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Synthesis, antiviral activity, and conformational studies of a P3 aza-peptide analog of a potent macrocyclic tripeptide HCV protease inhibitor

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Abstract—BILN 2061 is a macrocyclic tripeptide inhibitor of hepatitis C virus NS3-4A protease that has shown efficacy in the clinic for treating patients infected with HCV. We have synthesized a P3 aza-peptide analog of a potent macrocyclic tripeptide inhibitor closely related to BILN 2061. This aza-derivative was found to be >2 orders of magnitude less active than the parent macrocycle in both isolated enzyme (HCV NS3-4A) and HCV subgenomic replicon assays. NMR studies of P3 aza-peptides revealed these compounds adopt a β-turn conformation stabilized by an intramolecular H-bonding interaction. Molecular models of these structures indicate a D-like configuration of the P3 aza-residue. Thus, the configurationally undefined nature at P3 in the aza-peptide allows the compound to adopt an H-bond stabilized conformation that is substantially different from that necessary for tight binding to the active site of HCV NS3 protease.

Hepatitis C virus (HCV) infection is a chronic disease affecting 170 million people worldwide, according to current estimates. It is the primary cause of chronic liver disease and death due to liver disease in the United States today, a problem that is expected to grow with the aging population in the coming decades.[1] The current standard of care for treating patients with HCV infection is co-administration of pegylated interferon-α and ribavirin.[2] This therapy is not well tolerated by many patients during the long duration of treatment as the result of numerous side effects associated with its use. Furthermore, the sustained viral response (SVR) is <50% for patients with genotype 1a and 1b virus, which make up the majority of infections in the United States and throughout much of Europe and Asia. Thus, there is a growing need for improved therapies to combat HCV infection, particularly those effective in treating genotype 1 patients.[3]

Intensive research has focused on identifying small molecule inhibitors of the HCV NS3-4A serine protease.[4] NS3 is a dual function enzyme incorporating both a serine protease and RNA helicase domain that only becomes fully functional as a serine protease in a heterodimeric complex with NS4A. This enzyme complex, which is responsible for four of the five polyprotein cleavages necessary for viral replication, has been validated as a target for treating HCV infection through successful clinical studies using peptidic inhibitors. Initial clinical success was demonstrated using BILN 2061 (**1**), a 15-membered ring macrocyclic tripeptide that produced an impressive reduction in viral RNA levels in patients infected with HCV genotype 1 virus following oral administration.[5] This compound is a potent inhibitor of HCV NS3-4A protease in vitro, displaying single-digit nanomolar activity in both enzyme (IC_{50} = 3.0 nM) and replicon (EC_{50} = 1.2 nM) assays.[6]

Our interest in these compounds led us to investigate the properties of a P3 aza-peptide analog of macrocyclic compounds such as **1** (Fig. 1). The use of aza-peptide compounds in the treatment of HIV infection offers some evidence for the feasibility of the approach.[7] It was hoped that the macrocyclic core would reduce flexibility such that the aza-analog would adopt a similar conformation to **1**. Furthermore, a P3 aza-analog might provide some advantage from the standpoint of synthe-

