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Methods for high-throughput synthesis and screening of peptide libraries

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Abstract

To address unmet medical needs, pharmaceutical researchers are on a constant hunt for new molecules that can bind to clinically relevant targets. To find initial hits that can serve as starting points for further development, a significant number of drug discovery campaigns start with the high-throughput screening (HTS) of collections featuring thousands or millions of compounds. Therefore, there is a high demand for novel, large and chemically diverse chemical libraries. These compound collections can be assembled or generated through various means, however, if each library compound has to be synthesized and purified individually, creating libraries of sufficient size and chemical diversity can prove extremely time-consuming and costly. As peptides are a promising class of compounds for the modulation of several challenging disease targets, our group has aimed to address this challenge by taking advantage of the robustness and high synthetic throughput of automated, combinatorial solid-phase peptide synthesis (SPPS) and subsequent solution-phase diversification reactions to generate libraries of thousands of cyclic or linear peptides. In our direct-to-biology approach, each step of the synthetic procedure must be precisely optimized so that the final library compounds will be of sufficiently high quality, therefore purification steps can be omitted and the crude products can be screened directly.

The goal of my PhD research was to develop new methods for the efficient synthesis of such high-quality peptide libraries that can overcome drawbacks presented by the previously known procedures, and demonstrate the viability of such methods by screening libraries against model targets to identify potent binders.

In my first project, I aimed to modify the SPPS method used in our group to obtain pure peptides with free thiol groups. Even though a solid phase synthesis strategy based on a disulfide linker, followed by cyclative peptide release enabled the separation of the side chain deprotection step and the release of the peptide from the synthesis beads, its efficiency was found to be highly dependent on the specific peptide sequence, and the resulting cyclic disulfides had to be reduced before the thiols could be further diversified with electrophiles. By screening various conditions, I was able to identify 1,4-butanedithiol (BDT) as a potent reducing and peptide release agent, that has enabled us to directly obtain reduced mono- or dithiol peptides in high crude quality and yield. Since this reagent is also sufficiently volatile, it can be removed by evaporation, and the remaining thiol peptides can be easily diversified with electrophiles without interference from residues of the reducing agent.

In my second project, I developed a synthetic route for the diversification of the aminothiol linking element that anchors the peptide via a disulfide linker to the solid support during SPPS. With the new building block introduced, the available peptide backbone diversity has been

significantly expanded. To illustrate the utility of this tool, a peptide library of 2 688 macrocycles was synthesized, and novel inhibitors of the model target thrombin have been identified with nanomolar affinity.

In my third project, I applied the synthesis strategy based on a disulfide linker solid phase to generate a large library of small molecules. I synthesized and screened a library of 24 288 peptides that included only three randomized building blocks. Even with a limited possibility of favorable binding interactions, I was able to identify nanomolar inhibitors of thrombin, further highlighting the utility of our combinatorial synthesis approach based on combining solid phase and solution phase synthesis, and direct-to-biology screening.

Keywords

Peptide libraries, synthesis of thiol peptides, late-stage functionalization, direct-to-biology high-throughput chemistry, thrombin

Résumé

Pour répondre aux besoins médicaux non satisfaits, les chercheurs pharmaceutiques sont constamment à la recherche de nouvelles molécules capables de se lier à des cibles cliniquement pertinentes. Pour trouver les premiers résultats qui peuvent servir de points de départ à un développement ultérieur, un grand nombre de campagnes de découverte de médicaments commencent par le criblage à haut débit de collections comprenant des milliers ou des millions de composés. Il existe donc une forte demande de nouvelles chimiothèques de grande taille et chimiquement diversifiées. Ces bibliothèques de composés peuvent être assemblées ou générées par divers moyens, mais si chaque composé doit être synthétisé et purifié individuellement, la création de bibliothèques d'une taille et d'une diversité chimique suffisantes peut s'avérer extrêmement longue et coûteuse. Les peptides étant une classe de composés prometteurs pour la modulation de plusieurs cibles pathologiques difficiles, notre groupe a cherché à relever ce défi en tirant parti de la robustesse et du haut débit de la synthèse peptidique combinatoire automatisée en phase solide (SPPS) et des réactions de diversification ultérieures en phase de solution pour générer des bibliothèques de milliers de peptides cycliques ou linéaires. Dans notre approche directe de la biologie, chaque étape de la procédure synthétique doit être précisément optimisée pour que les composés de la bibliothèque finale soient d'une qualité suffisamment élevée, de sorte que les étapes de purification puissent être omises et que les produits bruts puissent être criblés directement.

L'objectif de ma recherche doctorale était de développer de nouvelles méthodes pour la synthèse de ces bibliothèques de peptides de haute qualité qui peuvent surmonter les inconvénients présentés par les procédures précédemment connues, et de démontrer la viabilité de ces méthodes en criblant les bibliothèques contre des cibles modèles afin d'identifier des liants puissants.

Dans mon premier projet, j'ai cherché à modifier la méthode SPPS utilisée dans notre groupe pour obtenir des peptides purs avec des groupes thiols libres. Bien qu'une stratégie de synthèse en phase solide basée sur un lien disulfure, suivie d'une libération cyclative du peptide, ait permis de séparer l'étape de déprotection de la chaîne latérale et la libération du peptide des billes de synthèse, son efficacité s'est avérée fortement dépendante de la séquence peptidique spécifique, et les disulfures cycliques résultants ont dû être réduits avant que les thiols ne puissent être diversifiés davantage avec des électrophiles. En examinant diverses conditions, j'ai pu identifier le 1,4-butanedithiol (BDT) comme un puissant agent de réduction et de libération des peptides, qui nous a permis d'obtenir directement des peptides mono- ou dithiols réduits, avec une qualité et un rendement bruts élevés. Comme ce réactif est également suffisamment volatil, il peut être éliminé par évaporation, et les peptides thiol

restants peuvent être facilement diversifiés avec des électrophiles sans interférence des résidus de l'agent réducteur.

Dans mon second projet, j'ai développé une voie synthétique pour la diversification de l'élément de liaison aminothiol qui ancre le peptide via une liaison disulfure au support solide pendant la SPPS. Avec le nouveau bloc de construction introduit, la diversité du squelette peptidique disponible a été considérablement élargie. Pour illustrer l'utilité de cet outil, une bibliothèque peptidique de 2 688 macrocycles a été synthétisée et de nouveaux inhibiteurs de la cible modèle qu'est la thrombine ont été identifiés avec une affinité nanomolaire.

Dans mon troisième projet, j'ai appliqué la stratégie de synthèse basée sur un lien disulfure en phase solide pour générer une grande bibliothèque de petites molécules. J'ai synthétisé et criblé une bibliothèque de 24 288 peptides qui comprenaient seulement trois blocs de construction aléatoires. Même avec une possibilité limitée d'interactions de liaison favorables, j'ai pu identifier des inhibiteurs nanomolaires de la thrombine, ce qui souligne encore l'utilité de notre approche de synthèse combinatoire basée sur la combinaison de la synthèse en phase solide et en phase de solution, et du criblage direct en biologie.

Mots-clés

Bibliothèques de peptides, synthèse de peptides thiolés, fonctionnalisation tardive, chimie à haut débit directe appliqué à la biologie, thrombine

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Abbreviations

Acm	acetamidomethyl
ADE	acoustic droplet ejection
AMC	7-amino-4-methyl coumarin
ASMS	affinity selection mass spectrometry
BDT	1,4-butanedithiol
b-ME	2-mercaptoethanol
Boc	tert-butyloxycarbonyl
BSA	bovine serum albumin
CAPA	chloroalkane penetration assay
CM	ChemMatrix
clogP	calculated water/1-octanol partition coefficient
COC	cyclic olefin copolymer
CuAAC	copper-catalyzed azide–alkyne cycloaddition
D2B-HTC	direct-to-biology high-throughput chemistry
DCM	dichloromethane
DDE	*N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl)
DEL	DNA-encoded libraries
DIPEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DODT	2,2'-(ethylenedioxy) diethanethiol
DOS	diversity-oriented synthesis
DTT	dithiothreitol
DVB	divinylbenzene
EDT	1,2-ethanedithiol
EVA	ethylene vinyl acetate
FDA	Food and Drug Administration
Fmoc	fluoren-9-ylmethyloxycarbonyl
HATU	(dimethylamino)-*N,N*-dimethyl(3H-[1,2,3]triazolo[4,5-b]pyridin-3-yloxy)methaniminium hexafluorophosphate
HBA	hydrogen bond acceptors

HBD	hydrogen bond donors
HBTU	(1H-Benzotriazol-1-yloxy)(dimethylamino)-*N,N*-dimethylmethaniminium hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	hydrogen fluoride
HMBA	hydroxymethyl benzamide
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HTE	high-throughput experimentation
HTS	high-throughput screening
IMCR	isocyanide-based multicomponent reaction
LC-MS	liquid chromatography – mass spectroscopy
LDV	low dead volume
LSF	late-stage functionalization
Mea	2-mercaptoethylamine
MeDbz	3-amino-4-(methylamino)benzoic acid
Mpa	3-mercaptopropionic acid
MW	molecular weight
NGS	next generation sequencing
NMM	*N*-methylmorpholine
NMP	*N*-methyl-2-pyrrolidone
NMR	nuclear magnetic resonance
OBOC	one bead-one compound
PAMPA	parallel artificial membrane permeability assay
Pbf	2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl
PCR	polymerase chain reaction
PEG	poly(ethylene glycol)
PP	polypropylene
PPI	protein-protein interaction
PROTAC	proteolysis targeting chimera
PS	polystyrene
PTFA	polytetrafluoroethylene

Ro5	rule of five
RP	reversed-phase
SAR	structure-activity relationship
SASRIN	Super Acid Sensitive ResIN
SCL	safety-catch linker
SID	surface-induced dissociation
SPPS	solid-phase peptide synthesis
SPR	surface plasmon resonance
SuFEx	sulfur (VI) fluoride exchange
TCEP	tris-(2-carboxyethyl)phosphine
TEA	triethylamine
TES	triethylsilane
TFA	trifluoroacetic acid
TG	TentaGel
THF	tetrahydrofurane
TIS or TIPS	triisopropylsilane
TLC	thin-layer chromatography
TOS	target-oriented synthesis
TR-FRET	time-resolved fluorescence energy transfer
Trt	triphenylmethyl, trityl

1. Introduction

1.1 Need for new chemical libraries in drug development

Despite the enormous advancement of pharmaceutical sciences in the past decades, there are still many medical conditions in the clinical practice for which no sufficient treatment is available. To develop a new drug against such diseases, researchers need to understand their molecular causes, then find compounds that can initiate a specific biochemical response on the target of interest. Even though initial hit compounds rarely meet the requirements to become a safe and efficacious drug, they can serve as starting points for discovery campaigns, where they undergo several iterative cycles of hit-to-lead optimization until their pharmacological properties are acceptable for clinical development. With the advance of laboratory automation and HTS, it has become possible to test thousands or millions of individual compounds, even though such large campaigns are still extremely costly. For this reason, chemical libraries are valuable sources of initial hits, and there is a constant demand for novel compound libraries that can be screened against relevant biological targets.[1]

The first screening libraries were composed of natural products, containing various extracts from plants, animals or fungi. Libraries can be constructed either directly from crude extracts, or the fractions obtained from them through chromatography.[2] Through natural products, it is possible to access a chemical space of highly complex molecules that would otherwise be difficult to synthesize. Once an extract is found to cause the desired biochemical effect on the target of interest, the exact structure and mode of action of the active compound is elucidated. However, this can be a challenging endeavor due to the high number of distinct constituents in each solution and their often-complicated structure.

To simplify hit validation and to increase the size and diversity of screening libraries, companies gradually shifted their attention to collections of pure and well-characterized compounds synthesized in their own previous research programs, as well as to other commercially available synthetic chemical libraries. One of the most prevalent strategies in the pharmaceutical industry is the screening of such compound collections, often featuring thousands or millions of well-defined molecules This makes confirming hits straightforward and establishing structure-activity relationship (SAR) less ambiguous than in the case of natural product libraries. However, assembling and screening such libraries can prove extremely expensive and time-consuming.

Here, I discuss the history of *de novo* library generation for drug development, a convenient approach to reduce the required costs and time of early hit discovery. These strategies include genetically encoded libraries (1.2.1), combinatorial synthesis on solid phase (1.2.2) and in solution phase (1.2.3), the combination of the two latter approaches that has been the primary focus of our research group (1.2.4).

1.2 Combinatorial *de novo* synthesis of libraries for HTS

To facilitate access to large and chemically diverse chemical compound collections quickly and at a relatively low cost, various methods have been developed for generating libraries *de novo*. A commonly used approach for generating these large and diverse chemical libraries is combinatorial chemistry, defined by Liu *et al.*[3] as the "systematic, repetitive and covalent linkage of various building blocks", is. For optimal library design, selecting these building blocks must account for the target of interest. If the biological target of interest or its close structural analogs are well characterized and there is plenty of information available on the key interactions in the binding pocket, it is possible to take this into consideration and maximize the chances of a successful HTS campaign by designing focused libraries.[4,5] This strategy is often termed as target-oriented synthesis (TOS).[6] Conversely, if little is known about the target, the path to success is usually through incorporating as much diversity in the library as possible, thereby sampling a large chemical space.[7] The systematic, modular assembly of the library compounds through combinatorial chemistry enables the design and synthesis of unbiased libraries with high level of structural and chemical diversity. These principles are the foundation of the diversity-oriented synthesis (DOS) approach, summarized in detail by Galloway *et al*.[8]

Libraries can be generated either through parallel synthesis or through the split-and-pool method. Using parallel synthesis, combinatorial libraries of hundreds or thousands of compounds can be generated that are position-addressable, therefore their screening directly provides information on the structure of hits and their SAR. In contrast, the split-and-pool method allows the synthesis of millions of unique compounds simultaneously. However, as the libraries are screened as mixtures, the structure of hits must be elucidated by genetic or chemical decoding. Various approaches in combinatorial chemistry have been extensively reviewed by others.[9–13]

The individual library compounds can be purified before screening, but this is usually a highly work-intensive procedure and can seriously limit the feasible library size. By applying highly optimized and robust methods for library synthesis, the emerging direct-to-biology (D2B) approach aims to overcome this challenge by generating libraries wherein the crude compounds are sufficiently pure for direct biological screening. In this approach. the risk of reaction byproducts and excess reagents interfering with biochemical assays can be minimized but cannot be eliminated, therefore hits must be analyzed and resynthesized to confirm that the biological activity was caused by the intended compound. Despite this challenge, the D2B strategy remains attractive due to its high synthetic throughput and its easy access to chemical diversity. It is discussed in further detail in section 1.2.2.

De novo chemical libraries can be genetically encoded (1.2.1), or synthesized on solid phase (see section 1.2.2), in solution (see section 1.2.3), or through a procedure combining solid- and solution-phase strategies (1.2.4).

1.2.1 Genetically encoded libraries

Even though there is enormous interest in large biomolecules like proteins and antibodies as therapeutic modalities, they are beyond the limitations of combinatorial synthesis strategies presented in later sections (1.2.2 and 1.2.3) and are therefore not discussed further in this introduction. To generate such libraries of biologics, *in vitro* peptide evolution techniques like phage,[14] bacterial,[15] yeast,[16] ribosomal[17] or mRNA[18] display have been developed, where genes encoding random sequences are cloned into the genome of phages, yeast or bacteria, which can express and present these sequences on their surface. During the screening of such a library, it is co-incubated with the desired biological target that is immobilized on solid support. After multiple rounds of affinity selection, the cells or viral particles expressing proteins with affinity for the desired target are enriched. Using polymerase chain reaction (PCR) amplification and DNA sequencing, the exact protein composition can be determined.[19] These techniques are valuable tools for generating libraries of billions of compounds and discovering potent binders. Furthermore, the methods established in these strategies laid the groundwork for the development DNA-encoded libraries (DELs), in which the chemical structure of the library compounds is encoded by a sequentially ligating a unique DNA barcode for each reaction step. This technology, reviewed in detail by Gironda-Martínez *et al.,*[20] is compatible with a wide range of unnatural building blocks various chemical transformations, making it especially attractive for generating diverse libraries.

Approximately twenty different types of reactions that are compatible with oligonucleotide moieties have been utilized to synthesize DELs, as reviewed by Fitzgerald and Paegel.[21] The wide range of available transformations has resulted in a high level of chemical diversity among the library compounds. In order to be applicable in DEL synthesis, a chemical transformation must satisfy similar criteria to those defined for click chemistry: it has to be robust, highly selective, use mild reaction conditions and be insensitive to water and oxygen. In addition to amide coupling reactions, the authors highlight the importance of reductive amination/alkylation, Suzuki-Miyaura, Buchwald-Hartwig and Ullmann cross-couplings, triazine-substitution, and Cu(I)-catalyzed azide-alkyne cycloaddition.

Apart from the wide range of reliable and DNA-compatible chemical reactions, the properties and the diversity of the building blocks used for DEL synthesis also play a major role in the overall library quality. As the number of possible combinations is extremely high (in the range of millions or tens of millions), different approaches have been published for optimizing the library design. Martín *et al*. developed a computational tool named eDESIGNER to virtually generate possible library compounds, profile them, and assist with choosing the most suitable subsets.[22]

Despite the extremely large library size and high structural diversity that can be achieved with the DEL technology, it is not without its drawbacks. Firstly, verifying the completion of the coupling reactions and the overall library quality is more challenging than in the case of position-addressable libraries. Secondly, as the selection is based on affinity, the screening does not provide information on the exact binding mode of hit compounds and compounds that bind to another site but do not elicit the desired biochemical response on the target may become enriched.

1.2.2 Combinatorial synthesis on solid phase and OBOC libraries

Solid-phase supported chemistry provides considerable advantages for combinatorial synthesis. Firstly, as the compounds are covalently bound to a solid support throughout the synthesis, reagents can be applied in a large excess in each synthesis step, and the unreacted molecules can be washed away with simple filtration. Secondly, the repetitive, cyclic nature of solid-phase synthesis, especially in the case of peptides, offers an excellent opportunity for automation and parallel synthesis.

The first examples for combinatorial peptide synthesis were reported in the 1980's. Geysen *et al*.[23] used polymer rods to synthesize hundreds of peptides in parallel (multi-pin technology), and screened them on solid support. Houghten[24] described a synthesis method that used solvent-permeable mesh packets filled with resin, and submerged them sequentially in solutions of activated building blocks (tea-bag method). In this research, peptides were cleaved from the solid support, then subjected to screening without chromatographic purification. By introducing a split-and-pool step between the coupling cycles, Furka *et al*.[25] developed a method for the rapid synthesis of multicomponent peptide mixtures. The procedure, illustrated in Figure 1, starts with dividing the synthesis resin into separate reaction vessels and coupling one building block, such as an amino acid, to each portion. The contents of each vessel are pooled and mixed, then redistributed, coupling a separate second building block to each mixture. The process can be repeated as many times as necessary, resulting in a combinatorial library where each synthesis bead features one specific sequence of building blocks. After cleavage, the result is a nearly equimolar mixture of a large number of peptides. This strategy was used by Lee *et al*.[26] to generate and screen a combinatorial library of 1,296 small molecule-like compounds by attaching to carboxylic acids to a diamino acid scaffold, in a similar concept as that described in Chapter 6 of this thesis.

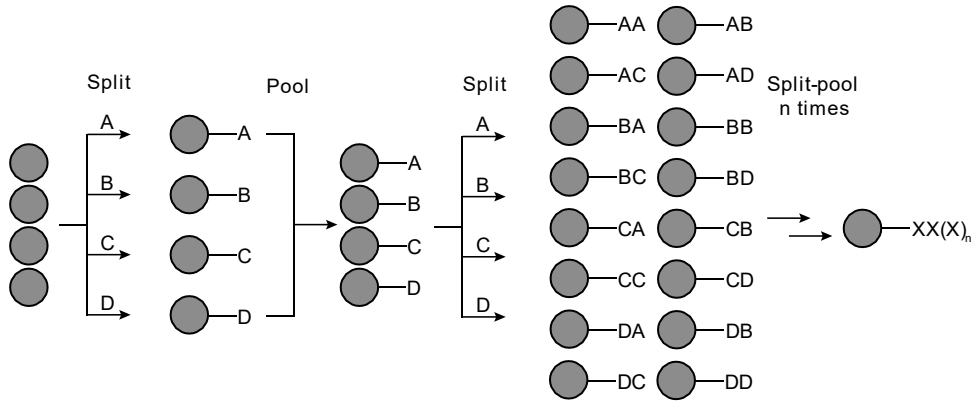

Figure 1: Illustration of the split-and-pool combinatorial synthesis strategy on solid support

In the same year, Lam *et al.*[27] reported a similar split-and pool solid-phase supported synthetic strategy. In their work, they took advantage of the fact that each individual bead displays multiple copies of a unique library compound, after which this these libraries are named one bead-one compound (OBOC) libraries. To identify the beads featuring binding sequences, they combined the solid support with a solution of the desired target labeled with an enzyme or fluorescein, resulting in a visible, intense coloration of a few individual beads. These are taken from the mixture and analyzed, initially through Edman peptide sequencing, but later with more convenient methods. As summarized by Pei and Kubi,[28] the sequence analysis can

also be performed by mass spectrometry.[29,30] A method that uses photocleavable chemical tags that can be identified by gas chromatography has been developed by Ohlmeyer *et al*.,[31,32] further facilitating the decoding process. This approach has been successfully applied for relevant targets.[33] The scope of the chemistry has been also expanded beyond peptides. OBOC libraries featuring peptidomimetics[30,34,35] and non-peptidic small molecules[36–40] have been reported, where among other reaction types, cross-couplings, reductive aminations, and alkylations were performed on solid phase. The potential of this method is exemplified by the successful identification of a hit compound that was later developed into sorafenib,[41] a multikinase inhibitor cancer drug that received approval from the Food and Drug Administration (FDA) in 2005.

Despite the improved strategies to decode OBOC libraries, analysis of single beads can still prove challenging. To overcome this difficulty, Ryba *et al*.[42] used modular disks as solid supports, called SynPhase lanterns, with a loading of 15-75 μmol on each disk, which are soaked in the reaction mixture. The disks can be easily washed, connected and disconnected between the split-and-pool cycles, and can be tagged either by color codes, radio frequency tags, or through the multipin array approach. This strategy retains the advantages of the split-and-pool approach in synthesizing large and diverse libraries, while scaling up the reactions and eliminating the need of chemical encoding. With this technology, Marcaurelle *et. al*.[43] synthesized a library of more than 30,000 macrocycles, and after cleaving them from the solid support, identified novel inhibitors of the histone deacetylase HDAC2 through HTS. Lowe *et. al*.[44] used a similar approach for the synthesis and screening of 1,976 spirocyclic azetidines.

Through automation, solid phase parallel synthesis has also become feasible. This approach offers the tremendous advantage that each library compound will have a defined position. Moreover, compared to the OBOC approach the amount of each synthesized peptide can be higher when they are prepared this way, which enables further chemical diversification.

The history of the development of automated peptide synthesizers was summarized by Tian *et al*.[45] These instruments rely on a computer-controlled pipetting system, and can dispense the desired building blocks, along with the necessary coupling reagents to their assigned position. Automated parallel synthesis can be performed in fritted syringes (e.g. 16 or 24 peptides), 96-well[46] or 384-well[47] synthesis plates. An example for the application of high-throughput SPPS in filter plates are the results published by Bhushan *et al.*,[48] where the most promising sequences found by mRNA display were synthesized in 96-well plates. Solid-phase

supported parallel synthesis is also applicable for small molecules, such as the heterocyclic library of 45,140 compounds reported by Ding *et al*.,[49] or the library of 98 cross-conjugated dienones reported by Kitade *et al*.[50] Automated parallel synthesis can also be scaled down using precision liquid handling, inkjet printing, or photolithography. This technology is used to generate libraries called peptide arrays, where each distinct region of the synthesis surface corresponds to one specific peptide sequence. SPPS can be performed on either a chip (microarray) or a cellulose or other membrane surface (macroarray, spot synthesis).[51] The technology of peptide arrays was reviewed in detail by Szymczak *et al.*,[52] and has various applications, such as antibody profiling, epitope mapping or investigating ligand-receptor interactions. The concept of arrays has been extended and applied for small molecules as well.[53–55]

While solid-phase-supported synthesis can offer a convenient path to combinatorial libraries, many chemical transformations from the enormous toolbox of traditional organic chemistry are incompatible with this approach, which can be a major limitation to chemical diversity. For example, performing reactions in inert atmosphere or at high temperatures can often prove challenging due to the technical limitations of solid-phase supported combinatorial chemistry. As highlighted by Brik, Wu, and Wong,[56] even if a chemical reaction is suitable for solid-phase synthesis, it must be thoroughly tested and potentially re-optimized for these special conditions to ensure reliable results.[57] Moreover, precise and small-scale handling of solids is far less amenable to laboratory automation than that of liquids.

1.2.3 Combinatorial synthesis in solution phase and D2B screening

To overcome the drawbacks presented by solid-phase supported combinatorial chemistry, an interest in solution-phase combinatorial chemistry has also emerged. However, while excess reagents can easily be removed by filtration during solid phase supported synthesis, this is far less trivial when the reactions are performed in solution. Even though techniques like solid-supported scavengers, fluorous synthesis or liquid-liquid extraction[58] have been developed to overcome the challenge of parallel purification, the solution-phase strategy is especially attractive if the crude compounds can be screened *in situ*. To apply this direct-to-biology (D2B) strategy, the synthetic transformations must be robust and highly efficient with minimal byproduct formation. Another crucial condition is that the reagents – or the side-products derived from them – must not interfere with the biological assay, even at high concentrations.

An early example was published by Brik *et al.*[59] in 2002, where they provided a solution for these challenges by developing a procedure for solution-phase combinatorial library synthesis in microtiter plates, using highly effective transformations to generate compounds on micromolar scale, with a purity that is suitable for *in situ* screening. They identified novel peptide inhibitors of the HIV protease by screening 62 crude compounds. The chemical scope of this approach was later greatly expanded. In their review, Brik, Wu and Wong[56] present several examples of library syntheses and discovery of inhibitors by direct screening through different chemical reactions, such as amide bond formation, Cu(I)-catalyzed 1,2,3-triazole formation, Pictet-Sprengler reaction, alkylation and epoxide opening.

With the development of nanoliter-scale liquid transfer technologies like mosquito liquid handling and acoustic droplet ejection, performing chemical reactions on micromolar and nanomolar scale has become possible. The former technique utilizes thin pins for liquid transfer and was successfully applied for the synthesis of small-molecule libraries and the screening of various reaction conditions.[60,61] Acoustic droplet ejection (ADE) relies on ultrasound impulses to transfer liquids with nanoliter precision.[62] The contactless nature of this technology also eliminates the need for pipette tips and minimizes the risk of cross-contamination during automated liquid transfers.

The advances in automation and small volume liquid transfer technologies made it possible to perform hundreds or thousands of solution phase reactions in parallel. On one hand, this approach called high-throughput experimentation (HTE) is extensively used for the screening and fast optimization of reaction conditions. [63–78] On the other hand, this technology is a powerful tool for synthesizing combinatorial libraries and applying them in the previously mentioned direct-to-biology high-throughput chemistry (D2B-HTC) strategy. Such libraries can be screened by affinity-based methods such as affinity selection mass spectrometry (ASMS)[79] or surface plasmon resonance (SPR),[80] but several examples have also been reported where enzymatic or even cell-based biochemical assays were used. The strategy is also of significant interest for the discovery and optimization of proteolysis targeting chimeras (PROTACs)[81,82] and molecular glues,[83] as emerging classes of therapeutics. An overview of combinatorial libraries subjected to D2B screening is presented in Table 1.

First author	Year	Reaction type	Synthesis scale, μmol	Liquid transfer method	Library size	Target	assay type
Brik[59]	2002	amide coupling	2.2	pipette	62	HIV-1 PR	enzymatic
Wu[84]	2003	amide coupling	0.1	pipette	60	α-fucosidase	enzymatic
Lee[85]	2003	CuAAC	2	pipette	85	Fuc-T VI	enzymatic
Brik[86]	2003	CuAAC	2	pipette	100	HIV-1 PR	enzymatic
Cheng[87]	2004	amide coupling	2	pipette	30	HIV-1 PR	enzymatic + cell-based
Best[88]	2004	amide coupling	2.5	pipette	126	β-AST-IV	enzymatic
Chang[89]	2004	amide coupling	0.1	pipette	60	α-fucosi-dase	enzymatic
Numa[90]	2005	Pictet-Sprengler	12.2	pipette	66	Anthrax LF	enzymatic
Brik[91]	2005	N-alkylation	3.2	pipette	30	β-AST-IV	enzymatic
Wu[92]	2005	N-alkylation and O-esterification	1	pipette	150	Cathepsin B	enzymatic
		O-esterification	1	pipette	84	Cathepsin B	enzymatic
		amide coupling	1	pipette	84	Cathepsin B	enzymatic
		N-alkylation	1	pipette	78	HIV-1 PR	enzymatic
Liang[93]	2006	epoxide ring opening	1.8	pipette	20	HIV-1 PR	enzymatic
Wang[94]	2006	CuAAC	52	pipette	96	MMP-7	enzymatic
Wu[95]	2006	amide coupling	10	pipette	180	SARS-CoV 3CL protease	enzymatic
Zhang[96]	2007	amide coupling	0.8	pipette	99	HeLa and HL-60 cell lines	cell-based
Srinivasan[97]	2009	CuAAC	0.25	pipette	325	PTP1B	enzymatic
Tang[98]	2011	aldehyde-amine condensation	5	pipette	270	HDAC2, 3, 8	enzymatic
Murray[80]	2014	amide coupling	500	pipette	13	HSP90	SPR
		Pd-catalysed coupling reactions	500	pipette	7	PIN1	SPR
Karageorgis[99]	2014	Intra-molecular metal-catalyzed carbenoid reactions	10	pipette	336	androgen receptor	TR-FRET
Karageorgis[100]	2015	Intra-molecular metal-catalyzed carbenoid reactions	10	pipette	326	androgen receptor	TR-FRET
Liu[101]	2018	SuFEx	0.1	pipette	39	MCF-7 ER+ breast cancer cell line	cell-based

Gesmundo[61]	2018	Pd-catalysed coupling reactions	0.1 or 0.12	mosquito liquid handler	384	CHK1	ASMS
Elías-Rodríguez[102]	2018	thiourea-forming click reaction	7	pipette	26	α-galacto-sidase	enzymatic
Benz[103]	2019	thiolactone opening + disulfide exchange	0.008	non-contact liquid dispenser	75	HEK293T cells	on-chip cell-based
van der Zouwen[104]	2019	aldehyde-amine condensation	0.05	pipette	24	BirA and CAT	enzymatic
Kitamura[105]	2020	SuFEx	4	pipette	460	SpeB	enzymatic
Leggott[106]	2020	Pd-catalysed coupling reactions	100	pipette	220	S. aureus ATCC29213	cell-based
Osipyan[107]	2020	IMCRs	0.21	non-contact liquid dispenser	1152	no screening	no screening
Green[108]	2020	intermolecular metal-catalyzed carbenoid reactions	10	pipette	346	MDM2	TR-FRET
Thomas[109]	2021	amide coupling	0.17 or 0.37l	pipette	1073	carbonic anhyd-rase I	intact protein LC-MS
Fu[110]	2021	amide coupling	1.2 μmol	pipette	192	UGM	enzymatic
Gao[111]	2021	IMCRs	0.5	ADE	1536	menin-MLL PPI	DSF
Sutanto[112]	2021	IMCRs	500	pipette	192	SARS-CoV-2 3CL protease	HT protein crystallo-graphy
		IMCRs	0.625	non-contact liquid dispenser	384	SARS-CoV-2 3CL protease	HT protein crystallo-graphy
Immel[113]	2021	flavin photocatalysis	2.33	pipette	96	thrombin	enzymatic
Gehrtz[114]	2022	CuAAC	0.02	ADE	768	U2OS cell line	cell-based
Hendrick[82]	2022	amide coupling	5	pipette	182	HiBiT-BRD4 cell line	cell-based
Francis[115]	2022	photoredox-catalysed alkylation	3	pipette	92	S. aureus ATCC29213	cell-based

Pingitore[116]	2022	CuAAC	11.25	pipette	20	β-N-acetylglu-cosamin-idase	Enzymatic
		thiourea-forming click reaction	7	pipette	19	β-N-acetylglu-cosamin-idase	enzymatic
		CuAAC	3	pipette	5	β-N-acetylglu-cosamin-idase	enzymatic
		thiourea-forming click reaction	3.8	pipette	5	β-N-acetylglu-cosamin-idase	enzymatic
Thomas[117]	2023	amide coupling	0.165	pipette	546	BCL6 and KRAS-G12D	intact protein LC-MS
Wang[83]	2023	IMCRs	0.625l	non-contact liquid dispenser	384	MM.1S cell line	cell-based
Gao[118]	2023	IMCRs	0.3, 0.375 or 0.5	ADE	1536	no screening	no screening
Stevens[81]	2023	amide coupling	0.15l	mosquito liquid handler	650	HiBiT-BRD4 cell line	cell-based
Kaguchi[119]	2023	serine/threonine ligation (STL)	0.2l	pipette	702	multiple bacterial strains	cell-based

Table 1: Overview of literature on combinatorial libraries subjected to D2B screening

Compounds synthesized by microfluidics (flow chemistry) have also been applied for D2B screening.[120–122] This approach enables high levels of automation, and it allows for reaction conditions (e.g. high pressure or temperature) that would be difficult to realize in parallel synthesis.

Another solution-phase combinatorial approach is target-directed dynamic combinatorial chemistry (tdDCC).[123,124] In this case, a mixture of fragments that can covalently react with each other in a reversible, biorthogonal fashion are co-incubated with the target protein of interest, and the fragment combinations with the highest affinity become enriched. In this case, the binding site of the target serves as a template for the formation of the most potent inhibitors, which can then be detected by various analytical methods. This approach has been successfully applied to discover novel inhibitors[125] and chemical probes.[104]

1.2.4 SPPS with subsequent solution-phase diversification

In our laboratory, we combine the advantages of automated SPPS such as robustness and high crude purity with the high synthetic throughput and easy automation of solution-phase combinatorial chemistry. Generating large peptide libraries with nanomole scale late-stage functionalization (LSF), we aim to perform to perform D2B-HTC and find binders to various targets.

Kale *et al.*[126] synthesized a library of 8,988 compounds by nanomolar-scale S-to-N macrocyclization of dipeptides purified by ether precipitation (Figure 2a). Screening this library resulted in potent thrombin inhibitors. The main limitation of this method was that the macrocycles consisted of only two building blocks, which was a major bottleneck to achieving high library diversity. To overcome this challenge, Mothukuri *et. al* [127] developed a novel synthesis strategy to generate 3,780 macrocycles from chromatographically purified short, linear peptides with an N-terminal bromoacetamide function. To achieve this, three consecutive "add-and-react" steps were performed, without any further purification between them (Figure 2b). Thrombin inhibitors with single-digit nanomolar potency were identified from this library. Capitalizing on the high precision of the ADE technology, Sangouard *et al.*[128] scaled down this synthesis strategy to a picomolar scale, then successfully synthesized and screened a pilot library of 2,700 cyclic peptides to identify novel inhibitors of the MDM2:p53 PPI with low micromolar affinity (Figure 2c). Even though the S-to-N cyclization strategy proved effective in these examples, it presented two major drawbacks. Firstly, peptides obtained from SPPS had to be chromatographically purified before combinatorial modification, which is a major limitation to library size and diversity. Secondly, the S-to-N cyclization reaction often yielded a significant quantity of undesired by-products, which made hit validation challenging in a D2B approach. This issue is well illustrated by the results of Kale *et al.*,[126] where the active inhibitor proved to be one of the cyclization by-products.

Merz *et al*.[129] aimed to solve these problems by synthesizing a library of 384 dithiol peptides on cysteamine SASRIN resin (see Chapter 1.3.3). In this case, the peptides were deprotected and cleaved with a TFA-based cleavage cocktail, and after evaporation of the acid, precipitated with ether. This method resulted in crude peptides that were sufficiently pure for S-to-S cyclization without chromatographic purification. After cyclization with bis-electrophilic linkers and nanomole-scale acylation with carboxylic acids a library of 8,448 peptide macrocycles (Figure 3a) was screened against thrombin. The hits identified from this screen could be later developed into orally bioavailable derivatives. This strategy greatly increased

the synthetic throughput of the SPPS step, however, the acidic cleavage and ether precipitation presented practical inconveniences such as releasing large amounts of corrosive vapors and decanting the ether supernatant from plates.

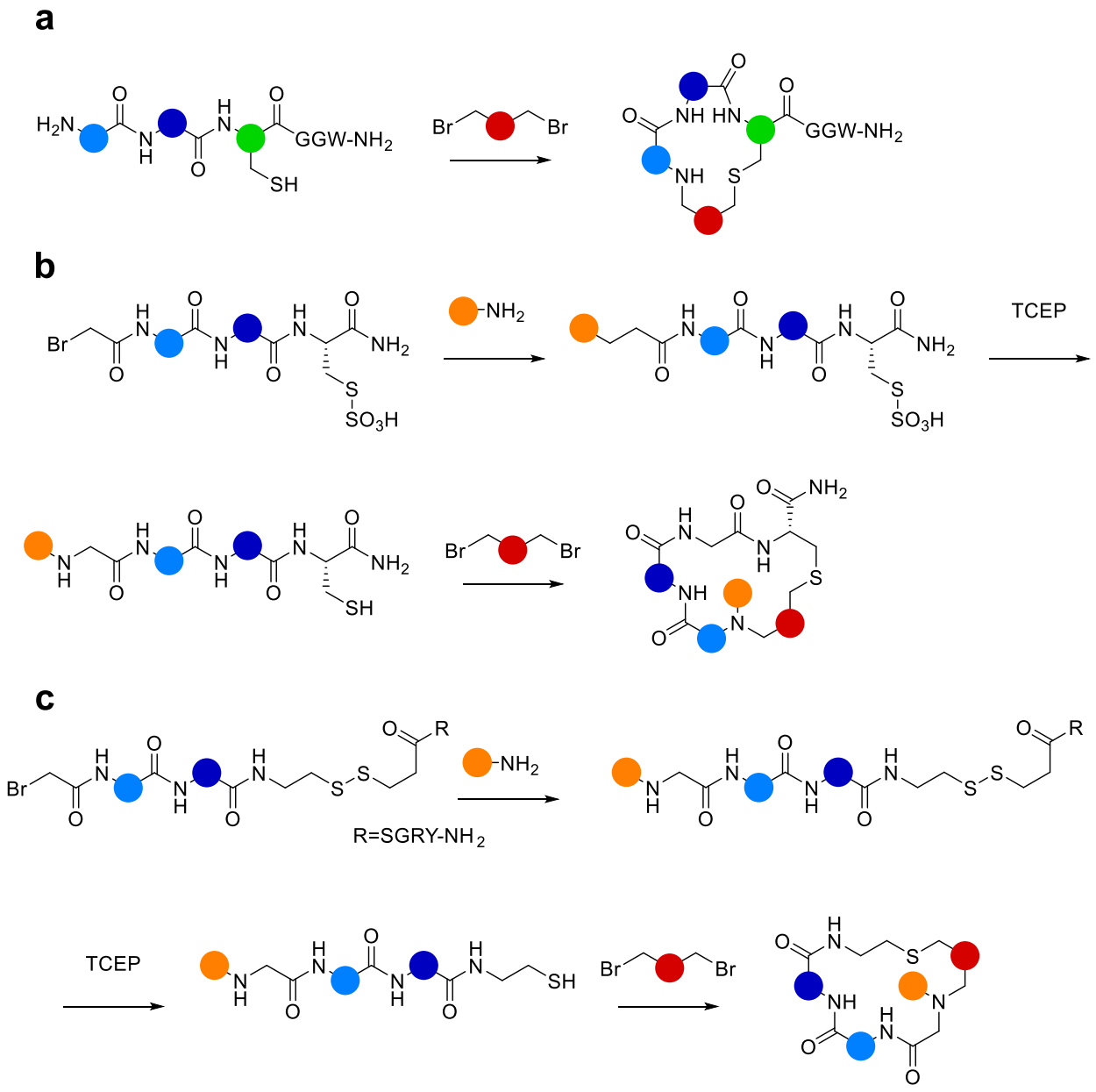

Figure 2: Synthetic strategies used in our group for the generation of combinatorial peptide libraries that require chromatographic purification after SPPS. (a) S-to-N cyclization used by Kale *et al.*,[126] (b) the add-and-react modification by Mothukuri *et al.*,[127] (c) and the S-to-N strategy used in picomole-scale synthesis by Sangouard *et al.*[128]

As a solution, Habeshian *et al.*[130] developed an SPPS strategy to obtain pure, disulfide-cyclized peptides through cyclative release. In this case, the release is initiated by a DMF solution containing a non-nucleophilic base, and the solvent can be removed by evaporation. Subsequently, he combined this approach[131] with the picomole-scale side chain acylation strategy reported by Merz *et al.*,[129] and synthesized libraries of 4,608 and 19,968 compounds (Figure 3b). These efforts resulted in novel inhibitors with nanomolar affinity for model targets such as the MDM2:p53 protein-protein interaction and thrombin.

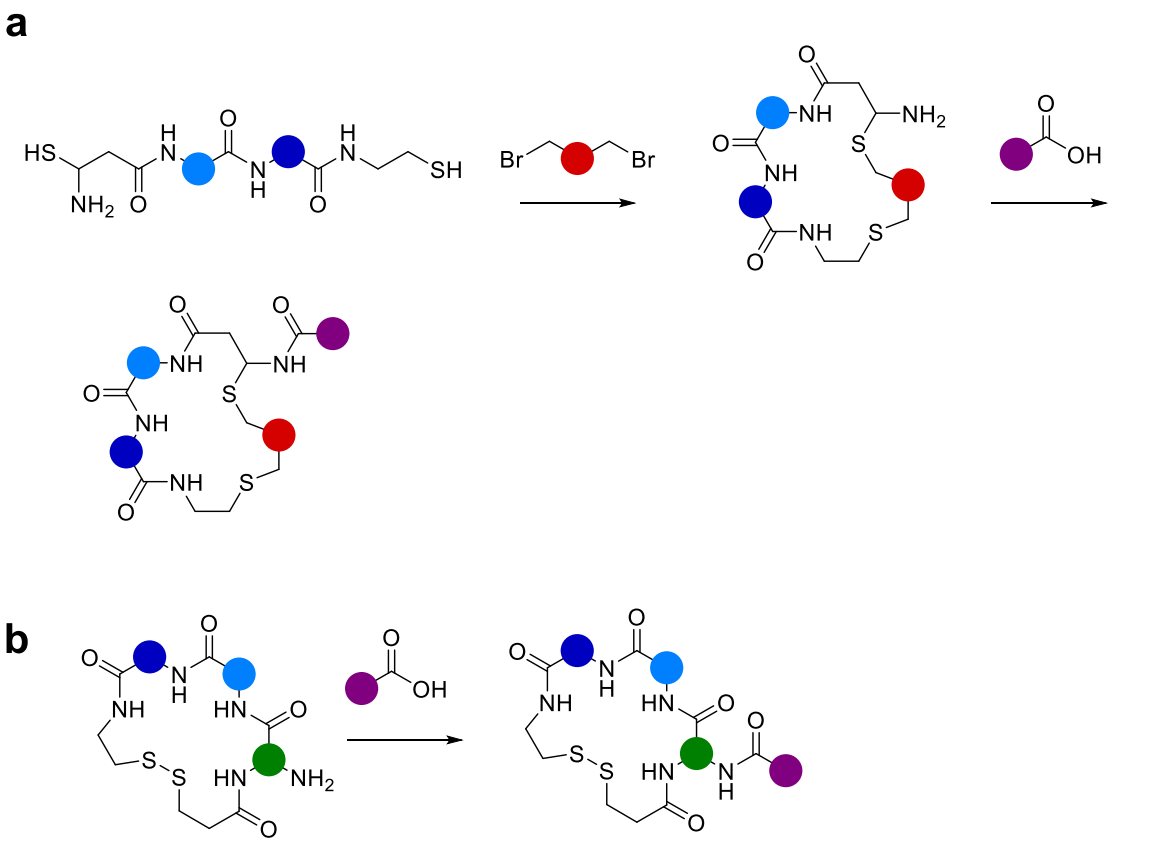

Figure 3: Schematic representations of the synthetic strategies used in our group for the generation of combinatorial peptide libraries that do not require chromatographic purification after SPPS. (a) S-to-S cyclization and subsequent picomole-scale side-chain acylation by Merz *et al.*[129] (b) picomole-scale side-chain acylation of disulfide-cyclized peptides by Habeshian *et al.*[131].

The cyclative release strategy was an efficient way to obtain pure and diverse peptides from SPPS that were suitable for subsequent modification. However, the disulfide-containing compounds are not ideal starting points for drug discovery as this bond can be easily broken in reducing biological environments like the cytosol or the gut, yielding linear thiol peptides that often lose their affinity to the target of interest. Furthermore, the efficiency of the release

reaction is highly dependent on the length and flexibility of the peptide chain. Short sequences that are of significant interest for drug discovery were difficult to obtain with this approach. Developing a method that can be used to efficiently to generate short dithiol peptides with a sufficient crude purity has been therefore a primary objective of my research. To achieve this, I built upon the existing SPPS strategies, that are described in subchapter 1.3.

1.3 Solid-phase peptide synthesis

Through SPPS, it is possible to rapidly generate a large number of chemically diverse compounds, especially considering the wide variety of commercially available building blocks that can be considered during the library design.[132] As the goal of my research was to develop methods for the efficient HTS of chemically diverse peptide libraries that can be subjected directly to biochemical screening without purification, we relied on established solid-phase peptide synthesis (SPPS) strategies. This key technology is introduced in this chapter.

When the goal is to assemble a custom sequence of amino acids, several challenges must be addressed. Firstly, to form an amide bond under mild conditions, the carboxylate function of the desired amino acid must be activated, and the amino function of the previous building block needs to be accessible. However, the amino group of the building block to be coupled must be rendered inactive by a protecting group to prevent oligomerization. It is also necessary to ensure that each coupling reaction is quantitative, otherwise the product will contain various deletion products that often pose a major challenge during purification. To achieve this, a high molar excess of reagents must be applied, but the unreacted material must be completely removed before the next coupling reaction can start.

An elegant solution for all of these difficulties was introduced by Merrifield and co-workers[133] by the invention of SPPS. By anchoring the peptide chain to polymer beads, it became possible to perform alternating cycles of coupling and amine deprotection with an excess of reagents. The unreacted compounds could be easily washed away after each step. When the desired peptide sequence is fully assembled, it can be cleaved from the solid support with the appropriate reagents. The cyclic nature of this method also allows for a high degree of automation, which made combinatorial, high-throughput application feasible. The key principles of SPPS are summarized in Figure 4.

