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Evaluation of a diverse set of potential P_1 carboxylic acid bioisosteres in hepatitis C virus NS3 protease inhibitors

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Abstract—There is an urgent need for more efficient therapies for people infected with hepatitis C virus (HCV). HCV NS3 protease inhibitors have shown proof-of-concept in clinical trials, which make the virally encoded NS3 protease an attractive drug target. Product-based NS3 protease inhibitors comprising a P_1 C-terminal carboxylic acid have shown to be effective and we were interested in finding alternatives to this crucial carboxylic acid group. Thus, a series of diverse P_1 functional groups with different acidity and with possibilities to form a similar, or an even more powerful, hydrogen bond network as compared to the carboxylic acid were synthesized and incorporated into potential inhibitors of the NS3 protease. Biochemical evaluation of the inhibitors was performed in both enzyme and cell-based assays. Several non-acidic C-terminal groups, such as amides and hydrazides, were evaluated but failed to produce inhibitors more potent than the corresponding carboxylic acid inhibitor. The tetrazole moiety, although of similar acidity to a carboxylic acid, provided an inhibitor with mediocre potencies in both assays. However, the acyl cyanamide and the acyl sulfinamide groups rendered compounds with low nanomolar inhibitory potencies and were more potent than the corresponding carboxylic acid inhibitor in the enzymatic assay. Additionally, results from a pH-study suggest that the P_1 C-terminal of the inhibitors comprising a carboxylic acid, an acyl sulfonamide or an acyl cyanamide group binds in a similar mode in the active site of the NS3 protease.

1. Introduction

The hepatitis C virus (HCV) is a global health threat affecting an estimated 123 million people worldwide.[1] An HCV infection becomes chronic in the majority of patients with cirrhosis and hepatocellular carcinoma as possible consequences.[2] Patients infected with HCV are currently treated with a combination of ribavirin and pegylated interferon-α. However, the present therapy regime possesses several drawbacks such as low response rates and severe side effects.[3] The pharmaceutical community is currently putting in major efforts in the search for new antiviral drugs targeting HCV.

Several possible virus-specific drug targets arise from the viral genome.[4] Among the most well-defined and well-studied targets is the virally encoded NS3 protease. The NS3 protease is one of two enzymatic domains of the NS3 protein, the other is a helicase/NTPase domain.[5] The NS3 serine protease is a key actor in the processing of the HCV encoded polyprotein and is essential for viral replication.[6,7] Recent studies suggest that the NS3 protease is also involved in viral persistence. Consequently, the inhibition of the NS3 protease could possibly, besides blocking the viral replication, also restore the host cell immune response, making it an attractive drug target.[8,9]

In 2003, the first report on clinical studies using an NS3 protease inhibitor was presented. A substantial reduction in viral-RNA blood levels was detected in HCV-infected patients treated with the inhibitor BILN-2061 (ciluprevir). This proof-of-concept study confirmed the NS3 protease as a valid drug target.[10] However, further clinical evaluation of BILN-2061 has been halted due to cardiac toxicity identified in animal studies.[11] Recently, two more NS3 protease inhibitors, VX-950 (telaprevir)

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and SCH 503034, have entered phase II clinical studies, both with very promising results.[12–15]

The emergence of drug resistance is a major problem in the use of antiviral drugs. Mutations conferring resistance to the three clinical candidates BILN-2061, VX-950, and SCH 503034 have been observed in the subgenomic HCV replicon assay.[16–19] If NS3 protease inhibitors are to be used in future therapy against hepatitis C, different inhibitors will certainly be needed in order to circumvent the problems with resistance. Thus, development of NS3 protease inhibitors comprising novel structural motifs is needed.

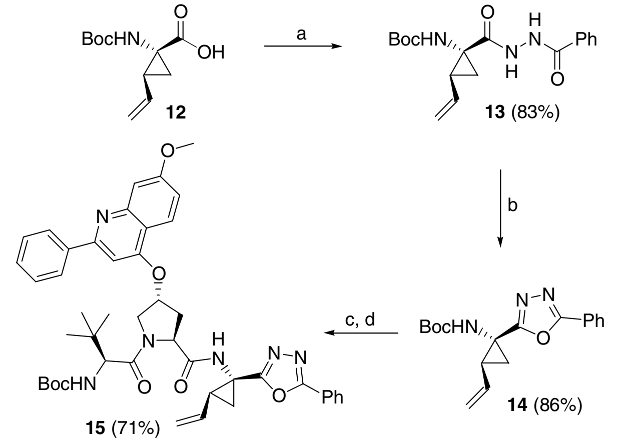
Our group has previously studied the acyl sulfonamide functionality as a very potent replacement to the P_1 C-terminal carboxylic acid found in product-based inhibitors of the NS3 protease such as BILN-2061.[20–22] We were interested in finding other alternatives to this carboxylic acid. Herein, we present the synthesis of inhibitors with diverse P_1 C-terminal functional groups and their inhibitory potential in both enzymatic and cell-based assays. Furthermore, the influence of different protonation states of the catalytic histidine on inhibitory potencies is studied. Some common carboxylic acid bioisosteres such as the tetrazole, the acyl cyanamide, and the hydroxamic acid are evaluated, as well as hydrazine- and amide-based groups.

2. Results

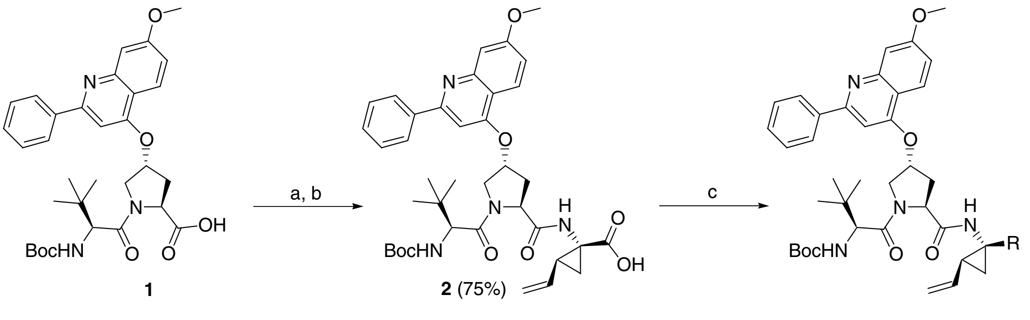
2.1. Chemistry

The target compounds **3**–**11** were synthesized in a linear fashion starting from dipeptide **1**[23] followed by the final coupling between tripeptide **2**[24] and the appropriate nucleophile (Scheme 1). The hydrazides **3** and **4** as well as the diacyl hydrazines **5** and **6** were synthesized in moderate yields employing standard peptide coupling conditions with *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)-methylene]-*N*- methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) or *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-yl- methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) as activating agents. The acyl sulfinamide **7** was prepared through activation with 1,1′-carbonyldiimidazole (CDI) followed by coupling with (*S*)-(+)-*p*-toluenesulfinamide. Partial epimerization at the sulfur occurred during synthesis of **7** which was obtained as a 2:1 mixture of diastereomers. Reaction between hydroxylamine hydrochloride and tripeptide **2** was enabled by the use of isobutyl chloroformate at low temperature to give hydroxamic acid **8** in good yield. The acyl cyanamide **9** was successfully prepared through the reaction between **2** and cyanamide using HATU as the activating agent. Three days of reaction at elevated temperature (40 °C) was required in order to obtain a reasonable yield of acyl cyanamide **9**. 2,2,2-Trifluoroethylamine was coupled to tripeptide **2**, again using HATU as the activating agent, to produce amide **10** in excellent yield. The poor nucleophile 2-aminothiazole was coupled to the tripeptide **2** by the use of HATU and dichloromethane as solvent to produce the heterocyclic amide **11**. However, **11** was obtained as a 3:1 mixture of isomers possibly due to the acylation of both the exocyclic and the endocyclic nitrogen of 2-aminothiazole.[25,26]

In order to synthesize the 1,3,4-oxadiazole **15** the P_1 building block **14** had to be prepared (Scheme 2). The diacyl hydrazine moiety in compound **13**, prepared from (1*R*,2*S*)-1-*tert*-butoxycarbonylamino-2-vinylcyclopropanecarboxylic acid (**12**), was successfully dehydrated using Burgess reagent to give the heterocyclic building block **14**.[27] N-terminal deprotection of **14** with



Scheme 2. Reagents and conditions: (a) benzhydrazide, HATU, DIEA, CH_2Cl_2, rt; (b) Burgess reagent, THF, 75 °C; (c) HCl/1,4-dioxane, rt; (d) **1**, HBTU, DIEA, DMF, rt.



3, R=CONHNHPh (44%)
4, R=CONHNHBn (43%)
5, R=CONHNHCOPh (32%)
6, R=CONHNHCOBn (67%)
7, R=CONHSO*p*tol (47%)
8, R=CONHOH (67%)
9, R=CONHCN (39%)
10, R=CONHCH$_2$CF$_3$ (82%)
11, R=CONH-2-thiazolyl (80%)

Scheme 1. Reagents and conditions: (a) HCl × (1*R*,2*S*)-1-amino-2-vinylcyclopropanecarboxylic acid ethyl ester, HATU, *N*,*N*′-diisopropylethylamine (DIEA), DMF, rt; (b) LiOH, THF, MeOH, H_2O, rt; (c) nucleophile, coupling reagent, base.

hydrochloric acid followed by amide coupling with dipeptide **1** afforded the target compound **15**.

