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Design and Synthesis of Bioisosteres of Acylhydrazones as Stable Inhibitors of the Aspartic Protease Endothiapepsin

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Acylhydrazone-based dynamic combinatorial chemistry (DCC) is a powerful strategy for the rapid identification of novel hits. Even though acylhydrazones are important structural motifs in medicinal chemistry, their further progression in development may be hampered by major instability and potential toxicity under physiological conditions. It is therefore of paramount importance to identify stable replacements for acylhydrazone linkers. Herein, we present the first report on the design and synthesis of stable bioisosteres of acylhydrazone-based inhibitors of the aspartic protease endothiapepsin as a follow-up to a DCC study. The most successful bioisostere is equipotent, bears an amide linker, and we confirmed its binding mode by X-ray crystallography. Having some validated bioisosteres of acylhydrazones readily available might accelerate hit-to-lead optimization in future acylhydrazone-based DCC projects.

Dynamic combinatorial chemistry (DCC) enables rapid screening of functionally diverse compounds against a target, circumventing the need for individual synthesis, purification and characterization.[1–7] Among many other prominent examples of DCC, reversible disulfide-bond formation was first introduced in DCC by the groups of Still,[8] Sanders,[5] and Lehn[9] in the late 1990s. Later on, in 1997, the group of Lehn first applied DCC to a protein target using imine formation/exchange.[1] Since then, its scope and wider applicability were demonstrated on a range of biological targets. Replacement of the reversible disulfide bond with thioether (-S-CH_2-)[10] or all-carbon (olefin, -CH_2-CH_2-)[11–15] and of the imine moiety with amines,[1] an ethyl linker[16] or with an amide linker[17] provides stable bioisosteres with potentially preserved binding mode, making DCC an enabling tool for medicinal chemistry and drug discovery (Figure 1 a).

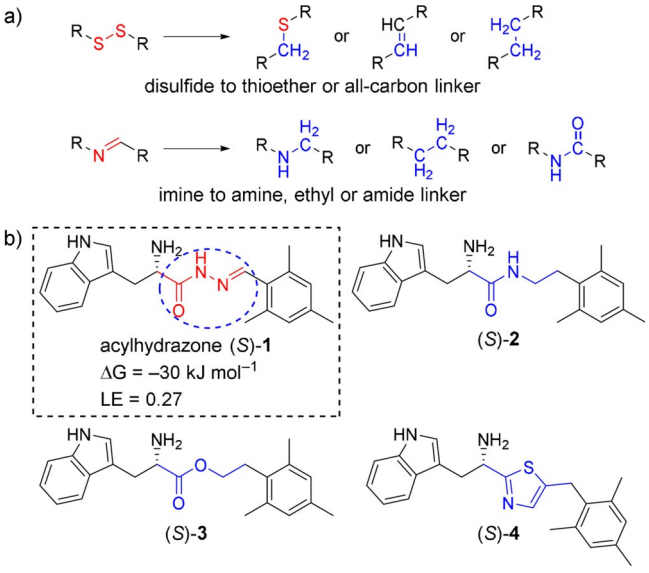

Figure 1. a) Previous examples of bioisosteres and b) proposed bioisosteres (**2**–**4**) of the acylhydrazone **1** as stable inhibitors of endothiapepsin.

We chose the target protein endothiapepsin, belonging to the family of pepsin-like aspartic proteases, which play a causative role in numerous diseases such as malaria, Alzheimer's disease, hypertension, and HIV-1.[18] Endothiapepsin is used as a representative enzyme due to its robustness, immense stability and similarity to the drug targets of the class of aspartic proteases. Moreover, it has been used as a model enzyme for mechanistic studies,[19–21] as it is readily available in large quantity and crystallizes easily and importantly remains active at room temperature for more than 20 days.

We previously discovered acylhydrazone-based inhibitors of endothiapepsin using DCC in combination with de novo structure-based drug design, which display a promising inhibitory

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profile ($IC_{50} = 12.8 \pm 0.4$ μM).[22] Acylhydrazones are considered to be important structural motifs in medicinal chemistry, as they hold the potential to interact with a range of biological targets in antiviral, anticancer and antibacterial drug discovery.

