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New method of peptide separation using electrostatic binding

Nová metoda separace peptidů za využití elektrostatické vazby

Diploma thesis

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Prague, 2020

Declaration

I declare that I have written my bachelor thesis by myself and that all the sources are listed in the bibliography. Neither this work, nor its significant part was used to obtain other academic title. I agree that this work may be lent and published.

Prague, 11.06.2020 Attila Palágyi

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Abstract

The diploma thesis deals with the development of a new method of synthetic peptide separation using electrostatic binding. It deals with the development of a multi-charged anchored linker attachable to a synthetic peptide after its synthesis on solid phase. Subsequently, it investigates the separation using electrostatic binding; however, after peptide cleavage from the anchored linker and Boc deprotection with TFA, the peptide formed so strong electrostatic interactions with the cation exchanger that we were unable to recover it. The thesis is finished with discussion and method improvement proposal.

Key words: peptide separation, solid-phase peptide synthesis, SPPS, electrostatic binding

Abstrakt

Diplomová práce se týká vývoje nové metody separace syntetických peptidů pomocí elektrostatické vazby. Zabývá se vývojem linkeru s vícenásobně nabitou kotvou, který má schopnost se zachytit na syntetický peptid po jeho přípravě na pevné fázi. Následně zkoumá separaci za využití elektrostatické vazby, avšak po odštěpení peptidu z linkeru s vícenásobně nabitou kotvou a odchránění Boc chránících skupin pomocí TFA, vytvořil peptid tak silné elektrostatické interakce s katexem, že se nepodařilo ho z katexu získat. Práce je zakončena diskusí a návrhem na zlepšení metody.

Klíčové slova: separace peptidů, syntéza peptidů na pevné fázi, SPPS, elektrostatické vázání

Contents

List of abbreviations

2-Br-Z – 2-bromobenzyloxycarbonyl

Acm – acetamidomethyl

BAL – backbone amide linker

Boc – *tert*-butyloxycarbonyl

BOP – (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafuorophosphate

Bzl – benzyl

COMU – (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate

COSY – correlated spectroscopy

CuAAC – copper(I)-catalyzed azide-alkyne cycloaddition

DBU – 1,8-diazabicyclo[5.4.0]undec-7-ene

DCC – *N,N'*-dicylohexylcarbodiimide

DCM – dichloromethane

de-Fmoc – removal of Fmoc protective group

DIC – *N*,*N*′-diisopropylcarbodiimide

DIPEA – *N*,*N*-diisopropylethylamine

DMF – *N,N*-dimethylformamide

DSC – *N*,*N*′-disuccinimidyl carbonate

EDC – *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimidehydrochloride

ESI – electrospray ionization

Fmoc – 9-fluorenylmethoxycarbonyl

HATU – 1-((dimethylamino)(dimethyl-iminio)methyl)-1*H*-[1,2,3] triazolo[4,5-b]pyridine 3-oxide hexafluorophosphate

HBTU – 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafuorophosphate

HCTU – 2-(6-chloro1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate

HFIP – hexafluoroisopropanol

HMBC – heteronuclear multiple bond coherence

HMPA – hexamethylphosphorotriamide

HOBt – *N*-hydroxybenzotriazole

HOSu – *N*-hydroxysuccinimide

HPLC – high-performance liquid chromatography

HSQC – heteronuclear single quantum coherence

IR – infrared radiation

MS – mass spectroscopy

Mtr – 4-methoxy-2,3,6-trimethyl-benzenesulfonyl

NMR – nuclear magnetic resonance

Oxyma – ethyl 2-cyano-2-(hydroxyimino)acetate

PyAOP – [(7-azabenzotriazol-1-yl)oxy]tris(pyrrolidino)phosphonium hexafuorophosphate

PyBOP – benzotriazol-1-yloxytri(pyrrolidino)phosphonium hexafuorophosphate

PyOxim (PyOxP) – (((1-cyano-2-ethoxy-2-oxoethylidene)amino)oxy)tri(pyro-lidin-1-yl)phosphornium hexafuorophosphate

RE – rotary evaporator

RP-HPLC – reverse-phased high-performance liquid chromatography

SPPS – solid phase peptide synthesis

TBTU – 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafuoroborate

t-Bu – *tert*-butyl

Tf - trifluoromethanesulfonate

THF - tetrahydrofurane

TIS - triisopropylsilane

TLC – thin layer chromatography

TOTU – *O*-[(ethoxycarbonyl)cyanomethylenamino]-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate

Trt – trityl

TSTU – 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate

UV – ultraviolet radiation

1 INTRODUCTION

Hundreds of amino acids have been discovered in Nature either in free form or bound up into larger molecules and complexes. Amino acids are present in every living organism starting with bacteria, through fungi, plants, animals and us, people. They are known as one of the most important building blocks of life utilized in living cells for peptide and protein synthesis under the control of genes. Peptides and proteins play a wide variety of roles in living organisms and display a range of properties useful not just for life itself, but also for us people in pharmacy, cosmetic industries and other sectors of everyday life. Since peptides and proteins found in Nature are hard to obtain in enough quantities, several methods for large-scale production have been developed.

The application of synthetic peptides is widely spread. For instance, there are more than 60 peptides already used in pharmacy, and hundreds are waiting for clinical or preclinical trials[1–4]. The most used method of peptide synthesis is on a solid support by stepwise addition (covalent binding) of amino-protected amino acids. Each addition of an amino acid usually requires three steps – coupling, capping and deprotection. Excess reagents and side products are washed off after each step of the synthesis. The final peptide is then purified using reversed-phase HPLC, resulting in using an enormous amount of chemicals, especially solvents. For example, to prepare 20 grams of a peptide consisting of 50 amino acids with 20% overall yield, at least 2 tons of organic solvents and chemicals are required.

To reduce the amount of waste generated during peptide synthesis, especially on a large scale, we have proposed a new method for peptide separation using electrostatic binding. Instead of classical separation on reversed-phase HPLC, a multiple-charged anchored linker is bound to the synthetic peptide and is separated by catching on a cation exchanger. By avoiding the solvent-consuming HPLC, this method requires significantly fewer organic solvents reducing the amount of waste.

This diploma thesis deals with the synthesis of a multiply-charged anchor bound to a linker and a peptide on a solid support. Subsequently, it investigates the peptide separation using electrostatic binding and compares it with the classical method of separation by reversed-phase HPLC.

2 STATE OF THE ART

2.1 Peptides

Peptides and proteins are essential components of cells that perform a huge range of important biological functions. Proteins are involved in DNA replication, serve as enzymes catalyzing metabolic reactions, provide structure to cells and organisms, respond to stimuli and transport molecules. Certain types of peptides play key roles in regulating the activities of other molecules, they are involved in gene expression, metabolism and other important biological processes.

Structurally peptides and proteins are very similar. They consist of chains of amino acids linked by amide (peptide) bonds containing N-terminal and C-terminal residues at the ends. The basic distinguishing factors are size and structure. Peptides are smaller, and in addition, they tend to be less well defined in structure when it comes to the comparison with proteins, which can adopt complex conformations known as secondary, tertiary and quaternary structures. Peptides can be subdivided into oligopeptides and polypeptides. While oligopeptides contain from 2 to 20 amino acid residues, polypeptides are larger, consisting of up to 50 amino acid monomers. Proteins are formed from one or more polypeptides joined together; hence, proteins fundamentally are very large peptides often containing other, non-peptidic residues such as coenzymes, cofactors or are bound to other macromolecules[5] (such as RNA, DNA or cell membrane).

Peptides are chemically synthesized by the condensation of the carboxyl group of one amino acid to the amino group of another. To prevent undesirable side reactions with the various amino acid side chains, protecting group strategies[6] are used. Chemical peptide synthesis usually begins at the C-terminus of the peptide (carboxyl end) and proceeds to the N-terminus (amino end), opposed to the biosynthesis in living organisms, which occurs in the opposite direction[7,8]. The chemical synthesis of peptides can be carried out by using the classical solution-phase technique; however, this method has been widely replaced by a more sophisticated solid phase synthesis method (chapter 2.2). Nevertheless, solution phase synthesis retains its usefulness in large-scale production of short oligopeptides for industrial purposes and ligation of peptide segments forming greater peptides and proteins[9].

2.2 Solid phase peptide synthesis

Solid-phase peptide synthesis (SPPS) is based on sequential addition of α-amino and side-chain protected amino acid residues to insoluble polymeric support that is swollen in a certain solvent, while reagents are added to the suspension in a solution state. This setup enables us to remove the excess of reagents and byproducts easily, only by filtration followed by washing of the solid support by different solvents[9].

The first-ever solid-phase peptide synthesis was published in 1963 by Robert Bruce Merrifield, where he demonstrated his new approach by synthesizing a tretrapeptide[10]. For his work and his ensuing research on SPPS, he was awarded the Nobel Prize in 1984. The original resin used by Merrifield was a chloromethylated copolymer of styrene and divinylbenzene and is named after him. In parallel to the SPPS on polystyrene solid support, He also developed the Boc (*tert*-butyloxycarbonyl)

protection strategy for synthesizing peptides. In this strategy, the growing peptide chain is attached to the resin via the C-terminus and the N-terminus is protected by an acid-labile Boc group.

In 1970 a new base-labile protecting group method was introduced to SPPS by Han and Carpino[11]. This method uses 9-fuorenylmethoxycarbonyl group (Fmoc) to protect the amines, which is orthogonal to many other protecting groups and greatly contributed in forming the Fmoc/*t*-Bu approach which is the most used SPPS method nowadays.

A typical SPPS consists of a series of repetitive and alternating reaction and washing steps. The first reaction step of SPPS is the attachment of the first amino acid to the solid support. Only one functional group of the bi- or more-functional amino acid can react with the active site of the resin, while all the others are protected to avoid unwanted side reactions. Subsequently, the unreacted active sites of the resin are being capped to avoid the attachment of a different amino acid in the following steps (explained in detail below). After, a second functionality is deprotected so that a second amino acid can be coupled to the first one bound to the resin. The peptide chain is then elongated by further deprotection and coupling cycles. After the synthesis of the full peptide chain, the peptide and all its remaining protection groups are cleaved. The above-explained general reaction scheme is exemplified in

Figure 1.

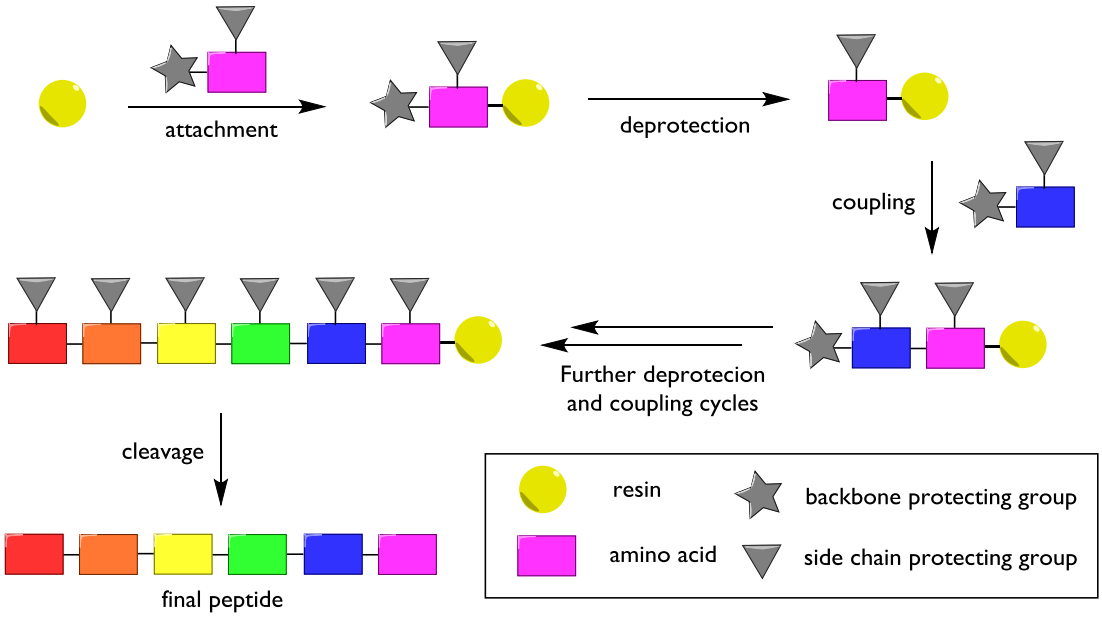


Figure 1 General reaction sequence of SPPS.

In-between the coupling and deprotection steps, a third reaction step called capping is often involved. Capping is used after the coupling step to block the reactivity of the unprotected growing peptide chains, which did not react during the previous coupling reaction, called failure sequences. The most used capping reagent is acetic anhydride, which readily reacts with the untouched amino groups of the failure sequences, thus preventing the reaction with a different amino acid in the next coupling step after the deprotection.

Linear SPPS is the fastest and most efficient ways of synthesizing peptides; however, it has some limitations. During the SPPS, several unexcepted difficulties can occur. The growing peptide chain can fold over into itself, or the neighboring chains can aggregate, thus making the synthesis more difficult or sometimes impossible. The result is difficult separation and low yield.

In 2003 B. L. Bray[12] summarized his observations on peptide length and ease of their synthesis and separation. The SPPS of peptides with around 15 amino acids or less is very efficient, and their separation is relatively easy. In the SPPS of peptides consisting of 20-30 amino acids, the purification becomes more difficult. The outcome of SPPS of peptides around 30-40 amino acids is very much sequence-dependent and is difficult to generalize, but the purification becomes gradually difficult, and the isolated yields significantly decrease. Peptides containing more than 40 amino acids might fail to produce and are prepared by combination of SPPS and solution phase synthesis. These observations are not absolute, and there are exceptions. There are peptides with less than 10 amino acids which could not be prepared by SPPS and successfully synthesized peptides above 50 amino acid residues using the same method.

2.2.1 Comparison of Boc and Fmoc methods

As mentioned above, there are two main strategies for SPPS – Boc/Bzl (*tert*-butyloxycarbonyl/benzyl) strategy and Fmoc/*t*-Bu (9-fuorenylmethoxycarbonyl/*tert*-butyl) strategy. The Fmoc/*t*-Bu strategy is currently the most used method in SPPS both for industrial and research purposes. Both protecting group strategies are shown in

Figure 2.

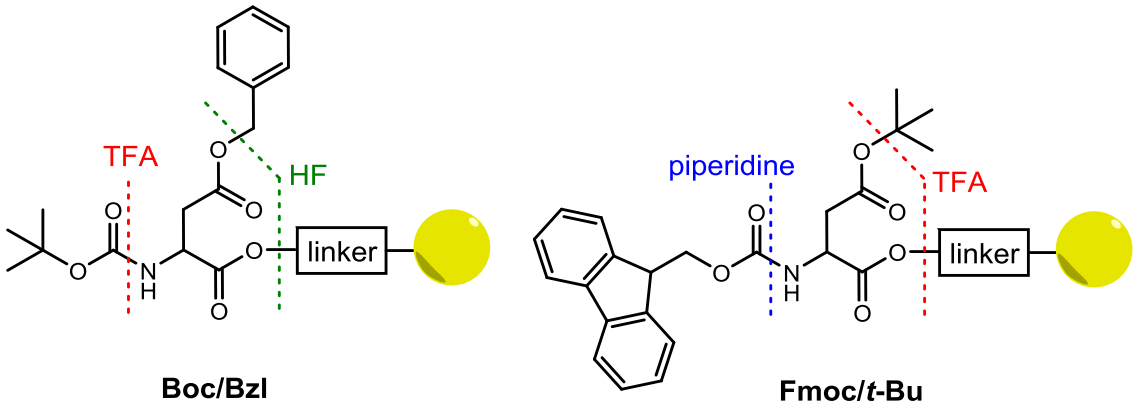


Figure 2 Protecting group strategies in SPPS.

In the Boc SPPS strategy, the temporary protecting group of the N^{α}-amino group is the acid-labile Boc, since it should be removed after every coupling step, while the permanent side-chain protecting group is benzyl, since they stay unharmed during the whole synthesis and are removed after the whole peptide synthesis is completed. Each temporary protecting group is removed after each coupling step using trifluoroacetic acid (TFA). The repetitive TFA treatment of the growing peptide chains can lead to side reactions and alteration of the peptide bonds. The final step is the cleavage of the peptide from the resin and all its permanent side chain protecting

groups. This step requires the use of dangerous hydrogen fluoride (HF) and expensive laboratory apparatus, which is not readily available to many researchers and may not be allowed in some countries by local safety regulations[13].

The development of the Fmoc strategy was triggered by the drawbacks of the Boc strategy. In Fmoc/*t*-Bu SPPS, the N^{α}-amino group is temporarily protected with a base-labile Fmoc group, which can be easily deprotected by mild base treatment using piperidine[11]. This leads to the formation of the desired free amino group and a side product – fulvene-piperidine adduct – as a consequence of the reaction between piperidine and dibenzofulvene[14] (Figure 3). It has to be pointed out that the Fmoc group is unsuitable for solution-phase peptide synthesis due to a number of potential side reactions caused by the free amino groups of the growing peptide chains in solution[15].

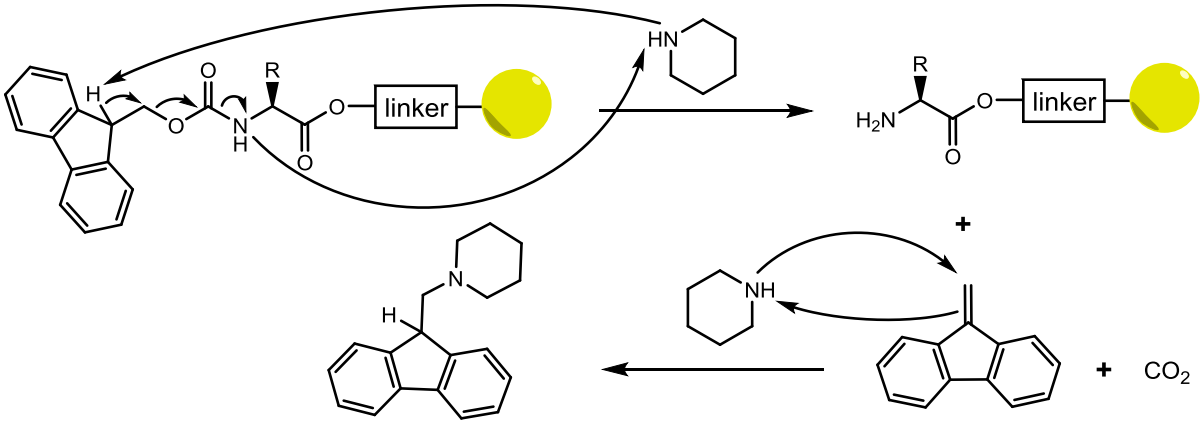


Figure 3 Piperidine mediated Fmoc removal.

Side chain protective groups are chosen to be orthogonal to the temporary protective group and can be removed simultaneously with the cleavage of the peptide from the resin. In the case of Fmoc, the side chains are mostly protected as *t*-Bu esters. The last step is the cleavage of the peptide from the resin and all of its permanent side chain protecting groups with the use of a TFA cocktail. The fully deprotected peptide is then precipitated form the TFA cocktail in cold ether[16].

Amino acids can be categorized into three groups with respect to the need of side chain protection:

1. Protection is not required – Ala, Gly, Ile, Leu, Met, Phe, Pro, Val.
2. Protection is optional but recommended to overcome unwanted side reactions – Asn, Gln, His, Trp.
3. Protection is needed – Arg, Asp, Cys, Glu, Lys, Orn, Ser, Thr, Tyr.

Recommended side chain protecting groups of some amino acids for both, Boc and Fmoc SPPS strategies are listed in Table 1[6].

Table 1 Recommended side chain protecting groups for Boc and Fmoc SPPS strategies.

Amino acid	Arg	Asn	Asp	Cys	Glu	Ser	Thr	Tyr
Boc strategy	Mtr	Trt	*t*-Bu	Trt	*t*-Bu	*t*-Bu	*t*-Bu	*t*-Bu
Fmoc strategy	Tos	—	Bzl	Acm	Bzl	Bzl	Bzl	2-Br-Z

2.3 Resins for SPPS

The whole concept of SPPS is based on attaching the first amino acid to the resin via a linker followed by peptide chain elongation. The most commonly used resins are based on polyethylene glycol cross-linked polystyrene polymers[17,18]. Other polymeric supports had been developed, including, for example, polyethylene glycol-acrylamide copolymers[19], polyamide supports[20], polyethylene glycol resins[21], polyethylene glycol-polystyrene graft resins[22] and other alternatives[23].

