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P4 and P1′ optimization of bicycloproline P2 bearing tetrapeptidyl α-ketoamides as HCV protease inhibitors

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Abstract—With the aim of improving HCV protease inhibitors reported in our previous manuscripts, we synthesized and evaluated a series of **1a**-based tetrapeptidyl α-ketoamides with additional P4 modification. The promising analog discovered through this SAR, **5a**, was further derivatized at P1′ or P1 position. As a result of these efforts, we found that replacement of the P4 valine as seen in **1a** with cyclohexylglycine (Chg) resulted in the discovery of **5a**, **5c**, and **5e** endowed with improved cellular activity in comparison to **1a**.

1. Introduction

Heptatitis C virus (HCV), the major etiological agent of the non-A non-B hepatitis, was identified at the end of 1980s. Currently, HCV has infected about 170 million people worldwide and has become a major health problem. About 70% of HCV infected patients become chronically infected, approximately 20% of those patients are at the risk of developing cirrhosis that can eventually lead to hepatocellular carcinoma.[1] Current interferon-based therapies are far less than ideal especially for those infected with HCV genotype 1. The suboptimal efficacy along with poor tolerance associated with current treatments necessitates the need for development of new therapeutics.[2] The HCV-encoded NS3 serine protease is essential for viral replication and thus has been considered as an attractive target for therapeutic intervention for the treatment of HCV-infected patients.[3–5] In order to address the unmet medical need, we became interested in the discovery of novel HCV protease inhibitors with therapeutic potentials. As documented in a series of recent publications from Lilly[6] and Vertex,[7] we focused our research efforts on the design and subsequent optimization of the bicycloproline P2 incorporated α-ketoamides as HCV protease inhibitors including a number of *tert*-butylglycine P3 bearing derivatives **1a**, **1b**, and **1c** as shown in Figure 1. We are pleased to find that compound **1a** exhibited very good in vitro potency (enzyme and cellular), toxicity profiles, as well as adequate liver exposure upon oral administration.[8] To further optimize this novel series inhibitors, we embarked on P4 modification either alone or in combination with additional P1′optimization. On the basis of substrate preference determined for a set of hexapeptides (P1–P6) by Pessi and his collaborators,[9] we decided to incorporate several aliphatic and aromatic residues (e.g., IIe, Cha, and Phg, etc.) at the P4 position as listed in Figure 1. Moreover, in order to maintain good membrane permeability, we excluded charged residues at that pocket. It should be mentioned that each of the tetrapeptidyl α-ketoamide inhibitor discussed in this manuscript incorporates a diastereomeric center at P1 position due to synthetic convenience.

In this communication, we report our recent progress achieved through such P4 optimization, which has led to the identification of a number of promising HCV NS3 protease inhibitors such as **5a**, **5c**, and **5e** endowed with improved activity in both the enzyme inhibition and replicon assays relative to **1a**.

2. Chemical synthesis

The representative synthetic route devised for the P4 modified peptidyl α-ketoamides **2a** through **7a** is

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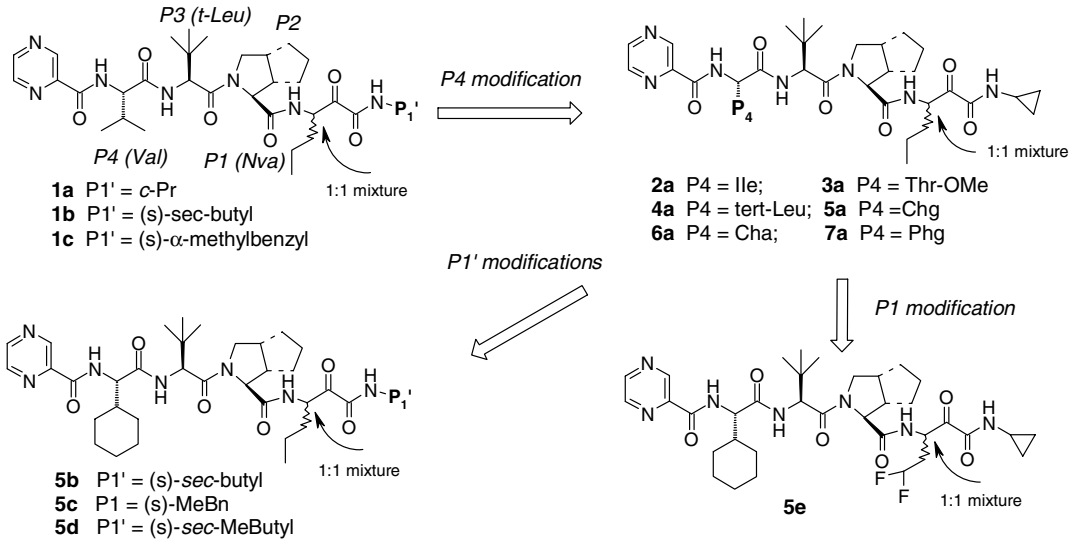


