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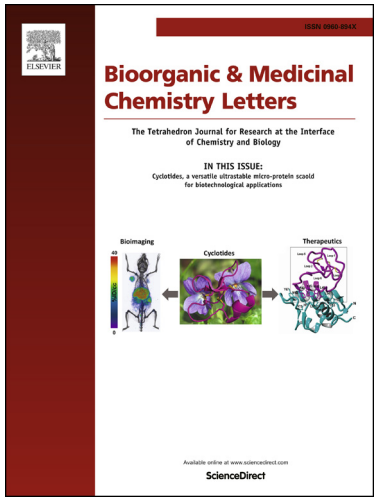

Discovery and validation of 2-styryl substituted benzoxazin-4-ones as a novel scaffold for rhomboid protease inhibitors

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PII: S0960-894X(18)30106-9
DOI: https://doi.org/10.1016/j.bmcl.2018.02.017
Reference: BMCL 25613

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 14 November 2017
Revised Date: 5 February 2018
Accepted Date: 8 February 2018

Please cite this article as: Goel, P., Jumpertz, T., Tichá, A., Ogorek, I., Mikles, D.C., Hubalek, M., Pietrzik, C.U., Strisovsky, K., Schmidt, B., Weggen, S., Discovery and validation of 2-styryl substituted benzoxazin-4-ones as a novel scaffold for rhomboid protease inhibitors, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.02.017

Bioorganic & Medicinal Chemistry Letters

Discovery and validation of 2-styryl substituted benzoxazin-4-ones as a novel scaffold for rhomboid protease inhibitors

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Keywords: rhomboid proteases, intramembrane proteases, benzoxazinones, molecular docking, inhibition

ABSTRACT

Rhomboids are intramembrane serine proteases with diverse physiological functions in organisms ranging from archaea to humans. Crystal structures analysis has provided a detailed understanding of the catalytic mechanism, and rhomboids have been implicated in various disease contexts. Unfortunately, the design of specific rhomboid inhibitors has lagged behind, and previously described small molecule inhibitors displayed insufficient potency and/or selectivity. Using a computer-aided approach, we focused on the discovery of novel scaffolds with reduced liabilities and the possibility for broad structural variations. Docking studies with the *E.coli* rhomboid GlpG indicated that 2-styryl substituted benzoxazinones might comprise novel rhomboid inhibitors. Protease *in vitro* assays confirmed activity of 2-styryl substituted benzoxazinones against GlpG but not against the soluble serine protease α-chymotrypsin. Furthermore, mass spectrometry analysis demonstrated covalent modification of the catalytic residue Ser201, corroborating the predicted mechanism of inhibition and the formation of an acyl enzyme intermediate. In conclusion, 2-styryl substituted benzoxazinones are a novel rhomboid inhibitor scaffold with ample opportunity for optimization.

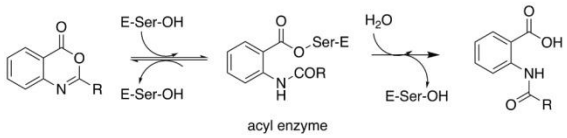
RESULTS AND DISCUSSION

Rhomboids are intramembrane serine proteases present in prokaryotic, archaeal and eukaryotic organisms [1]. In 2001, the first rhomboid was discovered in *Drosophila* and shown to perform a critical proteolysis step in EGF-receptor signaling [2, 3]. Since then, rhomboids have been implicated in a wide range of biological processes including bacterial quorum sensing [4], mitochondrial dynamics and integrity [5, 6], and protein quality control [7]. In addition, rhomboids have been identified as putative drug targets in the context of multiple diseases [8] such as cancer [9], diabetes [10, 11], parasitic diseases [12, 13], and Parkinson's disease [5]. The crystal structures of rhomboids from *E. coli* and *H. influenzae* have been solved and revealed that rhomboids are serine-histidine dyad proteases composed of 6 core transmembrane helices, which form a V-shaped cavity and expose the active site to a partially hydrophilic environment [14, 15]. These structures together with numerous biochemical studies have provided a detailed understanding of the catalytic mechanism of rhomboid proteases [16], but this has not yet translated into the development of potent, selective and drug-like inhibitors [17]. Through different strategies, from the testing of candidate molecules to rational synthesis to the screening of small molecule libraries, isocoumarins [3, 18, 19], fluorophosphonates [20], β-lactams [21], and β-lactones [22] were found to be effective against rhomboids, but these inhibitors generally displayed low potency and/or insufficient selectivity [18, 20, 21]. Effectively, inherent liabilities as exemplified by the high reactivity of isocoumarins likely preclude or limit further development of these compound classes [23, 24].

