Phage Selection of Bicyclic Peptide Ligands and Development of a New Peptide Cyclisation Method

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Résumé

Les peptides bicycliques sont des molécules pouvant se lier à une cible d'intérêt avec une grande affinité et de manière très spécifique. Ils peuvent être obtenus par une méthode appelée « phage display ». Au cours d'un premier projet, nous avons tiré profit de ces qualités pour générer des inhibiteurs spécifiques à certain membres de la famille des métalloprotéinases matricielles (MMPs), une famille de protéases ayant des structures très similaires et donc difficiles à cibler de manière sélective. A l'aide de librairies combinatoires de peptides présentés à la surface de phages, nous avons isolé un peptide bicyclique inhibant de manière spécifique MMP-2 avec une constante d'inhibition (K_i) de 2 μM. Cet inhibiteur présente une activité plus faible que l'inhibiteur peptidique ciblant MMP-2 le plus puissant découvert à ce jour. Il s'agit d'un peptide linéaire appelé APP-IP, possédant une K_i de 13 nM et étant dérivé de la protéine précurseur de l'amyloïde. Cependant, en raison de sa conformation plus rigide, le peptide bicyclique devrait être plus stable et donc plus approprié à des applications dans des systèmes biologiques. Il pourrait également être utilisé comme alternative au peptide monocyclique nommé CTT, largement utilisé en recherche malgré une activité inhibitrice faible (K_i de 142 μM). En résumé, le peptide bicyclique développé durant cette thèse devrait trouver de vastes applications en tant qu'outil de recherche. Une amélioration de son affinité par « phage display » pourrait également déboucher sur le développement de molécules thérapeutiques anti-cancer.

Au cours d'un deuxième projet, nous avons généré des peptides bicycliques pouvant se lier de manière spécifique à la protéine centriolaire SAS-6. Cette protéine remplit un rôle structurel important dans la formation des centrioles. Ces peptides sont actuellement testés *in cellulo* dans l'étude du processus de formation des centrioles par le laboratoire du Professeur Pierre Gönczy (EPFL).

Au cours du troisième projet de cette thèse, nous avons établi une nouvelle méthode enzymatique pour cycliser de manière sélective des peptides n'ayant aucun groupe protecteur sur leur chaîne latérale, ceci à l'aide d'une enzyme appelée transglutaminase (TGase). Cette enzyme catalyse la formation d'une liaison amide stable entre la chaîne latérale d'une glutamine et une amine primaire. Elle a été largement utilisée en biotechnologie pour lier des peptides à des protéines ou pour attacher des étiquettes sur des protéines. Nous avons découvert que l'enzyme d'origine microbienne provenant de la souche *Streptomyces*

mobaraensis peut cycliser intra-moléculairement de manière quantitative des peptides présentant une séquence comportant un acide aminé glutamine donneur en position N-terminale et une lysine en position C-terminale. Des expériences effectuées avec un substrat présentant une séquence minimale et un résidu glutamine donneur ont montré que des peptides contenant uniquement le dipeptide Ala-Leu en position N-terminale par rapport au résidu glutamine et une séquence d'acides aminés aléatoire entre la glutamine et la lysine peuvent être cyclisés de manière efficace. En combinant cette méthode de cyclisation avec une cyclisation chimique basée sur des thiols, nous avons pu générer des peptides avec une structure tricyclique. Cette méthode est actuellement testée pour déterminer si elle peut être utilisée pour cycliser une librairie combinatoire de peptides présentés à la surface de phages.

Au cours d'un quatrième et dernier projet, nous avons développé une méthode pour évaluer de manière qualitative et quantitative la modification chimique ou enzymatique de peptides présentés à la surface de phages. Pour cela, les peptides ont tout d'abord été modifiés chimiquement, puis séparés des phages à l'aide d'une protéase, purifiés, concentrés et finalement analysés par spectrométrie de masse. Cette méthode rend dès maintenant possible l'évaluation de nouvelles réactions à la surface des phages, telle que la cyclisation enzymatique établie au cours de cette thèse.

Mots-clés: « phage display », peptides bicycliques, métalloprotéinases matricielles (MMPs), inhibiteurs, SAS-6, ligands, macrocycles, cyclisation, transglutaminase (TGase), librairies combinatoires de peptides modifiés chimiquement présentés à la surface de phages.

Abstract

Bicyclic peptides binding to targets of interest can be isolated from combinatorial libraries using a phage display-based approach. In a first project of this thesis, we aimed at generating bicyclic peptide inhibitors of matrix metalloproteinases (MMPs), a family of proteases that share high structural similarities and have been difficult to target in a specific manner. From phage-encoded combinatorial libraries, we isolated a bicyclic peptide that inhibits specifically the gelatinase MMP-2 with a K_i of 2 μM. This inhibitor was less potent than the best peptidic MMP-2 inhibitor, the linear APP-derived inhibitory peptide (K_i = 13 nM). However, due to its conformational constraint, the bicyclic peptide is expected to be significantly more stable and hence more suitable for applications in biological systems. It may be used as a research tool alternatively to the broadly applied monocyclic peptide MMP-2 inhibitor CTT which is less potent (K_i of 142 μM). If affinity matured, the bicyclic peptide MMP-2 inhibitor might even be developed into an anti-cancer therapeutic.

In a second project, bicyclic peptide binders to the centriolar protein SAS-6 were generated. SAS-6 plays an important structural role in centrioles and bicyclic peptide binders are currently applied in cellular assays by the laboratory of Professor Pierre Gönczy (EPFL) to study the process of centriole formation.

In a third project of this thesis, we established an enzymatic method to selectively cyclise peptides with unprotected side chains using a transglutaminase (TGase). TGases catalyse the formation of stable amide bonds between the side chains of glutamine and primary amines. They have been used extensively in biotechnological applications to cross-link peptides and proteins or to attach labels to proteins. We found that the microbial transglutaminase (MTGase) of *Streptomyces mobaraensis* can cyclise quantitatively peptides with an N-terminal glutamine-donor sequence and a C-terminal lysine residue in an intramolecular reaction. Experiments with minimised glutamine-donor substrates revealed that peptides with only an Ala-Leu dipeptide N-terminal to the glutamine and a randomly chosen peptide between the glutamine and lysine residues are efficiently cyclised. By combining the MTGase-based cyclisation strategy with a chemical thiol-based cyclisation reaction, we were able to generate tricyclic peptide structures. It remains to be tested if this method can be applied to cyclise combinatorial peptide libraries on the surface of phage.

In a fourth and last project, we developed a method to follow qualitatively and quantitatively chemical or enzymatic modifications applied to peptides displayed on phage. In this method, peptides on phage were chemically modified, cleaved off by a protease, purified, concentrated and analysed by mass spectrometry. The method will help to assess new reactions applied to phage peptides such as the MTGase-based enzymatic cyclisation reaction.

Keywords: Phage display, bicyclic peptides, matrix metalloproteinases, MMP inhibitors, SAS-6, ligands, macrocycles, cyclisation, transglutaminase, MTGase, phage-encoded combinatorial chemical libraries.

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1. General introduction

1.1. Objectives of the thesis

The development of molecules binding with high affinity and selectivity to protein targets is an important scientific aim. Such molecules are needed for the development of therapeutics or as research tools as for example the detection and purification of biomolecules or the study of biological questions[1,2]. Phage display, a technology developed in the 1980s, has allowed the generation of polypeptide binders to many targets of interest and made critical contributions in these directions. Recently, combination of phage display and chemical cyclisation reactions allowed the generation of bicyclic peptide binders to proteins of interest[3].

The primary goal of this thesis was to generate bicyclic peptide inhibitors of two matrix metalloproteinases (MMPs), MMP-2 and MMP-9, which play an important role in cancer progression and other diseases. Inhibitors of these MMPs could serve as lead compounds for the development of new anti-cancer therapeutics.

A second goal was to develop bicyclic peptide binders to the centriolar protein SAS-6. This protein plays an important structural role in centrioles[4] and specific binders to SAS-6 are needed as tools for mechanistic studies of centriole formation. This project is performed in collaboration with the laboratory of Professor Pierre Gönczy[a].

A third aim of the thesis was to develop a strategy for the cyclisation of peptides with unprotected side chains. We intended to use this reaction for the cyclisation of peptides displayed on phage. In combination with a previously applied orthogonal chemical reaction, tricyclic peptides could be generated.

In a fourth project, we aimed at developing a procedure to qualitatively and quantitatively follow chemical modifications of peptides displayed on phage. Such information is valuable to establish new protocols for the chemical or enzymatic modifications of phage-encoded peptides.

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1.2. Cyclic peptides and phage display

Peptides have proven to be suitable for the development of therapeutics as well as research tools. One of the great strengths of peptides lies in the powerful approaches for discovering and screening binders to targets of interest. For example a large number of phage-displayed polypeptides binding to targets can be generated with little effort and a short time.

Most peptides used as therapeutics or research tools are based on cyclic structures. The cyclic configuration brings several advantages over the linear form: cyclic peptides are conformationally more constrained resulting in a smaller loss of entropy upon binding and hence higher binding affinities[5]. Moreover, the larger constraint limits the number of possible conformers leading to higher target specificity[6-8]. Furthermore, cyclic peptides are significantly more resistant to proteolytic activities[5,9]. In multicyclic peptides such as bicyclic peptides, the above described advantages are even more pronounced.

During the last decade, phage display has become an established technology for rapid and efficient high-throughput screening of protein and peptide binders to different targets (such as proteins, peptides or DNA). G. P. Smith had developed this technology in 1985. He first displayed a polypeptide on the surface of filamentous phage and later isolated linear peptides binding to an antibody from a peptide library displayed on phage[10,11]. This technology takes advantage of a bacteriophage to link the molecules exposed on the surface of the phage (phenotype) with their encoding DNA (genotype). This allows rapid screening and identification of the isolated binders by sequencing of the phage DNA.

Cyclic peptide ligands to targets of interest can be generated by phage display or other in vitro display techniques[12]. Typically, random peptides flanked by two cysteines are displayed on phage and cyclised through the formation of a disulfide bond[13,14]. O'Neil and co-workers were the first to construct such a library. They produced a library of hexapeptides flanked by cysteine residues capable of forming disulfide bridges[13]. They could identify cyclic peptides binding with dissociation constants in the nanomolar range to the platelet glycoprotein, IIb/IIIa, which mediates the aggregation of platelets through binding of fibrinogen.

1.3. Phage selection of bicyclic peptides

Christian Heinis and Sir Greg Winter had developed a strategy to generate phage-encoded combinatorial libraries of bicyclic peptides[3]. They speculated that bicyclic peptides could mimic the complementarity determining regions of antibodies and therefore bind to targets with high affinity and specificity (**Figure 1**).

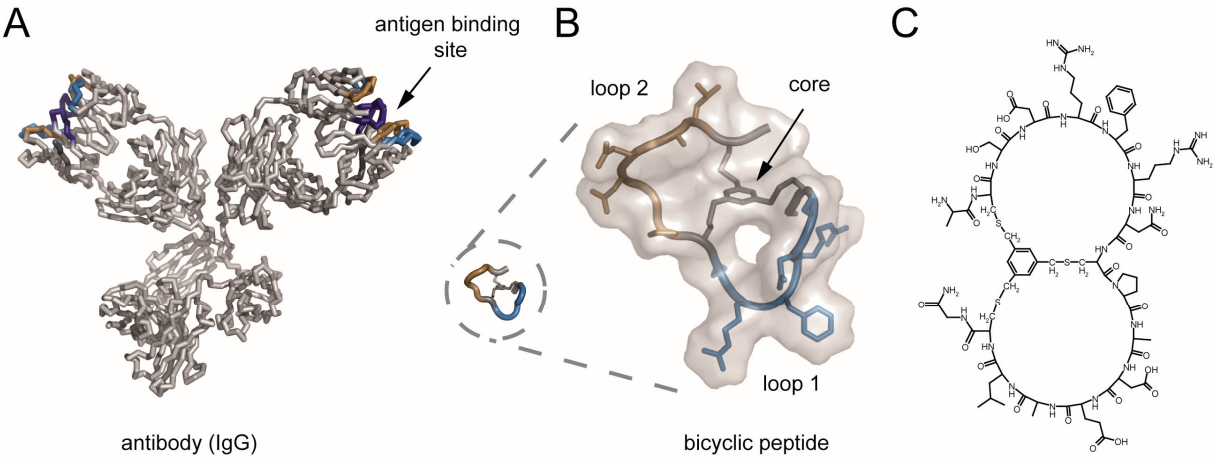

Figure 1. Comparison of a bicyclic peptide with an antibody. (**A**) Antigen binding sites of antibodies (150 kDa) are restricted to a small region here highlighted in colour. (**B**) In contrast, in the 100-fold smaller bicyclic peptides (1-3 kDa), the two binding loops represent the major part of the molecule. (**C**) Chemical structure of a bicyclic peptide isolated against human plasma kallikrein (PK15)[3]. Adapted from reference[15].

Large combinatorial repertoires of bicyclic peptides are generated by cyclising linear peptides displayed on phage using a chemical reaction. Libraries of linear peptides with three reactive cysteine residues, spaced apart by six random amino acids, are reacted via the cysteine side chains with a small molecule having three thiol-reactive groups such as tris(bromomethyl)benzene (TBMB)[3]. This reaction is quantitative and selective, and yields a single product. More than 10 billion different bicyclic peptides can be generated and subjected to iterative rounds (typically 2 or 3) of phage production, chemical cyclisation, affinity selection and amplification (**Figure 2**)[15].

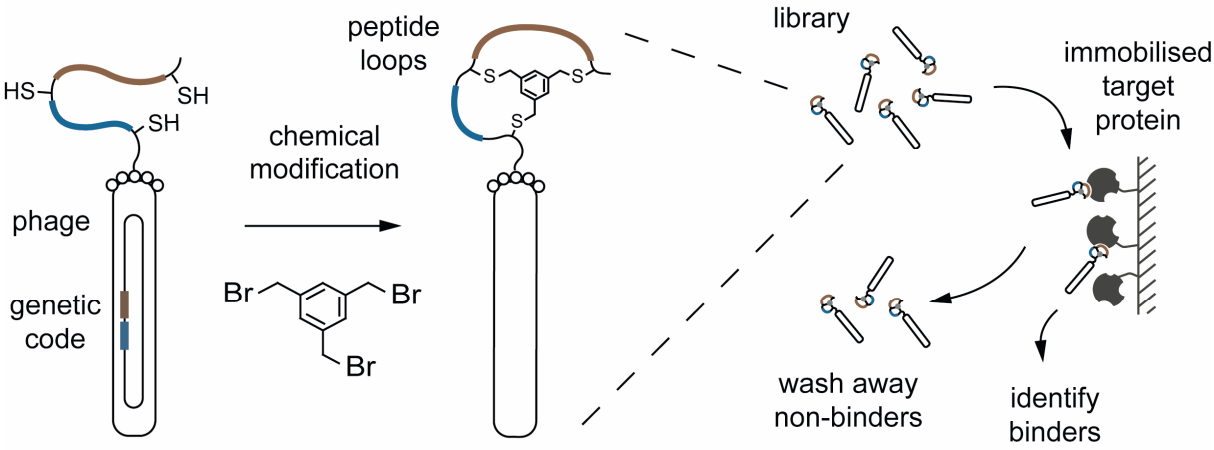

Figure 2. **Schematic representation of the bicyclic peptide phage selection strategy**. Linear peptides are displayed on phage particles to link them to their encoding DNA (**left**) and cyclised by reacting the three cysteine residues with the organic scaffold. The large library is then subjected to affinity selections (**right**). After 2 or 3 iterative rounds of selection, bicyclic peptide binders are isolated and identified by sequencing the DNA enclosed in the phage particles. From reference[15].

The binding affinities of the isolated peptides can be further improved by affinity maturation of one or both of the peptide loops. Bicyclic peptides were so far isolated against the serine proteases plasma kallikrein, cathepsin G and urokinase-type plasminogen activator (uPA) and bound all with affinities in the nanomolar range. The best binder is PK128, an inhibitor of human plasma kallikrein with a K_i of 0.3 nM[7]. All bicyclic peptides showed high target selectivity, generally not inhibiting homologous proteins.

2. Bicyclic peptide inhibitors of matrix metalloproteinases 2 and 9

2.1. Introduction

In this work we attempted to develop potent and selective inhibitors of matrix metalloproteinases (MMPs), a family of endopeptidases that was found to be difficult to target in a specific manner by small molecules[16]. The family of MMPs comprises more than 23 zinc-dependent endopeptidases that have important physiological functions in tissue homeostasis but also play a role in pathological conditions, such as cancer and inflammation[17,18]. They can be secreted by cells or remain membrane bound and are implicated in many physiological processes, such as embryo implantation, bone remodelling and organogenesis. MMPs are proteolytic enzymes regulating various cellular behaviours such as differentiation, apoptosis, migration, tumour growth, tumour angiogenesis and invasion[17].

MMPs are expressed with a signal sequence (typically comprising 18-30 residues) and a propeptide domain (containing around 80 residues). The signal sequence is excised during transit out of the cell and is not present in mature enzymes. The propeptide domain contains a sequence PRCXXPD (where X denotes any amino acid) that is highly conserved among different MMPs. In this conserved domain, the thiol group of the cysteine residue interacts with the zinc ion of the active site stabilising the enzyme in its inactive form. This mechanism is called the “cysteine switch”. During activation of the zymogen, the cysteine residue is displaced through a variety of means (oxidation, mercurial and gold compounds) and the propeptide domain is cut off autocatalytically or by the action of another MMP[19]. The typical MMP catalytic domain contains around 160-170 residues, including the binding sites for the structural (calcium and zinc) and catalytic (zinc) metal ions. A highly conserved sequence HEXXHXXGXXH is present at the C-terminus of the catalytic domain (active site of 50-54 residues). It includes a glutamic acid residue that promotes the nucleophilic attack of a water molecule on the carbonyl carbon of the scissile bond[19,20].

Members of the family of MMPs have been associated with many types and stages of cancer, and are thought to be essential for basement-membrane penetration during metastasis. MMPs regulate the tumour microenvironment and their expression and activation are increased in

almost all human cancers compared with normal tissue. While some MMPs are expressed by the cancer cells (e.g. MMP-7), other MMPs are produced by the tumour stromal cells, including fibroblasts, myofibroblasts, inflammatory cells and endothelial cells (e.g. MMP-2; **Figure 3**).

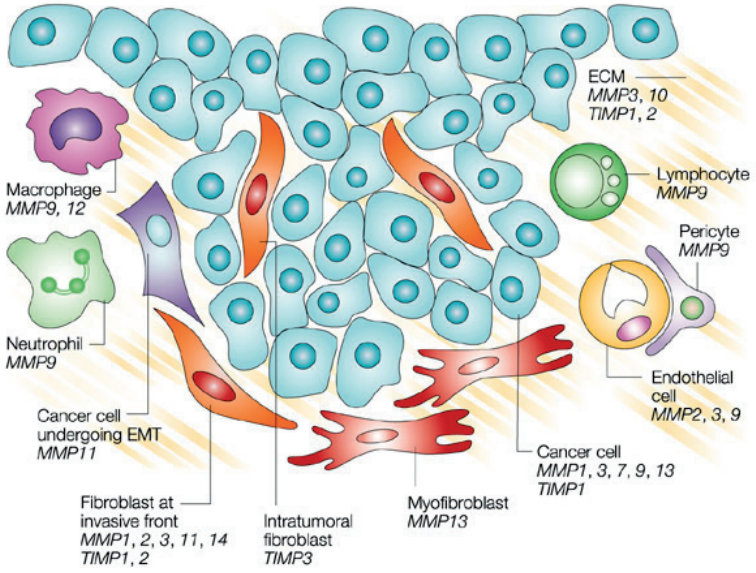

Figure 3. Expression of MMPs and TIMPs in breast tumours. In addition to cancer cells, tumours of the breast consist of stromal cells, which include fibroblasts, myofibroblasts, endothelial cells, pericytes, macrophages, neutrophils and lymphocytes. Different MMPs and tissue inhibitors of metalloproteinases (TIMPs) are synthesised by stromal cells, cancer cells and cancer cells undergoing the epithelial-to-mesenchymal transition (EMT). Figure and legend taken from reference[17].

Based on the association of MMPs with cancer cell growth and metastasis, several pharmaceutical companies had developed in the 1980s broad-spectrum MMP inhibitors wherein several of them have advanced to phase III clinical trials[21]. Unfortunately, to date, no MMP inhibitor has been successfully developed as anti-tumour drug. The failure is attributed to mainly two factors: firstly, the compounds were tested in patients with late-stage tumours where effects of MMP inhibitors are limited, as later verified in animal models. Secondly, the tested compounds were mostly broad-spectrum MMP inhibitors also acting on physiological targets, leading to toxicity and even pro-tumorigenic effects. In the last two decades, numerous studies with transgenic mouse models allowed to better understand the role of individual MMPs in tumour progression and to define targets and anti-targets among the MMP family of proteases[21]. However, the generation of MMP inhibitors with enhanced

selectivity has remained challenging. The most selective small molecule inhibitors were generated for MMP-13. They do not have, unlike most broad-spectrum MMP inhibitors, a zinc-binding functionality and inhibit MMP-13 in a non-substrate competitive manner[22]. Selective inhibitors of MMPs were recently developed based on antibodies. The antibody DX-2400 blocks MMP-14 with a K_i of 0.8 nM and reduces angiogenesis, tumour growth, and metastasis in MMP-14 expressing tumours in mice[23]. Selective and potent antibody inhibitors of MMP-2 and MMP-9 (also known as gelatinase A and B, respectively) as well as antibodies with dual specificities were also recently communicated (conference communication from Dyax Corp., Burlington, USA).

In this work, we aimed at generating specific inhibitors of MMPs based on bicyclic peptides. In comparison to small molecules, peptides can form larger binding interfaces with the MMPs and hence generate more tight and specific interactions. In contrast to antibodies, bicyclic peptides are much smaller and promise to penetrate tumour tissue better. As MMP targets, we chose MMP-2 and MMP-9 which are highly expressed in many human tumours and are associated with tumour invasion. The two gelatinases are structurally and functionally related. They degrade collagen IV present in the basement-membrane and denatured type I collagen, which is considered as an important activity in tumour invasion. While MMP-2 is considered as a clear therapeutic target, MMP-9 is considered as a tumour type and stage dependent target[21]. Many potent small molecule inhibitors of MMP-2 and MMP-9 have been developed, however the large majority are broad spectrum inhibitors. The most specific inhibitor of MMP-2 is a decapeptide coming from the endogenous amyloid precursor protein (APP; residues 586-595)[24-26]. The so called APP-derived inhibitory peptide (APP-IP) blocks efficiently MMP-2 (K_i = 13 nM) and other MMPs only weakly (K_i's > 2 μM). No data is available about the performance of APP-IP *in vivo* but a linear peptide is expected to be rapidly degraded. The most potent cyclic peptide inhibitor of MMP-2 developed to date has been isolated in phage panning experiments against MMP-9 and is termed CTT[27]. This disulfide-cyclised decapeptide inhibits the gelatinase MMP-2 with an IC_{50} of 10 μM when measured in a gelatin digestion assay, or an IC_{50} of 283 μM (K_i = 142 μM) when measured in a fluorogenic substrate-based activity assay[28]. Despite its relatively low potency, this peptidic inhibitor has been used extensively in biological studies including imaging applications.

2.2. Phage selection of bicyclic peptides binding to MMP-2

In order to allow the reader to follow best the experiments in this project and to understand the strategies taken during the course of it, the results are chronologically presented and divided in five subchapters, describing 5 different experiments to isolate bicyclic peptide MMP-2 inhibitors. Phage titres of each selection are reported in **Appendices subchapter 7.2**.

2.2.1. Expression and immobilisation of MMP-2

The protein target is a critical component in phage selections. We chose to use the full-length MMP-2 protein comprising the catalytic domain, the hinge region and the hemopexin-like domain as target for the phage selections. This strategy entailed the risk that not only binders to the catalytic domain could be isolated but also to other domains of MMP-2. However, since in previously performed phage selections with other protease targets, only bicyclic peptides binding to the active site were isolated[3,29], we reasoned that the use of full-length protease should not be a problem. A gene coding for the following protein sequences and domains was expressed (**Figure 4** and **Appendices subchapter 7.3**)[17,19,20,30]:

- Signal sequence

  ProMMP-2 is synthesised in the cell and secreted to the extracellular space. The signal sequence directs the protease to the endoplasmic reticulum and through the secretory pathway. This region is cut off during the transit out of the cell and is not observed in the mature enzyme.
- Propeptide domain

  As discussed in the introduction, the propeptide is composed of a conserved zinc-interacting thiol group that maintains the protease as inactive zymogen via a mechanism called the “cysteine switch”.
- Catalytic and fibronectin-like domains

  In addition to the typical MMP catalytic domain, including a catalytic zinc ion, MMP-2 and MMP-9 contain three tandem repeats of about 58 residues each related to collagen-binding type II domains of fibronectin. These domains are required to bind and cleave gelatin and gelatin-like substrates.

- Hinge region

  This region makes the connection between the catalytic domain and the hemopexin-like domain. This flexible linker does not have a secondary structure.

- Hemopexin-like domain:

  Four repeats of a hemopexin-like domain, each composed of approximately 48 residues are found at the C-terminus. These domains are not essential for catalytic activity but they mediate interactions with proteolytic substrates (e.g. heparin), cell-surface molecules (e.g. integrin) and tissue inhibitors of metalloproteinases (e.g. TIMP-2).

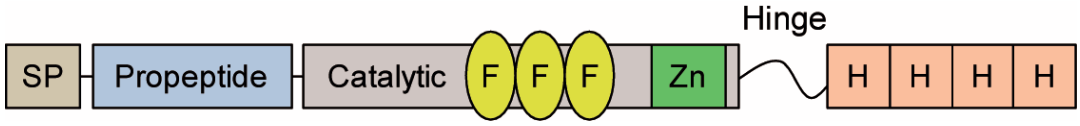

Figure 4. Structure of MMP-2. The important features of proMMP-2 are illustrated. The structure contains an N-terminal signal sequence (SP), a propeptide, a catalytic domain with a zinc-binding site (Zn) and three repeats homologous to the collagen-binding type II domains of fibronectin (F). At the C-terminus, four repeats of a hemopexin-like domain (H) are connected to the catalytic domain by a hinge.

The full-length proenzyme was expressed in mammalian cells, purified by size-exclusion chromatography and biotinylated to allow immobilisation on magnetic beads for the phage selection. Prior to the immobilisation, the zymogen was activated by incubation with the mercurial compound 4-aminophenylmercuric acetate (APMA, **Appendices subchapter 7.4**). This molecule is known to bind to the conserved cysteine residue found in the propeptide. The propeptide dissociates from the catalytic zinc ion and is autolytically processed by proMMP-2 itself resulting in an active form of MMP-2 (62 kDa) as verified by SDS-PAGE (**Figure 5A** and **B**). The activity of the protein was measured using an internally quenched fluorescent substrate. The specific activity was determined to be higher than 1000 pmol/min/µg.

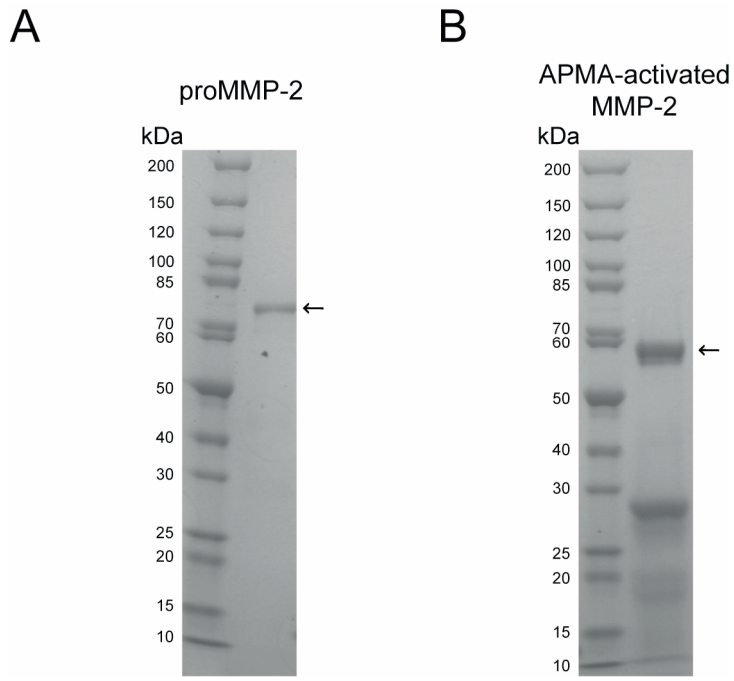

Figure 5. APMA-activation of purified proMMP-2. (**A**) The latent (72 kDa) and (**B**) the APMA-activated forms (62 kDa) of MMP-2 were analysed by SDS-PAGE to control the efficiency of the activation process.

2.2.2. Experiment 1: Standard procedure

Linear peptides from a phage peptide library previously generated and described[3] (ACX_6CX_6CG-phage; diversity > 4 billion peptides, library 6x6; **Figure 6A**) were cyclised by reacting the three cysteine residues with tris(bromomethyl)benzene (TBMB). The resulting phage-encoded bicyclic peptide library was then subjected to three iterative rounds of affinity selection with activated MMP-2. In all selection rounds, the captured phage were eluted with low pH buffer (pH 2.2). Acidic pH alters the ionisation state of target and bicyclic peptides as well as denatures the target, and hence disrupts the binding interaction. This strategy is routinely used in phage display for the elution of binders. The isolated peptides showed two different but related consensus sequences (**Figure 6B**). In the first consensus sequence the peptides shared similar amino acids in at least six positions (MMP2-1 to MMP2-3), which suggested that they form a specific interaction with the target. In the second consensus sequence, amino acids in the second loop showed high similarity to the 7-amino acid stretch of the MMP-2 propeptide (aa 21-27 of the 80 aa propeptide: PRCGNPD, identical amino acids are underlined). This sequence is known to block the active site through binding of the

cysteine residue to the catalytic zinc ion. At this point, we speculated that peptides of this consensus sequence mimic the role of the propeptide and therefore were isolated in the selections.