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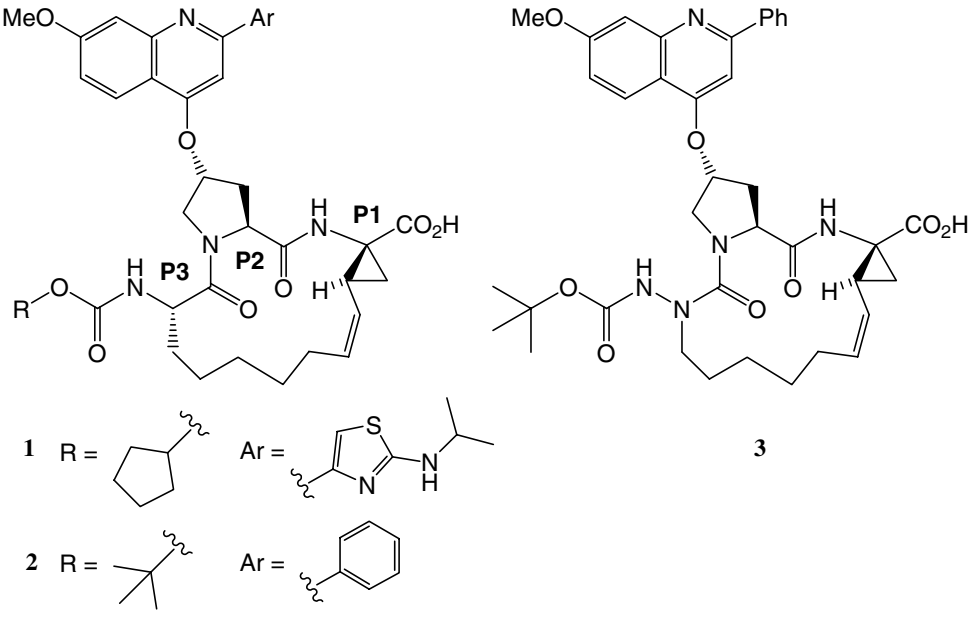
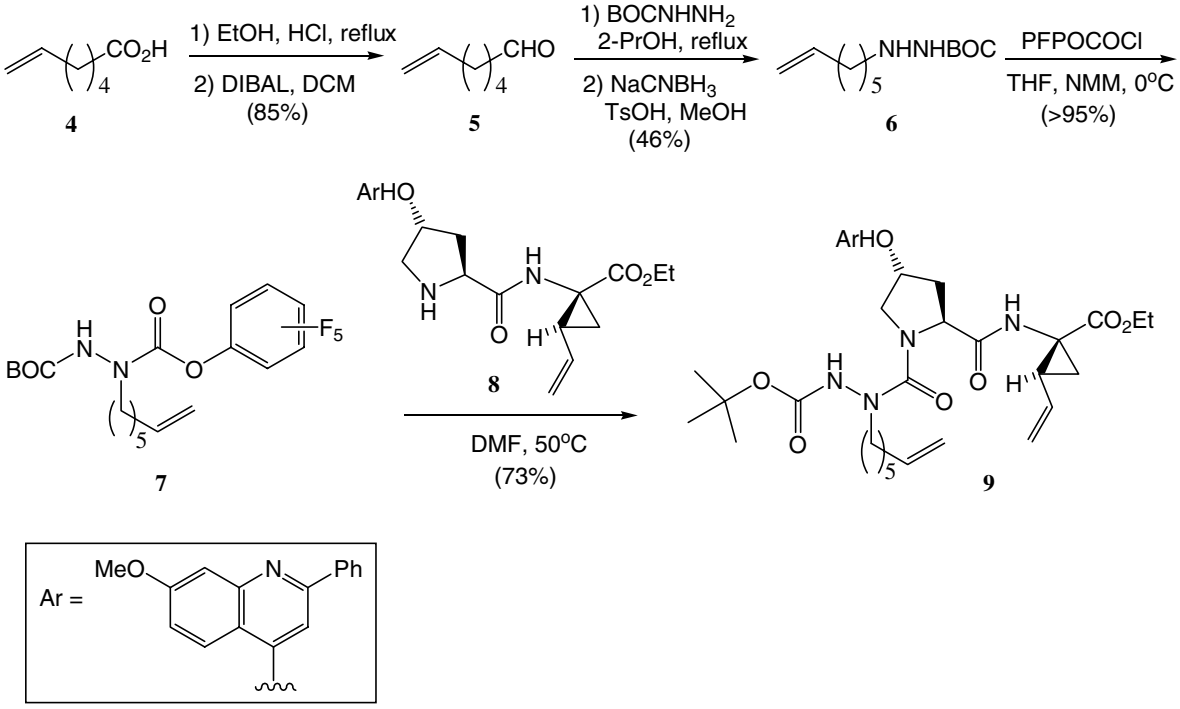


