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Novel inhibitors of hepatitis C NS3–NS4A serine protease derived from 2-aza-bicyclo[2.2.1]heptane-3-carboxylic acid

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Abstract—Prolonged hepatitis C infection is the leading cause for cirrhosis of the liver and hepatocellular carcinoma. The etiological agent HCV virus codes a single polyprotein of ~3000 amino acids that is processed with the help of a serine protease NS3A to produce structural and non-structural proteins required for viral replication. Inhibition of NS3 protease can potentially be used to develop drugs for treatment of HCV infections. Herein, we report the development of a series of novel NS3 serine protease inhibitors derived from 2-aza-bicyclo[2.2.1]-heptane carboxylic acid with potential therapeutic use for treatment of HCV infections.

Hepatitis C virus is the primary etiological agent responsible for chronic HCV infections of liver leading to cirrhosis and hepatocellular carcinoma. An estimated 170 million people worldwide are infected with HCV and up to 80% of these infections turn chronic.[1] Current therapy includes combination of α-interferon and ribavirin, which is effective only in 25–40% of patients for sustained response.[2] Introduction of pegylated interferon has reduced the frequency of injections enabling lower viral titers and improved histological growth particularly in relapse patients after interferon therapy.[2] Lack of efficient therapies necessitates discovery of new agents for treatment of chronic hepatitis C.

HCV, a Flaviviridae family virus, encodes a polyprotein of about 3000 amino acids from a positive strand RNA genome. This polyprotein is post translationally spliced to produce the various proteins essential for viral replication.[3] Autocatalytic cleavage at the NS2-NS3 junction followed by cleavage of NS3–NS4A, NS4A–NS4B, NS4B–NS5A, and NS5A–NS5B by the NS3 serine protease produces functionally mature virions. The inhibition of this pivotal protease would possibly impair the ability of the enzyme to process the polyprotein arresting maturation of virus and viral reproduction.[4]

HCV NS3 protease is a serine protease; with the assistance of co-factor NS4A catalyzes the hydrolysis of a cysteine serine amide bond. The hydrolysis proceeds via attack of Ser-139 followed by stabilization of tetrahedral intermediate in oxy-anion hole. Most potent inhibitors reported to date contain either a 4-substituted proline or a 3,4-disubstituted proline as P_2 amino acid residue.[5] In an attempt to depeptidize these inhibitors we decided to explore bicyclic amino acid 2-aza-bicyclo[2.2.1]-heptane carboxylic acid **4** as a P_2 surrogate. This amino acid could be visualized either as a *trans*-3,5-disubstituted proline or as a conformationally rigid pipecolinic acid derivative that presented the piperidine ring in a rigid boat form enabling maximum contact with the surface of the enzyme. Our previous studies with pipecolinic acid as the P_2 amino acid had produced inhibitors less potent than the inhibitors derived from proline.

Synthesis of amino acid 2-aza-bicyclo[2.2.1]-heptane carboxylic acid was accomplished following the procedure of Stella et al. as outlined in Scheme 1.[6] Thus, condensation of ethylglyoxalate with (*R*)-phenethylamine in refluxing benzene yielded chiral imine **2** that underwent hetero-Diels–Alder reaction with cyclopentadiene to yield aza-bicyclo[2.2.1]heptene derivative **3** as a mixture of diastereomers that were easily separable by chromatography. Hydrogenation of the double bond with concomitant removal of *N*-methylbenzyl group yielded the amino ester **4** that was coupled with Boc-*tert*-butylgly-

