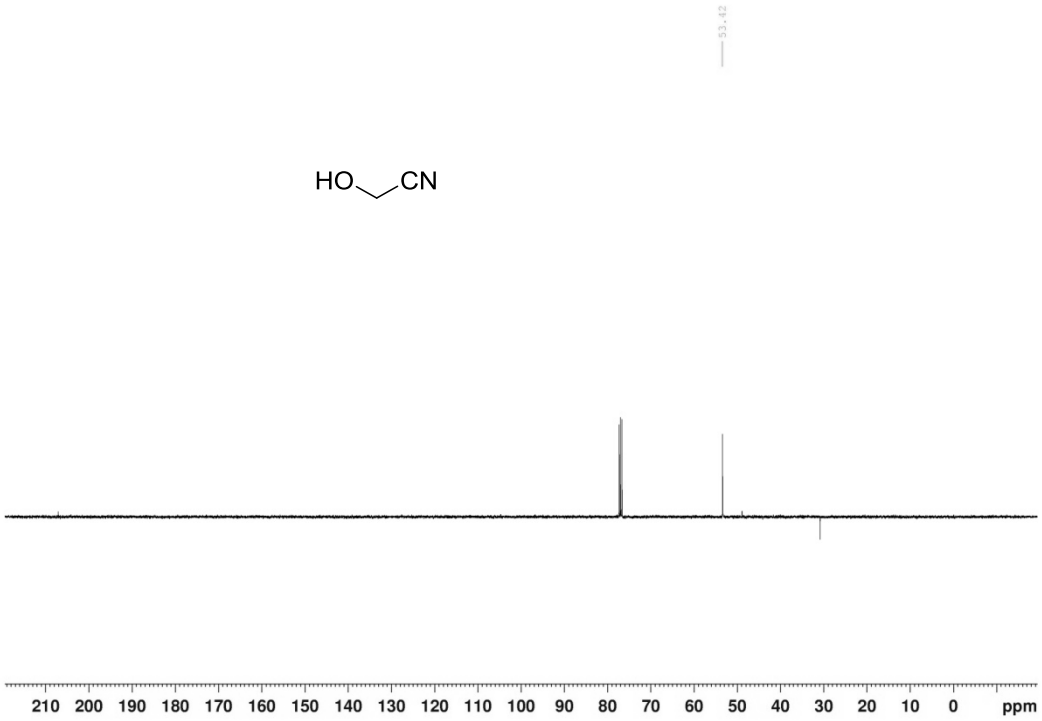
[3-^{13}C] Indole **53** (^{13}C; 100.61 MHz; 6d-DMSO)



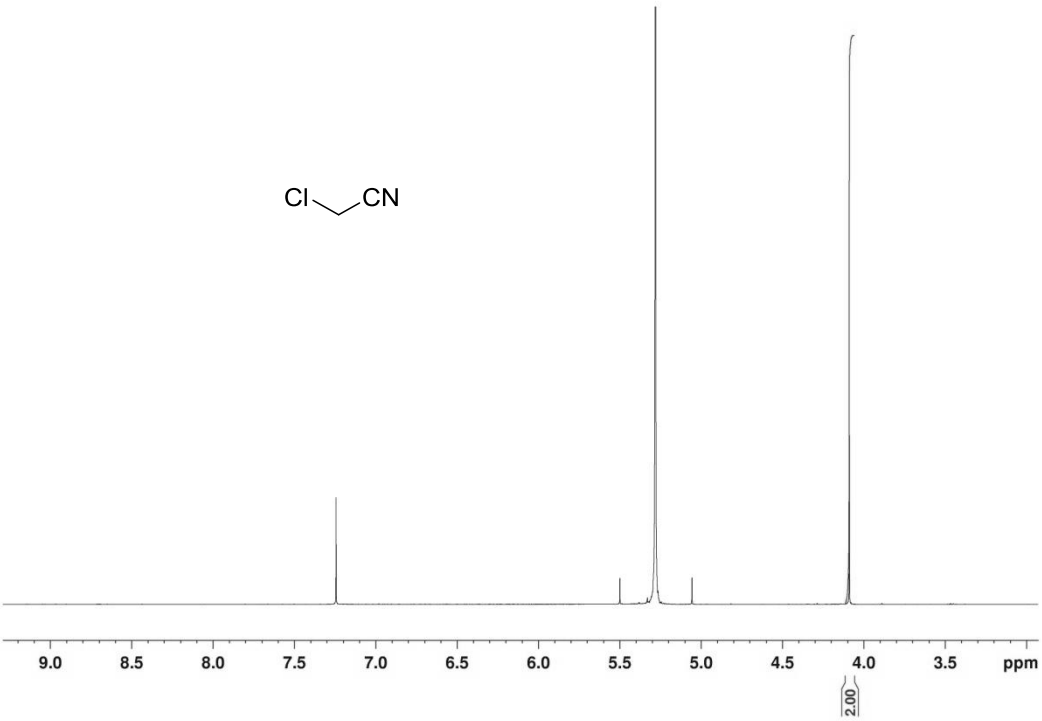
2-Hydroxyacetonitrile **56** (^{1}H; 400.13 MHz; $CDCl_3$)