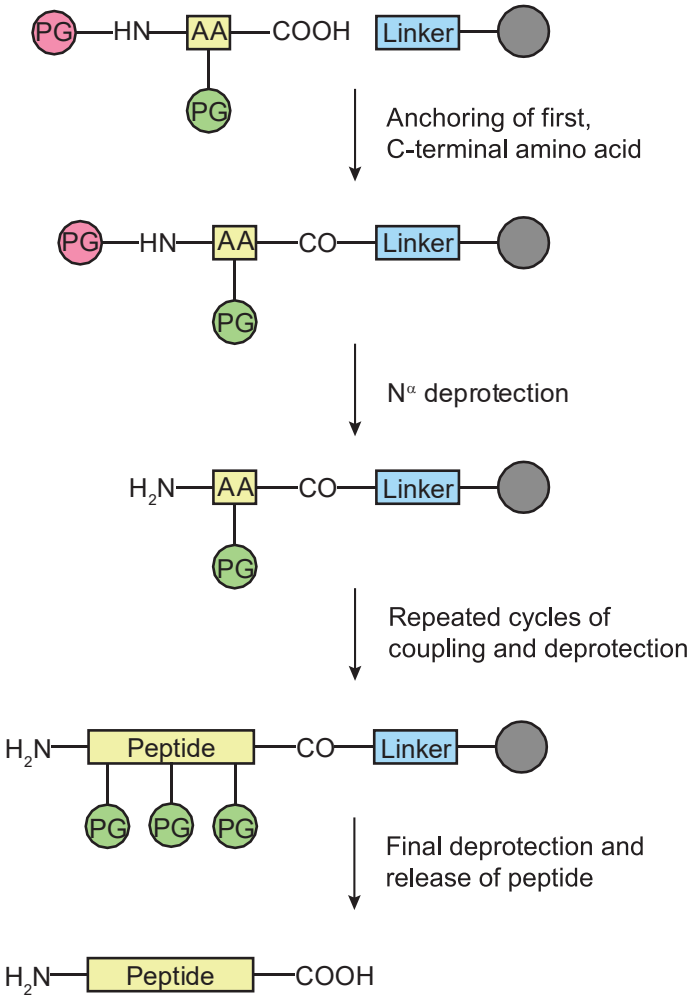

Figure 4 : Principles of solid-phase peptide synthesis (SPPS). Grey circle: polymer bead. AA : amino acid building block. PG, pink circle: protecting group of the N^{α} amino group. PG, green circle: side chain protecting groups. Figure adapted from Jensen et al.[134]

Since the birth of this concept in the 1960s, numerous improvements and variations of the method have been developed, which were reviewed in detail by Bunin[135] and by Jensen, Shelton and Pedersen,[134]. In this subchapter, I will give an overview of the wide range of options available to synthetic chemists to customize their synthesis strategies, with a focus on those that were important in my PhD project. These include available polymer supports (section 1.3.1), a number of different backbone and side chain protection strategies (section 1.3.2), various linking approaches (section 1.3.3), and a multitude of coupling reagents (1.3.4). These developments served as a basis for the development of the methodologies presented in chapters 4 and 5.

1.3.1 Resins used in SPPS

The type of polymer used as the solid support has a significant impact on the outcome of peptide synthesis. The ideal beads are stable under the synthesis conditions, but swell significantly when suspended in organic solvents. This increase in volume exposes their reactive functionalities and distances the peptide chains from one another during the synthesis, which is advantageous for the suppression of side reactions.

Chloromethyl-functionalized polystyrene (PS) cross-linked with divinylbenzene (DVB) reported by Merrifield in 1963 was the first resin used as solid support for solid-phase peptide synthesis.[133] Due to its favorable swelling properties, stability, high loading by weight and compatibility with a wide range of solvents except for water, PS-based resins are still extensively used, with modifications that enable a wide range of linkage and C-terminal modification strategies, as discussed in section 1.3.3. However, other types of resins can offer additional advantages in certain cases.

To increase achieve compatibility with aqueous media, TentaGel (TG) resins were developed by Rapp and Bayer[136,137] by attaching poly(ethylene glycol) (PEG) chains to the polystyrene beads. With this modification, there will also be larger distances between the peptides, which results in to higher product purity in the case of longer or more difficult sequences.

Resins that are completely PEG-based have also been reported, and are commercially available. The PEGA resin[138] is a PEG-poly-(N,N-dimethylacrylamide) copolymer, and is well-suited for flow synthesis conditions. Furthermore, it exhibits excellent swelling in water, making it suitable for immobilizing peptides on the bead surfaces to study interactions with proteins. The ChemMatrix (CM) resin is another example of a PS-free resin that was reported by Albericio and colleagues[139] in 2006. Featuring only primary ether bonds, this resin offers enhanced chemical stability. It also possesses practical properties as a free-flowing powder and has a relatively high loading. Studies demonstrate that CM resin is more efficient than PS in synthesizing highly structured, hydrophobic, and arginine-rich peptides in terms of peptide yield and purity. The three major categories of polymers used in SPPS, their usual loading range, and their swelling properties are summarized in Table 2.

			Approximate swelling (ml/g)						
Resin subgroups	**Commercial name**	**Initial loading (mmol/g)**	**DCM**	**DMF (NMP)**	**Et_2O**	**H_2O**	**TFA**	**THF**	**MeOH**
PS (1% DVB)	Aminomethylated PS	0.4–1.5	7	4	4	n.a.	2	9	1.6
PEG-PS	Amino TentaGel (TG)	0.15–0.3	6	5	2	3.6	n.a.	5	3.6
PEG based	Amino PEGA	0.2–0.4	13	11	n.a.	16	n.a.	13	13
	ChemMatrix (CM)	0.4–0.6	11	8	n.a.	11	14	n.a.	9

Table 2 : Loading and swelling characteristics of the major resin types. Table adapted from Jensen et al.[134]

In my PhD thesis projects, we relied on PS resins due to their high loading by weight, good swelling properties and relatively low cost.

1.3.2 Protecting groups

As previously mentioned, the amino group of the building block to be coupled must be protected to avoid oligomerization. Certain amino acids must also be protected on their side chain to prevent side reactions. In this section, I will present an overview of the common protection and deprotection strategies.

The choice protecting group for the amine function in the backbone (in the case of natural amino acids, the N^{α} nitrogen) is crucial for determining which reaction conditions, resins, linkers, and instrumentation can be considered for the synthesis. The first solutions introduced was the benzyloxycarbonyl (Cbz) group by Merrifield[133] and the *tert*-butyloxycarbonyl (Boc) group by Anderson and McGregor[140] and also by McKay and Albertson.[141] These groups can be removed in strongly acidic conditions by repeatedly incubating the resin with hydrogen bromide or trifluoroacetic acid (TFA). These highly corrosive substances are extremely difficult to apply in automated peptide synthesis, because their vapors pose a significant risk of damage to the instrumentation. Moreover, hydrogen fluoride (HF) is used in these strategies to cleave the peptide from the resin, which is also extremely corrosive and toxic, requiring specialized equipment and highly trained personnel for safe handling.

Due to these considerable technical difficulties, this strategy has been largely replaced by the application of the fluoren-9-ylmethyloxycarbonyl (Fmoc) protecting group. In this case, the deprotection can be conveniently performed under mild basic conditions, because upon

deprotonation, a dibenzofulvene residue is released. A 20% solution of piperidine in DMF is a common choice as deprotection reagent, as the nucleophilic base can react with the dibenzofulvene and therefore act as a scavenger.[142] The deprotection mechanism is illustrated in Figure 5. Other amines may be considered as alternatives if needed.[143]

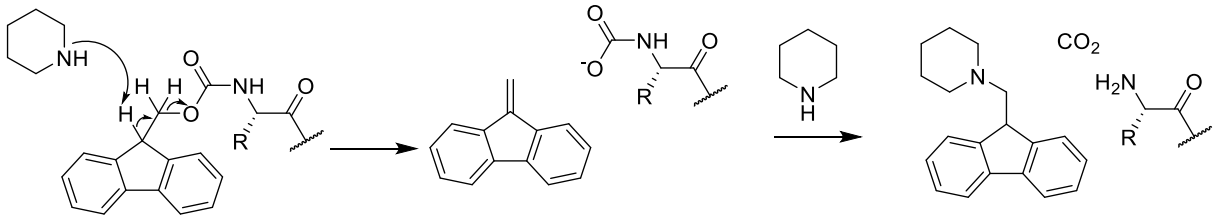

Figure 5: Reaction mechanism of piperidine-induced Fmoc deprotection.

Certain amino acids require protecting groups on their side chains during the synthesis to prevent side reactions. These groups must stay on the peptide during the entire synthesis (be semi-permanent), and only removed before or during the cleavage. As a result, deprotection reactions for side chains must have orthogonal chemistry compared to that of backbone protecting groups. Side chains requiring protection typically feature reactive nucleophilic functionalities like thiol, hydroxyl, or amino groups, or can be either protonated or deprotonated.

In Fmoc-SPPS, the most common side chain protecting groups[144] are the *tert*-butyl (ester or ether), Boc, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf),[145] and triphenylmethyl (Trt, trityl). These groups are all acid labile, and are usually removed with TFA. During the deprotection step, reactive species such as trityl and *tert*-butyl carbocations are formed. To prevent their reaction with the peptide product, nucleophilic scavengers must be added to the cleavage solution. After the cleavage, the crude peptide is usually obtained by evaporating the TFA, then adding cold diethyl ether to precipitate the residue. After centrifugation, the ether solution containing the scavengers and the remaining TFA can be decanted. The most common cocktails used for deprotection and cleavage are presented in Table 3.

Reagent	Recipe	Cleavage time, h
B	TFA/phenol/H_2O/TES (88/5/5/2)	1–4
K	TFA/phenol/H_2O/Thioanisole/EDT (82.5/5/5/5/2.5)	1–4
L	TFA/DTT/H_2O/TEA (88/5/5/2)	1–4
P	TFA/Phenol (95/5)	1–4
R	TFA/Thioanisole/EDT/anisole (90/5/3/2)	1–4
T	TFA/TES (95/5)	1–4
	TFA/H_2O/ (95/5)	1–4
	TFA/TES/H_2O (95/2.5/2.5)	1–4
	TFA/TES/EDT/H_2O (92.5/2.5/2.5/2.5)	1–4
	TFA/DODT (95/5)	2–3

Table 3: Common deprotection and cleavage cocktails. TFA trifluoroacetic acid, TES triethylsilane, EDT 1,2-ethanedithiol, DTT dithiothreitol, DODT 2,2'-(ethylenedioxy) diethanethiol. Table adatpted from Jensen et al.[134]

If the goal is to functionalize or modify a specific side chain group after the peptide synthesis but there are other residues in the sequence that would interfere with this reaction, it is possible to use acid-resistant protecting groups and remove them in a separate step. For example, the thiol of cysteine can be protected by the acetamidomethyl (Acm) group,[146] that can be removed either with mercury or thallium salts, or iodine. Allyl esters (for aspartate or glutamate) or allyl carbamates (Alloc, for lysine) can also be used. These groups can be eliminated through the use of Pd(0) compounds.[147] Additionally, amino groups such as in the side chain of lysine can be protected by the Dde (N-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl)) functional group, which can be removed with a 2% solution of hydrazine in DMF.[148] An overview of these functionalities is given in Figure 6.

In my PhD thesis project, I used the Fmoc strategy for the protection of backbone amines due to the mild and convenient reaction conditions it requires, and the wide variety of commercially available Fmoc-protected building blocks. In our case, it was not necessary to use acid-resistant side chain protecting groups, therefore derivatives featuring the above listed TFA labile groups were used.

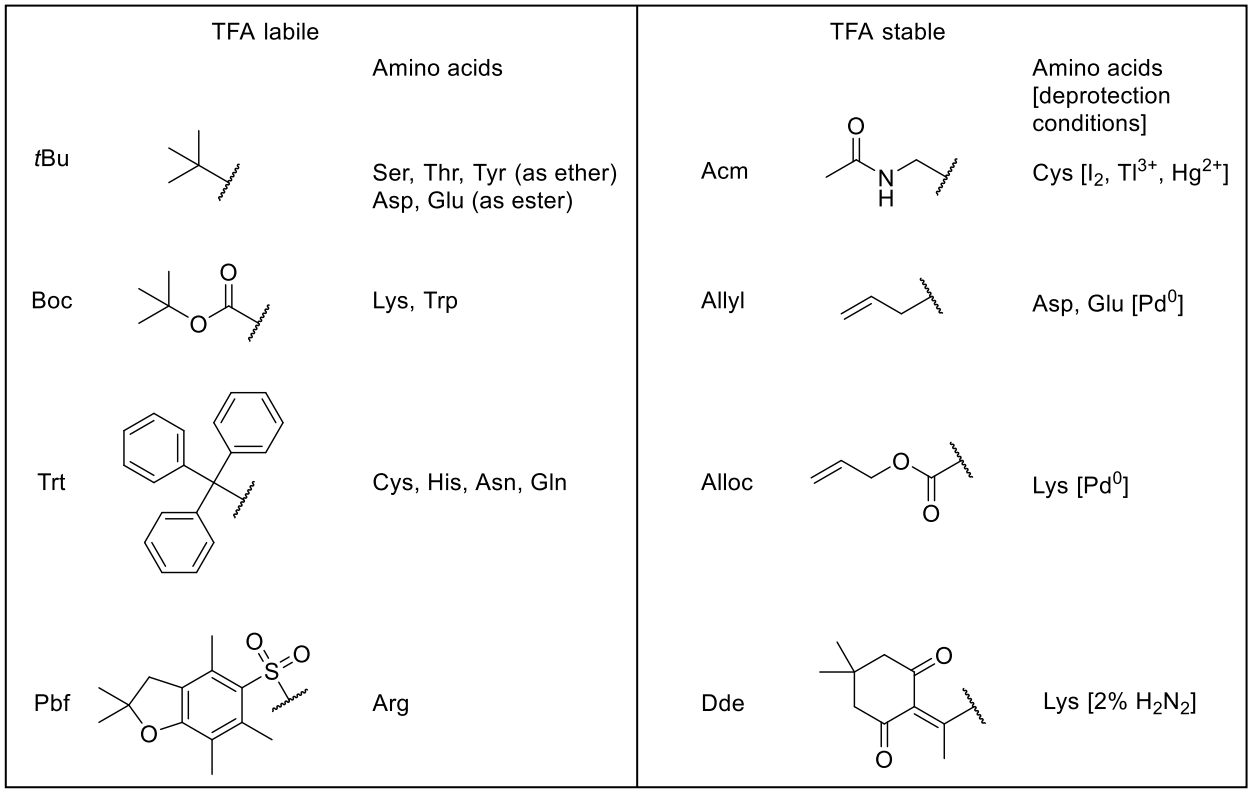

Figure 6: Common side chain protecting groups for natural amino acids in Fmoc-SPPS. TFA labile groups on the left are removed by an appropriate TFA cleavage cocktail. Figure adapted from Jensen et al.[134]

1.3.3 Linking strategies

As mentioned before, if the peptide synthesis was performed on Merrifield resin, the product must be cleaved with HF, which is a dangerous and cumbersome process. In order to enable milder, tunable cleavage conditions, as well as to customize the C-terminal functionality of the peptide product, various linking structures have been developed. Resins functionalized with these linkers are now commercially available and routinely used. This section will give an overview of common acid-labile and acid-stable linkers, and strategies for C-terminal functionalization. The examples below demonstrate that the linker is a key factor not only for choosing the applicable synthesis and cleavage strategies, but also of the chemical nature of the peptide's C-terminus, which determines what kind of chemical reactions can be used to modify, ligate or cyclize the peptide in this position. Recent advances in C-terminal modification strategies and their applications were reviewed in detail by Arbour *et al*.[149]

According to Pedersen and Jensen,[134] there are three main factors that determine the stability of the linker against acids: the stability of the carbocation intermediate, the chemical nature of the leaving group, and the steric effects. Fine-tuning the linker requires optimizing the first two

of these factors - for instance, incorporating electron donating functions like methoxy groups can increase carbocation stability. The most common acid-labile linkers possess a benzyl, benzhydryl or trityl core structure.

The first example of a benzyl-type linker was reported by Wang and co-workers.[150] The linker is based on a 4-alkoxybenzyl alcohol structure which is linked to the C-terminal amino acid by an ester bond. Protonation of the benzyl carbon with a strong acid such as TFA initiates the peptide release, resulting in a carboxylate function at the C-terminus. By introducing a methoxy group to the aromatic ring, Mergler *et al*.[151] was able to significantly increase the linker lability under acidic conditions, resulting in the Super Acid Sensitive ResIN (SASRIN). In this case, a dilute, 1% solution of TFA in DCM can be used to efficiently cleave the synthesized peptide. These examples are shown in Figure 7.

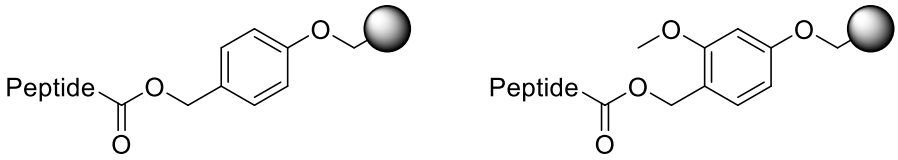

Figure 7: Acid labile benzyl-type linkers. Wang resin (left) and SASRIN resin (right).

As mentioned before in section 1.2.4, SASRIN resin modified with cysteamine (Figure 8) is also commercially available, and was successfully applied by Merz *et al*.[129] to obtain C-terminal thiol-functionalized peptides through acidic cleavage.

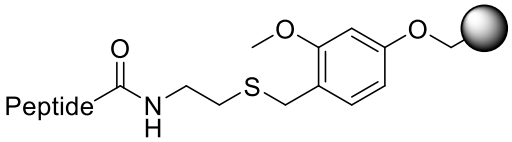

Figure 8: Cysteamine-functionalized SASRIN resin.

The most commonly used representative of the benzhydryl-type resins was reported by Rink.[152] These electron-rich structures possess either a hydroxyl (Rink-acid resin) or an amino (Rink-amide resin) group at the benzyl position. Upon cleavage with TFA, the resulting peptides can have either carboxylate or carboxamide residues at the C-terminus, respectively. A variation of this strategy was developed by Sieber[153] by employing a xanthenyl function as a linker, which is even more acid labile. The peptide can be released using 1% TFA in DCM, allowing access to the peptide with its side chains remaining protected. These examples are shown in Figure 9.

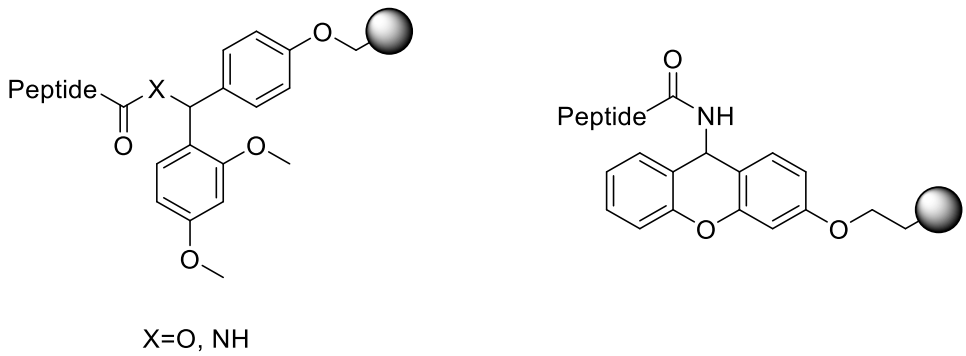

Figure 9: Acid labile benzhydryl-type linkers. Rink acid resin (left, X=O), Rink amide resin (left, X=NH), Sieber amide resin (right)

Trityl-type structures are known for their ability to form highly stable carbocations, which makes them an attractive option for acid-labile linkers. The first building block can be anchored by an ester bond, and the cleavage can be achieved with 1% TFA in DCM. In a detailed analysis conducted by Barlos *et al.,*[154] the researchers concluded that the 4-methoxy substitution results in the most labile derivative, followed by the 4-methyl, unsubstituted and 2-chloro linkers in that order. An example is shown in Figure 10.

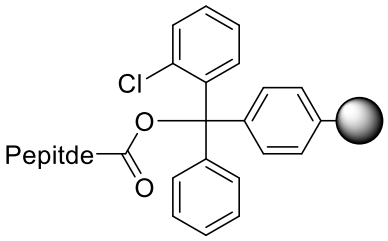

Figure 10: Acid labile 2-chlorotrityl resin.

The backbone amide linker (BAL) resin was developed by Boas *et. al*.[155] to enable access to additional C-terminal functionalities. The linker's core is a tris(alkoxy)benzylamide moiety, that is formed by linking the amino group of the C-terminal amino acid to the formyl group of the linker by reductive amination. In the following step, the next amino acid is coupled to this secondary amine. The advantage of this method is that various functions can be introduced at the C-terminus, such as dimethyl acetals (resulting in C-terminal aldehydes after cleavage), *t*Bu esters (giving peptide acids), *t*Bu ethers (giving peptide alcohols), or trithioortho- (TTO) esters (giving thioesters). Examples for this strategy are shown in Figure 11.

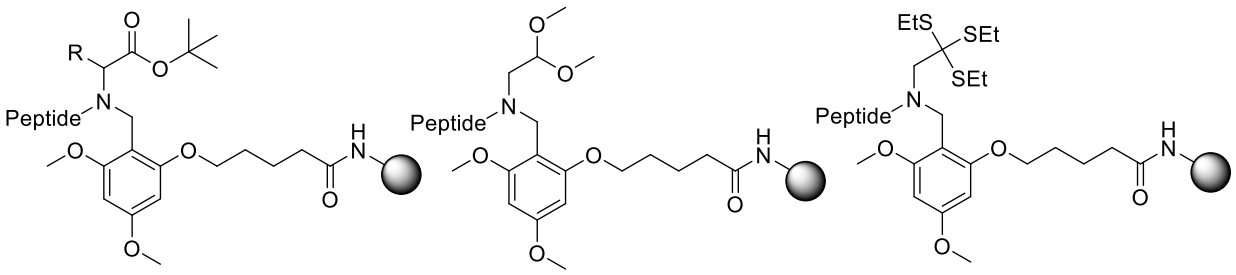

Figure 11: Common BAL resins. Cleavage of tBu esters (left) give peptide acids, dimethyl acetals (middle) give aldehydes, and trithioorthoesters (right) give thioesters).

In some cases, it was necessary to implement complete orthogonality in the acidic deprotection reaction and cleavage step. This offers the benefit that byproducts originating from side chain protecting groups can be washed away easily, and they will not be present in the crude cleaved peptide solution. As my ultimate goal has been to perform the biochemical assays on crude peptides, it was imperative to minimize impurities in the library compounds as much as possible. For this reason, we decided to rely on this orthogonal strategy.

An example for this approach is the safety-catch linker (SCL),[156] which is based on an aliphatic sulfonamide that can be acylated with the first amino acid building block.. After completing the SPPS (and, if desired, the acidic deprotection), the amide nitrogen is first alkylated by either TMS-diazomethane or iodoacetonitrile, then the cleavage is achieved by nucleophilic displacement with a thiol reagent such as ethyl-3-mercaptopropionate, benzyl mercaptan or sodium thiophenolate.[156,157] Another linker that features a safety-release function and that can be cleaved by reduction has been developed by Zitterbart and co-workers.[158] A similar strategy was developed by Blanco-Canosa and Dawson[159] through the MeDbz linker moiety. In their report, they used *p*-nitrophenyl chloroformate to form mildly activated aromatic *N*-acylurea functionalities on resin, which can be also cleaved by thiols. The structures of these linkers are shown in Figure 12. Both the SCL and the MeDbz approaches yield C-terminal thioester peptides, which are useful functionalities for joining two peptides through native chemical ligation.[160]

Figure 12: Resins with acid stabile sulfonamide-based safety-catch linker (left) and the MeDbz linker (right)

As discussed earlier, the electronic effects of substituents on aromatic linkers have a significant effect on their stability in acidic conditions. If the aromatic system is attached to the resin through an amide bond, its strong electron-withdrawing effects destabilize the carbocation, therefore the peptide will not be cleaved when the resin is suspended in TFA. The hydrazine benzoyl linker[161,162] is an example for such a benzamide structure, and it can be used for the orthogonalization of side chain deprotection and cleavage. The resin's hydrazine function is acylated by the first amino acid. The cleavage is facilitated by Cu(II) acetate catalysis, and an amine or alcohol as a nucleophile to give peptide amides or esters, respectively. The hydroxymethyl benzamide (HMBA) resin[163,164] is also an acid-stable resin that can be acylated with the first amino acid building block at the hydroxymethyl position. This resin offers the considerable advantage that a wide variety of nucleophiles can be used for cleavage, providing access to many C-terminal functionalities. Cleavage with amines yields peptide amides, alcohols give esters, hydrazine gives hydrazides, and peptide acids are obtained through aqueous hydrolysis. Thioesters can also be synthesized by applying thiols to cleave the ester bond. According to Hansen *et al.,*[165] the effectiveness of the cleavage is influenced by the steric accessibility of the ester bond, and bulky side chains on the first amino acid can significantly decrease the peptide yield. Examples for benzamide-type resins are shown in Figure 13.

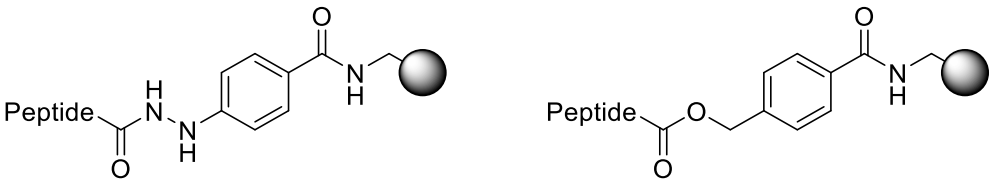

Figure 13: Resins with acid stabile benzamide-type linkers. Hydrazine benzoyl linker (left), HMBA linker (right).

Disulfide bonds can also be used to link the peptide to the solid phase during SPPS. This strategy provides access to peptides with C-terminal thiol functions, that can be convenient reactive handles for subsequent modification. This strategy was extensively used in my research, and the subsequent chapters provide in-depth information about this approach. An example for disulfide linkage is shown in Figure 14.

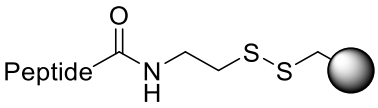

Figure 14: Example for an acid stabile disulfide-based linker.

Another convenient way to achieve complete orthogonality is through the use of photolabile linkers, in which case the peptides are released by irradiation with light. A vast number of such linker structures have been developed, and they were reviewed in detail by Mikkelsen *et al*.[166]

1.3.4 Coupling reagents

In addition to the polymer support, the protection-deprotection and the cleavage strategies, the appropriate coupling reagent must also be chosen for a successful SPPS, which activates the carboxylate function of the building blocks, thereby increasing their reactivity towards free amines.

In the early examples for SPPS; the activating agents of choice were carbodiimides, which carboxylate activation under mild conditions. However, N^{α}-racemization of amino was a common problem with this synthetic strategy. To suppress racemization, 1-hydroxybenzotriazole (HOBt)[167,168] and 1-hydroxy-7-azabenzotriazole (HOAt)[169] were introduced as additives.

Based on these compounds, convenient uronium-type coupling reagents such as (dimethylamino)-*N*,*N*-dimethyl(3H-[1,2,3]triazolo[4,5-b]pyridin-3-yloxy)methaniminium hexafluorophosphate (HATU) and (1H-benzotriazol-1-yloxy)(dimethylamino)-*N*,*N*-dimethylmethaniminium hexafluorophosphate (HBTU) were developed. They offer an efficient way to form amide bonds under mild conditions since they can form HOAt and HOBt esters with carboxylates respectively.[170] Since these functionalities are effective leaving groups, these activated esters are highly reactive towards nucleophiles such as free amines. The structure of these coupling reagents and the mechanism of the coupling reaction is illustrated in Figure 15.

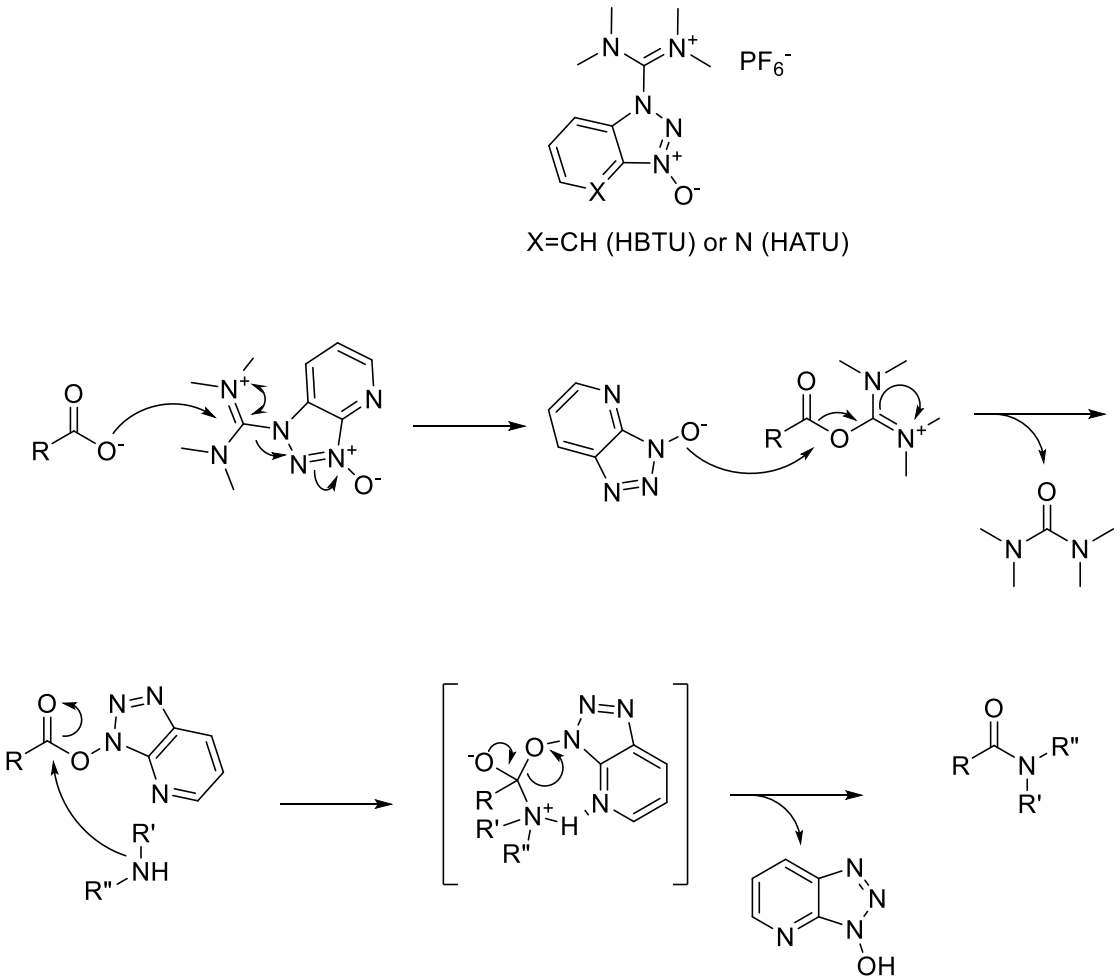

Figure 15 : Structure of the amide coupling reagents HBTU (X=CH) and HATU (X=N) (top); Reaction mechanism of the amide coupling reaction (bottom).

It is worth mentioning that in the case of HATU, the nitrogen atom of the pyridine ring can serve as a hydrogen bond acceptor in the transition state intermediate, allowing the formation of a seven membered ring. This property makes it more effective as a coupling reagent. In addition to HATU and HBTU, a large number of alternatives have been developed for peptide synthesis, and these compounds were reviewed by several authors.[171–173] However, the coupling reagent was not varied during the development of the methods presented in the chapters below, therefore they are not discussed in further detail here.

1.3.5 Synthesis strategy used in this work

In the following chapters, I describe experiments that use aminomethyl-functionalized PS resin, coupled to a mercaptopropionic acid moiety, resulting in a cheap thiol-functionalized resin. The thiol was then coupled to aminothiol building blocks through a disulfide bridge,

which enables orthogonal deprotection and cleavage steps and afford C-terminal thiol peptides. The SPPS was performed using conventional Fmoc chemistry and HATU as coupling reagent.

1.4 References

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2. Aim of the thesis

Drug discovery campaigns often start with identifying hits through HTS of chemical libraries. The size, quality and structural diversity of these libraries are known to be key determinants of screening success, therefore there is a continuous demand for novel compound collections and technologies to generate them. As peptides are oligomers of amino acid building blocks, automated, parallel, high-throughput SPPS offers a convenient way to generate large and diverse combinatorial peptide libraries in a quick and efficient manner. Furthermore, peptides are of great interest as therapeutic modalities, combining the synthetic accessibility and favorable pharmacological properties of small molecules and the ability of biologics to bind to challenging targets with high specificity.

The goal of my thesis was to develop robust high-throughput synthesis methods that afford crude products of cyclic or linear peptide compounds with a purity sufficiently high for direct-to-biology screening, then generate libraries and screen them against model targets to identify novel binders, providing a proof of concept to the new strategies.

In the first part of my work, detailed in Chapter 3, I developed a procedure for peptide cleavage through disulfide reduction with a volatile reducing agent, 1,4-butanedithiol. This protocol provides facile access to pure thiol containing peptides that can be directly reacted with electrophiles, resulting in high quality libraries of thioether peptides.

In the second part of this thesis, described in Chapter 4, my goal was the development of a novel method for the functionalization of thiol resins with various cysteamine analogues, introducing a new diversity element that was not variable in peptide libraries previously synthesized in our group. This was necessary because with the new reductive release procedure, very short dithiol peptides have become easily accessible that are good starting points for the development of orally available drugs. However, it is more challenging to include sufficient structural diversity with fewer diversity elements. The utility of this additional backbone diversity is illustrated by potent and novel thrombin inhibitors identified through the synthesis and high-throughput screening of a cyclic dithioether peptide library.

In the final part, detailed in Chapter 5, I aimed to apply the methods and combine them with the previously described solution-phase, nanomolar-scale diversification strategies for the

synthesis of a low MW small-molecule-like peptide library, and introduce large chemical diversity by introducing one diamino acid and two carboxylic acid building blocks for the construction of library compounds. Potent and novel inhibitors of the model target thrombin were identified from the HTS of this library with favorable properties. These results highlight that our methods can be also used for the discovery of compounds that resemble small molecules and provide starting points for the development of membrane permeable therapeutics.

3. Solid-phase peptide synthesis on disulfide-linker resin followed by reductive release affords pure thiol-functionalized peptides

3.1 Work contribution

Chapter 3 of this thesis is based on the following publication:

Solid-phase peptide synthesis on disulfide-linker resin followed by reductive release affords pure thiol-functionalized peptides

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Specific contributions:

I co-designed the research project and performed the experiments. C.H, G.K.M., Z.B. and M.L.M conceptualized this study. G.K.M. innovated the use of BDT as a removable reducing agent. A. L. N. and P.M.F.P tested the method for the synthesis of longer peptide sequences. C.H. and Z.B. wrote the manuscript, and all authors reviewed the final version.

3.2 Abstract

Thiol groups are suitable handles for site-selectively modifying, immobilizing or cyclizing individual peptides or entire peptide libraries. A limiting step in producing the thiol-functionalized peptides is the chromatographic purification, which is particularly laborious and costly if many peptides or even large libraries are to be produced. Herein, we present a strategy in which thiol-functionalized peptides are obtained in >90% purity and free of reducing agent, without a single chromatographic purification step. In brief, peptides are synthesized on a solid support linked via a disulfide bridge, the side-chain protecting groups are eliminated and washed away while the peptides remain on resin, and sufficiently pure peptides are released from the solid support by reductive cleavage of the disulfide linker. Application of a volatile reducing agent, 1,4-butanedithiol (BDT), enabled removal of the agent by evaporation. We demonstrate that the approach is suited for the parallel synthesis of many peptides and that peptides containing a second thiol group can directly be cyclized by bis-electrophilic alkylating reagents for producing libraries of cyclic peptides.

3.3 Introduction

Sulfhydryls, also called thiols, are widely used as functional groups in peptides for labeling, immobilizing or cyclizing individual peptides or libraries of peptides.[1] Several thiol-reactive chemical groups such as maleimides, haloacetamides, vinylsulfoxides, bromomethylbenzenes and pyridyl disulfides are suited to conjugate fluorophores, radionuclides, biotin and other functional molecules via sulfhydryl groups to peptides.[2] Cross-linker reagents containing the same thiol-reactive groups are used to conjugate peptides to surfaces, proteins, DNA strands or other macromolecules. Thiol groups are also used for peptide cyclization, wherein two or three thiol groups are introduced into peptides for reaction with bi- or tri-valent cross-linking agents to generate mono- or bicyclic peptides..[3] Our laboratory has cyclized thousands of cysteine-containing peptides that were identified in phage display selections against diverse protein targets.[4] We have also cyclized large libraries of short thiol-functionalized peptides for generating and screening sub-kilodalton macrocyclic compound libraries.[5]

The thiol groups can easily be introduced into peptides during solid-phase peptide synthesis (SPPS) by incorporating cysteines, cysteine analogs, or by appending non-amino acid thiol-building blocks. However, a major bottleneck in producing peptides is the chromatographic purification, in particular for generating large numbers of peptides. In order to omit peptide purification after SPPS, strategies were developed in which thiol-functionalized peptides are deprotected while still linked to the solid phase, allowing washing away the protecting groups and releasing peptides in a rather pure form.[6–8] Tegge and co-workers synthesized cyclic peptides on a solid support via a disulfide linker and released them by reducing the disulfide bond by dithiothreitol (DTT).[6] Gless and Olsen produced thiol-functionalized cyclic peptides applying Dawson's 3-amino-4-(methylamino)benzoic acid (MeDbz) linker[9] which yielded unprotected thioester peptides that were cyclized by native chemical ligation.[7] Our laboratory has recently developed a strategy for efficiently accessing disulfide-cyclized peptides by synthesizing peptides via a disulfide linker on solid phase and releasing them via a cyclative disulfide exchange reaction.[8] In peptides produced with all the described strategies, the thiol groups tend to oxidize during the production or storage, or are fully oxidized such as in the cyclative release approach, and thus require to be treated with reducing agents prior to conjugation reactions. The reducing agents applied for breaking the disulfide bridges, however, require to be removed afterwards as they would interfere with the chemical reactions, and this involves a cumbersome purification step. Phosphine-based reducing agents such as tris-(2-carboxyethyl)phosphine (TCEP) were reported to have a lower

reactivity towards thiol-reactive reagents and were used successfully in many bioconjugation applications without removal,[10] but they interfere with thiol-conjugation reactions too[11,12] and proved to not be a viable option for cyclizing thiol-containing peptides for peptide drug development applications in our laboratory.

Herein, we propose to synthesize peptides on a disulfide linker resin and to release them using a volatile reducing agent that can be removed by evaporation, and thus do not need a purification step prior to chemical modification of the thiol group(s). We show that this strategy allows for efficient production of thiol-functionalized peptides and enables the cyclization of the peptides by bis-electrophilic reagents without a single chromatographic purification step.

3.4 Results and discussion

We first tested if peptides synthesized via a disulfide linker on a solid support can be released by a volatile reducing agent and if the latter one can be quantitatively removed from the eluted peptide by evaporation (Fig. 1a). We synthesized the four peptides Ala–Trp–Mea, Tyr–Ala–Mea, Trp–Ala–Mea and Ala–Tyr–Mea that are linked to thiol-functionalized resin via the C-terminal 2-mercapto-ethylamine (Mea) group (Fig. 1b and Supplementary Fig. S1a). The peptides contain a tryptophan or tyrosine residue to allow precise determination of the amount of released peptide by absorbance measurement at 280 nm. We synthesized the peptides on polystyrene (PS) resin that is commonly used for peptide synthesis. We introduced the Mea building block in a disulfide exchange reaction using 2-(2-pyridyldithio)ethylamine hydrochloride and thiol-PS resin, as described before.[8] All peptides were synthesized in wells of a 96-well plate at a 5 μmol scale, in order to test the conditions at which we later planned synthesizing peptide libraries at high-throughput.

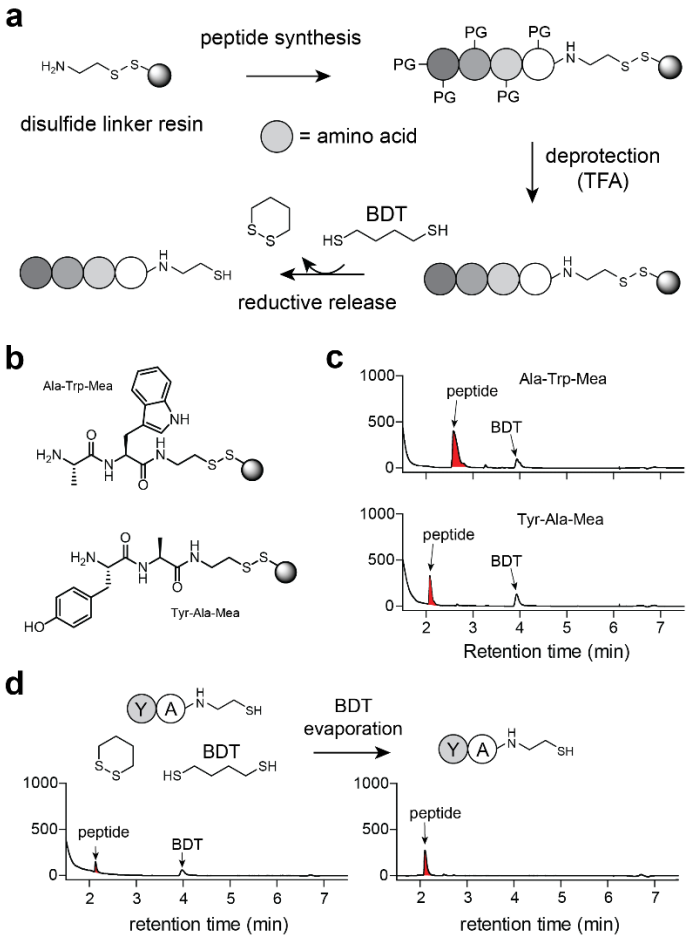


Figure 1: Strategy for the reductive release of peptides synthesized on solid phase via a disulfide bridge. (a) Overview of the synthetic steps. (b) Chemical structures of peptides used to test the reductive release of peptides from solid phase. Mea = mercaptoethylamine (c) HPLC chromatograms of peptides released by reduction with BDT. (d) Removal of reducing agent by centrifugal vacuum evaporation.

We tested the release of disulfide-immobilized peptide from PS resin by incubating the beads overnight with 200 μl DMF containing 20 equivalents of the volatile reducing agent β-mercaptoethanol (β-Me; 0.5 M). LC-MS analysis of the peptides showed efficient release but also that up to 40% of the product occurred disulfide-linked to β-Me (Supplementary Fig. S1b). We reasoned that the extent of adduct could potentially be reduced by using a larger molar excess of β-Me and/or by repeating the reduction, but as this would require additional working steps, we looked for a solution based on a different reducing agent. The reducing agent DTT reacts via a disulfide exchange reaction as β-Me but does not stop at the mixed-disulfide species because the second thiol of DTT has a high propensity to close, forming oxidized DTT (as a 6-membered ring) and leaving behind a reduced disulfide bond. Incubation of the resin carrying either peptide Ala–Trp–Mea or Tyr–Ala–Mea with four equivalents of DTT efficiently released the peptides without forming peptide-reducing agent adducts. However, we found that DTT could not be removed by vacuum evaporation in a standard rotary vacuum concentrator due to its rather high boiling point of 365 °C (Supplementary Fig. S2a).

A reducing agent that eliminates itself in the same way as DTT by forming a 6-membered ring in the oxidized form, but that evaporates at 195 °C and thus at a lower boiling point, is 1,4-butanedithiol (BDT).[13] For BDT, a boiling point of 106 °C was reported under vacuum at 40 mbar,[14] and we thus expected that it could be removed at a pressure of 0.1 mbar without elevating the temperature, using centrifugal vacuum evaporation. We incubated 5 μmol of the resin-linked peptides with 200 μl DMF containing four equivalents BDT (100 mM). The peptides were efficiently released by BDT as analyzed by LC-MS (Fig. 1c and Supplementary Fig. S2b). For all the four peptides, a major peak corresponding to the desired product was observed. The yields of the desired products were 3.4, 1.3, 4.2 and 4.3 μmol, respectively, which corresponded to 68, 25, 84 and 86% yield (based on 5 μmol resin loading). Pleasingly, the excess of BDT and oxidized BDT was efficiently removed by centrifugal evaporation under vacuum on a SpeedVac, yielding peptide as solid or viscous oil, and free of other molecules (Fig. 1d).

We next aimed at applying the reductive release strategy for synthesizing short dithiol peptides, which can be efficiently cyclized by bis-electrophile reagents such as **1**, and which are of great interest for the generation of small, membrane-permeable macrocycles (Fig. 2a). In previous attempts to produce dithiol peptides using a cyclative disulfide release strategy (Fig. 2b),[8] we had difficulties to obtain peptides in good yields if they were particularly short, likely due to steric hindrance of the intramolecular disulfide exchange reaction. With the new

approach releasing the peptides by disulfide bond reduction, we expected an efficient release as no cyclization was required. We synthesized a panel of eight short dithiol peptides of the format Mpa–Xaa–Mea (Mpa = 3-mercaptopropionic acid with the thiol group protected by a trityl group), wherein the Xaa amino acids were Trp, Tyr, Ser, His, Phe, Arg, Asp and Ala (Fig. 2c). Treatment of the resins with BDT indeed lead to efficient release of all peptides (Fig. 2d, left panels, and Supplementary Fig. S3). The main products were the desired dithiol peptides. Side products were found in only small quantities and were dithiol peptides that carried a tert-butyl group (tBu).

