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# Molecular modeling based approach, synthesis and in vitro assay to new indole inhibitors of hepatitis C NS3/4A serine protease

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ABSTRACT

In an attempt to identify potential HCV NS3 protease inhibitors lead compounds, a series of novel indoles (**10a–g**) was designed. Molecular modeling study, including fitting to a 3D-pharmacophore model of the designed molecules (**10a–g**), with HCV NS3 protease hypothesis using CATALYST program was fulfilled. Also, the molecular docking into the NS3 active site was examined using Discovery Studio 2.5 software. Several compounds showed significant high simulation docking score and fit values. The designed compounds with high docking score and fit values were synthesized and biologically evaluated in vitro using an NS3 protease binding assay. It appears that most of the tested compounds reveal promising inhibitory activity against NS3 protease. Of these, compounds **10a** and **10b** demonstrated potent HCV NS3 protease inhibitors with $IC_{50}$ values of 9 and 12 μg/mL, respectively. The experimental serine protease inhibitor activities of compounds **10a–g** were consistent with their molecular modeling results. Inhibitors from this class have promising characteristics for further development as anti-HCV agents.



## 1. Introduction

In 1989, a main causative virus of non-A, non-B posttransfusion hepatitis was first identified and named hepatitis C virus (HCV).[1] According to a press release from the World Health Organization, 170–200 million people are chronically infected with the HCV.[2] The positive single-stranded RNA virus of the *Flaviviridae* family was identified as the causative agent. This virus replicates primarily in the liver, and the disease progression is typically a slow process occurring over many years. Ultimately a significant fraction develops serious liver disease including cirrhosis and hepatocellular carcinoma.[3] HCV is currently a leading cause of death in HIV co-infected patients and the most common basis for liver transplantation surgery.[4] At the present, neither a vaccine against HCV nor an effective therapy with an acceptable broad spectrum of action against all genotypes of HCV is available.[5,6] A currently approved HCV therapy, comprised of pegylated α-interferon (PEG-INF-α), either alone or in combination with ribavirin (a broad spectrum antiviral agent). Unfortunately this treatment found limited patient compliance due to the severe side effects of these two drugs.[7–9] Therefore, the development of novel HCV antiviral agents with a high therapeutic index, reduced side effects, and easier route of administration will be paramount to meeting an urgently needed therapeutic Weapon against HCV.

Several promising antiviral targets for HCV have emerged in recent years with NS3/4A protease inhibitors showing perhaps the most dramatic antiviral effects.[10–14] The bifunctional nonstructural protein (NS3) from HCV is an interesting enzyme biochemically, and is of importance for anti-HCV drug discovery. It has an *N*-terminal domain that constitutes a serine protease with a typical chemotrypsinfold, and a *C*-terminal superfamily 2 DExH/D-box RNA helicase domain. Furthermore, the protease domain contains a structural zinc atom and needs to interact with the viral nonstructural protein 4A (NS4A) in order to be fully functional.[15] The protease activity of NS3 is responsible for cleaving the viral polyprotein of HCV, whereas the helicase is responsible for unwinding double-stranded RNA. Thus, the two enzyme activities of NS3 protein are involved in critical steps of viral replication, making them both attractive anti-HCV drug targets.[16,17]

The published crystal structure of the full-length NS3 revealed that the active site was situated in the interface between the helicase and the protease domains, creating a well-defined binding cleft.[18] A number of peptidic and nonpeptidic inhibitors of the NS3/4A protease have been reported. Harper and co-workers[19] proposed a structure–activity relationship (SAR) and disclosed enzyme bound crystal structure for indoline based peptidomimetic inhibitor **1a** in addition, they reported a new series of indole derivatives as HCV NS3 protease inhibitors **2** (Fig. 1).[20]

However, improvement of the activity was the next major challenge in the development of new anti-HCV drugs.

Our current investigation was based on; primarily, using a structure-guided strategy based on HCV NS3 protease is an

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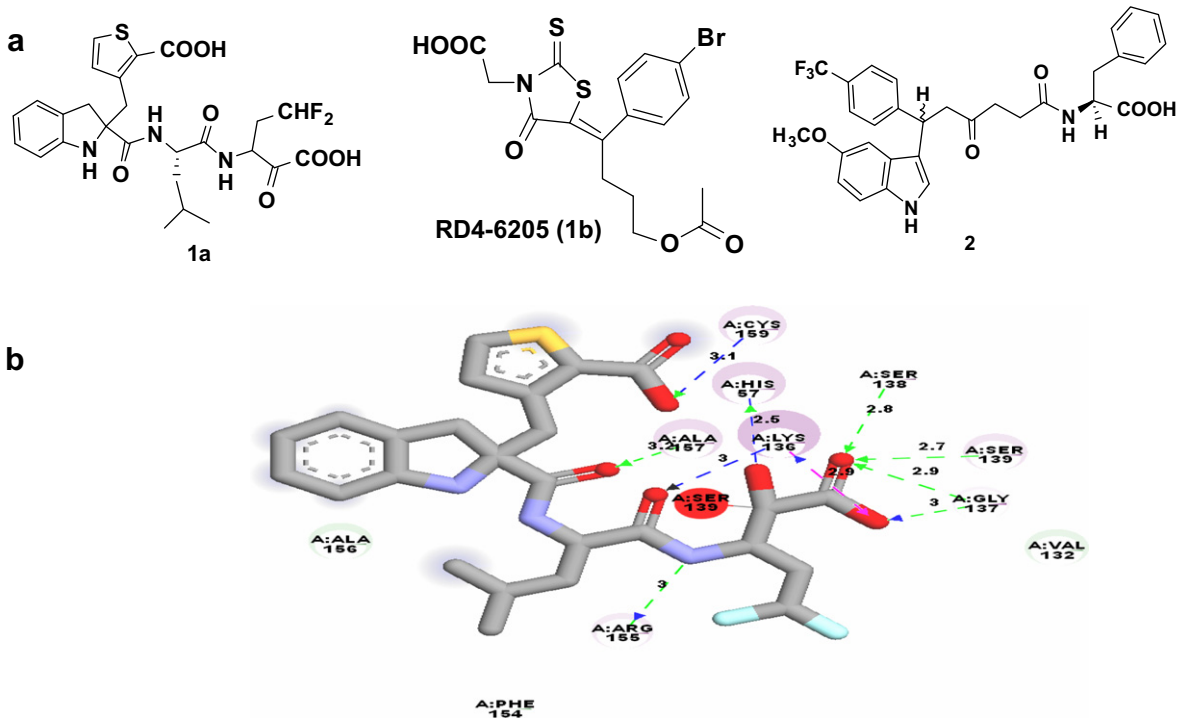



**Figure 1.** (a) Compounds **1a**,**b** and **2** are NS3/4A protease inhibitors. (b) 2D Ligand interaction diagram of indoline derivative (**1a**) co-crystallized with HCV NS3/4A protease enzyme (1W3C) using Discovery Studio program with the essential amino acid residues at the binding site are tagged in circles.

appropriate mean to generate HCV NS3 protease inhibitors that might prove useful for the therapy of HCV. Several cores have been reported as potent HCV NS3 protease inhibitors including thiazolidine, benzamides, indole and indoline.[19–25] The molecular modeling study, including fitting to a 3D-pharmacophore model and docking into the active site using crystal structure of NS3/4A protease in complex with ligand **1a** ($IC_{50}$ = 0.7 μM = 0.4 μg/mL) as a model system for inhibition studies, suggested an interesting binding mode (Fig. 2). based on these information we developed a strategy that involves synthesis of new indole derivatives by the replacement of the indoline ring of the lead compound **1a** with indole. The docking studies showed that (i) the nitrogen of indole ring system makes H-bond donor with Cys159; (ii) a large hydrophobic group lies on the enzyme surface, shields the stretch charged residues from solvent so that it can accommodate the requisite lipophilic interaction; (iii) the oxygen and amidic nitrogen side chain may interact with Lys136 and Arg138; (iv) carboxylic function makes H-bounds with Gly 137 & Ser 139; (v) replacement of the linker between indole moiety and amino acid residue in lead compound **2** with 4-formylphenoxyacetyl function demonstrated significant increase in the hydrophobic interaction.

In the present work, molecular modeling simulation studies were performed in order to predict the biological activity of the proposed compounds. Firstly, the reported pharmacophore model of HCV NS3 protease inhibitors was subjected to simulation compare/fit studies, using the best fit algorithm[26], with the conformational model of a test set of the proposed compounds (**10a–g**). Secondly, molecular docking analysis was performed by Using Discovery Studio 2.5 software, where the crystal structure of inhibitor (**1a**)/NS3 protease complex[19] was obtained from protein data bank website (pdb). The prepared protein complex structure was used in determination of the active site that is mentioned in the literature. Docking process was carried out for the test set of compounds (**10a–g**) using CDOCKER protocol. The designed compounds which showed high docking scores (CDOCKER interaction energy) and good binding mode were synthesized following the sequence outlined in Schemes 1 and 2, predicting that they would have potential HCV NS3 serine protease inhibitory activity.