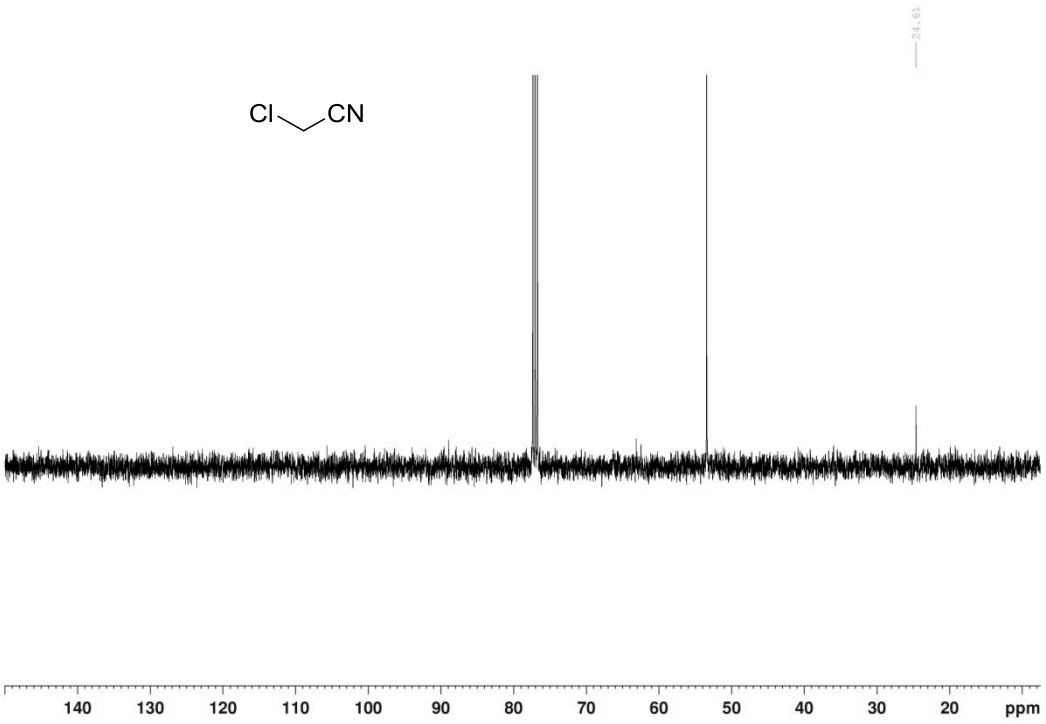
2-Hydroxyacetonitrile **56** (^{13}C; 100.61 MHz; $CDCl_3$)



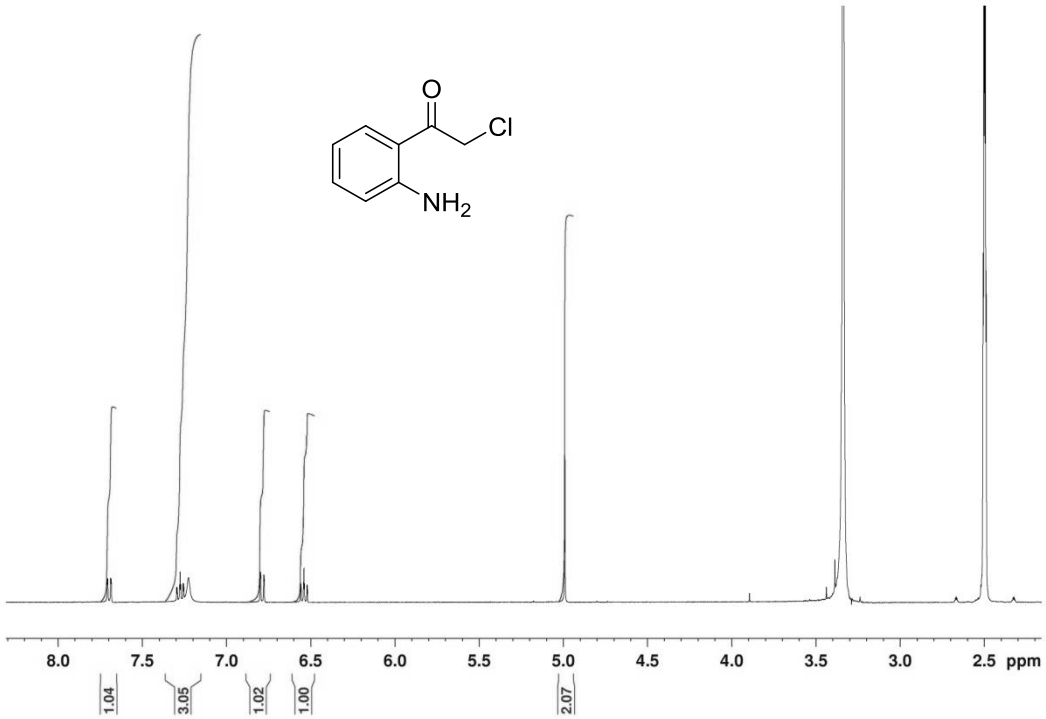
2-Chloroacetonitrile **57** (^{1}H; 400.13 MHz; $CDCl_3$)



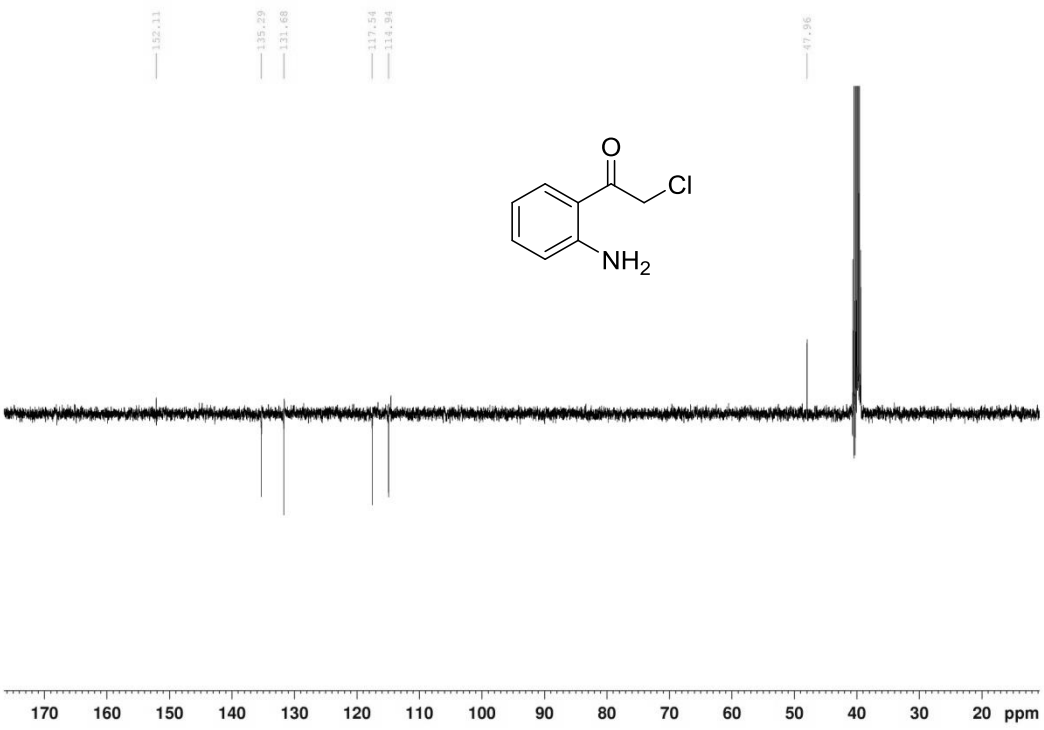
2-Chloroacetonitrile **57** (^{13}C; 100.61 MHz; $CDCl_3$)



