Letters

Phenethyl Amides as Novel Noncovalent Inhibitors of Hepatitis C Virus NS3/4A Protease: Discovery, Initial SAR, and Molecular Modeling

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Abstract: The discovery of novel, reversible and competitive tripeptide inhibitors of the Hepatitis C virus NS3/4A serine protease is described. These inhibitors are characterized by the presence of a C-terminal phenethyl amide group, which extends into the prime side of the enzyme. Initial SAR together with molecular modeling and data from site-directed mutagenesis suggest an interaction of the phenethyl amide group with Lys-136.

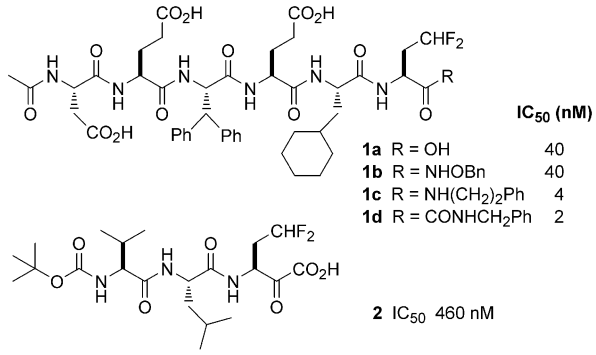
Introduction. Hepatitis C virus (HCV) is the leading cause of chronic non-A non-B hepatitis, an infection which can lead to liver cirrhosis and hepatocellular carcinoma. It is estimated that more than 170 million people worldwide are infected. Currently, the recommended therapy uses a combination of interferon-α and ribavirin, which achieves a sustained virologic response in only 50% of infected individuals and shows various side effects. Thus, there is a clear need for an effective and general therapy of HCV infection.[1,2]

HCV is an RNA virus, whose genome encodes a single polyprotein of about 3000 amino acids, which is processed by host and viral proteases to generate mature structural and nonstructural (NS) viral proteins. A serine protease contained within the NS3 protein is responsible for cleavage of the viral polyprotein at four distinct sites. It has also been demonstrated recently that NS3 protease activity is necessary for HCV infection in chimpanzee,[3] thus making the protease an attractive target for antiviral therapy.[4,5]

The serine protease forms a complex with the viral NS4A peptide, which is required for efficient processing.[5] Structural data for NS3 alone or for the NS3/NS4A complex in the absence or presence of bound inhibitor are available.[6−8] They reveal a shallow and solvent exposed substrate binding region, where the binding energy of the minimal polycarboxy decapeptide substrate is derived from a series of weak lipophilic and electrostatic interactions distributed along its contact surface.[9] The protease is subject to inhibition by its N-terminal hexapeptide cleavage products, which have been used as a starting point for the development of potent peptide inhibitors.[10−12] We have recently described the replacement of the canonical cysteine, found in the P1-position of substrates and product inhibitors with 2-amino-4,4-difluorobutanoic acid (difluoroAbu), leading to inhibitors such as **1a**.[13,14] Subsequently, this chemically nonreactive cysteine surrogate enabled the preparation of stable serine trap based inhibitors, which led to potent tripeptide ketoacids (i.e., **2**).[15]

While attempting to find a replacement for the C-terminal acid contained in product inhibitor **1a** we made the fortuitous observation that benzyl hydroxamate **1b**, prepared as a precursor for the parent hydroxamic acid, was as active as **1a**. The hydroxamate turned out to be a competitive, reversible inhibitor of NS3/4A, and was not a substrate for the enzyme. To answer the question whether **1b** was active as the deprotonated hydroxamate anion, and thus being essentially a productlike inhibitor, or as the neutral ester, the corresponding phenethyl amide **1c** was made, which proved to be 10-fold more potent than **1b**. In addition, **1c** also proved to be a competitive and reversible inhibitor of NS3/4A and was not a substrate for the enzyme.