## 2. Result and discussion

### 2.1. Chemistry

The intermediates **3**, **4** and **5** that were used to introduce the 4-aminobenzophenone-2′-carboxylic acid moiety to the phenoxyacetic acid derivative **8**, were synthesized according to the reported methods[27] (Scheme 1). For the synthesis of the target compounds **10a–g**, the following straightforward pathway was pursued: it involved condensation of 4-formylphenoxyacetic acid (**7**) and 3-acetylindole (**6**) using catalytic amounts of piperidine and acetic acid in benzene under reflux, with azeotropic removal of water using a Dean–Stark trap to give phenoxyacetic acid derivative **8**.[28] $^1$H NMR spectra of **8** exhibit the formation of *E*-form configuration ($J$ = 15.60 Hz). Then, the phenoxyacetic acid derivative **8** undergoes condensation reactions with different amines using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) to afford the corresponding amide derivatives **9a–g**.[29] Compounds **9a–f** were mildly hydrolyzed to the target carboxylic acids **10a–f** using lithium hydroxide at room temperature.[30] Acid-catalyzed hydrolysis of the nitrile (**9g**) resulted in the formation of **10g** in good yield.

### 2.2. Molecular modeling

Pharmacophore model was generated using CATALYST software (Accelrys Inc., San Diego, CA, USA). Molecular docking with the prepared enzyme was performed using the Discovery Studio 2.5.

#### 2.2.1. Pharmacophore modeling study for test set (10a–g)

The simulation compare/fit study of compounds **10a–g** was carried out using our recently reported common feature pharmacophore model that encompassed five features namely;

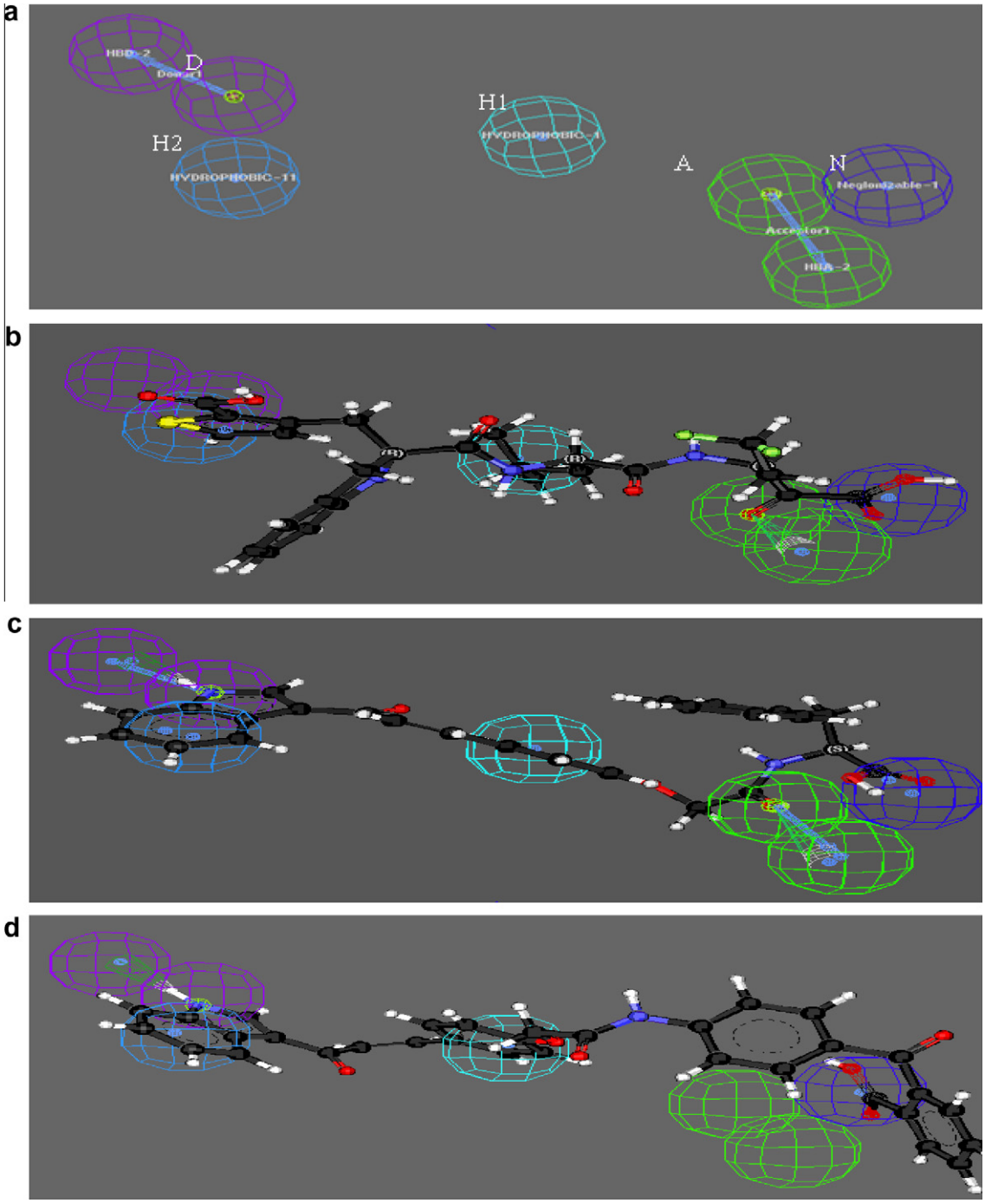


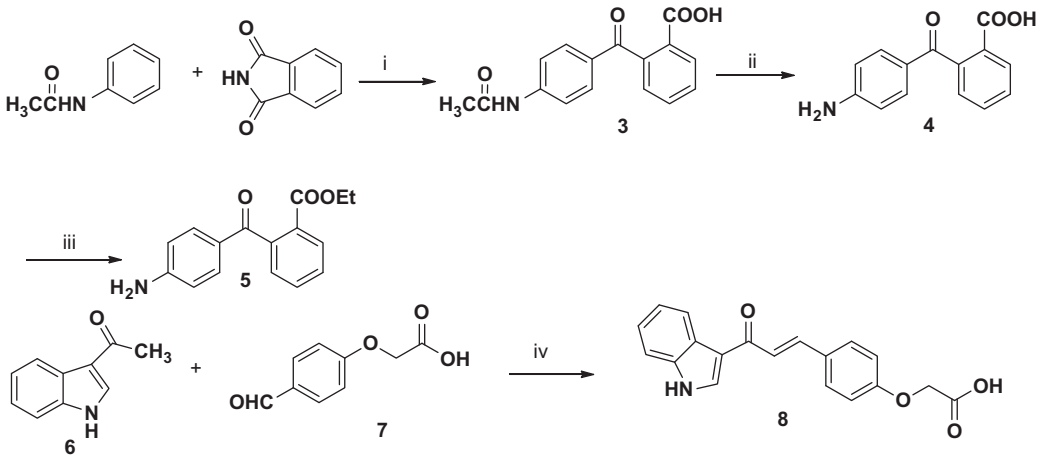

**Figure 2.** (a) Pharmacophore model of HCV NS3 protease inhibitors contains hydrogen-bond acceptor (A, green), hydrogen-bond donor (D, purple), two hydrophobic (H1, H2, light blue) and negative ionizable (N, blue). Mapping of (b) compound **1**, (c) compound **10a** and (d) compound **10e** with the generated HCV NS3 protease inhibitors hypothesis.

hydrogen-bond acceptor (A), hydrogen-bond donor (D), two hydrophobic (H1, H2) and negative ionizable (N) (Fig. 2).[20] our result were supported by Wei et al.[31] Hypogen-derived hypothesis generated from a set of HCV NS3 protease inhibitors.

Such a reported HypoGen hypothesis consisted of the same five pharmacophore features applied in our hypothesis. However, the dimensions between the features in this Hypogen hypothesis are not published. Crucially, we exploited our pharmacophore model to design a small number of novel and potent HCV NS3 protease inhibitors and those were evaluated in vitro. The structures of the test set of the target indoles were built using the CATALYST software, and their conformational models were generated in the energy rang of 20 kcal/mol above the estimated global energy minima). The fitting of the tested compound was performed using best fit during the compare/fit process. Different mappings for all the conformers of each compound of the test set to the hypothesis were visualized (Fig. 2), and the fit values of the best-fitting conformers listed in Table 1.

