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Letters

Synthesis and Evaluation of Keto-Glutamine Analogues as Potent Inhibitors of Severe Acute Respiratory Syndrome 3CL^{pro} †

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Abstract: The 3C-like proteinase (3CL^{pro}) of severe acute respiratory syndrome (SARS) coronavirus is a key target for structure-based drug design against this viral infection. The enzyme recognizes peptide substrates with a glutamine residue at the P1 site. A series of keto-glutamine analogues with a phthalhydrazido group at the α-position were synthesized and tested as reversible inhibitiors against SARS 3CL^{pro}. Attachment of tripeptide (Ac-Val-Thr-Leu) to these glutamine-based "warheads" generated significantly better inhibitors (**4a**−**c**, **8a**−**d**) with IC_{50} values ranging from 0.60 to 70 μM.

Human coronaviruses (HCoVs) are widespread causative agents of upper respiratory tract illness in humans.[1] It has recently been revealed that a previously unknown HCoV causes severe acute respiratory syndrome (SARS), a life-threatening form of atypical pneumonia that is rapidly spreading from its likely origin in southern China to several countries in other parts of the world.[2] SARS is characterized by high fever, malaise, rigor, headache, and nonproductive cough or dyspnea and may progress to generalized interstitial infiltrates in the lung, requiring intubation and mechanical ventilation.[3]

Because of the functional importance of SARS 3C-like proteinase (3CL^{pro}) in the viral life cycle, it has been recognized as a key target for structural-based drug design against SARS.[4] The 3CL^{pro} enzyme has a Cys-His catalytic dyad (Cys-145 and His-41).[4,6] The substrate specificity of 3CL^{pro} is determined mainly by the P1, P2, and P1′ positions.[5] The P1 position has a well-conserved Gln residue, and the P2 position has a hydrophobic amino acid residue.

Currently, no effective small molecule antiviral agent has been reported to treat SARS. A number of compounds have been shown to inhibit viral replication in vitro, including some drugs used to treat other viral infections. In most cases, the mode of action of these compounds is unknown. Several groups have developed compounds targeting either the spike protein[7] to inhibit viral entry or 3CL^{pro} to inhibit polyprotein processing.[6,8−10] For example, a recent screen of >10 000 compounds to inhibit viral replication identified two anti-HIV agents that target SARS viral entry and 3CL^{pro}, respectively.[8] The latter, a peptidic inhibitor designed as a transition state analogue of the HIV protease inhibited 3CL^{pro} with a K_i of 0.6 μM. Modeling studies indicate that this compound binds specifically to the active site of the SARS protease. CMK, another peptidic inhibitor, also targets the enzyme's active site but binds irreversibly.[6] In contrast, bifunctional aryl boronic acid compounds reversibly inhibit 3CL^{pro} and appear to target a cluster of serine residues close to the enzyme's active site.[9] Finally, Blanchard et al. identified five promising 3CL^{pro} inhibitors (IC_{50} values of 0.5−7 μM) from a library of 50 000 compounds.[10] However, it

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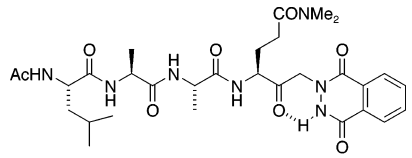
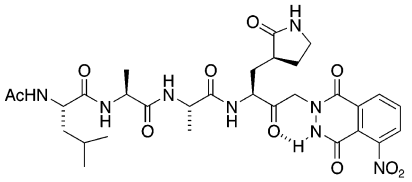
is unknown how these compounds interact with the protease. Further work on all of these and other compounds will facilitate the development of effective therapeutic agents to treat SARS.

Our previous studies on picornaviral 3C proteinases have focused on synthesis and evaluation of several glutamine analogues as effective inhibitors of these enzymes from human rhinovirus-14 (HRV-14) and hepatitis A virus (HAV).[11] In particular, we have recently reported[11a,b] that keto-glutamine analogues with the phthalhydrazido group at α-position (e.g. **1a**,**b**, Figure 1) are good reversible inhibitors of HAV 3C



1a, HAV 3C inhibitor
(IC_{50} = 13 μM)



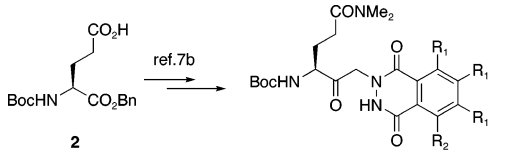
1b, HAV 3C inhibitor
(IC_{50} = 1.6 μM)

Figure 1. Keto-glutamine analogue as a potent inhibitor of HAV $3C^{pro}$.[11a,b]

proteinase, with IC_{50} values in the low micromolar range. This is probably due to the combined effect of the β and β' amino functionalities adjacent to the keto group as well as intramolecular hydrogen bonding to the carbonyl, which makes it more electrophilic and susceptible to hemithioacetal formation with the active site cysteine. In this paper, we report the evaluation of a series of new compounds of this class as potent inhibitors of SARS $3CL^{pro}$.