Figure 1. P4 and P1′-modified α-ketoamide HCV protease inhibitors.

outlined in Scheme 1. In general, a four-step sequence (*Step A–Step D*) was used for the preparation of all final products discussed in this manuscript. As shown in Scheme 1, the P4–P3 dipeptidyl acid unit **14** was assembled from HCl salt of H_2NChg-(*t*-Bu)GlyOMe **12** via N-acylation and subsequent ester hydrolysis. Compound **12** was in turn prepared by HOBt/EDCI mediated coupling[10] of BocHNChgOH **9** and amine **10**, followed by removal of the *N*-Boc protective group. Condensation of **14** (P4–P3 acid) with the previously described P2 amine **15**[6] was promoted by HOAt/DCC[11] and afforded the tripeptide ester **16**, thereafter the corresponding acid **17**, upon aqueous hydrolysis. PyBOP mediated coupling[12] of **17** with the requisite P1–P1′ hydroxylamine **18**[6] provided the expected adduct **19** (88%), which was then oxidized with Dess–Martin periodinane to afford the desired α-ketoamide inhibitor **5a** in 50% yield. The structure of **5a** along with other P4 modified analogs (**2a**, **3a**, **4a**, **6a**, and **7a**) were confirmed on the basis of their proton NMR and mass spectra analyses.

It should be pointed out that four P1′ or P1 modified analogs of **5a**, namely **5b**–**5e**, were designed according to the rationale articulated in the previous manuscripts.[6,8] The syntheses of these analogs were accomplished using appropriate P1–P1′ building blocks described in Ref. 8 cited in this manuscript.

