Expanding the toolbox of protein-templated reactions for early drug discovery
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Chapter 3

In situ Ugi four-component reaction for the protein-templated identification of inhibitors of endothiapepsin

The discovery of new bioactive compounds is a long and expensive process, which calls for the development of new techniques that can speed up hit identification and render it more efficient. In this context, a new protein-templated reaction for in situ selection of binders of protein targets would represent an invaluable addition to the portfolio available to medicinal chemists. In this work, we report the first example of an in situ Ugi reaction for the discovery of novel inhibitors of the aspartic protease endothiapepsin. Use of a protein-templated four-component reaction enables screening of a library of potential inhibitors in an efficient and rapid manner. This new protein-templated multicomponent reaction goes beyond known protein-templated reactions such as the in situ click reaction and opens up access to new regions of the chemical space. The Ugi products we identified are low-micromolar inhibitors, demonstrating the efficiency of this approach.

3.1 Introduction

Despite recent developments in drug discovery in terms of technology and techniques, there is a pressing need for the discovery of fast and efficient hit-identification strategies. Target guided synthesis (TGS) is a powerful approach to discover hit compounds by using the biological target itself in ligand selection. Two main methods, dynamic combinatorial chemistry (DCC) and kinetic target-guided synthesis (KTGS) have emerged. In KTGS, the target is actively involved in ligand selection by assembling its own inhibitors via an irreversible reaction from a library of complementary building blocks, whereas DCC assembles ligands via reversible process. Only a few protein-templated reactions for KTGS were reported so far because of the stringent requirements: The irreversible reaction needs to be compatible with physiological conditions, the building blocks need to be inert towards biomolecules and a substantial difference in reaction rate between the blank and biomolecule-templated reaction is required.

Multicomponent reactions (MCRs) are one-pot reactions in which more than two starting materials are incorporated to form a new adduct comprising most of the structural motifs of the starting building blocks. The Ugi four-component reaction (Ugi-4CR), discovered in 1959, is one of the most important MCRs, which affords dipeptide-like structures from isocyanides, carboxylic acids, aldehydes and amines. This elegant reaction has found numerous applications in drug discovery, including hit- and lead-identification as well as the generation of large libraries of analogues. Owing to the high exploratory power with regard to chemical space and biocompatibility, it represents an attractive reaction for KTGS. Herein, we describe the first use of the Ugi-4CR in KTGS for the identification of inhibitors.

The Ugi-4CR reaction represents a novel reaction for KTGS, in which the enzyme templates the synthesis of its own binders from a pool of four types of building blocks. It follows the same concept as other protein-templated reactions in terms of simultaneous binding of building blocks to adjacent pockets of the protein target, enabling the assembly of the corresponding binders. The great advantage of this method over other reported protein-templated reactions is the simultaneous screening of four subpockets and relative ease of accessing structurally complex binders and their derivatives for further optimization via a one-pot reaction, starting from simple and commercially available building blocks (Figure 1).
3.1 Introduction

Despite recent developments in drug discovery in terms of technology and techniques, there is a pressing need for the discovery of fast and efficient hit-identification strategies. Target guided synthesis (TGS) is a powerful approach to discover hit compounds by using the biological target itself in ligand selection. Two main methods, dynamic combinatorial chemistry (DCC) and kinetic target-guided synthesis (KTGS) have emerged. In KTGS, the target is actively involved in ligand selection by assembling its own inhibitors via an irreversible reaction from a library of complementary building blocks, whereas DCC assembles ligands via reversible process. Only a few protein-templated reactions for KTGS were reported so far because of the stringent requirements: The irreversible reaction needs to be compatible with physiological conditions, the building blocks need to be inert towards biomolecules and a substantial difference in reaction rate between the blank and biomolecule-templated reaction is required.

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Figure 1. Schematic representation of in situ Ugi 4-component reaction leading to a dipeptide-like Ugi product from a library of aldehydes, amines, carboxylic acids and isocyanides.

Endothiapepsin is a member of the class of pepsin-like aspartic proteases, which play a causative role in several diseases such as malaria, Alzheimer's disease, hypertension and HIV. The high degree of similarity makes endothiapepsin a convenient model enzyme for mechanistic studies and the identification of inhibitors of renin and β-secretase. For more detailed information about endothiapepsin, see Chapter 1.

To increase the power and scope of KTGS, it is necessary to expand the number of biocompatible reactions and building-block diversity. To the best of our knowledge, all the reports of KTGS represent examples of in situ reactions of two types of building blocks. In this work, for the first time, we described a new protein-templated reaction with four sets of fragments, which allows fast and efficient screening of a large portion of the chemical space.

3.2 Results and discussion

3.2.1 Design of the inhibitor with Ugi-4CR scaffold

We selected endothiapepsin as a model enzyme for our proof-of-principle work. After careful screening of the chemical structures of known inhibitors in terms of ease of modification to dipeptide-like compounds and affinity, we designed a novel, potential dipeptide inhibitor based on inhibitor which we had reported previously by using DCC and de novo structure-based drug design (IC₅₀ = 12.8 ± 0.4 μM, PDB: 4KUP). Starting from the X-ray crystal structure of inhibitor in complex with endothiapepsin, we used the molecular-modeling software Moloc and the FlexX docking module in the LeadIT suite for the structure-based design of inhibitor 1. For the first time, we described a new protein-templated reaction with four sets of fragments, which allows fast and efficient screening of a large portion of the chemical space.

The indole moiety of inhibitor functions as an anchor in the active site of the protein. Inhibitor occupies the S1 and S2 pockets of the protein and is involved in three H bonding interactions with the catalytic dyad (D35: 2.8 Å and 3.2 Å, D219: 2.9 Å) via its α-amino group. Additionally, while the NH of the acylhydrazone forms a H-bond with T222 (2.8 Å), the indolic
NH group engages in a H-bond with the carboxylate of D81 (3.0 Å).\textsuperscript{[20]} We designed inhibitor 2 as an Ugi product that should maintain the same interactions as inhibitor 1: H-bonding interactions with D35 (3.3 Å, 2.8 Å) and D219 (2.8 Å). The indolic NH group donates one H-bond to D81 (3.0 Å) and the carbonyl group of the amide bond accepts a H-bond from T222 (2.7 Å). An additional H-bonding interaction can be observed with G80 (2.7 Å) with the other carbonyl group, which should be hosted in the S2' pocket. By converting our parent inhibitor into a 4-CR scaffold, the imidazolyl group (amine component) can occupy the S2’ pocket where it engages in hydrophobic interactions with T79, G37, G221, while the phenyl group (isocyanide component) should be bound in between the S1’ and S2 pockets, benefiting from hydrophobic interactions with I302, I300 and I217 (Figure 2).