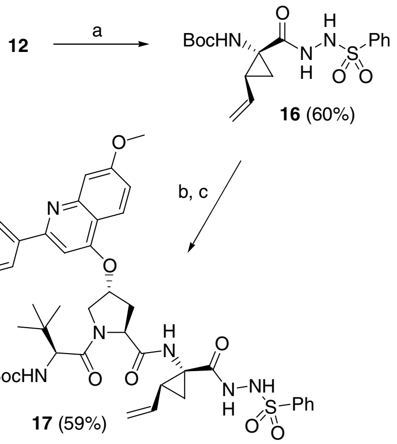
The carboxylic acid **12** was successfully reacted with benzenesulfonyl hydrazide in the presence of *N*,*N*′-diisopropylcarbodiimide to give the Boc protected P_1 building block **16** (Scheme 3). Deprotection followed by coupling with dipeptide **1** furnished the sulfonyl hydrazide **17** in good yield.

The target tetrazole **20** was synthesized in several steps (Scheme 4). The carboxylic acid **12** was converted to the corresponding primary amide which was subsequently dehydrated to give the nitrile **18**.[28] The conversion of the nitrile **18** to the tetrazole **19** was somewhat troublesome. However, a slight modification of the procedure presented by Koguro et al. was found to be successful.[29] A mixture of nitrile **18**, NaN_3, and triethylamine hydrochloride in toluene was gently heated in a closed reaction tube for 3 days to give the tetrazole **19** in good yield. Deprotection of **19** followed by amide coupling with dipeptide **1** delivered the final compound **20**.

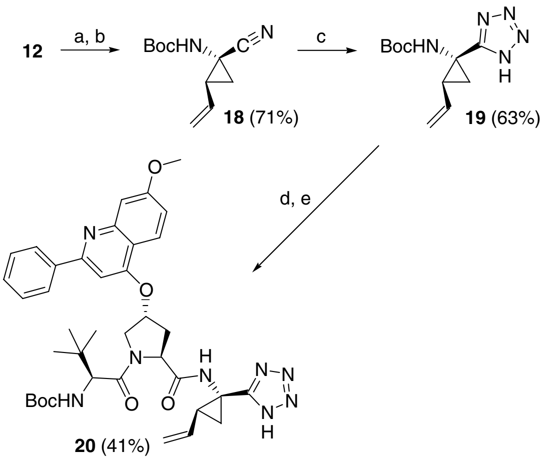
Syntheses of the acyl sulfonamides **21**–**23** have previously been reported by others.[23]

2.2. Biochemical evaluation

Biochemical evaluation of the target compounds **2**–**11**, **15**, **17**, and **20**–**23** was performed (Table 1). The inhibition constants (K_i) were determined in an enzymatic assay comprising the full-length NS3 protein.[30] Inhibition of viral replication (EC_{50}) was determined in a subgenomic HCV replicon assay.[31] The impact of pH variation on the inhibition of the NS3 protease for compounds **2**, **3**, **9**–**11**, and **23** was studied at three different pH-values (6.0, 7.0, and 8.0) in the enzymatic full-length NS3 assay (Table 2). Note that different assay buffers were used in the determination of the K_i-values presented in Table 1 and those presented in Table 2 (see Section 5 for details).



Scheme 3. Reagents and conditions: (a) benzenesulfonyl hydrazide, *N*,*N*′-diisopropylcarbodiimide, CH_2Cl_2, rt; (b) HCl/1,4-dioxane, rt; (c) **1**, HBTU, DIEA, DMF, rt.



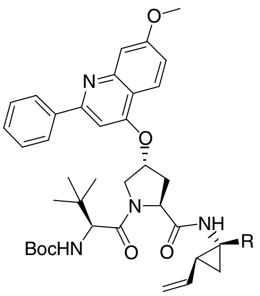
Scheme 4. Reagents and conditions: (a) i—CDI, THF, 70 °C; ii—NH_3, 0 °C to rt; (b) TsCl, pyridine, CH_2Cl_2, rt; (c) NaN_3, $HCl \times Et_3N$, toluene, rt to 50 °C; (d) HCl/1,4-dioxane, rt; (e) **1**, HATU, DIEA, DMF, rt.

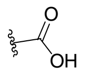
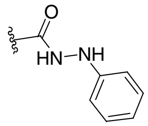
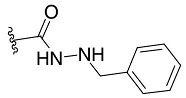
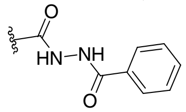
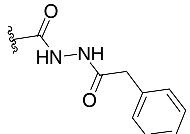
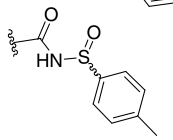
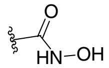
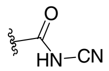
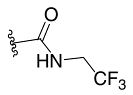
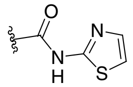
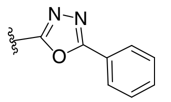
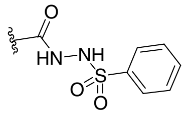
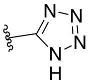
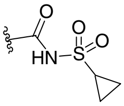
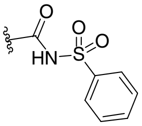
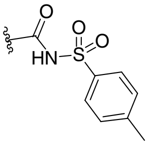
3. Discussion

Multiple interactions between an enzyme and an inhibitor are crucial for efficient enzyme inhibition. In addition, the presence of a strong anchor point is commonly seen. In the case of serine protease inhibitors, this anchor point is often an electrophilic atom capable of forming a covalent bond with the catalytic serine, as in the case of the clinical candidates VX-950 and SCH 503034.[12,14,32] For the HCV NS3 serine protease it was previously demonstrated that a carboxylic acid could function as an efficient non-covalent active site anchor point in NS3 protease inhibitors.[33,34] Our group has explored the replacement of this carboxylic acid with the acyl sulfonamide functionality, a strategy that proved to be successful.[20–22] In order to further explore the region around the active site of the NS3 protease, different C-terminal groups previously not used in NS3 protease inhibitors were evaluated. The C-terminal groups of the inhibitors were designed to be of different pK_a-values, contain different numbers of heteroatoms, and different numbers of H-bond donating/accepting atoms. The parent carboxylic acid **2** and the acyl sulfonamides **21**–**23** were included for comparison to the newly designed inhibitors.

The acyl hydrazines **3** and **4** and the diacyl hydrazines **5** and **6** all contain both H-bond donating and H-bond accepting atoms. Disappointingly, compounds **3**–**6** were mediocre inhibitors of the NS3 protease (K_i-values between 560 and 1600 nM), possibly due to their weak acidity. Ring closure of the diacyl hydrazine moiety in **5** provided the almost equipotent inhibitor **15** encompassing a 1,3,4-oxadiazole ring. The replacement of one of the two carbonyl groups in the diacyl hydrazine **5** with a sulfonyl group was successful. The sulfonyl analogue **17** with a K_i-value of 140 nM was more than 10 times as potent as the carbonyl counterpart **5** in the enzyme assay and it showed micromolar inhibition in the cellular assay (EC_{50} = 1.6 μM).

Table 1. HCV NS3 protease inhibition constants and EC_{50}-values



Compound	R	K_i ± SD[a] (nM)	EC_{50}[b] (μM)
2		20 ± 4	0.14
3		560 ± 97	>10
4		1100 ± 60	>10
5		1600 ± 140	>10
6		880 ± 130	>10
7[c]		9 ± 3	1.8
8		425 ± 36	3.8
9		6 ± 1	1.2
10		1690 ± 330	>2
11[d]		821 ± 71	>10
15		2200 ± 700	>10
17		140 ± 7	1.6
20		214 ± 53	2.8
21		0.058 ± 0.007	0.0009
22		0.34 ± 0.05	0.006
23		0.22 ± 0.02	0.006

SD, standard deviation.

[a] Determined in an enzymatic assay comprising the full-length NS3 protein.

[b] Determined in a subgenomic HCV replicon assay.

[c] 2:1 mixture of diastereomers.

[d] 3:1 mixture of isomers.

Table 2. HCV NS3 protease inhibition constants at different pH-values

Compound	K_i ± SD[a] (nM)		
	pH 6.0	pH 7.0	pH 8.0
2	0.12 ± 0.015	0.68 ± 0.11	8.7 ± 1.0
3	28 ± 5.8	113 ± 10	287 ± 23
9	0.14 ± 0.02	0.51 ± 0.08	10.8 ± 1.04
10	71 ± 6	349 ± 43	734 ± 72
11	7.7 ± 0.7	294 ± 41	343 ± 51
23	0.006 ± 0.002	0.056 ± 0.009	0.39 ± 0.06

SD, standard deviation.

[a] Determined in an enzymatic assay comprising the full-length NS3 protein.

The acyl sulfinamide **7** proved to be more potent than the carboxylic acid **2** in the enzyme assay. However, **7** was substantially less potent than **2** in the cellular assay despite its more lipophilic properties. In comparison to the reference acyl sulfonamide **23**, compound **7** was less active in both assays emphasizing the importance of both sulfonyl oxygens for excellent enzymatic and cell-based potencies.