Accordingly, using a computer-aided candidate approach, we focused on the discovery of novel scaffolds with reduced liabilities and the possibility for broad structural variations. One scaffold we selected was 2-substituted derivatives of 4*H*-3,1-benzoxazin-4-ones, which were previously used as heterocyclic acylating agents against serine proteases such as HLE, α-chymotrypsin, and cathepsin G [23, 25-28]. The mechanism of inhibition involves the formation of an O-acyl enzyme intermediate. The nucleophilic serine reacts with the C-4 carbonyl of the benzoxazinone, which results in opening of the heterocyclic ring and formation of the O-acyl enzyme intermediate (Fig. 1A) [23]. The enzyme selectivity and potency of acylating agents is promoted by fast acylation and slow deacylation, which is dependent on the substitution of the aromatic ring and the C-2 position in case of benzoxazinones [25, 29, 30]. A major advantage of benzoxazin-4-ones is that the core structure consists of two fused aromatic rings, which allows extensive structural modifications and optimization with respect to the target enzyme. For initial docking studies into the rhomboid active site, we assembled a molecular database of thirteen 2-alkyl or 2-aryl substituted benzoxazinones (Fig. 1B).

FIGURE 1

A



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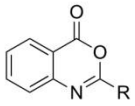
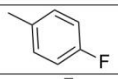
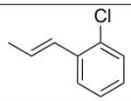
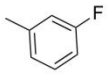
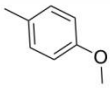
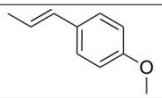
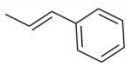
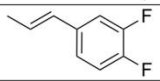
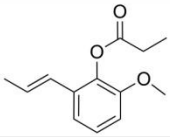
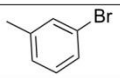


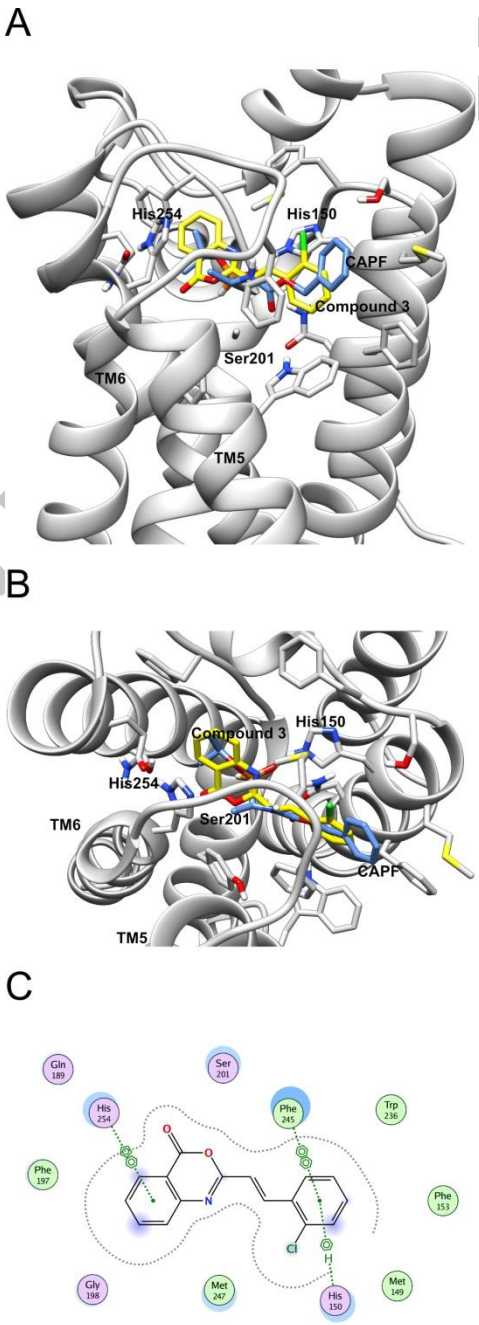
R = alkyl or aryl group

Compd	-R	Compd	-R
1		**8**	
2	$-CH_3$	**9**	
3		**10**	
4		**11**	
5		**12**	
6		**13**	
7			

