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P1 and P3 optimization of novel bicycloproline P2 bearing tetrapeptidyl α-ketoamide based HCV protease inhibitors

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Abstract—With the aim of discovering potent and selective HCV protease inhibitors, we synthesized and evaluated a series of **1a** based tetrapeptidyl ketoamides with additional modification(s) at P1′, P1, and P3 positions. As a result of this effort, we found that replacement of the P3 valine with *tert*-leucine resulted in the discovery of a series of inhibitors (e.g., **3a**, **3c**, and **4c**) endowed with improved enzyme and/or cellular activity relative to **1a**. When dosed to F-344 rats orally at 50 mg/kg, **3a** achieved 2.5× higher liver and plasma exposure in comparison to that detected with **1a**.

1. Introduction

Heptatitis C virus (HCV) infection has become a major health problem. It has been estimated that about 170 million people worldwide are infected with HCV, including 3.9 million (~1.8% population) in the USA. About 70% of HCV infections become chronically infected, approximately 20% of those patients will develop cirrhosis that can eventually lead to hepatocellular carcinoma.[1,2] The common routes of HCV transmission include blood transfusion, sexual activity, intravenous drug abuse, needle-stick infection in health care workers or those occurring as a result of acupuncture, tattoos, routine dental care, or surgery. The best treatment options for HCV are pegylated-INFs such as Pegasys and PEG-Intron.[3,4] The response rate thus obtained with HCV genotype-1 patients is far less than ideal. In view of this unmet medical need, we launched a research program aiming at designing potent and selective inhibitors to suppress the function of the HCV serine protease, an essential enzyme responsible for viral replication.[5,6] As disclosed in our previous publication, we focused our research efforts on the design and evaluation of a novel class of bicycloproline P2 bearing α-ketoamides as HCV NS3 protease inhibitors.[7] As documented in our previous paper detailing the comparative evaluation of various P2 modified bicycloproline bearing peptidyl ketoamides, we discovered that the deoxy bc-Proline P2 containing inhibitor LY514962 (**1a**) displayed best overall profiles (enzyme and cellular activity and liver drug exposure) within the inhibitors discussed therein.[7a]

To further improve upon **1a**, we decided to take advantage of the findings reported by Pessi and Narjes on P3 and P1 SAR, respectively. Recently Pessi discovered that other hydrophobic P3 moieties (e.g., IIe, Abu, Nva) can be used as P3 Val replacements.[8a] On the other hand, Narjes reported that diFAbu can mimic the P1 Cys moiety very well. Thus, a number of P1 diFAbu bearing peptidyl α-ketoacids exhibited very potent enzyme inhibitory activity against HCV NS3 protease inhibitors (see Fig. 1).[8b] As can be seen in Figure 1, we incorporated *tert*-leucine at P3 (as seen in **3** vs valine in **1**) and diFAbu at P1 (as seen in **2** vs nvaline in **1**) in hopes of increasing hydrolytic stability and/or inhibitory activity of the resulting HCV protease inhibitors. In conjunction with P1′ variation, further combination of P1 and P3 modifications led to another set of enzyme inhibitors **4a**–**c**.

In this communication, we report herein our recent progress achieved through **1a** based SAR modifications at P1, P3, and P1′ positions as depicted in Figure 1. These efforts have culminated in the discovery of LY526181 **3a**, a very promising HCV protease inhibitor endowed with balanced enzyme activity, enzyme selectivity, cellular activity, liver exposure and favorable toxicity profiles.

