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Discovery of achiral inhibitors of the hepatitis C virus NS3 protease based on 2(1*H*)-pyrazinones

Pernilla Örtqvist [a], Johan Gising [a], Angelica E. Ehrenberg [b], Aparna Vema [a], Anneli Borg [a], Anders Karlén [a], Mats Larhed [a], U. Helena Danielson [b], Anja Sandström [a,*]

[a] Department of Medicinal Chemistry, Organic Pharmaceutical Chemistry, Uppsala University, BMC, Box 574, SE-751 23 Uppsala, Sweden
[b] Department of Biochemisty and Organic Chemistry, Uppsala University, BMC, Box 576, SE-751 23 Uppsala, Sweden

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ABSTRACT

Herein, the design, synthesis and inhibitory potency of a series of novel hepatitis C virus (HCV) NS3 protease inhibitors are presented. These inhibitors are based on a 2(1*H*)-pyrazinone P3 scaffold in combination with either a P2 phenylglycine or a glycine, and they were evaluated on the wild type as well as on two resistant variants of the enzyme, A156T and D168V. Molecular modelling suggested that the aromatic side-chain of the P2 phenylglycine occupies the same space as the substituent in position 6 on the pyrazinone core. The versatile synthetic route applied for the pyrazinone synthesis made a switch between the two positions easily feasible, resulting in phenyl- or benzyl substituted pyrazinones and leaving glycine as the P2 residue. Of several P1–P1′ residues evaluated, an aromatic P1–P1′ scaffold was found superior in combination with the new P3–P2 building block. As a result, an entirely new type of achiral and rigidified inhibitors was discovered, with the best of the novel inhibitors having fourfold improved potency compared to the corresponding tripeptide lead. We consider these achiral inhibitors highly suitable as starting points for further optimization.

1. Introduction

With estimates of the total number infected ranging from 123 million[1] to 170 million[2] (i.e., 2–3% of the global population), hepatitis C virus (HCV) infection poses a considerable threat to the public health. Ultimately, the infection can cause fatal liver disease such as cirrhosis or cancer. The main transmission route is via percutaneous contact with contaminated blood, for example, from shared needles among injection drug abusers or transfusion of unscreened blood.[2] Today, the only available therapy consists of the broad-spectrum antiviral ribavirin in combination with pegylated interferon-α. In addition to draw-backs in the form of serious adverse effects, the efficacy of the current therapy is also dependent on the genotype of the virus. Best results are achieved in treatment of genotype 2 and 3, with sustained virological response in up to 80% of the cases.[2] The corresponding numbers for genotype 1 is 40–50%.[2] Potent and HCV specific drugs are therefore urgently needed.

One of the most studied potential HCV drug targets is the protease part of the non-structural (NS) protein 3, a bifunctional protein that also includes a helicase domain. Until today, several inhibitors have made it to clinical trials, of which the most advanced are shown in Figure 1.[3–6] One of these, ciluprevir, was however withdrawn due to cardiac toxicity observed in rhesus monkeys.[7]

Drug resistance will be an important issue in future anti-HCV therapy. The viral RNA-polymerase lacks a proof-reading function, which results in a high mutation rate and concomitant rapid emergence of resistant virus strains. Data from in vitro (enzyme assays and cell-based replicon assays)[8–10] as well as from the clinical studies,[11,12] point out three positions close to the active site where resistance-conferring substitutions occur: R155, A156 and D168. These positions are also in close proximity to the P2 residue, and the P2 has indeed been pointed out as a major resistance determinant.[13,14] The inhibitors in Figure 1 are affected negatively by one or several of these substitutions[10,15] (no resistance data available for TMC 435350 as yet), and they share one feature: the substituted P2 proline, or a proline mimic in the case of TMC 435350.

We have previously presented phenylglycine as an interesting alternative to proline in the P2 position, albeit with room for optimization.[16] Since the phenylglycine induces a different conformation in the P2–P1 area, inhibitors based on it are potentially less affected by mutants developed in the presence of proline-based inhibitors. To test the hypothesis, we included a phenylglycine-based inhibitor in an in vitro assay using the A156T and D168A NS3 mutants. There, it showed an interesting activity profile,[17] and inspired continued efforts aiming at the identification of non-proline P2 residues.

* Corresponding author.
E-mail address: Anja.Sandstrom@orgfarm.uu.se (A. Sandström).

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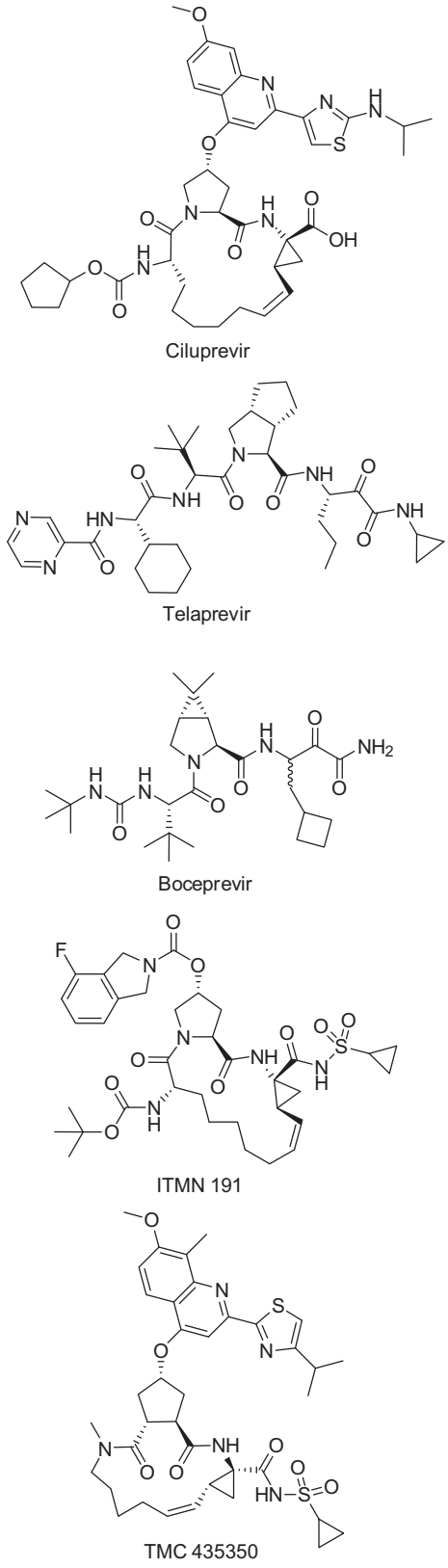


Figure 1. Examples of clinically evaluated inhibitors of the hepatitis C virus NS3 protease.

One option for increasing the inhibitory potency of a certain inhibitor is to preorganize it for binding to its biological target by incorporating a structural motif that locks the inhibitor in the optimal conformation. Since proteins and peptides generally can be assumed to adopt an extended conformation prior to binding to proteases,[18,19] we sought a β-strand inducing scaffold that would preferentially also decrease the overall peptide character of the inhibitors. Previously published examples of β-strand inducing elements from the HCV area of research include several macrocycles inspired by ciluprevir[20] (for instance ITMN-191 and TMC 435350, Fig. 1) as well as heterocycle-based inhibitors.[21–23] Among the heterocycles, substituted 2(1*H*)-pyrazinones are interesting from a medicinal chemistry point of view due to their inherent possibility of retaining the H-bonding pattern of a peptide back-bone, while simultaneously introducing rigidity and decreasing the overall peptide character (Fig. 2a). Since the pyrazinone core can be synthesised by cyclization of an α-aminonitrile, which in turn can be assembled from an aldehyde, a primary amine and a cyanide source in a Strecker reaction,[24] the substitution pattern of position 1 and 6 is easily varied (Fig. 2b).

The main draw-back of 3,5-dichloro-2(1*H*)-pyrazinone synthesis has long been the lengthy reaction times (several hours).[24] However, by employing a microwave-heated protocol[25] we have managed to reduce the reaction time to merely 2 × 10 min.[26] The large reactivity difference between the 3-halo and 5-halo groups makes selective introduction of substituents in the 3-position possible.[27]

We were inspired to investigate the effect of combining a pyrazinone core in the P3 position with a P2 phenylglycine. As starting point, we chose the simple tripeptide **A** (Fig. 3), although we knew that a large, aromatic P2 substituent improves the inhibitory potency of this type of inhibitors.[16] However, we feared that a bulky P2 would hamper the development of the synthetic protocol. Additionally, by leaving the large P2 substituents of previous inhibitors out, we would also decrease the molecular weight considerably, creating space for further optimization. Drawing on experience from our tetrapeptide series, we knew that an unsubstituted phenylglycine produced an inhibitor more than 20-fold more potent than its proline counterpart.[16]

Here, we describe the results of the optimization of tripeptide **A**, from incorporation of the P3 pyrazinone core, over evaluation of

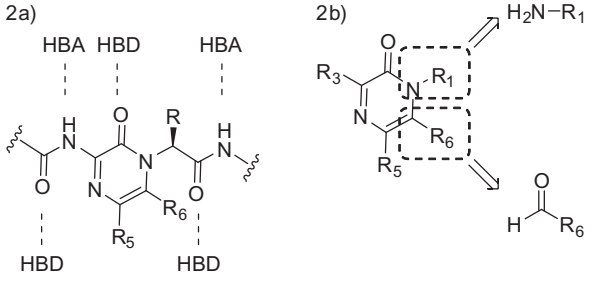


Figure 2. Starting points for the design of the novel, pyrazinone based inhibitors. (a) Schematic representation of the possibility of retained H-bond interactions after introduction of a 2(1*H*)-pyrazinone in a peptide back-bone. (b) The origin of the substituents in position 1 and 6 on the pyrazinone core. In our pyrazinones, $R_3 = R_5 = Cl$. The 3-Cl can be selectively substituted by nucleophiles in the presence of the 5-Cl.

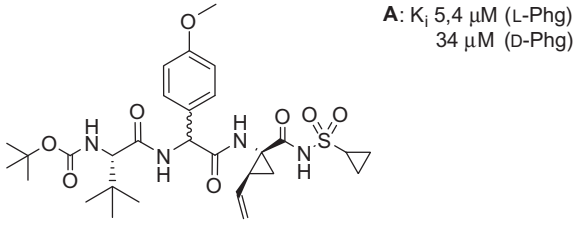


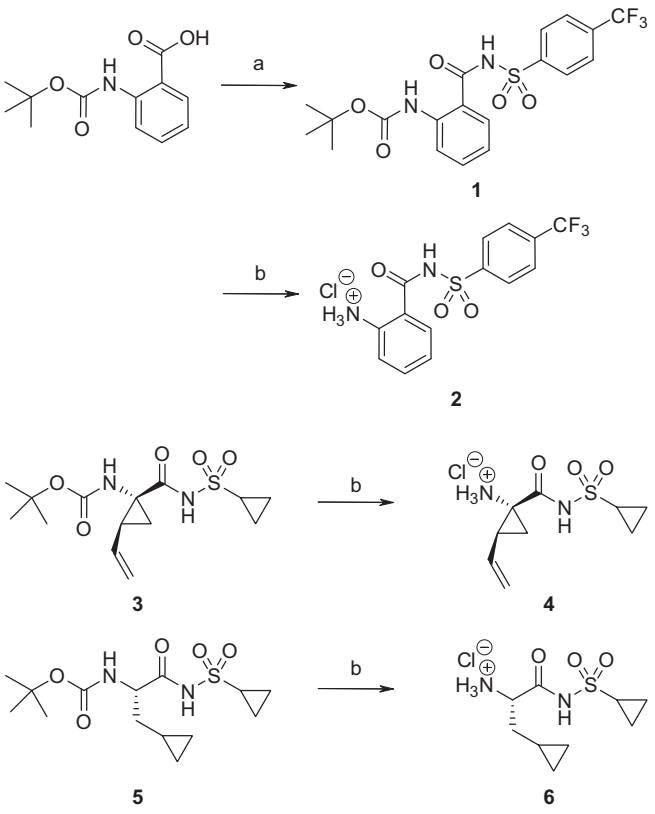
Figure 3. The lead, compound A, with K_i values ± standard deviations for the L- and D-P2 phenylglycine epimers.

different extensions in position 3 on that core (corresponding to the P3 capping group in **A**) and different P1–P1′ building blocks, ultimately resulting in achiral inhibitors with improved activity compared to the lead. Furthermore, the novel P3 pyrazinone based inhibitors display an intriguing inhibitory profile of the protease inhibitor resistant mutants A156T and D168A.

2. Chemistry

The tripeptide mimetic inhibitors **25**–**39** were synthesized in a convergent fashion with the final step being the peptide coupling between the P3 pyrazinone-P2 core and the P1–P1′ block. The synthesis of the *N*-Boc protected P1–P1′ building block **1** started from the commercially available 2-(*t*-butoxycarbonylamino)benzoic acid and *p*-trifluoro-methylbenzene sulfonamide, which were coupled using CDI and DBU in dry THF, as depicted in Scheme 1. The same procedure was used to synthesize the corresponding compounds **3**[28] and **5**. The *N*-Boc groups were removed using 4 M HCl in 1,4-dioxane. After removal of the solvent, the resulting hydrochlorides **2**, **4** and **6** were used in the subsequent coupling without further purification or characterization.

