Synthesis of modified, lipidated N-Ras C-terminus peptides

Diplomarbeit
vorgelegt von

Catherine Katzka
aus Sunnyvale, Kalifornien

Universität Dortmund

Contents

1. Introduction 2
2. General Background 3
   - 2.1 Protein Anchoring through Lipidation 3
   - 2.2 Ras Proteins 6
   - 2.3 Biophysical Investigation of the Membrane Insertion of Ras 8
   - 2.4 Chemical Synthesis of Peptides 11
     - 2.4.1 Coupling Reagents and Methods 11
     - 2.4.1.1 Carbodiimide Method 11
     - 2.4.1.2 Mixed Anhydride Method 12
     - 2.4.1.3 Phosphonium and Uronium Reagents 13
     - 2.4.1.4 Solid Phase Synthesis 14
     - 2.4.2 Protecting Groups in Chemical Peptide Synthesis 15
     - 2.4.2.1 Amino Protecting Groups 15
     - 2.4.2.2 Carboxy Protecting Groups 18
     - 2.4.3 Synthesis of Lipopeptides 19
3. Thesis 21
4. Results and Discussion 22
   - 4.1 Retrosynthetic Analysis of Lipidated Ras Peptides 22
   - 4.2 Synthesis of the unlabelled N-Ras Heptapeptide
     - 4.2.1 Synthesis of Lipidated Building Blocks 22
     - 4.2.2 Synthesis of PG-Met-Gly-Leu-Pro-OH 25
     - 4.2.3 Assembly of the Complete N-Ras Heptapeptide 26
   - 4.3 Synthesis of the Deuterium-Labelled Lipidated N-Ras Heptapeptide 29
     - 4.3.1 Preparation of Labelled Lipidated Building Blocks 29
     - 4.3.2 Peptide Assembly 30
5 Conclusion and Outlook 32
6 Experimental Section 35
   - 6.1 Materials and Methods 35
   - 6.2 Compounds from Chapter 4.2.1 36
   - 6.3 Compounds from Chapter 4.2.2 39
   - 6.4 Compounds from Chapter 4.2.3 45

6.5 Compounds from Chapter 4.3.1 49
6.6 Compounds from Chapter 4.3.2 53

Appendix A: Abbreviations 57
Appendix B: References 59
Appendix C: Acknowledgements 61

1. Introduction

The communication of cells with one another is absolutely necessary in order to coordinate their behavior for the benefit of the organism as a whole. Complex machinery in a cell interprets intercellular signals, enabling it to determine its' position and specialized role, for example that each cell only divides when its neighbor tells it to do so. As new powerful techniques become available to study cells, the intricacy and importance of these controls becomes apparent, especially when they fail, resulting in cancer.

The Ras proteins, H-, N-, and K-Ras, members of the superfamily of small GTP-binding proteins (also called GTP*ases* because of the GTP hydrolysis that they also catalyze), play a central role in the regulation of cell growth and differentiation. They are signaling proteins involved in the communication between cells as between different cellular compartments. Mutations in the Ras gene disrupt this delicate control and lead to cell proliferation. As in the case for many other signaling molecules, Ras´ activity is dependent on its lipidation state: lipidated, thus membrane bound, Ras is active. A better understanding of Ras´ function can potentially lead to new therapeutic treatments.

Along with biochemical methods, synthetic organic chemistry has become a helpful tool towards the understanding of these mechanisms. Small, well-defined molecules allow the observation of biological events on a molecular scale. Using lipidated peptides, structural information on membrane binding can be gained. For example, it is now possible with ^{1}H high resolution magic angle spinning NMR (^{1}H HR MAS NMR) to probe residues' membrane penetration depth or with deuterium NMR, the membrane mobility of peptides [40]. The chemical synthesis of isotopically and non-isotopically labeled lipidat-modified N-Ras C-terminus peptides for that purpose is the object of the presented work.

2. General background

2.1 Protein anchoring through lipidation

Membrane association is essential for the proper cellular distribution and functioning of many cell signaling proteins. Membrane localization and binding is achieved through co- and post-translational lipidation of amino acids at or near the N- or C-terminus of the protein. In contrast to the permanency of transmembrane binding through α-helices and β-barrels, lipidation offers an alternative, reversible binding modus. This reversibility permits the cell to regulate protein functions that only occur when the protein is membrane associated.

Stable membrane insertion requires the presence of at least two lipid groups or one lipid group and an attractive electrostatic peptide-membrane interaction [1, 2].

Four classes of lipids are found covalently attached to proteins (**Fig. 2.1**): (1) isoprenoid groups such as farnesyl and geranylgeranyl residues, (2) fatty acyl groups such as myristoyl and palmitoyl, (3) glycoinositol phospholipids (GPIs), and cholesterol.

Isoprenoid (or prenyl) **1**, **2**, moieties are attached to a cysteine via a thioether linkage. A common prenylation recognition sequence in proteins is the C-terminus -CaaX sequence, also known as –CaaX box, where *C* is cysteine and *a* can be any aliphatic amino acid. Although X may be any amino acid, its identity is a major prenylation determinant: when *X* is either alanine, methionine or serine, the protein is farnesylated. It is geranylgeranylated when *X* is leucine. Following prenylation, the *aaX* tripeptide is proteolytically excised and the newly exposed carboxy-group is methylated.

Other prenylation recognition sequences have been characterized and are predominantly found in the Ras-related Rab-family of GTPases: for the -CXC motif, both cysteine residues are geranylgeranylated and the C-terminus is then methylated [3], whereas for the –CC- and CCXX motives, one or both cysteines are geranylgeranylated but the carboxy-group is not methylated [4].

Protein prenylation has been viewed as a mechanism for post-translational attachement of proteins to the membrane. It is however becoming increasingly clear that prenylation does more than mediate non-specific modified protein association

with lipid bilayer. There is mounting evidence that prenylation plays a specific role in protein-protein interactions[5] including those that facilitate protein trafficking[6, 7] and subcellular localization.

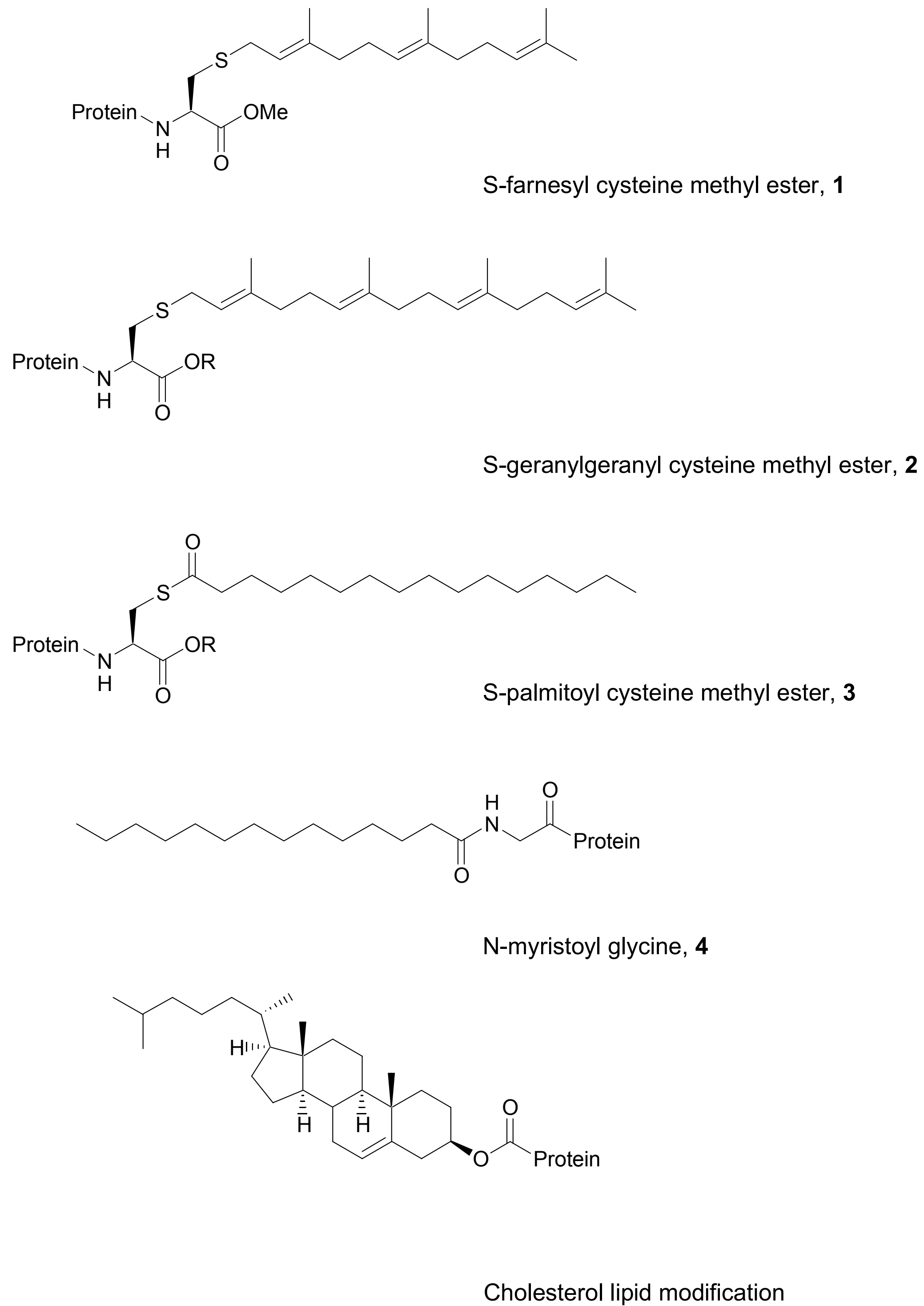


Figure 2.1: Various lipid classes.

The C_{14} myristoyl-group **4** is stably attached as an amide to a proteins' N-terminal glycine. This modification almost always occurs co-translationally [8].

The palmitoyl group, a common C_{16} fatty acid chain, is post-translationally bound to the protein via a cysteine thioester linkage **4**[9]. In contrast to other lipid modifications, palmitoylation is reversible under physiological conditions. This reversibility may be particularly important for modulating protein function during cycles of activation and deactivation. Palmitoylation can affect the affinity of proteins for membranes, subcellular localization and interaction with other proteins [10]. Similarly to prenyl moieties, palmitoylated groups are thought to function as membrane anchors. [1, 2, 9].

The biological relevance of lipid-modified proteins is highlighted by the role that Ras proteins play in maintaining the regular life cycle of cells.

2.2 The Ras proteins

The Ras genes produce a family of highly related proteins, the H-, N-, and K-Ras proteins which are differentiated from each other by the last 25 C-terminal amino acid residues (**Fig. 2.2**).

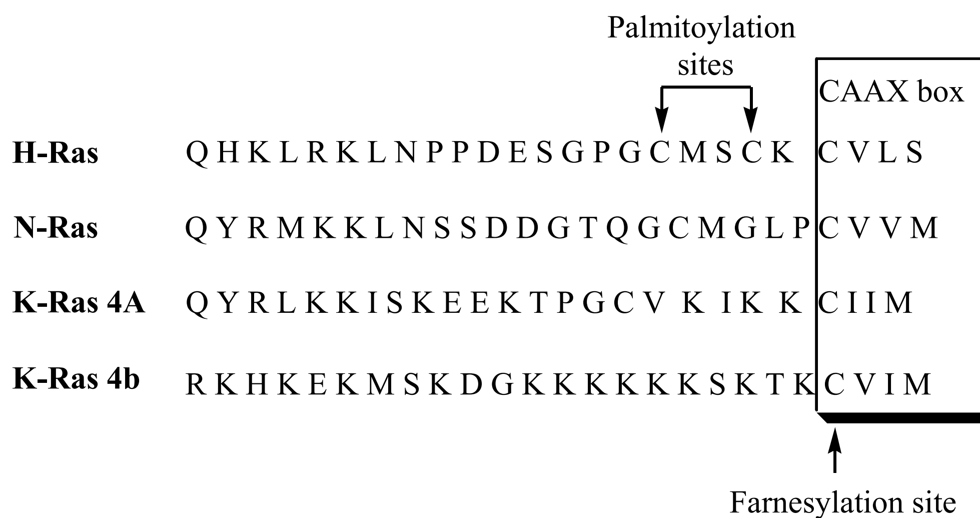


Figure 2.2:The variable domain of human Ras proteins. Indicated are the lipidation sites.

All Ras proteins are low molecular weight (21kD) GTP hydrolyzing proteins (GTPases) which cycle between an inactive GDP-bound state and an active GTP-bound state. Because the switch-on and switch-off rates are very slow, the process needs to be aided by guanine nucleotide exchange factors (GEFs) and GTP*ase* activating proteins (GAPs). In its inactive state, Ras binds to GDP. Ras activation by GEF catalyzes the dissociation of GDP, facilitating the loading of GTP. Only in this active state can Ras interact with effectors: proteins that interact specifically with the GTP-bound state and transmit a signal through, for example, phosphorylation (**Fig. 2.3**).

The signaling is terminated by the GAP-aided GTPase reaction of Ras. An artificially extended lifetime of the signal due to an extended GTP-bound active state, coming for example from a point mutation, whose biochemical consequence is to render the protein unable to hydrolyze GTP [11], may lead to unregulated biological responses, such as uncontrolled cell proliferation, thereafter to cancer. Activating Ras mutations

are particularly associated with myeloid malignancies and carcinomas of the colon, pancreas, lung and thyroid, but they have also been detected in other cancer types.[12]

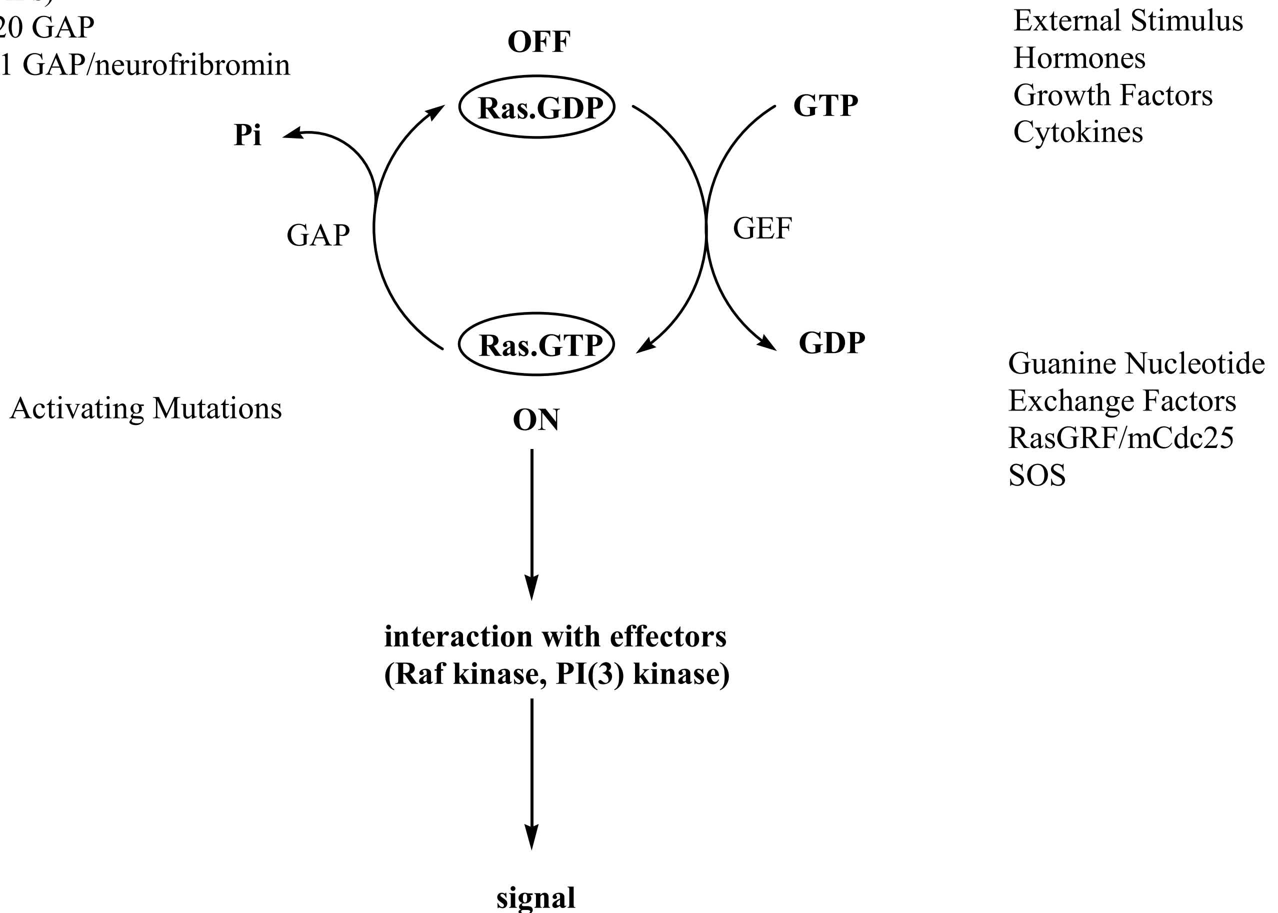


Figure 2.3: Schematic representation of Ras regulation and function.

Lipidation plays a crucial role in Ras´ activity: unlipidated, Ras is cytosolic and cannot transduce signals [13-15]. All the targeting information is contained in the variable domain of the Ras protein. Covalently attached farnesyl and palmitoyl residues localize Ras to the cystosolic side of the cell membrane, activating the protein.

2.3 Biophysical investigation of the membrane insertion of Ras

Although the three dimensional structure of truncated H-Ras protein has been determined (residues 1-166 or 1-171)[16-19] and crystals of the full length protein have been reported[19], the structure of full length proteins has not yet been solved. More precisely, these studies were undertaken in the absence of the C-terminus, therefore, the structure dynamics and details of membrane association of the membrane binding C-terminus remain unknown.

Unlike for soluble peptides and proteins, there are few standard techniques available for the study of lipid modified peptides to model membranes. Even if neutron diffraction allows the determination of the location of a peptide in a membrane, the rarity of a neutron source limits the use of this technique. While ^{1}H NMR has become a standard method to obtain high resolution molecular structures of peptides and proteins, it fails for membrane bound peptides because the samples are too large to tumble with a short enough correlation time to yield narrow and well-resolved resonance lines, as required for high resolution NMR. The ^{1}H NMR linewidths are homogenously broadened typically by several kHz due to strong anisotropic ^{1}H-^{1}H dipolar couplings (**Fig. 2.4**, top).

