Access to specifically stable isotope enriched, proteinogenic amino acids

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Chapter 5 General discussion and prospects

1

Introduction

1.1 GENERAL INTRODUCTION

"Amino acids are the building blocks of life" is a statement often seen in introductions to the topic of the structure and function of L-α-amino acids and is used to emphasise the importance of their role in the living cell as the building blocks used to form proteins. However, amino acids play pivotal roles in biological systems on many different levels. On a molecular basis, they are involved in roles as diverse as signal transmitter in the brain, energy source and building-block for more complex molecules (e.g. penicillin, haem). On another organisational level, they are the constituents of peptides, which can have amongst others hormonal, antibiotic, regulating and detoxifying functions. Finally, amino acids make up the building blocks of proteins, which have essential structural, transport, enzymatic, signalling and redox functions inside and outside the cell.[1] Due to their central role in myriad biological functions, amino acids are an important target and tool for researching protein function and interaction (proteomics), and for researching human (and animal) metabolism and catabolism (metabolomics) and how these processes relate to human (and animal) disease.

Nature provides us with the ultimate probe to investigate the targets mentioned above in the form of stable isotopes. Isotopes combine the same chemistry[2] with different physical properties[3] and the incorporation of isotopes in relevant proteins allows for the investigation of the unperturbed systems, using non-invasive and non-destructive techniques (e.g. FT-IR/FT-Raman and NMR). Furthermore, isotopically labelled L-α-amino acids allow the investigation of metabolic pathways by enabling mass-sensitive methods to trace isotopically labelled intermediates through consecutive steps in biosynthetic routes to more complex molecules. Access to all isotopomers of the DNA-encoded amino acids would be very beneficial to the investigation of the systems described above. Therefore the aim of this thesis is to develop enantioselective synthetic methods with which the full set of all isotopomers[4] of these chiral L-α-amino acids can be prepared.

1.2 ROLES OF AMINO ACIDS

The amino acids occurring in a cell are part of a fluctuating metabolic network. Proteins, hormones and ligands are formed and broken down and the waste products of all these processes are excreted. To maintain its health and structural integrity an organism should find a balance between the uptake of amino acid building blocks and the excretion of degradation products. Early studies (using stable isotopes) on animal metabolism showed that there is a rapid turn-over of proteins in the body.[5,6]

The mean turn-over rate of the total protein content of a rat-liver has been reported to be 4 to 5 days, though rates vary strongly from protein to protein.[6] In order to maintain a steady state, a human being requires an average daily protein uptake of 50 grams to fulfil the body's need for amino acids.[7] Of the 21 proteinogenic α-amino acids present in humans[8,9,10] and animals, 8 can not be obtained by *de novo* synthesis in the body itself and they must be present in human (and animal) nutrition (Appendix 1).[11] These amino acids are also called 'essential amino acids'; they are the L-enantiomers of valine, leucine, isoleucine, lysine, methionine, threonine, phenylalanine, tryptophan and histidine. Although it is not strictly essential, L-Arginine is sometimes also placed in this list, because the body's L-arginine synthesis has to be supplemented by L-arginine from nutrients for optimal growth.[7]
Surplus amino acids cannot be stored and are used as a metabolic fuel source.[1] First, they are deaminated via transamination to form α-keto acids. The α-keto acids are then metabolised into pyruvate or into intermediaries of the citric acid cycle. The amino group is converted to NH_3 in a few steps. In the urea cycle, NH_3 is subsequently coupled to ornithine, to form citrulline. This in turn is hydrolysed in two steps into ornithine and urea, the latter of which is secreted.
Amino acids can be incorporated or modified in many ways to form essential biochemicals for life. Examples are the hormones tyramine, histamine and GABA (γ-aminobutyric acid), which are prepared by decarboxylation of tyrosine, histidine and glutamic acid, respectively. Another example is haem, which is formed from glycine and glutamate and forms amongst others the oxygen-binding centre of hemoglobine. Apart from building block or energy source, glutamic acid has two more interesting biological functions as neurotransmitter[12] in the brain and as the taste molecule responsible for the taste umami.[13]

Amino acids can be coupled together to form short chains called peptides, which are generally up to 50 amino acids long. Many of these peptides have highly specific biological functions, serving as hormones, neurotransmitters, antibiotics and drugs. Moreover, peptides can be involved in signal-transduction and as such they are ligands for G-coupled receptors, where they have been detected in the binding pocket by SS-NMR.[14] There is even emerging evidence for a direct link between cellular (peptide) metabolism and the transcription process, by the discovery of transcriptional regulators whose activity can be regulated by metabolic cofactors such as SAM and acetyl-CoA.[15]
Other peptides, such as glutathion, are involved in detoxification of the body or are involved in gene regulation (S-adenosylmethionine). Another role of peptides is their function as taste compounds. The most well-known of these is the synthetic sweetener aspartame (L-aspartyl-L-phenylalanine methyl ester), which is produced

on a multi-ton scale. Others, such as the tri-peptide Leu-Pro-Trp produced by yeast during the production of beer have a bitter taste.[11]

1.3 PROTEINS

Proteins are long peptide chains (> 50 amino acid residues), which have, amongst others, structural, transport and enzymatic roles and which are usually dependent on their higher order structure for their functioning. There are four orders of protein structure. A protein's primary structure is simply the order in which the covalently linked amino acids follow each other in the peptide chain. Using the 21 'coded' amino acids, the number of different proteins made by translation in humans is roughly 30.000.[16] in addition, there are many instances of post-translational modifications of amino acids in proteins and peptides, boosting this number much higher.[11] Essential functions in human and animal metabolism and biology are taken care of by post-translationally modified proteins. Examples are the hormone insulin and the structural protein collagen. In insulin, 32 amino acids are excised from the peptide chain of proinsulin, to form a two-strand protein connected by disulphide bridges. To synthesise collagen, lysine and proline residues are hydroxylated to form hydroxylysine and hydroxyproline. Other post-translational modifications are amongst others phosphorylation and glycosylation of OH-groups, acetylation and methylation of the lysine NH_2 and oxidation of the cysteine -SH groups to from disulphide bridges. The secondary structure is the folded chain of helices, β-sheets and hairpin-loops. These structures are formed by hydrogen bond formation (between the NH of one peptide bond and the carbonyl of another peptide bond). The tertiary structure which emerges from here is the manner in which helices, sheets and loops spontaneously fold together (or are folded together with the help of chaperones), due to electrostatic interactions. hydrophobic interactions and the formation of disulphide bridges between cysteines, to form a functioning protein (e.g. the seven transmembrane helices of G-protein coupled receptors such as the protein rhodopsin, which are carefully aligned with regard to each other).[17,18] The quaternary structure is the way several proteins form one non-covalently bound complex together. Examples are the blue lobster pigment α-crustacyanin, which is an octamer of dimers of the protein β-crustacyanin.[19] Another example is the photosynthetic reaction centre of *Rhodobacter sphaeroides*, which is a transmembrane protein complex consisting of three subunits.[20] Ultimately, the protein is folded in such a way that certain essential substructures are formed (e.g. the catalytic and binding sites of an enzyme). The protein's folding is such that the substructure is available for close contact with a target, which can be a substrate molecule or another protein. This enables the protein

to fulfil its function, either as enzyme, transporter or part of a signalling cascade, with a tremendous rate and selectivity. It should be clear that in order to understand the function of a protein, knowledge of its structure is of paramount importance.

1.4 PROBING PROTEIN STRUCTURE AND CELLULAR METABOLISM USING ISOTOPES

The previous sections have shown that amino acids are present and essential at many organisational levels of a living being. To elucidate their roles, either as metabolite or building block, complex systems have to be analysed. This section describes the manner in which isotopes can be used to obtain the required structural and metabolic information.

There are many methods with which protein structural information can be obtained. Unfortunately, no method is without its drawbacks. In the following paragraphs several methods will be discussed. A very important method is X-ray diffraction. Using crystals of the desired protein, the complete structure of the protein can be resolved by analysis of its X-ray diffraction pattern. For this method, high quality crystals have to be made for the accurate analysis of the protein structure. This is not an easy task, especially for the lipophilic, membrane-spanning proteins that have important functions in signal transduction. Still, a high quality X-ray structure has at best a resolution of about 50 pm. This is approximately the length of a carbon-carbon bond. Moreover, due to the very low electron density, it is difficult to locate hydrogen atoms accurately. This means that the resulting information about the structure is incomplete and that essential information on hydrogen bonding and on protonation states of certain residues will not be available.

By using spectroscopic methods such as FT-IR, Raman and NMR, it is possible to investigate native, biologically active systems without perturbation, at the atomic level. FT-IR spectroscopy and (Resonance) Raman spectroscopy are techniques based on molecular vibrations and bond strengths. These techniques probe the electron density at atoms and the strength of chemical bonds of the molecule. A study with site-directed, highly enriched isotope labelling allows the determination of the whole force field of a system, based on the shifts in vibrational frequencies due to a difference between the masses of labelled and unlabelled atoms.[21,22,23]

A spectroscopic technique that gives another form of structural information is solution or solid-state NMR spectroscopy. This technique probes the electron density at nuclei having a nuclear spin. For carbon and nitrogen the stable isotopes that have a non-zero spin (I=1/2) are the ^{13}C and ^{15}N nuclei. The natural abundance of these isotopes is relatively low, being 1.1% for carbon-13 and 0.37% for nitrogen-15.

The more abundant ^{12}C (98.9%) and ^{14}N (99.63%) isotopes are NMR-silent. NMR-techniques allow the establishment of electronic charges on atoms, of protonation states and of configurations and conformations around bonds in the studied protein. Most proteins are large and complex systems, having molecular masses roughly between 10 and 500 kD. The determination of the characteristics of one or several atoms in such a surrounding is exceedingly difficult without a manner to boost the desired signals to a level that is noticeable above the background signal emitted by the other hundreds of ^{13}C-carbons and ^{15}N-nitrogens present in the protein at the natural abundance level. Introduction of stable isotopically labelled carbon or nitrogen atoms, enriched up to 99% ^{13}C or ^{15}N, can amplify their signals 90-fold and 270-fold, respectively. Many NMR techniques used for investigating protein systems are dependent on the introduction of stable isotope labels in the part of the protein or cofactor that is to be investigated.[19,24] Using isotope labels, even bond lengths can be resolved with 10 picometer resolution and torsion angles can be determined along selected bonds in a protein system.[24] Having access to the full set of possible site-directed isotopically enriched amino acids up to the uniformly labelled systems will greatly benefit structural and fundamental investigations. This is especially true since new peptide ligation techniques have made total organic synthesis of whole, folded proteins feasible.[25,26] The combination of peptide ligation and access to any and all amino acid isotopomers will make it possible to specifically introduce a stable isotope label at any position or combination of positions in the protein to be studied.

A technique that is utilised both for structural and metabolic analysis down to the attomole level is mass spectrometry. This technique is based on the difference between masses of molecules when they are ionised and accelerated through a magnetic field. The energy introduced during ionisation of the molecule or peptide often results in fragmentation of the compound, giving fragment peaks, that give additional structural information. The fragmentation patterns, originating from peptide fragments of different lengths, lead to the peptide structures. In this manner, high-speed screening of peptides and proteins can be performed. However, due to the presence of sets of isobaric amino acids (Leu, Ile and Hyp, Lys and Gln), ambiguities in the assignment remain. Growth of organisms on media containing specifically ($^{2}H/^{13}C/^{15}N$) isotope labelled amino acids can eliminate these problems by generating clearly distinct M+i peaks for the labelled species, where i is the number of isotopes introduced in the amino acid.[27] Another application of mass-spectrometric techniques is the elucidation of metabolic pathways. The problem of tracing the route of metabolic pathways and the metabolic flux under changing circumstances is eminently suitable to be tackled with selective isotope incorporation. The uptake of isotopically labelled compounds will introduce clearly distinguishable markers with which to follow the

progression of metabolites through consecutive steps in biochemical pathways.[28] The combined use of HPLC and mass-spectrometry will yield results in which the difference in mass between isotopically labelled and unlabelled compounds betrays the metabolite's origin.

1.5 INTRODUCING STABLE ISOTOPES IN AMINO ACIDS

The application of the techniques discussed in the previous section depends ultimately on the availability of stable isotope labelled amino acids. All uniformly ^{13}C and ^{15}N enriched L-α-amino acids are available via photosynthetic organisms that are grown in media containing $^{13}CO_2$ and $^{15}NH_3$.[29] However, the full set of site-directed isotopomers of amino acids cannot be obtained via biosynthesis. The only efficient way to obtain access to the whole set of desired isotopomers is via a modular synthetic approach such that one synthetic scheme can provide any specifically labelled L-amino acid. This approach may seem Herculean, however, only 21 different amino acids are needed. In the recent past this has already been realised for 9 of the 21 'coded' amino acids.[30,31,32,33] The two simplest amino acids, Gly and Ala are commercially available labelled in any position or combination of positions. For the remaining ten amino acids, this thesis presents schemes that allow the introduction of stable isotope labels in valine, isoleucine, leucine, methionine, cysteine, selenocysteine and lysine. Also presented are schemes for the preparation of metabolic intermediates ornithine (Orn), homocysteine, cystine and homoserine and for the seleno-analogues of cystine and methionine.

The amino acids that are translationally incorporated in the peptide chain by the ribosome all have the same basic structure (Figure 1). With the exception of L-proline, which is formally an imino acid, they contain an amino group, a carboxylic acid

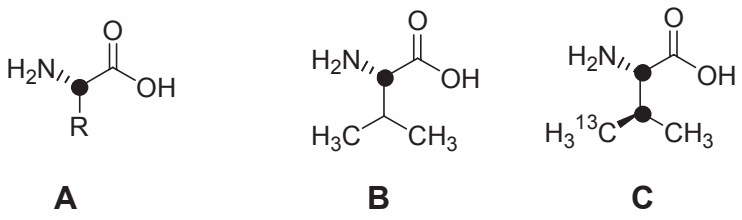

Figure 1. General L-α-amino acid structure (**A**), an amino acid (L-valine) with two prochiral methyl groups (**B**), introduction of a second chiral centre in L-valine through selective label incorporation in the 4(*S*)-methyl group (**C**). The dark dot marks a chiral centre.

group (the two functional groups from which they derive their name) and a side chain "R" attached to the α-carbon atom. This side chain R can be aliphatic, contain an amino group, a carboxylic acid or amide, contain a sulphur or selenium atom or can be aromatic.[34]

According to the Fisher-Rosanoff convention, all proteinogenic amino acids are defined as L-amino acids, as their structure can be traced to L-glyceraldehyde. The configuration around the central carbon atom is *S* for all amino acids except the chalcogen containing amino acids cysteine and selenocysteine[35,36] and except for glycine which is achiral. Two amino acids contain a second chiral centre, these amino acids are isoleucine with a (2*S*,3*S*) configuration and threonine with a (2*S*,3*R*) configuration. Two amino acids have a prochiral centre in their side-chain. These are L-valine and L-leucine. The chiral centre on the α-carbon gives rise to an inequivalency of the methyl groups attached to the C-3 and C-4 carbon, respectively.

There are several general considerations to keep in mind when designing schemes for the preparation of isotopically labelled compounds. First, there is only a very limited number of labelled precursor molecules commercially available. Therefore, any scheme has to be based on the simple, achiral starting materials available in isotopically enriched form. Secondly, the schemes should not allow dilution or scrambling of the isotope labels. This precludes symmetrical synthons or intermediates, because selective labelling of one half of the molecule would lead to an incorporation of 50% in the product. A third consideration is the high price of isotopically labelled starting materials (up to a factor of 10.000 more expensive than their unlabelled counterparts) which demands an efficient synthesis with a high overall yield. The fact that all α-amino acids share a basic structural element, the glycine backbone, can be used to one's advantage when designing efficient synthetic schemes. Different side-chains can be coupled to this shared structural element to prepare different amino acids. This suggests modular, convergent synthetic methods in which the glycine backbone and the side-chain are made separately and coupled at a late stage in the synthesis, after which only a deprotection step is needed. This methodology has the important advantage that the number of steps with isotopically labelled precursors is kept to a minimum. An added difficulty when designing schemes for the preparation of isotopically labelled amino acids is the chirality of the target molecules. Nature distinguishes between the *S* and *R* enantiomers of each amino acid and this forces one to pay careful attention to the synthetic schemes to ensure that the labelled amino acids have the correct chirality.

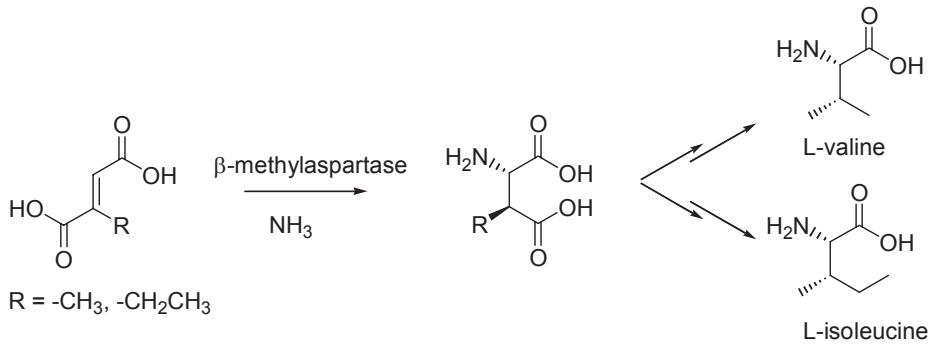

Figure 2. Introduction of chirality by β-methylaspartase.

Several strategies have been developed over the years to prepare chiral, isotopically labelled amino acids, but few strategies are generally applicable or without major drawbacks. One possibility is to let nature itself do the work, using enzymes whose active site will force the desired chirality upon the molecule. In this way an achiral precursor molecule is converted by an enzyme to yield only one enantiomer of an optically active compound. This approach has been chosen for the synthesis of L-valine and L-isoleucine, using the enzyme β-methylaspartase (Chapter 2), which introduces two chiral centres in one reaction step, by addition of ammonia to the double bond of 2-methyl- and 2-ethyl- fumaric acid (Figure 2). Unfortunately, the enzyme β-methylaspartase is fairly specific with regard to other substrates than the native one. Consequently, this method is not easily applicable to the synthesis of other amino acids.

A second approach is to introduce chirality via chemical methods. This can be achieved by using a chiral template, a molecule that functions as a chiral scaffold. In this case the molecule (or future part thereof) in which a chiral centre has to be introduced enantioselectively, is part of a larger molecular scaffold containing bulky side groups that induce the chirality at the desired position. A great advantage of this method is that it is possible to attach many different substituents to the scaffold. Thus one method could, in theory, suffice to make all 20 substituted, coded L-amino acids. Examples of scaffolds are the Williams and Schöllkopf templates (Figure 3).

The Williams template consists of a masked glycine moiety, that is part of a ring containing two bulky side-groups. Using a strong base, the glycine moiety can be deprotonated and a nucleophilic attack of the formed enolate will introduce a side-chain on the masked glycine. The steric hindrance of the bulky phenyl rings forces the alkylation to occur almost exclusively on one side of the enolate. After removal

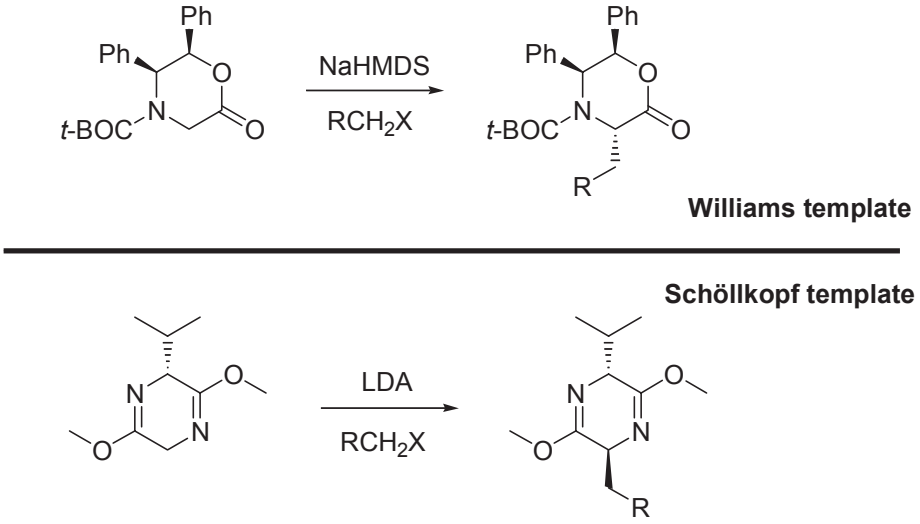

Figure 3. Introduction of chirality using the Williams and Schöllkopf templates.

via reduction of the scaffold the new amino acid can be isolated after purification. A similar procedure is used for the Schöllkopf template, which consists of two masked amino acids, one glycine and one D-valine, which are coupled to form a bis-lactim ether. In this case the steric hindrance, provided by the chiral D-valine, forces the reaction to form an L-amino acid. Both methods have several drawbacks however. The most important one is that to introduce isotope labels in the backbone of the amino acid, isotope labels have to be introduced into the glycine moiety of the scaffold via a relatively inefficient procedure. Another drawback of these methods is that they need a stoichiometric quantity of chiral inductor. After the alkylation reaction the template has to be hydrolysed to free the product. Therefore, for every reaction a stoichiometric amount of the expensive chiral template has to be used and subsequently dismantled, after which it can not be reused. A less important disadvantage of the Williams and Schöllkopf methods is the fact that the protons of the masked glycine are not acidic enough to be abstracted by aqueous base and that a strong base has to be used to generate the enolate. Therefore the reaction has to be carried out under careful exclusion of water. The Schöllkopf method has the additional drawback that the product is obtained as a mixture of the desired amino acid and D-valine, the other half of the former scaffold. The product must subsequently be isolated via a cumbersome separation using ion-exchange chromatography. Obviously, a last drawback of the Schöllkopf method is that it is not suitable for the production of isotopically labelled L-valine.

A second chemical manner to induce enantiospecificity is using a chiral inductor, a molecule that resembles an enzymatic pocket in its ability to influence chirality. One example is the class of cinchona-derived chiral phase transfer catalysts (CPTC) developed by the groups of O' Donnell and Corey (Figure 4).[37,38,39] This method also starts with a fully protected glycine, that can be deprotonated to form an enolate to which many substituents can be attached. The chirality is induced by the CPTC, a large chiral ammonium salt, that forms an ion pair with the enolate. The bulky side-chains of the cinchonidium ion induce a most favoured orientation of the enolate anion with regard to the ammonium cation (**1**) (Figure 5).[40]

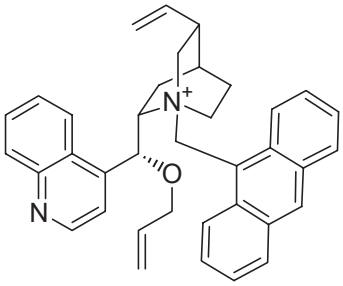

Figure 4. The chiral Cinchonidine based Phase Transfer Catalyst (CPTC).

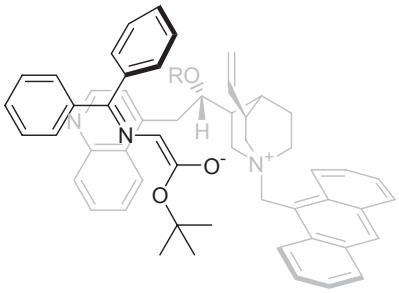

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Figure 5. The most favoured orientation of the CPTC-enolate complex.

This orientation is strongly favoured because of the smallest charge separation and least steric hindrance between the vinyl group on the catalyst and the bulky *t*-butyl ester compared with otherwise oriented CPTC-enolate complexes. In this way, the cinchonidine-derived catalyst shields the enolate in such a manner that reaction on the C-2 carbon of the glycinate can only take place from the *Si*-face. What makes this method special is that in this process the CPTC has the dual role of chiral inductor and phase transfer catalyst (Figure 6).

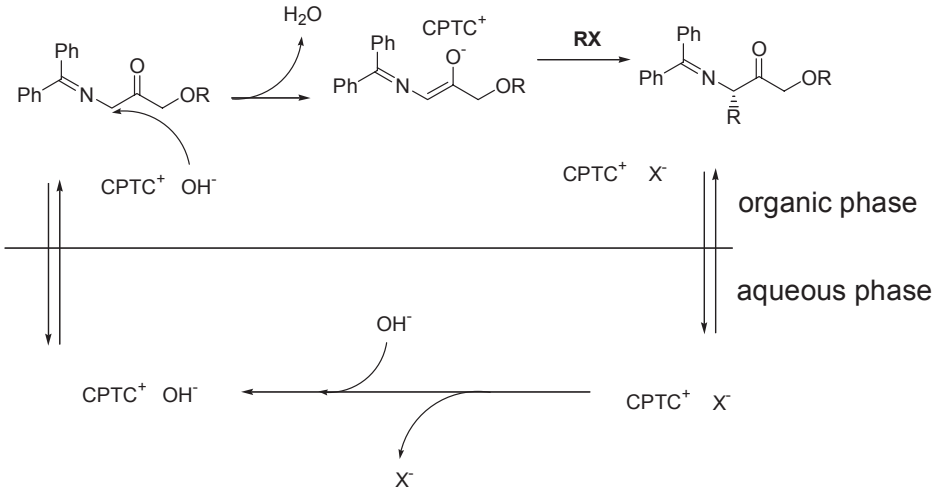


Figure 6. Schematic representation of the catalytic role of the phase transfer catalyst.

The CPTC-cation functions as the phase transfer catalyst by its capability to transfer a hydroxide-anion that shuttles the base across the interface between the aqueous phase and the organic phase. In this way the protected glycine is deprotonated and the enolate that is formed, forms a tight ion-pair with the positive charge of the nitrogen in the CPTC. Alkylation of, or Michael-addition on the enolate, results in an uncharged protected amino acid that diffuses out of the CPTC pocket. The CPTC subsequently forms a new ion-pair with the counter-ion produced during the alkylation, which shuttles back to the aqueous layer. After exchange of the anion for a new OH^- ion the CPTC becomes available for a new reaction cycle.
This method has several advantages over the scaffold methods described before. The most important one is that the catalyst can be easily recycled and reused. Moreover, the fact that aqueous base is used to form the enolate, means that no special precautions have to be taken with regard to moisture and that scaling up the reaction is relatively easy. Furthermore preparation of isotope labelled variants of the protected glycinate is relatively simple (see Chapter 2) as opposed to preparation of labelled scaffolds for the template-based methods. Considering these factors we decided to apply CPTC's for preparing isotope labelled amino acids.

1.6 SCOPE OF THIS THESIS

With the completion of the Human Genome Project, the primary structures of all human proteins have been elucidated. The fundamental challenge is now to study

in the native state, without perturbation, the role of these proteins in chemical processes at the atomic level. The ultimate probes for investigating these systems are stable isotope labels. This thesis deals with the development of a toolbox of isotopically labelled amino acids, labelled at any position or combination of positions, that can be used for studies into protein function, protein structure and human and animal metabolism.

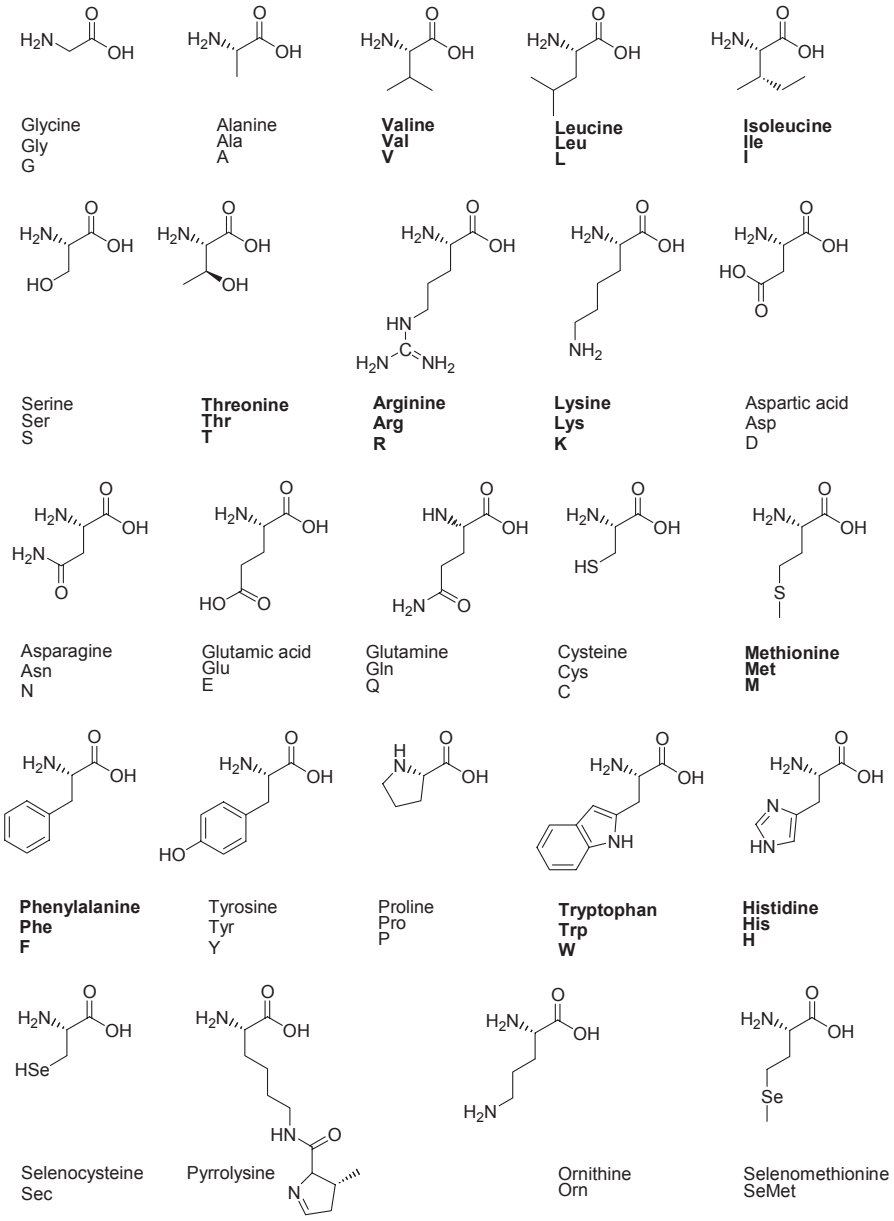
Chapter 2 describes the synthesis of the aliphatic amino acids valine, isoleucine and leucine. Schemes are described in which these amino acids can be labelled at all positions and combinations of positions with stable carbon and nitrogen isotopes. The developed schemes allow a distinction to be made between the two prochiral methyl groups of valine and leucine. Chirality is introduced in the valine and isoleucine syntheses using β-methyl aspartase, while the chiral quaternary ammonium salt developed by Corey et al. was used for synthesising L-leucine. The syntheses of [4-^{13}C]-L-valine and [5-^{13}C]-L-leucine are described in this Chapter.

Chapter 3 extends the CPTC methodology used for the synthesis of L-leucine to the synthesis of the chalcogen-containing amino acids. These amino acids are often involved in tuning redox properties of metalloproteins. Schemes for the preparation of all stable isotopomers of L-methionine, L-cysteine and their selenium derivatives are described. Also described are the synthesis of L-serine, L-homoserine and L-homocysteine and L-vinylglycine.

Chapter 4 contains the syntheses of L-proline and L-lysine. L-lysine plays a crucial role in visual signal transduction in the Schiff-base linkage with the retinylidene chromophore in rhodopsin.[41] Using efficient synthetic methods, [ε-^{15}N,ε-^{13}C]-L-lysine was prepared. Using an improved CPTC, L-ornithine, which is an important intermediate in the urea-cycle, was also prepared.

Chapter 5 contains a general discussion of the results presented in earlier chapters and a future outlook. Several ways are discussed to complete the set of all stable isotope labelled amino acids. Furthermore, the applicability of the developed toolbox on the investigation of several biological systems is discussed.

1.7 APPENDIX TO CHAPTER 1



Appendix 1. The structures, names and abbreviations of the 22 coded L-α-amino acids[34]. There are currently 22 amino acids known to be coded for by DNA. 20 of these are used throughout nature, the 21st, selenocysteine, which is also found in humans, is relatively rare and a stop-codon has been converted for its incorporation. The 22nd amino acid, pyrrolysine, has only recently been discovered, is exceedingly rare and has only been found in bacteria. The essential amino acids are denoted in bold typeface. The non-coded amino acids ornithine and selenomethionine are also depicted.

