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β-Amino acid substitutions and structure-based CoMFA modeling of hepatitis C virus NS3 protease inhibitors

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Abstract—In an effort to develop a new type of HCV NS3 peptidomimetic inhibitor, a series of tripeptide inhibitors incorporating a mix of α- and β-amino acids has been synthesized. To understand the structural implications of β-amino acid substitution, the P_1, P_2, and P_3 positions of a potent tripeptide scaffold were scanned and combined with carboxylic acid and acyl sulfonamide C-terminal groups. Inhibition was evaluated and revealed that the structural changes resulted in a loss in potency compared with the α-peptide analogues. However, several compounds exhibited μM potency. Inhibition data were compared with modeled ligand–protein binding poses to understand how changes in ligand structure affected inhibition potency. The P_3 position seemed to be the least sensitive position for β-amino acid substitution. Moreover, the importance of a proper oxyanion hole interaction for good potency was suggested by both inhibition data and molecular modeling. To gain further insight into the structural requirements for potent inhibitors, a three-dimensional quantitative structure–activity relationship (3D-QSAR) model has been constructed using comparative molecular field analysis (CoMFA). The most predictive CoMFA model has $q^2 = 0.48$ and $r^2_{pred} = 0.68$.

1. Introduction

Hepatitis C Virus (HCV) is the major cause of chronic liver disease and has an estimated world prevalence of 2–3%. Consequently, HCV is also the primary indication for liver transplantations.[1] Pegylated interferon-α and ribavirin constitute standard therapy for people infected with HCV, but the sustained virological response rate is only roughly 55% and is least effective for the most prevalent genotype (genotype 1).[2] Thus, considering the seriousness of the impact of HCV infection on global health, the development of more efficient therapies is extremely important. This is reflected by the rapidly increasing number of patents and publications from both academic and industrial groups devoted to HCV-related research.

Of several possible drug targets for HCV therapy, one thoroughly studied and promising target is the protease of the virally encoded HCV NS3 protein. The NS3 protein is a bifunctional enzyme with both helicase/NTPase and protease activities. The NS3 protease, in complex with the NS4A co-factor, is responsible for proteolytic processing at four sites of the HCV polyprotein, making it crucial for viral replication.[3] Additionally, recent results indicate that the NS3 protease is involved in the cellular mechanisms of viral persistence.[4] Thus, inhibition of the protease may have dual effects: both inhibition of viral replication as well as restoration of the host immune response. Indeed, HCV NS3 protease inhibitors have shown proof-of-concept in clinical trials in the cases of BILN 2061 (ciluprevir),[5] VX-950 (telaprevir),[6] SCH 503034 (boceprevir),[7] and ITMN-191[8] (Fig. 1).

Although ciluprevir has been subjected to clinical testing,[5] it has since been removed due to cardiac toxicity.[9] Telaprevir and boceprevir are currently in phase II trials[10,11] while InterMune is currently conducting phase 1b clinical trials on ITMN-191.[12] Ciluprevir and ITMN-191 are non-covalent product-based inhibitors,[13,14] containing a C-terminal carboxylic acid and carboxylic acid bioisostere (acyl sulfonamide), respec-

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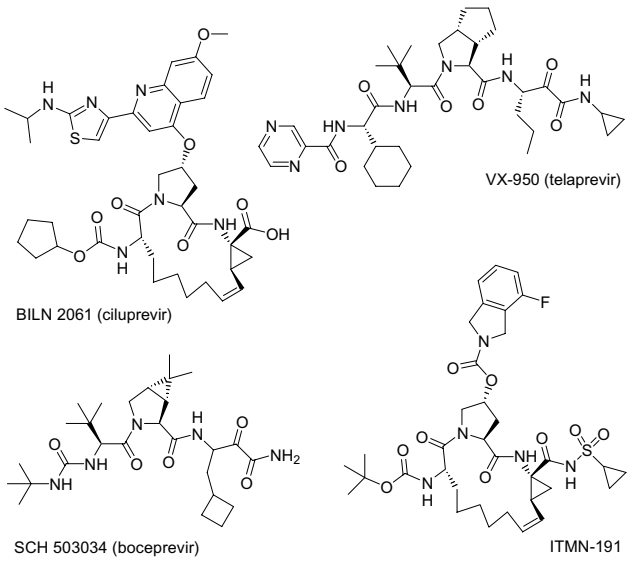


Figure 1. Structures of the HCV NS3 protease inhibitors that have entered clinical trials.

tively. Telaprevir and boceprevir are serine trap inhibitors[15] containing an electrophilic functional group (α-ketoamide), capable of forming a reversible covalent bond with the enzyme.

While some inhibitors of the HCV NS3 protease have been found using high throughput screening,[16] the majority have been discovered using peptidomimetic design with the natural peptide substrate as the starting point. The development of peptidomimetics has emerged as an attractive approach for overcoming the limitations inherent to peptides, seeking to improve their bioavailability and stability. Peptide truncation, the use of non-natural amino acids, side chain extension, and cyclizations are peptidomimetic techniques that have been used to reduce the peptide character of HCV NS3 protease inhibitors in order to produce more drug-like and potent compounds, as exemplified by the compounds subjected to clinical trials (Fig. 1).

As seen in Figure 1, all compounds currently in clinical trials are comprised of α-amino acids. However, backbone modifications, such as β-amino acid insertion, may be useful in reducing some negative attributes associated with peptide-like compounds.[17] β-Amino acids differ from their corresponding α-variants in the position where the side chain is attached to the peptide backbone. In the case of a β-amino acid, an extra methylene group is inserted into the backbone and the side chain is attached to one of the two methylene carbons, depending on the type of β-amino acid desired.[18] Figure 2 shows a comparison of α-, β^2-, and β^3-amino acids.

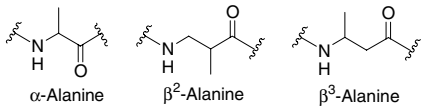


Figure 2. Depiction of α-, β^2- and β^3-amino acid backbone structures.

The insertion of an additional methylene group alters the electronic conditions of the amide bonds and adds additional degrees of conformational freedom. Moreover, peptide recognition and resistance to proteolysis may be affected. Although β-peptides are widely known to be more resistant to proteolysis,[19] studies have shown that peptides comprising a mix of α- and β-amino acids may also be more resistant to proteolytic degradation.[20]

The use of β-amino acids in the development of peptidase/protease inhibitors has previously been successful in both reducing proteolysis and retaining potency.[18,21–24] β-Amino acid substitutions have also been explored as an approach to obtain more selective serine protease inhibitors.[25] Additionally, it has been possible to design ligands more resistant to cleavage by exchanging native α-amino acid residues with a β-amino acid variant.[26,27]

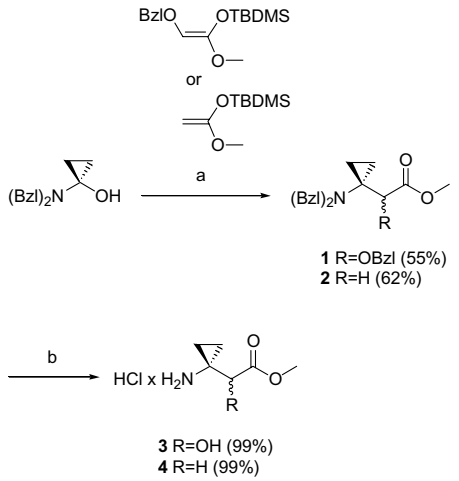
The accessibility of β-amino acids, their potentially enhanced resistance to proteolysis, and the fact that they, to the best of our knowledge, had not been previously used in HCV protease inhibitor design inspired us to explore their use in our ongoing HCV research endeavors. We began the study by selecting a tripeptide inhibitor whose corresponding β-amino acids are commercially available and/or synthetically accessible. A β-amino acid scan was performed, replacing the original α-amino acid with its β-amino acid counterpart in the P_1, P_2, and P_3 positions. Inhibitors comprising both the carboxylic acid and acyl sulfonamide C-terminal groups were prepared and subjected to inhibition studies. To study how the incorporation of β-amino acids would affect important protein–ligand interactions, molecular docking studies were performed. Based on the ligand poses, a Comparative Molecular Field Analysis[28] (CoMFA) model was calculated, also including inhibitors of the HCV NS3 protease previously synthesized by our group.

2. Results

2.1. Chemistry

The β-amino acid-comprising tripeptide compounds included in this study were generated by P_1–P_3 building block assembly followed by C-terminal modifications. The β-amino acid-based P_1 building blocks **3** and **4** were prepared by C-alkylation essentially following a procedure by Mertin and coworkers[29] outlined in Scheme 1. The hemiaminal 1-dibenzylamino-cyclopropanol,[29] was prepared from 1-ethoxy-1-trimethylsiloxycyclopropane,[30] and was treated with $TiCl_4$ followed by the addition of the corresponding ketene silyl acetals to give benzyl-protected **1** and **2**[29] in reasonable yields.

The benzyl-protected building blocks were deprotected by catalytic hydrogenation with 10% palladium on carbon and were isolated as the hydrochloride salts **3** and **4**. The methyl ester **2** was quantitatively deprotected in 3 h, whereas **1** required 40 h under hydrogen atmosphere to be fully deprotected.



Scheme 1. Reagents: (a) $TiCl_4$, DCM; (b) H_2, Pd/C 10%, HCl, MeOH.

The P_2 building block **8** (Scheme 2) was prepared starting from commercially available L-β-homohydroxyproline hydrochloride according to a method for the corresponding α-amino acid building block.[31] Treatment with Boc-anhydride in the presence of aqueous NaOH gave Boc-protected **5** that was further arylated with 4-chloro-7-methoxy-2-phenylquinoline to give the ether **6**. The carboxylic acid was converted to methyl ester with Cs_2CO_3 and methyl iodide to afford **7**, which was further Boc-deprotected to give the dihydrochloride **8**.

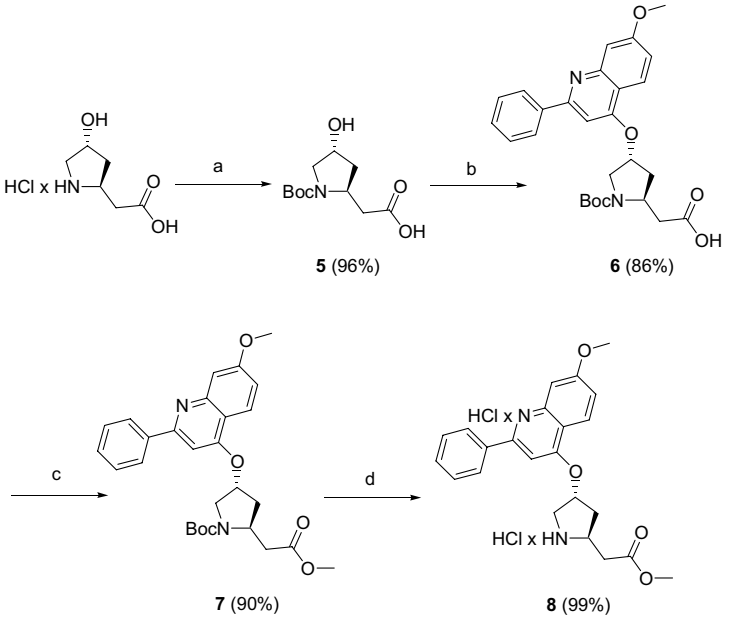
The target compounds **21**–**27**, containing a mix of α- and β-amino acids, were generated in a parallel fashion as described in Schemes 3–5. The assembly of carboxylic acids **21**–**23** is described in Scheme 3. The methyl ester **9**, previously described in the literature,[31] was coupled to Boc-L-valine using *N*-[(1*H*-benzotriazole-1-yl)-(dimethylamino)-methylene]-*N*- methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) in the presence of *N*,*N*-diisopropylethylamine (DIEA) to give dipeptide **10**. Coupling of the P_2 building block **8** with Boc-L-valine was conducted under the same conditions to give **11**. Also, **9** was allowed to react with commercially available Boc-L-β-homovaline and yielded **12**. Methyl esters **10**–**12** were subsequently hydrolyzed using LiOH, producing carboxylic acids **13**–**15**. The synthesis of intermediate **13** has been previously described by others.[31] The dipeptide **13** was coupled with P_1 building blocks **3** or **4** to give methyl ester-protected tripeptides **16** and **17**. Dipeptides **14** and **15** were also further coupled with P_1 building blocks to afford **18** and **19.** Deprotection with LiOH of methyl esters **16**–**19** gave carboxylic acids **20**–**23.**