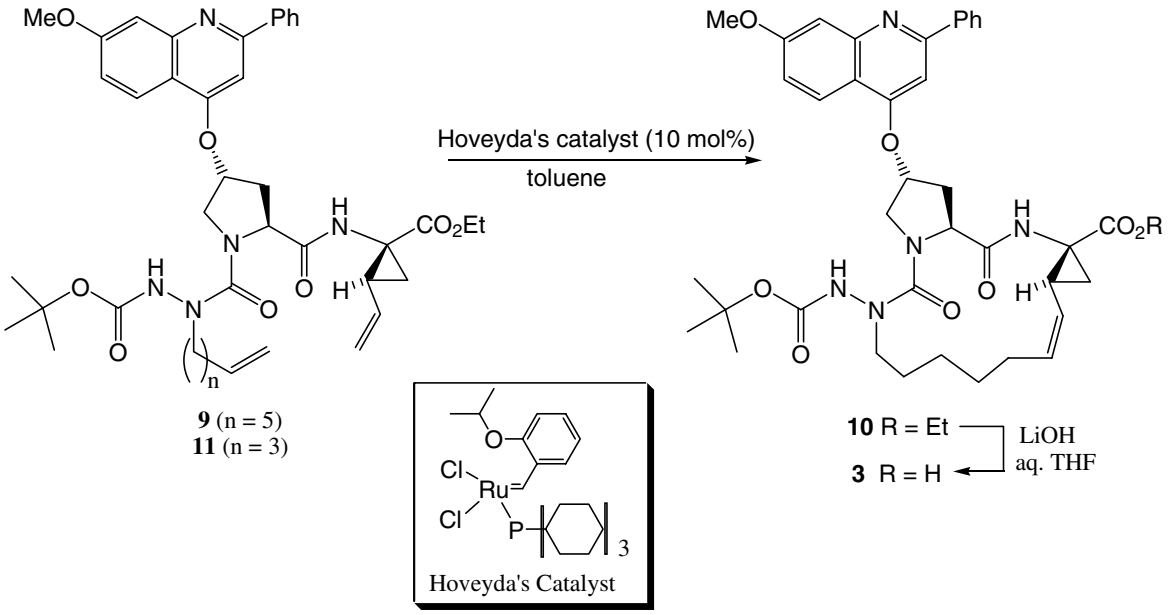
Figure 1. Macrocyclic tripeptide inhibitors of HCV NS3-4A protease. **1** = BILN 2061.

sis since it would avoid the chiral synthesis of the complex heptenylglycine P3 component in **1**.[8] In the effort, we selected the structurally similar macrocycle **2** as the parent compound for modification in order to simplify the problem from the point of synthesis. Thus, we targeted P3 aza-peptide macrocycle **3** for study.

Our strategy for the synthesis of **3** was analogous to that reported for the synthesis of **1** and **2**, which utilizes a late-stage ring-closing metathesis (RCM) reaction to form the macrocycle.[9,10] The di-olefin substrate for this macrocyclization was prepared as shown in Scheme 1. Esterification of heptenoic acid **4** followed by reduction with diisobutylaluminum hydride gave aldehyde **5**. Reaction of **5** with *tert*-butylcarbazate in refluxing 2-propanol afforded the hydrazone intermediate, which was reduced by the action of sodium cyanoborohydride in the presence of *p*-toluenesulfonic acid to give heptenyl analog **6**. Acylation of **6** with pentafluorophenyl chloroformate provided the carbazate derivative **7**, which reacted with the known dipeptide **8**,[11] to give RCM precursor tripeptide **9**.



Scheme 1. Synthesis of P3 aza-peptide.



Scheme 2. Macrocyclization of aza-peptide using RCM.