The pyrazinone-based P3–P2 scaffolds **7**–**12** (Scheme 2) were synthesized in two steps from the appropriate amine, aldehyde and trimethylsilyl cyanide, the first step being the formation of an α-aminonitrile in a Strecker-type reaction. In the second step, the cyclization of the intermediate α-aminonitrile to an N1, C6-disubstituted 3,5-dichloro pyrazinone was accomplished by HCl enrichment followed by heating in the presence of oxalyl chloride. Both steps were conveniently performed using microwave heating in sealed vials. Compounds **8**–**12** were synthesized according to this method and have recently been published by us.[26] Compound **7** was synthesized in the same fashion, but conventional heating was used instead of microwaves. To introduce the weakly nucleophilic carbamate selectively in position 3 on the pyrazinone core, a palladium-catalyzed, Buchwald N-arylation protocol was used,[29,30] resulting in compounds **13** and **14**. Unfortunately, the carbamate functionality underwent partial hydrolysis during the reaction and the purification process, which resulted in relatively low yields. The synthesis of compounds **15**–**23** was more straightforward, as a nucleophilic aromatic substitution effected by heating the pyrazinones, the appropriate amine (as the free base or the hydrochloride salt) and diisopropylethylamine (DIPEA) in MeCN for 1–3 h furnished the desired products in good to excellent isolated yields (ranging from 54% to 94%). The esters of compounds **15**–**23** were easily hydrolyzed by a novel method, employing K_2CO_3 in MeCN/H_2O and microwave heating at 120 °C for 45 min (except for compound **15**, which was heated for 55 min), which resulted in full conversion. When compounds **13** and **14**[26] were subjected to those conditions, the carbamate was completely hydrolyzed, resulting in the corresponding 3-amino substituted pyrazinone core. Hence, milder conditions were required and the hydrolysis was in both cases achieved by LiOH in THF/H_2O at room temperature, although partial hydrolysis of the carbamate could not be avoided even then. The benzyl ester-protected precursor of compound **24** suffered from poor solubility and was therefore not isolated at the ester stage, but as the carboxylic acid **24** after the hydrolysis (71% yield, two steps). In the final step of the synthesis of inhibitors **25**–**39**, a solution phase peptide coupling, the crude carboxylic acids derived from compounds **13**–**23** as well as the isolated acid **24** were coupled to the hydrochloride salts **2**, **4** or **6**, using *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) and DIPEA in DMF or DCM, either at room temperature or 40 °C. The inhibitors were purified by RP-HPLC in yields ranging from 8% to 43%.[31]



Scheme 1. Reagents: (a) *p*-trifluoromethylbenzene sulfonamide, CDI, DBU, THF; (b) 4 M HCl/1,4-dioxane.

3. Biochemical evaluation

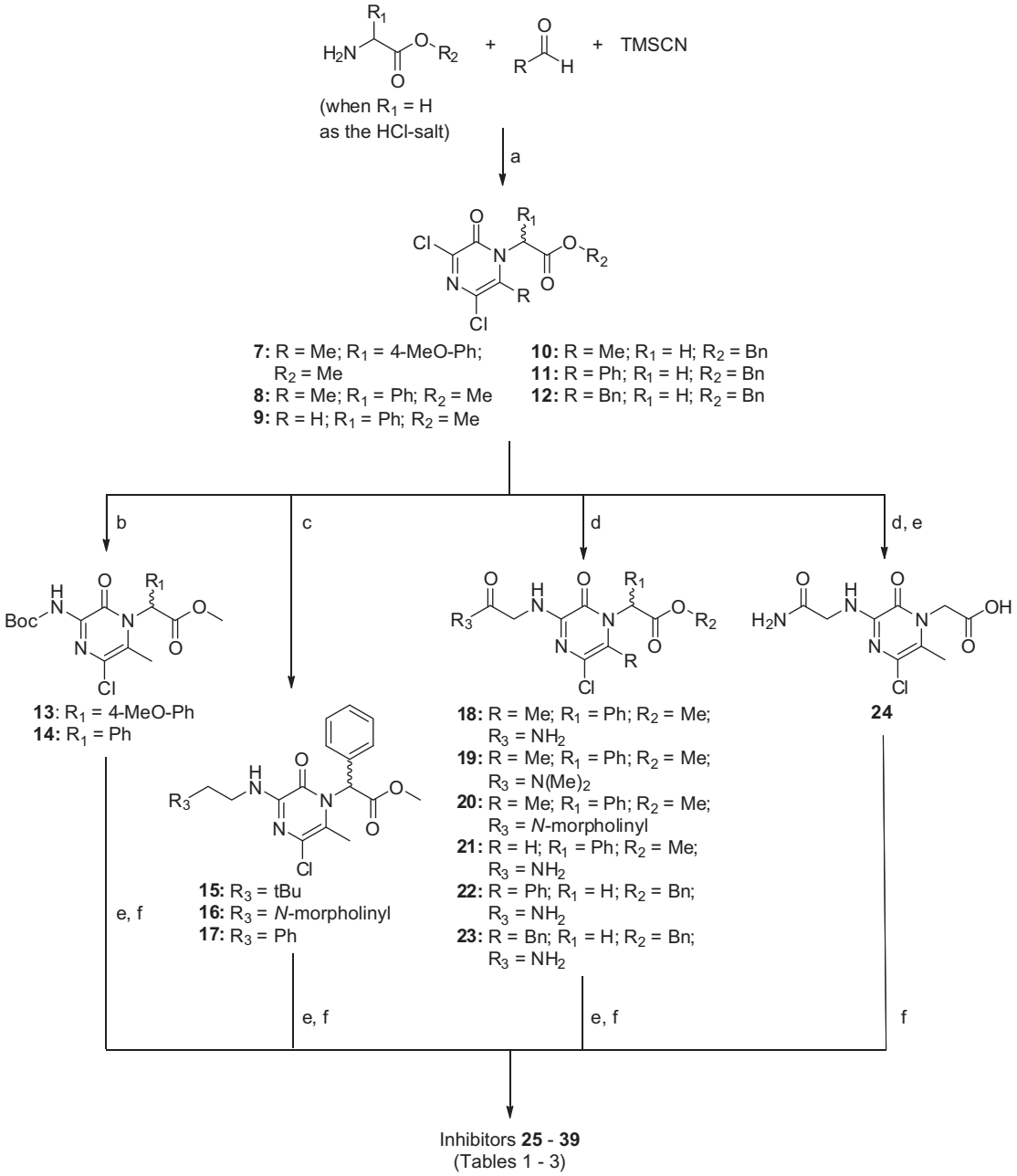
The inhibitory effects of inhibitors **25**–**39** were assessed in an in vitro assay, using wild type full length NS3 protein.[32] The K_i values are reported in Tables 1–3. The effects of inhibitors **26a**, **28** and **36**–**38** on the A156T and D168A substituted enzyme variants were also assessed in an in vitro assay using the full length protein with amino acid substitutions as stated.[17] The K_i values, along with vitality values, can be found in Table 4. The enzymatic assays were performed as described before.[17,32] Vitality values are normalized with respect to catalytic efficiency for the modified variants and the wild type enzyme, thus they enable comparison of the inhibitors′ performance on different enzyme variants. If $V > 1$, it indicates that the mutant enzyme has an advantage over the wild type in the presence of the inhibitor, vice versa if $V < 1$.[33]

4. Molecular modelling

Inhibitors **26a** (Fig. 4), **38** (Fig. 5) and **26a**, **37** and **38** (Fig. 6) were docked in the active site of the full-length NS3 protein. All the ligands were built in Maestro and geometry optimization was carried out using the OPLS-2005 force field. In this study, the crystal structure of the bifunctional HCV NS3 protein (PDB code: 1CU1)[34] was used. This full-length NS3 complex consists of the C-terminal helicase domain and the N-terminal protease domain with a covalently linked NS4A cofactor.

5. Results and discussion

Substituted 2(1*H*)-3,5-dihalopyrazinones are highly interesting as scaffolds in drug design mainly due to three factors: (1) their



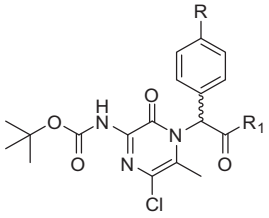
Scheme 2. Reagents: (a) (i) DIPEA (only if the HCl salt of the amine was used), DME, (ii) HCl, Et_2O, (iii) oxalyl chloride, DME; (b) *t*-butylcarbamate, $Pd(OAc)_2$, Xantphos, Cs_2CO_3, DME; (c) R_3NH_2, DIPEA, MeCN; (d) R_3NH_2·HCl, DIPEA, MeCN; (e) LiOH, THF, H_2O, MeOH or K_2CO_3, H_2O, MeCN; (f) **2**, **4** or **6** (except for **23a** and **23b**)[26], HATU, DIPEA, DMF or DCM.

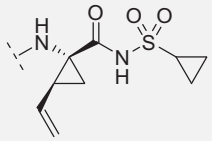
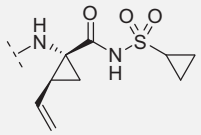
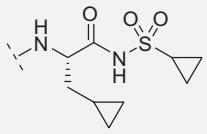
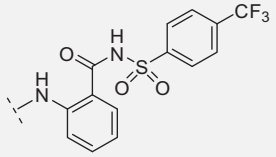
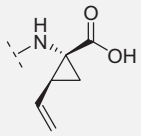
inherent β-strand inducing properties; (2) the vast number of possible substituents in position 1 and 6 on the pyrazinone core, since these originate from the amine and aldehyde starting material, respectively (Fig. 2b) and (3) the difference in reactivity between the 3- and 5-halo substituent, that renders the selective introduction of nucleophiles in position 3 possible. In the field of HCV NS3 protease inhibitors, P3 heterocycles fused to the P2 proline have been tested in combination with P1 boronic acids.[21–23] Other examples where incorporation of a pyrazinone scaffold have resulted in potent protease inhibitors include inhibitors of thrombin[35], tissue factor VIIa[36,37] and caspase-3.[38,39] Being already engaged in optimization of inhibitors based on the P2 phenylglycine, the introduction of a pyrazinone scaffold in the P3 position was an appealing alternative.

Compared to the lead compound **A** (Fig. 3, K_i = 5.4 μM for the L-phenylglycine epimer), the inhibitory potencies measured as K_i of the novel P3 pyrazinone-based inhibitors range from almost fourfold higher to fivefold lower (1.5–28 μM, Tables 1–3), indicating a successful exchange of the original P3 residue, *t*-leucine. Initially, we wanted to incorporate a *t*-butoxycarbonylamino in position 3 on the pyrazinone, since it would equal the Boc capping groups of compound **A** as well as those of our previously published tripeptide inhibitors.[16]

Synthetically, this could be achieved by using a modified Buchwald N-arylation protocol,[29] and inhibitors **25**, **26a** and **27**–**29b** (Table 1) were synthesized. Inhibitor **25** allows direct comparison with compound A, and the two are more or less equipotent, with K_i-values of 5.4 μM (P2 L-epimer of compound A) and 7.2 μM (P2 racemic inhibitor **25**). A range of different P1–P1′ blocks were evaluated, the well-known (1*R*,2*S*)-1-amino-2-vinylcyclopropanecarboxylic acid[40] (vinylACCA), β-cyclopropylalanine[41] and an aromatic P1–P1′

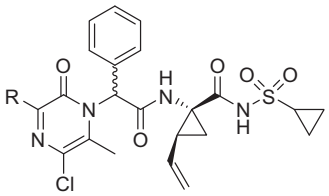
Table 1
Inhibition of the full length wild type NS3 (protease–helicase/NTPase) protease activity

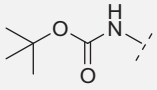
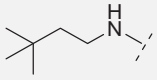
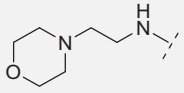
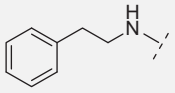
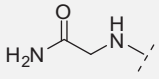
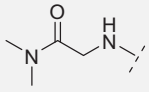
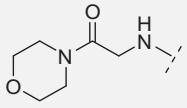


Compd	R	R_1	K_i (μM) ± SD[a]
25	MeO		7.2 ± 0.5
26a	H		4.3 ± 0.6
27	H		9.6 ± 1.2
28	H		3.8 ± 0.6
29a[b]	H		11 ± 1.1
29b[b]	H		13 ± 2.4

Evaluation of the P1–P1′ residue.
[a] SD = standard deviation.
[b] Inhibitors **29a** and **b** are stereochemically pure at the P2 α-carbon, the absolute configuration has however not been established.[26]

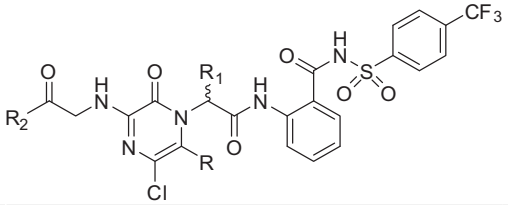
Table 2
Inhibition of the full length wild type NS3 (protease–helicase/NTPase) protease activity



Compd	R	K_i (μM) ± SD[a]
26a		4.3 ± 0.6
26b	H_2N–	11 ± 1.8
30		6.8 ± 1.1
31		11 ± 1.9
32		8.3 ± 1.6
33		12 ± 3.4
34		28 ± 5.6
35		10 ± 2.3

Evaluation of the substituent in position 3 on the pyrazinone core.
[a] SD = standard deviation.