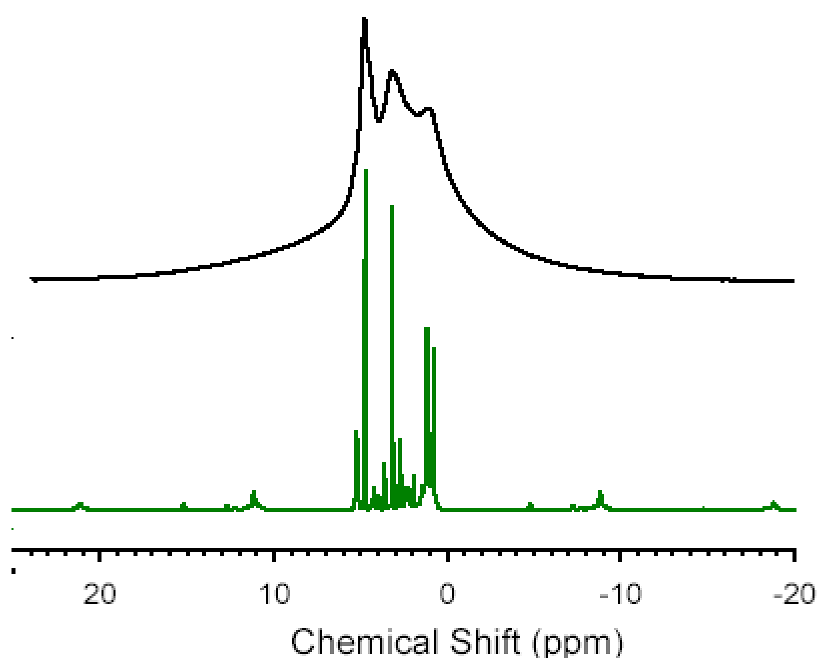


Figure 2.4: Comparison of MAS and non-MAS spun N-ras peptide in DMPC-d67 multilamellar liposomes. The broad, featureless top spectrum corresponds to the sample measured without MAS, the bottom spectrum is taken with MAS.[31]

By making use of the $1 - 3\cos^2\theta$ dependence of both the dipole-dipole and anisotropy and spinning the sample at $\theta = 54.7^o$ to the applied magnetic field (whereby $1 - 3\cos^2\theta = 0$), it is possible to average out all dipolar interactions and anisotropies.

This method has already been shown to be effective for obtaining almost solution-like spectra for pure lipid membrane systems[20] and used to study the structure and dynamics of these systems[21-25]. Unfortunately, membrane bound peptides only reorient themselves slowly, which broadens the NMR lines. This problem can be overcome by spinning the samples at very high speeds (10 to 15 kHz). Recently, Huster et al. showed that ^1H HR MAS NMR of bilayer bound lipidopeptides provide excellent spectral resolution for structural investigations (**Fig. 2.5**)[31]. Using the unlabelled lipid-modified C-terminus of the N-ras peptide, it was possible to estimate the location of the peptide backbone and the lipid side-chains.

The full assignment, quantitative analysis and dynamics of the peptide location and structure are only possible by using N-ras peptides that have been isotopically labeled either in the lipid side-chains, or within the backbone itself. The opportunity that ^1H HR MAS NMR offers to providing more insight into the mechanism of protein insertion into membranes by studying peptides in membrane bilayers could not be fully taken advantage of without possessing the know-how for the synthesis of sensitive lipidated peptides.

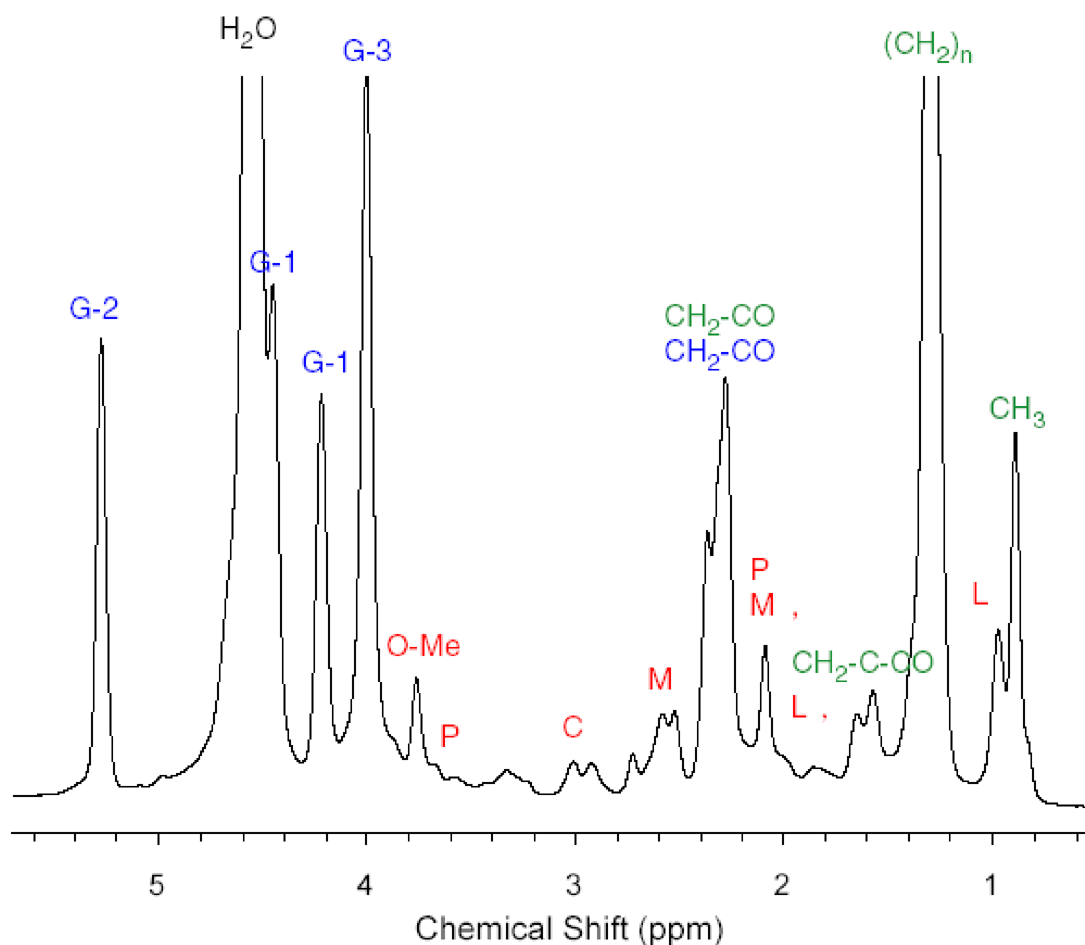


Figure 2.5: 600 MHz ^{1}H HR MAS NMR spectrum of the N-ras peptide in DMPC-d67 multilamellar liposomes at spinning speed 12 kHz. The peaks labeled G represent non-deuterium labeled glycerol signals of the phospholipid.[31]

2.4 The chemical synthesis of lipidated peptides

2.3.1 Coupling reagents and methods

The reaction coupling two amino acids is endergonic and therefore must be activated to obtain significant yields. Current coupling reagents and methods will be presented in the following section.

2.3.1.1 The carbodiimide method

The carbodiimide method emerged as one of the first of these methods [26]. The carboxy group of an amino-protected amino acid **6** adds to the carbodiimide **7**, creating an O-acyl urea intermediate **8**. The free amine of a carboxy protected amino acid **12** then nucleophilically attacks this intermediate, forming a new peptide bond **13** and a urea-derivative **10**. The reactive O-acyl urea intermediate can also rearrange to an unreactive N-acyl urea derivative **9**. Addition of a nucleophile, such as N-hydroxybenzotriazol (HOBt, **17**) or 1-hydroxy-7-azabenzotriazole (HOAt, **18**), leads not only to the formation of an ultra reactive species, which reacts, in the presence of a free amine, to form an amide bond, but also raises the acidity of the solution, minimizing the risk of amino acid racemization.

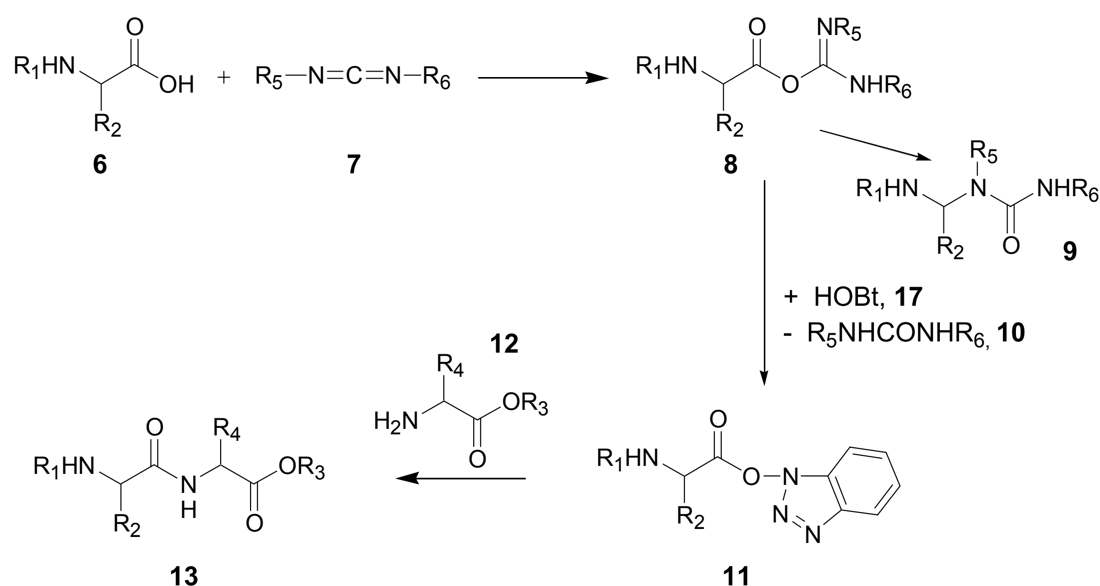


Figure 2.6: The mechanism of the carbodiimide mediated coupling.

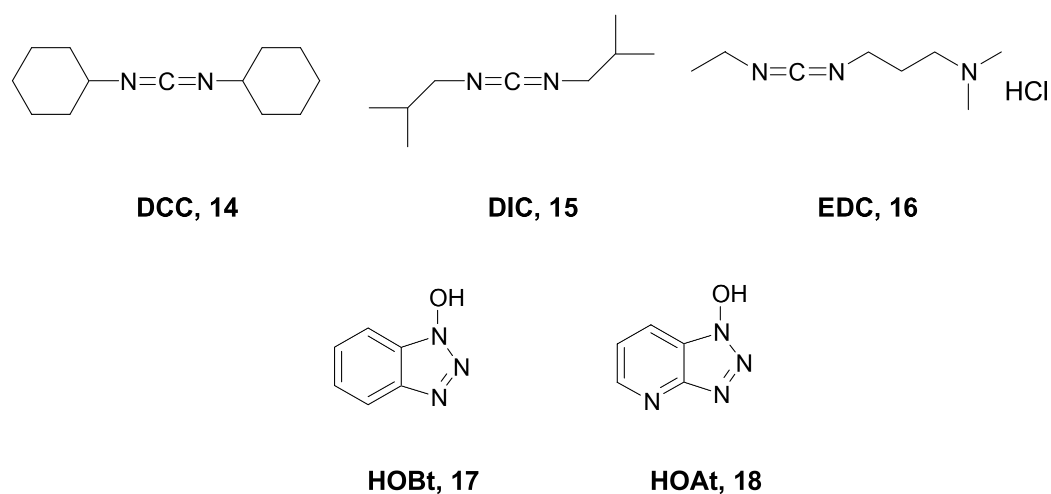


Figure 2.7: Various carbodiimides and benzotriazoles.

Couplings with N, N´-dicyclohexylcarboiimide (DCC, **14**) generate insoluble, difficult to remove urea by-products. Alternatively, N, N´-diisopropylcarboiimide (DIC, **15**) and N´-(3-dimethylamino-propyl)-N-ethylcarboiimide (EDC, **16**) induced couplings circumvent this problem by generating soluble ureas, the latter being water-soluble thus easily removable by mild acidic workup.

2.3.1.2 The mixed anhydride method

Amino acids are also efficiently activated by forming a mixed anhydride with 2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinoline (EEDQ, **18**)[27]. Upon reaction with the amino acid **11**, the chinoline looses ethanol, forming intermediate **19**, which undergoes a rearrangement, eliminating choline **20** to form the mixed anhydride **21**. The free amino group of a carboxy protected amino acid **16** can then nucleophilically attack, forming the peptide bond while releasing carbon dioxide and ethanol.

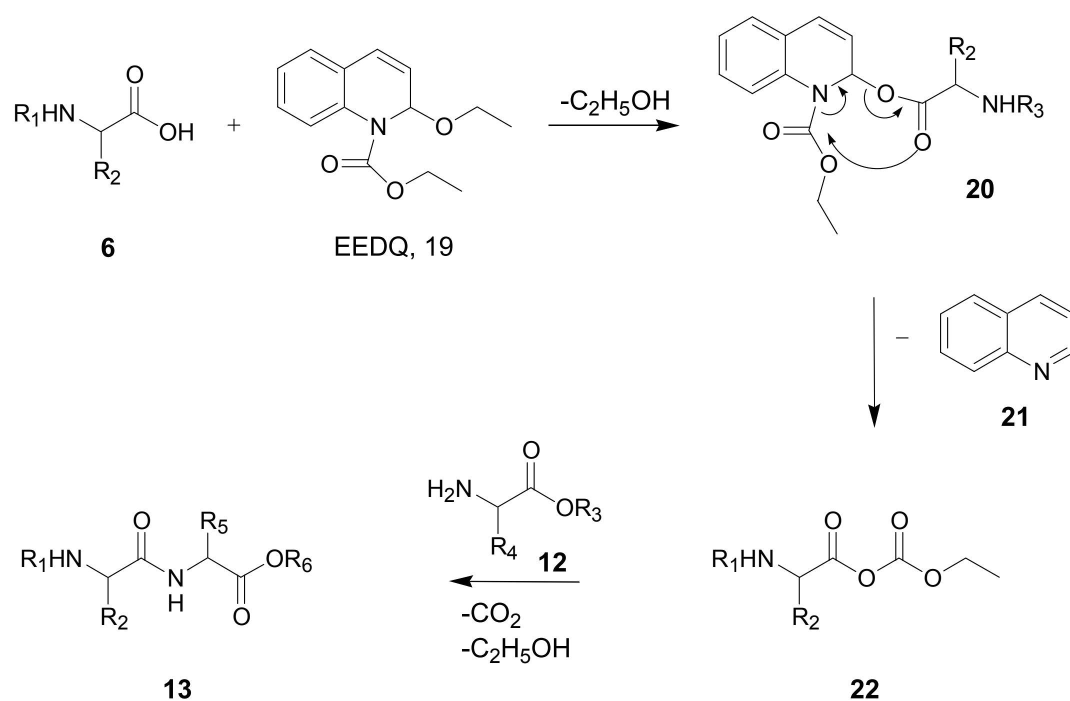


Figure 2.8: The mechanism of the EEDQ mediated coupling.

2.3.1.3 Methods involving phosphonium and uronium reagents

The phosphonium reagent benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP, **23**) was introduced in 1988[28] as a highly selective reagent for peptide couplings.

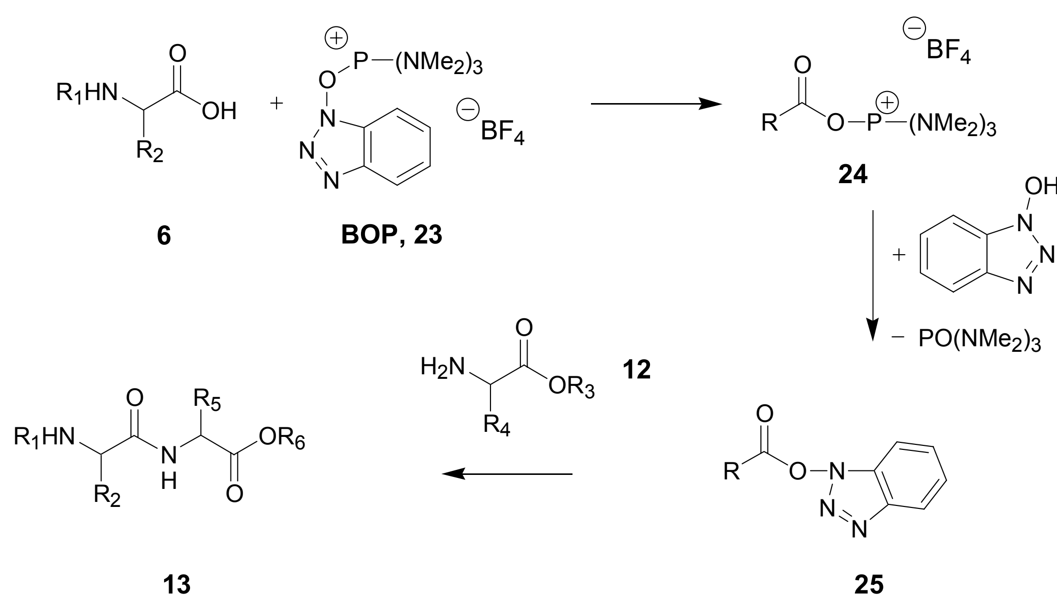


Figure 2.9: The mechanism of the BOP mediated coupling.

After deprotonation of the amino acid **6** by a tertiary amine, the resulting carboxylate anion nucleophilically attacks the phosphorous atom of BOP **23**. The formed intermediate **24** is further attacked by the benzotriazole forming the active ester **25**, which then reacts to form a peptide bond with the free amine of an amino acid. Due to the fact that one of the byproducts of this reaction, hexamethyl triamide phosphoric acid (HMPTA) is carcinogenic, reagents such as benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP, **26**), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, **27**) were developed. An even more reactive coupling reagent has also been developed: O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, **28**).

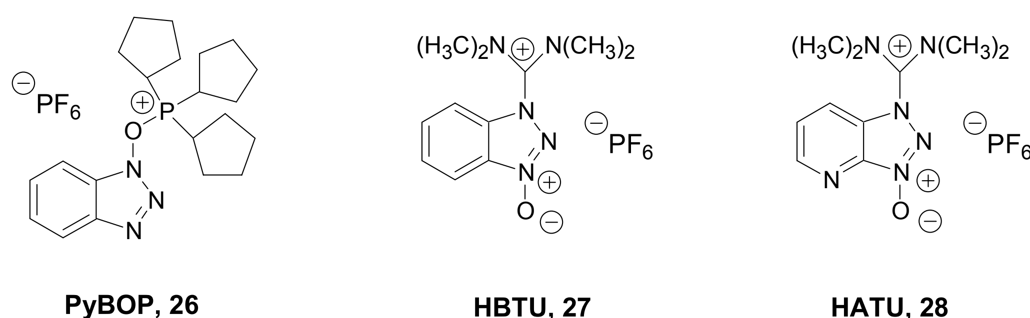


Figure 2.10: Commonly used benzotriazole coupling reagents.

2.3.2 Solid phase peptide synthesis

Merrifeld's successful synthesis of the nonapeptide bradykinin in 1964[29] marked the beginning of the solid phase peptide synthesis (SPPS) era.

The concept of SPPS is quite simple: synthesis is conducted in the C- to N-direction. The first amino acid is anchored to an insoluble polymer support by means of a flexible selectively cleavable linker group. The peptide chain is elongated through repetitive cycles of N-terminus deprotection and coupling. All soluble reagents are filtered away after each step. At the end of the synthesis, the peptide is selectively cleaved from the polymer support. The repetitive nature of SPPS lends itself ideally to automation. Furthermore, the large excess of reagents used ensures that the

reactions go to completion. This implies that for a synthesis of 100 steps, each step yielding 99% yield, one would obtain an overall yield of 36%. This yield would be entirely hopeless to achieve in solution.

2.4 Protecting groups in chemical peptide synthesis

Unambiguous formation of a peptide bond between two structurally similar amino acids requires that the amino group of one and the carboxy- group of the other are protected. It is equally important to protect the chemically reactive side chains of amino acids.

