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Synthesis and ion binding properties of cyclic homopeptides

PhD Thesis of

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ZAHVALE

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§ 1. INTRODUCTION

1.1. CYCLIC PEPTIDES

Cyclic peptides and peptidomimetics represent large and yet underexploited class of molecules for drug discovery. Cyclic peptides are present in hormones, antibiotics and toxins. Because of their reduced conformational freedom (compared to their linear precursors), cyclic peptides exhibit improved metabolic stability and binding affinity to their molecular targets. They are more resistant to enzymatic hydrolysis[1-3] and show an increased receptor selectivity and bioavailability[4] and are frequently endowed with biological activity.[5,6] These properties have inspired researchers to synthesize and screen large libraries of natural cyclic peptides to meet other medical needs and serve as biomedical research tools.

Two main classes of cyclic peptides can be described: in the frist one, heterodetic compounds, disulfide, ester or thioester bonds take place in the structure and connect amino acidic residues to each other.[7] In the second class, called homodetic, every amino acid is bonded exclusively through amide bond.

1.1.1. Synthesis of cyclic peptides

Due to the growing interest that cyclic peptides have generated, intense efforts have been invested in developing efficient methods for peptide cyclizations.[8]

There are four ways in which amino acids can cyclize in homodetic compounds (Figure 1). Head-to-tail, where condensation reaction happens between *C*- and *N*-termini, head-to-side chain, side chain-to-tail and side chain-to-side chain where the formation of amide bond involves the functional groups of the side chain of one or more residues that condensate with *C*-terminus, *N*-terminus or with each other.

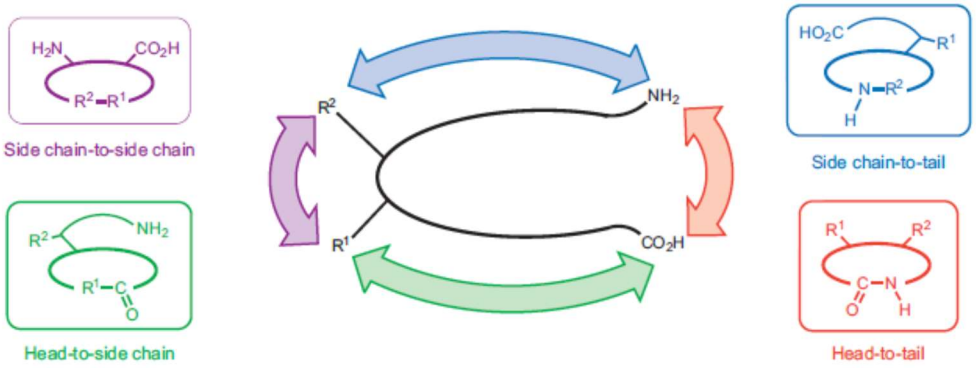


Figure 1. The four possible ways a peptide can be constrained in a macrocycle.[9]

Synthesis of cyclic peptides, in particular head-to-tail cyclisation, represents however a significant synthetic challenge. Most condensation methods usually used for linear peptides are applicable for ring closure. Anyway, cyclization requires particular conditions. For instance, high dilution is still the main route applicable to minimize unwanted intermolecular processes such as oligo- and polymerizations, making the peptide more prone to intramolecular reaction.

Selection of coupling reagents plays a very important role in the good outcome of the reaction, but the ring size is the main factor that governs the success of a macrocyclization.

Large peptides containing more than ten amino acids usually don't present difficulties and cyclize easily with good yields because intramolecular hydrogen bonds lower the free energy of loop closure.[10]

Smaller peptides, instead, can often be troublesome if not impossible, to cyclize; tri-, tetra-, and pentapeptides show the tendency to dimerization, trimerization and *C*-terminal epimerization.[11] In particular, pentapeptides tend to form dimers in solution that are hold together by hydrogen bonds in an antiparallel sheet-like conformation, and cyclodimerize.[12]

The success or failure of macrocyclization relies on the ability of a linear precursor to conformationally pre-organize its reactive ends bringing them close to each other before ring closure.[13-17] Such pre-arrangement results in fewer by-products from intermolecular processes. Over the years, various strategies for directing macrocyclizations using conformational pre-organization have been developed and reviewed.[18] These strategies can be devided in two groups:

1) 'internal' conformational elements, which include covalent modifications of the peptide chain to facilitate the union of its ends, and

1) 'external' conformational elements, which involve the use of molecular scaffolds that are neither covalently attached to the peptide, nor consumed during the course of the ring closure.

1) Internal conformational elements. The cyclization process is favoured when both termini of a linear precursor in the transition state have the least amount of strain. Daidone and Smith concluded that the loop-closure kinetics in the longer peptides (more than 10 amino acids) is determined by the formation of intra-peptide hydrogen bonds and transient β-sheet structure.[10] Intramolecular hydrogen bonds were found to lower the free energy of loop closure for longer peptides. The absence of intra-peptide hydrogen bonds for the shorter peptides provided evidence of intrinsic stiffness of the short polypeptide chains.

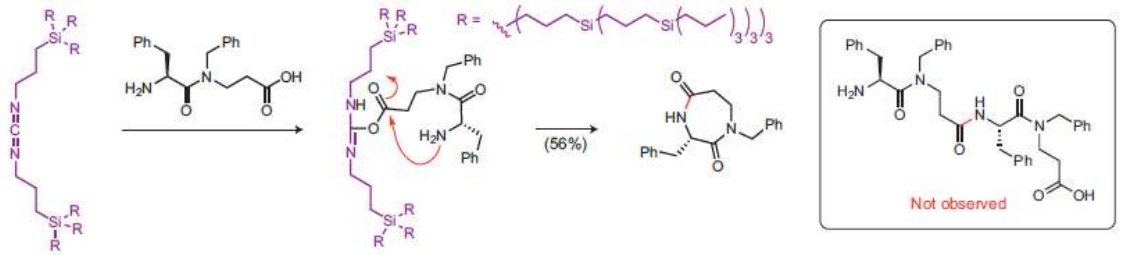
To help bring the ends of a linear peptide together, the secondary protein structures, particularly reverse turns have been taken into account.[19] An elegant way of achieving a minimal end-to-end distance is to introduce a *cis*-amide bond in the middle of the peptide chain, thus forming a motif analogous to a β-turn. This can be done, for example, by introducing proline in the sequence.

The incorporation of other D-amino acids into all-L peptides is also known to pursue turn-inducing effects.[20] For two hexapeptides that differ only in α-carbon configuration at the terminal residues, cyclization is favoured for the diastereomer that contains both a D- and an L-residue at its termini.[21] The presence of both D and L residues in the termini of linear precursor[22] is usually necessary to obtain cyclic product in particular for small (penta- and hexa-) peptides presumably because of less steric clashes among the side chains.

N-Methyl amino acids have a similar stereochemical impact on the backbone of peptides to that of proline. They have the potential to introduce *cis*-amide bonds into peptide sequences and are well suited to induce β-turns.[23,24]

2) External conformational elements. External templates for assisting macrocyclization operate on the basis of a site-isolation mechanism. Polymeric scaffolds can create reaction

cavities large enough for only one linear peptide molecule to enter and cyclize at a time. In this way peptide is isolated from the bulk solution and these internal cavities significantly decrease the probability of cyclooligomerization. Van Maarseveen and co-workers have applied this strategy through the development of carbosilane dendrimeric carbodiimides[25] (Scheme 1). The authors demonstrated that these dendrimeric carbodiimides could cyclize seven-membered bis-lactams (homodiketopiperazines), which are difficult to obtain through traditional lactamization methods.



Scheme 1. Carbosilane dendrimeric carbodiimides to cyclize homodiketopiperazines through site-isolation mechanism.[9]

Successful or inefficient cyclization depends also on the type of amino acid residues of the linear sequence. This sequence-dependent effect of cyclization often makes it neccesary to devise investigations to find adequate conditions that enable the formation of target cyclic structures.

One example that confirmes previuosly mentioned facts is the attempt of Schmidt and Langner to synthesize *cyclo*-[Pro-Val-Pro-Tyr], a known tyrosinase inhibitor. They were unable to prepare the all-L-isomer. Instead, 31% of the *C*-terminal epimerization product *cyclo*-[Pro-Val-Pro-D-Tyr] was obtained from the corresponding pentafluorophenyl (Pfp) ester. The authors also investigated all possible ring closures of the all-L-cyclic pentapeptide *cyclo*-[Pro-Ala-Ala-Phe-Leu] through Pfp ester activation, and observed a strong sequence dependence for successful cyclization. Only cyclization of H-Phe-Leu-Pro-Ala-Ala-OH resulted in the formation of the monomeric cyclic species with 21% yield (Figure 2). This example demonstrates the difficulties in a retrosynthetic analysis of a cyclic peptides. The ring disconnection must be chosen carefully and several guidelines have been developed to aid in this.[26]

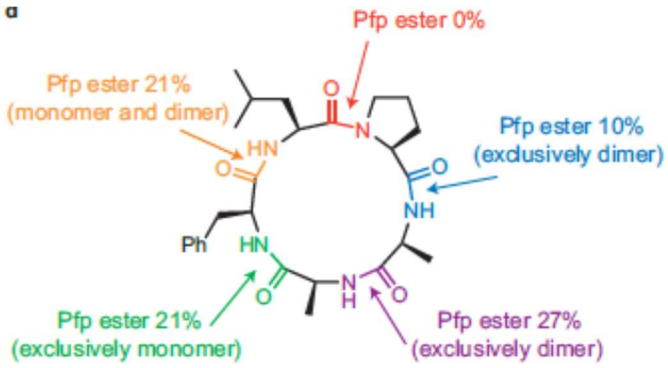


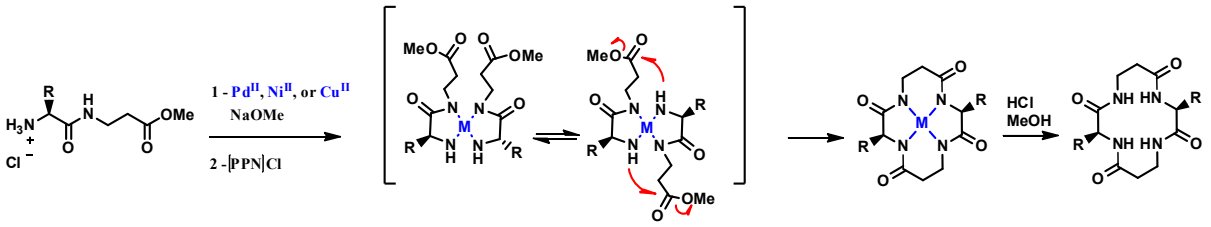
Figure 2. All possible ring disconnections of cyclo-[Pro-Ala-Ala-Phe-Leu] showing the isolated yields of the combined cyclomono- and/or dimerization through Pfp ester activation.[9]

It is also known that sequences rich in Arg, Lys and Thr as well as those that contain Arg-His-Ser motif next to the *N*-terminal are very difficult to cyclize.

In the next few pages the most significant cyclization methods will be reported.

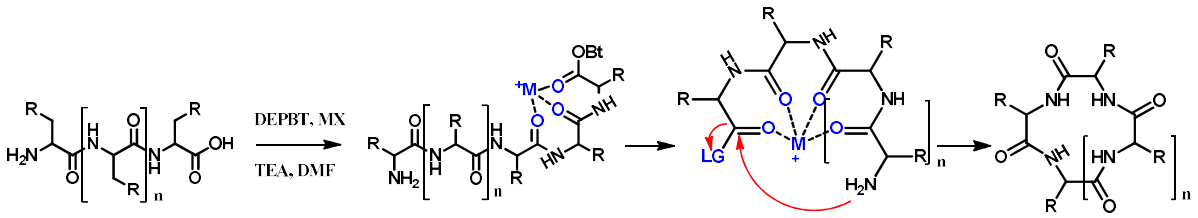
a) Metal-ion assisted cyclization

Tendency of peptide macrocycle to form stable complex with metal ions, found in natural compounds like gramicidin, valinomycin and anatamanide inspired the attempt to use metal ions to facilitate cyclisation. For instance, Beck and co-workers[27] used two un-activated dipeptide methyl esters coordinated to a metal center (Pd, Ni, Cu) allowing nucleophilic attack under basic condition of amino group to the carboxyl group to get the cyclic product. This reaction does not require high dilution, protection or activation (Scheme 2).



Scheme 2. Transition metal-mediated cyclodimerization.

Ye and co-workers later proposed that alkali metal ions[28] bind carbonyl groups, starting from *C*-terminus (Scheme 7), forcing the peptide to acquire a strong turn structure that bring *C*-terminus close to *N*-terminus allowing condensation reaction to occur.[29,30]



Scheme 3. Alkali metal ion assisted cyclisation

Various coupling reagents were used and proposed for cyclisation reactions, despite the fact that reagents usually used in linear synthesis could be also employed. A strong reactivity of the activated precursor is usually required because of disfavored intramolecular reaction due to the pre-cyclisation fashion the molecule must have. But on the other hand, an over activated peptide could cause epimerization and polymerization side-reactions.

Coupling reagents such as DCC paired with HOBt usually lead to very slow reactions causing high level of epimerization of *C*-terminus.[31-33] Other, commonly used coupling reagent for cyclisation, diphenylphosphoryl azide (DPPA), 2-(1H-benzotriazole-1-yl)-1,1,3,3 tetramethylaminium tetrafluoroborate[34] (TBTU) and (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate[35] (BOP) provide a fast cyclization[36] but still epimerization was observed at not negligible levels. Application of coupling reagents derived from 1-hydroxy-7-azabenzotriazole[37] (HOAt) such as *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate[38] (HApyU), (7-azabenzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate[38] (PyAOP) and *N*-[(dimethylamino)-H-1,2,3-triazolo[4,5]pyridin-1-yl-methylene]-*N*-methyl-methanaminium hexafluorophosphate[38] (HATU) have shown advantages over the corresponding HOBt-based reagents for solution and solid-phase peptide synthesis about both, speed and maintenance of chiral integrity.[38]

Among all 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one[39] (DEPBT) (Figure 3), due to its notable resistance to racemization and high yields noticed in peptide bond formation, is an useful tool for the synthesis of complex natural products[40, 41] and cyclisation reaction.[30] This coupling reagent was used in the previously mentioned example (Scheme 3).

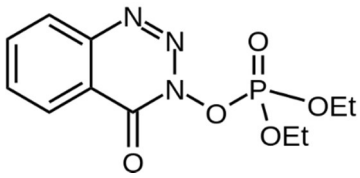


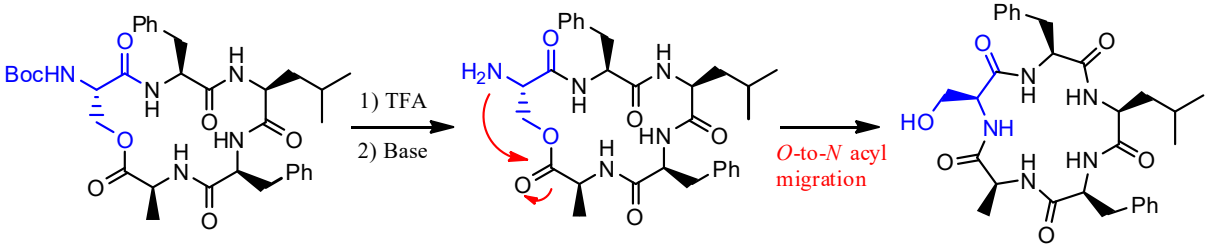
Figure 3. Structure of DEPBT.

b) Solid phase peptide synthesis (SPPS)

Cyclization of peptides on a solid support may be an interesting choice for removing the eventuality of side-reaction like polymerization facilitating also purification process as coupling reagents and by-products can be simply washed away. The linear precursor is most commonly anchored to the support through the side chain of a trifunctional amino acid. Anchoring a reactive molecule to an insoluble polymer can create a pseudodilution phenomenon.

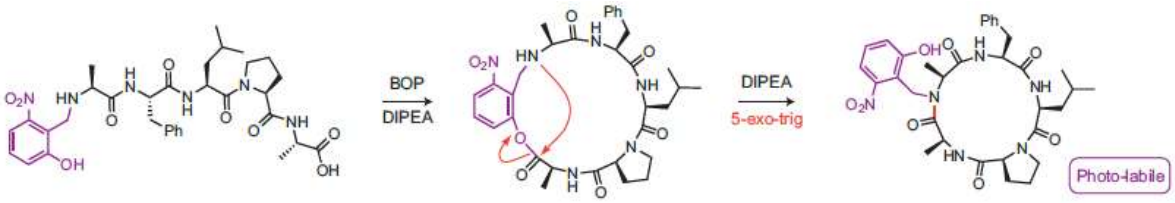
c) Lactamization

Amblard and co-workers[42] reported a synthetic strategy to obtain penta- and hexacyclopeptides trying to overcome the entropic penalty of peptide's macrocyclization. A *N*-Boc protected depsipeptide of serine was firstly synthetized through SPPS, then cyclized in solution with high dilution strategy and BOP as the coupling reagent, taking advantage of the greater flexibility of depsipeptides compared to linear ones. After the removal of Boc from the serine residue, and under basic conditions, the *O*-to-*N* migration occurred leading to a ring contraction as showed in Scheme 4. However, it was observed by the same authors that the acyl migration step did not proceed in the case of a more conformationally constrained tetrapeptide derivative.



Scheme 4. Ring-contraction strategy involving an O-to-N acyl transfer.

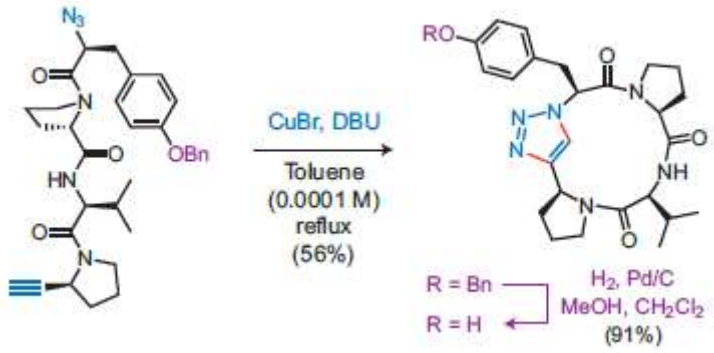
Another ring-contraction method involving lactones was developed by Meutermans, Smythe and co-workers and was applied to the head-to-tail cyclization of a pentapeptide.[43] A salicylaldehyde-derived auxiliary was attached to the *N*-terminus of the peptide chain through reductive amination. Activation of the *C*-terminus initially results in the cyclization to form the more accessible lactone. This brings the *N*-terminus into close proximity to the *C*-terminus and facilitates an *O*-to-*N* acyl transfer (Scheme 5). 2-hydroxy-6-nitrobenzyl (HnB) was chosen as the auxiliary because it is photo-labile and can be removed after macrocyclization.



Scheme 5. A photo-labile auxiliary used in a ring-contraction strategy.[9]

d) Azide-alkyne cycloadditions

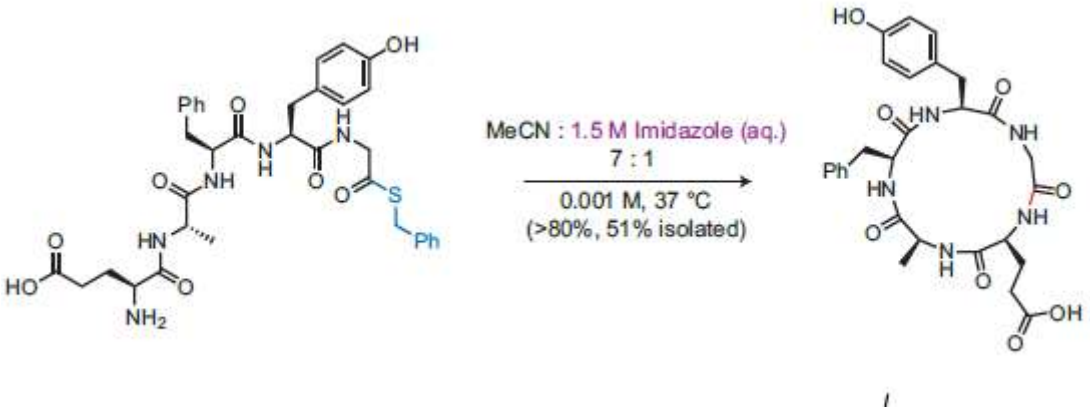
The incorporation of heterocycles into the backbone of cyclic peptides is growing in interest, especially from a medicinal chemistry perspective. The 1,2,3-triazole is both thermodynamically and physiologically stable. Depending on its substitution pattern it can serve as an effective isostere for a *trans*- or *cis*-amide bond. This heterocycle is readily obtained through a 1,3-dipolar cycloaddition between an *N*-terminal azide and a *C*-terminal alkyne (Scheme 6).[44]



Scheme 6. A click-mediated macrocyclization.[9]

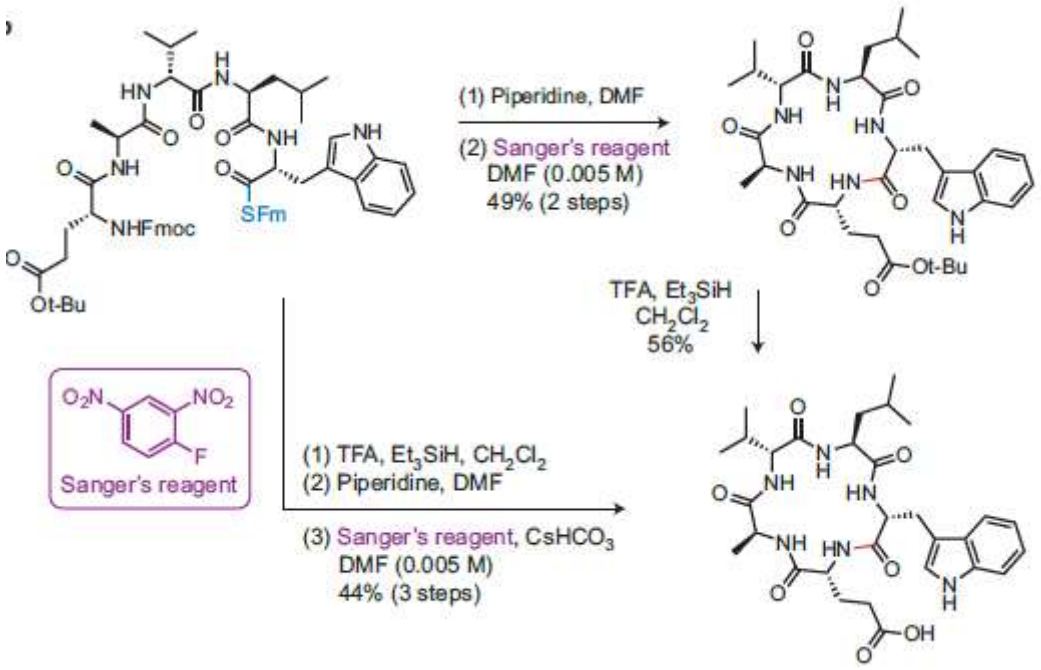
e) Sulfur-mediated cyclizations

Inspired by the prevalent role imidazole plays in the catalyzing the hydrolysis and transfer of activated acyl groups, Houghten and co-workers[45] have developed a method for the head-to-tail synthesis of cyclic peptides by the direct aminolysis of peptide thioesters in the presence of imidazole (Scheme 7). Imidazole acts as nucleophilic catalyst, attacking the carbonyl group of the thioester to form a reactive acyl imidazolyl intermediate, which is subsequently intercepted by another nucleophile.



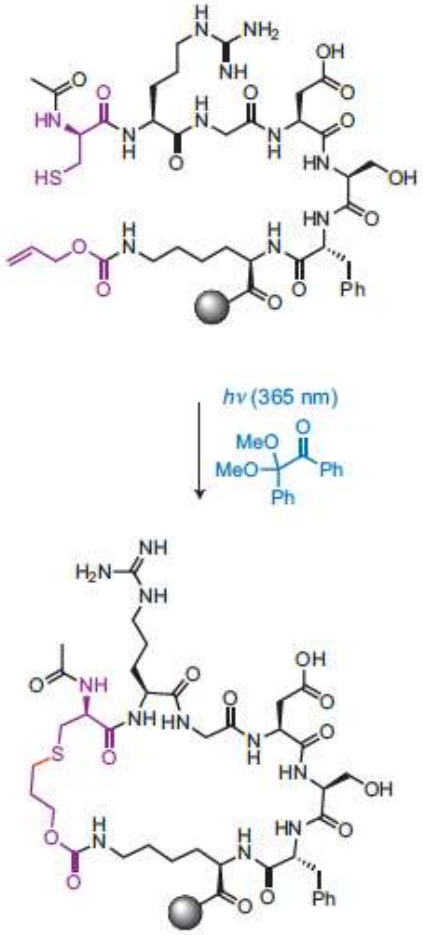
Scheme 7. Cyclization of a thioester catalysed by imidazole.[9]

Crich and Sasaki have developed an amide-bond-forming sequence employing peptide thioacids and Sanger's reagent that is viable for the cyclization of penta- and hexapeptides.[46] Treatment of an *N*-terminal Fmoc-protected and *C*-terminal 9-fluorenylmethylthioester peptide with piperidine (Scheme 8) gives a precursor, which cyclizes in the presence of Sanger's reagent. The reaction proceeds through an initial reaction with Sanger's reagent to generate a reactive thioester *in situ*.



Scheme 8. Cyclization of a thioester with Sanger's reagent.[9]

One of the most convenient and straightforward methods for constraining a peptide into a macrocycle is through the bridging of two internal cysteine thiol groups. The resulting intramolecular disulfide bridges can stabilize secondary structure motifs in peptides. This side chain-to-tail cyclization strategy was used by Anseth and co-workers.[47] The macrocyclization involves a radical addition of the thiol group of a cysteine residue at the *N*-terminus of the peptide to the alkene of an allyloxycarbonyl protecting group on the ε-NH_2 group of a lysine residue at the *C*-terminus (Scheme 9).



Scheme 9. Cylization through a thiolene reaction on solid-phase.[9]

1.1.2. Ion binding proprieties of cyclic peptides

Ionophores are molecules that bind and transport ions selectively.[48] To design useful ionophores, various factors such as host-guest size complementarity, rigidity of host molecule and ion dipolar moiety orientations in host-guest complexes are important.[49] These complexes are composed of molecules or ions held together by intermolecular forces such as electrostatic interactions, hydrogen bonding, dispersion interactions and solvophobic effects.[50] The first step in a formation of these complexes is the desolvation of the ion, followed by the interaction of the free ion and the ligand.[51] Thus, molecules of solvents that were part of the solvation spheres of ion are now free in the solution and replaced by one molecule of ligand, which is certainly entropically favoured. Furthermore, the conformational-electron compatibility of the ions and ligands can also result in a favorable enthalpy contribution. Binding processes, as well as other

chemical reactions, are characterized by thermodynamic parameters, such as equilibrium constant, reaction Gibbs energy, enthalpy and entropy. The above-mentioned parameters depend on the temperature and on the solvent in which the reaction takes place.[52] The solvent plays a significant role in these processes primarily due to the different solvation of the reactants and the complexes formed, as well as the inclusion of the solvent molecule in the host molecule, resulting in competition processes due to complexation of the host. Cyclic peptides were found to have high binding affinity toward various cations and anions[53-55] and it has been shown that such an affinity is strongly dependent on both structure and conformation.[56-59]

1.1.2.1. Anion binding

Anion recognition has become a hot theme in supramolecular chemistry due to its important roles in life sciences and in many medical and environmental settings. A number of neutral receptors containig amide, urea/thiourea or pyrrole groups have been developed in the recent years. Amongst them, cyclic peptides should be of particular interest because they belong to a familiy of natural macrocycles known for their importance in life processes. Research in this area is steadily increasing but still the development of neutral receptors of anions is quite slow compared to the abundance of articles about cation recognition. Strong complexation of anions is usually shown when all N-H groups converging towards the center of cavity binding *via* hydrogen bonds.[60]

1.1.2.2. Experimental studies of complexation reactions

The methodology of monitoring complexation reactions depends on the system being investigated. When designing the experiment, the concentration range that is to be used, the expected response, and the possible interference that may arise from the solvent should be taken into account. For example, if the ion complexation reaction is monitored by UV spectroscopy then solvents and counterions that absorb in the same region as the ligand and/or complex should be avoided. With NMR titration it should be noted that a much higher ligand concentration is required than for example in the case of fluorimetric titration, and problem can be an insufficient solubility. In calorimetry, higher concentrations of salt may result in high heat

dissipation which can disable obtaining of reliable results. In this work we used calorimetric and NMR titrations that will be explained more in detail in the following part of this section.

The complexatin reaction of an ion with a peptide can be represented by the equation (1), if the complex formed have a stoichiometry 1:1 (anion: ligand). Charges are omitted for simplicity.

$$A(sln) + L(sln) \rightleftarrows AL(sln) \tag{1}$$

The thermodynamic (standard) stability constant of the resulting complex is given by the following expression:

$$K° = \frac{a_{AL}}{a_A + a_L} = \frac{[\mathrm{AL}]\gamma_{AL}c°}{[\mathrm{A}]\gamma_A \cdot [L]\gamma_L} \approx \frac{[AL]c°}{[A]\cdot[L]} \tag{2}$$

The approximate equation (2) derives from the fact that the solutions are diluted and the ligand is a neutral species. For processes that would involve charged ligands, especially with multiple charges, it would be necessary to take into account activity coefficients. Also, if the ligand is neutral, activity coefficients of the free cation and complex are approximately equal and can be neglected.

In the case of calorimetric determinations of stability constants, isothermal microcalorimetric titration is the most frequently used approach. In our case, usually a solution of the salt is added to the solution of the ligand and the absorbed or emitted heat is monitored.[61] The extent of the reaction (1) can be expressed by the relation:

$$\xi = [\mathrm{AL}]V \tag{3}$$

where V is the volume of the reaction system.
Stochiometric equilibrium constant of the complexation reaction is defined as:

$$K = \frac{[AL]}{[A][L]} \quad (4)$$

From the stoichiometry of the complexation reaction, it follows:

$$L = c_L - [AL] \quad (5)$$

$$A = c_A - [AL] \quad (6)$$

Where c_L and c_A denote analytical (total) concentrations of the ligand and ion, respectively. Introducing the relations (5) and (6) into equation (4) results in:

$$K = \frac{[AL]}{(c_A - [AL])(c_L - [AL])} \quad (7)$$

By solving the above equation for complex equilibrium concentration and substraiting the obtained result into (3) one obtains:

$$\xi = [\frac{(Kc_A + Kc_L + 1) - \sqrt{(Kc_A + K\ _L + 1)^2 - 4K^2 c_A c_L}}{2K}]V \quad (8)$$

Since the reaction enthalpy is given by:

$$\Delta_r H = \Delta H / \xi \quad (9)$$

The dependence of a cumulative enthalpy change on the analytical concentrations of ion and ligand, A and L, can be expressed by the equation:

$$\Delta H = \Delta_r H[\frac{(Kc_A + Kc_L + 1) - \sqrt{(Kc_A + Kc_L + 1)^2 - 4K^2 c_A c_L}}{2K}]V \quad (10)$$

To avoid the influence of the experimental errors, it is common to express the successive enthalpy changes:

$$\Delta(\Delta H) = (\Delta H_n - \Delta H_{n-1}) = \Delta_r H(\xi_n - \xi_{n-1}) \quad (11)$$

where n represents the ordinal number of titrant addition. Extents ξ_{n-1} i ξ_n are given by equation (8) together with $c_{A(n)}$ and $c_{A(n-1)}$, and $c_{L(n)}$ and $c_{L(n-1)}$, respectively.

From $\Delta_r H$ and K values obtained from calorimetric titrations can, by fundamental thermodynamic equations, be reached values $\Delta_r G°$ and $\Delta_r S°$. The standard equilibrium constant is associated with standard Gibbs energy by equation:

$$\Delta_r G^\circ = -RT \ln K^\circ \quad (12)$$

and $\Delta_r G°$ is associated with $\Delta_r H°$ and $\Delta_r S°$ by equation:

$$\Delta_r G^\circ = \Delta_r H° - T\Delta_r S° \quad (13)$$

In NMR titrations the chemical shift of the signal of each nucleus in the NMR spectrum is the linear function of molar fractions of all species present in the solution. In the case of formation of 1:1 complex, this dependence is given by the equation:

$$\delta = \delta_L x(L) + \delta_{L\cdot A} x(L \cdot A) \quad (14)$$

where δ_L and δ_A denote chemical shift of the signal in NMR spectra of the ligand and ion, respectively.

By incorporating the mass balance in equation (14), the dependence of chemical shift δ on x_A and x_B can be obtained:

$$\delta = \delta_L c_L - (\delta_L - \delta_{L\cdot A}) \frac{x_L + x_A + \frac{1}{K} - \sqrt{(x_L + x_A + \frac{1}{K})^2 - 4x_L x_A}}{2} \quad (15)$$

It should be emphasized that equation (15) is applicable only when the presence of free anion does not affect the measured property of the solution, for example, when the only spectral-active species are receptor and complex.

It is also important to emphasize that equations (14) and (15) are only valid if the rate of the formation and dissociation reactions is high, *i.e.*, rate constant of the exchange reaction (*k*) is significantly greater than the difference in the resonance frequencies of the free receptor and complex nuclei:

$$k \gg |v_L - v_{L\cdot M}| \tag{16}$$

In the case of a slow exchange, separate signals of the species present in the solution appear in the spectrum, and the ratio of their molar fractions is given by the ratio of the area of the corresponding signals. In this case, if the total (analytical) concentrations of c_L and c_M in the solution are known, the equilibrium constant becomes very simple to be determined and for the simple case of the complex with stoichiometry 1:1 equation for the equilibrium constant is as follows:

$$K = \frac{1}{(c_A - \frac{f}{1+f} \times c_L)} \times f \tag{17}$$

where f is the ratio of integrals of the signal of the complex and the free ligand, i.e. the ratio of their equilibrium molar fractions (18).

$$f = \frac{x_{L\cdot A}}{x_A} \tag{18}$$

It should be noted that when the difference in the frequencies at which a particular nucleus resonates is comparable to the rate constant for the exchange reaction, it is not possible to use the above described approach of determining the equilibrium constant. In other words, at a temperature at which the following relation is valid:

$$k \approx |v_L - V_{L\cdot A}| \tag{19}$$

it is not possible to quantitatively characterize the equilibrium of such reaction by ^{1}H NMR spectroscopy.

As a confirmation of the importance of anion binding, there are numerous papers published in recent years on this topic. Cheng and coworkers[62] developed two new pseudopeptides (Figure 4) incorporating several H-bond donating and conformationally constrained structures (pyrrole, pyridine and cystine) into the macrocycle backbone. They found that both receptors possess good affinity and selectivity for fluoride and acetate ion in acetonitrile.

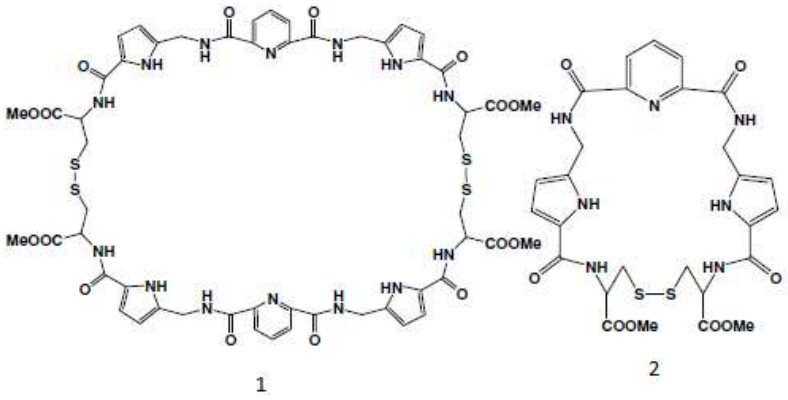


Figure 4. Structures of a pseudopeptides.[62]

Jolliffe with her colaboratores prepared a fluorescent chemosensing molecule based on a backbone modified cyclic peptide receptor (Figure 5). This product detects pyrophosphate in water and has a complete selectivity over monophopsphate anions.[63]

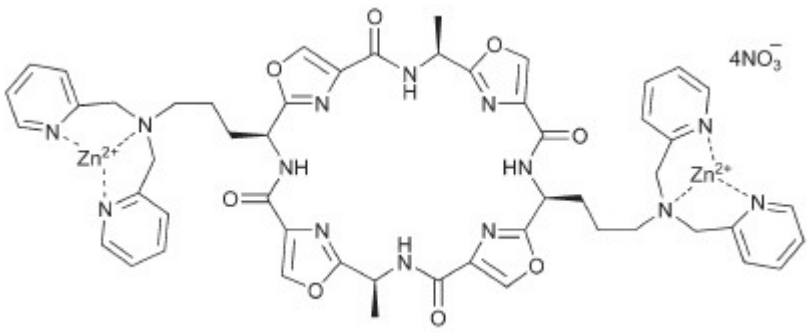


Figure 5. Structure of fluorescent chemosensing molecule.[63]

The same authors synthesized another two criptands (Figure 6) based on cyclic peptides featuring three conformationally constrained thiourea groups for anion binding.[64] Studies indicated that product **1** binds floride, chloride, bromide and acetate but not iodide, nitrate or hydrogensulfate. Product **2**, instead, is selective towards acetate ions. All measurments were done in 0.5 % H_2O/DMSO-d_6.

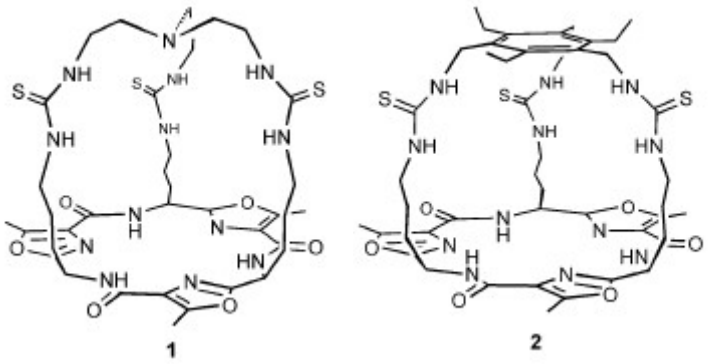


Figure 6. Structures of criptands.[64]

The most recent aricle is published by Kubik et el.[65] They synthesized two new pseudopeptides (Figure 7). Larger receptor **2** binds fully deprotonated sulfate ions with a lower affinity than **1**. Interestingly, receptor **2** make a sandwich-type binding mode with dihydrogenpyrophosphate and dihydrogenphosphhate. All measurments were done in 2.5 % H_2O/DMSO.

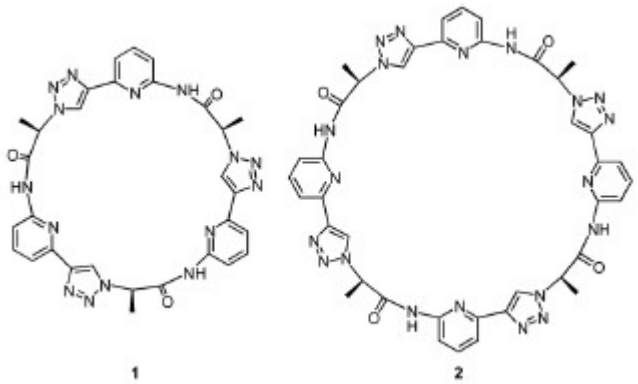


Figure 7. Structure of pseudopeptides.[65]

1.1.2.3. Cation binding

It is well known that cyclic peptides are also able to bind cations. All previously written equations are valid also to evaluate thermodynamic parameters for cation binding. For example, cyclododecapeptide c(L-Ala-Gly-D-Phe-L-Pro)$_3$, adopts in $CDCl_3$ a conformation which is able to bind various cations like Ba^{2+}, K^+, Rb^+, Cs^+, and Tl^+ forming 1:1 ion-peptide complexes while Na^+ and Li^+ form 1:2 complexes.[66]

Cereulide, produced by Bacillus cereus, is a cyclic dodecadepsipeptide containing three repeats of four amino acids: D-Oxy-Leu-D-Ala-L-Oxy-Val-L-Val and acts as ionophore with a high affinity for potassium cations.[67]

A cyclic peptide (Figure 8) having four cysteinyl residues was found to be very efficient ligand for Cd^{2+}, Zn^{2+} and Ni^{2+} ions.[68]

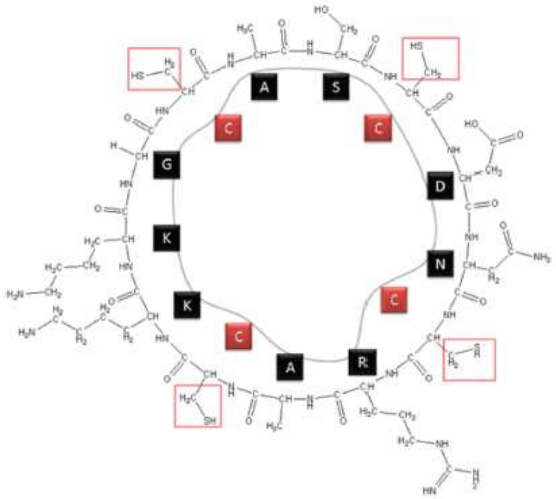


Figure 8. Structure of a cyclic peptide.[68]

More limited structural isomerism of cyclic ligands makes them a suitable choice for the design of ion receptors because the resulting metal complex is more predictable compared to the linear analogues.

Effects of modifications of histidine-based cyclic peptide sequences and ring dimensions (Figure 9) on Cu(II) binding studied by Aleksandra Kotynia and co-workers[69], indicate a great potential to apply these ligands in Cu-based systems. The presence of non-His residues in the sequence and its effect on binding capacity indicate that a rational peptide design may be applied to create a variety of cyclic peptide Cu complexes to serve different purposes.

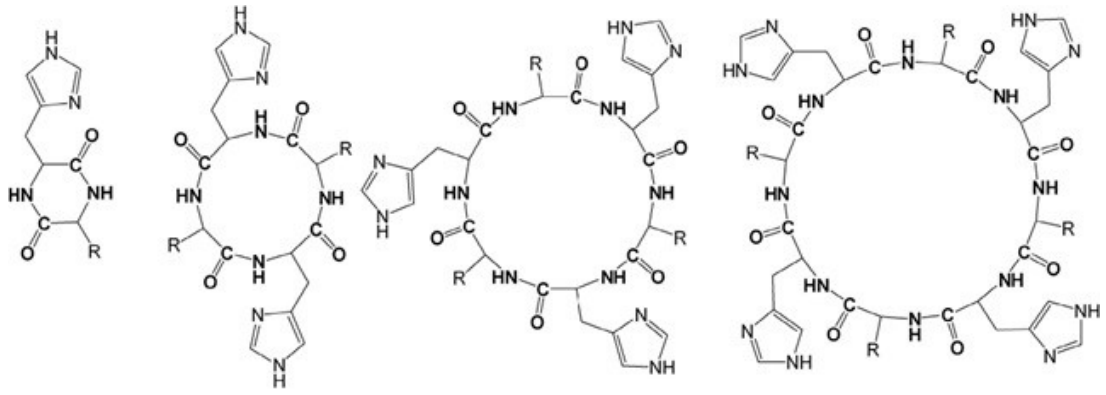
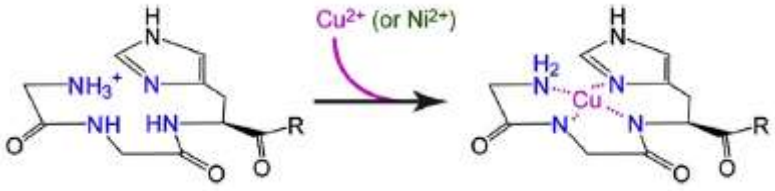


Figure 9. Hys-based cyclic peptides.

Cyclic peptides with two phosphoserines and two glutamic acids were developed by Delangle and co-workers to mimic high-affinity binding sites for uranyl found in proteins. They are found to chelate uranyl very efficiently even in a presence of calcium and they are relevant for uranyl binding *in vivo*.[70]

Kritzer et el.[71] found that a peptide shown in a figure coordinates Ni^{2+} and Cu^{2+} in a square planar geometry by the *N*-terminal amine, the imidazole and the two amide nitrogens in between.



Scheme 10. Structure of a tetrapeptide.[71]

Anion and cation binding can be succesfully predicted by DFT methods. Chermachini and co-workers published several articles on this topic. $C(Pro)_3$, $c(Pro)_4$, $c(Ala)_3$ and $c(Ala)_4$ were found to bind Be^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+} and Sr^{2+}.[72]

1.2. INTRODUCTION IN CALIXARENE'S CHEMISTRY

The development of calixarene's chemistry was initially closely related to the development of polymer chemistry. In attempts to find a better synthetic way to obtain bakelite resins (Figure 10), chemists have tried various conditions to condens phenol and formaldehyde.

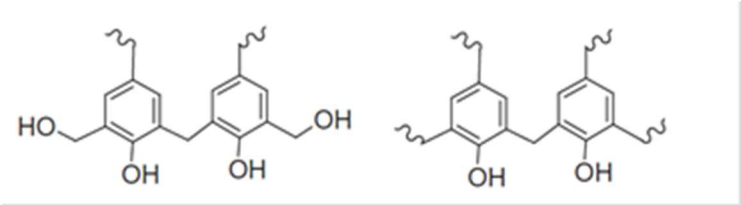


Figure 10. Structural fragments of Bakelite.

Zinke and Ziegler in 1942 came to the idea of blocking pairs of phenol groups with alkyl groups to reduce the possibility of branching, which is the first method of obtaining pure calixarenes.[73] Methods of preparation were published without structure because they were not yet sure whether they were cyclic or linear oligomers. Two years later, they proposed a cyclic tetramer structure. After that, petroleum oil company Petrolite became involved in the research, which became interested in these products as crude oil and water emulsion compounds. Researchers of the company in 1974 issue a patent for a new method of obtaining cyclic tetramers, but ten years later it becomes clear that their method actually generates octamer. Synthetic methods of preparation of these compounds were optimized by D. Gutsche, which examined a variety of phenol derivatives and searched for a wide range of reaction conditions to ensure the most efficient, obtaining a basic calixarene basket. The protocols mentioned in his works are nowadays used as reference methods for the synthesis of basic calixarenes.

Exist more than one structural conformations of calixarenes (Figure 11). The most stable one is called a cone.

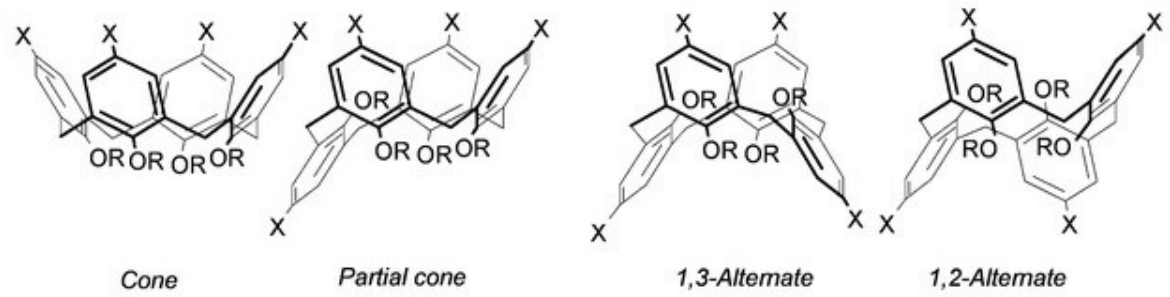


Figure 11. Structural conformations of Calix[4]arenes.

In this conformation all subunits are equally oriented and the lower rim, if the subunits are not substituted, is fully bonded by hydrogen bonds. NMR spectra of calixarenes in cone conformation are extremely simple because of the present Cn symmetry so that only one subunit is visible. The proton spectrum of the *p-tert*-butyl-calix[4]arene in deuterated chloroform is shown in Figure 12. The ^{13}C spectrum is also very simple and has only 7 signals. If other conformations are present, spectra become more complicated.[74] The spectrum appearance can be influenced by temperature and solvent, which is related to the conformations that the molecule may occupy.

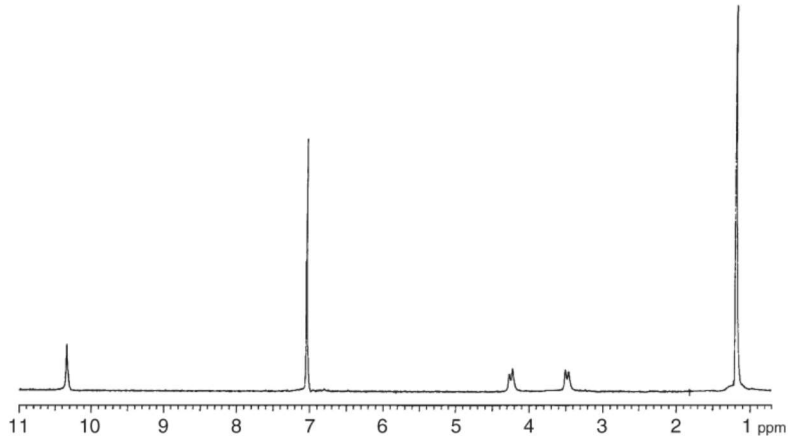
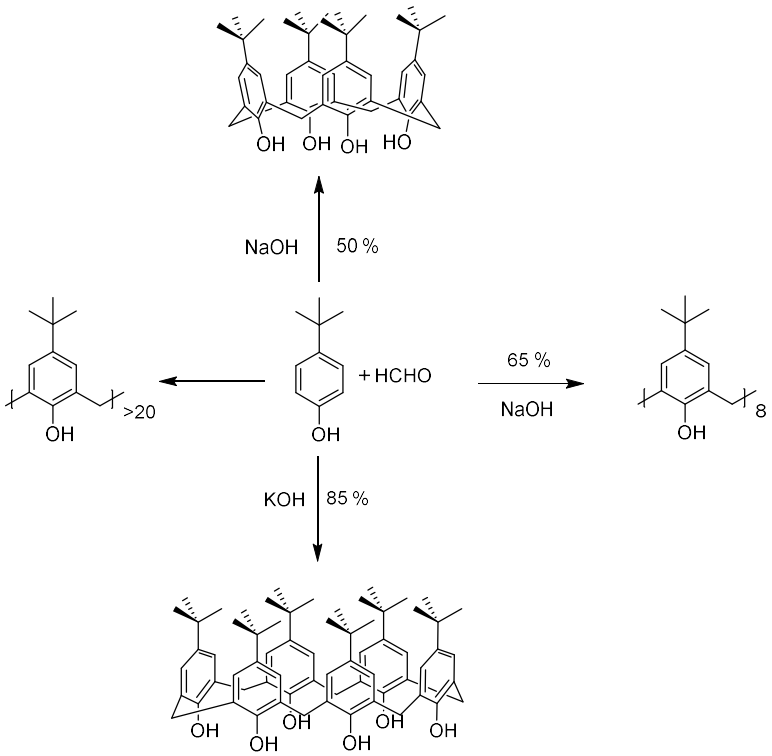


Figure 12. ^{1}H NMR spectrum of p-tert-butyl-calix[4]arene in chloroform.

1.2.1. The synthesis of basic "basket" and derivatization of calixarenes

1.2.1.1. Synthesis of basic "basket"

One of the reasons why calixarenes are so explored is certainly the simple formation of basic cyclic oligomers. Unlike other similar compounds such as cucurbits or porphyrins, basic calixarenes are obtained very easily and are now commercially available at an affordable price. The synthesis of calix[4]arenes, calix[6]arenes and calix[8]arenes is based on a base catalyzed condensation of formaldehyde and *p-tert*-butylphenol. As can be seen in Scheme 11, the reactants are equal in all syntheses, and various products are obtained in excellent yields. The difference is, of course, in the reaction conditions and the stoichiometric ratio of the reactants as shown by Gutsche.[75]

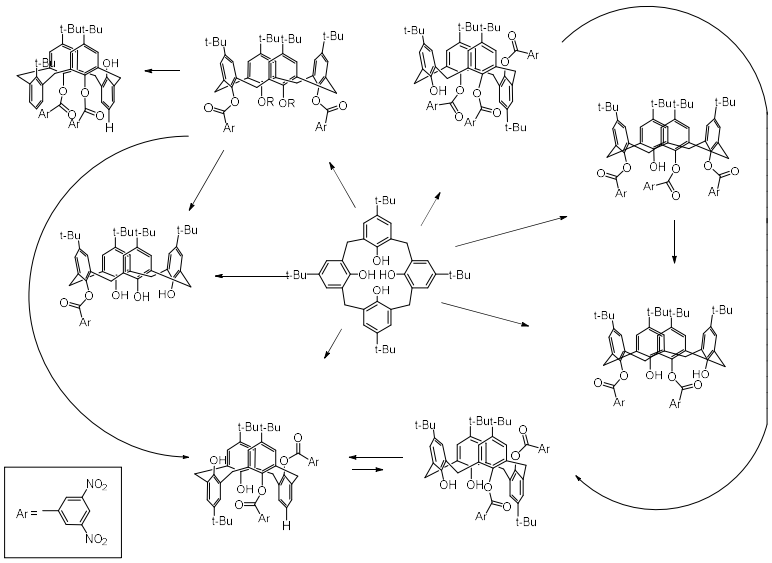


Scheme 11. Synthesis of basic Calixarenes.

1.2.1.2. Derivatization of calixarene's lower rim

Calixarenes with free hydroxyl groups do not exhibit significant receptor, catalytic and similar properties. To achieve such properties, it is necessary to make structural modifications to the

baskets or to functionalize the calixarenes. The two most common approaches are the modification of the hydroxyl group or the benzene ring pair relative to the hydroxyl group. In the hydroxyl group, the most important are reactions of esterification and translation into ether by various modifications of Williamson's synthesis. Ethers are much more frequent because the reactions can be better controlled, and the C-O-C bond is much more stable than the ester. The most commonly used is the reaction of sodium hydride and acyl chloride as well as the reaction of anhydride acid catalyzed by the addition of sulfuric acid.[76] The results of these reactions are quite unpredictable with regard to the degree of functionalization and conformation of the products, which is probably partly responsible for the steric influence of the ester fragments.[77] An interesting example of the reaction of the *p-tert*-butyl-calix[4]arenes with 3,5-dinitrobenzoyl chloride provides a whole range of products depending on minor changes in reaction conditions (Scheme 12).[78]



Scheme 12. Distribution of the reaction product of the p-tert-butyl-calix[4]arenes with 3,5-dinitrobenzoyl chloride depending on the reaction conditions.[78]

Using the imidazole as a base, a conical monoester is obtained, while using 1-methylimidazole, *syn* and *anti* 1,2-diesters may be formed depending on the solvent used. This example also shows one of the problems in the synthetic modifications of calixarenes, which is that besides the standard factors such as the compatibility of the reaction conditions and the present functional groups, has also been taken into account the stereochemical outcome of the reaction

as well as the functionalization degree. When choosing reaction conditions, it is best to rely on a large number of prior studies in which the reaction outcome was investigated in detail with respect to the reagents / conditions under which the reaction was conducted.

Much more frequent derivatization is carried out by alkylation than by acylation reactions. When introducing alkyl groups through ether bonds to the lower rim of the calix[4]arenes, a whole series of methods have been developed that for particular substrates result in the emergence of selectively functionalized products. When preparing monoalicylic ethers, sodium hydride is most frequently used in toluene with an alkylating reagent.[79] Although *p*Ka1 and *p*Ka2 are quite different for phenolic groups of calixarenes and is very difficult to avoid multiple functionalization. The reason is that *p*Ka1 of the starting compound and *p*Ka1 of monoalkylated product are comparable since monoanion of the single alkylated derivative is stabilized by hydrogen bonds with the two adjacent hydroxyl groups (Figure 13).[80]

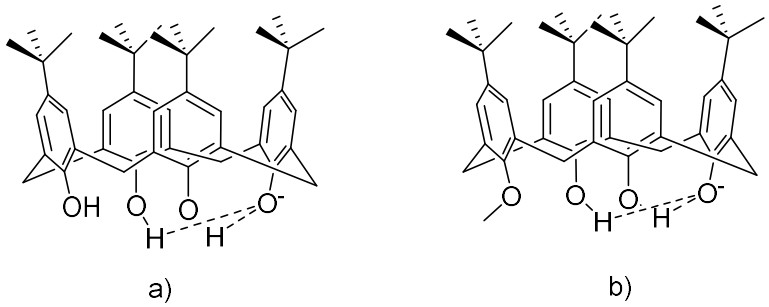


Figure 13. Hydrogen bonds in monoanion a) Unfunkcionalized and b) Monoalkylated calix[4]arenes.[80]

Tetrasubstituted alkyl calixarenes in the conformation of the cone are obtained by reaction with an excess of alkylating agent (about 4 equivalents per group) with sodium hydride as a base and tetrahydrofuran as a solvent. In these conditions, it has been found that the only tetra-substituted product is produced in the desired conformation. In addition to ordinary alkyl substituents, previously prepared branched esters such as bromoacetate esters, substituted bromoacetamides, substituted chloroacetones and the like can be introduced.[81] In such cases mild bases such as potassium carbonate in solvents such as acetone or acetonitrile are used. Alkylating reagents are then used in high excess and the reactions last for several days at reflux temperature.

1.2.2. Calixarenes as hosts

Calixarenes have been recognized as suitable parent compounds for the synthesis of a wide variety of ionophores and molecular receptors almost thirty years ago.[82-86] By the appropriate choice of the introduced donor atoms and the number of the repeating phenol units notable selectivity and high affinity towards variety of guests can be achieved.[85,87,88] The remarkable hosting abilities of calixarene derivatives have opened a path towards the design of specific receptors which could, for instance, be used in waste water treatment (the removal of toxic, heavy-metal ions).[89] Also, by properly choosing the substituents, one can prepare ion-selective ligands which may be useful for analytical purposes.[88,90] The receptor affinity towards particular cation is affected by several factors, the most important being the nature of the substituents on phenolic oxygen atoms forming the cation-binding site[85,86] and the compatibility of the size of this site with that of the metal ion.[85] In addition, the binding process is often strongly influenced by the solvation of the reactants and the complex(es) formed.[91-92] As shown in the literature,[93-96] the specific solvent-ligand and solvent-complex interactions, *i.e.* inclusion of the solvent molecule in a calixarene hydrophobic cavity, can play a very important role in determining the complexation equilibrium. In derivatives having substituents with both, a hydrogen-bond acceptor (carbonyl group) and a hydrogen-bond donor (*e.g.* –NH– group in secondary amides), intramolecular NH···O=C hydrogen bonds are formed.[97] These bonds need to be disrupted upon cation complexation and that was shown to have remarkable effect on the macrocycle binding abilities.[98]

1.3. PEPTIDOCALIXARENES

The ion-binding affinities of a cyclic peptides are often reduced by the inadequate orientation of amide functional groups. Moreover, the amide groups frequently participate in the formation of intramolecular hydrogen bonds and can be solvent exposed in the native conformation of cyclopeptide. A way to surpass these problems is to covalently bind cyclopeptide to a molecule with a well defined structure *via* amino acid side chains. In that way a proper orientation of amide groups and solvent-shielded binding site can be obtained. The molecules with a rigid geometry could be calixarenes and their derivatives. There have been reports in the literature on the calixarene amino acid derivatives as anion binders,[99-101] but, to the best of our knowledge,

there are no examples of derivatives with cyclopeptide substituents which are bound to calixarene moiety through amino acid side-chain groups. Most of the investigated compounds have amino acid substituents attached to separate subunits of calixarene molecule, and these substituents are not covalently connected to each other. The conjugation of peptides to calixarenes can be performed through terminal amino or carboxylic group. These two classes show different supramolecular properties as shown by Ungaro and co-workers[102] that synthesized compounds from both classes (Figure 14). The first difference appears in the conformational properties. ^{1}H NMR spectra of *N*-linked compounds are solvent independent with no evidence of intramolecular hydrogen bonding in a solution. On the contrary, ^{1}H NMR spectra of *C*-linked compounds in different solvents very clear demonstrate the shift from a closed to an open flattened cone conformation on switching from an apolar to a polar solvent. The two series of receptors have also different self-assembley properties in the solid state and they behave quite differently also in their molecular recognition properties. *N*-linked compounds are able to complex carboxylic acids and ammonium cations but not anionic guests. On the contrary, *C*-linked compounds don't bind primary ammonium cations but weakly bind carboxylate anions.

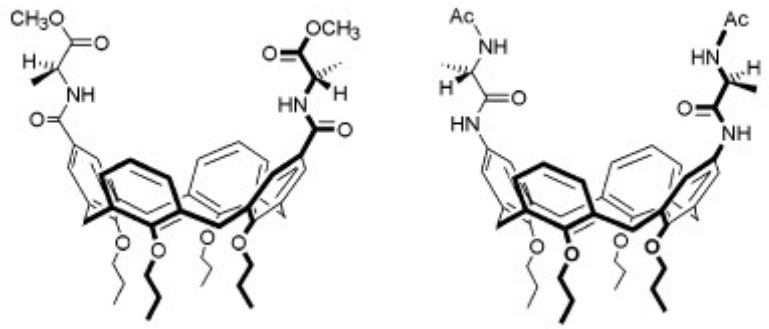


Figure 14. N- and C-linked peptidocalixarenes.[102]

The same group synthesized also bridged peptidocalixarenes[103] (Figure 15). *N*-bridged compounds contain a pseudopeptide bridge in 1,3 positions at the upper rim of a cone calix[4]arene consisting of α-amino acids linked through 1,3,5-diethylenetriamine spacer. This compound show formation of 1:1 complexes with peptides, simple amino acids and carboxylic

acids. On the other hand, *C*-bridged compounds were shown to possess high selectivity for carboxylate and no interaction with corresponding carboxylic acids.

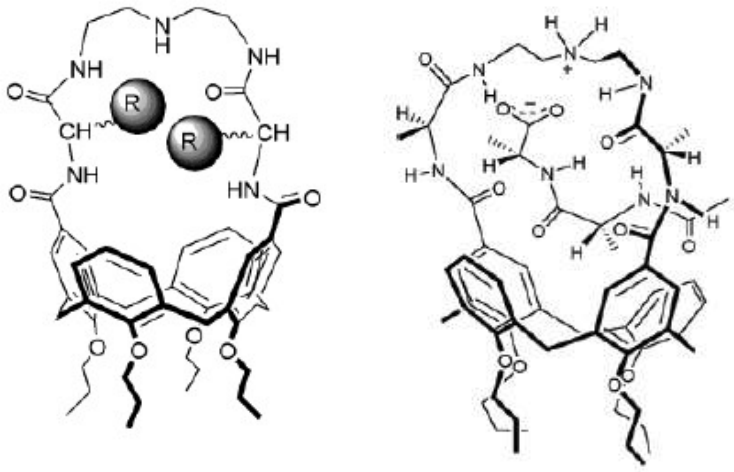


Figure 15. Structure of bridged peptidocalixarenes.[103] R= H, CH_3, $CH_2C_6H_5$.

Ungaro showed also that calix[4]arene amino acid can be easly incorporated in linear and cyclic peptides (Figure 16) *via* solid-phase synthetic protocols.[102]

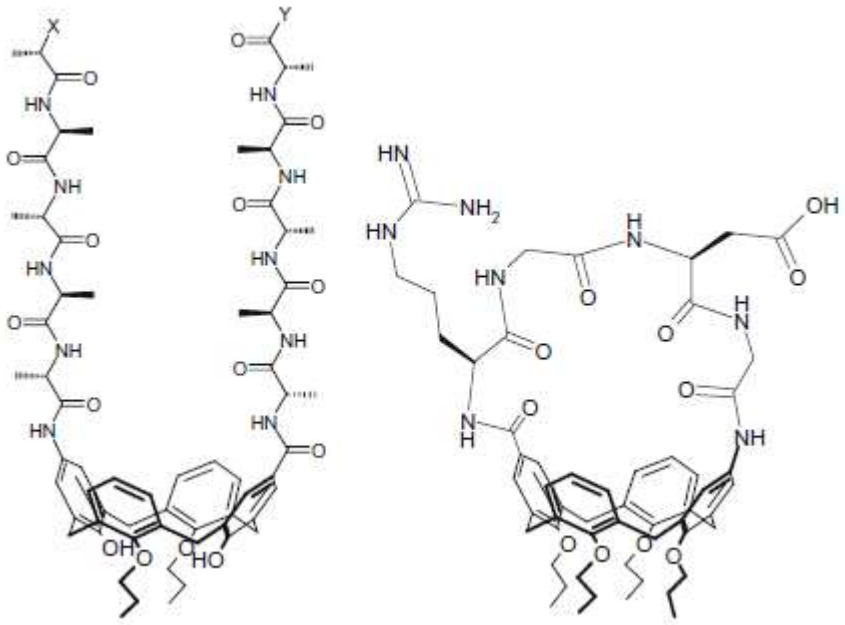


Figure 16. Peptidocalixarenes containing calix[4]arene amino acid.[102]

Soltani and co-workers[104] synthesized water-soluble peptidocalix[4]arenes (Figure 17) by incorporating arginine-rich short narrow groove binding residues on the lower rim of calixarene's scaffold with a goal of recognizing well-matched and mismatched DNA duplexes. By fluorescence measurments they confirmed the high afiinity of this compounds for DNA.

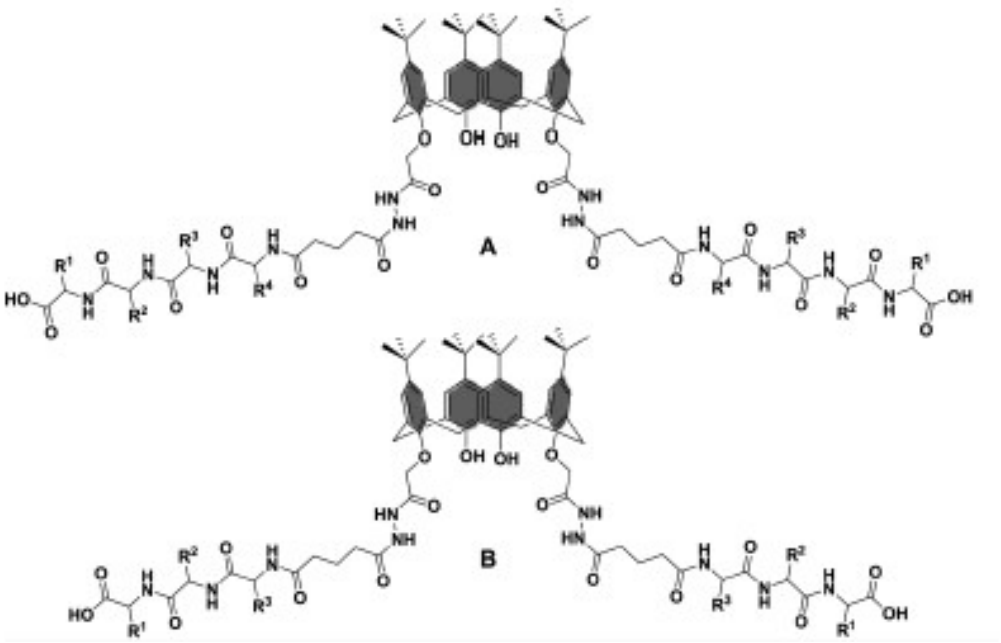


Figure 17. Structure of water-soluble, arginine-rich peptidocalix[4]arenes.[104]

Varma *et* el.[105] prepared series of tripeptoid and tetrapeptoid calix[4]arenes (Figure 18). By NMR and UV-VIS titrations they confirmed binding affinities toward different cations such as Cr^{3+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Hg^{2+}, Zn^{2+}. Ag^{+}, Pb^{2+}, Cd^{2+}, Na^{+}, K^{+}, Ca^{+}, Al^{3+} and Cu^{2+}.

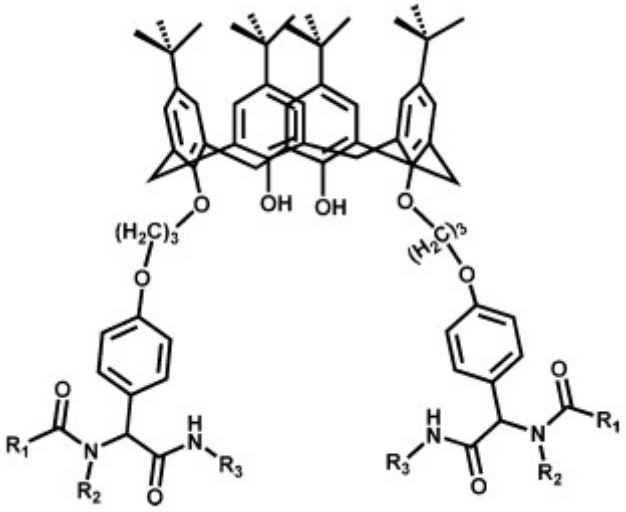


Figure 18. Structure of calixarene peptoid.[105]

§ 2. AIMS

In the last years, there has been a growing interest in the development of efficient and selective receptors of charged species and many cyclic and acyclic molelules with ion binding capacity have been investigated. Although most of the research regarding the complexation of ionic species by a variety of receptors has been focused on cation binding, recently anion recognition has become an intensively-studied area of supramolecular chemistry due to the ubiquitous role that anionic species play in biology, medicine, catalysis and the environment.[106]

The hydrogen bond is the most common non-covalent interaction employed in the design of anion receptors and organic compounds containing nitrogen atoms, such as amines, amides, as well as urea, thiourea and guanidine derivatives are used as hydrogen bond donors.[107]

Among them, cyclopeptides are of particular interest due to their occurrence in nature, the presence of peptide functional groups, and the possibility of introducing a range of amino-acid side-chain substituents able to give additional contributions to anion binding.[106,108]

A particular type of cyclopeptides are homocyclopeptides, *i.e.* peptides with multiple occurrences of a single amino acid residue. As these compounds may potentially form symmetric coordinating spheres around anion, which could result in receptors with high affinity for anions.

However, to the best of our knowledge they are still poorly investigated from the points of view of both synthesis and characterization and affinity towards anions.

Aim of this PhD thesis is the synthesis of homocyclopeptides bearing functional groups on the side chains able to enhance the binding affinity and selectivity of these receptors for anions.

Specific goals are:

1) To prepare linear precursors comprised of 4-6 amino acid residues by standard solution-phase peptide synthesis using three dimensional orthogonal protecting schemes (Figure 19).

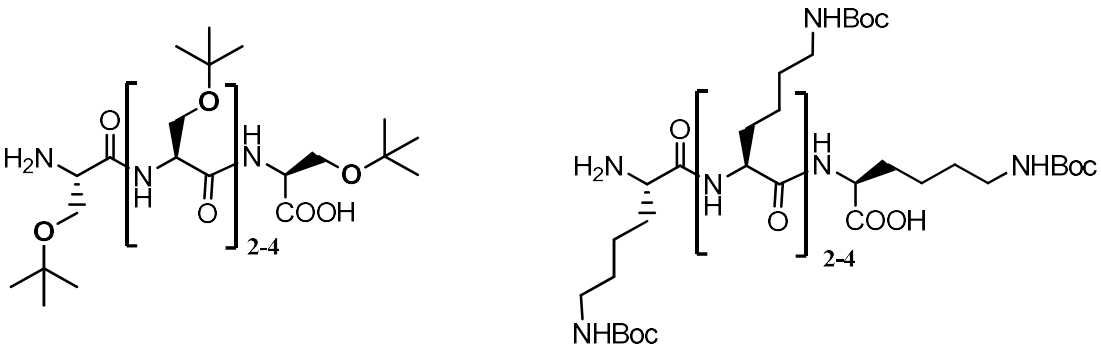


Figure 19. Structure of linear precursors

2) To investigate various strategies of head-to-tail macrocyclization and to find the optimal experimantal conditions to prepare cyclic tetra-, penta- and hexapeptides (Figures 20 and 21).

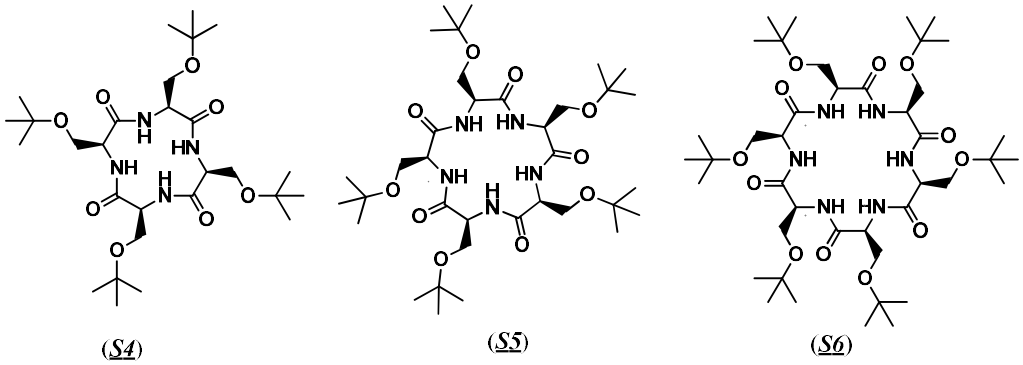


Figure 20. Structures of cyclic tetra-, penta- and hexaserines.

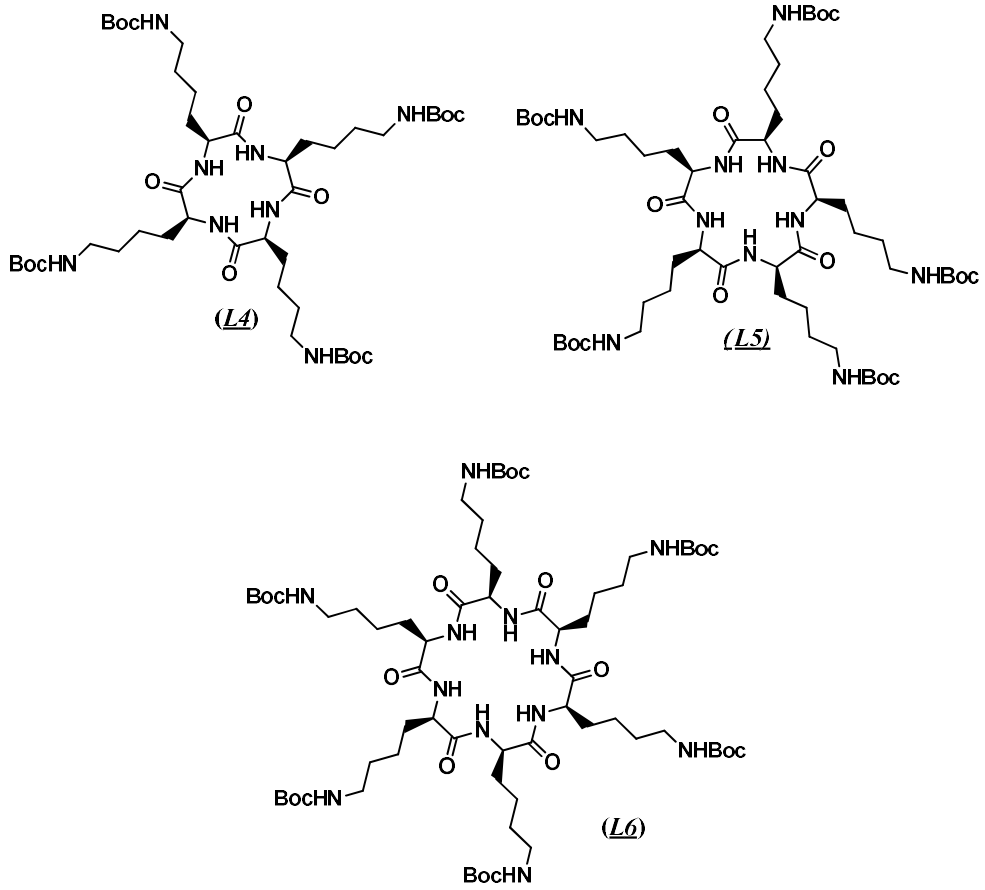


Figure 21. Structures of cyclic tetra-, penta- and hexalysines.

3) To study the complexation abilities of the above mentioned compounds towards various ions (cations, halides and molecular anions such as NO_3^-, ClO_4^-, HSO_4^-, $H_2PO_4^-$) by using an integrated approach including microcalorimetric, ^{1}H NMR and ESI-MS titrations as well as molecular dynamics simulations. Microcalorimetric measurements are extremely informative, providing the complexation enthalpy, and the standard entropy.[109] The groups forming binding site can be identified by ^{1}H NMR spectroscopy. Information about complex stoichiometry can be obtained by processing titration data and also by mass spectrometry.
4) To conjugate the cyclic peptides through side chain functional groups to rigid structures such as calixarene derivates in order to enhance their binding capacity and overcome their conformational freedom. The choice of serine, lysine and aminoalanine as starting materials is brought by the necessity to have a functionalized side chain in order to bind the cyclic compounds to calixarenes.

§ 3. EXPERIMENTAL SECTION

3.1. MATERIALS AND METHODS

All solvents and reagents were purchased from Sigma-Aldrich, Carbolution, Fluorochem or Merk and were used without further purification. Solvents were of HPLC grade.

Analytical Thin Layer Chromatography TLC was performed on silica gel 60 F254 precoated aluminum sheets (0.2 mm layer; Merck, Darmstadt, Germany); components were detected under an UV lamp (λ 254 nm) and by spraying with a cerium sulfate/ammonium molybdate solution or with a ninhydrin solution [5% (w/v) ninhydrin in ethanol], followed by heating at about 150 °C.

Silica Gel 60, 40-63 μm (Merk, Darmstadt, Germany) was used for flash chromatography.

NMR spectra were acquired at 400.13 and 100.61 MHz on a Bruker Advance 400 spectrometer (Bruker, Karlsruhe, Germany) interfaced with a workstation running a Windows operating system and equipped with a TOPSPIN software package. ^{1}H chemical shifts (δ) are given in parts per million (ppm) and are referenced to the solvent signals. Solutions of peptide complexes with ions were prepared by dissolving ligand in deuterated solvent followed by the addition of appropriate salt dissolved in the same deuterated solvent. Sonification of the solutions was used to ensure complete complexation. ^{1}H NMR titrations were processed by HypNMR program.

Matrix assisted laser desorption/ionization spectra (MALDI-TOF) were acquired on a Bruker Microflex LT Spectrometer. Electrospray ionization mass spectra (ESI-MS or ESI-Q-TOF-MS) were recorded on a Thermo Finnigan LCQ Advantage spectrometer (Hemel Hempstead, Hertfordshire, UK) and Agilent 1200.