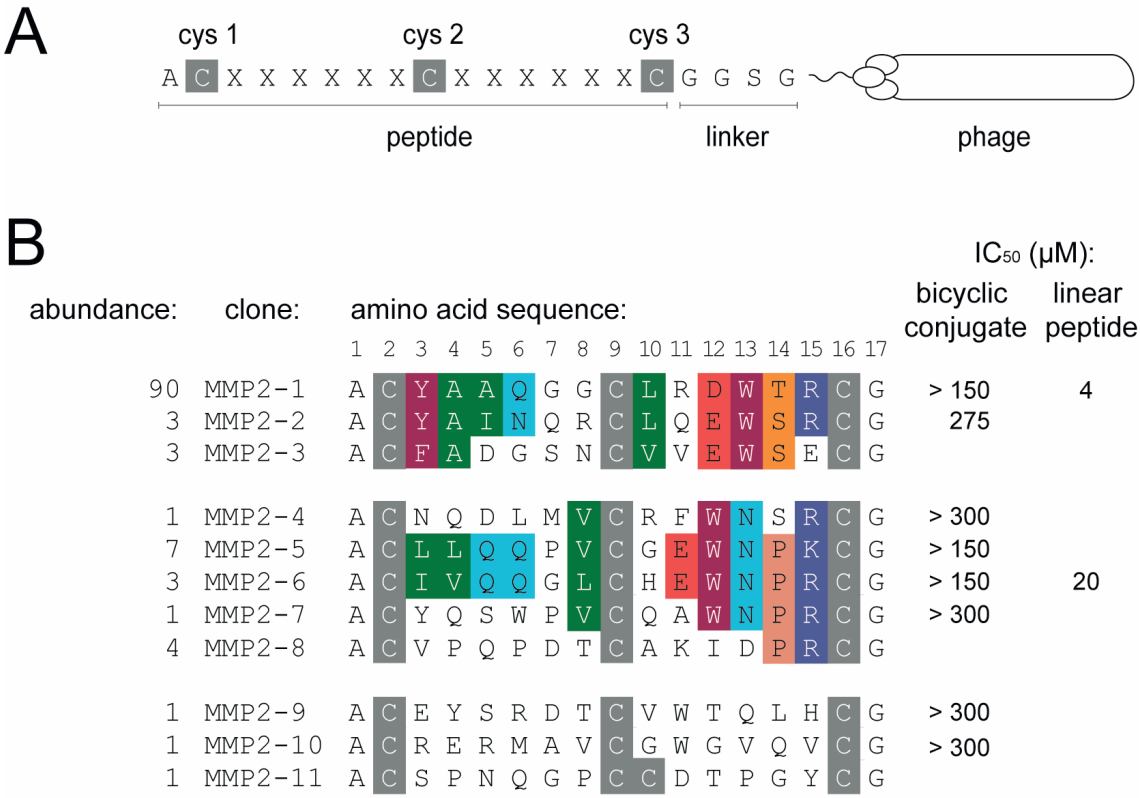

Figure 6. Peptides isolated in phage selections with MMP-2 (low pH elution). (**A**) Format of the phage peptide library that was cyclised prior to phage panning through reaction of the three highlighted cysteine residues (grey) with TBMB. (**B**) Amino acid sequences of clones isolated in phage selections using a low pH elution strategy. Sequence similarities are highlighted in colour (Rasmol colour code). The frequency each peptide was identified is indicated. The inhibitory activities (IC_{50}s) of TBMB-cyclised as well as linear peptides are shown.

Identified peptides were chemically synthesised on solid phase, cyclised with TBMB and the MMP-2 inhibition tested. The activity assay was based on an internally quenched fluorescent substrate. The bicyclic peptides inhibited MMP-2 at concentrations in the high micromolar range (**Figure 6B**) which is considered to be not a specific inhibition. A reason for the absence of potent inhibition could be that the peptides are not binding to the catalytic domain of MMP-2 or to surfaces of the catalytic domain that are distant from the active site (exosites). The similarity of the second consensus sequence with a motif of the propeptide (PRCG sequence) suggests that some of the peptides were isolated with the third cysteine

residue not modified. This observation prompted us to test the inhibitory activity of two clones as linear peptides (MMP2-1 and MMP2-6). Linear peptides were reduced, purified and their inhibitory activity tested in degassed buffer to prevent rapid oxidation. The peptides tested in the linear format were more potent than their bicyclic counterparts (**Figure 6B**). This finding suggested that the peptides were isolated in the linear form.

2.2.3. Experiment 2: Specific elution of active site binders by competitive elution

The phage selection experiment against MMP-2 was repeated wherein attention was paid to quantitatively cyclise the peptide library with TBMB. To enrich peptide ligands binding to the active site of MMP-2 and avoid the selection of exosite binders, we decided to perform three selection rounds in which phage were selectively eluted with a potent hydroxamate-based competitive inhibitor of MMP-2 (GM 6001; K_i = 0.5 nM, **Appendices subchapter 7.5**)[31].

Following this elution strategy, isolated peptides presented two new consensus sequences (**Figure 7**). In both consensus sequences two clones (1^{st} consensus: MMP2-12 and MMP2-13; 2^{nd} consensus: MMP2-14 and MMP2-15) shared 7 identical or highly similar amino acids that are distributed over both peptide loops. The three clones of the first consensus sequence contain a fourth cysteine residue. Overall, mostly different peptide sequences were found compared to experiment 1 in which phage were eluted with acidic pH. Two peptides were isolated in both of the two experiments: peptide MMP2-1 isolated 90 times by low pH elution and 3 times by competitive elution and MMP2-11 isolated once by low pH elution and 92 times by competitive elution. Peptides were synthesised and their inhibitory potential tested.

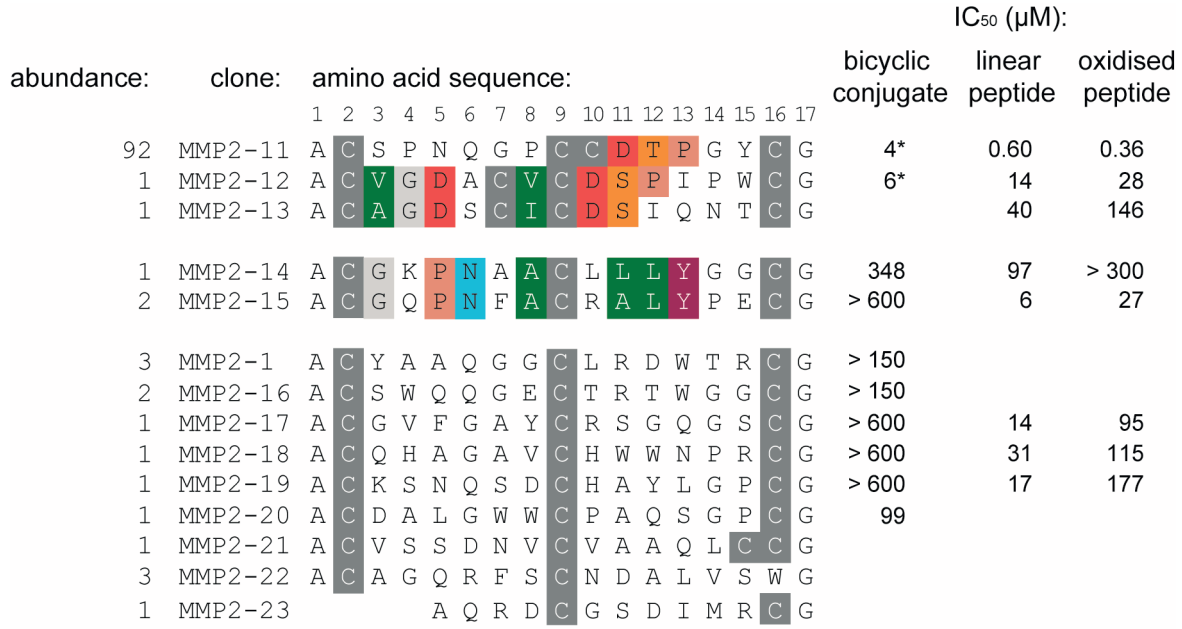

Figure 7. Peptides isolated in phage selections with MMP-2 (competitive elution). Amino acid sequences of clones isolated in phage selections using a competitive small molecule MMP inhibitor are presented. Sequence similarities are highlighted in colour (Rasmol colour code). The frequency each peptide was identified is indicated. The inhibitory activities (IC_{50}s) of TBMB-cyclised synthetic peptides, reduced-linear peptides and disulfide-cyclised peptides are shown. (*) For the peptides with 4 cysteine residues, the activity of the most potent structural isomer is indicated.

While all bicyclic peptides with three cysteine residues did not (or weakly) inhibit MMP-2, two bicyclic peptides having an additional cysteine residue in the positions 7 (MMP2-12) and 10 (MMP2-11) showed an inhibitory activity in the low micromolar range. We speculated that one cysteine residue remains free after reaction with TBMB and complexes the active-site zinc ion to foster the inhibitory activity. Since reaction of peptides having four cysteines with tris(bromomethyl)benzene yields a mixture of four different bicyclic peptide isomers, we applied the following synthetic strategy to generate the individual four possible structures (**Figure 8A** and **B**). Linear peptides were synthesised wherein the side chains of three cysteine residues were protected with the standard trityl (Trt) protecting group and one cysteine with the chemically orthogonal S-*tert*-butyl (*t*Bu). Release of the peptides from the solid phase with standard cleavage conditions (trifluoroacetic acid) left the cys(*t*Bu) residues unchanged and allowed selective reaction of three thiols with TBMB. The four structural isomers of peptide MMP2-11 and MMP2-12 showed significantly different inhibitory

activities, wherein for each peptide sequence, one isomer had a single-digit IC_{50} (**Table 1**; **Figure 8C**).

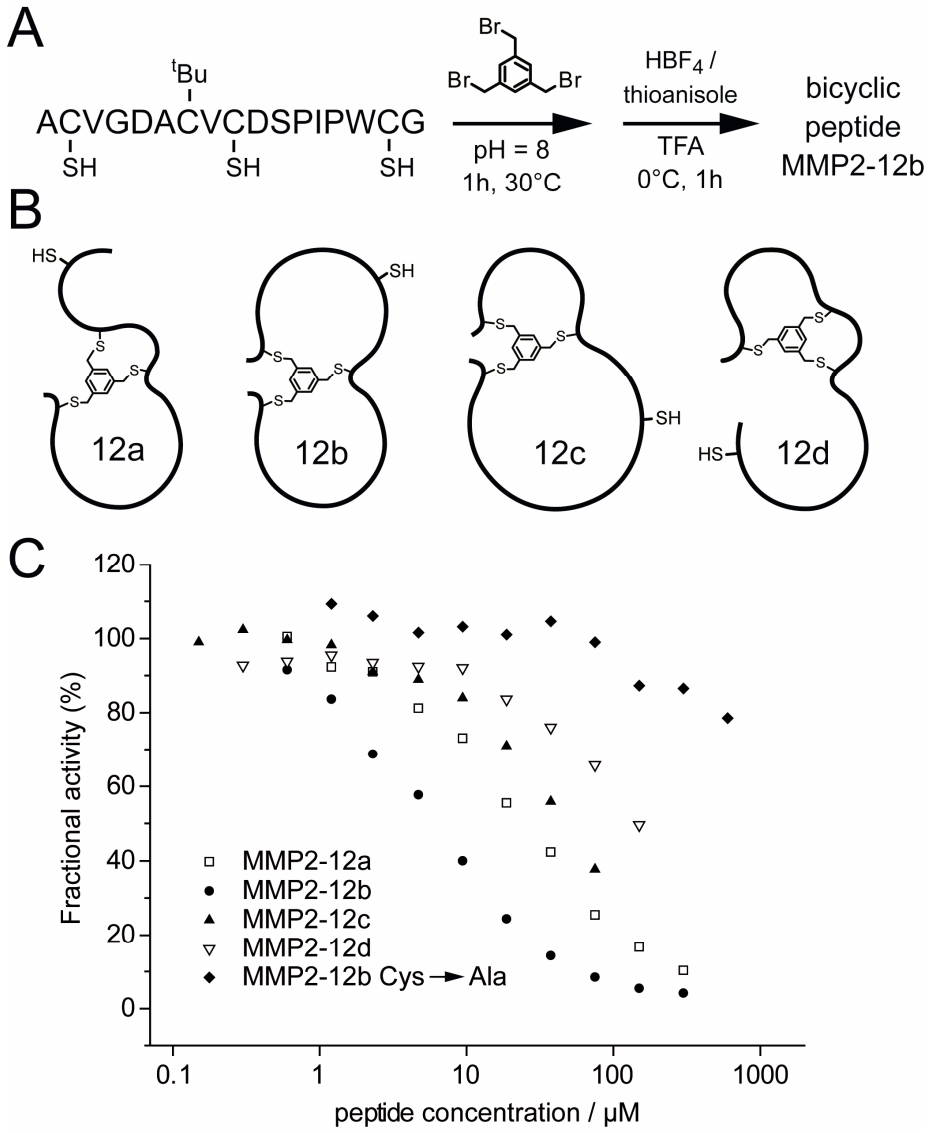

Figure 8. Structures and inhibitory activities of bicyclic peptides having a free cysteine residue. (**A**) The sequence of the linear peptide inhibitor corresponding to the clone MMP2-12 as well as the cyclisation strategy are indicated. (**B**) The four different structural isomers of MMP2-12 are schematically represented. (**C**) Inhibition of MMP-2 with different concentrations of four MMP2-12 bicyclic peptide isomers having one free cysteine or of a bicyclic peptide MMP2-12 Cys7Ala mutant. Fractional activities of MMP-2 measured with an internally quenched fluorescent peptide substrate are indicated.

Mutation of the free cysteine residues in the most potent structural isomers, MMP2-11b and MMP2-12b, to an alanine residue reduced the inhibitory activity 64-fold and more than 100-fold, respectively.

Peptide	Peptide sequence	IC_{50} (µM)				
		Position of free cysteine residue				
		a	b	c	d	Cys b → Ala
MMP2-11	H-A**C$_a$**SPNQGP**C$_b$C$_c$**DTPGY**C$_d$**G-NH_2	32	4	> 75	104	256
MMP2-12	H-A**C$_a$**VGDA**C$_b$**V**C$_c$**DSPIPW**C$_d$**G-NH_2	28	6	46	86	> 600

Table 1. Inhibitory activity of bicyclic peptides having a free cysteine residue. The cysteine residues in the amino acid sequences of the synthetic peptides MMP2-11 and MMP2-12 that are free in the different structural isomers are indicated with lower case letters a-d (in bold). The IC_{50}s are given for the four structural isomers with one free cysteine residue (the letter indicates the position of the free cysteine) as well as for a bicyclic peptide in which the free cysteine in position b was exchanged to alanine. If the IC_{50} was not reached, the highest concentration of bicyclic peptide used in the assay is indicated.

The activity of linear peptides was also tested. All the tested linear peptides inhibited MMP-2 with a better or similar activity than the corresponding bicyclic peptide (**Figure 7**). This result was unexpected and suggested that peptides were selected without being modified with TBMB.

Another possibility was that the peptides were isolated as disulfide-cyclised cyclic peptides. To test the inhibitory activity of this format, the linear peptides were oxidised with dimethylsulfoxide (DMSO) wherein at least three different isomers can potentially form. The oxidation reactions were followed by analytical HPLC (**Figure 9**). The inhibitory activity of a mixture of the oxidised forms was tested.

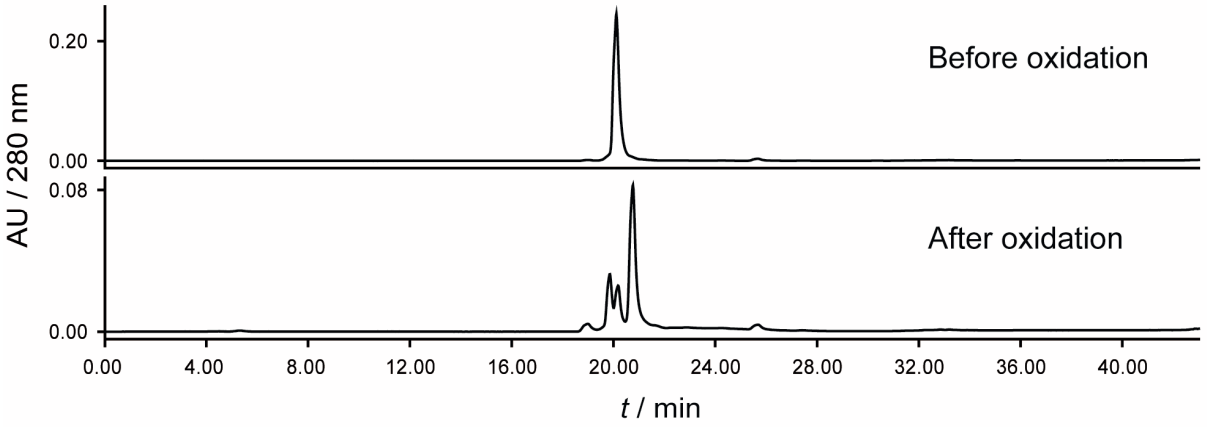

Figure 9. Example of RP-HPLC analysis of a linear peptide before and after DMSO-oxidation. Peptide MMP2-18 was analysed before (**top**) and after (**bottom**) 24 hours oxidation with DMSO. Three new peaks corresponding to the different oxidation forms appeared. The mixture of peptides after oxidation was tested in inhibition assays with MMP-2.

Peptide MMP2-11 containing four cysteines showed better inhibition (**Figure 7**, IC_{50} = 0.36 μM) after oxidation. Only one major oxidation product was observed by HPLC (24 hours oxidation, **Figure 10**) suggesting that one of the three possible disulfide-cyclised structural isomers is more favoured. The results suggest that peptides with four cysteines were most probably selected as bicyclic structures with two disulfide-bridges (not TBMB-modified).

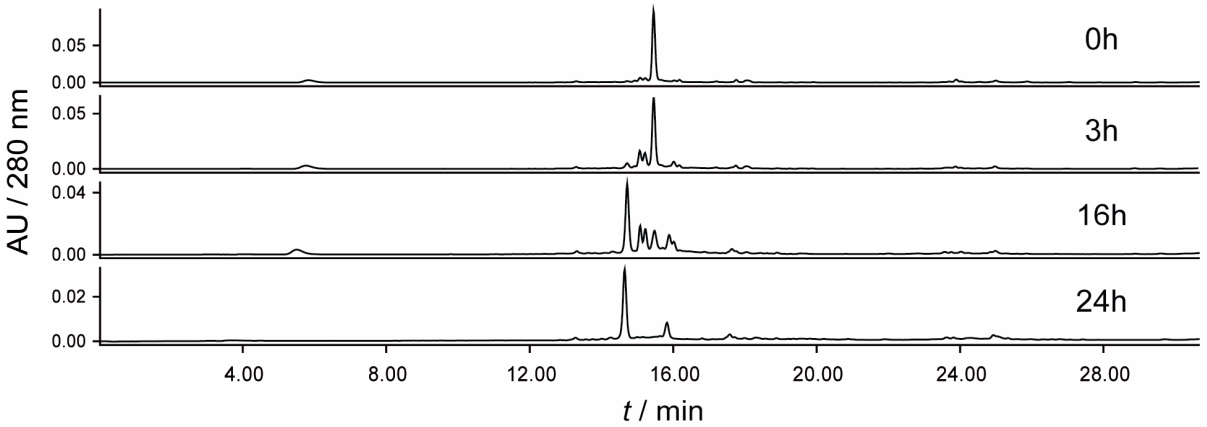

Figure 10. Monitoring of peptide MMP2-11 oxidation by RP-HPLC. The linear peptide was oxidised with DMSO and the reaction products were monitored at different time points (3h, 16h and 24h) by HPLC. After 24 hours incubation, one major peak was observed suggesting that one structural isomer is more stable and favoured over the others.

The ability of peptides MMP2-11 and MMP2-12 to inhibit the highly related gelatinase MMP-9 was assessed in inhibition assays. The bicyclic peptides MMP2-11b and MMP2-12b inhibit MMP-9 with IC_{50}s of 0.61 µM and 15 µM, respectively (**Table 2**). Therefore, they do not show selectivity toward MMP-2. MMP2-11b inhibited MMP-9 even better than MMP-2.

	IC_{50} (µM)					
	Bicyclic peptide		**Reduced-linear peptide**		**Oxidised peptide**	
	MMP2-11b	**MMP2-12b**	**MMP2-11**	**MMP2-12**	**MMP2-11**	**MMP2-12**
MMP-2	4	6	0.6	14	0.36	28
MMP-9	0.61	15	2.5	4	1	ND

Table 2. Selectivity of the best isolated peptide MMP-2 inhibitors. The inhibitory activity (IC_{50}) toward MMP-2 and MMP-9, which are structurally and functionally highly related, are indicated. Values are given for TBMB-modified peptides MMP2-11b and MMP2-12b as well as for their linear and disulfide-cyclised forms. ND, not determined.

2.2.4. Experiment 3: Selection after quenching of unpaired cysteine residues

Given the observation in experiment 2 that peptides were most likely isolated linear or in oxidised form, we performed new affinity selections using a sulfhydryl-specific reagent that could specifically react and quench thiols remaining unreacted after TBMB modification. Methyl methanethiosulfonate (MMTS) is known to quantitatively and reversibly sulfenylate thiol-containing molecules (**Figure 11**). This reaction is fast and selective and therefore suitable for our purpose[32-35].

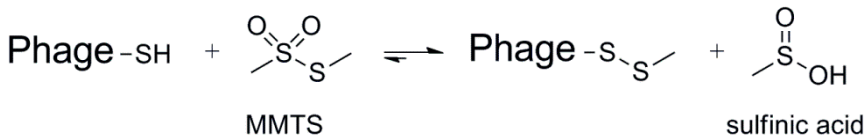

Figure 11. Reaction of unpaired cysteine residues on phage with the sulfhydryl-reactive compound MMTS. The large excess of MMTS over phage drives the reaction to the right side.

Phage of the library 6x6 described in **subchapter 2.2.2** were produced[3]. Additionally, two new phage libraries cloned in our laboratory were used. These libraries contain peptides with two sequences of three or four random amino acids between three constant cysteines and two random amino acids flanking the first and last cysteine (XCX_3CX_3CX-phage; library 3x3[7] and XCX_4CX_4CX-phage; library 4x4[36], respectively). The different libraries were produced separately, modified with TBMB and reacted with 10 mM MMTS. The phage infectivity was assessed to ensure that this treatment did not significantly affect their viability. The large excess of MMTS (10 mM) used over phage (pM range) should have quenched a large fraction of unreacted thiols. Libraries 3x3 and 4x4 were mixed together before the panning and a selection with library 6x6 was performed in parallel. Additionally, a control experiment was performed with library 6x6 in which the phage peptides were not modified with TBMB. For this latter control experiment, we expected to obtain mostly peptides with two or four cysteines based on results of other phage selections performed in the laboratory: in phage panning experiments performed with streptavidin or uPA as targets, it was found that selections with TBMB-cyclised phage peptide libraries predominantly yielded peptides with three cysteines, while selections with unmodified peptides yielded mostly peptides with four (and two) cysteines[37].

Most of the sequences isolated in this experiment contained two or four cysteines. Selections performed with libraries 3x3 and 4x4 modified with TBMB yielded 63 % of sequences with an even number of cysteines (2 or 4) (**Figure 12A**). Similarly, in selections with library 6x6 treated with TBMB, only two different sequences were found and the most abundant peptide (MMP2-32) contained four cysteines (**Figure 12B**). Finally, in the control experiment with library 6x6 not modified with TBMB, 78 % of the isolated sequences contained two or four cysteines (**Figure 12C**). These results were in contrast to our expectation that libraries modified with TBMB yield predominantly clones with three cysteines. This could indicate that the cyclisation of peptide libraries with TBMB was not efficient.

Interestingly, a consensus sequence present in more than 50 % of the isolated peptides in the selection with non-modified library 6x6 showed high similarity with the sequence of the published APP-IP MMP-2 inhibitor[24-26], suggesting a similar binding mode (APP-IP peptide: IS<u>YG</u>N<u>DAL</u>MP; identical amino acids are underlined; **Figure 12C** and **D**).

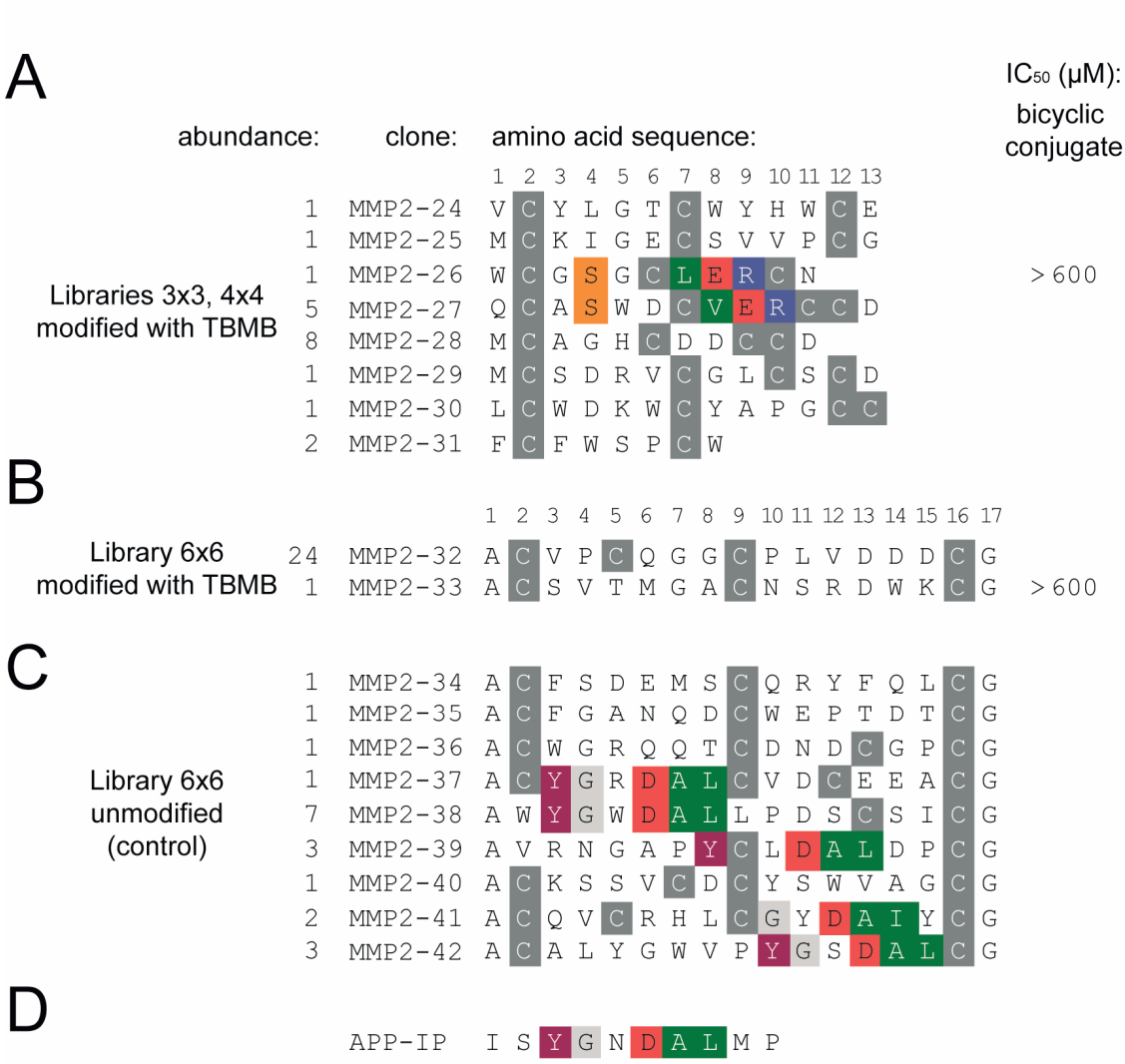

Figure 12. Peptides isolated in phage selections with MMP-2 after quenching of unpaired cysteine residues. Libraries were produced, TBMB modified and reacted with the sulfhydryl-specific reagent MMTS. Amino acid sequences of clones isolated in phage selections with libraries: (**A**) 3x3 and 4x4 produced separately and mixed together before panning, (**B**) 6x6 and (**C**) 6x6 not cyclised with TBMB (as control) are presented. Sequence similarities are highlighted in colour (Rasmol colour code). The frequency each peptide was identified is indicated. The inhibitory activities (IC_{50}s) of two TBMB-cyclised synthetic peptides are shown. (**D**) Amino acid sequence of the known MMP-2 inhibitor APP-IP is shown. Sequence similarities with isolated clones are highlighted.

Two clones containing three cysteines were synthesised, cyclised and tested for their activity. None of them showed MMP-2 inhibition.

The strategy used here to push the system toward the selection of TBMB-modified peptides over linear or disulfide-cyclised peptides was not successful. Furthermore, the TBMB-

cyclisation seems to have only partially modified the peptide libraries as only a minority of clones contained three cysteines.

2.2.5. Experiment 4: Selection with the purified active form of MMP-2

In experiments 1-3, the purified proMMP-2 was activated with APMA and immediately used for phage selections. In this experiment, proMMP-2 was activated by APMA and the active form purified by gel filtration prior to affinity selection. As a positive control, we performed in parallel a selection against human plasma kallikrein (hPK), a serine protease for which characteristic consensus sequences were previously identified in our laboratory[3,7]. A control experiment with phage peptides not modified with TBMB was performed in parallel as in experiment 3.

Panning of the TBMB-modified library against MMP-2 and hPK yielded a large number of peptides containing two or four cysteines, namely 71 % and 53 %, respectively (**Figure 13A** and **C**). In selections with library not modified with TBMB against MMP-2 and hPK, 87 % and 100 % of peptides contained an even number of cysteine residues (**Figure 13B** and **D**). As in experiment 3, these results suggest that the cyclisation of the peptide library with TBMB was not efficient and that a majority of peptides were not reacted with TBMB.

Furthermore, peptides isolated against MMP-2 and hPK did not share a consensus sequence. In the selections with hPK a high abundance of arginine residues was observed (1 out of 10 residues; **Figure 13C** and **13D**). The presence of this amino acid is characteristic for peptides isolated against hPK since this amino acid binds to the S1 site of the trypsin-like serine protease[3,7]. However, panning of peptide libraries against hPK typically yield peptides with consensus sequences, indicating that the affinity selection was not optimal with this positive control.