Some common carboxylic acid bioisosteres such as the hydroxamic acid **8**, the acyl cyanamide **9**, and the tetrazole **20** were also explored. The hydroxamic acid **8** (K_i = 425 nM) was approximately 20 times less potent than the carboxylic acid **2**, possibly due to its higher pK_a-value (pK_a ~ 8–9).[35] Interestingly, the acyl cyanamide **9** was very potent in the enzyme assay with a K_i-value of 6 nM. Unfortunately, **9** lost much of its potency in the cellular assay (EC_{50} = 1.2 μM). The tetrazole moiety, with a pK_a-value similar to a carboxylic acid, has previously been reported to be an efficient carboxylic acid bioisostere in hexa- and pentapeptide NS3 protease inhibitors.[20] However, the tetrazole inhibitor **20**, with a K_i-value of 214 nM and an EC_{50}-value of 2.8 μM, was less potent than the carboxylic acid **2** in both assays.

We envisioned that the introduction of electron withdrawing groups close to the nitrogen in an amide would increase the amide acidity and result in potent amide inhibitors. Consequently, compounds **10** and **11** comprising the trifluoromethyl group and the 2-thiazolyl groups, respectively, were prepared. Unfortunately, biochemical evaluation indicated that both **10** and **11** are modest inhibitors of the NS3 protease, with K_i-values of 1690 and 821 nM, respectively.

Our reference inhibitors **21**–**23**, encompassing the acyl sulfonamide functionality and with a pK_a-value similar to a carboxylic acid, display very impressive K_i- as well as EC_{50}-values. In particular, the cyclopropyl substituent previously designed by Campbell et al. used in compound **21** provided an extremely potent inhibitor displaying a K_i-value of 58 pM and an EC_{50}-value of 0.9 nM.[23]

The binding mode for the P_1 C-terminal carboxylic acid in product-based NS3 protease inhibitors has previously been established by X-ray crystallography and mutagenesis studies.[5,36] It has been found that the P_1 C-terminal carboxylate oxygens are engaged in hydrogen bonding to the catalytic His57 and the oxyanion hole. We have previously, from molecular modeling studies, proposed a possible binding mode of the P_1 C-terminal group in inhibitors comprising the acyl sulfonamide functionality where the P_1 carbonyl is positioned in the oxyanion hole and the adjacent nitrogen atom is pointing toward the catalytic His57.[21]

Our group recently conducted a mechanistic study on electrophilic and non-electrophilic NS3 protease inhibitors.[37] Therein, it was found that the inhibition constants for inhibitors comprising acidic P_1 C-terminal groups (carboxylic acid, ketoacid, and ketotetrazole) were highly affected by the protonation state of the catalytic His57 residue. We hoped that valuable information on the interactions between the P_1 C-terminal groups in our inhibitors and His57 could be obtained by conducting a similar pH-study. In order to see the effects from the different protonation states of His57, with a pK_a determined to be 6.85,[37] a pH-range covering its pK_a was chosen. Consequently, the inhibition constants for compounds **2**, **3**, **9**–**11**, and **23** were determined at pH-values 6.0, 7.0, and 8.0 (Table 2).

From Figure 1 it can be seen that the inhibition constant of the carboxylic acid **2** is highly pH-dependent. This observation is in line with the established binding mode for the P_1 C-terminal carboxylic acid, reflecting a stronger interaction between the negatively charged carboxylate with the protonated His57 (pH 6.0) compared to the non-protonated His57 (pH 8.0). In addition, this result is also in line with our previous findings on inhibitors comprising an acidic P_1 C-terminal functionality.[37] Interestingly, the K_i-values of the acyl cyanamide **9** and the acyl sulfonamide **23** were equally affected by the pH as the carboxylic acid **2**. Therefore, we reason that the P_1 C-terminal in compounds **9** and **23** binds in a similar mode as the carboxylic acid, where the P_1 carbonyl occupies the oxyanion hole and the adjacent nitrogen atom points toward His57 (Fig. 2).

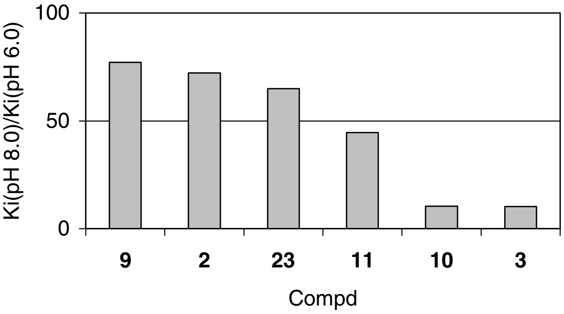


Figure 1. Ratios of HCV NS3 protease inhibition constants at pH 8.0 and pH 6.0. The compounds on the *X*-axis are arranged in a ratio-descending order. The exact K_i-values can be found in Table 2.

An increase in the inhibitory potencies of the carboxylic acid **2**, the acyl cyanamide **9**, and the acyl sulfonamide **23** with decreasing pH seems reasonable since these C-terminal groups are assumed to be partially deprotonated even at pH 6.0. However, we were surprised to see that the inhibition constants for the hydrazide **3** and the trifluoroethylamide **10**, assumed to be protonated at all pH-values examined, also were pH-dependent, although the effect was not as pronounced as for compounds **2**, **9**, and **23**. The assumption that the P_1 carbonyl in compounds **3** and **10** binds in the oxyanion hole makes these results hard to interpret. Noteworthy, our results are not in agreement with the results presented by Steinkühler et al.[34] They found that the inhibition constant for an inhibitor comprising a P_1 C-terminal amide was not pH-dependent when evaluated against the truncated NS3 (i.e., the protease domain only). One possible explanation of our results could be that another histidine residue, apart from His57, is engaged in interactions with our inhibitors and is also affected by changes in pH. The only histidine residue, with the exception of His57, in the vicinity of the protease binding site is His528 in the helicase domain, according to the scNS3-4A crystal structure.[5,38] The presence of His528 in our full-length NS3 protein may be responsible for the contradictory results although the possible influence of the helicase domain on the binding of protease inhibitors remains to be proven. On the other hand, the enhanced inhibition potencies observed at pH 6.0 for compounds **3** and **10** may also reflect an H-bond between the protonated His57 and the P_1 functionality in the inhibitors.

Our pH-study does not only reveal information about the enzyme–inhibitor interactions but it also emphasizes the importance of assay-conditions when comparing inhibition data from different laboratories.

During the preparation of this manuscript, the first X-ray structure of an acyl sulfonamide inhibitor in complex with the NS3 protease was presented.[39] Therein, the P_1 carbonyl is positioned in the oxyanion hole

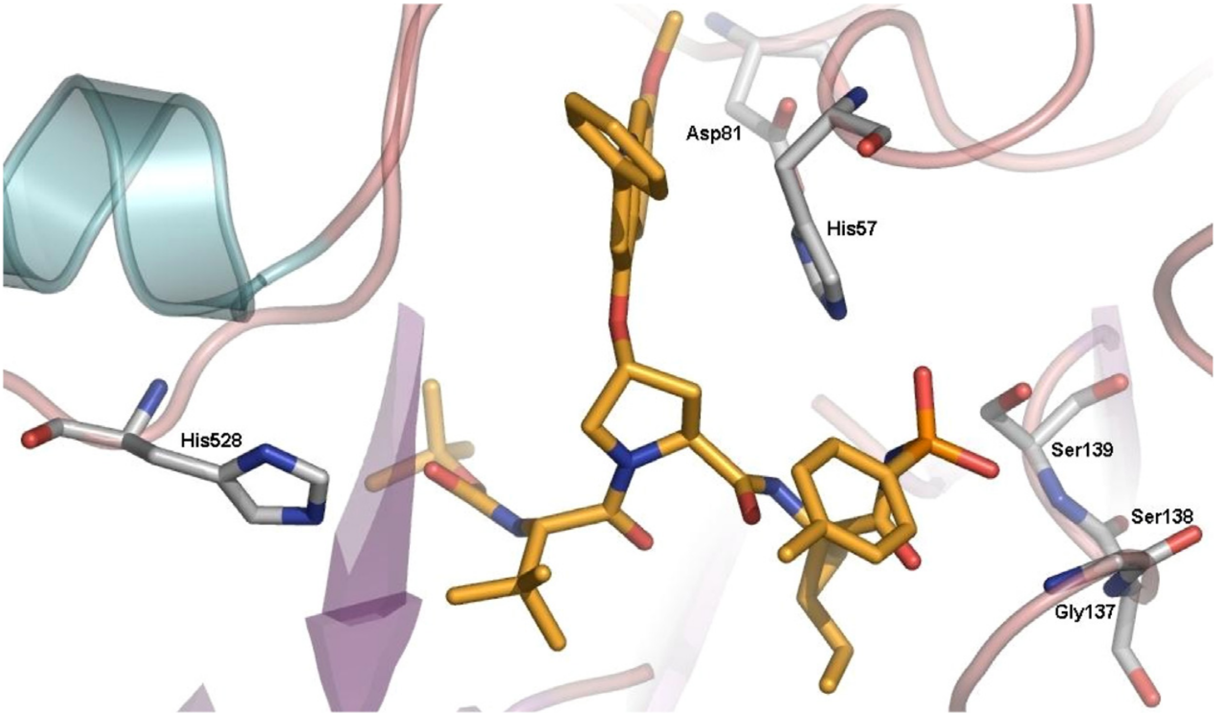


Figure 2. Compound **23** docked in the protease binding site, derived from the full-length NS3 protein (PDB code 1CU1).[5] Enzyme residues shown are: His57, Asp81, Gly137, Ser138, and Ser139 from the protease domain and His528 from the helicase domain. The P_1 carbonyl of **23** is positioned in the oxyanion hole (NHs of Ser139 and Gly137) and the adjacent nitrogen is pointing toward the catalytic His57. The carbonyl of the P_3 capping group of **23** is seen in close proximity to His528. Atoms are colored according to atom type (oxygen in red, nitrogen in blue, and sulfur in orange). Hydrogen atoms are omitted for clarity.

and the adjacent nitrogen points toward His57, findings that strengthen our hypothesis regarding the binding modes for inhibitors **2**, **9**, and **23**.