![Figure 2. X-ray crystal structure of endothiapepsin in complex with inhibitor 1 (PDB: 4KUP)\textsuperscript{[20]} superimposed with designed inhibitor 2. Color code: inhibitor 1 skeleton: C: cyan, N: blue, O: red; inhibitor 2 skeleton: C: yellow, N: blue, O: red; protein backbone: gray; dashed lines: H-bonding interactions below 3.3 Å.]

3.2.2 Generation of the library

To generate the combinatorial library, we selected two different building blocks for each of the four components (compounds 3–10, Scheme 1) with comparable reactivity and solubility. We used benzoic acid as one of the acid components, a negative control for our docking studies, to demonstrate that we maintain the same interactions with the catalytic dyad: H-bonding interactions with D35 and D219 as in inhibitor 1. The Ugi-4CR requires high concentrations (0.5–2.0 M) to afford the corresponding products. The Passerini reaction is a 3-CR in which an aldehyde, carboxylic acid and isocyanide react to form \(\alpha\)-acyloxyamides.\textsuperscript{[29]} This reaction is the main side reaction of the Ugi-4CR. The mechanism and the products formed are similar for both reactions, which should enable the simultaneous screening of a higher number of products.
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**Scheme 1.** Selection of building blocks for the in situ Ugi 4-CR, which affords sixteen possible Ugi and eight possible Passerini reaction products with their diastereomeric pairs.

### 3.2.3 In situ Ugi reaction

Having selected the building blocks, which are commercially available, we set up two reactions in parallel, a protein-templated reaction and a blank reaction, at pH=6.8 (phosphate buffer 0.1 M, 10% DMSO) by mixing two carboxylic acids 3 and 4 two amines 5 and 6, two aldehydes 7 and 8 and two isocyanides 9 and 10 (100 μM each) (Scheme 2).

**Scheme 2.** From top to bottom; in situ Ugi reaction, blank reaction.
The optimized reaction conditions have a building-block concentration of 100 μM to prevent product formation in the reference reaction and to reduce the amount of protein used. To the protein-templated reaction, we added a catalytic amount of endothiapepsin (25 μM) and analyzed both reaction mixtures after each day by using UPLC-TQD-SIR (SIR: selective-ion recording) for each of the possible 16 Ugi and 8 Passerini products. Although there are other possible side products derived from this library such as imine formation, Pictet-Spengler reaction, side products arising from the dual functionality of tryptophan (3) or cyclization products (each possibility was analyzed), we only focus on those products, which are most likely to bind and formed in the library mixture, for clarity. The SIR technique enables fast and sensitive screening of specific molecular weights (Mws, up to 8 Mws per injection) regardless of very low concentrations. By screening eight Mws per injection using the same concentrations for protein-templated and reference reactions, we analyzed each reaction in 3 SIR measurements (8-fold faster than commonly used SIM (selective ion monitoring) technique) and detected the formation of two Ugi products 2 and 11 only in the presence of endothiapepsin after 18 h (Figures 3-4).

**Figure 3.** UPLC-TQD-SIR analysis of compound 2 ([M+H]+ = 549). Formation of 2 by in situ Ugi-4CR was compared with the blank reaction and synthesized compound 2.
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Figure 3. UPLC-TQD-SIR analysis of compound 2 ([M+H]+ = 549). Formation of 2 by in situ Ugi-4CR was compared with the blank reaction and synthesized compound 2.

Figure 4. UPLC-TQD-SIR analysis of compound 11 ([M+H]+ = 485). Formation of 11 by in situ Ugi-4CR was compared with the blank reaction and synthesized compound 11.

3.2.4 Control experiments

To demonstrate that the active site of endothiapepsin is required for product formation, we repeated the reaction in the presence of 25 μM bovine serum albumin (BSA) instead of endothiapepsin and in the presence of a strong inhibitor of endothiapepsin, saquinavir (100 μM, Ki = 48 nM). No product formation was observed in these control experiments, indicating that binding of the components to the active site of endothiapepsin is essential for the formation (Figures 5 and 6).

Figure 5. Control reactions with BSA and saquinavir, compared with the synthesized compound 2.
To check for possible protein modification in the active site of endothiapepsin, we run several experiments in parallel under identical conditions: the whole library mixture 3–10 incubated with endothiapepsin, individual building blocks 3, 5–8, 10 incubated with endothiapepsin and as a reference only endothiapepsin in the reaction buffer. After 18 h, we evaluated the activity of the enzyme in each reaction using an adaptation from the fluorescence-based assay for HIV protease.[30] The activity was not affected, demonstrating that no modification occurred in the active site (Figure 7).

To investigate whether the individual building blocks bind to endothiapepsin, we used saturation-transfer difference (STD) NMR spectroscopy.[31] STD-NMR spectroscopy enables the characterization of target–ligand interactions in solution. The basic principle of the technique is that the protons, which are in close contact with the target protein receive a higher degree of saturation, resulting in stronger STD-NMR signals. Protons, which are not involved in the interaction or having weaker interaction with the target reveal no STD NMR signals. This technique is an excellent tool to investigate how a ligand interacts with the target. It is easy to implement and it requires small amount of the protein. STD-NMR experiments of building blocks 3, 6–8 and 10, which are comprised in the hit compound’s skeleton formed in the protein-templated reaction, showed that all building blocks except for aldehyde 7 have interactions with the target, which may be ascribed to its small size.