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2

Preparation and characterisation of (2*S*,4*R*)-[5-^{13}C]-leucine and (2*S*,3*S*)-[4-^{13}C]-valine. Establishing synthetic schemes to prepare any site-directed isotopomer of L-leucine, L-isoleucine and L-valine.

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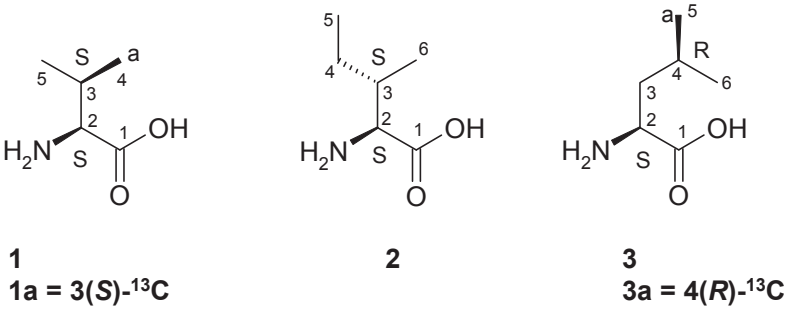
2.1 INTRODUCTION

The primary structures of all human proteins are now available with the completion of the human genome project.[1] Very rapidly, the total genomes of a plethora of other organisms will also become available in addition to mutants that lead to malfunctioning or non-functioning proteins leading to genetic diseases. Furthermore, efficient biotechnological procedures are available to obtain (membrane) proteins using these genetic codes.[2,3] The fundamental challenge now is to study the chemical processes of these proteins involving (bio)macromolecules without perturbation in the native states at the atomic level in time scales ranging from femtoseconds up to days. Nature provides us with the ultimate probe in the form of stable isotopes. Isotopes combine the same chemistry with different physical properties.[4] Study of a system with specific isotope labelling with a high incorporation allows the determination of the whole force field via vibrational techniques such as FT IR spectroscopy and (Resonance) Raman spectroscopy based on the difference in isotopic mass.[5,6,7] These techniques probe, for instance, the electron density in chemical bonds of the isotope-labelled molecule. Other spectroscopic methods are solution and solid-state magic angle spinning (MAS) NMR spectroscopy, which probe the electron density at the atoms. These techniques allow the establishment of electronic charges in the atoms, protonation states, and configurations and conformations around bonds of the stable isotopically labelled molecule.[8,9] Even bond lengths can be resolved with picometer resolution and torsion angles can be determined along selected bonds in a protein system.

Comparison of the structural parameters obtained by these techniques for intermediate I and intermediate I + 1 in the biochemical process of the studied system provides functional information, such as changes in protonation states, bond lengths, configuration, and conformation around bonds on the time scale involved.[7,8] When sufficient structural and functional information at the atomic level of the native form has been obtained, a whole new dimension can be attained by studying in a similar fashion systems with mutations in the protein chain and systems with rationally designed chemical changes in the cofactors.[9,10] These studies will lead to an even deeper understanding of the biochemical process. Implementation of the above-mentioned program is now of great urgency. Without this program, the increasingly available genetic information in the post-genomic era cannot be translated into the required structural and functional information that will lead to the expected quantum leap in the understanding of the various processes in human (and animal) health and diseases and the expected rational approach to treat these diseases. Access to the full set of possible site-directed isotopically enriched amino acids up to the uniformly labelled systems is a *conditio sine qua non* for the proposed structural

and fundamental investigations. All uniformly ^{13}C and ^{15}N enriched amino acids are commercially available through photosynthetic organisms that are grown in media containing $^{13}CO_2$ and $^{15}NH_3$.[11] However, all other possible site-directed isotopomers of amino acids cannot be obtained via biosynthesis. The only way to obtain access to the whole set of desired isotopomers is by a modular synthetic approach such that one synthetic scheme can give in a rational way any L-amino acid in a set of all isotopomers. The approach may seem Herculean, although, in fact, only 21 different amino acids are needed.

In the recent past this has already been realised for 9 of the 21 proteinogenic amino acids.[12,13,14,15] These are Glu, Gln, His, Phe, Pro, Ser, Thr, Trp and Tyr. Gly and Ala are commercially available labelled in any position or combination of positions. Of the remaining nine amino acids that are introduced in the protein chain by the translation process in the cell, this chapter focuses on the three amino acids with aliphatic side chains, namely valine, leucine and isoleucine. There are indications that the aliphatic side-chains of amino acids are involved protein stabilisation through interactions with aromatic residues of Trp, Tyr and Phe in the protein.[16] Previously, no method existed to obtain direct structural information about these interactions. This has now changed dramatically by the advent of ultra high field ^{1}H and ^{13}C solid state NMR techniques. The first solid-state ^{1}H and ^{13}C chemical shift study of $^{13}C_{20}$ rhodopsin showed that the largest shifts are due to the interaction of the chromophore with the aromatic side chains in the active site that occur on the 16 and 17 methyl groups of the aliphatic ring of 11-*Z* retinal. Access to valine, leucine and isoleucine with specific ^{13}C enrichment in the methyl groups will allow these interactions to be quantitatively established in any protein at the atomic level via ultra high field $^{1}H,^{13}C$ techniques. Another motive for the development of synthetic schemes for the synthesis of the full set of ^{13}C isotopomers of valine, leucine and isoleucine is the fact that these three are essential amino acids in human and animal nutrition. These amino acids have to be present in food because animals and humans cannot biosynthesise them. The access to isotopomers will allow nutritional and metabolic studies (metabolomics) in individual humans (and animals) at the physiological level without perturbation, based on a similar study for $^{13}C_{10}$ β-carotene which was recently published.[17] An additional motivation for the preparation of the isotopomers of leucine is that leucine is essential in gene regulation via the formation of leucine zippers and the regulation of protein turnover. The essential role of leucine in these vital processes will also be amenable to study at the atomic level without perturbation via site-directed isotopomers.
However, the presently most compelling reason to have access to the full set of isotopomers of leucine and isoleucine is that these amino acids are isobaric. This fact is



1
1a = 3(*S*)-^{13}C

2

3
3a = 4(*R*)-^{13}C

Figure 1. Structures of (2*S*)-valine (**1**), (2*S*)-isoleucine (**2**) and (2*S*)-leucine (**3**). (**1a**) and (**3a**) represent the site-specific ^{13}C enriched forms.

a strong handicap to the development of high-throughput mass spectral analysis as a tool in proteonomics, which is currently the method of choice to analyse the full set of proteins present in the cell. Leucine, isoleucine and hydroxyproline have the same molecular mass, such that in a protein the sequence containing these amino acids can only be easily analysed when the proteins are formed in media in which these residues differ in isotopic composition.[18,19]

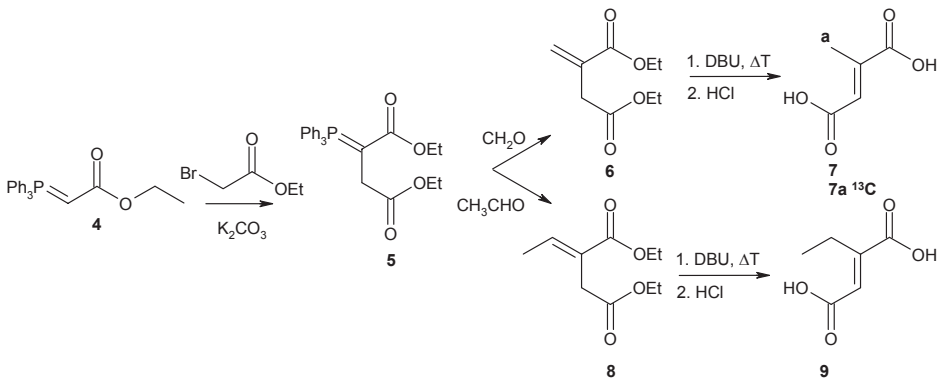
A complication for the synthetic schemes to prepare the whole set of isotopomers of these aliphatic amino acids is that valine and leucine each have two diastereotopic methyl groups. Their numbers are 4 and 5 for valine (**1**) and 5 and 6 for leucine (**3**) in Figure 1. If these groups differ in isotope incorporation, chirality is introduced, leading to a diastereotopic mixture. Any synthetic schemes should always be such that a precisely defined chirality will be present.

Isoleucine (**2**) has the complication that carbon atom 3 has the (*S*) chirality. Here, also, the synthetic scheme should only lead to the required stereochemistry. The starting point for the development of a synthetic scheme to prepare the full set of isotopomers of valine is the fact that in the literature[20] an enzymatic conversion of mesaconic into (2*S*,3*S*)-methylaspartic acid has been described, which could then be converted via subsequent chemical steps into (2*S*)-valine (**1**). Using this scheme Kluender *et al*.[20] were able to prepare just [4-^{13}C]-(2*S*,3*S*)-valine, because their scheme only allowed the preparation of mesaconic acid with ^{13}C enrichment in the methyl group. In this chapter the efficient synthesis of all isotopomers of mesaconic acid is described. For the preparation of (2*S*)-valine (**1**) the subsequent conversion of (2*S*,3*S*)-methylaspartate into valine was optimised. The synthetic scheme for the isotopomers of (2*S*)-isoleucine (**2**) could be based on a rational modification of the scheme for (2*S*)-valine. For access to all isotopomers of (2*S*)-leucine (**3**) a strategy based on a synthetic reaction involving optically active accessory materials was

devised.[21] For the preparation of isotopomers this leads to a well defined scheme via which any isotopomer is accessible without question. For (2*S*)-valine (**1**) and (2*S*)-leucine (**3**) the most critical question is a good differentiation in isotopic enrichment in the two diastereotopic methyl groups. In order to demonstrate the efficiency of the scheme in this regard [4-^{13}C]-(2*S*,3*S*)-valine (**1a**) and [5-^{13}C]-(2*S*,4*R*)-leucine (**3a**) were prepared. In these two systems the efficiency of the schemes in this regard can be easily established using ^{1}H and ^{13}C NMR techniques.

2.2 SYNTHETIC STRATEGY

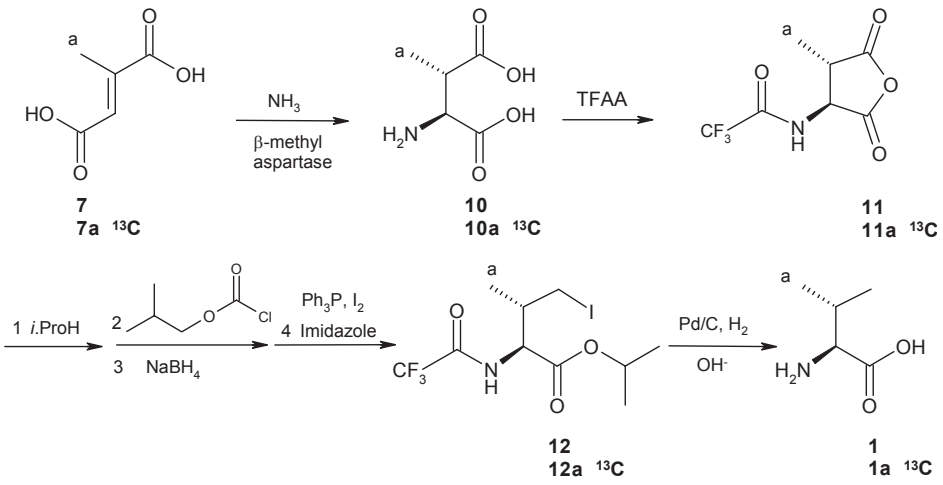
For the synthesis of the isotopomers of valine the scheme starting from mesaconic acid (**7**; Scheme 2) as has been pioneered by Kluender *et al.* was used. In order to obtain the full set of isotopomers of valine, the synthesis of mesaconic acid (**7**) which has the complete carbon skeleton of valine (**1**) is essential. Scheme 1 indicates how mesaconic acid (**7**) can be made, starting from the phosphorane **4**, which can be obtained in a few steps from acetic acid as previously described.[22] This scheme was first optimised by carrying out all the reactions with reagents with natural isotope abundance. Treatment of **4** with ethyl bromoacetate in the presence of solid K_2CO_3 gives the phosphorane **5**. Reaction of **5** with formaldehyde gives the itaconic diester **6**. Upon treatment with DBU the exo double bond isomerises to the main chain to give a mixture of citraconic and mesaconic diesters. Heating with concentrated hydrochloric acid hydrolysed the ester groups and effected isomerization of the double bond to give within experimental error only the thermodynamically more stable trans form, mesaconic acid (**7**). It is clear that the number of synthons and steps used is kept to a minimum. All synthons are commercially available in all isotopomeric



Scheme 1. Synthesis of the carbon skeleton of valine (**1**) and isoleucine (**2**).

forms. The reactions in these schemes give simple access to pure mesaconic acid in any site-directed isotopic form. The yield from **4** to **7** is 52 %.

Scheme 2 shows the conversion of mesaconic acid (**7**) into (2*S*)-valine (**1**) with chiral discrimination between the diastereotopic methyl groups. The first step in the stereo-selective conversion of **7** into (2*S*,3*S*)-3-methylaspartic acid (**10**) was carried out in aqueous solution by β-methyl aspartase, which was isolated from *Clostridium tetanomorphum*.[23] The yield after one pass was 65%; recovery of the starting material and two further reaction cycles gave a total yield of 92%. Introduction of a ^{15}N label is possible by using $^{15}NH_4Cl$ as the nitrogen source. Methyl aspartic acid (**10**) can be converted into valine (**1**) by selectively reducing the γ-carboxyl group. In order to make a distinction possible between the two carboxylic acid moieties the C-1 carboxylic acid was converted to an ester. Addition of trifluoroacetic anhydride in THF to the amino diacid resulted in the protection of the amine group by formation of a trifluoroacetamide function and formation of a cyclic anhydride **11**. Alcoholysis of the anhydride with 2-propanol at –5˚C gave exclusively the C-1 ester.[24] The protection of the C-1 ester leaves the C-4 acid open to reaction with isobutyl chloroformate and subsequent reduction by sodium borohydride. The resulting alcohol was converted into the iodide **12** using the adduct of elemental iodine and triphenylphosphine. Exchange of the iodine for hydrogen by palladium on coal and hydrogen gas gave the protected (2*S*)-valine. Deprotection could be accomplished using aqueous base, yielding the desired (2*S*)-valine (**1**). The yield from **7** to **1** is 40 %. The reactions described in Schemes 1 and 2 lead to any site-directed ^{13}C and ^{15}N isotopomer of (2*S*)-valine up to the uniformly labelled form. To show that these schemes allow the

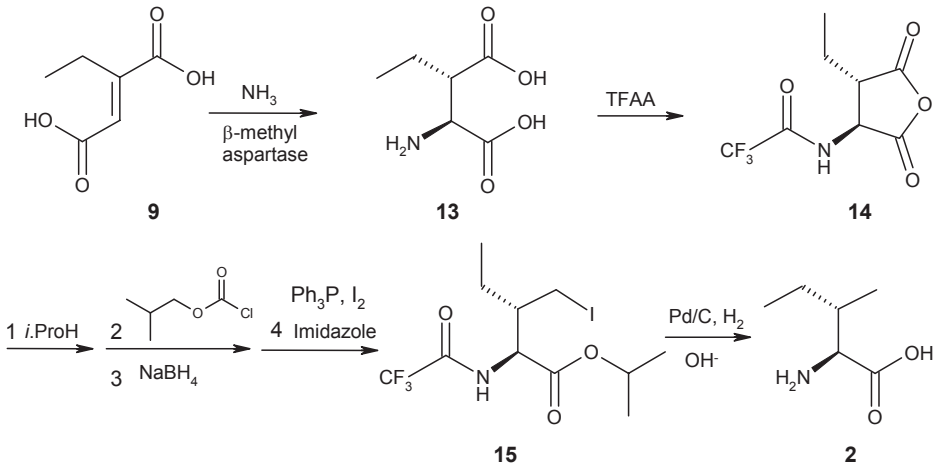


Scheme 2. Synthesis of (2*S*,3*S*)-valine (**1**) starting from achiral mesaconic acid (**7**).

chiral discrimination between the two diastereotopic methyl groups, [4-^{13}C]-valine was prepared. Starting from **7a**, [4-^{13}C]-(2*S*,3*S*)-valine (**1a**) could be prepared via the reactions in Scheme 2 in 35% yield. Spectral analysis showed that the isotope label was specifically and selectively incorporated in the (*S*)-methyl group (*vide infra*).

2.3 ISOLEUCINE

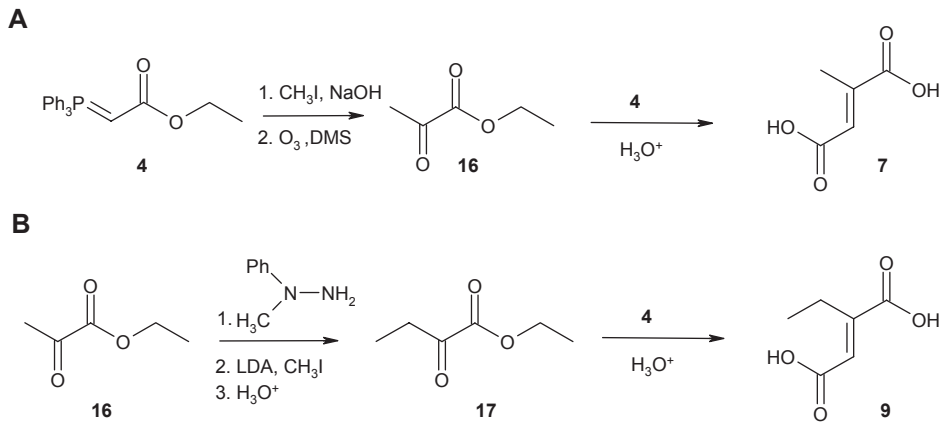
The synthesis of L-isoleucine (**2**) can be carried out in an analogous manner to the synthesis of L-valine (**1**). β-Methyl aspartase, the enzyme that converts the achiral mesaconic acid into the chiral (2*S*,3*S*)-methyl aspartic acid (**7**) is not restricted to one substrate.[25] Fumaric acid derivatives with side chains up to the length of a propyl group fit the binding pocket of the enzyme. Moreover, 2-chloro- and 2-fluorofumaric acid can be converted into the corresponding halo-aspartic acid analogues. This allows the extension of the methods in Schemes 1 and 2 to the synthesis of the isotopomers of (2*S*,3*S*)-isoleucine (**2**). Using 2-ethylfumaric acid as a substrate, (2*S*,3*S*)-ethylaspartic acid (**13**) could be prepared and converted into L-isoleucine (**2**) by the transformations in Scheme 3. The enzymatic substrate **10** (Scheme 2) was obtained by reacting acetaldehyde with **5** (Scheme 1) followed by isomerization of the double bond with DBU. Hydrolysis of the esters with concentrated HCl gave the free diacid while at the same time effecting the isomerization of ethylmaleic diacid to the desired 2-ethyl fumaric acid (**9**). Enzymatic conversion of the substrate gave after three passes a 90% yield of ethylaspartic acid (**13**). The chirality of the two stereocentres in the isoleucine skeleton is determined in the enzymatic step. The



Scheme 3. Synthesis of (2*S*,3*S*)-isoleucine (**2**) starting from achiral ethylfumaric acid (**9**).

formation of the (*S*) configuration at C-2 leads to the (2*S*) amino acid, while the (*S*) conformation at C-3 will result in the correct chirality at the branching point of the isoleucine side chain. Following the chain of reactions shown in Scheme 3, **13** could be converted into (2*S*)-isoleucine (**2**). The reactions in scheme 2 and 3 lead to any ^{13}C, ^{15}N isotopomer of (2*S*)-isoleucine (**2**). Because any atom in isoleucine (**2**) has a well-defined source and no complications arise from prochiral groups, there is no need to further check this scheme using isotope labels.

Before mesaconic acid (**7**) and 2-ethylfumaric (**9**) acid were prepared according to Scheme 1, the reactions in Scheme 4 were explored. Mesaconic acid (**7**; Scheme 4, A) could be prepared in the following fashion. Phosphorane **4** was treated with methyl iodide and then with base. In this way a substituted phosphorane was obtained, which could be converted into ethyl pyruvate (**16**) by treatment with ozone and subsequent reduction of the product with dimethylsulphide. Ethyl pyruvate (**16**) reacted with phosphorane **4** to give an (*E*)/(*Z*) mixture of mesaconic esters, which on aqueous HCl treatment gave within experimental error the pure *E* mesaconic acid (**7**). A similar conversion of **4** with ethyl iodide gives **17** in very low yields, due to the competing reaction of formation of ethene by elimination of HI. Compound **17** has been prepared by first converting ethyl pyruvate (**16**) into the corresponding methyl phenyl hydrazone. Subsequent treatment with LDA and S_N2 reaction with CH_3I followed by mild acidic work-up gave ethyl 2-oxobutyrate (**17**). The hydrazone protection can give access to a range of pyruvate analogues, if methyl iodide is replaced by other suitable electrophiles. Treatment of **17** with **4** gave an (*E*)/(*Z*) mixture of substituted maleic and fumaric esters. Hydrolysis of the esters and isomerization to the most stable form was effected by refluxing in concentrated hydrochloric acid,

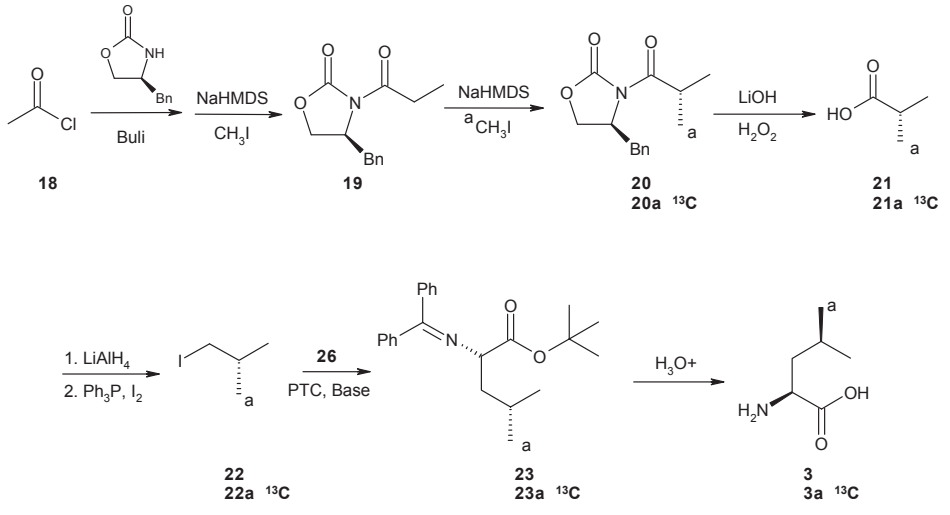


Scheme 4. Alternative synthesis of mesaconic acid (**7**) and 2-ethylfumaric acid (**9**).

giving exclusively 2-ethylfumaric acid (**9**). The synthetic route presented in Scheme 4 enables the synthesis of isotopically enriched **7** and **9** with a carbon label at each position or combination of positions.

2.4 LEUCINE

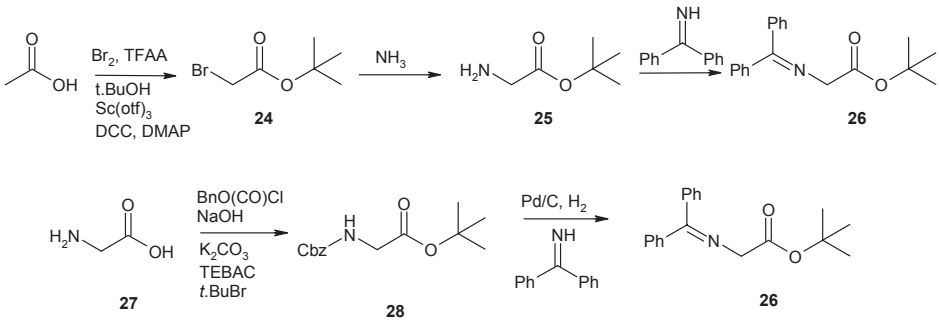
Isotopomers of (2*S*)-leucine (**3**) can not be prepared via a chemo-enzymatic method as described for (2*S*)-valine (**1**) and (2*S*)-isoleucine (**2**). Recently the asymmetric alkylation of *tert*-butyl *N*-(diphenylmethylene)glycinate under cinchona alkaloid derived phase transfer catalytic conditions has been optimised in literature.[26] The reaction conditions are mild with a high yield of α-amino acids and very high enantiomeric excess. Additional advantages of this method are that both the base, the chiral catalyst and *tert*-butyl *N*-(diphenylmethylene)glycinate are commercially available. In order to test the procedure commercial 2-methylpropyl iodide (**22**) was treated with the protected glycine ester (**26**). Reaction under phase transfer conditions with BTTP [BTTP = (*tert*-butylimino)-tripyrrolidinophosphorane] as base in dichloromethane at –50°C gave 76% of the protected leucine (**23**) which after deprotection gave (2*S*)-leucine (**3**) (95% e.e., after crystallisation 99% e.e). It is clear that for the preparation of the full set of isotopomers of leucine both reactants, namely 2-methylpropyl iodide (**22**) and the protected glycine derivative **23**, have to be available as the full set of well-defined isotopomers. The most critical requirement is to have the diastereotopic methyl groups in an exactly defined ^{13}C-enriched form. The latter requirement



Scheme 5. Synthesis of (2*S*)-leucine (**3**).

was effected first. Scheme 5 indicates how via the Evans template a propionic side chain can be converted into a ^{13}C-isobutyric side-chain which via hydrolysis, $LiAlH_4$ reduction and subsequent iodination can be converted into [^{13}C]-(2*S*)-methylpropyl iodide (**22a**) with 76% enantiomeric excess. This incorporation is sufficient for the present purpose. However it can be improved by using the Evans template with an isopropyl group.[27] This latter synthon is not commercially available in contrast to the benzyl system. The propionyl side chain on the Evans template can be prepared in all isotopomeric forms, starting from acetylchloride (commercially available in all isotopically enriched combinations), which after coupling with the template can be alkylated with CH_3I to give the propionic side chain.

The second step in the strategy is to prepare the complete set of ^{15}N and ^{13}C isotopomers of the protected glycine reagent. The introduction of the ^{13}C isotopes is straightforward. 2-bromoacetic acid is commercially available as the set of all possible ^{13}C isotopomers. It is converted into the required protected glycine derivative **26** by the reactions depicted in Scheme 6. Esterification with *tert*-butanol gave *tert*-butyl 2-bromoacetate. The bromine atom can be substituted by a $^{14}NH_2$ group by reaction with liquid ammonia, after which the resulting *tert*-butyl glycine is treated with benzophenoneimine to give **26** in all the possible ^{13}C isotopomers. A similar introduction of ^{15}N is prohibitively expensive, however. Therefore the synthesis of ^{15}N enriched protected glycine derivative **26** is carried out starting from glycine itself (which is commercially available in all combinations of ^{13}C and ^{15}N enrichment). Treatment of glycine with benzyloxycarbonylchloride to protect the amino function made it possible to convert the acid group into a *tert*-butyl ester by reacting it with *tert*-butyl bromide in the presence of K_2CO_3 and triethyl benzyl ammonium chloride as the phase transfer catalyst. Removal of the Cbz-protecting group was effected by reduction with palladium on coal and hydrogen gas, which allowed introduction of the benzophenoneimine group on the liberated amino function, giving **26**. Having



Scheme 6. Synthesis of the protected glycine backbone.

accomplished the preparation of the full set of isotopomers of the protected glycine synthon together with the full set of with different isotopomers of 2-methylpropyl iodide, access has been obtained to the full set of all isotopomers of (2*S*)-leucine (**3**). As far as known, leucine is the first α amino acid for which a synthetic scheme has been based on the O' Donnell strategy to obtain access to the full set of isotopomers.

Although the O' Donnell strategy is optimised for the preparation of optically active α-amino acids in which the side chain derives from a primary alkyl group, the protected glycine ester **22** was treated with 2-iodopropane to test if the system would also work with a side chain derived from secondary alkyl groups, such as occur in valine (**1**) and isoleucine (**2**). The best result that were obtained was a 7% yield of (2*S*)-valine (**1**) only. The yield of valine prepared in this way is too low to be a good substitute for the preparation of (2*S*)-valine and (2*S*)-isoleucine by a chemo-enzymatic method as described in this chapter. An experiment with 4-chlorobenzaldimine as the amino protecting group and the chiral quartenary ammonium catalyst devised by Ooi *et al*.[28], both chosen for the decreased steric hindrance, gave similar yields and literature shows no case of successful alkylation with a secondary halide using the O'Donnell method.

The full isotopomeric set of the glycine part in the bis-lactim ether of D-valine-glycine previously has been prepared.[29] This is the basic system of the Schöllkopf method for the preparation the full isotopomeric set of α-amino acids. These methods now can be compared. It is clear that the O' Donnell method for the preparation of optically active amino acids is superior both because of milder reaction conditions and easy work up of both catalyst and required amino acid, which in the case of the Schöllkopf method is always mixed with the same amount of D-valine which has to be separated with preparative ion-exchange chromatography.

2.5 SPECTROSCOPIC IDENTIFICATION

The samples of (2*S*)-valine (**1**), (2*S*)-isoleucine (**2**) and (2*S*)-leucine (**3**) prepared by the methods described in this chapter have all analytic characteristics of authentic samples. ^{1}H NMR and ^{13}C NMR spectroscopy is the method of choice to establish the location and amount of ^{13}C incorporation in a specifically ^{13}C enriched system. In Figure 2 the 400 MHz ^{1}H NMR spectra of [4*R*-^{13}C]-(2*S*,3*R*) valine (2A) and (2*S*)-valine (2B) are reproduced. The large 125.1 Hz splitting in the 4-H_3-(*R*) peak induced by the ^{13}C incorporation is obvious. From the intensities it is clear that the incorporation has to be high, however the precise incorporation can not be obtained from the spectra owing to the overlap of the peaks of the now enriched (4*R*) group and

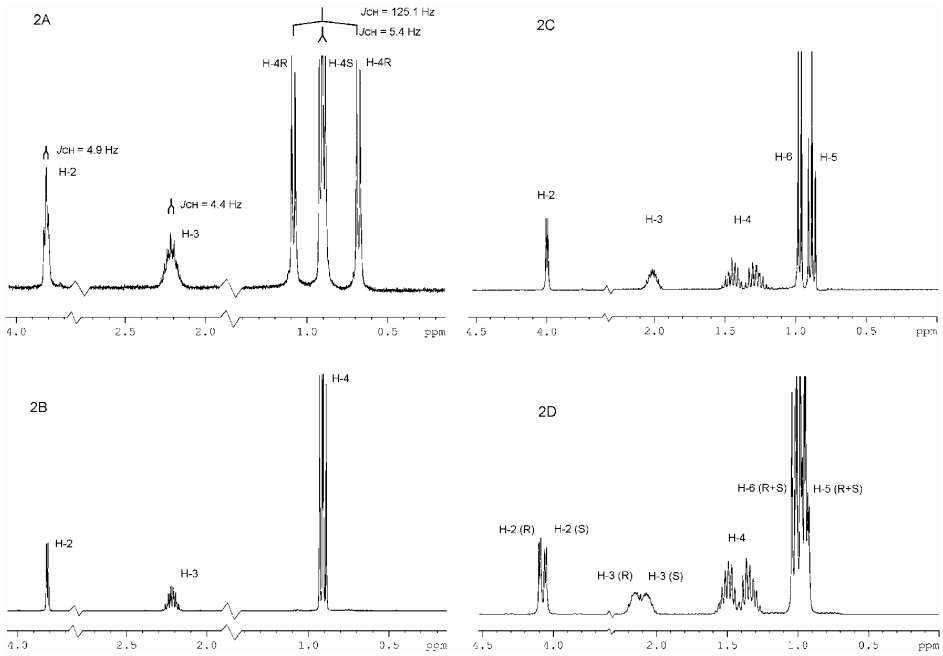


Figure 2. ^{1}H (400 MHz) spectra of [4-^{13}C]-(2*S*,3*S*)-valine (2A), natural abundance (2*S*)- valine (2B), (2*S*,3*S*)-isoleucine (2C) and (2*S*/*R*,3*S*)-isoleucine (2D).

the (4*S*) methyl group. It is also clear that the 2-H, 3-H and 4-H_3-(*S*) signals show additional splitting due to long-range $J_{C,H}$ coupling with the [4-^{13}C] (4.9 Hz, 4.4 Hz, 5.4 Hz respectively). Figure 2 also reproduces the 400 MHz spectra of (2*S*,3*S*) isoleucine made by the method presented in this chapter (2C) and commercial (2*S*/*R*,3*S*) isoleucine (2D). The spectrum of the (2*S*,3*S*) material shows within experimental error the signals of the correct material only, which has the same characteristics as commercial 99% (2*S*,3*S*)-isoleucine. When compared to a sample of commercial (2*S*/*R*,3*S*)-isoleucine the doubling of the peaks in the (2*S*/*R*,3*S*) isoleucine spectrum due to the presence of two diastereotopic molecules can be clearly seen. This leads to the conclusion that (2*S*,3*S*) isoleucine can be prepared selectively using the method described before (*vide supra*).