Nevertheless, there are problems associated with acylhydrazones. This class of compounds is considered by some as a member of the pan-assay interference compounds (PAINS).[23] They undergo photoinduced *E/Z* isomerization.[24] In addition, it is important to consider also the behavior of acylhydrazones in vivo. The major setback of acylhydrazones is their lack of stability due to hydrolysis into an aldehyde and a hydrazide under acidic pH. In spite of that, hydrazone and acylhydrazone linkages are used to develop pH-degradable drug-delivery systems for site-specific targeting.[25] Furthermore, some acylhydrazones, like PAC-1, are in clinical trials as a treatment for cancer.[26,27] Nevertheless, it is highly desirable to replace the labile acylhydrazone linker with stable and chemically benign analogues while maintaining the key interactions in the active site of the protein without significant changes in chemical structure.

Surprisingly, to the best of our knowledge, there are only few examples of bioisosteres of acylhydrazones,[16] but no report as a direct follow-up of a DCC experiment. In most cases, the binding mode of the bioisostere is not confirmed experimentally. Having suitable bioisosteres in hand, will establish 'acylhydrazone-based DCC' as a powerful hit/lead-identification strategy with the potential for further optimization.

Bioisosteres have been introduced as a fundamental strategy to improve the biocompatibility of the parent hit or lead compounds. As such, bioisosteres contribute to the field of medicinal chemistry, in terms of improving potency, enhancing selectivity, altering physicochemical properties, reducing or redirecting metabolism, eliminating or modifying toxicophores and acquiring novel intellectual property.[28] Herein, we describe the design, synthesis, and biochemical activity of three bioisosteres of the acylhydrazone (*S*)-**1**, the first acylhydrazone inhibitor of endothiapepsin. Importantly, unlike the parent acylhydrazone, bioisosteres (*S*)-**2** and (*S*)-**4** are not prone to hydrolysis, and all three do not liberate potentially toxic hydrazides.

We chose the X-ray crystal structure of endothiapepsin in complex with acylhydrazone (*S*)-**1** (PDB ID: 4KUP)[22] as a starting point for the design of stable bioisosteres of the labile acylhydrazone moiety. Hit (*S*)-**1** displays an IC_{50} value of 12.8 μM and a ligand efficiency (LE) of 0.27. It interacts with the catalytic dyad using H-bonding interactions (Asp35 (2.8 Å, 3.2 Å) and Asp219 (2.9 Å)) through its α-amino group.

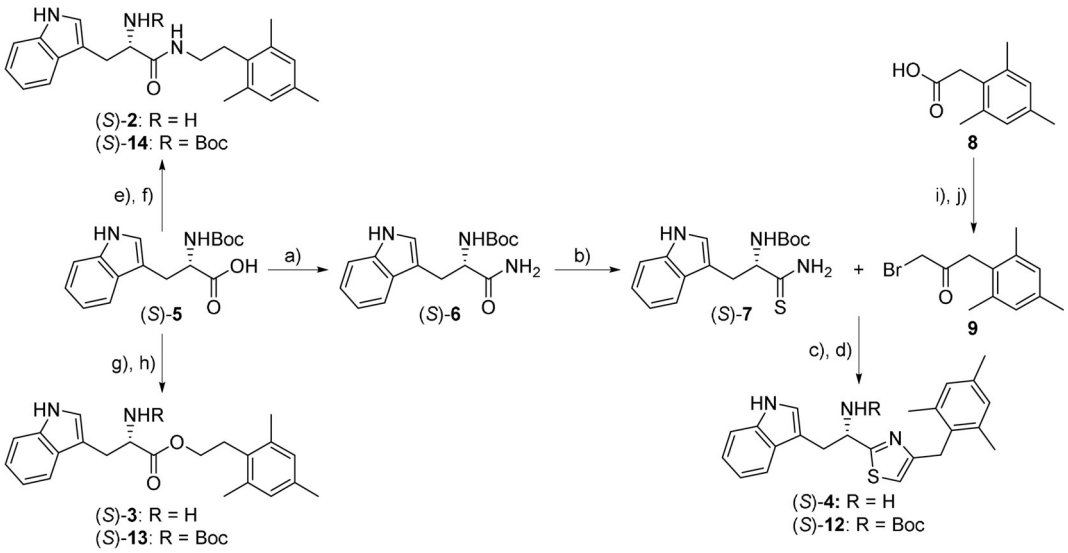
We designed bioisosteres using two different design approaches, namely Recore in the LeadIT suite[29] and the molecular modeling software Moloc[30] for molecular modeling and computation of the dipole moments. In Recore, a defined moiety of a molecule (the core) is replaced by fragments from a 3D database whilst keeping the rest of the molecule intact. To restrict the number of solutions, defined ligand-based pharmacophore constraints can be assigned. This modeling and docking resulted in various compounds displaying heterocyclic, ester or amide linkages. Among the various heterocycles (e.g., triazole, tetrazole, oxazole (Supporting Information Figure S8)), we chose the best three compounds (Figure 1 b) based on their dipole moments, their calculated ΔG, and predicted binding modes, which are similar to those of the parent acylhydrazone (*S*)-**1** and synthesized them as a proof-of-concept study. The predicted binding modes of three representative bioisosteres in the active pocket of endothiapepsin are shown in Figure S4 (Supporting Information).