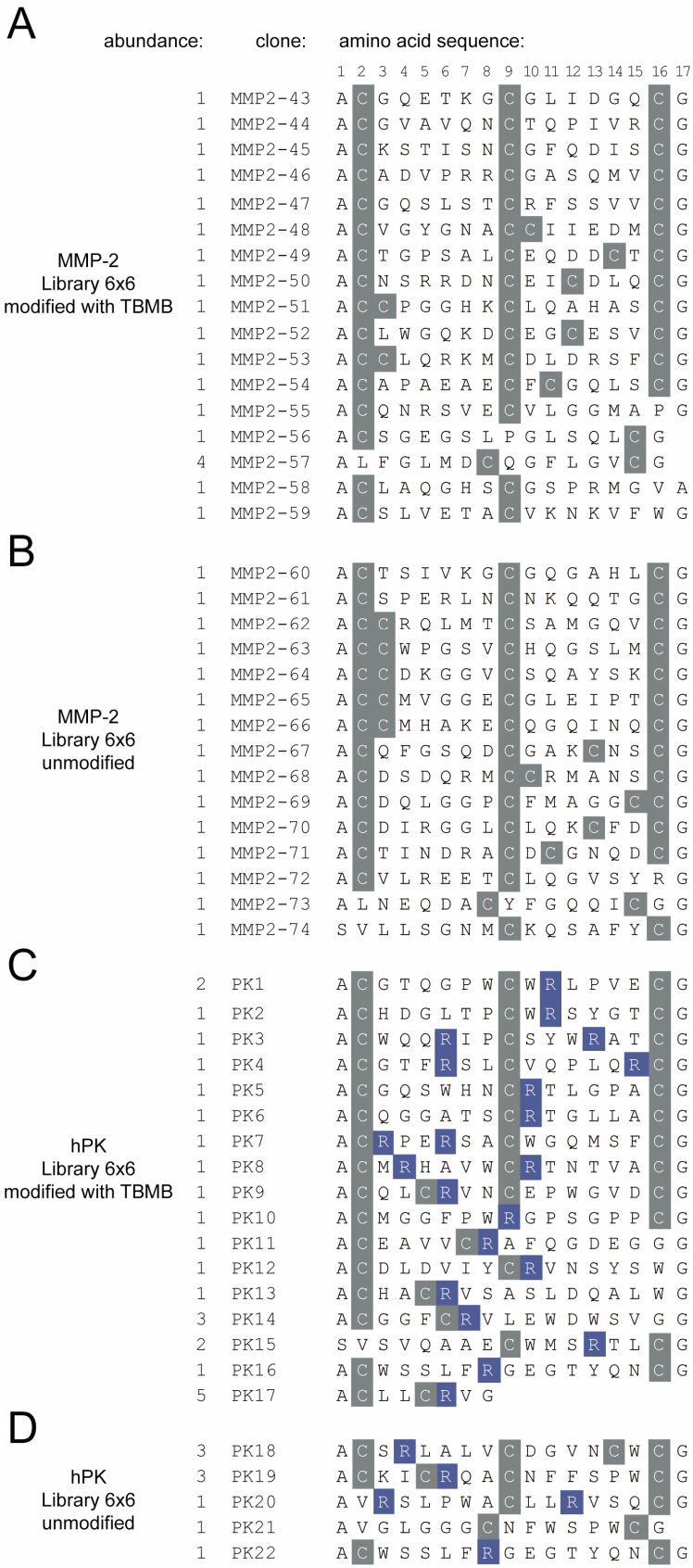

Figure 13. Peptides isolated in phage selections with purified active form of MMP-2 and the positive control hPK. Amino acid sequences of clones isolated in phage selections with MMP-2 (**A,**

B) and hPK (**C, D**) are presented. Library 6x6 was either TBMB-modified (**A, C**) or not cyclised (**B, D**). High abundance of arginine residues, characteristic of sequences isolated against hPK[3,7], are highlighted in colour (Rasmol colour code). The frequency each peptide was identified is indicated.

2.2.6. Experiment 5: Repetition of standard procedure

The four different experiments (1 to 4) showed that the phage selections can yield highly variable results even if similar conditions are applied. It appears that small variations in the procedure can highly influence the final outcome of phage selections. Particularly the negative results with the positive control (selections with plasma kallikrein, experiment 4) had prompted us to repeat the phage selection using the procedure described in experiment 1 (standard procedure, library 6x6, low pH elution, **subchapter 2.2.2**). The selections as well as the inhibitory assays were performed by Dr. Khan Maola, a new postdoctoral fellow in our laboratory, to whom I taught the different experimental procedures. In a control experiment performed in parallel, the phage-displayed library 6x6 was not cyclised with TBMB and panned also against MMP-2. The peptides isolated after two and three rounds of panning are shown in **Figure 14**.

In contrast to the selections in experiments 2 to 4, the selection with TBMB-modified peptide library yielded almost exclusively peptides with three cysteines. Selection with non-modified peptide library gave mostly peptides with an even number of cysteines. These results suggest that the peptide library was efficiently modified with TBMB in this experiment.

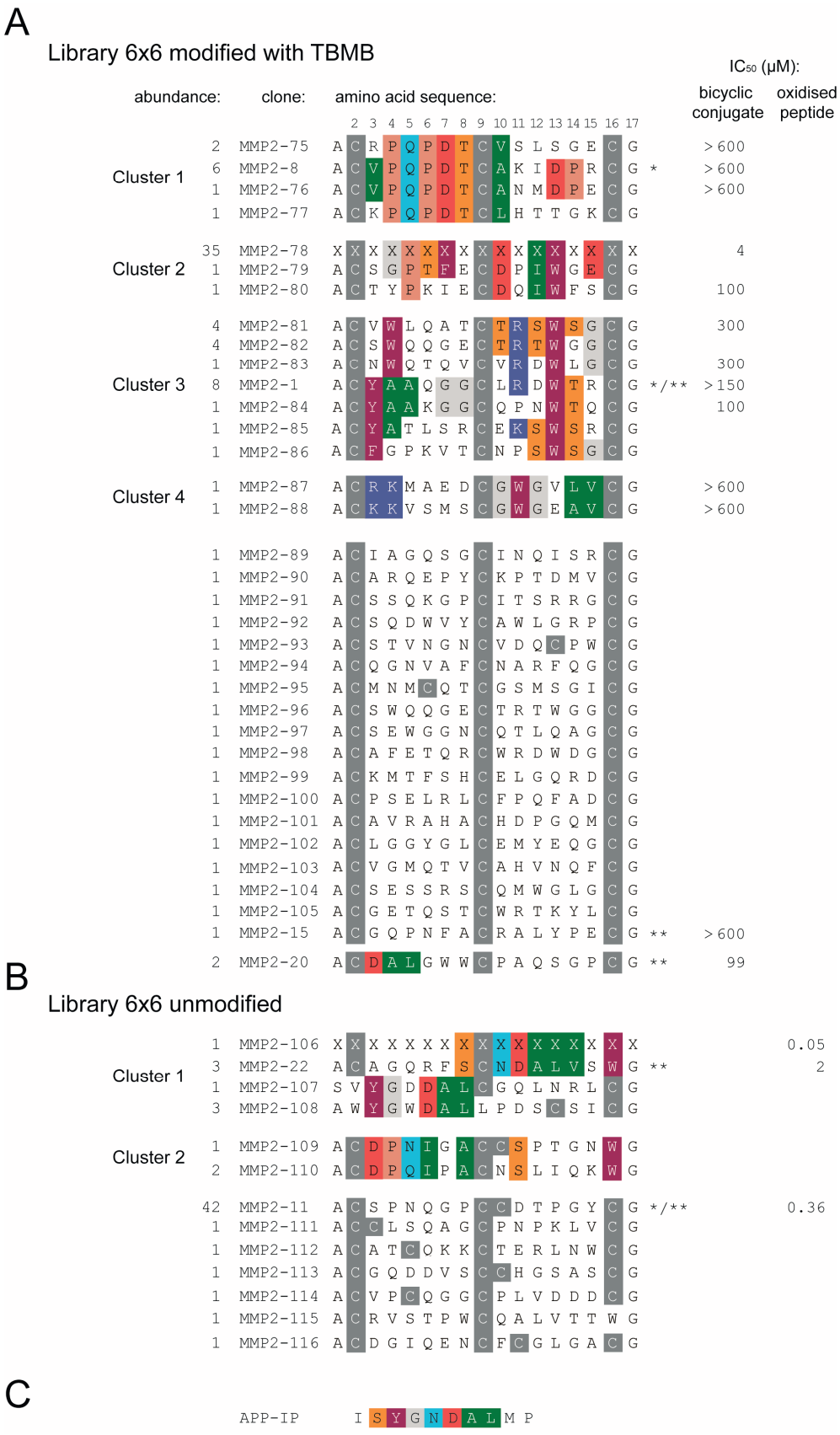

Figure 14. Peptides isolated in phage selections with MMP-2 (experiment 5). Library 6x6 was either TBMB-modified (**A**) or not modified (**B**) and subjected to affinity selections as in experiment 1.

Sequence similarities are highlighted in colour (Rasmol colour code). The frequency each peptide was identified is indicated. The inhibitory activities (IC_{50}s) of TBMB-cyclised synthetic peptides as well as oxidised peptides are shown. Clones already isolated after experiment 1 (*) or experiment 2 (**) are indicated. For patenting reasons, sequences of peptides MMP2-78 and -106 are hidden. (**C**) Amino acid sequence of the published MMP-2 inhibitor APP-IP. Sequence similarities with isolated clones are highlighted.

In the selection with the TBMB-modified peptide library, the isolated clones showed four different consensus sequences (**Figure 14A**). One of them (cluster 3) was already identified in experiment 1. In addition, three new consensus sequences (clusters 1, 2 and 4) were identified. Peptides of these clusters shared at least 6 (cluster 1), 4 (cluster 2) and 7 (cluster 4) identical or highly similar amino acids that are distributed over both peptide loops. Some of the peptides isolated in this experiment 5 were the same peptides as found previously (MMP2-1, MMP2-8, MMP2-15, MMP2-20). MMP2-20 shared sequence similarities with the APP-derived inhibitory peptide (**Figure 14A** and **C**). Peptides were synthesised and their inhibitory potential tested. One of the bicyclic peptides in cluster 2 inhibited efficiently MMP-2 activity (MMP2-78, IC_{50} = 4 μM, corresponding to a K_i of 2 μM). The specificity of the bicyclic peptide MMP2-78 was assessed by testing the inhibition of MMP-9. MMP2-78 inhibited MMP-9 with an IC_{50} higher than 100 μM. Hence, in addition to its good inhibitory activity against MMP-2, it also presents high specificity towards the gelatinases. Finally, peptides of clusters 1, 3 and 4 did not show inhibition or only at concentrations in the high micromolar range. Based on the strong consensus sequences, we speculated that these latter peptides bind to MMP-2 but do not inhibit the enzyme.

In the selection with the unmodified peptide library (control experiment), most of the isolated clones contained 2 or 4 cysteine residues (only peptide MMP2-109 had three cysteines). The isolated peptides showed two different consensus sequences (**Figure 14B**). Peptides of cluster 1 shared similarities with the APP-derived inhibitory peptide which is a potent inhibitor of MMP-2 (IC_{50} = 25 nM) (**Figure 14C**). The crystal structure of the APP-derived peptide bound to MMP-2 was recently solved and revealed that it binds to the active site into the substrate-binding cleft of the catalytic domain in the N to C direction opposite that of substrate[38]. The carboxylate group of the aspartic acid residue coordinates bidentately the catalytic zinc ion of the enzyme and the aromatic side chain of the tyrosine residue is accommodated by the S1' pocket of the protease. Given the similarity of peptides of cluster 1 with the APP-derived peptides (**Figure 14B**), we hoped that they also bind to the active site of

MMP-2 and inhibit the protease. MMP2-106 cyclised by a disulfide bridge was indeed found to inhibit MMP-2 with high potency (IC_{50} = 50 nM). Peptides of cluster 2 shared seven identical or highly similar amino acids that are distributed over both peptide loops. Noteworthy is also clone MMP2-11 which did not belong to a cluster but was isolated most frequently (42 times). This peptide was already found in experiment 1 and experiment 2 in which peptides were treated with TBMB prior to affinity selections (**Figures 6B** and **7**). The finding that this clone was here isolated from a library not modified with TBMB suggested that, in experiments 1 and 2, the clone was isolated as peptide not modified with TBMB.

2.2.7. Conclusions and outlook

Bicyclic peptide inhibitors of MMP-2 could finally be generated. The best bicyclic peptide inhibitor, MMP2-78, has an IC_{50} of 4 μM which corresponds to a K_i of 2 μM. Although the inhibitor is weaker than the APP-derived peptide (K_i = 13 nM), it is likely to be more resistant to degradation by proteases in biological systems. In a recently performed alanine scan (performed by Dr. Khan Maola), it was found that only 4 amino acids are essential for the inhibition. It is tempting to speculate that the bicyclic MMP inhibitor can be improved in an affinity maturation approach. In fact, such an affinity maturation attempt is currently performed by Dr. Khan Maola. Other potent peptide inhibitors isolated were the monocyclic peptide MMP2-106 having an IC_{50} of 50 nM corresponding to a K_i of 25 nM and the bicyclic peptide MMP2-11 having an IC_{50} of 360 nM which corresponds to a K_i of 180 nM. These peptides are cyclised by one or two disulfide bridges and may also be affinity matured. However, a limitation of the monocyclic peptide is the exocyclic linear tail, which is essential for the inhibition but most likely easily degraded by proteases. The limitation of the disulfide-linked bicyclic peptides (as MMP2-11) is the formation of multiple regioisomers, which complicates the characterisation and the synthesis of desired isomers.

2.3. Phage selection of bicyclic peptides binding to MMP-9

MMP-9 plays a role in several pathological processes including tumour growth and invasion. It is considered as a tumour type and stage dependent target. We performed selections against this protease in parallel to those against MMP-2 described before (**subchapter 2.2**).

2.3.1. Expression and immobilisation of MMP-9

Selections were performed on full-length MMP-9 (**Figure 15** and **Appendices subchapter 7.6**). This protease is structurally and functionally highly related to MMP-2. It is composed of the same domains with the exception that it has an additional type V collagen-like domain[19,30]. This extra region consists of 54 residues rich in proline. Its role is not clear.

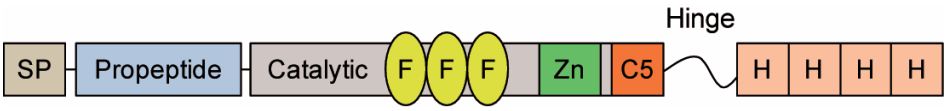

Figure 15. Structure of MMP-9. The different regions of proMMP-9 are schematically illustrated. The regions include an N-terminal signal sequence (SP), a propeptide, a catalytic domain containing a zinc-binding site (Zn) and three repeats homologous to the collagen-binding type II domains of fibronectin (F). Four repeats of a hemopexin-like domain (H) at the C-terminal part are connected to the catalytic domain by a hinge. In addition to MMP-2, MMP-9 has also a unique type V collagen-like domain (C5) before the linker region.

The full-length protein was expressed in mammalian cells, purified successively by affinity and size-exclusion chromatographies, and finally biotinylated (**Figure 16A**). Before the selections, the zymogen was activated, as MMP-2, by incubation with APMA. The activity of the protein was measured using the internally quenched fluorescent substrate used for MMP-2 (**Figure 16B**). The specific activity was measured to be 100 pmol/min/µg.

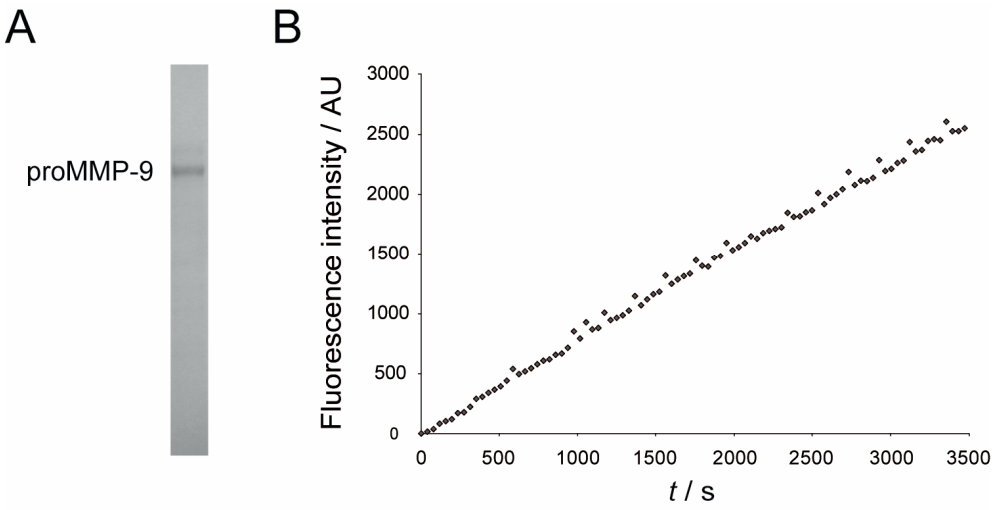

Figure 16. APMA-activation of purified proMMP-9. (**A**) The purity of proMMP-9 (92 kDa) was assessed by SDS-PAGE. (**B**) The activity of the activated form of MMP-9 was measured by monitoring the fluorescence intensity after incubation with an internally quenched fluorescent substrate.

2.3.2. Experiment 1: Standard procedure

Phage selections against activated MMP-9 were performed as previously described for MMP-2 (see experiment 1, **subchapter 2.2.2**). After three rounds of selection, the isolated clones were sequenced and aligned. Two different clusters of consensus sequences were found (**Figure 17**).

In the first cluster, we observed the presence of clones with proximal cysteine residues (positions 8-9 or 9-10) whereas the third cysteine fixed in the library design (position 16) was not present in most peptides. More than 72 % of the isolated sequences presented three cysteines suggesting that most of the peptides were isolated as bicyclic peptides (see discussion **subchapter 2.2**). Aspartic acid in position 6 was highly conserved as well as the phenylalanine in position 4. We speculated that, as for MMP-2, the aspartic residue could bind to the catalytic zinc ion. However, activity assays showed only weak inhibition for all the peptides. MMP9-1, the most abundant clone inhibited the protease with an IC_{50} of 66 μM when cyclised with TBMB (**Figure 17**). Surprisingly, the clone inhibited MMP-9 more efficiently as linear peptide (IC_{50} = 3 μM). Based on this latter observation, it was not clear if the peptides were isolated as TBMB-modified structures or unreacted.

In the second cluster, high similarities between isolated peptides were observed. MMP9-9 and MMP9-10 shared 7 identical or highly similar amino acids, distributed over both peptide loops. Unfortunately, synthetic peptides did not or only weakly inhibit MMP-9.

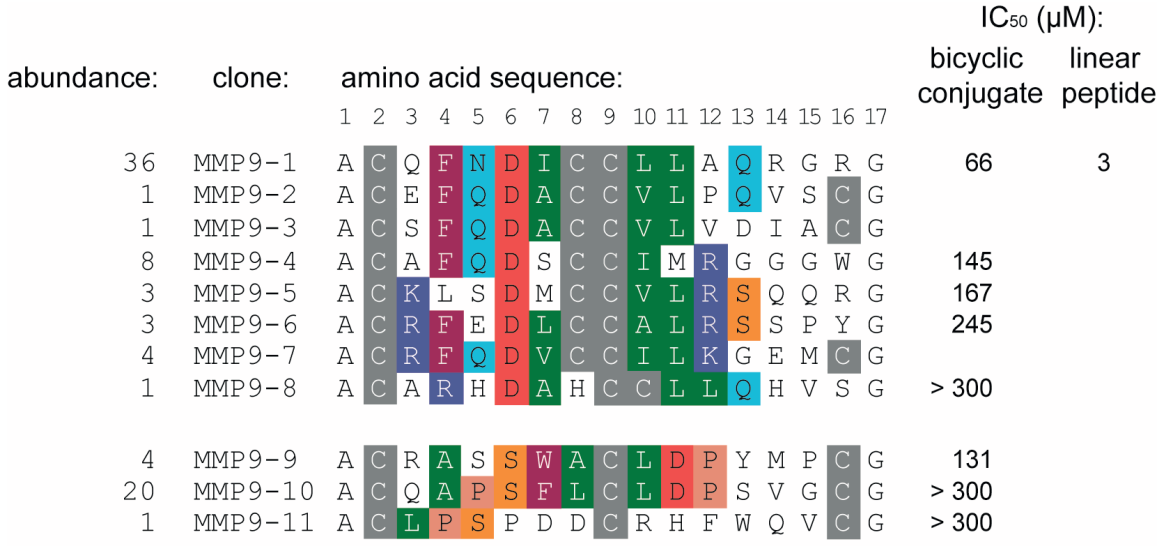

Figure 17. Peptides isolated in phage selections with MMP-9 (low pH elution). Amino acid sequences of clones isolated in phage selections using a low pH elution strategy are presented. Sequence similarities are highlighted in colour (Rasmol colour code). The frequency each peptide was identified is indicated. The inhibitory activities (IC_{50}s) of TBMB-cyclised synthetic peptides as well as reduced-linear peptide MMP9-1 are shown.

Despite the high structural similarities between the catalytic domains of MMP-2 and MMP-9, the sequences of isolated peptides were entirely different.

2.3.3. Experiment 2: Specific elution of active site binders by competitive elution

As for MMP-2, we decided in a second experiment to perform three rounds of affinity selection involving competitive elution of bound phage peptides. To specifically release active site binders we took advantage of a potent hydroxamate-based competitive inhibitor of MMP-9 (GM 6001; K_i = 0.2 nM).

Bicyclic peptides eluted with this strategy showed the same two clusters identified using a low pH elution (experiment 1, **subchapter 2.3.2**). All the isolated clones were previously selected except peptide MMP9-12. However this peptide is highly similar to the others and

did not show any MMP-9 inhibition (IC_{50} > 600 µM, **Figure 18**). All of the selected clones contained three cysteines, suggesting that the peptides were isolated as bicyclic structures modified by TBMB.

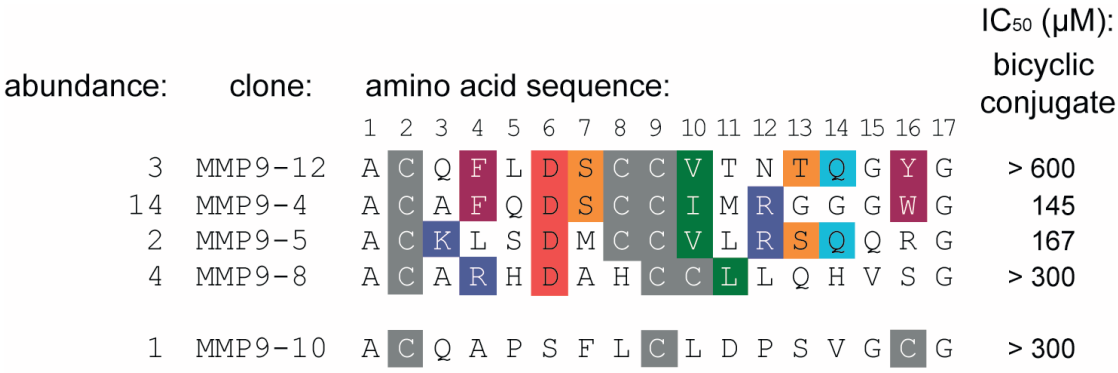

Figure 18. Peptides isolated in phage selections with MMP-9 (competitive elution). Amino acid sequences of clones isolated in phage selections using a competitive small molecule MMP inhibitor are presented. Sequence similarities are highlighted in colour (Rasmol colour code). The frequency each peptide was identified is indicated. The inhibitory activities (IC_{50}s) of TBMB-cyclised synthetic peptides are shown.

Both strategies (experiments 1 and 2) gave similar results and no better peptide hit was isolated after this second experiment.

2.3.4. Conclusions and outlook

Phage selections against MMP-9 yielded peptides with two strong consensus sequences that were reproducibly found in two entirely independent experiments. The peptides did not significantly inhibit MMP-9 when they were cyclised with TBMB. It remains unclear if they were isolated due to binding to a region not affecting the enzymatic activity or if they were isolated as unmodified peptides binding to the active site of MMP-9. In future experiments, the binding of the isolated peptides to MMP-9 may be assessed by enzyme linked immunosorbent assays (ELISA) or fluorescence polarisation. For the isolation of MMP-9 inhibitors, a promising strategy may be to perform affinity selections against the catalytic domain of MMP-9 alone. This would maximise the chances of isolating active site binders and therefore inhibitors.

2.4. Materials and methods

Expression and purification of the full-length of human MMP-2 and -9. Expression in mammalian cells of both proteases was performed by the protein expression core facility (PECF) at EPFL. The following protocol was used. Full-length cDNA of human MMP-2 and -9 cloned into pcDNA3 expression vector, a kind gift from Prof. Dr. Eric Howard[39], was used to transfect human embryonic kidney (HEK-293) cells using linear polyethylenimine (PEI, Polysciences, Eppenheim, Germany). The large scale expression of proMMP-2 and -9 (1 litre culture) was performed in suspension-adapted HEK-293 cells growing in serum-free ExCell 293 medium (SAFC Biosciences, St. Louis, MO, USA) in the presence of 4 mM glutamine. HEK-293 cells at high cell density (20 x 10^6 cells/ml) in 50 ml of RPMI 1640 medium were transferred to a 250-ml glass bottle (Schott Glass, Mainz, Germany) and transfected by sequential addition of DNA (62.5 µg/million cells) and PEI (187.5 µg/million cells)[40]. The cultures were incubated in an ISF-4-W incubator (Kühner AG, Birsfelden, Switzerland) at 37 °C with agitation at 110 rpm in the presence of 5 % CO_2[41]. After 2 h, the cultures were transferred to a 5-L glass bottle (Schott Glass) containing 950 ml Excell 293 medium. The histone deacetylase inhibitor valproic acid was added to a final concentration of 3.75 mM (2-propylypentanoic acid, sodium salt or VPA)[42] and the cultures were agitated as before. After 7 days, the cells were harvested by centrifugation at 2'500 rpm for 15 min at 4°C. Any additional cell debris was removed from the medium by filtration through 0.45 µm PES membranes (Filter-top 250 ml low protein binding TPP). The recombinant proteins were purified from the conditioned medium by size exclusion chromatography using a HiPrep 26/60 Sephacryl S-200 high resolution column (GE Healthcare, Uppsala, Sweden) and eluted in PBS buffer (pH 7.4). ProMMP-9 was purified sequentially by gelatin-Sepharose chromatography as described previously[43] and by gel filtration. The purity of both proteases was greater than 95 % as assessed by SDS-PAGE.

Biotinylation of human proMMP-2 and -9. The recombinant proteases (3 µM) in PBS (pH 7.4) were incubated with 50-fold excess of EZ-link Sulfo-NHS-LC-Biotin (150 µM; Pierce, Rockford, IL, USA) for 1 h at 25 °C. Excess of biotinylation reagent was removed by gel filtration with a PD-10 column (GE Healthcare) using TNC buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, 0.02 % (w/v) NaN_3 and 0.05 % (w/v) Brij-35) and the protein concentrations were determined spectrophotometrically by measuring the absorbance at 280 nm (GeneQuant 100, GE Healthcare). The ability of the biotinylated proMMPs to bind to

either streptavidin or neutravidin was verified by incubating the proteins with magnetic streptavidin and neutravidin beads respectively and analysing the bound and unbound protein fractions by SDS-PAGE. Similar protocol was used to biotinylate activated human plasma kallikrein purified from human plasma (Innovative Research, Novi, USA) as previously described[3].

Activation of human proMMP-2 and -9. The purified zymogens of both proteases (1 to 5 μM) were incubated 1 h (MMP-2) and 24 h (MMP-9) at 37 °C with freshly prepared 4-aminophenylmercuric acetate (APMA) at a final concentration of 2 mM as previously described[44]. APMA was used to activate the proenzymes to avoid inclusion of additional contaminating proteases in our phage selections. In *experiment 4*, MMP-2 was additionally purified after APMA-activation by size exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare) and eluted in TNC buffer.

Phage selection of bicyclic peptides. Bacterial cells of the library glycerol stock were inoculated in 500 ml of 2YT/chloramphenicol (30 μg/ml) medium to obtain an OD_{600} of 0.1. The culture was shaken (200 rpm) for 16 h at 30 °C. After 30 min of centrifugation at 8500 rpm and 4 °C, the phage were purified by precipitation with 0.2 volume of 20 % (w/v) polyethylene glycol 6000 (PEG6000), 2.5 M NaCl on ice and centrifugation at 8500 rpm for 30 min. PEG purified phage, typically 10^{11}-10^{12} t.u. (transducing units), were reduced in 20 ml of 20 mM NH_4HCO_3, 5 mM EDTA, pH 8.0 with 1 mM tris(2-carboxyethyl)phosphine (TCEP) at 42 °C for 1 h. The concentration of TCEP was subsequently reduced by repetitive concentration and dilution steps with reaction buffer (20 mM NH_4HCO_3, 5 mM EDTA, pH 8.0, degassed) in a Vivaspin-20 filter (MWCO of 100'000, Sartorius-Stedim Biotech GmbH, Goettingen, Germany) as described in Heinis *et al*., 2009[3]. The volume of the phage solution was adjusted to 32 ml with reaction buffer and 8 ml of 50 μM tris(bromomethyl)benzene (TBMB) in acetonitrile (ACN) was added to obtain a final TBMB concentration of 10 μM. For control libraries without TBMB-modification (*experiments 3-5*, non-modified peptide), 8 ml of ACN was added instead of TBMB. The reaction was incubated at 30 °C for 1 h before non-reacted TBMB was removed by precipitation of the phage with 0.2 volume of 20 % (w/v) PEG6000, 2.5 M NaCl on ice and centrifugation at 4000 rpm for 30 minutes. In *experiment 3*, 10 mM of methyl methanethiosulfonate (MMTS) was added to the phage (after TBMB reaction) and let incubated 30 min at 30 °C. The phage pellet was then dissolved in 3 ml washing buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$).

Biotinylated active MMP-2 (40 μg), MMP-9 (20 μg) or hPKK (6 μg) were added to 50 μl magnetic streptavidin beads (Dynabeads M-280 from Invitrogen Dynal Biotech AS, Oslo, Norway) in washing buffer and incubated on a rotating wheel for 10 min at room temperature (RT). The magnetic beads were then washed with 0.5 ml washing buffer and incubated for 30 min at RT with 0.5 ml washing buffer containing 1 % (w/v) BSA and 0.1 % (v/v) Tween 20. At the same time the chemically modified phage (typically 10^{10}-10^{11} t.u. dissolved in 3 ml washing buffer) were blocked by addition of 1.5 ml of washing buffer containing 3 % (w/v) BSA and 0.3 % (v/v) Tween 20 for 30 minutes. The blocked beads/antigen mixture (0.5 ml) and phage (4.5 ml) were mixed together and incubated for 30 minutes on a rotating wheel at room temperature. The beads (and antigen/phage bound to them) were washed eight times with washing buffer containing 0.1 % (v/v) Tween 20 and twice with washing buffer. The phage were eluted either by incubation with 100 μl of 50 mM glycine, pH 2.2 for 5 minutes (*experiments 1, 3, 4* and *5*; low pH elution), and then transferred to 50 μl of 1 M Tris-Cl, pH 8.0 for neutralisation or by incubation with 100 μl of 250 μM of the competitive hydroxamate inhibitor GM 6001 (Enzo Life Sciences, Inc., Farmingdale, New York, USA) in washing buffer (pH 7.4) for 30 minutes (*experiment 2*; competitive elution). The eluted phage were incubated with 30 ml TG1 cells at OD_{600} of 0.4 for 90 minutes at 37 °C and the cells were plated on large 2YT/chloramphenicol (30 μg/ml) plates. Second and third rounds of panning were performed following the same procedure but using in the second round neutravidin-coated magnetic beads instead of streptavidin in order to prevent the enrichment of streptavidin-specific peptide binders. Neutravidin beads were prepared by reacting 0.8 mg neutravidin (Pierce) with 0.5 ml tosyl-activated magnetic beads (2 x 10^9 beads/ml; Dynabeads M-280, Invitrogen Dynal Biotech AS) according to the supplier's instructions. DNA of individual clones was sequenced (Macrogen, Seoul, South Korea) after rounds two and three.

