Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 2267–2270

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Highly Potent and Selective Peptide-Based Inhibitors of the Hepatitis C Virus Serine Protease: Towards Smaller Inhibitors

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Received 9 June 2000; accepted 28 July 2000

Abstract—Structure–activity studies on a hexapeptide N-terminal cleavage product of a dodecamer substrate led to the identification of very potent and highly specific inhibitors of the HCV NS3 protease/NS4A cofactor peptide complex. The largest increase in potency was accomplished by the introduction of a (4*R*)-naphthalen-1-yl-4-methoxy substituent to the P2 proline. N-Terminal truncation resulted in tetrapeptides containing a C-terminal carboxylic acid, which exhibited low micromolar activity against the HCV serine protease.

Hepatitis C virus (HCV) infection is an important cause of chronic liver disease leading to cirrhosis and end-stage liver disease in humans. Liver failure from chronic hepatitis C is now the leading indication for liver transplantation in the United States. Over 150 million people world-wide are persistently infected with HCV and the number of deaths attributable to chronic infection is likely to rise dramatically over the next decade.[1] Current therapies for HCV disease involve treatment with interferon-α, either alone or in combination with the purine nucleoside analogue ribavirin. However, poor efficacy and relapse after cessation of therapy are frequent.[2] Therefore, hepatitis C is an important emerging infectious disease that warrants significant efforts in the development of novel and more effective treatment strategies.

One of the most intensively studied targets for antiviral therapy against HCV is the serine protease of the NS3 protein.[3] The NS3 protease domain, which constitutes the amino-terminal third of the NS3 protein, is a chymotrypsin/trypsin-like serine protease responsible for the proteolytic cleavage of the nonstructural NS3, NS4A, NS4B, NS5A, and NS5B proteins. The protease activity is enhanced by the NS4A protein, which acts as a cofactor essential to polyprotein maturation.[4] Recent work by Rice et al. has demonstrated that NS3 protease activity is required for HCV replication in the chimpanzee.[5]

We,[6] and others,[7] have shown that the NS3 protease complexed with NS4A cofactor peptide (NS3-4A$_{pep}$) is inhibited by N-terminal cleavage products of peptide substrates. Hexapeptide **1**, the N-terminal cleavage product of a peptide substrate corresponding to the NS5A/5B cleavage site, is an inhibitor of the enzyme (Table 1).[6] Capping the N-terminus of **1** with an acetyl group and replacing the cysteine at P1 with norvaline produced chemically stable hexapeptide **2**, which exhibited moderate potency. Introduction of a D-aspartic acid at the P5 position increased inhibitor potency by ~10-fold (compound **3**).[6,8] In contrast to what is observed with other serine proteases, peptide sequences containing electrophilic carbonyls such as trifluoromethyketone **4** did not offer an overall advantage over the corresponding carboxylic acid.[8] The carboxylic acid functionality at the C-terminus contributes considerably to potency and imparts great specificity to these peptide-based inhibitors of the HCV serine protease. In this Letter, we describe structure–activity studies on this series of C-terminal carboxylic acid inhibitors. These studies led to a substantial increase in potency allowing for a reduction in the peptidic nature of these compounds.

Table 1. Hexapeptides inhibitors of NS3-4A$_{pep}$ protease

Compound	Sequence[b]	IC_{50}, μM[a]
1	DDIVPC-OH	71
2	Ac-DDIVP-Nva-OH	150
3	Ac-DdIVP-Nva-OH	17
4	Ac-DdIVP-Nva-CF_3	22

[a]Values are the mean of at least four determinations.
[b]d = D-aspartic acid; Nva = Norvaline.

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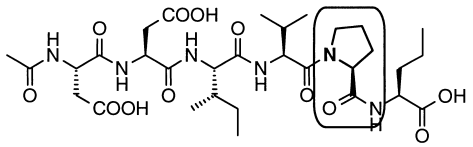
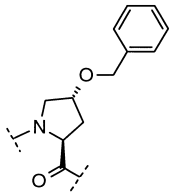
PII: S0960-894X(00)00465-0