The size of the resin beads can be described in different ways. The two most common are the Tyler mesh size and the actual diameter of the resin beads expressed in microns (μm). Both values are inversely correlated to each other. The smaller the beads are, the faster the reaction kinetics will be, as the specific surface area is larger and allows fast diffusion of the reagents into the resin. On the other side, if the beads are too small, the filtration time will be extended. For this reason, a reasonable compromise between the reaction kinetics and the filtration time must be made. The most used commercially available resins have two common particle sizes; 200-400 mesh (35-75 microns) and 100-200 mesh (75-150 microns)[9].

The resins are insoluble in all common solvents except in some aprotic solvents like *N,N*-dimethylformamide (DMF), dichloromethane (DCM) or tetrahydrofuran (THF) they can be solvated and swollen. One of the most important properties of the resins is swelling, as peptide synthesis takes place inside the bead's swollen network[24]. Good swelling properties are an important factor in the choice of the resin since reaction kinetics in SPPS is diffusion controlled. This means the reaction rates are higher the faster the reactants can diffuse into the resin. High swelling provides necessary space for the growing peptide chains and thus minimizes aggregation, otherwise coupling, capping and deprotection steps can be hindered or even completely inhibited by the resin leading to a low yield of the final peptide. Swelling of a cross-linked copolymer resin takes place when the molecules of the solvent occupy the void space between the polymer chains, increasing of the volume of the beads[18]. The resins are insoluble in all common solvents except in some aprotic solvents, such as *N,N*-dimethylformamide (DMF), dichloromethane (DCM) or tetrahydrofuran (THF), in which they can be solvated and swollen.

Another important characteristic of the resins is the quantity of available functional groups for the attachment of the amino acids. The quantity of these active sites is expressed in millimoles per gram ($mmol \cdot g^{-1}$) and is called the resin substitution[25]. The substitution of commercially available resins for SPPS usually fits within the range $0.1 – 2\ mmol \cdot g^{-1}$.

2.3.1 Immobilization of the first amino acid

The first amino acid is attached to the resin via a linker. The linker should fulfill several requirements, including high stability to the chemicals used during the SPPS and high yields in both, immobilization of the first amino acid to the resin and detaching of the full-length peptide from the solid support[9]. Linkers can be divided into four categories based on the type of the functional group that is used for the attachment of the first amino acid to the resin via its:

1. *C*-terminus (C- to N-SPPS strategy) – using amide, ester, hydrazide, thioester, *O*-substituted oxime bonds[26]
2. *N*-terminus (N- to C-SPPS strategy) – reverse SPPS uses resin-bound amine[27], carbamate[28] or silyl carbamate[29] bonds
3. Side chain (can be carried out both directions) – the amino acids are attached via their side chain's carboxy, hydroxy, sulfanyl, amino, guanidino and phenyl groups[30]
4. Backbone – using backbone amide linker (BAL) for cyclic or *C*-terminally modified peptides[31–33]

2.3.2 Resin substitution

Hands in hands with the attachment of the first amino acid comes the question about the amount of them bound to the resin expressed as substitution, *S* [mmol/g]. Since in SPPS, the nominal batch size is calculated using the mass of starting resin and its substitution; it is very important information to obtain. Substitution is also used for calculations for other important process parameters, such as the number of equivalents of amino acids and other substances used during the synthesis or the overall yield.

The most used procedure[25] for substitution determination after the attachment of the first Fmoc protected amino acid is spectrophotometric analysis. A small amount of resin (10 – 20 mg) is treated with 20% (*v*/*v*) piperidine in DMF, and the base-labile Fmoc protecting groups are cleaved and dibenzofulvene-piperidine adduct is generated. The adduct is then quantified by UV-VIS spectroscopy at and 289.8 nm. Using equation 2.1, derived from Lambert-Beer's law, the resin substitution *S* can be calculated:

$$S = \frac{A_{289.8\,\mathrm{nm}} \cdot 10^6 \mathrm{mmol} \cdot \mathrm{mol}^{-1} \cdot \mathrm{mg} \cdot \mathrm{g}^{-1} \cdot V \cdot D}{\varepsilon_{289.8\,\mathrm{nm}} \cdot m_{\mathrm{resin}} \cdot l} \tag{2.1}$$

In the equation, $A_{298\,\mathrm{nm}}$ is the absorbance of sample solution at 289.8 nm; V [L] is sample volume, D is dilution factor; $\varepsilon_{289.8\,\mathrm{nm}}$ [L·mol^{-1}·cm^{-1}] is molar absorption coefficient of dibenzofulvene-piperidine adduct at 289.8 nm with the value of $\varepsilon_{289.8\,\mathrm{nm}}$ = 6089 L·mol^{-1}·cm^{-1}; m_{resin} [mg] is the sample weight of the resin used in the experiment and l [cm] is the optical length of the cuvette. Using a volume of 0.1 L of 20% piperidine in DMF without any dilution steps and a cuvette with 1 cm optical path length, the formula can be simplified as equation 2.2 or, neglecting units in equation 2.3.

$$S = \frac{A_{289.8\,\mathrm{nm}} \cdot 10^5 \mathrm{mmol} \cdot \mathrm{mol}^{-1} \cdot \mathrm{mg} \cdot \mathrm{g}^{-1} \cdot \mathrm{L}}{\varepsilon_{289.8\,\mathrm{nm}} \cdot m_{\mathrm{resin}} \cdot 1\ \mathrm{cm}} \tag{2.2}$$

$$S[\mathrm{mmol} \cdot \mathrm{g}^{-1}] = \frac{10^5 \cdot A_{289.8\,\mathrm{nm}}}{\varepsilon_{289.8\,\mathrm{nm}} \cdot m_{\mathrm{resin}}} \tag{2.3}$$

2.3.3 Selected resins

This chapter shows some of the most used resins suitable not just for SPPS, but also in many other fields of organic chemistry. A brief selection of resins used in Fmoc and Boc chemistry is shown in Figure 4 and some of them are discussed below.

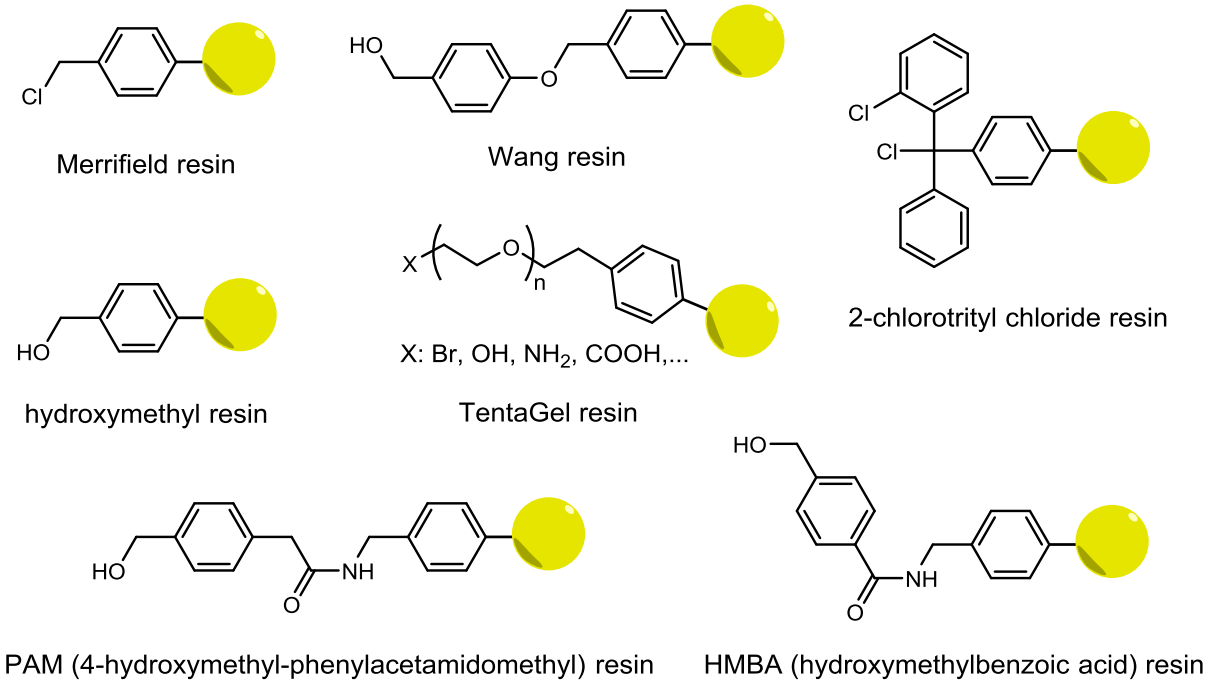


Figure 4 Selected resins used in SPPS.

Merrifield resin

The first resin used for SPPS by Merrifield is named after him. The Merrifield resin is a divinylbenzene-crosslinked polystyrene polymer carrying a chloromethane group attached to the polystyrene backbone as a linker. The reactive group of the resin is incorporated by copolymerization or electrophilic aromatic substitution. The standard Merrifield resin often serves as a basis for other resins that use linkers to introduce different functional groups for different synthetic strategies. The removal of the final peptide requires strongly acidic conditions[34]. Hydrofluoric acid, trifluoromethanesulfonic acid (TfOH) or microwave assisted TFA cleavage[35] can be used for detaching the peptide.

Wang resin

The Wang resin is very similar to the Merrifield resin; however, the cleavage conditions are significantly milder (50% TFA in DCM). This is caused by the alkoxy group of the *p*-alkoxybenzyl alcohol linker. The Wang resin is not limited just to peptide chemistry but found its application among organic chemists in general (attaching phenols, alcohols and more)[36,37].

Trityl-based resin

Resins functionalized with trityl based linkers are highly acid labile. The three phenyl rings of the trityl group stabilize the benzylic carbocation that is generated during the cleavage resulting in their ability to undergo acidolysis under extremely mild conditions. For the peptide cleavage, only 1% of TFA is needed, and even less acidic protocols using 20% hexafluoroisopropanol (HFIP) are enough. Peptides can, therefore, be cleaved from the solid support with all side chain protecting groups in place. This, if needed, can be used for further *C*- and *N*-terminal modifications, fragment condensation or head-to-tail cyclization. If glycine and proline are the first or second amino acid at the *C*-terminal, spontaneous cleavage from the peptide can occur due to diketopiperazine formation[38,39].

Various trityl-based linkers exist with different functionalization on the phenyls that fine-tune the cleavage conditions. The most used trityl-based resin in SPPS is the 2-chlorotrityl chloride resin, following by trityl, 4-methyltrityl and 4-methoxytrityl resins.

TentaGel resins

TentaGel resins have been developed for the use in polar solvents and are mostly incompatible with the traditional SPPS methods. TentaGel is produced by a process in which polyethylene glycol units of approximately 3 kDa are cografted onto low-cross-linked polystyrene. The result is a relatively hydrophilic resin which swells good both in polar protic and aprotic solvents like water, methanol or DMF, DCM or THF[40]. A variety of functional groups are available ranging from electrophilic bromine leaving groups[41] to nucleophilic groups like amino[42], hydroxy[43] or carboxy groups[44].

Due to these properties, the TentaGel resins found their application in synthesizing combinatorial libraries, where organic solvents are used, followed by bioassay in aqueous media[40].

2.4 Coupling methods in stepwise peptide chain elongation

Peptides are composed of amino acids linked by amide bonds. The formation of the amide bond is not easily accessible by treating the carboxylic acid with an amine, because before any nucleophilic substitution occurs, they undergo an acid-base reaction resulting in the formation of an ammonium salt[9,45].

The peptide chain elongation requires chemical activation of the carboxy functionality of the *N*-*α*-protected amino acid. The activation method must be chosen wisely to achieve very high coupling efficiency and avoid potential side reactions. The most frequently used coupling techniques employed currently in SPPS for stepwise addition of N^{α}-protected amino acids are listed below.

Carbodiimides

Carbodiimides have been some of the most popular *in situ* activating reagents in SPPS. In 1955 *N,N'*-dicylohexylcarbodiimide (DCC) was employed for the first time in SPPS[46]. DCC become one of the most popular coupling reagents for SPPS and SPS in general but has one main drawback. The downside of DCC is the formation of insoluble urea byproduct during the activation step slowing down the rate of the activation step so minimal quantities should be used. To overcome this problem, other carbodiimides like *N,N'*-diisopropylcarbodiimide (DIC) or the water-soluble *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were developed[47,48]. Selected carbodiimides are shown in Figure 5.

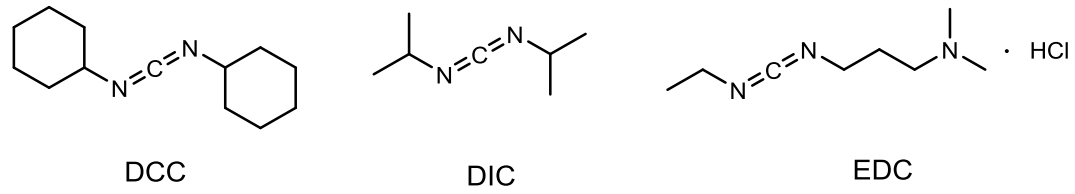
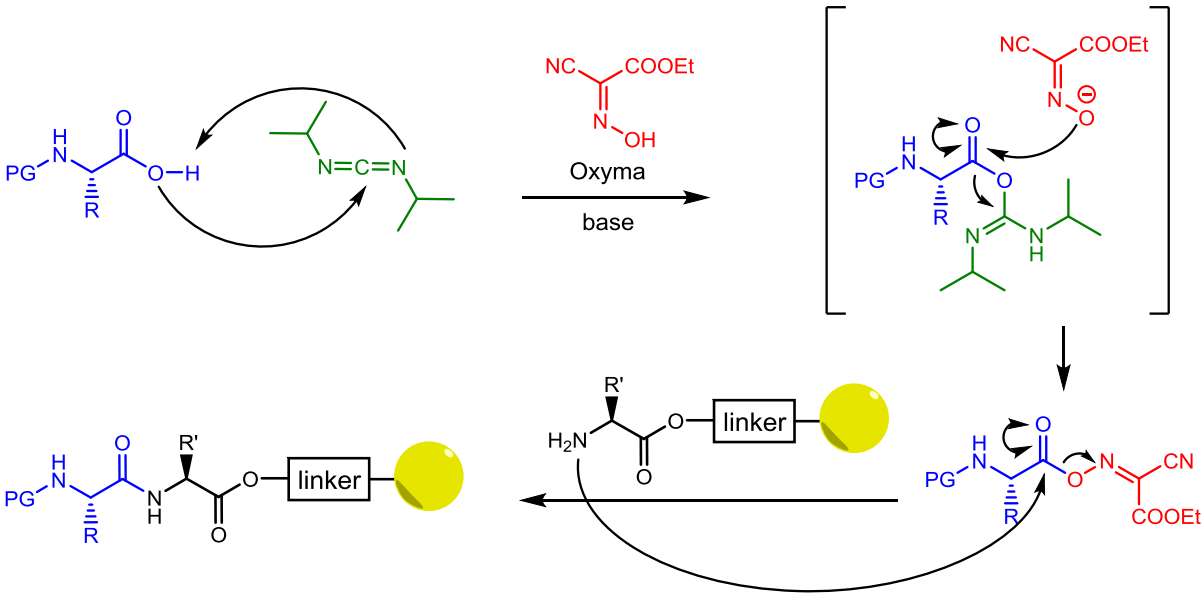


Figure 5 Selected carbodiimides.



Scheme 1 Proposed mechanism of peptide bond formation using Oxyma and DIC

El-Faham and Albericio reported[49,50] a safe and highly efficient additive, ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma), to be used mainly in the carbodiimide approach for peptide bond formation. Oxyma displays a remarkable capacity to suppress racemization and an impressive coupling efficiency in both manual and automated synthesis. The proposed reaction mechanism[51] of peptide bond formation via Oxyma and carbodiimides is shown in Scheme 1.

Active esters

Active esters are species that are highly susceptible toward nucleophilic attack. Active esters are mainly used as acylating agents and are prone to hydrolysis. Of great interest is the enhanced reactivity of active esters toward amines to give amides. The active ester can normally be obtained by reacting free carboxyl group of amino acids with either a substituted phenol or a substituted hydroxylamine, in the presence of a carbodiimide. The obtained active ester is then ready to react with the amino group of the growing peptide chain elongating it by one by forming a peptide bond.

The electrophilicity of the carbonyl carbon of the active ester is increased by the electron-withdrawing property of the leaving group. Commonly used coupling reagents include *N*-hydroxysuccinimide[52] (HOSu), *N*-hydroxybenzotriazole[53] (HOBt) and phenols with electron-withdrawing groups such as pentachlorophenol[54,55], pentafuorophenol[55] or *p*-nitrophenol[56]. Selected reagents for active ester preparation are shown in Figure 6.

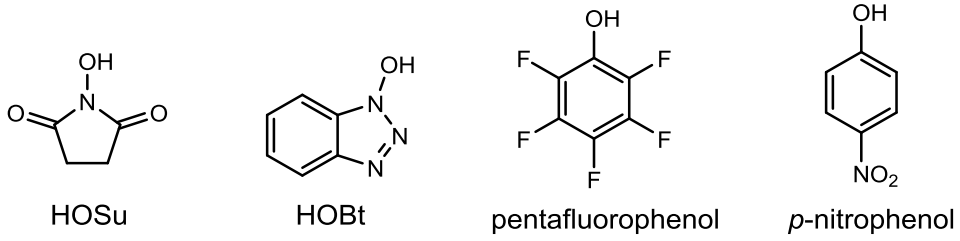


Figure 6 Selected reagents for active ester preparation.

Aminium and uronium salts

Aminium and uronium salts bear a positive carbon atom and were first introduced to SPPS by Gross and co-workers in 1978. They prepared 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafuorophosphate (HBTU) as an *N*-guanidium-type aminium salt suitable for SPPS[57]. Based on HBTU a various number of aminium salts were prepared including 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafuoroborate[58] (TBTU), 1-((dimethylamino)(dimethyl-iminio)methyl)-1*H*-[1,2,3] triazolo[4,5-b]pyridine 3-oxide hexafluorophosphate[59–61] (HATU) or 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate[62] (HCTU).

Hand in hands with the development of the aminium salts, the development of the related *O*-uronium salts took place. Prominent representatives for example are 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate[45,63] (TSTU) or the Oxyma-based *O*-[(ethoxycarbonyl)cyanomethylenamino]-*N*,*N*,*N*′,*N*′-tetramethyluronium

tetrafuoroborate[45,64] (TOTU) and (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)-dimethylamino-morpholino-carbenium hexafluorophosphate[65,66] (COMU).

In comparative studies in the coupling efficiency of different aminium salts, HATU has been found to give the best coupling yields with less isomerization. COMU mediates coupling reactions with efficiency even better than HATU. However, aminium and uronium coupling reagents have a disadvantage represented in their ability to react with free N^{α} -amino group of the incoming moiety. It means they cannot be added directly to the incoming moiety and carboxy component to be coupled. Preactivation of the carboxy group is required by the aminium/uronium salt, and the incoming moiety with the free N^{α} -amino group is added afterwards[9,66]. Selected aminium and uronium salts are shown in Figure 7.

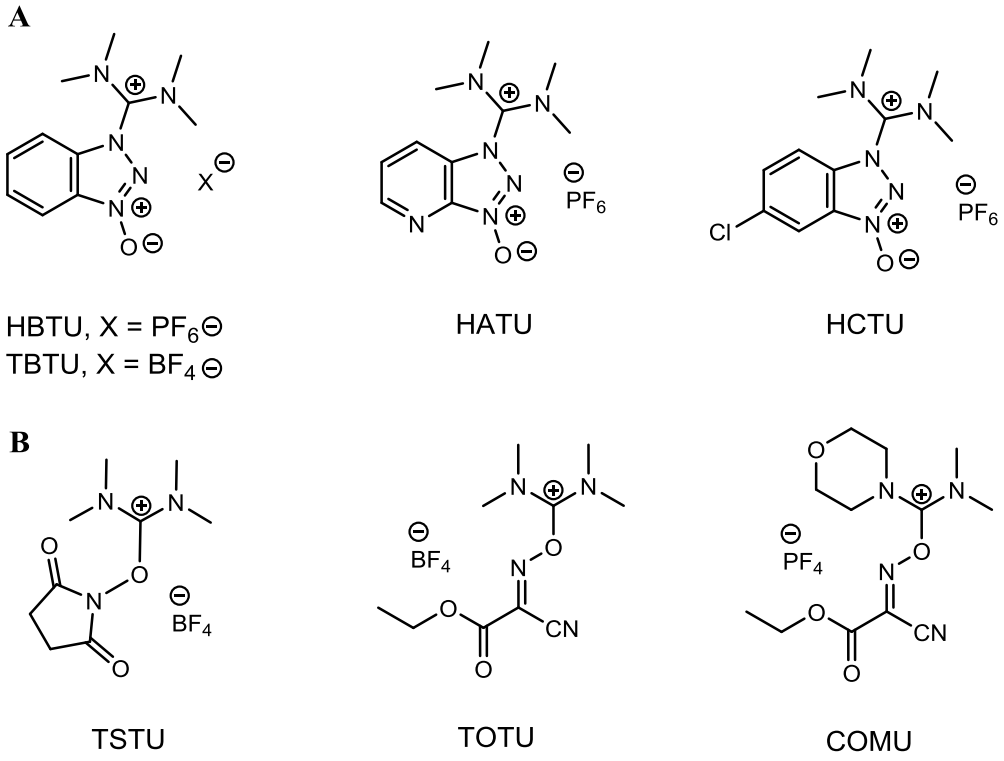


Figure 7 A: Selected aminium salts. **B:** Selected uronium salts.