Table 3
Inhibition of the full-length wild type NS3 (protease–helicase/NTPase) protease activity



Compd	R	R1	$R2_{exp}$[a]	$R2_{obs}$[b]	K_i (μM) ± SD[c]
36	H	Ph	NH_2	OH	2.3 ± 0.4
37	Ph	H	NH_2	OH	3.0 ± 0.6
38	Bn	H	NH_2	OH	1.5 ± 0.3
39	Me	H	NH_2	NH_2	18 ± 3.3

Evaluation of the transfer of the P2 side-chain to position 6 on the pyrazinone core.
[a] Exp = expected.
[b] Obs = observed.
[c] SD = standard deviation.

scaffold previously published by us.[42] Most successful was the introduction of vinylACCA in compound **26a** (K_i = 4.3 μM) and the aromatic P1 scaffold in compound **28** (K_i = 3.8 μM). The β-cyclopropylalanine P1 of inhibitor **27** has also been evaluated before but is not optimal in combination with the structural elements of our inhibitors, as evidenced by a K_i value of 9.6 μM. In analogy with our previous observations,[16,43,44] activity is gained when the P1 carboxylic acid is masked as an acyl sulfonamide (compare compounds **26a**, K_i = 4.3 μM, with **29a** and **29b**, K_i = 11 and 13 μM, respectively). From the comparison of compound **25** and **26a**, it can be deduced that nothing is to be gained from introduction of a *p*-methoxy group on the P2 phenylglycine, and consequently we decided to continue with the unsubstituted phenylglycine.

As mentioned before, the *t*-butylcarbamate functionality was not entirely stable, but on the other hand this gave us the opportunity to isolate a small amount of the 3-amino analogue **26b** and evaluate its enzymatic activity. We found that the amino functionality was not well tolerated in that position (K_i = 11 μM, Table 2).

Supported by the fact that computer-aided docking of compound **26a** (Fig. 4) did not reveal any crucial interactions with the oxygens of the Boc-group, we set out to find chemically stable replacements for the carbamate functionality. Hence, we synthesized compound **30** (Table 2), the 'oxygen-free' analogue of **26a**, to which it was more or less equipotent (the K_i values of inhibitors **30** and **26a** are 6.8 and 4.3 μM, respectively). To further probe the extension in the pyrazinone position 3, the morpholino and phenyl analogues **31** and **32** were synthesised.

Table 4
Inhibition of the protease activity of the A156T and D168A mutant forms of full length NS3 (protease–helicase/NTPase)

Compd	A156T		D168V	
	K_i (μM) ± SD[a]	V[b]	K_i (μM) ± SD[a]	V[b]
26a	5.9 ± 1.6	1.9	8.6 ± 1.2	2.1
28	1.5 ± 0.3	0.5	1.7 ± 0.3	0.5
36	1.8 ± 0.2	0.4	6.4 ± 1.1	2.1
37	5.6 ± 1.2	0.9	6.0 ± 0.6	1.6
38	2.5 ± 1.0	0.8	3.1 ± 0.8	1.6

[a] SD = standard deviation.
[b] V, vitality values calculated as described in Refs. 17 and 33.

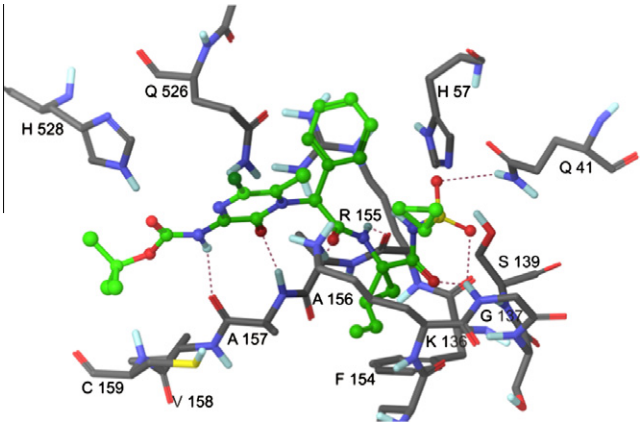


Figure 4. Inhibitor **26a** docked in the active site of the NS3 protease. H-bond interactions are shown as red dotted lines.

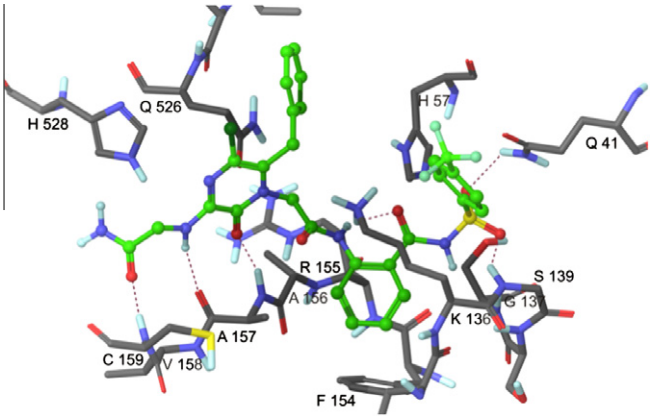


Figure 5. The expected, primary amide analogue of inhibitor **38** docked in the active site of the NS3 protease. H-bond interactions are shown as red dotted lines. Please note the interaction between the carbonyl of the primary amide and the NH of Cys 159.

While the morpholino group is clearly detrimental to activity (**31**, K_i = 11 μM), at least nothing is gained from the phenyl substituent in compound **32**, which has a K_i-value of 8.3 μM. Molecular modelling suggested that a carbonyl functionality one step further out compared to the Boc carbonyl of inhibitors **25**–**29b**, could have the possibility to pick up an interaction with the back-bone NH of Cys159 (Fig. 4). A carbonyl in that position on the extension would be achieved by reversed introduction of an α-amino acid (that is, in C→N direction) in position 3 on the pyrazinone core. We chose to start with the comparatively simple glycine derivatives in inhibitors **33**–**35** (Table 2). Docking studies indicated that the primary amide of inhibitor **33** would fit the enzyme better than the tertiary variants in inhibitors **34** and **35**; however, none of the glycine

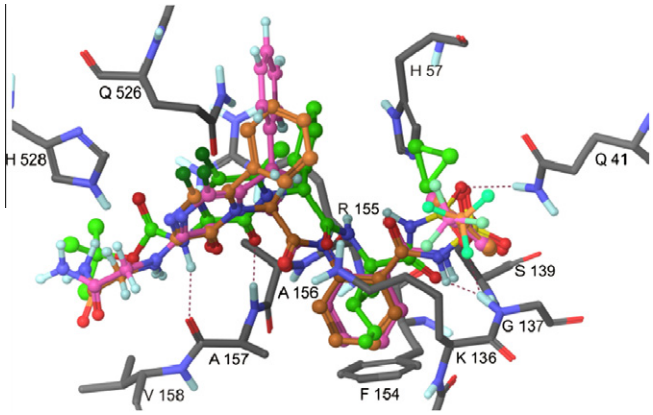


Figure 6. Compounds **26a** (green) and the expected primary amide analogues of **37** (orange) and **38** (pink) overlaid in the NS3 protease active site. H-bond interactions are shown as red dotted lines.

derivatives in inhibitors **33**–**35** led to an increase in the inhibitory potency of the inhibitors. The dimethylamide of compound **34** in particular showed a marked decrease in potency (K_i = 28 μM). Inhibitor **35** (K_i = 10 μM) demonstrated that the negative effect of the morpholino group in compound **31** (K_i = 11 μM) is not entirely attributable to the basic tertiary amine.

With the results from the P1–P1′ comparison in Table 1 in hand, we saw the possibility of further decreasing the peptide character by introducing the aromatic P1–P1′ block used in inhibitor **28**, which is entirely void of α-amino acid character. In addition, computer modelling suggested that the space normally occupied by the P2 side-chain could be reached from position 6 on the pyrazinone core if a bulky aldehyde was used in the synthesis (Fig. 5). Thus, we designed the inhibitors in Table 3 in order to explore the combined effects of the P2 side-chain and the substituent in position 6, and based on the docking results discussed above it was decided to use the primary glycine amide as the pyrazinone substituent in the side-chain transfer series.

It was highly surprising to find that the HRMS analyses of inhibitors **36**–**38** based on the found m/z-values suggested molecular formulas corresponding to the carboxylic acids rather than the intended primary amides. Thus, an unexpected hydrolysis appears to have occurred during the final coupling. During the preceding steps (d and e in Scheme 2), the primary amides had remained intact. In addition, inhibitors **33** and **39** were formed under the same conditions but without the above-mentioned hydrolysis.

A possible, beneficial influence from the carboxylic acids on the inhibitory potencies of inhibitors **36**–**38** cannot be excluded; indeed, the inhibitory potencies of inhibitors **33** and **36**–**39** could be an indication in that direction. When the methyl in position 6 on the pyrazinone was exchanged for a hydrogen, as in compound **36**, no significant effect on the inhibitory potency was observed (K_i of **36** is 2.3 μM, the same range as several other inhibitors in this study). However, the comparison is complicated by the amide hydrolysis of inhibitor **36**. In compounds **37** and **38**, the P2 phenylglycine side-chain was removed from the back-bone of the inhibitors, and instead a phenyl or a benzyl was introduced on the pyrazinone. Both substitutions were allowed with respect to inhibitory potency, as evidenced by the phenyl compound **37** being equipotent to compound **36** (K_i values 3.0 and 2.3 μM, respectively).

The benzyl-substituted compound **38** was the most potent in this series, and with a K_i-value of 1.5 μM it was also almost four times as potent as our tripeptide lead (K_i = 5.4 μM for the L-phenylglycine epimer of compound A). From Figure 6, showing inhibitors **26a**, **37** and **38** (**37** and **38** as they were intended, with an intact primary amide) overlaid in the active site, it can be seen how the benzyl of inhibitor **38** projects upwards in the S2 pocket.

Compound **39**, lacking the P2 side-chain as well as a bulky substituent in position 6, was designed to prove if the interactions between the enzyme and the aromatic groups, either attached to the back-bone or to the pyrazinone core, are necessary for inhibitory potency. However, as the HRMS analysis of inhibitor **39** did not indicate the above-mentioned hydrolysis, no such conclusions can be drawn today. The increased K_i-value of **39** (18 μM) compared with inhibitors **36**–**38** could reflect either the lack of P2–S2 interactions or a negative effect from the primary amide, or a combination of the two.

In spite of the, in our experience unprecedented amide hydrolysis under the employed conditions, it should be emphasized that the resulting inhibitors, **37**–**39**, are achiral and in addition the most potent in this series. Considering the impact of the large, optimized P2 substituent on the potency of the proline-based inhibitors, we believe that the pyrazinone core is a most promising scaffold for further lead optimization of a new class of hepatitis C virus NS3 protease inhibitors.

Five selected inhibitors, **26a**, **28** and **36**–**38**, were evaluated for inhibition of the inhibitor resistant variants A156T and D168A (Table 4). Inhibitor **28** displayed an overall improvement in potency on both mutants, whereas the other compounds experienced slightly decreased inhibition in terms of K_i-values. However, if the vitality values are considered, the performance of the inhibitors, especially on the A156T variant, is very promising as they for inhibitors **28** and **36**–**37** are <1. Since the vitality value also takes the relative catalytic efficiency of the enzymes into account, the potency loss of these inhibitors, which was small even in absolute terms, is virtually negligible.

6. Conclusion

We have evaluated a novel approach to the design of Hepatitis C NS3 protease inhibitors, combining a pyrazinone scaffold in the P3 position with a phenylglycine P2 residue. For inhibitors based on this scaffold, it will be possible to perform future optimization in a combinatorial fashion due to (a) the rapid synthetic scheme for pyrazinone synthesis previously developed by us,[26] (b) the easy access to structurally diverse starting materials for the pyrazinone synthesis, namely aldehydes, primary amines and a cyanide source, and (c) the short (five steps) overall synthetic route of the inhibitor synthesis. Indeed, we have shown that by changing the original combination of a small aldehyde and a bulky amine to a bulky aldehyde and a small amine, we can transfer the P2 side-chain to the P3 pyrazinone core. This structural modification did not only produce the best inhibitor in this series, with an almost fourfold improved potency compared to that of the tripeptide lead (1.5 vs 5.4 μM), but also a peptidomimetic inhibitor lacking stereocentres. Furthermore, the results from the evaluation on the resistant enzymes show an intriguing scope for further development. Consequently, we consider the obtained small-molecule inhibitor **38** a most promising lead for future Hepatitis C NS3 protease inhibitors. Further optimization of this novel class of inhibitors based on the pyrazinone scaffold as well as an investigation of the amide hydrolysis and the influence of a carboxylic acid in that position are in progress.