4. Conclusion

The design and synthesis of HCV NS3 protease inhibitors, comprising novel P_1 C-terminal functionalities as replacements of the common carboxylic acid, have been reported. The acyl sulfinamide and the acyl cyanamide were proven to be particularly potent P_1 C-terminal groups, providing inhibitors with nanomolar potencies in the enzyme assay. However, neither of the two inhibitors retained any significant potency in the cell-based assay. All of the non-acidic P_1 C-terminal functionalities evaluated herein failed to provide inhibitors more potent than the corresponding carboxylic acid inhibitor. Although the acidity of the P_1 C-terminal group seems important, it does not guarantee potent inhibitors, exemplified by the tetrazole-based inhibitor displaying lower potencies than the corresponding carboxylic acid-based inhibitor in both enzymatic and cell-based assays.

5. Experimental

5.1. Chemistry

TLC was performed using aluminum sheets precoated with silica gel 60 F_{254} (0.2 mm, Merck). Chromatographic spots were visualized using UV-detection and/or ethanolic ninhydrin solution (2%) followed by heating. Column chromatography was performed using silica gel 60 (40–63 μm, Merck) (or silica gel 60 RP-18 (40–63 μm, Merck) for compounds **8** and **9**). Analytical RP-HPLC-MS was performed on a Gilson–Finnigan ThermoQuest AQA system equipped with a C18 (Onyx Monolithic C18 (50 × 4.6 mm)) or a C4 (Hichrom ACE C4 (5 μm, 50 × 4.6 mm)) column using $MeCN/H_2O$ (0.05% HCOOH) as the mobile phase with UV (214 nm) and MS (ESI) detection. HPLC purity of the compounds was determined through integration of the UV-trace at 214 nm. Preparative RP-HPLC-MS was performed on a Gilson–Finnigan ThermoQuest AQA system equipped with a C8 [Sorbax SB-C8 (5 μm, 150 × 21.2 mm)] column using $MeCN/H_2O$ (0.05% HCOOH) as the mobile phase with UV (254 nm) and MS (ESI) detection. Elemental analyses were performed by Analytische Laboratorien, Lindlar, Germany, or by MikroKemi AB, Uppsala, Sweden. NMR spectra were recorded on a Varian Mercury plus spectrometer (^1H at 399.8 MHz, ^{13}C at 100.5 MHz) at ambient temperature. Chemical shifts (δ) are reported in ppm referenced indirectly to TMS via the solvent signals (^1H: $CHCl_3$ δ 7.26, CHD_2OD δ 3.31; ^{13}C: $CDCl_3$ δ 77.16, CD_3OD δ 49.00).

5.1.1. Compound 2.[24] A mixture of **1**[23] (200 mg, 0.346 mmol), the HCl salt of (1*R*,2*S*)-1-amino-2-vinylcyclopropanecarboxylic acid ethyl ester (100 mg, 0.522 mmol), HATU (158 mg, 0.416 mmol), DMF (5.0 mL), and DIEA (290 μL, 1.66 mmol) was stirred at room temperature for 3 h. The mixture was diluted with EtOAc and washed with aqueous NaOAc buffer (pH 4), 5% aqueous $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$), filtered, and evaporated. Purification by column chromatography (EtOAc/*i*-hexane 2:3) gave the corresponding tripeptide ester as a white solid. A solution of LiOH (97 mg, 4.1 mmol) in H_2O (3 mL) was added to a solution of the tripeptide ester (194 mg, 0.271 mmol) in THF (9 mL) and MeOH (2 mL). The resulting suspension was stirred at room temperature overnight and thereafter neutralized with 1.0 M aqueous HCl. The organic solvents were evaporated and the remaining aqueous phase was acidified to pH 3 using 1.0 M aqueous HCl. The aqueous phase was extracted with EtOAc and the organic layer was washed with brine, dried ($MgSO_4$), filtered, and evaporated to give **2**[24] (179 mg, 75%) as a white solid.

5.1.2. General procedure for the preparation of compounds 3–6. A mixture of **2**, the appropriate nucleophile, HBTU or HATU, CH_2Cl_2, and DIEA was stirred at room temperature for 1.5–3.5 h. The mixture was diluted with EtOAc or CH_2Cl_2 and washed with aqueous NaOAc buffer (pH 4), 5% aqueous $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$), filtered and evaporated. Purification by column chromatography and/or preparative RP-HPLC-MS gave the desired compounds **3**–**6**.

5.1.3. Compound 3. Prepared according to the general procedure using: **2** (30 mg, 0.044 mmol), phenylhydrazine (6.5 μL, 0.065 mmol), HBTU (20 mg, 0.053 mmol), CH_2Cl_2 (1.0 mL), and DIEA (15 μL, 0.086 mmol). Reaction time: 1.5 h. Purification by preparative RP-HPLC-MS followed by column chromatography (gradient elution: EtOAc/*i*-hexane 1:1 to 2:1) gave **3** (15 mg, 44%) as a light yellow solid. ^{1}H NMR (CD_3OD) δ 8.12 (d, $J = 9.2$ Hz, 1H), 8.07–8.05 (m, 2H), 7.57–7.48 (m, 3H), 7.40 (d, $J = 2.4$ Hz, 1H), 7.26 (s, 1H), 7.14–7.06 (m, 3H), 6.83–6.80 (m, 2H), 6.77–6.73 (m, 1H), 5.86 (ddd, $J = 9.3$, 10.2, 17.2 Hz, 1H), 5.59–5.57 (m, 1H), 5.26 (dm, $J = 17.2$ Hz, 1H), 5.03 (dm, $J = 10.2$ Hz, 1H), 4.61–4.55 (m, 2H), 4.22–4.20 (m, 1H), 4.10 (dd, $J = 3.3$, 11.8 Hz, 1H), 3.95 (s, 3H), 2.67 (ddm, $J = 6.9$, 14.0 Hz, 1H), 2.42–2.35 (m, 1H), 2.19–2.12 (m, 1H), 1.84 (dd, $J = 5.2$, 7.9 Hz, 1H), 1.38 (dd, $J = 5.2$, 9.6 Hz, 1H), 1.24 (s, 9H), 0.89 (s, 9H). ^{13}C NMR (CD_3OD) δ 175.0, 173.9, 172.0, 163.2, 161.8, 161.5, 158.0, 152.3, 149.8, 141.4, 135.4, 130.6, 129.8, 129.7, 129.0, 124.3, 120.7, 119.3, 117.4, 116.5, 114.4, 107.4, 100.1, 80.5, 78.1, 61.1, 60.5, 56.0, 55.4, 41.2, 35.8, 35.6, 34.7, 28.5, 26.8, 23.9. MS $[M+H]^+$ 777.5. HPLC purity (C18 column >99%, C4 column 97%). Anal. Calcd for $C_{44}H_{52}N_6O_7$: C, 68.02; H, 6.75; N, 10.82. Found: C, 67.82; H, 7.00; N, 10.44.

5.1.4. Compound 4. Prepared according to the general procedure using: **2** (34 mg, 0.050 mmol), benzylhydrazine dihydrochloride (15 mg, 0.077 mmol), HATU (23 mg, 0.060 mmol), CH_2Cl_2 (2.0 mL), and DIEA (43 μL, 0.25 mmol). Reaction time: 3.5 h. Purification by preparative RP-HPLC-MS followed by column chromatography (gradient elution: EtOAc/*i*-hexane 1:1 to 2:1) gave **4** (17 mg, 43%) as a white solid. ^{1}H NMR (CD_3OD) δ 8.10 (d, $J = 9.2$ Hz, 1H), 8.07–8.04 (m, 2H), 7.58–7.48 (m, 3H), 7.41–7.36 (m, 3H), 7.33–7.21 (m, 4H), 7.08 (dd, $J = 2.4$, 9.2 Hz, 1H), 5.86 (ddd, $J = 9.3$, 10.2, 17.2 Hz, 1H), 5.56–5.54 (m, 1H), 5.22 (dd, $J = 1.6$, 17.2 Hz, 1H), 5.04 (dd, $J = 1.6$, 10.2 Hz, 1H), 4.58–4.48 (m, 2H), 4.25–4.23 (m, 1H), 4.08 (dd, $J = 3.3$, 12.0 Hz, 1H), 3.96 (d, $J = 12.3$ Hz, 1H), 3.95 (s, 3H), 3.88 (d, $J = 12.3$ Hz, 1H), 2.60 (ddm, $J = 6.8$, 13.8 Hz, 1H), 2.33–2.25 (m, 1H), 2.09–2.02 (m, 1H), 1.81 (dd, $J = 5.2$, 7.9 Hz, 1H), 1.33 (dd, $J = 5.2$, 9.6 Hz, 1H), 1.29 (s, 9H), 1.07 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.8, 173.5, 170.8, 163.2, 161.9, 161.4, 158.0, 152.3, 141.4, 138.8, 135.4, 130.6, 130.0, 129.8, 129.4, 129.0, 128.4, 124.3, 119.3, 117.4, 116.5, 107.4, 100.1, 80.6, 78.2, 61.0, 60.5, 56.6, 56.0, 55.3, 41.1, 36.1, 35.5, 34.3, 28.6, 27.0, 23.1. MS $[M+H]^+$ 791.4. HPLC purity (C18 column 98%, C4 column 98%). Anal. Calcd for $C_{45}H_{54}N_6O_7 \cdot 1/2H_2O$: C, 67.56; H, 6.93; N, 10.51. Found: C, 67.76; H, 6.96; N, 10.40.