In Figure 3 the 400 MHz ^{1}H NMR spectra of [5-^{13}C]-(2*S*,4*R*)-leucine (3A) and natural abundance (2*S*)-leucine (3B) are reproduced. The large 125.3 Hz splitting in the 5-H_3-(*R*) peak induced by the ^{13}C incorporation is obvious. From the intensities it is clear that the label incorporation is divided between the C-5*R* (major) and the C-5*S* (minor) methyl group. Decoupling experiments show that the precise ratio of isotope

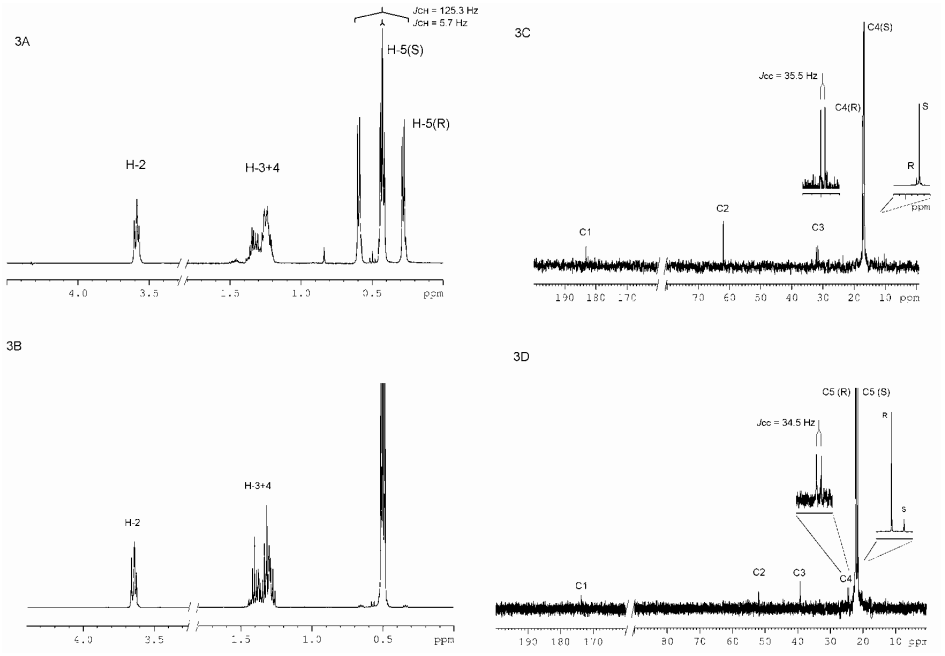


Figure 3. ^{1}H (400 MHz) spectra of [5-^{13}C]-(2*S*,4*R*) leucine (3A), natural abundance (2*S*)- leucine (3B) and the ^{13}C 100 MHz spectra of [4-^{13}C]-(2*S*,3*S*) valine (3C) and [5-^{13}C]-(2*S*,4R) leucine (3D).

incorporation between the C-5*R* and C-5*S* is 8.5:1 It is also clear that the 5-H_3-*S* shows additional splitting due to long-range $^{3}J_{C,H}$ coupling (5.7 Hz) with [5-^{13}C]-(*R*).

The inequivalency of the prochiral methyl groups in valine and leucine also shows itself in the ^{13}C spectra (Figure 3, C and D). Not only can separate peaks be discerned for each methyl group, but the incorporation of a stable carbon-13 isotope makes it clear that the synthetic schemes are selective, as can be witnessed by the selective magnification of the [4-^{13}C]-(3*S*) methyl signal of valine and the [5-^{13}C]-(4*R*) methyl signal of leucine: [^{13}C]-valine shows a large peak at δ=17.2 in the carbon spectrum, while the peak at δ=17.8 shows no significantly increased intensity (Figure 3), in agreement with literature. For [5-^{13}C]-L-leucine, a ratio of 8.5:1 between the [5-^{13}C]-(4*R*) and (4*S*) peaks is expected, due to the 8.5:1 R:S ratio in the chiral alkylation via the Evans' template **18**. Figure 3 (D) shows an increased intensity for both the [5-^{13}C]-(4*R*) and (4*S*) signals, with the former one having a much higher intensity (8.5:1), as expected. In both 3C and 3D a strong $^{1}J_{C,C}$ coupling between the carbon label and the adjacent C-atom can be seen. The splitting is 35.5 Hz in the case of valine and 34.5 Hz in the case of leucine.

2.6 CONCLUSION

Schemes have been successfully developed which allow the selective isotope labelling at each position or any combination of positions for (2*S*)-leucine (**3**), (2*S*)-isoleucine (**2**) and (2*S*)-valine (**1**). To prove that these schemes give the desired selectivity in discriminating between the prochiral methyl groups of leucine and valine, [4-^{13}C]-(2*S*,3*S*)-valine and [5-^{13}C]-(2*S*,4*R*)-leucine have been prepared in good yield and high enantiomeric excess. Spectral analysis of the products and comparison with literature data proved the validity and specificity of the schemes. For the preparation of (2*S*)-leucine in any stable isotopically enriched form a strategy has been developed based on the O' Donnell method for the preparation of optically active α-amino acids. The protected glycine scaffold that is central to the O' Donnell method can be obtained in all required isotopically labelled forms. Access to this isotopically labelled synthon will allow efficient schemes to be developed for the preparation of the remaining 7 α-amino acids that are introduced via translation processes into proteins. The preferred use of the O' Donnell strategy in the cases where amino acids have been earlier prepared via the Schöllkopf method is expected due to the easy recovery of the optically active catalyst, the milder reaction conditions and the fact that the final product is easily obtained without difficult HPLC separation from D-valine with the Schöllkopf method.

2.7 EXPERIMENTAL SECTION

General Remarks: ^{1}H NMR spectra were recorded with Jeol FX-200 and Bruker DPX-300 spectrometers, using tetramethylsilane (TMS: δ = 0 ppm) water (H_2O: δ = 4.8 ppm) or 3-(trimethylsilyl)tetradeuteriopropionic acid (TSP: δ = 0) as an internal standard. ^{13}C noise-decoupled NMR spectra were recorded with a Jeol FX-200 at 50.1 MHz and a Bruker DPX-300 spectrometer at 75.5 MHz, using $CDCl_3$ (δ = 77 ppm), $(CD_3)_2CO$ (δ = 206 ppm) or TSP (δ = 0) as internal standards. Column chromatography was performed on Merck silica gel 60 (0.040-0.063 mm 230-400 mesh) and spots were detected with UV-light, $KMnO_4$-spraying, Ninhydrin-staining (0.2% in ethanol) or by staining with with 4,4'-methylenebis(*N'N*-dimethylaniline) and ninhydrin (TDM-staining). Dry diethyl ether (ether, E) was obtained by distilling from P_2O_5. Dry petroleum ether 40-60 (PE) and dry dichloromethane (DCM) were obtained by distillation from CaH_2. Dry tetrahydrofuran (THF) was obtained by drying with sodium-benzophenone. Methyl iodide (99% ^{13}C) was purchased from Cambridge Isotope Laboratories, Inc. All other reagents were purchased from Aldrich Chemical Co. or Acros Chimica.

Enzyme isolation:

Clostridium tetanomorphum was obtained from the DSMZ micro-organism stock (Germany). For growth of the bacteria and harvesting of the enzyme, the original procedure by Barker[23] was applied. After purification with charcoal no further purification was performed, but the protein mixture was stored at –20°C until use. This seemed to have no detrimental effect on the activity of the enzyme.

[(ethoxycarbonyl)methyl]triphenylphosphonium Bromide

To 2.54 g (2.40 mL, 42.3 mmol) of glacial acetic acid in a dry 100 mL round bottomed flask equipped with a magnetic stirrer and cooled to 0 ºC, was carefully added using a dropping funnel, 2. equiv. of trifluoroacetic anhydride (21.0 g, 14.1 mL, 100 mmol). After stirring for 1 hour, Br_2 (6.83 g, 2.20 mL, 43 mmol) was added very slowly using a dropping funnel and stirring was continued overnight. The resulting pale orange solution was cooled to 0 ºC and 2.4 g (2.4 mL, 133 mmol) distilled water was slowly added using a dropping funnel. The solution was distilled using a mini-distillation setup with an oilbath heated to 120 ºC. After distilling off the bulk trifluoroacetic acid, the last traces were blown away with a soft nitrogen stream, giving 4.57 g of an off-white crystaline solid. A further 0.13 g was obtained after evaporation of the distillate using a steady nitrogen flow to give a total yield of 4.70 g (34 mmol) of bromo acetic acid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.9 (s, 2H, 2-H), 9.7 (Br. s, 1H, 1-H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 25.2 (C-2), 173.1 (C-1) ppm.

To 100 mL round-bottomed flask charged with a solution of 35 mmol bromoacetic acid in 5 mL dry diethyl ether and equipped with a $CaCl_2$-tube, $SOCl_2$ (42 mmol, 1.2 eq) was added at 0 ºC, using a dropping funnel. The mixture was stirred for 1 hours at 0 ºC, then for 2 hours at room temperature. Subsequently ether and excess $SOCl_2$ were distilled off using a mini-destillation setup. Dry ethanol (10 mL, 5 equiv.) was added and the mixture was stirred overnight. The reaction mixture was taken up in 50 mL ether, extracted once with 10 mL saturated bicarbonate solution, once with 10 mL water and then dried with anhydrous $MgSO_4$. After careful concentration the yield was 5.5 g (95 %).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.31 (t, $^3J_{HH}$ = 7.2 Hz, 3H, CH_3), 3.85 (s, 2H, 1-H), 4.21 (q, $^3J_{HH}$= 7.2 Hz, 2H, OCH_2) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ = 13.89 (CH_3), 25.86 (C-2), 62.23 (CH_2), 167.14 (C-1) ppm.

To a stirred solution of 2.75 g (10.5 mmol, 1.05 equiv.) triphenylphosphine in ethyl acetate (50 mL) a solution of ethyl bromoacetate (1.53 g, 10 mmol) in ethylacetate (10 mL) was slowly added using a dropping funnel. The reaction mixture was stirred overnight and the next day the white precipitate was filtered off, washed with 10 mL of ethyl acetate and dried *in vacuo* for 4 hours at 50°C. 4.05 g (9.7 mmol) phosphonium salt was obtained in a yield of 97 %.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.05 (t, $^3J_{HH}$ = 7.1 Hz, 3H, CH_3), 4.03 (q, $^3J_{HH}$ = 7.1 Hz, 2H, OCH_2), 5.51 (d, $^2J_{PH}$ = 13.8 Hz, 2H, 1-H), 7.93-7.61 (m, 15H, Ph) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ= 13.64 (CH_3), 33.1 (d, $^1J_{PC}$ 60.1 Hz, C-1), 62.8 (CH_2), 117.8 (d, $^1J_{PC}$ = 89 Hz, C'-1), 130.1 (d, $^3J_{PC}$ = 13 Hz, C'-3), 133.8 (d, $^2J_{PC}$ = 10 Hz, C'-2), 135.1 (C'-4), 164.37 (C-2) ppm.

[1,2-bis(ethoxycarbonyl)ethylidene]triphenylphosphorane 5

Phosphonium bromide (8.5 g, 20 mmol) was dissolved in 50 mL DCM and extracted with 2 equiv. NaOH dissolved in 50 mL water. The organic layer was collected and dried with $MgSO_4$, filtered and the solvent was evaporated. The resulting phosphorane **4** was redissolved in ethyl acetate, solid K_2CO_3 (5,52 g, 2 equiv.) was added and after addition of ethyl bromoacetate (2.4 mL, 1.1 equiv.) the mixture was refluxed for 4 hours. The reaction mixture was subsequently filtered and the solids rinsed with 25 mL ethyl acetate. The filtrate was concentrated giving **5** (6.57 g, 75%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.11 (t, $^3J_{HH}$ = 7.2 Hz, 6H, CH_3), 2.95 (d (broad), $^3J_{PH}$ = 20 Hz, 2H, H3), 3.95 (q, $^3J_{HH}$ = 7.2 Hz, 4H, OCH_2), 7.30-7.76 (m, 15H, 3*Ph) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 14.07 (CH_3), 57.3 (C-3), 59.72 (OCH_2), 128.23 (Ph), 128.84 (Ph), 131.5 (Ph), 133.59 (Ph), 175.02 (CO) ppm.

Diethyl Itaconate 6

To a stirred solution of 1.64 g (3.78 mmol) **5** in DCM a 37% formaldehyde solution was slowly added (3.65 mL, 1.2 equiv.). The mixture was stirred for 4 hours at R.T. and subsequently extracted with brine. the organic layer was dried with $MgSO_4$, filtered and and evaporated. The resulting raw product was purified by column chromatography (PE/E, 90:10). The yield was 0.59 g (85%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.26 (t, 3H, CH_3), 1.30 (t, 3H, CH_3), 3.35 (s, 2H, 3-H), 4.16 (q, 2H, OCH_2), 4.28 (q, 2H, OCH_2), 5.78 (d, 1H, 5-H) 6.37 (d, 1H, 5-H) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 14.2 (2*CH_3), 37.8 (C-3), 60.8 (OCH_2), 61.0 (OCH_2), 128.0 (C-5), 134.7 (C-2), 166.1 (CO), 170.6 (CO) ppm.

Mesaconic Acid 7

Diethyl itaconate (1.46 g, 7.89 mmol) was refluxed in toluene in the presence of DBU (1 equiv.) under the exclusion of water and the rearrangement of the double bond was followed by TLC (PE/E, 90:10). When the reaction was complete, the solution was extracted with 1M HCl, water, brine and the organic layer dried with $MgSO_4$. After evaporation of the solvent, concentrated HCl (10 mL) was added and the mixture was refluxed for 3 hours and subsequently cooled to 0°C using an ice bath. A white solid precipitated out of solution and was filtered over a glass filter after 15 minutes. The solid was rinsed carefully with 5 mL of cold concentrated hydrochloric acid, after which air was sucked through the filter for three hours. The yield of mesaconic acid was 0.79 g (6.1 mmol, 80 %)

^{1}H NMR (300 MHz, $(CD_3)_2CO$): δ = 2.24 (d, $^{4}J_{HH}$ = 1.7 Hz, 3H, 5-H), 6.78 (q, $^{4}J_{HH}$ = 1.7 Hz, 1H, 3-H) ppm.
^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 13.28 (C-5), 133.57 (C-3), 143.39 (C-2), 165.88 (CO), 167.07 (CO) ppm.

[5-^{13}C]-Mesaconic Acid 7a

Yield 81%
^{1}H NMR (300 MHz, $(CD_3)_2CO$): δ = 2.24 (dd, $^{1}J_{CH}$ = 129.41 Hz, $^{4}J_{HH}$ = 1.7 Hz, 3H, 5-H), 6.78 (dq, $^{4}J_{HH}$ = 1.7 Hz, $^{3}J_{CH}$ = 7.7 Hz, 1H, 3-H) ppm.
^{13}C NMR (75 MHz, $(CD_3)_2CO$): δ = 14.00 (C-5), 126.41 (C-3), 128.08 (d, $^{1}J_{CC}$ = 23.33 Hz, C-2), 166.5 (CO), 172.84 (CO) ppm.

(2S,3S)-3-Methylaspartic Acid 10

7 (5 g, 38.5 mmol) was dissolved in 15 mL of concentrated ammonia. The solution was then concentrated to dryness and the resulting ammonium salt of **7** was dissolved in 75 mL of a solution containing NH_4Cl (5.35 g per 100 mL), $MgCl_2.6H_2O$ (4.06 g per 100 mL) and KCl (0.75 g per 100 mL) and the pH was adjusted to pH 9 using concentrated ammonia. The solution was transferred to a stoppered 250 mL erlenmeyer and subsequently 250 µl enzyme solution was added. The mixture was put in a shaking bath at 32ºC and shaken overnight. After one night an aliquot was taken and ^{1}H-NMR indicated 65% conversion. The enzymes were denatured by acidifying to pH 1 and heating to 80ºC for 10 min. The mixture was filtered and extracted 2 times with 50 mL ether. The organic layer was evaporated and **7** (1.5 g) was recovered. The water layer was concentrated to half volume, 20 mL ethanol was added and the pH adjusted to 3.1. Standing overnight at –20ºC gave clear crystals which were subsequently collected. After drying *in vacuo* the yield was 3.4 g, 60%, Three cycles gave a total yield of 90%.
^{1}H NMR (200 MHz, H_2O): δ = 1.18 (d, $^{3}J_{HH}$ = 7.6 Hz, 3H, 5-H), 2.95, (dq, $^{3}J_{HH}$ = 7.6 Hz, $^{3}J_{HH}$ = 3.2 Hz, 1H, 3-H), 4.16 (dq, $^{3}J_{HH}$ = 3.2 Hz, $^{4}J_{HH}$ = 0.5 Hz, 1H, 2-H) ppm.
^{13}C NMR (75.5 MHz, H_2O): δ = 12.93 (C-5), 39.48 (C-3), 54.62 (C-2), 169.9 (CO), 175.8 (CO) ppm.

[5-^{13}C]-(2S,3S)-3-Methylaspartic Acid 10a

Prepared as **10**, Unconverted [5-^{13}C]-mesaconic acid could be recovered and reused. Total yield 92%
^{1}H NMR (300 MHz, H_2O, T= 300K): δ = 1.30 (ddd, $^{4}J_{HH}$ = 0.5 Hz, $^{3}J_{HH}$ = 7.6 Hz, $^{1}J_{CH}$ = 126.2 Hz, 3H, 5-H), 3.10, (ddq, $^{3}J_{HH}$ = 7.6 Hz, $^{3}J_{HH}$ = 3.2 Hz, $^{2}J_{CH}$ = 5.6 Hz, 1H, 3-H), 4.16 (ddq, $^{3}J_{HH}$ = 3.2 Hz, $^{3}J_{CH}$ = 6.5 Hz, $^{4}J_{HH}$ = 0.5 Hz, 1H, 2-H) ppm.
^{13}C NMR (75.5 MHz, H_2O): δ = 12.20 (C-5) ppm.

1-Isopropyl (2S,3S)-3-Methyl-*N*-(trifluoroacetamido)aspartate

Trifluoroacetic anhydride (5 g, 24 mmol) was added dropwise during ten minutes to 0.35 g of dried **10** in a flamedried 25 mL round-bottomed flask equipped with a pressure equalizing dropping funnel equipped with a calciumchloride drying tube and cooled to 0ºC. The reaction mixture was stirred for two hours while the temperature was allowed to reach room temperature. Subsequently the trifluoroacetic anhydride and the formed trifluoroacetic acid were removed using an oilpump. To the formed cyclic anhydride dried isopropanol (1.85 mL, 10 equiv.) was added and the mixture was stirred for two hours at room temperature. Excess isopropanol was removed using an oilpump. The crude product was taken up in 25 mL ether and washed with 5 mL $NaHCO_3$, 5 mL H_2O, brine, dried with $MgSO_4$ and subsequently filtered and concentrated to dryness. Yield 99%.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.27 (d, $^3J_{HH}$ = 5.9 Hz, 3H, CH_3), 1.29 (d, $^3J_{HH}$ = 5.9 Hz, 3H, CH_3), 1.36 (d, $^3J_{HH}$ = 7.3 Hz, 3H, 5-H), 3.08 (dq, $^3J_{HH}$ = 7.3 Hz, $^3J_{HH}$ = 4.0 Hz, 1H, 3-H), 4.80 (dd, $^3J_{HH}$ = 4.0 Hz, $^3J_{HH}$ = 8.0 Hz, 1H, 2-H), 5.11 (dq, $^3J_{HH}$ = 5.9 Hz, $^3J_{HH}$ = 5.9 Hz, 1H, OCH) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 13.17 (C-5), 21.49 (CH_3), 21.56 (CH_3), 41.68 (C-3), 54.23 (C-2), 71.0 (OCH), 118.75 (CF_3), 159.10 (CO), 168.26 (C-4), 177.68 (C-1) ppm.

1-Isopropyl [4-^{13}C]-(2S,3S)-3-Methyl-*N*-(trifluoroacetamido)aspartate

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.27 (d, $^3J_{HH}$ = 5.9 Hz, 3H, CH_3), 1.29 (d, $^3J_{HH}$ = 5.9 Hz, 3H, CH_3), 1.36 (dd, $^1J_{CH}$ = 129.41 Hz, $^3J_{HH}$ = 7.3 Hz, 3H, 5-H), 3.08 (m, 1H, 3-H), 4.80 (dd, $^3J_{HH}$ = 4.0 Hz, $^3J_{HH}$ = 8.0 Hz, 1H, 2-H), 5.11 (dq, $^3J_{HH}$ = 5.9 Hz, $^3J_{HH}$ = 5.9 Hz, 1H, OCH) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 13.17 (C-5), 21.49 (CH_3), 21.56 (CH_3), 41.68 (d, C-3), 54.23 (C-2), 71.0 (OCH), 118.75 (CF_3), 159.10 (CO), 177.68 (CO) ppm.

1-Isopropyl (2S,3S)-3-Methyl-*N*-(trifluoroacetamido)homoserinate

A solution of N-methyl morpholine (0.31 mL, 2.81 mmol) was added dropwise to a solution of the protected aspartate (0.73 g, 2.56 mmol) in 50 mL dry THF cooled to –50ºC in a three-necked round-bottomed flask, equipped with a pressure equalizing dropping funnel and under a dry argon atmosphere. Subsequently isobutyl chloroformate (0.35 mL) was added slowly using a syringe and the mixture was stirred for 5 minutes. The resulting suspension was quickly filtered into a solution of sodium borohydride (77 mg) in dry THF at –20ºC and was stirred for three hours. The reaction was quenched by addition of 1 mL of an 1:1 acetic acid/water mixture. The solvents were evaporated and the resulting oil was taken up in 50 mL of ether and extracted with water and brine and dried with $MgSO_4$.

The product was purified using column chromatography (PE/E 70/30) with five drops of acetic acid added to the eluent. Yield was 400 mg (58%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.77 (d, $^3J_{HH}$ = 7.1 Hz, 3 H, 5-H), 1.29 (d, $^3J_{HH}$ = 6.2 Hz, 3 H, CH_3), 1.31 (d, $^3J_{HH}$ = 6.2 Hz, 3 H, CH_3), 2.44 (m, 1 H, 3-H), 3.24 (dd, $^3J_{HH}$ = 12.1 Hz, $^3J_{HH}$ = 10.6 Hz, 1 H,

4-H), 3.57 (dd, $^3J_{HH}$ = 12.1 Hz, $^3J_{HH}$ = 4.9 Hz, 1 H, 4-H), 4.84 (dd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 2.8 Hz), 1 H, 1-H), 5.13 (h, $^3J_{HH}$ = 6.2 Hz, 1 H, CH), 7.28 (d, $^3J_{HH}$ = 7.8 Hz, 1H, NH) ppm.
^{13}C NMR (75.5 MHz, $CDCl_3$): δ= 10.44 (C-5), 21.69 (C-7/C-8), 38.74 (C-3), 53.50 (C-2), 63.91 (C-4), 70.57 (C-6), 115.61 (d, $^1J_{CF}$ = 287.3 Hz, C-9), 158.10 (d, $^2J_{CF}$ = 38.0 Hz, C-10), 170.06 (C-1) ppm.

1-Isopropyl [3-^{13}C]-(2S,3S)-3-Methyl-*N*-(trifluoroacetamido)homoserinate
The reaction was performed as for unlabelled homoserine. The yield was 65%.
^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.77 (dd, $^3J_{HH}$ = 7.1 Hz, $^1J_{CH}$ = 126.7 Hz, 3H, 5-H), 1.29 (d, $^3J_{HH}$ = 6.2 Hz, 3H, CH_3), 1.31 (d, $^3J_{HH}$ = 6.2 Hz, 3H, CH_3), 2.44 (m, 1H, 3-H), 3.24 (ddd, $^3J_{HH}$ = 12.1 Hz, $^3J_{HH}$ = 10.6 Hz, $^3J_{CH}$ 1.7 Hz, 1H, 4-H), 3.57 (ddd, $^3J_{HH}$ = 12.1 Hz, $^3J_{HH}$ = 4.9 Hz, $^3J_{CH}$ = 1.6 Hz, 1H, 4-H), 4.84 (ddd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 2.8 Hz, $^3J_{CH}$ = 2.9 Hz 1H, 1-H), 5.13 (H, $^3J_{HH}$ = 6.2 Hz, 1H, CH), 7.28 (d, $^3J_{HH}$ 7.8 Hz, 1H, NH) ppm.
^{13}C NMR (75.5 MHz, $CDCl_3$): δ =10.68 (C-5), 21.51 (C-7/C-8), 38.36 (d, $^1J_{CC}$ 35.15 Hz, C-3), 53.84 (C-2), 63.83 (C-4), 70.43 (C-6), 115.61 (d, $^1J_{CF}$ = 287.3 Hz, C-9), 158.10 (d, $^2J_{CF}$ = 38.0 Hz, C-10), 170.08 (C-1) ppm.

Isopropyl 4-Iodo-3-methyl-2-(trifluoroacetamido)butanoate 12
To a solution of 1.02 g (2 eq) triphenylphosphine in dry DCM was added dropwise a solution of iodine (0.99 g, 1.99 eq) in 10 mL of dry DCM. To the pale-coloured resulting liquid a mixture of 0.3 g imidazole (2.2 equiv.) and protected homeserine (0.5 g, 1.9 mmol) dissolved in 10 mL dry DCM was added dropwise. After two hours TLC indicated complete conversion. After filtering off the solids the product was extracted with 1M HCl solution, water and brine and dried with $MgSO_4$.
The resulting oil was purified using column chromatography (PE/E 85/15) with five drops of glacial acetic acid added to the eluens). The yield was 95%
^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.05 (d, $^3J_{HH}$ = 6.9 Hz, 3H, 5-H), 1.30 (d, $^3J_{HH}$ = 6.3 Hz, 3H, CH_3), 1.31 (d, $^3J_{HH}$ = 6.3 Hz, 3H, CH_3), 2.43 (m, 1H, 3-H), 2.96 (dd, $^3J_{HH}$ = 8.3 Hz, $^3J_{HH}$ = 10.2 Hz, 1H, 4-H), 3.20 (dd, $^3J_{HH}$ = 5.5. Hz, $^3J_{HH}$ = 10.2 Hz, 1H, 4-H), 4.83 (dd, $^3J_{HH}$ = 3.8 Hz, $^3J_{HH}$ = 8.6 Hz, 1H, 2-H), 5.11 (h, $^3J_{HH}$ = 6.3 Hz, 1H, CH), 6.95 (d, $^3J_{HH}$ = 8.6 Hz, 1H, NH) ppm.
^{13}C NMR (75.5 MHz, $CDCl_3$): δ= 8.2 (C-4), 15.32 (C-5), 21.65 (2*CH_3), 39.47 (C-3), 55.78 (C-2), 70.83 (OCH), 115.51 (q, $^1J_{CF}$ = 287.6 Hz, CF_3), 157.3 (q, $^2J_{CF}$ = 37.7 Hz, CO), 169.08 (C-1) ppm.

Isopropyl [^{13}C]-4-Iodo-3-methyl-2-(trifluoroacetamido)butanoate 12a
The same procedure was followed as for **12**. The yield was 92%.
^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.05 (dd, $^1J_{CH}$ = 127.35 Hz, $^3J_{HH}$ = 6.9 Hz, 3H, 5-H), 1.30 (d, $^3J_{HH}$ = 6.3 Hz, 3H, CH_3), 1,31 (d, $^3J_{HH}$ = 6.3 Hz, 3H, CH_3), 2.43 (m, 1H, 3-H), 2.96 (ddd, $^3J_{HH}$ = 8.3 Hz, $^3J_{HH}$ = 10.2 Hz, $^3J_{CH}$ = 3.7 Hz, 1H, 4-H), 3.20 (ddd, $^3J_{HH}$ = 5.5. Hz, $^3J_{HH}$ = 10.2 Hz, $^3J_{CH}$ = 5.3 Hz, 1H,

4-H), 4.83 (ddd, $^{3}J_{HH}$ = 3.8 Hz, $^{3}J_{HH}$ = 8.6 Hz, $^{3}J_{CH}$ = 4.7 Hz, 1H, 2-H), 5.11 (h, $^{3}J_{HH}$ 6.3 Hz, 1H, CH), 6.95 (d, $^{3}J_{HH}$ 8.6 Hz, 1H, NH) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ= 8.2 (C-4), 15.32 (C-5), 21.65 (2*CH_3), 39.47 (d, $^{1}J_{CC}$ = 35.3 Hz, C-3), 55.78 (C-2), 70.83 (OCH), 115.51 (q, $^{1}J_{CF}$ = 287.6 Hz, CF_3), 157.3 (q, $^{2}J_{CF}$ = 37.7 Hz, CO), 169.08 (C-1) ppm.

(2S)-Valine.HCl 1

11 (1.5 g) was dissolved in methanol (25 mL), 1.5 g 5% Pd/C was added and hydrogen gas was bubbled through. After TLC indicated complete disappearance of the starting material, the reaction mixture was flushed with argon and filtered through Celite under an argon blanket. Subsequently, the solution was concentrated to dryness and the protected valine was redissolved in 20 mL isopropanol. 10 mL of a 2 M KOH solution was added and the mixture was stirred for 4 hours. The solvent was evaporated and the residue was redissolved in water. Dowex H+ was added and the mixture was stirred gently for 1 hour. The Dowex was rinsed with 100 mL water and eluted with 0.2 M NH_3. The fractions giving a ninhydrin stain were collected and concentrated. 10 mL 1M HCl was added and the solution lyophilized giving a yield of 0.46 g valine.HCl (77 %).

^{1}H NMR (300 MHz, D_2O): δ = 1.02 (d, $^{3}J_{HH}$ = 7.2 Hz, 4-H), 1.04 (d, $^{3}J_{HH}$ = 7.1 Hz, 5-H), 2.35 (dqq, $^{3}J_{HH}$ = 7.2 Hz, $^{3}J_{HH}$ = 7.1 Hz, $^{3}J_{HH}$ = 4.4 Hz, 1H, 3-H), 3.94 (d, $^{3}J_{HH}$ = 4.4 Hz, 1H, 2-H) ppm.

^{13}C NMR (75.5 MHz, D_2O): δ = 17.47 (C-4), 17.96 (C-5), 32.58 (C-3), 62.61 (C-2), 183.83 (C-1) ppm.

[4-^{13}C]-(2S,3S)-Valine.HCl 1a

The procedure as described for the preparation of **1** was repeated with 0.8 g **11a**, giving **1a** in a yield of 81% (0.26 g, 1.7 mmol)

^{1}H NMR (300 MHz, D_2O): δ = 1.02 (dd, $^{1}J_{CH}$ = 125.1 Hz, $^{3}J_{HH}$ = 7.2 Hz, 3H, 4-H), 1.04 (dd, $^{3}J_{HH}$ = 7.1 Hz, $^{3}J_{CH}$= 5.4 Hz) 3H, 5-H), 2.35 (ddqq, $^{3}J_{HH}$ = 7.2 Hz, $^{3}J_{HH}$ = 7.1 Hz, $^{3}J_{HH}$ = 4.4 Hz, $^{2}J_{CH}$ = 4.4 Hz, 1H, 3-H), 3.94 (dd, $^{3}J_{CH}$ = 4.9 Hz, $^{3}J_{HH}$ = 4.4 Hz, 1H, 2-H) ppm.

^{13}C NMR (75.5 MHz, D_2O): δ = 17.47 (C-4), 17.96 (C-5), 32.58 (d, $^{1}J_{CC}$ = 35.5 Hz, C-3), 62.61 (C-2), 183.83 (C-1) ppm.

