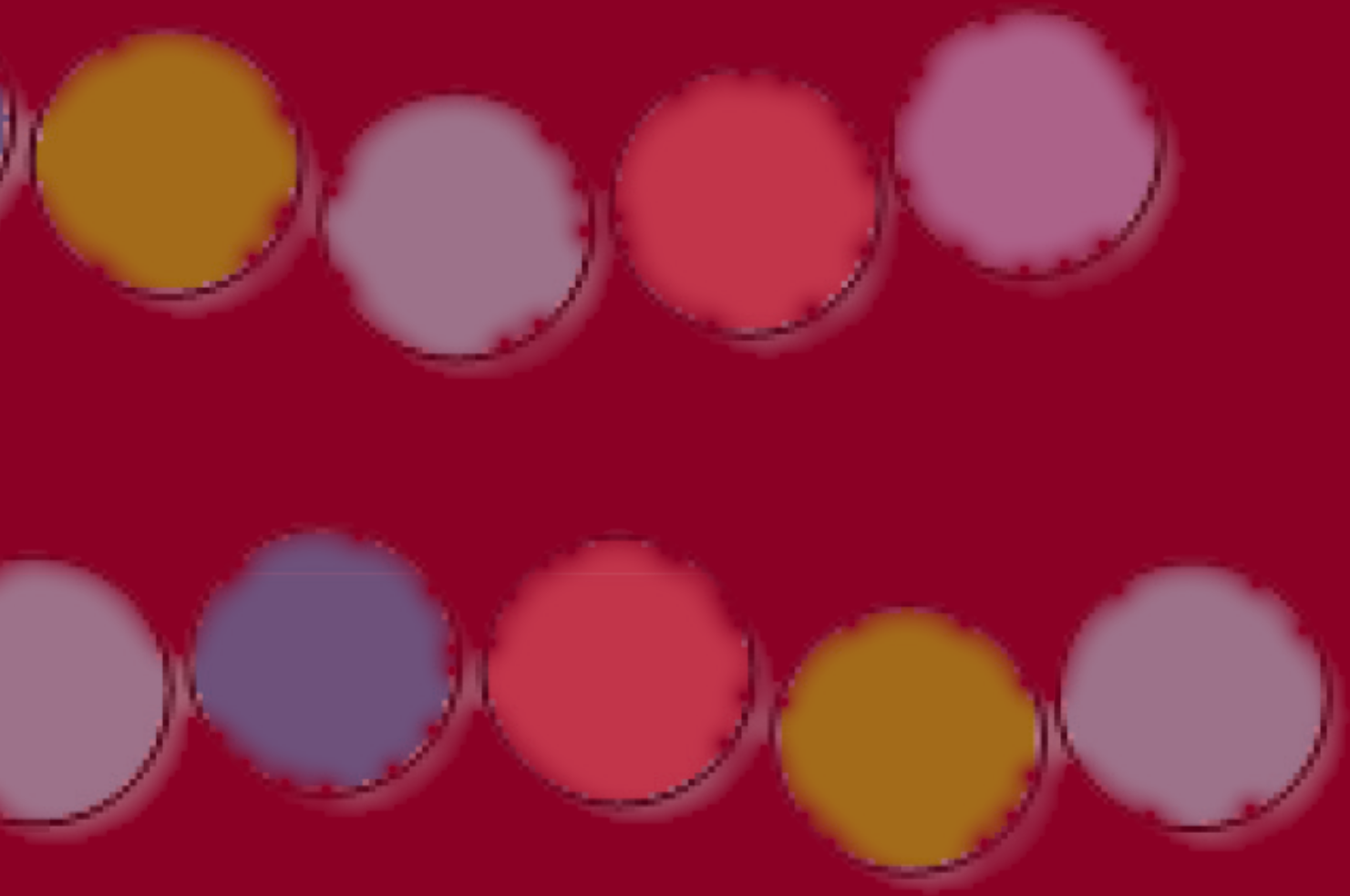
Stephen Middel

Photocleavable Templates for PNA Base Pairing Mediated Native Chemical Ligation



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Photocleavable Templates for PNA Base Pairing Mediated Native Chemical Ligation

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vorgelegt von

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Table of Contents

1 Introduction & Outline

The control of biological processes is a fundamental desire in medicinal chemistry because incorrect regulation causes diseases.[1] In the premodern era little was known about these processes and therefore, only few pharmaceuticals were established. Mainly anesthetics such as alcohol and opium but also other herbal ingredients were used. The first drugs that were synthesized on purpose were developed in the late 19th century. Admittedly, due to the lack of understanding biochemical functions, pharmaceuticals like the soporific chloral hydrate (1869) or the still very popular acetylsalicylic acid (1897) were designed after the principle of trial and error.[2] A rational design of pharmaceuticals was only possible after significant advances in protein crystallography and the development of nuclear magnetic resonance (NMR) spectroscopy. Proceedings in biochemical technologies allowed to understand biological processes, to find the cause of diseases, and to identify new targets for drugs.

Different approaches are made to develop new pharmaceuticals. For the rational design, a ligand, e.g. a natural product or a peptide that binds to the target, must be known. The ligand-target interactions are identified, and beginning from the ligand structure, the pharmaceutical structure is optimized in order to enhance the selectivity, the potency, the stability towards biological degradation mechanisms, and the bioavailability as well as in order to facilitate the synthetic access. This approach is often promising, but also very time-consuming and costly.[2] Further advances in technology offered additional options. Thus, in the 90s the high-throughput screening (HTS), a new technique that allowed to test an incredibly high number of compounds for their biological activity at one time, fuelled the hope that the search for lead structures could be dramatically facilitated.[3] Some positive examples are known, but altogether the output from HTS so far is disappointing measured against the affords that were made.[4]

The techniques are mostly used to develop small molecule drugs. However, due to their small size, a selective interaction with one target and not a whole target family is often difficult to achieve. The small size does not offer many interaction sites and these often flat molecules cannot efficiently fill the three dimensional pockets of enzymes or the binding sites of proteins. In terms of selectivity, natural products often achieve better results due

to their complex structure. Even so, their complexity is also the biggest disadvantage. It makes a synthetic production on an industrial scale impossible and can usually only be gained via extraction from plants or animals.[5] The best selectivity can be achieved by peptidic and protein based drugs. Yet, these drugs are very sensitive towards biological degradation processes. Peptides can be exchanged by peptidomimetics at the expense of a higher synthetic effort. Protein drugs often have the additional drawback that they usually cannot enter the cell, as a result they are mainly used for extracellular targets.[6]

A very interesting class of protein drugs is depicted by antibodies. These pharmaceuticals are mainly used in cancer treatment. Cancer cells differ from normal cells due to genomic mutations, which in some cases give raise to tumor-specific antigens. These cells can then be addressed selectively and destroyed by antibodies.[7] Monoclonal antibodies (mAbs) have already been successfully used in cancer treatment.[8] Nevertheless, many mAbs only have a low cytotoxic activity.[9] For this reason, a new class of anti-cancer drugs that combines the selectivity of antibodies with the potency of chemotherapeutic small molecules called antibody-drug conjugates (ADCs), has been developed.[10]

Besides the high selectivity towards certain antigens caused by the Fab region of the antibody, the long serum half-life also makes antibodies an interesting construct. The Fc region of the antibody is thought to be responsible for the high stability and is therefore an interesting scaffold for protein-binding domains or cell penetrating peptides that can be incorporated instead of the Fab region, and as a result a greater variety of targets can be attacked or cell permeability can be achieved.[11] Fc fusion proteins have already been marked as medicinal products, which shows their therapeutic potential.[12] The use of Fc regions has also been expanded by the design of a strand-exchange engineered domain (SEED) that delivers an Fc platform with two different Fc derived chains and therefore provides up to four different ligation sites.[13] This offers the possibility to design Fc conjugates with interesting combinations of recognition units (protein binding domains, CPPs), effector units (toxins) and reporter units (radiolabels, fluorophores). In order to introduce all kinds of cargo, a chemical modification method would be desirable.

Within the scope of this thesis, a novel ligation strategy shall be developed that later on would allow a chemical one-pot type modification of the Fc region at all ligation sites at the same time. This would open the possibility to design a library of different Fc conjugates.

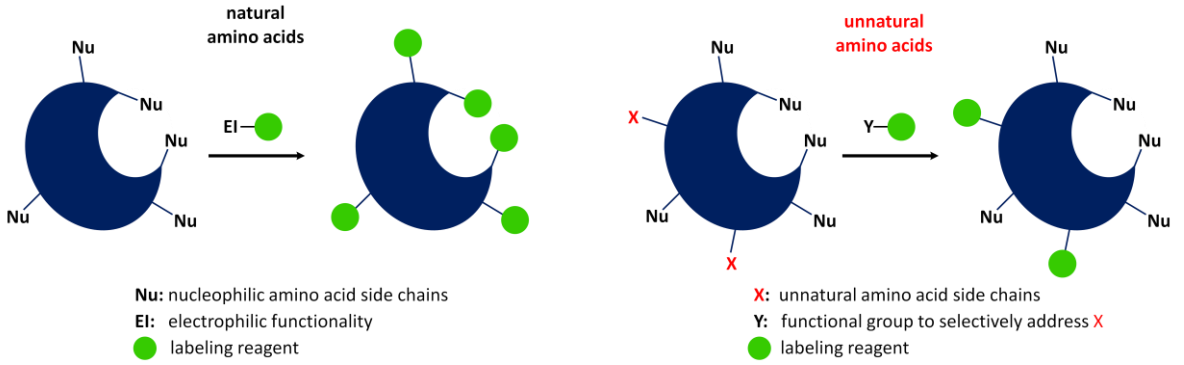
1.1 Protein Modification

Proteins conduct most of the biochemical functions in the cell. In order to understand these functions and to take influence on them, it is important to synthesize and modify proteins. Biochemists found ways to effectively produce proteins by expression techniques.[1] However, these methods are limited. Proteins that need complex post-translational modifications, like glycoproteins, are often excluded. Since only 20 genetically encoded amino acids can be readily incorporated, modifications, including post-translational ones are difficult to achieve and hard to control precisely.[14] Proteins can be modified when cargo with an electrophilic functionality is added, but these functionalities react with all surface nucleophiles of the protein. Therefore, the reactivity of the protein can be influenced in an undesired manner. Furthermore, all proteins abundant in the reaction medium will be labeled.[15]

Nevertheless, in some cases post-translational modifications can be conducted precisely. An *N*-terminal cysteine offers the possibility for a native chemical ligation (NCL)[16] or an *N*-terminal serine provides access to an aldehyde, via a specific sodium periodate oxidation, which can then be used for oxime ligation.[17] However, these examples are few and far between. A different approach is a purely synthetic route to produce proteins. Since the introduction of the solid phase peptide synthesis (SPPS) by MERRIFIELD in 1963, the synthesis of peptides with lengths of ≤ 50 amino acids has been strongly facilitated.[18] Nevertheless, a typical protein found in nature consists of about 300 amino acids.[14] By the development of powerful bioorthogonal ligation techniques, with native chemical ligation leading the way, bigger targets were accessible.[19] This method allows all imaginable kinds of modifications at every position in the protein. Unfortunately, the synthesis is very challenging and time consuming.

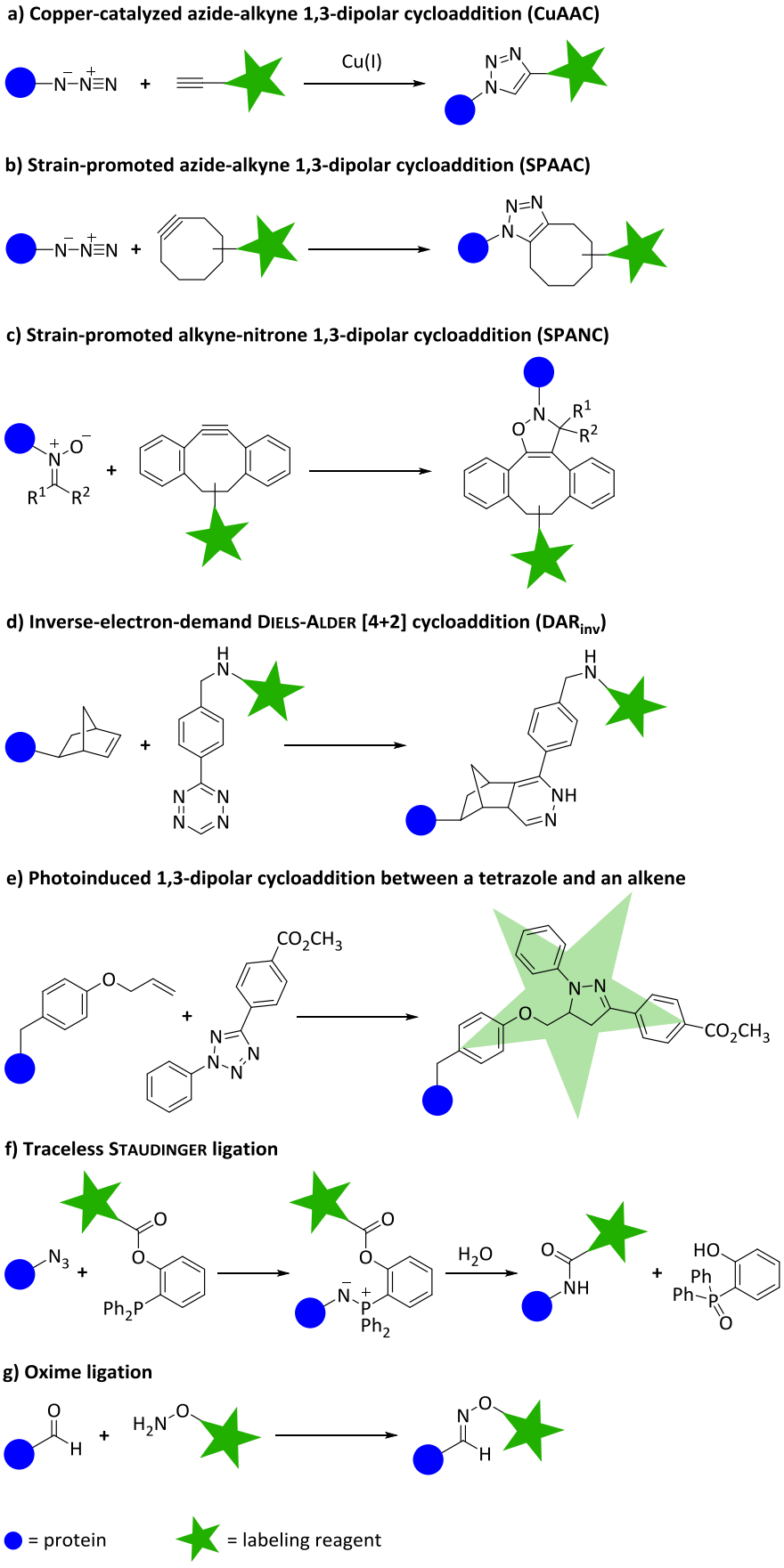
So, in the last few years two methodologies have been established and both can be seen as a compromise between the biological and the chemical approach. Semi-synthetic approaches are often used for big proteins that possess a modification, like glycoproteins, which cannot be added after translation. The part of the protein containing the modification is synthesized chemically, the other parts biologically and the fragments are ligated.[20] Another concept implies the incorporation of unnatural amino acids (UAAs) by expanding the genetic code within optimized protein biosynthetic machineries.[21] During

the last few years, it was possible to incorporate a whole variety of UAAs with different bioorthogonal groups, such as azides,[22] alkynes,[23] cyclooctynes,[24] alkenes,[25] tetrazoles,[26] ketones[27] or even iodides[27] and boronates.[28] These UAAs allow selective post-translational modifications on the proteins (Scheme 1). Bioorthogonal reagents have to perform a difficult balancing act between reactivity and stability. The reactants must be stable under physiologic conditions but still be reactive enough to assure a fast and efficient reaction. Also, the reaction must be chemoselective in a pool of countless different compounds.



Scheme 1. *On the left:* Modification of a natural protein using labeling reagents with electrophilic functionalities. *On the right:* Site-selective modification of a protein with incorporated UAAs (adapted from [15]).

Some reactions have frequently been used for protein modifications and are now established in this field of research. First of all, click reactions have to be named in this context. SHARPLESS' further development of HUISGEN'S azide-alkyne [3+2] cycloaddition[29] yielded the copper-catalyzed azide-alkyne [3+2] cycloaddition (CuAAC).[30] This reaction has become synonymous with the term of click chemistry, although many other reactions belong to this type of reaction (Scheme 2). The term of click chemistry was characterized by SHARPLESS and describes reactions that are high yielding, wide in scope, create only non-toxic by-products that can be removed without chromatography, are stereo- and regiospecific and are simple to perform.[31] These features make click reactions suitable for targeting molecules in complex biological environments. CuAAC reactions fit the requirements perfectly and therefore have been used for the modification of proteins.[32] Also interesting is that the resulting triazoles mimic *trans*-amide bonds because of their similar size, planarity, H-bonding capabilities and dipole moment.[33] Nevertheless, due to the cytotoxicity of copper, an alternative route has been developed.[34] Strain-promoted azide-alkyne 1,3-dipolar cycloadditions (SPAAC) offer a copper-free alternative. Although



Scheme 2. Frequently used bioorthogonal reactions for modification and labeling of proteins.

these reactions are slower compared to CuAAC, the fact that no additional catalysts or additives are needed makes them very attractive and SPAAC has been widely used for protein modifications.[35] Cyclooctyne derivatives do not only react with azides, but also with more reactive 1,3-dipoles such as nitrones via a very fast strain-promoted alkyne-nitrone cycloaddition (SPANC).[36] Inverse electron DIELS-ALDER [4+2] cycloadditions (DAR_{inv}) of tetrazines and strained alkenes belong to the fastest known click reactions (Figure 1). Different alkenes have been established and were used for protein modifications. Norbornene substrates are popular due to their stability against nucleophiles[37] and cyclopropenes offer a small-sized alternative,[38] but by far the most popular alkenes are *trans*-cyclooctene (TCO) derivatives due to their extremely high reaction rate.[39] The major advantage of the photoinduced 1,3-dipolar cycloaddition of tetrazoles and alkenes lies in its inducibility by light. This provides an exciting tool for spatiotemporal labeling reactions in living systems.[40] Besides click reactions, other bioorthogonal reactions like the STAUDINGER ligation[41] or the oxime ligation[42] have been approved for chemoselective bioconjugation reactions. However, it is important to note that these reactions are not useful for *in vivo* labeling, due to their comparable low reaction rate.

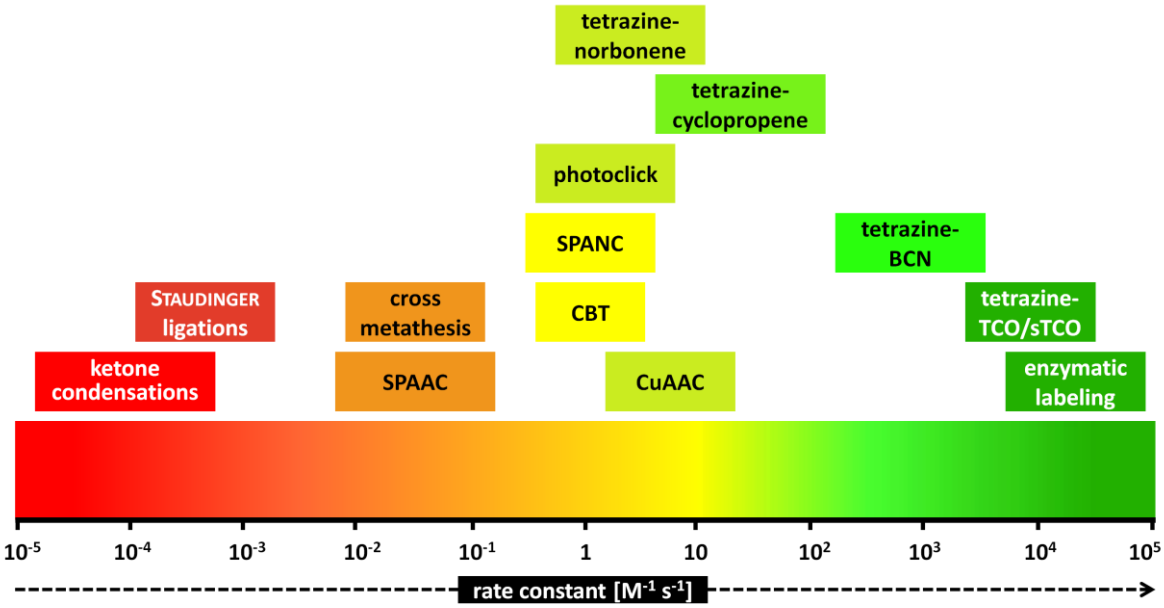
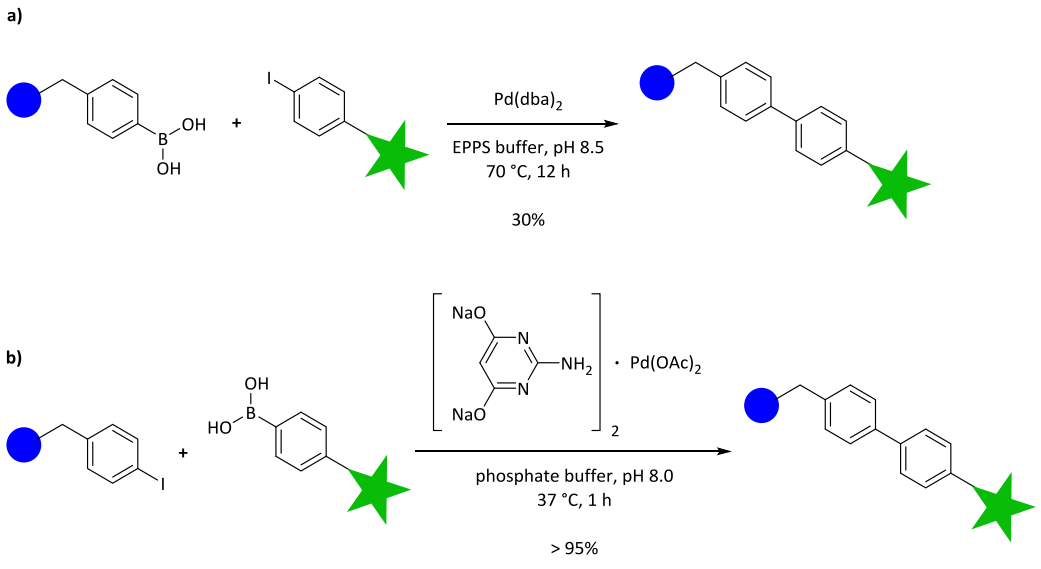


Figure 1. Rate constants of chemoselective reactions (CBT: 2-cyanobenzothiazole ; BCN: bicyclononyne; sTCO: strained *trans*-cyclooctene {adapted from [43]}).

Interestingly, in some examples it could be shown that many of these chemoselective reactions are also orthogonal to each other. This allows to modify proteins selectively at two or more sites. Orthogonality in bioorthogonal reactions could be shown among others

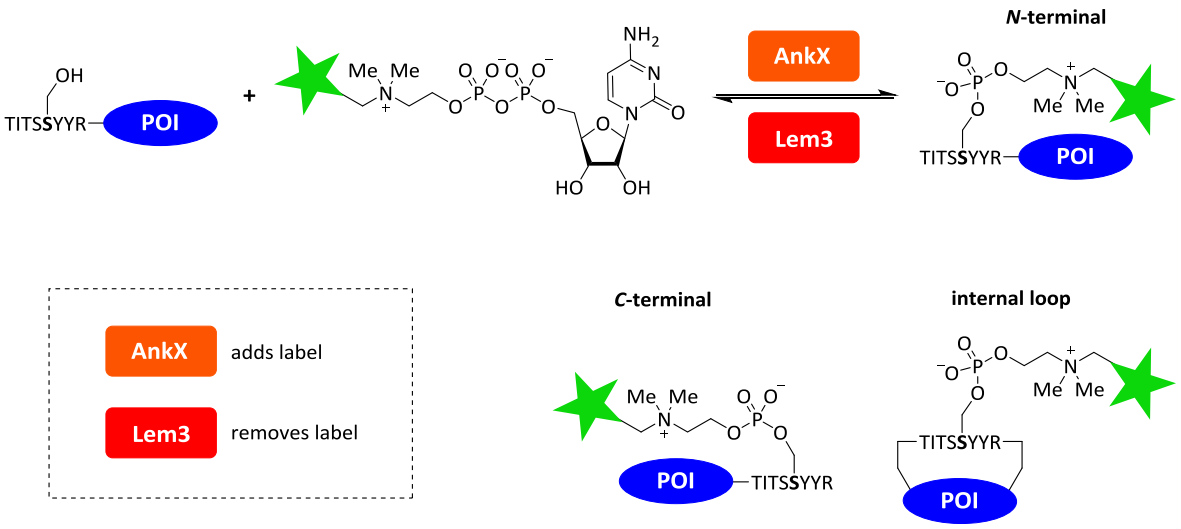
for DAR_{inv} and SPAAC reactions,[44,45] DAR_{inv} and oxime ligation[46] or oxime ligation and sortase A ligation.[47] In order to further exploit the toolbox of bioorthogonal reactions, a high-throughput screening was performed to identify new chemoselective reactions. Indeed, a series of novel 1,3-dipolar cycloadditions has been found that theoretically fits the requirements in terms of chemoselectivity, speed and biocompatibility.[48] Nevertheless, the applicability of these reactions still has to be proven. Besides the well-established concepts, metal-catalyzed cross coupling reactions are also increasing their popularity. Cross-metathesis is a very powerful carbon-carbon bond forming reaction and has been used for chemoselective protein modifications. However, at least a small amount of organic solvents had to be added and huge excesses of the ruthenium catalyst and the additives were necessary to achieve effective product formation.[49] Other alternatives are the palladium-mediated cross-coupling reactions. The absence of palladium from biological systems, its tolerance towards aqueous media and the excellent tolerance of its reaction partners towards nucleophiles make these reactions an interesting tool for bioorthogonal modifications.[43] The first attempts were not very promising, though. A MIZOROKI-HECK reaction between a *p*-iodophenylalanine and a labeled vinyl reagent only yielded 2% product after 50 h. Moreover, DMSO and high concentrations of additives had to be added.[50] A SONOGASHIRA reaction resulted in slightly better yields, but the reaction still did not proceed under physiological conditions.[51] The same can be stated for a SUZUKI reaction, where a reaction temperature of 70 °C was necessary to obtain the labeled product (Scheme 3a).[52]



Scheme 3. Palladium-catalyzed cross coupling reactions for the modification of proteins.[52,53]

However, things changed with the development of a new water-soluble 2-amino-4,6-dihydroxypyrimidine sodium salt (ADHP) ligand. After one hour reaction time, more than 95% conversion was obtained under physiological conditions (Scheme 3b).[53] Also, other Pd-catalyzed cross coupling reactions could be significantly improved by using the ADHP ligand.[54]

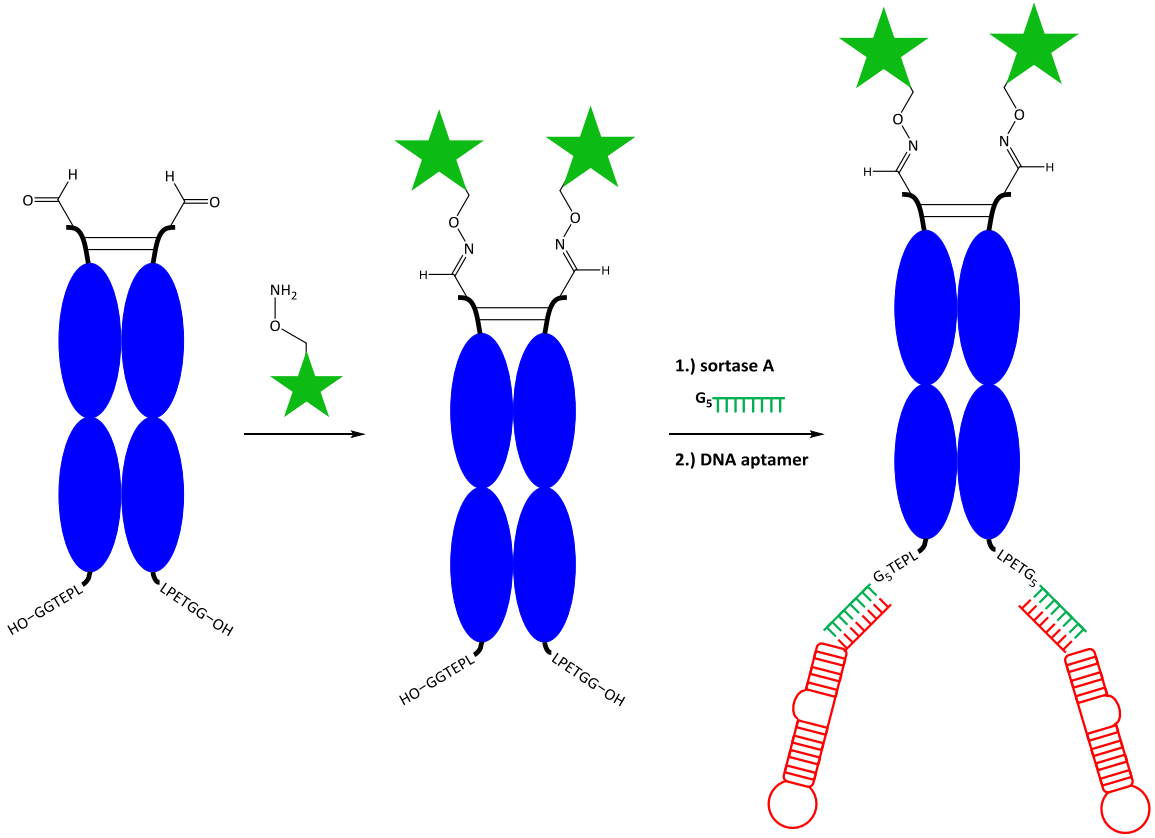
In another approach, enzymes are used to modify proteins. Owing to the very few ligases found in nature, proteases have been used for peptide ligation by reversing their proteolytic activity. Two basic strategies are known to manipulate proteases: the thermodynamically controlled[55] and the kinetically controlled approach[56]. A novel enzymatic protein labeling method was described by the ITZEN and the HEDBERG groups. They took advantage of the ability of the bacterial enzyme AnkX to identify a specific peptide sequence within proteins and to transfer a phosphocholine residue to it. By varying the natural substrate cytidine diphosphate choline and adding a fluorescent residue, it was possible to label the *C*-terminus, *N*-terminus or an internal loop of the protein of interest (POI). Furthermore, it was even possible to detach the label while adding the alkylphosphocholine resistance protein Lem3 (Scheme 4).[57]



Scheme 4. A novel enzymatic protein labeling method. Depending on the position of the octapeptide modification, the POI can be labeled at different positions (adapted from [57]).

One of the very few enzymes that are known to catalyze peptide ligations and have been successfully used for peptide ligations and protein modifications is the *trans*-peptidase sortase A. It cleaves a *C*-terminal LPxTG motif at the threonine residue and ligates it to a peptide containing at least one *N*-terminal glycine.[58] The KOLMAR group used a sortase A

ligation to introduce a peptide nucleic acid (PNA) strand to the *C*-terminus of an antibody Fc fragment and therefore converted it into a modular scaffold. Then, a DNA aptamer was added and attached to the Fc fragment via DNA/PNA base pairing. The *N*-terminus was modified with a fluorophore applying oxime ligation (Scheme 5).[47]

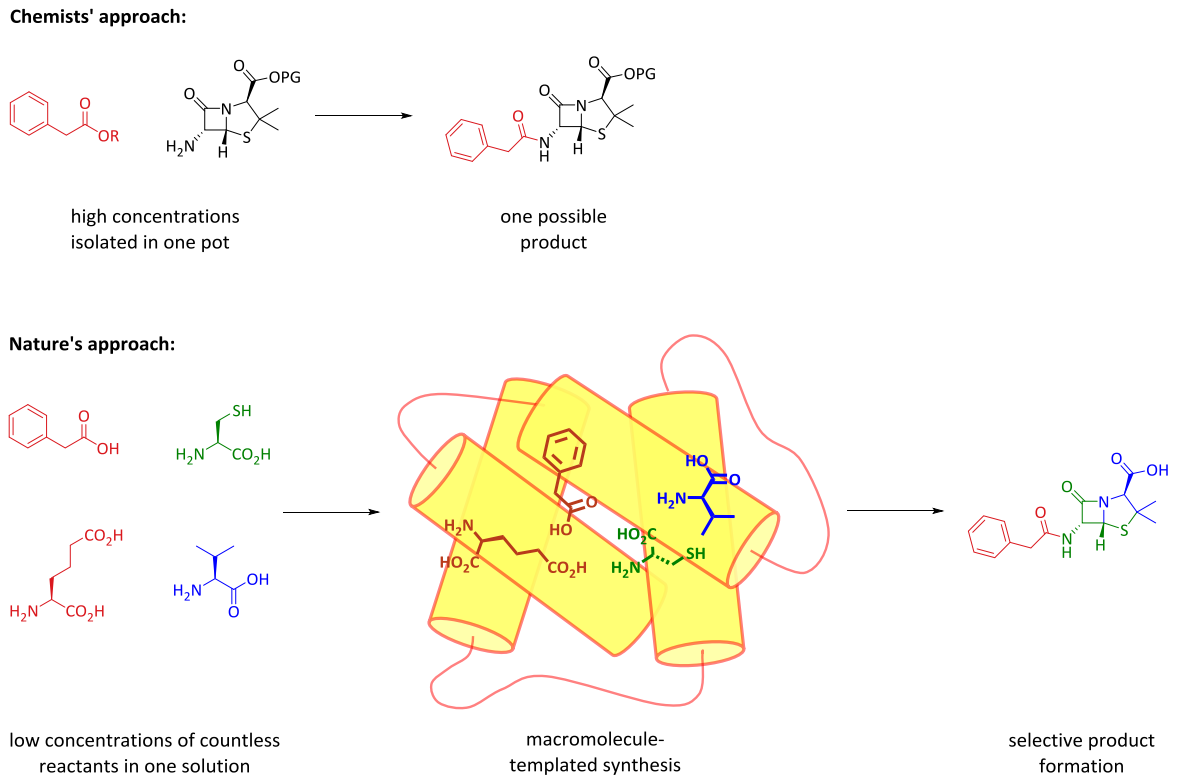


Scheme 5. Self-assembled hybrid aptamer-Fc conjugate (adapted from [47]).

In another attempt, antibody Fab fragments were equipped with PNA strands in order to create self-assembling multimers through PNA base-pairing. With this method, it was possible to access well-defined homo- and heterodimers, as well as higher-order multimers.[59] The WINNSINGER group used a DNA template as a modular scaffold for PNA encoded oligosaccharides that mimic the carbohydrate epitope of HIV.[60] The great advantage of templates lies in their ability to find each other within a medium of a great number of molecules. This ability was also used for covalent bond forming reactions, which will be discussed in the next chapter.

1.2 Template-Directed Reactions

The control of the selectivity and the rate of chemical reactions is a major concern of the natural sciences. In order to achieve this, chemists traditionally use a carefully picked pair of reactants in high concentrations, isolated in one vessel to obtain efficient product formation. Nature on the other hand can achieve highly efficient product formation in a medium with countless of different reactants in very low concentrations. The reason for this is that macromolecules can recognize reactants, bind them non-covalently, bring their reactive centers in close proximity and therefore increase the effective concentration of the reaction partners. These macromolecules are called templates (Scheme 6).[61]



Scheme 6. Chemists use a carefully picked pair of reactants in high concentrations in order to achieve an efficient reaction. Nature can accomplish this in the presence of many different reactants and low concentrations by using macromolecules as templates.[62]

This remarkably well operating machinery has gained a lot of attention from chemists who tried to copy this concept. Among these template based systems, nucleic acid templated synthesis plays an essential role in many biological processes. DNA and RNA, respectively, are polymeric structures that can sequence selectively bind complementary strands and form a great variety of different secondary structures like helices or hairpin structures.[1] They consist of a ribose-sugar unit with a phosphate residue that shapes the backbone and

nucleic acids that serve as recognition units. The four nucleic acids cytosine (C), guanine (G), adenine (A) and thymine (T, in DNA) or uracil (U, in RNA) can recognize each other and form two different WATSON-CRICK base pairs (C-G, A-T/U) (Figure 2).[63]

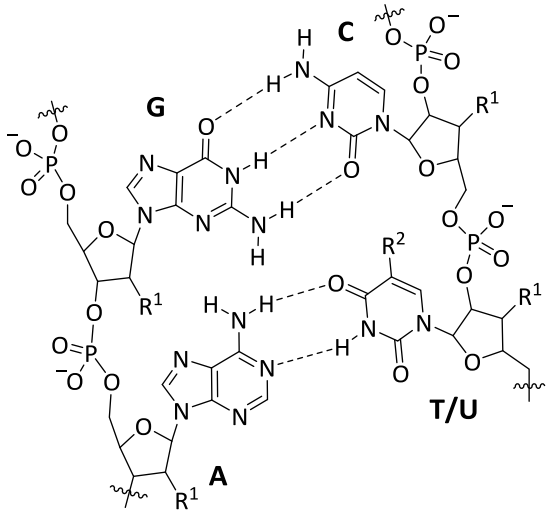
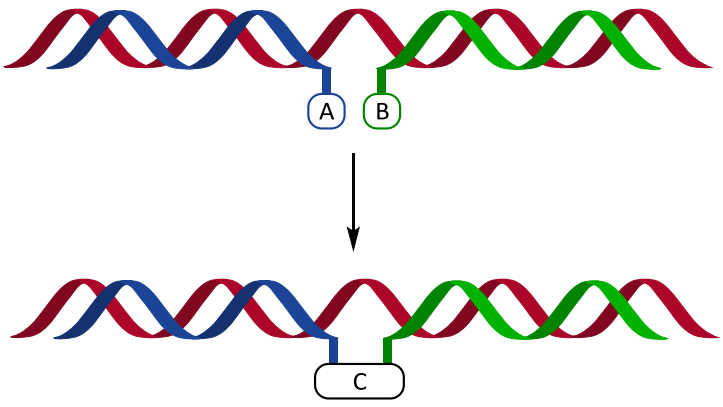


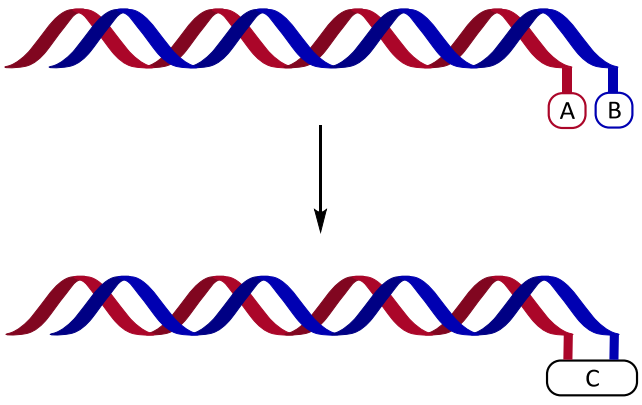
Figure 2. WATSON-CRICK base paring of DNA (R^1 = H, R^2 = CH_3) and RNA (R^1 = OH, R^2 = H).

The principle of template-directed reactions is applied for the DNA replication, the transcription of DNA into RNA and the translation of RNA into proteins.[1] The comparably easy adaptivity and the prevalent occurrence in biological systems made it very attractive for scientists. Therefore, this system was the first imitated for a template-directed reaction. Already in 1956, only three years after the structural elucidation of DNA,[63] synthetic polymers were explored as templates to direct the course of polymerization reactions.[64] One decade later, NAYLOR and GILHAM described the first attempt of a DNA-templated reaction. A leading DNA strand served as a template and two complementary lagging fragments were ligated in a condensation reaction (Scheme 7).[65]



Scheme 7. A leading DNA strand templates the ligation of two lagging strands.

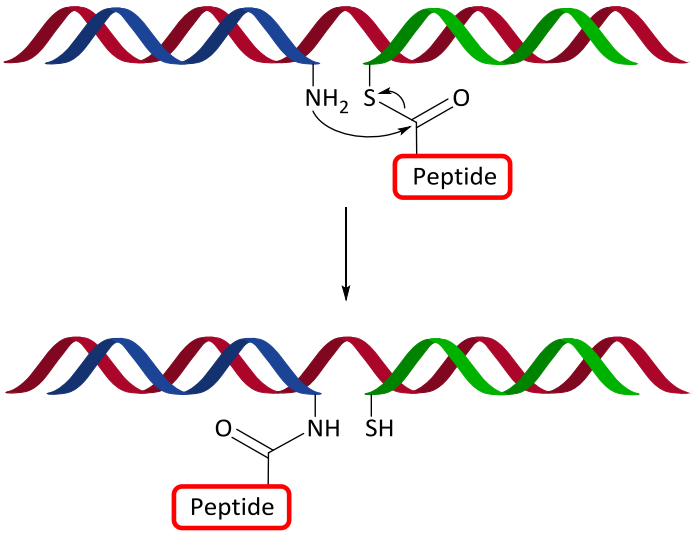
The yields were very low but the concept was copied, extended and optimized multiple times over the next decades.[61,66] Potential applications for template-directed reactions were mainly found in diagnostics. Accordingly, VON KIEDROWSKI could recently show a very fast and effective DNA templated disulfide exchange reaction. It resulted not only in the ligation of two lagging fragments, but also in the loss of a fluorescence quencher at one of the fragments, and therefore, in the occurrence of fluorescence.[67] This example shows very nicely that these simple systems can be used for DNA/RNA diagnostics. Besides DNA and RNA fragments, PNA fragments could also be ligated.[68] Apart from purely synthetic approaches, semi-synthetic approaches in combination with polymerase chain reactions (PCR) were established. The SEITZ group could show that PNA-peptide ligations can be performed on PCR DNA templates.[69] Later on, the method was optimized, so that the template-directed ligation could even be performed during the PCR process.[70] Aside from fragment condensation reactions, LYNN could show a DNA-catalyzed polymerization reaction, where a complementary DNA strand could be automatically synthesized from its monomers.[71] Also, DNA templates can be used to bind polyamides, arrange them and catalyze the ligation of two polyamides. They bind to the minor groove of dsDNA and can distinguish the two WATSON-CRICK base pairs.[72] LI and LIU demonstrated that not only the base pairing ability of nucleic acids makes them an interesting template, but also that the chiral nature of DNA offers some interesting possibilities. So, they showed that the chirality of the DNA template can be transferred to the ligated product.[73] In another approach, BROWN used an inorganic template additionally to a DNA template. Metal ions can be used to bring the reactive centers in the right conformation by coordination, which facilitates product formation even more.[74] A different concept was to ligate the leading strand to the lagging strand (Scheme 8).[61,66]



Scheme 8. Nucleic acid templated ligation of the leading and the lagging strand.

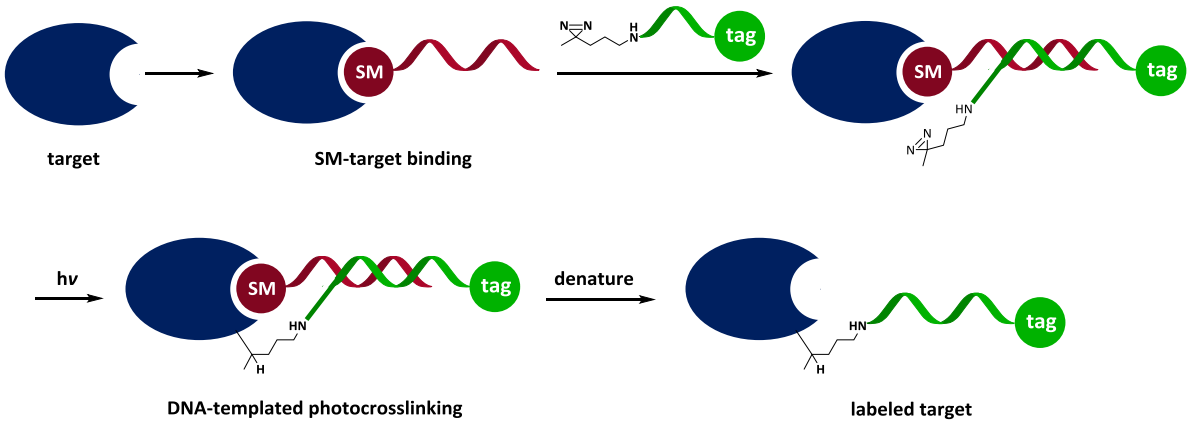
The LIU group could contribute a good deal of different bond-forming reactions that allowed the ligation of a leading and a lagging strand.[75]

In 1996, the JOYCE group introduced a novel idea. A peptide acyl chain was transferred in a nucleic acid templated ligation reaction from one lagging strand fragment to the other (Scheme 9).[76]



Scheme 9. Nucleic acid templated transfer reaction.

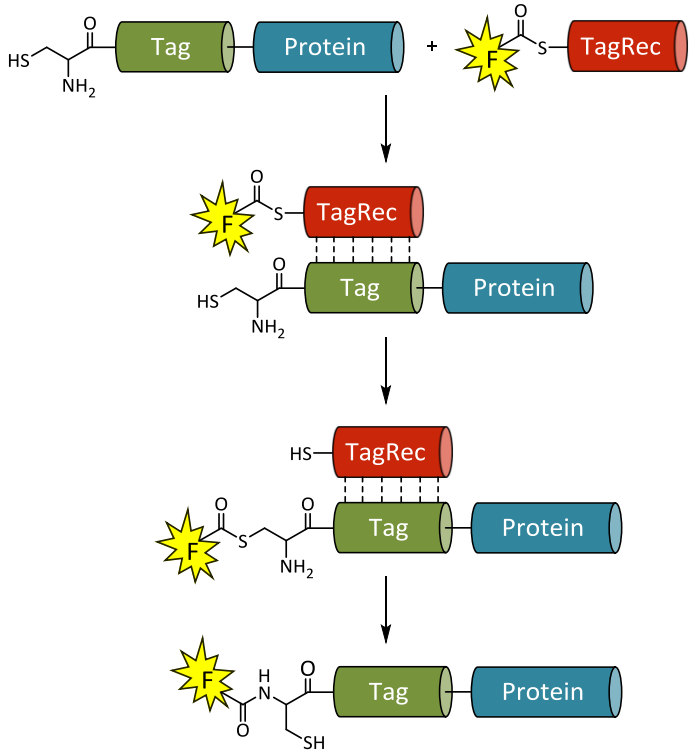
Within the last decade, chemists adapted this concept in order to expand the spectrum of template-directed reactions and to give more importance to it. This concept revealed a broader field of possible applications. Thus, it was possible to synthesize a bioactive peptide by a template-directed transfer reaction that can disrupt the caspase-9-XIAP interaction and therefore activate it. Caspase-9 is a protease that is involved in the initiation of apoptosis.[77] The LI group took the concept to the next level by using DNA-templated reactions to label small-molecule(SM)-binding proteins (Scheme 10).



Scheme 10. DNA-templated photoaffinity labeling of SM-binding proteins (adapted from [78]).

In their approach, small molecules were equipped with a DNA strand and brought together with SM-binding proteins. Then, a complementary DNA strand bearing a photoreactive group on the one side and a label on the other side was added. DNA/DNA base pairing arranged the photoreactive group near the protein and via irradiation a covalent bond to the protein was formed. After denaturation, the labeled protein was obtained.[78] In a similar but simplified approach, the HAMACHI group also utilized the specific protein-ligand interactions of SM-binding proteins and potent inhibitors. The small molecule was equipped with a cargo containing a tosylate linker that is prone to nucleophiles like thiols. Once the small molecule binds to the protein, a cysteine on the protein surface can attack and the cargo is transferred to the protein.[79]

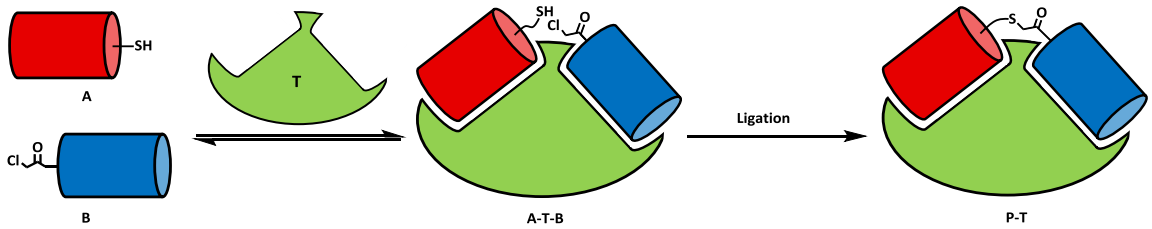
Besides nucleic acid templates, peptidic templates have been established. Alongside some extraordinary reports like the self-replication on amphiphilic β-sheet peptides,[80] coiled-coil motifs have been proven to be powerful templates.[81] This has also been demonstrated by the SEITZ and BECK-SICKINGER groups, who used a peptide-templated transfer reaction to label live cells (Scheme 11).



Scheme 11. Peptide-templated acyl transfer reaction for the labeling of membrane proteins (adapted from [82]).

Therefore, they equipped a membrane protein *N*-terminally with a genetically encoded peptide tag and a cysteine. Then, a fluorophore connected via a thioester bond to a

peptidic recognition tag (TagRec), was added. After coiled-coil formation, the fluorophore could be transferred to the membrane protein, and the cell therefore labeled.[82] Later on, they could expand and optimize the method.[83] Another remarkable example for template-directed ligations was introduced by the GROSSMANN group. They used the KIX domain of the CREB binding protein, which is known to bind two peptides close to each other simultaneously, as a template and therefore presented the first protein-templated ligation strategy. Two designed peptides could be ligated in a test reaction. Also, it could be shown that these peptides can be used as linkers and, when one peptide was fused to a protein and the other to a fluorophore, protein labeling was possible with this concept (Scheme 12).[84]

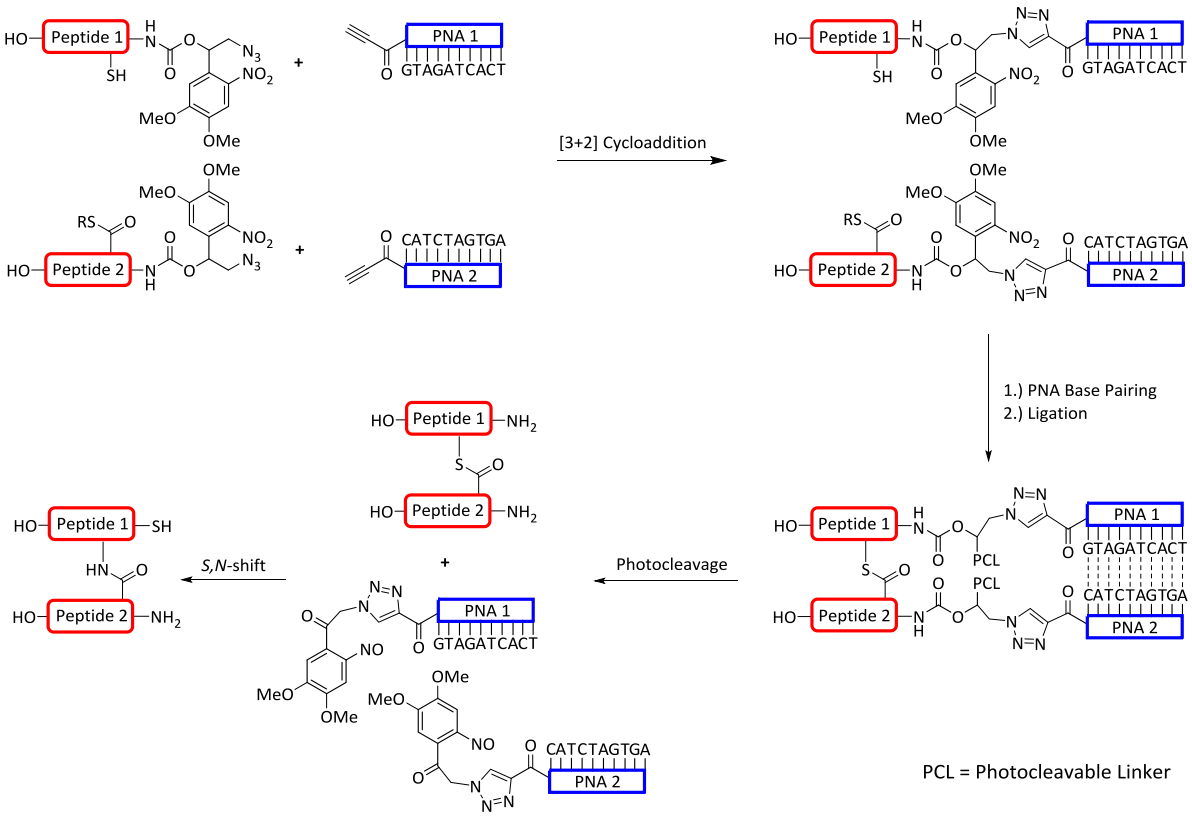


Scheme 12. Protein-templated peptide ligation (adapted from [84]).