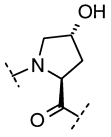
Table 2. SAR at the P2 proline



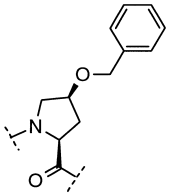
Compound **2** $IC_{50} = 150\mu M$



5 $IC_{50} = 7\ \mu M$

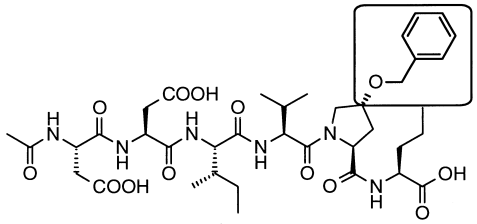


6 $IC_{50} = 93\ \mu M$

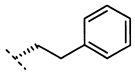


7 $IC_{50} = 206\ \mu M$

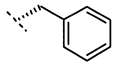
Table 3. Optimization of 4-*R*-Substituent in proline



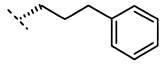
Compound **5** $IC_{50} = 7\ \mu M$



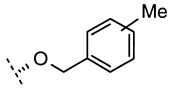
8 $IC_{50} = 10\ \mu M$



9 $IC_{50} = 60\ \mu M$



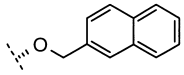
10 $IC_{50} = 3.3\ \mu M$



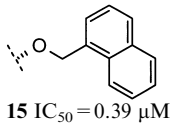
11 (*o*) $IC_{50} = 6.8\ \mu M$

12 (*m*) $IC_{50} = 3.5\ \mu M$

13 (p) $IC_{50} = 2.5\ \mu M$



14 $IC_{50} = 0.71\ \mu M$



15 $IC_{50} = 0.39\ \mu M$

Results and Discussion

Structure–activity relationship (SAR) studies on hexapeptide **2** revealed that the introduction of a benzyloxy group with the (*R*)-stereochemistry at the 4-position of the P2 proline (compound **5**, Table 2) resulted in a 21-fold increase in potency.[9] The importance of the benzyl ring and its stereochemistry is evidenced by compounds **6** and **7**. (*4R*)-Hydroxyproline analogue **6** and (*4S*)-benzyloxyproline derivative **7** have activities similar to the unsubstituted analogue **2**.

The oxygen in benzyloxy derivative **5** does not significantly contribute to the potency since the carbon analogue **8** is equipotent (Table 3). In order to study the optimal distance between the phenyl and proline rings, compounds **9** and **10** were synthesized. A shorter chain length, as represented by compound **9**, is detrimental, but a longer chain length (compound **10**) slightly increases the inhibitor potency.

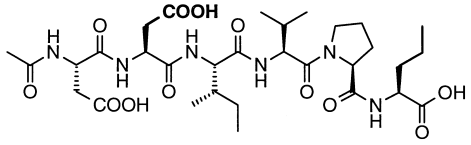
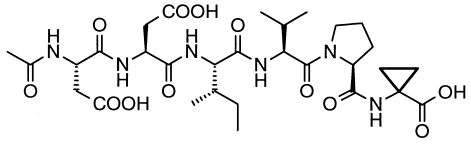
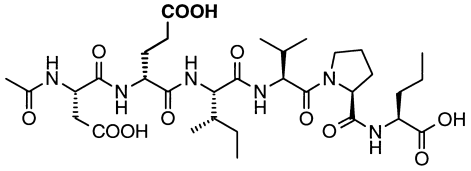
We have previously reported NMR and computational chemistry studies indicating that compound **5** binds the protease in a well-defined extended conformation.[10] In addition, a differential line broadening NMR study showed that the benzyloxy substituent, although quite flexible, is in direct contact with the protease.[10] Therefore, in an attempt to further increase the inhibitory potency, substituents were introduced onto the phenyl ring of compound **5** (Table 3). A methyl group at either the *ortho, meta,* or *para* position of the phenyl ring (compounds **11**, **12**, and **13**) was well tolerated and did not produce a significant change in the potency of these inhibitors. However, a substantial increase in potency was observed by the addition of an additional phenyl ring (compounds **14** and **15**). The naphthen-1-ylmethoxy derivative **15** was 18-fold more potent than the benzyloxy analogue **5** and ~400-fold more potent than analogue **2**, giving rise to inhibitors of the NS3-$4A_{pep}$ serine protease with submicromolar potency.