Diethyl Ethylidenesuccinate 8

To a stirred solution of **5** (5 g, 11.7 mmol) in DCM was slowly added 0.73 mL acetaldehyde in 5 mL DCM. The mixture was stirred for 4 hours at room temperature and subsequently extracted with brine. The organic layer was dried with $MgSO_4$ and evaporated. The resulting raw product was purified by column chromatography (PE/E). Yield: 90% (2.1 g).

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.25 (t, $^{3}J_{HH}$ = 7 Hz, 3H, CH_3), 1.28 (t, $^{3}J_{HH}$ = 7 Hz, 3H, CH_3), 1.84 (d, 3H, 6-H), 3.35 (s, 2H, 3-H) 4.15 (q, $^{3}J_{HH}$ = 7 Hz, 2H, OCH_2), 4.20 (q, $^{3}J_{HH}$ = 7 Hz, 2H, OCH_2), 6.13 (q, 1H, 5-H) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 14.1 (C-6), 14.5 (2*CH_3), 32.1 (C-3), 60.7 (2*CH2), 126.9 (C-5), 140.1 (C-2), 166.8 (CO), 170.7 (CO) ppm.

2-Ethylfumaric acid 9

1.5 g (7.5 mmol) **8** was refluxed in toluene in the presence of DBU (1 equiv.) and the rearrangement of the double bond was followed by TLC (PE/E 90/10). When only traces of **8** remained, the solution was extracted with 1M HCl, water, brine and the organic layer was dried with $MgSO_4$. After evaporation of the solvent, concentrated 10 mL HCl was added and the mixture was refluxed for 3 hours and subsequently cooled to 0ºC using an ice bath. A white solid precipitated out of solution and was filtered over a glass filter after 15 minutes. The solid was rinsed carefully with 5 mL of cold concentrated hydrochloric acid, after which air was sucked through the filter for three hours. The yield of 2-ethylfumaric acid was 0.87 g (6.1 mmol, 81 %)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.08 (t, $^3J_{HH}$ = 7.4 Hz, 3H, 6-H), 2.78 (q, $^3J_{HH}$ = 7.4 Hz, 2H, 5-H), 6.75 (s, 1H, 3-H) ppm.

3-Ethylaspartic Acid 13

5 g **9** (35 mmol) was dissolved in 15 mL of concentrated ammonia. The solution was then concentrated to dryness and the resulting ammonium salt of was dissolved in 75 mL of a solution containing NH_4Cl (5.35 g per 100 mL), $MgCl_2.6H_2O$ (4.06 g per 100 mL) and KCl (0.75 g per 100 mL) and the pH was adjusted to pH 9 using concentrated ammonia. The solution was transferred to a stoppered 250 mL erlenmeyer and subsequently 250 µl enzyme solution was added. The mixture was put overnight in a shaking bath at 32ºC and shaken vigorously. After one night an aliquot was taken and ^{1}H-NMR indicated 60% conversion. The enzymes were denatured by acidifying to pH 1 and heating to 80ºC for 10 min. The mixture was filtered and extracted 2 times with 50 mL ether. The organic layer was concentrated and 1.3 g **9** was recovered. The water layer was concentrated to half volume, 20 mL ethanol was added and the pH adjusted to 3.1. Standing overnight at –20ºC gave clear crystals which were subsequently collected. After drying *in vacuo* the yield was 3.38 g, 60%.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.99 (t, $^3J_{HH}$ = 7.4 Hz, 3H, 5-H), 1.7 (m, 2H, 4-H), 2.90 (p, $^3J_{HH}$ = 4.2 Hz, 1H, 3-H), 4.06 (d, $^3J_{HH}$ 4.2 Hz, 1H, 2-H) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 12.23 (C-5), 21.46 (C-4), 48.07 (C-3), 55.86 (C-2), 172.96 (C-1), 178.09 (C-6) ppm.

1-Isopropyl (2S,3S)-3-Ethyl-*N*-(trifluoroacetamido)aspartate

To 0.5 g (3.1 mmol) of dried **13** in a flamedried 25 mL round-bottomed flask equipped with a pressure equalizing dropping funnel equipped with a calciumchloride drying tube and cooled to 0ºC was added dropwise 6 g trifluoroacetic anhydride during ten minutes. The reaction mixture was stirred for two hours while the temperature was allowed to reach 20°C. Subsequently the trifluoroacetic anhydride and the formed trifluoroacetic acid were removed using an oilpump

equipped with a cold trap. To the cyclic anhydride formed 10 equiv. dried isopropanol was added and the mixture was stirred for two hours at room temperature. Excess isopropanol was removed using an oilpump. The crude product was taken up in 25 mL ether and washed with 5 mL $NaHCO_3$, 5 mL H_2O, brine, dried with $MgSO_4$ and subsequently filtered and concentrated to dryness. Yield 99% (0.92 g).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.95 (t, $^3J_{HH}$ = 7.51 Hz, 3H, 5-H), 1.20 (d, $^3J_{HH}$ = 6.3 Hz, 3H, CH_3), 1.22 (d, $^3J_{HH}$ = 6.3 Hz, 3H, CH_3), 1.71 (p, 7.5 Hz, 2H, 4-H), 2.93 (ddd, $^3J_{HH}$ = 6.3 Hz, $^3J_{HH}$ = 7.1 Hz, $^3J_{HH}$ = 8.7 Hz, 1H, 3-H), 4.79 (dd, $^3J_{HH}$ = 8.7 Hz, 1H, 2-H), 4.95 (H, $^3J_{HH}$ = 6.3 Hz, 1H, OCH), 8.6 (d, 1H, NH) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 10.34 (C-5), 20.68 (2* CH_3), 21.38 (C-4), 40.98 (C-3), 53.16 (C-2), 69.19 (OCH), 115.86 (q, $^1J_{CF}$ = 287 Hz, CF_3), 157.4 (q, $^2J_{CF}$ = 42 Hz), 168.29 (CO), 173.07 (CO) ppm.

Isopropyl (2*S*,3*S*)-3-Ethyl-*N*-(trifluoroacetamido)homoserinate

To a three-necked round-bottomed flask charged with a solution of 0.70 g (2.4 mmol) in 50 mL dry THF, equipped with a pressure equalizing dropping funnel, under a dry argon atmosphere and cooled to –50ºC, was added dropwise a solution of N-methyl morpholine (0.29 mL in 5 mL THF). Subsequently isobutyl chloroformate (0.32 mL) was added slowly using a syringe and the mixture was stirred for 5 minutes. The resulting suspension was quickly filtered into a solution of $NaBH_4$ (75 mg) in dry THF at –20ºC and was stirred for three hours. The reaction was quenched by addition of 1 mL of an 1:1 acetic acid/water mixture. The solvents were evaporated and the resulting oil was taken up in 50 mL of ether and extracted with water and brine and dried with $MgSO_4$. The product was purified using column chromatography (PE/E 70/30) with five drops of acetic acid added to the eluent. Yield was 450 mg (65%)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.96 (t, $^3J_{HH}$ = 7.4 Hz, 3H, 5-H), 1.24 (m, 2H, 4-H), 1.28 (d, $^3J_{HH}$ = 6.1 Hz, 3H, CH_3), 1.30 (d, $^3J_{HH}$ = 6.1 Hz, 3H, CH_3), 2.18 (m, 1H, 3-H), 3.55 (dd, $^2J_{HH}$ = 11.7 Hz, $^3J_{HH}$ = 10.3 Hz, 1H, 6-H), 3.76 (dd, $^2J_{HH}$ = 11.7 Hz, $^3J_{HH}$ = 4.4 Hz, 1H, 6-H), 4.82 (dd, $^3J_{HH}$ = 3.0 Hz, $^3J_{HH}$ = 7.9 Hz, 1H, 2-H), 4.99 (p, $^3J_{HH}$ = 6.2 Hz, 1H, CH), 7.69 (d, $^3J_{HH}$ = 7.9 Hz, 1H, NH) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 11.83 (C-5), 19.68 (C4), 21.51 (CH_3), 21.57 (CH_3), 45.06 (C-3), 53.74 (C-2), 62.29 (C-6), 70.33 (OCH), 115.64 (q, $^1J_{CF}$ = 287.3 Hz, CF_3), 157.82 (q, $^2J_{CF}$ = 37.8 Hz, CO), 170.12 (C-1) ppm.

Isopropyl (2*S*,3*S*)-4-Iodo-3-ethyl-2-(trifluoroacetamido)butanoate 15

To a solution of triphenylphosphine (0.82 g, 2 eq) in dry DCM was added dropwise a solution of iodine (0.80 g, 1.99 eq) in 10 mL of dry DCM. To the pale-coloured resulting liquid a mixture of 0.3 g imidazole and 0.45 g of the previously obtained alcohol dissolved in 10 mL dry DCM was added dropwise. After two hours TLC indicated complete conversion. After filtering off the solids the product was extracted with 1M HCl solution, water and brine and dried with $MgSO_4$.

The resulting oil was purified using column chromatography (PE/E 85/15) with five drops of glacial acetic acid added to the eluens). The yield was 95%

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.98 (t, $^{3}J_{HH}$ = 7.3 Hz, 3H, 5-H), 1.29 (d, $^{3}J_{HH}$ = 6.3 Hz, 3H, CH_3), 1.31 (d, $^{3}J_{HH}$ = 6.3 Hz, 3H, CH_3), 1.49 (m, 2H, 4-H), 2.08 (m, 1H, 3-H), 3.19 (m, 1H, 6-H), 4.82 (dd, $^{3}J_{HH}$ 4.16 Hz, $^{3}J_{HH}$ 8.3 Hz, 1H, 2-H), 5.11 (h, $^{3}J_{HH}$ 6.3 Hz, 1H, CH), 7.00 (d, $^{3}J_{HH}$ 8.6 Hz, 1H, NH) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ= 5.89 (C-6), 11.34 (C-5), 21.59 (2*CH_3), 23.66 (C-4), 45.06.47 (C-3), 55.31 (C-2), 70.66 (OCH), 115.66 (q, $^{1}J_{CF}$ = 287.6 Hz, CF_3), 157.15 (q, $^{2}J_{CF}$ = 38.2 Hz, CO), 169.36 (C-1) ppm.

(2S,3S)-Isoleucine·HCl 2

150 mg **15** was dissolved in dry methanol in a threenecked roundbottom flask and 2 eq triethyl amine and 0,75 g 5% Pd/C were added. Hydrogen was bubbled through the solution for three hours, after which the solution was purged with argon. The solution was filtered over celite and purified over a short column (50/50 PE/E). Subsequently 81 mg (82%) of the protected isoleucine was dissolved in 10 mL isopropanol. 5 mL of 2 M KOH solution was added and the mixture was stirred for 6 hours. The solvent was evaporated and the residue was redissolved in water. Dowex H+ was added and the mixture stirred gently for 0.5 hour. The Dowex was rinsed with 100 mL water and eluted with 0.2 M NH_3. The fractions giving a ninhydrin stain were collected and concentrated. 2 mL 1M HCl was added and the solution lyophilized giving a yield of 37 mg (73%) isoleucine.HCl.

^{1}H NMR (300 MHz, D_2O) : δ = 0.85 (dd, $^{3}J_{HH}$ = 7.35, $^{3}J_{HH}$ = 7.44 Hz, 3H, 5-H), 0.94 (d, $^{3}J_{HH}$ = 7.0 Hz, 3H, 6-H), 1.24 (m, 1H, 4-H), 1.42, (m, 1H, 4-H), 1.98 (m, 1H, 3-H), 3.97 (d, $^{3}J_{HH}$ = 3.9 Hz, 1H, 2-H) ppm.

^{13}C NMR (75.5 MHz, D_2O): δ= 11.70 (C-5), 14.92 (C-6), 25.90 (C-4), 36.53 (C-3), 57.97 (C-2), 182.68 (C-1) ppm.

Ethyl Pyruvate 16

To a 75 mL dichloromethane solution of 4.05 (9.7 mmol) **4** in a separation funnel 50 mL of a 2M NaOH solution was added. After vigorous shaking the two layers were separated and the dichloromethane layer was collected. The waterlayer was extracted twice with 25 mL dichloromethane and the combined organic layers were dried with $MgSO_4$ and concentrated in vacuo. The resulting ylide was redissolved in 90 mL of dichloromethane and transferred to a 100 mL flask equipped with stirring bar. 0.77 mL (12.6 mmol, 1.3 equiv.) methyl iodide was added using a syringe. After stirring overnight the mixture of non-, mono- and dimethylated product was concentrated in vacuo, giving a yield of 4.48 g Yield of the monomethylated product was 85% (8,0 mmol) according to ^{1}H-NMR.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.05 (t, $^{3}J_{HH}$ = 7.1Hz, 3H, CH_3), 1.69 (dd, $^{3}J_{HH}$ = 7.21Hz, $^{3}J_{PH}$ = 18.5 Hz, 3H, 3-H), 4.03 (q, $^{3}J_{HH}$ = 7.1Hz, 2H, CH_2O), 6.21 (dq, $^{2}J_{PH}$ = 14.2 Hz, $^{3}J_{HH}$ = 7.21Hz, 2H, 1-H), 7.68-7.98 (m, 15H, Ph) ppm.

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 12.0 (CH_3), 13.2 (s, CH_3), 33.1 (d, $^{1}J_{PC}$ = 60.1 Hz, C-1), 61.7 (OCH_2), 117.8 (d, $^{1}J_{PC}$ = 89 Hz, C'-1), 130.1 (d, $^{3}J_{PC}$ = 13 Hz C'-3), 133.8 (d, $^{2}J_{PC}$ = 10 Hz, C'-2), 135.1 (C'-4) 167.4 (C-2) ppm.

A 250 mL three-necked round-bottomed flask, equipped with stirring bar, non-pressure equalizing dropping funnel and a gas outlet connected to a bubble counter filled with a solution of acetic acid and potassium iodide in water was flame-dried under nitrogen. The flask was charged with a 150 mL solution of 5 g ylide (12.8 mmol) and cooled to -60°C, using an acetone/dry ice bath, while the dropping funnel was charged with a 25 mL solution of dimethylsulfide (2 equiv.) in dichloromethane. Ozone was bubbled through the solution in a steady stream until the solution turned blue. The excess ozone was purged from the solution using a nitrogen stream after which the dimethylsulfide solution was added dropwise. The solution was allowed to rise to 0°C, after which 50 mL of PE was added, resulting in a turbid solution. The solution was flashed over a glass filter filled with silica, which was rinsed with 50/50 PE/E. The solution was then concentrated in vacuo to 10 mL and purified over a short column (90/10 PE/E), giving ethyl pyruvate in 65% yield (0.95 g).

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.50 (t, $^{3}J_{HH}$ = 7.21Hz, 3H, CH_3), 2.44 (s, 3H, 3-H), 4.30 (q, $^{3}J_{HH}$ = 7.21Hz, 2H, OCH_2) ppm.

Ethyl 2-Oxobutyrate 17

ethyl pyruvate **16** (20 mmol, 2.22 mL) was dissolved in 50 mL ether and a solution of 2.31 mL (0.98 equiv.) N,N methyl,phenyl hydrazine in 10 mL was slowly added. After one night TLC showed almost complete conversion. The yellow solution was concentrated in vacuo and the product purified by column chromatography (PE/E 70/30), giving a yield of 95% hydrazone (4.25 g).

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.42 (t, $^{3}J_{HH}$ = 7.2 Hz, 3H, CH_3), 2.05 (s, H3, 3-H), 3.42 (s, 3H, NCH_3), 4.34 (q, $^{3}J_{HH}$ = 7.2 Hz, 2H, OCH_2), 6.96-7.34 (m, 5H, Ph) ppm.

2.2 g (10 mmol) of the hydrazone was dissolved in dry THF and added dropwise to a solution of 1.1 equiv. LDA in THF at –80°C, (prepared by addition of 7 mL 1.6M BuLi to a solution of 1.54 mL Diisopropylamine in dry THF). The reaction was quenched by addition of 1.5 equiv. MeI (0.93 mL) and stirred for 4 hours. The reaction mixture was extracted with water, brine and the collected organic layers dried with $MgSO_4$. Purification was performed by column chromatography (PE/E 70/30) to give a yield of 75% of the ethyl hydrazone. Addition of 1.2 equiv. 1M HCl to a solution of the hydrazone in 30 mL THF and stirring during 2 hours followed by addition of 3 mL 1M HCl and stirring for 10 minutes gave after extraction with ether and careful evaporation in vacuo of the solvents 80% of ethyl oxobutyrate.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.13 (t, $^3J_{HH}$ = 7.2 Hz, 3H, 4-H), 1.37 (t, $^3J_{HH}$ = 7.2 Hz, 3H, CH_3), 2.87 (q, $^3J_{HH}$ = 7.2 Hz, 2H, 3-H), 4.32 (q, $^3J_{HH}$ = 7.2 Hz, 2H, OCH_2) ppm.
^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 6.49 (C-4), 13.90 (CH_3), 32.69 (C-3), 62.24 (OCH_2), 161.09 (C-1), 195.04 (C-2) ppm.

***tert*-Butyl bromoacetate 24**

To 2.54 g (2.40 mL, 42.3 mmol) of glacial acetic acid in a dry 100 mL round bottomed flask equipped with a magnetic stirrer and cooled to 0 ºC, was carefully added using a dropping funnel, 2. equiv. of trifluoroacetic anhydride (21.0 g, 14.1 mL, 100 mmol). After stirring for 1 hour, 6.83 g (2.20 mL, 43 mmol) Br_2 was added very slowly using a dropping funnel and stirring was continued overnight. The resulting pale orange solution was cooled to 0 ºC and 2.4 g (2.4 mL, 133 mmol) distilled water was slowly added using a dropping funnel. The solution was distilled using a mini-distillation setup with an oilbath heated to 120 ºC. After distilling off the bulk trifluoroacetic acid, the last traces were blown away with a soft nitrogen stream, giving 4.57 g of an off-white crystaline solid. A further 0.13 g was obtained after evaporation of the distillate using a steady nitrogen flow to give a total yield of 4.70 g (34 mmol) of bromo acetic acid.
^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.9 (s, 2H, 2-H), 9.7 (s (broad), 1H, 1-H) ppm.
^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 25.2 (C-2), 173.1 (C-1) ppm.
A suspension of bromoacetic acid (1.18 g, 8.5 mmol), scandium triflate (2.55 g, 0.6 equiv.), *tert*-butanol (20 mL) and DMAP (5.2 g) in dry DCM was cooled to –5°C in an ice-salt bath for 40 minutes. 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (3.23 g) was added and the mixture stirred at –5°C for 30 minutes and then allowed to come to room temperature in 2 hours. The mixture was filtered, washed with 0.1 M HCl, 0.1 M Na_2CO_3 and water. The organic layer was dried with $MgSO_4$ and carefully concentrated to dryness, giving a yield of 1.33gr **24**.
^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.48 (s, 9H), 3.75 (s, 2H, 2-H) ppm.

***tert*-Butyl glycinate 25**

In a flask cooled to –40°C, ammonia (100mL) was condensed, and then diluted with anhydrous ether (100mL). To this solution, *tert*-butyl bromoacetate (50 g, 0,25 mol) in ether (50mL) was slowly added at –40°C and the temperature was maintained for 2 hours, then allowed to warm to room temperature. After stirring overnight, the ammonium bromide was filtered off and the solvent evaporated under reduced pressure, giving **25** in 87% yield.
^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.47 (s, 9H) 3.31 (s, 2H, 2-H) ppm.

***tert*-Butyl *N*-(diphenylmethylene) glycinate 26**

The glycine ester **25** was added to an equimolar amount of benzophenone imine in CH_2Cl_2 (20mL per g benzophenone imine) and was stirred at room temperature for 24 h under the exclusion of moisture ($CaCl_2$ tube). The reaction mixture was filtered to remove NH_4Cl and con-

centrated to dryness on a rotary evaporator. The residue was taken up in ether (equal amount as CH_2Cl_2), filtered, washed with water (equal amount as ether), and dried with $MgSO_4$. Filtration and solvent removal were followed by recrystallization (ethanol/PE) resulting in a yield of 98%.

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.44 (s, 9H); 4.12 (s, 2H, 2-H); 7.4 (m, 10H, Ph) ppm.

(4*S*)-4-Benzyl-3-(2-methyl-propionyl)-2-oxazolidinone 20

A solution of (4S)-4-benzyl-3-propanoyloxazolidin-2-one (700 mg, 3,0 mmol) in THF (6 mL) was added dropwise to a stirred solution of sodium hexamethyldisilazide (1.0M in THF, 3.3 mL, 3.3 mmol) in THF (15 mL) at –78°C. The reaction mixture was stirred at –78°C for 1 hour then iodomethane (0.37 mL, 6.0 mmol) was added dropwise. The reaction mixture was stirred at –78°C for 4 h then sat. ammonium chloride solution (15 mL) and water (6 mL) were added and the aqueous phase acidified to pH 2 with sulfuric acid. The product was extracted with ethylacetate (3 × 60 mL). The combined extracts were washed successively with sat. sodium hydrogen carbonate solution (15 mL), sodium thiosulfate solution (15 mL) and brine (15 mL), dried with $MgSO_4$, filtered and concentrated *in vacuo* to give an oil. The product was purified by column chromatography eluting with 2-10% ethyl acetate in PE to afford (4S)-Benzyl-3-(2-methyl-propionyl) oxazolidin-2-one as a pale yellow oil (536 mg, 72%)

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.19 (d, 3H, $^3J_{HH}$ = 6.8 Hz, 9-H (R)); 1.23 (d, 3H, $^3J_{HH}$ = 6.7 Hz, 9-H (S)); 2.77 (dd, 1H, $^2J_{HH}$ = 13.4 Hz, $^3J_{HH}$ = 9.5 Hz, 6-H); 3.24 (dd, 1H, $^2J_{HH}$ = 13.4 Hz, $^3J_{HH}$ = 3.3 Hz, 6-H); 3.75 (qq, 1H, $^3J_{HH}$ = 6.7 Hz, $^3J_{HH}$ = 6.8 Hz, 8-H); 4.15 (dd, 1H, $^2J_{HH}$ = 9.0 Hz, $^3J_{HH}$ = 3.5 Hz, 5-H); 4.19 (dd, 1H, $^2J_{HH}$ = 9.0 Hz, $^3J_{HH}$ = 7.4 Hz, 5-H); 4.67 (m, 1H, 4-H); 7.27 (m, 5H, Ph) ppm.

^{13}C-NMR (100 MHz, $CDCl_3$): δ 18.6 (C-9 (R)), 19.1 (C-9 (S)), 32.5 (C-8), 37.8 (C-6), 55.2 (C-4), 65.9 (C-5), 127-153 (Ar), 177.5 (C-7) ppm.

[2-^{13}C]-(4*S*)-4-Benzyl-3-(2-methyl-propionyl)-2-oxazolidinone 20a

The procedure was successfully reproduced starting with $^{13}CH_3I$ (4.5 g, 31.5 mmol), (4S)-4-benzyl-3-propanoyloxazolidin-2-one (5.3 g, 22.5 mmol), sodium hexamethyldisilazide (1.0M in THF, 25 mL, 25 mmol) and THF (80 mL) giving a 1:8.4 mixture of (4*S*)-4-Benzyl-3-(2*R*)-2-methyl-propionyloxazolidin-2-one and (4*S*)-4-Benzyl-3-(2*S*)-2-methyl-propionyloxazolidin-2-one as products (4.0 g, 72%)

^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.19 (dd, 0.2H, $^3J_{HH}$ = 6.8 Hz, $^1J_{CH}$ = 128.3 Hz, 9-H (R)); 1.19 (dd, 2.8H, $^3J_{HH}$ = 6.8 Hz, $^3J_{CH}$ = 5.1Hz, 9-H (R)), 1.23 (dd, 2.8H, $^3J_{HH}$ = 6.7 Hz, $^1J_{CH}$ = 128.3 Hz, 9-H (S)); 1.23 (dd, 0.2H, $^3J_{HH}$ = 6.7 Hz, $^3J_{CH}$ = 10.3 Hz, 9-H (S)); 3.75 (m, 1H, 8-H) ppm.

^{13}C-NMR (100 MHz, $CDCl_3$): δ 32.4 (d, $^1J_{CC}$= 33.8 Hz, C-8) ppm.

2-Methylpropionic acid 21

Hydrogen peroxide solution (30%, 2.7 mL, 27.9 mmol) and lithium hydroxide monohydrate (268 mg, 11.2 mmol in water 5 mL) were added successively to a solution of **20** (1.38 g, 5.58

mmol) in THF (50mL) and water (25 mL) at 0°C. After 2 h sodium sulfite (6.5 g in 25mL water) was added and the solution stirred at 0°C for a further 15 min. The solution was adjusted to pH 9-10 with sat. sodium hydrogen carbonate solution, the THF evaporated and the residual aqueous solution extracted with dichloromethane (2 x 50 mL). The organic extracts were dried with $MgSO_4$ and concentrated to yield (4*S*)-4-benzyloxazolidin-2-one (490 g, 97%)
The aqueous solution was acidified to pH 1-2 with 1 M sulfuric acid and extracted with ether (3 x 125 mL). The combined extracts were dried with $MgSO_4$ and concentrated to yield 2-methyl-propanoic acid (490 mg, quantitative) as a colorless liquid.
^{1}H-NMR (400 MHz, $CDCl_3$):δ 1.19 (d, 6H, $^3J_{HH}$ = 7.0 Hz, 3-H); 2.58 (qq, 1H, $^3J_{HH}$ = 7.0 Hz, H-2) ppm ^{13}C-NMR (100 MHz, $CDCl_3$): δ 18.6 (C-3), 33.7 (C-2), 183.1 (C-1) ppm.

[^{13}C]-(2*S*)-Methylpropionic acid 21a
The procedure was successfully reproduced starting with (4*S*)-4-Benzyl-3-(2*S*)-2-methyl-propionyloxazolidin-2-one (4.0 g, 16.1 mmol) giving (2*S*)-^{13}C-methylpropionic acid as product (1.4 g, near quantitative)
^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.19 (dd, 3H, $^3J_{HH}$ = 7.0 Hz, $^1J_{CH}$ 127.9 = Hz, 3-H); 1.19 (dd, 3H, $^3J_{HH}$ = 7.0 Hz, $^3J_{CH}$ = 5.2 Hz, 3-H); 2.58 (dqq, 1H, $^3J_{HH}$ = 7.0 Hz, $^2J_{CH}$ = 2.4 Hz, 2-H) ppm.
^{13}C-NMR (100 MHz, $CDCl_3$): δ 33.7 (d, $^1J_{CC}$= 34.5 Hz, C-2) ppm.

2-Methyl-1-propanol
2-methylpropionic acid (1.27g, 14.4 mmol) dissolved in dry ether was added to a slurry of $LiAlH_4$ (2.7g, 72 mmol) in ether at 0°C. After 6 hrs. the reaction was stopped with water, and the mixture was extracted with ether, washed with $NaHCO_3$ and brine, dried with $MgSO_4$ and concentrated *in vacuo* to yield 2-methyl-1-propanol as a colorless liquid (853 mg, 80%) .
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.91 (d, $^3J_{HH}$ = 6.7 Hz, 6H, 3-H); 1.75 (tqq, $^3J_{HH}$ = 6.7 Hz, $^3J_{HH}$ = 6.5 Hz, 1H, 2-H); 3.37 (d, $^3J_{HH}$ = 6.5 Hz, 2H, 1-H) ppm.
^{13}C-NMR (100 MHz, $CDCl_3$): δ = 18.6 (C-3), 30.1 (C-2), 68.7 (C-1) ppm.

[2-^{13}C]-(2*S*)2-Methyl-1-propanol
The procedure was successfully reproduced starting with (2*S*)-^{13}C-methylpropionic acid (1.4g, 16.1 mmol) giving (2*S*)-^{13}C-methyl-1-propanol (1.1g, 87%) as product.
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.91 (dd, 3H, $^3J_{HH}$ = 6.7 Hz, $^1J_{CH}$ = 104.3 Hz, 3-H [^{13}C]); 0.91 (dd, 3H, $^3J_{HH}$ = 6.7 Hz, $^3J_{CH}$ = 5.3 Hz, 3-H); 1.75 (dtqq, 1H, $^3J_{HH}$ = 6.7 Hz, $^3J_{HH}$ = 6.5 Hz, $^2J_{CH}$ = 3.6 Hz, 2-H); 3.37 (dd, 2H, $^3J_{HH}$ = 6.5 Hz, $^3J_{CH}$ = 3.8 Hz, 1-H) ppm.
^{13}C-NMR (100 MHz, $CDCl_3$): δ = 30.6 (d, $^1J_{CC}$= 34.5 Hz, C-2) ppm.

1-Iodo-2-methylpropane 22
Iodine (1.3g, 5.0 mmol) was added to a solution of triphenylphosphine (1.31g, 5.0 mmol) in nitrobenzene (25 mL). The brown color persisted and an orange solid precipitated after ca. 10

min. A solution of 2-methyl-1-propanol (370 mg, 5.0 mol) in nitrobenzene (1 mL) was added followed by quinoline (1.2 mL, 10 mmol) and the reaction mixture was stirred overnight. Distillation of the reaction mixture occurred at 50°C in 2 hours to a cold trap (-196°C) under reduced pressure (>1,0 mmHg). 2-methyl-1-iodo-propane was collected in 74% yield (1.5g).
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.00 (d, $^{3}J_{HH}$ = 6.6 Hz, 6H, 3-H); 1.73 (m, 1H, 2-H); 3.14 (d, $^{3}J_{HH}$ = 5.9 Hz, 2H, 1-H) ppm.
^{13}C-NMR (100 MHz, $CDCl_3$): δ = 18.4 (C-3), 22.6 (C-3), 30.4 (C-2) ppm.

[^{13}C]-1-Iodo-2-methylpropane 22a

The procedure was successfully reproduced starting with (2*S*)-^{13}C-methylpropanol (1.1 g, 16 mmol) giving (2*S*)-^{13}C-methyl-1-iodo-propane (2.1 g, 74%) as product.
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.00 (dd, 3H, $^{3}J_{HH}$ = 6.6 Hz, $^{1}J_{CH}$ = 125.8 Hz, 3-H), 1.00 (dd, 3H, $^{3}J_{HH}$ = 6.6 Hz, $^{3}J_{CH}$ = 5.0 Hz, 3-H); 1.73 (m, 1H, 2-H); 3.14 (dd, 2H, $^{3}J_{HH}$ = 5.9 Hz, $^{3}J_{CH}$ = 4.3 Hz, 1-H) ppm.

***tert*-Butyl *N*-Diphenylmethylidene-2-(2-methyl-propyl) glycinate 23**

2-methyl-1-iodo-propane (373 mg, 2.0 mmol) was added to a mixture of *tert*-butylglycinate benzophenone (500 mg, 1.69 mmol) and O-allyl-N-(9-anthracenylmethyl)cinchonidium bromide (200 mg, 0.34 mmol) in CH_2Cl_2 (8 mL). The reaction mixture was then cooled (-50°C), and BTTP (2.5 mL, 8.5 mmol) was added dropwise. After 24 hrs. the solvent was evaporated in vacuo and the residue was purified by flash chromatography on silica gel (PE / EtOAc, 12:1) yielding the **23** as a yellowish oil (455 mg, 76%)
^{1}H-NMR (300 MHz, $CDCl_3$): = δ 0.68 (d, 3H, $^{3}J_{HH}$ 6.5 Hz, 5-H (R)), 0.85 (d, 3H, $^{3}J_{HH}$ 6.6 Hz, 5-H (S)), 1.44 (s, 9H, 8-H); 1.60 (m, 1H, 4-H), 1.60 (m, 1H, H-3), 1.73 (ddd, 1H, $^{3}J_{HH}$ 8.8 Hz, $^{3}J_{HH}$ 5.2 Hz, $^{2}J_{HCH}$ 13.3 Hz, 3-H), 1.85 (ddd, 1H, $^{3}J_{HH}$ 4.9 Hz, $^{3}J_{HH}$ 8.4 Hz, $^{2}J_{HH}$ 13.3 Hz, 3-H), 3.95 (dd, 1H, $^{3}J_{HH}$ 4.9 Hz, $^{3}J_{HH}$ 8.7 Hz, 2-H), 7.4 (m, 10H, 2*Ph) ppm.
^{13}C-NMR (75 MHz, $CDCl_3$): δ = 21.7 (C-5 (R)), 23.2 (C-5 (S)), 24.7 (C-4), 28.0 (C-8), 42.7 (C-3), 64.6 (C-2), 80.7 (C-7), 127-139 (Ar), 169.7 (C-6), 172.0 (C-1) ppm.