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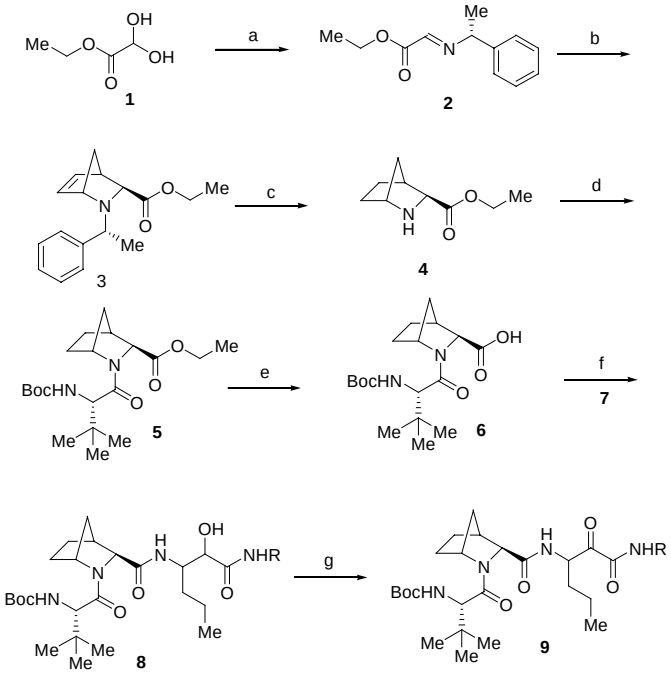


Scheme 1. Reagents and conditions: (a) (*R*)-phenethylamine, benzene, PPTS, reflux, 2 h; (b) cyclopentadiene, $BF_3{\cdot}O(C_2H_5)_2$, TFA, −78 °C; (c) H_2/Pd/C, CH_3OH, 3 h; (d) Boc-*tert*-butylglycine EDCI·HCl, HOOBt, NMM, CH_2Cl_2/DMF (1:1) 0 °C, 12 h; (e) LiOH·H_2O, H_2O/THF, rt; (f) NH_2-$CH(C_3H_7)$-CH(OH)-CONHR·HCl, (**7**) EDCI·HCl, HOOBt, NMM, CH_2Cl_2/DMF (1:1) 0 °C, 12 h; (g) Dess–Martin reagent, CH_2Cl_2, rt, 1 h (when R ≠ H) or EDCI·HCl, $Cl_2CHCOOH$, DMSO/toluene (when R = H).

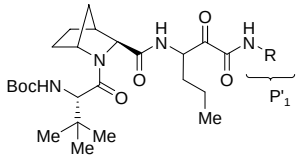
cine using standard peptide coupling conditions with EDCI and HOOBt to yield dipeptide **5** in 60% yield. Hydrolysis of ethyl ester **5** with aq LiOH resulted in acid **6**, which was coupled with norvaline segment **7**[7] using EDCI and HOOBt. The resulting hydroxy amides of type **8** were oxidized with either Dess–Martin reagent[8] or Moffat conditions to yield the ketoamide inhibitors of type **9**. The inhibitors synthesized were evaluated for their ability to inhibit the hydrolysis of chromogenic 4-phenylazophenyl (PAP) ester from the peptide fragment Ac-DTEDVVP(Nva)-*O*-4-PAP in a HCV protease continuous assay.[9] The dependence of inhibitory activity of the P′ residue is summarized in Table 1.

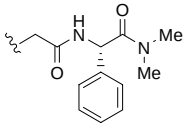
Replacement of the P'_1 hydrogen in **10** with allyl group resulted in compound **11** with a minimal improvement in binding from $K_i^* = 6.9$ to 5.5 μM. Incorporation of glycine benzyl ester at P'_1 gave compound **12** ($K_i^* = 1.5$ μM), which was 4-fold more potent than **10**. Hydrogenation of **12** gave glycine acid **13**, which was slightly more potent than **10** with a $K_i^* = 3.8$ μM.

To further improve the potency of our inhibitor, we decided to incorporate phenylglycine dimethylamide as a P'_2 residue, since it had been previously shown to be an excellent P'_2 group.[10] Functionalization of **9** with glycine-phenylglycine segment as the P'_1–P'_2 residues resulted in compound **14** with $K_i^* = 0.11$ μM, a 14-fold improvement in activity over inhibitor **12**.