In addition, we performed competition 1H-STD-NMR experiments between each of the above fragments and a known inhibitor of endothiapepsin (bisacylhydrazone 20, IC50 = 54 nM, Figure 9).[31] The results of the competition 1H-STD-NMR experiments revealed that the bisacylhydrazone displaces 3 and 6 from the binding site of the enzyme. Given that bisacylhydrazone 20 (PDB: 5HCT) is a strong inhibitor and binds in the active site of the enzyme, this experiment demonstrates that both 3 and 6 bind to the same pocket of the enzyme as the bisacylhydrazone inhibitor 20. On the other hand, the signals of fragments 8 and 10 did not disappear in the STD-NMR competition experiment with bisacylhydrazone 20, indicating that they occupy different subpockets of the enzyme’s active site. As a result, we conclude that the two fragments 3 and 6 bind in the core of the active site of endothiapepsin just like the bisacylhydrazone 20, whereas fragments 8 and 10 occupy alternative subpockets in proximity to enable the protein-templated formation of the products. A representative example of the 1H-STD-NMR competition experiments can be seen in Figures 9 and 10.

Figure 6. Control reactions with BSA and saquinavir, compared with the synthesized compound 11.

Figure 7. Protein modification control experiments.
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**Figure 8.** A representative example of STD-NMR studies for fragment 3.
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Figure 9. STD-NMR studies for the bisacylhydrazone 20.

Figure 10. A representative example for the 1H-STD-NMR competition study for fragment 3 and bisacylhydrazone 20.

3.2.6 Synthesis and biochemical evaluation of the inhibitors

In order to confirm that the Ugi products formed are indeed inhibitors of endothiapepsin, we synthesized both compounds. The Ugi-4CR reaction with Boc-protected tryptophan (12), amine 6, aldehydes 7 and 8 and isocyanide 10 followed by HCl-mediated deprotection of the Boc-group of the corresponding Ugi products afforded compounds 2 and 11 in 40% and 35% yield, respectively (Scheme 3). We isolated the final compounds as diastereomeric mixtures and used them without further separation.

Evaluation of the inhibitory potency of compounds 2 and 11 showed that compounds 2 and 11, which were formed in the presence of protein, are potent inhibitors with IC50 values of 1.3 ± 0.1 μM and 3.5 ± 0.1 μM, respectively (Figures 11).

We could separate only one diastereomer of inhibitor 2, which showed a slight improvement in activity (IC50 = 0.89 ± 0.9 μM. Further attempts to separate diastereomers of hit compounds by using various separation techniques failed, presumably owing to the polarity of the compounds.
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![Figure 11](image)

Figure 11. From left to right: IC$_{50}$ inhibition curves of 2 (IC$_{50}$ = 1.3 ± 0.1 μM) and 11 (IC$_{50}$ = 3.5 ± 0.1 μM). The inhibitors were measured in duplicate.

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Scheme 3. Synthetic strategy towards compounds 2, 11 and 13–19.

As the in situ Ugi-4CR reaction represents the first example in the field, we synthesized a library of possible compounds including the eight Ugi-4CR products 2, 11, 13–18 using L-Trp as the acid component and one Ugi-4CR product using benzoic acid 19 (Table 1).
Table 1. Synthesis and biochemical evaluation of selected compounds derived from the combinatorial library.

<table>
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<tr>
<th>Compound</th>
<th>Yield (%)</th>
<th>IC₅₀ (μM)</th>
<th>Compound</th>
<th>Yield (%)</th>
<th>IC₅₀ (μM)</th>
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<td>40</td>
<td>1.3 ± 0.1</td>
<td><img src="image2.png" alt="Image" /></td>
<td>40</td>
<td>10 ± 5</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td>35</td>
<td>3.5 ± 0.1</td>
<td><img src="image4.png" alt="Image" /></td>
<td>61</td>
<td>16 ± 3</td>
</tr>
<tr>
<td><img src="image5.png" alt="Image" /></td>
<td>60</td>
<td>6 ± 0.2</td>
<td><img src="image6.png" alt="Image" /></td>
<td>37</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td><img src="image7.png" alt="Image" /></td>
<td>60</td>
<td>129 ± 35</td>
<td><img src="image8.png" alt="Image" /></td>
<td>32</td>
<td>No inhibition</td>
</tr>
<tr>
<td><img src="image9.png" alt="Image" /></td>
<td>75</td>
<td>19 ± 3</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Synthesis and subsequent biochemical evaluation of the library demonstrated that all combinations with L-Trp as the acid component show activity against endothiapepsin in the range of 1.3–129 μM. Compounds 11 and 18 show activities in the same range as the hits and the analogue 19 with the benzoic acid component did not show any inhibition at the starting concentration of 250 μM as we expected. As can be concluded from Table 1, in situ Ugi-4CR is a powerful method for the selection of the best binders from this library. The slight preference of compound 11 over 18 could be due to the content ratio of the diasteromeric mixture.
3.2.7 Docking results

We tried to soak crystals of endothiapepsin with inhibitors 2 and 11 to confirm the binding mode but were unsuccessful due to the limited solubility of the compounds. Our docking studies for both diastereomers of 2 and 11 using the FlexX docking module in the LeadIT\textsuperscript{[28]} suite followed by evaluation using the scoring function HYDE in SEESAR\textsuperscript{[32]} show that the inhibitor 11 can occupy the same pockets as inhibitor 2. As can be seen in Figure 12, the inhibitor is involved in a H-bonding network with the catalytic dyad via its \( \alpha \)-amino group (D35: 3.3 Å, 2.8 Å, D219: 2.8 Å). The H-bonding between the NH of the indolyl moiety with D81 (3.1 Å), the carbonyl group in the S2' pocket with G80 (2.8 Å) and the interaction between the other carbonyl group with T222 (3 Å) should be retained (Figures 12 and 13).