***tert*-Butyl [^{13}C]-*N*-Diphenylmethylidene-2-(2-methyl-propyl)glycinate 23a**

The procedure was successfully reproduced starting with (2*S*)-^{13}C-1-iodopropane (2.1g, 11.4 mmol), *tert*-butylglycinate benzophenone (3.0 g, 10.4 mmol), catalyst (600 mg, 2.0 mmol) and BTTP (15.9 mL, 52 mmol) in CH_2Cl_2 (50mL), giving **23a** (2.7g, 75.6%) as product.
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.50-1.03 (dd's, 6H, 5-H), 1.60 (m, 1H, 4-H), 1.60 (m, 1H, 4-H), 1.73 (m, 1H, 3-H), 1.85 (m, 1H, 3-H) ppm.
^{13}C-NMR (100 MHz, $CDCl_3$): δ = 24.8 (d, $^{1}J_{CC}$= 35.0 Hz, C-4) ppm.

(2*S*)-Leucine 3

6N HCl (10 mL) was added to **21** and the mixture was refluxed for 6 hrs. The mixture was then diluted with water and extracted with ether to remove the organic waste. Water was evaporated *in vacuo* and the residue lyophilized three times.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.98 (d, $^{3}J_{HH}$ = 3.8 Hz, 3H, 6-H), 1.00 (d, $^{3}J_{HH}$ = 3.6 Hz, 5- H), 1.73 (m, 1H, 4-H), 1.83 (m, 2H, 3H), 3.94 (d, 1H, $^{3}J_{HH}$ = 5.6 Hz, $^{3}J_{HH}$ = 8.0 Hz, C(2)-H) ppm.

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 21.7 (C-5), 22.4 (C-6), 24.5 (C-4), 39.9 (C-3), 52.8 (C-2), 174.0 (C-1) ppm.

[5-^{13}C]-(2*S*,4*R*)-Leucine 3a

The procedure was successfully reproduced starting with **23a** (2.7g, 7.6 mmol) and giving [5-^{13}C]-(2*S*,4*R*)-Leucine (1.0g, quantitative) as product. The yield after two recrystallisations was 0.7 g (70 %), e.e. 99%.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.98 (dd, 3 H, $^{3}J_{HH}$ = 6.7 Hz, $^{3}J_{CH}$ = 3.0 Hz, 6-H), 1.00 (dd, 3 H, $^{3}J_{HH}$ = 6.7 Hz, $^{1}J_{CH}$ = 125.3 Hz, 5-H), 1.75 (m, 1 H, 4-H), 1.83 (m, 2H, 3-H) ppm.

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 24.5 (d, $^{1}J_{CC}$= 34.5 Hz, C-4) ppm.

Cbz-*tert*-Butyl Glycinate 28

Glycine (3 g, 40 mmol) was dissolved in an excess of 10% Na_2CO_3 solution to which was added benzyl chloroformate (2 equiv., 11.4 mL) dissolved in 25 mL THF. The mixture was allowed to stir at room temperature for 4 hours and subsequently extracted with 3 times with 50 mL ethyl acetate to remove excess benzyl chloroformate. The aqueous layers were concentrated in vacuo to give a white solid. The solid was redissolved in 50 mL water and the pH was brought to 1 using 1M HCl, after which the solution was extracted 3 times with 50 mL ethyl acetate to give N-Cbz-glycine (5.81 g 76%)

The protected glycine was dissolved in 75 mL dimethylacetamide in the presence of triethyl benzyl ammoniumchloride. Dried K_2CO_3 was added, followed by *tert*-buyl bromide and the mixture was stirred at 55°C for 24 hours. After cooling, cold water was added and the resulting oil was extracted with ethyl acetate to give after evaporation **28** (71%, 5.37 g).

28 was dissolved in 50 mL ethanol and the solution was purged with argon. Subsequently 10% Pd/C was added and the mixture was bubbled through with hydrogen gas until TLC indicated complete removal of the Z-protecting group. the solution was purged with argon and filtered over celite under an argon blanket. The celite was rinsed with 50 mL ethanol and the solvent was evaporated. The raw product was redissolved in 50 mL ethyl acetate and extracted twice with 50 mL 1M HCl. The waterlayers were collected and brought to PH 12 with 50% KOH solution. After extraction with twice 50 mL ethyl acetate, the organic layers were combined, dried with $MgSO_4$ and evaporated to give **25** in 92% yield (2.6 g).

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.47 (s, 9 H, *t*Bu) 3.31 (s, 2 H, 2-H) ppm.

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3

Access to any site-directed isotopomer of methionine, selenomethionine, cysteine and selenocysteine.

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3.1 INTRODUCTION

Methionine (Met), cysteine (Cys) and selenocysteine (Sec) are the three proteinogenic amino acids having a high mass chalcogen atom (S and Se) in their side chains (Figure 1). These amino acids are responsible for important protein functions that cannot be realised by other amino acid residues. The sulphur atoms of methionine and cysteine, for example, coordinate to metal ions involved in many redox and catalytic processes of metaloproteins.[1,2] Cysteines are involved in the disulphide bridges that constitute an important element in protein structure and peptide hormones such as insulin. Cys also coordinates to metal ions in metaloproteins, forms covalent bonds with the haem group in the active site of the protein part of cytochrome C and is the basis of the active site of hydrolytic enzymes such as papaine.[3]

A special case is selenocysteine, which is the 21st proteinogenic amino acid which is translationally incorporated in proteins via a special mechanism.[4] Most human selenoproteins have homologues in which Sec has been replaced by Cys. These sulphur proteins, though, are poor catalysts compared with selenoproteins.[5] In the meantime 18 different human selenoproteins have been described. Recently, it has been established that the human selenoproteonome consists of 25 selenoproteins.[6] Interestingly the genome sequences of *H. influenzae* and *M. jannaschii* indicate that their genomes code for Sec containing enzymes, which are involved in the biosyntheses of selenocysteine.[7,8] Dietary selenium is an essential element in human nutrition, playing important roles in cancer prevention, immunology, ageing, male reproduction and other physiological processes.[6,9] Selenoproteins are thought to be responsible for most biomedical effects of dietary selenium which is essential to mammals. The

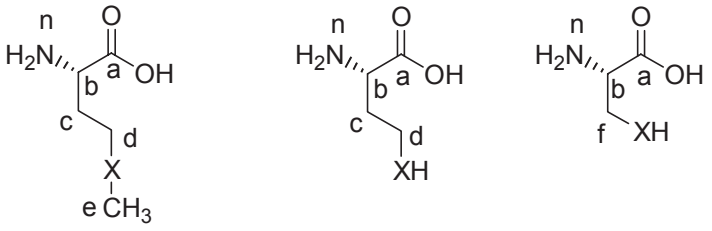


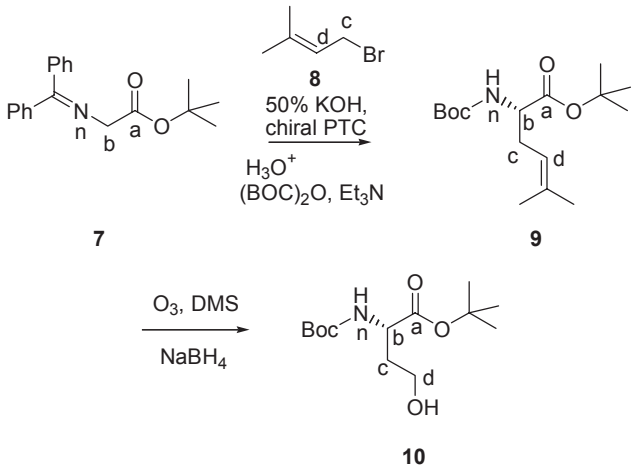
Figure 1. The structures of L-methionine (**1**), L-selenomethionine (**2**), L-homocysteine (**3**), L- homoselenocysteine (**4**), L-cysteine (**5**) and L-selenocysteine (**6**); The letters a, b, etc. indicate the source of the ^{13}C enriched starting materials: the 'a' and 'c' carbons are derived from the carboxylic group of acetic acid and 'b' and 'd' from the methyl group of acetic acid; 'e' originates from methyl iodide and 'f' from paraformaldehyde, while the source of ^{15}N, indicated by 'n' is $^{15}NH_3$.

reduction of incidence of cancer by dietary supplementation with selenomethionine via *Se*-(methyl) selenocysteine has been reported.[10] Among the sulphur-containing amino acids, methionine deserves special mention for another reason. One of the 9 essential amino acids that should be present in sufficient quantity in food[1], methionine reacts with ATP under formation of *S*-(adenosyl)methionine (SAM), which serves as the methyl donor in biological methylation. After the transfer of the methyl group, *S*-(adenosyl)homocysteine is formed and hydrolysed to homocysteine, the essential reagent in the biosynthesis of cysteine[1]. Homocysteine can be reconverted into methionine by methylation by *N*5-methyl tetrafolate. Impairment of this regeneration process leads to elevated plasma levels of homocysteine. It has been found that plasma levels of homocysteine are a causal risk factor for cardiovascular diseases of importance equal to that of hypercholesterimia, hypertension and smoking.[11,12] Selenomethionine mimics almost all the roles of methionine. [^{75}Se]-selenomethionine has a central place in medical diagnosis and therapy as a radiotracer in which temporal and spatial aspects of the interactions of various organs and tumours can be followed. Recently, the possibilities for [^{73}Se]-selenomethionine as a short lived positron emitter for *in vivo* positron emission tomography (PET) has been discussed. This technique is the method of choice for measurement of the rate of protein synthesis in the brain as an important tool to understand the function of the human brain.[13] A similar role for [^{11}C]-methionine has also been discussed.[14] The substitution of Met residues by selenomethionine, constituting an isomorphous substitution of S by the heavy Se, is of great importance in X-ray crystallography of proteins.[15,16] Besides its properties as heavy atom in X-ray structural analysis, natural Se contains 7.6% of the ^{77}Se isotope, a spin ½ nucleus with excellent properties for ^{77}Se spectroscopy.[17] In view of the importance of Cys, Sec, Met, and SeMet in various vital biological processes, access to the full set of ^{13}C and ^{15}N isotopomers of these amino acids is of great urgency. Via mass-spectrometry of these isotopomers their role in nutrition can be followed in exquisite detail by analysis of the serum after administration in the food. Site directed incorporation in proteins can elucidate the structural factors relating to each carbon, hydrogen, nitrogen and selenium atom with atomic resolution through ^{1}H, ^{13}C, ^{15}N and ^{77}Se NMR-spectroscopy. Vibrational spectroscopy of a set of different site-directed isotopomers should provide the force fields of the various chemical bonds in the desired residue in a protein. Even more detailed knowledge should be obtainable by comparison of pairs of proteins in which a sulphur atom has been replaced by a selenium atom. Quantitative biosynthetic replacement has been achieved via methionine auxotrophe *E. coli* strains.[16] In the meantime, the chemical incorporation of cysteine, methionine (via homocysteine), selenocysteine and selenomethionine (via homoselenocysteine) has also been achieved.[18,19,20,21] Two approaches for the synthesis of L-selenomethionine have been published[22,23]

and recently the preparation of L-selenocysteine and L-^{77}Se selenocysteine has been published.[24] The above mentioned syntheses for the sulphur analogues can not serve as basis for the synthesis of the site-directed isotopomers because they all use starting materials with a preformed carbon skeleton not amenable to simple stable ^{13}C and ^{15}N incorporation. For the preparation of the whole set of isotopomers of a system the optimal method is to develop a modular synthetic approach involving a minimal number of steps in a convergent fashion, each of them preferably in a high yield. These conditions and the use of relatively inexpensive isotopically enriched starting materials should provide the molecule in question in such a fashion that each atom derives in a unique way from the enriched starting material. Finally, the isotope-enriched L-amino acids should be prepared in the highest optical purity. Recently the synthetic scheme to allow access to any site-directed isotopomer of L-leucine, based on the O' Donnell strategy, was published.[25] A protected glycine derivative is treated in the presence of a chiral phase transfer catalyst with a primary alkyl halide to form the L-form of leucine with high enantiomeric excess. For that study a scheme was worked out to synthesise the protected glycine derivative in all its possible isotopomers. It is clear that for the preparation of the full set of isotopomers of methionine and selenomethionine it is necessary to prepare the full set of isotopomers of homoserine, which can subsequently be converted into Met and SeMet via homocysteine and homoselenocysteine respectively, followed by methylation with isotopically labelled methyl iodide. Similarly, the precursor for Cys and Sec, protected serine, would be prepared via the O' Donnell method. In this chapter these schemes have been optimised for the preparation of Cys, Sec, Met and SeMet with synthons in natural isotope abundance. The starting materials on which these conversions are based, are commercially available in any isotopically enriched form. In these schemes all atoms derive from a well-defined source. This approach has the advantage of a modular and efficient method, resulting in schemes in which any isotopomer is accessible, in high yield and in high enantiomeric excess (e.e.).

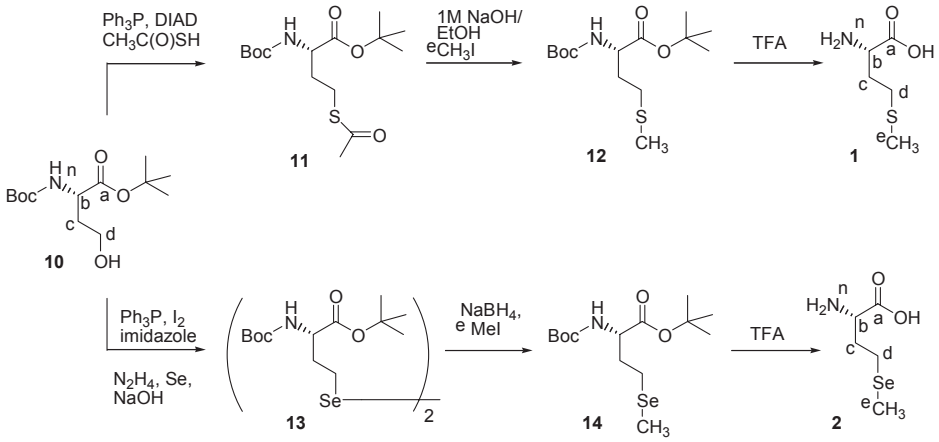
3.2 SYNTHESIS

The central synthon in the preparation of L-methionine (**1**), L-selenomethionine (**2**), L-homocysteine (**3**) and L-homoselenocysteine (**4**) is the protected homoserine **10** (Scheme 1). The reactions shown in Scheme 1 and all further schemes were optimised with synthons of natural isotopic abundance. The starting materials, *tert*-butyl benzophenoneimine glycinate (**7**), dimethylallyl bromide (**8**) and the chiral cinchona derived phase transfer catalyst[26,27] are all commercially available. In order to prepare homoserine **10**, **7** was dissolved in a toluene/chloroform mixture and



Scheme 1. Preparation of the protected homoserine (**10**) from the protected glycine derivative (**7**) and dimethylallyl bromide (**8**). The letters a, b, c, d and n indicate the ^{13}C or ^{15}N source (see legend of Figure 1).

treated with **8** in the presence of 0.01 equiv. chiral phase transfer catalyst (PTC) and base (50% KOH) to yield the alkylated species in 90% yield. Before treatment of the double bond of the side chain with O_3, the O_3-sensitive benzophenoneimine protecting group had to be removed with citric acid and the resulting free amine was reprotected with the *N*-Boc group, which is stable to ozonolysis. The Boc-group was introduced by treatment of the free amine with Boc-anhydride in the presence of triethylamine to give **9** in 92% over two steps. Subsequently, **9** was dissolved in dry dichloromethane and treated with ozone at –40°C. The resulting ozonide could be reduced with dimethylsulphide to the corresponding aldehyde, which was further reduced with $NaBH_4$ to give homoserine **10** in 78% yield. The preparation of all ^{13}C,^{15}N isotopomers of **7** as well as the full set of ^{13}C isotopomers of **8** is possible and has been reported in an earlier publication.[28] This means that all isotopomers of protected homoserine **10** are now accessible in a few steps, each in a high yield. **10** Was also prepared via alkylation of **7** with crotyl bromide, followed by the same conversions as described in scheme 1. Compound **10** was obtained in good yield, but the synthesis and purification of dimethylallyl bromide (**8**) and its isotopomers proved to be more efficient than the preparation of isotopically enriched crotylbromide. Scheme 2 describes the conversion of the protected homoserine into either Met (**1**) or SeMet (**2**). A Mitsunobu reaction of **10** with thioacetic acid, triphenylphosphine and diisopropyl diazodicarboxylate (DIAD) in dry THF gave **11** in 86% yield. Treatment of an ethanolic solution of **11** with aqueous NaOH in the presence of MeI gave saponification of the thioester immediately followed by methyl thioether formation to give the required protected methionine **12** in near quantitative yield. Subsequent



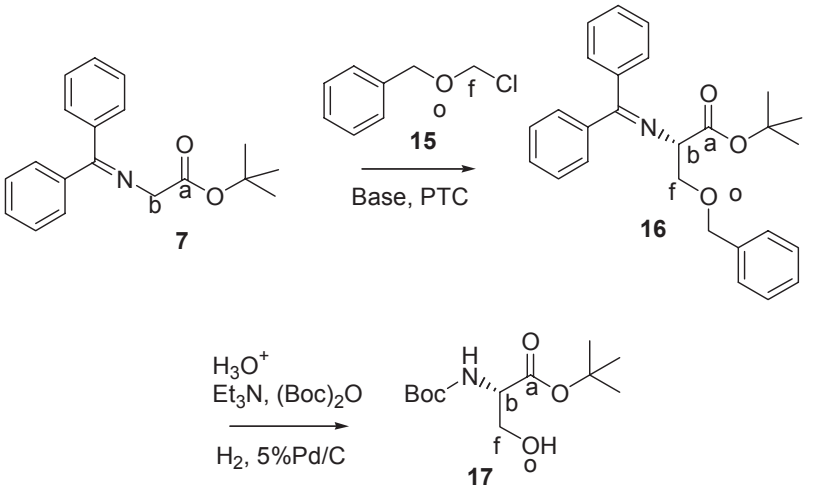
Scheme 2. preparation of L-methionine (**1**) and L-selenomethionine (**2**) from protected L-homoserine (**10**).

treatment of **12** with a 10% solution of trifluoroacetic acid in dichloromethane gave pure Met (**1**), which showed all the analytical characteristics of an authentic sample of commercial methionine after recrystallization. Carrying out the described reaction sequence with ^{13}C enriched methyl iodide will also give incorporation of a stable ^{13}C isotope label in the SCH_3 group of methionine.

The treatment of **11** with NaOH in the absence of CH_3I gave the protected homocysteine, which upon deprotection with trifluoroacetic acid gave homocysteine (**3**) with all the characteristics of the authentic material. For the preparation of selenomethionine (**2**), **10** was treated with triphenylphosphine, iodine and imidazole dissolved in dry dichloromethane to convert the alcohol function into the corresponding iodide in 86% yield. After this, the iodide was added to a DMF solution containing a mixture of elemental selenium, hydrazine monohydrate and sodium hydroxide. The reaction of selenium and hydrazine in the presence of NaOH gives Na_2Se_2 which reacted twice with the iodide to give the *N*-Boc protected homoselenocystine **13**. Treatment of **13** with $NaBH_4$ in the presence of methyl iodide resulted in the reduction of the diselenide bond followed by methylation of the resulting selenide to give the protected selenomethionine. Deprotection with trifluoroacetic acid gave selenomethionine (**2**), while deprotection of **13** with trifluoroacetic followed by reduction with $NaBH_4$ gave homoselenocysteine. Both products show all the analytical characteristics as described in literature.[29,30]

From the schemes presented above it is clear that the *N*-Boc serine *tert*-butyl ester (**17**) is the ideal starting material for the preparation of the full set of isotopomers of cysteine and selenocysteine. For their preparations the synthesis of L-serine was opti-

mised first (Scheme 3).[31] To prepare protected serine **17**, glycinate **7** was treated with benzyloxychloromethyl ether under chiral phase transfer conditions to give **16** in 70% yield. Benzyloxychloromethyl ether can be prepared in good yield in a one-pot reaction from paraformaldehyde, benzyl alcohol and HCl.[32] Via this scheme ^{18}O and ^{17}O can be incorporated from isotope enriched water and ^{13}C from isotope enriched paraformaldehyde.[32] The *O*-benzyl protecting group has to be removed by catalytic reduction (Pd/C and hydrogen), but the benzophenoneimine protecting group is not stable under reducing conditions. It was therefore replaced with the *N*-Boc protecting group by treatment of **16** with citric acid followed by a reaction using Boc-anhydride and triethylamine to give *N*-Boc, *O*-benzyl serine *tert*-butyl ester, which was finally converted into *N*-Boc serine *tert*-butyl ester **17** by catalytic hydrogenation.

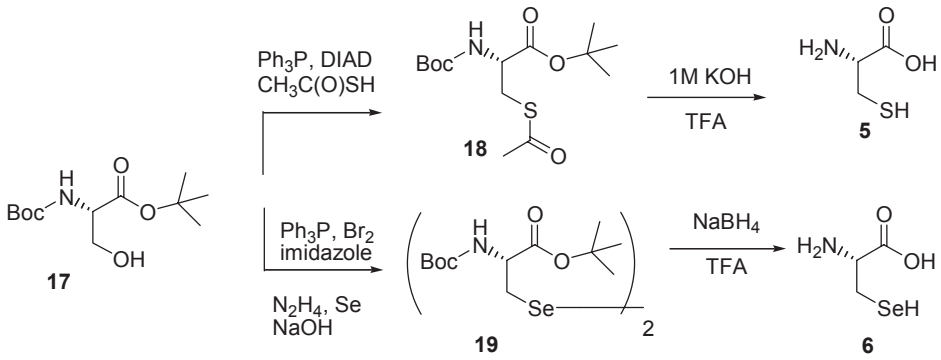


Scheme 3. A short and efficient route to protected serine **17**.

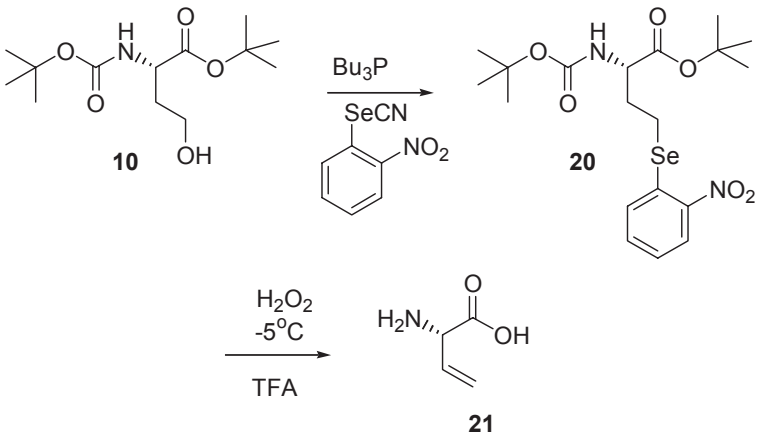
Scheme 4 depicts the conversions of the protected serine into Cys (**5**) and Sec (**6**). These reactions are essentially the same as discussed for scheme 2. The Cys (**5**) and Sec (**6**) obtained in this way both show, after recrystallization, the same analytical characteristics as the authentic materials. In this way the access is gained to the full set of ^{13}C and ^{15}N isotopomers for both Cys and Sec.

L-vinylglycine (**21**) is a potent inhibitor of β-cystathionase in the methionine biosynthetic pathway. It is also an irreversible inhibitor for pyridoxal phosphate dependent aminotransferases.[33] Again, the full cassette of isotopomers would be essential for investigation of these vital biological processes at atomic resolution. Oxidation of the protected selenomethionine **14** (Scheme 2) with hydrogenperoxide at low temperature and subsequent $CH_3Se(O)H$ elimination should give the protected

L-vinyl glycine derivative. Unfortunately, this reaction gives **21** in only 20% yield. It is known from literature, however, that formation of a terminal double bond by selenoxide elimination can be accomplished efficiently in high yield by use of an *o*-nitrophenyl selenide.[34,35] By this approach, **20** was prepared in 82% yield by treatment of **10** with $(Bu)_3P$ in dry THF (Scheme 5), followed by addition of *o*-nitrophenylselenocyanate. Subsequent treatment of **20** with aqueous H_2O_2 at –5°C yielded the protected vinylglycine **21** in 79% yield. Deprotection with trifluoroacetic acid gave L-vinylglycine (**21**) with the same analytical characteristics as given in literature. Protected vinylglycine can also serve as a starting material to form important novel chiral derivatives.



Scheme 4. Preparation of cysteine (**5**) and selenocysteine (**6**).



Scheme 5. Preparation of vinylglycine (**21**) starting from protected homoserine (**10**).

3.3 DISCUSSION

From the Mitsunobu reaction of the phenyl selenocyanate in Scheme 5 it is clear that these reagents can easily convert primary alcohols into the corresponding seleno and thioethers. There is no barrier to the extension of these reactions to the preparation of the corresponding tellurium systems.[24] These schemes also provide the building blocks for the preparation of cystathionine which is an intermediate in the biosynthesis of cysteine (it is the thioether with the same carbon skeleton as Cys) and lanthionine (the thioether made of two Cys carbon skeletons), which occur in proteins in the skin[36,37,38] and are formed during the biosynthesis of certain antimicrobial compounds.[39,40]

3.4 CONCLUSION

In this chapter the modular and convergent strategy for the preparation of L-Ser, Cys, Sec, Met, SeMet and vinylglycine in optically pure form such that all stable ^{13}C, ^{15}N, ^{17}O, ^{18}O, ^{77}Se as well as the radioactive isotopomers can be easily introduced in a site-specific manner, through the alkylation of a protected glycine derivative with alkyl halides in the presence of a chiral phase transfer catalyst, is described. After the reaction, the optically active catalyst can be easily isolated, ready to be reused. Via this method no difficult separation of the product from an optically active scaffold is needed.[41] The central step for the incorporation of S and Se is an efficient Mitsunobu reaction. Based on the scope of the Mitsunobu reaction it is to be expected that many important optically active α-amino acids with the serine and homoserine carbon skeleton can become easily available.

3.5 EXPERIMENTAL SECTION

General remarks: ^1H NMR spectra were recorded with a Jeol FX-200 and a Bruker DPX-300 spectrometer, using tetramethyl silane (TMS: δ = 0 ppm) or water (H_2O: δ = 4.8 ppm) as an internal standard. ^{13}C noise-decoupled NMR spectra were recorded with a Jeol FX-200 at 50.1 MHz and a Bruker DPX-300 spectrometer at 75.5 MHz , using $CDCl_3$ (δ = 7 ppm) $(CD_3)_2CO$ (δ = 206 ppm) or TSP (δ = 0 ppm) as internal standard. All spectra were recorded in $CDCl_3$, except where noted otherwise. Column chromatography was performed on Merck silica gel 60 (0.040-0.063 mm 230-400 mesh) and spots on thin-layer chromatography were detected with UV-light, $KMnO_4$-spraying, ninhydrin-staining (0.2% in ethanol) or staining with a mixture of 4,4'-methylenebis (*N*,*N*,dimethylaniline) and ninhydrin (TDM-staining). Chiral HPLC was per-

formed on a Chiralcel ODH column (25 cm) using hexane and isopropanol (*i*PrOH) as solvent system or on a Daicel crownpack CR(+) column (150 mm) using a $HClO_4$ solution as solvent system. Dry diethyl ether (ether, Et_2O) was obtained by distilling from P_2O_5. Dry petroleum ether (PE) 40-60 and dry dichloromethane (DCM) were obtained by distilling from CaH_2. Dry tetrahydrofurane (THF) was obtained by drying over sodium-benzophenone. Deaereated solvents were obtained by bubbling a steady stream of Argon or dry nitrogen through the liquid for one hour. All reagents were purchased from Aldrich Chemical Co., Acros Chimica or Fluka.

***tert*-Butyl 2-(benzhydrylidene-amino)-5-methylhex-4-enoate**

To a solution of **7** (1 g, 3.4 mmol) in 10 mL toluene at 0°C was added dimethyl-allylbromide **8** (0.55 g, 1.1 eq), catalyst (102 mg, 0.05 equiv.) and 10 equiv. of a 50% aqueous KOH solution. The mixture was stirred vigorously for 6 hours at 0°C and overnight at RT. Subsequently, the reaction mixture was diluted with 15 mL water and extracted 3 times with 20 mL ethylacetate. After evaporation the crude product was purified using column chromatography (PE/Et_2O, 80:20). After the product was collected, the column was eluted with DCM to recover the catalyst. The yield was 90 % (1.11 g), e.e. > 90 % (chiralcel ODH, 25 cm, Hexane/*i*PrOH 99.9/0.1 v/v, flow 0.8 mL/min. retention time (min.):15.26 (S), 16.47 (R)).

^{1}H NMR (300 MHz): δ = 1.44 (9 H, s, *t*Bu), 1.56 (3 H, s, H6), 1.64 (3 H, d, $^4J_{HH}$ = 0.75 Hz, H6), 2.57 (2 H, m, H3), 3.98 (1 H, dd, $^3J_{HH}$ = 5.62 Hz, $^3J_{HH}$ = 7.54 Hz, H4), 5.05 (1 H, tq, $^3J_{HH}$ = 7.5 Hz, $^4J_{HH}$ = 0.75 Hz, H4), 7.70-7.13 (10 H, m, arom) ppm.

^{13}C NMR (75.5 MHz): δ = 17.74 (CH_3), 25.60 (CH_3), 27.85 (*t*Bu), 32.24 (C3), 66.22 (C2), 80.42 (Cq), 120.11 (C4), 127-140 (C, Phe), 136.55 (C5), 169.33 (CN), 171.03 (C1) ppm.

***tert*-Butyl 2-amino-5-methylhex-4-enoate**

The alkylated glycinate (1.11 gr 3.05 mmol) was dissolved in 10 mL THF and stirred with 10 mL of a 10% citric acid solution for one night. TLC showed complete disappearance of the starting material and the mixture was extracted with ether. The water layer was brought to pH 12 and extracted twice with 30 mL ethyl acetate to give after evaporation the product in 98% yield (0.6 g, 3.0 mmol).

^{1}H NMR (300 MHz): δ = 1.46 (9 H, s, *t*Bu), 1.64 (3 H, s, H6c), 1.72 (3 H, d $^4J_{HH}$ = 1.0 Hz, H6t), 2.35 (2 H, m, H3), 3.37 (1 H, dd, $^3J_{HH}$ = 5.49 Hz, $^3J_{HH}$ = 6.87 Hz, H2), 5.10 (1 H, tq, $^3J_{HH}$ = 6.87 Hz, $^4J_{HH}$ = 1.0 Hz, H4) ppm.

^{13}C NMR (75.5 MHz): δ = 17.19 (CH_3), 25.60 (CH_3), 27.73 (*t*Bu), 33.33 (C3), 54.76 (C2), 80.36 (tBu), 119.00 (C4), 124.62 (C5), 174.61 (C1) ppm.

***tert*-Butyl 2-*tert*-butoxycarbonylamino-5-methylhex-4-enoate 9**

The aminoester prepared above, was dissolved in 5 mL DMF and 1 equiv. triethylamine (0.3 g, 0.42 mL) was added followed by 1.1 equiv. di-*tert*-butyl dicarbonate (0.71 mL) and the mixture was stirred until TLC showed completion of the reaction. The DMF was evaporated and 25

mL ethylacetate was added. The organic layer was washed thrice with 25 mL $KHSO_4$ solution (pH 2), water, brine and dried over $MgSO_4$. The crude product was purified over a short silica column (PE/Et_2O 80/20). The yield was 94% (0.85 g).

^{1}H NMR (300 MHz): δ = 1.44 (9 H, s, *t*bu), 1.45 (9 H, s, *t*bu), 1.61 (3 H, s, H6c), 1.70 (3H, d $^4J_{HH}$ = 1.0 Hz, H6t), 2.45 (2 H, m, H3), 4.21 (1 H, dd $^3J_{HH}$ 5.6 Hz, $^3J_{HH}$ 13.5 Hz, H2), 5.04 (1 H, m, H4), 5.04 (1 H, s, NH), ppm.