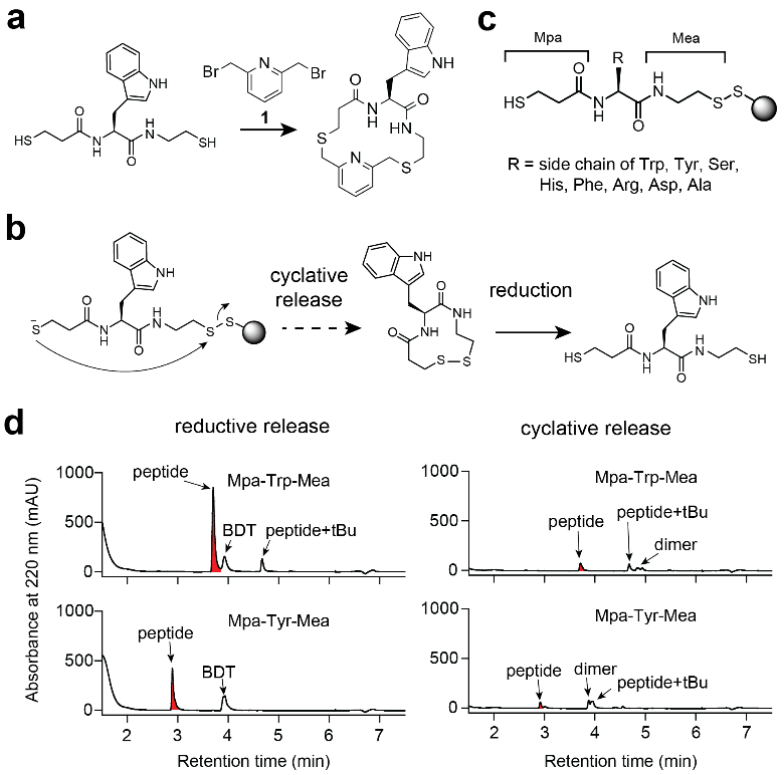


Figure 2: Synthesis of short dithiol peptides for the generation of macrocyclic compound libraries. (a) Cyclization reaction illustrated with the dithiol peptide Mpa–Trp–Mea and the bis-electrophile reagent 2,6-bis(bromomethyl)pyridine (**1**). (b) Recently developed "cyclative disulfide release" reaction.[8] Short peptides such as those containing only one amino acid between the two flanking thiol-containing structures (three building blocks) are not efficiently released due to conformational constraints (indicated by dashed line of arrow). (c) Structure of short dithiol peptides. (d) Comparison of yields obtained by reductive release (left) and cyclative release (right). The peaks of the desired peptides are highlighted in red. HPLC chromatograms of more dithiol peptides are shown in Supplementary Fig. S3.

Attempts to suppress the tert-butyl side product by thiol-based scavengers were not successful as they also broke the disulfide bridge. For comparison, we applied conditions to release the peptides via the cyclative release approach (Fig. 2b), which yielded the short peptides in around 10 to 100-fold smaller quantities (Fig. 2d, right panels, and Supplementary

Fig. S3) and showed the advantage of releasing the short peptides by disulfide reduction. As a positive control for the cyclative release method, we applied longer peptides such as Mpa–Lys–Trp–Gly–βAla–Mea which were satisfactorily released via the cyclative release mechanism, most likely due to the smaller conformational constraints (Supplementary Fig. S4). The yields of the short peptides Mpa–Trp–Mea and Mpa–Tyr–Mea released by BDT reduction were 3.6 and 4.3 µmol, respectively, which corresponded to 71% and 85% yield. Taken together, the reductive release strategy with BDT allowed accessing short dithiol peptides that could not be obtained in sufficient yields with the previously applied cyclative release approach.

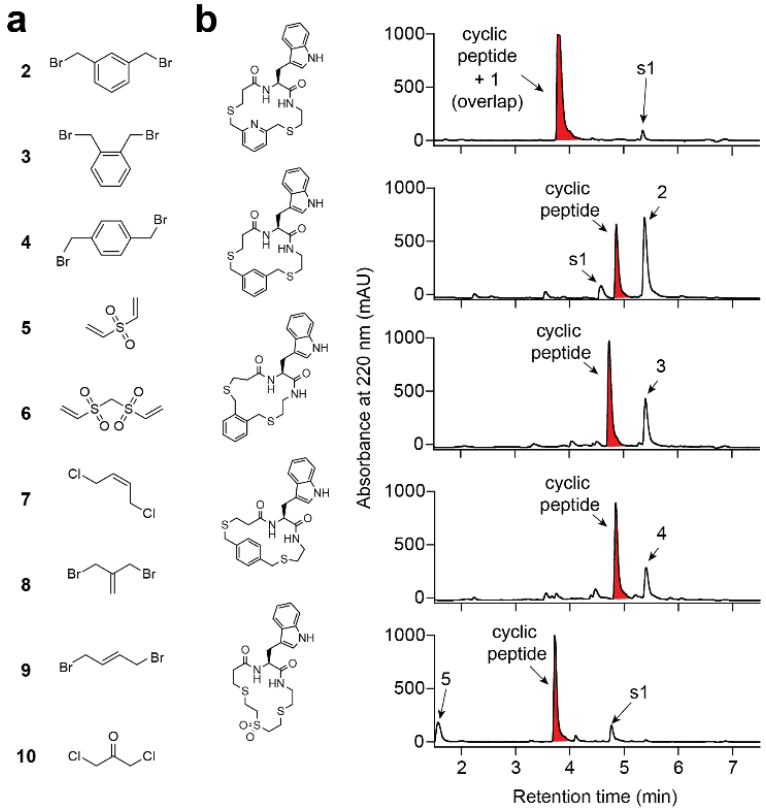


Figure 3: Cyclization of dithiol peptides by bis-electrophile reagents. (a) Bis-electrophile reagents **2–10**. (b) HPLC chromatographic analysis of Mpa–Trp–Mea cyclized by reagents **1–5**. The desired cyclic products are highlighted in red. s1 = side-product tert-butyl-peptide-linker (structure shown in ESI Fig. S8†). Data for cyclization with reagents 6–10 are shown in Supplementary Fig. S6.

We next tested if the reducing agent BDT can be removed from the dithiol peptides by centrifugation under vacuum, which was required for the subsequent cyclization of the peptides by bis-electrophile reagents. Peptides synthesized at a scale of 5 µmol and released in 200 µl DMF containing 100 mM BDT and 100 mM TEA were centrifuged at 0.1 mbar, 30 °C and at 400 × *g* in 96-well plates. The solvent was efficiently removed in less than one hour,

even if all wells of the microwell plate were filled. LC-MS analysis of the peptides showed that all BDT was removed. However, some peptides had up to 10% of back-oxidized product. We speculated that the oxidation was enabled by the alkaline pH, and we thus added two equivalents of TFA relative to TEA to each well prior to the centrifugal vacuum evaporation. With this acidification procedure, the fraction of oxidized peptide could be reduced efficiently (Supplementary Fig. S5).

We tested if the short dithiol peptides Mpa–Trp–Mea and Mpa–Tyr–Mea could be cyclized by a panel of ten bis-electrophile reagents (reagents **1** to **10**; Fig. 2a and 3a and Supplementary Fig. S6). The cyclization of peptides via two or three cysteines by electrophilic linker reagents is highly efficient and clean if performed at dilute concentrations, with peptides at concentrations of around 1 mM (or lower) and the cyclization reagents applied at a small excess.[15,16] We dissolved the peptides (5 mM) in 1 ml MeCN : water 1 : 1, added 3 ml of NH_4HCO_3 buffer (100 mM, pH 8.0) containing 10% acetonitrile, and immediately added 1 ml bis-electrophile reagents in MeCN (10 mM). The final concentrations of peptide and cyclization reagent were around 1 mM and 2 mM, respectively. The HPLC chromatograms of the cyclization reactions with the peptide Mpa-Trp-Mea are shown in Fig. 3b and Supplementary Fig. S6, and those with the peptide Mpa-Tyr-Mea in the Supplementary Fig. S7. In most of the reactions, the dithiol peptides were cyclized nearly quantitatively with yields higher than 90%. The small quantity of side product was mainly peptide that reacted with only one thiol group because one of them was modified with tert-butyl derived from side chain protecting groups (Supplementary Fig. S8). Addition of six equivalents of β-Me (relative to peptide) allowed efficient quenching of the excess of bis-electrophile reagents (Supplementary Fig. S9).

A second goal of this study was to assess if slightly longer peptides could be synthesized on a disulfide linker solid phase. In our previous work using the above described cyclative release strategy, we had tested the synthesis of rather short peptides having 2–3 amino acids, and it was not clear if the synthesis of longer peptides would be feasible, as the disulfide linker is exposed many more times to piperidine and could potentially break. In order to test the synthesis of longer peptides, we produced alanine-scan mutants of a cyclic peptide ligand of the cancer target KRAS, that was previously identified by phage display[17] and that is of interest to our laboratory (Fig. 4a). We synthesized the undecamer CPLYISYDPVC with Mpa and Mea in place of the N- and C-terminal cysteines. Despite the ten Fmoc deprotection steps that exposed the disulfide linker resin to the base piperidine, the peptides were obtained in good yields ranging from 28% to 59% based on resin loading, indicating that the disulfide

bridges resist to a large extent the repetitive treatment with the base (Fig. 4b and Supplementary Fig. S10). Centrifugal evaporation under vacuum again efficiently removed BDT and allowed efficient peptide cyclization with a bis-electrophilic cyclization reagent (10) (Fig. 4c and d). The example with several different peptides from the alanine-scan showed that peptides containing around 10 amino acids can be synthesized efficiently with the new method.

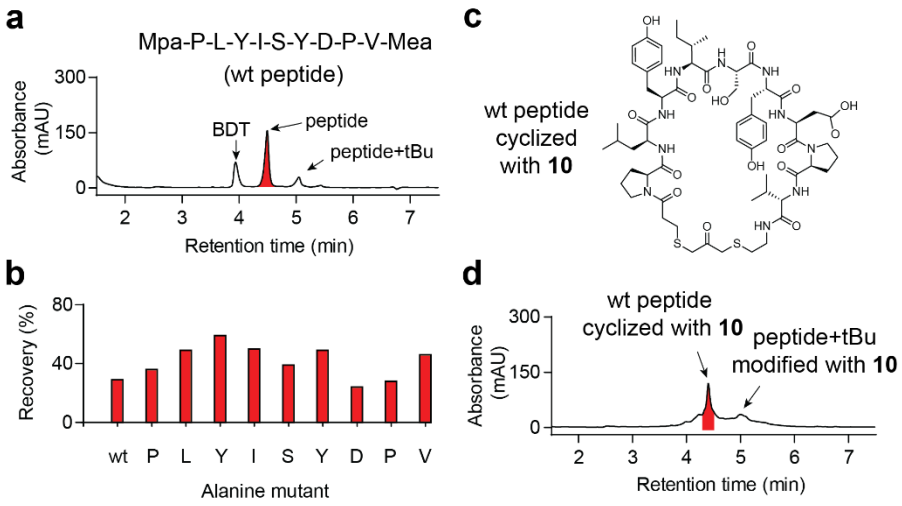


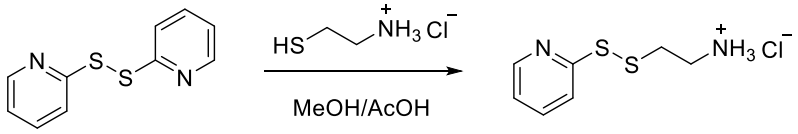
Figure 4: Synthesis of longer peptides, tested with sequences based on the phage display-selected KRAS-specific peptide KRpep-2d. (a) HPLC chromatogram of linear peptide containing the core sequence of KRpep-2d (wt peptide). (b) Yields of alanine mutants of KRpep-2d indicated as % recovery relative to the resin loading. HPLC chromatograms are shown in Supplementary Fig. S10. (c) Chemical structure of the wt peptide cyclized with linker **10**. (d) HPLC chromatogram of the wt peptide cyclized with linker **10**.

3.5 Conclusion

In conclusion, we were able to establish a SPPS and elution strategy that delivers thiol-functionalized peptides in high yields and purities, all without the need for a chromatographic purification step. Importantly, the strategy involves an easy and efficient step to remove the reducing agent needed for peptide release, allowing direct conjugation or cyclization of the thiol-functionalized peptides with electrophilic reagents. We show that the strategy can be applied for the synthesis of peptides having at least ten amino acids. Given the wide use of thiol-functionalized peptides in research, the new method may be applied for accessing peptides needed in diverse applications, ranging from peptide production for alanine scans, peptide array generation for linear and non-linear epitope scanning (similar to SPOT synthesis), synthesis of the numerous peptides identified by in vitro evolution using phage- or mRNA display selections, or the generation of cyclic peptide libraries. The herein presented volatile reducing agent BDT, that we report can be efficiently removed by centrifugal vacuum evaporation, might be used broadly, as for example for breaking disulfide bridges of proteins or for reducing thiol-functionalized DNA strands and carbohydrates.

3.6 Materials and methods

Synthesis of 2-(2-pyridyldithio)ethylamine hydrochloride



To a stirring solution of 2,2'-dipyridyldisulfide (4.41 g, 20 mmol) in MeOH (16 ml with 2% [v/v] AcOH) was added cysteamine hydrochloride (1.14 g, 10 mmol) dissolved in MeOH (10 ml, with 2% [v/v] AcOH dropwise over 15 min. The reaction mixture was stirred overnight at room temperature (RT) and concentrated under reduced pressure to afford a yellow oil. The residue was dissolved in MeOH (16 ml), distributed to eight 50-ml falcon tubes, and precipitated by addition of ice-cold diethyl ether (48 ml to each tube). The tubes were incubated at -20 °C for 30 min and centrifuged at 3400 × *g* (4000 rpm on an explosion proof Sigma centrifuge) at 4 °C for 30 min. The product was obtained as a colorless solid after repeating the precipitation 8 times (yield = 90%).

Preparation of cysteamine-polystyrene resin

The following procedure describes the preparation of polystyrene resin carrying around 2 mmol cysteamine immobilized via a disulfide linker, which is sufficient for the synthesis of 4 × 96 peptides at a 5 μmol scale. Into each one of four 20 ml plastic syringes was added 563 mg resin (Rapp Polymere Polystyrene A SH resin, 200-400 mesh, 0.95 mmol/gram loading). The resin was washed with DCM (15 ml), then swelled in MeOH/DCM (7:3; 15 ml) for 20 min. Pyridyl-cysteamine disulfide (2.10 grams, 9.42 mmol, 4.4 equiv.) was dissolved in MeOH (23 ml) and then DCM (53 ml). Then *N,N*-diisopropylethylamine (DIPEA; 410 μl) was added. A volume of 19 ml of this solution was pulled into each syringe and the syringes were then shaken at RT for 3 hours. The pyridyl-cysteamine solution was discarded and the resin was washed with MeOH/DCM (7:3; 2 × 20 ml), then with DMF (2 ×. 20 ml). The resin was combined into a single syringe as a suspension in DMF, washed with a solution of 1.2 M DIPEA in DMF (11.8 ml) for 5 min to ensure that all amines were neutralized. This solution was discarded, and the resin was washed with DMF (2 × 20 ml), then with DCM (4 × 20 ml), then kept under vacuum overnight to yield a free-flowing powder.

Fmoc peptide synthesis in 96-well plates

Peptides were synthesized at a 5 μmol scale in 96-well peptide synthesis filter plates (Orochem, cat. # OF1100) using an automated peptide synthesizer (Intavis MultiPep RSi).

Cysteamine-PS resin (around 5 mg, 0.95 mmol/g, 5 μmol scale) was distributed as powder to each well of the plate. The resin was washed with DMF (3 × 225 μl). In this and all the following washing steps, the resin was incubated for 1 min. The following reagents were transferred to tubes in the indicated order, mixed, incubated for 1 min, transferred to the resin in the microwell plate, and incubated for 45 min without shaking. Reagents: 50 μl HATU (500 mM in DMF, 5 equiv.), 5 μl *N*-methylpyrrolidone (NMP), 12.5 μl of *N*-methylmorpholine (NMM in DMF, 4 M, 10 equiv.) and 53 μl of amino acid (500 mM in DMF, 5.3 equiv.). The final volume of the coupling reaction was 120.5 μl and the final concentrations of reagents were 208 mM HATU, 415 mM NMM and 220 mM amino acid. Coupling was performed twice. The resin was washed with DMF (1 × 225 μl). Unreacted amino groups were capped by incubation with 5% acetic anhydride and 6% 2,6-lutidine in DMF (100 μl) without shaking for 5 min. The resin was washed with DMF (8 × 225 μl). Fmoc groups were removed by incubation twice DMF (120 μl) containing 20% (v/v) piperidine without shaking for 5 min each. For the synthesis of longer peptide sequences, the incubation time was reduced from 5 min to 2 min, in order to reduce exposure to the base. The resin was washed with DMF (8 × 225 μl). At the end of the peptide synthesis, the resin was washed with DCM (2 × 200 μl).

Peptide side chain deprotection in 96-well plates

For removing protecting groups from amino acid side chains as well as from Mpa, the bottom of the 96-well synthesis plate was sealed by pressing the plate onto a soft 6 mm thick ethylene-vinyl acetate pad, and the resin in each well was incubated with a solution of TFA:TIPS:H_2O (95:2.5:2.5 [v/v/v], around 300 μl). The plates were covered with an adhesive sealing film (iST scientific, QuickSeal Micro, cat. # IST-125-080-LS), then weighed down by placing a weight (1 kg) on top to prevent leakage. After 1 h incubation, the synthesis plate was placed onto a 2 ml deep-well plate, and the TFA mixture was allowed to drain. The synthesis plate was again sealed and the deprotection procedure was repeated. The wells were washed with DCM (3 × 500 μl; added with syringe) that was run through the wells by gravity flow. The resin was dried by placing the synthesis plate into a vacuum manifold for 5 min.

Reductive peptide release by β-Me, DTT or BDT

For releasing the peptides from the resin, the bottom of the 96-well synthesis plate was sealed by pressing the plate onto a soft 6 mm thick ethylene-vinyl acetate pad, and the resin in each well was incubated with a solution of 200 μl DMF containing 500 mM of β-Me, or 100 mM DTT, or 100 mM BDT, and 100 mM TEA for 4 h at RT. After this time, the samples were collected

in a 96-deep well plate by centrifugation at 250 × *g* (1100 rpm on a Thermo Scientific Heraeus Multifuge 3L-R centrifuge with a Sorvall 75006445 Rotor, radius = 19.2 cm rotor) for 2 min at RT.

Cyclative release of peptides

The 96-well synthesis plate was sealed as described above and the peptides were released from the resin by incubation with a solution of 200 μl DMSO containing 250 mM TEA (10 equiv.) overnight at RT. After this time, the samples were collected in a 96-deep well plate by centrifugation at 250 × *g* (1100 rpm on a Thermo Scientific Heraeus Multifuge 3L-R centrifuge with a Sorvall 75006445 Rotor, radius = 19.2 cm rotor) for 2 min at RT.

LC-MS analysis of peptides after solid phase release or cyclization

For peptides released from solid phase (concentration up to 25 mM in DMF or DMSO), 1 μl of peptide was diluted in 60 μl of milliQ H_2O containing 0.05% formic acid. For peptides from cyclization reactions (concentration around 1 mM), 10 μl of the reaction mixture was mixed with 10 μl of milliQ H_2O containing 0.05% formic acid. Samples (10 μl injection) were analyzed on a Shimadzu 2020 single quadrupole LC-MS system using a reverse phase C18 column (Phenomenex Kinetex®, 2.6 μm, 100 Å, 50 × 2.1 mm) and a linear gradient of solvent B (MeCN, 0.05% formic acid) over solvent A (H_2O, 0.05% formic acid) from 0 to 60% in 5 min at a flowrate of 1 ml/min. Absorbance was recorded at 220 nm and masses were analyzed in the positive mode.

Centrifugal vacuum evaporation of reducing agent and solvent

The following example describes a peptide that had a concentration of 20 mM after reductive release. Of the 200 μl peptide released from the solid phase by reduction (in DMF containing 100 mM BDT and 100 mM TEA), 5 μl (0.1 μmol) were transferred to a well of a V-bottom 96-well plate (Ratiolab, 6018321, PP, unsterile). A volume of 7 μl of 1% TFA in water (v/v) was added to each well to reach 2 equiv. of TFA over TEA. This sample was subjected to centrifugal vacuum evaporation using Christ RVC 2-33 CDplus IR instrument to remove the solvent (DMF) and reducing agent (BDT). Samples were centrifuged at 0.1 mbar, 30 °C and at 400 × *g* (1750 rpm in a Christ 124700 rotor with 124708 plate holder inserts, radius = 10.5 cm). The quantity of peptide expected was so low that it could not be expected to be seen by eye, which was the case.

Cyclization of peptides

The reduced and dried peptide (0.1 μmol) was dissolved in 20 μl of 50% acetonitrile, 50%H_2O to reach a concentration of 5 mM. To this solution 60 μl reaction buffer (100 mM ammonium bicarbonate, pH 8.0, containing 10% acetonitrile [v/v]) was added followed by 20 μl of 10 mM cyclization linker in acetonitrile (2 equiv.). The final concentrations in the reaction were 1 mM peptide, 2 mM cyclization linker, 60 mM ammonium bicarbonate buffer and 35% acetonitrile. The plate was covered with a foil and the reaction incubated for 2 h at RT.

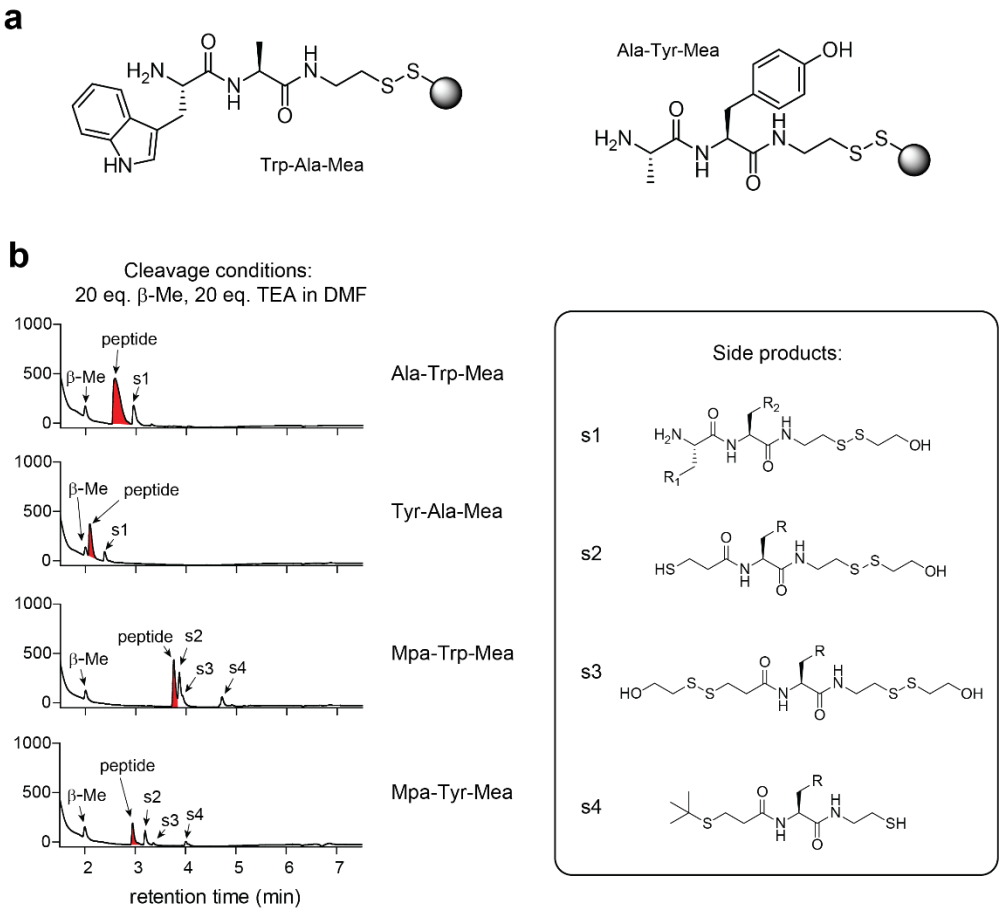
Quenching of linker reagents in cyclization reactions

After completion of the cyclization reaction, 4 μl of 150 mM β-Me in acetonitrile (0.6 μmol, 6 equiv. relative to the peptide) was added to the reaction mixture and incubated for 1 h at RT. The solvent (MeCN), buffer (bicarbonate) and excess β-Me were removed by centrifugal vacuum evaporation using Christ RVC 2-33 CDplus IR instrument. Samples were centrifuged at 0.1 mbar, 40 °C and at 400 × *g* (1750 rpm in a Christ 124700 rotor with 124708 plate holder inserts, radius = 10.5 cm). The quantity of peptide expected was so low that it could not be expected to be seen by eye, which was the case.

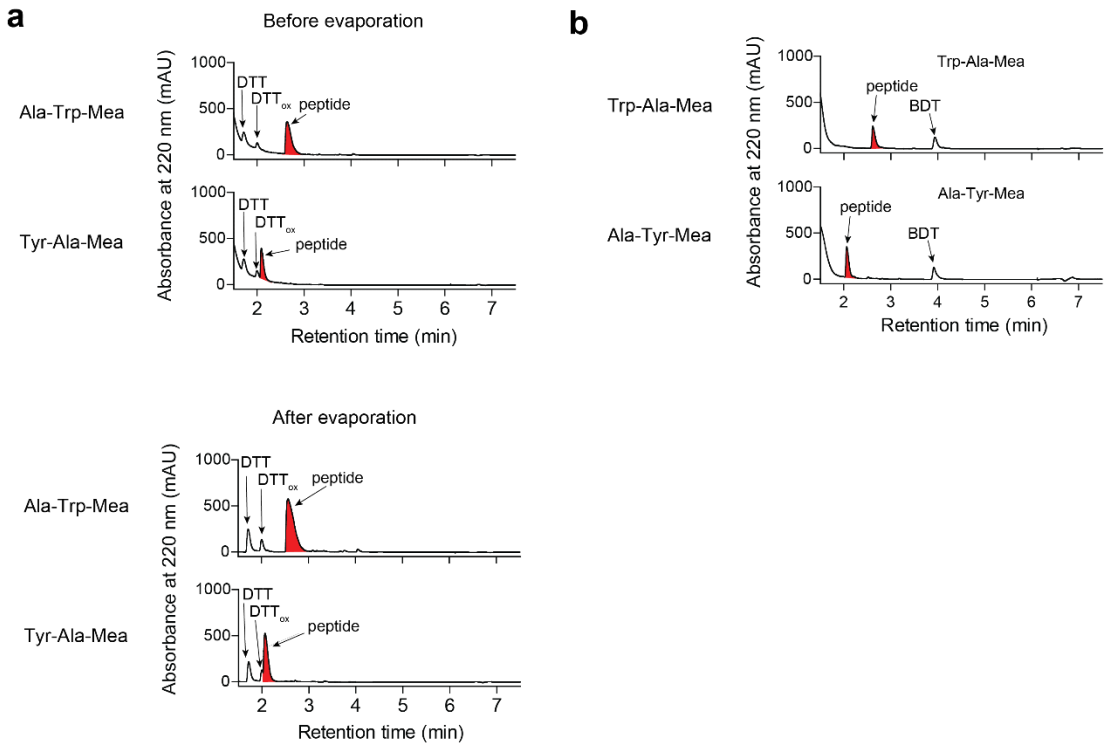
Concentration determination

The ThermoScientific NanoDrop 8000 spectrophotometer was blanked with milliQ H_2O. A baseline was measured at 280 nm with a mixture of 10 μl of cleavage mixture (250 mM TEA in DMSO for peptides released via cyclative release, and 100 mM TEA and 100 mM BDT in DMF for peptides released via reductive release) added to 90 μl DMSO. The average of three measurements was considered as a baseline value. For the concentration determination of peptide stocks, 1 μl of the peptide stock in the DMF solution obtained after the reductive release step was added to 9 μl of DMSO. The sample was measured three times, and the average was corrected by subtracting the baseline absorbance value. The extinction coefficients applied were ε_0(Trp)=5500 cm^{-1} M^{-1} for tryptophan containing sequences and ε_0(Tyr)=1490 cm^{-1} M^{-1} for tyrosine containing sequences. As BDT absorbs at 280 nm too, care was taken to ensure that the peptide concentrations measured were sufficiently high so the sample absorbance was several folds higher than the blank absorbance.

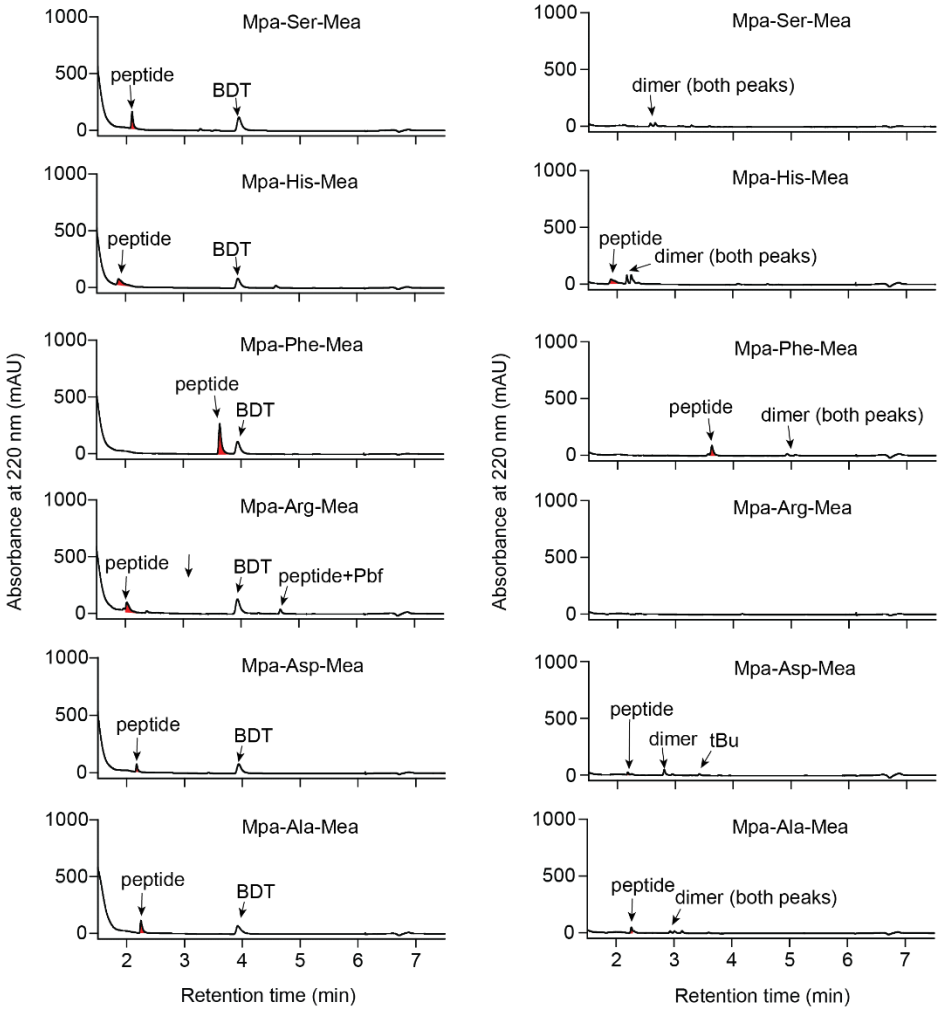
3.7 Supplementary information



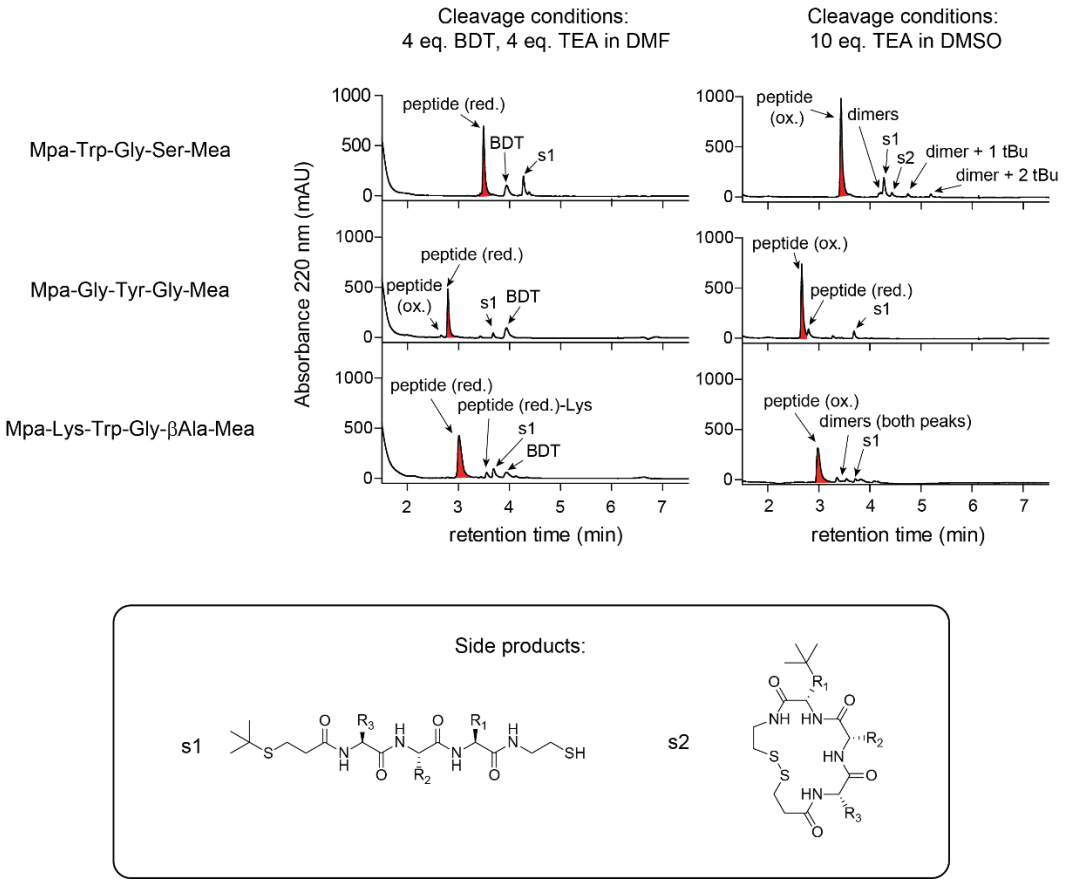
Supplementary Figure S1. SPPS of model peptides and release of disulfide-linked peptides from solid phase by β-Me. (a) SPPS of model peptides Ala-Trp-Mea and Tyr-Ala-Mea. (b) Reductive release by β-Me. The resin was incubated with 200 µl of DMF containing 500 mM β-Me (100 µmol, 20 equiv.) and 500 mM TEA (100 µmol, 20 equiv.) and incubated at room temperature overnight.



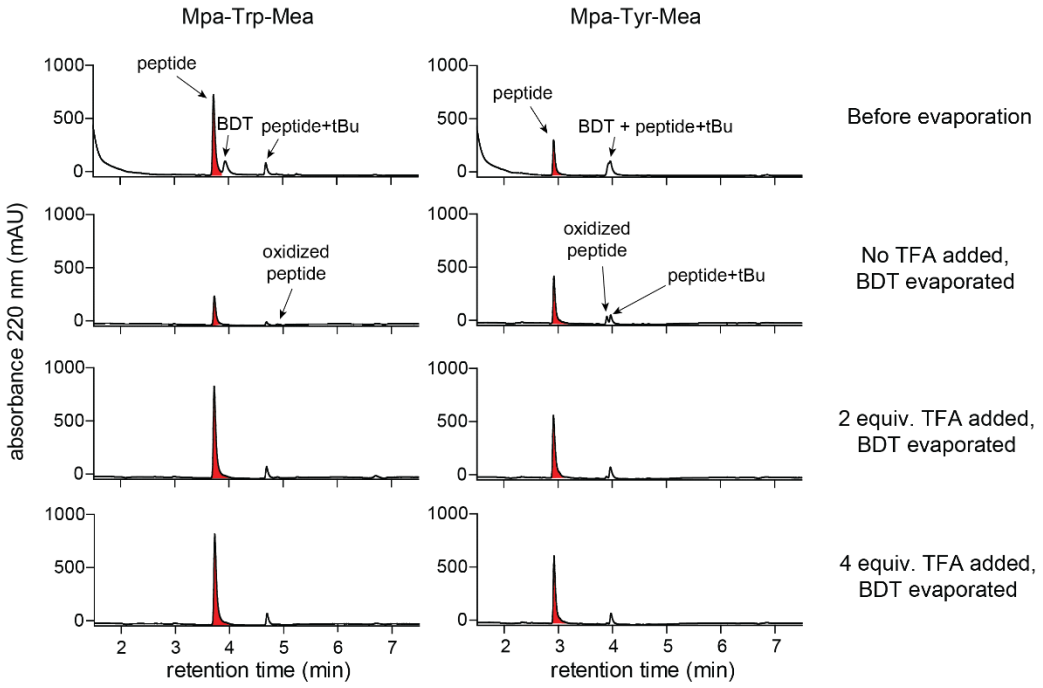
Supplementary Figure S2. Reductive release by DTT and BDT. (a) Release of disulfide-linked peptides from solid phase by 4 equiv. DTT and 4 equiv. TEA in DMF. The peptides were analyzed before (top) and after (bottom) evaporation of volatile agents by vacuum centrifugation. The resin was incubated with 200 μl of DMF containing 100 mM TEA (20 μmol, 4 equiv.) and 100 mM DTT (20 μmol, 4 equiv.) overnight at room temperature. (b) Release by BDT as shown in Figure 1c, tested with two additional peptides.



Supplementary Figure S3. Synthesis and release of diverse short dithiol peptides. Comparison of yields obtained by reductive release (left) and cyclative release (right). The peaks of the desired peptides are highlighted in red.



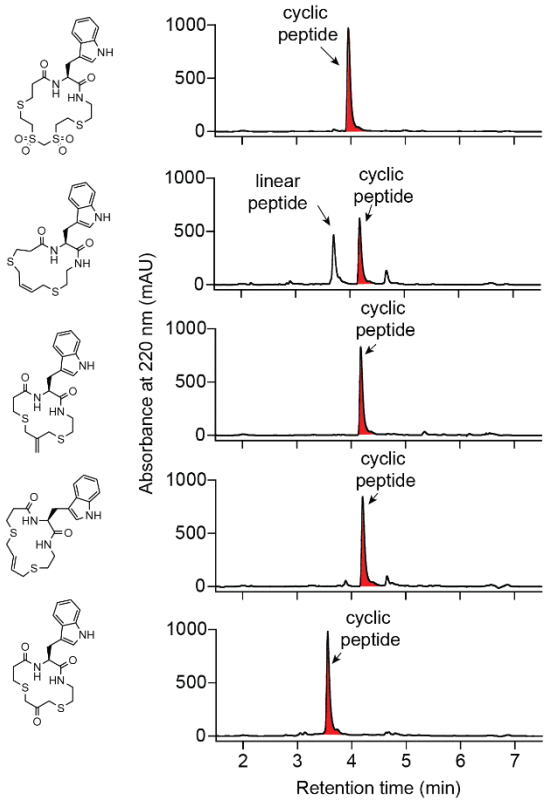
Supplementary Figure S4. Control peptides for cyclative disulfide release form solid phase. For reductive release, the resin was incubated with 200 μl of DMF containing 100 mM TEA (20 μmol, 4 equiv.) and 100 mM BDT (20 μmol, 4 equiv.), overnight at room temperature. For cyclative release, the resin was incubated with 200 μl DMSO containing 250 mM TEA (50 μmol, 10 equiv.) overnight at room temperature.



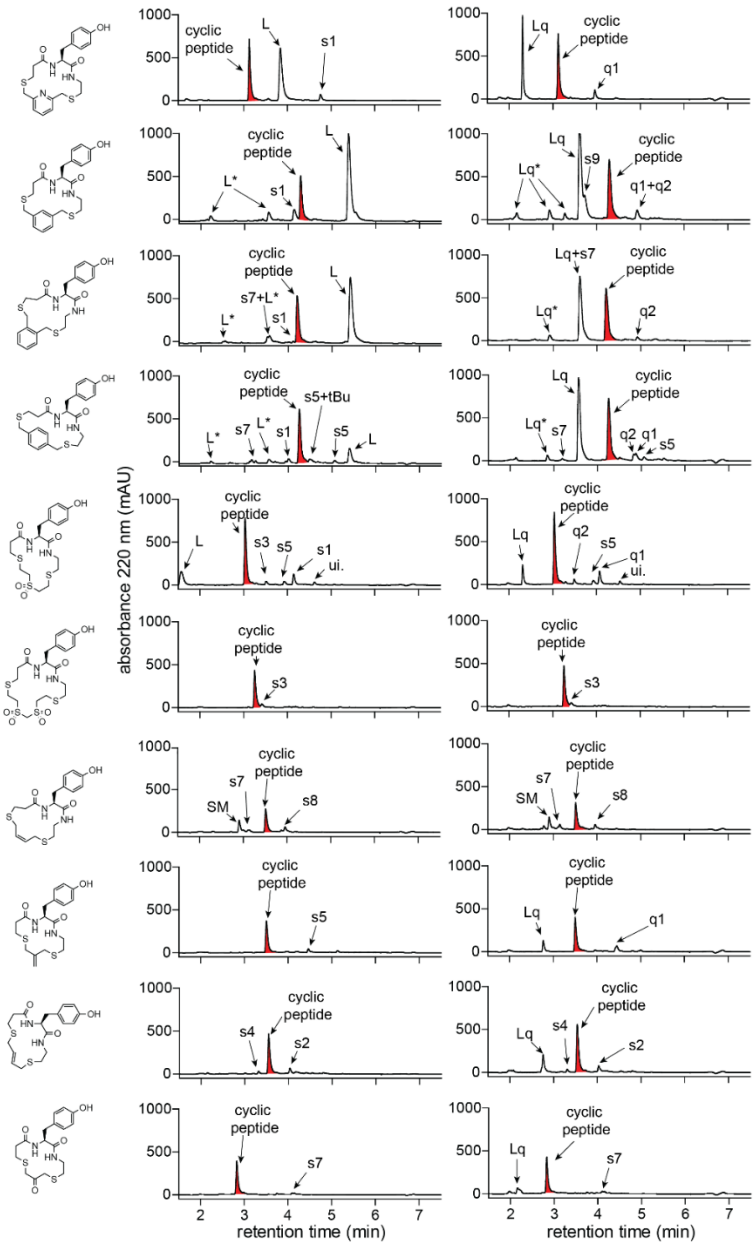
Relative peak areas

Peptide	TFA equiv. (rel. to base)	Peptide	Peptide+tBu	Peptide (ox)	Other
Mpa-Trp-Mea	Before evap.	87.9	11.2	0.3	0.7
	0	89.8	8.4	1.9	-
	2	88.7	9.6	0.3	1.4
	4	89.8	9	0.1	1.1
Mpa-Tyr-Mea	Before evap.	nd	nd	nd	nd
	0	74.9	14.4	9.3	1.4
	2	82.9	14.1	1.8	1.3
	4	85.9	12.3	1	0.8

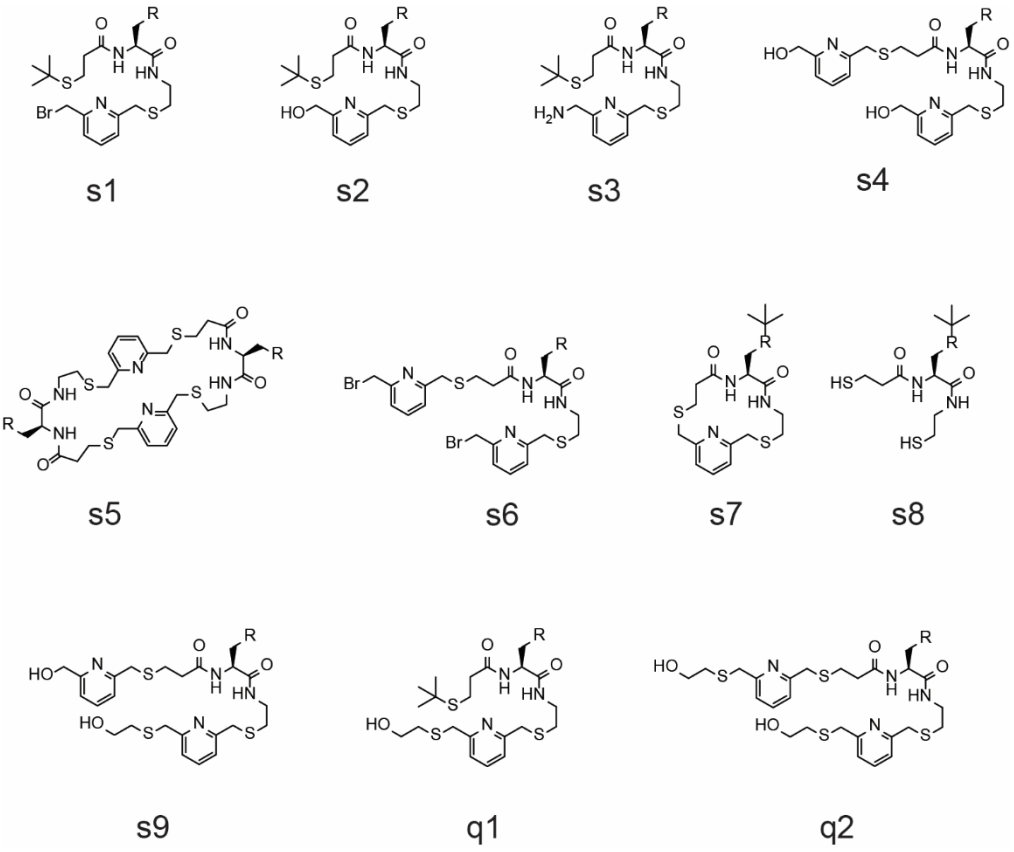
Supplementary Figure S5. Prevention of back-oxidation by addition of TFA prior to centrifugal vacuum evaporation. After reductive release, 0, 2 or 4 equiv. TFA were added to the peptide stocks as a 1% [v/v] (135 mM) solution in water. BDT was then removed by centrifugal vacuum evaporation at 0.1 mbar, 30 °C and at 400 × g for one hour.