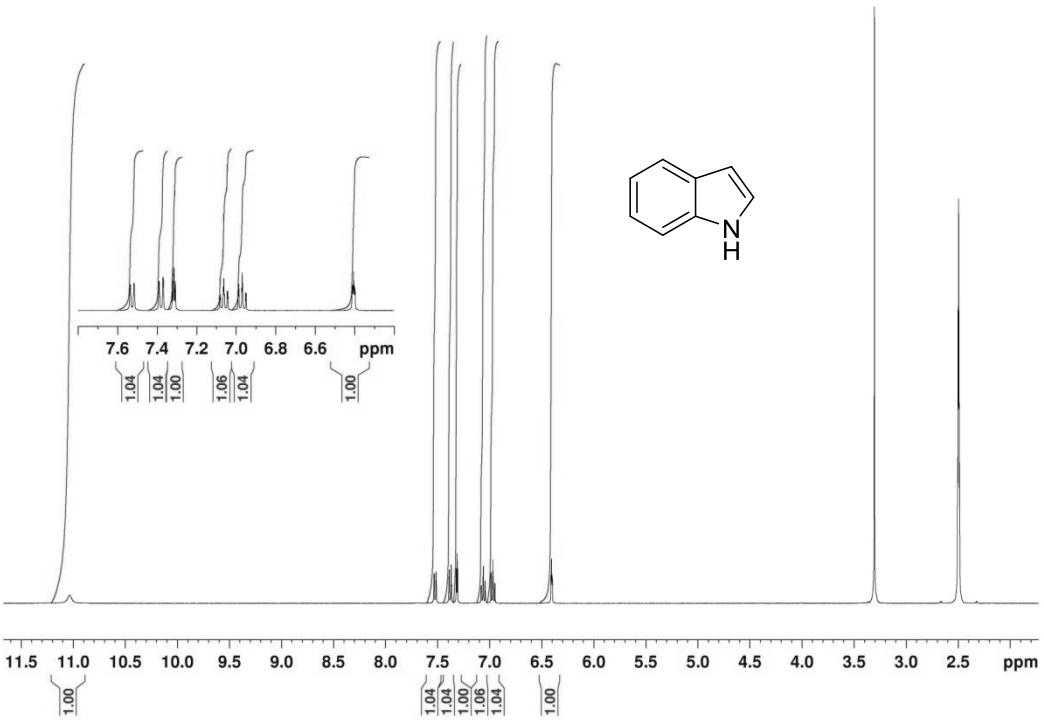
1-(2-Aminophenyl)-2-chloroethanone **58** (^{1}H; 400.13 MHz; 6d-DMSO)



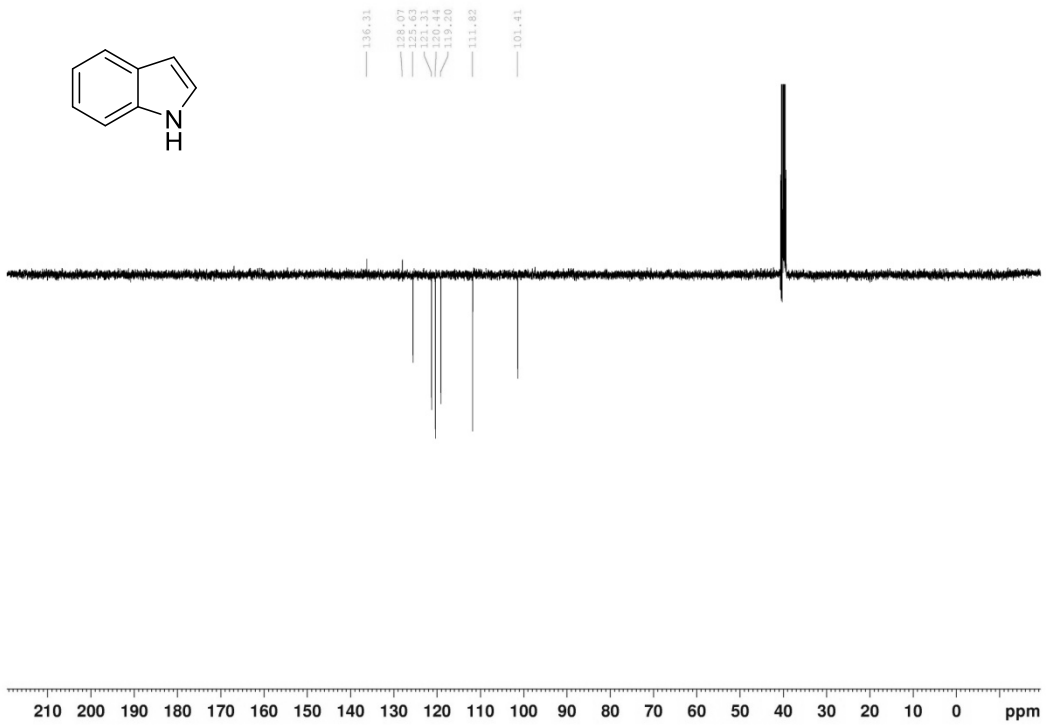
1-(2-Aminophenyl)-2-chloroethanone **58** (^{13}C; 100.61 MHz; 6d-DMSO)



