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Design, synthesis, and evaluation of 3C protease inhibitors as anti-enterovirus 71 agents

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ABSTRACT

Human enterovirus (EV) belongs to the picornavirus family, which consists of over 200 medically relevant viruses. A peptidomimetic inhibitor AG7088 was developed to inhibit the 3C protease of rhinovirus (a member of the family), a chymotrypsin-like protease required for viral replication, by forming a covalent bond with the active site Cys residue. In this study, we have prepared the recombinant 3C protease from EV71 (TW/2231/98), a particular strain which causes severe outbreaks in Asia, and developed inhibitors against the protease and the viral replication. For inhibitor design, the P3 group of AG7088, which is not interacting with the rhinovirus protease, was replaced with a series of cinnamoyl derivatives directly linked to P2 group through an amide bond to simplify the synthesis. While the replacement caused decreased potency, the activity can be largely improved by substituting the α,β-unsaturated ester with an aldehyde at the P1′ position. The best inhibitor **10b** showed EC_{50} of 18 nM without apparent toxicity (CC_{50} > 25 μM). Our study provides potent inhibitors of the EV71 3C protease as anti-EV71 agents and facilitates the combinatorial synthesis of derivatives for further improving the inhibitory activity.

1. Introduction

Enterovirus (EV), a member of *Picornaviridae* family, is a primary causative agent of hand, foot, and mouth diseases in humans and animals.[1] In severe cases, EV can damage the central nervous systems leading to viral meningitis, encephalitis, and severe myocarditis, as well as fatal pulmonary edema.[2–4] Children are particularly vulnerable due to their relative immunodeficiency.[5,6] There are over 5 billion cases of EV infections annually worldwide and several outbreaks have been reported.[7–9] In 1998, a severe outbreak by the EV71 (TW/2231/98) occurred in Taiwan causing about 120,000 cases of infection in children and 78 deaths.[10,11] Since then, a steady number of cases caused by this particular EV strain have been reported annually. In May 2008, Chinese health authorities reported a major outbreak of EV71 in Anhui province of China, which has led to 28 deaths as of May 7.

To date, no effective anti-viral therapy for the diseases caused by EV is available. Like other picornaviruses such as polioviruses, rhinoviruses (RV), coxsackieviruses, and hepatitis A viruses, a virally encoded chymotrypsin-like protease (3C protease) is required for the proteolytic processing of the large polyproteins translated from the viral RNA genomes and thus essential for viral replication.[12,13] The RV 3C protease was used as a target to develop inhibitor AG7088, aiming to treat the common cold.[14–16] This peptidomimetic inhibitor (see below for structure) contains a lactam ring to mimic Gln at the P1 position, fluoro-phenylalanine at P2, Val at P3 followed by 5-methyl-3-isoxazole, and an α,β-unsaturated ester at P1′ as a Michael acceptor to form a covalent bond with the active site Cys residue.[17,18] Recent research has shown that AG7088 is effective against EV6 and EV9 with EC_{50} of 43 and 15 nM, respectively, likely due to the structural similarity of their

Abbreviations: EV, enterovirus; RV, rhinovirus; SARS-CoV, severe acute respiratory syndrome-coronavirus; NiNTA, nickel nitrilo-tri-acetic acid; Dabcyl, 4-(4-dimethylaminophenylazo)benzoic acid; Edans, 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid; Boc, *tert*-butyloxycarbonyl; Cbz, benzyloxycarbonyl; MES, 2-*N*-morpholono-ethanesulfonic acid; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum.

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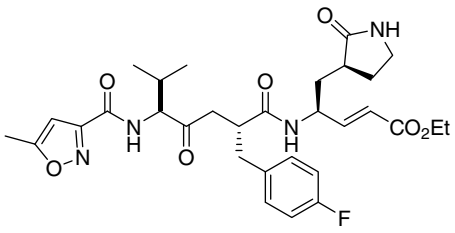
3C proteases judging from their sequence homology.[19] This provides a starting point for developing potent inhibitors against EV71.



AG7088

In this study we have expressed, purified, and characterized the recombinant EV71 3C protease and developed a fluorescence-based enzymatic assay according to the substrate specificity. Based on the crystal structure of RV 3C protease in complex with AG7088,[17] we designed and synthesized new compounds and evaluated their inhibitory activities in both enzymatic and cell-based assays. Our findings serve as a basis for anti-EV71 drug development.

2. Results

2.1. Preparation of the active protease

The EV71 3C protease expressed with pET16b vector contained N- or C-terminal hexa-His-tag and both forms were purified by using Ni–NTA column. In order to observe the effect of additional residues (hexa-His) on the protease activity, we also expressed the protease without any tag and purified it by using a cation-exchange column followed by a size-exclusion column. The three forms of the protein were purified to homogeneity as shown in the SDS–PAGE (Fig. 1A). The yields of the N- and C-terminal His-tagged forms were higher (2.2 and 3.6 mg/L medium, respectively) than that of the tag-free protease (0.8 mg/L medium).

The three forms of protease were compared for their activities using the fluorescence-based assay as described below. It was found that the protease with a C-terminus His-tag had specific activity equal to that of the tag-free enzyme, but the enzyme with N-terminal His-tag displayed 10-fold lower activity, indicating that additional N-terminal amino acids interfered with the protease activity. We thus used the protease with C-terminal His-tag in the following studies.

2.2. Substrate specificity and kinetics of the protease

The peptides corresponding to the autoprocessing sites by the 3C protease including RQAVTQ↓GFPTEL between vp2 and vp3, QTGTIQ↓GDRVAD between vp3 and vp1, DEAMEQ↓GVSDYI between 2A and 2C, IEALFQ↓GPPKFR between 3A and 3B, RTATVQ↓GPSLDF between 3B and 3C, YFCSEQ↓GEIQWN between 3C and 3D, and TSAVLQ↓SGFRKM derived from the N-terminal auto-processing site of a 3C-like protease from severe acute respiratory syndrome-coronavirus (SARS-CoV),[20,21] which all contain Gln followed by a small amino acid (Ser or Gly) to serve as the cleavage sites, were tested as substrates for 3C protease of EV71 (↓ indicates the cleavage site). Surprisingly, EV71 3C protease showed substantially better activity (60-fold) against TSAVLQ↓SGFRKM, the substrate of the SARS-CoV 3C-like protease, than aganist its own cleavage sites based on the HPLC assay (Fig. 1B). Therefore, the peptide Dabcyl-KTSAVLQSGFRKME-Edans with the fluorescence quenching pair (Dabcyl–Edans) was chosen for EV71 3C protease assay to monitor the fluorescence increase due to the peptide bond cleavage in real time. The K_m and k_{cat} of the enzyme in using this fluorogenic substrate were measured to be 5.8 ± 0.9 μM and 0.45 min^{-1}, respectively (Fig. 1C).

2.3. Synthesis of inhibitors

We used **3** (the intermediate in synthesizing AG7088)[22] as a starting material for making new AG7088 analogues (Fig. 2) by modifying the reported methods.[23–25] The protecting group *tert*-butyloxycarbony (Boc) in **3** was removed and the resulting species was reacted with Boc-protected Phe to make **4**. This molecule contained P1-lactam ring, P2-Boc-Phe, and P1′-α,β-unsaturated ethyl ester. To simplify the synthesis, a variety of different acyl chlorides were coupled with P2-Boc-Phe through an amide bond to make a library of compounds from **4**. Among them, we found that the best inhibition came from the compounds derived from cinnamoyl chlorides. As illustrated in Figure 2A, cinnamoyl chlorides **5a–c** with substituents of 3,4-(methylenedioxy), 4-dimethylamino, and H in the benzene ring, respectively, were incorporated into **4** to make **6a–c**.

To make the analogues containing aldehyde at the P1′ position, **2** was condensed with benzyloxycarbonyl (Cbz)-protected Phe to make **7** (Fig. 2B). The P1′-methyl ester in **7** was converted to aldehyde to make **8**. By adding cinnamoyl chlorides **5a–f** into **7**, the methyl esters **9a–f** were formed. Compounds **10a-f** were synthesized from the reduction, followed by oxidation of the methyl esters **9a–f**.

2.4. Evaluation of inhibitors

Recombinant 3C protease and the fluorogenic substrate were used to evaluate the IC_{50} of the synthesized compounds. Compound **4** with the basic features of AG7088 with P1-lactam ring, P1′-α,β-unsaturated ester, and P2-Boc-Phe did not inhibit the enzyme at 20 μM (the greatest concentration tested). With additional cinnamoyl groups, **6a–c** showed smaller IC_{50} values of 10.6, 8.2, and 10.0 μM against the enzyme, respectively, when they were not preincubated with the protease (Table 1). However, the time-dependent inhibition was observed in a prolonged time window for **6b** as an example (Fig. 3, top panel). From the replot (Fig. 3, lower panel), the k_{inact} was calculated to be 0.077 min^{-1} and the K_i was 2.6 μM. In agreement with their K_i values, **6a–c** showed EC_{50} of 1.8, 2.9 and 1.3 μM, respectively, in the anti-viral assay. However, **6b** and **6c** displayed some cellular toxicity with CC_{50} of 21 and 8 μM, respectively.

