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Evolution, Synthesis and SAR of Tripeptide α-Ketoacid Inhibitors of the Hepatitis C Virus NS3/NS4A Serine Protease

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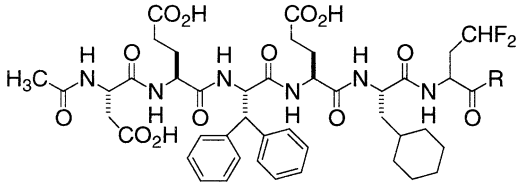
Abstract—N-Terminal truncation of the hexapeptide ketoacid **1** gave rise to potent tripeptide inhibitors of the hepatitis C virus NS3 protease/NS4A cofactor complex. Optimization of these tripeptides led to ketoacid **30** with an IC_{50} of 0.38 μM. The SAR of these tripeptides is discussed in the light of the recently published crystal structures of a ternary tripetide/NS3/NS4A complexes. © 2002 Elsevier Science Ltd. All rights reserved.

In the preceding paper, we described the design and successful implementation of 2-amino-4,4-difluorobutyric acid (difluoroAbu) as a substitute for the canonical cysteine found in substrates and product inhibitors of the hepatitis C virus (HCV) NS3/NS4A serine protease.[1] This protease is essential for the viral lifecycle and therefore presents a prime target for the development of antiviral drugs.[2,3] Avoiding the reactive thiol allowed the preparation of potent hexapeptide inhibitors **1**–**3** of this enzyme, from which ketoacid **1** emerged as an exceptionally potent inhibitor.[4] Since polypeptides, especially those containing multiple carboxylates, are unlikely to become orally bioavailable, cell-penetrant drugs, our next goal was to reduce the size and anionic nature of the hexapeptides.



1 R = CO_2H K_i* 0.01 nM
2 R = H K_i 0.5 nM
3 R = OH K_i 40 nM

In this letter, we describe the structure–activity studies leading from hexapeptide **1** to tripeptide inhibitors. Modeling these inhibitors into the active site of the enzyme guided the SAR, which led ultimately to the discovery of potent tripeptide keto acids, containing only one carboxylic acid. The findings will be discussed in conjunction with the crystal structures obtained from two tripeptides as ternary adducts with the protease and its NS4A co-factor.[5]

Synthesis

The synthesis of ketoacids has been described in part,[4] and examples are shown in Scheme 1. Condensation of **31**, readily obtained from the parent amino acid,[1] with (cyanomethylene)phosphorane by modifying the procedure by Wasserman and co-workers,[6] gave, after removal of the Cbz group, amine **32** in high overall yield. Coupling to CbzLeuOH and deprotection then furnished **33**, which was coupled to Boc-cyclopentyl glycine. Ozonolysis of this intermediate in dichloromethane in the presence of methanol led to the crude ketoester, which was hydrolyzed to ketoacid **30**.

Coupling amine **32** to dipeptide CbzIle-LeuOH followed by deprotection gave tripeptide phosphorane **34**, which was used for investigating the capping group SAR. For example, **34** was alkoxycarbonylated with the mixed succinimide carbonate obtained from methyl 4-(hydroxymethyl)-benzoate and *N*,*N*′-disuccinimidyl carbonate.[7] Ozonolysis and quenching the intermediate cyano ketone in water gave keto acid **17**. Hydrolysis of the N-terminal ester then led to **18**.