### 2.2.2. Molecular docking studies and binding mode

All dock runs were conducted using Discovery Studio 2.5. To investigate the detailed intermolecular interactions between the




i) $AlCl_3/C_2H_4Cl_2$ , ii) 75%HCl , iii) dryEtOH/$H_2SO_4$ , iv) piperidine, AcOH, dry benzene;

**Scheme 1.**

ligand and the target protein, an automated docking study was carried out using the crystal structure of inhibitor **1a** with the NS3/4A protease complex obtained from protein data bank website (pdb); having resolution of 2.0 Å.[19] The prepared protein was used in determination of the important amino acids in reported binding pocket.[19,20] Interactive docking using CDOCKER protocol was carried out for all the conformers of each compound of the test set (**10a**–**g**) to the selected active site, after energy minimization using the prepare ligand protocol.

Each docked compound was assigned a score according to its binding mode onto the binding site. The predicted binding energies of the compounds are listed in Table 1. From the interaction mode of the designed compounds with the predicated active site it has been noticed that, all target compounds **10a**–**g** make hydrogen bond donor interaction between NH function of indole ring system and C=O group of Cys 159 amino acid of the binding site. Additionally, the phenyl moiety of indole ring system of these compounds is responsible for hydrophobic interaction with the components of the binding pocket. Meanwhile, only compounds **10a** and **10b** exhibit hydrogen bonding acceptor interaction between the amino function of Lys 136 and the carbonyl oxygen of amide bond in side chain. However, compound **10a** (the most promising inhibitors of hepatitis C protease agent) have four hydrogen bonding due to mutual interactions taking place between its carboxylic oxygens and Lys 136 amino residue, Ser 139 amino function (Fig. 3).

These observations consistent with the experimentally binding assay of the synthesized indole analogs which support our assumption for designing of promising hits as Hepatitis C NS3/4A serine protease inhibitors

Alignment study of docked compound**10a** and ligand **1a** within the binding pocket of NS3/4A protease (Fig. 3 d) revealed that (i) carboxylic function of phenylalanine moiety of compound **10a** was perfectly aligned with carboxylic function of keto acid side chain of ligand **1a** and make the same hydrogen bonding interactions with Ser 139, Lys 136 and Gly 137 (ii) NH function of amide bond superimposed with ketonic C=O group of keto acid side chain of ligand **1a**, both functions interact with His 57 (iii) additionally, both the NH function of indole ring system of compound **10a** and OH function of carboxylic moiety at C2 of thiophen ring of ligand **1a** make same hydrogen bonding interaction with Cys 157.

### 2.3. HCV protease assay

A HCV protease binding assay was carried out by competitive displacement of the binding of peptide substrate (Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Abu-ψ). The fluorescence intensities were measured and the inhibition percentages were calculated.[20] The obtained data (Table 2) revealed that, we have synthesized some new potent HCV NS3/4A serine protease inhibitors, for example, compounds **10a**–**c**, in particular when the phenyl group is held constant to the terminal amino acid function, for example, **10a**,**b** (lowest docking energy and $IC_{50}$ value). Over 73% inhibitory effects on the HCV-PR activity were demonstrated by all target compounds (**10a**–**g**) at100 μg/mL. Of these, three compounds exhibited significant inhibitory activity (⩾82% inhibition), namely, compounds **10a**, **10b** and **10c** (Table 2). The study showed that compounds **10a** and **10b** were the most potent; their 50% inhibitory concentrations ($IC_{50}$) are 9 and 12 μg/mL, respectively (Table 2).

### 2.4. Structure–activity relationship (SAR)

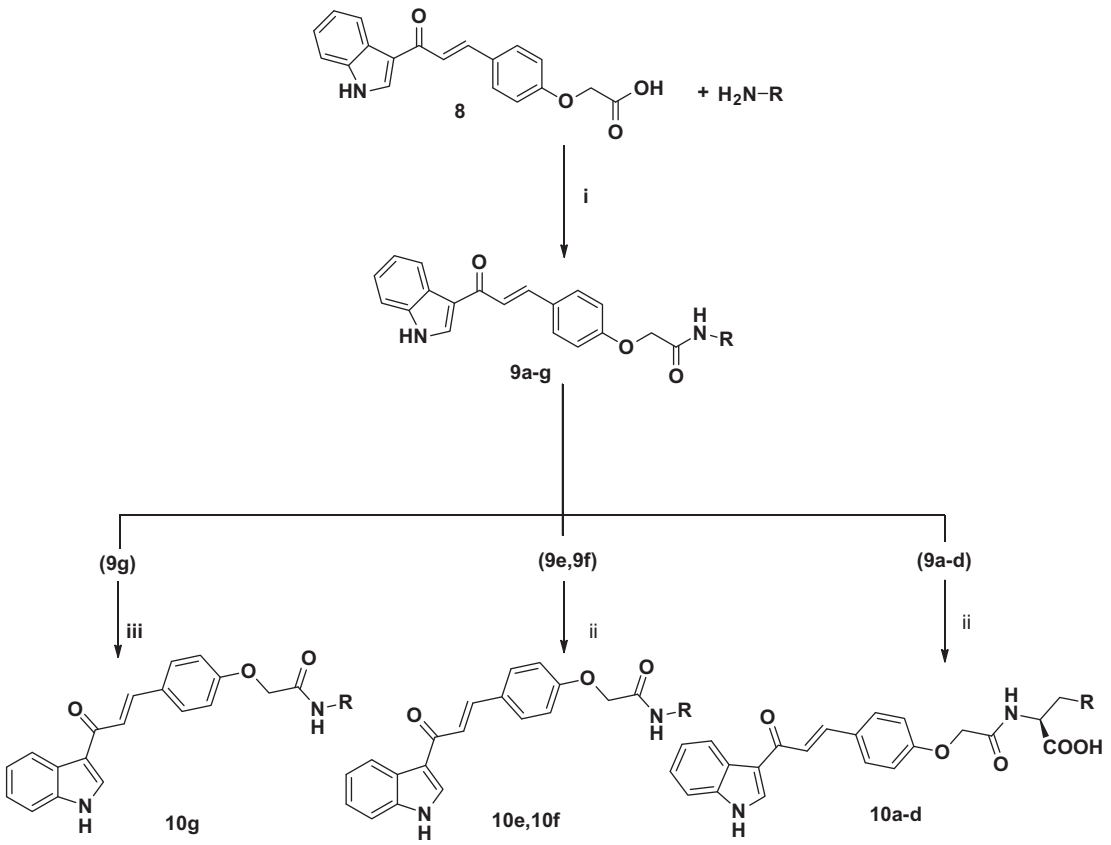
Based on the observed pharmacological data it could easily concluded that, attachment of the terminal amino acid group to the targeted chemical structure enhances the observed total potency against HCV NS3 protease enzyme. Additionally, attachment of (un) substituted phenyl group to the terminal amino acid function is considered the best choice for constructing a highly effective HCV NS3 protease inhibitor compared to the case when a heterocyclic moiety (indole, imidazole) was adopted.

## 3. Conclusion

The strategy used in this study has demonstrated that molecular modeling of inhibitor bound to NS3/4A protease structures proved a valuable tool in the design of a new series of potent NS3 protease inhibitors. Compounds **10a**, and **10b** showed high fit values and high docking scores. The synthesized compounds (**8, 9g** and **10a–g**) were biologically evaluated in vitro HCV protease binding assay and the result was consistent with molecular modeling study. Compounds **10a** and **10b** were the most potent; this suggests further study.

## 4. Experimental

All reagents and solvents were purchased from Aldrich, Wako and Tokyo Chemical Industry (TCI) and used as received. TLC was performed on Silica Gel 60 Partisil K6F plates (Whatman). Melting points are uncorrected and were measured on a Yanaco apparatus. IR spectra were recorded on a JASCO FT/IR-4100 spectrometer




**Scheme 2.**

using KBr disk. LC–MS ($ESI^+$) spectra were recorded with a Shimadzu LC–MS-2010EV spectroscopy. NMR spectral data were recorded on Bruker 400 MHz and Vaian-500 spectrometers; TMS was used as an internal standard for $^1H$ NMR and solvent peak was used as an internal standard for $^{13}C$ NMR. Elemental analyses were performed with Yanano CHN corder MT-5 element analyzer.