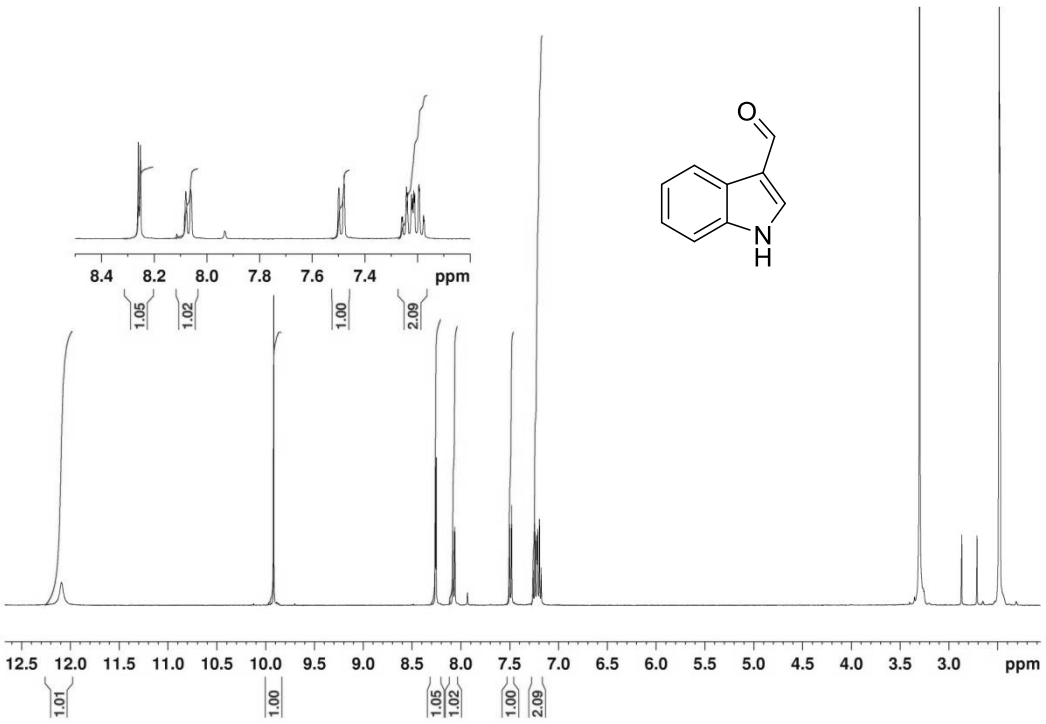
Indole **59** (^{1}H; 400.13 MHz; 6d-DMSO)



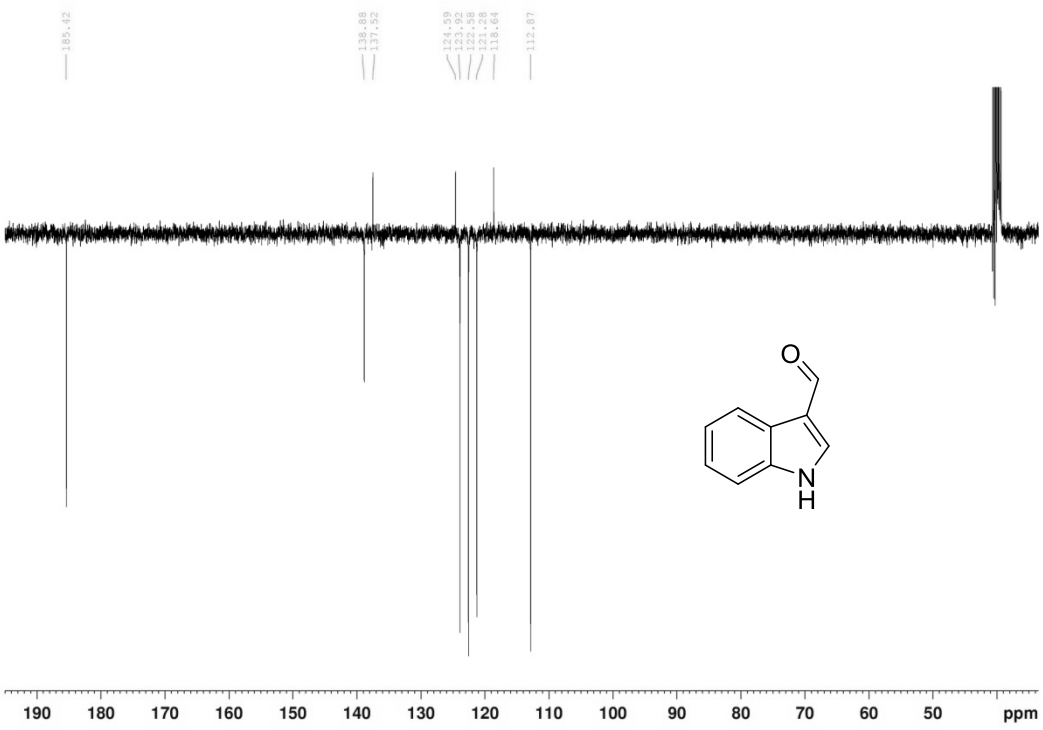
Indole **59** (^{13}C; 100.61 MHz; 6d-DMSO)