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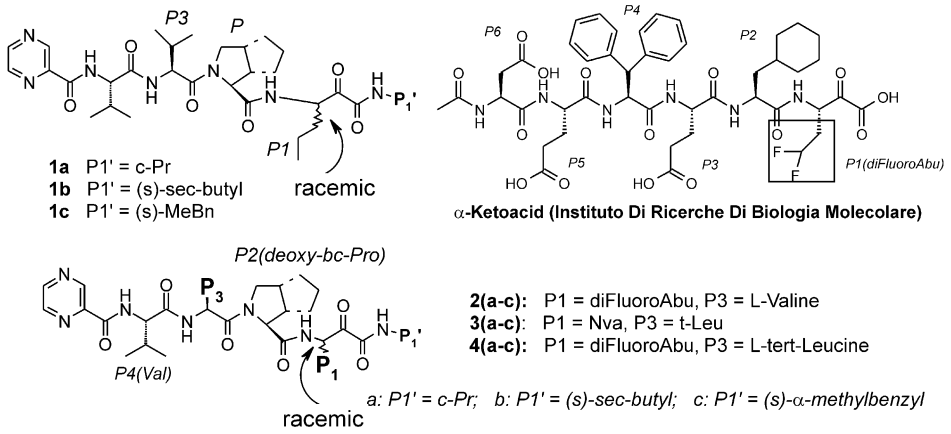


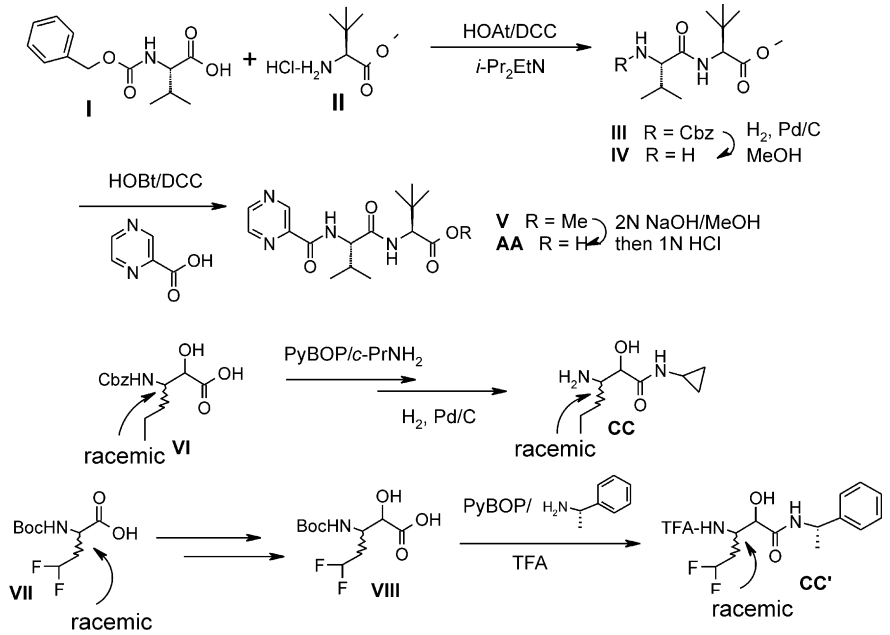
Figure 1. P1 and P3 modified bc-proline P2 bearing inhibitors.

The syntheses of both P1′ and/or P3 modified inhibitors were accomplished via sequential coupling of the building blocks **AA**, **BB**, and **CC**/**CC**′ as shown in Scheme 2. The preparation of the deoxy-bc-proline P2 unit (**BB**) as well as the *N*-pyrazine capped Val-Val-OH (**AA**′) were described previously.[6] The synthetic routes employed for the preparation of **AA** and **CC** and **CC**′ are outlined in Scheme 1. Towards these ends, HOAt/DCC mediated coupling[9] of *Z*-Val-OH and *tert*-Leu-OMe afforded **III**, thereafter the free amine **IV**, upon removal of the Cbz protective group. Acylation of the resulting amino moiety in **IV** with pyrazine carboxylic acid provided the methyl ester **V**, which was converted to the requisite acid **AA** via base hydrolysis. The P1–P1′ building blocks **CC** and **CC**′ were prepared in two steps (PyBOP promoted peptide coupling and subsequent hydrogenation) from the known α-hydroxyacids **VI**[10] and **VIII**,[11] respectively. The diFAbu bearing α-hydroxyacid **VIII** was prepared in turn from another known acid **VII** according to the published patent literature from Instituto Di Ricerche Di Biologia Melecolare.[11]

