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Identification of the fused bicyclic derivatives of pyrrolidine and

imidazolidinone as dengue virus-2 NS2B-NS3 protease inhibitors

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Abstract

A series of fused ring derivatives of pyrrolidine and imidazolidinone were designed, synthesized, characterized and assayed against the DENV-2 NS2B-NS3 protease and wild-type DENV-2 virus. The linear dipeptide compound **1** and the non-peptidic fused ring compound **2** show comparable activities against DENV-2 NS2B-NS3 protease and wild-type DENV-2 virus in the plaque assay. The preliminary SAR reveals that a substituent and its stereochemistry at C-3 position, substitution (X) at N-2 arene and a linker (Y) between C-3 position and its attached arene are important for the fused-ring scaffold of pyrrolidino[1,2-*c*]imidazolidinone to block the active site of NS2B-NS3 protease. This promising structural core will facilitate the discovery of non-peptidic, potent NS2B-NS3 protease inhibitors to stop dengue virus infections.

Keywords: Dengue virus NS2B-NS3 protease inhibitor; pyrrolidino[1,2-*c*]imidazolidinone; stereochemistry; substitution and substituent; linker; structure-activity relationship (SAR)

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Table of contents graphic \cap Ω H_2N -NO₂ [HN n NO₂ NO₂ 2 3 1 $\begin{array}{c} & & \text{NO}_2 \\ \text{IC}_{50} = 1.2 \pm 0.4 \ \mu\text{M to NS2B-NS3} \\ \text{EC}_{50} = 39.4 \pm 6.2 \ \mu\text{M to virus plaque} \end{array}$ ٠Ś O_2N IC_{50} = 1.2 \pm 0.4 μM to NS2B-NS3 EC_{50} = 38.7 \pm 5.4 μM to virus plaque inactive previous work this work

1. Introduction

Dengue virus (DENV), a member of the genus *Flavivirus* (family *Flaviviridae*), is one of the most rapidly spreading mosquito-borne human pathogens in the tropical and subtropical regions [1]. The dengue virus is transmitted by the *Aedes aegypti* and *Aedesalbopictus* mosquitos, and causes a wide spectrum of clinical manifestations in humans ranging from an epidemic disease, known as dengue fever (DF), to the more severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [1,2]. In recent decades, the number of occurring infections, severe cases, and regions reporting autochthonous infections for the first time has dramatically increased [3] and WHO estimates 50–100 million infections worldwide per year.

There are four distinctive, but closely related, serotypes of DENVs named as DENV-1, DENV-2, DENV-3 and DENV-4. It was observed that cross-immunity to the other serotypes after recovery is only partial and temporary, and more dangerously, subsequent infection by any of the other three serotypes may enhance the risk of developing severe dengue, which was called "antigen-dependent enhancement" effect (ADE) [1,4]. Due to ADE, the vaccine development against DENVs is extremely difficult and the first tetravalent vaccine against dengue virus was approved only recently in December, 2015 in Mexico.

The genome of DENV is comprised of a 10.7 kb, single, positive-stranded RNA encoding a single polypeptide, which is subsequently processed by the virus-encoded trypsin-like NS2B-NS3 protease and by host proteases to generate three structural proteins (capsid protein, precursor membrane protein, and envelope protein) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [5]. The N-terminal part of NS3 contains a protease domain (termed NS3pro) with a classic serine proteinase catalytic triad (His51, Asp75, and Ser135) and optimal catalytic activity of NS3 depends on the presence of NS2B [6]. Since the NS2B-NS3 protease is essential for the maturation and pathogenesis of dengue virus, it is considered to be a promising target for anti-dengue virus drug development [7]. Therefore, the inhibition of NS2B-NS3 protease

activity is an attractive antiviral therapeutic treatment against DENV infections and many efforts have been made to develop anti-dengue virus NS2B-NS3 agents [8,9].

We have recently reported a series of linear chain dipeptides as inhibitors of DENV-2 NS2B-NS3 protease, and compound **1** (Fig. 1) was demonstrated as the most potent NS2B-NS3 protease inhibitor among them [10]. As part of the ongoing research to develop small molecular entities targeting DENV NS2B-NS3 protease, structural modifications based on compound **1** were explored. In this work, we describe the design, synthesis, absolute configuration determination and bioassay of the fused bicyclic derivatives of pyrrolidine and imidazolidinone as DENV-2 NS2B-NS3 protease inhibitors. It was discovered that non-peptidic compound **2** (3α -isomer) (Fig. 1), with an IC₅₀ value of 1.2 ± 0.4 µM, showed comparable inhibitory activity to DENV-2 NS2B-NS3 protease as compound **1** (IC₅₀=1.2 ± 0.4 µM), which possesses K_i value of 4.9 µM as reported [10]; however, its C-3 epimeric counterpart **3** (3β -isomer) was inactive against NS2B-NS3 protease. Furthermore, both **1** and **2** are active against wild-type DENV-2 virus plaque formation with EC₅₀ values of around 39 µM.

<Fig. 1>

2. Results and Discussion

2.1. Design of cyclic modifications

The crystal structures of NS2B-NS3 without any ligand [11] and complexed with bovine pancreatic trypsin inhibitor (BPTI) [12] exhibit an "open state" (inactive form), in which the C-terminal of NS2B was mostly dissociated from NS3pro; however, NMR studies in solution suggested that inhibitors like BPTI and a *p*-guanidino-benzoyl group (esterified to the catalytic serine) induce a conformational change in NS2B-NS3pro from the "open state" (inactive form) to the "closed state" (active form). In the latter, one or more groups of the inhibitors can promote the association of NS2B with NS3 [13]. Experimental studies and molecular docking showed that some S-S disulphide bridged cyclopeptides improved the binding affinity to NS2B-NS3 [14]. Furthermore, it was recently uncovered that the head-to-tail connection of a cyclopeptide also enhanced the inhibitory activity

againstNS2B-NS3 [15]. These results suggest that an appropriate cyclic inhibitor may fit well into the binding cleft and effectively block the active site of NS2B-NS3 protease. In addition, cyclization avoids the entropic penalty that is associated with the binding of highly flexible (e.g., peptidic) inhibitors. Based on compound **1**, a linear dipeptide from our previous study [10], a series of fused bicyclic compounds of pyrrolidino[1,2-*c*] imidazolidinone derivatives (Fig. 1) were designed, synthesized and tested against DENV-2 NS2B-NS3 protease. It is expected that these non-peptidic compounds are resistant to peptide enzymes. As noted, the structural skeleton of pyrrolidino[1,2-*c*] imidazolidinone [16] is very stable and appears in natural products [16a], pharmaceutical entities [16b-16d], chiral organo-catalysts [16e] or chiral catalytic ligands [16f].

2.2. Chemical Synthesis

The general synthetic route is outlined in Scheme 1 and the reaction results are listed in Table 1.