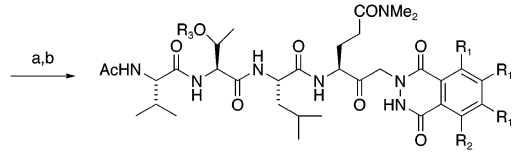
Initially, we focused on the *N*,*N*-dimethylglutamine analogues (**4a**−**c**, Scheme 1) with leucine, threonine, and valine at the P2, P3, and P4 positions, respectively.

Scheme 1[a]



2

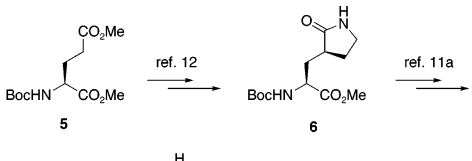
3a, $R_1 = R_2 = H$
3b, $R_1 = H$, $R_2 = NO_2$
3c, $R_1 = R_2 = F$



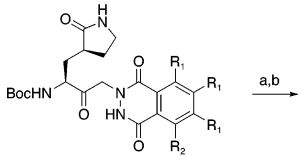
4a, $R_1 = R_2 = H$, R_3 = Bn ⟵ c
4b, $R_1 = R_2 = R_3 = H$
4c, $R_1 = H$, $R_2 = NO_2$, R_3 = Bn
4d, $R_1 = R_3 = H$, $R_2 = NO_2$ ⟵ d

[a] Reagents and conditions: (a) TFA:CH_2Cl_2 1:1, 0 °C, 1.5 h, quant. (b) Ac-Val-Thr(OBn)-Leu-OH, HBTU, DIPEA, DMF, rt, 28−33%. (c) H_2 (1 atm), Pd−C%, MeOH/H_2O, rt, 6 H, 80%. (d) TFA:TMS-OTf 2:1, 0 °C, 2 h, quant.

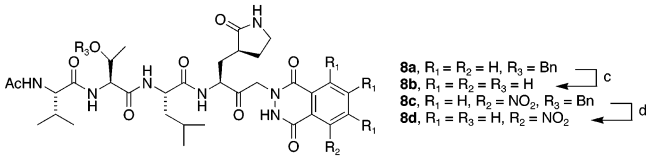
Scheme 2



5 **6**

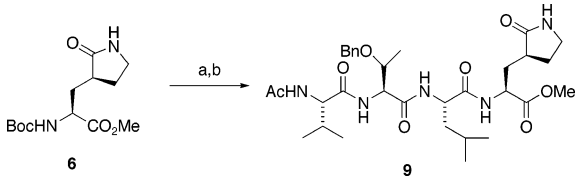


7a, $R_1 = R_2 = H$
7b, $R_1 = H$, $R_2 = NO_2$
7c, $R_1 = R_2 = F$



[a] Reagents and conditions: (a) TFA:CH_2Cl_2 1:1, 0 °C, 1.5 h, quant. (b) Ac-Val-Thr(OBn)-Leu-OH, HBTU, DIPEA, DMF, rt, 21−25% (c) H_2 (1 atm), Pd−C%, MeOH, rt, 6 h, quant. (d) TFA:TMS-OTf 2:1, 0 °C, 2 h, quant.

Scheme 3[a]



6 **9**

[a] Reagents and conditions: (a) TFA:CH_2Cl_2 1:1, 0 °C, 1.5 h, quant. (b) Ac-Val-Thr(OBn)-Leu-OH, HBTU, DIPEA, DMF, rt, 35%

The requisite "warheads" **3a**−**c** were synthesized as previously reported.[11a,b] Removal of Boc group in **3a**,**b** and coupling with the tripeptide Ac-Val-Thr(OBn)-Leu-OH generates the tetrapeptides **4a** (33% yield) and **4c** in (28% yield). Removal of benzyl protection in **4a** (H_2, Pd−C) and **4c** (TFA, TMS-OTf) affords tetrapeptides **4b** (80% yield) and **4d** (quant.) respectively, with free threonine at P3.

