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Rapid synthesis of internal peptidyl α-ketoamides by on resin oxidation for the construction of rhomboid protease inhibitors†

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Rhomboid proteases are intramembrane serine proteases, which are involved in a wide variety of biological processes and have been implied in various human diseases. Recently, peptidyl α-ketoamides have been reported as rhomboid inhibitors with high potency and selectivity – owing to their interaction with both the primed and non-primed site of the target protease. However, their synthesis has been performed by solution phase chemistry. Here, we report a solid phase strategy towards ketoamides as rhomboid protease inhibitors, allowing rapid synthesis and optimization. We found that the primed site binding part of inhibitors is crucial for potency.

Introduction

Rhomboid proteases are one of the most widespread families of intramembrane proteases (IMPs). They were originally discovered in *Drosophila melanogaster*,[1] but occur in virtually all sequenced organisms.[2,3] Their roles are diverse and include EGFR signaling in the fruitfly,[1] quorum sensing in specific bacteria[4] and endoplasmatic reticulum associated degradation in mammalian cells.[5] IMPs are potential drug targets,[6] but the exact biomedical role and the druggability of rhomboids are still under investigation. One of the bottlenecks in rhomboid research has been the availability of potent and selective inhibitors.

In the past decade, various electrophiles have been reported as scaffolds for the design and synthesis of rhomboid inhibitors.[7] These include 4-chloro-isocoumarins,[8,9] such as compound **1**, β-lactams, *e.g.* compound **2**,[10,11] benzoxazinones including compound **3**,[12] and fluorophosphonates such as compound **4** (ref. 13 and 14) (Fig. 1A). All of these form a covalent intermediate with active site residues by alkylation, phosphorylation or acylation. Unfortunately, the aforementioned compounds are not highly selective. Peptidyl α-ketoamides (Fig. 1B), however, were recently reported as potent and highly selective rhomboid inhibitors.[15] These compounds form a reversible covalent hemiketal intermediate, and elements at both sides of this electrophile contribute to interaction with the active site surroundings at the non-primed site and the primed site.

The peptidic nature of α-ketoamide rhomboid inhibitors makes it possible to utilize substrate preference information to rationally design effective rhomboid inhibitors. We therefore aimed at synthesizing α-ketoamides flanked with peptide sequences on each side by making use of solid phase peptide synthesis (SPPS). Various reports on the synthesis of internal peptidyl ketoamides by SPPS have been made in the past, for example by on-resin oxidation of α-hydroxyamides[17,18] or by using Fmoc-protected building blocks containing as dithioketal-protected[19] or acetal-protected

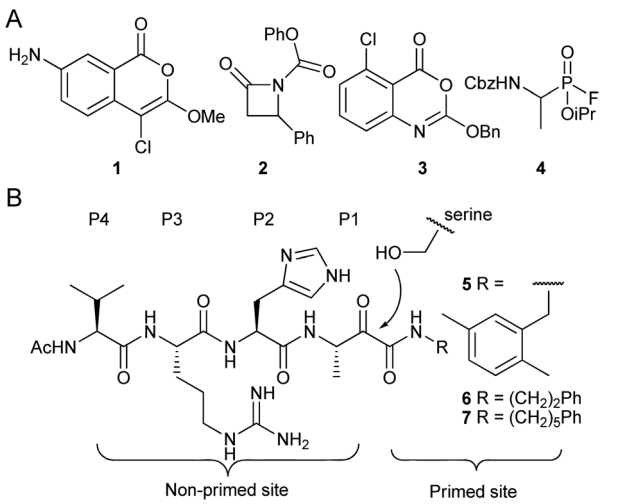

Fig. 1 Examples of rhomboid inhibitors. (A) 4-chloro-isocoumarins (**1**), β-lactams (**2**), benzoxazinones (**3**) and fluorophosphonates (**4**). (B) α-Ketoamide rhomboid inhibitors (**5**–**7**). The peptidic element in the non-primed site is indicated with the P1–P4 position according to the Schechter and Berger protease substrate nomenclature.[16]

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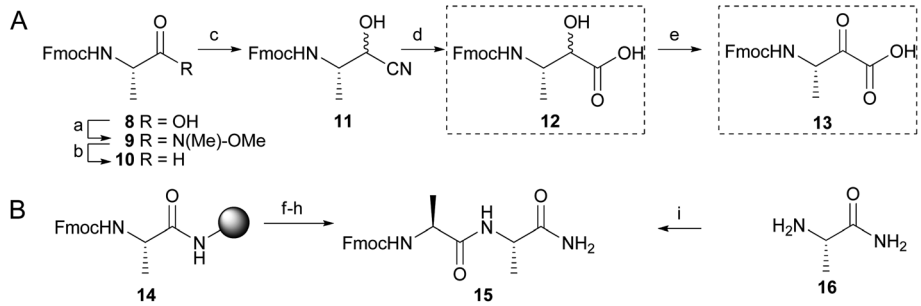


