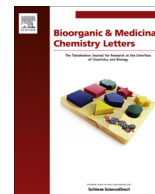




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Design, synthesis, and bioevaluation of viral 3C and 3C-like protease inhibitors



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ABSTRACT

A class of tripeptidyl transition state inhibitors containing a P1 glutamine surrogate, a P2 leucine, and a P3 arylalanines, was found to potently inhibit Norwalk virus replication in enzyme and cell based assays. An array of warheads, including aldehyde, α -ketoamide, bisulfite adduct, and α -hydroxyphosphonate transition state mimic, was also investigated. Tripeptidyls **2** and **6** possess antiviral activities against noroviruses, human rhinovirus, severe acute respiratory syndrome coronavirus, and coronavirus 229E, suggesting a broad range of antiviral activities.

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Viruses that belong to the *Picornaviridae*, *Caliciviridae*, and *Coronaviridae* families encode a 3C or 3C-like protease (3Cpro or 3CLpro, respectively), which cleaves the viral polyproteins into mature or intermediate proteins and is essential for viral replication.^{1–3} Viruses in the *Picornaviridae* family include enteroviruses (EV),⁴ coxsackieviruses (CV), human rhinoviruses (HRV),⁵ and poliovirus (PV); viruses in the *Caliciviridae* family include human noroviruses such as Norwalk virus (NV) and MD145,² and murine norovirus (MNV); and viruses in the *Coronaviridae* family include severe acute respiratory syndrome coronavirus (SARS-CoV)⁶ and human coronavirus 229E. These viruses can cause outbreaks of acute gastroenteritis (NV),⁷ severe systemic disease with high mortality (SARS-CoV),^{8,9} or a severe form of hand, foot and mouth disease (HFMD)¹⁰ associated with fatal encephalitis, paralysis, and myocarditis (EV71). Despite great efforts made in the discovery of preventive and therapeutic measures for these viruses,¹¹ no antiviral drug or vaccine (except for poliovirus) is currently available.

Viral 3Cpro and 3CLpro are cysteine proteases and share a typical chymotrypsin-like folding, a nucleophilic cysteine residue in the active site, and a preference for a glutamine or glutamic acid residue in the primary binding residue (P1 site) of the substrate proteins.¹² The 3Cpro or 3CLpro of these viruses are appealing

targets for the discovery of antiviral therapeutics, and the conservation of the proteases may provide an excellent platform for broad-spectrum antivirals for those important viruses.¹² We report herein our design, synthesis, and bio-evaluation of a class of broad-spectrum anti-viral tripeptidyl inhibitors as depicted in [Figure 1](#).

Our antiviral design focused on transition state inhibitors of 3Cpro and 3CLpro by incorporating a recognition element such as a peptidyl fragment that is compatible with the known substrate specificity of the targeted enzymes and a warhead such as an aldehyde, α -ketoamide, bisulfite adduct,^{13,14} or an α -hydroxy phosphonate transition state mimic. Since the primary substrate specificity residue of 3Cpro and 3CLpro is a glutamine residue, a glutamine surrogate^{15,16} was therefore utilized in the P1 site of the inhibitors. Like others,^{17,18} we found that a hydrophobic amino acid such as leucine residue at the P2 site enhances the recognition.

Results of our cell-based assays have shown that a hydrophobic residue at P3 in the tripeptidyl compounds is important for manifesting antiviral activity, and those with arylalanine at P3 are highly active in cell-based assays as well as enzyme assays, suggesting that arylalanine at P3 is important for cell penetration. Hence, a series of tripeptidyl molecules containing an aldehyde warhead of glutamine surrogate at P1, leucine at P2, and arylalanine at P3, such as **1–5**, was first synthesized and evaluated.