A series of cyclic glutamine analogues (**8a**−**d**) were also prepared by following an analogous route (Scheme 2). Thus, N-Boc-L-glutamic acid dimethylmethyl ester (**5**) can be converted into cyclic glutamine derivative **6** by a modified literature procedure (final cyclization step simplified: satd aq $NaHCO_3/CH_2Cl_2$, rt, 70% yield).[12] The phthalhydrazides **7a**−**c** were synthesized using a reaction sequence previously reported by us.[11a] Removal of Boc group in **7a**,**b** and coupling with the tripeptide Ac-Val-Thr(OBn)-Leu-OH produces the tetrapeptides **8a** and **8c** in 21−25% yield. Removal of benzyl protection in **8a** (H_2, Pd−C) and **8c** (TFA, TMS-OTf) affords tetrapeptides **8b** and **8d** respectively, in quantative yields and with free threonine at P3.

To probe the effect of tetrapeptide moiety in **8a**−**d**, compound **9** was prepared without the keto-phthalhydrazide "warhead", as outlined in Scheme 3.

Compounds **3c**, **4a**−**c**, **7a**−**c**, **8a**−**d**, and **9** were tested as inhibitors of SARS $3CL^{pro}$ using a continuous fluorometric assay and a His-tagged protease essentially as described previously.[10] Briefly, cleavage reactions (700 μL) were performed at 22 °C in a solution containing

Table 1. Evaluation of Compounds **3c**, **4a**−**c**, **7a**−**c**, **8a**−**d**, and **9** as Reversible Inhibitors of SARS 3CL^{pro}

entry	compounds	IC_{50} values,[a] μM	% inhibition[a,b]
1	**3c**		9 (±2)
2	**4a**	64	
3	**4b**	28	
4	**4c**	70	
5	**4d**	53	
6	**7a**		15 (±3)
7	**7b**		24 (±5)
8	**7c**		9 (±2)
9	**8a**	2.7	
10	**8b**	2.9	
11	**8c**	0.60	
12	**8d**	3.4	
13	**9**		33 (±7)

[a] 1.5 μM HAV 3C, 10 μM Abz-SVTLQSG-Tyr(NO_2)R, 2 mM EDTA, 100 mM KH_2PO_4/K_2HPO_4 at pH 7.5, 22 °C, no preincubation of the enzyme with inhibitor. The IC_{50} values reported are within ±10% error. [b] Inhibition at [I] = 100 μM.

100 mM KH_2PO_4/K_2HPO_4 at pH 7.5, 2 mM EDTA, 10 μM fluorogenic substrate (Abz-SVTLQSG-Tyr(NO_2)R, purity: 92.6% approximately), 1.5 μM of protease, and 1% DMSO. Increase in fluorescence (λ_{ex} 340 nm, λ_{em} 415 nm) was monitored using a Shimadzu RF5301 spectrofluorometer. Samples were not preincubated with inhibitor. Initial rates were calculated using the first 3 min of the progress curves. All tested componds displayed reversible inhibition. The results are summarized in Table 1.

As outlined in Table 1, the shorter keto-glutamine analogues **3c**, **7a**−**c** (entries 1, 6−8) as well as the tetrapeptide ester **9** (entry 13) show poor inhibition of 3CL^{pro}. However, the corresponding extended tetrapeptides **4a**−**d** and **8a**−**d** (entries 2−5, 9−12) are much more potent, indicating that both the tetrapeptide and keto-phthalhydrazide moieties are required for inhibition. Also, the cyclic glutamine analogues **8a**−**d** (entries 9−12) exhibit better inhibition compared to the acyclic derivatives (compounds **4a**−**d**, entries 2−5). The tetrapeptide with a benzyl protecting group on threonine (**8a**, entry 9) and the deprotected compounds **8d** and **8b** (entries 10 and 12) are approximately equipotent. Interestingly, in the case when the nitro group is introduced with the benzyl group present there is a 4-fold improvement of the inhibition constant (**8c**, entry 11).

Modeling studies (Figure 2) of 3CL^{pro} with covalently bound inhibitors **8a**−**d** were conducted based on the previously solved structures of a 3CL^{pro}/inhibitor complex,[6] a rhinovirus 3C protease/inhibitor complex,[13] and a glutamic acid specific serine protease/inhibitor complex.[14] Graphical manipulations were done using XtalView,[15] and energy minimizations were completed using CNS v1.1.[16] Additional force field parameters for the inhibitor were derived from the Cambridge Structural Database.[17] Graphics were produced using MolMol[18] and POV−Ray v3.5. As a comparison, the protease with no inhibitor was also modeled. The inhibitor is shown binding in an extended conformation, forming a partial β-sheet interaction with residues 163−166 in the proteinase and a hydrogen bond between residue His163 and the P1 side chain. This last hydrogen bond is responsible for the protease specificity for glutamine in the P1 position. The modeling studies indicate that the active site of the enzyme has enough room to accommodate the bulky phthalhydrazide group. Minimal rearrangements of the protein, in particular residue