Inspection of the soaked crystal structure of endothiapepsin with acylhydrazone (*S*)-**1** in the active site shows that the aromatic parts of the compound such as indolyl and/or mesityl moieties are able to form π–π-stacking interactions with the amino acid residues of the protein backbone. In all of the structures (Figure 1 b), the binding modes of the indolyl and mesityl moieties are preserved. It was computationally observed that the α-amino groups of all bioisosteres (*S*)-**2**-(*S*)-**4** form charge-assisted H bonds to the catalytic dyad (Asp35 and Asp 219) as well as additional H-bonding interactions with Asp81, and Gly221. The indolyl NH forms H bonds either with Asp81 or Asp33, the NH group of the amide donates an H bond to Gly221 in (*S*)-**2**. In addition to these, the thiazolyl ring of (*S*)-**4** is involved in several hydrophobic interactions with the protein backbone. The main building blocks required for the synthesis of bioisosteres (*S*)-**2**-(*S*)-**4**, are *N*-α-Boc-L-tryptophan (**5**) and the 2-mesitylene-derived compounds (*S*)-**5**, **8** and **11** (see Schemes S1–S4 in the Supporting Information and Scheme 1).

Very mild peptide-coupling conditions afforded the bioisostere (*S*)-**2** with the amide linker, followed by deprotection of the Boc group. Starting from *N*-Boc-L-tryptophan (**5**) and 2-mesitylethanamine hydrochloride (**10**) in presence of the weak base carbonyldiimidazole, furnished the corresponding amide (*S*)-**14** in 80% yield, and after deprotection with TFA, the test compound (*S*)-**2** in quantitative yield. The ester (*S*)-**3** was accessible through the Steglich esterification.[31] We synthesized the bioisostere (*S*)-**4** from the building blocks thioamide (*S*)-**7** and ketobromide **9**, which can be both accessed in two steps from *N*-α-Boc-L-tryptophan (**5**) and mesitylacetic acid (**8**),[32,33] respectively.

Subsequent deprotection of the Boc group of compound (*S*)-**12** afforded bioisostere (*S*)-**4** in quantitative yield. The first step to obtain thioamide (*S*)-**7** consists of the synthesis of amide (*S*)-**6** followed by thionation using Lawesson's reagent. On the other hand, using modified Arndt–Eistert reaction conditions, starting from mesitylacetic acid (**8**), afforded intermediate **9**. To investigate the biochemical activity of the designed bioisosteres (*S*)-**2** to (*S*)-**4**, we performed a fluorescence-based assay adapted from the HIV-protease assay (see Figures S1–S3 for the IC_{50} curves, Supporting Information).[34]

The three designed bioisosteres inhibit the activity of endothiapepsin to a different extent. The most potent inhibitor, the amide bioisostere (*S*)-**2**, displays a K_i value of 6.1 μM, very similar to the parent acylhydrazone (*S*)-**1** ($K_i = 6.0$ μM, Table 1). We calculated the K_i values from experimental IC_{50} values using the Cheng–Prusoff equation.[35] To verify the predicted binding mode of the bioisosteres, we soaked crystals of endothiapepsin with the most potent bioisostere (*S*)-**2** and determined the