7. Experimental

7.1. Chemistry

Commercially obtained reagents and solvents were used without further purification. The microwave reactions were performed in a Smith Synthesizer™ single mode cavity with controlled irradiation at 2450 MHz with a power of 0–300 W. The reaction temperature was determined using the built-in on-line IR-sensor and the reactions were executed in septum sealed 2.0–5.0 mL process vials. Thin layer chromatography (TLC) was performed using aluminum sheets precoated with Silica Gel 60 F254 (0.2 mm, E. Merck) or RP-TLC RP-18 F254S, (E. Merck). Chromatographic spots were visualized using UV-detection or by spraying with a 2% ethanolic ninhydrin solution followed by heating, or both. Column chromatography was performed using commercially available Silica Gel 60 (particle size: 0.040–0.063 mm) or Merck Silica Gel 60 RP-18 (40–63 μm). Analytical RP-HPLC–MS analysis was performed on a Gilson HPLC system with a Finnigan AQA quadropole mass spectrometer using an Onyx Monolithic C18 4.6 × 50 mm (Phenomenex) and UV detection (DAD) or ELSD in combination with MS (ESI+) detection, using a MeCN/H_2O gradient (0.05% HCOOH). Preparative RP-HPLC purification of the final products was performed on a system equipped with a Sorbax SB-C8 column (21.2 × 150 mm) and UV detection at 220 nm. The purity of each of the inhibitors was determined by RP-HPLC in two of the following systems: A: ACE 5 C8-A3071, MeCN/H_2O (0.1% TFA), UV detection at 220 nm; B: Allure Biphenyl (5 μm, 50 × 4.6 mm), UV detection at 220 nm; C: HPLC–MS ACT C4, 4.6 × 50 mm, 5 μm, UV detection at 214 nm; or D: HPLC–MS Onyx monolithic C18, 4.6 × 50 mm (Phenomenex), UV detection at 214 nm. ^1H and ^{13}C NMR spectra were recorded on Varian Mercury Plus instruments; ^1H at 399.9 MHz and ^{13}C at 100.6 MHz. Chemical shifts are reported as δ values (ppm) indirectly referenced to TMS via the solvent signal (^1H: CHD_2OD δ 3.31, $CHCl_3$ δ 7.26, CHD_2COCD_3 δ 2.05, CHD_2SOCD_3 δ 2.50, CHD_2CN δ 1.94; ^{13}C: CD_3OD δ 49.0, $CDCl_3$ δ 77.16, $(CD_3)_2CO$ δ 49.0, $(CD_3)_2SO$ δ 39.52, CD_3CN δ 118.26. Exact molecular masses were determined on Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source. Elemental analysis was performed by Analytische Laboratorien, Lindlar, Germany.

7.1.1. *t*-Butyl 2-(4-(trifluoromethyl)phenylsulfonyl-carbamoyl) phenylcarbamate (1)

All solid chemicals used were dried in vacuum over P_2O_5 over night. *N*-BocAbzOH (0.960 g, 4.0 mmol) and CDI (0.992 g, 6.1 mmol) was dissolved in dry THF (15 mL) and was stirred at room temperature for 3 h. 4-Trifluoromethylbenzene sulfonamide (1.04 g, 4.6 mmol) and DBU (0.90 mL, 6.0 mmol) was added and stirring was continued at room temperature over night. The solvent was removed in vacuo, H_2O was added and pH adjusted to ≈2 by addition of 1 M HCl. The aqueous phase was extracted with EtOAc (4 × 25 mL). The organic phases were combined, the solvent was removed in vacuo and the crude product vacuum dried. Purification by column chromatography (isohexane/EtOAc 4:1, 2:1) yielded the title compound as a solid (0.841 g, 47%). ^1H NMR (CD_3OD) δ 8.19–8.13 (m, 3H), 8.07 (dd, J = 8.0, 1.7 Hz, 1H), 7.77 (m, 2H), 7.35 (ddd, J = 8.4, 7.2, 1.7 Hz, 1H), 6.93 (ddd, J = 8.0, 7.2, 1.2 Hz, 1H), 1.42 (s, 9H). ^{13}C (CD_3OD) δ 174.7, 154.8, 148.7, 141.7, 134.3 (q, J = 32.3 Hz), 133.3, 132.0, 129.2, 126.7 (q, J = 3.8 Hz), 124.8 (q, J = 271.1 Hz), 123.8, 122.5, 119.9, 81.2, 28.7. LC–MS ($C_{19}H_{19}F_3N_2O_5S$, M+H$^+$) 445.1.

7.1.2. General method A, for the preparation of hydrochlorides 2-(4-(trifluoromethyl)phenyl-sulfonyl-carbamoyl)benzeneammonium chloride (2), (1*R*,2*S*)-1-(cyclopropylsulfonylcarbamoyl)-2-vinylcyclopropan-ammonium chloride (4) and (*S*)-1-(cyclopropane-sulfonamido)-3-cyclopropyl-1-oxopropan-2-ammonium chloride (6)

The P1–P1′ building blocks **1**, **3** or **5** was dissolved in 4 M HCl/1,4-dioxane and stirred at room temperature until LC–MS analysis showed that the reaction was complete. The solvent was evaporated in vacuo, and the residue was dried under vacuum. The resulting hydrochlorides **2**, **4** and **6** were used in the subsequent

coupling reactions without further purification or characterization except for LC–MS (**2**: LC–MS ($C_{14}H_{11}F_3N_2O_3S$, M+H$^+$) 345.1; **4**: LC–MS ($C_9H_{14}N_2O_3S$, M+H$^+$) 231.1; **6**: LC–MS ($C_9H_{16}N_2O_3S$, M+H$^+$) 233.0.

7.1.3. (1*R*,2*S*)-*t*-Butyl-1-(cyclopropylsulfonyl-carbamoyl)-2-vinylcyclopropylcarbamate (3)

Compound **3** has previously been described by us[16] and others.[28]

7.1.4. (*S*)-*t*-Butyl-1-(cyclopropanesulfonamido)-3-cyclopropyl-1-oxopropan-2-ylcarbamate (5)

The synthesis and characterization of compound **5** has recently been described by us.[45]

7.1.5. 2-(3,5-Dichloro-6-methyl-2-oxopyrazin-1(2*H*)-yl)-2-(4-methoxyphenyl)acetic acid methyl ester (7)

(*S*)-*t*-Butoxycarbonylamino(4-methoxyphenyl)acetic acid methyl ester[16] (8.59 g, 29.1 mmol) was dissolved in 4 M HCl/1,4-dioxane and stirred at room temperature for 2.5 h. The solvent was removed in vacuo, the residue was dissolved in DCM and washed with saturated $NaHCO_3$-solution (3 × 100 mL). The organic phase was dried (K_2CO_3), filtered and the solvent was removed in vacuo to yield (*S*)-2-amino-2-(4-methoxy-phenyl) acetic acid methyl ester as a yellow oil, of which (0.19 g, 0.98 mmol) was weighed into a 2.0–5.0 mL microwave process vial and dissolved in DME (3 mL). Acetaldehyde (0.066 mL, 1.2 mmol) was added and the mixture was stirred for 30 s. Me_3SiCN (0.15 mL, 1.2 mmol) was added, the vial was sealed and the reaction was stirred at room temperature over night. An additional amount of acetaldehyde (0.033 mL, 0.59 mmol) and Me_3SiCN (0.075 mL, 0.60 mmol) were added and the reaction mixture was stirred for another 7 h at room temperature. The solvent was removed and the resulting yellow oil was dissolved in Et_2O. A white yellowish precipitate was formed upon enrichment with HCl gas. The solvent was evaporated and the precipitate was dissolved in DME (3 mL). Oxalyl chloride (0.21 mL, 2.5 mmol) was added, the vial was sealed and air was exchanged for N_2. The mixture was subjected to microwave heating at 170 °C for 10 min in a 2.0–5.0 mL microwave process vial and reached a stable pressure of 9 bar. The solvent and the excess oxalyl chloride were removed in vacuo. The resulting black oil was dissolved in a small amount of DCM and was purified by column chromatography (isohexane/EtOAc 9:1, isohexane/EtOAc 4:1, isohexane/EtOAc 2:1. The solvent was removed in vacuo to yield compound **7** (0.11 g, 32%). ^{1}H NMR ($CDCl_3$) δ 7.32 (m, 2H), 6.88 (m, 2H), 6.31 (s, 1H), 3.82 (s, 3H), 3.80 (s, 3H), 2.40 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 167.2, 160.2, 152.8, 144.3, 135.8, 130.2, 124.8, 124.4, 114.4, 63.2, 55.5, 53.5, 17.7. Anal. Calcd for $C_{15}H_{14}Cl_2N_2O{\cdot}1H_2O$, C: 48.01; H: 4.30; N: 7.47. Found: C, 47.60; H, 4.55; N, 6.79.

7.1.6. 2-(3,5-Dichloro-6-methyl-2-oxopyrazin-1(2*H*)-yl)-2-phenylacetic acid methyl ester (8), 2-(3,5-dichloro-2-oxopyrazin-1(2*H*)-yl)-2-phenylacetic acid methyl ester (9), 2-(3,5-dichloro-6-methyl-2-oxopyrazin-1(2*H*)-yl)acetic acid benzyl ester (10), 2-(3,5-dichloro-2-oxo-6-phenylpyrazin-1(2*H*)-yl)acetic acid benzyl ester (11) and 2-(6-denzyl-3,5-dichloro-2-oxopyrazin-1(2*H*)-yl)acetic acid benzyl ester (12)

The synthesis of compounds **8**–**12** has recently been published by us.[26]

7.1.7. 2-(3-(*t*-Butoxycarbonylamino)-5-chloro-6-methyl-2-oxopyrazin-1(2*H*)-yl)-2-(4-methoxyphenyl)-acetic acid methyl ester (13)

t-Butylcarbamate (0.19 g, 1.6 mmol), $Pd(OAc)_2$ (0.012 g, 0.052 mmol), Xantphos (0.036 g, 0.062 mmol) and Cs_2CO_3 (0.21 g, 0.64 mmol) were added into a reaction tube. Compound **7** (0.11 mg, 0.32 mmol) dissolved in DME (4 mL) was added. The air was exchanged to N_2-gas and the tube was sealed. The reaction was run at 80 °C for 47 h. The palladium was removed by addition of 3-(1-thioureido)propyl functionalized silica gel (0.14 g, 1,1 mmol/g) that was filtered off after 1 h of stirring at room temperature. The solvent was removed, the residue dissolved in DCM and put onto a silica column and eluted with DCM. The solvent was removed in vacuo. The crude product was dissolved in EtOAc (10 ml) and washed with water (9 × 15 mL). The solvent was removed in vacuo, yielding compound **13** (0.018 g, 13%, some contamination by *tert*-butylcarbamate). The crude product was dissolved in MeCN and purified on an RP-silica column (MeCN/H_2O 50:50, MeCN:H_2O 75:25). The solvent was removed in vacuo yielding the title compound as a solid (0.010 g, 7%). ^{1}H NMR ($CDCl_3$) δ 8.29 (s, 1H), 7.29 (m, 2H), 6.86 (m, 2H), 6.23 (s, 1H), 3.79 (s, 3H), 2.36 (s, 3H), 1.51 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 167.6, 160.1, 151.1, 149.6, 143.3, 129.9, 126.4, 126.0, 124.6, 114.4, 82.3, 62.5, 55.5, 53.3, 28.3, 17.0. Anal. Calcd for $C_{20}H_{24}ClN_3O_6$: C, 54.86; H, 5.52; N, 9.60. Found: C, 55.09; H, 5.82; N, 9.44.

7.1.8. 2-(3-(*t*-Butoxycarbonylamino)-5-chloro-6-methyl-2-oxopyrazin-1(2*H*)-yl)-2-phenylacetic acid methyl ester (14)

The synthesis and characterization of compound **14** has recently been published by us.[26]

7.1.9. General method B, for the preparation of compounds 15–24

The pyrazinone/P2 cores **8**–**12** and the amine, as free base or hydrochloride salt, were dissolved in MeCN in a 2.0–5.0 mL microwave process vial and DIPEA was added. The vial was sealed with a septum and the reaction was subjected to microwave heating, after which the solvent was removed in vacuo. Purification by column chromatography yielded the title compounds **15**–**24** after evaporation of the solvent in vacuo.

7.1.10. 2-(5-Chloro-3-(3,3-dimethylbutylamino)-6-methyl-2-oxopyrazin-1(2*H*)-yl)-2-phenylacetic acid methyl ester (15)

Compound **15** was prepared according to General method B, using the pyrazinone/P2 core **8** (0.101 g, 0.31 mmol), 3,3-dimethylbutylamine (0.051 mL, 0.38 mmol), DIPEA (0.066 mL, 0.38 mmol) and MeCN (3.0 mL). Microwave heating, 60 min at 100 °C. Column eluent: isohexane/EtOAc 7:1, 4:1. Yield: 0.108 g, 89%, solid material. ^{1}H ($CDCl_3$) δ 7.33 (m, 5H), 6.17 (s, 1H), 6.03 (m, 1H), 3.78 (s, 3H), 3.40 (m, 2H), 2.23 (s, 3H), 1.55 (m, 2H), 0.97 (s, 9H). ^{13}C ($CDCl_3$) δ 168.0, 151.7, 148.4, 133.2, 128.8, 128.7, 128.2, 126.9, 118.4, 62.0, 53.1, 42.8, 37.8, 30.1, 29.6, 16.3. Anal. Calcd for $C_{20}H_{26}ClN_3O_3$: C, 61.29; H, 6.69; N, 10.72. Found: C, 61.52; H, 6.78; N, 10.60.