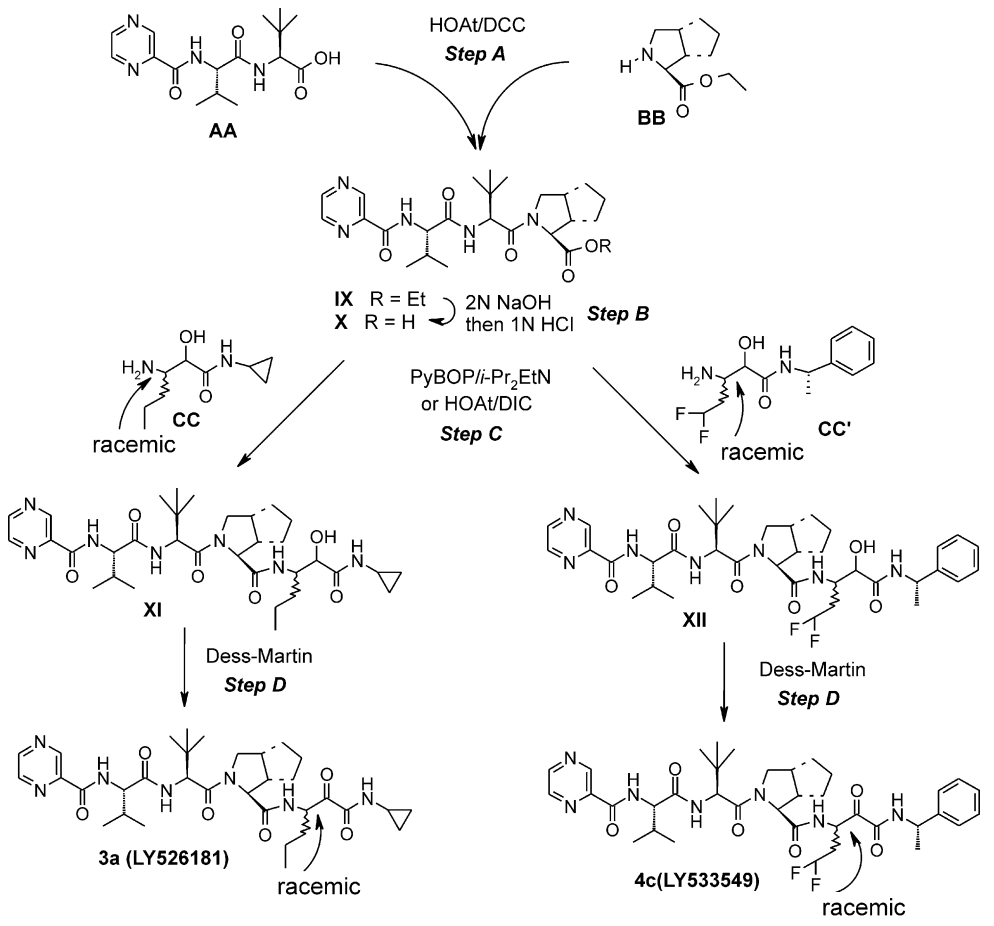
With the key building blocks in hand, we then moved on to assembling various α-ketoamides depicted in Figure 1. In general, all of the HCV protease inhibitors discussed herein (**1**–**4**) were synthesized via a four-step successive coupling procedure using **AA**, **BB**, and **CC** (or their closely related analogues) as the key building blocks. The overall yields of the final products and their respective intermediates are listed in ref 16. The structures of the HCV NS3 protease inhibitors disclosed in this manuscript were secured on the basis of their proton NMR and mass spectra analyses.