HPLC were performed using an Amersham pharmacia biotech (P900) liquid chromatographer connected to an UV-VIS detector; chromatographic conditions were set as follows: column for analytical HPLC, Jupiter RP-18 (10 μm proteo 90A size: 250x4.60 mm, Phenomenex); column

for semipreparative HPLC, Jupiter RP-18 (10 μm proteo 90A size: 250x10 mm, Phenomenex); detector λ 226 and 280 nm; mobile phase: A (0.1 % TFA/H_2O) and B (CH_3CN) gradient elution from 5 % to 100 % B in 60 min.

The following solvents were used for physico-chemical measurements: mQ water, methanol (J.T. Baker, HPLC grade and Sigma-Aldrich, spectroscopic grade), CH_3CN (Sigma-Aldrich, spectroscopic grade). In the ^1H NMR spectroscopy, deuterated DMF (Sigma-Aldrich > 99.8%) and deuterated CD_3CN (Sigma-Aldrich > 99.8) were used as solvents.

Microcalorimetric measurements were conducted with an isothermal titration calorimeter Microcal VP-ITC at 25.0 °C. The calorimeter was calibrated electrically, and its reliability was additionally checked by carrying out the complexation of barium(II) by 18-crown-6 in aqueous medium at 25 °C. The results obtained (log K = 3.75, $\Delta_r H$ = –31.7 kJ mol^{-1}) were in excellent agreement with the literature values (log K = 3.73, $\Delta_r H$ = –31.5 kJ mol^{-1}).[1] Thermograms were processed using the Microcal OriginPro 7.0 program.

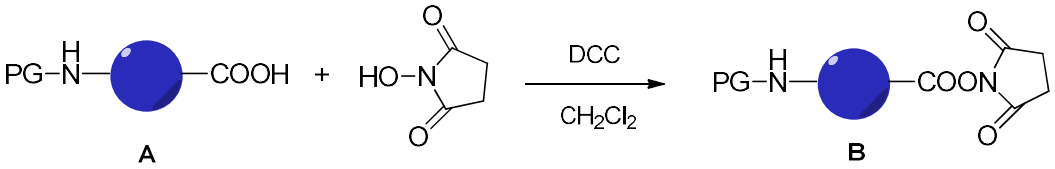
In the calorimetric studies, the enthalpy changes were recorded upon stepwise additions of acetonitrile solution of salts into solution of ligand (V_0 = 1.4182 cm^3). The heats measured in the titration experiments were corrected for heats of titrant dilution obtained by blank experiments. The dependence of successive enthalpy changes on the titrant volume was processed by non-linear least-squares fitting procedure using OriginPro 7.5 program. All measurements were repeated three or more times.

The molecular dynamics simulations were carried out by means of the GROMACS package (version 2016.4). Intramolecular and nonbonded intermolecular interactions were modelled by the OPLS-AA (Optimized Parameters for Liquid Simulations-All Atoms) force field. The linear and cyclic peptide ligands, and their anion complexes were solvated in cubical boxes containing between 1800 and 2500 molecules of acetonitrile or DMF, with periodic boundary conditions. The solvent boxes were equilibrated prior to inclusion of ligands and their complexes with the box density after equilibration in all cases being close to the experimental one within 2 %. During the simulations of the systems comprising anion-ligand complexes, tetramethylammonium (TMA) cation was included to neutralize the box. The TMA counterion was kept fixed at the box periphery whereas the complex was initially positioned at the box

center. In all simulations an energy minimization procedure was performed followed by a molecular dynamics simulation in *NpT* conditions for 50.5 ns, where the first 0.5 ns were not used in data analysis. The Verlet algorithm with a time step of 1 fs was employed. The cutoff radius for nonbonded van der Waals and short-range Coulomb interactions was 16 Å. Long-range Coulomb interactions were treated by the Ewald method as implemented in the PME (Particle Mesh Ewald) procedure. The simulation temperature was kept at 298 K with the Nosé-Hoover algorithm using a time constant of 1 ps. The pressure was kept at 1 bar by Martyna-Tuckerman-Tobias-Klein algorithm and the time constant of 1 ps.

3.2. GENERAL PROCEDURES

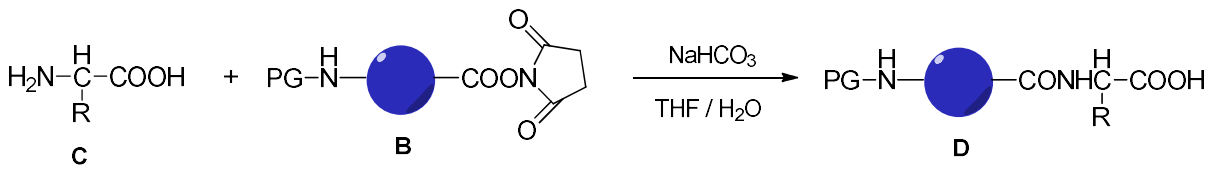
3.2.1. General procedure A: synthesis of succinimidyl ester[110]



PG = Fmoc, CbZ

A round-bottom flask was filled with *N*-protected amino acid A (1.0 mmol), NHS (1.1 mmol), dissolved in CH_2Cl_2 (DCM, 15 mL). A solution of DCC (1.1 mmol) in DCM (10 mL) was added dropwise. The resulting mixture was stirred at 0° C for 4 h and left in a refrigerator overnight. The next day, the *N*, *N*′-Dicyclohexylurea (DCU) precipitated was filtered off and the solvent was removed on a rotary evaporator. The residue was dissolved in ethyl acetate (EtOAc) and the remaining precipitate was filtered off. The solution was washed with $NaHCO_3$ (0.5 M, 15 mL), H_2O (15 mL), HCl (0.5 M, 15 mL), and H_2O (15 mL), dried over anhydrous Na_2SO_4, filtered and the solvent was removed on a rotary evaporator. The crude succinimidyl ester **B** was used in the coupling step without additional purification.

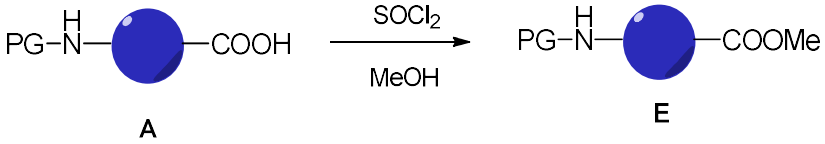
3.2.2. General procedure B: peptide bond formation through succinimidyl ester[110]



PG = Fmoc, CbZ

A round-bottom flask was charged with amino acid **C** (1.0 mmol), $NaHCO_3$ (2.2 mmol) and THF-H_2O (1:1, 10 mL). A solution of succinimidyl-activated ester **B** (1.1 mmol) in THF (7 mL) was added dropwise to the mixture, and the reaction was stirred at RT for 1–2 days. THF was removed under reduced pressure and the reaction mixture was acidified with HCl (0.1 M) to pH 7. The product was extracted with EtOAc (3×20 mL). The reunited organic layers were washed with water (15 mL) and dried over anhydrous Na_2SO_4. After filtration and evaporation of the solvent, the product **D** was purified by flash column chromatography on silica gel.

3.2.3. General procedure C: synthesis of methyl ester[111]

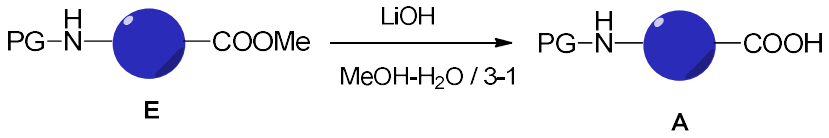


PG = CbZ

A solution of MeOH (5 mL) and $SOCl_2$ (2.0 mmol) was stirred for 30 minutes at 0°C. Reagent **A** (1.0 mmol) was then added and the mixture was stirred at RT overnight.

Solvents were removed under reduced pressure; obtained crude solid was dissolved in EtOAc and washed with a saturated solution of $NaHCO_3$ (5 mL) and H_2O (5 mL). Organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to obtain product **E**.

3.2.4. General procedure D: cleavage of methyl ester[112]



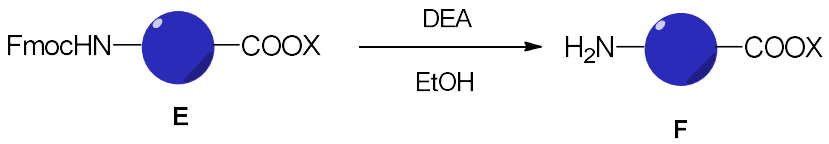
E A

PG = CbZ

Reagent **E** (1.0 mmol) and $LiOH \times H_2O$ (2.0 mmol) were added to a solution of $MeOH/H_2O$ (3:1, 12 mL). Reaction mixture was stirred at RT for 2 hours and monitored by TLC until the complete consume of reagent **E**.

The obtained crude solid, after removal of solvents, was dissolved in EtOAc (5 mL) and washed with HCl (0.1 M, 5 mL) and H_2O (2×5 mL). Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure to give product **A**.

3.2.5. General procedure E: cleavage of Fmoc protecting group[113]

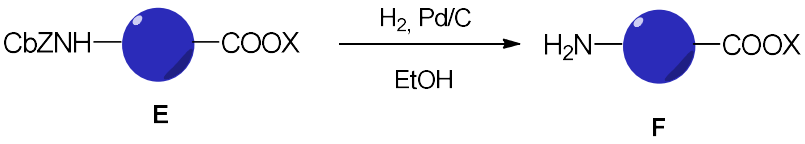


E **F**

X = H, OMe

To a solution of reagent **E** (1.0 mmol) in EtOH (50 mL), DEA (10.0 mmol) was added dropwise. Reaction mixture was stirred at RT overnight and monitored by TLC. EtOH and DEA were removed under reduced pressure to obtain a crude product that was then dissolved in H_2O (10 mL). Remaining DEA and by-product of the reaction were extracted with DCM (2×10 mL) and inorganic layer was then freeze-dried to give the product **F** that was used in the next step without further purification.

3.2.6. General procedure F: cleavage of CbZ protecting group[114]

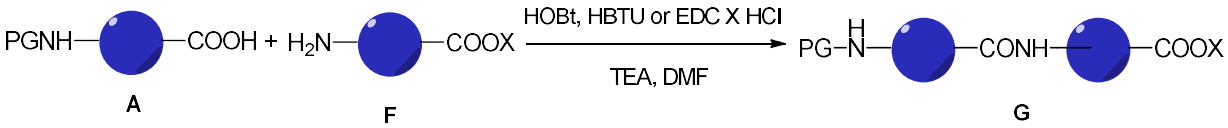


E **F**

X = H, Me, *t*Bu, Bn

Mixture of reagent **E** (1.0 mmol) and Pd/C (10wt. %) in EtOH (15 mL) was maintained under H_2 flow for 2 hours. Catalyst was then filtered through celite bed and solvent was removed under reduced pressure to obtain pure product **F**.

3.2.7. General procedure G: peptide bond formation through HOBt ester[115]

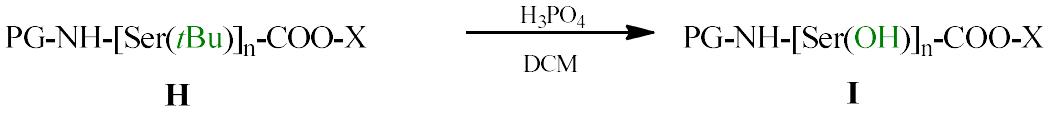


PG = CbZ, X = Me, *t*Bu

Reagent **A** (1.1 mmol) and HOBt (1.5 mmol) were dissolved in DMF (10 mL), cooled at 0 °C and maintained under stirring for 15 minutes. Were then added **F** (1.0 mmol.), HBTU or EDC × HCl (1.5 mmol) and after 5 minutes TEA (5 mmol).

Reaction mixture was stirred at RT for 24 hours and monitored by TLC until the complete consume of reagent **F**. Product **G** was precipitated adding cold water (10 mL) dropwise under stirring. Further purifications were performed by extraction with DCM (3×10 mL) and flash column chromatography in isocratic flow (10 % MeOH/DCM).

3.2.8. General procedure H: cleavage of tBu protecting group[116]



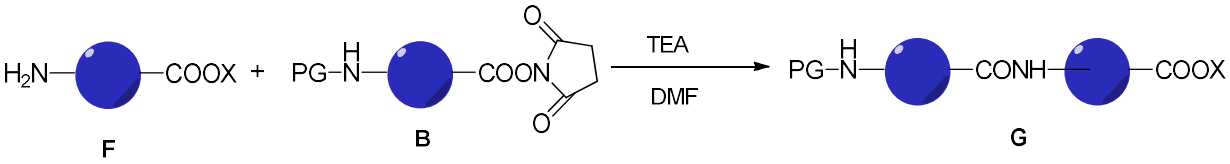
H **I**

PG = CbZ, Boc, Fmoc; X = H, Me

To a solution of **H** (1.0 mmol) in DCM (1 mL) aqueous phosphoric acid (10.0 mmol) was added dropwise. The mixture was stirred for 4 h at room temperature. Then, the solution was neutralized with $NaHCO_3$ (0.5 M, 5 mL) and water (10 mL) was added. The mixture was

extracted with EtOAc (3×5 mL). Combined organic phases were dried over Na_2SO_4 and concentrated in vacuum to give the desired product **I**.

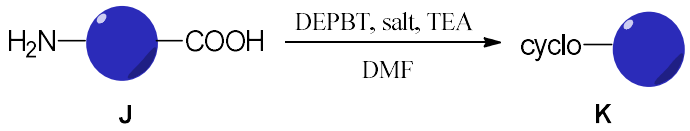
3.2.9. General procedure I: peptide bond formation through succinimidyl ester and protected amino acid on C-terminus[107]



PG = CbZ, X = Me, *t*Bu

Succinimidyl ester **B** (1.0 mmol) was dissolved in DMF (5 mL) and resulting mixture was cooled at 0 °C. TEA (2.0 mmol) and amino acid **F** were added and reaction mixture was stirred at RT overnight. Cold H_2O (5 mL) was added to the solution to precipitate **G** as a white powder, which was filtered using sintered funnel (D4), washed several times with cold water and dried under reduced pressure.

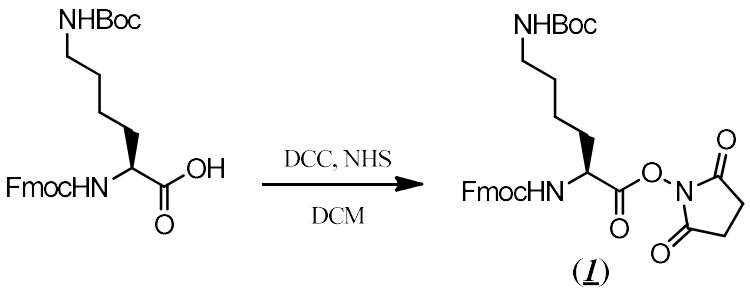
3.2.10. General procedure J: ion-assisted head-to-tail cyclization through DEPBT ester[29]



salt = LiCl, NaCl, NaTPB, TEACl, TBAOAc

To a solution of reagent **J** (1.0 mmol) and DEPBT (1.1 mmol) in DMF (500 mL) were added salt (5 or 100 mmol) dissolved in the minimum amount possible of H_2O, and TEA (at least 2 mmol) to reach pH 8. Reaction mixture was stirred at RT 3-5 days. Solvents were removed under reduced pressure and obtained crude solid was dissolved in EtOAc (10 mL) and washed with saturated solution of $NaHCO_3$ (5 mL), H_2O (5 mL), HCl (0.1 M, 5 mL), and H_2O (5 mL) H_2O. Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure to obtain product **K**. Further purifications were performed by flash column chromatography (gradient flow of 1-10%.MeOH/DCM).

3.3. Synthesis of *N*-alpha-(9-Fluorenylmethyloxycarbonyl)-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine succinimidyl ester (*1*)

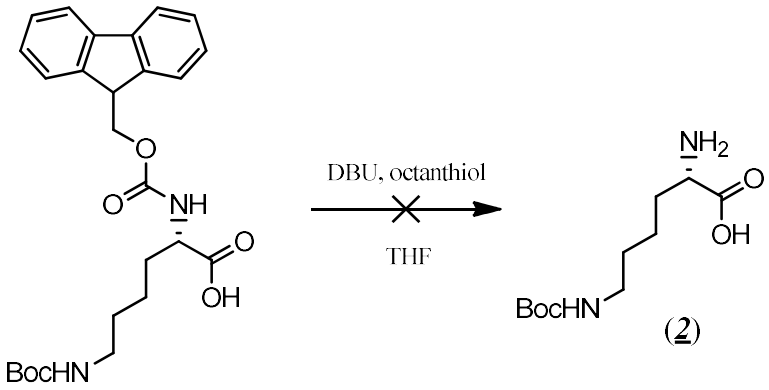


DCC (3.39 g, 16.4 mmol) dissolved in DCM (15 mL) was added dropwise to a solution of Fmoc-Lys(Boc)-COOH (7.00 g, 14.9 mmol) and NHS (1.89 g, 16.4 mmol) in DCM (15 mL). Following General procedure A, product ***1*** was obtained as a white powder (8.17 g, 14.5 mmol, 97 %) and was used in the next step without further purification.

R_f: 0.75 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.09 (d, 1H), 7.90 (d, 2H), 7.72 (t, 2H), 7.43 (t, 2H), 7.34 (t, 2H), 6.81-6.77 (m, 1H), 4.40-4.37 (m, 1H), 4.36-4.34 (m, 2H), 4.31-4.26 (m, 1H), 2.92 (br. s, 2H), 2.82 (br. s, 4H), 1.92-1.79 (m, 2H), 1.41 (br. s, 9H), 1.38-1.19 (m, 2H), one signal (2H) is covered by signal of Boc.

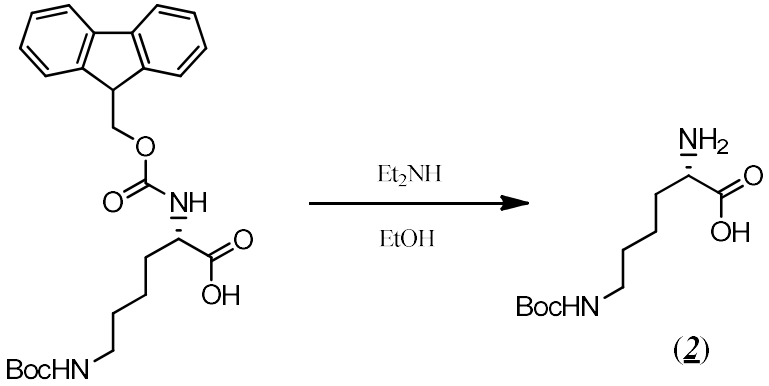
3.4. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysine



(***2***)

Fmoc-Lys(Boc)-COOH (0.10 g, 0.2 mmol) was dissolved in THF (10 mL) and under magnetic stirring 1-octanthiol (3.64 mL, 2.1 mmol) and catalytical amount of DBU (0.01 mL, 0.006 mmol) were added. Resulting mixture was stirred at RT for 24 hours and solvent was then removed under reduced pressure. Crude was dissolved in EtOAc and, after extraction, inorganic phase was evaporated. In this way was not possible to obtain the desired product.

3.5. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysine (*2*)

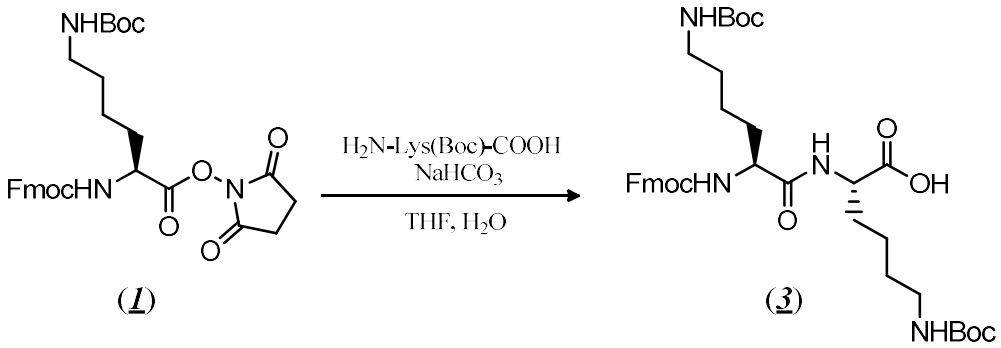


Fmoc-Lys(Boc)-COOH (0.10 g, 0.2 mmol) was dissolved in EtOH (20 mL) and under magnetic stirring Et_2NH (2.0 mL, 0.02 mmol) was added. Resulting mixture was stirred overnight at RT and solvent was then removed under reduced pressure. Crude was dissolved in EtOAc and, after extraction, inorganic phase was evaporated. In this way the desired product ***2*** (0.04 g, 0.18 mmol, 85 %) was obtained as a white powder.

R_f: 0.05 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ/ppm: 3.68 (t, *J* = 6.1 Hz, 1H), 3.04 (t, *J* = 6.7 Hz, 2H), 1.92 – 1.73 (m, 2H), 1.49 (dt, *J* = 13.7, 6.9 Hz, 2H), 1.39 (br s, 9H), 1.35-1.30 (m, 2H).

3.6. Synthesis of *N*-alpha-(9-Fluorenylmethyloxycarbonyl)-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine (*3*)



(*1*) (*3*)

H_2N-Lys(Boc)-COOH (*2*) (3.32 g, 13.5 mmol) and $NaHCO_3$ (2.58 g, 30.7 mmol) were dissolved in water (38 mL) and THF (76 mL). The rsulting mixture was stirred 10 min at 0 °C and after amino acid ***1*** (7.63 g, 13.5 mmol) dissolved in THF (38 mL) was added dropwise. Reaction mixture was left 24 hours at RT. The resulting solution was acidified until pH 6 using HCl (1.0 M) and THF was removed under reduced pressure. Extraction with DCM (3×100 mL) was performed and the reunited organic phases were washed with water (100 mL), dried over $MgSO_4$ and evaporated under reduced pressure. Resulting crude product was purified by flash column chromatography (gradient flow 0-10% MeOH/DCM) to obtain ***3*** as a white powder (7.15 g, 12.6 mmol, 93 %).

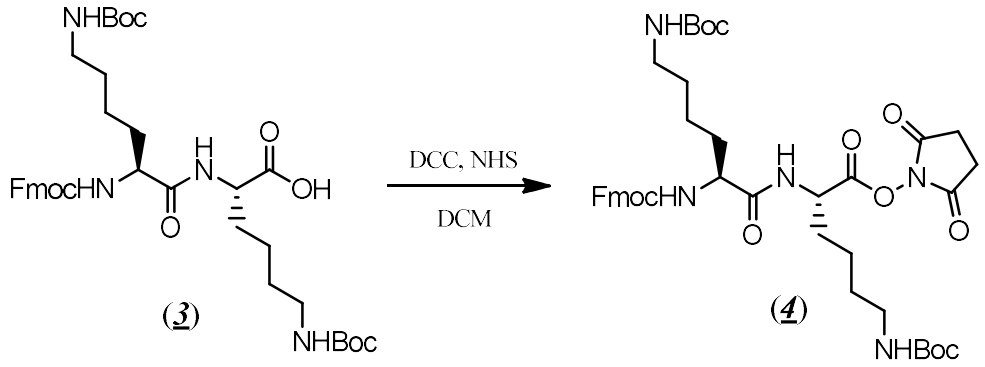
R_f: 0.53 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.90 (d, J = 7.2 Hz, 2H), 7.73 (t, J = 7.6 Hz, 2H), 7.56 (t, 1H), 7.42 (t, J = 7.6 Hz, 2H), 7.34 (t, J = 7.6 Hz, 2H), 6.76 (t, 1H), 6.69 (t, 1H), 4.32-4.23 (m, 3H), 3.96-3.90 (m, 2H), 2.89-2.84 (m, 4H), 1.70-1.65 (m, 2H), 1.54-1.51 (m, 2H), 1.37 (br. s, 9H), 1.35 (br. s, 9H) 1.35-1.21 (m, 8H).

^{13}C NMR (300 MHz, DMSO-d_6) δ/ppm: 171.1, 155.9, 155.5, 143.9, 143.7, 140.7, 128.9, 127.6, 127.3, 127.1, 125.4, 121.4, 120.1, 77.3, 65.6, 58.1, 54.9, 53.1, 46.7, 31.7, 29.5, 29.3, 28.3, 22.9, 22.5.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 719.36, found 719.48.

3.7. Synthesis of *N*-alpha-(9-Fluorenylmethyloxycarbonyl)-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine succinimidyl ester (*4*)

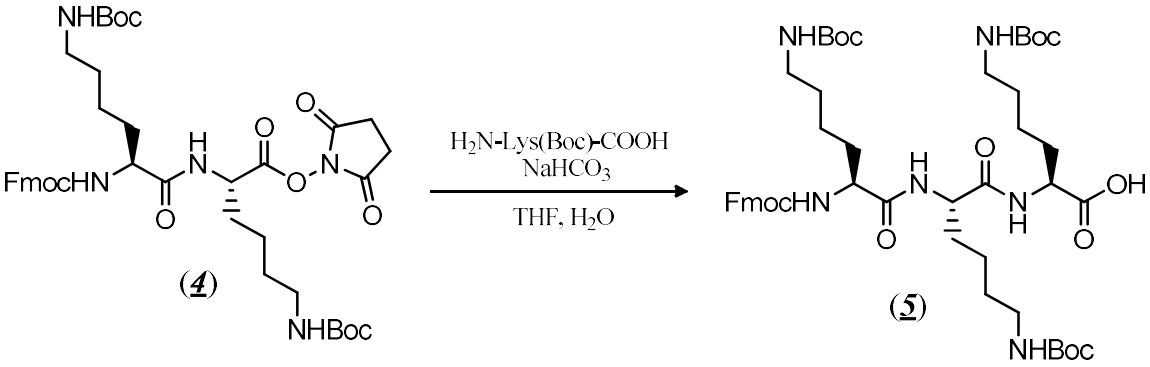


DCC (2.33 g, 11.3 mmol) dissolved in DCM (20 mL) was added dropwise to a solution of Fmoc-Lys(Boc)-Lys(Boc)-COOH (*3*) (7.15 g, 10.3 mmol) and NHS (1.31 g, 11.3 mmol) in DCM (60 mL). Following General procedure A product *4* was obtained as a white powder (7.07 g, 8.9 mmol, 86 %) that was used in the next step without further purification.

R_f: 0.93 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.57 (d, 1H), 7.90 (d, 2H), 7.73 (d, 2H), 7.50 (d, 1H), 7.43 (t, 2H), 7.34 (t, 2H), 6.81-6.77 (m, 2H), 4.62-4.55 (m, 1H), 4.30-4.18 (m, 3H), 4.12-3.96 (m, 1H), 2.98-2.92 (m, 4H), 2.82 (br. s, 4H), 1.86-1.77 (m, 2H), 1.72-1.63 (m, 4H), 1.55-45 (m, 2H), 1.37 (br. s, 9H), 1.34(br. s, 9H), 1.28-1.21 (m, 6H).

3.8. Synthesis of *N*-alpha-(9-Fluorenylmethyloxycarbonyl)-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine (*5*)



H_2N-Lys(Boc)-COOH (*2*) (2.41 g, 9.8 mmol) and $NaHCO_3$ (1.64 g, 19.6 mmol) were dissolved in water (38 mL) and THF (76 mL). The rsulting mixture was stirred 10 min at 0 °C and after ester *4* (7.07 g, 8.9 mmol) dissolved in THF (38 mL) was added dropwise. Following General procedure B, crude product was obtained and purified by flash column chromatography (10% MeOH/DCM) to obtain *5* as a white powder (4.22 g, 4.4 mmol, 50 %).

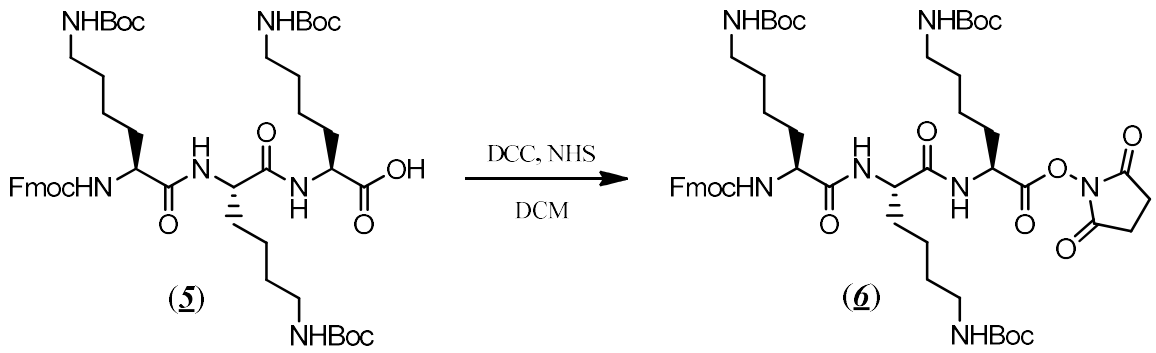
R_f: 0.34 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.12 (s, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.73 (t, *J* = 6.4 Hz, 2H), 7.50 (s, 1H), 7.41 (t, *J* = 7.3 Hz, 2H), 7.32 (t, *J* = 7.4 Hz, 2H), 6.79 (d, *J* = 76.4 Hz, 2H), 4.23 (d, *J* = 7.0 Hz, 3H), 4.14 (s, 1H), 4.00 (s, 1H), 3.77 (s, 1H), 2.86 (d, *J* = 18.6 Hz, 6H), 1.65 (s, 3H), 1.50 (s, 3H), 1.36 (br s, 18H), 1.33 (br s, 9H), 1.33-1.10 (m, 3H).

^{13}C NMR (300 MHz, DMSO-d_6) δ/ppm: 129.0, 127.4, 127.3, 121.4, 120.1, 108.8, 76.7, 46.8, 28.4. Some peaks are not visible.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 947.51, found 947.72.

3.9. Synthesis of *N*-alpha-(9-Fluorenylmethyloxycarbonyl)-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine succinimidyl ester (***6***)

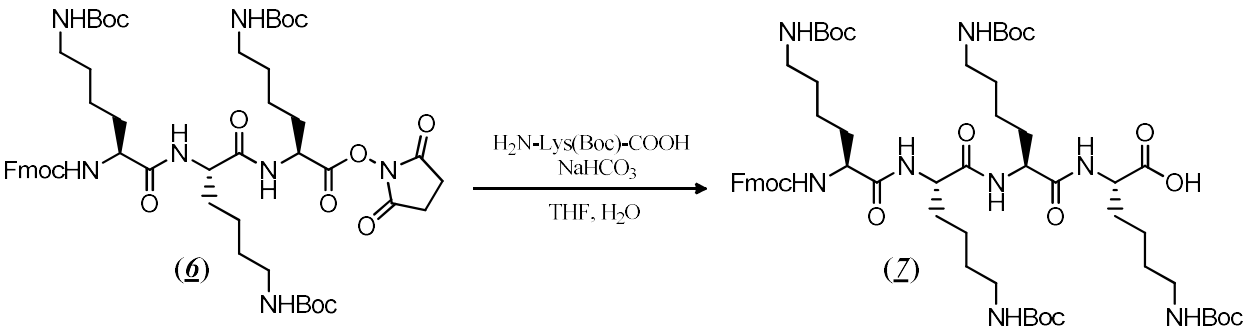


DCC (1.01 g, 4.9 mmol) dissolved in DCM (20 mL) was added dropwise to a solution of ***5*** (4.20 g, 4.4 mmol) and NHS (0.6 g, 4.9 mmol) in DCM (50 mL). Following General procedure A product ***6*** was obtained as a white powder (3.65 g, 3.6 mmol, 81 %) that was used in the next step without further purification.

R_f: 0.91 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.57-8.52 (m, 1H), 7.89 (d, *J*=7.4 Hz, 2H), 7.72 (t, *J*=6.1 Hz, 2H), 7.53-7.48 (m, 1H), 7.42 (t,*J*= 7.5 Hz, 2H), 7.33 (t, *J*= 7.4 Hz,2H), 6.80-6.67 (m, 3H), 4.62-4.50 (m, 1H), 4.33-4.18 (m, 4H), 2.93-2.92 (m, 6H), 2.91-2.75 (m, 4H), 1.86-1.77 (m, 3H), 1.75-1.55 (m, 5H), 1.52-1.45 (m, 3H), 1.37 (br. s, 18H), 1.34 (br. s, 9H), 1.29-1.21 (m, 7H), one signal is covered by solvent.

3.10. Synthesis of *N*-alpha-(9-Fluorenylmethyloxycarbonyl)-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine (*7*)



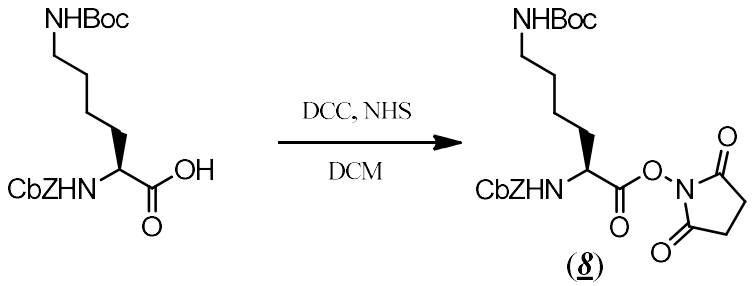
H_2N-Lys(Boc)-COOH (*2*) (1.06 g, 5.2 mmol) and $NaHCO_3$ (0.87 g, 10.3 mmol) were dissolved in water (30 mL) and THF (60 mL). The rsulting mixture was stirred 10 min at 0 °C and after ester *6* (4.90 g, 4.7 mmol) dissolved in THF (30 mL) was added dropwise. Following a General procedure B crude product was obtained and purified by flash column chromatography (10% MeOH/EtOAc) to obtain *7* as a white powder (1.00 g, 0.8 mmol, 18%).

R_f: 0.31 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.57-8.52 (m, 1H), 7.89 (d, *J*=7.4 Hz, 2H), 7.72 (t, *J*= 6.1 Hz, 2H), 7.53-7.48 (m, 1H), 7.42 (t,*J*= 7.5 Hz, 2H), 7.33 (t, *J*= 7.4 Hz, 2H), 6.80-6.67 (m, 4H), 4.37-4.09 (m, 6H), 3.95-3.91 (m, 1H), 2.95-2.91 (m, 8H), 1.78-1.60 (m, 6H), 1.59-1.42 (m, 6H),1.37 (br. s, 36H), 1.34-1.15 (m, 12H).

ESI-Q-TOF: $[M+Na]^+$ Calcd: 1175.66, found: 1176.15.

3.11. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine succinimidyl ester (*8*)



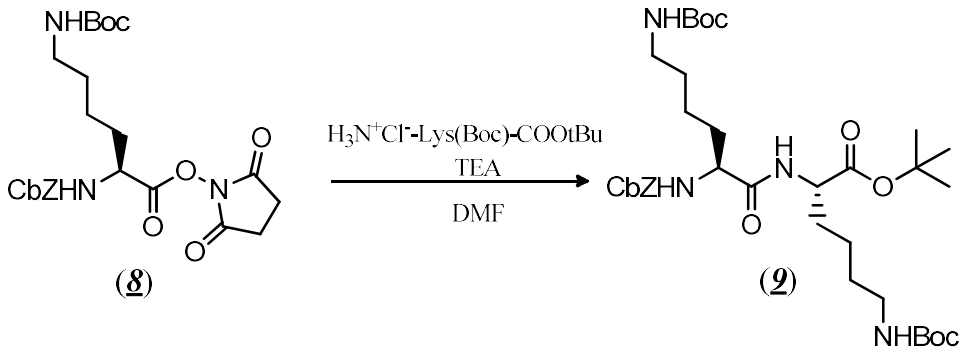
(*8*)

DCC (8.95 g, 43.4 mmol) dissolved in DCM (100 mL) was added dropwise to a solution of CbZ-Lys(Boc)-COOH (15.00 g, 39.4 mmol) and NHS (4.99 g, 43.4 mmol) in DCM (350 mL). Following a General procedure for a synthesis of succinimidyl ester, product ***8*** was obtained as a white powder (17.86 g, 37.4 mmol, 95 %) and was used in the next step without further purification.

R_f: 0.99 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.04 (d, *J* = 7.5 Hz, 1H), 7.42 – 7.27 (m, 5H), 6.77 (t, *J* = 4.9 Hz, 1H), 5.07 (s, 2H), 4.45 – 4.35 (m, 1H), 2.89 (d, *J* = 5.5 Hz, 2H), 2.81 (s, 4H), 1.88 – 1.69 (m, 2H), 1.44-1.36 (m, 4H), 1.37 (s, 9H), one peak is covered by Boc.

3.12. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine *tert*-butyl ester (*9*)



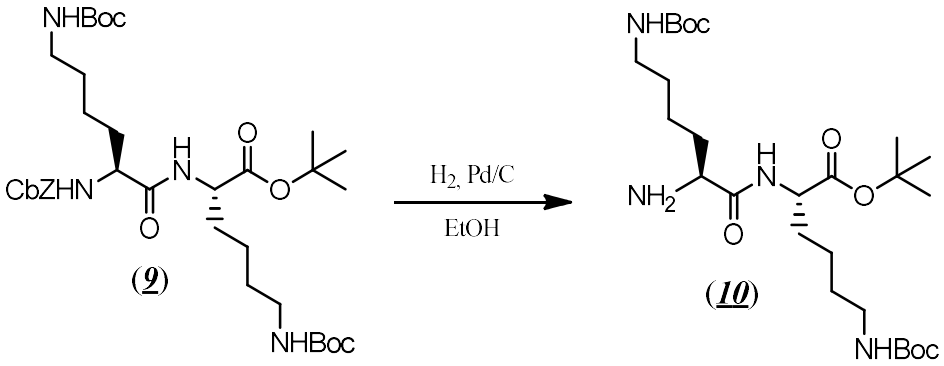
Compound ***8*** (5.40 g, 11.3 mmol) was dissolved in DMF (40 mL) and resulting mixture was cooled at 0 °C. TEA (3.6 mL, 20.6 mmol) and $NH_3^+Cl^-$Lys(Boc)-COOtBu (3.50 g, 10.3 mmol) were added and reaction mixture was stirred at RT overnight. As described in a General procedure I, product ***9*** (6.05 g, 9.1 mmol, 81 %) was isolated as a white powder and used in the next step without further purification.

R_f: 0.93 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.07 (d, J = 6.9 Hz, 1H), 7.38 – 7.22 (m, 6H), 6.67 (br s, 1H), 5.00 (s, 2H), 4.08 – 3.93 (m, 2H), 1.69 – 1.44 (m, 4H), 1.36 (br s, 9H), 1.34 (br s, 18H), 1.29 – 1.16 (m, 8H), one signal is covered by signal of DMF.

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 172.6, 171.6, 163.0, 137.3, 128.8, 128.3, 128.0,88.0, 80.0, 65.9, 54.8, 53.0, 32.1, 31.1, 29.6, 29.4, 28.6, 28.0, 23.1, 22.9.

3.13. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine *tert*-butyl ester (***10***)



Peptide ***9*** (6.05 g, 7.7 mmol) was dissolved in EtOH (150 mL) and 10wt. % Pd/C (0.61 g) was added. In a way described in General procedure F product ***10*** (5.29 g, 7.5 mmol, 98 %) was obtained as a yellowish oil and used in the next step without further purification.

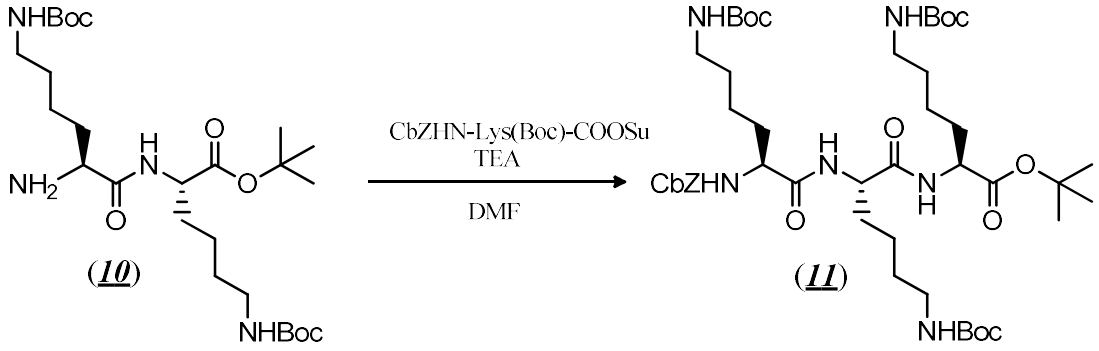
R_f: 0.03 (10% MeOH/DCM)

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.04 (d, *J* = 7.7 Hz, 1H), 6.78 – 6.69 (m, 2H), 4.09 (d, *J* = 5.8 Hz, 1H), 3.15 (t, *J* = 6.0 Hz, 1H), 2.89 (q, *J* = 5.7 Hz, 4H), 1.71-1.61 (m, 1H), 1.60-1.52 (m, 2H), 1.40 (br s, 9H), 1.37 (br s, 18H), 1.35 – 1.20 (m, 7H) one signal is covered by signal of Boc.

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 73.6, 72.7, 68.7, 49.8, 43.7, 43.2, 42.7, 38.3, 37.4, 36.7, 36.5, 35.2, 34.2.

ESI-Q-TOF: $[M+H]^+$ Calcd: 530.70, found: 531.28.

3.14. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine *tert*-butyl ester (***11***)



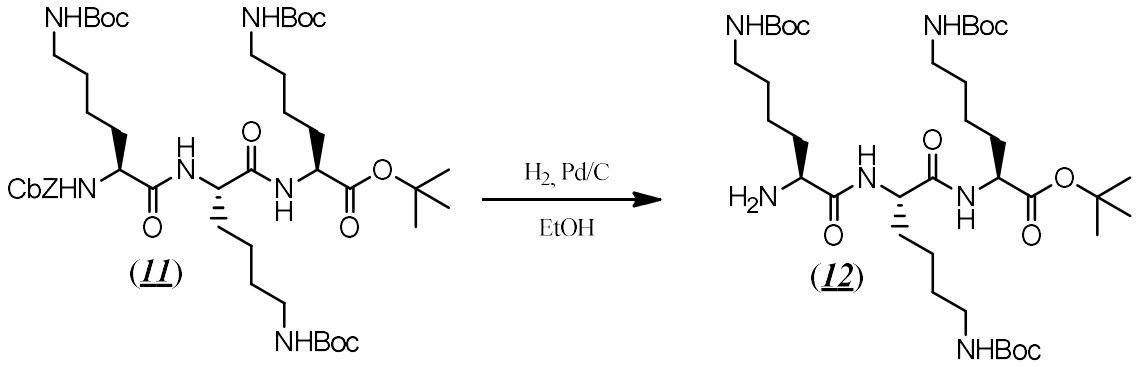
Succinimidyl ester ***8*** (3.68 g, 7.7 mmol) was dissolved in DMF (25 mL) and the resulting mixture was cooled at 0 °C. TEA (1.9 mL, 14.0 mmol) and peptide ***10*** (4.00 g, 7.0 mmol) were added and reaction mixture was stirred at RT overnight. As described in a general procedure I, product ***11*** (5.42 g, 6.4 mmol, 83 %) was isolated as a white powder and used in the next step without further purification.

R_f: 0.87 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.09 (d, *J* = 5.8 Hz, 1H), 7.84 (d, *J* = 7.5 Hz, 1H), 7.42 – 7.24 (m, 6H), 6.73 (br s, 3H), 5.02 (br s, 2H), 4.29-4.24 (m, 1H), 4.11 – 3.99 (m, 2H), 1.70 – 1.44 (m, 7H), 1.38 (br s, 9H), 1.36 (br s, 27H), 1.29-1.20 (m, 9H), two signals are covered by others.

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 72.8, 72.7, 72.6, 70.4, 64.0, 61.2, 61.7, 49.8, 46.0, 44.6, 43.3, 42.8, 42.7, 38.6, 37.6, 37.3, 36.7, 36.3, 35.2.

3.15. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine *tert*-butyl ester (*12*)



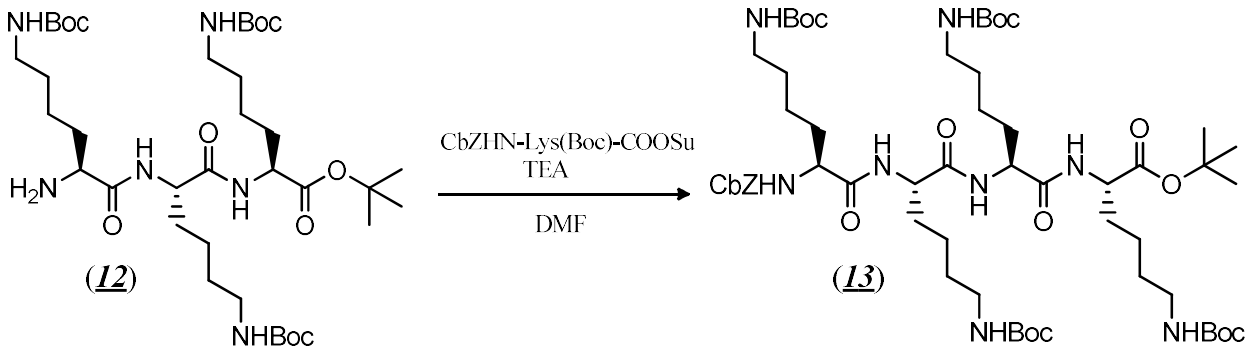
Tripeptide ***11*** (5.42 g, 5.8 mmol) was dissolved in EtOH (100 mL) and 10wt. % Pd/C (0.54 g) was added. In a way described in General procedure F product ***12*** (4.54 g, 5.7 mmol, 98 %) was obtained as a yellowish oil and used in the next step without further purification.

R_f: 0.02 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.18 (d, *J* = 7.4 Hz, 1H), 6.81 – 6.67 (m, 3H), 4.44 – 4.25 (m, 2H), 3.12 (dd, *J* = 7.4, 4.8 Hz, 1H), 2.89 – 2.83 (m, 6H), 1.69 – 1.58 (m, 3H), 1.58 – 1.45 (m, 4H), 1.39 (br s, 9H), 1.37 (br s, 27H), 1.34 – 1.18 (m, 11H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 73.5, 72.7, 72.6, 70.4, 68.7, 49.8, 49.0,43.7, 43.3, 42.8, 42.5, 38.6, 36.9, 36.7, 34.2.

3.16. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine *tert*-butyl ester (***13***)



Succinimidyl ester ***8*** (0.66 g, 1.4 mmol) was dissolved in DMF (7 mL), the resulting mixture was cooled at 0 °C. TEA (0.3 mL, 2.5 mmol) and peptide ***12*** (1.00 g, 1.3 mmol) were added and reaction mixture was stirred at RT overnight. As described in a general procedure I, product ***13*** (1.26 g, 1.1 mmol, 86 %) was isolated as a white powder and used in the next step without further purification.

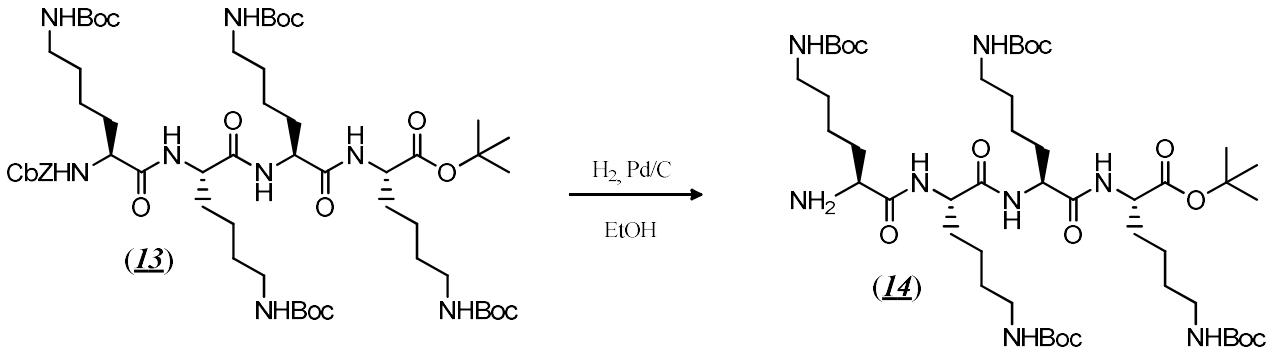
R_f: 0.85 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.05 (s, 1H), 7.88 (s, 1H), 7.82 (s, 1H), 7.39-7.31 (m, 6H), 6.70 (s, 4H), 5.02 (s, 2H), 4.24 (s, 2H), 4.05 (s, 1H), 3.97 (s, 1H), 2.89 – 2.79 (m, 8H), 1.69 – 1.55 (m, 4H), 1.53-1.39 (m,4H), 1.38 (brs, 9H), 1.36 (brs, 36H), 1.34 – 1.17 (m, 12H), one signal is covered by a signal of Boc.

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 72.8, 72.7, 72.6, 70.4, 64.0, 61.8, 61.7, 49.8, 49.0, 46.0, 38.6, 37.3, 36.5, 35.9, 36.7, 35.2.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 1143.69, found: 1144.35.

3.17. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine *tert*-butyl ester (***14***)



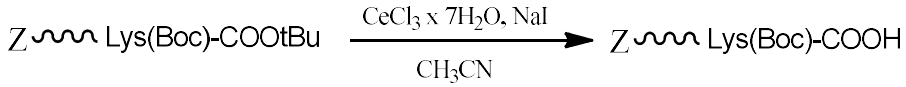
(***13***) (***14***)

Tetrapeptide ***14*** (1.26 g, 1.1 mmol) was dissolved in EtOH (60 mL) and 10wt. % Pd/C (0.13 g) was added. In a way described in General procedure F, ***14*** (1.16 g, 1.1 mmol, 99 %) was obtained as a yellowish oil and used in the next step without further purification.

R_f: 0.01 (10% MeOH/DCM).

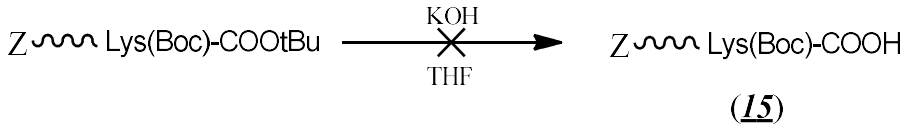
^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 4.43 – 4.31 (m, 2H), 4.31 – 4.24 (m, 1H), 3.42-3.36 (m, 1H), 3.10 – 3.02 (m, 8H), 1.90 – 1.76 (m, 3H), 1.74-1.64 (m, 4H), 1.56-1.49 (m, 11H), 1.48 (br s, 9H), 1.45 (br s, 36H), 1.42 – 1.33 (m, 6H).

3.18. Selective removal of *tert*-buthyl ester in a presence of Boc protecting group



(***15***)

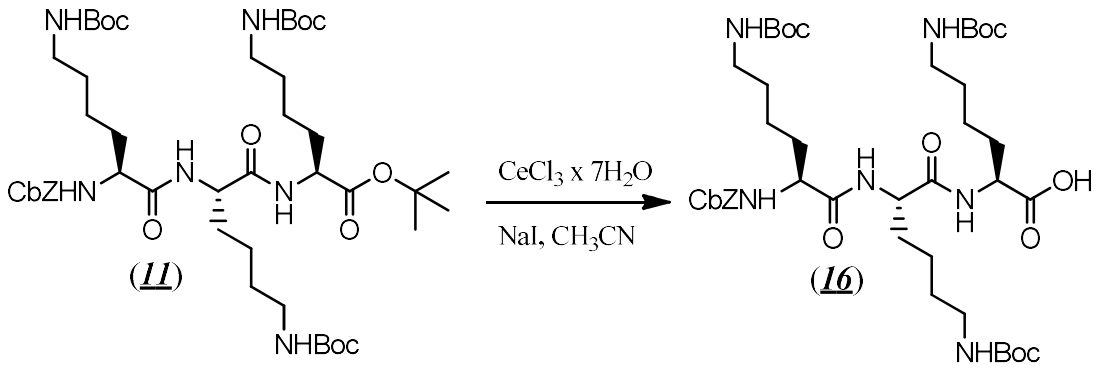
$CeCl_3$ x $7H_2O$ (0.04 g, 0.1 mmol) and NaI (0.01 g, 0.1 mmol) were dissolved in CH_3CN (5 mL) and the resulting mixture was refluxed for 24 hours. After cooling to the RT, peptide (0.06 g, 0.1 mmol) was added and reflux was continued. Reaction was monitored by TLC (10 % MeOH/ DCM) until a spot of the starting material didn't dissapear. Solvent was reduced under reduced pressure, resulting crude was dissolved in EtOAc (10 mL) and extracted with water (5 mL). Aquous phase, that was containig a product, was evaporated under reduced pressure to obtain product ***15*** (0.05 g, 0.1 mmol, 80 %).



(***15***)

Peptide (0.05 g, 0.1 mmol) was dissolved in THF (5 mL) and KOH (0.08 g, 0.2 mmol) was added. The resulting mixture was stirred at RT for 3 hours and monitored by TLC (hexane/EtOAc = 20/1). Solvent was then removed under the reduced pressure, H_2O (5 mL) was added and extraction with EtOAc (3 x 10 mL) was done. Reunited organic phases were dried over $MgSO_4$ and evaporated. In this way was not possible to obtain wanted product ***15***.

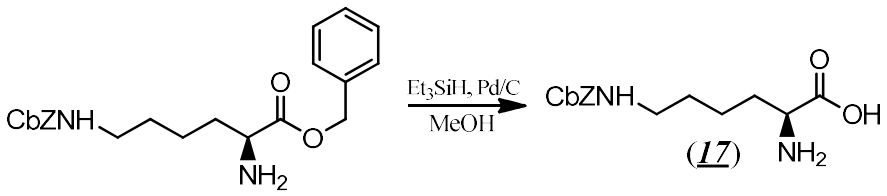
3.19. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine (***16***)



(***11***) (***16***)

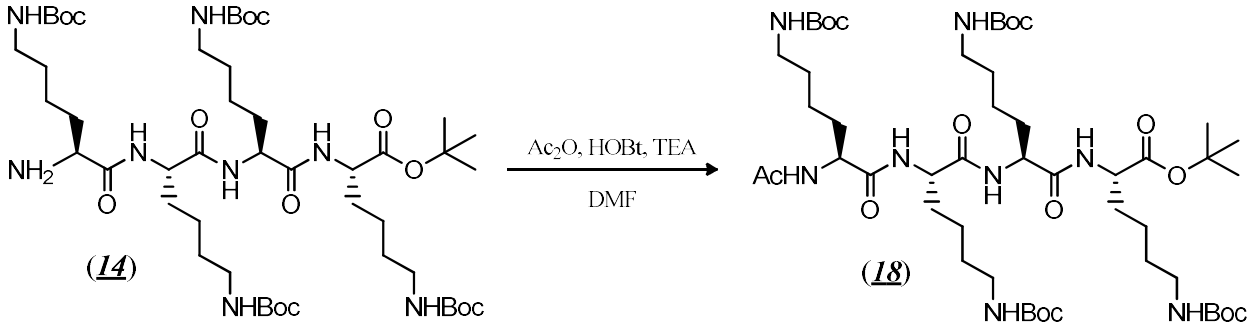
$CeCl_3$ x $7H_2O$ (1.67 g, 4.5 mmol) and NaI (0.60 g, 3.9 mmol) were dissolved in CH_3CN (200 mL) and the resulting mixture was refluxed for 24 hours. After cooling to the RT, peptide ***11*** (3.50 g, 2.9 mmol) was added and reflux was continued. Reaction was monitored by TLC (10 % MeOH/ DCM) until a spot of the starting material didn't dissapear. Solvent was reduced under reduced pressure, resulting crude was dissolved in EtOAc (100 mL) and extracted with water (150 mL). Aquous phase, that was containig a product, was evaporated under reduced pressure to give a product ***16*** (0.67g, 0.6 mmol, 20 %).

3.20. Selective removal of benzyl ester in a presence of CbZ protecting group



Protected lysine (0.05 g, 0.2 mmol) was dissolved in MeOH (5 mL) and Et_3SiH (33 μL, 0.3 mmol) and 10wt. % Pd/C were added. Reaction was monitored by TLC (10 % MeOH/DCM). Catalyst was filtered out and supernatant evaporated under the reduced pressure to give ***17*** (0.01 g, 0.1 mmol, 50%).

3.21. Synthesis of *N*-alpha-acetyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine *tert*-butyl ester (***18***)



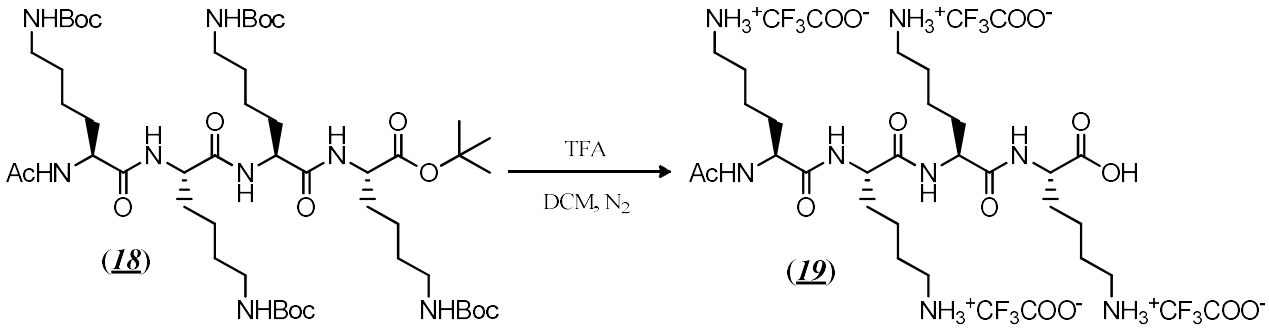
Peptide ***14*** (1.15 g, 1.1 mmol), HOBt (0.21 g, 1.6 mmol), Ac_2O (0.21 mL, 2.2 mmol) and TEA (0.3 mL, 2.2 mmol) were dissolved in DMF (10 mL) and sonicated for 2 minutes. Enother 2 equivalents of Ac_2O were added and the resulting mixture was sonicated for enother 2 minutes. This action was repeated for 5 times. Cold H_2O (10 mL) was added to the solution to precipitate ***18*** (0.75 g, 0.7 mmol, 63 %) as a white powder, which was filtered using sintered funnel (D4), washed several times with cold water and dried under reduced pressure.

R_f: 0.85 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.02 (d, *J* = 7.5 Hz, 1H), 7.95 (d, *J* = 8.0 Hz, 1H), 7.87 (d, *J* = 7.3 Hz, 1H), 7.76 (d,*J* = 7.2 Hz, 2H), 6.85-6.68 (m, 4H), 4.2-4.15 (m, 3H), 4.08-4.01 (m, 1H), 2.95 – 2.81 (m, 8H), 1.85 (s, 3H), 1.69-1.56 (m, 4H), 1.54 – 1.43 (m, 3H), 1.39 (br s, 9H), 1.37 (br s, 36H), 1.35-1.30 (m, 5H), 1.28-1.15 (m, 7H), one signal is covered by a signal of Boc.

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 172.3, 171.9, 171.8, 156.0, 80.9, 77.8, 53.1, 52.9, 52.4, 28.7, 28.1, 23.0, some peaks are not visible.

3.22. Synthesis of *N*-alpha-acetyl-L-lysyl-*N*-epsilon-L-lysyl-*N*-epsilon-L-lysyl-*N*-epsilon -L-lysine tetra-trifluoroacetate salt(***19***)



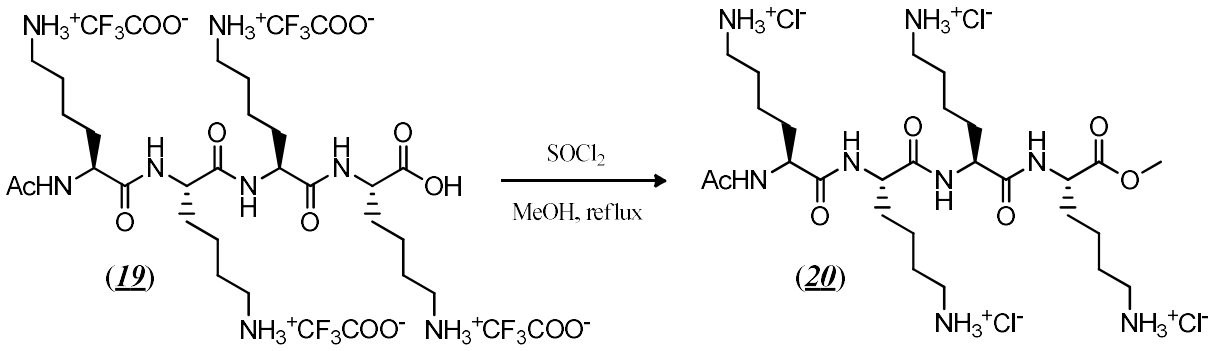
Peptide ***18*** (0.75 g, 0.7 mmol) was dissolved in dry DCM (10 mL) and TFA (15 mL) was added dropwise within 45 minutes. The resulting mixture was stirred under inert atmosphere 30 minutes at 0 °C and enother 60 minutes at RT. Reaction was monitored by TLC (10 % MeOH/ DCM). Solvent was removed under the reduced pressure and cold EtOEt (35 mL) was added to precipitate ***19*** (0.70 g, 0.7 mmol, 98 %) as a white powder that, after filtration, was dried in exicator over KOH and P_2O_5 for 36 hours.

R_f: 0.01 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.15 (d, *J* = 7.4 Hz, 1H), 8.08 (d, *J* = 7.8 Hz, 1H), 7.97 (d, *J* = 7.9 Hz, 1H), 7.88 (d, *J* = 7.4 Hz, 1H), 4.30 – 4.20 (m, 2H), 4.20 – 4.10 (m, 2H), 2.85 – 2.68 (m, 8H), 1.86 (s, 3H), 1.77-1.62 (m, 4H), 1.59 – 1.43 (m, 12H), 1.43 – 1.22 (m, 8H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 173.8, 172.3, 172.0, 171.8, 158.8, 158.5, 53.0, 52.6, 52.5, 52.2, 31.9, 31.7, 31.6, 30.8, 27.1, 27.0, 22.8, 22.6, 22.6, some peaks are not visible.

3.23. Synthesis of *N*-alpha-acetyl-*N*-epsilon-L-lysyl-*N*-epsilon-L-lysyl-*N*-epsilon-L-lysyl-*N*-epsilon-L-lysine tetrachloride salt methyl ester (***20***)



MeOH (30 mL) was cooled at 0°C and $SOCl_2$ (0.2 mL, 2.1 mmol) was added dropwise. Resulting mixture was stirred at 0 °C for 30 minutes. Peptide ***19*** (0.60 g, 1.1 mmol) was then added and mixture was stirred at RT overnight and enother 3 hours on reflux. Solvent was removed under the reduced pressure and the resulting crude was dissolved in EtOAc (30 mL) and washed with saturated solution of $NaHCO_3$ (20 mL) and H_2O (20 mL). Organic layer was dried over $MgSO_4$ and solvent was removed under the reduced pressure to give ***20*** (0.55 g, 0.8 mmol, 78 %) as a white solid.

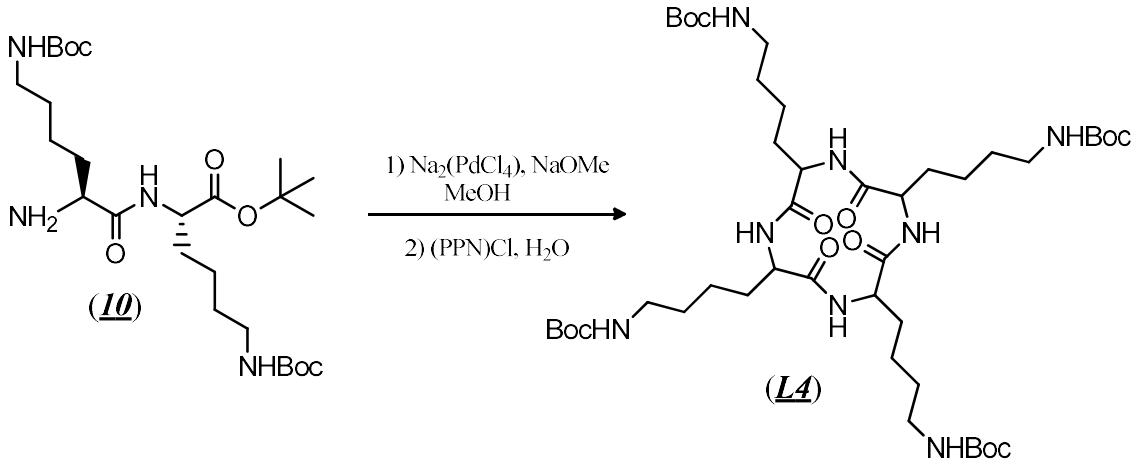
R_f: 0.02 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 4.39 – 4.17 (m, 4H), 3.63 (s, 3H), 2.83-2.70 (m, 8H), 1.88 (s, 3H), 1.78-1.75 (m, 2H), 1.4-1.65 (m, 3H), 1.64-1.50 (m, 11H), 1.50 – 1.27 (m, 8H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 52.71, 52.00, 51.87, 48.66, 38.48, 38.47, 38.34, 26.48, 26.39, 22.27, some peaks are not visible.

ESI-Q-TOF: $[M+H]^+$ Calcd: 586.68, found: 587.38.

3.24. Synthesis of cyclic *N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysine (***L4***)

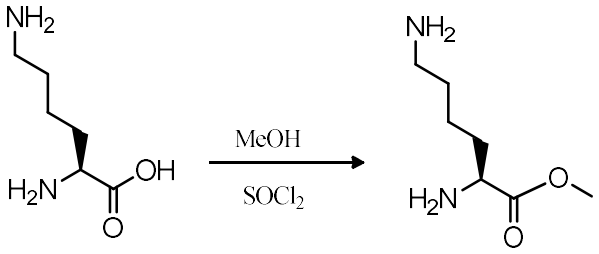


Dipeptide ***10*** (0.10 g, 0.2 mmol) and $Na_2(PdCl_4)$ (0.29 g, 0.1 mmol) were dissolved in MeOH (4 mL) and NaOMe (0.1 mL, 0.5 mmol) was added dropwise. The resulting mixture was stirred 24 hours at 65 °C. Cold H_2O (4 mL) and (PPN)Cl (0.11 g, 0.2 mmol) were added to precipitate product ***L4*** (0.01 g, 0.01 mmol, 5 %) as a white solid.

R_f: 0.73 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ/ppm: 8.23 – 7.66 (m, 4H), 6.88 – 6.62 (m, 4H), 4.32 – 3.88 (m, 4H), 2.96 – 2.76 (m, 8H), 1.76 – 1.44 (m, 9H), 1.37 (br s, 36H), 1.25-0.87 (m, 11H), one signal is covered by a signal of Boc.

3.25. Synthesis of L-lysine methyl ester (*21*)



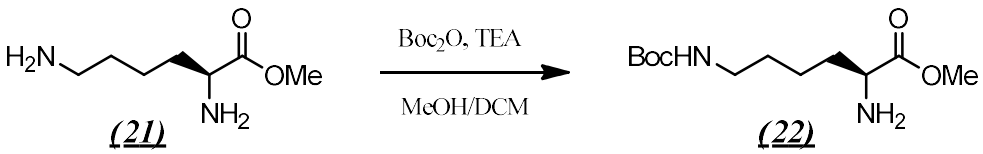
(*21*)

MeOH (200 mL) was cooled at 0 °C and $SOCl_2$ (3.9 mL, 54.0 mmol) was added dropwise. Resulting mixture was stirred at 0 °C for 30 minutes. L-Lysine (6.75 g, 37.0 mmol) was then added and mixture was stirred at RT overnight and another 3 hours on reflux. Solvent was removed under the reduced pressure and the resulting crude was dissolved in EtOAc (150 mL) and washed with saturated solution of $NaHCO_3$ (100 mL) and H_2O (100 mL). Organic layer was dried over $MgSO_4$ and solvent was removed under the reduced pressure to give ***21*** (4.62 g, 28.9 mmol, 78 %) as a white solid.

R_f: 0.35 (10%MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 3.97 – 3.92 (m, 1H), 3.74 (s, 3H), 2.78 – 2.68 (m, 2H), 1.90 – 1.76 (m, 2H), 1.65 – 1.54 (m, 2H), 1.53 – 1.31 (m, 2H).

3.26. Synthesis of N-epsilon-t-butyloxycarbonyl-L-lysine methyl ester (22)



(21) *(22)*

In a round bottom flask reagent ***21*** (4.40 g, 27.5 mmol) and TEA (15.4 mL, 110.0 mmol) were dissolved in MeOH (200 mL) and cooled at 0°C under stirring. Solution of Boc_2O (6.59 g, 30.3

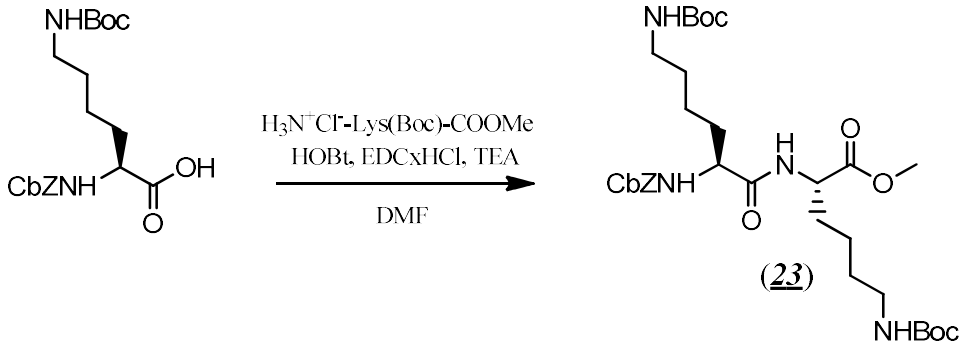
mmol), in DCM (200 mL) was then added dropwise within 10 minutes. After 6 hours at 0°C, solvents were removed under reduced pressure. Crude was purified by flash column chromatography (isocratic flow of 10% MeOH/DCM). In this way product ***22*** (4.00 g, 15.4 mmol, 56%) was isolated as a yellow oil.

R_f: 0.60 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d$_6$) δ/ppm: 3.61 (s, 3H), 3.27 (dd, *J* = 7.3, 5.7 Hz, 1H), 2.88 (dd, *J* = 12.8, 6.6 Hz, 2H), 1.54 (m, 1H), 1.46 – 1.21 (m, 5H), 1.37 (s, 9H).

^{13}C NMR (400 MHz, CDCl$_3$,) δ/ppm: 175.77, 155.04, 76.77, 53.38, 50.82, 33.77, 28.78, 27.74, 21.96, singnal of **C**H$_{2\varepsilon}$ is covered by DMSO.

3.27. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine methyl ester (*23*)



The product was obtained according to General procedure G using HCl×NH$_2$-Lys(Boc)-COOMe (3.50 g, 13.4 mmol) and Z-Lys(Boc)-COOH (5.63 g, 14.8 mmol), EDC×HCl (3.85 g, 20.1 mmol) and HOBt (2.72 g, 20.1 mmol), TEA (7.8 mL 67.0 mmol) and DMF (25 mL) as solvent.

After precipitation of a crude product, flash chromatography was performed in isocratic flow of 10% MeOH/DCM and the product ***23*** was isolated as white powder (7.91 g, 12.7 mmol, 95%).

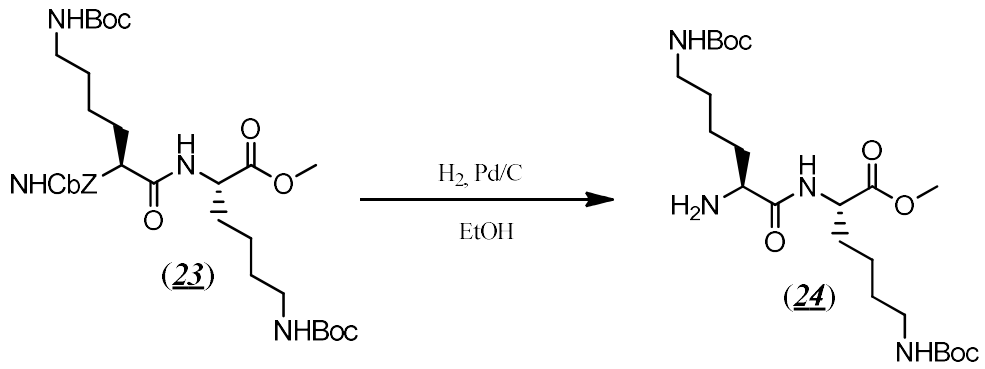
R_f: 0.70 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.21 (d, *J* = 7.4 Hz, 1H), 7.40 - 7.25 (m, 6H), 6.75 (br s, 2H), 5.02 (s, 2H), 4.20 (dd, *J* = 13.3, 7.9 Hz, 1H), 4.01 (dd, *J* = 13.0, 8.4 Hz, 1H), 3.61 (s, 3H), 2.95 – 2.84 (m, 4H), 1.75 – 1.44 (m, 4H), 1.37 (s, 18H), 1.35 – 1.31 (m, 4H), 1.31 – 1.19 (m, 4H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 172.62, 172.34, 155.97, 155.66, 137.14, 128.42, 127.86, 127.80, 77.44, 65.43, 54.39, 51.97, 51.84, 48.69, 31.70, 30.60, 29.32, 29.13, 28.36, 22.80, 22.72.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 645.76, found: 645.25.

3.28. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine methyl ester (*24*)



Dipeptide ***23*** (7.91 g, 12.7 mmol) was dissolved in EtOH (200 mL) and 10wt. % Pd/C (0.60 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***24*** (6.14 g, 12.6 mmol, 99 %) was obtained as a yellowish oil and used in the next step without further purification.

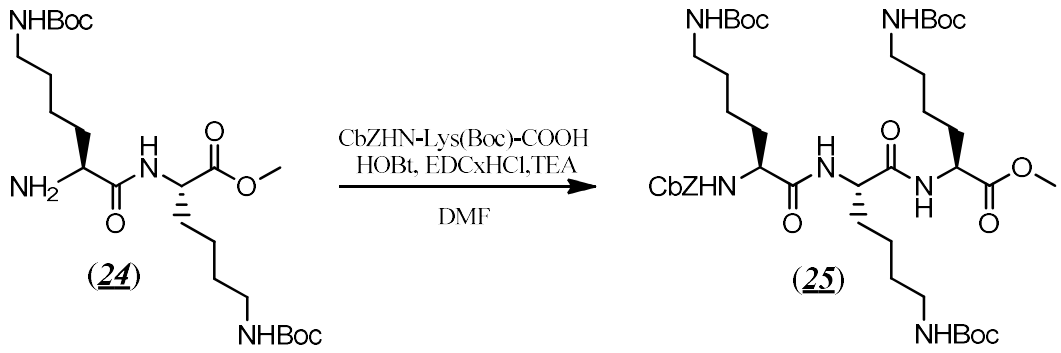
R_f: 0.01 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ/ppm: 8.13 (d, *J* = 7.6 Hz, 1H), 6.84 – 6.69 (m, 2H), 4.35 (s, 1H), 4.22 (dd, *J* = 13.5, 8.0 Hz, 1H), 3.62 (s, 3H), 2.94 – 2.84 (m, 4H), 1.75 – 1.44 (m, 4H), 1.37 (s, 18H), 1.35 - 1.22 (m, 4H).

^{13}C NMR (400 MHz, DMSO-*d*$_6$) δ/ppm: 175.49, 172.71, 167.97, 155.67, 77.44, 77.40, 56.12, 55.00, 54.36, 54.00, 51.86, 51.68, 45.29, 34.93, 30.86, 29.54, 29.16, 28.37, 22.69, 22.59, 18.65.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 511.31, found: 511.34.

3.29. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine methyl ester (***25***)



The product was obtained according to General procedure G starting with a product ***24*** (5.63 g, 11.5 mmol) and Z-Lys(Boc)-COOH (3.96 g, 10.4 mmol), HOBt (2.35 g, 17.3 mmol) and HBTU (6.55 g, 17.3 mmol), TEA (6.7 mL 57.6 mmol) and DMF (30 mL) as solvent.

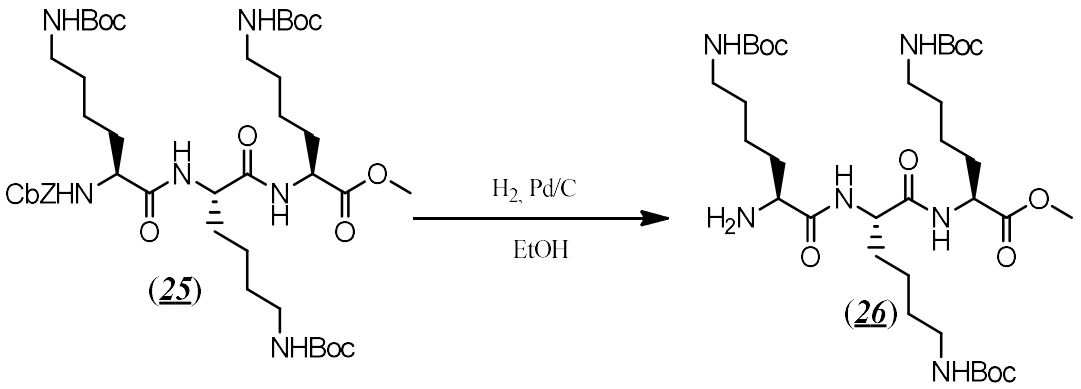
After precipitation of a crude product, flash chromatography was performed in isocratic flow of 10% MeOH/DCM and the product ***25*** was isolated as white powder (8.81 g, 11.4 mmol, 99%).

R_f: 0.74 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.22 (d, *J* = 7.1 Hz, 1H), 7.84 (d, *J* = 8.0 Hz, 1H), 7.42 – 7.21 (m, 6H), 6.80 – 6.70 (m, 3H), 5.02 (s, 2H), 4.27 (dd, *J* = 13.5, 8.2 Hz, 1H), 4.19 (dd, *J* = 13.1, 7.8 Hz, 1H), 3.97 (dd, *J* = 13.1, 8.4 Hz, 1H), 3.61 (s, 3H), 2.93 – 2.85 (m, 6H), 1.78 – 1.45 (m, 6H), 1.37 (s, 27H), 1.33 – 1.30 (m, 5H), 1.29-1.23 (m, 7H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 172.52, 171.87, 155.66, 137.12, 128.43, 127.86, 127.77, 77.44, 65.47, 54.00, 52.13, 51.84, 45.80, 28.37, two peaks are covered bby peak of DMSO.