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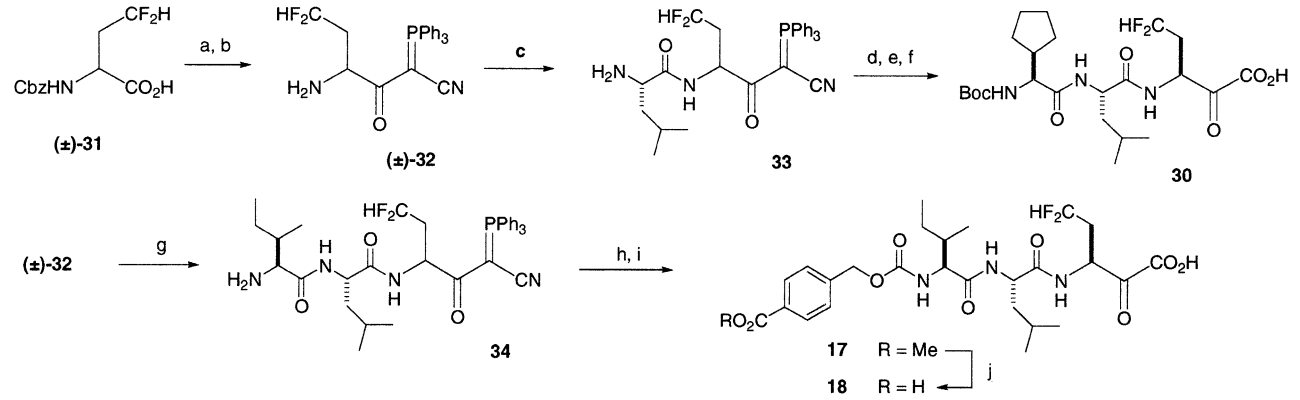
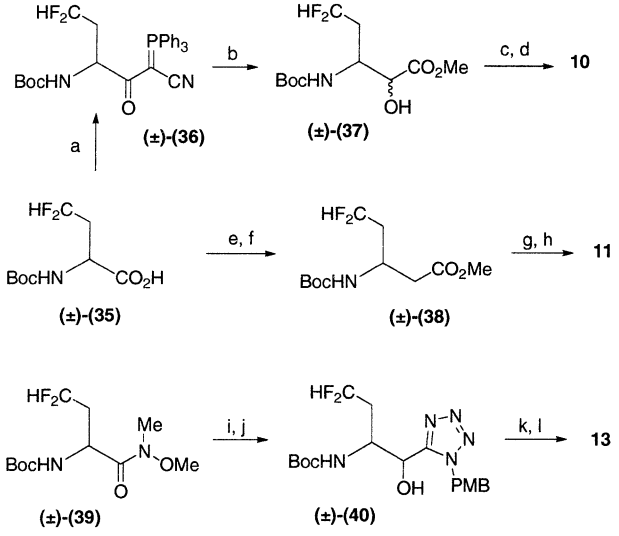


Scheme 1. (a) Ph_3P=CHCN, EDCI, HOBt, DCM, 82%; (b) NH_4CO_2, MeOH, Pd/C, 80%; (c) CbzLeuOH, EDCI, HOBt, DCM, 88%; NH_4CO_2, MeOH, Pd/C, 78%; (d) Boc–*c*-pentylglycine, EDCI, HOBt, DCM; (e) O_3, DCM, MeOH −78 °C; 68%; (f) MeOH, 1 N NaOH; RP-HPLC; (g) CbzIleLeuOH, EDCI, HOBt, DCM, 80%; NH_4CO_2, MeOH, Pd/C, 87%; (h) methyl 4-(hydroxymethyl)benzoate, DSC, NEt_3, MeCN; evaporate, add **34**, DCM, 55%; (i) O_3, DCM,−78 °C; THF, H_2O; RP-HPLC, 21%; (j) 1 N NaOH, MeOH; RP-HPLC, 56%.



Scheme 2. (a) Ph_3P=CHCN, EDCI, DMAP DCM, 54%; (b) O_3, DCM, MeOH −78 °C; $NaBH_4$, MeOH, 62%; (c) HCl/EtOAc; CbzLeuOH, EDCI, HOBt, *i*-Pr_2NEt DCM, 76%; (d) NaOH, MeOH, quant; (e) IBCF, NMM, THF; CH_2N_2, 79%; (f) AgBz, NEt_3, MeOH, 78%; (g) HCl/EtOAc; CbzLeuOH, EDCI, HOBt, *i*-Pr_2NEt DCM, 92%; (h) NaOH, MeOH, 27%; (i) PMB-tetrazole, BuLi, THF, TMEDA, −98 °C, 30%; (j) $NaBH_4$, EtOH, 88%; (k) HCl/EtOAc; CbzIleLeuOH, HATU, 2,6-lutidine, DCM, 92%; (l) CAN, MeCN, H_2O; DMP, *t*-BuOH, MeCN; RP-HPLC, 15%.

All crude ketoacids were purified by HPLC with separation of the diastereomers resulting from the use of racemic difluoroAbu. The more active diastereomer eluted first and was later shown by X-ray crystallography to have the L-configuration at P_1.[5] The other diastereomer was usually one order of magnitude less active (results not shown).

The building block for aldehyde **12** was synthesized as described.[1] Hydroxyester **10** was prepared as shown in Scheme 2. Homologation at **35** using diazomethane[8] led to acid **11**. Keto tetrazole **13** was prepared by reacting amide **39** with the anion of the PMB-protected tetrazole.[9] Reduction and deprotection led to hydroxy amine **40**, which after coupling, removal of the PMB group and oxidation of the alcohol, furnished **13**.

