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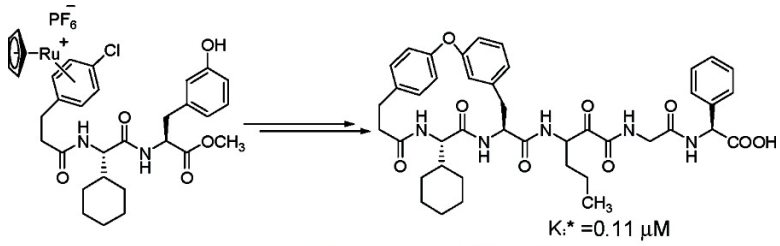

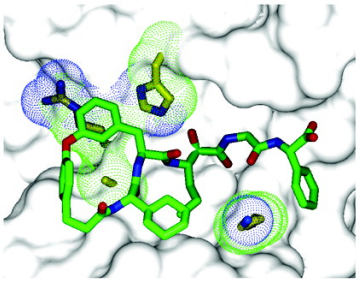

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Design and Synthesis of Depeptidized Macrocyclic Inhibitors of Hepatitis C NS3-4A Protease Using Structure-Based Drug Design

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Abstract: Hepatitus C virus (HCV) NS3, when bound to NS-4A cofactor, facilitates development of mature virons by catalyzing cleavage of a polyprotein to form functional and structural proteins of HCV. The enzyme has a shallow binding pocket at the catalytic site, making development of inhibitors difficult. We have designed, preorganized, and depeptidized macrocyclic inhibitors from P_4 to P_2' and optimized binding to 0.1 μM. The structure of an inhibitor bound to the enzyme was also solved.

Chronic hepatitis C virus (HCV) infections are the leading cause of liver cirrhosis and hepatocellular carcinoma.[1] An estimated 170 million people worldwide are infected with HCV, making it a major public health concern. More than 80% of HCV infections turn chronic, of which 20% develop hepatocellular carcinoma, making it one of the leading reasons for liver transplantions. In the U.S., ∼72% of the patients are infected with genotype 1, a more resistant strain for treatment using existing therapy that includes three or four injections of α-interferon (IFN) per week and combination therapy of IFN with ribavirin.[2] Although approximately 30−50% of infected patients initially respond to IFN monotherapy, only 8−20% have sustained response. Administration of a combination of ribavirin and interferon has increased the sustained response rate to 14−40% particularly in patients who relapse after IFN therapy. Introduction of pegylated interferon has reduced the frequency of injection to once a week with a better pharmacokinetics profile while providing sustained response to 50%. Patients administered with a combination of ribavirin and pegylated interferon have markedly lower viral titers and enhanced histological growth. The use of pegylated interferon has a very high response rate of up to 80% in patients infected with genotypes 2 and 3 and is less effective with patients infected with genotype 1 (40%). Even though IFN and combination therapies have allowed the management of HCV infections in a number of patients, an effective treatment of existing genotypes of HCV that could be orally administered is vital.

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HCV, a *Flaviviridae* family virus, produces a single positive strand of RNA genome with a single open reading frame of 9.6 KB and encodes a polyprotein of approximately 3000 amino acids.[3] The encoded polyprotein is cleaved cotranslationally and posttranslationally to produce the core proteins, envelope proteins, and nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B.[4] Posttranslational modification leading to mature virus is accomplished by autocatalytic cleavage of the NS2−NS3 junction followed by cleavage of NS3−NS4A, NS4A−NS4B, NS4B−NS5A, and NS5A−NS5B with the assistance of a single NS3 serine protease. These processed proteins are indispensable for development of mature virus, and inhibition of any one of these enzymes makes it an attractive target for development of a drug for hepatitis C.[3b,5] However, the central role of NS3 protease in the replication cycle makes it particularly attractive for development of inhibitors for the treatment of chronic hepatitis C.[6]