7.1.11. 2-(5-Chloro-6-methyl-3-(2-morpholinoethylamino)-2-oxopyrazin-1(2*H*)-yl)-2-phenylacetic acid methyl ester (16)

Compound **16** was prepared according to general method B, using the pyrazinone/P2 core **8** (0.100 g, 0.31 mmol), 4-(2-aminoethyl)morpholine (0.050 mL, 0.38 mmol), DIPEA (0.066 mL, 0.38 mmol) and MeCN (3.0 mL). Microwave heating, 60 min at 100 °C. Column eluent isohexane/EtOAc 1:5, 100% EtOAc, EtOAc/MeOH 99:1. Yield: 0.112 g, 86%, solid material. Anal. Calcd for $C_{20}H_{25}ClN_4O_4$: C, 57.07; H, 5.99; N, 13.31. Found: C, 57.28; H, 6.31; N, 13.09. ^{1}H ($CDCl_3$) δ 7.33 (m, 5H), 6.59 (dd, J = 5.2, 5.2 Hz, 1H), 6.21 (s, 1H), 3.79 (s, 3H), 3.70 (m, 4H), 3.49 (m, 2H), 2.59 (m, 2H), 2.47 (m, 4H), 2.22 (s, 3H). ^{13}C ($CDCl_3$) δ 168.0, 151.6, 148.4, 133.2, 128.8, 128.7, 128.2, 126.7, 118.8, 67.0, 62.0, 56.7, 53.4, 53.1, 37.4, 16.4.

7.1.12. 2-(5-Chloro-6-methyl-2-oxo-3-(phenethylamino)pyrazin-1(2*H*)-yl)-2-phenylacetic acid methyl ester (17)

Compound **17** was prepared according to General method B, using the pyrazinone/P2 core **8** (0.102 g, 0.31 mmol), 2-phenyleth-

ylamine (0.048 mL, 0.38 mmol), DIPEA (0.066 mL, 0.38 mmol) and MeCN (3.0 mL). Microwave heating, 60 min at 100 °C, LC–MS showed considerable amount of starting material left. Addition of 2-phenylethylamine (0.024 mL, 0.19 mmol), DIPEA (0.033 mL, 0.19 mmol) followed by another 60 min at 100 °C of microwave heating, after which the starting material was consumed. Column eluent 100% EtOAc. Yield: 0.071 g, 65%, solid material. Anal. Calcd for $C_{16}H_{17}ClN_4O_4$: C, 52.68; H, 4.70; N, 15.36. Found: C, 52.81; H, 5.04; N, 15.08. ^{1}H ($CDCl_3$) δ 7.34–7.29 (m, 8H), 7.24 (m, 2H), 6.21 (m, 1H), 6.19 (s, 1H), 3.78 (s, 3H), 3.69 (m, 2H), 2.94 (m, 2H), 2.21 (s, 3H). ^{13}C ($CDCl_3$) δ 168.1, 151.6, 148.3, 138.8, 133.2, 128.9, 128.8, 128.7, 128.2, 126.8, 126.4, 118.9, 62.1, 53.1, 50.9, 42.4, 35.2, 16.4.

7.1.13. 2-(3-(2-Amino-2-oxoethylamino)-5-chloro-6-methyl-2-oxopyrazin-1(2*H*)-yl)-2-phenylacetic acid methyl ester (18)

Compound **18** was prepared according to general method B, using the pyrazinone/P2 core **8** (0.097 g, 0.30 mmol), HCl × $HGlyNH_2$ (0.041 g, 0.37 mmol), DIPEA (0.12 mL, 0.69 mmol) and MeCN (3.0 mL). Microwave heating, 1 h at 100 °C, after which LC–MS showed considerable amount of starting material left. Addition of HCl × $HGlyNH_2$ (0.037 g, 0.34 mmol) and DIPEA (0.060 mL, 0.34 mmol) was followed by another 60 min of microwave heating at 100 °C, but still much starting material was left. The procedure was repeated once more, HCl × $HGlyNH_2$ (0.041 g, 0.37 mmol) and DIPEA (0.060 mL, 0.34 mmol) was added followed by 1 h of heating at 100 °C after which the starting material was consumed. Column eluent isohexane/EtOAc 4:1. Yield: 0.120 g, 94%, solid material. Anal. Calcd for $C_{22}H_{22}ClN_3O_3$: C, 64.15; H, 5.38; N, 10.20. Found: C, 64.37; H, 5.52; N, 10.17. ^{1}H (CD_3OD) δ 7.35 (m, 5H), 6.30 (s, 1H), 4.06 (d, J = 17.1 Hz, 1H), 4.00 (d, J = 17.1 Hz, 1H), 3.75 (s, 3H), 2.60 (s, 3H). ^{13}C (CD_3OD) δ 174.8, 170.0, 153.0, 150.0, 134.9, 129.7, 129.6 (two peaks), 127.3, 121.7, 63.8, 53.5, 44.7, 16.4.

7.1.14. 2-(5-Chloro-3-(2-(dimethylamino)-2-oxoethylamino)-6-methyl-2-oxopyrazin-1(2*H*)-yl)-2-phenylacetic acid methyl ester (19)

Compound **19** was prepared according to general method B, using the pyrazinone/P2 core **8** (0.103 g, 0.31 mmol), 2-amino-*N*,*N*-dimethyl-acetamide hydrochloride (0.152 g, 1.1 mmol), DIPEA (0.24 mL, 1.4 mmol) and MeCN (3.0 mL). Microwave heating, 150 min at 100 °C. Column eluent 100% EtOAc. Yield: 0.098 g, 80%, solid material. Anal. Calcd for $C_{18}H_{21}ClN_4O_4$: C, 55.03; H, 5.39; N, 14.27. Found: C, 54.89; H, 5.45; N, 14.17. ^{1}H (CD_3OD) δ 7.36 (m, 5H), 6.29 (s, 1H), 4.25 (d, J = 17.4 Hz, 1H), 4.19(d, J = 17.4 Hz, 1H), 3.75 (s, 3H), 3.10 (s, 3H), 2.99 (s, 3H), 2.27 (s, 3H). ^{13}C (CD_3OD) δ 170.5, 170.0, 153.1, 149.8, 135.0, 129.7 (two peaks), 129.6, 127.2, 121.3, 63.9, 53.4, 43.5, 36.6, 36.1, 16.3.

7.1.15. 2-(5-Chloro-6-methyl-3-(2-morpholino-2-oxoethylamino)-2-oxopyrazin-1(2*H*)-yl)-2-phenylacetic acid methyl ester (20)

Compound **20** was prepared according to General method B, using the pyrazinone/P2 core **8** (0.103 g, 0.31 mmol), 2-Amino-1-morpholin-4-yl-ethanone hydrochloride (0.172 g, 0.95 mmol), DIPEA (0.22 mL, 1.3 mmol) and MeCN (3.0 mL). Microwave heating, 150 min at 100 °C. Column eluent 100% EtOAc. Yield: 0.102 g, 76%, solid material. Anal. Calcd for $C_{20}H_{23}ClN_4O_5$: C, 55.23; H, 5.33; N, 12.89. Found: C, 55.08; H, 5.49; N, 12.68. ^{1}H (CD_3OD) δ 7.36 (m, 5H), 6.29 (s, 1H), 4.27 (d, J = 17.2 Hz, 1H), 4.21 (d, J = 17.2 Hz, 1H), 3.75 (s, 3H), 3.71 (m, 4H), 3.59 (m, 4H), 2.27 (s, 3H). ^{13}C (CD_3OD) δ 170.0, 169.3, 153.0, 149.8, 134.9, 129.7 (two peaks), 129.6, 127.2, 121.4, 67.9, 67.7, 63.9, 53.5, 46.5, 43.8, 43.3, 16.3.

7.1.16. 2-(3-(2-Amino-2-oxoethylamino)-5-chloro-2-oxopyrazin-1(2*H*)-yl)-2-phenylacetic acid methyl ester (21)

Compound **21** was prepared according to general method B, using the pyrazinone/P2 core **9** (0.108 g, 0.34 mmol), HCl × $HGlyNH_2$ (0.111 g, 1.0 mmol), DIPEA (0.24 mL, 1.4 mmol) and MeCN (3.0 mL). Microwave heating, 150 min at 100 °C. Column eluent 100% EtOAc. Yield: 0.090 g, 76%, solid material. Anal. Calcd for $C_{15}H_{15}ClN_4O_4$: C, 51.36; H, 4.31; N, 15.98. Found: C, 51.06; H, 4.52; N, 15.68. ^{1}H (CD_3OD) δ 7.48 (m, 3H), 7.38 (m, 2H), 6.45 (s, 1H), 6.34 (s, 1H), 4.07 (d, J = 17.1 Hz, 1H), 4.01 (d, J = 17.1 Hz, 1H), 3.82 (s, 3H). ^{13}C (CD_3OD) δ 174.5, 170.3, 152.0, 151.4, 133.7, 131.1, 130.8, 130.7, 127.8, 112.3, 63.9, 53.7, 44.7.

7.1.17. 2-(3-(2-Amino-2-oxoethylamino)-5-chloro-2-oxo-6-phenylpyrazin-1(2*H*)-yl)acetic acid benzyl ester (22)

Compound **22** was prepared according to general method B, using the pyrazinone/P2 core **10** (0.201 g, 0.52 mmol), HCl × $HGlyNH_2$ (0.170 g, 1.5 mmol), DIPEA (0.36 mL, 2.1 mmol) and MeCN (3.0 mL). Microwave heating, 150 min at 100 °C. Column eluent 100% EtOAc. Yield: 0.158 g, 71%, solid material. Anal. Calcd for $C_{21}H_{19}ClN_4O_4$: C, 59.09; H, 4.49; N, 13.13. Found: C, 59.30; H, 4.69; N, 12.84. ^{1}H NMR (CD_3CN) δ 7.48–7.41 (m, 3H), 7.36 (m, 3H), 7.25 (m, 4H), 7.01 (m, 1H), 5.08 (s, 2H), 4.39 (s, 2H), 3.99 (m, 2H). ^{13}C NMR (CD_3CN) δ 172.0, 168.3, 151.9, 150.0, 136.5, 132.6, 131.4, 130.7, 130.1, 129.6, 129.4, 129.3, 126.1, 125.3, 68.1, 48.6, 44.4.

7.1.18. 2-(3-(2-Amino-2-oxoethylamino)-6-benzyl-5-chloro-2-oxopyrazin-1(2*H*)-yl)acetic acid benzyl ester (23)

Compound **23** was prepared according to general method B, using the pyrazinone/P2 core **11** (0.179 g, 0.44 mmol), HCl × $HGlyNH_2$ (0.147 g, 1.3 mmol), DIPEA (0.30 mL, 1.8 mmol) and MeCN (3.0 mL). Microwave heating, 150 min at 100 °C. Column eluent 100% EtOAc, EtOAc/MeOH 95:5. Yield: 0.112 g, 58%, solid material. Anal. Calcd for $C_{22}H_{21}ClN_4O_4$: C, 59.93; H, 4.80; N, 12.71. Found: C, 60.21; H, 5.02; N, 12.49. ^{1}H NMR (CD_3CN) δ 7.37 (m, 3H), 7.30 (m, 5H), 7.17 (m, 2H), 5.00 (s, 2H), 4.60 (s, 2H), 4.06 (s, 2H), 3.96 (m, 2H). ^{13}C NMR (CD_3CN) δ 171.4, 167.8, 152.6, 149.5, 137.2, 136.6, 129.9, 129.6, 129.4, 129.1, 128.8, 128.0, 127.4, 123.0, 68.0, 47.4, 44.2, 35.4.

