Novel macrocyclic HCV NS3 protease inhibitors derived from α-amino cyclic boronates

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ARTICLE INFO

ABSTRACT

A novel series of P2–P4 macrocyclic HCV NS3/4A protease inhibitors with α-amino cyclic boronates as warheads at the P1 site was designed and synthesized. When compared to their linear analogs, these macrocyclic inhibitors exhibited a remarkable improvement in cell-based replicon activities, with compounds **9a** and **9e** reaching sub-micromolar potency in replicon assay. The SAR around α-amino cyclic boronates clearly established the influence of ring size, chirality and of the substitution pattern. Furthermore, X-ray structure of the co-crystal of inhibitor **9a** and NS3 protease revealed that Ser-139 in the enzyme active site traps boron in the warhead region of **9a**, thus establishing its mode of action.

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Hepatitis C virus (HCV) infection is a major cause of human liver disease throughout the world, leading to cirrhosis, carcinoma and liver failure.[1] Worldwide over 200 million people are estimated to be chronically infected. The current standard of care for HCV infection is a combination therapy of injectable pegylated interferon-α (PEG IFN-α) plus oral ribavirin. However, adverse side effects are commonly associated with this treatment, such as flu-like symptoms, leukopenia, thrombocytopenia, depression from interferon, as well as anemia induced by ribavirin.[2] In addition, approximately 50% of genotype 1 patients do not respond well to this treatment regimen (while genotype 1a is most abundant in the U.S., the majority of sequences in Europe and Japan are from genotype 1b).[3] Therefore, there is an enormous unmet medical need for developing new therapies against HCV infection.

Recently, HCV NS3/4A protease inhibitors have emerged as a promising potential treatment for HCV infection.[4] Unlike interferons, which work by stimulating the immune system's response to viral infection, NS3 protease inhibitors target the virus directly by inhibiting NS3/4A serine protease, a key enzyme involved in HCV replication.

Two major classes of inhibitors have been developed for HCV NS3 protease (Fig. 1). The first class is comprised of reversible covalent inhibitors, also known as serine-trap inhibitors. The most promising drugs are α-ketoamides such as VX-950 (telaprevir)[5] and SCH-503034 (boceprevir),[6] currently in phase III clinical trials. The second class is represented by reversible noncovalent inhibitors, such as the P1–P3 macrocyclic inhibitor BILN-2061, the first compound in its class to achieve clinical proof of concept.[7] The subsequent search for NS3 protease inhibitors incorporating new structural motifs led to the discovery of other P1–P3 macrocyclic inhibitors including ITMN-191[8] and TMC-435350[9] currently in phase II trials. Recently, scientists at Merck identified a novel class of P2–P4 macrocyclic inhibitors of NS3 protease exemplified by