3.30. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine methyl ester (***26***)



Tripeptide ***25*** (8.81 g, 12.6 mmol) was dissolved in EtOH (200 mL) and 10wt. % Pd/C (0.88 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***26*** (8.85 g, 12.3 mmol, 98 %) was obtained as a yellowish oil and used in the next step without further purification.

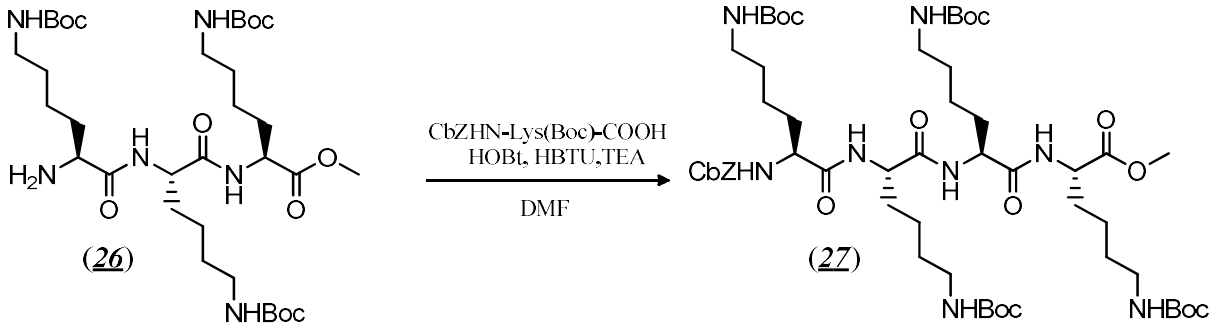
R_f: 0.01 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ/ppm: 8.31 (d, *J* = 7.3 Hz, 1H), 7.97 (d, *J* = 8.3 Hz, 1H), 6.75 (t, *J* = 9.1 Hz, 3H), 4.31 (d, *J* = 6.8 Hz, 1H), 4.25 – 4.13 (m, 1H), 3.62 (s, 3H), 3.11 (dd, *J* = 7.4, 4.7 Hz, 1H), 2.88 (t, *J* = 6.4 Hz, 6H), 1.58 (dtt, *J* = 35.9, 13.6, 6.5 Hz, 6H), 1.37 (s, 27H), 1.34 (d, *J* = 2.0 Hz, 6H), 1.31 – 1.11 (m, 6H).

^{13}C NMR (400 MHz, DMSO-*d*$_6$) δ/ppm: 175.37, 172.89, 172.31, 156.03, 77.82, 54.92, 52.38, 52.23, 51.99, 35.12, 32.79, 30.86, 29.87, 29.75, 29.48, 28.48, 23.14, 22.98, 22.74.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 739.46, found: 739.32.

3.31. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine methyl ester (*27*)



The product was obtained following General procedure G starting with a product ***26*** (5.51 g, 7.7 mmol) and Z-Lys(Boc)-COOH (3.14 g, 8.2 mmol), HOBt (1.52 g, 11.5 mmol) and HBTU (4.26 g, 11.5 mmol), TEA (4.4 mL 38.5 mmol) and DMF as solvent (40 mL).

After precipitation of a crude product, flash chromatography was performed in isocratic flow of 10% MeOH/DCM and the product ***27*** was isolated as white powder (6.71 g, 6.1 mmol, 79%).

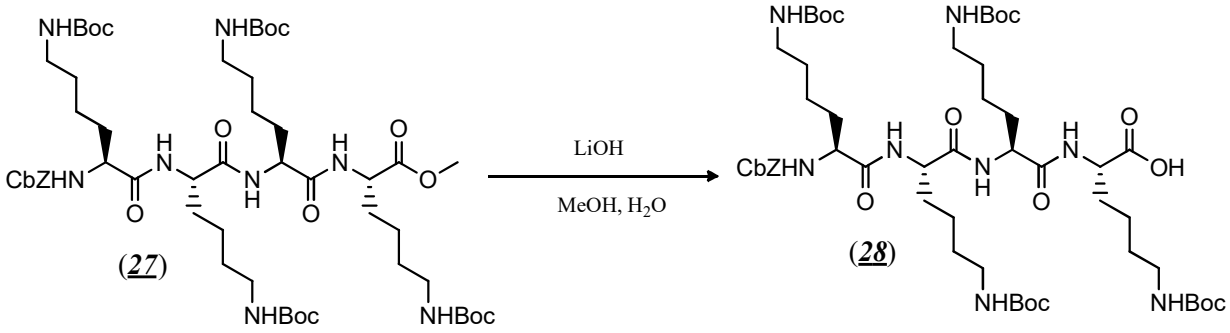
R_f: 0.78 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.19 (d, *J* = 7.3 Hz, 1H), 7.89 (d, *J* = 8.1 Hz, 1H), 7.83 (d, *J* = 7.9 Hz, 1H), 7.41 – 7.26 (m, 6H), 6.82 – 6.63 (m, 4H), 5.03 (s, 2H), 4.22 (tt, *J* = 15.9, 13.1, 6.0 Hz, 3H), 3.97 (td, *J* = 8.5, 4.6 Hz, 1H), 3.61 (s, 3H), 2.94 – 2.80 (m, 8H), 1.73 – 1.55 (m, 5H), 1.50 (t, *J* = 10.4 Hz, 4H), 1.37 (s, 36H), 1.33 (d, *J* = 6.7 Hz, 6H), 1.24 (dt, *J* = 12.3, 6.5 Hz, 9H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 172.86, 172.35, 172.13, 171.75, 156.43, 156.01, 137.48, 128.79, 128.13, 77.80, 65.86, 55.20, 52.77, 52.60, 52.38, 52.19, 32.30, 32.18, 32.04, 30.96, 29.67, 29.55, 28.73, 23.30, 23.06, 23.00, 22.93.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 1101.68, found: 1101.72.

3.32. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine (***28***)



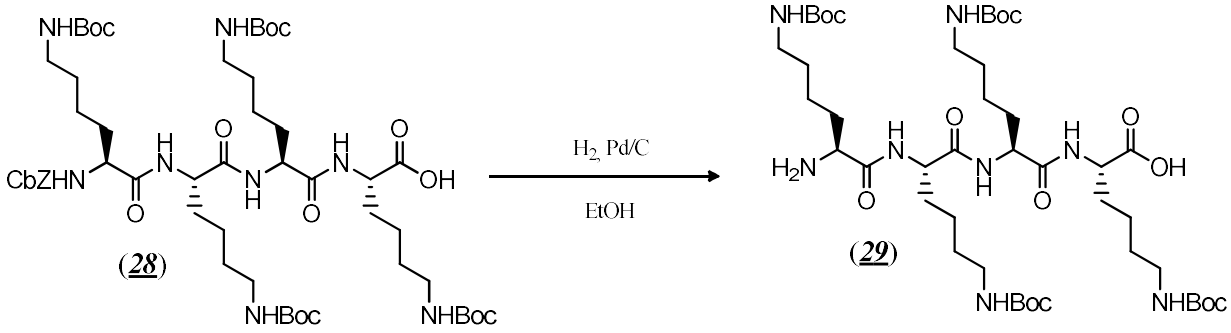
The product was obtained according to General procedure D starting with compound ***27*** (1.70 g, 1.5 mmol) and LiOH (0.13 g, 3.0 mmol) in MeOH (24 mL) and H_2O (8 mL) as solvents. After work-up product ***28*** was isolated as white powder (1.65 g, 1.5 mmol, 99%).

R_f: 0.43 (10% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 8.13 (s, 2H), 8.07 (s, 1H), 7.44 – 7.27 (m, 5H), 7.27 – 7.13 (m, 1H), 5.12 (s, 2H), 4.48 – 4.24 (m, 3H), 4.12 (s, 1H), 3.04 (t, *J* = 6.6 Hz, 8H), 1.86 (d, *J* = 19.6 Hz, 4H), 1.71 (dd, *J* = 15.1, 9.2 Hz, 4H), 1.49 (d, *J* = 9.8 Hz, 9H), 1.44 (d, *J* = 2.2 Hz, 36H), 1.44-1.33 (m, 7H).

^{13}C NMR (400 MHz, Methanol-d_4) δ/ppm: 165.87, 165.68, 164.69, 149.13, 128.76, 120.12, 119.65, 119.47, 70.48, 58.36, 47.22, 45.22, 44.20, 31.82, 31.63, 23.43, 23.35, 23.23, 23.00, 21.10, 19.45, 14.73.

3.33. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine (***29***)



Tetrapeptide ***28*** (1.65 g, 1.5 mmol) was dissolved in EtOH (40 mL) and 10wt. % Pd/C (0.17 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***29*** (1.40 g, 1.5 mmol, 100 %) was obtained as a white powder and used in the next step without further purification.

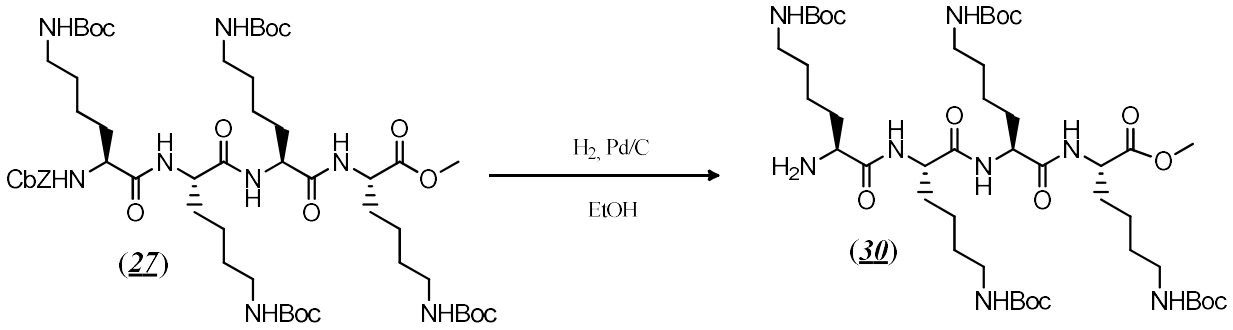
R_f: 0.00 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.07 (d, *J* = 7.9 Hz, 2H), 7.75 (s, 1H), 6.83 (s, 1H), 6.75 (dd, *J* = 8.7, 4.7 Hz, 3H), 4.27 (s, 1H), 4.22 – 4.14 (m, 1H), 4.10 (s, 1H), 3.95 (s, 1H), 2.87 (q, *J* = 6.5 Hz, 8H), 1.65 (s, 3H), 1.61 – 1.44 (m, 4H), 1.38 (s, 9H), 1.37 (s, 27H), 1.34 (s, 5H), 1.29 – 1.13 (m, 7H), some signals are covered by Boc.

^{13}C NMR (300 MHz, Methanol-d_4) δ/ppm: 155.55, 144.03, 77.30, 57.53, 54.44, 53.72, 52.91, 51.92, 31.99, 31.55, 29.66, 29.20, 28.31, 22.60.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 954.16, found: 955.07.

3.34. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine methyl ester (***30***)

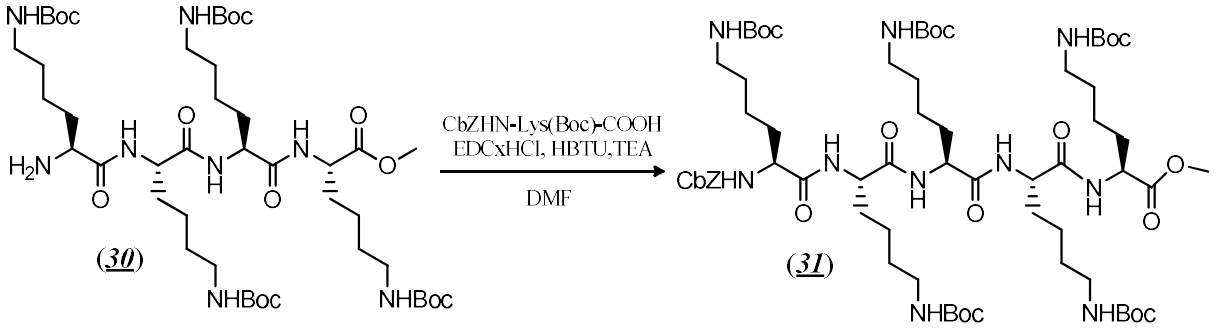


Fully protected tetrapeptide ***27*** (4.10 g, 3.7 mmol) was dissolved in EtOH (200 mL) and 10wt. % Pd/C (0.41 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***30*** (3.60 g, 3.7 mmol, 100 %) was obtained as a yellowish oil and used in the next step without further purification.

R_f: 0.01 (10% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 4.42 (d, J = 6.6 Hz, 1H), 4.39 – 4.30 (m, 2H), 3.73 (s, 3H), 3.12 – 2.99 (m, 8H), 1.95 – 1.77 (m, 3H), 1.70 (s, 4H), 1.50 (s, 5H), 1.45 (s, 36H), 1.43-1.31 (m, 12H).

3.35. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine methyl ester (***31***)



The product was obtained according to General procedure G starting from a product ***30*** (3.6 g, 3.8 mmol) and Z-Lys(Boc)-COOH (1.09 g, 4.2 mmol), HOBt (0.77 g, 5.7 mmol) and EDC×HCl (1.00 g, 5.7 mmol), TEA (1.7 mL 19.0 mmol) and DMF as solvent (25 mL).

After precipitation of a crude product, flash chromatography was performed in isocratic flow of 10% MeOH/DCM and the product ***31*** was isolated as white powder (3.93 g, 3.3 mmol, 87%).

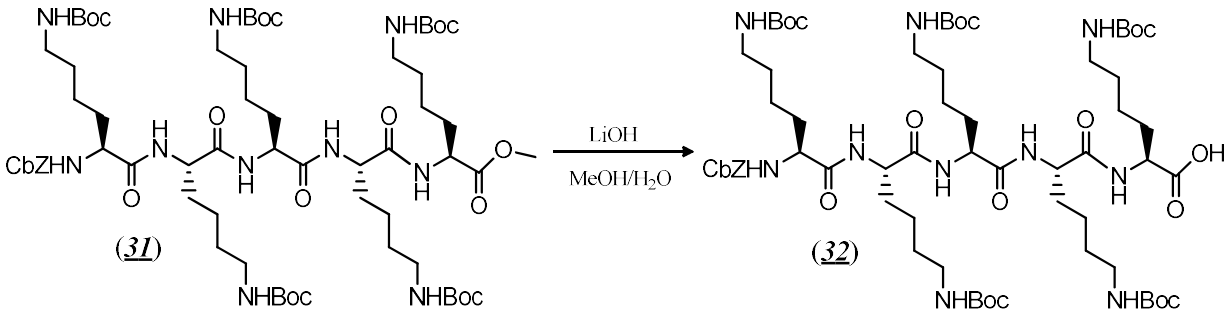
R_f: 0.82 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.19 (d, *J* = 7.2 Hz, 1H), 7.96 – 7.85 (m, 2H), 7.80 (d, *J* = 7.8 Hz, 1H), 7.44 – 7.22 (m, 6H), 6.72 (q, *J* = 9.3, 5.2 Hz, 5H), 5.03 (s, 2H), 4.22 (dt, *J* = 14.7, 7.2 Hz, 4H), 3.96 (t, *J* = 6.6 Hz, 1H), 3.61 (s, 3H), 2.87 (t, *J* = 6.4 Hz, 10H), 1.60 (dd, *J* = 15.2, 8.0 Hz, 6H), 1.50 (t, *J* = 9.7 Hz, 5H), 1.43-1.41 (m, 4H), 1.40 – 1.35 (m, 45H), 1.33 (d, *J* = 7.2 Hz, 7H), 1.24 (s, 8H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 155.67, 128.45, 127.81, 77.45, 65.48, 55.01, 52.48, 28.36, 22.70, some peaks are not visible.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 1329.79, found: 1330.50.

3.36. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine (*32*)



The product was obtained according to General procedure D starting with compound ***31*** (2.14 g, 1.6 mmol) and LiOH (0.13 g, 3.3 mmol) in MeOH (36 mL) and H_2O (12 mL) as solvents. After work-up product ***32*** was isolated as white powder (2.12 g, 1.6 mmol, 97%).

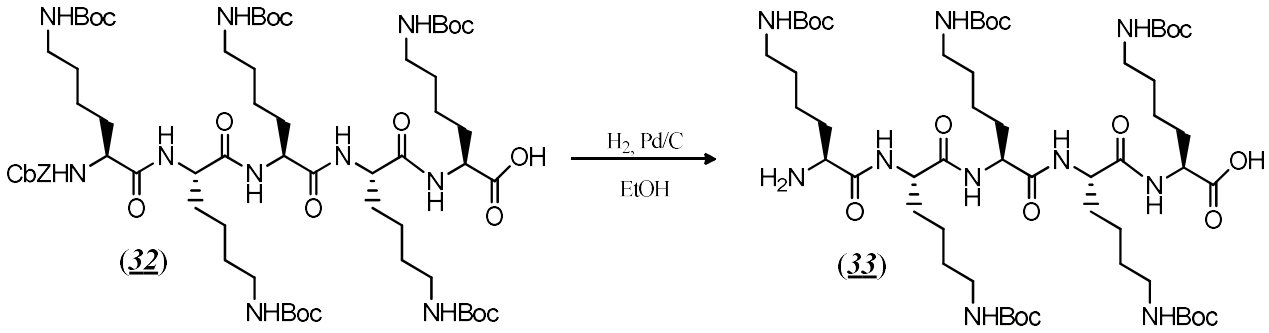
R_f: 0.40 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 12.52 (s, 1H), 7.98 (s, 1H), 7.89 (d, *J* = 7.8 Hz, 2H), 7.82 (s, 1H), 7.46 – 7.19 (m, 6H), 6.73 (q, *J* = 6.4, 5.9 Hz, 5H), 5.03 (s, 2H), 4.23 (d, *J* = 9.2 Hz, 3H), 4.12 (s, 1H), 3.97 (d, *J* = 5.9 Hz, 1H), 2.87 (p, *J* = 6.4 Hz, 10H), 1.64 (d, *J* = 22.9 Hz, 5H), 1.56 – 1.43 (m, 5H), 1.37 (d, *J* = 1.5 Hz, 45H), 1.33 (s, 10H), 1.30 – 1.17 (m, 10H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 173.84, 172.39, 171.93, 171.72, 156.44, 156.02, 77.83, 65.87, 55.20, 52.86, 52.69, 52.35, 32.27, 32.12, 31.42, 31.24, 29.69, 28.73, 23.28, 23.11, 23.05.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 1315.77, found: 1316.61.

3.37. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine (*33*)



Pentapeptide ***32*** (2.04 g, 1.6 mmol) was dissolved in EtOH (60 mL) and 10wt. % Pd/C (0.21 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***33*** (1.83 g, 1.6 mmol, 100 %) was obtained as a white powder and used in the next step without further purification.

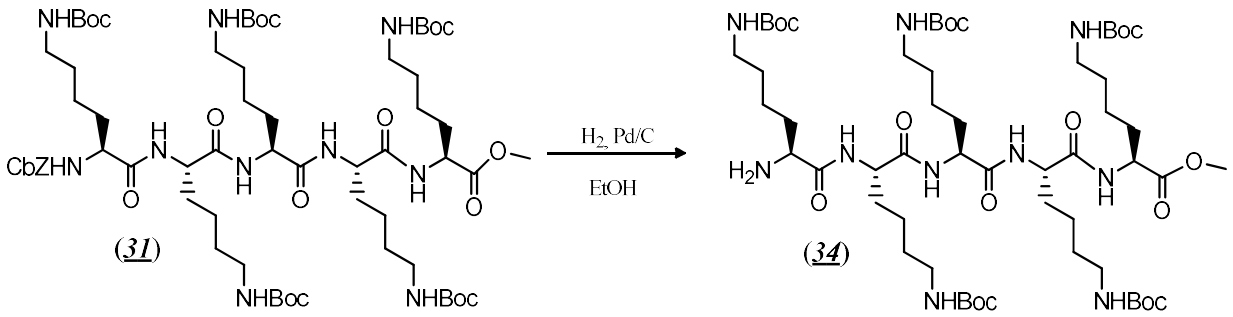
R_f: 0.00 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.19 (s, 1H), 8.01 (d, *J* = 7.9 Hz, 1H), 7.89 (s, 2H), 6.82 – 6.67 (m, 5H), 4.27 (s, 1H), 4.21 (s, 3H), 2.87 (d, *J* = 6.8 Hz, 10H), 1.75 – 1.57 (m, 5H), 1.52 (dd, *J* = 13.0, 8.0 Hz, 5H), 1.37 (s, 45H), 1.34 (d, *J* = 6.9 Hz, 10H), 1.24 (s, 10H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 173.72, 173.37, 171.81, 171.74, 171.62, 156.01, 77.80, 54.31, 52.95, 52.90, 52.79, 52.66, 32.53, 32.23, 32.09, 31.55, 28.74, 23.04.

ESI-Q-TOF: $[M+H]^+$ Calcd: 1159.61, found: 1159.67.

3.38. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine methyl ester (*34*)



Fully protected pentapeptide ***31*** (1.79 g, 1.4 mmol) was dissolved in EtOH (50 mL) and 10wt. % Pd/C (0.18 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***34*** (1.60 g, 1.4 mmol, 99 %) was obtained as a colourless oil and used in the next step without further purification.

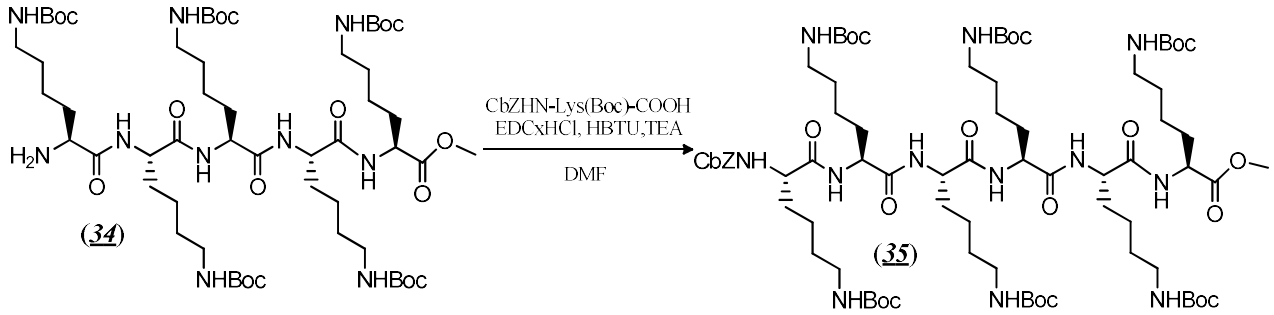
R_f: 0.01 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.18 (d, *J* = 7.2 Hz, 1H), 7.99 (d, *J* = 7.9 Hz, 2H), 7.79 (d, *J* = 7.9 Hz, 1H), 6.73 (q, *J* = 7.2, 5.7 Hz, 5H), 4.32 – 4.13 (m, 4H), 3.61 (s, 3H), 3.12 (t, *J* = 6.0 Hz, 1H), 2.88 (p, *J* = 8.0, 7.2 Hz, 10H), 1.62 (q, *J* = 10.8, 7.0 Hz, 5H), 1.50 (t, *J* = 10.3 Hz, 4H), 1.37 (s, 54H), 1.35 – 1.30 (m, 10H), 1.30 – 1.14 (m, 11H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 172.86, 172.15, 171.68, 156.00, 77.80, 56.49, 55.00, 52.38, 52.21, 35.16, 29.90, 29.73, 29.65, 29.56, 28.73, 23.07, 22.91.

ESI-Q-TOF: $[M+H]^+$ Calcd: 1173.84, found: 1173.90.

3.39. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine methyl ester (***35***)



The product was obtained following General procedure G starting from a product ***34*** (1.60 g, 1.4 mmol) and Z-Lys(Boc)-COOH (0.39 g, 1.5 mmol), HOBt (0.28 g, 2.1 mmol) and EDC×HCl (0.39 g, 2.1 mmol), TEA (0.8 mL 6.8 mmol) and DMF as solvent (15 mL).

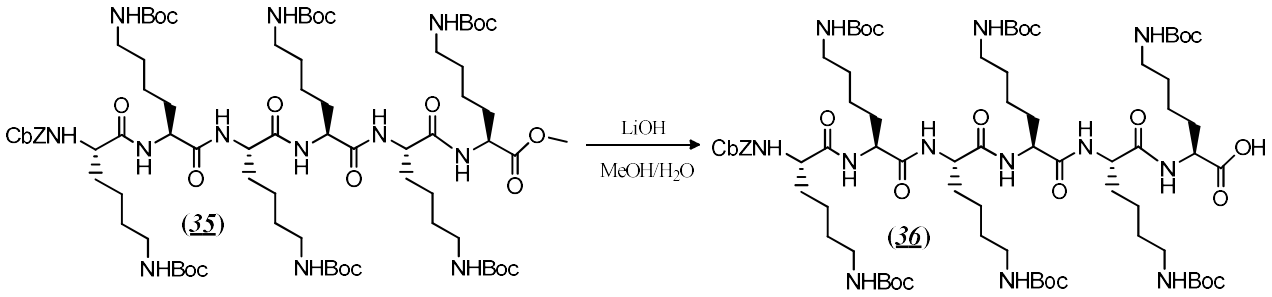
After precipitation of a crude product, flash chromatography was performed in isocratic flow of 10% MeOH/DCM and the product (***35***) was isolated as white powder (1.63 g, 1.1 mmol, 78%).

R_f: 0.85 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.18 (d, *J* = 7.1 Hz, 1H), 7.89 (s, 3H), 7.79 (s, 1H), 7.45 – 7.26 (m, 6H), 6.72 (dt, *J* = 11.7, 7.4 Hz, 6H), 5.03 (s, 2H), 4.22 (s, 5H), 3.97 (s, 1H), 3.61 (s, 3H), 2.96 – 2.78 (m, 12H), 1.61 (s, 7H), 1.55 – 1.43 (m, 7H), 1.37 (s, 54H), 1.33 (d, *J* = 7.8 Hz, 9H), 1.23 (d, *J* = 15.7 Hz, 13H).

ESI-Q-TOF: $[M+Na]^+$ Calcd: 1557.94, found: 1557.95.

3.40. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-t-butyloxycarbonyl-L-lysyl-*N*-epsilon-t-butyloxycarbonyl-L-lysyl-*N*-epsilon-t-butyloxycarbonyl-L-lysyl-*N*-epsilon-t-butyloxycarbonyl-L-lysyl-*N*-epsilon-t-butyloxycarbonyl-L-lysine (36)



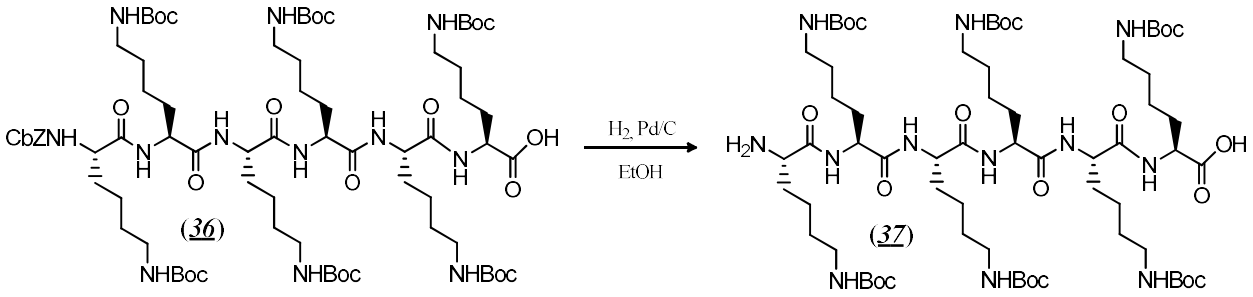
The product was obtained according to General procedure D starting from compound ***35*** (1.63 g, 1.1 mmol) and LiOH (0.09 g, 2.2 mmol) in MeOH (27 mL) and H_2O (9 mL) as solvents. After work-up product ***36*** was isolated as white powder (1.53 g, 1.0 mmol, 95%).

R_f: 0.45 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 12.50 (s, 1H), 7.98 (s, 1H), 7.88 (s, 2H), 7.80 (s, 1H), 7.42 – 7.26 (m, 6H), 6.72 (t, *J* = 8.9 Hz, 6H), 5.03 (s, 2H), 4.22 (s, 4H), 3.97 (s, 1H), 2.87 (t, *J* = 6.6 Hz, 12H), 1.61 (s, 7H), 1.55 – 1.43 (m, 6H), 1.37 (d, *J* = 1.6 Hz, 54H), 1.33 (d, *J* = 6.4 Hz, 10H), 1.30 – 1.15 (m, 13H), one signal is covered by solvents.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 1543.83, found: 1543.98.

3.41. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-L-lysine (*37*)



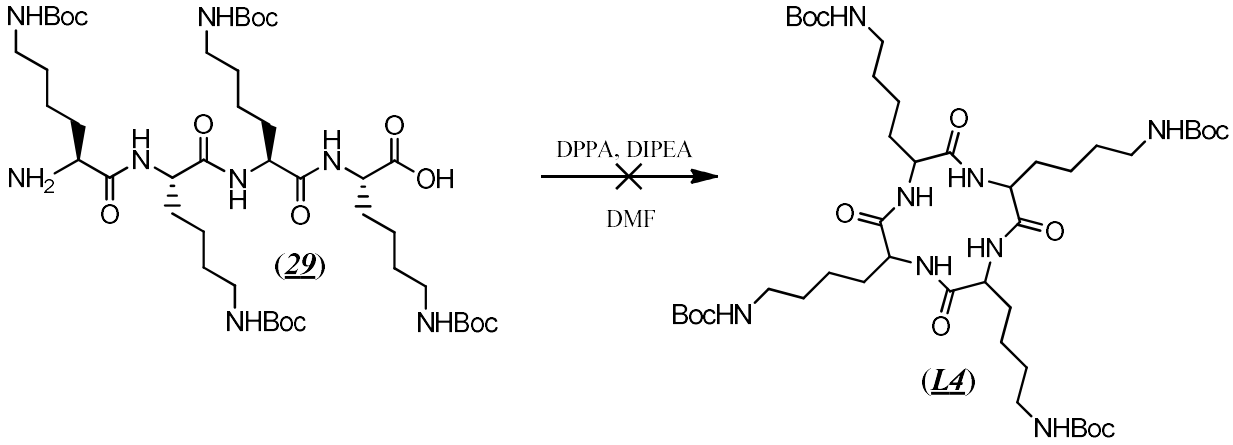
Hexapeptide ***36*** (1.53 g, 1.0 mmol) was dissolved in EtOH (50 mL) and 10wt. % Pd/C (0.15 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***37*** (1.39 g, 1.0 mmol, 100 %) was obtained as a white powder and used in the next step without further purification.

R_f: 0.00 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.26 (s, 1H), 8.08 (s, 1H), 7.88 (s, 3H), 6.78-6.68 (m, 6H), 4.32-4.14 (m, 4H), 4.13-3.97 (m, 2H), 2.87 (d, J = 6.7 Hz, 12H), 1.73 – 1.56 (m, 6H), 1.55-1.43 (m, 7H), 1.37 (s, 54H), 1.34-1.29 (m, 10H), 1.29-1.10 (m, 13H).

ESI-Q-TOF: $[M-H]^-$ Calcd: 1385.89, found: 1386.11.

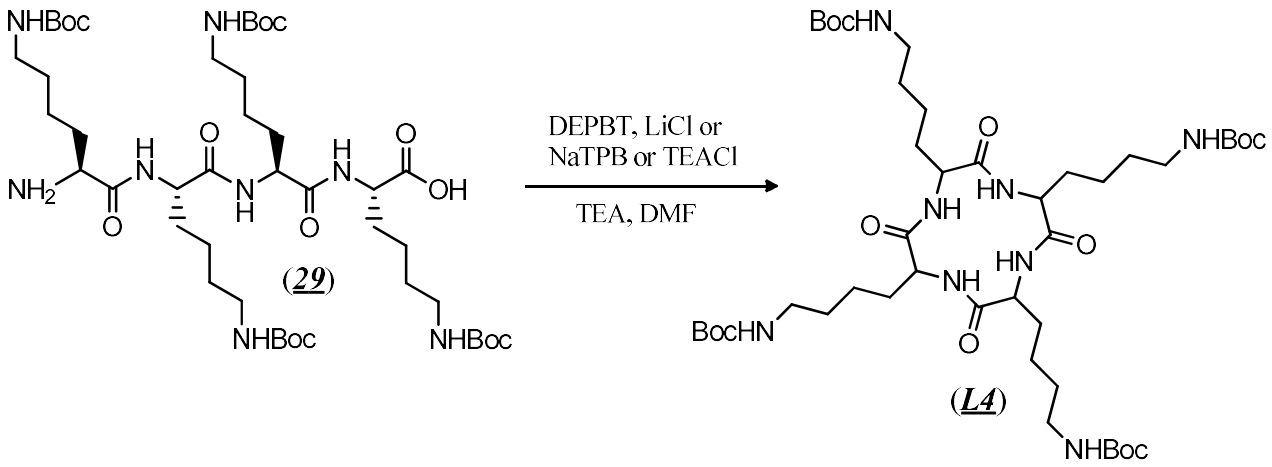
3.42. Synthesis of cyclic *N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysine (***L4***)



Under inert atmosphere DPPA (0.01 mL, 0.06 mmol) and DIPEA (0.01 mL, 0.06 mmol) were dissolved in dry DMF (10 mL). Product ***29*** (0.05 g, 0.05 mmol), previously dissolved in dry DMF (5 mL), was slowly added to the obtained solution in a 3 hours.

Reaction mixture was stirred at RT under inert atmosphere for 4 days and monitored by TLC.

3.43. Synthesis of cyclic *N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysine (***L4***)



Product ***L4*** was obtained through general procedure ***J*** using compound ***29*** (0.20 g, 0.2 mmol), DEPBT (0.08 g, 0.2 mmol), LiCl (0.04 g, 1.0 mmol) or NATPB (0.74 g, 20.0 mmol) or TEACl (0.36 g, 20.0 mmol) dissolved in 0.125 mL of H_2O, TEA until pH 8 and DMF (200 mL).

After work-up flash chromatography was performed in isocratic flow of 10 % MeOH/DCM and the product ***L4*** was isolated as white powder (0.04 g, 0.05 mmol, 21%; 0.02 g, 0.02 mmol, 8 %; 0.09 g, 0.11 mmol, 47 %).

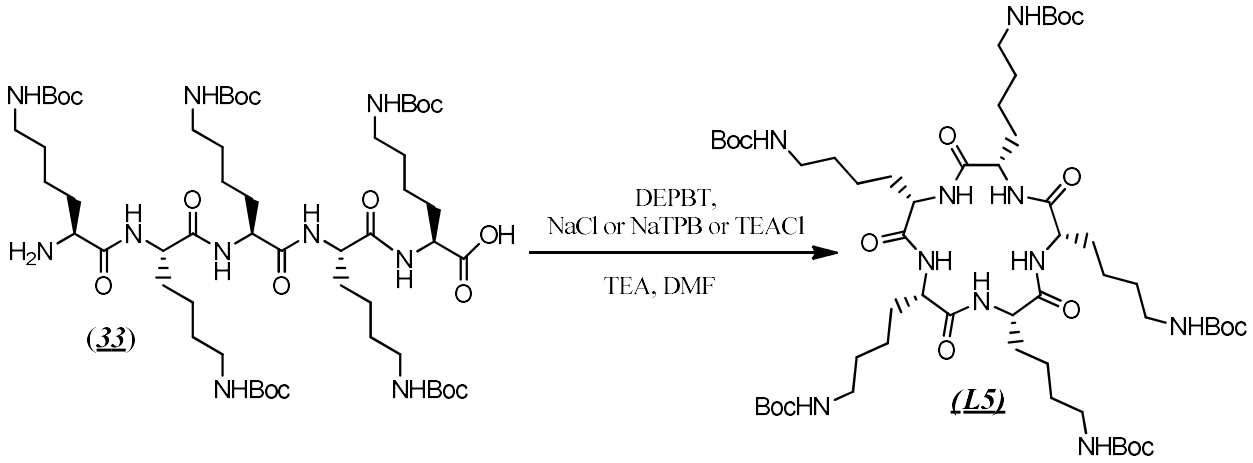
R_f: 0.37 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.04 (d, J = 9.7 Hz, 4H), 6.76 (d, J = 5.8 Hz, 4H), 4.09 (q, J = 5.2 Hz, 4H), 2.88 (q, J = 6.4 Hz, 8H), 1.76-1.55 (m, 4H), 1.55-1.43 (m, 2H), 1.37 (s, 9H), 1.30 – 1.14 (m, 10H).

^{13}C NMR (400 MHz, Methanol-d_4) δ/ppm: 148.61, 69.93, 47.55, 39.95, 31.21, 27.06, 21.40, 20.67, one peak is not visible.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 936.16, found: 936.47.

3.44. Synthesis of cyclic *N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysine (***L5***)



Product ***L5*** was obtained through general procedure J using compound ***33*** (0.20 g, 0.2 mmol), DEPBT (0.06 g, 0.2 mmol), NaCl (0.06 g, 1.0 mmol) or NATPB (0.58 g, 2.0 mmol) or TEACl (0.28 g, 2.0 mmol) dissolved in 0.125 mL of H_2O, TEA until pH 8 and DMF (200 mL).

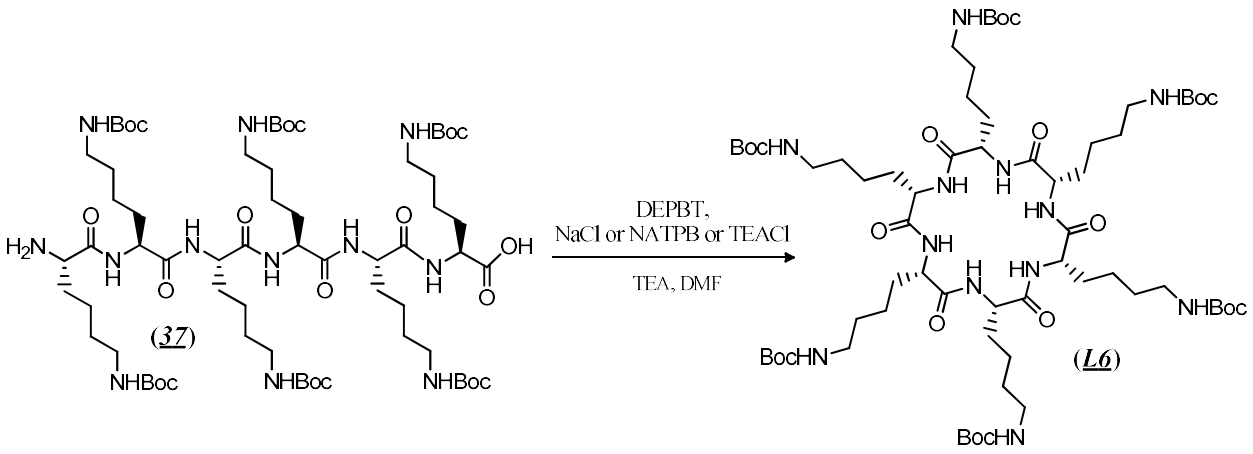
Solvents were removed under reduced pressure; the obtained crude solid was dissolved in AcOEt (10 mL) and washed with $NaHCO_3$ (sat. 5 mL), H_2O (5 mL), 0.1 M HCl (5 mL) and H_2O (5 mL). Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. Flash chromatography was performed in isocratic flow of 10 % MeOH/DCM and the product ***L5*** was isolated as white powder (0.08 g, 0.07 mmol, 35%; 0.06 g, 0.05 mmol, 26%; 0.1 g, 0.08 mmol, 43 %).

R_f: 0.41 (10% MeOH/DCM).

^{1}H NMR (400 MHz, Acetonitrile-d_3) δ/ppm: 7.19 (d, J = 7.8 Hz, 5H), 5.43 (s, 5H), 4.02 (q, J = 7.8, 7.3 Hz, 5H), 3.11 – 2.97 (m, 10H), 1.91 – 1.71 (m, 10H), 1.59 – 1.44 (m, 10H), 1.42 (s, 45H), 1.40 – 1.25 (m, 10H).

ESI-Q-TOF: $[M+Na]^+$ Calcd: 1163.73, found: 1163.75.

3.45. Synthesis of cyclic *N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysyl-*N*-epsilon-*t*-butyloxycarbonyl-lysine (*L6*)



Product ***L6*** was obtained following General procedure ***J*** using compound ***37*** (0.20 g, 0.1 mmol) as a starting material, DEPBT (0.05 g, 0.2 mmol), NaCl (0.04 g, 0.7 mmol) or NATPB (0.49 g, 14.4 mmol) or TEACl (0.24 g, 14.4 mmol) dissolved in 0.125 mL of H_2O, TEA until pH 8 and DMF (200 mL).

Solvents were removed under reduced pressure; the obtained crude solid was dissolved in AcOEt (10 mL) and washed with $NaHCO_3$ (sat. 5 mL), H_2O (5 mL), 0.1 M HCl (5 mL) and H_2O (5 mL). Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. Flash chromatography was performed in isocratic flow of 10 % MeOH/DCM and the product ***L5*** was isolated as white powder (0.03 g, 0.03 mmol, 15 %; 0.02 g, 0.02 mmol, 10 %; 0.03 g, 0.03 mmol, 17 %).

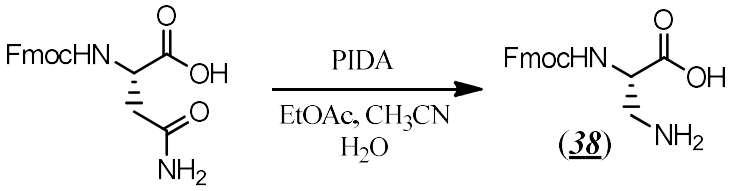
R_f: 0.46 (10% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 4.07 – 3.96 (m, 6H), 2.94 (t, J = 6.9 Hz, 12H), 1.90 – 1.65 (m, 12H), 1.41 (q, J = 7.0 Hz, 12H), 1.33 (s, 54H), 1.31 – 1.17 (m, 12H).

^{13}C NMR (400 MHz, Methanol-d_4) δ/ppm: 172.60, 78.45, 54.72, 39.79, 30.46, 29.22, 27.46, 23.08, one peak is not visible.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 1391.87, found: 1391.81.

3.46. Synthesis of *N*-alpha-(9-Fluorenylmethyloxycarbonyl)-L-β-aminoalanine (***38***)



Fmoc-L-Asn-COOH (6.76 g, 19.1 mmol) and PIDA (7.36 g, 22.9 mmol) were dissolved in EtOAc:CH_3CN:H_2O = 2:2:1 (350 mL). Reaction mixture was stirred 30 min at 10 °C and enother 3 hours at 20 °C. Reaction was monitored by TLC. Solvents were then evaporated under reduced pressure and product ***38*** (3.11 g, 9.5 mmol, 50 %) was precipitated as a white solid by addition of EtOAc (100 mL), filtered out and dryed in exicator.

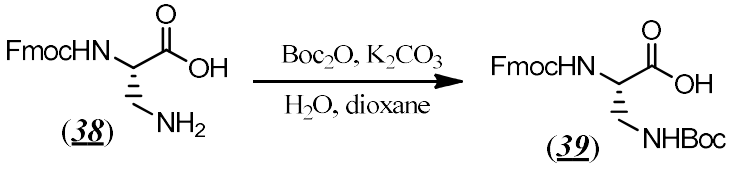
R_f: 0.00 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ/ppm: 7.90 (d, *J* = 7.5 Hz, 2H), 7.71 (d, *J* = 7.5 Hz, 2H), 7.54 (d, *J* = 8.3 Hz, 1H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.34 (d, *J* = 6.8 Hz, 2H), 4.38 – 4.30 (m, 1H), 4.27 (d, *J* = 6.9 Hz, 2H), 4.25 – 4.20 (m, 1H), 2.57 (dd, *J* = 15.4, 5.3 Hz, 1H), 2.47 – 2.42 (m, 1H).

^{13}C NMR (400 MHz, DMSO-*d*$_6$) δ/ppm: 171.11, 155.79, 143.84, 140.73, 127.65, 127.11, 125.23, 120.13, 65.74, 46.64, one signal is covered by DMSO.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 349.12, found 349.10.

3.47. Synthesis of *N*-alpha-(9-Fluorenylmethyloxycarbonyl)-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine (***39***)



K_2CO_3 (3.96 g, 28.7 mmol) was dissolved in a mixture of dioxane:H_2O = 1:1 (45 mL) and temperature was lowered to 0 °C. Starting material ***38*** (3.11 g, 9.6 mmol) and Boc_2O (2.09 g, 9.6 mmol), previuosly dissolved in dioxane (15 mL), were added in a mixture and reaction was stirred at RT overnight. pH of the reaction mixture was then brought to 4 by adding $KHSO_4$ (sat) and extraction with EtOAc (3×25 mL) was performed. Organic layer was dried over anhydrous $MgSO_4$ and solvent was removed under reduced pressure. In this way product ***39*** (4.05 g, 9.6 mmol, 100 %) was obtained as a white powder and used in the next step without further purification.

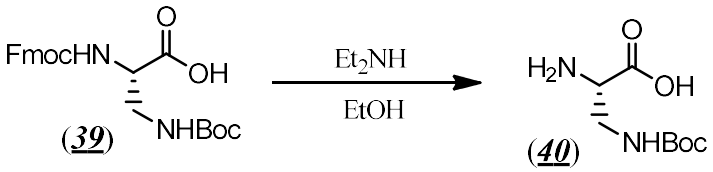
R_f: 0.25 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 12.68 (s, 1H), 7.90 (d, *J* = 7.5 Hz, 2H), 7.72 (d, *J* = 7.5 Hz, 2H), 7.47 (d, *J* = 8.0 Hz, 1H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.33 (t, *J* = 7.4 Hz, 2H), 6.84 (s, 1H), 4.30 (d, *J* = 7.0 Hz, 2H), 4.26 – 4.17 (m, 1H), 4.08 (q, *J* = 6.7 Hz, 1H), 3.31-3.26 (m, 2H), 1.37 (s, 9H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 172.05, 156.04, 155.61, 143.82, 140.74, 127.80, 127.22, 125.31, 120.04, 78.12, 65.73, 54.11, 46.63, 28.20.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 449.17, found 449.30.

3.48. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine (***40***)



Starting material ***39*** (0.10 g, 0.2 mmol) was dissolved in EtOH (100 mL) and under magnetic stirring Et_2NH (0.3 mL, 0.02 mmol) was added. Resulting mixture was stirred overnight at RT and solvent was then removed under reduced pressure. Crude was dissolved in EtOAc and, after extraction, inorganic phase was evaporated. In this way the desired product ***40*** (0.05 g, 0.2 mmol, 100 %) was obtained in a form of white powder.

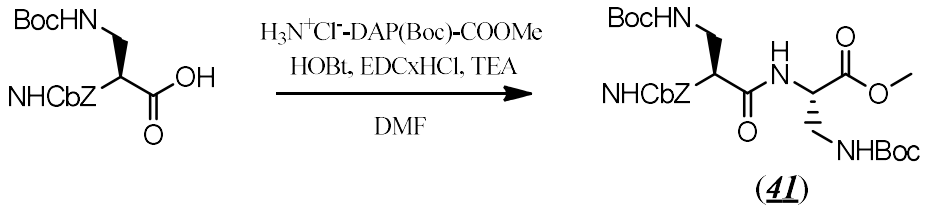
R_f: 0.00 (10% MeOH/DCM).

^{1}H NMR (400 MHz, Deuterium Oxide-d_2) δ/ppm: 3.75 (dd, J = 6.9, 3.8 Hz, 1H), 3.57 (dd, J = 15.0, 3.8 Hz, 1H), 3.39 (dd, J = 15.1, 6.9 Hz, 1H), 1.35 (s, 9H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 174.33, 155.56, 80.46, 42.97, 54.21, 28.20.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 227.10, found 227.21.

3.49. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine methyl ester (***41***)



(***41***)

The product was obtained according to General procedure G using HCl×NH_2-DAP(Boc)-COOMe (3.50 g, 16.0 mmol) and Z-Lys(Boc)-COOH (5.97 g, 17.6 mmol), EDC×HCl (3.25 g, 24.1 mmol) and HOBt (4.61 g, 24.1 mmol), TEA (11.2 mL 87.0 mmol) and DMF as solvent (30 mL).

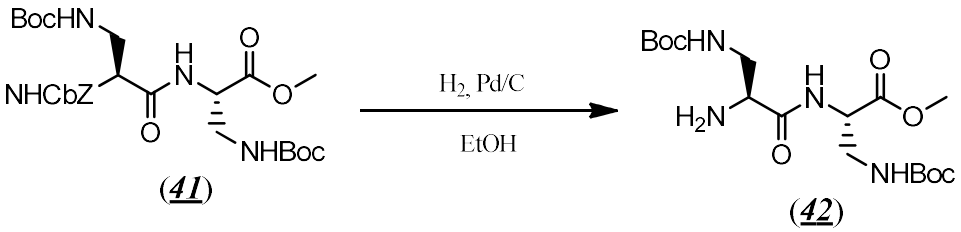
After precipitation product ***41*** was isolated as white powder (8.62 g, 16.0 mmol, 100%).

R_f: 0.97 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.23 (d, *J* = 7.6 Hz, 1H), 7.40 – 7.28 (m, 5H), 7.19 (d, *J* = 8.1 Hz, 1H), 6.86 (d, *J* = 6.7 Hz, 1H), 6.67 (s, 1H), 5.11 – 4.96 (m, 2H), 4.32 (t, *J* = 6.6 Hz, 1H), 4.14 (q, *J* = 7.2 Hz, 1H), 3.61 (s, 3H), 3.14 (dt, *J* = 13.9, 7.2 Hz, 1H), 3.08 – 2.98 (m, 1H), 1.37 (s, 18H).

ESI-Q-TOF: $[M+Na]^+$ Calcd: 561.49, found 561.50.

3.50. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-aminoalanine methyl ester (*42*)



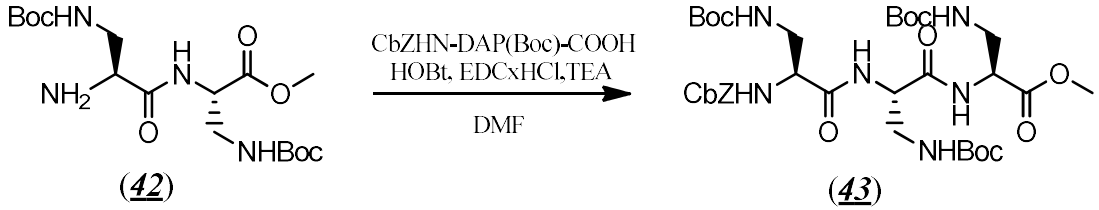
Dipeptide ***41*** (6.54 g, 12.1 mmol) was dissolved in EtOH (150 mL) and 10wt. % Pd/C (0.65 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***42*** (4.91 g, 12.1 mmol, 100 %) was obtained as a yellowish oil and used in the next step without further purification.

R_f: 0.72 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.30 (s, 1H), 6.98 (t, *J* = 6.4 Hz, 1H), 6.71 (s, 1H), 4.32 (d, *J* = 5.5 Hz, 1H), 3.62 (s, 3H), 3.26 (dt, *J* = 5.6, 2.1 Hz, 1H), 3.21 – 3.08 (m, 1H), 3.02 – 2.91 (m, 1H), 1.39 (s, 9H), 1.38 (s, 9H), one signal is covered by signal of H_2O.

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 173.00, 171.11, 166.55, 156.21, 124.93, 123.66, 118.91, 111.01, 78.56, 78.36, 56.50, 55.17, 52.88, 52.51, 44.58, 41.54, 28.62, 18.99.

3.51. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine methyl ester (***43***)



(***42***) (***43***)

The product was obtained according to General procedure G starting with a product ***42*** (4.91 g, 12.1 mmol) and Z-DAP(Boc)-COOH (4.50 g, 13.3 mmol), EDC×HCl (3.48 g, 18.5 mmol) and HOBt (2.45 g, 18.5 mmol), TEA (3.5 mL 30.3 mmol) and DMF as solvent (30 mL).

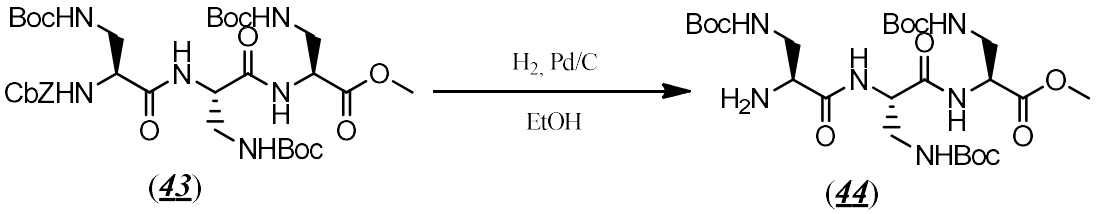
After precipitation product ***43*** was isolated as white powder (8.50 g, 11.7 mmol, 97%).

R_f: 0.99 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.16 (d, *J* = 7.6 Hz, 1H), 8.02 (d, *J* = 8.0 Hz, 1H), 7.37 (d, *J* = 4.8 Hz, 5H), 6.90 – 6.79 (m, 1H), 6.75 (s, 1H), 6.56 (s, 1H), 5.14 – 4.96 (m, 2H), 4.41 – 4.25 (m, 1H), 4.10 (dd, *J* = 24.2, 6.6 Hz, 2H), 3.61 (s, 3H), 3.20 (dq, *J* = 21.2, 7.7, 6.7 Hz, 4H), 1.37 (s, 27H), one signal is covered by signal of H_2O.

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 170.54, 169.96, 169.60, 156.05, 155.88, 155.65, 136.79, 128.42, 127.94, 127.81, 78.22, 78.20, 78.10, 65.81, 56.11, 55.58, 52.91, 52.55, 52.06, 41.71, 41.07, 28.24, 28.20, 18.62.

3.52. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine methyl ester (***44***)



(***43***) (***44***)

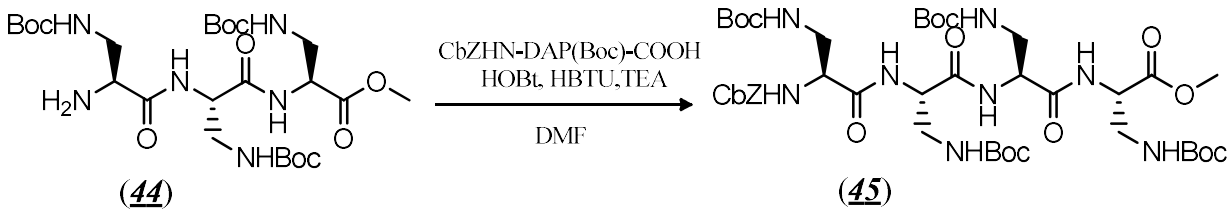
Tripeptide ***43*** (8.50 g, 11.7 mmol) was dissolved in EtOH (150 mL) and 10wt. % Pd/C (0.85 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***44*** (6.70 g, 11.3 mmol, 97 %) was obtained as a yellowish oil and used in the next step without further purification.

R_f: 0.62 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.31 (d, *J* =7.5 Hz, 1H), 6.89 – 6.84 (m, 1H), 6.72 (s, 1H), 6.66 (d, *J* = 6.2 Hz, 1H), 4.43 – 4.23 (m, 2H), 3.62 (s, 3H), 3.50 – 3.36 (m, 2H), 3.14 (dt, *J* = 12.2, 6.5 Hz, 2H), 2.96 (dt, *J* = 13.9, 7.5 Hz, 1H), 1.39 (s, 9H), 1.38 (s, 9H), 1.37 (s, 9H), one signal is covered by a signal of H_2O.

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 169.80, 78.22, 60.21, 56.10, 55.28, 52.65, 52.47, 52.08, 28.22, 14.11, some paks are covered by a solvent peak.

3.53. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine methyl ester (***45***)



The product was obtained following General procedure G starting from a product ***45*** (5.51 g, 9.3 mmol) and Z-Lys(Boc)-COOH (3.47 g, 10.2 mmol), HOBt (1.89 g, 14.0 mmol) and EDC×HCl (2.68 g, 14.0 mmol), TEA (5.5 mL, 46.7 mmol) and DMF as solvent (30 mL).

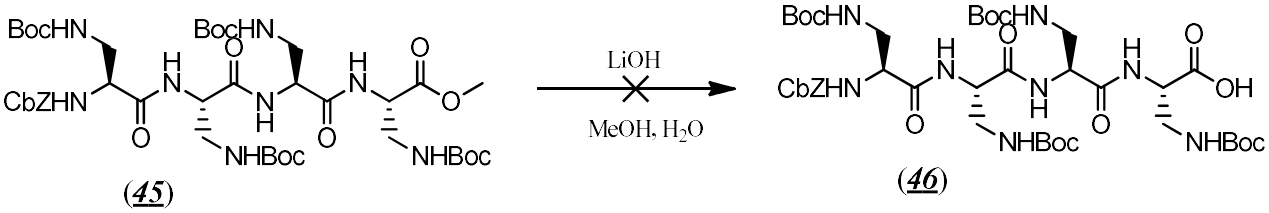
After precipitation of a crude product, flash chromatography was performed in isocratic flow of 10% MeOH/DCM and the product ***45*** was isolated as white powder (6.65 g, 7.3 mmol, 79%).

R_f: 0.98 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.14 (d, *J* = 7.7 Hz, 1H), 8.05 (d, *J* = 7.4 Hz, 1H), 7.95 (d, *J* = 9.7 Hz, 1H), 7.40 – 7.19 (m, 6H), 6.80 (s, 1H), 6.75 (s, 1H), 6.66 (d, *J* = 6.4 Hz, 1H), 6.56 (s, 1H), 5.19 – 4.93 (m, 2H), 4.41 – 4.21 (m, 3H), 4.09 (s, 1H), 3.60 (s, 3H), 3.45-3.32 (s, 4H), 3.30 – 3.04 (m, 4H), 1.37 (s, 36H).

ESI-Q-TOF: $[M+Na]^+$ Calcd: 933.44, found 933.76.

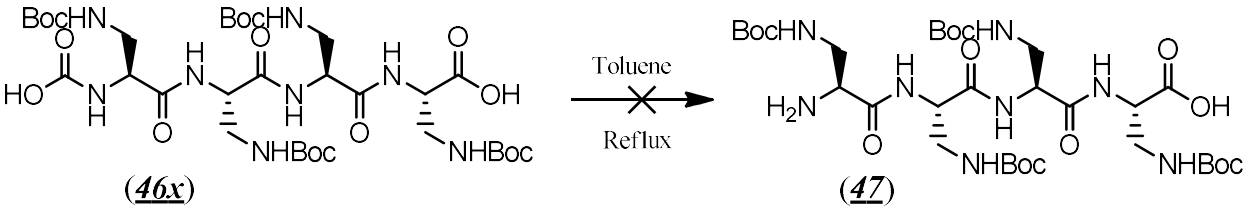
3.54. Synthesis of *N*-alpha-carbobenzoxy-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine (***46***)



(***45***) (***46***)

Compound ***45*** (6.65 g, 7.3 mmol) and LiOH (0.60 g, 14.6 mmol) were dissolved in MeOH (120 mL) and H_2O (30 mL) as solvents. Reaction was monitored by TLC and standard work-up was performed. In this way was not possible to obtain the desired product ***46***.

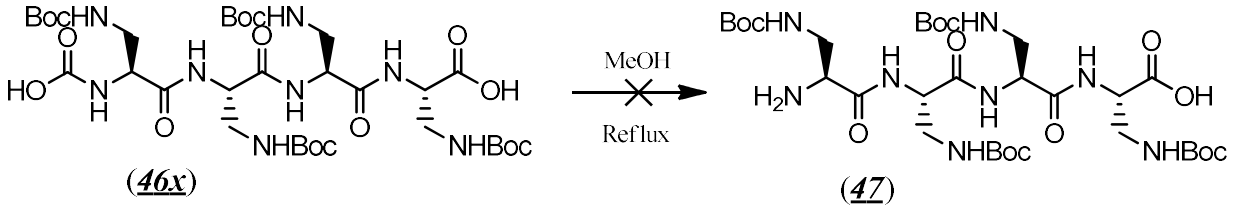
3.55. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine (***47***)



(***46x***) (***47***)

Compound ***46x*** (0.10 g, 0.1 mmol) was dissolved in toluene (20 mL) and reaction mixture was stirred under reflux of toluen for 48 hours. Solvent was then removed under reduced pressure. In this way was not possible to obtain the desired product ***47***.

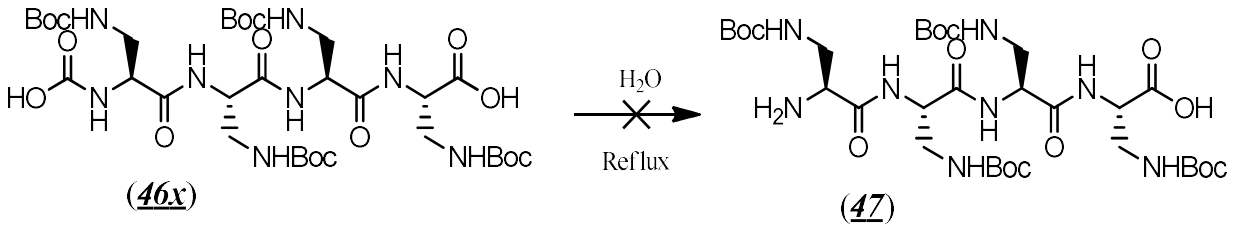
3.56. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine (*47*)



(*46x*) (*47*)

Compound ***46x*** (0.10 g, 0.1 mmol) was dissolved in methanol (20 mL) and reaction mixture was stirred under reflux for 48 hours. Solvent was then removed under reduced pressure. In this way was not possible to obtain the desired product ***47***.

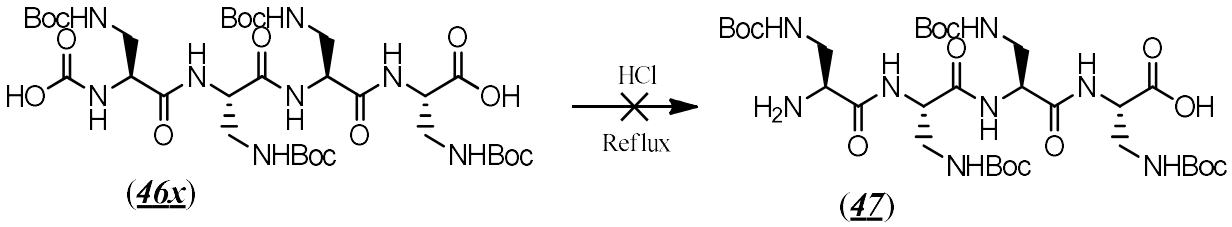
3.57. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine (*47*)



(*46x*) (*47*)

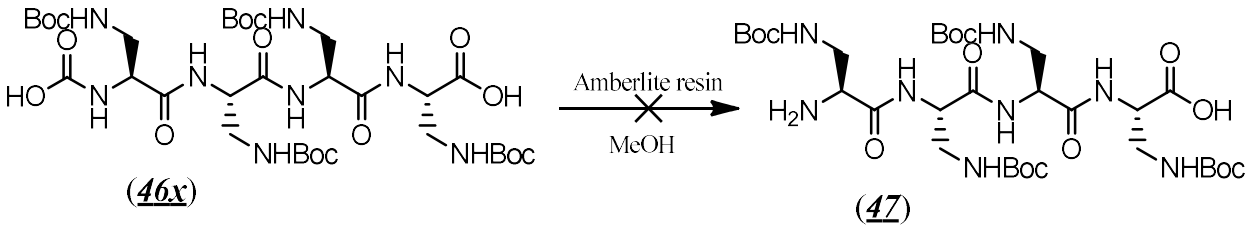
Compound ***46x*** (0.10 g, 0.1 mmol) was dissolved in H_2O (20 mL) and reaction mixture was stirred under reflux for 48 hours. Solvent was then removed under reduced pressure. In this way was not possible to obtain the desired product ***47***.

3.58. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine (*47*)



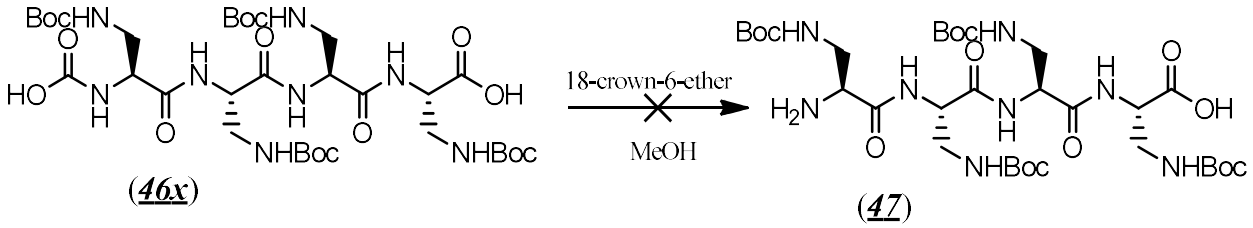
Compound ***46x*** (0.10 g, 0.1 mmol) was dissolved in HCl (0.1 M, 20 mL) and reaction mixture was stirred under reflux for 48 hours. Solvent was then removed under reduced pressure. In this way was not possible to obtaine the desired product ***47***.

3.59. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine (*47*)



Compound ***46x*** (0.10 g, 0.1 mmol) was dissolved in MeOH (20 mL) and amberlite resin (0.02 g) was added. Reaction mixture was stirred at RT for 24 hours. Resin was removed by filtration and solvent was evaporated under reduced pressure. In this way was not possible to obtaine the desired product ***47***.

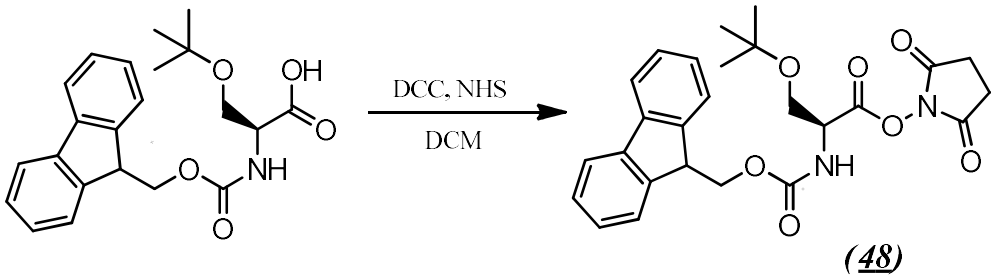
3.60. Synthesis of *N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanyl-*N*-epsilon-*t*-butyloxycarbonyl-L-β-aminoalanine (*47*)



(*46x*) (*47*)

Compound ***46x*** (0.10 g, 0.1 mmol) was dissolved in MeOH (20 mL) and 18-crown-6-ether (0.02 g) was added. Reaction mixture was stirred at RT for 24 hours. Solvent was then evaporated under reduced pressure. In this way was not possible to obtaine the desired product *47*.

3.61. Synthesis of *N*-(9-Fluorenylmethyloxycarbonyl)-*O*-*t*-butyl-L-serine succinimidyl ester (*48*)



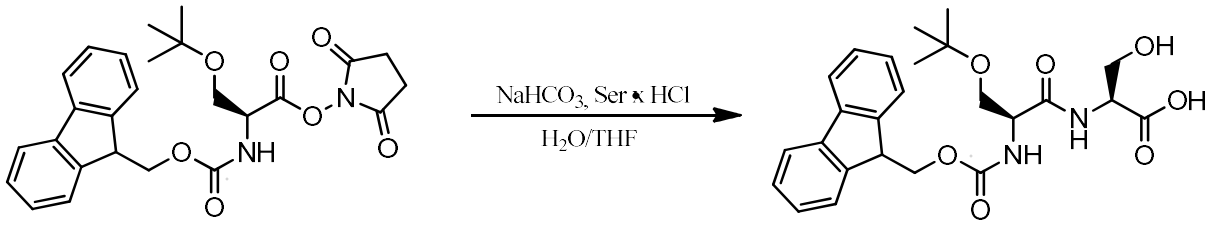
(48)

The product was obtained through General procedure A using Fmoc-Ser(*t*Bu)-COOH (10.00 g, 26.1 mmol) commercially available, DCC (6.45 g, 31.3 mmol), NHS (3.60 g, 31.3 mmol) and DCM (80 mL) as solvent. The obtained crude solid was dissolved in EtOAc (50 mL) and washed with $NaHCO_3$ (0.5 M, 50 mL), H_2O (50 mL), HCl (0.1 M, 50 mL) and H_2O (50 mL). Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure to give product ***48*** (11.60 g, 24.1 mmol, 92%) that was used in the next step without further purification.

R_f: 0.80 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d$_6$,) δ/ppm: 8.09 (d, *J* = 8.3 Hz, 1H), 7.90 (d, *J* = 7.5 Hz, 2H), 7.74 (m, 2H), 7.42 (t, *J* = 7.4 Hz, 2H), 7.33 (t, *J* = 7.4 Hz, 2H), 4.62 (dd, *J* = 13.4, 5.4 Hz, 1H), 4.38 – 4.21 (m, 3H), 3.76 – 3.63 (m, 2H), 2.81 (s, 4H), 1.16 (s, 9H).

3.62. Synthesis of *N*-(9-Fluorenylmethyloxycarbonyl)-*O*-*t*-butyl-L-seryl-L-serine (*49*)



(48) *(49)*

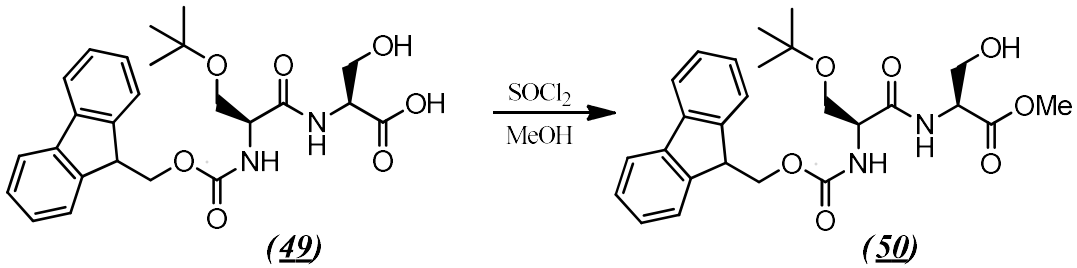
The product was obtained following General procedure B using compound ***3*** (11.50 g, 23.9 mmol), Ser×HCl (3.08 g, 21.8 mmol), $NaHCO_3$ (4.02 g, 47.9 mmol) and THF (90 mL)/H2O (60 mL) as solvents.

The crude product was dissolved in DCM (50 mL) and washed with HCl (0.1 M, 2×30 mL) and H_2O (2×30 mL); the crude solid was dissolved in Et_2O (70 mL) and then were added water (30 mL) and hexane until the product start precipitating. The inorganic phase was separated and the product was extracted with DCM. Solvents were removed under reduced pressure. Flash chromatography was performed in gradient flow of 2%-10% MeOH/DCM and the product ***49*** was isolated as white powder (8.10 g, 17.2 mmol, 79%).

R_f: 0.25 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.89 (d, *J* = 7.5 Hz, 2H), 7.75 – 7.72 (m, 2H), 7.66 (d, *J* = 6.2 Hz, 1H), 7.49 (d, *J* = 8.4 Hz, 1H), 7.42 (t, *J* = 7.4 Hz, 2H), 7.36 – 7.29 (m, 2H), 4.35 – 4.21 (m, 3H), 4.11 (dd, *J* = 13.1, 6.2 Hz, 1H), 3.96 (dd, *J* = 10.4, 5.0 Hz, 1H), 3.67 (dd, *J* = 9.7, 3.7 Hz, 1H), 3.56 – 3.44 (m, 3H), 1.12 (s, 9H).

3.63. Synthesis of *N*-(9-Fluorenylmethyloxycarbonyl)-*O*-*t*-butyl-L-seryl-L-serine methyl ester (***50***)



(49) ***(50)***

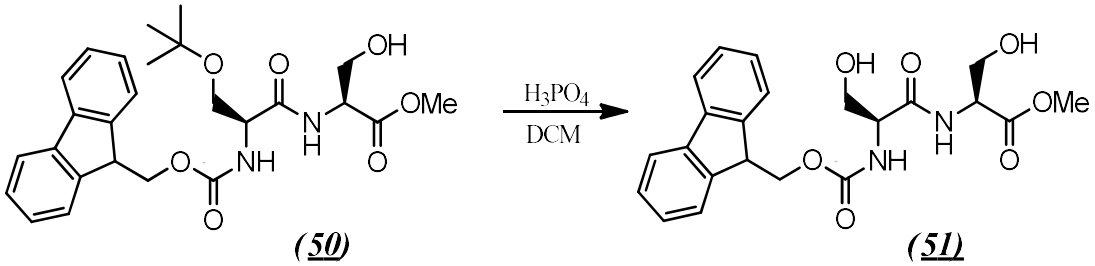
The product was obtained through general procedure C using compound ***49*** (0.80 g, 1.7 mmol), $SOCl_2$ (0.24 mL, 3.4 mmol) and MeOH (30 mL).

Solvents were removed under reduced pressure; the obtained crude solid was dissolved in EtOAc (20 mL) and washed with a saturated solution of $NaHCO_3$ (20 mL) and H_2O (2×20 mL). Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure to obtain product ***50*** as white powder (0.82 g, 1.6 mmol, 95%).

R_f: 0.50 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6,) δ/ppm: 8.19 (d, *J* = 7.5 Hz, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.73 (d, *J* = 7.2 Hz, 2H), 7.42 (t, *J* = 7.3 Hz, 2H), 7.32 (t, *J* = 7.4 Hz, 2H), 4.37 (m, 1H), 4.29 – 4.19 (m, 3H), 3.73 (dd, *J* = 11.9, 4.6 Hz, 1H), 3.62 (s, 3H), 3.67 – 3.57 (m, 1H), 3.54 – 3.43 (m, 2H), 1.13 (s, 9H).

3.64. Synthesis of *N*-(9-Fluorenylmethyloxycarbonyl)-L-seryl-L-serine methyl ester (*51*)



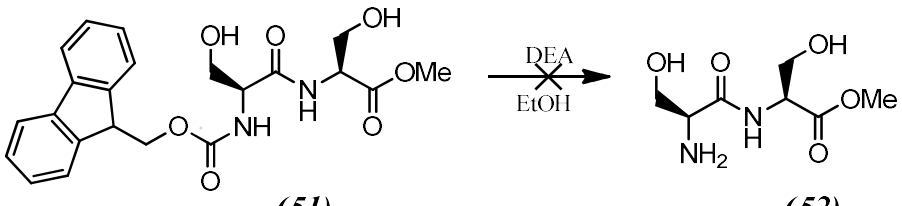
(50) *(51)*

To a solution of ***50*** (0.78 g, 1.6 mmol) in DCM (1 mL) aqueous phosphoric acid (0.94 mL, 16.1 mmol) was added dropwise. The mixture was stirred for 4 h at room temperature. Then the solution was neutralized with $NaHCO_3$ (0.5 M, 5 mL) and water (10mL) was added. The mixture was extracted with ethyl acetate (3×5 mL). The combined ethyl acetate phases were dried over Na_2SO_4 and concentrated in vacuum to give the desired product ***6*** as yellow powder (0.60 g, 1.4 mmol, 87%).

R_f: 0.40 (10% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 7.82 (d, J = 7.5 Hz, 2H), 7.69 (t, J = 6.69 Hz, 2H), 7.41 (t, J = 7.5 Hz, 2H), 7.33 (t, J = 7.5 Hz, 2H), 4.57 (t, J = 4.1 Hz, 1H), 4.43-4.37 (m, 2H), 4.34-4.24 (m, 2H), 3.94 (dd, J = 11.3, 4.3 Hz, 1H), 3.86-3.79 (m, 3H), 3.76 (s, 3H).

3.65. Synthesis of L-seryl-L-serine methyl ester (*52*)

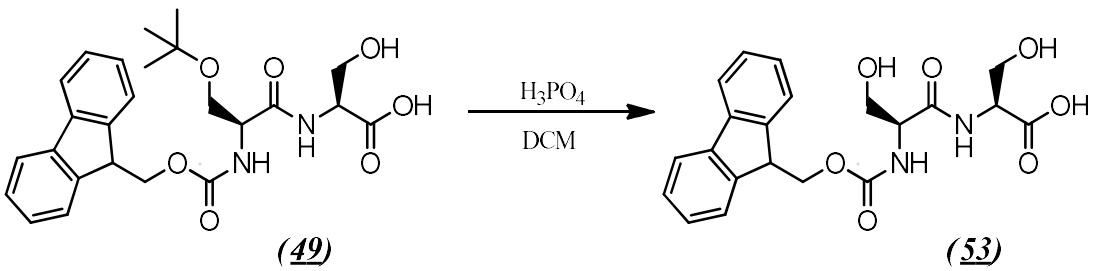


(51) *(52)*

The reaction was performed through General procedure E using compound ***51*** (0.30 g, 0.7 mmol), DEA (0.72 mL, 7.0 mmol) and EtOH (30 mL).

Solvents were removed under reduced pressure. The crude product was dissolved in EtOAc (10 mL) and extracted with H_2O (3×10 mL). Inorganic phase was then freeze-dried but in this way was not possible to obtain the desired product ***52***.

3.66. Synthesis of *N*-(9-Fluorenylmethyloxycarbonyl)-L-seryl-L-serine (*53*)



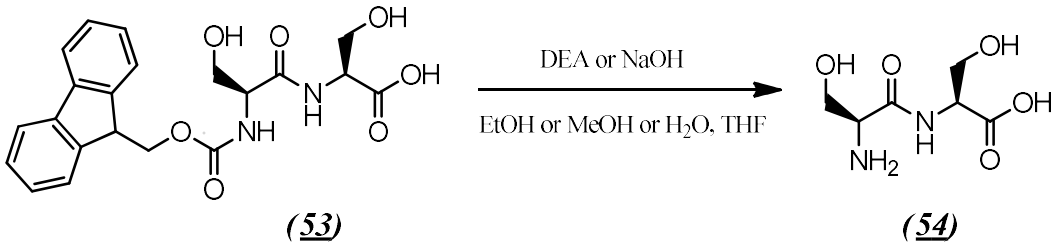
(49) *(53)*

To a solution of ***49*** (0.65 g, 1.4 mmol) in DCM (2 mL) aqueous phosphoric acid (0.84 mL, 14.5 mmol) was added dropwise. The mixture was stirred for 4 h at room temperature. Then the solution was neutralized with $NaHCO_3$ (0.5 M, 5 mL) and water (10 mL) was added. The mixture was extracted with ethyl acetate (3×5 mL). The combined ethyl acetate phases were dried over Na_2SO_4 and concentrated in vacuum to give the desired product ***53*** as yellow powder (0.50 g, 1.21 mmol, 83%).

