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Authors: Jian Yang, Luis A.R. Carvalho, Shanping Ji, Suyuan Chen, Rui Moreira, and Steven Verhelst

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RESEARCH ARTICLE

4-Oxo-β-Lactams as Novel Inhibitors for Rhomboid Proteases

Jian Yang,[a] Luís A. R. Carvalho,[b] Shanping Ji,[a] Suyuan Chen,[c] Rui Moreira[b] and Steven H. L. Verhelst*[a,c]

Dedicated to Prof. Dr. Matthew Freeman for his 10 year anniversary at Oxford University

[a] Dr. Jian Yang, Prof. Dr. Steven H. L. Verhelst,
Laboratory of Chemical Biology, Department of Cellular and Molecular Medicine
KU Leuven – University of Leuven
Herestraat 49, box 901b, 3000 Leuven, Belgium
E-mail: steven.verhelst@kuleuven.be
[b] Dr. Luís A. R. Carvalho and Prof. Dr. Rui Moreira
Department of Pharmaceutical Sciences and Medicines, Research Institute for Medicines (iMed.ULisboa)
Faculdade de Farmácia, Universidade de Lisboa
Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal
[c] Suyuan Chen, Prof. Dr. Steven Verhelst
Leibniz Institut für Analytische Wissenschaften – ISAS – e.V
Otto-Hahn Strasse 6b, 44227 Dortmund, Germany

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Abstract: Intramembrane serine proteases (rhomboid proteases) are involved in a variety of different biological processes and are implicated in several diseases. Here, we report 4-oxo-β-lactams as a novel scaffold for inhibition of rhomboids. We here show that they covalently react with the active site and that the covalent bond is sufficiently stable for detection of the covalent rhomboid-lactam complex. 4-Oxo-β-lactams may therefore find future use as both inhibitors and activity-based probes for rhomboid proteases.

Introduction

Rhomboid proteases are evolutionary conserved intramembrane proteases, whose active sites reside – in contrast to their soluble counterparts – within the lipid bilayer of a membrane. Intramembrane proteases are attractive drug targets,[1] as they are involved in a variety of human pathologies. Also rhomboid proteases have been linked to several diseases, such as Parkinson's disease, due to the involvement of the mitochondrial rhomboid PARL,[2] and infection by the malaria parasite, where rhomboid proteases cleave adhesin molecules to finalize entry into red blood cells.[3–5]

Rhomboid proteases utilize a serine-histidine catalytic dyad for attack onto the peptide backbone of transmembrane substrates (Figure 1A). In the past two decades, protocols have been developed to express and purify rhomboid proteases by using different expression systems, such as *Escherichia coli* (*E. coli*),[6,7] *Pichia pastoris* (*P. pastoris*)[8] and in vitro translation,[9] in combination with different solubilization methods, including mild detergents,[6,7] liposomes[9,10] and lipid nanodiscs.[9,11] These efforts enabled inhibitor development resulting in the discovery of various covalent electrophilic scaffolds for rhomboid inhibition: 4-chloro-isocoumarins,[12,13] fluorophosphonates,[14,15] β-lactones,[16] *N*-sulfonyl- and *N*-oxycarbonyl-β-lactams,[17,18] benzoxazinones,[19,20] saccharins,[21] and peptide derived aldehydes,[22] chloromethylketones,[23] ketoamides[9,24] and boronates[5] (Figure 1B).

β-Lactams occur in a variety of approved drugs. However, for rhomboid proteases, the lactam scaffold is limited to those with electron-withdrawing substituents on the nitrogen atom. These substituents are necessary to increase the electrophilicity of the carbonyl in the lactam ring. Recently, Moreira and co-workers reported 4-oxo-β-lactams (Figure 1C) as novel inhibitors for leukocyte elastase[25–27] as well as dipeptidyl peptidases 8 and 9.[28] We reasoned that the electron withdrawing effect of the oxo-substituent in the lactam ring, leading to a higher reactivity, could make this scaffold suitable for rhomboid protease inhibition as well. Here, we report the 4-oxo-β-lactam as novel scaffold for covalent, irreversible inhibition of bacterial rhomboid proteases.