Scheme 1. Synthesis of bioisosteres: a) $ClCO_2Et$, Et_3N, dry THF, aq. NH_3; b) Lawesson's reagent, dry CH_2Cl_2; c) EtOH, reflux, 4 h; d) TFA, CH_2Cl_2; e) 2-mesitylethanamine hydrochloride (**10**), 1,1′-carbonyldiimidazole, THF, RT, 15 h; f) TFA, CH_2Cl_2, 0 °C→RT, 1.5 h; g) 2-mesitylethanol (**11**), DCC, DMAP (5%) CH_2Cl_2, 8 h; h) HCl/Et_2O 1 M, 24 h; i) $SOCl_2$, dry toluene, reflux, 3 h; j) a) TMS-diazomethane, Et_2O, b) 47.5% aq. HBr.

Table 1. Biochemical evaluation of acylhydrazone (*S*)-**1** and its bioisosteres (*S*)-**2**–(*S*)-**4**. Each experiment was carried out in duplicate.

Inhibitor	IC_{50} [μM][a]	K_i [μM][b]	ΔG_{EXPT} [kJ mol^{-1}][b]	ΔG_{HYDE} [kJ mol^{-1}][c]
(*S*)-**1**	12.8 ± 0.4	6.0 ± 0.2	−30	−32
(*S*)-**2**	12.9 ± 0.7	6.1 ± 0.4	−30	−27
(*S*)-**3**	28.7 ± 4.1	13.5 ± 1.9	−28	−28
(*S*)-**4**	193.7 ± 11.4	91.2 ± 5.4	−23	−31

[a] Eleven different concentrations of inhibitor were used; errors are given in standard deviations (SD). [b] Values indicate the inhibition constant (K_i) and the Gibbs free energy of binding (ΔG) derived from IC_{50} values using the Cheng–Prusoff equation.[35] [c] Values indicate the calculated Gibbs free energy of binding (ΔG_{HYDE}; calculated by the HYDE scoring function in the LeadIT suite).

crystal structure of (*S*)-**2** in complex with endothiapepsin at 1.58 Å resolution (PDB ID: 5OJE). The structure features clear electron density for the ligand, as shown in Figure 2b.

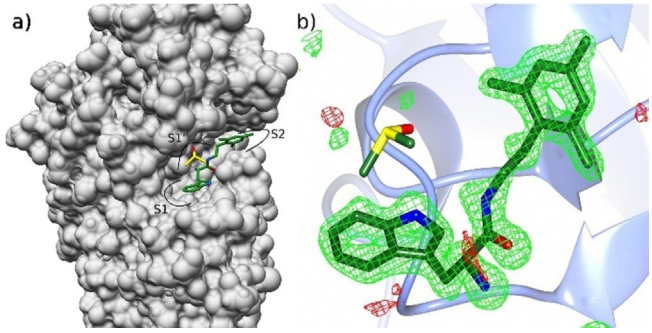


Figure 2. a) Zoomed-out view of the protein shown as surface. b) Electron density omit-map of the crystal structure of endothiapepsin in complex with compound (*S*)-**2** and a coordinated DMSO molecule. F_o-F_c map contoured at 3.3 σ (color code: protein cartoon: light blue, C: green, O: red, N: blue, S: yellow).

Upon closer examination, the location of the ligand is similar to the docked pose shown in Figure S4 (See Supporting Information). The amino group of the ligand forms two H bonds with Asp35 (2.9 Å) and Asp219 (3.0 Å). The indolyl nitrogen atom forms an H bond with Asp81 (3.2 Å). The hydrophobic part of the indolyl moiety is engaged in hydrophobic interactions with Phe116, Leu125, Tyr79 and Gly221. The mesityl substituent is involved in hydrophobic interactions with Ile300, Ile304, Tyr226, Gly80 and Asp81. The oxygen atom of the amide linkage forms water-mediated H bonds to the carbonyl oxygen of Gly37 and the amide nitrogen of Gly80. The mediating water molecules are conserved between the crystal structures in complex with (*S*)-**1** and (*S*)-**2** (PDB IDs: 4KUP and 5OJE, respectively, Supporting Information Figure S7).