**Figure 12.** Compounds formed in presence of endothiapepsin. Top: top-ranked pose of inhibitor 11 generated by docking using the FlexX docking module in the LeadIT\textsuperscript{[28]} suite followed by evaluation using the scoring function HYDE in SEESAR.\textsuperscript{[32]} Bottom: superimposition of top-ranked poses of inhibitors generated by docking using the FlexX docking module in the LeadIT\textsuperscript{[28]} suite followed by hydescoring in SEESAR.\textsuperscript{[32]} Color code: inhibitor 2 skeleton: C: yellow, N: blue, O: red; inhibitor 11 skeleton: C: green, N: blue, O: red; protein backbone: gray; dashed lines: H-bonding interactions below 3.3 Å.
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Figure 13. Predicted binding mode of compounds (S,S)-2 and (S,S)-11. These binding modes are the result of a docking run using the FlexX docking module with 30 poses and represent the top-scoring pose after HYDE scoring with SEESAR and careful visual inspection to exclude poses with significant inter- or intra-molecular clash terms or unfavorable conformations. The figures were generated with PoseView as implemented in the LeadIT suite. Docking studies were performed for both diastereomers of the inhibitors 2 and 11.

3.3 Conclusions

In conclusion, herein, we describe the in situ Ugi-4CR, which represents the first example in drug discovery. As a result, the mechanism of the in situ reaction needs to be studied in detail. We screened 24 compounds (16 Ugi+8 Passerini) products by using commercially available building blocks and a catalytic amount of endothiapepsin in 18 h, which is comparatively fast for KTGS (6 h–2 weeks). Our efficient strategy circumvents the need for synthesis, purification and evaluation of each individual compound. Both inhibitors identified were synthesized in two synthetic steps. Thanks to the use of the Ugi-4CR, the inhibitors identified can be optimized in a straightforward manner. Our novel protein-templated 4-CR strategy could find application in the early stages of drug discovery, namely hit identification, on a range of drug targets.
3.4 Experimental section

3.4.1 Fluorescence-based inhibition assay

For fluorescence-based inhibition assay, see Chapter 2, Section 2.4.1.

3.4.2 Modeling and docking

The X-ray crystal structure of the complex of endothiapepsin (PDB code: 4KUP) with compound 1 was used for our modeling studies. The energy of the system was minimized using the MAB force field as implemented in the computer program MOLOC.[27] Taking inspiration from the cocrystal structure of endothiapepsin with compound 1, as well as from hot-spot analysis[34] of the active site of endothiapepsin, a new Ugi scaffold was designed and subsequent energy minimization (MAB force field) was done using MOLOC. All types of interactions (H-bonding, lipophilic and repulsive interactions) between designed Ugi products and protein were analyzed in MOLOC. Designed compounds were subsequently docked into the active site of endothiapepsin by using the FlexX docking module in the LeadIT suite.[28] During the docking, the binding site in the protein was restricted to 6.5 Å around the cocrystallized ligand 1, and the 30 top (FlexX)-scored solutions were retained, subsequently post-scored with SEESAR,[32] and the best scored pose was selected.

3.4.3 Experimental procedures

In situ Ugi reaction

Endothiapepsin (25 μL, 1.0 mM in phosphate buffer 0.1 M, pH 6.8), the seven building blocks 4–10 (1 μL each, 100 mM in DMSO) and L-tryptophan (3) (2.5 μL, 25 mM in DMSO) were added to a mixture of DMSO (91.5 μL) and phosphate buffer (900 μL, 0.1 M, pH 6.8). The reaction mixture was allowed to rotate at room temperature with 10 rpm. After 18 h, the library was analyzed by UPLC-TQD-SIR (electro-spray ionization, (ESI+)) measurement because of its higher sensitivity and greater reliability for product identification.

In situ Ugi-4CR experiments in presence of saquinavir

Endothiapepsin (25 μL, 1.0 mM in phosphate buffer 0.1 M, pH 6.8), the 7 building blocks 4–10 (1 μL each, 100 mM in DMSO), L-tryptophan (3) (2.5 μL, 25 mM in DMSO) and saquinavir (1 μL, 100 mM in DMSO) were added to a mixture of DMSO (91.5 μL) and phosphate buffer (900 μL, 0.1 M, pH 6.8). The reaction mixture was allowed to rotate at room temperature with 10 rpm. After 18 h, the library was analyzed by UPLC-TQD-SIR (electro-spray ionization, (ESI+)) measurement because of its higher sensitivity and greater reliability for product identification.
**Blank reaction, negative control:**
The 7 building blocks 4–10 (1 μL each, 100 mM in DMSO) and L-tryptophan (3) (2.5 μL, 25 mM in DMSO) were added to a mixture of DMSO (91.5 μL) and phosphate buffer (900 μL, 0.1 M, pH 6.8). The reaction mixture was allowed to rotate at room temperature with 10 rpm. After 18 h, the library was analyzed by UPLC-TQD-SIR (electro-spray ionization, (ESI+)) measurement because of its higher sensitivity and greater reliability for product identification.

**In situ Ugi experiments using BSA**
BSA (25 μL, 1.0 mM in phosphate buffer 0.1 M, pH 6.8), the 7 building blocks 4–10 (1 μL each, 100 mM in DMSO) and L-tryptophan (3) (2.5 μL, 25 mM in DMSO) were added to a mixture of DMSO (91.5 μL) and phosphate buffer (900 μL, 0.1 M, pH 6.8). The reaction mixture was allowed to rotate at room temperature with 10 rpm. After 18 h, the library was analyzed by UPLC-TQD-SIR (electro-spray ionization, (ESI+)) measurement because of its higher sensitivity and greater reliability for product identification.

**Protein**
Endothiapepsin (25 μL, stock solution 1.0 mM in phosphate buffer 0.1 M, pH 6.8) was added to 75 μL of DMSO and 900 μL phosphate buffer 0.1 M, pH 6.8. After 18 h, the enzyme solution was analyzed by UPLC-TQD-SIR (ESI+) measurements and compared with the positive hits identified from in situ Ugi product formation.