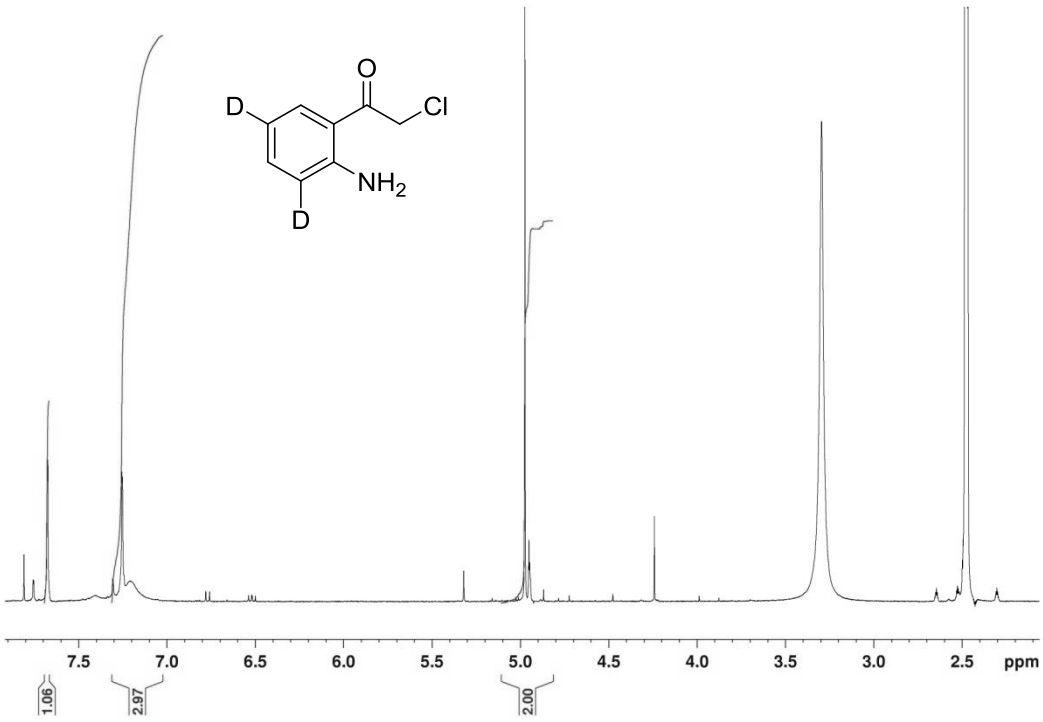
Indole-3-carboxaldehyde **60** (^{1}H; 400.13 MHz; 6d-DMSO)



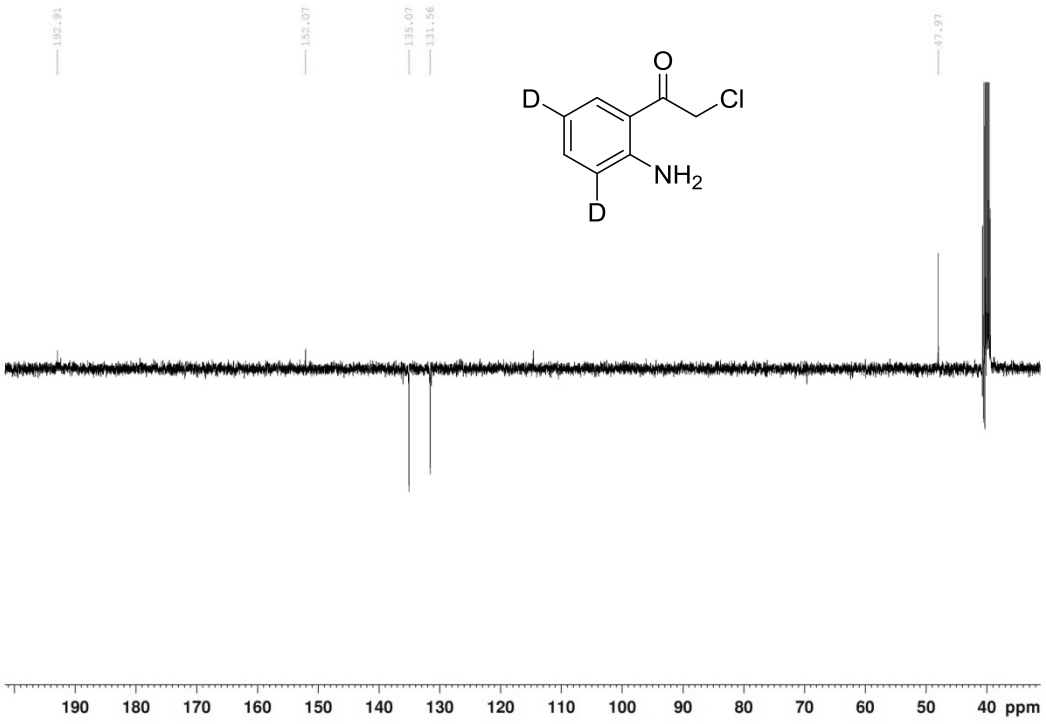
Indole-3-carboxaldehyde **60** (^{13}C; 100.61 MHz; 6d-DMSO)



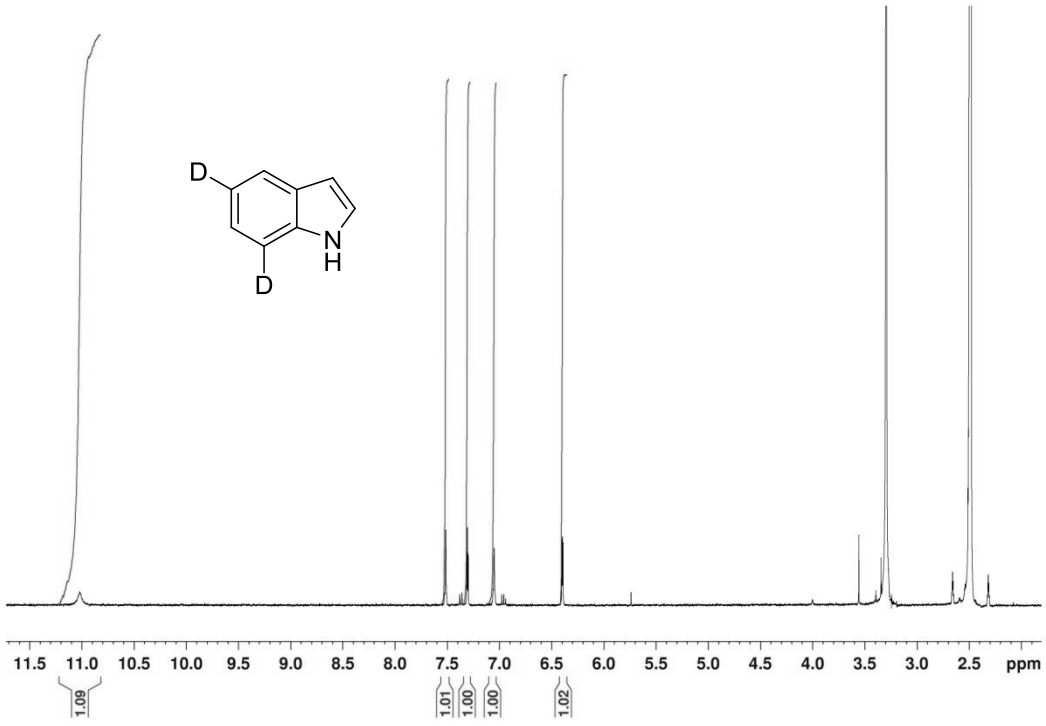
1-(2-Aminophenyl-3,5-dideuterio)-2-chloroethanone **63** (^{1}H; 400.13 MHz; 6d-DMSO)