R_f: 0.45 (20% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.95 (d, J = 6.9 Hz, 1H), 7.90 (d, J = 7.5 Hz, 2H), 7.77 – 7.70 (m, 2H), 7.42 (t, J = 7.2 Hz, 2H), 7.36 – 7.30 (m, 2H), 4.39 – 4.12 (m, 5H), 3.77 – 3.40 (m, 4H).

3.67. Synthesis of L-seryl-L-serine (*54*)



(53) *(54)*

The reaction was performed through General procedure E starting from compound ***53*** (0.05 g, 0.1 mmol) and using different bases and reaction conditions:

I. Using DEA (0.12 mL, 1.2 mmol) as base and EtOH (5 mL) as solvent obtaining product ***54*** as white powder (0.01 g, 0.1 mmol, 60%)

II. Using DEA (0.12 mL, 1.2 mmol) as base and MeOH (5 mL) as solvent obtaining product ***54*** as white powder (0.02 g, 0.1 mmol, 78%)

Purification was performed in the same way for both reactions: solvents were removed under reduced pressure; the crude solid was dissolved in H_2O (10 mL) and washed with DCM (2×10 mL); inorganic phase was then freeze-dried to obtain product ***54***.

III. Using NaOH (5 mL from a solution 1M, 0.20 g 5.0 mmol) as base and THF/H_2O as solvent (1:1, 5 mL) obtaining product ***54*** as white powder (0.01 g, 0.05 mmol, 43%).

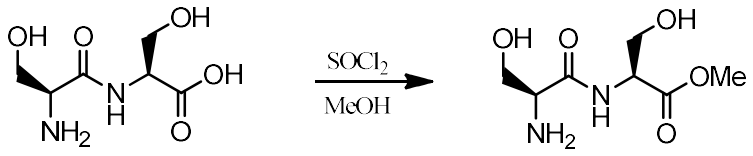
The solution was neutralized with a solution of HCl (1 M), and washed with hexane (5 mL). Solvents were removed under reduced pressure; the crude product was dissolved in EtOAc (15 mL) and NaCl was filtered out through a sintered funnel. EtOAc was then removed under reduced pressure to obtain product ***54***.

Best yield was encountered using reaction condition II and reaction was repeated starting from compound ***53*** (0.30 g, 0.7 mmol) in MeOH (20 mL) and product ***54*** was obtained as white powder (0.12 g, 0.6 mmol, 86%).

R_f: 0.50 (35% *t*BuOH/ 23% H2O/ 35% $(CH_3)_2CO$/ 7% AcOH).

^{1}H NMR (400 MHz, Deuterium Oxide-d_2) δ/ppm: 4.29 (dd, *J* = 6.0, 3.9 Hz, 1H), 4.08 (t, *J* = 4.9 Hz, 1H), 3.95 (d, *J* = 4.8 Hz, 2H), 3.86 (dd, *J* = 11.5, 3.8 Hz, 1H), 3.80 (dd, *J* = 11.6, 6.2 Hz, 1H).

3.68. Synthesis of L-seryl-L-serine methyl ester (55)



(54) *(52)*

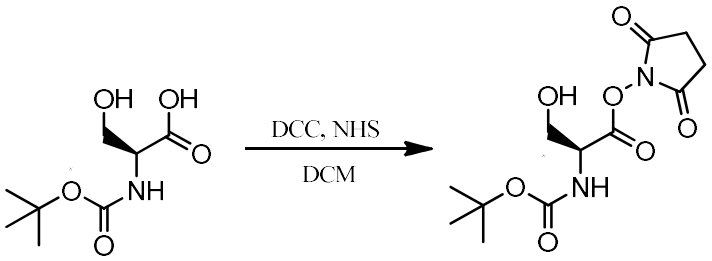
The product was obtained through General procedure C starting from compound ***54*** (3.80 g, 2.0 mmol), $SOCl_2$ (0.29 mL, 4.0 mmol) and MeOH (15 mL).

Solvents were removed under reduced pressure. The obtained crude solid was dissolved in EtOAc (10 mL) and washed with a saturated solution of $NaHCO_3$ (10 mL) and H_2O (2×10 mL). Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure to give product ***52*** as white powder (0.35 g, 1.7 mmol, 86%).

R_f: 0.40 (20% MeOH/DCM).

^{1}H NMR (400 MHz, Deuterium Oxide-d_2) δ/ppm: 4.65 (t, *J* = 4.4 Hz, 1H), 4.20 (dd, *J* = 5.8, 4.2 Hz, 1H), 4.04 (dd, *J* = 12.4, 4.2 Hz, 1H), 3.99 – 3.93 (m, 2H), 3.88 (dd, *J* = 11.8, 4.0 Hz, 1H), 3.76 (s, 3H).

3.69. Synthesis of *N*-*t*-butyloxycarbonyl-L-serine succinimidyl ester *(55)*



(55)

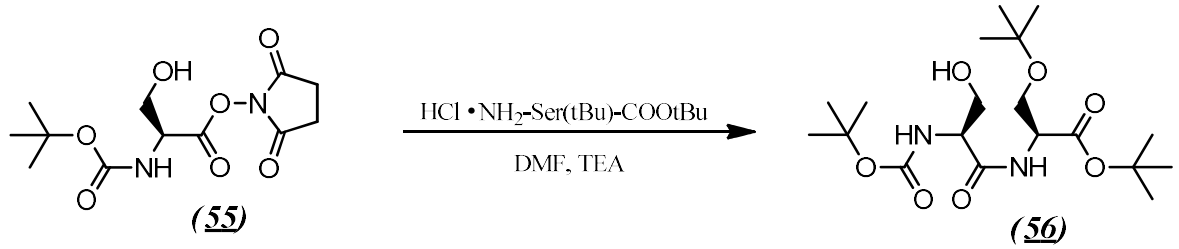
The product was obtained through General procedure A using Boc-Ser(OH)-COOH (4.00 g, 19.6 mmol) previously synthetized by our research group, DCC (4.44 g, 21.6 mmol), NHS (2.48 g, 21.6 mmol) and DCM (50 mL) as solvent.

The obtained crude solid was dissolved in EtOAc (50 mL) and washed with $NaHCO_3$ (0.5 M, 50 mL), H_2O (50 mL), HCl (0.1 M, 50 mL) and H_2O (50 mL). Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure to obtain product ***55*** (5.30 g, 17.5 mmol, 90%) that was used in the next step without further purification.

R_f: 0.90 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 4.45 (dd, J = 12.2, 6.5 Hz, 1H), 3.86 – 3.67 (m, 2H), 2.81 (s, 4H), 1.40 (s, 9H).

3.70. Synthesis of *N*-*t*-butyloxycarbonyl-L-seryl-*O*-*t*-butyl-L-serine *tert*-butyl ester (*56*)



(*55*) (*56*)

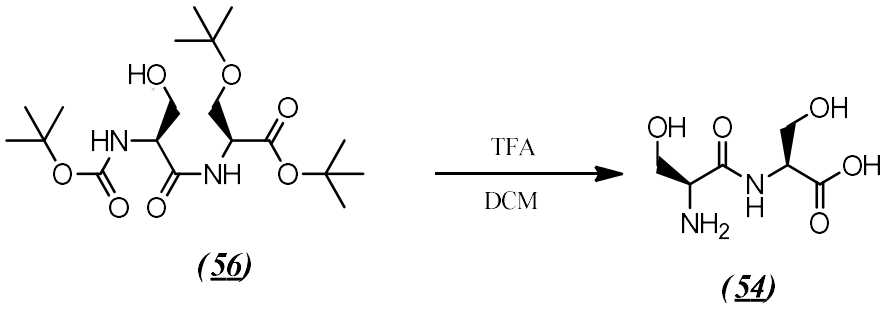
The product was obtained through General procedure I using compound ***55*** (2.00 g, 6.6 mmol), NH_2-Ser(*t*Bu)-COO*t*Bu (1.31 g, 6.0 mmol) previously synthetized by our research group, TEA (1.85 mL, 13.2 mmol) and DMF (50 mL) as solvent.

TEA was removed under reduced pressure and the precipitation was performed adding cold water dropwise, under stirring. The product ***56*** was isolated as white powder (1.45 g, 3.6 mmol, 54%).

R_f: 0.7 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 4.31 (dt, *J* = 7.3, 3.5 Hz, 1H), 4.05 (dd, *J* = 12.7, 6.5 Hz, 1H), 3.62 (dd, *J* = 8.9, 4.2 Hz, 1H), 3.60 – 3.51 (m, 2H), 3.44 (dd, *J* = 9.0, 4.2 Hz, 1H), 1.41 (s, 9H) 1.39 (s, 9H), 1.12 (s, 9H).

3.71. Synthesis of L-seryl-L-serine (*54*)



(56) *(54)*

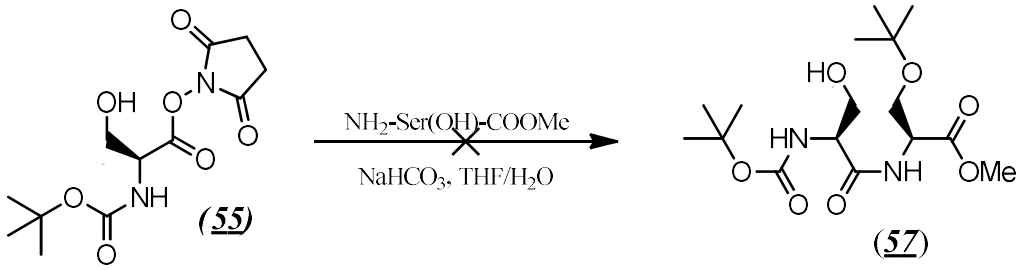
To a solution of ***56*** (1.2 g, 3.0 mmol) in DCM (50 mL) TFA (35 mL, 457.1 mmol) was added dropwise. The mixture was stirred for 4 h at room temperature. The solution was reduced to half its volume under reduced pressure and then cooled at 0°C. The product ***54*** was then precipitated slowly adding Et_2O (40 mL).

The solution was left at -20°C overnight and next day filtered out through a sintered funnel obtaining product ***54*** as white powder (0.51 g, 2.7 mmol, 90%).

R_f: 0.1 (20% MeOH/DCM).

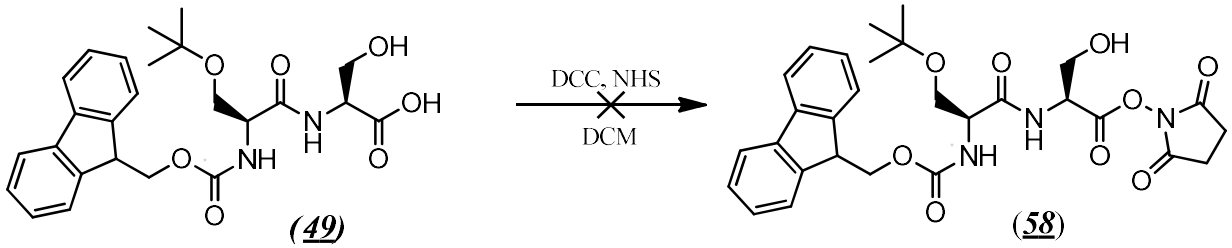
^{1}H NMR (400 MHz, Deuterium Oxide-d_2) δ/ppm: 4.29 (dd, J = 6.0, 3.9 Hz, 1H), 4.08 (t, J = 4.9 Hz, 1H), 3.95 (d, J = 4.8 Hz, 2H), 3.86 (dd, J = 11.5, 3.8 Hz, 1H), 3.80 (dd, J = 11.6, 6.2 Hz, 1H).

3.72. Synthesis of *N-t*-butyloxycarbonyl-L-seryl-*O-t*-butyl-L-serine methyl ester (*57*)



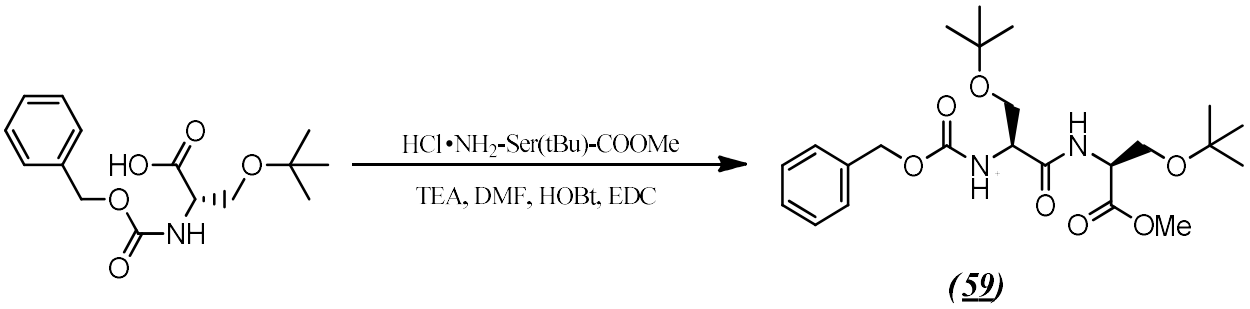
The reaction was performed through general procedure B using compound ***55*** (2.80 g, 9.3 mmol), NH_2-Ser(OH)-COOMe (1.46 g, 8.4 mmol), $NaHCO_3$ (1.72 g, 20.5 mmol) and THF (90 mL)/H_2O (60 mL) as solvents. In this way was not possible to obtain the desired product ***57***.

3.73. Synthesis of *N*-(9-Fluorenylmethyloxycarbonyl)-*O-t*-butyl-L-seryl-L-serine succinimidyl ester *(58)*



The reaction was performed through General procedure A using compound ***49*** (5.00 g, 10.6 mmol), DCC (2.63 g, 12.8 mmol), NHS (1.47 g, 12.8 mmol) and DCM (200 mL) as solvent. In this way was not possible to obtain the desired produt ***58***.

3.74. Synthesis of *N*-carbobenzoxy-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-serine methyl ester (*59*)



(59)

The product was obtained through General procedure G using HCl×NH_2-Ser(*t*Bu)-COOMe (2.44 g, 12.2 mmol) and Z-Ser(*t*Bu)-COOH (4.00 g, 13.5 mmol) commercially available, EDC×HCl (3.97 g, 20.3 mmol) and HOBt (2.78 g, 20.3 mmol), TEA (9.53 mL 67.5 mmol) and DMF as solvent (30 mL).

Solvent was removed under reduced pressure. The crude solid was dissolved in DCM (50 mL) and washed with water (3×50 mL). Flash chromatography was performed in isocratic flow of 10% MeOH/DCM and the product ***59*** was isolated as orange oil (5.16 g, 11.4 mmol, 92%).

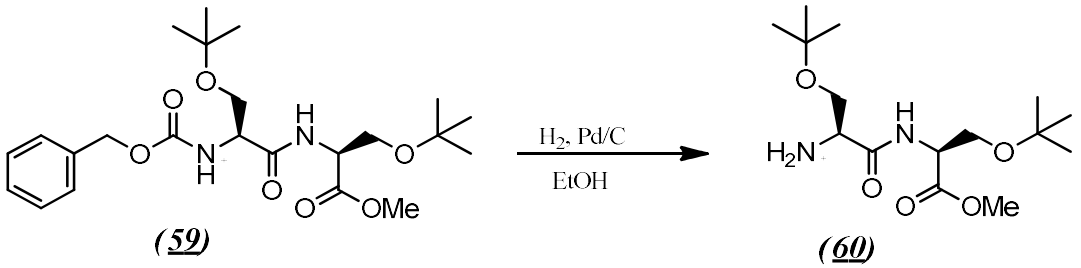
R_f: 0.85 (10% MeOH/DCM).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.64 (d, *J* = 7.3 Hz, 1H), 7.42 – 7.30 (m, 6H), 5.15-5.13 (m, 2H), 4.73 (dt, *J* = 8.6, 3.0 Hz, 1H), 4.31 (m, 1H), 3.86 (dd, *J* = 9.1, 2.9 Hz, 1H), 3.83-3.81 (m, 1H), 3.75 (s, 3H), 3.56 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.46 (t, *J* = 8.5 Hz, 1H), 1.26 (s, 9H), 1.15 (s, 9H).

^{13}C NMR (400 MHz, $CDCl_3$) δ/ppm: 170.94, 170.76, 156.45, 136.73, 128.95, 128.57, 128.51, 73.88, 67.37, 62.43, 53.55, 52.65, 27.79, 27.74.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 475.24, found: 475.25.

3.75. Synthesis of O-*t*-butyl-L-seryl-O-*t*-butyl-L-serine methyl ester (***60***)



(59) ***(60)***

The product was obtained folowwing General procedure F using compound ***59*** (7.30 g, 16.1 mmol), 10wt. % Pd/C (0.73 g) and EtOH as solvent (200 mL).

Catalyst was filtered through celite bed and solvent was removed under reduced pressure to give product ***60*** as a white powder (4.77 g, 15.0 mmol, 93%).

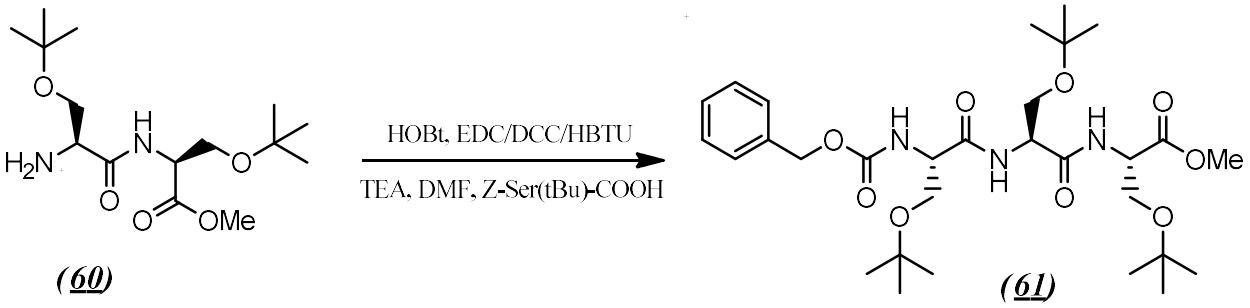
R_f: 0.5 (10% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 4.64 (t, *J* = 3.5 Hz, 1H), 3.86 (dd, *J* = 9.3, 3.6 Hz, 1H), 3.75 (s, 3H), 3.62 (dd, *J* = 9.3, 3.5 Hz, 1H), 3.58 (d, *J* = 5.5 Hz, 2H), 3.52 – 3.47 (m, 1H), 1.24 (s, 9H), 1.19 (s, 9H).

^{13}C NMR (400 MHz, DMSO-d_6,) δ/ppm 173.28, 171.11, 73.40, 73.05, 64.35, 62.27, 55.06, 52.67-52.37, 27.68.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 341.21, found: 341.24.

3.76. Synthesis of N-carbobenzoxy-O-*t*-butyl-L-seryl-O-*t*-butyl-L-seryl-O-*t*-butyl-L-serine methyl ester (61)



(60) *(61)*

The product was obtained through General procedure G using compound ***60*** (0.10 g, 0.3 mmol) and Z-Ser(*t*Bu)-COOH (0.10 g, 0.3 mmol), HOBt (0.07 g, 0.5 mmol), DMF as solvent (5 mL) and three different coupling reagents:

I. DCC (0.10 g, 0.5 mmol) obtaining product ***61*** as yellow powder (0.08 g, 0.1 mmol, 44%).

II. HBTU (0.20 g, 0.5 mmol), TEA (0,24 mL 1.7 mmol) obtaining product ***61*** as yellow powder (0.13 g, 0.2 mmol, 72%).

III. EDC×HCl (0.10 g, 0.5 mmol), TEA (0,24 mL 1.7 mmol) obtaining product ***61*** as yellow powder (0.15 g, 0.3 mmol, 83%).

Solvents were removed under reduced pressure; the crude solid was dissolved in Et_2O (5 mL) and washed with water (3×5 mL). Flash column chromatography was performed in isocratic flow of 10% MeOH/DCM and the product ***61*** was isolated as yellow powder.

Scale up was performed using conditions II following General procedure G starting from compound ***60*** (4.27 g, 13.4 mmol, 50 mL of DMF). Product ***61*** was obtained as a yellow powder (5.73 g, 9.6 mmol, 72%)

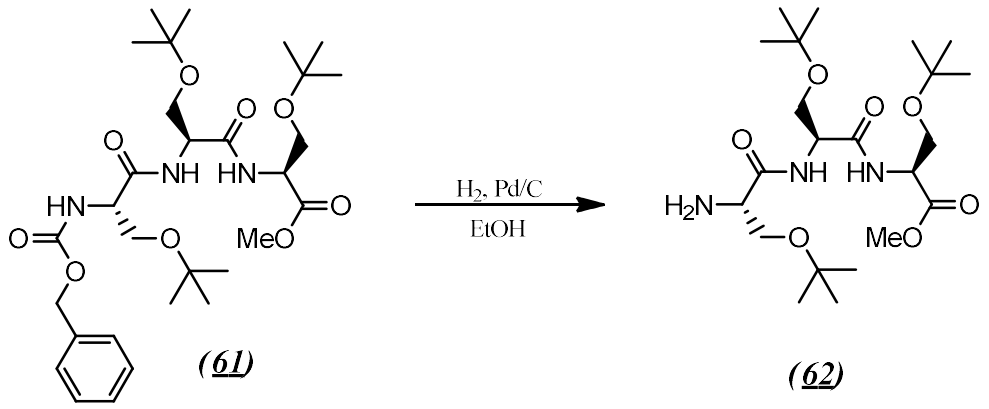
R_f: 0.80 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6,) δ/ppm: 8.05 (d, *J* = 8.2 Hz, 1H), 7.82 (d, *J* = 7.9 Hz,1H), 7.39 – 7.30 (m, 6H), 5.05 (s, 2H), 4.49 (dt, *J* = 8.6, 4.5 Hz, 1H), 4.43 (m, 1H), 4.14 (dd, *J* = 14.1, 6.0 Hz, 1H), 3.63 (s, 3H), 3.68 – 3.60 (m, 1H), 3.54 – 3.38 (m, 5H), 1.13 (s, 18H), 1.12 (s, 18H), 1.10 (s, 9H).

^{13}C NMR (400 MHz, Methanol-d_4) δ/ppm: 137.45, 128.79, 128.22, 128.10, 73.45, 73.10, 71.98, 65.94, 62.21, 62.02, 55.76, 53.47, 53.28, 53.09, 52.31, 26.15.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 618.34, found: 618.29.

3.77. Synthesis of *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine methyl ester (*62*)



(61) *(62)*

The product was obtained through General procedure F using compound ***61*** (5.70 g, 9.6 mmol), 10wt. % Pd/C (0.57 g) and EtOH as solvent (300 mL).

Catalyst was filtered through celite bed and solvent was removed under reduced pressure to obtain product ***62*** as a white powder (4.34 g, 9.4 mmol, 98%).

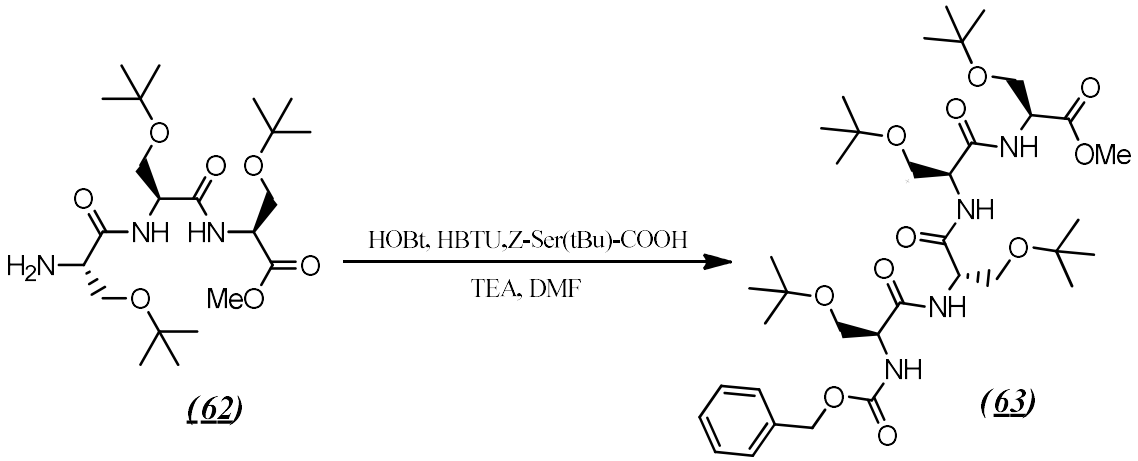
R_f: 0.50 eluted in (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6,) δ/ppm: 8.07 (d, *J* = 8.1 Hz, 2H), 4.52 – 4.45 (dt, *J* = 8.2, 4.3 Hz, 1H), 4.41 (dd, *J* = 11.9, 6.4 Hz, 1H), 3.70 – 3.60 (m, 1H), 3.64 (s, 3H), 3.53 (dd, J = 8.8, 4.6 Hz, 1H), 3.51 (dd, J = 8.8, 4.6 Hz, 1H) 3.64 (m, 2H), 1.13 (s, 18H), 1.11 (s, 9H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 172.35, 172.17, 170.56, 169.87, 172.17, 170.56, 169.87, 73.08, 72.70, 63.98, 61.97, 61.64, 54.96, 52.88, 52.31, 51.99, 27.35, 27.21, 21,26.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 484.30, found: 484.25.

3.78. Synthesis of *N*-carbobenzoxy-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-serine methyl ester (*63*)



The product was obtained following General procedure G using compound ***62*** (3.42 g, 7.4 mmol) and Z-Ser(*t*Bu)-COOH (1.50 g, 8.2 mmol), commercially available, HBTU (4.21 g, 11.1 mmol) and HOBt (1.90 g, 11.1 mmol), TEA (5.17 mL 37.0 mmol) and DMF as solvent (50 mL). Solvent was removed under reduced pressure; the crude solid was dissolved in DCM (50 mL) and washed with water (3×50 mL). Flash chromatography was performed in isocratic flow of (10% MeOH/DCM) and the product ***63*** was isolated as yellow powder (3.00 g, 4.1 mmol, 55 %).

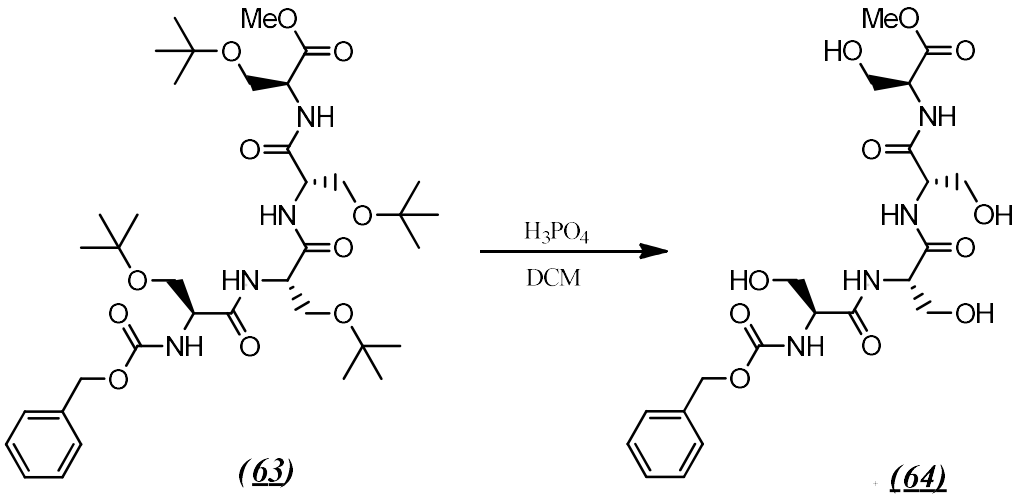
R_f: 0.70 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6,) δ/ppm: 8.03 (d, *J* = 8.1 Hz, 1H), 7.84 – 7.79 (m, 2H), 7.39 – 7.29 (m, 6H), 5.05 (s, 2H), 4.49 (dt, *J* = 8.2, 4.2 Hz, 1H), 4.45– 4.42 (m, 1H), 4.38 (dt, *J* = 7.6, 5.9 Hz, 1H), 4.14 (dd, *J* = 14.4, 6.1 Hz, 1H), 3.66 – 3.60 (m, 1H), 3.64 (s, 3H), 3.55 – 3.39 (m, 7H), 1.12 (s, 27H), 1.10 (s, 9H).

^{13}C NMR (400 MHz, $CDCl_3$) δ/ppm: 170.86, 170.18, 169.98, 169.74, 156.39, 137.45, 128.79, 128.22, 128.10, 73.47, 65.94, 62.21, 62.02, 55.76, 53.47, 53.28, 53.09, 52.31, 27.57.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 761.43, found: 761.41.

3.79. Synthesis of *N*-carbobenzoxy-L-seryl-L-seryl-L-seryl-L-serine methyl ester (*64*)



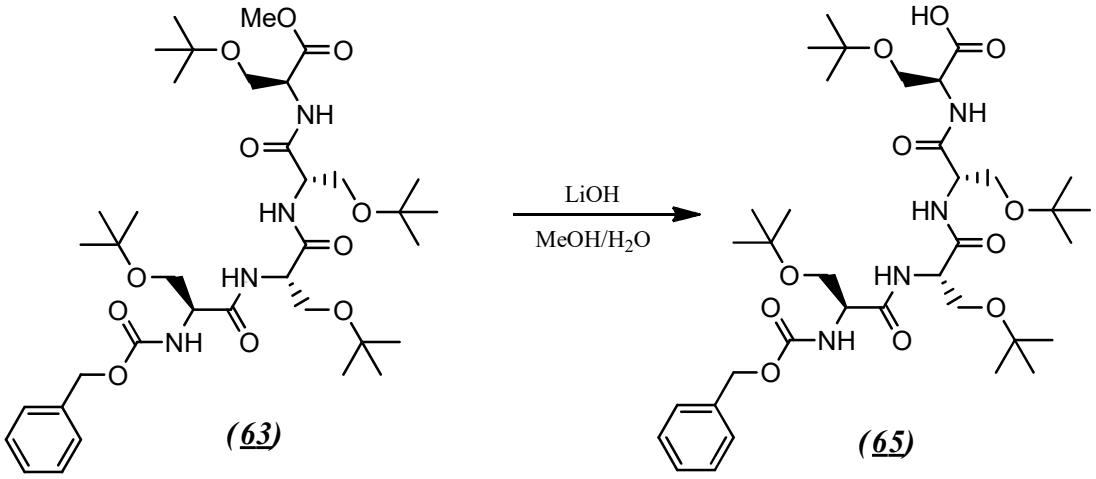
To a solution of ***63*** (0.10 g, 0.2 mmol) in DCM (1 mL) aqueous phosphoric acid (0.08 mL, 1.4 mmol) was added dropwise. The mixture was stirred for 4 h at room temperature.

Solvents were removed under reduced pressure; the crude product was dissolved in AcOEt (5 mL) and extracted with H_2O (5 mL). Inorganic phase was then freeze-dried to obtain product ***64*** as yellow powder (0.06 g, 0.1 mmol, 86%).

R_f: 0.50 (20% MeOH/DCM).

^{1}H NMR (DMSO-d$_6$, 400 MHz) δ/ppm: 8.11 – 8.04 (m, 2H), 8.02 (d, *J* = 8.3 Hz, 1H), 7.41 – 7.26 (m, 6H), 5.04 (s, 2H), 4.42 – 4.29 (m, 3H), 4.16 (dd, *J* = 13.9, 5.9 Hz, 1H), 3.74 – 3.49 (m, 8H), 3.62 (s, 3H).

3.80. Synthesis of *N*-carbobenzoxy-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-serine (***65***)



The product was obtained following General procedure D starting with compound ***63*** (0.80 g, 1.1 mmol), LiOH×H_2O (0.10 g, 1.65 mmol) and MeOH (30 mL) / H_2O (10 mL).

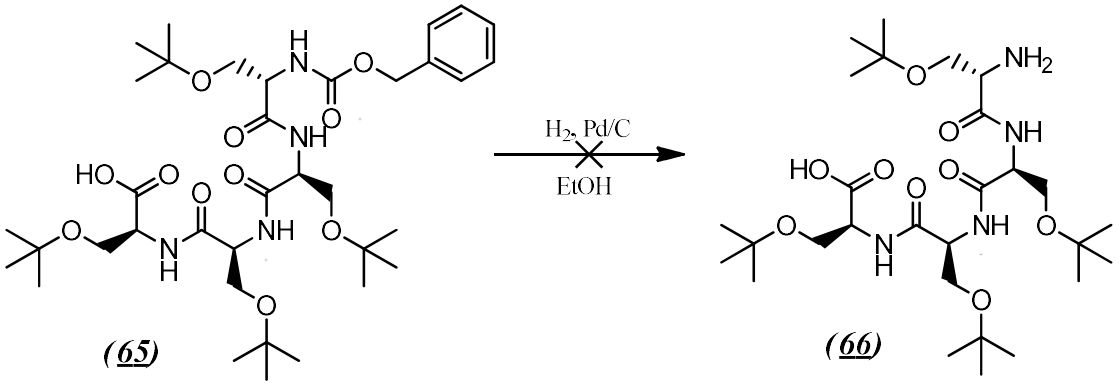
Solvents were removed under reduced pressure; the crude product was dissolved in AcOEt (5 mL) and washed with HCl (0.1 M, 2×20 mL) and H_2O (2×20 mL). Organic layer was dried

over Na_2SO_4 and evaporated under reduced pressure to obtain product ***65*** as a white powder (0.65 g, 0.9 mmol, 83%).

R_f: 0.40 (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6,) δ/ppm: 7.83 (d, *J* = 7.6 Hz, 1H), 7.81 (d, *J* = 7.8 Hz, 1H), 7.64 (d, *J* = 7.2 Hz, 1H), 7.39 – 7.27 (m, 6H), 5.04 (s, 2H), 4.38 (dt, *J* = 7.8, 5.8 Hz, 1H), 4.34 – 4.32 (m, 1H), 4.15 (dd, J = 13.9, 6.3, 1H), 4.09 (dt, J = 7.2, 3.4 Hz, 1H), 3.46 (m, 8H), 1.12 (s, 27H), 1.07 (s, 9H). **ESI-Q-TOF**: $[M+Na]^+$ Calcd: 747.87, found: 748.78.

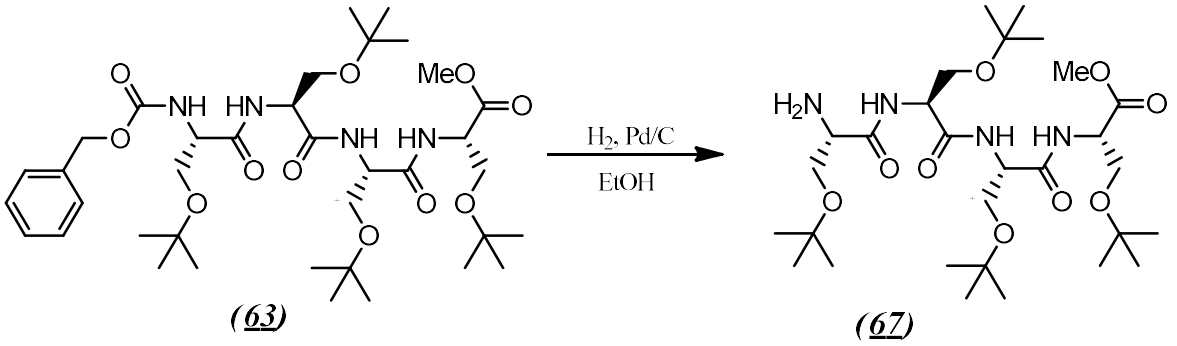
3.81. Synthesis of *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine (*66*)



The reaction was performed through General procedure F using compound ***65*** (0.63 g, 0.9 mmol), 10wt. % Pd/C (0.06 g) and EtOH as solvent (50 mL).

Catalyst was filtered through celite bed and solvent was removed under reduced pressure but the product didn't decarboxylate and desired compound ***66*** was not obtained.

3.82. Synthesis of *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine methyl ester (*67*)



(63) *(67)*

The product was obtained through General procedure F using compound ***63*** (2.30 g, 3.1 mmol), 10wt. % Pd/C (0.23 g) and EtOH as solvent (50 mL).

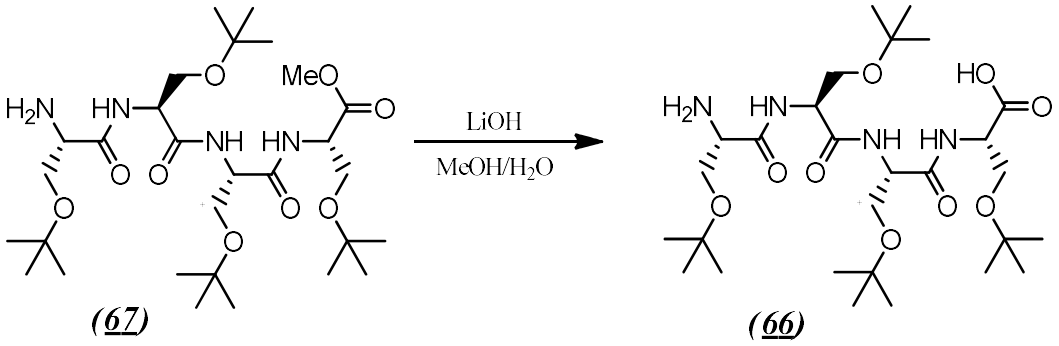
Catalyst was filtered through celite bed and solvent was removed under reduced pressure to obtain product ***67*** as white powder (1.78 g, 2.9 mmol, 95%).

R_f: 0.5 eluted in (10% MeOH/DCM).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 8.11 (d, *J* = 7.2 Hz, 1H), 7.61 – 7.45 (m, 3H), 4.72 (dt, *J* = 6.5, 2.7 Hz, 1H), 4.55 – 4.44 (m, 2H), 3.88-3.36 (m, 9H), 3.74 (s, 3H), 1.27 – 1.23 (m, 18H), 1.22 (s, 9H), 1.15 (s, 9H).

^{13}C NMR (400 MHz, $CDCl_3$,) δ/ppm 173.14, 170.56, 170.26, 170.19, 73.18, 73.57, 73.41, 63.74, 62.06, 61.56, 61.26, 55.39, 53.22-53.11, 55.39, 27.41.

3.83. Synthesis of *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine (*66*)



(67) *(66)*

The product was obtained through General procedure D using compound ***67*** (0.50 g, 0.8 mmol), LiOH×H_2O (0.07 g, 1.7 mmol) and MeOH (9 mL) / H_2O (3 mL).

Solution was neutralized using HCl (0.1 M) and solvent was removed under reduced pressure; the crude product was dissolved in cold MeOH (5 mL) and LiCl was filtered out through a sintered funnel. Solvent was removed under reduced pressure to obtain product ***66*** as white powder (0.37 g, 0.6 mmol, 75%).

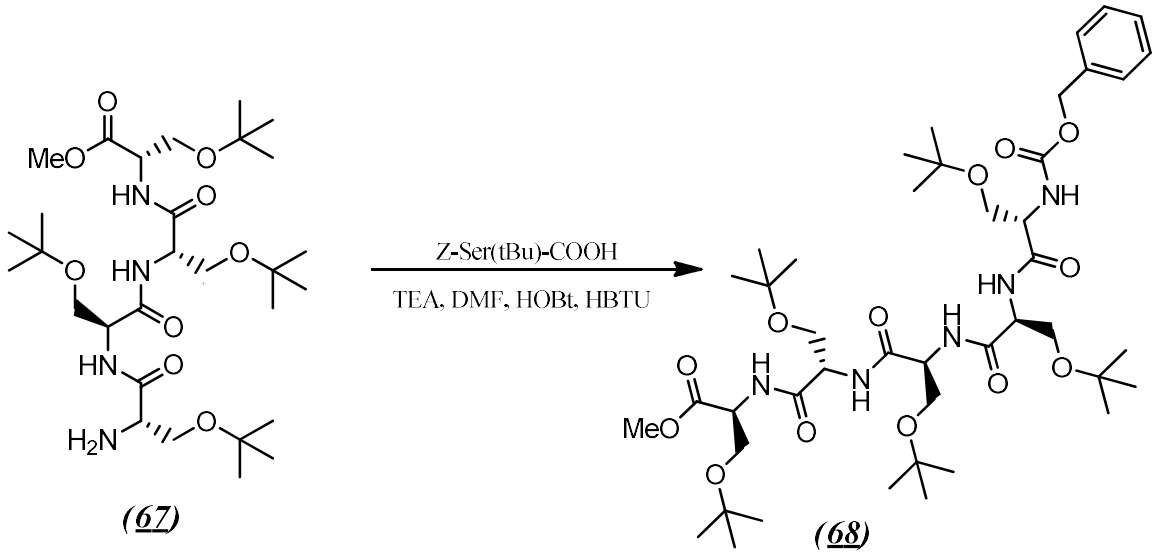
R_f: 0.20 eluted in (20% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 4.65 – 4.50 (m, 3H), 4.12 (dd, *J* = 16.2, 9.2 Hz, 1H), 3.98 – 3.54 (m, 8H), 1.26 (s, 9H), 1.25 – 1.22 (br, 18H), 1.20 (s, 9H).

^{13}C NMR (400 MHz, Methanol-d_4) δ/ppm: 174.34, 173.89, 173.01, 172.21, 75.51, 75.35, 75.07, 74.91, 63.33, 63.13, 62.00, 57.37-55.09, 28.06.

ESI-Q-TOF: $[M+H]^+$ Calcd: 591.40, found: 591.73.

3.84. Synthesis of *N*-carbobenzoxy-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-serine methyl ester (***68***)



(67) ***(68)***

The product was obtained through General procedure G using compound ***67*** (1.00 g, 1.7 mmol) and Z-Ser(*t*Bu)-COOH (0.54 g, 1.8 mmol), commercially available, HBTU (0.94 g, 2.5 mmol) and HOBt (0.34 g, 2.5 mmol), TEA (1.15 mL 8.3 mmol) and DMF as solvent (50 mL). Solvent was removed under reduced pressure. The crude solid was dissolved in DCM (50 mL) and washed with H_2O (3×50 mL). Flash chromatography was performed in isocratic flow of (10% MeOH/DCM) and the product ***68*** was isolated as yellow powder (0.80 g, 4.9 mmol, 55 %).

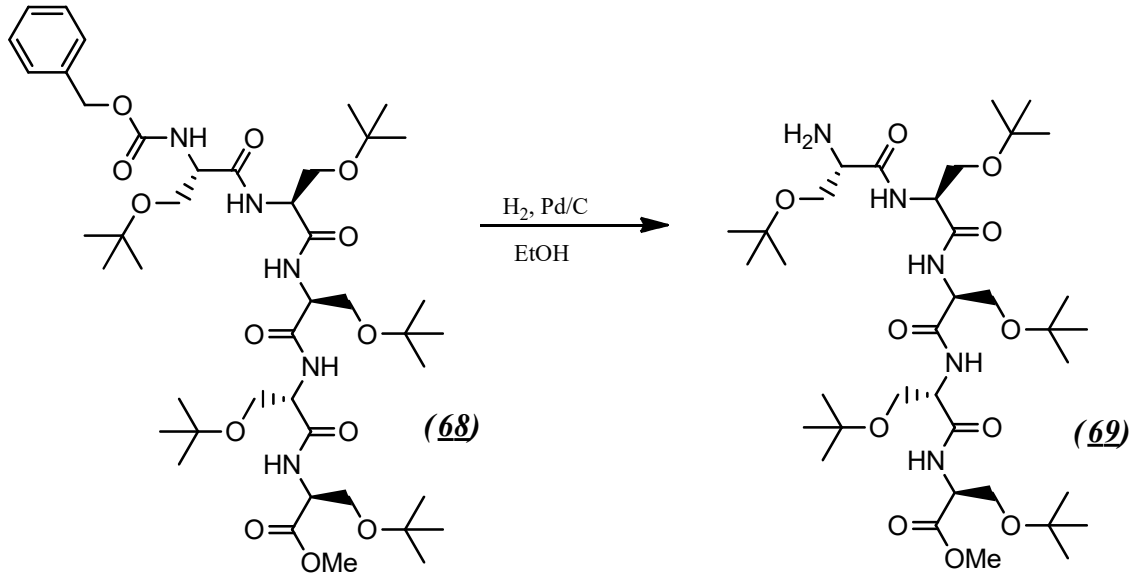
R_f: 0.65 (10% MeOH/DCM).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.61 (d, *J* = 5.7 Hz, 1H), 7.56 (d, *J* = 8.5 Hz, 1H), 7.47 – 7.42 (m, 2H), 7.41 – 7.31 (m, 6H), 5.14 (s, 2H), 4.72 (dt, *J* = 8.3, 2.7 Hz, 1H), 4.53 – 4.45 (m, 3H), 4.30 (m, 1H), 3.89 – 3.77 (m, 5H), 3.73 (s, 3H), 3.55 (dd, *J* = 9.1, 2.9 Hz, 1H), 3.50 – 3.36 (m, 4H), 1.25 (s, 9H), 1.23 (s, 9H), 1.23 (s, 18H), 1.15 (s, 9H).

^{13}C NMR (400 MHz, $CDCl_3$) δ/ppm: 170.55, 170.25, 170.09, 169.81, 156.03, 136.31, 128.51, 128.11, 74.31, 74.20, 73.39, 66.98, 62.06, 61.88, 61.46, 61.32, 54.63, 53.62, 53.56, 53.16, 53.06, 52.10, 27.38.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 905.08, found: 905.70.

3.85. Synthesis of *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine methyl ester (***69***)



The product was obtained through General procedure F starting from compound ***68*** (0.75 g, 0.9 mmol), 10wt. % Pd/C (0.09 g) and EtOH as solvent (60 mL).

Catalyst was filtered through celite bed and solvent was removed under reduced pressure to obtain product ***69*** as white powder (0.59 g, 0.8 mmol, 93%).

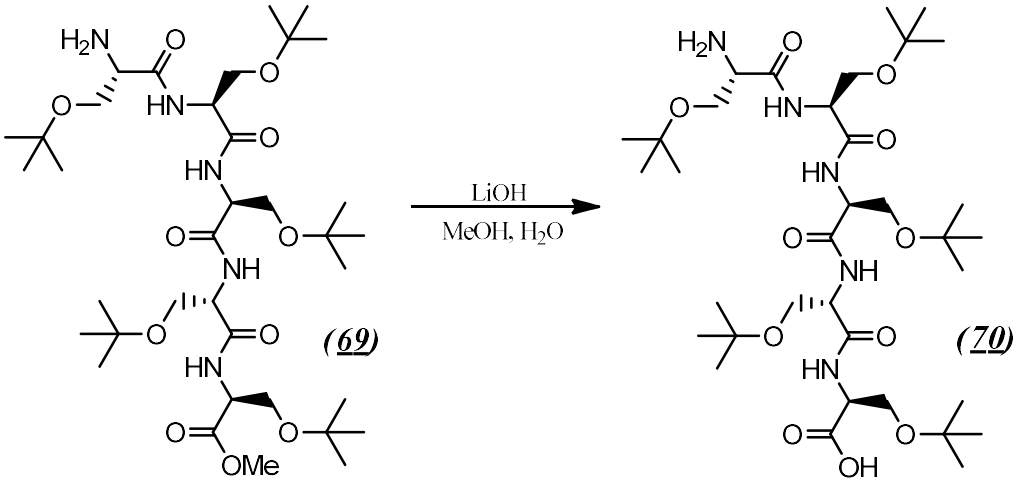
R_f: 0.55 (10% MeOH/DCM).

^{1}H NMR (400 MHz, $CDCl_3$,) δ/ppm: 8.14 (d, *J* = 7.1 Hz, 1H), 7.57 (d, *J* = 8.4 Hz, 1H), 7.51 (d, *J* = 6.9 Hz, 1H), 7.48 (d, *J* = 6.6 Hz, 1H), 4.71 (dt, *J* = 8.5, 3.0 Hz, 1H), 4.55 – 4.44 (m, 3H), 3.90 – 3.77 (m, 4H), 3.73 (s, 3H), 3.66 (dd, *J* = 8.2, 4.8 Hz, 1H) 3.61 – 3.37 (m, 6H), 1.25 (s, 9H), 1.23 (s, 18H). 1.22 (s, 9H). 1.14 (s, 9H).

^{13}C NMR (400 MHz, $CDCl_3$) δ/ppm 173.27, 170.83, 170.51, 170.38, 170.23, 74.49, 73.87, 73.67, 63.89, 62.31, 61.79, 61.65, 55.61, 55.61, 53.81, 53.44, 52.40, 27.65.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 748.51, found: 748.84.

3.86. Synthesis of *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine (*70*)



The product was obtained through general procedure D using compound ***69*** (0.57 g, 0.8 mmol), LiOH×H_2O (0.06 g, 1.5 mmol) and MeOH (9 mL) / H_2O (3 mL).

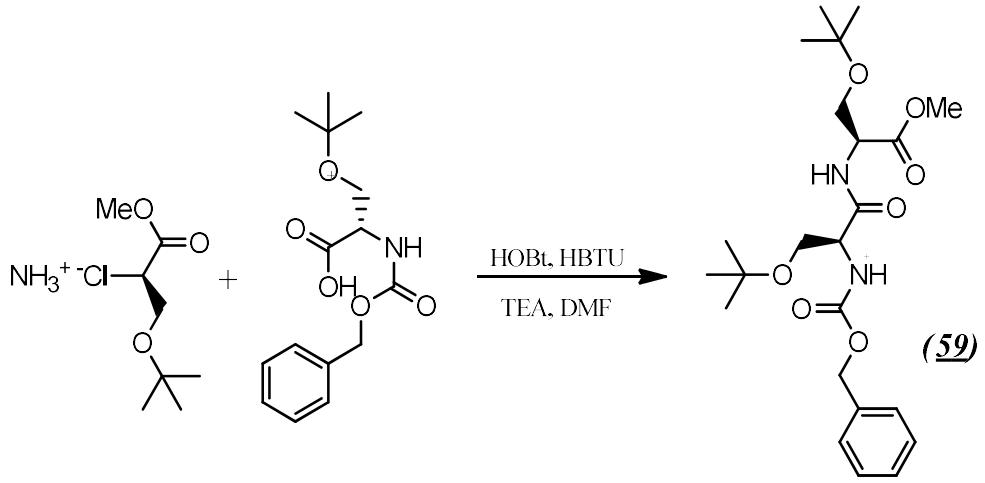
Solution was neutralized using HCl (0.1 M) and solvent was removed under reduced pressure; the crude product was dissolved in cold MeOH (5 mL) and LiCl was filtered out through a sintered funnel. Solvent was removed under reduced pressure to obtain product ***70*** as white powder (0.44 g, 0.6 mmol, 79%).

R_f: 0.20 (20% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 4.59 – 4.47 (m, 3H), 4.44 – 4.40 (m, 1H), 4.13 – 4.05 (m, 1H), 3.93 – 3.56 (m, 10H), 1.27 (s, 9H), 1.25 (s, 9H), 1.25 (s, 9H), 1.24 (s, 9H), 1.18 (s, 9H).

ESI-Q-TOF: $[M+H]^+$ Calcd: 756.47, found: 756.80.

3.87. Synthesis of *N*-carbobenzoxy-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-serine methyl ester (*59*)



The product was obtained through General procedure G using HCl×NH_2-Ser(*t*Bu)-COOMe (6,53 g, 30.9 mmol) and Z-Ser(*t*Bu)-COOH (10.00 g, 34.0 mmol), commercially available, HBTU (17.56 g, 46.3 mmol) and HOBt (6.26 g, 46.3 mmol), TEA as base (21.45 mL 154.3 mmol) and DMF as solvent (50 mL).

Precipitation was unsuccessful; after the removal of solvent under reduced pressure, the crude solid was dissolved in hexane (50 mL) and washed with water (3×30 mL).

Flash chromatography was performed in isocratic flow of (10% MeOH/DCM) and the product ***59*** was isolated as orange oil (11.20 g, 24.8 mmol, 80%).

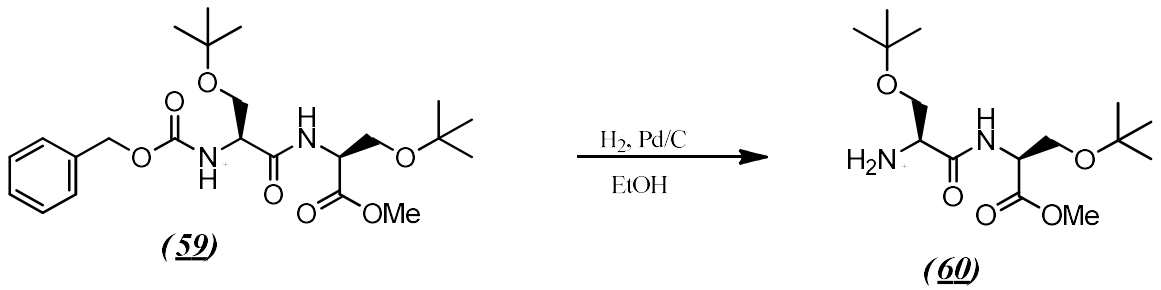
R_f: 0.85 eluted in (10% MeOH/DCM).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.64 (d, *J* = 7.3 Hz, 1H), 7.42 – 7.30 (m, 6H), 5.15-5.13 (m, 2H), 4.73 (dt, *J* = 8.6, 3.0 Hz, 1H), 4.31 (m, 1H), 3.86 (dd, *J* = 9.1, 2.9 Hz, 1H), 3.83-3.81 (m, 1H)., 3.75 (s, 3H), 3.56 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.46 (t, *J* = 8.5 Hz, 1H), 1.26 (s, 9H), 1.15 (s, 9H).

^{13}C NMR (400 MHz, $CDCl_3$) δ/ppm: 170.94, 170.76, 156.45, 136.73, 128.95, 128.57, 128.51, 73.88, 67.37, 62.43, 53.55, 52.65, 27.79, 27.74.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 475.24, found: 475.25.

3.88. Synthesis of *O-t*-butyl-L-seryl-*O-t*-butyl-L-serine methyl ester (***60***)



(59) ***(60)***

The product was obtained through General procedure F using compound ***59*** (6.00 g, 13.3 mmol), 10wt. % Pd/C (0.60 g) and EtOH as solvent (100 mL).

Catalyst was filtered through celite bed and solvent was removed under reduced pressure to give product ***60*** as a white powder (4,10 g, 12.9 mmol, 97%).

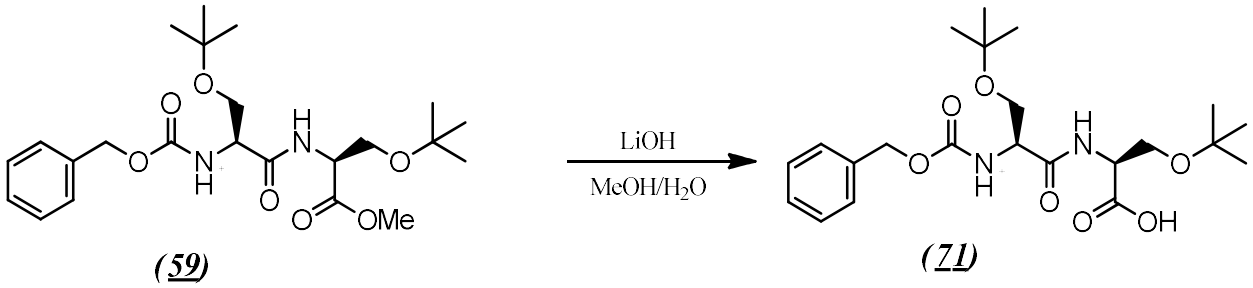
R_f: 0.5 eluted in (10% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 7.92 (s, 1H), 4.64 (t, J = 3.5 Hz, 1H), 3.86 (dd, J = 9.3, 3.6 Hz, 1H), 3.75 (s, 3H), 3.62 (dd, J = 9.3, 3.5 Hz, 1H), 3.58 (d, J = 5.5 Hz, 2H), 3.52 – 3.47 (m, 1H), 1.24 (s, 9H), 1.19 (s, 9H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm 173.28, 171.11, 73.40, 73.05, 64.35, 62.27, 55.06, 52.67, 52.37, 27.68.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 341.21, found: 341.24.

3.89. Synthesis of *N*-carbobenzoxy-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-serine (*71*)



(59) *(71)*

The product was obtained through general procedure D using compound ***59*** (5.20 g, 11.5 mmol), LiOH×H_2O (0.96 g, 23.0 mmol) and MeOH (30 mL) / H_2O (10 mL).

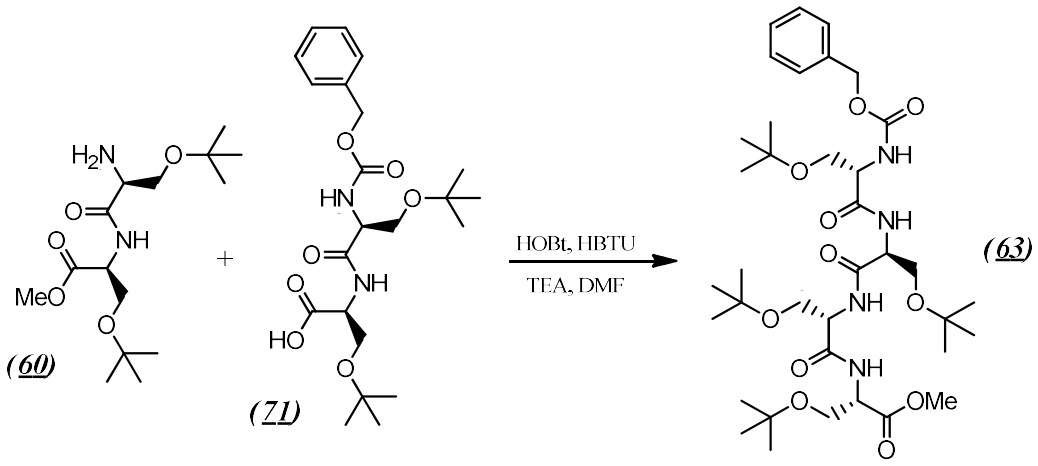
Solution was neutralized using HCl (0.1 M), solvent was removed under reduced pressure; the crude product was dissolved in cold MeOH (10 mL) and LiCl was filtered out through a sintered funnel. Solvent was removed under reduced pressure to obtain product ***71*** as orange oil (4.30 g, 9.8 mmol, 85%).

R_f: 0.50 (20% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 7.97 (d, J = 7.5 Hz, 1H), 7.90 (d, J = 8.4 Hz, 1H), 7.44 – 7.22 (m, 6H), 5.13 (s, 2H), 4.58 (s, 1H), 4.32 (dd, J = 6.6, 3.3 Hz, 1H), 3.88 – 3.85 (m, 1H), 3.71 – 3.62 (m, 3H), 1.22 (s, 9H), 1.18 (s, 9H).

ESI-Q-TOF: $[M-H]^-$ Calcd: 437.23, found: 437.63.

3.90. Synthesis of *N*-carbobenzoxy-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-serine methyl ester (*63*)



(*60*) (*71*) (*63*)

The product was obtained through General procedure G using compound ***71*** (3.03 g, 6.9 mmol) and compound ***60*** (2.00 g, 6.3 mmol), HBTU (3.57 g, 9.4 mmol), HOBt (1.27 g, 9.4 mmol), TEA (4.38 mL 31.4 mmol) and DMF as solvent (50 mL).

The product was precipitated adding water dropwise under stirring at RT, the crude solid was dissolved in AcOEt (50 mL) and washed with H_2O (3×50 mL). Flash chromatography was performed in isocratic flow of (10% MeOH/DCM) and the product ***63*** was isolated as an orange powder (1.80 g, 2.4 mmol, 39%).

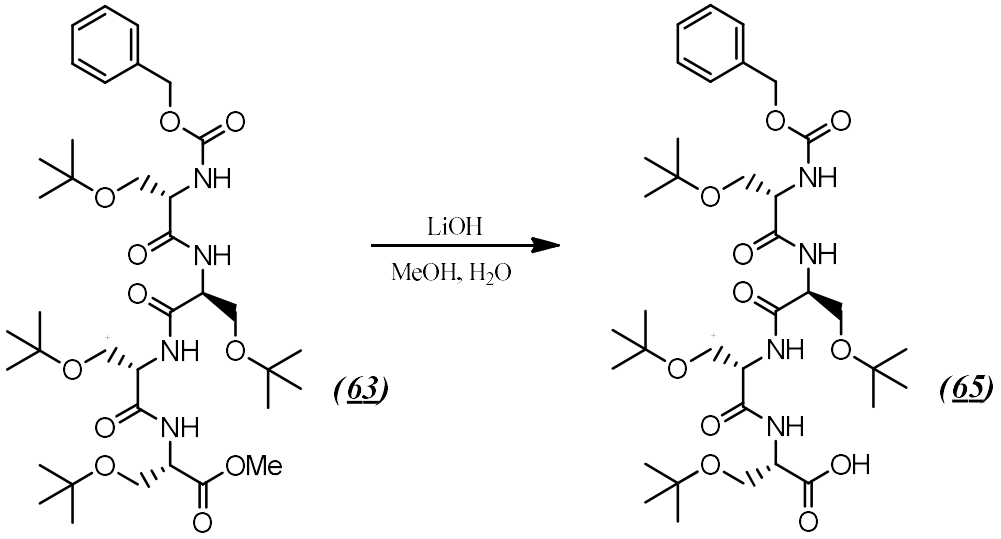
R_f: 0.70 eluted in (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.03 (d, *J* = 8.1 Hz, 1H), 7.84 – 7.79 (m, 2H), 7.39 – 7.29 (m, 6H), 5.05 (s, 2H), 4.49 (dt, *J* = 8.2, 4.2 Hz, 1H), 4.45– 4.42 (m, 1H), 4.38 (dt, *J* = 7.6, 5.9 Hz, 1H), 4.14 (dd, *J* = 14.4, 6.1 Hz, 1H), 3.66 – 3.60 (m, 1H), 3.64 (s, 3H), 3.55 – 3.39 (m, 7H), 1.12 (s, 27H), 1.10 (s, 9H).

^{13}C NMR (400 MHz, $CDCl_3$) δ/ppm: 170.86, 170.18, 169.98, 169.74, 156.39, 137.45, 128.79, 128.22, 128.10, 73.47, 65.94, 62.21, 62.02, 55.76, 53.47, 53.28, 53.09, 52.31, 27.57.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 761.43, found: 761.41.

3.91. Synthesis of *N*-carbobenzoxy-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-serine (*65*)



The product was obtained through General procedure D using compound ***63*** (1.70 g, 2.3 mmol), LiOH×H_2O (0.19 g, 4.6 mmol) and MeOH (30 mL) / H_2O (10 mL).

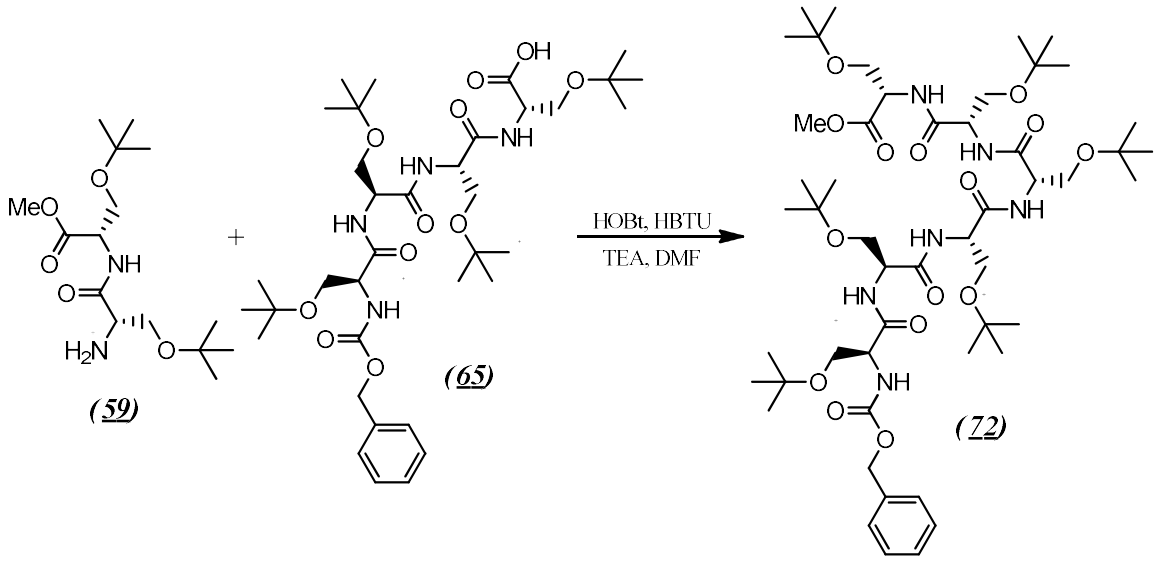
Solution was neutralized using HCl (0.1 M), solvent was removed under reduced pressure; the crude product was dissolved in cold MeOH (10 mL) and LiCl was filtered out through a sintered funnel. Solvent was removed under reduced pressure to give product ***65*** as an orange powder (1.30 g, 1.8 mmol, 78%).

R_f: 0.30 eluted in (20% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 7.98 (d, J = 7.9 Hz, 1H), 7.90 (d, J = 8.2 Hz, 1H), 7.44 – 7.22 (m, 6H), 5.13 (s, 2H), 4.58 (s, 1H), 4.32 (s, 1H), 3.89 – 3.82 (m, 1H), 3.72 – 3.60 (m, 3H), 1.22 (d, J = 3.5 Hz, 9H), 1.18 (d, J = 6.2 Hz, 9H).

ESI-Q-TOF: $[M-H]^-$ Calcd: 437.23, found: 437.63.

3.92. Synthesis of *N*-carbobenzoxy-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-serine methyl ester (*72*)



The product was obtained through General procedure G using compound ***59*** (0.51 g, 1.6 mmol) and compound ***65*** (1.29 g, 1.8 mmol), HBTU (0.92 g, 2.4 mmol), HOBt (3.27 g, 2.4 mmol), TEA (1.13 mL 8.1 mmol) and DMF as solvent (25 mL)

Precipitation was unsuccessful; after the removal of solvent under reduced pressure, the crude solid was dissolved in AcOEt (50 mL) and washed with water (3×50 mL).

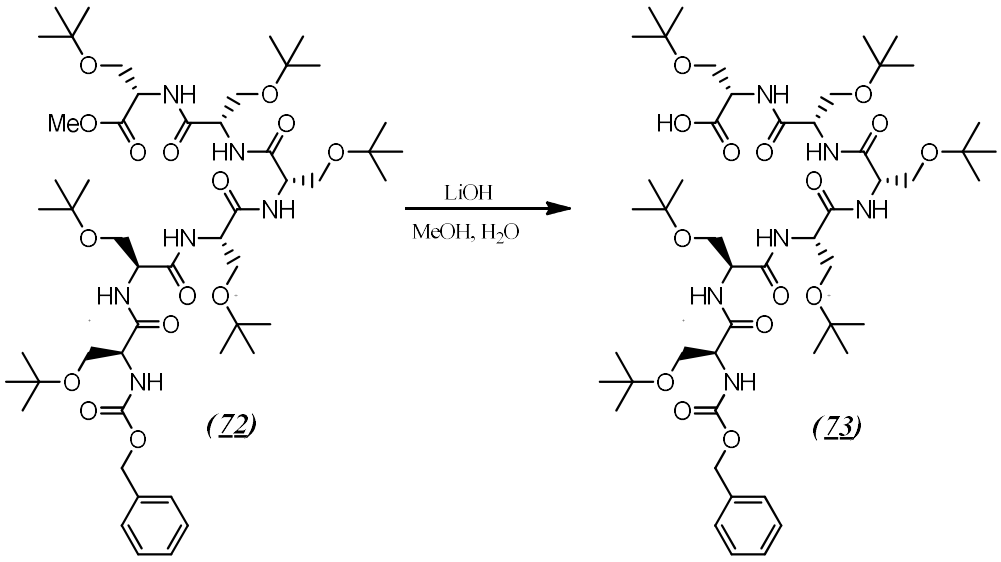
Flash chromatography was performed in isocratic flow of (10% MeOH/DCM) and the product *72* was isolated as an orange powder (0.80 g, 0.8 mmol, 48%).

R_f: 0.70 eluted in (10% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.36 (br s, 6H), 5.05 (s, 2H), 4.51 – 4.55 – 4.31 (br s, 4H), 4.23 – 4.09 (br s, 2H), 3.62 (s, 3H), 3.68 – 3.60 (br s, 1H), 3.56 – 3.37 (br s, 11H), 1.14 – 1.09 (br s, 54H).

ESI-Q-TOF: $[M+Na]^+$ Calcd: 1047.62, found: 1048.34.

3.93. Synthesis of *N*-carbobenzoxy-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-seryl-*O*-*t*-butyl-L-serine (*73*)



The product was obtained through general procedure D using compound ***72*** (0.80 g, 0.8 mmol), LiOH×H_2O (0.07 g, 1.6 mmol) and MeOH (30 mL) / H_2O (10 mL).

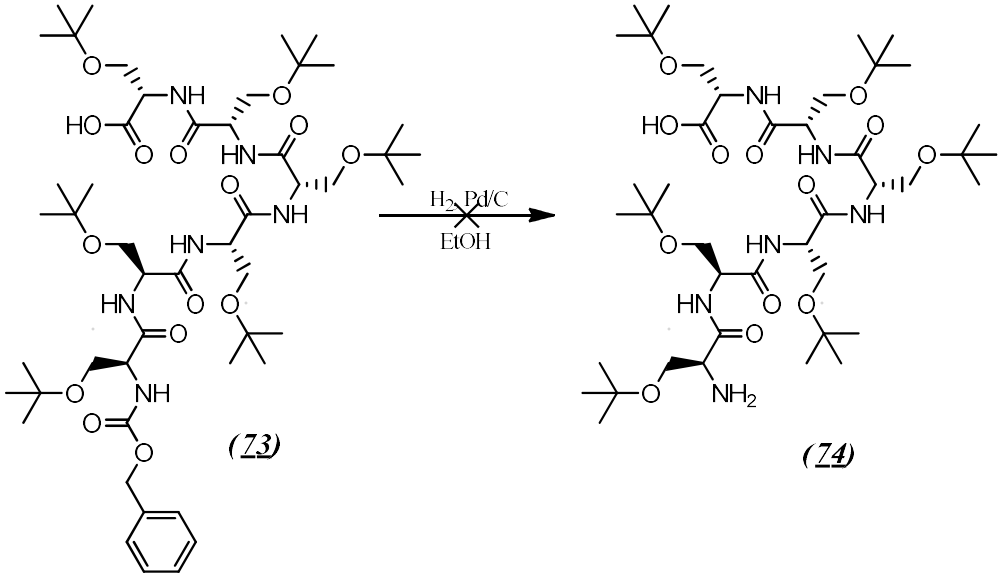
Solution was neutralized using HCl (0.1 M), solvent was removed under reduced pressure; the crude product was dissolved in cold MeOH (10 mL) and LiCl was filtered out through a sintered funnel. Solvent was removed under reduced pressure to obtain product ***73*** as an orange powder (0.75 g, 0.7 mmol, 95%).

R_f: 0.30 (20% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 7.38 – 7.27 (m, 5H), 4.54 – 4.47 (br, 2H), 4.36 – 4.28 (br s, 2H), 4.16 – 4.12 (br s, 1H), 4.04 – 3.98 (br s, 1H), 3.82 – 3.54 (br s, 12H), 1.24 – 1.18 (br s, 54H).

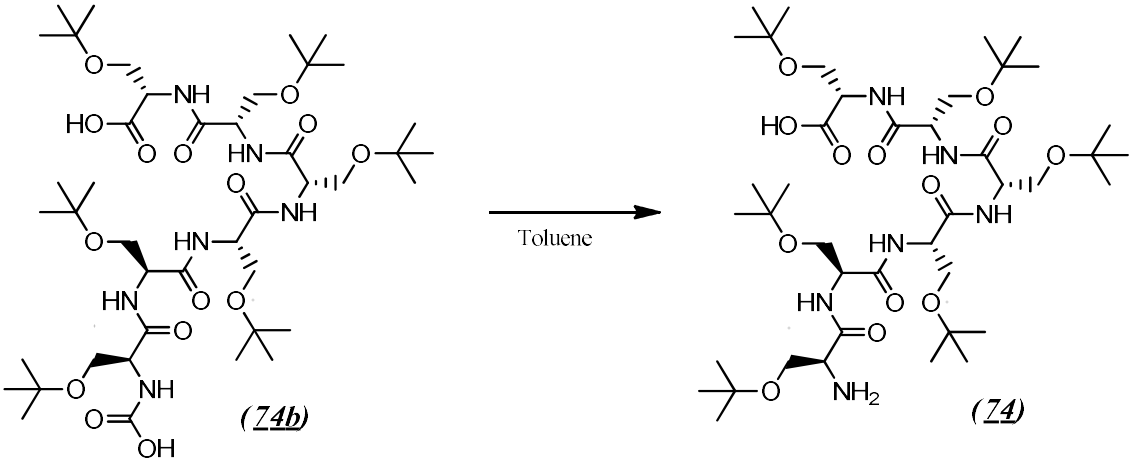
ESI-Q-TOF: $[M+Na]^+$ Calcd: 1033.60, found: 1033.86.

3.94. Synthesis of *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine (*74*)



The reaction was performed through General procedure F using compound *73* (0.75 g, 0.7 mmol), 10wt. % Pd/C (0.08 g) and EtOH as solvent (30 mL). Catalyst was filtered through celite bed and solvent was removed under reduced pressure. In this way was not possible to obtain the desired product *74*.

3.95. Synthesis of *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine (*74*)



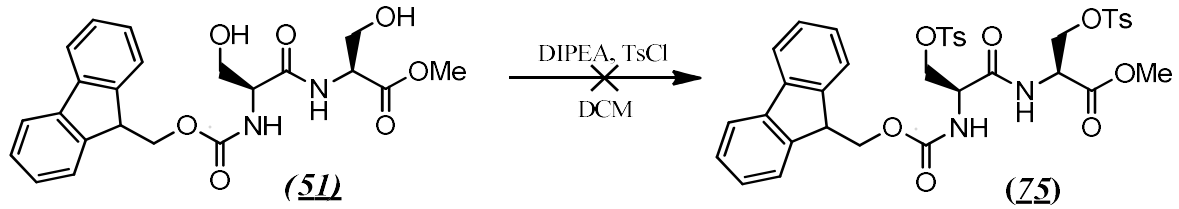
A solution of compound ***74b*** (0.25 g, 0.3 mmol) in toluene (50 mL) was stirred at 120° C overnight. After removal of a solvent under reduced pressure, product ***74*** as obtained as an orange powder (0.21 g, 0.2 mmol, 88%).

R_f: 0.40 (20% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 4.50 – 4.13 (br s, 6H), 3.62 – 3.39 (br s, 12H), 1.11 (s, 654H).

ESI-Q-TOF: $[M+H]^+$ Calcd: 877.59, found: 877.42.

3.96. Synthesis of *N*-(9-Fluorenylmethyloxycarbonyl)-*O*-tosyl-L-seryl-*O*-tosyl-L-serine methyl ester (*75*)

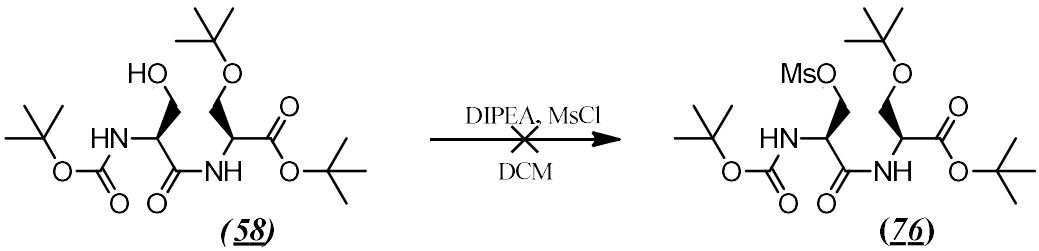


Compound ***51*** (0.05 g, 0.1 mmol) and DIPEA (0.14 mL, 0.8 mmol) were dissolved under inert atmosphere in DCM (2 mL). The mixture was then cooled at 0°C and a solution of TsCl (0.07 g, 0.4 mmol) in DCM (1 mL) was slowly added.

Reaction mixture was stirred at RT for 3 hours and 30 minutes and monitored by TLC.

Solvents were removed under reduced pressure, the crude solid was dissolved in EtOAc and washed with H_2O. Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. In this way was not possible to obtain the desired product ***75***.

3.97. Synthesis of *N*-*t*-butyloxycarbonyl-*O*-*t*-mesyl-L-seryl-*O*-mesyl-L-serine *tert*-butyl ester (76)

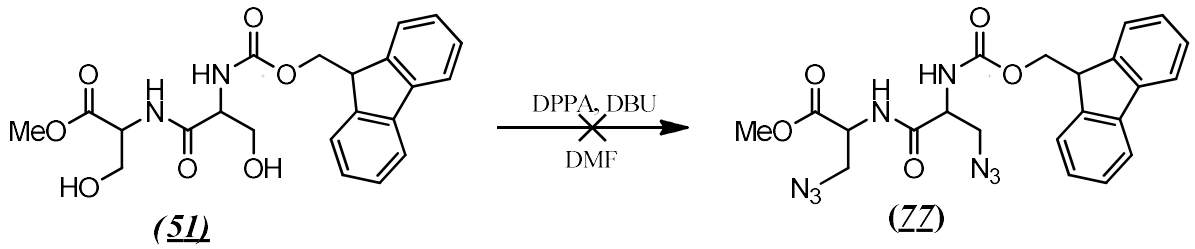


Reaction was performed under inert atmosphere. Compound ***58*** (0.10 g, 0.2 mmol) and DIPEA (0.17 mL, 1.0 mmol) were dissolved in dry DCM (2 mL) and cooled at 0°C under stirring. Was then slowlly added a solution of MsCl (0,057 mL, 0.7 mmol) in DCM (1 mL).

Reaction mixture was stirred at RT for 3 hours and 30 minutes and monitored by TLC. Solvents were removed under reduced pressure, the crude solid was dissolved in EtOAc and washed with H_2O. Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure.

In this way was not possible to obtain the desired product ***76***.