Chemical synthesis of peptides. Peptides with a free amine at the N-terminus and an amide at the C-terminus were synthesised either on a 25 mg scale by solid-phase chemistry (JPT Peptide Technologies, Berlin, Germany or GL Biochem Ltd., Shanghai, China) or at a 0.03 mmol scale with standard Fmoc chemistry on an automated peptide synthesiser (Advanced ChemTech 348Ω, Louisville, USA). Peptides were first deprotected and cleaved from the Rink-4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin with trifluoroacetic acid (TFA) (90 %, v/v), thioanisole (2.5 %, v/v), phenol (2.5 %, w/v), 1,2-ethanedithiol (2.5 %, v/v) and H_2O (2.5 %, v/v) and then precipitated three times in chilled diethylether. The crude peptides (1 mM) in 1 ml 70 % (v/v) 0.1 M NH_4HCO_3 (pH 8) and 30 % (v/v) acetonitrile were

reacted with TBMB (1.2 mM) for 1 h at 30 °C. The reaction product was purified by semi-preparative reversed-phase HPLC using a XBridge Prep BEH300 C18 (5 µm) column (10 x 250 mm) (Waters, Milford, MA, USA) and a linear gradient elution with a mobile phase composed of eluent A (99.9 % (v/v) H_2O and 0.1 % (v/v) TFA) and eluent B (94.9 % (v/v) ACN, 5 % (v/v) H_2O and 0.1 % (v/v) TFA) at flow rate of 6 ml/min. The purified peptides were freeze-dried and dissolved in water and 10 % (v/v) DMSO for activity measurements.

Peptides (MMP2-11 and -12) containing four cysteines were synthesised as above using orthogonal cysteine protecting groups (three Cys(Trt) and one Cys(*t*Bu)) in order to perform selective cyclisation. Peptides were cleaved from the resin with TFA (88 %, v/v), thioanisole (2.5 %, v/v), phenol (2.5 %, w/v), 1,2-ethanedithiol (2.5 %, v/v), H_2O (2.5 %, v/v) and triisopropylsilane (2 %, v/v) and precipitated three times in chilled diethylether. The crude peptides were TBMB-cyclised and purified as above. The remaining S-*tert*-butyl protected cysteine was deprotected with 1M HBF_4-thioanisole with scavengers dissolved in TFA as previously described[45] and precipitated again three times in cold diethylether. The bicyclic peptides having a free cysteine residue were purified by HPLC as above and peptide stocks made in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$ for activity measurements.

Linear peptides were synthesised and crude extracts purified by RP-HPLC as described above. Freeze-dried peptides were dissolved in degassed water and immediately used for activity measurements. Disulfide-cyclised peptides were obtained after incubation of the HPLC-purified linear peptides with 4.5 % DMSO in TNC buffer for 24 h at RT. Mixtures of peptide oxidation forms were directly used for activity measurements.

The purity of the modified and unmodified peptides as well as the oxidation pattern of disulfide-cyclised peptides were assessed by analytical RP-HPLC on an Agilent 1260 system (Agilent Technologies, Santa Clara, California, USA), using a C18 column and the same buffer system as for semi-preparative RP-HPLC. The molecular mass of peptides was determined by MALDI-TOF mass spectrometry (Axima-CFR plus, Kratos, Manchester, UK).

Protease inhibition assays. The inhibitory activity of the synthetic peptides was determined by incubating active MMP-2 (1 nM) or MMP-9 (10 nM) with various concentrations of peptides (two fold dilutions) ranging from 600 µM to 0.005 µM (or from 4 nM to 0.008 nM for the hydroxamate inhibitor GM 6001 used as positive control). Residual activity was measured in TNC buffer containing 0.1 % (w/v) BSA and 1 % (v/v) DMSO (DMSO was not

used with linear peptides and peptides MMP2-11 and -12 after selective cyclisation) with the fluorogenic substrate Mca-Lys-Pro-Leu-Gly-Leu-Dap-Ala-Arg-NH_2 (Bachem, Bubendorf, Switzerland) at a concentration of 20 µM. The fluorescence intensity was recorded during 1 h at 37 °C on a Spectramax Gemini fluorescence plate reader (excitation at 325 nm, emission at 400 nm; Molecular Devices, Sunnyvale, CA, USA). IC_{50} values were determined using OriginPro 8G software (OriginLab Corporation, Northampton, USA). The inhibitory constant K_i was calculated according to Cheng and Prusoff equation $K_i = IC_{50} / (1+([S]_0/K_m)$[46] wherein IC_{50} is the functional strength of the inhibitor (half-maximal inhibitory concentration), $[S]_0$ is the initial substrate concentration and Km is the Michaelis-Menten constant.

3. Bicyclic peptide binders of SAS-6

3.1. Introduction

The centrosome is the primary microtubule organising centre (MTOC) in animal cells. It regulates a number of processes including cell motility, adhesion and polarity in interphase[47]. During mitosis, it mediates assembly and organisation of the mitotic spindle, an event required for correct chromosome segregation[48]. In the heart of the centrosome, two centrioles are found. In addition to their role in mitotic spindle organisation, they are fundamental for the assembly of cilia and flagella in eukaryotic cells. Each centriole duplicates only once every cell cycle, during S phase, to generate a new daughter centriole. This new centriole arises orthogonally and very close to the old mother centriole[49,50]. In most species, the centriole adopts a 9-fold symmetry. It is organised around a cartwheel that comprises a central hub (25 nm in diameter) from which nine spokes radiate outward and connect to nine microtubules blades[4]. The centrioles are composed of many proteins. The spindle assembly abnormal 6 protein, abbreviated SAS-6, is at the structural basis of the 9-fold symmetry of the cartwheel[51]. Two crystal structures of both the crN-dimer and the crCC-dimer of SAS-6 from the green alga *Chlamydomonas reinhardtii* (cr) were solved and a structural model of the ring oligomer of CrSAS-6 was built (**Figure 19**)[4].

Self-assembly of high-order SAS-6 oligomeric structures occurs in two steps. Firstly, SAS-6 homodimers assemble via strong interactions between the helices of the two-stranded parallel coiled coil (crCC-dimer). The stability of the dimer was estimated by circular dichroism (CD) and revealed a dissociation constant (K_d) of 0.5 μM[4]. Secondly, oligomers of SAS-6 homodimers assemble, driven by weak interactions (K_d of 60 μM, determined by isothermal titration calorimetry (ITC))[4] between pairs of N-terminal globular domains (crN-dimer). The crystal structure shows that this interaction is mediated by the residue F145 (in Chlamydomonas) which is inserted deeply into a hydrophobic cavity of the second monomer. Mutation of this single amino acid disrupts this interaction. Both F145 and the residues shaping the hydrophobic pocket are well conserved among SAS-6 orthologs (residue F131 in human SAS-6).

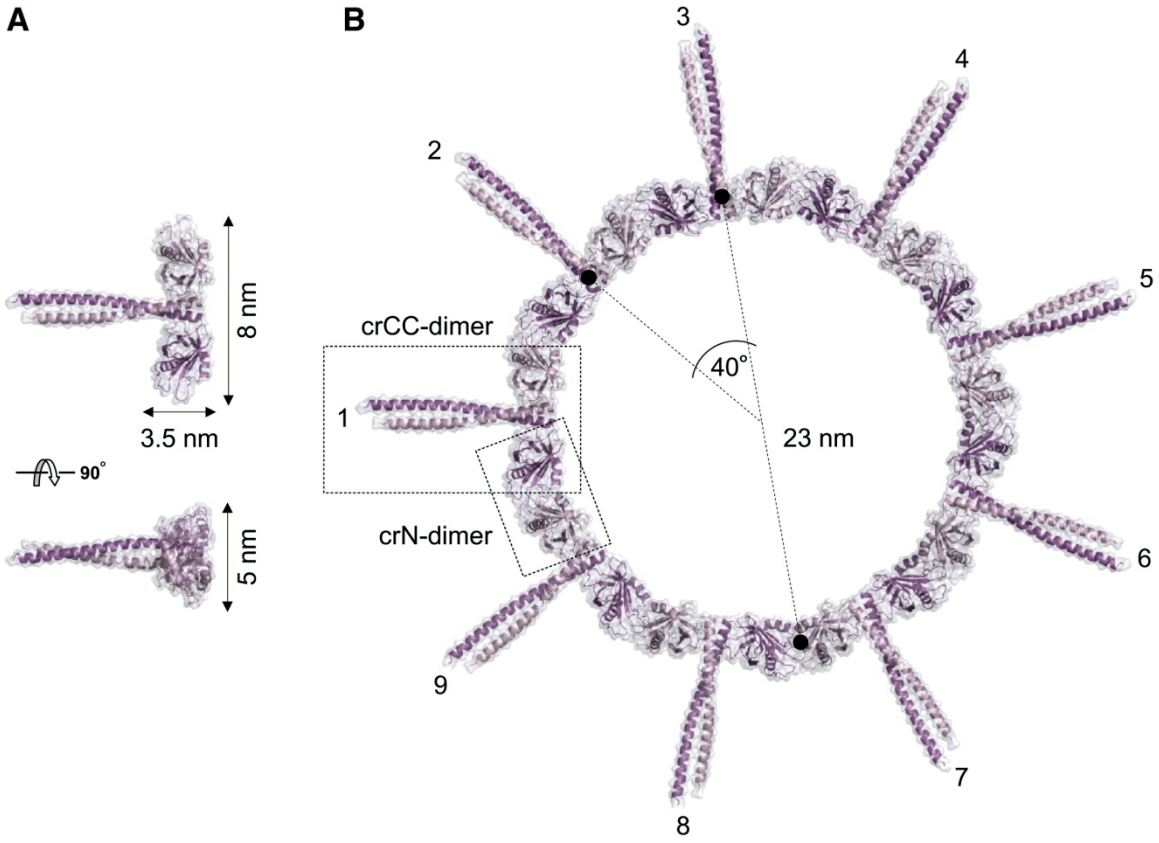

Figure 19. Model of CrSAS-6 oligomerisation. (**A**) Views of the crCC-dimer 90° apart. (**B**) SAS-6 monomers initially dimerise via the coiled-coil interaction and the resulting dimers can then associate via the N-domain interaction to form the 9-fold symmetric ring oligomer. From reference[4].

Abnormalities in centrosome number, size and morphology were observed in almost all human tumour types. This has been associated with genomic instability because extra and often irregular centrosomes can lead to aberrant cell division. In addition to cancer, aberrations in centriole structure or function are implicated in a wide variety of human diseases, including ciliopathy, male sterility and primary microcephaly[47-49,52]. Therefore, increased understanding of centriole biology should result in important clinical implications.

In this study, we attempted to develop bicyclic peptide binders to *C. reinhardtii* (CrSAS-6) and *H. sapiens* SAS-6 (HsSAS-6). Peptides binding with high affinity and specificity to SAS-6 are needed as research tools by the laboratory of Professor Pierre Gönczy (at EPFL) that is studying the structure and function of centrioles[4]. Bicyclic peptides that inhibit the formation of the SAS-6 ring structure should be used to study the mechanism of centriole duplication during cell cycle. This should be done by using specific bicyclic peptides to block the formation of new SAS-6 ring with temporal resolution *in vitro* and *in vivo*.

3.2. Phage selection of bicyclic peptides binding to SAS-6

We aimed at generating bicyclic peptides binding to the dimerising region of the SAS-6 CC-dimer head-groups. Binding to this region should prevent or disrupt ring formation (**Figure 19**). Toward this end, recombinantly expressed protein comprising the N-terminal domain of SAS-6 extended by the first six heptad repeats of the coiled coil was used as target (N-6HR; **Figure 20** and **Appendices subchapter 7.7**). The resulting monomer is known to dimerise (CC-dimer) via coiled coil interaction and to form higher-order oligomeric species at high protein concentration via N-terminal domain interactions (N-dimer) (as shown by analytical ultracentrifugation (AUC) experiments at 150 μM SAS-6 concentration)[4]. We chose to generate bicyclic peptides binding to the SAS-6 fragments of *C. reinhardtii* from which the ring model was built, and of the *H. sapiens* version where no model is currently available, but a similar structure is expected.

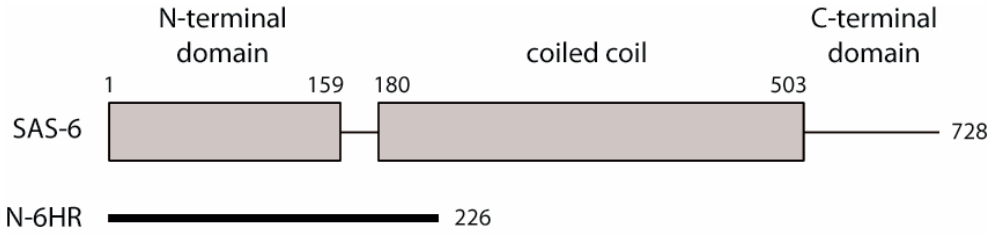

Figure 20. Schematic representation of SAS-6 and the fragment used for selections. N-6HR, N-terminal domain extended by 6 heptad repeats of the adjacent coiled coil. Numbers correspond to CrSAS-6 amino acids. Adapted from reference[4].

3.2.1. Immobilisation of SAS-6 proteins

Both proteins were expressed and purified by our collaborators in the laboratory of Professor Pierre Gönczy. We performed an additional step of purification by size exclusion chromatography (**Figure 21**). CrSAS-6 and HsSAS-6 CC-dimers were biotinylated, immobilised on magnetic streptavidin or neutravidin beads and subjected to three iterative rounds of phage selection. Phage of the libraries 4x4 and 6x6 were produced separately and mixed together before affinity panning. Phage titres of each selection are reported in **Appendices subchapter 7.8**. Sequences of isolated bicyclic peptides as well as their binding

assessed by enzyme linked immunosorbent assays (ELISA) are presented and discussed in the following subchapters.

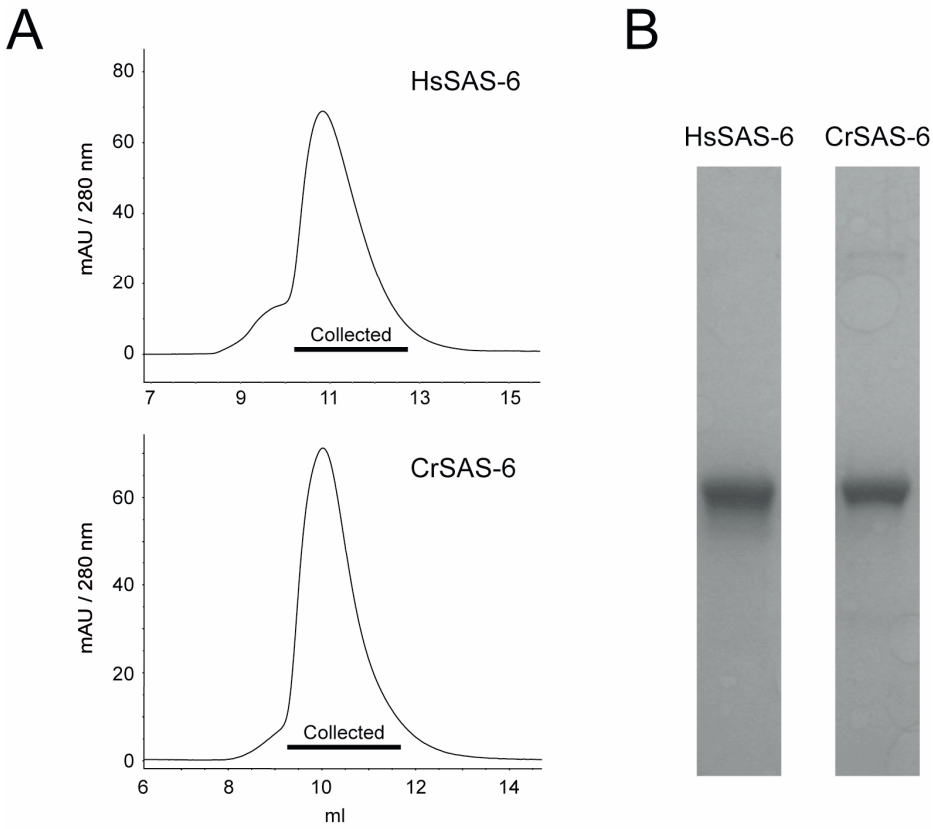

Figure 21. Purification of SAS-6 proteins. (**A**) Chromatograms from the size exclusion purification are shown. The collected fractions are indicated (black line). (**B**) The purity of HsSAS-6 (**left**) and CrSAS-6 (**right**) used for selections was assessed by SDS-PAGE.

3.2.2. Bicyclic peptides isolated against human SAS-6 (HsSAS-6)

Affinity selections yielded bicyclic peptides from both of the two libraries (**Figure 22**). A comparison of the isolated peptide sequences revealed three different consensus sequences. A first consensus sequence was shared by the two peptides SAS6-1 and SAS6-2 ($GL(X)_4CSXGQ^{L}/_{V}{}^{L}/_{V}$; cluster 1). Some sequence similarities were shared by the three peptides SAS6-4, SAS6-5 and SAS6-6 in the first ($PQ^{L}/_{V}$) and the second ring (ELF) (cluster 2). A strong consensus comprising four amino acids ($^{Y}/_{F}R^{L}/_{V}Y$) was found in the second (cluster 3a) and first ring (cluster 3b) of 4x4 bicyclic peptides.

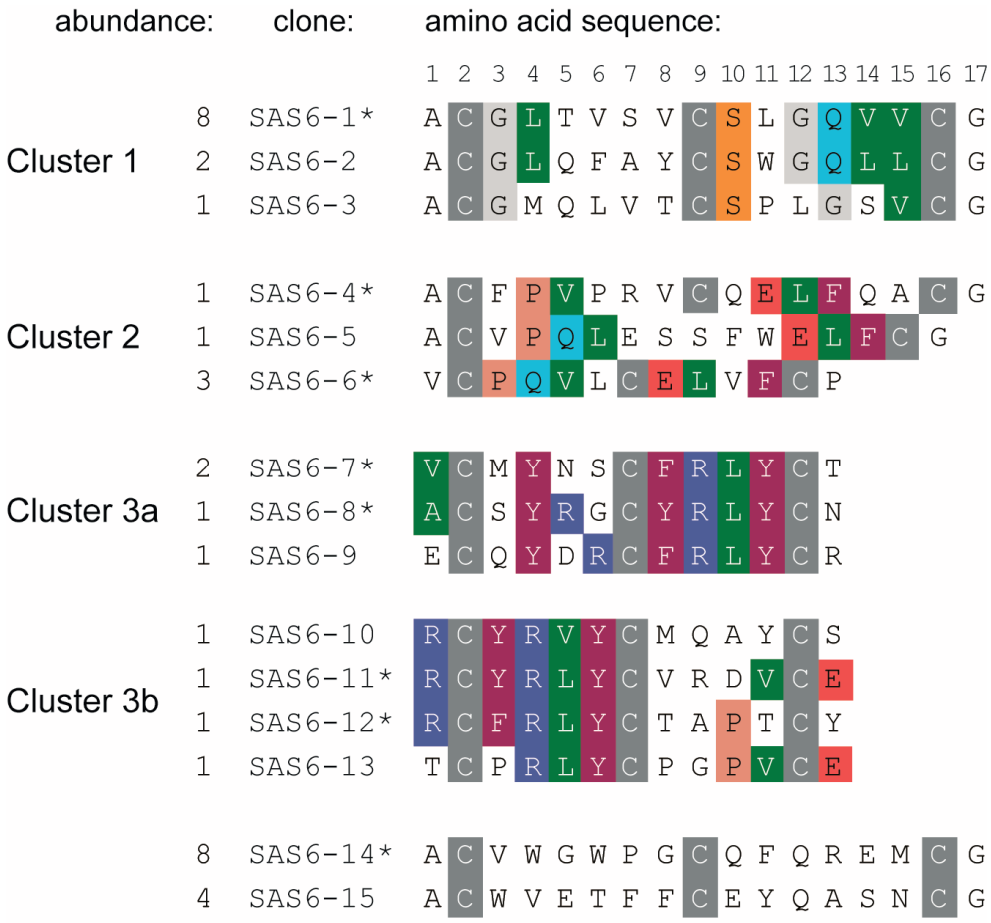

Figure 22. Peptides isolated in phage selections with HsSAS-6. Amino acid sequences of clones isolated in phage selections using a mixture of the libraries 6x6 and 4x4. Sequence similarities are highlighted in colour (Rasmol colour code). The frequency each peptide was identified is indicated. Bicyclic peptides that were further characterised by ELISA assays are indicated (*).

3.2.3. Bicyclic peptides isolated against Chlamydomonas SAS-6 (CrSAS-6)

Bicyclic peptides isolated in selections against the HsSAS-6 ortholog, CrSAS-6, shared fewer sequence similarities. Highlighted in **Figure 23** are sequence similarities between peptides that share identical or similar amino acids in at least four amino acid positions. A total of six consensus sequences were identified (clusters 1 to 6) coming from both libraries. The different motifs of conserved amino acids were either distributed over both peptide loops (clusters 1, 2 and 6) or mostly localised to one of the two loops (clusters 3 to 5).

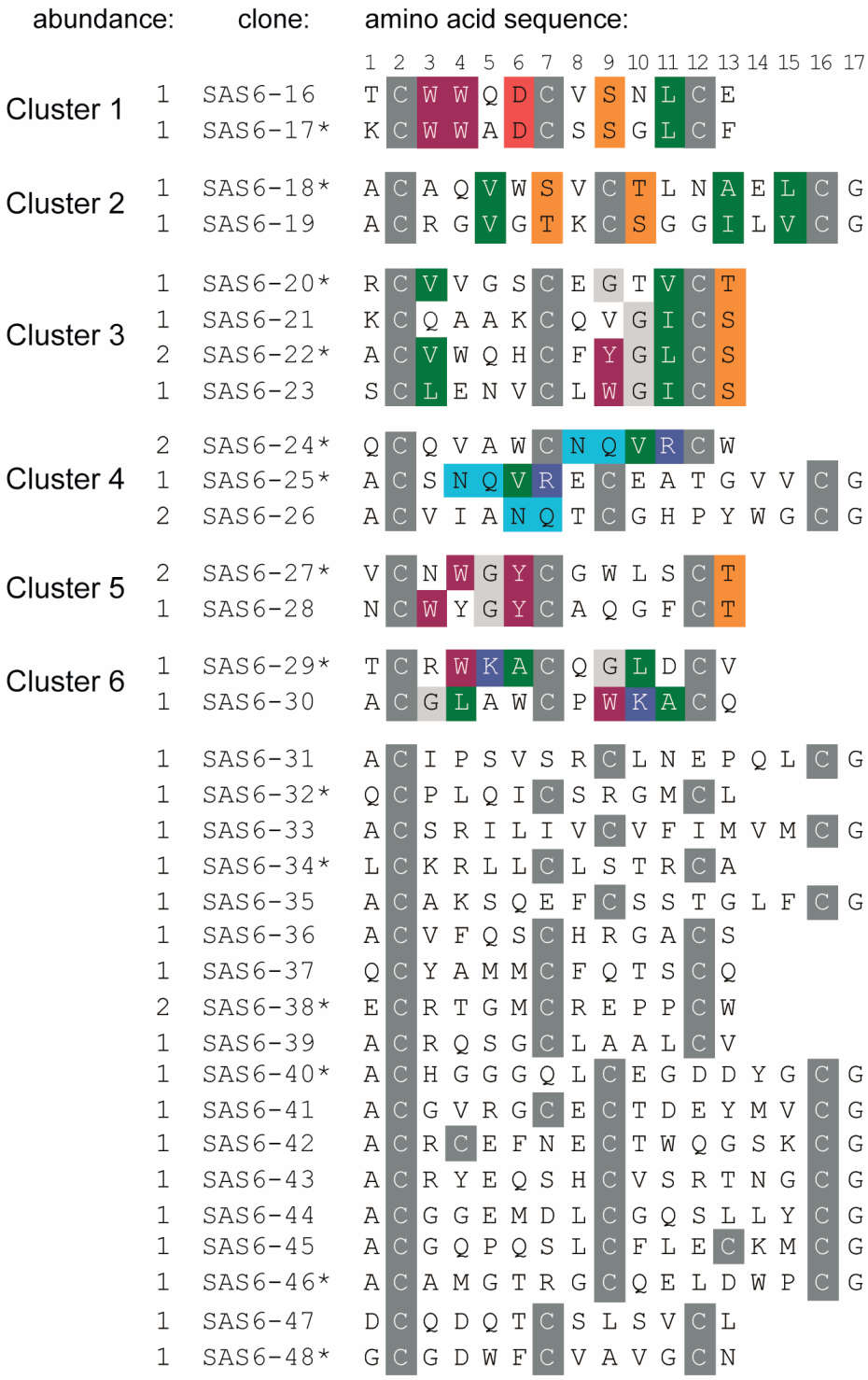

Figure 23. Peptides isolated in phage selections with CrSAS-6. Amino acid sequences of clones isolated in phage selections using mixture of libraries 6x6 and 4x4. Sequence similarities are highlighted in colour (Rasmol colour code). The frequency each peptide was identified is indicated. Bicyclic peptides that were further characterised by ELISA are indicated (*).

3.3. Assessment of binding by ELISA

Twenty-two bicyclic peptides were chemically synthesised and their binding to either the human (8 peptides) or the green alga SAS-6 (14 peptides) assessed by ELISA. All peptides were synthesised with a biotin moiety at the C-terminal end for immobilisation or detection. A linker of three amino acids was inserted between the peptide and the biotin to omit interference with the binding. Three different ELISA formats were applied:

- *Format 1*: SAS-6 was immobilised on an ELISA plate. Peptide was added and the unbound fraction washed away. Bound peptide was detected with a neutravidin-peroxidase conjugate and a chromogenic substrate.
- *Format 2*: SAS-6 was immobilised on an ELISA plate. Peptides, tetramerised by premixing with neutravidin-peroxidase (ratio 1:20 for neutravidin:peptide), were added. Bound peroxidase was detected with a chromogenic substrate.
- *Format 3*: Peptides were immobilised on a neutravidin-coated plate. SAS-6 was added and binding detected via its histidine tag using an anti-His6 antibody-peroxidase and a chromogenic substrate.

Experiments according to *formats 1* and *2* were performed in parallel. Binding signals obtained with *format 2* (**Figures 24B** and **25B**) were more intense than those obtained with *format 1* (**Figures 24A** and **25A**). This can be explained by the avidity effect and the resulting slower dissociation of the tetrameric peptides.

3.3.1. Bicyclic peptides isolated against human SAS-6

Seven out of the eight tested peptides bound to human SAS-6 in a concentration dependent manner. In the assay *format 1*, three of these peptides also bound significantly to albumin which was used as a negative control (peptides 7, 12 and 14; **Figure 24A**). In these controls, no target was immobilised and albumin was used to block the plate and avoid unspecific interactions. A significantly better signal over background was observed in the assay *format 2*. Similar results were found in the experiment applying assay *format 3*. An unrelated bicyclic peptide (ACGQPNFACRALYPECGB with B = Lys(biotin)) used as a negative control did not bind at all to human SAS-6 in all assay formats (peptide control; **Figure 24**).

Peptides 4 and 6 with similar sequences (cluster 2; **Figure 22**) showed the most specific binding to HsSAS-6 in experiments following the ELISA *formats 2* and *3* (**Figure 24B** and **C**). Peptides 7-8 and 11-12 presenting a strong consensus sequence in either of the two rings (clusters 3a and 3b respectively) showed the highest signals in assay *formats 2* and *3*. However, a significant signal was also observed in the negative controls where no target was immobilised (control BSA; **Figure 24B** and **C**). It is known that peptides with multiple aromatic amino acids bind to hydrophobic surfaces of albumin[53]. It could be that the hydrophobic amino acids in these peptides might result in the unspecific interactions with albumin. Other proteins may be used as negative controls to assess in detail the specificity profile of these peptides.

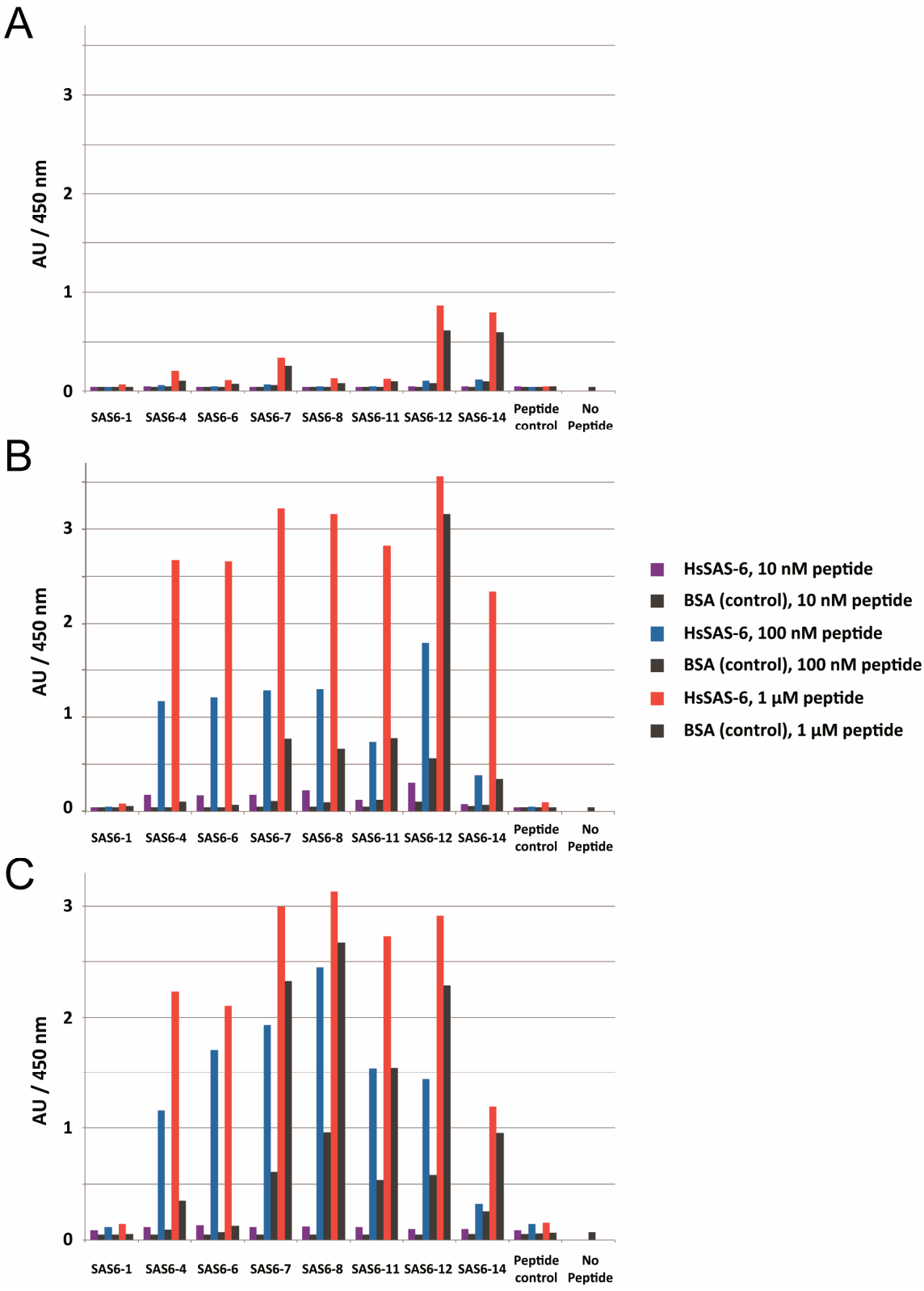

Figure 24. ELISAs of peptides selected against HsSAS-6. Binding of peptides isolated in phage selections with human SAS-6 was assessed using three different ELISA formats. (**A**) The target was first immobilised on an ELISA plate (high binding capacity). The biotinylated peptides (10 nM, 100 nM and 1 µM) and the neutravidin-peroxidase were added sequentially (*format 1*). (**B**) The target was

immobilised and biotinylated peptides pre-mixed with neutravidin-peroxidase were added to the plate (*format 2*). (**C**) Peptides were first immobilised on a neutravidin-coated plate. HsSAS-6 was added and detected with an anti-His6 antibody (*format 3*). Negative controls performed with a control peptide, without peptide or without target (replaced by bovine serum albumin, BSA) are indicated. ELISAs (**A**) and (**B**) were performed in parallel.