Macrocyclization was performed by RCM as shown in Scheme 2. Initially, **9** was subjected to conditions reported and used for the synthesis of **2** (Hoveyda's catalyst, 40 °C in CH_2Cl_2). This resulted in only 50% macrocyclization after 3 days, giving a 4:1 ratio of cis:trans olefin products. When the temperature was raised to 50 °C using toluene as solvent, only a trace amount of **9** remained after 3 days. The resulting macrocyclic product was obtained in a 62% yield as a 3:2 ratio of cis:trans olefin isomers. These isomers were readily separated by column chromatography on silica gel to give macrocyclic tripeptide **10**. Saponification of the ester gave the target inhibitor **3**. The unexpectedly sluggish reactivity of **9** in the RCM reaction, and the resulting poor cis:trans ratio of products are likely the result of conformational bias in this intermediate. NMR studies of **9** revealed a β-turn conformation for this compound resulting from intramolecular H-bonding between the *tert*-butylcarbamate carbonyl oxygen and the proton on the P1-P2 amide nitrogen (Fig. 2). Evidence for this intramolecular H-bond was obtained by standard methods comparing chemical shifts for the NH protons in $CDCl_3$ and DMSO-d_6.[12] The difference in chemical shifts observed in the two solvents for the P3 NH (H_a) was 3.1 ppm, the strong solvent effect indicating the availability of this proton to interact with solvent. In the case of the P1 NH (H_b), the observed solvent effect on chemical shift is almost negligible, with a chemical shift difference of only 0.2 ppm between the two solvents. This small solvent effect is typical of a proton involved in a stable intramolecular H-bonding interaction.[13] Furthermore, a positive NOE observed between the *tert*-butyl group of the N-terminal BOC and protons on both the cyclopropane ring and the olefin of the vinyl cyclopropane (VCP) group is further support for the proposed β-turn conformation. Finally, the lack of an NOE between the P1 NH (H_b) and the proton at C-1 of the P2 proline (H_c) indicates compound **9** does not adopt an extended conformation typical for tripeptide HCV PIs.[14] All of these NMR findings point to a β-turn conformation for **9**. A model of **9** showing the β-turn conformation is shown in Figure 3. The conformational constraints imparted by intramolecular H-bonding in **9** would be expected to affect reactivity during RCM, which is consistent with our experimental findings.

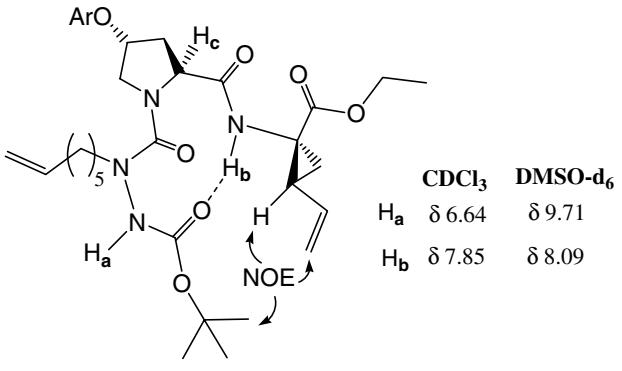


Figure 2. NMR evidence for β-turn conformation of **9**.

Molecular modeling was used to examine the extent to which intramolecular H-bonding could affect RCM in these systems. A model of compound **9** in the β-turn conformation revealed that enough flexibility remained such that RCM could still occur (i.e., the two olefins could still be brought close enough to one another such that bonding would not be unexpected). However, these models made clear the added difficulty of RCM in a β-turn constrained system, thus explaining the need for higher temperature and longer reaction time to achieve macrocyclization of **9**. This modeling work indicated that the intramolecular H-bond is made possible by the P3 aza group adopting a D-like configuration (this center is inverted relative to the L-amino acids in the parent tripeptide inhibitors).[15] In order to determine the extent to which the β-turn conformation might affect