In the docking studies, we focused on the initial interactions between the benzoxazinones and the active site of the rhomboid protease rather than the final reaction product. For preparation of the docking receptor, we used the co-crystal structure of the *E.coli* rhomboid GlpG and the fluorophosphonate inhibitor CAPF (PDB ID: 3UUB), in which the active site Ser201 is covalently bound to CAPF [31]. The molecular modelling experiments were performed in the molecular operating environment software (MOE) with the DOCK module and the MMFF94x force field, and scored by London dG and Affinity dG followed by energy minimization within the enzyme active site cleft [32, 33]. The output data was ranked based on the calculated ligand efficiencies (cLE = docking score / number of heavy atoms) [34], which revealed that the 2-styryl substituted compound **3** was the most favorable of all 2-substituted benzoxazinones (cLE=-0.3164). A comparative analysis of the protein/ligand docking results

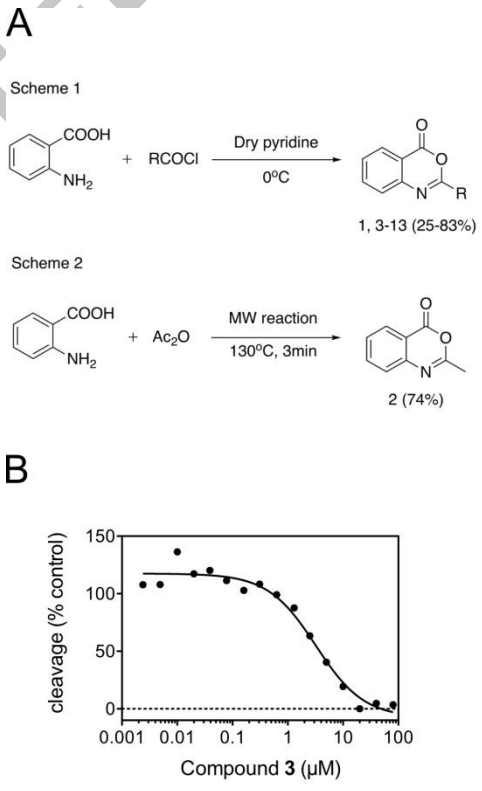
of compound **3** and CAPF indicated that **3** was adequately fitting into the binding pocket of the enzyme and was not exposed to the external environment (Fig. 2A and B). The core heterocyclic ring of **3** was oriented towards the S1 subsite while the 2-styryl extension pointed towards the S2' subsite of the rhomboid, which had been defined in previous structures of GlpG in complex with different inhibitors [31, 35]. Moreover, close interactions of **3** with the neighbouring residues His254 and Phe245 as shown in the ligand interaction map were observed and suggested to further explore the scaffold (Fig. 2C).

FIGURE 2



To validate the docking results, all derivatives listed in Table 1 were synthesized by methods shown in schemes 1 and 2 (Fig 3A) [36]. The benzoxazinone derivatives were then evaluated for their inhibitory potency in an established *in vitro* enzyme activity assay with the *E.coli* rhomboid GlpG and the transmembrane domain 2 of the *Drosophila* protein Gurken as a substrate [21, 37, 38, 39]. Each of the benzoxazinones were pre-incubated with GlpG at a single concentration of 250 μM for 30 min at 37°C. Subsequently, the Gurken substrate was added, the reaction was continued for another 90 min at 37°C, and the N-terminal Gurken substrate cleavage fragment was visualized by SDS-PAGE and quantified using ImageJ. Only the 2-styryl substituted benzoxazinones **3**, **5** and **11** showed activity at this concentration. For IC_{50} determinations, fluorogenic rhomboid substrates were applied as described previously [40, 41, 42]. Correlating well with the docking results, compound **3** was a potent rhomboid inhibitor with an IC_{50} value of 4.4 ± 1.6 μM (Figure 3B). Compound **5** was equally potent (IC_{50} 3.7 ± 1.3 μM) while **11** displayed around 10-fold lower activity (IC_{50} 48 ± 14.1 μM). Among these three compounds with a single substitution at the aromatic ring of the styryl substituent, an electron withdrawing group appeared to increase the potency, which could be further explored in subsequent studies.