3.3.2. Bicyclic peptides isolated against Chlamydomonas SAS-6

Nine out of the fourteen tested peptides bound to CrSAS-6 in a concentration dependent manner. In the assay *formats 1* and *3*, two and six of these peptides, respectively, also bound significantly to albumin which was used as a negative control (**Figure 25A** and **C**). A significantly better signal over background was observed in the assay *format 2*. The unrelated bicyclic peptide used as negative control did not bind at all to CrSAS-6.

Peptides 17, 22, 24, 29, 32, 46 and 48 showed specific binding to CrSAS-6 in ELISA *format 2* (**Figure 25B**). Peptides 27 and 34 showed the highest signals in experiments following the assay *formats 1* and *2*. However, strong signals were also observed in the negative controls where albumin was immobilised (control BSA; **Figure 25A** and **B**), indicating unspecific interactions.

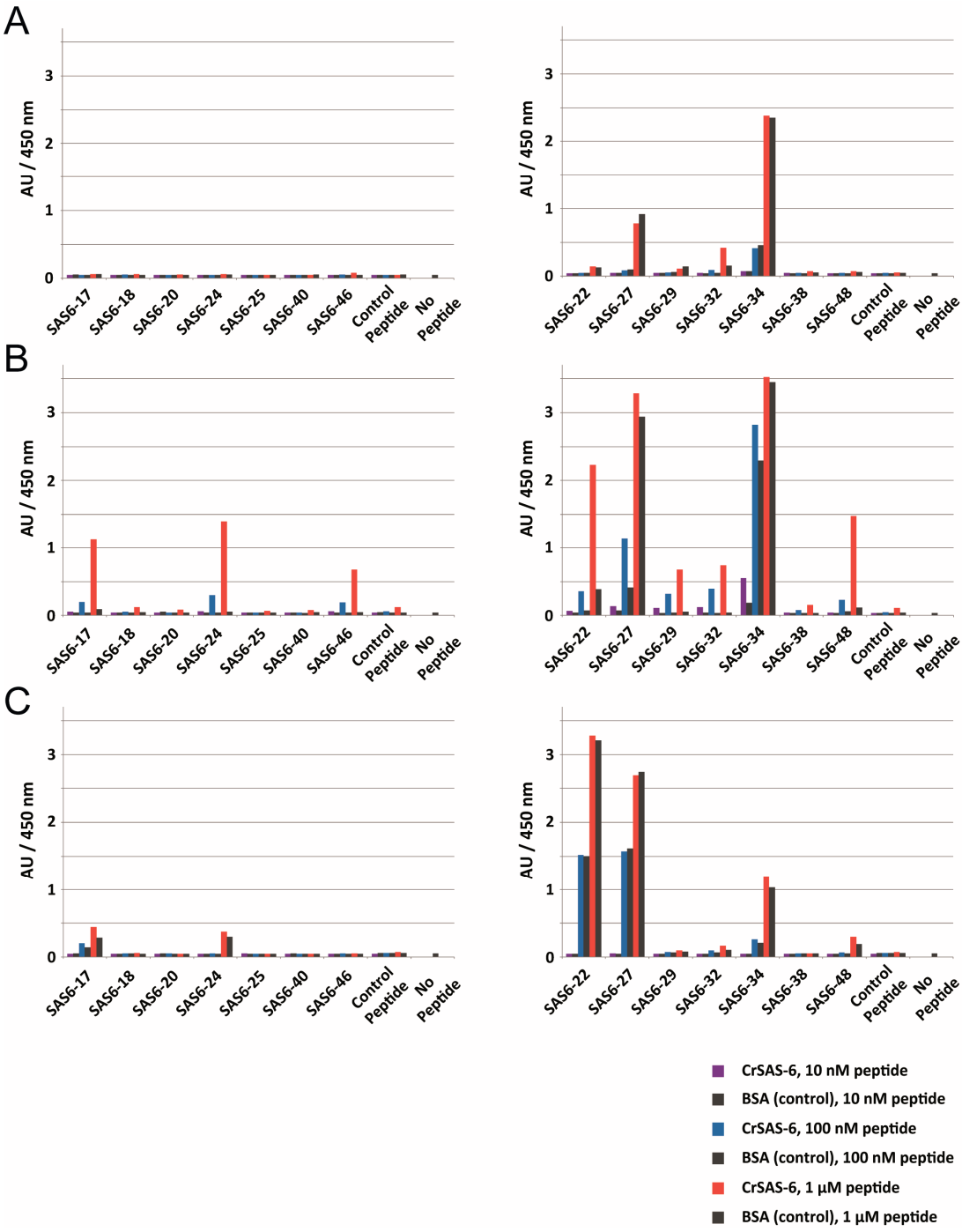

Figure 25. ELISAs of peptides selected against CrSAS-6. Binding of peptides isolated in phage selections with CrSAS-6 was assessed using three different ELISA formats. (**A**) The target was first immobilised on an ELISA plate (high binding capacity). The biotinylated peptides (10 nM, 100 nM and 1 µM) and the neutravidin-peroxidase were added sequentially (*format 1*). (**B**) The target was immobilised and biotinylated peptides pre-mixed with neutravidin-peroxidase were added to the plate (*format 2*). (**C**) Peptides were first immobilised on a neutravidin-coated plate. CrSAS-6 was added and detected with an anti-His6 antibody (*format 3*). Negative controls performed with a control peptide, without peptide or without target (replaced by BSA) are indicated. ELISAs (**A**) and (**B**) were performed in parallel.

3.4. Conclusions and outlook

Bicyclic peptide binders to the centriolar protein SAS-6 of human and green alga could be generated. For both targets, peptides belonging to two (HsSAS-6; clusters 2 and 3a, b) or four (CrSAS-6; clusters 1, 3, 4 and 6) different consensus sequences showed significant binding. It is likely that peptides belonging to these different consensus sequences bind with different orientations and possibly even to different epitopes. It remains to be seen if the peptides can disrupt the interaction between SAS-6 CC-dimer head-groups needed to form the circular oligomer structure.

Based on the weak signals in the ELISA assays, the bicyclic peptides are expected to bind with a binding constant in the micromolar range. For cellular studies, it will be crucial to apply the bicyclic peptides at high micromolar concentrations. Alternatively, bicyclic peptides being able to disrupt the dimerisation of SAS-6 N-terminal domain (N-dimer) may be affinity matured to allow application of lower concentrations.

In vitro and *in vivo* works are currently made in the laboratory of Professor Pierre Gönczy to assess if the affinity of the isolated binders is high enough to observe any phenotype during the formation of centrioles. The following experiments are currently performed or planned:

- For HsSAS-6 binders: In a first experiment, high concentration of bicyclic peptides (100 μM) are incubated (8 and 24 hours) with U2OS cells and the resulting phenotypes followed by confocal microscopy. In a second experiment, peptides are microinjected into human cells and the phenotype observed by microscopy. Lead compounds will be further characterised by isothermal titration calorimetry (ITC).

- For CrSAS-6 binders: In a first experiment, the same assay as done with HsSAS-6 is performed, but using a *C. reinhardtii* cell wall-minus strain. In a second experiment, an *in vitro* assay in which stacked SAS-6 ring can form tubules on lipid monolayer platform is developed. Destabilisation of the tubule formation upon incubation with peptides is monitored by microscopy. Lead compounds will be further characterised by ITC.

3.5. Materials and methods

Expression and purification of HsSAS-6 and CrSAS-6 proteins. Both proteins were cloned, expressed and purified by our collaborators in the laboratory of Professor Pierre Gönczy as previously described[4]. The fragments HsN-6HR (residues 1-212) and CrN-6HR (residues 1-226) were purified by immobilised metal-affinity chromatography (IMAC) on HisTrap HP Ni^{2+}-Sepharose columns (GE Healthcare) at 4 °C according to manufacturer's information. We performed an additional step of purification by size exclusion chromatography using a Superdex 75 10/300 GL column (GE Healthcare). Proteins were eluted in PBS buffer. The purity was greater than 95 % for both proteins as assessed by SDS-PAGE.

Biotinylation of SAS-6 proteins. The recombinant proteins (10 μM) were incubated with 4-fold excess of EZ-link Sulfo-NHS-LC-Biotin (40 μM; Pierce) in PBS (pH 7.4) for 1 h at 25 °C. Excess of biotinylation reagent was removed by gel filtration with a PD-10 column (GE Healthcare) using PBS buffer. Protein concentrations were determined spectrophotometrically by absorbance measurements at 280 nm (GeneQuant 100, GE Healthcare). The ability of the biotinylated SAS-6 proteins to bind to either streptavidin or neutravidin was verified by incubating the proteins with magnetic streptavidin and neutravidin beads respectively and analysing the bound and unbound protein fractions by SDS-PAGE.

Phage selections of bicyclic peptides. The same protocol as described in **subchapter 2.4** (standard procedure) was used. Briefly, peptide libraries 4x4 (250 ml per target) and 6x6 (1 litre per target) were produced and cyclised separately. Libraries were mixed together before biopanning. 10 μg of biotinylated SAS-6 proteins were immobilised on magnetic streptavidin beads. Bound phage were eluted by incubation with 100 μl of 50 mM glycine, pH 2.2 for 5 minutes and then transferred to 50 μl of 1 M Tris-Cl, pH 8.0 for neutralisation. Three iterative rounds of selection were performed. Second and third rounds of panning were performed following the same procedure but using in the second round neutravidin-coated magnetic beads instead of streptavidin in order to prevent the enrichment of streptavidin-specific peptide binders.

Chemical synthesis of biotinylated peptides. Peptides with a free amine at the N-terminus and an amide at the C-terminus were synthesised and purified using the same protocol as

described in **subchapter 2.4**. Peptides were biotinylated at the C-terminal end by incorporating a Fmoc-Lys(biotin)-OH residue (GL Biochem Ltd.). The purity of the peptides was assessed by analytical RP-HPLC on an Agilent 1260 system (Agilent Technologies) using a C18 column and the molecular mass of peptides was determined by MALDI-TOF mass spectrometry (Axima-CFR plus, Kratos).

ELISA *format 1*. SAS-6 proteins (or BSA in control wells without target) were immobilised (80 µl at 7 µg/ml) overnight at 4 °C on a NUNC-Immuno^{TM} 96 MicroWell^{TM} MaxiSorp plate (Thermo Fisher Scientific Inc., Waltham, MA, USA). The wells were washed with buffer RT (buffer R (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$) containing 0.1 % (v/v) Tween 20), blocked for 2 h at RT with 300 µl of buffer RTB (Buffer R containing 0.1 % (v/v) Tween 20 and 5 % (w/v) BSA) and then washed twice with buffer RT. In parallel, biotinylated peptides and neutravidin^{TM}-horseradish peroxidase (HRP) conjugate (Invitrogen Molecular Probes, Paisley, UK) were blocked for 1 h in buffer RTB. 80 µl of peptides (1 µM, 100 nM, 10 nM) were added to the wells and incubated with a slight shaking for 1 h at room temperature (buffer RTB was added in control wells without peptide). The wells were washed twice with buffer RT and 80 µl of neutravidin-HRP at dilution 1:2000 in buffer RTB were added to the wells and incubated for 15 min. The wells were washed six times with buffer RT and once with buffer R. 80 µl of 1-step Ultra TMB (Thermo Fisher Scientific Inc.) substrate were added to the wells and incubated for 5 to 30 minutes. The reaction was stopped with 80 µl of 2 M sulphuric acid, and the absorbance measured at 450 nm on a Spectramax 340 fluorescence plate reader (Molecular Devices). All the experiments were performed twice in duplicate.

ELISA *format 2*. SAS-6 proteins (or BSA in control wells without target) were immobilised (80 µl at 7 µg/ml) overnight at 4 °C on a NUNC-Immuno^{TM} 96 MicroWell^{TM} MaxiSorp plate (Thermo Fisher Scientific Inc.). The wells were washed with buffer RT, blocked for 2 h at RT with 300 µl of buffer RTB and then washed twice with buffer RT. Biotinylated peptides were pre-incubated for 1h with neutravidin-HRP (Invitrogen Molecular Probes) in buffer RTB with a ratio 1:20 (neutravidin:peptide). 80 µl of peptides (1 µM, 100 nM, 10 nM) bound to neutravidin were added to the wells and incubated with a slight shaking for 1 h at room temperature (buffer RTB was added in control wells without peptide). The wells were washed six times with buffer RT and once with buffer R. 80 µl of 1-step Ultra TMB (Thermo Fisher Scientific Inc.) substrate were added to the wells and incubated for 5 to 30 minutes. The

reaction was stopped with 80 µl of 2 M sulphuric acid, and the absorbance measured at 450 nm on a Spectramax 340 fluorescence plate reader (Molecular Devices). All the experiments were performed twice in duplicate.

ELISA *format 3*. Neutravidin™ biotin binding protein (Thermo Fisher Scientific Inc.) was immobilised (80 µl at 5 µg/ml) overnight at 4 °C on a NUNC-Immuno™ 96 MicroWell™ MaxiSorp plate (Thermo Fisher Scientific Inc.). The wells were washed with buffer RT, blocked for 2 h at RT with 300 µl of buffer RTB and then washed twice with buffer RT. In parallel, biotinylated peptides and Anti-6X His tag antibody (HRP) (Abcam plc, Cambridge, UK) were blocked for 1 h at RT in buffer RTB. 80 µl of peptides (1 µM, 100 nM, 10 nM) were added to the wells and incubated with a slight shaking for 15 minutes at room temperature (buffer RTB was added in control wells without peptide). The wells were washed twice with buffer RT and 80 µl of SAS-6 proteins (or BSA in control wells without target) at 4 µg/ml in buffer RTB were added to the wells and incubated for 1 h at room temperature with a slight shaking. The wells were washed twice with buffer RT and 80 µl of Anti-6X His tag antibody (HRP) (Abcam plc) at dilution 1:10'000 in buffer RTB were added to the wells and incubated for 1 h at RT. The wells were washed six times with buffer RT and once with buffer R. 80 µl of 1-step Ultra TMB (Thermo Fisher Scientific Inc.) substrate were added to the wells and incubated for 5 to 30 minutes. The reaction was stopped with 80 µl of 2 M sulphuric acid, and the absorbance measured at 450 nm on a Spectramax 340 fluorescence plate reader (Molecular Devices). All the experiments were performed twice in duplicate.

4. Enzymatic cyclisation of peptides with a transglutaminase

4.1. Introduction

Peptide macrocycles are ring structures that can bind with high affinity and specificity to biological targets, which make them an attractive class of molecules for pharmaceutical applications[5]. Synthetic cyclic peptides are typically produced by connecting the ends of side chain-protected peptides which allows the use of a wide range of ligation chemistries. In some cases, the linear peptide precursors have to be produced by recombinant expression as for example polypeptides with long chains (> 50 amino acids) that are difficult to synthesise chemically or combinatorial peptide libraries that are generated by phage display, ribosome display, yeast display or other display techniques[12,14]. The cyclisation of such unprotected peptides is more challenging and requires ligation strategies and reagents that are orthogonal to the peptide side chains.

Peptides with unprotected side chains are predominantly cyclised by chemically linking two cysteine side chains that flank the peptide. Linking reactions with cysteines are particularly suited because of the unique chemical reactivity of thiols and the relatively low abundance of cysteine residues in peptides. The cysteine residues are introduced by mutagenesis at both ends of the peptides and connected through the formation of disulfide bridges in oxidation reactions[54] or through treatment with thiol-specific linking reagents[3,55]. Cyclisation through disulfide-formation has, for example, been used for the generation of macrocycle peptide libraries on phage and has led to the affinity-selection of numerous cyclic peptide ligands[14]. While the thiol-based cyclisation reactions work well in many applications, alternative cyclisation strategies would be useful for a number of situations. For example, reactions that do not involve thiols could be used for the cyclisation of peptide sequences containing cysteines or for the generation of redox-insensitive cyclic peptides[56]. Furthermore, alternative cyclisation reactions could be combined with thiol-based reactions for the generation of multicyclic peptide structures[57]. In fact, a range of cyclisation strategies that are based on amino acids other than cysteines have been described, but most of them are rarely used due to their limitations. For example, lysine residues have been linked to the N-terminal α-amino group of peptides by using iodoacetic anhydride[58] or disuccinimidyl glutarate[56,59], but some of

the reagents were also found to react to a certain extent with other amino acid side chains (e.g. methionine, arginine)[58]. Cyclisation strategies based on enzymes are much more selective, but many of them show limitations such as the need for ester precursors, low cyclisation yields, restriction to a narrow range of peptide lengths and the need for specific amino acid sequences within the peptide cycle. Subtiligase[60], the thioesterase (TE)[61] domain of tyrocidine synthetase, inteins[62] and sortase[63] have all been used for peptide cyclisation.

In this work, we tested whether peptides with varying lengths and amino acid sequences can be quantitatively cyclised with a microbial transglutaminase. In nature, transglutaminases (TGases) catalyse the acyl transfer reaction between the carboxyamide group of glutamines in peptides and a variety of primary amines[64]. This reaction, which is widely observed in plants, animals and microorganisms, leads to post-translational cross-linking of a protein with either another protein or a small molecule through the formation of isopeptide bonds. The ligation activity of TGases has been exploited in the food industry by cross-linking proteins and by binding low-molecular weight compounds to carrier proteins using mammalian transglutaminase from guinea pig or microbial TGase[65]. TGases have also been used for the labelling of proteins with small molecule tags (e.g. the attachment of biotin[66] or fluorescent probes[67] to antibodies) or for the cross-linking of proteins[68]. However, TGases as a class have so far not been used for the cyclisation of peptides. Although it has been found that in human saliva 1 % of the natural peptide statherin is transformed by the action of transglutaminase 2 into a cyclic derivative[69], it was initially unclear whether peptides with varying amino acid sequences and lengths could be cyclised efficiently by TGases.

4.2. Quantitative cyclisation of peptides with a microbial transglutaminase

We used the 38 kDa microbial transglutaminase (MTGase) of *Streptomyces mobaraensis* to test the enzymatic cyclisation of peptides[65]. The MTGase was reported to be relatively stable in food processing and other applications, and, in contrast to mammalian TGases, it is active in the absence of Ca^{2+}. Phage selections with random peptide libraries had revealed that MTGase of *Streptomyces mobaraensis* accepts a broad range of substrates wherein certain peptides are preferred as glutamine-donor substrates[70]. We designed a peptide with the MTGase substrate sequence WALQRPH (the glutamine-donor residue is underlined)[70], a flexible 3-amino acid spacer (GGG) and a lysine-acceptor residue (peptide **1**, H-WALQRPHGGGKS-NH_2; **Table 3**). We chose to place the glutamine-donor substrate peptide (WALQRPH) at the N-terminus and the lysine residue at the C-terminus of the peptide because a model of a peptide-MTGase complex suggested that only in this configuration, the peptide linker could bend back to bring the lysine and glutamine side chains into close proximity (M.J. Hinner, A.H. de Vries and K. Johnsson, unpublished data). Incubation of peptide **1** (3.8 μM) with MTGase (30 nM) and subsequent mass spectrometric analysis revealed a single product with a mass 17 Da smaller; this suggests that an ammonia molecule was eliminated and the peptide was cyclised (**Figure 26**). The formation of dimers was observed at significantly higher peptide concentrations (data not shown).

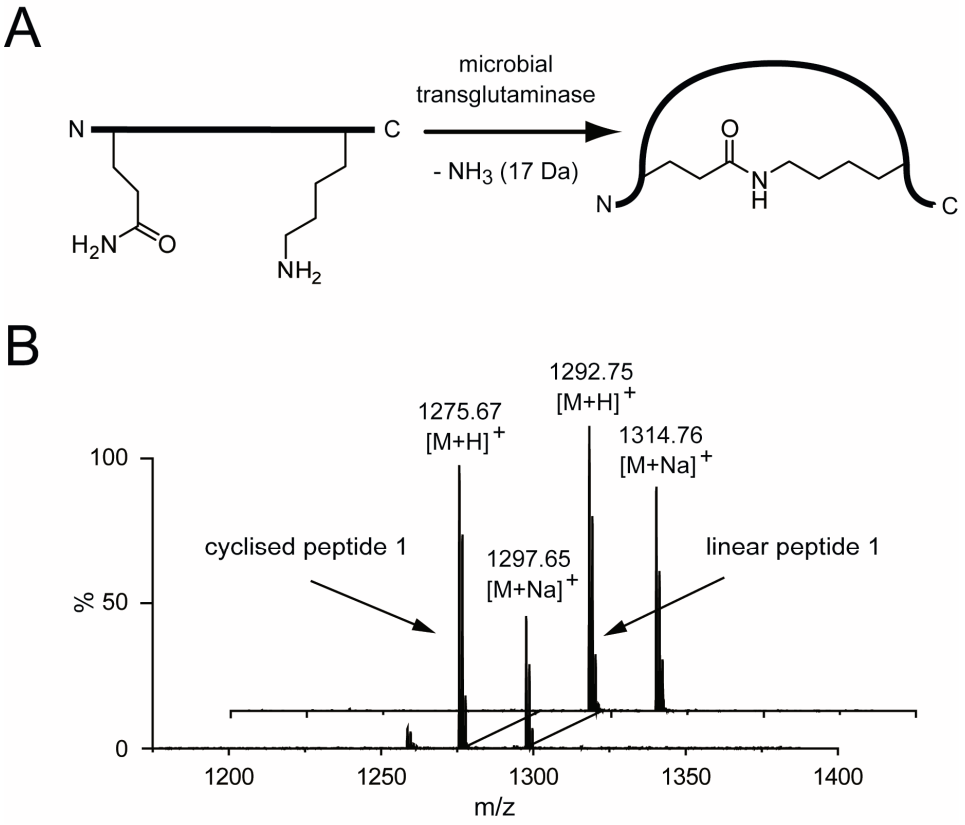

Figure 26. Cyclisation of linear peptides by microbial transglutaminase (MTGase). (**A**) Schematic representation of the enzyme catalysed reaction. The side chains of the glutamine and lysine residues of a linear peptide are ligated by the MTGase to yield a cyclic peptide with a stable amide bond. (**B**) Mass spectra of peptide **1** (H-WALQRPHGGGKS-NH_2; the glutamine and lysine residues that participate in the reaction are underlined) before and after incubation with MTGase.

4.2.1. Substrate specificity of MTGase in cyclisation reactions

In order to determine the minimal amino acid sequence that is accepted by MTGase as a substrate in a cyclisation reaction, we synthesised a range of peptides in which the MTGase glutamine-donor substrate sequence WALQRPH was truncated (**Table 3**) and measured the extent of cyclisation upon incubation with MTGase. While a peptide without the three N-terminal amino acids Trp-Ala-Leu (peptide **2**; H-GQRPHGGGKS-NH_2) was not cyclised at all, the substitution of the three amino acids Arg-Pro-His C-terminal to the reactive glutamine residue to Ser-Gly-Ser yielded a substrate (peptide **3**, H-WALQSGSGGGKS-NH_2) that was cyclised efficiently. This result was pleasing since it suggested that cyclic peptides with variable sequences in the ring can be generated in MTGase catalysed reactions. Experiments with peptides truncated partially at the N-terminus revealed that a peptide with Ala-Leu at the

N-terminal side of the glutamine residue is cyclised (peptide **4**, H-ALQSGSGGGKS-NH_2) but not a shorter peptide (peptide **5**, H-LQSGSGGGKS-NH_2; **Table 3**). In contrast to a previous study[70] that showed that amino acids in positions -3, -1, +1, +2 and +3 are the main determinants of the glutamine donor substrate, these results suggest that the amino acids in positions -2 and -1 are the most important ones. The relatively small sequence requirement of MTGase determined in this set of experiments should allow the generation of cyclic peptides that are not much compromised in their design.

Peptide	Amino acid sequence	MTGase cyclisation	Mass (Da)
1	H-*WALQRPH*GGGKS-NH_2	Yes	1291.68
2	H-G*QRPH*GGGKS-NH_2	No	978.50
3	H-*WALQ*SGSGGGKS-NH_2	Yes	1132.55
4	H-*ALQ*SGSGGGKS-NH_2	Yes	946.47
5	H-*LQ*SGSGGGKS-NH_2	No	875.44
6	H-*ALQ*SGSRGGGKS-NH_2	Yes	1102.57
7	H-*WALQ*SGSGGGGS-NH_2	No	1061.48
8	H-*ALQ*AYDGWLPWEIHVKS-NH_2	Yes	2011.02
9	H-*ALQ*ACSDRFRNCPADEALCAKS-NH_2	Yes	2481.08
10	H-*WALQ*ACSDRFRNCPADEALCAKS-NH_2	Yes	2667.16

Table 3. Sequences of peptides. Peptide sequences with the amino acids used for cyclisation (glutamine, lysine and cysteine) underlined. Amino acids of the MTGase glutamine-donor substrate WALQRPH are in italics. The monoisotopic masses of the linear (1–8) and bicyclic (9, 10) peptides as well as whether they are cyclised by MTGase are indicated.

4.2.2. Catalytic activity of MTGase in cyclisation reactions

To quantify the catalytic activity of MTGase in cyclisation reactions we incubated the two substrate peptides **3** (H-WALQSGSGGGKS-NH_2) and **4** (H-ALQSGSGGGKS-NH_2) (90 μM) with different enzyme concentrations (1 nM to 6 μM) and quantified the extent of cyclisation by LC/MS and MALDI-TOF MS[71] (**Figure 27**). After incubation at room temperature for 5

hours, the peptides **3** and **4** were quantitatively cyclised at MTGase concentrations of 60 nM and 3 µM, respectively. Peptide **4** having a shorter glutamine-donor substrate sequence was cyclised by MTGase around 5-times slower than peptide **3** (225 and 45 catalysed cyclisation reactions per enzyme per hour for peptides **3** and **4**, respectively).

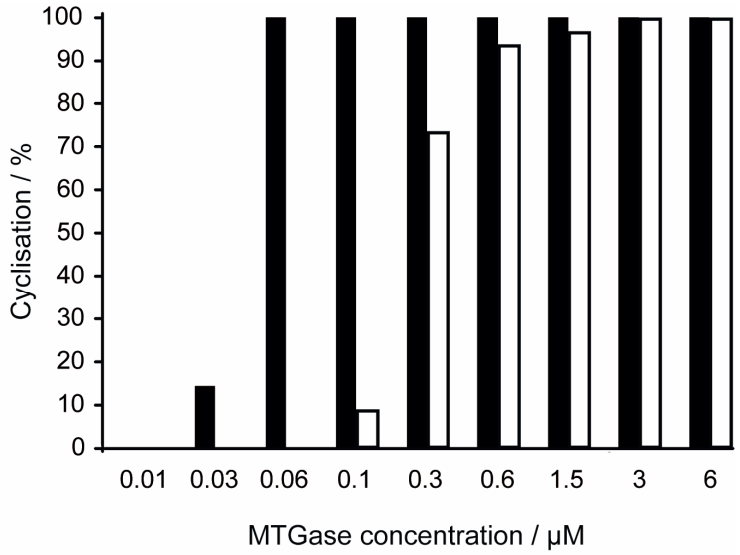

Figure 27. Activity of MTGase. Peptides 3 (black) and 4 (white) were incubated with different MTGase concentrations. The cyclisation efficiency indicated as a percent of cyclic peptide was determined by LC/MS.

4.2.3. Non-specific ligation of glutamine and lysine residues

To assess the non-specific ligation of random glutamine and lysine residues by MTGase, we incubated 5 µg of bovine serum albumin (BSA) with enzyme concentrations ranging from 60 nM to 6 µM and analysed the extent of protein cross-linking by SDS-PAGE. Although BSA has around 15 and 58 solvent accessible glutamine and lysine residues corresponding to concentrations of 56 µM and 215 µM respectively, no unspecific cross-linking of protein was observed (**Figure 28**).

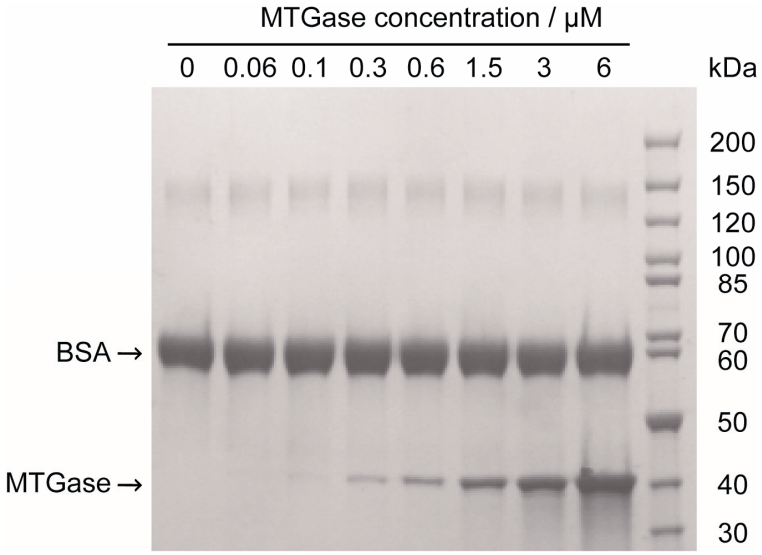

Figure 28. SDS-PAGE of bovine serum albumin (BSA; 5 µg) treated with different MTGase concentrations. No cross-linking of BSA through linkage of surface-exposed glutamine and lysine residues was observed.