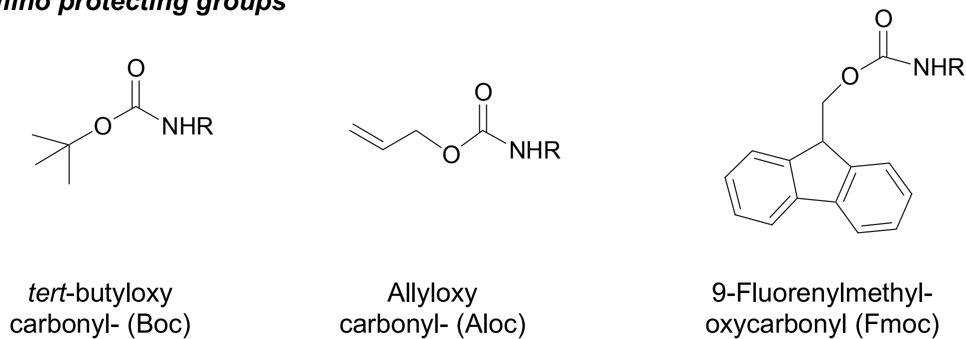
Protecting groups must be easily introduced, reduce the reactivity of amino or carboxy groups, be chemically stable under peptide coupling conditions and finally be easily removable at the end or intermediate phases of the synthesis, leaving the newly created amide link intact.

Described in the coming section are the deprotection methods of the most commonly used amino- and carboxyl- protecting groups.

2.4.1 Amino-protecting groups

Urethane derivatives are suitable for amino protection: they satisfy the above-mentioned protecting group requirements and the urethane nitrogen is usually chemically inert to peptide synthesis conditions. Commonly used protecting groups are the *tert*-butyloxy carbonyl (Boc), the fluorenylmethoxycarbonyl (Fmoc) and the allyloxycarbonyl group.

Amino protecting groups



Carboxyl protecting groups

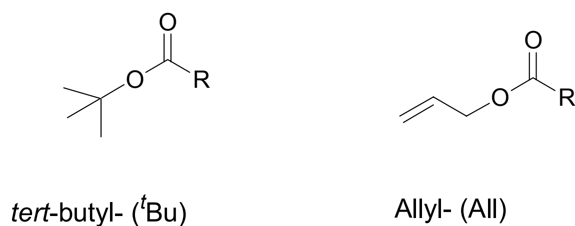


Figure 2.11: Commonly used protecting groups in peptide synthesis.

2.4.1.1 The *tert*-butyloxycarbonyl (Boc) protecting group[30]

The Boc group is introduced onto the amine functionality of the amino acid with Boc_2O. It is inert under basic conditions and unaffected by catalytic hydrogenation. Its´ acid lability is driven by the relative stability of the cleavage product, the *tert*-butyl cation. Trifluoroacetic acid or HCl/diethyl ether are commonly used to cleave this group.

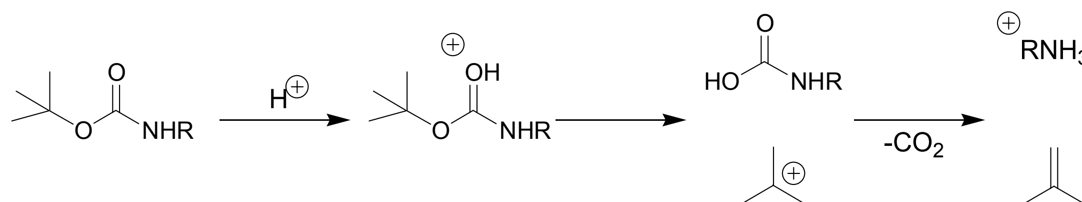


Figure 2.12: Cleavage mechanism of the Boc group under acidic conditions.

2.4.1.2 The fluorenylmethoxycarbonyl (Fmoc) protecting group.[31]

The Fmoc group is inert to acids. It is usually prepared from the chloroformate or the derived hydroxysuccinamide ester and is quickly and cleanly removed by secondary amines in DMF. The Fmoc group owes its base lability to the acidity of the 9-hydrogen atom of **29** due to the resonance stabilization of the cyclopentadiene anion **30**. The abstraction of this proton initiates a β-elimination.
The resulting dibenzofulvene **31** generally reacts further with the basic reagent, usually piperidine. The fluorenyl product, **32**, of this last reaction has a strong absorbance in the ultraviolet region (λ_{max} 301nm), providing the possibility for spectroscopic monitoring of solid phase synthesis.

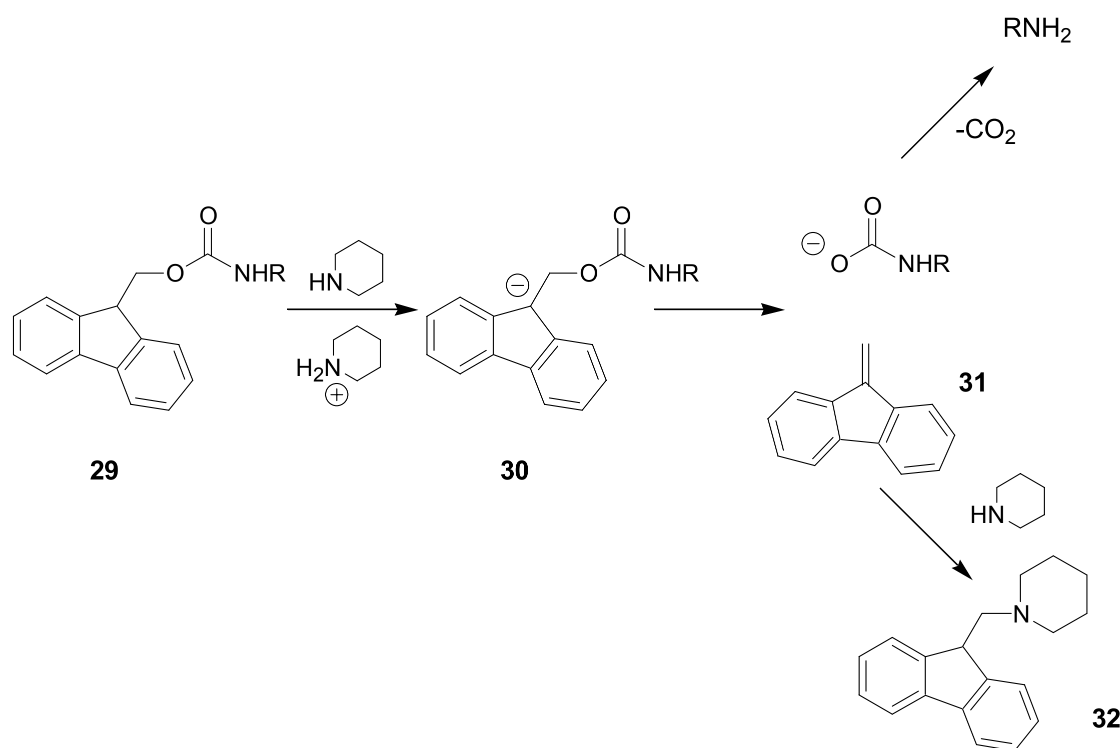


Figure 2.13: Piperidine catalyzed Fmoc deprotection.

2.4.1.3 The allyloxycarbonyl protecting group[32]

The allyloxycarbonyl protecting group is stable to acidic and basic conditions. It is introduced by coupling the amino acid with allyl chloroformate under Schotten-Baumann conditions.

The deprotection proceeds through the formation of an $\eta^3\pi$ allylic intermediate, which is then nucleophilically attacked by, for example, dimethyl barbituric acid or morpholine.

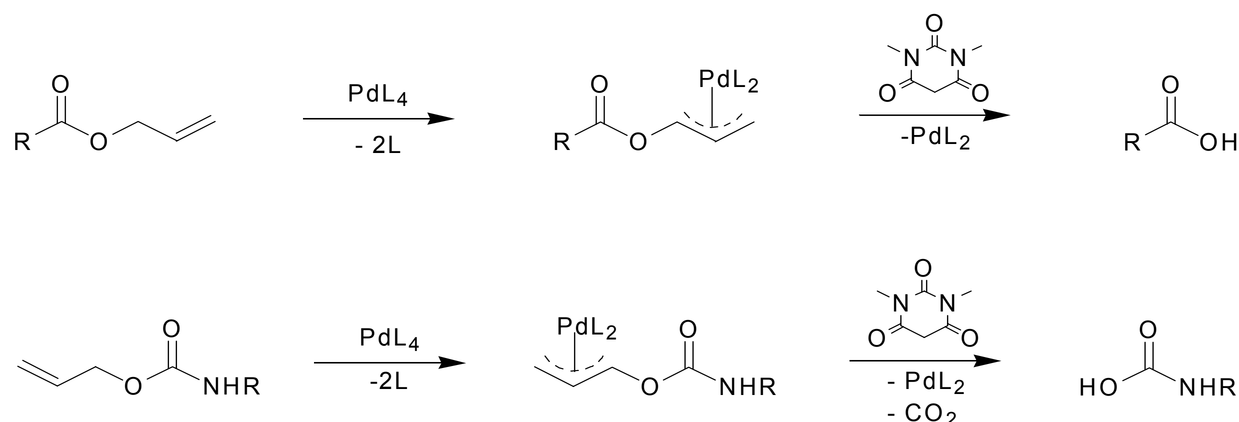


Figure 2.14: Mechanism of Pd(0)-mediated cleavage of N- and C-terminal allylic protecting groups with dimethyl barbituric acid.

2.4.2 Carboxy-protecting groups

The carboxy function was initially protected with methyl or ethyl esters though their cleavage by aqueous alkaline hydrolysis was sometimes difficult. Alternative protecting groups such as *tert*-butyl or allyl esters are cleaved under analogous conditions (acid treatment or Pd(0) mediated) to the corresponding urethanes used for amino protection.

2.4.2.1 The *tert*-butyl ester protecting group

The *tert*-butyl ester protecting group is one of only two carboxy protecting group that is stable to base hydrolysis. It is introduced by acid catalyzed esterification with t-butyl acetate[33]. Acid induced cleavage forms a gaseous byproduct, thus commonly eliminating the need for classical aqueous workup and purification.

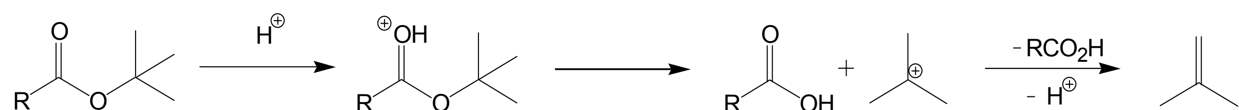


Figure 2.15: Acid catalyzed *tert*-butyl ester deprotection.

2.4.2.2 Allyl ester

Allyl esters are mainly introduced by azeotropic esterification[34]. They are elegantly removed under palladium (0) catalyzed allylic substitution by a nucleophile such as dimethyl barbituric acid or morpholine[32].
The cleavage mechanism is depicted in **Figure 14**.

2.5 Synthesis of lipopeptides

Farnesylated and palmitoylated peptides are respectively acid and base sensitive thus precautions need to be taken in order to undertake their synthesis.
The isolated double bonds of isoprenoid residues are prone to attack under acidic conditions (**Fig. 16**)[35]. The Boc and tert-butyl groups are thus unsuitable protecting groups. Instead, the Fmoc and Aloc groups have been proven to be safe and effective.

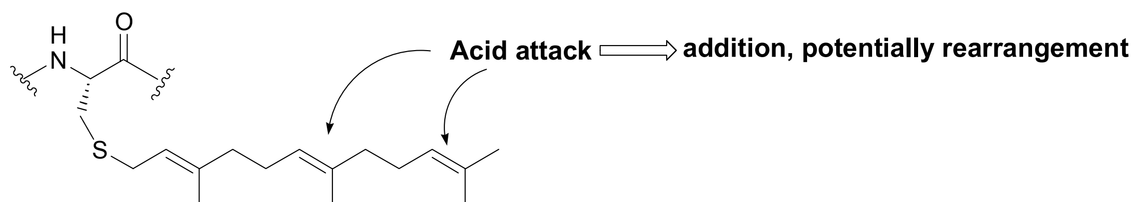


Figure 2.16: Side reactions of the farnesyl group in the presence of acid.

The palmitoyl group is base and nucleophile sensitive and undergoes N-S-acyl shifts (**Fig. 17**)[36]. It spontaneously hydrolyzes at pH>7[37]. Under basic conditions, it runs the risk of undergoing β-elimination after dehydroalanine formation[38]. Synthesis involving Fmoc protecting groups is unsuitable. Boc, allyl ester or enzyme labile protecting groups are more appropriate.

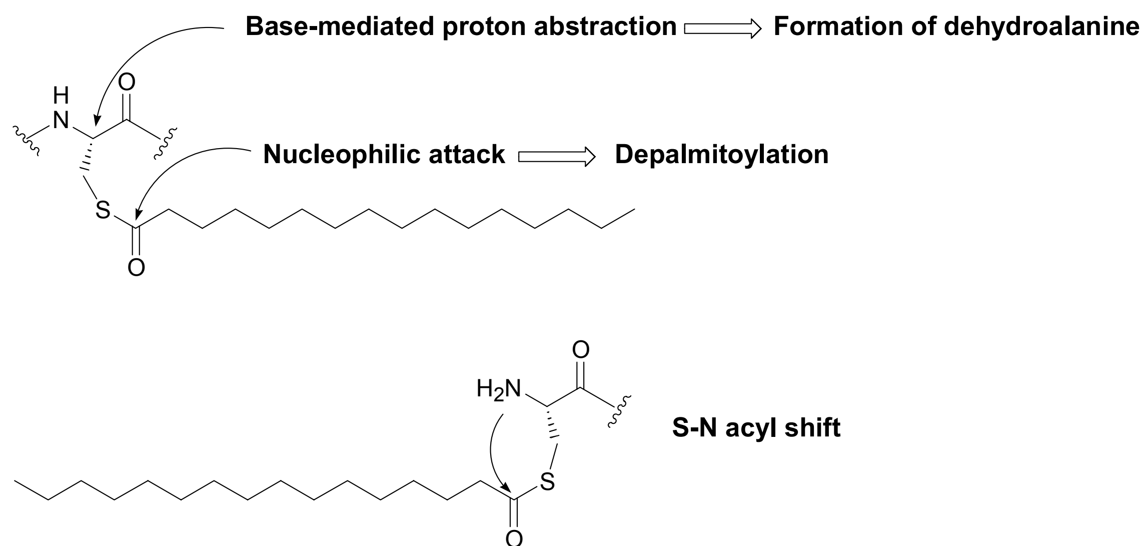
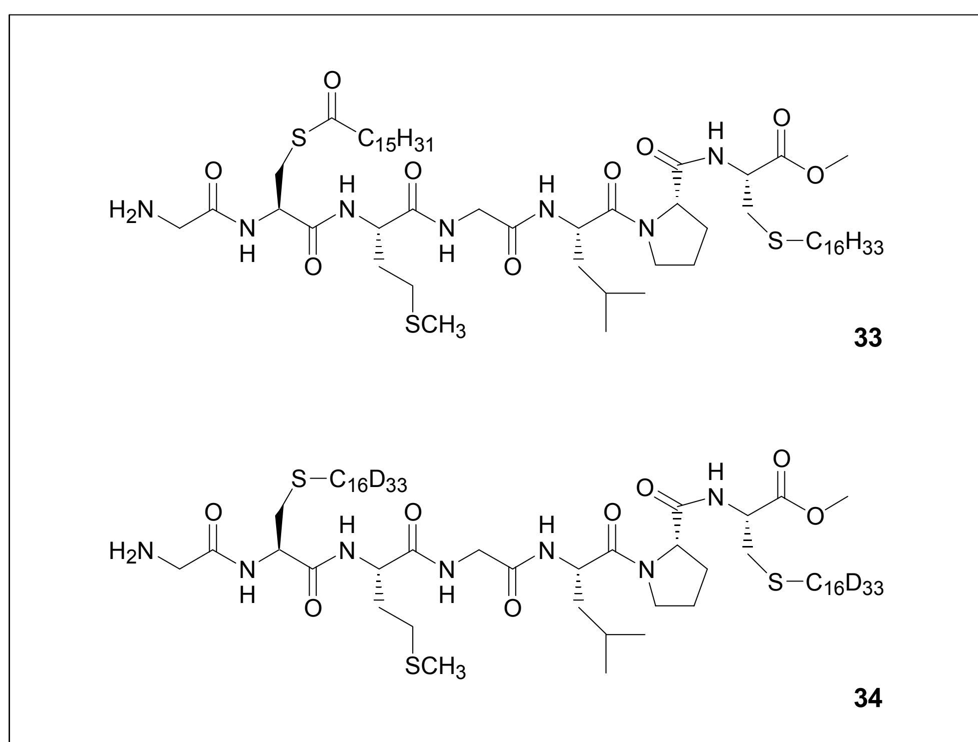


Figure 2.17: Side-reactions of the palmitoyl group.

3. Objectives

Membrane-bound lipidated proteins play a central role in cell signaling pathways which make them attractive research targets. As the activity of some of these proteins seems to be linked to their lipidation state (and perhaps their localization), it is therefore essential to understand how these proteins bind to the membrane. Already proven to be effective tools for Ras localization studies to the plasma membrane[39], lipidated N-Ras peptides, which possess the characteristic lipidation pattern of their parent lipidated proteins, can also be used to study their structure and dynamics in a model lipid membrane by ^{1}H HR MAS NMR[40].

The object of this work is to synthesize peptides **33** and **34**, hexadecyl modified, lipidated C-terminus fragment peptides of the N-Ras protein. The labile farnesyl moiety of the original protein has been substituted for a hexadecyl chain in order to simplify the interpretation of the spectra from the NMR experiments and improve signal separation.



4. Results and Discussion:

4.1 Retrosynthetic Analysis of Lipidated Ras Peptides 33 and 34

The modified N-Ras peptide was synthesized according to Kuhn[41]. The peptide was modularly built from three building blocks, namely, the PG-Gly-Cys(R)-OH dipeptide (**35**), the central PG-Met-Gly-Leu-Pro-OH (**36**), and the C-terminal H-Cys(R)-OMe methyl ester (**37**). This synthetic strategy permits the rapid assembly of the heptapeptide with different cysteine R side chains.

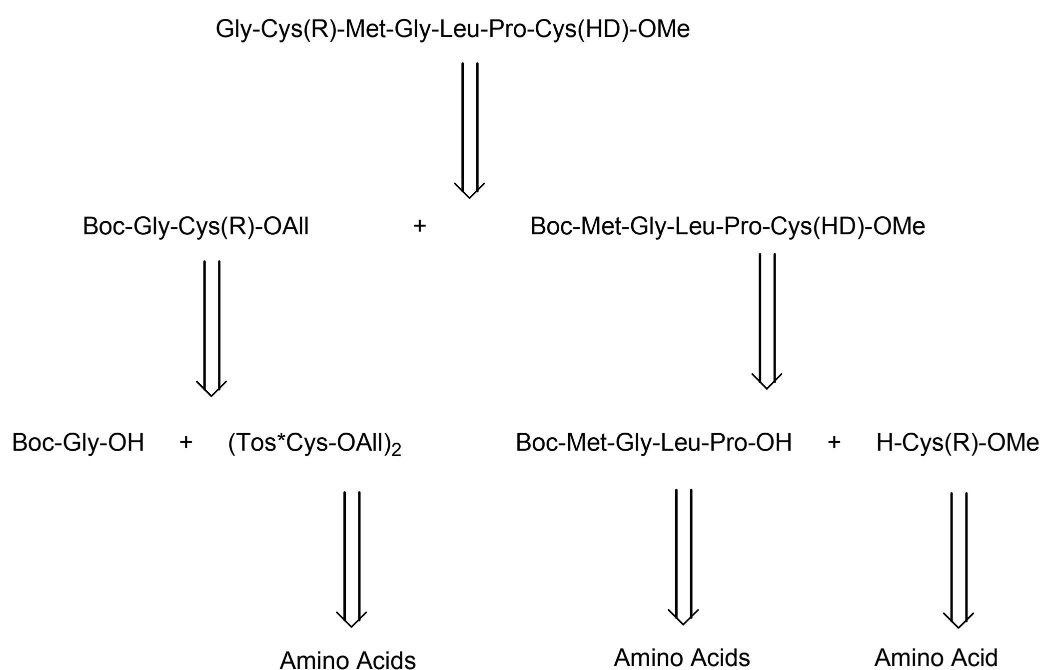


Figure 4.1: Retrosynthetic strategy for the N-Ras lipidated peptide.