5.1.5. Compound 5. Prepared according to the general procedure using: **2** (35 mg, 0.051 mmol), benzhydrazide (10 mg, 0.073 mmol), HBTU (23 mg, 0.061 mmol), CH_2Cl_2 (2.0 mL), and DIEA (36 μL, 0.21 mmol). Reaction time: 3 h. Purification by preparative RP-HPLC-MS gave **5** (13 mg, 32%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 10.02 (d, $J = 4.2$ Hz, 1H), 8.34 (d, $J = 4.2$ Hz, 1H), 8.06–8.03 (m, 2H), 7.99 (d, $J = 9.1$ Hz, 1H), 7.77–7.75 (m, 2H), 7.54–7.40 (m, 7H), 7.13 (s, 1H), 7.07 (dd, $J = 2.5$, 9.1 Hz, 1H), 7.00 (s, 1H), 5.85 (ddd, $J = 8.8$, 10.4, 17.2 Hz, 1H), 5.40–5.37 (m, 1H), 5.25 (d, $J = 9.8$ Hz, 1H), 5.21 (dd, $J = 1.7$, 17.2 Hz, 1H), 5.08 (dd, $J = 1.7$, 10.4 Hz, 1H), 4.60 (dm, $J = 11.6$ Hz, 1H), 4.47 (dd, $J = 7.6$, 9.2 Hz, 1H), 4.29 (d, $J = 9.8$ Hz, 1H), 4.08 (dd, $J = 3.8$, 11.6 Hz, 1H), 3.96 (s, 3H), 2.67–2.53 (m, 2H), 2.08–2.01 (m, 1H), 1.97 (dd, $J = 5.4$, 8.1 Hz, 1H), 1.66 (s, 9H), 1.45 (dd, $J = 5.4$, 9.9 Hz, 1H), 1.38 (s, 9H). ^{13}C NMR (CD_3OD) δ 175.0, 173.7, 170.8, 169.0, 163.2, 161.8, 161.5, 158.0, 152.3, 141.5, 135.2, 133.8, 133.2, 130.6, 129.8, 129.6, 129.1, 128.7, 124.2, 119.3, 117.6, 116.5, 107.4, 100.2, 80.5, 78.1, 61.1, 60.3, 56.0, 55.3, 41.1, 35.9, 35.5, 35.1, 28.5, 26.8, 24.2. MS $[M+H]^+$ 805.5. HPLC purity (C18 column 97%, C4 column 98%). Anal. Calcd for $C_{45}H_{52}N_6O_8 \cdot 1/2H_2O$: C, 66.40; H, 6.56; N, 10.33. Found: C, 66.15; H, 6.54; N, 9.99.

5.1.6. Compound 6. Prepared according to the general procedure using: **2** (34 mg, 0.050 mmol), phenylacetic hydrazide (11 mg, 0.073 mmol), HATU (23 mg, 0.060 mmol), CH_2Cl_2 (2.0 mL), and DIEA (35 μL, 0.20 mmol). Reaction time: 3.5 h. Purification by column chromatography (gradient elution: EtOAc/*i*-hexane 1:2 to 2:1) gave **6** (27 mg, 67%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 9.66 (d, $J = 3.1$ Hz, 1H), 8.04–8.01 (m, 2H), 7.97 (d, $J = 9.1$ Hz, 1H), 7.63 (d, $J = 3.1$ Hz, 1H), 7.53–7.43 (m, 3H), 7.42 (d, $J = 2.5$ Hz, 1H), 7.35–7.21 (m, 6H), 7.05 (dd, $J = 2.5$, 9.1 Hz, 1H), 6.97 (s, 1H), 5.84 (ddd, $J = 9.1$, 10.3, 17.3 Hz, 1H), 5.34–5.32 (m, 1H), 5.21 (d, $J = 9.8$ Hz, 1H), 5.17 (dd, $J = 1.6$, 17.3 Hz, 1H), 5.05 (dd, $J = 1.6$, 10.3 Hz, 1H), 4.53 (dm, $J = 11.6$ Hz, 1H), 4.40 (dd, $J = 7.2$, 9.5 Hz, 1H), 4.24 (d, *J* = 9.8 Hz, 1H), 4.03 (dd, *J* = 3.6, 11.6 Hz, 1H), 3.95 (s, 3H), 3.57 (s, 2H), 2.62–2.56 (m, 1H), 2.51–2.44 (m, 1H), 2.01–1.95 (m, 1H), 1.86 (dd, *J* = 5.6, 8.1 Hz, 1H), 1.42–1.40 (m, 1H), 1.39 (s, 9H), 0.91 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.9, 173.5, 172.5, 170.5, 163.1, 161.8, 161.4, 158.0, 152.3, 141.4, 136.0, 135.1, 130.6, 130.4, 129.8, 129.5, 129.0, 128.0, 124.2, 119.3, 117.6, 116.4, 107.4, 100.1, 80.5, 78.1, 61.0, 60.2, 56.0, 55.2, 41.6, 41.0, 35.9, 35.5, 35.0, 28.5, 26.8, 24.0. MS $[M+H]^+$ 819.5. HPLC purity (C18 column 98%, C4 column 95%). Anal. Calcd for $C_{46}H_{54}N_6O_8$: C, 67.46; H, 6.65; N, 10.26. Found: C, 67.11; H, 6.70; N, 9.95.

5.1.7. Compound 7. A mixture of **2** (25 mg, 0.036 mmol), CDI (18 mg, 0.11 mmol), and dry THF (1.5 mL) was stirred at 70 °C under N_2 atmosphere in a dried, sealed reaction tube for 2 h. After cooling to room temperature, (*S*)-(+)-*p*-toluenesulfinamide (17 mg, 0.11 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (16 μL, 0.11 mmol) were added to the mixture whereafter it was stirred at room temperature for 3 h. Evaporation followed by purification by column chromatography (gradient elution: EtOAc/*i*-hexane 2:3 to 1:1) gave **7** (14 mg, 47%) as a white solid (2:1 mixture of diastereomers). ^{1}H NMR (CD_3OD) δ (major diastereomer reported) 8.08–8.03 (m, 3H), 7.66–7.63 (m, 1H), 7.60–7.48 (m, 4H), 7.42–7.37 (m, 3H), 7.24 (s, 1H), 7.07 (dm, *J* = 9.1 Hz, 1H), 5.88–5.79 (m, 1H), 5.56–5.55 (m, 1H), 5.30 (dm, *J* = 17.1 Hz, 1H), 5.11 (dm, *J* = 10.2 Hz, 1H), 4.51–4.40 (m, 2H), 4.19–4.17 (m, 1H), 4.09–4.05 (m, 1H), 3.95 (s, 3H), 2.60 (ddm, *J* = 6.9, 14.2 Hz, 1H), 2.43 (s, 3H), 2.32–2.15 (m, 2H), 1.86–1.83 (m, 1H), 1.51–1.47 (m, 1H), 1.32 (s, 9H), 1.03 (s, 9H). ^{13}C NMR (CD_3OD) δ (major diastereomer reported) 174.8, 173.2, 173.0, 163.2, 161.9, 161.4, 157.8, 152.3, 143.5, 141.3, 134.8, 134.4, 130.8, 130.6, 129.8, 129.0, 126.6, 124.3, 119.4, 118.3, 116.5, 107.4, 100.1, 80.8, 78.1, 60.9, 60.7, 56.0, 55.4, 42.2, 36.4, 35.9, 35.5, 28.6, 27.2, 24.3, 21.6. MS $[M+H]^+$ 824.2. HPLC purity (C18 column 96%, C4 column 99%). Anal. Calcd for $C_{45}H_{53}N_5O_8S{\cdot}1/2H_2O$: C, 64.88; H, 6.53; N, 8.41. Found: C, 64.50; H, 6.59; N, 8.06.