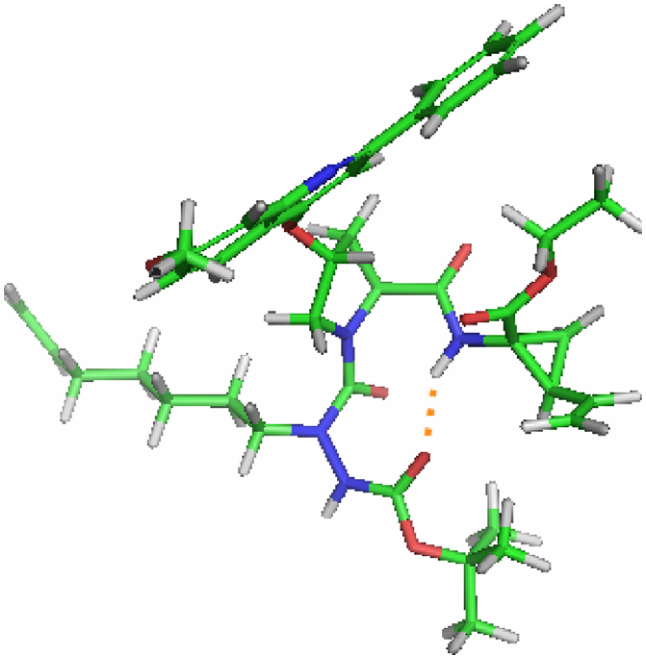


Figure 3. Molecular model of β-turn conformation of **9** showing the close proximity of *tert*-butyl ester and vinylcyclopropyl groups.[21]

reactivity, pentenyl analog **11** was prepared using the methods described for the synthesis of **9**. Molecular modeling studies of **11** suggested that, in the β-turn form, RCM would be highly difficult if not impossible due to conformational constraints preventing cyclization of the intermediate ruthenium alkylidene. Subjecting **11** to RCM conditions used to cyclize **9** (50 °C for 3 days in toluene) resulted in no observed macrocyclization. Raising the temperature to 60 °C resulted primarily in slow decomposition of **11**. After 2 days, a cyclic product was obtained in low yield (7% isolated) that was identified as the trans-olefin macrocycle (the cis-olefin was not obtained).

Compound **3** was tested to determine the effect of the P3 aza-peptide modification on in vitro potency.[16] Activity in the biochemical assay against NS3-4A protease (J4) was in the low-micromolar range (IC_{50} = 3.8 μM). Activity in the HCV subgenomic replicon assay was further attenuated 10-fold (EC_{50} = 31 μM). Activity data reported for parent macrocyclic inhibitor **2** are NS3-4A protease IC_{50} = 0.011 μM and replicon EC_{50} = 0.077 μM.[6] Thus, the P3 aza-modification resulted in a loss in activity in both the enzyme inhibition and replicon assays of 350- and 400-fold, respectively. This large loss in potency for **3** is likely the result of a significant difference in overall conformation relative to **2**.

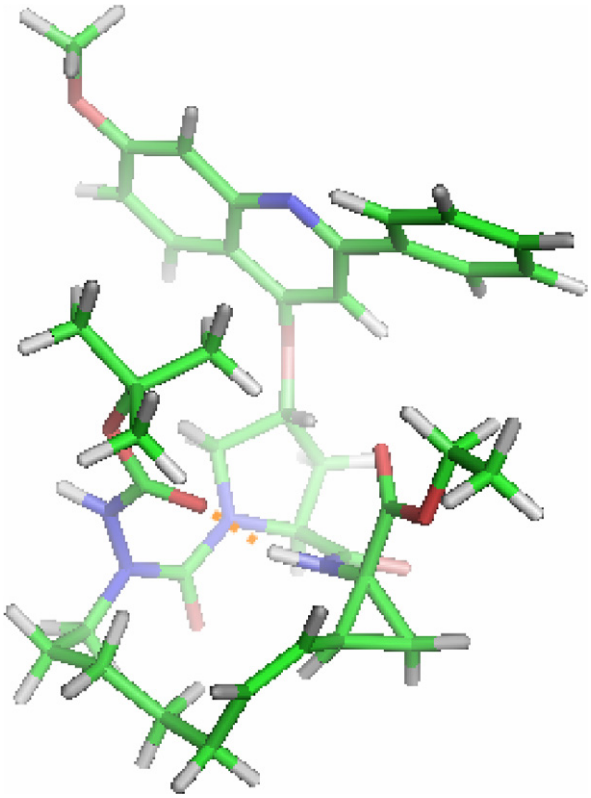


Figure 5. Molecular model of **10** showing that an intramolecular H-bond can exist in the 16-membered ring macrocycle.[21]