4.2 Synthesis of the unlabelled N-Ras Heptapeptide

4.2.1 Synthesis of lipidated building blocks

4.2.1.1 Synthesis of HD-X and H-Cys(HD)OMe

The synthesis of hexadecylated building block (**37**) following the method described by Xue et al.[42] gave the desired product in unacceptable yields of 23-26%. The yields were substantially increased to 75% by improving the work-up procedures and

by using the more reactive hexadecyl iodide instead of hexadecyl bromide. The literature described workup procedure involves evaporation of the solvent, and the resulting crude oil be brought up in an 1N HCl solution followed by product extraction in dichloromethane. Instead of bringing up the crude oil in acid, bringing it up in $NaHCO_3$ dramatically increases the yield: non-ionic, it is easier to extract out of the aqueous phase into organic solvents.

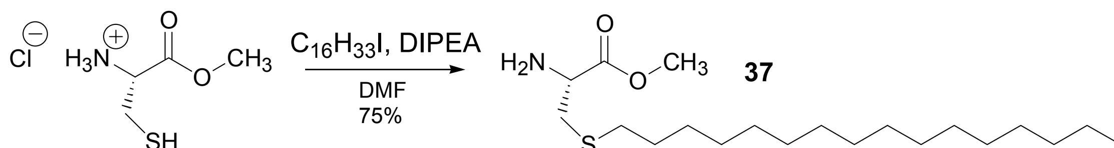


Figure 4.2: Synthesis of hexadecylated Cysteine building block **37**.

4.2.1.2 Synthesis of lipidated dipeptide Boc-Gly-Cys(R)-OH

To prevent S-N-acyl shift of the palmitoyl group during the synthesis, the palmitoylated building block needs to be synthesized as a dipeptide: **35.** The coupling of Boc-Gly-OH with $(Tos*Cys\text{-}OAll)_2$ was first tried, without success, with EDC as coupling reagent. **38** was synthesized from the coupling of Boc-Gly-OH and $(Tos*Cys\text{-}OAll)_2$ with EEDQ. After reducing the resulting disulfide dimer with DTT, palmitoylation was carried out. Palladium(0) catalyzed cleavage of the allyl protecting group generated (**35**) in 49% overall yield.

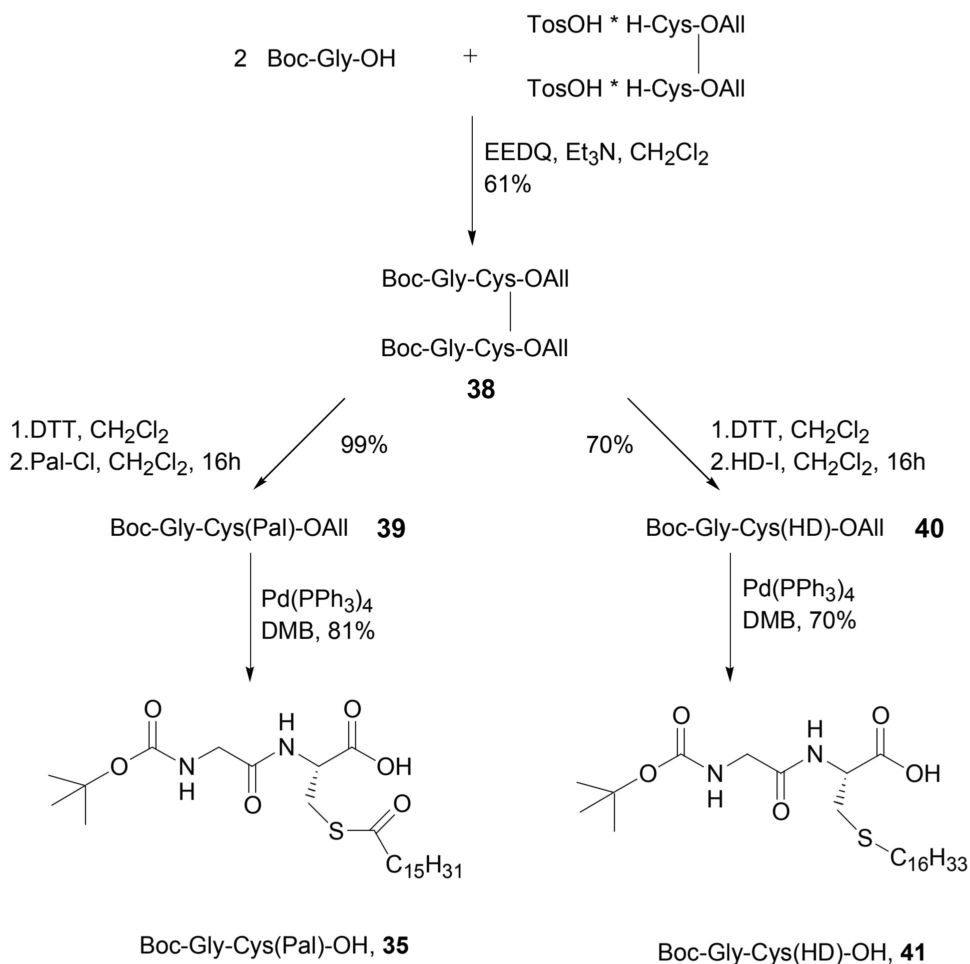


Figure 4.3: Synthesis of the lipidated dipeptides **35** and **40.**

The corresponding hexadecylated building block was produced in a similar manner, yielding **40** in 30% yield.

4.2.2 Synthesis of PG-Met-Gly-Leu-Pro-OH (36)

The core tetrapeptide Fmoc-Met-Gly-Leu-Pro-OH (**42**) was initially rapidly assembled on solid support using the 2-chlorotrityl resin, HBTU/HOBt coupling methodology and Fmoc-protected amino acids (**Fig. 4.4**).

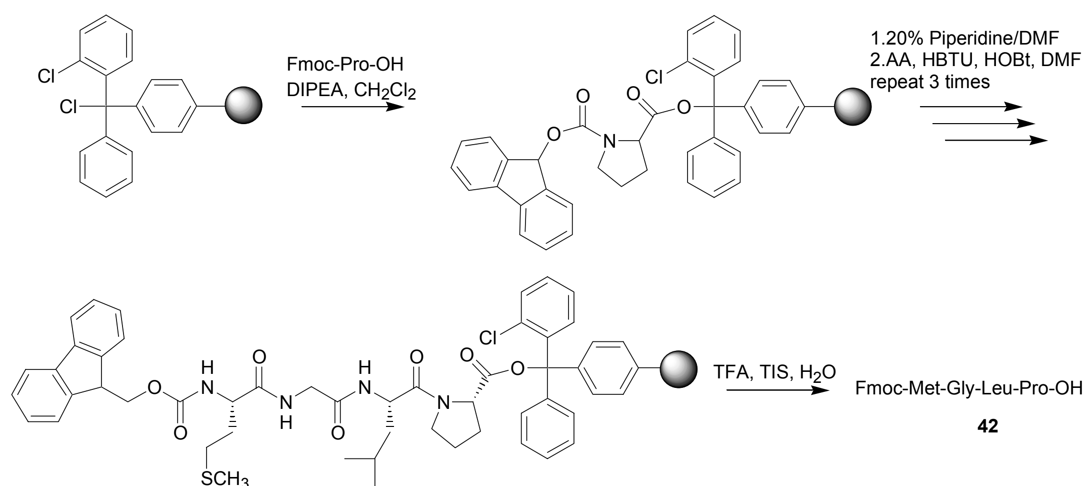


Figure 4.4: Solid phase synthesis of Fmoc-Met-Gly-Leu-Pro-OH, **42**.

Although rapid, the solid phase synthesis of the tetrapeptide in large quantities is not economically advantageous. In solution, Boc-Met-Gly-Leu-Pro-OH (**43**) was synthesized from the coupling of Boc-Met-Gly-OH **44** and TFA*H-Leu-Pro-OAll **45**. Boc-Met-Gly-OH was synthesized by first forming the hydroxysuccinimide active ester, and then reacting it with glycine. The H-Leu-Pro-OAll building block was assembled by first coupling Boc-Leu-OH with Tos*H-Pro-OAll using the EEDQ method, then cleaving the Boc group with TFA.

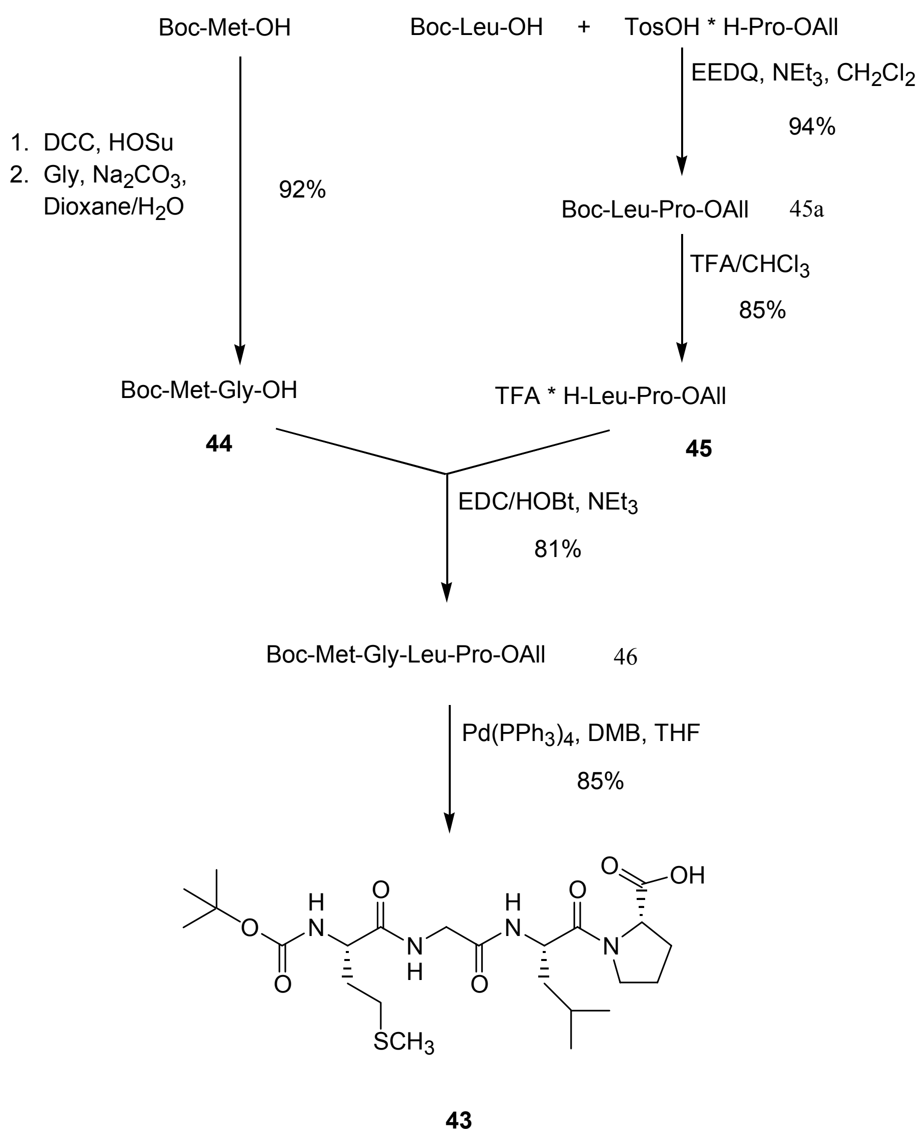


Figure 4.5: Synthesis of tetrapeptide Boc-Met-Gly-Leu-Pro-OH, **43**.

4.2.3 Assembly of lipidated peptide 33

Tetrapeptide **42** is first coupled to hexadecylated **37** with standard EDC methodology. After Fmoc deprotection, **47** is coupled to dipeptide **35**, generating **48**. Final Boc deprotection of the N- terminal glycine affords the target peptide **33 (Fig. 3.6**).

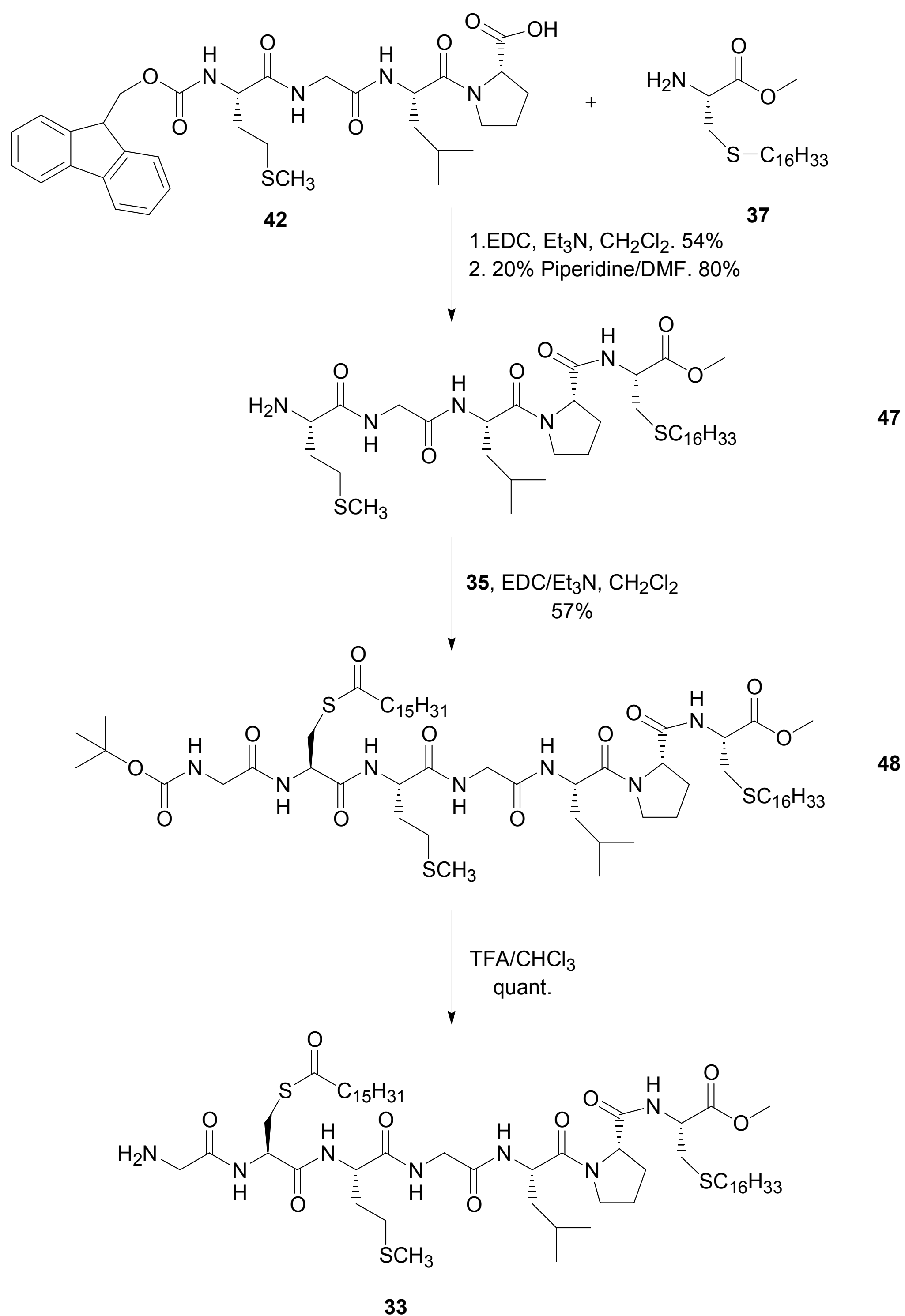


Figure 4.6: Assembly of the target peptide, **33**.

4.3 Synthesis of the Deuterium-Labelled Lipidated N-Ras Heptapeptide

4.3.1 Preparation of Labelled Lipidated Building Blocks

4.2.1.1 Synthesis of HD-d_{33}-I (49)

Due to the encouraging yields in the preparation of the unlabeled compounds, it was decided to undertake the synthesis of the deuterated hexadecyl cysteine with hexadecyl-d_{33} iodide (**49**). As hexadecyl-d_{33} alcohol, and not hexadecyl-d_{33} iodide, can be purchased, different methods for the efficient transformation of hexadecyl alcohol to hexadecyl iodide were probed on unlabelled substrate (Table 1). The most efficient synthesis involved the Appel reaction [43] (PPH_3, I_2, imidazole), yielding hexadecyl iodide in 98% yield.

Product	Reagents	Yield
HD-Br	PBr_5, toluene	24%
HD-Br	$MeSO_2Cl$, pyr, DCM; NaBr, DMSO, Δ	61%
HD-Br	PPH_3, CBr_4, DCM	94%
HD-I	$CeCl_3 \cdot H_2O$, NaI, acetonitrile, Δ	30%
HD-I	$MeSO_2Cl$, pyr, DCM; NaI, DMSO, Δ	59%
HD-I	PPh_3, imidazole, I_2, ether/acetonitrile	98%

Table 1: Halogenation of hexadecyl alcohol.

The desired hexadecyl-d_{33} iodide was thus prepared from hexadecan-d_{33}-ol in 85% yield using the Appel reaction.

4.2.1.2 Synthesis of H-Cys(HD-d_{33})-OMe (50)

H-Cys(HD-d_{33})-OMe (**50**) was prepared following the optimized procedures worked out for the non-labelled building block **37** (**Fig. 4.7**). The yields for the deuterated compounds ranged consistently between 50-60%.

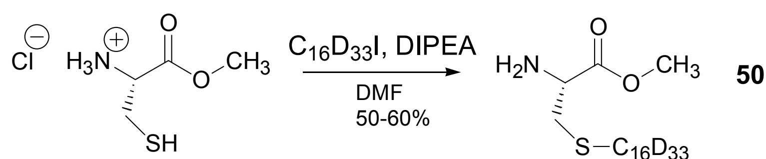


Figure 4.7: Synthesis of labelled cysteine methyl ester, **50**.

4.2.1.2 Synthesis of lipidated dipeptide Boc-Gly-Cys(HD-d_{33})-OH (51)

Dipeptide **51** was synthesized similarly to the non-labelled substrate (Fig. 4.3) begining from $(Boc\text{-}Gly\text{-}Cys\text{-}OAll)_2$ (**38**) in an overall yield of 57%.

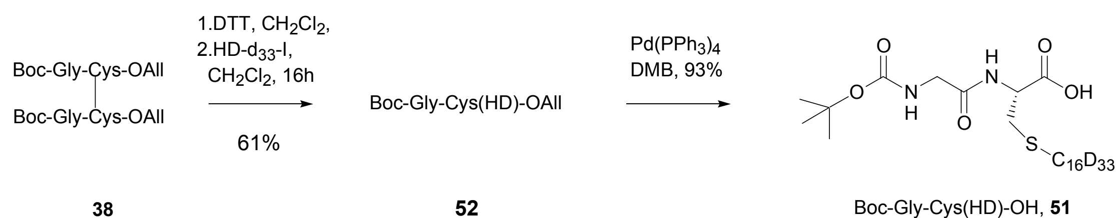


Figure 4.8: Synthesis of **51**.