5.1.8. Compound 8. A mixture of **2** (25 mg, 0.036 mmol), dry THF (0.40 mL), and *N*-methylmorpholine (4.0 μL, 0.036 mmol) was cooled to −15 °C under N_2 atmosphere prior to the addition of isobutylchloroformate (5.0 μL, 0.039 mmol). The mixture was stirred for 2 min before the addition of hydroxylamine hydrochloride (3.0 mg, 0.043 mmol) in dry DMF (0.20 mL) and Et_3N (6.0 μL, 0.043 mmol) whereafter it was stirred for an additional 40 min at −15 °C. The mixture was diluted with H_2O and extracted with EtOAc. The organic layer was washed with brine, dried ($MgSO_4$), filtered, and evaporated. Purification by column chromatography (CH_2Cl_2/MeOH 96:4) followed by column chromatography using RP-18 silica gel (gradient elution: MeCN/H_2O 30:70 to 100:0) gave **8** (17 mg, 67%) as a white solid. ^{1}H NMR (CD_3OD) δ 8.09 (d, *J* = 9.2 Hz, 1H), 8.05–8.03 (m, 2H), 7.56–7.47 (m, 3H), 7.39 (d, *J* = 2.4 Hz, 1H), 7.23 (s, 1H), 7.07 (dd, *J* = 2.4, 9.2 Hz, 1H), 5.90 (ddd, *J* = 9.4, 10.3, 17.1 Hz, 1H), 5.55–5.54 (m, 1H), 5.23 (dd, *J* = 1.3, 17.1 Hz, 1H), 5.03 (dd, *J* = 1.3, 10.3 Hz, 1H), 4.59 (dm, *J* = 11.8 Hz, 1H), 4.52 (dd, *J* = 6.7, 10.7 Hz, 1H), 4.23 (s, 1H), 4.08 (dd, *J* = 3.3, 11.8 Hz, 1H), 3.95 (s, 3H), 2.63 (ddm, *J* = 6.7, 13.8 Hz, 1H), 2.35 (ddd, *J* = 3.9, 10.7, 13.8 Hz, 1H), 2.09–2.02 (m, 1H), 1.83 (dd, *J* = 5.3, 8.0 Hz, 1H), 1.32 (dd, *J* = 5.3, 9.6 Hz, 1H), 1.27 (s, 9H), 1.05 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 173.3, 172.1, 167.0, 161.1, 159.9, 159.4, 156.1, 151.6, 140.3, 133.7, 129.5, 129.0, 127.7, 123.0, 118.6, 117.5, 115.2, 107.5, 98.1, 80.1, 76.2, 60.0, 59.2, 55.7, 54.5, 39.4, 35.3, 34.0, 33.7, 28.4, 26.5, 22.5. MS $[M+H]^+$ 702.5. HPLC purity (C18 column > 99%, C4 column 97%). Anal. Calcd for $C_{38}H_{47}N_5O_8{\cdot}1/2H_2O$: C, 64.21; H, 6.81; N, 9.85. Found: C, 64.17; H, 6.88; N, 9.70.

5.1.9. Compound 9. A mixture of **2** (25 mg, 0.036 mmol), HATU (17 mg, 0.045 mmol), cyanamide (6.0 mg, 0.14 mmol), dry DMF (1.0 mL), and DIEA (32 μL, 0.18 mmol) was stirred at 40 °C under N_2 atmosphere in a sealed reaction tube for 3 days. The solution was diluted with EtOAc and washed with NaOAc buffer (pH 4), 5% aqueous $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$), filtered, and evaporated. Purification by column chromatography (gradient elution: CH_2Cl_2/MeOH 97:3 to 95:5) followed by column chromatography using RP-18 silica gel (gradient elution: MeCN/H_2O 30:70 to 100:0) gave **9** (10 mg, 39%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 8.03–8.00 (m, 3H), 7.53–7.46 (m, 3H), 7.43–7.42 (m, 1H), 7.04 (dd, *J* = 2.6, 9.1 Hz, 1H), 6.98–6.97 (m, 2H), 5.89–5.78 (m, 1H), 5.38–5.36 (m, 1H), 5.23 (dm, *J* = 17.2 Hz, 1H), 5.20–5.17 (m, 1H), 5.13 (dm, *J* = 10.3 Hz, 1H), 4.63 (dm, *J* = 11.6 Hz, 1H), 4.34–4.30 (m, 1H), 4.25 (d, *J* = 9.3 Hz, 1H), 4.08 (dm, *J* = 11.6 Hz, 1H), 3.94 (s, 3H), 2.64 (ddm, *J* = 6.8, 13.8 Hz, 1H), 2.47–2.40 (m, 1H), 2.15–2.09 (m, 1H), 2.03–1.99 (m, 1H), 1.54–1.50 (m, 1H), 1.42 (s, 9H), 1.04 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.8, 174.0, 164.0, 163.4, 160.6, 158.1, 150.0, 139.3, 134.6, 131.5, 130.14, 130.06, 129.35, 129.28, 124.8, 120.0, 118.3, 116.4, 105.6, 100.7, 80.5, 78.9, 61.0, 60.7, 56.3, 55.3, 42.4, 35.9, 35.8, 35.5, 28.5, 27.0, 24.7. MS $[M+H]^+$ 711.2. HPLC purity (C18 column 98%, C4 column 96%). Anal. Calcd for $C_{39}H_{46}N_6O_7{\cdot}21/2H_2O$: C, 61.97; H, 6.80; N, 11.12. Found: C, 62.10; H, 6.69; N, 11.01.

5.1.10. Compound 10. A mixture of **2** (25 mg, 0.036 mmol), HATU (17 mg, 0.045 mmol), CH_2Cl_2 (1.0 mL), 2,2,2-trifluoroethylamine (5.0 μL, 0.063 mmol), and DIEA (25 μL, 0.14 mmol) was stirred at room temperature overnight. Evaporation followed by purification by column chromatography (EtOAc/*i*-hexane 2:3) gave **10** (23 mg, 82%) as a white solid. ^{1}H NMR (CD_3OD) δ 8.09 (d, *J* = 9.1 Hz, 1H), 8.06–8.03 (m, 2H), 7.56–7.47 (m, 3H), 7.39 (d, *J* = 2.5 Hz, 1H), 7.22 (s, 1H), 7.08 (dd, *J* = 2.5, 9.1 Hz, 1H), 6.57 (d, *J* = 9.2 Hz, 0.6H), 5.81 (ddd, *J* = 9.2, 10.2, 17.2 Hz, 1H), 5.55–5.53 (m, 1H), 5.23 (dd, *J* = 1.8, 17.2 Hz, 1H), 5.04 (dd, *J* = 1.8, 10.2 Hz, 1H), 4.58–4.52 (m, 2H), 4.30–4.19 (m, 2H), 4.10–4.06 (m, 1H), 3.95 (s, 3H), 3.53 (dq, *J* = 9.0, 14.9 Hz, 1H), 2.64 (ddm, *J* = 7.1, 13.8 Hz, 1H), 2.32 (ddd, *J* = 4.0, 10.9, 13.8 Hz, 1H), 2.14–2.08 (m, 1H), 1.84 (dd, *J* = 5.3, 8.1 Hz, 1H), 1.36 (dd, *J* = 5.3, 9.6 Hz, 1H), 1.27 (s, 9H), 1.04 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.9, 173.5, 172.3, 163.2, 161.8, 161.4, 158.0, 152.3, 141.4, 135.1, 130.6, 129.8, 129.0, 125.6 (q, $^1J_{CF}$ = 278 Hz), 124.3, 119.3, 117.5, 116.5, 107.4, 100.1, 80.5, 78.1, 60.9, 60.5, 56.0, 55.3, 42.0, 41.7 (q, $^2J_{CF}$ = 35 Hz), 35.9, 35.5, 34.6, 28.6, 26.9, 23.3. MS $[M+H]^+$ 768.4. HPLC purity (C18 column > 99%, C4 column 99%). Anal. Calcd for $C_{40}H_{48}F_3N_5O_7$: C, 62.57; H, 6.30; N, 9.12. Found: C, 62.22; H, 6.44; N, 8.95.

5.1.11. Compound 11. A mixture of **2** (20 mg, 0.029 mmol), HATU (13 mg, 0.034 mmol), 2-aminothiazole (4.4 mg, 0.044 mmol), dry CH_2Cl_2 (1.0 mL), and DIEA (19 μL, 0.11 mmol) was stirred at 45 °C under N_2 atmosphere in a sealed reaction tube for 4 days. Purification by column chromatography (gradient elution: EtOAc/*i*-hexane 2:3 to 1:1) gave **11** (18 mg, 80%) as a white solid (3:1 mixture of isomers). ^{1}H NMR (CD_3OD) δ (major isomer reported) 8.08–8.03 (m, 3H), 7.57–7.48 (m, 3H), 7.44 (d, *J* = 3.7 Hz, 1H), 7.41–7.40 (m, 1H), 7.27 (s, 1H), 7.12 (d, *J* = 3.7 Hz, 1H), 7.10–7.07 (m, 1H), 5.86 (ddd, *J* = 9.2, 10.2, 17.2 Hz, 1H), 5.62–5.59 (m, 1H), 5.28 (dd, *J* = 1.5, 17.2 Hz, 1H), 5.08 (dd, *J* = 1.5, 10.2 Hz, 1H), 4.58 (dd, *J* = 6.4, 10.8 Hz, 1H), 4.43 (dm, *J* = 11.9 Hz, 1H), 4.33–4.29 (m, 1H), 4.16–4.10 (m, 1H), 3.96 (s, 3H), 2.66 (ddm, *J* = 6.6, 14.5 Hz, 1H), 2.41–2.34 (m, 1H), 2.28–2.21 (m, 1H), 1.99 (dd, *J* = 5.4, 8.2 Hz, 1H), 1.52 (dd, *J* = 5.4, 9.6 Hz, 1H), 1.33 (s, 9H), 0.87 (s, 9H). ^{13}C NMR (CD_3OD) δ (major isomer reported) 175.0, 173.0, 169.8, 163.2, 161.8, 161.4, 160.1, 158.3, 152.3, 141.3, 137.8, 134.7, 130.6, 129.8, 129.0, 124.0, 119.4, 118.3, 116.4, 114.8, 107.5, 100.2, 80.4, 78.2, 61.2, 60.3, 56.0, 55.4, 41.9, 36.6, 36.0, 35.4, 28.6, 26.8, 24.4. MS $[M+H]^+$ 769.2. HPLC purity (C18 column >99%, C4 column >99%). Anal. Calcd for $C_{41}H_{48}N_6O_7S\cdot 1/2H_2O$: C, 63.30; H, 6.35; N, 10.80. Found: C, 63.29; H, 6.59; N, 10.44.