Phosphonium salts

Phosphonium salts to promote peptide coupling by acylphosphonium salt formation were wide-spread after the development of the (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) reagent by Castro[67] in 1975. One of the drawbacks of the utilization of BOP as a coupling reagent is that it produces hexamethylphosphorotriamide (HMPA) as a toxic byproduct. Since then many other phosphonium salts were synthesized including benzotriazol-1-yloxytri(pyrrolidino)phosphonium hexafuorophosphate[68] (PyBOP), [(7-azabenzotriazol-1-yl)oxy]tris(pyrrolidino)phosphonium hexafuoro-phosphate[69] (PyAOP), or the Oxyma-based (((1-cyano-2-ethoxy-2-oxoethylidene)amino)oxy)tri-(pyrrolidin-1-yl)phosphonium hexafuorophosphate[70] (PyOxim or PyOxP).

One of the advantages of phosphonium salts is that they can be added directly to the amino group of the incoming moiety and carboxy component to be coupled. Unlike aminium and uronium salts, phosphonium salts do not react with free N^{α} - amino group of the incoming moiety, meaning that these reagents can yield the active ester *in situ*. Selected phosphonium salts are shown in Figure 8.

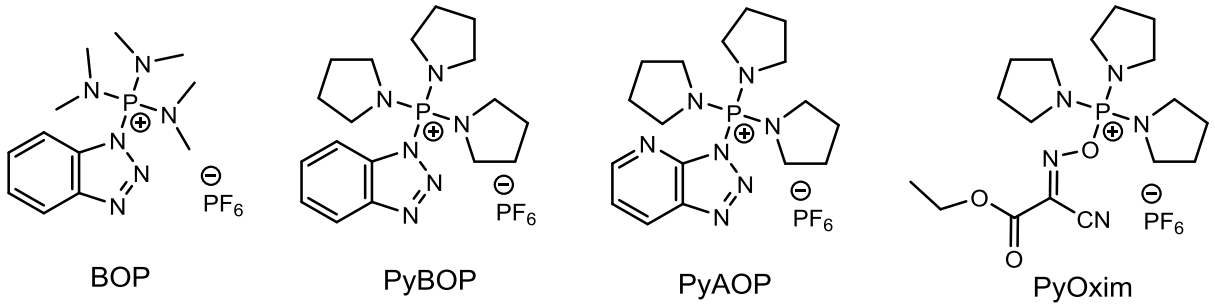


Figure 8 Selected phosphonium salts.

2.5 Purification of peptides

The previous chapters show that the most widely used method for peptide synthesis is on a solid support by stepwise addition of amino-protected amino acid monomers. Each addition of an amino acid usually requires three steps – coupling, capping and deprotection. Excess reagents and side products are washed off after each step of the synthesis. After the peptide synthesis, the product is cleaved from the solid support and fully deprotected by TFA cocktail, e.g., TFA/TIS/H_2O (92.5/2.5/5.0 *v*/*v*/*v*). The role of TIS (triisopropylsilane) in the TFA cocktail is to terminate the reactivity of the deprotected Boc groups.

Alongside the desired full-length peptide, the major impurities in the crude product are salts, scavengers, deprotected side-chain protecting groups, and, most importantly, the so-called failure sequences – peptides with shorter lengths compared to the desired product. The removal of the small molecules from the crude product is relatively easy. For example, precipitation can be used from diethyl ether from a trifluoroacetic acid solution. The purification of the full-length peptide from the failure sequences is more difficult due to their very similar properties, and other methods must be used[71].

Currently, the most used separation tool for peptides on an industrial scale is reversed-phase high-performance liquid chromatography (RP-HPLC). Other methods for peptide purification include antigen-antibody affinity purification[72], covalent capture with a solid matrix[73], fluorous affinity purification[74], and lipophilic tag assisted chromatography[75].

2.5.1 Purification of peptides by RP-HPLC

Reversed-phase HPLC has become an essential tool in the separation and analysis of proteins and peptides. It plays a vital role in the separation of peptides from digested proteomes, during investigative studies, in biotechnology and is used for

large scale purification of peptide therapeutic drugs. RP-HPLC has found a central role in peptide and protein studies because of its versatility, sensitive detection and its ability to work together with other techniques such as MS. Moreover, RP-HPLC is widely used because of its ability to separate proteins and peptides of nearly identical structure[76].

In RP-HPLC, the particle surface is very hydrophobic due to the chemical attachment of hydrocarbon groups (C_{18}, C_8, C_4) to the surface. Peptides are retained by the adsorption of their hydrophobic parts to the hydrophobic surface. Since peptides are large compared to the thickness of the hydrophobic surface, only a portion of the peptides adsorbs to the hydrophobic surface, and the rest of the peptide stays in contact with the mobile phase. The net interaction caused by this hydrophobic adsorption is very strong, resulting in the peptide remaining adsorbed to the surface until a specific concentration of organic solvent is reached, at which time the peptide desorbs from the surface and elutes from the column. The concentration of the organic solvent required to desorb the peptide is highly specific and is a function of the size of the hydrophobic part of the molecule[77,78].

For semi-preparative and preparative HPLC, it is necessary to achieve purities of >95%. The choice of RP silica depends on the peptide's size and hydrophobicity – packings of C_{18}, C_8, C_4 or biphenyl are recommended. Polypeptides are almost always eluted using a solvent gradient where the relative concentration of organic solvent is slowly increased during the separation. The most used eluents are H_2O/MeCN, H_2O/MeOH, H_2O/isopropanol gradient with acidic ion-pairing reagents like TFA, heptafluorobutyric acid (HFBA), phosphoric acid or buffers like NH_4OAc, triethylammonium phosphate (TEAP). After the purification, the fractions containing the product are collected and lyophilized[79,80].

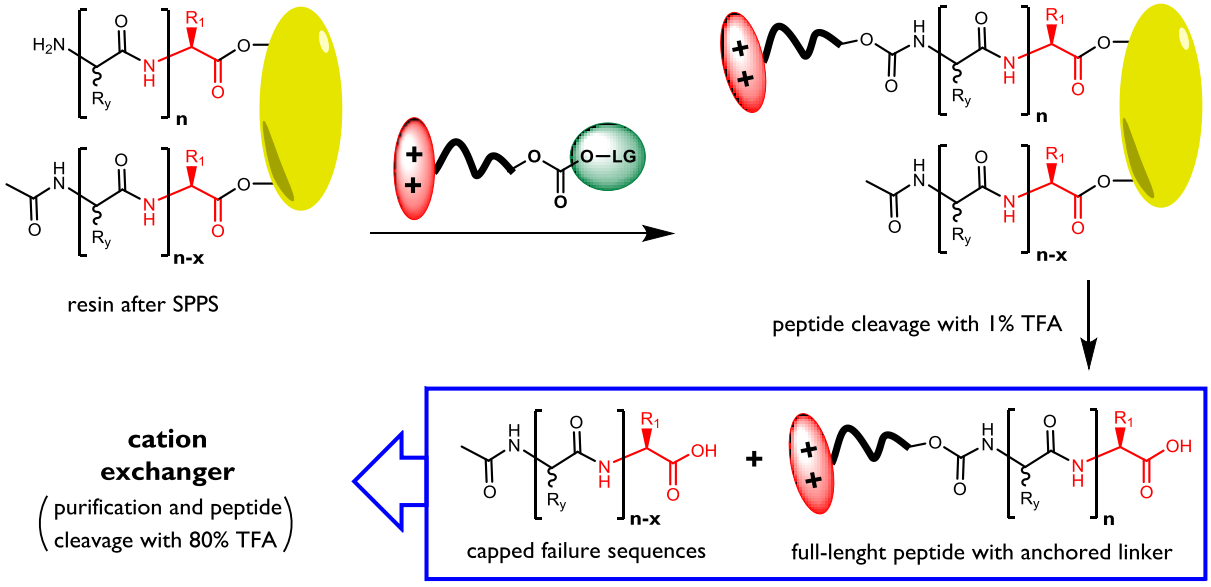
2.5.2 Purification using a polymerizable methacrylamide tag

In 2014, a new approach to peptide synthesis and purification was published[71] by Zhang et al., in which during the automated solid-phase peptide synthesis the failure sequences were capped with acetic anhydride. This capping blocked the growth of the failed peptide, so it was unable to react with another amino acid in the following steps of the synthesis. After synthesis, a polymerizable methacrylamide tag was attached to the full-length sequences, while capped failure sequences with the acetic anhydride were unable to react with the methacrylamide tag. Later the peptides were cleaved from the solid phase, and the purification was achieved by polymerizing the full-length sequences, washing away impurities, and cleaving the peptide product from the polymer.

3 THE AIMS OF THE WORK

Currently, the most used separation tool for peptides on an industrial scale is RP-HPLC, but it also has its limitations. In general, the disadvantages of HPLC for large-scale purification include high costs on instrumentation and columns, labor intensiveness, high energy demand for solvent evaporation, and inability to resolve peptides with multiple higher order structures. Most importantly, HPLC consumes large volumes of harmful solvents. This results in high waste-to-product ratios, usually more than 1,000. The waste of solvents during the synthesis and purification is one of the most significant driving forces to improve and tune the current peptide synthesis methods.

With this work, we would like to contribute with a new approach to the world of peptide synthesis and purification. We proposed a new separation method using electrostatic binding for the immobilization of the full-length peptides to a solid support (e.g., cation exchanger). For instance, electrostatic binding was used in case of binding a series of monosubstituted tetraalkylammonium derivatives of α-, β-, and γ-cyclodextrins with 1, 2 and 3 permanent positive charges to negatively charged solid surface[81,82]. The molecules electrostatically attached to the surface can be washed off with concentrated aqueous salt solutions and this way the surface and the charged molecules can be used multiple times.



Scheme 2 New separation method using electrostatic binding

The whole idea is that to the amino group to the full-length peptide, after the classical SPPS including Ac_2O capping in-between the coupling and deprotection steps, a linker with a positively charged anchor is attached. The peptide is then cleaved from the resin with its side-chain protecting groups attached using 1% TFA solution and electrostatically bound to a cation exchanger. The following step will be washing off the excess of reagents, side products and capped failure sequences and receiving the pure peptide from the solid support afterwards. The whole concept is shown in Scheme 2.

3.1 Plans of research

We prepared the plans of research divided into four parts which are discussed in detail in chapter 4 RESULTS AND DISCUSSION:

1. Synthesis of a series of neopentyl-based multiply positively charged anchor molecules that will attach the peptide to the negatively charged solid surface through a linker.

2. Synthesis of a linker bound to a multiply-charged anchor, containing a moiety capable to covalently bind peptides (called “anchored linker”) suitable for the reaction conditions used during peptide cleavage from the resin and deprotection. The linker will be attached to an anchor via click reaction.

3. Synthesis of a peptide on a solid phase and its purification in a standard way using RP-HPLC

4. Separation of the peptide using the newly proposed method based on electrostatic binding and comparing it with the RP-HPLC method.

4 RESULTS AND DISCUSSION

4.1 Synthesis

The synthetic part of the work was divided into three parts. The first part of the work includes the synthesis of a series of neopentyl-based positively charged anchor molecules, which can be used for the attachment of the desired peptide to the negatively charged solid surface.

The second part includes the preparation of a linker containing a previously synthesized multiply-charged anchor and containing a moiety suitable to covalently bind peptides through their terminal amino group. Such "anchored linker" must be stable under the reaction conditions used during peptide cleavage from the resin and deprotection.

The third part of the synthetic work was done in APIGENEX s.r.o. and included the synthesis of a peptide using the SPPS method under the supervision of Mgr. Tomáš Warzecha. The company is a contract research organization based in Prague, Czech Republic. Besides peptide synthesis, they are experts in chemistry, experimental pharmacology and clinical pharmacology.

4.1.1 Charged anchors

The methods for the preparation of the charged anchors were developed in cooperation with Petr Kasal's dissertation project as a part of the project MPO Trio - FV10082 and Czech patent application[83] (PV 2019-731). The synthetic routes of the charged anchors were developed by Petr Kasal and were simplified and scaled up in this work to prove that the anchors can be readily prepared on a large scale (100 g of the starting material) for the further possible industrial use. Notable is the fact that during the synthesis of the anchors, no chromatographic separations were used.

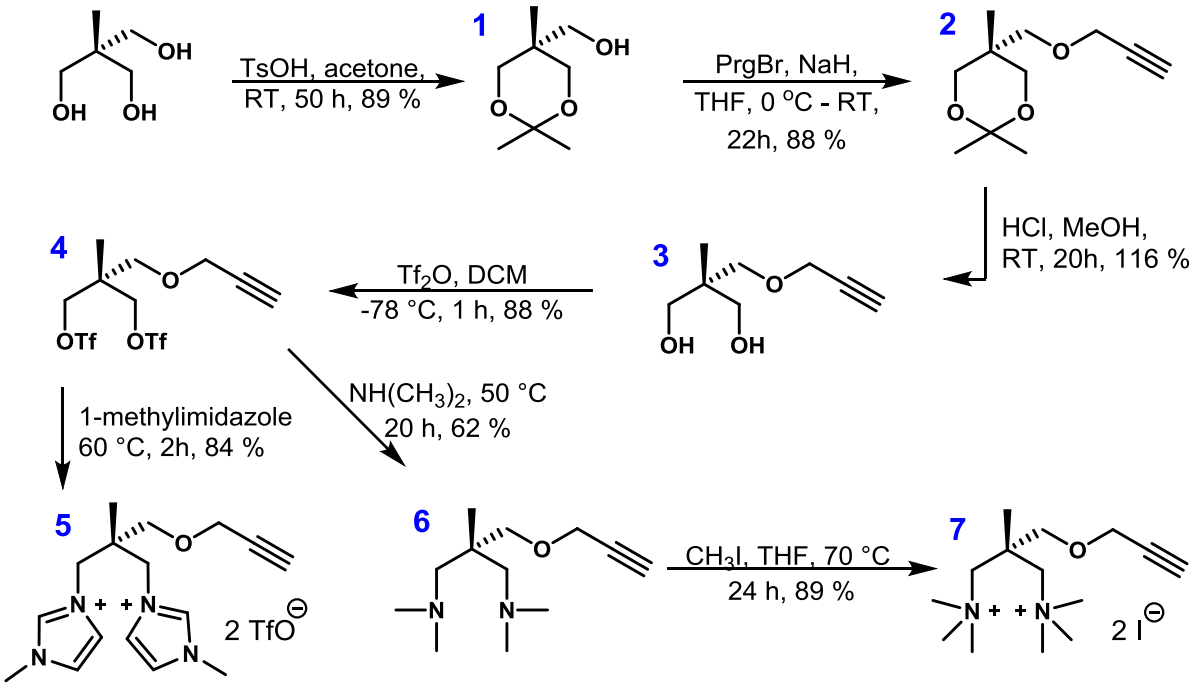
Two series of neopentyl-based positively charged anchor molecules were prepared with two and three positive charges, respectively. The individual steps of the synthesis of compounds **5** and **7** bearing two positive charges are represented in Scheme 3, while Scheme 4 shows the synthetic route of compounds **12** and **14** with three positive charges.

Synthesis of the charged anchors bearing two positive charges

As stated before, the preparation of the anchors was performed on a large scale to prove that they can be efficiently prepared for further possible industrial use. As the starting material 100 g of 1,1,1-tris(hydroxymethyl)ethane was used. The first step of the synthesis was the protection of two of three alcohol functionalities of the starting material as acetals using acetone[84]. Product **1** was obtained by distillation of the crude product.

Compound **1** was used in the following step, where propargyl group was attached to the remaining unprotected alcohol using propargyl bromide[85]. Compound **2** was obtained as colorless oil.

This was followed by the acidic hydrolytic deprotection of the two protected alcohol groups[85]. Compound **2** was treated with methanolic solution of hydrochloric acid until the full hydrolysis of the acetals occurred. The final compound **3** had clean NMR spectrum, however, it was contaminated with NaCl due to neutralization with NaOH. Luckily it could be used for the next reaction without any complications.



Scheme 3 Synthesis of anchors with two permanent positive charges

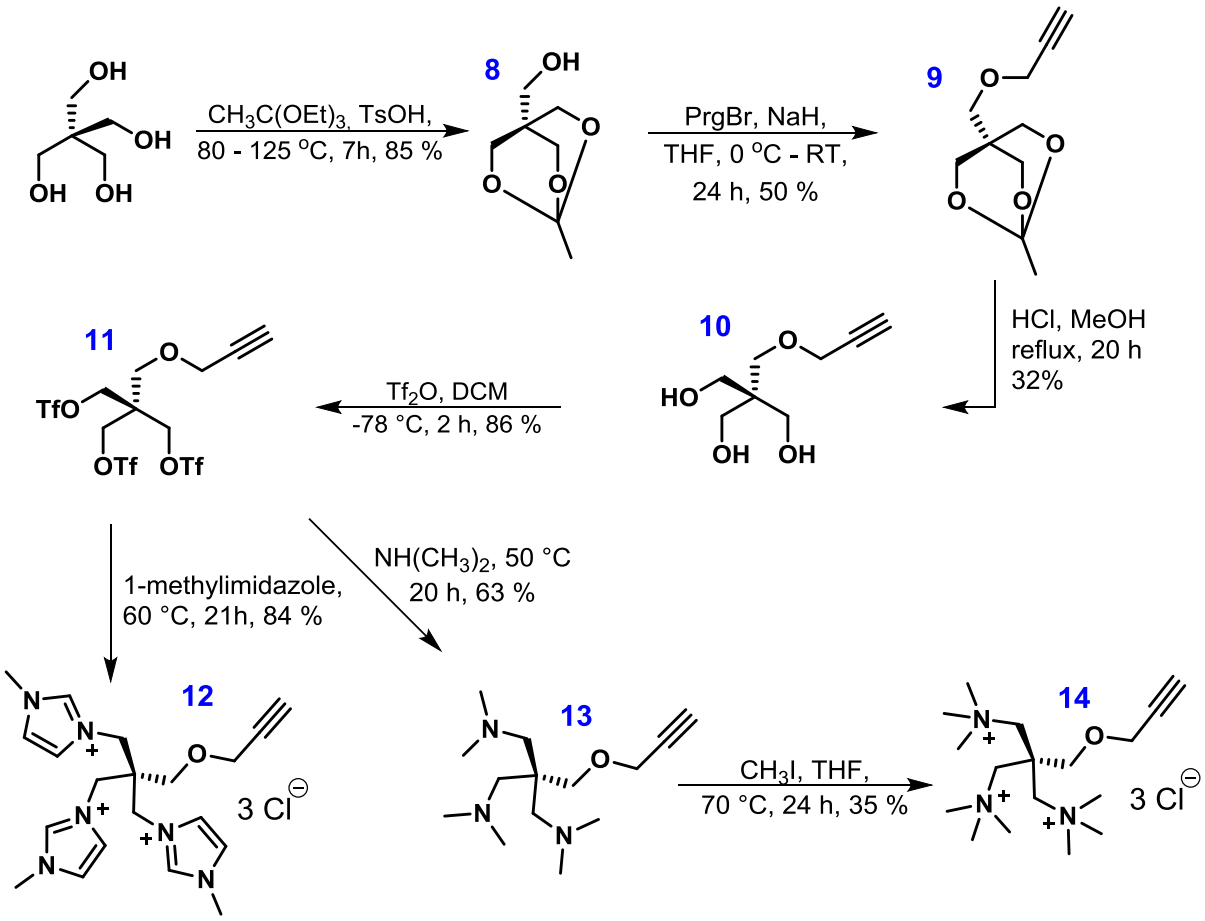
The synthesis[86] of compound **4** was accomplished via the nucleophilic substitution reaction of Tf_2O with **3** using 2,6-lutidine as base. Product was obtained as brown oil. It was used for the preparation of the final double-charged anchors – **5** with 1-methylimidazole ammonium salts and **7** bearing quaternal trimethylamine ammonium salts.