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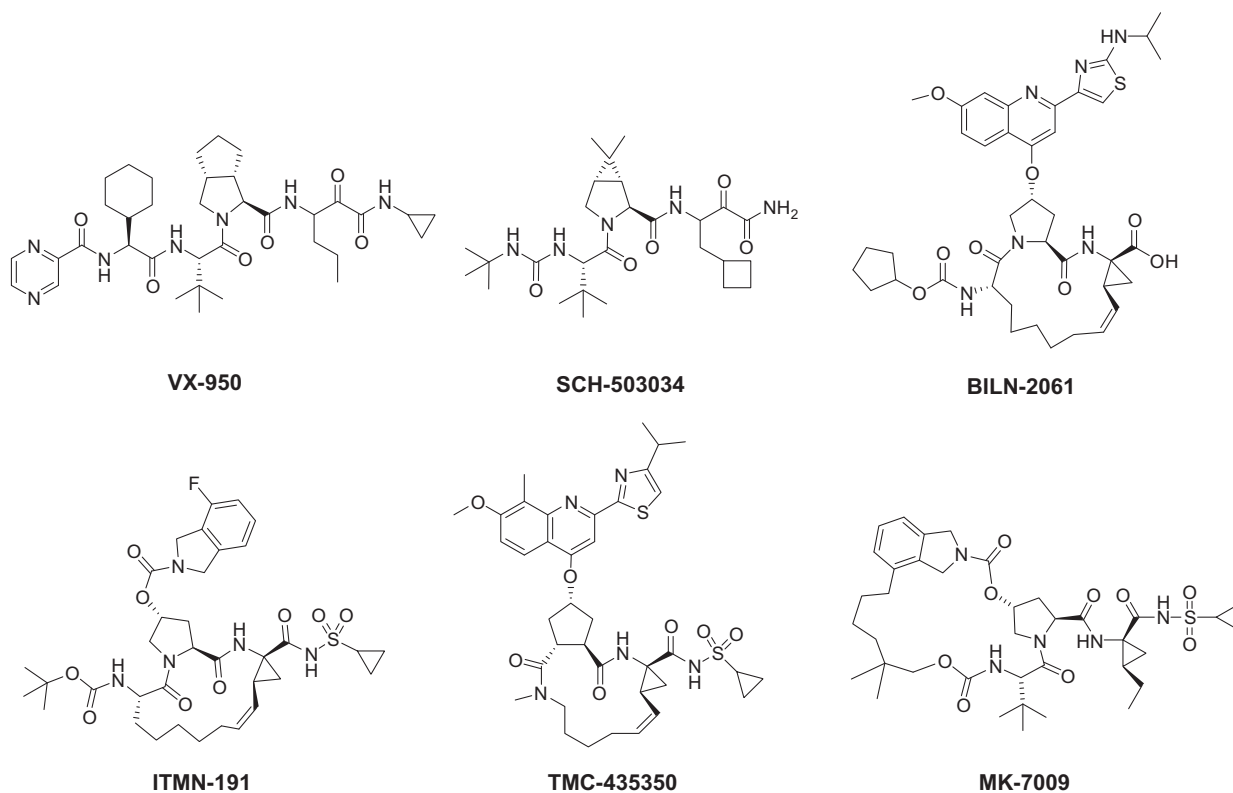
Figure 1. Selected inhibitors of NS3 protease in advanced clinical trials.

MK-7009 (Fig. 1),[10] currently in phase II trials. In general, the macrocyclic HCV NS3 protease inhibitors feature improved binding and pharmacokinetic properties versus their linear counterparts.

We have recently reported a new series of acyclic inhibitors of HCV NS3 protease in which α-amino cyclic boronates are incorporated as warheads at the P1 position (**1**, Fig. 2).[11] The α-amino cyclic boronate group has been shown to be a good α-ketoamide replacement in serine-trap inhibitors. From the X-ray structure of a co-crystal of a cyclic boronate inhibitor with NS3 protease, the boron warhead is covalently linked to the hydroxyl group of Ser-139 in the enzyme active site. These acyclic inhibitors were found to exhibit good enzymatic potency, but weak cellular replicon activity with EC_{50} of >1.0 μM. For example, compound **1** showed poor potency in the replicon 1a and 1b assay with EC_{50} of 8.7 and >10 μM, respectively. To further improve cellular potency of these compounds, we turned our attention to the macrocyclic series and decided to attach the boron warhead to MK-7009–derived P2–P4 macrocycle.[10]

The representative synthesis of macrocyclic inhibitor **9a** derived from five-membered α-amino oxaborole is outlined in Scheme 1. The two building blocks include (R) α-amino boronate **7a** and P2-P4 macrocyclic acid **8**. Compound **7a** was prepared via Matteson asymmetric homologation chemistry[12] and as described in our previous publication.[11] The macrocyclic acid **8** was prepared according to the literature procedure.[10] Incorporation of (R) α-amino oxaborole into a macrocyclic product was achieved by a one-pot procedure. This included the coupling of **8** with boronate **7a** using HATU/DIEA, removal of pinanediol and spontaneous formation of cyclic boronate in the presence of HCl and isobutyl boronic acid. The final product **9a** was isolated as a white solid,[13] with (R) α-amino oxaborole incorporated at the P1 position.