**Adaptation protocol for protein-modification test experiment**
Seven reactions were started in parallel, an in situ Ugi reaction with all library members 3–10 and individual fragments 3, 6–8 (100 μM) were incubated with endothiapepsin (25 μM) in phosphate buffer (0.1 M, pH 6.8). Finally, pure protein was incubated in the same reaction buffer as a reference. After 18 h, reaction mixtures were diluted to 4 nM and were directly used as protein stock solutions. Endothiapepsin was purified from Suparen® (kindly provided by DSM Food Specialties) by exchanging the buffer to potassium phosphate buffer (0.1 M, pH 6.8) using a Vivaspin 500 with a molecular weight cut-off at 10,000 Da. Measurement of the absorption at 280 nm, assuming an extinction coefficient of 1.15 for 1 mg/mL solutions, afforded the protein concentration. The final reaction volume was 200 μL containing 0.4 nM endothiapepsin (from each test reaction), 1.8 μM substrate and 2.1% DMSO. As substrate, Abz-Thr-Ile-Nle-p-nitro-Phe-Gln-Arg-NH2 (purchased from Bachem) was used for the fluorescence screening assay. The assay was performed with flat bottom 96-well microplates (purchased from Greiner Bio-One) using a Synergy Mx microplate reader at an excitation wavelength of 337 nm and an emission wavelength of 414 nm. The $K_m$ of the substrate toward endothiapepsin was known, 1.6 μM. [35]
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The assay buffer (phosphate buffer 0.1 M, pH 6.8, containing 0.001% Tween 20) was premixed with the substrate, endothiapepsin was added directly before the measurement. As the substrate is a fluorogenic substrate, during measurement the fluorescence increased because of substrate hydrolysis by endo-thiapepsin. The initial slopes of the fluorescence in the wells containing different protein solutions were compared for data analysis. Each compound was measured in duplicate.

UPLC-TQD-SIR method

UPLC-TQD was performed using a Waters Acquity UPLC H-class system coupled to a Waters TQD. All analyses were performed using a reversed-phase UPLC column (ACQUITY HSS T3 Column, 130 Å, 1.8 μm, 2.1 mm x 150 mm). Positive-ion mass spectra were acquired using ES ionization, injecting 10 μL of sample; column temperature 35 °C; flow rate 0.3 mL/min. The eluents, acetonitrile and water contained 0.1% of formic acid. The library components were eluted with a gradient from 95% → 30% over 20 min, then at 5% over 1 min, followed by 5% for 2 min.

The UPLC-TQD-SIR method was used to analyze the formation of Ugi products in in situ and blank reactions. SIR measurements are highly sensitive, where a minute amount of compound can be detected by the mass spectrometer. [M+H]^+ were monitored using the full mass range to ensure correct isotope patterns for all possible potential Ugi products both for in situ Ugi and blank reactions. The Ugi products in the protein-templated reaction were identified by comparison of their retention time with those synthesized using conventional methods.

^1H-STD-NMR binding experiments

All ^1H-STD-NMR experiments were performed at 25 °C on a Varian 600 MHz spectrometer. The on-resonance irradiation on endo-thiapepsin was set to 0 ppm. In each experiment, one ligand (3 mM) and endo-thiapepsin (10 μM) were present.

All fragments of two Ugi hit compounds 2 and 11, identified in the protein-templated Ugi reaction, were tested to verify their interaction with the protein by STD-NMR technique.

First, the ^1H-NMR spectra of the fragments Trp.HCl (3), histamine (6), 3-phenylpropanal (8), cyclopropanecarboxaldehyde (7), benzylisocyanide (10) in NaOAc buffer (100 mM, NaOAc of pD=4.7 in deuterated water (pH = 4.3)) and 5% DMSO-δ6, except for 3-phenylpropanal (8) (10% DMSO-δ6 was used for the solubility reason) in the presence of protein (off-resonance ^1H NMR spectra) were recorded.
Subsequently, on-resonance $^1$H-NMR spectra were recorded with minimum 512 scans. $^1$H-STD-NMR spectra were obtained by subtracting the on-resonance spectrum from the corresponding off-resonance spectrum. $^1$H-STD-NMR analysis revealed that all five fragments except for the cyclopropanecarboxaldehyde (7) bind to the protein with good to moderate interaction realized by the intensity of the peaks in the STD NMR.

$^1$H-STD-NMR competition experiments

First, the $^1$H-STD-NMR of each fragment (final concentration 3 mM), was recorded as mentioned above. Subsequently, bisacylhydrazone 20 (reported inhibitor, IC$_{50}$ = 54 nM) was added to the same NMR tube in order to have a final concentration of 3 mM, subsequently a second $^1$H-STD NMR spectrum was recorded showing the appearance of bisacylhydrazone peaks and disappearance (or reduced intensity) of peaks of some fragments such as TrpHCl and histamine, indicating that they are displaced by the bisacylhydrazone 20 and bind in the active site of endothiapepsin where bisacylhydrazone is hosted (PDB: 5HCT). This experiment confirms that fragments TrpHCl and histamine bind to the same binding pocket of the enzyme.

3.4.4 General experimental details

For general experimental conditions, see Chapter 2, Section 2.4.4

Chemical shifts ($\delta$) are reported relative to the residual solvent peak. The $^1$H-NMR spectra except for compound 19 (mixture of diastereomers and rotamers, see NMR spectra) were not interpreted due to their complexity. High-resolution mass spectra were recorded with an FTMS orbitrap (Thermo Fisher Scientific) mass spectrometer.

3.4.5 Synthesis of the Ugi products

General procedure for Ugi/Deprotection reaction

To a 10-mL round-bottomed flask charged with MeOH (4 mL), the corresponding aldehyde 7 or 8 (1 mmol, 1 eq.), amine 5 or 6 (1 mmol, 1 eq.), carboxylic acid 12 or 4 (1 mmol, 1 eq.) and isocyanide 9 or 10 (1 mmol, 1 eq.) were added. The reaction mixture was stirred at room temperature for 24 h. The reaction mixture was concentrated in vacuo and a quick purification over silica gel using EtOAc/pentane (1:1) afforded the corresponding N-Boc-protected Ugi product, which was directly used for the following deprotection step. The crude product was dissolved in DCM (4 ml), and HCl/diethyl ether (1 M, 10 mL) was added. After 24 h stirring at r.t., the resulting white precipitate was collected and washed with Et$_2$O. Complete removal of the solvent afforded the HCl salt of the desired compounds.
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2-((S)-N-(2-(1H-Imidazol-4-yl)ethyl)-2-amino-3-(1H-indol-3-yl)propanamido)-N-benzyl-4-phenylbutanamide hydrochloride (2)

General procedure starting from commercially available Boc-L-Trp (12, 304 mg, 1 mmol), histamine (6, 101 mg, 1 mmol), 3-phenylpropenal (8, 134 mg, 1 mmol) an benzyl isocyanide (10, 117 mg, 1 mmol) in MeOH (4 mL) afforded the desired product 2 as a white solid as a diastereomic mixture in 40% yield.