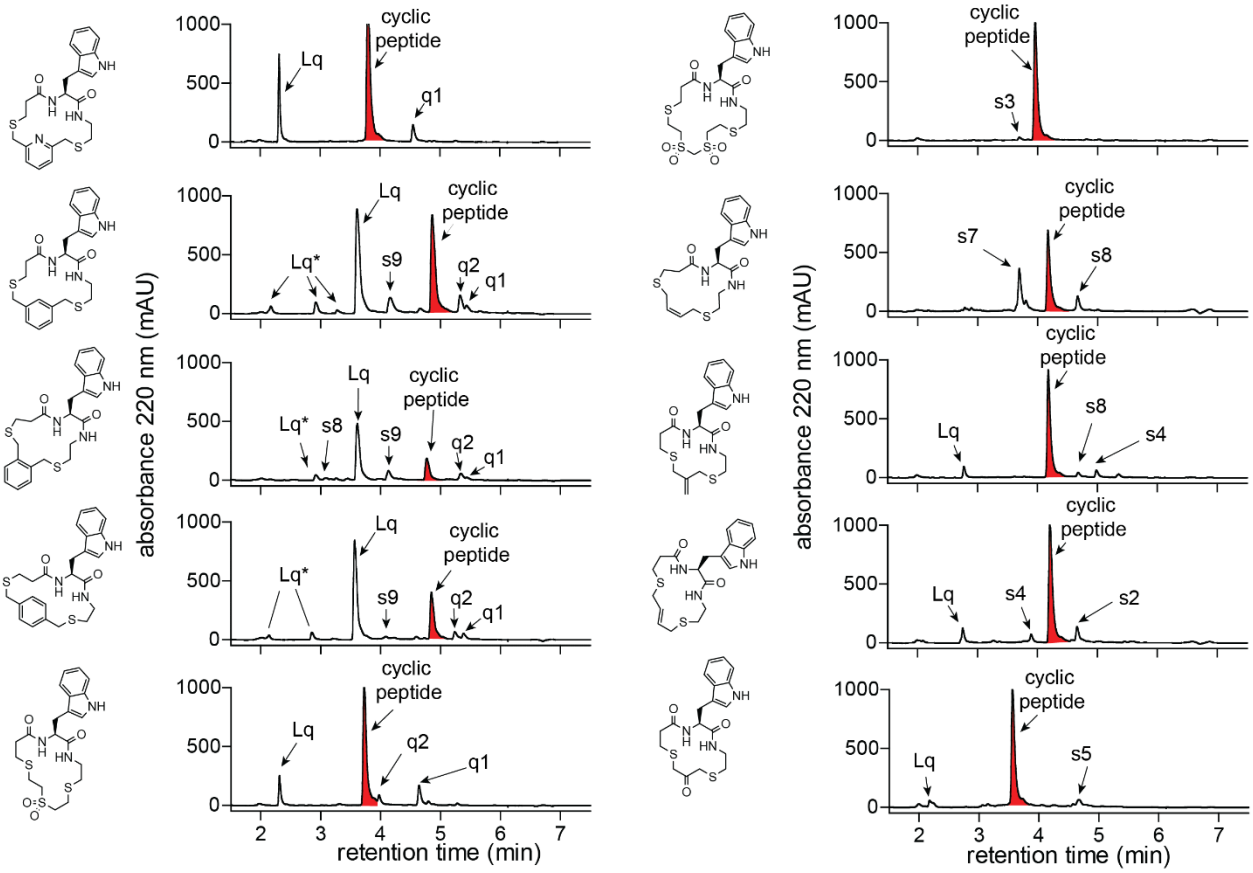
Supplementary Figure S6. Cyclization of dithiol peptide Mea-Trp-Mpa by bis-electrophile reagents **6**–**10**. The desired cyclic products are highlighted in red. L = bis-electrophile cyclization reagent. s1 = side-product tert-butyl-peptide-linker (structure shown in Supplementary Figure 8).



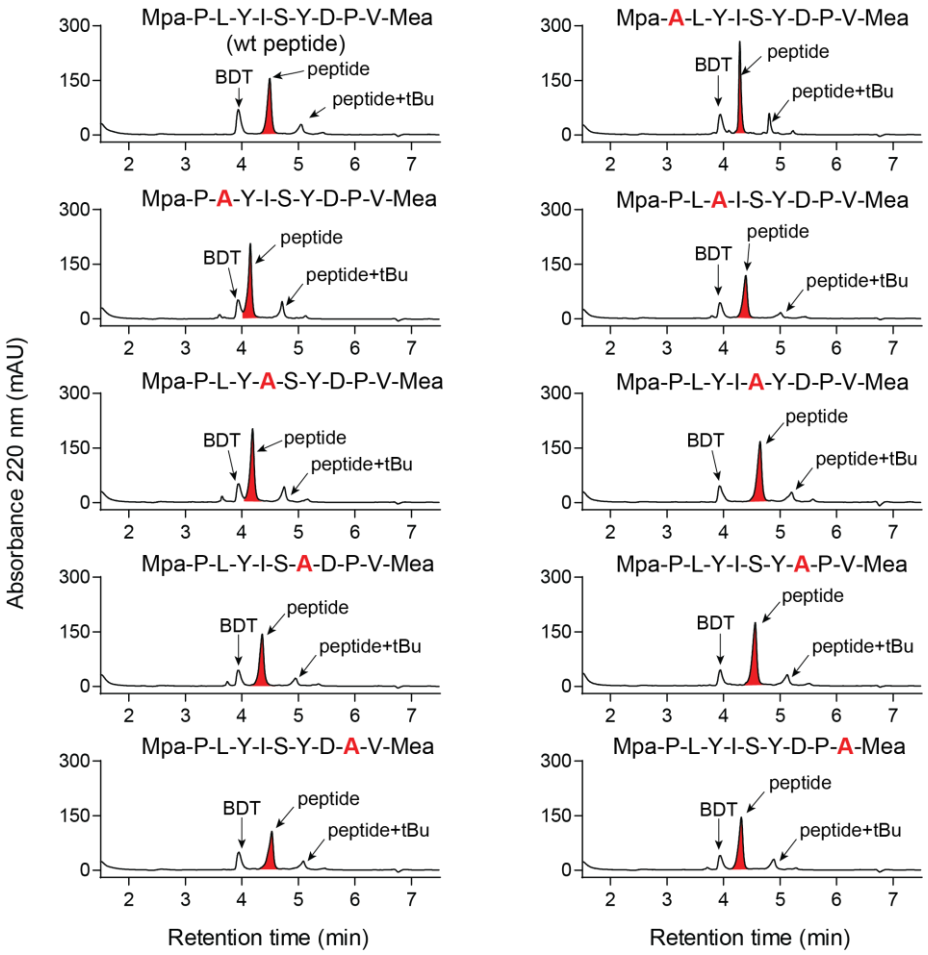
Supplementary Figure S7. Cyclization reactions with Trp and Tyr peptides. (a) Chemical structures of macrocycles and HPLC chromatographic analysis of the cyclization reactions before (left) and after (right) quenching of the bis-electrophile reagents with βMe. The desired cyclic products are highlighted in red. Side products s1 to s9 and q1 to q2 are shown in Supplementary Figure 7. L = bis-electrophile cyclization reagent. L* is hydrolyzed L. Lq = bis-electrophile cyclization reagent reacted with βMe. Lq* is hydrolyzed Lq.



Supplementary Figure S8. Structures of side products indicated in Supplementary Figures 6 and 8.



Supplementary Figure S9. Cyclization reactions with Trp peptides and quenching with βMe. The desired cyclic products are highlighted in red. Side products s2 to s9 and q1 to q2 are shown in Supplementary Figure 7. L = bis-electrophile cyclization reagent. L* is hydrolyzed L. Lq = bis-electrophile cyclization reagent reacted with βMe. Lq* is hydrolyzed Lq.



Supplementary Figure S10. Synthesis of longer peptides, tested with sequences based on the phage display-selected KRAS-specific peptide KRpep-2a. HPLC chromatograms of linear peptides containing the core sequence of KRpep-2a (wt peptide) and alanine mutants thereof.

3.8 References

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4. Accessing thiol peptides with high skeletal diversity by introducing novel aminothiol linkers in solid-phase peptide synthesis

4.1 Work contribution:

Chapter 4 of this thesis is based on the following manuscript, but describes only the parts to which I made major contributions:

Synthesis and screening large libraries of structurally diverse macrocycles suitable for membrane permeation

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Specific contributions:

I performed the experiments to establish the synthesis of the aminothiols and I tested them in SPPS. My experimental procedures were optimized and up-scaled by A.L.N., and are described in this chapter. G.K.M innovated the synthetic strategy for synthesizing aminothiol reagents; M.C., L.C. and A.A. generated the co-crystals and solved the X-ray structures; A.L.N. and C.H. wrote the original draft of the manuscript, and all authors reviewed the final version.

4.2 Abstract

Macrocycles offer an attractive format for drug development due to their ability to bind to challenging targets and potential to cross cell membranes. To efficiently identify macrocyclic ligands for new targets, methods for the synthesis and screening of large combinatorial libraries of small cyclic peptides were developed, many of them using thiol groups for efficient peptide macrocyclization. However, a weakness of these libraries is that invariant thiol-containing building blocks such as cysteine are used, resulting in a region that does not contribute to library diversity and likely not to target binding. Herein, we synthesized a panel of structurally diverse thiol-containing elements and tested them. In a subsequent work not detailed in this thesis, the elements were applied for the synthesis of a 2,688-member library of small, structurally diverse peptide macrocycles with previously unseen skeletal diversity. This library was used to discover nanomolar thrombin and plasma kallikrein inhibitors, some also demonstrating favorable membrane permeability. X-ray structure analysis of macrocycle-target complexes showed that the size and shape of the newly developed thiol elements are key for binding. The strategy presented in this work significantly enhances structural diversity by allowing combinatorial modifications to a previously invariant region of peptide macrocycles, which may be broadly applied in the development of therapeutics for challenging intracellular disease targets.

4.3 Introduction

Traditional drug discovery modalities have predominantly focused on small molecules and monoclonal antibodies; however, small molecules lack the requisite surface area to bind to complex biological targets, and antibodies are not membrane-permeable, limiting the applicability of both classes in certain therapeutic areas. In contrast, macrocycles are of growing interest due to their improved ability over small molecules to bind challenging targets, such as flat and featureless protein surfaces.[1] Macrocycles bind well to these targets because their cyclic structure provides high conformational stability for strong and specific target binding. Their slightly larger size compared to classical small molecules also allows for more molecular contact with the target, increasing the overall interaction surface area.[2] In contrast with impermeable antibodies, there are also several examples of permeable macrocycles that can reach intracellular targets after oral application,[3] despite their often slightly larger size that falls outside the range of conventional druglikeness models such as Lipinski's rule of five (Ro5).[4] Overall, their favorable binding properties and ability to cross membrane barriers offer enormous opportunities for macrocycles in drug development, such as for generating inhibitors of the numerous intracellular protein-protein interactions that have been difficult to target with classical modalities.

Most existing macrocyclic drugs are based on natural products or derivatives[1,5] though for the majority of disease targets, natural ligands do not exist and must therefore be developed *de novo* using synthetic approaches. However, this discovery process is currently hindered by a lack of techniques for developing and screening libraries of permeable macrocyclic ligands to new targets. Cyclic peptide libraries screened by phage or mRNA display usually yield peptides longer than ten amino acids that typically are not membrane permeable, though an exception to this was recently reported being a heavily *N*-methylated cyclic peptide ligand of KRAS that is cell-active.[6] Most compound collections that fulfill the Ro5 used in classical high-throughput screening by pharmaceutical companies contain only a small number of macrocyclic compounds. The generation of larger collections of macrocyclic compounds is hindered by typically low-yielding cyclization reactions that necessitate chromatographic purifications of individual macrocycles prior to screening.[7,8] Consequently, creating large numbers of macrocycles is extremely time-consuming and resource-intensive. Overall, the difficulty of generating these libraries has drastically limited the potential applicability of macrocyclic compounds in therapeutics.

To address these existing issues with macrocyclic drug development, methods for generating and screening large libraries comprising tens of thousands of small peptide-based macrocycles were developed.[9,10] The approaches are based on screening crude products without purification, which is made possible by high-throughput solid-phase peptide synthesis (SPPS) of short peptides that can be efficiently cyclized via two thiol end groups[11,12] and combinatorial late-stage diversification of the peptides using efficient and selective reactions.[10,13] However, a major limitation of current macrocycle libraries is that they have a structurally identical region that is required for macrocyclization (colored groups in Figures 1 and 2a), which compromises the structural diversity of the library and impairs the chances of identifying good binders.

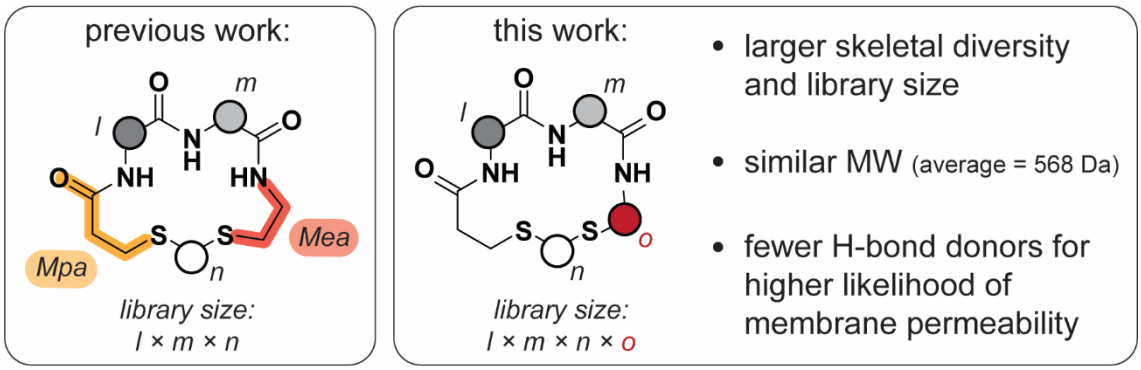


Figure 16: Macrocycle structures and building blocks that can be varied combinatorially to generate large libraries. Gray circles indicate amino acids, Mea and Mpa are the thiol-containing building blocks, and white circles are cyclization linkers. The red circle indicates the herein-developed varied C-terminal aminothiol element. In the chemical structures, standard amide bonds are shown for simplicity although some of them are N-methylated.

Not surprisingly, ligands identified form such libraries were all binding via the variable region and the constant groups are pointing away from the targets.[9,10] The same structural limitation is also found in disulfide- or thioether-cyclized peptide collections produced by cyclizing peptides via disulfide bridges, where the random amino acids are flanked by two constant cysteines.[14–17]

In macrocycle libraries produced most recently in our lab, we cyclized peptides via the two thiol building blocks, 3-mercaptopropionic acid (Mpa; at the N-terminal end) and 2-mercaptoethylamine (Mea; also named cysteamine). They contribute 89 and 76 Da to the molecular mass, respectively, which is substantial considering that this comprises 33% of the molecule weight target of <500 Da for the best chances of membrane permeability. Hence, to

increase the structural diversity of our macrocyclic libraries while remaining within the realm of likely permeable compounds, it is essential to diversify one or both of these constant elements.

Accordingly, in this work, we developed a synthetic strategy to efficiently produce short peptides with altered C-terminal thiol-containing elements (Figures 1 and 2a) to contribute to the development of macrocyclic ligands with a high probability of being membrane-permeable. Adding six structurally different C-terminal thiol-containing elements could increase the skeletal diversity of the generated libraries 7-fold compared to existing libraries, all without significantly increasing the size of the macrocycles that could negatively affect membrane permeability. Several of the developed thiol-containing elements lack an H-bond donor, reducing the overall polarity of the macrocycles further increasing the chances of membrane permeability. In a subsequent work not detailed here, synthesis and screening of structurally diverse libraries of 2,688 macrocycles containing varied C-terminal thiols yielded nanomolar inhibitors of thrombin and plasma kallikrein, several of which exhibited high cellular permeabilities. The herein-developed approach for synthesizing small and structurally diverse macrocycles suitable for membrane permeation is broadly applicable and may offer a solution for developing therapeutics for some of the most challenging intracellular disease targets.

4.4 Results and discussion

To synthesize large libraries of short peptides with altered C-terminal thiol-containing elements, we build on the procedure described in Chapter 3, in which peptides are synthesized by SPPS via a disulfide linker that can be reduced to release the peptides.[12] In this strategy, peptides are obtained in high purity without a purification step because we can first deprotect all side chains on-resin using trifluoroacetic acid (TFA) and then release the washed and deprotected peptides from the resin via a disulfide reduction with 1,4-butanedithiol (BDT). BDT is volatile and can be removed under reduced pressure by rotary vacuum concentration (RVC) to afford only the remaining dithiol peptides in excellent crude purity. The C-terminal peptide building block used for this synthesis so far was the aminothiol group **1** (Mea; Figure 1) that was conjugated to thiol-functionalized resin in a disulfide exchange reaction using pyridyldithioethylamine.[10–12] To generate peptides having different thiol-containing groups at the C-terminus (Figure 2a), our initial plan was to use analogous pyridylthiol-activated aminothiols, but we soon realized that their synthetic access was restricted due to the lack of commercial availability of structurally diverse aminothiols or high costs of the material.

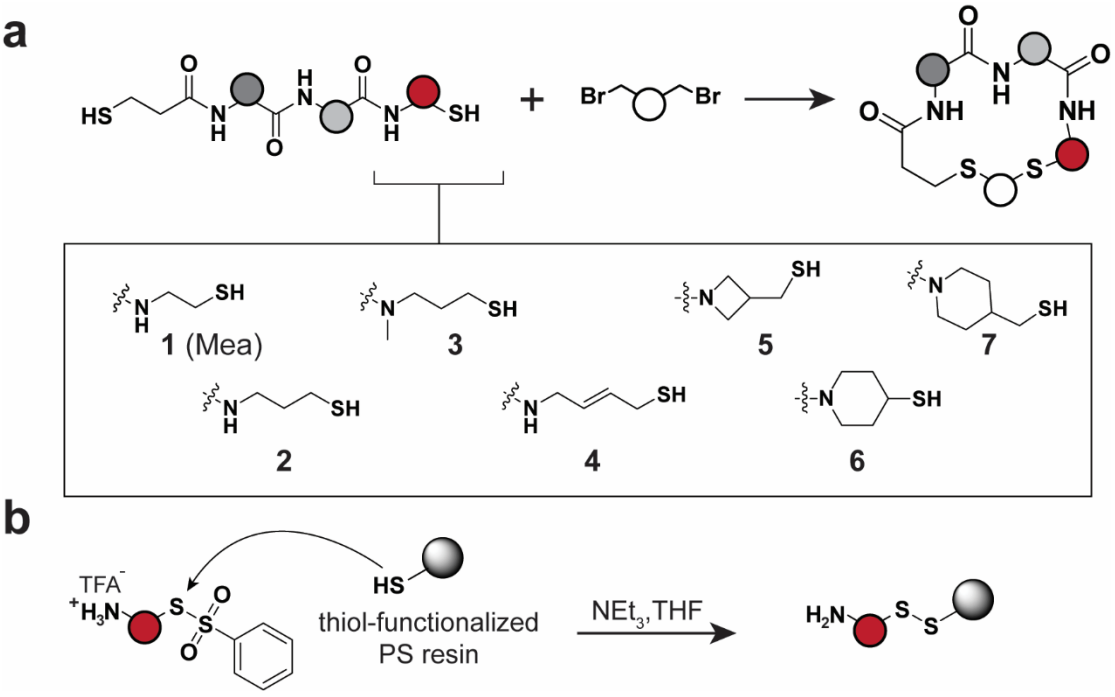


Figure 17: Macrocycle synthesis strategy. a) Schematic representation of the thiol-thiol cyclization strategy. Chemical structures of diverse aminothiols are shown. b) Schematic representation of the linkage of aminothiols to resin via a disulfide bridge using thiosulfonate building blocks. PS = polystyrene.

As an alternative approach, we conjugated different aminothiols onto thiol resin using these reagents with phenylsulfone as leaving group (Figure 2b) that is equally suited for disulfide exchange reactions.[18–20] Such building blocks can easily be accessed by reacting

thiosulfonates with halogenated *N*-Boc protected amines that are commercially available and cheap (Figure 3a).

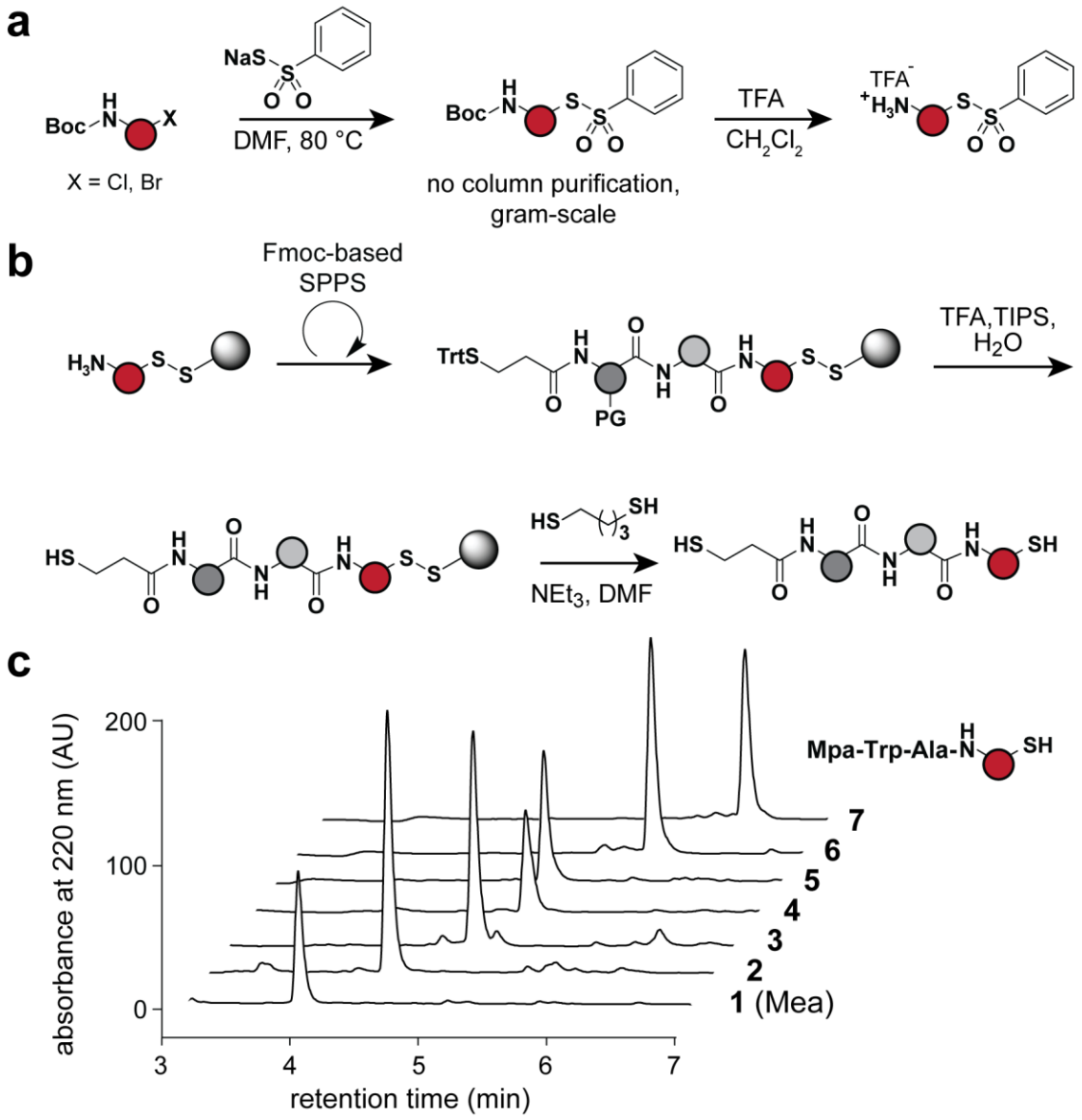


Figure 18: Preparation of aminothiol building blocks. a) Strategy for the “no purification” synthesis of aminothiol compounds activated with a phenylsulfone leaving group. b) SPPS of dithiol peptides using resin carrying a disulfide-linked aminothiol group. c) Stacked HPLC chromatograms of the quality of crude model peptides (Mpa-Trp-Ala-aminothiol X) synthesized with aminothiols **1**–**7**.

Importantly, the thiosulfonate precursors were separated from the excess thiosulfonate by extraction without the need of chromatographic purification, which facilitated the preparation of such reagents enormously. Through nucleophilic substitution with sodium benzenethionosulfonate in DMF at 80°C, we prepared six new *N*-Boc thiosulfonate building blocks on a gram-scale, ready for testing under library-synthesis conditions (Supporting

Figure S1; see Supporting Information for the synthesis).

To immobilize the aminothiols **2**–**7** onto the solid support, we first performed Boc deprotection of the *N*-Boc thiosulfonate building blocks and then conjugated the crude mixtures to thiol-resin via a disulfide exchange reaction (Figure 2b and Supporting Scheme S1). To test the quality of the new aminothiol-functionalized resins, we synthesized on them the model peptide Mpa-Trp-Ala by SPPS and liberated the resulting dithiol peptides using the reducing agent BDT (Figure 3b). HPLC-MS analysis showed purities ranging between 85 and 98% (Figure 3c and Supporting Figure S2), indicating the new aminothiol building blocks generate sufficiently pure crude products for the preparation and screening of macrocycle libraries.

To test whether potent binders could be identified using the new diversification elements, Alexander L. Nielsen designed and synthesized a library of 2,688 macrocycles by cyclizing 384 dithiol peptides with 7 bis-electrophilic linker reagents. He screened the crude library against trypsin-like serine protease thrombin using a final concentration of 10 μM crude compounds. To identify hit compounds based on activity and not just binding, he measured the ability of the macrocycles to inhibit enzymatic cleavage of fluorogenic 7-amino-4-methylcoumarin (AMC) substrates. Structure-activity analysis showed that all aminothiol building blocks generated hits in the thrombin screen, underscoring the importance of skeletal diversity in libraries. After resynthesis and purification, several macrocycles were found to have nanomolar affinity for thrombin (Figure 4), and the one with the highest affinity (**T2**) had a measured K_i of 10.9 ± 5.6 nM. This value makes this inhibitor around 4-fold more potent than those identified previously in other random libraries (best K_i = 42 nM),[9,10] suggesting that the higher structural diversity directly translated into the isolation of better inhibitors.

X-ray crystallography confirmed that in contrast to inhibitors identified in previous screens, the aminothiol groups are buried in the binding pocket (Figure 5). This supports the initial theory that they can form significant interactions with biological targets, and that their shapes and dimensions enable the hit compounds to adapt biologically active conformations. In summary, the introduction of the novel aminothiols resulted in an increased structural diversity in the macrocycle backbones, which contributed to the discovery of potent new inhibitors that fit tightly in the desired binding pocket. Some hits also showed promising properties in parallel artificial membrane permeability assay (PAMPA) and chloroalkane penetration assay (CAPA), which validated the strategy of diversifying a previously constant element in macrocycle libraries, yielding more potent inhibitors that are also membrane-permeable.

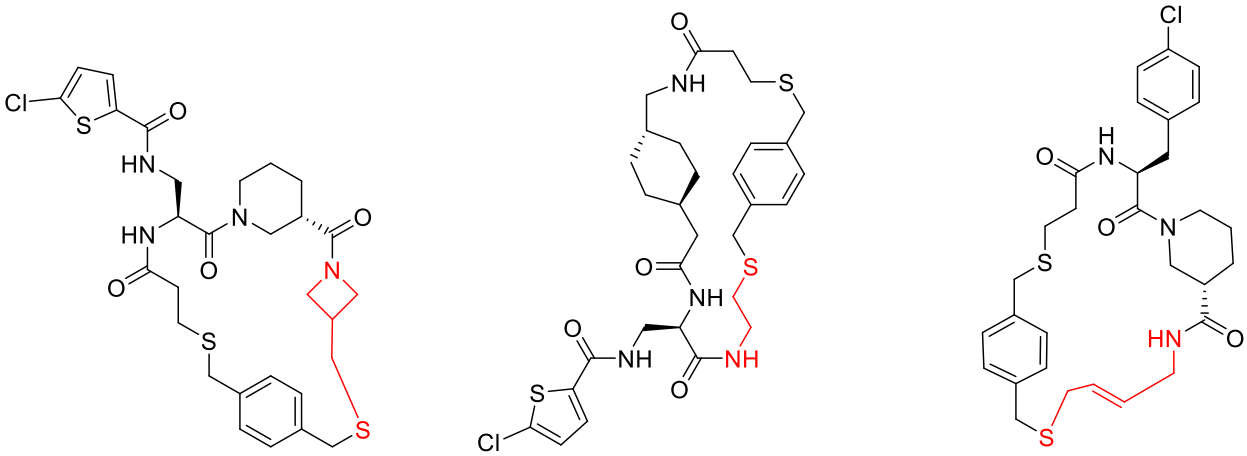


Figure 19: Structures thrombin inhibitors **T1**-**T3** and their respective binding affinities, identified by screening the peptide library featuring the new aminothiol elements (highlighted in red).

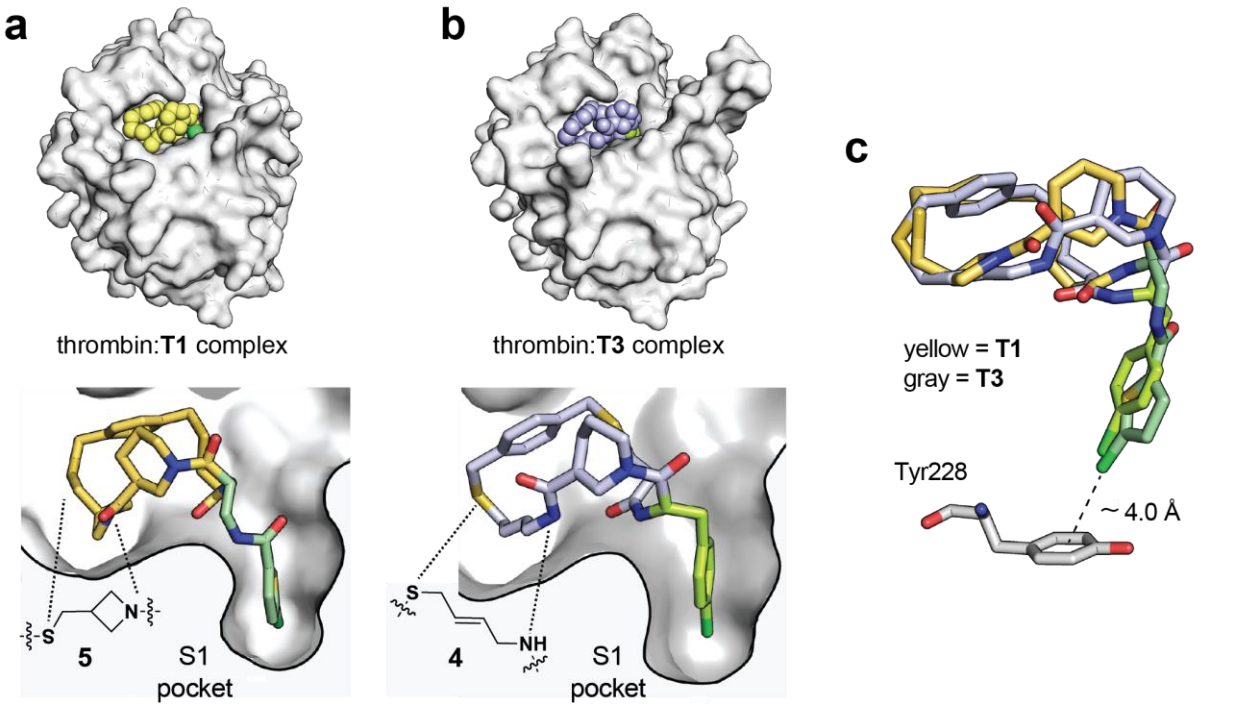


Figure 20: X-ray structures of thrombin-macrocycle complexes. a,b) Surface view of thrombin (grey) with bound **T1** (a) or **T3** (b). The lower parts of the panels show the macrocycles as sticks, wherein the S1 binding anchor is highlighted in green. The regions of the aminothiol building blocks are indicated. c) Superposition of **T1** and **T3** in the active site and highlighting of the halogen-π interaction with Tyr228.

4.5 Conclusion

In this work, we developed a strategy for efficiently synthesizing large libraries of peptide macrocycles that have a considerably higher structural diversity than previous libraries, important for identifying binders to targets, and yet are sufficiently small sized to achieve good membrane permeability. We achieved this by diversifying elements of cyclic peptides that are typically kept constant in library formation such as the cysteines or cysteamines used for cyclizing peptides via thioether or disulfide linkages. This diversification became possible via the development of an efficient synthesis pathway for generating structurally diverse, phenylsulfone-activated aminothiol building blocks that could be incorporated into peptides by SPPS. In a later work not presented in this thesis, we tested our strategy in a proof-of-concept study, making a library of 2,688 compounds that culminated in the discovery of nanomolar inhibitors of two proteases, including some that displayed good membrane permeability.

The high structural diversity directly translated into the isolation of better binders from random libraries, with the best thrombin inhibitor (K_i = 10.9 nM) identified being 4-fold more potent than those isolated previously from other libraries (best K_i = 42 nM).[9] The importance of the expanded structural diversity is supported by the X-ray structural analysis of two of the macrocycles bound to thrombin, which showed that the specific size and shape of the aminothiol groups are needed to optimally accommodate the neighboring macrocycle building blocks for forming optimal contacts with the thrombin's active site. Importantly, several of the identified binders showed good membrane permeability in artificial membranes (PAMPA) and in live cells, which was likely a result of keeping the size of the macrocycles rather small, close to 500 Da. Such a result would likely not have been possible if the library diversity had been increased through adding an additional amino acid position for diversification due to the increase in size and the addition of an extra amide bond, the latter typically reducing the membrane permeability considerably due to its polar nature.

While the new strategy was only applied to synthesize a rather small library of only 2,688 macrocycles in this proof-of-concept study, much larger libraries can be generated with the applied methods. Our group has recently modified a commercial 96-well plate peptide synthesizer to make peptides in 384-well plates.[23] The instrument holds four 384-well reactor plates allowing us to synthesize 1,536 peptides in one run and in less than two days. As mentioned above, we also have preliminary data that shows that the cyclization reactions and procedures established herein for 384-well plates are applicable to 1,536-well plates, allowing the synthesis of ten-thousands or even over hundred-thousand small cyclic peptides with the

described expanded skeletal diversity. Such larger libraries may be beneficial for developing macrocycle-based ligands to more challenging targets than the herein-applied proteases, such as protein-protein interactions.

Finally, the the *N*-terminal thiol-containing building block Mpa, which was kept constant in this work, may be diversified in the future too. Building blocks analogous to Mpa may be prepared by reacting halogenated carboxylic acids with trityl mercaptan. Together with the herein developed aminothiols, even more diverse macrocyclic libraries can be generated to access membrane permeable ligands. We are optimistic that the herein developed new approach for synthesizing small and structurally highly diverse, membrane permeable macrocycles will be broadly applicable to many proteins and may offer a solution to develop therapeutics to some of the most challenging intracellular disease targets.

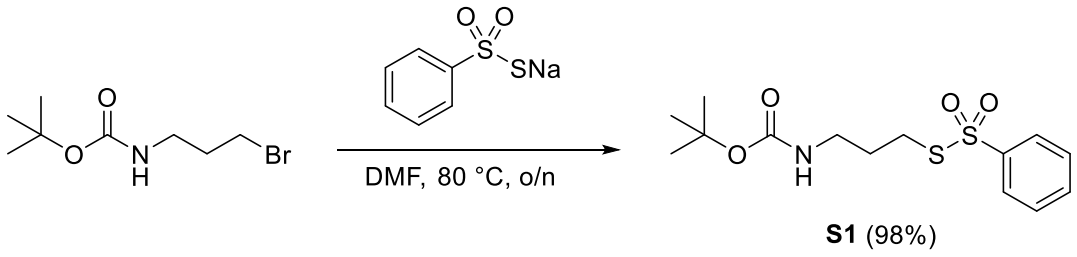
4.6 Materials and methods

Chemical synthesis of building blocks and amino acids

All reagents and solvents were of analytical grade and used without further purification as obtained from commercial suppliers. Reactions were monitored by thin-layer chromatography (TLC) using silica gel coated plates (analytical SiO_2-60, F-254) and/or by HPLC-MS analysis. TLC plates were visualized under UV light or by dipping into a solution of potassium permanganate (10 g/L in water) followed by visualization with a heat gun. Rotary evaporation of solvents was carried out under reduced pressure at a temperature below 40 °C. HPLC-MS analyses were performed with a UHPLC and single quadrupole MS system (Shimadzu LCMS-2020) using a C18 reversed phase (RP) column (Phenomenex Kinetex 2.1×50 mm C18 column, 100 Å pore, 2.6 μm particle). A linear gradient of solvent B (0.05% HCOOH in MeCN) over solvent A (0.05% HCOOH in water) rising from 0% to 60% during t = 1.00–6.00 min was applied at a flow rate of 1.00 mL/min. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III (^{1}H NMR and ^{13}C NMR recorded at 400 and 101 MHz, respectively) equipped with a cryogenically cooled probe. All spectra were recorded at 298 K. Chemical shifts are reported in ppm relative to deuterated solvent as internal standard (δ_H DMSO-d_6 2.50 ppm; δ_C DMSO 39.52 ppm; δ_H $CDCl_3$ 7.26 ppm; δ_C $CDCl_3$ 77.16 ppm).

High-resolution mass spectrometry (HRMS) measurements were recorded on a Xevo G2-XS QTof time-of-flight (TOF) mass spectrometer.

S-(3-((tert-butoxycarbonyl)amino)propyl) benzenesulfonothioate (**S1**)

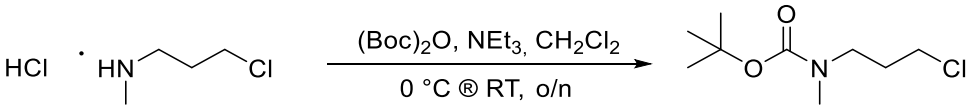


S1 (98%)

To a stirring solution of 3-(Boc-amino)propyl bromide (6.02 g, 25.3 mmol, 1.0 equiv.) in DMF (120 mL) was added sodium benzenethionosulfonate (7.48 g, 38.0 mmol, 1.5 equiv.; tech. 85%) and the solution was stirred overnight at 80 °C. After cooling down, the reaction mixture was concentrated under reduced pressure, resuspended in water (100 mL) and extracted with EtOAc:hexanes (2×150 mL, 10:1, v/v). The combined organic layers were washed with water (3×150 mL) and brine (150 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford crude **S1** (8.21 g, 24.8 mmol, 98%) as a yellow-tainted oil. The

applied extraction procedure would co-purify also the starting material 3-(Boc-amino)propyl bromide but this molecule was not detected in the extracted product. TLC (25% EtOAc in hexanes): R_f = 0.25 ($KMnO_4$ stain). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98–7.90 (m, 2H), 7.70–7.61 (m, 1H), 7.60–7.52 (m, 2H), 3.16 (q, *J* = 6.4 Hz, 2H), 3.02 (t, *J* = 7.2 Hz, 2H), 1.83 (p, *J* = 6.8 Hz, 2H), 1.43 (s, 9H).

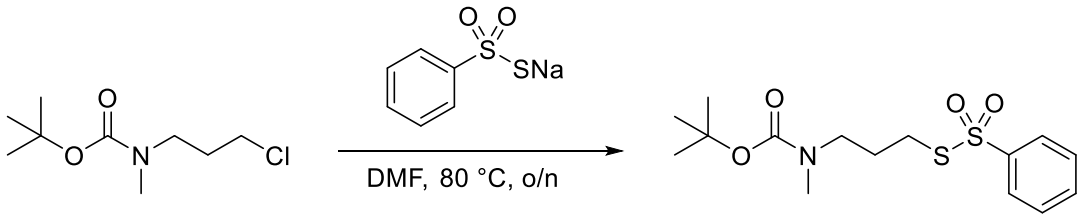
tert-butyl (3-chloropropyl)(methyl)carbamate (**S2**)



S2 (quant.)

A stirring solution of 3-chloropropyl-*N*-methylamine hydrochloride (4.32 g, 30.0 mmol, 1.0 equiv.) and di-*tert*-butyl dicarbonate (6.54 g, 30.0 mmol, 1.0 equiv.) in CH_2Cl_2 (150 mL) was cooled to 0 °C under argon atmosphere. NEt_3 (4.18 mL, 30.0 mmol, 1.0 equiv.) was added dropwise over 5 min and the solution was stirred overnight going towards ambient temperature. The reaction mixture was concentrated under reduced pressure and resuspended in EtOAc (120 mL). The solution was washed with aq. HCl (1 M, 2×120 mL), sat. $NaHCO_3$ (120 mL) and brine (120 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford crude **S2** (6.22 g, 30.0 mmol, quant.) as a colorless oil.* TLC (25% EtOAc in hexanes): R_f = 0.45 ($KMnO_4$ stain). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.55 (t, *J* = 6.5 Hz, 2H), 3.36 (t, *J* = 6.8 Hz, 2H), 2.87 (s, 3H), 2.06–1.91 (m, 2H), 1.46 (s, 9H). CAS RN: 114326-14-6. *Product is volatile, so limit time under low pressure.

S-(3-((tert-butoxycarbonyl)(methyl)amino)propyl) benzenesulfonothioate (**S3**)

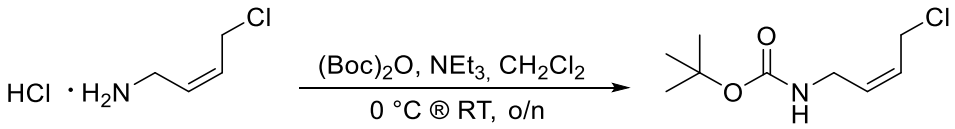


S2 **S3** (87%)

To a stirring solution of **S2** (3.51 g, 16.9 mmol, 1.0 equiv.) in DMF (25 mL) was added sodium benzenethionosulfonate (5.47 g, 27.9 mmol, 1.65 equiv.; tech. 85%) and the solution was stirred overnight at 80 °C. After cooling down, the reaction mixture was concentrated under reduced pressure, resuspended in water (100 mL) and extracted with EtOAc:hexanes (2×75 mL, 10:1, v/v). The combined organic layers were washed with water (3×75 mL) and

brine (75 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford crude **S3** (5.07 g, 14.7 mmol, 87%) as a yellow-tainted oil. The applied extraction procedure would co-purify also the starting material **S2** but this molecule was not detected in the extracted product. TLC (12.5% EtOAc in hexanes): R_f = 0.30 ($KMnO_4$ stain). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01–7.88 (m, 2H), 7.69–7.50 (m, 3H), 3.24 (t, *J* = 6.7 Hz, 2H), 2.96 (t, *J* = 7.3 Hz, 2H), 2.77 (s, 3H), 1.85 (p, *J* = 6.8 Hz, 2H), 1.42 (s, 9H).

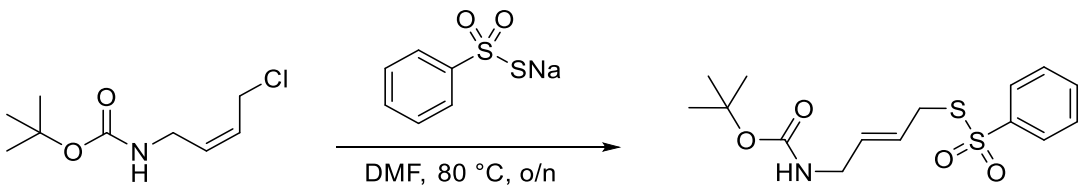
tert-butyl (Z)-(4-chlorobut-2-en-1-yl)carbamate (**S4**)



S4 (98%)

A stirring solution of *cis*-4-chloro-2-butenylamine hydrochloride (2.50 g, 17.6 mmol, 1.0 equiv.) and di-*tert*-butyl dicarbonate (3.84 g, 17.6 mmol, 1.0 equiv.) in CH_2Cl_2 (75 mL) was cooled to 0 °C under argon atmosphere. NEt_3 (2.45 mL, 17.6 mmol, 1.0 equiv.) was added dropwise over 5 min and the solution was stirred overnight going towards ambient temperature. The reaction mixture was concentrated under reduced pressure and resuspended in CH_2Cl_2 (100 mL). The solution was washed with aq. HCl (1 M, 2×100 mL), sat. $NaHCO_3$ (100 mL) and brine (100 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford crude **S4** (3.55 g, 17.3 mmol, 98%) as a light-brown solid. TLC (25% EtOAc in hexanes): R_f = 0.30 ($KMnO_4$ stain). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.82–5.70 (m, 1H), 5.63 (dt, *J* = 10.8, 6.9 Hz, 1H), 4.59 (br s, 1H), 4.12 (d, *J* = 7.8 Hz, 2H), 3.83 (t, *J* = 6.6 Hz, 2H), 1.44 (s, 9H). CAS RN: 123642-28-4. Spectral data is in agreement with literature.[18]

(Z)-S-(4-((tert-butoxycarbonyl)amino)but-2-en-1-yl) benzenesulfonothioate (**S5**)

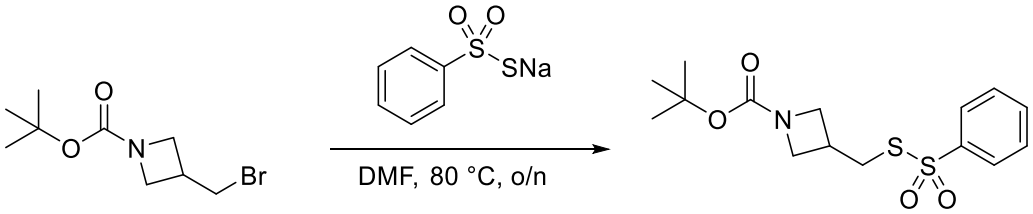


S4 **S5** (85%)

To a stirring solution of **S4** (3.55 g, 17.3 mmol, 1.0 equiv.) in DMF (70 mL) was added sodium benzenethionosulfonate (6.77 g, 34.5 mmol, 2.0 equiv.; tech. 85%) and the solution was stirred overnight at 80 °C. After cooling down, the reaction mixture was concentrated under reduced pressure, resuspended in water (400 mL) and extracted with EtOAc (3×100 mL). The

combined organic layers were washed with water (3×200 mL) and brine (200 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain crude **S5** (5.03 g, 14.6 mmol, 85%*) as a brown oil. The applied extraction procedure would co-purify also the starting material **S4** but this molecule was not detected in the extracted product. TLC (25% EtOAc in hexanes): R_f = 0.20 ($KMnO_4$ stain). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94–7.87 (m, 2H), 7.68–7.60 (m, 1H), 7.60–7.49 (m, 2H), 5.62 (dt, *J* = 15.4, 5.6 Hz, 1H), 5.46 (dtt, *J* = 15.4, 7.0, 1.5 Hz, 1H)**, 4.43 (br s, 1H), 3.67 (dq, *J* = 7.0, 1.1 Hz, 2H), 3.60 (t, *J* = 6.1 Hz, 2H), 1.43 (s, 9H). *The crude compound purity is lower than for other thiosulfonate building blocks, but still provides excellent resin quality in the subsequent resin loading steps (see chromatogram in Figure S2). Albeit not necessary prior to resin loading, the crude product can be purified by silica column chromatography.**During the reaction, the *cis*-alkene converts into the *trans*-alkene. This is confirmed by the increase in the coupling constant between the two alkene protons (from 10.8 to 15.4 Hz), and was also observed from the observed X-ray co-crystal structure of macrocycle **T3** in complex with thrombin (Figures 6b and S14).