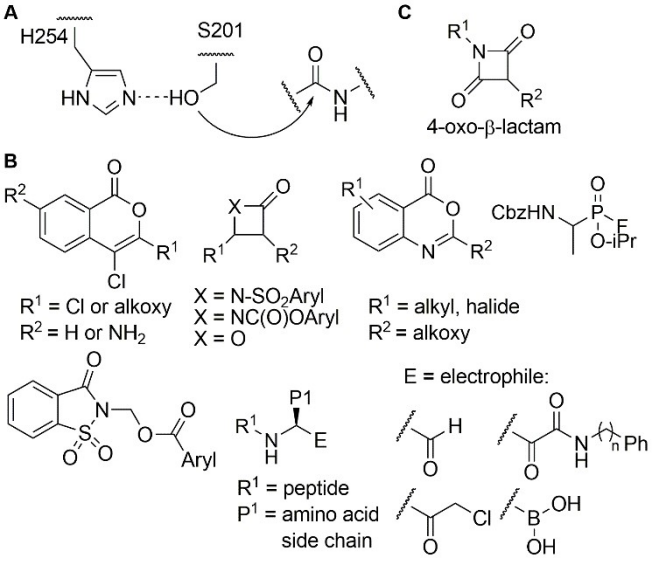

Figure 1. Rhomboid proteases and inhibitors. (A) The catalytic dyad of rhomboid proteases consists of an active site serine activated by a histidine side chain (indicated amino acid numbering of *E. coli* GlpG). (B) Reported rhomboid inhibitors. Top row: 4-chloro-isocoumarins, β-lactones and β-lactams, benzoxazine-4-ones, fluorophosphonate. Bottom row: Saccharins and peptide-derivatives with a C-terminal electrophile: aldehydes, ketoamides, chloromethyl

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ketones and boronates. (C) The 4-oxo-β-lactam scaffold reported as leukocyte elastase inhibitor.

Results and Discussion

In order to evaluate 4-oxo-β-lactams as possible rhomboid protease inhibitors, we utilized a recently reported library of compounds with the majority having a general structure displayed in Figure 2A (full library details in Figure S1).[28] We screened these compounds for inhibition of *E. coli* rhomboid GlpG by making use of a commercial FRET peptide that was recently reported to be cleaved by GlpG.[29] To this end, we first incubated recombinantly expressed EcGlpG, solubilized and purified as before,[13] at 37 °C with a high concentration (100 μM) of each library member and then measured residual protease activity by adding the reporter peptide. A sample with only DMSO vehicle was utilized as control and the slope of the progress curve was set to 100% activity (Figure 2B). 7-Amino-3-butynoxy-4-chloro-isocoumarin (JLK6), a known irreversible inhibitor of GlpG,[13] was used as positive control and led to no residual GlpG activity. To our delight, several 4-oxo-β-lactam library members displayed inhibition of GlpG, with more than 10 compounds leading to less than 20% residual activity (Figure 2C).

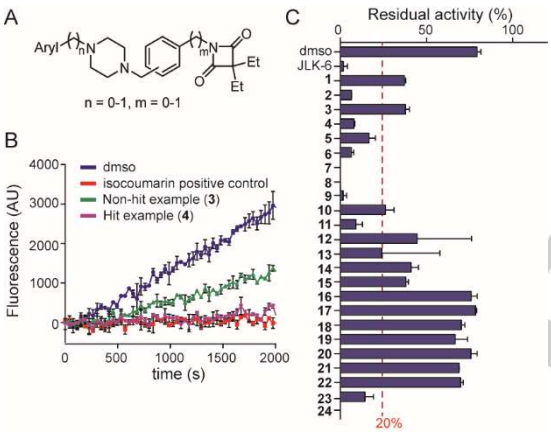

Figure 2. Screening of 4-oxo-β-lactams against EcGlpG. (A) Structures of the tested compounds (full details in Figure S1). (B) Progress curves of some selected compounds including DMSO control, a known 4-chloro-isocoumarin inhibitor, a compound that didn't make the cut-off for maximum residual activity (**3**) and a hit compound (**4**). (C) Bar graph of residual activity of GlpG after inhibition with 4-oxo-β-lactam library members or positive control S006 (all 100 μM). Values represent means of normalized slopes and error bars represent standard deviations of two replicates.