Table 1. Truncation of inhibitor **1**

Compd		IC_{50} (μM)
4	Ac-Dif-Glu-Cha-N(H)-C(CHF$_2$)(CO$_2$H)-O	5.3
5	Ac-Dif-Glu-Cha-N(H)-C(CHF$_2$)(H)-O	>50
6	Ac-Dif-Glu-Cha-N(H)-C(CHF$_2$)(OH)-O	>50
7	Cbz-Ile-Cha-N(H)-C(CHF$_2$)(CO$_2$H)-O	1.4
8	Cbz-Ile-Leu-N(H)-C(CHF$_2$)(CO$_2$H)-O	1.7

Results and Discussion

Data for inhibition of the NS3/NS4A complex were obtained after 30-min incubation[10] with the inhibitor.

Truncation of the P5 and P6 acid anchor, which is usually crucial for activity in the hexapeptide product-based inhibitors, was uniquely successful in the keto acid series, giving rise to tetrapeptide ketoacid **4** (IC_{50} 5.3 μM). The corresponding aldehyde **5** and product acid **6** were essentially inactive (Table 1).

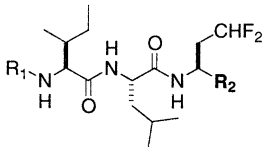
Encouraged by this result, further truncation was attempted. Initially the Cbz-protected compounds **7** and **8** were made, with the intention to partially mimic the P_4 residue with the Cbz-capping group. They proved to be more active than tetrapeptide **4**, probably due to a better contact of the phenyl ring in the S_4-site. More importantly these inhibitors contain only a single

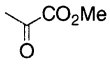
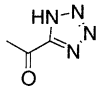
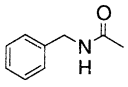
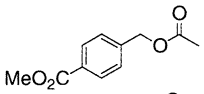
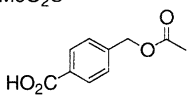
carboxylate and have reduced molecular weight. As with ketoacid **1**, the potency of these tripeptides is due to the slow dissociation rate of the covalent adduct, whereas the affinity of product inhibitors **2,** and also aldehyde **3** is determined by high electrostatically driven association rates.[11]

Compound **8** was then used as a starting point for optimization of the tripeptide series.

Attempts to modify or replace the ketoacid moiety were unsuccessful (Table 2). Ketoester **9**, hydroxy-acid **10**, homologated acid **11** and aldehyde **12** were inactive, pointing to a crucial role played by the carbonyl group *and* the acid in the ketoacid moiety.

Table 2. C- and N-terminal SAR of tripeptide ketoacids



No.	R_1	R_2	IC_{50} (μM)
8	Cbz		1.7
9	Cbz		> 50
10	Cbz		> 50[a]
11	Cbz		> 50
12	Cbz		> 50[b]
13	Cbz		> 50[c]
14			3.3
15	Boc		1.0
16	Ac		16
17			1.7
18			0.44

[a]Mixture of four diastereomers.
[b]Compound contains Cha in P_2.
[c]2:1 mixture of diastereomers.

Also, ketotetrazole **13**, envisioned as a stable and pharmacologically more acceptable replacement of the ketoacid, was inactive.

These observations can be explained on the basis of the properties of the active site. In the X-ray crystal structure of NS3/4A bound with inhibitor **8**, the expected covalent bond between the catalytic serine (S139) and the ketone is formed. However, the configuration at the ketal carbon is different from that usually observed, where the oxyanion is bound in the oxyanion hole.[5] Here this cavity, formed by the backbone of Ala137, Ser138 and Ser139, is occupied by the carboxylate. The conserved Lys136 proximal to the active site favors this interaction (Fig. 1),[5] which may explain the specific need for a ketoacid at this position. Due to the limited size of the oxyanion hole and its rigidity, the tetrazole moiety can not be accommodated there in this orientation.

The SAR in the capping group was investigated only briefly (Table 2). Conversion of the benzyl carbamate to the benzyl urea gave a less active compound. The Cbz group could be replaced by the Boc group, without loss of potency, as in **15**. That the lipophilic part of the capping group makes a significant contribution to binding was shown with acetyl amide **16**, which is 16 times less active than **15**. In the X-ray crystal structure the capping group interacts with Val158 (Fig. 1), thus explaining the preference for hydrophobic residues. Modeling suggested that an acid in the *para*-position of the Cbz ring could exploit the same electrostatic interactions of the P_6-Glu with Arg123. Consistent with this hypothesis, acid **18** showed an improvement in potency (IC_{50} 0.44 μM), while methyl ester **17** retained the same potency as **8**.