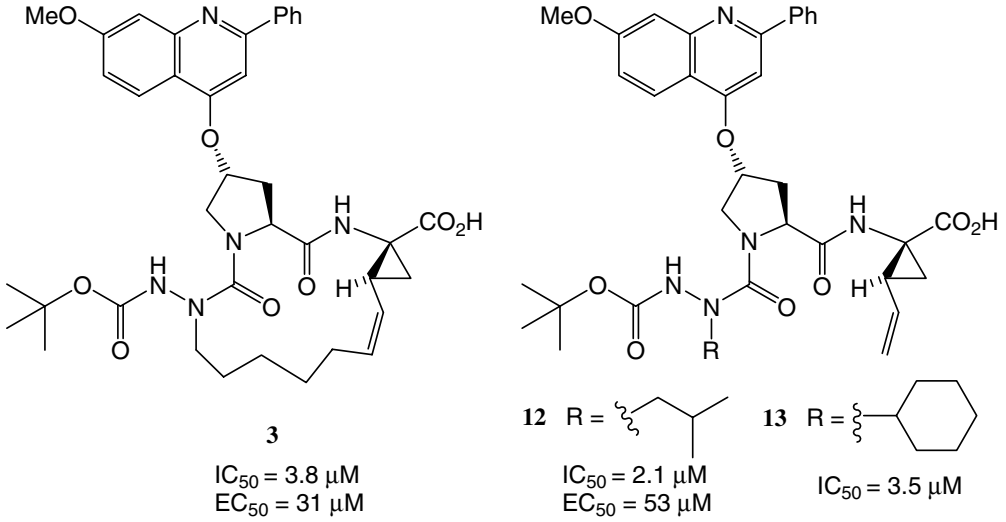
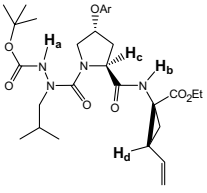
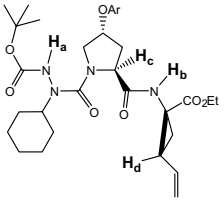
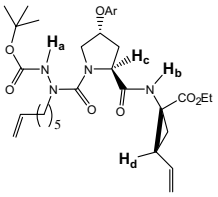
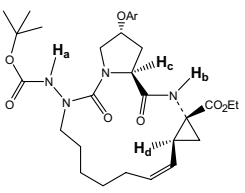
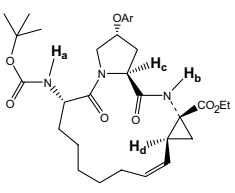
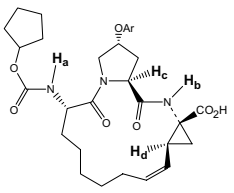


Figure 4. In vitro activity of P3 aza-peptide analogs.