7.1.19. 2-(3-(2-Amino-2-oxoethylamino)-5-chloro-6-methyl-2-oxopyrazin-1(2*H*)-yl)acetic acid (24)

Compound **24** was prepared according to general method B, using the pyrazinone/P2 core **12** (0.173 g, 0.53 mmol), HCl × $HGlyNH_2$ (0.240 g, 2.2 mmol), DIPEA (0.37 mL, 2.1 mmol) and MeCN (3.0 mL). Microwave heating, 180 min at 100 °C. The solvent was removed in vacuo, after which the residue was dissolved in EtOAc. The organic phase was washed with 0.1 M $NaHSO_4$ (2 × 20 mL), the solvent was removed in vacuo and the residue was dried under vacuum. The crude product was dissolved in MeCN (1.25 mL) and H_2O (1.25 mL). K_2CO_3 (0.148 g, 1.1 mmol) was added and the mixture was subjected to microwave heating at 120 °C for 45 min. The solvent was evaporated, and the residue was dissolved in EtOAc (5 mL) and H_2O (5 mL). The aqueous phase was acidified to pH ≈2 by addition of 1 M HCl. The aqueous phase was extracted with EtOAc (3 × 10 mL). The organic phases were combined, the solvent was removed in vacuo and the residue was dried under vacuum. Yield: 0.104 g, 71%, solid material. ^{1}H (DMSO-d_6) δ 7.41 (s, 1H), 7.33 (t, J = 5.9 Hz, 1H), 7.09 (s, 1H), 4.75 (s, 2H), 3.80 (d, J = 5.9 Hz, 2H), 2.21 (s, 3H). ^{13}C (DMSO-d_6) δ 170.3, 168.9, 150.8, 147.6, 123.5, 119.8, 46.1, 43.2, 15.2. LC–MS ($C_9H_{11}ClN_4O_4$, M+H$^+$) 275.1.

7.1.20. General method C, for the ester hydrolysis of compounds 15–23

Compounds **15**–**23** and K_2CO_3 were dissolved in MeCN and H_2O in a 2.0–5.0 mL microwave process vial. The vial was sealed with a

septum and the mixture was subjected to microwave heating at 120 °C, after which it was checked by LC–MS. The MeCN was evaporated in vacuo, EtOAc and water was added and the pH was adjusted to ≈2 by addition of 1 M HCl. The aqueous phase was extracted repeatedly with EtOAc, the organic phases were combined and the solvent removed in vacuo. The product was vacuum dried and thereafter used in the subsequent coupling reaction without further purification or characterization.

7.1.21. General method D, final step in the synthesis of inhibitors 25–39

The crude carboxylic acid derived from compounds **15**–**23** (general method C) or compound **24**, the hydrochloride **2**, **4**, or **6**, HATU and DIEA was dissolved in DCM. The pH of the solution was controlled to be >10 and the progress of the reaction was monitored by LC–MS. When the reaction was complete, more DCM (to approximately twice the volume) was added and the organic phase was washed with 0.1 M $NaHSO_4$ solution. The organic solvent was removed in vacuo, and the crude product was purified on RP-HPLC (Sorbax SB-C8 column (21.2 × 150 mm), MeCN/H_2O gradient with 0.1% TFA). The title compounds were isolated as solids after freeze-drying.

7.1.22. Compound 25

Compound **13** (0.050 g, 0.11 mmol) was dissolved in THF (2.3 mL) and a solution of LiOH (0.030 g, 1.3 mmol) in H_2O (1.5 mL) was added. The reaction mixture was stirred at room temperature for 6.5 h and was monitored by LC–MS. When the reaction was complete, the mixture was acidified with 1 M HCl to pH 1. H_2O (3 mL) was added prior to extraction with EtOAc (3 × 10 mL). The organic phases were combined and the solvent was removed in vacuo yielding the corresponding carboxylic acid. Compound **25** was prepared according to general method D using the carboxylic acid derived from **13**, hydrochloride **4** (0.064 g, 0.24 mmol), HATU (0.055 g, 0.14 mmol) and DIEA (0.13 g, 1.0 mmol) in DCM (2 mL). The reaction was stirred at room temperature for 42 h after which it was heated to 40 °C for another 4 h. Purification by RP-HPLC. Yield: (0.013 g, 18%) of the title compound as a white solid. ^{1}H NMR ($CDCl_3$) (mixture of epimeres, ratio 1:1) δ 10.80 (s, 1H), 10.68 (s, 1H), 8.57 (s, 1H), 8.55 (s, 1H), 7.29 (m, 4H), 6.97 (m, 4H), 6.26 (s, 1H), 6.24 (s, 1H), 5.85 (m, 2H), 5.50 (s, 2 × 1H, overlapping signals), 5.36 (dd, 1H, J = 17.0, 1.8), 5.21 (dd, 1H, J = 17.1, 1.8) 5.16 (dd, 1H, J = 10.2, 1.8), 5.11 (dd, J = 10.3, 1.7), 3.86 (s, 3H), 3.84 (s, 3H), 2.98 (m, 2H), 2.41(s, 3H), 2.40 (s, 3H), 2.23 (m, 1H), 2.02 (dd, 1H, J = 8.3, 5.1), 1.93 (m, 1H), 1.86 (dd, 1H, J = 8.7, 5.4), 1.46 (m, 2H), 1.41 (dd, 1H, J = 9.4, 5.1), 1.23 (dd, 1H, J = 9.6, 5.4), 1.11 (m, 4H), 0.99 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 168.6, 168.4, 168.4, 168.3, 161.2, 161.1, 151.1, 151.0, 149.8, 149.7, 144.1, 143.9, 132.7, 132.5, 130.0, 129.8, 126.5, 126.4, 123.0, 122.8, 119.0, 118.6, 115.7 (overlapping signals), 82.3, 82.2, 67.4, 67.2, 55.7 (overlapping signals), 41.6 (overlapping signals), 37.0, 35.6, 31.2, 30.9, 28.2 (overlapping signals), 25.2, 23.6, 16.3 (overlapping signals), 6.5 (overlapping signals), 6.3, 6.0. RP-HPLC purity; column A: 98.9%; column B: 98.9%). HRMS calcd for $C_{28}H_{34}ClN_5O_8S$ ($M+H^+$) 636.1895, found: 636.1898.

7.1.23. Compounds 26a and 26b

The hydrolysis of the ester of compound **14** has been described by us.[26] Compound **26a** was prepared according to general method D, using the carboxylic acid derived from **14** (0.064 g, 0.16 mmol), hydrochloride **4** (0.072 g, 0.27 mmol), HATU (0.073 g, 0.19 mmol) and DIEA (0.31 mL, 1.8 mmol) in DCM (2 mL). The reaction was stirred at room temperature for 36 h. Purification by column chromatography (DCM/MeOH 95:5) followed by RP-HPLC. Yield: 0.042 g, 43% of the title compound as a white solid. Compound **26b**: 0.005 g isolated as a white solid after repurification of **26a**, the same RP-HPLC system as above. Compound **26a**: ^{1}H NMR ($CDCl_3$) δ 10.75 (s, 1H), 10.64 (s, 1H), 8.61 (s, 1H), 8.57 (s, 1H), 7.49–7.46 (m, 6H), 7.35–7.30 (m, 4H), 6.37 (br s, 1H), 6.35 (br s, 1H), 5.84 (ddd, J = 17.3, 10.3, 7.1 Hz, 1H), 5.82 (ddd, J = 17.3, 10.3, 7.0 Hz, 1H), 5.62 (s, 1H), 5.60 (s, 1H), 5.35 (dd, J = 17.3, 2.0 Hz, 1H), 5.20 (dd, J = 17.3, 1.7 Hz, 1H), 5.15 (dd, J = 10.3, 2.0 Hz, 1H), 5.11 (dd, J = 10.3, 1.7 Hz, 1H), 3.00 (m, 1H), 2.94 (m, 1H), 2.40 (s, 3H), 2.38 (s, 3H), 2.24 (m, 1H), 1.97 (m, 1H), 1.86 (dd, J = 8.7, 5.5 Hz, 1H), 1.53 (s, 9H), 1.52 (s, 9H), 1.46–1.42 (m, 2H), 1.24 (dd, J = 9.7, 5.5 Hz, 1H), 1.16–1.06 (m, 4H), 1.05–0.98 (m, 4H). RP-HPLC purity (column C: 94.8%; column B: 90.2%). HRMS calcd for $C_{27}H_{33}ClN_5O_7S$ ($M+H^+$) 606.1789, found: 606.1786. Compound **26b** ^{1}H NMR ($(CD_3)_2CO$) δ 10.74 (s, 1H), 10.73 (s, 1H), 8.01 (s, 1H), 7.94 (s, 1H), 7.47–7.36 (m, 10H), 6.50 (m, 2H), 6.05 (s, 1H), 6.03 (s, 1H), 5.83–5.71 (m, 2H), 5.17 (dd, J = 17.2, 1.9 Hz, 1H), 5.16 (dd, J = 17.2, 1.9 Hz, 1H), 5.04 (dd, J = 10.3, 1.9 Hz, 1H), 5.00 (dd, J = 10.3, 1.9 Hz, 1H), 2.95 (m, 1H), 2.93 (m, 1H), 2.35 (s, 3H), 2.32 (s, 3H), 2.24 (m, 1H), 2.19 (m, 1H), 1.76 (dd, J = 8.4, 5.8 Hz, 1H), 1.72 (dd, J = 8.2, 5.2 Hz, 1H), 1.42 (dd, J = 9.5, 5.2 Hz, 1H), 1.40 (dd, J = 9.6, 4.8 Hz, 1H), 1.26–1.18 (m, 4H), 1.16–0.98 (m, 4H). RP-HPLC purity: column C: >99%; column D: >99%). HRMS calcd for $C_{22}H_{25}ClN_5O_5S$ ($M+H^+$) 506.1265, found: 506.1246.

7.1.24. Compound 27

The hydrolysis of the ester of compound **14** has been described by us.[26] Compound **27** was prepared according to general method D, using the carboxylic acid derived from **14** (0.065 g, 0.17 mmol), hydrochloride **6** (0.063 g, 0.27 mmol), HATU (0.075 g, 0.20 mmol) and DIEA (0.31 mL, 1.8 mmol) in DCM (2.5 mL). The reaction was stirred at room temperature for 48 h followed by 24 h at 40 °C. Purification by column chromatography (DCM/MeOH 95:5) followed by RP-HPLC. Yield: 0.008 g, 8% of the title compound as a white solid. ^{1}H NMR, approx. 3:1 diastereomeric ratio, major stereoisomer reported ($CDCl_3$) δ 10.05 (s, 1H), 8.37 (s, 1H), 7.50–7.47 (m, 3H), 7.29 (m, 2H), 7.36 (m, 1H), 5.72 (s, 1H), 4.29 (m, 1H), 2.89 (m, 1H), 2.41 (s, 3H), 1.93 (m, 1H), 1.70 (m, 1H), 1.53 (s, 9H), 1.37 (m, 1H), 1.17–1.06 (m, 3H), 0.60 (m, 1H), 0.43 (m, 1H), 0.31 (m, 1H), 0.01 (m, 2H). ^{13}C ($CDCl_3$) δ 170.0, 167.5, 151.3, 149.7, 143.7, 131.8, 130.5, 130.3, 128.5, 127.1, 126.3, 82.5, 66.9, 56.0, 34.7, 31.1, 28.2, 16.5, 7.4, 6.4, 6.1, 4.6, 4.1. RP-HPLC purity: column A: 96.6%; column B: 97.2%. HRMS calcd for $C_{27}H_{35}ClN_5O_7S$ ($M+H^+$) 608.1946, found: 608.1934.

7.1.25. Compound 28

The hydrolysis of the ester of compound **14** has been described by us.[26] Compound **28** was prepared according to General method D, using the carboxylic acid derived from **14** (0.045 g, 0.11 mmol), hydrochloride **2** (0.046 g, 0.12 mmol), HATU (0.050 g, 0.13 mmol) and DIEA (0.17 mL, 0.98 mmol) in DCM (2 mL). The reaction was stirred at 40 °C for 22 h. Purification by column chromatography (isohexane/EtOAc 1:2; 1:3) followed by RP-HPLC. Yield: 0.021 g, 26% of the title compound as a white solid. ^{1}H ($(CD_3)_2CO$) δ 10.61 (s, 1H), 8.48 (dd, J = 8.5, 1.2 Hz, 1H), 8.31 (s, 1H), 8.23 (m, 2H), 8.06 (m, 2H), 7.93 (dd, J = 8.0, 1.5 Hz, 1H), 7.62–7.53 (m, 4H), 7.48 (m, 2H), 7.18 (ddd, J = 8.0, 7.3, 1.2 Hz, 1H), 6.43 (s, 1H), 2.42 (s, 3H), 1.51 (s, 9H). ^{13}C ($(CD_3)_2CO$) δ 168.1, 166.1, 152.2, 150.0, 144.3, 144.2, 141.0, 135.1 (q, J = 32.3), 135.1, 133.8, 130.3 (two peaks), 130.1, 130.0, 129.9, 128.4, 127.1 (q, J = 4.0), 125.6, 124.6 (q, J = 272.9), 124.1, 122.2, 119.1, 81.8, 67.0, 28.2, 17.0. RP-HPLC purity: column C: 95.8%; column D: 94.8%. HRMS calcd for $C_{32}H_{30}ClF_3N_5O_7S$ ($M+H^+$) 720.1507, found: 720.1527.