<Scheme 1>

Firstly, the intermediates of 4 and 5 were synthesized according to the procedure reported previously [10]. Briefly, the condensation of Boc-L-proline with p-nitroaniline provided 4, which was followed by the deprotection of Boc group in the presence of trifluoroacetic acid (TFA) to provide the salt of 5. Then, various aldehydes were refluxed in ethanol or toluene with compound 5 in the presence of triethylamine (TEA) to afford compounds 2, 3, 6a series and 6b series, respectively. Specifically, the reaction of linear aliphatic aldehydes (not including phenylacetaldehydes) (Table 1, entries 1-4) with 5 in refluxing ethanol or refluxing toluene gave the thermodynamic products of 3α -diastereoisomers (*trans*); meanwhile, the reaction of phenylacetaldehydes (Table 1, entries 5 and 6) with 5 happened only in refluxing toluene and yielded 3α -diastereoisomers (trans), which is a similar result as with TFA catalysis [16a]. It is interesting that the reaction of 5 with aromatic aldehydes without substitution (Table 1, entry 7) or with electron-donating group (EDG) at the p-position (Table 1, entries 8 and 9) in refluxing toluene led to only 3α -diastereoisomers (*trans*); whereas, starting from aromatic aldehydes bearing electron-withdrawing group (EWG) at the p-position (Table 1, entries 10-15) in refluxing toluene afforded two diastereoisomers in the ratio of around 4 to 1 of

thermodynamic 3α -diastereoisomer (*trans*) [16e] to 3β -diastereoisomer (*cis*), which could be separated by column chromatography. Even though chlorine is a weak electron-withdrawing substituent, the corresponding products obeyed the above rule and these results suggest that the electron-deficiency of aromatic ring and aldehyde group dominates the reaction mechanism. However, due to the hindrance at the *m*-position, *m*-nitro phenyl aldehyde only gave *trans*-isomer **6a12** (Table 1, entry 16). It is valued that free bases and salts of these compounds are stable as expected in our study.

<Table 1>

2.3. Determination of stereochemistry

Some absolute configurations of pyrrolidino[1,2-c]imidazolidinone compounds were analyzed in relevant references [16] but the determination of C-3 stereochemistry by ¹H NMR assignment is still very difficult. Therefore, X-ray crystallography analysis and 2D-NOESY NMR study were used here to determine the relationship between the absolute configurations of C-3 and their ¹H NMR data (Table 1). The absolute configuration of the chloro analog **6a11** (*p*-Cl-Ph is α -oriented) was determined by X-ray diffraction analysis (Fig. 2a), indicating that the reaction condition used in this work did not affect the stereochemistry of position C-7a, which originates from L-proline; accordingly, the absolute configuration of compound 2 in crystals (Fig. 2b) is trans isomer as 6a11 and the aromatic ring of p-NO₂-Ph in position 3 is α -oriented. Therefore, p-NO₂-Ph and p-Cl-Ph at C-3 in **3** and **6b11**, respectively, are β -oriented. Comparing the chemical shifts of $7\alpha\alpha$ -Hs and 3β-Hs of **2** and **6a11** (4.01 and 5.90, 3.98 and 5.76, respectively) with those of $7a\alpha$ -Hs and 3α -Hs of **3** and **6b11** (4.05-4.28 and 6.55, 3.98-4.20 and 6.40, respectively), we conclude that 3β -Hs (*trans*-isomers) are less than 6.0 ppm but 3α -Hs (*cis*-isomers) are higher than 6.0 ppm. Based on these results, we deduce that the 3-aromatic compounds of 2, 6a7, 6a8, 6a9, 6a10 and 6a11 are trans-isomers and those of 3, 6b10 and 6b11 are cis-isomers.

<Fig. 2>

The detailed assignment of **2** was also analyzed by a NOESY experiment (Fig. s1, Supporting Materials). The NOESY data of **2** showed that no obvious NOE correlation

between 3β-H (5.90 ppm) and 7*a*α-H (4.01 ppm) was observed while there were strong NOE correlations between 7*a*α-H with 2'-H (7.71 ppm) and 2"-H (7.50 ppm) of two aromatic rings (Fig. s1, Supporting Materials), supporting that 3β-H and 7*a*α-H are in *trans* configuration. The observations that 3β-H (5.90 ppm) exhibited strong NOE correlations with both 5-Hs (3.55 and 2.94 ppm) but there was lack of NOE correlation between 7*a*α-H and any one of 5-Hs propose the α-orientation of the lone-pair electrons of N-4. Meanwhile, the presence of strong NOE correlation between 3β-H with 2'-H and the absence of NOE correlation between 7*a*α-H and 2'-H indicated that the *p*-nitro-phenyl group at N-2 is β-oriented. These results lead to the deduction that 3β-H is surrounded by two adjacent nitrogen atoms and shielded by an aromatic ring at N-2, which are in agreement to the crystal structures of **2** and **6a11**.

In order to deduce the stereochemistry of **6a5**, which was derived from phenylacetaldehyde, NOESY experiment (Fig. s2, Supporting Materials) was conducted. The strong NOE correlation between $7a\alpha$ -H (3.61 ppm) with benzylic H (3.00 ppm) and 2"-H (7.18 ppm) and the absence of a NOE between $7a\alpha$ -H with 3β-H (5.15 ppm) support that benzylic group is *trans*-configuration to 1-carbonyl. Similar to **2**, both 5α -H (3.26 ppm) and 5β -H (2.61 ppm) have NOE correlations with 3β-H and there is no NOE correlation between $7a\alpha$ -H and 5α -Hs; meanwhile, no NOE correlation was observed between $7a\alpha$ -H with 2'-H. It was found that benzylic Hs (α -oriented) and 3β-H have NOE correlations with 2'-H (7.84 ppm) but stronger NOE correlation of 3β-H with 2'-H was observed than that of benzylic H with 2'-H, indicating that the *p*-nitro-phenyl group at N-2 is more β-oriented. Based on these results, it is deduced that the aliphatic Rs in **6a2**, **6a3**, **6a4** and the *p*-nitro-benzylic group in **6a6** are in α -orientation as **6a5**.

2.4. Inhibitory activity against DENV-2 NS2B-NS3 protease

The inhibitory activity of these compounds to DENV-2 NS2B-NS3 protease was assayed according to our previous procedure [10] and the activity results are listed in Table 1.

<Fig. 3>

Initially, compounds **6a1**, **6a2**, **6a3** and **6a4**, derived from aliphatic aldehydes, were assayed and it was discovered that they showed moderate inhibitory activities and

established good structure-activity relationship (SAR) against DENV-2 NS2B-NS3 protease (Table 1, entries 1-4 and Fig. 3). Along with the increase of R group from H (**6a1**) to Me (**6a2**) and Et (**6a3**), the inhibitory activity was stepwise evaluated; however, branched alkyl R of bulky *i*-Pr (**6a4**) decreased the inhibitory activity, as seen by comparison of **6a4** with **6a3**. These findings created a new and non-peptidic scaffold as DENV-2 NS2B-NS3 protease inhibitors. Based upon these results, diverse compounds with this scaffold were explored as DENV-2 NS2B-NS3 protease inhibitors.

In order to enhance the inhibitory activity, the replacement of the linear aliphatic group with a benzylic group was conducted and compound **6a5** (Table 1, entry 5) exhibited a moderate inhibition as **6a4** with *i*-Pr group; however, compound **6a6** bearing a *p*-nitro-benzylic group sharply lost the inhibitory activity (Table 1, entry 6). It seems that the *p*-nitro group seriously diverts the conformation of whole molecule, which negatively affects the binding between NS2B-NS3 with compound **6a6**.

<Table 2>

Further studies by the replacement of linear aliphatic group with aromatic groups were conducted and it was discovered that compound **6a7** with phenyl group (Table 1, entry 7) exhibited very weak inhibitory activity to NS2B-NS3 protease and compounds **6a8** (Table 1, entry 8) and **6a9** (Table 1, entry 9) bearing *p*-EDG phenyl groups did not show any activity against NS2B-NS3 protease. Then, *p*-EWG phenyl groups were introduced and the results showed that 4-chloro-phenyl and 4-trifluoromethyl phenyl derivatives (Table 1, entries 10-13) were also not active against NS2B-NS3 protease. Interestingly, it was disclosed that compound **2**, with R = p-nitro-phenyl, of *trans*-isomer (Table 1, entry 14) exhibited good inhibitory activity (IC₅₀ value of $1.2 \pm 0.4 \mu$ M) and almost the same inhibition to NS2B-NS3 protease as parent compound **1** [10] (Table 1, entry 17). However, compound **3**, the corresponding *cis*-isomer (Table 1, entry 15) did not exhibit inhibitory activity at NS2B-NS3 protease, and compound **6a12** (*trans*-isomer) with *m*-nitro phenyl group (Table 1, entry 16) showed no activity. These activity differences suggest that the stereochemistry of the 3α -(*p*-nitro) phenyl group provides an appropriate binding demand of **2** to NS2B-NS3.