Attempts to determine the conformation of **3** by NMR were complicated due to the complexity of the spectra, especially in $CDCl_3$.[17] For this reason, we were unable to establish the presence of an intramolecular H-bond using the method described for the study of **9**. However, two non-cyclic P3 aza-peptide compounds **12** and **13** (Fig. 4) gave similar biochemical activity to **3** and were determined by NMR study to adopt a β-turn conformation.[18] While acyclic azapeptides such as **9**, **12**, and **13** adopt the β-turn structure, it was unknown whether the additional constraints imparted by the macrocyclic structure of **3** would destabilize the intramolecular H-bond, resulting in a different conformation in solution. We were able to observe evidence for an intramolecular H-bond between the P1 NH and *tert*-butylcarbamate carbonyl oxygen in compound **10**, the ethyl ester of **3**.[19] The chemical shift for P3 NH (H_a) in **10** differs by 2.65 ppm between spectra in $CDCl_3$ and DMSO-d_6, while the P1 NH (H_b) proton is only shifted 0.60 ppm from 7.70 ppm in $CDCl_3$ to 8.30 ppm in DMSO-d_6. The small solvent effect on H_b chemical shift is an evidence that it is involved in an intramolecular H-bond, indicating **10** adopts a β-turn conformation. The somewhat larger solvent effect on H_b in macrocycle **10** in comparison to the solvent effect on H_b in **9** may indicate a weaker H-bonding interaction in the case of **10**, most likely due to conformational constraints in macrocycle **10** not present in acyclic peptide **9**. A weak NOE observed between the P1 NH and the Hα proton at P2 in **10** suggests the overall conformation of this compound differs from that of **9**, where no such NOE is observed. A model of **10** showing a conformation stabilized by an intramolecular H-bond is shown in Figure 5.

Table 1 shows a comparison of NMR data collected for P3 aza-peptide analogs **9**, **10**, **12** (ethyl ester), and **13** (ethyl ester) and macrocyclic tripeptides **1** and **2** (ethyl ester).[20] The difference in chemical shift between $CDCl_3$ and DMSO-d_6 for the P1–P2 amide NH (H_b) for each of the aza-analogs ranges from 0.1 to 0.6 ppm, indicating involvement of this proton in a strong intramolecular H-bonding interaction in each case. Such is not the case for macrocycle **2**, where the chemical shift difference was measured to be 1.69 ppm. The strong NOE observed between H_b and H_c for macrocycles **1** and **2** is absent from NOE spectra for the aza-peptides, with the exception of a weak NOE observed in the case of **10**. Likewise, the NOE observed between the BOC *tert*-butyl and the VCP group (both H_d and one of the olefinic protons on the terminal carbon of the vinyl group) for aza-peptides **9**, **12**, and **13** is absent in the case of **1** and **2**. The NMR data for P3 aza-peptides consistently support a β-turn

Table 1. Comparison of NMR data for P3 aza-peptides and macrocyclic tripeptide HCV PIs

Compound ID	**12** (ethyl ester)	**13** (ethyl ester)	**9**
Structure			
Evidence for β turn	Yes	Yes	Yes
δH_b ($CDCl_3$)	7.82 ppm	8.09 ppm	7.85 ppm
δH_b (DMSO-d_6)	7.99 ppm	8.20 ppm	8.09 ppm
δH_a ($CDCl_3$)	6.57 ppm	6.51 ppm	6.64 ppm
δH_a (DMSO-d_6)	9.55 ppm	9.51 ppm	9.71 ppm
NOE: H_b-H_c ($CDCl_3$)	No	No	No
NOE: H_b-H_c (DMSO-d_6)	No	No	No
NOE: BOC-VCP ($CDCl_3$)	Yes	Yes	Yes
NOE: BOC-VCP (DMSO-d_6)	Yes	Yes	Yes
	10	**2** (ethyl ester)	**1**
Structure			
Evidence for β turn	Yes	No	No
δH_b ($CDCl_3$)	7.70 ppm	7.03 ppm	NA
δH_b (DMSO-d_6)	8.30 ppm	8.65 ppm	8.48 ppm
δH_a ($CDCl_3$)	6.60 ppm	5.30 ppm	NA
δH_a (DMSO-d_6)	9.25 ppm	6.99 ppm	7.00 ppm
NOE: H_b-H_c ($CDCl_3$)	NA	Strong	NA
NOE: H_b-H_c (DMSO-d_6)	Weak	Strong	Strong
NOE: BOC-VCP ($CDCl_3$)	NA	No	NA
NOE: BOC-VCP (DMSO-d_6)	NA	No	No

conformation that is substantially different from the conformation of macrocyclic HCV PIs such as **1** and **2**. Thus, the poor activity of P3 aza-peptide analogs, including macrocycle **3**, s likely the result of conformational constraints due to intramolecular H-bonding preventing the compounds from adopting the necessary extended conformation for tight binding to the active site of NS3 protease.