5.1.12. Compound 13. A mixture of (1*R*,2*S*)-1-*tert*-butoxycarbonylamino-2-vinylcyclopropanecarboxylic acid (**12**) (130 mg, 0.572 mmol), benzhydrazide (117 mg, 0.859 mmol), HATU (261 mg, 0.686 mmol), CH_2Cl_2 (6 mL), and DIEA (399 μL, 2.29 mmol) was stirred at room temperature for 3.5 h. The mixture was diluted with EtOAc and washed with NaOAc buffer (pH 4), 5% aqueous $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$), filtered, and evaporated. Purification by column chromatography (gradient elution: EtOAc/*i*-hexane 2:3 to 1:1) gave **13** (164 mg, 83%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 9.51–9.50 (m, 1H), 9.43 (m, 1H), 7.82–7.80 (m, 2H), 7.48–7.44 (m, 1H), 7.38–7.33 (m, 2H), 5.90 (br s, 1H), 5.72 (m, 1H), 5.23 (ddm, *J* = 1.3, 17.2 Hz, 1H), 5.03 (dm, *J* = 10.5 Hz, 1H), 2.17–2.11 (m, 1H), 1.86 (dd, *J* = 5.6, 7.6 Hz, 1H), 1.45 (s, 9H), 1.34 (dd, *J* = 5.6, 9.5 Hz, 1H). ^{13}C NMR (CD_3OD) δ 171.9, 168.8, 158.0, 135.5, 133.8, 133.2, 129.6, 128.7, 117.3, 81.2, 41.8, 35.4, 28.7, 23.1. MS $[M+H]^+$ 346.1. HPLC purity (C18 column 97%). Anal. Calcd for $C_{18}H_{23}N_3O_4$: C, 62.59; H, 6.71; N, 12.17. Found: C, 62.49; H, 7.03; N, 11.76.

5.1.13. Compound 14. A mixture of **13** (70 mg, 0.20 mmol), (methoxycarbonylsulfamoyl)-triethylammonium hydroxide inner salt (Burgess reagent) (145 mg, 0.608 mmol), and dry THF (4 mL) was stirred at 75 °C under N_2 atmosphere in a dried, sealed reaction tube for 2 h. Evaporation and purification by column chromatography (EtOAc/*i*-hexane 2:3) gave **14** (57 mg, 86%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 8.00–7.97 (m, 2H), 7.52–7.44 (m, 3H), 5.68 (br s, 1H), 5.60 (ddd, *J* = 8.4, 10.3, 17.2 Hz, 1H), 5.29 (dm, *J* = 17.2 Hz, 1H), 5.08 (ddd, *J* = 0.7, 1.6, 10.3 Hz, 1H), 2.41–2.34 (m, 1H), 2.03 (dd, *J* = 6.0, 7.9 Hz, 1H), 1.77–1.69 (m, 1H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 166.1, 164.9, 155.5, 133.0, 131.7, 129.1, 126.8, 124.0, 118.6, 80.8, 35.3, 33.9, 28.4, 22.5. MS $[M+H]^+$ 328.0. HPLC purity (C18 column 97%). Anal. Calcd for $C_{18}H_{21}N_3O_3\cdot H_2O$: C, 62.59; H, 6.71; N, 12.17. Found: C, 62.98; H, 6.33; N, 11.78.

5.1.14. Compound 15. Compound **14** (28 mg, 0.086 mmol) was stirred in a 4 M solution of HCl in 1,4-dioxane (1.0 mL) at room temperature overnight. Coevaporation with MeOH gave the corresponding HCl salt as a pale yellow solid that was used in the next step without further purification. A mixture of **1** (26 mg, 0.045 mmol), the HCl salt described above (18 mg, 0.068 mmol), HBTU (21 mg, 0.055 mmol), DMF (1.5 mL), and DIEA (32 μL, 0.18 mmol) was stirred at room temperature for 3 h. The mixture was diluted with CH_2Cl_2 and washed with aqueous NaOAc buffer (pH 4), 5% aqueous $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$), filtered, and evaporated. Purification by column chromatography (EtOAc/*i*-hexane 1:1) gave **15** (25 mg, 71%) as a white solid. ^{1}H NMR ($CDCl_3$) δ (6:1 mixture of rotamers, major rotamer reported) 8.06–8.02 (m, 3H), 7.98–7.91 (m, 2H), 7.90 (br s, 1H), 7.53–7.42 (m, 7H), 7.05 (dd, *J* = 2.5, 9.1 Hz, 1H), 7.03 (s, 1H), 5.61 (ddd, *J* = 8.1, 10.3, 17.1 Hz, 1H), 5.37–5.35 (m, 1H), 5.30 (dm, *J* = 17.1 Hz, 1H), 5.24 (d, *J* = 9.6 Hz, 1H), 5.11 (dm, *J* = 10.3 Hz, 1H), 4.83 (dd, *J* = 7.5, 7.8 Hz, 1H), 4.49 (dm, *J* = 11.8 Hz, 1H), 4.35 (d, *J* = 9.6 Hz, 1H), 3.97 (dd, *J* = 4.7, 11.8 Hz, 1H), 3.95 (s, 3H), 2.96 (ddd, *J* = 5.2, 6.9, 12.2 Hz, 1H), 2.58–2.47 (m, 1H), 2.38–2.32 (m, 1H), 2.05 (dd, *J* = 6.2, 7.8 Hz, 1H), 1.68 (dd, *J* = 6.2, 9.5 Hz, 1H), 1.42 (s, 9H), 1.02 (s, 9H). ^{13}C NMR ($CDCl_3$) δ (6:1 mixture of rotamers, major rotamer reported) 172.9, 171.1, 165.04, 165.03, 161.5, 160.2, 159.3, 155.9, 151.5, 140.3, 132.7, 131.7, 129.4, 129.1, 129.0, 127.7, 127.0, 124.0, 123.2, 119.0, 118.6, 115.2, 107.5, 98.2, 80.1, 76.1, 59.2, 58.6, 55.7, 54.1, 35.5, 34.5, 33.5, 32.5, 28.4, 26.6, 22.1. MS $[M+H]^+$ 787.3. HPLC purity (C18 column 98%, C4 column 99%). Anal. Calcd for $C_{45}H_{50}N_6O_7$: C, 68.68; H, 6.40; N, 10.68. Found: C, 68.43; H, 6.34; N, 10.28.

5.1.15. Compound 16. A mixture of (1*R*,2*S*)-1-*tert*-butoxycarbonylamino-2-vinylcyclopropanecarboxylic acid (**12**) (50 mg, 0.22 mmol), benzenesulfonyl hydrazide (57 mg, 0.33 mmol), CH_2Cl_2 (2 mL), and *N*,*N*′-diisopropylcarbodiimide (34 μL, 0.22 mmol) was stirred at room temperature for 2 h. Evaporation followed by purification by column chromatography (EtOAc/*i*-hexane 1:1) gave **16** (50 mg, 60%) as a white solid. ^1H NMR ($CDCl_3$) δ 8.94 (br s, 1H), 7.92–7.89 (m, 2H), 7.61–7.56 (m, 1H), 7.50–7.46 (m, 3H), 5.41 (br s, 1H), 5.25–5.12 (m, 2H), 4.93 (ddm, $J = 2.7$, 9.0 Hz, 1H), 2.05–1.98 (m, 1H), 1.61 (dd, $J = 5.6$, 8.0 Hz, 1H), 1.45 (s, 9H), 1.25 (dd, $J = 5.6$, 9.6 Hz, 1H). ^{13}C NMR (CD_3OD) δ 171.0, 157.8, 139.7, 135.1, 134.2, 129.9, 129.6, 117.5, 81.1, 41.6, 34.8, 28.7, 22.5. MS $[M+H]^+$ 382.0. HPLC purity (C18 column 96%). Anal. Calcd for $C_{17}H_{23}N_3O_5S$: C, 53.53; H, 6.08; N, 11.02. Found: C, 53.36; H, 5.91; N, 10.85.