These examples illustrate the development of template-directed reactions and their possible applications. Despite of the notable progress in this field of research, a very limited set of accepted reactants, the difficult cleavage of the template or an overly great distance between the ligated products, caused by long linkers, are still significant problems that inhibit the exploitation of its full potential.

1.3 Project Outline

Within the scope of this thesis, a novel template-directed ligation strategy shall be established. Due to their easy accessibility, modularity, high affinity, chemical stability and low mismatch tolerance, PNA based templates are chosen for this method.[85] Additionally, a photocleavable linker is envisioned to connect the template to the ligation partners. This offers the possibility to release the templates after the ligation. For the actual ligation reaction, native chemical ligation (NCL) is picked, since it is well established in peptide and protein chemistry and provides a natural amide bond.[86] The concept is displayed in Scheme 13.



Scheme 13. A novel ligation strategy applying photocleavable PNA strands as guiding units.

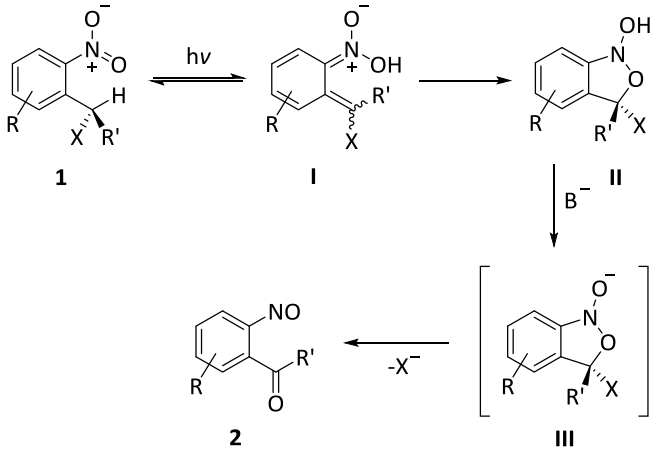
A nitrobenzene compound is chosen as the linker, since these compounds are known to have suitable photochemical properties[87] and have already been used as caging groups for peptides.[88] After attachment to a peptide, an implemented azide function in the linker allows the synthesis of PNA/peptide hybrids via copper-catalyzed azide-alkyne cycloaddition. When two of these hybrids are brought together, PNA-PNA recognition brings the reactive centers of two peptides in close proximity to each other, so that the

ligation of these peptides is facilitated. After the ligation, the PNA strands can be cleaved by irradiation and after an *S,N*-shift, an amide bond is formed. The development and the optimization of this method will be the core of this work. In future projects, this method could be applied for combinatorial chemistry approaches, taking advantage of the modularity of the PNA templates. Moreover, some features make it an interesting method for protein modifications as well. Properly designed templates would allow multiple and selective modifications at different positions at the same time in a one pot reaction. Additionally, it is a big advantage that the guiding units can be removed after the ligation reaction as the protein activity or the interactions of the introduced cargo with other biological compounds will not be disturbed by the bulky templates.

2 Results & Discussion

2.1 Development of the Photocleavable Linker

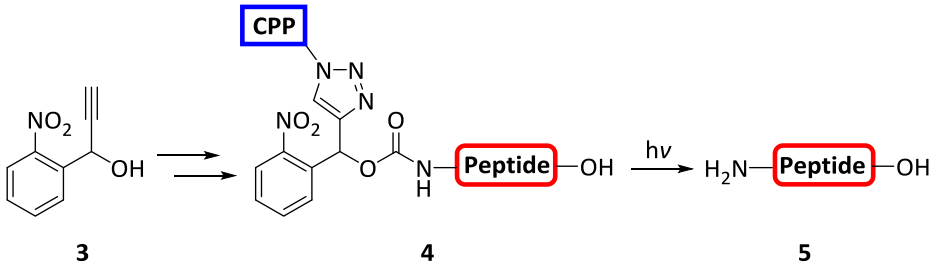
Photochemistry has become an essential part in our daily life. We are surrounded by products that are produced with the aid of photochemistry or need photochemistry to trigger their function (e.g. computer chips, skin protection or solar energy storage). Also, many biochemical processes depend on photochemical reactions (e.g. photosynthesis or vitamin D synthesis). A broad spectrum of chemical reactions can be catalyzed by photochemical processes, like rearrangement, isomerization, cycloaddition, reduction or fragmentation reactions.[89] In the last decades, photoactivatable groups became increasingly important in biochemistry.[90] This comes with the fact that photochemical reactions are clean and traceless and no additional chemicals have to be added to biological systems. These groups can be divided into three subgroups: photoreactive crosslinkers (PRC), photocleavable protecting groups (PPG) and photocleavable linkers (PCL). Photoreactive crosslinkers have been used for photoaffinity labeling of proteins.[78] Photocleavable protecting groups are used to block the reactivity of a (bio)molecule. This gives temporal and spatial control of the reactivity of the caged molecules, which e.g. could be used to analyze neurological processes. The uncaging and therefore reactivity of these molecules can be triggered by irradiation.[91] Photocleavable linkers work the other way round. They connect the molecule of interest to a helper molecule which can be cleaved after finishing its task.[88] Various different classes of photoactivatable groups are literature known, but particular attention has been paid to the *o*-nitrobenzyl groups.[90,92]



Scheme 14. Simplified mechanism of the uncaging of *o*-nitrobenzyl compounds.[93]

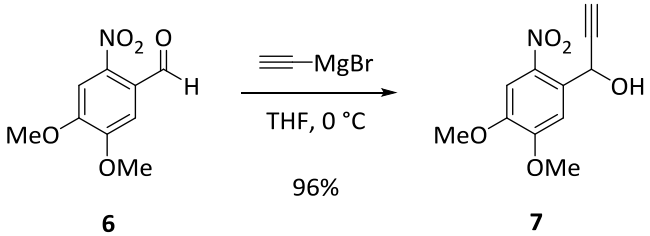
These groups are well known since they are easy to handle and still feature good quantum yields. A lot of effort has been expended in order to elucidate the reaction mechanism and many transition states could be identified.[87,93–97] A simplified mechanism of the photoinduced uncaging reaction is shown in Scheme 14.

In the first part of chapter 2 the development of a photocleavable linker will be discussed, which was a collaboration with CORNELIA PANSE. Parts of this cooperation have already been published in her doctoral thesis.[98] As a model for the design of the photocleavable linker served the work of FRANK ROSSI.[88] He used nitrobenzene compound **3** to link a cell penetrating peptide (CPP) to a bioactive peptide (**4**). After entering the cell, the bioactive peptide was uncaged by irradiation with UV light (Scheme 15).



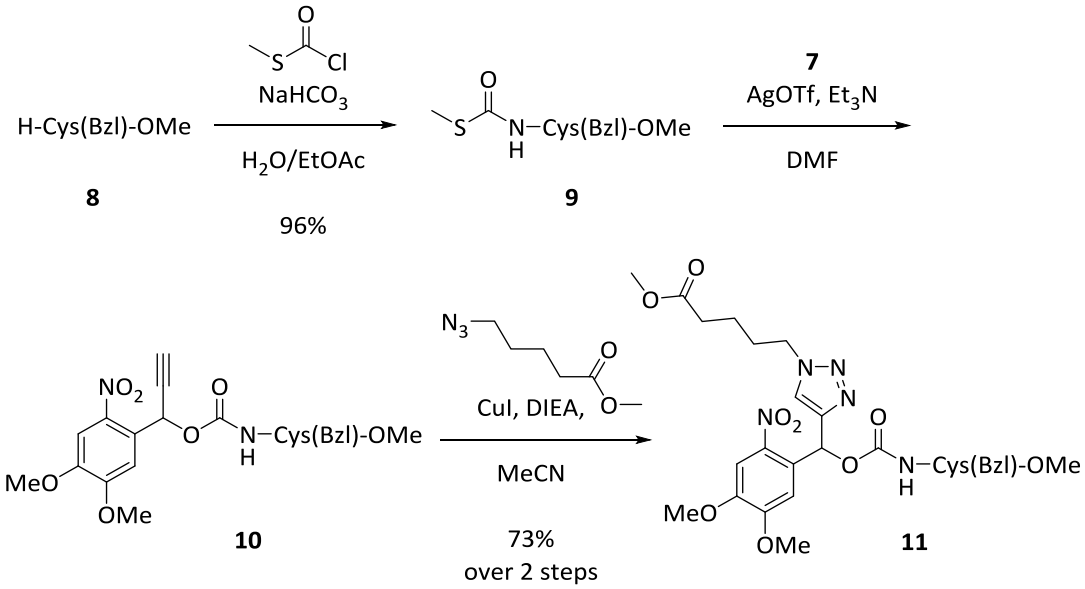
Scheme 15. A modular synthesis of photocleavable peptide conjugates.[88]

Unfortunately, ROSSI's PCL has an absorption maximum at 260 nm, a wavelength which is also absorbed by aromatic amino acids and especially by nucleobases of PNA. This might cause side product formation during the photocleavage. In order to avoid this, two methoxy groups were introduced to the aromatic ring, since it could be shown that increasing the electron density in the aromatic ring leads to a shift of the absorption maximum to a longer wavelength.[99] The photocleavable linker (**7**, PCL1) could be synthesized in one step in a GRIGNARD reaction of 6-nitroverataldehyde (**6**) with ethynylmagnesium bromide (Scheme 16). The same reaction conditions as for ROSSI's unsubstituted linker **3** could be applied.[88]



Scheme 16. Synthesis of photocleavable linker 1 (PCL1).

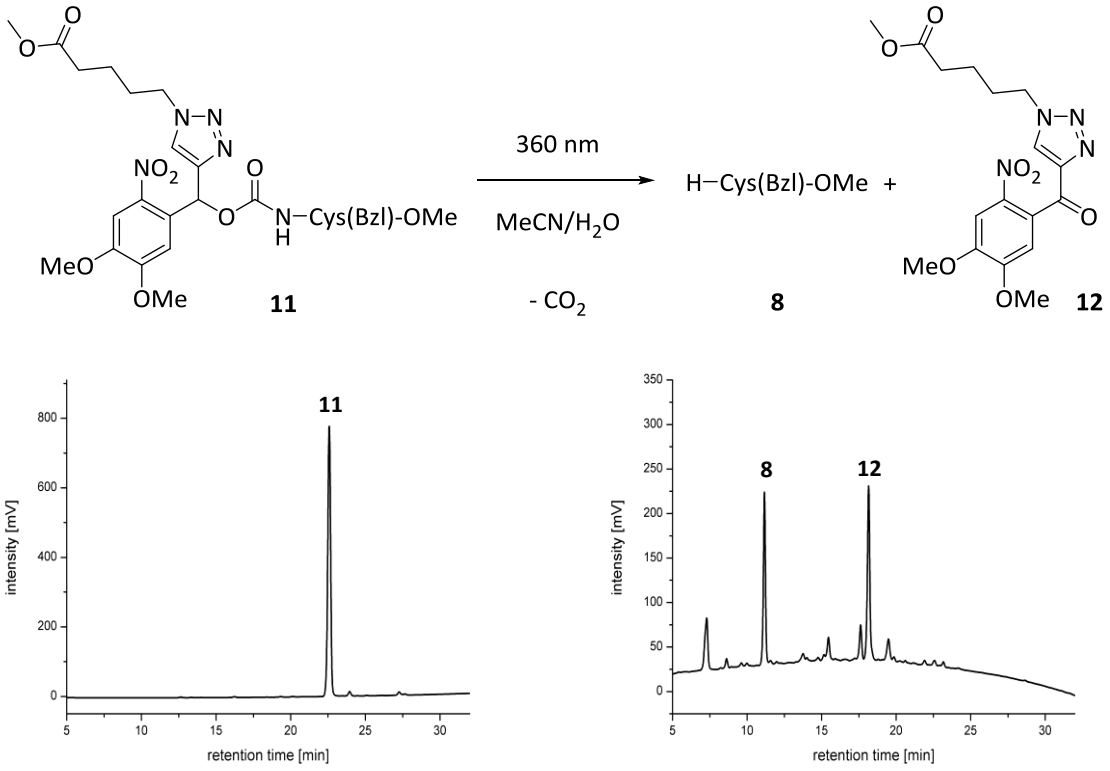
Next, it was important to activate PCL1, so that it can be attached to the *N*-terminus of a peptide. Several attempts to activate alcohol **7** failed,[98] so that a different strategy had to be used. Instead of activating PCL1, the amino group of the *N*-terminal amino acid was altered with methyl chlorothiolformate. The activation of amino acid **8** and the following attachment of PCL1 via carbamate formation proceeded in high yields (Scheme 17). So, in principle this approach depicts a useful alternative, but the lack of modularity is a significant drawback. The varying *N*-terminal amino acids have to be activated separately. In order to test the photochemical properties of PCL1, **10** was then clicked to an aliphatic azide to give compound **11**.



Scheme 17. Activation of H-Cys(Bzl)-OMe **8**, followed by attachment of PCL1 via carbamate formation and subsequent introduction of an aliphatic residue by CuAAC gave compound **11**.

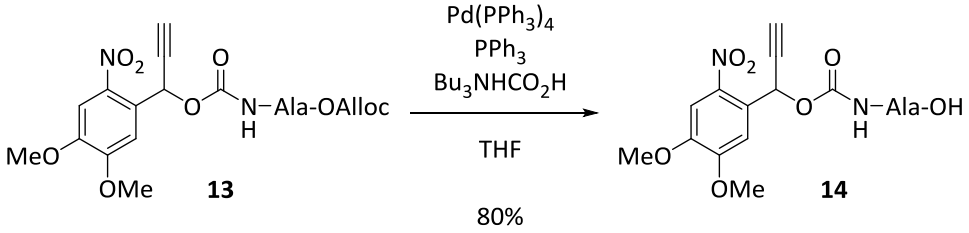
Finally, compound **11** was tested for its photochemical properties. 30 min of irradiation with UV light led to complete uncaging. Both, the uncaged amino acid **8** and the nitrobenzene compound **12** could be identified by mass spectrometry. Only little side product formation was observed, whereas the main peaks were the desired products (Scheme 18).

Unfortunately, PCL1 turned out to not be stable under acidic conditions. As a result, the amino acid that is linked to PCL1 cannot have an acid cleavable *C*-terminal protecting group because deprotection of the *C*-terminus is essential for the attachment to a peptide.



Scheme 18. Uncaging of H-Cys(Bzl)-OMe **8** after 30 min of irradiation with UV light. *Left*: HPLC chromatogram of caged compound **11** before irradiation. *Right*: HPLC chromatogram of uncaged compound **8** and **12**.

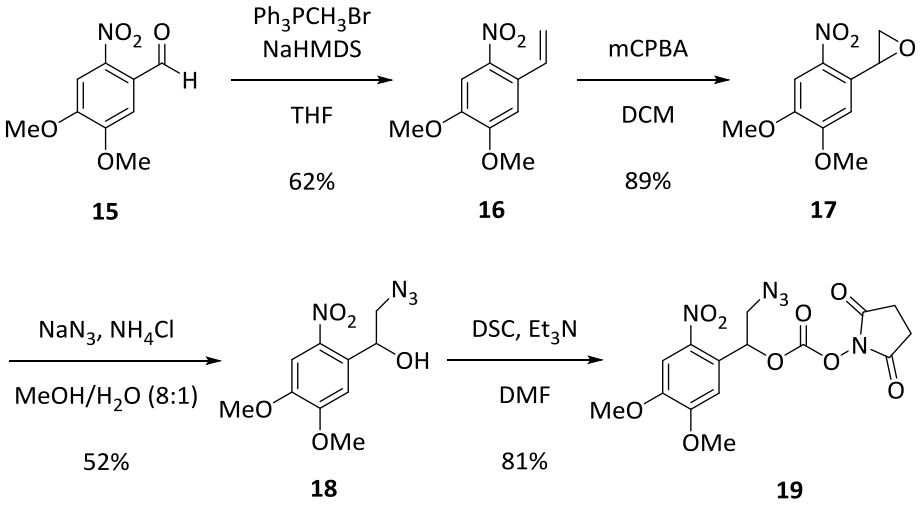
In order to attach PCL1 to an amino acid that can be used for SPPS, a different non-acid-cleavable *C*-terminal protecting group that is also orthogonal to Fmoc was needed. Therefore, the Alloc protecting group was chosen. Only very few Fmoc amino acids with a *C*-terminal Alloc protecting group are commercially available, so that for most amino acids the time consuming introduction of an Alloc protecting group would be necessary in the first place. Nevertheless, as a prove of principle it should be shown that deprotection of PCL1-Ala-OAlloc (**13**) can be performed to obtain a building block that can be attached to a peptide. Indeed, Pd-catalyzed Alloc deprotection led to **14** with a yield of 80% (Scheme 19).



Scheme 19. Pd-catalyzed Alloc deprotection.

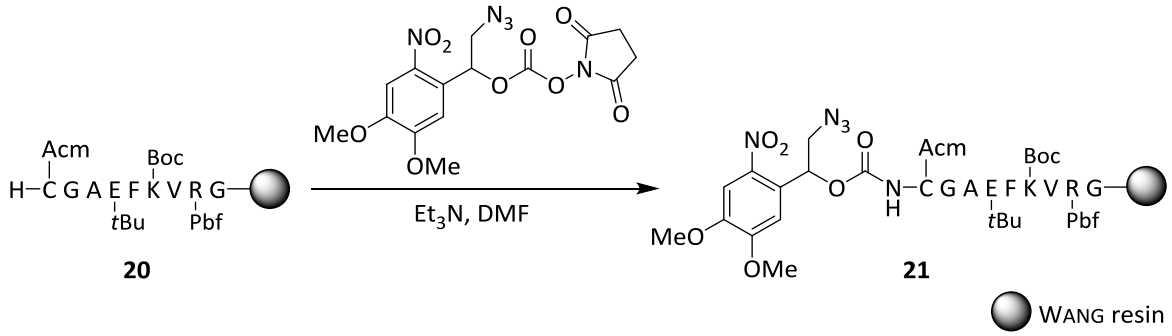
Building block **14** would now be ready for incooperation into a peptide chain. Then, CuAAC could be applied on-resin and afterwards the product could be cleaved from the resin, since it could be shown that PCL1 is stable under acidic conditions as soon as the triazole was formed.[98] So, in conclusion, a photocleavable linker (PCL1) with suitable photochemical properties could be synthesized. The design of the linker itself was simple, but the attachment to an amino acid turned out to be very complicated. Especially, because standard Fmoc amino acids cannot be used and the reaction steps have to be repeated for each different *N*-terminal amino acid. This makes the use of PCL1 generally possible, but unfunctional.

As a consequence, a new linker with an azide function instead of an alkyne, which was first conceived by CORNELIA PANSE, was synthesized.[98] The initial design was based on a linker described by VAN DER DONK, with the difference that two methoxy groups were added in order to shift the absorption maximum to a higher wavelength.[100] Thus, copper-catalyzed click chemistry can still be applied to build PNA/peptide hybrids. 6-Nitroverataldehyde (**15**) served as the starting material and was consumed in a WITTIG type reaction to give alkene **16** in 62% yield. In the next step, epoxidation with mCPBA was performed and epoxide **17** was obtained with 89% yield. Afterwards, nucleophilic ring opening of the epoxide with sodium azide was performed. Both regioisomers were obtained in a 2:1 mixture in favor of the desired product **18**. Now, it was possible to activate the photocleavable linker 2 (PCL2) with *N,N'*-disuccinimidyl carbonate (DSC), giving **19** in 81% yield (Scheme 20).



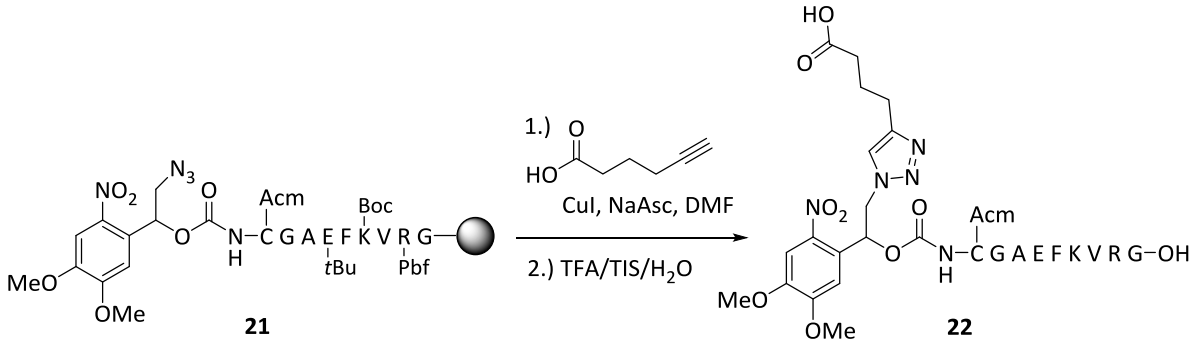
Scheme 20. Synthesis and activation of photocleavable linker 2 (PCL2).

The synthesis and activation of PCL2 could be achieved in a considerable amount of time on gram scale. The possibility of activating PCL2 is an essential advantage. Building block **19** could now directly be reacted with the free *N*-terminus of an amino acid or a peptide. A complicated activation process was not necessary anymore, making PCL2 a convenient linker. First of all, the uncomplicated attachment of PCL2 to peptide **20** was demonstrated. The reaction occurred on solid support in DMF with a small excess of PCL2 and triethylamine as an additive (Scheme 21). Complete conversion was monitored after 12 h. Since the reaction proceeded without significant side product formation and was carried out on solid support, no complex purification steps were necessary. Test cleavage of caged peptide **21** also showed that PCL2 was stable under highly acidic conditions (95% TFA).



Scheme 21. Attachment of PCL2 to peptide **20**.

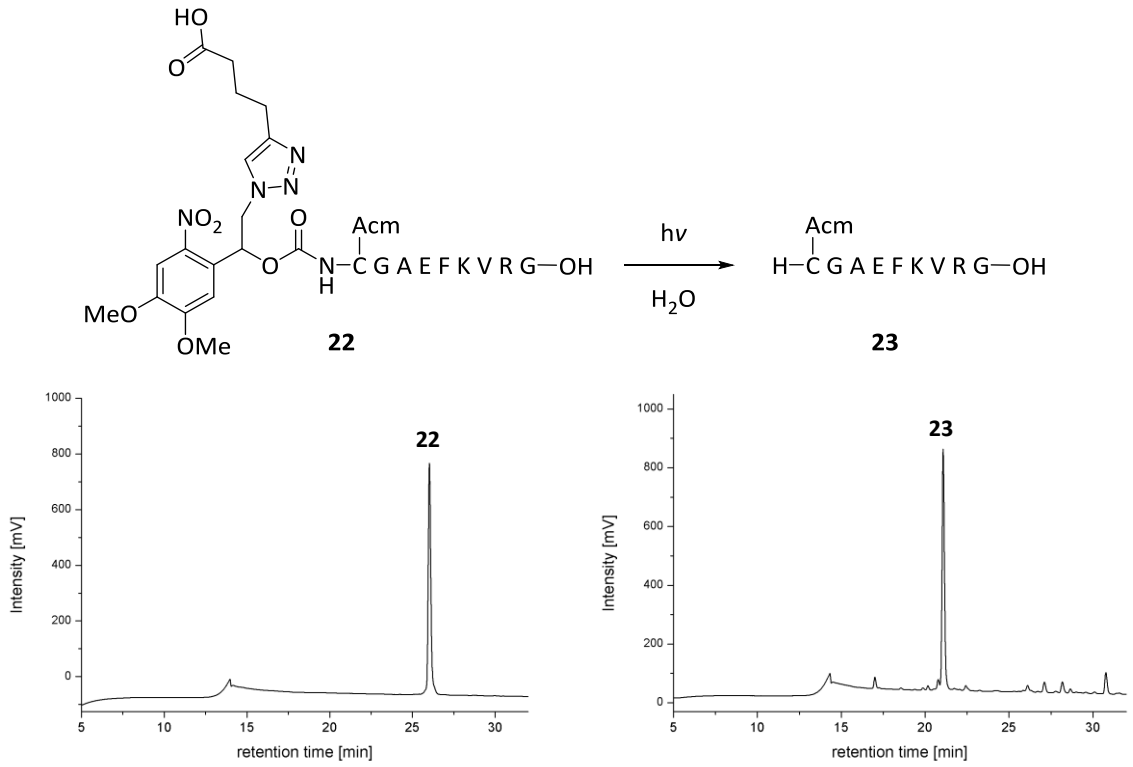
In the following step, 5-hexynoic acid was clicked to **21** via CuAAC on-resin (Scheme 22). This reaction again proceeded without significant side product formation.



Scheme 22. Triazole formation by CuAAC, followed by cleavage from resin.

Despite of its structural resemblance to PCL1, the photochemical properties of PCL2 should be tested again. Therefore, **22** was dissolved in water and irradiated with UV light. Complete uncaging could be accomplished after 1 h. The reaction proceeded in a very clean manner, so that beside the uncaged peptide **23** and the cleaved caging group no

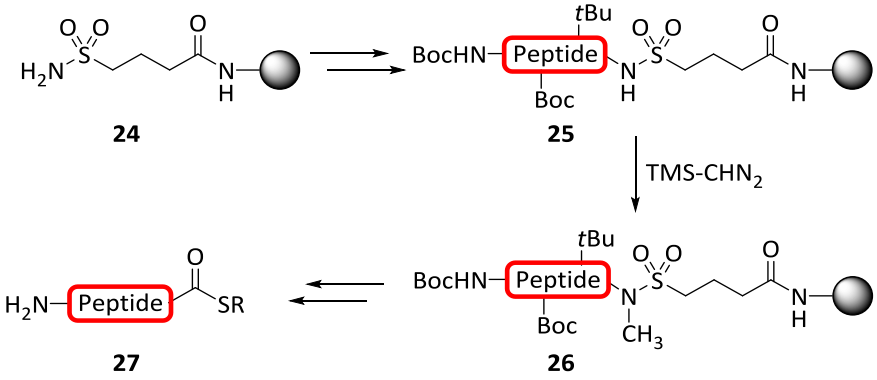
considerable peaks were found in the HPLC chromatogram (Scheme 23). This proved the usability of PCL2, which was therefore used from now on.



Scheme 23. Uncaging of peptide **22** after 1 h of irradiation with UV light. *Left*: HPLC chromatogram of caged compound **22** before irradiation. *Right*: HPLC chromatogram of uncaged peptide **23**.

2.2 Synthesis of the Thioester Precursor

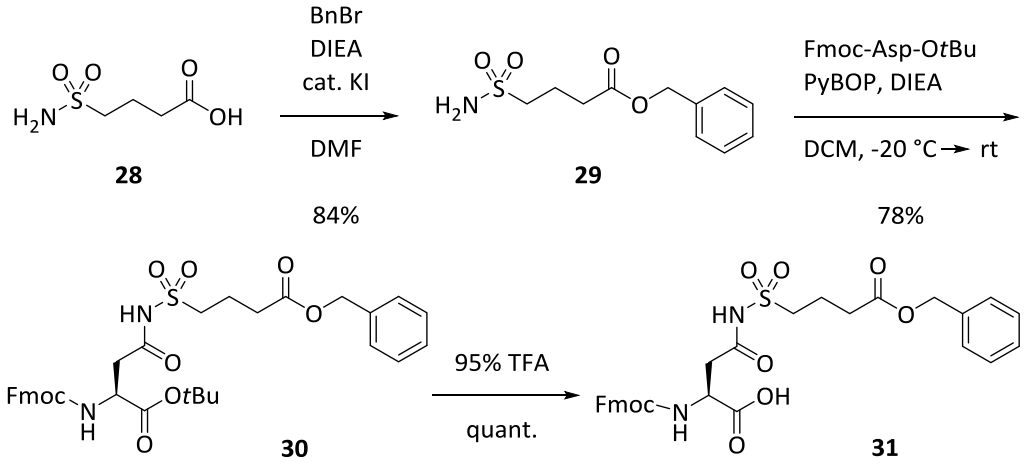
One reactant for the native chemical ligation is a thioester. Incorporating a thioester into a peptide chain by Fmoc-SPPS is not trivial, since thioesters are prone to basic conditions used during the deprotection steps. Thus, different ways are necessary in order to obtain the desired thioester. While biochemists often use protein splicing technologies to equip their POI with a thioester,[101] chemists had to find different concepts for synthetic approaches. A common approach makes use of precursors, which can be selectively transformed into thioesters after peptide synthesis. However, it turned out that most of them suffer some significant drawbacks, such as difficult synthesis or attachment of the precursors, low turnover rates or racemization.[102–107] A completely different strategy was used by MACMILLAN, who forced an *N,S*-shift of an internal cysteine in a peptide to the thioester form and after transthioesterification could isolate the corresponding thioester.[108] Nevertheless, very harsh reaction conditions were necessary to make this happen. However, a different method is widely used and established that uses a special linker on the solid support. The safety-catch linker was first introduced by KENNER and allows, according to a specific cleavage protocol, to cleave the peptide with a *C*-terminal thioester from resin (Scheme 24).[109,110]



Scheme 24. After resin loading and peptide synthesis, the linker can be activated by alkylation. Cleavage from resin can then be achieved by adding a nucleophile.

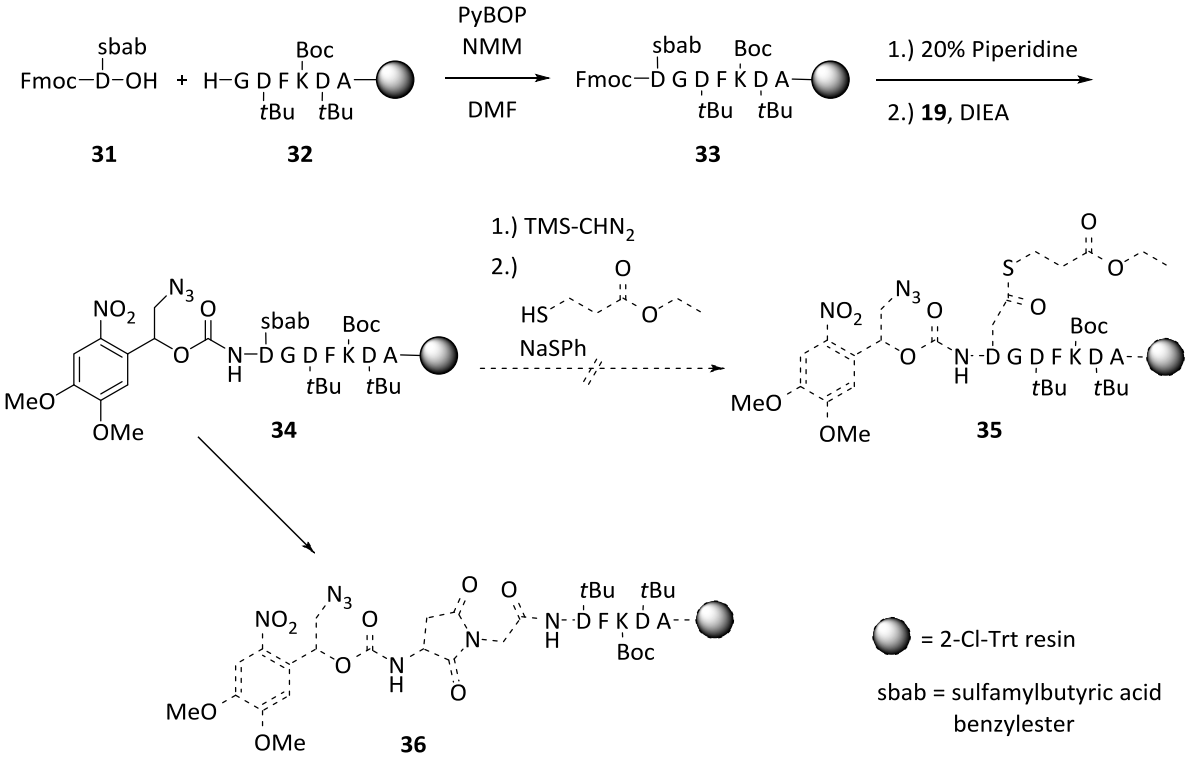
Certainly, this and all other discussed examples only lead to *C*-terminal thioesters. This already indicates that creating a thioester at the side chain of an amino acid, which is necessary for the goal of this project, is even more challenging. First of all, it was tried to adopt the concept of the safety-catch linker and design a sulfonamide side chain protecting group.

For this purpose, a sulfonamide was introduced to the side chain of aspartic acid. Starting from the commercially available 3-carboxypropanesulfonamide (**28**), the carboxyl group was protected as a benzyl ester in the first step, followed by the coupling to the side chain of Fmoc-Asp-O*t*Bu. Finally, the *tert*-butyl group of **30** was removed by acidic cleavage. Thus, **31** was obtained in only three steps with an overall yield of 66% and was ready for SPPS (Scheme 25).



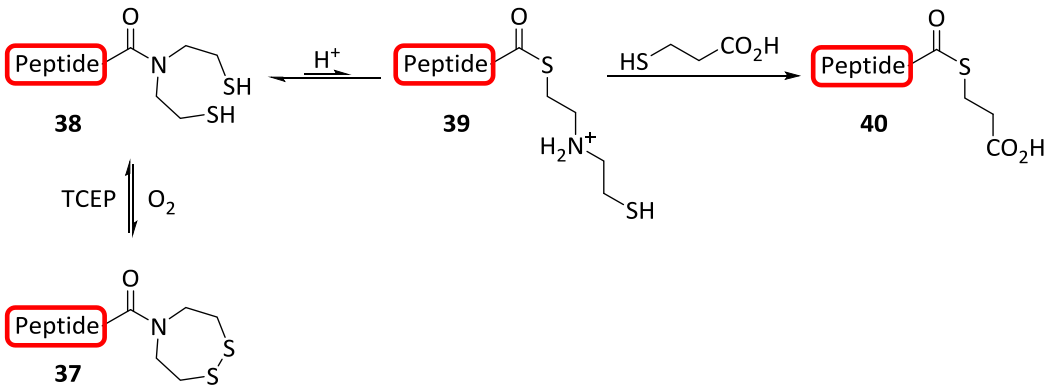
Scheme 25. Synthesis of Fmoc-Asp(sbab)-OH (**31**).

Next, the new building block was attached to a peptide (**32**) by SPPS. Here, it was necessary to screen different coupling conditions, since standard coupling conditions did not provide an adequate result. At last, coupling with PyBOP as activator and NMM as activator base in DMF led to a satisfying result (**33**). Also, subsequent Fmoc-cleavage and attachment of PCL2 was accomplished to give **34**. Then, transformation into the corresponding thioester was tested. Therefore, **34** was treated with trimethylsilyldiazomethane (TMS-CHN_2) as an alkylation reagent to activate the sulfonamide group. After alkylation, the sulfonamide is prone to nucleophilic attack. So, treatment with a thiol should lead to the corresponding thioester (Scheme 26). However, it was found that the reaction did not work as predicted. MS/MS studies revealed that after treatment with TMS-CHN_2, the sulfonamide group was so reactive that it already reacted with a neighboring amide group which led to cyclization. Since this reaction occurs *in situ*, it was not possible to isolate the intermediate and therefore, building block **31** turned out not to be a useful thioester precursor for this purpose.



Scheme 26. Incorporation of **31** into a peptide followed by activation of the sulfonamide group leads to cyclization and therefore prevents thioester formation.

In the meantime, a thioester precursor that can also be used as an aspartic or glutamic acid side chain protecting group, was presented by MELNYK.[111] He first described the bis(2-sulfanylethyl)amido (SEA) ligation in 2010.[112] Scheme 27 shows the concept of the SEA group.

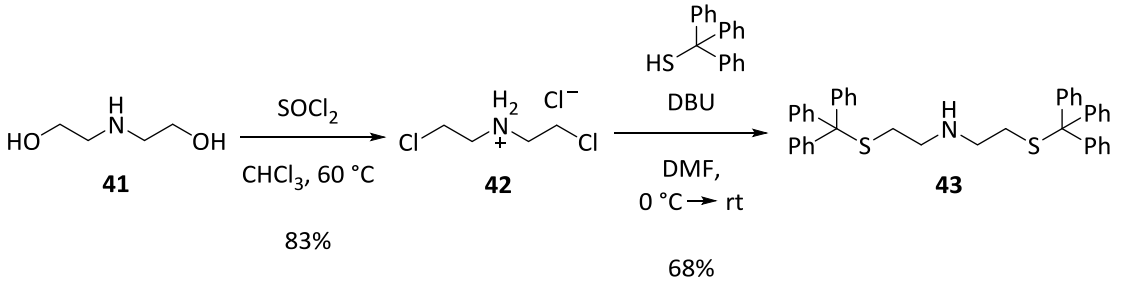


Scheme 27. Activation of SEA^{off} (**37**), followed by *N,S*-shift and transthioesterification.

The activation of this precursor takes place under reductive conditions, so that in principle, it can be used directly for native chemical ligation. Thereby, the inactive disulfide form **37** (SEA^{off}) is converted into the active dithiol form **38** (SEA^{on}). Then, an *N,S*-shift can take place and a thioester **39** is formed *in situ*. The equilibrium of the reaction still lies on the

side of amide **38**, but thioester formation is way more favored compared to an internal peptidic cysteine. One reason is that there are two thiols and therefore one is always in the needed *cis* conformation. On the other hand, a peptidic cysteine has usually *trans*-conformation, so that an energy-costly *trans-cis* isomerization must take place first. Secondly, in contrast to an *N,S*-shift in an internal peptidic cysteine, not a primary but a secondary amine is formed, which can be protonated and therefore trapped much more easily. Additionally, transthioesterification can help by withdrawing **39** from the equilibrium. However, MELNYK reported that an effective ligation is possible without using additives.[113] The *N,S*-shift is strongly pH dependent but even at pH 7 acceptable reaction rates could be measured.[114]

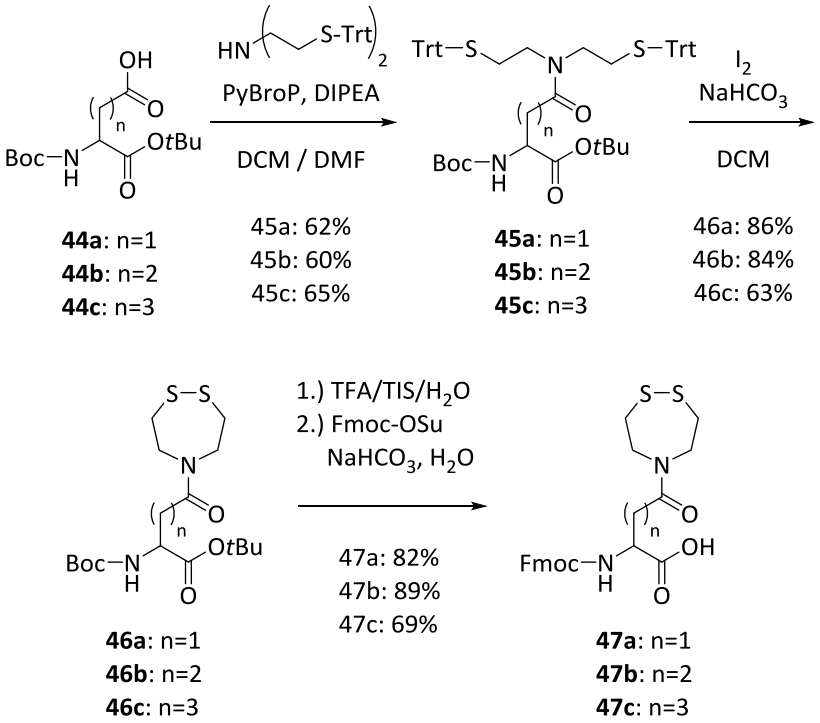
In the context of this thesis, aspartic acid, glutamic acid and 2-aminoadipic acid (Aad) were modified with the SEA group at the side chain. Therefore, at first bis({2[triphenylmethyl]sulfanyl}ethyl)amine (**42**) needed to be synthesized. Bis(2-hydroxyethyl)amine (**41**) was converted with $SOCl_2$ to bis(2-chloroethyl)amine (**42**) via an S_Ni-mechanism with a yield of 83%. Then, **42** was reacted with triphenylmethanethiol and DBU in an S_N2 reaction to give **43** in 68% yield (Scheme 28).



Scheme 28. Synthesis of bis({2[triphenylmethyl]sulfanyl}ethyl)amine **43**.

This compound could now be used for side chain modification of the acidic amino acids (Aaa). The modifications of aspartic acid and glutamic acid were already literature known and only few minor alterations to the established procedures were made.[111] The synthesis of Fmoc-Aad(SEA^{off})-OH **47c** was new, but similar reaction conditions were used. In the first step, **43** was coupled to Boc-Aaa-O*t*Bu (**44**). Coupling a secondary amine is more demanding than coupling a primary amine due to sterical hindrance. As a consequence, standard coupling reagents could not be used. The thereby formed active esters are too bulky, which is why PyBrOP was used as a coupling reagent. This reagent also forms an active ester in the first place, which is then converted to an acid bromide *in situ*.[115] With

this strategy, moderate yields could be accomplished. The next step was an intramolecular ring formation by oxidative cleavage of the trityl groups with iodine. Good yields were obtained. Finally, deprotection and protection steps followed to give **47** in good yields (Scheme 29).



Scheme 29. Side chain modifications of different acidic amino acids with the SEA group.[111]

So, the different Fmoc-Aaa(SEA^{off})-OH **47** were obtained in three steps with overall yields between 29% and 45%. In the SEA^{off}-state, the amino acids can be coupled under standard SPPS conditions.

2.3 Synthesis of PNA/Peptide Hybrids

Peptide nucleic acid (PNA) is a DNA mimic consisting of a pseudopeptidic backbone and the corresponding nucleobases. Therefore, it combines recognition and structural properties of DNA with the opportunities of peptide chemistry.[116] It was first described in 1991 and was initially designed to target double stranded DNA by binding in the major groove of a DNA double helix via HOOGSTEEN base pairing.[117] However, it was found that PNA is a better DNA mimic than expected and therefore much effort has been devoted to explore the full potential of PNA.[118] It could be shown that PNA hybridizes to complementary DNA or RNA strands by WATSON-CRICK base pairing. These hybrids bind more strongly than their DNA/DNA or RNA/RNA analogues because PNA has a neutral backbone and therefore no electronic repulsion of the backbones occurs.[119,120] Stable DNA-like double helices are formed by PNA as well. Even though PNA itself is achiral, chirality of the formed double helix can be triggered by a neighboring *C*-terminal amino acid and thus the helix adopts the same handedness as in DNA/DNA duplexes.[121] Structural and binding properties of PNA duplexes have been accurately studied.[122] Duplex formation is achieved by WATSON-CRICK base pairing of the nucleobases adenine (A), cytosine (C), guanine (G) and thymine (T) (Figure 3).

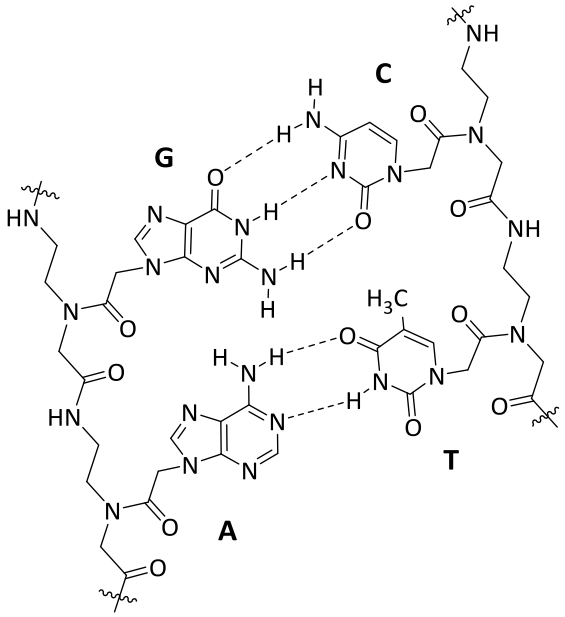


Figure 3. PNA duplex formation via WATSON-CRICK base pairing.

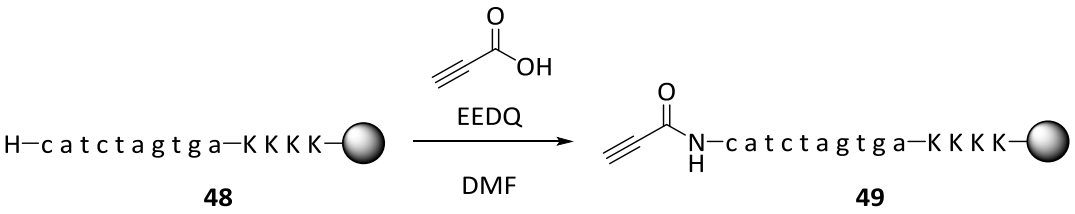
Also, the pharmacological potential of PNA has been recognized and it has gained interest in genetic diagnostics, molecular recognition and drug discovery.[85,123] For this project,

PNA is of great interest because of its stability, low mismatch tolerance and the possibility of creating nearly unlimited PNA pairs that, by proper engineering only bind with each other. For the method development, a PNA pair was chosen that was first described by NIELSEN (Figure 4).[121] With a melting temperature of 45.5 °C this PNA pair forms a stable helix with parallel strand orientation under physiological conditions.

PNA1 H–g t a g a t c a c t–OH **PNA2** H–c a t c t a g t g a–OH

Figure 4. The PNA strands that were used for this project.

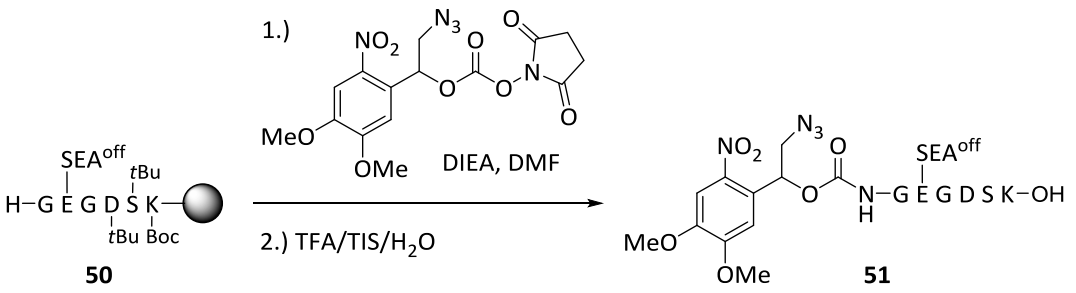
The *C*-termini of PNA1 and PNA2 were equipped with 1-4 lysines in order to enhance the solubility. For the solid support, a RINK amide MBHA resin was chosen, with the result that after cleavage from resin a neutral *C*-terminal amide is obtained. The PNA strands were coupled manually following an optimized protocol (see Experimental Part). Then, the *N*-terminus was functionalized with an alkyne moiety, so that CuAAC can be applied to attach a PCL2-peptide to the PNA strand. Propiolic acid was predestined as the alkyne source. Due to the fact that the alkyne terminal proton is very acidic and deprotonation led to significant side product formation, standard coupling conditions, including base activators, could not be applied here. Hence, different base-free coupling conditions were screened, whereas coupling with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) gave the best results. Indeed, no side product formation was observed and even very small excess of reactant and activator (both 1.2 eq.) led to complete product formation. This reaction was performed on solid support (Scheme 30).



48 **49**

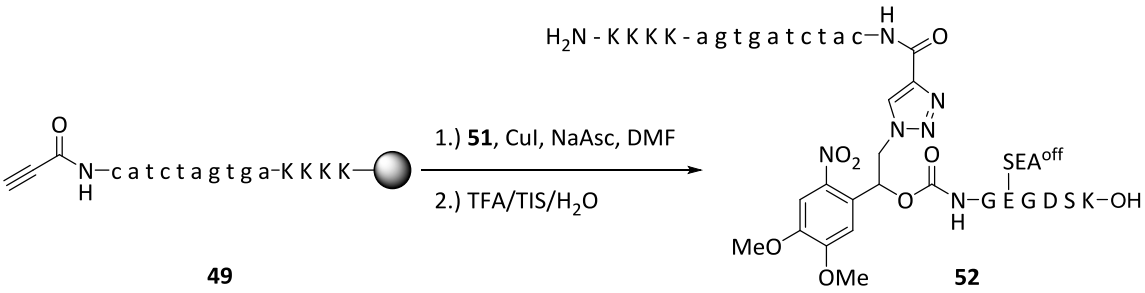
Scheme 30. *N*-terminal functionalization of PNA2 with propiolic acid and EEDQ on solid support (a, c and t nucleobases are protected with Bhoc, lysine side chains are protected with Boc protecting groups).

Peptide **50**, including coupling of Fmoc-Asp(SEA^{off})-OH (**47a**), was synthesized under standard Fmoc-SPPS coupling conditions. Activated PCL2 (**19**) was then attached on resin to give **51** in an excellent purity. Thus, after cleavage from resin, it could be directly used for the next step without further purification (Scheme 31).



Scheme 31. Synthesis of PCL2-SEA peptide **51**.

In the next step, the PNA/peptide hybrid was synthesized. Different reaction conditions for copper-catalyzed alkyne-azide cycloaddition were screened. The most straight forward method was using CuI as copper source and sodium ascorbate as an additive in DMF directly on solid support. Due to the electron-poor and therefore relatively unreactive alkyne, the reaction time was comparatively long. Nevertheless, after 48 h the starting material was consumed and the product was obtained in good purity (Scheme 32).



Scheme 32. Synthesis of PNA/peptide **52** by on resin CuAAC.

It is noteworthy, that during the whole synthesis of the PNA/peptide hybrid only one HPLC purification step was necessary. The complete synthesis was optimized to the point that it was possible to synthesize the hybrids in a reasonable amount of time. The synthesis of PCL2 and the SEA amino acid can be performed in gram scale, so that one batch lasts for many hybrids. The reactions on resin all proceed in a very clean manner, so that good yields can be obtained. To clarify this, HPLC chromatograms of all important on-resin reactions are summarized in Figure 5. It is important to mention that PCL2 was synthesized as a racemic mixture and the PNA/peptide hybrids therefore have a diastereomeric character. That is why they sometimes happen to appear as double peaks in HPLC chromatograms.

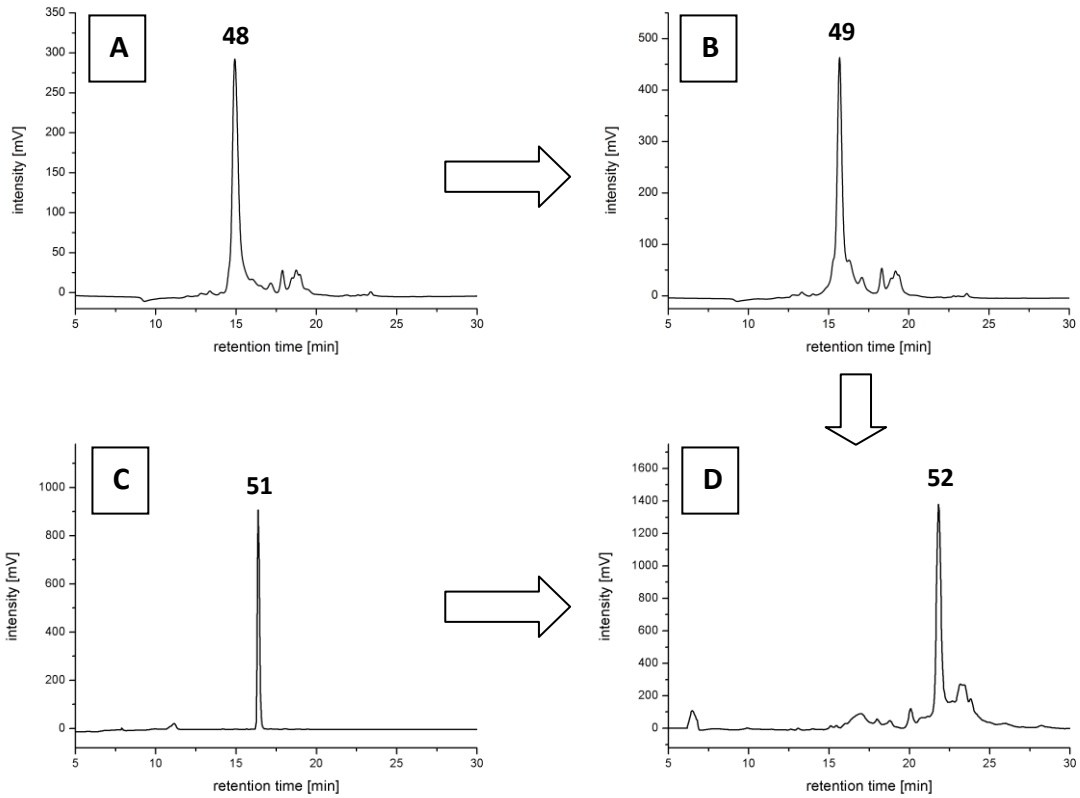


Figure 5. HPLC chromatograms of crude products throughout the PNA/peptide hybrid synthesis. *A*: Chromatogram of H-PNA2-KKKK-NH_2 (**48**). *B*: Chromatogram of **49** after *N*-terminal modification of **48** with propiolic acid and EEDQ. *C*: Chromatogram of PCL2-peptide **51**. *D*: Chromatogram of the final PNA/peptide hybrid **52** after CuAAC.