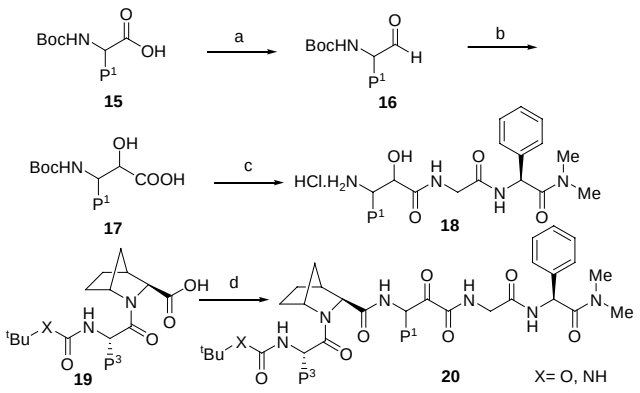
Having established inhibitors of type **14** containing glycine phenylglycine derivative gave potent inhibitors against HCV NS3 protease, we decided to modify the P_1 and P_3 residues to improve potency. The syntheses

Table 1.



Compound	R	K_i^* (μM)
10	H	6.9
11		5.5
12		1.5
13		3.8
14		0.11

of these inhibitors are outlined in Scheme 2. Thus, Boc-protected P_1 amino acids **15** were converted to their corresponding Weinreb amides and reduced to corresponding aldehydes **16** using $LiAlH_4$. These aldehydes were converted to hydroxy acids **17** by treatment with acetone cyanohydrin followed by acid hydrolysis. Coupling of hydroxy acid with glycine-phenylglycine dimethylamide yielded P_1–P'_2 segment **18**, which was coupled with P_2 acid **19** and oxidized using Dess–Martin reagent to yield inhibitors **22**–**39**. The effect of variation of P_1 and P_3 groups is shown in Table 2.

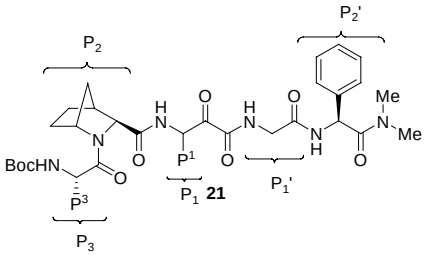


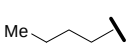
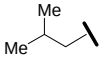
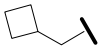
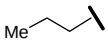
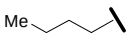
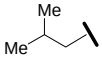
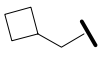
Scheme 2. Reagents and conditions: (a) i. EDCI·HCl, $CH_3ON(CH_3)$-H·HCl, HOOBt, NMM, CH_2Cl_2, rt, 12 h; ii. $LiAlH_4$, Et_2O, 0 °C to rt, 1 h; (b) i. $(CH_3)_2C(OH)CN$, Et_3N, CH_2Cl_2, rt; ii. 6 M methanolic HCl reflux; iii. aq NaOH, THF, Boc_2O; iv. aq LiOH, THF rt; (c) i. EDCI, HOOBt, NMM, HCl·H_2N-Gly-Phg-$CON(Me)_2$, DMF/CH_2Cl_2, rt; ii.4 M HCl/dioxane; (d) i. **18**, EDCI, HOOBt, NMM, DMF/CH_2Cl_2, rt; ii. Dess–Martin periodinane, CH_2Cl_2 rt, 1 h.