FIGURE 3



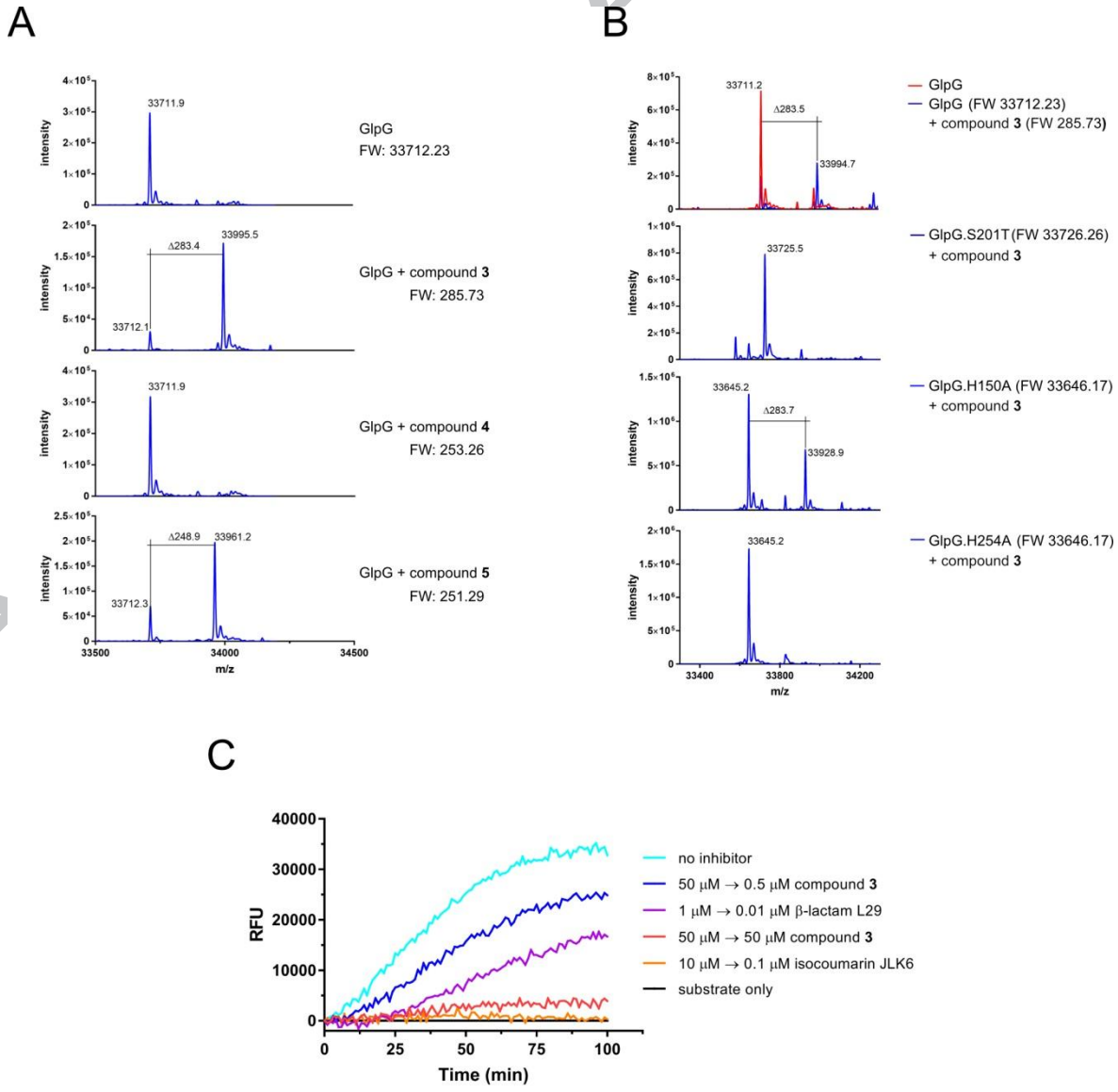
Next, we evaluated the active benzoxazinones in a well-established *in vitro* activity assay