Following this pathway, a good many of different PNA/peptide hybrids have been synthesized within the scope of this thesis. The most important ones are summarized in Table 1. Having these PNA/peptide hybrids in hand, it was important to demonstrate that the bulky residues do not influence the base pairing abilities.

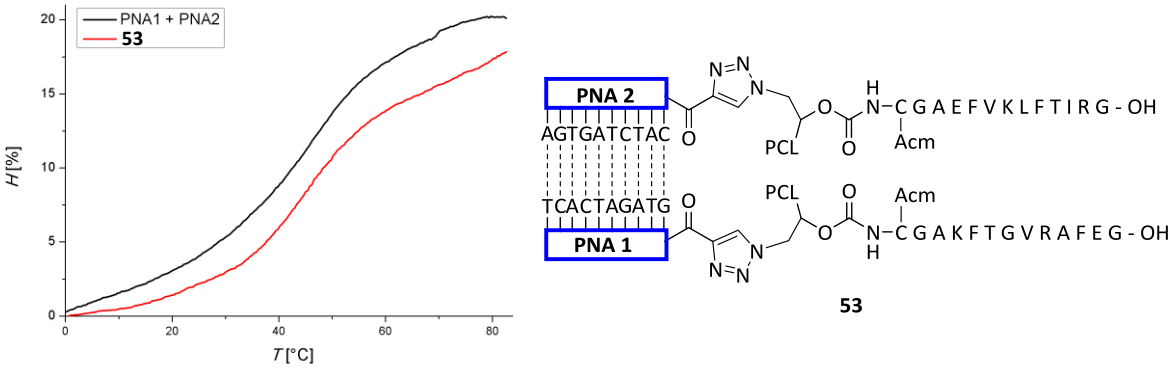


Figure 6. Melting curves of PNA1 and PNA2 compared with PNA/peptide hybrids **53** (both 46 °C).

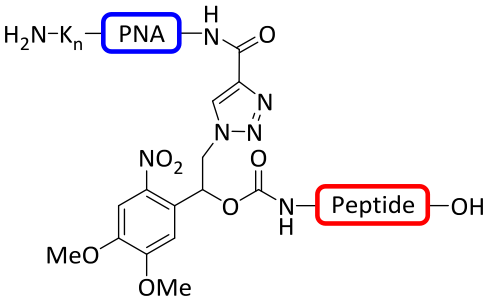
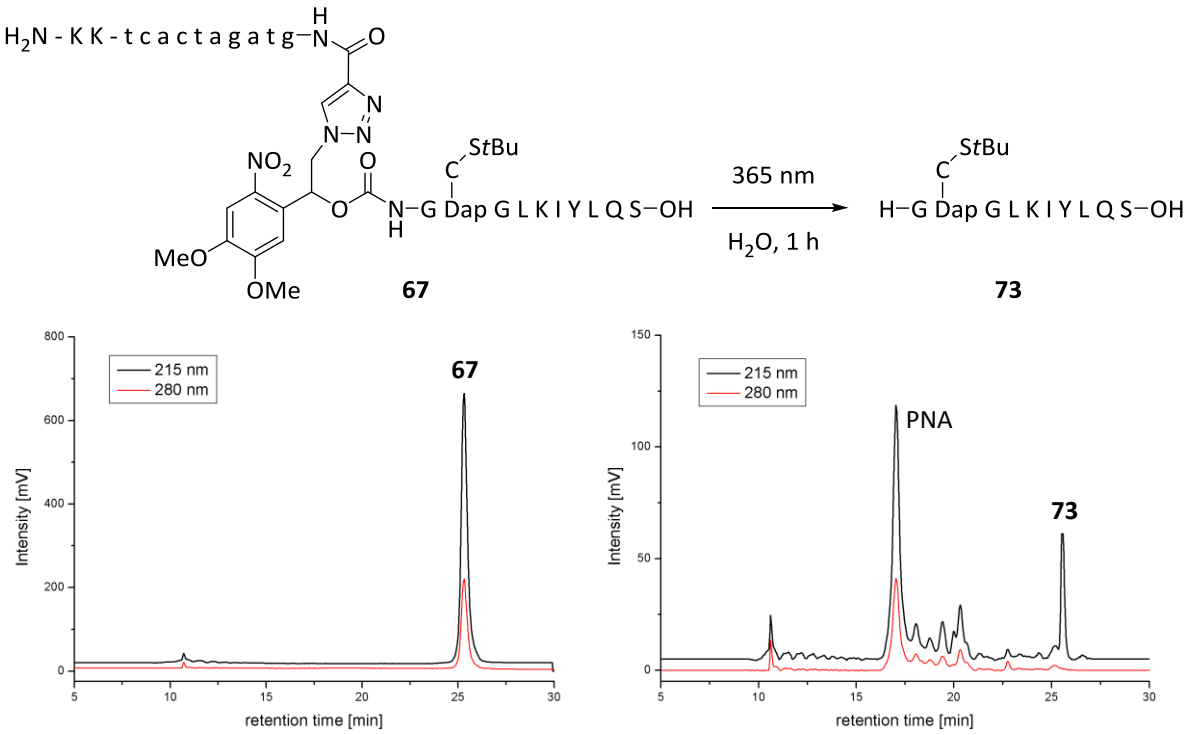


Table 1. Most important synthesized PNA/peptide hybrids are highlighted.

Entry	No.	n	PNA	Peptide
1	**52**	4	2	G E(SEA^{off}) G D S K
2	**53a**	1	1	C(Acm) G A K F T G V R A F E G
3	**53b**	1	2	C(Acm) G A E F V K L F T I R G
4	**54**	1	2	D(SEA^{off}) G D F K D L
5	**55**	1	1	C(S*t*Bu) G K T L
6	**56**	1	2	E(SEA^{off}) G K E A K K K
7	**57**	1	1	Hcy G K S G K K
8	**58**	1	1	Hcy G K T L
9	**59**	2	1	Hcy G K T L
10	**60**	4	2	E(SEA^{off}) G K E A K K K
11	**61**	2	1	E(SEA^{off}) G K E A K K K
12	**62**	4	2	D(SEA^{off}) G E S F K K K
13	**63**	2	1	G K(C) G F K S
14	**64**	4	2	E(SEA^{off}) G A K K
15	**65**	2	1	G Dap(C{S*t*Bu}) G T Y V A K L F S
16	**66**	4	2	G E(SEA^{off}) A D S K
17	**67**	2	1	G Dap(C{S*t*Bu}) G L K I Y L Q S
18	**68**	2	1	G E(SEA^{off}) G D S K
19	**69**	4	2	A E(SEA^{off}) A D S K
20	**70**	4	2	A D(SEA^{off}) V S K K
21	**71**	2	1	G Dap(C{S*t*Bu}) A E Y A K L F S
22	**72**	4	2	G Dap(C{S*t*Bu}) A E Y A K L F S

Consequently, melting curves of two complementary PNA/peptide hybrids were measured, the melting temperature was defined and compared with the data from literature (Figure 6).[98,121] No significant difference can be observed comparing the melting curves (both 46 °C), which clearly shows that the PNA base pairing ability is not influenced by the bulky residues.

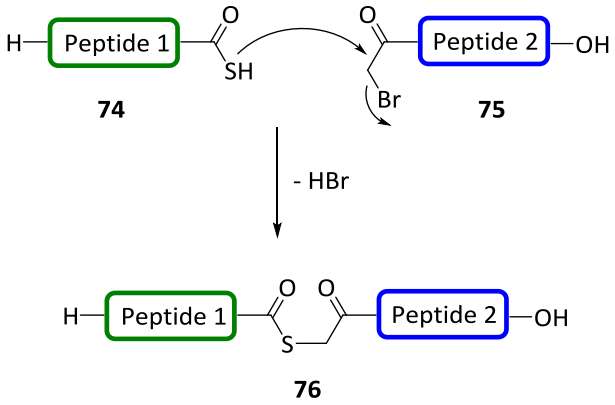
Next, the photochemistry for the PNA/peptide hybrids needed to be optimized. While uncaging of peptide **23** did undergo in a very clean reaction (Scheme 23), the same result cannot automatically be expected for the PNA/peptide hybrids. Nucleobases absorb light much better than peptides, therefore side reactions are more likely to occur.[124] Indeed, more side product formation, derived from the PNA template, was observed. Nevertheless, after 1 h of irradiation, complete uncaging was received and the main peaks were the expected products (Scheme 33).



Scheme 33. Uncaging of peptide **73** after 1 h of irradiation with UV light. *Left*: HPLC chromatogram of caged compound **67** before irradiation. *Right*: HPLC chromatogram of uncaged peptide **73**.

2.4 Template-Directed Ligation Reactions

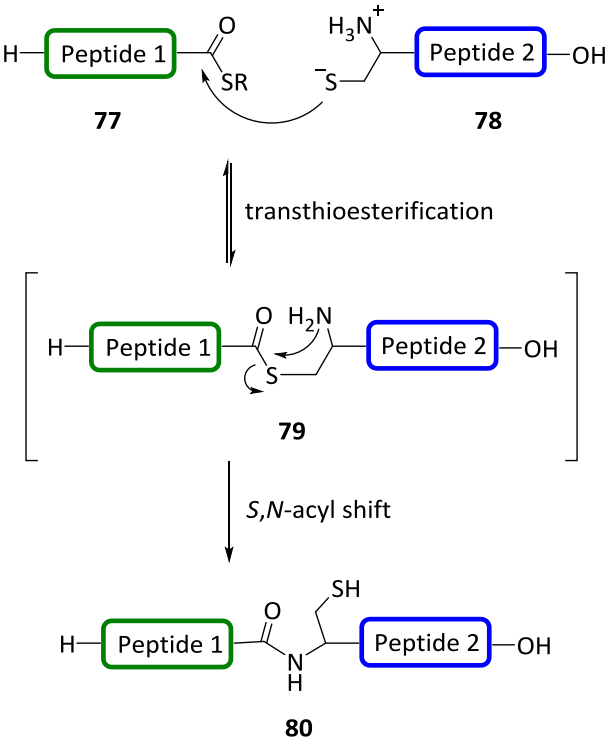
Since the introduction of the solid phase peptide synthesis by MERRIFIELD in 1963, the synthesis of medium sized peptide chains has been strongly facilitated.[18] Nevertheless, the length of these chains was still limited, so that peptide chains with about 50 amino acids could be obtained in acceptable purity by applying this method. Still, many interesting polypeptides or proteins are significantly longer. In 1992, the chain length of chemically synthesized proteins and polypeptides dramatically increased after KENT published a new method that allowed the ligation of unprotected peptide chains. For the first chemical ligation experiments he reacted two peptides, one with a *C*-terminal thioacid (**74**) and the other with an *N*-terminal bromoacetyl group (**75**), in an S_N2 reaction to form product **76** with a thioester bond (Scheme 34).[125]



Scheme 34. KENT'S chemical ligation approach.

Two years later, KENT optimized the method and called it native chemical ligation (NCL). Instead of using thioacids, thioesters were used because they react with thiols in thiol-exchange reactions. These reactions are reversible but it was found that the amino group of an *N*-terminal cysteine can attack the thioester and irreversibly form an amide bond (Scheme 35).[86] The second step is possible due to the fact that a favored 5-membered transition state is formed. This step is also responsible for the chemoselectivity of the reaction, since only *N*-terminal cysteines can undergo the irreversible *S*,*N*-shift while internal cysteines can only react in a reversible manner with thioesters.[126] The high selectivity, the easy applicability, the high efficiency, the formation of a native amide bond and the possibility to link unprotected peptide chains are the reasons why NCL has become a very important tool in protein and peptide chemistry.[14] Throughout the years, the

method could be further optimized by investigating the influence of different additives or the presence of internal cysteines.[127,128]

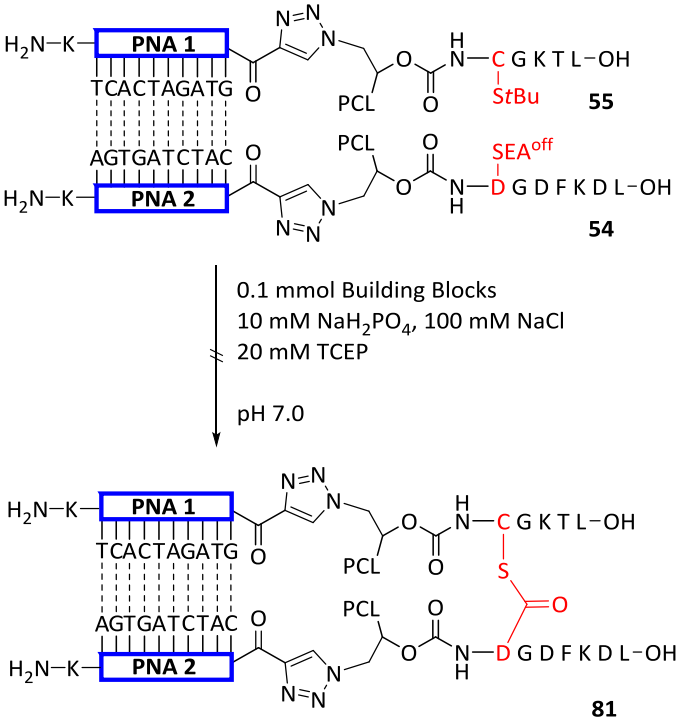


Scheme 35. Simplified reaction mechanism of the native chemical ligation.

The most stated point of criticism in the context of NCL is that an *N*-terminal cysteine is essential but the natural abundance of cysteines in proteins is very low. Therefore, in most cases the sequence of the synthesized proteins has to be altered. Even for this point solutions have been found in the last two decades. Either by converting the cysteine of the ligated product into an alanine by desulfurization[129] or into a lysine mimic by alkylation.[130] Another method is to use auxiliaries that mimic a cysteine and can be cleaved after the ligation.[131–136] Due to all these positive aspects, NCL was the method of choice for the ligation of two PNA/peptide hybrids.

2.4.1 Native Chemical Ligation Approaches

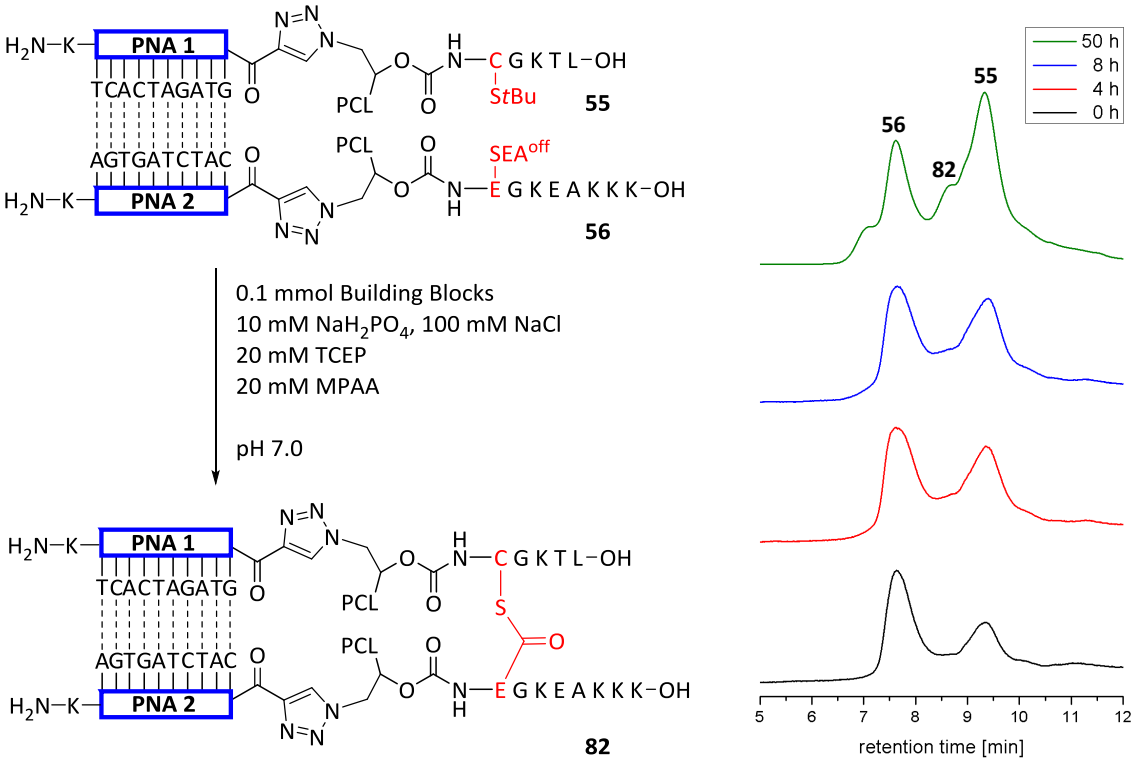
All ligation reactions that were performed within this thesis were conducted in an aqueous NaH_2PO_4 buffer with TCEP as a reducing agent. For long reactions sodium ascorbate was added as a radical scavenger in order to prevent desulfurization of cysteine or homocysteine.[137] For some ligation reactions thiol additives were used as they are known to significantly increase the reaction rate during an NCL. Two different additives were employed. On the one side, 4-mercaptophenylacetic acid (MPAA) was used, which can form aryl thioesters by transthioesterification. These thioesters are very reactive and undergo ligation reactions rapidly, but due to the high reactivity they are also prone to side reactions like hydrolysis.[127] Also, the solubility of MPAA in aqueous solution is strongly pH dependent and is very low at pH < 7. On the other side, sodium 2-mercaptoethanesulfonate (MesNa) was added which gives less reactive thioesters. The reactivity still allows good reaction rates and the *in situ* formed MesNa-thioesters are much less prone to side reactions compared to MPAA-thioesters.[138] Additionally, the solubility in water is good. The ligation reactions were monitored by LC-MS and the depicted HPLC chromatograms were recorded at 215 nm. For the first ligation reaction, Cys-hybrid **55** and Asp(SEA^{off})-hybrid **54** were synthesized (Scheme 36).



Scheme 36. Template-directed NCL reaction of **54** and **55**.

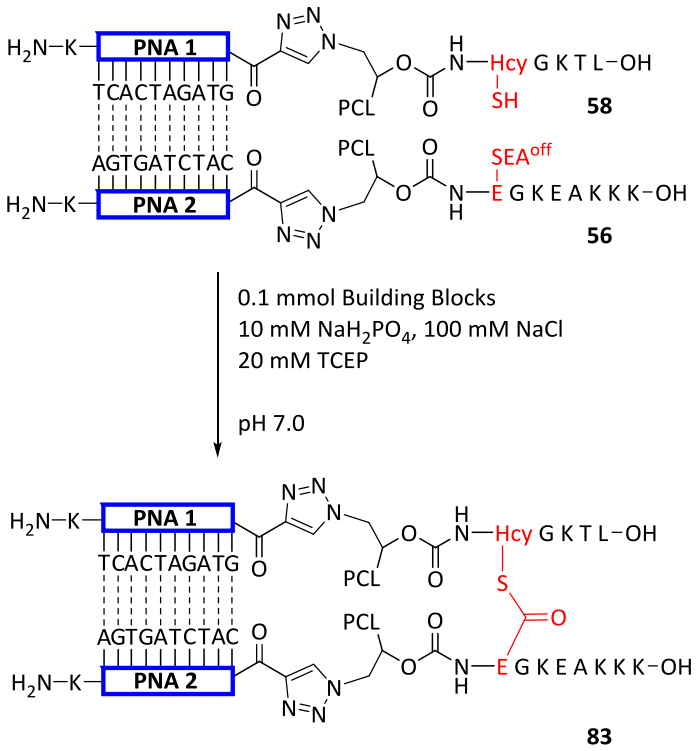
No additives were used for the reaction since it was reported that SEA^{on} itself can undergo ligation reactions.[112] Additives increase the reaction rate but it was anticipated that the template effect would absorb the reduced reactivity. However, after 24 h reaction time no product formation was detected. It was assumed that the *N,S*-shift of SEA^{on} is the rate limiting step in this reaction and therefore responsible for the negative result. MELNYK could show that the *N,S*-shift is strongly pH dependent and for this reason the reaction was repeated with a lower pH (pH 6).[112] MesNa was also added but still, even after 96 h reaction time no product was formed (Table 3, Entry 2).

Realizing that the right distance between the PNA/peptide hybrids is crucial, a new Glu(SEA^{off}) building block (**56**) with a one CH_2 unit longer side chain was synthesized. Thereafter, **56** was reacted with Cys building block **55** at pH 7 with MPAA as an additive. Finally, LC-MS analysis showed the formed product **82** (Scheme 37). After 4 h and 8 h, the HPLC chromatogram did not show a proper new peak for the product, although it could already be detected in the mass spectrum. After 50 h a new peak occurred and could be identified as the product peak.



Scheme 37. *Left-hand side:* NCL of **55** and **56**. *Right-hand side:* HPLC chromatograms of the reaction mixture at different points of time.

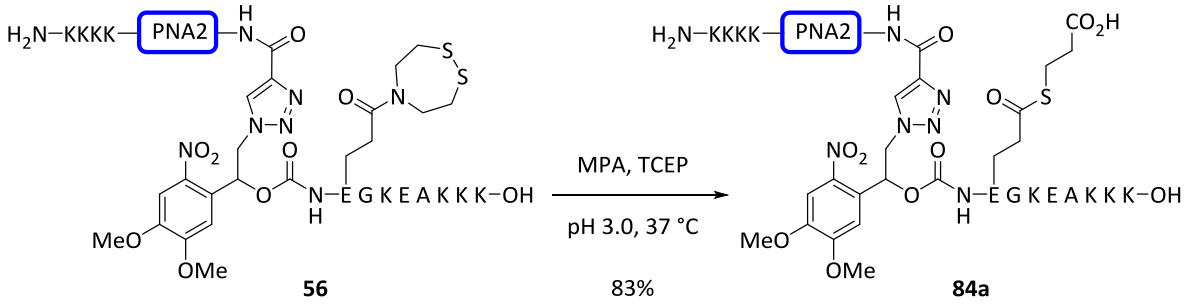
Nevertheless, the peak remained relatively small and longer reaction times did not lead to further product formation. So, this result can be seen as a prove of principle that the proposed concept works. Yet, the reaction rate was too low for a template-directed reaction and the conversion was not satisfactory. Again, it was tested if a lower pH value would favor the *N,S*-shift, and therefore, enhance the reaction rate. However, at pH 6 similar results were obtained and no improvement could be observed (Table 3, Entry 4). Further decreasing the pH was not an option because the PNA base pairing ability would be disturbed. For these reasons, all following ligation reactions were conducted at pH 7. Next, it was tried whether longer side chains would lead to a more favored array of reactive centers and therefore increase the reactivity. For this purpose, the homocysteine (Hcy) building block **58** was designed and reacted with **56** for 48 h (Scheme 38).



Scheme 38. Template-directed NCL approach with Hcy hybrid **58**.

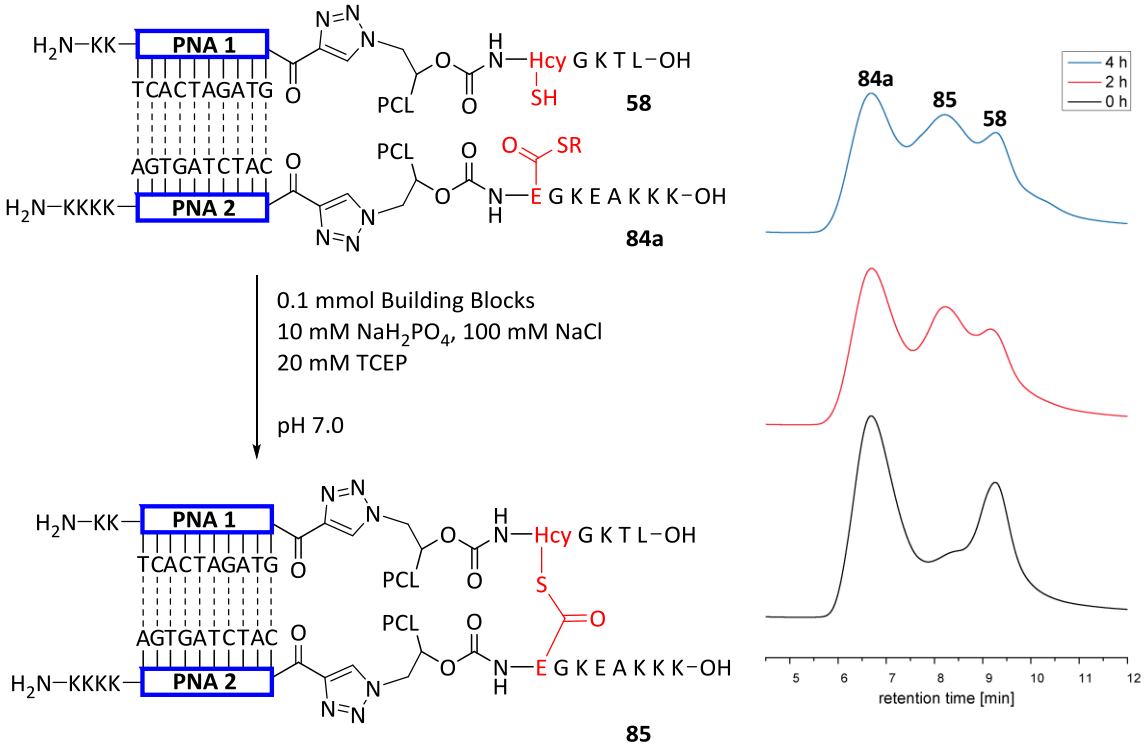
Once more, product formation was observed but still, only very slow and no significant enhancement considering reaction rate and product yield was determined. In the absence of thiols desulfurization occurred slowly. In order to avoid this, sodium ascorbate was added as a radical scavenger for long reactions without thiol additives.[137] Dublicating the reaction time and addition of MPAA did not lead to faster product formation (Table 3, Entry 6). Hence, it was anticipated that the *N,S*-shift is the rate-limiting step. Therefore, the

SEA-building block **56** was transformed into the corresponding 3-mercatopropionic acid (MPA) thioester (Scheme 39).



Scheme 39. Transformation of SEA hybrid **56** to its corresponding MPA thioester **84a**.

The reaction conditions were adapted from MELNYK.[139] A reaction time three times longer and a lower pH were necessary to achieve complete conversion, though. Besides a very long reaction time and acidic conditions, a higher temperature was also important. Having this in mind, the long reaction times and the poor conversion for the conducted ligation reactions so far are not surprising. MPA thioester **84a** could be synthesized with a yield of 83% (Scheme 39). Next, **84a** was tested in a template-directed NCL reaction. Here, the reaction was much faster, so that already after 2 h a considerable amount of product was formed. After 4 h, no further product formation was observed (Scheme 40).



Scheme 40. Template-directed NCL with MPA thioester **84a**.

The formed thioester bond in product **85** turned out to be unstable and already slowly decomposed while new product was formed. Nevertheless, the immensely increased reaction rate clearly showed the advantage of MPA thioesters towards the SEA ligation. On that score, from here on all SEA groups were converted into thioesters before the ligation (Table 2).

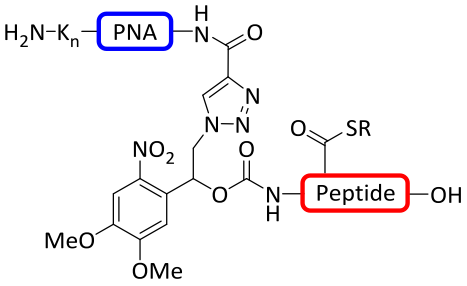


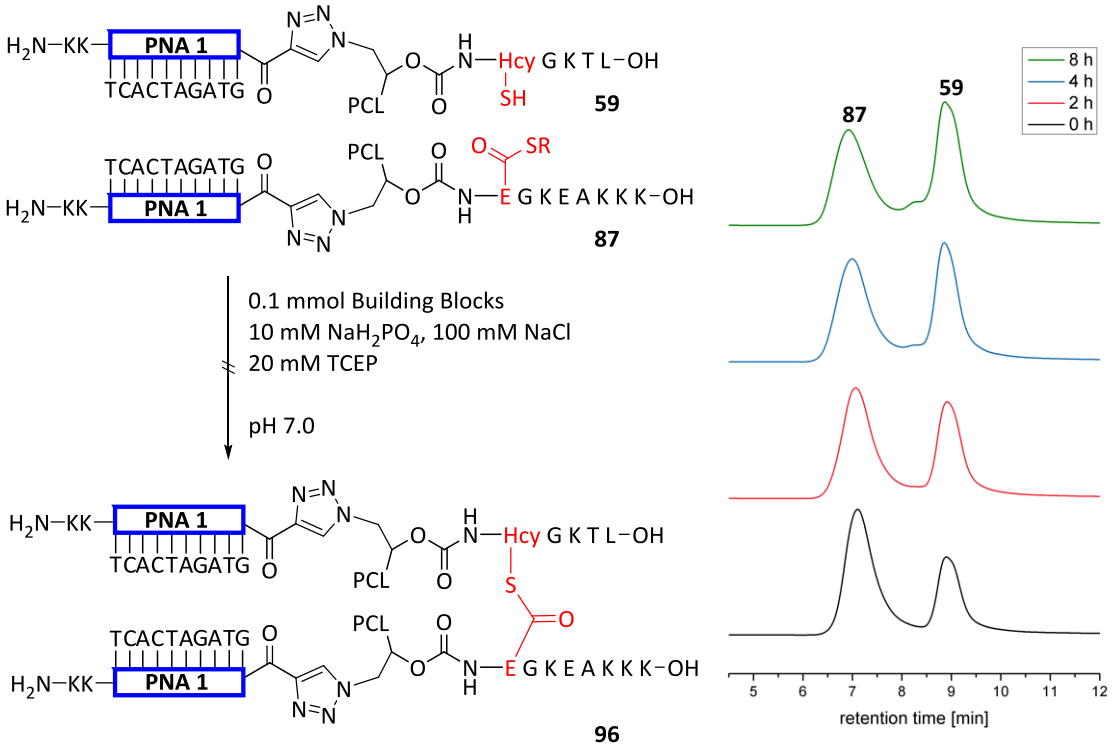
Table 2. Formed thioesters during the course of this thesis. [a] Diastereomer 1, [b] Diastereomer 2, [c] Low yield due to side product formation.

Entry	No.	n	PNA	Peptide	Yield
1	**84a**	4	2	E(MPA thioester) G K E A K K K[a]	83%
2	**84b**	4	2	E(MPA thioester) G K E A K K K[b]	80%
3	**86**	1	2	E(MPA thioester) G K E A K K K	78%
4	**87**	2	1	E(MPA thioester) G K E A K K K	n. d.
5	**88**	4	2	D(MPA thioester) G E S F K K K	45%[c]
6	**89**	4	2	E(MPA thioester) G A K K	78%
7	**90**	4	2	G E(MPA thioester) A D S K	78%
8	**91**	4	2	G E(MPA thioester) G D S K	79%
9	**92**	2	1	G E(MPA thioester) G D S K	81%
10	**93**	4	2	G E(MesNa thioester) G D S K	72%
11	**94**	4	2	A E(MPA thioester) A D S K	78%
12	**95**	4	2	A D(MPA thioester) V S K K	75%

As previously mentioned, the diastereomeric character of the PNA/peptide hybrids leads to the fact that they sometimes occur as double peaks on the HPLC. In terms of clarity, the diastereomers were separated. In the last reaction (Scheme 40), diastereomer 1 of the thioester building block was used. In order to demonstrate that both diastereomers react

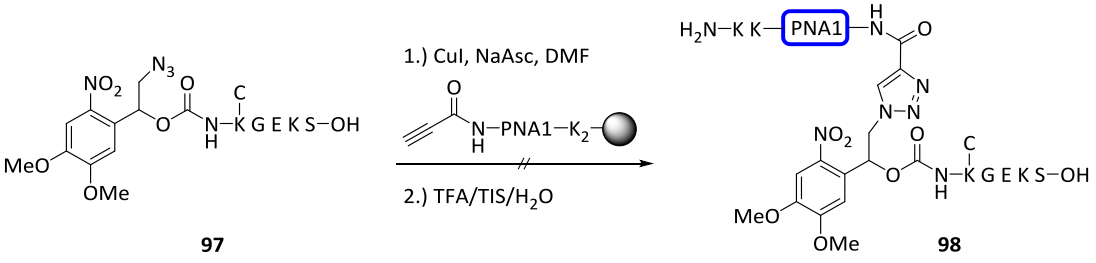
in a comparable manner, the reaction was repeated with diastereomer 2 of the thioester (Table 3, Entry 9). Again, a fast reaction could be seen, showing that both diastereomers can be used for the ligation.

In the next step, the template effect should be demonstrated. Therefore, a thioester building block with a non-complementary PNA strand was synthesized (Table 2, Entry 4) and used for a non-templated NCL (Scheme 41). Here, no product formation was detected by LC-MS even though the reaction time was doubled. This showed very nicely the importance of the template.



Scheme 41. NCL with non-complementary templates.

Nevertheless, the fact that template-directed NCL led to an unstable product that could not be isolated was a major drawback that needed to be corrected. For this purpose, a novel PNA/peptide hybrid with a cysteine linked to the side chain of a lysine should be synthesized (Scheme 42).

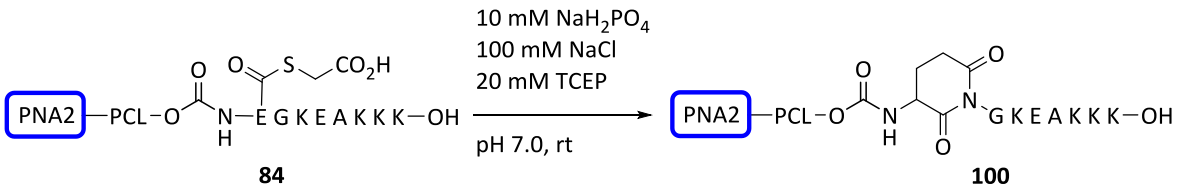


Scheme 42. Failed CuAAC due to sterical hindrance.

The advantage of a cysteine incorporated into the side chain of a peptide is that the *N*-terminus is free and the *S,N*-shift can take place directly during the ligation reaction and form a stable amide bond. A first attempt to synthesize hybrid **98** by CuAAC failed due to sterical hindrance.

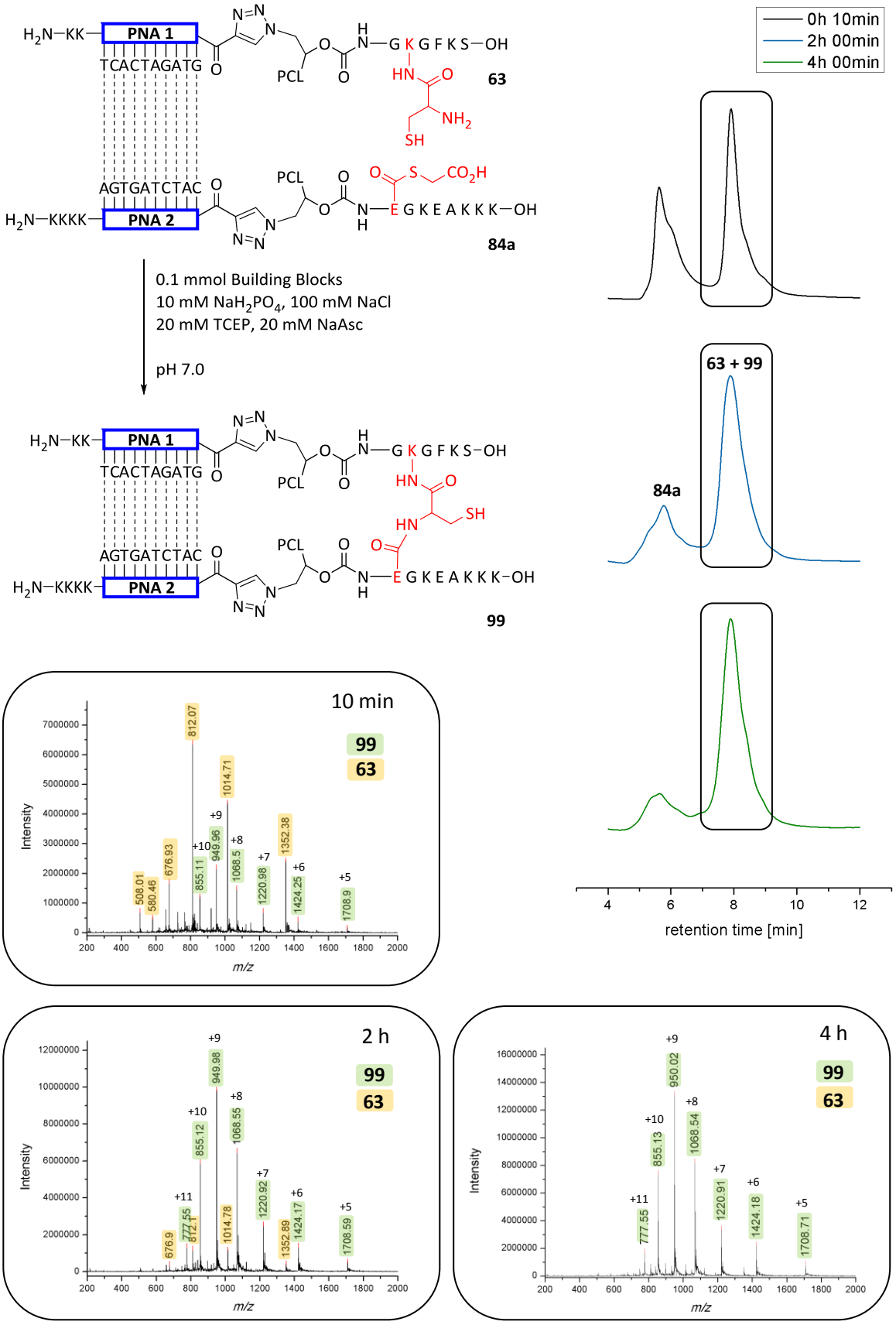
Consequently, for the next attempt a glycine spacer was installed between PCL2 and the branch point. Then, CuAAC proceeded as usual and gave PNA/peptide hybrid **63** which could be used for NCL. The template-directed NCL reaction was very fast, so that already after 10 min a considerable amount of product **99** was found (Scheme 44). After 4 h, the reaction was completed and a conversion of 67% of the thioester building block was detected. The obtained product was linked by a stable amide bond and could be isolated. Due to the significantly longer side chain of the cysteine building block, the reactive centers of the ligation partners are more accessible and therefore, background reactions are more likely to occur.

For this reason, again a control reaction with two non-complementary PNA strands had to be conducted (Table 3, Entry 11). Indeed, background reaction was observed. Clearly slower than the templated reaction, but still 20% conversion of the thioester was observed after 4 h. Besides the background reaction a competing side reaction also raised trouble. Similar to the intramolecular attack at the activated sulfamylbutyryl group described in Chapter 2.2 (Scheme 26), the thioester is attacked by the neighboring amide bond of the glycine and a 6-membered ring is formed (Scheme 43). Thus, the conversion during the NCL was limited by the slow cyclization of the thioester building block.



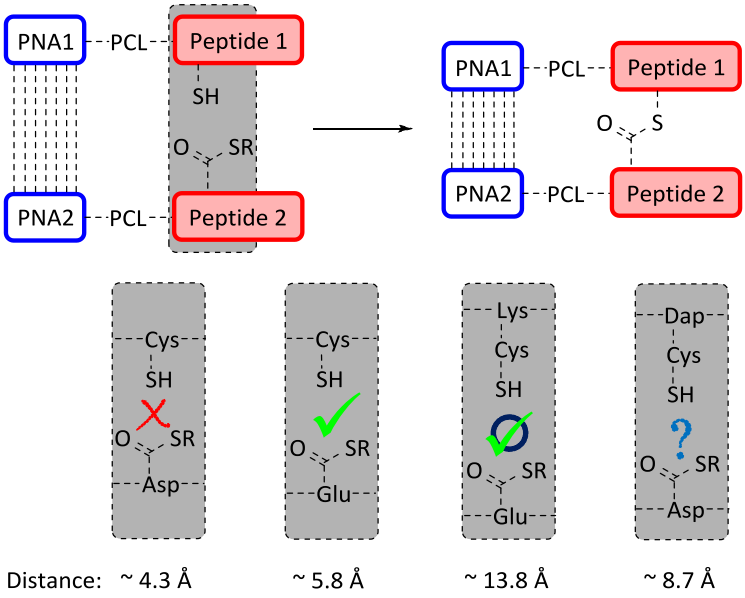
Scheme 43. Intramolecular cyclization reaction competing with NCL.

In order to avoid this side reaction, Fmoc-Aad(SEA^{off})-OH (**47c**) was synthesized. As a result of the additional CH_2 group a less favored 7-membered ring would be formed by an intramolecular cycloaddition. Since the 6-membered ring formation was already significantly slower than the NCL, it was anticipated that this side reaction would not be noteworthy anymore.



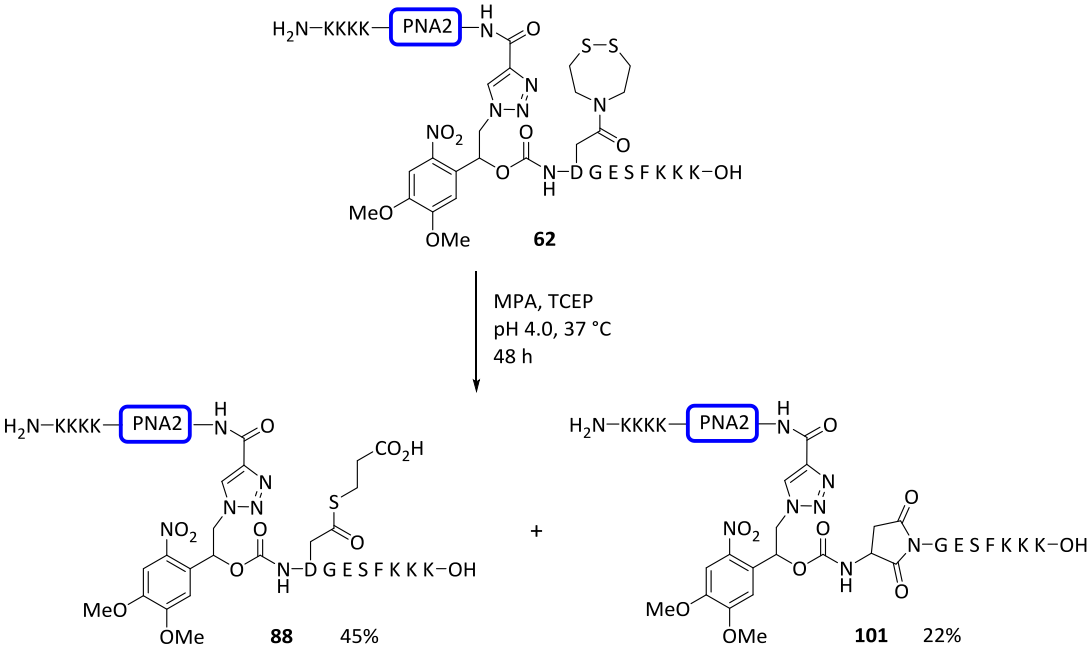
Scheme 44. *Left-hand side:* NCL of **63** and **84a**. *Right-hand side:* HPLC chromatograms of the reaction mixture after different points of time. The framed peak is both starting material **63** and product **99**. *Bottom:* ESI-MS spectra of the product/educt peak at different points of time.

Nevertheless, in order to avoid background reactions of non-complementary hybrids, the distance between reaction partners was decreased. The shortest possible pair of reactants that still allows an *S,N*-shift before the photocleavage would be Asp(SR) on the one side and Dap-Cys on the other side (Scheme 45).



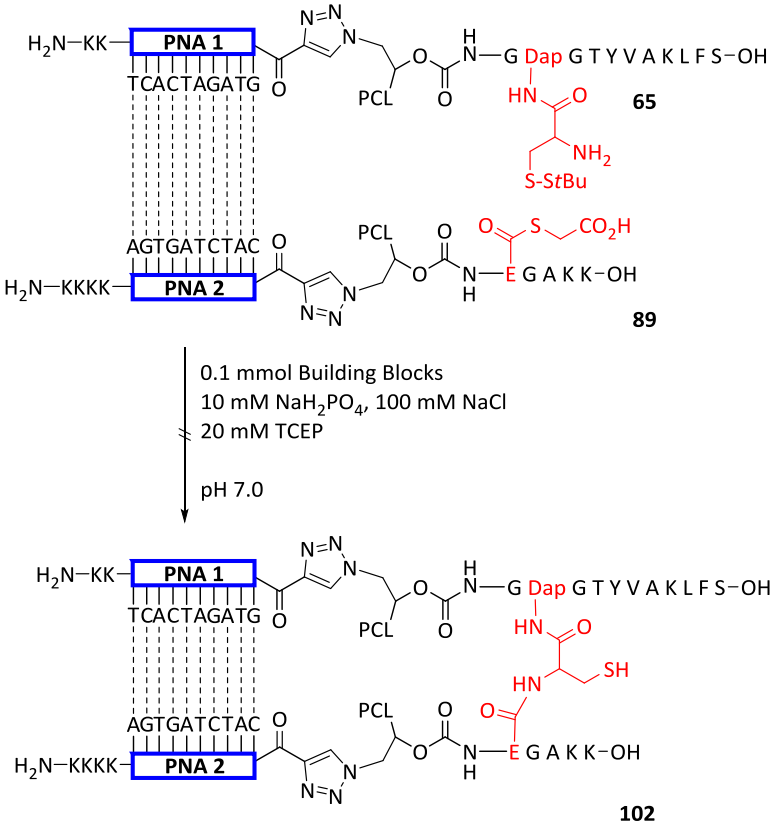
Scheme 45. Distances between the different ligation partners. A distance of ~ 5.8 Å is required to obtain product formation while at a distance of ~ 13.8 Å background reactions are observed.

For this purpose, a PNA/peptide hybrid with Asp(SEA^{off}) (**62**) was synthesized. The following transthioesterification yielded not only the desired product **88**, but also cyclization was observed (Scheme 46).



Scheme 46. Thioester formation partially resulted in intramolecular cyclization.

The formation of a 5-membered ring proceeded already in a significant manner at a low pH which indicated that massive cyclization would take place during NCL conditions. So for now, Glu thioester hybrids were used for the next ligation reactions. Next, the Dap-Cys hybrid should be synthesized. Since it has three CH_2 units less compared to the Lys-Cys hybrid, it was tried to synthesize the hybrid without a glycine linker. Yet again, the CuAAC of the alkyne functionalized PNA strand and the PCL2-peptide could not be accomplished due to steric hindrance. It was also important to introduce an S*t*Bu protecting group at the cysteine, since the peptide tended to form disulfide bonds. Finally, Dap-Cys hybrid **65** was synthesized in good purity. This hybrid was then used for an NCL approach with the Glu thioester hybrid **89** (Scheme 47). However, no product was formed in this reaction. This can be explained with the unsymmetrical array of the reactive groups. While the Glu thioester was directly linked to PCL2, a glycine spacer was in between PCL2 and the Dap-Cys group at the other hybrid. This type of reaction was possible when the Lys-Cys hybrid was used (Scheme 44). With the three additional CH_2 units, the side chain was long and flexible enough to reach the Glu thioester, even though it was not opposite to it. When these CH_2 units are missing, the side chain is not long enough anymore.



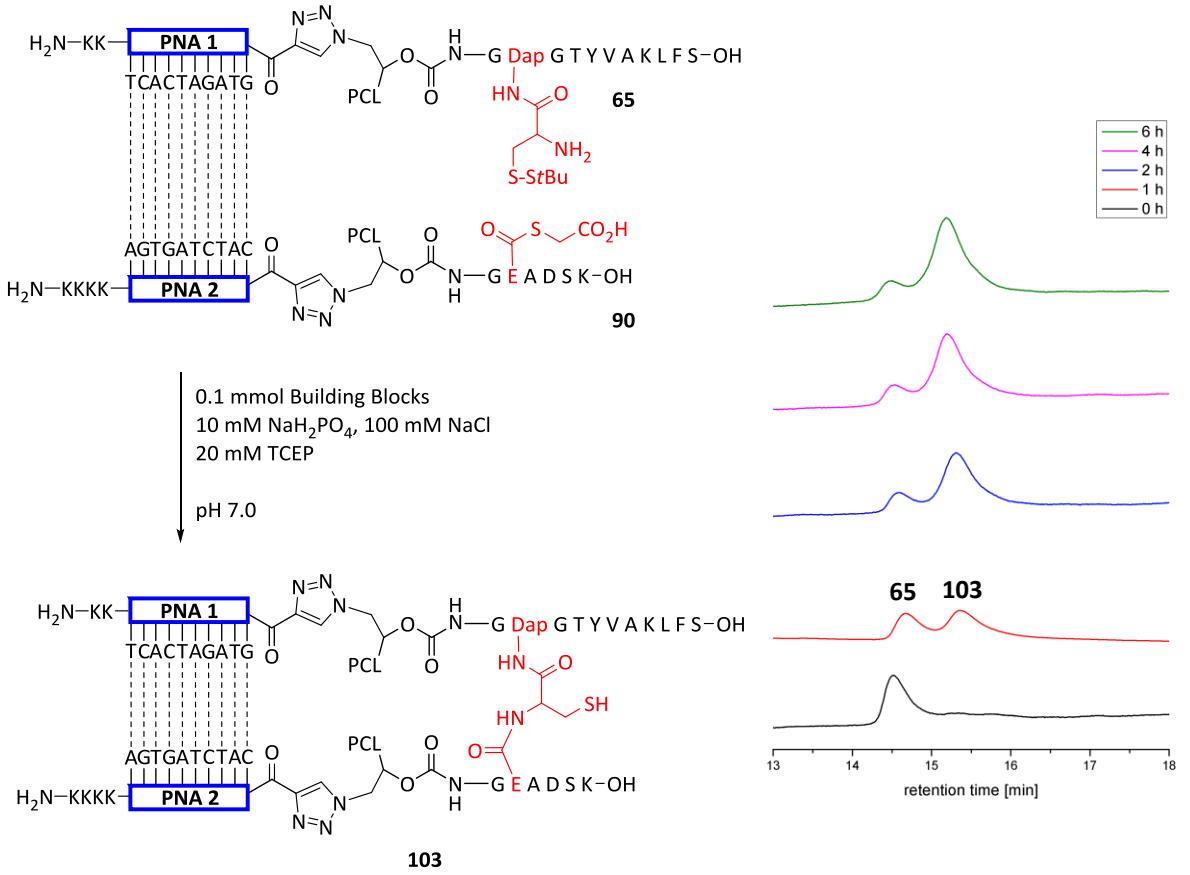
Scheme 47. Unsymmetrical NCL approach did not yield product formation.

As a consequence, a glycine spacer was also introduced for the thioester hybrid to reestablish symmetry. This time a fast ligation was observed. After 1 h, already 48% of the Cys hybrid was consumed and after 2 h the reaction was almost completed. Only little progress was made during the next hours. After 6 h, 71% of the Cys hybrid was consumed and all thioester hybrid was either consumed by NCL or by cyclization (Scheme 48).

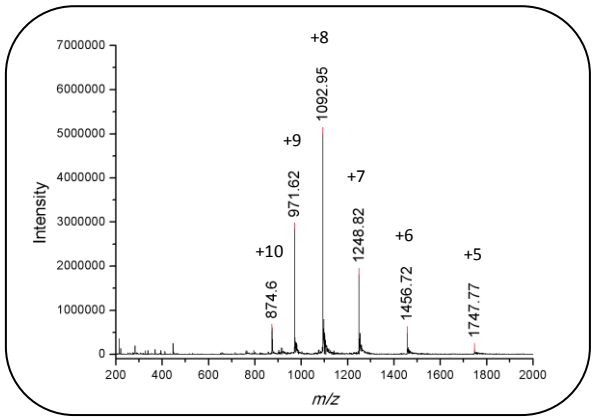
The fact that the reaction only works when the reactive groups are located directly opposite to each other, allows the assumption that background reaction only plays a minor part in this system. In order to test this hypothesis, the NCL was repeated with non-complementary reaction partners (Table 3, Entry 15). Contrary to the expectations, again product formation was observed. A similar conversion compared to the control reaction with Lys-Cys (Table 3, Entry 11) was obtained (22%), however the reaction time was twice as long. So, carefully a trend can be discerned that shorter side chains lead to slower product formation.