It is interesting and valuable that the SARs of NS2B-NS3 protease inhibitors determined by X and Y (Table 2) in fused ring derivatives of **2** and **6a7**, **6a5** and **6a6** also existed in our previous report [10] of linear dipeptides (Table 2), in which a *p*-nitro group negatively affects the activity when Y is methylene whereas *p*-nitro positively exerts the activity when Y is empty, indicating an elementary molecular recognition requirement for these series of NS2B-NS3 protease inhibitors. These results imply that the suitable combination of X and Y can determine optimal orientation of a specific group, which is crucial for the binding and interaction of an inhibitor with the enzyme.

2.5. Antiviral activity of 1 and 2 against wild-type DENV-2 virus

As 1 and 2 possess the most potent inhibitory activity to DENV-2 NS2B-NS3 protease among these compounds, antiviral testing of 1 and 2 was conducted in wild-type DENV-2 virus plaque assays and the results are listed in Table 3. Both of 1 and 2 are moderately active against wild-type DENV-2 virus plaque formation with similar EC₅₀ values of 38.7 \pm 5.4 µM and 39.4 \pm 6.2 µM, respectively; meanwhile, the CC₅₀ values of 1 and 2 are higher than 100 µM at Huh-7 cells, indicating that they have no cytotoxic effect in the tested concentration range. Importantly, these observations illustrate that these series compounds could be as lead compounds to discover more potent inhibitors to treat and prevent dengue virus infections.

<Table 3>

2.6. Docking analysis of inhibitory activity to DENV-2 NS2B-NS3 protease

To understand the relationship of the structural feature with the binding mode, the putative binding poses of compounds 1 [10], 2 and 3 to DENV-2 NS2B-NS3 protease were compared by means of molecular docking. The docking model was generated from the chain A of the crystal structure of NS2B-NS3 (PDB: 2FOM [11]) by using Discovery Studio 3.5 and the results are shown in Fig. 4. In the fitted low energy conformation, 1 and 2 are binding close to the catalytic pocket (His51, Asp75 and Ser135) of NS2B-NS3 protease (Fig. 4a and Fig. 4b) even though there are slight binding variations in detail between compound 2 and dipeptide 1. *p*-Nitro phenyl in 2 to substitute pyrrole ring in 1 occupies the cleft of Tyr 150, Ser135 and Pro132, and the oxygen of the *p*-nitro phenyl

group in 2 interacts with Ser135 from the catalytic triad via a hydrogen bond. Importantly, the fused ring of 2 is close to Asp75 and His51 of the catalytic triad, and its N-4 nitrogen atom donates its lone-pair electrons to imidazole of His51 to form a hydrogen bond. Both p-nitro-anilide moieties in 1 and 2 are oriented toward the channel of Gly133, Ile36 and Val52, but the p-nitro-anilide in 1 is more towards the cleft of Ile36 and Val52 while the p-nitro-anilide in 2 is more towards the cleft of Ile36 and Gly133. The hydrogen bond network could be an important factor to stabilize the binding and block the catalytic activity that both of two hydrogen bonds by the binding of 2 are related with His51 and Ser135 from the catalytic triad; meanwhile, one of three hydrogen bonds formed by 1 is toward Ser135 from the catalytic pocket. The comparable inhibitory activity of 1 and 2 at NS3 suggests that their predicted binding poses are complementary and the binding discrepancy between 1 and 2 is compensated by hydrogen bonds with the catalytic pocket. These results will be helpful for us to design more potent NS3 inhibitors.

<Fig. 4>

It is noted that the putative binding conformation of 2 (Fig. 4d) is different from the free conformation of 2 in crystal structure (Fig. 2b and Fig. 4e), especially the relative orientations of two phenyl groups and the fused ring, illustrating that the conformational change of 2 may be necessary to generate more favorable interactions between 2 and NS3.

In comparison of the docking results of **2** and **3** to NS3, it is proposed that their different configurations at 3-position lead to a modified orientation of *p*-nitro-phenyl at C-3 and *p*-nitro aniline at N-2 in **2** and **3**, in which *p*-nitro-phenyl and *p*-nitro aniline in **3** (Fig. 4c) are oriented towards the channel of Gly133, Ile36 and Val52, and the cleft of Tyr 150, Ser135 and Pro132, respectively. There are two hydrogen bonds between **3** and NS3 and the fused ring is located very close to His51; however, the twisted conformation of the fused ring in **3** renders the N-4 nitrogen unable to form a hydrogen bond with the imidazole ring of His51 from the catalytic pocket due to unfavorable chirality of the C-3 position in **3**, which might explain at least in part the loss of its binding and inhibitory activity to NS3 observed in **3**.

3. Conclusions

In summary, a series of the fused ring derivatives of hexahydropyrrolo[1,2-c]imidazol-1-one were designed, synthesized, determined and assayed against DENV-2 NS2B-NS3 protease and wild-type DENV-2 virus. The data in Table 1 and Table 2 show that non-peptidic fused-ring compound 2 (CLogP = 3.7286, calculated from Chem3D) has similar potency against DENV-2 NS2B-NS3 protease as linear dipeptide 1 (CLogP = 1.4050, calculated from Chem3D). It is noteworthy that 1 and 2 possess moderate activity against wild-type DENV-2 virus plaque formation (Table 3). From the point of molecular docking, 1 and 2 have a similar putative binding mode even though their bindings to NS3 slightly differ in some aspects. The preliminary SAR reveals that R and its stereochemistry at C-3 position, X of a substituent at arene and Y of a linker between C-3 position and arene are important for the fused-ring scaffold to block the active site of NS2B-NS3 protease. Although these inhibitors are not very potent at the current stage, it appears likely that the further discovery of highly active non-peptidic NS2B-NS3 protease inhibitors will be facilitated, based on the promising structural core element of the compounds. Further studies will focus on designing more potent inhibitors for the treatment and prevention of dengue virus infections and to decipher the underlying structural features and binding mechanism.

4. Experimental

4.1. Chemistry

Unless stated, materials were obtained from commercial suppliers and used without further purification. ¹H and ¹³C NMR spectra were recorded on a Bruker AV-400 spectrometer at 400 and 101 MHz, respectively. Coupling constants (*J*) are expressed in hertz (Hz). Chemical shifts (δ) of NMR are reported in parts per million (ppm) units relative to the solvent. The high resolution of MS (HRMS) was recorded on an Applied Biosystems Q-STAR Elite ESI-LC-MS/MS mass spectrometer. Melting points were measured using a YRT-3 melting point apparatus (Shanghai, China) and were uncorrected.

4.1.1. Preparation of intermediates 4 and 5

To the solution of Boc-*L*-Proline (2.0 g, 9.3 mmol) and *p*-nitroamine (1.2 g, 9.0 mmol) in 60 ml anhydrous dichloromethane (DCM), dicyclohexylcarbodiimide (DCC) (1.9 g, 9.3 mmol) was added and the reaction mixture was stirred for 5h. After resulting precipitate was filtered and the solvent was evaporated, the residue was recrystallized from ethanol to

afford intermediate *tert*-butyl (S)-2-((4-nitrophenyl)carbamoyl)pyrrolidine-1-carboxylate (4) (2.4 g, 72% yield).