When replacing α,β-unsaturated ester with an aldehyde at the P1′ position, **10a–e** displayed 10- to 100-fold smaller EC_{50} values compared to **6a–c** (Table 1). Both **10b** and **10d** displayed potent inhibitory activity with EC_{50} of 18 and 26 nM, respectively, without cytotoxicity at the concentration approximately 1000-fold greater than their EC_{50}. The anti-viral activity of **10d** was further demonstrated by using a western blot analysis. As shown in Figure 4, the confluent cells infected with EV71 (EV71 TW/2231/98) showed less quantity of viral protein (3C protease) as detected by using its antibody in the presence of increased concentrations of **10d** at 0.01, 0.025, 0.05, 0.1, 0.5, and 1 μM. The data were consistent with the EC_{50}. Compound **10e** showed the smallest EC_{50} of 7 nM with toxicity CC_{50} of 5 μM, giving the selective index (CC_{50}/EC_{50}) of 714. Compound **8** with a more flexible Cbz group also showed potent anti-viral activity (EC_{50} = 0.031 μM) but slight toxicity (CC_{50} = 24 μM). Compound **10f** containing 5-methyl-3-isoxazole showed less inhibitory activity (EC_{50} = 0.94 μM) in the anti-viral assay, indicating importance of the benzene ring in inhibiting the virus. In the enzymatic assay, these compounds including **10f** inhibited the protease potently, so their K_i could not be accurately deter-

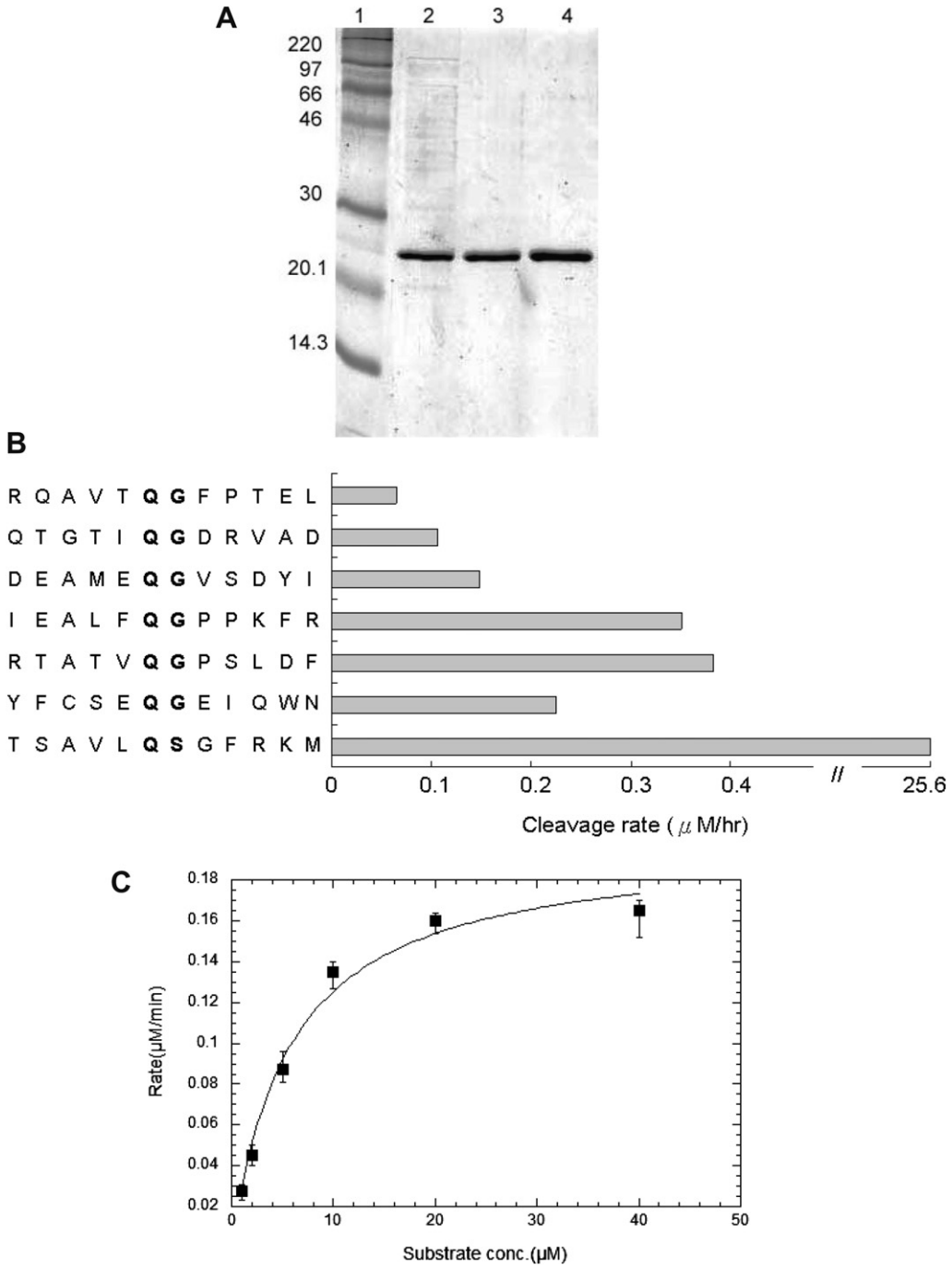


Figure 1. Purification and characterization of EV71 3C protease. (A) SDS–PAGE analysis of the purified proteins from different constructs. Lane 1 represents the MW markers. Lanes 2, 3, and 4 show the purified proteins without tag, with N-terminal His-tag, and with C-terminal His-tag, respectively. (B) Compared to the peptides derived from its own auto-activation sites, EV71 3C protease showed the best activity toward the preferred substrate, TSAVLQSGFRKM, of the SARS-CoV 3C-like protease. (C) Using the peptide containing a fluorescence quenching pair, the initial rates of the protease versus substrate concentrations were measured to obtain the k_{cat} of 0.45 min^{-1} and K_m of 5.8 ± 0.9 μM.

mined under the assay condition as limited by the 0.5 μM enzyme used to detect the activity. When aldehyde at the P1′ position of **10b** was replaced with CH_2OH or CO_2Me, the IC_{50} values became 40 and 50 μM, respectively, and EC_{50} > 25 μM (data not shown), indicating the requirement of aldehyde in inhibiting the 3C protease.

To rationalize the different inhibitory activities of these compounds, a computer program was used to dock **6b** (in cyan), **10b** (in yellow), and AG7088 (in salmon) into the active site of EV71 3C protease structure simulated using the structure of RV 3C protease as a template. From the computer modeling (Fig. 5), we found in order to accommodate the α,β-unsaturated ester of **6b** (a poor inhibitor) at the S1′ site, its cinnamoyl group is forced away from the S3 site (see the top view in the left panel and side view in the right panel) and could not make proper interaction with the nearby amino acids (e.g., Phe170), leading to significant loss of binding affinity and inhibitory activity. However, with the aldehyde of much smaller size in **10b** (a potent inhibitor), the binding becomes favorable. The aldehyde group of **10b** occupies a similar position to that of the α-carbon of AG7088 in the active site, and is readily accessible by the nucleophile Cys147.