$^{13}$C NMR (101 MHz, CD$_3$OD) δ 172.2, 171.8, 171.6, 171.4, 171.2, 171.1 170.9, 170.7, 142.1, 142.0, 141.6, 141.5, 139.9, 139.6, 139.5, 139.4, 138.1, 138.0, 137.9, 134.8, 132.6, 132.5, 130.2, 129.9, 129.8, 129.6, 129.5, 129.5, 129.5, 129.4, 129.3, 129.2, 128.9, 128.8, 128.7, 128.5, 128.4, 128.4, 128.3, 128.3, 128.2, 128.2, 127.6, 127.2, 127.1, 126.1, 126.0, 125.9, 125.6, 123.1, 123.0, 120.7, 120.6, 120.5, 120.3, 119.2, 119.1, 119.0, 117.4, 117.1, 113.0, 112.9, 112.8, 112.8, 112.6, 107.9, 107.8, 107.6, 107.2, 61.5, 61.0, 60.7, 59.2, 52.5, 52.4, 52.1, 51.8, 49.9, 49.7, 49.5, 49.3, 49.1, 48.9, 44.5, 44.4, 44.3, 43.9, 43.8, 33.7, 33.5, 33.3, 32.9, 32.5, 32.2, 31.9, 29.4, 29.1, 28.5, 27.6, 25.6, 24.3 HRMS (ESI) calcd for C$_{28}$H$_{32}$N$_{6}$O$_{2}$: 549.2972, found: 549.2940. calcd for y$_2$ fragment (m/z): 363.2179, found 363.2161, calcd for b$_2$ fragment (m/z): 442.2243, found 442.2213.

(2S)-N-(2-(1H-Imidazol-4-yl)ethyl)-2-amino-N-(2-benzylamino)-1-cyclopropyl-2-oxoethyl)-3-(1H-indol-3-yl)propanamido hydrochloride (11)

General procedure starting from commercially available Boc-L-Trp (12, 304 mg, 1 mmol), histamine (6, 101 mg, 1 mmol), cyclopropanecarboxyraldehyde (7, 70 mg, 1 mmol) and benzyl isocyanide (10, 117 mg, 1 mmol) in MeOH (4 mL) afforded the desired product 11 as a white solid as a diastereomic mixture in 35% yield.

$^{13}$C NMR (101 MHz, CD$_3$OD) δ 173.7, 172.7, 172.5, 172.0, 171.7, 171.1, 170.5, 139.9, 139.8, 139.7, 138.2, 138.1, 138.0, 134.8, 129.7, 129.6, 129.5, 129.4, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 126.2, 125.9, 125.6, 123.3, 123.1, 123.0, 122.9, 120.6, 120.5, 120.4, 120.3, 118.9, 119.8, 117.6, 117.3, 117.1, 113.81, 112.9, 112.6, 107.9, 107.7, 107.6, 67.4, 66.8, 66.7, 52.6, 52.4, 52.0, 51.9, 49.9, 49.7, 49.5, 49.3, 49.1, 48.6, 48.6, 46.9, 44.7, 44.2, 44.1, 43.7, 29.2, 29.1, 25.9, 24.5, 14.2, 11.7, 11.3, 10.9, 7.7, 7.3, 6.2, 5.9, 5.2, 4.8, 4.1HRMS (ESI) calcd for C$_{28}$H$_{32}$N$_{6}$O$_{2}$ [M+H]: 485.2659, found: 485.2640, calcd for y$_2$ fragment (m/z): 299.1866, found 299.1859, calcd for b$_2$ fragment (m/z): 378.1925, found 378.1912.
(2S)-2-Amino-N-(2-(tert-butylamino)-1-cyclopropyl-2-oxoethyl)-3-(1H-indol-3-yl)-N-phenethylpropanamide hydrochloride (13)

General procedure starting from commercially available Boc-L-Trp (12, 304 mg, 1 mmol), phenylethylamine (5, 121 mg, 1 mmol), cyclopropanecarboxaldehyde (7, 70 mg, 1 mmol) and tert-Butyl isocyanide (9, 83 mg, 1 mmol) in MeOH (4 mL) afforded the desired product 13 as a white solid as a diastereomeric mixture in 80% yield.

$^{13}$C NMR (101 MHz, CD$_3$OD) δ 171.2, 170.3, 170.2, 170.0, 169.7, 169.6, 169.6, 169.3, 169.2, 169.1, 139.3, 139.0, 138.0, 137.7, 137.7, 136.8, 136.7, 136.6, 136.6, 128.3, 128.3, 128.7, 128.2, 128.1, 128.0, 127.4, 127.0, 126.9, 126.6, 126.3, 126.2, 126.1, 125.9, 125.9, 124.6, 124.5, 124.4, 124.3, 124.1, 121.9, 121.7, 121.6, 121.6, 121.3, 119.1, 119.1, 118.7, 117.9, 117.5, 117.5, 117.5, 117.4, 117.1, 111.4, 111.1, 106.7, 106.7, 106.3, 106.3, 106.3, 105.7, 65.5, 65.2, 65.1, 65.0, 51.5, 51.4, 51.3, 51.2, 51.1, 50.9, 50.6, 50.5, 48.3, 48.1, 45.6, 45.5, 35.7, 35.6, 34.3, 34.1, 27.8, 27.8, 27.7, 27.6, 27.5, 27.5, 27.5, 26.5, 26.3, 13.3, 10.6, 10.5, 10.1, 5.3, 5.2, 4.3, 4.3, 4.2, 4.1, 3.9, 3.2. HRMS (ESI-) calcd for C$_{28}$H$_{32}$N$_6$O$_2$ [M - H]: 459.2754, found: 459.2764.