^{13}C NMR (75.5 MHz): δ =18.64 (CH_3), 26.58 (CH_3), 28.69 (*t*Bu), 29.05 (*t*Bu), 31.94 (C3), 54.57 (C2), 80.14 (*t*Bu), 85.81 (*t*Bu), 118.80 (C4), 136.17 (C5), 155.91 (CO), 172.19 (C1) ppm.

_N_-Boc-homoserine _tert_-butyl ester 10

A solution of **9** (0.84 g, 2.8 mmol) in 150 mL of DCM in a three-necked round bottomed flask equipped with a dropping funnel with dimethylsulfide (0.37 mL, 1.5 equiv.) in 10 mL DCM was cooled to -50°C using an acetone/dry ice bath. Ozone was led through the solution until the liquid had a persistent blue color. The excess ozone was purged from the solution using a soft nitrogen stream and subsequently the dimethylsulfide was added dropwise while the temperature was kept below -40°C. The reaction mixture was allowed to warm to RT during two hours and the solvents were evaporated (careful, stench) *in vacuo*. The crude product was taken up in 60 mL of ethanol and the solution cooled to 0°C. $NaBH_4$ (0.21 g, 2 equiv.) was added and the mixture was stirred during 4 hours. Work-up was accomplished by addition of 10 mL 1 M HCl solution, followed by evaporation of the bulk of the ethanol. 40 mL ether was added and the organic layer was extracted with $NaHCO_3$ solution, water, brine and the organic layers were subsequently dried over $MgSO_4$. the crude product was purified using column chromatography (gradient 20% to 40% Et_2O in PE). The yield was 78% (611 mg, 2.2 mmol).

^{1}H NMR (300 MHz): δ = 1.49 (18 H, s, 2**t*Bu), 2.04 (2 H, m, H3), 3.58 (2 H, m, H4), 4.26 (1 H, m, H2), 5.38 (1 H, d $^3J_{HH}$ = 7.8 Hz, NH) ppm.

^{13}C NMR (75.5 MHz): δ = 28.17 (*t*Bu), 27.87 (*t*Bu), 36.16 (C3), 50.93 (C2), 58.14 (C3), 80.12 (*t*Bu), 82.11 (*t*Bu), 156.40 (CO), 171.72 (C1) ppm.

_S_-Acetyl-_N_-boc-homocysteine _tert_-butyl ester 11

To a solution of triphenylphosphine (418 mg, 1.6 mmol, 2 equiv.) in 10 mL dry THF in a flame-dried roundbottomed flask at 0°C and under a dry nitrogen atmosphere, was added DIAD (0.317 mL, 2 equiv.) and the mixture was stirred until a white solid appeared. Stirring was continued for 10 minutes at 0°C after which **10** (0.22 g, 0.8 mmol) was addded in 2 mL dry THF. After 45 min stirring, thioacetic acid (0.114 mL, 1.6 mmol) was added and stirring continued for 3 hours. 30 mL ether was added to the reaction mixture and the mixture was extracted with 25 mL water followed by drying of the organic layer over $MgSO_4$. After evaporation of the solvents, the resulting solid was taken up in 3 mL DCM and flashed over silica, which was then rinsed with 50 mL ether. The product concentrated *in vacuo* and was further purified by chromatography (PE/Et_2O 85/15). The yield was 86% (0.23 g, 0.68 mmol).

^{1}H NMR (300 MHz): δ = 1.46 (9 H, s, *t*Bu), 1.48 (9 H, s, *t*Bu), 1.88 (1 H, m, H3), 2.04 (1 H, m, H3), 2.33 (3 H, s, CH3), 2.90 (2 H, m, H4), 4.22 (1 H, m, H2), 5.14 (1 H, d, NH) ppm.
^{13}C NMR (75.5 MHz): δ = 25.04 (C5), 27.93 (*t*Bu), 28.29 (*t*Bu), 30.54 (C4), 33.07 (C3), 53.31 (C2), 80.20 (*t*Bu), 82.28 (*t*Bu), 155.40 (CO), 171.09 (C1), 196.20 (SCO) ppm.

_N_-Boc-methionine _tert_-butyl ester 12

Compound **11** (0.23 g, 0.68 mmol) was redissolved in 10 mL ethanol and 5 mL 2M NaOH solution was added to the stirred solution, which was kept under an argon atmosphere. The hydrolysis was followed by TLC. After 3 hours, 2 equiv. CH_3I (90 µL) was added and the solution was stirred overnight. The solution was concentrated *in vacuo* to 4 mL and 50 mL ethylacetate was added. The organic layer was washed with 10 mL water, dried with brine and dried over $MgSO_4$. After evaporation the product was purified over a silica column (PE/Et_2O 80/20), giving 91% yield (190 mg).
^{1}H NMR (300 MHz): δ = 1.45 (18 H, s, 2**t*Bu), 1.99 (1 H, m, H3), 2.11 (3 H, s, H5), 2.15 (1 H, m, H3), 2.58 (2 H, m, H4), 4.45 (1 H, H2), 5.25 (1 H, NH) ppm.
^{13}C NMR (75.5 MHz): δ = 15.36 (C5), 28.28 (6* CH_3), 29.95 (C4), 31.72 (C3), 52.63 (C2), 80.35 (*t*Bu), 82.60 (*t*Bu), 155.58 (CO), 170.80 (C1) ppm.

L-Methionine trifluoroacetate 1

The protected methionine (190 mg) **12** was dissolved in DCM and 10% trifluoroacetic acid (TFA) was added. The mixture was stirred at RT for 1 night and then extracted with water to give after evaporation the methionine.TFA salt in 82% starting from **12** (135 mg, 0.51 mmol). Recrystallization of L-methionine from a water/ethanol mixture (pH 3.9) gave L-methionine in >98% e.e. ($HClO_4$ (pH 1.5), 15% meOH, flow 0.6 mL/min, retention-time (min) 3.31 (R), 6.39 (S)).
^{1}H NMR (300 MHz, D_2O): δ = 2.13 (3 H, s, H5), 2.20 (2 H, m, H3), 2.70 (2 H, t ($^{3}J_{HH}$ = 7.38), H4), 4.20 (1 H, dd, $^{3}J_{HH}$ = 6.3, $^{3}J_{HH}$ = 6.5, H2) ppm.
^{13}C NMR (75.5 MHz D_2O): δ = 14.69 (C5), 29.39 (C4), 29.76 (C3), 52.79 (C2), 172.79 (C1) ppm.

***tert*-Butyl 2-*tert*-butoxycarbonylamino-4-iodobutanoate**

Triphenylphosphine (1.04 g, 4.0 mmol, 2 equiv.) was dissolved in 15 mL dry dichloromethane, the solution cooled to 0°C and stirred under a dry nitrogen atmosphere while iodine (0.99 g, 1.99 equiv.) was added in portions. After 30 minutes, a mixture of **10** (0.55 g, 2.0 mmol) and imidazole (0.28 g, 2.05 equiv.) was slowly added to the dark purple solution. After one minute a white solid became visible. The reaction mixture was stirred for another two hours, while the temperature was maintained at 0°C. Subsequently, the solids were filtered off and the solvent was evaporated. The resulting solid was redissolved in 1 mL DCM and purified by column chromatography (PE/Et_2O 90/10) to give the iodide in 86% yield (0.66 g, 1.74 mmol)

^{1}H NMR (300 MHz): δ = 1.45 (9 H, s, *t*Bu) 1.48 (9 H, s, *t*Bu), 2.17 (1 H, m, H3), 2.35 (1 H, m, H3), 3.43 (2 H, m, H4), 4.24 (1 H, m, H2), 5.13 (1 H, broad s, NH) ppm.

_N_-Boc-homoselenocystine _tert_-butyl ester 13

To a suspension of Se (0.15 g, 1.87 mmol, 1.1 equiv.) and 0.1 g NaOH in 5 mL DMF was added $N_2H_4.H_2O$ (100 µL, 1.1 equiv.). The mixture was stirred under an argon atmosphere for 3 hours at 60°C. Subsequently the iodide prepared above (0.65 g, 1.70 mmol) was added dropwise and the mixture was stirred for three hours. TLC indicated complete disappearance of the starting material and the solvent was evaporated *in vacuo*. The resulting oil was dissolved in 25 mL ether and extracted with 5 mL water, 5 mL brine and dried over $MgSO_4$ to give **13,** which was obtained in 71% yield (0.41 g) after column chromatography (PE/Et_2O 85/15).

^{1}H NMR (300 MHz): δ = 1.45 (18 H, s, 2**t*Bu) 1.48 (18 H, s, 2**t*Bu), 1.96-2.32 (4 H, 2*H3), 2.89 (4 H, m, 2*H4), 4.19 (2 H, m, H1), 5.14 (2 H, broad s, NH) ppm.

_N_-Boc-selenomethionine _tert-butyl_ ester 14

Diselenide **13** (0.41 g, 0.60 mmol) was dissolved in 10 mL EtOH, cooled to 0°C and $NaBH_4$ (50 mg, 1.33 mmol) was added. The mixture was stirred at 0°C for 15 minutes and CH_3I (0.18 mL, 2.9 mol) was added. After 30 minutes 2 mL 1M HCl was added, followed by 20 mL water and the mixture was extracted thrice with 25 mL EtOAc. The collected organic fractions were dried over $MgSO_4$ and the solvent was evaporated *in vacuo*. The resulting oil was purified using column chromatography to give the title compound in 90% yield (0.38 g).

^{1}H NMR (300 MHz): δ = 1.44 (s, 9 H, *t*Bu), 1.47 (s, 9 H, *t*Bu), 1.97 (1 H, m, H3) 1.99 (3 H, s, H5), 2.16 (1 H, m, H3), 2.53 (2 H, m, H4), 4.25 (1 H, m, H2), 5.19 (1 H, broad s, NH) ppm.

^{13}C NMR (75.5 MHz): δ = 4.06 (C5), 20.26 (C4), 28.35 (6* CH_3), 33.56 (C3), 54.13 (C2), 79.69 (*t*Bu), 82.62 (cq), 155.24 (CO), 171.79 (C1) ppm.

Selenomethionine trifluoroacetate 2

The protected selenomethionine **14** was dissolved in 15 mL 10% TFA in dry DCM and the mixture was stirred for 3 hours. When TLC indicated complete deprotection, the mixture was extracted twice with 15 mL water and the collected water layers were evaporated under reduced pressure to give 0.31 g selenomethionine trifluoroacetic acid salt (91% yield).

^{1}H NMR (200 MHz, D_2O, pH = 7): δ = 2.0 (3 H, s, H5), 2.1-2.3 (2 H, m, H3), 2.6 (2 H, t ($^3J_{HH}$ = 7.14), H4), 3.8 (1 H, m, H2) ppm.

L-Homocysteine.trifluoroacetate 3

11 (0.5 mmol, 167 mg) was redissolved in 5 mL deaereated ethanol and 3 mL deaereated 1M NaOH solution was added to the stirred solution, which was subsequently kept under argon. After 6 hours, the mixture was extracted twice with 30 mL deaereated DCM and the collected organic layers were dried over $MgSO_4$ in an erlenmeyer filled with argon atmosphere. The sol-

vent was then evaporated and the product was redissolved in 10 mL of a deareated 10% TFA in DCM solution. After 12 hours the solvent and acid were extracted with 2 * 10 mL deaereated water and the waterlayers were evaporated under reduced pressure. The yield of **3** (as the trifluoroacetate) was 73% (90 mg)

^{1}H NMR (200 MHz, D_2O): δ = 2.2 (2 H, m, H3) 2.6 (2 H, m, H4), 3.9 (1 H, m, H2) ppm.

L-Homoselenocystine trifluoroacetate 4

The diselenide **13** (0.3 g, 0.44 mmol) was dissolved in 10 mL of a 10% TFA in DCM solution and the mixture was stirred for 18 hours without stopper. The solvent and TFA were evaporated *in vacuo*. The resulting oily solid was taken up in 5 mL water and washed twice with 5 mL DCM to give after evaporation of the water layer the title compound in 79% yield (208 mg).

^{1}H NMR (300 MHz, D_2O): δ = 1.72-1.91 (2* 2 H, m, H3), 2.42 (2* 2 H, m, H4), 3.62 (2* 1 H, d, $^3J_{HH}$ = 6.45 Hz, H1) ppm.

***tert*-Butyl 2-(Benzhydrylidene)amino-3-(benzyloxy)-propionate 16**

N-Benzophenoneimine *tert*-butyl glycinate **7** (200 mg, 0.67 mmol) was dissolved in 3 mL dry DCM. The solution was cooled to -5˚C and benzyl chloromethylether (1.5 equiv. 70µL), *O*-allyl *N*-methylanthracenyl cinchonidium bromide (20%, 80 mg) and BEMP (2-*tert*-butylimino-2-diethylamino-1,3-dimethyl-perhydro-1,3,2-diazaphosphorine) (0.58 mL, 3 equiv.) were added. The mixture was stirred vigorously at this temperature for 18 hours. TLC indicated almost complete disappearance of the starting material and 5 mL water and 20 mL DCM were added. The organic layer was washed with 5 mL water, dried over $MgSO_4$ and evaporated. The crude product was purified over a silica column (PE/ethyl acetate 95/5) giving **16** in 70% yield (193 mg). E.e. > 81% (Chiralcel ODH, 25 cm, Hexane/*i*PrOH 99.5/0.5 v/v, flow 0.8 mL/min. retention times (min.) 16.45 (R), 18.81 (S)).

^{1}H NMR (300 MHz): δ = 1.36 (9 H, s, *t*Bu), 3.79 (1H, dd $^2J_{HH}$ = 9.64 Hz, $^3J_{HH}$ = 7.27 Hz, H-3), 3.90 (1 H, dd $^2J_{HH}$ = 9.64 Hz, $^3J_{HH}$ = 4.76 Hz, H-3), 4.23 (1 H, dd $^3J_{HH}$ = 7.27 Hz, $^3J_{HH}$ = 4.76 Hz, H-2), 4.51 (1 H, d $^2J_{HH}$ = 12.34 Hz, OCH_2), 4.52 (1 H, d $^2J_{HH}$ = 12.34 Hz, OCH_2), 7.21-7.66 (15 H, m, 3*Phe) ppm.

^{13}C NMR (75.5 MHz): δ = 28.01 (*t*Bu), 66.51 (C2), 71.55 (C3), 73.18 (C4), 81.23 (*t*Bu), 125.64 - 139.68 (arom), 169.42 (C=N), 171.22 (C1) ppm.

***O*-Benzyl-*N*-boc-serine *tert*-butyl ester**

The *O*-benzyl protected serinate ester **16** was dissolved in 5 mL THF and stirred with 5 mL of a 10% citric acid solution for one night. TLC showed complete disappearance of the starting material and the mixture was washed twice with 15 mL ether. The water layer was brought to pH 13 and extracted twice with 30 mL ethyl acetate to give after evaporation the product in 95% yield (112 mg). The aminoester prepared above, was dissolved in 5 mL DMF and triethylamine (136 µL, 2 equiv.) was added followed by 1.5 equiv. di-*tert*-butyl dicarbonate (153 µL) and the

mixture was stirred until TLC showed completion of the reaction. The DMF was evaporated and 25 mL ethylacetate was added. The organic layer was washed with 2*15 mL $KHSO_4$ solution (pH 2), water and dried over $MgSO_4$. The crude product was purified over a short silica column (PE/Et_2O 85/15). The yield was 82% (128 mg).
^1H NMR (200 MHz): δ = 1.5 (18 H, s, 2**t*Bu), 3.7 (1 H, m, H-3), 3.9 (1 H, m, H-3), 4.3 (1 H, m, H-2), 4.4-4.6 (2 H, m, OCH_2,), 5.4 (1 H, broad s, NH), 7.2-7.4 (5 H, m, Phe) ppm.

_N_-Boc-serine _tert_-butyl ester 17

The *N*-Boc protected serine derivative (128 mg, 0.36 mmol) was dissolved in 10 mL of a mixture of deaereated methanol and acetic acid (97/3 v/v) to which a catalytic amount of 10% Pd/C was added. Hydrogen was bubbled through at normal pressure for 18 hours. The solution was flushed with nitrogen gas and 10 mL water and 50 mL ethylacetate were added. The organic layer was extracted twice with 5 mL water and was evaporated *in vacuo*. The crude product was purified using column chromatography (PE/Et_2O 80/20) giving **17** in a yield of 79% (75 mg, 0.29 mmol).
^1H NMR (200 MHz): δ = 1.45 (9 H, s, *t*Bu), 1.49 (9 H, s, *t*Bu), 3.9 (2 H, m, H3), 4.3 (1 H, broad s, H2), 5.4 (1 H, broad s, NH) ppm.

_S_-Acetyl-_N_-boc-cysteine _tert_-butyl ester 18

To a a solution of triphenylphosphine (0.44 g, 2.0 equiv.) in a flame-dried roundbottomed flask in 10 mL dry THF at 0°C under a dry nitrogen atmosphere, was added DIAD (0.317 mL, 2 equiv.) and the mixture was stirred until a white solid appeared. stirring was continued for 10 minutes at 0°C after which **17** (0.22 g 0.84 mmol) was addded in 2 mL dry THF. After 45 min stirring, thioacetic acid (0.120 mL, 1.68 mmol) was added and stirring continued for 3 hours. 30 mL ether was added to the reaction mixture and the mixture was extracted with 15 mL water and the organic layer dried over $MgSO_4$. After evaporation of the solvents, the resulting solid was taken up in 5 mL DCM and flashed over a glass filter filled with silica, which was then rinsed with 50 mL ether. After evaporation of the ether, the product was further purified by chromatography (PE/Et_2O 85/15). The yield was 76% (0.2 g).
^1H NMR (300 MHz): δ = 1.44 (9 H, s, *t*Bu), 1.47 (9 H, s, *t*Bu), 2.35 (3 H, s, H4), 3.34 (2 H, m, H3), 4.50 (1 H, broad s, H2), 5.21 (1 H, broad s, NH) ppm.

L-Cysteine 5

Compound **18** (0.2 g) was dissolved in 10 mL deaereated ethanol and 5 mL deaereated NaOH solution was added to the solution which was stirred under an argon atmosphere. The hydrolysis was followed by TLC. After 5 hours the bulk of the ethanol was evaporated *in vacuo* (care was taken to keep the product under an argon atmosphere as much as possible) and the resulting solution was extracted twice with 25 mL deaereated DCM. The organic layers were collected and dried over $MgSO_4$ and the solution was filtered without vacuum suction, but with an Argon

blanket, created by a steady stream of Argon coming from an inverted funnel suspended over the glass filter, covering the solution. 5 mL TFA was added to the collected organic layers and the mixture was allowed to stir under an argon atmosphere overnight. The reaction mixture was extracted twice with 10 mL deaereated water to give after evaporation of the water 0.133 g cysteine.TFA salt (89%, 0.57 mmol).

^{1}H NMR (300 MHz): δ = 2.58 (2 H, m, H3), 3.78 (1 H, t, $^{3}J_{HH}$ = 4.8 Hz, H2) ppm.

^{13}C NMR (75.5 MHz): δ = 24.14 (C-3), 54.70 (C-2), 170.17 (C-1) ppm.

_N_-Boc selenocystine _tert_-butyl ester 19

Triphenylphosphine (0.44 g, 2 equiv.) was dissolved in 5 mL dry dichloromethane, the solution cooled to 0°C and stirred under a dry nitrogen atmosphere while 86 µl bromine dissolved in 2 mL DCM was added dropwise. After 20 minutes, a mixture of **17** (0.22 g, 0.84 mmol) and imidazole (0.12 g, 2 eq) dissolved in 5 mL DCM was slowly added to the pale yellow solution. After one minute a white solid became visible. The reaction mixture was stirred for another two hours, while the temperature was maintained at 0°C. Subsequently, the solids were filtered off and the solvent was evaporated. The resulting solid was redissolved in 1 mL DCM and purified by column chromatography (PE/Et_2O 90/10) to give the bromide in 81% yield (0.22 g, 0.68 mmol)

^{1}H NMR (200 MHz): δ = 1.45 (9H, s, *t*Bu) 1.47 (9H, s, *t*Bu), 3.5 (1H, m, H3), 3. 6 (1H, m, H3), 4.5 (1H, m, H2), 5.2 (1H, broad s, NH) ppm.

To a suspension of Se (62 mg, 1.1 equiv.) and 0.1 g pulverized NaOH in DMF was added 40 µL of a $N_2H_4.H_2O$ solution. The mixture was stirred under an argon atmosphere for 3 hours at 60°C. Subsequently the bromide (0.22 g, 0.68 mmol) was added dropwise and the mixture was stirred for three hours. TLC indicated complete disappearance of the starting material and the mixture was poured into 5 mL 1M HCl, which was subsequently extracted with 3 * 15 mL DCM after which the solvent was dried over $MgSO_4$ and evaporated *in vacuo* to give **19** in 74% (161 mg, 0.25 mmol).

^{1}H NMR (300 MHz): δ = 1.44 (9 H, s, *t*Bu), 1.47 (9 H, s, *t*Bu), 3.34 (2 H, m, H3), 4.50 (1 H, broad s, H2), 5.21 (1 H, broad s, NH) ppm.

L-Selenocysteine 6

To a solution of the protected selenocystine **19** (0.26 g, 0.25 mmol) dissolved in 4 mL ethanol was added $NaBH_4$ (30 mg, 3 equiv.) at 0°C and the reaction mixture was allowed to proceed for 30 minutes at 0°C. The solution was then concentrated to 1 mL *in vacuo*, deaereated water (5 mL) was added and the solution was extracted twice with 20 mL deaereated DCM. The organic layers were collected and dried over $MgSO_4$ and the solution was filtered without vacuum suction, but with an argon blanket. 2 mL TFA was added to the solution and the whole was stirred overnight. The bulk of the acid and solvent were distilled off using a high vacuum pump equipped with a cold-trap and the oily product was taken up in 10 mL deaereated water.

Evaporation of the water *in vacuo* gave the selenocystine.TFA salt as an off-white solid in 85% yield (70 mg, 0.42 mmol).

^{1}H NMR (300 MHz, D_2O): δ = 3.05 (1 H, dd, $^{3}J_{HH}$ = 5.55 Hz, $^{2}J_{HH}$ = 14.17 Hz, H-3), 3.14 (1 H, dd, $^{3}J_{HH}$ = 4.41 Hz, $^{2}J_{HH}$ = 14.17 Hz, H-3), 4.41 (dd, $^{3}J_{HH}$ = 5.55 Hz, $^{3}{}_{HH}$ = 4.41 Hz, 1 H, H-2) ppm.

^{13}C NMR (75.5 MHz, D_2O): δ = 15.64 (C3), 54.14 (C2), 170.23 (C1) ppm.

tert-_B_utyl 2-_tert_-butoxycarbonylamino-4-(2-nitro-phenylselenyl) butanoate 20

To a solution of **10** (100 mg, 0.36 mmol) and 2-nitrophenylselenocyanate (0.25 g 3 equiv.)in 5 mL dry THF under a dry nitrogen atmosphere was added dropwise 95% Bu_3P (93 mg, 1.2 equiv., 113 µL) dissolved in 3 mL dry THF. After 45 minutes TLC indicated almost complete dissappearance of the starting material and the mixture was concentrated *in vacuo* and purified by column chromatography (PE/Et_2O 90/10) to give **20** in 82% yield (137 mg, 0.30 mmol).

^{1}H NMR (300 MHz): δ = 1.45 (9 H, s, tBu), 1.47 (9 H, s, *t*Bu), 2.05 (1 H, m, H3), 2.20 (1 H, m, H3), 2.93 (2 H, m, H4) 4.27 (1 H, broad s, H2), 5.19 (1 H, broad s, NH) 7.32-8.30 (4 H, m, Phe) ppm.

***N*-Boc vinyl glycine *tert*-butyl ester**

Compound **20** (137 mg) was dissolved in 10 mL THF, cooled to –5°C and treated with 3 mL 30% H_2O_2. After 30 minutes the solution was extracted thrice with 50 mL ether and the collected organic layers were concentrated *in vacuo*. The crude product was purified using column chromatography (PE/Et_2O, 93:7) to give 61 mg of the desired product (79% yield).

^{1}H NMR (200 MHz): δ = 1.45 (9 H, s, *t*Bu), 1.47 (9 H, s, *t*Bu), 4.8 (1 H, broad s, H-2), 5.2-5.4 (3 H, m, H4 + NH), 5.9 (1 H, m, H3) ppm.

^{13}C NMR (50.1 MHz): δ = 27.9 (*t*Bu), 28.3 (*t*Bu), 56.3 (C2), 82.4 (Cq), 116.7 (C4), 133.2 (C3) ppm.

L-Vinyl glycine 21

The protected vinylglycine (61 mg, 0.24 mmol) was dissolved in 5 mL of a 10% TFA in DCM solution and stirred overnight. The acid and solvent were distilled off and the product was taken up in water. Evaporation of the water *in vacuo* gave pure vinylglycine trifluoroacetic acid salt in 94% yield (48 mg).

^{1}H NMR (200 MHz): δ = 4.6 (1H, d ,$^{3}J_{HH}$ = 6 Hz, H2), 5.6 (2H, m, H4), 6.1 (1H, m, H3) ppm.

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4

Synthesis of ε-^{13}C,ε-^{15}N enriched L-lysine. Establishing schemes for the preparation of all possible ^{13}C and ^{15}N isotopomers of L-lysine, L-ornithine and L-proline.

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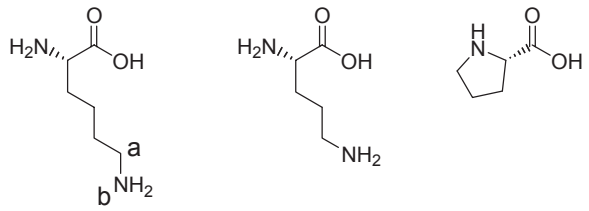
4.1 INTRODUCTION

Rhodopsin is the photosensitive protein of the rod photoreceptor in the vertebrate retina, that mediates dim light vision. Rhodopsin represents a paradigm for the large and diverse family of G-protein coupled membrane receptors (GPCR's).[1] Lysine-296 in the active site of rhodopsin connects the 11-Z protonated Schiff base of retinal to the protein.[2] In order to study the role of this residue in the rhodopsin photoreceptor process with ^{1}H and ^{13}C solid state NMR techniques access to [ε-^{13}C,ε-^{15}N]-lysine (Figure 1, compound **1a**) was necessary. Lysine is also an essential amino acid in human (and animal) nutrition and access to the set of site-directed isotopomers of lysine will be essential to elucidate the metabolic role of this amino acid by mass-spectral techniques. In this chapter a modular strategy is described that is able to give access to any site-directed isotopically labelled L-lysine, L-ornithine and L-proline, with the use of commercially available stable isotopically enriched synthons or synthons that are available in any site-directed enriched form by strategies described before.[3,4] For the ^{1}H,^{13}C and ^{15}N MAS NMR study, next to ($^{13}C_{20}$)-11-(*Z*) retinal, (ε-^{15}N,ε-^{13}C)-lysine is also needed.[5] The one-step synthesis of the protected L-glutamate ester via the O' Donnell method[6] has been published for the natural abundance materials.[7] Moreover, schemes were worked out to prepare the required synthons in any site-directed isotopomeric form.[3,4] Glutamic acid has the complete carbon skeleton of other proteinogenic amino acids such as proline and glutamine. For the preparation of lysine only one additional carbon and nitrogen atom are needed. The basis of using glutamic acid as building block for the preparation of the aforementioned amino acids in each site-directed isotopically enriched form is the possibility of the selective conversion of the 5-carboxylic acid ester group into other functional groups. The protected glutamate obtained by the O' Donnell method has proved to be the perfect intermediate in this synthesis.



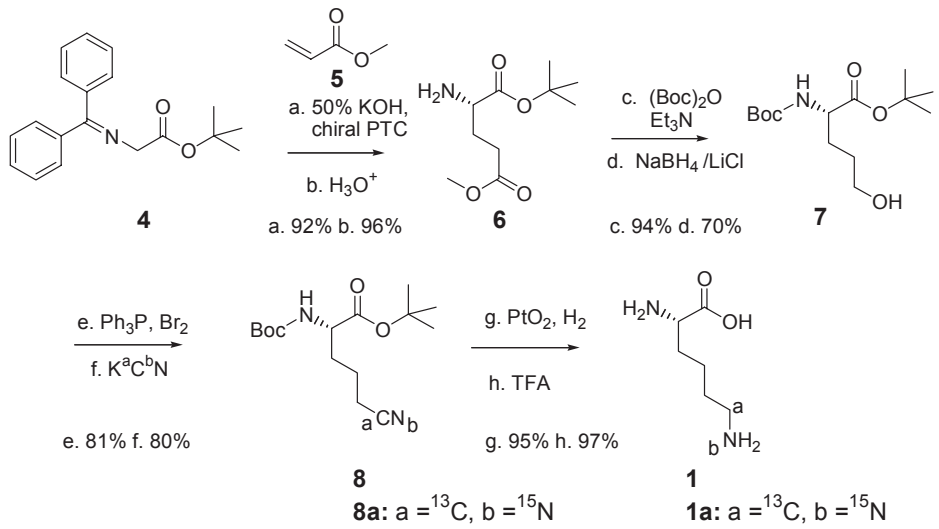
1 **2** **3**

1a: a = ^{13}C, b = ^{15}N

Figure 1: L-lysine (**1**), L-ornithine (**2**) and L-proline (**3**).

4.2 SYNTHETIC STRATEGY

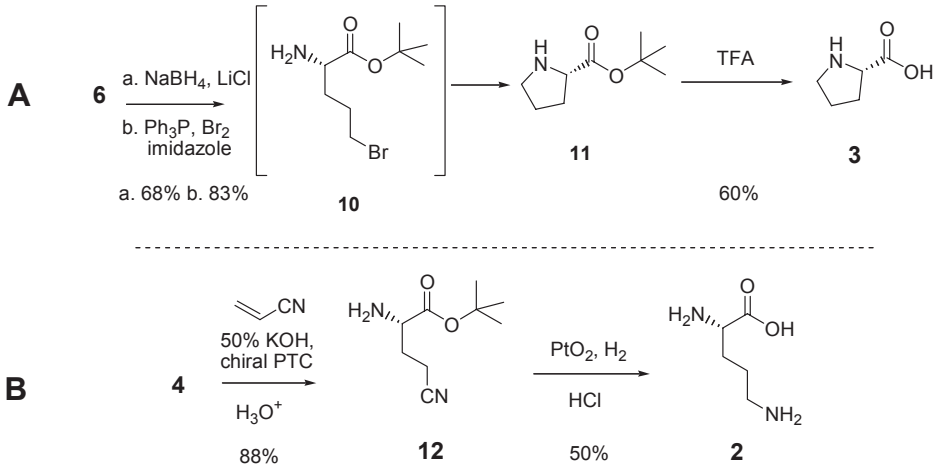
In Scheme 1, the strategy to prepare any site directed enriched isotopomer of L-lysine **1** is indicated. The first step is the O' Donnell coupling of the protected glycine derivative[3] **4** with methyl acrylate **5**, which can be prepared in any isotopomeric form, as has been previously published.[4] The product of the coupling gives after treatment with aqueous citric acid, the *tert*-butyl, methyl ester of glutamic acid **6**. The free amino group in **6** is subsequently converted into the Boc derivative in 85% yield. This fully protected glutamate is then treated with $NaBH_4$/LiCl in ethanol at 0˚C.[8] This reagent is a mild and selective reducing agent, capable of selectively reducing the methyl ester to the alcohol **7**, without concomitant reduction of the *tert*-butyl ester. Conversion of the alcohol function in **7** with triphenylphosphine and bromine in dichloromethane (DCM) gives the corresponding bromide in 81% yield. Subsequent treatment of the bromide with KCN in refluxing acetone gives the protected adiponitrile **8** in 80% yield. Catalytic reduction with Adam's catalyst (PtO_2) in acidified ethanol and subsequent deprotection with 10% trifluoroacetic acid leads to L-lysine **1** with all characteristics of the authentic material. Using $K^{13}C^{15}N$, **8a** can be prepared in 76% yield from the bromide. Reduction of the labelled nitrile and subsequent deprotection gave [ε-^{13}C,ε-^{15}N] L-Lysine in 91% yield. Introducing $K^{13}C^{15}N$ in the final steps gives [ε-^{13}C,ε-^{15}N]-lysine in 69% yield with regard to $K^{13}C^{15}N$. It is clear from Scheme 1 that L-lysine can be prepared in any isotopomeric form.