In conclusion, we have modified a potent macrocyclic tripeptide inhibitor of HCV protease (**2**) by replacing the P3 alpha-carbon with nitrogen, thereby producing a P3 aza-macrocyclic peptide analog. This structural modification resulted in a large loss in inhibitory activity in vitro, as aza-analog **3** was orders of magnitude less active than **2** in both the biochemical and replicon assays. It is believed that the loss in potency is due to conformational constraints on these aza-analogs preventing an extended conformation required for tight binding to the protease active site. Solution NMR studies of P3 aza-tripeptide HCV protease inhibitors indicate these compounds adopt a β-turn structure resulting from intramolecular H-bonding between the P1 NH and N-terminal *tert*-butylcarbamate at P3. Molecular modeling of an aza-peptide analog in the β-turn conformation indicate a D-like configuration at P3. Thus, it is believed that the increased flexibility at the aza-center of **3** relative to the absolute configuration of the P3 L-amino acid in **2**, allowed **3** to adopt an overall conformation that was stabilized by an intramolecular H-bond.

Acknowledgment

The authors thank Kent Stewart for his help in generating molecular models of compounds **9** and **10**.

Supplementary data

Experimental procedures for the synthesis of compound **3**, and detailed NMR data, including 2D spectra in both $CDCl_3$ and DMSO-d_6 for compound **9**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.02.053.

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14. We have observed a strong NOE between the P1 NH and P2 Hα in NMR spectra of non-aza tripeptide HCV PIs, including **1** and **2**. This NOE is consistent with an extended conformation for the peptide portion of these compounds.
15. Our model is consistent with X-ray crystal studies of aza-Asx-Pro dipeptides that reveal a β-turn structure in which the aza-residue exhibits a D-like chirality.[13]
16. For assay procedures, see: Lu, L.; Pilot-Matias, T. J.; Stewart, K. D.; Randolph, J. T.; Pithawalla, R.; He, W.; Huang, P. P.; Klein, L. L.; Mo, H.; Molla, A. *Antimicrob. Agents Chemother.* **2004**, *48*, 2260.
17. The NMR spectra for **3** in both $CDCl_3$ and DMSO-d_6 were characterized by broad signals at room temperature. It was necessary to heat the DMSO-d_6 sample to 60 °C to obtain useful NMR data for analysis.
18. Huang, P. P.; Randolph, J. T.; Flentge, C.; Klein, L. L.; Kurtz, K.; Konstantinidis, A.; Kati, W.; Zhang, X.; Stoll, V. S.; Kempf, D. J. 232nd ACS National Meeting, San Francisco, CA, Sept, 2006; MEDI-176.
19. The P1 NH (H_b) and P3 NH (H_a) in **10** are clearly observed and assigned by 2D NMR in DMSO-d_6 at 60 °C, but are too broad to be assigned directly in $CDCl_3$. The five points titration at 0%, 5%, 10%, 20%, and 50% DMSO in $CDCl_3$, including variable temperature (from 30° C to 80 °C) at each titration point, was used to determine the chemical shift of P1 NH (H_b) and P3 NH (H_a) in $CDCl_3$.
20. Data for ethyl ester analogs are reported due to the relative ease of obtaining quality NMR data in $CDCl_3$ for these compounds compared to the corresponding carboxylic acids. The NMR of **1** in $CDCl_3$ was not obtained.
21. The structures corresponding to compounds **9** (Fig. 3) and **10** (Fig. 5) were energy minimized using the CFF force field within Insight software (Accelrys, San Diego).