Figure 3 lists all α-amino boronates **7a–i** which were used to synthesize P2–P4 macrocyclic inhibitors **9a–i** shown in Table 1. The synthesis of boronates **7a**, **7c**, and **7f** has been reported previously.[11] The β-substituted boronates **7b** and **7g** were prepared by a similar route to **7c**. The previously inaccessible chiral boronates **7d**,

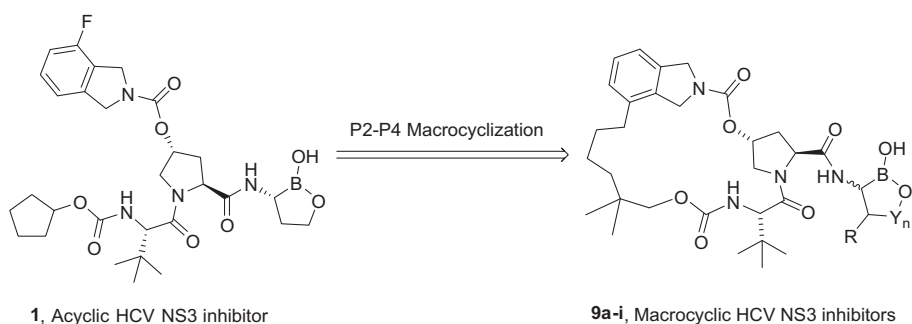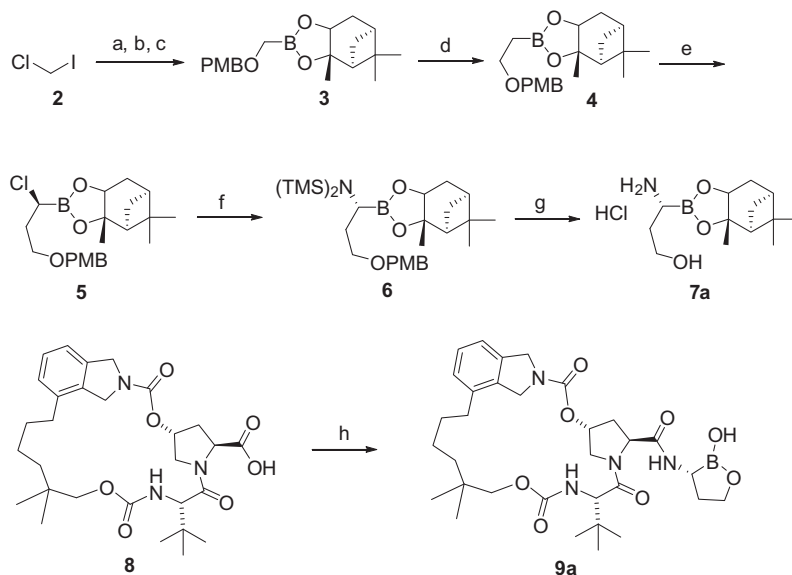**1**, Acyclic HCV NS3 inhibitor   **9a-i**, Macrocyclic HCV NS3 inhibitors

Figure 2. α-Amino cyclic boronate-based P2–P4 macrocyclic HCV NS3/4A protease inhibitors.

Scheme 1. Reagents and conditions: (a) B(OiPr)$_3$, *n*-BuLi, THF, −78 °C, 50%; (b) (+)-pinanediol, Et$_2$O, 98%; (c) PMB-OH, *n*-BuLi, DMSO, 58%; (d) CH$_2$ICl, *n*-BuLi, THF, −78 °C, 47%; (e) LiCHCl$_2$, THF, −100 °C, then ZnCl$_2$, 75%; (f) LiN(TMS)$_2$, THF, −78 °C, 90%; (g) 4 N HCl in dioxane, hexane, 98%; (h) **7a**, HATU, DIEA, DMF, then *i*-BuB(OH)$_2$, MeOH/hexane, HCl, 35%.
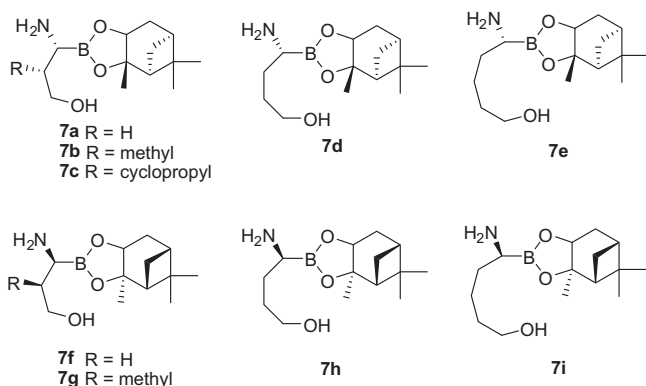

Figure 3. Key α-amino boronates **7a–i** prepared.