In the next step, it should be demonstrated that the results of the NCL with the Dap-Cys hybrid (Scheme 48) can be reproduced. To this end, the reaction was repeated with the same system and arrangement of the reactive groups and only minor changes in the peptide sequence (Table 3, Entry 14). Indeed, a similar result was obtained, even with a slightly better conversion.

The obtained results were already very satisfactory but since further conversion seemed to be inhibited by the slow cyclization of the thioester, a way to avoid this side reaction had to be developed. Therefore, a different thioester was utilized. Studies showed that MesNa thioesters are less prone to hydrolysis than MPA thioesters.[140] Hence, it was anticipated that MesNa thioesters might be less prone to nucleophilic attacks in general and therefore also to an internal cyclization. Consequently, a MesNa thioester hybrid was synthesized and employed for NCL with a Dap-Cys hybrid (Table 3, Entry 16). However, contrary to the expectations, an even faster cyclization was observed so that all thioester was consumed after 6 h and only 58% conversion was obtained. As a result, only MPA thioesters were utilized for the following NCL reactions.



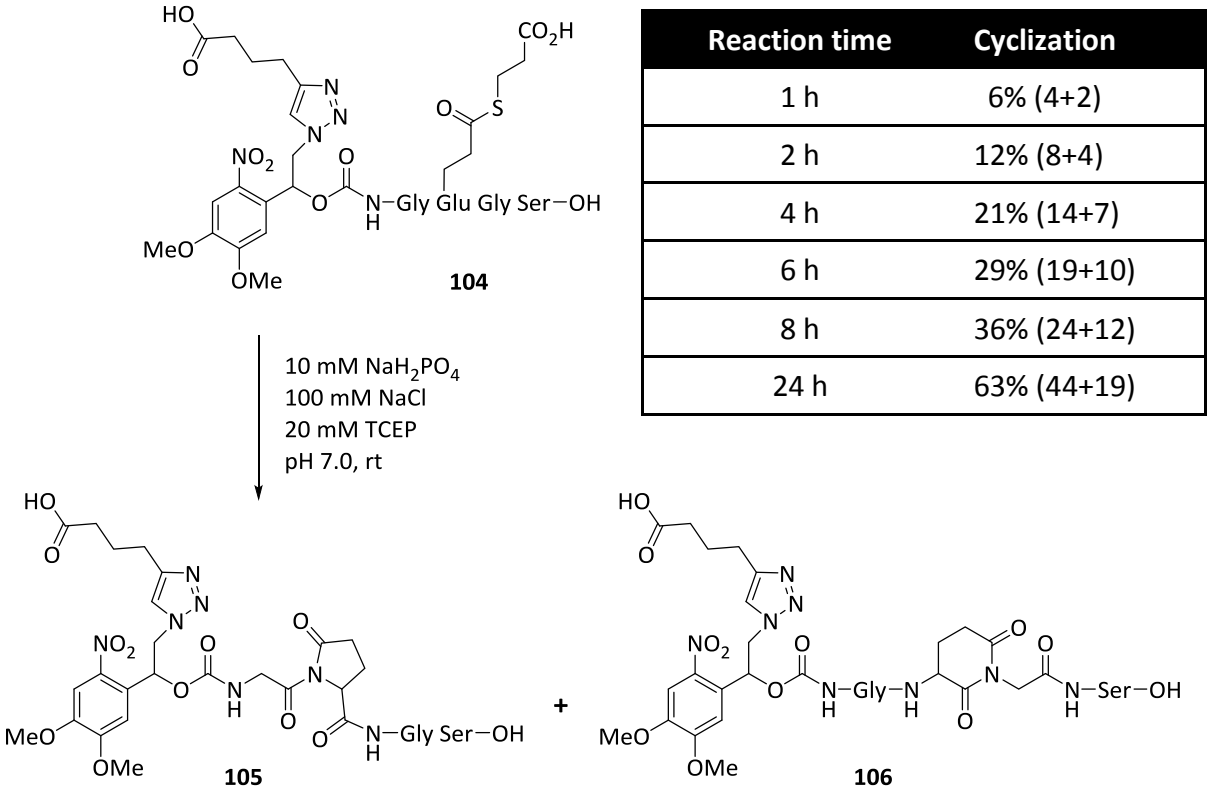
Reaction time	Conversion
1 h	48%
2 h	65%
4 h	69%
6 h	71%



Scheme 48. *Left top:* Native chemical ligation of **65** and **90**. *Right top:* HPLC chromatograms of the reaction mixture after different points of time. *Left bottom:* Conversion of the starting material **65** at different points of time. *Right bottom:* ESI-MS spectrum of the product peak.

Subsequently, the cyclization of the thioester hybrid was further analyzed. Therefore, a short peptide, containing a Gly-thioester-Gly sequence was synthesized. This was attached to PCL2 and CuAAC was applied for triazole formation in order to design a small-sized thioester hybrid mimic **104**. It was then stirred under NCL conditions and cyclization was monitored by HPLC. Cyclization is possible from two different sides in order to either form a 5- or a 6-membered ring. Indeed, two new peaks occurred that displayed the same mass

spectra. One product was formed two times faster than the other and it is not far to seek that the formation of the 5-membered ring is faster (Scheme 49).



Reaction time	Cyclization
1 h	6% (4+2)
2 h	12% (8+4)
4 h	21% (14+7)
6 h	29% (19+10)
8 h	36% (24+12)
24 h	63% (44+19)

Scheme 49. Cyclization of thioester **104** can occur from two different sites.

After the for NCL relevant reaction time of 8 h, a combined yield of 36% cyclized product was formed. This is in accordance with the results from previous NCL reactions. Next, it was tested whether sterical crowdedness has an influence on the cyclization. Indeed, already the exchange from the smallest amino acid glycine to the second smallest amino acid alanine, flanking the thioester, had an immense effect (Figure 7).

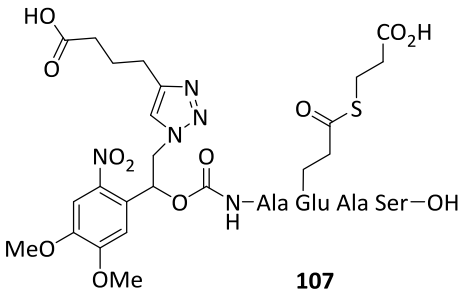
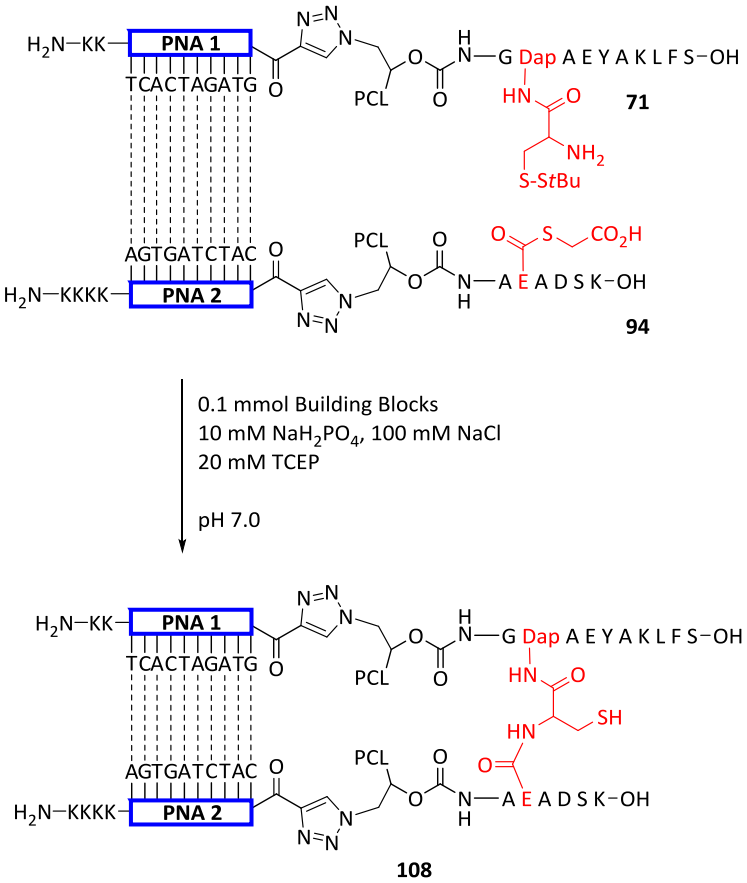
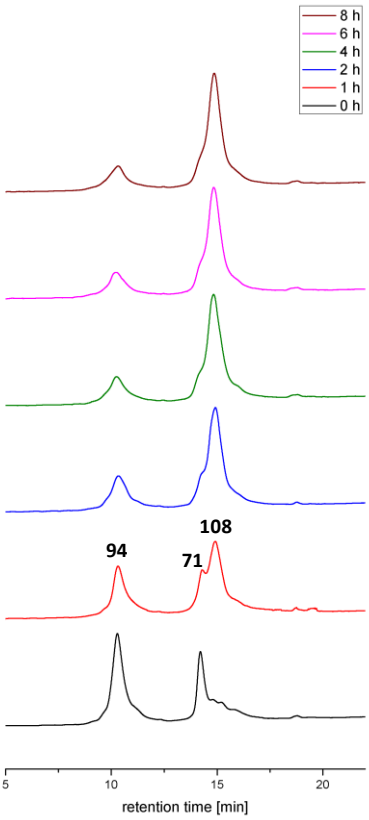


Figure 7. A small increase of crowdedness on the neighboring amino acids already prevented cyclization.

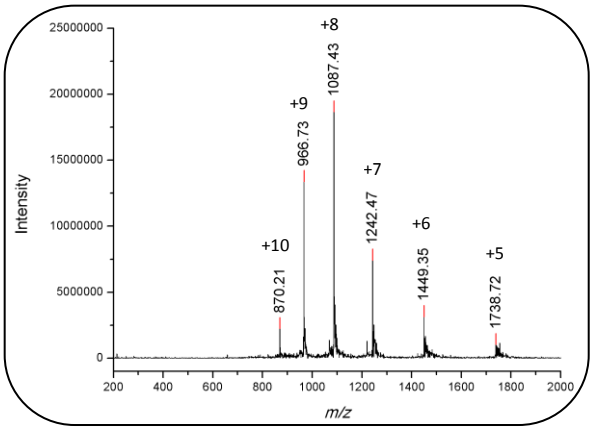
After 24 h only traces of cyclized products were observed. So, cyclization only seemed to occur when one or more neighboring amino acids are glycines. Even then, good

conversions of about 70% were obtained. So, in the next step a PNA/peptide thioester hybrid was synthesized with two alanines flanking the thioester (**94**). This hybrid was then used in a native chemical ligation reaction. The reaction proceeded similar to previous template-directed NCLs and after 8 h a conversion of 67% of **94** was observed (Scheme 50).





Reaction time	Conversion
1 h	38%
2 h	53%
4 h	62%
6 h	64%
8 h	67%



Scheme 50. Template-directed NCL with the stable thioester hybrid **94**.

Thus, no improvement in terms of conversion was achieved, although, even after 8 h the thioester remained completely intact and no cyclization occurred. Also, the shoulder at the product peak indicated that there was still Cys hybrid abundant, albeit it could not be

detected by mass spectrometry anymore. Apparently, at some point the reaction seems to be inhibited by an unknown factor.

The possibility to design cyclization resistant thioester hybrids offered the option to use aspartic acid thioesters, which show a greater proneness towards cyclization with neighboring glycine residues. An aspartic acid derivative on the one side and a Dap-Cys hybrid on the other side would give the shortest possible linkage between two hybrids where an *S,N*-shift is achievable before the photoclavage. Thereby, a decreased background reaction would be expected. In order to examine this hypothesis, a thioester hybrid with an aspartic acid thioester, flanked by valine and alanine, was synthesized (**95**). This thioester was then used for an NCL reaction. The reaction proceeded similar to ligation reactions with a Glu thioester. However, the conversion was not determined since the thioester was accidently added in excess and the Cys hybrid **71** and product **109** peak overlap. Mass spectra of the Cys hybrid/product peak showed that already after 1 h the majority of the Cys hybrid was consumed and after 4 h no Cys hybrid could be detected anymore (Scheme 51).

Subsequently, a test reaction with two non-complementary PNA strands was performed in order to elucidate the background reaction. Herein, a conversion of 29% was measured after 8 h reaction time. This is even slightly more background reaction compared to ligations with glutamic acid derivatives, but still in the same range. Here again, no cyclization of the thioester was observed. Nevertheless, the Cys hybrid decomposed and an unindentified product appeared, displaying a mass of Cys hybrid + 26 Da.

In the next step, it should be proven that the predicted *S,N*-shift really proceeds during the ligation. Therefore, the ligated product **110** was stirred in an aqueous buffer (pH 10) at room temperature for 1 h (Figure 8).

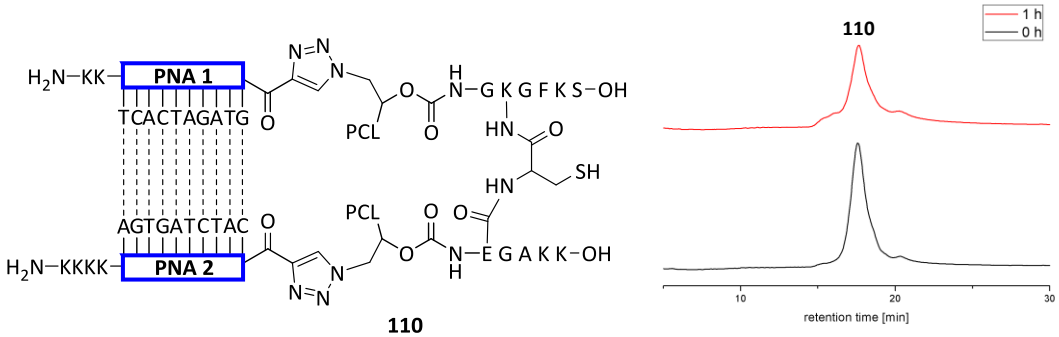
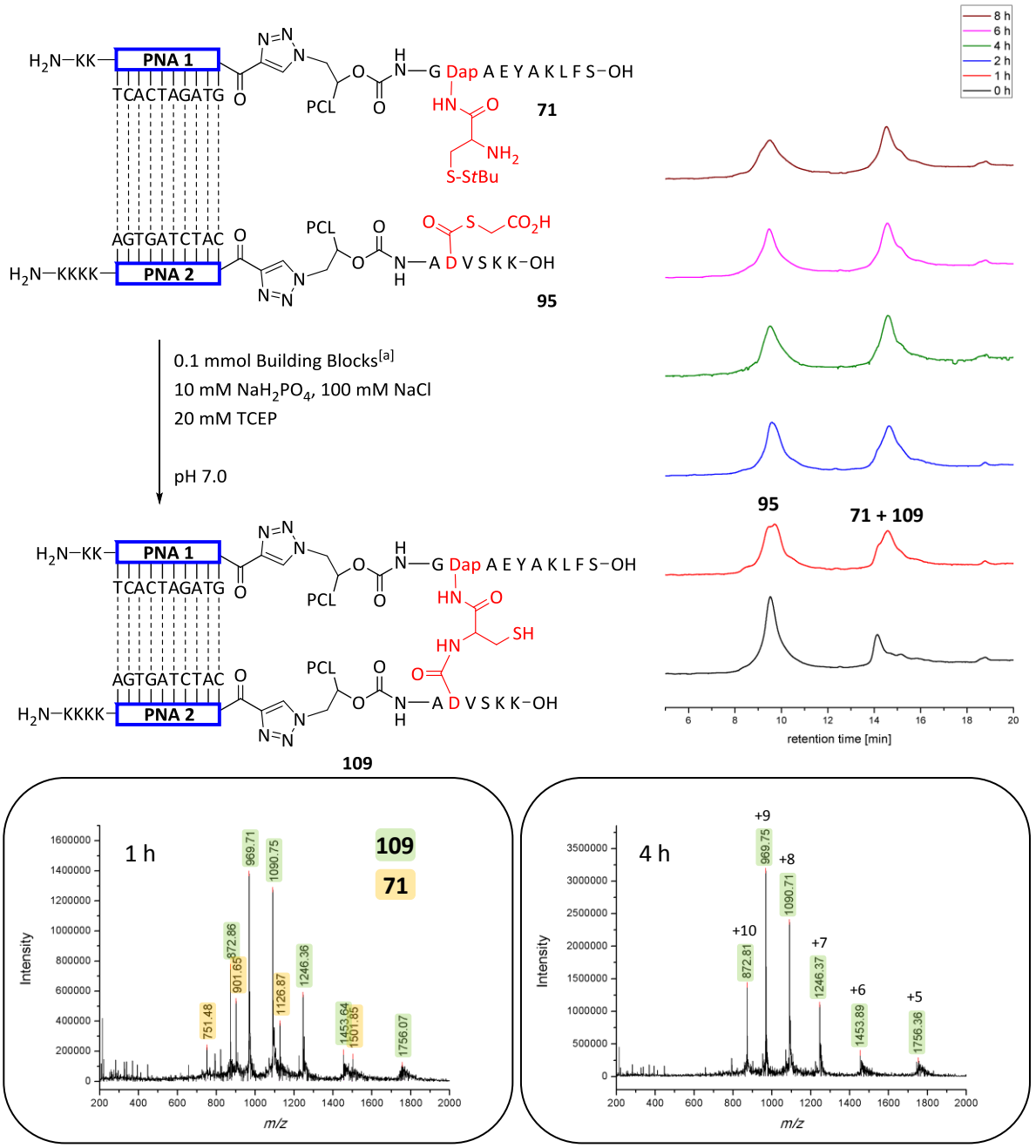


Figure 8: The ligated product **110** was stirred in an aqueous buffer (10 mM NaH_2PO_4, 100 mM NaCl, 20 mM TCEP, pH 10) for 1 h at room temperature.

As expected, **110** remained stable under these conditions. A thioester bond is very unstable under basic conditions, so this result clearly shows that the two hybrids are linked by an amide bond.



Scheme 51. Templated NCL with an aspartic acid derived thioester. After 4 h no Cys hybrid could be detected by mass spectrometry. [a] **95** was added in excess.

No further optimization steps were taken from this on because the developed method constantly showed a fast reaction with good yields in the range of 70%. Side reactions could be prevented and background reactions were found to be significantly slower. It can be expected that in a combinatorial chemistry approach with different PNA pairs

background reactions will not be relevant because the complementary PNA strands will bind very fast and tight and will therefore not be available for background reactions.

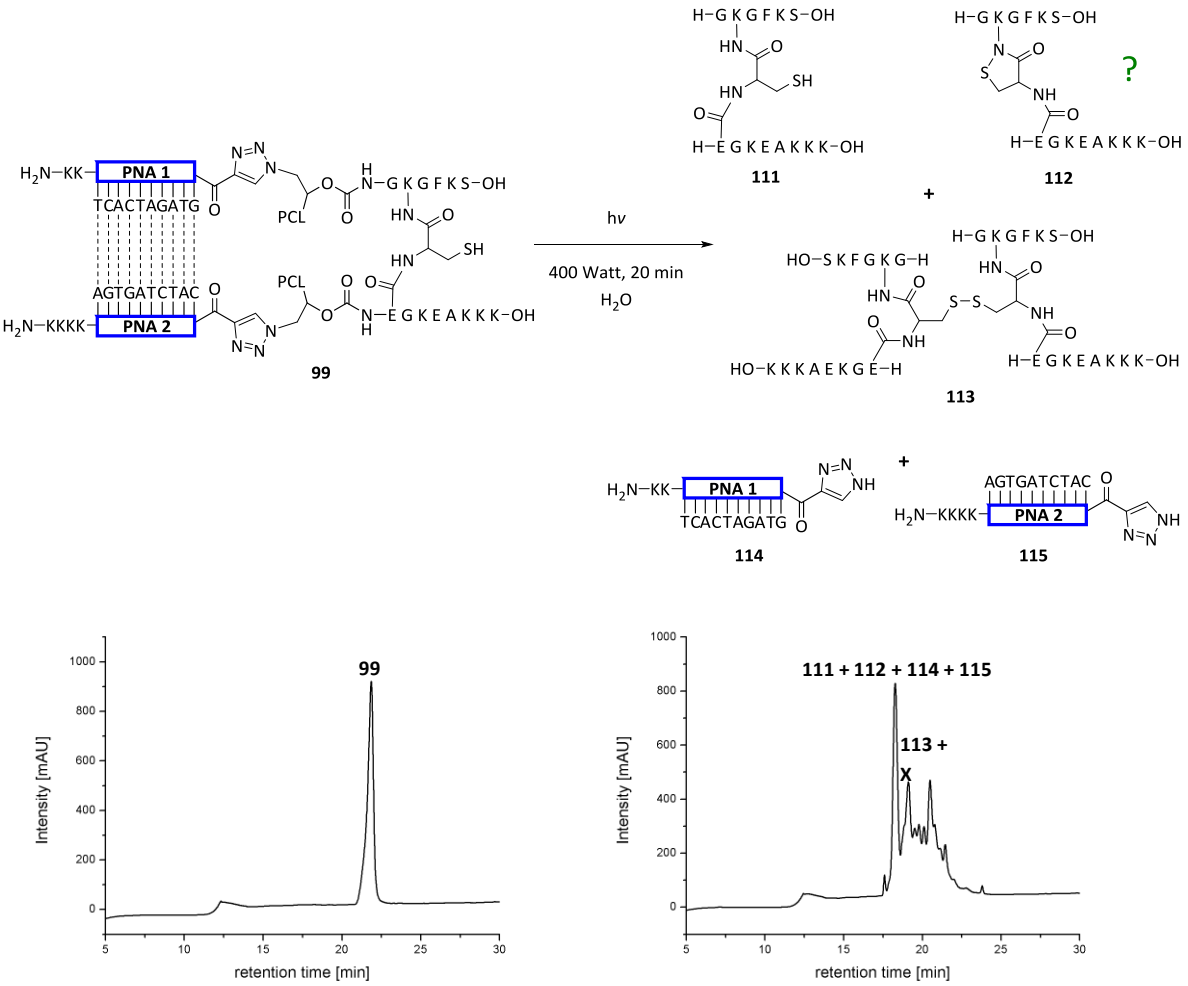
All important ligation reactions carried out within the framework of this thesis are summarized in Table 3.

Table 3. Results for the templated ligation reactions. Conversions of the Cys hybrids are displayed. [a] R = 3-mercaptopropionic acid, R' = sodium 2-sulfanylethanesolfonate. [b] All reactions were carried out in aqueous buffer (10 mM NaH_2PO_4, 100 mM NaCl) with 20 mM TCEP at rt. [c] Control reaction with non-complementary PNA strands. [d] A different set of stereoisomers was used. [e] Conversion of the thioester hybrid is displayed. – = no conversion, + = low conversion, ++ = moderate conversion, high conversion.

Entry	Peptide 1	Peptide 2[a]	Conditions[b]	Conv.
1	**C(*St*Bu)** G K T L	**D(SEA^{off})** G D F K D L	pH 7.0, 24 h	–
2	**C(*St*Bu)** G K T L	**D(SEA^{off})** G D F K D L	pH 6.0, 96 h, MesNa	–
3	**C(*St*Bu)** G K T L	**E(SEA^{off})** G K E A K K K	pH 7.0, 50 h, MPAA	+
4	**C(*St*Bu)** G K T L	**E(SEA^{off})** G K E A K K K	pH 6.0, 50 h, MesNa	+
5	**Hcy** G K T L	**E(SEA^{off})** G K E A K K K	pH 7.0, 48 h	+
6	**Hcy** G K T L	**E(SEA^{off})** G K E A K K K	pH 7.0, 96 h MPAA	+
7	**Hcy** G K T L	**E(SR)** G K E A K K K	pH 7.0, 4 h	++
8	**Hcy** G K T L	**E(SR)** G K E A K K K	pH 7.0, 4/8 h	–[c]
9	**Hcy** G K T L	**E(SR)** G K E A K K K	pH 7.0, 4 h	++[d]
10	G K(**C**) G F K S	**E(SR)** G K E A K K K	pH 7.0, 4 h	67%[e]
11	G K(**C**) G F K S	**E(SR)** G K E A K K K	pH 7.0, 4 h	20%[c, e]
12	G Dap{**C(*St*Bu)**} G T Y V A K L F S	**E(SR)** G A K K	pH 7.0, 8 h	–
13	G Dap{**C(*St*Bu)**} G T Y V A K L F S	G **E(SR)** A D S K	pH 7.0, 6 h	71%
14	G Dap{**C(*St*Bu)**} G T Y V A K L F S	G **E(SR)** G D S K	pH 7.0, 8 h	84%
15	G Dap{**C(*St*Bu)**} G T Y V A K L F S	G **E(SR)** G D S K	pH 7.0, 8 h	22%[c]
16	G Dap{**C(*St*Bu)**} G T Y V A K L F S	G **E(SR')** G D S K	pH 7.0, 6 h	58%
17	G Dap{**C(*St*Bu)**} A E Y A K L F S	A **E(SR)** A D S K	pH 7.0, 8 h	67%[e]
18	G Dap{**C(*St*Bu)**} A E Y A K L F S	A **D(SR)** V S K K	pH 7.0, 8 h	++
19	G Dap{**C(*St*Bu)**} A E Y A K L F S	A **D(SR)** V S K K	pH 7.0, 8 h	29%[c]
20	G K(**C**) G F K S	**E(SR)** G A K K	pH 7.0, 16 h	73%[e]

2.4.2 Photolytic Cleavage Reactions

The last part of this thesis focused on the uncaging of the ligated product. In a first attempt, **99** was uncaged upon irradiation with 400 Watt for 20 min (Scheme 52).



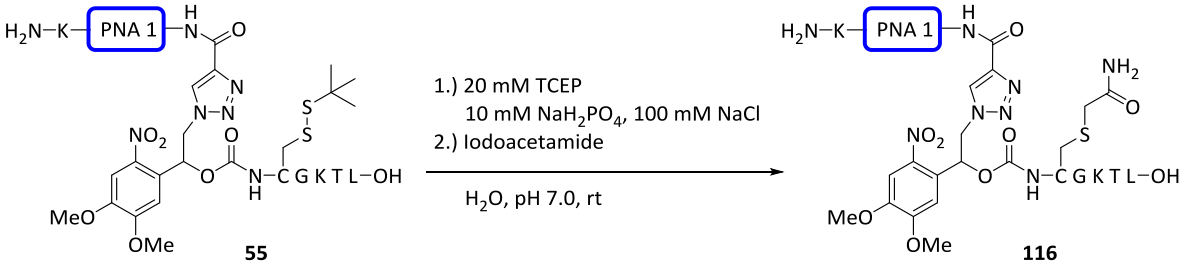
Scheme 52: Uncaging of **99** resulted in the formation of multiple products.

Only small amounts of the desired product **111** were obtained. Moreover, the uncaged peptide had the same retention time as the cleaved PNA strand, therefore, the amount of formed product could not be quantified. In addition, two more compounds were identified that can be associated with the uncaged peptide. On the one hand, the corresponding disulfide **113** and on the other hand, the uncaged peptide minus two protons **112**. Thiols easily form radicals induced by reactions with other radicals, irradiation or heat.[141] Radicals, as electron poor reactants, are prone to nucleophilic attacks. Hence, it was assumed that a thiol radical was attacked by an intramolecular nucleophile under cyclization. This would explain the difference of two protons compared to the uncaged

peptide. Furthermore, the cleaved PNA strands appeared to have a different structure than expected from the classic mechanism. Instead of a PNA/PCL conjugate cleaved at the carbamate function, the PNA strands (**114** and **115**) were cleaved at the triazole function. Caging groups, with leaving groups at the β-carbon to the aromatic ring, are literature known.[90] Additionally, NIELSEN has recently shown that triazoles can be convenient leaving groups for photolytic cleavage reactions.[96] Thus, it can be presumed that due to the special design of PCL2 not one, but multiple competitive reaction mechanisms lead to the uncaging. On top of this, it is likely that after the first photolytic reaction a second reaction takes place since the obtained products (PNA strands and uncaged peptide) cannot both be explained by one mechanistic route.

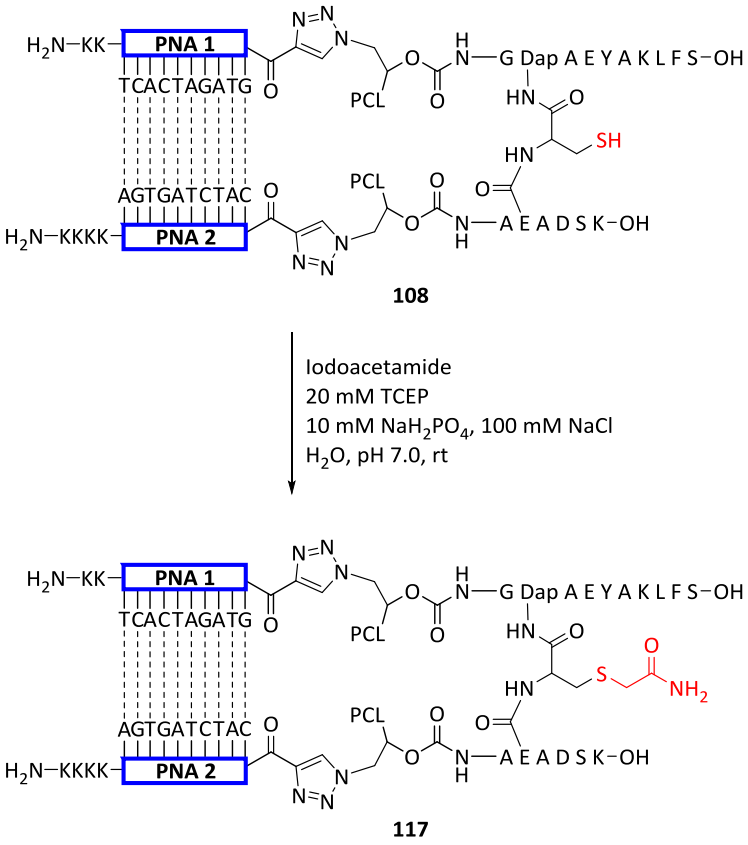
In the following, various optimization approaches were conducted in order to improve the uncaging outcome. Therefore, different light sources (see Experimental Part), wavelength ranges (347-400 nm, 280-400 nm, 350-375 nm), power (190 mW, 400 W, 650 W), solvents (H_2O, H_2O/MeCN) and additives (hydrazine, ascorbic acid) were tried, but unfortunately none of the tested combinations led to a satisfying result. The uncaging of **99** showed that several by-products are obtained from the tendency of unprotected thiols to form reactive radicals (Scheme 52), whereas prior uncaging attempts of hybrids with protected thiols (Scheme 23 and Scheme 33) proceeded in a clean manner. Consequently, the free thiol was identified as the disturbing factor during the uncaging process.

For that reason, a way to cap the thiol function after the ligation was searched. It was important that the capping reaction proceeded fast and selectively. Also, it was important that it could be conducted in the ligation medium so that no additional purification step was necessary. Iodoacetamide is known to react rapidly with thiols but also, significantly slower, with other nucleophiles like amines.[142] In a test reaction, the cysteine containing hybrid **55** was capped with iodoacetamide under ligation conditions. The hybrid was first stirred in the TCEP/buffer solution for 30 min in order to cleave the S*t*Bu protecting group. Then, iodoacetamide was added in excess and after 30 min the reaction was quenched with MPA. Complete conversion was monitored and only the monoacetylated product was observed (Scheme 53).



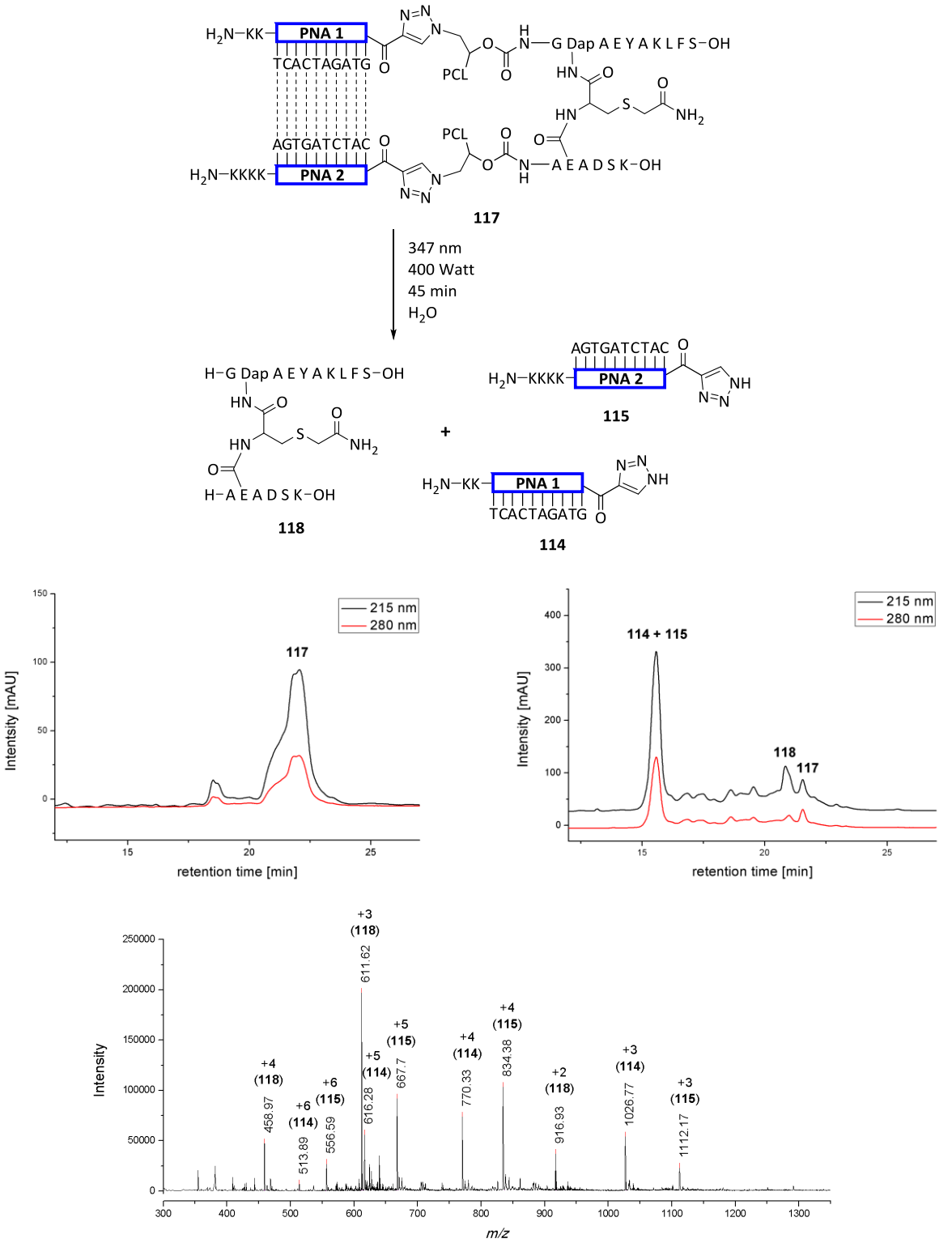
Scheme 53. Selective capping of the thiol function in **55**.

Next, the reaction conditions were used to cap the ligated product **108**. Again, the reaction proceeded fast and selective, so that after HPLC purification a ligated hybrid with a protected thiol funtion was obtained (Scheme 54).



Scheme 54. Thiol protection of the ligated hybrid **108**.

Subsequently, the product was employed in a photolysis reaction. This time uncaging delivered the desired peptide **118** in good purity. Minor by-products were still abundant, but the major peaks were classified as the product and the cleaved PNA strands (Scheme 55). After 45 min reaction time, 91% of the starting material **117** was uncaged leading to 68% defined products. Indeed, the unprotected thiol was the critical factor during the photolytic cleavage reaction.

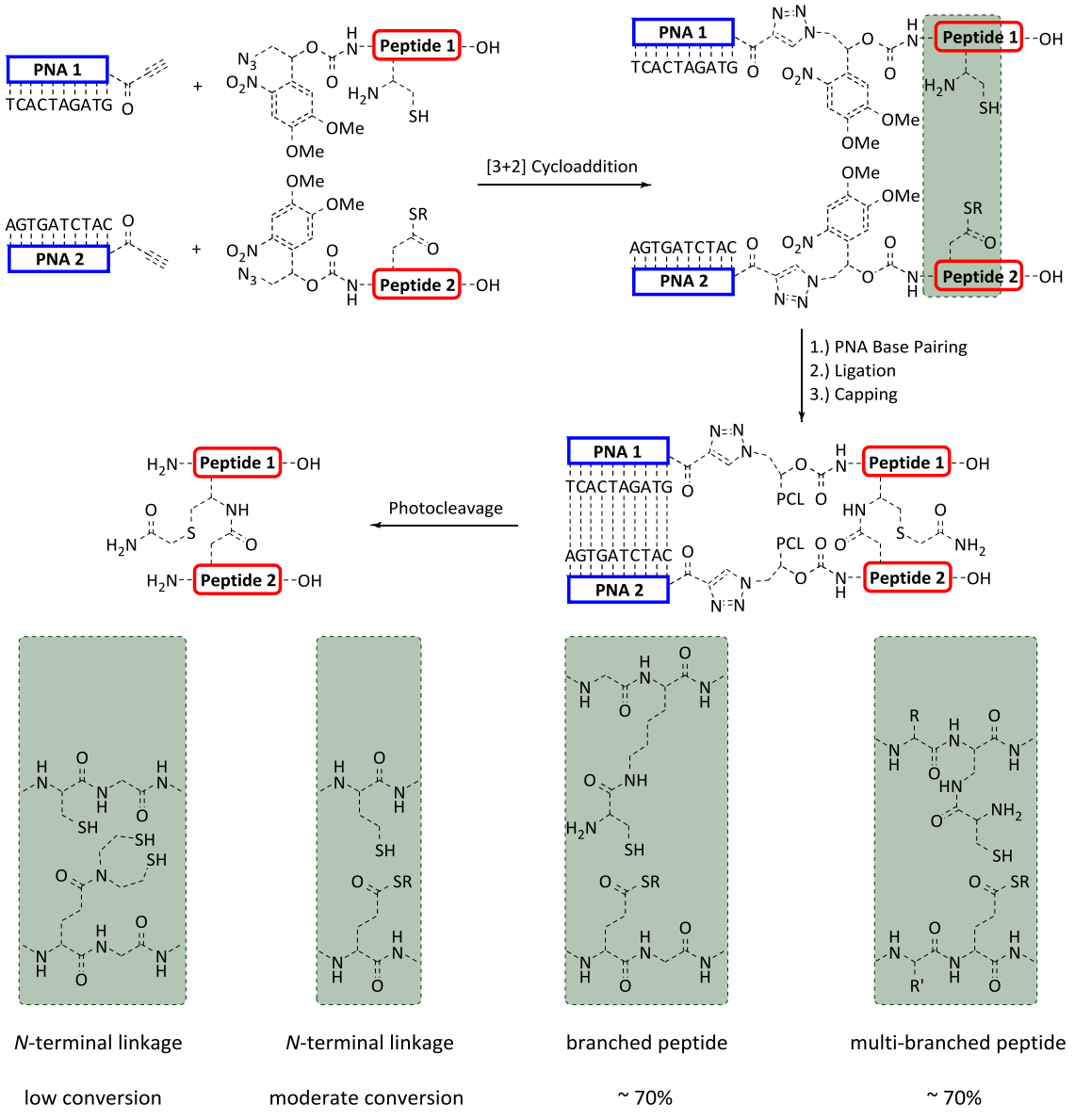


Scheme 55. Uncaging of **117** led to the desired product **118**. *Left:* HPLC chromatogram of the starting material. *Right:* HPLC chromatogram after irradiation. *Bottom*: ESI-MS spectrum of the reaction mixture.

Thus, having solved the last problematic aspect, finally an efficient and easily accessible protocol for the template-directed NCL approach under application of photocleavable PNA strands was available.

3 Summary & Outlook

The modification of proteins is a fundamental task in natural sciences in order to control and to understand biochemical processes. To address more complicated targets and to widen the spectrum of possible modifications, it is necessary to develop new strategies and to expand the established techniques. Within the scope of this thesis a novel template-directed ligation strategy applying photocleavable PNA strands, was originated. The final concept and the most important results are depicted in Scheme 56.



Scheme 56. PNA base pairing guided ligation strategy. The concept allowed linkage of *N*-terminal amino acids as well as the synthesis of branched and multi-branched peptides.

The first part of the thesis comprised the design of a new photocleavable linker. Herein, two different linkers were synthesized that both met the requirements. Nevertheless, PCL2

benefited from its easy and modular attachment to peptides on-resin. Having the linker in hand, PNA/peptide hybrids were synthesized from alkyne modified PNA strands and PCL2-peptide conjugates via CuAAC in good yields and purity. For the ligation of two hybrids, MELNYK'S SEA building blocks were used as thioester precursors.[112] It was reported that these building blocks can be directly used for NCL, but unfortunately they showed insufficient reactivity during the template-directed ligation experiments. Therefore, the SEA hybrids were transformed into MPA thioester hybrids which resulted in a dramatically increased reaction rate. The original concept envisioned that the *S,N*-shift takes place after the photocleavage, but it was found that the thioester bond between the ligated hybrids was not stable enough to isolate the product. Therefore, the next step was to incorporate a cysteine in the side chain of a lysine. As a consequence, both, thiol and amine function, were available for the ligation so that the *S,N*-shift could take place during the ligation. This led to stable ligation products which could be isolated. Further optimization steps, including variation of the linker length, thioester reactivity and the sterical environment of the reactive groups were taken in order to decrease side and background reactions. This led to a stable and reliable system where conversions of about 70% were obtained constantly. Moreover, different junctures could be addressed. As a result peptides were ligated with their *N*-terminal amino acids and in addition, branched as well as multi-branched peptides were synthesized within this thesis.

The final part of this work focused on the uncaging of the ligated hybrids. First photolytic experiments were not satisfying since many by-products were observed. Finally, the breakthrough was accomplished by capping the free thiol function of the ligated products. Hence, the thiol protected hybrids were uncaged and delivered the cleaved PNA strands and the desired peptide in good purity. With that, all problems having emerged throughout the project were solved and an efficient protocol for this new ligation strategy was established.

This strategy can now be used for combinatorial chemistry approaches to build peptide libraries or for protein modifications. Here, this template-directed ligation approach is a powerful tool, as different ligation sites can be addressed selectively in a one pot reaction and the bulky guiding units can be cleaved by irradiation afterwards. A protein with different addressable ligation sites, as recently described by KOLMAR, could serve as a modular scaffold and allow to build a library with different protein-conjugates.[47]

4 Experimental Part

4.1 General Information

Unless otherwise noted, all reactions were carried out under argon atmosphere and under exclusion of light. All reagents, including anhydrous solvents, were purchased from commercial suppliers (*ABCR*, *Acros-Organics, Alfa Aesar, Bachem, Fisher Scientific, GL Biochem, IRIS Biotech, Merck, Nova Biochem, Roth, Sigma-Aldrich, TCI* and *VWR*) without further purification. Technical solvents were distilled prior to use. Water was purified using a *Simplicity* water purification system from *Millipore*. Synthesized chemicals were stored at -20 °C under light exclusion.

Photolytic cleavage reactions were conducted in *a)* a transparent, polystyrene 6 well tissue culture test plate using a *biostep* UV transilluminator (*UST-20L-8E*, 360 nm, filter size 20 x 20 cm, 100% intensity), *b)* a UV cuvette (1 cm) using a UV (365 nm) mounted LED (*M365L2*, 700 mA, 190 mW) and an LED Driver (*LEDD1B*) from *Thorlabs* or *c)* a UV cuvette (1 cm) using an Ark Lamp Source (*66924*) from *Newport*, consisting of a 1000 W *Arc Housing-IGN F/1.0* (*66921*), a 1000 W Hg-Xe lamp (*6295NS*), a power supply (*69920*), a dichroic mirror (280-400 nm, *66226*) and a 347 nm longwave cut-on filter (*20CGA-345*).

Reactions were monitored by thin-layer chromatography (**TLC**) carried out on aluminium backed plates of silica gel 60 F_{254} (layer thickness: 0.20 mm) from *Merck*. Spots were detected by fluorescence quenching at 254 nm. Amino groups were dyed with 3% ninhydrin in EtOH. Purification by **flash chromatography** was conducted using *Merck* Silica Gel 60 (particle size: 40-62 µm).

Semi-preparative **HPLC purification** was performed on *Amersham Pharmacia Biotech* systems (*Äkta basic*, pump type *P-900*, variable wavelength UV detector 900). UV absorption was detected at 215 nm, 254 nm and 280 nm. An *MN Nucleodur 100* column (RP-C18, 250 x 10.0 mm, 5 µm) was used with a flow rate of 3 mL/min. All runs were conducted with a linear gradient of eluent A (H_2O, 0.1% TFA) to B (MeCN/H_2O/TFA 80:20:0.1).

Method A: gradient: 0-60% B in 30 min.

Method B: gradient: 30-90% B in 30 min.

Analytical HPLC purification was performed on a *JASCO* system (communications interface: *LC-Net II/ADC*, multiwavelength detector: *MD-2010 Plus*, autosampler: *AS-2055 Plus*, pumps: *PU-2085 Plus*) or on an *Amersham Pharmacia Biotech* system (*Äkta basic*, pump type *P-900*, variable wavelength UV detector 900). UV absorption was detected at 215 nm, 254 nm and 280 nm. An *MN Nucleodur 100* column (RP-C18, 250 x 4.6 mm, 5 µm) was used with a flow rate of 1 mL/min. All runs were conducted with a linear gradient of eluent A (H_2O, 0.1% TFA) to B (MeCN/H_2O/TFA 80:20:0.1) or C (MeCN, 0.1% TFA).

Method C: gradient: 0-50% C in 30 min.

Method D: gradient: 10-90% C in 30 min.

Method E: gradient: 0-60% B in 30 min.

Method F: gradient: 30-90% B in 30 min.

LC-MS measurements were either conducted on a *Thermo Finnigan MAT* LCQ ion trap mass spectrometer connected to a *Thermo ACCELA Autosampler* with a *Thermo ACCELA Pump* and a *Thermo Finnigan Surveyor PDA* detector or a *Bruker micrOTOF* spectrometer connected to an *Agilent Technologies* system (*1200 Series* consisting of a degasser {*G1322A*}, a binary pump {*Bin Pump SL, G1312B*}, an autosampler {*ALS SL, G1329B*}, a thermostatted column compartment {*TCC SL, G1316B*} and a variable wavelength detector {*VWD SL, G1314C*}). 10 µL of sample solution were injected. As eluents, H_2O + 0.05% FA (eluent A‘) and MeCN (LC-MS grade) + 0.05% FA (eluent B‘) were used.

Method G: gradient: 5-45% B' in 20 min; column: *MN Nucleodur* (RP-C18, 100 Å, 100 · 2 mm, 3 µm); flow rate: 300 µL/min.

Method H: gradient: 5-15% B' in 10 min, then 15-45% B in 10 min; column: *MN Nucleodur* (RP-C18, 100 Å, 150 · 2 mm, 3 µm); flow rate: 200 µL/min.

Method I: gradient: 10-20% B' in 10 min, then 20-45% B in 10 min; column: *MN Nucleodur* (RP-C18, 100 Å, 150 · 2 mm, 3 µm); flow rate: 200 µL/min.

High resolution ESI spectra were obtained with a *Bruker APEX-Q IV 7T* or *Bruker micrOTOF* spectrometer. The values are given as *m/z* relation.

NMR spectra were recorded at *Varian* instruments (*Mercury 300, Mercury VX 300, Unity 300, VNMRS-300*) and *Bruker* instruments (*Avance III 400, Avance III HD 400*) using chloroform-*d* or DMSO-d_6 as the internal standard.[143] Chemical shifts are quoted in ppm. Multiplicities are abbreviated as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, td = triplet of doublets, q = quartet, quin. = quintet, m = multiplet. Also, the abbrevations u = upfield and d = downfield are used.

IR spectra were recorded on a *JASCO FT/IR-4100* spectrometer. Characteristic absorption bands are classified as strong (s), medium (m) or weak (w).

UV-Vis spectra were measured on a *JASCO V-550* spectrophotometer using a UV cuvette (1 cm). For melting curve measurements an *ETC-505T* temperature controller from *JASCO* was added and the sample cell was floated with nitrogen.

4.2 General Procedures (GP)

GP1: Microwave assisted resin loading

The resin loading was performed with a *CEM Discover* microwave reaction cavity. RINK amide MBHA resin (1.0 eq.) was swollen in NMP for 1 h. Deprotection was performed with 20% piperidine in NMP (30 s, 50 °C, 25 W; then 180 s, 50 °C, 25 W). For the coupling, a solution of the amino acid (5.0 eq.; final concentration: 0.2 M), HOBt (4.9 eq.) and DIC (5.0 eq.) in NMP was used (2 · 10 min, 40 °C, 20 W). 20% Acetic anhydride in NMP was used for capping (2 · 5 min, no microwave). The resin was washed with NMP (5 · 1 mL/10 µmol), DCM (5 · 1 mL/10 µmol) and NMP (5 · 1 mL/10 µmol) between each step.

GP2: Estimation of the resin loading

The resin loading was estimated by measuring the UV absorption. The loaded resin (5–10 mg) was placed in a graduated flask (10 mL), and 2 mL of a 2% solution of DBU in DMF was added. After gentle shaking for 2 h, the flask was filled to 10 mL with MeCN. The solution was further diluted with MeCN (1/10) and transferred to a UV cuvette (d = 1 cm). The absorption of the cleaved Fmoc-dibenzofulven species was detected at 304 nm with a *JASCO V-550* UV/VIS spectrometer. The resin loading L was calculated on basis of the *Lambert-Beer's* law.

$$L = \frac{(A - A_0) \cdot 100\,mL}{\varepsilon_{304} \cdot d \cdot m}$$

with A = absorption of the probe, A_0 = absorption of the blank probe, ε_{304} = extinction coefficient at 304 nm (7624 $Lmol^{-1}cm^{-1}$), d = diameter of the cuvette [cm] and m = mass of the loaded resin [mg].[144]

GP3: Manual solid phase peptide synthesis

Solid phase peptide synthesis was carried out in *BD* syringes according to Fmoc-strategy at room temperature. Unless otherwise noted, the reactions were carried out on a preloaded WANG resin. The resin was swollen for 1 h in NMP before starting. The Fmoc cleavage of the resin-bound amino acid was performed with 20% piperidine in NMP (2 · 10 min, 1 mL/10 µmol) and washed with NMP (5 · 1 mL/10 µmol), DCM (5 · 1 mL/10 µmol) and NMP (5 · 1 mL/10 µmol). Further coupling of the amino acid building blocks was performed

after the following protocol: *Coupling*: After preactivation of the building block (5.0 eq./ final concentration: 0.25 mol/L) with HBTU (4.9 eq.), HOBt (5.0 eq.) and DIEA (10 eq.), the solution was added to the resin and agitated (45 min). Afterwards, the resin was washed with NMP (5 · 1 mL/10 µmol), DCM (5 · 1 mL/10 µmol) and NMP (5 · 1 mL/10 µmol). *Capping*: After treatment with acetic anhydride/NMP (2:8, 2 · 5 min, 1 mL/10 µmol), the resin was washed with NMP (5 · 1 mL/10 µmol), DCM (5 · 1 mL/10 µmol) and NMP (5 · 1 mL/10 µmol). *Fmoc cleavage*: 20% piperidine in NMP (2 · 10min, 1 mL/10 µmol) was added to the resin and then, the resin was washed with NMP (5 · 1 mL/10 µmol), DCM (5 · 1 mL/10 µmol) and NMP (5 · 1 mL/10 µmol).

GP3-2: Manual solid phase synthesis of branched peptides

Synthesis of the linear peptide part was carried out following GP3. The branching point was marked by Fmoc-Lys(Mmt)-OH, Fmoc-Orn(Mtt)-OH or Fmoc-Dap(Mtt)-OH, respectively. Side chain deprotection was performed with DCM/TIS/TFA (94:5:1, 1 mL/10 µmol, 3 · 2 min). After each step, the resin was washed with DCM (10 · 1 mL/10 µmol) and after the final deprotection step with NMP (5 · 1 mL/10 µmol), DCM (5 · 1 mL/10 µmol) and NMP (5 · 1 mL/10 µmol). Then, Boc-Cys(Trt)-OH (5.0 eq.) or Boc-Cys(S*t*Bu)-OH (5.0 eq.) was coupled to the side chain with PyBOP (5.0 eq.) and DIEA (10 eq.) (45 min). Afterwards, the resin was washed with NMP (5 · 1 mL/10 µmol), DCM (5 · 1 mL/10 µmol) and NMP (5 · 1 mL/10 µmol). The further synthesis of the main chain was performed following GP3.