For compounds that were below the cut off of 20% residual activity in our screen, we determined apparent IC_{50} values by competitive activity-based protein profiling (ABPP) using pan serine hydrolase fluorophosphonate probe FP-Rh[30,31] (Table 1). To this end, purified EcGlpG was incubated with a serial dilution of hit compound, residually active EcGlpG was labelled by Fp-Rh and detected by SDS-PAGE and fluorescence scanning. We found that the 4-oxo-β-lactam displayed submicromolar activity for the most active compound (0.48 μM for compound **4**) to 19 μM for compound **24**. Based on the limited amount of compounds it is difficult to derive a defined structure-activity relationship. However, the substitution pattern on the phenyl group attached to the 3-position of the lactam scaffold seems to have an influence, as para- and meta-substituted phenyl rings are active (with para being most active), but ortho substitutions are not (compare active compounds **2**, **11** with inactive compound **16**; Figure 3A). Such relationship can also be seen with para-substituted compound **4** and **7**, meta-substituted **12** and **15** and ortho-substituted **17** and **21** (see Figure S1 and Figure 2). Additionally, the length of the substituent also seems to play a role, as compound **4**, which is one methylene shorter than compound **2**, is one order of magnitude more potent. To gain some more insight into the binding mode of the compounds, we synthesized the alkyne-containing analog **25** (Figure 3B; apparent IC_{50} = 3.8 μM, see Figure S3) and subjected it to a reaction with EcGlpG followed by click chemistry-mediated introduction of an azide-TAMRA conjugate. Gel-based analysis revealed a covalent complex, whose intensity was similar to the pan-selective rhomboid protease ABP FP-Rh (Figure 3C).

For the soluble serine protease pancreatic elastase, 4-oxo-β-lactams are covalent inhibitors with a reversible mechanism: after acylation of the active site serine, the acyl group can be hydrolyzed to reform the active protease species (Figure 3D).[25,26] To investigate the binding mechanism of the 4-oxo-β-lactams with rhomboids, we evaluated the inhibition mechanism of the most potent compounds **4**, **5** and **9**, as well as compounds **2**, **7** and **8**. To this end, EcGlpG was incubated with these inhibitors, followed by removal of excess inhibitor by a gel filtration column. The enzyme was then allowed to incubate for up to 4h to allow the possible acyl hydrolysis to happen, followed by measurement of residual GlpG activity by activity-based protein profiling using the pan serine hydrolase probe FP-Rh. Interestingly, EcGlpG only very slowly regains activity over time. Only after 24h, approximately 20-30% of active GlpG is reformed by ester hydrolysis (Figure 3E). We therefore conclude that the 4-oxo-β-lactams are rhomboid protease inhibitors with a covalent, and slowly reversible mechanism.

For the most active compound **4**, we performed covalent docking with AutodockFR 1.0[32] in order to gain some insight into its binding mode. We used the reported crystal structure of EcGlpG with a covalently bound β-lactam inhibitor (PDB IC: 3ZMI),[18] as this reactive group is closely related to the oxo-lactam scaffold. This compound carries a phenyl carbamate substituent on its ring nitrogen, which interacts with the S2' pocket of GlpG (Figure 4A). Redocking of the reported lactam led to satisfactory overlay (Figure S3), suggesting that the docking method results in acceptable models. Covalent docking of the ring-opened form of inhibitor **4** attached to the active site serine S201 resulted in a structure presented in Figure 4B and 4C. The substituent on the nitrogen of compound **4** is too large to fit in the S2' pocket and protrudes between helices TM5 and TM2, as the phenyl substituent of the original inhibitor (Figure 4B). There also seems to be some hydrophobic interaction between the bromophenyl ring and methionine M149 in helix TM2 (Figure 4C).

Table 1. Apparent IC_{50} values of selected compounds against EcGlpG[a,b]

Compound	IC_{50}^{Aop} (μM)	Compound	IC_{50}^{Aop} (μM)
2	3.9 ± 0.5	**8**	10.4 ± 1.9
4	0.48 ± 0.04	**9**	1.9 ± 0.28
5	0.67 ± 0.11	**11**	6.5 ± 0.72

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6	7.0 ± 2.3	**13**	10 ± 1.6
7	9.2 ± 0.6	**24**	19 ± 2.3

[a]Apparent IC_{50} values were determined by competitive ABPP by preincubation of EcGlpG with different concentrations of the indicated compound (1h at rt) followed by labelling of residually active EcGlpG by FP-Rh with gel-based fluorescence detection (see also Figure S5).
[b]Note that some of the IC_{50}^{App} values are different from the ranking in the screen of Figure 2C. We attribute this to the low signal-to-noise of the screening assay with the FRET peptide.