for the soluble serine protease α-chymotrypsin [43,44]. The compounds were pre-incubated with bovine α-chymotrypsin for 30 min at 25°C. Subsequently the substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide was added, which under alkaline conditions is turned over by α-chymotrypsin to p-nitroanilline, a yellow compound that can be detected spectroscopically at 410 nm. DCI was used as a positive control and inhibited α-chymotrypsin with an IC_{50} value of 3.5 μM, comparable to previously reported values [21]. In contrast, the benzoxazinones **3** and **5** did not display any inhibitory activity in the α-chymotrypsin *in vitro* activity assay at the highest concentration of 250 μM (data not shown). In addition, we examined the active benzoxazinones in analogous *in vitro* protease activity assays for bovine trypsin, human neutrophil elastase, and human cathepsin G [45]. At a concentration of 10 μM, none of the benzoxazinones inhibited trypsin or neutrophil elastase, while DCI almost completely abolished the activity of both enzymes. At a concentration of 50 μM, trypsin activity was reduced around 50% by compound **3**, and neutrophil elastase activity was reduced around 50% by compound **11** (Supplemental Figures 1 and 2). In contrast, the compounds were more effective against cathepsin G. Both **3** and **5** but not **11** caused significant enzyme inhibition at 10 μM (Supplemental Figure 3). Overall, these results indicated that 2-styryl substituted benzoxazinones might possess some selectivity for the rhomboid GlpG over other soluble serine proteases. Notably, in an accompanying paper, Yang, Verhelst and colleagues presented benzoxazinones with a 2-alkoxy substituent as rhomboid inhibitors [46]. These derivatives displayed slightly higher potency for GlpG but no apparent selectivity over bovine chymotrypsin and trypsin, suggesting potential advantages of the docking approach to optimize the scaffold for this particular target.

The inhibitory activity of 2-styryl substituted benzoxazinones in the GlpG *in vitro* activity assay indicated that this scaffold contained active rhomboid inhibitors. However, to ensure that the reduced GlpG activity was not caused by non-specific effects and that the mechanism of inhibition conformed to the known reaction mechanism of benzoxazinones with soluble serine proteases [23], we used mass spectrometry to study the residues in the rhomboid's active site that were reacting with the benzoxazinones. GlpG mutants were generated, expressed and purified, in which Ser201 was exchanged to threonine (S201T) or in which His150 or His254 were exchanged to alanine. The recombinant proteins were then reacted *in vitro* with the 2-styryl substituted benzoxazinones **3** or **5**, and binding of the compounds to the rhomboid was examined by electrospray mass spectrometry. When the 2-styryl substituted benzoxazinones were reacted with wild type GlpG, two major peaks were observed in the spectra: the first smaller peak corresponding to the free unbound GlpG and the second major peak corresponding to the inhibitor-bound enzyme (Fig. 4A). Importantly, the shifts in the rhomboid's mass for compounds **3** (283.4 Da) and **5** (248.9 Da) were close to the theoretical mass differences of 285.73 and 251.29 Da that would occur when the

benzoxazinones reacted with the enzyme according to the mechanism shown in Figure 1. In contrast, no binding and mass shift was seen when the compounds were omitted (vehicle control) or when the rhomboid was reacted with the inactive benzoxazinone compound **4**, supporting the specificity of the mass spectrometry analysis (Fig. 4A). Further analysis showed that compound **3** was not able to bind to either the S201T or the H254A mutant (Fig. 4A). This indicated that the benzoxazinone indeed reacted with the catalytic Ser201 to form an O-acyl enzyme intermediate (Fig. 4B). In the catalytic Ser201-His254 dyad, the histidine is required to properly activate the serine [16]. Hence, in the H254A mutant the serine is likely not nucleophilic enough to react with the C-4 carbonyl of the benzoxazinone, which could explain that **3** failed to bind this mutant. However, mutating His150, which contributes to the oxyanion hole of the active site and is covalently modified by some isocoumarins [19, 47], did not prevent binding of **3** to the rhomboid as expected (Fig. 4B).