The first double-charged anchor was prepared from one third of compound **4**. 1-Methylimidazole was used as the reactant and the solvent as well. After full conversion, the solvent was distilled off and the crude product was purified by extraction. The final product **5** was obtained as light brown oil with 55% yield after five steps of synthesis. It must be noted that the triflic leaving group functioned as the counterion in the final product[83].

The second third of compound **4** was used in the next reaction[83]. Compound **4** and anhydrous dimethylamine were sealed in a thick-walled ampoule and heated overnight. After, the reaction mixture was extracted between DCM and aqueous solution of NaOH. Organic phase was evaporated on RE and the crude product was codistilled with water. The intermediate **6** was purified by extraction from water to DCM.

The aim of the following reaction was the quaternization of the two amino groups of **6** to obtain two positive charges relatively close to each other[83]. Twenty equivalents of methyl iodide had to be used in THF to push the reaction rate to its maximum. Product was precipitated as solid and was purified by extraction with THF. Compound **7** bearing two positive charges was obtained as a light pink crystalline with 36% yield (12.0 g) after six steps of synthesis. This time the counterion to the trimethylammonium cation is the iodide from the used iodomethane. The lower overall yield compared to **5** was caused by the extra step in the synthetic route, where intermediate **6** was obtained in 62% yield.

Synthesis of the charged anchors bearing three positive charges



Scheme 4 Synthesis of anchors with three permanent positive charges

For the preparation of the anchors with three permanent positive charges, 100 g (735 mmol) of pentaerythritol was used as starting material. This time three of the four alcohols were protected as orthoesters by using triethyl orthoacetate[87]. The crude product was purified by sublimation on RE with oil bath at 210 °C obtaining compound **8**.

The remaining free -OH group of compound number **8** was propargylated using propargyl bromide analogically to the reaction conditions[85] used for the preparation of **2**. Compound **9** was obtained by purification of the crude product by sublimation on RE with oil bath at 180 °C.

The treatment of **9** by methanolic solution of hydrochloric acid resulted in the deprotection of the orthoester protective group[88], obtaining compound **10** in 37% yield due to poor isolation. This was followed by the preparation of the compound for the S_N2 reaction with the nitrogen compounds later. Tf_2O was used under the same conditions[86] to obtain compound **11** as in the case of preparation of **4**.

The first triple-charged anchor was prepared from one third of compound **11**[83]. 1-Methylimidazole was used as the reactant and the solvent as well. Crude product was purified on weak cation exchanger, eluting with water and aqueous solutions of NH_4HCO_3 and neutralized with HCl. After six steps of synthesis compound **12** was

obtained as a salt with chloride anions with 11% yield (13.5 g).

The second third of compound **10** was used in a reaction with anhydrous dimethylamine in a thick-walled ampoule and heated overnight[83]. After extraction between organic and aqueous phase, the organic phase was evaporated on RE and the crude product was codistilled with water. The intermediate **12** was obtained by extraction of the water with organic solvent.

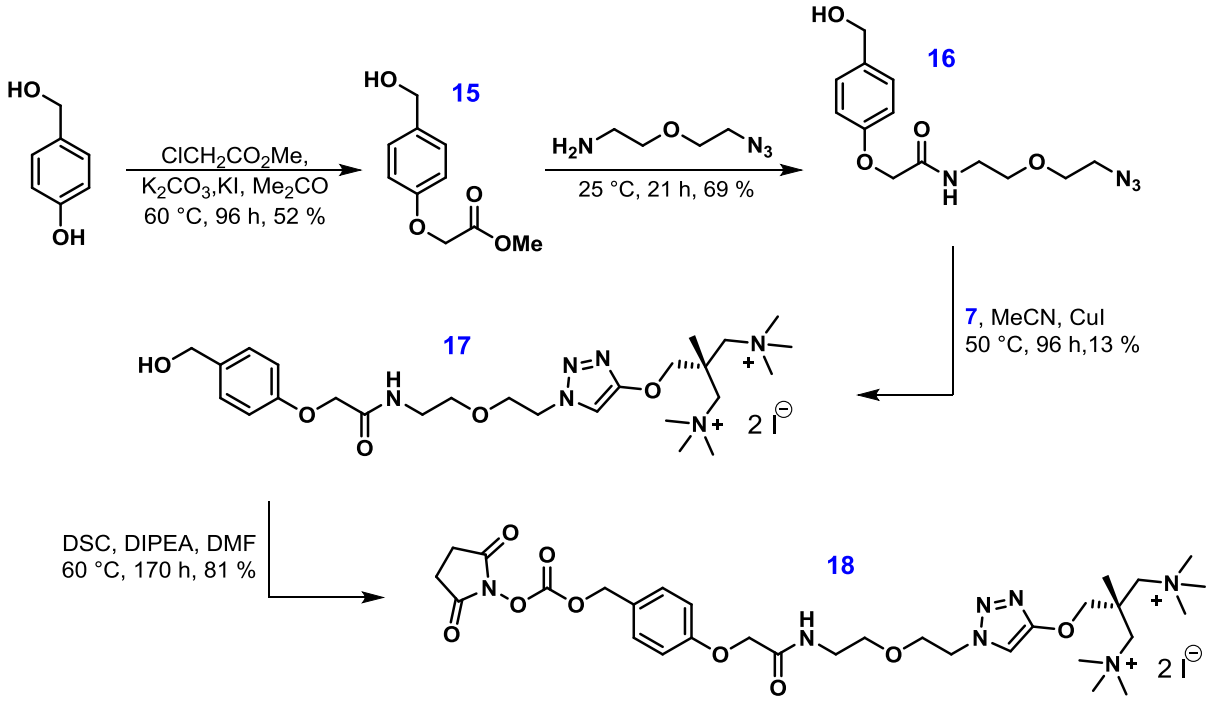
Compound **13**, the last of the charged anchors in this series, was synthesized by quaternization of the three dimethylamino groups of **12** using iodomethane[83]. However, using even 30 equivalents of methylating agent couldn't push the reaction to full conversion and besides the desired product a side product was formed with only two methylated amino functionalities. The mixture was purified on weak cation exchanger, eluting with water and aqueous solutions of NH_4HCO_3 and neutralized with HCl. Compound **14** was obtained as a salt with chloride anions with 3% yield (4.8 g) after six steps of synthesis.

4.1.2 Anchored linker

To allow us using the charged anchors for peptide separation, we had to design a linker connecting the anchor with the peptide. The linker should be stable under the reaction conditions used during peptide cleavage from the resin and deprotection, on the other hand it should be easily detachable from the peptide after it is purified on solid phase.

Based on a publication[71] from 2014 by Zhang et al., we designed an acid-labile linker containing a reactive succinimidyl carbonate function able to react with the amino group on the peptide and an azido functionality allowing to connect it with an anchor via click reaction (Scheme 5). Because as a solid support for SPPS we plan to use 2-chlorotrityl chloride resin, the linker must be stable in 1% TFA, which are the conditions for cleaving peptides from the resin[12,38,89]. On the other hand, the linker must be readily cleavable under more acidic conditions to obtain the peptide in good recovery yield after the purification process. These goals were achieved by careful tuning the electron density of the benzene ring in the linker. The molecule was also designed to be easily synthesizable for further possible industrial use. Scheme 5 shows the synthetic route of the chosen anchored linker.

The first reaction was carried out by the modification of a published procedure[90] using 4-hydroxybenzyl alcohol and methyl chloroacetate as starting materials. The reaction conditions remained unchanged; however, we changed the separation method. In the article flash column chromatography eluted with $CHCl_3$/MeOH (4/1) was used, but we had to make some changes, since the described method in the article did not work for us. We obtained the product using two chromatographic columns. The first column was eluted with pure Et_2O, where most of the impurities were removed. The second column eluting with $CHCl_3$/MeOH gradient (100/0 → 100/1 → 50/1) removed the impurities having the same R_f as the product in the first column. On a small scale, compound **15** was obtained without impurities, however, on a large scale, product was obtained as a mixture with 8-9% impurities according to NMR. Luckily, these impurities stayed untouched during the following step and could be easily removed.



Scheme 5 Synthetic route of the anchored linker

The following step is a modified procedure[71] from Zhang et al., where they use triethylene glycol diamine as reactant and flash column chromatography eluting with Et_2O/MeOH/MeCN/Et_3N (5/2/2/1) for purification. In our case, a 2-(2-azidoethoxy)ethanamine spacer was attached to compound **15** to give **16,** allowing to click the anchors to its azido moiety in the upcoming step. The crude product was purified on column using silica gel eluting with Et_2O/MeOH 10/1.

To compound **16** the anchor with number **7** was clicked via click reaction resulting in compound **17**. A click reaction is a copper(I)-catalyzed azide alkyne cycloaddition (CuAAC) resulting in a 1,2,3-triazole ring with 1,4-regioselectivity[91]. The double-charged anchor **7** was chosen because of its higher overall yield compared to the triple-charged anchors. The whole reaction was provided under argon atmosphere in acetonitrile bubbled with argon to remove oxygen, since the O_2 would oxidize the Cu^+ catalyst.

The last step was the activation of the almost ready anchored linker, so it can readily react with the amino moiety of the full-length peptide later. First, we tried the activation with *p*-nitrophenyl chloroformate, just as in the article[71] cited before, but the result was pure disappointment. Howsoever hard we tried by drying all the glassware and chemicals used, we could not detect any product neither by TLC methods, nor ESI MS. After providing an orientation reaction with the set reaction mixture by benzylamine, we observed a compound with *p*-nitrophenyl chloroformate proving the reactant is not decomposed. The formed product had to be too reactive and was decomposed during the TLC and ESI MS detection or the reaction did not work at all.

Keeping this in mind, we decided to try an another activation method using *N*,*N*′-disuccinimidyl carbonate (DSC), which is widely used in peptide synthesis. The reaction was successful with full conversion by ESI MS. Crude product was purified by extraction and the activated anchored linker **18** was obtained with 4% yield after four steps of synthesis.

4.1.3 Peptide by SPPS

The third part of the synthetic work was performed in APIGENEX s.r.o. and included the synthesis of a peptide using the SPPS method. We decided to prepare a hybrid peptide, cecropin A (1-7) – mellitin (2-9), containing 15 amino acids (H-Lys-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-COOH)[92] and is shown in Figure 9. This peptide sequence is a hybrid consisting of the first seven amino acids of the antimicrobial peptide cecropin A and residues 2 – 9 of the bee venom peptide mellitin. The peptide has a highly basic N-terminal domain and a quite hydrophobic C-terminal domain and in synthesis it's known for its difficulty. The peptide was chosen for its high aggregation potential, since it contains several hydrophobic Ala, Val, Leu, and Ile residues and because of the relatively large number of Boc protection groups present on the Lys and Trp amino acid residues[93]. The higher aggregation potential was expected to generate more failure sequences and to better show the effectiveness of the new separation method later.

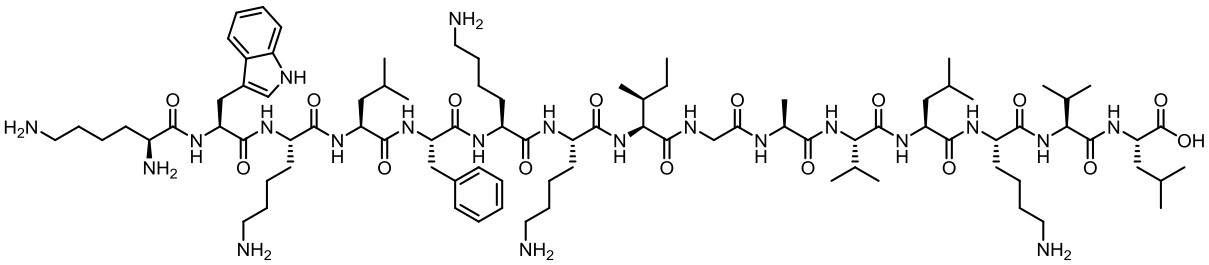


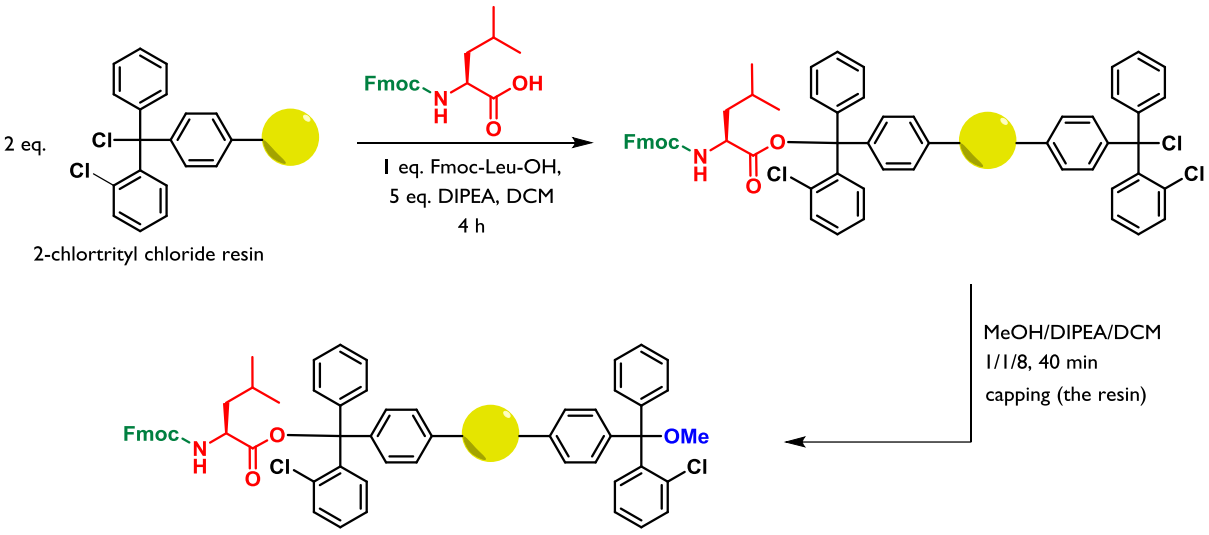
Figure 9 Structure of cecropin A (1-7) – mellitin (2-9)

Manual SPPS of cecropin A (1-7) – mellitin (2-9)

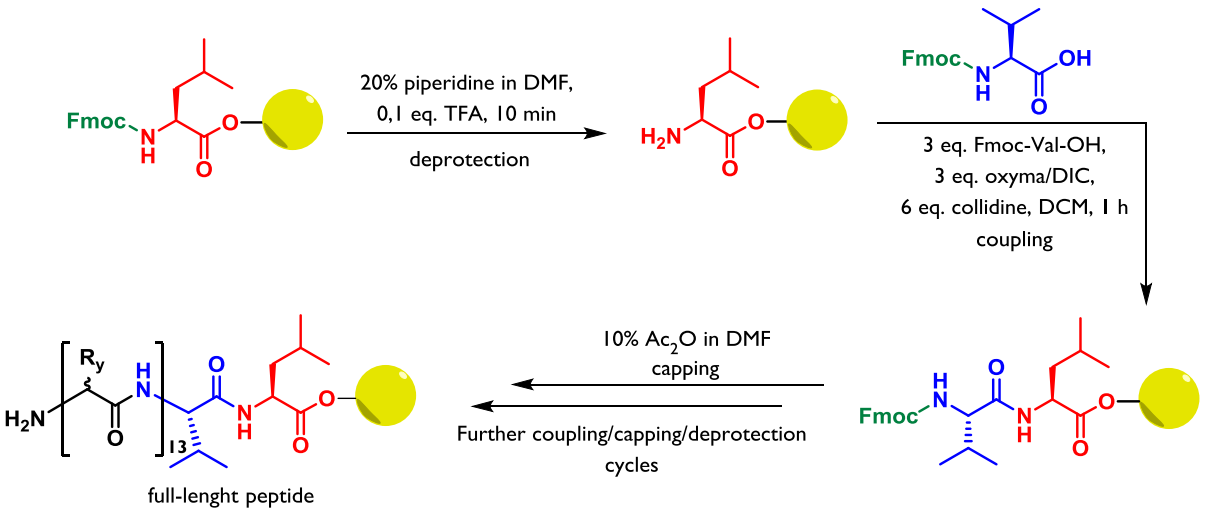
The chosen hybrid peptide synthesized using a solid-phase method on 2-chlorotrityl chloride resin using Fmoc chemistry. Amongst the 8 used amino acids monomers (Lys, Trp, Leu, Phe, Ile, Gly, Ala, Val), Lys and Trp were protected on the side chain by Boc protecting group. The synthesis was carried out manually in a peptide reactor.

The attachment of the first amino acid, leucine, to the resin by its C-terminal was performed by S_N1 on the 2-chlorotrityl moiety. Half equivalent of the Fmoc-Leu-OH was used compared to the loading capacity of the resin. After the reaction the remaining active 2-chlorotrityl sites were capped by methoxy groups (Scheme 6). Resin substitution was determined as S = 0.478 mmol/g by the method described in chapter 2.3.2 using equation 2.3 (see chapter 6.3.1). The following 14 amino acids were coupled to the growing peptide chain by using three equivalents of amino acid monomers alongside coupled using 3-fold excess of Oxyma and DIC with 6 eq. of collidine used as base (Scheme 7). Each coupling was aborted after one hour (instead of leaving it for three hours) to generate more failure sequences.

After coupling, failure sequences were capped with excess acetic anhydride to prevent their growth and attachment of the charged anchor during separation (Scheme 7). Deprotection of the Fmoc group was carried out, in a normal way with piperidine solution two times for over 10 and 15 min.



Scheme 6 Attachment of the first amino acid to the resin and its capping



Scheme 7 SPPS – deprotection, coupling, capping cycles

SPPS monitoring

The synthesis was monitored after each coupling by a ninhydrin test and an UPLC-MS analysis. For both tests a small amount of resin was washed with DMF, and DCM. Half of it was used for the ninhydrin test by adding a few drops of KCN in pyridine and ethanolic solutions of phenol and ninhydrin and suspension was heated for 5 min at 100 °C. The ninhydrin reacts with the free amino groups of the growing peptide chains causing a coloring range from light purple to dark blue. The more unreacted -NH_2 groups are present, the darker the color is. If the sample remains colorless, no or little peptidic amino groups remain unreacted and the coupling is done. The coloring of ninhydrin tests provided after every coupling was in purple range, meaning most of the growing peptide chains were coupled, but enough failure sequences were left for our purpose.

Before the UPLC-MS analysis, fast peptide cleavage was performed on the second half of the prepared resin by adding a TFA/TIS/H_2O (95/2.5/2.5) mixture and heating for 2 min at 100 °C. The harsh acidic conditions detach the peptide and capped failure sequences from the 2-chlorotrityl resin and remove the Boc protective groups. TIS is added as an agent to neutralize the fallen Boc groups. It must be noted that Fmoc protective group attached on the N-terminal stays on the peptide, since it's not acid labile. After, the suspension was filtered and aqueous solutions of NH_4OAc and MeCN are added to neutralize excess of TFA and better solubilize the peptide. The mixture is then chromatographed and analyzed by UPLC-MS. The results show the *m*/*z* of the Boc-deprotected growing peptide chain with Fmoc attached on the N-terminal, capped failure sequences, fallen protective groups and other agents used during the fast cleavage (Figure 10).