4.3.2 Peptide Assembly

The target deuterated N-Ras peptide was assembled in solution analogously to the non-labelled peptide starting from Boc-Met-Gly-Leu-Pro-OH (**43**).

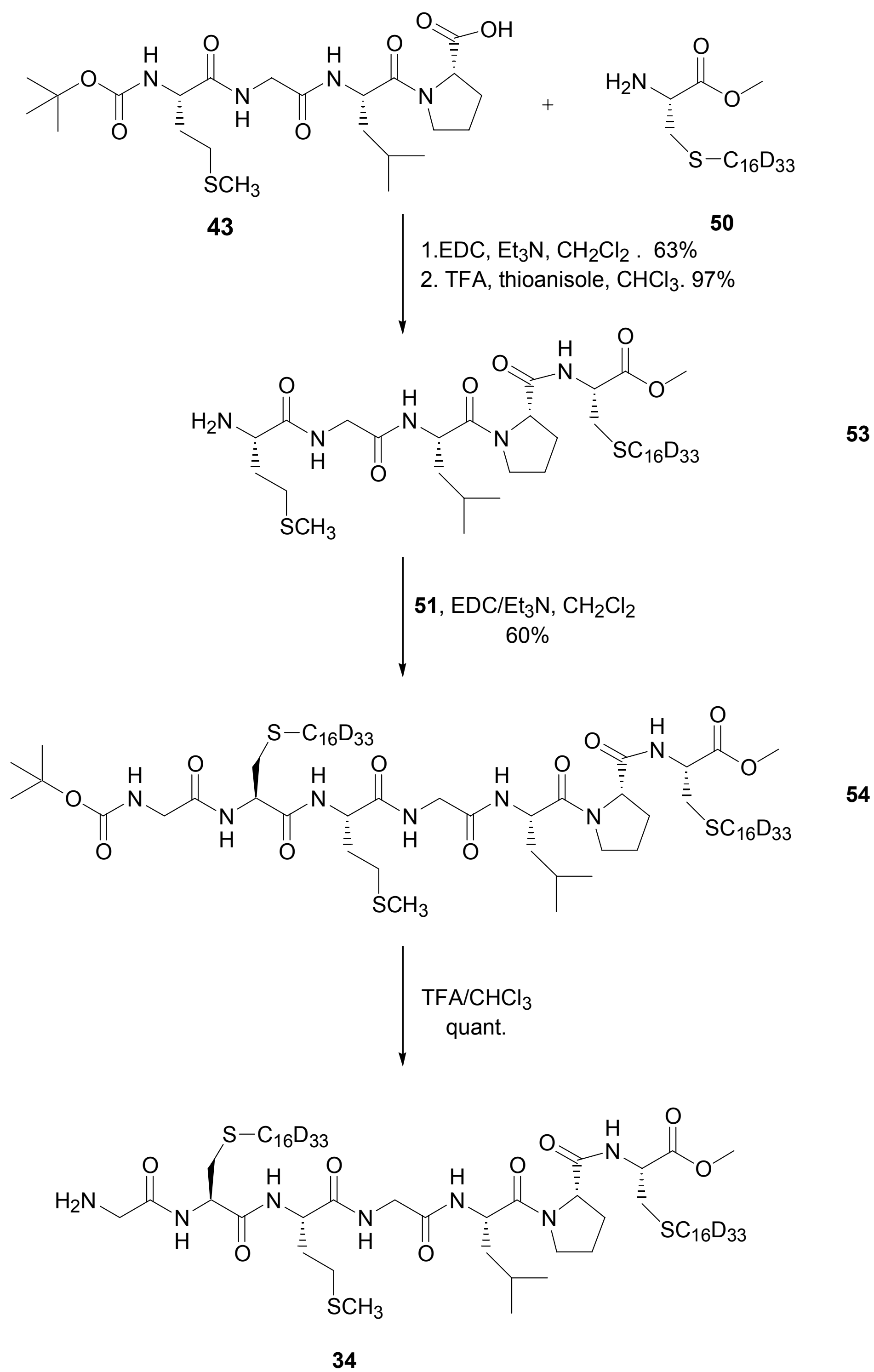


Figure 4.9: Assembly of **34**.

5. Conclusion and Outlook

The study of the structural and dynamical properties of lipidated peptides and proteins to membrane has long been limited by a lack of methods for the synthesis of lipopeptides and permitting biophysical techniques. The synthetic problems have been mostly overcome and there now exist new ^{1}H HR MAS NMR methods which would make these structural and dynamical studies possible[40].

The dually lipidated, modified, N-Ras peptide **33** was and will be used as a model compound in developing this NMR method. Dually deuterium-labelled N-Ras **34** will also serve useful.

The modified N-Ras peptide contains a hexadecyl group instead of the original farnesyl group to improve the interpretation of the spectra from NMR experiments.

The deuterated peptide **34** contains two hexadecyl groups instead of one in order to cut costs and simplify the synthesis.

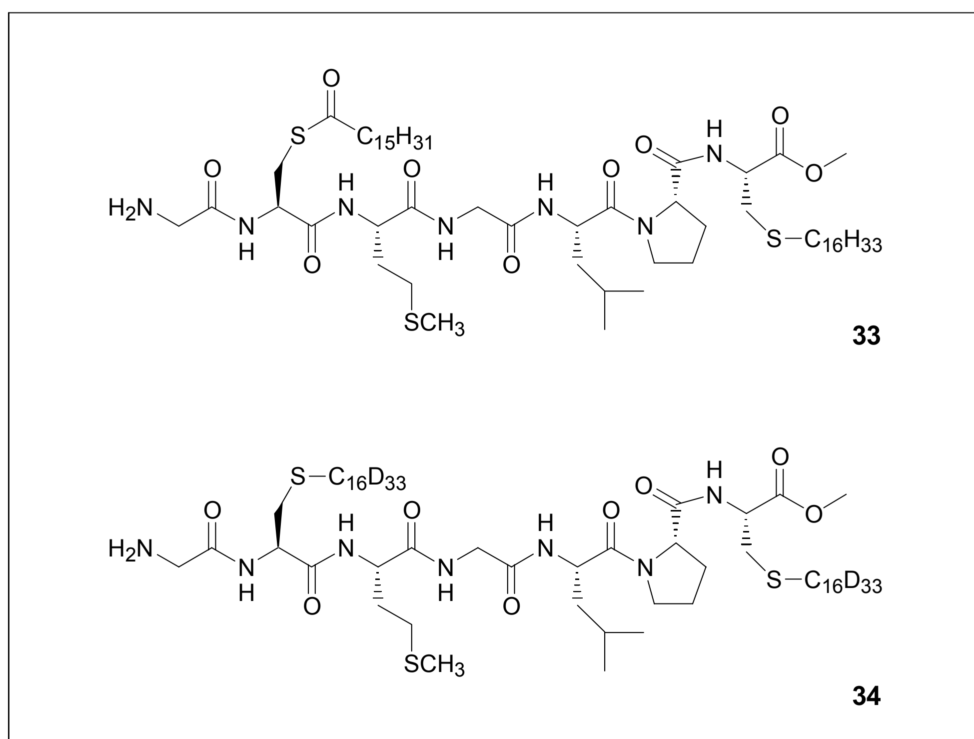


Figure 5.1: The target lipidated-modified N-Ras heptapeptides **33** and **34**.

The lipid groups were introduced onto dipeptide **38** to prevent possible S-N acyl shifts that might occur when the cysteine's N-terminus is deprotected (**Fig. 5.2**).

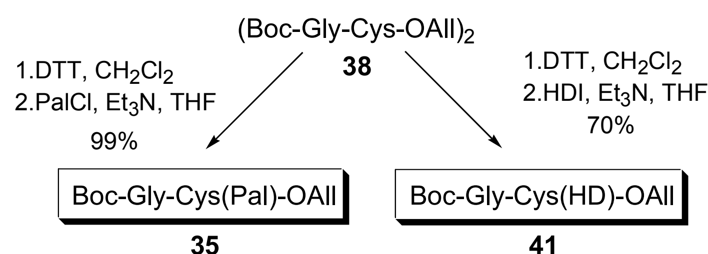


Figure 5.2: Lipidation of **38**.

Due to its' quicker accessibility, the core tetrapeptide **36** was prepared on 2-chlorotrityl resin with an overall yield of 52%.

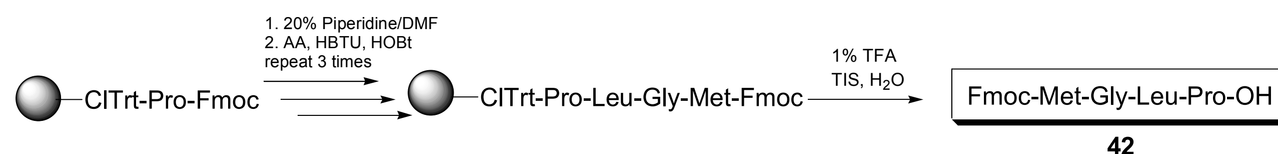


Figure 5.3: Solid Phase synthesis of Fmoc-Met-Gly-Leu-Pro-OH, **42**.

As excess reagents is used in SPPS, it is not a suitable method when the reagents are excessively expensive (e.g. when isotopically labeled reagents are required). For that reason, the core tetrapeptide was also prepared in solution.

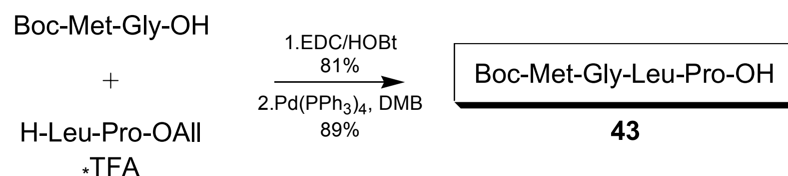


Figure 5.4: Solution synthesis of core tetrapeptide, **43**.

The final assembly steps for the target peptide **33** were carried out in solution using standard EDC coupling methods. After TFA deprotection of the Boc group, the target peptide **33** was obtained in 6.3% yield.

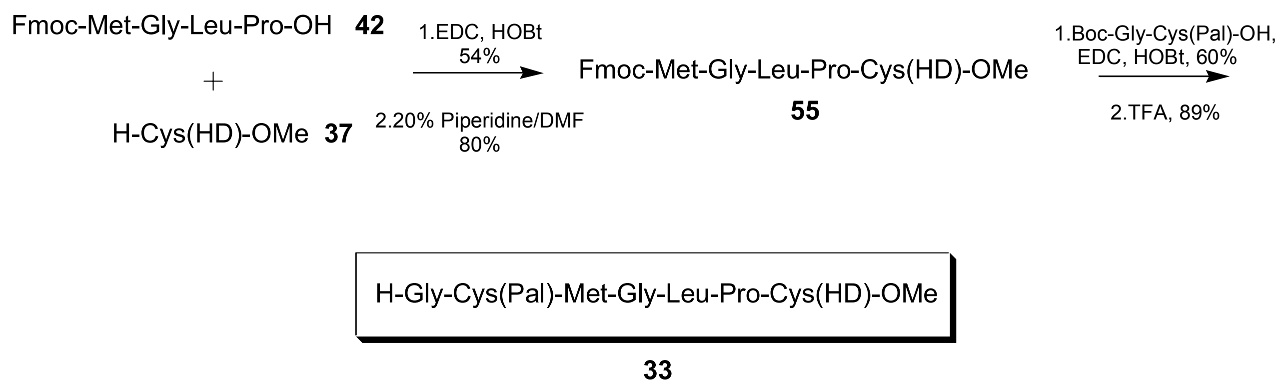


Figure 5.5: Assembly of the target heptapeptide, **33**.

In preparation for the synthesis of deuterated heptapeptide **34** for deuterium membrane dynamics NMR experiments, iodination (**Fig 5.7**) and hexadecylation reactions (**Fig. 5.8**) were successfully optimized on non-labeled substrate.

As only hexadecyl-d-$_{33}$-ol is commercially available, hexadecyl-d_{33} iodide needs to be synthesized in order to lipidate H-Cys-OMe. The best iodination procedure involved the use of PPh_3, I_2 and imidazole [44].

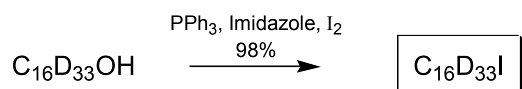


Figure 5.7: Iodination of hexadecyl-d_{33}-ol.

The hexadecylation reaction of **37** was undertaken under the same conditions as the palmitoylation (**Fig. 5.2**).
The deuterated target peptide **34** was prepared with an overall yield of 20%.

6. Experimental section

6.1 Materials and methods

NMR spectroscopy

The ^{1}H and ^{13}C NMr spectra were recorded on the following instrument:

400 MHz ^{1}H and 100.6 MHz ^{13}C NMR Varian Mercury 400

The chemical shifts were referenced against CHCl3 solvent peak and are given in ppm. The coupling constants J are given in Hz and the multiplicities are abbreviated as follows: s = singlet; d = doublet; t = triplet; m = multiplet.

Mass spectrometry

The MALDI-TOF spectra were measured on a Voyager-DE Pro BioSpectrometry™ Workstation from PerSeptive Biosystems using 2,5-dihydroxy benzoic acid as matrix.

Optical rotation:

The optical rotation $[\alpha]_D^{20}$ was measured using a Perkin-Elmer polarimeter 241. The concentrations and solvents used are noted in parenthesis.

Chromatography:

Thin layer chromatography plates were obtained from Merck (Silica gel 60, F_{254}). Tlcs were visualized under UV light (λ = 254 nm, 366 nm). The tlcs were stained using the following stains:

a) 2.5 g molybdate phosphoric acid, 1 g cerium (IV)-sulfate, 6 ml conc. H_2SO_4 in 94 ml H_2O.

b) 0.3 g ninhydrin, 3 ml acetic acid, 100 ml ethanol.

c) 1.6 g potassium permanganate, 10 g potassium carbonate, 2.5 ml 5% sodium hydroxide, 200 ml H_2O

The solvent system and Rf values are noted for all synthesized substances.

Silica gel column chromatography was undertaken on silica gel from Baker (40-60 μm).

Chemicals

All chemicals were purchased from the following suppliers and used without further purification: Aldrich, Bachem, Fluka, Senn Chemicals.

Solvents

The solvents were distilled prior to use following standard protocols.[45]

6.2 Compounds from chapter 4.2

L-Cystin-bis-(allyl ester)

(Tos·Cys-OAll)$_2$

Cystin (5 g, 20.8 mmol), p-Tos-OH (15.83 g, 82.22 mmol), allyl alcohol (17 ml, 249.6 mmol) and 300 ml benzene were combined in a 500 ml round bottom flask equiped with a Dean-Stark condensor. The mixture was heated to 90°C for 16h. The benzene and allyl alcohol were evaporated under reduced pressure. The product was precipitated from the resulting oil with diethyl ether and then dried in vacuo.

White solid.

Yield: 14.1g (100%).

^{1}H-NMR (400 MHz, MeOD):

δ = 7.74 (d, J= 8.24Hz, 4H, CH p-Tos-OH), 7.27 (d, J= 8.4Hz, 4H, CH p-Tos-OH), 6.04 (m, 2H, $\underline{C}H=CH_2$), 5.45 (dd, 2H, J=17.2Hz, J=, $CH=\underline{C}H_2$), 5.34 (dd, 2H, J=10.36Hz, J=, $CH=\underline{C}H_2$), 4.80 (m, 4H, OCH_2), 4.51 (m, 2H, α-CH Cys), 3.37 (m, 4H, β-CH_2 Cys), 2.41 (s, 6H, Me).

Bis-(N-*tert*-butyloxycarbonyl-L-glycyl)-L-cystin-bis-(allyl ester)

((Boc-Gly-Cys-OAll)$_2$) (38)

(TosOH·Cys-OAll)$_2$ (2.5 g, 3.75 mmol) was first dissolved in 100 ml of DCM and 20 ml TFE. Boc-Gly-OH (1.31 g, 7.7 mmol) was then added. The solution was then cooled to 0oC before EEDQ (2.4 g, 9.75 mmol) and triethylamine (1.04 ml, 7.5

mmol) were added. The reaction was brought up to room temperature overnight. After 18h, the reaction organic phase was washed 3 times each with 1N HCl, sat. Sln $NaHCO_3$, brine followed by drying over Na_2SO_4. The crude oil was purified by flash chromatography in 1:1 ethyl acetate/cyclohexane.

Yellow oil.
Yield: 1.463 g (61 %)
Rf value: 0.17 (1:1 ethyl acetate/cyclohexane)
$[\alpha]_D^{20}$: -51.8° (c = 1.1, MeOH)

^{1}H-NMR (400 MHz, $CDCl_3$):
δ = 7.16 (m, 2 H, CONH), 5.88 (m, 2 H, C<u>H</u>=CH_2), 5.52 (m, 2 H, OCONH), 5.35 (dd, J = 17.3 Hz, ^{3}J = 1.56 Hz, 2 H, CH=C$\underline{H}_{2a}$), 5.28 (dd, J = 10.56, J = 1.4 Hz, 2 H, CH=C$\underline{H}_{2b}$), 4.88 (m, 2 H, α-CH Cys), 4.66 (m, 4 H, OCH_2), 3.87 (m, 4 H, α-CH_2 Gly), 3.20 (m, 4 H β-CH_2 Cys), 1.46 (s, 18 H, $C(CH_3)_3$).

^{13}C-NMR (125 MHz, $CDCl_3$):
δ =169.84 (6*C=O), 131.28 (2* <u>C</u>H=CH_2), 119.29 (2* CH=<u>C</u>H_2), 66.57 (2* CH_2-O, Allyl), 52.07 (cys), 41.10 (gly), 28.31 (6* CH_3, Boc).

N-*tert*-butyloxycarbonylglycyl-S-palmitoyl-L-cysteine allyl ester
(Boc-Gly-Cys(Pal)-OAll) (39)

DTT (1.36 g, 8.82 mmol) and triethylamine (0.613 ml, 4.4 mmol) were added to a degassed solution of $(Boc\text{-}Gly\text{-}Cys\text{-}OAll)_2$ in 50 ml DCM. After 1.5 hours, the reaction was extracted three times with H_2O and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to a colorless oil. The oil was then dissolved in 10 ml freshly distilled THF and cooled to 0°C before adding triethylamine (0.754 ml, 5.41 mmol) and palmitoyl chloride (2.08 ml, 6.86 mmol). After 2 hours, the reaction was filtered over celite, and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography in 2:5 ethyl acetate/cyclohexane.

White solid

Yield: 1.21 g (99 %)

Rf Value: 0.53 (1:1 ethyl acetate/cyclohexane)

$[\alpha]_D^{20}$: -14.0° (c = 1.0, MeOH)

^{1}H-NMR (400 MHz, $CDCl_3$):

δ =5.88 (m, 1H, $CH=CH_2$), 5.34 (dd, ^3J = 17.2 Hz, ^2J = 1.4 Hz, 2H, $CH=C\underline{H}_{2a}$), 5.27 (dd, ^3J = 10.5 Hz, ^2J = 1.0 Hz, 1H, $CH=C\underline{H}_{2b}$), 4.82 (m, 1H, α-CH Cys), 3.80 (m, 2H, α-CH_2 Gly, β-CH_2 Cys), 2.56 (t, ^3J = 7.2 Hz, α-CH_2 Pal), 1.60-1.65 (m, 2H, β-CH_2 Cys), 1.46 (s, 9H, $C(CH_3)_3$), 1.23 (s, 24 H, $(CH_2)_{12}$ Pal), 0.87 (t, ^3J = 6.5 Hz, ω-CH_3 Pal).