FIGURE 4



Formation of the O-acyl enzyme intermediate should be reversible over time (Fig. 1). To test this, we used the rapid dilution method [48,49] and a previously described fluorogenic peptidic rhomboid substrate [40]. Compound **3** (50 μM) was pre-incubated with GlpG for 1 h. Subsequently, the reaction mixture was rapidly diluted 100-fold and the substrate KSp76 was added. Cleavage of this substrate by the rhomboid leads to the release of a quencher peptide and activation of a red fluorophore, which indicates recovery of enzyme activity. This showed that GlpG activity largely recovered over a time period of 100 min demonstrating reversibility of the inhibition mechanism (Fig. 4C). Similar results were obtained with the β-lactam L29, a known reversible inhibitor of rhomboids [21]. Conversely, pre-incubation with the isocoumarin JLK-6, an irreversible inhibitor that forms a double-bonded end product with the rhomboid [19], did not allow recovery of enzyme activity (Fig. 4C).

In summary, by combining molecular docking studies with enzyme activity assays, we found that 2-styryl substituted benzoxazin-4-ones are a novel template to generate inhibitors for rhomboid proteases. Mechanistic studies indicated that benzoxazinone inhibitors covalently modified the catalytic serine residue in the rhomboid active site, analogous to the reaction mechanism of benzoxazinones with soluble serine proteases. While the identified active 2-styryl substituted benzoxazinones at present have moderate potency, this new scaffold allows extensive structural variations with considerable potential to increase potency and specificity, towards the goal of improved small molecule inhibitors for rhomboid proteases.

ACKNOWLEGEMENTS

We thank Professors Matthew Freeman (University of Oxford) and Ya Ha (Yale School of Medcine) for bacterial expression constructs. P.G. was supported by a scholarship of the iGRASPseed graduate school of the Heinrich-Heine-University Duesseldorf. K.S. was a recipient of the Purkyne Fellowship of the Academy of Sciences of the Czech Republic and also acknowledges support from EMBO (Installation Grant no. 2329), Ministry of Education, Youth and Sports of the Czech Republic (projects no. LK11206 and LO1302), Marie Curie Career Integration Grant (project no. 304154), and the National Subvention for Development of Research Organisations (RVO: 61388963) to the Institute of Organic Chemistry and Biochemistry.

CONFLICTS OF INTEREST: none

FIGURE LEGENDS

Figure 1. (A) Mechanism of inhibition of soluble serine proteases by 2-substituted benzoxazin-4-one derivatives [30]. (B) Molecular database of 2-alkyl or aryl substituted benzoxazin-4-ones assembled for docking studies into the rhomboid active site.

Figure 2. Molecular docking model of the rhomboid protease GlpG in complex with a benzoxazinone-based inhibitor. (A) Front view of GlpG docked with the 2-styryl substituted compound **3** (yellow) and the known rhomboid inhibitor CAPF (blue). The model indicated that both compounds occupied a similar space in the rhomboid pocket and were not exposed to the external surface. (B) Top view of compound **3** and CAPF in the GlpG pocket. (C) Ligand interaction map of compound **3** in the GlpG pocket. Significant interactions were observed with neighbouring residues His254 and Phe245. Green: hydrophobic, pink: polar, red: exposed.

Figure 3. (A) Synthesis of 2-substituted benzoxazin-4-ones. Scheme 1 shows the synthesis of 2-alkyl/aryl substituted benzoxazinones (compounds **1** and **3**-**13**) obtained by the reaction of corresponding acyl/benzoyl chlorides with anthranilic acid. Scheme 2 shows the synthesis of 2-methyl benzoxazin-4-one (compound **2**) obtained by a microwave reaction of anthranilic acid with acetic anhydride. (B) Biological evaluation of 2-alkyl and 2-aryl substituted benzoxazinones inhibitors in rhomboid *in vitro* activity assays. All compounds were first screened in a gel-based activity assay for the *E.coli* rhomboid GlpG at a single concentration of 250 μM as described [39]. Only the 2-aryl substituted benzoxazinones were found to be active at this concentration and were selected for IC_{50} determinations. In these assays, the rhomboid GlpG was pre-incubated with increasing concentrations of the compounds **3**, **5** and **11** for 1 h at 37°C. Subsequently, the reaction was started by the addition of fluorogenic substrates [40]. These quenched fluorescent peptides are cleaved by the rhomboid, leading to the activation of a fluorophore. Fluorescence intensities were normalized to the DMSO control condition and plotted against log (inhibitor concentration) in GraphPad Prism software. The figure shows a representative dose-response curve for compound **3**. A nonlinear regression curve fit was used to determine apparent IC_{50} values as described [40]. Compounds **3** (4.4 ± 1.6 μM) and **5** (3.7 ± 1.3 μM) were equally potent while **11** (48 ± 14.1 μM) displayed around 10-fold lower activity. Two independent IC_{50} determinations were performed for each compound, and IC_{50} values represent averages ± SD.