In a round-bottomed flask, compound 4 (2.9 mmol) was dissolved in 30 ml dry DCM. The solution was cooled to 0 $^{\circ}$ C and treated with trifluoroacetic acid (TFA) (TFA/DCM (V/V) = 1/4) and stirred for 2h. Upon completion, the solvent was evaporated and the residue was compound (S)-2-((4-nitrophenyl)carbamoyl)pyrrolidin-1-ium trifluoroacetate (5) and used for the next reaction without further purification.

4.1.2. General preparation of compounds 6a1-6a4

Intermediate **5** (349 mg, 1.0 mmol) was dissolved in 8 ml ethanol. The solution was treated with 0.15 ml triethylamine and distinct aldehyde (1.0 mmol). The reaction mixture was stirred at 85 \square for 30 min. Upon completion, the solution was evaporated under the reduced pressure and the residue was dissolved in ethyl acetate. The organic layer was washed with water (3 × 50 mL) and brine (50 ml), dried over anhydrous Na₂SO₄ and filtered and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (petroleum ether/ethyl acetate 10/1 to 6/1) to give **6a1-6a4**.

4.1.2.1. (*S*)-2-(4-Nitrophenyl)hexahydro-1*H*-pyrrolo[1,2-*c*]imida-zol-1-one (6a1). 60% yield, white solid, m.p. 197.5-203.3 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.35-8.16 (m, 2H, Ar-*H*), 7.87-7.71 (m, 2H, Ar-*H*), 5.03 (d, *J* = 8.0 Hz, 1H, 3-*H*), 4.67 (d, *J* = 8.0 Hz, 1H, 3-*H*), 3.96 (dd, *J* = 4.0, 8.8 Hz, 1H, 7*a*-*H*), 3.34-3.29 (m, 1H, 5-*H*), 2.69 (dd, *J* = 16.0, 8.0 Hz, 1H, 5-*H*), 2.36-2.11 (m, 2H, 7-*H*), 1.99-1.79 (m, 2H, 6-*H*); ¹³C NMR (101 MHz, CDCl₃) δ 175.1 (*C*=O), 143.6 (Ar-*C*), 143.4 (Ar-*C*), 125.0 (Ar-*C*), 118.0 (Ar-*C*), 70.0 (7*a*-*C*), 66.5 (3-*C*), 55.9 (5-*C*), 27.9 (7-*C*), 25.2 (6-*C*); HRMS (ESI) *m*/*z* 248.1033 [M+H]⁺, calculated for C₁₂H₁₄N₃O₃, 248.1035.

4.1.2.2. (*3R*,7*aS*)-3-Methyl-2-(4-nitrophenyl)hexahydro-1*H*-pyrrolo[1,2-*c*]imidazol-1one (6a2). 74% yield, white solid, m.p. 154.2-157.3 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.31-8.22 (m, 2H, Ar-*H*), 7.87-7.76 (m, 2H, Ar-*H*), 5.06 (q, *J* = 8.0 Hz, 1H, 3-*H*), 4.06 (dd, *J* = 12.0, 4.0 Hz, 1H, 7*a*-*H*), 3.34-3.29 (m, 1H, 5-*H*), 2.63 (dd, *J* = 16.0, 8.4 Hz, 1H, 5-*H*), 2.33-2.08 (m, 2H, 7-*H*), 1.94-1.80 (m, 2H, 6-*H*), 1.49 (d, *J* = 6.0 Hz, 3H, C<u>*H*</u>₃); ¹³C NMR (101 MHz, CDCl₃) δ 174.7 (*C*=O), 143.7 (Ar-*C*), 142.3 (Ar-*C*), 125.0 (Ar-*C*), 119.7 (Ar-*C*), 77.2 (3-*C*), 64.5 (7*a*-*C*), 55.3 (5-*C*), 27.2 (7-*C*), 24.7 (6-*C*), 20.5 (*C*H₃); HRMS (ESI) *m*/z 262.1188 [M+H]⁺, calculated for C₁₃H₁₆N₃O₃, 262.1192.

4.1.2.3. (3*R*,7*aS*)-3-Ethyl-2-(4-nitrophenyl)hexahydro-1*H*-pyrrolo[1,2-*c*]imidazol-1one (6a3). 70% yield, white solid, m.p. 151.1-157.3 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.30-8.21 (m, 2H, Ar-*H*), 7.83-7.74 (m, 2H, Ar-*H*), 4.80 (dd, *J* = 8.0, 4.0 Hz, 1H, 3-*H*), 4.01 (dd, *J* = 8.0, 4.0 Hz, 1*H*, 7*a*-*H*), 3.34-3.29 (m, 1H, 5-*H*), 2.71-2.65 (m, 1H, 5-*H*), 2.32-2.18 (m, 1H, 7-*H*), 2.10 (m, 1H, 7-*H*), 1.96-1.72 (m, 3H, 6-*H* and one of C<u>*H*</u>₂CH₃), 1.61 (m, 1H, one of C<u>*H*</u>₂CH₃), 1.03 (t, *J* = 7.4 Hz, 3H, CH₂C<u>*H*</u>₃); ¹³C NMR (101 MHz, CDCl₃) δ 175.3 (*C*=O), 143.7 (Ar-*C*), 143.1 (Ar-*C*), 124.94 (Ar-*C*), 120.1 (Ar-*C*), 82.9 (3-*C*), 65.0 (7*a*-*C*), 56.5 (5-*C*), 27.8 (7-*C*), 27.2 (\underline{C} H₂CH₃), 24.9 (6-*C*), 9.2 (CH₂ \underline{C} H₃); HRMS (ESI) *m*/*z* 276.1346 [M+H]⁺, calculated for C₁₄H₁₈N₃O₃, 276.1348.

4.1.2.4. (*3R*,7*aS*)-3-Isopropyl-2-(4-nitrophenyl)hexahydro-1*H*-pyrrolo[1,2-*c*]imidazol -1-one (6a4). 66% yield, white solid, m.p. 145.0-148.8 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.30-8.22 (m, 2H , Ar-*H*), 7.78-7.66 (m, 2H, Ar-*H*), 4.77 (d, *J* = 4.0 Hz, 1H, 3-*H*), 3.98 (dd, *J* = 5.0, 4.8 Hz, 1H, 7*a*-*H*), 3.36-3.31 (m, 5-*H*), 2.80-2.69 (m, 1H, 5-*H*), 2.29-2.17 (m, 1H, C<u>*H*</u>(CH₃)₂), 2.08-1.95 (m, 2H, 7-*H*), 1.89-1.75 (m, 2H, 6-*H*), 1.04 (d, *J* = 6.8 Hz, 3H, one C<u>*H*</u>₃ of CH(C<u>*H*</u>₃)₂), 0.80 (d, *J* = 6.8 Hz, 3H, one C<u>*H*</u>₃ of CH(C<u>*H*</u>₃)₂); ¹³C NMR (101 MHz, CDCl₃) δ 174.8 (*C*=O), 144.0 (Ar-*C*), 142.8 (Ar-*C*), 124.9 (Ar-*C*), 121.6 (Ar-*C*), 87.0 (3-*C*), 66.4 (7*a*-*C*), 58.6 (5-*C*), 31.4(7-*C*), 29.0 (<u>C</u>H(CH₃)₂), 25.2 (6-*C*), 18.4 (one <u>C</u>H₃ of CH(<u>C</u>H₃)₂), 14.8 (one <u>C</u>H₃ of CH(<u>C</u>H₃)₂); HRMS (ESI) *m*/*z* 290.1498 [M+H]⁺, calculated for C₁₅H₂₀N₃O₃, 290.1505.