GP4: Manual solid phase PNA synthesis

Solid phase chemistry was carried out in *BD* syringes according to Fmoc/Bhoc-strategy at room temperature. The reactions were carried out on a RINK amide MBHA resin, which was preloaded with Fmoc-Lys(Boc)-OH. The resin was swollen for 1 h in NMP before starting. The Fmoc cleavage of the resin-bound amino acid was performed with 20% piperidine in NMP (2 · 10 min, 1 mL/10 µmol) and the resin was washed with NMP (5 · 1 mL/10 µmol), DCM (5 · 1 mL/10 µmol) and NMP (5 · 1 mL/10 µmol). Further coupling of the PNA building blocks was performed after the following protocol: *Coupling*: After preactivation of the building block (5.0 eq.; final concentration: 0.25 mol/L) with HATU (4.9 eq.), HOAt (5.0 eq.), DIEA (10 eq.) and 2,6-lutidine (10 eq.), the solution was added to the resin and shaken (2 · 45 min). After each step, the resin was washed with 5% DIEA in NMP

(5 · 1 mL/10 µmol), DCM (5 · 1 mL/10 µmol) and NMP (5 · 1 mL/10 µmol). *Capping*: After treatment with acetic anhydride/2,6-lutidine/NMP (1:2:7, 1 mL/10 µmol, 2 · 5 min), the resin was washed with 5% DIEA in NMP (5 · 1 mL/10 µmol), diethyl ether (5 · 1 mL/10 µmol) and NMP (5 · 1 mL/10 µmol). *Fmoc cleavage*: 20% piperidine in NMP (2 · 4 min, 1 mL/10 µmol) was added to the resin and then, the resin was washed with NMP (5 · 1 mL/10 µmol), DCM (5 · 1 mL/10 µmol) and NMP (5 · 1 mL/10 µmol).

GP5: Solid phase alkyne functionalization of PNA

Coupling: Propiolic acid (1.2 eq.; final concentration: 0.05 mol/L) was dissolved in DMF and EEDQ (1.2 eq.) was added. The resulting solution was added to the resin-bound PNA and shaken at room temperature. After 12 h, the resin was washed with DMF (5 · 1 mL/10 µmol) and DCM (5 · 1 mL/10 µmol). *Test cleavage*: The cleavage cocktail (1.0 mL) was added to the resin (1.0 mg). After 1.5 h, the TFA solution was concentrated *in vacuo* before addition of cold diethylether. The resulting precipitate was washed with cold diethylether (5x).

GP6: Attachment of PCL2 to peptides

Coupling: The caging group **19** (2.0 eq.), $DIEA_{abs}$ (5.0 eq.) and DMF_{abs} (1 mL/20 µmol) were added to the resin-bound peptide (1.0 eq.). The mixture was shaken for 18 h at room temperature. The resin was washed with DMF (5 · 1 mL/20 µmol) and DCM (5 · 1 mL/20 µmol). *Cleavage*: The cleavage cocktail was added to the resin. After 1.5 h, the resin was washed with TFA (3 · 1 mL/30 µmol) and the combined TFA solutions were concentrated *in vacuo* before addition of cold diethylether. The resulting precipitate was washed with cold diethylether (5x). The crude product was purified by HPLC (Method B). Products were marked with * when diastereomers were separated on HPLC.

GP7: Click reactions (Table 1)

Acetylene functionalized resin-bound PNA (1.0 eq.), the caging group containing peptide (2.0 eq.), CuI (1.4 eq.) and NaAsc (1.5 eq.) were suspended in degassed DMF_{abs} (1 mL/10 µmol). The mixture was shaken for 48 h at room temperature. The resin was washed with DMF (5 · 1 mL/10 µmol), diethyl ether (5 · 1 mL/10 µmol) and DCM (5 · 1 mL/10 µmol). Then, the cleavage cocktail was added to the resin. After 1.5 h, the resin was washed with TFA (3 · 1 mL/10 µmol) and the combined TFA solutions were

concentrated *in vacuo* before addition of cold diethylether. The resulting precipitate was washed with cold diethylether (5x), dissolved in MeCN/H_2O (1:1) and purified by HPLC (Method A). Products were marked with * when diastereomers were separated on HPLC.

GP8: Thioester formation (Table 2)[114]

The thioester precursor was dissolved in degassed buffer (1.0 ml/0.5 µmol; 10 mM NaH_2PO_4, 100 mM NaCl, 20 mM TCEP) containing 5% MPA (v/v). The pH was adjusted to 3 and the solution was stirred for 48 h at 37°C. The solvent was removed *in vacuo* and the crude product was purified by HPLC (method A).

GP9: Template-directed native chemical ligation (Table 3)

The thioester building block (100 nmol, 1.0 eq.) and the thiol building block (100 nmol, 1.0 eq.) were dissolved in 1.0 mL degassed buffer (10 mM NaH_2PO_4, 100 mM NaCl, 20 mM TCEP, pH 7.0) and stirred under argon atmosphere and light exclusion at room temperature. The progress was monitored by LC-MS. If the thiol building block was equipped with an S*t*Bu protecting group, it was stirred for 30 min in the buffer before the thioester building block was added. The crude product was purified by HPLC (method A).

GP10: PNA/PNA melting curve measurements

Stock solutions of PNA oligomers in HEPES buffer (4.0 µm in 100 mM NaCl, 20 mM HEPES, 1.0 mM EDTA, pH 7.4) were added to a UV cuvette and the UV thermal melting analysis was recorded at 260 nm applying the following program:

20 °C → 80 °C (15 min) → 80 °C (2 min) → 0 °C (25 min) → 0 °C (3 min) → 80 °C (120 min) → 80 °C (2 min) → 20 °C (10 min)

with a heating rate of 0.4 °C/min. The hyperchromicity *H*(%) was calculated according to the following equation:

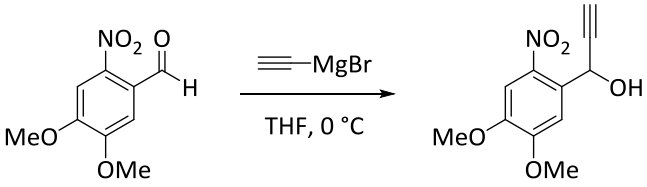
$$H(\%) = \frac{A(T) - A_0}{A_0} \times 100\%$$

with *A*(T) (absorbance at any given temperature) and A_0 (minimum absorbance). The melting temperature T_m is the point of inflection of the resulting curve.

4.3 Synthetic Procedures

4.3.1 Photocleavable Linker

Synthesis of 1-(4,5-Dimethoxy-2-nitrophenyl)prop-2-yn-1-ol (7)[88]



6 **7**

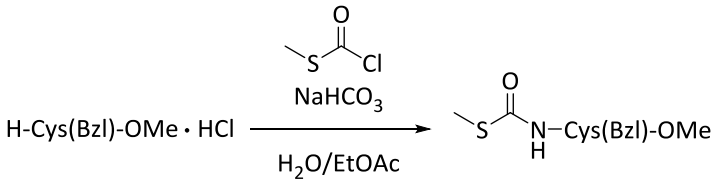
4,5-Dimethoxy-2-nitrobenzaldehyde (2.00 g, 9.47 mmol, 1.0 eq.) was dissolved in THF_{abs} and cooled to 0 °C. Then, ethynylmagnesium bromide (0.5 M in THF, 28.4 mL, 14.2 mmol, 1.5 eq.) was added slowly and the solution was stirred for 2 h at 0 °C. After that, the solution was allowed to warm to room temperature and stirred for another 16 h. The reaction was quenched with aqueous saturated NH_4Cl solution (20 mL) and THF was removed *in vacuo*. The residue was dissolved in EtOAc (200 mL) and washed with water (100 mL) and brine (100 mL). The organic layer was dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to give 2.16 g (9.09 mmol, 96%) of a yellow solid.

^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.61 (s, 1H, CH_{ar}), 7.43 (s, 1H, CH_{ar}), 6.41 (d, $^3J_{H\text{-}H}$ = 5.7 Hz, 1H, O*H*), 5.94 (dd, $^3J_{H\text{-}H}$ = 5.7 Hz,$^4J_{H\text{-}H}$ = 2.1 Hz, 1H, Ph-C*H*), 3.91 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3) 3.39 (d, $^3J_{H\text{-}H}$ = 2.1 Hz, 1H, CC*H*).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 153.0 (*C*H_{ar}), 147.7 (*C*H_{ar}), 139.0 (*C*H_{ar}), 131.5 (*C*H_{ar}), 109.3 (*C*H_{ar}), 108.1 (*C*H_{ar}), 83.9 (*alkyne-C*), 74.8 (*alkyne-C*H), 58.5 (*C*H-OH), 56.1 (O*C*H_3), 56.0 (O*C*H_3).

ESI (HRMS): calcd. for $C_{11}H_{11}NO_5Na^+$ $[M+Na]^+$: 260.0529, found: 260.0530; calcd. for $C_{11}H_{10}NO_5^-$ $[M–H]^-$: 236.0564, found: 236.0558.

Synthesis of *S*-Methyl thiocarbonyl Cys(Bzl)-OMe (9)



8 **9**

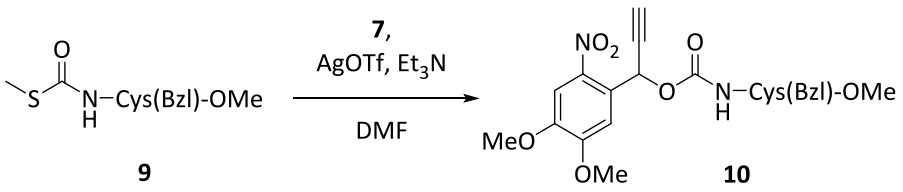
H-Cys(Bzl)-OMe · HCl (0.50 g, 1.91 mmol, 1.0 eq.) was dissolved in a saturated aqueous solution of $NaHCO_3$ and EtOAc (1:1, 30 mL) and methyl chlorothiolformate (162 µL, 0.21 g, 1.91 mmol, 1.0 eq.) was added. The resulting mixture was stirred for 30 min at room temperature and then, the reaction was quenched with methanol. The organic layer was dried over $MgSO_4$, filtered and the solvent was removed *in vacuo* to give 0.55 g (1.83 mmol, 96%) of a colorless solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.33–7.20 (m, 5H, C*H*$_{ar}$), 6.13 (d, $^3J_{H\text{-}H}$ = 7.3 Hz, 1H, N*H*), 4.80–4.74 (m, 1H, α-C*H*), 3.73 (s, 3H, C*H*$_3$), 3.69 (s, 2H, C*H*$_2$), 2.90 (dd, $^2J_{H\text{-}H}$ = 14.0 Hz,$^3J_{H\text{-}H}$ = 5.0 Hz, 1H, β-C*H*$_2^d$), 2.82 (dd, $^2J_{H\text{-}H}$ = 14.0 Hz,$^3J_{H\text{-}H}$ = 5.0 Hz, 1H, β-C*H*$_2^u$), 2.34 (s, 3H, C*H*$_3$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.7 (*C*O_2Me), 168.0 (S-*C*O-N), 137.4 (*C*H$_{ar}$), 128.8 (*C*H$_{ar}$), 128.5 (*C*H$_{ar}$), 127.2 (*C*H$_{ar}$), 53.3 (α-*C*H), 52.7 (CO_2-*C*H$_3$), 36.5 (Ph-*C*H$_2$), 33.2 (β-*C*H$_2$), 12.3 (S-*C*H$_3$).

ESI (HRMS): calcd. for $C_{13}H_{18}NO_3S_2^+$ $[M+H]^+$: 300.0723, found: 300.0725; calcd. for $C_{13}H_{17}NO_3S_2Na^+$ $[M+Na]^+$: 322.0542, found: 322.0543; calcd. for $C_{13}H_{16}NO_3S_2^-$ $[M–H]^-$: 298.0577, found: 298.0577.

Synthesis of PCL1-Cys(Bzl)-OMe (10)



1-(4,5-Dimethoxy-2-nitrophenyl)prop-2-yn-1-ol (**7**, 0.20 g, 0.84 mmol, 1.00 eq.) was dissolved in DMF_{abs} (20 mL) and then, consecutively silver triflate (0.28 g, 1.10 mmol, 1.30 eq.), *S*-methyl thiocarbonyl Cys(Bzl)-OMe (0.27 g, 0.89 mmol, 1.05 eq.) and Et_3N (158 µL, 1.14 mmol, 1.35 eq.) were added. The reaction mixture was stirred for 17 h at room temperature. Afterwards, the mixture was filtered through Celite® and washed with EtOAc (150 mL). The organic layer was washed with aqueous saturated $NaHCO_3$ solution (2 · 80 mL), H_2O (80 mL) and brine (80 mL), dried over $MgSO_4$, filtered and the solvent was removed *in vacuo* to give 0.41 g (0.84 mmol, quant.) of a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.62 (d, *diastereomers*, 1H, C*H*$_{ar}$), 7.32–7.18 (m, 6H, C*H*$_{ar}$), 7.07 (dd, $^4J_{H\text{-}H}$ = 2.4 Hz + *diastereomers*, 1H, Ph-C*H*), 5.68–5.65 (m, 1H, N*H*), 4.58–4.50 (m, 1H, α-C*H*), 3.98 (d, *diastereomers*, 3H, OC*H*$_3$), 3.92 (d, *diastereomers*, 3H, OC*H*$_3$), 3.72–3.68 (m, 5H, Ph-C*H*$_2$ + CO_2C*H*$_3$), 2.91–2.76 (m, 2H, β-C*H*$_2$), 2.63 (dd, $^4J_{H\text{-}H}$ = 2.4 Hz + *diastereomers*, 1H, CC*H*).

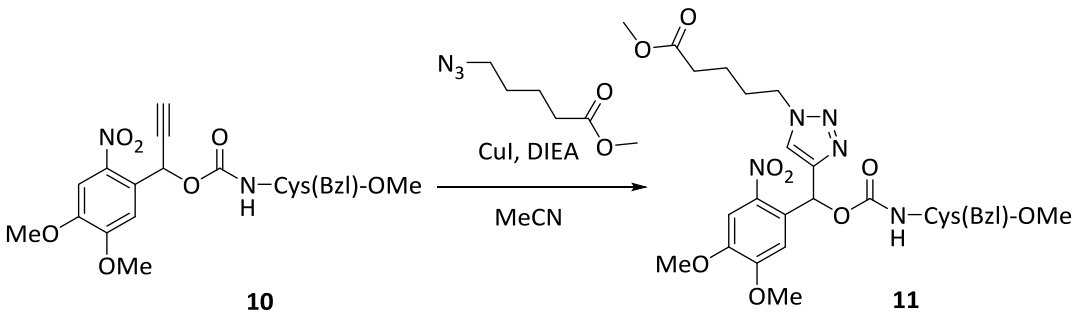
^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.9 (*C*O_2Me), 170.9 (*C*O_2Me*), 156.1 (O-*C*O-N), 153.8 (*C*H$_{ar}$), 153.4 (*C*H$_{ar}$*), 148.9 (*C*H$_{ar}$), 148.8 (*C*H$_{ar}$*), 139.9 (*C*H$_{ar}$), 139.8 (*C*H$_{ar}$*), 137.9 (*C*H$_{ar}$), 137.4 (*C*H$_{ar}$*), 128.9 (*C*H$_{ar}$), 128.9 (*C*H$_{ar}$*), 128.8 (*C*H$_{ar}$), 128.6 (*C*H$_{ar}$*), 128.5 (*C*H$_{ar}$), 128.5 (*C*H$_{ar}$*), 127.3 (*C*H$_{ar}$), 127.2 (*C*H$_{ar}$*), 110.0 (*C*H$_{ar}$), 109.7 (*C*H$_{ar}$*), 108.1 (*C*H$_{ar}$), 79.4 (*C*CH), 75.5 (C*C*H), 75.3 (C*C*H*), 62.7 (Ph-*C*H), 62.6 (Ph-*C*H*), 56.5 (O*C*H$_3$), 56.4 (O*C*H$_3$), 53.5 (α-*C*H), 53.4 (α-*C*H*), 52.7 (CO_2-*C*H$_3$), 52.6 (CO_2-*C*H$_3$*), 36.7 (Ph-*C*H$_2$), 36.5 (Ph-*C*H$_2$*), 33.5 (β-*C*H$_2$), 33.3 (β-*C*H$_2$*).

** Second set of signals due to different diastereomers.*

TLC: R_f = 0.73 (pentane/EtOAc 1:1).

ESI (HRMS): calcd. for $C_{23}H_{28}N_3O_8S^+$ $[M+NH_4]^+$: 506.1592, found: 506.1584; calcd. for $C_{23}H_{24}N_2O_8SNa^+$ $[M+Na]^+$: 511.1146, found: 511.1138; calcd. for $C_{23}H_{23}N_2O_8S^-$ $[M–H]^-$: 487.1181, found: 487.1180.

Synthesis of Methyl 5-(4-{PCL1-Cys(Bzl)-OMe}-1*H*-1,2,3-triazol-1-yl)pentanoate (11)



PCL1-Cys(Bzl)-OMe (**10**, 213 mg, 0.46 mmol, 1.0 eq.) was dissolved in degassed MeCN (10 mL). Then, DIEA (78 µL, 59 mg, 0.46 mmol, 1.0 eq.), CuI (9 mg, 0.05 mmol, 0.1 eq.) and methyl 5-azidopentanoate (87 mg, 0.55 mmol, 1.2 eq.) were added and the resulting solution was stirred for 18 h at room temperature. The solvent was removed *in vacuo* and the crude product was purified by column chromatography on silica (pentane/EtOAc 3:1) to give 215 mg (0.33 mmol, 73%) of a yellow solid.

^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.22 (d, $^{3}J_{H\text{-}H}$ = 8.4 Hz, 1H, N*H*), 8.09 (s, 1H, CH_{ar}), 7.69 (d, *diastereomers*, 1H, CH_{ar}), 7.41–7.17 (m, 7H, 6x CH_{ar} + Ph-C*H*), 4.35–4.20 (m, 3H, CH_2 + α-C*H*), 3.98–3.57 (m, 14H, 2x OCH_3 + 2x CO_2CH_3 + Ph-CH_2), 2.87–2.62 (m, 2H, β-CH_2), 2.35–2.29 (m, 2H, CH_2), 1.85–1.75 (m, 2H, CH_2), 1.51–1.41 (m, 2H, CH_2).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 172.9 (*C*O_2Me), 170.9 (*C*O_2Me), 154.8 (N-*C*O-O), 154.7 (N-*C*O-O*), 153.7 (*C*H_{ar}), 147.9 (*C*H_{ar}), 144.6 (*C*H_{ar}), 144.5 (*C*H_{ar}*), 139.1 (*C*H_{ar}), 139.1 (*C*H_{ar}*), 138.0 (*C*H_{ar}), 137.8 (*C*H_{ar}*), 129.5 (*C*H_{ar}), 128.8 (*C*H_{ar}), 128.7 (*C*H_{ar}*), 128.3 (*C*H_{ar}), 128.2 (*C*H_{ar}*), 126.8 (*C*H_{ar}), 126.8 (*C*H_{ar}*), 124.0 (*C*H_{ar}), 109.4 (*C*H_{ar}), 109.4 (*C*H_{ar}*), 108.0 (*C*H_{ar}), 65.9 (Ph-*C*H), 65.8 (Ph-*C*H*), 56.1 (O*C*H_3), 56.0 (O*C*H_3), 53.6 (α-*C*H), 53.4 (α-*C*H*), 52.1 (CO_2-*C*H_3), 52.0 (CO_2-*C*H_3*), 51.1 (CO_2-*C*H_3), 49.0 (*C*H_2), 35.2 (Ph-*C*H_2), 35.1 (Ph-*C*H_2*), 32.4 (*C*H_2), 32.4 (β-*C*H_2), 28.9 (*C*H_2), 21.2 (*C*H_2).

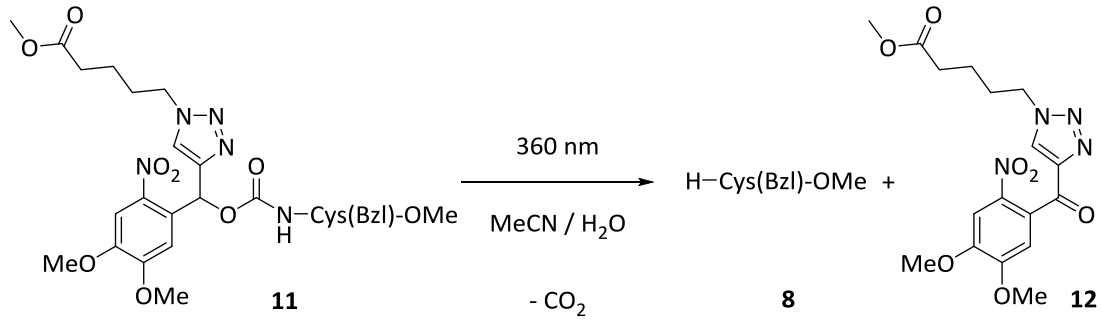
**Second set of signals due to different diastereomers.*

TLC: R_f = 0.43 (pentane/EtOAc 3:1).

HPLC (Method D): t_R = 22.58 min.

ESI (HRMS): calcd. for $C_{29}H_{36}N_5O_{10}S^+$ $[M+H]^+$: 646.2177, found: 646.2173; calcd. for $C_{29}H_{35}N_5O_{10}SNa^+$ $[M+Na]^+$: 668.1997, found: 668.1998; calcd. for $C_{29}H_{34}N_5O_{10}S^-$ $[M–H]^-$: 644.2032, found: 644.2029.

Uncaging of Methyl 5-(4-{PCL1-Cys(Bzl)-OMe}-1*H*-1,2,3-triazol-1-yl)pentanoate (11)

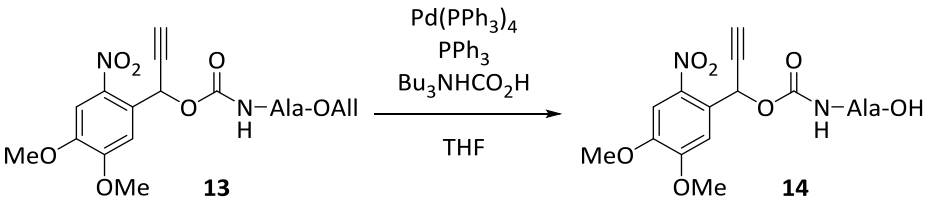


Methyl 5-(4-{PCL1-Cys(Bzl)-OMe}-1*H*-1,2,3-triazol-1-yl)pentanoate (**11**, 1.5 mg, 2.32 µmol) was dissolved in MeCN/H_2O 1:1 (1 mL) and irradiated for 30 min at 360 nm [lamp setup: *a)*].

HPLC (Method D): t_R = 11.19 min (**8**), 18.15 min (**12**).

ESI (HRMS): calcd. for $C_{11}H_{16}NO_2S^+$ [M(**8**)+H]$^+$: 226.0896, found: 226.0897; calcd. for $C_{17}H_{21}N_4O_7^+$ [M(**12**)+H]$^+$: 393.1405, found: 393.1397; calcd. for $C_{17}H_{20}N_4O_7Na^+$ [M(**12**)+Na]$^+$: 415.1224, found: 415.1217.

Synthesis of PCL1-Ala-OH (14)[145]



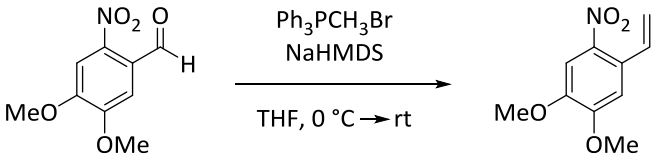
$Pd(PPh_3)_4$ (45 mg, 0.04 mmol, 3.5 mol%) and PPh_3 (288 mg, 1.10 mmol, 1.0 eq.) were dissolved in degassed THF (5 mL) and stirred for 10 min at room temperature. Then, Bu_3NHCO_2H (1.2 M in THF, 3.67 mL, 4.40 mmol, 4.0 eq.) and PCL1-Ala-OAll (in 10 mL THF, 430 mg, 1.10 mmol, 1.0 eq.) were added and the solution was stirred for 1 h at room temperature. The solvent was removed under reduced pressure, the residue was dissolved in Et_2O (100 mL) and extracted with aqueous saturated $NaHCO_3$ (2 · 100 mL). The aqueous solution was adjusted to pH 2 with 2 M HCl and then, extracted with EtOAc solution (2 · 100 mL). The organic layer was dried over $MgSO_4$, filtered and the solvent was removed *in vacuo* to give 310 mg (0.88 mmol, 80%) of a yellow solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.62 (d, *diastereomers*, 1H, CH_{ar}), 7.29–7.22 (m, 1H, CH_{ar}), 7.07 (d, $^4J_{H\text{-}H}$ = 2.1 Hz, 1H, Ph-C*H*), 5.40 (d, $^3J_{H\text{-}H}$ = 6.7 Hz, 1H, N*H*), 4.44–4.34 (m, 1H, α-C*H*), 3.99 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 2.64 (dd, $^4J_{H\text{-}H}$ = 2.1 Hz + *diastereomers*, 1H, CC*H*), 1.49–1.43 (m, 3H, β-CH_3).

TLC: R_f = 0.73 (pentane/EtOAc 1:1).

ESI (HRMS): calcd. for $C_{15}H_{20}N_3O_8^+$ $[M+NH_4]^+$: 370.1245, found: 370.1251; calcd. for $C_{15}H_{16}N_2O_8Na^+$ $[M+Na]^+$: 375.0799, found: 375.0809; calcd. for $C_{15}H_{15}N_2O_8^-$ $[M–H]^-$: 351.0834, found: 351.0833.

Synthesis of 1,2-Dimethoxy-4-nitro-5-vinylbenzene (16)



15 **16**

Methyl(triphenyl)phosphonium bromide (4.39 g, 12.3 mmol, 1.3 eq.) was suspended in THF_{abs} (20 mL) and cooled to 0 °C. Then, sodium bis(trimethylsilyl)amide (1.0 M in THF, 12.3 mL, 12.3 mmol, 1.3 eq.) was added dropwise and the reaction mixture was stirred for 1 h at 0 °C. 4,5-Dimethoxy-2-nitrobenzaldehyde (2.00 g in 20 mL THF_{abs}, 9.47 mmol, 1.0 eq.) was added and the reaction mixture was allowed to warm to room temperature. The reaction mixture was stirred for 18 h and then quenched with aqueous saturated NH_4Cl solution (50 mL). THF was removed under reduced pressure and the residue was extracted with DCM (100 mL). The organic layer was dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica (pentane/EtOAc 4:1) to give 1.23 g (5.88 mmol, 62%) of a yellow solid.

IR (neat, cm^{-1}): 3010 (w), 2947 (w), 2839 (w), 1569 (m), 1510 (s), 1322 (s), 1264 (s), 1218 (s), 1074 (m), 1040 (s), 972 (m), 792 (m).

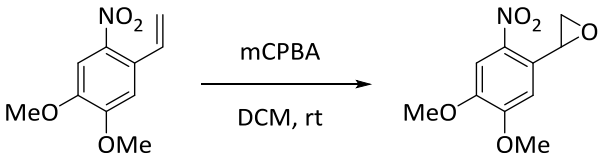
^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.56 (s, 1H, CH_{ar}), 7.28 (dd, $^3J_{H\text{-}H}$ = 17.2 Hz, 10.9 Hz, 1H, Ph-C*H*), 6.95 (s, 1H, CH_{ar}), 5.62 (dd, $^3J_{H\text{-}H}$ = 17.2 Hz, $^2J_{H\text{-}H}$ = 1.0 Hz, 1H, CH-CH_2^d), 5.42 (dd, $^3J_{H\text{-}H}$ = 10.9 Hz, $^2J_{H\text{-}H}$ = 1.0 Hz, 1H, CH-CH_2^u), 3.98 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 153.2 (*C*H_{ar}), 148.5 (*C*H_{ar}), 140.2 (*C*H_{ar}), 133.7 (*alkene-C*), 128.9 (*C*H_{ar}), 117.6 (*alkene-C*), 109.8 (*C*H_{ar}), 107.7 (*C*H_{ar}), 56.5 (O*C*H_3), 56.5 (O*C*H_3).

TLC: R_f = 0.60 (pentane/EtOAc 4:1).

HRMS (ESI): calcd. for $C_{10}H_{12}NO_4^+$ $[M+H]^+$: 210.0761, found 210.0767, calcd. for $C_{10}H_{11}NO_4Na^+$ $[M+Na]^+$: 232.0580, found 232.0586.

Synthesis of 2-(4,5-Dimethoxy-2-nitrophenyl)oxirane (17)



16 **17**

To a solution of 1,2-dimethoxy-4-nitro-5-vinylbenzene (**16**, 0.80 g, 3.82 mmol, 1.0 eq.) in DCM (20 mL), *meta*-chloroperbenzoic acid (70% w/w, 1.41 g in 20 mL DCM, 5.72 mmol, 1.5 eq.) was added slowly. The resulting mixture was stirred for 24 h at room temperature. Then, the organic phase was washed with saturated Na_2SO_4 solution (2 · 50 mL), aqueous saturated $NaHCO_3$ solution (2 · 50 mL) and brine (2 · 50 mL). The organic layer was dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica (pentane/EtOAc 5:1) to give 0.77 g (3.40 mmol, 89%) of a yellow solid.

IR (neat, cm^{-1}): 3084 (w), 2835 (w), 1576 (m), 1506 (s), 1319 (m), 1279 (s), 1261 (s), 1221 (s), 1063 (m), 1004 (m).

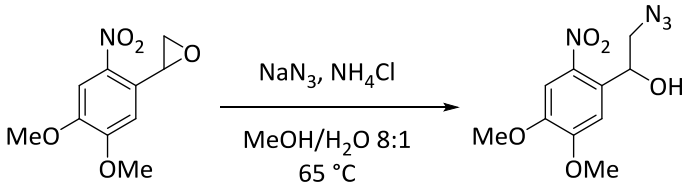
^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.71 (s, 1H, C*H*$_{ar}$), 7.02 (s, 1H, C*H*$_{ar}$), 4.51 (dd, $^3J_{H\text{-}H}$ = 4.4 Hz, 2.6 Hz, 1H, Ph-C*H*), 3.96 (s, 3H, OC*H*$_3$), 3.94 (s, 3H, OC*H*$_3$), 3.28 (dd, $^2J_{H\text{-}H}$ = 5.5 Hz, $^3J_{H\text{-}H}$ = 4.4 Hz, 1H, CH-C*H*$_2^d$), 2.63 (dd, $^2J_{H\text{-}H}$ = 5.5 Hz, $^3J_{H\text{-}H}$ = 2.6 Hz, 1H, CH-C*H*$_2^u$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 154.3 (*C*H$_{ar}$), 148.3 (*C*H$_{ar}$), 140.2 (*C*H$_{ar}$), 129.9 (*C*H$_{ar}$), 108.1 (*C*H$_{ar}$), 107.9 (*C*H$_{ar}$), 56.6 (O*C*H$_3$), 56.5 (O*C*H$_3$), 51.3 (*epoxide-C*), 50.7 (*epoxide-C*).

TLC: R_f = 0.41 (pentane/EtOAc 5:1).

HRMS (ESI): calcd. for $C_{10}H_{12}NO_5^+$ $[M+H]^+$: 226.0710, found 226.0714, calcd. for $C_{10}H_{11}NO_5Na^+$ $[M+Na]^+$: 248.0529, found 248.0534.

Synthesis of 2-Azido-1-(4,5-dimethoxy-2-nitrophenyl)ethanol (18)



17 **18**

To a solution of 2-(4,5-dimethoxy-2-nitrophenyl)oxirane (**17**, 1.20 g, 5.33 mmol, 1.0 eq.) and NH_4Cl (0.57 g, 10.7 mmol, 2.0 eq.) in MeOH/water 8:1 (20 mL) sodium azide (2.78 g, 42.8 mmol, 8.0 eq.) was added. The reaction mixture was heated to 65 °C and stirred for 6 h. The solvent was removed under reduced pressure, the residue was diluted with water (30 mL) and extracted with Et_2O (3 · 30 mL). The organic layer was dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica (pentane/EtOAc 5:1) to give 0.74 g (2.76 mmol, 52%) of a yellow solid.

IR (neat, cm^{-1}): 3513 (m), 3000 (w), 2850 (w), 2094 (s), 1576 (m), 1517 (s), 1322 (s), 1264 (s), 1208 (s), 1082 (s), 1031 (s).

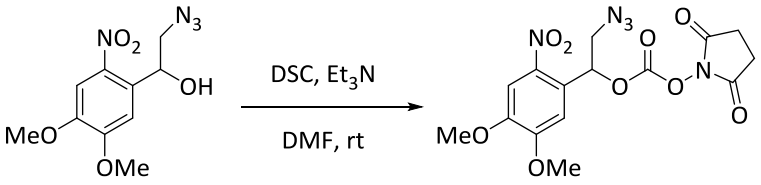
^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.64 (s, 1H, C*H*$_{ar}$), 7.36 (s, 1H, C*H*$_{ar}$), 5.65 (dd, $^3J_{H\text{-}H}$ = 7.6 Hz, 2.8 Hz, 1H, Ph-C*H*), 4.02 (s, 3H, OC*H*$_3$), 3.95 (s, 3H, OC*H*$_3$), 3.74 (dd, $^2J_{H\text{-}H}$ = 12.5 Hz, $^3J_{H\text{-}H}$ = 2.8 Hz, 1H, C*H*$_2$-N_3^d), 3.39 (dd, $^2J_{H\text{-}H}$ = 12.5 Hz, $^3J_{H\text{-}H}$ = 7.6 Hz, 1H, C*H*$_2$-N_3^u).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 154.0 (*C*H$_{ar}$), 148.5 (*C*H$_{ar}$), 139.7 (*C*H$_{ar}$), 131.6 (*C*H$_{ar}$), 109.5 (*C*H$_{ar}$), 108.0 (*C*H$_{ar}$), 69.4 (Ph-*C*H), 57.3 (*C*H$_2$-N$_3$), 56.7 (O*C*H$_3$), 56.6 (O*C*H$_3$).

TLC: R_f = 0.15 (pentane/EtOAc 5:1).

HRMS (ESI): calcd. for $C_{10}H_{16}N_5O_5^+$ $[M+NH_4]^+$: 286.1146, found 286.1146, calcd. for $C_{10}H_{12}N_4O_5Na^+$ $[M+Na]^+$: 291.0700, found 291.0705, calcd. for $C_{10}H_{11}N_4O_5^-$ $[M-H]^-$: 267.0735, found 267.0738.

Synthesis of 2-Azido-1-(4,5-dimethoxy-2-nitrophenyl)ethyl 2,5-dioxo-pyrrolidin-1-yl carbonate (19)



18 **19**

To a solution of 2-azido-1-(4,5-dimethoxy-2-nitrophenyl)ethanol (**18**, 0.32 g, 1.19 mmol, 1.0 eq.) and *N,N'*-disuccinimidyl carbonate (0.50 g, 1.96 mmol, 1.6 eq.) in DMF (7 mL) Et_3N (0.50 mL, 3.57 mmol, 3.0 eq.) was added. The reaction mixture was stirred for 20 h at room temperature. Then, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica (pentane/EtOAc 1:1) to give 0.40 g (0.97 mmol, 81%) of a yellow solid.

IR (neat, cm^{-1}): 2103 (m), 1800 (m), 1781 (m), 1739 (s), 1576 (m), 1336 (m), 1276 (s), 1221 (s), 1059 (s), 1025 (s).

UV VIS (MeCN, nm): λ_{max} = 347, 304, 244, 221, 205.

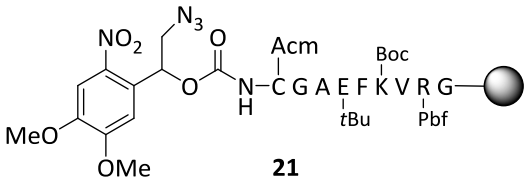
^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.68 (s, 1H, C*H*$_{ar}$), 7.09 (s, 1H, C*H*$_{ar}$), 6.53 (dd, $^3J_{H\text{-}H}$ = 6.3 Hz, 2.7 Hz, 1H, Ph-C*H*), 4.08 (s, 3H, OC*H*$_3$), 3.98–3.83 (m, 4H, OC*H*$_3$ + C*H*$_2$-N$_3^d$), 3.72 (dd, $^2J_{H\text{-}H}$ = 13.6 Hz, $^3J_{H\text{-}H}$ = 6.3 Hz, 1H, C*H*$_2$-N$_3^u$), 2.80 (s, 4H, C*H*$_2$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 168.3 (*C*ON), 154.4 (O-*C*O-O), 150.6 (*C*H$_{ar}$), 149.2 (*C*H$_{ar}$), 139.7 (*C*H$_{ar}$), 126.0 (*C*H$_{ar}$), 108.4 (*C*H$_{ar}$), 108.3 (*C*H$_{ar}$), 78.3 (Ph-*C*H), 56.8 (O*C*H$_3$), 56.6 (O*C*H$_3$), 54.2 (*C*H$_2$-N$_3$), 25.5 (*C*H$_2$).

TLC: R_f = 0.41 (pentane/EtOAc 1:1).

HRMS (ESI): calcd. for $C_{15}H_{19}N_6O_9^+$ $[M+NH_4]^+$: 427.1208, found 427.1210, calcd. for $C_{15}H_{15}N_5O_9Na^+$ $[M+Na]^+$: 432.0762, found 432.0759.

Synthesis of PCL2-C(Acm)GAEFKVRG-WANG resin (21)



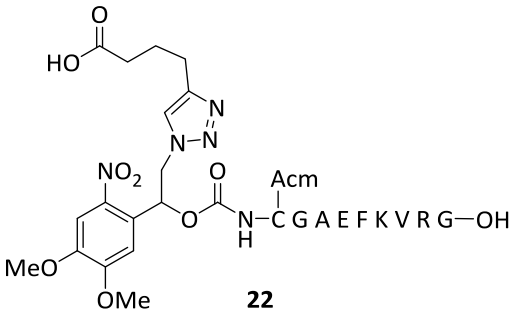
21

The peptide (100 µmol) was coupled to a preloaded WANG resin (glycine, 0.38 mmol/g) following GP3. PCL2 was attached following GP6. Test cleavage was performed with TFA/TIS/H_2O (95:2.5:2.5, 1 mL cleavage solution, 1.5 h).

HPLC (Method C): t_R = 26.058 min.

ESI (HRMS): calcd. for $C_{55}H_{84}N_{18}O_{19}S^{2+}$ $[M+2H]^{2+}$: 666.2935, found: 666.2936; calcd. for $C_{55}H_{83}N_{18}O_{19}S^{+}$ $[M+H]^{+}$: 1331.5797, found: 1331.5795.

Synthesis of PCL2({1*H*-1,2,3-triazol-4-yl}butanoic acid)-C(Acm)GAEFKVRG-OH (22)



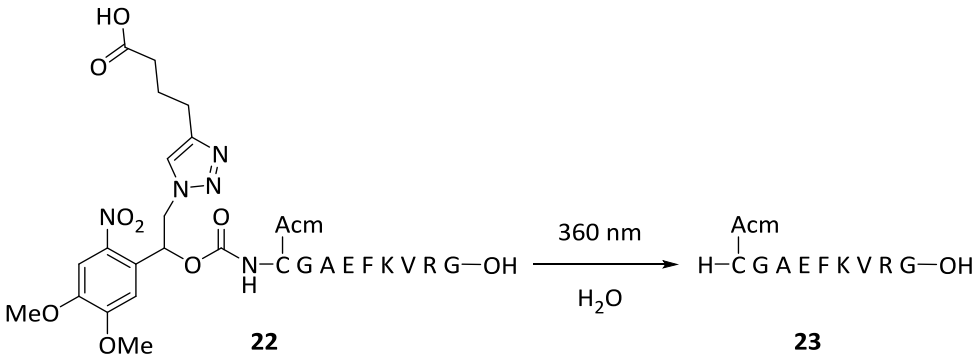
22

Resin-bound **21** (20 µmol, 1.0 eq.), 5-hexynoic acid (4.5 µL, 40 µmol, 2.0 eq.), CuI (5.3 mg, 28 µmol, 1.4 eq.) and NaAsc (5.9 mg, 30 µmol, 1.5 eq.) were suspended in degassed DMF_{abs} (1.5 mL). The mixture was agitated for 18 h at room temperature. The resin was washed with DMF (5 · 1 mL), diethyl ether (5 · 1 mL) and DCM (5 · 1 mL). Then, TFA/TIS/H_2O (95:2.5:2.5, 1 mL/10 µmol) was added to the resin. After 1.5 h, the resin was washed with TFA (3 · 1 mL) and the combined TFA solutions were concentrated *in vacuo*. The resulting precipitate was washed with cold diethylether (5x), dissolved in H_2O/MeCN 3:1 and purified by HPLC (method A).

HPLC (Method C): t_R = 26.025 min.

ESI (HRMS): calcd. for $C_{61}H_{93}N_{18}O_{21}S^{3+}$ $[M+3H]^{3+}$: 481.8822, found: 481.8813; calcd. for $C_{61}H_{92}N_{18}O_{21}S^{2+}$ $[M+2H]^{2+}$: 722.3197, found: 722.3191; calcd. for $C_{61}H_{91}N_{18}O_{21}S^{+}$ $[M+H]^{+}$: 1443.6321, found: 1443.6332.

Uncaging of PCL2({1*H*-1,2,3-triazol-4-yl}butanoic acid)-C(Acm)GAEFKVRG-OH (23)



22 **23**

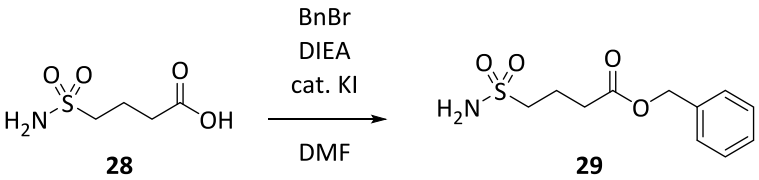
PCL2({1*H*-1,2,3-triazol-4-yl}butanoic acid)-C(Acm)GAEFKVRG-OH (**22**, 0.7 mg, 0.49 µmol) was dissolved in H_2O (1.5 mL) and irradiated for 1 h with UV light [lamp setup: *a)*].

HPLC (Method C): t_R = 21.075 min.

ESI (HRMS): calcd. for $C_{44}H_{74}N_{14}O_{13}S^{2+}$ $[M+2H]^{2+}$: 519.2635, found: 519.2619; calcd. for $C_{44}H_{73}N_{14}O_{13}S^{+}$ $[M+H]^{+}$: 1037.5197, found: 1037.5193.

4.3.2 Thioester Precursor

Synthesis of Sulfamylbutyric acid benzylester (29)[146]

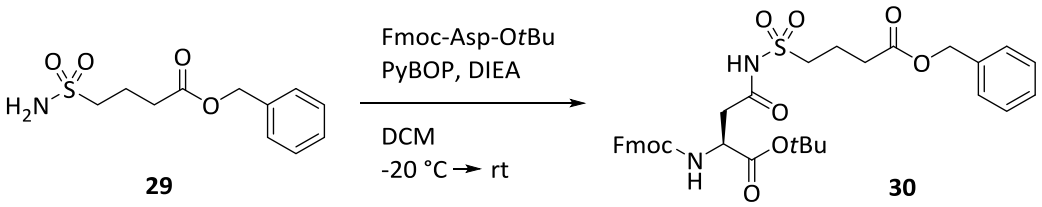


3-Carboxy propane sulfonamide (**28**, 3.00 g, 17.9 mmol, 1.00 eq.), KI (0.89 g, 5.38 mmol, 0.30 eq.) and DIEA (3.20 mL, 2.44 g, 18.8 mmol, 1.05 eq.) were dissolved in DMF (30 mL). Then, benzyl bromide (2.24 mL, 3.22 g, 18.8 mmol, 1.05 eq.) was added slowly and the solution was stirred for 18 h at room temperature. Subsequently, EtOAc (200 mL) was added and the organic layer was washed with H_2O (2 · 100 mL), 1 M HCl (2 · 100 mL) and brine (2 · 100 mL). The organic layer was dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. The crude product was purified by recrystallization from toluene/hexane (1:1) to give 4.08 g (15.9 mmol, 84%) of a colorless solid. The ESI mass spectrometric data were in accordance with the data from literature.[146]

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.39–7.24 (m, 5H, CH_{ar}), 5.19 (s, 2H, NH_2), 5.02 (s, 2H, Bn–CH_2), 3.16 (t, $^3J_{H\text{-}H}$ = 7.2 Hz, 2H, CH_2), 2.52 (t, $^3J_{H\text{-}H}$ = 7.2 Hz, 2H, CH_2), 2.13 (q, $^3J_{H\text{-}H}$ = 7.2 Hz, 2H, CH_2).

^{13}C NMR (75 MHz, $CDCl_3$): δ =172.5 (CH_2-(*C*O)-OBn), 135.6 (*C*H_{ar}), 128.6 (*C*H_{ar}), 128.3 (*C*H_{ar}), 128.2 (*C*H_{ar}), 66.6 (*C*H_2), 53.9 (*C*H_2), 32.0 (*C*H_2), 19.3 (*C*H_2).

Synthesis of Fmoc-Asp(sbab)-O*t*Bu (30)



Sulfamylbutyric acid benzylester (**29**, 2.25 g, 8.78 mmol, 1.00 eq.) was dissolved in DCM_{abs} (100 mL) and cooled to -20 °C. Then, DIEA (4.48 mL, 3.40 g, 26.3 mmol, 3.00 eq.), PyBOP (5.48 g, 10.5 mmol, 1.20 eq.) and Fmoc-Asp-O*t*Bu (3.80 g, 9.22 mmol, 1.05 eq.) were added. The solution was stirred for 2 h at -20 °C and after that, stirred for 40 h at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in EtOAc (200 mL). The organic layer was washed with 1 M HCl (2 · 100 mL) and the aqueous layer was extracted with EtOAc (2 · 30 mL). The combined organic layers were dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica (pentane/EtOAc 2:1 → 1:1) to give 4.46 g (6.85 mmol, 78%) of a colorless solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 10.03 (s, 1H, N*H*-S(O_2)), 7.72 (d, $^3J_{H\text{-}H}$ = 7.4 Hz, 2H, CH_{ar}), 7.58 (d, $^3J_{H\text{-}H}$ = 7.4 Hz, 2H, CH_{ar}), 7.38–7.25 (m, 9H, CH_{ar}), 6.12 (d, $^3J_{H\text{-}H}$ = 8.2 Hz, 1H, N*H*), 5.06 (s, 2H, Bn-CH_2), 4.54–4.49 (m, 1H, α-C*H*), 4.42–4.26 (m, 2H, Fmoc-CH_2), 4.19 (t, $^3J_{H\text{-}H}$ = 7.1 Hz, 1H, Fmoc-C*H*), 3.45 (t, $^3J_{H\text{-}H}$ = 7.2 Hz, 2H, CH_2), 3.42–2.81 (m, 2H, β-CH_2), 2.47 (t, $^3J_{H\text{-}H}$ = 7.2 Hz, 2H, CH_2), 2.11 (q, $^3J_{H\text{-}H}$ = 7.2 Hz, 2H, CH_2), 1.43 (s, 9H, CH_3).

^{13}C NMR (125 MHz, $CDCl_3$): δ =171.9 (CH_2-(*C*O)-O), 171.3 (CH_2-(*C*O)-O), 169.3 (CH_2-(*C*O)-N), 156.1 (O-(*C*O)-N), 143.6 (*C*H_{ar}), 143.5 (*C*H_{ar}), 141.0 (*C*H_{ar}), 135.4 (*C*H_{ar}), 128.4 (*C*H_{ar}), 128.3 (*C*H_{ar}), 128.0 (*C*H_{ar}), 127.6 (*C*H_{ar}), 126.9 (*C*H_{ar}), 125.0 (*C*H_{ar}), 119.8 (*C*H_{ar}), 82.7 (O-*C*(CH_3)$_3$), 67.2 (*C*H_2), 66.4 (*C*H_2), 51.9 (*C*H), 50.7 (*C*H_2), 46.9 (*C*H), 38.22 (*C*H_2), 31.7 (*C*H_2), 27.6 (*C*H_3), 18.4 (*C*H_2).

TLC: R_f = 0.43 (DCM/MeOH 30:1 + 0.5% AcOH).

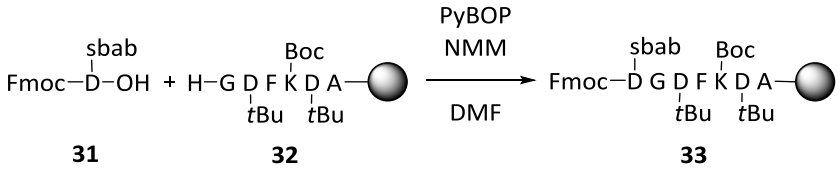
ESI (HRMS): calcd. for $C_{34}H_{38}N_2O_9SNa^+$ $[M+Na]^+$: 673.2190, found: 673.2180; calcd. for $C_{34}H_{37}N_2O_9S^-$ $[M–H]^-$: 649.2225, found: 649.2223.

Synthesis of Fmoc-Asp(sbab)-OH (31)

Fmoc-Asp(sbab)-O*t*Bu (**30**, 555 mg, 0.85 mmol) was dissolved in TFA/TIS/H_2O (95:2.5:2.5) (5 mL) and cooled to 0 °C. The solution was stirred for 1 h at 0 °C and then for 1 h at room temperature. The solvent was concentrated under reduced pressure and the crude product was washed with cold ether (5x) to give 506 mg (0.85 mmol, quant.) of a colorless solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.65 (d, $^3J_{H\text{-}H}$ = 7.4 Hz, 2H, CH_{ar}), 7.52 (d, $^3J_{H\text{-}H}$ = 7.4 Hz, 2H, CH_{ar}), 7.34–7.18 (m, 9H, CH_{ar}), 6.37 (d, $^3J_{H\text{-}H}$ = 8.1 Hz, 1H, N*H*), 4.98 (s, 2H, Bn-CH_2), 4.71–4.62 (m, 1H, α-C*H*), 4.35–4.23 (m, 2H, Fmoc-CH_2), 4.17–4.09 (m, 1H, Fmoc-C*H*), 3.42 (t, $^3J_{H\text{-}H}$ = 7.1 Hz, CH_2), 3.09–2.83 (m, 2H, β-CH_2), 2.41 (t, $^3J_{H\text{-}H}$ = 7.1 Hz, CH_2), 2.10–2.00 (m, 2H, CH_2).
^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.4 (*C*O_2H), 172.6 (*C*O_2-CH_2), 170.6 (*C*O-NH), 156.7 (O-*C*O-NH), 143.6 (*C*H_{ar}), 143.5 (*C*H_{ar}), 141.1 (*C*H_{ar}), 135.4 (*C*H_{ar}), 128.5 (*C*H_{ar}), 128.3 (*C*H_{ar}), 128.1 (*C*H_{ar}), 127.7 (*C*H_{ar}), 127.1 (*C*H_{ar}), 125.1 (*C*H_{ar}), 119.9 (*C*H_{ar}), 67.9 (*C*H_2), 66.7 (*C*H_2), 52.0 (*C*H), 50.2 (*C*H_2), 46.8 (*C*H), 38.1 (*C*H_2), 31.8 (*C*H_2), 18.4 (*C*H_2).
TLC: R_f = 0.07 (DCM/MeOH 30:1 + 0.5% AcOH).
ESI (HRMS): calcd. for $C_{30}H_{30}N_2O_9SNa^+$ $[M+Na]^+$: 617.1564, found: 617.1563; calcd. for $C_{30}H_{29}N_2O_9S^-$ $[M–H]^-$: 593.1599, found: 593.1599.

Synthesis of Fmoc–D(sbab)-G-D(*t*Bu)-F-K(Boc)-D(*t*Bu)-A–2-Cl-Trt resin (33)

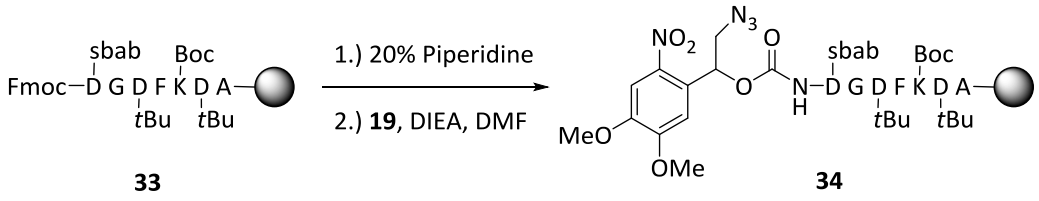


Peptide **32** was synthesized following GP3 (25 µmol, H-Ala-2-Cl-Trt resin, 0.64 mmol/g). Peptide **32** was swollen in DMF for 1 h. Then, Fmoc-Asp(sbab)-OH (60 mg, 100 µmol, 4.0 eq.), PyBOP (52 mg, 100 µmol, 4.0 eq.) and NMM (22 µL, 200 µmol, 8.0 eq.) were dissolved in DMF (0.5 mL), added to the resin and agitated for 1 h. Afterwards, the resin was washed with DMF (8x) and DCM (8x). Test cleavage was performed with 30% HFIP in DCM (45 min).