Lipophilic contact with the enzyme is also essential in the P_2-position, as replacement of Cha or Leu in **7** and **8** shows (Table 3). The glycine compound **19** is devoid of activity, probably also for conformational reasons. Going from Ala (**20**, IC_{50} 16 μM) to the more hydrophobic Abu (**21**, IC_{50} 3.0 μM) and then to **7** and **8**, it

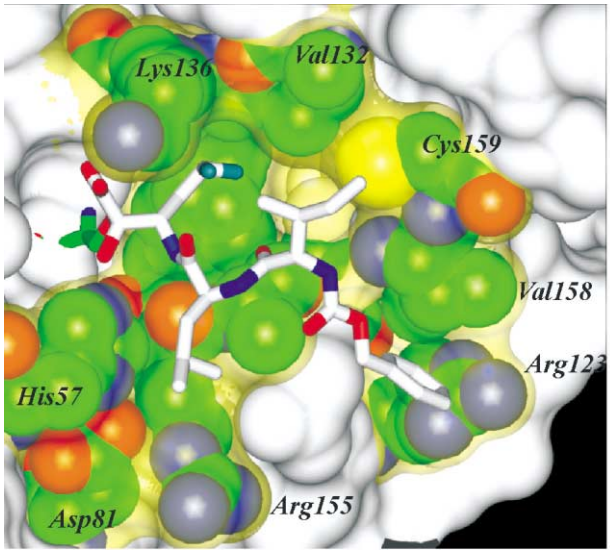
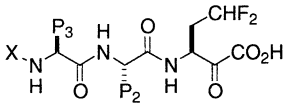


Figure 1. Inhibitor **8** bound in the active site of the NS3/NS4A complex.

Table 3. P_2 and P_3 SAR of tripeptide ketoacids



No.	X	P_3	P_2	IC_{50} (μM)
19	Cbz	Ile	Gly	>100
20	Cbz	Ile	Ala	15.6
21	Cbz	Ile	Abu	3.28
22	Cbz	Ile	Phe	9.4
23	Cbz	Ile	Val	16.0
24	Boc	Glu	DifluoroAbu	0.30
25	Boc	Glu	Leu	0.33
26	Boc	Val	Leu	0.46
27	Boc	Gln	Leu	2.10
28	Boc	Asp	Leu	1.63
29	Boc	Ala	Leu	8.90
30	Boc	*c*-Pentylgly	Leu	0.38

becomes clear that activity is related to the hydrophobicity of the side chain. Alkyl is preferred over phenyl (cf., **7** to **22**, IC_{50} 9.4 μM), and β-branched hydrophobic amino acids such as Val (**23**, IC_{50} 16 μM) are excluded by the architecture of this site. These observations are consistent with earlier SAR in the product inhibitor hexapeptide series.[12]

Recalling that the NS5A/5B substrate for NS3 has cysteine in P_1 and P_2, substitution with our cysteine mimetic was also tried in this position. Inhibitor **24** proved to be equipotent to **25**. The difluoromethyl group probably acts here as a purely hydrophobic group, although electrostatic components may have a role in binding.[13,14]

In the crystal structure (Fig. 1), the lipophilic P_2 group covers the catalytic ion pair His57-Asp81, thus stabilizing it in two ways: (1) by reducing its conformational flexibility, and (2) by shielding it from solvent. We presume that this may influence active site binding through subtle changes in the pK_a of the amino acids involved.[15]

Both hydrophobic and hydrophilic groups are accepted in P_3. Replacing isoleucine in **8** with valine or glutamic acid, both found in substrates of NS3, increased potency (Table 3). The interactions in the S_3 site were quite specific. Converting glutamic acid into glutamine as in **27** or aspartic acid as in **28** resulted in a 6- or 10-fold decrease in potency, respectively. Likewise, replacement of Val with Ala gave a much weaker compound (**29**, IC_{50} 8.9 μM).

Modeling studies, confirmed by the X-ray structure, showed that the lipophilic groups interact with Cys159 and Val132, and the carboxylate groups can have electrostatic interactions with Lys136 (Fig. 1). The longer side-chain of Glu versus Asp allows the carboxylate to be in close vicinity to Lys136 and the extra methylene augments the hydrophobic contact.

To optimize the hydrophobic interactions in the S_3 site, a variety of natural and non-natural hydrophobic amino acids were screened. Cyclopentylglycine emerged as the optimal substituent in this position (**30**, IC_{50} 0.38 μM), being nearly equipotent to the glutamic acid containing compound **24**.

In summary, we have described the evolution of tripeptide ketoacid inhibitors from a polyanionic hexapeptide, showing that the P_5 and P_6 acids are not needed for activity. Also the P_4 residue could be eliminated, although the capping group fulfills part of the role of this group. The structural data collected helped in understanding the observed SAR. We believe that these results may provide a basis for the difficult task of finding more potent and less peptidic inhibitors of NS3/NS4A protease.

Acknowledgements

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References and Notes

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