3.98. Synthesis of *N*-alpha-(9-Fluorenylmethyloxycarbonyl)-*N*-beta-azido-L-seryl-*N*-beta-azido-L-serine methyl ester (*77*)



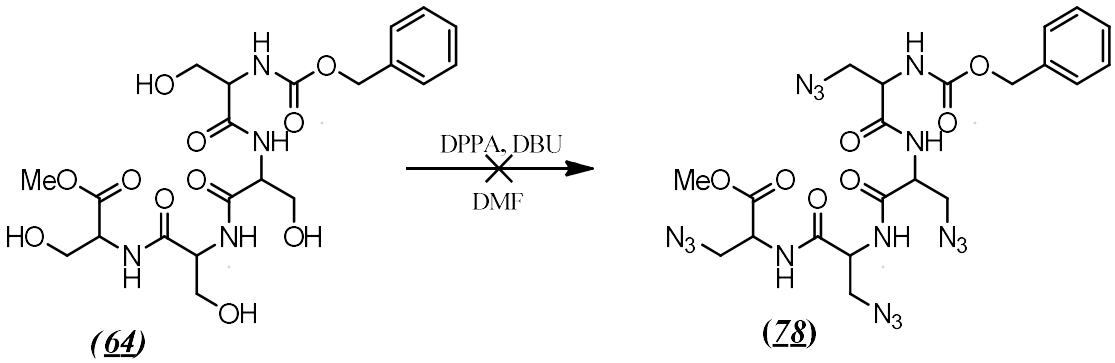
(51) ***(77)***

Under inert atmosphere compound ***51*** (0.05 g, 0.1 mmol) was dissolved in dry DMF (0.1 mL) and cooled at 0°C under stirring. Was then slowly added DBU (0.02 mL, 0.2 mmol), DPPA (0.03 mL, 0.2 mmol) and NaN_3 (0.01 g, 0.2 mmol).

Reaction mixture was stirred at RT for 8 hours and monitored by TLC.

EtOAc was added to the solution (3 mL) and the mixture was washed with H_2O (3×3 mL). Organic layer was separated, dried over Na_2SO_4 and evaporated under reduced pressure. In this way was not possible to synthesize the desired product *77*.

3.99. Synthesis of *N*-alpha-carbobenzoxy-*N*-beta-azido-L-seryl-*N*-beta-azido-L-seryl-*N*-beta-azido-L-seryl-*N*-beta-azido-L-serine methyl ester (*78*)

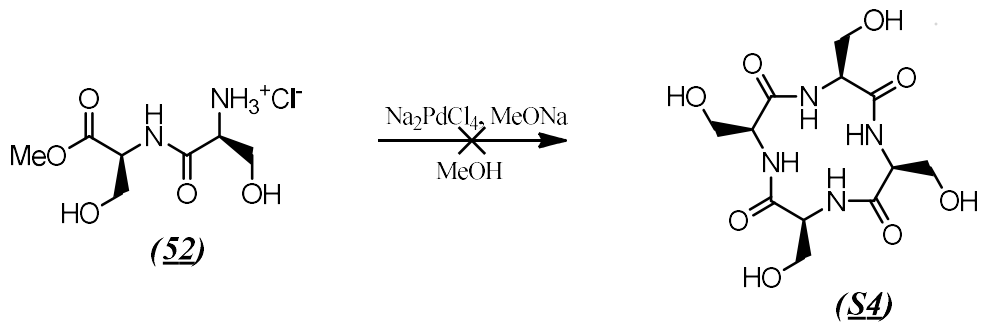


(*64*) (*78*)

Under inert atmosphere compound ***64*** (0.05 g, 0.1 mmol) was dissolved in dry DMF (0.1 mL) and cooled at 0°C under stirring. Was then slowly added DBU (0.02 mL, 0.1 mmol), DPPA (0.03 mL, 0.1 mmol) and NaN_3 (0.01 g, 0.1 mmol). Reaction mixture was stirred at RT for 8 hours and monitored by TLC. EtOAc was added to the solution (3 mL) and the mixture was washed with H_2O (3×3 mL). Organic layer was separated, dried over Na_2SO_4 and evaporated under reduced pressure.

In this way was not possible to obtain the desired product ***78***.

3.100. Synthesis of cyclo L-seryl-L-seryl-L-seryl-L-serine (*S4*)



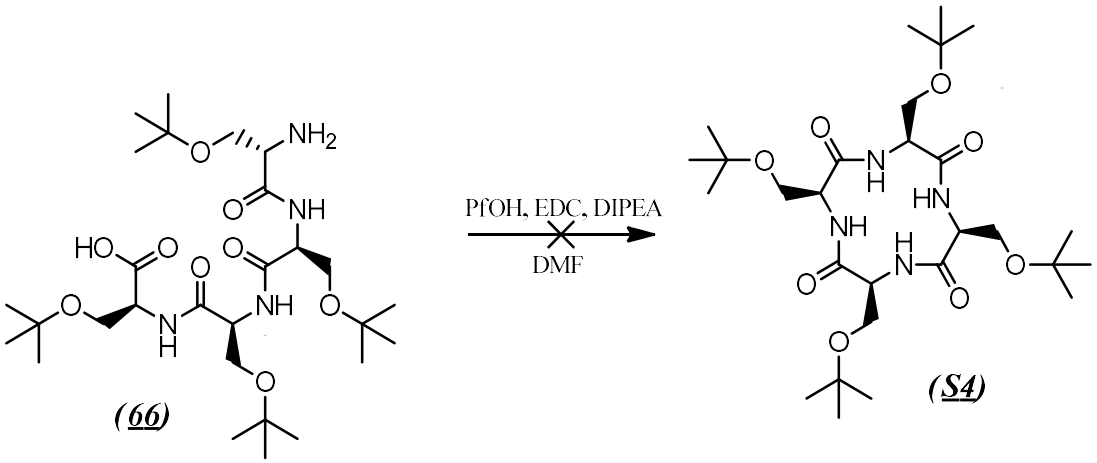
Compound ***52*** was dissolved in a solution of HCl (0.5 M, 2 mL) and MeOH (2 mL) to obtain hydrochloride salt of product ***52***.

Under inert atmosphere product ***52*** (0.05 g, 0.2 mmol) and Na_2PdCl_4 (0.12 g, 0.4 mmol) were dissolved in MeOH (3 mL) under stirring. Was then slowly added MeONa (0.14 mL, 1.2 mmol from a solution 25 wt. % in methanol).

Reaction mixture was stirred at RT under inert atmosphere overnight and monitored by TLC.

In this way was not possible to obtain cyclic product ***S4***.

3.101. Synthesis of cyclo *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine (*S4*)

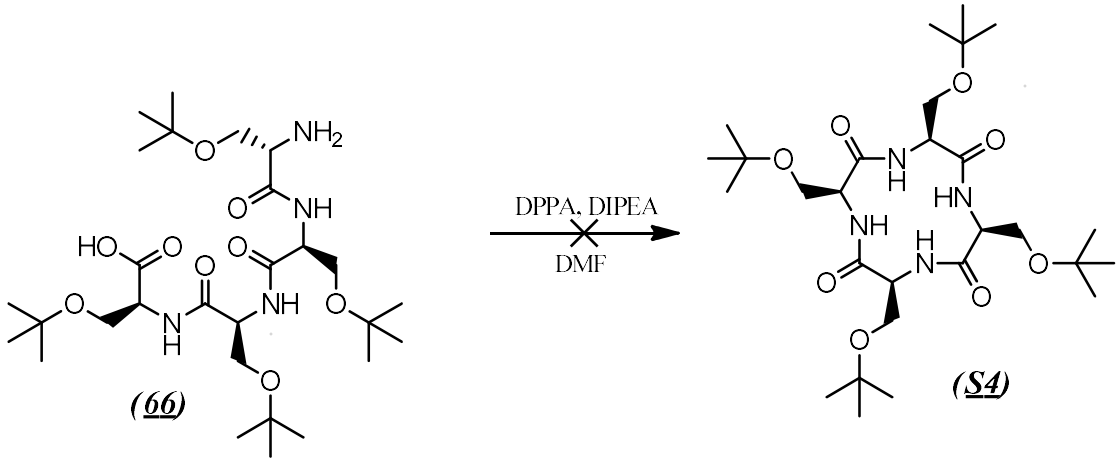


Under inert atmosphere product ***66*** (0.05 g, 0.1 mmol) and DIPEA (0.74 mL, 4.2 mmol) were dissolved in dry DMF (3 mL) under stirring. Was then slowly added to a solution of PfOH (0.41 g, 2.1 mmol) and EDC×HCl (0.39 g, 2.1 mmol) in dry DMF (7 mL) in 3 hours.

Reaction mixture was stirred at RT under inert atmosphere for 4 days and monitored by TLC.

In this way was not possible to obtain cyclic product ***S4***.

3.102. Synthesis of cyclo *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine (*S4*)

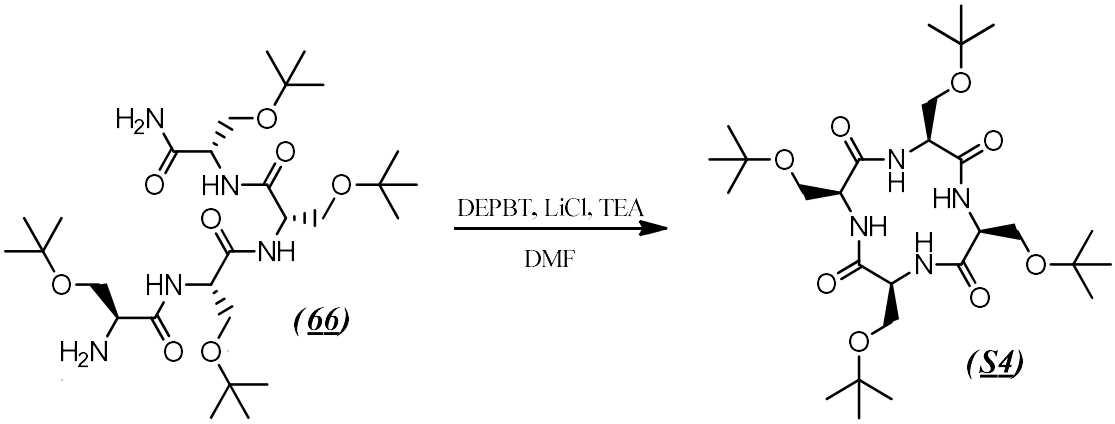


Under inert atmosphere product ***66*** (0.05 g, 0.1 mmol) was dissolved in dry DMF under stirring. Was then slowly added to a solution of DPPA (0.03 mL, 0.1 mmol) and DIPEA (0.03 mL, 0.2 mmol) in dry DMF in 3 hours.

Reaction mixture was stirred at RT under inert atmosphere for 4 days and monitored by TLC.

In this way was not possible to obtain cyclic product ***S4***.

3.103. Synthesis of cyclo *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine (*S4*)



The product was obtained following general procedure J starting from compound ***66*** (0.05 g, 0.1 mmol), DEPBT (0.03 g, 0.1 mmol), LiCl (0.02 g, 0.4 mmol) dissolved in 0.125 mL of H_2O, TEA until pH 8 and DMF (50 mL).

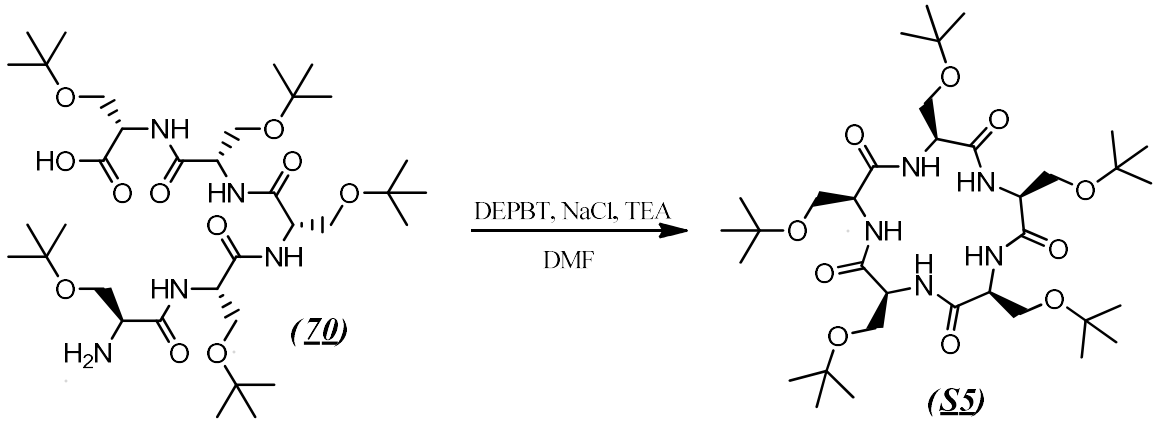
Solvents were removed under reduced pressure; the obtained crude solid was dissolved in EtOAc (5 mL) and washed H_2O (5×5 mL). Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. Flash chromatography was performed in gradient flow of MeOH/DCM (from 1% to 10%) and the product ***S4*** was isolated as white powder (~0.01 g, ~10%).

R_f: 0.30 (10% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 7.41 – 7.33 (br s, 2H), 4.62 – 4.37 (br s, 4H), 3.94 – 3.70 (br s, 8H), 1.33 – 1.18 (br s, 36H).

ESI-Q-TOF: $[M+Na]^+$ Calcd: 595.37, found: 595.42.

3.104. Synthesis of cyclo *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine (*S5*)



The product was obtained through general procedure J using compound ***70*** (0.17 g, 0.1 mmol), DEPBT (0.07 g, 0.3 mmol), NaCl (0.07 g, 1.0 mmol) dissolved in 0.125 mL of H_2O, TEA until pH 8 and DMF (170 mL). Solvents were removed under reduced pressure; the crude solid obtained was dissolved in DCM (10 mL) and washed H_2O (5×10 mL). Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. Flash chromatography was performed in gradient flow from 1% to 10% MeOH/DCM and the product ***S5*** was isolated as white powder (0.04 g, 0.05 mmol, 21%).

R_f: 0.50 (10% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 8.63 (d, *J* = 5.4 Hz, 1H), 8.22 (d, *J* = 8.2 Hz, 1H), 8.16 (d, *J* = 6.5 Hz, 1H), 8.08 (d, *J* = 9.0 Hz, 1H), 7.46 (d, *J* = 8.4 Hz, 1H), 4.69 – 4.62 (m, 1H), 4.51 (dt, *J* = 7.6, 3.6 Hz, 1H), 4.41 (dd, *J* = 12.4, 6.5 Hz, 2H), 4.20 (dt, *J* = 6.8, 3.6 Hz, 1H), 3.91 (dd, *J* = 9.3, 4.1 Hz, 1H), 3.80 (dt, *J* = 10.2, 3.0 Hz, 2H), 3.75 – 3.65 (m, 3H), 3.63 – 3.54 (m, 4H), 1.35 (s, 9H), 1.24 (s, 9H), 1.23 (s, 9H), 1.21 (s, 9H), 1.18 (s, 9H).

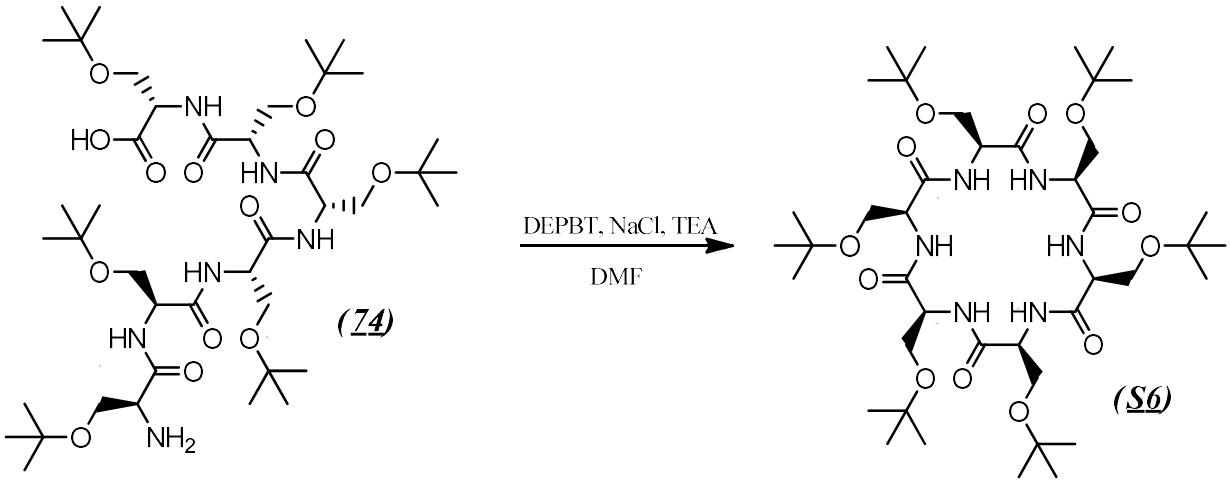
^{13}C NMR (400 MHz, Methanol-d_4) δ/ppm: 171.81, 171.66, 171.50, 171.02, 170.74, 74.12, 73.39, 73.11, 73.03, 72.87, 61.73, 60.72, 60.24, 59.68, 56.39, 55.28, 54.36, 53.12, 52.40, 26.13.

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 4.57 (br s, 5H), 3.74 (br s, 10H), 1.22 (br s, 45H).

^{13}C NMR (400 MHz, $CDCl_3$) δ/ppm: 171.77, 74.05, 60.64, 54.88, 27.44.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 738.46, found: 738.54

3.105. Synthesis of cyclo *O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-seryl-*O-t*-butyl-L-serine (***S6***)



The product was obtained following General procedure J starting from compound ***74*** (0.20 g, 0.2 mmol), DEPBT (0.08 g, 0.3 mmol), NaCl (0.07 g, 1.1 mmol) dissolved in 0.5 mL of H_2O, TEA until pH 8 and DMF (200 mL).

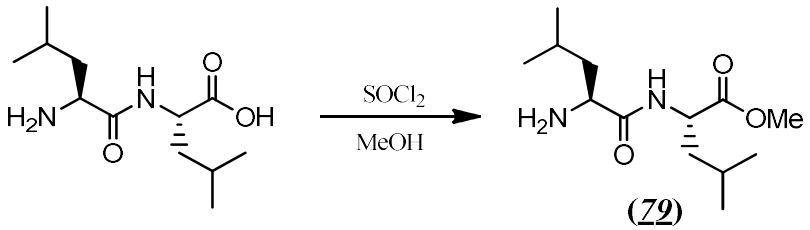
Solvents were removed under reduced pressure; the obtained crude solid was dissolved in DCM (10 mL) and washed H_2O (5×10 mL). Organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. Flash chromatography was performed in gradient flow from 1% to 10% MeOH/DCM and the product ***S6*** was isolated as white powder (0.04 g, 0.04 mmol, 15%).

R_f: 0.50 (10% MeOH/DCM).

^{1}H NMR (400 MHz, Methanol-d_4) δ/ppm: 4.59 – 4.41 (br s, 6H), 3.85 – 3.61 (br s, 12H), 1.26 – 1.17 (br s, 54H).

ESI-Q-TOF: $[M+Na]^+$ Calcd: 881.56, found: 881.81.

3.106. Synthesis of L-leucyl- L-leucine-methyl ester (*79*)



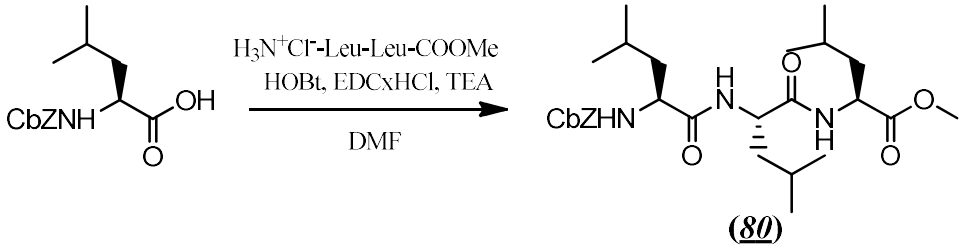
(*79*)

MeOH (200 mL) was cooled at 0 °C and $SOCl_2$ (3.9 mL, 53.4 mmol) was added dropwise. Resulting mixture was stirred at 0 °C for 30 minutes. L-Lysine (7.00 g, 26.7 mmol) was then added and mixture was stirred at RT overnight and enother 3 hours on reflux. Solvent was removed under the reduced pressure and the resulting crude was dissolved in EtOAc (150 mL) and washed with saturated solution of $NaHCO_3$ (100 mL) and H_2O (100 mL). Organic layer was dried over $MgSO_4$ and solvent was removed under the reduced pressure to give ***79*** (5.80 g, 22.5 mmol, 84 %) as a white solid.

R_f: 0.38 (10%MeOH/DCM).

^{1}H NMR (400 MHz, DMSO- d_6) δ/ppm: 8.12 (d, *J* = 7.9 Hz, 1H), 4.38 – 4.25 (m, 1H), 3.62 (s, 3H), 3.18 (dd, *J* = 8.8, 5.5 Hz, 1H), 1.79 – 1.45 (m, 4H), 1.43 – 1.36 (m, 1H), 1.26 – 1.15 (m, 1H), 0.89 (dd, *J* = 6.5, 3.6 Hz, 6H), 0.85 (d, *J* = 6.5 Hz, 6H).

3.107. Synthesis of *N*-carbobenzoxy-L-leucyl-L-leucyl-L-leucine methyl ester (***80***)



(***80***)

The product was obtained according to General procedure G starting from CbZ-Leu-COOH (5.87 g, 11.4 mmol) and H_2N-Leu-Leu-COOMe (2.95 g, 10.3 mmol), EDC×HCl (3.28 g, 17.1 mmol) and HOBt (2.31 g, 17.1 mmol), TEA (5.2 mL 57.1 mmol) and DMF as solvent (30 mL).

After precipitation product ***80*** was isolated as white powder (4.05 g, 8.0 mmol, 78 %).

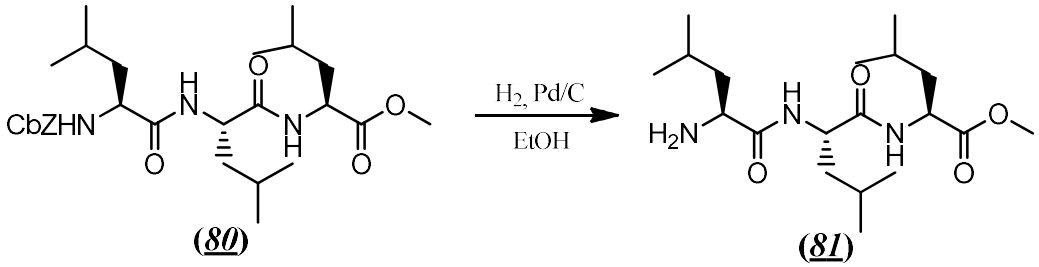
R_f: 0.99 (5% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO- d_6) δ/ppm: 8.19 (d, J = 7.6 Hz, 1H), 7.89 (d, J = 8.4 Hz, 1H), 7.41 (d, J = 8.3 Hz, 1H), 7.37 – 7.29 (m, 5H), 5.03 (s, 2H), 4.40 – 4.24 (m, 2H), 4.10 – 3.99 (m, 1H), 3.61 (s, 3H), 1.74 – 1.33 (m, 9H), 0.99 – 0.75 (m, 18H).

^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 172.38, 171.65, 155.50, 136.73, 127.95, 127.30, 126.56, 64.94, 52.74, 51.41, 50.20, 49.76, 40.49, 40.38, 24.12, 23.49, 23.04, 22.24, 21.47, 21.16, 20.83.

ESI-Q-TOF: $[M+Na]^{+-}$ Calcd: 528.62, found: 528.66.

3.108. Synthesis of L-leucyl- L-leucyl- L-leucine methyl ester (*81*)



Tripeptide ***80*** (7.30 g, 16.1 mmol) was dissolved in EtOH (200 mL) and 10wt. % Pd/C (0.73 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***81*** (4.77 g, 15.0 mmol, 93 %) was obtained as a white powder and used in the next step without further purification.

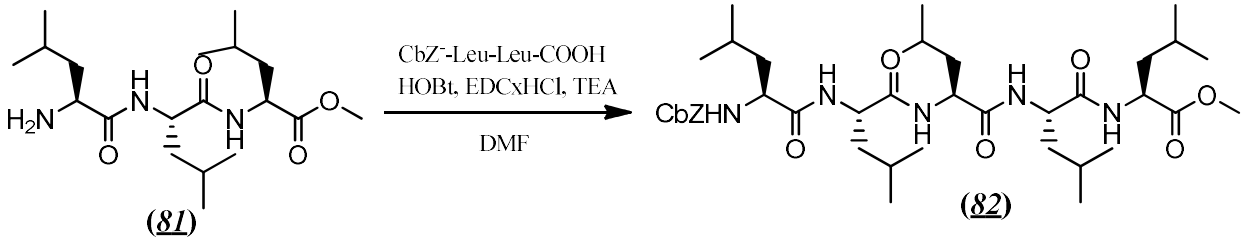
R_f: 0.43 (5% MeOH/DCM).

^{1}H NMR (400 MHz, MeOD- d_4) δ/ppm: 4.51 – 4.46 (m, 2H), 3.72 (s, 3H), 3.39 (dd, J = 8.0, 6.3 Hz, 1H), 1.81 – 1.46 (m, 8H), 1.38 – 1.36 (m, 1H), 1.06 – 0.88 (m, 18H).

^{13}C NMR (400 MHz, MeOD-d_4) δ/ppm: 176.43, 173.28, 173.03, 53.03, 51.24, 50.60, 44.26, 40.70, 39.99, 24.41, 22.01, 21.96, 21.90, 21.16, 20.90, 20.36.

ESI-Q-TOF: $[M+H]^{+-}$ Calcd: 372.18, found: 372.35.

3.109. Synthesis of *N*-carbobenzoxy-L-leucyl- L-leucyl- L-leucyl- L-leucyl-L-leucine methyl ester (*82*)



(*81*) (*82*)

The product was obtained according to General procedure G starting from ***81*** (4.83 g, 12.7 mmol) and CbZ-Leu-Leu-COOH (4.70 g, 11.6 mmol), EDC×HCl (3.33 g, 17.4 mmol) and HOBt (2.35 g, 17.4 mmol), TEA (8.1 mL 57.9 mmol) and DMF as solvent (30 mL).

After precipitation product ***82*** was isolated as white powder (5.30 g, 7.4 mmol, 64 %).

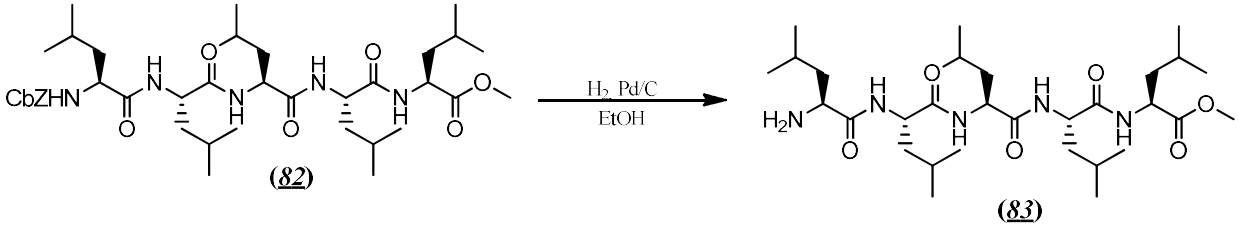
R_f: 0.98 (5% MeOH/DCM).

^{1}H NMR (400 MHz, DMSO- d_6) δ/ppm: 8.14 (d, *J* = 7.6 Hz, 1H), 7.95 (d, *J* = 7.9 Hz, 1H), 7.88 (d, *J* = 8.2 Hz, 1H), 7.81 (d, *J* = 8.3 Hz, 1H), 7.43 (d, *J* = 8.1 Hz, 1H), 7.39 – 7.28 (m, 5H), 5.03 (s, 2H), 4.40 – 4.22 (m, 4H), 4.09 – 3.99 (m, 1H), 3.60 (s, 3H), 1.61 – 1.39 (m, 15H), 0.94 – 0.75 (m, 30H).

^{13}C NMR (400 MHz, DMSO) δ/ppm: 172.71, 172.16, 171.88, 171.60, 171.32, 155.90, 137.06, 65.30, 64.90, 53.13, 51.77, 50.82, 50.46, 50.33, 50.07, 40.96, 40.86, 40.86, 40.55, 40.25, 39.33, 39.16, 38.97, 24.14, 24.10, 24.05, 24.01, 23.97, 22.99, 22.97, 22.78, 21.71, 21.68 , 21.50, 21.16, 15.16.

ESI-Q-TOF: $[M+Na]^{+-}$ Calcd: 754.89, found: 754.10.

3.110. Synthesis of L-leucyl- L-leucyl- L-leucyl- L-leucyl- L-leucyl- L-leucine methyl ester (*83*)



(*82*) (*83*)

Hexapeptide ***82*** (5.30 g, 7.4 mmol) was dissolved in EtOH (100 mL) and 10wt. % Pd/C (0.53 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***82*** (4.26 g, 7.3 mmol, 98 %) was obtained as a white powder and used in the next step without further purification.

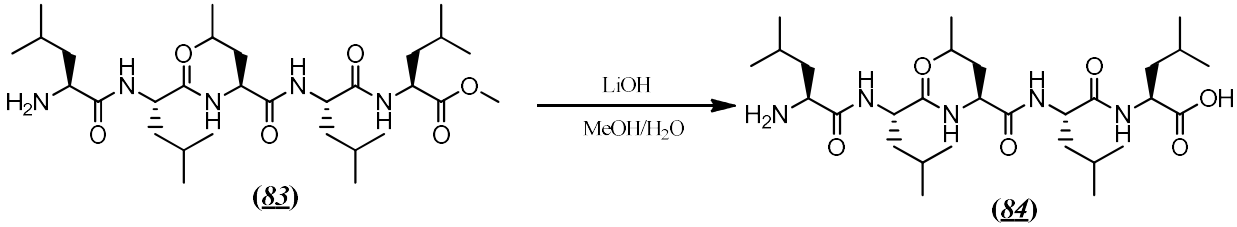
R_f: 0.41 (5% MeOH/DCM).

^{1}H NMR (400 MHz, MeOD- d_4) δ/ppm: 4.50 – 4.40 (m, 4H), 3.72 (s, 3H), 3.39 (dd, *J* = 8.2, 6.1 Hz, 1H), 1.78 – 1.51 (m, 15H), 0.96 – 0.89 (m, 30H).

^{13}C NMR (400 MHz, MeOD) δ/ppm: 178.24, 174.70, 174.67, 174.52, 174.35, 54.62, 53.15, 53.10, 52.90, 52.73, 52.13, 45.77, 42.04, 41.93, 41.87, 41.82, 41.48, 32.85, 25.91, 23.80, 23.66, 23.39, 22.62, 22.43, 22.07, 21.88, 14.53.

ESI-Q-TOF: $[M-H]^-$ Calcd: 597.45, found: 597.25.

3.111. Synthesis of L-leucyl- L-leucyl- L-leucyl- L-leucyl- L-leucyl- L-leucine (*84*)



(*83*) (*84*)

The product was obtained through General procedure D using compound ***83*** (4.26 g, 7.3 mmol), LiOH×H_2O (0.63 g, 14.6 mmol) and MeOH (39 mL) / H_2O (13 mL).

Solution was neutralized using HCl (0.1 M), solvent was removed under reduced pressure; the crude product was dissolved in cold MeOH (10 mL) and LiCl was filtered out through a sintered funnel. Solvent was removed under reduced pressure to give product ***84*** as an white powder (3.99 g, 7.1 mmol, 96%).

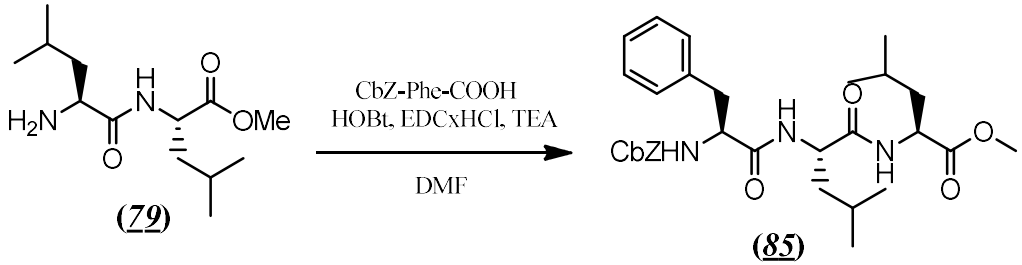
R_f: 0.26 (5% MeOH/DCM).

^{1}H NMR (400 MHz, MeOD- d_4) δ/ppm: 4.52 – 4.45 (m, 4H), 3.91 (t, J = 6.9 Hz, 1H), 1.79 – 1.54 (m, 15H), 1.06 – 0.87 (m, 30H).

^{13}C NMR (400 MHz, MeOD) δ/ppm: 175.81, 174.50, 174.24, 173.95, 170.59, 53.27, 52.72, 52.02, 42.05, 41.90, 41.75, 26.01, 25.92, 25.81, 25.42, 23.56, 23.44, 23.27, 22.33, 22.25, 22.21, 22.15, 21.93.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 584.51, found: 584.76.

3.112. Synthesis of *N*-carbobenzoxy-L-phenylalanyl-L-leucyl-L-leucine methyl ester (*85*)



The product was obtained according to General procedure G starting from ***79*** (2.95 g, 10.3 mmol) and CbZ-Phe-COOH (3.42 g, 11.4 mmol), EDC×HCl (3.28 g, 17.1 mmol) and HOBt (2.31 g, 17.1 mmol), TEA (5.23 mL 57.1 mmol) and DMF as solvent (40 mL).

After precipitation product ***85*** was isolated as white powder (4.96 g, 9.2 mmol, 89 %).

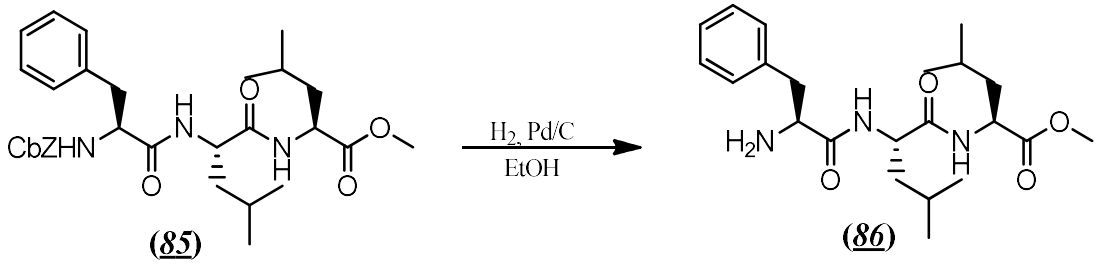
R_f: 0.98 (5% MeOH/DCM).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm:7.40 – 7.13 (m, 11H), 6.52 (d, *J* = 7.5 Hz, 1H), 6.39 (d, *J* = 7.2 Hz, 1H), 5.09 (s, 2H), 4.59 (td, *J* = 8.6, 5.2 Hz, 1H), 4.45 (td, *J* = 8.5, 5.9 Hz, 2H), 3.75 (s, 3H), 3.18 – 3.03 (m, 2H), 1.73 – 1.42 (m, 6H), 1.00 – 0.85 (m, 12H).

^{13}C NMR (101 MHz, $CDCl_3$) δ/ppm: 173.04, 171.16, 170.81, 135.99, 129.29, 128.82, 128.61, 128.35, 128.13, 127.22, 67.28, 56.12, 52.32, 51.76, 50.81, 41.33, 40.83, 37.91, 24.84, 24.57, 22.83, 21.98, 21.91.

ESI-Q-TOF: $[M+Na]^{+-}$ Calcd: 562.53, found: 562.86.

3.113. Synthesis of -L-phenylalanyl- L-leucyl- L-leucine methyl ester (*86*)



Tripeptide ***85*** (2.89 g, 5.4 mmol) was dissolved in EtOH (200 mL) and 10wt. % Pd/C (0.29 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***86*** (2.00 g, 4.9 mmol, 92 %) was obtained as a white powder and used in the next step without further purification.

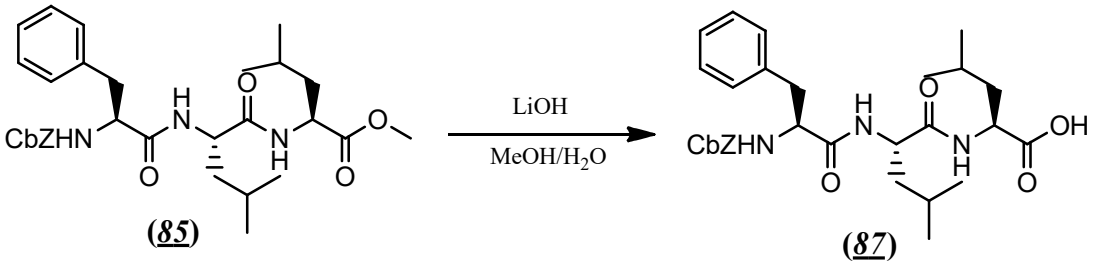
R_f: 0.42 (5% MeOH/DCM).

^{1}H NMR (400 MHz, MeOD- d_4) δ/ppm: 7.33 – 7.20 (m, 5H), 4.50 – 4.46 (m, 2H), 3.72 (s, 3H), 3.67 – 3.60 (m, 1H), 3.06 (dd, *J* = 13.6, 5.1 Hz, 1H), 2.81 (dd, *J* = 13.6, 7.7 Hz, 1H), 1.82 – 1.48 (m, 6H), 1.06 – 0.86 (m, 12H).

^{13}C NMR (400 MHz, MeOD) δ/ppm: 176.46, 174.78, 174.52, 174.25, 138.73, 130.62, 129.62, 127.84, 57.29, 52.74, 52.72, 52.17, 42.40, 42.13, 41.82, 40.82, 25.96, 25.96, 25.77, 23.48, 23.42, 22.36, 21.98.

ESI-Q-TOF: $[M-H]^-$ Calcd: 404.52, found: 404.78.

3.114. Synthesis of *N*-carbobenzoxy-L-phenylalanyl- L-leucyl- L-leucine (*87*)



(*85*) (*87*)

The product was obtained through General procedure D using compound ***85*** (2.89 g, 5.4 mmol), LiOH×H_2O (0.45 g, 10.7 mmol) and MeOH (27 mL) / H_2O (9 mL).

Solution was neutralized using HCl (0.1 M), solvent was removed under reduced pressure; the crude product was dissolved in cold MeOH (10 mL) and LiCl was filtered out through a sintered funnel. Solvent was removed under reduced pressure to give product ***87*** as an white powder (2.75 g, 5.2 mmol, 97%).

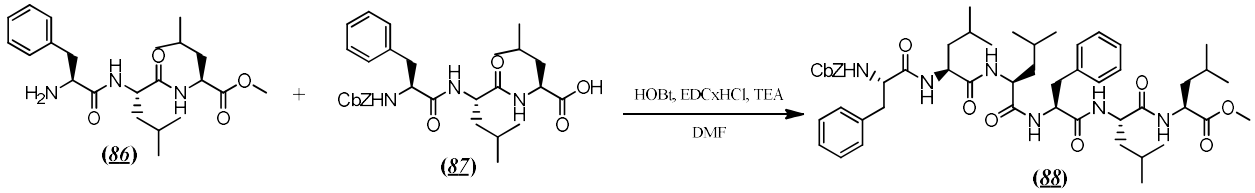
R_f: 0.28 (5% MeOH/DCM).

^{1}H NMR (400 MHz, MeOD- d_4) δ/ppm: 8.23 (d, *J* = 7.7 Hz, 1H), 8.12 (d, *J* = 7.7 Hz, 1H), 7.38 – 7.15 (m, 10H), 7.17 (d, *J* = 8.4 Hz, 1H), 5.03 (s, 2H), 4.53 – 4.39 (m, 3H), 3.14 (dd, *J* = 14.0, 9.5 Hz, 1H), 2.86 (dd, *J* = 14.0, 9.7 Hz, 1H), 1.81 – 1.55 (m, 6H), 1.02 – 0.88 (m, 12H).

^{13}C NMR (400 MHz, $CDCl_3$) δ/ppm: 207.36, 175.61, 172.14, 171.46, 156.17, 135.98, 129.30, 128.67, 128.57, 128.28, 128.04, 127.10, 67.24, 60.44, 56.00, 51.88, 51.15, 40.80, 38.16, 24.87, 24.56, 22.81, 22.74, 22.05, 21.86, 20.64, 14.20.

ESI-Q-TOF: $[M+Na]^+$ Calcd: 548.63, found: 548.74.

3.115. Synthesis of *N*-carbobenzoxy-L-phenylalanyl- L-leucyl- L-leucyl-L-phenylalanyl- L-leucyl- L-leucine methyl ester (***88***)



(***86***) (***87***) (***88***)

The product was obtained according to General procedure G starting from ***86*** (2.00 g, 4.9 mmol) and ***87*** (2.86 g, 5.4 mmol), EDC×HCl (1.42 g, 7.4 mmol) and HOBt (1.00 g, 7.4 mmol), TEA (3.45 mL 24.7 mmol) and DMF as solvent (40 mL).

After precipitation product ***88*** was isolated as white powder (3.60 g, 3.9 mmol, 79 %).

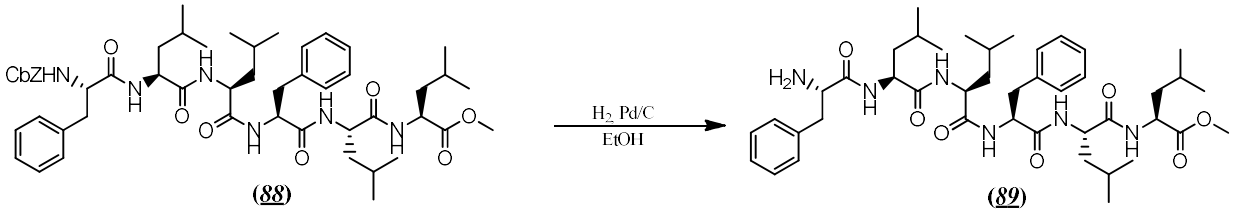
R_f: 0.89 (5% MeOH/DCM).

^{1}H NMR (400 MHz, MeOD- d_4) δ/ppm: 7.38 – 7.19 (m, 15H), 5.07 (dd, *J* = 29.4, 12.6 Hz, 2H), 4.61 (dd, *J* = 9.7, 4.6 Hz, 1H), 4.46 (dd, *J* = 9.8, 5.1 Hz, 2H), 4.40 (dd, *J* = 8.7, 5.2 Hz, 1H), 4.25 (ddd, *J* = 15.0, 9.6, 5.1 Hz, 2H), 3.71 (s, 3H), 3.23 (dd, *J* = 14.2, 4.6 Hz, 1H), 3.13 (dd, *J* = 14.0, 5.1 Hz, 1H), 2.97 (dt, *J* = 14.0, 8.2 Hz, 2H), 1.82 – 1.39 (m, 12H), 1.01 – 0.88 (m, 24H).

^{13}C NMR (400 MHz, MeOD) δ/ppm: 175.39, 174.98, 174.71, 173.37, 138.62, 138.26, 130.45, 130.40, 129.62, 129.57, 129.15, 128.77, 127.99, 127.84, 67.87, 58.60, 56.27, 54.34, 54.29, 54.19, 53.23, 52.73, 52.28, 46.19, 45.74, 41.97, 41.39, 41.34, 38.69, 38.26, 30.77, 25.88, 25.87, 25.84, 25.78, 23.75, 23.56, 23.50, 22.22, 22.06, 21.99, 21.90.

ESI-Q-TOF: $[M+Na]^{+-}$ Calcd: 936.24, found: 936.35.

3.116. Synthesis of L-phenylalanyl- L-leucyl- L-leucyl-L-phenylalanyl- L-leucyl- L-leucine methyl ester (*89*)



(*88*) (*89*)

Peptide ***88*** (3.60 g, 3.9 mmol) was dissolved in EtOH (200 mL) and 10wt. % Pd/C (0.36 g) was added. In a way described in General procedure for a cleavage of CbZ protecting group product ***89*** (2.85 g, 3.7 mmol, 94 %) was obtained as a white powder and used in the next step without further purification.

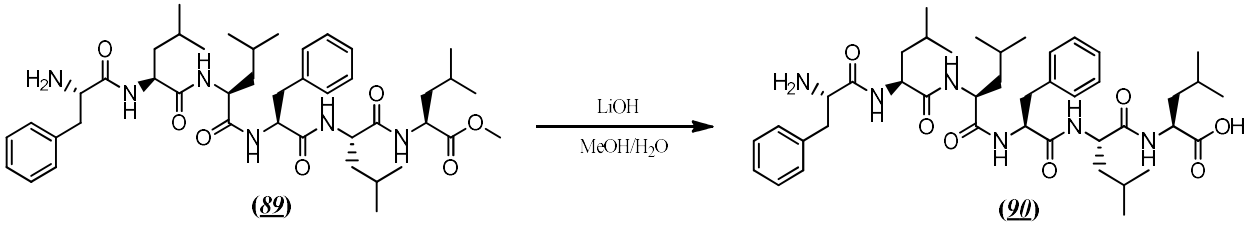
R_f: 0.35 (5% MeOH/DCM).

^{1}H NMR (400 MHz, MeOD- d_4) δ/ppm: 7.39 – 7.15 (m, 10H), 4.63 (dd, *J* = 8.9, 5.0 Hz, 1H), 4.47 (t, *J* = 5.0 Hz, 2H), 4.31 (dt, *J* = 15.0, 7.3 Hz, 2H), 3.72 (s, 3H), 3.64 (dd, *J* = 7.2, 5.2 Hz, 1H), 3.21 (dd, *J* = 14.3, 5.1 Hz, 1H), 3.05 – 3.00 (m, 2H), 2.84 (dd, *J* = 13.5, 7.7 Hz, 1H), 1.81 – 1.44 (m, 12H), 1.04 – 0.84 (m, 24H).

^{13}C NMR (400 MHz, MeOD) δ/ppm: 176.88, 176.72, 175.09, 174.70, 174.67, 174.65, 174.47, 173.24, 173.09, 138.62, 138.52, 130.64, 130.39, 129.72, 129.58, 127.97, 127.87, 61.64, 57.39, 55.95, 53.84, 53.65, 53.11, 52.74, 52.53, 52.26, 42.07, 41.98, 41.93, 41.67, 41.42, 38.35, 25.93, 25.91, 25.88, 25.79, 23.69, 23.57, 23.52, 23.45, 22.24, 22.20, 22.16, 22.13, 21.95, 20.96, 14.57.

ESI-Q-TOF: $[M-H]^-$ Calcd: 778.20, found: 778.35.

3.117. Synthesis of L-phenylalanyl- L-leucyl- L-leucyl-L-phenylalanyl- L-leucyl- L-leucine (*90*)



(*89*) (*90*)

The product was obtained through General procedure D using compound ***89*** *(*2.85 g, 3.7 mmol), LiOH×H_2O (0.31 g, 7.4 mmol) and MeOH (18 mL) / H_2O (6 mL).

Solution was neutralized using HCl (0.1 M), solvent was removed under reduced pressure; the crude product was dissolved in cold MeOH (10 mL) and LiCl was filtered out through a sintered funnel. Solvent was removed under reduced pressure to give product ***90*** as an white powder (2.60 g, 3.4 mmol, 92%).

R_f: 0.24 (5% MeOH/DCM).

^{1}H NMR (400 MHz, MeOD- *d*$_4$) δ/ppm: 7.40 – 7.09 (m, 10H), 4.67 (dd, *J* = 9.9, 4.6 Hz, 1H), 4.46 (dd, *J* = 9.3, 5.4 Hz, 1H), 4.34 – 4.23 (m, 3H), 3.67 (dd, *J* = 7.6, 4.9 Hz, 1H), 3.27 (dd, *J* = 14.3, 4.5 Hz, 1H), 3.08 – 3.04 (m, 2H), 2.86 (dd, *J* = 13.6, 7.6 Hz, 1H), 1.79 – 1.40 (m, 12H), 1.02 – 0.81 (m, 24H).

^{13}C NMR (400 MHz, MeOD) δ/ppm: 180.49, 177.17, 175.09, 174.68, 173.55, 173.37, 138.76, 138.67, 130.65, 130.43, 129.63, 129.55, 127.92, 127.82, 127.74, 57.47, 55.93, 54.98, 53.74, 53.69, 53.65, 53.52, 42.07, 41.98, 41.95, 41.88, 41.70, 38.39, 30.83, 26.16, 25.88, 24.33, 23.97, 23.85 , 23.59, 23.49, 22.63, 22.21, 22.16, 22.12, 22.00.

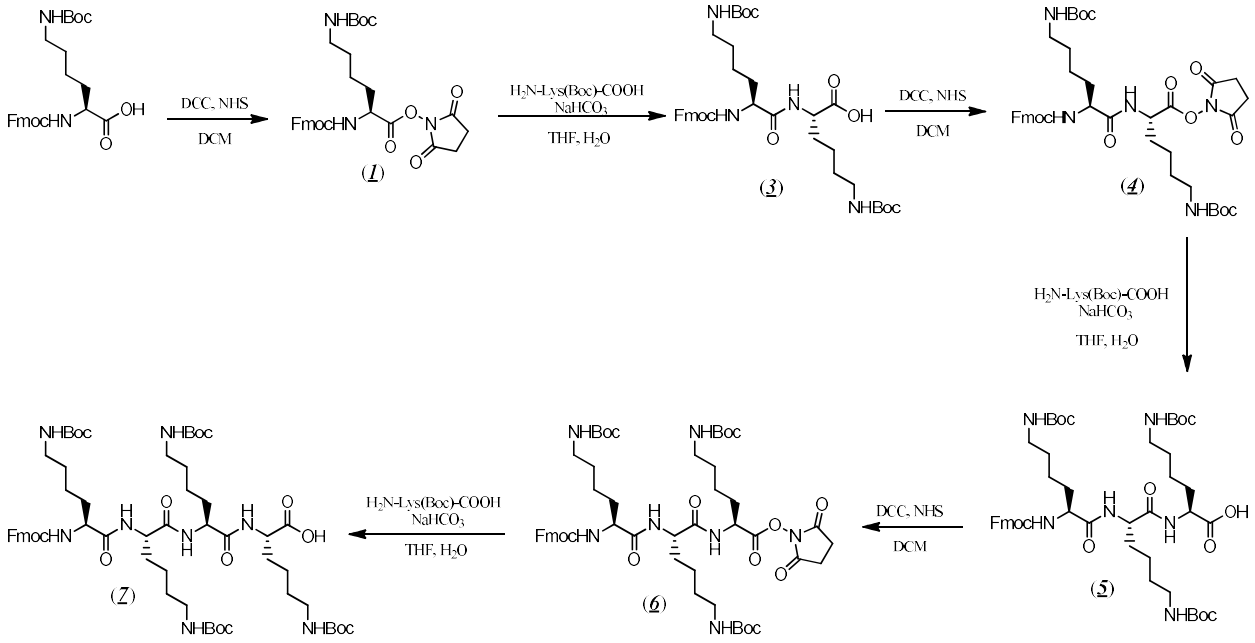
ESI-Q-TOF: $[M-H]^-$ Calcd: 764.09, found: 764.10.

§ 4. RESULTS AND DISCUSSION

4.1. SYNTHESIS AND CYCLIZATION OF TETRA-HEXALYSINE

4.1.1. Fmoc synthetic strategy

As a first attempt to synthesize cyclic products ***L4-L6,*** it was decided to use orthogonal Fmoc-Boc protecting groups (Scheme 13). The first step was the synthesis of activated succinimidyl ester starting from Fmoc-Lys(Boc)-COOH using NHS and DCC as the condensing agents. Activation of the carboxyl group is a key step in the peptide synthesis. The carboxylic group can be activated *in situ* or an activated ester can be prepared and isolated as is the case here reported. It is necessary for the activation step to be sufficiently rapid and to preserve the optical purity of the starting compound. After activation of a starting amino acid, crude succinimidyl ester ***1*** was obtained and was not purified but was used as it is in further synthesis. Confirmation of the formation of the activated compound ***1*** is the disappearance of the corresponding carboxyl group signal in ^{1}H NMR in DMSO-d_6 and a singlet appearance at 2.82 ppm that belongs to the succinimidyl ester. Activated ester ***1*** was converted into dipeptide ***3*** by reaction with the corresponding unprotected amino acid in a mildly basic conditions in THF as a solvent. The formation of the dipeptide ***3*** was confirmed by the disappearance of the succinimide signal in the NMR spectra and the emergence of one signal at 3.96-3.90 ppm in the ^{1}H NMR spectrum corresponding to the a second CH group and a singlet at around 1.35 ppm that corresponds to another Boc group. Mass analysis also confirmed formation of the desired product. These reactions were repeated until the formation of the compound ***7***. Since the isolation of the compound ***7*** was difficult, the overall yield of this synthetic route was very low (5.6 %) and the removal of Fmoc in solution rather problematic, we decided to try another synthetic strategy in order to synthesise the desired precursors.



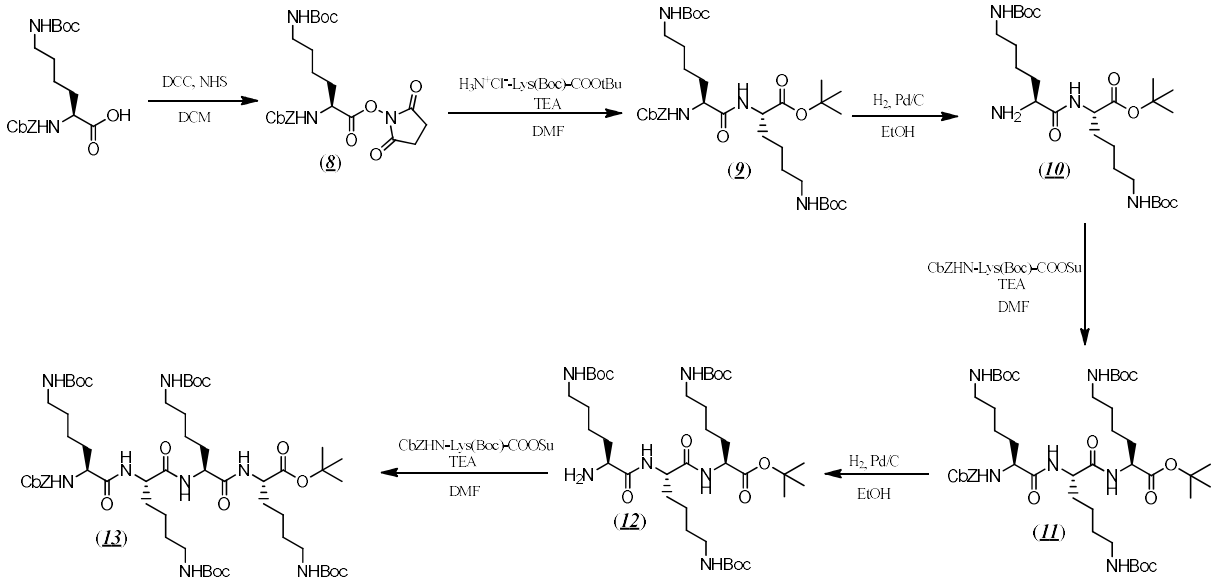
*Scheme 13. Synthesis of a compound **7**. Overall yield 5.6 %.*

In fact, the use of octanthiol and DBU as a catalyst was not successful. By changing reaction conditions *i.e*. using Et_2NH it was possible to remove Fmoc in 85% yield, but purification and isolation of the products were quite complicated.[113]

4.1.2. CbZ - OtBu synthetic strategy

The second strategy was to protect lysine using other two orthogonal protecting groups: CbZ and Boc (Scheme 14). Activation of a carboxyl group was done as before by converting it in succinimidyl ester. Succesful synthesis of a precursor ***8*** was confirmed by ^{1}H NMR spectra (singlet integrating 4H at 2.81 ppm corresponding to CH_2 of succinimidyl group). The next step was the condensation with the second amino acid protected on *C*-terminus as *tert*-butyl ester. NMR and mass analysis confermed formation of the compound ***9***. Since the synthesis this time was done from *C*- to *N*-terminus, in the next step it was neccesary to remove the CbZ protecting group by standard hydrogenolysis in the presence of Pd/C as a catalyst. The resulting product ***10*** was coupled with ester ***8*** to give a fully protected tripepeptide ***11*** (singlets at 1.38 and 1.36 ppm corresponding to three Boc groups and at 1.29-1.20 ppm for a *tert*-butyl ester in the ^{1}H

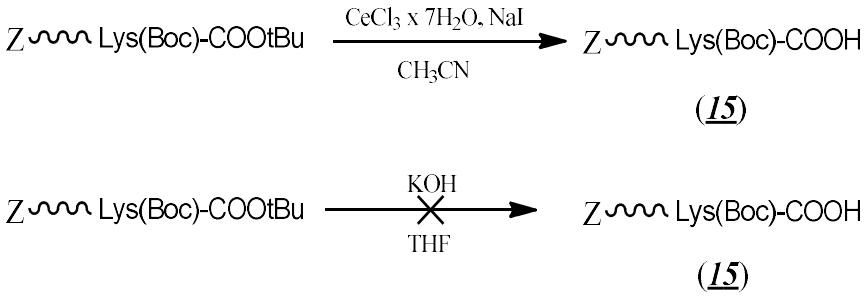
NMR spectrum). Removal of CbZ by hydrogenolysis and further condensation with the succinimidyl ester afforded the tetrapeptide ***13***.



*Scheme 14: Synthesis of a tetrapeptide **13**. Overall yield: 48.8 %.*

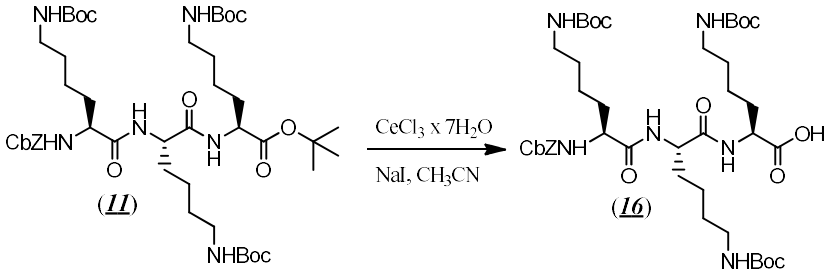
For a synthesis of compounds ***L4***-***L6*** it was necessary to prepare precursors having free *N*- and *C*-termini but protected on the side chains, *i.e.* in the previously described synthetic strategy to selectively remove *tert*-butyl ester in presence of a Boc protecting group.

To this aim, before starting the synthesis, we used a peptide containing these protecting groups, previously synthesized by our research group, as the model compound (Scheme 15).



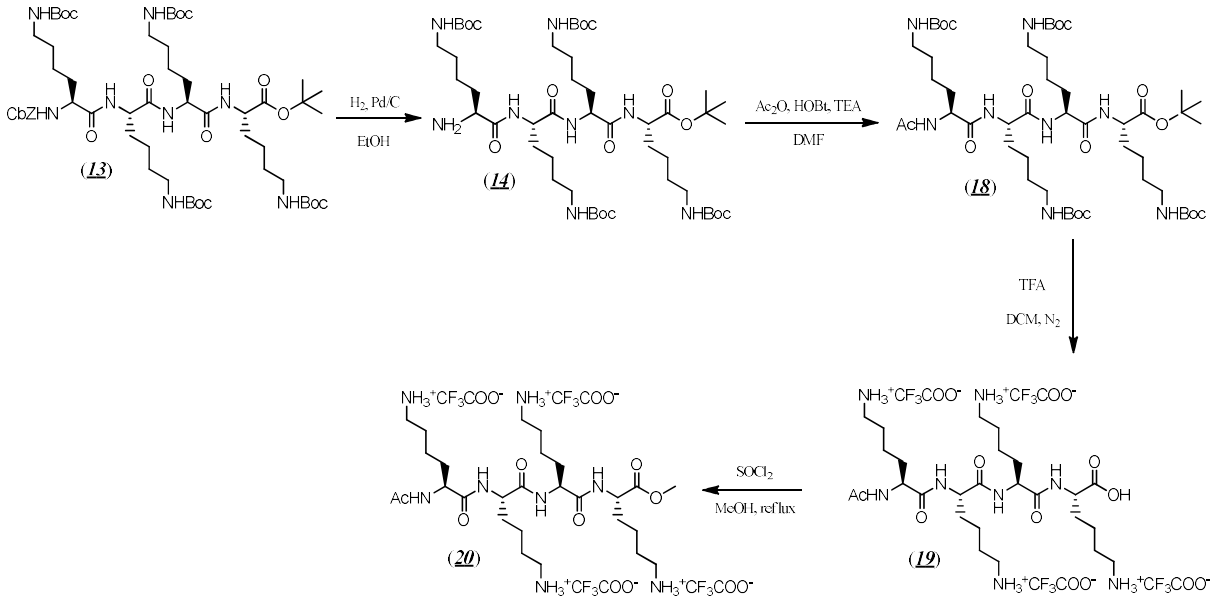
Scheme 15. Selective removal of a tert-butyl ester.

We tried two different synthetic pathways described in the literature, using both basic conditions and Lewis acid. Reaction with KOH wasn't successful while reaction with $CeCl_3$ resulted in the formation of the desired product ***15***. But when the same reaction conditions were repeated using tripeptide ***11*** as the substrate (Scheme 16), reaction yield was only 20 % and contemporary removal of one or more Boc groups was observed. Thus, the synthetic strategy for the preparation of linear precursors was changed again. However, tetrapeptide ***13*** was used as it was converted into the capped tetrapeptide ***20*** used later for a coupling with calixarenes.



*Scheme 16. Synthesis of a compound **16**. Yield: 20 %.*

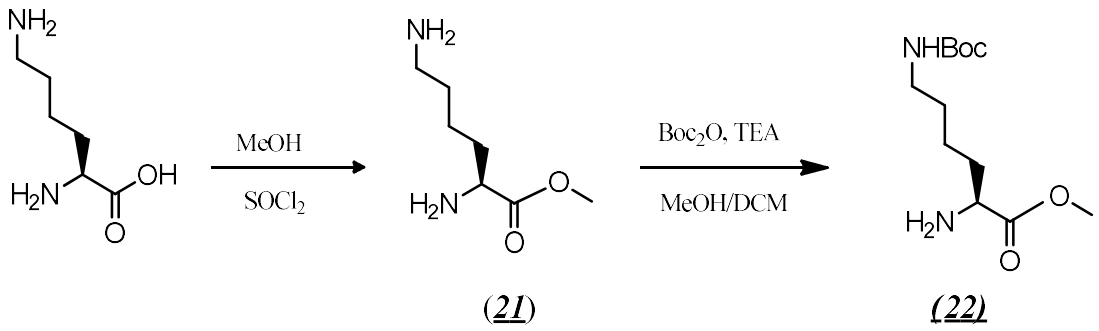
In order to obtain product ***20***, CbZ protecting group was succesfully removed from compound ***13*** by catalytic hydrogenolysis to give ***14*** which was used without purification. In the next step acetyl group was introduced as a protecting group since it is hard to remove and it is stable in the conditions used for the coupling with calixarenes. Then, Boc protecting groups were removed by treatment of ***18*** with TFA to obtain a product (***19***) with free amino groups on side chains able to react with carboxyl groups of calixarene. Since TFA is a strong organic acid, in this reaction also *tert*-butyl ester was hydrolysed. The last step was introduction of methyl ester to protect *C*-terminus to obtain compound ***20*** (Scheme 17).



*Scheme 17. Synthesis of a compound **20**. Overall yield: 47.7 %*

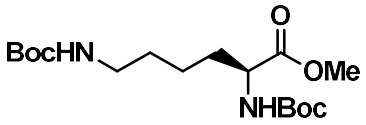
4.1.3. CbZ – OMe synthetic strategy

After two unsuccesful trials, we decided to use three-dimensional orthogonal protection scheme to build the linear peptides, then to deprotect the *N*- and *C*-termini and to cyclize them in a head-to-tail fashion obtaining final products ***L4***-***L6***. Specifically, we planned to protect the first amino acid as CbZ – Boc on amino groups and the second as Boc on the side chain and as methyl ester on the carboxyl group. The first step was the preparation of the latter. L-lysine was converted into methyl ester (Scheme 18) through acyl chloride in quantitative yield. Singlet at 3.74 ppm in ^{1}H NMR spectra confirmed the formation of this product.



*Scheme 18. Synthesis of amino acid **22**. Overall yield: 63.2 %*

The second step was selective protection of only side chain amino group. Since $NH_{2\varepsilon}$ (pKa 10.67) is more basic than $NH_{2\alpha}$ (pKa 9.16) and less sterically hindered, it generally reacts faster. Yield (63 %) was lowered by contemporary formation of the di-protected lysine methyl ester ***22b*** as by-product (18 %).



22b

A singlet at 1.37 ppm that integrate ~ 9 in ^{1}H NMR spectrum of product ***22*** corresponds to the C**H**$_3$ of a Boc protecting group showing the good outcome of the reaction. ^{1}H NMR spectra of product ***22*** and byproduct ***22b*** were compared in Figure 22 to prove that was selectively protected only NH_2 of the side-chain.

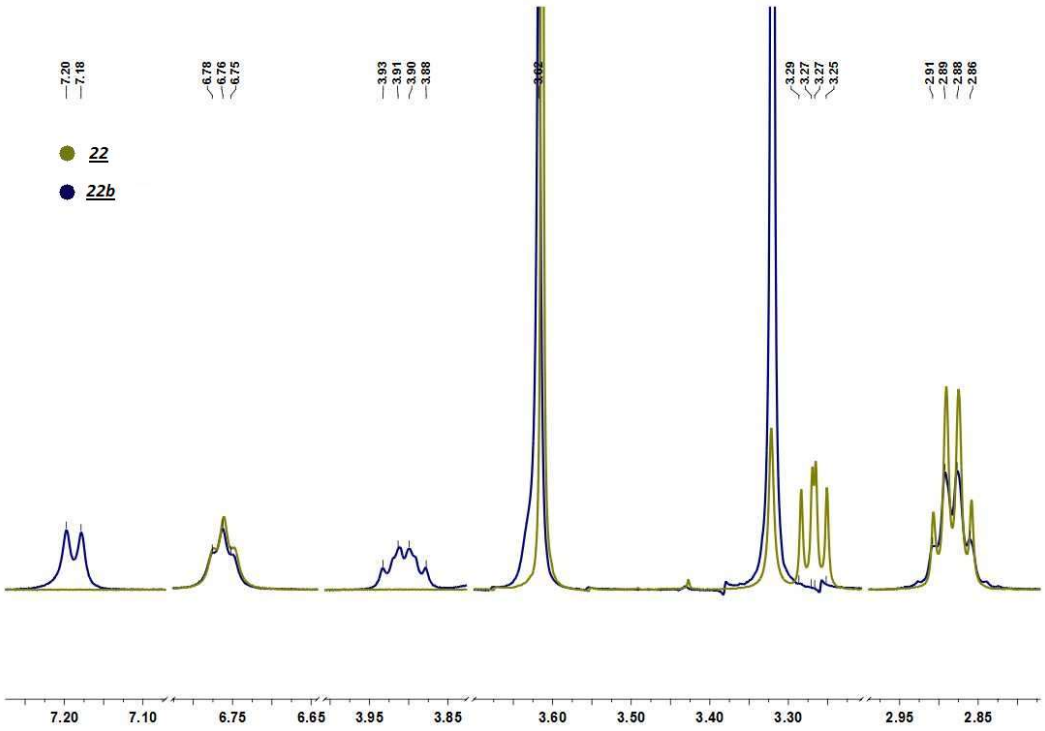
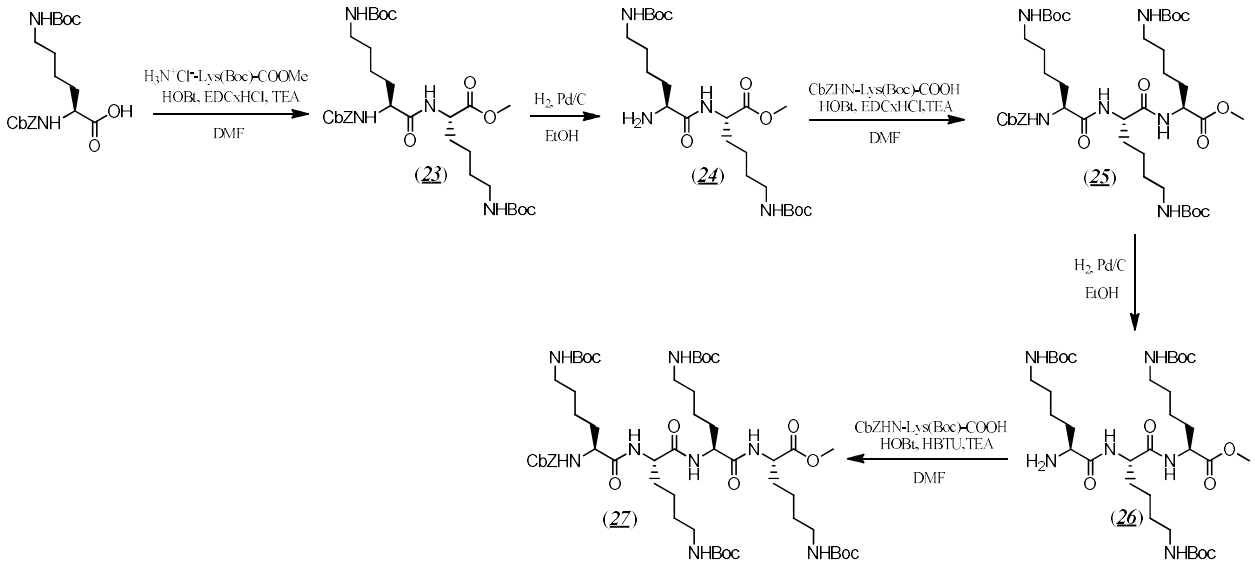


*Figure 22. ^{1}H NMR comparison between product **22** and byproduct **22b.***

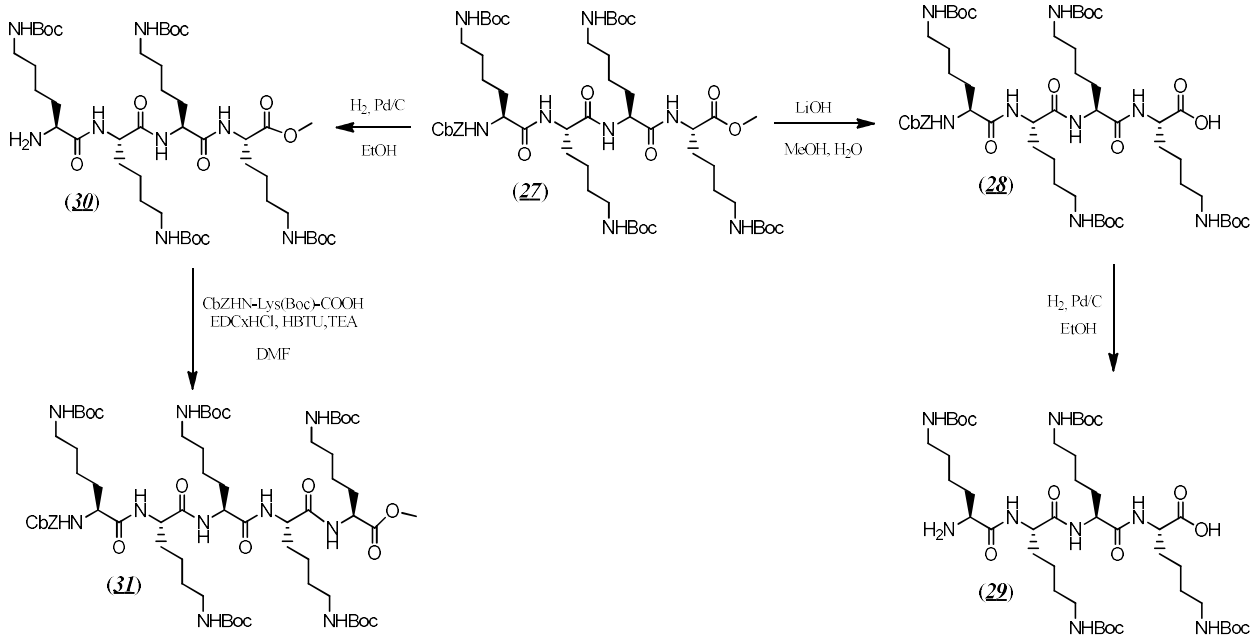
$CH_{2\varepsilon}$ peaks of ***22*** and ***22b*** have the same chemical shift (2.88 ppm), while signal of CH_α of ***22*** is at higher fields (3.27 ppm) compared to CH_α of byproduct ***22b*** (3.90 ppm). NH_ε-Boc of both compounds at 6.76 ppm are shown as triplets corresponding to an NH coupled to a CH_2. NH_α-Boc of byproduct ***22b*** instead is coupled with a CH_α and it is a doublet at lower fields (7.19 ppm).

Synthesis of target molecule ***23*** was accomplished by reaction of hydrocloride salt of compound ***22*** with commercially available CbZ-Lys(Boc)-COOH, in the presence of HBTU and EDC×HCl as condensation reagents and TEA as a base in DMF (Scheme 19). To obtain ***24*** it was neccesary to remove CbZ protection as already described. Repeating these two reactions, fully protected tetrapeptide ***27*** was obtained with an overall yield of 72.1 %.



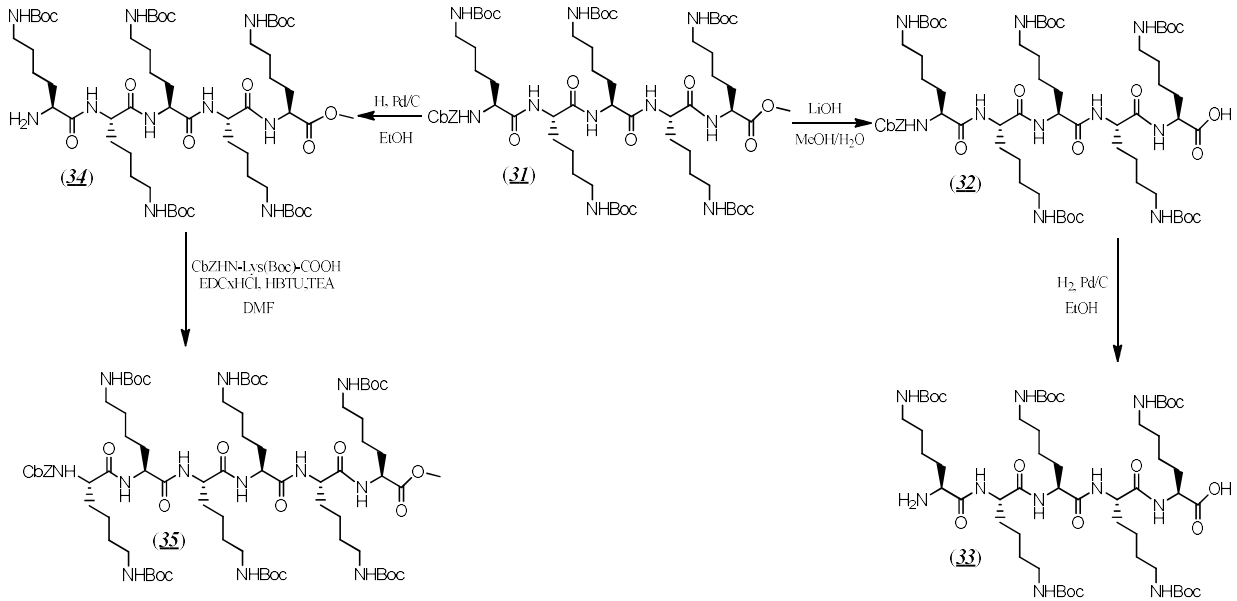
*Scheme 19. Synthesis of tetrapeptide **27**. Overall yield: 72.1 %.*

Tetrapeptide ***27*** was used as a precursor of other two products ***29*** and ***31*** (Scheme 20). For the synthesis of product ***29***, the methyl ester of ***27*** was hydrolyzed in basic conditions (LiOH in MeOH-water, 3:1) and the resulting product ***28*** was subjected to hydrogenolysis to give ***29***. From the other side, removal of CbZ protection from ***27*** to give ***30***, followed by coupling with H-Lys(Boc)-OMe led to the protected pentapeptide ***31*** whose structure was confirmed by NMR and mass analysis.



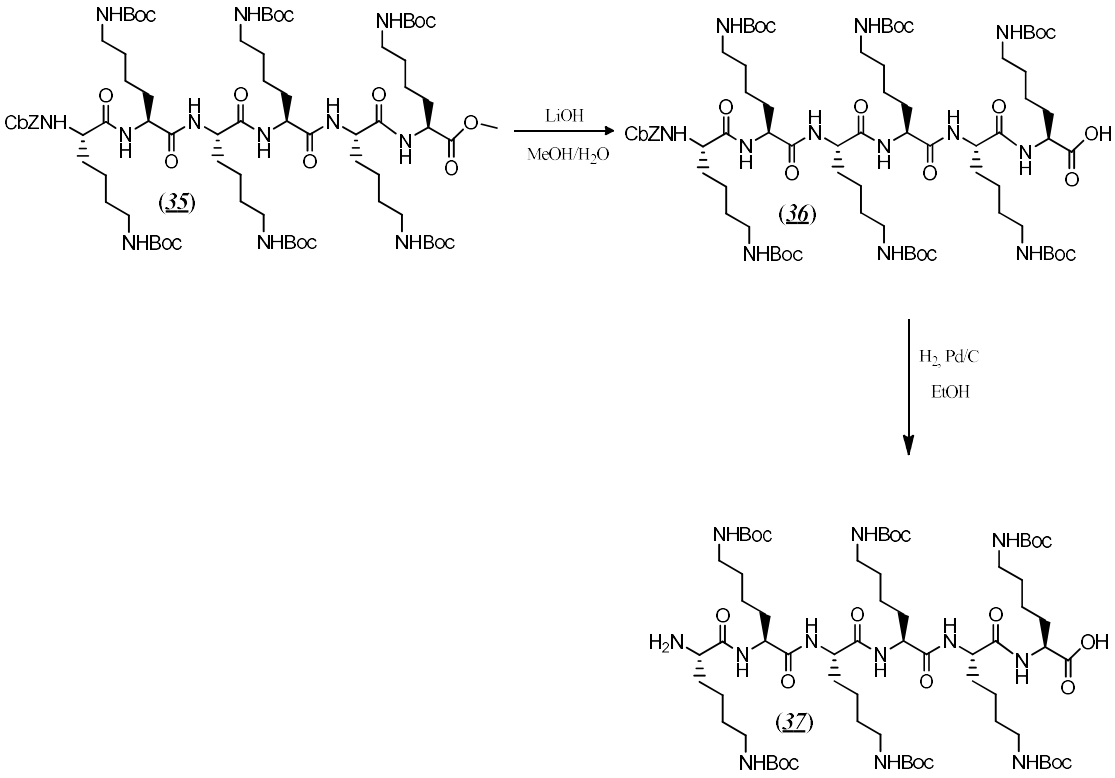
*Scheme 20. Synthesis of products **29** and **31**. Overall yields: 99.0 % and 87.0 % respectively.*

In the same way the pentalysine ***33*** and the hexalysine ***35*** were synthesized from precursor ***31*** in 97.0 % and 77.2 %, respectively (Scheme 21). NMR and mass analysis were performed to confirm their structures.