The benzene sulfonamide compounds were prepared as described in Scheme 4. The corresponding carboxylic acids **21–23** were preactivated with *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridine-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) and DIEA followed by the addition of benzene sulfonamide, 4-dimethylaminopyridine (DMAP) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU).[32]

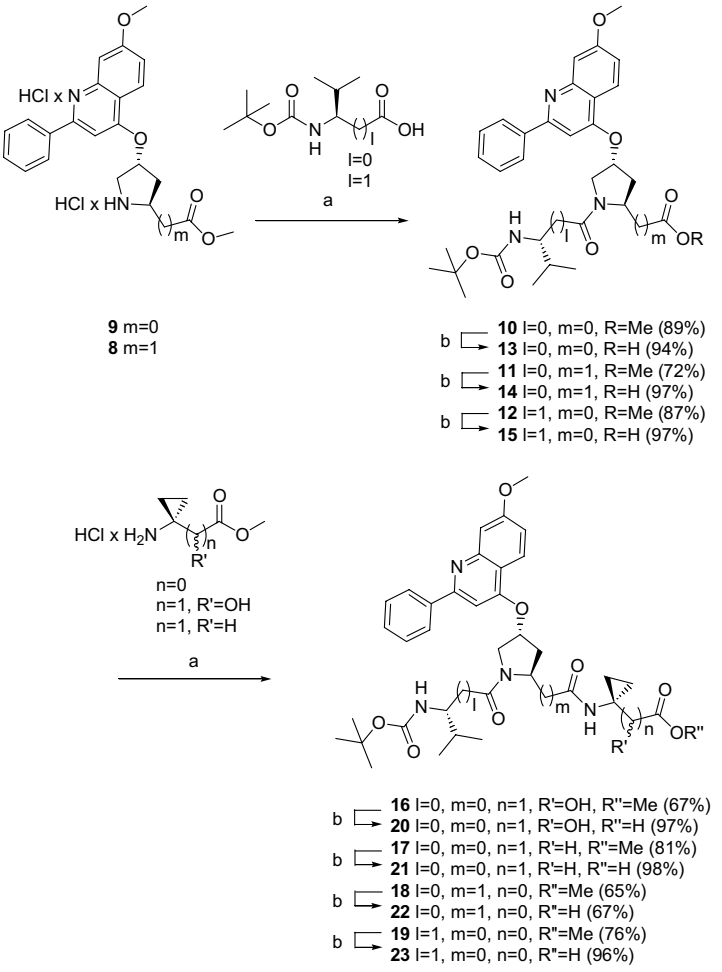
Finally, the α-hydroxy-β-amino acid-containing compound **20** was oxidized using Dess–Martin reagent to give the α-ketoacid **27** as depicted in Scheme 5.

2.2. Molecular modeling

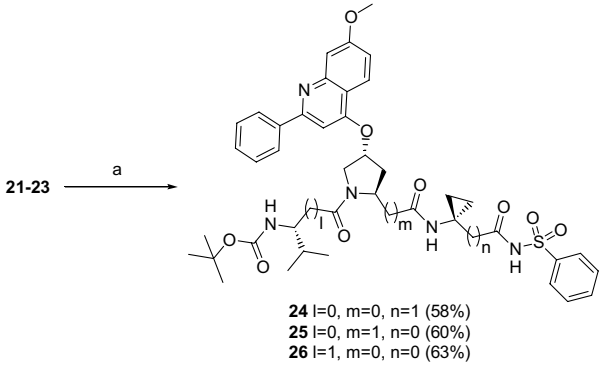
Docking studies were performed with the inhibitors presented herein as well as those from our previous work on non-covalent proline-based inhibitors.[33–35] Ligand structures in their predicted binding conformation were used to develop CoMFA models. By using default



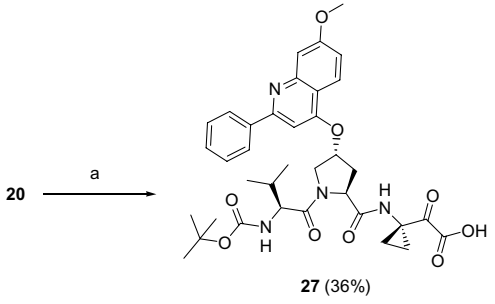
Scheme 2. Reagents: (a) Boc_2O, NaOH, 1,4-dioxane, H_2O; (b) 4-Chloro-7-methoxy-2-phenylquinoline, KO*t*-Bu, DMSO; (c) MeI, Cs_2CO_3, DMF; (d) HCl/1,4-dioxane.



Scheme 3. Reagents: (a) HBTU, DIEA, DMF; (b) LiOH, THF, MeOH, H_2O.



Scheme 4. Reagents: (a) Benzenesulfonamide, HATU, DIEA, DMAP, DBU, DMF.



Scheme 5. Reagents: (a) Dess–Martin periodinane, DCM.

CoMFA parameters, it was possible to calculate a 7-component model with internal (q^2) and external (r^2_{pred}) predictive abilities of 0.31 and 0.56, respectively, and a fit (r^2) of 0.99. This model was further refined using our CoMFA parameter optimization methodology,[36] resulting in a 4-component model with $q^2 = 0.48$, $r^2_{pred} = 0.68$, and $r^2 = 0.97$. Settings for the improved model differed from the default settings in steric and electrostatic cutoff (60 and 15, respectively), transform (Indicator), switch function (No), volume averaging type (Box) and the drop electrostatics setting (No).

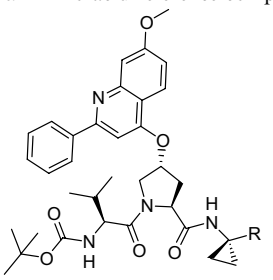
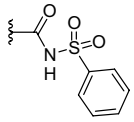
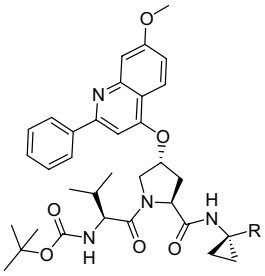
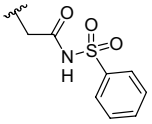
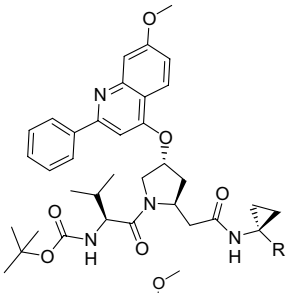
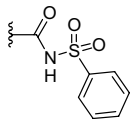
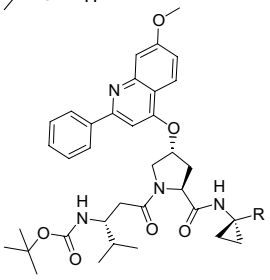
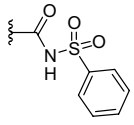
2.3. Biochemical evaluation

Target compounds **21**–**27** were tested in an HCV NS3 protease enzymatic assay comprising the full-length NS3 protein.[37] Structures and inhibition data (expressed as K_i values) are shown in Table 1.

3. Discussion

In an effort to further reduce the peptide character of HCV NS3 protease inhibitors and to provide insight into the structural requirements for potency, we have

Table 1. Structures and inhibition constants for tripeptide inhibitors of HCV NS3

Scaffold	Compound	R	$K_i \pm$ SD (μM)
α-Amino acid reference compounds			
	**28**		0.32 ± 0.03[a]
	29		0.055 ± 0.007[a]
β-Amino acid-comprising compounds			
	**21**		18 ± 5
	24		14 ± 7
	27		1.0 ± 0.6
	**22**		61 ± 13
	25		1.8 ± 0.5
	**23**		17 ± 3
	26		1.2 ± 0.4

[a] Inhibition data reported earlier by Rönn et al. [35]

explored the possibility of substituting the more commonly used α-amino acids with their β^3-counterparts using a previously developed tripeptide inhibitor as a reference. Thus, we have substituted the α-amino acid in each position with the corresponding β^3-amino acid. Inhibition analysis, docking, and 3D-QSAR studies have been performed to understand the effect of this structural modification on potency.

The tripeptide scaffolds, **28**[31,35] and **29**[35] (Table 1), selected for β-amino acid modification, were previously found to be potent inhibitors of the HCV NS3 protease and were selected partly due to the building block accessibility. Both Boc-L-β-homovaline and L-β-homohydroxyproline were obtained commercially, while (1-aminocyclopropyl)-acetic acid methyl ester (**4**) was obtained by synthesis. Although previous studies by our research group indicated that the acyl sulfonamide moiety in the C-terminal position is beneficial for potency and selectivity,[35,38–40] inhibitor scaffolds with both carboxylic acid and acyl sulfonamide C-terminal groups were studied. Insertion of a β-amino acid in the inhibitor

backbone will result in longer, more flexible inhibitors with potentially different structure–activity relationships.

According to observations from docking, reference inhibitors **28** and **29** form hydrogen-bonding interactions as depicted in Figure 3. These interactions have been observed between the natural cleavage product and the protease in the protein crystal structure[41] and are consistent with other published structure–activity relationship studies. Thus, the product peptide in the crystal structure forms an extended anti-parallel β-strand with the protease backbone, allowing the NH and carbonyl moieties of the P_3 residue to form hydrogen bonds with the A157 residue in the protease domain. The hydrogen bonds between the C-terminal carboxylic acid and the backbone amides of G137 and S139 have also been noted in the crystal structure. G137 and S139 form what is commonly referred to as the oxyanion hole[42,43] and is an important binding site for the oxyanion in natural substrate cleavage for serine proteases. In contrast to the natural cleavage product in the crystal structure, our docking model indicates that the NH of the inhibitors P_1 residue tends to form hydrogen-bonding interactions with the R155 backbone rather than the K136 side chain (Fig. 3). This hydrogen bond is a common feature in other published crystal structures of protease inhibitors.[44,45]

The P_1, P_2, and P_3 positions were explored by substituting β^3-amino acids in place of the original α-amino acids. All of the β-amino acid-comprising compounds **21**–**26** (Table 1) were found to be less potent than their α-amino acid counterpart (**28** or **29**) but μM potency was still achieved. As in previous studies, inhibitors with an acyl sulfonamide C-terminal group were found to be of higher potency than their corresponding carboxylic acid analogues (compare **24**, **25**, **26** with **21**, **22**, and **23**). In the acyl sulfonamide series, β-amino acid substitution in the P_3 and P_2 positions were most successful, rendering inhibitors **25** and **26** with K_i values of 1.8 μM and 1.2 μM, respectively. The corresponding compound with β-amino acid in P_1 position (**24**) had a K_i value of 14 μM.

In contrast to the acyl sulfonamide series, β-amino acid substitution in the P_3 and P_1 position was best tolerated in the C-terminal carboxylic acid series, rendering inhibitors **23** and **21** with K_i values of 17 and 18 μM, respectively. Use of a β-amino acid in the P_2 position as in compound **22** resulted in a significant loss of potency, yielding a K_i value of 61 μM.

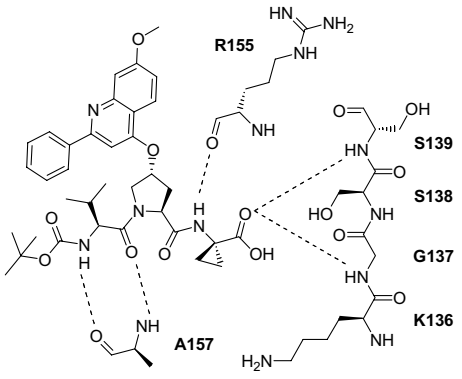


Figure 3. Protein–ligand hydrogen-bonding interactions for reference compound **28**, as proposed by docking.