Tripeptidyl aldehydes **1–5** were readily synthesized using a five-step reaction sequence starting from glutamine surrogate

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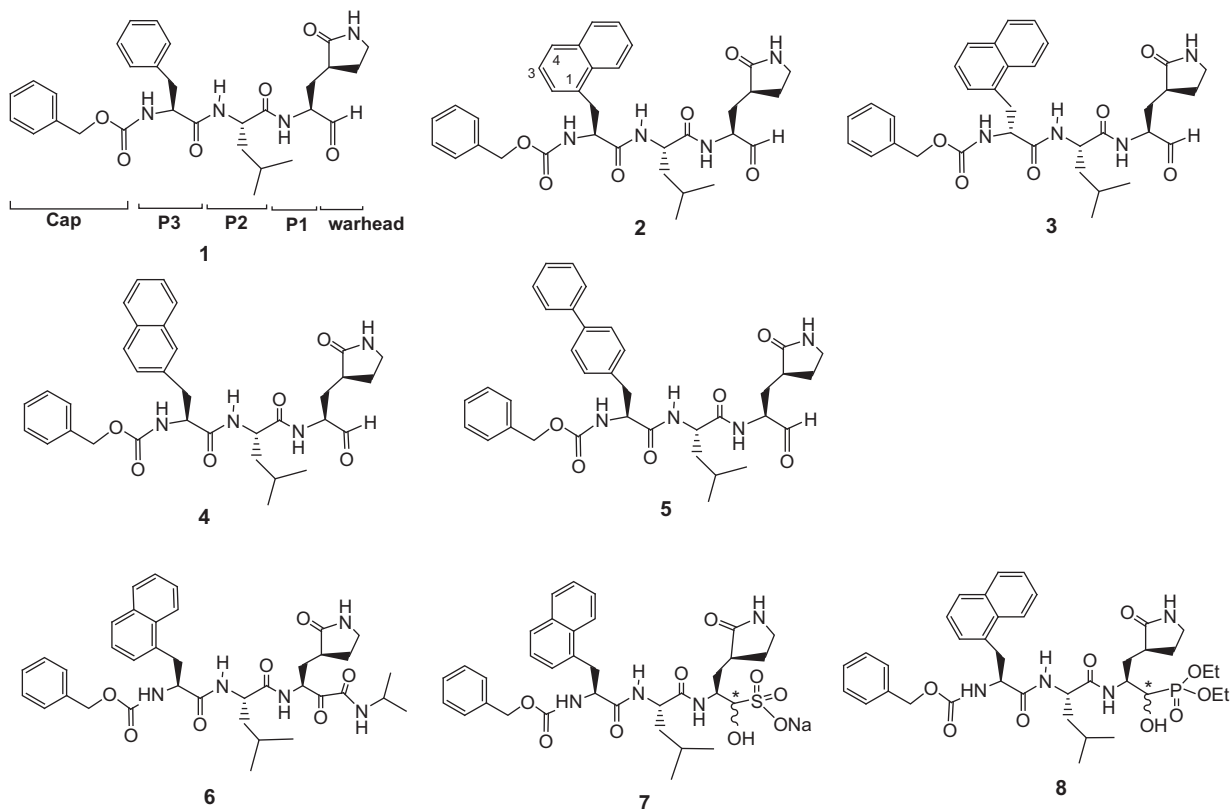
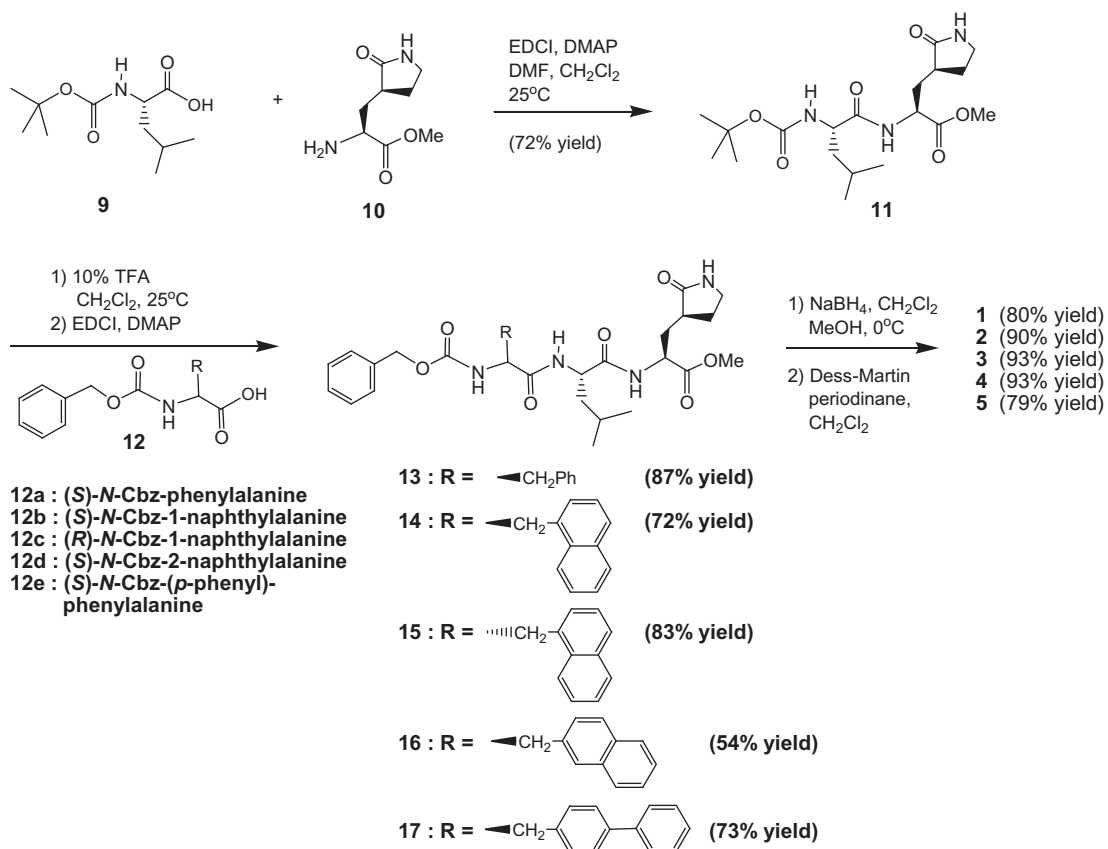
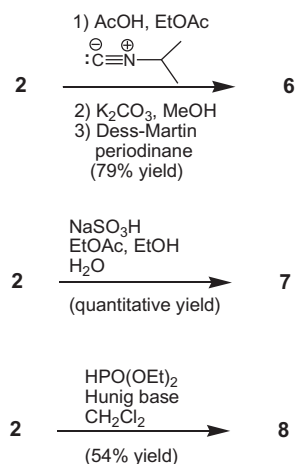