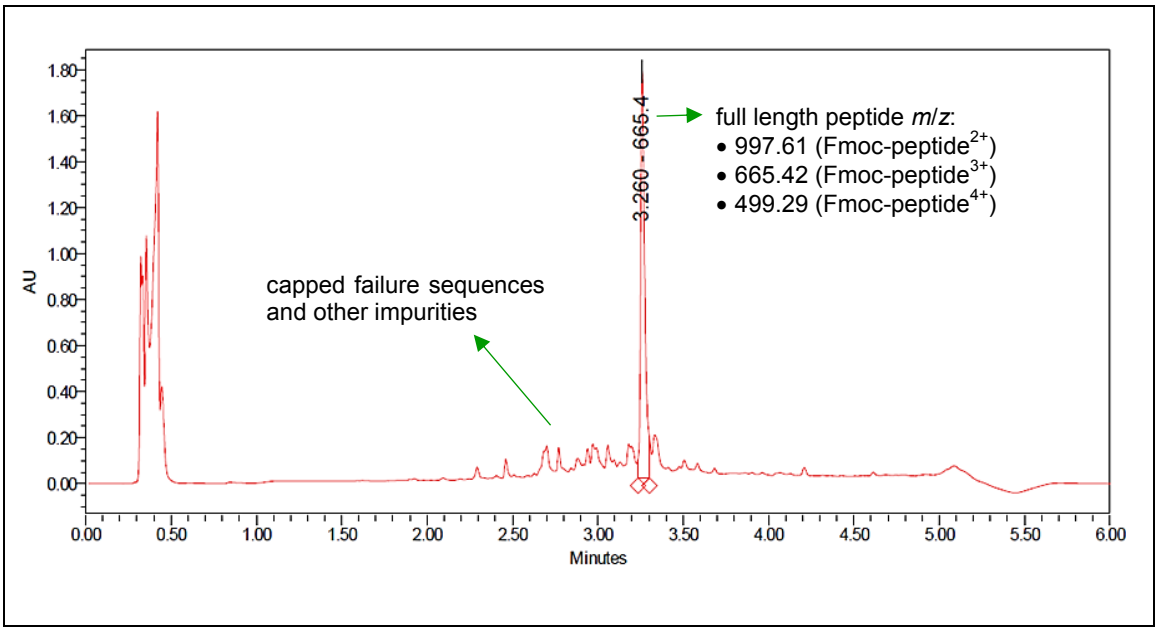


Figure 10 UPLC-MS analysis of the Fmoc-protected peptide cleaved from the resin after SPPS; the peak at 3.26 min corresponds to the Fmoc-protected full-length peptide

Peptide purification on RP-HPLC

After the synthesis had been completed, the peptide from 250 mg resin was purified in classic way on RP-HPLC. Prior to separation, the resin was treated with a TFA cleaving cocktail releasing the full-length peptide, the 14-mer and all the capped failure sequences and removing Boc protecting groups. After filtration the filtrate was precipitated in cold Et_2O and the crude product was obtained by filtration. Crude product (104 mg) was purified on RP-HPLC eluting with MeCN/H_2O/0.1% TFA gradient 10 % → 50 % (Figure 11). The product was obtained in two fractions; one in 94.6 % purity (17.49 mg) with 12% yield and the other in 76.6% purity (13.16 mg) with 9% yield.

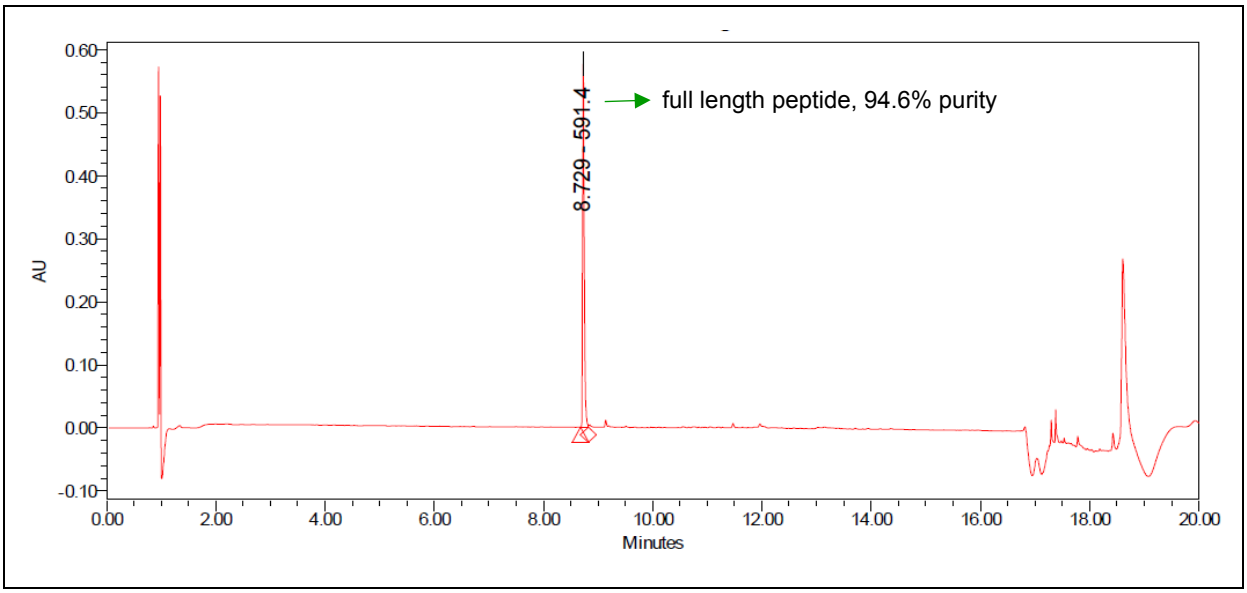


Figure 11 Chromatogram of cecropin A (1-7) – mellitin (2-9) purified on RP-HPLC

4.1.4 Failed Fmoc deprotection of Fmoc-Lys[10](Boc)-peptide

By committing a crime and not analyzing the outcome of de-Fmoc steps, because we were so sure of its positive outcome, we've fallen into a trap. As stated before, this peptide is relatively hydrophobic with high aggregation potential during the synthesis. After analyzing the outcome of the eleventh coupling with Fmoc-Phe[11]-OH on UPLC-MS, instead of one, two main peaks were present (Figure 12) – one containing the desired Fmoc-protected growing peptide chain, while the other peak corresponded to Fmoc-protected peptide ending with Lys[10](Boc). This means de de-Fmoc of the tenth amino acid, Lysine, was not finished by treating it with piperidine solution two times for 15 min, most probably caused by some steric hindering due to the aggregation of the peptide chains on the resin.

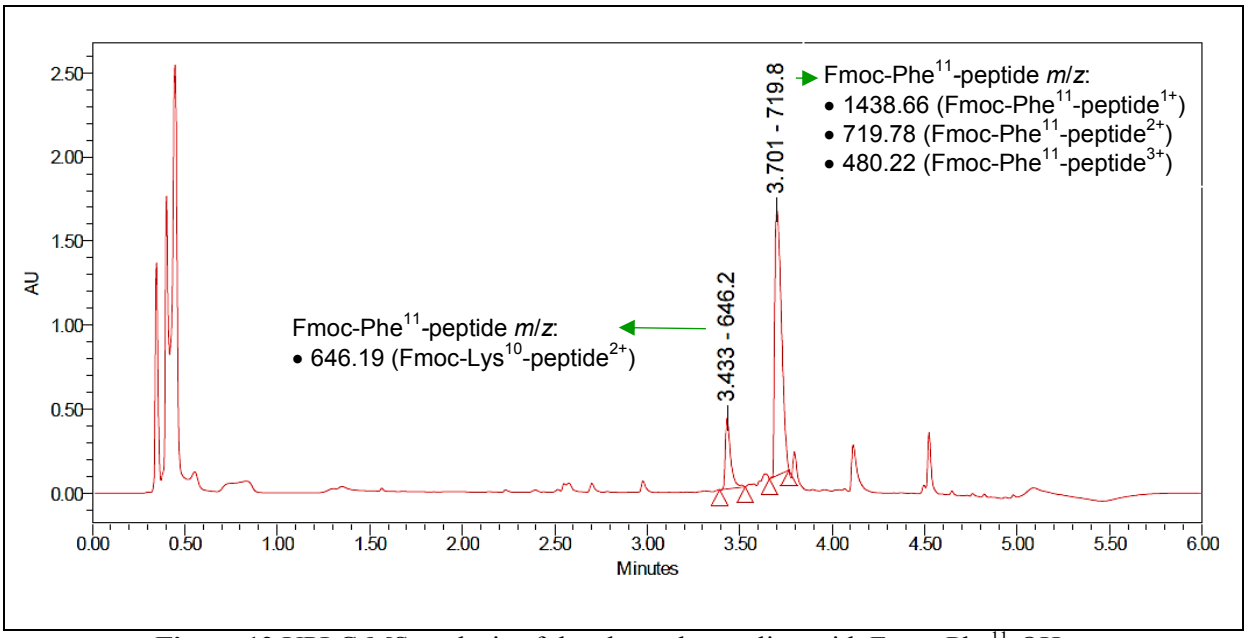


Figure 12 UPLC-MS analysis of the eleventh coupling with Fmoc-Phe[11]-OH

After coupling with Fmoc-Phe^{11}-OH and capping, de-Fmoc was performed with solution of piperidine two times for 30 minutes. The UPLC-MS analysis showed the deprotection was completed and besides the main growing peptide chain leading to the product, another growing peptide chain was formed with one Phe^{11} lesser. The SPPS was continued as described before and after the last coupling two main peptide chains were present, one with 15 and the other with 14 amino acid residues.

This leads us to a problem connected with the purity of the peptide after the purification on cation exchanger, since the anchored linker can react both with the full-length peptide and with the peptide one Phe^{11} shorter. The newly proposed peptide purification is not able to separate peptides which differ due to not properly finished deprotection during a difficult peptide synthesis, and HPLC must be used for final purification.

However, we've decided to use the peptide for the experiments for purification and decided to synthesize the peptide once again with proper deprotection after the tenth coupling and capping. Automated peptide synthesizer was used, and the process will be described later. The new peptide would be used later to clearly show the effectiveness of our method in the case it works.

Automated SPPS of cecropin A (1-7) – mellitin (2-9)

The second synthesis of cecropin A (1-7) – mellitin (2-9) was provided on automated peptide synthesizer. The first amino acid was attached to the resin manually using the same protocols as described in the manual synthesis. Resin substitution was determined as S = 0.475 mmol/g and is described in detail in experimental part 6.3.1. The coupling, capping and deprotection cycles were also the same as before, but provided automatically.

The synthesis after the tenth coupling and capping was stopped and the de-Fmoc was performed manually using a stronger base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). After proving the full deprotection, the synthesis continued automatically until the end. The final UPLC-MS analysis showed the presence of one main peak corresponding to the full-length peptide and peaks for capped failure sequences. Purification on RP-HPLC resulted in 90.0% purity (30.0 mg) with 22.7% yield.

4.2 Peptide separation on cation exchanger

Finishing with the synthesis of a charged anchor and peptide allowed us to move on to the last part of work, where the newly proposed synthetic peptide separation method based on electrostatic binding is tested (Scheme 2).

4.2.1 Separation experiment No.1

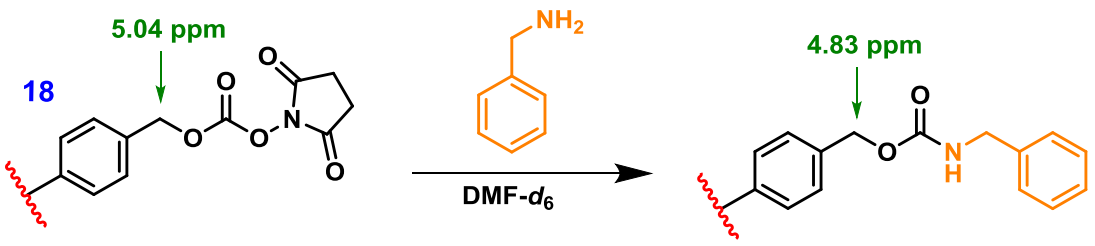
For the first orientation experiment a part of the resin with peptide prepared by manual SPPS was de-Fmoc-ed using piperidine solution in DMF. The deprotection exposed the amino groups of the full-length peptide and the peptide one Phe[11] shorter, while the rest of the failed sequences remained capped with acetyl moiety.

In DMF two equivalents of the double-charged anchored linker **18** were dissolved and the pH value of the mixture was tuned to basic by adding collidine. Subsequently the de-Fmoc-ed resin beads were added, and suspension was carefully stirred overnight at 50 °C. A part of resin beads was submitted to ninhydrin test and UPLC-MS analysis as described in chapter 4.1.3. The blue coloring of the ninhydrin test showed most of the amino groups were unreacted. This was proven also by UPLC-MS analysis, since main peptide peaks stayed unchanged. The UPLC-MS analysis of the reaction mixture itself showed the presence of unreacted anchored linker **18**. Reaction mixture was stirred at 50 °C for another 72 hours and the tests were repeated once again with approximately the same results with lower amount of peptide peaks on the chromatogram. However, no adduct of the anchored linker with the peptide was present, but we've assumed it can be electrostatically bound to the reversed-phase column on UPLC.

The whole situation became suspicious, since no product was observed on UPLC-MS and the ninhydrin test proved the presence of most of the peptidic amino groups. We rumored the reaction could be very slow and we decided to test the reactivity of the anchored linker by kinetic studies on NMR.

4.2.2 Kinetic studies by NMR

After the first failed attempt of peptide purification we decided to test the reactivity of the anchored linker with benzylamine to have an approximate idea about its behavior (Scheme 8). An experiment was set, where the reaction of benzylamine with **18** was monitored by ^{1}H NMR spectroscopy at 25 °C and 50 °C and the half-lives of the reactions were calculated.



Scheme 8 Study of reactivity of anchored linker **18** with benzylamine

For both experiments about 8 mg anchored linker was dissolved in deuterated DMF-d_6 and 1 eq. of benzylamine was added. The reaction was monitored for 65 h at 25 °C and for 92 hours at 50 °C with acquisition every 5 minutes. Using the program MestreNova, two plots were built from the obtained data showing the dependency of decrease of reactant signal at 5.04 ppm and increase of the product signal at 4.83 ppm. It must be noted that the decrease of the reactant signal is inverse to the increase of product signal and they carry the same information about the reaction half-life. Figure 13 shows the decrease of reactant signals and increase of product signals during time-lapse at 50 °C and Figure 14 shows the corresponding plot of the course of reaction kinetics.

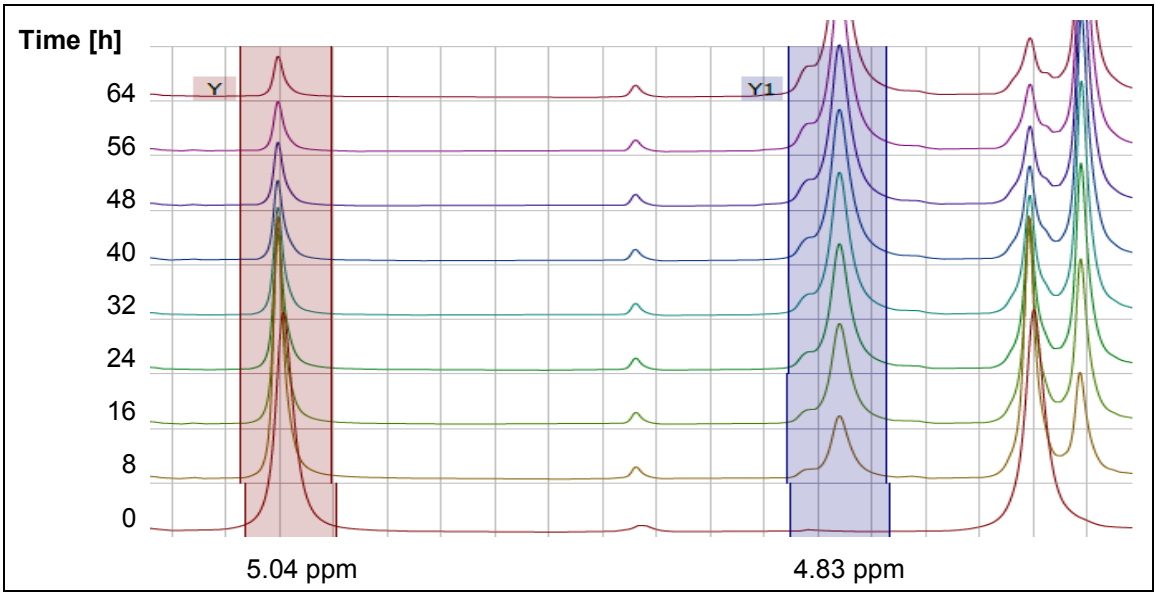


Figure 13 Decrease of reactant signal at 5.04 ppm and increase of the product signal at 4.83 ppm during time-lapse at 50 °C in the reaction of anchored linker with benzylamine

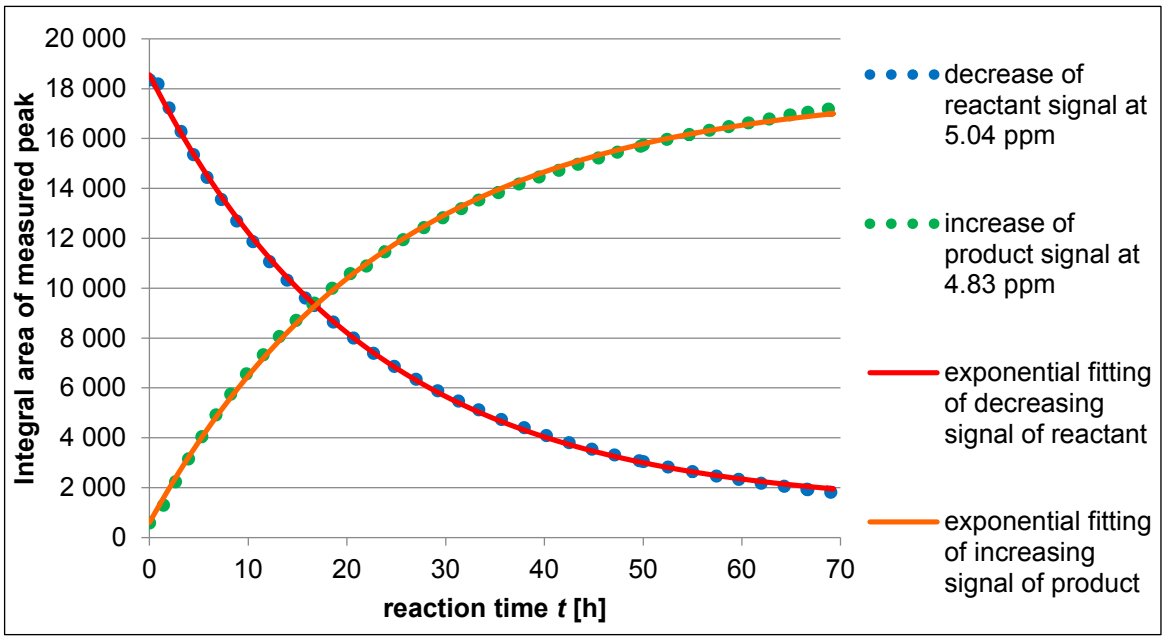


Figure 14 The course of reaction kinetics of anchored linker with benzylamine at 50 °C

The obtained data points were fit with exponential functions and expressed in equation 4.1, where I_0 is the intensity of the measured peak on the beginning of the experiment, I represents the intensity of the measured peak, t_0 is the starting time of the experiment, t is the time of the measurement of peak I, A is a constant and τ is the time constant.

$$I = I_0 + Ae^{\frac{-(t-t_0)}{\tau}} \tag{4.1}$$

$$t_{1/2} = \tau \cdot \ln 2 \tag{4.2}$$

From the equations of the exponential fittings the time constants of the reaction, τ, were obtained Table 2. Using equation 4.2, the half-lives of the reactions, $t_{1/2}$, were calculated using the time constants Table 2.

Table 2 Time constants and half-lives of the reactions of anchored linker with benzylamine at 25 °C and 50 °C

Reaction studied at	τ [h]	$t_{1/2}$ [h]
25 °C	81	56
50 °C	23	16

The obtained data showed the reaction of the double charged anchored linker with benzaldehyde is not as fast as expected, which could explain the results from the separation experiment No. 1. Reaction kinetics in SPPS is diffusion controlled, which means it depends on diffusion of the reagents into the resin. The relatively slow reactivity of **18** demonstrated with benzylamine can get significantly slower with the peptide in the resin. To overcome this problem, the reaction time of the attachment of **18** to the peptide on the resin must be prolonged or the succinimidyl carbonate function on the anchored linker must be changed to a more reactive carbonate moiety. For further possible industrial use, the second option is required, but for testing the newly proposed separation method we continue using **18** with prolonged reaction time.

4.2.3 Separation experiment No.2

For the second separation experiment resin with de-Fmoc-ed peptide was used in reaction with **18** with pH of the reaction mixture adjusted to 8 using collidine. Based on the conclusions got from the reactivity studies of the anchored linker with benzylamine, the reaction conditions were changed. The reaction was slowly stirred at 60 °C for 21 days and was monitored by ninhydrin test and UPLC-MS analysis. Since after two weeks no adduct of the anchored linker bound to the peptide was present on UPLC-MS analysis, TFA was added to the studied sample and the analysis showed the presence of the full-length peptide (Figure 15). This proved our assumption about the attachment of the linker-bound peptide to the reversed-phase column on UPLC.