*Scheme 21. Synthesis of compounds **33** and **35**. Overall yields: 97.0 % and 77.2 % respectively.*

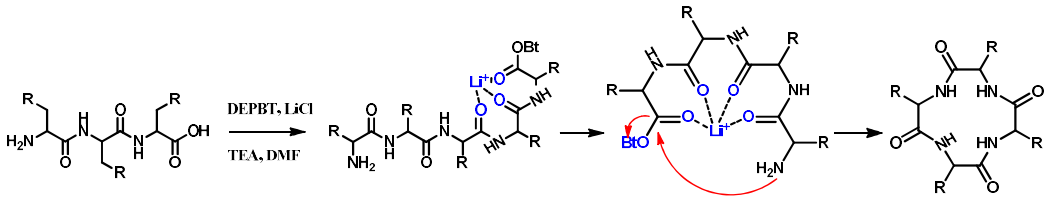
Removal of protecting groups from *N*- and *C*-termini of the hexapeptide ***35*** afforded ***37*** in a very good yield (95%) (Scheme 22).



*Scheme 22. Synthesis of a compound **37**. Overall yield: 95.0 %.*

4.1.4. Cyclization

After preparation of linear precursors, the next step was head-to-tail cyclization. We followed a procedure described by Ye and co-workers[29] in which DEPBT is used as the coupling reagent in DMF as a solvent, TEA as the base and LiCl or NaCl as a source of metal cations that should promote cyclization step. The proposed mechanism is shown in Scheme 23. According to the mechanism, the cation coordinate the activated *C*-terminal carboxyl group of the linear peptide and make it closer to the *N*-terminal amino group facilitating the subsequent ring closure.



Scheme 23. Metal assisted head-to-tail cyclisation mechanism.

With a goal to determine the equilibrium constants of reactions of linear peptides with Na^+, microcalorimetric titrations were performed using the methyl esters of linear tetra-, penta- and hexapeptides as ligands and sodium perchlorate ($NaClO_4$) as a salt. Titrations were carried out at 25 °C by adding a salt solution in *N,N*-dimethylformamide to the ligand solution. The measured heats are reported in Figures 23-28.

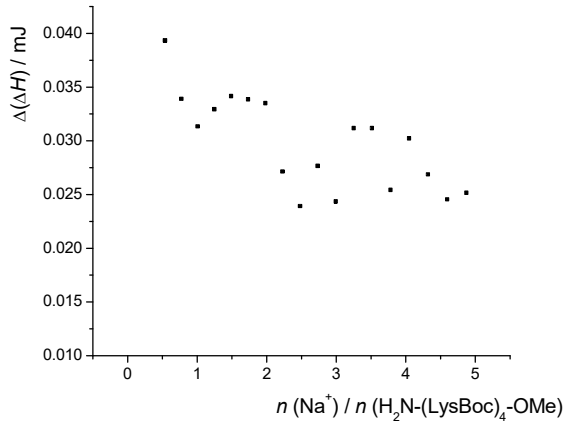


Figure 23. Dependence of successive enthalpy change on n(Na^+) / n(H_2N-Lys(Boc)$_4$-OMe) in the microcalorimetric titration of linear H_2N-Lys(Boc)$_4$-OMe (c = 4.597·10^{-3} mol dm^{-3}) with $NaClO_4$ (c = 0.098 mol dm^{-3}) in N, N-dimethylformamide in the presence of $TBAClO_4$ (c = 5,010·10^{-2} mol dm^{-3}) at ϑ = 25 ° C.

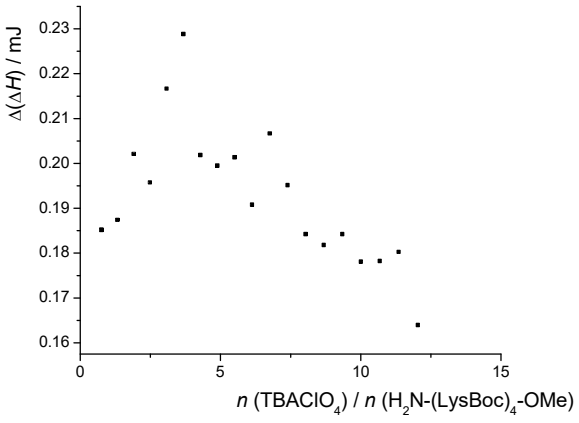


Figure 24. Microcalorimetric titration of linear H_2N-Lys(Boc)$_4$-OMe with $TBAClO_4$ (c = 0.250 mol dm^{-3}) in N,N-dimethylformamide at ϑ = 25 ° C.

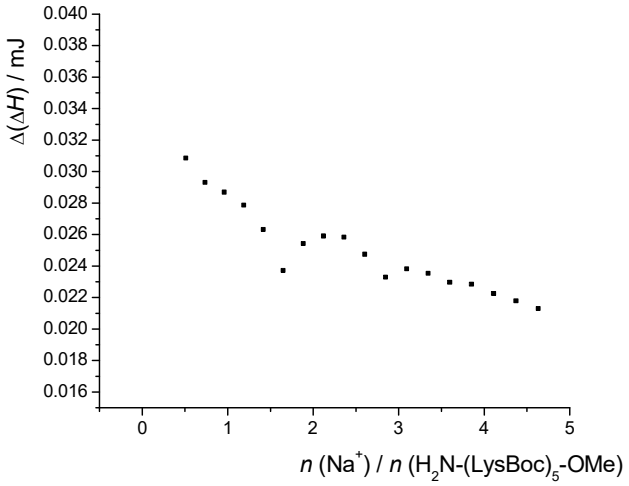


Figure 25. Dependence of successive enthalpy change on n(Na^+) / n(H_2N-Lys(Boc)$_5$-OMe) in the microcalorimetric titration of linear H_2N-Lys(Boc)$_5$-OMe (c = 4.948·10^{-3} mol dm^{-3}) with $NaClO_4$ (c = 0.102 mol dm^{-3}) in N,N-dimethylformamide in the presence of $TBAClO_4$ (c = 5,002·10^{-2} mol dm^{-3}) at ϑ = 25 ° C.

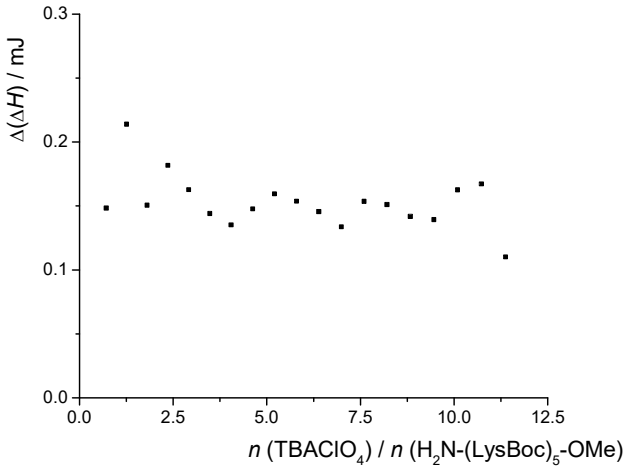


Figure 26. Microcalorimetric titration of linear H_2N-Lys(Boc)$_5$-OMe (c = 4.944·10^{-3} mol dm^{-3}) with $TBAClO_4$ (c = 0.249 mol dm^{-3}) in N, N-dimethylformamide at ϑ = 25 ° C.

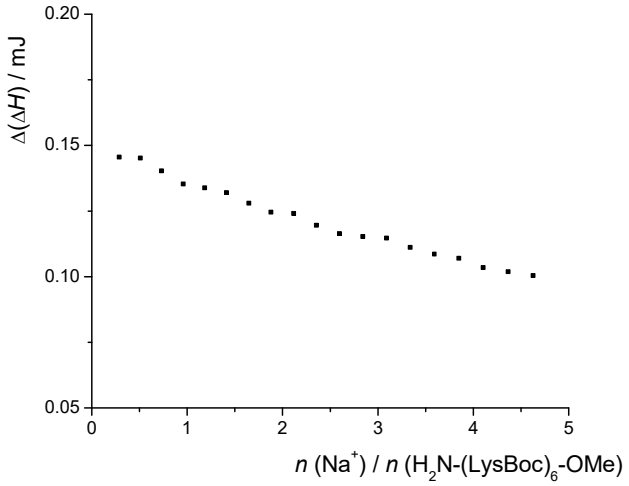


Figure 27. Dependence of successive enthalpy change on n(Na^+) / n(H_2N-Lys(Boc)$_6$-OMe) in the microcalorimetric titration of linear H_2N-Lys(Boc)$_6$-OMe (c = 4.979·10^{-3} mol dm^{-3}) with NaTPB (c = 0.102 mol dm^{-3}) in N,N-dimethylformamide in the presence of TBAClO$_4$ (c = 5,002·10^{-2} mol dm^{-3}) at ϑ = 25 ° C.

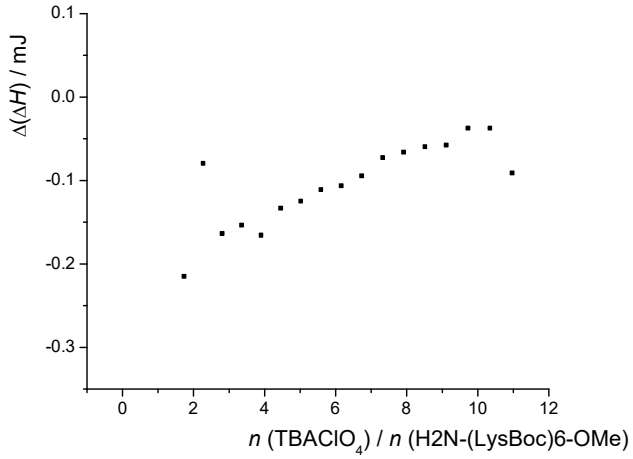


Figure 28. Microcalorimetric titration of linear H_2N-Lys(Boc)$_6$-OMe (c = 5,155·10^{-3} mol dm^{-3}) with TBAClO$_4$ (c = 0.249 mol dm^{-3}) in N,N-dimethylformamide at ϑ = 25 ° C.

Analysis of microcalorimetric data clearly indicated that there is no binding between linear peptides and Na^+. As a consequence, the mechanism described before of Na-induced cyclization is not completely correct. Since the anion recognition capacity of peptides has been reported[62-

[65], a possible role of chloride ion to promote cyclization could be envisaged. With the goal to understand the reaction mechanism we carried out a series of experiments in which tetraethylammonium chloride, containing a very large cation, was used as the salt in the titrations

The dependence of ΔH on the amount of salt added is shown in Figures 29-31. In Table 1 the thermodynamic parameters of the complexation reaction are reported.

An inert electrolyte, tetrabutylammonium perchlorate, was added to the system in order to reduce the heats of dissolution. The evidence of the inert behavior of perchlorate ion comes from the microcalorimetric titrations of linear peptides with perchlorate ion (Figures 24, 26, and 28). In these titrations the measured successive enthalpy changes were negligible and inapt for stability constant determination.

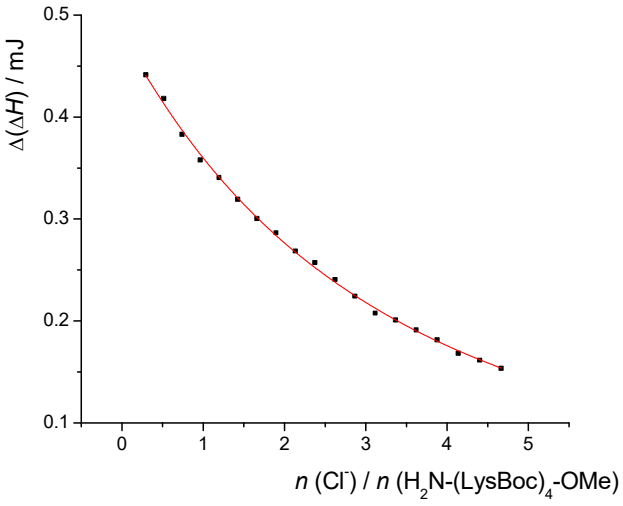


Figure 29. Dependence of successive enthalpy change on n(Cl^-) / n(H_2N-Lys(Boc)$_5$-OMe) in the microcalorimetric titration of linear H_2N-Lys(Boc)$_4$-OMe (c = 4.597·10^{-3} mol dm^{-3}) with TEACl (c = 0.095 mol dm^{-3}) in N,N-dimethylformamide in the presence of $TBAClO_4$ (c = 5.010·10^{-2} mol dm^{-3}) at ϑ = 25 ° C. ■ measured values, — calculated values.

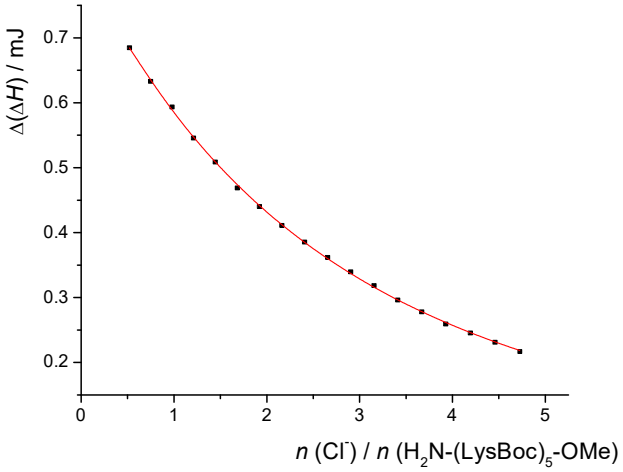


Figure 30. Dependence of successive enthalpy change on n(Cl^-) / n(H_2N-Lys(Boc)$_5$-OMe) in the microcalorimetric titration of linear H_2N-Lys(Boc)$_5$-OMe (c = 4.902·10^{-3} mol dm^{-3}) with TEACl (c = 0.103 mol dm^{-3}) in N,N-dimethylformamide in the presence of $TBAClO_4$ (c = 4.980·10^{-2} mol dm^{-3}) at ϑ = 25 °C. ■ measured values, — calculated values.

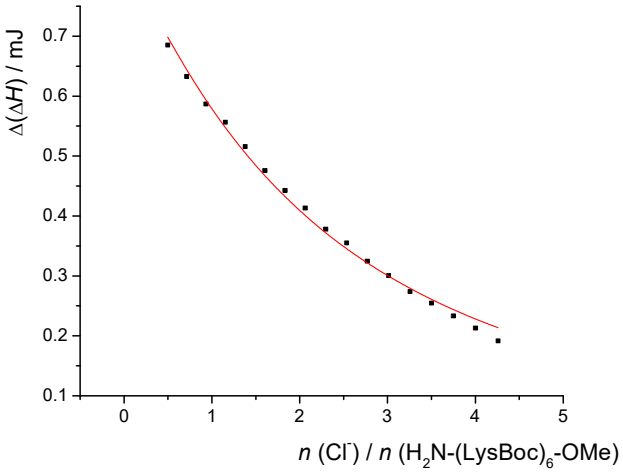


Figure 31. Dependence of successive enthalpy change on n(Cl^-) / n(H_2N-Lys(Boc)$_6$-OMe) in the microcalorimetric titration of linear H_2N-Lys(Boc)$_6$-OMe (c = 4,979·10^{-3} mol dm^{-3}) with TEACl (c = 0.991 mol dm^{-3}) in N,N-dimethylformamide in the presence of $TBAClO_4$ (c = 5,002·10^{-2} mol dm^{-3}) at ϑ = 25 °C. ■ measured values, — calculated values.

Table 1: Thermodynamic parameters of complexation reactions of linear tetra-, penta- and hexalysine with chloride anion in N,N-dimethylformamide at 25 °C obtained by microcalorimetric titrations.

solvent	peptide	log K	($\Delta_r G^\theta$ ± SE) / kJ mol^{-1}	($\Delta_r H^\theta$ ± SE) / kJ mol^{-1}	($\Delta_r S^\theta$ ± SE) / kJ mol^{-1}
DMF	H_2N-(LysBoc)$_4$-OMe	1,51 ± 0,01	-8,61 ± 0,05	2,50 ± 0,09	37,3 ± 0,2
	H_2N-(LysBoc)$_5$-OMe	1,47 ± 0,07	-8,4 ± 0,4	4,4 ± 0,9	43 ± 2
	H_2N-(LysBoc)$_6$-OMe	1,68 ± 0,03	-9,6 ± 0,2	2,8 ± 0,3	41,7 ± 0,4

SE = standard error of the mean (N = 3)

These data, clearly indicating a binding affinity of the linear peptides toward chloride ions, were confirmed by ^{1}H NMR titrations of ligands with NaTPB and TEACl salts in deuterated *N*,*N*-dimethylformamide. In Figures 32 and 33 the ^{1}H NMR spectra (α-hydrogen region) of linear pentapeptide in the presence of increasing amount of salts are shown.

In the titration with NaTPB only small shifts of α-hydrogen signals were observed; by contrast, in the titration with TEACl much more significant shifts occured. This was in agreement with a preferred binding of chloride by the ligands investigated.

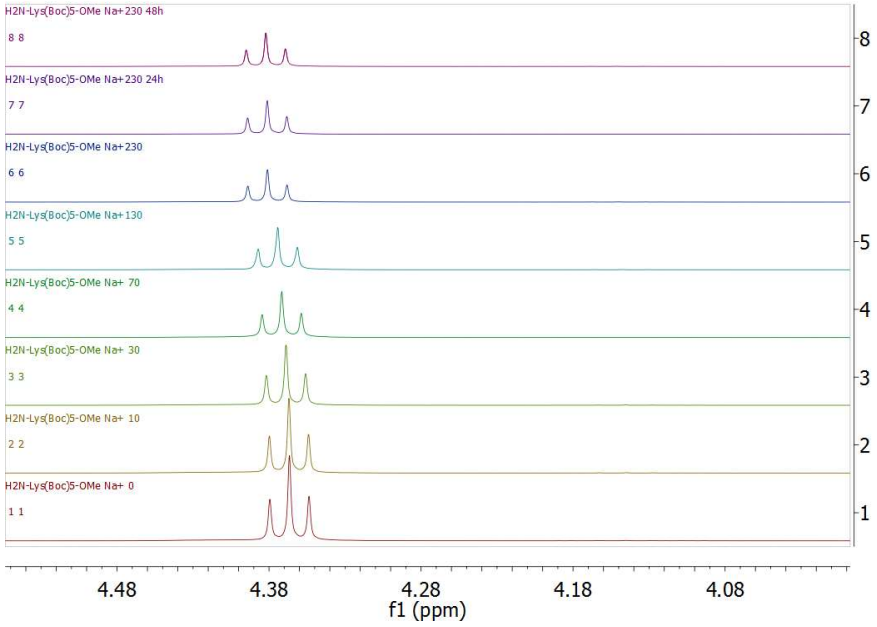


Figure 32. ^{1}H NMR titration of H_2N-Lys(Boc)$_5$-OMe (c = 1,08·10^{-3} mol dm^{-3}) with NaTPB (c = 0,19952 mol dm^{-3}) in deuterated N,N-dimethylformamide at θ = 25 °C.

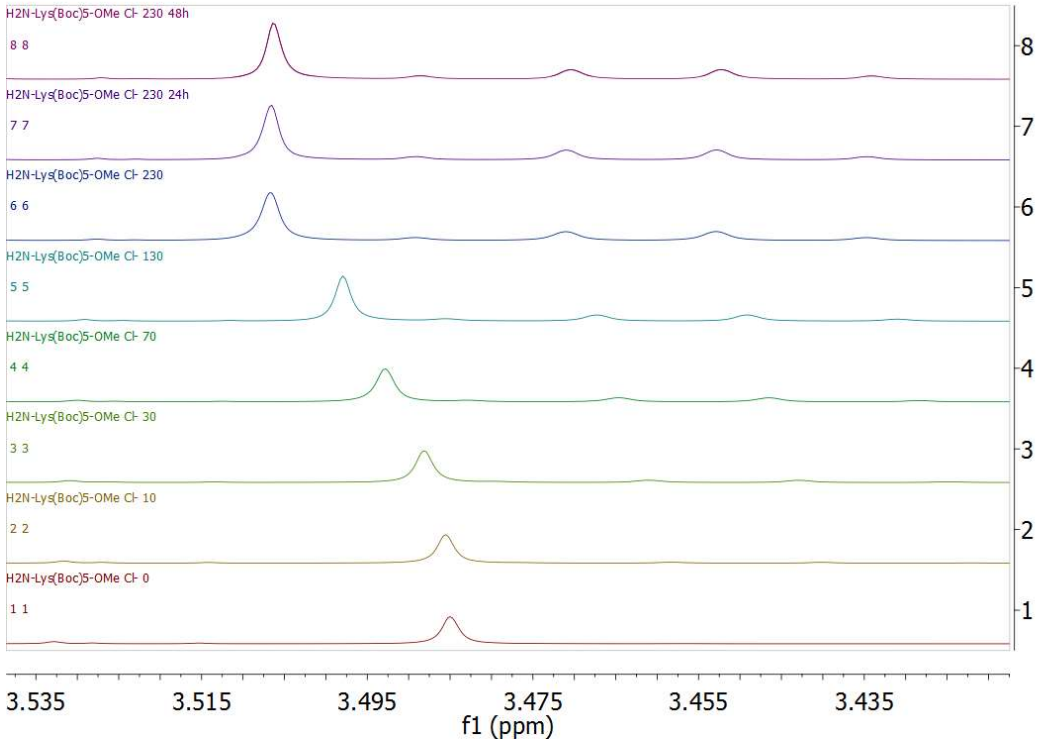


Figure 33. ^{1}H NMR titration of H_2N-Lys(Boc)$_5$-OMe (c = 1,08·10^{-3} mol dm^{-3}) with TEACl (c = 8,075·10^{-2} mol dm^{-3}) in deuterated N,N-dimethylformamide at θ = 25 °C.

The results of molecular dynamics simulations, performed on tetra-, penta- and hexalysine, are consistent with microcalorimetric and NMR titrations, indicating that anions, not cations, can help linear precursors to adopt favourable conformation and bring two termini closer. Figure 34 show structures of stable complexes of linear peptides with Cl^-.

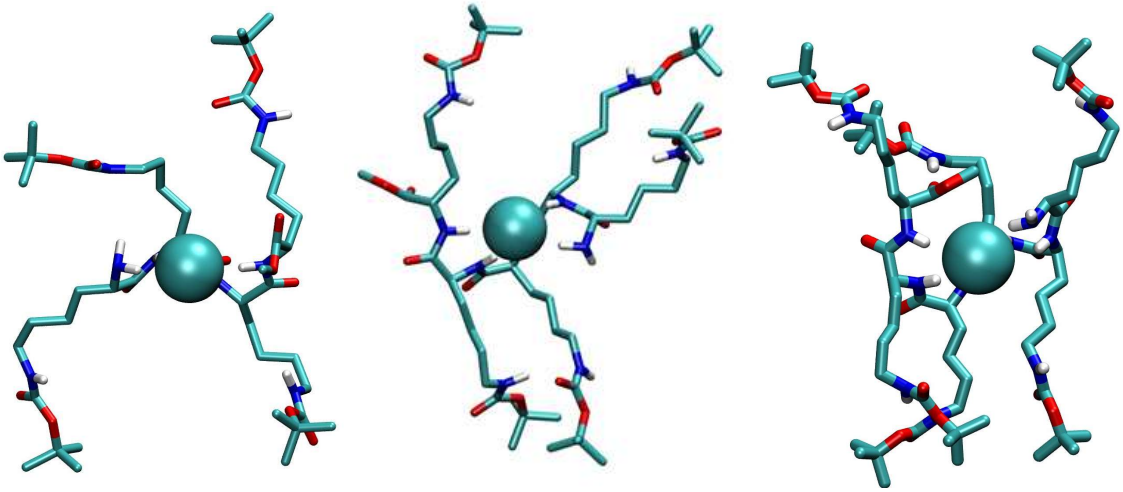
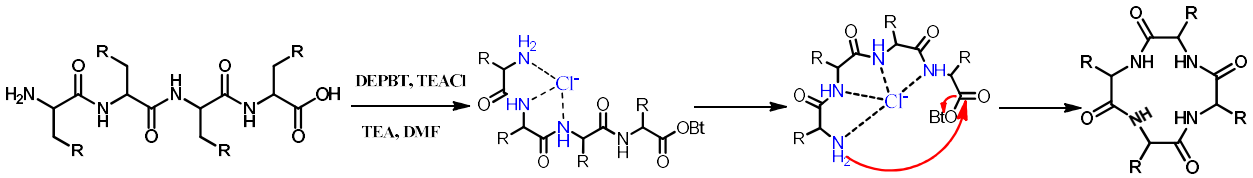


Figure 34. MDs simulations of a complexes of H_2N-Lys(Boc)$_4$-OMe, H_2N-Lys(Boc)$_5$-OMe and H_2N-Lys(Boc)$_6$-OMe with Cl^- in N,N-dimethylformamide.

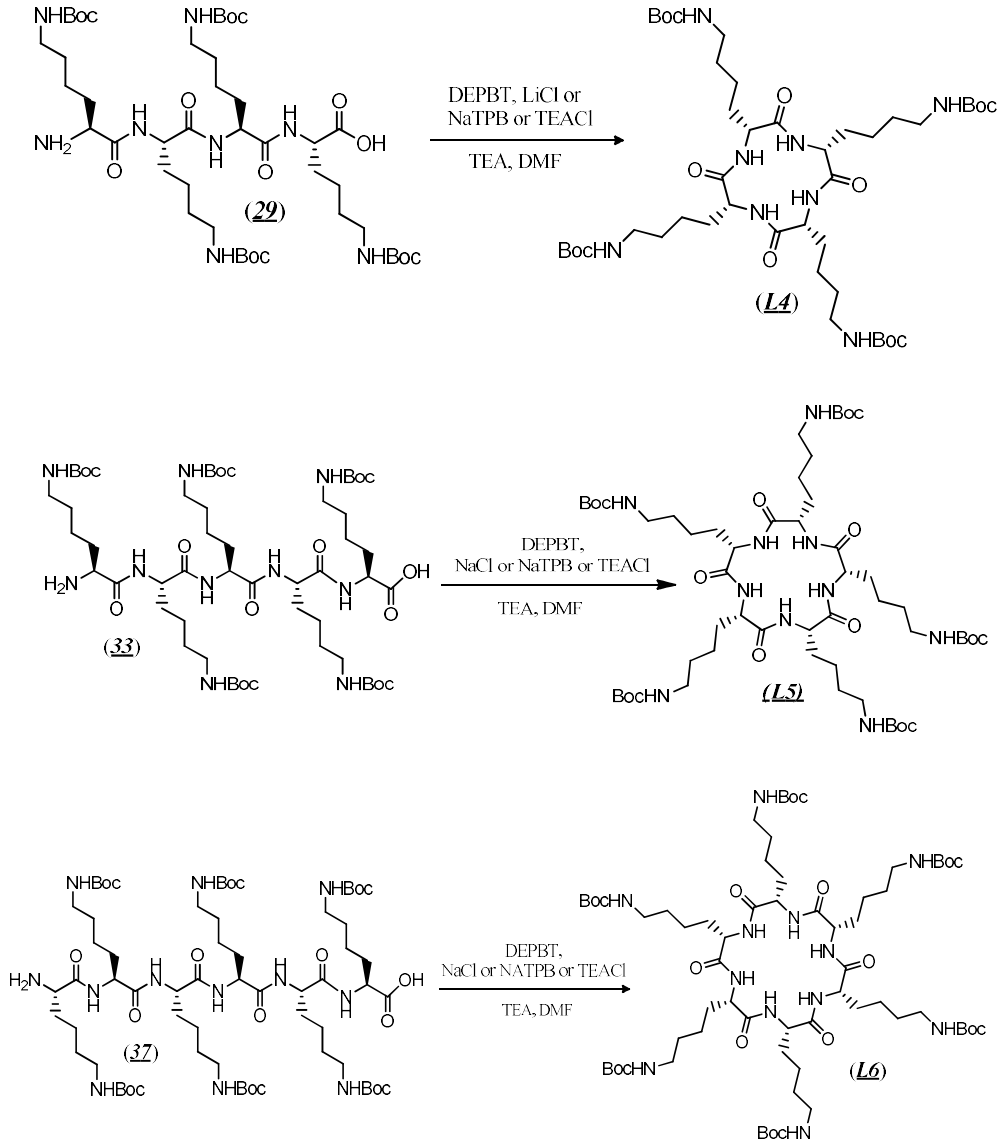
When the same simulations were carried out with complexes of Na^+ with linear peptides only the complex dissociation was observed. Thus MDs simulations can be used to predict which ion forms the most stable complex with a linear precursor and is able to conformationally pre-organize its reactive ends bringing them close to each other, a position suitable for the ring closure. Such pre-arrangement results in a fewer by-products from intermolecular processes and higher reaction yields. This is a very important result since cyclization step usually has a very low yield. Based on this finding, it is reasonable to conclude that the most likely mechanism for ion assisted peptide macrocyclization is that shown in Scheme 24.



Scheme 24. Proposed mechanism of the ion assisted macrocyclization of peptides.

With these results in hands, synthesis of cyclic peptides ***L4***, ***L5*** and ***L6*** was repeated by treating the linear precursors ***29***, ***33*** and ***37*** with the coupling reagent DEPBT in the presence of both NaTPB and TEACl salts to enhance the head-to-tail cyclization, under high dilution conditions

for 3-5 days at room temperature (Scheme 25). The obtained results, reported in Table 2, were in line with our expectations. Yield in the cyclisation of tetrapeptide ***29*** increased from 8% (in the presence of NaTPB) to 47% when TEACl was used as the salt. For pentapeptide ***33*** difference was a little bit smaller: 26 % in the reaction with NaTPB *vs* 43 % with TEACl; for the cyclization of hexapeptide ***37*** we obtained 10 % yield in the reaction with NaTPB and 17 % in a reaction with TEACl. Yields in the reactions with LiCl and NaCl were in the middle respect to those with NaTPB and TEACl probably due to the competition between anion and cation in coordination to the peptide chain.



Scheme 26. Ion-assisted cyclisation of linear precursors.

Table 2. Outcome of the macrocyclization reaction in the presence of different salts.

Linear precursor	Yield of cyclic peptides in the presence of			
	NaTPB	TEACl	LiCl	NaCl
L5	8 %	47 %	21 %	----------
33	26 %	43 %	----------	35 %
37	10 %	17 %	----------	15 %

4.1.5. Anion binding studies

To evaluate the binding properties of cyclic penta- and hexalysine (***L5*** and ***L6***) toward several anions (Cl^-, Br^-, I^-, NO_3^-, AcO^-, HSO_4^-, $H_2PO_4^-$) in acetonitrile (MeCN) microcalorimetric and ^1H NMR titrations were performed. ESI–MS spectra of ligand-anion solutions were recorded in order to identify the stoichiometry of the formed cyclopeptide-anion complexes as well.

4.1.5.1. Cyclic pentalysine

a) Calorimetric measurements

Stasbility constants of the complexes of ***L5*** with the Cl^- (Figure 35), Br- (Figure 36), NO_3^- (Figure 38), OAc^- , HSO_4^- (Figure 40) and $H_2PO_4^-$ (Figure 41) in acetonitrile were determined by microcalorimetric titrations. Ligand ***L5*** forms complexes of 1:1 stoichiometry with Cl^-, I^- and NO_3^- anions. In the titrations with bromide and hydrogensulfate anions in addition to the formation of the complexes with 1:1 cyclopeptide/anion stoichiometry, the formation of 2:1 complexes was also observed. In the titrations with I^- (Figure 37) no signifacant enthalpy changes were observed, although the NMR measurements indicated the binding of the anion in the solution. These data are a clear indicator that the binding process is almost isoenthalpic. With that assumption, standard entropy of complexation was calculated directly from the binding constant obtained by ^1H NMR measurements (Table 3).

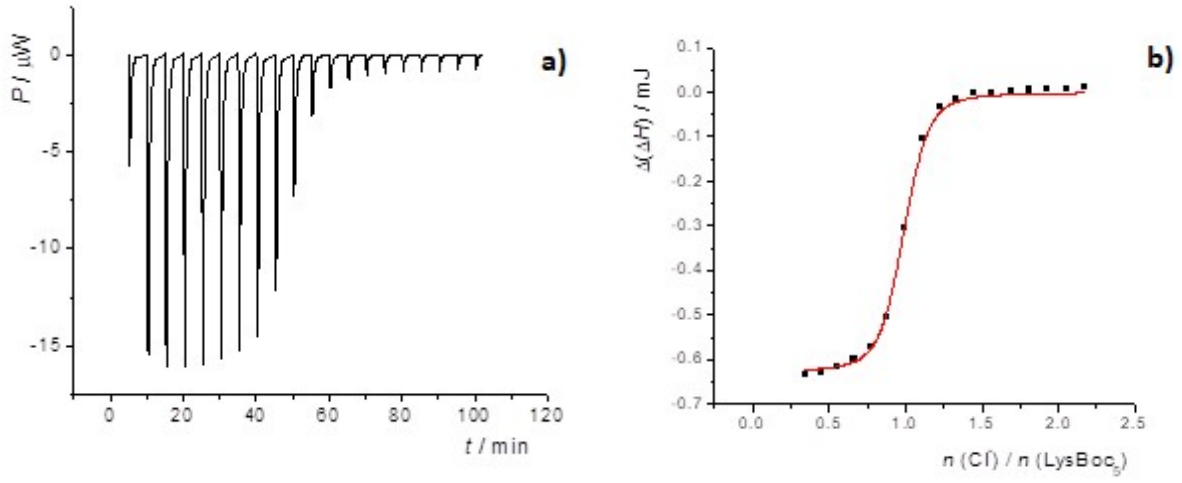


Figure 35. ***a)*** *Microcalorimetric titration of c[Lys(Boc)$_5$] (c = 5,137 · 10^{-4} mol dm^{-3}) with TEACl (c = 4,925 · 10^{-3} mol dm^{-3}) in acetonitrile at ϑ = 25 ° C.* ***b)*** *Dependence of successive enthalpy change on n(Cl$^-$) / n(c[Lys(Boc)$_5$]) ratio. ■ measured values, —calculated values.*

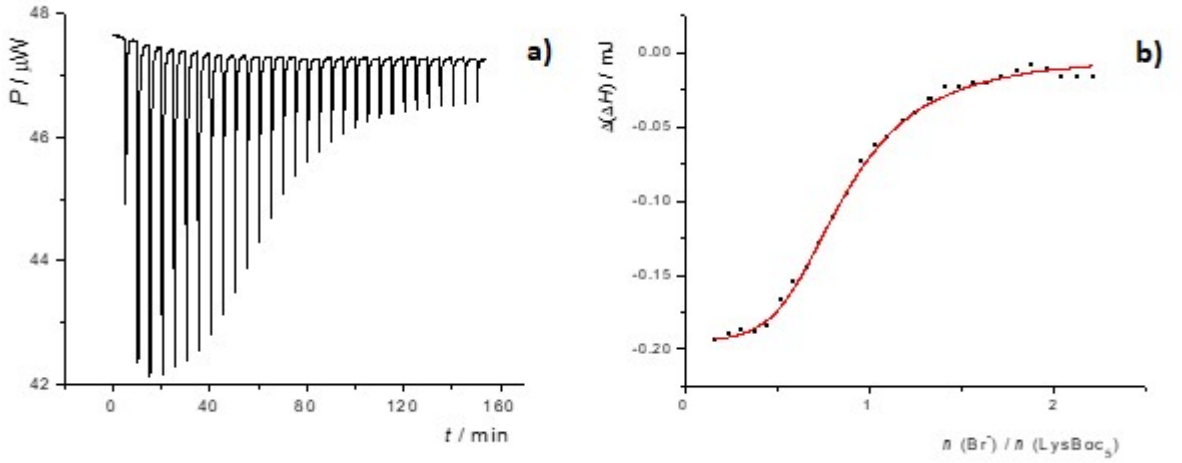


Figure 36. ***a)*** *Microcalorimetric titration of c[Lys(Boc)$_5$] (c = 5,137 · 10^{-4} mol dm^{-3}) with TBABr (c = 4,920 · 10^{-3} mol dm^{-3}) in acetonitrile at ϑ = 25 ° C.* ***b)*** *Dependence of successive enthalpy change on n(Br$^-$)/n(c[Lys(Boc)$_5$]) ratio. ■ measured values, —calculated values*

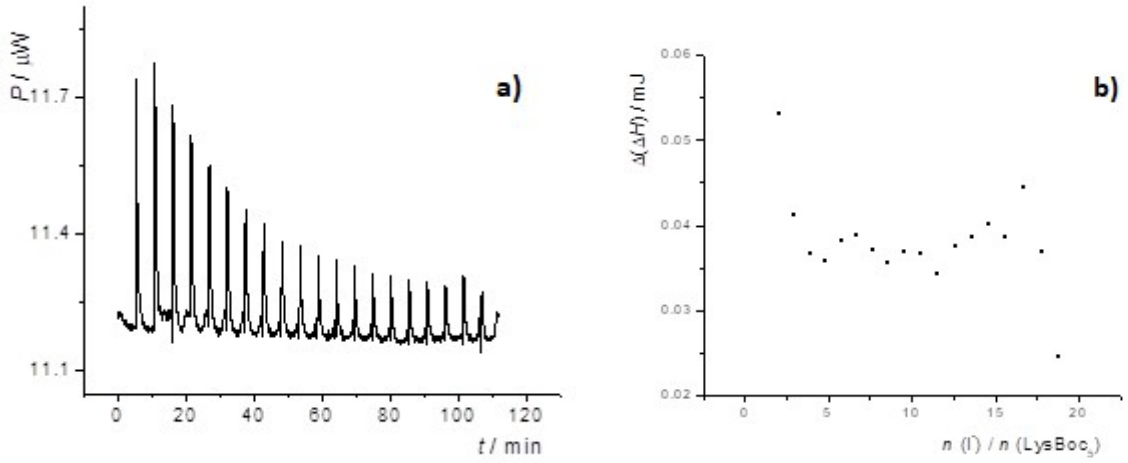


Figure 37. ***a)*** *Microcalorimetric titration of c[Lys(Boc)$_5$] (c = 5,355 · 10^{-4} mol dm^{-3}) with TBAI (c = 4,444 · 10^{-3} mol dm^{-3}) in acetonitrile at ϑ = 25 ° C.* ***b)*** *Dependence of successive enthalpy change on n(I$^-$) / n(c[Lys(Boc)$_5$]) ratio. ■ measured values.*

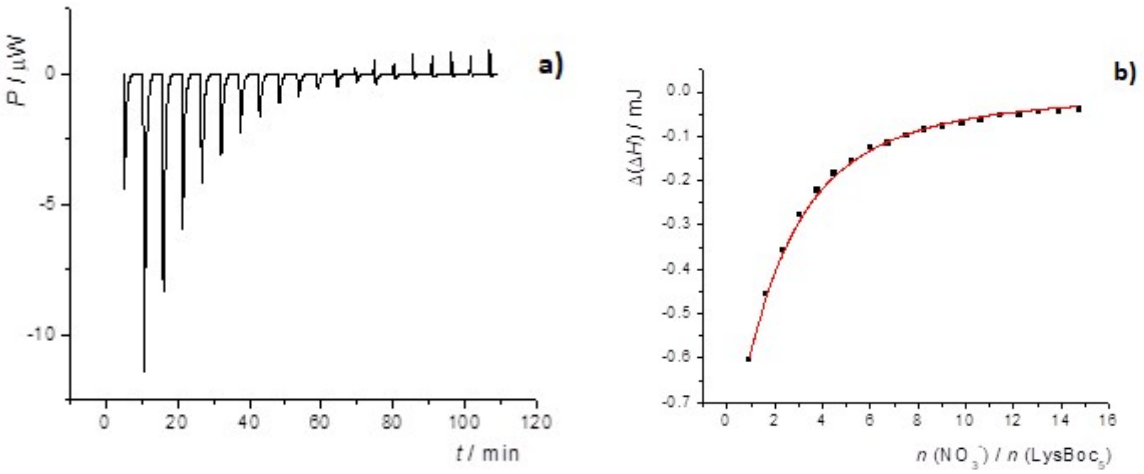


Figure 38. ***a)*** *Microcalorimetric titration of c[Lys(Boc)$_5$] (c = 5,355 · 10^{-4} mol dm^{-3}) with TBANO$_3$ (c = 3,499 · 10^{-3} mol dm^{-3}) in acetonitrile at ϑ = 25 ° C.* ***b)*** *Dependence of successive enthalpy change on n(NO$_3^-$) / n(c[Lys(Boc)$_5$]) ratio. ■ measured values, —calculated values.*

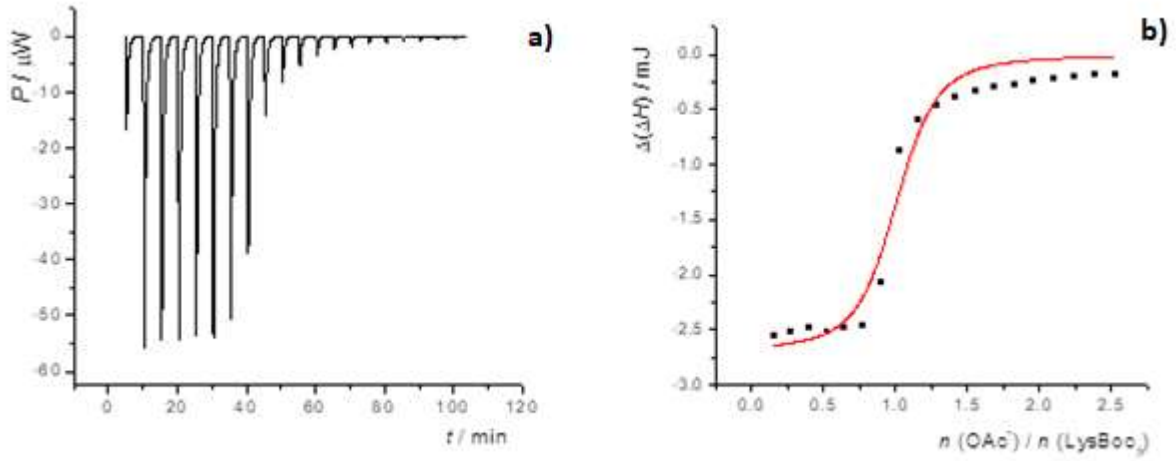


Figure 39. ***a)*** *Microcalorimetric titration of c[Lys(Boc)$_5$] (c = 5,113 · 10^{-4} mol dm^{-3}) with TBAOAc(c = 5,730 · 10^{-3} mol dm^{-3}) in acetonitrile at ϑ = 25 ° C.* ***b)*** *Dependence of successive enthalpy change on n(OAc$^-$) / n(c[Lys(Boc)$_5$]) ratio. ■ measured values, — calculated values.*

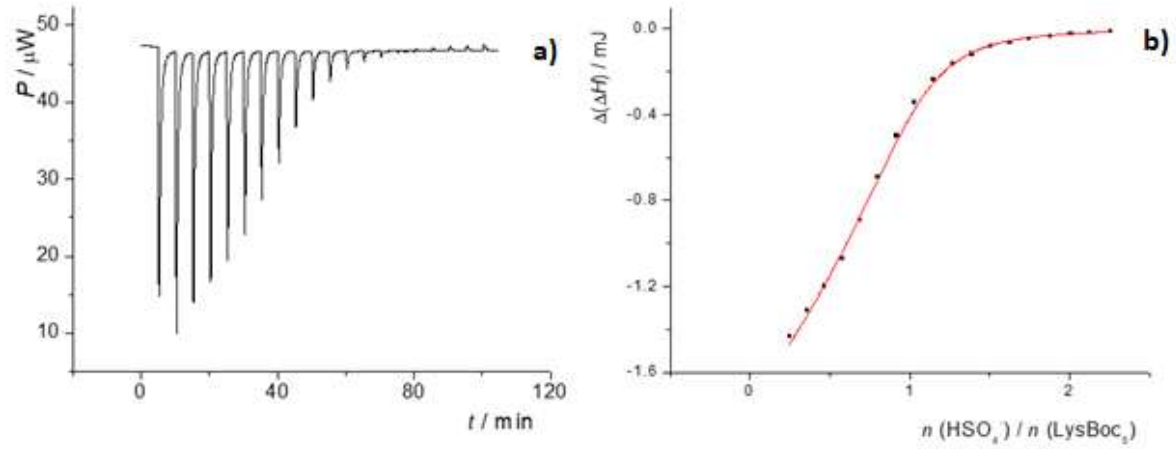


Figure 40. ***a)*** *Microcalorimetric titration of c[Lys(Boc)$_5$] (c = 5,110 · 10^{-4} mol dm^{-3}) with TBAHSO$_4$ (c = 5,070 · 10^{-3} mol dm^{-3}) in acetonitrile at ϑ = 25 ° C.* ***b)*** *Dependence of successive enthalpy change on n(HSO$_4^-$) / n(c[Lys(Boc)$_5$]) ratio. ■ measured values, — calculated values.*

The ITC isotherm obtained by titrating ***L5*** with $TBAH_2PO_4$ in acetonitrile (Figure 41) is of characteristic shape for strong binding event and has an inflection point at the value of $n(H_2PO_4^-)/n(\mathbf{L5})$ ratio being ≈ 2. This is a clear indicator for the formation of $\mathbf{L5}\cdot(H_2PO_4)_2^{2-}$ complex.

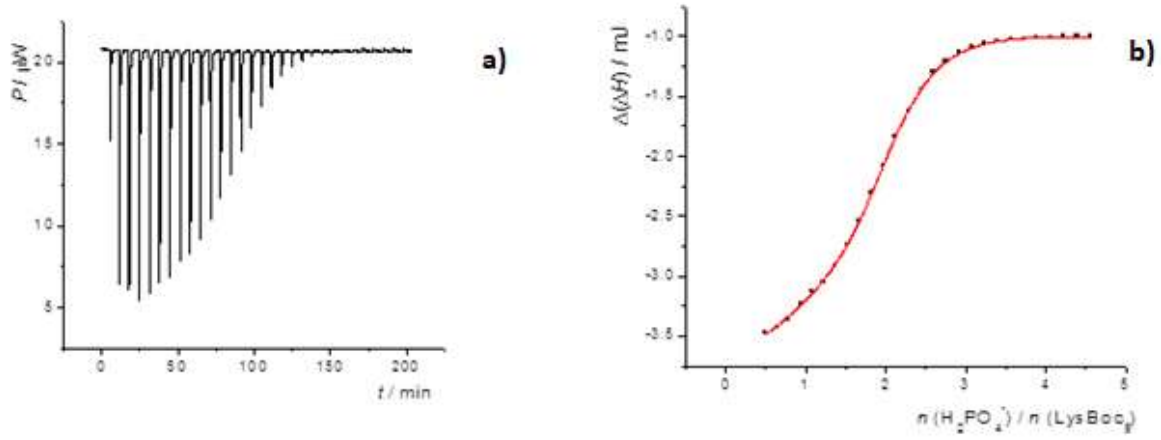


Figure 41. ***a)*** *Microcalorimetric titration of c[Lys(Boc)$_5$] (c = 4,827 · 10^{-4} mol dm^{-3}) with $TBAH_2PO_4$ (c = 9,498 · 10^{-3} mol dm^{-3}) in acetonitrile at ϑ = 25 ° C.* ***b)*** *Dependence of successive enthalpy change on n($H_2PO_4^-$) / n(c[Lys(Boc)$_5$]) ratio. ■ measured values, —calculated values.*

Ligands ***L5*** in acetonitrile forms the most stable 1:1 complex with the chloride anion, followed by hydrogensulfate and bromide anions, dihydrogenphosphate, and finally with iodide and nitrate ions (Table 5). The binding of halide anions is almost solely entropy driven which can be attributed to the desolvation of the anion upon complexation. This is most evident in the case of the formation of the complexes with bromide and iodide anions. On the other hand, the binding of structural oxoanions has a more pronounced enthalpic contribution to the standard reaction Gibbs energy, with the exception for the formation of ***L5***-nitrate complex (Table 5). In the case of $H_2PO_4^-$ anion, the formation of 1:1 complex is accompanied by a favourable enthalpy change while the complexation entropy is unfavourable. The formation of the 1:2 complex has a similar cumulative entropy change indicating that the complexation of the second anion by 1:1 complex is isoentropic. Successive equilibrium constants for the formation of both 1:1 and 1:2 complex were determined from the titrations data, being log K_1 = 4,04 for the formation of 1:1 complex and log K_2 = 4.57 for the 1:2 complex. It should be noted that the

second constant is larger than first one, indicating the cooperative binding of the second dihydrogenphosphate anion. In the model used for the titration data processing, the dimerization of $H_2PO_4^-$ was taken into account.[117]

b) NMR measurements

Addition of 1 equivalent of TEACl to a solution of ***L5*** in deuterated acetonitrile induced a downfield shift of NH amide signals by ≈ 1 ppm and of α-CH signals by ≈ 0.3 ppm while no shift was observed for NH-Boc signals (Figure 42). This indicates a very strong binding of Cl^- by NH amide group while NH-Boc does not contribute to the interaction, which is in agreement with results obtained by MD simulations (Figure 56). Further addition of TEACl did not cause shift of signals. A very rough estimation of the maximum value of the stability constant of the 1: 1 complexes that can be reliably determined by NMR is 10^4. The stability constant for chloride binding is log K_1 = 5.72 and therefore could not be determined by NMR titrations.

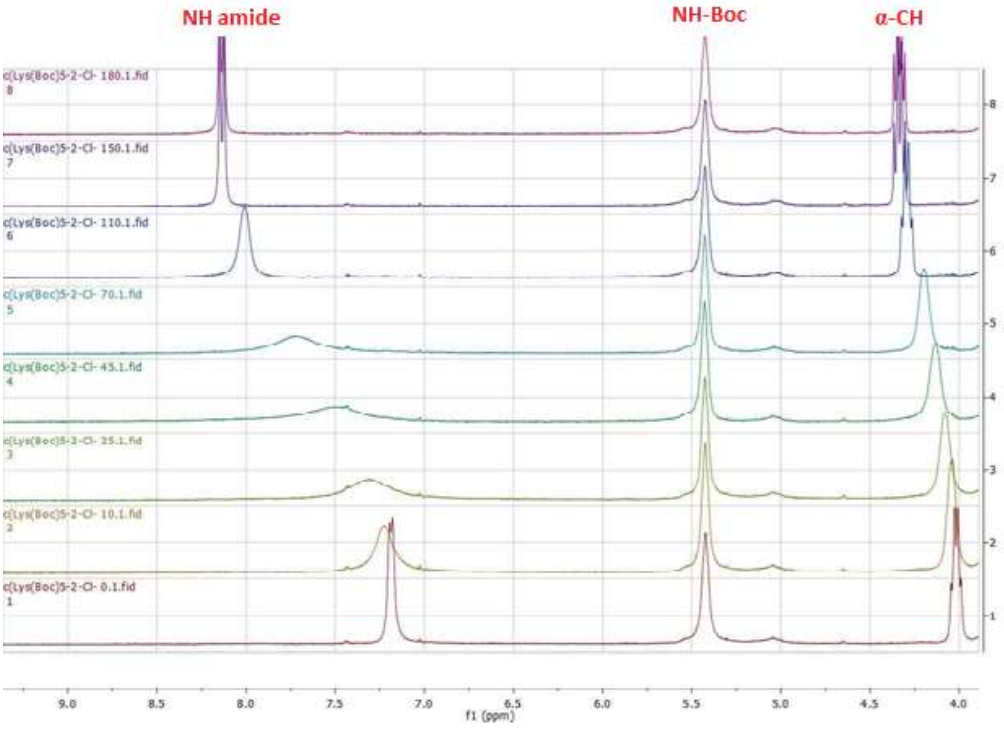


Figure 42. ^{1}H NMR titration of c[Lys(Boc)]$_5$ (c = 0,003 mol dm^{-3}) with TEACl (c = 0,01 mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

Analogously to titration with TEACl, by adding TBABr (Figure 43), TBAI (Figure 44) and $TBANO_3$ (Figure 45) to a solution of ***L5*** in deuterated acetonitrile a downfield shifts of NH amide signals (≈ 0.65, ≈ 0.35 and 0.25 ppm, respectively) of α-CH signals (≈ 0.3 ppm) was observed, whereas no shift of NH-Boc signals occured. This confirms that interaction of the cyclopeptide with halide ions involves NH groups of the peptide backbone, whereas NH-Boc does not contribute to complex formation.

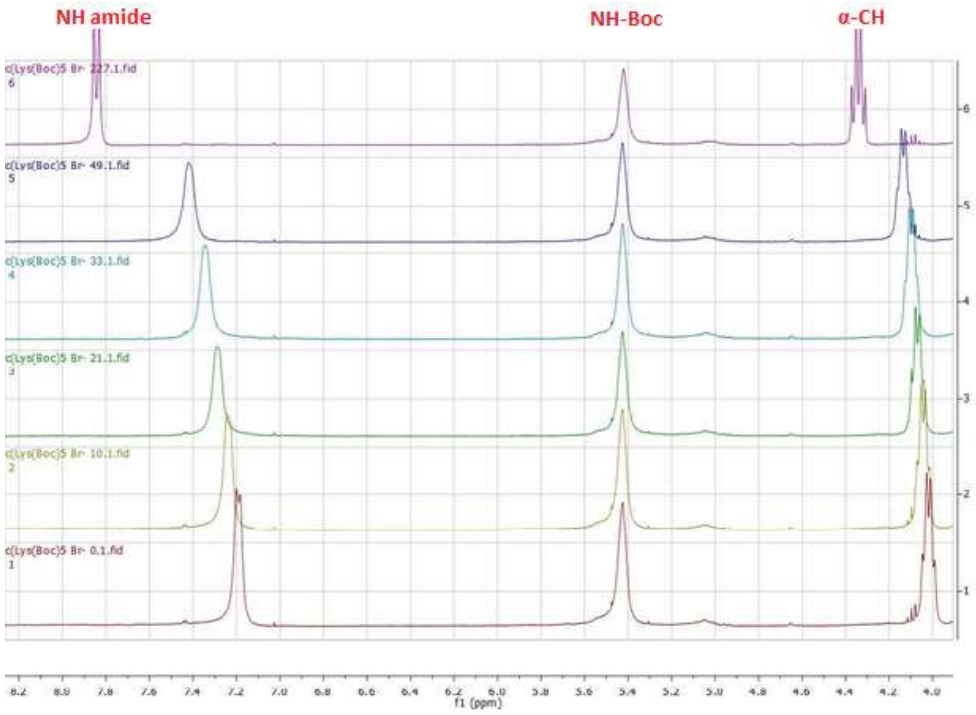


Figure 43. ^{1}H NMR titration of c[Lys(Boc)]$_5$ (c = 0,003 mol dm^{-3}) with TBABr (c = 8,79 · 10^{-3} mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

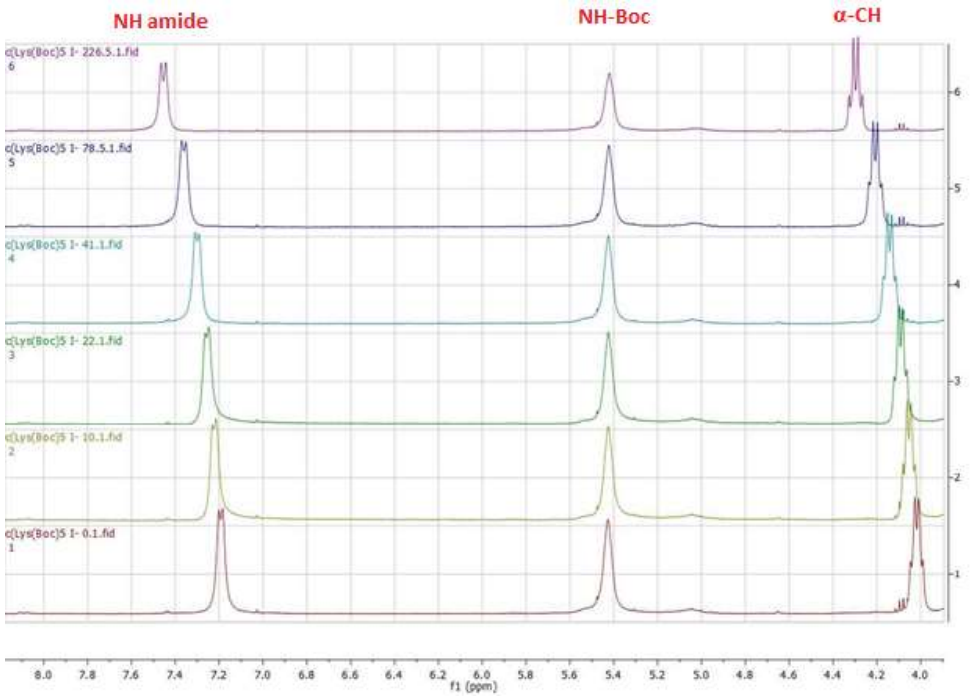


Figure 44. ^{1}H NMR titration of c[Lys(Boc)]$_5$ (c = 0,003 mol dm^{-3}) with TBAI (c = 0,021 mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

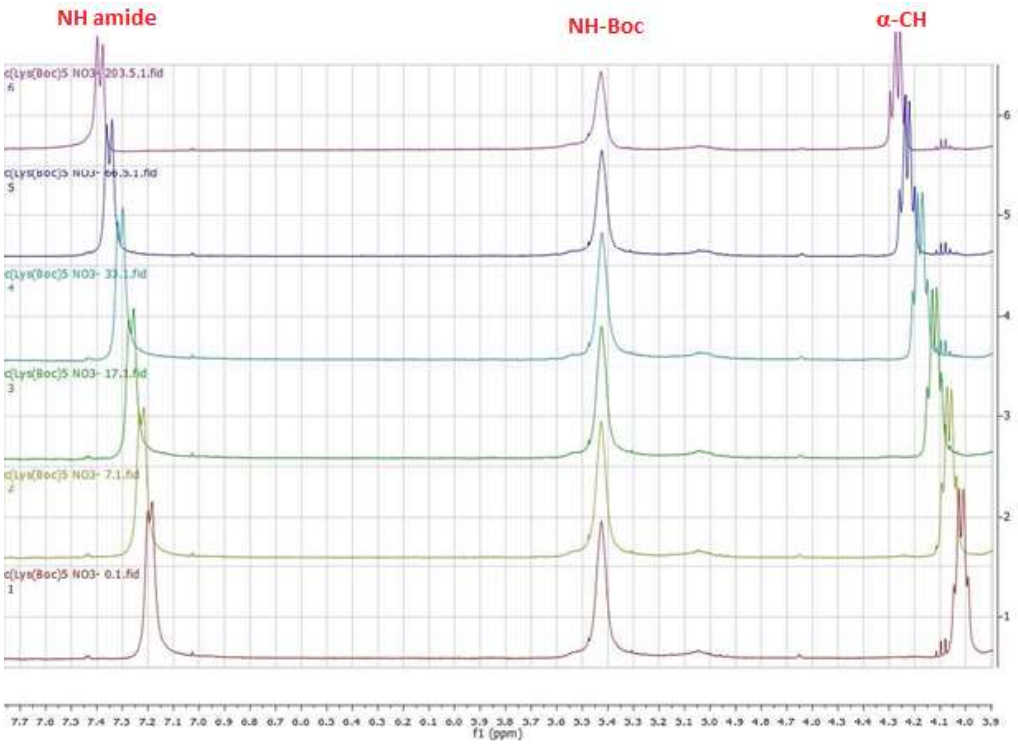


Figure 45. ^{1}H NMR titration of c[Lys(Boc)]$_5$ (c = 0,003 mol dm^{-3}) with TBANO$_3$ (c = 0,047 mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

By contrast, addition of TBAOAc (1 eq.), $TBAHSO_4$ (1.3 eq.) and $TBAH_2PO_4$ (1.3 eq.) to a solution of ***L5*** in deuterated acetonitrile (Figures 46-48) causes the appearance of a new set of signals growing in intensity, while simultanously resonances of the free ligand decrease and broaden. This indicates that the formation of the complex is slow on the NMR time scale. Both NH amide signals and α-CH signals exhibited a downfield shift, which is very significant in the case of H_2PO_4 (about 2 ppm). Moreover, signals of NH-Boc groups also appeared slightly downfield shifted indicating that they contribute to AcO^-, HSO_4^- and $H_2PO_4^-$ anions complexation.

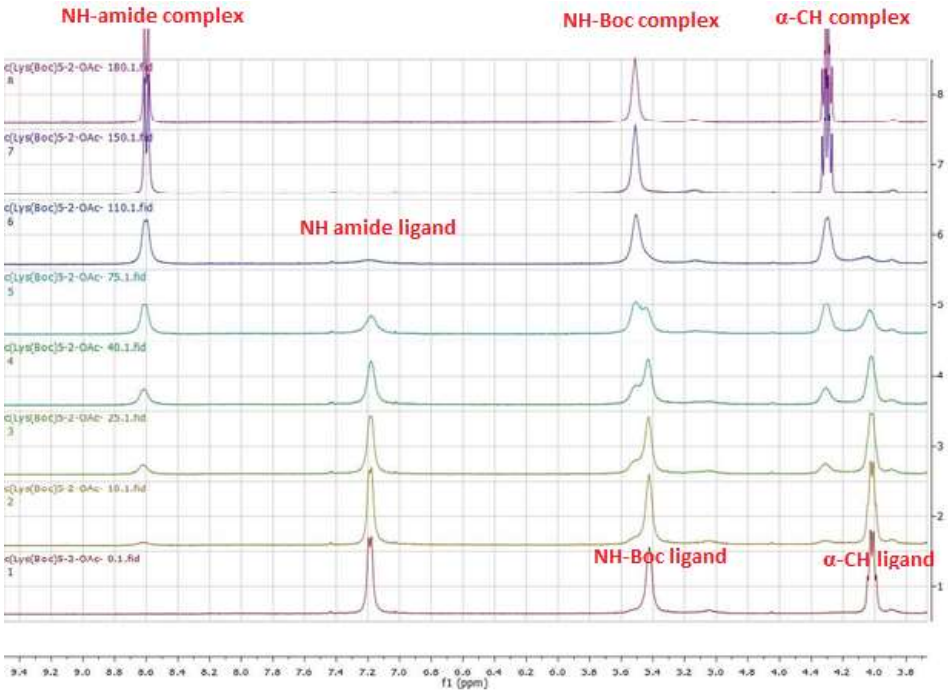


Figure 46. ^1H NMR titration of c[Lys(Boc)]$_5$ (c = 0,003 mol dm^{-3}) with TBAOAc (c = 0,01mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

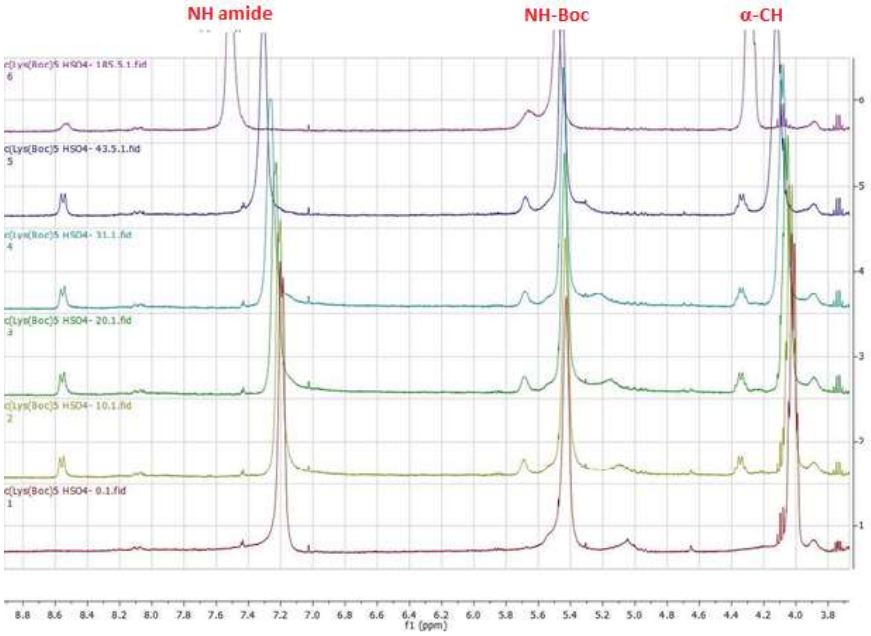


Figure 47. ^{1}H NMR titration of c[Lys(Boc)]$_5$ (c = 0,003 mol dm-3) with TBAHSO$_4$ (c = 0,011 mol dm-3) in deuterated acetonitrile at θ = 25 °C.

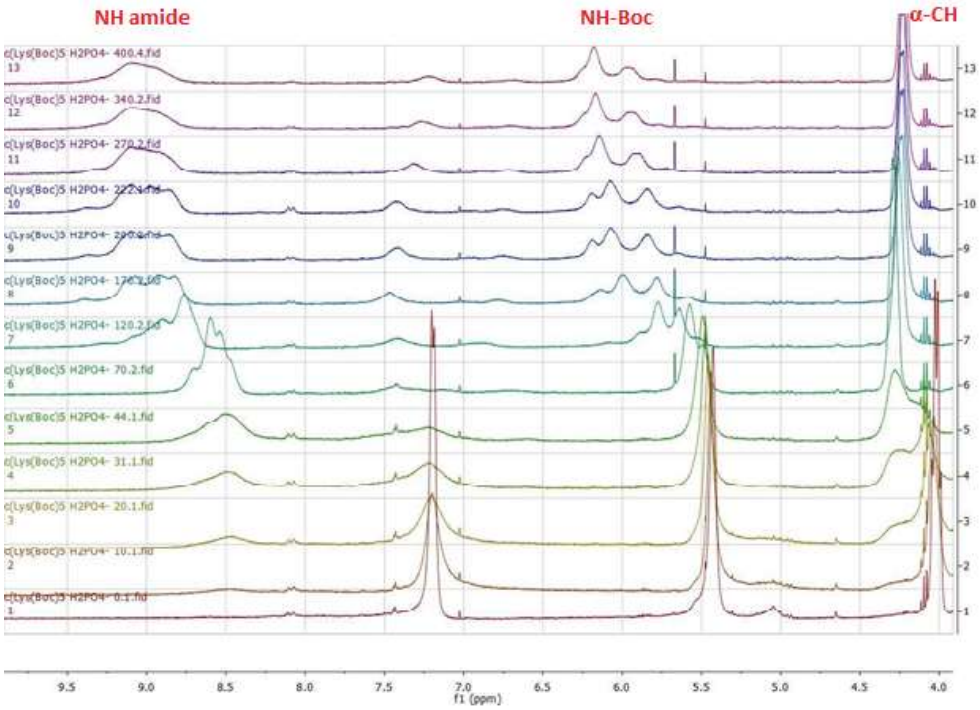


Figure 48. ^{1}H NMR titration of c[Lys(Boc)]$_5$ (c = 0,003 mol dm^{-3}) with TBAH$_2$PO$_4$ (c = 0,011 mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

c) ESI titrations

The ESI mass spectra (negative mode) of ***L5*** in acetonitrile containing 0.5, 1 and 2 equivalents of TEACl, TBABr, TBAI, $TBANO_3$ and TBAOAc show two main peaks (Figures 49-53). One signal belongs to the 1:1 complex of ***L5*** with corresponding anion and the other one to the ***L5*** complex with formic acid. No evidence for presence of higher complexes could be detected.

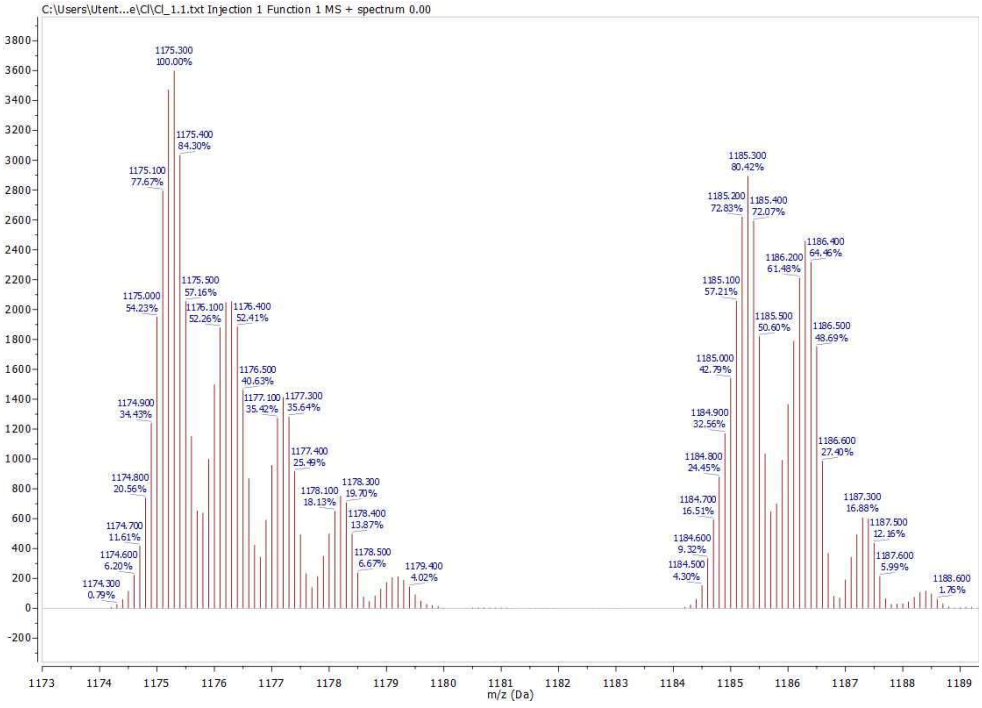


*Figure 49. ESI MS spectrum (negative mode) of **L5** after addition of 1 equivalent of TEACl. Signals assigned to 1:1 complex **L5**·Cl^- and complex with formic acid are shown.*

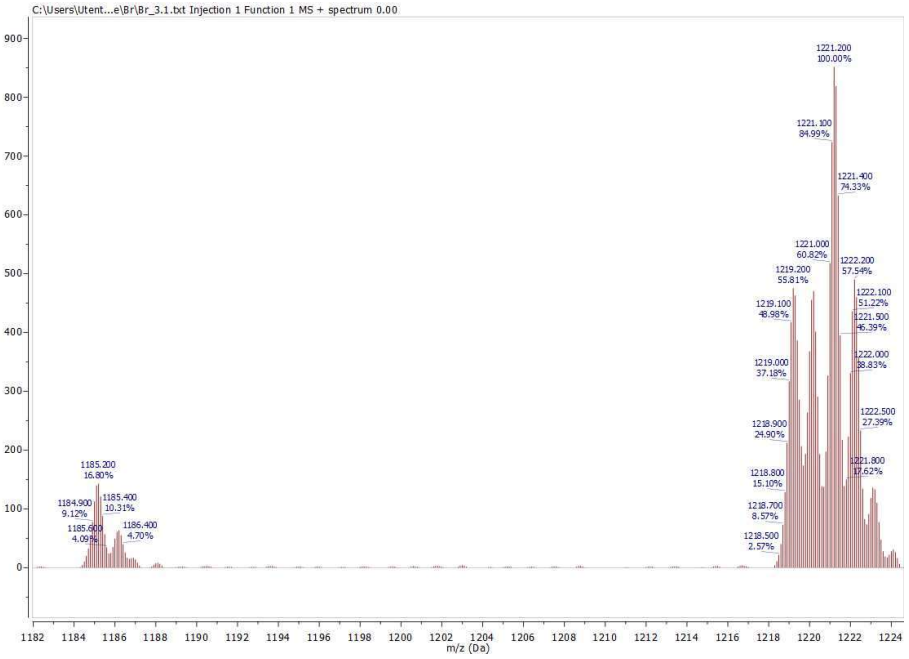


*Figure 50. ESI MS spectrum (negative mode) of **L5** after addition of 1 equivalent of TBABr. Signals assigned to complex with formic acid and 1:1 complex **L5**·Br⁻ are shown.*

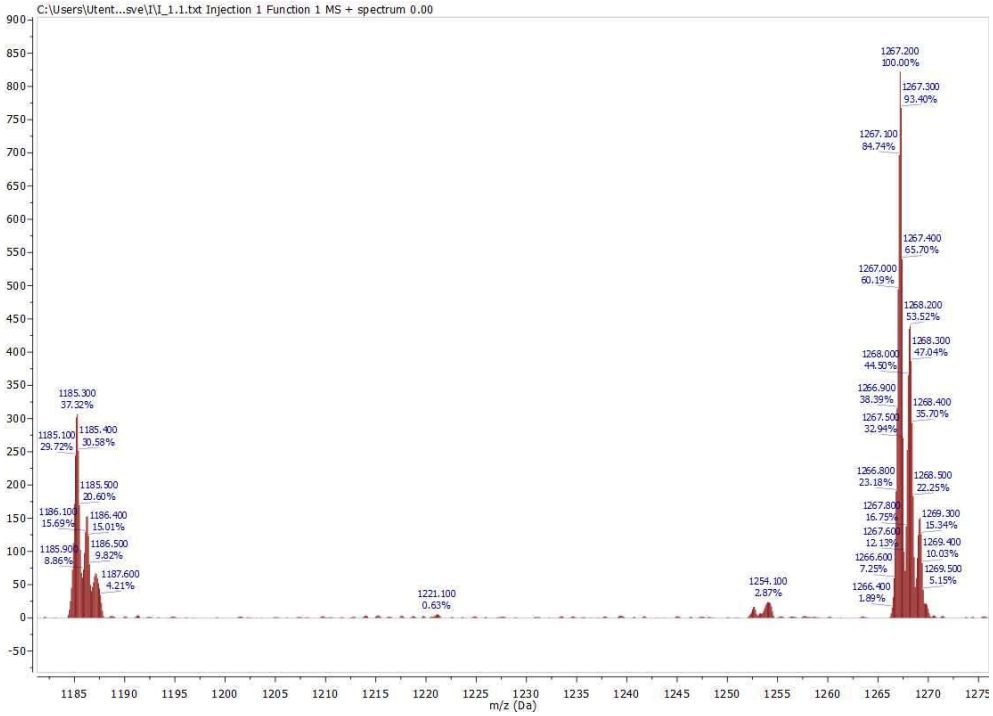


*Figure 51. ESI MS spectrum (negative mode) of **L5** after addition of 1 equivalent of TBAI. Signals assigned to a complex with formic acid and 1:1 complex **L5**·I⁻ are shown.*

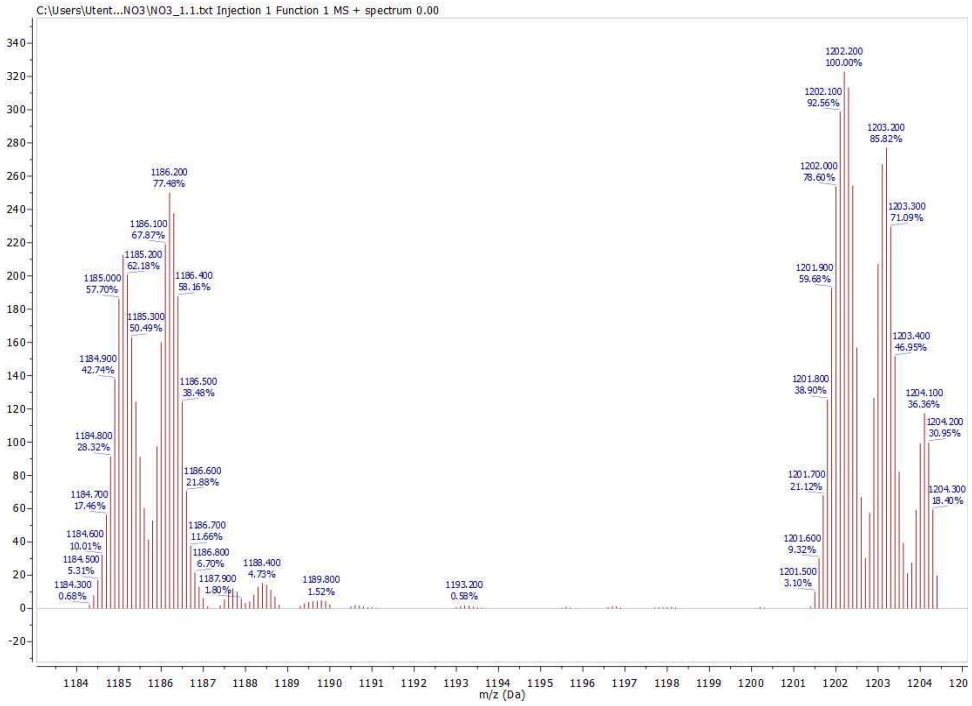


*Figure 52. ESI MS spectrum (negative mode) of **L5** after addition of 0.5 equivalents of $TBANO_3$. Signals assigned to a complex with formic acid and 1:1 complex **L5**·NO_3^- are shown.*

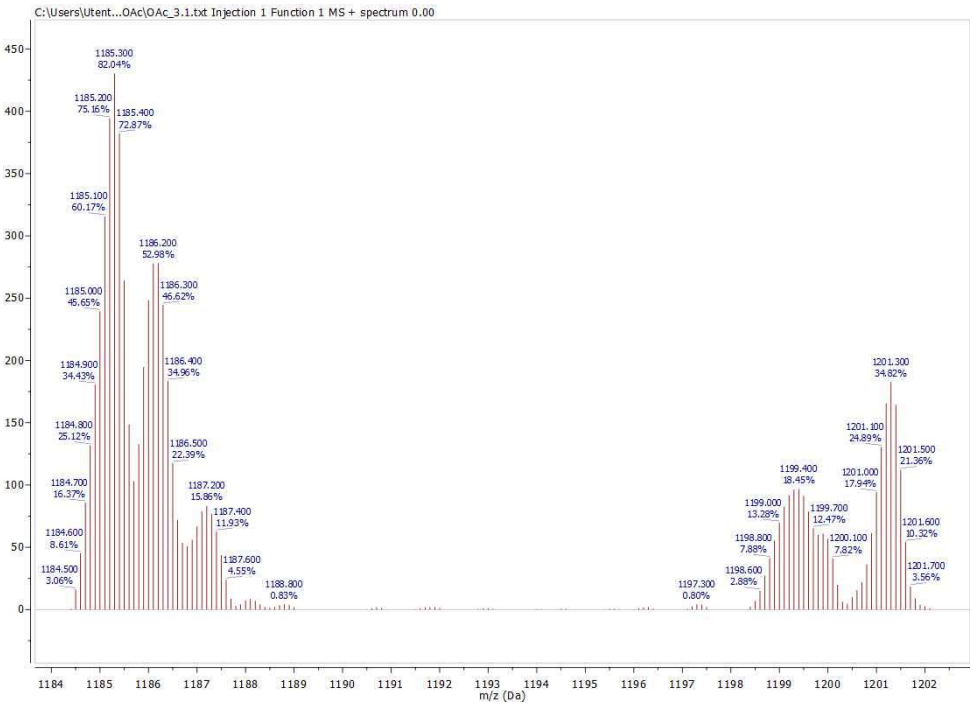


*Figure 53. ESI MS spectrum (negative mode) of **L5** after addition of 2 equivalents of TBAOAc. Signals assigned to a complex with formic acid and 1:1 complex **L5**·OAc^- are shown.*

The ESI mass spectra (negative mode) containing $TBAHSO_4$ show five signals. Two signals belong to the single and doubly charged 1:1 complex ***L5***·HSO_4^-, one to the ***L5*** complex with formic acid, one to the doubly charged 2:1 complex ***L5***·HSO_4^- and the fifth one to the 1:1 complex with one TBA cation (Figure 54).