β-Amino acid substitutions in the P_3 position provided μM inhibitors using both carboxylic acid and acyl sulfonamide C-terminal groups. Docking studies were conducted to understand the experimental inhibition data and showed a close similarity in interactions seen in the corresponding reference inhibitors (Fig. 3). Substitution in the P_3 position did not generally prevent the inhibitors from forming a similar hydrogen bond network with A157 observed in the reference inhibitor but may have introduced some ligand strain. Compared with the P_3 side chain of the reference inhibitors, the valine side chain of both P_3 β-amino acid inhibitors was found in closer proximity to the C159 side chain and may be a sign of steric clash. Since the compounds with a β-amino acid in the P_3 position provided μM inhibitors using both carboxylic acid and acyl sulfonamide C-terminal groups, this position shows potential for further structure optimization and studies of stability, selectivity, and pharmacokinetic properties.

According to docking studies, insertion of a β-amino acid in the P_2 position resulted in the disruption of the P_1 NH to R155 carbonyl hydrogen bond for both the acyl sulfonamide and the carboxylic acid compounds **22** and **25**. In the case of **22**, where a carboxylic acid C-terminal group was used, interactions with the oxyanion hole were also lost. This was likely not the case for **25**, where the acyl sulfonamide C-terminal was used. Docking experiments indicated hydrogen bonds between the sulfonyl oxygens and the Q41 and S139 side chains, which may have helped position the acyl sulfonamide carbonyl oxygen in the oxyanion hole. Although **25** may interact favorably with residues in the oxyanion hole, docking suggests disruption of hydrogen bonding with A157 to a greater extent than was observed in the corresponding carboxylic acid inhibitor **22**. These observations may explain the loss in potency for carboxylic acid **22** and show why acyl sulfonamide **25** remained fairly potent.

Molecular docking indicated that insertion of a β-amino acid in the P_1 position did not preclude the formation of hydrogen bonds between the terminal carbonyl and the G137 and S139 NHs. Although **21** adopted a conformation permitting hydrogen bonding with G137 and S139 with less strain than was seen in **24**, its hydrogen bond to R155 may be disrupted to a greater extent. Inhibition measurements showed that **24** exhibited the greatest loss in potency. The corresponding reference inhibitor **29** has a K_i of 0.055 μM, while **24** is 250 times less potent (14 μM). This dramatic loss of potency may be attributed to the size of the bulky acyl sulfonamide and the resulting ligand strain necessary to form important interactions in the oxyanion hole.

To sum up, a general loss in potency was seen for inhibitors **21**–**26**. Docking suggests that the 20- to 60-fold loss

in potency seen for inhibitors **21**, **23**, **25**, and **26** is most likely due to unfavorable conditions for the formation of hydrogen bonds between the inhibitor backbone and A157 or R155. The more dramatic loss in potency of compounds **22** and **24** may be explained by poor oxyanion hole interactions.

In our past publications we have speculated that compounds with an electrophilic C-terminal α-ketoacid group and a neighboring cyclopropane P_1 side chain may not function as a covalent serine-trap inhibitor due to the steric hindrance introduced by the bulky α,α-disubstituted side chain.[38,39,46] Instead, the high potencies observed for this type of inhibitor were thus expected to be due to the carboxylic acid (positioned as in a β-amino acid), similar to a non-covalent product-based inhibitor. Thus, we were surprised to see the loss in potency for compound **21** comprising a β-amino acid with a free carboxylic acid in the C-terminus. Docking indicated that hydrogen bonding to the oxyanion hole in the P_1β-amino acid inhibitors required some backbone ligand strain. This inspired the modification of the P_1 cyclopropane-based β-amino acid in compound **21** to its α-ketoacid analogue **27**, supposed to be a non-covalent inhibitor as discussed above. Inhibition studies showed **27** (K_i = 1.0 μM) to be roughly 20-fold more potent than **21** (K_i = 18 μM). This may indicate that it is the keto function adjacent to the P_1-side chain and not the terminal carbonyl that is important for the non-covalent oxyanion hole interactions. The proposed binding pose of **27** is shown in Figure 4, where it can be seen that the α-keto moiety is positioned appropriately for efficient interaction with the oxyanion hole as in a non-covalent product-based inhibitor. This allowed for interaction with the oxyanion hole without the introduction of any notable ligand strain, allowing for better interactions with R155 and A157.

Based on the results obtained from docking, it was possible to develop 3D-QSAR models using CoMFA. In addition to the compounds presented in this work, the CoMFA model included 47 previously published non-covalent proline-based compounds comprising various C-terminal groups and different P_1 side chains (structures available in the Supplementary data). Due to the structural similarity in P_2 and P_3, we have assumed that the inhibitors bind in a similar fashion. Docking was used to predict the biologically active conformation and also suggested a similar binding mode for this set of compounds. Although it was possible to derive an acceptable CoMFA model (q^2 = 0.31, r^2_{pred} = 0.56) using only default settings, predictive performance was enhanced by adjusting settings (q^2 = 0.48, r^2_{pred} = 0.68). Figure 5 shows a comparison of experimental versus predicted activities for compounds included in the external validation set, where the general improvement in predictive performance of the improved CoMFA model can be seen. The high degree of predictive accuracy of the CoMFA model suggests a clear correlation between ligand structure and measured inhibitory effects and supports information obtained from docking.

In addition to its value as a predictive tool, CoMFA fields may be visualized and overlaid with the protein to show regions correlated with changes in activity. CoMFA models are developed without knowledge of the protein structure and correspondence of contour surfaces with the protein structure supports structure–

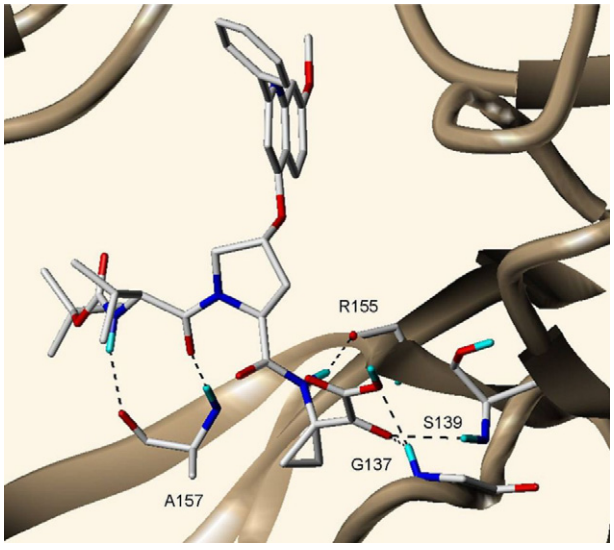


Figure 4. Depiction of the hydrogen-bonding network observed in docking studies of **27**. The P_3 valine residue is seen lying antiparallel to the protease, forming hydrogen bonds with A157. The P_1 NH is located in close proximity to the R155 carbonyl while the α-keto C-terminal group is seen hydrogen bonding with the NHs of G137 and S139. The terminal carboxylic acid moiety may participate in hydrogen-bonding interactions with G137. Hydrogen bond distances varied between 2.8 Å and 3.4 Å.

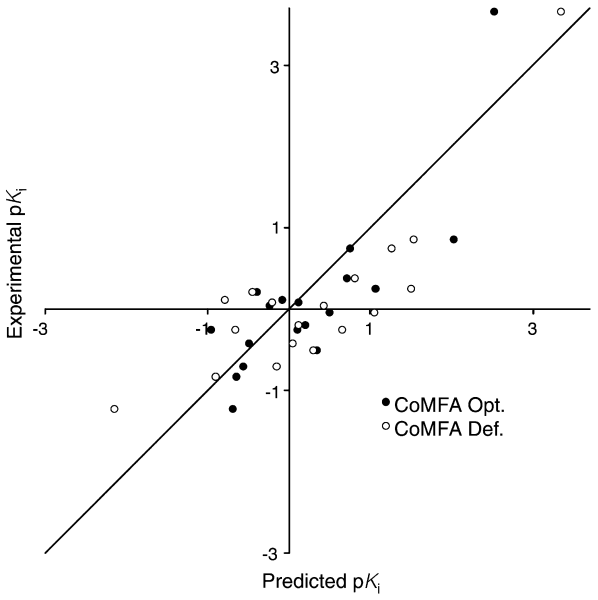


Figure 5. Experimental versus predicted pK_i values for test set compounds showing a comparison of the predictive quality of the default and improved CoMFA models. The placement of points relative to the line gives an indication of the predictive accuracy of each model. The experimental and predicted pK_i values are available in the supplementary material.

activity relationships. Figure 6 shows the steric and electrostatic contour maps overlaid with the protein surface. Some of the more notable features include the red surface between the S139 and Q41 side chains, the blue surface near the R155 backbone carbonyl and the green surfaces near the M485 and K136 side chains. Greater potency is correlated with the presence of electron withdrawing groups near red surfaces and in this case corresponds with the acyl sulfonamide C-terminal group in many of the inhibitors of the training set. In the docking poses of nearly all C-terminal acyl sulfonamides, one of the sulfone oxygens is located in or near this red surface. The model expects the presence of electropositive groups in the ligand near blue regions. In this case, the blue region near the R155 backbone carbonyl and the P_1 backbone nitrogen may indicate the importance of hydrogen bonding between these groups. The green surfaces near M485 and K136 indicate the importance of hydrophobic interactions in these areas.

Unfortunately, inhibition studies showed that introduction of β-amino acids resulted in an overall loss in potency. Docking indicated that, depending on the insertion position of the β-amino acid, some electrostatic interactions may have been affected. In such cases, ligand strain may have prevented the inhibitor from forming the hydrogen bonds seen in the relevant reference compound. This would result in unsatisfied hydrogen-bond partners being buried within the protein, which is known to be detrimental to potency.[47] Additionally, insertion of an extra methylene group to the inhibitor backbone results in greater conformational flexibility. Upon inhibitor binding, this would result in a greater loss of entropy and may have also contributed to the observed loss of potency. Despite these detrimental effects on potency for this particular series, several inhibitors were still of μM potency. This study has shown that it is possible to produce inhibitors of modest potency by inserting β-amino acids into the peptide backbone and has shown that optimization of the β-amino acid backbone will likely enhance potency. Further ligand optimization will be required to obtain more potent inhibitors.

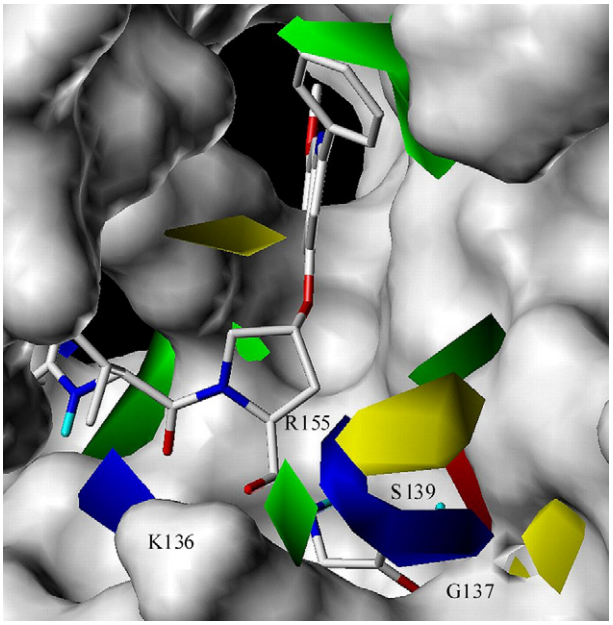


Figure 6. Steric and electrostatic contour maps derived from our improved CoMFA model and overlaid with the protein surface and **27**. Electrostatic maps are shown in red and blue; red regions are those in which the presence of electronegative groups are associated with improved potency and blue contour maps indicate the opposite. Steric maps are shown in green and yellow; the green areas are those, where the presence of steric bulk is associated with greater potency and the yellow areas are those, where steric bulk has a negative impact on potency.

4. Conclusion

A series of tripeptide protease inhibitors comprising β-amino acids have been synthesized and evaluated in a full-length HCV NS3 assay. Although experimental data showed a loss in potency, inhibitors with μM potency were obtained. This study has provided important information regarding structural requirements in the P_1, P_2, and P_3 positions for inhibitors comprising carboxylic acid and acyl sulfonamide C-terminal groups. First, the importance of good oxyanion hole interactions has been confirmed. Also, the P_3-position seems to be least affected by β-amino acid substitution, something that can be exploited in future inhibitor optimization. Molecular docking has provided a qualitative understanding of which protein–ligand interactions that may be affected at each position. A predictive CoMFA model has been derived using docked ligand poses, allowing the prediction of potency for future compounds and suggesting a correlation between changes in ligand structure and observed potency.