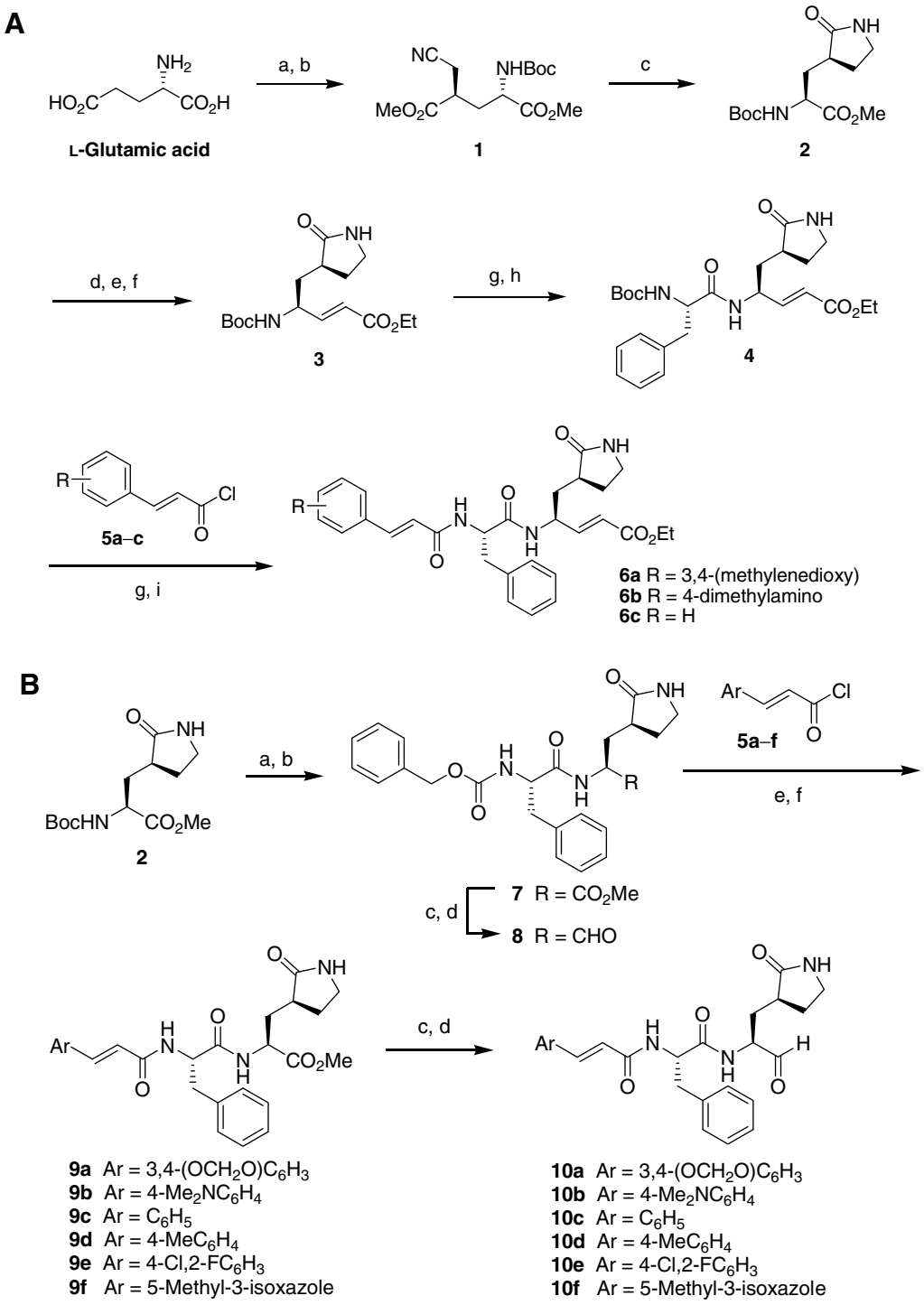


Figure 2. Synthesis of EV71 3C protease inhibitors with α,β-unsaturated ester (A) and aldehyde (B) at the P1′ position. (A) Reagents and conditions: (a) Me_3SiCl, MeOH, 0–25 °C, 18 h; then Boc_2O, Et_3N, 0–25 °C, 4 h; 96%. (b) $LiN(SiMe_3)_2$, THF, −78 °C, 3 h; then $BrCH_2CN$, 3.5 h; 82%. (c) H_2, cat. PtO_2, MeOH, $CHCl_3$, 25 °C, 12 h; then NaOAc, reflux, 12 h; 81%. (d) $NaBH_4$, LiCl, THF, EtOH, 25 °C, 18 h; 89%. (e) Pyridine-SO_3, Me_2SO, CH_2Cl_2, Et_3N, −10 °C, 3 h. (f) $[EtO_2CCHPO(OEt)_2]^-Na^+$, THF, −78 °C, 1 h; 75% for two steps. (g) HCl, 1,4-dioxane, 25 °C, 2 h. (h) Boc-L-Phe, HOBt, EDCI, $(i\text{-Pr})_2NEt$, DMF, 0–25 °C, 18 h; 72% for two steps. (i) Prepared cinnamoyl chloride **5a–c**, *N*-methylmorpholine, THF, 0–25 °C, 5 h; 53–66% for two steps. (B) Reagents and conditions: (a) HCl, 1,4-dioxane, 25 °C, 2 h. (b) Cbz-L-Phe, HOBt, EDCI, $(i\text{-Pr})_2NEt$, DMF, 0–25 °C, 20 h; 75% for two steps. (c) $LiBH_4$, THF, CH_2Cl_2, 0 °C, 3 h. (d) Dess–Martin periodinane, CH_2Cl_2, 0–25 °C, 3 h; 55–71% for two steps. (e) H_2, Pd/C, MeOH, 25 °C, 3 h. (f) Prepared cinnamoyl chloride **5a–f**, *N*-methylmorpholine, THF, 0–25 °C, 5 h; 45–68% for two steps.

3. Discussion

In this study, we have verified that EV71 3C protease is a target for anti-EV 71 drug discovery based on the enzyme- and cell-based assays. The compounds, which inhibited the protease activity, also blocked the viral replication in the cell-based assay with similar activity. In order to set up an assay for finding the protease inhibitors, we have expressed, purified, and characterized the recombinant EV71 3C protease. By testing the potential substrates, we unexpectedly found that the protease showed the best activity

Table 1
IC_{50}, EC_{50}, and CC_{50} of the peptidomimetic inhibitors against the EV71 3C protease

Compound	IC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
4	>20	>20	n.d.
6a (JMF1528)	10.6 ± 0.4	1.8 ± 0.1	>25
6b (JMF1810)	8.2 ± 1.7 (2.6 μM[a])	2.9 ± 0.1	21.4 ± 1.6
6c (JMF1813)	10.0 ± 1.3	1.3 ± 0.1	8.0 ± 0.2
8 (JMF1897)	<0.5	0.031 ± 0.001	23.6 ± 0.4
10a (JMF1807)	<0.5	0.16 ± 0.02	>25
10b (JMF1860)	<0.5	0.018 ± 0.003	>25
10c (JMF1857)	<0.5	0.096 ± 0.006	>25
10d (JMF1859)	<0.5	0.026 ± 0.002	>25
10e (JMF1900)	<0.5	0.007 ± 0.001	5.1 ± 0.7
10f (JMF1904)	<0.5	0.94 ± 0.36	>25

n.d., not determined.
[a] K_i measured from time-dependent inhibition.

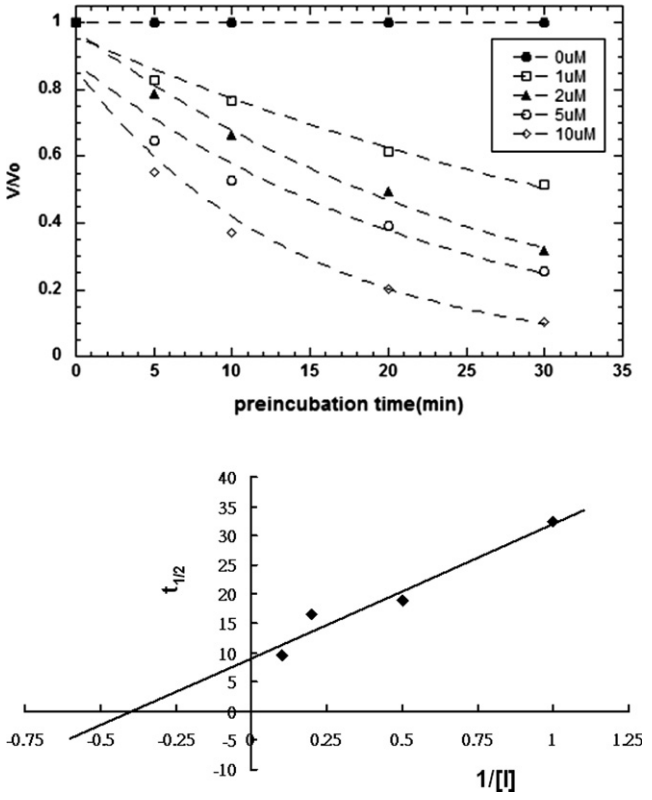


Figure 3. Enzyme inhibition studies of **6b**. Drop of enzymatic activities against the incubation time in the presence of 1–10 μM **6b** is shown (upper panel). The lower panel shows the replot of the half-life ($t_{1/2}$) of enzyme inactivation as a function of the reciprocal of the slow inactivator concentration. The k_{inact} is 0.077 min^{-1} and K_i is 2.6 μM for the time-dependent inactivator **6b** based on the kinetic data.

against a substrate peptide derived from the N-terminal autocleavage site of SARS-CoV 3C-like protease. Using this fluorogenic peptide, we evaluated a series of AG7088 analogues as inhibitors of EV71 3C protease, which were further tested for their anti-viral activities.

Compared to the complicated procedure to synthesize AG7088, which requires synthesis and assembly of three moieties, we report potent AG7088 analogues that can be easily synthesized and used as anti-EV71 agents in this study. In contrast to the tripeptide aldehyde inhibitors of RV 3C protease, which contain Gln or unnatural amino acid at P1, Phe at P2, and Cbz-Leu at the P3 position, showing moderate EC_{50} in the μM range,[26] our inhibitors **10b** and **10d** with the lactam ring at the P1 position, Phe at P2, cinnamoyl derivatives at P3, and aldehyde at P1′ exhibit great inhibitory activities in enzymatic and anti-viral assays without cytotoxicity.

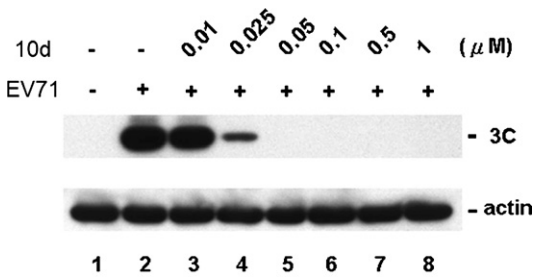


Figure 4. Inhibition of EV71 protein accumulation in RD cells by **10d** treatment. Cell lysates (40 μg protein per lane) were prepared from either mock-infected (lane 1) or EV71-infected (lanes 2–8) RD cells at 48 hours post infection and resolved with 12% SDS–PAGE. Western blot analysis for 3C protease or β-actin was conducted as described in Section 2. Lane 1, mock-infected cells not treated with **10d**; lanes 2–8, cells were treated with 0, 0.01, 0.025, 0.05, 0.1, 0.5, or 1 μM **10d**, respectively; The bands corresponding to EV71 3C protease (20 kDa) are indicated. Expression of β-actin was used to control equal protein loading.

Therefore, P1-lactam group is important and Leu or Val at the P3 position seems not important for the anti-virus activity. However, with only P1-lactam, P2-Phe, and P1′-α,β-unsaturated ester, the key features of AG7088, do not guarantee the protease-binding affinity (no enzyme inhibition was found for compound **4** at 20 μM). Addition of cinnamoyl group at P3 and simultaneous replacement of P1′-α,β-unsaturated ester with aldehyde yield potent inhibition as rationalized by the computer modeling. Aldehyde group, which can form reversible covalent bond with the catalytic Cys, is important for inhibition since other compounds with CH_2OH or CO_2Me at P1′ did not effectively inhibit the 3C protease. Our study provides potent EV71 3C protease inhibitors effective as anti-EV71 agents and facilitates the combinatorial synthesis of derivatives for further improving the inhibitory activity.

4. Experimental

4.1. General methods

All the reagents were commercially available and used without further purification unless indicated otherwise. All solvents were of anhydrous grade unless indicated otherwise. All non-aqueous reactions were carried out in oven-dried glassware under a slight positive pressure of argon unless otherwise noted. Reaction mixtures were magnetically stirred and monitored by thin-layer chromatography on silica gel. Flash chromatography was performed on silica gel of 60–200 μm particle size. Yields are reported for spectroscopically pure compounds. Melting points were recorded on an Electrothermal MEL-TEMP® 1101D melting point apparatus. NMR spectra were recorded on Bruker AVANCE 600, 400 and 300 spectrometers. Chemical shifts are given in δ values relative to tetramethylsilane (TMS); coupling constants *J* are given in Hz. Internal standards were $CDCl_3$ ($\delta_H = 7.24$) or DMSO-d_6 ($\delta_H = 2.49$) for ^{1}H-NMR spectra; $CDCl_3$ ($\delta_C = 77.0$) or DMSO-d_6 ($\delta_C = 39.5$) for ^{13}C-NMR spectra. The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad), and dd (double of doublets). IR spectra were recorded on a Thermo Nicolet 380 FT-IR spectrometer. High resolution ESI mass spectra were recorded on a Bruker Daltonics spectrometer.