Figure 4. Reaction mechanism of 2-styryl substituted benzoxazinone inhibitors. (A) Wild type GlpG was recombinantly expressed and purified as described previously [40], reacted *in vitro*

with compounds 3 or 5, and binding to the rhomboid was examined by electrospray mass spectrometry. A second major peak was observed in the spectra corresponding to the inhibitor-bound enzyme with the expected mass shift compared to the free enzyme. In contrast, no binding was seen when the compounds were omitted (vehicle control) or when the rhomboid was reacted with the inactive benzoxazinone 4. The results of one of two independent experiments are shown. (B) The 2-styryl substituted benzoxazinone 3 was incubated with different rhomboid mutants and binding was analyzed as in A. Compound 3 was able to bind to wild type GlpG and the H150A mutant with identical mass shifts between the inhibitor-bound and the free enzyme. However, no binding was observed to either the S201T or the H254A mutant. This indicated that Ser201 and proper activation of this catalytic serine by His254 was required for binding of the benzoxazinone to the rhomboid and formation of the O-acyl enzyme intermediate. The results of one of two independent experiments are shown. (C) Reversibility of the reaction was tested by the rapid dilution method. Compound 3 (50 μM) was pre-incubated with the rhomboid GlpG for 1 h. Subsequently, the reaction mixture was rapidly diluted 100-fold with reaction buffer containing a fluorogenic rhomboid substrate (10 μM). GlpG activity largely recovered over a time period of 120 min demonstrating reversibility of the inhibition mechanism. No recovery was observed when the reaction mixture was diluted into buffer containing substrate and 50 μM compound 3. The known reversible and irreversible rhomboid inhibitors β-lactam L29 and isocoumarin JLK-6 were used as controls and displayed the expected behaviour. The results of one of two independent experiments are shown.

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38. Bacterial expression constructs for N-terminally His-tagged GlpG and C-terminally His-tagged Gurken chimeric substrate (MBP-GurkenTMD-Trx-His6) were kind gifts of Ya Ha (Yale School of Medicine) and Matthew Freeman (University of Oxford). Both enzyme and substrate were expressed in E. coli C43(DE3) cells. After induction with IPTG, GlpG was expressed overnight at 18°C, while Gurken was expressed for 3h at 37°C. The bacterial cells were broken with a nitrogen cavitation bomb, membranes were isolated by differential centrifugation and proteins were solubilized with 1.5% dodecyl-beta-maltoside (DDM, Glycon Biochemicals). GlpG and Gurken were purified via immobilized metal-ion affinity chromatography using a Talon®Crude 1 ml HiTrap column and an Akta prime plus chromatography system (GE Healthcare). The recombinant proteins were further concentrated using 10 or 30 kDa molecular weight

cut-off Amicon® Ultra Centrifugal filters (Millipore). Protein purity was estimated by Coomassie Brilliant Blue (Merck) stained SDS-PAGE. Enzyme activity assays were performed in 20 µl reaction buffer [50 mM HEPES-NaOH (pH 7.5); 5 mM EDTA; 0.4 M NaCl; 10% Glycerol (v/v); 0.05% DDM] containing GlpG (0.35 µM) and Gurken substrate (1.8 µM). Benzoxazinone inhibitors were pre-incubated with GlpG in reaction buffer for 30 min at 37°C with gentle shaking. The substrate was added and the reaction was continued for another 90 min at 37°C. Subsequently, the reaction mixture was separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The N-terminal Gurken cleavage fragment was quantified using ImageJ, normalized to the DMSO vehicle control condition, and the values were plotted in GraphPad Prism software.