5. Experimental

5.1. Chemistry

Reagents and solvents were obtained commercially and used without further purification. TLC was performed using aluminium sheets precoated with silica gel 60 F_{254} (Merck) with visualization of spots using UV-detection and/or ninhydrin treatment followed by heating. Column chromatography was performed using silica gel 60 (40–63 μm) (Merck). LC-MS was performed on a Gilson-Finnigan AQA system in ESI mode using a Chromolith SpeedROD RP-18e 4.6 × 50 mm column (Merck) and a CH_3CN/H_2O linear gradient with 0.05% HCOOH. Preparative RP-HPLC was performed on an ACE-Phenyl, 5 μm, (21.2 × 150 mm) column or a Zorbax SB-C8, 5 μm, (21.2 × 150 mm) column using a CH_3CN/H_2O linear gradient with 0.1% TFA. Selected fractions were pooled and lyophilized. The purity of the inhibitors was determined by analytical RP-HPLC in the following systems (UV detection at 220 nm): column 1 (ACE 5 C18, 50 × 4.6 mm, H_2O/MeCN gradient with 25 mM NH_4OAc, pH 6.3) and column 2 (Thermo Hypersil C4, 50 × 4.6 mm, 5 μm, H_2O/MeCN gradient with 0.1% TFA). NMR spectra were recorded on a Varian Mercury plus spectrometer (^1H at 399.8 MHz, ^{13}C at

100.5 MHz) at ambient temperature unless otherwise stated. Chemical shifts are reported as δ values in ppm and are indirectly referenced to TMS via the solvent signal (^{1}H: $CHCl_3$ δ 7.26, CHD_2OD δ 3.31, DMSO-d_5 δ 2.50; ^{13}C: $CDCl_3$ δ 77.16, CD_3OD δ 49.00, DMSO-d_6 δ 39.50). Exact molecular masses were determined on a Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source. Elemental analyses were performed by Analytische Laboratorien, Lindlar, Germany.

5.1.1. Benzyloxy-(1-dibenzylamino-cyclopropyl)-acetic acid methyl ester (1). A solution of 1-dibenzylamino-cyclopropanol (1.0 g, 4.0 mmol) in dry DCM (60 mL) was stirred at −78°C under N_2 atmosphere. $TiCl_4$ (0.48 mL, 4.4 mmol) was slowly added and the reaction mixture was stirred for 30 min. (*Z*)-2-Benzyloxy-1-methoxy-1-(*tert*-butyldimethylsiloxy)ethene[48] (~8 mmol), prepared from benzyloxyacetic acid methyl ester and used as crude product, was added dropwise. After 1 h the reaction mixture was poured into saturated aqueous $NaHCO_3$ (50 mL) and extracted three times with diethylether (30 mL). The organic layers were dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by column chromatography (gradient elution, *i*-hexane/EtOAc 12:1 followed by *i*-hexane/EtOAc 9:1) to give **1** (0.91 g, 55%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.42–7.30 (m, 5H), 7.23–7.13 (m, 10H), 4.68 (d, J = 11.6 Hz, 1H), 4.38 (d, J = 11.6 Hz, 1H), 4.33 (s, 1H), 3.86 (d, J = 13.7 Hz, 1H), 3.73 (s, 3H), 3.73 (d, J = 13.7 Hz, 1H), 0.90–0.84 (m, 1H), 0.67–0.61 (m, 1H), 0.58–0.51 (m, 1H), 0.46–0.39 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 172.2, 140.2, 137.3, 129.0, 128.4, 128.3, 127.9, 127.8, 126.6, 79.5, 72.2, 56.9, 51.7, 45.6, 15.0, 12.3. HRMS calcd for $C_{27}H_{30}NO_3$ ($M+H^+$) 416.2226, found: 416.2222.

5.1.2. (1-Dibenzylamino-cyclopropyl)-acetic acid methyl ester (2)[29]. A solution of 1-dibenzylamino-cyclopropanol (1.0 g, 4.0 mmol) in dry DCM (60 mL) was stirred at −78 °C under N_2 atmosphere. $TiCl_4$ (0.48 mL, 4.4 mmol) was slowly added and the reaction mixture was stirred for 30 min. 1-(*tert*-Butyl-dimethylsilyloxy)-1-methoxyethene (1.8 mL, 8.0 mmol) was added dropwise. After 1 h the reaction mixture was poured into saturated aqueous $NaHCO_3$ (50 mL) and extracted three times with diethylether (30 mL). The organic layers were dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by column chromatography (gradient elution, *i*-hexane/EtOAc 9:1 followed by *i*-hexane/EtOAc 4:1) to give **2** (0.77 g, 62%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.16–7.26 (m, 10H), 3.76 (s, 4H), 3.70 (s, 3H), 2.61 (s, 2H), 0.57–0.53 (m, 2H) 0.52–0.48 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 173.2, 140.1, 128.9, 128.0, 126.7, 56.4, 51.6, 41.2, 36.3, 15.1. MS ($M+H^+$) 310.0.

5.1.3. (1-Amino-cyclopropyl)-hydroxy-acetic acid methyl ester hydrochloride (3). To a solution of **1** (0.35 g, 0.84 mmol) in MeOH (10 mL) was added 4 M HCl in 1,4-dioxane (1.5 mL). Coevaporation with MeOH gave a white solid. To the residual were added MeOH (25 mL) and Pd/C 10% (70 mg). The reaction mixture was stirred under H_2 atmosphere for 40 h. The catalyst was filtered off and the solvent was removed by rotary evaporation to give **3** (0.15 g, 99%) as colorless syrup. ^{1}H NMR (DMSO-d_6): δ 8.34 (br s, 3H), 6.35 (d, J = 5.3 Hz, 1H), 4.01 (d, J = 5.3 Hz, 1H), 3.33 (s, 3H), 1.05–0.95 (m, 2H), 0.95–0.87 (m, 1H), 0.87–0.76 (m, 1H). ^{13}C NMR (DMSO-d_6): δ 171.1, 71.6, 52.1, 36.1, 8.8, 7.8. Anal. Calcd for $C_6H_{12}ClNO$: C, 39.68; H, 6.66; N, 7.71. Found: C, 39.42; H, 6.68; N, 7.71.

5.1.4. (1-Aminocyclopropyl)-acetic acid methyl ester hydrochloride (4). To a solution of **2** (0.49 g, 1.6 mmol) in MeOH (30 mL) was added Pd/C 10% (49 mg). The reaction mixture was stirred under H_2 atmosphere for 3 h. The catalyst was filtered off and HCl in EtOH was added to pH 3. The solvent was removed by rotary evaporation to give **4** (0.26 g, 99%) as a colorless syrup. ^{1}H NMR ($CDCl_3$): δ 8.70 (br s, 3H), 3.79 (s, 3H), 2.77 (s, 2H), 1.48–1.41 (m, 2H), 0.81–0.74 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 171.3, 52.6, 39.3, 31.7, 10.6. Anal. Calcd for $C_6H_{12}ClNO_2$: C, 43.51; H, 7.30; N, 8.46. Found: C, 43.32; H, 7.20; N, 8.42.

5.1.5. (2*S*,4*R*)-2-Carboxymethyl-4-hydroxy-pyrrolidine-1-carboxylic acid *tert*-butyl ester (5). A solution of L-β-homohydroxyproline hydrochloride (0.36 g, 2.0 mmol) in 1,4-dioxane (4.0 mL) was treated with 1.0 M aqueous NaOH (4.0 mL). Di-*tert*-butyldicarbonate (0.65 g, 3.0 mmol) in 1,4-dioxane (4.0 mL) was added and the reaction mixture was stirred at room temperature overnight. The dioxane was evaporated and the aqueous phase was acidified to pH 2 with 10% aqueous $KHSO_4$. The acidic solution was extracted with EtOAc (3 × 10 mL), dried with $MgSO_4$ and concentrated by rotary evaporation to give **5** (0.47 g, 96%) as a colorless syrup which was used without further purification. ^{1}H NMR (DMSO-d_6, 70° C): δ 11.89 (br s, 1H), 4.69 (br s, 1H), 4.22–4.17 (m, 1H), 4.08–3.99 (m, 1H), 3.30–3.25 (m, 2H), 2.90–2.72 (m, 1H), 2.28 (dd, J = 9.1, 15.2 Hz, 1H), 2.05–1.98 (m, 1H), 1.85–1.76 (m, 1H), 1.41 (s, 9H). HRMS calcd for $C_{11}H_{20}NO_5$ ($M+H^+$) 246.1341, found: 246.1343.

5.1.6. (2*S*,4*R*)-2-Carboxymethyl-4-(7-methoxy-2-phenyl-quinolin-4-yloxy)-pyrrolidine-1-carboxylic acid *tert*-butyl ester (6). To a solution of **5** (0.20 g, 0.82 mmol) in dry DMSO (3.0 mL) was added KO*t*Bu (0.23 g, 2.1 mmol) in portions. The reaction mixture was stirred under N_2 atmosphere for 2.5 h followed by the addition of a solution of 4-chloro-7-methoxy-2-phenylquinoline (0.24 g, 0.90 mmol) in dry DMSO (4.0 mL). The reaction mixture was stirred for additional 22 h and then cooled to 0 °C. Thereafter cold H_2O (25 mL) was added and the suspension was washed with diethyl ether (5 × 20 mL), acidified to pH 4.7 using 1.0 M aqueous HCl, filtered and dried to give **6** (0.34 g, 86%) as beige solid. ^{1}H NMR (DMSO-d_6, 70 °C): δ 12.0 (br s, 1H), 8.28–8.24 (m, 2H), 7.94 (d, J = 9.1 Hz, 1H), 7.57–7.47 (m, 3H), 7.44 (s, 1H), 7.39 (d, J = 2.6 Hz, 1H), 7.16 (dd, J = 2.6, 9.1 Hz, 1H), 5.54–5.49 (m, 1H), 4.28–4.20 (m, 1H), 3.94 (s, 3H), 3.81 (dm, J = 12.5 Hz, 1H), 3.66 (dd, J = 4.1, 12.5 Hz, 1H), 2.85 (dm, J = 15.3 Hz, 1H), 2.58–2.50 (m, 2H), 2.32–2.23 (m, 1H), 1.38 (s, 9H).

HRMS calcd for $C_{27}H_{31}N_2O_6$ ($M+H^+$) 479.2182, found: 479.2173.

5.1.7. (2*S*,4*R*)-2-Methoxycarbonylmethyl-4-(7-methoxy-2-phenyl-quinolin-4-yloxy)-pyrrolidine-1-carboxylic acid *tert*-butyl ester (7). To a solution of **6** (0.25 g 0.52 mmol) in dry DMF (15 mL) was added Cs_2CO_3 (0.20 g, 0.63 mmol). The reaction mixture was stirred under N_2 atmosphere for 10 min followed by the addition of MeI (0.049 mL, 0.78 mmol). After 6 h the reaction mixture was diluted with EtOAc (30 mL), washed with 5% aqueous $NaHCO_3$ (2 × 15 mL), H_2O (15 mL), and brine (15 mL). The organic layer was dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by column chromatography (*i*-hexane/EtOAc 2:1) to give **7** (0.23 g, 90%) as white foam. ^1H NMR (DMSO-d_6, 70 °C): δ 8.27–8.24 (m, 2H), 7.93 (d, J = 9.1 Hz, 1H), 7.57–7.46 (m, 3H), 7.43 (s, 1H), 7.39 (d, J = 2.5 Hz, 1H), 7.15 (dd, J = 2.5, 9.1 Hz, 1H), 5.53–5.48 (m, 1H), 4.31–4.23 (m, 1H), 3.94 (s, 3H), 3.81 (dm, J = 12.2 Hz, 1H), 3.66 (dd, J = 3.8, 12.2 Hz, 1H), 3.63 (s, 3H), 2.89 (dd, J = 4.1, 15.3 Hz, 1H), 2.65 (dd, J = 8.3, 15.3 Hz, 1H), 2.57–2.49 (m, 1H), 2.30–2.21 (m, 1H), 1.37 (s, 9H). HRMS calcd for $C_{28}H_{33}N_2O_6$ ($M+H^+$) 493.2339, found: 493.2349.