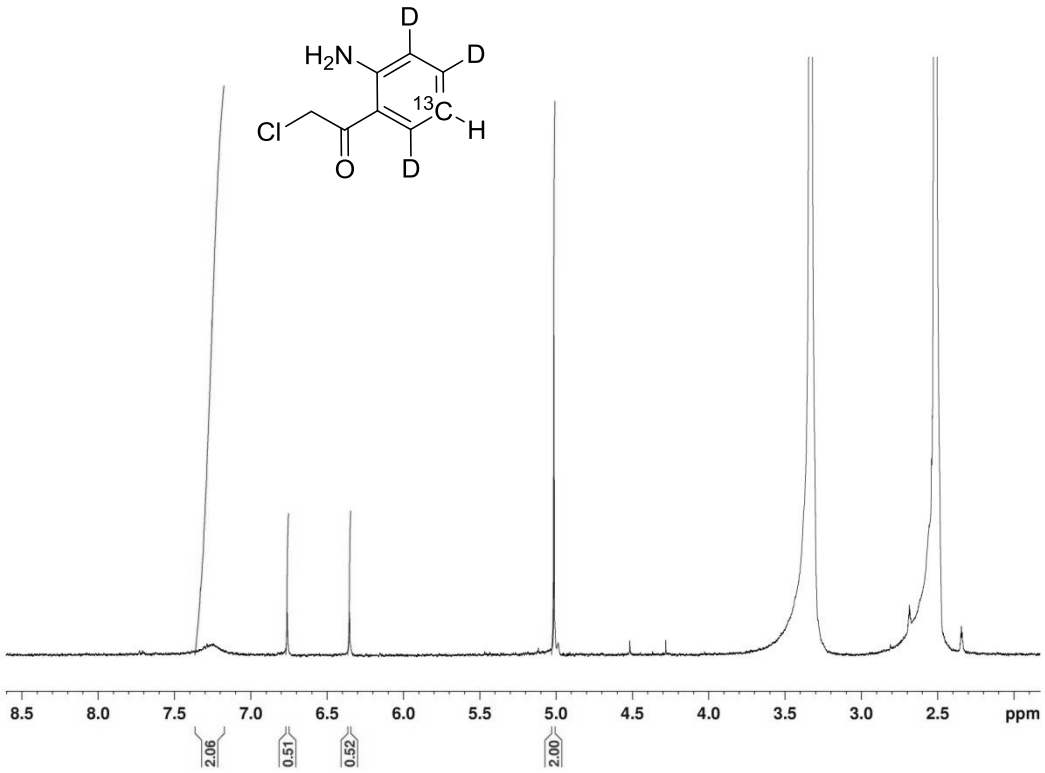
1-(2-Aminophenyl-3,5-dideuterio)-2-chloroethanone **63** (^{13}C; 100.61 MHz; 6d-DMSO)



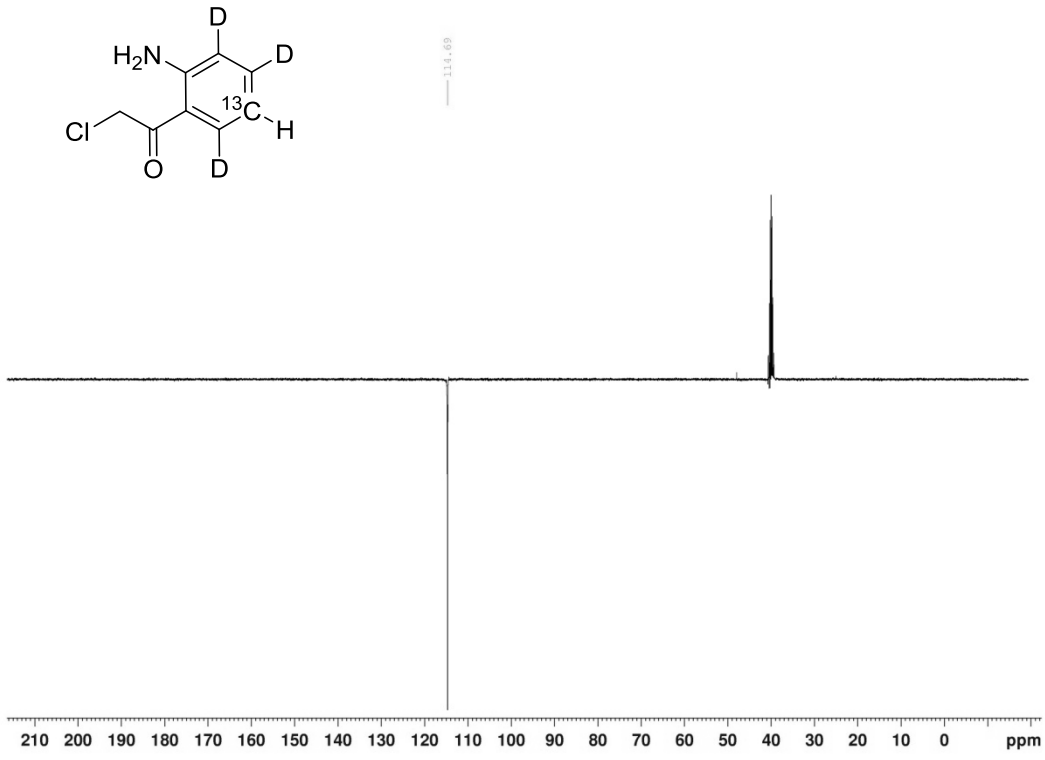
6,8-Dideuterioindole **64** (^{1}H; 400.13 MHz; 6d-DMSO)