7.1.26. Compound 29a

The synthesis and characterization of **29a** has recently been published by us.[26]

7.1.27. Compound 29b

The synthesis and characterization of **29b** has recently been published by us.[26]

7.1.28. Compound 30

Compound **15** (0.069 g, 0.18 mmol) was subjected to ester hydrolysis according to general method C using K_2CO_3 (0.049 g, 0.36 mmol), MeCN (0.75 mL) and H_2O (0.50 mL). Microwave heating for 55 min followed by work-up produced the corresponding carboxylic acid (0.0962 g, 100%). Compound **30** was prepared according to general method D, using the carboxylic acid derived from **15** (0.047 g, 0.13 mmol), hydrochloride **4** (0.051 g, 0.19 mmol), HATU (0.059 g, 0.15 mmol) and DIEA (0.20 mL, 1.1 mmol) in DCM (2.0 mL). The reaction was stirred at 40 °C over night. Purification by RP-Si column chromatography (MeCN/H_2O 1:1, 100% MeCN) followed by RP-HPLC. Yield: 0.017 g, 23% of the title compound as a white solid. ^{1}H NMR (1:1 diastereomeric ratio, $CDCl_3$) δ 11.0 (s, 1H), 10.9 (s, 1H), 7.47 (m, 6H), 7.33 (m, 2H), 7.29 (m, 2H), 6.51 (m, 2H), 6.17 (s, 1H), 6.15 (s, 1H), 5.88–5.77 (m, 2H), 5.54 (s, 1H), 5.53 (s, 1H), 5.37 (ddd, J = 17.2, 1.8, 0.7 Hz, 1H), 5.19 (ddd, J = 17.2, 1.7, 0.7 Hz, 1H), 5.15 (ddd, J = 10.3, 1.8, 0.7 Hz, 1H), 5.10 (ddd, J = 10.3, 1.7, 0.7 Hz, 1H), 3.38 (m, 4H), 2.95 (m, 2H), 2.29 (s, 3H), 2.27 (s, 3H), 2.24 (m, 1H), 2.04 (dd, J = 8.3, 5.4 Hz, 1H), 1.88 (m, 1H), 1.85 (dd, J = 8.6, 5.4 Hz, 1H), 1.62–1.49 (m, 4H), 1.42 (m, 1H), 1.22 (dd, J = 9.6, 5.4 Hz, 1H), 1.15–1.05 (m, 4H), 1.02–0.95 (m, 4H), 0.97 (s, 9H), 0.96 (s, 9H). ^{13}C NMR (1:1 diastereomeric ratio, $CDCl_3$) δ 169.0, 168.9, 168.7, 168.6, 151.9, 151.8, 148.9, 148.7, 132.8, 132.4, 131.9, 131.6, 130.3 (two peaks), 130.2 (two peaks), 128.5, 128.3 (two peaks), 128.1, 118.9, 118.6, 118.3, 118.1, 67.1, 66.8, 42.6 (two peaks), 41.7, 41.6, 38.0 (two peaks), 37.2, 35.5, 31.2, 30.9, 30.1, 30.0, 29.6 (two peaks), 25.2, 23.7, 15.8, 15.7, 6.7, 6.6, 6.2, 5.8. RP-HPLC purity: column C: 96.7%; column D: 98.3%. HRMS calcd for $C_{28}H_{37}ClN_5O_5S$ ($M+H^+$) 590.2204, found: 590.2227.

7.1.29. Compound 31

Compound **16** (0.102 g, 0.24 mmol) was subjected to ester hydrolysis according to General method C using K_2CO_3 (0.100 g, 0.72 mmol), MeCN (1.5 mL) and H_2O (1.0 mL). Microwave heating for 45 min followed by work-up produced the corresponding carboxylic acid. Compound **31** was prepared according to general method D, using the carboxylic acid derived from **16** (0.049 g, 0.12 mmol), hydrochloride **4** (0.048 g, 0.18 mmol), HATU (0.055 g, 0.15 mmol) and DIEA (0.20 mL, 1.1 mmol) in DCM (2.0 mL). The reaction was stirred at 40 °C over night. Purification by RP-Si column chromatography (MeCN/H_2O 1:4, 100% MeCN) followed by RP-HPLC. Yield: 0.023 g, 26% of the title compound as its TFA salt, as a white solid. $^{1\,H\ NMR}$ (1:1 diastereomeric ratio, $CDCl_3$) δ 10.84 (s, 1H), 10.82 (s, 1H), 7.37 (m, 6H), 7.28 (m, 4H), 7.18 (m. 1H), 7.11 (m, 1H), 5.91 (ddd, J = 17.1, 10.4, 8.4 Hz, 1H), 5.68 (ddd, J = 17.1, 10.3, 8.7 Hz, 1H), 5.56 (s, 1H), 5.54 (s, 1H), 5.32 (dd, J = 17.1, 2.1 Hz, 1H), 5.21 (dd, J = 17.1, 2.0 Hz, 1H), 5.12–5.07 (m, 2H), 4.02–3.58 (m, 18H), 3.02–2.78 (m, 8H), 2.29 (s, 2 × 3H, overlapping), 2.23 (m, 2H), 1.98 (dd, J = 8.4, 5.6 Hz, 1H), 1.82 (dd, J = 8.7, 5.1 Hz, 1H), 1.50 (dd, J = 9.6, 5.1 Hz, 1H), 1.47 (dd, J = 9.5, 5.6 Hz, 1H), 1.41–1.34 (m, 2H), 1.32–1.24 (m, 2H), 1.1–1.02 (m, 4H). ^{13}C NMR (1:1 diastereomeric ratio, $CDCl_3$) δ 169.9, 169.4, 167.9, 167.7, 152.3 (two peaks), 149.5 (two peaks), 133.3, 133.1 (two peaks), 132.9, 129.2 (two peaks), 129.1, 129.0, 128.7, 128.6, 127.4, 127.2, 121.5, 121.4, 118.4, 118.3, 66.0, 65.6, 64.1 (two peaks), 63.8, 63.7, 55.7, 55.1, 53.9, 53.7, 51.7, 51.4, 42.1, 41.2, 36.2, 35.7 (two peaks), 35.0, 31.4, 31.3, 25.3, 22.4, 15.8 (two peaks), 6.6, 6.4, 6.3, 6.2. RP-HPLC purity: column C: >99%; column D: >99%. HRMS calcd for $C_{28}H_{36}ClN_6O_6S$ ($M+H^+$) 619.2106, found: 619.2108.

7.1.30. Compound 32

Compound **17** (0.109 g, 0.27 mmol) was subjected to ester hydrolysis according to General method C using K_2CO_3 (0.114 g, 0.82 mmol), MeCN (1.5 mL) and H_2O (1.0 mL). Microwave heating for 45 min followed by work-up produced the corresponding carboxylic acid (0.106 g, 99%). Compound **32** was prepared according to general method D, using the carboxylic acid derived from **17** (0.054 g, 0.14 mmol), hydrochloride **4** (0.054 g, 0.20 mmol), HATU (0.062 g, 0.16 mmol) and DIEA (0.22 mL, 1.3 mmol) in DCM (2.0 mL). The reaction was stirred at 40 °C over night. Purification by RP-Si column chromatography (MeCN/H_2O 1:4, 1:1, 100% MeCN) followed by RP-HPLC. Yield: 0.021 g, 26% of the title compound as a white solid. ^{1}H NMR (approx. 10:1 diastereomeric ratio, major stereoisomer reported, $CDCl_3$) δ 10.99 (s, 1H), 7.49–7.46 (m, 3H), 7.34–7.20 (m, 7H), 6.70 (m, 1H), 6.12 (s, 1H), 5.84 (ddd, J = 17.1, 10.3, 8.4 Hz, 1H), 5.33 (s, 1H), 5.37 (ddd, J = 17.1, 1.8, 0.8 Hz, 1H), 5.17 (ddd, J = 10.3, 1.8, 0.7 Hz, 1H), 3.65 (m, 2H), 2.95 (m, 2H), 2.91 (m, 1H), 2.29 (s, 3H), 2.23 (m, 1H), 1.86 (dd, J = 8.6, 5.4 Hz, 1H), 1.42 (m, 1H), 1.21 (dd, J = 9.7, 5.4 Hz, 1H), 1.57 (m, 1H), 1.07 (m, 1H), 0.98 (m, 1H). ^{13}C NMR (approx. 10:1 diastereomeric ratio, major stereoisomer reported, $CDCl_3$) δ 168.9, 168.4, 151.8, 148.9, 139.0, 123.7, 131.9, 130.3, 130.2, 128.9, 128.6, 128.5, 128.3, 126.5, 118.9, 118.6, 67.1, 42.8, 41.6, 37.1, 35.1, 31.3, 23.6, 15.8, 6.7, 6.2. RP-HPLC purity: column C: 97.6%; column D: >99%. HRMS calcd for $C_{30}H_{33}ClN_5O_5S$ ($M+H^+$) 610.1891, found: 610.1888.

7.1.31. Compound 33

Compound **18** (0.058 g, 0.16 mmol) was subjected to ester hydrolysis according to general method C using K_2CO_3 (0.045 g, 0.32 mmol), MeCN (0.75 mL) and H_2O (0.75 mL). Microwave heating for 45 min followed by work-up produced the corresponding carboxylic acid. Compound **33** was prepared according to general method D, using the carboxylic acid derived from **18**, hydrochloride **4** (0.054 g, 0.20 mmol), HATU (0.075 g, 0.20 mmol) and DIEA (0.22 mL, 1.3 mmol) in DCM (2.5 mL). The reaction was stirred at 40 °C for 27 h followed by room temperature for 36 h. Purification by column chromatography (isohexane/EtOAc 4:1) followed by RP-HPLC. Yield: 0.025 g, 28% (two steps) of the title compound as a white solid. ^{1}H NMR (CD_3OD) δ 7.43–7.30 (m, 10H), 6.44 (s, 1H), 6.43 (s, 1H), 5.67 (m, 1H), 5.31 (ddd, J = 17.2, 1.8, 0.7 Hz, 1H), 5.30 (ddd, J = 17.2, 1.8, 0.7 Hz, 1H), 5.13 (dd, J = 10.3, 1.8 Hz, 1H), 5.12 (dd, J = 10.3, 1.8 Hz, 1H), 4.09 (d, J = 16.8 Hz, 1H), 4.08 (d, J = 16.8 Hz, 1H), 4.02 (d, J = 16.8 Hz, 1H), 4.01 (d, J = 16.8 Hz, 1H), 2.94 (m, 1H), 2.29 (m, 1H), 2.24 (s, 3H), 1.88 (dd, J = 8.2, 5.5 Hz, 1H), 1.39 (m, 1H), 1.21 (m, 2H), 1.06 (m, 2H). ^{13}C NMR (CD_3OD) δ 174.0, 170.8, 170.6, 153.1 (two peaks), 150.0, 149.9, 135.1 (two peaks), 134.3, 129.5, 129.4, 129.3, 126.8, 122.1, 118.6, 63.3 (two peaks), 45.3, 43.1, 43.0, 35.3 (two peaks), 32.1, 22.7, 16.5 (two peaks), 6.5 (two peaks). RP-HPLC purity: column C: 97.7%; column D: >99%. HRMS calcd for $C_{24}H_{28}ClN_6O_6S$ ($M+H^+$) 563.1480, found: 563.1494.

7.1.32. Compound 34

Compound **19** (0.063 g, 0.16 mmol) was subjected to ester hydrolysis according to general method C using K_2CO_3 (0.046 g, 0.33 mmol), MeCN (1.25 mL) and H_2O (1.0 mL). Microwave heating for 45 min followed by work-up produced the corresponding carboxylic acid. Compound **34** was prepared according to general method D, using the carboxylic acid derived from **19** (0.061 g, 0.16 mmol), hydrochloride **4** (0.059 g, 0.22 mmol), HATU (0.075 g, 0.20 mmol) and DIEA (0.22 mL, 1.3 mmol) in DCM (2.5 mL). The reaction was stirred at 40 °C for 27 h followed by room temperature for 36 h. Work-up by extractions followed by RP-HPLC. Yield: 0.032 g, 34% (two steps) of the title compound as a white solid. ^{1}H NMR (1:1 diastereomeric ratio, CD_3OD) δ 7.43–7.34 (m, 10H), 6.00

(s, 2 × 1H, overlapping), 5.85–5.74 (m, 2H), 5.31–5.25 (m, 2H), 5.11–5.06 (m, 2H), 4.31 (m, 2H), 4.24 (m, 2H), 3.09 (s, 2 × 3H, overlapping), 2.98 (s, 2 × 3H, overlapping), 2.98–2.90 (m, 2H), 2.33 (s, 3H), 2.29 (s, 3H), 2.28–2.19 (m, 2H), 1.84–1.78 (m, 2H), 1.50 (ddd, J = 9.5, 5.1, 1.0 Hz, 1H), 1.45 (dd, J = 9.6, 5.2 Hz, 1H), 1.32–1.28 (m, 3H), 1.20 (m, 1H), 1.10–0.95 (m, 4H). ^{13}C NMR (1:1 diastereomeric ratio, CD_3OD) δ 171.3 (two peaks), 171.1, 170.8, 170.5, 170.4, 153.7, 153.4, 150.4, 150.3, 134.7, 134.6, 134.5, 134.1, 130.2 (two peaks), 130.1 (two peaks), 130.0, 129.8, 128.6, 128.3, 121.9, 121.8, 118.6 (two peaks), 66.8, 66.7, 43.5 (two peaks), 43.1, 42.9, 36.9, 36.8, 36.7 (two peaks), 36.2 (two peaks), 32.1 (two peaks), 24.9, 24.4, 16.2, 16.1, 6.8 (two peaks), 6.6, 6.5. RP-HPLC purity: column C: 98.2%; column D: >99%. HRMS calcd for $C_{26}H_{32}ClN_6O_6S$ ($M+H^+$) 591.1793, found: 591.1779.