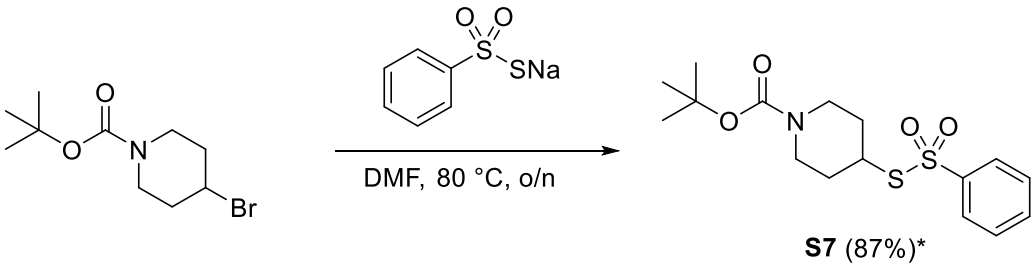
tert-butyl 3-(((phenylsulfonyl)thio)methyl)azetidine-1-carboxylate (**S6**)



S6 (quant.)

To a stirring solution of 1-Boc-3-bromomethylazetidine (1.93 g, 7.71 mmol, 1.0 equiv.) in DMF (25 mL) was added sodium benzenethionosulfonate (2.41 g, 12.3 mmol, 1.6 equiv.; tech. 85%) and the solution was stirred overnight at 80 °C. After cooling down, the reaction mixture was concentrated under reduced pressure, resuspended in water (100 mL) and extracted with EtOAc:hexanes (2×75 mL; 10:1, v/v). The combined organic layers were washed with water (2×75 mL), sat. $NaHCO_3$ (75 mL) and brine (75 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain crude **S6** (2.65 g, 7.71 mmol, quant.) as a yellow-tainted oil. The applied extraction procedure would co-purify also the starting material 1-Boc-3-bromomethylazetidine but this molecule was not detected in the extracted product. TLC (33% EtOAc in hexanes): R_f = 0.30 ($KMnO_4$ stain). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, *J* = 7.5 Hz, 2H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.58 (t, *J* = 7.6 Hz, 2H), 3.96 (t, *J* = 8.6 Hz, 2H), 3.52 (dd, *J* = 9.0, 5.3 Hz, 2H), 3.23 (d, *J* = 7.8 Hz, 2H), 2.85–2.70 (m, 1H), 1.41 (s, 9H).

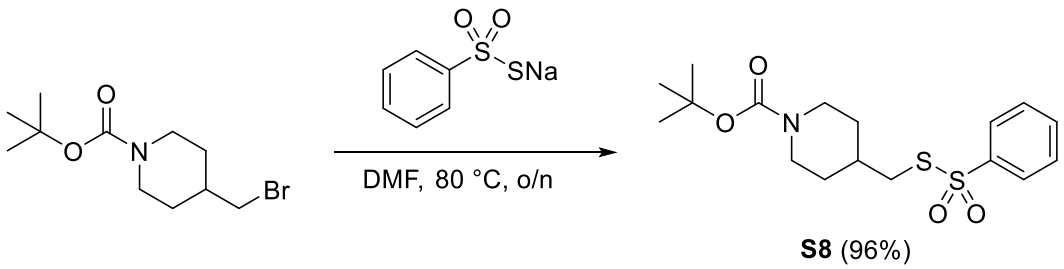
tert-butyl 4-((phenylsulfonyl)thio)piperidine-1-carboxylate (**S7**)



S7 (87%)*

To a stirring solution of 1-*N*-Boc-4-bromopiperidine (2.25 g, 8.51 mmol, 1.0 equiv.) in DMF (25 mL) was added sodium benzenethionosulfonate (2.75 g, 14.0 mmol, 1.65 equiv.; tech. 85%) and the solution was stirred overnight at 80 °C. Due to incomplete overnight reaction (monitored by TLC), additional sodium benzenethionosulfonate (1.38 g, 7.00 mmol, 0.83 equiv.; tech. 85%) was added and the reaction was stirred another 24 h at 80 °C. After cooling down, the reaction mixture was concentrated under reduced pressure, resuspended in water (100 mL) and extracted with EtOAc:hexanes (2×75 mL; 10:1, v/v). The combined organic layers were washed with water (2×75 mL), sat. $NaHCO_3$ (75 mL) and brine (75 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain crude **S7** (2.65 g, 7.41 mmol, 87%*) as a yellow-tainted oil. The applied extraction procedure would co-purify also the starting material 1-*N*-Boc-4-bromopiperidine but this molecule was not detected in the extracted product. TLC (33% EtOAc in hexanes): R_f = 0.44 ($KMnO_4$ stain). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01–7.89 (m, 2H), 7.71–7.61 (m, 1H), 7.60–7.50 (m, 2H), 3.89–3.63 (m, 2H), 3.55–3.39 (m, 1H), 3.11–2.91 (m, 2H), 1.96–1.86 (m, 2H), 1.64–1.51 (m, 2H), 1.42 (s, 9H). *The substitution reaction progresses significantly slower than for the preparation of the other thiosulfonate building blocks. Additionally, the crude purity is lower, but still provides good resin quality in the subsequent resin loading steps (see chromatogram in Supporting Figure S2). Albeit not necessary prior to resin loading, the crude product can be purified by silica column chromatography.

tert-butyl 4-(((phenylsulfonyl)thio)methyl)piperidine-1-carboxylate (**S8**)

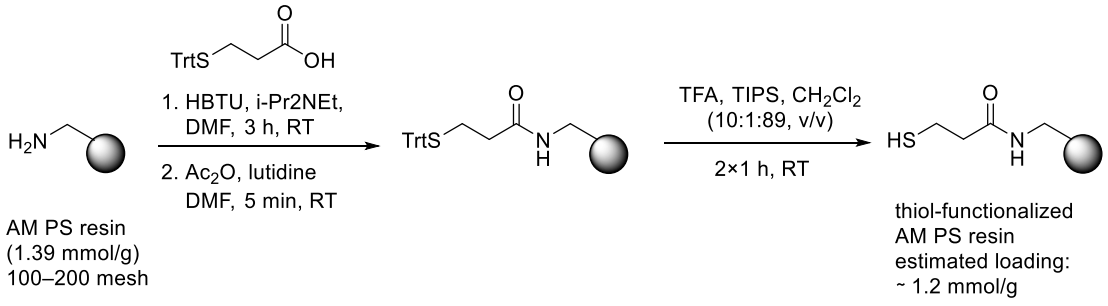


S8 (96%)

To a stirring solution of 1-*N*-Boc-4-(bromomethyl)piperidine (2.16 g, 7.76 mmol, 1.0 equiv.) in DMF (50 mL) was added sodium benzenethionosulfonate (2.52 g, 12.8 mmol, 1.65 equiv.; tech. 85%) and the solution was stirred overnight at 80 °C. After cooling down, the reaction mixture was concentrated under reduced pressure, resuspended in water (100 mL) and extracted with EtOAc:hexanes (2×75 mL; 10:1, v/v). The combined organic layers were washed with water (2×75 mL), sat. $NaHCO_3$ (75 mL) and brine (75 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain crude **S8** (2.76 g, 7.42 mmol, 96%) as a clear oil. The applied extraction procedure would co-purify also the starting material 1-*N*-Boc-4-(bromomethyl)piperidine but this molecule was not detected in the extracted product. TLC (25% EtOAc in hexanes): R_f = 0.30 ($KMnO_4$ stain). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01–7.87 (m, 2H), 7.70–7.61 (m, 1H), 7.61–7.51 (m, 2H), 4.20–3.94 (m, 2H), 2.91 (d, *J* = 6.5 Hz, 2H), 2.58 (t, *J* = 12.8 Hz, 2H), 1.73–1.57 (m, 3H), 1.43 (s, 9H), 1.14–0.98 (m, 2H).

Resin preparation

The following procedure described the protocol to generate thiol resin applied in this work. In later experiments, we found that commercially offered thiol-functionalized PS (e.g. Polystyrene A SH from Rapp Polymere GmbH, product code HA40004.0, ~0.85 mmol/g and 200–400 mesh) is equally suited, and we recommend the latter one as it can be purchased.



AM PS resin
(1.39 mmol/g)
100–200 mesh

thiol-functionalized
AM PS resin
estimated loading:
~ 1.2 mmol/g

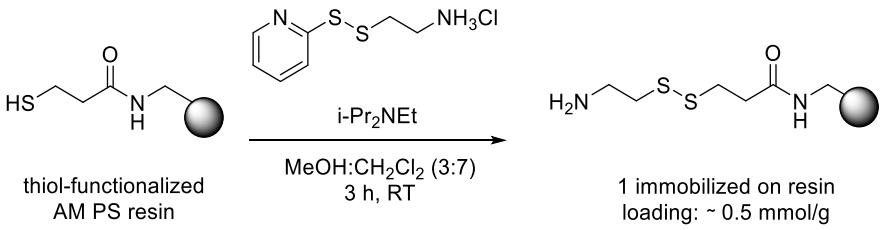
Pre-washing: Each 25 mL-fritted syringe was loaded with ~0.8 g (1.11 mmol) aminomethyl)polystyrene resin (AM PS resin; 1.39 mmol/g, 100–200 mesh; Aapptec, cat. #RAZ001) and pre-washed using MeOH (2×10 mL), CH_2Cl_2 (3×10 mL), 1% (v/v) TFA in CH_2Cl_2 (2×10 mL), *i*-Pr_2NEt in CH_2Cl_2 (1.2 M; 2×10 mL for 5 min), CH_2Cl_2 (2×10 mL) and DMF (2×10 mL).

Coupling: A solution of 3-(tritylthio)propionic acid (1.16 g, 3.33 mmol, 3.0 equiv.) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; 1.27 g, 3.33 mmol, 3.0 equiv.) in DMF (10 mL) was activated with *i*-Pr_2NEt (1.16 mL, 6.66 mmol, 6.0 equiv.) and added to the fritted syringe and agitated for 3 h at ambient temperature. The resin was filtered and washed with DMF (3×10 mL) and CH_2Cl_2 (3×10 mL) followed by drying the beads (first under mild suction and then under reduced pressure overnight (<0.5 mbar)). The loading of the Mpa(Trt) resin was determined to ~1.2 mmol/g (weight based).

Capping: A solution of 5% Ac_2O and 6% lutidine in DMF (12 mL; v/v/v) was added to the resin and incubated it for 5 min at ambient temperature. The resin was drained and washed with DMF (3×10 mL) and CH_2Cl_2 (3×10 mL).

Deprotection: A solution of 10% TFA and 1% TIPS in CH_2Cl_2 (15 mL, v/v/v) was added to the resin and agitated for 1 h at ambient temperature. The resin was washed with CH_2Cl_2 (3×10 mL) and the procedure was repeated once to afford high-loaded thiol-functionalized polystyrene resin that was utilized for subsequent disulfide exchange and loading of aminothiol derivatives (termed SH PS resin; *see next page*).

*Immobilization of aminothiol **1** (Mea) onto resin*



thiol-functionalized AM PS resin

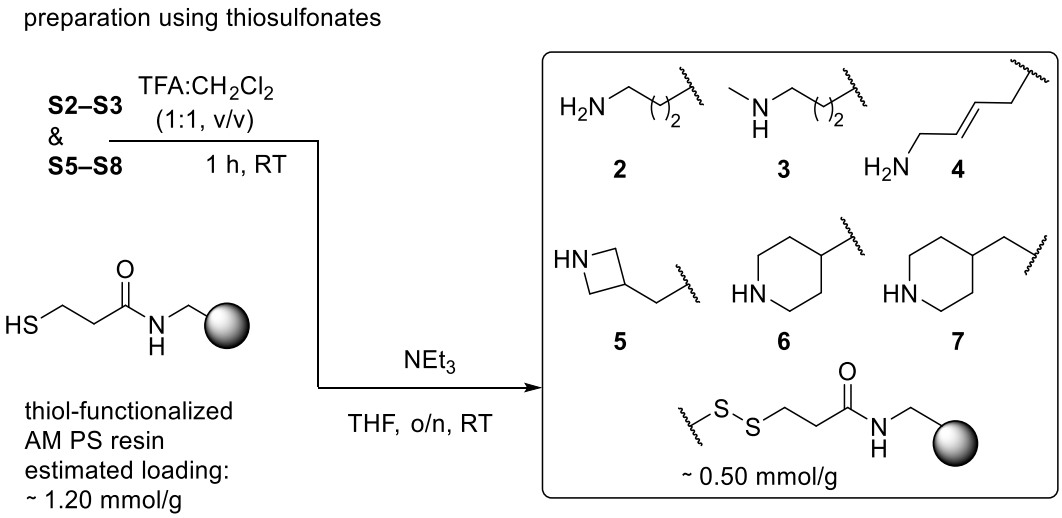
1 immobilized on resin
loading: ~ 0.5 mmol/g

Dithiol exchange: Each 25 mL-fritted syringe was loaded with ~0.4 g (0.48 mmol). SH PS resin was swelled in CH_2Cl_2 (10 mL) and then drained. 2-pyridylthio cysteamine hydrochloride salt[11] (0.21 g, 0.96 mmol, 2.0 equiv.) was dissolved in MeOH:CH_2Cl_2 (19 mL, 3:7, v/v) followed by addition of *i*-Pr_2NEt (167 μL, 0.96 mmol, 2.0 equiv.). The solution was added to the resin and agitated for 3 h at ambient temperature. The resin was drained and washed with MeOH:CH_2Cl_2 (2×10 mL, 3:7, v/v), DMF (2×10 mL), *i*-Pr_2NEt in DMF (1.2 M; 10 mL for 5 min), DMF (3×10 mL) and CH_2Cl_2 (2×10 mL) followed by drying the beads (first under mild suction

and then under reduced pressure overnight (<0.5 mbar). Average resin loading (free aminothiol derivative loaded onto resin) was determined to ~0.5 mmol/g by Fmoc-quantitation from coupling Fmoc-Gly-OH using litterature procedure.[19]

Qualitative controls: (1) Kaiser test;[20] complete purple/blue coloration of the beads. (2) Ellman's reagent on beads;[21,22] no coloration.

*Immobilization of aminothiols **2–7** onto resin*



Deprotection: N-Boc protected thiosulfonate intermediate (**S2**, **S3**, **S5**, **S6**, **S7** or **S8**; ~7.0 mmol) was dissolved in CH_2Cl_2 (10 mL) followed by dropwise addition of TFA until CO_2 bubbling was observed (~10 mL). The solution was stirred for another 1 h at ambient temperature whereafter solvent was removed under a stream of nitrogen. Excess TFA was removed under reduced pressure by co-evaporation with CH_2Cl_2:MeOH solution (1:1, v/v) to afford the TFA salts of the thiosulfonates.

Dithiol exchange: Each 25 mL-fritted syringe was loaded with ~0.8 g (0.96 mmol) SH PS. The resin was swelled in THF (15 mL) and then drained. The desired thiosulfonate TFA salt (2.40–2.88 mmol, 2.5–3.0 equiv.) was dissolved in THF (15 mL), and NEt_3 (803 μL, 5.76 mmol, 6.0 equiv.) was added. The solution was added to the resin and agitated overnight at ambient temperature. The resin was drained, washed with THF (3×15 mL) and CH_2Cl_2 (2×15 mL) followed by drying of the beads (first under suction and then under reduced pressure overnight (<0.5 mbar). Resin loading (free aminothiol derivative loaded onto resin) was even between the different aminothiol building blocks and averaged to ~0.5 mmol/g by Fmoc-quantitation from coupling Fmoc-Gly-OH using literature procedure.[19] Due to sufficient yields to our application, optimization towards higher resin loading was not pursued in this work.

Qualitative controls: (1) Kaiser test;[20] complete purple/blue coloration of the beads. (2) Ellman's reagent on beads;[21,22] no coloration.

Automated solid-phase peptide synthesis (SPPS)

SPPS was performed on an Intavis Multipep RSi synthesizer. Polypropylene (PP) 96-well filter plates were equipped with ~2 μmol/well of resin (**1**–**7** immobilised onto AM PS resin via a disulfide bridge), and washed with DMF (6×225 μL). Coupling was performed with 53 μL of amino acids (500 mM in DMF, 26.5 μmol, 13.3 equiv.), 50 μL of 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU; 500 mM in DMF, 25 μmol, 12.5 equiv), 13 μL of *N*-methylmorpholine (NMM; 4 M in DMF, 52 μmol, 26.0 equiv.), and 5 μL *N*-methylpyrrolidone (NMP). For couplings of expensive and/or synthesized amino acids (*t*-Acha, APipAc, 2Amb and Thio; consult Supporting Table S4 for information on amino acid abbreviations), 75 μL of amino acid (170 mM in DMF, 12.75 μmol, 6.4 equiv.), 25 μL HATU (500 mM in DMF, 12.5 μmol, 6.25 equiv.), 7 μL of *N*-methylmorpholine (4 M in DMF, 28 μmol, 14 equiv.), and 5 μL NMP were used. All components were pre-mixed for one minute, then added to the resin (two hour reaction, no shaking). Coupling was performed twice and resin was washed with DMF (6×225 μL). Fmoc deprotection was performed using 20% (v/v) piperidine in DMF (120 μL, 2×2 min) and the resin was washed with DMF (6×225 μL). At the end of the peptide synthesis, the resin was washed with CH_2Cl_2 (2×200 μL) and the resin beads were dried under suction.

Macrocyclization procedure

Transfer to microtiter plates: Based on the determined concentration of each di-thiol peptide in DMSO, 40 nmol of dithiol peptide in DMSO was transferred into 384-well PP plates (one plate per linker) using ADE.

Peptide reduction: As dithiol peptides oxidize in DMSO over time, we ensured that the peptides were fully reduced by adding a solution of BDT and NEt_3 in DMF (both 100 mM; 20 μL, 2 μmol, 50 equiv.) to each well, followed by incubation for 30 min at ambient temperature. A solution of TFA in milliQ-water (10% (v/v); 3 μL, 2 equiv. relative to NEt_3) was added and the peptides were dried by RVC (30 °C, 1750 rpm, 0.1 mbar) to afford the fully reduced dithiol peptides as dried pellets.

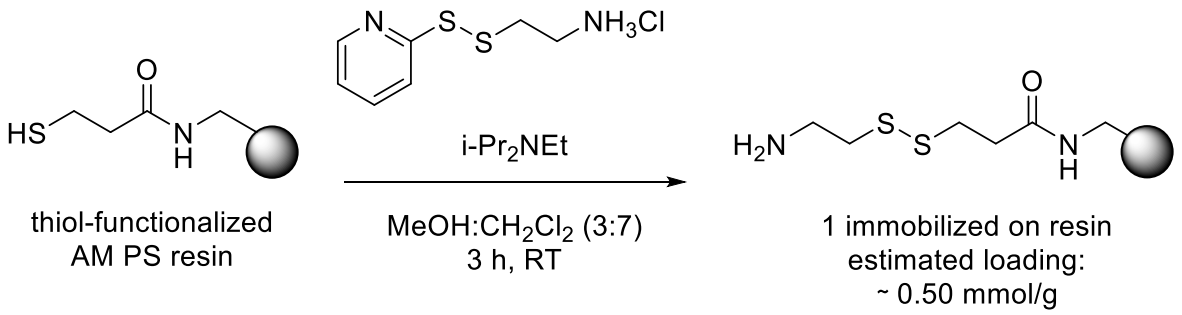
Cyclization: Biselectrophilic linkers (**L1**−**L7**, Supporting Table S4) were dissolved in a degassed 60 mM solution of NH_4HCO_3 in MeCN:H_2O (1:1, v/v, pH 8) to a final concentration of 4 mM. The prepared linker solutions (40 μL, 160 nmol, 4 equiv. relative to dithiol peptide) was added to the 384-well PP plates by bulk dispensing, which were sealed with adhesive PP lids and agitated for 2 h at ambient temperature.

Linker quenching: β-mercaptoethanol (β-ME) was dissolved in the cyclization buffer to a final concentration of 32 mM. The prepared solution (20 μL/well, 4 equiv. relative to linker) was added by bulk dispensing and incubated for 1 h at ambient temperature without plate lids.

Removal of solvent and β-ME, and resolubilization: Solvent was removed by RVC (40 °C, 1750 rpm, 0.1 mbar) to afford the peptide macrocycles as pellets, which were dissolved in DMSO (20 μL) to afford 2 mM macrocyclic peptide libraries that could immediately be applied in subsequent protease screening assays.

4.7 Supplementary information

preparation using dipyridyl-disulfide



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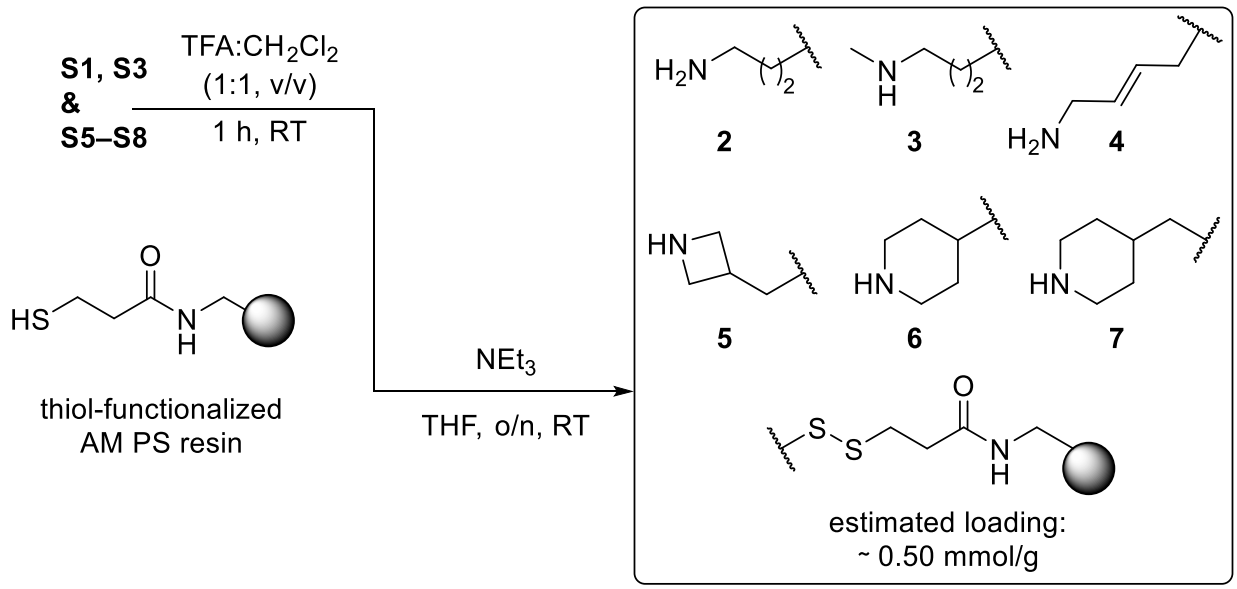
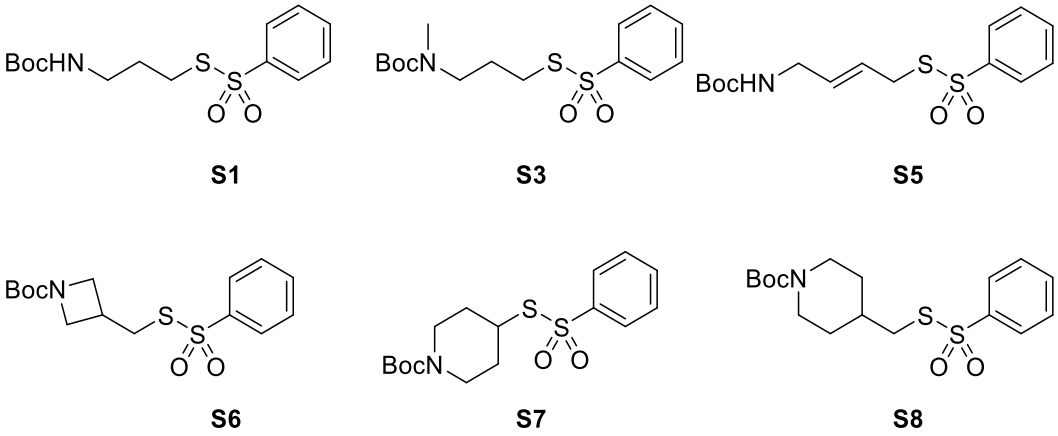
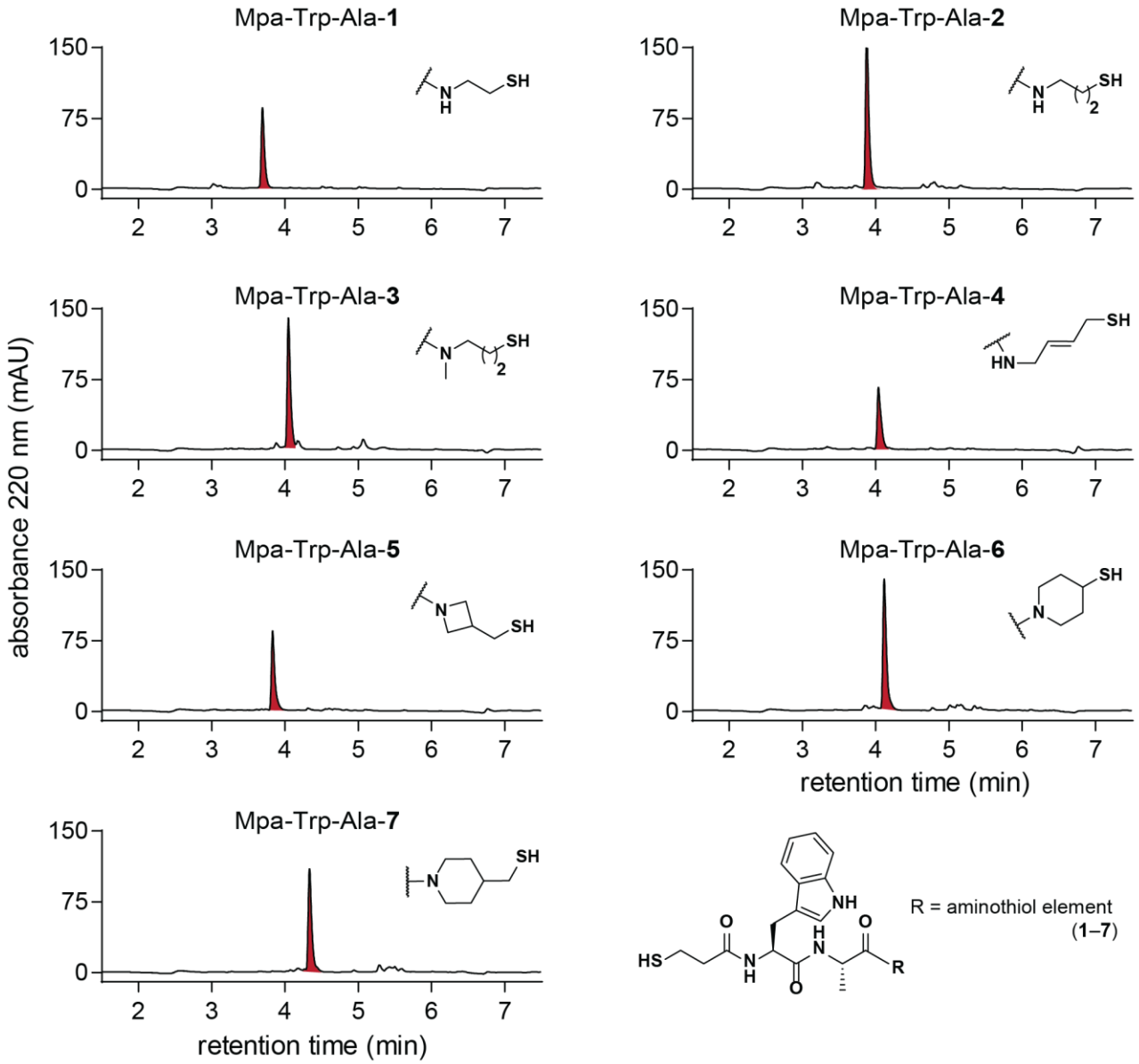
preparation using thiosulfonates



Supporting Scheme S1. Preparation of aminothiols (1–7) and their immobilization onto resin via a disulfide bridge. Method for functionalizing aminothiol derivatives onto solid support.



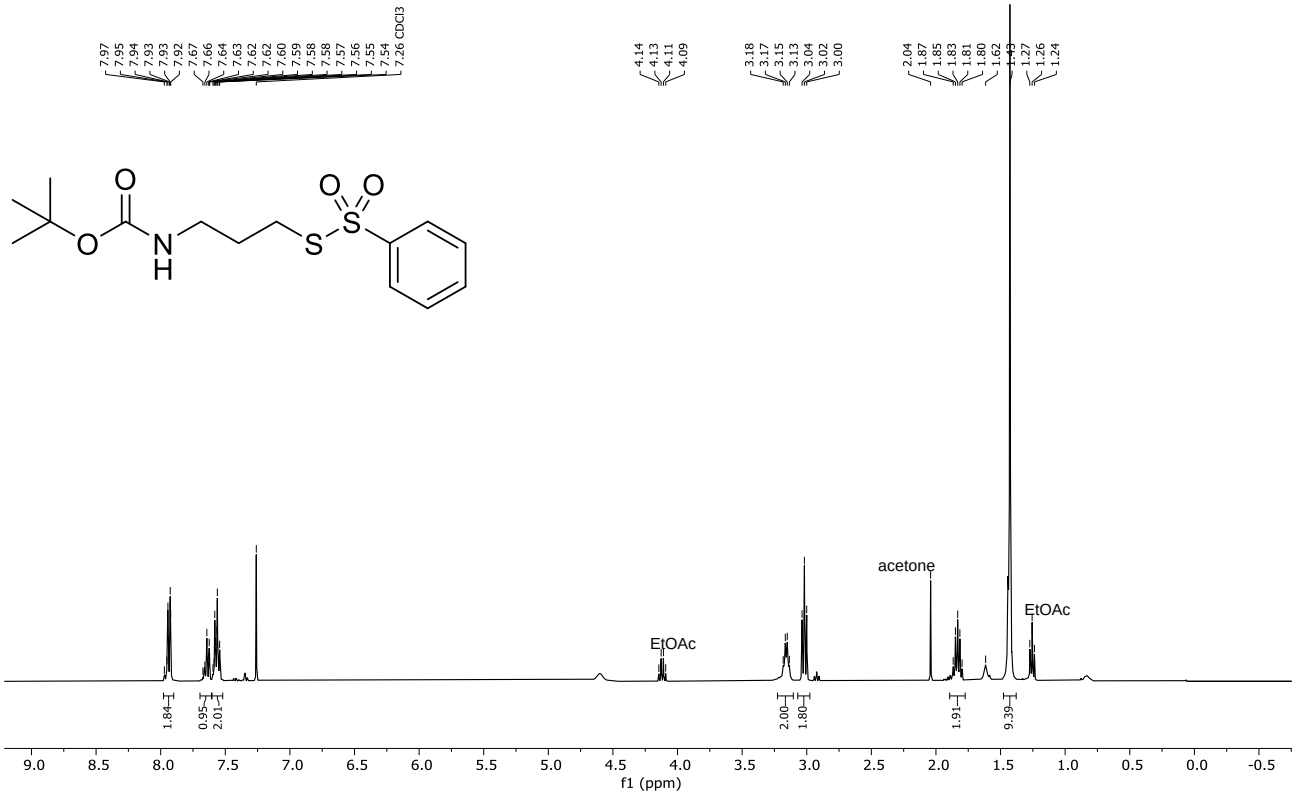
Supporting Figure S1. Synthesized thiosulfonate building blocks.



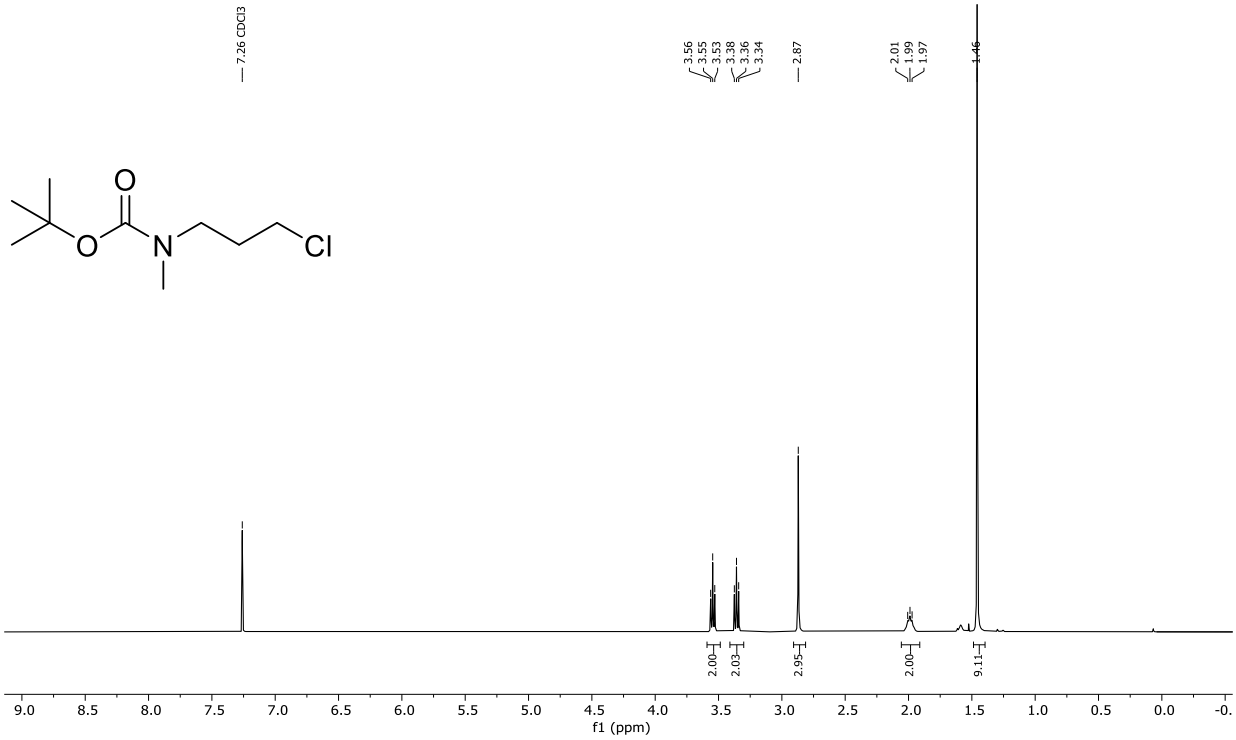
Supporting Figure S2. Quality assessment of linear peptides prepared with resin derivatives. UV_{220} chromatograms of crude peptide controls utilizing different aminothiols (**1**–**7**) after reductive release and RVC from synthesis in 96-well plates (5 μmol scale). Highlighted in red are the desired linear peptide species.

NMR SPECTRA

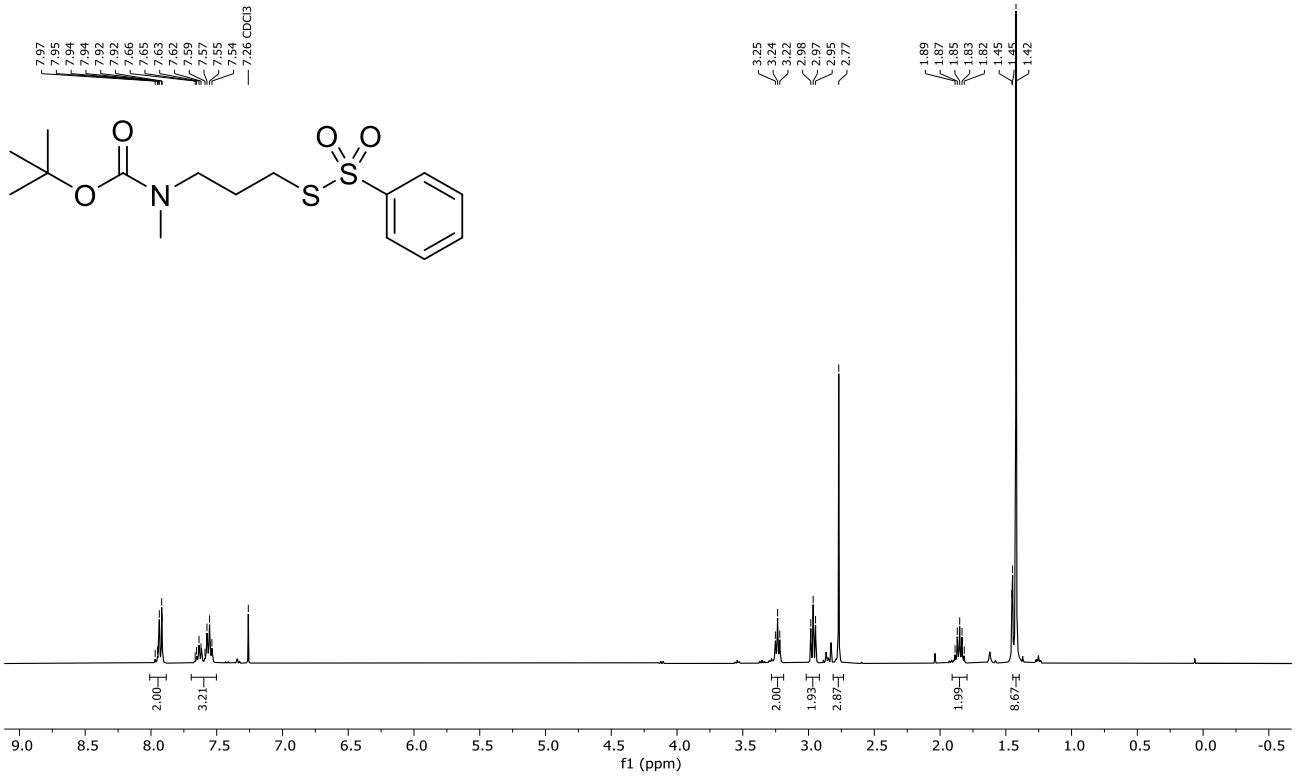
^{1}H NMR (400 MHz, $CDCl_3$) of **S1** (crude)



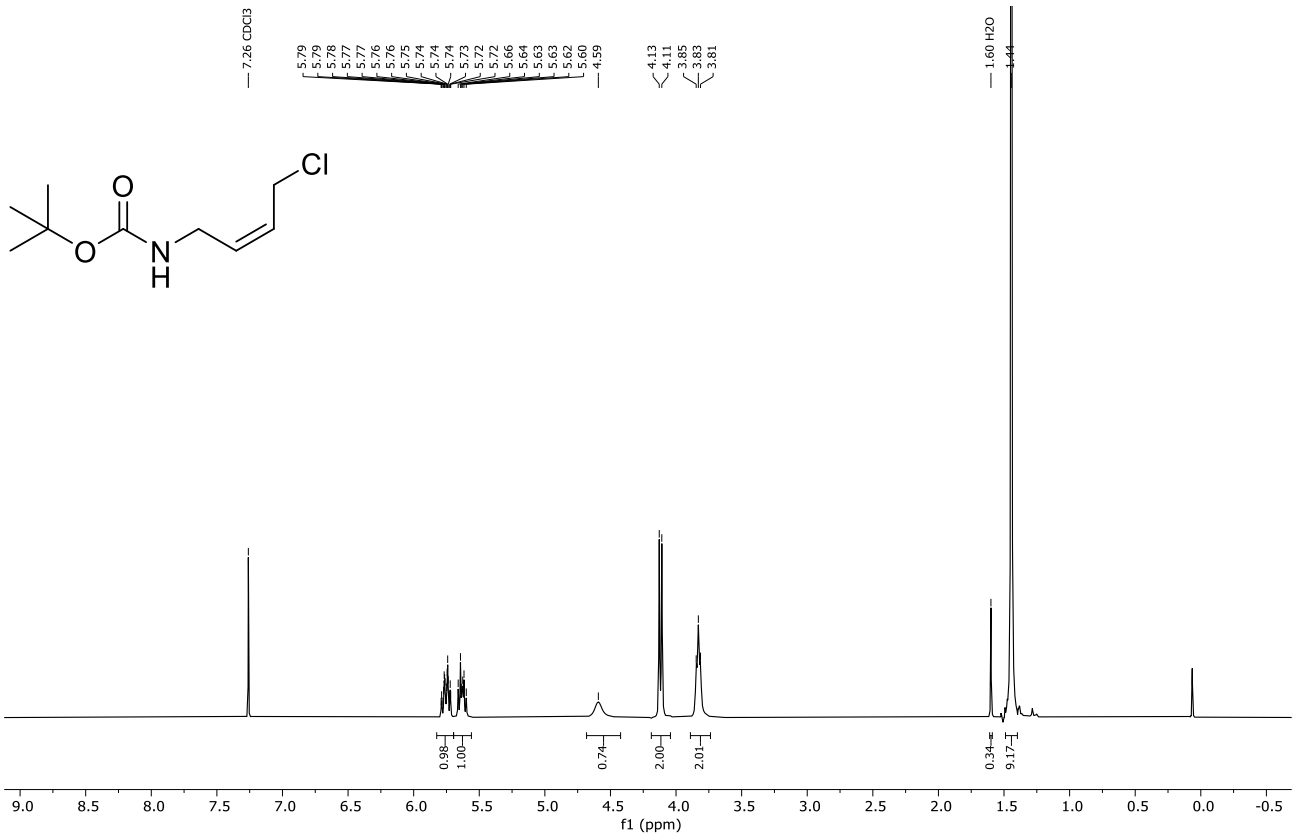
^{1}H NMR (400 MHz, $CDCl_3$) of **S2** (crude)



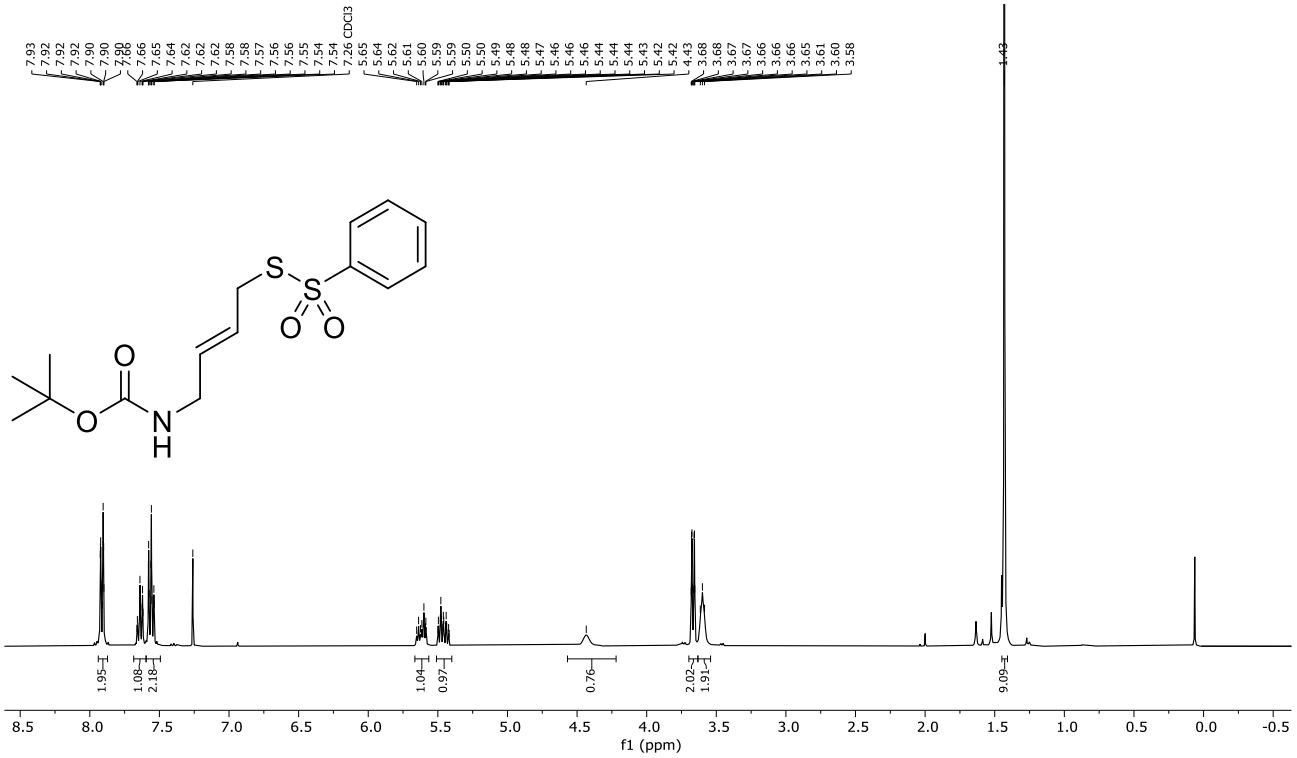
^{1}H NMR (400 MHz, $CDCl_3$) of **S3** (crude)