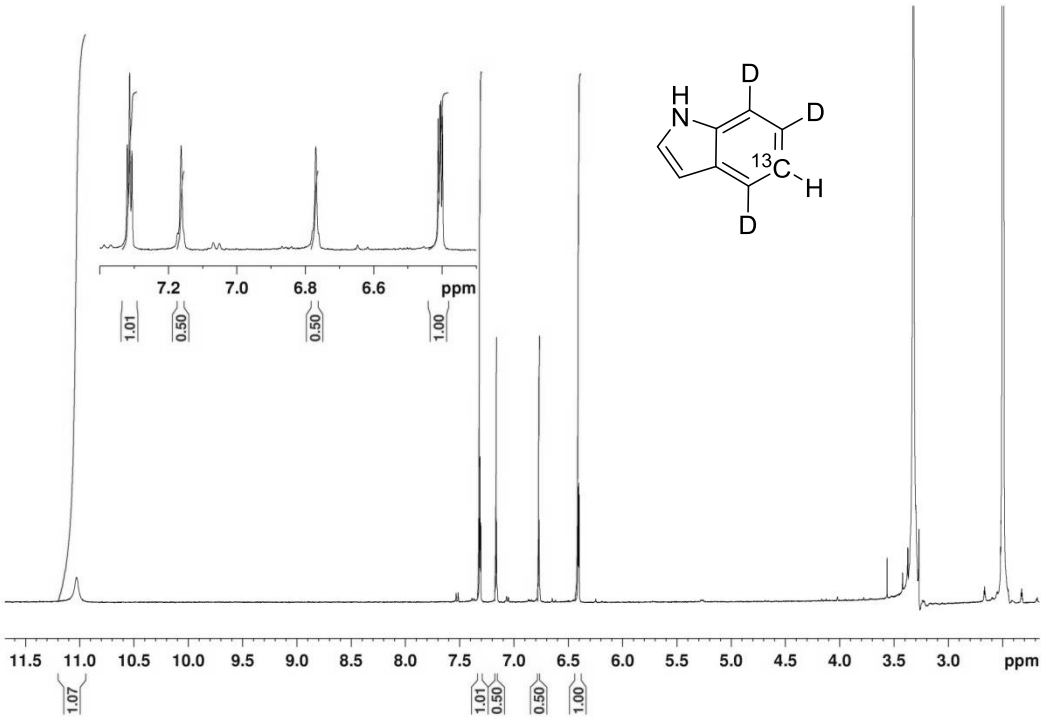
1-(2-Aminophenyl-5-^{13}C-3,4,6-trideuterio)-2-chloroethanone**70**(^{1}H;400.13 MHz;6d-DMSO)



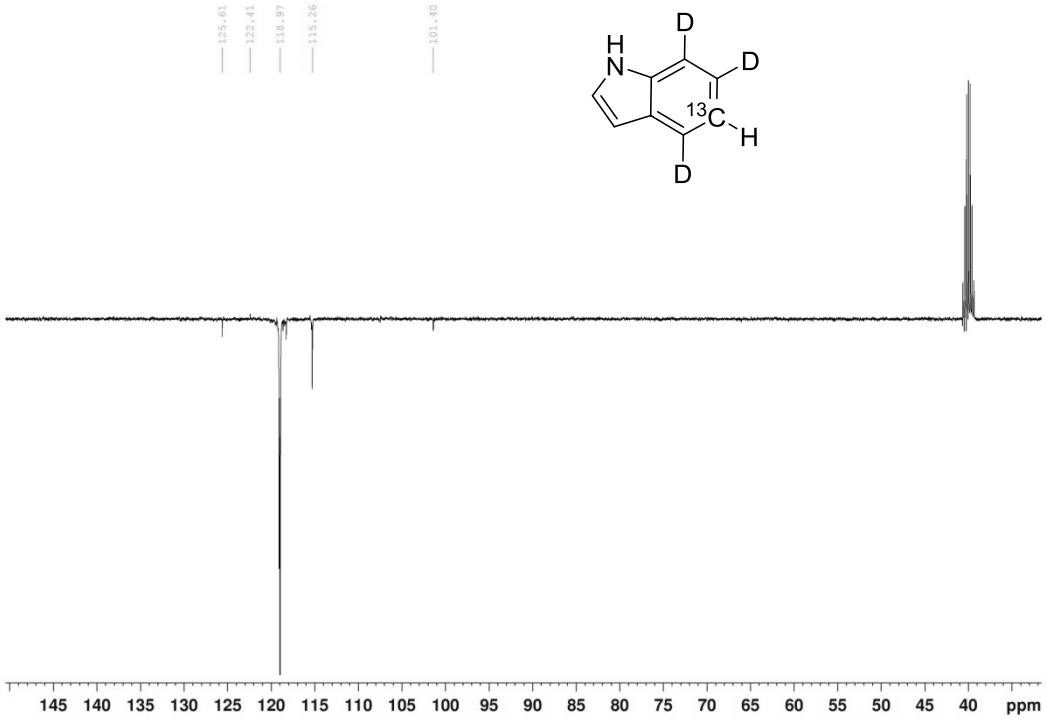
1-(2-Aminophenyl-5-^{13}C-3,4,6-trideuterio)-2-chloroethanone **70** (^{13}C; 100.61 MHz; 6d-DMSO)