Scheme 1. The synthesis of L-lysine (**1**) starting from the achiral scaffold N-diphenylmethylene glycine *tert*-butyl ester (**4**).

Before the present synthetic scheme was developed, **1a** was prepared via the O' Donnell method using [4-^{13}C,4-^{15}N]-4-iodobutyronitrile. Labelled iodobutyronitrile was prepared by S_N2 substitution of the iodo-substituent of 3-chloro-1-iodopropane with K^{13}C^{15}N, followed by treatment of the resulting chlorobutyronitrile with KI in refluxing acetone. Based on this method the overall yield of **1a** from K^{13}C^{15}N is 37%. This method does allow access to the full set of isotopomers[9], but gives a lower yield with regard to the incorporated isotopes, hence the development of the synthesis presented in Scheme 1. In Scheme 1 the α-aminoadiponitrile derivative **8** is the intermediate that can be easily hydrolysed to α-aminoadipic acid.[10] α-Aminoadipic acid is an essential intermediate in the biosynthesis of L-lysine in micro-organisms[11] and access to the full set of isotopomers will be beneficial to metabolic research.

For the synthesis of proline **3**, a route using the internal alkylation of the α-amino group with a Cδ-Br leads quickly to the desired molecule (Scheme 2). The reduction of **6** with $NaBH_4$/LiCl to the amino alcohol is directly followed by treatment with the adduct of triphenylphosphine and bromine in dichloromethane in the presence of imidazole as proton scavenger. The resulting bromide **10** reacts intramolecularly with the amino group to form the proline *tert*-butyl ester. Removal of the ester group with 10% TFA in DCM gives L-proline (**3**). This scheme thus gives access to all isotopomers of L-proline (**3**) in a simpler and more convergent fashion than existing methods.[12]



Scheme 2. A - The synthesis of L-proline (**3**) starting from chiral *tert*-butyl methyl glutamate (**6**); **B** - the synthesis of L-ornithine (**2**) starting from achiral glycinate (**4**).

The simple catalytic reduction of adiponitrile **8** to form the protected lysine was a motivation to prepare L-ornithine in a similar way via reduction of glutaronitrile **12**. The protected glycinate **4** was treated under O' Donnell conditions with acrylonitrile, which can be prepared in all isotopomeric forms[13], to form the protected glutaronitrile derivative, which upon treatment with aqueous citric acid gives **12**. Catalytic reduction and acid-mediated deprotection leads to L-ornithine **2** with the analytical properties of the authentic material. Access to all stable carbon-13 and nitrogen-15 isotopomers of ornithine allows preparation of the isotopomers of other amino acids that share the same carbon skeleton. In this way, starting from ornithine, arginine and citrulline can easily be prepared in a few simple steps. Treatment of L-ornithine with cyanate anion gives citrulline.[14] Earlier, all isotopomers of cyanate and prepared site-directed ^{15}N and ^{13}C enriched urea derivatives were prepared from primary amines.[15] The conversion of L-ornithine into L-arginine with ^{13}C and ^{15}N isotopes has also been published.[16,17] The ability to prepare all ^{15}N and ^{13}C isotopomers of the three aforementioned amino acids ornithine, arginine and citrulline, will be a boon to the metabolomics studies of the vertebrate urea-cycle since all intermediates of this cycle can now be easily prepared in any desired isotopomeric form.

4.3 CONCLUSION

In this chapter the modular and convergent strategy to prepare L-lysine (**1**), L-ornithine (**2**) and L-proline (**3**) in optically pure form is described, such that all site-directed ^{13}C, and ^{15}N isotopomers can be easily prepared by the alkylation of a protected glycine derivative using Michael-type reactions with methyl acrylate and acrylonitrile in the presence of a chiral phase-transfer catalyst. After the reaction, the optically active catalyst can be easily isolated, ready to be reused. Via this method, no difficult separation of the product from an optically active scaffold is needed.[18] This scheme can easily be adapted to the synthesis of a whole class of new non-proteinogenic α-amino acids in high optical purity.

4.4 EXPERIMENTAL SECTION

General remarks: ^1H NMR spectra were recorded with a Jeol FX-200, a Bruker DPX-300 or a Bruker DPX 400 spectrometer, using tetramethyl silane (TMS: $\delta = 0$ ppm) or water (H_2O: $\delta =$ 4.8 ppm) as an internal standard. ^{13}C noise-decoupled NMR spectra were recorded with a Jeol FX-200 at 50.1 MHz a Bruker DPX-300 spectrometer at 75.5 MHz and a Bruker DPX-400 at 100.7 MHz, using $CDCl_3$ ($\delta = 77$ ppm), $(CD_3)_2CO$ ($\delta = 206$ ppm) or TSP (3-(trimethylsilyl)tetr

adeuteropropionic acid sodium salt, δ = 0 ppm) as internal standard. A saturated solution of ammonium nitrate was used as an external standard ($^{15}NH_4NO_3$ δ = 22.3 ppm relative to NH_3 (l) δ = 0.0 ppm) for the ^{15}N-NMR spectra. All spectra were recorded in $CDCl_3$, except where noted otherwise. Column chromatography was performed on Merck silica gel 60 (0.040-0.063 mm 230-400 mesh) and spots on thin-layer chromatography were detected with UV-light, $KMnO_4$-spraying, ninhydrin-staining (0.2% in ethanol) or staining with a mixture of 4,4′-methylenebis (*N*,*N*,dimethylaniline) and ninhydrin (TDM-staining). Chiral HPLC was performed on a Chiralcel ODH column (25 cm) using hexane and isopropanol (*i*PrOH) as solvent system. Dry diethyl ether (ether, Et_2O) was obtained by distilling from P_2O_5. Dry petroleum ether (PE) 40-60 and dry dichloromethane (DCM) were obtained by distilling from CaH_2. All commercially available chemicals were purchased from Sigma–Aldrich, Across or Fluka. All chemicals were used without further purification, unless stated otherwise. [^{13}C, ^{15}N]-potassium cyanide (> 99% isotope enriched) was purchased from Cambridge Isotope Laboratories.

_N_-(benzophenoneimine) of 1-*tert*-butyl 5-methyl glutamate

Methylacrylate **5** (1.98 mL, 22 mmol) dissolved in toluene (3 mL) was added dropwise to a solution of **4** (5 gr, 17 mmol) in toluene (15 mL) and a 50% KOH solution (1/3 volume of toluene, 6mL) with O-allyl N-9-anthracenyl methylcinchonidium bromide (0.05 equiv.) added as chiral phase transfer catalyst. The solution was stirred very vigorously for 14h. The mixture was then diluted with 10 mL water and extracted with dichloromethane (3x 20 mL). The collected organic layers were dried over $MgSO_4$ and evaporated. The product was further purified over a silica column (90/10 PE/Et_2O) to yield the product in 92% (5.97g, 15.6 mmol). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.48 (s, 9 H, *t*Bu), 2.23 (m, 2 H, 4-H), 2.41 (m, 2 H, 3-H), 3.56 (s, 3 H, OMe), 4.01 (dd, $^3J_{HH}$ = 7.28 Hz, $^3J_{HH}$ = 5.50 Hz, 1 H, 2-H), 7.14-7.66 (m, 10 H, arom) ppm.
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 27.77 (*t*Bu), 28.4 (C-4), 30.21 (C-3), 51.18 (OMe), 64.54 (C-2), 80.84 (*t*Bu), 127.52-139.19 (aromatic), 170.44 (C-1 + CN), 173.20 (C-5) ppm.

1-*tert*-Butyl 5-methyl glutamate 6

The alkylated protected glycinate (5.97g, 15.6 mmol) was dissolved in 10 mL THF and stirred with 20 mL of a 10% citric acid solution for one night. TLC showed complete disappearance of the starting material and the mixture was extracted twice with 25 mL ether. The water layer was then brought to pH 12 and extracted twice with 30 mL ethyl acetate to give after evaporation the product **6** in 96% yield (3.26 g).
^{1}H NMR (300 MHz): δ = 1.47 (s, 9 H, *t*Bu), 1.81 (m, 1 H, 3-H), 2.03 (m, 1 H, 3-H), 2.36 (dd, 2 H, 4-H), 3.34 (dd $^3J_{HH}$ = 5.3 Hz, $^3J_{HH}$ = 8.2 Hz, 1 H, 2-H), 3.68 (s, 3 H, OMe) ppm.
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 27.35 (*t*Bu), 29.60 (C-4) 29.80 (C-3), 50.90 (C-2), 53.65 (OMe), 80.44 (*t*Bu), 172.95 (C-1), 174.11 (C-5) ppm.

1-*tert*-Butyl 5-methyl *N*-boc-glutamate

The aminoester was subsequently dissolved in 10 mL DMF and 1 equiv. (2.1 mL) triethylamine was added followed by 1.1 equiv. di-*tert*-butyl dicarbonate (3.53 mL) and the mixture was stirred until TLC showed completion of the reaction. The DMF was evaporated and 25 mL ethylacetate was added. The organic layer was washed with 3*15 mL $KHSO_4$ solution (PH 2), water, brine and dried over $MgSO_4$. The raw product was purified over a short silica column (PE/Et_2O 80/20). The yield was 94% (4.48g, 14.1 mmol).

^{1}H NMR (300 MHz): δ = 1.44 (s, 9 H, *t*Bu), 1.47 (s, 9 H, *t*Bu), 1.91 (m, 1 H, 3-H), 2.16 (m, 1 H, 3-H), 2.34 (m, 2 H, 4-H), 3.68 (s, 3 H, OMe), 4.19 (m, 1 H, 2-H), 5.10 (d, $^3J_{HH}$ = 8.1 Hz, 1 H, N-H) ppm.

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 27.87 (*t*Bu), 27.96 (C-4) 28.20 (*t*Bu), 30.00 (C-3), 51.61 (OMe), 53.31 (C-2), 79.63 (*t*Bu), 82.04 (*t*Bu), 155.27 (NCO), 171.20 (C-1), 173.20 (C-5) ppm.

***tert*-Butyl *N*-Boc-2-amino-5-hydroxypentanoate 7**

To a suspension of LiCl (4 equiv., 311 mg) in 10 mL THF/EtOH 1/1 was added $NaBH_4$ (4 equiv., 278 mg) and the mixture was stirred for 10 minutes. After cooling to 0˚C a solution of the glutamate (1,8 mmol, 580 mg) dissolved in THF/EtOH (5mL) was added dropwise. The reaction mixture was stirred overnight and the temperature was allowed to rise to room temperature. Water was added (30 mL) and the reaction mixture was extracted thrice with 30 mL EtOAc. The collected organic layers were extracted with brine and dried over $MgSO_4$. Subsequently the solvents were evaporated and the raw product was purified using column chromatography (15% to 25% EtOAc in PE) to give **7** in 70% yield (361 mg, 1.26 mmol).

^{1}H NMR (300 MHz): δ = 1.47 (2*s, 18 H, 2**t*Bu), 1.54-1.95 (m, 4 H, 3-H, 4-H), 3.66 (m, 2 H, 5-H), 4.20 (m, 1 H, 2-H), 5.15 (d, $^3J_{HH}$ = 8.3 Hz, 1 H, NH) ppm.

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 27.88 (*t*Bu), 27.90 (C-4) 28.23 (*t*Bu), 29.53 (C-3), 53.55 (C-2), 61.95 (C-5), 79.63 (*t*Bu), 81.80 (*t*Bu), 155.88 (NCO), 171.88 (C-1) ppm.

***tert*-Butyl *N*-Boc-2-amino-5-cyanopentanoate 8**

Triphenylphosphine (0.66 g, 2 equiv.) was dissolved in 5 mL dry dichloromethane, the solution was cooled to 0°C and stirred under a dry nitrogen atmosphere while bromine (128 µL, 1.99 equiv.) dissolved in 2 mL DCM was added dropwise. After 20 minutes, a mixture of **7** (361 mg, 1.26 mmol) and imidazole (0.18 g, 2 equiv.) dissolved in 5 mL DCM was slowly added to the pale yellow solution. After one minute a white solid became visible. The reaction mixture was stirred for another two hours, while the temperature was maintained at 0°C. Subsequently, the solids were filtered off and the solvent was evaporated. The resulting product was redissolved in 10 mL DCM and flashed over a glass-filter with silica which was rinsed with 50 mL EtOAc to give the bromide in 81% yield (0.35 g, 1.0 mmol)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.43 (s, 9 H, *t*Bu), 1.47 (s, 9 H, *t*Bu), 1.78-199 (m, 4 H, 3-H,4-H), 3.41 (m, 2 H, 5-H), 4.18 (m, 1 H, 2-H), 5.31 (d, 1 H, NH) ppm.

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 28.0 (*t*Bu), 28.3 (*t*Bu), 30.3 (C-3), 31.6 (C-4), 33.0 (C-5), 53.2 (C-2), 79.8 (*t*Bu), 82.14 (*t*Bu), 155.3 (CO), 171.5 (C-1) ppm.

The bromide (0.35 g, 1.0 mmol) was dissolved in ethanol (30 mL) and after adding potassium cyanide (1.1 equiv., 74 mg) dissolved in ethanol/water 90/10 v/v (5 mL), the mixture was refluxed for 6 hours. The solution was allowed to cool to room temperature and concentrated to a quarter of its volume, after which the precipitate was filtered off and the solution was extracted with twice with dichloromethane (2*40 mL). Subsequently, the collected organic layers were washed with water (20 mL). The solution was dried over $MgSO_4$ and after removing the solvent *in vacuo* the raw product was purified by column chromatography (PE/Et_2O 85/15) to yield **8** in 80% yield (237 mg, 0.8 mmol).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.45 (s, 9 H, *t*Bu), 1.47 (s, 9 H, *t*Bu), 1.63 (m, 2 H, 4-H), 1.72 (m, 2 H, 3-H), 2.40 (m, 2 H, 5-H), 4.20 (m, 1 H, 2-H), 5.05 (m, 1 H, NH) ppm.

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 16.44 (C-5), 21.26 (C-4), 28.0 (*t*Bu), 28.3 (*t*Bu), 31.12 (C-3), 52.8 (C-2), 79.5 (*t*Bu), 82.0 (*t*Bu), 118.70 (C-6), 155.2 (CO), 171.5 (C-1) ppm.

***tert*-Butyl [5-^{13}C, 5-^{15}N]-*N*-boc-2-amino-5-cyanopentanoate 8a**

Prepared as described for **8**. Yield: 260 mg, 0.87 mmol **8a**. Starting from 0.36 g bromide (1.04 mmol) and 77 mg (1.1 equiv.) K^{13}C^{15}N. The yield is 76% in regard to the labelled KCN and 84% in regard to the bromide.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.45 (s, 9 H, *t*Bu), 1.47 (s, 9 H, *t*Bu), 1.63 (m, 2 H, 4-H), 1.72 (m, 2 H, 3-H), 2.40 (m, 2 H, 5-H), 4.20 (m, 1 H, 2-H), 5.05 (m, 1 H, NH) ppm.

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 16.44 (d, $^1J_{CC}$ = 56.6 Hz, C-5), 21.26 (d, $^2J_{CC}$ = 2.6 Hz, C-4), 28.0 (*t*Bu), 28.3 (*t*Bu), 31.12 (C-3), 52.8 (C-2), 79.5 (*t*Bu), 82.0 (*t*Bu), 118.70 (d, 1.6 Hz, $^1J_{CN}$ = 16.8 Hz, C-6), 155.2 (CO), 171.5 (C-1) ppm.

L-Lysine.TFA 1

The purified protected nitrile (237 mg, 0.8 mmol) was dissolved in isopropanol (30 mL) together with concentrated HCl (2 mL) and a catalytic amount of platinum oxide. Using a Parr-apparatus, the mixture was then shaken vigorously at 50 psi H_2 atmosphere for 16 hours. There after the mixture was filtered over celite to remove the platinum catalyst and concentrated *in vacuo* to give the free amine in 95% yield (226 mg, 0.76 mmol). The raw product was redissolved in a mixture of 10% trifluoroacetic acid in dry DCM (20 mL) and stirred overnight. The mixture was subsequently extracted 3 times with 15 mL water and the combined waterlayers were evaporated and the sample was lyophilized to give the Lysine.TFA salt **1** in 97% yield (190 mg, 0.73 mmol).

^{1}H-NMR (400 MHz, D_2O): δ = 1.55 (m, 2 H, 4-H), 1.75 (m, 2 H, 3-H), 1.99 (m, 2 H, 5-H), 3.2 (dt, $^3J_{HH}$ = 7.6 Hz, 2 H, 6-H), 3.7 (dd, $^3J_{HH}$ = 6.4 Hz, 1 H, 2-H) ppm.

^{13}C-NMR (100 MHz, D_2O): δ = 22.0 (C-4), 26.83 (C-5), 29.8 (C-3), 39.7 (C-6), 53.7 (C-2), 117.1 (q, CF_3), 163.6 (q, CO), 175.3 (C-1) ppm.

[ε-^{13}C,ε-^{15}N]-L-Lysine 1a

Prepared as described above. The yield was 91% (220 mg, 0.84 mmol) starting from **8a**.

^{1}H-NMR (400 MHz, D_2O): δ = 1.55 (m, 2 H, 4-H), 1.75 (m, 2 H, 3-H), 1.99 (m, 2 H, 5-H), 3.2 (dt, $^{3}J_{HH}$ = 7.6 Hz, $^{1}J_{CH}$ = 143 Hz, 2 H, 6-H), 3.7 (dd, $^{3}J_{HH}$ = 6.4 Hz, 1 H, 2-H) ppm.

^{13}C-NMR (100 MHz, D_2O): δ = 22.0 (s, C-4), 26.83 (d, $^{1}J_{CC}$ = 35.2 Hz, C-5), 29.8 (d, $^{3}J_{CC}$ = 5.3 Hz, C-3), 39.7 (d, $^{1}J_{CN}$ = 4.9 Hz, C-6), 53.7 (C-2), 175.3 (C-1) ppm.

[4-^{13}C,^{15}N]-1-Chloro-butyronitrile

A mixture of 1-bromo-3-chloropropane (22.0 g, 14.0mL, 140 mmol) and (^{13}C,^{15}N) potassium cyanide (2.39 g, 35.6 mmol) dissolved in ethanol/water 90/10 vv (100mL) was refluxed for 3h. The solution was allowed to cool to room temperature, the precipitate was filtered off and the solution was extracted with dichloromethane and washed with water (3x). After removing the solvent *in vacuo* the solution was further purified by vacuum distillation to remove the excess 1-bromo,3-chloropropane. The product (a yellow oil) was obtained in 76% yield (2.92 g, 27 mmol).

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 2.11 (ddt, $^{3}J_{HH}$ = 6.0 Hz, $^{3}J_{HH}$ = 7.0 Hz, $^{3}J_{CH}$ = 6.6 Hz, 2 H, 2-H), 2.57 (ddt, $^{3}J_{HH}$ = 7.0 Hz, $^{2}J_{CH}$ = 10 Hz, $^{3}J_{NH}$ = 1.6 Hz, 2 H, 3-H), 3.67 (t, $^{3}J_{HH}$ = 6.0 Hz, 2 H, 1-H), ppm.

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 14.59 (dd, $^{1}J_{CC}$ = 56.7 Hz, $^{2}J_{CN}$ = 3.0 Hz, C-3), 28.9 (d, $^{2}J_{CC}$ = 2.5Hz, C-2), 42.5 (d, $^{3}J_{CC}$ = 3.8 Hz, C-1), 118.4 (d, $^{1}J_{CN}$ = 16.9 Hz, C-1) ppm.

^{15}N-NMR (40 MHz, $CDCl_3$): δ = 248.6 (dt, $^{3}J_{NH}$ = 1.6 Hz, $^{1}J_{CN}$ = 16.8 Hz, ^{15}N) ppm.

[4-^{13}C, ^{15}N]-1-Iodobutyronitrile

1-chlorobutyronitrile (2.92 g, 27 mmol) and NaI (3 equiv.) were dissolved in warm acetone (150 mL) and refluxed for 24h, during which the mixture gradually became yellow. The mixture was allowed to cool to 20°C. After distilling off the solvent, the residue was diluted with water and extracted with dichloromethane. The organic layer was successively washed with 1 M Na_2SO_4 and dried with $MgSO_4$. The product was concentrated *in vacuo* and purified over a silica column (PE/Et_2O 90/10) to yield a colorless liquid (5.05 g, 19.4 mmol) in 94%.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 2.09 (dt, $^{3}J_{HH}$ = 7.0 Hz, $^{3}J_{HH}$ = 6.0 Hz, $^{3}J_{CH}$ = 6.6 Hz, 2 H, 2-H), 2.50 (ddt, $^{2}J_{CH}$ = 9.7 Hz, $^{3}J_{HH}$ = 6.8 Hz, $^{3}J_{NH}$ = 1.8 Hz, 2 H, 3-H), 3.22 (t, $^{3}J_{HH}$ = 6.4 Hz, 2 H, 1-H) ppm.

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 3.3 (d, $^{3}J_{CC}$ = 3.7 Hz, C-1), 17.8 (dd, $^{1}J_{CC}$ = 55.7 Hz, $^{2}J_{CN}$ = 3.0 Hz, C-3), 28.1 (d, $^{2}J_{CC}$ = 2.9 Hz, C-2) 117.8 (d, $^{1}J_{CN}$ = 16.9 Hz, C-4) ppm.

^{15}N-NMR (40 MHz, $CDCl_3$): δ = 248.7 (dt, $^{3}J_{NH}$ = 1.6 Hz, $^{1}J_{CN}$ = 16.8 Hz, ^{15}N) ppm.

***tert*-Butyl [5-^{13}C,5-^{15}N]-2-(Benzhydrylidene)amino-5-cyanopentanoate**

1-Iodobutyronitrile (5.05 g, 25.6 mmol) was dropwise added to a solution of *tert*. butyl N-(diphenylmethylene)-glycinate (1.1 equiv., 8.2 g, 28.1 mmol) in toluene (40 mL) and 50% KOH (1/3 volume of toluene) in the presence of O-allyl N-9-antracenylmethylcinchonidium bromide (0.1equiv.) as a phase transfer catalyst. The solution was stirred very vigorously for

12h. The mixture was then diluted with water and extracted with dichloromethane (3x20 mL) and dried over $MgSO_4$. The product was further purified over a silica column (PE/Et_2O 90/10) to yield a mixture of unreacted 4-iodonitrile (which could be recovered en reused) and product in 50% yield (4.6 g, 12.8 mmol).

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.44 (s, 9 H, *t*Bu), 1.65 (m, 2 H, 4-H), 1.98 (m, 2 H, 3-H), 2.31 (m, 2 H, 5-H), 3.94 (dd, $^3J_{HH}$ = 5.0 Hz, $^3J_{HH}$ = 7.1 Hz, 1 H, 2-H), 7.15–7.68 (m, 10 H, arom.) ppm.

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ = 16.8 (dd, $^1J_{CC}$ = 55.98 Hz, $^2J_{CN}$ = 2.8 Hz, C-5), 21.9 (d, $^2J_{CC}$ = 2.46 Hz, C-4), 27.81 (*t*Bu), 32.2 (d, $^3J_{CC}$ = 3.68 Hz, C-3), 64.7 (C-2), 81.09 (*t*Bu), 117-139 (arom) 119.4 (d, $^1J_{CN}$ = 16.9 Hz, C-6), 170.44 (CN), 178.10 (C-1) ppm.

[ε-^{13}C, ε-^{15}N]-*tert*-Butyl 2-amino-5-cyanopentanoate

The protected amino acid (4.6 g, 12.8 mmol) and unreacted 4-iodobutyronitril were dissolved in THF (50 mL) and diluted with a 15% citric acid solution (20equiv.). After stirring for 3 hours the mixture was diluted with water and extracted with ether to remove the benzophenone and the unreacted iodonitrile. The water phase was then neutralised with K_2CO_3 (pH 11-12) and extracted with ethyl acetate (3x 20mL). The combined organic phases were dried over $MgSO_4$ and concentrated *in vacuo*. The mixture was further purified over a silica column (PE/EtOAc 85/15) to obtain the product in 96% yield (2.8 g, 12.2mmol).

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.47 (s, 9 H, *t*Bu), 1.64 (m, 1 H, 3-H), 1.8 (m, 3 H, 3-H + 4-H), 2.4 (m, 2 H, 5-H), 3.32 (m, 1 H, H-2) ppm.

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 16.8 (dd, $^1J_{CC}$ = 56.02 Hz, $^2J_{CN}$ = 3.0 Hz, C-5), 21.76 (d, $^2J_{CC}$ = 2.7 Hz, C-4), 27.75 (*t*Bu), 33.4 (d, $^3J_{CC}$ = 1.6 Hz, C-3), 81.14 (*t*Bu), 119.27 (d, $^1J_{CN}$ 16.9 Hz, C-6), 174.5 (s, CO) ppm.

^{15}N-NMR (40 MHz, $CDCl_3$): δ 246 (dt, $^1J_{CN}$ = 16.93 Hz, $^3J_{HN}$ = 1.61 Hz, ^{15}N) ppm.

[ε-^{13}C, ε-^{15}N]-L-lysine *tert*-butyl ester

The purified nitrile (2.80 g, 12.3 mmol) was dissolved in isopropanol (50 mL) together with concentrated HCl (3 mL) and a catalytic amount of platinum oxide. The mixture was then stirred vigorously under 50 psi H_2 atmosphere for 16 hours using a Parr-aparatus. Thereafter the mixture was filtered over celite to remove the platinum catalyst, which was rinsed with 25 mL isopropanol and concentrated *in vacuo* to give the amine in 98% yield (2.4 g, 12 mmol).

^{1}H-NMR (400 MHz, D_2O): δ = 1.40 (s, 9 H, *t*Bu), 1.45 (m, 2 H, 4-H), 1.65 (m, 2 H, 5-H), 1.85 (m, 2 H, 5-H), 2.9 (dt, $^3J_{HH}$ 7.5 Hz, $^1J_{CH}$ 143 Hz, 2 H, 6-H), 4.0 (m, 1 H, 2-H) ppm.

^{13}C-NMR (100 MHz, D_2O): δ = 22.16 (s, C-4), 27.04 (d, $^1J_{CC}$ 35.9 Hz, C-5), 27.80 (*t*Bu), 30.5 (d, $^3J_{CC}$ = 5.3 Hz, C-3), 39.7 (d, $^1J_{CN}$ = 4.9 Hz, C-6), 53.6 (s, C-2), 82.52 (*t*Bu), 173.0 (C-1) ppm.

[ε-^{13}C, ε-^{15}N]-L-lysine.HCl (1a)

L-lysine *tert*-butyl ester was dissolved in 100 mL 6M HCl. The mixture was then refluxed for 24h. After removing the solvent *in vacuo*, the yellow residue was taken up in water (100 mL)

and treated with a small amount of charcoal (Norit) and refluxed for 2 hours to decolorize the solution. After filtering off the charcoal the solution was concentrated *in vacuo*. recrystallyzation from water/ethanol yielded L-lysine monohydrochloride as a white hygroscopic powder in 90% yield (1.95 g, 10.8 mmol, (37% yield based on ^{13}C,^{15}N KCN)). $[\alpha]_D^{23}$ = + 24.8 (c = 2, 6N HCl), lit. $[\alpha]_D^{20}$ = +25.2 (c = 2, 6N HCl).

^{1}H-NMR (400 MHz, D_2O): δ = 1.55 (m, 2 H, 4-H), 1.75 (m, 2 H, 3-H), 1.99 (m, 2 H, 5-H), 3.2 (dt, $^3J_{HH}$ = 7.6 Hz, $^2J_{CH}$ = 143 Hz, 2 H, 6-H), 3.7 (dd, $^3J_{HH}$ = 6.4 Hz, 1 H, 2-H) ppm.

^{13}C-NMR (100 MHz, D_2O): δ = 22.0 (s, C-4), 26.83 (d, $^1J_{CC}$ = 35.2 Hz, C-5), 29.8 (d, $^3J_{CC}$ = 5.3 Hz, C-3), 39.7 (d, $^1J_{CN}$ = 4.9 Hz, C-6), 53.7 (C-2), 175.3 (C-1) ppm.

tert-Butyl 2-(benzhydrylidene)amino-4-cyanobutanoate

acrylonitrile **5** (116 µL, 1.77 mmol) dissolved in 2 mL toluene was added dropwise to a mixture of N-(diphenylmethylene) *tert*-butyl glycinate (400 mg, 1.36 mmol) in toluene (4 mL) and 50% KOH (2 mL) with O-allyl N-9-anthracenyl methylcinchonidium bromide (41 mg, 0.05 equiv.) added as chiral phase transfer catalyst. The solution was stirred very vigorously for 14h. The mixture was then diluted with 10 mL water and extracted with dichloromethane (3x 10 mL). The collected organic layers were dried over $MgSO_4$ and evaporated. The product was further purified over a silica column (PE/ether 90/10) to yield the product in 92% (434 mg, 1.25 mmol). e.e. = 91 % (chiralcel ODH, 25 cm, hexane/*i*PrOH 99.9/0.1 v/v, flow 0.8 mL/min. retention time (min.): 19.05 (R), 27.35 (S))

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.43 (s, 9 H, tBu), 2.13-2.37 (m, 2 H, 3-H), 2.44-2.54 (m, 2 H, 4-H), 4.05 (dd, $^3J_{HH}$ = 7.55 Hz, $^3J_{HH}$ = 4.80 Hz, 1H, 2-H), 7.17–7.68 (m, 10 H, aromatic) ppm.

^{13}C-NMR (50.1 MHz, $CDCl_3$): δ 13.57 (C-3), 27.96 (*t*Bu), 29.39 (C-4), 63.69 (C-2) 81.76 (*t*Bu), 119.32 (C-5), 127.59-135.99 (arom), 158.10 (CN), 169.76 (C-1) ppm.

Proline _tert_-butyl ester 11

To a suspension of LiCl (4 equiv., 483 mg) in 10 mL THF/EtOH 1/1 was added $NaBH_4$ (4 equiv., 430 mg) and the mixture was stirred for 10 minutes. After cooling to 0°C a solution of **6** (2,7 mmol, 600 mg) dissolved in THF/EtOH (5mL) was added dropwise. The reaction mixture was stirred overnight and the temperature was allowed to rise to room temperature. a KOH solution (pH 13) was added (30 mL) and the reaction mixture was extracted thrice with 30 mL EtOAc. The collected organic layers were dried over $MgSO_4$. Subsequently the solvents were evaporated and the raw product was purified using column chromatography (15% to 35% EtOAc in PE) to give the product in 68% yield (340 mg, 1.9 mmol).

Triphenylphosphine (1.0 g, 2 equiv.) was dissolved in 5 mL dry dichloromethane, the solution was cooled to 0°C and stirred under a dry nitrogen atmosphere while bromine (193 µL, 1.99 equiv.) dissolved in 2 mL DCM was added dropwise. After 20 minutes, a mixture of the alcohol (340 mg, 1.9 mmol) and imidazole (0.27 g, 2 equiv.) dissolved in 5 mL DCM was slowly added to the pale yellow solution. After one minute a white solid became visible. The reaction

mixture was stirred for another three hours, while the temperature was maintained at 0°C. Subsequently, an 0.1 M KOH solution (10 mL) and 10 mL DCM were added, the organic layer was separated and the solvent was evaporated. The resulting product was redissolved in 10 mL DCM and flashed over a glass-filter with silica which was rinsed with 50 mL EtOAc to give the proline *tert*-butyl ester in 83% yield (0.52 g, 1.57 mmol)

^{1}H-NMR (200 MHz, D_2O/DCl): δ = 1.50 (s, 9 H, *t*Bu), 2.12 (m, 3 H, 3-H, 4-H), 2.43 (m, 1 H, 4-H), 3.42 (m, 2 H, 5-H), 4.35 (m, 1 H, H-2) ppm.

L-Proline.HCl 3

11 was redissolved in 10 mL 6N HCl and refluxed overnight. The mixture was subsequently concentrated *in vacuo*, treated with DEAE Sephadex (acetate form), concentrated and transferred on a Dowex W x 8 (H^+-form) column. Hydrochloric acid was first eluted with water and the amino acid subsequently with 2N ammonia. The eluate was evaporated to dryness, dissolved in water and the solution was adjusted to pH 3. Ethanol was then added until crystallization started. After one night at -20°C the crystals were collected to give 142 mg proline.HCl with all the characteristics of an authentic sample (60%, 0.9 mmol). $[\alpha]_D^{23}$ = -83.7 (c = 4, H_2O), lit. (L-proline) $[\alpha]_D^{20}$ = -84 (c = 4, H_2O).