4.1.3. General preparation of compounds 6a5-12, 6b10, 6b11, 2 and 3

To a stirred solution of intermediate **5** (349 mg, 1.0 mmol) and triethylamine (0.15 ml) in toluene, phenylacetaldehyde or aromatic aldehyde (1.0 mmol) was added. The reaction mixture was refluxed for 2h and then evaporated under reduced pressure to remove the volatile solvents. The obtained crude was dispersed by water (50 mL) and extracted with ethyl acetate (3×50 mL). The resulting organic phase was washed with water (3×50 mL) and brine (50 mL), dried over Na₂SO₄, filtered, and concentrated under vacuum to afford a yellow powder. The crude solid was purified by flash silica gel column chromatography (dichloromethane/ethyl acetate 10/0 to 10/1) to afford **6a5-12**, **6b10**, **6b11**, **2** and **3**.

4.1.3.1. (*3R*,7*aS*)-3-Benzyl-2-(4-nitrophenyl)hexahydro-1*H*-pyrrolo[1,2-*c*]imidazol-1one (6a5). 68% yield, white solid, m.p. 145.1-150.4 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.36-8.27 (m, 2H, Ar-*H*), 7.91-7.83 (m, 2H, Ar-*H*), 7.35-7.17 (m, 5H, Ar-*H*), 5.15 (t, *J* = 4.8 Hz, 1H, 3-*H*), 3.61 (dd, *J* = 9.2, 4.4 Hz, 1H, 7*a*-*H*), 3.32-3.25 (m, 1H, 5-*H*), 3.00 (d, *J* = 4.9 Hz, 2H, Ar-C<u>*H*</u>₂), 2.64-2.57 (m, 1H, 5-*H*), 2.20-2.11 (m, 1H, 7-*H*), 2.10-1.97 (m, 1H, 7-*H*), 1.92-1.75 (m, 2H, 6-*H*); ¹³C NMR (101 MHz, CDCl₃) δ 174.9 (*C*=O), 143.8 (Ar-*C*), 143.1 (Ar-*C*), 135.5 (Ar-*C*), 129.8 (Ar-*C*), 128.5 (Ar-*C*), 127.2 (Ar-*C*), 125.0 (Ar-*C*), 120.1 (Ar-*C*), 82.1 (3-*C*), 64.7 (7*a*-*C*), 56.3 (5-*C*), 39.9 (Ar-<u>*C*</u>H₂), 27.8 (7-*C*), 24.8 (6-*C*); HRMS (ESI) *m*/z 338.1499 [M+H]⁺, calculated for C₁₉H₂₀N₃O₃, 338.1505.

4.1.3.2. (3*R*,7*aS*)-3-(4-Nitrobenzyl)-2-(4-nitrophenyl)hexahydro-1*H*-pyrrolo[1,2-*c*]imidaz-ol-1-one (6a6). 64% yield, red brown solid, m.p. 159.7-166.4 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.29-8.18 (m, 2H, Ar-*H*), 8.00-7.93 (m, 2H, Ar-*H*), 7.92-7.84 (m, 2H, Ar-*H*), 7.69 (d, *J* = 13.8 Hz, 1H, one of Ar-C*H*₂), 7.34-7.24 (m, 2H, Ar-*H*), 5.21 (d, *J* = 13.2 Hz, 1H, one of Ar-C*H*₂), 4.40 (dd, *J* = 8.4, 3.2 Hz, 1H, 3-*H*), 3.54-3.43 (m, 1H, 7*a*-*H*), 3.39-3.34 (m, 1H, 5-*H*), 2.38-2.24 (m, 1H, 5-*H*), 2.08-1.92 (m, 4H, 7-*H* and 6-*H*); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 171.9 (*C*=O), 148.5 (Ar-*C*), 145.0 (Ar-*C*), 142.3 (Ar-*C*), 141.2 (Ar-*C*), 124.9 (Ar-*C*), 124.2 (Ar-*C*), 122.3 (Ar-*C*), 119.1 (Ar-*C*), 96.4 (3-*C*), 63.8 (7a-C), 48.7 (5-C), 40.1 (Ar-<u>C</u>H₂), 30.7 (7-C), 23.3 (6-C); HRMS (ESI) m/z 383.1354 $[M+H]^+$, calculated for C₁₉H₁₉N₄O₅, 383.1355.

4.1.3.3. (*3R*,7*aS*)-2-(4-Nitrophenyl)-3-phenylhexahydro-1*H*-pyrrolo[1,2-*c*]imidazol-1one (6a7). 50% yield, white solid, m.p. 126.7-131.9 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.19-8.13 (m, 2H, Ar-*H*), 7.77-7.70 (m, 2H, Ar-*H*), 7.44-7.33 (m, 3H, Ar-*H*), 7.32-7.27 (m, 2H, Ar-*H*), 5.80 (s, 1H, 3-*H*), 4.07 (t, *J* = 6.4 Hz, 1H, 7*a*-*H*), 3.61-3.49 (m, 1H, 5-*H*), 2.91 (m, 1H, 5-*H*), 2.30-2.20 (m, 2H, 7-*H*), 2.01-1.88 (m, 2H, 6-*H*); ¹³C NMR (101 MHz, CDCl₃) δ 175.6 (*C*=O), 143.6 (Ar-*C*), 143.4 (Ar-*C*), 137.5 (Ar-*C*), 129.4 (Ar-*C*), 129.1 (Ar-*C*), 125.8 (Ar-*C*), 124.8 (Ar-*C*), 119.5 (Ar-*C*), 82.9 (3-*C*), 64.1 (7a-*C*), 56.0 (5-*C*), 27.4 (7-*C*), 24.7 (6-*C*); HRMS (ESI) *m*/*z* 324.1344 [M+H]⁺, calculated for C₁₈H₁₈N₃O₃, 324.1348.

4.1.3.4. (3*R*,7*aS*)-3-(4-Isopropylphenyl)-2-(4-nitrophenyl)-hexa-hydro-1*H*-pyrrolo [1,2-*c*]imidazol-1-one (6a8). 75% yield, white solid, m.p. 100.7-105.3 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.18-8.15 (m, 2H, Ar-*H*), 7.75-7.73 (m, 2H, Ar-*H*), 7.24-7.21 (m, 4H, Ar-*H*), 5.81 (s, 1H, 3-*H*), 4.20-4.05 (m, 1H, 7*a*-*H*), 3.80-3,55 (m, 1H, C<u>*H*</u>(CH₃)₂), 2.92-2.86 (m, 2H, 5-H), 2.28-2.25 (m, 2H,7-H), 1.96 (s, 2H, 6-H), 1.21 (dd, *J* = 7.2, 2 Hz, 6H, CH(C<u>*H*</u>₃)₂); ¹³C NMR (101 MHz, CD₃OD) δ 177.1 (*C*=O), 151.1 (Ar-*C*), 145.2 (Ar-*C*), 144.5 (Ar-*C*), 137.4 (Ar-*C*), 128.2 (Ar-*C*), 127.5 (Ar-*C*), 125.5 (Ar-*C*), 121.7 (Ar-*C*), 83.8 (3-*C*), 65.41 (7a-*C*), 56.7 (5-*C*), 35.1 (<u>C</u>H(CH₃)₂), 28.5 (7-*C*), 25.6 (6-*C*), 24.3 (CH(<u>C</u>H₃)₂); HRMS (ESI) *m*/*z* 366.1811 [M+H]⁺, calculated for C₂₁H₂₄N₃O₃, 366.1818. **4.1.3.5.**