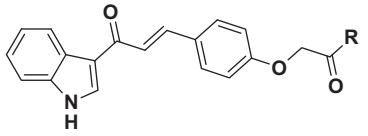
### 4.1. Synthesis of (*E*)-2-[4-(3-(1*H*-Indol-3-yl)-3-oxoprop-1-enyl)phenoxy]acetic acid (8)

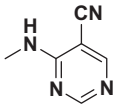
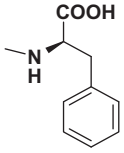
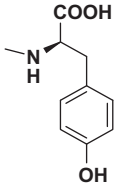
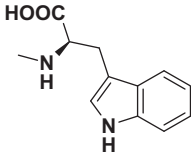
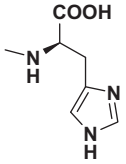
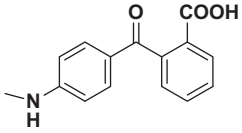
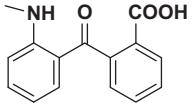
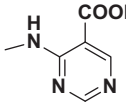
Equimolar amounts of 3-acetylindole (**6**) (30 mmol) and 4-formylphenoxyacetic acid (**7**) were dissolved in dry benzene (100 mL) containing acetic acid (3 mL) and piperidine (1 mL). The solution was heated under reflux using Dean–Stark water trap under argon until the theoretical amount of water was collected (≈6 h, TLC monitoring using 7% methanol/$CHCl_3$ for elution) indicated disappearance of the starting material. The solvent was evaporated in vacuo and after cooling the solid product was washed twice with 10 mL of diethyl ether and then twice with 15 mL of 2 N HCl, dried and recrystallized from benzene affording compound **8** as a yellow solid. Yield 81%, mp 242–243 °C. IR: $\nu_{max}/cm^{-1}$ 3016, 2985, 2843, 1714, 1682, 1657, 1598, 1570, 1511, 1423, 1401, 1372, 1250. $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 4.76 (s, 2H), 6.99 (d, $J$ = 8.76 Hz, 2H), 7.18–7.21 (m, 3H), 7.49 (d, $J$ = 7.12 Hz, 1H), 7.58 (d, $J$ = 15.52 Hz, 1H), 7.72 (d, $J$ = 15.54 Hz, 1H), 7.80 (d, $J$ = 8.76 Hz, 2H), 8.33 (d, $J$ = 6.98 Hz, 1H), 8.70 (d, $J$ = 5.88 Hz, 1H), 12.08 (br s, 1H). $^{13}C$ NMR (125 MHz, DMSO-$d_6$): $\delta$ 64.50, 112.09, 114.77, 117.72, 121.68, 121.73, 122.56, 122.95, 125.87, 128.29, 129.94, 134.37, 136.78, 139.14, 159.07, 169.72, 183.67. MS ($EI^+$): $m/z$: 322.1 $[M{\cdot}H]^+$; HRMS ($EI^+$) 322.1079 $[M{\cdot}H]^+$, found 322.1094. Anal. Calcd for $C_{19}H_{15}NO_4$: C, 71.02; H, 4.71; N, 4.36, Found: C, 71.22; H, 4.99; N, 4.60.

### 4.2. Synthesis of (*E*)-2-[4-(3-(1*H*-Indol-3-yl)-3-oxoprop-1-enyl)phenoxy]acetamides 9a–g (general procedure)

A mixture of compound **8** (31.0 mmol), the corresponding amine (34.9 mmol) and 1-hydroxybenzotriazole hydrate (38.5 mmol)

**Table 1**
Best fit and docking conformer for each compound in the test set (**8**, **9g** and **10a**–**g**) mapped with generated hypothesis and active site of HCV protease



| Entry | R | Compound | Fit value | Docking score (kcal/mol) |
|---|---|---|---|---|
| 1 | OH | **8** | 4.05 | −56.16 |
| 2 |  | **9g** | 4.21 | −59.15 |
| 3 |  | **10a** | 4.71 | −70.96 |
| 4 |  | **10b** | 4.64 | −70.48 |
| 5 |  | **10c** | 4.59 | −68.71 |
| 6 |  | **10d** | 4.09 | −70.07 |
| 7 |  | **10e** | 4.41 | −67.91 |
| 8 |  | **10f** | 4.11 | −66.78 |
| 9 |  | **10g** | 4.53 | −67.1 |

were dissolved in dimethylformamide (60 mL). The resulting solution was then placed in an ice bath at 0 °C and treated with triethylamine (14 mL), followed by stirring for 10 min. To the resulting mixture 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (38.5 mmol) was added. After removing the ice bath, the mixture was stirred for 18 h at room temperature. The reaction was diluted with water (200 mL), extracted with ethyl acetate, dried over sodium sulfate anhydrous, concentrated under reduced pressure and purified by silica gel column chromatography using chloroform/methanol as 10:1 (v/v) to obtain the product.

#### 4.2.1(*S*,*E*)-Methyl-2-[2-[4-(3-(1*H*-indol-3-yl)-3-oxoprop-1-enyl)-phenoxy]acetamido]-3-phenyl propanoate (9a)

Yield 76%, mp 105–106 °C. IR: $\nu_{max}/cm^{-1}$ 3312, 3009, 2944, 2908, 1746, 1716, 1680, 1659, 1486, 1324, 1165, 1119, 1067. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 3.08–3.13 (m, 2H), 3.67 (s, 3H), 4.29–4.31 (m, 1H), 4.76 (s, 2H), 6.99 (d, $J$ = 8.76 Hz, 2H), 7.18–7.23 (m, 5H), 7.29–7.34 (m, 3H), 7.48 (d, $J$ = 7.32 Hz, 1H), 7.58 (d, $J$ = 15.52 Hz, 1H), 7.72 (d, $J$ = 15.54 Hz, 1H), 7.80 (d, $J$ = 8.74 Hz, 2H), 8.33 (d, $J$ = 7.12 Hz, 1H), 8.70 (d, $J$ = 2.56 Hz, 1H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ ppm 22.17, 43.43, 51.10, 65.86, 111.05, 113.44, 114.75, 116.77, 120.65, 122.52, 123.24, 123.57, 123.81, 125.03, 125.06, 125.40, 126.47, 126.72, 127.96, 128.36, 130.03, 134.97, 149.78, 155.74, 157.37, 170.26, 173.68, 183.71. MS (EI$^+$): $m/z$: 483 [M·H]$^+$; HRMS (EI$^+$) 483.1920 [M·H]$^+$, found 483.1908. Anal. Calcd for $C_{29}H_{26}N_2O_5$: C, 72.18; H, 5.43; N, 5.81. Found: C, 72.39; H, 5.31; N, 5.98.

#### 4.2.2. (*S*,*E*)-Ethyl-2-[2-[4-(3-(1*H*-indol-3-yl)-3-oxoprop-1-enyl)-phenoxy]acetamido]-3-(4-hydroxy phenyl)propanoate (9b)

Yield 78%, mp 87–88 °C. IR: $\nu_{max}/cm^{-1}$ 3346, 2994, 2935, 2933, 1732, 1716, 1656, 1612, 1511, 1484, 1442, 1373, 1246, 1217, 1175, 1103, 1030. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 1.12 (t, $J$ = 7.09, Hz, 3H), 2.87–2.96 (m, 2H), 3.90–4.10 (m, 1H), 4.09 (q, $J$ = 7.03, Hz, 2H), 4.72 (s, 2H), 6.69 (d, $J$ = 8.74 Hz, 2H), 6.99 (m, 5H), 7.22–7.28 (m, 2H), 7.49 (d, $J$ = 7.26 Hz, 1H), 7.58 (d, $J$ = 15.52 Hz, 1H), 7.72 (d, $J$ = 15.54 Hz, 1H), 7.82 (d, $J$ = 8.74 Hz, 2H), 8.34 (d, $J$ = 6.89 Hz, 1H), 8.70 (d, $J$ = 2.56 Hz, 1H), 9.33 (br s, 1H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 13.91, 21.75, 43.43, 54.51, 65.36, 100.50, 110.92, 112.02, 116.68, 122.88, 123.29, 124.91, 125.44, 126.16, 126.32, 126.42, 126.58, 127.99, 128.39, 129.11, 129.24, 129.83, 131.49, 137.96, 150.03, 152.69, 170.26, 173.69, 183.71. MS (EI$^+$): $m/z$: 513 [M·H]$^+$; HRMS (EI$^+$) 513.2025 [M·H]$^+$, found 513.2024. Anal. Calcd for $C_{30}H_{28}N_2O_6$: C, 70.33; H, 5.51; N, 5.47. Found: C, 70.73; H, 5.81; N, 5.36.

#### 4.2.3. (*S*,*E*)-Methyl-2-[2-[4-(3-(1*H*-indol-3-yl)-3-oxoprop-1-enyl)- phenoxy]acetamido]-3-(1*H*-indol-3-yl)propanoate (9c)

Yield 71%, mp 93–94 °C. IR: $\nu_{max}/cm^{-1}$ 3371, 3001, 2950, 2832, 1738, 1716, 1660, 1509, 1484, 1456, 1439, 1247, 1218, 1175, 772. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 3.28–3.41 (m, 2H), 3.66 (s, 3H), 4.24–4.35 (m, 1H), 4.76 (s, 2H), 6.99–7.13(m, 3H), 7.06 (t, $J$ = 6.65 Hz, 2H), 7.22–7.28 (m, 2H), 7.37 (d, $J$ = 7.72 Hz, 2H), 7.49 (d, $J$ = 7.26 Hz, 2H), 7.58 (d, $J$ = 15.52 Hz, 1H), 7.72 (d, $J$ = 15.54 Hz, 1H), 7.80 (d, $J$ = 8.74 Hz, 2H), 8.32 (d, $J$ = 6.95 Hz, 1H), 8.70 (d, $J$ = 2.56 Hz, 1H), 11.09 (s, 1H). $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 27.43, 47.77, 55.36, 64.53, 101.59, 109.75, 109.82, 111.26, 111.82, 112.09, 113.85, 118.49, 118.81, 119.59, 121.96, 122.03, 122.10, 122.14, 123.06, 126.99, 127.57, 128.61, 131.76, 136.06, 153.77, 157.37, 170.87, 173.41, 183.68. MS (EI$^+$): $m/z$: 522 [M·H]$^+$; HRMS (EI$^+$) 522.1951 [M·H]$^+$, found 522.1958.