HCV NS3 protease is a serine enzyme with a catalytic site that is located on the surface and is characteristically featureless, solvent-exposed, and shallow.[7] The enzyme catalyzes the cleavage of a cysteine−serine amide bond with the assistance of a 52 amino acid cofactor NS4A. The enzyme comprises of eight-stranded distorted β-barrels with the NS4A bound between two β-strands in the N-terminal subdomain. The active site of the enzyme consists of the catalytic triad Ser-139, His-57, and Asp-81 that is characteristic of all serine proteases. The P_1 amino acid cysteine and P_1' serine residue are conserved in all the enzyme substrates.[5c,8] The S_1 pocket is hydrophobic, containing Phe-154, Ile-135, and Ala-157 where the sulfhydryl group of cysteine from the substrate makes an efficient interaction with the aromatic group of Phe-154. The scission of the cysteine−serine amide bond is initiated by a nucleophilic attack of serine-139 with the formation of a tetrahedral intermediate, which is stabilized by the histidine present in the oxyanion hole. The hydrolytic cycle is completed by the collapse of the tetrahedral intermediate followed by nucelophilic attack of water to hydrolyze the acylserine ester. A suitably placed electrophilic trap such as trifluoromethyl ketones, tricarbonyls, or ketoamides in inhibitors could reversibly trap serine by the formation of a tetrahedral adduct, thus inhibiting the enzyme catalytic cycle.[9] This strategy has been well-explored and used for the identification of various serine protease inhibitors.

In our HCV program we identified the α-ketoamide functionality as a good serine trap that provided potent inhibitors of HCV NS3−NS4A protease. In an attempt to depetidize these inhibitors, various strategies were explored. A close analysis of the X-ray structure of the NS3 protease revealed the proximity of the S_2 and the S_4 pockets. We reckoned that a suitable linker that would tether residues, which efficiently bound to the S_2 and S_4 pockets, could potentially provide depeptidized

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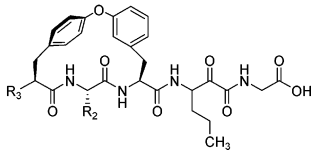


Figure 1.

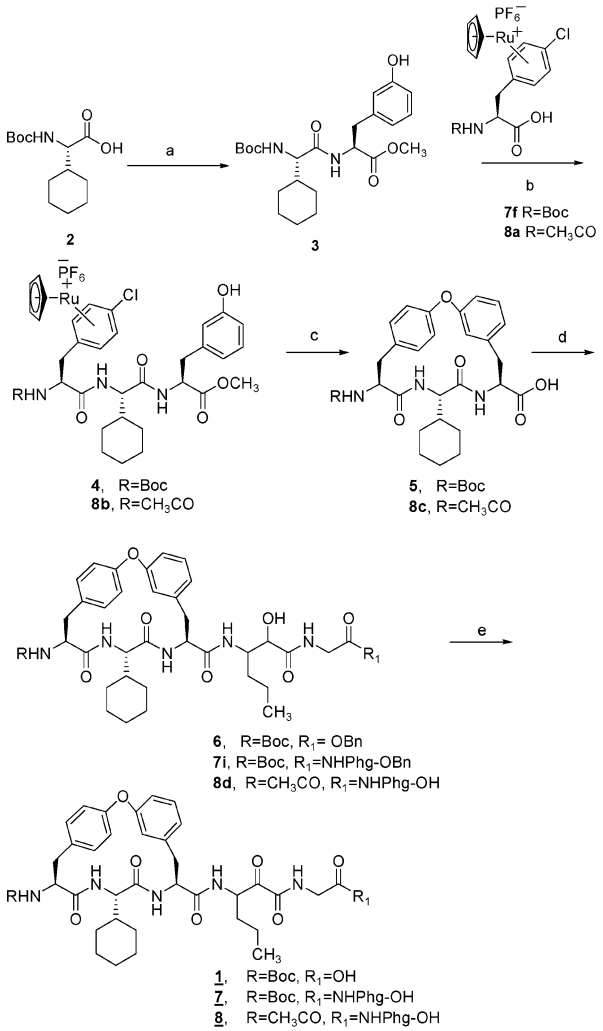
inhibitors with high affinity and selectivity to HCV protease. Further modeling and structure−activity relationship studies suggested biaryl ether of type **1** (Figure 1) as an excellent scaffold for the generation of NS3 protease inhibitors. Previously, a group from IRBM reported a biaryl scaffold as potential HCV NS3 protease inhibitors that spanned from P_4 to P_1, which lacked the electrophilic ketoamide moiety. However, the group, to our knowledge, did not disclose the inhibitory activity of these compounds. We therefore wanted to further explore this scaffold by incorporating a ketoamide trap and to evaluate their potential as HCV inhibitors.[10]