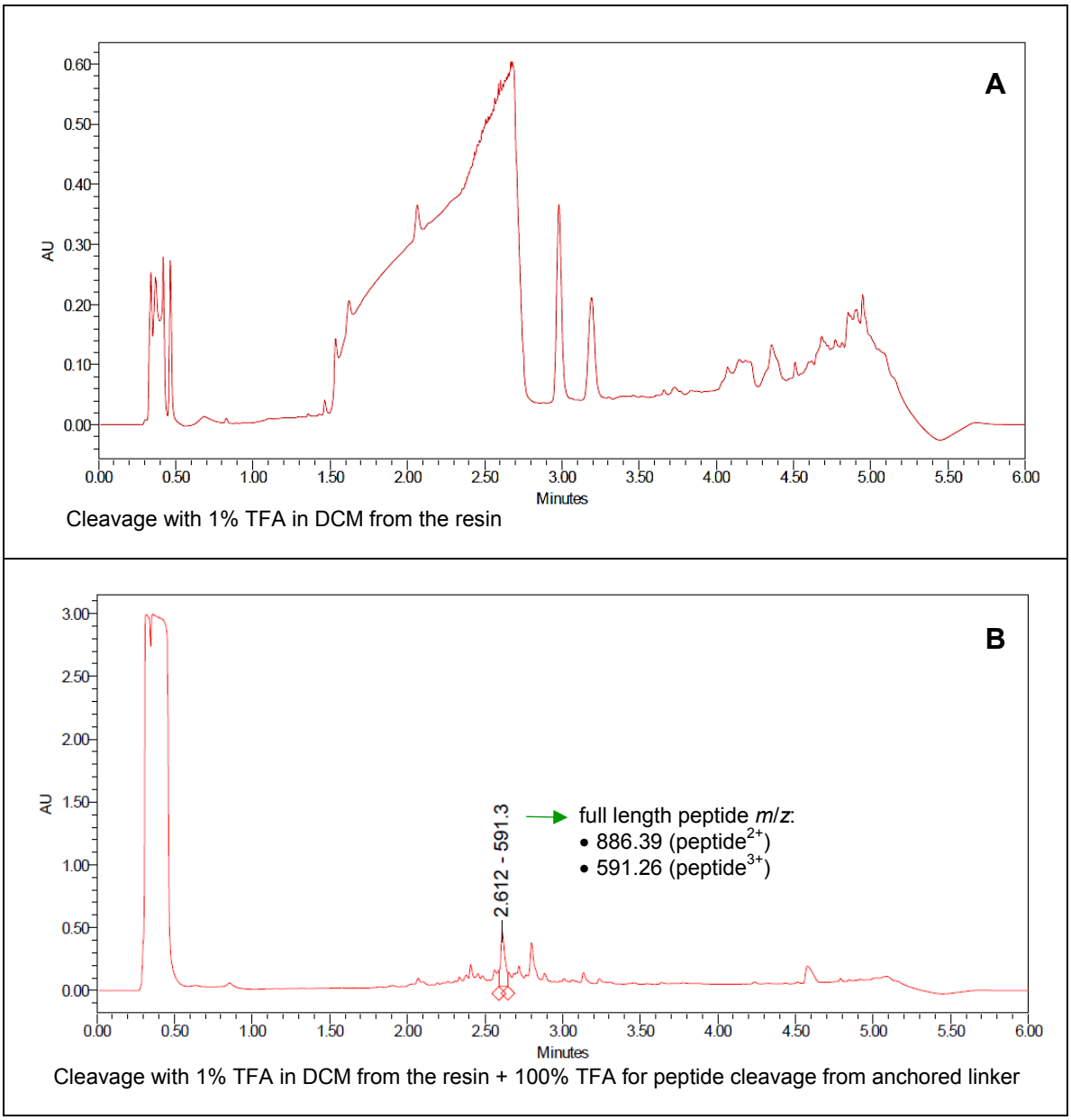


Figure 15 A) Chromatogram of peptide cleavage with 1% TFA in DCM – no adduct of the anchored linker bound to the peptide present. B) Chromatogram of the previous mixture treated with 100% TFA.

To cleave the peptide and capped failure sequences with the side chain protecting Boc groups attached from the resin, it was treated with 1% TFA in DCM and TIS. Adduct of peptide and anchored linker was not detected on UPLC-MS analysis, but after treating the sample with TFA, the full-length peptide was observed.

For separation experiment strong cation exchanger in H^+ cycle was used. DCM was evaporated from cleaved mixture from resin and residue was dissolved in 1% TFA in MeOH. Cation exchanger was added to the methanolic solution and mixture was monitored by TLC, which showed that one spot disappears after addition of cation exchanger. We assumed the disappearing spot was the adduct peptide and anchored linker which got electrostatically attached to the cation exchanger. Subsequently the cation exchanger was washed by several solvents before adding TFA cocktail for

cleavage of the peptide from the anchored linker bound electrostatically to the cation exchanger. After 1 h the TFA cocktail was filtered and precipitated in cold Et_2O, however no precipitate was observed, and UPLC-MS analysis showed no traces of the desired peptide. This could be explained by the fact, that strongly acidic conditions not just cleave the peptide from the anchored linker, but also expose the Boc-protected side chain amino moieties generating up to 7 positive charges in the case of the used peptide. Every single one of the charges is able to electrostatically interact with the cation exchanger forming a very strong binding.

To recover the peptide from the cation exchanger we tried washing it with aqueous 0.01%, 0.1%, 1% and 10% solution of NH_4HCO_3, concentrated aqueous ammonia solution, 10% and 50% methanolic solution of TEA. Solutions were analyzed by UPLC-MS. The double-charged anchored linker was washed from the cation exchanger by the 50% methanolic solution of TEA. The peptide stayed attached electrostatically on the cation exchanger and we were unable to recover it.

4.2.4 Discussion and method improvement proposal

The newly proposed separation method of peptides using electrostatic binding did not work as expected and we were unable to recover the full-length peptide from the cation exchanger. This problem has occurred due to the deprotection of Boc side chain protecting groups generating up to 7 positive charges able to form strong electrostatic interactions with the cation exchanger. Since the deprotection of Boc and cleaving the peptide from the anchored linker require high concentration of TFA, and cannot be done selectively, other sorbents should be used, which would not form such strong electrostatic interactions with the peptide. For example, weak cation exchanger, silica gel, or reversed-phase silica gel could be used, since the anchored linker is able to interact with those mediums and the impurities could be washed away easily.

When it comes to the anchored linker itself, there is more room for improvement. The succinimidyl carbonate function on the anchored linker must be changed to a more reactive carbonate moiety, since its reactivity is relatively low. In the case of SPPS, reaction kinetics depends on diffusion of the reagents into the resin and the reaction with the peptide inside the resin takes several days or weeks. Prolongation of the anchored linker would make it more flexible and solution-like which would enable faster diffusion to the resin. The anchored linker can be easily prolonged by changing the diethylene glycol section to a longer tetra- or octaethylene glycol moiety.

Lastly it must be mentioned that this method would not be able to separate failure sequences formed due to not completely finished Fmoc deprotection during a difficult peptide synthesis and non-acetylated failure sequences and HPLC must be used for final purification.

5 CONCLUSION

The research discussed in this thesis deals with a newly proposed separation method of peptides trying to circumvent the use of RP-HPLC. The research process was divided in four parts and is summed up below.

In the first part, a series of neopentyl-based multiply positively charged anchor molecules were synthesized to attach the peptide to the negatively charged solid surface through a linker. The preparation of the anchor molecules was provided on large scale to show the effectiveness of the process for further possible industrial use. The anchor is used for attachment of peptide to a negatively charged solid surface through a linker.

In the second part, a linker bound to a double-charged anchor, containing a succinimidyl carbonate function capable to covalently bind peptides was designed and successfully synthesized. The anchored linker is suitable for the reaction conditions used during peptide cleavage from the resin and deprotection.

In the following part, a hybrid peptide, cecropin A (1-7) – mellitin (2-9) was synthesized using the SPPS method, both manually and on an automated peptide synthesizer. A part of the peptides was purified in a classical way on RP-HPLC.

The last part deals with the newly proposed separation method of peptides on cation exchanger. The anchored linker is bound to the full-length peptide on the resin and after cleaving with 1% TFA, the linker-bound peptides are electrostatically bound to cation exchanger and impurities are washed away. However, after peptide cleavage from the anchored linker and Boc deprotection with TFA, the peptide formed strong electrostatic interactions with the cation exchanger, and we were unable to recover it. Nevertheless, the findings and experiences gained from the previous parts of this thesis enabled us to propose an improvement in the method which could overcome the problems faced in the last part of the thesis.

6 EXPERIMENTAL SECTION

6.1 Instruments, general methods and chemicals

^{1}H NMR, ^{13}C NMR, 2D NMR (^{1}H,^{1}H-COSY, HSQC and HMBC) were measured on Varian ^{UNITY}INOVA 400 (400 MHz for ^{1}H and 101 MHz for ^{13}C) and simple ^{1}H NMR spectra for fast characterization were obtained on Varian VNMRS 300 (300 MHz for ^{1}H). Kinetic studies were measured on Bruker AVANCE III 600 MHz (600.17 MHz for ^{1}H, 150.04 MHz for ^{13}C). Samples were dissolved in deuterated solvents – $CDCl_3$, CD_3OD, D_2O or DMF-d_6. The chemical shift values, δ, are given in ppm and the values of the interaction constants, J, in Hz.

The mass spectra were measured by the Bruker ESQUIRE 3000 ES-ion trap and the samples were ionized using an electrospray technique (ESI). High resolution mass spectra (HRMS) were measured on an Agilent 6530Q-TOF MS spectrometer. The samples were dissolved in methanol (20-40 μg/mL). For IR measurements, samples (5-10 mg) were mixed with KBr powder and measured on a Thermo Nicolet AVATAR 370 FT-IR spectrometer using DRIFT method.

Peptide synthesis was monitored using UPLC-MS analysis. Prior to the UPLC-MS analysis the studied peptide was cleaved from the resin by adding H_2O (10 μL), TIS (10 μL) and TFA (100 μL) to a few resin beads (1-3 mg) from SPPS and heating for 100 °C for 5 min. Subsequently 0.5 mL of aqueous solution of NH_4OAc (1,03 g/mL) and 0.5 mL of 70% aqueous MeCN were added and resin beads were filtered. The obtained solution (1-10 μL) was chromatographed on Waters Acquity Ultra Performance LC and was analyzed by Waters Micromass ZQ mass spectrometer using an electrospray technique (ESI).

Ninhydrin test was provided by treating a few resin beads (1-3 mg) with 2-3 drops of ninhydrin solution (5 g in 100 mL of EtOH), KCN solution in pyridine (2 mL of 0.001M aqueous KCN in 98 mL of pyridine) and solution of phenol (80 g in 20 mL of EtOH) and heating for 100 °C for 2 min.

Purification of peptides on RP-HPLC was provided on Gilson Model 322-H1 HPLC using XSelect CSH Prep C18 30x150 mm column for peptide purification after manual SPPS and Gemini 5 μm NX-C18 110 Å LC column for peptide separation after automated SPPS.

For the peptide separation experiments Dowex 50WX8 H^+-form (Sigma-Aldrich) strong cation exchanger was used. Cation exchanger was transformed to H^+ cycle by washing with aqueous 5% NaOH solution (3 column volumes), washing with water until neutral pH, washing with 5% HCl (3 column volumes) and washing with water until neutral pH.

Silica gel 60 (0.040–0.063 mm) was used for column chromatography and TLC was performed on DC-Alufolien Kiesegel 60 F254 (Merck, Darmstadt, Germany) silica gel plates. Plates were developed in a saturated chamber; the mobile phases are given at each procedure in volume/volume ratio. Spots on TLC plates were detected by the following methods:

- using an UV lamp (λ = 254 nm) by Mouser Electronics
- dipping in cerium sulfate tetrahydrate (0.5 g), ammonium molybdate tetrahydrate (2.5 g), sulfuric acid (5 mL) and water (45 ml) and heating with a heat gun.
- dipping in basic $KMnO_4$ and subsequently heating with a heat gun. The basic

$KMnO_4$ was prepared by dissolving of $KMnO_4$ (1.5 g), K_2CO_3 (10 g), 10% NaOH (1.25 mL) in H_2O (100 mL).

- dipping in ninhydrin solution and subsequently heating with a heat gun. The ninhydrin solution was prepared by dissolving ninhydrin (200 mg) in butanol (95 mL) and adding 10% aqueous AcOH (5 mL).

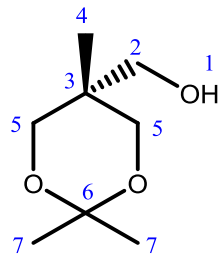
Organic solvents were distilled before use. Chemicals were purchased from common commercial sources and used without further purification (Sigma-Aldrich, Penta). Argon was used as an inert gas. Products were dried using oil pump at 0.5-10 mbar.

6.2 Synthesis of compounds

6.2.1 Synthesis of the charged anchors

Synthesis of the charged anchors bearing two positive charges

(2,2,5-Trimethyl-1,3-dioxan-5-yl)methanol (1)



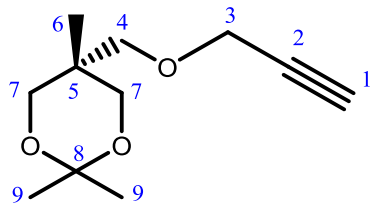
Compound **1** was prepared by modification of a previously published procedure[84]. 1,1,1-Tris(hydroxymethyl)-ethane (100 g, 825 mmol) and *p*-toluenesulfonic acid (100 mg, 525 µmol) were dissolved in dry acetone (1000 mL) under argon atmosphere. Reaction mixture was stirred for 50 hours at room temperature. The reaction was monitored by TLC with CHCl3/MeOH 20/1 mixture. Spots on TLC were detected by immersion into mixture of cerium sulfate tetrahydrate, ammonium molybdate tetrahydrate, sulfuric acid and water and heating with heat gun. Mixture was neutralized by potassium carbonate (2.5 g, 18 mmol), filtered and evaporated on rotary evaporator (RE) at 40 ºC. Product was purified by vacuum distillation (130 ºC, 1.5 mbar). The final product was obtained as colorless oil, yield 89 % (117.9 g). NMR and MS spectra are in accordance with literature[84].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.61 (m, 6H, **H2**, **H5**), 2.50 (t, J = 5.6 Hz, 1H, **H1**), 1.41 (s, 3H, **H7**), 1.36 (s, 3H, **H7**), 0.80 (s, 3H, **H4**) ppm

^{13}C NMR (101 MHz, $CDCl_3$): δ = 98.07 (**C6**), 66.38 (**C5**), 65.77 (**C2**), 34.84 (**C3**), 27.3 (**C7**), 20.23 (**C7**), 17.67 (**C4**) ppm

ESI MS: for $C_8H_{16}O_3$ found 198.2 $[M+K]^{1+}$

2,2,5-Trimethyl-5-((prop-2-yn-1-yloxy)methyl)-1,3-dioxane (2)



Compound **2** was prepared by modification of a previously published procedure[85]. Compound **1** (117.9 g, 736 mmol) was dissolved in dry THF (1000 mL) and cooled to 0 ºC under argon atmosphere. Sodium hydride (44.3 g, 1.10 mol; 60% dispersion in oil) was added to solution during 30 min. Suspension was stirred at 0 ºC for 2 hours, then it was cooled to -78 ºC and propargyl bromide (123 ml, 1.10 mol; 80% solution in toluene) was added dropwise during 30 minutes. The mixture was allowed to warm up to room

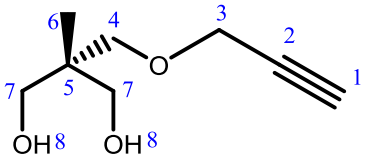
temperature and stirred for 22 hours. The reaction was monitored by TLC with hexane/EtOAc 5/1 mixture. Spots on TLC were detected by immersion into mixture of cerium sulfate tetrahydrate, ammonium molybdate tetrahydrate, sulfuric acid and water and heating with heat gun. Mixture was filtered and evaporated on RE at 40 ºC. Product was purified by vacuum distillation (115 ºC, 1.5 mbar). The final product was obtained as colorless oil, yield 88 % (129.1 g). NMR and MS spectra are in accordance with literature[83].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.16 (d, J = 2.4 Hz, 2H, **H3**), 3.70 (d, *J* = 12.0 Hz, 2H, **H7**), 3.55 (d, *J* = 12.0 Hz, 2H, **H7**), 3.52 (s, 2H, **H4**), 2.41 (t, *J* = 2.4 Hz, 1H, **H1**), 1.43 (s, 3H, **H9**), 1.40 (s, 3H, **H9**), 0.88 (s, 3H, **H6**) ppm

^{13}C NMR (101 MHz, $CDCl_3$): δ = 98.01 (**C8**), 80.06 (**C2**), 74.30 (**C1**), 73.07 (**C4**), 66.63 (**C7**), 58.85 (**C3**), 34.34 (**C5**), 26.58 (**C9**), 21.20 (**C9**), 18.32 (**C6**) ppm

ESI MS: for $C_{11}H_{18}O_3$ found 221.1 $[M+Na]^{1+}$

2-Methyl-2-((prop-2-yn-1-yloxy)methyl)propane-1,3-diol (3)



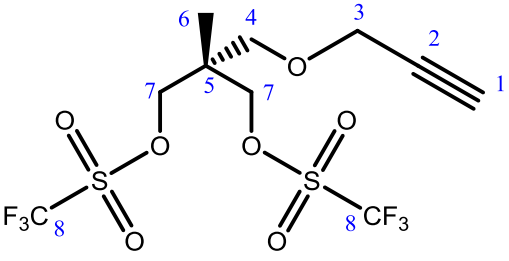
Compound **3** was prepared by modification of a previously published procedure[85]. Compound **2** (129.1 g, 651 mmol) was dissolved in MeOH (770 mL) and concentrated hydrochloric acid (52.5 ml, 651 mmol) was added. Mixture was stirred at room temperature for 20 hours. The reaction was monitored by TLC with hexane/EtOAc 1/2 mixture. Spots on TLC were detected by basic permanganate solution. After full conversion the mixture was neutralized by 40% aqueous solution of NaOH and the NaCl suspended from reaction mixture was filtered off and MeOH was evaporated. The oily residue with crystalline precipitate was dissolved in $CHCl_3$, NaCl was filtered off. Filtrate was evaporated on RE at 40 ºC. The previous step was repeated twice. Product was dried by vacuum distillation (115 ºC, 1.5 mbar). The crude product (containing NaCl) was obtained as a slightly yellow oil (119 g). The NMR was clean, and the mixture was used in the next reaction without further purification. NMR and MS spectra are in accordance with literature[94].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.13 (d, *J* = 2.4 Hz, 2H, **H3**), 3.64 (d, *J* = 11.0 Hz, 2H, **H7**), 3.55 (d, *J* = 11.0 Hz, 2H, **H7**), 3.50 (s, 2H, **H4**), 2.98 (bs, 2H, **H8**), 2.45 (t, *J* = 2.4 Hz, 1H, **H1**), 0.82 (s, 3H, **H6**) ppm

^{13}C NMR (101 MHz, $CDCl_3$): δ = 79.65 (**C2**), 74.87 (**C1**), 74.72 (**C4**), 67.66 (**C7**), 58.86 (**C3**), 40.86 (**C5**), 17.17 (**C6**) ppm

ESI MS: for $C_8H_{14}O_3$ found 181.0 $[M+Na]^{1+}$

2-Methyl-2-((prop-2-yn-1-yloxy)methyl)propane-1,3-diyl bis(trifluoromethanesulfonate) (4)



Compound **4** was prepared by modification of a previously published procedure[86]. Compound **3** (40 g, 253 mmol) was dissolved in dry DCM (1150 mL) and 2,6-lutidine (59 mL, 506 mmol) was added. Mixture was cooled to -78 ºC and Tf_2O (85

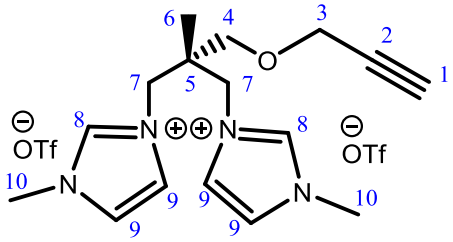
mL, 506 mmol) was added dropwise and mixture was stirred at this temperature for 1 hour. The reaction was monitored by TLC with hexane/EtOAc 10/1 mixture. Spots on TLC were detected by basic permanganate solution. Reaction mixture was extracted between Et_2O (2 L) and 1M HCl (1.5 L). Organic phase was subsequently extracted with saturated aqueous solution of sodium bicarbonate (1 L) and brine (1 L). Organic phase was dried over $MgSO_4$ (20 g), filtered and evaporated at RT on RE. Product was dried at RT on vacuum pump and obtained as brown oil in 88% yield (94.2 g). NMR and MS spectra are in accordance with literature[83].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.45 (s, 4H, **H7**), 4.17 (d, J = 2.4 Hz, 2H, **H3**), 3.48 (s, 2H, **H4**), 2.48 (t, J = 2.4 Hz, 1H, **H1**), 1.17 (s, 3H, **H6**) ppm

^{13}C NMR (101 MHz, $CDCl_3$): δ = 118.72 (q, J = 319.7 Hz, **C8**), 78.44 (**C2**), 76.68 (**C7**), 75.76 (**C1**), 69.74 (**C4**), 58.80 (**C3**), 40.63 (**C5**), 16.34 (**C6**) ppm

ESI MS: for $C_{10}H_{12}F_6O_7S_2$ found 445.0 $[M+Na]^{1+}$

3,3'-(2-Methyl-2-((prop-2-yn-1-yloxy)methyl)propane-1,3-diyl)bis(1-methyl-1*H*-imidazol-3-ium) trifluoromethanesulfonate (5)



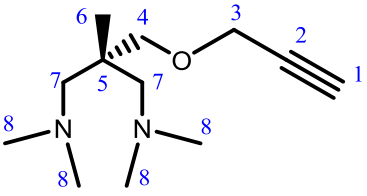
Compound **5** was prepared according to literature[83]. Compound **4** (25.7 g, 61.1 mmol) was dissolved in 1-methylimidazole (250 mL) and mixture was heated to 60 ºC and stirred for 2 hours. The reaction was monitored by TLC (MeOH/HOAc/1% aq. NH_4OAc 10/1/9 – detection by basic permanganate solution). 1-Methylimidazole was distilled off under reduced pressure at 100 ºC. $CHCl_3$ (2 × 500 ml) was added to solid crude product to get rid of the rest of 1-methylimidazole. Then crude product was extracted between water (500 mL) and $CHCl_3$ (200 ml). Aqueous phase was extracted with another $CHCl_3$ (3 × 200 ml). Aqueous phase was lyophilized. Product obtained as light brown oil in 84% yield (30.1 g). NMR and MS spectra are in accordance with literature[83].