7h (for making six-membered cyclic boronates), and **7e**, **7i** (for making seven-membered cyclic boronates) were prepared according to Scheme 2 and Scheme 3, respectively.

As shown in Scheme 2, the preparation of (*S*) α-amino boronate **7h** started with *n*-butyl boronate ester **10**.[11] Transesterification of compound **10** with (−)-pinanediol gave pinanediol ester **11**. Alcohol **11** was protected with TBS group to give boronate **12**. Subsequently, methylene chloride insertion of **12** afforded (*R*) chloroboronate **13**. Reaction of **13** with LiHMDS produced the TMS-protected α-amino boronate **14**, with inversion of stereochemistry. Treatment of **14** with anhydrous HCl resulted in the formation of (*S*) α-amino boronate **7h** as its HCl salt. The corresponding (*R*) α-amino boronate **7d** was prepared using the same procedure that incorporates (+)-pinanediol boronate ester of **11** instead.

Commercially available pinacol boronate ester **15** was used to prepare boronates **7e** and **7i**, as shown in Scheme 3. Compound **15** was transesterified with (+)-pinanediol to give the pinanediol boronate ester, followed by hydrogenation to yield the saturated boronate **16**. Methylene chloride insertion of boronate **16** gave (*S*) α-chloroboronate **17**. Treatment of **17** with LiHMDS gave the TMS-protected amino boronate **18**, with inversion of stereochemistry. The treatment of **18** with anhydrous HCl led to the formation

of (*R*) α-amino boronate **7e** as its HCL salt. The corresponding (*S*) α-amino isomer **7i** was also prepared using (−)-pinanediol as a chiral auxiliary via the same route.

In addition to cell-based 1a and 1b HCV replicon assays,[14] compounds **9a–i** were tested in FRET assay with NS3/4A 1a protease domain[11] in a new buffer containing 20% sucrose (Table 1). We developed this sucrose-based assay to improve the enzyme–cell assay correlation for boron containing compounds. For example, highly potent boronic acid-based NS3/4A inhibitors reported by several research groups[15a–e] were much less potent or inactive in the replicon assay. Thus, Schering's boronic acid inhibitors failed to translate into replicon potency (EC$_{90}$ >5 μM)[15a] and Phenomix's inhibitors showed poor translation of enzymatic potency to replicon potency (EC$_{50}$/IC$_{50}$ >48),[15b] even though these compounds were nanomolar inhibitors in the glycerol-based assay. We found that the presence of glycerol in the testing media enhanced in vitro enzymatic potency of boronates or boronic acids by 1- to 10-fold. This glycerol effect was not observed for non-boron containing inhibitors. In addition, the X-ray structure of a co-crystal of a cyclic boronate inhibitor with NS3 protease S139A mutant[11] suggests the presence of oxaborole glycerol adduct, which can reach into the S1 binding site and thus may contribute to inhibitor potency. Subsequently, we identified that sucrose is a suitable glycerol substitute in the enzyme assay and it enables more predictive enzymatic potencies, that correlate better with replicon activity. Therefore, the macrocyclic inhibitors **9a–i** were evaluated using FRET assay in a new buffer system containing 20% sucrose.

As shown in Table 1, compound **9a** inhibited NS3 1a enzyme with an IC$_{50}$ of 0.043 μM in the sucrose assay and it exhibited good replicon 1a and 1b potency with EC$_{50}$ values of 0.78 and 0.52 μM, respectively. This encouraging result, stimulated further syntheses of six- and seven-membered cyclic boronates **9d** and **9e** to explore the influence of ring size on potency. Data in Table 1 suggest a minimal influence of the ring size of α-amino cyclic boronate on the enzymatic and replicon activities for this series of compounds. Compound **9e**, derived from seven-membered cyclic boronate, showed comparable enzymatic potency with IC$_{50}$ of 0.047 μM and was marginally more potent in replicon 1a and 1b assay with EC$_{50}$ of 0.38 and 0.22 μM, respectively. Therefore, inhibitors **9a** and **9e** exhibited comparable replicon activity compared to two serine-

Table 1
In vitro activity of P2–P4 macrocyclic inhibitors against HCV NS3/4A 1a, and replicon 1a and 1b