2-((S)-2-Amino-3-(1H-indol-3-yl)-N-phenethylpropanamido)-N-(tert-butyl)-4-phenylbutanamide hydrochloride (14)

General procedure starting from commercially available Boc-L-Trp (12, 304 mg, 1 mmol), phenylethylamine (5, 121 mg, 1 mmol), 3-phenylpropanal (8, 134 mg, 1 mmol) and tert-Butyl isocyanide (9, 83 mg, 1 mmol) in MeOH (4 mL) afforded the desired product 14 as a white solid as a diastereomeric mixture in 60 % yield.

$^{13}$C NMR (101 MHz, CD$_3$OD) δ 171.9, 171.2, 171.2, 171.0, 171.0, 170.9, 170.9, 170.7, 170.7, 169.9, 142.2, 142.1, 141.8, 141.4, 140.4, 139.0, 138.8, 138.5, 138.3, 138.2, 138.0, 137.9, 137.8, 137.3, 130.3, 129.9, 129.9, 129.9, 129.8, 129.7, 129.7, 129.7, 129.6, 129.6, 129.6, 129.5, 129.4, 129.4, 129.4, 129.4, 129.3, 129.3, 129.3, 129.1, 128.5, 128.4, 128.2, 128.2, 128.1, 128.1, 128.0, 127.9, 127.6, 127.5, 127.3, 127.3, 127.2, 127.1, 127.1, 127.0, 126.6, 125.8, 125.8, 125.6, 125.5, 125.4, 123.1, 122.9, 122.9, 120.5, 120.5, 120.3, 119.2, 119.2, 118.9, 118.9, 112.8, 112.8, 112.8, 112.6, 107.9, 107.9, 107.9, 107.7, 107.1, 83.7, 81.2, 62.2, 61.3, 61.0, 60.9, 59.6, 59.0, 52.9, 52.8, 52.7, 52.7, 52.6, 52.5, 52.4, 51.6, 49.8, 49.5, 49.5, 49.2, 49.1, 48.4, 46.9, 46.4, 43.3, 37.12, 36.78, 35.41, 35.32, 34.92, 34.76, 33.45, 33.36, 33.25, 33.19, 33.03, 32.9, 32.6, 32.4, 32.4, 32.2, 32.1, 31.8, 29.3, 29.2, 29.1, 28.8, 28.7, 28.7, 28.1, 27.7, 27.6. HRMS (ESI-) calcd for C$_{38}$H$_{33}$N$_4$O$_2$ [M - H]: 523.3067, found: 523.3079.
2-((S)-2-Amino-3-(1H-indol-3-yl)-N-phenethylpropanamido)-N-benzyl-4-phenylbutanamide hydrochloride (15)

General procedure starting from commercially available Boc-\textit{L}-Trp (12, 304 mg, 1 mmol), phenylethylamine (5, 121 mg, 1 mmol), 3-phenylpropanal (8, 134 mg, 1 mmol) and benzyl isocyanide (10, 117 mg, 1 mmol) in MeOH (4 mL) afforded the desired product 15 as a white solid as a diastereomeric mixture in 75% yield.

\[ ^{13} \text{C NMR (101 MHz, CD}_3\text{OD) \delta 172.0, 171.7, 171.3, 171.0, 170.9, 170.8, 164.3, 142.1, 142.1, 141.6, 141.4, 140.3, 140.2, 139.9, 139.6, 139.6, 139.4, 139.0, 138.8, 138.2, 138.1, 138.0, 137.8, 130.2, 130.0, 129.9, 129.7, 129.6, 129.6, 129.5, 129.5, 129.5, 129.4, 129.4, 129.3, 129.1, 129.0, 128.8, 128.8, 128.6, 128.4, 128.4, 128.3, 128.3, 128.2, 128.1, 128.0, 127.6, 127.6, 127.4, 127.4, 127.2, 127.1, 127.1, 126.1, 125.8, 125.6, 125.5, 123.2, 123.2, 123.0, 120.8, 120.6, 120.5, 119.2, 119.1, 118.8, 118.8, 112.9, 112.8, 112.8, 107.9, 107.8, 107.7, 107.7, 107.5, 61.4, 60.8, 59.1, 52.8, 52.5, 51.8, 49.5, 49.4, 49.2, 49.0, 47.1, 47.0, 44.5, 44.4, 44.3, 44.1, 36.9, 36.7, 35.4, 34.7, 33.5, 33.4, 32.8, 32.5, 32.1, 32.1, 29.3, 29.2, 29.1, 28.6. HRMS (ESI-) calcd for C\textsubscript{30}H\textsubscript{37}N\textsubscript{6}O\textsubscript{2} [M-H]-: 513.2972 found: 513.2985.]

2-((S)-N-(2-(1H-Imidazol-4-yl)ethyl)-2-amino-3-(1H-indol-3-yl)propanamido)-N-(tert-butyl)-4-phenylbutanamide hydrochloride (16)

General procedure starting from commercially available Boc-\textit{L}-Trp (12, 304 mg, 1 mmol), histamine (5, 101 mg, 1 mmol), 3-phenylpropanal (8, 134 mg, 1 mmol) and tert-Butyl isocyanide (9, 83 mg, 1 mmol) in MeOH (4 mL) afforded the desired product 18 as a white solid as a diastereomeric mixture in 37% yield.