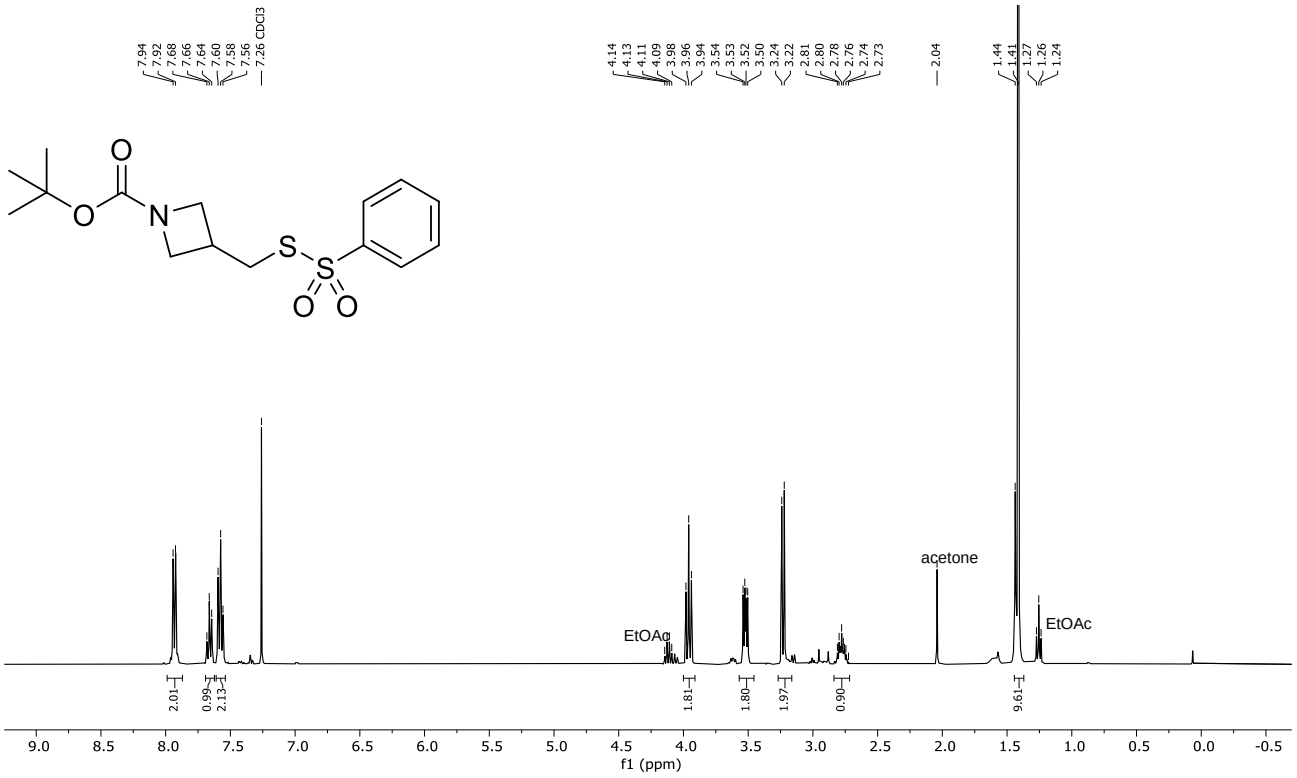
^{1}H NMR (400 MHz, $CDCl_3$) of **S4** (crude)



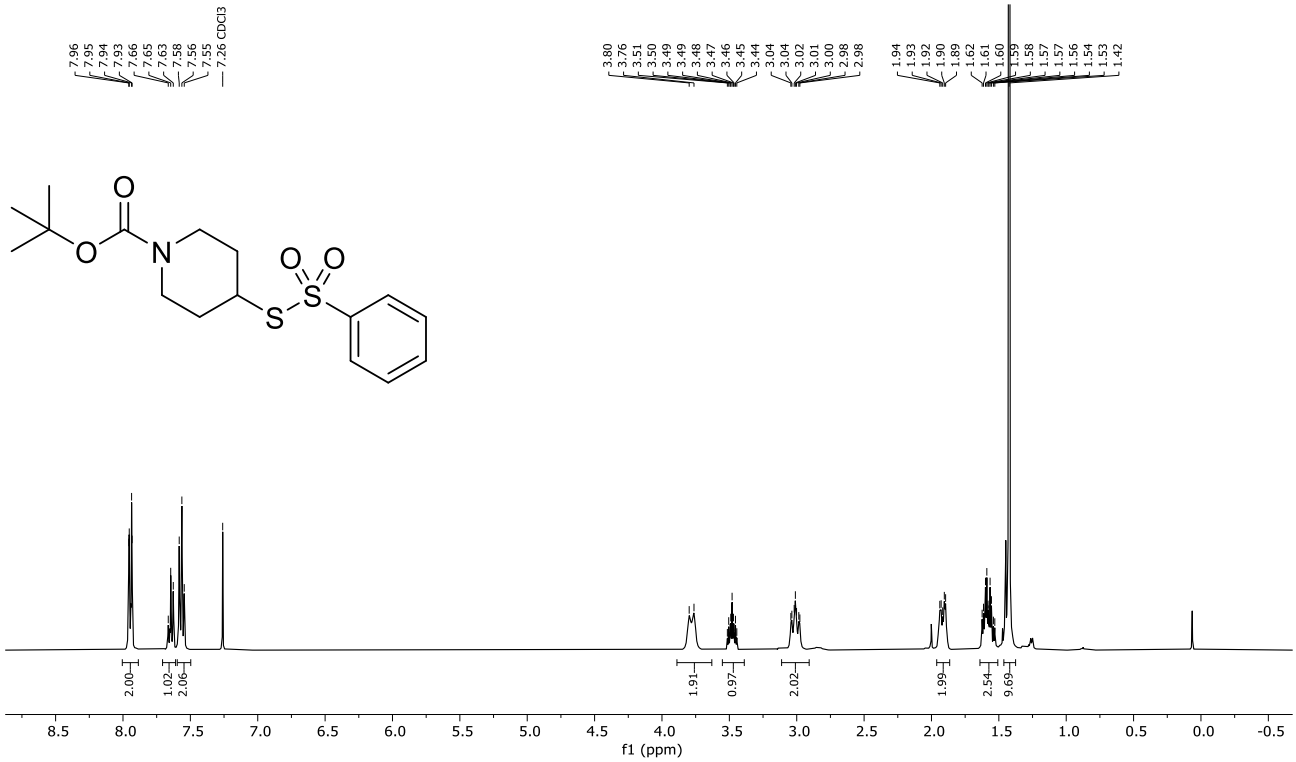
^{1}H NMR (400 MHz, $CDCl_3$) of **S5**



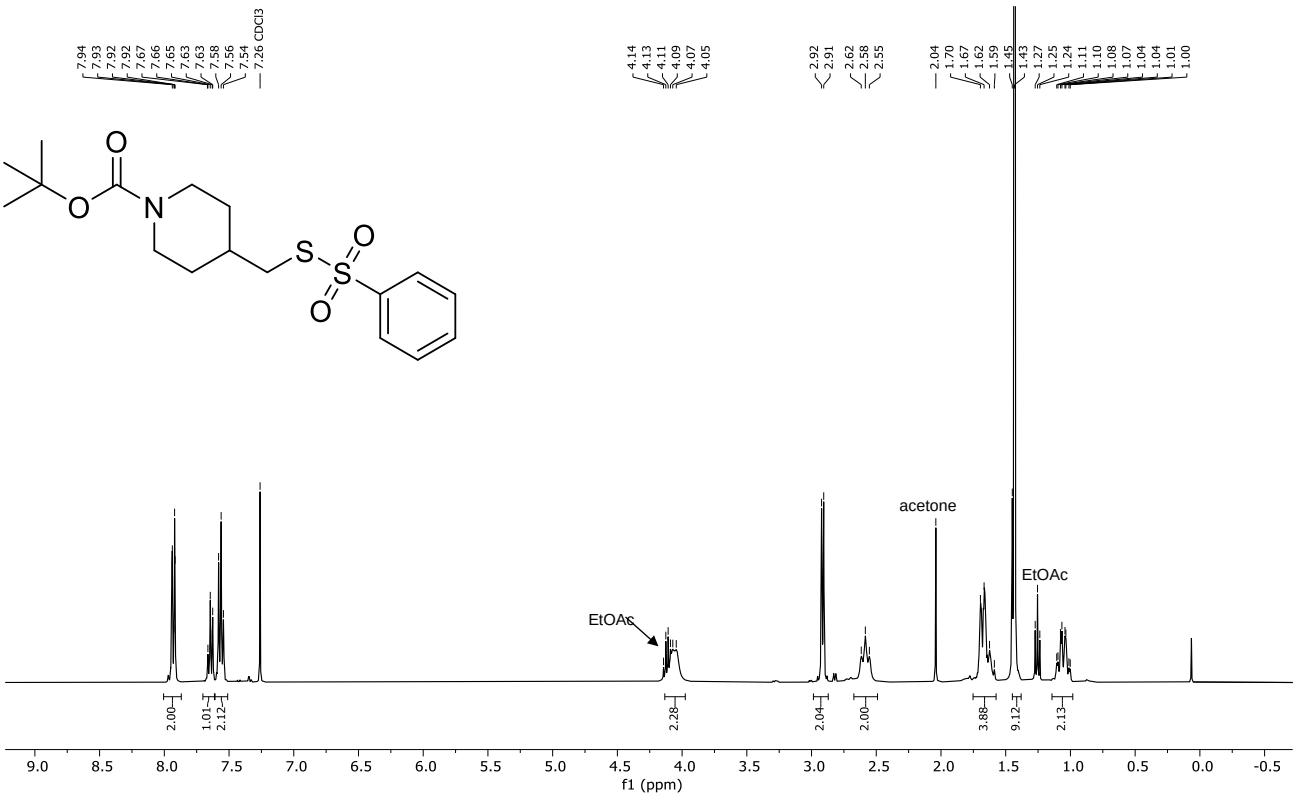
^{1}H NMR (400 MHz, $CDCl_3$) of **S6** (crude)



^{1}H NMR (400 MHz, $CDCl_3$) of **S7**



^{1}H NMR (400 MHz, $CDCl_3$) of **S8** (crude)



Equipment	Model and/or cat. #	Supplier
96-well deep well plates	Fisherbrand 96-well deepwell TM polypropylene microplates (2.0 mL)	Thermo Fisher Scientific (Waltham, MA, USA)
384-well assay plate	polystyrene, Fbottom, μClear, black (cat. #781096)	Greiner Bio-One GmbH (Frickenhausen, Germany)
384-well LDV plate	Echo qualified 384-well COC source plate (cat. #LP-0200)	Labcyte (San José, CA, USA)
384-well PP plate	Echo qualified 384-well polypropylene source plate (cat. #P-05525)	Labcyte (San José, CA, USA)
1536-well OptiPlate	OptiPlate-1536F, untreated (cat. #6004270)	PerkinElmer (Waltham, MA, USA)
Adhesive metal plate lids	Silverseal sealer, aluminum (cat. #676090)	Greiner Bio-One GmbH (Frickenhausen, Germany)
Adhesive PP plate lids	Adhesive polypropylene film (cat. #IST-125-080LS)	IST scientific (Farnham, United Kingdom)
Automated bulk dispenser	CERTUS Flex	Fritz Gyger AG (Gwatt, Switzerland)
Automated SPPS synthesizer	MultiPep 2 Rsi	Intavis AG (Tübingen, Germany)
Centrifuge	Sigma 4-16KHS centrifuge (equipped for plates or falcon tubes)	Sigma Laborzentrifugen GmbH (Osterode, Germany)
HPLC-MS system	Single quadrupole MS system (Shimadzu LCMS-2020)	Shimadzu (Kyoto, Japan)
pH meter	Mettler Toldeo FiveEasy pH meter	Mettler (Columbus, OH, USA)
Preparative HPLC	Waters system (2489 UV detector, 2535 pump, fraction Collector III)	Waters AG (Baden, Switzerland)
Rotary vacuum concentration	Maxi concentrator RVC 2-33 CDplus attached to Alpha 2-4 LSCbasic freeze dryer	Martin Christ Gefriertrocknungs-anlagen GmbH (Osterode, Germany)
Seal pad for filter plate	6 mm ethylen-vinyl acetate pad (cat. #7826301)	Rayher GmbH (Laupheim, Germany)
SPPS reactor syringes	5 mL BD syringe, polypropylene with 25 μm PE frit	Carl Roth GmbH (Karlsruhe, Germany)

SPPS 96-well synthesis plates	Polypropylene 96-well filter plates (cat. #OF1100)	Orochem (Naperville, IL, USA)

Supporting Table S5. List of applied laboratory equipment and utilities

4.8. References

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5. Small molecule inhibitors development by nanoscale library synthesis and functional screening of crude product

5.1 Work contribution

Chapter 5 of this thesis is based on the following manuscript in preparation:

Small molecule inhibitors development by nanoscale library synthesis and functional screening of crude product

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Specific contributions:

I co-designed the research project and performed the experiments. Z.B., X.D. and C.H. conceived the strategy for synthesizing and screening the combinatorial library and planned the experiments. Z.B and X.D. established the procedures to cap thiol groups of the compounds. Z.B. designed and synthesized the thrombin library and characterized the hits. Z.B. and A.Z. screened the thrombin library. G.M. analyzed inhibitor-thrombin complexes by native MS. A.L.N., E.W. and L.F.-S. helped with peptide synthesis. C.H. and Z.B wrote the manuscript. All authors discussed the results and edited/commented the manuscript.

5.2 Abstract

Combinatorial synthesis of chemical compounds at small scale followed by high-throughput screening of crude products in biological assays, also abbreviated by the term ‘direct-to-biology’, allows rapid access to chemical diversity and identification of new bioactive molecules and pharmacophores. An efficient strategy for library synthesis, that was successfully applied for cyclic peptide and macrocycle ligands development, is the combination of solid- and solution phase methods. Herein, we applied the same principles for synthesizing and functionally screening of small molecule compounds of interest for small molecule drug development. We conceived and tested a workflow in which ‘m’ diamino acid core structures are combinatorially modified with ‘n’ carboxylic acids on solid phase (via the first amino group), and diversified with ‘o’ carboxylic acids in solution phase at nanomole-scale via the second amino group to generate and screen ‘m×n×o’ small-molecule-weight compounds in 1,536-well plates. Application of the approach to the protease thrombin identified potent inhibitors with nanomolar affinity (K_i = 93±3 nM). The approach allowing rapid generation of target-tailored small molecule libraries for functional screening is general and might be applied to a broad range of targets for which bioassays can be performed in microwell plates at high-throughput.

5.3 Introduction

The development of small molecule drugs requires the identification of molecules that specifically engage and modulate disease targets, which often involves a step in which large collections of compounds are tested at high-throughput. In recent years, methods were established to combinatorially synthesize large libraries of compounds at a small scale and test them directly without prior purification.[1–3] Such strategies, now also named 'direct-to-biology' methods,[4] have the advantage that large libraries can be produced due to omission of time and resource demanding chromatographic purification, and due to the small scale of the reactions and thus a minimal reagent use. Direct-to-biology were applied for affinity ranking of small molecule compounds,[5] screening products of multi-component reactions,[6] cyclic peptide and macrocyclic compounds,[2,3,7] PROTACs,[8,9] and molecular glues.[10] Reported ligands identified with such methods are inhibitors of thrombin[2,3,7] (also see chapter 4 of this thesis), tissue kallikrein,[2] p53:MDM2,[3] as well as binders of menin,[6] degraders of BRD4,[8,9] and GSPT1/2.[10]

For generating large numbers of cyclic peptide or macrocyclic compound libraries, solid phase synthesis and solution phase synthesis methods were combined, as described in the following.[3,7]By solid phase synthesis, hundreds of short cyclic peptides are produced in individual wells of 96- or 384-well plates. A strategy in which the amino acid protecting groups are removed and washed away before release of the peptide from the solid phase offer access to large numbers of peptides with high purity that did not require further purification.[11,12] By solution phase synthesis at a small scale in wells of 1,536-well plates, the cyclic peptides are subsequently diversified by acylation of an amino group that is present in each peptide.[3] For example 192 cyclic peptides were combinatorially reacted in individual wells of microwell plates with 104 carboxylic acids to generate 19,968 different macrocyclic compounds.[3] The solution phase reaction was performed at a nanomole or even picomole scale using acoustic reagent dispensing[3,13] to allow a high throughput and minimize reagent use. Crude products are directly screened using functional assays and yield bioactive compounds with nanomolar potency and high target selectivity.[3,7]

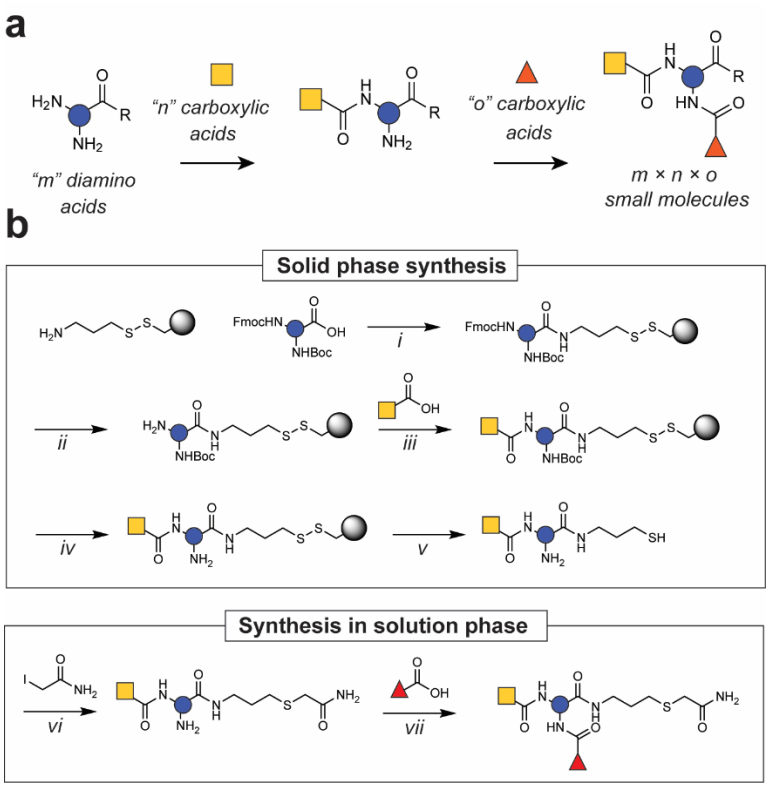


Figure 21: Library synthesis strategy. (a) Overview of approach for the combinatorial synthesis of small molecule compound libraries. The number of building blocks applied are indicated by the variables m, n and o. (b) Chemical transformation and conditions. Solid-phase synthesis: i) HATU, NMM, ii) piperidine, iii) HATU, NMM, iv) TFA, H_2O, TIPS, v) BDT, Et_3N. Solution phase: vi) MeCN, NH_4HCO_3 buffer pH 8, vii) HBTU, DIPEA.

Herein, we test if the combination of solid phase compound synthesis and solution phase diversification is applicable to the development of small molecule ligands too. As a molecule format, we choose to develop small molecules with a central diamino acid as core that is diversified at the two amine positions by carboxylic acids (Figure 1a). For the first diversification step, we apply solid phase peptide synthesis and for the second one solution phase conditions (Figure 1b). We take advantage of methods previously developed for nanoscale cyclic peptide library synthesis based on a disulfide-linker solid-phase, and develop new procedures to obtain linear rather than cyclic compounds. Application of the methods to the protease thrombin, a target for thrombotic disorders, yield inhibitors with nanomolar potency (Ki = 93±3 nM), demonstrating the suitability of the presented molecule format and library synthesis and screening strategy for the development of bioactive small molecule ligands.

5.4 Results and discussion

Chromatography-free synthesis of small-molecule compounds

We first established methods for synthesizing large libraries of small molecules composed of the following three variable components: a diamino acid (blue) and two carboxylic acids (yellow, red) (Figure 1a). By reacting “m” diamino acids with “n” and “o” carboxylic acids in a combinatorial fashion, we aimed at generating libraries comprising “m×n×o” different molecules. The small molecules obtained have a similar format to DNA-encoded libraries that were recently developed by Oehler, S. *et al.* in which structurally diverse carboxylic acid-based fragments were conjugated to 4-amino-proline cores.[14] We aimed at obtaining the compounds in wells of 1,536-plates at millimolar concentrations suitable for high-throughput screening using functional assays.

For conjugating the two first building blocks, the diamino acid (blue) and the first carboxylic acid (yellow), we used solid phase peptide synthesis (SPPS). An automated parallel peptide synthesizer, recently modified in our lab to produce peptides in 384- instead of 96-well plates, can produce peptides in four plates in parallel and thus 1,536 peptides in one run.[12] We used a disulfide linker resin as solid phase that we had recently developed for the high-throughput synthesis of peptides.[11,15] With this resin, acid-labile side chain protecting groups such as Boc or Trt can be cleaved and washed away before the peptide is released from the resin by disulfide reduction to obtain rather pure peptide that does do not need chromatographic purification (> 80% purity).[15] The disulfide resin was ideal for the small molecule library synthesis because the diamino acid building blocks (blue), having one amine protected by Boc, could be modified with a carboxylic acid (yellow) before removing the Boc group and release of the two-building block product from the solid phase (Figure 1b). The use of disulfide liker resin adds an appendix (aminopropylthiol) that is present in the functional screen but can be omitted in the final compound.

Before the coupling of the second carboxylic acid (red) to the second amino group of the diamino acid core, we had to cap the thiol group that could react with activated carboxylic acid. We tested a range of reagents and conditions and found that iodoacetamide applied at 4-fold molar excess over 1.5 mM peptide in ammonium bicarbonate buffer (pH 8) containing 50% acetonitrile was most suited. The excess of iodoacetamide was quenched with 3-fold excess (over iodoacetamide) of β-mercaptoethanol (β-ME), that was volatile and could be removed by centrifugational vacuum evaporation. In this latter step, the solvents were removed too and

the library intermediate products were dissolved in DMSO. The thiol-quenched alkylating agent is not volatile, but it was not found to interfere with subsequent reaction steps or the assay used for library screening.

Next, the library intermediates (blue-yellow product in Figure 1) were diversified by appending diverse carboxylic acids.[3,7] This step required a large number of parallel reactions, as all "m×n" intermediates were acylated with "o" different carboxylic acids in separate reaction wells. We performed these reactions in wells of 1,536-well plates at a 4-nmol scale and using acoustic dispensing for reagent transfer. For efficient acylation reactions, we transferred the intermediates in 200 nL DMSO and the carboxylic acids in 200 nL DMSO (5-fold molar excess over intermediate), and added 1.6 μL of HBTU activating agent and DIPEA base in DMF. The excess of activated carboxylic acid could efficiently be quenched by addition of 2-methoxyethylamine in 5-fold molar excess over acid. The products were dried by centrifugational vacuum evaporation and dissolved in DMSO to obtain stocks of around 1 mM small molecule compound. The quenching reagent was chosen due to its good nucleophilicity as a primary amine, its volatility being in an optimal range that allows both convenient handling and easy removal by evaporation, and an ether functionality that decreases the risk of forming amide byproducts with low solubility that could precipitate in the aqueous media used for the subsequent biochemical assay.

Synthesis of 24,288-member small molecule library

We tested the synthesis strategy by generating a library and analyzing the quality of randomly picked intermediate products and final library compounds (Figure 2). As diamino acid building blocks (blue), we used 23 different commercially available Fmoc amino acids that contain a Boc-protected amino group in addition to an Fmoc-protected amine (Figure 3; D1-D23). These building blocks are all unnatural amino acids with the exception of L-lysine that was included. As carboxylic acids, we used 66 diverse chemical structures in the first diversification step on solid-phase (C1-C66; Figure 2a, yellow building block), and 16 of these carboxylic acids also in the second that was performed in solution (C1-C16; Figure 2a, red building block). The combination of 23×66×16 building blocks produced a library of 24,288 small molecule compounds. Computational analysis of key physicochemical properties for these molecules (without the capped thiol-containing tail) showed that they have in average MW of 433 Da, a clogP of 1.7, a polar surface of 125 $Å^2$, 2.9 and 7.8 H-bond donors and acceptors, respectively, and thus properties suitable for small molecule drug development (Figure 2b).

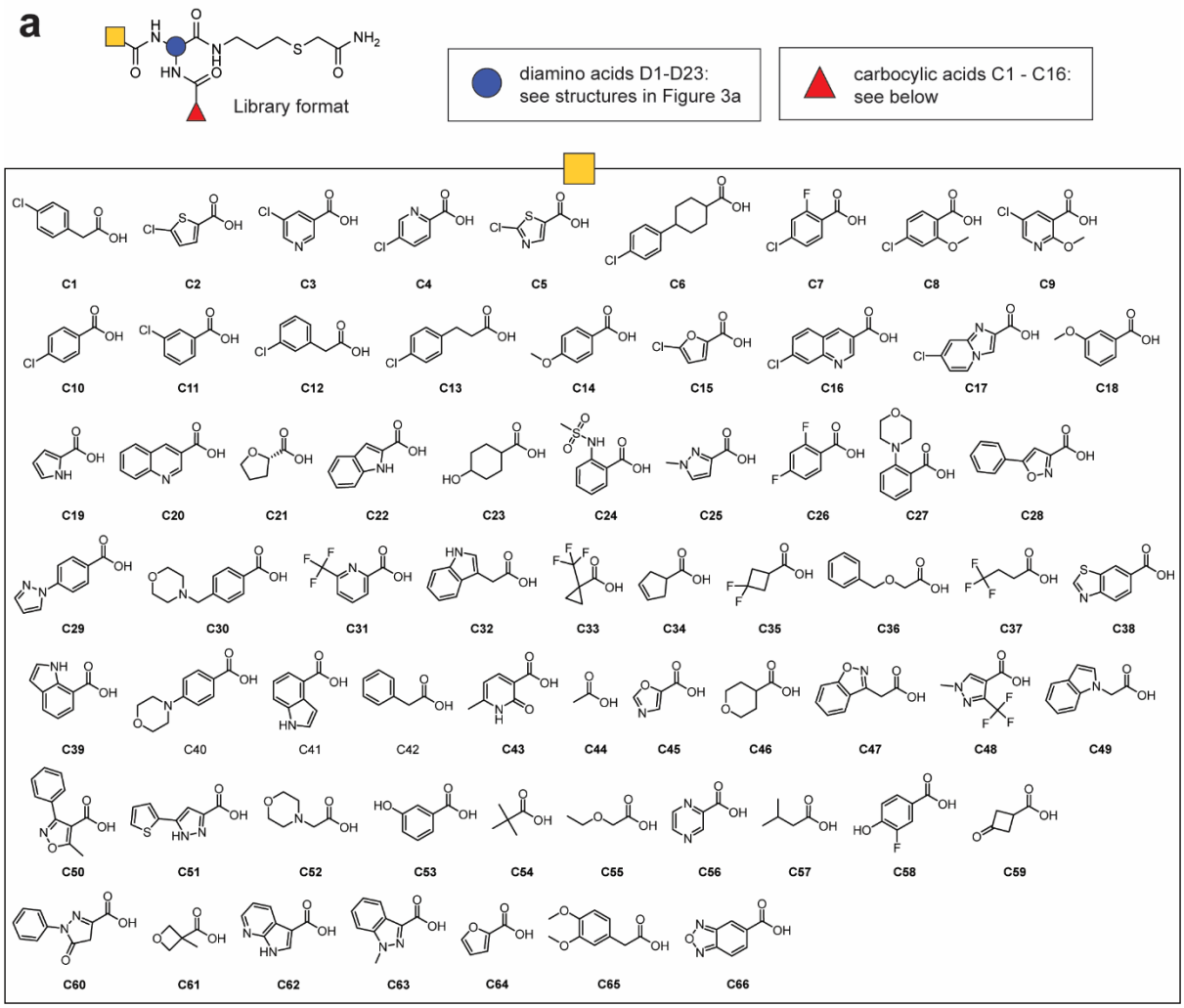


Figure 2: Library synthesis and quality assessment. (a) Chemical building blocks used. (continued on next page).

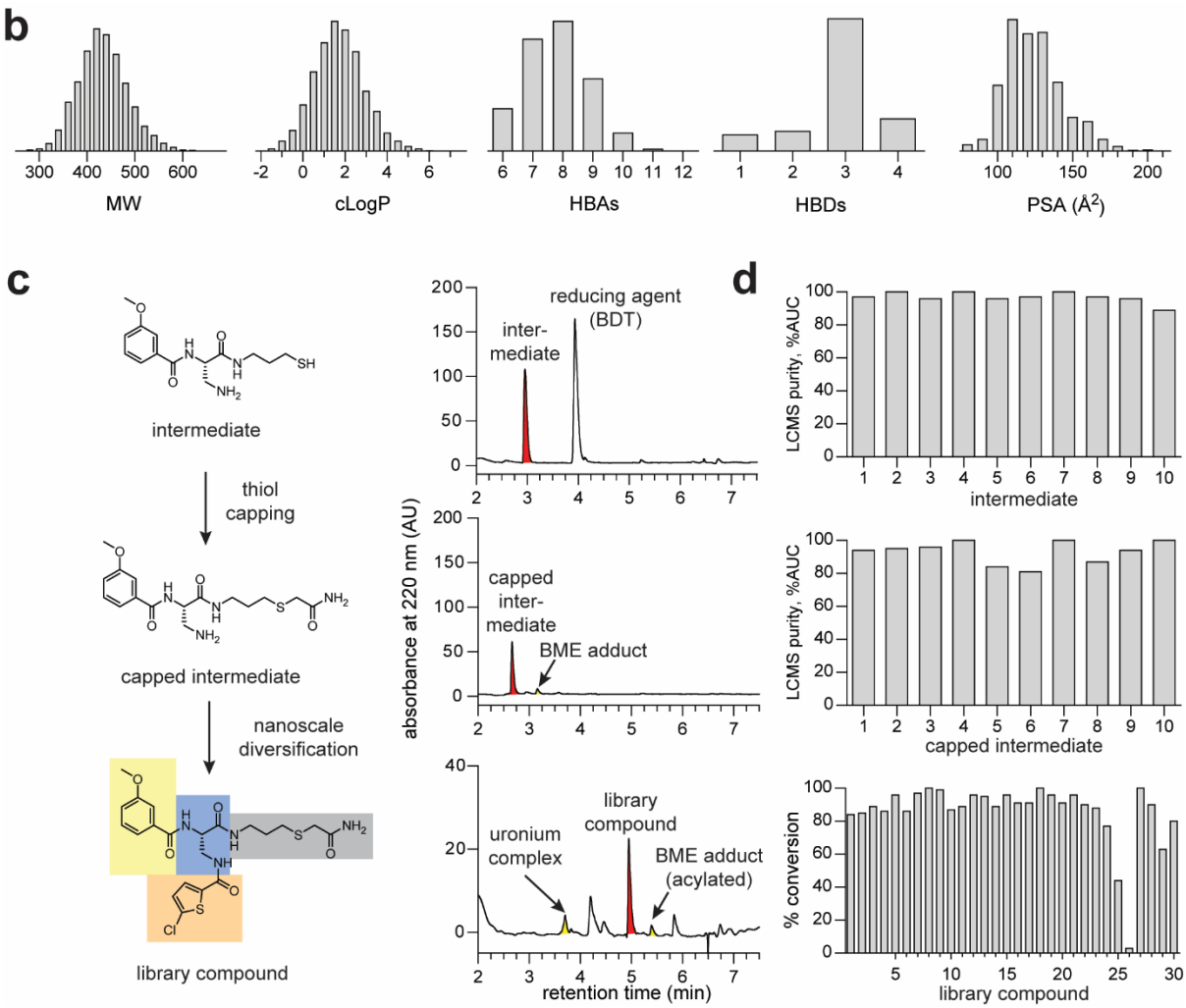


Figure 2, continued. Library synthesis and quality assessment. (b) Physicochemical properties of library members (excluding the capped C-terminal appendix) calculated based on the chemical structures. (c) Solution-phase transformations shows for an example compound (D1-C18-C2). The different building blocks are highlighted with the same colors used in the synthesis schemes. HPLC chromatograms are shown. (d) Purity of intermediate and final product for randomly picked library compounds, extracted from HPLC chromatograms (Figures S2 and S3).

We analyzed the efficiency of the chemical transformations and the quality of the library by LC-MS of randomly picked intermediate products after i) the solid-phase synthesis or ii) after capping of the thiol group, and of products after nanoscale diversification with a second carboxylic acid (Figure 2c. 2d and Supplementary Fig. S1-S3). The HPLC chromatograms showed mostly the desired products along with reagents used in the reaction and small quantities of side-products, as shown for an example compound (Figure 2c). After the solid phase synthesis, the analysis showed the desired products along with a peak from the reducing agent BDT that was used to release the product by disulfide reduction and was present as the products were analyzed before BDT evaporation. The solid-phase synthesis

yielded around 0.6 μmol of intermediate library product (Figure 2d, top panel; Supplementary Fig. S1). LC-MS analysis of the same peptides after thiol group capping showed selective and quantitative modification and an average purity of 84% (Figure 2d, middle panel; Supplementary Fig. S2). Analysis of 30 randomly picked final product showed efficient acylation for most library members, as shown by more than 80% acylation yield for 25 compounds (Figure 2d, bottom panel; Supplementary Fig. S3). The analysis of the final products showed also the presence of quenched acylation reagents that were expected as carboxylic acid and activating agent were applied in 5-fold excess. Taken together, the synthesis strategy was successfully applied to generate an around 25-thousand-member library obtained in DMSO stocks of 1 mM in 1,536-well plates, wherein the main products were the desired library compounds for most reactions (typically >80%, measured by LC absorbance peak area at 220 nm, considering only peptidic impurities).

HTS identifies nanomolar thrombin inhibitors

We screened the library against human thrombin, a trypsin-like serine protease that is implicated in several thrombotic disorders. For thrombin, the small molecule drug dabigatran etexilate was already developed and is successfully applied as oral drug for thrombosis prevention. However, due to two charged groups in the active inhibitor, the molecule is applied as pro-drug which displays a rather small oral availability of 6.5% that leaves much room for improvement.[16] In the synthesized libraries, we used building blocks that were previously identified to form favorable binding interactions with the S1-specificity pocket of thrombin, such as 5-chlorothiophene (C2) or other chlorinated aromatic carboxylic acids, and we thus expected that the library contains inhibitors with low micromolar affinity or even better. We thus screened the library at a final compound concentration of 10 μM. Inhibition of thrombin was quantified using a fluorogenic coumarin substrate and measurement of substrate conversion over 15 minutes for each compound.

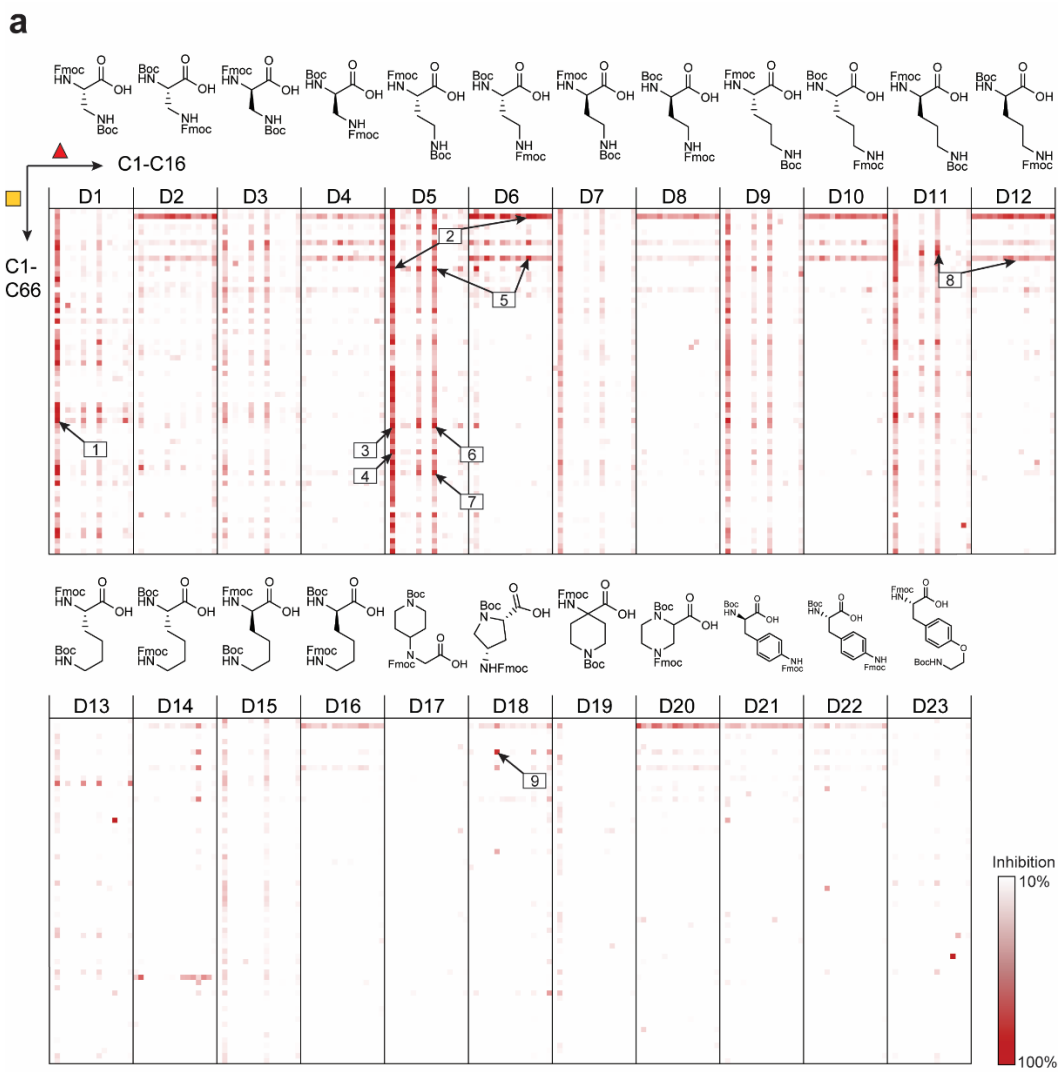
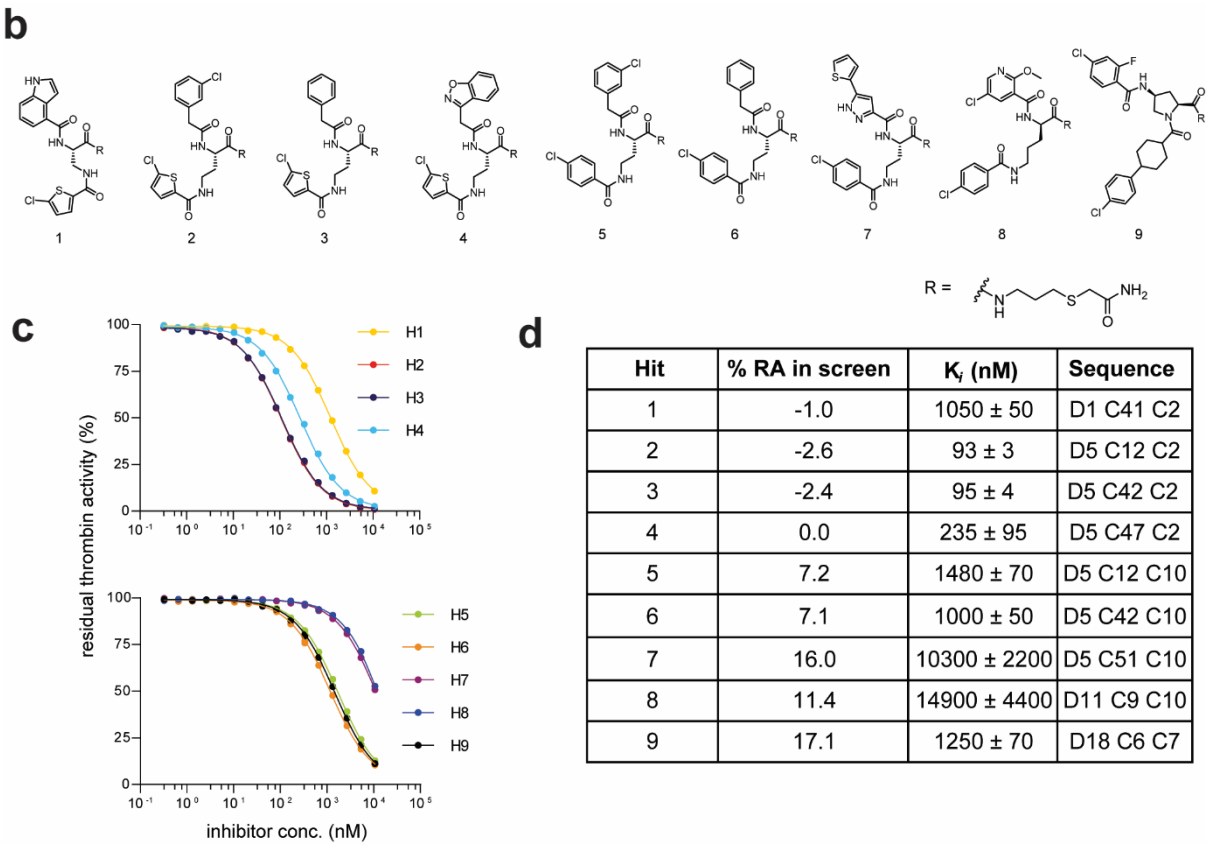


Figure 3. Library screen and thrombin inhibitors. (a) Heatmap showing thrombin inhibition measured in HTS. Each panel shows compounds based on the same diamino acid, with the structure of the diamino acid shown above. The compounds in each panel are ordered based on the first carboxylic acid (yellow, y-axis) and the second carboxylic acid (red, x-axis). Hits chosen for re-synthesis, purification and characterization are labeled with numbers. (continued on next page).



Hit	% RA in screen	K_i (nM)	Sequence
1	-1.0	1050 ± 50	D1 C41 C2
2	-2.6	93 ± 3	D5 C12 C2
3	-2.4	95 ± 4	D5 C42 C2
4	0.0	235 ± 95	D5 C47 C2
5	7.2	1480 ± 70	D5 C12 C10
6	7.1	1000 ± 50	D5 C42 C10
7	16.0	10300 ± 2200	D5 C51 C10
8	11.4	14900 ± 4400	D11 C9 C10
9	17.1	1250 ± 70	D18 C6 C7

Figure 3 (continued). (b) Chemical structures of hits. (c) Inhibition of human thrombin measured using a fluorogenic substrate. (d) Building blocks of hit compounds, activity in primary screen, IC_{50} of confirmation screen with crude product using four different inhibitor concentrations, K_i of purified compound. Mean values and SDs of two independent measurements are shown for the K_is.

The HTS identified 291 compounds that showed > 50% thrombin inhibition (1.2%) and 29 compounds with more than 90% inhibition (0.12%) (Figure 3a). Arrangement of the compounds in groups according to the diamino acid core (blue) and display of the activity in heatmaps showing the first variable carboxylic acid (yellow) on the Y-axis and the second one (red) on the X-axis revealed a strong structure activity relationship. Most decisive for the activity was the presence of specific carboxylic acids in combination with some highly preferred diamino acids, as seen by horizontal or vertical “lines” in the heatmap (Figure 3a). Compounds with 5-chlorothiophene-2-carboxylic acid (C2), 3-chlorphenylacetic acid (C12) and phenylacetic acid (C42) showed the highest activities in combination with several diamino acids. The diamino acids N_α-Fmoc-N_γ-Boc-L-2,4-diaminobutyric acid (D5) and N_α-Boc-N_γ-Fmoc-L-2,4-diaminobutyric acid (D6) yielded the best inhibitors. The two amino acids provide identical cores but their two amino groups are acylated in different orders during the library synthesis. In case of D5, the α-amino group (protected with Fmoc) is first acylated, and in case of D6, the side chain amine (Fmoc-protected) is first acylated. As the carboxylic acids

C1-C16 are applied to both amino groups, some of the library members (2,848 compounds, 11.7%) and hits are identical compounds. For example, the hits **2** and **5** are present in the library twice and were thus also identified twice (Figure 3b). In fact, while we synthesized 24,288 compounds, only 21,440 are unique compounds and the remaining 2,848 each occur twice. The symmetry can also be seen by the horizontal and vertical "red lines" of neighboring core compounds. The compound pairs were synthesized through different paths and the finding of equivalent activities further confirmed the good quality of the library and the suitability of the library synthesis approach.

To identify the most active compounds for purification and characterization, we repeated the activity screen for the 96 best hits, but this time measured activities at four different inhibitor concentrations (10 μM, 3.3 μM, 1 μM and 0.33 μM; Supplementary Figure S5). Of these 96 compounds, 80 contained the 5-chlorothiophene-2-carboxylic acid (C2). The four best inhibitors **1**-**4** featuring the C2 building block contained the either the diamino acid D1 (**1**) or D5 (**2**-**4**) that differ only in the length of the side chain (one carbon difference), C2 linked to the side chain, and diverse aromatic carboxylic acids linked to the N-terminal amine (Figure 3b). Of the five most active compounds not having a 5-chlorothiophene-2-carboxylic acid, three shared some structural similarity being the diamino acid D5 and a 4-chlorobenzoic acid linked to the side chain of the core amino acid (**5**-**7**; Figure 3b). Resynthesis of the nine molecules at a scale of 50 μmol followed by HPLC-purification and characterization showed that the best inhibitor (**2**) has a K_i of 93±3 nM, and the second best one (**3**) a K_i of 95±4 nM (Figures 3c, 3d and Supplementary Fig. 5). Synthesis of these inhibitors **2** and **3** without the aminopropyl-thio-acetamide appendix (**2b** and **3b**) inhibited thrombin too, though with lower inhibitory constants of 1.7±0.1 μM and 1.5±0.1 μM, respectively (Supplementary Figure S7).

Inhibition mode analysis by native MS

The 5-chlorothiophene-2-carboxyl and 4-chlorobenzoyl groups present in the most active compounds are found in previously developed macrocyclic inhibitors of thrombin,[3,7] suggesting that herein identified small molecule inhibitors bind in a similar way, occupying the protease's active site and pointing with these carboxylic acids into the S1 specificity pocket. Given the linear nature of the inhibitors, we wondered if they bind to the active site similar as peptide substrates of thrombin. If so, they could potentially form an acyl-enzyme complex which may be an advantage if this complex is stable and locks the enzyme in an inactive form, but which may also be a disadvantage if the intermediate is hydrolyzed and is consumed. To shed light onto the mechanism, we analyzed the thrombin/inhibitor complex for compounds **2**

and **3** by native MS. The masses of the complexes were 546.22 and 511.18 Da larger than thrombin, which corresponded to the masses of the two inhibitors (Figure 4). The detection of these peaks confirms that compounds **2** and **3** bind to thrombin in a non-covalent manner, i.e. without the formation of an acyl-enzyme complex.

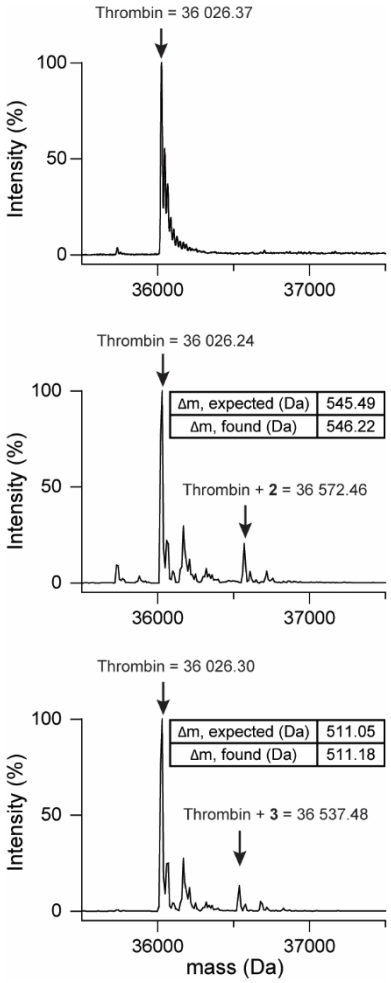


Figure 4. Binding mode of thrombin inhibitors. Native MS analysis of thrombin alone (top), thrombin incubated with 3-fold excess of inhibitor **2** (middle), and thrombin incubated with 3-fold excess of inhibitor **3** (bottom). Peaks corresponding to the mass of thrombin+inhibitor indicate that the small molecules bind non-covalently and do not form acyl-enzyme complexes.