7.1.33. Compound 35

Compound **20** (0.066 g, 0.15 mmol) was subjected to ester hydrolysis according to general method C using K_2CO_3 (0.043 g, 0.31 mmol), MeCN (1.0 mL) and H_2O (1.0 mL). Microwave heating for 45 min followed by work-up produced the corresponding carboxylic acid. Compound **35** was prepared according to general method D, using the carboxylic acid derived from **20**, hydrochloride **4** (0.053 g, 0.20 mmol), HATU (0.075 g, 0.20 mmol) and DIEA (0.22 mL, 1.3 mmol) in DCM (2.5 mL). The reaction was stirred at 40 °C for 27 h followed by room temperature for 36 h. Work-up by extractions followed by RP-HPLC. Yield: 0.012 g, 13% (two steps) of the title compound as a white solid. ^{1}H NMR (1:1 diastereomeric ratio, CD_3OD) δ 7.38 (m, 10H), 6.00 (m, 2H), 5.80 (ddd, J = 17.3, 10.4, 7.2 Hz, 1H), 5.78 (ddd, J = 17.4, 10.4, 7.2 Hz, 1H), 5.34–5.24 (m, 2H), 5.15–5.06 (m, 2H), 4.34 (m, 2H), 4.27 (m, 2H), 3.76–3.68 (m, 8H), 3.62–3.57 (m, 8H), 2.97–2.92 (m, 2H), 2.32 (s, 3H), 2.30 (s, 3H), 2.27–2.20 (m, 2H), 1.83–1.79 (m, 2H), 1.49 (m, 1H), 1.42 (m, 1H), 1.33–1.25 (m, 2H), 1.23–1.16 (m, 2H), 1.10–0.90 (m, 4H). ^{13}C NMR (1:1 diastereomeric ratio, CD_3OD) δ 171.4, 171.3, 171.0, 170.9, 169.3, 1692, 153.7, 153.4, 150.4, 150.3, 134.7, 134.6, 134.4, 134.1, 130.2, 130.1, 129.9, 129.8, 129.7 (two peaks), 128.6, 128.3, 122.0, 121.9, 118.7, 118.6, 67.9 (two peaks), 67.7 (two peaks), 66.9, 66.8, 46.6 (two peaks), 43.9 (two peaks), 43.3 (two peaks), 43.1, 42.9, 36.8 (two peaks), 32.1 (two peaks), 24.9, 24.4, 16.1 (two peaks), 6.8, 6.7, 6.6, 6.5. RP-HPLC purity: column C: >99%; column D: >99%. HRMS calcd for $C_{28}H_{34}ClN_6O_7S$ ($M+H^+$) 633.1898, found: 633.1891.

7.1.34. Compound 36

Compound **21** (0.057 g, 0.16 mmol) was subjected to ester hydrolysis according to general method C using K_2CO_3 (0.043 g, 0.31 mmol), MeCN (1.0 mL) and H_2O (0.75 mL). Microwave heating for 45 min followed by work-up produced the corresponding carboxylic acid. Compound **36** was prepared according to general method D, using the carboxylic acid derived from **21**, hydrochloride **2** (0.076 g, 0.20 mmol), HATU (0.073 g, 0.19 mmol) and DIEA (0.20 mL, 1.1 mmol) in DCM (2.5 mL). The reaction was stirred at 40 °C for 8 h followed by room temperature for 60 h. Work-up by extractions followed by purification on RP-HPLC. Yield: 0.019 g, 19% (two steps) of the title compound as a greyish solid. ^{1}H NMR (CD_3OD) δ 8.38 (dd, J = 8.4, 1.2 Hz, 1H), 8.32 (m, 2H), 7.96 (m, 2H), 7.74 (dd, J = 8.0, 1.5 Hz, 1H), 7.55 (ddd, J = 8.4, 7.4, 1.5 Hz, 1H), 7.49 (m, 5H), 7.19 (ddd, J = 8.0, 7.4, 1.2 Hz, 1H), 6.56 (s, 1H), 6.35 (s, 1H), 4.16 (d, J = 17.3 Hz, 1H), 4.01 (d, J = 17.3 Hz, 1H). ^{13}C NMR (CD_3OD) δ 171.2, 170.7, 169.2, 152.1, 151.4, 144.4, 140.4 (two peaks), 136.2 (q, J = 32.6 Hz), 135.4, 133.8, 131.1, 130.8, 130.7, 130.5, 127.5 (q, J = 4.0 Hz), 124.9, 124.8 (q, J = 272.1 Hz), 122.7, 120.7, 113.1, 64.1, 46.5. RP-HPLC purity: column C: 98.8%; column D: 98.3%. HRMS calcd for $C_{28}H_{23}ClF_3N_5O_7S$ ($M+H^+$) 664.0881, found: 664.0880. LC–MS ($M+H^+$) 664.2.

7.1.35. Compound 37

Compound **22** (0.067 g, 0.16 mmol) was subjected to ester hydrolysis according to general method C using K_2CO_3 (0.042 g, 0.30 mmol), MeCN (1.0 mL) and H_2O (0.75 mL). Microwave heating for 45 min followed by work-up produced the corresponding carboxylic acid. Compound **37** was prepared according to general method D, using the carboxylic acid derived from **22**, hydrochloride **2** (0.076 g, 0.20 mmol), HATU (0.073 g, 0.19 mmol) and DIEA (0.20 mL, 1.1 mmol) in DCM (2.5 mL). The reaction was stirred at 40 °C for 8 h followed by room temperature for 60 h. Work-up by extractions followed by RP-HPLC purification. Yield: 0.019 g, 19% (two steps) of the title compound as a greyish solid. ^{1}H NMR (CD_3OD) δ 8.40 (dd, J = 8.4, 1.4 Hz, 1H), 8.31 (m, 2H), 7.97 (m, 2H), 7.74 (dd, J = 8.0, 1.5 Hz, 1H), 7.57 (ddd, J = 8.4, 7.4, 1.5 Hz, 1H), 7.52–7.46 (m, 3H), 7.40–7.36 (m, 2H), 7.20 (ddd, J = 8.0, 7.4, 1.4 Hz, 1H), 4.49 (s, 2H), 4.13 (s, 2H). ^{13}C NMR (CD_3OD) δ 170.8, 170.5, 169.2, 152.6, 150.7, 144.3, 140.5, 136.3 (q, J = 33.0 Hz), 135.4, 133.4, 131.9, 130.9 (two peaks), 130.3, 130.2, 127.5 (q, J = 3.9 Hz), 126.9, 126.8, 124.9, 124.8 (q, J = 271.9 Hz) 122.9, 120.6, 46.6, 43.4. RP-HPLC purity: column C: 98.1%; column D: 95.3%. HRMS calcd for $C_{28}H_{23}ClF_3N_5O_7S$ ($M+H^+$) 664.0881, found: 664.0950. LC–MS ($M+H^+$) 664.2.

7.1.36. Compound 38

Compound **23** (0.065 g, 0.15 mmol) was subjected to ester hydrolysis according to General method C using K_2CO_3 (0.043 g, 0.31 mmol), MeCN (0.75 mL) and H_2O (0.75 mL). Microwave heating for 45 min followed by work-up produced the corresponding carboxylic acid. Compound **38** was prepared according to general method D, using the carboxylic acid derived from **23**, hydrochloride **2** (0.076 g, 0.20 mmol), HATU (0.072 g, 0.19 mmol) and DIEA (0.20 mL, 1.1 mmol) in DCM (2.5 mL). The reaction was stirred at 40 °C for 8 h followed by room temperature for 60 h. Work-up by extractions followed by RP-HPLC purification. Yield: 0.012 g, 11% (two steps) of the title compound as a greyish solid. ^{1}H NMR (CD_3OD) δ 8.37 (dd, J = 8.4, 1.2 Hz, 1H), 8.29 (m, 2H), 7.95 (m, 2H), 7.74 (dd, J = 8.0, 1.5 Hz, 1H), 7.58 (ddd, J = 8.4, 7.4, 1.6 Hz, 1H), 7.33–7.29 (m, 2H), 7.26–7.16 (m, 4H), 4.69 (s, 2H), 4.12 (s, 2H), 4.10 (s, 2H). ^{13}C NMR (CD_3OD) δ 170.8, 170.2, 169.2, 153.3, 150.2, 144.4, 140.3, 137.5, 135.4, 130.8, 130.4, 130.3, 129.0, 128.2, 128.0, 127.3 (q, J = 3.8 Hz), 125.0, 124.3, 122.9, 121.0, 47.7, 46.5, 36.0. N.B.! In this case is the quartet at approximately 125 ppm, $J \approx 272$ Hz, too weak for detection. RP-HPLC purity: column C: >99%; column D: 97.9%. HRMS calcd for $C_{29}H_{25}ClF_3N_5O_7S$ ($M+H^+$) 678.1037, found: 678.1039. LC–MS ($M+H^+$) 678.2.

7.1.37. Compound 39

Compound **39** was prepared according to general method D, using compound **24** (0.043 g, 0.16 mmol), hydrochloride **2** (0.076 g, 0.20 mmol), HATU (0.077 g, 0.20 mmol) and DIEA (0.20 mL, 1.1 mmol) in DCM (2.5 mL). The reaction was stirred at 40 °C for 2 days followed by room temperature for 1 day. Work-up by extractions followed by RP-HPLC purification. Yield: 0.016 g, 17% of the title compound as a greyish solid. ^{1}H NMR (DMSO-d_6) δ 8.15 (m, 2H), 7.96 (m, 2H), 7.83 (m, 1H), 7.78 (m, 1H), 7.49 (m, 1H), 7.39 (s, 1H), 7.31 (m, 1H), 7.18 (m, 1H), 7.11 (s, 1H), 4.77 (s, 2H), 3.82 (m, 2H), 2.15 (s, 3H). RP-HPLC purity: column C: >99%; column D: >99%. HRMS calcd for $C_{23}H_{21}ClF_3N_6O_6S$ ($M+H^+$) 601.0884, found: 601.0892.

7.2. Enzyme inhibition

The protease activity of the HCV NS3 protein (full-length protease–helicase/NTPase) was assessed in a FRET-assay as described.[32,46] Briefly, 1 nM enzyme was incubated for 10 min at 30 °C in 50 mM HEPES pH 7.5, 10 mM DTT, 40% glycerol, 0.1%

n-octyl-β-D-glucoside, 3.3% DMSO with 25 μM of the peptide cofactor 2K-NS4A (KKGSVVIVGRIVLSGK), and inhibitor. The reaction was started by the addition of 0.5 μM substrate (Ac-DED(Edans) EEAbuψ[COO]ASK(Dabcyl)-NH_2) obtained from AnaSpec Inc. (San Jose, USA). Non-linear regression analysis of the data was made using Grafit 5.0.8 (Erithacus software limited).

7.3. Computational methodology

The crystal structure used (PDB code: 1CU1) shows the protease active site occupied by the C-terminal residues of the cleavage product. The active site was made available for the docking studies by deleting these terminal residues (624–631). All the crystallographic waters were removed and the protein was prepared for docking using the protein preparation tool implemented in the Schrödinger Suite 2008.[47] The generated structures were subjected to restrained minimization using the OPLS-2005 force field with 'normal' BatchMin cutoffs (7.0 Å VDW; 12.0 Å ELE).

To account for the conformational changes in the protein, induced fit docking was carried out using FLO (also called QXP).[48] A truncated protein structure that included only amino acids within 9 Å from the residues 624–631 (C-terminus) was used in the docking studies using FLO. Protein flexibility is accounted for by allowing crucial amino acid residues in the binding pocket to move freely up to 0.2 Å. Movement larger than 0.2 Å was penalized by 20.0 kJ/mol/Å.[48] Flexible residues occupying the active site viz., Arg155, Gln526 and Lys136 were given full conformational freedom. Constraints were applied to the inhibitor to restrict its translation away from the active site: H-bonds to the back-bone carbonyl and NH of Ala157 to the corresponding back-bone atoms of P3, between the back-bone carbonyl of Arg155 and the NH of the P1, and between the NHs of Gly137 and Ser139 and the P1 carbonyl). For each inhibitor, 10 unique binding poses were generated using 2000 Monte Carlo perturbation cycles. These poses were further subjected to 20 steps of simulated annealing followed by energy minimization. Each cycle involved 400 rapid Monte Carlo steps generating unique conformations within an energy window of 50 kJ/mol/Å and an RMSD >0.5 Å. Each of the complexes was submitted to a 3 fs dynamics at 600 K after 3000 fs of equilibration steps. The maximum movement of an atom in any single step was limited to 0.1 Å. Hydrogen vibrations were damped by assigning an atomic weight of 10. Among the 10 best poses generated, the most plausible binding mode conformation for each inhibitor was selected based on visual inspection.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.101. These data include MOL files and InChiKeys of the most important compounds described in this article.

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