HPLC (Method D): t_R = 26.867 min.

ESI (HRMS): calcd. for $C_{71}H_{94}N_9O_{21}S^+$ $[M+H]^+$: 1440.6279, found: 1440.6279; calcd. for $C_{71}H_{93}N_9O_{21}SNa^+$ $[M+Na]^+$: 1462.6099, found: 1462.6101.

Synthesis of PCL2–D(sbab)-G-D(*t*Bu)-F-K(Boc)-D(*t*Bu)-A–2-Cl-Trt resin (34)

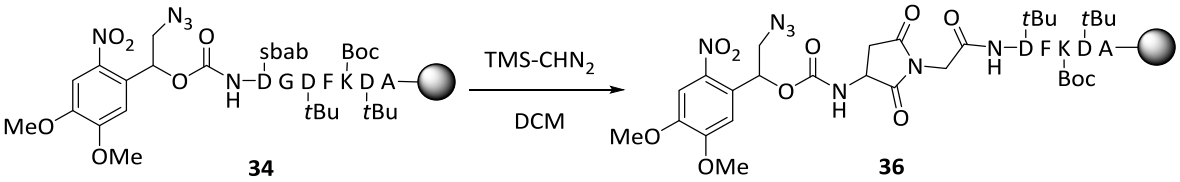


Peptide **34** (25 µmol) was swollen for 1 h in NMP and then treated with 20% piperidine in NMP (2 · 10 min). The resin was washed with NMP (8x) and DCM (8x) and dried *in vacuo*. PCL2 was attached following GP6. Test cleavage was performed with 30% HFIP in DCM (45 min).

HPLC (Method D): t_R = 25.533 min.

ESI (HRMS): calcd. for $C_{67}H_{94}N_{13}O_{25}S^+$ $[M+H]^+$: 1512.6199, found: 1512.6200; calcd. for $C_{67}H_{93}N_{13}O_{25}SNa^+$ $[M+Na]^+$: 1534.6018, found: 1534.6020.

Synthesis of 3-PCL2-1-(G-D(*t*Bu)-F-K(Boc)-D(*t*Bu)-A–2-Cl-Trt resin)-pyrrolidine-2,5-dion (36)



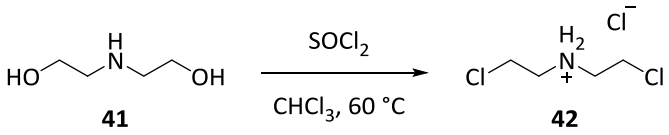
Peptide **34** (30 mg,10 µmol, 1.00 eq.) was swollen in DCM for 1 h. Then, TMS-CHN_2 (2 M in hexane, 0.50 mL, 1.00 mmol, 100 eq.) was added and the mixture was agitated for 21 h at room temperature. Afterwards, the resin was washed with DMF (8x) and DCM (8x). Test cleavage was performed with 30% HFIP in DCM (45 min).

HPLC (Method D): t_R = 23.808 min.

ESI (HRMS): calcd. for $C_{56}H_{79}N_{12}O_{21}^+$ $[M+H]^+$: 1255.5477, found: 1255.5496; calcd. for $C_{56}H_{78}N_{12}O_{21}Na^+$ $[M+Na]^+$: 1277.5297, found: 1277.5277.

ESI (MSMS): calcd. for $C_{21}H_{22}N_7O_{12}$ $[M\text{-}FKDA]^+$: 564.1, found: 564.1; calcd. for $C_{22}H_{34}N_5O_7$ $[FKDA]^+$: 480.2, found: 480.3; calcd. for $C_{30}H_{31}N_8O_{13}$ $[M\text{-}KDA]^+$: 711.2, found: 711.2; calcd. for $C_{13}H_{25}N_4O_6$ $[KDA]^+$: 333.2, found: 333.2.

Synthesis of Bis(2-chloroethyl)amine hydrochloride (42)[147]

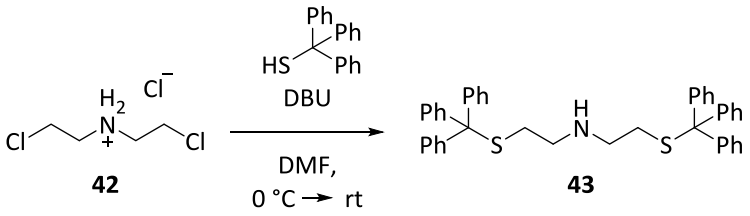


Diethanolamine (**41**, 2.49 g, 23.7 mmol, 1.0 eq.) was dissolved in CH_3Cl (10 mL) and cooled to 0 °C. Then, thionyl chloride (6.01 mL, 82.9 mmol, 3.5 eq.) in CH_3Cl (10 mL) was added slowly. The solution was heated slowly to 60 °C and stirred for 2 h. Afterwards, the solution was cooled and the precipitated crystals were filtered and washed with ice-cooled CH_3Cl and Et_2O to give 3.49 g (19.6 mmol, 83%) of a colorless solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.69 (s, 2H, NH_2), 3.95 (t, $^3J_{H\text{-}H}$ = 6.4 Hz, 4H, CH_2), 3.35 (t, $^3J_{H\text{-}H}$ = 6.4 Hz, 4H, CH_2).

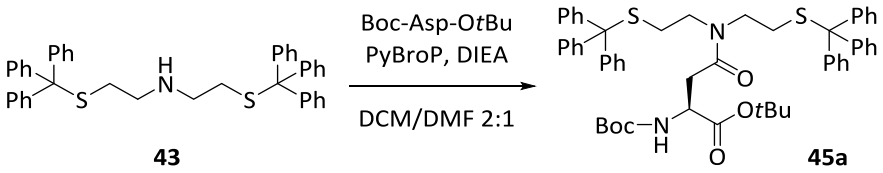
ESI (HRMS): calcd. for $C_4H_{10}Cl_2N^+$ $[M+H]^+$: 142.0185, found: 142.0184.

Synthesis of Bis({2-[triphenylmethyl]sulfanyl}ethyl)amine (43) [112]



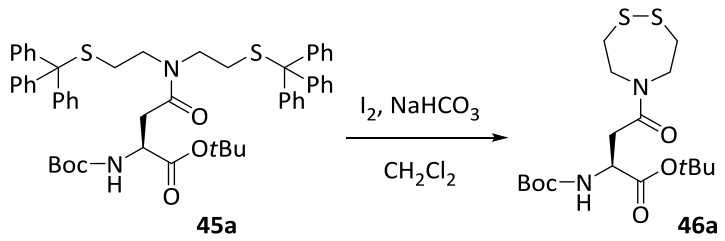
To a solution of ice-cold bis(2-chloroethyl)amine hydrochloride (**42**, 3.00 g, 16.8 mmol, 1.0 eq.) and triphenylmethanethiol (9.30 g, 32.6 mmol, 2.0 eq.) in DMF_{abs} (50 mL), DBU (10.06 mL, 67.2 mmol, 4.0 eq.) was added dropwise. Then, the solution was stirred for 12 h at room temperature and after that, the solvent was removed under reduced pressure. The residue was dissolved in DCM (100 mL) and washed with 5% aqueous KH_2PO_4 solution (3 · 100 mL). The organic layer was dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. Subsequently, the crude product was purified by column chromatography on silica (pentane/EtOAc/TEA 8:2:0.1) to give 6.97 g (11.2 mmol, 68%) of a colorless solid. The NMR spectroscopic data were in accordance with the data from literature.[112]

Synthesis of Boc-Asp(SEA{Trt}$_2$)-O*t*Bu (45a)[111]



Boc-Asp-O*t*Bu (0.64 g, 2.21 mmol, 1.0 eq.) was dissolved in degassed DMF (5 mL) and bis({2-[triphenylmethyl]sulfanyl}ethyl)amine (**43**, 2.75 g, 4.42 mmol, 2.0 eq.) in DCM_{abs} (10 mL) was added. Then, PyBrOP (1.03 g, 2.21 mmol, 1.0 eq.) and DIEA (1.13 mL, 6.63 mmol, 3.0 eq.) was added and the resulting solution was stirred for 20 h at room temperature. The solvent was removed *in vacuo* and the crude product was purified by column chromatography on silica (pentane/EtOAc/Et_3N 8:2:0.1) to give 1.24 g (1.39 mmol, 63%) of a colorless solid. The NMR spectroscopic data were in accordance with the data from literature.[111]

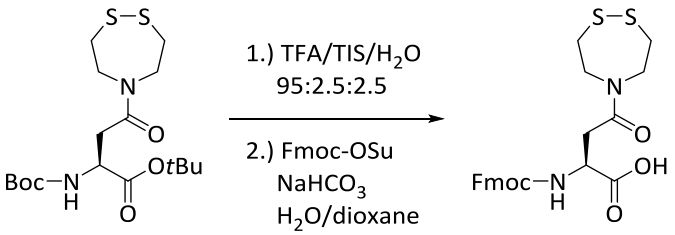
Synthesis of Boc-Asp(SEA^{off})-O*t*Bu (46a)[111]



Boc-Asp(SEA{Trt}$_2$)-O*t*Bu (**45a**, 1.13 g, 1.27 mmol, 1.0 eq.) and $NaHCO_3$ (0.32 g, 3.80 mmol, 3.0 eq.) were dissolved in DCM (50 mL) and iodine (0.96 g, 3.80 mmol, 3.0 eq.) was added slowly. The resulting solution was stirred for 30 min and was then quenched with 3 M aqueous $Na_2S_2O_3$ solution (25 mL). Afterwards, the aqueous layer was extracted with DCM (2 · 30 mL), the combined organic layers were dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*. The crude product was purified by column chromatography on silica (pentane/EtOAc 4:1) to give 0.44 g (1.09 mmol, 86%) of a colourless solid. The NMR spectroscopic data were in accordance with the data from literature.[111]

TLC: R_f = 0.16 (pentane/EtOAc 4:1).

Synthesis of Fmoc-Asp(SEA^{off})-OH (47a)[111]

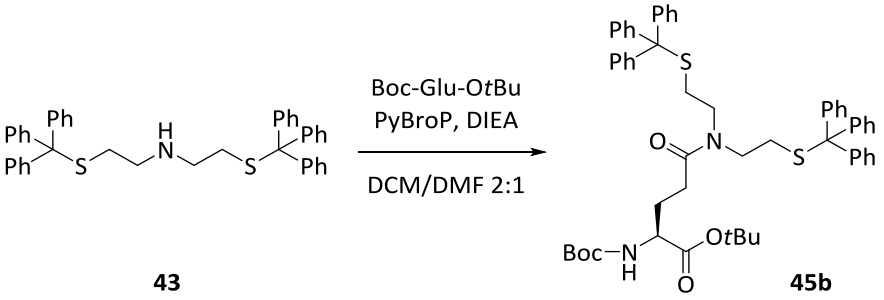


46a **47a**

Boc-Asp(SEA^{off})-O*t*Bu (**46a**, 200 mg, 0.49 mmol, 1.0 eq.) was dissolved in TFA/TIS/H_2O (95:2.5:2.5, 10 mL) and stirred for 1 h at room temperature and then evaporated to dryness. The residue was dissolved in H_2O (20 mL), washed with DCM (3 · 10 mL) and the solvent was removed *in vacuo*. The residue and $NaHCO_3$ (165 mg, 1.96 mmol, 4.0 eq.) were dissolved in H_2O (5 mL) and the solution was cooled to 0 °C. Then, Fmoc-OSu (250 mg, 0.74 mmol, 1.5 eq.) in dioxane (10 mL) was added and the pH was adjusted to 8.5 with 0.1 M aqueous $NaHCO_3$ solution. The solution was warmed to room temperature and stirred for 18 h. Afterwards, the solution was washed with Et_2O (2 · 30 mL). The aqueous phase was adjusted to pH 1 with 1 M HCl and extracted with EtOAc (3 · 30 mL). Subsequently, the organic layer was washed with H_2O (2 · 30 mL), dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica (pentane/EtOAc 2:1 + 0.5% AcOH → pentane/EtOAc 3:2 + 0.5% AcOH) to give 190 mg (0.40 mmol, 82%) of a colorless solid. The NMR spectroscopic data were in accordance with the data from literature.[111]

TLC: R_f = 0.09 (pentane/EtOAc 2:1 + 0.5% AcOH).

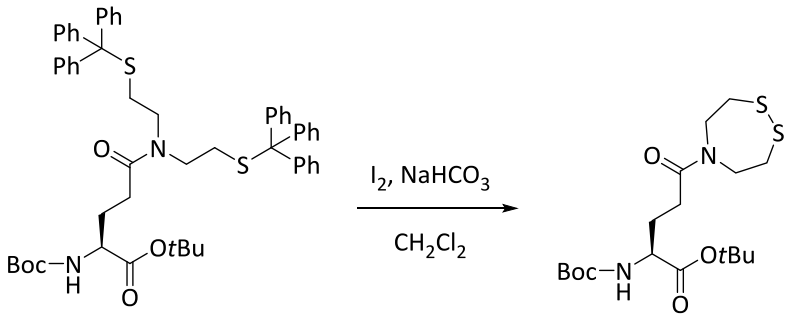
Synthesis of Boc-Glu(SEA{Trt}$_2$)-O*t*Bu (45b)[111]



43 **45b**

Boc-Glu-O*t*Bu (1.50 g, 4.94 mmol, 1.0 eq.) was dissolved in degassed DMF (15 mL) and bis({2-[triphenylmethyl]sulfanyl}ethyl)amine (**43**, 6.15 g, 9.89 mmol, 2.0 eq.) in DCM_{abs} (30 mL) was added. Then, PyBrOP (2.30 g, 4.94 mmol, 1.0 eq.) and DIEA (2.52 mL, 14.8 mmol, 3.0 eq.) were added and the resulting solution was stirred for 20 h at room temperature. The solvent was removed *in vacuo* and the crude product was purified by column chromatography on silica (pentane/EtOAc/Et_3N 8:2:0.1) to give 2.67 g (2.96 mmol, 60%) of a colorless solid. The NMR spectroscopic data were in accordance with the data from literature.[111]

Synthesis of Boc-Glu(SEA^{off})-O*t*Bu (46b)[111]

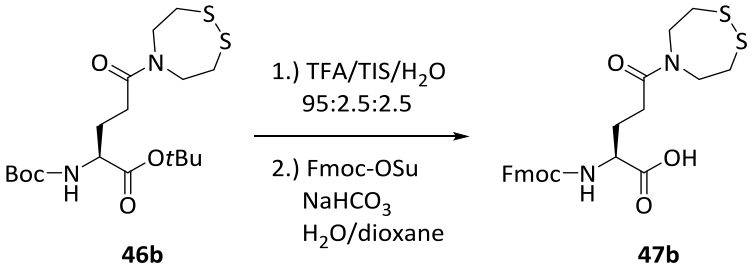


45b **46b**

Boc-Glu(SEA{Trt}$_2$)-O*t*Bu (**45b**, 5.04 g, 5.56 mmol, 1.0 eq.) and $NaHCO_3$ (1.40 g, 16.7 mmol, 3.0 eq.) were dissolved in DCM (300 mL) and iodine (4.24 g, 16.7 mmol, 3.0 eq.) was added slowly. The resulting solution was stirred for 30 min and was then quenched with 3 M aqueous $Na_2S_2O_3$ solution (200 mL). Afterwards, the aqueous layer was extracted with DCM (3 · 100 mL), the combined organic layers were dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*. The crude product was purified by column chromatography on silica (pentane/EtOAc 2:1) to give 1.78 g (4.23 mmol, 84%) of a colorless solid. The NMR spectroscopic data were in accordance with the data from literature.[111]

TLC: R_f = 0.24 (pentane/EtOAc 2:1).

Synthesis of Fmoc-Glu(SEA^{off})-OH (47b)[111]

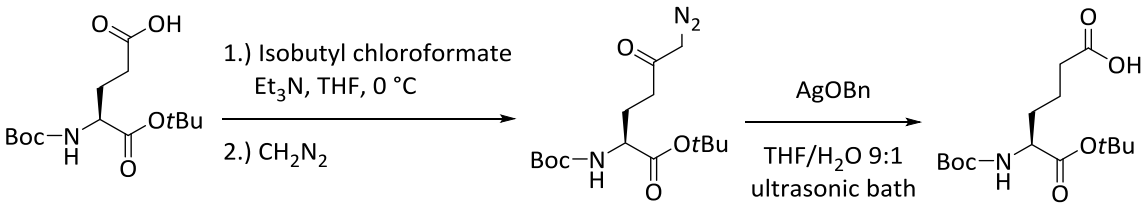


46b **47b**

Boc-Glu(SEA^{off})-O*t*Bu (**46b**, 1.78 g, 4.23 mmol, 1.0 eq.) was dissolved in TFA/TIS/H_2O (95:2.5:2.5, 30 mL) and stirred for 1 h at room temperature and then evaporated to dryness. The residue was dissolved in H_2O (30 mL), washed with DCM (3 · 15 mL) and the solvent was removed *in vacuo*. The residue and $NaHCO_3$ (1.42 g, 16.9 mmol, 4.0 eq.) were dissolved in H_2O (50 mL) and the solution was cooled to 0 °C. Then, Fmoc-OSu (2.14 g, 6.35 mmol, 1.5 eq.) in dioxane (80 mL) was added and the pH was adjusted to 8.5 with 0.1 M aqueous $NaHCO_3$ solution. The solution was warmed to room temperature and stirred for 20 h. Afterwards, the solution was washed with Et_2O (2 · 100 mL). The aqueous phase was adjusted to pH 1 with 1 M HCl and extracted with DCM (3 · 100 mL). Subsequently, the organic layer was washed with H_2O (2 · 100 mL), dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica (pentane/EtOAc 1:1 + 0.5% AcOH) to give 1.84 g (3.78 mmol, 89%) of a colorless solid. The NMR spectroscopic data were in accordance with the data from literature.[111]

TLC: R_f = 0.30 (pentane/EtOAc 1:1 + 0.5% AcOH).

Synthesis of Boc-Aad-O*t*Bu (44c)



119 **120** **44c**

Boc-Glu-O*t*Bu (4.00 g, 13.2 mmol, 1.0 eq.) was dissolved in THF_{abs} (60 mL) and cooled to 0 °C. Then, Et_3N (2.21 mL, 14.5 mmol, 1.1 eq.) and isobutyl chloroformate (1.88 mL, 14.5 mmol, 1.1 eq.) were added and the solution was stirred for 30 min at room temperature. Afterwards, diazomethane (0.7 M in Et_2O, 37.7 mL, 26.4 mmol, 2.0 eq.) was added. The solution was allowed to warm to room temperature and stirred for 18 h at room temperature. Subsequently, the reaction was quenched with acetic acid (1.6 mL) and 6% aqueous $NaHCO_3$ solution (100 mL) was added. The reaction mixture was extracted with EtOAc (3 · 100 mL), the combined organic layers were washed with saturated aqueous NH_4Cl solution (3 · 60 mL) and brine (60 mL), dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was used without further purification for the next step. It was dissolved in THF/H_2O 9:1 (80 mL), silver benzoate (0.30 g, 1.32 mmol, 0.1 eq.) was added and the reaction mixture was left in an ultrasonic bath for 4 h. After that, H_2O (50 mL) was added and the reaction mixture was carefully adjusted to pH 2 with 1 M HCl. The reaction mixture was extracted with EtOAc (3 · 100 mL), the combined organic layers were dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. The crude product was purified by column chromatography on silica (pentane/EtOAc/AcOH 8:1:0.01) to give 1.29 g (4.05 mmol, 31%) of a colorless solid.

IR (neat, cm^{-1}): 2976 (w), 2933 (w), 2856 (w), 1707 (s), 1556 (w), 1525 (w), 1366 (m), 1252 (w), 1227 (w), 1147 (s), 1045 (w), 1023 (w).

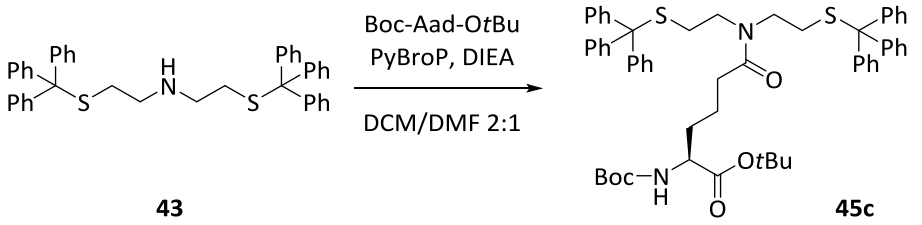
^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.13 (d, $^3J_{H\text{-}H}$ = 8.2 Hz, 1H, N*H*), 4.27–4.11 (m, 1H, α-C*H*), 2.45–2.32 (m, 2H, CH_2), 1.74–1.61 (m, 4H, CH_2), 1.46 (s, 9H, CH_3), 1.43 (s, 9H, CH_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 178.5 (*C*O_2H), 171.7 (*C*O_2*t*Bu), 155.4 (O-*C*O-N), 82.0 (*C*$(CH_3)_3$), 79.7 (*C*$(CH_3)_3$), 53.6 (*C*H), 33.4 (*C*H_2), 32.1 (*C*H_2), 28.3 (*C*H_3), 27.9 (*C*H_3), 20.3 (*C*H_2).

TLC: R_f = 0.14 (pentane/EtOAc/AcOH 8:1:0.01).

ESI (HRMS): calcd. for $C_{15}H_{27}NO_6Na^+$ $[M+Na]^+$: 340.1731, found: 340.1729; calcd. for $C_{15}H_{27}NO_6K^+$ $[M+K]^+$: 356.1470, found: 356.1477; calcd. for $C_{15}H_{26}NO_6^-$ $[M–H]^-$: 316.1766, found: 316.1768.

Synthesis of Boc-Aad(SEA{Trt}$_2$)-O*t*Bu (45c)



Boc-Aad-O*t*Bu (1.20 g, 3.79 mmol, 1.0 eq.) was dissolved in DMF_{abs} (6 mL) and degassed with argon for 30 min. Then, bis({2[triphenylmethyl]sulfanyl}ethyl)amine (**43**, 5.15 g, 7.58 mmol, 2.0 eq.) in DCM_{abs} (12 mL) was added. Afterwards, PyBrOP (1.93 g, 3.79 mmol, 1.0 eq.) and DIEA (2.16 mL, 14.4 mmol, 3.0 eq.) were added and the resulting solution was stirred for 16 h at room temperature. The solvent was removed *in vacuo* and the crude product was purified by column chromatography on silica (pentane/EtOAc/Et_3N 8:2:0.1) to give 2.28 g (2.48 mmol, 65%) of a colorless solid.

IR (neat, cm^{-1}): 3058 (w), 2973 (w), 2928 (w), 2859 (w), 1714 (s), 1647 (m), 1488 (m), 1443 (m), 1363 (m), 1150 (s), 1033 (m).

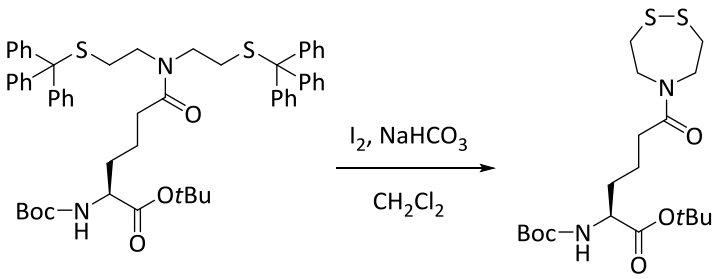
^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.39–7.31 (m, 12H, C*H*$_{ar}$), 7.29–7.15 (m, 18H, C*H*$_{ar}$), 5.04 (d, $^3J_{H\text{-}H}$ = 8.2 Hz, 1H, N*H*), 4.13–4.03 (m, 1H, α-C*H*), 2.77–2.72 (m, 4H, CH_2), 2.32–2.25 (m, 2H, CH_2), 2.15–2.09 (m, 2H, CH_2), 1.86–1.79 (m, 2H, CH_2), 1.67–1.58 (m, 2H, CH_2), 1.57–1.53 (m, 2H, CH_2), 1.43 (s, 9H, CH_3), 1.42 (s, 9H, CH_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.7 (*C*O_2*t*Bu), 171.1 (*C*O-N), 155.4 (O-*C*O-N), 144.7 (*C*H$_{ar}$), 144.5 (*C*H$_{ar}$), 129.6 (*C*H$_{ar}$), 129.5 (*C*H$_{ar}$), 128.0 (*C*H$_{ar}$), 127.9 (*C*H$_{ar}$), 126.9 (*C*H$_{ar}$), 126.7 (*C*H$_{ar}$), 81.7 (*C*(CH$_3$)$_3$), 79.5 (*C*(CH$_3$)$_3$), 66.8 (*C*Ph$_3$), 53.8 (*C*H), 47.3 (*C*H$_2$), 45.3 (*C*H$_2$), 32.2 (*C*H$_2$), 31.9 (*C*H$_2$), 30.3 (*C*H$_2$), 29.6 (*C*H$_2$), 28.3 (*C*H$_3$), 28.0 (*C*H$_3$), 20.6 (*C*H$_2$).

TLC: R_f = 0.62 (pentane/EtOAc/TEA 8:2:0.1).

ESI (HRMS): calcd. for $C_{57}H_{65}N_2O_5S_2^+$ $[M+H]^+$: 921.4329, found: 921.4323; calcd. for $C_{57}H_{64}N_2O_5S_2Na^+$ $[M+Na]^+$: 943.4149, found: 943.4140; calcd. for $C_{57}H_{64}N_2O_5S_2K^+$ $[M+K]^+$: 959.3888, found: 959.3882.

Synthesis of Boc-Aad(SEA^{off})-O*t*Bu (46c)



45c **46c**

Boc-Aad(SEA{Trt}$_2$)-O*t*Bu (**45c**, 2.26 g, 2.45 mmol, 1.0 eq.) and $NaHCO_3$ (0.51 g, 7.35 mmol, 3.0 eq.) were dissolved in DCM (100 mL) and iodine (1.54 g, 7.35 mmol, 3.0 eq.) was added slowly. The resulting solution was stirred for 30 min and was then quenched with 3 M aqueous $Na_2S_2O_3$ solution (100 mL). Afterwards, the aqueous layer was extracted with DCM (3 · 100 mL), the combined organic layers were dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*. The crude product was purified by column chromatography on silica (pentane/EtOAc 50:50:1) to give 0.67 g (1.54 mmol, 63%) of a colorless solid.

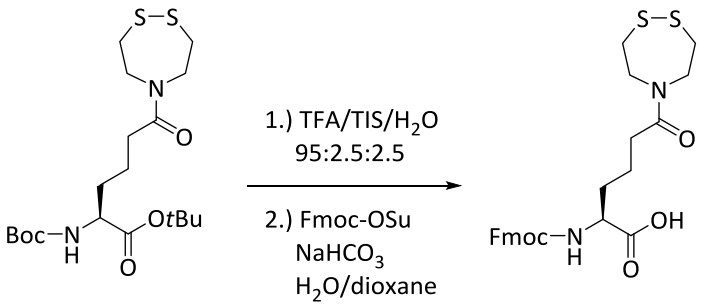
^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.06 (s, $^3J_{H\text{-}H}$ = 8.0 Hz, 1H, N*H*), 4.17–4.11 (m, 1H, α-C*H*), 3.89–3.84 (m, 2H, CH_2), 3.80–3.76 (m, 2H, CH_2), 3.05 (t, $^3J_{H\text{-}H}$ = 5.7 Hz, 2H, CH_2), 2.90 (t, $^3J_{H\text{-}H}$ = 5.7 Hz, 2H, CH_2), 2.38–2.25 (m, 2H, CH_2), 1.80–1.61 (m, 4H, 2x CH_2), 1.44 (s, 9H, CH_3), 1.41 (s, 9H, CH_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.4 (*C*O-N), 171.8 (*C*O$_2$*t*Bu), 155.4 (O-*C*O-N), 81.9 (*C*(CH$_3$)$_3$), 79.6 (*C*(CH$_3$)$_3$), 53.3 (*C*H), 52.4 (*C*H$_2$), 50.1 (*C*H$_2$), 39.7 (*C*H$_2$), 35.6 (*C*H$_2$), 32.4 (*C*H$_2$), 32.0 (*C*H$_2$), 28.3 (*C*H$_3$), 28.0 (*C*H$_3$), 20.7 (*C*H$_2$).

TLC: R_f = 0.32 (pentane/EtOAc/TEA 50:50:1)

HRMS (ESI): calcd. for $C_{19}H_{35}N_2O_5S_2^+$ $[M+H]^+$: 435.1982, found: 435.1982; calcd. for $C_{19}H_{34}N_2O_5S_2Na^+$ $[M+Na]^+$: 457.1801, found: 457.1801; calcd. for $C_{19}H_{34}N_2O_5S_2K^+$ $[M+K]^+$: 473.1541, found: 473.1546; calcd. for $C_{19}H_{33}N_2O_5S_2^-$ $[M–H]^-$: 433.1836, found: 433.1830.

Synthesis of Fmoc-Aad(SEA^{off})-OH (47c)



46c **47c**

Boc-Aad(SEA^{off})-O*t*Bu (**46c**, 0.63 g, 1.45 mmol, 1.0 eq.) was dissolved in TFA/TIS/H_2O (95:2.5:2.5, 20 mL) and stirred for 1 h at room temperature and then evaporated to dryness. The residue was dissolved in H_2O (20 mL), washed with DCM (3 · 10 mL) and the solvent was removed *in vacuo*. The residue and $NaHCO_3$ (0.54 g, 6.42 mmol, 4.0 eq.) were dissolved in H_2O (20 mL) and the solution was cooled to 0 °C. Then, Fmoc-OSu (0.79 g, 2.34 mmol, 1.5 eq.) in dioxane (30 mL) was added and the pH was adjusted to 8.5 with 0.1 M aqueous $NaHCO_3$.solution The solution was warmed to room temperature and stirred for 16 h. Afterwards, the solution was washed with Et_2O (3 · 50 mL). The aqueous phase was adjusted to pH 2 with 4 M HCl and extracted with EtOAc (3 · 50 mL). Subsequently, the organic layer was washed with H_2O (2 · 50 mL), dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica (pentane/EtOAc 7:3 + 0.5% AcOH) to give 0.49 g (0.98 mmol, 68%) of a colorless solid.

IR (neat, cm^{-1}): 2951 (w), 2921 (w), 2853 (w), 1717 (s), 1635 (m), 1520 (w), 1448 (m), 1418 (w), 1252 (w), 1212 (m), 1048 (m), 738 (s).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.72 (d, $^3J_{H\text{-}H}$ = 7.4 Hz, 2H, C*H*$_{ar}$), 7.58 (d, $^3J_{H\text{-}H}$ = 6.8 Hz, 2H, C*H*$_{ar}$), 7.36 (t, $^3J_{H\text{-}H}$ = 7.4 Hz, 2H, C*H*$_{ar}$), 7.30–7.24 (m, 2H, C*H*$_{ar}$), 5.91 (d, $^3J_{H\text{-}H}$ = 7.6 Hz, 1H, N*H*), 4.41–4.28 (m, 3H, Fmoc-C*H*$_2$ + α-C*H*), 4.19 (t, $^3J_{H\text{-}H}$ = 7.2 Hz, 1H, Fmoc-C*H*), 3.88 (t, $^3J_{H\text{-}H}$ = 5.5 Hz, 2H, C*H*$_2$), 3.79–3.74 (m, 2H, C*H*$_2$), 3.04 (t, $^3J_{H\text{-}H}$ = 5.7 Hz, 2H, C*H*$_2$), 2.88 (t, $^3J_{H\text{-}H}$ = 5.5 Hz, 2H, C*H*$_2$), 2.41–2.32 (m, 2H, C*H*$_2$), 1.94–1.67 (m, 4H, 2x C*H*$_2$).

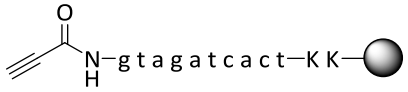
^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.2 (*C*O), 174.0 (*C*O), 156.3 (O-*C*O-N), 144.0 (*C*H$_{ar}$), 143.9 (*C*H$_{ar}$), 141.4 (*C*H$_{ar}$), 127.9 (*C*H$_{ar}$), 127.2 (*C*H$_{ar}$), 125.3 (*C*H$_{ar}$), 120.1 (*C*H$_{ar}$), 67.2 (Fmoc-*C*H$_2$), 53.5 (α-*C*H), 52.7 (*C*H$_2$), 50.6 (*C*H$_2$), 47.3 (Fmoc-*C*H), 39.3 (*C*H$_2$), 35.3 (*C*H$_2$), 32.2 (*C*H$_2$), 20.4 (*C*H$_2$).

TLC: R_f = 0.10 (pentane/EtOAc 1:1 + 0.1% AcOH).

ESI (HRMS): calcd. for $C_{25}H_{29}N_2O_5S_2^+$ $[M+H]^+$: 501.1512, found: 501.1501; calcd. for $C_{25}H_{28}N_2O_5S_2K^+$ $[M+K]^+$: 539.1071, found: 539.1061; calcd. for $C_{25}H_{27}N_2O_5S_2^-$ $[M–H]^-$: 499.1367, found: 499.1362.

4.3.3 Alkyne-PNAs

Synthesis of alkyne-PNA1-KK-RINK amide MBHA resin (PNA1', 121)



121

A RINK amide MBHA resin (0.36 mmol/g) was loaded with Fmoc-Lys(Boc)-OH following GP1 and the resin loading was estimated following GP2. Then, the peptide sequence (25 µmol) was coupled following GP3 and the PNA sequence following GP4. Alkyne functionalization was accomplished following GP5. Test cleavage was performed with TFA/TIS/H_2O (95:2.5:2.5, 1 mL cleavage solution, 2 mg resin, 1.5 h).

HPLC (Method C): t_R =16.725 min.

ESI (HRMS): calcd. for $C_{123}H_{166}N_{62}O_{33}^{6+}$ $[M+6H]^{6+}$: 506.5531, found: 506.5538; calcd. for $C_{123}H_{165}N_{62}O_{33}^{5+}$ $[M+5H]^{5+}$: 607.6622, found: 607.6622; calcd. for $C_{123}H_{164}N_{62}O_{33}^{4+}$ $[M+4H]^{4+}$: 759.3260, found: 759.3265; calcd. for $C_{123}H_{163}N_{62}O_{33}^{3+}$ $[M+3H]^{3+}$: 1012. 0989, found: 1012.0996.

Synthesis of alkyne-PNA2-K-RINK amide MBHA resin (PNA2, 122)

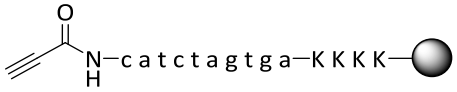
122

A RINK amide MBHA resin (0.36 mmol/g) was loaded with Fmoc-Lys(Boc)-OH following GP1 and the resin loading was estimated following GP2. Then, the PNA sequence (25 µmol) was coupled following GP4. Alkyne functionalization was accomplished following GP5. Test cleavage was performed with TFA/TIS/H_2O (95:2.5:2.5, 1 mL cleavage solution, 2 mg resin, 1.5 h).

HPLC (Method C): t_R = 16.883 min.

ESI (HRMS): calcd. for $C_{117}H_{153}N_{60}O_{32}^{5+}$ $[M+5H]^{5+}$: 582.0432, found: 582.0435; calcd. for $C_{117}H_{152}N_{60}O_{32}^{4+}$ $[M+4H]^{4+}$: 727.3022, found: 727.3025; calcd. for $C_{117}H_{151}N_{60}O_{32}^{3+}$ $[M+3H]^{3+}$: 969.4005, found: 969.3998.

Synthesis of alkyne-PNA2-KKKK-RINK amide MBHA resin (PNA2', 49)



49

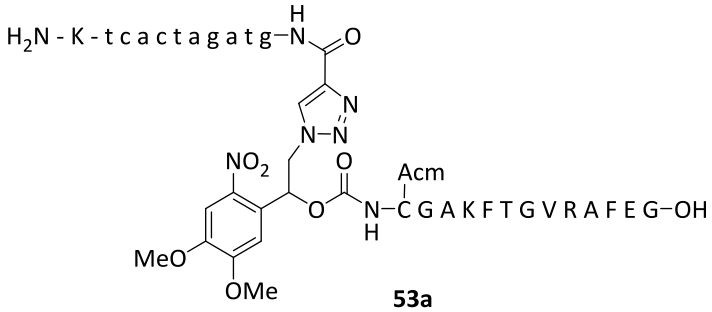
A RINK amide MBHA resin (0.36 mmol/g) was loaded with Fmoc-Lys(Boc)-OH following GP1 and the resin loading was estimated following GP2. Then, the peptide sequence (25 µmol) was coupled following GP3 and the PNA sequence following GP4. Alkyne functionalization was accomplished following GP5. Test cleavage was performed with TFA/TIS/H_2O (95:2.5:2.5, 1 mL cleavage solution, 2 mg resin, 1.5 h).

HPLC (Method E): t_R = 16.275 min.

ESI (HRMS): calcd. for $C_{135}H_{190}N_{66}O_{35}^{6+}$ $[M+6H]^{6+}$: 549.2514, found: 549.2535; calcd. for $C_{135}H_{189}N_{66}O_{35}^{5+}$ $[M+5H]^{5+}$: 658.9002, found: 658.9008; calcd. for $C_{135}H_{188}N_{66}O_{35}^{4+}$ $[M+4H]^{4+}$: 823.3735, found: 823.3741; calcd. for $C_{135}H_{187}N_{66}O_{35}^{3+}$ $[M+3H]^{3+}$: 1097.4955, found: 1097.4960.

4.3.4 PCL2-Peptides and PNA/Peptide Hybrids

Synthesis of PCL2(PNA1)-C(Acm)GAKFTGVRAFEG-OH (53a)



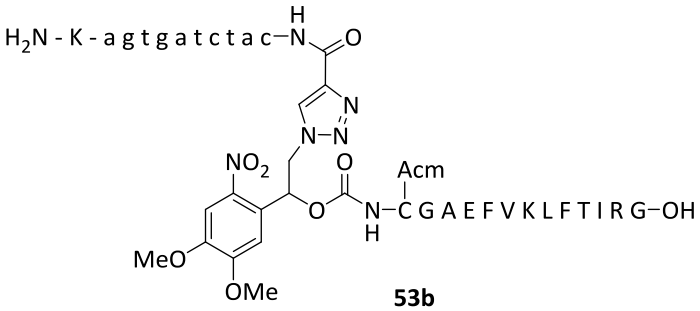
53a

The caged peptide was attached to PNA1 (10 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method C): t_R = 23.625 min.

ESI (HRMS): calcd. for $C_{190}H_{258}N_{82}O_{56}S^{4+}$ $[M+4H]^{4+}$: 1153.9890, found: 1153.9925; calcd. for $C_{190}H_{257}N_{82}O_{56}S^{3+}$ $[M+3H]^{3+}$: 1538.3162, found. 1538.3158.

Synthesis of PCL2(PNA2)-C(Acm)GAEFVKLFTIRG-OH (53b)



53b

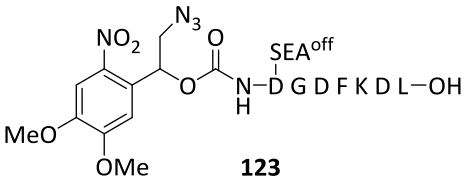
The caged peptide was attached to PNA2 (**122**, 10 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method C): t_R = 26.867 min.

ESI (HRMS): calcd. for $C_{197}H_{273}N_{82}O_{56}S^{5+}$ $[M+5H]^{5+}$: 943.0146, found: 943.0195; calcd. for $C_{197}H_{272}N_{82}O_{56}S^{4+}$ $[M+4H]^{4+}$: 1178.5164, found: 1178.5180.

PCL2-peptide & PNA1 used for **53a** and PCL2-peptide for **53b** were synthesized by CORNELIA PANSE.[98]

Synthesis of PCL2-D(SEA^{off})GDFKDL-OH (123)



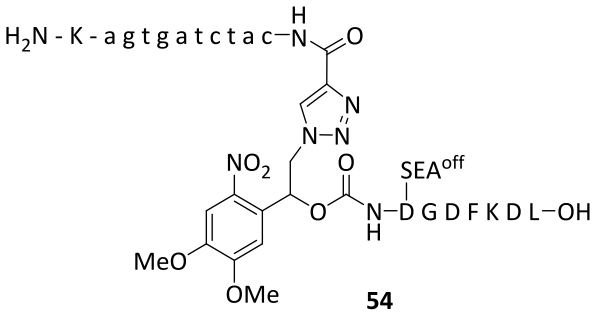
123

The peptide (35 µmol) was coupled to a preloaded WANG resin (leucine, 0.38 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method D): t_R = 18.092 min.

ESI (HRMS): calcd. for $C_{50}H_{70}N_{13}O_{19}S_2^+$ $[M+H]^+$: 1220.4347, found: 1220.4350; calcd. for $C_{50}H_{69}N_{13}O_{19}S_2Na^+$ $[M+Na]^+$: 1242.4166, found: 1242.4172.

Synthesis of PCL2(PNA2)-D(SEA^{off})GDFKDL-OH (54)



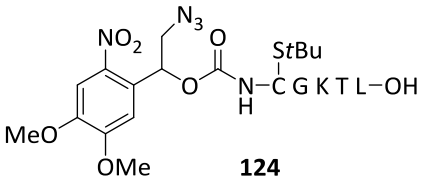
54

123 was attached to PNA2 (**122**, 10 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method C): t_R = 24.292 min (*ds*1), 24.967 min (*ds*2).

ESI (HRMS): calcd. for $C_{167}H_{221}N_{73}O_{51}S_2^{4+}$ $[M+4H]^{4+}$: 1032.1591, found: 1032.1602.

Synthesis of PCL2-C(S*t*Bu)GKTL-OH (124)



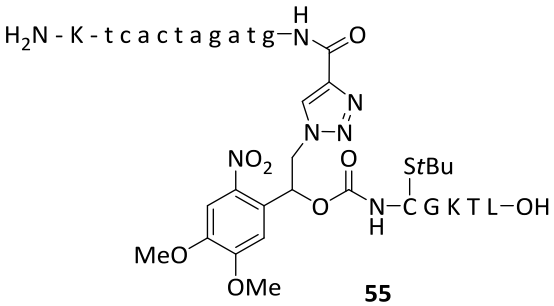
124

The peptide (50 µmol) was coupled to a preloaded WANG resin (leucine, 0.38 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method D): t_R = 17.925 min (*ds*1), 18.308 min (*ds*2).

ESI (HRMS): calcd. for $C_{36}H_{59}N_{10}O_{13}S_2^+$ $[M+H]^+$: 903.3699, found: 903.3696; calcd. for $C_{36}H_{58}N_{10}O_{13}S_2Na^+$ $[M+Na]^+$: 925.3518, found: 925.3516.

Synthesis of PCL2(PNA1)-C(S*t*Bu)GKTL-OH (55)



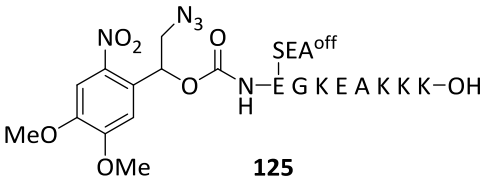
55

124 was attached to PNA1 (10 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method C): t_R = 24.333 min (*ds*1), 24.033 min (*ds*2).

ESI (HRMS): calcd. for $C_{153}H_{209}N_{70}O_{45}S_2^{3+}$ $[M+3H]^{3+}$: 1270.1881, found: 1270.1895.

Synthesis of PCL2-E(SEA^{off})GKEAKKK-OH (125)



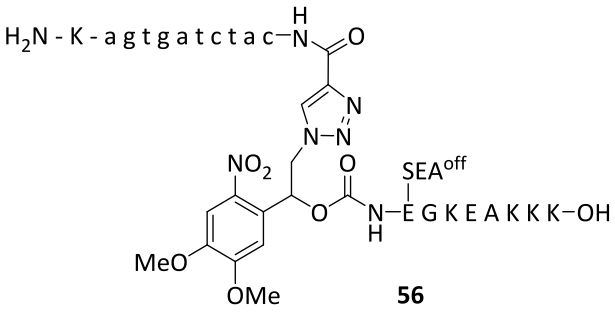
125

The peptide (35 µmol) was coupled to a preloaded WANG resin (lysine, 0.31 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method D): t_R = 12.167 min (*ds*1), 12.692 min (*ds*2).

ESI (HRMS): calcd. for $C_{54}H_{91}N_{17}O_{18}S_2^{2+}$ $[M+2H]^{2+}$: 664.8079, found: 664.8079; calcd. for $C_{54}H_{90}N_{17}O_{18}S_2^{+}$ $[M+H]^{+}$: 1328.6086, found: 1328.6087.

Synthesis of PCL2(PNA2)-E(SEA^{off})GKEAKKK-OH (56)



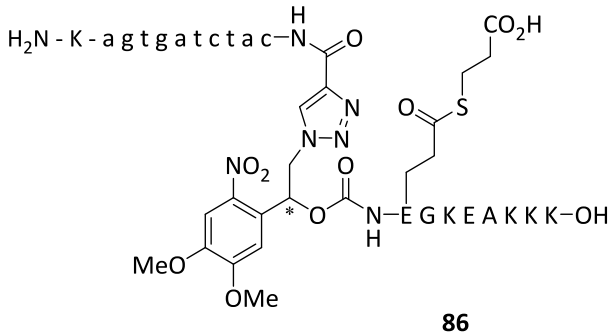
56

125 was attached to PNA2 (**122**, 10 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method C): t_R = 20.058 min (*ds*1), 20.433 min (*ds*2).

ESI (HRMS): calcd. for $C_{171}H_{244}N_{77}O_{50}S_2^{7+}$ $[M+7H]^{7+}$: 605.6903, found: 605.6903; calcd. for $C_{171}H_{243}N_{77}O_{50}S_2^{6+}$ $[M+6H]^{6+}$: 706.4708, found: 706.4714; calcd. for $C_{171}H_{242}N_{77}O_{50}S_2^{5+}$ $[M+5H]^{5+}$: 847.5635, found: 847.5644; calcd. for $C_{171}H_{241}N_{77}O_{50}S_2^{4+}$ $[M+4H]^{4+}$: 1059.2026, found: 1059.2038.

Synthesis of PCL2*(PNA2)-E(MPA thioester)GKEAKKK-OH (86)



86

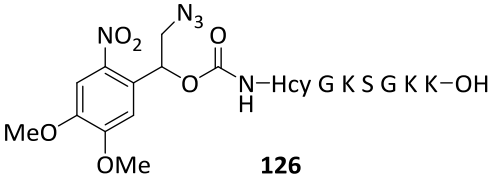
56*ds2* (1.8 mg, 0.42 µmol) was converted to the corresponding MPA thioester following GP8 to give 1.4 mg (0.33 µmol, 79%) of a colorless solid.

HPLC (Method G): t_R = 7.22 min.

ESI–MS *m/z* (rel.%): 602.05 (28) $[M+7H]^{7+}$, 702.16 (70) $[M+6H]^{6+}$, 842.32 (100) $[M+5H]^{5+}$, 1052.57 (39) $[M+4H]^{4+}$, 1402.82 (12) $[M+3H]^{3+}$.

ESI (HRMS): calcd. for $C_{170}H_{241}N_{76}O_{52}S^{7+}$ $[M+7H]^{7+}$: 601.5462, found: 601.5466; calcd. for $C_{170}H_{240}N_{76}O_{52}S^{6+}$ $[M+6H]^{6+}$: 701.6360, found: 701.6368; calcd. for $C_{170}H_{239}N_{76}O_{52}S^{5+}$ $[M+5H]^{5+}$: 841.7617, found: 841.7630; calcd. for $C_{170}H_{238}N_{76}O_{52}S^{4+}$ $[M+4H]^{4+}$: 1051.9504, found: 1051.9527.

Synthesis of PCL2-HcyGKSGKK-OH (126)



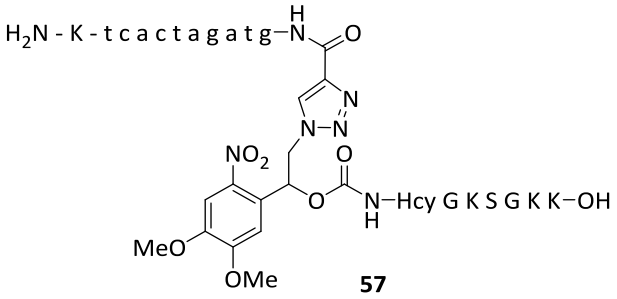
126

The peptide (35 µmol) was coupled to a preloaded WANG resin (lysine, 0.31 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/EDT/TIS/H_2O (95:2.5:2.5).

HPLC (Method D): t_R = 11.200 min (*ds*1), 11.492 min (*ds*2).

ESI (HRMS): calcd. for $C_{40}H_{68}N_{14}O_{15}S^{2+}$ $[M+2H]^{2+}$: 508.2349, found: 508.2354; calcd. for $C_{40}H_{67}N_{14}O_{15}S^{+}$ $[M+H]^{+}$: 1015.4626, found: 1015.4615.

Synthesis of PCL2(PNA1)-HcyGKSGKK-OH (57)



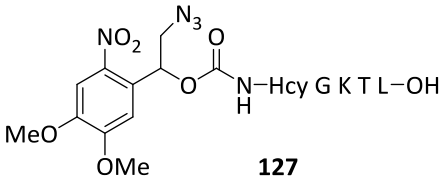
57

126 was attached to PNA1 (10 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/EDT/TIS/H_2O (94:2.5:2.5:1).

HPLC (Method E): t_R = 15.340 min (*ds*1), 15.590 min (*ds*2).

ESI (HRMS): calcd. for $C_{157}H_{220}N_{74}O_{47}S^{6+}$ $[M+6H]^{6+}$: 654.2798, found: 654.2807; calcd. for $C_{157}H_{219}N_{74}O_{47}S^{5+}$ $[M+5H]^{5+}$: 784.9343, found: 784.9355; calcd. for $C_{157}H_{218}N_{74}O_{47}S^{4+}$ $[M+4H]^{4+}$: 980.9160, found: 980.9179.

Synthesis of PCL2-HcyGKTL-OH (127)



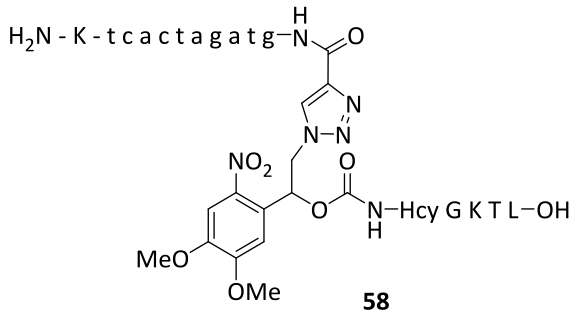
127

The peptide (35 µmol) was coupled to a preloaded WANG resin (leucine, 0.35 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/EDT/TIS/H_2O (94:2.5:2.5:1).

HPLC (Method D): t_R = 15.208 min (*ds*1), 15.517 min (*ds*2).

ESI (HRMS): calcd. for $C_{33}H_{53}N_{10}O_{13}S^{+}$ $[M+H]^{+}$: 829.3509, found: 829.3511; calcd. for $C_{33}H_{52}N_{10}O_{13}SNa^{+}$ $[M+Na]^{+}$: 851.3328, found: 851.3332; calcd. for $C_{33}H_{51}N_{10}O_{13}S^{-}$ $[M–H]^{-}$: 827.3363, found: 827.3352.

Synthesis of PCL2(PNA1)-HcyGKTL-OH (58)



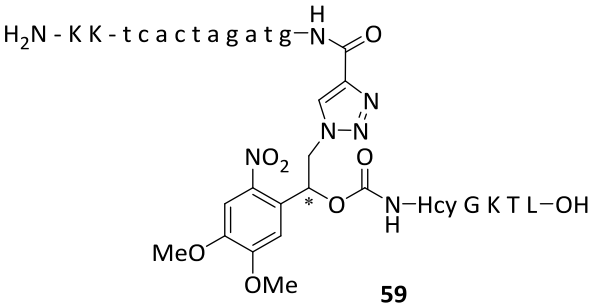
58

127 was attached to PNA1 (10 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/EDT/TIS/H_2O (94:2.5:2.5:1).