The structure shown in Figure 1 contains a 17-membered biaryl macrocyclic ether derived from (L)-tyrosine and (L)-*m*-tyrosine as the P_4 and P_2 residues. Cyclohexylglycine was chosen as the P_3 amino acid because this moiety had been shown to be an excellent group at this position in our acyclic series of inhibitors (data not shown). Similarly, norvaline and glycine were deemed as appropriate P_1 and P_1' residues, respectively, thus making **1** the desired macrocyclic inhibitor candidate. Although various methods have been reported for the construction of cyclic biaryl ethers reported in the context of the total syntheses of vancomycin, ristocetin A, teicoplanin, OF4949III, and K-1310 macroetherification using η^6-ruthenium chemistry pioneered by Pearson and Rich was our method of choice.[11]

The synthesis of macrocycle **1** began with the coupling of Boc protected cyclohexylglycine **2** with *m*-tyrosine using the mixed-anhydride method to produce the dipeptide **3** in 73% yield (Scheme 1). The Boc group of **3** was deprotected using 4 M HCl in dioxane, and the resulting amine was coupled with the η^6-ruthenium complex of Boc-4-chlorophenylglycine to yield **4** in 69%. Macrocyclicliization of **4** was effected using Cs_2CO_3 as the base followed by photolytic decomplexation of Ru at $\lambda = 350$ nm, giving macrocycyclic biaryl ether **5** in 47% yield as a colorless solid. Completion of the synthesis of **1** was achieved by hydrolysis of the methyl ester with aqueous LiOH followed by coupling with a norvaline-hydroxyamide[12] segment using EDCI and HOOBt. Dess−Martin oxidation[13] of the hydroxyamide **6** yielded α-ketoamide **1** in excellent yield.

The synthesized macrocycle **1** was evaluated for its 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.[14] The inhibitory activity of **1** was found to be $K_i^* = 12\ \mu$M. Modeling studies clearly indicated that the arene rings of P_2 *m*-tyrosine and P_4 tyrosine occupied the S_2 and S_4 pockets of the enzyme appropriately. However, attempts to obtain a crystal structure of **1a** bound to the HCV NS3 protease were unsuccessful. Further modeling and SAR studies from our acyclic inhibitors prompted us to incorporate a phenylglycine at the P_2' site of inhibitor,[15] resulting in the synthesis

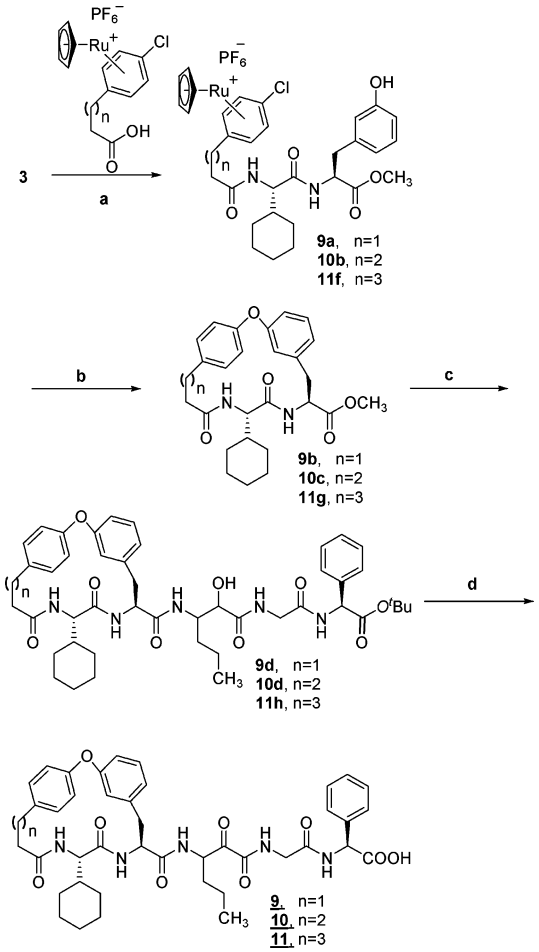
Scheme 1[a]