5.5 Conclusion

We have successfully combined solid- and solution-phase synthesis methods to generate large libraries of small molecules that are arrayed in wells of 1,536-well plates and can be screened for desired bioactivities using functional assays. In a proof-of-concept study synthesizing 24,288 small molecule compounds and screening against human thrombin, we identified nanomolar inhibitors that bound and blocked the protease at its active site. The synthesis and screening of the 24K library could be done in a short time of less than two weeks. The synthesis of the 1,536 intermediate products done in four 384-well plates by a modified peptide synthesizer took only a week, including reagent preparation (1 day), solid-phase synthesis (2 days) and workup (2 days). The combinatorial diversification of the intermediate products done by automated acoustic liquid transfer in nanoliter volumes took one day, and the activity screen was also done in one day, making the entire process fast and highly economical.

For the library generation, a thiol group was used for the solid-phase synthesis as handle to immobilize the molecules but was impractical for the solution-phase reaction where we capped it with iodoacetamide to prevent reaction with activated acids. In future, advantage may be taken from the thiol group also in the solution-phase step where it may be used to further diversify the compounds by reacting with structurally diverse thiol-reactive building blocks. For example, ‘m’ diamino acid core structures modified with ‘n’ carboxylic acids on solid phase may be diversified with ‘o’ different thiol reactive groups before modification of the second amine with ‘p’ carboxylic acids to generate and screen ‘m×n×o×p’’ small-molecule-weight compounds. For modification of the thiol group, many building blocks are commercially offered, such as reagents having chloro- or bromoacetamide groups, bromomethylaryls or acrylamides. The structural and chemical diversity of the molecule library may also be expanded by using additional building blocks not yet used here, such as another around 30 diamino acids and hundreds of carboxylic acids which are commercially available at reasonable prices.

Compared to the DEL molecule libraries reported by Bassi, Neri and co-workers, which have a similar format but contain over 4 million molecules, the number of molecules that can be generated and screened with the presented approach is much smaller. An interesting

possibility would be to combine the DEL approach with the single-well compound synthesis and screening approach. For example, it would be conceivable to use a DEL library with the same molecular format and up to 1 billion molecules to identify potential binders followed by the synthesis and screen of the approximately 10,000 most enriched molecules using the approach presented here. With this combination, very large libraries could not only be sampled but also tested for the desired activity in a functional screen, that is easily possible with the herein developed strategy. This combination could also eliminate a weakness of DEL libraries and selections, where many of the identified compounds cannot be validated as hits and turn out to be inactive.

In conclusion, we present an approach that combines solution- and solid-phase synthesis methods to rapidly generate ten-thousands of structurally diverse compounds. The compounds arrayed in 1,536-well plates can be screened by functional assays which allows reliable identification of bioactive compounds. The building blocks of the ten-thousands of compounds can be chosen from several dozens of commercial diamino cores and hundreds to even thousands of carboxylic acids and are individually programmable which allows generating of target-focused libraries, or potentially the synthesis of compounds enriched in selections with DELs.

5.6 Materials and methods

All reagents and solvents were of analytical grade and used as obtained from commercial suppliers without further purification. Reactions were monitored by UHPLC-MS analysis. Rotary vacuum concentration of peptides was carried out using a Christ RVC 2-33 CDplus IR. UHPLC-MS analyses were performed with a UHPLC and single quadrupole MS system (Shimadzu LCMS-2020) using a C18 reversed phase (RP) column (Phenomenex Kinetex 2.1×50 mm C18 column, 100 Å pore, 2.6 µm particle). A linear gradient of solvent B (0.05% HCOOH in MeCN) over solvent A (0.05% HCOOH in water) rising from 0% to 60% during t = 1.00–6.00 min was applied at a flow rate of 1.00 mL/min.

Automated solid-phase peptide synthesis (SPPS)

The resin was assumed to have a loading of 1.2 mmol/g aminomethyl group (this is the functional group of the commercial resin used) or 0.5 mmol/g aminopropylthiol (this is the group appended to install the disulfide linker), as obtained before using the same preparation procedure. Automated solid-phase peptide synthesis was performed on an Intavis Multipep RSi synthesizer (equivalent to CEM MultiPep 2) that was modified for the automated SPPS in 384-well synthesis plates as reported by Schüttel *et. al.*[12] Commercially available 384-well filter plates were used (201035-10 PE 25 UM, Agilent). For each 384-well synthesis plate, around 1.1 g of dry resin was washed with 10 ml DMF. After discarding the solvent, another 10 ml DMF was pulled into the synthesis syringe, and allowed to stand for 5 minutes. The excess solvent was then removed by vacuum filtration. Around 3 µmol resin/well (loading based on aminomethyl group) was loaded into the wells using the appropriate solid dispenser. This amount of resin corresponded to around 1.25 µmol aminopropylthiol group per well.

For the coupling of the Fmoc diamino acids, a reagent rack with 59 derivative positions was used. All reactions, reagent transfers and washing steps were performed at room temperature. All the equivalents of reagents indicated for the peptide synthesis are relative to the aminomethyl loading (1.25 µmol aminopropylthiol per well). The resin in each well was washed with 6 × 70 µl DMF. Coupling was performed with 30 µl of Fmoc diamino acid (500 mM in NMP, 15 µmol, 5 equiv.), 28 µl HATU (500 mM in NMP, 14 µmol, 4.7 equiv.), 8 µl of N-methylmorpholine (4 M in NMP, 32 µmol, 10.7 equiv.) and 1 µl NMP. All components were premixed for 1 min, then added to the appropriate well containing resin and incubated for 60 min without shaking. The final volume of the coupling reaction was 67 µl, and the final

concentrations of reagents were 224 mM amino acid, 209 mM HATU, and 478 mM N-methylmorpholine. Coupling was performed twice, followed by resin washing with 6 × 70 μl/well of DMF. Fmoc deprotection was performed twice, each time using 35 μl/well of piperidine in DMF (1:4, v/v) for 2 min. After Fmoc deprotection, the resin was washed with 8 × 70 μl DMF. When the coupling cycle was finished, 500 mM NMP solutions of carboxylic acids were placed in the synthesizer using a reagent rack with 107 derivative positions, and the same coupling procedure was performed, omitting the Fmoc deprotection step. At the end of the peptide synthesis, the resin was washed with 2 × 70 μl/well of DCM. The names and CAS numbers of amino acid building blocks D1-D23 and carboxylic acids C1-C66 are detailed in Supplementary tables 1 and 2.

Boc group deprotection of resin-bound intermediates

After DCM washing, the 384-well plates were dried (4 h, room temperature on air) and the protecting groups removed by incubation with 2 × 1 h deprotecting solution (TFA/TIS/H_2O, 38:1:1, 50 μl/well). The cocktail was transferred using a multichannel pipette (8 channels, 30-300 μl, VWR) with activated charcoal-protected tips (5469, MBP 200 Solvent Safe TM) while the outlets of the wells were blocked by pressing the tips of the plate onto a 10 mm thick EVA foam pad (76899, Creotime). During incubation, the 384-well synthesis plate were placed in a semi-closed box in a fume hood. The deprotection solution was allowed to drain by gravity, and the resin was washed with DCM (3 × 50 μl/well) using a manual plate vacuum filtration station and dried on air for a few hours.

Reductive release of intermediates from solid support

The 384-well reactor plates containing the deprotected peptides on resin (theoretical: ~3 μmol/well, 1.0 equiv.) were stacked onto 384-well deep well plates (CLS3347, Corning). The peptides were released by applying release solution (60 μl/well, 200 mM TEA and 200 mM BDT, 4.0 equiv. in DMF) using a multichannel pipette (8 channels, 30-300 μl, VWR) with activated charcoal-protected tips (5469, MBP 200 Solvent Safe TM). The plates were placed in a semi-closed box and incubated overnight at room temperature in a fume hood. Subsequently, the synthesis plates were sealed with a polypropylene adhesive film (G070-N, Kisker Biotech GmbH & Co.), stacked on 384-well deep-well plates (CLS3347, Corning), then centrifuged at 860 × g (2000 rpm on a Thermo Scientific Heraeus Multifuge 3L-R centrifuge with a Sorvall 75006445 Rotor, radius = 19.2 cm rotor) for 2 min at RT. The release was repeated once more (5 hours incubation only), then the two release filtrates were combined

(120 μl in total). The peptide plate was covered with aluminum adhesive film (AB-0626, Thermo Scientific), then stored at 4 °C.

LC-MS analysis of intermediate products

Peptide samples were analyzed by LC-MS analysis with a UHPLC and single quadrupole MS system (Shimadzu LCMS-2020) using a C18 reversed phase column (Phenomenex Kinetex 2.1 mm × 50 mm C18 column, 100 Å pore, 2.6 μm particle size) and a linear gradient of solvent B (MeCN, 0.05% formic acid) over solvent A (H2O, 0.05% formic acid) at a flow rate of 1 ml min^{-1}. For all samples, a gradient of 0 to 60% MeCN within 5 min was applied. Absorbance was measured by UV light at 220 nm. Mass analysis was performed in positive ion mode. 100 μl polypropylene (PP) HPLC microvial (Shimadzu, 980-14379) with PP and teflon caps (Shimadzu, 980-18425) were used for all samples. For analyzing peptides after reductive release and final linear peptide stock solutions in DMSO, a 0.5 μl sample was transferred into 30 μl solvent (MeCN/water/TFA, 50:49.95:0.05%, v/v/v) and analyzed using an injection volume of 4 μl.

Quantification of intermediate products

The peptide stock solution concentrations were determined based on the eight copies of the Ac-Tyr and eight copies of Ac-Trp containing reference sequences in the library. As a calibration reference, 25 mM solutions of Ac-Tyr-OH (CAS No. 537-55-3) and Ac-Trp-OH (CAS No. 1218-34-4) were prepared in the reductive cleavage mixture described above. These solutions correspond to 100% of the theoretical peptide yield. A dilution series was prepared from each according to the scheme in Table 4.

Volume from stock, μl	Volume from cleavage mixture, μl	Concentration, mM	Yield, % of theoretical
60	10	21.43	85.7
50	20	17.86	71.4
40	30	14.29	57.1
30	40	10.71	42.9
20	50	7.14	28.6
10	60	3.57	14.3

Table 4: Preparation of the calibration stock solutions for concentration determination by absorbance

5 μl was taken from each of the library reference peptide stock solutions, the blank wells containing no peptide and the calibration samples, and transferred into separate wells of a 96-well V-Bottom PP-plate (6018321, Ratiolab). The solutions were acidified with 3 μl of 5 v/v% TFA in milliQ water (2 equiv. acid relative to the TEA in the stocks), then the plate was concentrated by centrifugal vacuum evaporation using Christ RVC 2-33 CDplus IR instrument. Samples were centrifuged at 0.1 mbar, 30 °C and at 400 × g (1750 rpm in a Christ 124700 rotor with 124708 plate holder inserts, radius = 10.5 cm). The residual samples were dissolved in 25 μl of DMSO containing 0.05 v/v% TFA: The ThermoScientific NanoDrop 8000 spectrophotometer was blanked with milliQ water. For every measurement, 2 μl solution was pipetted onto the instrument pedestals. An average of two absorbance readings at 280 nm were considered as the measurement result. A baseline was measured with the peptide-free synthesis blanks, then the calibration samples and the library reference samples. The concentrations were determined based on the absorbance of the samples minus the absorbance of the blank solution. The absorbances of the calibration points, the fitted calibration curves, the absorbances of the library reference peptide samples, and the calculated stock concentrations is are shown in Supplementary figure S4.

For the subsequent reactions, the concentration of the cleaved peptide stocks was considered to be 5 mM, which corresponds to 20% synthesis yield.

Alternatively, the concentration can be determined in a similar fashion through LC-MS measurements, by comparing the areas under the appropriate peaks in the UV absorbance spectrum.

Centrifugal vacuum evaporation of reducing agent and solvent from intermediates

Of the 120 μl peptide released from the solid phase by reduction (in DMF containing 100 mM BDT and 100 mM TEA), 40 μl (0.2 μmol) were transferred to a well of a V-bottom 96-well plate (Ratiolab, 6018321, PP, unsterile) using an Integra ViaFlo 384 handheld electronic pipette. A volume of 23.7 μl of 5% TFA in MilliQ water (v/v) was added to each well to reach 2 equiv. of TFA over TEA. This sample was subjected to centrifugal vacuum evaporation using Christ RVC 2-33 CDplus IR instrument to remove the solvent (DMF) and reducing agent (BDT). Samples were centrifuged at 0.1 mbar, 30 °C and at 400 × g (1750 rpm in a Christ 124700 rotor with 124708 plate holder inserts, radius = 10.5 cm). The quantity of peptide expected was so low that it could not be expected to be seen by eye, which was the case.

Thiol alkylation of intermediate products

The reduced and dried peptide (0.2 μmol) was dissolved in 133 μl of reaction buffer (50% acetonitrile, 50% milliQ water containing 60 mM ammonium bicarbonate and 6 mM iodoacetamide (4 equiv.), adjusted to pH 8.0). The final peptide concentration in the reaction was 1.5 mM. The buffer was dispensed using a CERTUS Flex automated bulk dispenser (Fritz Gyger AG). The plates were covered with a polypropylene adhesive film (G070-N, Kisker Biotech GmbH & Co. KG) and the reaction incubated for 2 h at RT. After completion of the alkylation reaction, 16 μl of 150 mM β-Me in acetonitrile (2.4 μmol, 12 equiv. relative to the peptide) was added to the reaction mixture and incubated for 1 h at RT. Note that the quenching reaction time should not exceed 1 hour, as this will result in insufficient conversion during the subsequent acylation step. The solvent (MeCN), buffer (bicarbonate) and excess β-Me were removed by centrifugal vacuum evaporation using Christ RVC 2-33 CDplus IR instrument. Samples were centrifuged at 0.1 mbar, 40 °C and at 400 × g (1750 rpm in a Christ 124700 rotor with 124708 and 124705 plate holder inserts, radius = 10.5 cm). The quantity of peptide expected was so low that it could not be expected to be seen by eye, which was the case. The residual peptides were then dissolved in 10 μl DMSO added with bulk dispensing to afford stock solutions with an average peptide concentration of 20 mM, covered with polypropylene adhesive film, centrifuged at 484 × g (1500 rpm on a Thermo Scientific Heraeus Multifuge 3L-R centrifuge with a Sorvall 75006445 Rotor, radius = 19.2 cm rotor) for 2 min at RT and transferred to 384-well LDV Echo qualified 384-well COC source plates (Labcyte, cat. #LP-0200). Peptide samples were analyzed by LC-MS analysis using the same protocol described above for the thiol peptides, except an injection volume of 2 μl was applied. The peptide plate was covered with aluminum adhesive film (AB-0626, Thermo Scientific), then stored at -20 °C.

Combinatorial library diversification by acylation

Using the Echo® 650 Liquid handler (Labcyte, cat. #001-16079), 200 nl (4 nmol, 1 equiv.) of the capped peptide stock solution was transferred to a 1536-well LDV Echo qualified 1536-well COC source plate (Labcyte, cat. #LP-0400), 16 copies. 100 mM DMSO stock solutions were prepared from carboxylic acids **C1**-**C16**, plated on an Echo qualified 384-well PP source plate (Labcyte, cat. #P-05525) (multiple wells of 50 μl solution from each acid), then 200 nl (20 nmol, 5 equiv) was transferred to each well of the plates containing the peptides. With bulk dispensing, 1.6 μl of a DMF solution with 12.5 mM HBTU (20 nmol, 5 equiv.) and 62.5 mM DIPEA (100 nmol, 25 equiv.). The plates were covered with polypropylene adhesive films, centrifuged at 484 × g (1500 rpm on a Thermo Scientific Heraeus Multifuge 3L-R centrifuge

with a Sorvall 75006445 Rotor, radius = 19.2 cm) for 2 minutes, then left to stand for 5 h at RT. The reaction was then quenched by bulk dispensing a 1 μl DMF solution of 100 mM 2-methoxyethylamine (100 nmol, 25 equiv.) to each well. The above described centrifugation procedure was repeated, and the plate were left to stand for at RT overnight. The plates were concentrated by centrifugal vacuum evaporation at 0.1 mbar, 30 °C and at 400 × g (1750 rpm in a Christ 124700 rotor with 124708 and 124705 plate holder inserts, radius = 10.5 cm), then 4 μl of DMSO was added to each well by bulk dispensing to result in stock solutions with an average peptide concentration of 1 mM. For the LC-MS analysis of peptides after acylation, a 0.5 μl sample was transferred into 20 μl solvent (MeCN/water/TFA, 50:49.95:0.05%, v/v/v) and analyzed using an injection volume of 10 μl. The peptide plate was covered with aluminum adhesive film (AB-0626, Thermo Scientific), then stored at -20 °C.

Thrombin screen

Enzyme inhibition of compound libraries was assessed by measuring the residual enzyme activity in presence of peptides (10 μM average concentration) at 1.0% final DMSO concentration. 75 nl of library compounds (1 mM DMSO stocks in 1536-well LDV plates) was transferred into wells of black 1536-well microtiter OptiPlates via ADE. An assay buffer containing 50 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.01% Triton-X, 0.1% BSA, pH=7.4 was prepared. Assays were initiated by addition of thrombin (3.71 μl/well, 8.09 nM in assay buffer, prepared from Innovative research cat. #IHUTHRA, filtered through 0.22 μm PTFE syringe filters) by bulk dispensing. Plates were incubated for 10 min at ambient temperature before fluorogenic substrate (3.71 μl/well, 101.1 μM in assay buffer, diluted from a 100 mM DMSO stock solution of Cbz-G-G-R-AMC, Bachem, cat. #4002155 and filtered through 0.22 μm PTFE syringe filters) was added by bulk dispensing. The total assay volume was therefore 7.5 μl/well with final concentrations of 10 μM library compound, 4 nM thrombin, 50 μM substrate and 1% DMSO. Plates were centrifuged at 769 × g (BioNex HiG3 centrifuge, 2500 rpm, radius = 11.0 cm) for 2 minutes and fluorescence intensity was measured using a PHERAstar FSX plate reader (excitation 384 nm, emission 440 nm) in time increments of 150 s over 15 min. Slopes of fluorescence increase (m) were calculated with Microsoft Excel (vers. 16.56), as well as the coefficients of determination for the linear regressions (R^2). In every assay plate, 3 wells of negative controls (75 nl DMSO) and 3 wells of positive controls (75 nl of a 1 mM DMSO solution of **T2** shown in chapter 4 of this thesis, IC_{50}= 13±7 nM) An average of 3 negative and 3 positive controls was used to calculate inhibitions using the following formula:

$$\text{inhibition (\%)} = \left(1 - \frac{m_{sample} - m_{positive\ control}}{m_{DMSO\ control} - m_{positive\ control}}\right) \cdot 100\%$$

Hit validation

Repeated assay:

The 96 most active hits were assayed a second time using the same protocol as above, but volumes of 75 nl, 25 nl, 7.5 nl and 2.5 nl were transferred from the library source plate, to give assay concentrations of 10 μM, 3.3 μM, 1 μM, 0.33 μM respectively. The results are summarized in Supplementary figure S5.

Native mass spectrometry:

Using a pipette, approximately 0.15 μl of 1 mM DMSO solution was added to 50 μl of buffer solution containing 1 μM thrombin, 50 mM HEPES and 120 mM NaCl at pH 7.5, resulting in a final compound concentration of 3 μM. The mixture was incubated at 4°C for one hour. A Zeba Spin Desalting Column with a molecular weight cut-off of 7 kDa (Thermo Fisher Scientific, cat. #89882) was pre-washed twice with 300 μl of milliQ water, centrifuged each time for 1 minute at 1500 × *g* using a Thermo Scientific Heraeus Pico 17 centrifuge, then equilibrated three times with 300 μl of a solution containing 50 mM ammonium acetate with the same centrifugation step between the addition of each portion. Subsequently, the peptide mixtures were loaded onto the column, then centrifuged 2 minutes at 1500 × *g.*

The resulting filtrates were analyzed by an Orbitrap Exploris™ 240 Mass Spectrometer (resolution 15,000 (FWHM), nanoelectrospray ionization by a Triversa NanoMate® automated ion source platform with SID energy of 135 V). Spectra obtained were deconvoluted utilizing UniDec[17] (version 6.0.2), with specified parameters as follows: m/z range: full, charge range: 1-20, mass range: 30000-40000, Sample Mass Every (Da): 0.1, Smooth Nearby Points: None, Suppress Artifacts: Some, Peak FWHM (Th): 0.5, Peak Detection Range (Da):10, Peak Detection Threshold: 0.1. Other parameters were maintained at their default values.

Hit resynthesis

SPPS:

To each 5 mL syringe reactor (5 mL BD syringe, polypropylene with 25 μm PE frit, Carl Roth GmbH, Art. No. 7927.1) was added 75 mg resin **2** (~75 μmol/syringe) for hits **1**-**9**, or 167 mg Rink amide MBHA resin (~50 μmol/syringe, GL Biochem Shanghai Ltd., cat. #49101) for **2b** and **3b**. The synthesis was performed on a 50 μmol scale, but due to the relatively low yields observed during the library synthesis in plates, an excess of resin **2** was used. The Rink amide resins were subjected to Fmoc deprotection by 5 ml of 20% piperidine in DMF, for 2×5 minutes. The resins were washed with DMF (6×2000 μl). Coupling was performed with 530 μl of amino acid or carboxylic acid (500 mM, 265 μmol, 5.3 equiv.), 500 μl HATU (500 mM, 250 μmol, 5.0 equiv.) and 125 μl of NMM (4.0 M, 500 μmol, 10.0 equiv.). All components were pre-mixed for one minute, then added to the resin (60 minutes reaction, with shaking). Couplings were performed twice, then the resin was washed with DMF (2×2000 μl). Fmoc deprotection was performed using using 20% piperidine in DMF (900 μl, 2×2 min), and the resin was washed with DMF (8×2000 μl). At the end of the peptide synthesis, the resin was washed with CH_2Cl_2 (2×1200 μl) and resin beads were dried under suction.

On-resin deprotection, cleavage and solution-phase functionalization

For hits **1**-**9**:

After automated SPPS (50 μmol scale), the fritted syringe containing the resin was incubated with TFA/TIPS/H2O (3 mL, 95:2.5:2.5, v/v/v) for 1 h at ambient temperature. The TFA solution was discarded, and the procedure was repeated with an additional 3 ml of deprotection mixture. The resin was washed with CH_2Cl_2 (3×3 mL) and DMF (3 mL).

For the coupling of the second carboxylic acid, DMF solutions of 150 mM carboxylic acids and 300 mM DIPEA were prepared. 1 ml of this solution (150 μmol, 3 equiv. carboxylic acid and 300 μmol, 6 equiv. DIPEA) was added to 57 mg HATU (150 μmol, 3 equiv.) After 1 minute, the mixture was pulled into the syringe reactor and agitated for 1 h at RT. The solution was discarded, and the resin washed with 3x2 ml DMF. The coupling was repeated 1 more time, and the resin was washed again with 3x3 ml DMF.

To perform the reductive cleavage, 1 ml of 200 mM BDT and 200 mM TEA solution in DMF was pulled into each syringe (200 μmol, 4 equiv. each) and the syringes were shaken at RT overnight. The next day, the contents were pushed into a 50 ml Falcon tube, the cleavage was repeated with an additional 1 ml of the mixture with a 4 h reaction time, and the fractions were combined. The quality of the thiol peptides was confirmed by LC-MS analysis by combining 0.5 μl of the cleavage solution with 30 μl MeCN/water/TFA, 50:49.95:0.05%, v/v/v, and injecting 4 μl for analysis using the same method as described above (chromatograms not shown). 1.19 ml of 5% TFA in water was added to the peptide stocks, and the solutions were concentrated by centrifugal vacuum evaporation at 0.1 mbar, 30 °C and at 400 × g (1750 rpm in a Christ 124629 rotor with tube holder inserts, radius = 10.5 cm).

For the thiol capping, a peptide yield of 30 μmol was assumed. To each crude thiol peptide residue, 20 ml of MeCN/milliQ water (50/50% v/v) containing 60 mM ammonium bicarbonate (pH adjusted to 8.0) and 6 mM iodoacetamide (120 μmol, 4 equiv.), was added. The peptides were shaken at RT for 2.5 h. The completion of the reaction was confirmed by LC-MS analysis. 5 μl reaction mixture added to 25 μl MeCN/water/TFA, 50:49.95:0.05%, v/v/v in an Eppendorf tube. The samples were centrifuged for 1 minute at 2156× g (5000 rpm on Eppendorf 5418 tabletop centrifuge with 09918 rotor, radius = 7.7 cm). 25 μl of supernatant transferred to LCMS vial, then an injection volume of 2 μl was analyzed using the same method as described above. All thiols were fully alkylated (chromatograms not shown), therefore the reactions were quenched by the addition of 0.72 ml of 500 mM (360 μmol, 12 equiv.) solution of β-Me in water. The mixtures were shaken at RT for 1 h, then frozen in liquid nitrogen and lyophilized.

For hits **2b** and **3b**:

After automated SPPS (50 μmol scale), the fritted syringe containing the resin was incubated with TFA/TIPS/H2O (5 mL, 95:2.5:2.5, v/v/v) for 2 h at RT, then the solution was pushed in a 50 ml Falcon tube. The TFA was evaporated under a flow of nitrogen in a fume hood, then 5 ml water was added to each tube. The mixtures were frozen in liquid nitrogen and lyophilized. The peptide residues were dissolved in 2 ml DMF, then their quality was confirmed by LC-MS analysis by combining 0.5 μl DMF solution with 40 μl MeCN/water/TFA, 50:49.95:0.05%, v/v/v, and injecting 2 μl for analysis using the same method as described above (chromatograms of crude compounds not shown).

For the acylation reaction, a peptide yield of 30 μmol was assumed. To each solution, 24 mg **C2** (150 μmol, 5 equiv.) and 131 μl DIPEA (750 μmol, 25 equiv.), then 54 mg HATU (142.5 μmol, 4.75 equiv.) was added. The solution was shaken at RT for 5 h. The reaction was monitored by LC-MS with the same sample preparation steps as described for the thiol capping procedure. H2x was still not completely reacted, therefore and the same amounts of reagents were added again and the mixture was shaken at RT for an additional 2 h. Subsequently, 65 μl of 2-methoxyethylamine (750 μmol, 25 equiv.) was added, the solutions were shaken at RT overnight, then concentrated by centrifugal vacuum evaporation at 0.1 mbar, 30 °C and at 400 × g (1750 rpm in a Christ 124629 rotor with tube holder inserts, radius = 10.5 cm).

Purification:

1 ml DMSO was added to the peptide residues, then the tubes were vortexed. 1 ml MeCN was added, the tubes were vortexed again, then 8 ml H_2O with 0.1% v/v TFA was added. The tubes were vortexed and sonicated. The solutions were filtered through a 0.22 μm Hydrophilic PTFE filter (SF2509-1, BGB Analytik AG) and purified by Waters preparative HPLC system (2489 UV detector, 2535 pump, fraction Collector III) equipped with a C18 RP Waters OBD column. A linear gradient of solvent B (0.1% TFA in MeCN) over solvent A (0.1% TFA in water) rising linearly from 20% to 50% during t = 3.00–32.00 (for **9**, the gradient was 30% to 60% during t = 3.00–32.00 min) was applied at a flow rate of 14.0 mL/min. In the case of **2**, **5**, **6** and **8**, a large amount of precipitate was visible after adding the solvents. In this case, the tube was centrifuged at 3000 × g (3735 rpm on a Thermo Scientific Heraeus Multifuge 3L-R centrifuge with a Sorvall 75006445 Rotor, radius = 19.2 cm) for 1 min. The supernatant was filtered and injected. The precipitate was dissolved according to the same protocol. In the second dilution, only negligible amounts of precipitate were visible, therefore the solution was filtered and injected. Pure fractions containing the desired product were unified and lyophilized to afford the products as colorless fluffy materials.

1-2 mg amounts of purified peptides were measured and transferred into 2 mL centrifuge tubes, then dissolved in DMSO to afford 10 mM compound stocks. The pure stocks were analyzed by LC-MS by combining 0.5 μl of the stock solution with 40 μl MeCN/water/TFA, 50:49.95:0.05%, v/v/v, and injecting 2 μl for analysis using the same method as described above See Supplementary figures S6-S7 for HPLC chromatograms.

IC_{50} and K_i determination

The half maximal inhibitor concentration (IC_{50}) values were determined by measuring thrombin inhibition using a similar assay as for the library screening, in two independent measurements. Three dilutions were prepared from the 10 mM DMSO stocks of the hit compounds. The first dilution was prepared by combining 10 μl of the 10 mM solution with 90 μl DMSO, resulting in a concentration of 1000 μM. By combining 25 μl of the first dilution with 775 μl DMSO, the concentration of the second dilution was 31.25 μM. By combining 25 μl of the second dilution with 775 μl DMSO, the concentration of the third dilution was 0.98 μM. From these solutions, 8 μl was transferred to the wells of a 384-well LDV Echo qualified 384-well COC source plate (Labcyte, cat. #LP-0200). Volumes of 2.5 nl, 5 nl, 10 nl, 20 nl, 40 nl and 80 nl were transferred from each dilution into 1536-well OptiPlates (OptiPlate-1536F, untreated, PerkinElmer, cat. #6004270) by ADE, then each well was complemented with DMSO to a total volume of 80 nl. The assay was performed according to the procedure described for the library screening, including the control wells and the data analysis. IC_{50} values were obtained by fitting the resulting residual activity data to a variable slope (three parameters) concentration–response equation using GraphPad Prism (version 6.0.1) and Ki values were calculated based on the IC_{50} using the Cheng-Prusoff equation:[18]

$$K_{\mathrm{i}} = \frac{\mathrm{IC}_{50}}{1 + \frac{[S]_0}{K_M}}$$

where $[S]_0$ is the initial substrate concentration (50 μM) and K_M is the Michaelis−Menten constant[19] for the enzyme and substrate (305 ± 46 μM)[20].

5.7 Supplementary information

Diamino acid	Name	CAS number
D1	Fmoc-L-Dap-Boc-OH	162558-25-0
D2	Boc-L-Dap-Fmoc-OH	122235-70-5
D3	Fmoc-D-Dap-Boc-OH	198544-42-2
D4	Boc-D-Dap-Fmoc-OH	131570-56-4
D5	Fmoc-L-Dab-Boc-OH	125238-99-5
D6	Boc-L-Dab-Fmoc-OH	117106-21-5
D7	Fmoc-D-Dab-Boc-OH	114360-56-4
D8	Boc-D-Dab-Fmoc-OH	131570-57-5
D9	Fmoc-L-Orn-Boc-OH	109425-55-0
D10	Boc-L-Orn-Fmoc-OH	150828-96-9
D11	Fmoc-D-Orn-Boc-OH	118476-89-4
D12	Boc-D-Orn-Fmoc-OH	163336-15-0
D13	Fmoc-L-Lys-Boc-OH	71989-26-9
D14	Boc-L-Lys-Fmoc-OH	84624-27-1
D15	Fmoc-D-Lys-Boc-OH	92122-45-7
D16	Boc-D-Lys-Fmoc-OH	115186-31-7
D17	Fmoc-(1-Boc-piperidin-4-yl)-Gly-OH	269078-80-0
D18	N-Boc-*cis*-4-Fmoc-amino-L-Pro-OH	174148-03-9
D19	1-*N*-Boc-4-*N*-Fmoc-aminopiperidine-4-carboxylic acid	183673-66-7
D20	1-*N*-Boc-4-*N*-Fmoc-piperazine-2-carboxylic acid	218278-58-1
D21	Boc-D-Phe(4-NHFmoc)-OH	173054-11-0
D22	Boc-L-Phe(4-NHFmoc)-OH	114346-31-5
D23	Fmoc-L-Phe(4-OEt-NHBoc)-OH	1013883-02-7

Supplementary table 1: Names and CAS numbers of diamino acids **D1**-**D23** used for library synthesis.

Carboxylic acid	Name	CAS number
C1	4-Chlorophenylacetic acid	1878-66-6
C2	5-Chlorothiophene-2-carboxylic acid	24065-33-6
C3	5-Chloronicotinic acid	22620-27-5
C4	5-Chloropyridine-2-carboxylic acid	86873-60-1
C5	2-Chloro-1,3-thiazole-5-carboxylic acid	101012-12-8
C6	4-(4-Chlorophenyl)cyclohexanecarboxylic acid	95233-37-7
C7	4-Chloro-2-fluorobenzoic acid	446-30-0
C8	4-Chloro-2-methoxybenzoic acid	57479-70-6
C9	5-Chloro-2-methoxynicotinic acid	54916-65-3
C10	4-Chlorobenzoic acid	74-11-3
C11	3-Chlorobenzoic acid	535-80-8
C12	3-Chlorophenylacetic acid	1878-65-5
C13	3-(4-Chlorophenyl)propionic acid	2019-34-3
C14	4-Methoxybenzoic acid	0100-09-04
C15	5-Chlorofuran-2-carboxylic acid	618-30-4
C16	7-Chloroquinoline-3-carboxylic acid	892874-49-6
C17	6-Chloro-imidazo[1,2-a]pyridine-2-carboxylic acid	182181-19-7
C18	3-Methoxybenzoic acid	586-38-9
C19	1H-Pyrrole-2-carboxylic Acid	634-97-9
C20	Quinoline-3-carboxylic acid	6480-68-8
C21	(S)-(-)-Tetrahydro-2-furoic Acid	87392-07-2
C22	Indole-2-carboxylic acid	1477-50-5
C23	*Cis*-4-Hydroxycyclohexanecarboxylic acid	3685-22-1
C24	2-[(Methylsulphonyl)amino]benzoic acid	162787-61-3
C25	1-Methyl-1H-Pyrazole-3-carboxylic acid	25016-20-0
C26	2,4-Difluorobenzoic acid	1583-58-0
C27	2-Morpholinobenzoic acid	42106-48-9
C28	5-Phenylisoxazole-3-carboxylic acid	14441-90-8
C29	4-(1H-pyrazol-1-yl)benzoic acid	16209-00-0
C30	4-(Morpholinomethyl)benzoic acid	62642-62-0
C31	2-Trifluoromethyl-6-pyridinecarboxylic acid	131747-42-7

C32	3-Indoleacetic acid	87-51-4
C33	1-(Trifluoromethyl)cyclopropane-1-carboxylic acid	277756-46-4
C34	3-Cyclopentene-1-carboxylic acid	7686-77-3
C35	3,3-Difluorocyclobutanecarboxylic acid	107496-54-8
C36	Benzyloxyacetic acid	30379-55-6
C37	4,4,4-Trifluorobutyric acid	406-93-9
C38	Benzothiazole-6-carboxylic acid	3622-35-3
C39	Indole-7-carboxylic acid	1670-83-3
C40	4-Morpholinobenzoic acid	7470-38-4
C41	Indole-4-carboxylic acid	2124-55-2
C42	Phenylacetic acid	103-82-2
C43	2-Hydroxy-6-methylnicotinic acid	38116-61-9
C44	Acetic acid	64-19-7
C45	5-Oxazolecarboxylic acid	118994-90-4
C46	Tetrahydropyran-4-carboxylic acid	5337-03-01
C47	2-(1,2-Benzisoxazol-3-yl)acetic acid	4865-84-3
C48	1-Methyl-3-(trifluoromethyl)-1H-pyrazole-4-carboxylic acid	113100-53-1
C49	Indol-1-yl-acetic acid	24297-59-4
C50	5-Methyl-3-phenylisoxazole-4-carboxylic acid	1136-45-4
C51	5-Thien-2-yl-1H-pyrazole-3-carboxylic acid	182415-24-3
C52	2-morpholinoacetic acid hydrochloride	89531-58-8
C53	3-Hydroxybenzoic acid	99-06-9
C54	2,2-Dimethylpropanoic acid	75-98-9
C55	2-Ethoxyacetic acid	0627-03-02
C56	Pyrazine-2-carboxylic acid	98-97-5
C57	3-Methylbutanoic acid	503-74-2
C58	3-Fluoro-4-hydroxybenzoic acid	350-29-8
C59	3-Oxocyclobutanecarboxylic acid	23761-23-1
C60	5-Oxo-1-phenyl-2-pyrazolin-3-carboxylic acid	119-18-6
C61	3-Methyloxetane-3-carboxylic acid	28562-68-7
C62	7-Azaindole-3-carboxylic acid	156270-06-3
C63	1-Methylindazole-3-carboxylic acid	50890-83-0

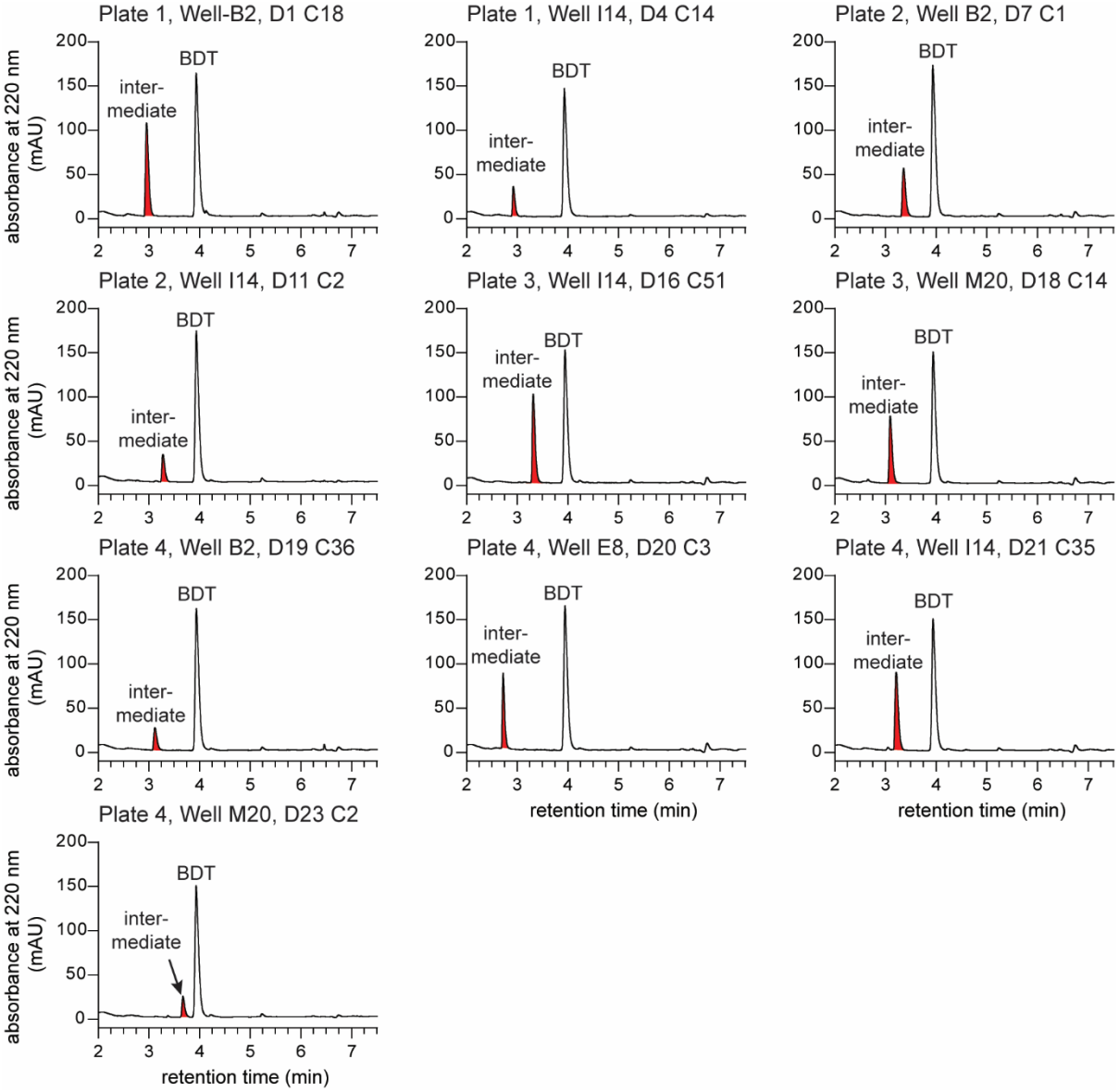
C64	2-Furoic acid	88-14-2
C65	3,4-Dimethoxyphenylacetic acid	93-40-3
C66	2,1,3-Benzoxadiazole-5-carboxylic acid	19155-88-5

Supplementary table 2: Names and CAS numbers of carboxylic acids **C1**-**C66** used for library synthesis

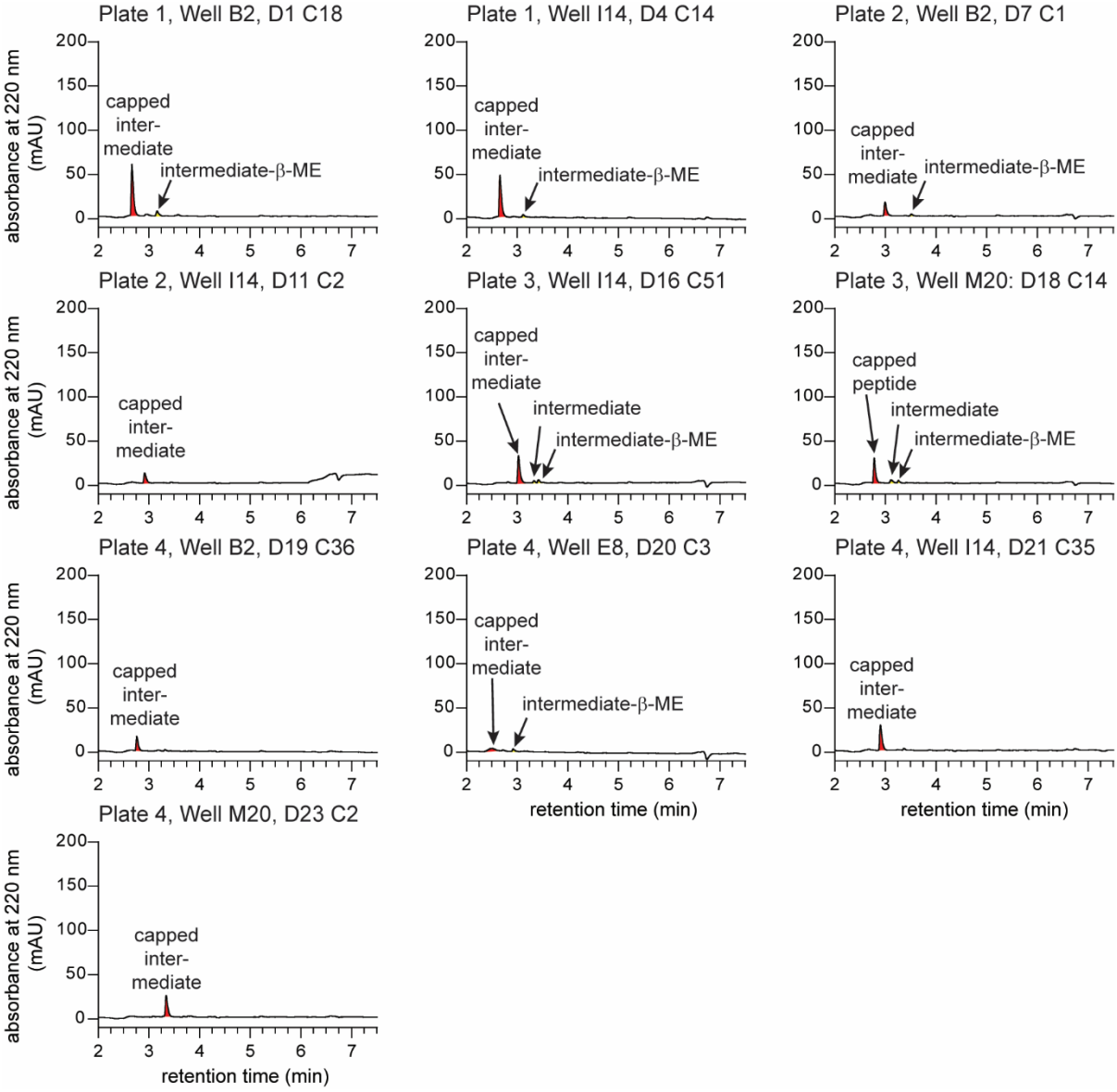
Equipment	Model and/or cat. #	Supplier
96-well V-bottom plate	96-Well micro test plates, V-bottom (cat. #6018321)	Ratiolab GmbH (Dreieich, Germany)
384-well LDV plate	Echo qualified 384-well COC source plate (cat. #LP-0200)	Labcyte (San José, CA, USA)
384-well PP plate	Echo qualified 384-well polypropylene source plate (cat. #P-05525)	Labcyte (San José, CA, USA)
1536-well OptiPlate	OptiPlate-1536F, untreated (cat. #6004270)	PerkinElmer (Waltham, MA, USA)
Acoustic liquid transfer	Echo® 650 Liquid handler (cat. #001-16079)	Labcyte (San José, CA, USA)
Activated charcoal-protected tips	Solvent Safe™ MBP 200 tips (cat. #5469)	Thermo Fisher Scientific (Waltham, MA, USA)
Adhesive metal plate lids	Adhesive PCR sealing foil sheets, aluminum (cat. #AB-0626)	Thermo Fisher Scientific (Waltham, MA, USA)
Adhesive PP plate lids	QuickSeal Micro adhesive polypropylene film (cat. #G070-N)	Kisker Biotech GmbH & Co. KG (Steinfurt, Germany)
Automated bulk dispenser	CERTUS Flex	Fritz Gyger AG (Gwatt, Switzerland)
Automated pipette	ViaFlo 384 Handheld Electronic Pipette	INTEGRA Biosciences AG (Zizers, Switzerland)
Automated SPPS synthesizer	MultiPep 2 Rsi	Intavis AG (Tübingen, Germany)
Centrifuge	Heraeus Multifuge 3L-R centrifuge (equipped for plates or falcon tubes)	Thermo Fisher Scientific (Waltham, MA, USA)
Centrifuge	Eppendorf 5418 tabletop centrifuge	Eppendorf SE (Hamburg, Germany)
Centrifuge	HiG3 automated cetrifuge	BioNex Solutions, Inc. (San José, CA, USA)