The syntheses of the HCV NS3 protease inhibitors **1a**–**1c** were reported previously.[7] The representative synthetic routes used for the preparation of inhibitors **3** and **4** are exemplified for compounds **3a** and **4c**. Inhibitors **2a**–**c** were prepared in a similar fashion to that described for **4**.

As can be seen in Scheme 2, the HOAt/DCC mediated coupling between the dipeptidyl acid **AA** and the bc-



Scheme 1. Syntheses of the key building blocks **AA**, **CC**, and **CC**′.



Scheme 2. Synthesis of **3a** and **4c**.

proline P2 unit **BB** provided the desired adduct **IX**, which was next converted to its corresponding tripeptidyl acid **X** via base hydrolysis. Subsequent PyBOP mediated coupling of the acid **X** with the P1-Nva bearing building block **CC** afforded the α-hydroxyamide intermediate **XI**, which was then oxidized (Dess–Martin reagent) to the final α-ketoamide inhibitor **3a**. On the other hand, the coupling of the tripeptidyl acid **X** with the diFluroAbu-P1 bearing unit **CC′** gave rise to the corresponding intermediate **XII**, which was further converted to the final product **4c** upon Dess–Martin periodinane mediated oxidation.

All peptidyl ketoamides synthesized (1–4) were evaluated in the following bioassays: (1) enzyme inhibition assay against truncated NS3 enzyme; (2) selected potent HCV NS3 protease inhibitors were evaluated against a panel of relevant cellular proteases; (3) HCV subgenomic replicon assay for cellular activity; (4) cytotoxicity assay in a liver cell line (Huh-7 cells). Promising inhibitors emerging from this set of testing were further evaluated in vivo (rat) for drug exposure level in liver and systemic circulation.

2. Enzyme inhibition assay[12]

Careful analysis of the K_i values generated from NS3 enzyme inhibition assay revealed the following trends: (1) Generally speaking, the cyclopropyl-P1′ bearing inhibitors (**1a**, **2a**, and **3a**) displayed higher potency relative to their respective *sec*-butyl and α-MeBn P1′ bearing counterparts. (2) Similar K_i values obtained for inhibitors bearing different P1 moieties [e.g., **3a**–**c** vs **4a**–**c**] suggest that there is no clear preference at P1 position (diFAbu vs Nva). (3) As far as P3 SAR is concerned, the results obtained from P1-Nva series indicate that P3-*t*-Leu bearing inhibitors **3a**–**c** exhibited higher potency relative to the P3-Val containing counterparts **1a**–**c**. On the other hand, the results obtained with P1-diFluoroAbu series (**2** and **4**) did not show any preference at P3 (Table 1).

3. Enzyme selectivity[13]

Three P3 *tert*-Leu containing and one diFAbu P1′ bearing inhibitors (**3a**–**c**, and **4a**) were selected, on the basis of their activities demonstrated in enzyme and replicon assays as shown in Table 1, for further testing against a panel of related cellular serine/cysteine proteases. As can be seen in Table 2, with the exception of **3c**, all of the HCV NS3 protease inhibitors exhibited at least 10-fold selectivity against chymotrypsin, elastase, cathepsins G and L. Lower selectivity (except for **3c**) against cathepsin B (an enzyme invoked in cancer metastases) was observed for our inhibitors. In addition, no significant inhibition against kallikrein, thrombin, plasmin, and trypsin was detected at 100 μM for all

Table 1. Enzyme binding affinity K_i, replicon IC_{50} and XTT cytotoxicity

Compd	HCV K_i PNA (μM)	Replicon IC_{50} (μM)	Cytotoxicity CC_{50} (μM)	CC_{50}/IC_{50} ratio
1a	0.12	7.0	>100	>14
1b	1.80	>10	>100	N/A
1c	2.66	3.36	>100	>30
2a	0.22	3.78	>100	>26
2b	0.38	3.94	>100	>25
2c	0.40	0.91	>100	>110
3a	*0.084*	*2.21*	*>100*	*>45*
3b	0.21	3.48	>100	>29
3c	0.19	0.78	>100	>128
4a	0.20	2.90	>100	>34
4b	0.48	2.61	>100	>38
4c	0.12	0.63	>100	>159

five compounds listed in Table 3 (data not shown). In view of the enzyme selectivity data generated thus far, it is fair to say that the peptidyl ketoamide based inhibitors discussed in this manuscript are quite selective against HCV protease.