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42. Rhomboid inhibition assays were performed in black 96-well plates. Increasing concentrations of the 2-styryl substituted benzoxazinones **3**, **5**, or **11** were pre-incubated with 0.4 µM GlpG in reaction buffer [20 mM HEPES-NaOH (pH 7.4); 150 mM NaCl; and 0.05% (w/v) DDM] for 1 h. Subsequently, the reaction was started by the addition of 10 µM of the fluorogenic substrates KSp35 (for compounds 5 and 11) or KSp76 (for compound 3). Both substrates have comparable kinetic properties and are interchangeable for IC_{50} determinations. IC_{50} values were determined as described previously [40].
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44. The ability of the benzoxazinone derivatives to inhibit the classical serine protease α-chymotrypsin was determined in an *in vitro* activity assay. The compounds were pre-incubated in concentrations up to 250 µM with 4 µM of bovine α-chymotrypsin (PanReac AppliChem) in reaction buffer [50 mM HEPES-NaOH (pH 7.5); 5 mM EDTA; 0.4 M NaCl; 10 % Glycerol (v/v); 10 % DMSO] for 30 min at 25°C. Subsequently, 2.5 µl of this mixture were added to 100 µl reaction buffer containing 0.12 mM of the substrate N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Suc-AAPF-pNA, Merck). Cleavage of the substrate by α-chymotrypsin resulted in 4-nitroaniline, which is yellow under alkaline conditions. A microtiter plate reader (Beckman Coulter) was used to record the increase in absorbance at 410 nm over 5 min at 25°C. IC_{50} values were determined by fitting a nonlinear regression curve to a plot of log (compound concentration) versus % α-chymotrypsin activity using GraphPad Prism software.
45. Enzyme *in vitro* activity assays were used to determine the inhibitory activity of the 2-styryl substituted benzoxazinones against the soluble serine proteases trypsin, neutrophil elastase and cathepsin G. In the trypsin assay, increasing concentrations of the benzoxazinones or DMSO vehicle were pre-incubated with 10 nM trypsin from bovine pancreas (Sigma-Aldrich) for 30 min at 30°C in a total volume of 90 µl assay buffer (PBS). The reaction was started by the addition of 10 µl substrate solution (1 mM Z-Gly-Gly-Arg-aminomethylcoumarin in PBS, Bachem), and the release of the fluorescent cleavage product aminomethylcoumarin was monitored at 340 nm excitation/ 440 nm emission with a microplate reader (Tecan Safire). In the cathepsin G assay, increasing concentrations of the compounds or DMSO vehicle were pre-incubated with 200 nM human neutrophil cathepsin G (Enzo Life Sciences) for 30 min at 37°C in a total volume of 90 µl assay buffer [100 mM HEPES; 500 mM NaCl (pH 7.4)]. The reaction was started by the addition of 10 µl substrate solution (2 mM N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide in assay buffer, Bachem). Enzymatic

cleavage of the substrate resulted in the release of the yellow coloured p-nitroaniline, and absorbance was recorded at 410 nm. To measure the activity of the benzoxazinones against human neutrophil elastase, the Neutrophil Elastase Colorimetric Drug Discovery Kit (Enzo Life Sciences) was used according to the manufacturer`s protocol. In brief, increasing concentrations of the benzoxazinones or DMSO vehicle were pre-incubated with 0.22 mU neutrophil elastase for 20 min at 37°C in a total volume of 95 µl assay buffer [100 mM HEPES (pH 7.25); 500 mM NaCl; 0.05 % Tween-20]. The reaction was started by the addition of 5 µl substrate solution (2 mM N-Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilid in assay buffer), and the formation of p-nitroaniline was recorded as described for the cathepsin G assay. In all three assays, changes in absorbance/fluorescence were measured at 1 min time intervals for 5 min, and the initial reaction slopes were used to determine the reaction velocity (V). To calculate the % remaining protease activity, the formula (V inhibitor/ V DMSO control) x 100 was used.

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49. Rhomboid activity recovery assays were performed in black 96-well plates. Compound 3 (100 μM), β-lactam L29 (1 μM) or isocoumarin JLK-6 (10 μM) were pre-incubated for 1 h with 0.4 μM GlpG in reaction buffer [20 mM HEPES-NaOH (pH 7.4); 150 mM NaCl; 0.05% (w/v) DDM]. Afterwards, this solution was rapidly diluted 100-fold into reaction buffer containing 10 μM of the fluorogenic substrate KSp76. This quenched fluorescent peptide is cleaved by the rhomboid leading to activation of a red fluorophore. Substrate cleavage and enzyme activity recovery were monitored continuously by measuring fluorescence over a time period of 120 min with excitation at 553 nm and emission at 583 nm using a microplate reader (Tecan Infinite M1000).