 $(3R,7\alpha S)$ -3-(4-Methoxyphenyl)-2-(4-nitrophenyl)hexa-hydro-1*H*-pyrrolo[1,2-*c*]

imidazol-1-one (**6a9**). 67% yield, white solid, m.p. 142.7-145.3 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.26-8.14 (m, 2H, Ar-*H*), 7.80-7.70 (m, 2H, Ar-*H*), 7.28-7.20 (m, 2H, Ar-*H*), 6.97-6.88 (m, 2H, Ar-*H*), 5.78 (s, 1H, 3-*H*), 4.14-4.03 (m, 1H, 7*a*-*H*), 3.81 (s, 3H, C<u>H₃</u>), 3.56-3.46 (m, 1H, 5-*H*), 2.91-2.85 (m, 1H, 5-*H*), 2.32-2.22 (m, 2H, 7-*H*), 1.96-1.91 (m, 2H, 6-*H*); ¹³C NMR (101 MHz, CDCl₃) δ 176.0 (*C*=O), 160.0 (Ar-*C*), 143.5 (Ar-*C*), 132.0 (Ar-*C*), 130.1 (Ar-*C*), 127.1 (Ar-*C*), 124.7 (Ar-*C*), 119.5 (Ar-*C*), 114.7 (Ar-*C*), 82.6 (3-*C*), 64.1 (7*a*-*C*), 55.8 (5-*C*), 55.4 (*C*H₃), 27.4 (7-*C*), 24.8 (6-*C*); HRMS (ESI) *m*/z 354.1450 [M+H]⁺, calculated for C₁₉H₂₀N₃O₄, 354.1454.

4.1.3.6. (3*R*,7*aS*)-2-(4-Nitrophenyl)-3-(4-(trifluoromethyl)-phenyl)-hexahydro-1*H*pyrrolo[1,2-*c*]-imidazol-1-one (6a10). 57% yield, white solid, m.p. 139.1-143.5 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.23-8.14 (m, 2H, Ar-*H*), 7.75-7.69 (m, 2H, Ar-*H*), 7.65 (d, *J* = 8.2 Hz, 2H, Ar-*H*), 7.42 (d, *J* = 8.4 Hz, 2H, Ar-*H*), 5.85 (s, 1H, 3-*H*), 3.99 (dd, *J* = 6.0, 7.6 Hz, 1H, 7*a*-*H*), 3.55-3.50 (m, 5-*H*), 2.92-2.86 (m, 1H, 5-*H*), 2.28-2.19 (m, 2H, 7-*H*), 1.99-1.87 (m, 2H, 6-*H*); ¹³C NMR (101 MHz, CDCl₃) δ 175.8 (*C*=O), 143.8 (Ar-*C*), 143.1 (Ar-*C*), 142.2 (Ar-*C*), 131.3 (q, 131.7⁹, 131.4⁶, 131.1⁴, 130.8¹, *J* = 33 Hz, 4"-*C*), 126.3⁹ (126.4⁴, 126.4¹, 126.3⁷, the last peak overlapped with the peak at 126.3 ppm, *J* = 3 Hz, 3"-*C*), 126.3 (Ar-*C*), 126.4 (Ar-*C*), 123.7 (q, 127.7⁷, 125.0⁵, 122.3⁵, 119.6³, J = 272 Hz, *C*F₃), 119.4 (Ar-*C*), 82.2 (3-*C*), 64.1 (7*a*-*C*), 56.1 (5-*C*), 27.5 (7-*C*), 24.8 (6-*C*); HRMS (ESI) *m*/*z* 392.1217 [M+H]⁺, calculated for C₁₉H₁₇F₃N₃O₃, 392.1222.

4.1.3.7. (3*S*,7*aS*)-2-(4-Nitrophenyl)-3-(4-(trifluoromethyl)-phenyl)hexahydro-1*H*pyrrolo[1,2-*c*]-imidazol-1-one (6b10). 12% yield, white solid, m.p. 82.1-83.5 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.18-8.12 (m, 2H, Ar-*H*), 7.62 (d, *J* = 8.4 Hz, 2H, Ar-*H*), 7.46-7.35 (m, 4H, Ar-*H*), 6.48 (s, 1H, 3-*H*), 4.20-4.05 (m, 1H, 7*a*-*H*), 2.53-2.20 (m, 4H, 5-H and 7-*H*), 1.85-1.70 (m, 2H, 6-*H*); ¹³C NMR (101 MHz, CDCl₃) δ 175.9 (*C*=O), 143.9 (Ar-*C*), 143.2 (Ar-*C*), 142.2 (Ar-*C*), 131.3 (q, 131.8², 131.5¹, 131.1⁷, 130.8⁶, *J* = 31 Hz, 4"-*C*), 126.4⁴ (126.4⁹, 126.4⁶, 126.4¹, the last peak overlapped with the peak at 126.4 ppm, *J* = 3 Hz, 3"-*C*), 126.5 (Ar-*C*), 126.4 (Ar-*C*), 123.7 (q, 127.7⁸, 125.0⁹, 122.3⁹, 119.6⁸, *J* = 271 Hz, *C*F₃),119.7 (Ar-*C*), 82.2 (3-*C*), 64.1 (7*a*-*C*), 56.1 (5-*C*), 27.5 (7-*C*), 24.9 (6-*C*); HRMS (ESI) *m*/z 392.1218 [M+H]⁺, calculated for C₁₉H₁₇F₃N₃O₃, 392.1222.

4.1.3.8. (*3R*,7*aS*)-3-(4-Chlorophenyl)-2-(4-nitrophenyl)hexahydro-1*H*-pyrrolo[1,2-*c*]imidazol-1-one (6a11). 63% yield, white solid, m.p. 146.0-151.8 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.24-8.15 (m, 2H, Ar-*H*), 7.78-7.69 (m, 2H, Ar-*H*), 7.42-7.34 (m, 2H, Ar-*H*), 7.30-7.20 (m, 2H, Ar-*H*), 5.76 (s, 1H, 3-*H*), 3.98 (dd, *J* = 13.2, 5.4 Hz, 1H, 7*a*-*H*), 3.55-3.45 (m, 1H, 5-*H*), 2.84 (m, 1H, 5-*H*), 2.34-2.18 (m, 2H, 7-*H*), 2.01-1.87 (m, 2H, 6-*H*); ¹³C NMR (101 MHz, CDCl₃) δ 175.9 (*C*=O), 143.7 (Ar-*C*), 143.2 (Ar-*C*), 136.7 (Ar-*C*), 134.9 (Ar-*C*), 129.5 (Ar-*C*), 127.3 (Ar-*C*), 124.8 (Ar-*C*), 119.5 (Ar-*C*), 82.2 (3-*C*), 64.1 (7*a*-*C*), 55.9 (5-*C*), 27.4 (7-*C*), 24.7 (6-*C*); HRMS (ESI) *m*/*z* 358.0953 [M+H]⁺, calculated for C₁₈H₁₇ClN₃O₃, 358.0958.