5.1.16. Compound 17. Compound **16** (36 mg, 0.094 mmol) was stirred in a 4 M solution of HCl in 1,4-dioxane (1.0 mL) at room temperature overnight. Coevaporation with MeOH gave the corresponding HCl salt as a pale yellow solid that was used in the next step without further purification. A mixture of **1** (28 mg, 0.048 mmol), the HCl salt described above (23 mg, 0.072 mmol), HBTU (22 mg, 0.058 mmol), DMF (1.5 mL), and DIEA (34 μL, 0.20 mmol) was stirred at room temperature for 3.5 h. The mixture was diluted with CH_2Cl_2 and washed with aqueous NaOAc buffer (pH 4), 5% aqueous $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$), filtered, and evaporated. Purification by column chromatography (EtOAc/*i*-hexane 1:1) gave **17** (24 mg, 59%) as a white solid. ^1H NMR ($CDCl_3$) δ 9.87 (d, $J = 5.2$ Hz, 1H), 8.04–8.01 (m, 2H), 7.95 (d, $J = 9.1$ Hz, 1H), 7.89–7.86 (m, 2H), 7.53–7.38 (m, 7H), 7.29 (d, $J = 5.2$ Hz, 1H), 7.05 (dd, $J = 2.5$, 9.1 Hz, 1H), 6.97 (s, 1H), 6.82 (br s, 1H), 5.36–5.34 (m, 1H), 5.30 (d, $J = 9.8$ Hz, 1H), 5.03–4.99 (m, 2H), 4.79–4.76 (m, 1H), 4.64 (dm, $J = 11.7$ Hz, 1H), 4.38 (dd, $J = 6.8$, 10.1 Hz, 1H), 4.30 (d, $J = 9.8$ Hz, 1H), 4.06 (dd, $J = 3.9$, 11.7 Hz, 1H), 3.96 (s, 3H), 2.59 (ddm, $J = 6.8$, 13.9 Hz, 1H), 2.45 (dddm, $J = 4.1$, 10.1, 13.9 Hz, 1H), 1.86–1.80 (m, 1H), 1.74 (dd, $J = 5.6$, 8.1 Hz, 1H), 1.40 (s, 9H), 1.27 (dd, $J = 5.6$, 9.5 Hz, 1H), 1.06 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 173.2, 172.1, 168.6, 161.5, 159.8, 159.3, 156.0, 151.5, 140.2, 137.1, 133.3, 132.7, 129.5, 128.94, 128.89, 128.8, 127.6, 122.9, 118.6, 117.7, 115.1, 107.5, 98.0, 80.0, 76.1, 59.9, 58.9, 55.7, 54.4, 40.1, 35.2, 34.3, 33.9, 28.3, 26.5, 23.3. MS $[M+H]^+$ 841.2. HPLC purity (C18 column 96%, C4 column 96%). Anal. Calcd for $C_{44}H_{52}N_6O_9S{\cdot}1/2H_2O$: C, 62.17; H, 6.28; N, 9.89. Found: C, 62.28; H, 6.50; N, 9.79.

5.1.17. Compound 18. A mixture of (1*R*,2*S*)-1-*tert*-butoxycarbonylamino-2-vinylcyclopropanecarboxylic acid (**12**) (210 mg, 0.92 mmol), CDI (300 mg, 1.85 mmol), and dry THF (11 mL) was stirred at 70 °C under N_2 atmosphere in a dried, sealed reaction tube for 2 h. The mixture was cooled to 0 °C and NH_3 (g) was bubbled through the solution for 45 min. The remaining mixture was stirred at room temperature overnight. The solvent was evaporated and the mixture was diluted with EtOAc and washed with H_2O, 5% aqueous citric acid, 5% aqueous $NaHCO_3$, and brine. The combined aqueous phases were extracted with EtOAc and the combined organic phases were washed with brine, dried ($MgSO_4$), filtered, and evaporated. The crude product was dissolved in EtOAc and MeOH, and filtered through a short plug of silica to give the corresponding primary amide as white crystals after evaporation that were used in the next step without further purification. A mixture of the primary amide described above (174 mg, 0.769 mmol), *p*-toluenesulfonyl chloride (293 mg, 1.54 mmol), CH_2Cl_2 (7.0 mL), and pyridine (311 μL, 3.85 mmol) was stirred at room temperature for 5 days. Saturated aqueous $NaHCO_3$ (5.5 mL) was added and the resulting mixture was rigorously stirred for 1.5 h. The organic layer was evaporated and thereafter purified using column chromatography (gradient elution: EtOAc/*i*-hexane 1:4 to 2:3) to give **18** (114 mg, 71%) as a white solid. ^1H NMR (CD_3OD) δ 5.73–5.64 (m, 1H), 5.36 (ddd, $J = 0.9$, 1.4, 17.0 Hz, 1H), 5.27 (ddd, $J = 0.7$, 1.4, 10.3 Hz, 1H), 2.13–2.07 (m, 1H), 1.55–1.49 (m, 2H), 1.47 (s, 9H). ^{13}C NMR (CD_3OD) δ 157.3, 134.8, 120.0, 119.2, 81.6, 32.4, 29.4, 28.6, 22.8. MS $[M+H]^+$ 209.0. HPLC purity (C18 column 99%). Anal. Calcd for $C_{11}H_{16}N_2O_2$: C, 63.44; H, 7.74; N, 13.45. Found: C, 63.77; H, 7.90; N, 13.10.

5.1.18. Compound 19. A solution of **18** (71 mg, 0.34 mmol) in toluene (4.5 mL) was added to a reaction tube containing triethylamine hydrochloride (141 mg, 1.02 mmol) and NaN_3 (66 mg, 1.02 mmol). The tube was sealed and the mixture was stirred at room temperature for 40 min and thereafter at 50 °C for 3 days. The mixture was repeatedly extracted with H_2O. The aqueous phase was acidified to pH 2 with 1 M aqueous HCl and extracted with EtOAc. The organic layer was washed with brine, dried ($MgSO_4$), filtered, and evaporated. Purification by column chromatography (gradient elution: CH_2Cl_2:MeOH:HCOOH 98:2:0.2 to 97:3:0.2) gave **19** (54 mg, 63%) as white crystals. ^1H NMR (CD_3OD) δ 5.52–5.43 (m, 1H), 5.23 (dm, $J = 17.0$ Hz, 1H), 5.01 (dm, $J = 10.2$ Hz, 1H), 2.38–2.32 (m, 1H), 1.91 (dd, $J = 6.0$, 7.7 Hz, 1H), 1.64 (dd, $J = 6.0$, 9.5 Hz, 1H), 1.43 (s, 9H). ^{13}C NMR (CD_3OD) δ 158.9, 157.9, 134.5, 118.5, 81.2, 34.8, 34.0, 28.6, 23.3. MS $[M+H]^+$ 252.0. HPLC purity (C18 column 99%). Anal. Calcd for $C_{11}H_{17}N_5O_2$: C, 52.58; H, 6.82; N, 27.87. Found: C, 52.25; H, 7.01; N, 26.93.

5.1.19. Compound 20. Compound **19** (43 mg, 0.17 mmol) was stirred in a 4 M solution of HCl in 1,4-dioxane (3.0 mL) at room temperature for 3.5 h. Coevaporation with MeOH gave the corresponding HCl salt as a pale yellow solid that was used in the next step without further purification. A mixture of **1** (30 mg, 0.052 mmol), HATU (24 mg, 0.063 mmol), the HCl salt described above (15 mg, 0.080 mmol), DMF (1.5 mL), and DIEA (36 μL, 0.21 mmol) was stirred at room temperature for 3 h. The mixture was diluted with EtOAc and washed with NaOAc buffer (pH 4), 5% aqueous $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$), filtered, and evaporated. Purification by preparative RP-HPLC-MS gave **20** (15 mg, 41%) as a white solid. ^1H NMR (CD_3OD) δ 8.13 (d, $J = 9.2$ Hz, 1H), 8.06–8.04 (m, 2H), 7.58–7.50 (m, 3H), 7.40 (d, $J = 2.5$ Hz, 1H), 7.27 (s, 1H), 7.10 (dd, $J = 2.5$, 9.2 Hz, 1H), 5.72 (ddd, $J = 9.0$, 10.1, 17.1 Hz, 1H), 5.59–5.57 (m, 1H), 5.24 (dm, $J = 17.1$ Hz, 1H), 5.05 (dm, $J = 10.1$ Hz, 1H), 4.65–4.59 (m, 2H), 4.24 (s, 1H), 4.09 (dd, $J = 3.5$, 12.0 Hz, 1H), 3.95 (s, 3H), 2.72 (ddm, $J = 7.2$, 14.2 Hz, 1H), 2.43 (ddd, $J = 4.0$, 10.2, 14.2 Hz, 1H), 2.40–2.33 (m, 1H), 1.92 (dd, $J = 5.9, 7.8$ Hz, 1H), 1.72 (dd, $J = 5.9, 9.6$ Hz, 1H), 1.28 (s, 9H), 1.03 (s, 9H). ^{13}C NMR (CD$_3$OD) δ 174.9, 174.1, 163.4, 162.3, 161.2, 158.6, 158.1, 151.6, 140.7, 134.8, 130.8, 129.9, 129.1, 124.5, 119.5, 118.4, 116.5, 106.8, 100.3, 80.5, 78.4, 60.9, 60.8, 56.1, 55.4, 35.9, 35.6, 34.9, 34.0, 28.6, 26.9, 24.3. MS [M+H]$^+$ 711.3. HPLC purity (C18 column >99%, C4 column >99%). Anal. Calcd for $C_{38}H_{46}N_8O_6 \cdot H_2O$: C, 62.62; H, 6.64; N, 15.37. Found: C, 62.69; H, 6.69; N, 15.01.

5.2. Enzyme inhibition

The protease activity of the full-length HCV NS3 protein (protease-helicase/NTPase) was measured using a FRET-assay as previously described.[30,38] In short, for the determination of K_i-values at pH 7.5 presented in Table 1, 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, 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). For the determination of K_i-values at pH 6.0, 7.0, and 8.0 presented in Table 2, a mixture of 25 mM MES, 25 mM MOPS, and 25 mM BICINE replaced the HEPES buffer.[37] The non-linear regression analysis was made using Grafit 5.0.8 (Erithacus software limited).

5.3. Computational methodology

Compound **23** was docked in the protease binding site, derived from the full-length NS3 protein (scNS3-NS4A, PDB code 1CU1)[5] using FLO as previously described.[21]

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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.2007.03.089.

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