^{13}C-NMR (125 MHz, $CDCl_3$):

δ =169.62 (C=O), 35.28, 31.91, 29.66, 29.65, 29.63, 29.61, 29.57, 29.55, 29.38, 29.34, 29.23, 29.18, 24.22 (13*CH_2 Pal), 22.67 (CH_2 Pal), 14.09 (ω-CH_3 Pal)

N-*tert*-butyloxycarbonylglycyl-S-palmitoyl-L-cysteine

(Boc-Gly-Cys(Pal)-OH) (35)

Boc-Gly-Cys(Pal)-OAll (1.2 g, 2.15 mmol) and DMB (0.673 g, 4.31 mmol) were dissolved in 100 ml of freshly distilled THF and the solution was degassed. After addition of palladium triphenyl phosphine tetrakis (50 mg, 0.043 mmol), the reaction was stirred in the dark under argon atmosphere for two hours. The solvent was evaporated under reduced pressure, and the crude substance was redissolved in DCM. The organic phase was washed three times with 1M HCl, and then dried over Na_2SO_4. After evaporation, the crude solid was purified by flash chromatography first with 1:2 ethyl acetate/cyclohexane; the product was eluted with 1:7 ethanol/DCM.

Pale yellow solid.

Yield: 81%

Rf value: 0.3 (10:1 ethyl acetate/methanol, + 1% vol. acetic acid)

$[\alpha]_D^{20}$: -5.0 (c = 1.1, MeOH)

^{1}H-NMR (400 MHz, $CDCl_3$):

δ = 4.37-4.41 (m, 1H, α-CH Cys), 3.84 (d, J = 17 Hz, 1H, α-CH2a Gly), 3.69 (d, J = 17 Hz, 1H, α-CH2a Gly), 3.42-3.46 (m, 1H, β-CH_2 Cys), 2.55 (t, 3J = 7.3 Hz, α-CH_2 Pal), 1.46 (s, 9H, $C(CH_3)_3$), 1.24 (s, 24H, $(CH_2)_{12}$ Pal), 0.86 (t, ^3J = 6.5 Hz, ω-CH_3 Pal).

S-hexadecyl-L-Cysteine methyl ester
(H-Cys(HD)-OMe) (37)

Diisopropylethylamine (101 µl, 0.582 mmol) and hexadecyl iodide (205 mg, 0.582 mmol) were added to a stirring solution of Cys-OMe·HCl (50 mg, 0.291 mmol) in DMF. After 18 hours, the solvent was removed under reduced pressure. The remaining solid was brought up in diethyl ether and saturated $NaHCO_3$. The aqueous phase was extracted three times with diethyl ether. The combined organic phases were washed with brine and dried over Na_2SO_4. The crude oil was further purified by flash chromatography in 1:1 ethyl acetate/cyclohexane.

White solid.
Yield: 78 mg (75 %)
Rf value: 0.26 (1:1 ethyl acetate/cyclohexane)

^{1}H-NMR (400 MHz, $CDCl_3$):
δ = 3.75 (s, 3H, OCH_3), 3.68 (m, 1H, α-CH Cys), 2.89 (m, 1H, β-CH_2 Cys), 2.51 (t, J = 7 Hz, 2H, α-CH_2 HD), 1.50 (m, 2H, β-CH_2 HD), 1.18 (br s, 26H, $(CH_2)_{13}$ HD), 0.81 (t, J = 7 Hz, 3H, ω-CH_3 HD)

^{13}C-NMR (125 MHz, $CDCl_3$):
δ =52.73 (α-CH Cys), 34.92 (β-CH_2 Cys), 31.91, 31.90, 29.69, 29.65, 29.53, 29.50, 29.35, 29.22, 28.78 (9* CH_2 HD), 22.68 (CH_2 HD), 14.10 (ω-CH_3 HD).

MALDI-TOF m/z:	calc.: 360.61 [M+H]+	found: 360.71
	calc.: 382.60 [M+Na]+	found: 382.69

6.3 Compounds from chapter 4.2.2

N-tert-butyloxycarbonylleucyl-L-proline allyl ester

(Boc-Leu-Pro-OAll) (45a)

Boc-leucine (1.36g, 5.0 mmol) and Tos·Pro-Oall (1.93 g, 5.9 mmol) were dissolved in 100 ml of freshly distilled dichloromethane. The clear, colorless solution was cooled to 0°C before adding triethylamine (0.821 ml, 5.9 mmol) and EEDQ (1.9 g, 7.67 mmol). The solution was left to come up to room temperature overnight. After 16 hours, the organic phase was extracted twice each with 1M HCl, saturated $NaHCO_3$, and then once with brine. The combined organic phases were dried over Na_2SO_4 and evaporated under reduced pressure. The crude oil was purified by flash chromatography in 1:3 ethyl acetate:cyclohexane.

Colorless oil.

Yield: 2.06 g (94%).

Rf value: 0.56 (1:1 ethyl acetate/cyclohexane)

$[\alpha]_D^{20}$: -72.5° (c = 1.0, $CHCl_3$)

^{1}H-NMR (400 MHz, $CDCl_3$):

δ = 5.88 (m, 1H, <u>C</u>H=CH_2), 5.33 (dd, J= 17.2Hz, J= 1.4Hz, 1H, CH=CH_2a), 5.29 (dd, J=10.4Hz, J=4.1Hz, 1H, CH=CH_2b), 5.11 (d, J= 9Hz, 1H, OCONH), 4.60 (m, 2H, OCH_2), 4.54 (m, 1H, α-CH), 4.46 (m, 1H, α-CH), 3.76 (m, 1H, δ-CH_{2a} Leu), 3.59 (m, 1H, δ-CH_{2b}), 2.21 (m, 1H, β-CH_{2a} Pro), 2.01 (m, 3H, β-CH_{2b} Pro, γ-CH_2 Pro), 1.74 (m, 1H, γ-CH Leu), 1.49 (m, 2H, β-CH_2 Leu), 1.41 (s, 9H, $C(CH_3)_3$), 0.98 (d, J= 6.45Hz, 3H, ω-CH_3 Leu), 0.94 (d, J= 6.06Hz, 3H, ω-CH_3).

^{13}C-NMR (125 MHz, $CDCl_3$):

δ = 171.6 (3* C=O), 131.78 (<u>C</u>H=CH_2), 118.4 (CH=<u>C</u>H_2), 79.4 (<u>C</u>$(CH_3)_3$), 65.6 (CH_2-O, Allyl), 58.7 (α-CH Pro), 51.2 (α-CH Leu), 46.7(δ-CH Pro), 42.0 (β-CH Leu), 28.9 (β-CH Pro), 28.3 (C(<u>C</u>$H_3)_3$), 24.8 (γ-CH Pro), 24.5(γ-CH Leu), 23.3, 21.8 (2*ω-CH).

L-Leucyl-L-proline allyl ester

(H-Leu-Pro-OAll) (45)

Boc-Pro-Leu-OAll(181 mg) was dissolved in 2 ml chloroform. The clear, colorless solution was cooled to 0oC before adding 2 ml TFA. After 15 min, the mixture was brought up to room temperature and stired for an additional 1.5h. The TFA and chloroform were then co-evaporated with toluene under reduced pressure.

Colorless oil.
Yield: 0.171 g (91%).
$[\alpha]_D^{20}$: -53.6^o (c = 1.0 $CHCl_3$).

^{1}H-NMR (400 MHz, $CDCl_3$):
δ = 5.84 (m, 1H, $\underline{C}H=CH_2$), 5.27 (dd, $^3J_{trans}$=17.3Hz, ^{2}J= 1.4Hz, 1H, $CH=C\underline{H}_{2a}$), 5.22 (dd, $^3J_{cis}$=10.4Hz, ^{2}J=1.1Hz, 1H, $CH=C\underline{H}_{2b}$), 4.58 (m, 2H, OCH2), 4.46 (m, 1H, α-CH), 3.68 (m, 1H, δ-CH_{2b}), 3.48 (m, 1H, δ-CH_{2b}), 2.23 (m, 1H, β-CH_{2a} Pro), 2.02 (m, 3H, β-CH_{2b} Pro, γ-CH_2 Pro), 1.72 (m, 1H, γ-CH Leu), 1.52 (m, 2H, β-CH_2 Leu), 0.97 (d, J= 6.65Hz, 3H, ω-CH_3 Leu), 0.94 (d, J= 6.45Hz, 3H, ω-CH_3).

N-tert-butyloxycarbonylmethionylglycine

(Boc-Met-Gly-OH) (44)

Boc-Met-OH (2 g, 8.02 mmol) was dissolved in 20 ml of dry THF and cooled to 0°C in an ice water bath before adding N-hydroxysuccinimide (0.923 g, 8.02 mmol) and dicyclohexyl carbodiimide (1.72 ml, 8.34 mmol). The mixture was stirred at room temperature for 16h after which the dicyclohexyl urea byproduct was filtered away. The filtrate was then evaporated under reduced pressure to a white solid, which was redissolved in 10 ml dioxane and added to a clear, colorless solution of glycine (0.903 g, 12.03 mmol) and NaOH (0.320 g, 8.02 mmol) in 40 ml water and 10 ml dioxane. The mixture was stirred at room temperature for 3 days before the dioxane was evaporated under reduced pressure. The pH of the remaining aqueous solution was adjusted to 8 and then twice extracted with dichloromethane and once with diethyl ether. The aqueous phase was then acidified to pH 2, extracted three times with ethyl acetate, affording **5** as a white amorphous solid (2.26 g, 92% yield).

White amorphous solid.
Yield: 2.26 g (92%).
Rf value: 0.1 (1:1 cyclohexane/ethyl acetate, 1% acetic acid).
$[\alpha]_D^{20}$: -16.6° (c = 1.1, MeOH).

^{1}H NMR (400 MHz, MeOD):
δ = 4.17-4.25 (m, 1H, α-CH_2 Met), 3,98 (d, J = 17.5 Hz, 1H, α-CH_{2a} Gly), 3.85 (d, J= 17.5 Hz, 1H, α-CH_{2b} Gly), 2.50-2.58 (m, 2H, γ-CH_2 Met), 2.09 (s, 3H, SCH_3), 1.98-2.08 (m, 1H, β-CH_{2a} Met), 1.81-1.94 (m, 1H, β-CH_{2b} Met), 1.43 (s, 9H, $C(CH_3)_3$).

^{13}C (100.6 MHz, MeOD)
δ = 172.72, 172.50 (2* C=O), 41.54, 32.14, 30.29, 28.52 ($C(\underline{C}H_3)_3$), 25.65, 15.51 (SCH_3).

N-tert-butyloxycarbonylmethionylglycyl-L-leucyl-L-proline allyl ester (Boc-Met-Gly-Leu-Pro-OAll) (46)

Boc-Met-Gly-OH (120 mg, 0.392 mmol) and Leu-Pro-OAll·TFA (150 mg, 0.392 mmol) were dissolved in DCM in a warm water bath. EDC (97 mg, 0.509 mmol), triethylamine (54.7 µl, 0.392 mmol) and HOBt (90 mg, 0.588 mmol) were added to the 0oC cooled slightly yellow colored solution. The mixture was left to come up to room temperature overnight. After 16h, the organic phase was washed twice each with 1M HCl and sat. $NaHCO_3$. The organic phases were combined and dried over Na_2SO_4 and evaporated under reduced pressure. The resulting oil was chromatographed in straight ethyl acetate.

White solid.
Yield: 177 mg (81%)
Rf value: 0.41 (100% ethyl acetate)
$[\alpha]_D^{20}$: -36.2° (c = 1.0 $CHCl_3$)

^{1}H-NMR (400 MHz, $CDCl_3$):

δ = 5.85 (m, 1H, <u>C</u>H=CH_2), 5.31 (dd, J= 16Hz, J= 1.4Hz, 1H, CH=CH_{2a}), 5.23 (dd, J=10.36Hz, J=4.1Hz, 1H, CH=CH_{2b}), 4.77 (m, 1H, α-CH), 4.60 (m, 1H, OCH_2), 4.55 (m, 1H, OCH_2), 4.26 (m, 1H, α-CH), 3.99 (m, 1H, α-CH_{2a} Gly), 3.88 (m, 1H, α-CH_{2b} Gly), 3.80 (m, 1H, δ-CH_{2b} Pro), 3.60 (m, 1H, δ-CH_{2a} Pro), 2.56 (t, J=7.24Hz, 2H, γ-CH_2 Pro), 2.09 (s, 3H, SCH_3), 1.97 (m, 1H, γ-CH Leu), 1.55 (m, 2H, β-CH_2 Leu), 1.43 (s, 9H, $C(CH_3)_3$), 0.96 (d, J= 6.64Hz, 3H, ω-CH_3 Leu), 0.93 (d, J= 5.67Hz, 3H, ω-CH_3 Leu).

^{13}C-NMR (125 MHz, $CDCl_3$):

δ = 171.93, 171.43, 171.24, 169 (4* C=O), 155.68 (OCONH), 131.73 (<u>C</u>H=CH_2), 118.56 (CH=<u>C</u>H_2), 79 (<u>C</u>$(CH_3)_3$), 65.72 (CH_2-O, Allyl), 58.91 (α-CH Pro), 53.54 (α-CH Met), 49.20 (α-CH Leu), 46.90 (δ-CH Pro), 42.72 (α-CH Gly), 41.43 (β-CH Leu), 30.18, 30.13 (CH_2 Met), 28.97 (β-CH Pro), 28.31 (C(<u>C</u>H_3)$_3$), 24.87 (γ-CH_2 Pro), 24.56 (γ-CH_2 Leu), 23.26, 21.82 (2* ω-CH_3 Leu), 15.30 (SCH_3).

N-tert-butyloxycarbonylmethionylglycyl-L-leucyl-L-proline allyl ester
Boc-Met-Gly-Leu-Pro-OAll

Boc-Met-Gly-Leu-Pro-OAll and DMB were dissolved in freshly distilled THF and the solution was then thoroughly degassed before the addition of palladium triphenylphosphine tetrakis. The clear yellow colored solution was allowed to stir at room temperature for 2 hours before evaporating the solvent under reduced pressure. The

Fluorenyl-L-methionylglycyl-L-leucyl-L-proline
(Fmoc-Met-Gly-Leu-Pro-OH) (42)

Amino acid loading proceedure: the proline loaded resin was prepared from chloro-chloro tritylchloride resin (1.08 mmol/g), by adding Fmoc proline, dissolved in DCM to preswelled resin. DIPEA was added to the slurry and was shaken for 2.5 hours at room temperature. The unreacted chloride groups were then displaced by the addtion of 6 ml MeOH with 1 ml DIPEA, and shaken for another 20 minutes. The resin was then washed 4 times each with DCM, DMF and once more with DCM. The Fmoc group of the resulting loaded resin was promptly removed with 20% piperidine in

DMF (20 minutes) in order to quench any possible acid that would result in premature cleavage.

Coupling proceedure: (repeated 4 times): the Fmoc group was first removed by shaking for 20 minutes 20 ml of a solution of 20% piperidine in DMF. The resin is washed 4 times each with 20 ml DCM and DMF. The resin was then qualitatively tested with the kaiser reagent test to ensure that the N-terminus is free. The amino acids are dissolved in DMF and then preactivated by the addition of HBTU, HOBt and DIPEA. The preactivated amino acid is added to a slurry of resin and shaken for 2 hours at room temperature, time afterwhich the resin is once again washed 4 times each with DCM and DMF.

Resin cleavage proceedure: The resin was first preswelled in DCM. After transfering to a round bottom flask, the cleavage cocktail is added (1% TFA, 1% TIS in 10 ml DCM). The slurry is shaken for 1 hour at room temperature, and the resin then filtered out. The filtrate is evaporated and the resulting oil chromatographed in ethyl acetate / MeOH (3:2).

Yield: 200 mg (52%).

Rf value: 0.35 (3:2 ethyl acetate/MeOH)

$[\alpha]_D^{20}$: -20.8° (c = 1.1, $CHCl_3$)

^{1}H-NMR (400 MHz, $CDCl_3$):

δ = 8.04 (d, J= , 1H, CONH), 7.76 (d, J= , 2H, arom. CH), 7.57-7.53 (m, 3H, arom. CH, CONH), 7.38 (t, J= , 2H, arom. CH), 7.23 (t, 2H, arom. CH), 5.61 (d, J= 8.0 Hz, 1H, CONH), 5.00-4.98 (m, 1H, α-CH), 4.40 (m, 4H, 2 * α-CH, OCH_2), 4.21 (t, 3J = 6.0 Hz, 1H, Fmoc), 4.00 (m, 2H, α-CH_2 Gly), 3.77 (m, 1H, δ-CH_2 Pro), 3.65 (m, 1H, δ-CH_2 Pro), 2.53 (m, 2H, γ-CH_2 Met), 2.35 (m, 1H, β-CH_2), 2.08 (m, 2H, β-CH_2), 2.04 (s, 3H, SCH_3), 1.97 (m, 3H, β-CH_2, γ-CH2 Pro), 1.53 (m, 3H, γ-CH2 Leu, β-CH Leu), 0.93 (t, J = 6.44 Hz, 6H, 2*ω-CH_3 Leu).

^{13}C-NMR (125 MHz, $CDCl_3$):

δ = 173.63, 172.02, 171.42 (5*C=O), 156.26 (OCONH), 144, 143.85, 141.51 (3* arom. Quart. C), 128.32, 127.95, 125.50, 120.20 (4 * arom. CH), 60.63 (α-CH Pro),

49.31 (α-CH Leu), 47.90 (δ-CH Pro), 47.36 (Fmoc CH), 43.06 (α-CH_2 Gly), 42.78 (β-CH_2 Leu), 31.96, 30.29 (2 * CH_2 Met), 29.09 (β-CH_2 Pro), 25.34 (γ-CH_2 Pro), 24.64 (γ-CH_2 Leu), 23.45, 22.32 (2 * ω-CH_3 Leu), 15.52 (SCH_3).

MALDI-TOF m/z: calc.: 638.28 $[M+Na]^+$ found: 661.59

6.4 Compounds from chapter 4.2.3

Fluorenyl-L-methionylglycyl-L-leucyl-L-prolyl-S-hexadecyl-L-cysteine methyl ester

(Fmoc-Met-Gly-Leu-Pro-Cys(HD)-OMe) (47a)

EDC (39 mg, 0.202 mmol), triethylamine (21.7 µl, 0.156 mmol) and HOBt (36 mg, 0.234 mmol) were added to a cooled, stirring solution of Fmoc-Met-Gly-Leu-Pro-OH (100 mg, 0.156 mmol) and Cys(HD)-OMe (56 mg, 0.156 mmol). The reaction was brought up to room temperature overnight. After 18h, the reaction organic phase was extracted two times each with 1N HCl, sat. $NaHCO_3$, brine followed by drying over Na_2SO_4. The crude oil was purified by flash chromatography in straight ethyl acetate.