HPLC (Method C): t_R = 21.885 min (*ds*1), 22.259 min (*ds*2).

ESI (HRMS): calcd. for $C_{150}H_{205}N_{70}O_{45}S^{5+}$ $[M+5H]^{5+}$: 747.7120, found: 747.7116; calcd. for $C_{150}H_{204}N_{70}O_{45}S^{4+}$ $[M+4H]^{4+}$: 934.3881, found: 934.3883; calcd. for $C_{150}H_{203}N_{70}O_{45}S^{3+}$ $[M+3H]^{3+}$: 1245.5151, found: 1245.5157.

Synthesis of PCL2*(PNA1')-HcyGKTL-OH (59)



59

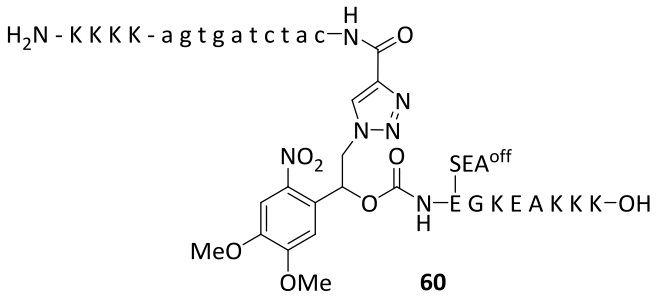
127*ds2* was attached to PNA1' (**121**, 5 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/EDT/TIS/H_2O (94:2.5:2.5:1).

HPLC: t_R = 8.81 min (Method G), 21.759 min (Method E).

ESI–MS *m/z* (rel.%): 553.00 (16) $[M+7H]^{7+}$, 645.11 (43) $[M+6H]^{6+}$, 773.86 (85) $[M+5H]^{5+}$, 966.97 (100) $[M+4H]^{4+}$, 1288.83 (70) $[M+3H]^{3+}$, 1932.70 (10) $[M+2H]^{2+}$.

ESI (HRMS): calcd. for $C_{156}H_{217}N_{72}O_{46}S^{5+}$ $[M+5H]^{5+}$: 773.3310, found: 773.3322; calcd. for $C_{156}H_{215}N_{72}O_{46}S^{3+}$ $[M+3H]^{3+}$: 1288.2134, found: 1288.2150.

Synthesis of PCL2(PNA2')-E(SEA^{off})GKEAKKK-OH (60)



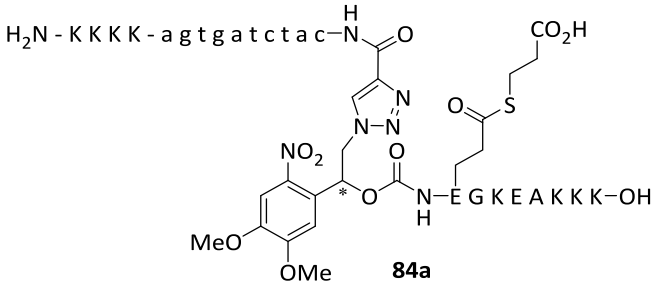
60

125 was attached to PNA2' (**49**, 10 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 19.001 min (*ds*1), 19.467 min (*ds*2).

ESI (HRMS): calcd. for $C_{189}H_{280}N_{83}O_{53}S_2^{7+}$ $[M+7H]^{7+}$: 660.5881, found: 660.5874; calcd. for $C_{189}H_{279}N_{83}O_{53}S_2^{6+}$ $[M+6H]^{6+}$: 770.5183, found: 770.5184; calcd. for $C_{189}H_{278}N_{83}O_{53}S_2^{5+}$ $[M+5H]^{5+}$: 924.4205, found: 924.4209; calcd. for $C_{189}H_{277}N_{83}O_{53}S_2^{4+}$ $[M+4H]^{4+}$: 1155.2738, found: 1155.2756.

Synthesis of PCL2*(PNA2')-E(MPA thioester)GKEAKKK-OH (84a)



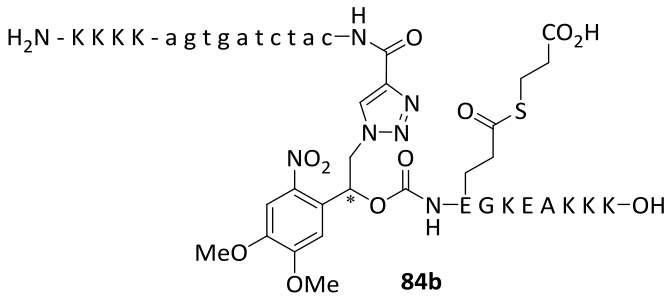
84a

60*ds1* (5.0 mg, 1.08 µmol) was converted to the corresponding MPA thioester following GP8 to give 4.0 mg (0.87 µmol, 83%) of a colorless solid.

HPLC (Method E): t_R = 18.521 min.

ESI (HRMS): calcd. for $C_{188}H_{279}N_{82}O_{55}S^{9+}$ $[M+9H]^{9+}$: 510.7914, found: 510.7902; calcd. for $C_{188}H_{278}N_{82}O_{55}S^{8+}$ $[M+8H]^{8+}$: 574.5144, found: 574.5139; calcd. for $C_{188}H_{277}N_{82}O_{55}S^{7+}$ $[M+7H]^{7+}$: 656.4440, found: 656.4440; calcd. for $C_{188}H_{276}N_{82}O_{55}S^{6+}$ $[M+6H]^{6+}$: 765.6835, found: 765.6839; calcd. for $C_{188}H_{275}N_{82}O_{55}S^{5+}$ $[M+5H]^{5+}$: 918.6187, found: 918.6195.

Synthesis of PCL2*(PNA2')-E(MPA thioester)GKEAKKK-OH (84b)



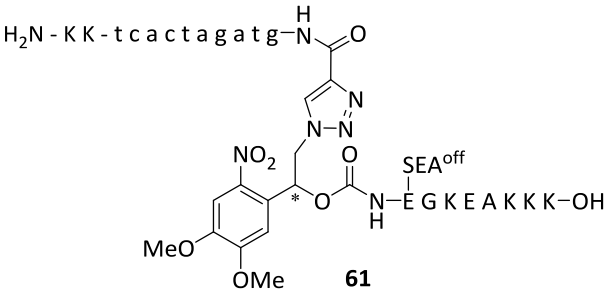
84b

60*ds2* (0.9 mg, 0.19 µmol) was converted to the corresponding MPA thioester following GP8 to give 0.7 mg (0.16 µmol, 80%) of a colorless solid.

HPLC: t_R = 18.966 min (Method E), 6.28 min (Method G).

ESI (HRMS): calcd. for $C_{188}H_{278}N_{82}O_{55}S^{8+}$ $[M+8H]^{8+}$: 574.5144, found: 574.5144; calcd. for $C_{188}H_{277}N_{82}O_{55}S^{7+}$ $[M+7H]^{7+}$: 656.4440, found: 656.4449; calcd. for $C_{188}H_{276}N_{82}O_{55}S^{6+}$ $[M+6H]^{6+}$: 765.6835, found: 765.6838; calcd. for $C_{188}H_{275}N_{82}O_{55}S^{5+}$ $[M+5H]^{5+}$: 918.6187, found: 918.6198; calcd. for $C_{188}H_{274}N_{82}O_{55}S^{4+}$ $[M+4H]^{4+}$: 1148.0216, found: 1148.0240.

Synthesis of PCL2*(PNA1')-E(SEA^{off})GKEAKKK-OH (61)



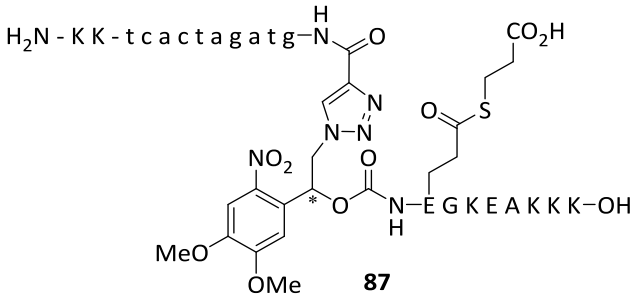
61

125*ds2* was attached to PNA1' (**121**, 5 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 19.311 min.

ESI (HRMS): calcd. for $C_{177}H_{256}N_{79}O_{51}S_2^{7+}$ $[M+7H]^{7+}$: 623.9896, found: 623.9897; calcd. for $C_{177}H_{255}N_{79}O_{51}S_2^{6+}$ $[M+6H]^{6+}$: 727.8200, found: 727.8204; calcd. for $C_{177}H_{254}N_{79}O_{51}S_2^{5+}$ $[M+5H]^{5+}$: 873.1825, found: 873.1833; calcd. for $C_{177}H_{253}N_{79}O_{51}S_2^{4+}$ $[M+4H]^{4+}$: 1091.2263, found: 1091.2279.

Synthesis of PCL2*(PNA1')-E(MPA thioester)GKEAKKK-OH (87)



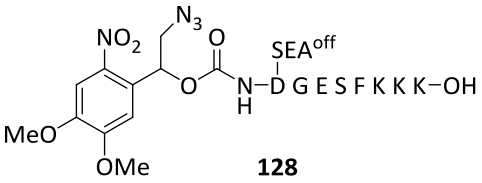
87

61*ds2* (2.2 mg, 0.50 µmol) was converted to the corresponding MPA thioester following GP8 to give 1.8 mg (0.42 µmol, 83%) of a colorless solid.

HPLC (Method E): t_R = 18.526 min.

ESI (HRMS): calcd. for $C_{176}H_{254}N_{78}O_{53}S^{8+}$ $[M+8H]^{8+}$: 542.4907, found: 542.4916; calcd. for $C_{176}H_{253}N_{78}O_{53}S^{7+}$ $[M+7H]^{7+}$: 619.8455, found: 619.8458; calcd. for $C_{176}H_{252}N_{78}O_{53}S^{6+}$ $[M+6H]^{6+}$: 722.9852, found: 722.9857; calcd. for $C_{176}H_{251}N_{78}O_{53}S^{5+}$ $[M+5H]^{5+}$: 867.3807, found: 867.3820; calcd. for $C_{176}H_{250}N_{78}O_{53}S^{4+}$ $[M+4H]^{4+}$: 1083.9741, found: 1083.9760.

Synthesis of PCL2-D(SEA^{off})GESFKKK-OH (128)



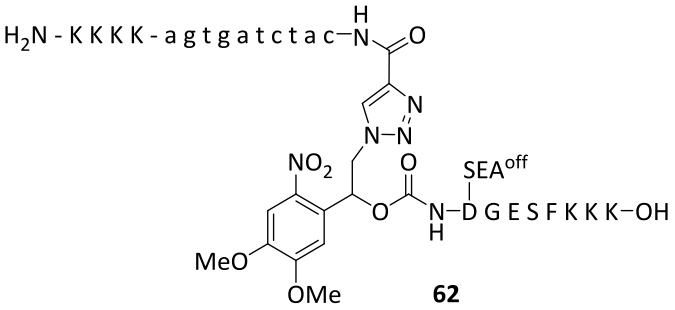
128

The peptide (100 µmol) was coupled to a preloaded WANG resin (lysine, 0.31 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method D): t_R = 16.308 min.

ESI (HRMS): calcd. for $C_{56}H_{87}N_{16}O_{19}S_2^{3+}$ $[M+3H]^{3+}$: 450.5253, found: 450.5152; calcd. for $C_{56}H_{86}N_{16}O_{19}S_2^{2+}$ $[M+2H]^{2+}$: 675.2843, found: 675.2848, calcd. for $C_{56}H_{85}N_{16}O_{19}S_2^{+}$ $[M+H]^{+}$: 1349.5613, found: 1349.5614.

Synthesis of PCL2(PNA2')-E(SEA^{off})GESFKKK-OH (62)



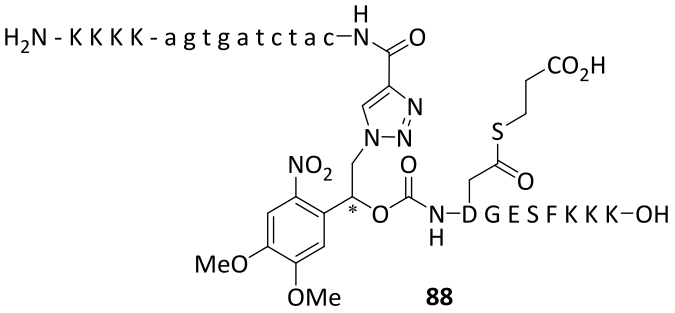
62

128 was attached to PNA2' (**49**, 10 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 21.153 min (*ds*1), 21.656 min (*ds*2).

ESI (HRMS): calcd. for $C_{191}H_{275}N_{82}O_{54}S_2^{7+}$ $[M+7H]^{7+}$: 663.5814, found: 663.5809; calcd. for $C_{191}H_{274}N_{82}O_{54}S_2^{6+}$ $[M+6H]^{6+}$: 774.0104, found: 774.0119; calcd. for $C_{191}H_{273}N_{82}O_{54}S_2^{5+}$ $[M+5H]^{5+}$: 928.6110, found: 928.6117; calcd. for $C_{191}H_{272}N_{82}O_{54}S_2^{4+}$ $[M+4H]^{4+}$: 1160.5120, found: 1160.5137.

Synthesis of PCL2*(PNA2')-D(MPA thioester)GESFKKK-OH (88)



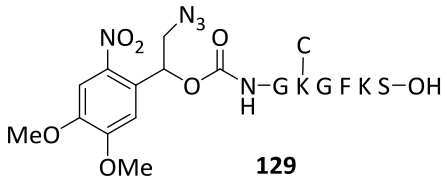
88

62*ds2* (2.0 mg, 0.43 µmol) was converted to the corresponding MPA thioester following GP8 to give 0.9 mg (0.19 µmol, 45%) of a colorless solid.

HPLC (Method E): t_R = 20.968 min.

ESI–MS *m/z* (rel.%): 514 (29) $[M+9H]^{9+}$, 578 (43) $[M+8H]^{8+}$, 660 (64) $[M+7H]^{7+}$, 770 (100) $[M+6H]^{6+}$, 923 (95) $[M+5H]^{5+}$, 1154 (77) $[M+4H]^{4+}$, 1538 (37) $[M+3H]^{3+}$.

Synthesis of PCL2-GK(C)GFKS-OH (129)



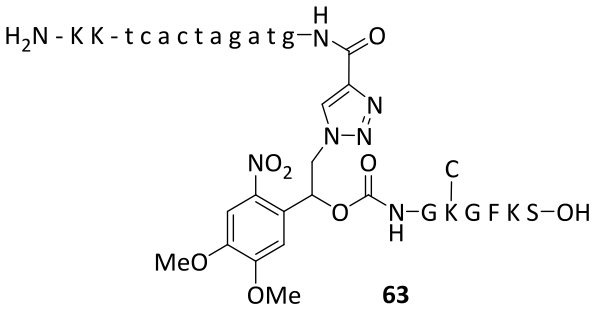
129

The peptide (100 µmol) was coupled to a preloaded WANG resin (serine, 0.31 mmol/g) following GP3-2. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/TIS/EDT/H_2O (94:2.5:2.5:1).

HPLC (Method D): t_R = 26.575 min.

ESI (HRMS): calcd. for $C_{42}H_{62}N_{13}O_{15}S^+$ $[M+H]^+$: 1020.4204, found: 1020.4203.

Synthesis of PCL2(PNA1')-GK(C)GFKS-OH (63)



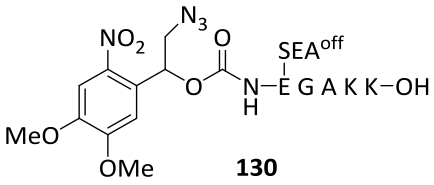
63

129 was attached to PNA1' (**121**, 8.5 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/EDT/TIS/H_2O (94:2.5:2.5:1).

HPLC (Method E): t_R = 19.990 min.

ESI (HRMS): calcd. for $C_{165}H_{228}N_{75}O_{48}S^{7+}$ $[M+7H]^{7+}$: 579.9627, found: 579.9627; calcd. for $C_{165}H_{227}N_{75}O_{48}S^{6+}$ $[M+6H]^{6+}$: 676.4553, found: 676.4554; calcd. for $C_{165}H_{226}N_{75}O_{48}S^{5+}$ $[M+5H]^{5+}$: 811.5448, found: 811.5457; calcd. for $C_{165}H_{225}N_{75}O_{48}S^{4+}$ $[M+4H]^{4+}$: 1014.1792, found: 1014.1815; calcd. for $C_{165}H_{224}N_{75}O_{48}S^{3+}$ $[M+3H]^{3+}$: 1351.9032, found: 1351.9052.

Synthesis of PCL2-E(SEA^{off})GAKK-OH (130)



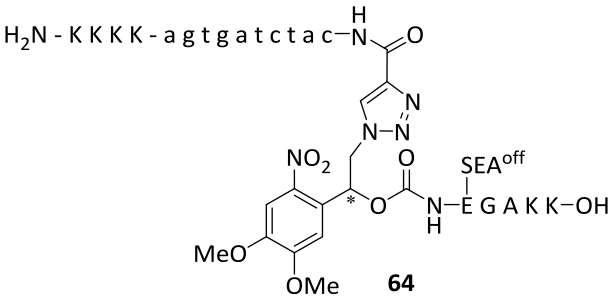
130

The peptide (100 µmol) was coupled to a preloaded WANG resin (lysine, 0.31 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method D): t_R = 17.242 min (*ds*1), 17.550 min (*ds*2).

ESI (HRMS): calcd. for $C_{37}H_{59}N_{12}O_{13}S_2^+$ $[M+H]^+$: 943.3760, found: 943.3739; calcd. for $C_{37}H_{58}N_{12}O_{13}S_2Na^+$ $[M+Na]^+$: 965.3580, found: 965.3555.

Synthesis of PCL2(PNA2')-E(SEA^{off})GAKK-OH (64)



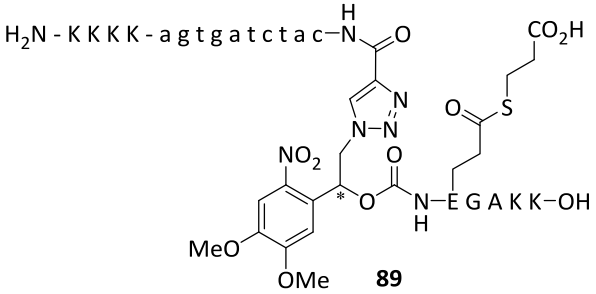
64

130*ds1* was attached to PNA2' (**49**, 10 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 20.154 min.

ESI (HRMS): calcd. for $C_{172}H_{249}N_{78}O_{48}S_2^{7+}$ $[M+7H]^{7+}$: 605.5549, found: 605.5543; calcd. for $C_{172}H_{248}N_{78}O_{48}S_2^{6+}$ $[M+6H]^{6+}$: 706.3129, found: 706.3130; calcd. for $C_{172}H_{247}N_{78}O_{48}S_2^{5+}$ $[M+5H]^{5+}$: 847.3740, found: 847.3741; calcd. for $C_{172}H_{246}N_{78}O_{48}S_2^{4+}$ $[M+4H]^{4+}$: 1058.9656, found: 1058.9662; calcd. for $C_{172}H_{245}N_{78}O_{48}S_2^{3+}$ $[M+3H]^{3+}$: 1411.6184, found: 1411.6179.

Synthesis of PCL2*(PNA2')-E(MPA thioester)GAKK-OH (89)



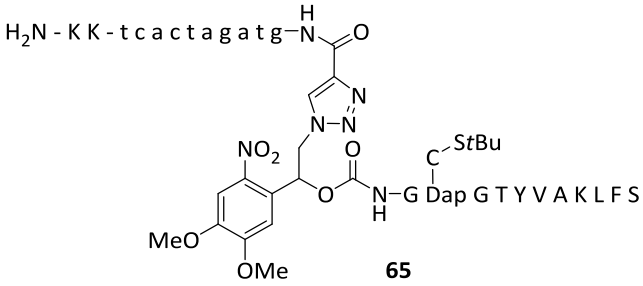
89

64*ds1* (4.0 mg, 0.94 µmol) was converted to the corresponding MPA thioester following GP8 to give 3.1 mg (0.74 µmol, 79%) of a colorless solid.

HPLC (Method E): t_R = 19.324 min.

ESI (HRMS): calcd. for $C_{171}H_{246}N_{77}O_{50}S^{7+}$ $[M+7H]^{7+}$: 601.4108, found: 601.4115; calcd. for $C_{171}H_{245}N_{77}O_{50}S^{6+}$ $[M+6H]^{6+}$: 701.4781, found: 701.4787; calcd. for $C_{171}H_{244}N_{77}O_{50}S^{5+}$ $[M+5H]^{5+}$: 841.5722, found: 841.5733; calcd. for $C_{171}H_{243}N_{77}O_{50}S^{4+}$ $[M+4H]^{4+}$: 1051.7134, found: 1051.7152; calcd. for $C_{171}H_{242}N_{77}O_{50}S^{3+}$ $[M+3H]^{3+}$: 1401.9488, found: 1401.9500.

Synthesis of PCL2(PNA1')-GDap(C{S*t*Bu})GTYVAKLFS-OH (65)



65

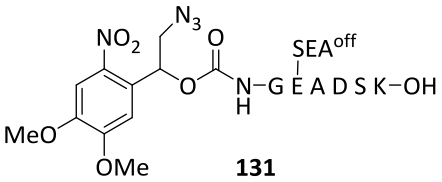
The caged peptide was attached to PNA1' (**121**, 5 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 26.173 min.

ESI (HRMS): calcd. for $C_{193}H_{271}N_{80}O_{55}S_2^{7+}$ $[M+7H]^{7+}$: 665.0046, found: 665.0049; calcd. for $C_{193}H_{270}N_{80}O_{55}S_2^{6+}$ $[M+6H]^{6+}$: 775.6708, found: 775.6718; calcd. for $C_{193}H_{269}N_{80}O_{55}S_2^{5+}$ $[M+5H]^{5+}$: 930.6035, found: 930.6048; calcd. for $C_{193}H_{268}N_{80}O_{55}S_2^{4+}$ $[M+4H]^{4+}$: 1163.2537, found: 1163.2549; calcd. for $C_{193}H_{267}N_{80}O_{55}S_2^{3+}$ $[M+3H]^{3+}$: 1550.6692, found: 1550.6709.

PCL2-peptide used for **65** was synthesized by SWANTJE NAWRATIL.

Synthesis of PCL2-GE(SEA^{off})ADSK-OH (131)



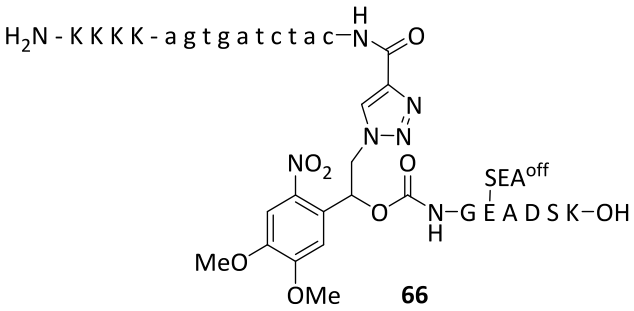
131

The peptide (50 µmol) was coupled to a preloaded WANG resin (lysine, 0.31 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method F): t_R = 16.017 min.

ESI (HRMS): calcd. for $C_{38}H_{58}N_{12}O_{17}S_2^{2+}$ $[M+2H]^{2+}$: 509.1737, found: 509.1732; calcd. for $C_{38}H_{57}N_{12}O_{17}S_2^{+}$ $[M+H]^{+}$: 1017.3401, found: 1017.3402.

Synthesis of PCL2(PNA2')-GE(SEA^{off})ADSK-OH (66)



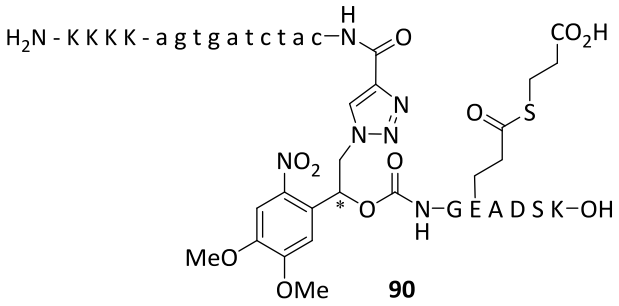
66

131 was attached to PNA2' (**49**, 5 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 21.414 min.

ESI (HRMS): calcd. for $C_{173}H_{246}N_{78}O_{52}S_2^{6+}$ $[M+6H]^{6+}$: 718.6402, found: 718.6413; calcd. for $C_{173}H_{245}N_{78}O_{52}S_2^{5+}$ $[M+5H]^{5+}$: 862.1668; found: 862.1684; calcd. for $C_{173}H_{244}N_{78}O_{52}S_2^{4+}$ $[M+4H]^{4+}$: 1077.4566, found: 1077.4589.

Synthesis of PCL2*(PNA2')-GE(MPA thioester)ADSK-OH (90)



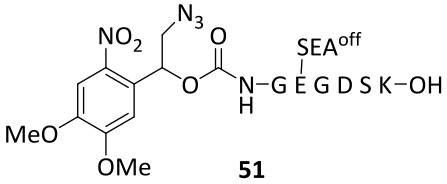
90

66*ds2* (2.0 mg, 0.46 µmol) was converted to the corresponding MPA thioester following GP8 to give 1.5 mg (0.36 µmol, 78%) of a colorless solid.

HPLC (Method E): t_R = 19.316 min.

ESI (HRMS): calcd. for $C_{172}H_{244}N_{77}O_{54}S^{7+}$ $[M+7H]^{7+}$: 611.9771, found: 611.9766; calcd. for $C_{172}H_{243}N_{77}O_{54}S^{6+}$ $[M+6H]^{6+}$: 713.8054, found: 713.8052; calcd. for $C_{172}H_{242}N_{77}O_{54}S^{5+}$ $[M+5H]^{5+}$: 856.3650, found: 856.3650; calcd. for $C_{172}H_{241}N_{77}O_{54}S^{4+}$ $[M+4H]^{4+}$: 1070.2033, found: 1070.2055; calcd. for $C_{172}H_{240}N_{77}O_{54}S^{3+}$ $[M+3H]^{3+}$: 1426.6035, found: 1426.6047.

Synthesis of PCL2-GE(SEA^{off})GDSK-OH (51)



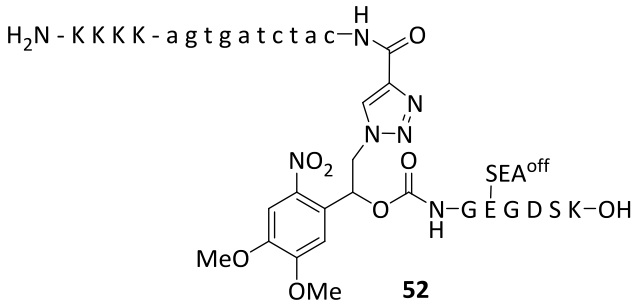
51

The peptide (50 µmol) was coupled to a preloaded WANG resin (lysine, 0.30 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method F): t_R = 16.377 min.

ESI (HRMS): calcd. for $C_{37}H_{56}N_{12}O_{17}S_2^{2+}$ $[M+2H]^{2+}$: 502.1658, found: 502.1653; calcd. for $C_{37}H_{55}N_{12}O_{17}S_2^{+}$ $[M+H]^{+}$: 1003.3244, found: 1003.3244.

Synthesis of PCL2(PNA2')-GE(SEA^{off})GDSK-OH (52)



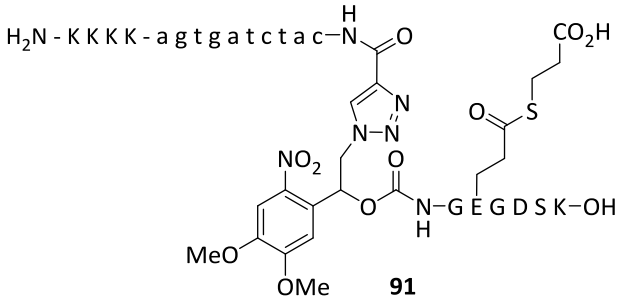
52

The caged peptide **51** was attached to PNA2' (**49**, 5 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 20.996 min.

ESI (HRMS): calcd. for $C_{172}H_{245}N_{78}O_{52}S_2^{7+}$ $[M+7H]^{7+}$: 614.1190, found: 614.1193; calcd. for $C_{172}H_{244}N_{78}O_{52}S_2^{6+}$ $[M+6H]^{6+}$: 716.3042, found: 716.3042; calcd. for $C_{172}H_{243}N_{78}O_{52}S_2^{5+}$ $[M+5H]^{5+}$: 859.3636, found: 859.3631; calcd. for $C_{172}H_{242}N_{78}O_{52}S_2^{4+}$ $[M+4H]^{4+}$: 1073.9527, found: 1073.9528.

Synthesis of PCL2(PNA2')-GE(MPA thioester)GDSK-OH (91)



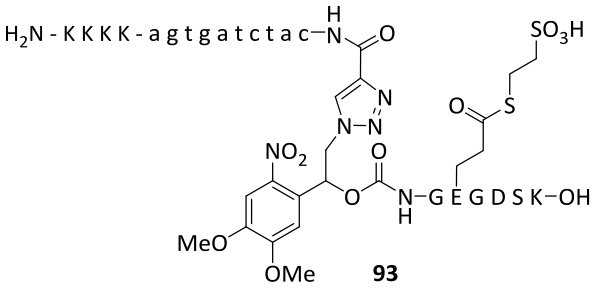
91

52 (1.8 mg, 0.42 µmol) was converted to the corresponding MPA thioester following GP8 to give 1.4 mg (0.33 µmol, 79%) of a colorless solid.

HPLC (Method E): t_R = 18.712 min.

ESI (HRMS): calcd. for $C_{171}H_{241}N_{77}O_{54}S^{6+}$ $[M+6H]^{6+}$: 711.4694, found: 711.4702.

Synthesis of PCL2(PNA2')-GE(MesNa thioester)GDSK-OH (93)



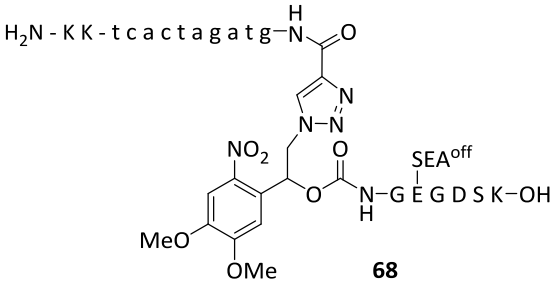
93

52 (1.3 mg, 0.30 µmol) was dissolved in 1 mL degassed Na_2HPO_4 buffer (containing 100 mM NaCl, 20 mM TCEP, 5% w/w MesNa, pH 3) and stirred for 48 h at 37 °C. HPLC purification (Method A) provided 1.0 mg (0.23 µmol, 78%) of a colorless solid.

HPLC (Method E): t_R = 18.432 min.

ESI (HRMS): calcd. for $C_{170}H_{241}N_{77}O_{55}S^{6+}$ $[M+6H]^{6+}$: 717.4639, found: 717.4631; calcd. for $C_{170}H_{240}N_{77}O_{55}S^{5+}$ $[M+5H]^{5+}$: 860.7553, found: 860.7552; calcd. for $C_{170}H_{239}N_{77}O_{55}S^{4+}$ $[M+4H]^{4+}$: 1075.6923, found: 1075.6934; calcd. for $C_{170}H_{238}N_{77}O_{55}S^{3+}$ $[M+3H]^{3+}$: 1433.9206, found: 1433.9219.

Synthesis of PCL2(PNA1')-GE(SEA^{off})GDSK-OH (68)



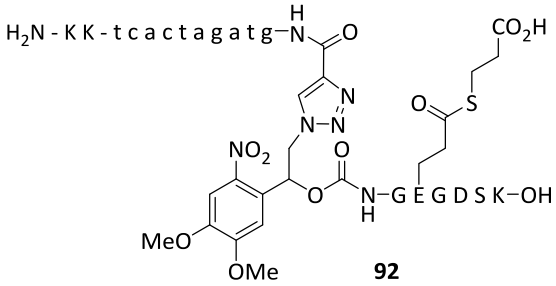
68

51 was attached to PNA1' (**121**, 5 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 21.233 min.

ESI (HRMS): calcd. for $C_{160}H_{219}N_{74}O_{50}S_2^{5+}$ $[M+5H]^{5+}$: 808.1257, found: 808.1252; calcd. for $C_{160}H_{218}N_{74}O_{50}S_2^{4+}$ $[M+4H]^{4+}$: 1009.9053, found: 1009.9057; calcd. for $C_{160}H_{217}N_{74}O_{50}S_2^{3+}$ $[M+3H]^{3+}$: 1346.2046, found: 1346.2045.

Synthesis of PCL2(PNA1')-GE(MPA thioester)GDSK-OH (92)



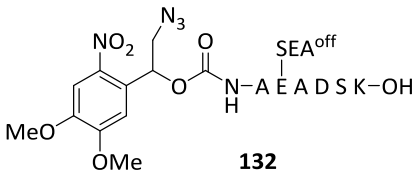
92

68 (1.5 mg, 0.37 µmol) was converted to the corresponding MPA thioester following GP8 to give 1.2 mg (0.30 µmol, 81%) of a colorless solid.

HPLC (Method E): t_R = 19.783 min.

ESI (HRMS): calcd. for $C_{159}H_{217}N_{73}O_{52}S^{6+}$ $[M+6H]^{6+}$: 668.7711, found: 668.7722; calcd. for $C_{159}H_{216}N_{73}O_{52}S^{5+}$ $[M+5H]^{5+}$: 802.3239, found: 802.3249; calcd. for $C_{159}H_{215}N_{73}O_{52}S^{4+}$ $[M+4H]^{4+}$: 1002.6531, found: 1002.6552.

Synthesis of PCL2-AE(SEA^{off})ADSK-OH (132)



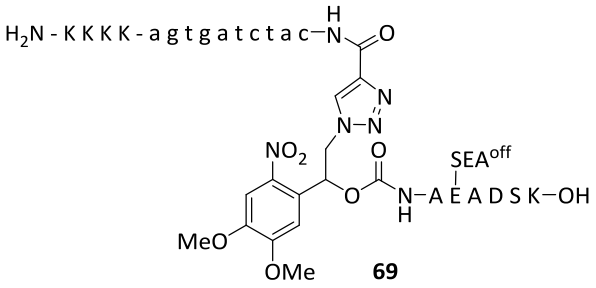
132

The peptide (35 µmol) was coupled to a preloaded WANG resin (lysine, 0.30 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method F): t_R = 16.523 min.

ESI (HRMS): calcd. for $C_{39}H_{59}N_{12}O_{17}S_2^+$ $[M+H]^+$: 1031.3557, found: 1031.3553.

Synthesis of PCL2(PNA2')-AE(SEA^{off})ADSK-OH (69)



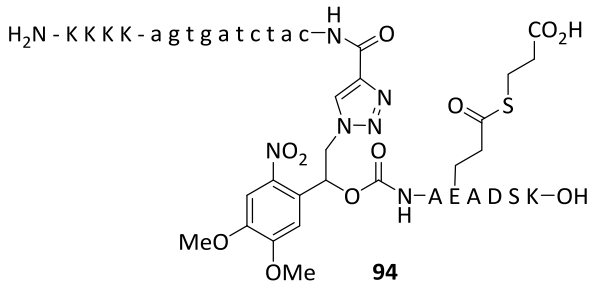
69

132 was attached to PNA2' (**49**, 5 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 21.401 min.

ESI (HRMS): calcd. for $C_{174}H_{247}N_{78}O_{52}S_2^{5+}$ $[M+5H]^{5+}$: 865.3712, found: 865.3702; calcd. for $C_{174}H_{246}N_{78}O_{52}S_2^{4+}$ $[M+4H]^{4+}$: 1081.4621, found: 1081.4659; calcd. for $C_{174}H_{245}N_{78}O_{52}S_2^{3+}$ $[M+3H]^{3+}$: 1441.6137, found: 1441.6155.

Synthesis of PCL2(PNA2')-AE(MPA thioester)ADSK-OH (94)



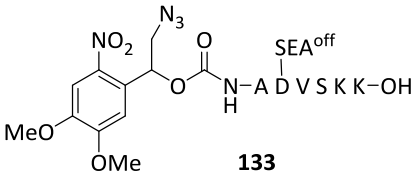
94

69 (2.2 mg, 0.51 µmol) was converted to the corresponding MPA thioester following GP8 to give 1.7 mg (0.40 µmol, 78%) of a colorless solid.

HPLC (Method E): t_R = 19.904 min (*ds*1), 20.128 (*ds*2).

ESI (HRMS): calcd. for $C_{173}H_{246}N_{77}O_{54}S^{7+}$ $[M+7H]^{7+}$: 614.2659, found: 614.2661; calcd. for $C_{173}H_{245}N_{77}O_{54}S^{6+}$ $[M+6H]^{6+}$: 716.4757, found: 716.4764; calcd. for $C_{173}H_{244}N_{77}O_{54}S^{5+}$ $[M+5H]^{5+}$: 859.5694, found: 859.5700; calcd. for $C_{173}H_{243}N_{77}O_{54}S^{4+}$ $[M+4H]^{4+}$: 1074.2099, found: 1074.2105; calcd. for $C_{173}H_{242}N_{77}O_{54}S^{3+}$ $[M+3H]^{3+}$: 1431.9442, found: 1431.9449.

Synthesis of PCL2-AD(SEA^{off})VSKK-OH (133)



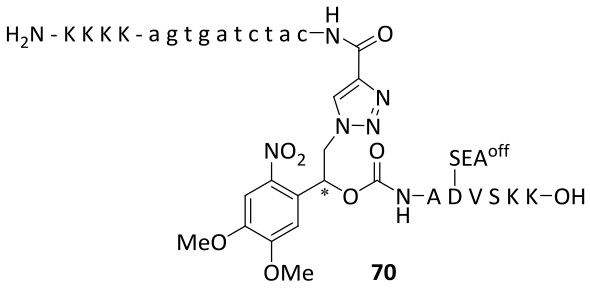
133

The peptide (35 µmol) was coupled to a preloaded WANG resin (lysine, 0.30 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method F): t_R = 14.747 min (*ds*1), 15.062 (*ds*2).

ESI (HRMS): calcd. for $C_{42}H_{69}N_{13}O_{15}S_2^{2+}$ $[M+2H]^{2+}$: 529.7233, found: 529.7234.

Synthesis of PCL2*(PNA2')-AD(SEA^{off})VSKK-OH (70)



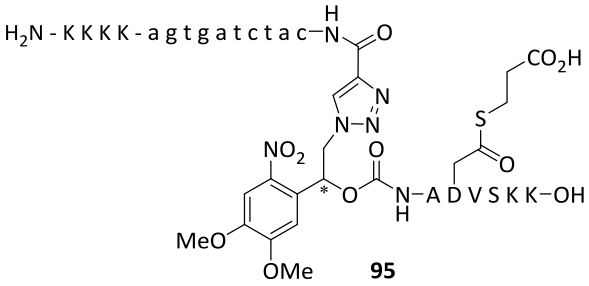
70

133*ds*2 was attached to PNA2' (**49**, 5 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 21.018 min.

ESI (HRMS): calcd. for $C_{177}H_{259}N_{79}O_{50}S_2^{8+}$ $[M+8H]^{8+}$: 544.6202, found: 544.6207; calcd. for $C_{177}H_{258}N_{79}O_{50}S_2^{7+}$ $[M+7H]^{7+}$: 622.2791, found: 622.2793; calcd. for $C_{177}H_{257}N_{79}O_{50}S_2^{6+}$ $[M+6H]^{6+}$: 725.8245, found: 725.8247; calcd. for $C_{177}H_{256}N_{79}O_{50}S_2^{5+}$ $[M+5H]^{5+}$: 870.7879, found: 870.7887; calcd. for $C_{177}H_{255}N_{79}O_{50}S_2^{4+}$ $[M+4H]^{4+}$: 1088.2330, found: 1088.2344; calcd. for $C_{177}H_{254}N_{79}O_{50}S_2^{3+}$ $[M+3H]^{3+}$: 1450.6416, found: 1450.6419;

Synthesis of PCL2*(PNA2')-AD(MPA thioester)VSKK-OH (95)



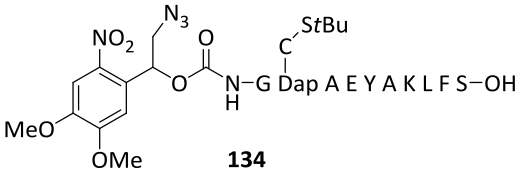
95

70 (2.8 mg, 0.64 µmol) was converted to the corresponding MPA thioester following GP8 to give 2.1 mg (0.49 µmol, 76%) of a colorless solid.

HPLC (Method E): t_R = 19.612 min.

ESI (HRMS): calcd. for $C_{176}H_{251}N_{78}O_{52}S^{3+}$ $[M+3H]^{3+}$: 1440.9721, found: 1440.9734; calcd. for $C_{176}H_{252}N_{78}O_{52}S^{4+}$ $[M+4H]^{4+}$: 1080.9809, found: 1080.9828; calcd. for $C_{176}H_{253}N_{78}O_{52}S^{5+}$ $[M+5H]^{5+}$: 864.9861, found: 864.9872; calcd. for $C_{176}H_{254}N_{78}O_{52}S^{6+}$ $[M+6H]^{6+}$: 720.9897, found: 720.9901; calcd. for $C_{176}H_{255}N_{78}O_{52}S^{7+}$ $[M+7H]^{7+}$: 618.1350, found: 618.1350; calcd. for $C_{176}H_{256}N_{78}O_{52}S^{8+}$ $[M+8H]^{8+}$: 540.9941, found: 540.9935.

Synthesis of PCL2-GDap(C{S*t*Bu})AEYAKLFS-OH (134)



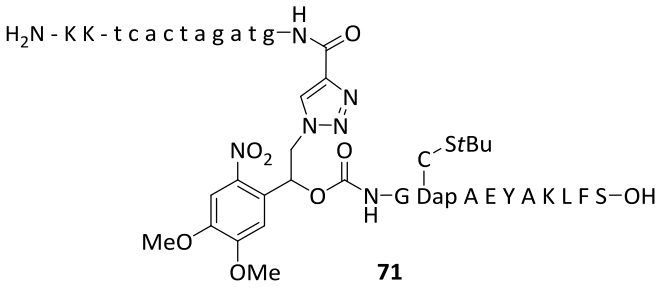
134

The peptide (50 µmol) was coupled to a preloaded WANG resin (serine, 0.31 mmol/g) following GP3. PCL2 was attached following GP6. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method F): t_R = 17.254 min.

ESI (HRMS): calcd. for $C_{67}H_{99}N_{17}O_{22}S_2^{2+}$ $[M+2H]^{2+}$: 778.8291, found: 778.8294; calcd. for $C_{67}H_{98}N_{17}O_{22}S_2^{+}$ $[M+H]^{+}$: 1556.6508, found: 1556.6498;

Synthesis of PCL2(PNA1')-GDap(C{S*t*Bu})AEYAKLFS-OH (71)



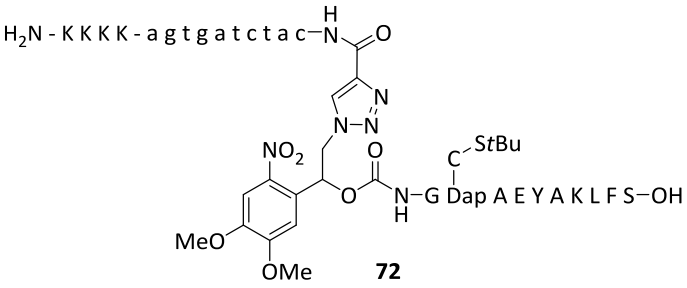
71

134 was attached to PNA1' (**121**, 5 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 25.143 min.

ESI (HRMS): calcd. for $C_{190}H_{264}N_{79}O_{55}S_2^{7+}$ $[M+7H]^{7+}$: 656.8535, found: 656.8528; calcd. for $C_{190}H_{263}N_{79}O_{55}S_2^{6+}$ $[M+6H]^{6+}$: 766.1611, found: 766.1612; calcd. for $C_{190}H_{262}N_{79}O_{55}S_2^{5+}$ $[M+5H]^{5+}$: 919.1919, found: 919.1923; calcd. for $C_{190}H_{261}N_{79}O_{55}S_2^{4+}$ $[M+4H]^{4+}$: 1148.7381, found: 1148.7395; calcd. for $C_{190}H_{260}N_{79}O_{55}S_2^{3+}$ $[M+3H]^{3+}$: 1531.3150, found: 1531.3158.

Synthesis of PCL2(PNA2')-GDap(C{S*t*Bu})AEYAKLFS-OH (72)



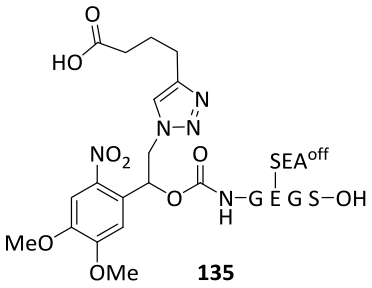
72

The caged peptide **134** was attached to PNA2' (**49**, 4 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method E): t_R = 24.756 min.

ESI (HRMS): calcd. for $C_{202}H_{288}N_{83}O_{57}S_2^{7+}$ $[M+7H]^{7+}$: 693.4520, found: 693.4520; calcd. for $C_{202}H_{287}N_{83}O_{57}S_2^{6+}$ $[M+6H]^{6+}$: 808.8595, found: 808.8599; calcd. for $C_{202}H_{286}N_{83}O_{57}S_2^{5+}$ $[M+5H]^{5+}$: 970.4299, found: 970.4308; calcd. for $C_{202}H_{285}N_{83}O_{57}S_2^{4+}$ $[M+4H]^{4+}$: 1212.7856, found: 1212.7869; calcd. for $C_{202}H_{284}N_{83}O_{57}S_2^{3+}$ $[M+3H]^{3+}$: 1616.7117, found: 1616.7130

Synthesis of 4-(1-{PCL2-GE[SEA^{off}]GS}-1*H*-1,2,3-triazol-1-yl)butanoic acid (135)



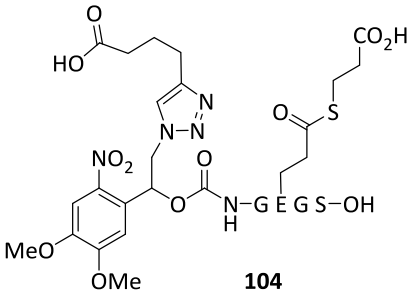
135

The peptide (20 µmol) was coupled to a preloaded WANG resin (serine, 0.31 mmol/g) following GP3. PCL2 was attached following GP6. Then, hexynoic acid (4.4 µL, 40 µmol, 2.0 eq.), CuI (5.3 mg, 28 µmol, 1.4 eq.) and NaAsc (5.9 mg, 30 µol, 1.5 eq.) were dissolved in degassed DMF_{abs} (1.5 mL) and added to the resin. The mixture was agitated for 18 h at room temperature. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method F): t_R = 14.442 min.

ESI (HRMS): calcd. for $C_{33}H_{46}N_9O_{15}S_2^+$ $[M+H]^+$: 872.2549, found: 872.2552; calcd. for $C_{33}H_{45}N_9O_{15}S_2Na^+$ $[M+Na]^+$: 894.2369, found: 894.2374.

Synthesis of 4-(1-{PCL2-GE[MPA thioester]GS}-1*H*-1,2,3-triazol-1-yl)butanoic acid (104)



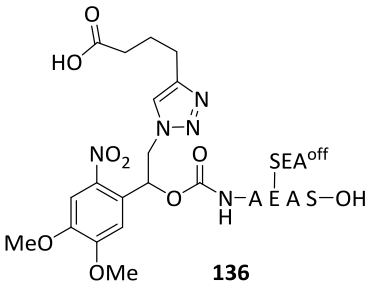
104

135 (5.0 mg, 5.73 µmol) was converted to the corresponding MPA thioester following GP8 to give 4.2 mg (4.98 µmol, 87%) of a colorless solid.

HPLC (Method E): t_R = 26.352 min.

ESI (HRMS): calcd. for $C_{32}H_{42}N_8O_{17}SNa^+$ $[M+Na]^+$: 865.2281, found: 865.2285.

Synthesis of 4-(1-{PCL2-AE[SEA^{off}]AS}-1*H*-1,2,3-triazol-1-yl)butanoic acid (136)



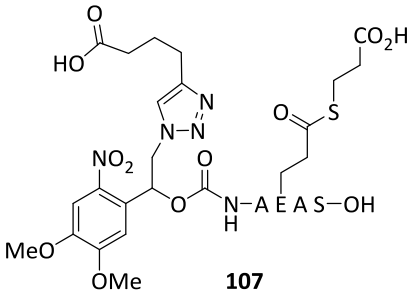
136

The peptide (20 µmol) was coupled to a preloaded WANG resin (serine, 0.31 mmol/g) following GP3. PCL2 was attached following GP6. Then, hexynoic acid (4.4 µL, 40 µmol, 2.0 eq.), CuI (5.3 mg, 28 µmol, 1.4 eq.) and NaAsc (5.9 mg, 30 µol, 1.5 eq.) were dissolved in degassed DMF_{abs} (1.5 mL) and added to the resin. The mixture was agitated for 18 h at room temperature. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method F): t_R = 14.680 min (ds1), 15.037 (*ds*2).

ESI (HRMS): calcd. for $C_{35}H_{50}N_9O_{15}S_2^+$ $[M+H]^+$: 900.2862, found: 900.2866; calcd. for $C_{35}H_{49}N_9O_{15}S_2Na^+$ $[M+Na]^+$: 922.2682; found: 922.2679.

Synthesis of 4-(1-{PCL2-AE[MPA thioester]AS}-1*H*-1,2,3-triazol-1-yl)butanoic acid (107)



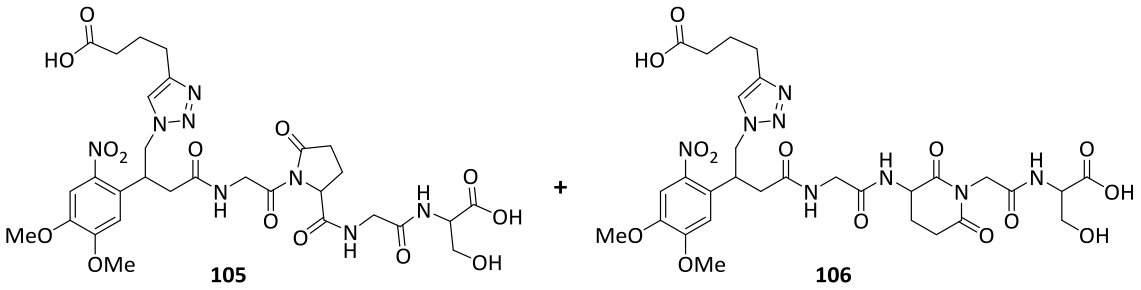
107

136 (4.5 mg, 5.00 µmol) was converted to the corresponding MPA thioester following GP8 to give 3.8 mg (4.36 µmol, 87%) of a colorless solid.

HPLC (Method E): t_R = 26.279 min (*ds*1), 26.742 (*ds*2).

ESI (HRMS): calcd. for $C_{34}H_{46}N_8O_{17}SNa^+$ $[M+Na]^+$: 893.2594, found: 893.2601.

Cyclization of 104 (105+106)



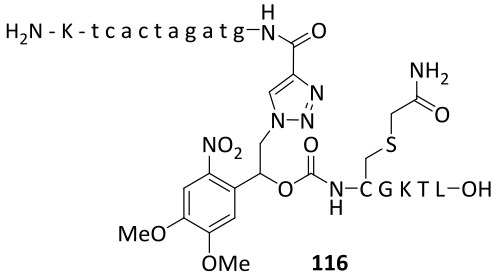
105 + **106**

104 (2.1 mg, 2.50 µmol) was dissolved in 1.5 mL degassed aqueous buffer (10 mM NaH_2PO_4, 100 mM NaCl, 20 mM TCEP, pH 7.0) and stirred at room temperature.

HPLC (Method E): t_R = 20.435 min (*cycl*1), 20.973 min (*cycl*2).

ESI (HRMS): calcd. for $C_{29}H_{36}N_8O_{15}Na^+$ $[M+Na]^+$: 759.2192, found: 759.2189; calcd. for $C_{29}H_{35}N_8O_{15}Na_2^+$ $[M+Na]^+$: 781.2012, found: 781.1988; calcd. for $C_{29}H_{34}N_8O_{15}^{2-}$ $[M-2H]^{2-}$: 367.1077, found: 367.1067; calcd. for $C_{29}H_{35}N_8O_{15}^-$ $[M-H]^-$: 735.2227, found: 735.2206; calcd. for $C_{29}H_{34}N_8O_{15}Na^-$ $[M-H]^-$: 757.2047, found: 757.2014; calcd. for $C_{29}H_{33}N_8O_{15}Na_2^-$ $[M-H]^-$: 779.1866, found: 779.1840.