HPLC-MS system	Single quadrupole MS system (Shimadzu LCMS-2020)	Shimadzu (Kyoto, Japan)
HRMS system	Orbitrap ExplorisTM 240 Mass Spectrometer (cat. # BRE725535)	Thermo Fisher Scientific (Waltham, MA, USA)
Lyophilizer	Alpha 2-4 LDplus freeze dryer	Martin Christ Gefriertrocknungs-anlagen GmbH (Osterode, Germany)
Nanodrop	Nanodrop 8000 spectrophotometer	Thermo Fisher Scientific (Waltham, MA, USA)
pH meter	Mettler Toldeo FiveEasy pH meter	Mettler (Columbus, OH, USA)
Plate reader	PHERAstar FSX	BMG Labtech (Ortenberg, Germany)
Preparative HPLC	Waters system (2489 UV detector, 2535 pump, fraction Collector III)	Waters AG (Baden, Switzerland)
Rotary vacuum concentration	Maxi concentrator RVC 2-33 CDplus attached to Alpha 2-4 LSCbasic freeze dryer	Martin Christ Gefriertrocknungs-anlagen GmbH (Osterode, Germany)
RP column (PrepHPLC)	C18 Xterra OBD column (19×250 mm, 125 Å, 10 µm, cat. #186002259)	Waters AG (Baden, Switzerland)
Seal pad for filter plate	10 mm ethylene-vinyl acetate pad, (cat. # 76899)	Creotime
SPPS reactor syringes	5 mL BD syringe, polypropylene with 25 µm PE frit	Carl Roth GmbH (Karlsruhe, Germany)
SPPS 384-well synthesis plates	384 PP filter plate with a 25 µm pore PE frit (cat. # PN 201035-100)	Agilent Technologies (Santa Clara, CA, USA)

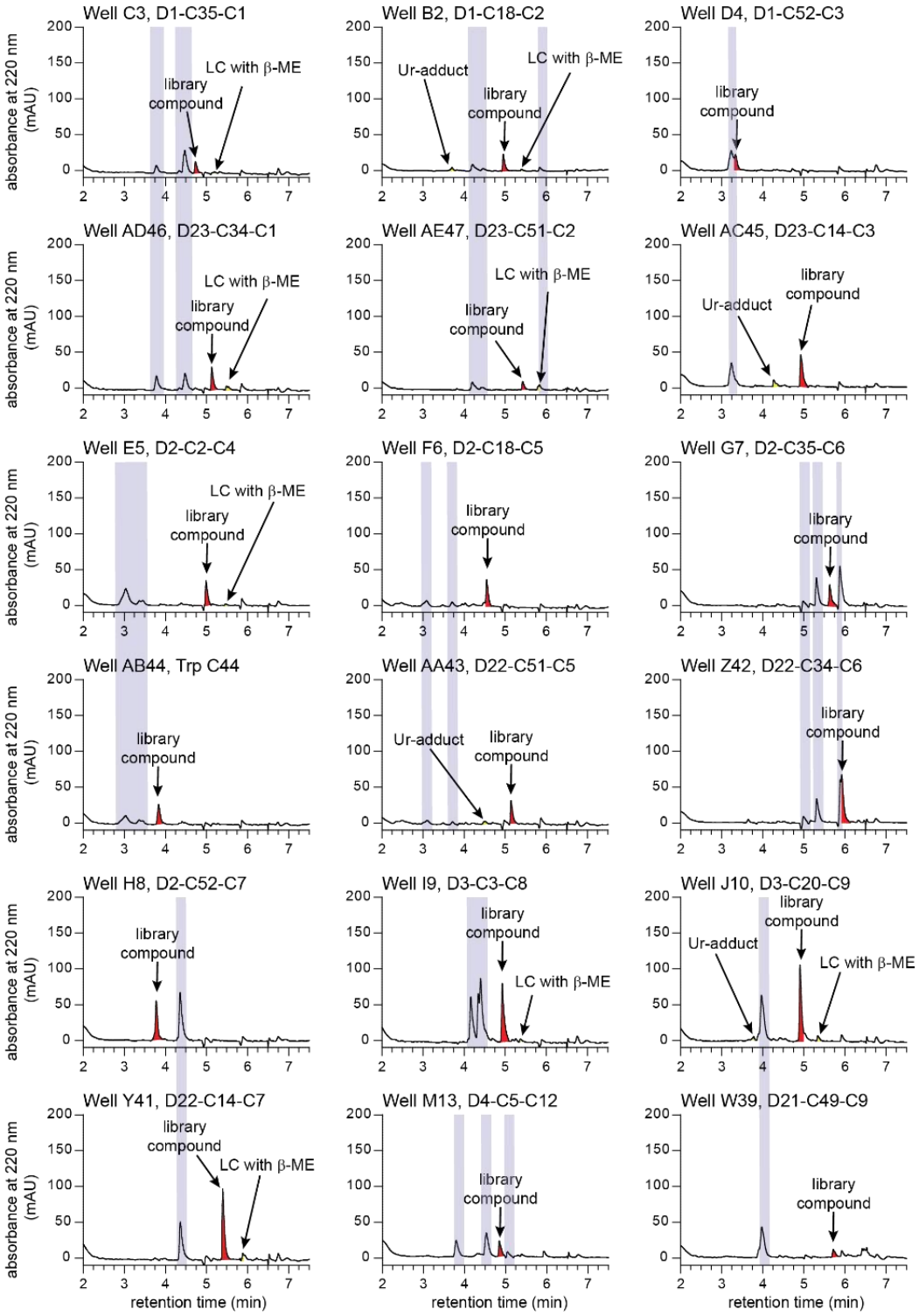
Supplementary table 3: List of applied laboratory equipment and utilities



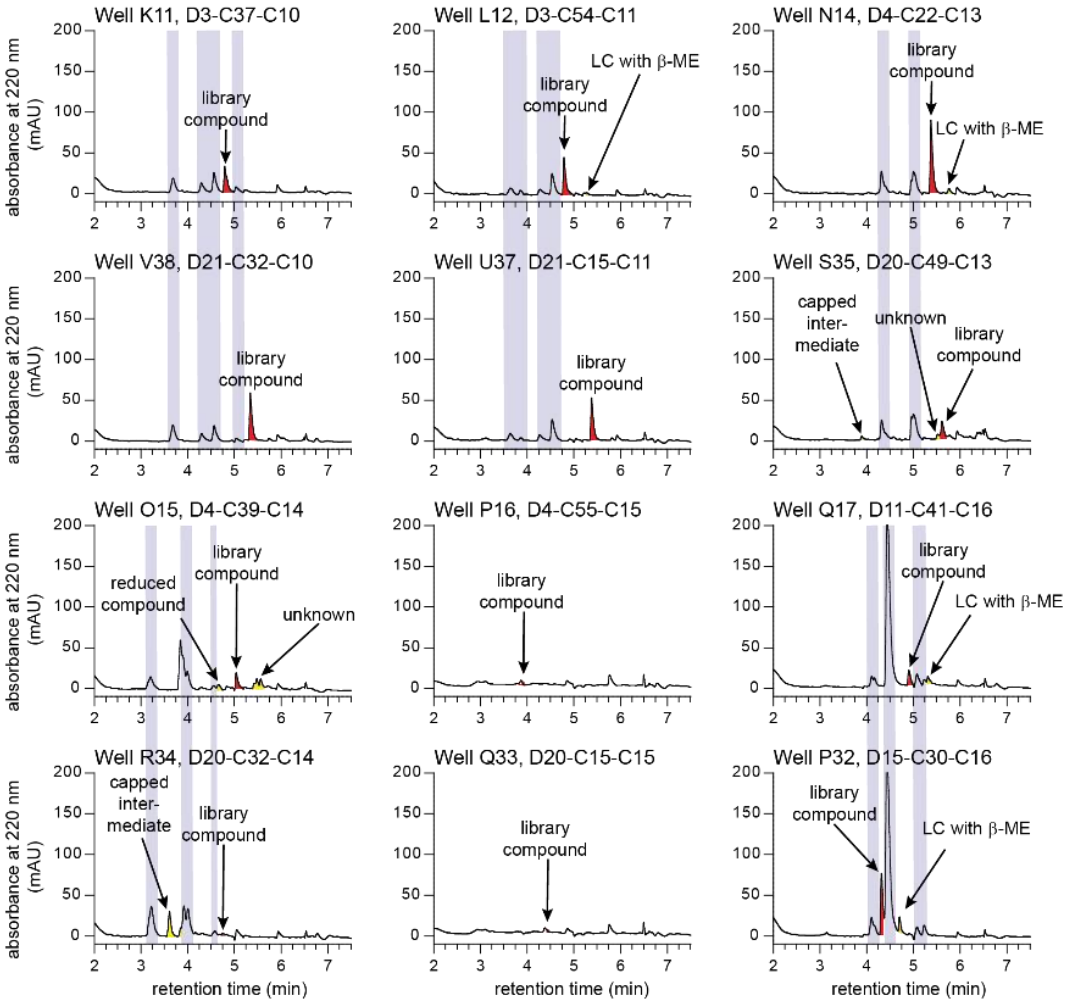
Supplementary figure S1: LC-MS analysis of library intermediates produced by SPPS. From each one of four plates, four wells were randomly chosen (positions B2, E8, I14, M20 lying on a diagonal) and products analyzed. Of the 16 intermediate products, ten were detected by UV and showed the expected mass, four did not absorb but showed the expected mass, and two showed side products that were present due to impurities in C49. The ten products that were detected by UV and had the expected mass are shown in the figure (UV traces) and were used to quantify the purity, that is shown in Figure 2d (top panel).



Supplementary figure S2: LC-MS analysis of intermediate products with capped thiol groups. UV spectra of ten capped intermediate products are shown (the same wells as in Figure S1) and were used to quantify the purity, that is shown in Figure 2d (middle panel).



Supplementary figure S3: LC-MS analysis of library compounds. UV spectra are shown. From the library of 24,288 compounds, 32 were semi-randomly picked as follows: two compounds on the diagonals of 16 different 1,536 well plates wherein the position chosen on the diagonal was shifter by one well from plate to plate) and analyzed. As a result of this choice, always two compounds are acylated with the same carboxylic acid (the excess of carboxylic acid and quenched carboxylic acid is highlighted in violet). The purity was quantified based on the UV spectra and is shown in Figure 2d (bottom panel).



Supplementary figure S3 (continued).

a

Ac-Tyr-OH calibration

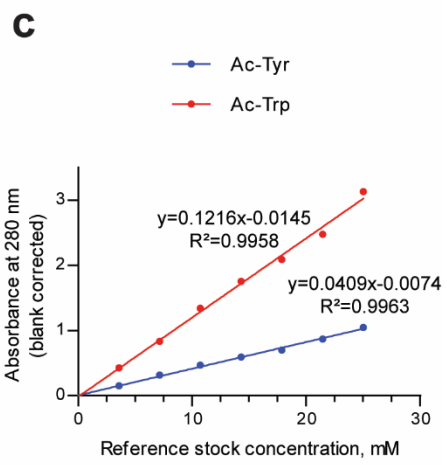
Blank, first reading	Blank, second reading	Blank average	Sample, first reading	Sample, second reading	Sample average	(Sample average) - (Blank average)	Reference stock concentration, mM
0.028	0.028	0.028	1.09	1.061	1.0755	1.0475	25.00
0.036	0.038	0.037	0.901	0.913	0.907	0.87	21.43
0.034	0.036	0.035	0.731	0.742	0.7365	0.7015	17.86
0.034	0.04	0.037	0.632	0.631	0.6315	0.5945	14.29
0.033	0.039	0.036	0.508	0.506	0.507	0.471	10.71
0.035	0.038	0.0365	0.358	0.356	0.357	0.3205	7.14
0.036	0.038	0.037	0.192	0.19	0.191	0.154	3.57
0.042	0.037	0.0395	0.029	0.026	0.0275	-0.012	0.00

b

Ac-Trp-OH calibration

Blank, first reading	Blank, second reading	Blank average	Sample, first reading	Sample, second reading	Sample average	(Sample average) - (Blank average)	Reference stock concentration, mM
0.028	0.028	0.028	3.165	3.157	3.161	3.133	25.00
0.036	0.038	0.037	2.558	2.472	2.515	2.478	21.43
0.034	0.036	0.035	2.114	2.133	2.1235	2.0885	17.86
0.034	0.04	0.037	1.806	1.782	1.794	1.757	14.29
0.033	0.039	0.036	1.394	1.362	1.378	1.342	10.71
0.035	0.038	0.0365	0.868	0.874	0.871	0.8345	7.14
0.036	0.038	0.037	0.459	0.47	0.4645	0.4275	3.57
0.042	0.037	0.0395	0.02	0.025	0.0225	-0.017	0.00

c



d

Ac-Tyr library peptides

Blank, first reading	Blank, second reading	Blank average	Sample, first reading	Sample, second reading	Sample average	(Sample average) - (Blank average)	Concentration, mM
0.028	0.028	0.028	0.238	0.234	0.236	0.208	5.27
0.036	0.038	0.037	0.232	0.232	0.232	0.195	4.95
0.034	0.036	0.035	0.257	0.265	0.261	0.226	5.71
0.034	0.04	0.037	0.247	0.254	0.2505	0.2135	5.40
0.033	0.039	0.036	0.23	0.234	0.232	0.196	4.97
0.035	0.038	0.0365	0.235	0.239	0.237	0.2005	5.08
0.036	0.038	0.037	0.251	0.254	0.2525	0.2155	5.45
0.042	0.037	0.0395	0.229	0.238	0.2335	0.194	4.92

e

Ac-Trp library peptides

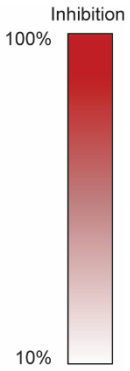
Blank, first reading	Blank, second reading	Blank average	Sample, first reading	Sample, second reading	Sample average	(Sample average) - (Blank average)	Concentration, mM
0.028	0.028	0.028	0.585	0.588	0.5865	0.5585	4.71
0.036	0.038	0.037	0.626	0.629	0.6275	0.5905	4.98
0.034	0.036	0.035	0.724	0.73	0.727	0.692	5.81
0.034	0.04	0.037	0.61	0.625	0.6175	0.5805	4.89
0.033	0.039	0.036	0.733	0.737	0.735	0.699	5.87
0.035	0.038	0.0365	0.501	0.5	0.5005	0.464	3.94
0.036	0.038	0.037	0.48	0.477	0.4785	0.4415	3.75
0.042	0.037	0.0395	0.552	0.553	0.5525	0.513	4.34

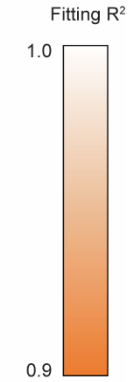
f

	Ac-Tyr	Ac-Trp	**Total**
Average concentration. mM	5.22	4.79	**5.00**
Standard deviation, mM	0.28	0.78	**0.61**

Supplementary figure S4: Concentration determination of the cleaved peptide mixtures based on UV_{280} absorbance. a) absorbance values of the Ac-Tyr-OH calibration series. b) absorbance values of the Ac-Trp-OH calibration series. c) fitting of the calibration line based on data from a) and b). d) absorbance of the Ac-Tyr library peptides and the stock concentration based on data from c). e) absorbance of the Ac-Trp library peptides and the stock concentration based on data from c). f) Average concentrations and standard deviations calculated from d) and e).

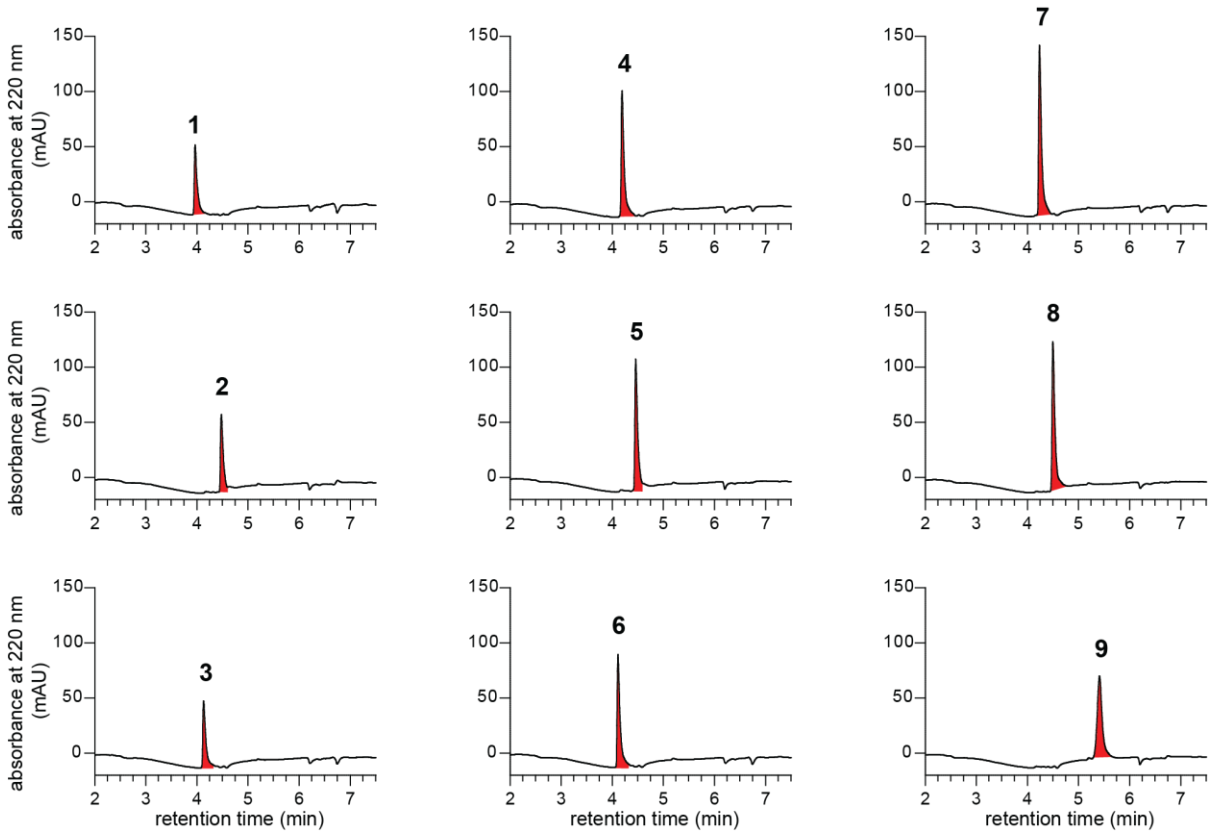
C2?	D	Acid (SPPS)	Acid (ADE)	% Inh. 10 µM	R^2 10 µM	% Inh. 10 µM	R^2 10 µM	% Inh. 3.33 µM	R^2 3.33 µM	% Inh. 1 µM	R^2 1 µM	% Inh. 0.33 µM	R^2 0.33 µM	IC_{50} µM	
	D6	C2	C12	100.7	0.961	101.1	0.991	92.8	0.541	92.8	1.000	77.3	0.997	0.11	H2
	D5	C42	C2	102.4	0.886	100.8	0.995	98.5	0.999	90.9	1.000	72.7	1.000	0.12	H3
	D5	C12	C2	102.6	0.891	101.0	0.992	98.6	0.999	90.9	1.000	73.4	1.000	0.15	H2
	D1	C41	C2	101.0	0.990	99.4	0.993	93.5	1.000	73.9	1.000	37.9	1.000	0.23	H1
	D5	C47	C2	100.0	0.983	98.9	0.998	92.6	0.999	74.6	1.000	44.3	1.000	0.30	H4
	D6	C2	C4	97.9	0.993	98.1	0.999	91.0	1.000	70.9	1.000	40.1	1.000	0.34	
	D5	C4	C2	99.7	0.998	98.0	0.999	89.6	0.999	67.1	1.000	33.6	1.000	0.41	
	D5	C59	C2	99.2	0.997	97.5	0.999	87.7	1.000	59.2	1.000	20.7	0.999	0.46	
	D6	C2	C1	98.5	0.994	98.9	0.999	89.0	1.000	60.0	1.000	25.6	1.000	0.53	
	D1	C30	C2	97.8	0.999	96.4	0.999	87.1	1.000	63.2	1.000	33.5	0.997	0.57	
	D5	C1	C2	100.1	0.997	98.3	0.997	87.5	1.000	57.3	1.000	22.2	1.000	0.60	
	D2	C2	C11	76.0	1.000	74.4	1.000	114.3	0.096	5.2	0.999	-11.6	1.000	0.63	
	D1	C50	C2	97.6	0.996	95.4	0.999	83.3	0.999	54.3	1.000	19.9	1.000	0.65	
	D5	C42	C10	92.9	0.999	92.6	1.000	77.2	1.000	47.0	1.000	15.8	1.000	0.87	H6
	D6	C2	C7	92.1	0.998	92.5	0.999	76.4	1.000	43.3	1.000	11.2	0.999	0.90	
	D5	C12	C10	92.8	0.999	93.2	0.999	76.6	1.000	42.9	1.000	10.1	1.000	0.90	H5
	D5	C37	C2	94.7	0.997	94.6	1.000	78.0	1.000	46.7	1.000	15.0	1.000	0.92	
	D2	C2	C16	87.2	1.000	86.0	1.000	61.8	1.000	25.1	1.000	-13.5	1.000	1.09	
	D11	C40	C2	88.6	0.687	89.8	0.999	68.4	1.000	32.1	1.000	0.3	1.000	1.18	
	D6	C2	C10	90.8	0.998	90.6	0.999	71.3	1.000	35.1	1.000	5.0	1.000	1.18	
	D5	C61	C2	91.1	0.999	89.7	0.999	70.4	1.000	35.3	1.000	6.6	0.999	1.27	
	D5	C49	C2	94.5	0.999	92.3	1.000	71.9	1.000	26.6	1.000	-5.3	1.000	1.28	
	D1	C51	C2	87.8	0.999	85.1	1.000	61.4	1.000	22.9	1.000	-10.9	1.000	1.33	
	D5	C7	C2	98.8	0.513	95.6	0.999	87.5	1.000	31.2	1.000	13.1	1.000	1.36	
	D5	C10	C2	92.3	1.000	90.1	1.000	69.6	1.000	33.5	1.000	5.4	1.000	1.41	
	D6	C2	C8	83.3	0.999	87.0	0.999	64.1	1.000	27.2	1.000	-0.2	1.000	1.42	
	D6	C10	C12	89.3	0.999	89.4	1.000	66.3	1.000	30.6	0.999	1.6	1.000	1.44	H5
	D5	C32	C2	92.7	0.999	90.5	1.000	70.4	1.000	34.3	1.000	8.0	1.000	1.48	
	D9	C41	C2	88.4	1.000	85.7	1.000	61.5	1.000	22.9	1.000	-8.1	1.000	1.50	
	D12	C2	C8	83.2	1.000	86.4	1.000	63.5	1.000	26.5	1.000	0.6	1.000	1.52	
	D5	C2	C2	91.0	0.999	88.8	1.000	66.8	1.000	29.1	0.999	1.8	1.000	1.55	
	D5	C8	C2	89.6	0.999	87.1	1.000	63.8	1.000	27.4	1.000	-1.1	1.000	1.57	
	D1	C20	C2	89.9	0.999	87.3	1.000	64.7	1.000	27.3	1.000	-0.4	1.000	1.58	
	D5	C13	C2	91.0	0.999	88.9	1.000	65.4	1.000	29.0	1.000	1.2	1.000	1.62	
	D1	C27	C2	88.1	0.999	85.9	1.000	62.8	1.000	25.4	1.000	-1.5	1.000	1.65	
	D6	C2	C2	90.0	0.999	87.7	1.000	64.3	1.000	26.0	1.000	0.7	1.000	1.78	
	D1	C40	C2	86.2	0.999	83.8	1.000	59.2	1.000	22.0	1.000	-4.3	1.000	1.80	
	D5	C15	C2	77.8	1.000	79.4	1.000	61.3	1.000	7.0	1.000	-9.1	1.000	1.81	
	D6	C2	C5	85.1	0.999	84.3	1.000	59.8	1.000	22.0	1.000	-2.9	1.000	1.83	
	D1	C39	C2	92.9	0.996	89.3	1.000	65.1	1.000	27.1	1.000	1.3	1.000	1.85	
	D1	C62	C2	89.8	1.000	86.5	1.000	62.4	1.000	23.2	1.000	-2.1	1.000	1.86	
	D11	C8	C2	85.4	1.000	83.7	1.000	58.7	1.000	21.9	1.000	-3.6	1.000	1.86	
	D5	C51	C2	92.7	0.998	89.5	1.000	64.5	0.999	20.7	1.000	-4.6	1.000	1.90	
	D6	C2	C13	87.2	1.000	86.3	1.000	60.2	1.000	22.5	1.000	-3.1	1.000	1.91	
	D5	C26	C2	86.9	0.999	84.5	1.000	60.1	1.000	22.8	1.000	-1.8	1.000	1.91	
	D5	C20	C2	88.8	1.000	85.1	1.000	58.8	1.000	20.5	1.000	-5.0	1.000	2.04	
	D2	C2	C8	81.3	1.000	84.5	1.000	56.8	1.000	17.5	1.000	-5.3	1.000	2.07	
	D5	C5	C2	87.6	0.999	85.3	1.000	58.3	1.000	19.0	1.000	-5.7	1.000	2.09	
	D1	C8	C2	85.7	1.000	83.8	1.000	54.8	1.000	17.7	1.000	-8.7	1.000	2.13	
	D6	C12	C2	87.5	1.000	85.0	1.000	58.6	1.000	19.2	1.000	-4.4	1.000	2.13	
	D5	C33	C2	85.5	0.999	83.8	0.999	56.2	0.999	18.4	1.000	-5.4	1.000	2.20	
	D5	C66	C2	85.5	0.999	83.1	1.000	56.7	1.000	17.7	1.000	-3.5	1.000	2.33	
	D5	C35	C2	84.5	1.000	81.8	1.000	53.4	1.000	16.3	1.000	-7.5	1.000	2.33	
	D5	C4	C10	83.2	1.000	81.5	0.999	53.8	1.000	17.1	1.000	-5.4	1.000	2.34	
	D6	C10	C4	82.2	1.000	80.3	1.000	52.1	1.000	15.8	1.000	-7.4	1.000	2.34	
	D1	C38	C2	85.1	0.999	82.5	1.000	54.3	1.000	18.5	1.000	-5.1	1.000	2.35	
	D18	C7	C6	82.9	1.000	82.8	1.000	54.0	1.000	15.6	1.000	-6.5	1.000	2.38	H9
	D5	C42	C7	78.6	1.000	78.5	1.000	50.3	0.999	14.9	1.000	-7.2	1.000	2.38	



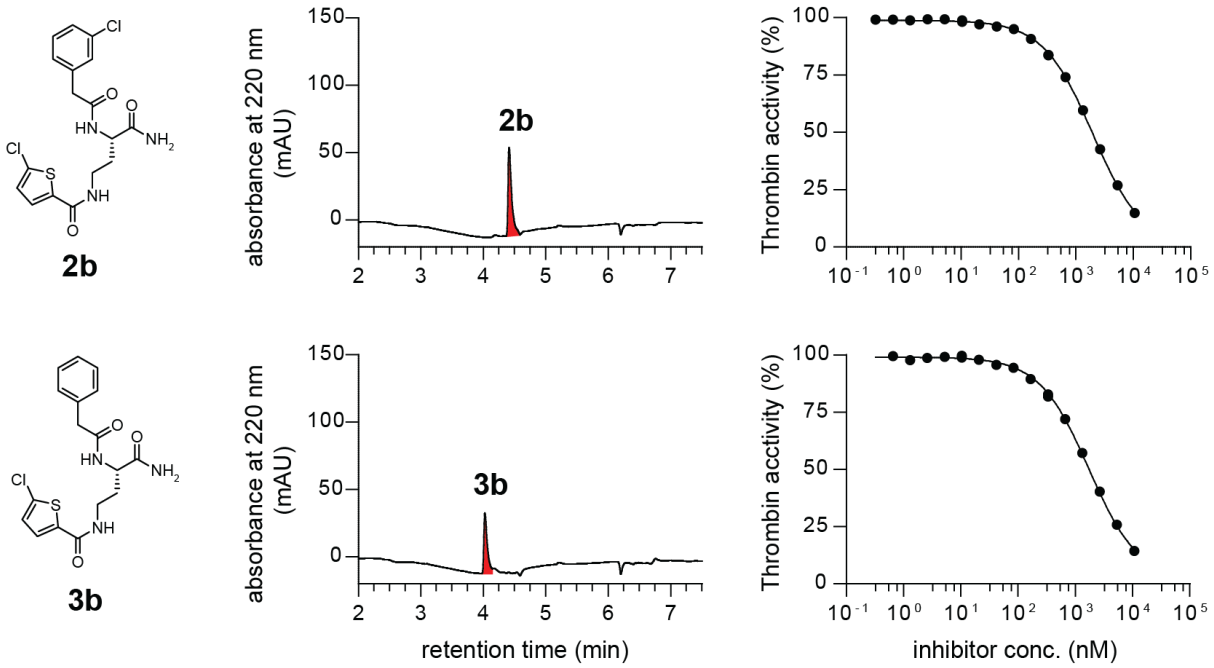


C2?	D	Acid	Acid	% Inh.	R^2	% Inh.	R^2	% Inh.	R^2	% Inh.	R^2	% Inh.	R^2	IC_{50}	
		(SPPS)	(ADE)	10 µM	10 µM	10 µM	10 µM	3.33 µM	3.33 µM	1 µM	1 µM	0.33 µM	0.33 µM	µM	
	D12	C2	C14	80.3	0.999	80.7	1.000	50.3	1.000	15.7	0.999	-8.3	1.000	2.41	
	D1	C22	C2	82.9	0.999	79.3	1.000	51.6	1.000	14.5	1.000	-8.2	1.000	2.41	
	D5	C12	C7	80.3	0.999	80.1	1.000	52.0	1.000	16.5	1.000	-4.9	1.000	2.44	
	D1	C63	C2	85.1	1.000	81.8	1.000	55.9	1.000	15.4	1.000	-3.9	1.000	2.46	
	D12	C2	C7	80.4	0.999	80.6	1.000	51.5	1.000	15.3	1.000	-5.4	1.000	2.57	
	D1	C53	C2	85.8	1.000	81.9	1.000	52.7	1.000	15.2	1.000	-7.1	1.000	2.60	
	D11	C7	C2	80.4	1.000	78.8	1.000	49.4	0.999	11.7	1.000	-8.4	1.000	2.72	
	D20	C2	C1	74.7	1.000	72.0	1.000	41.7	1.000	10.9	0.992	-11.7	1.000	2.84	
	D11	C9	C10	88.6	1.000	85.8	1.000	54.4	1.000	8.8	1.000	-9.7	1.000	2.90	H8
	D1	C7	C2	77.6	1.000	76.1	1.000	44.9	0.999	11.1	1.000	-9.0	1.000	3.07	
	D5	C51	C10	84.0	1.000	81.0	1.000	48.3	1.000	6.7	1.000	-11.5	1.000	3.23	H7
	D5	C36	C2	80.6	1.000	77.0	1.000	45.6	1.000	9.8	1.000	-9.3	1.000	3.27	
	D6	C2	C14	78.2	1.000	78.4	1.000	45.4	1.000	9.3	1.000	-8.6	1.000	3.37	
	D5	C14	C2	81.0	0.999	78.0	1.000	46.0	1.000	8.2	1.000	-9.5	1.000	3.39	
	D11	C26	C2	77.5	1.000	73.5	0.999	43.4	1.000	8.4	1.000	-9.0	1.000	3.40	
	D1	C26	C2	76.5	0.999	74.5	1.000	42.5	1.000	7.1	1.000	-10.9	1.000	3.41	
	D5	C56	C2	75.8	1.000	73.2	1.000	41.2	1.000	9.9	0.979	-10.1	1.000	3.41	
	D12	C2	C10	74.9	1.000	74.9	1.000	42.3	0.999	6.5	1.000	-10.8	1.000	3.43	
	D1	C18	C2	77.4	0.999	74.6	1.000	42.8	1.000	7.5	1.000	-10.3	1.000	3.48	
	D11	C14	C2	79.3	1.000	75.6	1.000	43.8	1.000	8.5	1.000	-9.7	1.000	3.48	
	D5	C20	C10	75.3	1.000	72.7	1.000	39.7	0.999	5.2	1.000	-12.5	1.000	3.70	
	D5	C64	C2	79.9	1.000	83.1	1.000	45.3	1.000	8.7	1.000	-8.3	1.000	3.94	
	D10	C2	C16	75.0	1.000	73.2	1.000	37.8	1.000	3.7	1.000	-14.3	0.999	4.00	
	D11	C10	C2	77.4	1.000	73.5	1.000	40.1	0.999	5.5	1.000	-11.2	1.000	4.02	
	D9	C20	C2	77.4	0.999	71.9	1.000	39.5	1.000	4.4	1.000	-11.8	1.000	4.03	
	D5	C34	C2	76.7	1.000	72.7	1.000	39.6	1.000	3.8	1.000	-11.0	1.000	4.20	
	D11	C9	C2	77.3	1.000	72.2	1.000	36.5	0.999	-0.4	1.000	-13.1	1.000	5.04	
	D5	C52	C2	74.6	1.000	69.3	1.000	33.6	1.000	-0.3	1.000	-14.2	1.000	5.20	
	D5	C1	C10	75.7	0.999	75.3	1.000	34.5	1.000	0.0	0.999	-14.6	1.000	5.51	
	D5	C19	C2	77.5	1.000	75.4	1.000	43.5	1.000	9.8	1.000	2.7	1.000	5.71	
	D5	C16	C2	74.4	0.999	70.1	1.000	28.7	0.989	-1.3	1.000	-13.2	1.000	8.15	
	D11	C9	C7	76.5	0.999	69.4	1.000	24.1	0.986	-1.6	1.000	-15.7	1.000	11.36	
	D5	C41	C2	75.9	0.999	78.5	1.000	27.8	0.738	11.2	1.000	-11.5	1.000	11.42	
	D5	C45	C2	77.1	1.000	73.1	1.000	41.6	1.000	5.4	1.000	14.0	0.993	12.47	
	D1	C14	C2	125.1	0.342	59.8	1.000	21.6	0.999	-3.4	1.000	-14.5	1.000	79.78	
	D13	C20	C13	101.3	sat*	3.4	1.000	-12.9	1.000	-19.6	1.000	-24.9	0.999	~ 5372	
	D23	C46	C13	112.0	0.199	5.3	1.000	-11.1	1.000	-19.7	1.000	-21.5	1.000	~ 5821	
	D11	C61	C15	77.8	0.999	5.9	1.000	-15.2	0.805	-19.1	1.000	-20.6	1.000	~ 8176	

Supplementary figure S5: Hit validation by repeated screening of the 96 most active initial hits at four different concentrations. The calculations of inhibition (% Inh.) and coefficients of determination for the linear regressions (R^2) were made in the same way as described for the screening. The compounds are ranked by their crude IC_{50} values, obtained by fitting the resulting inhibition data to a variable slope (three parameters) concentration–response equation using GraphPad Prism (version 6.0.1). The first column of the table ("C2?") is colored yellow if the peptide contains the carboxylic acid **C2**. Sat*: in this assay well, the detector got saturated, likely due to an error in liquid dispensing.



Supplementary figure S6: LC-MS analysis of hit compounds **1**-**9**, obtained after preparative HPLC purification.



Supplementary Figure S7: Inhibitors without appendix, **2b** (top) and **3b** (bottom).: Chemical structures, UV_{220} spectra of purified compounds, inhibition curves.

5.8 References

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6. Conclusion and outlook

In this thesis, I aimed to develop methods for the efficient and robust generation of peptide and small molecule libraries that can be directly screened without purification. As described in Chapter 3, we developed a novel SPPS method, where the TFA-stable disulfide linkage allows the side chain deprotection step to be performed separately from the peptide elution. The latter is achieved by a reductive release strategy that uses the potent reducing agent 1,4-butanedithiol (BDT), that does not form significant amounts of undesired disulfide byproducts due to its ability to form stable six-membered rings during the reaction. We demonstrated that the volatile nature of this reagent enables its removal by simple rotational vacuum concentration, providing facile access to high purity thiol peptides with good yields. The sulfhydryl functionality can serve as a handle for late-stage diversification or cyclization with electrophilic reagents. This is demonstrated by the synthesis of several examples of short dithiol peptides featuring a variety of side chain functions and their subsequent cyclization with bis-electrophilic linker reagents, resulting in thioether macrocyclic peptides of good quality.

Unlike in the case of the previously established cyclative release method, the efficiency of the cleavage was found to be independent of the peptide sequence or length, therefore both long and short peptides can be synthesized through this strategy. This versatility is demonstrated with the synthesis of short dithiol peptides and the direct comparison of the two approaches for peptide release. The stability of the disulfide bond during SPPS and the method's applicability for the synthesis of longer peptide sequences is also demonstrated by the successful generation of various dithiol decapeptides. Due to its robustness, the method presented herein was suitable for use in the projects described in the subsequent chapters, and it is also routinely used by others in our group for the generation of various peptide libraries.

When we only had the cyclative release strategy at our disposal, our efforts were primarily focused on libraries of slightly longer peptides, featuring at least 3-4 amino acid building blocks. In these cases, it was not too difficult to achieve high library diversity due to the number of possible diversification points. However, the newly established reductive release method enabled us to generate very short thiol peptides with high crude purity. As chemical diversity is a crucial characteristic of chemical libraries and a major determinant of screening success rate, we had to find new ways to incorporate novel structural elements in our sequences to ensure a sufficient sampling of the conformational space, especially concerning the backbones of cyclic peptides.

To this end, I turned my attention to the aminothiol moiety used for linking the peptides to solid phase during SPPS, because it has become a major fraction of the peptide backbone in the case of shorter sequences. However, the established linking strategy relied on mercaptoethylamine, and due to the lack of commercially available aminothiol derivatives, another synthetic route had to be investigated. In the approach described in Chapter 4, we utilized haloalkyl amines, of which a large variety is commercially available at a low price, and reacted them with the key reagent, sodium benzenethionosulphonate. In the resulting set of compounds, the phenylsulfone functionality could serve as an efficient leaving group when they were reacted with thiol functionalized PS resin, leading to the formation of a disulfide linkage between the aminothiol derivatives and the solid support.

The method's compatibility with the previously described reductive release strategy was successfully demonstrated by synthesizing short peptide sequences featuring the new aminothiols. We found that the quality of these peptides is acceptable even without performing chromatographic purification of the aminothiol reagents, which is likely the result of the additional selectivity achieved by performing the synthesis on solid support and the release strategy being specific for disulfide bonds. For this reason, possible impurities in the crude thionosulfonate esters will either be unable to react with the thiol functions of the resin and washed away, or the resulting impurity will not be cleaved from the resin by BDT.

Following the establishment of the synthetic method, a model library was synthesized and screened against the serine protease thrombin. This campaign resulted in potent, structurally novel inhibitors of the model target, and the elucidation of their binding mode through X-ray crystallography revealed that the aminothiol derivative is a key factor in creating a ring conformation that can fit in the desired pocket. These results illustrate well the advantages of the increased macrocycle skeletal diversity. Some of the active compounds also presented favorable pharmacological characteristics in membrane permeability assays, further highlighting the possibilities that this new strategy opens up regarding the structural optimization peptides.

In the project described in Chapter 5, I applied the new methods to generate a peptide library with physicochemical properties resembling small molecules, such as low molecular weight and low polar surface area. These favorable characteristics make them interesting starting

points for the development of orally bioavailable drugs. In this case, a single diamino acid was anchored to thiol-functionalized resin by the new mercaptopropylamine linker, then the high chemical diversity was ensured by subsequent incorporation of two carboxylic acid building blocks. These compounds are highly advantageous because they can be easily accessed at cost that is considerably lower than that of Fmoc-protected unnatural amino acids and allow the inclusion of small, drug-like and highly diverse chemical moieties. One of the carboxylic acids was coupled on solid phase, and the second one was added after cleavage in solution phase, using nanomole scale late-stage functionalization. The resulting chemical library was confirmed by LC-MS analysis to be of sufficient purity for direct biological screening.

By screening the library against our model target thrombin, several crude mixtures showed activity by inhibiting the proteolytic cleavage of a fluorogenic substrate. The hits were validated by repeating the screening at lower concentrations, and the presence of the desired library compounds in the crude mixtures was confirmed by LC-MS analysis. The non-covalent binding of selected hits was also confirmed by native MS analysis. As expected, the most active hits featured the 2-chlorothiophene moiety, which is a known binding fragment of thrombin. The most promising hit compounds were synthesized on a larger scale and purified, showing dose-dependent inhibition of the target. The affinity of the most potent binders was found to be in the nanomolar range.

The methods described in this thesis allow for the rapid and robust generation of diverse libraries of peptides, with a yield and purity that enables screening them directly without chromatographic purification. Due to its high synthetic throughput, this approach allows for the quick identification of binders to biological targets of interest and the efficient generation of large structure-activity datasets that can support subsequent structure optimization efforts. The strategies presented herein can later be used to find inhibitors of more challenging targets such as protein-protein interactions, and also to aid in lead development by generating and screening focused libraries, shortening the experimental cycles in this iterative process. The strategy described in Chapter 5 can be used as a powerful tool in fragment-based drug discovery, especially for fragment linking and fragment expansion, due to the efficient coverage of possible steric conformations by the structurally diverse diamino acid scaffolds. This can serve as a practical strategy for finding an optimal linker motif between two fragments, which is often a challenge in the case of hit validation from dual-display DEL screens or the design of targeted protein degradation-inducing compounds.

7. Curriculum Vitae

zb

Zsolt Bognar

PhD Scientist in Chemistry

Professional Summary

Five years of experience in organic synthesis and peptide and small molecule drug discovery. Co-inventor of a patented high-throughput peptide synthesis method. A passionate innovator and team player recognized for reliability and insight for designing, executing, documenting complex experiments.

Work History

2020-03 - 2024-04

Doctoral assistant

Swiss Federal Institute of Technology-Lausanne (EPFL), Lausanne, Switzerland

- Developed novel and robust methods for automated parallel synthesis (CEM Multipep SPPS platform), analysis (LC-MS) and late-stage nanoscale diversification of combinatorial peptide libraries with more than 20,000 distinct compounds
- Co-inventor in patent covering these technologies, which are now routinely used by spin-off company
- Established biochemical assays (TR-FRET, FP, kinetic)
- Identified novel biologically active compounds by high-throughput screening, routinely using automated liquid handling (Access Workstation, ECHO acoustic liquid handling, Certus Flex).
- Mentored Master students to achieve impactful scientific results

2018-09 - 2019-09

Medicinal Chemistry Intern

F. Hoffmann - La Roche AG, Basel, Switzerland

- Planned and independently performed multi-step synthesis and chromatographic purification of hundreds of small molecules and PROTACs
- Monitored reaction progression and characterized products by LC-MS and NMR
- Recorded all experimental data in Electronic Laboratory Notebook (ELN) system with perfect precision, enabling easy replication for others
- Analyzed activity (SAR) and ADMET data from biological assays, and participated in structural optimization of lead compounds for an oncology-related project

Personal information

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06/18/1994
Nationality
Hungarian
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+41 78 772 1414
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LinkedIn
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Skills

- Organic synthesis
- Peptide chemistry (SPPS)
- High-throughput screening (HTS)
- Preparative HPLC, LC-MS, NMR
- Chromatography
- Medicinal chemistry
- Drug discovery
- Laboratory automation
- Cheminformatics, molecular modeling
- Troubleshooting
- Research data management
- Fast learner
- Team player

2017-07 - 2020-01

Student researcher

Hungarian Academy of Sciences, Budapest, Hungary

- Identified novel inhibitors of an enzyme involved in hematologic malignancies (SETD2) by designing and conducting Virtual Screening campaign with more than 5 million compounds (Schrödinger, KNIME)

2016-07 - 2016-08

Intern

Gedeon Richter Plc., Budapest, Hungary

- Performed organic synthesis reactions for process development and scale-up

Education

2020-03 - 2024-04

Ph.D. in Chemistry

Swiss Federal Institute of Technology (EPFL)
Lausanne, Switzerland

2017-02 - 2020-01

Master of Science in Pharmaceutical Engineering

Budapest University of Technology and Economics
Budapest, Hungary
Diploma: Excellent with highest honors

2013-09 - 2017-01

Bachelor of Science in Chemical Engineering

Budapest University of Technology and Economics
Budapest, Hungary
Diploma: Excellent with highest honors

Publications and Patents

(1) Bognar, Z.; Mothukuri, G. K.; Nielsen, A. L.; Merz, M. L.; Pânzar, P. M. F.; Heinis, C. Solid-Phase Peptide Synthesis on Disulfide-Linker Resin Followed by Reductive Release Affords Pure Thiol-Functionalized Peptides. Org. Biomol. Chem. 2022, 20 (29), 5699–5703.

(2) Bajusz, D.; Bognar, Z.; Ebner, J.; Grebien, F.; Keseru, G. M. Discovery of a Non-Nucleoside SETD2 Methyltransferase Inhibitor against Acute Myeloid Leukemia. Int. J. Mol. Sci. 2021, 22 (18), 10055.

(3) Habeshian, S.; Merz, M. L.; Sangouard, G.; Mothukuri, G. K.; Schüttel, M.; Bognar, Z.; Díaz-Perlas, C.; Vesin, J.; Bortoli Chapalay, J.; Turcatti, G.; Cendron, L.; Angelini, A.; Heinis, C. Synthesis and Direct Assay of Large Macrocycle Diversities by Combinatorial Late-Stage Modification at Picomole Scale. Nat. Commun. 2022, 13 (1), 1–14.

(4) Heinis, C.; Habeshian, S.; Mothukuri, G. K.; Schuttel, M.; Merz, M. L.; Sangouard, G.; Bognar, Z.; Nielsen, A. L. Methods for Preparing a Library of Peptides or a Peptide. WO2022242993, 2022.

(5) Nielsen, A. L.; Bognar, Z.; Mothukuri, G. K.; Zarda, A.; Schuttel, M.; Merz, M. L.; Ji, X.; Will, E.; Chinellato, M.; Bartling, C. R. O.; Stromgaard, K.; Cendron, L.; Angelini, A.; Heinis, C. Large Libraries of Structurally Diverse Macrocycles Suitable for Membrane Permeation. Angew. Chemie Int. Ed. 2024, e202400350.

Software

- KNIME
- MS Office
- Schrödinger
- DataWarrior
- GraphPad Prism
- Adobe Illustrator
- ChemDraw

Languages

English ●●●●●
German ●●●○○
French ●●●○○
Hungarian ●●●●●