5.1.8. [(2*S*,4*R*)-4-(7-Methoxy-2-phenyl-quinolin-4-yloxy)-pyrrolidin-2-yl]-acetic acid methyl ester dihydrochloride (8). To a solution of **7** (0.21 g, 0.43 mmol) in anhydrous 1,4-dioxane (1.5 mL) was added 4.0 M HCl in 1,4-dioxane (2.2 mL). The reaction mixture was stirred at room temperature for 8 h. Coevaporation with MeOH gave **8** (0.20 g, 99%) as yellow solid. ^1H NMR (CD_3OD): δ 8.47 (d, J = 9.3 Hz, 1H), 8.13–8.07 (m, 2H), 7.80–7.70 (m, 3H), 7.67 (s, 1H), 7.61 (d, J = 2.3 Hz, 1H), 7.50 (dd, J = 2.3, 9.3 Hz, 1H), 6.00–5.95 (m, 1H), 4.43–4.32 (m, 1H), 4.08 (s, 3H), 3.96 (dm, J = 13.1 Hz, 1H), 3.88 (dm, J = 13.1 Hz, 1H), 3.78 (s, 3H), 3.09 (dd, J = 3.8, 18.0 Hz, 1H), 3.00 (dd, J = 9.4, 18.0 Hz, 1H), 2.80 (ddm, J = 6.0, 14.9 Hz, 1H), 2.44–2.32 (m, 1H). ^{13}C NMR (CD_3OD): δ 172.3, 167.4, 166.7, 158.4, 143.7, 134.0, 133.2, 130.8, 130.1, 126.5, 122.1, 116.0, 102.3, 100.6, 80.7, 57.1, 56.7, 52.8, 51.8, 37.6, 36.4. HRMS calcd for $C_{23}H_{25}N_2O_4$ ($M+H^+$) 393.1814, found: 393.1811.

5.1.9. Compound 10[31]. To a suspension of (2*S*,4*R*)-4-(7-Methoxy-2-phenyl-quinolin-4-yloxy)-pyrrolidin-2-carboxylic acid methyl ester dihydrochloride (**9**) (0.13 g, 0.28 mmol) in dry DMF (4.0 mL) were added Boc-L-valine (0.12 g, 0.55 mmol), HBTU (0.13 g, 0.33 mmol), and DIEA (0.19 mL, 1.1 mmol). The resulting solution was stirred at room temperature for 5 h, diluted with EtOAc (30 mL), and washed with 5% aqueous $NaHCO_3$ (10 mL), 35 mM aqueous $NaHSO_4$ (2 × 10 mL), H_2O (10 mL), and brine (10 mL). The organic layer was dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by column chromatography (gradient elution, *i*-hexane/EtOAc 2:1 followed by *i*-hexane/EtOAc 1:1) to give **10** (0.14 g, 89%) as white foam. ^1H NMR (CD_3OD): δ 8.08 (d, J = 9.1 Hz, 1H), 8.05–8.01 (m, 2H), 7.56–7.46 (m, 3H), 7.37 (d, J = 2.5 Hz, 1H), 7.18 (s, 1H), 7.08 (dd, J = 2.5, 9.1 Hz, 1H), 5.52–5.48 (m, 1H), 4.69 (dd, J = 7.6, 10.1 Hz, 1H), 4.65 (dm, J = 11.5 Hz, 1H), 4.08–4.01 (m, 2H), 3.93 (s, 3H), 3.74 (s, 3H), 2.78 (ddm, J = 7.5, 14.2 Hz, 1H), 2.36 (ddd, J = 4.3, 10.1, 14.2 Hz, 1H), 1.99 (dh, J = 6.7, 9.0 Hz, 1H), 1.20 (s, 9H), 1.01 (d, J = 6.7 Hz, 3H), 0.97 (d, J = 6.7 Hz, 3H). ^{13}C NMR (CD_3OD): 174.2, 173.5, 163.1, 161.9, 161.4, 158.0, 157.9, 152.3, 141.5, 130.5, 129.8, 129.1, 124.4, 119.3, 116.4, 107.4, 100.1, 80.3, 78.1, 59.5, 59.4, 56.0, 54.2, 52.8, 35.9, 31.7, 28.5, 19.4, 19.1. MS ($M+H^+$) 578.3.

5.1.10. Compound 11. To a suspension of **8** (0.13 g, 0.28 mmol) in dry DMF (4.0 mL) were added Boc-L-valine (0.12 g, 0.55 mmol), HBTU (0.13 g, 0.33 mmol), and DIEA (0.19 mL, 1.1 mmol). The resulting solution was stirred at room temperature for 5 h, diluted with EtOAc (30 mL), and washed with 5% aqueous $NaHCO_3$(10 mL), 35 mM aqueous $NaHSO_4$ (2 × 10 mL), H_2O (10 mL), and brine (10 mL). The organic layer was dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by column chromatography (gradient elution, *i*-hexane/EtOAc 2:1 followed by *i*-hexane/EtOAc 1:1) to give **11** (0.12 g, 72%) as white foam. ^1H NMR (CD_3OD): δ (~5:1 mixture of rotamers, major rotamer reported) 8.08 (d, J = 9.2 Hz, 1H), 8.06–8.00 (m, 2H), 7.57–7.46 (m, 3H), 7.38 (d, J = 2.5 Hz, 1H), 7.19 (s, 1H), 7.09 (dd, J = 2.5, 9.2 Hz, 1H), 5.45–5.40 (m, 1H), 4.56 (dm, J = 12.2 Hz, 1H), 4.56–4.50 (m, 1H), 4.04 (d, J = 8.8 Hz, 1H), 3.96 (dd, J = 3.7, 12.2 Hz, 1H), 3.94 (s, 3H), 3.66 (s, 3H), 2.99 (dd, J = 3.7, 16.2 Hz, 1H), 2.80 (dd, J = 7.5, 16.2 Hz, 1H), 2.67 (ddm, J = 7.6, 14.2 Hz, 1H), 2.36 (ddd, J = 4.5, 9.2, 14.2 Hz, 1H), 1.97 (dh, J = 6.8, 8.8 Hz, 1H), 1.21 (s, 9H), 0.95 (d, J = 6.8 Hz, 3H), 0.93 (d, J = 6.8 Hz, 3H). ^{13}C NMR (CD_3OD): δ (~5:1 mixture of rotamers, major rotamer reported) 174.0, 173.2, 163.2, 162.2, 161.4, 157.9, 152.2, 141.5, 130.5, 129.8, 129.1, 124.5, 119.3, 116.5, 107.3, 100.2, 80.3, 77.8, 59.9, 56.0, 54.6, 54.3, 52.1, 37.6, 37.3, 31.8, 28.5, 19.6, 18.9. HRMS calcd for $C_{33}H_{42}N_3O_7$ ($M+H^+$) 592.3023, found: 592.3035.

5.1.11. Compound 12. To a suspension of (2*S*,4*R*)-4-(7-Methoxy-2-phenyl-quinolin-4-yloxy)-pyrrolidin-2-carboxylic acid methyl ester dihydrochloride (**9**) (0.13 g, 0.28 mmol) in dry DMF (4.0 mL) were added Boc-L-β-homovaline (0.13 g, 0.55 mmol), HBTU (0.13 g, 0.33 mmol) and DIEA (0.19 mL, 1.1 mmol). The resulting solution was stirred at room temperature for 3 h, diluted with EtOAc (30 mL), and washed with 5% aqueous $NaHCO_3$ (10 mL), 35 mM aqueous $NaHSO_4$ (2 × 10 mL), H_2O (10 mL), and brine (10 mL). The organic layer was dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by column chromatography (gradient elution, *i*-hexane/EtOAc 2:1 followed by *i*-hexane/EtOAc 1:1) to give **12** (0.14 g, 87%) as white foam. ^1H NMR (CD_3OD): δ (~4:1 mixture of rotamers, major rotamer reported) 8.10–8.00 (m, 3H), 7.56–7.46 (m, 3H), 7.39 (d, J = 2.5 Hz, 1H), 7.23 (s, 1H), 7.14 (dd, J = 2.5, 9.2 Hz, 1H), 5.59–5.53 (m, 1H), 4.67–4.62 (m, 1H), 4.14 (dd, J = 3.7, 11.8 Hz, 1H), 4.08 (dm, J = 11.8 Hz, 1H), 3.94 (s, 3H), 3.80–3.75 (m, 1H), 3.75 (s, 3H), 2.75 (ddm,

J = 8.1, 14.0 Hz, 1H), 2.53–2.48 (m, 2H), 2.40 (ddd, J = 4.7, 9.0, 14.0 Hz, 1H), 1.83–1.70 (m, 1H), 1.40 (s, 9H), 0.86 (d, J = 6.7 Hz, 3H), 0.80 (d, J = 6.7 Hz, 3H). ^{13}C NMR (CD_3OD): δ (~4:1 mixture of rotamers, major rotamer reported) 173.9, 172.6, 163.2, 162.0, 151.3, 157.9, 152.3, 141.3, 130.6, 129.8, 129.0, 124.2, 119.5, 116.4, 107.5, 100.0, 79.8, 78.0, 59.3, 56.0, 54.3, 54.0, 52.9, 38.3, 25.8, 32.9, 28.8, 19.9, 18.2. HRMS calcd for $C_{33}H_{42}N_3O_7$ ($M+H^+$) 592.3023, found: 592.3019.

5.1.12. Compound 13[31]. To a solution of **10** (0.13 g, 0.23 mmol) in THF (10 mL) and MeOH (1.5 mL) was added a solution of LiOH (0.055 g, 2.3 mmol) in H_2O (4.0 mL). The reaction mixture was stirred at room temperature for 2 h and neutralized with 1.0 M aqueous HCl. The organic solvents were removed by rotary evaporation, H_2O (10 mL) was added, and the aqueous phase was acidified with 1.0 M aqueous HCl to pH 3 and extracted with EtOAc (3 × 40 mL). The organic layers were washed with brine (20 mL), dried with $MgSO_4$, and concentrated by rotary evaporation to give **13** (0.12 g, 94%) as white solid. ^{1}H NMR (CD_3OD): δ 8.14 (d, J = 9.2 Hz, 1H), 8.08–8.02 (m, 2H), 7.60–7.51 (m, 3H), 7.40 (d, J = 2.5 Hz, 1H), 7.28 (s, 1H), 7.14 (dd, J = 2.5, 9.2 Hz, 1H), 5.59–5.54 (m, 1H), 4.70–4.61 (m, 2H), 4.08–4.03 (m, 2H), 3.96 (s, 3H), 2.82 (ddm, J = 7.4, 14.1 Hz, 1H), 2.47–2.38 (m, 1H), 2.00 (dh, J = 6.7, 9.0 Hz, 1H), 1.19 (s, 9H), 1.03 (d, J = 6.7 Hz, 3H), 0.97 (d, J = 6.7 Hz, 3H). ^{13}C NMR (CD_3OD): δ 175.2, 174.0, 163.7, 163.0, 160.9, 157.9, 150.8, 140.1, 131.1, 129.9, 129.2, 124.8, 119.8, 116.4, 106.2, 100.5, 80.3, 78.7, 59.4, 56.1, 54.1, 52.8, 36.1, 31.8, 28.5, 19.6, 19.1. MS ($M+H^+$) 564.3.