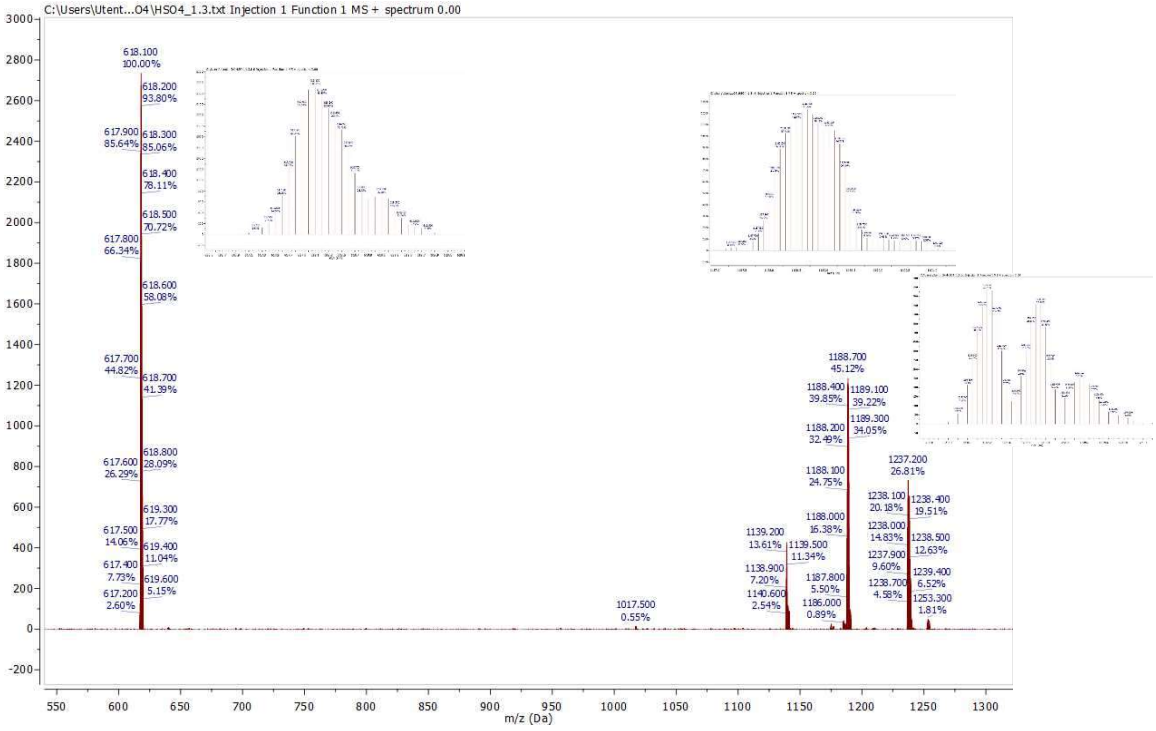


*Figure 54. ESI MS spectrum (negative mode) of **L5** after addition of 1 equivalent of $TBAHSO_4$. Signals assigned to a doubly charged 1:1 complex **L5**·HSO_4^-, doubly charged 2:1 complex **L5**·HSO_4^-, 1:1 complex **L5**·HSO_4^- are shown.*

Similarly, spectra after addion of $TBAH_2PO_4$ show three signals: complex with formic acid, 1:1 complex ***L5***·$H_2PO_4^-$ and doubly charged 2:1 complex ***L5***·$H_2PO_4^-$ (Figure 55).

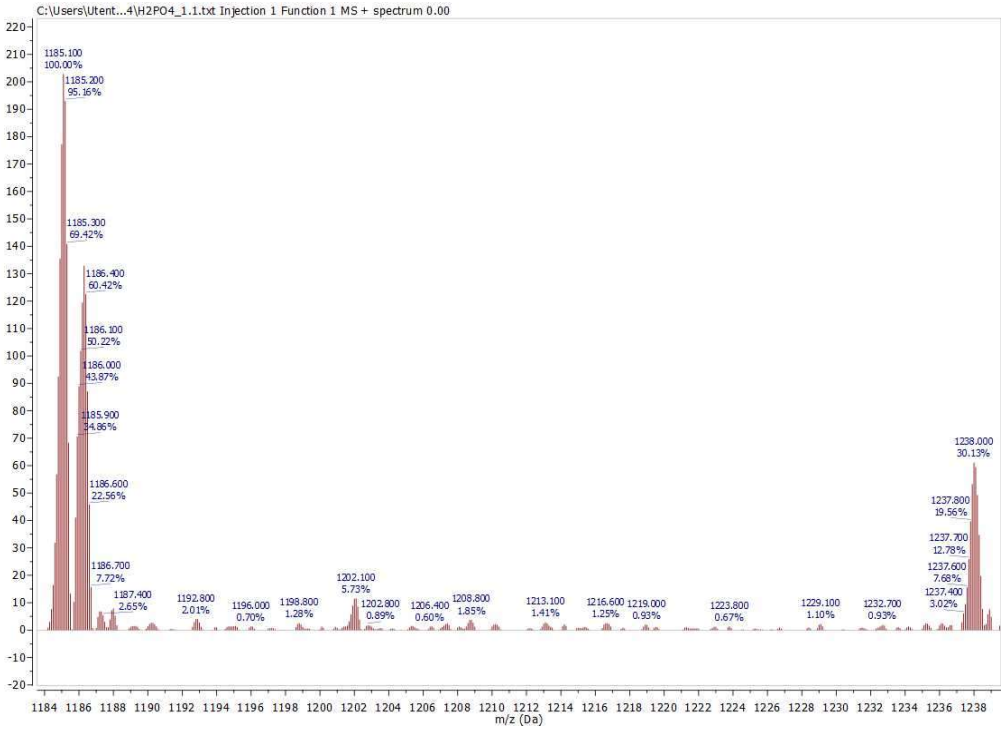


*Figure 55. ESI MS spectrum (negative mode) of **L5** after addition of 1 equivalent of $TBAH_2PO_4$. Signals assigned to a complex with formic acid and 1:1 complex **L5**·$H_2PO_4^-$ are shown.*

*Table 3. **L5**-anion complexes in ESI-MS spectrum (negative mode).*

	Ion (ESI-)	m/z
No salt	$[M+FA]^-$	1185.1
Cl^-	$[M+Cl]^-$	1175.2
Br^-	$[M+Br]^-$	1218.9
I^-	$[M+I]^-$	1267.2
NO_3^-	$[M+NO_3]^-$	1203.3
OAc^-	$[M+OAc]^-$	1201.9
HSO_4^-	$[M+HSO_4-1]^{2-}$	617.9
	$[2M+HSO_4-1]^{2-}$	1188.9
	$[M+HSO_4]^-$	1237.2
	$[M+HSO_4]^- TBA^+$	1479.3
$H_2PO_4^-$	$[2M+H_2PO_4-1]^{2-}$	1189.5
	$[M+H_2PO_4]^-$	1238.2

d) Molecular dynamics simulations

The structure of ***L5***-anion 1:1 complexes (Figures 56-59) were obtained by molecular dynamics simulations in acetonitrile with explicit solvent molecules. In the MD simulations studied anions were bound by all five amide protons of ***L5*** (Table 4) and in some cases with the protons from the NH Boc groups (AcO^-, HSO_4^- and H_2PO_4-). These results are backed by NMR spectra of the complexes from which the participation of NH Boc groups in the anion binding can be clearly deduced (Figures 46-48). Structures and stabilities of complexes with higher stoichiometries are under further investigation.

*Table 4: The average number of bonds formed between protons of **L5** and complexed anion in acetonitrile obtained by MD simulations.*

	N(amide-anion bonds)	*N*(carbamate-anion bonds)
Cl^-	5.0	0.1
Br^-	5.0	0.1
I^-	5.0	0.5
NO_3^-	7.4	1.4
OAc^-	7.32	0.85
HSO_4^-	7.2	2.4
$H_2PO_4^-$	7.4	2.4

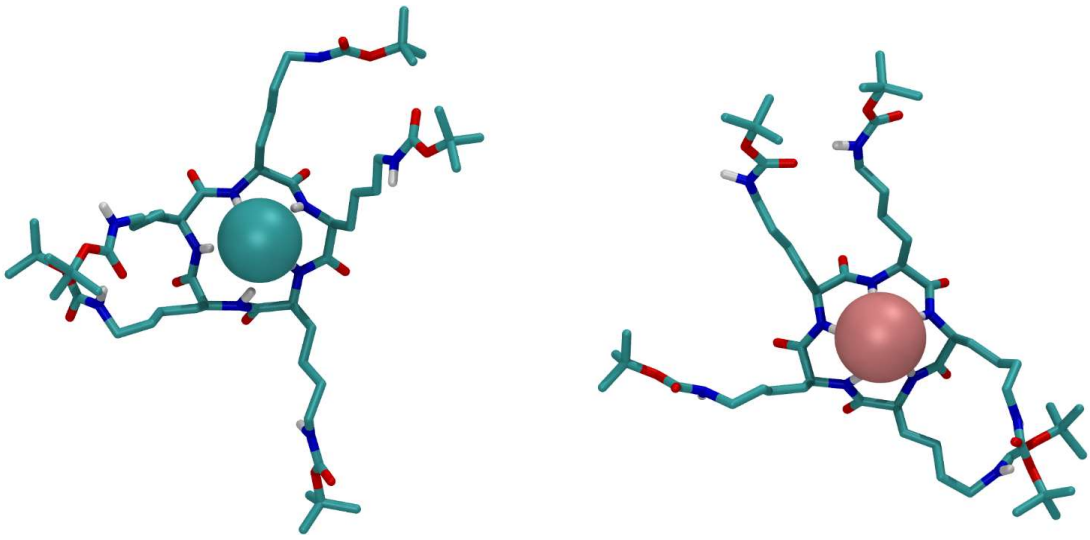


*Figure 56. Structures of a complexes of c[Lys(Boc)]$_5$ with Cl^- (**left**) and Br^- (**right**) obtained by MDs simulations in acetonitrile at 25 °C.*

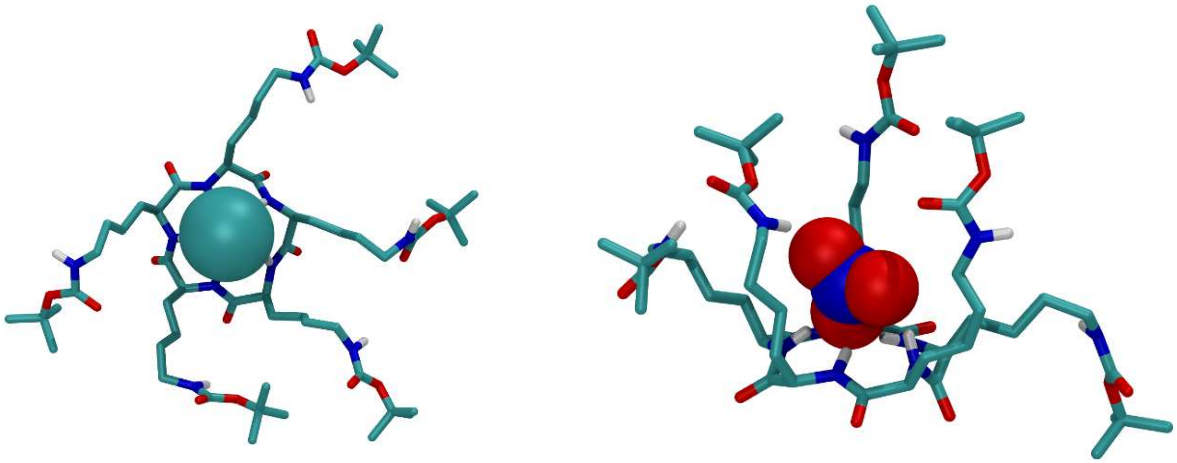


*Figure 57. Structures of a complexes of c[Lys(Boc)]$_5$ with I^- (**left**) and NO_3^- (**right**) obtained by MDs simulations in acetonitrile at 25 °C.*

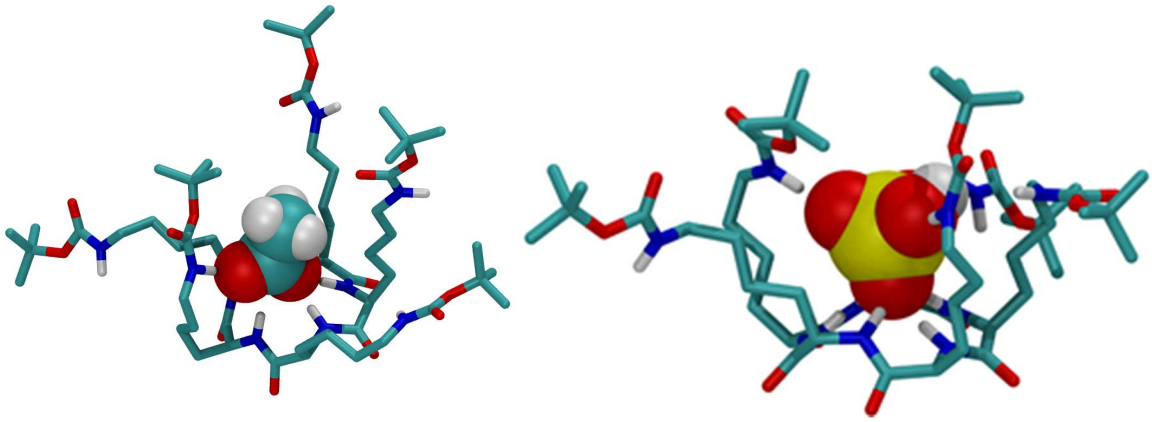


*Figure 58. Structures of a complexes of c[Lys(Boc)]$_5$ with AcO^- (**left**) and HSO_4^- (**right**) obtained by MDs simulations in acetonitrile at 25 °C.*

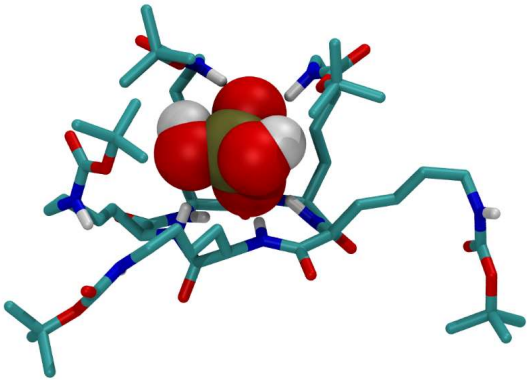


Figure 59. Structures of a complexes of c[Lys(Boc)]$_5$ with $H_2PO_4^-$ obtained by MDs simulations in acetonitrile at 25 °C.

*Table 5. Thermodynamic parameters for complexation reaction of cyclic pentalysine **L5** with different anions in acetonitrile at 25 ° C.*

solvent	anion	log β_n[a] ± SE	($\Delta_r G^\Theta$ ± SE) / kJ mol^{-1}	($\Delta_r H^\Theta$ ± SE) / kJ mol^{-1}	($\Delta_r S^\Theta$ ± SE) / J mol^{-1} K^{-1}
MeCN	Cl^-	5,72 ± 0,02[b] (1:1) > 4[c] (1:1)	-32,7 ± 0,1	-8,6 ± 0,1	80,6 ± 0,3
	Br^-	4,95 ± 0,05 (1:1) 9,05 ± 0,07 (1:2) > 4[c] (1:1)	-28,3 ± 0,3 -51,6 ± 0,4	-4,2 ± 0,1 -3,98 ± 0,06	80,8 ± 0,3 159,7 ± 0,4
	I^-	- [d] 2,90 ± 0,02[c] (1:1)	-16,55 ± 0,04	≈ 0	55,5 ± 0,1
	NO_3^-	2,79 ± 0,01[b] (1:1) 3,05 ± 0,03[c] (1:1)	-15,86 ± 0,08 -17,4 ± 0,2	-6,0 ± 0,2	33 ± 1
	$H_2PO_4^-$	4,04 ± 0,02[c] (1:1) 8,607 ± 0,005[f] (1:2) > 4[b] (1:1)	-23,03 ± 0,09 -49,13 ± 0,03	-49,7 ± 0,9 -75,9 ± 0,2	-89,5 ± 0,9 -89,8 ± 0,2
	HSO_4^-	4,98[b] (1:1) 8,24[b] (2:1) > 4[c] (1:1)	-28,42 -47,03	-15,43 -29,22	43,6 59,7
	CH_3COO^-	In progress			

[a] Cumulative stability constants of 1:1 and 1:2 (ligand:anion) complexes. [b] Determined by microcalorimetric measurments, [c] Determined by ^{1}H NMR titrations, [d] Isenthalpic process,
SE = standard error of the mean (N = 3)

4.1.5.2. Cyclic hexalysine

Very similar results were obtained when the binding of cyclic hexalysine ***L6*** to the anions was investigated by ^{1}H NMR and ESI titrations. Microcalorimetric titrations are still in progress.

a) NMR titrations

Addition of TEACl to a solution of ***L6*** in deuterated acetonitrile produced downfield shifts of NH amide and α-CH signals while no shift was observed for NH-Boc signals (Figure 60). As confirmed by MD simulations (Figure 74), NH Boc does not participate in anion binding. Calculated binding constant amounts log K_1 = 3.41. The analysis of titrations with TBABr and TBAI resulted in binding constants which amount log K_1 = 2.79 and log K_1 = 1.54, respectively (Figures 61 and 62). It is worth to note that similar results were obtained also for pentapeptide, but binding constants were higher which means that pentapeptide binds more strongly these anions.

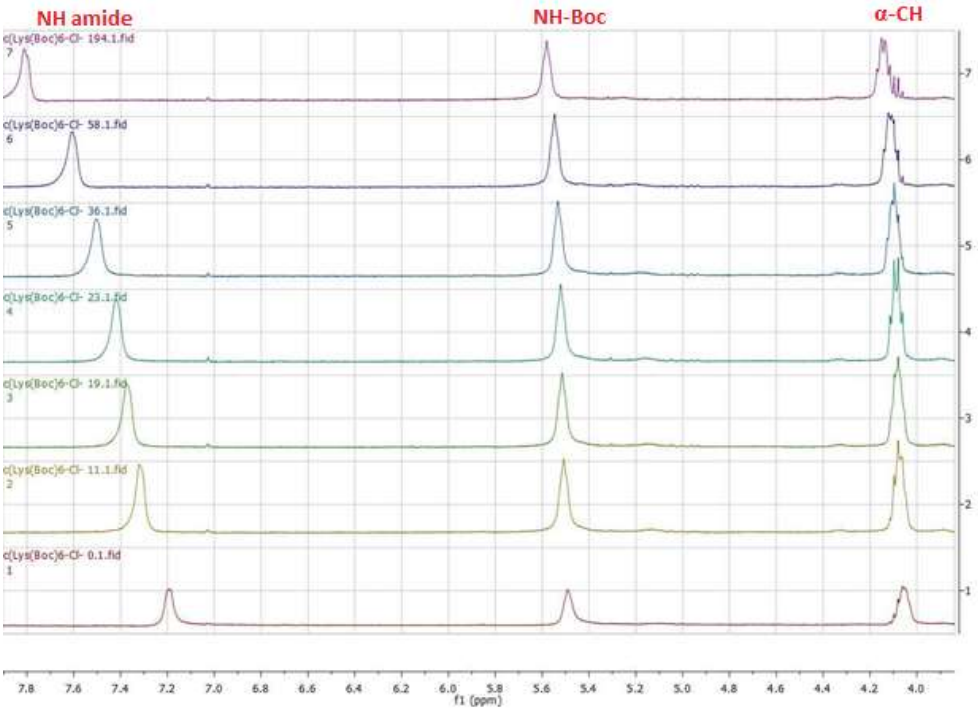


Figure 61. ^{1}H NMR titration of c[Lys(Boc)]$_6$ (c = 9,90 · 10^{-4} mol dm^{-3}) with TEACl (c = 0,01 mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

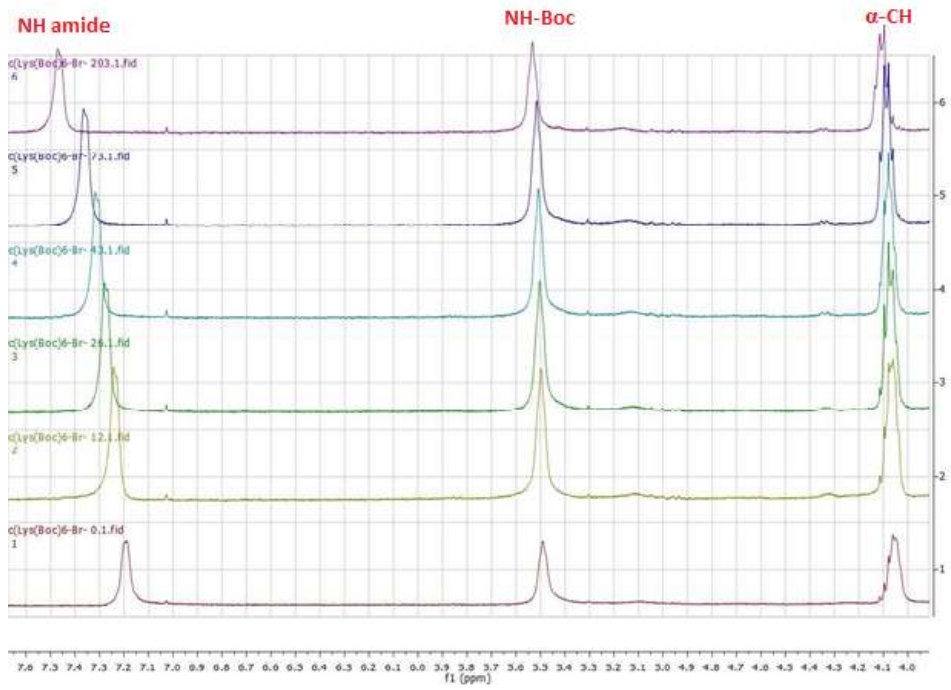


Figure 62. ^{1}H NMR titration of c[Lys(Boc)]$_6$ (c = 9,90 · 10^{-4} mol dm^{-3}) with TBABr (c = 0,02 mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

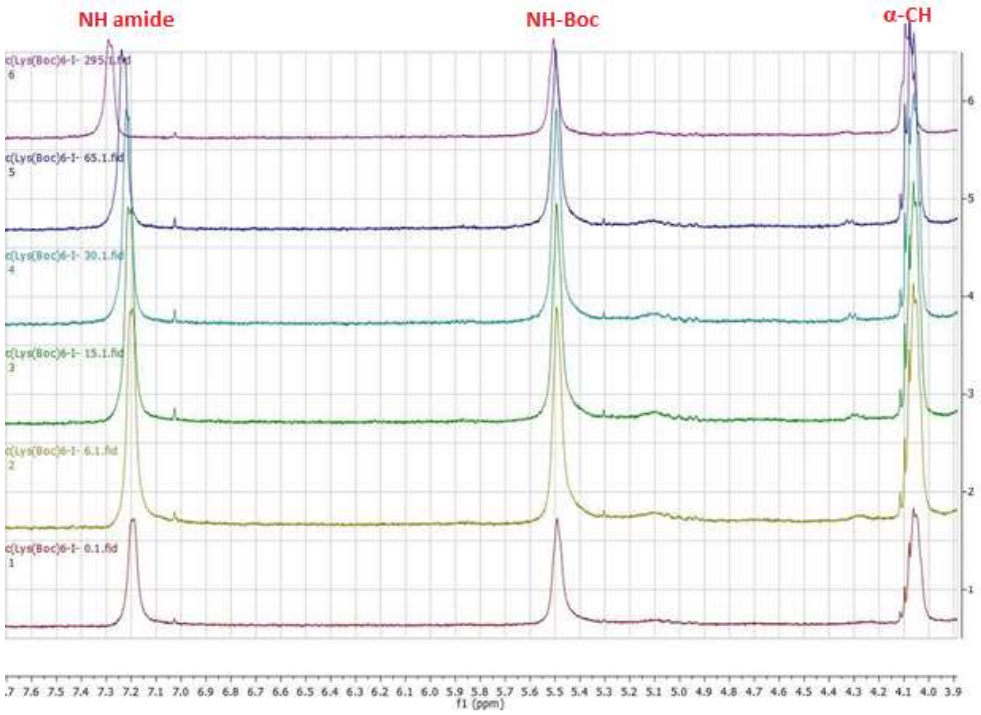


Figure 63. ^{1}H NMR titration of c[Lys(Boc)]$_6$ (c = 9,90 · 10^{-4} mol dm^{-3}) with TBAI (c = 0,0498 mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

NMR spectra recorded after the addition of TBAOAc (3.9 eq.), $TBAHSO_4$ (2.8 eq.), and $TBAH_2PO_4$ (2.2 eq.) to a solution of ***L6*** in deuterated acetonitrile show downfield shift of NH amide signals, NH-Boc signals and α-CH signals (Figures 64-66). That indicates a binding of all anions by NH amide group but with a contribution of also NH-Boc. In addition, in the case of $TBAH_2PO_4$ the appearance of a new set of signals can be noted. Binding constant calculated from the titration data corresponding to TBAOAc amounts to log $K > 4$ and indicate a very strong binding of this anion. Actually, the strongest binding of hexapeptide is with acetate anion, unlike pentapeptide which binds Cl^- more strongly. From these results we can conclude that peptides with larger binding site (like hexapeptide) bind more efficiently larger anions.

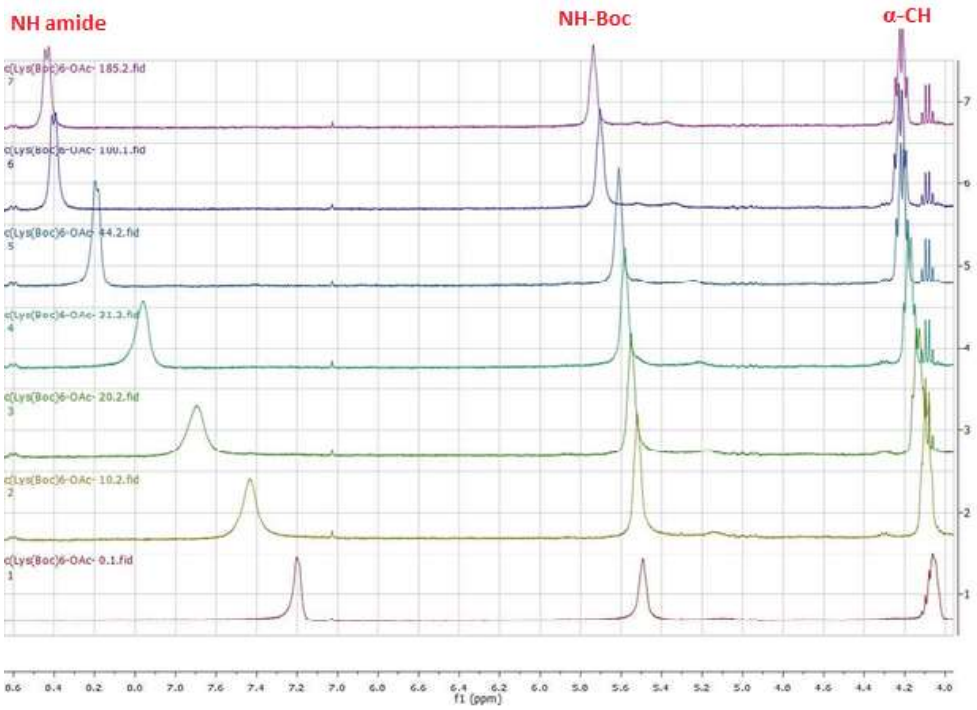


Figure 64. ^1H NMR titration of c[Lys(Boc)]$_6$ (c = 9,90 · 10^{-4} mol dm^{-3}) with TBAOAc (c = 0,0105 mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

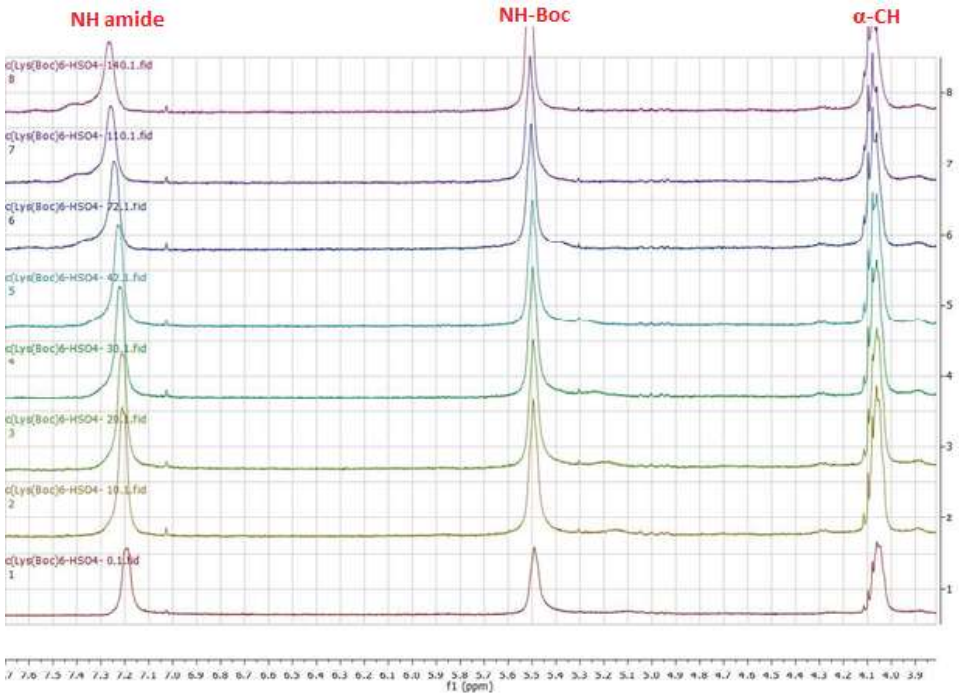


Figure 65. ^{1}H NMR titration of c[Lys(Boc)]$_6$ (c = 9,90 · 10^{-4} mol dm^{-3}) with $TBAHSO_4$ (c = 0,0102 mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

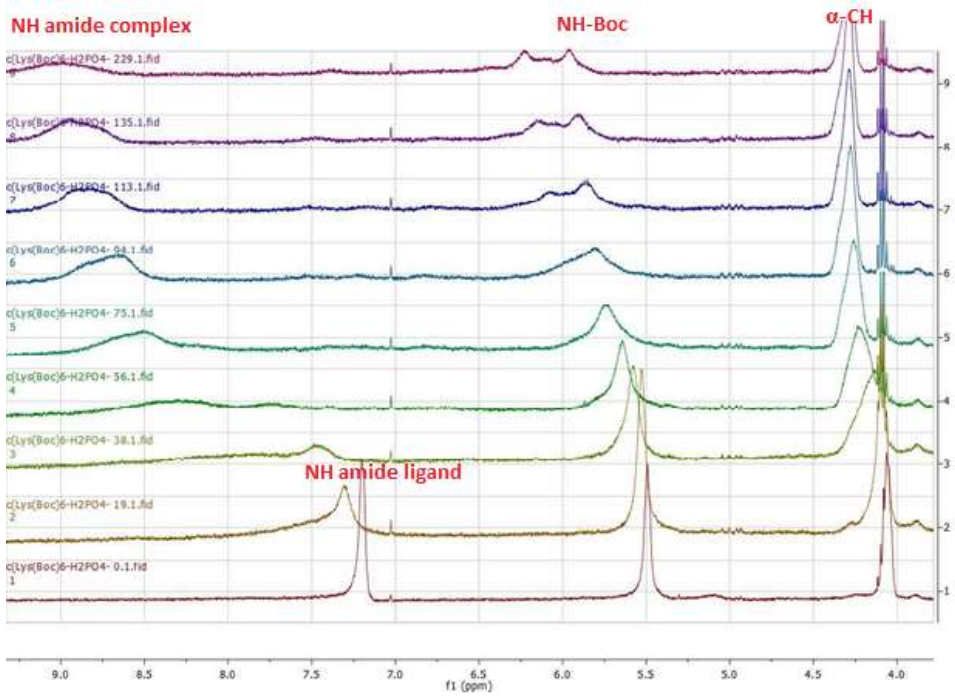


Figure 66. ^{1}H NMR titration of c[Lys(Boc)]$_6$ (c = 9,90 · 10^{-4} mol dm^{-3}) with $TBAH_2PO_4$ (c = 9,96 · 10^{-3} mol dm^{-3}) in deuterated acetonitrile at θ = 25 °C.

b) ESI titrstions

In ESI mass spectrum (negative mode), after addition of TEACl, two signals could be detected; one can be atributed to the ***L6*** complex with formic acid (Figure 67) and the other one to the 1:1 complex ***L6***·Cl^- (Figure 68). Similar results were obtained using TBABr and TBAI (Figures 69-70).

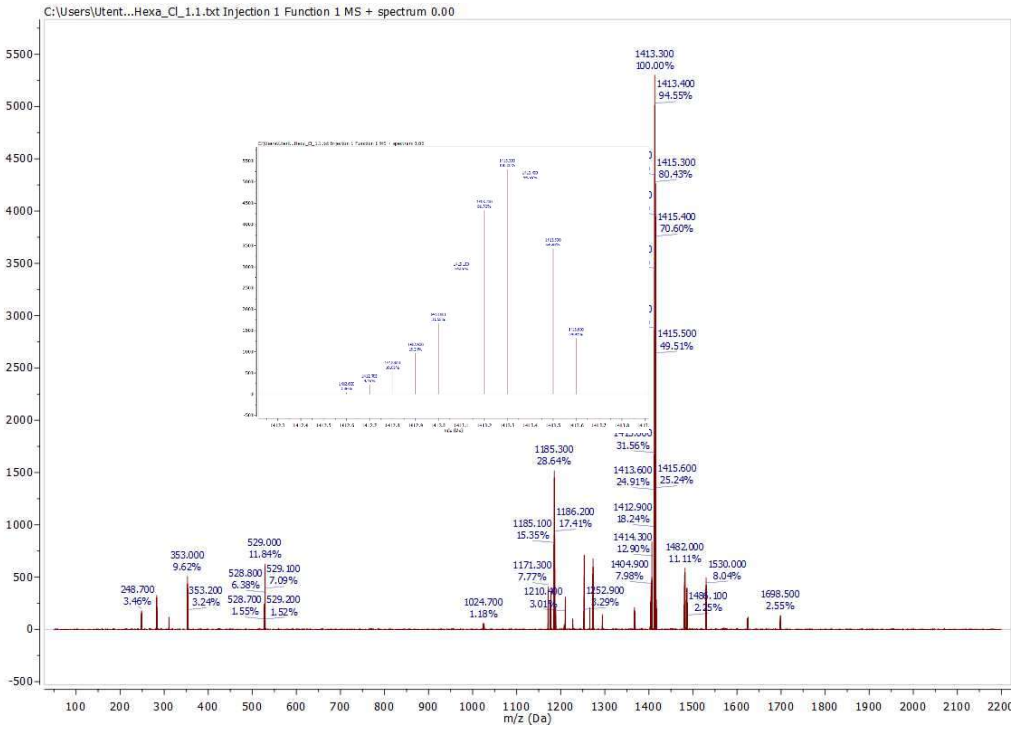


*Figure 67. ESI MS spectrum (negative mode) of **L6** ater addition of 0.5 equivalents of TEACl. Signal assigned to a complex with formic acid is shown.*

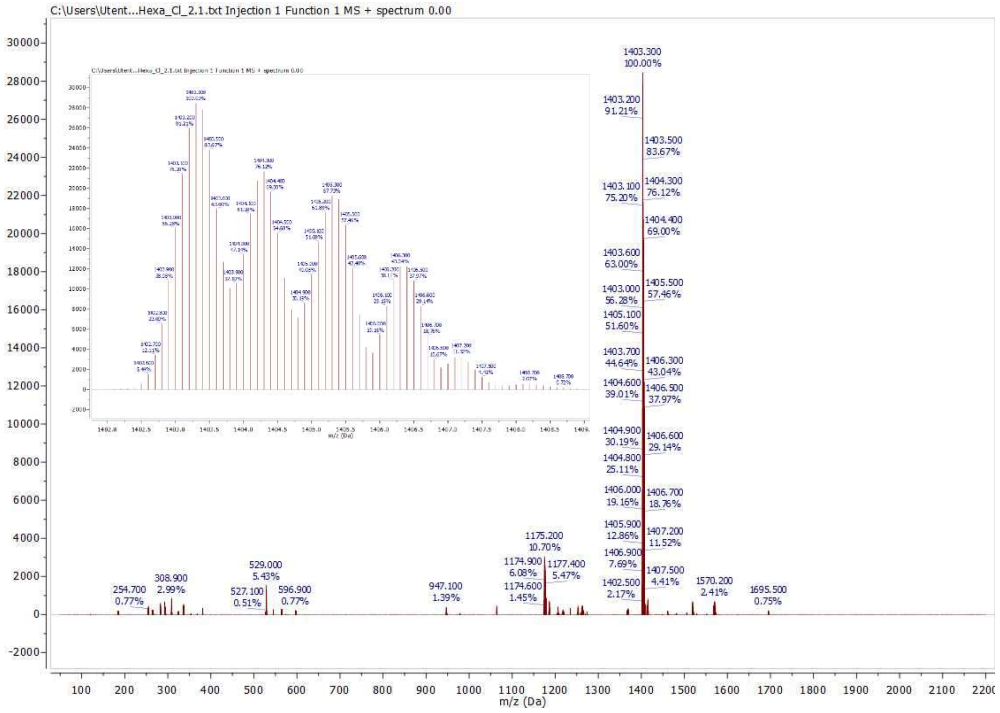


*Figure 68. ESI MS spectrum (negative mode) of **L6** ater addition of 1 and 2 equivalents of TEACl. Signal assigned to a 1:1 complex **L6**·Cl^- is shown.*

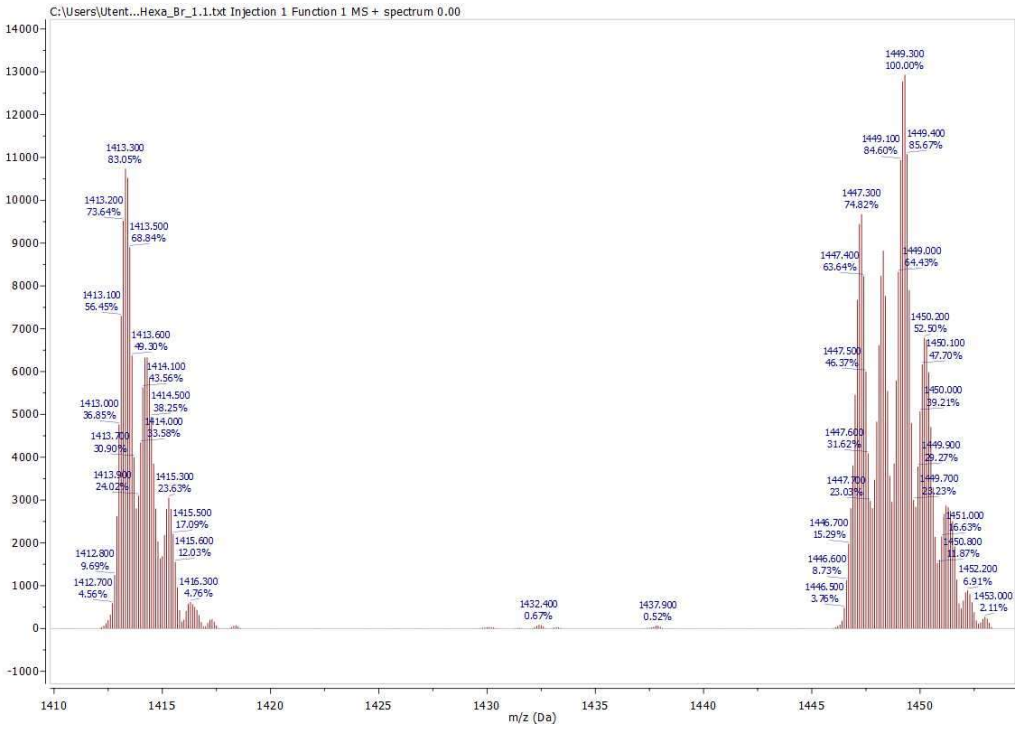


*Figure 69. ESI MS spectrum (negative mode) of **L6** ater addition of 0.5 equivalents of TBABr. Signals assigned to a complex with formic acid and 1:1 complex **L6**·Br^- are shown.*

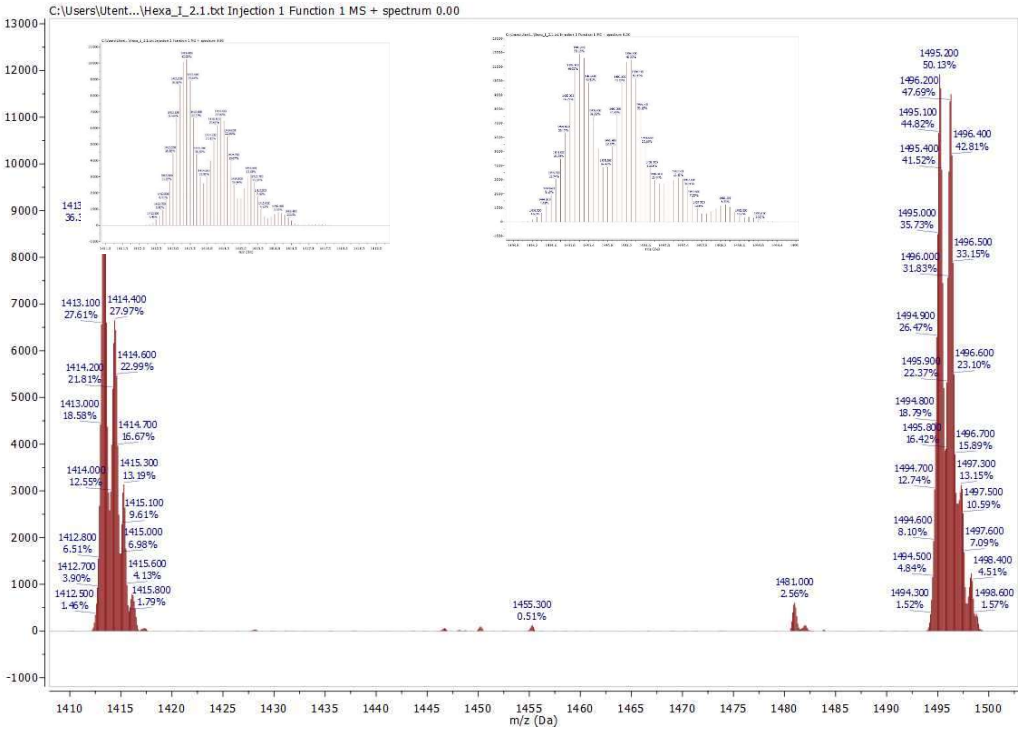


*Figure 70. ESI MS spectrum (negative mode) of **L6** ater addition of 1 equivalent of TBAI. Signals assigned to a complex with formic acid and 1:1 complex **L6**·I^- are shown.*

The ESI mass spectrum (negative mode) of ***L6*** in acetonitrile containing TBAOAc, show two main signals that can be attributed to a deprotonated ligand and complex with formic acid. Only small signals that belong to the 1:1 complex ***L6***·$OAc^-$and ***L6***·$2OAc^-$ can be detected (Figure 71). In the case of $TBAHSO_4$, four signals can be noted (Figure 72); two of them belong to the single and double charged 1:1 complex ***L6***·HSO_4^-, one to the doubly charged 2:1 complex 2***L6***·HSO_4^- and the fourth one to the 1:1 complex with one TBA cation. ESI spectra with $TBAH_2PO_4$ show the analogous signals as with HSO_4 with addition of a signal that can be attributed to the double charged 2:2 complex ***L6***·$H_2PO_4^-$ with TBA cation (Figure 73).

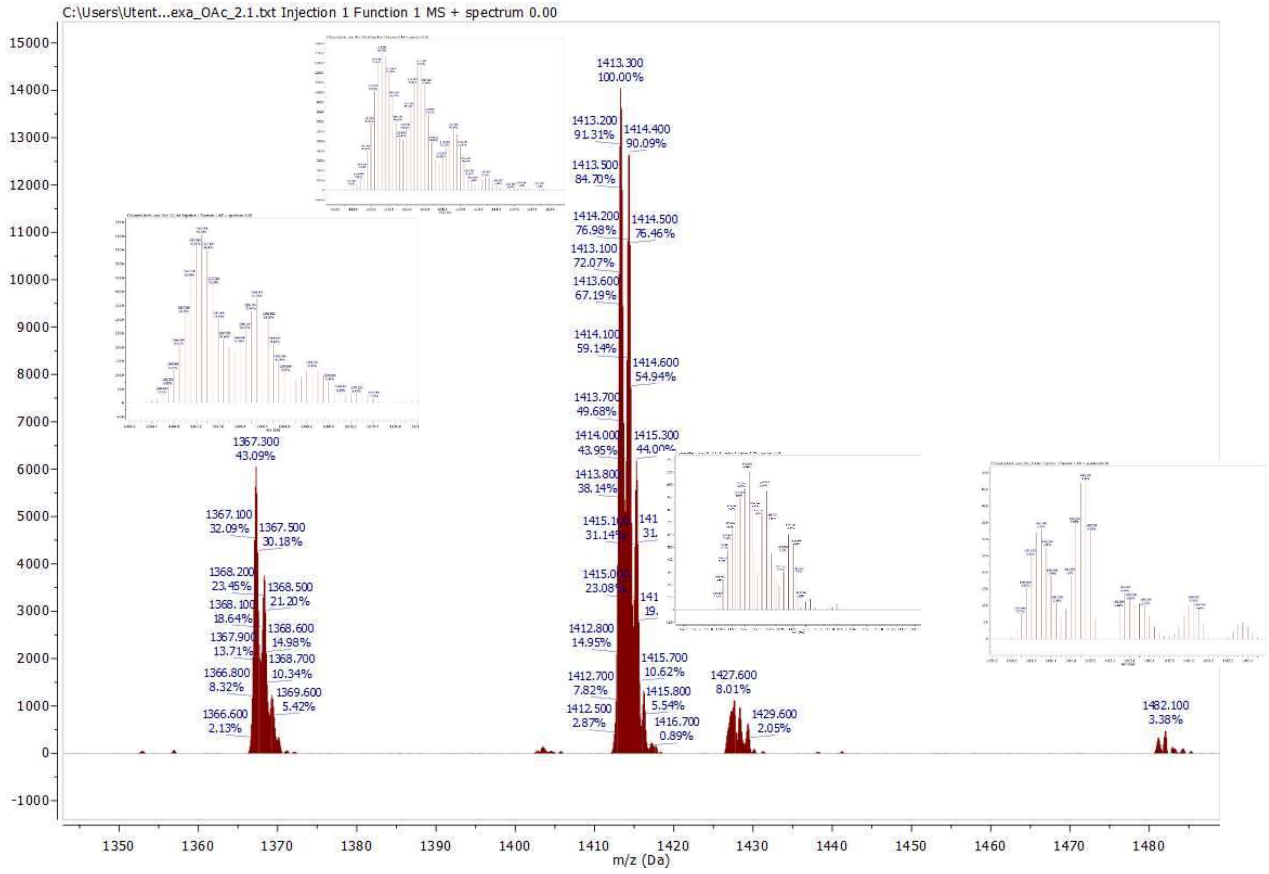


*Figure 71. ESI MS spectrum (negative mode) of **L6** ater addition of 1 equivalent of TBAOAc. Signals assigned to a deprotonated ligand, complex with formic acid, 1:1 complex **L6**·OAc⁻ and 1:2 complex **L6**·OAc⁻ are shown.*

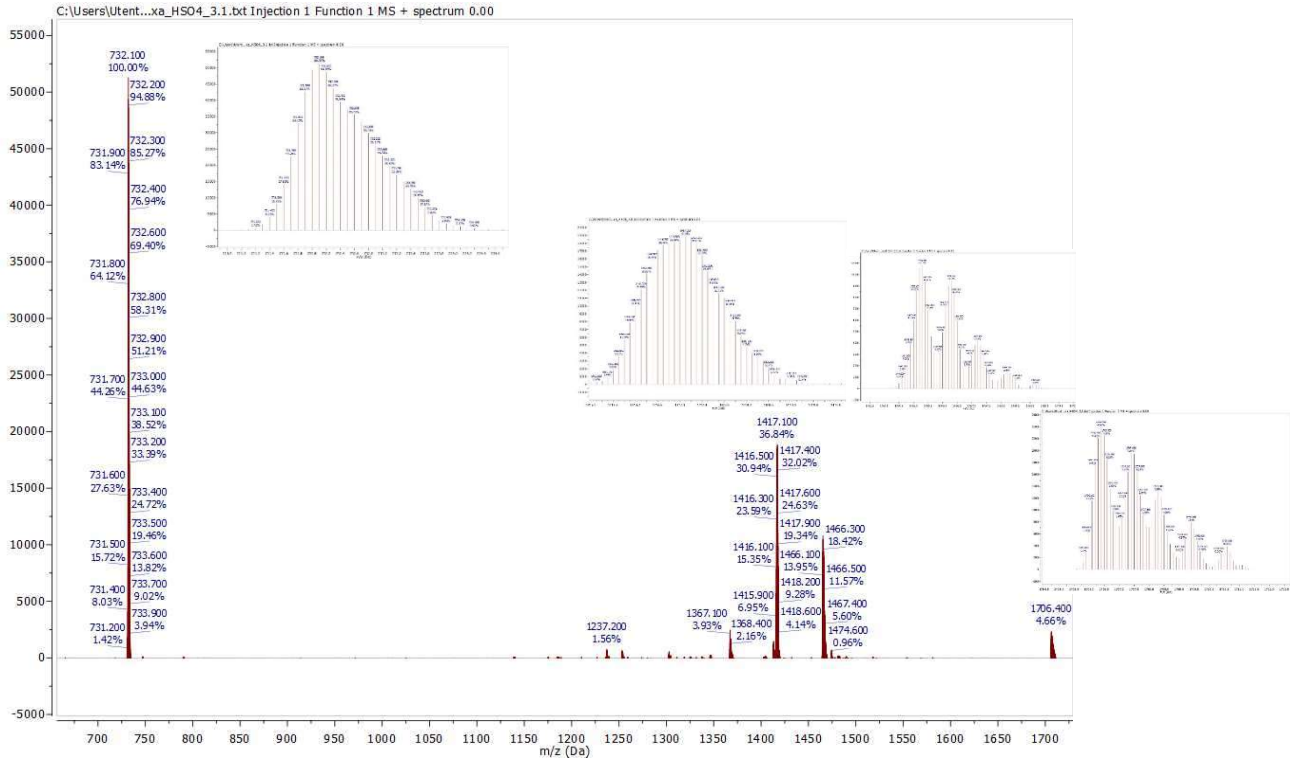


*Figure 72. ESI MS spectrum (negative mode) of **L6** ater addition of 1 equivalent of $TBAHSO_4$.Signals assigned to a doubly charged 1:1 complex **L6**·HSO_4^-, doubly charged 2:1 complex **L6**·HSO_4^-, 1:1 complex **L6**·HSO_4^- and 1:1 complex **L6**·HSO_4^- with TBA cation are shown.*

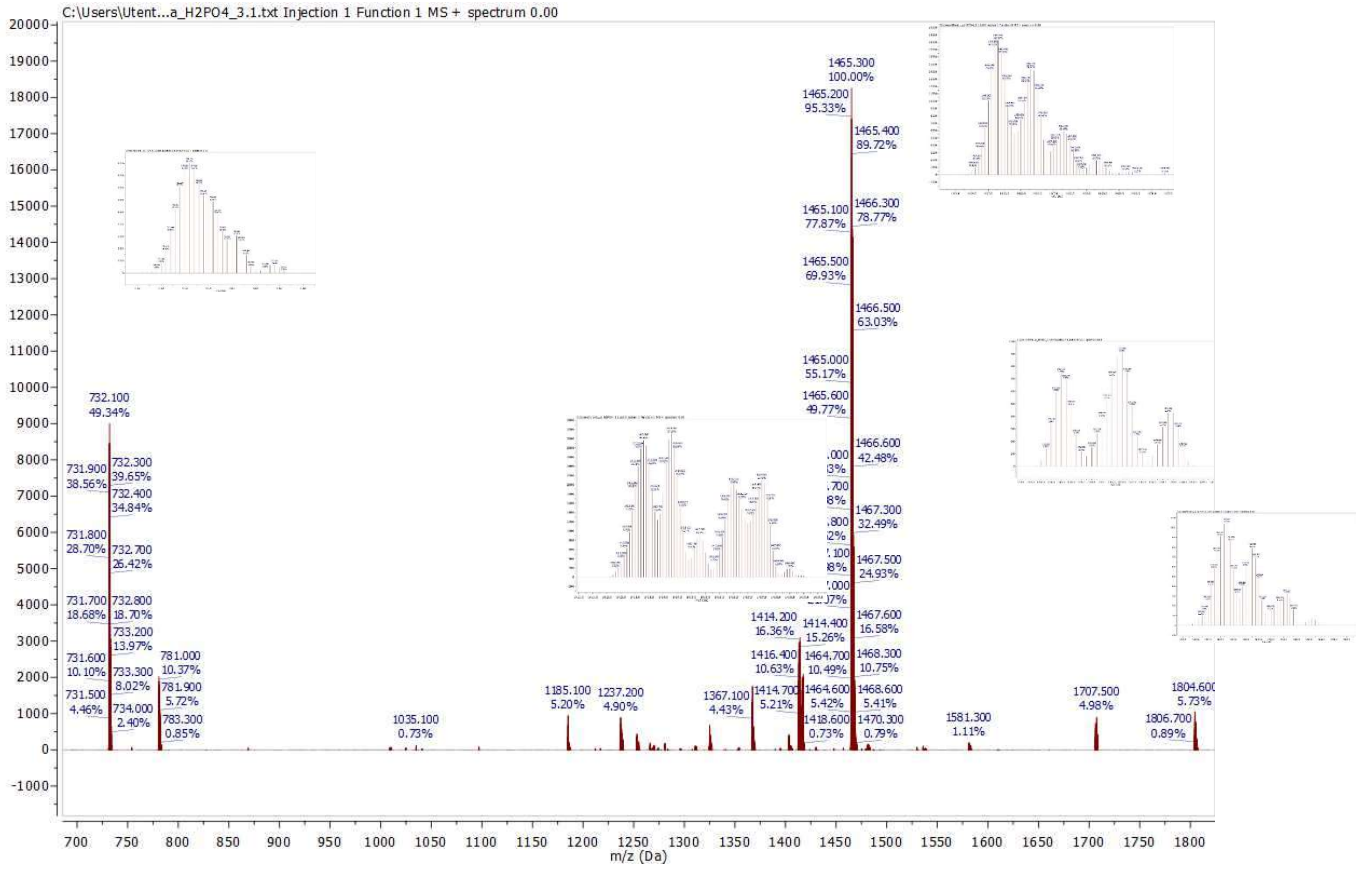


*Figure 73. ESI MS spectrum (negative mode) of **L6** ater addition of 1 equivalent of $TBAH_2PO_4$.Signals assigned to a doubly charged 1:1 complex **L6**·$H_2PO_4^-$, doubly charged 2:1 complex **L6**·$H_2PO_4^-$, 1:1 complex **L6**·$H_2PO_4^-$, 1:1 complex **L6**·$H_2PO_4^-$ with TBA cation and doubly charged 2:2 complex **L6**·$H_2PO_4^-$ with TBA cation.*

*Table 6. **L6**-anion complexes in ESI-MS spectrum (negative mode).*

	Ion (ESI-)	m/z
No salt	$[M+FA]^-$	1413.3
Cl^-	$[M+Cl]^-$	1403.3
Br^-	$[M+Br]^-$	1449.3
I^-	$[M+I]^-$	1495.2
NO_3^-	$[M+NO_3]^-$	1430.3
OAc^-	$[M+OAc]^-$	1426.9
	$[M+2OAc]^-$	1492.1
HSO_4^-	$[M+HSO_4\text{-}1]^{2-}$	732.1
	$[2M+HSO_4\text{-}1]^{2-}$	1416.9
	$[M+HSO_4]^-$	1465.3
	$[M+HSO_4]^- TBA^+$	1706.4
$H_2PO_4^-$	$[M+H_2PO_4\text{-}1]^{2-}$	732.1
	$[2M+H_2PO_4\text{-}1]^{2-}$	1416.7
	$[M+H_2PO_4]^-$	1465.3
	$[M+H_2PO_4]^- TBA^+$	1708.4
	$[[M+H_2PO_4]_2 TBA]^{2-}$	1804.4

c) Molecular dynamics simulations

Molecular dynamics simulation of 1:1 complexes of ***L6*** with anions were also performed (Figures 74-76). Similar as for the pentapeptide, the formation of complexes of 1:1 stoichiometry was confirmed which correlates well with the results of NMR titrations.

Monoatomic anions are preferably coordinated by amide protons of ***L6*** while in the coordination sphere of the larger cations the participation of carbamatic groups is observed (Table 7). It should be noted the average number of bonds between ***L6*** and halogen anions is smaller than in the corresponding complexes of ***L5*** ligand. That can at least partially explain the reduced stability of halogen anion ***L6*** complexes compared to the ones of ***L5***. The MD simulations of complexes with higher stoichiometries are planned to be performed in future investigations.

*Table 7: The average number of bonds formed between protons of **L6** and complexed anion in acetonitrile obtained by MD simulations.*

	N(amide-anion bonds)	*N*(carbamate-anion bonds)
Cl^-	4.9	0.1
Br^-	4.6	0.2
I^-	3.0	0.2
NO_3^-	7.9	1.3
OAc^-	9.1	0.7
HSO_4^-	8.5	1.4

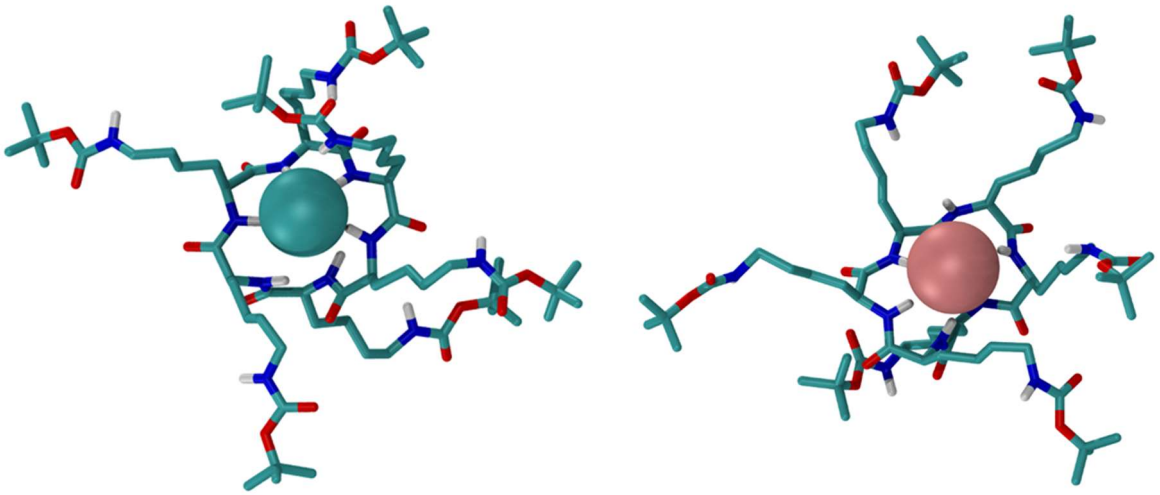


*Figure 74. Structures of a complexes of c[Lys(Boc)]$_6$ with Cl^- (**left**) and Br^- (**right**) obtained by MDs simulations in acetonitrile at 25 °C.*

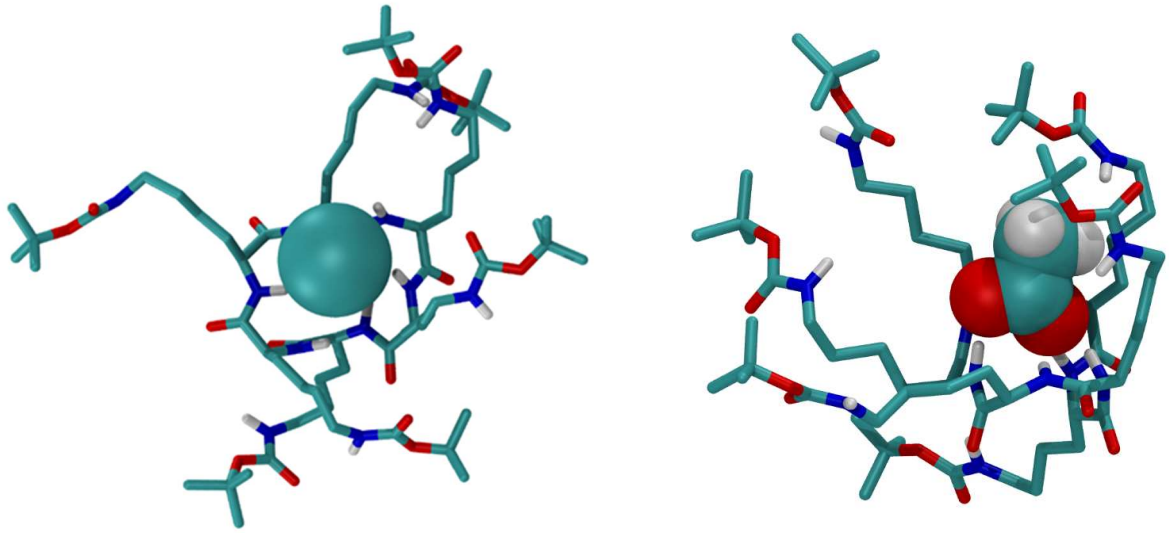


*Figure 75. Structures of a complexes of c[Lys(Boc)]$_6$ with I^- (**left**) and OAc^- (**right**) obtained by MDs simulations in acetonitrile at 25 °C.*

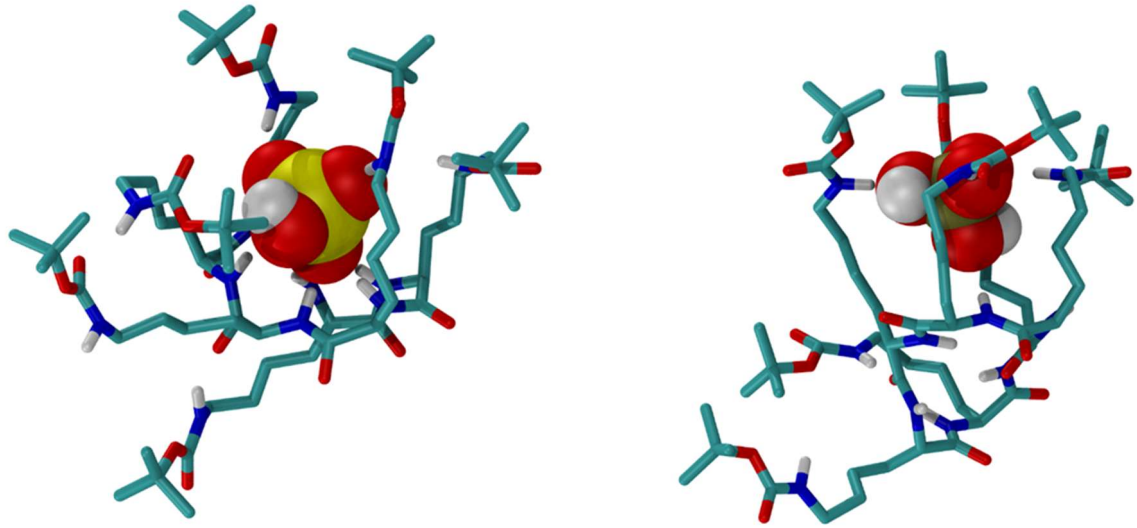
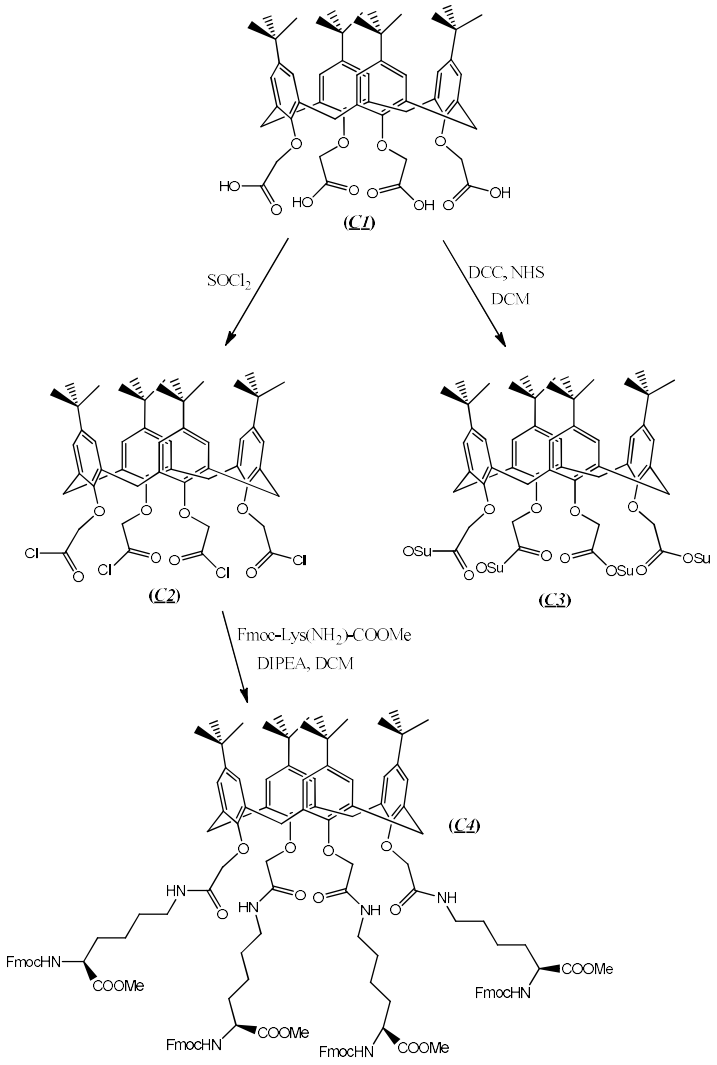


*Figure 76. Structures of a complexes of c[Lys(Boc)]$_6$ with HSO_4^- (**left**) and $H_2PO_4^-$ (**right**) obtained by MDs simulations in acetonitrile at 25 °C.*

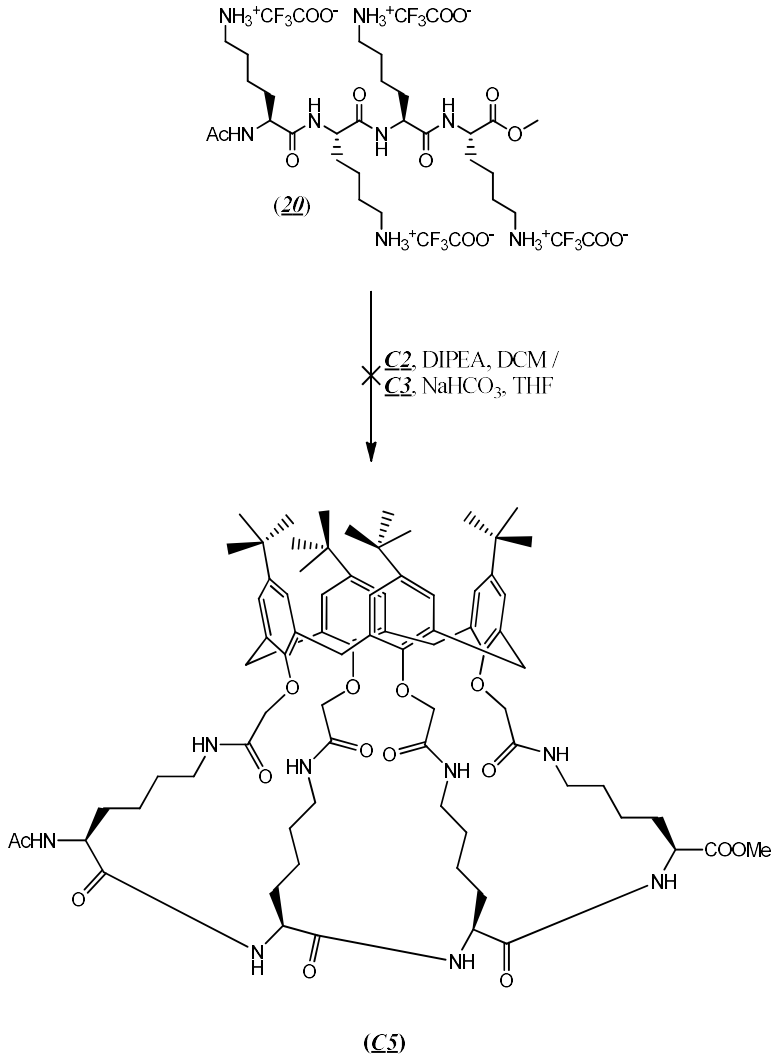
4.1.6. Synthesis of a peptidocalix[4]arenes

With the aim to synthesize a novel class of anion recepetors, namely calixarene homo-cyclopeptide derivatives, we needed to set-up a simple and efficient method to directly conjugate cyclopeptides ***L4-L6*** to calixarenes through the amino acid side chains. Specifically, the *p-tert*-butyl-lower-rim-functionalized calix[4]arenes ***C1,*** previously synthesized at University of Zagreb, was activated both to the acyl chloride ***C2*** by reaction with thionyl chloride and to the succinimidyl ester by reaction with NHS/DCC. By coupling ***C2*** with Fmoc-Lys(NH_2)-COOMe, used as the model compound, in the presence of DIPEA as a base in DCM as a solvent we obtained the tetralysine-calix[4]arene derivative ***C4*** (Scheme 27) in 59% yield.



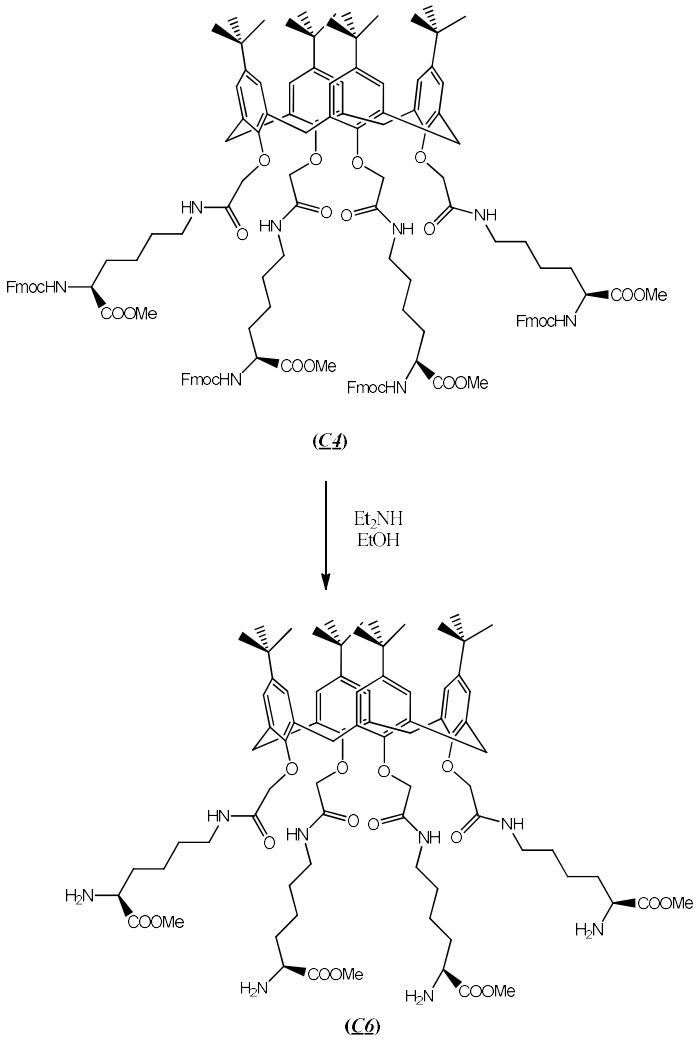
*Scheme 27. Synthesis of a compounds **C2**, **C3** and **C4**.*

Unfortunately, any attempt to conjugate the capped linear tetrapeptide ***20*** to the calix[4]arene derivative ***C1*** under the same experimental conditions was unsuccessfull. Also reaction of ***20*** with the activated tetraester ***C3*** didn't lead to the desired product ***C5*** (Scheme 28).



Scheme 28. Synthesis of a compound ***C5***.

As an alternative approach, we explored the possibility to use the tetralysine derivative ***C4*** as the precursor of ***C5***, *i.e.* to obtain the cyclic tetrapeptide ***C5*** by reaction of amino and carboxyl groups of the lysines already conjugated to calixarene. In this way calixarene could serve as a template for the cyclization reaction.



(*C4*)

(*C6*)

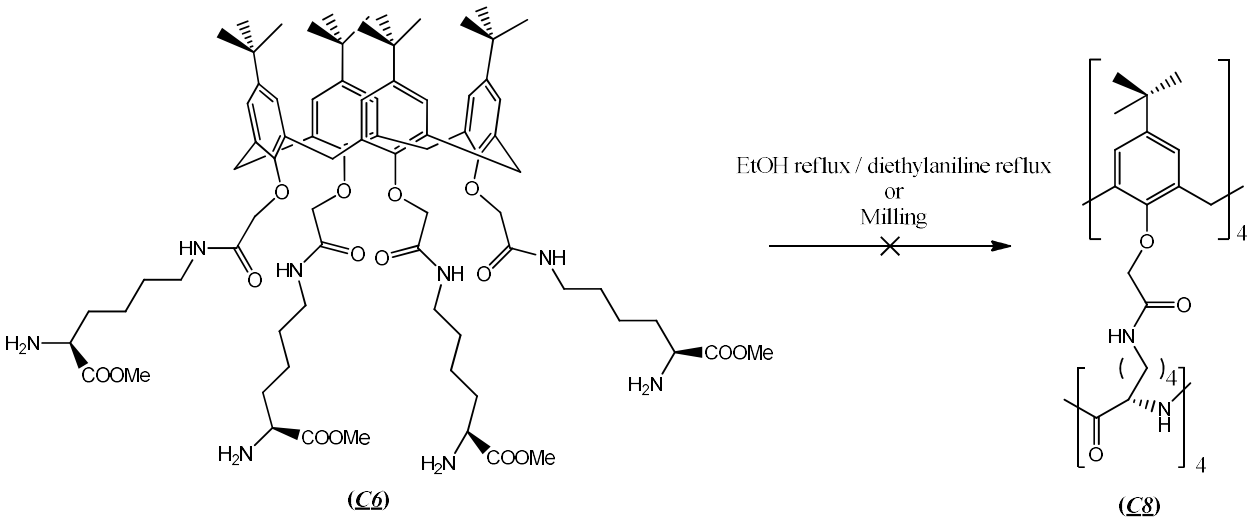
*Scheme 29. Synthesis of a compound **C6**.*

The first step was the removal of Fmoc protecting group from ***C4*** *N*-terminus (Scheme 29).

Then, we tried to get peptide bonds through intramolecular aminolysis of methyl esters by heating the precursor ***C6*** under reflux in EtOH and diethylaniline (Scheme 30), but in both cases the reaction was not succsesful.

In the last few years mechanochemistry, *i.e*., chemical transformations initiated or sustained by mechanical force, has become a very attractive field. This approach reduce significantly use of organic solvents and it is a very useful for a reagents with reduced solubility. Mechanochemical reactions are carried out in sealed vessels or jars of materials like stainless steel, carbide, agate, *etc*. Since procedures for amide bond formation by mechanocemical milling has been reported in the literature[118,119] we decided to try this alternative route. Specifically, precursor ***C6*** was

milled for 2 hours with NaCl to obtain diluted conditions (Scheme 30). Even this reaction didn't lead to the desired product.