Amide inhibitors of the NS3 protease have been described recently in the context of noncleavable decapeptide substrates,[16,17] which contain P1-prime substitutions such as proline or tetrahydroisoquinoline-3-carboxylic acid. Also described is the conversion of the C-terminal acid of a product inhibitor into the less potent benzyl amide.[18]



Amide **1c** is structurally related and nearly as potent as the recently described ketoamide **1d**.[14] We were intrigued by the fact that **1c** does not contain the electrophilic group that covalently interacts with the active site serine. We report here the further development of **1c**, which led to the discovery of tripeptide phenethyl amide inhibitors.

We first investigated the SAR in the hexapeptide series, using commercial available amines and exchanging difluoroAbu in P1 for readily available cysteine. Standard peptide coupling procedures were applied for the synthesis of the compounds shown in Tables 1 and

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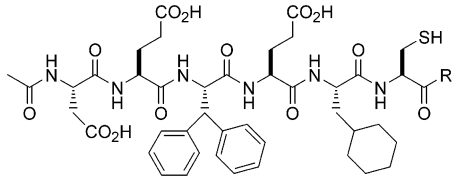
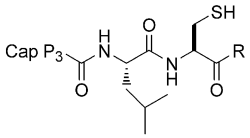
Table 1. Hexapeptide Amide Inhibitors of the HCV NS3/NS4A Protease



compd	R	IC_{50} (μM)[a]
3a	$NH(CH_2)_2Ph$	0.007
3b	$NHCH_2Ph$	0.25
3c	$NH(CH_2)_3Ph$	0.21
3d	$NH(CH_2)_2C_6H_{11}$	1.54
3e	NH-2-indanyl	1.70
3f	NH-*trans*−2-(cyclopropyl)Ph	1.50
3g	$NMe(CH_2)_2Ph$	70.0
3h	$NH(CH_2)_2$(2-Cl-Ph)	0.002

[a] Data are the means of two independent experiments.

Table 2. Truncation to Tripeptide Inhibitors and Initial SAR



compd	Cap-P3	R	IC_{50} (μM)[a]
4a	Cbz-Val	$NH(CH_2)_2Ph$	>400
4b	Cbz-Glu	$NH(CH_2)_2Ph$	40
4c	Boc-Glu	$NH(CH_2)_2Ph$	27
5a	Boc-Glu	$NHCH_2Ph$	>200
5b	Boc-Glu	$NH(CH_2)_2$(2-Cl−Ph)	6.5
5c	Boc-Glu	OH	>200

[a] Data are the means of two independent experiments.

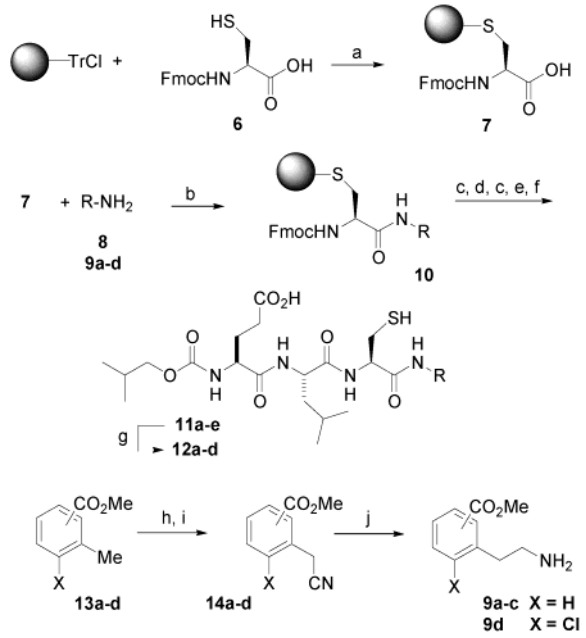
2 (see Supporting Information). They were evaluated using the HCV NS3 protease domain and an NS4A cofactor peptide (see Supporting Information).