6-^{13}C-5,7,8-Trideuterioindole **71** (^{1}H; 400.13 MHz; 6d-DMSO)



6-^{13}C-5,7,8-Trideuterioindole **71** (^{13}C; 100.61 MHz; 6d-DMSO)



6.2. Abbreviations and Acronyms

Å	Angstroem
Ac_2O	Acetic anhydride
aq.	Aqueous
ATP	Adenosine triphosphate
b	Broadened signal
BCP	Bromocresol purple
CAS	Chemical Abstracts Service
conc.	Concentrated
COSY	Correlated spectroscopy
d	Duplet
DCM	Dichloromethane
dd	Duplet of duplets
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
dt	duplet of triplet
E. coli	*Escherichia coli*
e. g.	Exempli gratia (par example)
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
EtOAc	Ethyl acetate
Et_2O	diethyl ether
GB1	Protein GB1
His	Histidine
HMQC	Heteronuclear Multiple Quantum Coherence
Ile	Isoleucine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
J	Coupling constant
kDa	Kilodalton
LB	Lysogeny broth

Leu	Leucine
lit.	Literature
m	Multiplet
M	Molecular Mass
MHz	Megahertz
MS	Mass Spectrum
N	Normality
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect Spectroscopy
OAc	Acetate
OD_{600}	Optical density of a sample measured at a wavelength of 600 nm
P. pastoris	*Pichia pastoris*
Pd/C	Palladium on carbon
PE	Petrol ether
Phe	Phenylalanine
ppm	Parts Per Million
q	Quartet
R_f	Retention factor
s	Singlet
SAIL	Stereo Array Isotope Labeling
t	Triplet
TEA	Triethylamine
TLC	Thin layer chromatography
TOCSY	Total correlated spectroscopy
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet

Val	Valine
δ	Chemical shift

6.3. REFERENCES

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6.4. DEUTSCHE ZUSAMMENFASSUNG

Selektive Isotopenmarkierung von Molekülen wird eingesetzt um komplexe NMR Spektren von Proteinen zu vereinfachen, indem man unwichtige Signale eliminiert und somit Signalerkennung ermöglicht. In unseren Synthesewegen werden unterschiedliche Muster von ^{13}C, ^{2}H und ^{15}N in den Zielmolekülen erzeugt, die im Folgenden als Bausteine in der Proteinexpression eingesetzt werden können. Die durchgeführten Synthesewege basieren auf kostengünstigen Isotopenquellen und der Verknüpfung literaturbekannter Reaktionen.

Zum einen wurden eine α-Ketosäure und zum anderen Indol als Vorstufen für Tryptophan in *E.Coli* Expressionsmedium synthetisiert. Nach erfolgreichem Einbau der α-Ketosäure wurden unterschiedliche Synthesen für den Indolprecursor mit komplizierteren Isotopenmustern durchgeführt, die ^{13}C-^{1}H Spinsyteme in einem deuterierten Umfeld enthielten.

Darüber hinaus wurde eine Syntheseroute entwickelt, die für mehrere enantiomerenreine Aminosäuren (Val, Leu, His, Try, Trp, Phe) anwendbar ist. Das chirale Zentrum dieser markierten Aminosäuren wurde enzymatisch generiert, wobei sowohl L- als auch D-Aminosäuren zugänglich sind. Diese markierten Aminosäuren können in der Festphasensynthese, sowie bei zellfreier Proteinsynthese eingesetzt werden.

6.5. CURRICULUM VITAE

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Date of Birth: 19th February 1991

Place of Birth: Amstetten

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Education

1997-2001	Primary School in Stadt Haag, NOE
2001-2009	Stiftsgymnasium Seitenstetten, NOE

Studies

2009-2014	Studies of Chemistry at the University of Vienna, Austria.
Apr.-Jun. 2011	Bachelor thesis under the supervision of Dr. Claudia Bello and Prof. Christian Becker at the University of Vienna.
Oct. 2013-Aug. 2014	Master thesis under the supervision of Dr. Roman Lichtenecker and Prof. Walther Schmid at the University of Vienna.

Research

Master Thesis	"New Concepts in Isotopic Labeling of Proteins"
Bachelor Thesis	"Nucleophilic Aromatic Substitution of the Methoxy Group of Nitroanisoles"

Fields of practical laboratory experience during the Master studies:

- Synthese von potentiell biologisch aktiven Phosphonsäuren (Prof. Friedrich Hammerschmidt)
- Studien zur Isotopenmarkierung von Valin und Isoleucin (Dr. Roman Lichtenecker)
- Herstellung von Komplexen mit einem Indolochinolin-Grundgerüst als Cytostatika (Dr. Lukas Filak)
- Further Studies on Nucleophilic Aromatic Substitution (Dr. Claudia Bello)

Scientific Publications and Presentations

Lichtenecker, R. J.; Weinhaupl, K.; Reuther, L.; Schoerghuber, J.; Schmid, W.; Konrat, R. **Independent valine and leucine isotope labeling in Escherichia coli protein overexpression systems** *J Biomol Nmr* **2013**, *57*, 205-209.

Schörghuber, J.; Lichtenecker, R. J.; Konrat, R.; Schmid, W. **Synthesis of Small Molecules to be used in Protein Labelling.** Poster; First Symposium of the Institute of Organic Chemistry at University of Vienna, 18th February 2014, Vienna, Austria.

Schörghuber, J.; Vogt, M. **Die Entstehung der Erde – die Erde atmet auf.** Poster Presentation; Bioanorganisches Symposium, 19th and 20th December 2012, Vienna, Austria.

Extra-Curricular Activities

Summer term 2014	I advised the practical course „Chemische Übungen für Biologen“ as tutor at the University of Vienna.

Professional Career

2005-2013	I was working as waitress in the zoo of Stadt Haag on weekends and during the summer months.
2012	I acquired practical experience working in BMW Steyr.
2011-2013	In the summer months I was working in the research sector of voestalpine Stahl AG in Linz.