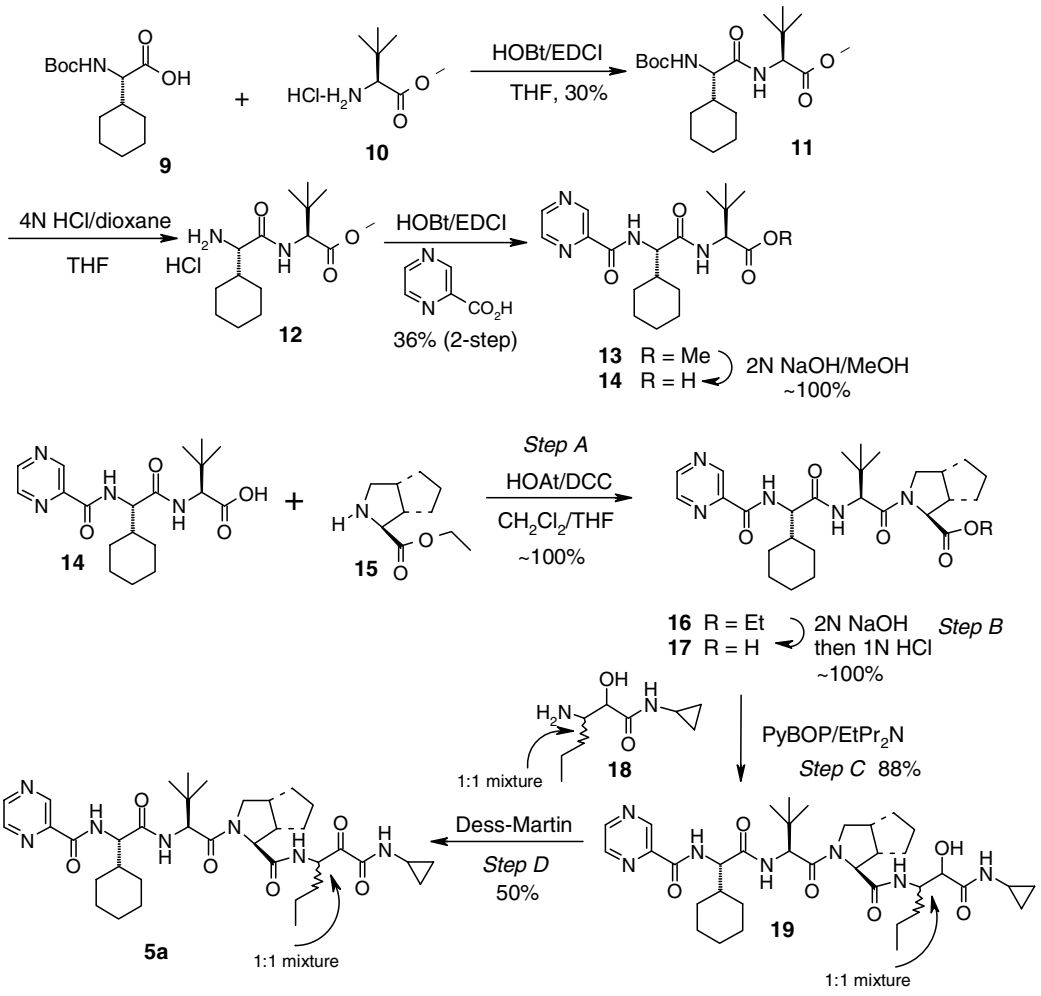
3. Biological evaluation

All P4 modified peptidyl α-ketoamides synthesized (**2**–**7**) were evaluated in the following bioassays: (1) enzyme inhibition assay against truncated NS3 enzyme;[13] (2) enzyme selectivity assays against a panel of relevant cellular proteases;[14] (3) HCV replicon assay for cellular activity;[15] (4) cytotoxicity assay in a liver cell line (Huh-7 cells).[16]

Enzyme inhibition assay:[13] Careful comparison of the K_i values determined in the pNA based inhibition assay for various P4 modified inhibitors listed in Table 1 revealed the following SAR trends: (1) replacement of the P4 Val. in **1a** with IIe or cyclohexylglycine (Chg) led to **2a** and **5a** with similar NS3 binding affinity; (2) incorporation of either ThrOMe or *t*-Leu at the P4 pocket produced inhibitors **3a**, or **4a** displaying 2-fold reduced enzyme binding affinity; and (3) replacing of the Val moiety at P4 with either Cha or Phg yielded inhibitors **6a** or **7a**, both of which showed at least 8-fold weaker enzyme inhibitory potency relative to that found with **1a**.

Further P1′ SAR: Having completed the first round of P4 SAR exploration, we synthesized and evaluated a few more tetrapeptidyl α-ketoamides with additional modification at either P1′ or P1 position. Judging from the data shown in Table 2, it is clear that incorporation of (*S*)-*sec*-butyl (**5b**) or (*S*)-MeBn (**5c**) functionality as the P1′ group retained the enzyme binding affinity in comparison to that obtained with **5a**. Introduction of an additional methyl group at the P1′ position as seen in **5d** resulted in ~3x reduction in enzyme potency relative to **5a**. In contrast to this finding, replacing the P1 Nva in **5a** with difluoroAbu led to **5e** displaying ~3x enhanced enzyme binding affinity (K_i = 25 nM).

Replicon and cytotoxicity assays:[15,16] All newly synthesized HCV NS3 protease inhibitors disclosed in this manuscript were also evaluated in a modified replicon assay as well as XTT cytotoxicity assay in Huh-7 liver cells. Careful inspection of the IC_{50} values determined in the replicon assay as listed in Table 1 showed the following trends: (1) three out of seven inhibitors, **2a**, **4a**, and **5a**, demonstrated improved cellular potency relative to **1a**. The P4 Chg incorporated inhibitor, **5a**, demonstrated the best potency with IC_{50} value of 0.55 μM, which is about 4-fold more potent than that obtained with **1a**; (2) consistent with the reduced enzyme affinity, inhibitors **3a**, **6a**, and **7a** showed weaker potencies in the replicon assay relative to **1a**; (3) when compared with **5a**, whilst two P1′ modified compounds **5b** and **5d** showed 2-fold weaker activity than **1a**, inhibitors **5c**



Scheme 1. Synthesis of **5a**.

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

Comp	HCV K_i pNA (μM)	Replicon IC_{50} (μM)	Cytotoxicity CC_{50} (μM)	CC_{50}/IC_{50} ratio
1a	0.084	2.21	>100	>45
2a	0.089	1.44	>100	>69
3a	0.151	4.35	>100	>22
4a	0.162	1.29	>100	>77
5a	0.070	0.55	86.5	157
6a	0.655	2.81	45.5	16
7a	0.655	2.81	58.6	21

Table 2. 5a based P1′ or P1 modifications

Comp	HCV K_i pNA (μM)	Replicon IC_{50} (μM)	Cytotoxicity CC_{50} (μM)	CC_{50}/IC_{50} ratio
5a	0.070	0.55	86.5	157
5b	0.084	1.07	69.2	65
5c	0.090	0.21	22	105
5d	0.254	1.0	31.7	32
5e	0.025	0.40	>100	250

(IC_{50} = 0.21 μM) and **5e** (IC_{50} = 0.40 μM) exhibited enhanced cellular potency (see Table 2).