9a-i
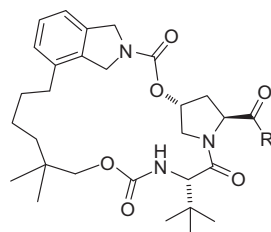
| Compound	Key α-amino borate 7 used	Cyclic boronate R	NS3/4A 1a IC$_{50}$[a] (μM)	HCV replicon 1a EC$_{50}$[b] (μM)	HCV replicon 1b EC$_{50}$ (μM)[b]
MK-7009			⩽0.023[c]	⩽0.001[d]	⩽0.001[d]
Acyclic analog **1**			⩽0.023[c]	8.7	>10
9a	**7a**		0.043	0.78	0.52
9b	**7b**		0.13	1.9	1.7
9c	**7c**		0.11	0.98	4.2
9d	**7d**		0.062	1.3	2.8
9e	**7e**		0.047	0.38	0.22
9f	**7f**		1.4	11	>50
9g	**7g**		25	33	30
9h	**7h**		1.3	12	>25
9i	**7i**		0.30	3.7	3.9

[a] FRET assay with 100 nM HCV NS3 1a protease domain as described in Ref. 11, however, 20% sucrose substituted for 20% glycerol in the assay buffer.

[b] Replicon assay performed as described in Ref. 14.

[c] Value determined as described in Ref. 11, using FRET assay with 100 nM HCV NS3 1a protease domain in the buffer containing 20% glycerol. Potency approaching limit of detection of assay therefore modifier ⩽ used.

[d] Compound was more potent than the maximum concentration tested.

trap inhibitors, VX-950 (replicon 1b, EC$_{50}$ = 0.354 μM)[5a,b] and SCH-503034 (replicon 1b, EC$_{50}$ = 0.20 μM).[4b] It is not surprising that inhibitors **9a** and **9e** are less potent than MK-7009, since the non-covalent inhibitor MK-7009 contains an optimized cyclopropyl acylsulfonamide moiety[16] at the P1′ site which significantly enhances inhibitor binding.

As the cyclic boronates in these compounds presumably provide critical interactions with the enzyme, we also investigated the influence of chirality at the α-position of these boronates. As shown in Table 1, (R) α-amino cyclic boronates were much more potent than the corresponding (S) α-amino isomers. For example,

(R) α-amino oxaborole **9a** was 30-fold more active against NS3 1a, and over 14-fold more potent in replicon 1a and 1b assay, compared to its (S) isomer **9f**. Similarly, (R) six- and seven-membered cyclic boronates (**9d** and **9e**) were significantly more active than their (S) α-amino isomers (**9h** and **9i**) in both enzyme and cell assays. These results suggest that these boronates interact with the HCV NS3 protease in the expected, enantiospecific fashion.

Further investigation of β-substitution on cyclic boronates of these macrocyclic inhibitors provided SAR consistent with the acyclic series observed previously. Introducing a small methyl group at the β-position of α-amino cyclic boronate in compound **9b** re-
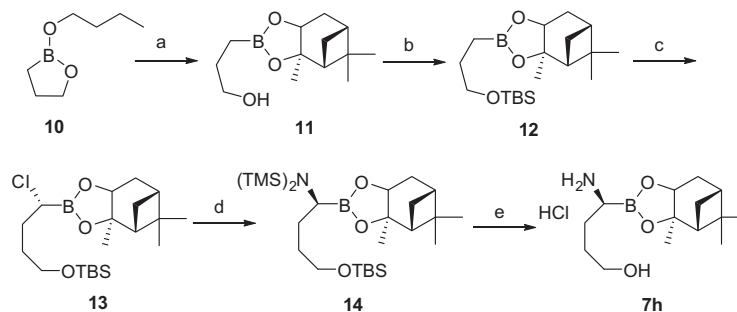
Scheme 2. Reagents and conditions: (a) (−)-pinanediol, THF, reflux, 100%; (b) TBSCl, imidazole, DMF, 86%; (c) LiCHCl$_2$, THF, −100 °C, then ZnCl$_2$, 89%; (d) LiN(TMS)$_2$, THF, −78 °C, 100%; (e) 4 N HCl in dioxane, hexane, 95%.
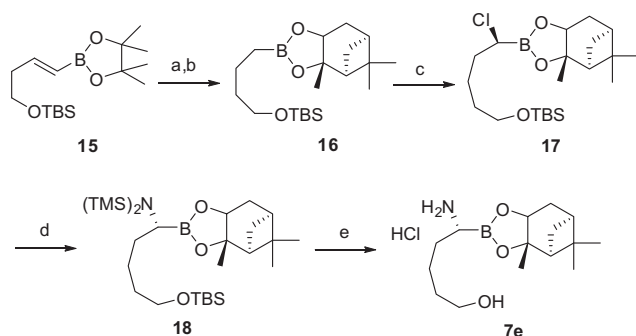
Scheme 3. Reagents and conditions: (a) (+)-pinanediol, Et$_2$O, 96%; (b) Pd/C, H$_2$, MeOH, 84%; (c) LiCHCl$_2$, THF, −100 °C, 93%; (d) LiN(TMS)$_2$, THF, −78 °C, 94%; (e) 4 N HCl in dioxane, hexane, 93%.