Figure 1. Synthesized and bioevaluated viral cysteine protease inhibitors 1-8.



Scheme 1. Synthesis of viral cysteine protease inhibitors 1-5.



Scheme 2. Synthesis viral cysteine protease inhibitors **6–8**.

Table 1

IC₅₀ and EC₅₀ values of compounds **1–8** from NV 3CLpro FRET-based assay and NV replicon cells, respectively²⁴

Compound	IC ₅₀ (μM)	EC ₅₀ (μM)
1	0.21 ± 0.1	0.06 ± 0.03
2	0.14 ± 0.2	0.04 ± 0.02
3	0.7 ± 0.3	0.45 ± 0.4
4	0.15 ± 0.1	0.08 ± 0.03
5	0.18 ± 0.06	0.05 ± 0.05
6	2.6 ± 1.5	4.2 ± 2.4
7	0.24 ± 0.1	0.04 ± 0.03
8	35.5 ± 5.7	0.1 ± 0.3

Table 2

IC₅₀ values of two active tripeptidyl compounds **2** and **6** against various 3Cpro and 3CLpro using FRET-based enzyme assays²⁴

Compound	NV (μM)	MD145 (μM)	HRV (μM)	SARS-CoV (μM)
2	0.14 ± 0.2	0.51 ± 0.2	0.15 ± 0.05	0.23 ± 0.1
6	2.6 ± 1.5	3.1 ± 1.6	0.12 ± 0.2	0.61 ± 0.2

methyl ester **10**¹⁵ (Scheme 1). Hence, condensation of **10** and (*S*)-*N*-Boc-leucine (**9**) in the presence of 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDCI) afforded dipeptide **11**, which upon removal of the Boc group and coupling with various *N*-Cbz-amino acids **12a–e** gave tripeptides **13–17**, respectively in good yields. Reduction of the methyl ester function with sodium borohydride followed by oxidation with Dess–Martin periodinane (DMP) furnished aldehydes **1–5**. Compounds **12a–d** are commercially available, while **12e** was made by following a reported procedure¹⁹ via a Suzuki coupling of (*S*)-*N*-Boc-4-bromophenylalanine and phenylboronic acid followed by removal of the Boc group and installation of the Cbz group with benzyl chloroformate.