Pale yellow solid.
Yield: 83 mg (54 %)
Rf value: 0.68 (10:1 ethyl acetate/methanol + 1% vol. acetic acid)

^{1}H-NMR (400 MHz, $CDCl_3$):
δ = 8.18 (d, J = 5.28 Hz, 1H, CONH), 7.74 (d, J = 7.76 Hz, 2H, arom. CH), 7.57-7.53 (m, 3H, arom. CH, CONH), 7.38 (t, J = 7.32 Hz, 2H, arom. CH), 7.29 (t, J = 7.48 Hz, 2H, arom. CH), 6.28-5.85 (br s, 1H, OCONH), 4.93-4.84 (m, 1H, α-CH), 4.63 (m, 2H, 2 * α-CH), 4.40 (m, 2 H, α-CH, OCH_2), 4.20 (m, 1 H, Fmoc CH), 4.09 (m, 2 H, α-CH_2 Gly), 3.81 (m, 1H, δ-CH_2 Pro), 3.71 (s, 3H, OCH_3), 3.64 (m, 1H, δ-CH_2 Pro), 2.91 (m, 1H, β-CH_2 Cys), 2.81 (m, 1H, β-CH_2 Cys), 2.52 (m, 2H, γ-CH_2 Met), 2.44 (m, 2H, β-CH_2 Met), 2.21 (m, 2H, CH_2 Pro), 2.08 (s, 3H, SCH_3), 1.97 (m, 2H, CH_2 Pro), 1.57 (m, 4H, γ-CH_2 Leu, β-CH_2 Leu), 1.50 (m, 2H, β-CH_2 HD), 1.45 (s, 9H, $C(CH_3)_3$), 1.24 (s, 24H, $(CH_2)_{13}$ HD), 0.95 (t, ^3J = 6.0 Hz, 2 * ω-CH_3 Leu), 0.88 (t, ^3J = 7.0 Hz, 3H, ω-CH_3 HD)

MALDI-TOF m/z: calc.: 1002.54 $[M+Na]^+$ found: 1002.81

calc.: 1018.65 $[M+K]^+$ found: 1018.77

L-Methionylglycyl-L-leucyl-L-prolyl-S-hexadecyl-L-cysteine methyl ester (H-Met-Gly-Leu-Pro-Cys(HD)-OMe) (47)

Piperidine (0.5 ml) was added to a solution of Fmoc-Met-Gly-Leu-Pro-Cys(HD)-OMe (80 mg, 82µmol) in 2.5 ml chloroform. After stirring the solution two hours, the mixture was first evaporated, and then coevaporated with toluene, and chloroform. The crude mixture was purified via flash chromatography first with straight ethyl acetate. The product was then eluted with 7:1 ethyl acetate/methanol.

Yield: 51 mg (80 %)

Rf value: 0.05 (10:1 ethyl acetate/methanol)

^{1}H-NMR (400 MHz, $CDCl_3$):

δ = 8.02 (m, 1H), 7.68 (d, J = 7.36 Hz, 1H, CONH), 7.61 (d, J = 8.8 Hz, 1H, CONH), 4.90-4.80 (m, 1H, α-CH), 4.68-4.56 (m, 1H, α-CH), 4.14-4.02 (m, 2 H), 3.86-3.76 (m, 1H), 3.70 (s, 3H, OCH3), 3.67-3.54 (m, 1H, δ-CH_{2b} Pro), 2.83 (dd, J = 13.76 Hz, J = 7.64 Hz, 2H), 2.58 (t, J = 6.92 Hz, 2H, CH_2 Met), 2.42 (t, J = 7.4 Hz, 2H, α-CH_2 HD), 2.07 (s, 3H, SCH_3), 2.20-2.00 (m, 6H, β-CH_2 Met, β-CH_2 Pro, γ-CH_2 Pro), 1.80-1.75 (m, 1H, γ-CH Leu), 1.70-1.60 (m, 4H, β-CH_2 Leu, β-CH_2 HD); 1.50-1.45 (m, 2H, CH_2 HD), 1.34-1.12 (s, br, 24H, $(CH_2)_{12}$ HD), 0.91 (t, J = 6.44 Hz, 6H, 2*ω-CH_3 Leu), 0.84 (t, J = 7.04 Hz, 3H, ω-CH_3 HD).

^{13}C-NMR (125 MHz, $CDCl_3$):

δ = 172.15, 171.40, 171.19, 168.64, 163.66 (5*C=O), 59.80 (α-CH Pro), 54.10, 54.42 (α-CH Met, α-CH Cys), 52.18 (OCH_3), 48.79 (α-CH Leu), 47.47 (δ-CH Pro), 42.82 (α-CH_2 Gly), 42.82 (β-CH_2 Leu), 42.79 (β-CH_2 Cys), 41.78, 33.58, 33.52, 32.32, 31.87, 30.46, 29.63, 29.61, 29.57, 29.50, 29.46, 29.18 (β-CH_2 Met, β-CH_2 Pro, γ-CH

Met, 9* CH_2 HD), 24.56 (γ-CH Pro), 23.24 (γ-CH Leu), 22.63 (ω-CH_3 Leu), 21.86 (CH_2 HD), 15.28 (SCH_3), 14.06 (ω-CH_3 HD).

MALDI-TOF m/z:	calc: 780.47 $[M+Na]^+$	found: 780.77
	calc.: 796.58 $[M+K]^+$	found: 796.75

N-*tert*-butyloxycarbonylglycyl-S-palmitoyl-L-cysteine-L-methionylglycyl-L-leucyl-L-prolyl-S-hexadecyl-L-cysteine methyl ester
(Boc-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(HD)-OMe) (48)

EDC (17 mg, 87 μmol), triethylamine (9.3 μl, 67 μmol) and HOBt (15 mg, 0.101 mmol) were added to a cooled, stirring solution of H-Met-Gly-Leu-Pro-Cys(HD)-OMe (51 mg, 67 μmol) and Cys(HD)-OMe (35 mg, 67 μmol). The reaction was brought up to room temperature overnight. After 18h, the reaction organic phase was extracted two times each with 1N HCl, sat. $NaHCO_3$, brine followed by drying over Na_2SO_4. The crude oil was purified by flash chromatography in straight ethyl acetate.

Yield: 48 mg (57%)
Rf value: 0.16 (20:1 ethyl acetate/methanol)
$[\alpha]_D^{20}$: -52.3° (c = 1.0, $CHCl_3$)

^{1}H-NMR (400 MHz, $CDCl_3$):
δ = 4.60-4.64 (m, 2H, 2*α-CH), 4.53 (dd, ^3J= 12.32 Hz, ^2J= 1.36 Hz, 1H, α-CH_2 Leu), 4.45 (m, 1H, α-CH Cys_{HD}), 4.19-4.14 (m, 1H, α-CH Met), 3.99 (d, J = 16.8 Hz, 1H, α-CH_{2a} Gly), 3.75-3.53 (m, 4H, δ-CH_{2a} Pro, α-CH_{2b} Gly, α-CH_{2b} Gly, δ-CH_{2a} Pro), 3.27-3.23 (m, 1H, β-CH_{2a} Cys_{Pal}), 3.14 (dd, ^2J = 13.56 Hz, ^3J = 8.44 Hz, 1H, β-CH_{2a} Cys_{Pal}), 2.88 (dd, ^2J = 13.68 Hz, ^3J = 5.48 Hz, 1H, β-CH_{2a} Cys_{HD}), 2.78 (dd, ^2J = 13.88 Hz, ^3J = 7.04 Hz, β-CH_{2a} Cys_{HD}), 2.51-2.41 (m, 6H, α-CH_2 Pal, α-CH_2, α-CH_2 HD, γ-CH_2 Met), 2.20-2.10 (m, 1H, β-CH_{2a} Met), 2.10 (s, 3H, SCH_3), 2.05-1.90 (m, 5H, β-CH_{2b} Met, β-CH_2 Pro, γ-CH_2 Pro), 1.75-1.61 (m, 2H, γ-CH Leu, β-CH_2 Leu), 1.61-1.52 (m, 2H, β-CH_2 Pal), 1.51-1.43 (m, 3H, β-CH_2 HD, β-CH_2 Leu), 1.37 (s, 9H, $C(CH_3)_3$), 1.18 (br, 50H, $(CH_2)_{12}$ Pal, $(CH_2)_{13}$ HD), 0.87 (t, ^3J = 6.24 Hz, 6H, 2*ω-CH_3 Leu), 0.79 (t, ^3J = 7.04, 6H, ω-CH_3 Pal, ω-CH_3 HD).

MALDI-TOF m/z: calc.: 1279.84 $[M+Na]^+$ found: 1279.04

calc.: 1295.95 $[M+K]^+$ found: 1295.03

Glycyl-S-palmitoyl-L-cysteine-L-methionylglycyl-L-leucyl-L-prolyl-S-hexadecyl-L-cysteine methyl ester

(H-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(HD)-OMe) (33)

A solution of Boc-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(HD)-OMe (16 mg, 12.7 µmol) in 2 ml chloroform was cooled to 0^oC before adding 5 µl thioanisole and 1 ml TFA. After 10 minutes, the reaction was brought up to room temperature, and left to react for another 50 minutes. The solvents were removed under reduced pressure and the remaining TFA was coevaporated two times with toluene. The crude product was purified by flash chromatography in 5:1 DCM/MeOH.

Yield: quantitative.

Rf value: 0.35 (1:5 MeOH, DCM).

$[\alpha]_D^{20}$: -50.3° (c = 1.0, $CHCl_3$).

^{1}H-NMR (400 MHz, $CDCl_3$/MeOH 1:1):

δ = 4.61-4.68 (m, 2H, 2*α-CH), 4.53 (dd, ^3J = 5.9 Hz, ^3J = 5.6 Hz, α-CH), 4.43 (dd, ^3J = 6.8 Hz, ^3J = 6.7 Hz, 1H, α-CH), 4.14-4.18 (m, 2H, α-CH Met, α-CH_{2a} Gly), 3.85 (d, ^2J = 16.5 Hz, 1H, α-CH_{2a} Gly), 3.81-3.84 (m, 1H, δ-CH_{2a} Pro), 3.76 (s, 3H, OCH_3), 3.72 (d, ^2J = 16.4 Hz, 1H, α-CH Gly), 3.60-3.65 (m, 2H, δ-CH_{2b} Pro, α-CH_{2b} Gly), 3.27 (dd, ^2J = 14 Hz, ^3J = 6.8 Hz, 1H, β-CH_{2a} Cys_{Pal}), 3.23 (dd, ^2J = 14 Hz, ^3J = 6.8 Hz, 1H, β-CH_{2b} Cys_{Pal}), 3.01 (dd, ^2J = 14 Hz, ^3J = 5.3 Hz, 1H, β-CH_{2a} Cys_{HD}), 2.90 (dd, ^2J = 14 Hz, ^3J = 5.3 Hz, 1H, β-CH_{2b} Cys_{HD}), 2.60-2.67 (m, 1H, γ-CH_{2a} Met), 2.61 (t, ^3J = 7.8 Hz, 2H, α-CH_2 Pal), 2.50-2.60 (m, 3H, γ-CH_2 Met, α-CH_2 HD), 2.27-2.35 (m, 1H, γ-CH_2 Met), 2.10 (s, 3H, SCH_3), 2.01-2.25 (m, 5H, β-CH_2 Pro, β-CH_2 Met, γ-CH_2 Pro), 1.75-1.90 (m, 2H, β-CH_2 Met, γ-CH_2 Leu), 1.65-1.70 (m, 2H, β-CH_2 Pal), 1.53-1.59 (m, 2H, β-CH_2 HD), 1.46-1.50 (m, 1H, β-CH_2 Leu), 1.30 (s, 50H, $(CH_2)_{12}$ Pal, $(CH_2)_{13}$ HD), 0.98 (t, ^3J = 6.24 Hz, 6H, 2*ω-CH_3 Leu), 0.88 (t, ^3J = 7.04, 6H, ω-CH_3 Pal, ω-CH_3 HD).

MALDI-TOF m/z:	calc.: 1155.74 [M+H]	found: 1156.79
	calc.: 1178.74 $[M+Na]^+$	found: 1178.81
	calc.: 1194.84 $[M+K]^+$	found: 1194.75

6.5 Compounds from chapter 4.3.1

Hexadecyl bromide

HD-Br

Triphenylphosphine (162 mg, 0.618 mmol), dissolved in 1 ml DCM, was added to a cooled solution of hexadecyl alcohol (100 mg, 0.412 mmol) and carbon tetrabromide (170 mg, 0.515 mmol) in 1 ml DCM. The reaction was stirred at room temperature overnight. The solvent was removed under reduced pressure, leaving behind a white solid. The crude material was purified with column chromatography (gravity) in straight cyclohexane.

Colorless oil.

Yield: 118 mg (94 %)

Rf value: 0.77 (1:2 ethyl acetate/cylclohexane)

^{1}H-NMR (400 MHz, $CDCl_3$):

δ = 3.40 (t, J = 6.8 Hz, 2 H, α-CH_2 HD), 1.85 (m, 2 H, β-CH_2 HD), 1.25 (m, 26 H, $(CH_2)_{13}$ HD), 0.88 (t, J = 6.4, ω-CH_3 HD)

^{13}C-NMR (125 MHz, $CDCl_3$):

δ = 34.21, 33.08, 32.15, 29.93, 29.92, 29.91, 29.90, 29.89, 29.88, 29.87, 29.86, 29.85, 29.84, 29.83, 28.77, 29.66, 29.60, 29.59, 29.58, 29.01, 29.00, 28.41, 22.91 (CH_2 HD), 14.32 (ω-CH_3 HD)

Hexadecyl iodide

HD-I

Under slight heating, hexadecyl alcohol (0.5 g, 2.06 mmol) was dissolved in 500 µl diethyl ether and 300 µl acetonitrile. Triphenylphosphine (0.734 g, 2.80 mmol) and imidazole (0.182 g, 2.68 mmol) were then added. The reaction was cooled to 0^oC before batchwise adding I_2 (0.746 g, 2.94 mmol). The solid reaction was then warmed up to room temperature and diluted with 30 ml diethyl ether. The organic phase was washed with a saturated solution of $Na_2S_2O_3$, brine and then dried over Na_2SO_4. Gravity chromatography in straight cyclohexane yielded product.

Colorless oil
Yield: 0.718 g (98 %)
Rf value: 0.85 (1:2 ethyl acetate/cyclohexane)

^1H-NMR (400 MHz, $CDCl_3$):
δ =3.18 (t, J = 7.04 Hz, 2H, α-CH_2 HD), 1.85-1.78 (m, 2H, β-CH_2 HD), 1.30-1.20 (m, 26 H, $(CH_2)_{13}$ HD), 0.88 (t, J = 7.00 Hz, 3H, ω-CH_3 HD).

^{13}C-NMR (125 MHz, $CDCl_3$):
δ =33.58, 31.93, 31.92, 31.91, 30.51, 29.68, 29.66, 29.64, 29.63, 29.61, 29.54, 29.42, 29.35, 28.54, 26.91, 22.68 (CH_2 HD), 14.10 (ω-CH_3 HD)

1-hexadecyl-d_{33}- iodide
(HD-d_{33}-I) (49)
1-hexadecyl-d_{33}-ol (50 mg, 0.182 mmol) was dissolved under light heating in freshly distilled diethyl ether : acetonitrile 5:3. Triphenyl phosphine (65 mg, 0.247 mmol) and imidazole (19 mg, 0.278 mmol) were then added before cooling the mixture to 0^oC. Iodine (66 mg, 0.260 mmol) was then added portionwise to the solution and brought up to room temperature. The yellow colored mixture was stirred for an additional hour before being diluted with diethyl ether. The organic phase was washed with NaS_2O_3, then with brine and finally dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the resulting white crude material was chromatographed in 100% cyclohexane.

Colorless oil.

Yield: 60 mg (85%).
Rf value: 0.85 (1:2 ethyl acetate/cyclohexane)

S-Hexadecyl-d_{33}-L-cysteine methyl ester
(H-Cys(HD-d_{33})-OMe) (50)

Diisopropylethylamine (61 µl, 0.349 mmol) and hexadecyl-d_{33} iodide (134 mg, 0.349 mmol) were added to a stirring solution of Cys-OMe·HCl (30 mg, 0.174 mmol) in DMF. After 18 hours, the solvent was removed under reduced pressure. The remaining solid was brought up in diethyl ether and saturated $NaHCO_3$. The aqueous phase was extracted three times with diethyl ether. The combined organic phases were washed with brine and dried over Na_2SO_4. The crude oil was further purified by flash chromatography in 1:1 ethyl acetate/cyclohexane.

Colorless oil.
Yield: 38 mg (56%).
Rf value: 0.26 (1:1 ethyl acetate/cyclohexane).

^{1}H-NMR (400 MHz, $CDCl_3$/MeOH 1:1):
δ = 3.67 (s, 3H, OCH_3), 3.57 (dd, $^3J_1$ = 7.2 Hz, $^3J_2$ = 4.7 Hz, 1H, α-CH Cys), 2.84 (dd, $^3J_1$ = 13.5 Hz, $^3J_2$ = 4.7 Hz, 1H β-CH_{2a} Cys), 2.69 (dd, $^3J_1$ = 13.5 Hz, $^3J_2$ = 7.4 Hz, 1H β-CH_{2b} Cys), 1.68 (s, br, 2H, NH_2).

N-*tert*-butyloxycarbonylglycyl-S-hexadecyl-d_{33}-L-cysteine allyl ester
(Boc-Gly-Cys(HD-d_{33})-OAll) (52)

DTT (58.3 mg, 378 µmol) and triethylamine (26 µl, 0.189 mmol) were added to a degassed solution of $(Boc\text{-}Gly\text{-}Cys\text{-}OAll)_2$ in 5 ml DCM. After 1.5 hours, the reaction was extracted three times with H_2O and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to a colorless oil. The oil was then dissolved in 5 ml DMF and cooled to 0°C before adding triethylamine (32 µl, 0.23 mmol) and hexadecyl-d33 iodide (113 mg, 0.294 mmol). After 20 hours, the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography in 2:5 ethyl acetate/cyclohexane.

Colorless oil.

Yield: 64 mg (61%).

Rf value: 0.39 (1:1 ethyl acetate/cycloheaxane)

^{1}H-NMR (400 MHz, $CDCl_3$/MeOH 1:1):

δ = 6.87 (d, J = 7.4 Hz, 1H, CONH), 5.96-5.86 (m, 1H, $CH=CH_2$), 5.37-5.32 (m, 1H, $CH=CH_{2a}$), 5.29-5.25 (m, 1H, $CH=CH_{2b}$), 5.14 (s, br, CONH), 4.85-4.81 (m, 1H, α-CH Cys), 4.66 (t, $^3J_1$ = 1.4 Hz, 1H, OCH_{2a}), 4.65 (t, $^3J_1$ = 1.4 Hz, 1H, OCH_{2b}), 3.91-3.79 (m, 2H, α-CH_2 Gly), 2.98 (dd, $^3J_1$ = 5.3 Hz, $^3J_2$ = 1.0 Hz, 2H, β-CH_2 Cys), 1.46 (s, 9H, $C(CH_3)_3$).

^{13}C-NMR (125 MHz, $CDCl_3$):

δ = 170.27, 169.26 (2 * C=O), 131.29 ($\underline{C}H=CH_2$), 119.09 ($CH=\underline{C}H_2$), 66.32 (OCH_2), 51.87 (α-CH Cys), 34.02 (β-CH_2 Cys), 28.26 ($C(\underline{C}H_3)_3$).

MALDI-TOF m/z:	calc.: 598.57 $[M+Na]^+$	found: 598.85
	calc.: 614.67 $[M+K]^+$	found: 614.82

N-*tert*-butyloxycarbonylglycyl-S-palmitoyl-L-cysteine

(Boc-Gly-Cys(HD-d_{33})-OH) (51)

Boc-Gly-Cys(HD-d_{33})-OAll (64 mg, 0.11 mmol) and DMB (8.7 mg, 0.055 mmol) were dissolved in 100 ml of freshly distilled THF and the solution was degassed. After addition of palladium triphenyl phosphine tetrakis (2.5 mg, 2.2 μmol), the reaction was stirred in the dark under argon atmosphere for two hours. The solvent was evaporated under reduced pressure, and the crude substance was redissolved in DCM. The organic phase was washed three times with 1M HCl, and then dried over Na_2SO_4. After evaporation, the crude solid was purified by flash chromatography with 1:2 ethyl acetate/cyclohexane.