4.2. Expression and purification of EV71 3C protease

The gene encoding EV71 3C protease was cloned from viral cDNA obtained from Chang-Gung memorial hospital (Tao-Yuan, Taiwan) by using a polymerase chain reaction (PCR). The forward primer 5′-CATGCCATGGCATCATCATCATCATCATGGCCGAGCTTGGAC-3′ and the backward primer 5′-GCGCTCGAGTCATTGTTCACT

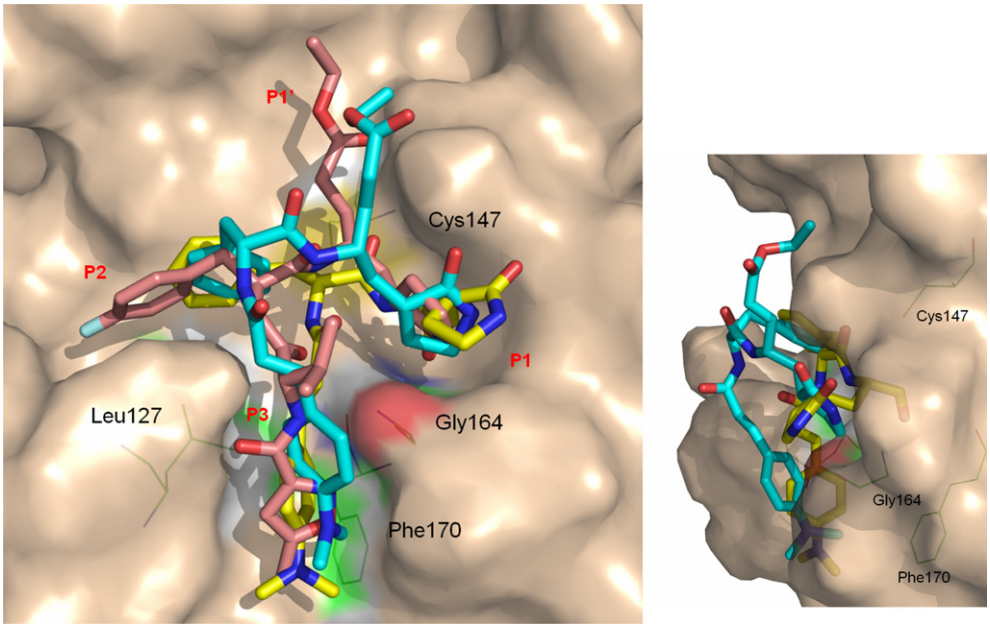


Figure 5. Computer modeling of the complex structures of EV71 protease with **6b** and **10b** based on the published structure (PDB code 1CQQ) of RV protease with AG7088 bound. The carbon skeletons of **6b**, **10b**, and AG7088 are shown in cyan, yellow, and salmon, respectively. The oxygen atoms are shown in red and nitrogen atoms are shown in blue. The side view is shown in the right panel.

GCAAAAGTATCC-3′, which contain NcoI and XhoI restriction sites (underlined), were used to express N-terminal His-tagged protease; and the forward primer 5′-CATGCCATGGGGCCGAGCTTGGAC-3′ and the backward primer 5′- GCGCTCGAGTCAATGATGATG ATGATGATGTTGTTCACTGCAAAAGTATCC- 3′) were used for C-terminal His-tagged protease. For expression of tag-free protease, the forward primer 5′-CATGCCATGGGGCCGAGCTTGGAC-3′ and backward primer 5′-GCGCTCGAGTCATTGTTCACTGCAAAAGTATCC-3′ containing the same restriction sites were used. The PCR products were digested with NcoI and XhoI, and the DNA fragments were cloned into pET16b (Novagen). The plasmid containing protease gene was used to transform *E. coli* JM109 competent cells and the transformed cells were streaked on a Luria-Bertani (LB) agar plate containing 100 μg/mL ampicillin. Ampicillin-resistant colonies were selected from the agar plate and grown in 5 mL LB culture containing 100 μg/mL ampicillin overnight at 37 °C. The correct constructs based on gene sequencing were subsequently transformed into *E. coli* Origami B(DE3) (Novagen) for protein expression. A 5-mL overnight culture of a single colony was used to inoculate 500 mL of fresh LB medium containing 25 μg/mL kanamycin and 50 μg/mL ampicillin. The cells were grown to A_{600} = 0.6 and induced with 1 mM isopropyl-β-thiogalactopyranoside. After 4–5 h, the cells were harvested by centrifugation at 7000*g* for 15 min.

Purification of His-tagged protease was conducted at 4 °C. The cell paste obtained from 2 L cell culture was suspended in an 80 mL lysis buffer containing 25 mM Tris–HCl, pH 7.5, and 150 mM NaCl. A French-press instrument (AIM-AMINCO spectronic Instruments) was used to disrupt the cells at 12,000 psi. The lysis solution was centrifuged and the debris was discarded. The cell-free extract was loaded onto a 20-mL Ni–NTA column, which was equilibrated with 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 5 mM imidazole. The column was washed with 5 mM imidazole followed by a 30 mM imidazole-containing buffer. His-tagged protease was eluted with 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 300 mM imidazole. The protein solution was dialyzed against a 2× 2 L buffer containing 12 mM Tris–HCl, pH 7.5, 120 mM NaCl, 0.1 mM EDTA, 7.5 mM β-mercaptoethanol, and 1 mM DTT. The purified protein was confirmed by N-terminal sequencing and mass spectrometry. The protein concentration used in all experiments was determined from the absorbance at 280 nm. For preparing untagged protein, SP sepharose, a cation exchange column, was applied to purify the protease. The untagged protein was eluted by a linear gradient of NaCl from 200 to 1000 mM. Subsequently, the eluted sample was subjected to a size-exclusion chromatography (Sephadex 200, 10/300 GL column) for further purification.

4.3. Protease activity assay

The peptides for testing as the protease substrates were synthesized via solid phase using a 433A peptide synthesizer (Applied Biosystems, USA). Starting with 0.10 mmol (0.101 g) of *p*-hydroxymethyl phenoxymethyl polystyrene resin (1.01 mmol/g), the synthesis was performed using a stepwise FastMoc protocol (Applied Biosystems, USA). The amino acids were introduced using the manufacturer's prepacked cartridges (1 mmol each). For examining the substrate specificity of the protease, each peptide at 50 μM was incubated with 0.5 μM protease for 6 and 12 h, and the resulting mixture was injected into C-18 reverse-phase HPLC for analysis using the above-mentioned conditions. The areas of the product peaks were integrated to calculate the reaction rate of each peptide substrate under the catalysis of the protease.

The best peptide substrate was attached with a fluorescence quenching pair, and the fluorogenic peptide (Dabcyl-KTSAVLQ SGFRKME-Edans) was used to measure the protease activity. The kinetic constants for the fluorogenic peptide were measured in 10 mM MES at pH 6.5 (the optimal pH for EV71 3C protease) at 25 °C. Enhanced fluorescence due to the cleavage of the peptide was monitored at 538 nm with excitation at 355 nm using a fluorescence plate reader (Fluoroskan Ascent from ThermoLabsystems, Finland). The enzyme concentration used in measuring K_m and k_{cat} values was 0.5 μM and the substrate concentrations were from 0.5- to 5-fold of an estimated K_m value. Substrate concentration was determined by using the extinction coefficients 5438 M^{-1} cm^{-1} at 336 nm (Edans) or 15,100 M^{-1} cm^{-1} at 472 nm (Dabcyl). The initial rates within 10% substrate consumption at different substrate

concentrations were used to calculate the kinetic parameters using Michaelis–Menten equation fitted with the KaleidaGraph computer program.

4.4. Synthesis of AG7088 analogues

Known compounds **1**–**3** and **6a** were prepared as previously reported.[22–24] The synthesis of new compounds is described below in details.

4.4.1. (2*S*,4*S*)-2-*tert*-Butoxycarbonylamino-4-cyanomethylpentanedioic acid dimethyl ester (1)

Oil; TLC (EtOAc/hexane, 3:7) R_f = 0.13; IR (neat) 3371, 2982, 2263, 1743, 1719, 1520, 1441, 1370, 1250, 1169, 1054 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.09 (1H, d, J = 8.6 Hz), 4.37 (1H, dd, J = 13.6, 8.6 Hz), 3.74 (3H, s), 3.73 (3H, s), 2.82 (3H, m), 2.14 (2H, m), 1.42 (9H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.4,169.3, 153.3, 117.1, 82.3, 52.7, 52.6, 51.0, 38.2, 33.8, 28.2 (3×), 19.0; HRMS calcd for $C_{14}H_{23}N_2O_6$ (M^++H): 315.1478; found: 315.1554.

4.4.2. 2*S*-*tert*-Butoxycarbonylamino-3-(2-oxopyrrolidin-3*S*-yl)-propionic acid methyl ester (2)

White solid; TLC (EtOAc) R_f = 0.27; IR (KBr) 3304, 2981, 1745, 1699, 1524, 1260 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.03 (1H, br s, NH), 5.49 (1H, d,J = 7.9 Hz), 4.32–4.29 (1H, m), 3.27 (3H, s), 3.34–3.29 (2H, m), 2.47–2.41 (2H, m), 2.15–2.05 (1H, m), 1.88–1.75 (2H, m), 1.41 (9H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 175.7,172.7, 155.5, 79.3, 51.9, 45.3, 40.1, 38.0, 33.4, 27.9 (3×), 27.6; HRMS calcd for $C_{13}H_{23}N_2O_5$ (M^++H): 287.1529; found: 287.1611.

4.4.3. 4*S*-*tert*-Butoxycarbonylamino-5-(2-oxopyrrolidin-3*S*-yl)-pent-2-enoic acid ethyl ester (3)

White foam; TLC (EtOAc/hexane, 1:1) R_f = 0.32; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.82 (1H, dd, J = 15.6, 5.1 Hz), 6.31 (1H, br s, NH), 5.92 (1H, dd, J = 15.6, 1.4 Hz), 5.27 (1H, d, J = 8.1 Hz), 4.37–4.26 (1H, m), 4.15 (2H, q, J = 7.2 Hz), 3.34–3.29 (2H, m), 2.48–2.44 (2H, m), 2.01–1.91 (2H, m), 1.81–1.74 (1H, m), 1.59–1.53 (1H, m), 1.27 (9H, s), 1.24 (3H, t, J = 7.2 Hz); HRMS calcd for $C_{16}H_{27}N_2O_5$ (M^++H): 327.1842; found: 327.1912.