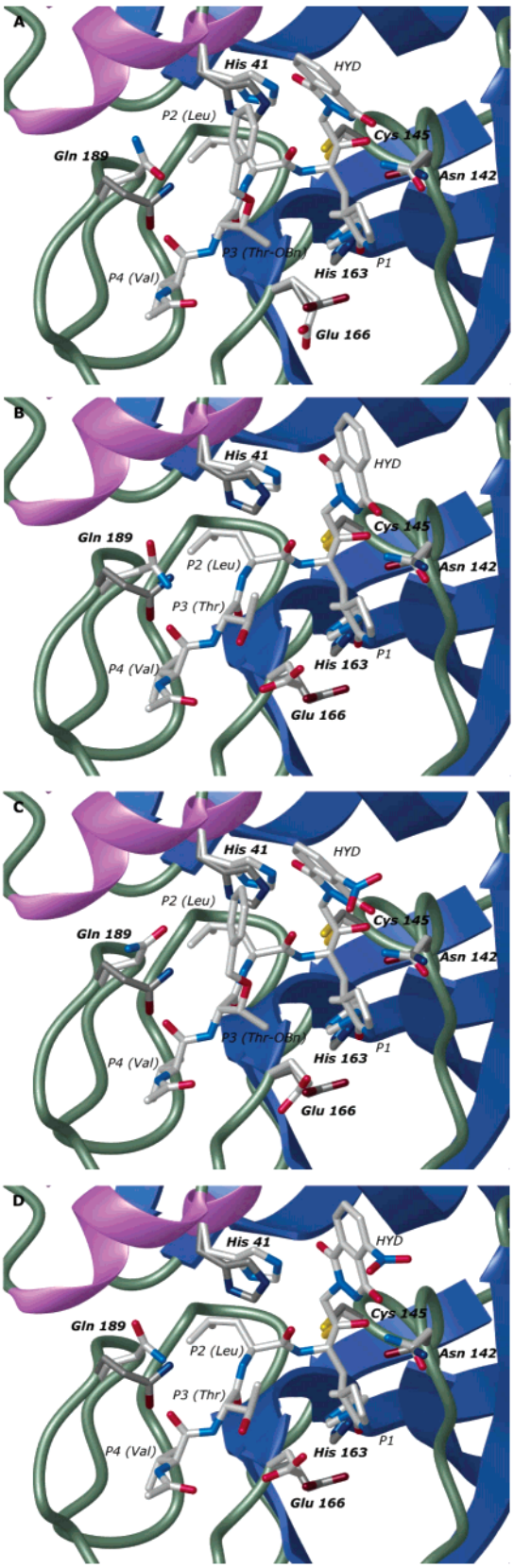


Figure 2. Modeling studies indicating inhibitors **8a**−**d** (A−D, respectively) in the active site of 3CL^{pro}. Key active site side chains are shown in two shades: lighter for the protease/inhibitor complex and darker for the enzyme in the absence of inhibitor. Side chains from the protein are also labeled in bold.

Glu166, are required to accommodate the extra bulk on the P1 residue. Gln189, near the P3 side chain, also moves slightly to accommodate the different substituents.

Specific interactions relating to the different substituents on the inhibitors were noted during the modeling, particularly with the nitro group attached to the phthalhydrazide (**8c**,**d**) and the benzyl group on the threonine (**8a**,**c**). The oxygens on the nitro group are in a position to hydrogen bond to the side chain nitrogen of Asn142. The benzyl group, attached to the threonine at P3, fits into a small pocket above the leucine at P2. In addition to filling a small hydrophobic pocket, the phenyl ring forms a favorable aromatic−aromatic stacking interaction with the phthalhydrazide group of the inhibitor. For the inhibitors with no benzyl group (**8b**,**d**), the free hydroxy group on the threonine side chain is in a position to form a hydrogen bonding interaction with Glu166.

In light of the interactions in the models, it is possible to rationalize the increased inhibition of **8c** relative to **8b**. The nitro group on the phthalhydrazide, in addition to an interaction with Asn142, may contribute to the binding of **8c** by presenting the hydrophilic atoms toward the solvent. These three major effects, the increased hydrophilicity of the phthalhydrazide moiety, the packing of a hydrophobic pocket, and the aromatic−aromatic stacking may explain the synergistic contributions to inhibition parameters for these two chemical groups.

In conclusion, the present work provides access to several electrophilic keto-glutamine analogues that may allow development of potent inhibitors of $3CL^{pro}$ as therapeutic agents for SARS infections. Although the K_i values of these reversible inhibitors remain to be determined, earlier studies[7b] have shown that the parent compound **1** (Figure 1) is a competitive reversible inhibitor of the HAV 3C enzyme with a K_i of 9 μM. Additional studies with modified analogues and other $3C^{pro}$ will be reported in the future.

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Supporting Information Available: (1) Experimental procedures; (2) NMR spectra of the final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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