Figure 4. X-ray crystal structure of macrocyclic inhibitor **9a** complexed with HCV NS3 serine protease.

sulted in several fold decrease in both enzyme and cell-based replicon potency. Compound **9c** with a cyclopropyl group at the β-position was also less active in the enzyme and replicon assay. Overall, these results suggest that β-alkyl substituents are not well tolerated due to either steric interaction with the S1 pocket and/or undesirable stereochemistry.

When compared to their linear analogs, these P2–P4 macrocyclic inhibitors exhibited a significant improvement in replicon 1a and 1b potency. For example, compound **9a** is 10-fold more active in the replicon 1a assay and 20-fold more potent in the replicon 1b assay, in comparison to its acyclic inhibitor **1**. Similar trends were also observed for other macrocyclic inhibitors in this series, such as **9d** and **9e**. We speculate that this enhancement may be due to two reasons. First of all, macrocyclic inhibitors are more potent against NS3 enzyme than their linear analogs. One potential explanation is that the former are conformationally more rigid with less inactive conformers that would lead to more productive binding to the enzyme. Secondly, the $c \log P$ increase in the macrocyclic molecule may result in an improved membrane penetration (macrocyclic inhibitor **9a** $c \log P = 5.95$[17] versus linear **1** $c \log P = 4.50$) and thus the cellular potency. Significant improvements in replicon potency have also been reported for other P2–P4 macrocyclic inhibitors of NS3 protease.[18]

X-ray crystal structure of inhibitor **9a** complexed with NS3 serine protease was obtained,[19] by soaking preformed crystals of NS3 protease domain/synthetic 4A co-factor with inhibitor **9a** in 10–20% DMSO.[20] As shown in Figure 4, boronate **9a** binds at the HCV NS3 active site in the extended β-strand conformation. The density, which is at low resolution, as expected is consistent with a model where the boron warhead is covalently linked to the catalytic Ser-139, and is locked in the negatively-charged tetrahedral form mimicking the transition state. The oxygen–boron bond inside the five-membered oxaborole ring of inhibitor **9a** is cleaved resulting in the
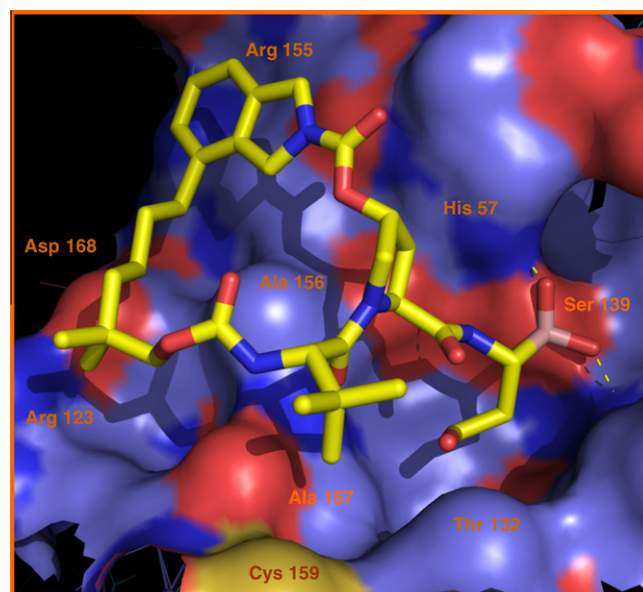
hydroxyethyl side chain in the S1 pocket, similar to the previously described acyclic series.[11] When the catalytic Ser-139 is absent, the co-crystal structures of these inhibitors with HCV NS3 protease S139A mutant clearly show the intact cyclic boronate ring at the S1 subsite. Presumably inhibitor **9a** binds to the enzyme active site as a closed form to afford an initial covalent adduct, which is subsequently hydrolyzed to release a hydroxyethyl side chain into the S1 pocket.[21] The methylene linker connecting the P2 and P4 moieties is not well ordered in the structure which suggests it has minimum hydrophobic contact with the flat protein surface. The P2 and P4 cyclization might have resulted in more rigid conformations and adds lipophilicity to the molecule, which translate well in improved cellular potency compared to the acyclic series. It is interesting to note that the carbamate linkage in the ring adopts a non-planar conformation. Relieving the constraint could further improve inhibitor and enzyme binding.