4.1.3.9. (3*S*,7*aS*)-3-(4-Chlorophenyl)-2-(4-nitrophenyl)hexa-hydro-1*H*-pyrrolo[1,2-*c*] imidazol-1-one (6b11). 15% yield, white solid, m.p. 80.3-91.5 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.18-8.11 (m, 2H, Ar-*H*), 7.46-7.39 (m, 2H, Ar-*H*), 7.36-7.29 (m, 2H, Ar-*H*), 7.19 (d, *J* = 7.9 Hz, 2H, Ar-*H*), 6.40 (s, 1H, 3-*H*), 4.20-3.98 (m, 1H, 7*a*-*H*), 2.54-2.18 (m, 4H, 5-*H* and 7-*H*), 1.85-1.70 (m, 2H, 6-H); ¹³C NMR (101 MHz, CDCl₃) δ 176.0 (*C*=O), 143.6 (Ar-C), 143.2 (Ar-C), 136.7 (Ar-C), 134.9 (Ar-C), 129.5 (Ar-C), 127.3 (Ar-C), 124.8 (Ar-C), 119.4 (Ar-C), 82.2 (3-C), 64.1 (7*a*-C), 55.9 (5-C), 27.4 (7-C), 24.8 (6-C); HRMS (ESI) *m/z* 358.0954 [M+H]⁺, calculated for C₁₈H₁₇ClN₃O₃, 358.0958.

4.1.3.10. (*3R*, *7aS*)-2, 3-bis(4-Nitrophenyl)hexahydro-1*H*-pyrrolo-[1,2-*c*]imidazol-1-one (2). 60% yield, white solid, m.p. 125.3-142.1 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.25 (d, *J* = 8.8 Hz, 2H, Ar-*H*), 8.22-8.16 (m, 2H, Ar-*H*), 7.76-7.65 (m, 2H, Ar-*H*), 7.49 (d, *J* = 8.8 Hz, 2H, Ar-*H*), 5.90 (s, 1H, 3-*H*), 4.01 (dd, *J* = 7.8, 5.2 Hz, 1H, 7*a*-*H*), 3.56 (m, 1H, 5-*H*), 2.93 (m, 1H, 5-*H*), 2.33-2.19 (m, 2H, 7-*H*), 2.02-1.89 (m, 2H, 6-*H*); ¹³C NMR (101 MHz, CDCl₃) δ 175.3 (*C*=O), 148.3 (Ar-*C*), 144.9 (Ar-*C*), 144.0 (Ar-*C*), 142.8 (Ar-*C*), 127.1 (Ar-*C*), 125.0 (Ar-*C*), 124.6 (Ar-*C*), 119.5 (Ar-*C*), 81.8 (3-*C*), 64.1 (7*a*-*C*), 56.2 (5-*C*), 27.5 (7-*C*), 24.9 (6-*C*); HRMS (ESI) m/z 369.1194 [M+H]⁺, calculated for C₁₈H₁₇N₄O₅, 369.1199.

4.1.3.11. (3*S*,7*aS*)-2,3-bis(4-Nitrophenyl)hexahydro-1*H*-pyrrolo-[1,2-*c*]imidazol-1-one (3). 15% yield, white solid, m.p. 65.0-70.1 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.25-8.20 (m, 2H, Ar-*H*), 8.19-8.13 (m, 2H, Ar-*H*), 7.48 (d, *J* = 7.7 Hz, 2H, Ar-*H*), 7.45-7.39 (m, 2H, Ar-*H*), 6.55 (s, 1H, 3-*H*), 4.28-4.05 (m, 1H, 7*a*-*H*), 2.54-2.19 (m, 4H 5-*H* and 7-*H*), 1.86 (s, 2H, 6-*H*); ¹³C NMR (101 MHz, CDCl₃) δ 175.4 (*C*=O), 148.2 (Ar-*C*), 144.9 (Ar-*C*), 144.0 (Ar-*C*), 142.8 (Ar-*C*), 127.1 (Ar-*C*), 125.0 (Ar-*C*), 124.6 (Ar-*C*), 119.4 (Ar-*C*), 81.8 (3-*C*), 64.1 (7*a*-*C*), 56.2 (5-*C*), 27.5 (7-*C*), 24.9 (6-*C*); HRMS (ESI) *m*/*z* 369.1198 [M+H]⁺, calculated for C₁₈H₁₇N₄O₅, 369.1199.

4.1.3.12. (*3R*,7*aS*)-3-(3-Nitrophenyl)-2-(4-nitrophenyl)hexahydro-1*H*-pyrrolo[1,2-*c*] imidazol-1-one (6a12). 55% yield, white solid, m.p. 109.9-126.1 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.35-8.30 (m, 1H, Ar-*H*), 8.26-8.24 (m, 1H, Ar-*H*), 8.22-8.18 (m, 2H, Ar-*H*), 7.79-7.70 (m, 2H, Ar-*H*), 7.66-7.57 (m, 2H, Ar-*H*), 6.01 (s, 1H, 3-*H*), 4.15 (m, 1H, 7*a*-*H*), 3.71 (m, 1H, 5-*H*), 3.05 (m, 1H, 5-*H*), 2.41-2.29 (m, 2H, 7-*H*), 2.12-1.98 (m, 2H, 6-*H*); ¹³C NMR (101 MHz, CDCl₃) δ 175.5 (*C*=O), 148.8 (Ar-*C*), 144.0 (Ar-*C*), 142.9 (Ar-*C*), 140.7 (Ar-*C*), 131.5 (Ar-*C*), 130.4 (Ar-*C*), 125.0 (Ar-*C*), 124.0 (Ar-*C*), 121.6 (Ar-*C*), 119.7 (Ar-*C*), 81.8 (3-*C*), 64.1 (7*a*-*C*), 56.1 (5-*C*), 27.5 (7-*C*), 24.9 (6-*C*); HRMS (ESI) *m*/*z* 369.1195 [M+H]⁺, calculated for C₁₈H₁₇N₄O₅, 365.1199.

4.2. General information for bioassay

All compounds were dissolved in DMSO and diluted into gradient stock solution before use, and the final concentration of DMSO in every well including control well was 0.5%.

4.2.1. Inhibitory activity assay against DENV-2 NS2B-NS3 protease

DENV-2 NS2B-NS3 protease was expressed with the plasmid kindly provided by Dr. Siew Phengsuozeg Lim, Novartis Institute for Tropical Diseases, Singapore [17]. The assay was conducted as our previous report [10,15]. Briefly, the inhibitory activity of a compound against DENV-2 NS2B-NS3 protease were measured at 37 °C in the reaction buffer (50 mM Tris–HCl, pH 9.0, 10 mM NaCl, 20% glycerol, 1 mM CHAPS, and 0.04% NaN₃) and Benzoyl-Nle-Lys-Arg-Arg-AMC (33 μ M) was used as a substrate. The protease was incubated with different concentrations of an inhibitor at 37 °C for 3 min before the substrate (33 μ M) was added. The remaining enzyme activity was measured with a fluorescence spectrophotometer using 356 and 438 nm as the excitation and emission wavelengths, respectively. Vehicle control was normalized as 100% remaining NS3 enzymatic activity and active compound **1** reported in our previous work¹⁰ was used as a reference (Table 1, entry 17). IC₅₀ value of an inhibitor was calculated based on the relationship of its remaining NS3 enzymatic activity vs its corresponding concentration. The assay data were averaged from triplicate measurements and the results are shown in Table 1, Table 2 and Fig 3.

4.2.2. Cytotoxicity assay for compounds 1 and 2 to Huh-7 cells

All cells were cultivated in Dulbecco's Modified Eagle Medium(DMEM) supplemented with 10 % fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin at 37 °C, 5 % CO₂ and 95 % relative humidity. For assessing cytotoxicity, Huh-7 cells were seeded into 96-well plates with a density of 10⁴ cells per well in presence of increasing concentrations of the tested compounds. After overnight incubation, medium was removed and 50 μ l of fresh DMEM was added to the cells. Cell viability was determined using Cell-Titer Glo Luminescent Viability Assay (Promega) by recording luminescence for 2 s with a FluoStar Omega microplate luminometer (BMG Labtech). All concentrations were measured in triplicates and compared to an untreated control.