4.4.4. Ethyl 4-[2-(*tert*-butoxycarbonyl)amino-1-oxo-3-phenyl]propylamino-5-(2-oxo-3-pyrrolidyl)-2-pentenoate (4)

A solution of HCl in 1,4-dioxane (4.0 M, 10 mL) was added to a solution of **3** (652 mg, 2 mmol) in 1,4-dioxane (5 mL) at room temperature. The mixture was stirred for 2 h, and then concentrated under reduced pressure to give a crude ammonium salt. The material and Boc-L-Phe (584 mg, 2.2 mmol) were dissolved in DMF (20 mL) and cooled to 0 °C, followed by the addition of *N*,*N*-diisopropylethylamine (1.0 mL, 6 mmol), HOBt (338 mg, 2.5 mmol), and EDCI (479 mg, 2.5 mmol). The mixture was removed from the ice bath, stirred for 18 h at room temperature, and diluted with CH_2Cl_2 (50 mL). The mixture was washed with 10% aqueous citric acid (20 mL), water (20 mL), and brine (20 mL). The organic phase was dried over Na_2SO_4, filtrated, and concentrated. The residual oil was purified by flash column chromatography (EtOAc/hexane, 2:1) to afford the desired product **4** (682 mg, 72% for two steps). White foam; TLC (EtOAc) R_f = 0.45; IR (neat) 3257, 2910, 1659, 1421, 1209 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.09 (1H, d, J = 8.7 Hz), 7.55 (1H, s), 7.26–7.16 (5H, m), 7.01 (1H, d, J = 8.1 Hz), 6.76 (1H, dd, J = 15.7, 4.7 Hz), 5.63 (1H, dd, J = 15.7, 1.4 Hz), 4.55–4.45 (1H, m), 4.13–4.08 (3H, m), 3.13 (1 H, t, J = 9.2 Hz), 3.03 (1H, dd, J = 16.4, 9.1 Hz), 2.87 (1 H, dd, J = 13.5, 6.0 Hz), 2.78 (1H, dd, J = 13.5, 9.2 Hz), 2.32–2.29 (1H, m), 2.12–2.07 (1H, m), 1.85–1.80 (1H, m), 1.64–1.60 (1H, m), 1.48–1.40 (1H, m), 1.31 (9H, s), 1.20 (3H, t, J = 7.1 Hz); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 178.3, 171.5, 165.5, 155.3, 149.3, 137.8, 129.1 (2×), 128.0 (2×), 126.3, 119.5, 78.0, 59.8, 56.2, 47.2, 38.2, 37.4, 37.2, 34.9, 28.0, 27.2, 14.1 (3×); HRMS calcd for $C_{25}H_{35}N_3NaO_6$: 496.2424 (M^++Na); found: 496.2427.

4.4.5. Ethyl 4-{2-[3,4-(methylenedioxy)cinnamoyl]amino-1-oxo-3-phenyl}propylamino-5-(2-oxo-3-pyrrolidyl)-2-pentenoate (6a)

To an ice cooled suspension of 3,4-(methylenedioxy)cinnamic acid (96 mg, 0.5 mmol) in THF (3 mL), PCl_5 (125 mg, 0.6 mmol) was added. The mixture was stirred for 10 min at 0 °C, and then for 30 min at room temperature to prepare an acid chloride solution.[25] To a solution of HCl in 1,4-dioxane (4.0 M, 2 mL) was added a solution of **4** (190 mg, 0.4 mmol) in 1,4-dioxane (2 mL) at room temperature. The mixture was stirred for 2 h, and then concentrated under reduced pressure to yield a crude ammonium salt. The material was dissolved in THF (15 mL) and DMF (1 mL) and cooled to 0 °C. The above acid chloride solution and *N*-methylmorpholine (0.33 mL, 3 mmol) were added sequentially, and the ice bath was removed. After being stirred for 5 h at room temperature, the reaction mixture was partitioned between CH_2Cl_2 (20 mL) and water (10 mL). The organic layer was washed with water (2× 10 mL), and brine (10 mL), dried over Na_2SO_4, filtrated, and concentrated. The residue was purified by flash column chromatography (MeOH/CH_2Cl_2, 1:99) to provide **6a** (144 mg, 58%). White solid; mp 103–105 °C; TLC (MeOH/CH_2Cl_2, 1:99) R_f = 0.24; IR (KBr) 3283, 2919, 1653, 1447, 1241 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.81 (1H, d, J = 7.0 Hz), 7.50 (1H, d, J = 15.5 Hz), 7.29–7.20 (4H, m), 7.00–6.94 (2H, m), 6.79–6.67 (3H, m), 6.31–6.26 (2H, m), 5.99 (2H, s), 5.75 (1H, d, J = 15.5 Hz), 5.10–5.00 (1H, m), 4.57–4.40 (1H, m), 4.18 (2H, q, J = 7.1 Hz), 3.31–3.19 (3H, m), 3.08–3.06 (1H, m), 2.23–2.12 (2H, m), 2.10–1.81 (2H, m), 1.80–1.40 (2H, m), 1.30 (3H, t, J = 7.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.2, 166.2, 165.8, 165.7, 149.2, 148.2, 146.8, 141.3, 136.5, 129.7, 129.1, 128.5 (2×), 126.9 (2×), 124.1, 121.2, 118.3, 108.5, 106.4, 101.4, 60.5, 54.8, 54.3, 49.5, 40.7, 38.9, 34.8, 29.7, 14.2; HRMS calcd for $C_{30}H_{34}N_3O_7$: 548.2397 (M^++H); found: 548.2399; Anal. calcd for $C_{30}H_{33}N_3O_7$: C 65.80, H 6.07, N 7.67; found: C 65.71, H 6.17, N 7.65.

4.4.6. Ethyl 4-{2-[4-(dimethylamino)cinnamoyl]amino-1-oxo-3-phenyl}propyl-amino-5-(2-oxo-3-pyrrolidyl)-2-pentenoate (6b)

Compound **6b** was prepared by following the procedure for preparing **6a**, except using 4-(dimethylamino)cinnamic acid. (Yield: 53%) Light yellow solid; mp 92–94 °C; TLC (MeOH/CH_2Cl_2, 1:99) R_f = 0.21; IR (KBr) 3275, 2910, 1665, 1421, 1221 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.30 (1H, d, J = 8.1 Hz), 8.20–8.12 (1H, m), 7.58 (1H, s), 7.39–7.18 (8H, m), 6.91–6.75 (2H, m), 6.71 (2H, d, J = 8.9 Hz), 5.67 (1H, d, J = 15.8 Hz), 4.69–4.60 (1H, m), 4.58–4.49 (1H, m), 4.11 (2H, q, J = 7.0 Hz), 3.18–2.80 (10H, m), 2.35–2.27 (1H, m), 2.12–2.07 (1H, m), 1.91–1.81 (1H, m), 1.68–1.57 (1H, m), 1.51–1.43 (1H, m), 1.20 (3H, t, J = 7.0 Hz); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 178.8, 171.5, 167.9, 166.0, 151.5, 149.6, 140.7, 138.2, 129.6, 129.3 (2×), 128.5 (2×), 126.7 (2×), 122.7, 120.3, 117.0, 112.3 (2×), 60.3, 54.9, 48.1, 40.1 (2×), 38.5, 38.0, 37.8, 35.3, 27.8, 14.6; HRMS calcd for $C_{31}H_{38}N_4NaO_5$: 569.2740 (M^++Na); found: 569.2756; Anal. calcd for $C_{31}H_{38}N_4O_5$: C 68.11, H 7.01, N 10.25; found: C 68.07, H 7.13, N 10.19.

4.4.7. Ethyl 4-[2-(4-cinnamoyl)amino-1-oxo-3-phenyl]propylamino-5-(2-oxo-3-pyrrolidyl)-2-pentenoate (6c)

Compound **6c** was prepared by following the procedure for preparing **6a**, except using cinnamoyl chloride. (Yield: 66%) Colorless solid; mp 87–89 °C; TLC (MeOH/CH_2Cl_2, 1:99) R_f = 0.28; IR (KBr) 3271, 2925, 1666, 1456, 1241 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.47–8.40 (1H, m), 8.35 (1H, d, J = 8.6 Hz), 7.58–7.54

(3H, m), 7.43–7.37 (4H, m), 7.28–7.25 (4H, m), 7.18 (1H, d, J = 6.1 Hz), 6.87–6.70 (2H, m), 5.67 (1H, dd, J = 15.8, 1.5 Hz), 4.72–4.61 (1H, m), 4.58–4.50 (1H, m), 4.11 (2H, q, J = 7.1 Hz), 3.28–2.95 (3H, m), 2.93–2.87 (1H, m), 2.25–2.34 (1H, m), 2.15–1.79 (2H, m), 1.69–1.40 (2H, m), 1.20 (3H, t, J = 7.1 Hz); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 178.8, 171.3, 166.1, 165.3, 149.5, 139.4, 137.9, 135.2, 129.9, 129.6 (2×), 129.4 (2×), 128.6 (2×), 127.9 (2×), 126.9, 122.3, 120.2, 60.4, 55.0, 48.0, 38.3, 38.0, 37.8, 35.3, 27.8, 14.6; HRMS calcd for $C_{29}H_{33}N_3NaO_5$: 526.2318 (M^++Na); found: 526.2323; Anal. calcd for $C_{29}H_{33}N_3O_5$: C 69.17, H 6.60, N 8.34; found: C 69.01, H 6.69, N 8.37.