4.2.4. Confirmation of cyclisation by tandem mass spectrometry

Because the mass change of 17 Da in all the MTGase catalysed reactions suggests, but does not directly prove the proposed cyclisation reaction, we tested whether a peptide presumably cyclised by MTGase could be re-opened with a protease. Stepwise treatment of peptide **6** (H-AL<u>Q</u>SGSRGGG<u>K</u>S-NH_2, expected mass: 1102.57 Da; measured mass: 1102.47 Da), which contains a trypsin cleavage site (Arg), with MTGase and trypsin yielded intermediate and final products with masses expected for cyclisation and re-linearisation reactions (expected masses: 1085.54 and 1103.56 Da; measured masses: 1085.43 and 1103.46 Da, respectively). Tandem mass spectrometry analysis of the re-linearised product confirmed the expected sequences at the new N and C termini (**Figure 29**).

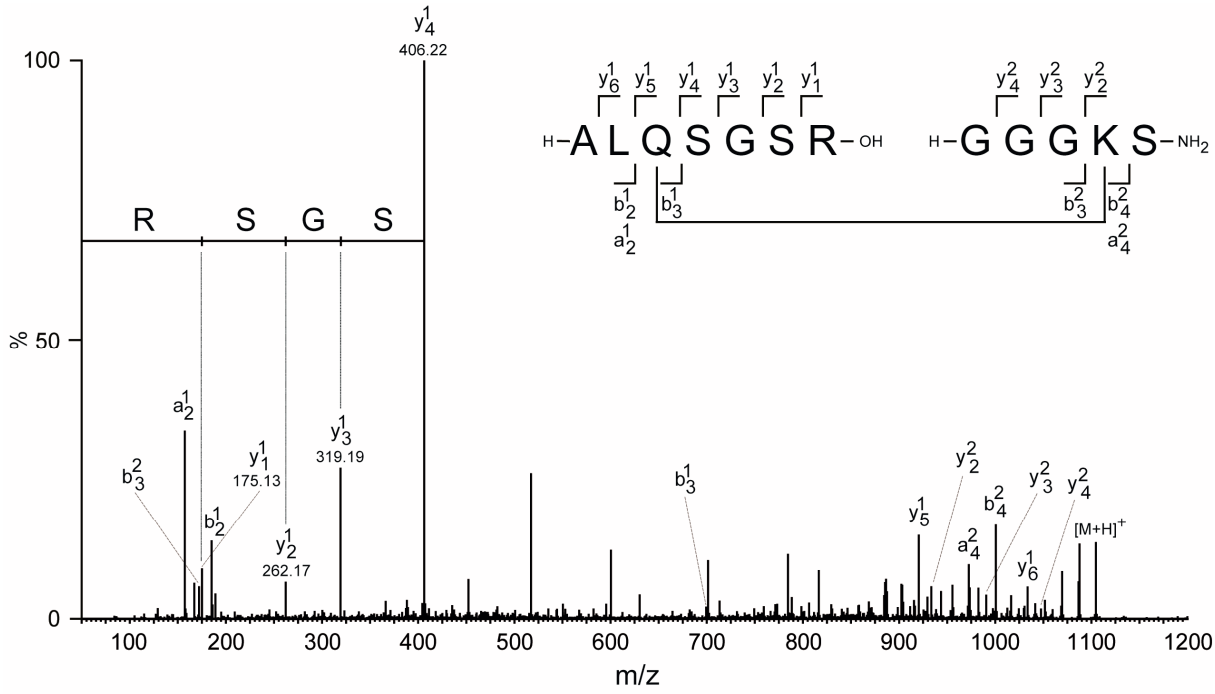

Figure 29. Trypsin cleavage of an MTGase cyclised peptide. The tandem mass spectrum obtained for MTGase and trypsin treated peptide **6** and a schematic drawing of the expected product are shown. The fragments obtained in tandem mass spectrometry are indicated in the schematically drawn re-linearised peptide **6**. Singly charged species, $[M+H]^+$, are shown in the spectrum. Non-assigned fragments are expected to have derived from internal fragmentation.

4.2.5. MTGase catalysed deamidation of glutamine

In a further control experiment, we incubated peptide **7** (H-WALQSGSGGGGS-NH_2; **Table 3**; 28 to 280 μM), which does not have a lysine-acceptor residue at the C-terminal side of the peptide, with MTGase (6 μM), expecting that it would not be cyclised. MALDI-TOF analysis showed reproducibly a product with a mass of 1062.43 Da which is 1 Dalton larger than the mass of the untreated peptide **7** (1061.42 Da; **Figure 30**). Such a mass shift is expected for a deamidation reaction in which the glutamine residue of peptide **7** is attacked by the active site thiol of MTGase and the intermediate is hydrolysed and a glutamate residue formed. The transformation of glutamine into glutamate was confirmed by sequencing of the reaction product using tandem mass spectrometry (data not shown). Deamidation of the glutamine residues in peptides **1-6** was not detected; this suggests that cyclisation through transamidation is much faster.

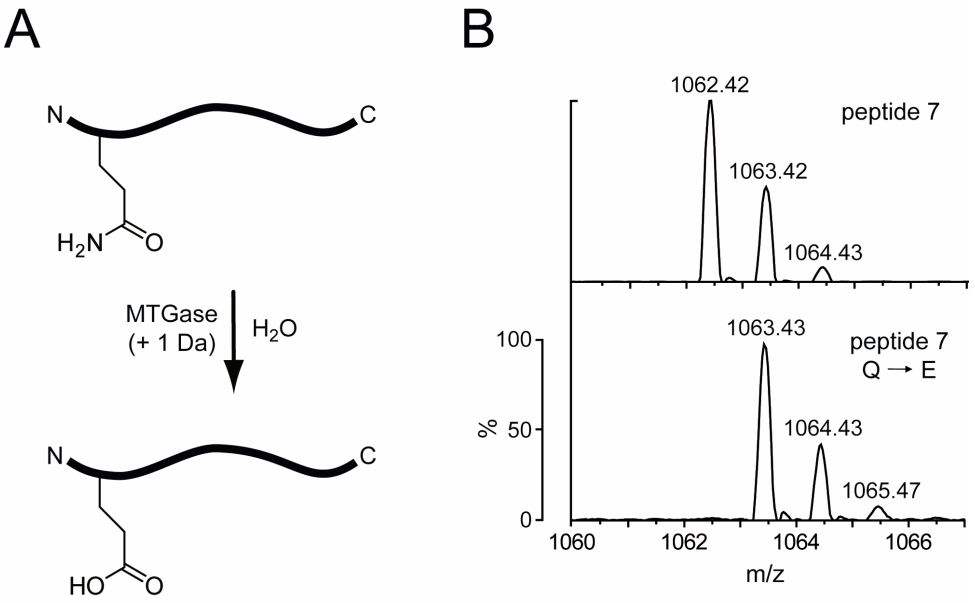

Figure 30. Deamidation of glutamine residue. (**A**) Schematic representation of the transformation of glutamine into glutamate residue in peptide **7** by MTGase. (**B**) Mass spectra of peptide **7** before (**upper panel**) and after (**lower panel**) incubation with MTGase. The gain of 1 Da in the molecular weight corresponds to the deamidation of the glutamine residue of peptide **7** into glutamate. Singly charged species, $[M+H]^+$, are shown in each spectrum.

4.3. General applicability of the method

To assess the general applicability of the MTGase-based approach to peptide cyclisation, we designed peptide **8** (H-*AL*QAYDGWLPWEIHVKS-NH_2), which has a different size (12 amino acids between the Gln and Lys residues) and a diverse amino acid content (Ala, Val, Leu, Ile, Gly, Asp, Glu, Tyr, Trp, His, Pro), and incubated it with MTGase. Analysis of the reaction products by reversed-phase HPLC showed a main product with a retention time different from that of peptide **8** and mass spectrometric analysis of this product showed a mass corresponding to the cyclised peptide **8** (fraction 4, **Figure 31**). Analysis of the minor peaks revealed that in this reaction not all peptide was cyclised (fraction 1) and that a small fraction of peptide appeared deamidated (fraction 2). Although the cyclisation was not as efficient (about 50 % of the peptide was cyclised) as with peptides **1**, **3**, **4** and **6**, this experiment showed that MTGase can catalyse the cyclisation of peptides with different sequences and lengths.

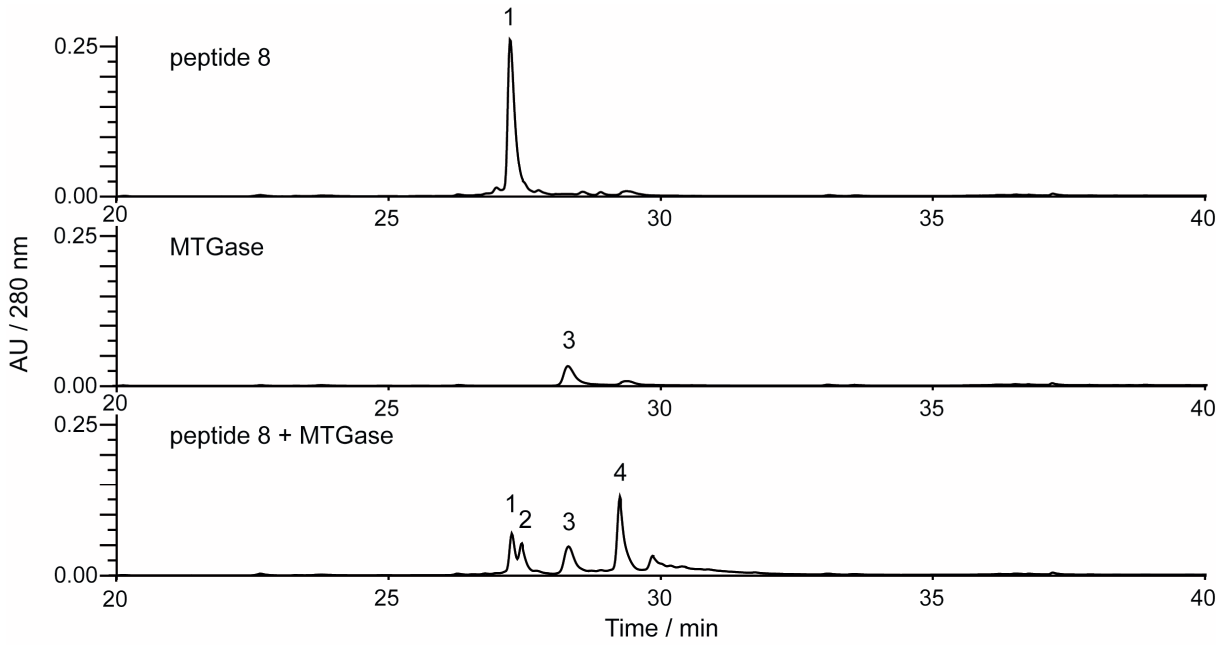

Figure 31. RP-HPLC analysis of peptide 8 before and after the reaction. Mass spectrometric analysis of fractions suggests the presence of the following molecules: linear peptide **8** (1), deamidated peptide **8** (2), MTGase (3) and cyclic peptide **8** (4).

4.4. Generation of tricyclic peptides

Finally, we tested whether a thiol-based chemical cyclisation reaction that was previously used to generate bicyclic peptides[3,55], could be combined with the enzymatic cyclisation to obtain a tricyclic peptide (**Figure 32A**). We synthesised the bicyclic peptide inhibitor of human plasma kallikrein PK15[3] with the exocyclic peptide appendices Ala-Leu-Gln-Ala at the N-terminus and Ala-Lys-Ser at the C-terminus by linking linear peptide **9** (H-ALQACSDRFRNCPADEALCAKS-NH_2; **Table 3**) via the three cysteine side chains to the small organic compound tris(bromomethyl)benzene. Incubation of the bicyclic peptide with MTGase yielded a single product with a mass of 2464.02 Dalton, which is 17 Dalton smaller than that of the bicyclic peptide, thus suggesting that the two ends had been linked in a transamidation reaction (**Figure 32B** and **C**). An identical experiment on peptide **10** (H-WALQACSDRFRNCPADEALCAKS-NH_2; **Table 3**) with an additional tryptophan residue at the N-terminus gave an equivalent result (data not shown).

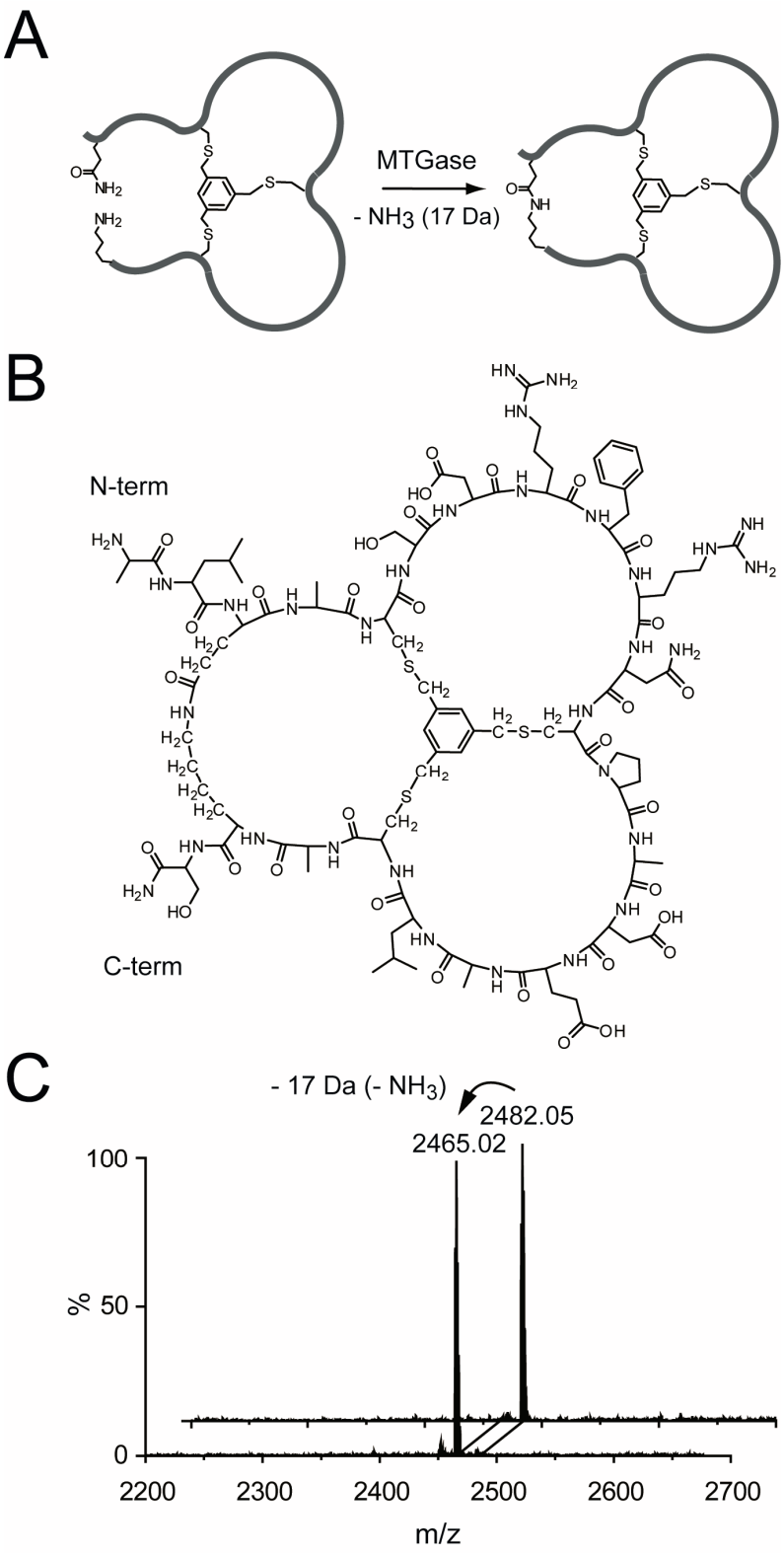

Figure 32. Generation of tricyclic peptides. (**A**) Schematic representation of the transglutamination reaction linking the terminal appendices of a bicyclic peptide. (**B**) Chemical structure of a tricyclic peptide obtained by subsequently treating peptide **9** with TBMB and with MTGase. (**C**) Mass spectra of the peptide **9**-TBMB conjugate before and after incubation with MTGase. Singly charged species, $[M+H]^+$, are shown.

To confirm the cyclisation of the third loop, bi- and tricyclic peptides **9** were treated with the leucyl aminopeptidase from *Aeromonas proteolytica* (EC 3.4.11.10), which cleaves amino acids from the N-terminus of easily accessible peptide chains. Mass spectrometric analysis of the reaction products showed that the unconjugated glutamine residue in the bicyclic peptide was degraded together with the other exocyclic N-terminal amino acids at small amounts of exopeptidase (70 ng and 352 ng) (**Figure 33A**). In contrast, the lysine-linked glutamine residue of the tricyclic peptide resisted higher concentrations of exopeptidase (**Figure 33B**). Together with the observed mass change of 17 Dalton upon incubation of the bicyclic peptide with MTGase, these results supported the tricyclic peptide configuration shown in **Figure 32B**. This experiment not only showed that the MTGase and thiol based cyclisation strategies can be combined to obtain multicyclic structures, but also that peptides with relatively constrained spacers between the glutamine-donor and lysine-acceptor residues (in this case a bicyclic peptide structure) can be cyclised with MTGase.

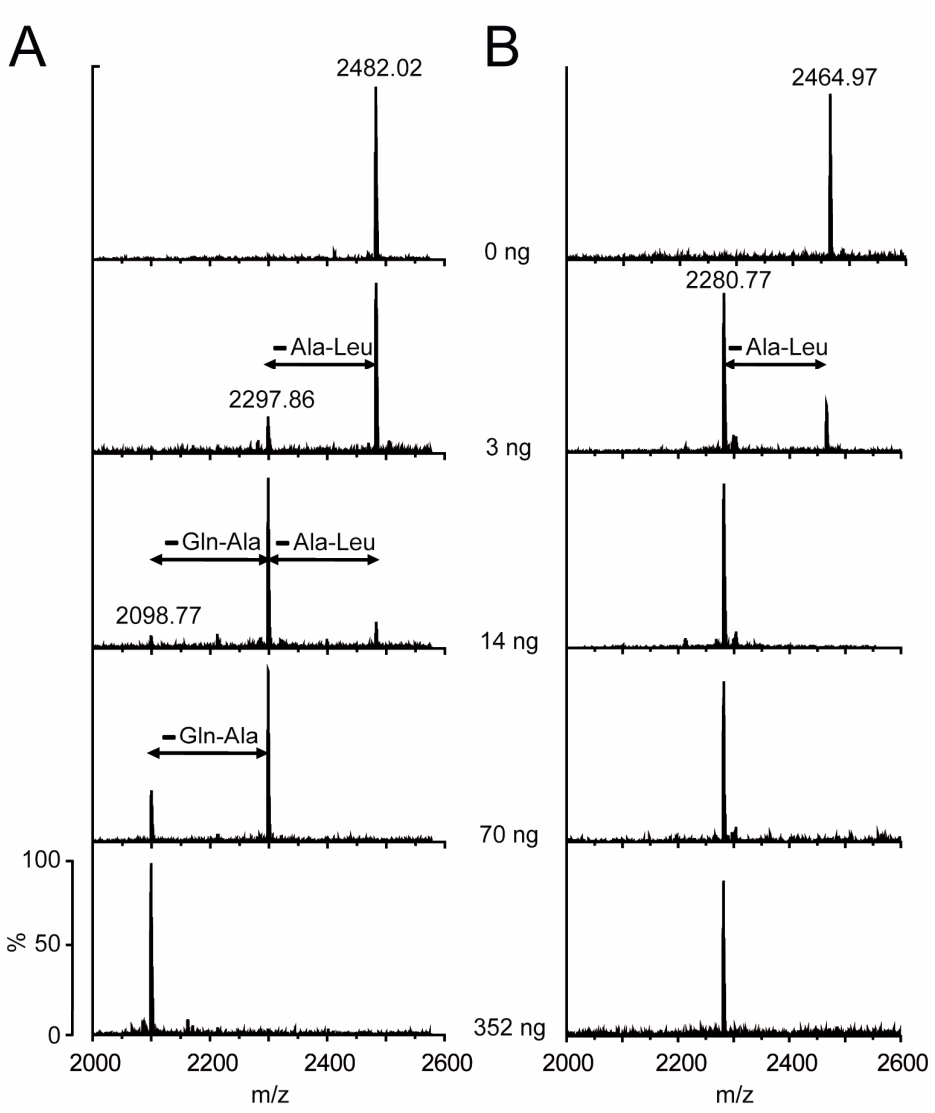

Figure 33. Susceptibility of bi- and tricyclic peptides to aminopeptidase cleavage. Mass spectrometric analysis of bicyclic (**A**) and tricyclic (**B**) peptides **9** treated with different amounts of leucyl aminopeptidase from *Aeromonas Proteolytica*. The mass differences correspond to the loss of exocyclic amino acids present at the N-terminus of the peptide. The quantity of aminopeptidase added to the reactions (in ng) is indicated. Singly charged species, $[M+H]^+$, are shown in each spectrum.

4.5. Conclusions and outlook

We have found that MTGase is a suitable tool for the cyclisation of unprotected peptides of varying lengths and amino acid sequences (except glutamine and lysine residues). The enzyme displays an optimal balance between selectivity and promiscuity: it ligates exclusively peptides that contain the two amino acids glutamine and lysine wherein the glutamine residue needs to be flanked N-terminally by the Ala-Leu dipeptide. At the same time, the enzyme has a promiscuous activity towards peptide substrates with varying amino acid sequences between the donor and acceptor amino acids. The microbial enzyme may be used as an alternative to thiol-based cyclisation reactions for the cyclisation of recombinantly expressed peptides such as genetically encoded peptide libraries. The MTGase cyclisation strategy may also be used in combination with thiol-based cyclisation reactions to generate constrained multicyclic peptide structures.

4.6. Materials and methods

Peptide synthesis. Peptides **1-7** with free N-terminus and amidated C-terminus were synthesised at a 0.05 mmol scale with standard Fmoc chemistry on an automated peptide synthesiser (Advanced ChemTech 348 Ω). After cleavage from the Rink-4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin with trifluoroacetic acid (TFA) (95 %), triisopropylsilane (2.5 %) and H_2O (2.5 %), the peptides were precipitated two times in chilled diethylether and the dried peptides were further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Vydac 218TP1022 C18 column (22 x 250 mm) (Hesperia, USA) with a solvent system of 99.9 % H_2O/0.1 % TFA and 94.9 % ACN/5 % H_2O/0.1 % TFA, and a flow rate of 20 ml/min. The peptides were lyophilised and dissolved in 100 mM Tris-HCl, pH 7.4 and 100 mM NaCl. Peptides **8-10** with free N-terminus and amidated C-terminus were synthesised on a 25 mg scale by solid-phase chemistry (JPT Peptide Technologies GmbH, Berlin, Germany or GL Biochem Ltd., Shanghai, China).

Peptide cyclisation. Peptides **1-7** (1 µM – 1 mM) in 10 mM Tris-HCl, pH 7.4 and 10 mM NaCl (reaction buffer) were tested for cyclisation by incubation with microbial transglutaminase of *Streptomyces mobaraensis* (38 kDa, 30 nM – 6 µM; Zedira, Darmstadt, Germany) for 5 hours at RT. Peptide **8** (25 µM) in reaction buffer was incubated with MTGase (1.5 µM) for 6 hours. Cyclisation was quantified by RP-HPLC on a Vydac 218TP54 C18 column (4.6 x 250 mm; Hesperia, USA) using a linear ACN/0.1 % TFA gradient (0-50 %) in water/0.1 % TFA over 30 minutes at a flow rate of 1 ml/min. The peaks were collected and analysed by MALDI-TOF mass spectrometry. Peptides **9** and **10** were reacted with 1,3,5-tris(bromomethyl)benzene (TBMB) by incubating peptide (800 µM) with 1 mM TBMB in 5 ml 66.5 % (v/v) NH_4HCO_3 (100 mM, pH 8), 28.5 % acetonitrile and 5 % DMSO for 1 hour at 30 °C and purified by RP-HPLC. The HPLC-purified bicyclic peptides **9** and **10** (150 µM) in 10 mM Tris-HCl, pH 7.4 and 10 mM NaCl were incubated with MTGase (1.5 µM) for 6 hours at RT.

Mass spectrometric analysis of peptides. The mass of peptides was determined by MALDI-TOF mass spectrometry (Axima-CFR plus, Kratos, Manchester, UK) as follows. Peptides in 0.1 % TFA/10-30 % acetonitrile in water or MTGase reaction buffer were mixed with the same volume of matrix solution (10 mg α-cyano-4-hydroxycinnamic acid (α-CHCA) in 1 ml

of 50 % acetonitrile/49.9 % H_2O/0.1 % TFA), 1 µl of the mixture was transferred to the carrier plate and mass measurements were performed in the positive ionisation mode. The extent of conversion of the peptides **3** and **4** into the cyclic products was quantified by LC/MS (Micromass ZQ 4000, Waters, Milford, USA). The reactions (20 µl) were mixed with 80 µl of 0.1 % formic acid and 0.02 % TFA in water and 50 µl was separated by RP-HPLC on a Vydac 218TP54 C18 column (4.6 x 250 mm) (Hesperia, USA) and subjected to mass analysis. The quantification was performed by extraction of the ion chromatogram (EIC) and by integration of the peaks. The results were confirmed by MALDI-TOF mass spectrometry[71]. The deamidation of glutamine to glutamate residue in peptide **7** was analysed by MALDI-TOF and the results were confirmed by sequencing using tandem mass spectrometry (MS/MS) (Micromass Q-Tof Ultima™, Waters, Milford, USA).

Calculation of the velocity of MTGase catalysed cyclisation reactions. The concentrations of MTGase needed to cyclise 50 % of the linear peptides in a given time were determined by extracting the values of **Figure 27**. The concentration of generated cyclic peptide was divided by the MTGase concentration and the reaction time to express the number of reactions catalysed per enzyme per hour.

Treatment of BSA with MTGase. 5 µg of bovine serum albumin (68 kDa; Applichem, Darmstadt, Germany) in 20 µl of MTGase reaction buffer (corresponding to a BSA concentration of 3.7 µM) was incubated with MTGase (concentrations ranging from 60 nM to 6 µM) for 5 hours at RT. Samples were analysed by SDS-PAGE.

Trypsin cleavage of MTGase cyclised peptide. Peptide **6** (100 µM) before and after MTGase treatment (1.5 µM) was incubated with 0.2 µM trypsin for 90 minutes at 37 °C in reaction buffer. The products before and after trypsin digestion were analysed by MALDI-TOF mass spectrometry. The MTGase and trypsin treated peptide **6** was additionally analysed by tandem mass spectrometry. Electrospray-ionisation MS data were acquired on a Q-Tof Ultima mass spectrometer (Waters, Milford, USA) fitted with a standard Z-spray ion source and operated in the positive ionisation mode. The sample was introduced into the mass spectrometer by infusion at a flow rate of 10 ml/min with a solution of ACN/H2O/HCOOH 50:49.8:0.2 (v:v:v). Single MS analysis were followed by MS/MS experiments on the selected precursor ions. The collision energy was manually adjusted for proper fragmentation. The multiply-charged spectrum was deconvoluted into a singly-charged axis using the Maxent3

tool. External calibration was carried out with a solution of phosphoric acid at 0.01 %. Data were processed using the MassLynx 4.1 software.

Aminopeptidase treatment of peptides. Peptide **9** (TBMB-conjugate), before (300 µM) and after (75 µM) MTGase treatment in reaction buffer, was incubated with different amounts of leucyl aminopeptidase from *Aeromonas proteolytica* (EC 3.4.11.10, A8200, Sigma-Aldrich, St. Louis, USA) (3 to 352 ng, corresponding to concentrations of about 50 nM to 6 µM) in a final volume of 2 µl for 30 min at RT. The mass of the peptides was determined by MALDI-TOF mass spectrometry.

5. Monitoring chemical reactions on phage

5.1. Introduction

The chemical modification of peptides or proteins on phage allows the generation of phage-encoded libraries containing building blocks beyond the 20 natural amino acids and hence the generation of larger and chemically more diverse molecule libraries[72]. Furthermore, different polypeptide architectures such as bicyclic peptides can be generated. Towards the establishment of new modification reactions, methods are required to monitor the modification of phage-displayed polypeptides in a qualitative and quantitative manner.

In this thesis, we generated bicyclic peptide ligands using a methodology in which peptide libraries displayed on phage were chemically cyclised prior to affinity selection (**chapters 2** and **3**). The reaction conditions for the peptide cyclisation were originally elaborated and optimised on peptides in solution being present at micromolar concentration. It was assumed that exactly the same conditions could be applied to modify peptides displayed on phage having picomolar concentrations. The method proved to work efficiently, as demonstrated by the selection of several potent bicyclic peptide inhibitors against different targets (as PK15 and UK18)[3,29]. However we previously noticed (**chapter 2**) that for some selections the extent of peptides cyclised with TBMB on phage appears to vary.

To date only two characterisation methods were developed to assess reactions directly on phage. The first method was reported in a patent by Winter and co-workers where they used Western blot and fluorescent densitometry analysis of phage pIII protein, isolated from M13 virion using SDS-PAGE[73]. In the second method, Derda and co-workers could distinguish individually modified and non-modified phage particles using a capture reagent (aminooxy-biotin) which undergoes covalent ligation with aldehydes displayed on phage particles. Upon incubation with streptavidin magnetic beads, biotinylated clones were captured and the remaining non-biotinylated clones quantified as plaque forming units[74]. Both of these methods can only detect if the desired product is formed but they cannot detect any side product. Moreover, these methods are restricted to the modifications in which molecules are ligated to the phage peptide.

In this work we developed a new method to follow any type of chemical reaction applied to phage-displayed peptides.

5.2. Strategy

We proposed a strategy in which peptide displayed on phage is subjected to a chemical reaction of choice, cleaved off by a protease and analysed by mass spectrometry. This strategy is based on studies in which phage peptide libraries were used for the mapping of protease substrates[75,76] or for detection of protease activity[77]. In the work of Ratnikov and co-workers, the substrate displayed on phage pIII protein was cleaved by the protease, identified from nucleotide sequencing, and the position of the scissile bond was determined by mass spectrometric analysis[76].

5.3. Mass spectrometric detection of chemical reaction products on phage

5.3.1. TBMB-modification on phage

The approach was tested using the cyclisation reaction based on TBMB[3] well-established in our laboratory. Phage displaying a linear peptide with three reactive cysteine residues (sequence: LCQLDCTWQC) were prepared. The peptide was linked to the phage protein 3 by an Arg-Ser-His-Ser linker containing a trypsin cleavage site (Arg). Phage were produced, purified and chemically modified with TBMB as described before (**subchapter 2.4**). An additional step of gel filtration was used in order to remove the residual polyethylene glycol used for purification as well as contaminant proteins co-purified with the phage. This step was essential for optimal MS analysis[78]. The modified peptide was then cleaved from the phage with trypsin and the remaining phage particle removed by filtration. The resulting peptide solution was concentrated and analysed by MALDI-TOF MS (**Figure 34**).