Conclusion

In conclusion, we here report the 4-oxo-β-lactam scaffold as a new type of rhomboid inhibitor. Future tests of the 4-oxo-β-lactam scaffold against other rhomboid proteases, such as those of the malaria parasite or the mitochondrial rhomboid PARL, will be interesting, as these rhomboids have been implicated in human disease. Because the β-lactam scaffold occurs in many antibiotics, rhomboid inhibitors based on this scaffold may also have good drug-like properties.

The ease of synthesis of 4-oxo-β-lactams from malonic acid chlorides and amines,[25] will allow future optimization of substituents on both the nitrogen as well as the carbon in the 4-oxo-β-lactam scaffold. Such optimization will be necessary to allow for highly selective inhibitors, as the current 4-oxo-β-lactams with 3,3-diethyl substituents also inhibit human neutrophil elastase[25–27] and dipeptidyl peptidases (DPP) 8 and 9.[28] The computational model presented here may guide future choices for these substituents, although it will be worth pursuing crystallization of GlpG with compound **4** to obtain a better view on the molecular interactions. Furthermore, reported computational models[25,26] and crystal structures[28] of this compound class with neutrophil elastase and DPP8/9 may help in selecting substituents that disfavour interaction with substrate recognition pockets of these proteases.

As shown in this paper, the 4-oxo-β-lactam acts as a covalent inhibitor, which is sufficiently stable over time to allow detection of the covalent complex by ABPP. Although we here used a small propargyl biorthogonal tag, it seems that the substituent on the piperazine ring is exposed to solvent or membrane, and therefore may be functionalized with larger tags, such as biotin or fluorophores as well. This scaffold may therefore be a valuable starting point for the development of novel ABPs for rhomboid proteases.