^{1}H NMR (400 MHz, D_2O): δ = 8.84 (s, 2H, **H8**), 7.52 (s, 4H, **H9**), 4.48 (d, J = 14.3 Hz, 2H, **H7**), 4.28 – 4.25 (m, 4H, H-3, **H7**), 3.95 (s, 6H, **H10**), 3.23 (s, 2H, **H4**), 3.02 (t, J = 2.4 Hz, 1H, **H1**), 1.03 (s, 3H, **H6**) ppm

^{13}C NMR (101 MHz, CD_3OD): δ = 139.19 (**C8**), 125.40 – 125.03 (**C9**), 79.91 (**C2**), 77.61 (**C1**), 71.06 (**C4**), 59.28 (**C3**), 54.39 (**C7**), 41.46 (**C5**), 37.02 (**C10**), 18.37 (**C6**) ppm

ESI MS: for $C_{16}H_{24}N_4O^{2+}$ found 144.0 $[M]^{2+}$

N^1,*N*^{1},*N*^{3},*N*^{3},2-Pentamethyl-2-((prop-2-yn-1-yloxy)methyl)propane-1,3-diamine (6)



Compound **6** was prepared according to literature[83]. Dimethylamine (65 mL) was added to **4** (22.4 g, 11.8 mmol) at -78 ºC and mixture was sealed in a thick-walled ampoule and heated to 60 ºC for 20 h. Thick-walled ampoule was cooled to -78 ºC and opened. The reaction was checked by TLC

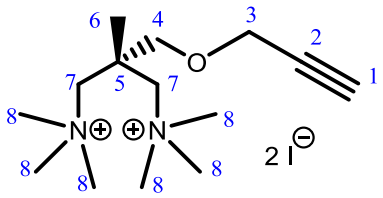
($CHCl_3$/MeOH/conc. aq. NH_3 90/10/0.5), detected basic permanganate solution. Mixture was extracted between DCM (760 mL) and 5% aq. NaOH (2 × 500 mL). Organic phase was evaporated on RE at 23 °C. Crude product was dissolved in water (700 mL) and codistilled at 110 ºC. Distillate was then extracted with DCM (2 × 250 mL). Organic phase was dried over $MgSO_4$ (10 g), filtered and evaporated on RE at 23 °C. Product was obtained as colorless oil in 62% yield (7.1 g). NMR and MS spectra are in accordance with literature[83].

^{1}H NMR (400 MHz, D_2O): δ = 4.34 (d, J = 2.4 Hz, 2H, **H3**), 3.77 (s, 2H, **H4**), 3.51 (d, J = 14.1 Hz, 2H, **H7**), 3.36 (d, J = 14.1 Hz, 2H, **H7**), 3.01 (d, J = 4.1 Hz, 12H, **H8**), 2.99 (t, J = 2.4 Hz, 1H, **H1**), 1.28 (s, 3H, **H6**) ppm

^{13}C NMR (101 MHz, D_2O, *t*BuOH): δ = 79.21 (**C2**), 76.68 (**C7**), 77.73 (**C1**), 72.26 (**C4**), 70.51 (*t*BuOH), 64.88 (**C7**), 59.03 (**C3**), 47.92 (**C8**), 47.52 (**C8**), 38.75 (**C5**), 30.29 (*t*BuOH), 17.97 (**C6**) ppm

ESI MS: for $C_{12}H_{24}N_2O$ found 212.2 $[M+H]^{1+}$

*N*¹,*N*¹,*N*¹,*N*³,*N*³,*N*³,2-Heptamethyl-2-((prop-2-yn-1-yloxy)methyl)propane-1,3-diaminium iodide (7)



Compound **7** was prepared according to literature[83]. Compound **6** (6.8 g, 32 mmol) was dissolved in THF (180 ml) and MeI (39 mL, 626 mmol) was added. Mixture was heated to reflux for 24 hours. The reaction was checked by TLC ($CHCl_3$/MeOH/conc. aq. NH_3 90/10/0,5 – for starting compound, detection by basic permanganate solution; MeOH/HOAc/1% aq. NH_4OAc 10/1/9 – for product, detection by basic permanganate solution). THF was filtered off and solid phase was extracted by THF (3 × 100 ml). Product was dried at 50 ºC on vacuum pump. Product was obtained as a light pink crystalline in 89% yield (12.0 g). NMR and MS spectra are in accordance with literature[83].

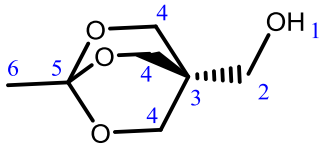
^{1}H NMR (400 MHz, D_2O): δ = 4.36 (d, J = 2.4 Hz, 2H, **H3**), 3.86 (s, 2H, **H4**), 3.84 (d, J = 14.2 Hz, 2H, **H7**), 3.63 (d, J = 14.2 Hz, 2H, **H7**), 3.34 (s, 18H, **H8**), 2.98 (t, J = 2.4 Hz, 1H, **H1**), 1.54 (s, 3H, **H6**) ppm

^{13}C NMR (101 MHz, MeOD): δ = 78.97 (**C2**), 78.13 (**C1**), 73.37 (**C7**), 72.78 (**C4**), 59.19 (**C3**), 56.89 (**C8**), 44.98 (**C5**), 22.14 (**C6**) ppm

ESI MS: for $C_{14}H_{30}N_2O^{2+}$ found 121.0 $[M]^{2+}$, for $C_{14}H_{30}IN_2O^{1+}$ found 370.0 $[M+I^-]^{1+}$

Synthesis of the charged anchors bearing three positive charges

(1-Methyl-2,6,7-trioxabicyclo[2.2.2]octan-4-yl)methanol (8)



Compound **8** was prepared by modification of a previously published procedure[87]. Pentaerythritol (100 g, 735 mmol) was suspended in toluene (75 mL) and triethyl orthoacetate (119 g, 735 mmol) and *p*-toluenesulfonic acid (350 mg, 1.84 mmol) were added. Mixture was heated and resulting EtOH was distilled off, subsequently the temperature was raised and toluene was distilled off. Resulting solid was put in a RE with oil bath and product was sublimated (210 ºC). Product was dissolved in MeOH, evaporated on RE and dried on

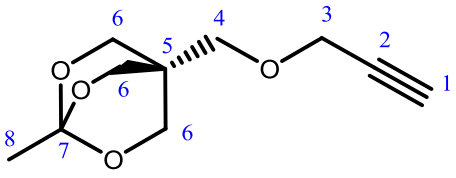
vacuum (50 °C, 1.5 mbar). Product was obtained as white solid in 85% yield (100.2 g). NMR and MS spectra are in accordance with literature[87].

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.02 (s, 6H, **H4**), 3.47 (d, J = 4.7 Hz, 2H, **H2**), 1.51 (t, J = 4.7 Hz, 1H, **H1**), 1.46 (s, 3H, **H6**) ppm

^{13}C NMR (101 MHz, $CDCl_3$): δ = 108.65 (**C5**), 69.41 (**C4**), 61.40 (**C2**), 35.71 (**C3**), 23.51 (**C6**) ppm

ESI MS: for $C_7H_{12}O_4$ calcd: *m/z* 160.1, found 161.1 $[M+H]^{1+}$

1-Methyl-4-((prop-2-yn-1-yloxy)methyl)-2,6,7-trioxabicyclo[2.2.2]octane (9)



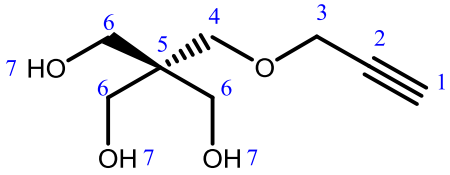
Compound **9** was prepared by modification of a previously published procedure[85]. Compound **8** (100.2 g, 628 mmol) was dissolved in THF (600 mL) and cooled to 0 ºC. Then NaH (27.5 g, 688 mmol, 60% dispersion in mineral oil) was added and the mixture was stirred for 2 hours at 0 ºC. Then propargyl bromide (71 mL, 688 mmol, 80% dispersion in toluene) was added dropwise and mixture was stirred at RT overnight. The reaction was monitored by TLC (Hexane/EtOAc 5/1 – detection by basic permanganate solution). Mixture was filtered and evaporated on RE at 50 ºC. The solid residue was put in a RE with oil bath and product was sublimated (180 ºC). Product was obtained as white solid in 50% yield (61.1 g). NMR and MS spectra are in accordance with literature[95].

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.09 (s, 2H, **H3**), 4.00 (s, 2H, **H6**), 3.29 (s, 2H, **H4**), 2.44 (s, 1H, **H1**), 1.45 (s, 3H, **H8**) ppm

^{13}C NMR (101 MHz, $CDCl_3$): δ = 108.70 (**C7**), 78.96 (**C2**), 75.34 (**C1**), 69.54 (**C6**), 68.11 (**C4**), 58.87 (**C3**), 34.84 (**C5**), 23.56 (**C8**) ppm.

ESI MS: for $C_{10}H_{14}O_4$ found 199.0 $[M+H]^{1+}$

2-(Hydroxymethyl)-2-((prop-2-yn-1-yloxy)methyl)propane-1,3-diol (10)



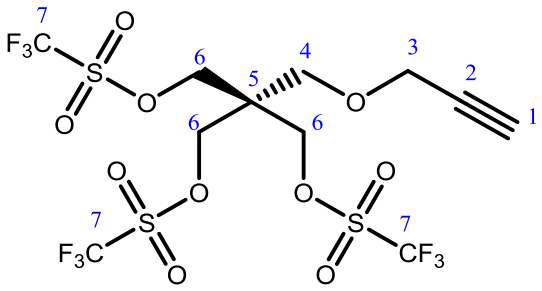
Compound **10** was prepared by modification of a previously published procedure[88]. Compound **9** (61.1 g, 308 mmol) was dissolved in MeOH (1200 mL) and concentrated HCl (16.3 g, 154 mmol) was added. Mixture was heated to reflux and stirred overnight. The reaction was monitored by TLC ($CHCl_3$/MeOH 15/1 – detection by basic permanganate solution). Mixture was cooled to RT, neutralized by 5% aqueous NaOH and evaporated on RE at 50 ºC. The crude product was extracted between water (700 mL) and $CHCl_3$ (3 × 700 mL). Aqueous phase was evaporated on RE at 50 ºC and the solid product was extracted to acetone (800 mL). Solution was filtered and evaporated on RE at 40 ºC. Product was dried at 60 ºC on vacuum pump and obtained as yellowish oil in 37% yield (19.6 g). NMR and MS spectra are in accordance with literature[95].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.15 (d, *J* = 2.4 Hz, 2H, **H3**), 3.72 (s, 6H, **H6**), 3.57 (s, 2H, **H4**), 2.47 (t, *J* = 2.4 Hz, 1H, **H1**) ppm

^{13}C NMR (125 MHz, $CDCl_3$): δ = 79.41 (**C2**), 75.16 (**C1**), 71.59 (**C4**), 64.57 (**C6**), 59.04 (**C3**), 45.15 (**C5**) ppm

ESI MS: for $C_8H_{14}O_4$ found 175.0 $[M+H]^{1+}$

2-((Prop-2-yn-1-yloxy)methyl)-2-((((trifluoromethyl)sulfonyl)oxy)methyl)-propane-1,3-diyl bis(trifluoromethanesulfonate) (11)



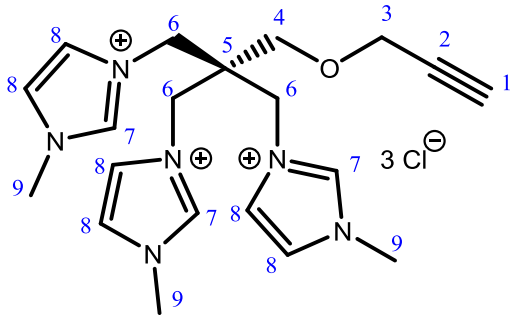
Compound **11** was prepared by modification of a previously published procedures[86]. Compound **10** (18.2 g, 104 mmol) was dissolved in DCM (550 mL) and 2,6-lutidine (33.7 g, 314 mmol) was added. Mixture was cooled to -78 ºC and Tf_2O (91.7 g, 325 mmol) was added dropwise and reaction was stirred at this temperature for 2 hours. The reaction was monitored by TLC ($CHCl_3$/MeOH 15/1 – detection by basic permanganate solution; hexan/EtOAc 10/1 – for product, detection by 1% ethanolic solution of 4-(4-nitrobenzyl)pyridine and concentrated ammonia). Mixture was extracted between Et_2O (1100 mL) and 1M HCl (700 mL). Organic phase was then washed by saturated solution of $NaHCO_3$ (700 mL) and brine (700 mL), dried over $MgSO_4$ (5 g), filtered and evaporated on RE at 30 °C. Product was dried at 23°C by vacuum pump and obtained as brown solid in 86% yield (53.8 g). NMR and MS spectra are in accordance with literature[83].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.57 (s, 6H, **H6**), 4.22 (d, J = 2.4 Hz, 2H, **H3**), 3.64 (s, 2H, **H4**), 2.54 (t, J = 2.4 Hz, 1H, **H1**) ppm

^{13}C NMR (101 MHz, $CDCl_3$): δ = 118.65 (q, J = 319.9 Hz, **C7**), 77.36 (**C2**), 76.75 (**C1**), 71.43 (**C6**), 64.61 (**C4**), 59.05 (**C3**), 44.84 (**C5**) ppm

ESI MS: for $C_{11}H_{11}F_9O_{10}S_3$ found 587.9 $[M+NH_4]^{1+}$

3,3'-(2-((1-Methyl-1H-imidazol-3-ium-3-yl)methyl)-2-((prop-2-yn-1 yloxy)methyl)propane-1,3-diyl)bis(1-methyl-1H-imidazol-3-ium) (12)



Compound **12** was prepared according to literature[83]. Compound **11** (20.0 g, 35 mmol) was dissolved in 1-methylimidazole (235 mL) and the mixture was stirred at 60 ºC for 21 hours. The reaction was monitored by TLC (MeOH/HOAc/1% aq. sol. NH_4OAc 10/10/9 – detection by basic permanganate solution). 1-Methylimidazole was distilled off at 80 ºC under vacuum. Crude product was dissolved in water (500 ml) and extracted with $CHCl_3$ (4 × 400 ml). Crude product was then dissolved in water and purified on weak cation exchanger Amberlite (20 g dry, NH_4^+ cycle). Solutions for elution were water, 1% aq. sol. NH_4HCO_3, 5% aq. sol. NH_4HCO_3 and 10% aq. sol. NH_4HCO_3. Fractions with pure product were evaporated on RE at 50 ºC. Product was extracted with MeOH (300 mL), solution was

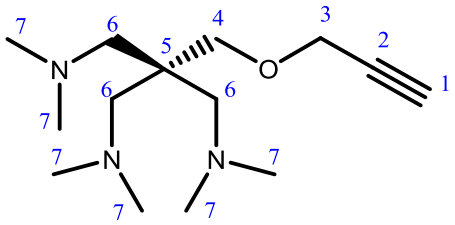
filtrated, neutralized with 1M HCl and evaporated at 50 ºC on RE. Product was dried at 50 ºC using oil vacuum pump and obtained as glassy oil compound in 84% yield (13.5 g). NMR and MS spectra are in accordance with literature[83].

^{1}H NMR (400 MHz, DMSO-*d*6): δ = 9.52 (s, 3H, **H7**), 7.80 (s, 6H, **H8**), 4.61 (s, 6H, **H6**), 4.13 (d, *J* = 2.4 Hz, 2H, **H3**), 3.90 (s, 9H, **H9**), 3.64 (t, *J* = 2.3 Hz, 1H, **H1**), 3.61 (s, 2H, **H4**) ppm

^{13}C NMR (101 MHz, DMSO-*d*6): δ = 138.47 (**C7**), 123.91 – 123.49 (**C8**), 79.21 (**C2**), 78.38 (**C1**), 68.36 (**C4**), 57.95 (**C3**), 49.87 (**C6**), 42.26 (**C5**), 36.03 (**C9**) ppm.

ESI MS: $C_{20}H_{28}ClN_6O^{1+}$ found 403.2 $[M\text{-}H^+\text{+}Cl^-]^{1+}$

2-((Dimethylamino)methyl)-*N*^{1},*N*^{1},*N*^{3},*N*^{3}-tetramethyl-2-((prop-2-yn-1-yloxy)methyl)propane-1,3-diamine (13)



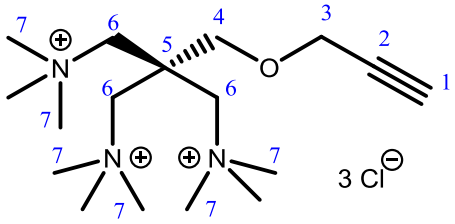
Compound **13** was prepared according to literature[83]. Dimethylamine (85 ml) was added to compound **11** (29.6 g, 116 mmol) at -78 ºC and mixture was sealed in a thick-walled ampoule and heated to 60 ºC overnight. Thick-walled ampoule was cooled to -78 ºC and opened. The reaction was checked by TLC ($CHCl_3$ /MeOH/conc. aq. NH_3 90/10/0.5 – detection by basic permanganate solution). Mixture was extracted between DCM (500 mL) and 5% aq. NaOH (500 mL). Organic phase was evaporated on RE at 23 °C. Crude product was dissolved in water (500 mL) and codistilled at 120 ºC. Distillate was then extracted with $CHCl_3$ (300 mL). Organic phase was dried over $MgSO_4$ (4 g), filtered and evaporated on RE at 23 °C. Product was dried at RT on vacuum oil pump and obtained as colorless oil in 63% yield (8.3 g). NMR and MS spectra are in accordance with literature[83].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.10 (d, *J* = 2.4 Hz, 2H, **H3**), 3.46 (s, 2H, **H4**), 2.38 (d, *J* = 2.4 Hz, 1H, **H1**), 2.37 (s, 6H, **H6**), 2.27 (s, 18H, **H7**) ppm

^{13}C NMR (101 MHz, $CDCl_3$): δ = 80.12 (**C2**), 77.32 (**C1**), 72.55 (**C4**), 61.86 (**C6**), 58.42 (**C3**), 49.11 (**C7**), 46.98 (**C5**) ppm

ESI MS: for $C_{14}H_{29}N_3O$ found 256.3 $[M+H]^{1+}$

N^1,*N*^{1},*N*^{1},*N*^{3},*N*^{3},*N*^{3}-Hexamethyl-2-((prop-2-yn-1-yloxy)methyl)-2-((trimethylammonio)methyl)propane-1,3-diaminium bicarbonate (14)



Compound **14** was prepared according to literature[83]. Compound **13** (7.3 g, 28.7 mmol) was dissolved in DMF (215 mL) and MeI (122 g, 861 mmol) was added. Mixture was heated to 70 ºC and stirred for 24 hours. The reaction was monitored by TLC (MeOH/HOAc/1% aq. sol. NH_4OAc 10/10/9 – detection by basic permanganate solution). Mixture was filtrated and DMF was filtered off. Crude product was then dissolved in water and purified on weak cation exchanger Amberlite (20 g dry, NH_4^+ cycle). Solutions for elution were water, 1% aq. sol. NH_4HCO_3, 2% aq. sol. NH_4HCO_3, 4% aq. sol. NH_4HCO_3 and 10% aq. sol. NH_4HCO_3. Fractions with

pure product were evaporated on RE at 50 ºC. Product was extracted with MeOH (150 mL), solution was filtrated, neutralized with 1M HCl and evaporated again at 50 ºC on RE. Product was dried at 50 ºC using oil vacuum pump and obtained as brown glassy compound in 35% yield (4.8 g). NMR and MS spectra are in accordance with literature[83].