4.4.8. Methyl 2-[2-(benzyloxycarbonyl)amino-1-oxo-3-phenyl]propylamino-3-(2-oxo-3-pyrrolidyl)-propionate (7)

A solution of HCl in 1,4-dioxane (4.0 M, 10 mL) was added to a solution of **2** (573 mg, 2 mmol) in 1,4-dioxane (5 mL) at room temperature. The mixture was stirred for 2 h, and then concentrated under reduced pressure to yield a crude ammonium salt. The material and Cbz-L-Phe (660 mg, 2.2 mmol) were dissolved in DMF (20 mL) and cooled to 0 °C, followed by the addition of *N*,*N*-diisopropylethylamine (1.0 mL, 6 mmol), HOBt (340 mg, 2.5 mmol), and EDCI (482 mg, 2.5 mmol). The mixture was removed from the ice bath, stirred for 20 h at room temperature, and diluted with CH_2Cl_2 (50 mL). The mixture was washed with 10% aqueous citric acid (20 mL), water (20 mL), and brine (20 mL). The organic phase was dried over Na_2SO_4, filtrated, and concentrated. The residual oil was purified by flash column chromatography (EtOAc/hexane, 2:1) to afford the desired product **7** (702 mg, 75% for two steps). White foam; TLC (EtOAc) R_f = 0.40; IR (neat) 3222, 2935, 1622, 1437 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.58 (1H, d, J = 7.8 Hz), 7.64 (1H, s), 7.50 (1H, d, J = 8.4 Hz), 7.49–7.15 (10H, m), 4.94 (1H, d, J = 12.8 Hz), 4.92 (1H, d, J = 12.8 Hz), 4.37–4.34 (1H, m), 4.30–4.26 (1H, m), 3.60 (3H, s), 3.14–2.98 (3H, m), 2.77–2.70 (1H, m), 2.33–2.26 (1H, m), 2.09–2.05 (2H, m), 1.63–1.57 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 178.4, 172.7, 172.4, 156.3, 138.5, 137.5, 129.6 (2×), 128.7 (2×), 128.5 (2×), 128.1, 127.8 (2×), 126.7, 65.6, 56.5, 52.4, 50.7, 38.7, 38.0, 37.7, 32.9, 27.6; HRMS calcd for $C_{25}H_{29}N_3NaO_6$: 491.1954 (M^++Na); found: 491.1960.

4.4.9. 2-[2-(Benzyloxycarbonyl)amino-1-oxo-3-phenyl]propylamino-3-(2-oxo-3-pyrrolidyl)-propan-1-al (8)

Compound **7** (235 mg, 0.5 mmol) was dissolved in CH_2Cl_2 (10 mL). $LiBH_4$ solution (1 M in THF, 0.6 mL, 0.75 mmol) was added to the above solution at 0 °C. After the reaction mixture was stirred for 3 h at 0 °C, the reaction was quenched by adding saturated NH_4Cl solution (5 mL). All volatiles were removed under reduced pressure and the aqueous residue was extracted with CH_2Cl_2 (5× 10 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtrated, and concentrated. The crude product (225 mg) was used in the next step without further purification. The above alcohol (225 mg) was dissolved in CH_2Cl_2 (20 mL) and was treated with Dess–Martin periodinane (640 mg, 1.5 mmol) at 0 °C. The reaction mixture was warmed to room temperature and stirring was continued for 3 h. The reaction was quenched with a mixture of $Na_2S_2O_3$ (1.26 g) in aqueous saturated $NaHCO_3$ (20 mL). The resulting mixture was stirred until the organic layer turned clear. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (3× 10 mL). The combined organic layers were dried over Na_2SO_4, filtrated, and concentrated. The residue was purified by flash column chromatography (MeOH/CH_2Cl_2, 1:19) to afford the desired product **8** (148 mg, 68% for two steps). White foam; TLC (MeOH/CH_2Cl_2, 1:9) R_f = 0.55; IR (neat) 3433, 2922, 1744, 1656, 1402 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.32 (1H, s), 8.57 (1H, d, J = 6.8 Hz), 8.00 (1H, d, J = 7.5 Hz), 7.80 (1H, br s), 7.33–7.21 (10H, m), 4.95 (1H, d, J = 13.7 Hz), 4.92 (1H, d, J = 13.7 Hz), 4.40–4.35 (1H, m), 4.25–4.13 (1H, m), 3.23–3.02 (3H, m), 2.85–2.81 (1H, m), 2.26–2.14 (2H, m), 1.91–1.88 (1H, m), 1.65–1.62 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 201.3 178.7, 172.7, 156.3, 138.3, 137.5, 129.7 (2×), 128.7 (2×), 128.5 (2×), 128.1, 127.9 (2×), 126.7, 65.7, 62.1, 56.8, 56.7, 38.0, 37.6, 29.7, 27.7; HRMS calcd for $C_{24}H_{28}N_3O_3$: 438.2029 (M^++H); found: 438.2031; Anal. calcd for $C_{24}H_{27}N_3O_3$: C 65.89, H 6.22, N 9.60; found: C 65.76, H 6.53, N 9.51.

4.4.10. 3-[5-Methylisoxazol-3-yl] acrylic acid ethyl ester

To a solution of 5-methylisoxazole-3-carboxaldehyde (1.0 g, 9 mmol) and (carbethoxymethylene) triphenylphosphorane (4.70 g, 13.5 mmol) in toluene (30 mL) was refluxed for 5 h and concentrated under reduced pressure to give a crude product. The residual solid was purified by flash column chromatography (EtOAc/hexane, 1:9) to afford the α,β-unsaturated ethyl ester (1.32 g, 81%) as a white solid mp 35–37 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.60 (1H, d, J = 16.1 Hz), 6.42 (1H, d, J = 16.1 Hz), 6.14 (1H, s), 4.25 (2H, q, J = 7.1 Hz), 2.43 (3H, s), 1.31 (3H, t, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 170.3, 165.7, 159.8, 131.9, 125.2, 99.3, 60.9, 14.2, 12.2; HRMS calcd for $C_9H_{12}NO_3$: 182.0817 (M^++H); found: 182.0816.

4.4.11. 3-[5-Methylisoxazol-3-yl] acrylic acid

The above α,β-unsaturated ethyl ester (1.32 g, 7.3 mmol) was dissolved in THF (40 mL), and was added aqueous LiOH (1 N, 15 mL, 15 mmol). The reaction mixture was stirred for 1 h at room temperature, and then 10% aqueous citric acid was added to make the pH 2–3. The mixture was extracted with EtOAc (5× 20 mL), and the combined organic layers were washed with brine. The organic layer was dried over $MgSO_4$, filtrated, and concentrated under reduced pressure. The residual solid (1.11 g) was used directly without further purification. Colorless solid, mp 175–177 °C; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 7.38 (1H, d, J = 16.0 Hz), 6.74 (1H, s), 6.67 (1H, d, J = 16.0 Hz), 2.43 (3H, s); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 170.8, 167.1, 160.3, 131.0, 127.7, 100.4, 12.2; HRMS calcd for $C_7H_8NO_3$: 154.0504 (M^++H); found: 154.0506.

4.4.12. Methyl 2-{2-[3,4-(methylenedioxy)cinnamoyl]amino-1-oxo-3-phenyl}propyl-amino-3-(2-oxo-3-pyrrolidyl)-propionate (9a)

To an ice cooled suspension of 3,4-(methylenedioxy)cinnamic acid (96 mg, 0.5 mmol) in THF (3 mL), PCl_5 (125 mg, 0.6 mmol) was added. The mixture was stirred for 10 min at 0 °C, and then 30 min at room temperature to prepare an acid chloride solution (25). Compound **7** (187 mg, 0.4 mmol) in CH_3OH (10 mL) was treated with Pd/C (20 mg) and then put under a hydrogen atmosphere at room temperature for 3 h. The catalyst was then removed by filtration through Celite followed by washing with CH_3OH. The filtrate was concentrated in vacuo to afford the deprotecting amine product (131 mg). The material was dissolved in THF (15 mL) and DMF (1 mL) and cooled to 0 °C. The above acid chloride solution and *N*-methylmorpholine (0.33 mL, 3 mmol) were added sequentially, and the ice bath was removed. After being stirred for 5 h at room temperature, the reaction mixture was partitioned between CH_2Cl_2 (20 mL) and water (10 mL). The organic layer was washed with water (2× 10 mL), and brine (10 mL), dried over Na_2SO_4, filtrated, and concentrated. The residue was purified by flash column chromatography (MeOH/CH_2Cl_2, 1:99) to provide **9a** (124 mg, 61%). White foam; TLC (MeOH/CH_2Cl_2, 1:19) R_f = 0.20; IR (neat) 3501, 2934, 1756, 1655, 1417 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.64 (1H, d, J = 8.7 Hz), 8.17 (1H, d, J = 8.9 Hz), 7.63 (1H, s), 7.27–7.16 (6H, m), 7.10 (1H, s), 7.04–7.02 (1H, m), 6.94–6.92 (1H, m), 6.52 (1H, d, J = 15.7 Hz), 6.05 (2H, s), 4.71–4.68 (1H, m), 4.37–4.31 (1H, m), 3.62 (3H, s), 3.29–3.04 (2H, m), 2.85–2.78 (1H, m), 2.32–2.27 (2H, m), 2.14–1.95 (2H, m), 1.64–1.59 (2H, m); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 178.2, 172.5, 172.0, 165.3,

148.8, 148.2, 139.1, 138.1, 129.4 (2×), 128.3 (2×), 126.6, 123.5, 120.3, 120.2, 108.8, 106.5, 101.7, 54.2, 52.2, 50.6, 40.4, 37.9, 37.8, 32.7, 27.5; HRMS calcd for $C_{27}H_{29}N_3NaO_7$: 530.1903 (M^++Na); found: 530.1910.