To avoid oxidative degradation and improve absorption and in vivo pharmacokinetics, various warheads were installed^{13,14} by modifying the aldehyde function of compound **2**.²⁰ α-Ketoamide **6** was synthesized from a nucleophilic addition reaction of aldehyde **2** and isopropylisocyanide followed by hydration and oxidation

with DMP (Scheme 2).¹³ Bisulfite adduct **7** and α-hydroxy phosphonate **8** were readily prepared as pairs of alcohol diastereomers at the carbon adjacent to S or P from the addition of sodium bisulfite¹⁴ and diethylphosphite,²¹ respectively, in ~1.3:1 ratio in both compounds based on ¹³C NMR spectral data.

The inhibitory activities of compounds **1–8** against NV were studied using a FRET-based enzyme²² and cell-based assays using the NV replicon cell system,²³ and results are summarized in Table 1. The half maximum inhibitory concentration (IC₅₀) or half maximum effective concentration (EC₅₀) values of these compounds, except for compound **6**, were found to be in the submicromolar ranges. Among different P3 residues, that is, compounds **1–5**, compound **2** displayed the highest inhibitory activities both in the enzyme and cell-based assays. The *D*-amino acid analog **3** exhibits lower activities than that of **2**, and 2-naphthyl **4** and biphenyl **5** showed slightly weaker activity than that of **2**. Among different warheads, compounds **2** and **7** possess the strongest activities. Bisulfite adduct **7** is a precursor and pro-drug of **2** through equilibrium in aqueous solution.¹⁴ It is possible that the activity of α-hydroxy phosphonate (warhead) varies in different conditions in enzyme and cell-based assays. Nevertheless, compound **8** showed strong activity in NV replicon cells.

We further examined the broad-spectrum inhibitory activities of compounds **2** and **6** against 3Cpro or 3CLpro from NV, MD145, HRV, and SARS-CoV in the FRET-based enzyme assays (Table 2), and against NV, MNV-1, HRV18, and coronavirus 229E in cell culture systems (Table 3). Both compounds effectively inhibited tested proteases and viruses. Moreover, tripeptidyl **2** showed nanomolar inhibitory activity against all viruses, suggesting that a broad-spectrum inhibitor against multiple viruses can be achieved based on the conservation of the substrate binding pocket of 3Cpro and 3CLpro.

The enzyme selectivity of a representative inhibitor, compound **2**, was assessed using a panel of proteases, including human neutrophil elastase, α-chymotrypsin, trypsin, and thrombin. Inhibitor **2**, up to 10 μM, was found to display minimal or no activity against these enzymes.

Highly potent inhibitors against noroviruses, picornaviruses and coronaviruses were discovered through the design of tripeptidyl transition state inhibitors and transition state mimics capable of binding to the active site of viral 3Cpro or 3CLpro. The cell-based results suggest that a naphthylalanine residue at P3 site is important for antiviral activity of the tripeptidyl compounds. These inhibitors could be further developed as broad-spectrum antivirals against these important viruses.

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Supplementary data

Supplementary data (synthetic procedure, analysis data, cell line and cell culture, and protocols for cell-viability assay)

Table 3

EC₅₀ values of two active tripeptidyl compounds **2** and **6**, against various viruses in the cell-based assays and CC₅₀ (concentration that causes 50% cell death) values²⁴

Compound	NV (μM)	MNV-1 (μM)	HRV18 (μM)	Corona-virus 229E (μM)	CC ₅₀ (μM)
2	0.04 ± 0.02	0.6 ± 0.2	0.015 ± 0.03	0.2 ± 0.1	87 ± 6.3
6	4.2 ± 2.4	4.2 ± 2.1	0.03 ± 0.04	0.5 ± 0.2	>100

associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2013.09.070>.

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