The only difference compared to the docked pose is at the amide linkage. In contradiction to the computational modeling, the nitrogen atom of the amide does not form an H bond with the oxygen atom of Gly221, the distance is 4.2 Å. Instead, the hydroxy group of Thr222 acts as an H-bond acceptor and forms an H bond (2.9 Å) with the amide nitrogen atom of the ligand, which is also shown in Figure 3.

Due to the slightly bent shape of the coordinated ligand, both aromatic groups are able to form hydrophobic interactions with one DMSO molecule, shown in Figure 2. This DMSO molecule is well-coordinated and seems to displace several water molecules. This may be important for the stabilization of the ligand bound to the protein. A similar DMSO molecule can be observed in previous crystal structures (e.g., PDB ID: 4KUP).[22]

The single bond connecting the mesityl unit to the rest of the acylhydrazone (*S*)-**1** is part of a conjugated system and prefers a planar orientation. It is twisted out of planarity to an unfavorable angle of 34.4° compared to the more favored angle of 107.0° as in bioisostere (*S*)-**2** (Supporting Information Figure S6).

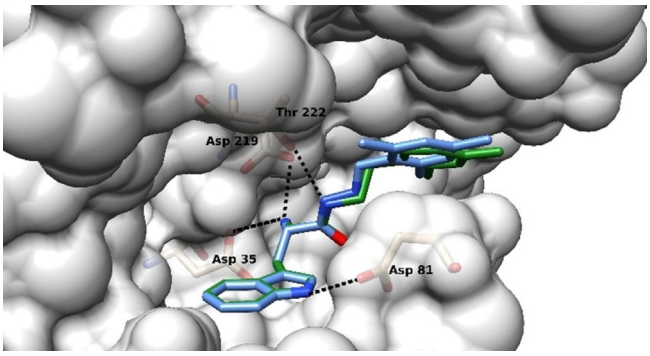


Figure 3. Superimposition of the acylhydrazone inhibitor (*S*)-**1** (cyan) and the amide bioisostere (*S*)-**2** (green). H bonds below 3.0 Å are shown as black dashed lines (color code: protein backbone: C: gray, O: red, N: blue, (*S*)-**1**: C: cyan and (*S*)-**2**: C: green).

The bioisostere (*S*)-**2**, however, contains a peptidic bond in the linker, which also prefers planarity. This forces the C–N bond, its third bond, counting from the mesityl substituent, into an unfavorable torsional angle of 122° compared to the preferred 170° of the acylhydrazone (Figure S6). In conclusion, both ligands have to adopt a slightly unfavorable conformation to bind in the pocket of the enzyme, which is reflected in their binding affinities. Based on our observations, it might be difficult to design a linker with improved binding affinity, which would need to be more flexible with respect to the torsional angles, while the H-bond donor and –acceptor functions of the peptidic nitrogen and oxygen atoms should ideally be preserved.

We report the successful replacement of the acid-sensitive and hydrolyzable acylhydrazone linker of parent hit (*S*)-**1**, affording stable and equipotent inhibitors of endothiapepsin. We designed and synthesized three bioisosteres and evaluated them for their inhibitory potency against endothiapepsin. Compounds (*S*)-**2** and (*S*)-**3**, possessing amide and ester linkers, respectively display similar K_i values as the parent hit (*S*)-**1**, while compound (*S*)-**4** is an order of magnitude weaker than the parent hit. The crystal structure of amide (*S*)-**2** ($K_i = 6.1$ μM) in complex with endothiapepsin validates the predicted binding mode. In this proof-of-concept study, we identified molecular interactions that should be taken into consideration if further modifications are done to achieve a more druglike replacement for the acylhydrazone linker. Taken together, we demonstrate that acylhydrazones can be replaced without affecting the binding mode and whilst preserving the activity, demonstrating that acylhydrazone-based DCC is a powerful tool to identify hits, which can then be optimized to stable lead compounds in a straightforward manner.

Experimental Section

Full experimental details are provided in the Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: acylhydrazones · aspartic proteases · bioisosteres · drug design · dynamic combinatorial chemistry

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