4.4.13. Methyl 2-{2-[4-(dimethylamino)cinnamoyl]amino-1-oxo-3-phenyl} propyl-amino-3-(2-oxo-3-pyrrolidyl)-propionate (9b)

Compound **9b** was prepared by following the procedure to prepare **9a**, except using 4-(dimethylamino)cinnamic acid (Yield: 45%). Light yellow foam; TLC (MeOH/CH_2Cl_2, 1:19) R_f = 0.12; IR (neat) 3528, 2901, 1733, 1627, 1459 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 8.61 (1H, d, J = 7.9 Hz), 8.10 (1H, d, J = 8.3 Hz), 7.64 (1H, s), 7.34 (2H, d, J = 8.8 Hz), 7.28–7.22 (3H, m), 7.18 (1H, d, J = 15.6 Hz), 7.28–7.22 (2H, m), 6.69 (2H, d, J = 8.8 Hz), 6.39 (1H, d, J = 15.6 Hz), 4.68–4.64 (1H, m), 4.35–4.31 (1H, m), 3.61 (3H, s), 3.14–3.02 (3H, m), 2.93 (6H, s), 2.79 (1H, dd, J = 13.9, 10.1 Hz), 2.32–2.25 (1H, m), 2.12–2.04 (2H, m), 1.64–1.59 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 178.4, 172.7, 171.6, 162.8, 151.5, 138.1, 133.2, 132.4 (2×), 129.7 (2×), 129.6 (2×), 128.5, 126.8, 120.2, 118.5, 111.9 (2×), 55.3, 52.4, 50.8, 38.7 (2×), 38.0, 37.4, 32.9, 27.6; HRMS calcd for $C_{28}H_{34}N_4NaO_5$: 529.2427 (M^++Na); found: 529.2431.

4.4.14. Methyl 2-[2-(cinnamoyl)amino-1-oxo-3-phenyl]propylamino-3-(2-oxo-3-pyrrolidyl)-propionate (9c)

Compound **9c** was prepared by following the procedure to prepare **9a**, except using cinnamoyl chloride (Yield: 68%). White foam; TLC (MeOH/CH_2Cl_2, 1:19) R_f = 0.20; IR (neat) 3530, 2912, 1750, 1621, 1421 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 8.72 (1H, d, J = 7.9 Hz), 8.38 (1H, d, J = 8.3 Hz), 7.68 (1H, s), 7.54 (2H, d, J = 7.1 Hz), 7.41–7.26 (8H, m), 7.20–7.17 (1H, m), 6.70 (1H, d, J = 15.8 Hz), 4.75–4.71 (1H, m), 4.39–4.32 (1H, m), 3.64 (3H, s), 3.15–3.06 (3H, m), 2.84–2.80 (1H, m), 2.35–2.28 (1H, m), 2.12–2.09 (2H, m), 1.64–1.61 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 178.4, 172.7, 172.1, 165.2, 139.4, 138.2, 135.2, 129.9, 129.6 (2×), 129.4 (2×), 128.5 (2×), 128.0 (2×), 126.7, 122.3, 54.4, 52.4, 50.7, 39.7, 38.1, 38.0, 32.8, 27.6; HRMS calcd for $C_{26}H_{29}N_3NaO_5$: 486.2005 (M^++Na); found: 486.2008.

4.4.15. Methyl 2-{2-[4-(methyl)cinnamoyl]amino-1-oxo-3-phenyl}propylamino-3-(2-oxo-3-pyrrolidyl)-propionate (9d)

Compound **9d** was prepared by following the procedure to prepare **9a**, except using 4-methylcinnamic acid (yield: 58%). White foam; TLC (MeOH/CH_2Cl_2, 1:19) R_f = 0.14; IR (neat) 3512, 2921, 1746, 1681, 1421 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 8.72 (1H, d, J = 7.9 Hz), 8.33 (1H, d, J = 8.3 Hz), 7.68 (1H, s), 7.43 (2H, d, J = 8.0 Hz), 7.33–7.17 (8H, m), 6.64 (1H, d, J = 15.8 Hz), 4.76–4.68 (1H, m), 4.40–4.32 (1H, m), 3.63 (3H, s), 3.15–3.06 (3H, m), 2.84–2.80 (1H, m), 2.35–2.28 (4H, m), 2.12–2.09 (2H, m), 1.65–1.60 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 178.4, 172.7, 172.2, 165.4, 139.7, 139.3, 138.3, 132.5, 129.9 (2×), 129.6 (2×), 128.5 (2×), 127.9 (2×), 126.7, 121.2, 54.4, 52.4, 50.7, 40.1, 38.09, 38.02, 32.8, 27.6, 21.4; HRMS calcd for $C_{27}H_{31}N_3NaO_5$: 500.2161 (M^++Na); found: 500.2167.

4.4.16. Methyl 2-{2-[(4-chloro-2-fluoro)cinnamoyl]amino-1-oxo-3-phenyl}propyl amino-3-(2-oxo-3-pyrrolidyl)-propionate (9e)

Compound **9e** was prepared by following the procedure to prepare **9a**, except using 4-chloro-2-fluorocinnamic acid (yield: 50%). White foam; TLC (MeOH/CH_2Cl_2, 1:19) R_f = 0.16; IR (neat) 3529, 2918, 1752, 1670, 1413 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 8.69 (1H, d, J = 7.8 Hz), 8.50 (1H, d, J = 8.2 Hz), 7.67–7.64 (2H, m), 7.51 (1H, d, J = 9.1 Hz), 7.38–7.17 (7H, m), 6.80 (1H, d, J = 15.9 Hz), 4.75–4.70 (1H, m), 4.40–4.33 (1H, m), 3.64 (3H, s), 3.16–3.06 (3H, m), 2.84–2.80 (1H, m), 2.32–2.29 (1H, m), 2.13–2.07 (2H, m), 1.65–1.61 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 178.4, 172.7, 172.0, 164.8, 138.2, 135.07, 135.00, 130.79, 130.75, 129.6 (2×), 128.5 (2×), 126.7, 125.8, 122.1, 117.2, 117.1, 54.5, 52.4, 50.7, 38.7, 38.1, 38.0, 32.8, 27.6; HRMS calcd for $C_{26}H_{27}ClFN_3NaO_5$: 538.1521 (M^++Na); found: 538.1527.

4.4.17. Methyl 2-{2-[3-(5-methylisoxazol-3-yl)acryloyl]amino-1-oxo-3-phenyl}propyl-amino-3-(2-oxo-3-pyrrolidyl)-propionate (9f)

Compound **9f** was prepared by following the procedure to prepare **9a**, except using the prepared 3-(5-methylisoxazol-3-yl) acrylic acid. (Yield: 48%) White foam; TLC (MeOH/CH_2Cl_2, 1:19) R_f = 0.14; IR (neat) 3321, 1702, 1668, 1532, 1328, 1180 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 8.71 (1H, d, J = 7.6 Hz), 8.57 (1H, d, J = 8.2 Hz), 7.65 (1H, s), 7.30–7.18 (6H, m), 6.78 (1H, d, J = 15.9 Hz), 6.47 (1H, s), 4.72–4.68 (1H, m), 4.38–4.34 (1H, m), 3.64 (3H, s), 3.17–3.08 (3H, m), 2.85–2.81 (1H, m), 2.42 (3H, s), 2.31–2.29 (1H, m), 2.12–2.09 (2H, m), 1.66–1.61 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 178.4, 172.6, 171.9, 170.8, 164.2, 160.3, 138.1, 129.6, 129.5, 128.5 (2×), 126.9, 126.79, 126.71, 100.2, 54.5, 52.4, 50.8, 40.5, 38.0, 37.9, 32.8, 27.6, 12.2; HRMS calcd for $C_{24}H_{28}N_4NaO_6$: 491.1907 (M^++Na); found: 491.1912.

4.4.18. 2-{2-[3,4-(Methylenedioxy)cinnamoyl]amino-1-oxo-3-phenyl}propyl amino-3-(2-oxo-3-pyrrolidyl)-propan-1-al (10a)

White foam; TLC (MeOH/CH_2Cl_2, 1:9) R_f = 0.45; IR (neat) 3321, 2909, 1721, 1638, 1472 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 9.30 (1H, s), 8.67 (1H, d, J = 7.6 Hz), 8.30 (1H, d, J = 8.2 Hz), 7.65 (1H, s), 7.31–7.18 (7H, m), 7.12 (1H, s), 6.94 (1H, d, J = 8.0 Hz), 6.55 (1H, d, J = 15.8 Hz), 6.06 (2H, s), 4.78–4.69 (1H, m), 4.17–4.13 (1H, m), 3.23–3.06 (3H, m), 2.92–2.90 (1H, m), 2.25–2.10 (2H, m), 1.95–1.85 (1H, m), 1.63–1.60 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 201.3, 178.6, 172.5, 165.4, 148.9, 148.4, 139.3, 138.1, 129.6 (2×), 128.5 (2×), 128.4, 126.8, 123.7, 120.3, 109.0, 106.6, 101.9, 60.2, 56.8, 54.6, 38.3, 37.6, 29.6, 27.7; HRMS calcd for $C_{26}H_{27}N_3NaO_6$: 500.1798 (M^++Na); found: 500.1800; Anal. calcd for $C_{26}H_{27}N_3O_6$: C 65.40, H 5.70, N 8.80; found: C 65.31, H 5.85, N 8.79.

4.4.19. 2-{2-[4-(Dimethylamino)cinnamoyl]amino-1-oxo-3-phenyl}propyl amino-3-(2-oxo-3-pyrrolidyl)-propan-1-al (10b)

Light yellow foam; TLC (MeOH/CH_2Cl_2, 1:19) R_f = 0.20; IR (neat) 3411, 2934, 1728, 1681, 1432 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 9.29 (1H, s), 8.62 (1H, d, J = 7.6 Hz), 8.20 (1H, d, J = 8.1 Hz), 7.63 (1H, s), 7.34 (2H, d, J = 8.7 Hz), 7.28–7.15 (6H, m), 6.68 (2H, d, J = 8.7 Hz), 6.43 (1H, d, J = 15.5 Hz), 4.73–4.66 (1H, m), 4.16–4.09 (1H, m), 3.22–3.19 (1H, m), 3.10–3.03 (3H, m), 2.92 (6H, s), 2.15–2.11 (2H, m), 2.02–2.06 (2H, m), 1.66–1.50 (1H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 201.0, 178.2, 170.3, 165.6, 151.1, 139.5, 137.8, 129.1 (2×), 128.8 (2×), 128.1 (2×), 126.3, 122.2, 116.2, 111.9 (2×), 59.7, 57.4, 56.4, 39.7 (2×), 37.9, 37.3, 28.3, 27.2; HRMS calcd for $C_{27}H_{32}N_4NaO_4$: 499.2321 (M^++Na); found: 499.2325; Anal. calcd for $C_{27}H_{32}N_4O_4$: C 68.05, H 6.77, N 11.76; found: C 68.12, H 6.69, N 11.70.