5.1.13. Compound 14. To a solution of **11** (0.099 g, 0.17 mmol) in THF (10 mL) and MeOH (1.5 mL) was added a solution of LiOH (0.040 g, 1.7 mmol) in H_2O (4.0 mL). The reaction mixture was stirred at room temperature for 4 h and neutralized with 1.0 M aqueous HCl. The organic solvents were removed by rotary evaporation, H_2O (10 mL) was added, and the aqueous phase was acidified with 1.0 M aqueous HCl to pH 3 and extracted with EtOAc (3 × 40 mL). The organic layers were washed with brine (20 mL), dried with $MgSO_4$, and concentrated by rotary evaporation to give **14** (0.093 g, 97%) as white solid. ^{1}H NMR (CD_3OD): δ (~5:1 mixture of rotamers, major rotamer reported) 8.11 (d, J = 9.2 Hz, 1H), 8.05–8.01 (m, 2H), 7.59–7.48 (m, 3H), 7.39 (d, J = 2.5 Hz, 1H), 7.23 (s, 1H), 7.11 (dd, J = 2.5, 9.2 Hz, 1H), 5.54–5.43 (m, 1H), 4.58 (dm, J = 12.2 Hz, 1H), 4.58–4.50 (m, 1H), 4.04 (d, J = 8.8 Hz, 1H), 3.99 (dd, J = 3.3, 12.2 Hz, 1H), 3.95 (s, 1H), 3.04 (dd, J = 3.3, 16.3 Hz, 1H), 2.76–2.67 (m, 2H), 2.39 (ddd, J = 4.5, 9.3, 14.0 Hz, 1H), 1.98 (dh, J = 6.8, 8.8 Hz, 1H), 1.21 (s, 9H), 0.95 (d, J = 6.8 Hz, 1H), 0.94 (d, J = 6.8 Hz, 1H). ^{13}C NMR (CD_3OD): δ (~5:1 mixture of rotamers, major rotamer reported) 174.8, 173.9, 163.4, 162.7, 161.1, 157.9, 151.4, 140.8, 130.8, 129.9, 129.0, 124.7, 119.5, 116.5, 106.7, 100.3, 80.3, 78.1, 59.9, 56.1, 54.7, 54.3, 37.8, 37.4, 31.9, 28.5, 19.6, 19.0. HRMS calcd for $C_{32}H_{40}N_3O_7$ ($M+H^+$) 578.2866, found: 578.2855.

5.1.14. Compound 15. To a solution of **12** (0.13 g, 0.23 mmol) in THF (10 mL) and MeOH (1.5 mL) was added a solution of LiOH (0.055 g, 2.3 mmol) in H_2O (4.0 mL). The reaction mixture was stirred at room temperature for 2.5 h and neutralized with 1.0 M aqueous HCl. The organic solvents were removed by rotary evaporation and the remaining aqueous phase was acidified with 1.0 M aqueous HCl to pH 3 and extracted with EtOAc (3 × 20 mL). The organic layers were washed with brine (20 mL), dried with $MgSO_4$, and concentrated by rotary evaporation to give **15** (0.12 g, 97%) as white solid. ^{1}H NMR (CD_3OD): δ (~3:2 mixture of rotamers, major rotamer reported) 8.18 (d, J = 9.2 Hz, 1H), 8.08–8.01 (m, 2H), 7.65–7.55 (m, 3H), 7.43 (d, J = 2.5 Hz, 1H), 7.35 (s, 1H), 7.22 (dd, J = 2.5 9.2 Hz, 1H), 5.66–5.61 (m, 1H), 4.61–4.59 (m, 1H), 4.20 (m, 2H), 3.98 (s, 3H), 3.81–3.74 (m, 1H), 2.78 (ddm, J = 7.9, 14.1 Hz, 1H), 2.60–2.35 (m, 3H), 1.86–1.75 (m, 1H), 1.40 (s, 9H), 0.86 (d, J = 6.8 Hz, 3H), 0.81 (d, J = 6.8 Hz, 3H). ^{13}C NMR (CD_3OD): δ (~3:2 mixture of rotamers, major rotamer reported) 175.5, 173.4, 164.5, 164.2, 160.3, 157.9, 149.7, 138.9, 131.6, 130.1, 129.3, 125.0, 120.2, 116.3, 105.4, 100.7, 79.8, 78.9, 59.5, 56.3, 54.1, 53.9, 38.4, 36.1, 32.8, 28.8, 19.9, 18.2. HRMS calcd for $C_{32}H_{40}N_3O_7$ ($M+H^+$) 578.2866, found: 578.2876.

5.1.15. Compound 16. To a solution of **13** (0.10 g, 0.18 mmol) and **3** (0.048 g, 0.27 mmol) in dry DMF (2.0 mL) were added HBTU (0.081 g, 0.21 mmol) and DIEA (0.12 mL, 0.71 mmol). The resulting solution was stirred at room temperature for 3 h, diluted with EtOAc (15 mL), and washed with aqueous NaOAc buffer pH 4 (2 × 10 mL), 5% aqueous $NaHCO_3$ (10 mL), and brine (10 mL). The organic layer was dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by column chromatography (gradient elution, *i*-hexane/EtOAc 1:1 followed by *i*-hexane/EtOAc 1:3) to give **16** (0.82 g, 67%) as white solid. ^{1}H NMR (CD_3OD): δ (1:1 mixture of diasteromers) 8.13–8.04 (m, 3H), 7.58–7.48 (m, 3H), 7.42–7.39 (m, 1H), 7.273 & 7.268 (s, 1H), 7.12–7.07 (m, 1H), 5.58–5.52 (m, 1H), 4.60–4.51 (m, 2H), 4.10–4.03 (m, 2H), 3.96 (s, 3H), 3.89 & 3.84 (s, 1H), 3.73 & 3.72 (s, 3H) 2.73–2.61 (m, 1H), 2.45–2.36 (m, 1H), 2.04–1.94 (m, 1H), 1.25 & 1.24 (s, 9H), 1.25–1.09 (m, 2H), 1.03–0.94 (m, 6H), 0.93–0.73 (m, 2H). ^{13}C NMR (CD_3OD): δ (1:1 mixture of diasteromers) 176.0 & 174.7, 174.4 & 174.2, 173.9 & 173.8, 163.0, 161.9, 161.2, 157.9, 152.2, 141.3, 130.5, 129.8, 129.0, 124.4, 119.2, 116.4, 107.3, 100.0, 80.3, 78.0, 76.5 & 74.5, 60.4 & 60.2, 59.5 & 59.4, 56.0, 54.4, 52.6, 37.0 & 36.5, 36.2 & 36.0, 31.7, 28.6, 19.8 & 19.0, 19.1 & 19.0, 12.8, 12.4 & 11.8. HRMS calcd for $C_{37}H_{47}N_4O_9$ ($M+H^+$) 691.3343, found: 691.3331.

5.1.16. Compound 17. To a solution of **13** (0.11 g, 0.20 mmol) and **4** (0.050 g, 0.30 mmol) in dry DMF (2.0 mL) were added HBTU (0.092 g, 0.24 mmol) and DIEA (0.14 mL, 0.81 mmol). The resulting solution was stirred at room temperature for 3 h, diluted with EtOAc (20 mL), and washed with aqueous NaOAc buffer pH 4 (5 × 8 mL), 5% aqueous $NaHCO_3$ (8 mL), and

brine (8 mL). The organic layer was dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by column chromatography (gradient elution, *i*-hexane/EtOAc 1:1 followed by *i*-hexane/EtOAc 1:2) to give **17** (0.11 g, 81%) as white solid. ^{1}H NMR (CD_3OD): δ 8.09 (d, J = 9.2 Hz, 1H), 8.07–8.02 (m, 2H), 7.57–7.47 (m, 3H) 7.38 (d, J = 2.6 Hz, 1H), 7.23 (s, 1H), 7.08 (dd, J = 2.6, 9.2 Hz, 1H), 5.55–5.49 (m, 1H), 4.57 (dm, J = 11.8 Hz, 1H), 4.55 (dd, J = 7.4, 9.5 Hz, 1H), 4.05 (dd, J = 3.9, 11.8 Hz, 1H), 4.04 (d, J = 8.9 Hz, 1H) 3.94 (s, 3H), 3.66 (s, 3H), 2.73 (d, J = 15.7 Hz, 1H), 2.64 (ddm, J = 7.4, 13.9 Hz, 1H), 2.47 (d, J = 15.7 Hz, 1H), 2.36 (ddd, J = 4.4, 9.5, 13.9 Hz, 1H), 1.99 (dh, J = 6.7, 8.9 Hz, 1H), 1.23 (s, 9H), 1.01 (d, J = 6.7 Hz, 3H), 0.96 (d, J = 6.7 Hz, 3H), 0.91–0.76 (m, 4H).^{13}C NMR (CD_3OD): δ 174.3, 173.9, 173.6, 163.2, 162.1, 161.4, 157.9, 152.2, 141.4, 130.6, 129.8, 129.1, 124.4, 119.3, 116.5, 107.3, 100.1, 80.3, 78.1, 60.4, 59.6, 56.0, 54.4, 52.1, 41.2, 36.1, 31.7, 31.0, 28.6, 19.7, 19.1, 14.1, 13.4. HRMS calcd for $C_{37}H_{47}N_4O_8$ ($M+H^+$) 675.3394, found: 675.3383.

5.1.17. Compound 18. To a solution of **14** (0.086 g, 0.15 mmol) and the hydrochloric salt of 1-amino-cyclopropanecarboxylic acid methyl ester (0.034 g, 0.22 mmol) in dry DMF (3.0 mL) were added HBTU (0.068 g, 0.18 mmol) and DIEA (0.10 mL, 0.60 mmol). The resulting solution was stirred at room temperature for 3 h diluted with EtOAc (30 mL), and washed with aqueous NaOAc buffer pH 4 (3 × 12 mL) and brine (10 mL). The organic layer was dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by column chromatography (*i*-hexane/EtOAc 1:3) to give **18** (0.065 g, 65%) as white solid. ^{1}H NMR (CD_3OD): δ (~5:1 mixture of rotamers, major rotamer reported) 8.17 (d, J = 9.3 Hz, 1H), 8.08–8.01 (m, 2H), 7.62–7.53 (m, 3H), 7.42 (d, J = 2.5 Hz, 1H), 7.33 (s, 1H), 7.17 (dd, J = 2.5, 9.3 Hz, 3H), 5.56–5.50 (m, 1H), 4.60 (dm, J = 12.6 Hz, 3H), 4.60–4.55 (m, 1H), 4.04 (d, J = 8.9 Hz, 1H), 4.01 (dd, J = 8.8, 12.6 Hz, 1H), 3.98 (s, 3H), 3.59 (s, 3H), 3.04 (dd, J = 3.9, 14.5 Hz, 1H), 2.71 (ddm, J = 8.0, 14.5 Hz, 1H), 2.50–2.41 (m, 2H), 2.00 (dh, J = 6.6, 8.9 Hz, 1H), 1.51–1.46 (m, 2H), 1.23 (s, 9H) 1.13–1.08 (m, 2H), 0.97 (d, J = 6.6 Hz, 3H), 0.95 (d, J = 6.6 Hz, 3H). ^{13}C NMR (CD_3OD): δ (~5:1 mixture of rotamers, major rotamer reported) 174.4, 174.2, 173.9, 163.2, 162.3, 161.3, 157.9, 152.1, 141.3, 130.6, 129.8, 129.1, 124.5, 119.3, 116.5, 107.2, 100.2, 80.4, 77.8, 59.9, 56.0, 55.3, 54.2, 52.9, 39.5, 37.0, 34.2, 31.9, 28.6, 19.7, 19.0, 17.8, 17.7. HRMS calcd for $C_{37}H_{47}N_4O_8$ ($M+H^+$) 675.3394, found: 675.3398.

5.1.18. Compound 19. To a solution of **15** (0.097 g, 0.17 mmol) and the hydrochloric salt of 1-amino-cyclopropanecarboxylic acid methyl ester (0.038 g, 0.25 mmol) in dry DMF (3.0 mL) were added HBTU (0.077 g, 0.20 mmol) and DIEA (0.12 mL, 0.67 mmol). The resulting solution was stirred at room temperature for 4 h, diluted with EtOAc (30 mL), and washed with aqueous NaOAc buffer pH 4 (4 × 12 mL) and brine (15 mL). The organic layer was dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by column chromatography (gradient elution, *i*-hexane/EtOAc 1:2 followed by *i*-hexane/EtOAc 1:3) to give **19** (0.086 g, 76%) as white solid ^{1}H NMR (CD_3OD): δ (~ 2:1 mixture of rotamers, major rotamer reported) 8.10–8.01 (m, 3H), 7.57–7.47 (m, 3H), 7.41 (d, J = 2.5 Hz, 1H), 7.30 (s, 1H), 7.15 (dd, J = 2.5, 9.1 Hz, 1H), 5.62–5.57 (m, 1H), 4.61–4.55 (m, 1H), 4.14 (dd, J = 4.3, 11.7 Hz, 1H), 4.06 (dm, J = 11.7 Hz, 1H), 3.96 (s, 3H), 3.82–3.74 (m, 1H), 3.66 (s, 3H), 2.73–2.65 (m, 1H), 2.63–2.51 (m, 1H), 2.55 (dd, J = 5.5, 15.2 Hz, 1H), 2.44 (dd, J = 7.8, 15.2 Hz, 1H), 1.81–1.71 (m, 1H), 1.57–1.49 (m, 1H), 1.49–1.42 (m, 1H), 1.41 (s, 9H), 1.25–1.14 (m, 2H), 0.86 (d, J = 6.8 Hz, 3H), 0.81 (d, J = 6.8 Hz, 3H). ^{13}C NMR (CD_3OD): δ (~ 2:1 mixture of rotamers, major rotamer reported) 175.3, 174.3, 172.9, 163.3, 162.1, 161.4, 158.0, 152.3, 141.3, 130.6, 129.8, 129.0, 124.2, 119.5, 116.4, 107.5, 100.1, 79.9, 77.6, 60.3, 56.0, 54.3, 54.1, 53.0, 38.6, 36.0, 34.4, 33.1, 28.8, 19.7, 18.2, 18.0, 17.4. HRMS calcd for $C_{37}H_{47}N_4O_8$ ($M+H^+$) 675.3394, found: 675.3413.