As far as in vitro cytotoxicity is concerned, all P4 modified inhibitors listed in Table 1, except for **6a**, were considered to be noncytotoxic with CC_{50} values > 50 μM and therapeutic indexes of greater than 20-fold. In addition, in light of the cytotoxicity data obtained with a set of **5a** based P1′ or P1 modified analogs, it is interesting to note that although the absolute CC_{50} values determined for **5c** and **5d** were in the 20–30 μM range, the actual CC_{50}/IC_{50} (replicon) ratio reached at least 30-fold. It is encouraging to see that compound **5e** with difluoroAbu incorporated at P1 exhibited minimal cytotoxicity (CC_{50} > 100 μM) and an excellent selectivity index of 250 (see Table 2). As also shown in Table 2, the therapeutic indexes (CC_{50}/ IC_{50}) values obtained with **6a** and **7a** were found to be around 20.

Enzyme selectivity:[14] Based upon the encouraging activities determined in the enzyme and replicon assays, seven P4 modified inhibitors were chosen for further evaluation in the enzyme selectivity assays against a panel of related cellular enzymes. As shown in Table 3, all of the P2 bicycloproline bearing α-ketoamides discussed herein exhibited excellent selectivity against kallikrein, thrombin, plasmin, trypsin, and chymotrypsin with K_i values greater than 50 μM. Generally

Table 3. Enzyme binding affinity K_i's (μM) or *x*-fold of enzyme selectivity

Compd	HCVNS3	Elastase	Cathep. B	Cathep. L	Thromb.	Chymotrp.	Trypsin	Plasmin	Kallikrein
1a	0.084	139x	1.6x	154x	>50	>50	>50	>50	>50
2a	0.089	36x	10x	15x	>50	>50	>50	>50	>50
3a	0.151	126x	7.3x	146x	>50	>50	>50	>50	>50
4a	0.162	102x	3.9x	123x	>50	>50	>50	>50	>50
5a	0.070	62	3x	134x	>50	>50	>50	>50	>50
5b	0.084	81x	13x	107x	>50	>50	>50	>50	>50
5c	0.090	29x	283x	51x	>50	>50	>50	>50	>50
5e	0.025	392	40x	760x	>50	>50	>50	>50	>50

speaking, all P4 modified inhibitors (except for **2a**) showed at least 30-fold or 50-fold selectivity against elastase or cathepsin L. On the other hand, relatively low selectivity (⩽10x) towards cathepsin B, an enzyme involved in cancer metastases, was observed with **1a** through **5a**. Interestingly, replacing the P1′ *c*-Pr moiety in **5a** with a more bulky (S)-MeBn as seen in **5c** resulted in significant improvement (90x) in selectivity against cathepsin B. Likewise, the P1 difluoroAbu bearing analog **5e** was found to be ~6-fold more selective towards elastase, cathepsin B and cathepsin L in comparison to its P1 Nva containing counterpart **5a**. Thus, in light of the data listed in Table 3, it is reasonable to claim that the P4 modified tetrapeptidyl α-ketoamides discussed in this manuscript are rather specific for HCV NS3 protease.

4. Conclusions

Starting from the previously identified P4 Val. bearing HCV NS3 protease inhibitor **1a**, we incorporated further structural modification at P4 alone or in combination with additional modification at P1′ or P1. On the basis of biological data shown in Table 1, it is clear that the P4 Chg bearing analog **5a** exhibited improved enzyme (K_i = 70 nM) and cellular potency (IC_{50} = 0.55 M) relative to **1a**, thus it was selected for further P1′ and P1 modification. This continued effort led to the discovery of **5c** and **5e** endowed with excellent enzyme and cellular potency and desirable therapeutic indexes (see Table 2). Thus, in view of the data presented in Tables 1–3, it is evident that the newly prepared P4 Chg bearing inhibitors **5a**, **5c**, and **5e** represent new promising P4 modified α-ketoamide based HCV protease inhibitors endowed with very good enzyme binding affinity, enzyme specificity, and replicon activity.

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