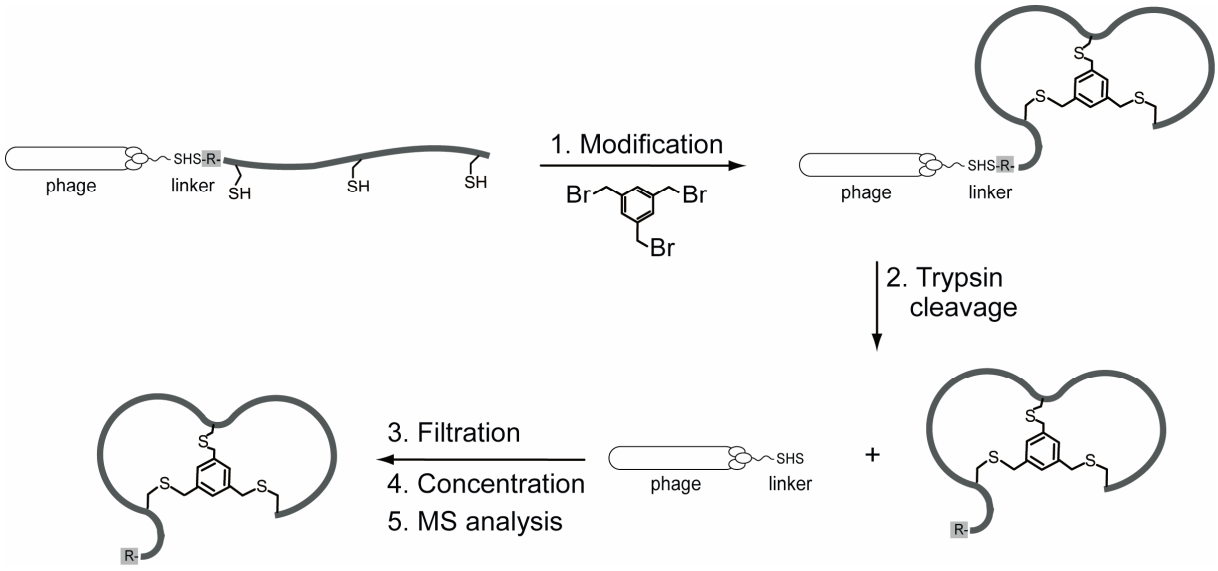

Figure 34. Strategy for the detection of chemical modifications of phage-displayed peptides. A linear peptide displayed on phage particle is modified (as an example: TBMB-cyclised), cleaved from the phage with trypsin and filtrated to remove the remaining phage particle. The filtrate is then concentrated and analysed by mass spectrometry. The trypsin cleavage site (Arg) is highlighted.

5.3.2. Analysis of reaction products

To assess the sensitivity of the mass spectrometric detection, we synthesised a peptide and modified it with TBMB (**Table 4**). The peptide was passed through a filter which was later used to remove phage particles. The peptide was detectable by MALDI-TOF MS. With an optimised filter the quantity of peptide that could be detected was as small as 0.01 pmol if the peptide was cyclised with TBMB and 0.1 pmol if the peptide was not modified and kept linear (**Table 4**).

Synthetic peptide	**MS detection limits**	
Amino acid sequence	**Linear**	**TBMB-conjugate**
H-LCQLDCTWQCR-NH_2	0.1 pmol	0.01 pmol

Table 4. Mass spectrometry limits of peptide detection. The corresponding phage-encoded peptide was chemically synthesised and TBMB-modified. The detection limits of the linear and TBMB-conjugate peptides were determined on a MALDI-TOF mass spectrometer. The peptide sequence as well as the minimum amount of detectable peptide are indicated.

0.1 pmol of peptide corresponds to 6 x 10^{10} peptides. In phage cultures, typically 10^{11} phage are produced per milliliter and at least 10^{10} phage can be purified from one milliliter. We therefore estimated that the mass spectrometric detection is sufficiently sensitive for our purpose. Phage were produced in 500 ml, modified with TBMB, purified and peptides cleaved off with an excess of trypsin. After filtration, the filtrate was lyophilised and resuspended in a smaller volume of ultrapure water. The solution was finally analysed by MALDI-TOF MS.

Displayed peptide	**Monoisotopic masses (Da), $[M+H]^+$**	
Amino acid sequence	**Linear peptide**	**TBMB-conjugate**
H-LCQLDCTWQCR-OH	1368.6	1482.6

Table 5. Masses of the expected phage-encoded peptides after trypsin cleavage. Amino acid sequence of the resulting peptide as well as the expected monoisotopic masses, $[M+H]^+$, of the linear and TBMB-conjugate are indicated.

The MS spectrum of the reaction product showed a single peak with the mass of the expected TBMB-modified peptide (**Table 5**; **Figure 35**). Remarkably, we did not observe the presence of the unmodified peptide. This result showed the applicability of the method to monitor peptide modification on phage. At the same time it confirmed the efficiency of the TBMB-modification on phage routinely used in our laboratory.

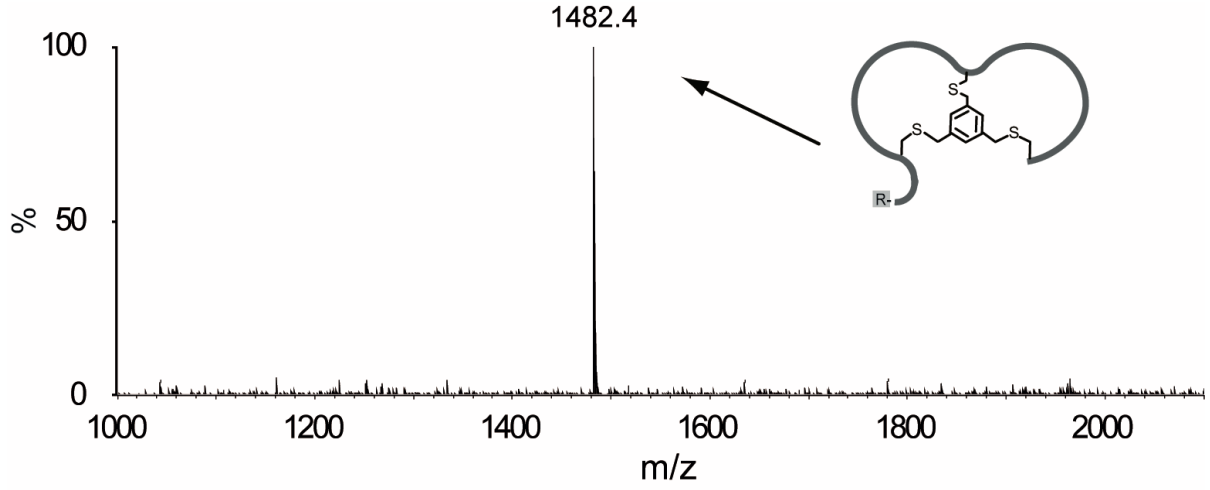

Figure 35. Mass spectrometric analysis of the TBMB-reaction product cleaved from the phage. The modified phage were treated with trypsin, filtrated and reaction product analysed by MALDI-TOF MS. Mass spectrum of the product after reaction with TBMB is shown. The schematic structure of the expected bicyclic peptide is represented. Singly charged species, $[M+H]^+$, are shown.

5.3.3. Application of the detection method to new chemical reactions on phage

The methodology was applied by Shiyu Chen, a PhD student who developed two new reagents for the cyclisation of cysteine-rich peptides. Both molecules contain three thiol-reactive groups with the following functional groups (**Figure 36**)[6]:

- 1,3,5-triacryloyl-1,3,5-triazinane (TATA):
  This molecule is composed of three acrylamide functional groups linked to a triazinane core. Its α,β-unsaturated carbonyl groups can react with thiols through an ionic thiolene reaction mechanism.

- N,N',N''-(benzene-1,3,5,-triyl)tris(2-bromoacetamide) (TBAB):
  This molecule contains three bromoacetamide functional groups, linked to a benzene ring, which are known to react specifically with thiols in aqueous buffer at moderate temperature.

As for the reaction with TBMB, these reactions were previously optimised and tested only on synthetic peptides and on peptide-fusion proteins. These molecules were applied to modify peptide libraries on phage, which were further used for selections. However, so far it was never proved that these reaction conditions result in a quantitative modification of peptides displayed on phage.

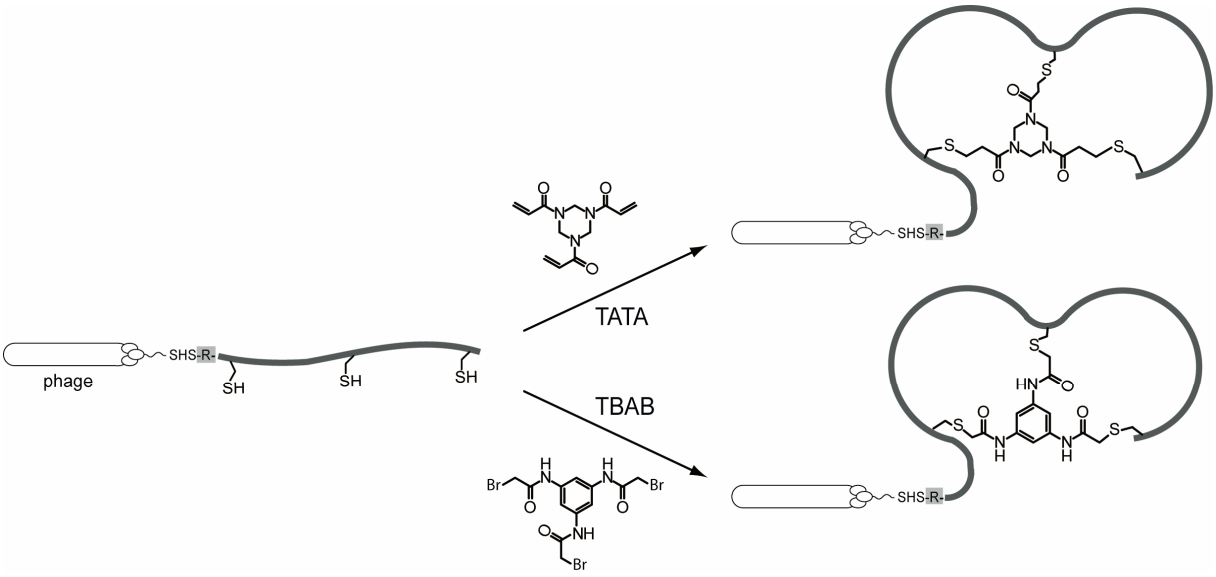

Figure 36. Characterisation of two different chemical modifications on phage. A phage-encoded peptide was cyclised via its three cysteine residues using the two scaffolds: TATA and TBAB. The resulting peptide products were characterised by mass spectrometry.

Phage were produced, modified, purified and the peptide products cleaved off by trypsin and analysed by mass spectrometry as described above. The two MS spectra showed a major peak with the mass of the expected modified peptide products (**Table 6**). Remarkably we did not observe the presence of the linear peptide (**Figure 37**).

Displayed peptide	Monoisotopic masses (Da), $[M+H]^+$		
Amino acid sequence	Linear peptide	TATA-conjugate	TBAB-conjugate
H-LCQLDCTWQCR-OH	1368.6	1617.6	1611.6

Table 6. Masses of the expected phage-encoded peptides after trypsin cleavage. Amino acid sequence of the resulting peptide as well as the expected monoisotopic masses, $[M+H]^+$, of the linear, TATA- and TBAB-conjugates are indicated.

However, several identical minor peaks were observed (**Figure 37A** and **B**) in the spectra. They probably correspond to fragments of phage capsid proteins generated by trypsin digestion or contaminants of bacteria that were co-purified with the phage.

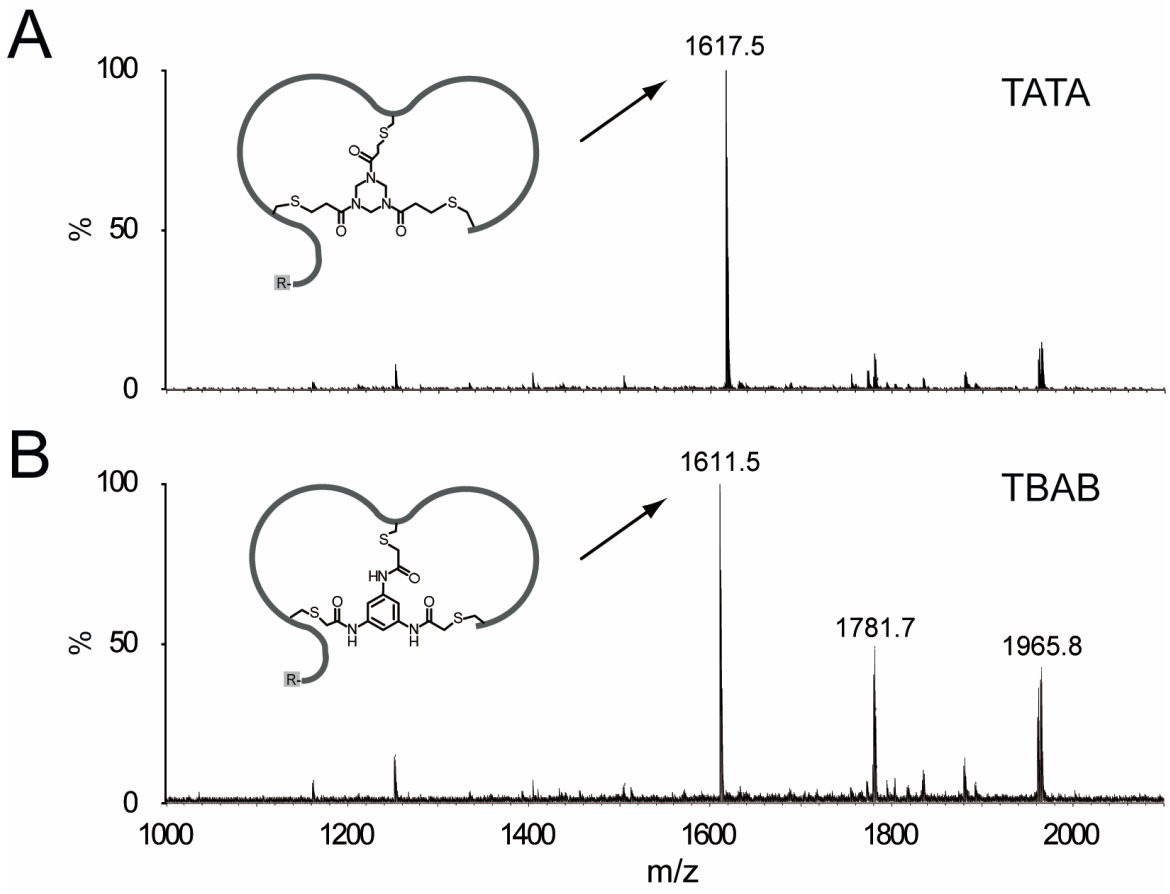

Figure 37. Mass spectrometric analysis of the reaction products cleaved from the phage. The modified phage were treated with trypsin, filtrated and reaction products analysed by MALDI-TOF MS. Mass spectra of the products, obtained after reactions with TATA (**A**) and TBAB (**B**), are shown. The schematic structures of the expected bicyclic peptides are represented. Singly charged species, $[M+H]^+$, are shown in each spectrum.

These results showed the general applicability of the method with different peptide modifications on phage. At the same time they confirmed the efficiency of the different chemical reactions applied on phage.

5.4. Conclusions and outlook

We developed a successful approach to characterise chemical reactions applied on phage surface. For the first time, the efficiency of the reactions can be validated in the context of intact M13 virion. We could in this way confirm the quantitative yield of the chemical reactions developed and routinely used in our laboratory to create diverse peptide libraries.

The method is simple and does not require sophisticated protocols and materials. The only requirement is the presence of a trypsin cleavage site in the linker between the phage and the displayed peptide. However, once such a clone is designed, it can be used to characterise similar chemical reactions.

This method may be applied to any kind of modifications on phage including enzymatic reactions. We are currently applying the enzymatic peptide cyclisation, developed in **chapter 4**, on phage surface.

In order to precisely quantify the efficiency of the reactions (modified versus unmodified products), this method could be combined with nano-LC/MS analysis, a technique where quantification of the product ions can be performed by extraction of the ion chromatograms and by integration of the peaks. This technique is more quantitative than MALDI-TOF MS.

Finally, we believe that the methodology described herein will find broad applications in the elaboration of new chemical reactions on phage which would certainly facilitate the development of new chemically modified phage libraries for the discovery of functional ligands.

5.5. Materials and methods

Phage production and modification. Bacterial cells of a monoclonal phage (clone isolated in phage selection against plasma kallikrein with library 3x3 and having the following peptide sequence: LCQLDCTWQCR) were inoculated in 500 ml of 2YT/chloramphenicol (30 μg/ml) medium to obtain an OD_{600} of 0.1. The culture was shaken (200 rpm) for 16 h at 30 °C. After 30 min of centrifugation at 8500 rpm and 4 °C, the phage were purified by precipitation with 0.2 volume of 20 % (w/v) polyethylene glycol 6000 (PEG6000), 2.5 M NaCl on ice and centrifugation at 8500 rpm for 30 min. PEG purified phage, typically 10^{11}-10^{12} t.u., were reduced in 20 ml of 20 mM NH_4HCO_3, 5 mM EDTA, pH 8.0 with 1 mM TCEP at 42 °C for 1 h. The concentration of TCEP was subsequently reduced by repetitive concentration and dilution steps with reaction buffer (20 mM NH_4HCO_3, 5 mM EDTA, pH 8.0, degassed) in a Vivaspin-20 filter (MWCO of 100'000, Sartorius-Stedim Biotech GmbH) as described in Heinis *et al*., 2009[3]. The volume of the phage solution was adjusted to 32 ml with reaction buffer. 8 ml of either 50 μM TBMB, 750 μM TATA or 200 μM TBAB in acetonitrile were added to obtain a final concentration of 10 μM for TBMB, 150 μM for TATA and 40 μM for TBAB. The reactions were incubated at 30 °C for 1 h before non-reacted TBMB, TATA or TBAB were removed by precipitation of the phage with 0.2 volume of 20 % (w/v) PEG6000, 2.5 M NaCl on ice and centrifugation at 4000 rpm for 30 minutes. The phage pellets were then dissolved in 1 ml of 10 mM NH_4HCO_3 at pH 8.0 and additionally purified by size exclusion chromatography using a HiPrep 16/60 Sephacryl S-500 high resolution column (GE Healthcare) and eluted in 10 mM NH_4HCO_3 (pH 8.0). After the purification, the phage were concentrated in a Vivaspin-20 filter (MWCO of 100'000, Sartorius-Stedim Biotech GmbH) to 0.5 ml and stored at -20 °C.

Trypsin digestion. The phage solution (500 μl in 10 mM NH_4HCO_3, pH 8.0), typically 10^{10}-10^{11} t.u. per ml, were incubated for 24 hours at 37 °C with 5 μl of trypsin endoproteinase at 1 mg/ml in water (modified, TPCK treated, MS Grade, Thermo Fisher Scientific Inc.) to obtain a final concentration of 10 μg/ml (430 nM).

Filtration. The phage solution (500 μl) was filtered using a Nanosep OMEGA filter (MWCO of 10'000, Pall Corporation, Port Washington, NY, USA). The filter was previously washed successively three times with 0.1 M NaOH and three times with ultrapure water. These washing steps are important in order to remove the trace amounts of preservative on the

membrane filter that could interfere with the following mass spectrometric analysis. The filtrate was then freeze-dried and dissolved in 10 µl of ultrapure water for mass spectrometric analysis.

Mass spectrometric analysis. The filtrated solution was analysed by MALDI-TOF mass spectrometry (Axima-CFR plus, Kratos) as follows. The solution was mixed with the same volume of matrix solution (10 mg α-cyano-4-hydroxycinnamic acid (α-CHCA) in 1 ml of ACN/H2O/TFA 50:49.9:0.1 % v/v/v). An aliquot (2 µl) of the mixture was transferred to the carrier plate and mass measurements were performed in the positive-ionisation mode.

Chemical synthesis of peptide H-LCQLDCTWQCR-NH_2. The linear and the TBMB-modified peptides having a free amine at the N-terminus and an amide at the C-terminus were synthesised and purified using the same protocol as described in **subchapter 2.4.**

6. Acknowledgements

I would like to sincerely thank:

- Prof. Dr. Eric Howard (University of Oklahoma) for sharing cDNA of human proMMP-2 and proMMP-9 cloned into pcDNA3 expression vectors.

- The members of the Lausanne protein expression core facility (PECF) for performing the expression of human MMP-2 and MMP-9.

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- Olivier Sallin for his contribution to the MMP project during his master thesis.

7. Appendices

7.1. Abbreviations

aa	Amino acid
ACN	Acetonitrile
Ala or A	Alanine
APMA	4-aminophenylmercuric acetate
APP	Amyloid precursor protein
APP-IP	APP-derived inhibitory peptide
Arg or R	Arginine
Asn or N	Asparagine
Asp or D	Aspartic acid
AU	Arbitrary unit
BSA	Bovine serum albumin
CD	Circular dichroism
cDNA	Complementary DNA
Cys or C	Cysteine
Da	Dalton
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
Gln or Q	Glutamine
Glu or E	Glutamic acid
Gly or G	Glycine
h	Hour
His or H	Histidine
hPK	Human plasma kallikrein
HRP	Horseradish peroxidase
IC_{50}	Half-maximal inhibitory concentration
Ile or I	Isoleucine
ITC	Isothermal titration calorimetry

kDa	Kilodalton
LC	Liquid chromatography
Leu or L	Leucine
Lys or K	Lysine
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time of flight
Met or M	Methionine
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
MMP	Matrix metalloproteinase
MMTS	Methyl methanethiosulfonate
MS	Mass spectrometry
ng	Nanogram
nm	Nanometer
nM	Nanomolar
O/N	Overnight
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
Phe or F	Phenylalanine
PK	Plasma kallikrein
pM	Picomolar
pmol	Picomole
Pro or P	Proline
RP-HPLC	Reversed phase high-performance liquid chromatography
rpm	Rotation per minute
RT	Room temperature
SAS-6	Spindle assembly abnormal 6 protein
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser or S	Serine
TATA	1,3,5-triacryloyl-1,3,5-triazinane
TBAB	N,N',N''-(benzene-1,3,5-triyl)tris(2-bromoacetamide)
TBMB	1,3,5-tris(bromomethyl)benzene
TCEP	Tris(2-carboxyethyl)phosphine

TFA	Trifluoroacetic acid
Thr or T	Threonine
TIMP	Tissue inhibitor of metalloproteinases
Trp or W	Tryptophan
t.u.	Transducing unit
Tyr or Y	Tyrosine
U2OS	Human osteosarcoma cells
µg	Microgram
µm	Micrometer
µM	Micromolar
uPA	Urokinase-type plasminogen activator
Val or V	Valine
v/v	Percentage by volume
w/v	Percentage by weight

7.2. Phage titres: MMP-2 and MMP-9 selections

The "input phage" used for every selection was always between 10^{10} and 10^{11} phage.

The number of phage isolated after every round of selection was determined (output phage). Negative controls (affinity selections performed without immobilised target on magnetic beads) were performed only for the first two experiments. Results are summarised in the following tables:

EXPERIMENT 1	Round 1	Round 2	Round 3
Control (No target)	$2.40 \cdot 10^5$	$4.20 \cdot 10^4$	$1.70 \cdot 10^5$
MMP-2	$4.50 \cdot 10^5$	$4.30 \cdot 10^5$	$8.00 \cdot 10^7$
MMP-9	$1.50 \cdot 10^6$	$6.00 \cdot 10^5$	$2.20 \cdot 10^7$

EXPERIMENT 2	Round 1	Round 2	Round 3
Control (No target)	$1.95 \cdot 10^4$	$1.20 \cdot 10^4$	$1.05 \cdot 10^4$
MMP-2	$3.15 \cdot 10^4$	$1.95 \cdot 10^6$	$2.85 \cdot 10^8$
MMP-9	$5.25 \cdot 10^4$	$4.50 \cdot 10^7$	$5.40 \cdot 10^8$

EXPERIMENT 3	Round 1	Round 2	Round 3
MMP-2 Library 6x6 TBMB-modified	$1.05 \cdot 10^4$	$1.50 \cdot 10^5$	$2.70 \cdot 10^8$
MMP-2 Library 6x6 Unmodified	$2.40 \cdot 10^4$	$2.10 \cdot 10^6$	$4.50 \cdot 10^8$
MMP-2 Libraries 3x3 and 4x4 mixed TBMB-modified	< 1500	$2.85 \cdot 10^4$	$1.80 \cdot 10^6$

EXPERIMENT 4	Round 1	Round 2	Round 3
MMP-2 Library 6x6 TBMB-modified	$6.00 \cdot 10^4$	$6.00 \cdot 10^4$	$2.40 \cdot 10^5$
MMP-2 Library 6x6 Unmodified	$2.10 \cdot 10^5$	$1.65 \cdot 10^5$	$2.85 \cdot 10^5$
hPK Library 6x6 TBMB-modified	$1.05 \cdot 10^4$	$1.35 \cdot 10^7$	$1.35 \cdot 10^8$
hPK Library 6x6 Unmodified	$4.50 \cdot 10^3$	$2.40 \cdot 10^6$	$2.55 \cdot 10^8$

EXPERIMENT 5	Round 1	Round 2	Round 3
MMP-2 Library 6x6 TBMB-modified	$1.10 \cdot 10^4$	$1.60 \cdot 10^5$	$2.60 \cdot 10^8$

7.3. Amino acid and DNA sequences of proMMP-2 used for selections

- Amino acid sequence of the protein. The colours correspond to the ones used in **Figure 4**.

MEALMARGALTGPLRALCLLGCLLSHAVAAPSPIIKFPGDVAPKTDKELAVQYLNTF
YGCPKESCNLFVLKDTLKKMQKFFGLPQTGDLDQNTIETMRKPRCGNPDVANYNFFP
RKPKWDKNQITYRIIGYTPDLDPETVDDAFARAFQVWSDVTPLRFSRIHDGEADIMIN
FGRWEHGDGYPFDGKDGLLAHAFAPGTGVGGDSHFDDDELWTLGEGQVVRVKYG
NADGEYCKFPFLFNGKEYNSCTDTGRSDGFLWCSTTYNFEKDGKYGFCPHEALFTM
GGNAEGQPCKFPFRFQGTSYDSCTTEGRTDGYRWCGTTEDYDRDKKYGFCPETAMS
TVGGNSEGAPCVFPFTFLGNKYESCTSAGRSDGKMWCATTANYDDDRKWGFCPDQ
GYSLFLVAAHEFGHAMGLEHSQDPGALMAPIYTYTKNFRLSQDDIKGIQELYGASPDI
DLGTGPTPTLGPVTPEICKQDIVFDGIAQIRGEIFFFKDRFIWRTVTPRDKPMGPLLVAT
FWPELPEKIDAVYEAPQEEKAVFFAGNEYWIYSASTLERGYPKPLTSLGLPPDVQRVD
AAFNWSKNKKTYIFAGDKFWRYNEVKKKMDPGFPKLIADAWNAIPDNLDAVVDLQ
GGGHSYFFKGAYYLKLENQSLKSVKFGSIKSDWLGC

- DNA sequence of the gene used to express proMMP-2.

ATGGAGGCGCTAATGGCCCGGGGCGCGCTCACGGGTCCCCTGAGGGCGCTCTGTCTCCTGGGCTGCCTGCTGAGC
CACGCCGTCGCCGCGCCGTCGCCCATCATCAAGTTCCCCGGCGATGTCGCCCCCAAAACGGACAAAGAGTTGGCA
GTGCAATACCTGAACACCTTCTATGGCTGCCCCAAGGAGAGCTGCAACCTGTTTGTGCTGAAGGACACACTAAAG
AAGATGCAGAAGTTCTTTGGACTGCCCCAGACAGGTGATCTTGACCAGAATACCATCGAGACCATGCGGAAGCCA
CGCTGCGGCAACCCAGATGTGGCCAACTACAACTTCTTCCCTCGCAAGCCCAAGTGGGACAAGAACCAGATCACA
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GGCCGCAGCGATGGCTTCCTCTGGTGCTCCACCACCTACAACTTTGAGAAGGATGGCAAGTACGGCTTCTGTCCC
CATGAAGCCCTGTTCACCATGGGCGGCAACGCTGAAGGACAGCCCTGCAAGTTTCCATTCCGCTTCCAGGGCACA
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TTCCCCTTCACTTTCCTGGGCAACAAATATGAGAGCTGCACCAGCGCCGGCCGCAGTGACGGAAAGATGTGGTGT
GCGACCACAGCCAACTACGATGACGACCGCAAGTGGGGCTTCTGCCCTGACCAAGGGTACAGCCTGTTCCTCGTG
GCAGCCCACGAGTTTGGCCACGCCATGGGGCTGGAGCACTCCCAAGACCCTGGGGCCCTGATGGCACCCATTTAC

ACCTACACCAAGAACTTCCGTCTGTCCCAGGATGACATCAAGGGCATTCAGGAGCTCTATGGGGCCTCTCCTGAC
ATTGACCTTGGCACCGGCCCCACCCCCACACTGGGCCCTGTCACTCCTGAGATCTGCAAACAGGACATTGTATTT
GATGGCATCGCTCAGATCCGTGGTGAGATCTTCTTCTTCAAGGACCGGTTCATTTGGCGGACTGTGACGCCACGT
GACAAGCCCATGGGGCCCCTGCTGGTGGCCACATTCTGGCCTGAGCTCCCGGAAAAGATTGATGCGGTATACGAG
GCCCCACAGGAGGAGAAGGCTGTGTTCTTTGCAGGGAATGAATACTGGATCTACTCAGCCAGCACCCTGGAGCGA
GGGTACCCCAAGCCACTGACCAGCCTGGGACTGCCCCCTGATGTCCAGCGAGTGGATGCCGCCTTTAACTGGAGC
AAAAACAAGAAGACATACATCTTTGCTGGAGACAAATTCTGGAGATACAATGAGGTGAAGAAGAAAATGGATCCT
GGCTTCCCCAAGCTCATCGCAGATGCCTGGAATGCCATCCCCGATAACCTGGATGCCGTCGTGGACCTGCAGGGC
GGCGGTCACAGCTACTTCTTCAAGGGTGCCTATTACCTGAAGCTGGAGAACCAAAGTCTGAAGAGCGTGAAGTTT
GGAAGCATCAAATCCGACTGGCTAGGCTGCTGA

7.4. APMA

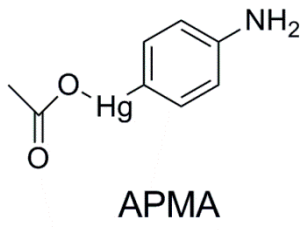

Figure A.1. Chemical structure of the mercurial compound 4-aminophenylmercuric acetate (APMA).