5.1.19. Compound 20. To a solution of **16** (0.074 g, 0.11 mmol) in THF (10.0 mL) and MeOH (1.5 mL) was added a solution of LiOH (0.026 g, 1.1 mmol) in H_2O (4.0 mL). The reaction mixture was stirred at room temperature for 3 h and neutralized with 1.0 M aqueous HCl. The organic solvents were removed by rotary evaporation, H_2O (10 mL) was added, and the aqueous phase was acidified with 1.0 M aqueous HCl to pH 3 and extracted with EtOAc (3 × 15 mL). The organic layers were washed with brine (10 mL), dried with $MgSO_4$, and concentrated by rotary evaporation to give **20** (0.070 g, 97%) as white solid. ^{1}H NMR (CD_3OD): δ (1:1 mixture of diasteromers) 8.12 (d, J = 9.2 Hz, 1H), 8.09–8.03 (m, 2H), 7.59–7.50 (m, 3H), 7.40 (d, J = 2.5 Hz, 1H), 7.28 (s, 1H), 7.12 & 7.11 (dd, J = 2.5, 9.2 Hz, 1H), 5.59–5.53 (m, 1H), 4.63–4.53 (m, 2H), 4.10–4.00 (m, 2H), 3.95 (s, 3H), 4.06 (detected by HSQC) & 3.81 (s, 1H), 2.74–2.63 (m, 1H), 2.51–2.38 (m, 1H), 2.04–1.93 (m, 1H), 1.24 (s, 9H), 1.04–0.73 (m, 4H), 1.01 (d, J = 6.8 Hz, 3H), 0.96 (d, J = 6.8 Hz, 3H). ^{13}C NMR (CD_3OD): δ (1:1 mixture of diasteromers) 176.3 & 175.8, 176.0 & 174.9, 174.0 & 173.9, 163.6, 162.8, 160.9, 157.9, 151.0, 140.2, 131.0, 129.9, 129.2, 124.7, 119.6, 116.4, 106.4, 100.4, 80.3, 78.6 & 78.5, 77.1 & 73.7, 60.5 & 60.2, 59.6, 56.1, 54.4, 37.2 & 36.3, 36.2 & 36.1, 31.7, 28.6, 19.74 & 19.67, 19.09 & 19.07, 13.1 & 12.9, 12.0 & 11.1. HRMS calcd for $C_{36}H_{45}N_4O_9$ ($M+H^+$) 677.3187, found: 677.3199.

5.1.20. Compound 21. To a solution of **17** (0.097 g, 0.14 mmol) in THF (7.0 mL) and MeOH (1.5 mL) was added a solution of LiOH (0.035 g, 1.4 mmol) in H_2O (2.0 mL). The reaction mixture was stirred at room temperature overnight and neutralized with 1.0 M aqueous HCl. The organic solvents were removed by rotary evaporation, H_2O (10 mL) was added, and the aqueous phase was acidified with 1.0 M aqueous HCl to pH 3 and extracted with EtOAc (3 × 35 mL). The organic layers were washed with brine (40 mL), dried with $MgSO_4$ and concentrated by rotary evaporation to give **21** (0.093 g, 98%) as white solid. ^{1}H NMR (CD_3OD): δ

8.11 (d, J = 9.2 Hz, 1H), 8.07–8.03 (m, 2H), 7.58–7.49 (m, 3H) 7.40 (d, J = 2.5 Hz, 1H), 7.25 (s, 1H), 7.10 (dd, J = 2.5, 9.2 Hz, 1H), 5.55–5.53 (m, 1H), 4.59 (dm, J = 11.8 Hz, 1H), 4.56 (dd, J = 7.6, 9.8 Hz, 1H), 4.06 (dd, J = 3.7, 11.8 Hz, 1H), 4.05 (d, J = 8.9 Hz, 1H) 3.96 (s, 3H), 2.79 (d, J = 15.7 Hz, 1H), 2.67 (ddm, J = 7.6, 14.1 Hz, 1H), 2.38 (d, J = 15.7 Hz, 1H), 2.38 (ddd, J = 4.0, 9.8, 14.1 Hz, 1H), 1.99 (dh, J = 6.8, 8.9 Hz, 1H), 1.23 (s, 9H), 1.02 (d, J = 6.8 Hz, 3H), 0.97 (d, J = 6.8 Hz, 3H), 0.90–0.76 (m, 4H). ^{13}C NMR (CD_3OD): δ 175.3, 174.3, 173.9, 163.3, 162.3, 161.3, 157.9, 151.8, 141.0, 130.7, 129.8, 129.1, 124.5, 119.4, 116.5, 107.0, 100.2, 80.3, 78.2, 60.4, 59.6, 56.0, 54.5, 41.4, 36.1, 31.7, 31.0, 28.6, 19.7, 19.1, 14.2, 13.5. HRMS calcd for $C_{36}H_{45}N_4O_8$ ($M+H^+$) 661.3237, found: 661.3242. RP-HPLC purity (column 1: 99%, column 2: 99%).

5.1.21. Compound 22. To a solution of **18** (0.057 g, 0.084 mmol) in THF (7.0 mL) and MeOH (1.5 mL) was added a solution of LiOH (0.020 g, 0.84 mmol) in H_2O (2.0 mL). The reaction mixture was stirred at room temperature for 4 h and neutralized with 1.0 M aqueous HCl. The organic solvents were removed by rotary evaporation, H_2O (10 mL) was added, and the aqueous phase was acidified with 1.0 M aqueous HCl to pH 3 and extracted with EtOAc (3 × 25 mL). The organic layers were washed with brine (20 mL), dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by preparative RP-HPLC (MeCN/H_2O gradient with 0.1% TFA) to give the TFA salt of **22** (0.044 g, 67%) as white solid. ^{1}H NMR (CD_3OD): δ (~5:1 mixture of rotamers, major rotamer reported) 8.33 (d, J = 9.3 Hz, 1H), 8.09–8.04 (m, 2H), 7.74–7.67 (m, 3H), 7.55 (s, 1H), 7.49 (d, J = 2.4 Hz, 1H), 7.34 (dd, J = 2.4, 9.3 Hz, 1H), 5.71–5.67 (m, 1H), 4.71 (dm, J = 12.2 Hz, 1H), 4.66–4.58 (m, 1H), 4.06–4.00 (m, 1H), 4.03 (s, 3H), 3.98 (d, J = 8.7 Hz, 1H), 3.04 (dd, J = 3.8, 14.6 Hz, 1H), 2.77 (ddm, J = 7.6, 14.6 Hz, 1H), 2.57–2.49 (m, 2H), 1.97 (dh, J = 6.7, 8.7 Hz, 1H), 1.51–1.47 (m, 2H), 1.17 (s, 9H), 1.12–1.08, (m, 2H), 0.97 (d, J = 6.7 Hz, 3H), 0.95 (d, J = 6.7 Hz, 3H). ^{13}C NMR (CD_3OD): δ (~5:1 mixture of rotamers, major rotamer reported) 175.8, 174.2, 174.1, 167.5, 166.1, 158.6, 158.0, 144.7, 134.6, 133.4, 130.7, 129.8, 126.4, 121.5, 116.2, 102.1, 101.3, 80.8, 80.1, 60.0, 56.8, 55.2, 53.9, 39.2, 37.3, 34.0, 31.8, 28.5, 19.7, 19.2, 17.7, 17.6. HRMS calcd for $C_{36}H_{45}N_4O_8$ ($M+H^+$) 661.3237, found: 661.3238. RP-HPLC purity (column 1: 99%, column 2: 99%).

5.1.22. Compound 23. To a solution of **19** (0.073 g, 0.11 mmol) in THF (7.0 mL) and MeOH (1.5 mL) was added a solution of LiOH (0.026 g, 1.1 mmol) in H_2O (2.0 mL). The reaction mixture was stirred at room temperature for 5 h and neutralized with 1.0 M aqueous HCl. The organic solvents were removed by rotary evaporation and the remaining aqueous phase was acidified with 1.0 M aqueous HCl to pH 3 and extracted with EtOAc (3 × 30 mL). The organic layers were washed with brine (25 mL), dried with $MgSO_4$ and concentrated by rotary evaporation to give **23** (0.069 g, 96%) as white solid. ^{1}H NMR (CD_3OD): δ (~2:1 mixture of rotamers, major rotamer reported) 8.09 (d, J = 9.2 Hz, 1H), 8.08–8.02 (m, 2H), 7.58–7.48 (m, 3H), 7.41 (d, J = 2.5 Hz, 1H), 7.31 (s, 1H), 7.16 (dd, J = 2.5, 9.2 Hz, 1H), 5.63–5.57 (m, 1H), 4.61–4.55 (m, 1H), 4.14 (dd, J = 4.2, 11.7 Hz, 1H), 4.05 (dm, J = 11.7 Hz, 1H), 3.96 (s, 3H), 3.81–3.75 (m, 1H), 2.75–2.67 (m, 1H), 2.61–2.52 (m, 2H), 2.43 (dd, J = 7.7, 15.2 Hz, 1H), 1.81–1.71 (m, 1H), 1.58–1.50 (m, 1H), 1.58–1.50 (m, 1H), 1.41 (s, 9H), 1.26–1.10 (m, 2H), 0.86 (d, J = 6.8 Hz, 1H), 0.80 (d, J = 6.8 Hz, 1H). ^{13}C NMR (CD_3OD): δ (~2:1 mixture of rotamers, major rotamer reported) 176.0, 175.1, 172.8, 163.4, 162.5, 161.0, 158.0, 151.6, 140.6, 130.8, 129.9, 129.0, 124.3, 119.6, 116.3, 107.0, 100.2, 79.9, 77.9, 60.3, 56.1, 54.2, 54.1, 38.5, 36.0, 34.3, 33.1, 28.8, 19.9, 18.2, 17.9, 17.3. HRMS calcd for $C_{36}H_{45}N_4O_8$ ($M+H^+$) 661.3237, found: 661.3258. RP-HPLC purity (column 1: 98%, column 2: 99%).