[a] Reagents and conditions: (a) *m*-Tyr-OH, IBCF, NMM, −20 → 5 °C, 1 h, 53%; (b) (i) 4 M HCl in dioxane, (ii) **7f** or **8a**, EDCI, HOBt, DMF, room temp, 24 h; (c) (i) 5.0 equiv of Cs_2CO_3, DMF, 12 h, room temp, (ii) CH_3CN, $h\nu$, $\lambda = 350$ nm, 48 h, 47%, (iii) $LiOH{\cdot}H_2O$, THF/H_2O, 100%; (d) EDCI, HOOBt, H−Nva(OH)-C(O)-Gly-OBn•HCl, $(^iPr)_2EtN$, (e) (i) Dess−Martin reagent, CH_2Cl_2, (ii) $H_2/Pd/C$, CH_3OH.

of **7** (Scheme 1). Inhibitor **7** had a binding of $K_i^* = 7.8\ \mu$M. Because the incorporation of phenylglycine at P_2' slightly improved the binding, which was encouraging, we therefore decided to further refine our subsequent targets. To further improve potency, various possible sites for modification of **1** were evaluated and changes at the P_3 site and P_4 capping group seemed appropriate. The BocNH (P_4 capping) group of **1** was replaced with an acetamide (AcNH) moiety to afford inhibitor **8** (Scheme 1). Synthesis of **8** was initiated from acetyl protected 4-chlorophenylalanine **8a** and synthesized as shown in Scheme 1 in good yields. Inhibitor **8** was found to have a binding of $K_i^* = 0.8\ \mu$M, which was 15 times more potent than **1**. This improvement in activity by the replacement of the Boc group with an acetamide group was ascribed to the smaller steric requirement of the acetyl group in comparison to the bulky *tert*-butoxycarbonyl moiety. We therefore reasoned that the complete removal of the P_4 capping group would further enhance binding efficiency, and thus, we designed macrocyclic inhibitor **9** (Scheme 2).

Scheme 2[a]



[a] Reagents and Conditions: (a) (i) 4 M HCl in dioxane, (ii) EDCI, HOBt, DMF, room temp, 24 h, (b) (i) 5.0 equiv of Cs_2CO_3, DMF, 12 h, room temp, (ii) CH_3CN, $h\nu$, λ = 350 nm, 48 h; (d) (i) $LiOH{\cdot}H_2O$, THF/H_2O, 100%, (ii) EDCI, HOOBt, H-Nva(OH)-C(O)-Gly-Phg-O^{t}Bu·HCl, $(^i Pr)_2EtN$; (e) (i) Dess−Martin reagent, CH_2Cl_2; (ii) 4 M HCl, dioxane.

The synthesis of **9** was initiated with 4-chlorophenylpropionic acid and is shown in Scheme 2. Inhibitor **9** was assayed for its inhibitory activity and was found to have a $K_i^* = 0.11$ μM, which was 100 times more potent than **1**. To evaluate the specificity of binding of macrocycle **9** to HCV protease in comparison with other serine proteases, binding studies with a structurally closely related protease, human neutrophil elastase (HNE), was evaluated.[16] The binding constant of macrocycle **9** to HNE was 2.9 μM, providing a selectivity of HNE/HCV of 27. In addition, selectivities against other serine and cysteine proteases chymotripsin, cathepsin G, and cathepsin H were determined to be BPC/HCV = 26, HNC-G/HCV = 250 and HLC−H/HCV > 380, respectively.

After a potent, selective HCV protease inhibitor was developed, the correlation of inhibitory activity (K_i^*) on the macrocyclic ring size was evaluated. The 18-membered inhibitor **10** and 19-membered inhibitor **11** were synthesized as shown in Scheme 2 starting from 4-chlorophenylbutyric acid and 4-chlorophenylpentanoic acid, respectively. These inhibitors were also very potent, with a binding activity of $K_i^* = 0.3$ μM and $K_i^* = 0.16$ μM, respectively. Thus, changing the ring size did not enhance the binding efficiency of the resulting inhibitor.