#### 4.2.4. (*S*,*E*)-Methyl-2-[2-[4-(3-(1*H*-indol-3-yl)-3-oxoprop-1-enyl)- phenoxy]acetamido]-3-(1*H*-imidazol-4-yl)propanoate (9d)

Yield 75%, mp 96–97 °C. IR: $\nu_{max}/cm^{-1}$ 3360, 3037, 2951, 2908, 2830, 1746, 1716, 1680, 1659, 1486, 1324, 1218, 1165, 1119, 1067. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 3.29–3.42 (m, 2H), 3.73 (s, 3H), 4.43–4.51 (m, 1H), 4.76 (s, 2H), 6.98 (d, $J$ = 8.74 Hz, 2H), 7.23–7.30 (m, 2H), 7.49 (d, $J$ = 7.26 Hz, 2H), 7.51 (s, 1H), 7.58 (d, $J$ = 15.52 Hz, 1H), 7.72 (d, $J$ = 15.54 Hz, 1H), 7.80 (d, $J$ = 8.74 Hz, 2H), 8.32 (d, $J$ = 6.95 Hz, 1H), 8.71 (d, $J$ = 2.72 Hz, 1H), 8.77 (br s, 1H), 9.07 (s, 1H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 27.59, 48.55, 52.65, 64.52, 111.05, 113.44, 114.75, 116.77, 120.65, 122.52, 123.24, 123.57, 123.81, 125.03, 125.06, 125.40, 126.47, 126.72, 127.95,128.36, 130.03, 134.97, 149.78, 155.64, 170.90, 173.44,

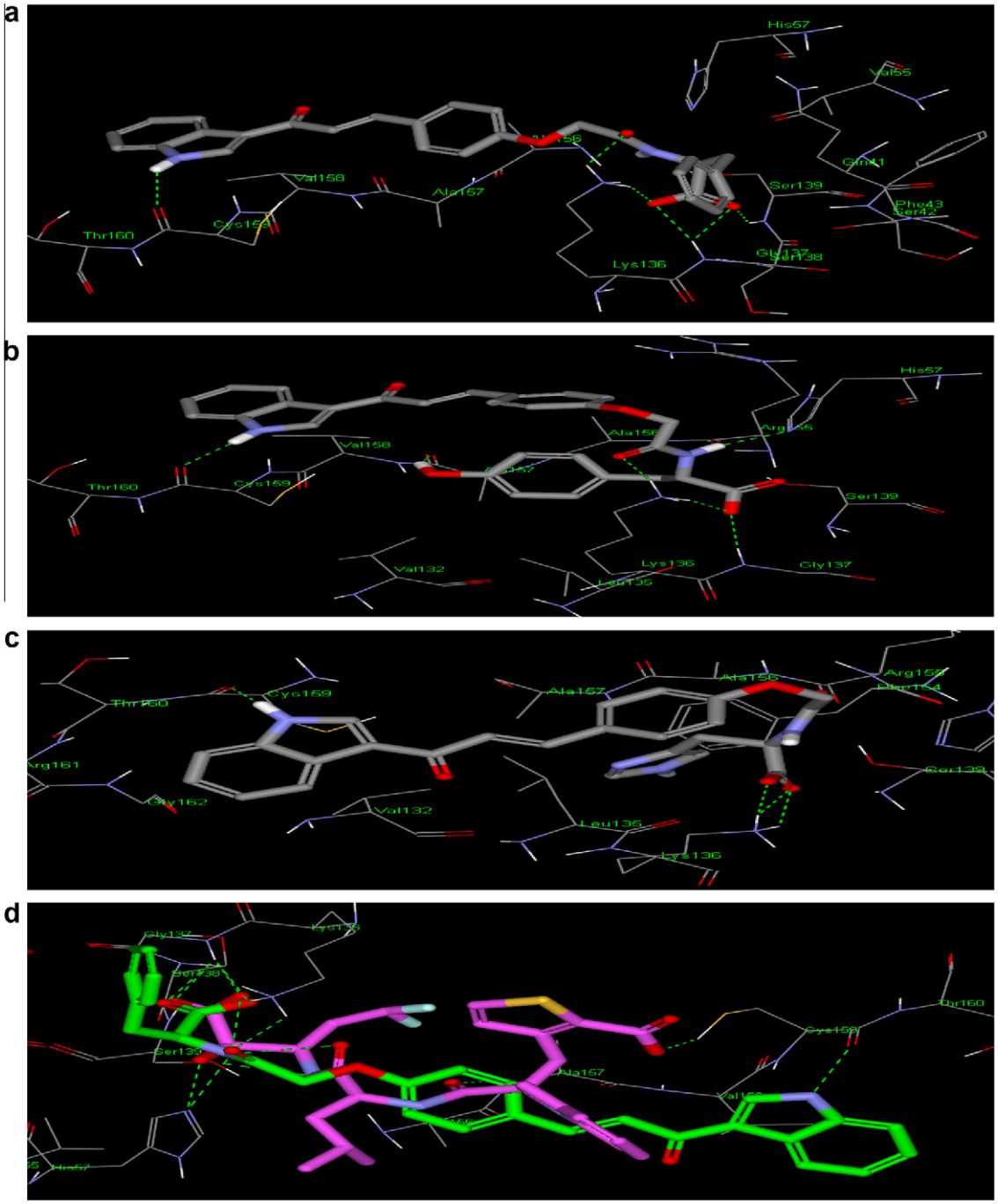



**Figure 3.** the proposed binding mode of (a) compound **10a**, (b) compound **10b** and (c) compound **10d**, inside the active site of different representation for Y-shaped active site of HCV NS3/4A protease enzyme. The most important amino acids are shown together with their respective numbers. (d) Alignment of docked compound **10a** (green color) and ligand **1a** with the binding pocket.

183.67. MS ($EI^+$): *m/z*: 473 $[M{\cdot}H]^+$; HRMS ($EI^+$) 473.1747 $[M{\cdot}H]^+$, found 473.1758.

#### 4.2.5. (*E*)-Ethyl-2-[4-[2-(4-(3-(1*H*-indol-3-yl)-3-oxoprop-1-enyl) phenoxy)acetamido]-benzoyl] benzoate (9e)

Yield 68%, mp 126–127 °C. IR: $\nu_{max}/cm^{-1}$ 3029, 2950, 2840, 1743, 1716, 1658, 1509, 1455, 1246, 1218, 1175. $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 1.03 (t, $J$ = 7.11, Hz, 3H), 4.05 (q, $J$ = 7.03, Hz, 2H), 4.76 (s, 2H), 6.39 (t, $J$ = 7.48 Hz, 1H), 6.83 (d, $J$ = 8.33 Hz, 1H), 6.87 (d, $J$ = 8.07 Hz, 1H), 7.00 (d, $J$ = 8.69 Hz, 2H), 7.24–7.32 (m, 4H), 7.39 (d, $J$ = 7.47 Hz, 1H), 7.50 (d, $J$ = 7.88 Hz, 1H), 7.59 (d, $J$ = 15.60 Hz, 1H), 7.64 (d, $J$ = 7.62 Hz, 1H), 7.72–7.81 (m, 2H), 7.80 (d, $J$ = 8.71 Hz, 2H), 7.96 (d, $J$ = 7.67 Hz, 1H), 8.33 (d, $J$ = 7.18 Hz, 1H), 8.71 (d, $J$ = 3.04 Hz, 1H). $^{13}C$ NMR (125 MHz, DMSO-$d_6$): $\delta$ 15.08, 55.30, 64.50, 100.61, 109.40, 110.92, 111.38, 112.00, 116.67, 117.93, 118.35, 120.90, 122.66, 123.27, 123.63, 124.90, 124.93,125.44, 126.32, 126.41, 126.56, 127.05, 128.38, 128.95, 131.47, 136.03, 136.51, 137.44, 137.59, 150.01, 152.88, 158.95, 171.27, 171.29, 172.37, 183.71. MS ($EI^+$): *m/z*: 573 $[M{\cdot}H]^+$; HRMS ($EI^+$) 573.1947 $[M{\cdot}H]^+$, found 573.1950.