Scheme 1 Synthesis and peptide coupling of building block **13**, which leads to CO extrusion. (A) Synthesis of compound **13**. (a) MeONHMe, HBTU, DIEA, DMF/DCM, 97%, (b) $LiAlH_4$, THF, (c) NaCN, AcOH, DCM/MeOH, (d) conc HCl (aq)/dioxane 1/1, reflux, 48% (over three steps), (e) Dess-Martin reagent, DCM, 43%. (B) Peptide coupling with building block **13**. (f) 20% piperidine in DMF, (g) **13** (3 eq.), HBTU (3 eq.), DIEA (6 eq.), DMF, (h) TFA/H_2O/TIS 95/2.5/2.5, (i) **13** (1 eq.), HBTU (1 eq.), DIEA (2 eq), DMF.

ketoacids.[20] We here report rhomboid substrate-derived ketoamides by Fmoc-based SPPS combined with an on-resin oxidation by IBX. Inhibition tests revealed that the precise nature of the primed site binding elements are crucial for effective rhomboid inhibition.

Results and discussion

Most rhomboid substrates contain an alanine at the P1 position. Consequently, the thusfar reported peptide-derived inhibitors for rhomboid proteases also display an alanine residue in this position.[15,21,22] In order to explore a solid phase peptide synthesis of α-ketoamide inhibitors of rhomboid proteases, we first synthesized Fmoc-protected, alanine-derived α-keto-acid **13** with α-hydroxy-acid **12** as an intermediate (Scheme 1A). Known Fmoc L-alanine aldehyde **10** was generated from commercially available Fmoc-L-alanine (**8**) by conversion into Weinreb amide **9** and subsequent reduction under influence of $LiAlH_4$. Cyanohydrin **11** was formed as a diastereomeric mixture by reaction of aldehyde **10** with hydrogen cyanide. Acidic hydrolysis of the nitrile function to a carboxylic acid furnished compound **12**, which was oxidized to ketoacid **13** with Dess–Martin periodinane (Scheme 1A). With these building blocks in hand, we explored the synthesis of potential rhomboid inhibitors with peptide elements on both sides of the ketoamide moiety. In the primed site, a single alanine amino acid or an alanine–phenylalanine dipeptide was chosen. Coupling of keto-acid **13** to the free amine of alanine on a Rink resin (**14**), followed by TFA cleavage, led to formation of alanine dimer **15** (Scheme 1B and Fig. S1†) by CO extrusion, as reported before.[19] The elimination of CO has previously been attributed to a reaction under influence of TFA.[19] However, we also observed CO extrusion when building block **13** was coupled in solution to alanine amide (**16**) (Scheme 1B and Fig. S2†). Although the mechanism of the CO extrusion during peptide coupling remains elusive, we reasoned that coupling of hydroxy-acid **12** would not lead to loss of CO during coupling. This could then be followed by on-resin oxidation of the α-hydroxyamide, which has been reported for products from an on-resin Passerini reaction,[23] as well as for solution and solid phase approaches using various α-hydroxy-acids, followed by treatment with Dess–Martin periodinane.[18,24] In these cases, CO extrusion did not take place after cleavage from the solid support. Hence, we reasoned that the proposed strategy may successfully lead to the formation of the desired products as potentially novel rhomboid inhibitors.

As P4–P1 residues, the desired compounds **18–20** contain the tetrapeptide element VRHA, as a most optimally binding sequence for GlpG[15,21] or IATA, derived from the rhomboid substrate TatA.[4] For the primed site, we chose an alanine or an alanine–phenylalanine dipeptide, as found in TatA (Scheme 2). The sequence was synthesized on a Rink amide resin to ensure a non-charged, C-terminal amide. After elongation of the peptide with the respective Fmoc amino acid building blocks, including building block **12**, on resin oxidation was performed by using IBX in DMF : DMSO (1 : 1) (Scheme 2), which yielded target compounds **18–20** after cleavage from resin. Note that no CO extrusion was observed on the crude material (Fig. S3–S5†).