4.4.20. 2-[2-(Cinnamoyl)amino-1-oxo-3-phenyl]propylamino-3-(2-oxo-3-pyrrolidyl)-propan-1-al (10c)

White foam; TLC (MeOH/CH_2Cl_2, 1:9) R_f = 0.55; IR (neat) 3412, 2911, 1742, 1602, 1412 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 9.31 (1H, s), 8.67 (1H, d, J = 7.5 Hz), 8.43 (1H, d, J = 8.1 Hz), 7.63 (1H, s), 7.55 (2H, d, J = 7.2 Hz), 7.42–7.18 (9H, m), 6.73 (1H, d, J = 15.8 Hz), 4.73–4.71 (1H, m), 4.18–4.16 (1H, m), 3.17–3.08 (3H, m), 2.93–2.89 (1H, m), 2.23–2.12 (2H, m), 1.92–1.88 (1H, m), 1.66–1.61 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 201.3, 178.6, 172.4, 165.2, 139.4, 138.1, 135.2, 129.9, 129.6 (2×), 129.4 (2×),

128.6 (2×), 127.9 (2×), 126.8, 122.3, 56.9, 54.6, 39.5, 38.3, 37.6, 29.6, 27.7; HRMS calcd for $C_{25}H_{27}N_3NaO_4$: 456.1899 (M^++Na); found: 456.1901; Anal. calcd for $C_{25}H_{27}N_3O_4$: C 69.27, H 6.28, N 9.69; found: C 69.51, H 6.42, N 9.57.

4.4.21. 2-{2-[4-(Methyl)cinnamoyl]amino-1-oxo-3-phenyl}propylamino-3-(2-oxo-3-pyrrolidyl)-propan-1-al (10d)

White foam; TLC (MeOH/CH_2Cl_2, 1:9) R_f = 0.50; IR (neat) 3372, 2928, 1751, 1621, 1487 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 9.32 (1H, s), 8.67 (1H, d, J = 7.4 Hz), 8.39 (1H, d, J = 8.0 Hz), 7.63 (1H, s), 7.44 (2H, d, J = 7.6 Hz), 7.36 (1H, d, J = 15.8 Hz), 7.44–7.14 (7H, m), 6.67 (1H, d, J = 15.8 Hz), 4.75–4.71 (1H, m), 4.18–4.16 (1 H, m), 3.17–3.09 (3H, m), 2.94–2.90 (1H, m), 2.31 (3H, s), 2.22–2.12 (2H, m), 1.94–1.89 (1H, m), 1.67–1.60 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 201.3, 178.7, 172.4, 165.4, 139.4, 138.1, 129.9, 129.6 (2×), 129.3, 128.6 (2×), 128.5, 127.9 (2×), 126.8, 125.7, 121.3, 56.9, 54.6, 38.3, 37.6, 29.6, 27.7, 21.5, 21.3; HRMS calcd for $C_{26}H_{29}N_3NaO_4$: 470.2056 (M^++Na); found: 470.2059; Anal. calcd for $C_{26}H_{29}N_3O_4$: C 69.78, H 6.53, N 9.39; found: C 69.59, H 6.61, N 9.31.

4.4.22. 2-{2-[(4-Chloro-2-fluoro)cinnamoyl]amino-1-oxo-3-phenyl}propyl amino-3-(2-oxo-3-pyrrolidyl)-propan-1-al (10e)

White foam; TLC (MeOH/CH_2Cl_2, 1:9) R_f = 0.45; IR (neat) 3421, 2900, 1732, 1677, 1521 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 9.32 (1H, s), 8.69 (1H, d, J = 7.4 Hz), 8.59 (1H, d, J = 8.0 Hz), 7.67–7.63 (2H, m), 7.50–7.16 (8H, m), 6.83 (1H, d, J = 15.9 Hz), 4.80–4.72 (1H, m), 4.21–4.13 (1H, m), 3.16–3.08 (3H, m), 2.93–2.90 (1H, m), 2.24–2.13 (2H, m), 1.91–1.89 (1H, m), 1.66–1.61 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 201.2, 178.7, 172.3, 164.9, 138.0, 130.85, 130.81, 129.6, 129.3 (2×), 128.64, 128.60, 126.8, 125.7, 122.1, 122.0, 117.2, 117.0, 56.9, 54.7, 38.3, 37.6, 29.6, 27.7, 21.5; HRMS calcd for $C_{25}H_{26}ClFN_3O_4$: 486.1596 (M^++H); found: 486.1599; Anal. calcd for $C_{25}H_{25}ClFN_3O_4$: C 61.79, H 5.19, N 8.65; found: C 61.69, H 5.43, N 8.61.

4.4.23. 2-{2-[3-(5-Methylisoxazol-3-yl)acryloyl]amino-1-oxo-3-phenyl}propyl amino-3-(2-oxo-3-pyrrolidyl)-propan-1-al (10f)

White foam; TLC (MeOH/CH_2Cl_2, 1:9) R_f = 0.60; IR (neat) 3441, 2918, 1706, 1672, 1410 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 9.31 (1H, s), 8.69–8.65 (2H, m), 7.63 (1H, s), 7.28–7.18 (6H, m), 6.80 (1H, d, J = 14.9 Hz), 6.48 (1H, s), 4.78–4.64 (1H, m), 4.20–4.13 (1H, m), 3.15–3.09 (3H, m), 2.92–2.88 (1H, m), 2.38 (3H, s), 2.22–2.13 (2H, m), 1.92–1.88 (1H, m), 1.65–1.62 (2H, m); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 201.2, 178.6, 172.1, 170.8, 164.2, 160.3, 138.0, 129.6, 129.3 (2×), 128.6 (2×), 126.7, 125.7, 100.2, 62.1, 56.9, 54.7, 38.2, 37.6, 29.6, 27.7, 12.2; HRMS calcd for $C_{23}H_{26}N_4NaO_5$: 461.1801 (M^++Na); found: 461.1209; Anal. calcd for $C_{23}H_{26}N_4O_5$: C 63.00, H 5.98, N 12.78; found: C 63.01, H 6.02, N 12.75.

4.5. Enzyme inhibition assay

For IC_{50} measurements, the reactions were performed with 0.5 μM protease and 10 μM fluorogenic peptide in a buffer of 10 mM MES at pH 6.5 and 25 °C. The fluorescence change resulted from the reaction was followed with time using a 96-well fluorescence plate reader. The initial velocities of the enzymatic reaction in the first 5 min of reactions were plotted against the different inhibitor concentrations to obtain the IC_{50} by fitting with Eq. 1.

$$A(I) = A(0) \times \{1 - [[I]/([I] + IC_{50})]\} \quad (1)$$

In Eq. 1, $A(I)$ is the enzyme activity with inhibitor concentration [I], A(0) is the enzyme activity without inhibitor, and [I] is the inhibitor concentration. For the K_i measurements from time-dependent inhibition, 1–10 μM of inhibitor **6b** was used to inhibit 0.5 μM enzyme. The reduction of enzyme activity with the incubation time of the enzyme with the inhibitor was fitted with a single exponential equation to obtain the k_{obs} of inactivation at different inhibitor concentrations. From the k_{obs} of inactivation, the half-life ($t_{1/2}$) can be calculated ($t_{1/2} = \ln 2/k_{obs}$). The half-life for inactivation at each inactivator concentration was plotted against 1/[inactivator] to yield a line. The intersection at the y-axis was $\ln 2/k_{inact}$, from which the value of k_{inact} was determined. The extrapolated negative x-axis intercept was $-1/K_i$.

4.6. Anti-viral assay

This assay measured the ability of a tested compound to prevent the infection of EV71 on human embryonic rhabdomyosarcoma cells (RD cells). The 96-well tissue culture plates were seeded with 200 μL of RD cells at a concentration of 1.1×10^5 cells/mL in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). The plates were incubated for 24–30 h at 37 °C and were used at about 90% confluence. Virus (100 $TCID_{50}$) mixed with different concentrations of the tested compounds was added to the cells and incubated at 35 °C for 1 h. After adsorption, the infected cell plates were overlaid with 50 μL of DMEM plus 2% FBS and 0.1% DMSO. The plate was incubated at 35 °C for 72 h. At the end of incubation, the plates were fixed by the addition of 100 μL of 4% formaldehyde for 1 h at room temperature. After the removal of formaldehyde, the plates were stained with 0.1% crystal violet for 15 min at room temperature. The plates were washed and dried, and the density of the remaining cells in the wells was measured at 570 nm. The concentration required for a tested compound to reduce the virus-induced cell death by 50% relative to the virus control was defined as EC_{50}. All assays were performed in triplicate and at least twice.

4.7. Western blot analysis

The ability of **10d** to inhibit viral protein accumulation was performed by using RD cells grown in DMEM supplemented with 10% fetal calf serum. The confluent cells in 12-well plates were infected with EV71 (EV71 TW/2231/98) at a multiplicity of infection (MOI) of 0.01 in the absence or presence of **10d** at 0.01, 0.025, 0.05, 0.1, 0.5 or 1 μM. After 48 h post infection, total cell lysate was harvested and subjected to 12% SDS–PAGE. Protein samples were subsequently transferred to PVDF membrane and probed with a mouse monoclonal antibody against EV71 3C protease and anti-β-actin antibody. A rabbit anti-mouse antibody conjugated with horseradish peroxidase (1:2000; Amersham biosciences) was used as the secondary antibody. The horseradish peroxidase was detected by an enhanced chemiluminescence detection system (ECL; Amersham Pharmacia, Freiburg, Germany).

4.8. Computer modeling

Construction of EV71 3C protease structural model was based on the X-ray crystal structure of RV 3C protease with AG7088 bound (PDB code 1CQQ) as template using the software of Discovery studio 1.7 (Accelrys, Inc.). Docking of **6b** and **10b** in the active site was performed with an automated ligand-docking program of Discovery studio 1.7. The docking utilized a genetic algorithm and a set of parameters to control the precise operation via defined binding sites, specified ligand conformations, energy grid parameters, variable numbers of Monte Carol trials, and selected score type.

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