Results for hexapeptides **3** are shown in Table 1. As expected, compound **3a**, having cysteine instead of difluoroAbu in P1, was equipotent to **1c**. Activity strongly depends on the length of the amide chain, the benzyl and the phenylpropyl analogues **3b** and **3c** being significantly less active. The phenyl substituent is important, since its replacement by cyclohexane resulted in 220-fold loss in potency (**3d**, IC_{50} 1.54 μM). This level of activity was also observed when the ethylamine chain forms part of an Indane (**3e**, IC_{50} 1.70 μM) or cyclopropyl (**3f**, IC_{50} 1.50 μM) ring system. *N*-Methylation of the amide is detrimental to activity as **3g** shows. Chlorine and methoxy groups were tolerated in all positions of the phenyl ring, but only the *o*-chloro derivative **3h** was more potent than **3a**.

We incorporated the phenethyl amide in place of the α-keto acid found in compounds similar to **2**. Surprisingly, amide **4a** did not display any activity up to 400 μM (Table 2).

Hypothesizing that a carboxylic acid might be needed to facilitate binding to the polycationic surface of the enzyme, we prepared **4b** and **4c**, exchanging the valine residue of **4a** for glutamic acid. Both amides were weak inhibitors with IC_{50}'s of 40 μM and 27 μM, respectively. On the basis of the SAR of the hexapeptide series, some selected amide derivatives of **4c** were prepared, which confirmed the previous SAR. The benzyl amide **5a** is

Scheme 1. Solid-Phase Synthesis of Tripeptide Amides[a]



[a] Reagents and conditions: (a) 7% TFA in DCM, rt; (b) 5 equiv of RNH_2, EDCI, HOBt, i-Pr_2NEt, DCM, rt; (c) 20% piperidine, DMF; (d) 5 equiv of FmocLeuOH, EDCI, HOBt, DCM; (e) 5 equiv of *i*-BocGlu(O^{t}Bu)OH, EDCI, HOBt, DCM; (f) TFA, Et_3SiH, DCM (g) for **11b**−**e**: NaOH, MeOH, THF; (h) NBS, CCl_4; (i) NaCN, DMSO, for **13d** *n*-Bu_4NCN, MeCN; (j) H_2, Pd/C, for **13d** $CoCl_2$, $NaBH_4$, MeOH, rt.

essentially inactive, and the *o*-chloro phenyl derivative **5b** is 4-fold more active (IC_{50} 6.5 μM) than the parent phenyl. Product inhibitor **5c** is essentially inactive.

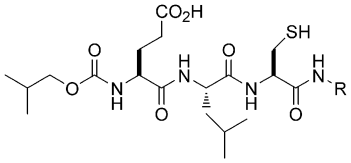
Encouraged by this result we decided to further probe the SAR in this series. To achieve this in a rapid way, we took advantage of the presence of the cysteine thiol in our molecules and developed the solid-phase approach shown in Scheme 1. The cysteine derivative **6** was loaded onto polystyrene trityl chloride resin to give resin **7**. That alkylation took place exclusively on the thiol and not on the acid was evidenced after coupling of amines. Cleavage from the resulting resin **10** produced only the desired cysteine amide coupling products in nearly quantitative yield. No trace of **6** could be detected by HPLC or NMR.

Our first round of optimization concentrated on the amide portion and kept the tripeptide fragment of **5c** constant. As N-terminal capping group we chose isobutyl carbamate, because of its stability to trifluoroacetic acid used in the final cleavage step.

Thus, after sequential coupling of the leucine and glutamic acid residues, the tripeptides **11a**−**e** were obtained in good yield and purity. The ester groups in **11b**−**e** were hydrolyzed using sodium hydroxide, giving the acids **12a**−**d**. All final compounds were then purified by RP-HPLC. The 2-aminoethyl benzoic acids **9a**−**d** used in this study were prepared as shown in Scheme 1.