**Table 2**
Inhibitory effects in different concentration and $IC_{50}$ values of the target compounds **8**, **9g** and **10a–g** against hepatitis C virus (HCV) serine protease

| Compd | HCV-PR Inhibition (%) at100 μg/mL | HCV-PR Inhibition (%) at 50 μg/mL | HCV-PR Inhibition (%) at 10 μg/mL | $IC_{50}$ (μg/mL) |
|---|---|---|---|---|
| **1a** | – | – | – | 0.4 |
| **1b** | – | – | – | 6.4 |
| **8** | 51.8 ± 1 | 22.9 ± 2.7 | – | >100 |
| **9g** | 58.4 ± 0.9 | 29.4 ± 5.8 | – | >100 |
| **10a** | 84.7 ± 0.2 | 82.4 ± 0.5 | 53.7 ± 3.5 | 9 |
| **10b** | 83.9 ± 0.7 | 81.2 ± 1.5 | 49.5 ± 5 | 12 |
| **10c** | 82.9 ± 0.1 | 67.2 ± 0.8 | 44.5 ± 2.8 | 19 |
| **10d** | 78.7 ± 6.6 | 48.5 ± 8 | 36.4 ± 5 | 21 |
| **10e** | 80.9 ± 2.3 | 72.05 ± 1.1 | 40.1 ± 4 | 19 |
| **10f** | 81.4 ± 0.3 | 70.15 ± 1.5 | 40.4 ± 3.2 | 26 |
| **10g** | 73.7 ± 3 | 57.2 ± 8 | 22.4 ± 3 | 29 |

The results are the mean ± SE, $P \leq 5$ ($n = 3$).

#### 4.2.6. (*E*)-Ethyl-2-[2-[2-(4-(3-(1*H*-indol-3-yl)-3-oxoprop-1-enyl)-phenoxy)acetamido]-benzoyl] benzoate (9f)

Yield 68%, mp 126–127 °C. IR: $\nu_{max}/cm^{-1}$ 3029, 2950, 2840, 1743, 1716, 1658, 1509, 1455, 1246, 1218, 1175. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 1.03 (t, $J$ = 7.11, Hz, 3H), 4.05 (q, $J$ = 7.03, Hz, 2H), 4.76 (s, 2H), 6.39 (t, $J$ = 7.48 Hz, 1H), 6.83 (d, $J$ = 8.33 Hz, 1H), 6.87 (d, $J$ = 8.07 Hz, 1H), 7.00 (d, $J$ = 8.69 Hz, 2H), 7.24–7.32 (m, 4H), 7.39 (d, $J$ = 7.47 Hz, 1H), 7.50 (d, $J$ = 7.88 Hz, 1H), 7.59 (d, $J$ = 15.60 Hz, 1H), 7.64 (d, $J$ = 7.62 Hz, 1H), 7.72–7.78 (m, 2H), 7.80 (d, $J$ = 8.71 Hz, 2H), 7.96 (d, $J$ = 7.67 Hz, 1H), 8.33 (d, $J$ = 7.18 Hz, 1H), 8.71 (d, $J$ = 3.04 Hz, 1H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 15.08, 55.30, 64.50, 100.61, 109.40, 110.92, 111.38, 112.00, 116.67, 117.93, 118.35, 120.90, 122.66, 123.27, 123.63, 124.90, 124.93,125.44, 126.32, 126.41, 126.56, 127.05, 128.38, 128.95, 131.47, 136.03, 136.51, 137.44, 137.59, 150.01, 152.88, 158.95, 171.27, 171.29, 172.37, 183.71. MS (EI$^+$): $m/z$: 573 [M·H]$^+$; HRMS (EI$^+$) 573.1947 [M·H]$^+$, found 573.1950.

#### 4.2.7. (*E*)-2-[4-(3-(1*H*-Indol-3-yl)-3-oxoprop-1-enyl)phenoxy]-*N*-(5-cyanopyrimidin-4-yl) acetamide (9g)

Yield 71%, mp 163–164 °C. IR: $\nu_{max}/cm^{-1}$ 3154, 2923, 1714, 1646, 1585, 1547, 1458, 1414, 1362, 1324, 1215, 1165, 1118, 1067, 1018. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 4.76 (s, 2H), 6.99 (d, $J$ = 8.74 Hz, 2H), 7.23–7.31 (m, 2H), 7.49 (d, $J$ = 7.26 Hz, 2H), 7.58 (d, $J$ = 15.60 Hz, 1H), 7.72 (d, $J$ = 15.59 Hz, 1H), 7.80 (d, $J$ = 8.74 Hz, 2H), 8.33 (d, $J$ = 7.18 Hz, 1H), 8.57 (s, 1H), 8.64 (s, 1H), 8.71 (d, $J$ = 3.04 Hz, 1H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 64.50, 109.75, 109.82, 111.26, 111.82, 112.09, 113.86, 115.12, 118.49, 118.81, 119.59, 121.96, 122.03, 122.10, 122.14, 123.06, 126.99, 127.57, 128.61, 131.76, 136.06, 153.77, 158.01, 171.51, 183.66. MS (EI$^+$): $m/z$: 424 [M·H]$^+$; HRMS (EI$^+$) 424.1409 [M·H]$^+$, found 424.1396.

### 4.3. Synthesis of (*S,E*)-2-[2-[4-(3-(1*H*-indol-3-yl)-3-oxoprop-1-enyl)phenoxy]acetamido]-propanoic acid derivatives 10a–f (general procedure)

To a solution of alkyl esters **9a–f** (0.742 mmol) in THF (20 mL) and ethanol (10 mL) at room temperature (20–25 °C) was added an aqueous solution of lithium hydroxide (2.92 mmol). The reaction was stirred at room temperature for 3 h. The progress of the reaction was monitored by TLC. After the solution was concentrated in vacuo to one-third of its original volume, ethyl acetate (60 mL), 1 N HCl (10 mL) and water (20 mL) were added and the organic layer was separated. The aqueous solution was extracted with ethyl acetate (2 × 50 mL). Organic solutions were combined, dried with sodium sulfate anhydrous, filtered, and concentrated to afford the corresponding **10a–f**.

#### 4.3.1. (*S,E*)-2-[2-[4-(3-(1*H*-Indol-3-yl)-3-oxoprop-1-enyl)-phenoxy]acetamido]]-3-phenyl propanoic acid (10a)

Yield 88%, mp 189–190 °C; $[\alpha]_D^{22} = -33.35$ ($c$ 0.43%, methanol). IR: $\nu_{max}/cm^{-1}$ 3403, 3033, 3000, 2954, 2833, 1716, 1667, 1609, 1584, 1509, 1484, 1455, 1247, 1218, 1175, 1031, 772. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 3.12–3.320 (m, 2H), 4.29–4.37 (m, 1H), 4.76 (s, 2H), 6.98 (d, $J$ = 8.74 Hz, 2H), 7.27–7.34 (m, 8H), 7.49 (d, $J$ = 6.98 Hz, 1H), 7.56 (d, $J$ = 15.60 Hz, 1H), 7.73 (d, $J$ = 15.60 Hz, 1H), 7.81 (d, $J$ = 8.74 Hz, 2H), 8.33 (d, $J$ = 7.12 Hz, 1H), 8.70 (d, $J$ = 3.03 Hz, 1H), 12.10 (br s, 1H). $^{13}$C NMR (125 MHz, DMSO-$d_6$); $\delta$ 21.74, 43.43, 65.86, 100.60, 110.92, 112.02, 116.68, 123.29, 124.91, 125.44, 126.16, 126.32, 126.42, 126.58, 127.99, 128.38, 129.11, 129.24, 129.83, 131.49, 137.96, 150.03, 152.89, 170.47, 183.71. MS (EI+): $m/z$: 469 [M·H]+; HRMS (EI$^+$) 469.1763 [M·H]$^+$, found 469.1752. Anal. Calcd for $C_{28}H_{24}N_2O_5$: C, 71.78; H, 5.16; N, 5.98. Found: C, 71.73; H, 5.47; N, 6.16.