Synthesis of PCL2(PNA1)-C(acetamide)GKTL-OH (116)



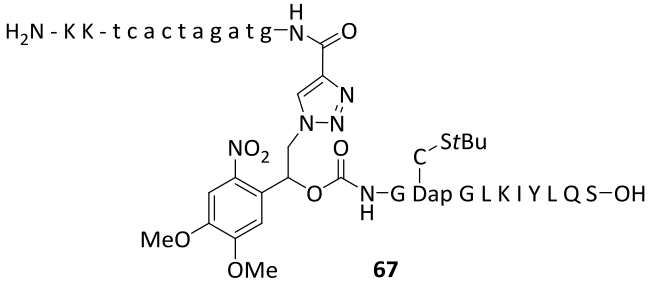
116

55 (0.5 mg,0.13 µmol) was dissolved in 1 mL degassed aqueous buffer (10 mm NaH_2PO_4, 100 mm NaCl, 20 mm TCEP, pH 7.0) and iodoacetamide (1.0 mg, 5.4 µmol) was added. The solution was stirred at room temperature for 30 min, then it was quenched with 3-mercaptopropionic acid (25 µL, 288 µmol).

HPLC (Method C): t_R = 20.692 min.

ESI (HRMS): calcd. for $C_{151}H_{207}N_{71}O_{46}S^{6+}$ $[M+6H]^{6+}$: 630.7632, found: 630.7627; calcd. for $C_{151}H_{206}N_{71}O_{46}S^{5+}$ $[M+5H]^{5+}$: 756.7144, found: 756.7147; calcd. for $C_{151}H_{205}N_{71}O_{46}S^{4+}$ $[M+4H]^{4+}$: 945.6412, found: 945.6418; calcd. for $C_{151}H_{204}N_{71}O_{46}S^{3+}$ $[M+3H]^{3+}$: 1260.5191, found: 1260.5197.

Synthesis of PCL2(PNA1')-GDap(C{S*t*Bu})GLKIYLQS-OH (67)



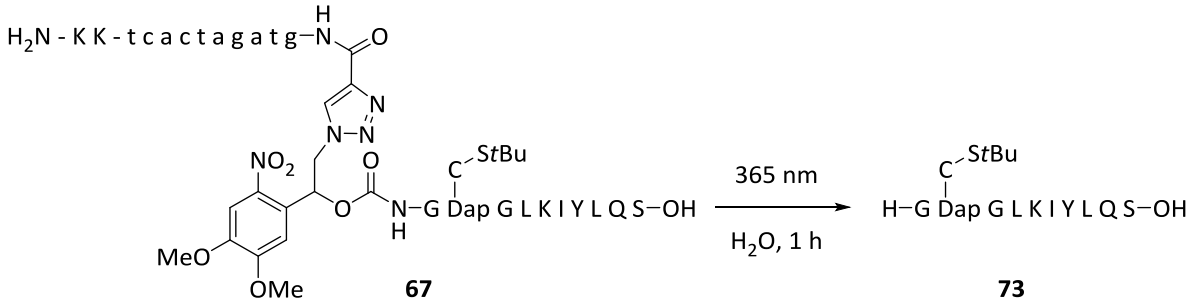
67

The caged peptide was attached to PNA1' (**121**, 4 µmol) on resin following GP7. Cleavage from the resin was performed with TFA/TIS/H_2O (95:2.5:2.5).

HPLC (Method C): t_R = 24.623 min.

ESI (HRMS): calcd. for $C_{189}H_{271}N_{80}O_{54}S_2^{7+}$ $[M+7H]^{7+}$: 655.5760, found: 655.5753; calcd. for $C_{189}H_{270}N_{80}O_{54}S_2^{6+}$ $[M+6H]^{6+}$: 764.6708, found: 764.6709; calcd. for $C_{189}H_{269}N_{80}O_{54}S_2^{5+}$ $[M+5H]^{5+}$: 917.4035, found: 917.4044; calcd. for $C_{189}H_{268}N_{80}O_{54}S_2^{4+}$ $[M+4H]^{4+}$: 1146.5026, found: 1146.5042; calcd. for $C_{189}H_{267}N_{80}O_{54}S_2^{3+}$ $[M+3H]^{3+}$: 1528.3344, found: 1528.3353.

Uncaging of PCL2(PNA1')-GDap(C{S*t*Bu})GLKIYLQS-OH (73)



67 **73**

67 (0.5 mg, 0.11 µmol) was dissolved in H_2O (1.0 mL) and irradiated for 1 h with UV light [lamp setup: *b)*].

HPLC (Method E): t_R = 25.567 min.

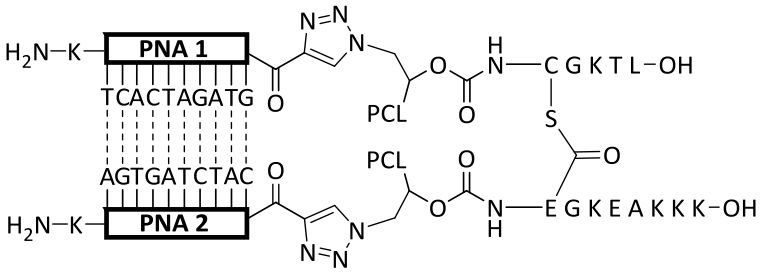
ESI (HRMS): calcd. for $C_{55}H_{96}N_{14}O_{15}S_2^{2+}$ $[M+2H]^{2+}$: 628.3305, found: 628.3303; calcd. for $C_{55}H_{95}N_{14}O_{15}S_2^{+}$ $[M+H]^{+}$: 1255.6537, found: 1255.6548.

PCL2-peptide used for **67** was synthesized by SWANTJE NAWRATIL.

4.3.5 Template-Directed Ligation Reactions

All ligation reactions were carried out following GP9. Reaction conditions, conversion and reaction times are summarized in Table 3 (page 54).

Synthesis of 82

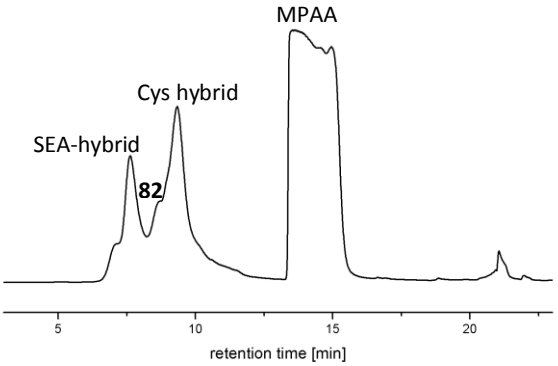


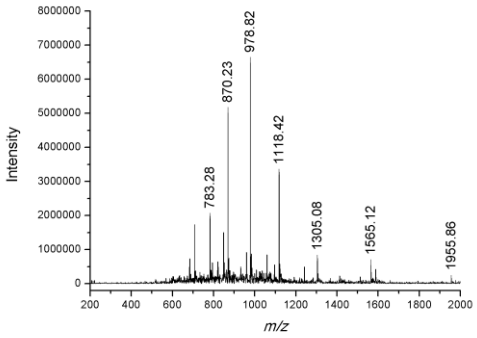
82

Templated ligation reaction of **55** and **56** following GP9. Table 3, Entry 3.

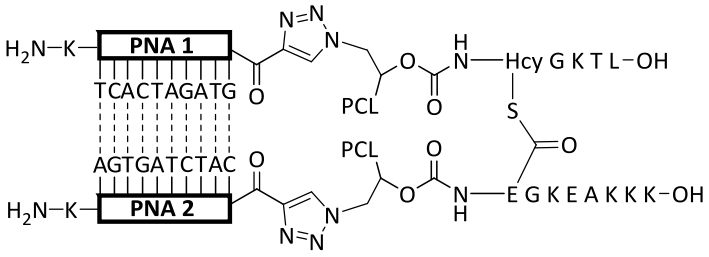
HPLC (Method G): t_R = 8.61 min.

ESI–MS *m/z* (rel.%): 783.28 (30) $[M+10H]^{10+}$, 870.23 (77) $[M+9H]^{9+}$, 978.82 (100) $[M+8H]^{8+}$, 1118.42 (49) $[M+7H]^{7+}$, 1305.08 (12) $[M+6H]^{6+}$, 1565.12 (10) $[M+5H]^{5+}$, 1955.87 (3) $[M+4H]^{4+}$.





Synthesis of 83



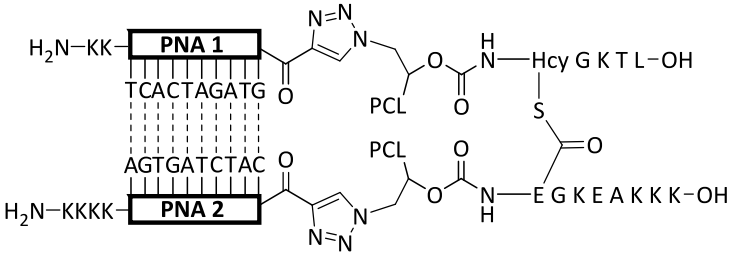
83

Templated ligation reaction of **58** and **56** following GP9.Table 3, Entries 5 & 6.

HPLC (Method G): t_R = 8.70 min.

ESI–MS *m/z* (rel.%): 748.48 (37) $[M+11H]^{11+}$, 823.13 (57) $[M+10H]^{10+}$, 914.50 (85) $[M+9H]^{9+}$, 1028.64 (100) $[M+8H]^{8+}$, 1175.37 (34) $[M+7H]^{7+}$.

Synthesis of 85

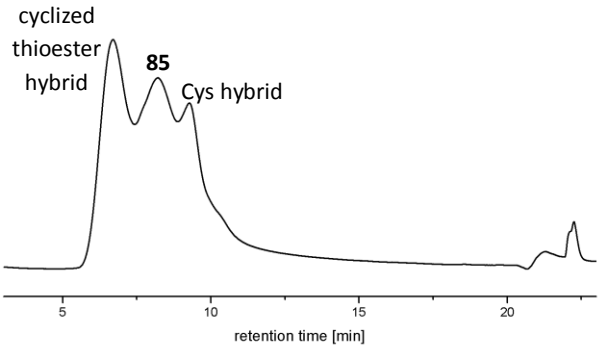


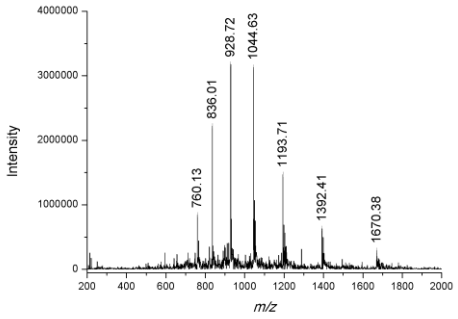
85

Templated ligation reaction of **58** and **84** following GP9. Table 3, Entry 7.

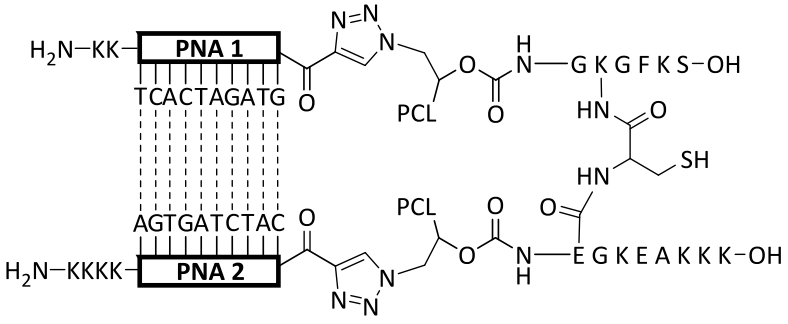
HPLC (Method G): t_R = 8.23 min.

ESI–MS *m/z* (rel.%): 760.13 (27) $[M+11H]^{11+}$, 836.01 (71) $[M+10H]^{10+}$, 928.72 (100) $[M+9H]^{9+}$, 1044.63 (98) $[M+8H]^{8+}$, 1193.71 (48) $[M+7H]^{7+}$, 1392.41 (21) $[M+6H]^{6+}$, 1670.38 (10) $[M+5H]^{5+}$.





Synthesis of 99



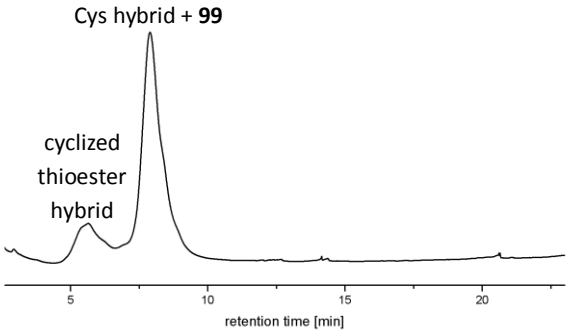
99

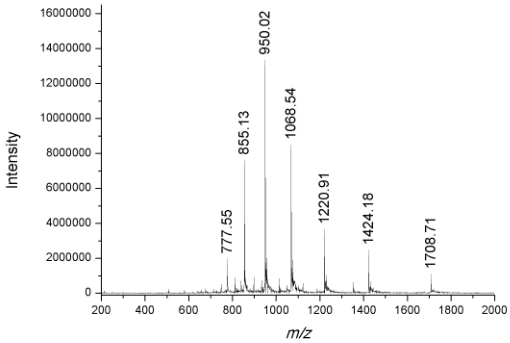
Templated ligation reaction of **63** and **84** following GP9. Table 3, Entry 10.

HPLC (Method G): t_R = 7.89 min.

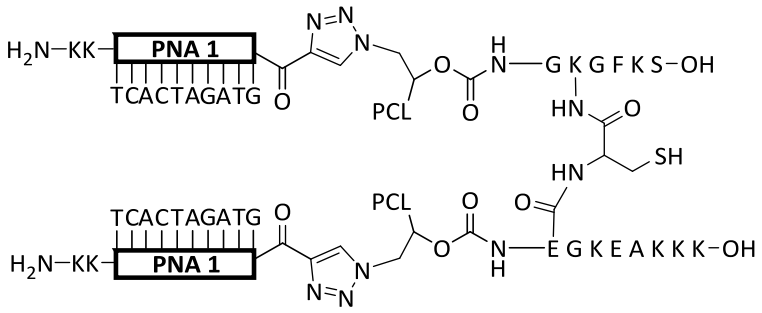
ESI–MS *m/z* (rel.%): 777.55 (12) $[M+11H]^{11+}$, 855.13 (56) $[M+10H]^{10+}$, 950.02 (100) $[M+9H]^{9+}$, 1068.54 (64) $[M+8H]^{8+}$, 1220.91 (26) $[M+7H]^{7+}$, 1424.18 (19) $[M+6H]^{6+}$, 1708.71 (8) $[M+5H]^{5+}$.

ESI (HRMS): calcd. for $C_{350}H_{494}N_{157}O_{101}S^{9+}$ $[M+9H]^{9+}$: 949.7569, found: 949.7577; calcd. for $C_{350}H_{493}N_{157}O_{101}S^{8+}$ $[M+8H]^{8+}$: 1068.3505, found: 1068.3526; calcd. for $C_{350}H_{492}N_{157}O_{101}S^{7+}$ $[M+7H]^{7+}$: 1220.8282, found: 1220.8312.





Synthesis of 137

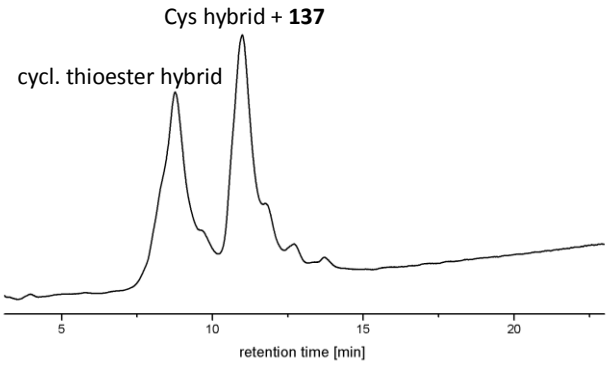


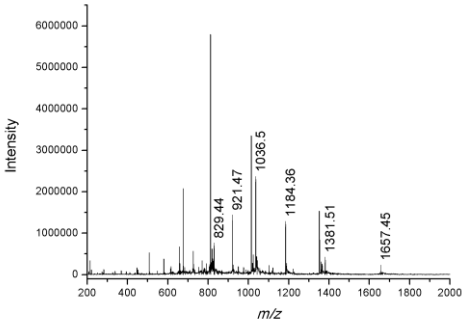
137

Templated ligation reaction of **63** and **87** following GP9. Table 3, Entry 11.

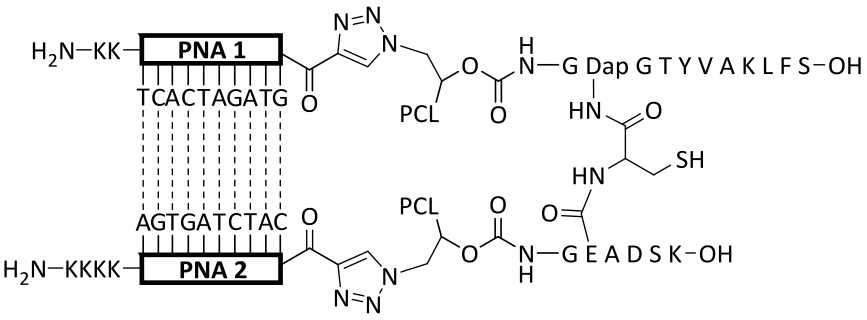
HPLC (Method H): t_R = 10.99 min.

ESI–MS *m/z* (rel.%): 829.44 (13) $[M+10H]^{10+}$, 921.47 (24) $[M+9H]^{9+}$, 1036.49 (39) $[M+8H]^{8+}$, 1184.36 (21) $[M+7H]^{7+}$, 1381.51 (6) $[M+6H]^{6+}$, 1657.45 (3) $[M+5H]^{5+}$.





Synthesis of 103

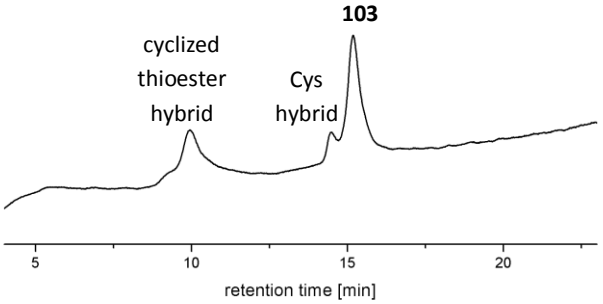


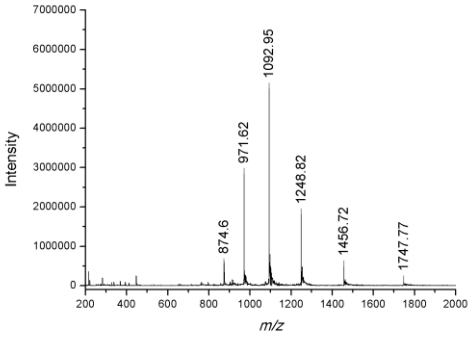
103

Templated ligation reaction of **65** and **90** following GP9. Table 3, Entry 13.

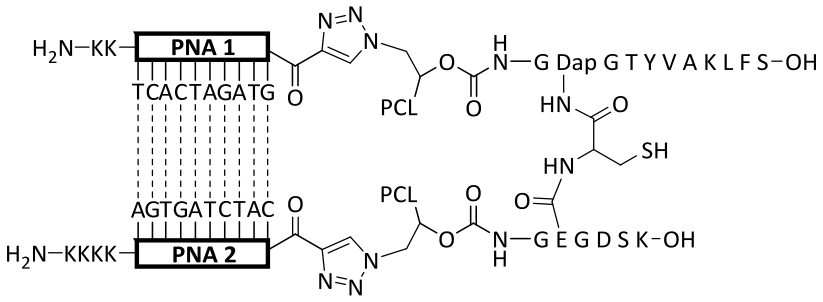
HPLC (Method H): t_R = 15.19 min.

ESI–MS *m/z* (rel.%): 795.14 (2) $[M+11H]^{11+}$, 874.60 (12) $[M+10H]^{10+}$, 971.62 (57) $[M+9H]^{9+}$, 1092.95 (100) $[M+8H]^{8+}$, 1248.82 (37) $[M+7H]^{7+}$, 1456.72 (11) $[M+6H]^{6+}$, 1747.77 (3) $[M+5H]^{5+}$.





Synthesis of 138



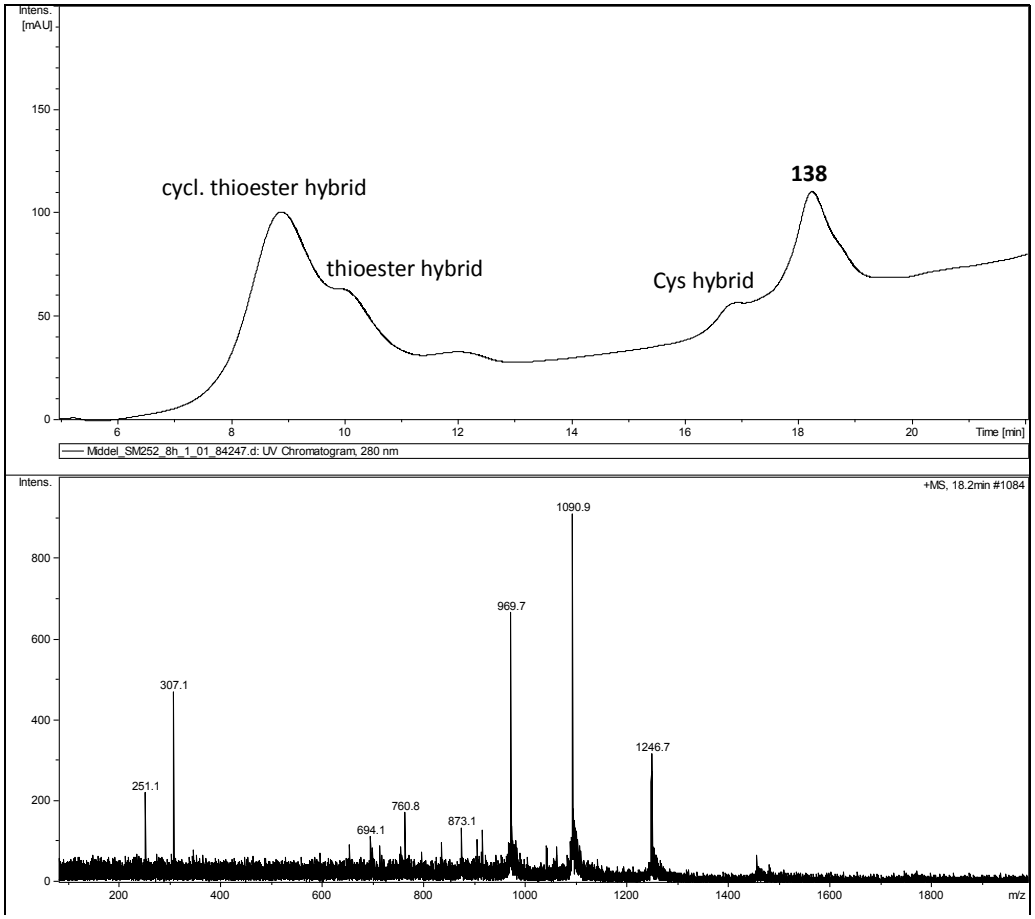
138

Templated ligation reaction of **65** and **91** following GP9. Table 3, Entry 14.

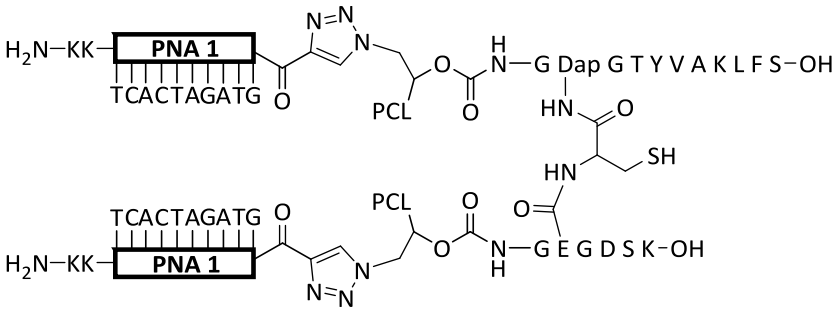
HPLC (Method I): t_R = 18.21 min.

ESI–MS *m/z* (rel.%): 873.1 (15) $[M+10H]^{10+}$, 969.7 (74) $[M+9H]^{9+}$, 1090.9 (100) $[M+8H]^{8+}$, 1246.7 (35) $[M+7H]^{7+}$.

ESI (HRMS): calcd. for $C_{357}H_{493}N_{157}O_{107}S^{8+}$ $[M+8H]^{8+}$: 1090.8467, found: 1090.8502; calcd. for $C_{357}H_{492}N_{157}O_{107}S^{7+}$ $[M+7H]^{7+}$: 1246.5381, found: 1246.5400; calcd. for $C_{357}H_{491}N_{157}O_{107}S^{6+}$ $[M+6H]^{6+}$: 1454.1266, found: 1454.1314.



Synthesis of 139

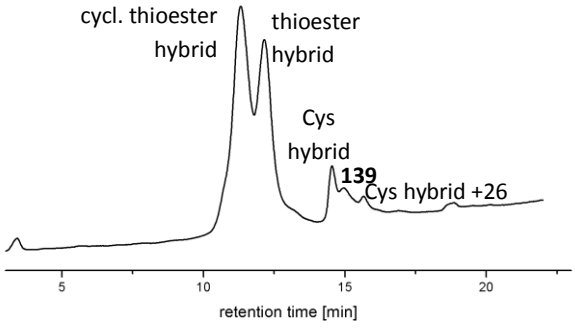


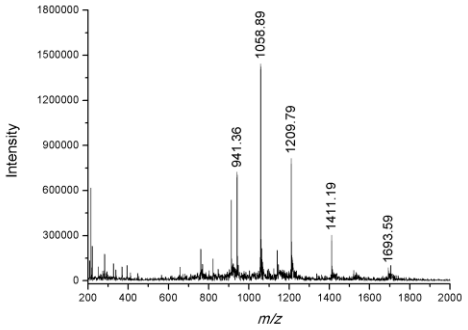
139

Templated ligation reaction of **65** and **92** following GP9. Table 3, Entry 15.

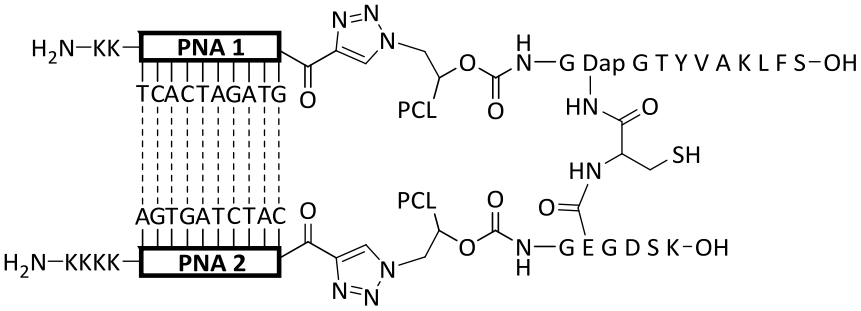
HPLC (Method H): t_R = 14.95 min.

ESI–MS *m/z* (rel.%): 941.36 (47) $[M+9H]^{9+}$, 1058.89 (100) $[M+8H]^{8+}$, 1209.79 (55) $[M+7H]^{7+}$, 1411.19 (19) $[M+6H]^{6+}$, 1693.59 (4) $[M+5H]^{5+}$.





Synthesis of 140

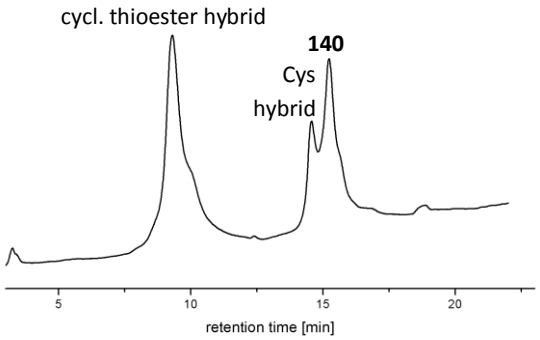


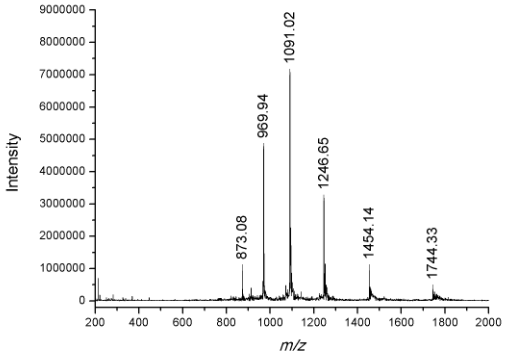
140

Templated ligation reaction of **65** and **93** following GP9. Table 3, Entry 16.

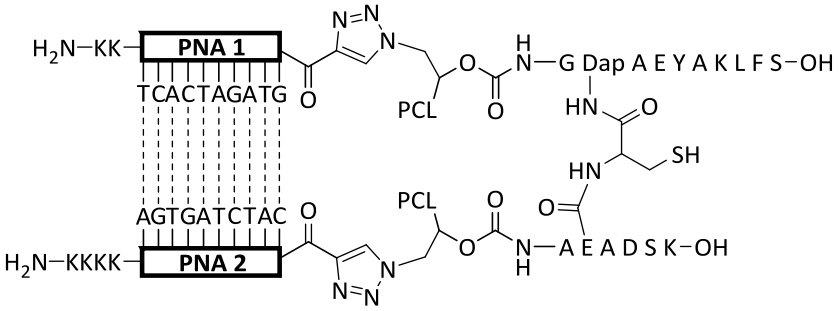
HPLC (Method I): t_R = 15.23 min.

ESI–MS *m/z* (rel.%): 873.07 (16) $[M+10H]^{10+}$, 969.94 (67) $[M+9H]^{9+}$, 1091.02 (100) $[M+8H]^{8+}$, 1246.65 (44) $[M+7H]^{7+}$, 1454.12 (16) $[M+6H]^{6+}$, 1744.35 (6) $[M+5H]^{5+}$.





Synthesis of 108



108

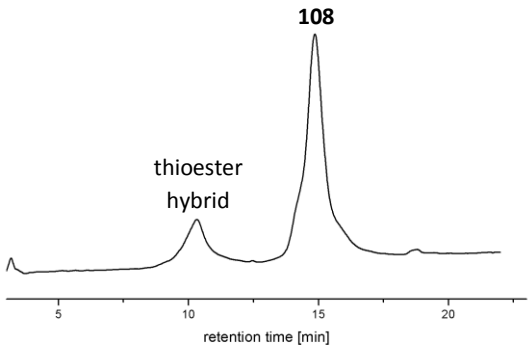
Templated ligation reaction of **71** and **94** following GP9. Table 3, Entry 17.

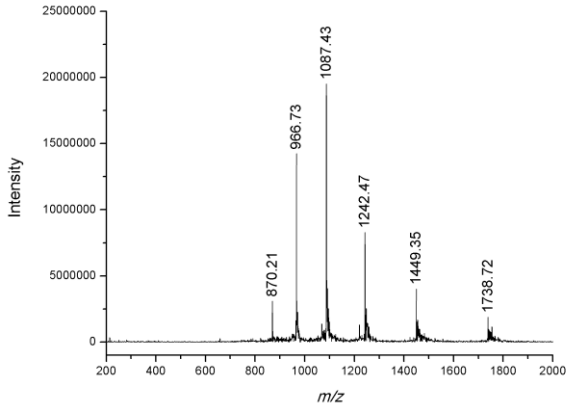
Additional information for HPLC purification: 0.05% FA instead of 0.1% TFA was used.

HPLC (Method I): t_R = 14.87 min.

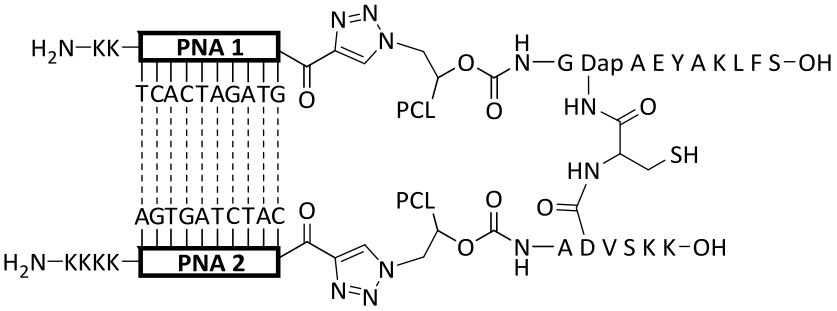
ESI–MS *m/z* (rel.%): 870.21 (16) $[M+10H]^{10+}$, 966.73 (68) $[M+9H]^{9+}$, 1087.43 (100) $[M+8H]^{8+}$, 1242.47 (39) $[M+7H]^{7+}$, 1449.35 (16) $[M+6H]^{6+}$, 1738.72 (6) $[M+5H]^{5+}$.

ESI (HRMS): calcd. for $C_{356}H_{490}N_{156}O_{107}S^{8+}$ $[M+8H]^{8+}$: 1087.2184, found: 1087.2186; calcd. for $C_{356}H_{489}N_{156}O_{107}S^{7+}$ $[M+7H]^{7+}$: 1242.5346, found: 1242.5380; calcd. for $C_{356}H_{488}N_{156}O_{107}S^{6+}$ $[M+6H]^{6+}$: 1449.2888, found: 1449.2918.





Synthesis of 109

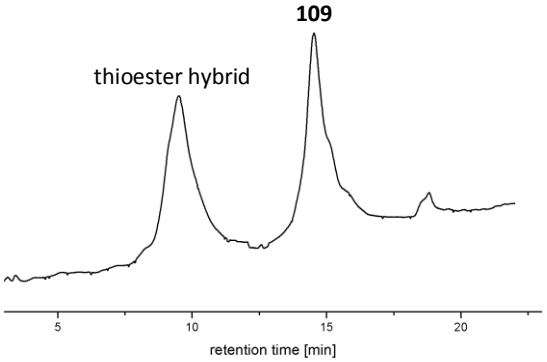


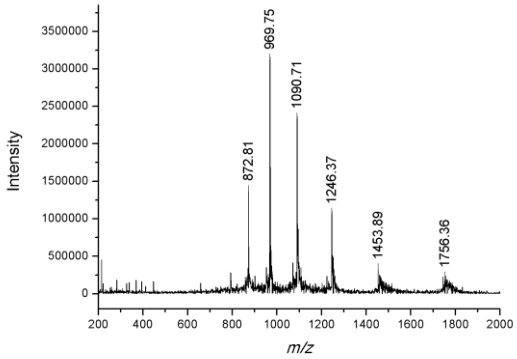
109

Templated ligation reaction of **71** and **95** following GP9. Table 3, Entry 18.

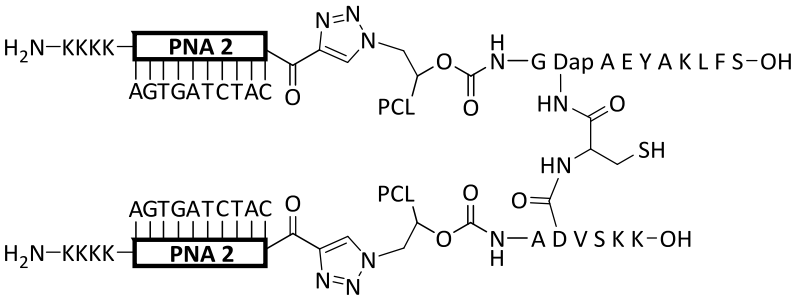
HPLC (Method I): t_R = 14.53 min.

ESI–MS *m/z* (rel.%): 872.81 (48) $[M+10H]^{10+}$, 969.75 (100) $[M+9H]^{9+}$, 1090.71 (87) $[M+8H]^{8+}$, 1246.37 (38) $[M+7H]^{7+}$, 1453.89 (11) $[M+6H]^{6+}$, 1756.36 (9) $[M+5H]^{5+}$.





Synthesis of 141



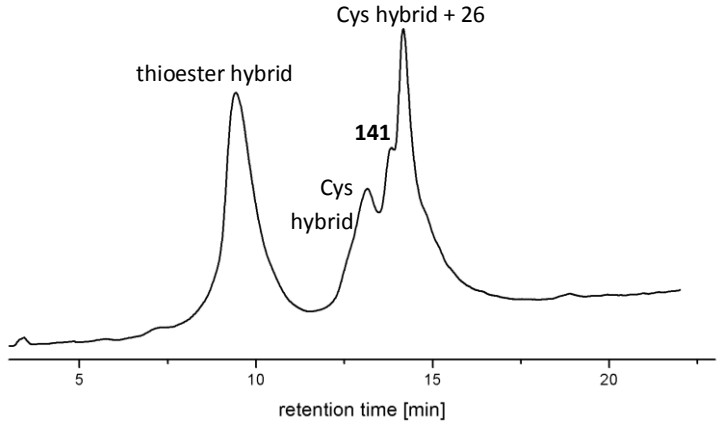
141

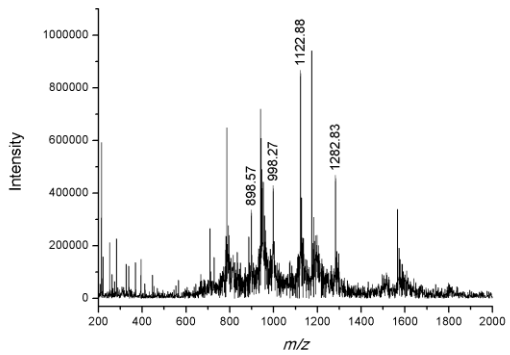
Templated ligation reaction of **72** and **95** following GP9. Table 3, Entry 19.

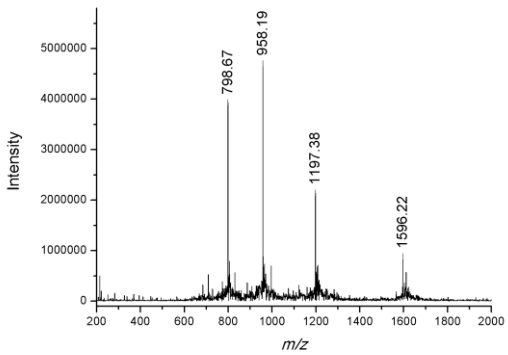
HPLC (Method I): t_R = 13.830 min (**141**), 14.173 min (Cys+26).

ESI–MS (Product) *m/z* (rel.%): 898.57 (34) $[M+10H]^{10+}$, 998.27 (45) $[M+9H]^{9+}$, 1122.88 (92) $[M+8H]^{8+}$, 1282.83 (48) $[M+7H]^{7+}$.

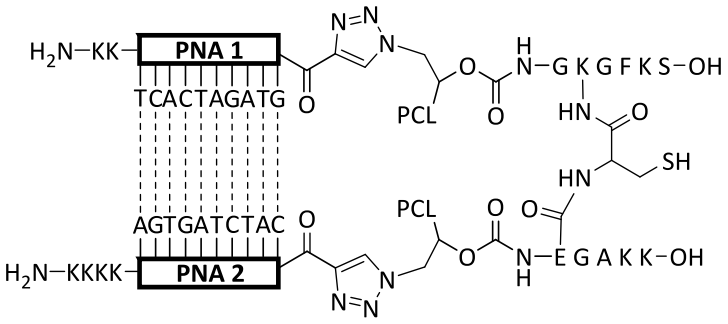
ESI–MS (Cys+26) *m/z* (rel.%): 798.67 (84) $[M+6H]^{6+}$, 958.19 (100) $[M+5H]^{5+}$, 1197.38 (46) $[M+4H]^{4+}$, 1596.22 (17) $[M+3H]^{3+}$.







Synthesis of 142

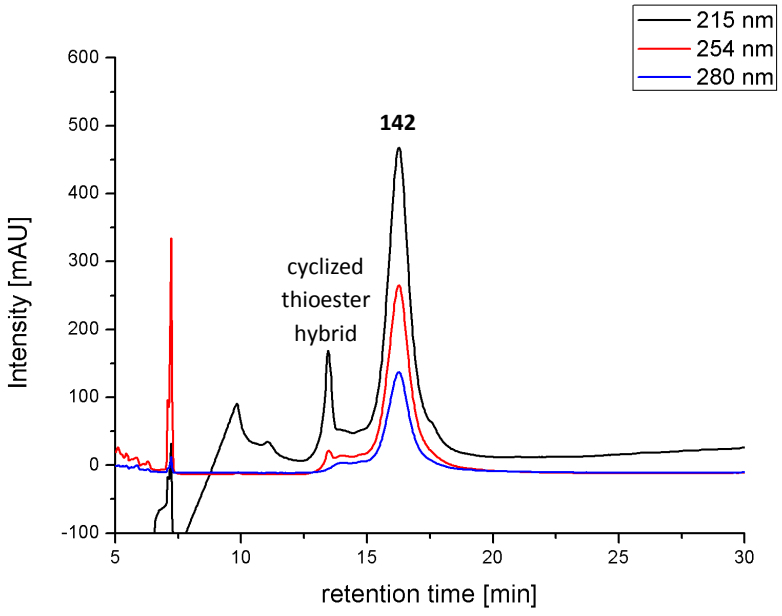


142

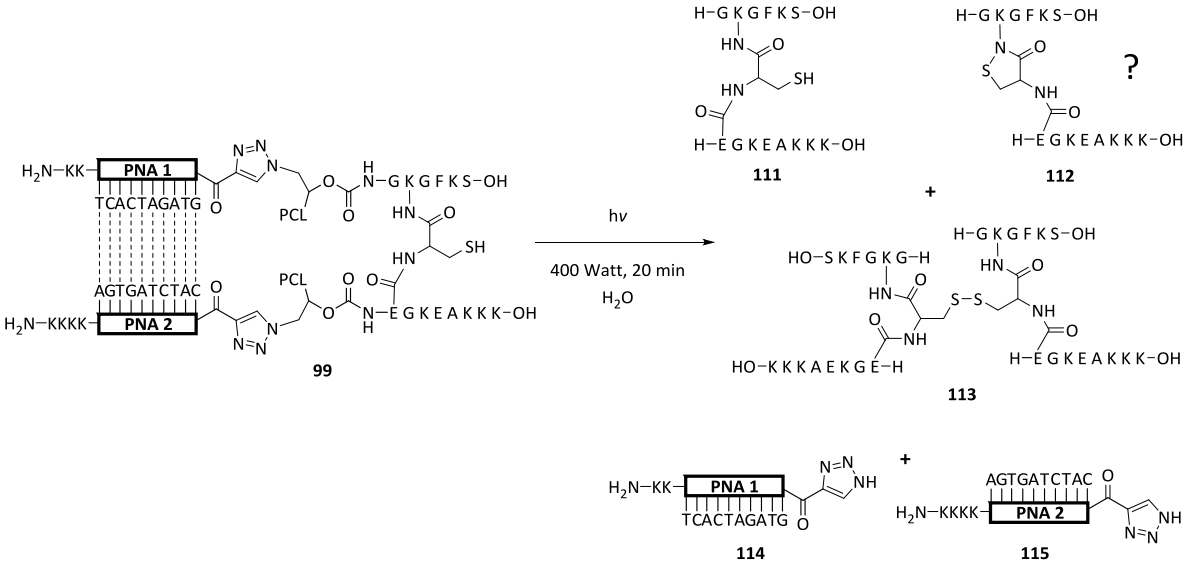
Templated ligation reaction of **63** and **89** following GP9. Table 3, Entry 20.

HPLC (Method E): t_R = 16.243 min.

ESI (HRMS): calcd. for $C_{333}H_{462}N_{152}O_{96}S^{8+}$ $[M+8H]^{8+}$: 1020.1965, found: 1020.1970; calcd. for $C_{333}H_{461}N_{152}O_{96}S^{7+}$ $[M+7H]^{7+}$: 1165.7949, found: 1165.7980; calcd. for $C_{333}H_{460}N_{152}O_{96}S^{6+}$ $[M+6H]^{6+}$: 1359.9262, found: 1359.9305.



Uncaging of 99 (111)



99 (0.2 mg, 0.02 µmol) was dissolved in H_2O (0.4 mL) and irradiated for 20 min with UV light. No cut-on filter was used [lamp setup *c)*].

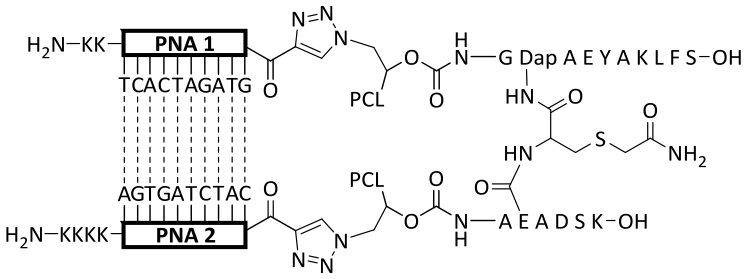
HPLC (Method C): t_R = 18.275 min (**111**, **112**{**111**-2H}, **114** and **115**), 19.117 (**113**).

ESI (HRMS): calcd. for $C_{70}H_{125}N_{21}O_{21}S^{4+}$ [M(**111**)+4H]$^{4+}$: 406.9764, found: 406.9776; calcd. for $C_{70}H_{124}N_{21}O_{21}S^{3+}$ [M(**111**)+3H]$^{3+}$: 542.2995, found: 542.2981;

calcd. for $C_{140}H_{246}N_{42}O_{42}S_2^{6+}$ [M(**113**)+6H]$^{6+}$: 542.1307, found: 542.1296; calcd. for $C_{140}H_{245}N_{42}O_{42}S_2^{5+}$ [M(**113**)+5H]$^{5+}$: 650.5561, found: 650.5567;

calcd. for $C_{70}H_{123}N_{21}O_{21}S^{4+}$ [M(**112**)+4H]$^{4+}$: 406.4725, found: 406.4721; calcd. for $C_{70}H_{122}N_{21}O_{21}S^{3+}$ [M(**112**)+3H]$^{3+}$: 541.6276, found: 541.6281.

Synthesis of 117



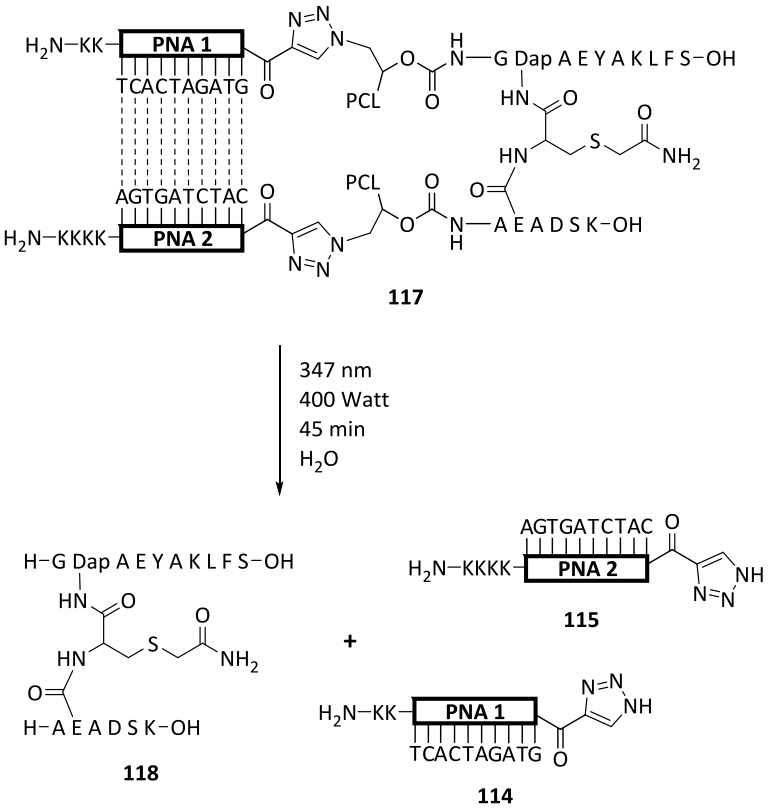
117

Iodoacetamide (1.0 mg, 5.4 µmol) was added to the ligation solution of **108**. The solution was stirred at room temperature for 30 min, then it was quenched with 3-mercaptopropionic acid (25 µL, 288 µmol). The product was purified by HPLC chromatography (Method A).

HPLC (Method C): t_R = 22.058 min.

ESI (HRMS): calcd. for $C_{358}H_{492}N_{157}O_{108}S^{7+}$ $[M+7H]^{7+}$: 1250.6806, found: 1250.6800; calcd. for $C_{358}H_{491}N_{157}O_{108}S^{6+}$ $[M+6H]^{6+}$: 1458.7924, found: 1458.7947.

Uncaging of 117 (118)



117 (0.2 mg, 0.02 µmol) was dissolved in H_2O (0.4 mL) and irradiated for 45 min with UV light [lamp setup: *c)*].

HPLC (Method C): t_R = 20.792 min.

ESI (HRMS): calcd. for $C_{78}H_{125}N_{21}O_{28}S^{4+}$ [M(**118**)+4H]$^{4+}$: 458.9675, found: 458.9666; calcd. for $C_{78}H_{124}N_{21}O_{28}S^{3+}$ [M(**118**)+3H]$^{3+}$: 611.6210, found: 611.6202; calcd. for $C_{78}H_{123}N_{21}O_{28}S^{2+}$ [M(**118**)+2H]$^{2+}$: 916.9278, found: 916.9261;

calcd. for $C_{135}H_{190}N_{69}O_{35}^{5+}$ [M(**115**)+5H]$^{5+}$: 667.7041, found: 667.7032; calcd. for $C_{135}H_{189}N_{69}O_{35}^{4+}$ [M(**115**)+4H]$^{4+}$: 834.3783, found: 834.3783; calcd. for $C_{135}H_{188}N_{69}O_{35}^{3+}$ [M(**115**)+3H]$^{3+}$: 1112.1687, found: 1112.1689;

calcd. for $C_{123}H_{165}N_{65}O_{33}^{4+}$ [M(**114**)+4H]$^{4+}$: 770.3308, found: 770.3306; calcd. for $C_{123}H_{164}N_{65}O_{33}^{3+}$ [M(**114**)+3H]$^{3+}$: 1026.7720, found: 1026.7722.

5 List of Abbrevations

δ	chemical shift
Å	Ångström
°C	degree Celsius
Aad	L-2-aminoadipic acid
Acm	acetamidomethyl
AcOH	acetic acid
ADHP	2-amino-4.6-dihydroxypyrimidine
Alloc	allyloxycarbonyl
Bhoc	(*N*6-(Benzhydryloxycarbonyl)aden-9-yl)acetyl
Bn	benzyl
Boc	*N*-*tert*-butoxycarbonyl
calcd.	calculated
dba	dibenzylideneacetone
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
DIC	*N*,*N*'-diisopropylcarbodiimde
DIEA	*N*,*N*'-diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
ds	diastereomer
EDT	1,2-ethanedithiol
EDTA	ethylenediaminetetraacetic acid
EPPS	3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid
eq.	equivalent(s)
ESI	electrospray inonization
Et_2O	diethylether
EtOAc	ethyl acetate
EtOH	ethanol
Fmoc	fluorenylmethyloxycarbonyl
h	hour
HATU	1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	hydroxybenzotriazole
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry

LC-MS	liquid chromatography-mass spectrometry
M	molar concentration
mA	milliampere
mAU	milli-arbitrary units
Me	methyl
MeCN	acetonitrile
MeOH	methanol
MHz	megahertz
min	minutes
Mmt	4-methoxytrityl
MS	mass spectrometry
Mtt	4-methyltrityl
mW	milliwatt
m/z	mass-to-charge ratio
NaHMDS	sodium bis(trimethylsilyl)amide
NMM	*N*-methylmorpholine
NMP	*N*-methyl-2-pyrrolidone
NMR	nuclear magnetic resonance
Orn	Ornithine
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
Ph	phenyl
PPh_3	triphenylphosphine
ppm	parts per million
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
PyBrOP	bromotripyrrlidinophosphonium hexafluoriphosphate
quant.	quantitative
R_f	retention factor
RNA	ribonucleic acid
Su	succinimide
*t*Bu	*tert*-butyl
TCEP	*tris*(2-carboxyethyl)phosphine
TEA	triethylamine
Tf	trifluoromethanesulfonate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIS	triisopropylsilyl ether
t_R	retention time
Trt	triphenylmethyl
UV	ultraviolet

6 Literature

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