Of a wide variety of substituents that we introduced in the phenyl ring of **11a** (IC_{50} 19 μM, Table 3), the benzoic acid derivatives proved to be the most interesting. The *ortho* benzoic acid **12a** is not active up to 100 μM, whereas the corresponding methyl ester **11b** is tolerated (IC_{50} 54 μM). The *meta* ester **11c** and its *para* isomer **11d** are equipotent with **11a**, but for the corre-

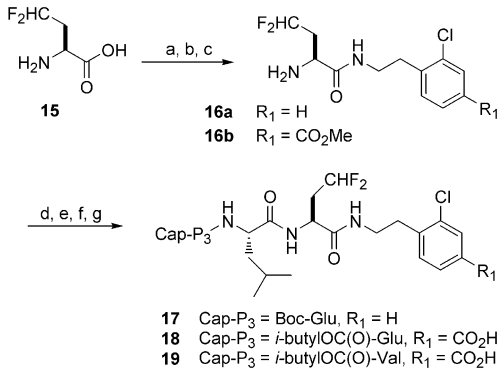
Table 3. Inhibition of NS3/4A by Tripeptide Phenethyl Amides



compd	R	NS3 IC_{50} (μM)[a]
11a	$(CH_2)_2$-Ph	19
11b	$(CH_2)_2$-(Ph-2-CO_2Me)	54
11c	$(CH_2)_2$-(Ph-3-CO_2Me)	25
11d	$(CH_2)_2$-(Ph-4-CO_2Me)	17
11e	$(CH_2)_2$-(2-Cl-Ph-4-CO_2Me)	3.2
12a	$(CH_2)_2$-(Ph-2-CO_2H)	>100
12b	$(CH_2)_2$-(Ph-3-CO_2H)	9.2
12c	$(CH_2)_2$-(Ph-4-CO_2H)	2.0
12d	$(CH_2)_2$-(2-Cl-Ph-4-CO_2H)	0.7

[a] Data are the means of two independent experiments.

Scheme 2[a]



[a] (a) CbzCl, Na_2CO_3, dioxane, water; (b) RNH_2, EDCI, HOBt, DCM; (c) TfOH, DCM; (d) BocLeuOH, EDCI, HOBt, DCM; (e) HCl, MeOH; (f) for **17**: BocGlu(OBn)OH EDCI, HOBt, DCM; for **18**: *i*-Boc-Glu(OMe)OH, EDCI, HOBt, DCM; for **19**: FmocValOH, EDCI, HOBt, DCM; pip/DMF; *i*-BuOCOCl; (g) NaOH, MeOH.

Table 4. [a,b]

enzyme	**17** K_i (μM)	**18** K_i (μM)	**19** K_i (μM)
WT	7.3 ± 1.0	0.60 ± 0.1	2.1 ± 0.4
HLE	>100	>100	–
K136R	8.3 ± 1.8	0.9 ± 0.2	2.3 ± 0.8
K136M	13.5 ± 3.2	1.3 ± 0.1	4.0 ± 1.5

[a] For structures, see Scheme 2. [b] Data are the means of three to four independent experiments.

sponding acids an improvement in potency is observed, indicative of an interaction of the acid with a basic amino acid of the enzyme. The *meta* isomer gains 2-fold with respect to **11a**, whereas a 9-fold improvement in potency is observed for the *para* acid **12c**. Finally, introduction of the *o*-chloro substituent gave **12d** with sub-micromolar activity against NS3/4A.

We then exchanged the cysteine for difluoroAbu in some active compounds. These were prepared from L-difluoroAbu as shown in Scheme 2.[14,19] As established previously in other series, **17** and **18** were equipotent with their cysteine counterparts (Table 4). Compound **19**, containing valine instead of glutamic acid in P3, was 3-fold less potent than **18**, but now contains only one carboxylic acid.

The activity of **17** and **18** against human leukocyte elastase (HLE) was determined, since this enzyme also