In conclusion, we have designed and synthesized a novel series of P2–P4 macrocyclic HCV NS3/4A serine protease inhibitors derived from α-amino cyclic boronates at the P1 site. When compared to the linear analogs, these macrocyclic inhibitors exhibited a remarkable improvement in cell-based replicon activities, with compounds **9a** and **9e** reaching sub-micromolar potency in replicon assay. We established key SAR around α-amino cyclic boronates by examining the influence of ring size, chirality and of ring substitution pattern. The X-ray structure of the co-crystal of inhibitor **9a** and HCV NS3

protease revealed that Ser-139 in the enzyme active site traps the warhead boron. Since the five-membered oxaborole ring is cleaved at the enzyme active site resulting in unfavorable interaction of liberated hydroxyethyl moiety in the hydrophobic S1 pocket, the next step in the optimization of these macrocyclic inhibitors will be to stabilize the cyclic boronate ring against opening and to install an appropriate P1′ substitution in the warhead region. The boronate-based warheads described here could be useful for further design and development of HCV NS3 and perhaps other serine protease inhibitors.[22]

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13. *Typical experimental procedure for the coupling of α-amino boronate* **7a** *to macrocyclic acid* **8**: To a stirred solution of boronate **7a** (88 mg, 0.304 mmol) and macrocyclic acid **8** (82.6 mg, 0.152 mmol) in 5 mL of anhydrous DMF was added HATU (116 mg, 0.304 mmol). Subsequently, DIEA (53 μL, 0.304 mmol) in 1 mL of anhydrous THF was added dropwise to the reaction mixture. After stirred for 4 h, the reaction mixture was diluted with ethyl acetate and washed with brine. The organic layer was dried over Na_2SO_4, filtered and concentrated to give a yellow residue. Subsequently, a solution of this residue in 5 mL of hexane and 5 mL of MeOH was treated with isobutyl boronic acid (34 mg, 0.334 mmol) and HCl (0.6 mL, 6 N), respectively. The reaction was stirred at room temperature for 16 h. The mixture was concentrated in vacuo, diluted with ethyl acetate and washed with brine. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The crude residue was purified on a reversed-phase column eluted with ACN and H_2O. The pure fractions were collected and ACN was removed in vacuo. The aqueous solution was extracted with ethyl acetate three times. The organic layer was dried over Na_2SO_4, filtered and concentrated to give the desired product **9a** as a white solid (33.6 mg, yield 35%). MS *m/z* 627.3 [M+1]$^+$, 625.4 [M−1]$^−$ (calcd MS 626.4). ^{1}H NMR (300 MHz, CD_3OD) δ 7.22 (1H, t), 7.13 (1H, d), 7.08 (1H, d), 6.94 (d, 1H), 5.40 (1H, t), 4.70–4.50 (5H, m), 4.41 (1H, d), 4.32 (1H, d), 4.26 (1H, d), 3.96 (1H, m), 3.84 (1H, t), 3.44 (1H, m), 3.25 (1H, d), 2.94 (1H, d), 2.76–2.24 (4H, m), 1.98–1.20 (8H, m), 1.05 (9H, s), 0.98 (3H, s), 0.76 (3H, s). Two protons assumed to be exchanged with solvent. Yields for the step from **7a–i** to **9a–i** were in the range of 6–47% and not optimized. The final compounds were found to be stable after over 1 year of storage in air.
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