4. Replicon assay and cytotoxicity assay

All NS3 protease inhibitors discussed herein (**1**–**4**) were also tested in a modified HCV subgenomic replicon assay[14] as well as in the XTT cytotoxicity assay in Huh-7 liver cells.[15] Careful reviewing of the IC_{50} values from replicon assay shown in Table 1 indicated the following trends: (1) In all cases, the P1′ (s)-MeBn bearing inhibitors (**1c**–**4c**) were found to be the most potent ones within each series (**c** vs **a** or **b**). Compounds **2c**, **3c**, and **4c** demonstrated impressive IC_{50} values less then 1 uM. (2) In terms of P1 SAR, the diFAbu P1 bearing inhibitors **2a**–**c** were proved to be superior to their Nva P1 bearing counterparts **1a**–**c**. In contrast to this finding, however, we did not observe any P1 preference for the *t*-Leu P3 bearing series inhibitors (**3** and **4**). (3) When compared with their P3 Val bearing counterparts (**1** and **3**), all *t*-Leu P3 containing inhibitors (**2** and **4**) exhibited improved activity in replicon assay. For example, compound **3a** ($IC_{50}=2.2$ μM) demonstrated ~3-fold higher potency than **1a** ($IC_{50}=7.0$ μM). Less pronounced improvements were noted with the P1 diFAbu bearing pairs (**4** vs **2**). (4) The XTT cytotoxicity assay indicated that none of the inhibitors evaluated had significant cytotoxicity. All IC_{50} values were greater than 100 μM. (5) Based on their cellular activity and cytotoxicity data, compounds **2c**, **3a**, **3c**, and **4c** were identified as potent HCV NS3 protease inhibitors with at least >45-fold selectivity index.

In vivo target tissue drug exposure. With the aim of identifying orally active HCV protease inhibitors with improved drug exposures in liver relative to that found with **1a**, compounds **2a**, **3a**, **3b**, and **4a** were dosed orally to male Sprague–Dawley rats at a dose of 50 mg/kg. The compounds were dosed as suspensions in a wet granulation vehicle (povidone/lactose/polysorbate). Blood was collected by orbitakl sinus and cardiac puncture (terminal) bleeding procedures at selected time points following dosing. Liver samples were obtained after intracardiac perfusion of the livers with saline to remove residual blood in the liver. Plasma and liver drug concentrations were determined by LC/MS/MS.

Analysis of the exposure data listed in Table 3 revealed the following in vivo SAR trends: (1) for P3 site: comparison of the exposure data (both liver and plasma) obtained with **1a**/**3a** as well as **2a**/**4a** indicated that the P3-*tert*-Leu bearing inhibitors (**3a** and **4a**) achieved at least 2.5-fold higher liver exposure than their corresponding P3-Val bearing counterparts (**1a** and **2a**). (2) for P1 site: comparing the exposure data generated for two sets of inhibitors (**1a** vs **2a** and **3a** vs **4a**) clearly showed that P1-Nva bearing inhibitors (**1a** and **3a**) were able to deliver at least 4-fold more drug to liver than their respective P1-diFAbu bearing inhibitors (**2a** and **4a**). Similar SAR trend was also observed in systemic circulation. (3) for P1′ site: when compared with its P1′-cyclopropyl bearing inhibitor **3a**, the P1′-(s)-*sec*-butyl bearing derivative **3b** achieved similar liver and plasma drug exposure. (4) in order to identify promising HCV protease