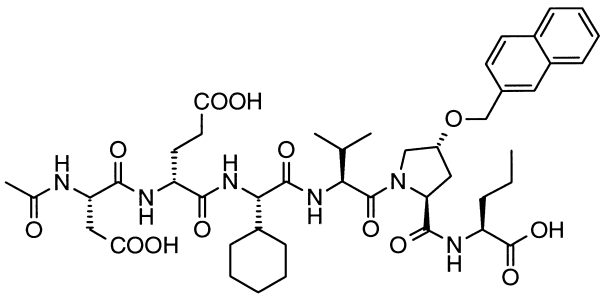
Table 4 presents further optimizations of compound **2**. 1-Amino-cyclopropylcarboxylic acid was identified as a good replacement for the P1 norvaline (compound **16**). In addition, D-glutamic acid at the P5 position was found to be a better substitution than the D-aspartic acid (compound **17** vs **3**, Table 1).

Additivity of beneficial substitutions

Combining the optimal substitutions at each position in the hexapeptide resulted in very potent and competitive inhibitors of the enzyme. Compound **18** (Fig. 1), which contains a (4*R*)-naphthen-2-yl- methoxyproline at P2, a cyclohexylglycine at P4[11] and a D-glutamic acid at P5, has an IC_{50} of 0.027 μM. Kinetic studies showed that compound **18** is a competitive inhibitor of the NS3-$4A_{pep}$ protease with a $K_i = 0.003\ \mu M$.[9] In addition,

Table 4. Optimization of compound **2**

Compound		IC_{50} (μM)
2		150
16		54
17		7.5



Compound 18
IC_{50} = 0.027 μM

Competitive inhibitor K_i = 0.003 μM

Specificity Profile

Human Leukocyte Elastase (HLE)	IC_{50} > 300 μM
Porcine Pancreatic Elastase (PPE)	IC_{50} > 300 μM
α-Chymotrypsin	IC_{50} > 300 μM
Cathepsin B	IC_{50} > 300 μM

Figure 1.

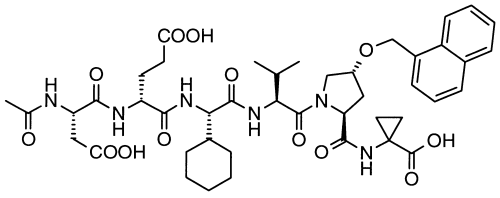
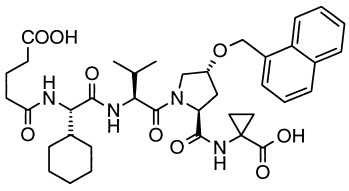
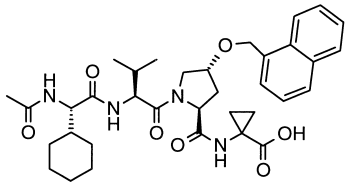
compound **18** is very specific for the HCV serine protease. As shown in Figure 1, compound **18** does not significantly inhibit other serine or cysteine proteases.

Compound **19** (Table 5), which contains a 1-aminocyclopropylcarboxylic acid at P1 in addition to other optimal substitutions, is also a very potent inhibitor of the enzyme.

Size reduction

Having identified low nanomolar NS3-4A_{pep} protease inhibitors, we decided to explore reducing their size.

Table 5. Size reduction

Compound		IC_{50} (μM)
19		0.013
20		0.9
21		3.5

Previous NMR studies[10] showed that compound **5** binds to the protease in a well-defined extended conformation from P1 to P4 with little to no contact observed between the P5 and P6 side chains and the protein. This suggested that N-terminal truncation could be possible. Table 5 shows the effect on inhibitory potency upon N-terminal truncation.