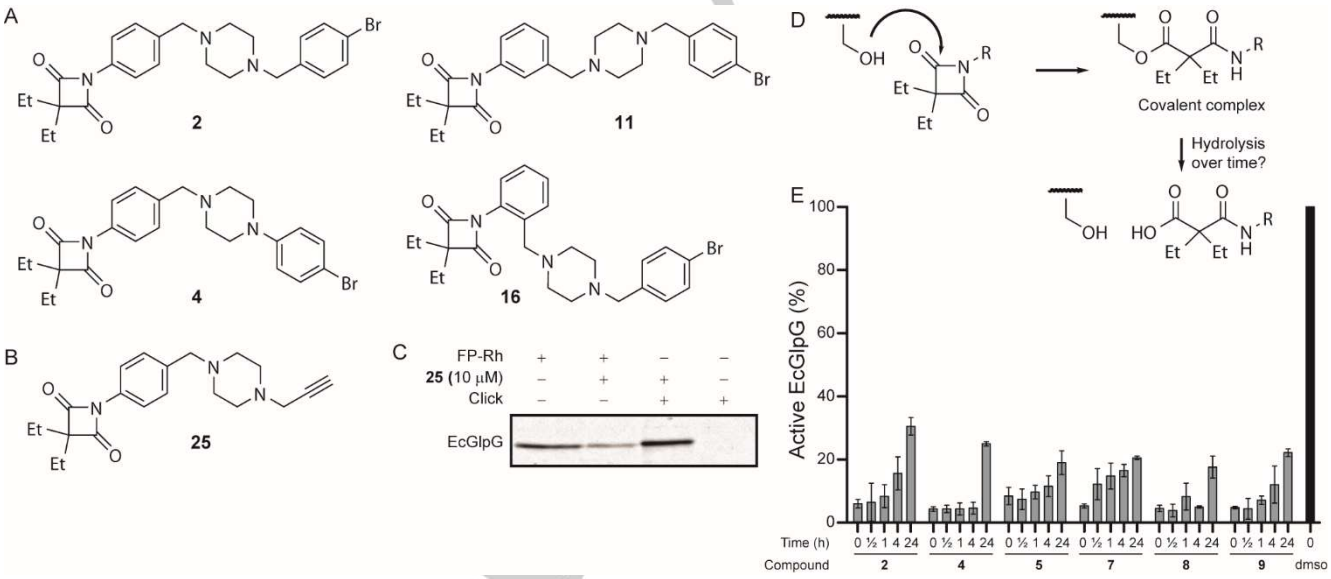

Figure 3. (A) Structures of some analogous compounds with different activities. (B) Structure of alkyne-conjugated oxo-β-lactam **25**. (C) ABPP with 2-step click chemistry detection of the covalent complex of EcGlpG-**25** (uncropped gel in Figure S6). (D) Mechanism of covalent inhibition and hydrolysis of the acyl-bound inhibitor. (E) Plot of the activity of EcGlpG at several time points after removal of the indicated inhibitor and detection of the residually active GlpG by labeling with the ABP FP-Rh followed by in-gel fluorescence quantification. These experiments reveal very slow hydrolysis rate of the acyl intermediate. Values represent mean and standard deviation of two replicates.

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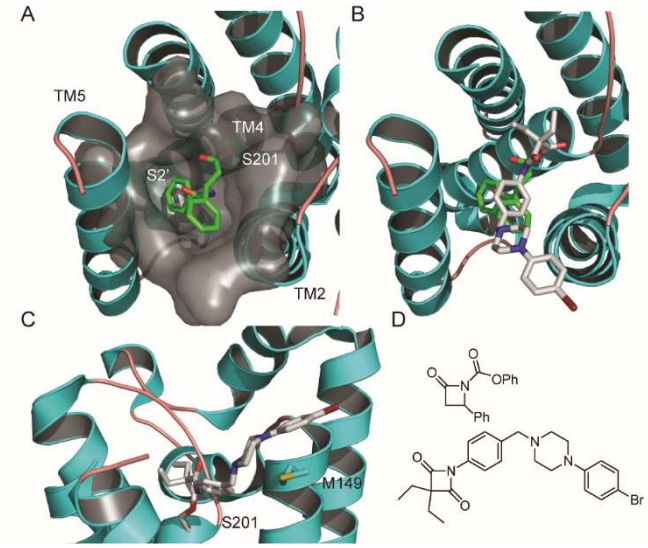

Figure 4. Covalent docking of compound **4** into EcGlpG. Protein is depicted in cartoon mode; helices are colored in cyan, random coil in pink. Small molecules and some selected residues are in stick model. Pictures rendered with PyMol.[33] (A) Close-up of the active site of GlpG of a reported crystal structure (PDB code: 3ZMI),[18] viewed from the top, with a lactam bound to active site serine S201, which is located at the top of helix TM4. Note that the phenoxy substituent interacts with the S2' pocket. The phenyl substituent of the lactam inhibitor locates between helices TM2 and TM5. Lactam structure given in panel D. (B) Overlay of the lactam inhibitor with the covalent docking pose of compound **4**. Note that the phenyl ring connected to the oxolactam nitrogen takes a similar position as the phenyl ring on the lactam inhibitor in the crystal structure. (C) Side view of the docked structure with indication of M149, which may hydrophobically interact with the bromophenyl substituent. (D) Chemical structures of the lactam inhibitor and the oxolactam inhibitor **4**.

Experimental Section

Compound screening. Purified recombinant *E.coli* GlpG[13] (500 nM) in 50 μL reaction buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 0.1 mM NaCl, with 10% (v/v) glycerol and 0.05% (w/v) DDM) was incubated for 1 h at room temperature with 100 μM compound or 1% DMSOvehicle. Next, 50 μL of a 100 μM solution of the quenched fluorescent substrate Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2 trifluoroacetate salt was added at 37 °C. Progress curves were immediately read for 50 minutes using the SpectraMax iD3 multi-mode plate reader (Molecular Devices) with an excitation wavelength of 320 nm and an emission wavelength of 400 nm. Protease activity was calculated from the slope of the linear part of the curve. The DMSO negative control was defined as 100% activity, and any negative slope was considered as no activity.

Apparent IC_{50} determination by ABPP. Purified recombinant *E.coli* GlpG[13] was diluted into 30 μL of reaction buffer as described in the previous section, and incubated for 1 h at room temperature with a series of different compound concentrations (0.1-100 μM). 7-amino-3-butynoxy-4-chloro-isocoumarin was used as a positive control[12] and an equal volume of DMSO (1% v/v) was used as a negative control. Residual GlpG activity was measured by adding FP-Rh to a final concentration of 1 μM with a 1h incubation time. The reaction was stopped by addition of 4× Laemmli buffer and 20 μL of the reaction mixture was resolved by 12% SDS-PAGE. Gels were scanned on a Typhoon FLA 9500. Residual rhomboid activity was quantified by gel band densitometry using ImageJ[34]. IC_{50} was calculated with GraphPad Prism after normalization of the data (the DMSO negative control was defined as 100% activity).