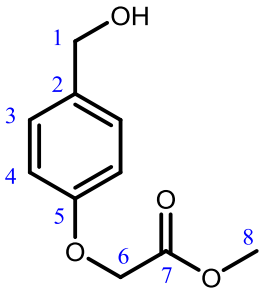
^{1}H NMR (400 MHz, D_2O, *t*BuOH): δ = 4.49 (d, J = 2.4 Hz, 2H, **H3**), 4.42 (s, 2H, **H4**), 4.12 (s, 6H, **H6**), 3.47 (s, 27H, **H7**), 3.05 (t, J = 2.4 Hz, 1H, **H1**), 1.25 (s, *t*BuOH) ppm

^{13}C NMR (101 MHz, D_2O, *t*BuOH): δ = 78.86 (**C1**), 77.52 (**C2**), 70.40 (*t*BuOH), 70.24 (**C6**), 68.65 (**C4**), 58.81 (**C3**), 57.54 (**C7**), 52.67 (**C5**) ppm

ESI MS: for $C_{17}H_{38}IN_3O^{2+}$ found 213.7 $[M+I^-]^{2+}$, for $C_{17}H_{38}I_2N_3O^{1+}$ found 554.1 $[M+2I^-]^{1+}$

6.2.2 Synthesis of the anchored linkers

Methyl 2-(4-(hydroxymethyl)phenoxy)acetate (15)



Compound **15** was prepared according to a previously published procedure[90] with some modifications. 4-(Hydroxymethyl)phenol (12.2 g, 98 mmol) and methyl 2-chloroacetate (11.7 g, 108 mmol) were dissolved in acetone (200 mL), then K_2CO_3 (13.5 g, 98 mmol) and KI (1.6 g, 9.8 mmol) were added and the mixture was stirred at 60 °C for 96 hours. was heated to 70 ºC and stirred for 24 hours. The reaction was monitored by two TLC elution mixtures ($CHCl_3$/MeOH 10/1 for starting compound and Et_2O for product – detection by UV light). After full conversion the reaction mixture was filtrated and evaporated. The crude product was purified on two columns using 20 g of silica gel per 1 g of reaction mixture, eluting the first with Et_2O and the second with $CHCl_3$/MeOH gradient 100/0 → 100/1 → 50/1. Fractions with product were evaporated on RE at 50 ºC, product was dried at 50 ºC using oil vacuum pump and obtained in 52% yield (9.9 g) with 8-9% impurities according to NMR. NMR and MS spectra are in accordance with literature[90].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.32 – 7.27 (m, 2H, **H3**), 6.91 – 6.87 (m, 2H, **H4**), 4.63 (s, 2H, **H6**), 4.61 (d, J = 4.9 Hz, 2H, **H1**), 3.80 (s, 3H, **H8**), 1.75 (t, J = 5.5 Hz, 1H, **OH**) ppm

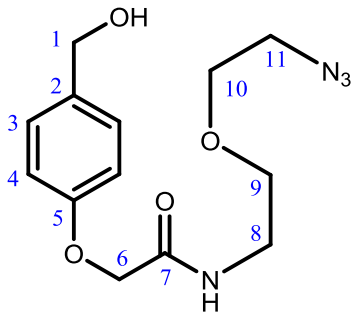
^{13}C NMR (101 MHz, $CDCl_3$): δ = 169.40 (**C7**), 157.36 (**C2**), 134.34 (**C5**), 128.67 (**C3**), 114.71 (**C4**), 65.38 (**C6**), 64.87 (**C1**), 52.30 (**C8**) ppm

ESI MS: for $C_{10}H_{12}O_4$ found 219.10 $[M+Na]^{1+}$

HRMS: for $C_{10}H_{12}O_4$ calcd: m/z 196.2020, found 219.0629 $[M+Na]^{1+}$

IR: υ = 3342, 3525, 2959, 2923, 2863, 1769, 1613, 1518, 1440, 1299, 1219, 1078, 821, 635, 516 cm^{-1}

N-(2-(2-Azidoethoxy)ethyl)-2-(4-(hydroxymethyl)phenoxy)acetamide (16)



Compound **16** was prepared according to a modification of a previously published procedure[71]. Compound **15** (3.0 g, 15.3 mmol) was dissolved in 2-(2-azidoethoxy)ethanamine (2.0 g, 15.3 mmol) and stirred at 25 °C for 21 hours. The reaction was monitored by two TLC elution mixtures ($CHCl_3$/MeOH 10/1 for starting compound and Et_2O/MeOH 10/1 for product – detection by UV light). After full conversion the reaction mixture was purified on column using 150 g silica gel eluting with Et_2O/MeOH 10/1. The Fractions with product were evaporated on RE at 30 ºC, product was dried at 50 ºC using oil vacuum pump and obtained in 69% yield (3.1 g)

^{1}H NMR (400 MHz, MeOD): δ = 7.36 – 7.27 (m, 2H, **H4**), 7.02 – 6.94 (m, 2H, **H3**), 4.55 (s, 2H, **H1**), 4.52 (s, 2H, **H6**), 3.67 – 3.58 (m, 4H, **H9**, **H10**), 3.50 (t, J = 5.5 Hz, 2H, **H11**), 3.36 (d, J = 2.9 Hz, 2H, **H8**).

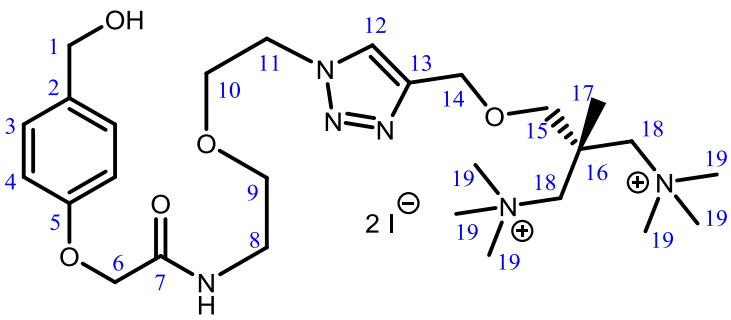
^{13}C NMR (101 MHz, MeOD): δ = 169.88 (**C7**), 157.06 (**C5**), 134.76 (**C2**), 128.27 (**C4**), 114.34 (**C3**), 69.57 – 68.90 (**C9**, **C10**), 66.90 (**C6**), 63.35 (**C1**), 50.35 (**C8**), 38.53 (**C7**) ppm

ESI MS: for $C_{13}H_{18}N_4O_4$ found 317.20 $[M+Na]^{1+}$

HRMS: for $C_{13}H_{18}N_4O_4$ calcd: m/z 294.3110, found 317.1220 $[M+Na]^{1+}$

IR: υ = 3443, 3330, 2944, 2863, 2104, 1646, 1553, 1509, 1290, 1245, 1123, 1072, 1033, 833, 719, 582 cm^{-1}

1-((1-(2-(2-(2-(4-(Hydroxymethyl)phenoxy)acetamido)ethoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-*N*,*N*,*N*,*N*',*N*',*N*'-hexamethylpropane-2,2-diaminium (17)



Compound **16** was prepared according to a modification of a previously published procedure[91]. Acetonitrile (60 mL) was bubbled with argon to remove oxygen. Compound **6** (2.0 g, 6.8 mmol), compound **15** (1.6 g, 3.4 mmol) and CuI (2.6 g, 13.6 mmol) were added and stirred at 50 °C for 96 hours under argon atmosphere. The reaction was monitored by ESI MS and TLC (MeOH/HOAc/1% aq. sol. NH_4OAc 10/10/9 – detection by basic permanganate solution). The reaction was filtrated, and the brown precipitate was ultrasounded in water (250 mL). The mixture was filtrated and evaporated and the crude product was purified on reverse phase silica gel eluting with water and 10% MeOH. The product was lyophilized and obtained in 13% yield (350 mg).

^{1}H NMR (400 MHz, D_2O): δ = 8.10 (s, 1H, **H12**), 7.40 – 7.36 (m, 2H, **H4**), 7.02 – 6.98 (m, 2H, **H3**), 4.61 (s, 2H, **H1**), 4.59 (s, 2H, **H14**), 4.58 (s, 2H, **H11**), 4.57 (s, 2H, **H6**), 3.91 (t, 2H, **H10**), 3.77 (d, J = 14.1 Hz, 2H, **H18**), 3.71 (s, 2H, **H15**), 3.61 (t, J = 5.2 Hz, 2H, **H9**), 3.57 (d, J = 14.2 Hz, 2H, **H18**), 3.44 (t, J = 5.1 Hz, 2H, **H8**), 3.25 (s, 18H, **H19**), 1.47 (s, 3H, **H17**) ppm

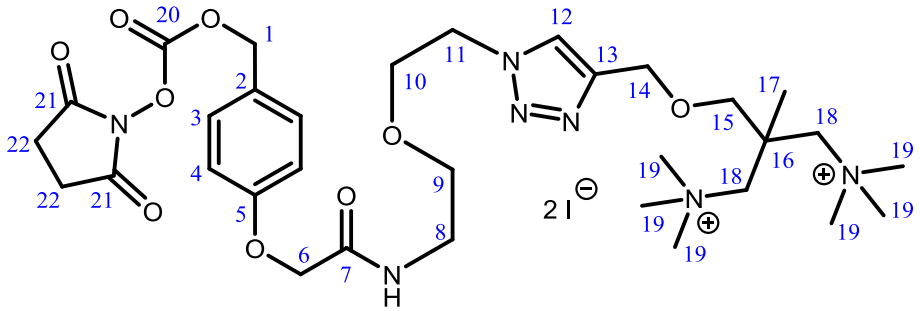
^{13}C NMR (101 MHz, D_2O): δ = 171.12 (**C7**), 156.61 (**C5**), 142.61 (**C13**), 133.92 (**C2**), 129.36 (**C4**), 125.81 (**C12**), 114.84 (**C3**), 72.16 (**C18**), 71.38 (**C15**), 68.72 (**C9**), 68.40 (**C10**), 66.64 (**C6**), 63.29 (**C11**), 62.67 (**C1**), 55.86 (**C19**), 50.17 (**C14**), 43.18 (**C16**), 38.52 (**C8**), 20.97 (**C17**) ppm

ESI MS: for $C_{27}H_{48}I_2N_6O_4$ found 521.40 $[M\text{-}Me]^{1+}$ and 268.35 $[M]^{2+}$

HRMS: and for $[C_{27}H_{48}N_6O_4]^{2+}$ calcd: m/z 268.1838, found 268.1838 $[M]^{2+}$

IR: υ = 3384, 3010, 2938, 2866, 1670, 1610, 1539, 1512, 1476, 1237, 1108, 917, 833, 585 cm^{-1}

2-(((1-(2-((2-(4-(((((2,5-Dioxopyrrolidin-1-yl)oxy)carbonyl)oxy)methyl)-phenoxy)acetamido)methoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-methyl)-*N*^{1},*N*^{1},*N*^{1},*N*^{3},*N*^{3},*N*^{3},2-heptamethylpropane-1,3-diaminium (18)



Compound **17** (187 mg, 0.236 mmol), DSC (100 mg, 0.391 mmol) and DIPEA (90 µL, 0.520 mmol) were dissolved in dry DMF (12 mL) and stirred at 60 °C for 170 hours under argon atmosphere. The reaction was monitored by ESI MS. After full conversion the DMF was distilled at 60 °C under vacuum. The crude product was extracted with DCM (6 × 15 mL). The product was obtained in 81% yield (179 mg).

^{1}H NMR (600 MHz, DMF-*d*7): δ = 8.33 (s, 1H, **H12**), 8.21 (t, J = 5.9 Hz, 1H, **NH**), 7.52 – 7.46 (m, 2H, **H3**), 7.08 – 7.01 (m, 2H, **H4**), 5.02 (s, 2H, **H1**), 4.73 (s, 2H, **H14**), 4.64 (t, J = 5.1 Hz, 2H, **H11**), 4.60 (s, 2H, **H6**), 4.11 (d, J = 2.8 Hz, 4H, **H18**), 3.92 (s, 2H, **H15**), 3.90 (t, J = 5.1 Hz, 2H, **H10**), 3.57 (t, J = 5.9 Hz, 2H, **H9**), 3.51 (s, 18H, **H19**), 3.42 (t, J = 5.9 Hz, 2H, **H8**), 2.69 (s, 4H, **H22**), 1.66 (s, 3H, **H17**) ppm

^{13}C NMR (125 MHz, DMF-*d*7): δ = 172.85 (**C20**), 172.40 (**C21**), 168.31 (**C7**), 158.91 (**C5**), 143.34 (**C13**), 131.59 (**C3**), 127.77 (**C2**), 125.17 (**C12**), 115.05 (**C4**), 78.02 (**C1**), 72.78 (**C15**), 72.25 (**C18**), 69.34 (**C9**), 69.18 (**C10**), 67.57 (**C6**), 64.23 (**C14**), 55.84 (**C19**), 50.13 (**C11**), 43.95 (**C16**), 38.68 (**C8**), 25.94 – 25.69 (**C22**), 21.53 (**C17**) ppm

ESI MS: for $C_{32}H_{51}I_2N_7O_9$ found 317.0

HRMS: for $[C_{32}H_{51}N_7O_9]^{2-}$ calcd: m/z 338.6869, found 332.7051 $[M\text{-}Me]^{2+}$

IR: υ = 3396, 2929, 1718, 1536, 1509, 1479, 1213, 1075, 815, 653 cm^{-1}

6.2.3 Synthesis of the peptide

Manual SPPS of Cecropin A (1-7) – Mellitin (2-9) hybrid peptide (19)

The 15-mer[92] H-Lys-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-$CONH_2$ was synthesized using a solid-phase method on 2-chlorotrityl chloride resin (1.53 g, loading 1.46 mmol/g, 1% DVB, 100–200 mesh, Hecheng, China) using Fmoc chemistry, based on protocols from APIGENX s.r.o. Tryptophan and lysine were protected on the side chain by Boc group.

The attachment of the first amino acid to the resin was performed by treating the resin with a solution of Fmoc-Leu-OH (0.33 g, 0.93 mmol) and DIPEA (0.813 mL, 4.67 mmol) in dry DCM (20 mL) for 4 hours. After the resin was washed with 10% solution of DIPEA in dry DCM (5 × 20 mL). The resin was end-capped with a solution of MeOH/DIPEA/DCM 1/1/8 (40 mL) for 40 min. The obtained resin substitution was 0.478 $mmol \cdot g^{-1}$ and its determination is described in chapter 6.3.1.

Deprotection of the Fmoc group was carried out two times over 10 and 15 min with 20% piperidine in DMF with 0.1 eq. TFA (30 mL). All the amino acids were coupled using a three-fold molar excess of the protected amino acid (Fmoc-AA), Oxyma, DIC and collidine dissolved in DMF (25 mL) for 1 h (Fmoc-AA/Oxyma/DIC/collidine 3/3/3/6). After each coupling, the resin was treated with capping mixture (10% Ac_2O in DMF at pH 7.5 set by collidine) for 15 min. After each coupling, capping and deprotection step the resin was washed with DMF (6 × 50 mL). The extent of each coupling step was monitored by UPLC and using the ninhydrin test.

After the synthesis had been completed, the peptide was cleaved from the resin (250 mg) with a TFA/TIS/H_2O (92.5/5/2.5) mixture for 1 h. The cleaved peptide was precipitated with cold Et_2O (50 ml), filtrated and dried in exicator for 24 h. The crude product (104 mg) was purified using reverse phase C18 column on HPLC eluting with MeCN/H_2O/0.1% TFA gradient 10 % → 50 %. The product was obtained by lyophilization of two fractions in 94.6% purity (17.49 mg) and 76.6% purity (13.16 mg) with 21% combined yield.

Automated SPPS of Cecropin A (1-7) – Mellitin (2-9) hybrid peptide (19)

The automated SPPS was provided on Symphony – UV IR automatic peptide synthesizer in APIGENEX s.r.o. The first amino acid was attached to the resin manually using the same protocols as described above and substitution was 0.475 mmol/g. The reaction conditions of coupling, capping and deprotection cycles were also the same stated above. The synthesis after the tenth coupling and capping was stopped and the de-Fmoc was performed manually using 20% DBU in DMF (30 mL) two times for 20 min and was continued until the end. Purification on RP-HPLC resulted in 90.0% purity (30.00 mg) with 22.7% yield.

6.3 Other experiments

6.3.1 Resin substitution determination

Resin substitutions for the resins used for manual and automatic SPPS were determined using the method[25] described in chapter 2.3.2 using equation 2.3, where $A_{290\ nm}$ is the average of absorptions of sample solutions measured at 290 nm ($A_1 - A_5$); $\varepsilon_{289.8\ nm}$ [L·mol^{-1}·cm^{-1}] is molar absorption coefficient of dibenzofulvene-piperidine adduct at 289.8 nm with the value of $\varepsilon_{289.8\ nm}$ = 6089 L·mol^{-1}·cm^{-1}; m_{resin} [mg] is the sample weight of the resin used in the experiment

$$S[\text{mmol} \cdot \text{g}^{-1}] = \frac{10^5 \cdot A_{289.8\ nm}}{\varepsilon_{289.8\ nm} \cdot m_{resin}} \quad (2.3)$$

The 2-chlorotrityl resin with Fmoc-Lys attached was suspended in 20% (*v*/*v*) piperidine in DMF (100.0 mL) for 1 h. The absorbance of the dibenzofulvene-piperidine adduct in the solution was measured 5 times by UV-VIS spectroscopy at 290 nm. The substitution was calculated using eq. 2.3 and the data in Table 3. The substitution of the resin used for manual SPPS was 0.478 mmol/g and for the resin used for automatic SPPS was 0.475 mmol/g.

Table 3 Substitution determination

SPPS	m_{resin} [mg]	A_1	A_2	A_3	A_4	A_5	$A_{290\ nm}$	S [mmol/g]
Manual	10.88	0.31884	0.31674	0.31624	0.31569	0.31594	0.31669	0.478
Automatic	19.05	0.55224	0.55092	0.54982	0.55135	0.55035	0.55093	0.475

6.3.2 Attachment of the anchored linker to the peptide

Resin with peptide prepared by manual SPPS (76 mg) was swelled in DMF (3 mL) for 20 min and was treated with 20% piperidine in DMF with 0.1 eq. TFA (2 mL) two times for 15 minutes for Fmoc deprotection. In DMF (1.5 mL) two equivalents of the double-charged anchored linker **18** (50 mg) were dissolved and pH was set to 8 using collidine. The resin beads were added to the mixture, and suspension was stirred at 60 °C for 21 days. Reaction was monitored by ninhydrin test and UPLC-MS analysis. After reaction was stopped, the resin was washed with excess of DMF, DCM and Et_2O and was treated with 1% TFA in DCM (3 mL) and TIS (20 µL) for 1 h for peptide cleavage from the resin. Mixture was evaporated (17.5 mg), dissolved in 1% TFA in MeOH (4 mL) and used for separation on cation exchanger.

6.3.3 Peptide separation on cation exchanger

For separation Dowex 50WX8 cation exchanger in H^+ cycle (130 mg) was added to the methanolic solution of cleaved peptides (chapter 6.3.2). The attachment of the adduct of the full-length peptide with anchored linker to the cation exchanger was monitored by TLC (MeCN/H_2O 7/3 – detection by ninhydrin solution); one spot (R_f = 0.4) disappears after addition of cation exchanger. Cation exchanger was washed 1% TFA in MeOH, H_2O, DMF, DCM and Et_2O. TFA cocktail (TFA/TIS/H_2O 92.5/2.5/5.0 *v*/*v*/*v*) was added for 1 h to cleave the peptide from the anchored linker. To recover the peptide, cation exchanger was washed with aqueous 0.01%, 0.1%, 1% and 10% solution of NH_4HCO_3, 24% aqueous solution of NH_3, 10% and 50% methanolic solution of TEA. UPLC-MS analysis showed the double-charged anchored linker was washed by 50% methanolic solution of TEA, but no traces of the peptide.

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