#### 4.3.2. (*S,E*)-2-[2-[4-(3-(1*H*-Indol-3-yl)-3-oxoprop-1-enyl)-phenoxy]acetamido]-3-(4-hydroxy phenyl)propanoic acid (10b)

Yield 85%, mp 176–177 °C; $[\alpha]_D^{26} = -9.73$ ($c$ 0.95%, methanol). IR: $\nu_{max}/cm^{-1}$ 3347, 3063, 3009, 2938, 1707, 1655, 1616, 1515, 1484, 1441, 1417, 1364, 1325, 1217, 1165, 1117, 1067. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 2.86–2.91 (m, 2H), 3.88–3.93 (m, 1H), 4.71 (s, 2H), 6.68 (d, $J$ = 8.769 Hz, 2H), 6.99–7.10 (m, 5H), 7.24–7.31 (m, 2H), 7.48 (d, $J$ = 7.53 Hz, 1H), 7.60 (d, $J$ = 15.60 Hz, 1H), 7.70 (d, $J$ = 15.60 Hz, 1H), 7.79 (d, $J$ = 8.74 Hz, 2H), 8.34 (d, $J$ = 6.89 Hz, 1H), 8.70 (d, $J$ = 3.02 Hz, 1H), 9.33 (br s, 1H), 12.09 (br s, 1H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 22.17, 43.43, 65.86, 111.05, 113.44, 114.75, 116.77, 120.65, 122.52, 123.24, 123.57, 123.81, 125.03, 125.06, 125.40, 126.47, 126.72, 127.95, 128.36, 130.03, 134.97, 149.78, 155.74, 157.37, 170.26, 173.68, 183.71. MS (EI$^+$): $m/z$: 485 [M·H]$^+$; HRMS (EI$^+$) 485.1712 [M·H]$^+$, found 485.1718. Anal. Calcd for $C_{28}H_{24}N_2O_6$: C, 69.41; H, 4.99; N, 5.78. Found: C, 69.03; H, 4.75; N, 5.81.

#### 4.3.3. (*S,E*)-2-[2-[4-(3-(1*H*-Indol-3-yl)-3-oxoprop-1-enyl)-phenoxy]acetamido]-3-(1*H*-indol-3-yl)propanoic acid (10c)

Yield 83%, mp 166–167 °C; $[\alpha]_D^{27} = -29.38$ ($c$ 0.51%, methanol). IR: $\nu_{max}/cm^{-1}$ 3394, 2934, 2832, 1715, 1666, 1648, 1509, 1485, 1455, 1363, 1218, 1175, 1114, 1032, 772. 1H NMR (400 MHz, DMSO-$d_6$): $\delta$ 3.29–3.32 (m, 2H), 4.24–4.31 (m, IH), 4.76 (s, 2H), 6.99–7.01 (m, 3H), 7.03 (t, $J$ = 6.72 Hz, 2H), 7.23–7.30 (m, 2H), 7.38 (d, $J$ = 7.76 Hz, 2H), 7.49 (d, $J$ = 7.65 Hz, 2H), 7.58 (d, $J$ = 15.62 Hz, 1H), 7.72 (d, $J$ = 15.61 Hz, 1H), 7.81 (d, $J$ = 8.71 Hz, 2H), 8.32 (d, $J$ = 6.95 Hz, 1H), 8.70 (d, $J$ = 3.04 Hz, 1H), 11.09 (s, 1H), 12.13 (br s, 1H). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ ppm 27.46, 47.77, 64.53, 101.59, 109.75, 109.82, 111.26, 111.82, 112.09,

113.85, 118.49, 118.81, 119.59, 121.96, 122.03, 122.10, 122.14, 123.06, 126.99, 127.57, 128.61, 131.76, 136.06, 153.77, 157.37, 170.87, 173.41, 183.68. MS ($EI^+$): *m/z*: 508 $[M{\cdot}H]^+$; HRMS ($EI^+$) 508.1872 $[M{\cdot}H]^+$, found 508.1876. Anal. Calcd for $C_{30}H_{25}N_3O_5$: C, 70.99; H, 4.96; N, 8.28. Found: C, 71.02; H, 4.71; N, 7.99.

#### 4.3.4. (*S,E*)-2-[2-[4-(3-(1*H*-Indol-3-yl)-3-oxoprop-1-enyl)-phenoxy]acetamido]-3-(1*H*-imidazol-4-yl)propanoic acid (10d)

Yield 81%, mp 147–148 °C; $[\alpha]_D^{26} = +17.18$ (*c* 0.81%, methanol). IR: $\nu_{max}/cm^{-1}$ 3257, 3141, 3034, 2936, 2840, 1715, 1673, 1655, 1619, 1543, 1485, 1416, 1325, 1218, 1165, 1120, 1067. $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 3.29–3.32 (m, 2H), 4.39–4.43 (m, IH), 4.76 (s, 2H), 6.99 (d, *J* = 8.74 Hz, 2H), 7.22–7.30 (m, 2H), 7.49 (d, *J* = 7.26 Hz, 2H), 7.52 (s, 1H), 7.59 (d, *J* = 15.62 Hz, 1H), 7.71 (d, *J* = 15.60 Hz, 1H), 7.80 (d, *J* = 8.74 Hz, 2H), 8.32 (d, *J* = 6.95 Hz, 1H), 8.71 (d, *J* = 2.72 Hz, 1H), 9.08 (s, 1H), 12.16 (br s, 1H). $^{13}C$ NMR (125 MHz, DMSO-$d_6$): $\delta$ 27.59, 48.55, 64.52, 111.05, 113.44, 114.75, 116.77, 120.65, 122.52, 123.24, 123.57, 123.81, 125.03, 125.06, 125.40, 126.47, 126.72, 127.95,128.36, 130.03, 134.97, 149.78, 155.64, 170.90, 173.44, 183.67. MS ($EI^+$): *m/z*: 459 $[M{\cdot}H]^+$; HRMS ($EI^+$) 459.1668 $[M{\cdot}H]^+$, found 459.1678. Anal. Calcd for $C_{25}H_{22}N_4O_5$: C, 65.49; H, 4.84; N, 12.22. Found: C, 65.36; H, 4.60; N, 12.43.

#### 4.3.5. (*E*)-2-[4-[2-[4-(3-(1*H*-Indol-3-yl)-3-oxoprop-1-enyl)-phenoxy]acetamido]benzoyl]-benzoic acid (10e)

Yield 80%, mp 181–182 °C. IR: $\nu_{max}/cm^{-1}$ 3282, 3224, 3141, 3005, 2936, 2840, 1720, 1670, 1655, 1619, 1530, 1485, 1436, 1416, 1325, 1218, 1165, 1120, 1067. $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 4.76 (s, 2H), 6.38 (t, *J* = 7.58 Hz, 1H), 6.82 (d, *J* = 8.36 Hz, 1H), 6.88 (d, *J* = 8.14 Hz, 1H), 6.99 (d, *J* = 8.69 Hz, 2H), 7.24–7.31 (m, 4H), 7.39 (d, *J* = 7.47 Hz, 1H), 7.49 (d, *J* = 7.88 Hz, 1H), 7.59 (d, *J* = 15.60 Hz, 1H), 7.63 (d, *J* = 7.62 Hz, 1H), 7.73–7.79 (m, 2H), 7.83 (d, *J* = 8.71 Hz, 2H), 7.96 (d, *J* = 7.67 Hz, 1H), 8.33 (d, *J* = 7.18 Hz, 1H), 8.70 (d, *J* = 3.01 Hz, 1H), 12.11 (br s, 1H). $^{13}C$ NMR (125 MHz, DMSO-$d_6$): $\delta$ 64.50, 100.61, 109.40, 110.92, 111.38, 112.00, 116.67, 117.93, 118.35, 120.90, 122.66, 123.27, 123.63, 124.90, 124.93,125.44, 126.32, 126.41, 126.56, 127.05, 128.38, 128.95, 131.47, 136.03, 136.51, 137.44, 137.59, 150.01, 152.88, 158.95, 171.27, 171.29, 172.37, 183.71. MS ($EI^+$): *m/z*: 545 $[M{\cdot}H]^+$; HRMS ($EI^+$) 545.1712 $[M{\cdot}H]^+$, found 545.1726. Anal. Calcd for $C_{31}H_{32}N_2O_6.5H_2O$: C, 72.78; H, 4.44; N, 5.14. Found: C, 72.56; H, 4.71; N, 5.23.