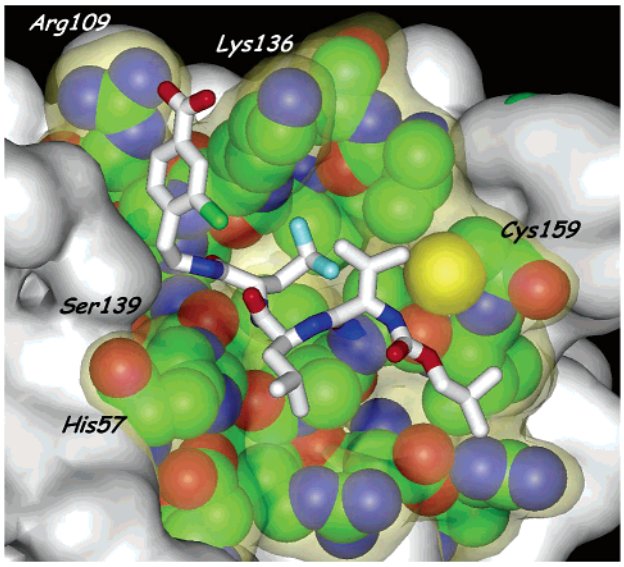


Figure 1. Model of compound **19** bound in the active site of NS3/NS4A.

prefers small hydrophobic side-chains in its S1-pocket.[20] No inhibition was observed up to 100 μM (Table 4).

The SAR obtained for the Cap-P3−P2−P1-fragment in this series is consistent with the previous tripeptide ketoacid SAR.[15] This indicated that the P3 to P1 residues of the phenethyl amide peptide bind in their respective subsites, while the phenylethyl amide part engages in favorable interactions in the prime side of the protease. Active site binding is also in accordance with kinetic data: **18** was a reversible, competitive inhibitor with K_i = 0.60 μM. Under pre-steady-state conditions, the compound quantitatively displaced an active site fluorescent probe[13] with $k_{on} = 3.6\ 10^6\ M^{-1}\ s^{-1}$ and $k_{off} = 3.2\ s^{-1}$, from which a $K_i = 0.88\ \mu$M can be calculated. Similar results were obtained with **19**.

Molecular modeling of the tripeptides into the active site of NS3 suggested Lys136 as a possible residue for interaction with the phenethyl amide. This amino acid is conserved in all strains of HCV and is crucial for recognition and selectivity observed with product inhibitors and α-ketoacids.[10,13]

To test this hypothesis, the activity of **17**, **18**, and **19** was determined on two NS3 mutants, Lys136Arg and Lys136Met.[10]

The results in Table 4 show that the hydrophobic part of the side-chain of Lys136 is as important as its amino group for binding of phenethyl amides. Compounds **17**−**19** retain activity on the Lys136Arg mutant and lose only 2-fold on the mutant enzyme containing the neutral methionine instead of Lys136.

Based on these data and the cocrystal structures of NS3/NS4A with ketoacid inhibitors,[8] a model for **19** bound in the active site was constructed. The P1, P2, and P3 residues as well as the capping group bind in the same way as in the corresponding ketoacid structures. The phenyl ring of the phenethyl amide is stacked against the aliphatic part of Lys136 and interacts also with Arg109. These interactions are mainly of a lipophilic nature although cation−π interactions with Arg109 may play a role. In the Lys136Met enzyme, which is inhibited by amides **17**−**19** to nearly the same extent as the wild-type NS3, these favorable lipophilic interac-

tions are maintained. The model also shows that introduction of the carboxylate into the para position as in **19** is most favored due to close contacts with the charged groups of both Lys136 and Arg109. Introduction of the carboxylate into the *ortho*-position places it into an apolar environment where the energy penalty for desolvation is detrimental for activity. Compared to the substrate, the amide function of the phenethyl amide is slightly shifted out of the active site into the direction of Lys136 (Figure 1), and we speculate that this shift may increase the reaction barrier sufficiently to suppress cleavage.

In summary, a new series of reversible, competitive inhibitors of HCV NS3 protease has been developed, which are characterized by the presence of a phenethyl amide group in the P1-prime position. While tripeptide ketoacid **2** (IC_{50} 0.46 μM) is more potent than phenethyl amides **18** or **19**, these inhibitors lack the electrophilic carbonyl and explore novel interactions in the prime side of the NS3/4A protease, which renders them highly specific. The SAR, mutagenesis data, and molecular modeling suggest a precise binding arrangement for the phenethyl amide ring against the lipophilic chain of lysine-136.

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Supporting Information Available: Experimental section including information on synthesis and biological evaluation for compounds **3a**, **4c**, **5b**, **11e**, **12d**, **17**, **18**, **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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