Having optimized the ring size of the macrocyclic inhibitor, we wanted to understand the mode of binding of these macrocycles to HCV NS3A. The X-ray crystal structure of inhibitor **9** bound to the protease was obtained (Figure 2).

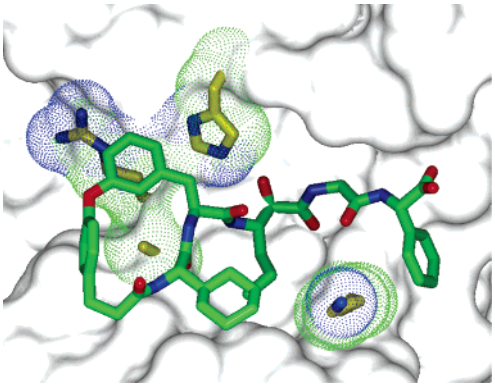


Figure 2. X-ray crystal structure of inhibitor **9** bound to active site of HCV NS3 protease.

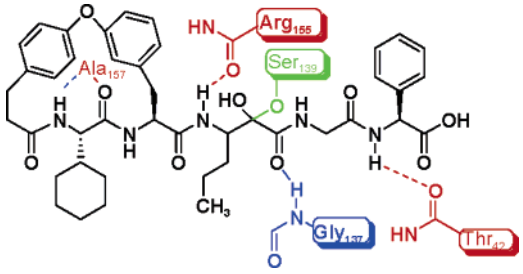


Figure 3. Pictorial representation of hydrogen bonding network of inhibitor **9** to HCV NS3 protease.

As shown in the Figure 2, the inhibitor **9** bound to the protease with the aryl groups of *m*-tyrosine in the S_2 domain and the phenylpropionic acid in the S_4 pocket. The arene rings effectively made contact with the surface of the protein and wrap around the methyl group side chain of alanine-156, thereby maximizing surface contact. The biaryl ether adopted a 90° conformation, with the two aryl rings being orthogonal to each other. The cyclohexyl ring of cyclohexylglycine (P_3 amino acid) adopted a chair conformation with excellent van der Waals contact with the protein in the S_3 pocket. The propyl chain of the norvaline (P_1 residue) was buried deeply into the S_1 pocket, making excellent contact with Phe-154 located at the base of the S_1 pocket. The P_2' phenylglycine and P_1' glycine form a part of a "C clamp" around the extended aliphatic chain of lysine-136, thus minimizing the mobility of the lysine side chain residue. The arene ring of P_2' phenylglycine also made excellent hydrophobic contact with the carbon chain of lysine, thus maximizing binding of the individual residues.

Additionally, the inhibitor **9** is held in close contact to the surface of the protein by an array of specific hydrogen bonds to the backbone. A schematic representation of these specific hydrogen bonds are shown in Figure 3.

The amide nitrogens of P_2' phenylglycine, P_3 cyclohexylglycine, and P_1 norvaline donate hydrogen bonds to threonine-42, alanine-157, and arginine-155 of the protease, and the carbonyl group of the ketoamide is attacked by the hydroxyl group of serine-139. In addition, the oxygen of the ketoamide accepts a hydrogen bond from the NH group of glycine-137 from the backbone, making the binding of **9** extremely specific.

In conclusion we have developed a novel biaryl P_2-P_4 macrocyclic depeptidized inhibitor of HCV NS3 protease with excellent *in vitro* binding potencies. The binding efficiency of the inhibitor was optimized from 12 to 0.11 μM activity through the optimization of appropriate moieties. An X-ray crystal structure of the inhibitor **9** bound to the protease provided information about the various hydrogen bondings and hydrophobic interactions of the inhibitor with the protease. The X-ray clearly showed a "C clamp" formation between the phenylglycine and glycine that immobilizes lysine-139, thus enhancing binding efficiency. The P_2-P_4 macrocyclic inhibitors are selective toward HCV protease and are being further evaluated for its pharmacokinetics properties.

Supporting Information Available: Experimental and characterization data for intermediates and final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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