Covalent docking. The 3D protein structure of *E. coli* GlpG in complex with monobactam L29 was downloaded from the RCSB Protein Data Bank (PDB code 3ZMI). Waters, ions and detergents were removed using PyMol. The complex was converted into a.pdbqt file and missing hydrogens of residues were added using AutoDockFR suite 1.0.[32] A target file (.trg) was generated by specifying the docking box (center: Ser201, padding size: 10,0 x 10.0 x 10.0) and covalent docking atoms (carbon and oxygen of Ser201 residue) using AutoGridFR 1.0. The 3D structures of the ring-opened forms of monobactam L29 and 4-oxo-β-lactam inhibitor **4**, with the partial serine residue attached, were generated as .mol2 file using ChemOffice 3D (2019). The .mol2 file of ligands were converted into .pdbqt and the root atom series number was reset at the overlapped atom using AutoDockFR suite (1.0). 15 independent searches of covalent docking (each using up to 5 million evaluations of the scoring function) were performed by submitting the target file (.trg) and ligand file (.pdbqt) to AutoDockFR suite (1.0). The covalent docking output was analysed using PyMol.[33]

Gel-based ABPP. *E. coli* GlpG was diluted into 30 μL of reaction buffer as described above, and incubated at room temperature with compound **25** (10 uM) or 1% DMSO for 1h. Next, FP-Rh was added to a final concentration of 1 uM or the sample was subjected to click chemistry for 1h with TAMRA-azide (final concentration 50 μM), 1 mM $CuSO_4$, 1 mM sodium ascorbate and 50 μM ligand (THPTA). Reactions were stopped by addition of 4× Laemmli buffer. Samples were resolved by 12% SDS-PAGE and gels were scanned on a Typhoon FLA 9500.

Reversibility test. *E. coli* GlpG was diluted into 30 μL of reaction buffer as described above, and incubated for 1h at room temperature with compound to be tested (100 μM), JLK-6 (100 μM; positive control) or an equal volume of DMSO (as negative control). Excess compound was removed from the solution by a ZebaTM spin desalting column (7K MWCO, 0.5 mL), which was equilibrated with the rhomboid reaction buffer. The flow through was collected and incubated for various times (0 h, 0.5 h, 1 h, 4h). Next, FP-Rh was added to a final concentration of 1 μM and incubated for 1h at room temperature in the dark. 4× Laemmli buffer was added to stop the reaction. 20 μL of the reaction mix were resolved by 12% SDS-PAGE. Gels were scanned on a Typhoon FLA 9500. The reaction was stopped by addition of 4× Laemmli buffer and 20 μL of the reaction mixture was resolved by 12% SDS-PAGE. Gels were scanned on a Typhoon FLA 9500. Residual rhomboid activity was quantified by gel band densitometry using ImageJ[34] (the DMSO negative control was defined as 100% activity).

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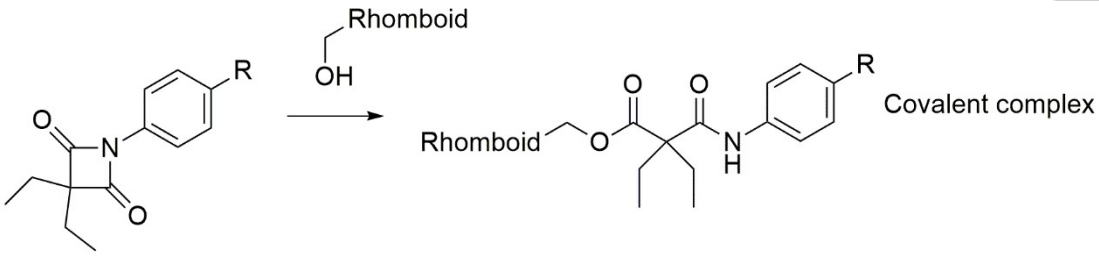

Rhomboid proteases are serine proteases that reside inside the lipid bilayer of a membrane. They are implicated in several diseases. We here report 4-oxo-β-lactam as a new scaffold for covalent rhomboid inhibitors and activity-based probes.

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