As shown in Table 2 changing the P_1 residue from norvaline to norleucine yielded **22** which marginally improved the binding from $K_i^* = 0.11$ to 0.08 μM. Incorporation of a β-branched amino acid leucine yielded **23** with further improvement in $K_i^* = 0.040$ μM. This enhancement in binding was encouraging and prompted us to investigate cyclopropylalanine and cyclobutylalanine at P_1 position. The binding of cyclopropylalanine derived inhibitor **24** and that of cyclobutylalanine derived inhibitor **25** were superior to that of norvaline, providing compounds with $K_i^* = 0.019$ and 0.027 μM, respectively, a 4- to 5-fold improvement in potency in comparison to **14**. The poor binding of cyclobutylalanine compared to that of cyclopropylalanine suggested that the S_1 pocket was shallow and tolerated small changes in sterics. Having established cyclopropylalanine as the optimal P_1 residue, we explored the effect of varying the P_3 amino acid to cyclohexylglycine (inhibitors **26**–**30**) from *tert*-butylglycine. As shown in Table 2, this provided little improvement in binding activity. In P_3-cyclohexylglycine-derived series norleucine-derived inhibitor **27** was more potent than cyclobutylalanine derived inhibitor **30** in contrast to P_3 *tert*-butylglycine series. Once again incorporation of cyclopropylalanine at P_1 provided an inhibitor with activity better than that of noravline or norleucine; thus, compound **29** with $K_i^* = 0.020$ μM was more potent than compound **26** with a $K_i^* = 0.090$ μM.

To establish the mode of binding of these inhibitors, X-ray crystal structure of inhibitor **27** bound to NS3 protease was solved.[11] From Figure 1 it is clear that the P_2 bicyclic amino acid binds to the enzyme with the concave face of piperidine making surface contact to the enzyme and the bridging methylene of bicyclic amino acid is solvent exposed. The P_1'–P_2' glycine-phenylglycine segment forms a 'C-clamp' around lysine-136 restricting its mobility. The carbonyl oxygen of phenylglycine dimethylamide forms a hydrogen bond with Thr-42 of protein backbone and aryl group of phenylglycine forms hydrophobic interactions with alkyl chain of lysine-136. The ketoamide moiety makes covalent bond with serine-139 with amide carbonyl occupying oxy-anion hole. The concave portion of the bicyclic P_2 overlaps with alanine-156 further enhancing binding.

Table 2.



Compound	P^1	P^3	K_i^* (μM)
22			0.081
23			0.040
24			0.019
25			0.027
26			0.090
27			0.041
28			0.053
29			0.020
30			0.051

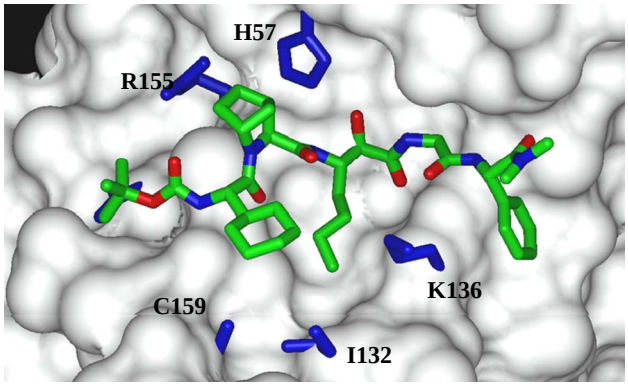


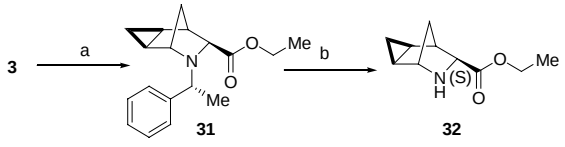
Figure 1. X-ray structure of inhibitor 27 bound to NS3 protease.

Analysis of the X-ray of inhibitor **27** bound to the enzyme suggested that functionalization at 4 or 5 position of the bicyclic ring with aliphatic groups could potentially make additional contact with Arg-155 and Ala-156 with a possibility of improving potency. To evaluate the effect of substitution at 4 and 5 positions cyclopropanation of **3** was explored. Intermediate **3** obtained from the cycloaddition of ethylglyoxalate and imine **2** was cyclopropanated using $Pd(OAc)_2$ and diazomethane to form the amino ester **32** (Scheme 3). The cyclopropyl group was exclusively delivered from the less hindered *exo*-face.

This tricyclic amino ester **32** was incorporated into the ketoamide inhibitors using a similar procedure as outlined in Scheme 2 to provide inhibitors **33** and **34** (Fig. 2).