Yellow oil.

Yield: 56 mg (93%).

Rf value: 0.21 (20:1 ethyl acetate/methanol).

^{1}H-NMR (400 MHz, $CDCl_3$/MeOH 1:1):

δ = 4.89-4.72 (m, 1H, α-CH Cys), 3.97 (d, J= 17 Hz, 1H, α-CH_{2a} Gly), 3.80 (d, J= 15 Hz, 1H, α-CH_{2b} Gly), 3.02-2.98 (m, 2H, β-CH_2 Cys), 1.45 (s, 9H, $C(CH_3)_3$).

6.6 Compounds from chapter 4.3.2

N-*tert*-Butyloxycarbonyl-L-methionylglycyl-L-leucyl-L-prolyl-S-hexadecyl-d_{33}-L-cystein-methylester

(Boc-Met-Gly-Leu-Pro-Cys(HD-d_{33})-OMe) (53a)

EDC (31 mg, 0.165 mmol), triethylamine (17.7 µl, 0.127 mmol) and HOBt (29 mg, 0.190 mmol) were added to a cooled, stirring solution of Boc-Met-Gly-Leu-Pro-OH (81 mg, 0.127 mmol) and H-Cys(HD-d33)-OMe (50 mg, 0.127 mmol). The reaction was brought up to room temperature overnight. After 18h, the reaction organic phase was extracted two times each with 1N HCl, sat. $NaHCO_3$, brine followed by drying over Na_2SO_4. The crude oil was purified by flash chromatography in 10:1 ethyl acetate/methanol.

Amorphous solid.

Yield: 71 mg (63 %).

Rf value: 0.68 (10:1 ethyl acetate/methanol).

^{1}H-NMR (400 MHz, $CDCl_3$/MeOH 1:1):

δ =7.75 (d, ^2J = 8.8 Hz, 1H, CONH), 7.76 (d, ^2J = 7.2 Hz, 1H, CONH), 7.47 (d, ^2J = 9.7 Hz, 1H, CONH), 6.96 (s, 1H, CONH), 5.34-5.26 (m, 1H, α-CH), 4.91-4.86 (m, 1H, α-CH), 4.71-4.66 (m, 1H, α-CH), 4.37-4.28 (m, 1H, α-CH), 4.11 (d, ^2J = 5.0 Hz, 2H, α-CH_2 Gly), 3.76 (s, 3H, OCH_3), 3.70-3.64 (m, 1H, δ-CH_{2b} Pro), 2.97 (dd, $^3J_1$ = 13.9 Hz, $^3J_2$ = 5.2 Hz, 1H, β-CH_{2a} Cys), 2.84 (dd, $^3J_1$ = 13.9 Hz, $^3J_2$ = 6.6 Hz, 1H, β-CH_{2b} Cys), 2.43-2.34 (m, 2H, γ-CH_2 Met), 2.19-2.00 (m, 4H, β-CH_2 Met, β-CH_2 Pro), 2.11 (s, 3H, SCH_3), 1.98-1.93 (m, 1H, γ-CH_2 Pro), 1.74-1.66 (m, 1H, γ-CH_2 Leu), 1.65-1.56 (m, 2H, β-CH_2 Leu), 0.99-0.97 (m, 6H, 2 * ω-CH_3 Leu).

^{13}C-NMR (125 MHz, $CDCl_3$):

δ = 172.12, 171.37, 171.34, 168.52 (4 * C=O), 60.09 (α-CH Pro), 52.68 (α-CH Cys), 52.46 (α-CH Met), 52.29 (OCH_3), 49.09 (α-CH Leu), 48.26 (δ-CH_2 Pro), 28.31 ($C(CH_3)_3$), 24.74 (γ-CH_2 Pro), 23.49 (γ-CH_2 Leu), 22.12 (ω-CH_3 Leu), 15.54 (SCH_3).

MALDI-TOF m/z: calc.: 913.73 $[M+Na]^+$ found: 914.01
calc.: 929.83 $[M+K]^+$ found: 930.00

L-Methionylglycyl-L-leucyl-L-prolyl-S-hexadecyl-d_{33}-L-cysteine methyl ester

(H-Met-Gly-Leu-Pro-Cys(HD-d_{33})-OMe) (53)

Peptide **53a** was dissolved in 1.5 ml chloroform and cooled to 0^oC before adding thioanisole and TFA. The reaction was stirred for 3 hours before first twice coevaporating with toluene and then with chloroform, resulting in a coloress oil.

Colorless oil.
Yield: 60 mg (97%).
Rf value: 0.03 (10:1 ethyl acetate/methanol).

^{1}H-NMR (400 MHz, $CDCl_3$/MeOH 1:1):
δ = 4.73-4.66 (m, 1H, α-CH), 4.56-4.36 (m, 1H, α-CH), 4.24-4.19 (m, 1H, α-CH), 3.79 (s, 3H, OCH_3), 3.78-3.62 (m, 4H, α-CH_2 Gly, δ-CH_2 Pro), 3.10 (dd, $^3J_1$ = 13.1 Hz, $^3J_2$ = 5.0 Hz, 1H, β-CH_{2a} Cys), 3.01 (dd, $^3J_1$ = 13.5 Hz, $^3J_2$ = 6.5 Hz, 1H, β-CH_{2b} Cys), 2.66-2.58 (m, 2H, γ-CH_2 Met), 2.24-2.16 (m, 6H, β-CH_2 Pro, γ-CH_2 Pro, β-CH_2 Met), 2.09 (s, 3H, SCH_3), 2.09-2.04 (m, 1H, γ-CH_2 Leu), 1.89 (m, 2H, β-CH_2 Leu), 0.90 (m, 6H, 2 * ω-CH_3 Leu).

^{13}C-NMR (125 MHz, $CDCl_3$):
δ = 60.75 (α-CH Pro), 55.46 (α-CH Met), 53.02 (α-CH Cys), 52.79 (OCH_3), 50.97 (α-CH Leu), 50.34 (δ-CH_2 Pro), 33.10 (β-CH_2 Cys), 29.25 (γ-CH_2 Pro), 24.68 (γ-CH_2 Leu), 23.04 (ω-CH_3 Leu), 21.05 (ω-CH_3 Leu), 15.19 (SCH_3).

MALDI-TOF m/z: calc.: 813.68 $[M+Na]^+$ found: 813.77

N-*tert*-butyloxycarbonylglycyl-S-hexadecyl-d$_{33}$-L-cysteine-L-methionylglycyl-L-leucyl-L-prolyl-S-hexadecyl-L-cysteine methyl ester

(Boc-Gly-Cys(HD-d$_{33}$)-Met-Gly-Leu-Pro-Cys(HD-d$_{33}$)-OMe) (54)

EDC (13 mg, 70.2 µmol), triethylamine (7.5 µl, 54.4 µmol) and HOBt (12.4 mg, 81.6 µmol) were added to a cooled, stirred solution of H-Met-Gly-Leu-Pro-Cys(HD-d33)-OMe (43 mg, 54.4 µmol) and Boc-Gly-Cys(HD-d33)-OH (29 mg, 54.4 µmol). The reaction was brought up to room temperature overnight. After 18h, the reaction organic phase was extracted two times each with 1N HCl, sat. $NaHCO_3$, brine followed by drying over Na_2SO_4. The crude oil was purified by flash chromatography in 20:1 ethyl acetate/methanol.

Colorless oil.
Yield: 60%
Rf value: 0.38 (20:1 ethyl acetate/methanol).

^{1}H-NMR (400 MHz, $CDCl_3$/MeOH 1:1):
δ = 8.21 (d, ^2J = 7.2 Hz, 1H, CONH), 7.85 (d, ^2J = 8.6 Hz, 1H, CONH), 7.64 (d, ^2J = 6.4 Hz, 1H, CONH), 7.55 (d, ^2J = 8.02 Hz, 1H, CONH), 7.35 (d, ^2J = 8.4 Hz, 1H, CONH), 4.52-4.46 (m, 1H, α-CH), 4.44-4.42 (m, 1H, α-CH), 4.35-4.32 (m, 1H, α-CH), 4.29-4.24 (m, 1H, α-CH), 4.04-3.98 (m, 2H, α-CH_2 Gly), 3.94-3.92 (δ-CH_2 Pro)3.90-3.88 (m, 2H, α-CH_2 Gly), 3.53 (s, 3H, OCH_3), 2.74 (dd, $^3J_1$ = 13.9 Hz, $^3J_2$ = 5.5 Hz, 2H, β-CH_2 Cys), 2.68-2.56 (m, 1H, γ-CH_2 Met), 2.42-2.30 (m, 1H, γ-CH_2), 2.08-2.02 (m, 1H, β-CH_{2a} Met), 1.95-1.80 (m, 5H, β-CH_{2b} Met, β-CH_2 Pro, γ-CH_2 Pro), 1.44-1.33 (m, 1H, β-CH_{2b} Leu), 1.22 (s, 9H, $C(CH_3)_3$), 0.72 (m, 6H, 2 * ω-CH_3 Leu).

MALDI-TOF m/z: calc.: 1331.22 $[M+Na]^+$ found: 1331.15
calc.: 1347.32 [M+K]+ found: 1347.11

Glycyl-S-hexadecyl-d33-L-cysteine-L-methionylglycyl-L-leucyl-L-prolyl-S-hexadecyl-d33-L-cysteine methyl ester
(H-Gly-Cys(HD-d33)-Met-Gly-Leu-Pro-Cys(HD-d_{33})-OMe) (34)
A solution of Boc-Gly-Cys(HD-d33)-Met-Gly-Leu-Pro-Cys(HD-d33)-OMe (17 mg, 12.9 µmol) in 1.5 ml chloroform was cooled to 0^oC before adding 7.6 µl thioanisole and 0.5 ml TFA. After 10 minutes, the reaction was brought up to room temperature, and left to react for another 2 hours. The solvents were removed under reduced pressure and the remaining TFA was twice coevaporated with toluene.

Yellow oil.
Yield: 15mg (96%).
Rf value:

^{1}H-NMR (400 MHz, $CDCl_3$/MeOH 1:1):
δ = 8.51 (d, ^2J = 6.8 Hz, 1H, CONH), 7.82 (d, ^2J = 5.2 Hz, 1H, CONH), 7.41 (d, ^2J = 7.4 Hz, 1H, CONH), 4.37-4.34 (m, 1H, α-CH), 4.27-4.24 (m, 2H, α-CH), 4.13-4.09 (m, 1H, α-CH), 3.96-3.90 (m, 1H, α-CH), 3.66-3.59 (m, 2H, α-CH_2 Gly), 3.56-3.44 (m, 3H, δ-CH_{2a} Pro, α-CH_2 Gly), 3.40-3.32 (m, 1H, δ-CH_{2b} Pro), 2.95-2.76 (m, 2H, γ-CH_2 Met), 2.76-2.66 (m, 2H, β-CH_2 Cys), 2.65 (m, 2H, β-CH_2 Cys), 2.45 (m, 1H, β-CH_{2b} Met), 2.10-2.04 (m, 1H, β-CH_2 Cys), 1.96-1.78 (m, 5H, β-CH_{2b} Met, β-CH_2 Pro, γ-CH_2 Pro), 1.63-1.53 (m, 2H, γ-CH_2 Leu, β-CH_{2a} Leu), 1.46-1.20 (m, 1H, β-CH_{2b} Leu), 0.71-0.68 (m, 6H,2 * ω-CH_3 Leu).

MALDI-TOF m/z:	calc.: 1231.16 $[M+Na]^+$	found: 1231.47
	calc.: 1247.26 $[M+K]^+$	found: 1247.45

Appendix A: Abbreviations.

GPI glycoinositol phospholipid

GTP guanosine triphosphate

GDP guanosine diphosphate

EEDQ 2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinoline

DCC N, N´-dicyclohexylcarboiimide

DIC N, N´-diisopropylcarboiimide

EDC N´-(3-dimethylaminopropyl)-N-ethylcarboiimide·HCl

BOP benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate

PyBOP benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate

HBTU 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HATU O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HOBt N-hydroxybenzotriazole·H_2O

HOAt 1-hydroxy-7-azabenzotriazole

Fmoc 9-fluorenylmethoxy carbonyl

Boc tert-butyloxy carbonyl

Alloc allyloxy carbonyl

Tert tertiary

Pal palmitoyl

HD hexadecyl

All allyl

NMR nuclear magnetic resonnance

TIS triisopropyl silane

TFA trifluoroacetic acid

TFE trifluoro ethanol

Me methyl

Sat. Saturated

^{t}Bu *tert*-butyl

DTT 1,4-dithiothreithol

DMF N, N-dimethyl formamide

DMB N,N-dimethyl barbituric acid

Appendix B: References

1. Pool, C.T. and Thompson, Biochemistry, 1998. **37**: p. 10246-10255.
2. Shahinian, S.H. and J.R. Silvius, Biochemistry, 1995. **34**: p. 3813-3822.
3. Novick, P. and P. Brennwald, Cell, 1993. **75**: p. 597.
4. Farnsworth, C.C., et al., Proc. Natl. Acad. Sci. USA, 1994. **91**: p. 11963.
5. Sinensky, M., *Functional aspects of polyisoprenoid protein substituents: roles in protein-protein interaction and trafficking.* Biochemica et Biophysica Acta, 2000. **1529**: p. 203-209.
6. Appolloni, A.P., I.A.; Lindsay, M.; Parton, R.G.; Hancock, J.F., Mol. Cell. Bio., 2000. **20**: p. 2475-2487.
7. Choy, E.C., V.K; Silletti, J.; Feokistov, M.; Morimoto, T.; Michealson, D.; Ivanov, I.E., Philips, M.R., Cell, 1999. **98**: p. 69-80.
8. Gorden, D.R., et al., Ann. Rev. Biochem., 1994. **63**: p. 869.
9. Casey, P.J., Science, 1995. **268**: p. 221-225.
10. Dunphy, J.T.L.M.E., *Signalling functions of protein palmitoylation.* Biochemica et Biophysica Acta, 1998. **1436**: p. 245-261.
11. Lowry, D.R. and B.M. Willumsen, Ann. Rev. Biochem., 1993. **62**: p. 851-891.
12. Beaupre, D.M.K., R., *RAS and leukemia: from basic mechanisms to gene-directed therapy.* Journal of Clinical Oncology, 1999. **17**: p. 1071-1079.
13. Boguski, M., Nature, 1993. **366**: p. 643.
14. Egan, S.E. and R.A. Weinberg, Nature, 1993. **365**: p. 781.
15. Hall, A., Science, 1994. **264**: p. 1413.
16. Pai, E.F.K., W.; Krengel, U.; Holmes, K.C.; John, J.; Wittinghofer, A., Nature, 1989. **341**: p. 209-214.
17. Milburn, M.V.T., L.; DeVos, A.M.; Bruenger, A.; Yamaizumi, Z.; Nishimura, S.; Kim, S.-H., Science, 1990. **247**: p. 939-945.
18. Pai, E.F.K., U.; G.A. Petsko; R.S. Goody; Kabsch, W.; Wittinghofer, A., EMBO, 1990. **9**: p. 2351-2359.
19. Prive, G.G.M., M.V; Tong, L.; DeVos, A.M.; Bruenger, A.; Yamaizumi, Z.; Nishimura, S.; Kim, S.-H., Proc. Natl. Acad. Sci. USA, 1992. **80**: p. 3649-3653.
20. Forbes, J.H., C.; Oldfield, E, J. Am. Chem. Soc., 1988. **110**: p. 1059-1065.
21. Davis, J.H.A., M.; Hodges, R.S., Biophysical Journal, 1995. **69**: p. 1917-1932.
22. Volke, F.P., A., Biophysical Journal, 1995. **68**: p. 1960-1965.
23. Zhou, Z.S., B.G; Stark, R.E.; Epand, R.M., Chem. Phys. Lipids, 1997. **90**: p. 45-53.
24. Huster, D.A., K.; Gawrich, K., Biochemistry, 1998. **37**: p. 17299-17308.
25. Huster, D.A., K.; Gawrich, K., J. Phys. Chem. B, 1999. **103**: p. 243-251.
26. Schehan, J.C. and G.P. Hess, J. Am. Chem. Soc., 1955. **114**: p. 2145.
27. Bellau, B. and G. Malek, J. Am. Chem. Soc., 1968. **90**: p. 1651.
28. Castro, B. and D. Ngyen, in *Peptide Chemistry*, T. Shiba and S. Sakakibara, Editors. 1988, Protein Research Foundation.
29. Merrifeld, R.B., Biochemistry, 1964. **3**: p. 1385.
30. McKay, F. and N.F. Albertson, J. Am. Chem. Soc., 1957. **79**: p. 4686-4690.
31. Carpino, C.A. and G.Y. Han, J. Org. Chem., 1972. **37**: p. 3404.
32. Kunz, H. and C. Unverzagt, Angew. Chem. Int. Ed., 1984. **96**: p. 426-427.
33. Taschner, E.C., A.; Bator, B.; Sokolowska, T., *Darstellung von tert.-butylestern freier aminosaeuren.* Liebigs Ann. Chem., 1961. **646**: p. 134-136.

34. Waldmann, H. and H. Kunz, Liebigs Ann. Chem., 1983: p. 1724.
35. Stöber, P., et al., Bioorg. Med. Chem., 1997. **5**: p. 75.
36. Hiskey, R.G., T. Mizigushi, and T. Inui, J. Org. Chem., 1966. **31**: p. 1192.
37. Schelhaas, M., et al., Angew. Chem. Int. Ed., 1996. **35**: p. 106.
38. Wakabayashi, K. and W. Pigman, Carbohyd. Res., 1974. **35**: p. 3.
39. Bader, B.K., K.; Owen, D.J.; Waldmann, H.; Wittinghofer, A.; Kuhlmann, J., *Bioorganic synthesis of lipid-modified proteins for the study of signal transduction.* Nature, 2000. **403**: p. 223-226.
40. Huster, D.K., K.; Kadereit, D.; Waldmann, H.; Arnold, K., *1H High Resolution Magic Angle Spinning NMR Spectroscopy for the Investigation of Ras Lipopeptide in a Lipid Membrane.* Angew. Chem. Int. Ed., 2001. **40**: p. 1056-1058.
41. Kuhn, K., *Synthese unterschidlich lipidmodifizierter Peptide und Proteine*. 1999, Universitaet Karlsruhe.
42. Xue, C.-B.E., A.; Becker, J.M.; Naider, F., Int. J. Peptide Protein Res., 1990. **37**: p. 513.
43. Appel, R., Angew. Chem. Int. Ed., 1975. **14**: p. 801.
44. Cleary, M., J. Org. Chem., 1986. **51**: p. 862.
45. Perrin, D.E.A., W.L.F, *Purification of Laboratory Chemicals*. 3 ed. 1988, Oxford: Perfamon.

Appendix C: Acknowledgements

I would like to thank Prof. Waldmann for guidance and support in this particularly exciting project.

My thanks extends to Michael Manger, Claudia Rosenbaum, Reinhard Reents, and Michael Scheck for carefully going through this thesis.

Not forgotten, but too numerous to be listed here, are all those at the MPI in Dortmund who have contributed to making my first two years in the Pott more than bearable!

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides Statt, daß ich die vorliegende Arbeit selbstständig und ohne unerlaubte Hilfsmittel durchgeführt habe.

Dortmund, den

(Catherine Katzka)