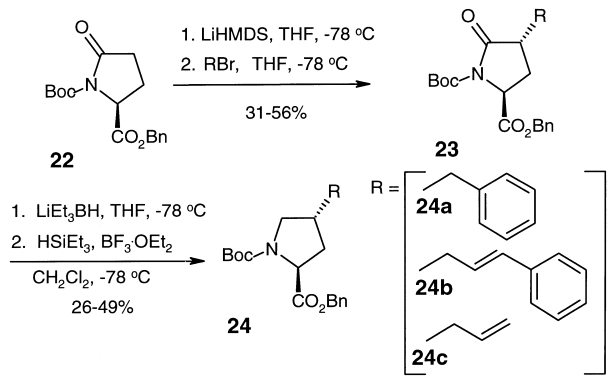
Replacing the Ac-Asp-D-Glu- residues in compound **19** with a 4-carboxybutanoyl moiety afforded compound **20** that is ~70-fold less potent. The 4-carboxybutanoyl capping group in **20** did not contribute much to inhibitor potency as shown by the acetyl tetrapeptide derivative **21**. Compound **21**, with an IC_{50} of 3.5 μM, represents a low micromolar, monocharged inhibitor of the protease. These tetrapeptides are also highly specific as demonstrated by the lack of activity against HLE (IC_{50} >600 μM), a serine protease with related substrate recognition. The NMR-derived bound conformation of one such HCV serine protease inhibitor is reported in the next Letter.

SAR directed to improve the potency of this new series is currently underway.

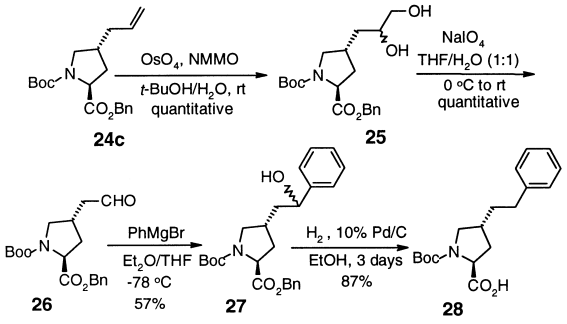
Chemistry

The synthesis of inhibitors was carried out by standard solid-phase or solution-phase peptide chemistry.[12,13] Various 4-substituted prolines were prepared as follows:

a. 4-Arylmethoxyproline derivatives required for the synthesis of compounds **5**, **7**, and **11**–**15** were prepared from the sodium alkoxide of *N*-Boc-(4*R*)-hydroxyproline and various aryl bromides according to the procedure of Smith.[14]
b. The synthesis of (4*R*)-benzyl and (4*R*)-(3-phenyl)-propylproline derivatives (for compounds **9** and **10**, respectively) was achieved via alkylation of pyroglutamate ester **22** according to the procedure of Ezquerra et al. (Scheme 1).[15] The lithium enolate of protected pyroglutamic ester could be stereoselectively alkylated with reactive electrophiles without epimerization of the α-center. The resulting lactams **23** could then be reduced chemoselectively with lithium triethylborohydride followed by further reduction with triethylsilane/borontrifluoride etherate to yield (4*R*)-substitued prolines **24**.[16]
c. The 4-phenethylproline derivative incorporated into compound **9** was prepared as shown in Scheme 2. Starting from allyl derivative **24c**, osmylation of the double bond followed by oxidative cleavage of the resulting diol **25** with sodium periodinate, gave the aldehyde **26** in quantitative yield. A Grignard reaction with phenylmagnesium bromide gave the expected benzylic alcohol **27** in 57% yield. Hydrogenation over palladium on charcoal resulted in hydrogenolysis of both the benzyl alcohol and the benzyl ester to yield the desired (4*R*)-phenethylproline **28**.



Scheme 1.



Scheme 2.

Summary

Structure–activity studies on hexapeptide **2** led to the identification of very potent, specific and competitive inhibitors of the HCV serine protease. The largest increase in potency (~400-fold) was obtained by the introduction of a (4*R*)-naphthen-1-ylmethoxy group to the P2 proline. The combination of (4*R*)-naphthen-1-ylmethoxy proline at P2, D-glutamic acid at P5, cyclohexyl glycine at P4 and 1-amino-cyclopropyl carboxylic acid at P1 generated compound **19,** a low nanomolar inhibitor with an IC_{50} of 13 nM. These compounds are competitive inhibitors of the enzyme as demonstrated for compound **18** with a K_i of 3 nM. The large increase in potency has allowed a reduction in the size and peptidic nature of these compounds. A new series of potent and specific tetrapeptide inhibitors of the NS3-4A$_{pep}$ protease has been introduced with compound **21** which displays an IC_{50} of 3.5 μM.

Acknowledgements

We thank P. Bonneau, C. Plouffe and H. Li for specificity assays, the analytical chemistry department for assistance and S. Kawai for proof reading the manuscript. We also thank P. Anderson, M. Bös, and M. Cordingley for encouragement and support.

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