Table 2. Enzyme binding affinity K_i's (nM) or *x*-fold of selectivity

Compd	HCV NS3	Elastase	Cathepsin B	Cathepsin G	Cathepsin L	Chymotrypsin
1a	123	38*x*	4*x*	683*x*	12*x*	583*x*
3a	*84*	*41x*	*1.6x*	*492x*	*154x*	*$IC_{50}>100\,\mu M$*
3b	210	36*x*	3.7*x*	1061*x*	52*x*	$IC_{50}>100$ μM
3c	190	6*x*	52*x*	229*x*	29*x*	62*x*
4a	200	15*x*	5*x*	1370*x*	30*x*	303*x*

Table 3. In vivo exposure of **1a**, **3a**, **3b**, and **4a** following oral administration @50 mg/kg

Compd	Liver exposure		Plasma exposure		C liver/IC_{50} (replicon)
	$C_{ave.(0-8\ h)}$ (μM)	$C_{8\ h}$ (μM)	AUC (0–8 h) (μg×h/mL)	$C_{8\ h}$ (μg/mL)	
1a	6.29	0.50	2.86	0.01	0.9
2a	≤0.88	BDL[a]	0.145	0.02	≤0.23
3a	16	3.67	25.83	0.52	7.2
3b	14	8.68	22.27	0.92	4.0
4a	3.64	0.67	1.88	0.04	1.3

[a] BDL, Below Detection Limit.

inhibitors for further ADME and toxicology evaluations, we ranked the compounds on the basis of the ratio of $C_{liver\ ave.(0\text{–}8\ h)}/IC_{50}$(replicon) calculated for inhibitors included in Table 3. Our preference decreases according to the following order: **3a** > **3b** > **4a** > **1a** > **2a**. The best compound disclosed in this manuscript, **3a**, was about 7 times better than the previously reported inhibitor **1a**.

5. Conclusion

Starting from the previously identified bicycloproline P2 bearing HCV protease inhibitor **1a**, we incorporated further structural modifications at P1 (diFAbu as replacement for Nva) or/and at P3 (*tert*-Leu as replacement for Val). On the basis of enzyme inhibition data shown in Table 2, it is clear that neither P1 nor P3 modification had significant impact on enzyme binding affinity (K_i). However, when compared with their P1-Nva bearing counterparts **1a**–**c**, all three P1-diFAbu bearing inhibitors **2a**–**c** exhibited improved (2-fold) activity in the replicon assay. Parallel with this finding, all three P3-*t*-Leu containing inhibitors **3a**–**c** demonstrated enhanced cellular activity (3-fold) than their corresponding P3-Val analogues **1a**–**c**. In view of the exposure data shown in Table 3, it is evident that incorporation of P1-diFAbu moiety resulted in 4-(**3a** vs **4a**) to 7-(**1a** vs **2a**) fold reduction in liver drug exposure. In contrast to this observation, introduction of the *t*-Leu at P3 (as seen in **3a**) led to ~2.5-fold improvement in drug exposure in liver relative to that found with the P3-Val bearing counterpart **1a**. More pronounced enhancement in liver drug exposure was detected for **4a** relative to **2a**. Careful inspection of the data presented in Table 2 clearly indicates that compound **3a** was the most promising one endowed with good enzyme binding affinity, replicon activity, acceptable enzyme specificity, and excellent drug liver exposure yet without inherent cytotoxicity. On the basis of these data, compound **3a** was selected for further evaluation at NovaScreen. When tested against 80 different receptors and enzyme targets, compound **3a** only showed >50% inhibitory effect against elastase (at 10 μM). Thus, in light of the encouraging data generated for **3a**, we are convinced that further optimization of the bicycloproline P2 bearing peptidyl ketoamide series could lead to novel HCV protease inhibitors with therapeutic utilities.

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