\[ ^{13} \text{C NMR (101 MHz, CD}_3\text{OD) \delta 172.3, 171.5, 171.4, 171.3, 171.2, 171.1, 171.1, 170.8, 170.1, 142.4, 142.2, 142.1, 142.0, 141.8, 141.7, 138.0, 137.9, 137.9, 137.8, 137.8, 137.7, 137.7, 134.7, 132.7, 132.5, 131.1, 131.0, 129.8, 129.6, 129.5, 129.5, 129.4, 129.4, 129.3, 129.3, 129.2, 129.1, 128.5, 128.3, 128.2, 128.1, 127.6, 127.3, 127.2, 127.1, 127.0, 126.1, 126.0, 125.9, 125.8, 125.6, 125.6, 123.0, 122.9, 122.3, 122.8, 120.5, 120.4, 120.3, 120.2, 119.2, 119.1, 119.0, 117.4, 117.3, 117.2, 117.19, 117.05, 116.8, 112.9, 112.8, 112.8, 112.6, 108.0, 107.8, 107.7, 107.7, 107.5, 106.7, 61.9, 61.4, 61.0, 60.9, 60.5, 59.2, 54.8, 52.9, 52.9, 52.8, 52.7, 52.7, 52.5, 52.6, 52.6, 52.4, 52.4, 52.1, 51.8, 51.6, 49.8, 47.4, 45.1, 44.9, 43.8, 43.2, 34.8, 33.7, 33.4, 33.4, 33.2, 32.9, 32.7, 32.6, 32.1, 31.9, 30.8, 30.7, 30.6, 30.3, 29.3, 29.1, 28.8, 28.7, 28.7, 28.7, 28.7, 27.9, 27.7, 27.6, 25.7, 25.5, 24.9, 24.4, 24.1, 23.6. HRMS (ESI-) calcd for C\textsubscript{36}H\textsubscript{37}N\textsubscript{4}O\textsubscript{2} [M-H]-: 513.2972 found: 513.2985.\]
(2S)-2-Amino-N-(2-(benzylamino)-1-cyclopropyl-2-oxoethyl)-3-(1H-indol-3-yl)-N-
phenethylpropanamide hydrochloride (17)

General procedure starting from commercially available Boc-L-Trp (12, 304 mg, 1 mmol), phenylethylamine (5, 121 mg, 1 mmol), cyclopropanecarboxaldehyde (7, 70 mg, 1 mmol) and benzyl isocyanide (10, 117 mg, 1 mmol) in MeOH (4 mL) afforded the desired product 16 as a white solid as a diastereomeric mixture in 75% yield.

General procedure starting from commercially available Boc-L-Trp (12, 304 mg, 1 mmol), phenylethylamine (5, 121 mg, 1 mmol), cyclopropanecarboxaldehyde (7, 70 mg, 1 mmol) and benzyl isocyanide (10, 117 mg, 1 mmol) in MeOH (4 mL) afforded the desired product 16 as a white solid as a diastereomeric mixture in 40% yield.

(2S)-2-Amino-N-(2-(1H-Imidazol-4-yl)ethyl)-2-amino-N-(2-(tert-butylamino)-1-cyclopropyl-2-
oxoethyl)-3-(1H-indol-3-yl)propanamide hydrochloride (18)

General procedure starting from commercially available Boc-L-Trp (12, 304 mg, 1 mmol), histamine (6, 101 mg, 1 mmol), cyclopropanecarboxaldehyde (7, 70 mg, 1 mmol) and tert-Butyl isocyanide (9, 83 mg, 1 mmol) in MeOH (4 mL) afforded the desired product 17 as a white solid as a diastereomeric mixture in 61% yield.

(2S)-2-Amino-N-(2-(1H-Imidazol-4-yl)ethyl)-2-amino-N-(2-(tert-butylamino)-1-cyclopropyl-2-
oxoethyl)-3-(1H-indol-3-yl)propanamide hydrochloride (18)

(2S)-2-Amino-N-(2-(1H-Imidazol-4-yl)ethyl)-2-amino-N-(2-(tert-butylamino)-1-cyclopropyl-2-
oxoethyl)-3-(1H-indol-3-yl)propanamide hydrochloride (18)
N-(2-(1H-Imidazol-4-yl)ethyl)-N-(1-(benzylamino)-1-oxo-4-phenylbutan-2-yl)benzamide (19)

General procedure starting from commercially available benzoic acid (4, 122 mg, 1 mmol), histamine (5, 101 mg, 1 mmol), 3-phenylpropanal (8, 134 mg, 1 mmol) and benzyl isocyanide (10, 117 mg, 1 mmol) in MeOH (4 mL) afforded the desired product 19 as a white solid in 80% yield.

\(^1\)H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 8.58 (brs, 1H), 8.17 (brs, 1H), 7.43 – 6.11 (m, 17H), 4.74 – 4.17 (m, 3H), 3.87 – 3.30 (m, 2H), 3.18 – 1.86 (m, 6H).

\(^13\)C NMR (101 MHz, CDCl\textsubscript{3}) \(\delta\) 173.2, 171.2, 140.8, 138.3, 135.8, 134.4, 133.3, 129.6, 128.5, 128.4, 127.8, 127.3, 126.2, 117.4, 58.9, 49.0, 43.6, 32.9, 30.2, 25.9. HRMS (ESI) calcd for C\textsubscript{29}H\textsubscript{30}N\textsubscript{4}O\textsubscript{2} [M+H]\(^+\): 467.2441 found: 467.2443.

3.5 Contributions from co-authors

STD-NMR experiments and part of the synthesis were performed by Dr. V. R. Jumde. M. Witte participated in the discussion of the results and commented on the manuscript.
N-(2-(1H-Imidazol-4-yl)ethyl)-N-(1-(benzylamino)-1-oxo-4-phenylbutan-2-yl)benzamide (19)

General procedure starting from commercially available benzoic acid (4, 122mg, 1 mmol), histamine (5, 101mg, 1 mmol), 3-phenylpropanal (8, 134mg, 1 mmol) and benzyl isocyanide (10, 117mg, 1 mmol) in MeOH (4 mL) afforded the desired product 19 as a white solid in 80% yield.

1H NMR (400 MHz, CDCl3) δ 8.58 (brs, 1H), 8.17 (brs, 1H), 7.43 – 6.11 (m, 17H), 4.74 – 4.17 (m, 3H), 3.87 – 3.30 (m, 2H), 3.18 – 1.86 (m, 6H).

13C NMR (101 MHz, CDCl3) δ 173.2, 171.2, 140.8, 138.3, 135.8, 134.4, 133.3, 129.6, 128.5, 128.4, 127.8, 127.3, 126.2, 117.4, 58.9, 49.0, 43.6, 32.9, 30.2, 25.9.


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