5.1.23. Compound 24. A solution of **21** (0.030 g, 0.045 mmol), HATU (0.021 g, 0.055 mmol), and DIEA (0.032 mL, 0.18 mmol), in dry DMF (2.0 mL) was stirred at room temperature for 1.5 h. Benzenesulfonamide (0.029 g, 0.18 mmol), DMAP (0.022 g, 0.18 mmol) and DBU (0.028 mL, 0.18 mmol) in dry DMF (1.0 mL) were added and the mixture was stirred at room temperature overnight, diluted with EtOAc (25 mL), and washed with aqueous NaOAc buffer pH 4 (2 × 12 mL), 5% aqueous $NaHCO_3$ (12 mL) and brine (15 mL). The organic layer was dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by preparative RP-HPLC (MeCN/H_2O gradient with 0.1% TFA) to give the TFA salt of **24** (0.024 g, 58%) as white solid. ^{1}H NMR (CD_3OD): δ 8.35 (d, J = 9.3 Hz, 1H), 8.10–8.07 (m, 2H), 8.02–7.99 (m, 2H) 7.76–7.69 (m, 3H), 7.66–7.61 (m, 1H), 7.60 (s, 1H) 7.58–7.53 (m, 2H), 7.52 (d, J = 2.4 Hz, 1H), 7.38 (dd, J = 2.4, 9.3 Hz, 1H), 5.78–5.74 (m, 1H), 4.76 (dm, J = 12.3 Hz, 1H), 4.55 (dd, J = 7.5, 10.0 Hz, 1H), 4.08 (dd, J = 3.4, 12.3 Hz, 1H), 4.05 (s, 3H), 3.96 (d, J = 9.0 Hz, 1H), 2.73 (d, J = 15.7 Hz, 1H), 2.70 (ddm, J = 7.5, 14.3 Hz, 1H), 2.45–2.35 (m, 1H), 2.36 (d, J = 15.7 Hz, 1H), 1.97 (dh, J = 6.7, 9.0 Hz, 1H), 1.17 (s, 9H), 1.03 (d, J = 6.7 Hz, 3H), 0.98 (d, J = 6.7 Hz, 3H), 0.83–0.78 (m, 2H), 0.75–0.69 (m, 2H). ^{13}C NMR (CD_3OD): δ 174.1, 174.0, 171.3, 167.9, 166.5, 158.4, 158.0, 143.9, 141.0, 134.8, 133.3, 133.6, 130.8, 130.0, 129.9, 129.2, 126.4, 121.9, 116.2, 102.5, 100.6, 81.3, 80.1, 60.3, 59.7, 56.9, 54.2, 43.0, 36.3, 31.6, 30.8, 28.5, 19.6, 19.3, 13.7, 13.6. HRMS calcd for $C_{42}H_{50}N_5O_9S$ ($M+H^+$) 800.3329, found: 800.3323. RP-HPLC purity (column 1: >99%, column 2: 99%).

5.1.24. Compound 25. A solution of **22** (0.025 g, 0.038 mmol), HATU (0.017 g, 0.045 mmol), and DIEA (0.026 mL, 0.15 mmol) in dry DMF (2.0 mL) was stirred at room temperature for 1.5 h. Benzenesulfonamide (0.024 g, 0.15 mmol), DMAP (0.019 g, 0.18 mmol), and DBU (0.023 mL, 0.15 mmol) in dry DMF (1.0 mL) were added and the mixture was stirred at room temperature overnight, diluted with EtOAc (25 mL), and washed with aqueous NaOAc buffer pH 4 (2 × 12 mL), 5% aqueous $NaHCO_3$ (12 mL), and brine (15 mL). The organic layer was dried with $MgSO_4$ and concentrated by rotary

evaporation. The resulting product was purified by preparative RP-HPLC (MeCN/H_2O gradient with 0.1% TFA) to give the TFA salt of **25** (0.021 g, 60%) as white solid. ^{1}H NMR (CD_3OD): δ 8.37 (d, J = 9.3 Hz, 1H), 8.10–8.05 (m, 2H), 8.03–7.97 (m, 2H), 7.78–7.64 (m, 4H), 7.60 (s, 1H), 7.57–7.53 (m, 2H), 7.52 (d, J = 2.5 Hz, 1H), 7.38 (dd, J = 2.5, 9.2 Hz, 1H), 5.76–5.71 (m, 1H), 4.77–6.67 (m, 1H), 4.61 (dm, J = 12.7 Hz, 1H), 4.13 (d, J = 8.2 Hz, 1H), 4.12–4.07 (m, 1H), 4.05 (s, 3H), 2.78 (ddm, J = 6.9, 14.6 Hz, 1H), 2.63 (dd, J = 7.7, 15.3 Hz, 1H), 2.48 (dd, J = 3.0, 15.3 Hz, 1H), 2.30 (ddd, J = 3.8, 10.6, 14.6 Hz, 1H), 2.10 (dh, J = 6.7, 8.2 Hz, 1H), 1.54–1.46 (m, 1H), 1.40–1.33 (m, 1H), 1.20 (s, 9H), 1.15–1.07, (m, 2H), 1.06 (d, J = 6.7 Hz, 3H), 1.04 (d, J = 6.7 Hz, 3H). ^{13}C NMR (CD_3OD): δ 175.6, 174.5, 173.5, 167.7, 166.4, 158.4, 158.2, 144.2, 140.7, 134.8, 133.9, 133.7, 130.7, 129.9, 129.9, 129.2, 126.4, 121.8, 116.2, 102.3, 100.9, 80.4, 80.3, 59.5, 56.9, 55.7, 54.1, 42.3, 38.5, 36.0, 32.1, 28.5, 19.9, 19.8, 19.4, 19.0. HRMS calcd for $C_{42}H_{50}N_5O_9S$ ($M+H^+$) 800.3329, found: 800.3328. RP-HPLC purity (column 1: >99%, column 2: 98%).

5.1.25. Compound 26. A solution of **23** (0.030 g, 0.045 mmol), HATU (0.021 g, 0.055 mmol) and DIEA (0.032 mL, 0.18 mmol), in dry DMF (2.0 mL) was stirred at room temperature for 1.5 h. Benzenesulfonamide (0.029 g, 0.18 mmol), DMAP (0.022 g, 0.18 mmol) and DBU (0.028 mL, 0.18 mmol) in dry DMF (1.0 mL) were added and the mixture was stirred at room temperature overnight, diluted with EtOAc (25 mL), and washed with aqueous NaOAc buffer pH 4 (2 × 12 mL), 5% aqueous $NaHCO_3$ (12 mL), and brine (15 mL). The organic layer was dried with $MgSO_4$ and concentrated by rotary evaporation. The resulting product was purified by preparative RP-HPLC (MeCN/H_2O gradient with 0.1% TFA) to give the TFA salt of **26** (0.026 g, 63%) as white solid. ^{1}H NMR (CD_3OD): δ 8.38 (d, J = 9.3 Hz, 1H), 8.09–8.04 (m, 2H), 8.03–7.98 (m, 2H) 7.76–7.65 (m, 4H), 7.61–7.54 (m, 3H), 7.53 (d, J = 2.5 Hz, 1H), 7.43 (dd, J = 2.5, 9.3 Hz, 1H), 5.89–5.84 (m, 1H), 4.24–4.19 (m, 2H), 4.06 (s, 3H), 3.76–3.68 (m, 1H), 2.71 (ddm, J = 6.8, 13.8 Hz, 1H), 2.69–2.65 (ddd, J = 4.0, 10.4, 13.8 Hz, 1H), 2.02–1.91 (m, 1H), 1.48–1.39 (m, 2H), 1.41 (s, 9H), 1.15–1.06 (m, 2H), 0.95, (d, J = 6.8 Hz, 3H), 0.89 (d, J = 6.8 Hz, 3H). ^{13}C NMR (CD_3OD): δ 174.9, 173.7, 172.9, 167.8, 166.6, 158.2, 158.1, 143.6, 140.5, 134.8, 133.9, 133.3, 130.8, 129.9, 129.9, 129.2, 126.4, 122.0, 116.0, 102.2, 100.5, 81.2, 79.3, 61.2, 57.0, 54.8, 54.4, 38.3, 35.8, 35.7, 32.6, 28.8, 20.0, 19.6, 19.2, 19.0. HRMS calcd for $C_{42}H_{50}N_5O_9S$ ($M+H^+$) 800.3329, found: 800.3304. RP-HPLC purity (column 1: 99%, column 2: 99%).

5.1.26. Compound 27. Compound **20** (0.024 g, 0.35 mmol) was dissolved in dry DCM (1.5 mL), stirred under N_2 atmosphere, and cooled to 0 °C. Dess–Martin periodinane (0.022 g, 0.53 mmol) in dry DCM (1.5 mL) was added and the resulting mixture was stirred at room temperature for 4 h. The mixture was cooled to 0 °C and quenched with saturated aqueous $Na_2S_2O_4$ (2.0 mL) and saturated aqueous $NaHCO_3$ (2.0 mL). The reaction mixture was extracted with DCM (3 × 10 mL), dried with $MgSO_4$, and concentrated by rotary evaporation. The resulting product was purified by preparative RP-HPLC (MeCN/H_2O gradient with 0.1% TFA) to give the TFA salt of **27** (0.010 g, 36%) as white solid. ^{1} NMR (DMSO-d_6): δ 8.88 (s, 1H), 8.25–8.13 (m, 3H), 7.71–7.57 (m, 4H), 7.53–7.46 (m, 1H), 7.25–7.17 (m, 1H), 7.03 (d, J = 7.9 Hz, 1H), 5.81–5.75 (m, 1H), 4.57–4.51 (m, 1H), 4.47–4.41 (m, 1H), 3.96 (s, 3H), 3.96–3.91 (m, 1H), 3.87–3.81 (m, 1H), 2.59–2.52 (m, 1H), 2.27–2.18 (m, 1H), 1.93–1.82 (m, 1H), 1.64–1.58 (m, 1H), 1.47–1.41 (m, 1H), 1.29–1.22 (m, 1H), 1.19 (s, 9H), 1.13–1.06 (m, 1H), 0.90 (d, J = 6.8 Hz, 3H), 0.87 (d, J = 6.8 Hz, 3H). HRMS calcd for $C_{36}H_{43}N_4O_9$ ($M+H^+$) 675.3030, found: 675.3035. RP-HPLC purity (column 1: 96%, column 2: 96%).

5.2. Inhibition analysis

Protease inhibition of full-length HCV NS3 protein was determined essentially as described previously.[37] The hydrolysis of a depsipeptide substrate, Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Abu-ψ-[COO]Ala-Ser-Lys(DABCYL)-NH2 (AnaSpec, San Jose, CA, USA), was measured continuously over time with a fluorescence plate reader (Fluoroskan Ascent Labsystems, Stockholm, Sweden). Enzyme at a concentration of 1 nM was pre-incubated in 50 mM HEPES, pH 7.5, 10 mM DTT, 40% (w/v) glycerol, 0.1% *n*-octyl-β-D-glucoside, 3.3% (v/v) DMSO, 25 μM NS4A peptide cofactor (KKGSVVIVGRIVLSGK), and inhibitor. The reaction was started by the addition of 0.5 μM substrate and the initial rate velocities were used to determine the K_i using nonlinear regression analysis.[49]

5.3. Molecular docking

FLO+[50] was used for all docking calculations and was chosen partly due to its ability to allow protein flexibility, allowing a better approximation of protein–ligand interactions.[51] Like the assay used for inhibition analysis, the active site used in docking is comprised of both the protease and helicase domains of the NS3 protein and is derived from the crystal structure of Yao et al. (PDB code 1CU1).[41] This active site has been used previously and described in greater detail in our earlier work.[35]

Ligand conformations within the active site were explored using limited Monte Carlo perturbation and simulated annealing was used to explore local conformational space. To limit conformational change induced by simulated annealing, an energy penalty of 20 kJ/(mol Å^2) was applied when the similarity distance between two sequential conformations differed by more than 0.2 Å. Residues R155 and K136 of the active site were allowed full conformational freedom while movement of all other residues by more than 0.2 Å was penalized by 20 kJ/(mol Å^2). Zero-order bonds to hold the ligand in the vicinity of the catalytic site of the protein were used.

5.4. Comparative molecular field analysis

A 3D-QSAR model was derived using comparative molecular field analysis (CoMFA)[28] using all of our previously synthesized and published proline-based inhibi-

tors[33–35] as well as the β-amino acid- comprising peptides presented herein. Docking was used to propose bioactive conformation and provide molecular alignment. Docking poses for subsequent use in CoMFA were selected based on docking score as well as subjective analysis. Prior to use in CoMFA, ligands were subjected to 10 steps of minimization using the Tripos force field[52] and partial atomic charges were calculated using the Gasteiger-Hückel[53,54] method. A carbon sp^3 atom with a +1 charge and the default CoMFA region were used for all CoMFA modeling.

In total, 54 proline-based inhibitors of HCV NS3 protease were available for use in CoMFA. To allow for model validation using external prediction, the compounds were divided into training and test sets. This was done using the Selector module in Sybyl. CoMFA fields and biological activity data (expressed as pK_i) were used as descriptors and hierarchical clustering was used to select the 36 (two-thirds) training set compounds. The remaining 18 compounds were withheld from the CoMFA model for use as an external validation set. Sybyl 7.2[55] was used for training and test set selection as well as CoMFA modeling. The structures and inhibition data for all compounds included in the training and test sets are available as Supplementary material. Predictive performance was enhanced by optimizing CoMFA parameters.[36]

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Supplementary data

Structures and inhibition data for training and test set compounds and experimental and predicted pK_i values used to produce Figure 6, are available as supplementary material. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.04.005.

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