#### 4.3.6. (*E*)-2-[2-[2-[4-(3-(1*H*-indol-3-yl)-3-oxoprop-1-enyl)-phenoxy]acetamido]benzoyl]-benzoic acid (10f)

Yield 80%, mp 181–182 °C. IR: $\nu_{max}/cm^{-1}$ 3282, 3224, 3141, 3005, 2936, 2840, 1720, 1670, 1655, 1619, 1530, 1485, 1436, 1416, 1325, 1218, 1165, 1120, 1067. $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 4.76 (s, 2H), 6.38 (t, *J* = 7.58 Hz, 1H), 6.82 (d, *J* = 8.36 Hz, 1H), 6.88 (d, *J* = 8.14 Hz, 1H), 6.99 (d, *J* = 8.69 Hz, 2H), 7.24–7.30 (m, 4H), 7.39 (d, *J* = 7.47 Hz, 1H), 7.49 (d, *J* = 7.88 Hz, 1H), 7.59 (d, *J* = 15.60 Hz, 1H), 7.63 (d, *J* = 7.62 Hz, 1H), 7.73–7.80 (m, 2H), 7.83 (d, *J* = 8.71 Hz, 2H), 7.96 (d, *J* = 7.67 Hz, 1H), 8.33 (d, *J* = 7.18 Hz, 1H), 8.70 (d, *J* = 3.01 Hz, 1H), 12.11 (br s, 1H). $^{13}C$ NMR (125 MHz, DMSO-$d_6$): $\delta$ 64.50, 100.61, 109.40, 110.92, 111.38, 112.00, 116.67, 117.93, 118.35, 120.90, 122.66, 123.27, 123.63, 124.90, 124.93,125.44, 126.32, 126.41, 126.56, 127.05, 128.38, 128.95, 131.47, 136.03, 136.51, 137.44, 137.59, 150.01, 152.88, 158.95, 171.27, 171.29, 172.37, 183.71. MS ($EI^+$): *m/z*: 545 $[M{\cdot}H]^+$; HRMS ($EI^+$) 545.1712 $[M{\cdot}H]^+$, found 545.1726.

### 4.4. Synthesis of (*E*)-4-[2-[4-(3-(1*H*-Indol-3-yl)-3-oxoprop-1-enyl)phenoxy]acetamido]-pyrimidine-5-carboxylic acid (10g)

To cyanopyrimidine derivative (**9g**) (2.00 g, 10.35 mmol), glacial acetic acid (20 mL), concd sulfuric acid (98%, 20 mL) and water (40 mL) was added then, the reaction mixture was heated under reflux for 18 h. The reaction mixture was poured into water (100 mL), the aqueous layer was extracted with ethyl acetate (3 × 100 mL) and the combined organic extracts were washed with brine (50 mL) and with sodium sulfate anhydrous. The ethyl acetate solution was concentrated in vacuo, to give bright yellow solid crystallized from ethyl acetate/*n*-hexane mixture as 4:6 v/v. Yield 80%, mp 163–164 °C. IR: $\nu_{max}/cm^{-1}$ 3339, 3050, 3000, 2934, 2833, 1713, 1678, 1609, 1509, 1484, 1455, 1247, 1218, 1031, 772. $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 4.76 (s, 2H), 6.99 (d, *J* = 8.74 Hz, 2H), 7.24–7.29 (m, 2H), 7.49 (d, *J* = 7.16 Hz, 2H), 7.58 (d, *J* = 15.64 Hz, 1H), 7.72 (d, *J* = 15.60 Hz, 2H), 7.80 (d, *J* = 8.74 Hz, 2H), 8.33 (d, *J* = 6.66 Hz, 1H), 8.57 (s, 1H), 8.64 (s, 1H), 8.71 (d, *J* = 3.04 Hz, 1H), 12.10 (br s, 1H). $^{13}C$ NMR (125 MHz, DMSO-$d_6$): $\delta$ 64.50, 109.75, 109.82, 111.26, 111.82, 112.09, 113.86, 118.49, 118.81, 119.59, 121.96, 122.03, 122.10, 122.14, 123.06, 126.99, 127.57, 128.61, 131.76, 136.06, 153.77, 158.01, 171.51, 172.31, 183.66. MS ($EI^+$): *m/z*: 443 $[M{\cdot}H]^+$; HRMS ($EI^+$) 443.1355 $[M{\cdot}H]^+$, found 443.1362. Anal. Calcd for $C_{24}H_{18}N_4O_5$: C, 65.15; H, 4.10; N, 12.66, Found: C, 65.49; H, 4.28; N, 12.92.

### 4.5. Synthesis of 2-(4-acetamidobenzoyl)benzoic acid (3)

To a solution of phthalic anhydride (10 mmol) in dichloroethane (50 mL) was added acetylaniline (15 mmol). The solution was cooled in an ice bath; aluminum chloride (100 mmol) was added portionwise. The ice bath was removed after 10 min and warmed to room temperature over 1 h. The reaction mixture was refluxed for 16 h, cooled to room temperature and poured carefully into a stirred solution of ice/1 N HCl (100 mL). The organic layer was separated, and the aqueous layer was extracted with dichloroethane (3 × 50 mL). The combined organic layer was extracted with cold aqueous 1 N NaOH (100 mL). The aqueous layer was extracted with dichloroethane (1 × 50 mL). The organic layers were discarded and the cold aqueous basic layer was acidified with concentrated HCl (12 mL) resulting in a milky white suspension which was extracted with dichloroethane (3 × 50 mL), dried over anhydrous $Na_2SO_4$ and concentrated affording **3**. Yield 72%, mp 170–171 °C. $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 3.25 (s, 3H), 6.36–6.39 (m, 1H), 6.79–6.81 (m, 1H), 6.86–6.88 (m, 1H), 7.19–7.24 (m, 1H), 7.27 (br s, 1H), 7.30–7.32 (m, 1H), 7.56–7.60 (m, 1H), 7.65–7.69 (m, 1H), 7.94–7.96 (m, 1H); MS ($EI^+$): *m/z*: 284 $[M{\cdot}H]^+$.

### 4.6. Synthesis of 2-(4-aminoobenzoyl)benzoic acid (4)

To 2-(4-acetamidobenzoyl) benzoic acid (**3**) (2.83 g, 10 mmol) was added hydrochloric acid 37% (50 mL) and the reaction mixture was heated under reflux for 6 h then, the reaction mixture was poured into water (100 mL). The aqueous layer was extracted with ethyl acetate (3 × 100 mL) and the combined organic extracts were washed with brine (50 mL) and dried over sodium sulfate anhydrous. The ethyl acetate solution was concentrated in vacuo, to give yellow solid recrystallized from hot concentrated hydrochloric acid affording **4**. Yield 72%, mp 203–204 °C. $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 6.36–6.40 (t, *J* = 7.20 Hz, 1H), 6.82 (d, *J* = 8.26 Hz, 1H), 6.88–6.91(m, 1H), 7.21–7.25 (m, 1H), 7.32 (br s,1H), 7.39 (d, *J* = 7.07 Hz, 1H), 7.60–7.64 (m, 1H), 7.71–7.73 (m, 1H), 7.96 (d, *J* = 7.75 Hz, 1H). MS ($EI^+$): *m/z*: 242 $[M{\cdot}H]^+$.

### 4.7. Synthesis of ethyl 2-(4-aminobenzoyl)benzoate (5)

To a solution of 2-(4-aminobenzoyl) benzoic acid (**4**) (15.5 mmol) in absolute ethanol (150 mL) concd $H_2SO_4$ (2 mL) was added and the mixture was refluxed for 6 h. The reaction mixture was cooled to room temperature and concentrated. The

residue was taken in cold water, solid sodium bicarbonate was added until the solution became basic and the layer was extracted with diethyl ether (2 × 100 mL). The combined ether layer was dried over sodium sulfate anhydrous and evaporated under vacuum. The crude product was purified by silica gel column chromatography, using chloroform/methanol mixture as 10:0.5(v/v) for elution affording **5**. Yield 83%, mp 143–144 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 1.03 (t, $J$ = 7.11 Hz, 3H), 4.04 (q, $J$ = 7.11 Hz, 2H), 6.36–6.40 (t, $J$ = 7.20 Hz, 1H), 6.82 (d, $J$ = 8.26 Hz, 1H), 6.88 (m, 1H), 7.21–7.25 (m, 1H), 7.32 (br s, 2H), 7.39 (d, $J$ = 7.07 Hz, 1H), 7.60–7.64 (m, 1H), 7.71–7.73 (m, 1H), 7.96 (d, $J$ = 7.75 Hz, 1H). MS (EI$^+$): $m/z$: 270 $[M{\cdot}H]^+$.

### 4.8. In vitro assay procedure

HCV NS3/4A protease (lot# 046-047 for the screening and lot# 046-079 for the mechanism study) and SensoLyte™520 HCV Protease Assay Kit *Fluorimetric* (lot# AK 71147-1005) were purchased from *Anaspec*, San Jose, CA, USA. The substrate was a 5-FAM/QXL™520 FRET peptide based on the sequence of Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Abu-ψ –[COO]Ala-Ser-Lys-(DABCYL)-$NH_2$. All compounds were dissolved in DMSO for the assay. To each well were added 2 μL of respective compound solution and 8 μL of freshly diluted enzyme (0.5 μg/mL). The reaction was started by adding 10 μL of freshly diluted substrate (100 times dilution of a DMSO stocking solution). After being incubated at room temperature (28 °C) for 30 min, the fluorescence intensities were measured at $E_x/E_m$ = 485 nm. Inhibition percentages were calculated as $100 \times (F_{vehicle} - F_{sample})/F_{vehicle}$ = % inhibition, where $F$ is the fluorescence value of vehicle control or of compound minus the fluorescence of the substrate control.

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