7.5. GM 6001

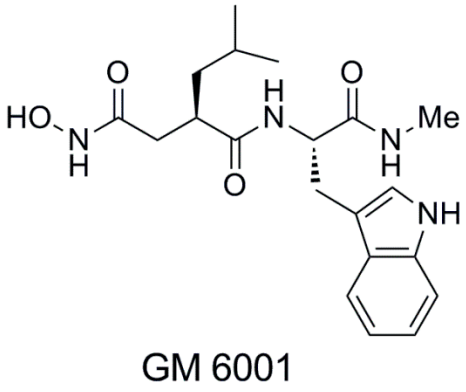

Figure A.2. Chemical structure of the potent broad-spectrum hydroxamate-based inhibitor of matrix metalloproteinases.

7.6. Amino acid and DNA sequences of proMMP-9 used for selections

- Amino acid sequence of the protein. The colours correspond to the ones used in **Figure 15**.

MSLWQPLVLVLLVLGCCFAAPRQRQSTLVLFPGDLRTNLTDRQLAEEYLYRYGYTR
VAEMRGESKSLGPALLLLQKQLSLPETGELDSATLKAMRTPRCGVPDLGRFQTFEGD
LKWHHHNITYWIQNYSEDLPRAVIDDAFARAFALWSAVTPLTFTRVYSRDADIVIQF
GVAEHGDGYPFDGKDGLLAHAFPPGPGIQGDAHFDDDELWSLGKGVVVPTRFGNAD
GAACHFPFIFEGRSYSACTTDGRSDGLPWCSTTANYDTDDRFGFCPSERLYTRDGNA
DGKPCQFPFIFQGQSYSACTTDGRSDGYRWCATTANYDRDKLFGFCPTRADSTVMG
GNSAGELCVFPFTFLGKEYSTCTSEGRGDGRLWCATTSNFDSDKKWGFCPDQGYSLF
LVAAHEFGHALGLDHSSVPEALMYPMYRFTEGPPLHKDDVNGIRHLYGPRPEPEPRP
PTTTTPQPTAPPTVCPTGPPTVHPSERPTAGPTGPPSAGPTGPPTAGPSTATTVPLSPVD
DACNVNIFDAIAEIGNQLYLFKDGKYWRFSEGRGSRPQGPFLIADKWPALPRKLDSVF
EEPLSKKLFFFSGRQVWVYTGASVLGPRRLDKLGLGADVAQVTGALRSGRGKMLLF
SGRRLWRFDVKAQMVDPRSASEVDRMFPGVPLDTHDVFQYREKAYFCQDRFYWRV
SSRSELNQVDQVGYVTYDILQCPED

- DNA sequence of the gene used to express proMMP-9.

ATGAGCCTCTGGCAGCCCCTGGTCCTGGTGCTCCTGGTGCTGGGCTGCTGCTTTGCTGCCCCCAGACAGCGCCAG
TCCACCCTTGTGCTCTTCCCTGGAGACCTGAGAACCAATCTCACCGACAGGCAGCTGGCAGAGGAATACCTGTAC
CGCTATGGTTACACTCGGGTGGCAGAGATGCGTGGAGAGTCGAAATCTCTGGGGCCTGCGCTGCTGCTTCTCCAG
AAGCAACTGTCCCTGCCCGAGACCGGTGAGCTGGATAGCGCCACGCTGAAGGCCATGCGAACCCCACGGTGCGGG
GTCCCAGACCTGGGCAGATTCCAAACCTTTGAGGGCGACCTCAAGTGGCACCACCACAACATCACCTATTGGATC
CAAAACTACTCGGAAGACTTGCCGCGGGCGGTGATTGACGACGCCTTTGCCCGCGCCTTCGCACTGTGGAGCGCG
GTGACGCCGCTCACCTTCACTCGCGTGTACAGCCGGGACGCAGACATCGTCATCCAGTTTGGTGTCGCGGAGCAC
GGAGACGGGTATCCCTTCGACGGGAAGGACGGGCTCCTGGCACACGCCTTTCCTCCTGGCCCCGGCATTCAGGGA
GACGCCCATTTCGACGATGACGAGTTGTGGTCCCTGGGCAAGGGCGTCGTGGTTCCAACTCGGTTTGGAAACGCA
GATGGCGCGGCCTGCCACTTCCCCTTCATCTTCGAGGGCCGCTCCTACTCTGCCTGCACCACCGACGGTCGCTCC
GACGGCTTGCCCTGGTGCAGTACCACGGCCAACTACGACACCGACGACCGGTTTGGCTTCTGCCCCAGCGAGAGA
CTCTACACCCGGGACGGCAATGCTGATGGGAAACCCTGCCAGTTTCCATTCATCTTCCAAGGCCAATCCTACTCC
GCCTGCACCACGGACGGTCGCTCCGACGGCTACCGCTGGTGCGCCACCACCGCCAACTACGACCGGGACAAGCTC
TTCGGCTTCTGCCCGACCCGAGCTGACTCGACGGTGATGGGGGGCAACTCGGCGGGGGAGCTGTGCGTCTTCCCC
TTCACTTTCCTGGGTAAGGAGTACTCGACCTGTACCAGCGAGGGCCGCGGAGATGGGCGCCTCTGGTGCGCTACC
ACCTCGAACTTTGACAGCGACAAGAAGTGGGGCTTCTGCCCGGACCAAGGATACAGTTTGTTCCTCGTGGCGGCG

CATGAGTTCGGCCACGCGCTGGGCTTAGATCATTCCTCAGTGCCGGAGGCGCTCATGTACCCTATGTACCGCTTC
ACTGAGGGGCCCCCCTTGCATAAGGACGACGTGAATGGCATCCGGCACCTCTATGGTCCTCGCCCTGAACCTGAG
CCACGGCCTCCAACCACCACCACACCGCAGCCCACGGCTCCCCCGACGGTCTGCCCCACCGGACCCCCCACTGTC
CACCCCTCAGAGCGCCCCACAGCTGGCCCCACAGGTCCCCCCTCAGCTGGCCCCACAGGTCCCCCCACTGCTGGC
CCTTCTACGGCCACTACTGTGCCTTTGAGTCCGGTGGACGATGCCTGCAACGTGAACATCTTCGACGCCATCGCG
GAGATTGGGAACCAGCTGTATTTGTTCAAGGATGGGAAGTACTGGCGATTCTCTGAGGGCAGGGGGAGCCGGCCG
CAGGGCCCCTTCCTTATCGCCGACAAGTGGCCCGCGCTGCCCCGCAAGCTGGACTCGGTCTTTGAGGAGCCGCTC
TCCAAGAAGCTTTTCTTCTTCTCTGGGCGCCAGGTGTGGGTGTACACAGGCGCGTCGGTGCTGGGCCCGAGGCGT
CTGGACAAGCTGGGCCTGGGAGCCGACGTGGCCCAGGTGACCGGGGCCCTCCGGAGTGGCAGGGGGAAGATGCTG
CTGTTCAGCGGGCGGCGCCTCTGGAGGTTCGACGTGAAGGCGCAGATGGTGGATCCCCGGAGCGCCAGCGAGGTG
GACCGGATGTTCCCCGGGGTGCCTTTGGACACGCACGACGTCTTCCAGTACCGAGAGAAAGCCTATTTCTGCCAG
GACCGCTTCTACTGGCGCGTGAGTTCCCGGAGTGAGTTGAACCAGGTGGACCAAGTGGGCTACGTGACCTATGAC
ATCCTGCAGTGCCCTGAGGACTAG

7.7. Amino acid sequences of SAS-6 proteins used for selections

The sequences of both proteins are shown below. An additional His6 tag is attached at the N-terminus of the proteins.

- **HsSAS-6** (HsN-6HR, residues 1-212)

MSQVLFHQLVPLQVKCKDCEERRVSIRMSIELQSVSNPVHRKDLVIRLTDDTDPFFLY
NLVISEEDFQSLKFQQGLLVDFLAFPQKFIDLLQQCTQEHAKEIPRFLLQLVSPAAILD
NSPAFLNVVETNPFKHLTHLSLKLLPGNDVEIKKFLAGCLKCSKEEKLSLMQSLDDAT
KQLDFTRKTLAEKKQELDKLRNEWASHTAALTNKHSQ

- **CrSAS-6** (CrN-6HR, residues 1-226)

MPLLLDDGDPKAQTGFDLSTATTLFWRPVPVHVKQQDREDVLEELTFRILTGVAKQN
HNLRILRIHISSDSDLFFLHTLEVSEEDFQSLKNDQGILVDFASFPGKIISLLEKCILAQP
GDSPRFQAVLTIRGGESVFKIVEINDFKQLPHITLAFRPGNDSVVKQFLAFRLSEVKGT
CHDLSDDLSRTRDDRDSMVAQLAQCRQQLAQLREQYDKHLLEVQAQAKT

7.8. Phage titres: SAS-6 selections

The "input phage" used for every selection was always between 10^{10} and 10^{11} phage.

The number of phage isolated after every round of selection was determined (output phage). Titres are summarised in the following table:

	Round 1	**Round 2**	**Round 3**
HsSAS-6 Libraries 4x4 and 6x6 mixed	$7.80 \cdot 10^4$	$6.75 \cdot 10^6$	$5.55 \cdot 10^7$
CrSAS-6 Libraries 4x4 and 6x6 mixed	$1.00 \cdot 10^3$	$2.55 \cdot 10^5$	$4.50 \cdot 10^4$

8. References

1. RENTERO, I. & HEINIS, C. SCREENING OF LARGE MOLECULE DIVERSITIES BY PHAGE DISPLAY. *CHIMIA (AARAU)* **65**, 843-845 (2011).
2. BARBAS, C.F., BURTON, D.R., SCOTT, J.K. & SILVERMAN, G.J. PHAGE DISPLAY: A LABORATORY MANUAL. (COLD SPRING HARBOR LABORATORY PRESS, NEW YORK, 2001).
3. HEINIS, C., RUTHERFORD, T., FREUND, S. & WINTER, G. PHAGE-ENCODED COMBINATORIAL CHEMICAL LIBRARIES BASED ON BICYCLIC PEPTIDES. *NAT CHEM BIOL* **5**, 502-507 (2009).
4. KITAGAWA, D.*, ET AL.* STRUCTURAL BASIS OF THE 9-FOLD SYMMETRY OF CENTRIOLES. *CELL* **144**, 364-375 (2011).
5. DRIGGERS, E.M., HALE, S.P., LEE, J. & TERRETT, N.K. THE EXPLORATION OF MACROCYCLES FOR DRUG DISCOVERY--AN UNDEREXPLOITED STRUCTURAL CLASS. *NAT REV DRUG DISCOV* **7**, 608-624 (2008).
6. CHEN, S., MORALES-SANFRUTOS, J., ANGELINI, A., CUTTING, B. & HEINIS, C. STRUCTURALLY DIVERSE CYCLISATION LINKERS IMPOSE DIFFERENT BACKBONE CONFORMATIONS IN BICYCLIC PEPTIDES. *CHEMBIOCHEM* **13**, 1032-1038 (2012).
7. BAERISWYL, V.*, ET AL.* BICYCLIC PEPTIDES WITH OPTIMIZED RING SIZE INHIBIT HUMAN PLASMA KALLIKREIN AND ITS ORTHOLOGUES WHILE SPARING PARALOGOUS PROTEASES. *CHEMMEDCHEM* (2012).
8. ANGELINI, A. & HEINIS, C. POST-TRANSLATIONAL MODIFICATION OF GENETICALLY ENCODED POLYPEPTIDE LIBRARIES. *CURR OPIN CHEM BIOL* **15**, 355-361 (2011).
9. CASCALES, L.*, ET AL.* IDENTIFICATION AND CHARACTERIZATION OF A NEW FAMILY OF CELL-PENETRATING PEPTIDES: CYCLIC CELL-PENETRATING PEPTIDES. *J BIOL CHEM* **286**, 36932-36943 (2011).
10. SMITH, G.P. FILAMENTOUS FUSION PHAGE: NOVEL EXPRESSION VECTORS THAT DISPLAY CLONED ANTIGENS ON THE VIRION SURFACE. *SCIENCE* **228**, 1315-1317 (1985).
11. PARMLEY, S.F. & SMITH, G.P. ANTIBODY-SELECTABLE FILAMENTOUS FD PHAGE VECTORS: AFFINITY PURIFICATION OF TARGET GENES. *GENE* **73**, 305-318 (1988).
12. DOWER, W.J. & MATTHEAKIS, L.C. IN VITRO SELECTION AS A POWERFUL TOOL FOR THE APPLIED EVOLUTION OF PROTEINS AND PEPTIDES. *CURR OPIN CHEM BIOL* **6**, 390-398 (2002).
13. O'NEIL, K.T.*, ET AL.* IDENTIFICATION OF NOVEL PEPTIDE ANTAGONISTS FOR GPIIB/IIIA FROM A CONFORMATIONALLY CONSTRAINED PHAGE PEPTIDE LIBRARY. *PROTEINS* **14**, 509-515 (1992).
14. MCLAFFERTY, M.A., KENT, R.B., LADNER, R.C. & MARKLAND, W. M13 BACTERIOPHAGE DISPLAYING DISULFIDE-CONSTRAINED MICROPROTEINS. *GENE* **128**, 29-36 (1993).
15. HEINIS, C. BICYCLIC PEPTIDE ANTAGONISTS DERIVED FROM GENETICALLY ENCODED COMBINATORIAL LIBRARIES. *CHIMIA (AARAU)* **65**, 677-679 (2011).
16. TURK, B. TARGETING PROTEASES: SUCCESSES, FAILURES AND FUTURE PROSPECTS. *NAT REV DRUG DISCOV* **5**, 785-799 (2006).
17. EGEBLAD, M. & WERB, Z. NEW FUNCTIONS FOR THE MATRIX METALLOPROTEINASES IN CANCER PROGRESSION. *NAT REV CANCER* **2**, 161-174 (2002).
18. HU, J., VAN DEN STEEN, P.E., SANG, Q.X. & OPDENAKKER, G. MATRIX METALLOPROTEINASE INHIBITORS AS THERAPY FOR INFLAMMATORY AND VASCULAR DISEASES. *NAT REV DRUG DISCOV* **6**, 480-498 (2007).
19. WOESSNER, F., J. & NAGASE, H. *MATRIX METALLOPROTEINASES AND TIMPS*, (2000).

20. PARKS, W.C., WILSON, C.L. & LOPEZ-BOADO, Y.S. MATRIX METALLOPROTEINASES AS MODULATORS OF INFLAMMATION AND INNATE IMMUNITY. *NAT REV IMMUNOL* **4**, 617-629 (2004).

21. OVERALL, C.M. & KLEIFELD, O. VALIDATING MATRIX METALLOPROTEINASES AS DRUG TARGETS AND ANTI-TARGETS FOR CANCER THERAPY. *NAT REV CANCER* **6**, 227-239 (2006).

22. LI, J.J. & JOHNSON, A.R. SELECTIVE MMP13 INHIBITORS. *MEDICINAL RESEARCH REVIEWS*, N/A-N/A (2010).

23. DEVY, L.*, ET AL.* SELECTIVE INHIBITION OF MATRIX METALLOPROTEINASE-14 BLOCKS TUMOR GROWTH, INVASION, AND ANGIOGENESIS. *CANCER RES* **69**, 1517-1526 (2009).

24. MIYAZAKI, K., HASEGAWA, M., FUNAHASHI, K. & UMEDA, M. A METALLOPROTEINASE INHIBITOR DOMAIN IN ALZHEIMER AMYLOID PROTEIN PRECURSOR. *NATURE* **362**, 839-841 (1993).

25. HIGASHI, S. & MIYAZAKI, K. IDENTIFICATION OF AMINO ACID RESIDUES OF THE MATRIX METALLOPROTEINASE-2 ESSENTIAL FOR ITS SELECTIVE INHIBITION BY B-AMYLOID PRECURSOR PROTEIN-DERIVED INHIBITOR. *JOURNAL OF BIOLOGICAL CHEMISTRY* **283**, 10068-10078 (2008).

26. HIGASHI, S. & MIYAZAKI, K. IDENTIFICATION OF A REGION OF BETA-AMYLOID PRECURSOR PROTEIN ESSENTIAL FOR ITS GELATINASE A INHIBITORY ACTIVITY. *J BIOL CHEM* **278**, 14020-14028 (2003).

27. KOIVUNEN, E.*, ET AL.* TUMOR TARGETING WITH A SELECTIVE GELATINASE INHIBITOR. *NAT BIOTECHNOL* **17**, 768-774 (1999).

28. HANAOKA, H.*, ET AL.* CHEMICAL DESIGN OF A RADIOLABELED GELATINASE INHIBITOR PEPTIDE FOR THE IMAGING OF GELATINASE ACTIVITY IN TUMORS. *NUCL MED BIOL* **34**, 503-510 (2007).

29. ANGELINI, A.*, ET AL.* BICYCLIC PEPTIDE INHIBITOR REVEALS LARGE CONTACT INTERFACE WITH A PROTEASE TARGET. *ACS CHEM BIOL* **7**, 817-821 (2012).

30. POLETTE, M., NAWROCKI-RABY, B., GILLES, C., CLAVEL, C. & BIREMBAUT, P. TUMOUR INVASION AND MATRIX METALLOPROTEINASES. *CRIT REV ONCOL HEMATOL* **49**, 179-186 (2004).

31. GALARDY, R.E.*, ET AL.* LOW MOLECULAR WEIGHT INHIBITORS IN CORNEAL ULCERATION. *ANN N Y ACAD SCI* **732**, 315-323 (1994).

32. SMITH, D.J., MAGGIO, E.T. & KENYON, G.L. SIMPLE ALKANETHIOL GROUPS FOR TEMPORARY BLOCKING OF SULFHYDRYL GROUPS OF ENZYMES. *BIOCHEMISTRY* **14**, 766-771 (1975).

33. BODWELL, J.E., HOLBROOK, N.J. & MUNCK, A. SULFHYDRYL-MODIFYING REAGENTS REVERSIBLY INHIBIT BINDING OF GLUCOCORTICOID-RECEPTOR COMPLEXES TO DNA-CELLULOSE. *BIOCHEMISTRY* **23**, 1392-1398 (1984).

34. KENYON, G.L. & BRUICE, T.W. NOVEL SULFHYDRYL REAGENTS. *METHODS ENZYMOL* **47**, 407-430 (1977).

35. STAUFFER, D.A. & KARLIN, A. ELECTROSTATIC POTENTIAL OF THE ACETYLCHOLINE BINDING SITES IN THE NICOTINIC RECEPTOR PROBED BY REACTIONS OF BINDING-SITE CYSTEINES WITH CHARGED METHANETHIOSULFONATES. *BIOCHEMISTRY* **33**, 6840-6849 (1994).

36. BAERISWYL, V. & HEINIS, C. UNPUBLISHED DATA. (2012).

37. CHEN, S., RENTERO, I., MORALES-SANFRUTOS, J., TOUATI, J. & HEINIS, C. SUBMITTED. (2012).

38. HASHIMOTO, H.*, ET AL.* STRUCTURAL BASIS FOR MATRIX METALLOPROTEINASE-2 (MMP-2)-SELECTIVE INHIBITORY ACTION OF BETA-AMYLOID PRECURSOR PROTEIN-DERIVED INHIBITOR. *J BIOL CHEM* **286**, 33236-33243 (2011).

39. CHEN, E.I.*, ET AL.* A UNIQUE SUBSTRATE RECOGNITION PROFILE FOR MATRIX METALLOPROTEINASE-2. *J BIOL CHEM* **277**, 4485-4491 (2002).

40. Backliwal, G., Hildinger, M., Hasija, V. & Wurm, F.M. High-density transfection with HEK-293 cells allows doubling of transient titers and removes need for a priori DNA complex formation with PEI. *Biotechnol Bioeng* **99**, 721-727 (2008).

41. Muller, N., Girard, P., Hacker, D.L., Jordan, M. & Wurm, F.M. Orbital shaker technology for the cultivation of mammalian cells in suspension. *Biotechnol Bioeng* **89**, 400-406 (2005).

42. Backliwal, G.*, et al.* Valproic acid: a viable alternative to sodium butyrate for enhancing protein expression in mammalian cell cultures. *Biotechnol Bioeng* **101**, 182-189 (2008).

43. O'Connell, J.P., Willenbrock, F., Docherty, A.J., Eaton, D. & Murphy, G. Analysis of the role of the COOH-terminal domain in the activation, proteolytic activity, and tissue inhibitor of metalloproteinase interactions of gelatinase B. *J Biol Chem* **269**, 14967-14973 (1994).

44. Clark, I.M. *Matrix metalloproteinase protocols*, (Humana Press, Totowa, N.J., 2001).

45. Akaji, K.*, et al.* Tetrafluoroboric acid as a useful deprotecting reagent in Fmoc-based solid-phase peptide syntheses (Fmoc = fluoren-9-ylmethoxycarbonyl). *Journal of the Chemical Society, Chemical Communications*, 288-290 (1990).

46. Cheng, Y. & Prusoff, W.H. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**, 3099-3108 (1973).

47. Bettencourt-Dias, M. & Glover, D.M. Centrosome biogenesis and function: centrosomics brings new understanding. *Nat Rev Mol Cell Bio* **8**, 451-463 (2007).

48. Badano, J.L., Teslovich, T.M. & Katsanis, N. The centrosome in human genetic disease. *Nat Rev Genet* **6**, 194-205 (2005).

49. Rodrigues-Martins, A., Riparbelli, M., Callaini, G., Glover, D.M. & Bettencourt-Dias, M. From centriole biogenesis to cellular function: centrioles are essential for cell division at critical developmental stages. *Cell Cycle* **7**, 11-16 (2008).

50. Gonczy, P. Towards a molecular architecture of centriole assembly. *Nat Rev Mol Cell Biol* **13**, 425-435 (2012).

51. Cottee, M.A., Raff, J.W., Lea, S.M. & Roque, H. SAS-6 oligomerization: the key to the centriole? *Nat Chem Biol* **7**, 650-653 (2011).

52. van Breugel, M.*, et al.* Structures of SAS-6 suggest its organization in centrioles. *Science* **331**, 1196-1199 (2011).

53. Vodnik, M., Zager, U., Strukelj, B. & Lunder, M. Phage display: selecting straws instead of a needle from a haystack. *Molecules* **16**, 790-817 (2011).

54. Andreu, D.*, et al.* Formation of disulfide bonds in synthetic peptides and proteins. *Methods Mol Biol* **35**, 91-169 (1994).

55. Timmerman, P., Beld, J., Puijk, W.C. & Meloen, R.H. Rapid and quantitative cyclization of multiple peptide loops onto synthetic scaffolds for structural mimicry of protein surfaces. *Chembiochem* **6**, 821-824 (2005).

56. Millward, S.W., Fiacco, S., Austin, R.J. & Roberts, R.W. Design of cyclic peptides that bind protein surfaces with antibody-like affinity. *ACS Chem Biol* **2**, 625-634 (2007).

57. Avrutina, O.*, et al.* Head-to-tail cyclized cystine-knot peptides by a combined recombinant and chemical route of synthesis. *Chembiochem* **9**, 33-37 (2008).

58. Wood, S.J. & Wetzel, R. Novel cyclization chemistry especially suited for biologically derived, unprotected peptides. *Int J Pept Protein Res* **39**, 533-539 (1992).

59. MILLWARD, S.W., TAKAHASHI, T.T. & ROBERTS, R.W. A GENERAL ROUTE FOR POST-TRANSLATIONAL CYCLIZATION OF MRNA DISPLAY LIBRARIES. *J AM CHEM SOC* **127**, 14142-14143 (2005).

60. JACKSON, D.Y., BURNIER, J. & WELLS, J.A. ENZYMATIC CYCLIZATION OF LINEAR PEPTIDE ESTERS USING SUBTILIGASE. *J AM CHEM SOC* **117**, 819-820 (1995).

61. BORDUSA, F. ENZYMES FOR PEPTIDE CYCLIZATION. *CHEMBIOCHEM* **2**, 405-409 (2001).

62. XU, M.Q. & EVANS, T.C., JR. INTEIN-MEDIATED LIGATION AND CYCLIZATION OF EXPRESSED PROTEINS. *METHODS* **24**, 257-277 (2001).

63. TSUKIJI, S. & NAGAMUNE, T. SORTASE-MEDIATED LIGATION: A GIFT FROM GRAM-POSITIVE BACTERIA TO PROTEIN ENGINEERING. *CHEMBIOCHEM* **10**, 787-798 (2009).

64. BENINATI, S. & PIACENTINI, M. THE TRANSGLUTAMINASE FAMILY: AN OVERVIEW: MINIREVIEW ARTICLE. *AMINO ACIDS* **26**, 367-372 (2004).

65. YOKOYAMA, K., NIO, N. & KIKUCHI, Y. PROPERTIES AND APPLICATIONS OF MICROBIAL TRANSGLUTAMINASE. *APPL MICROBIOL BIOTECHNOL* **64**, 447-454 (2004).

66. JOSTEN, A., HAALCK, L., SPENER, F. & MEUSEL, M. USE OF MICROBIAL TRANSGLUTAMINASE FOR THE ENZYMATIC BIOTINYLATION OF ANTIBODIES. *J IMMUNOL METHODS* **240**, 47-54 (2000).

67. MINDT, T.L., *ET AL.* MODIFICATION OF DIFFERENT IGG1 ANTIBODIES VIA GLUTAMINE AND LYSINE USING BACTERIAL AND HUMAN TISSUE TRANSGLUTAMINASE. *BIOCONJUG CHEM* **19**, 271-278 (2008).

68. TAKAZAWA, T., KAMIYA, N., UEDA, H. & NAGAMUNE, T. ENZYMATIC LABELING OF A SINGLE CHAIN VARIABLE FRAGMENT OF AN ANTIBODY WITH ALKALINE PHOSPHATASE BY MICROBIAL TRANSGLUTAMINASE. *BIOTECHNOL BIOENG* **86**, 399-404 (2004).

69. CABRAS, T., *ET AL.* HPLC-MS CHARACTERIZATION OF CYCLO-STATHERIN Q-37, A SPECIFIC CYCLIZATION PRODUCT OF HUMAN SALIVARY STATHERIN GENERATED BY TRANSGLUTAMINASE 2. *J SEP SCI* **29**, 2600-2608 (2006).

70. SUGIMURA, Y., YOKOYAMA, K., NIO, N., MAKI, M. & HITOMI, K. IDENTIFICATION OF PREFERRED SUBSTRATE SEQUENCES OF MICROBIAL TRANSGLUTAMINASE FROM STREPTOMYCES MOBARAENSIS USING A PHAGE-DISPLAYED PEPTIDE LIBRARY. *ARCH BIOCHEM BIOPHYS* **477**, 379-383 (2008).

71. GREIS, K.D., *ET AL.* MALDI-TOF MS AS A LABEL-FREE APPROACH TO RAPID INHIBITOR SCREENING. *J AM SOC MASS SPECTROM* **17**, 815-822 (2006).

72. NG, S., JAFARI, M.R. & DERDA, R. BACTERIOPHAGES AND VIRUSES AS A SUPPORT FOR ORGANIC SYNTHESIS AND COMBINATORIAL CHEMISTRY. *ACS CHEMICAL BIOLOGY* **7**, 123-138 (2012).

73. JESPERS, L.S.A.T., WINTER, G.P., BONNERT, T.P. & SIMON, T.M. BACTERIOPHAGE LIBRARY DISPLAYING IMMUNOGLOBULIN REPERTOIRES WITH A CHEMICAL MOIETY COVALENTLY BOUND WITHIN THE BINDING SITE: PRODUCTION AND SELECTION THEREOF. (1995).

74. NG, S., JAFARI, M.R., MATOCHKO, W.L. & DERDA, R. QUANTITATIVE SYNTHESIS OF GENETICALLY ENCODED GLYCOPEPTIDE LIBRARIES DISPLAYED ON M13 PHAGE. *ACS CHEM BIOL* (2012).

75. SCHOLLE, M.D., *ET AL.* MAPPING PROTEASE SUBSTRATES BY USING A BIOTINYLATED PHAGE SUBSTRATE LIBRARY. *CHEMBIOCHEM* **7**, 834-838 (2006).

76. RATNIKOV, B., CIEPLAK, P. & SMITH, J.W. HIGH THROUGHPUT SUBSTRATE PHAGE DISPLAY FOR PROTEASE PROFILING. *METHODS MOL BIOL* **539**, 93-114 (2009).

77. CAPEK, P., KIRKCONNELL, K.S. & DICKERSON, T.J. A BACTERIOPHAGE-BASED PLATFORM FOR RAPID TRACE DETECTION OF PROTEASES. *J AM CHEM SOC* **132**, 13126-13128 (2010).

78. LAVIGNE, R., CEYSSENS, P.J. & ROBBEN, J. PHAGE PROTEOMICS: APPLICATIONS OF MASS SPECTROMETRY. *METHODS MOL BIOL* **502**, 239-251 (2009).

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2009	**Master's of Science in Molecular and Biological Chemistry** EPFL
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RESEARCH EXPERIENCE:

03/2009 – 10/2012	**Doctoral Research in Chemical Biology** Laboratory of Therapeutic Peptides and Proteins, EPFL Advisor: Prof. Christian Heinis *"Phage selection of bicyclic peptide ligands and development of a new peptide cyclisation method."*
09/2008 – 02/2009	**Master Thesis in "Drug Discovery"** MRC, Centre for Protein Engineering, Cambridge (UK) Advisor: Prof. Sir Alan Fersht *"Screening and biophysical characterization of small-molecule stabilizers of WT-p53, a protein involved in cancer."*
03/2008 – 06/2008	**Master Semester Project in Chemical Biology** Laboratory of Protein Engineering, EPFL Advisor: Prof. Kai Johnsson *"Deletion study on the activity of Microbial Transglutaminase."*

AWARDS:

2010	EPFL best teaching assistant in Chemistry during doctoral studies
2007 – 2008	Excellence scholarship awarded by EPFL for marks achieved during bachelor studies

PUBLICATIONS:

Touati, J., Angelini, A., Hinner, M. J., and Heinis, C. (2011) Enzymatic cyclisation of peptides with a transglutaminase, *Chembiochem 12*, 38-42.

Angelini, A., Cendron, L., Chen, S., **Touati, J.**, Winter, G., Zanotti, G., and Heinis, C. (2012) Bicyclic Peptide inhibitor reveals large contact interface with a protease target, *ACS Chem Biol 7*, 817-821.

Chen, S., Rentero, I., Morales-Sanfrutos, J., **Touati, J.**, and Heinis, C. (2012) Phage selection of bicyclic peptides formed by two disulfide bridges. *Submitted*.

PATENT:

Heinis, C., **Touati, J.**, and Winter, G. P. **(2010)** Structured polycyclic peptide. (WO 2010089117)