As it can be seen from Figure 2 incorporation of cyclopropyl ring on the aza-bicyclo[2.2.1]heptane ring provided compounds **33** and **34** with HCV protease activity of $K_i^* = 0.090$ and 0.027 μM, respectively. This was a marginal improvement in potency in comparison to compounds **26** and **27**. Analysis of X-ray structure of these inhibitors bound to NS3 protease clearly showed that derivatization of the bicyclic ring system from the *exo*-face placed the cyclopropyl functionality away from the protein into the solvent, thus rendering minimal overlap to the surface of the enzyme. The effect of this interaction was small and made a low contribution to the binding that is clearly reflected in the case of the inhibitors **33** and **34**. Since the *exo*-side was the sterically more accessible face most functionalizations of the double bond would form *exo*-product providing small improvement in potency.

Further efforts were focused on modification of capping functionality on our most potent inhibitors. Replacement of *tert*-butyl carbamate to *tert*-butyl urea, an isosteric replacement that changes hydrogen bond acceptor oxygen to hydrogen bond donor nitrogen lead to inhibitors **36**–**39** (Table 3).



Scheme 3. Reagents and conditions: (a) CH_2N_2, $Pd(OAc)_2$, ether, 0 °C to rt; (b) H_2/Pd/C, CH_3OH, 3 h.

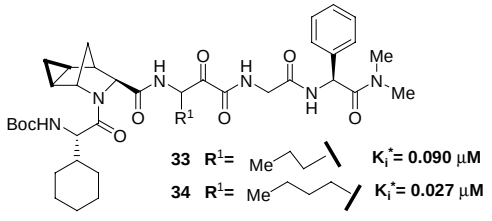
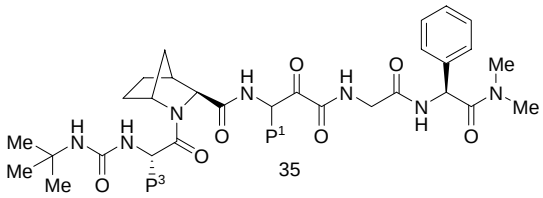


Figure 2.

Table 3.



Compound	P^1	P^3	K_i^* (μM)
36			0.019
37			0.010
38			0.021
39			0.013

From the results indicated in Table 3 it was clear that the replacement of the Boc group with a urea enhanced the binding affinity by 2- to 3-fold. Inhibitor **37** containing cyclopropylalanine at P_1 had $K_i^* = 0.010$ μM and inhibitor **39** with cyclobutylalanine at P_1 and *tert*-butylglycine at P_3 had a $K_i^* = 0.013$ μM. Both these inhibitors were 10-fold better than **14**.

The selectivity of inhibitor **37** and **39** to HCV protease was evaluated by comparing its binding affinity to a structurally homologous serine protease, human neutrophil elastase (HNE). Inhibitor **37** had a HNE activity of $K_i^* = 340$ nM with a HNE/HCV selectivity of 35 and inhibitor **39** had a HNE activity of $K_i^* = 5300$ nM with a HNE/HCV selectivity of 420. In a replicon-based cellular assay, inhibitor **27** demonstrated a $EC_{50} = 0.6$ μM and inhibitor **39** had a $EC_{50} = 2.0$ μM.

In conclusion, we have effected the development of potent HCV protease inhibitors with non-proline P_2 amino acid obtained from a 2-aza-bicyclo[2.2.1]-heptane carboxylic acid. The binding affinity of these inhibitors was optimized from high micromolar to low double digit nanomolar potencies. The constriction of pipecolinic acid with a bridged methylene group constrains the piperidine ring in a six-membered boat conformation enabling it to overlap effectively with the enzyme. Further modifications and evaluation of DMPK properties of inhibitors **36**–**39** are in progress.

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11. Crystallographic data for the inhibitor 27-HCV NS3 protease complex shown in Figure 1 have been deposited with the RCSB (code rcsb035631) Protein Data Bank as PDB ID 2F9U. The structural details can be viewed at www.rscb.org using the ID number above.