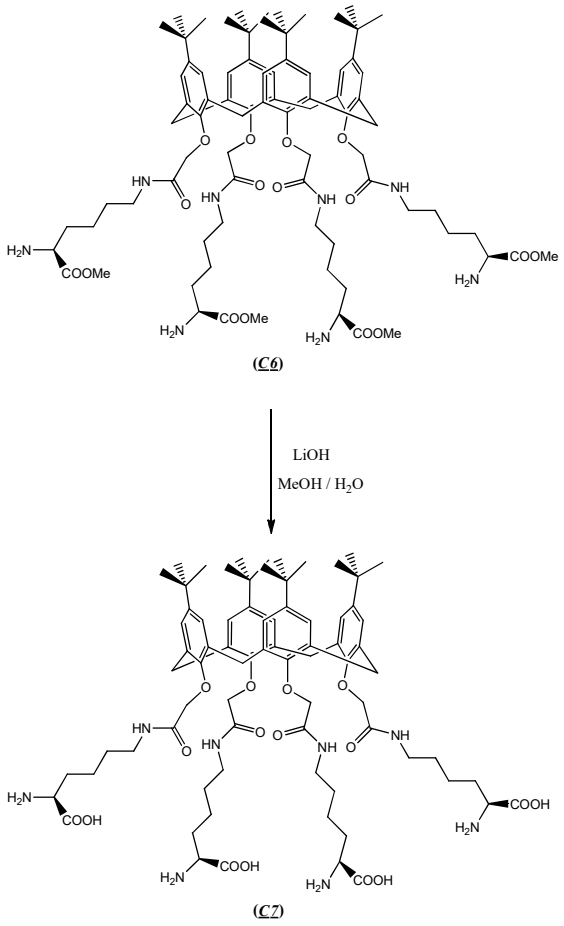


*Scheme 30. Attempt of a synthesis of a product **C8**.*

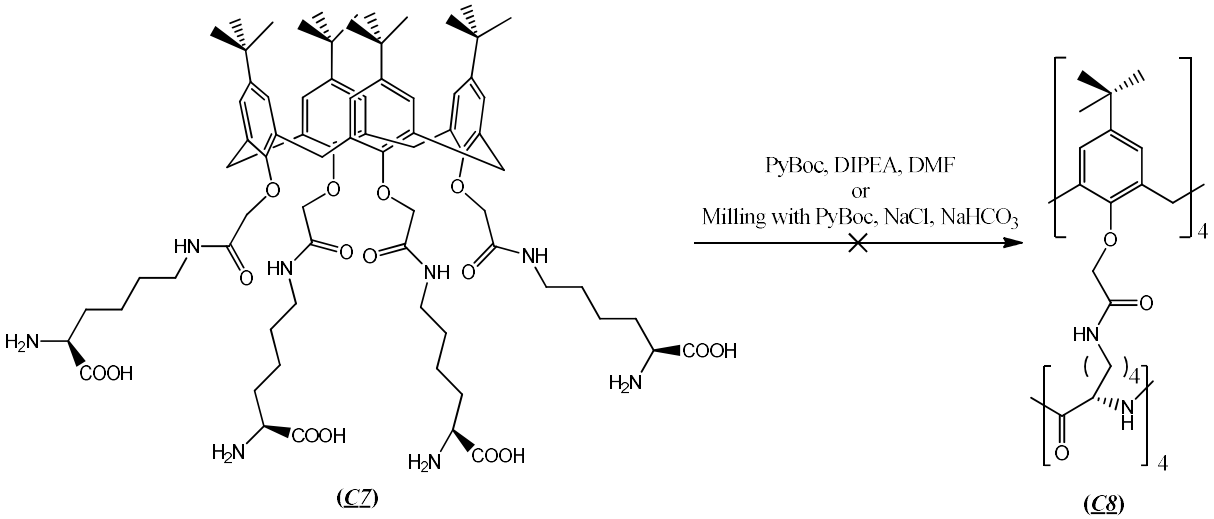
A possible explanation of the unsuccessful outcome of the previous reactions could be the not sufficient activation of the carboxylic groups. For this reason, the ester groups of compound ***C6*** were hydrolyzed with LiOH (Scheme 31) and the resulting product ***C7*** was treated with a very strong coupling reagent PyBop both in solution (DMF) in the presence of DIPEA and by milling in the presence of $NaHCO_3$ as a base and NaCl to obtain diluted conditions (Scheme 32).

In both cases, no formation of the cyclic peptidocalixarene ***C8*** was observed.

Work is in progress to find the proper conditions for the conjugation reaction.



Scheme 31. Hydrolysis of the methyl ester.



*Scheme 32. An attempt of a synthesis of **C8**.*

4.2. AMINOALANINE

4.2.1. Synthesis of aminoalanine

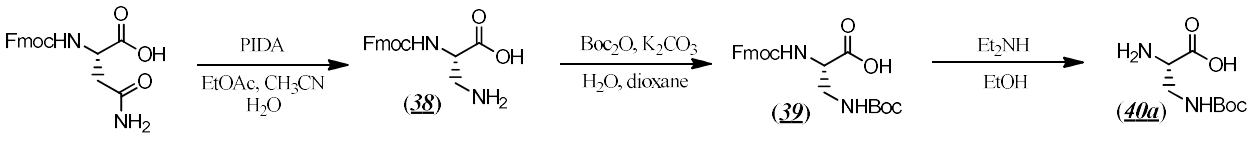
As a first approach to prepare poly-β-aminoalanines we decide to use the synthesized polyserines (see chapter 4.3) as staring material. Unfortunately, any attempt to convert the side-chain-OH groups of the into NH_2 (using $TsCl/NaN_3$, $MsCl/NaN_3$, $DPPA/NaN_3$) was unsuccessful.

Probably the presence of inter- or intramolecular hydrogen bonds makes the hydroxyl group less reactive inhibiting the formation of the desired products.

β-amino-L-alanine is commercially available but it is prohibitively expensive (CbZ-β-amino-L-alanine(Boc)-COOH (***40b***): 500 mg, 132 €, Sigma Aldrich).

A number of synthesis of β-amino-L-alanine derivatives are reported in the literature.[120-122] Among them we selected the route based on the Hofmann rearrangement of protected asparagine mediated by iodosobenzene diacetate (PIDA).

PIDA is a hypervalent iodine chemical reagent used as an oxidizing agent in organic chemistry. Usually, (bis(trifluoroacetoxy)iodo)benzene (PIFA) is used in this kind of reaction, but PIDA offers some advantages: it is possible to conduct the reaction under less acidic conditions and it is possible to avoid basic catalysis with pyridine. Additionally, reaction is faster and there is no formation of urea. For this reason, it is easier to isolate pure product. Since PIDA is commercially available and cheap reagent, this reaction is widely used in academic and industrial fields.



*Scheme 33. Synthesis of a compound **40**. Overall yield: 50 %.*

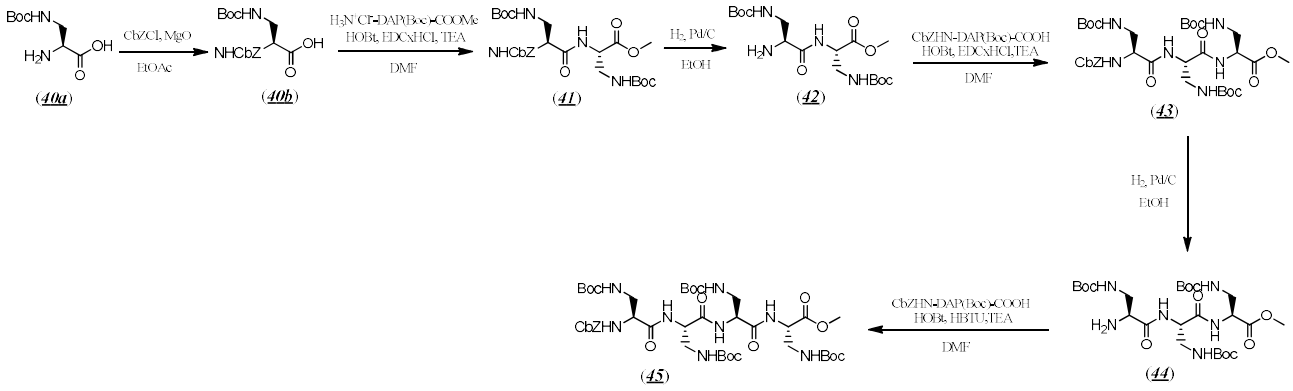
Fmoc-L-Asn-COOH was subjected to a Hoffmann rearrangement with PIDA (1.1 eq) in a mixture of in ethyl acetate, acetonitrile, and water (39/39/22 v/v/v) to give β-amino-L-alanine derivative ***38*** (Scheme 33). The formation of the product was confirmed by the significant upfield shift of protons bound to β-carbon going from starting materiam to ***38***.

The next step was the introduction of Boc group on the side chain to give ***39*** in quantitative yield as confirmed ^{1}H NMR spectrum showing the presence of the diagnostic peak relative to the 9 methyl protons of the *tert*-butyl group as well as of triplet at 6.8 ppm to NHBoc.

Removal of Fmoc protecting group with diethylamine afforded ***40a*** in pure form as indicated by ^{1}H NMR (no peaks in the resonance area of the aromatic protons) and MS analysis.

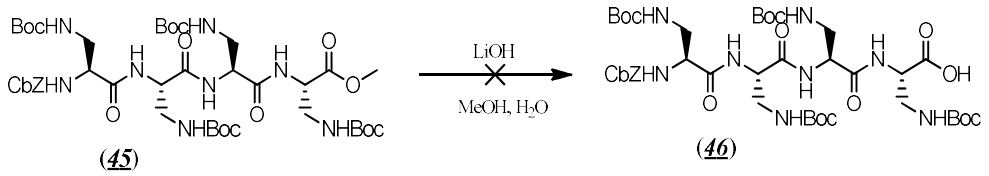
4.2.2. Synthesis of tetra- β-amino-L-alanine

Fully protected tetra-β-amino-L-alanine ***45*** was synthesized (Scheme 34) in the same way as described in the chapter 4.1.3 for polylysines using CbZ-β-amino-L-alanine(Boc)-COOH (***40b***) prepared from ***40a***, as a starting material. The intermediate formation of dimers (***41*** and ***42***) and trimers (***43*** and ***44***) was confirmed by NMR and mass analysis.



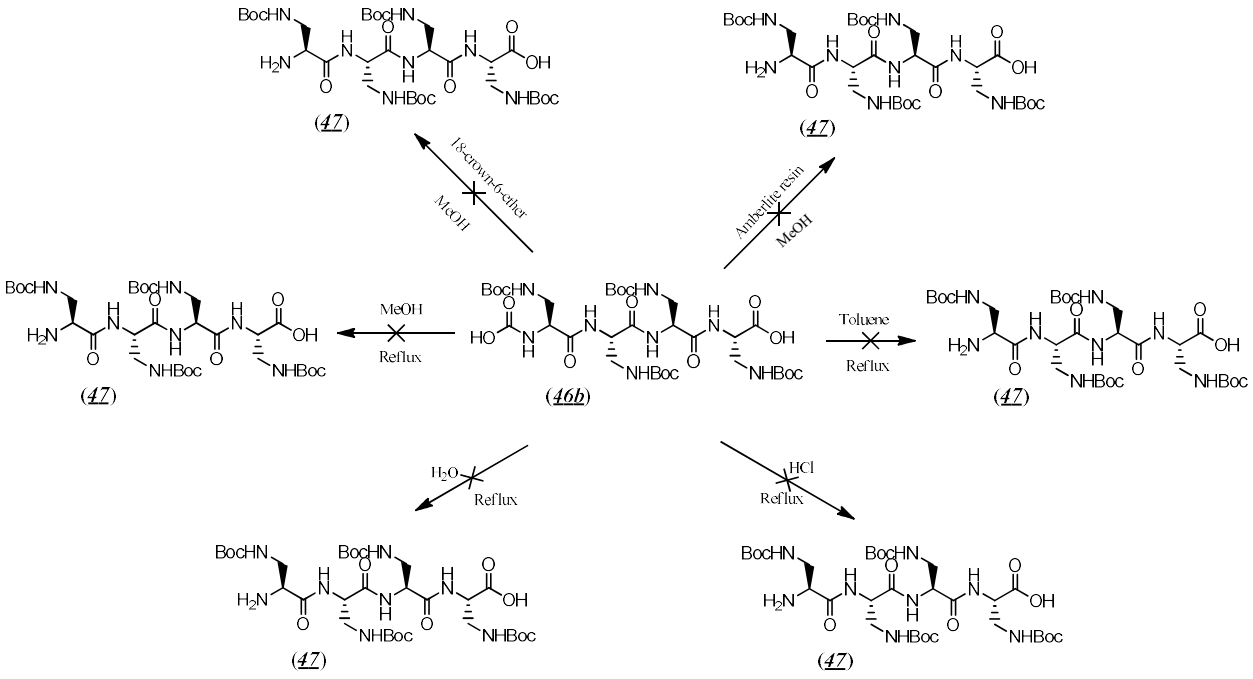
*Scheme 34. Synthesis of a compound **45**. Overall yield: 74.3 %.*

Compound ***45*** was subjected to the hydrolysis of the methyl ester using, as previously described, LiOH as a base (Scheme 35). Unexpectedly, under these reaction conditions, hydrolysis of the benzyl carbamate and formation of the carbamic acid ***46b*** occur. ***46b*** was found to be unusually stable as it did not decarboxylate even by heating under the reflux of methanol, toluene or water (acid solution) (Scheme 36).



(***45***) (***46***)

*Scheme 35. Synthesis of a compound **46**.*



(***47***) (***47***) (***47***) (***46b***) (***47***) (***47***) (***47***)

*Scheme 36. Unsuccessful attempts to synthesize compound **47**.*

Results of MDs simulations suggest stabilization of this product by intramolecular hydrogen bonds between the carbamate and CO and NH groups of Boc as well as NH group of the peptide backbone (Figure 77).

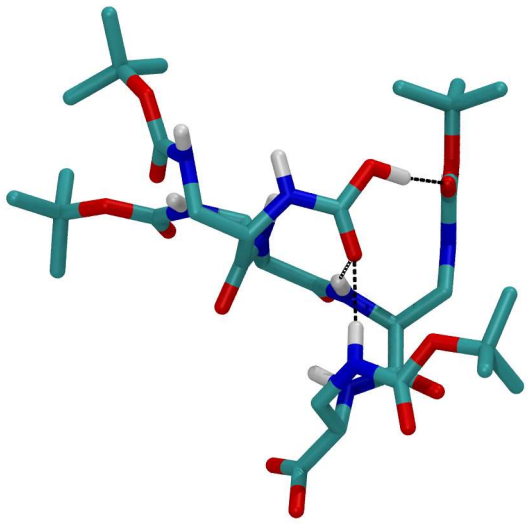


*Figure 77. Structure of a compound **46b** obtained by MDs simulations in N,N-dimethylformamide at 25 °C.*

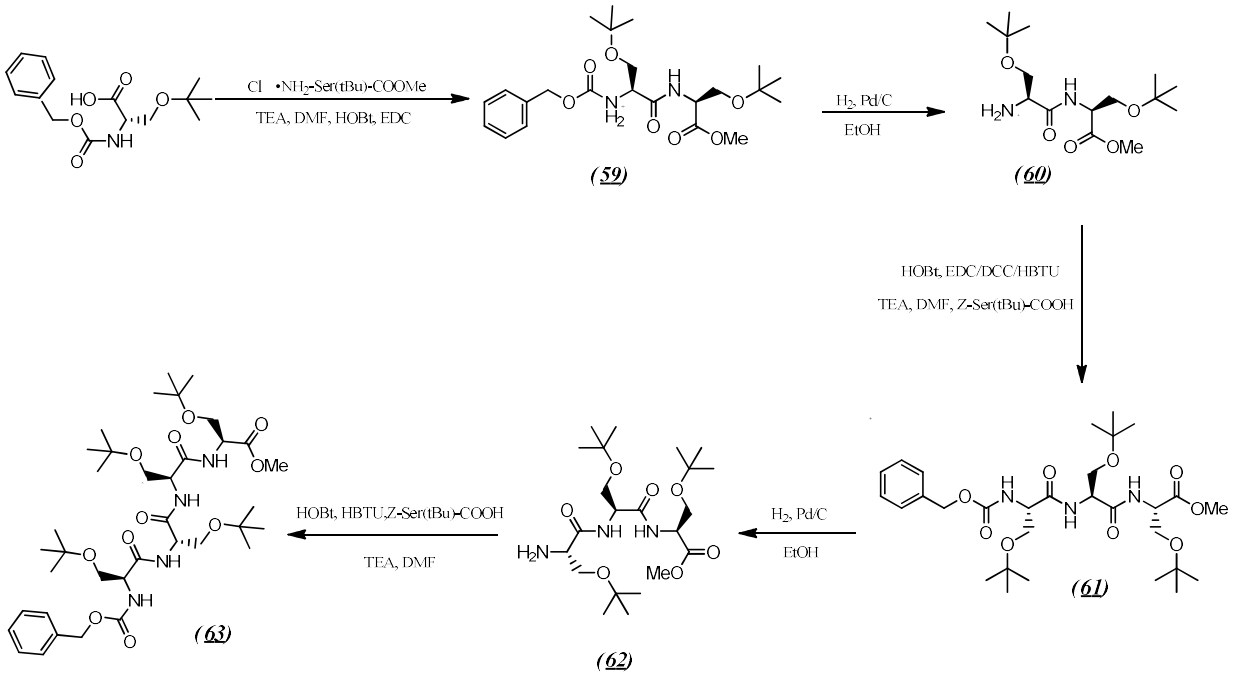
4.3. SERINE

With the aim to prepare cyclic peptides functionalized on the side chain with OH groups, we planned the synthesis of polyserine derivatives.

This task was found to be more difficult than expected. Most of the planned reactions were unsuccessful and this forced us to change several times the synthetic route.

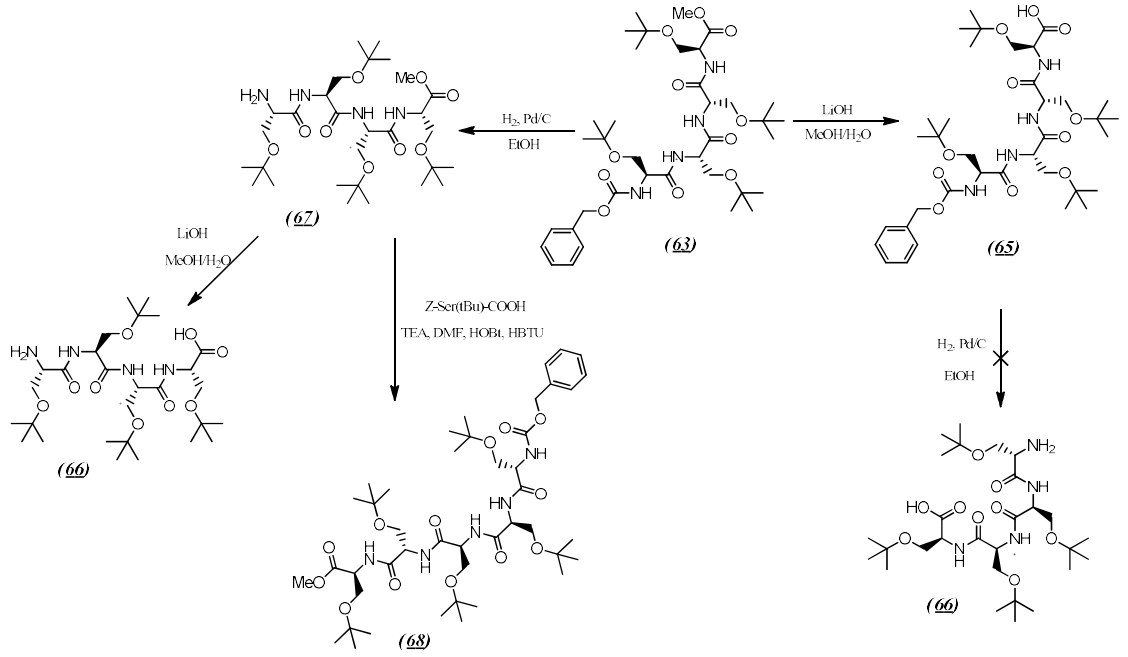
We succeeded in preparing tetra-, penta-, and hexaserine (***66***, ***70*** and ***74***, respectively), linear precursors of the corresponding cyclic peptides, as reported in the Schemes 38, 39 and 43.

In the case of tetra- and pentaserine, a linear approach analogous to that used for polylysine and poly-β-aminoalanine, was followed (Scheme 37).



*Scheme 37. Synthesis of a compound **63**. Overall yield: 33.2 %.*

Specifically, reaction of CbzNH-Ser(tBu)-COOH and H_2N-Ser(tBu)-COOMe in the presence of HOBt and EDC as coupling reagents, TEA as a base in DMF, afforded dipeptide ***59*** (92 % yield) which was CbZ deprotected by hydrogenolysis and coupled with another unit of CbZ-Ser(tBu)-COOH. Deprotection of the resulting tripeptide ***61*** and a further coupling led to fully protected tetraserine ***63*** in 55 % yield.



*Scheme 38. Synthesis of a compounds **66** and **68**. Overall yields: 71.2 % and 52.3 % respectively.*

Starting from ***63***, the linear tetrapeptide ***66***, deprotected at both *N*-and *C*-termini, could be prepared (Scheme 38) by hydrogenolys followed by removal of methyl ester with LiOH. It is worth noting that, by changing the order of reactions it was not possible to obtain ***66*** in that the hydrogenolysis of ***65*** led to the formation of a stable carbamic acid ***66b*** (Figure 78). Any attempt to decarboxylate product ***66b*** in neutral, basic or mild acidic conditions at 50 °C didn't lead to the desired product.

Using the *N*-terminus deprotected tetrapeptide ***67***, the fully protected pentaserine ***68*** was prepared by coupling with CbZ-Ser(tBu)-COOH.

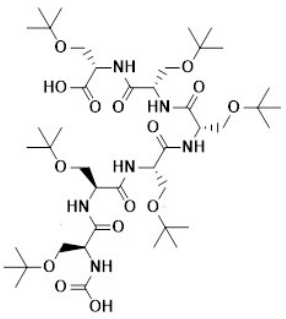
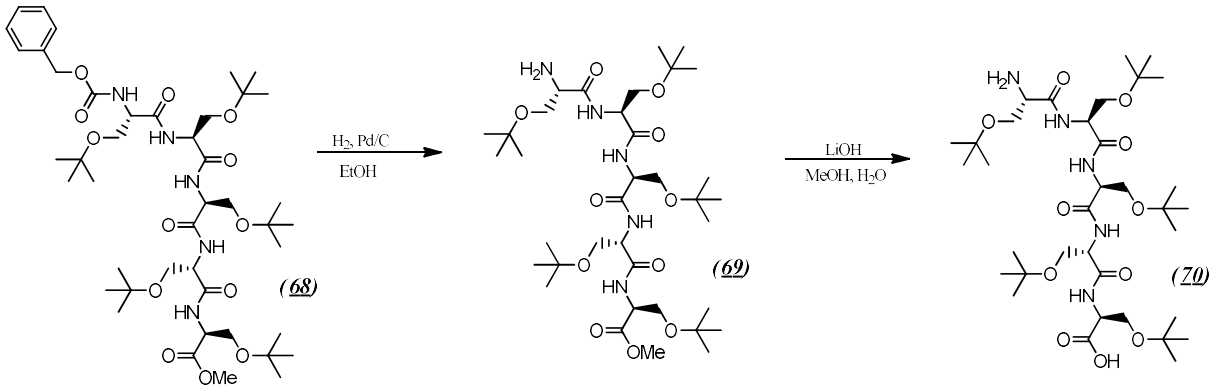


*Figure 78. Structure of a by-product **66b**.*

Removal of both *N*- and *C*-termini protection from ***68*** led to the pentapeptide ***70*** which was used later in the cyclization reaction (Scheme 39). Its structure was confirmed by MS and NMR analysis.

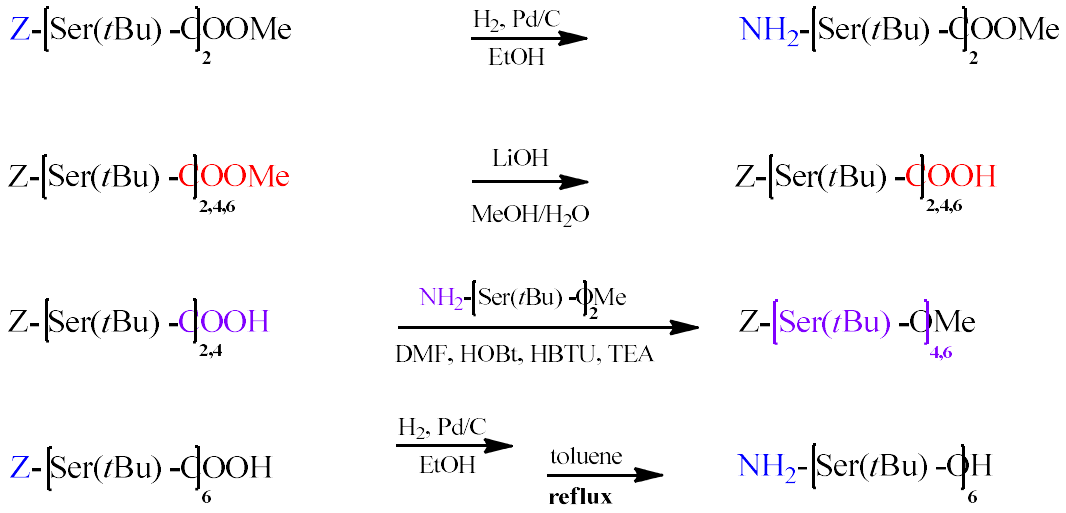


*Scheme 39. Synthesis of a compound **70**. Overall yield: 73.5 %.*

Considering two facts a) significant amounts of linear precursors needed for cyclization reactions:

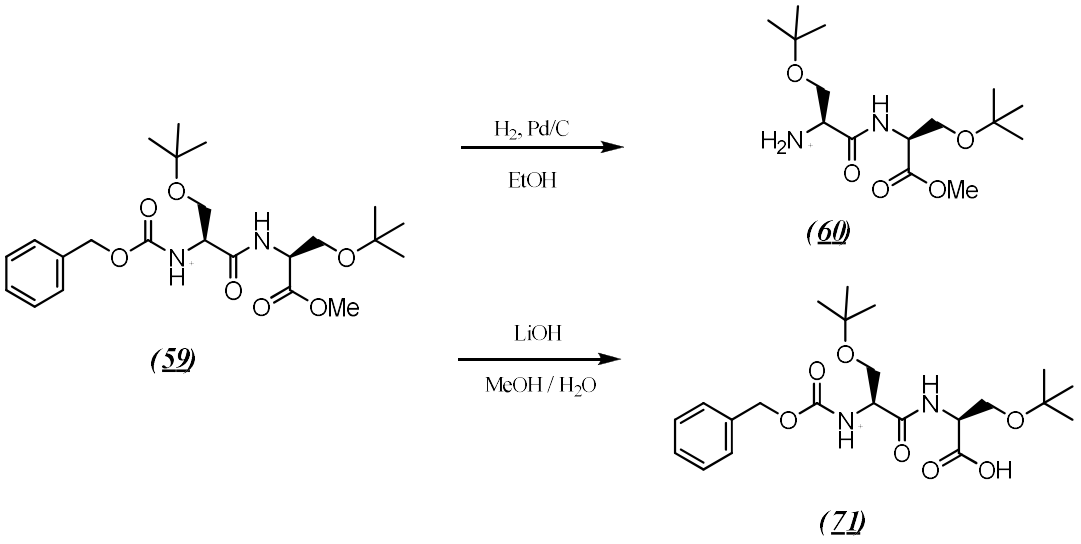
b) the significant decreasing of yield with increasing of the length of the peptide chain

we decide to move from a linear to a convergent approach for the preparation of the hexapeptide ***74*** according to the following strategy of assembly of intermediate segments: condensation of two dipeptides to give a tetrapeptide which is then coupled with another dipeptide segment (Scheme 40).



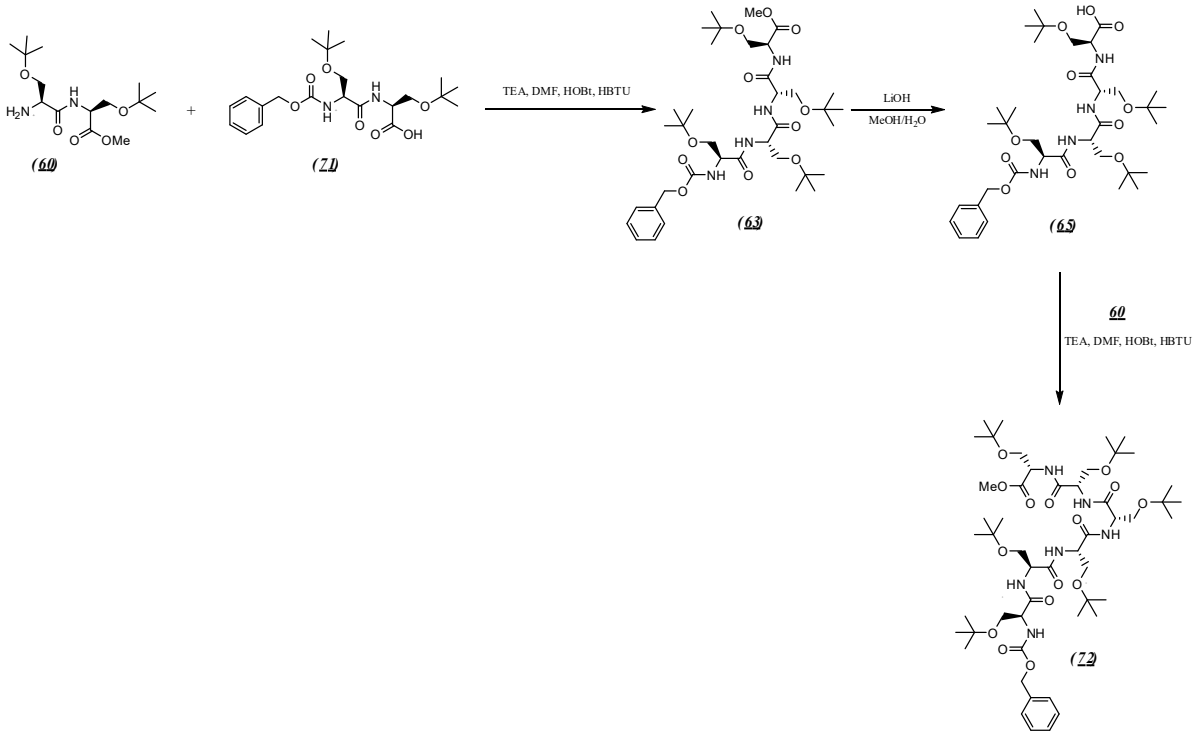
*Scheme 40. Convergent synthesis of **74**.*

Deprotection of dipeptide ***59*** from either –OMe or –CbZ to give ***60*** and ***71*** proceeded without particular issues as previously described (Scheme 41). ^{1}H NMR and mass spectrometry both confirmed the structure of products.



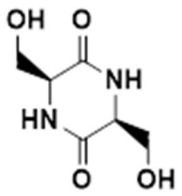
Scheme 41. Deprotection of either methyl ester and CbZ protecting group.

Dipetides ***60*** and ***71*** were coupled under the usual experimental conditions to give ***63*** that after deprotection of methyl ester was in turn coupled with ***60*** to afford the fully protected linear hexaserine (***72***) (Scheme 42) in an overall yield of 8%.



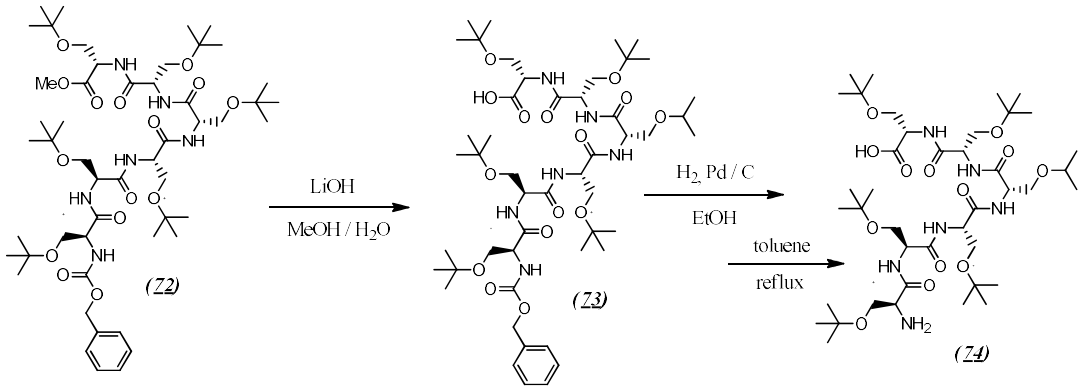
*Scheme 42. Synthesis of a compound **72**. Overall yield: 8 %.*

The dramatic decrease of yield is certainly attributable to the formation of a by-product ***52b*** arising from the intramolecular condensation of the amine at one terminus and the ester at the other one in ***60***. The basic conditions enhance this tendency to a rapid cyclization driven by the stability of the resulting six membered ring of the diketopiperazine ***52b***.



(***52b***)

Cleavage of methyl ester and CbZ in ***72*** led to the precursor ***74*** which was characterized by NMR and mass spectrometry.



Scheme 43. Cleavage of OMe and CbZ protecting groups and decarboxylation.

It is worth noting that also during the removal of CbZ in ***73***, the formation of an intermediate carbamic acid ***74b*** was observed, presumably due to stabilization by intramolecular hydrogen bonds. Decarboxylation of product ***74b*** was however successful operating at reflux of toluene overnight and hexaserine ***74*** was obtained in 85% yield (Scheme 43).

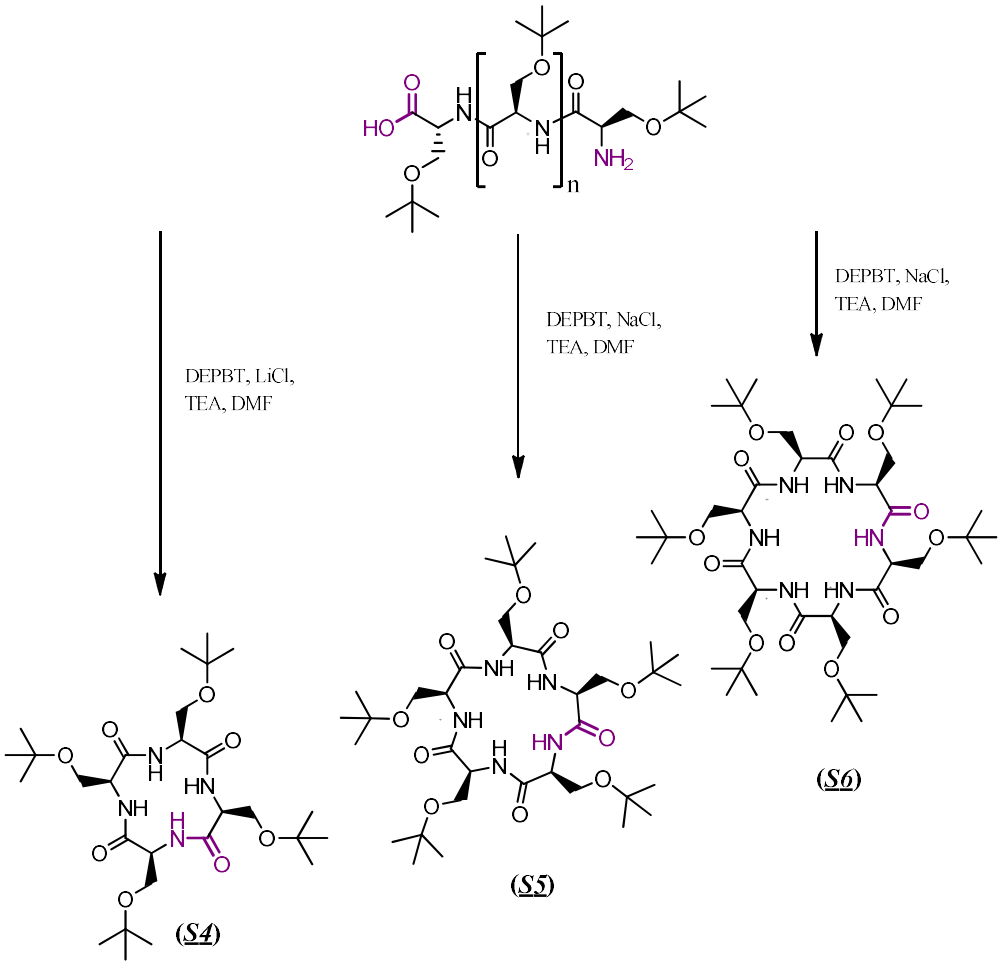
4.3.1. Cyclization

A number of different coupling reagents (PfOH, EDC or DPPA) and experimental conditions were tested to perform head-to-tail cyclization of linear precursors ***66***, ***70***, and ***74***.

Frequently TLC analysis during the monitoring of the reactions showed the presences of activated intermediates. However, cyclization reaction didn't proceed and only starting material was found upon the addition of water and the extraction with EtOAc.

Analogously to the case of poly-lysines, successful cyclization of ***66***, ***70***, and ***74*** was obtained using the Ye and co-workers procedure, *i.e.* DMF as the solvent, DEPBT as the coupling reagent, TEA as the base in the presence of LiCl or NaCl.

Cyclo[Ser(*t*Bu)]$_4$ (***S4***), cyclo[Ser(*t*Bu)]$_5$ (***S5***) and cyclo[Ser(*t*Bu)]$_6$(***S6***) were isolated in 10 %, 21 %, and 15 % yield, respectively (Scheme 44) and characterized by NMR ad MS analysis.



Scheme 44. Products of head-to-tail cyclization.

ESI-Q-TOF mass spectrometry confirmed the good outcome of the cyclization as reported in Figure 79.

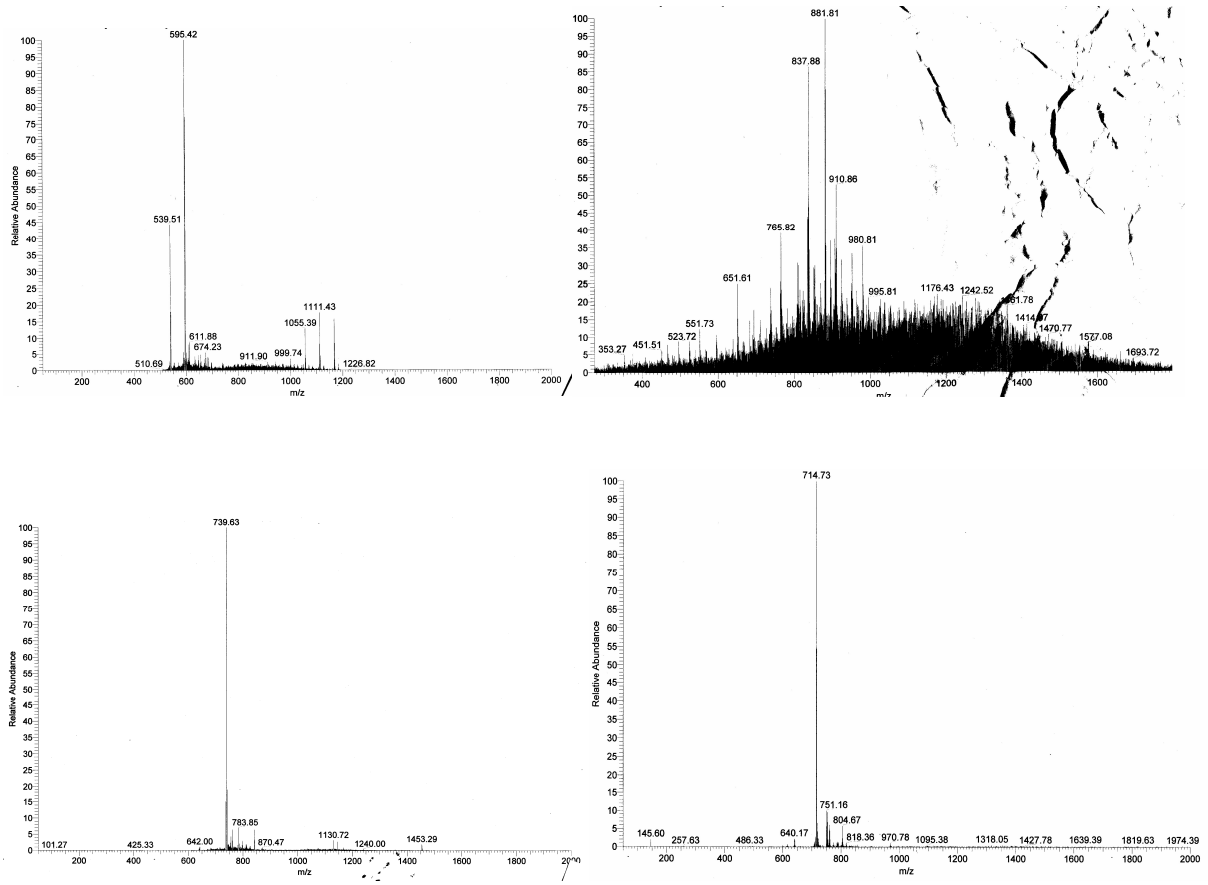


*Figure 79. ESI-Q-TOF of products **S4**, **S5** and **S6**.*

^{1}H NMR spectrum of cyclo[Ser(tBu)]$_5$ displayed a strong dependence on solvents used for analysis. In deuterated chloroform at RT all signals appeared very broad (Figure 80).

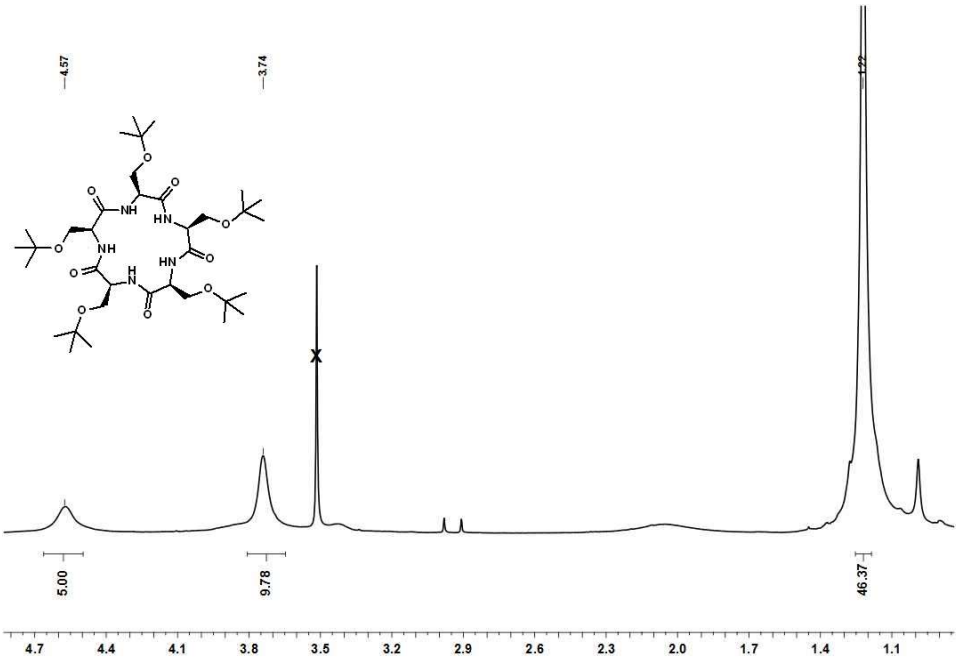


*Figure 80. ^{1}H NMR spectra of a compound **S5** in $CDCl_3$.*

In deuterated methanol (Figure 81), a significant sharpening of all signals was observed. It is worth noting that 5 doublets integrating each 1H, one for each NH group, were observed in the range 7.4-8.7 ppm, indicating that no exchange with the solvent occurs. In addition 4 multiplets, 3 of which integrating 1H and the last one 2H, are present in the range typical of the α-hydrogens and 5 broad singlets, each integrating 9H, corresponding to the five *t*Bu groups, in the range 1.15-1.4 ppm.

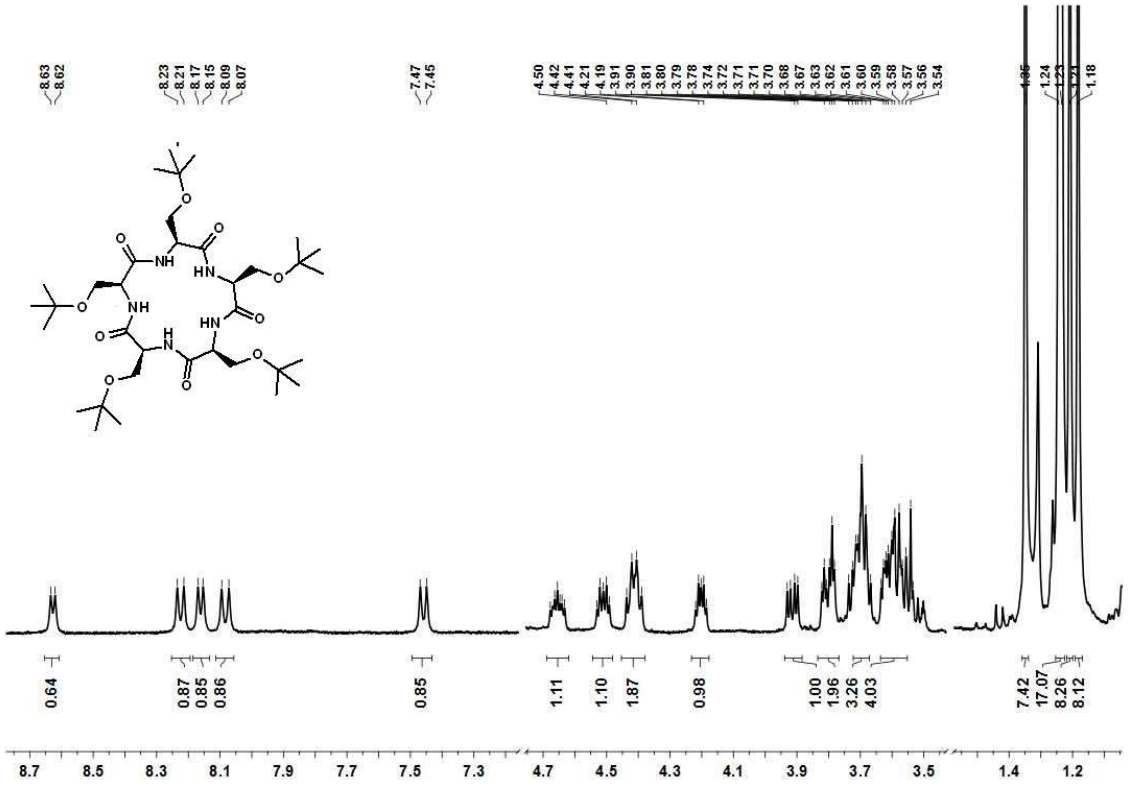


*Figure 81. ^{1}H NMR spectra of compound **S5** in CD_3OD.*

Comparison of ^{1}H NMR spectra of compound ***S5*** in CD_3OD recorded at various temperatures in the range -70 °C to 50 °C are shown in Figures 82 and 83.

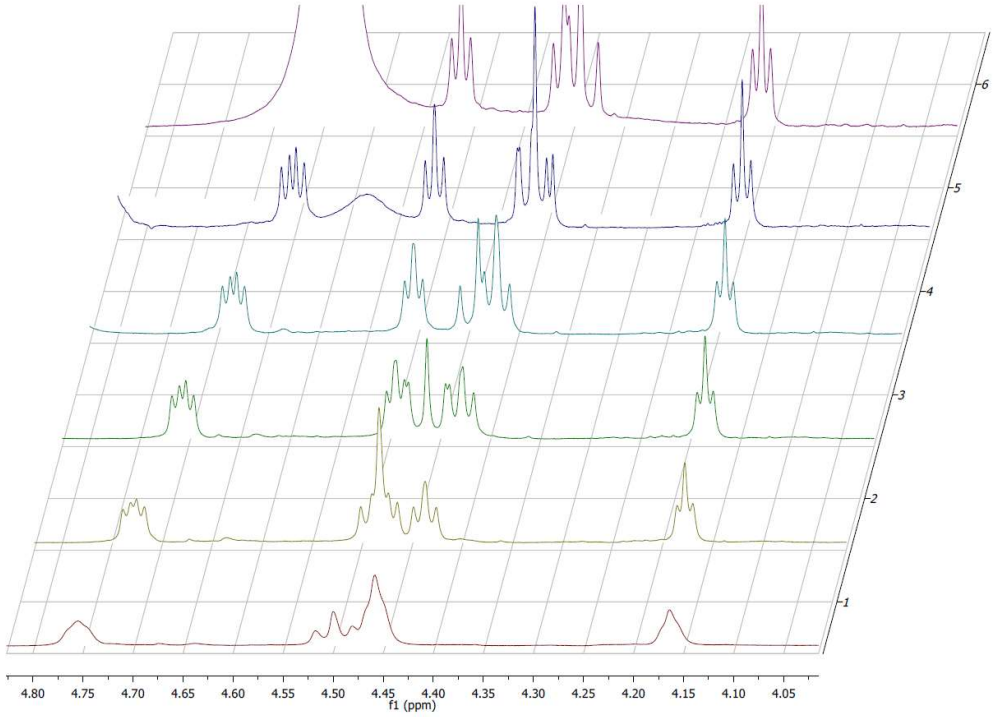


*Figure 82. Temperature dependence of the ^{1}H NMR spectrum of compound **S5** in CD_3OD (alpha region).*

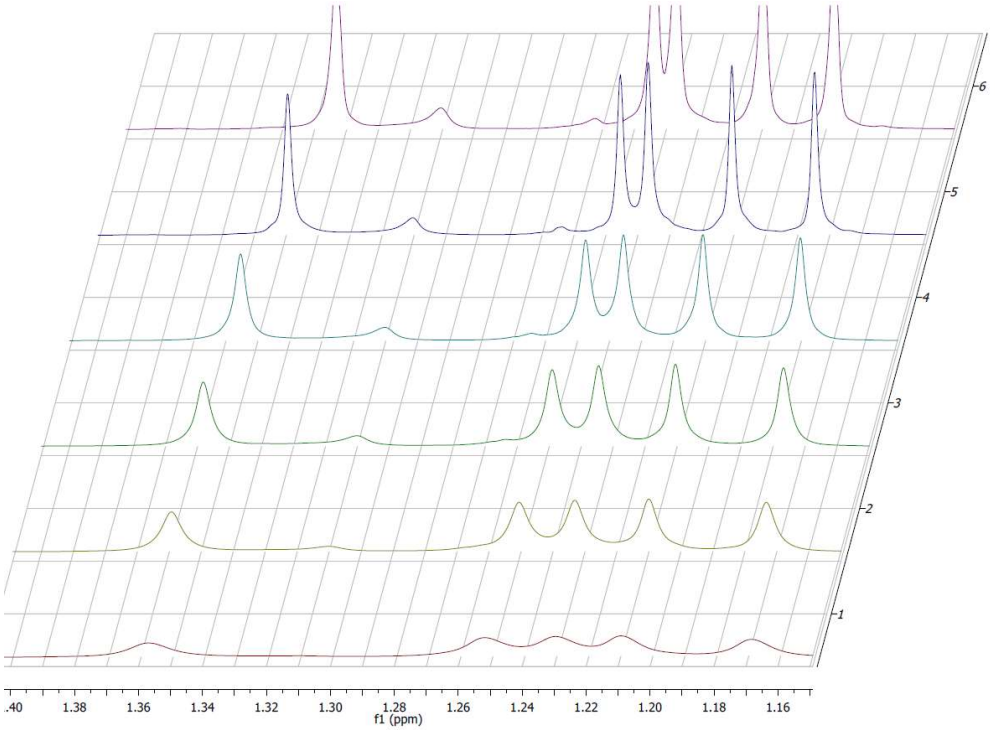
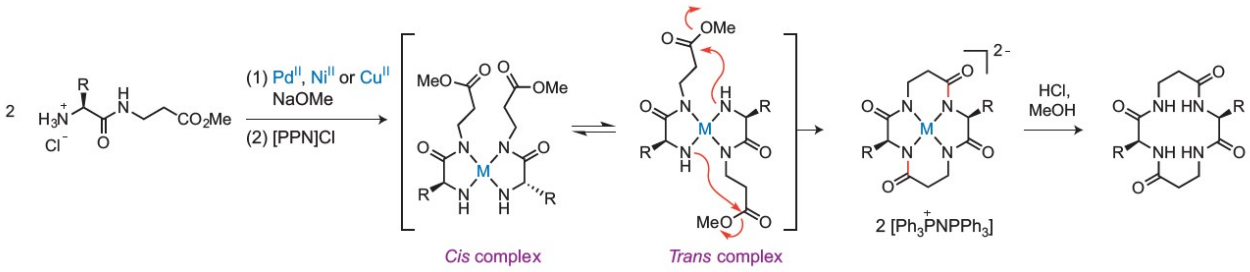


*Figure 83. Temperature dependence of the ^{1}H NMR spectrum of compound **S5** in CD_3OD (tBu region).*

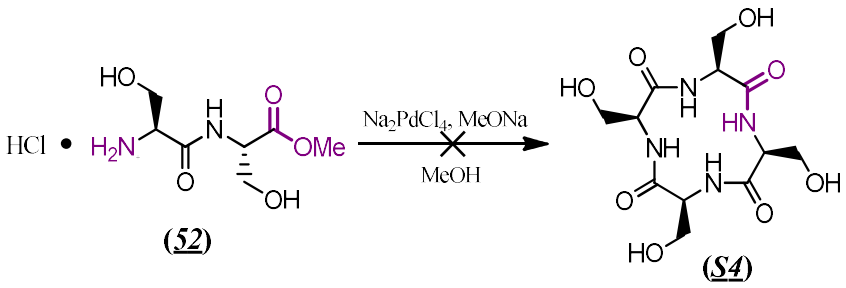
As Addendum to the above reported preparation of cyclic poly-serines, it has to be mentioned that we tried a number of alternative synthetic routes to these compounds, in particular the one based on the use of [L-Serine]$_2$ methyl ester as starting material in the conditions of Beck and co-workers.[27]

These authors reported that a facile synthesis of 12-, 14-.16- and 18-membered cyclic tetrapeptide can be through a transition metal assisted cyclodimerization of nonactivated dipeptide ester precursors (Scheme 45).



Scheme 45. Synthesis of cyclic tetrapeptide by transition metal-mediated cyclodimerization of unactivated dipetide esters.[9]

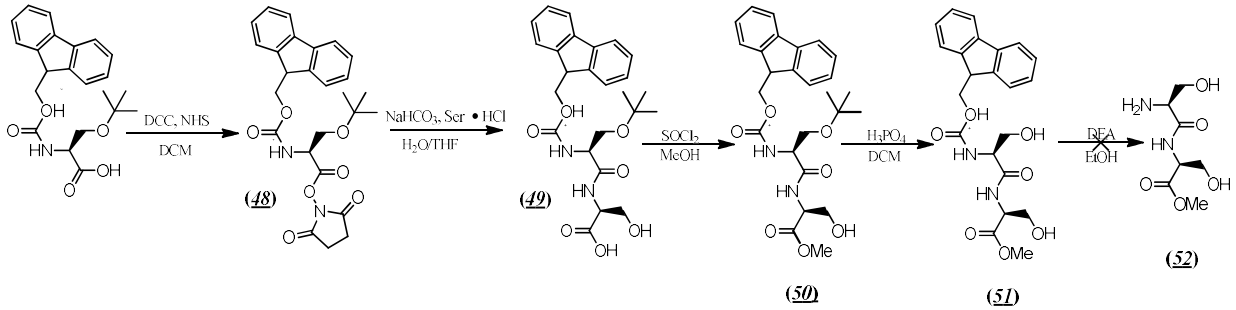
Unfortunately, any attempt to perform the same reaction using [L-Serine]$_2$ methyl ester as starting material didn't lead to the desired cyclic product (Scheme 46).



Scheme 46. Cyclisation of dipeptide methyl ester.

Preparation of compound ***52*** was not straightforward and different routes were followed to prepare it (Schemes 47, 48 and 49).

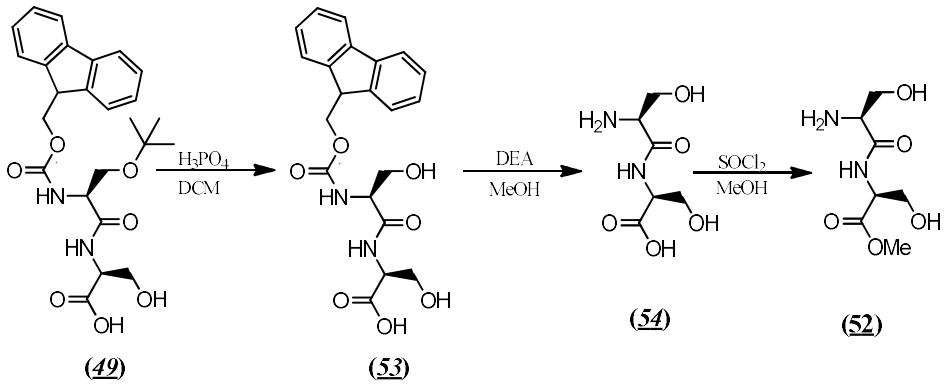
a)



*Scheme 47. Synthesis of compound **52**.*

Fmoc-L-Serine-(*O*-*t*Bu)-Serine was converted into succinimidyl ester ***48*** that in a reaction with Ser gave a dipeptide ***49***. Methyl ester ***50*** was then obtained following an already described procedure. Removal of *t*Bu protecting groups led to the product ***51***. Cleavage of Fmoc protecting group from compound ***51*** didn't led to the desired product ***52***. Instead, diketopiperazine ***52b*** was obtained.

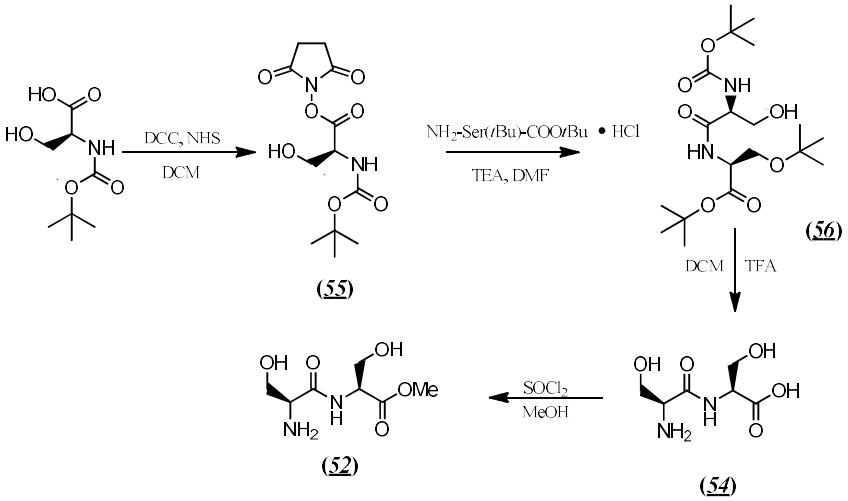
b)



*Scheme 48. Synthesis of compound **52**. Overall yield: 58.2 %.*

Since previously described synthesis was unsuccessful, we tried to invert the order of reactions *i.e.* first removal of *t*Bu ether to ***49*** and then of Fmoc to give compound ***54***. Finally, methyl ester was introduced leading to the desired precursor ***52*** in 58,2% overall yield (Scheme 48).

c)



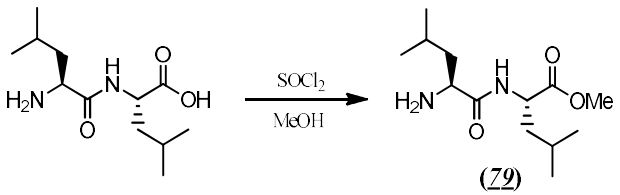
*Scheme 49. Synthesis of compound **52**. Overall yield: 38.1 %.*

Boc-Ser-COOH was converted into the succinimidyl ester ***55*** that in a coupling reaction with NH_2-Ser(*t*Bu)-COO*t*Bu gave dipeptide ***56***. Protecting groups were then removed to give diserine ***54*** which was protected as methyl ester ***52*** in 38.1 % overall yield (Scheme 49).

4.4. H_2N-(Leu)$_5$-COOH and H_2N-Phe-Leu-Leu-Phe-Leu-Leu-COOH

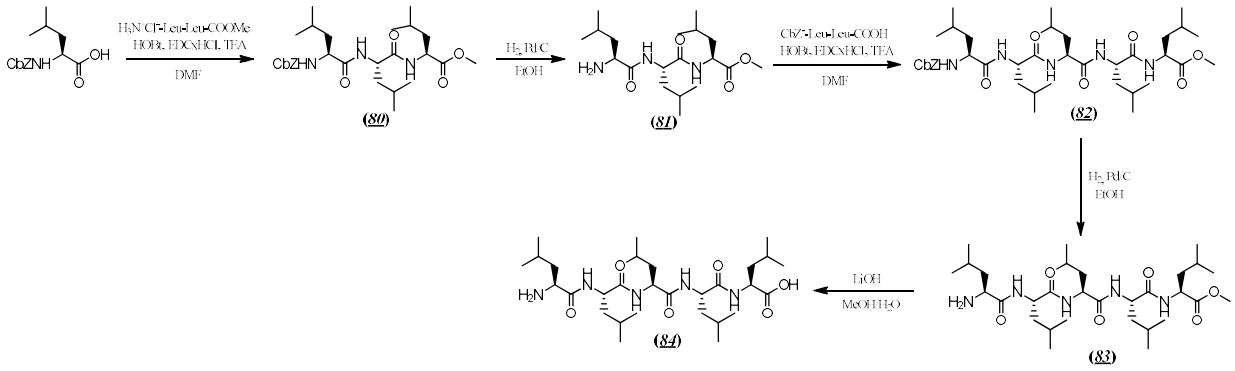
To confirm the role of chloride anion in assisting macrocyclization of peptides, two additional linear precursors were prepared: pentaleucine ***84*** and the hexapeptide ***90*** (Phe-Leu-Leu-Phe-Leu-Leu).

The first step in the synthesis of the linear precursor ***84*** was the conversion of carboxylic group of dileucine into methyl ester ***79*** with a yield of 84.2 % (Scheme 50).



*Scheme 50. Synthesis of compound **79**. Yield: 84.2 %.*

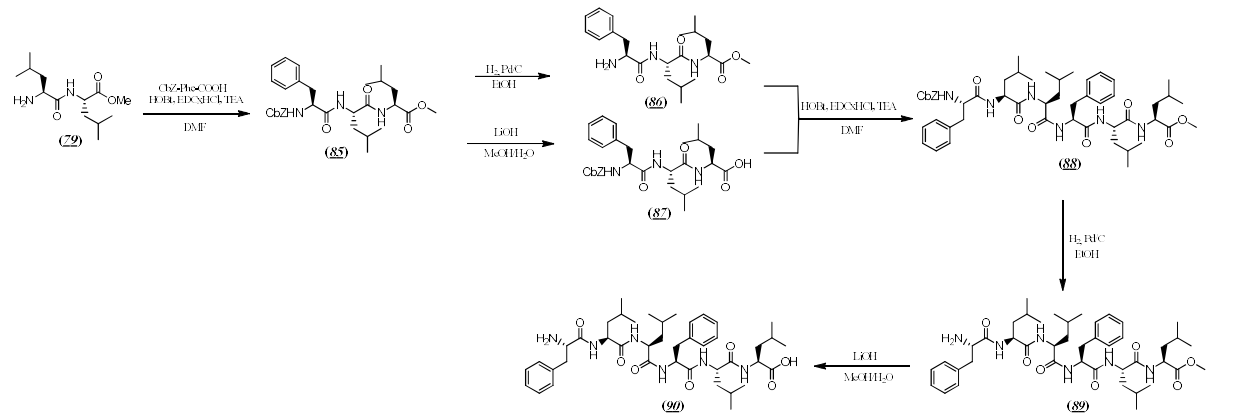
Then the same procedure already described for the synthesis of linear precursors protected as methyl esters and CbZ was followed and the desired product ***84*** was obtained in an overall yield of 64.1 % (Scheme 51).



Scheme 51. Synthesis of compound ***84***. Overall yield: 64.1 %.

To prepare hexapeptide ***90***, dipeptide ***79*** was condensed with CbZ-Phe-COOH to give the fully protected tripeptide ***85*** (Scheme 52). ***85*** was then converted into two compounds: ***86*** by removal of CbZ protecting group and ***87*** by hydrolysis of methyl ester.

Condensing these two tripeptides the fully protected hexapeptide ***88*** was obtained. Its deprotection from both CbZ and OMe to give sequentially ***89*** and ***90*** proceeded without particular issues as previously described (Scheme 52).



*Scheme 52. Synthesis of compound **90**. Overall yield: 55.1 %.*

The binding of peptides methyl esters ***83*** and ***89*** with Na^+ and Cl^- ions was studied by means of microcalorimetric titrations using $NaClO_4$ and TEACl salts. Titrations were carried out at 25 °C by adding a salt solution in *N,N*-dimethylformamide to the ligand solution. The measured heats for titration with Na^+ cation are reported in Figures 84-87.

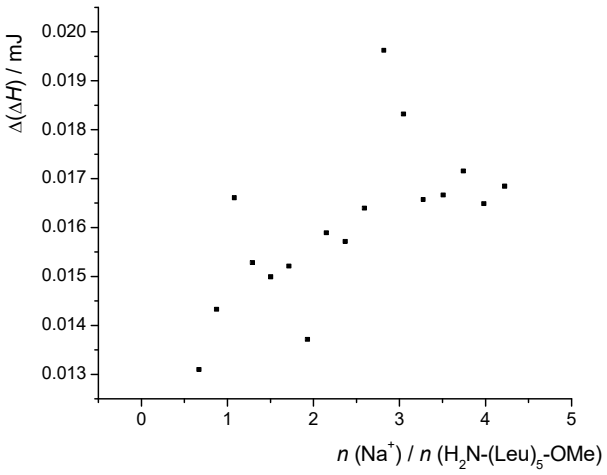


Figure 84. Dependence of successive enthalpy change on n(Na^+) / n(H_2N-$(Leu)_5$-OMe) in the microcalorimetric titration of linear H_2N-$(Leu)_5$-OMe (c = 5,117 · 10^{-3} mol dm^{-3}) with $NaClO_4$ (c =0,095 mol dm^{-3}) in N,N-dimethylformamide in the presence of $TBAClO_4$ (c = 5,020·10^{-2} mol dm^{-3}) at ϑ = 25 ° C.

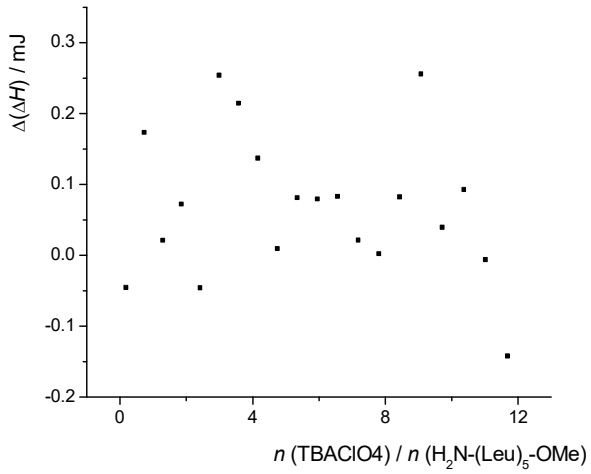


Figure 85. Microcalorimetric titration of linear (H_2N-(Leu)$_5$-OMe) with $TBAClO_4$ (c = 4,824 mol dm^{-3}) in N,N-dimethylformamide at ϑ = 25 ° C.

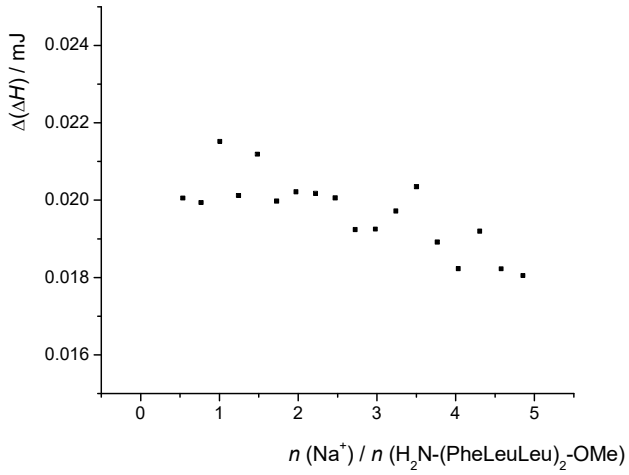


Figure 86. Dependence of successive enthalpy change on n(Na^+) / n(H_2N-(Phe-Leu-Leu)$_2$-OMe) in the microcalorimetric titration of linear H_2N-(Leu)$_5$-OMe (c = 4,445 · 10^{-3} mol dm^{-3}) with $NaClO_4$ (c = 0,095· 10^{-2} mol dm^{-3}) in N,N-dimethylformamide in the presence of $TBAClO_4$ (c = 5,020·10^{-2} mol dm^{-3}) at ϑ = 25 ° C.

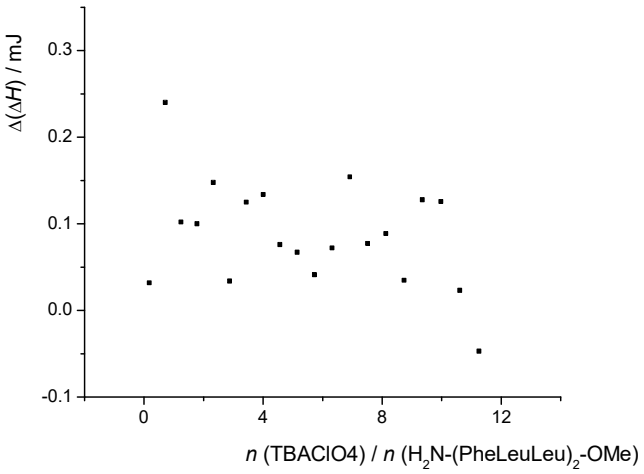


Figure 87. Microcalorimetric titration of linear (H_2N-(Phe-Leu-Leu)$_2$-OMe) with $TBAClO_4$ (c = 0,250 mol dm^{-3}) in N,N-dimethylformamide at ϑ = 25 ° C.

Analysis of microcalorimetric data indicated that there was no binding between linear peptides and Na^+.

On the contrary, microcalorimetric measurments clearly showed that compounds ***83*** and ***89*** bind chloride ions (Figures 88 and 89). By processing the corresponding titration data, the thermodynamic quantities listed in Table 6 were calculated.

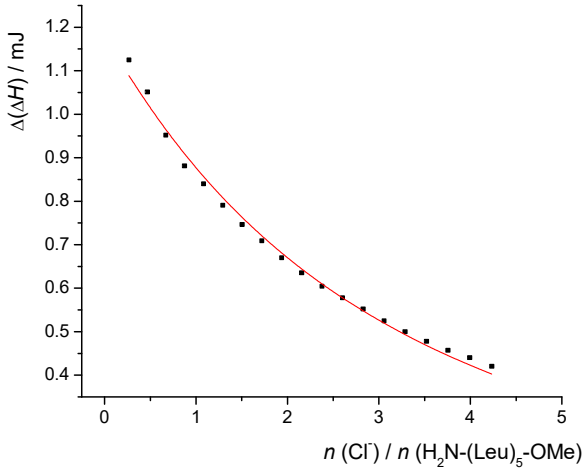


Figure 88. Dependence of successive enthalpy change on n(Cl^-) / n(H_2N-$(Leu)_5$-OMe) in the microcalorimetric titration of linear H_2N-$(Leu)_5$-OMe (c = 5.117·10^{-3} mol dm^{-3}) with TEACl (c = 0.096 mol dm^{-3}) in N,N-dimethylformamide in the presence of $TBAClO_4$ (c = 5,020·10^{-2} mol dm^{-3}) at ϑ = 25 ° C. ■ measured values, — calculated values.

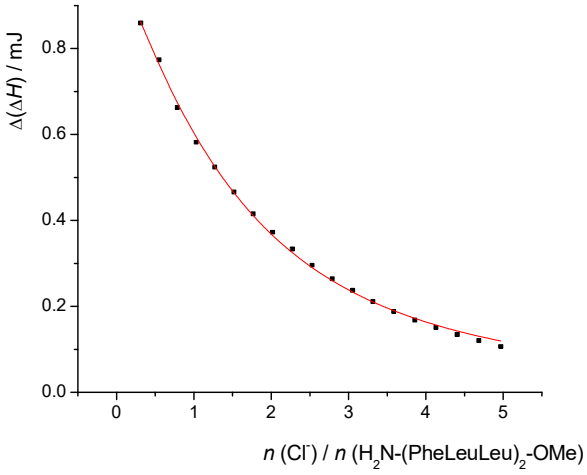


Figure 89. Dependence of successive enthalpy change on n(Cl^-) / n(H_2N-$(Phe\text{-}Leu\text{-}Leu)_2$-OMe) in the microcalorimetric titration of linear H_2N-$(Phe\text{-}Leu\text{-}Leu)_2$-OMe (c = 4,445·$10^{-3}$ mol dm^{-3}) with TEACl (c = 0.097 mol dm^{-3}) in N,N-dimethylformamide in the presence of $TBAClO_4$ (c = 5,020·10^{-2} mol dm^{-3}) at ϑ = 25 ° C. ■ measured values, — calculated values.

*Table 6. Thermodynamic parameters of complexation reactions of **83** and **89** with chloride anion in N,N-dimethylformamide at 25 ° C obtained by microcalorimetric titrations.*

solvent	peptide	log K_1 ± SE	($\Delta_r G^\Theta$ ± SE) / kJ mol^{-1}	($\Delta_r H^\Theta$ ± SE) / kJ mol^{-1}	($\Delta_r S^\Theta$ ± SE) / J K^{-1} mol^{-1}
DMF	H_2N-(Leu)$_5$-OMe	1,46 ± 0,01	-8,33 ± 0,05	6,2 ± 0,1	48,7 ± 0,3
	H_2N-(PheLeuLeu)$_2$-OMe	2,06 ± 0,03	-11,8 ± 0,2	1,9 ± 0,2	46,0 ± 0,1

SE = standard error of the mean (N = 3)

§ 5. CONCLUSIONS

In conclusion, in the course of this PhD thesis a number of novel synthetic cyclopeptides have been prepared by head-to-tail coupling-based lactamization and their complexation abilities have been investigated through an integrated approach which includes thermodynamic, computational and NMR studies.

Specifically:

1. Linear homopeptides containing three different side-chain functionalized amino acids, namely lysine, serine and aminoalanine, were prepared by solution phase peptide synthesis in a moderate to good yields and in amounts up to 3 g. An orthogonal protection scheme was adopted involving the use of the following protecting groups: methyl ester for carboxyl group, CbZ for *N*-terminus, Boc and *tert*-butyl ether for the side chains. EDC/HBTU and HOBt were used as coupling reagents in DMF as a solvent and TEA as a base.

All products were purified by flash column chromatography and characterized by ^{1}H NMR, ^{13}C NMR spectroscopy and ESI-Q-TOF mass spectrometry.

2. Macrocyclization reactions were carried out on completely protected peptide precursors under high dilution conditions in the presence of salts (NaCl, LiCl, NaTPB and TEACl) to promote the head-to-tail condensation and DEPBT as the condensing reagent in order to have a strongly activated acyl component.

Microcalorimetric titrations using H_2N-[Lys(Boc)]$_4$-OMe, H_2N-[Lys(Boc)]$_5$-OMe and H_2N-[Lys(Boc)]$_6$-OMe as ligands and $NaClO_4$ and TEACl as salts indicated a very weak binding of the peptides with Na^+ and, on the contrary, a strong binding with Cl^-. The binding constants for the complexation of chloride ions were evaluated to be Log *K*=1.51, Log *K*=1.47 and Log *K*=1.68, for tetra-, penta- and hexapeptide, respectively. These findings suggest that the mechanism commonly accepted for the so called metal-ion assisted cyclization has to be revised indicating that coordination of chloride (and not alkali metals) with amidic groups along the

chain is the factor that predominantly brings *N*- and *C*-termini closer, forcing them to react. Accordingly, the highest cyclization yields were obtained when TEACl was used as the salt to bring the reactive functionalities in close proximity. Finally, MD simulations show the formation of stable complexes of linear peptides with Cl^- while in complexes with sodium dissociation of the cation occurs.

3. Binding proprieties of cyclic pentalysine toward different anions were investigated by means of microcalorimetric titrations in CH_3CN. In complexation reactions with halides (Cl^-, Br^-, I^-) as well as with NO_3^-, inflection was observed at a ratio $n(A^-)/n(L) \approx 1$ which indicates that the stoichiometry of the resulting complex is 1:1. In contrast, with $H_2PO_4^-$ we observed inflection at the ratio $n(L)/n(A^-) \approx 2$ *i.e.* two ligands are needed to coordinate one $H_2PO_4^-$ anion. Calorimetric titrations showed also that the most efficient binding of cyclopentalysine is with Cl^- with the binding constant of Log K=5.72 while that of cyclohexalysine is with OAc^- (Log $K > 4$, obtained by NMR titration). In general, cyclic pentapeptide binds stronger all investigated anions than cyclic hexapeptide.

Complexation reactions of cyclic penta- and hexalysine were also investigated by ^1H NMR and ESI-MS titrations. The results obtained by these two methods are in excellent agreement to each other as well as with results arising from microcalorimetric titrations of pentapeptide.

Cyclic homoserine derivatives were also prepared as well as linear H_2N-$(Leu)_5$-COOH and H_2N-Phe-Leu-Leu-Phe-Leu-Leu-COOH to confirm the role of chloride anion in assisting macrocyclization of peptides.

With the ultimate goal to prepare a novel class of anion receptors, namely calixarene homo-cyclopeptide derivatives, work is in progress to set-up a simple and efficient method to directly conjugate the prepared homocyclopeptides to calixarenes by exploiting side chain functional groups.

§ 6. ABBREVATIONS

Boc: *tert*-butoxycarbonyl

Boc_2O: Di-tert-butyl dicarbonate

Cbz: carboxybenzyl

DBU: 1,8-Diazabicyclo(5.4.0)undec-7-ene

DCC: *N,N*'-dicicloesilcarbodiimmide

DCM: dichloromethane

DCU: Dicyclohexylurea

DEA: Diethylamine

DEPBT: 3-(Diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one

DIPEA: *N,N*-diisopropylamine

DMF: dimethylformamide

DMSO: dimethylsulphoxyde

DPPA: Diphenylphosphoryl azide

EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

EtOAc: ethyl acetate

EtOEt: diethyl ether

EtOH: ethanol

Fmoc: fluorenylmethyloxycarbonyl

HBTU: *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate

HOBt: hydroxybenzotriazole

MeOH: methanol

MeONa: Sodium methoxide

MsCl: Methanesulfonyl chloride

NaTPB: sodium tetraphenyl borate

NHS: *N*-hydroxysuccinimide

OMe: Methyl ester

OSu: Succinimidyl ester

O*t*Bu: *Tert*-butyl ester

PfOH: 2,3,4,5,6-Pentafluorophenol

Ser: Serine

SPPS: Solid-phase peptide synthesis

*t*Bu: *Tert*-butyl ether

TEA: triethylammine

TEACl: tetraethylammonium chloride

TFA: trufluoroacetic acid

THF: Tetrahydrofuran

TsCl: 4-Toluenesulfonyl chloride

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