We next performed an inhibition assay of *E. coli* rhomboid protease GlpG by using competitive activity-based protein profiling (competitive ABPP; Fig. 2A). To this end, GlpG was pretreated with 50 μM of compounds **18–20**, known ketoamide inhibitors **5–7**, or 100 μM dichloroisocoumarin (DCI) as a general rhomboid inhibitor control. Residually active GlpG was then labeled by the general serine hydrolase probe FP-Rh,[25]

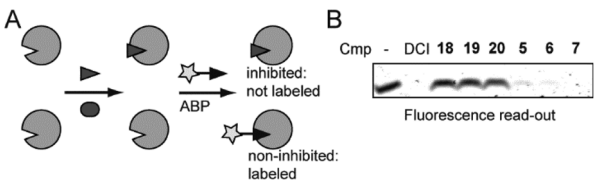
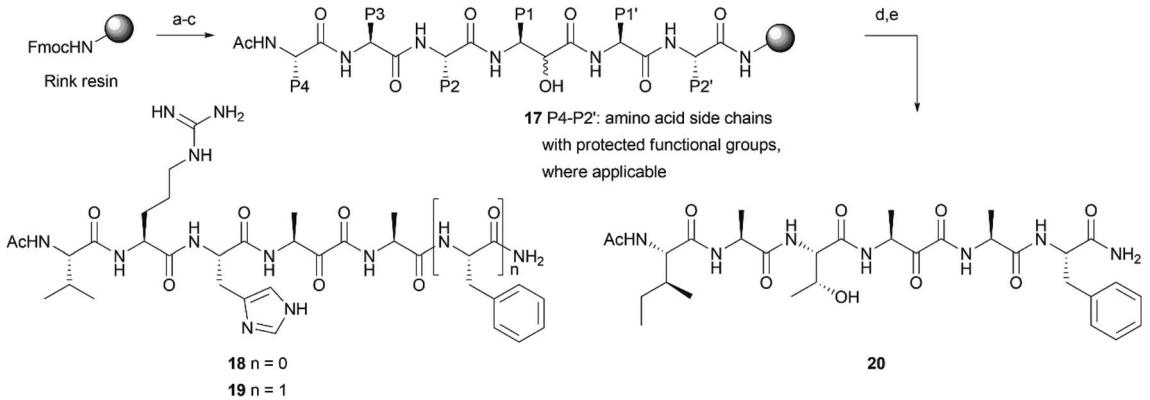


Fig. 2 Evaluation of peptide α-ketoamides as inhibitors of the *E. coli* rhomboid GlpG. (A) Schematic representation of competitive ABPP. Pretreatment of a serine protease with a small molecule may lead to inhibition (above) or not (below). Subsequent addition of an activity-based probe (ABP) will lead to fluorescent labeling of the residually active enzyme, which can be measured by in-gel fluorescent scanning. (B) *E. coli* GlpG was incubated with the indicated ketoamides, the pan rhomboid inhibitor 3,4-dichloroisocoumarin (DCI) or dmso control for 30 min. Residually active GlpG was determined by incubation with the general serine hydrolase activity-based probe FP-Rh. Disappearance of fluorescent bands indicate full inhibition.



Scheme 2 On resin synthesis of peptidyl α-ketoamides. (a) Fmoc-based solid phase peptide synthesis: (1) 20% piperidine in DMF, (2) Fmoc-amino acid (5 eq.), HBTU (5 eq.), DIEA (10 eq.), DMF (couple twice; except for building block **12**, which was coupled in a single step); repeat from step 1 for every amino acid. Utilized amino acids: Fmoc-Phe-OH, Fmoc-Ala-OH, Fmoc-His(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Ile-OH (b) (1) 20% piperidine in DMF, (2) Ac_2O (5 eq.), Et_3N (5 eq.), pyridine (5 eq.), DMF, (c) 1 M KOH in MeOH (d) IBX (3 eq.), H_2O (3 eq.) in DMF : DMSO (1 : 1) (e) TFA/H_2O/TIS 95/2.5/2.5.

which is also a general probe for rhomboid proteases.[26] To our surprise, the ketoamide compounds **18–20** did not display any inhibition in the competitive ABPP assay, while the reference compounds **5–7** as well as DCI gave full inhibition.

Conclusions

In this paper, we have described a synthesis of peptides with an internal α-ketoamide electrophile that can be fully executed on solid support. Crucial to the synthesis is an α-hydroxy-β-amino acid derivative, exemplified by compound **12**, which is oxidized on resin after elongation of the peptide sequence. Importantly, this enables a synthesis of peptidyl α-ketoamides that can be fully executed on resin, and circumvents the need for building blocks with protected ketone function.[19,20] The synthesized α-ketoamides, despite close resemblance of rhomboid substrate sequences and previously reported inhibitors, turned out to be inactive. This indicates that the structure located at the C-terminal side of the α-ketoamide is crucial for potent inhibition of rhomboids. It also underlines the difficulty of using specificity information from both primed and non-primed sites of rhomboid protease substrates, which may complicate the design of inhibitors based on substrate sequences. Since α-ketoamides show a broad range of biological activities against proteases and other targets, we expect that the presented solid support strategy will find application in the future synthesis of biologically active peptidyl ketoamide derivatives.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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