4.2.3. Wild-type DENV-2 virus plaque assay for compounds 1 and 2

Antiviral activity of compounds **1** and **2** were determined by plaque assay. First, Huh-7 cells were seeded into 96-well plates with a density of 10^4 cells per well and infected with wild type DENV serotype 2 with a multiplicity of infection (MOI) of 1. After one hour, a gradient of non-toxic concentrations of the compounds were added to the infected cells and plates were incubated for 48 h at 37 °C. Each concentration was assayed in triplicates. Following this, the supernatant was harvested and used for determination of the virus yield reduction by plaque assay using Vero E6 cells. Therefore Vero E6 cells were seeded in 24-well plates with a density of 2.5 x 10^5 cells per well and incubated overnight at 37 °C. The virus containing medium was diluted with DMEM ranging from 10^{-1} to 10^{-6} . Afterwards 100 µl of these dilutions were used to infect the Vero E6 cells with agitation for 1 h. Then, the medium was removed and 1 ml of plaque medium was added. After further incubation for 7 days at 37 °C the cells were fixed with 5 % (v/v) formaldehyde for 2 h, stained with crystal violet and plaques were counted. Reduction of the viral titer was monitored by comparison to an untreated control.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <u>http://dx.doi.org/</u>. These data include 2D NOESY spectra of compounds **2** and **6a5**, ¹H NMR and ¹³C NMR spectra of new compounds, MOL file of reported compound of **1** and new compound **2** described in this article.

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Fig. 1 The chart of NS2B-NS3 protease inhibitor design



Fig. 2 Crystal structure diagrams of **6a11** (a) and **2** (b). The crystal structures of **6a11** and **2** were deposited at the Cambridge Crystallographic Data Centre (CCDC) with deposition numbers1474483 and1474481, respectively.



Fig. 3 Relationships between the substituents (R) in 3-position of compounds 6a1 to 6a4 and their inhibitory activity at the dengue NS2B-NS3 protease.

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Fig. 4 Three-dimensional (3D) diagrams of putative binding poses of 1 (a), 2 (b) and 3 (c) in the NS2B-NS3 active site (His51, Asp75 and Ser135). The figures were generated from chain A of 2FOM [11] from the same view. Hydrogen bonds are indicated by green dashed lines and critical amino acid residues of the binding pocket are labelled. The docking conformation (d) of 2 was extracted from (b) and the crystal conformation (e) of 2 was produced from Fig. 2b.



Reagents and conditions: (a) DCM, DCC, rt, 5h. (b) DCM, TFA, 0 °C, 2h. (c) aliphatic aldehydes (including phenylacetaldehydes) or aromatic aldehydes, TEA, reflux in EtOH or toluene, 30min.

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	cmpd No.	R (α or β)	Chemical			
entry			3-Н	7а-Н	Yield (%) ^b	Activity to NS3 ^{c,d}
1	6a1	Н	NA	3.96 (dd, <i>J</i> = 4.0, 8.8 Hz)	60	61% (16)
2	6a2	Me (a)	5.06 (q, J = 8.0 Hz)	4.06 (dd, <i>J</i> = 4.0, 12.0 Hz)	74	58% (16)
3	6a3	Et (α)	4.80 (dd, <i>J</i> = 4.0, 8.0 Hz)	4.01 (dd, <i>J</i> = 4.0, 8.0 Hz)	70	40% (16)
4	6a4	<i>i</i> -Pr (α)	4.77 (d, <i>J</i> = 4.0 Hz)	3.98 (dd, <i>J</i> = 4.8, 5.0 Hz)	66	66% (16)
5	6a5	Bn (α)	5.15 (t, <i>J</i> = 4.8 Hz)	3.61 (dd, <i>J</i> = 4.4, 9.2 Hz)	68	58% (20)
6	6a6	p -NO ₂ -Bn (α)	4.40 (dd, <i>J</i> = 3.2, 8.4 Hz)	3.43-3.54 (m)	64	1
7	6a7	Ph (α)	5.80 (s)	4.07 (t, $J = 6.4$ Hz)	50	33% (200)
8	6a8	p -(i -Pr)-Ph (α)	5.81 (s)	4.05-4.20 (m)	75	/
9	6a9	p -OMe-Ph (α)	5.78 (s)	4.03 -4.14 (m)	67	/
10	6a10	p -CF ₃ -Ph (α)	5.85 (s)	3.99 (dd, <i>J</i> = 6.0, 7.6 Hz)	57	/
11	6b10	p -CF ₃ -Ph (β)	6.48 (s)	4.05-4.20 (m)	12	/
12	6a11	p -Cl-Ph (α)	5.76 (s)	3.98 (dd, <i>J</i> = 5.4, 13.2 Hz)	63	/
13	6b11	p -Cl-Ph (β)	6.40 (s)	3.98-4.20 (m)	15	/
14	2	p -NO ₂ -Ph (α)	5.90 (s)	4.01 (dd, <i>J</i> = 5.2, 7.8 Hz)	60	$IC_{50} = 1.2 \pm 0.4$
15	3	p -NO ₂ -Ph (β)	6.55 (s)	4.05-4.28 (m)	15	/
16	6a12	m -NO ₂ -Ph (α)	6.01 (s)	4.15 (t, $J = 8.0$ Hz)	55	/
17	1		NA	f		$IC_{50} = 1.2 \pm 0.4$

Table 1 Reaction results and the inhibitory activity to NS2B-NS3 protease

^a NMR experiments were conducted in CDCl₃ except entry 6 (**6a6** in DMSO-d6) due to insolubility of **6a6** in chloroform. ^b Isolated yield. ^c Percent residual activity of DENV-2 NS2B-NS3 (inhibitor concentration, μ M) or IC₅₀ (μ M). ^d "/" means that there is higher than 50% remaining activity of DENV-2 NS2B-NS3 up to 200 μ M of inhibitor. ^f Not applicable.

		Line	ar dipeptides	Fused	ring derivatives
para	meters		N-Y-X-X NH ₂ -X S-		
х	Y	cmpd ^a	activity to NS3 ^b	cmpd ^c	activity to NS3 ^b
NO_2	empty	1 (1)	31% (15)	2	32% (15)
Н	empty	(12d)	inactive	6a7	33% (200)
NO_2	CH ₂	(12e)	36% (200)	6a6	inactive
Н	CH_2	(12f)	58% (15)	6a5	58% (20)

Table 2 SARs determined by substituent (X) and linker (Y) for two series of NS2B-BS3 inhibitors

^a Compounds and their inhibitory activity, compound identifiers in parentheses from our previous report [10]. ^b Percent residual activity of DENV-2 NS2B-NS3 (inhibitor concentration, μ M), and "inactive" means that there is higher than 50% remaining activity of DENV-2 NS2B-NS3 at inhibitor concentrations up to 200 μ M. ^c From Table 1.

ntry	cmpd No.	$EC_{50}\left(\mu M\right)$ against wild-type DENV-2 virus	$CC_{50}(\mu M)$ in Huh-7 cells
	1	38.7 ± 5.4	> 100
2	2	39.4 ± 6.2	> 100
			$ \rightarrow $
		Q '	
	- X		

Hightlights:

Fused compounds of pyrrole and imidazole were synthesized and studied against NS2B-NS3 protease.

Fused compound **2** and linear compound **1** show activity against DENV-2 virus and NS2B-NS3 protease.

Substituent and its stereochemistry at C-3 position are important for the inhibitory activity.

Both of substitution (X) and a linker (Y) affect the inhibitory activity.

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