^{1}H-NMR (300 MHz, D_2O): δ = 2.13 (m, 3 H, 3-H, 4-H), 2.44 (m, 1 H, 3-H), 3.40 (m, 2 H, 5-H), 4.42 (dd, 1 H, 2-H) ppm.

^{13}C-NMR (75.5 MHz, D_2O): δ = 24.1 (C-4), 29.1 (C-3), 47.0 (C-5), 60.4 (C-2), 172.7 (C-1) ppm.

***tert*-Butyl 2-amino-4-cyanobutanoate 12**

The protected amino acid (434 g, 1.25 mmol) was dissolved in THF (10 mL) and a 15% citric acid solution (5 mL) was added. After stirring the mixture for 3 hours it was diluted with 10 mL water and extracted twice with 25 mL ether. The water phase was then neutralised with K_2CO_3 to pH 12-13 and extracted with ethyl acetate (3x 20mL). The combined organic phases were dried over $MgSO_4$ and concentrated *in vacuo*. The mixture was further purified over a silica column (PE:ethylacetate / 85:15) to obtain the product in 96% yield (221 mg, 1.20 mmol).

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.47 (s, 9 H, *t*Bu), 1.82 (m, 1 H, 3-H), 2.09 (m, 1 H, 3-H), 2.54 (m, 2 H, 4-H), 3.39 (m, 1 H, 2-H) ppm.

^{13}C-NMR (50.1 MHz, D_2O) δ = 14.19 (C-4), 28.02 (C-3), 54.03 (C-2), 84.04 (*t*Bu), 121.43 (C5), 175.34 (C1) ppm.

L-ornithine.HCl

The purified protected nitrile (1.0 g, 5.3 mmol) was dissolved in acetic acid (10 mL) and platinum oxide (300 mg) was added. The mixture was then shaken vigorously under H_2 atmosphere for 16 hours. Thereafter the mixture was filtered to remove the platinum catalyst and concentrated *in vacuo*. The raw product was redissolved in 10 mL 6N HCl and refluxed overnight. The mixture was subsequently concentrated *in vacuo*, treated with DEAE Sephadex (acetate form),

concentrated and transferred on a Dowex W x 8 (H^+-form) column. Hydrochloric acid was first eluted with water and the amino acid subsequently with 2N ammonia. The eluate was evaporated to dryness, dissolved in water and the solution was adjusted to pH 3. Ethanol was then added until crystallization started. After one night at -20˚C the crystals were collected to give 450 mg ornithine.HCl with all the characteristics of an authentic sample (50%, 2.7 mmol).
$[\alpha]_D^{20}$ = +20.5 (c = 1, 6N HCl), ref. $[\alpha]_D^{20}$ = + 22.0 (c = 4, 6N HCl).
NMR (50.1 MHz, D_2O): δ = 23.32 (C-4), 27.96 (C-3), 39.44 (C-5), 54.64 (C-2), 174.58 (C-1) ppm.
FTIR (neat, fingerprint region): 350.2, 397.8, 458.4, 555.7, 669.9, 757.7, 780.6, 845.6, 878.6, 935.1, 981.9, 1039.7, 1098.9, 1131.0, 1146.1, 1243.3, 1287.1, 1329.4, 1345.9, 1363.4, 1419.8, 1438.3, 1474.4 cm^{-1}

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5

General discussion and prospects

5.1 GENERAL DISCUSSION

In this thesis the synthetic schemes are presented that give access to any isotopomer of the 22 proteinogenic α-amino acids. After incorporation into proteins, the labelled amino acids can be used as probes to study biological processes at the atomic level with isotope-sensitive techniques such as (solid state) NMR, ESR, FT-IR and resonance Raman spectroscopy. Moreover, the access to site-directed isotopomers of amino acids will allow a fundamental study of the metabolism of these compounds with mass-sensitive techniques. In this thesis modular synthetic schemes have been worked out that give access to any ^{13}C and ^{15}N isotopomer of the L-enantiomers of valine, leucine, isoleucine, methionine, cysteine, serine, selenomethionine, selenocysteine, lysine, proline and ornithine. Together with earlier work, 20 of the 22 proteinogenic amino acids can now be obtained in any site-directed (^{13}C,^{15}N) isotopically labelled form. Only for L-arginine and L-pyrrolysine this step has not been reached. However, in the case of arginine, the synthetic method that has been described in this thesis for any isotopomer of ornithine can be extended to give easy access to any isotopomeric form of L-arginine.[1] For the case of the recently discovered pyrrolysine there are still uncertainties about the chemical structure.[2] If the structure turns out to be the one depicted in the appendix to Chapter 1, the current scheme for the preparation of L-lysine combined with a modified scheme for L-proline, in which the protected glycinate would be treated with crotonaldehyde, after which deprotection of the amino moiety would lead to ring closure, might allow a modular preparation of any isotopomer of L-pyrrolysine.

The schemes presented in this thesis for most of the site-directed isotopically labelled amino acids are based on the O' Donnell method in which a protected glycine derivative is coupled with a primary iodide or reacted with an acrylic ester or acrylonitrile in the presence of a chiral phase transfer catalyst.[3,4] In this manner two achiral synthons form the carbon skeleton of the amino acid in question, while the newly formed chiral centre of the L-α-amino acid derivative is formed in a high enantiomeric excess (e.e.) in one step. Also, the corresponding D-α-amino acids are accessible via the other enantiomer of the chiral phase transfer catalyst. The greatest advantage of the O' Donnell method is the possibility it offers to develop modular synthetic schemes. Other advantages are the fact that no isotopes have to be incorporated into a temporary chiral scaffold, as in the case of the Schöllkopf and Williams templates, where the glycine moiety is covalently bound to a chiral inductor that has to be removed at a later stage and that cannot be reused, unlike the O' Donnell catalyst which can be easily recovered and reused. Other, more practical, advantages are the relatively mild reaction conditions and the simple purification and isolation of the

product. A disadvantage of this system is that secondary alkyl halides react only with great difficulty, making this method unsuitable for the modular synthesis of valine and isoleucine.

The aliphatic amino acids valine and leucine contain two prochiral methyl groups, while isoleucine, like threonine, has two chiral centres. A synthetic scheme leading to selective isotope introduction in the aliphatic amino acids should therefore allow distinction between the two prochiral methyl groups of valine and leucine and in the case of isoleucine yield an enantiomerically pure product with two well-defined chiral centres. When prochiral methyl groups have a different isotope composition a new chiral centre is introduced that, without special precautions, will lead to generation of diastereomeric mixtures. Both valine and isoleucine have been prepared via a chemo-enzymatic route in which the enzyme β-methyl aspartase was used to introduce two chiral centres in one step. For valine and isoleucine, methyl- and ethylfumaric acid respectively were converted to (2*S*,3*R*) methyl- and ethylaspartic acid. The achiral methyl- and ethylfumaric acid were prepared using Wittig chemistry followed by rearrangement of the *exo* double bond to form the fumaric acid derivatives. The γ-carboxylic acid groups of the aspartic acid derivatives yielded by β-methyl aspartase were then in a few steps selectively reduced to give the desired amino acids in good yield and high e.e. To show that this scheme is indeed highly specific in distinguishing between the prochiral methyl groups of L-valine, [4-^{13}C]-(2*S*,3*S*)-valine was prepared with excellent selectively. For the full set of isotopomers of L-leucine enzymatic schemes are not available. For the case of L-leucine the O' Donnell method could be used by reacting 2-methylpropyl iodide with the protected glycine synthon. Using an Evans template (2*S*)-[3-^{13}C]-methylpropyl iodide has been prepared in good optical purity as described in Chapter 2.

Using the method described in this thesis it is also possible to synthesise the chalcogen containing amino acids cysteine, methionine and their selenium containing counterparts in high yield and high e.e. in such a way that all possible ^{13}C and ^{15}N isotopomers can be obtained. Moreover, this method also led to a more efficient synthetic route for all isotopomers of L-serine. Besides these coded amino acids, we were also able to prepare the important metabolic intermediates L-homocysteine and L-homoserine in high yield with the possibility of introducing a carbon or nitrogen label at any position or combination of positions. Further extension of the same modular strategy made it possible to prepare efficient schemes with which all isotopomers of L-lysine, L-proline and L-ornithine can be synthesised. Using this scheme [ε-^{15}N,ε-^{13}C]-labelled L-lysine was prepared starting from K^{13}C^{15}N in high yield and high e.e.

A recent patent by DSM details another way of preparing a protected glycine moiety in high yields starting from a glyoxalic acid ester and an amine.[5] The resulting Schiff-base has the C=N bond towards the Cα (Figure 1), but functions as the glycinate used in the O' Donnell reaction as described in chapters 2-4. This scheme might provide a cheaper and more efficient way to prepare all isotopomers of the protected glycine ester. It could also provide a more efficient manner of introducing a nitrogen-15 label in the glycine derivative by performing a reductive amination reaction on acetophenone in the presence of ^{15}N-labeled ammonium formate. Schemes for the preparation of any ^{13}C-label in glyoxalate esters have been devised in our group before.[6]

Recently, Belokon *et al.* have published a paper in which the synthesis of α-amino acids via alkylation of achiral nickel-(II) complexes of glycine derived Schiff bases via asymmetric phase transfer catalysis has been reported.[7] In this way, L-valine could be prepared in a high yield (95% after one hour), efficient one step synthesis from 2-iodopropane (Figure 2). In this method glycine can be used directly to form the protected derivative that is used in the chiral alkylation reaction, without previous or further protection steps. Presumably this method will also allow the synthesis of α-amino acids with other sterically demanding secondary alkyl halides. 2-Iodopropane and 2-iodobutane are accessible in any isotopically labelled form. In this way isotope enriched valine and isoleucine may also be prepared in high yield using modular and convergent schemes. A recent improvement of the synthesis of the protected glycinate has been reported, which allows application of the reaction on a >100 gram scale.[8]

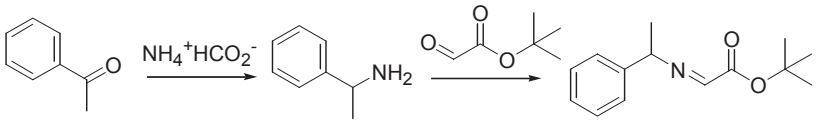

Figure 1. An alternative synthesis of a protected glycine derivative in the O' Donnell method for the access to L-α-amino acids.

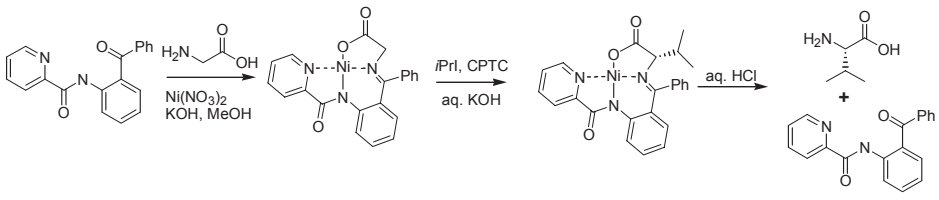

Figure 2. The modular synthesis of L-valine using the nickel-(II) based glycine synthon developed by Belokon *et al.*

In conclusion, there are no essential restrictions to the access of any stable site-directed isotopomer of the proteinogenic amino acids. This means that, in principle, the proteins that are formed by the translational processes are now accessible via heterologous expression techniques to specific label incorporation with the restriction that, as yet, one type of amino acid residue at any position in the peptide chain will have identical site-directed isotope incorporation.
Another drawback of heterologous expression in isotope-enriched growth media is that except for essential amino acids sufficiently high concentrations of isotopically labelled L-α-amino acids have to be used in order to prevent isotope dilution via *de novo* biosynthesis. Especially amino acids that are involved in amino transferase catalysed metabolism will lead to isotope scrambling and isotope dilution. The use of cell-free protein synthesis methods can alleviate the problems of isotope label scrambling and dilution, but this technique still labels all residues of one type of amino acid.[9] Adaptation of the cell-free synthesis technique with the amber mutation/amber suppressor tRNA method, using tRNA preloaded with a site-directedly isotope enriched amino acid, might lead to isotope labeled proteins, specifically enriched at one position in the protein.[9] However, especially for membrane proteins the site-directed incorporation of (isotopically labelled) amino acids is far from trivial and there is as yet no generally effective method for site-directed isotope incorporation in proteins.

Earlier in our group, access to any isotopomer of the DNA-building blocks A,C and T has been reached.[10] With minor adjustments based on the synthesis of the adenine base as described in literature,[11] this can also be reached for the fourth base G. For the synthesis of hexoses, schemes have been published[12] which can be adapted to schemes which will give access to any stable isotopomer of hexose carbohydrates. It is to be expected that this strategy can be extended to any monosaccharide system. It is gratifying to realise that for three groups of important biopolymers, namely proteins, DNA systems and polysaccharides for each system the building blocks are now accessible in any site-directed isotopomeric labelled form.

5.2 PROSPECTS

During the work described in this thesis, intermediates have been prepared via methods that allow site-directed stable isotope incorporation. With a few well known chemical steps these intermediates can be converted to any isotopomer of Ala, Asp, Asn, Glu and Gln. For His, Trp, Phe and Tyr, methods have been worked out earlier to give access to the whole set of isotopomers. Since the scheme for His was based

on the Schöllkopf method, there should be no difficulty in converting this scheme to one based on the O' Donnell method. For the preparation of isotopically labelled aromatic residues of Trp, Phe and Tyr no modular synthetic method is yet available to prepare any of their isotopomers. As soon as such synthetic schemes have been devised for the aromatic ring systems, schemes based on the O' Donnell method and giving access to all isotopomers of Trp, Phe and Tyr will likely be easy to develop, as information from preliminary experiments and literature shows that benzylic electrophiles react in high yield and enantiomeric excess.[13]

With the availability of the full set of isotopomers of the proteinogenic amino acids, all peptides composed of these amino acids can be labelled at any position or combination of positions. This means that receptor-ligand interactions in which the ligand is a peptide can now be mapped in exquisite detail. Of special interest are the interactions between aliphatic and aromatic residues, which recently have also been found in the rhodopsin system[14,15] and which now also appear to play an important role in enantiomeric resolution.[16] The total synthesis of site-directed isotopomers of the hexadecapeptaibol Zervamicin IIB, shows that there are in principle no limits to the total synthesis of site-directed isotopically labelled peptides.[17]

A decade ago, the syntheses of proteins by the so called native chemical ligation method without protecting groups have been described.[18,19] Very recently, D. Bang and S.B.H. Kent have described a one-pot total synthesis of crambin using native chemical ligation techniques.[20] Crambin is a low-molecular weight, hydrophobic protein with three disulphur bonds, a α-helix, a β-sheet and a reverse turn. The authors report that they have prepared 42 mg pure crambin in 40% overall yield using a three peptide segments one-pot synthesis with only one purification step. The synthesis took place under denaturing conditions and after purification and renaturing, crambin was obtained with all characteristics of the native material. The optimised crambin synthesis took only 48 hours total reaction time. In 2003 the total chemical synthesis of a functional 166 amino acid protein H-Ras based on the ligation method has been published.[21] Together these experiments show the scope the native ligation method already possesses. For the selective incorporation of isotope labels in proteins, these are very promising recent breakthroughs. The advantage of the native ligation method is that any particular amino acid can be selectively introduced isotopically enriched, at any particular position in the protein and not as part of the whole set of that amino acid in the protein. Further development of these techniques might allow their application to the total organic synthesis of membrane-bound proteins, which often need a lipophilic environment for a correct folding. However, already in 1987 Khorana *et al*. were able to correctly refold the denatured membrane protein bacteriorhodopsin.[22] Therefore, total chemical synthesis

of functional proteins seems the most direct and practical method to incorporate isotope labels selectively in proteins. Most of the cell's receptor proteins are membrane bound and the study of cellular signal transduction processes and the interaction of drugs with their receptor would be greatly benefited by methods to selectively introduce isotope labels in these important research targets. The combination of selective labelling schemes described in this thesis and the possible selective introduction of a specific isotope labelled amino acid in a protein allows complete control over the isotope labelling patterns in proteins. This will greatly facilitate measurement of intra-protein distances, torsion, bond angles and aliphatic-aromatic interactions. Thus, organic synthesis will be a pre-eminent technique in the process of translating (human) genomic information into spectroscopic information which will result in structural and functional biological information at the atomic level of the protein coded by the genome.

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SUMMARY

Amino acids are often called the building blocks of life to emphasise their biological importance. For humans 8 of the 22 amino acids encoded by DNA are called essential, as we are unable to synthesise them ourselves and need to obtain them from nutrients. Amino acids are biomolecules that occupy essential roles in many biological processes and structures. They are involved as signal transmitter in the brain, energy source, building block for hormones and sweeteners (as part of peptides) and linked together in large numbers they constitute proteins, which have structural, transport, enzymatic and signalling functions amongst others. Due to their central role in life amino acids are an important target and tool for researching protein structure and function and for researching human (and animal) metabolism and catabolism and how these processes related to human (and animal) disease. The targets mentioned can very well be investigated using stable isotopes. Isotopes combine the same chemistry with different physical properties and the incorporation of isotopes in relevant proteins allows the investigation of the system using non-invasive, non-destructive spectroscopic techniques such as FTIR and NMR. Furthermore, because isotopes differ in mass, mass-sensitive techniques can be used to investigate metabolic routes by tracing isotopes through different intermediates in the route. To carry out these investigations it is necessary to have access to the full set of isotopomers of the DNA-coded amino acids. The L-α-amino acids all contain two identical structure elements, the amino and carboxylic acid moieties from which they derive their name, and all are based on the simplest amino acid, glycine, to which different side chains are attached. Moreover, all amino acids except glycine are chiral and isoleucine and threonine have two chiral centres. A general synthetic approach to obtain isotopically labelled amino acids should therefore be modular and convergent by starting from a glycine-containing synthon to which the different side-chains can be coupled with the right enantioselectivity. The O' Donnell chiral alkylation method was chosen to accomplish this general scheme. The method is based on a chiral cinchonidinium-based phase transfer catalyst which transports base from an aqueous layer to a protected glycinate in an organic layer, so that the the glycinate is deprotonated. The formed enolate-anion forms a tight ion-pair with the chiral phase transfer catalyst and the bulky side groups of the catalyst allow the subsequent reaction of the enolate with an electrophile that is also present in the organic layer to take place on only on side of the enolate (the *Si*-face). The newly formed protected amino acid is then hydrolysed to give the desired amino acid in high yield and enantiomeric excess. In this manner one general method can be used to obtain the labelled L-α-amino acids provided that schemes can be developed to selectively incorporate isotope labels in the different side-chains and in the protected glycinate.

In Chapter 2 a chemo-enzymatic method has been developed that gives access to any isotopomer of the essential amino acids isoleucine and valine. The method gives the correct introduction of the second chiral centre in (2*S*,3*S*)-isoleucine and allows for discrimination between the two prochiral methyl groups in valine as shown by the preparation of [4-^{13}C]-(2*S*,3*S*)-valine. For the preparation of (2*S*)-leucine in any isotopomeric form, the O'Donnell method has been used to prepare optically active amino acids. The protected glycine scaffold that is used in this method has been prepared via a strategy that allows access to any isotopomeric form. The preparation of [5-^{13}C]-(2*S*,4*R*)-leucine shows that the O'Donnell method in combination with the Evans method to obtain chiral 2-methylpropyl iodide leads to a good discrimination between the two prochiral methyl groups. This strategy for the preparation of α-amino acids has preference over other methods since the reaction conditions are mild, the chiral auxiliary can be easily recovered and the optically active product, which is obtained in high enantiomeric excess, can be easily separated. For the preparation of isotopically enriched valine and isoleucine the O' Donnell method is not suitable due to the fact that the alkyl substituents involved have a secondary halide which is too sterically hindered to give an effective reaction with the protected glycine.

In Chapter 3 simple modular reaction schemes have been worked out that allow access to any isotopomer of serine and homoserine. Via Mitsunobu chemistry, these systems could simply be converted into cysteine, selenocysteine, homocysteine, homoselenocysteine, the essential amino acids methionine and selenomethionine. These sulphur and selenium containing amino acids fulfil many essential roles in the living organism. In addition homoserine could be converted via a few steps into optically active L-vinylglycine. Beside the introduction of the stable isotopes ^{13}C, ^{15}N, ^{17}O and ^{18}O, also the radioactive isotopes of sulphur, selenium can be easily and site-directedly introduced. In view of the large scope of the Mitsunobu reaction, it is felt that many more important systems with the carbon skeleton of serine and homoserine can be prepared via this method.

Chapter 4 describes a simple synthetic strategy, based on the O' Donnell method, that with the correct rational adaptation, gives direct access to any ^{13}C/^{15}N isotopomer of L-glutamate, L-ornithine, L-proline, L-lysine and L-α amino adipic acid. This strategy also allows access to non-proteinogenic amino acids such as L-citrulline in high yield and optical purity.

Chapter 5 contains the general discussion and future prospects. Methods are described to gain access to all isotopomers of amino acids for which no labelling schemes currently exist and the O' Donnell method is compared with earlier methods for labelling amino acids. Recent developments in the synthesis of a new glycine-based starting materials are discussed as well as the possibilities they offer for the modular synthesis of the amino acids valine and isoleucine. The section on

prospects describes how recent advances in protein synthesis through total synthetic methods using native ligation techniques, combined with access to the full set of labelled amino acids, can give full control over label incorporation in proteins and how this in turn will allow the investigation of numerous biological systems using isotope sensitive techniques.

SAMENVATTING

Aminozuren worden vaak de bouwstenen van het leven genoemd om hun belangrijke biologische rol te benadrukken. Van de 22 door DNA gecodeerde aminozuren zijn er 8 essentieel voor mensen, aangezien we ze niet zelf kunnen synthetiseren en dus uit onze voeding moeten halen. Aminozuren zijn biomoleculen die essentiële rollen vervullen in menig biologisch proces. Ze hebben rollen als signaalmoleculen in de hersenen, als energiebron, als bouwsteen voor hormonen en smaakstoffen (in de vorm van peptiden) en in grote aantallen aan elkaar geketend vormen ze eiwitten, die onder andere structuur-, transport-, enzymatische en signaalfuncties hebben. Dankzij de centrale rol die aminozuren in processen van levende organismen hebben, zijn zij een belangrijk doel voor onderzoek naar de structuur en functies van eiwitten en voor onderzoek naar het metabolisme en katabolisme van mens en dier en hoe deze samenhangen met ziektes die mens en dier treffen. De hierboven genoemde systemen kunnen goed worden bestudeerd met behulp van stabiele isotopen. Isotopen hebben onderling dezelfde chemische eigenschappen, maar andere fysische eigenschappen en de incorporatie van isotopen in relevante eiwitten maakt het mogelijk om deze systemen te bestuderen middels niet-invasieve, niet-destructieve technieken als FT-IR en NMR. Omdat isotopen in massa verschillen, kunnen bovendien massagevoelige technieken als massa-spectrometrie gebruikt worden om metabole routes te onderzoeken door de isotopen van tussenproduct naar tussenproduct te volgen. Om dergelijk onderzoek te kunnen uitvoeren is het noodzakelijk dat er toegang is tot de gehele set van isotopomeren van de door DNA gecodeerde aminozuren. De L-α-aminozuren hebben alle twee identieke structuurelementen, nl. de carboxylzuur-groep en de amino-groep, waar ze hun naam ook aan ontlenen. Allemaal zijn ze bovendien afgeleid van het simpelste aminozuur, glycine, waaraan verschillende zijketens zijn bevestigd. Verder zijn alle aminozuren, behalve glycine, chiraal en hebben isoleucine en threonine zelfs twee chirale centra. Een algemene synthetische aanpak om isotoop-gelabelde aminozuren te verkrijgen zou daarom gebruik kunnen maken van een modulaire methode, door uit te gaan van een glycine bevattend synthon, waaraan vervolgens enantioselectief verschillende zijketens bevestigd kunnen worden. De O' Donnell chirale alkyleringsmethode is gekozen om deze algemene aanpak te verwezenlijken. De methode is gebaseerd op een chirale fase-transfer katalysator, gebaseerd op een cinchonidium-ion, die base kan transporteren vanuit de waterlaag naar een beschermd glycinaat in een aangrenzende organische laag, waarna het glycinaat gedeprotoneerd wordt. Het gevormde enolaat vormt een hecht ionen-paar met de chirale fase-transfer katalysator. De grote zijketens van de katalysator zorgen er vervolgens voor dat het enolaat slechts van een kant (de *Si*-zijde) door een electrofiel benaderd kan worden. Het op deze manier gevormde

beschermde chirale aminozuur wordt vervolgens gehydrolyseerd om zodoende het gewenste vrije aminozuur te krijgen. Dit alles met hoge opbrengst en een hoog enantiomeer overschot. Op deze manier kan een algemene methode worden gebruikt om verschillende L-α-aminozuren te verkrijgen, mits er schema's kunnen worden ontwikkeld die het mogelijk maken om selectief isotoop-labels in de verschillende zijketens en het beschermde glycinaat te incorporeren.

In Hoofdstuk 2 is een chemo-enzymatische methode ontwikkeld die toegang geeft tot elk willekeurig isotopomeer van de essentiële aminozuren isoleucine en valine. Deze methode geeft de juiste introductie van het tweede chirale centrum in (2*S*,3*S*)-isoleucine en maakt het mogelijk om onderscheid te maken tussen de prochirale methylgroepen van valine, zoals aangetoond door de synthese van [4-^{13}C]-(2*S*,3*S*)-valine. Om (2*S*)-leucine in elke mogelijke isotoop-verrijkte vorm te kunnen verkrijgen is de O' Donnell methode ter bereiding van chirale aminozuren gebruikt. Het in deze methode gebruikte beschermde glycine-derivaat is gesynthetiseerd via een schema dat de introductie van elke combinatie van koolstof- en stikstofisotopen mogelijk maakt. De synthese van [5-^{13}C]-(2*S*,4*R*)-leucine laat zien dat de O' Donnell methode, in combinatie met de Evans methode om chiraal 2-methylpropyl jodide te maken, leidt tot een goed onderscheid tussen de twee prochirale methylgroepen. De hier beschreven strategie is verkiesbaar boven andere methodes om α-aminozuren te synthetiseren, aangezien de reactieomstandigheden mild zijn, de chirale hulpstof eenvoudig teruggewonnen kan worden en het optisch actieve product, dat met hoog enantiomeer overschot wordt verkregen, eenvoudig geïsoleerd kan worden. De O' Donnell methode is niet geschikt voor de synthese van L-valine en L-isoleucine, aangezien de secundaire halides die voor deze synthese nodig zijn te veel sterische hindering ondervinden om een effectieve reactie mogelijk te maken.

In Hoofdstuk 3 zijn eenvoudige modulaire reactieschema's uitgewerkt die toegang geven tot elke isotopomeer van serine en homoserine. Via Mitsunobu chemie kunnen deze systemen eenvoudig worden omgezet in cysteine, selenocysteine, homocysteine, homoselenocysteine en de essentiële aminozuren methionine en selenomethionine. Deze zwavel en seleen bevattende aminozuren vervullen vele essentiële rollen in levende organismen. Bovendien kan homoserine in enkele stappen worden omgezet in optisch actief vinylglycine. Afgezien van de introductie van stabiele ^{13}C, ^{15}N, ^{17}O en ^{18}O isotopen kunnen ook de radioactieve isotopen van zwavel en seleen makkelijk worden geïntroduceerd. Gezien de grote reikwijdte van de Mitsunobu reactie moet het mogelijk zijn om meer belangrijke systemen met het koolstofskelet van serine en homoserine te synthetiseren via deze methode.

Hoofdstuk 4 beschrijft een eenvoudige synthetische strategie, gebaseerd op de O' Donnell methode, die het mogelijk maakt om elke ^{13}C/^{15}N isotopomeer van L-glutamaat, L-glutamine, L-ornithine, L-proline, L-lysine en L-α-aminohexaandizuur te be-

reiden. Deze strategie maakt het ook mogelijk om een belangrijk niet-proteïnogeen als L-citrulline te maken in hoge opbrengst en optische zuiverheid.

Hoofdstuk 5 bevat een algemene discussie en een vooruitblik. Er worden mogelijke methodes in beschreven om toegang te krijgen tot alle isotopomeren van aminozuren waar tot op heden geen synthetische routes voor zijn. Verder wordt de O' Donnell methode vergeleken met eerdere methodes om α-aminozuren te synthetiseren. Recente ontwikkelingen op het gebied van nieuwe, op glycine gebaseerde, uitgangsstoffen worden besproken evenals de mogelijkheden die deze stoffen kunnen bieden voor de modulaire syntheses van valine en isoleucine. De vooruitblik beschrijft hoe recente vooruitgang op het gebied van de eiwitsynthese, door middel van totaal-synthetische methodes gebaseerd op ligatie technieken, gecombineerd kan worden met de toegang tot de volledige set gelabelde aminozuren. De combinatie van ligatiemethodes en isotoop-gelabelde aminozuren maakt het mogelijk om volledige controle over labelincorporatie in eiwitten uit te oefenen en dit zal vervolgens het onderzoek in talrijke biologische systemen mogelijk maken of vergemakkelijken.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 24 februari 1974 geboren in Hengelo (O.). Na in 1993 het VWO diploma aan het Gymnasium Celeanum te Zwolle te hebben behaald, werd in september van dat jaar begonnen met de studie Scheikunde aan de Universiteit Leiden. Het propedeutisch examen werd behaald in augustus 1994. Van februari 1996 tot en met mei 1998 werd gewerkt aan een afstudeeronderzoek bij de werkgroep Bio-organische Fotochemie onder leiding van Prof. Dr. J. Lugtenburg en onder dagelijkse supervisie van Dr. Ir. T.M. Werkhoven, getiteld 'The synthesis of ^{13}C-labelled isoprene'. Het afstudeeronderzoek werd van augustus 1997 tot maart 1998 onderbroken voor een onderzoeksstage onder leiding van Prof. Dr. P. Garegg en Dr. S. Oscarson, met als onderwerp 'Investigations concerning the reversed anomeric effect' in de vakgroep Organiska Kemi in Stockholm, Zweden. Het doctoraaldiploma werd behaald in augustus 1998. Tussen november 1998 en januari 2003 werd het in dit proefschrift beschreven onderzoek uitgevoerd, onder directe leiding van Prof. Dr. J. Lugtenburg en Dr. J. Raap. In 2000 en 2002 werd deelgenomen aan respectievelijk 'the 9th international Conference on retinal proteins' in Szeged, Hongarije en 'the 10th international conference on retinal proteins' in Seattle, de Verenigde Staten van Amerika. Het in dit proefschrift beschreven werk werd ook op jaarlijkse NWO/CW bijeenkomsten in Lunteren gepresenteerd, waar het in maart 2003 met een poster-prijs werd gewaardeerd. Vanaf oktober 2003 is de auteur werkzaam als post-doctoraal onderzoeker in de biokatalyse, onder leiding van Prof. Dr. A.P.G. Kieboom.

NAWOORD

Het werk dat beschreven is in dit proefschrift is gelukkig niet in een menselijk vacuüm tot stand gekomen. Er zijn veel mensen die er in meer of mindere mate hebben bijgedragen en die het verdienen genoemd te worden. Allereerst Thekla Werkhoven, wier voorbeeld mij op het kronkelige AIO-pad heeft gezet. Vervolgens mijn geweldige lab-genoten Ineke van der Hoef en Arjan van Wijk die mij op zaal en daarbuiten hebben gestimuleerd na te denken over chemie, boeken en muziek en over de vele aspecten van het leven (en die dat gelukkig nog steeds doen). Natuurlijk wil ik Amparo, Robert, Mohammed en vooral Wei Sein noemen voor hun noeste geploeter op de syntheses en voor hun bijdrages aan de publicaties en het aan lab-gevoel. Verder wil ik vele collega's en studenten van de BOF (en BOF-annex A.R.C.) en SSNMR noemen voor de discussies en goede sfeer (met name Alain, Anna, Els, Michiel, Niels, Prashant, Reinier, Robin, Richard, Sylvia en Yolanda en bovenal Rob van der Steen, die er nog een extra taak heeft bijgekregen). Kees, Johan en Fons wil ik noemen voor hun hulp en tijd bij NMR-metingen en meet-gerelateerde problemen. Martina de Ruijter voor het laten zien hoe je een enzym in handen krijgt en Rian van den Nieuwendijk voor zijn hulp bij de chirale HPLC analyses.
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