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Application of Dually Activated Michael Acceptor to the Rational Design of Reversible Covalent Inhibitor for Enterovirus 71 3C Protease

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KEYWORDS: Enterovirus 71, 3C protease, reversible targeted covalent inhibitor, Michael acceptor, time-dependent inhibition.

ABSTRACT: Targeted covalent inhibitors (TCIs) have attracted growing attention from the pharmaceutical industry in recent decades because they have potential advantages in terms of efficacy, selectivity and safety. TCIs have recently evolved into a new version with reversibility that can be systematically modulated. This feature may diminish the risk of haptenization and help optimize the drug-target residence time as needed. The enteroviral 3C protease ($3C^{pro}$) is a valuable therapeutic target, but the development of $3C^{pro}$ inhibitors is far from satisfactory. Therefore, we aimed to apply a reversible TCI approach to the design of novel $3C^{pro}$ inhibitors. The introduction of various substituents onto the α-carbon of classical Michael acceptors yielded inhibitors bearing several classes of warheads. Using steady-state kinetics and biomolecular mass spectrometry, we confirmed the mode of reversible covalent inhibition and elucidated the mechanism by which the potency and reversibility were affected by electronic and steric factors. This research produced several potent inhibitors with good selectivity and suitable reversibility; moreover, it validated the reversible TCI approach in the field of viral infection, suggesting broader applications in the design of reversible covalent inhibitors for other proteases.

INTRODUCTION

Most of the early covalent drugs such as penicillin, omeprazole and aspirin were discovered by serendipity or resulted from phenotypic screening.[1] Although these drugs proved to be great successes and are being widely used today, the pharmaceutical industry has long been reluctant to develop covalent drugs because of safety concerns that are associated with the nonspecificity and irreversibility of covalent inhibition. In recent years, the growing fields of structural biology and bioinformatics have greatly promoted the rational design of targeted covalent inhibitors (TCIs).[1-2] Over the past two decades, several TCIs have been approved by the FDA, while more are in clinical trials or being used as chemical tools in drug discovery for various classes of proteins.[3] Among the benefits specific to these TCIs are high biochemical potency, improved selectivity and reduced drug resistance.[2, 4]

In general, the mechanism by which a TCI inhibits its target occurs in two steps. The inhibitor initially recognizes and noncovalently binds to the target site, then forms bond with certain nucleophilic residue (Figure 1A).[5] Accordingly, the optimization of a TCI takes into account both the initial binding affinity (K_i) and the chemical reactivity (k_{inact}). Sometimes the chemical reaction may be reversible, resulting in two types of reversible TCIs. The first type of inhibitor, bearing warhead like aldehyde or nitrile, can form unstable adduct with target which readily break down on the biological timescale.[2] In contrast, the adduct formed by the second type of inhibitor is metastable. The reversal of such adduct is relatively slow and can be systematically modulated, as exemplified by some recently developed electron-deficient alkenes.[6] These inhibitors were thought to possess a reduced risk of hapten formation and to enable fine-tuning of the inhibitor-target residence time. The potency of a reversible TCI is determined by the overall inhibition constant K_i^* that depends not only on the K_i but also on the relative magnitudes of the values for k_5 and k_6 (Figure 1A).

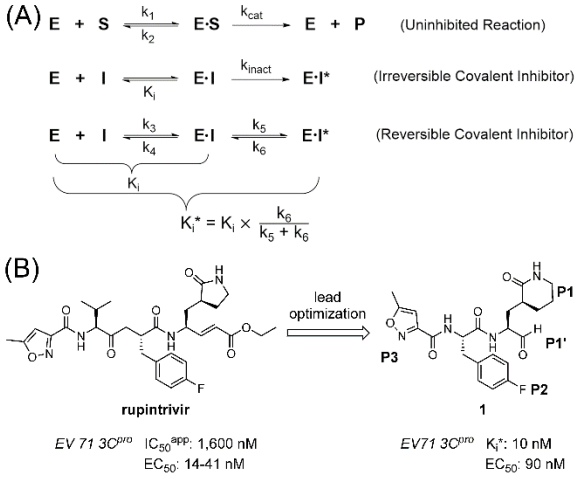


Figure 1. (A) Generic two-step mechanism of irreversible/reversible covalent inhibition. k_{inact}/K_i is usually used for evaluation of irreversible inhibitors, while K_i^* measures the potency of reversible covalent inhibitor that conforms to a two-step mechanism. The relationship between K_i, K_i^*, k_5 and k_6 are described. (B) Structures and bioactivity data of rupintrivir and compound **1**. Apparent IC_{50} or K_i^* values are indicative of their inhibitory effects on EV71 $3C^{pro}$. EC_{50} values represent their antiviral activity in EV71-infected human rhabdomyosarcoma (RD) cells.

Enterovirus 71 (EV71) is one of the causative agents of hand, foot and mouth disease (HFMD), which tends to infect children and may be associated with severe neurological and cardiopulmonary disorders.[7] The 3C cysteine protease ($3C^{pro}$) is an essential viral enzyme that is now considered an attractive anti-EV71 target. Currently, noncovalent inhibitors obtained from screening methods produced disappointing inhibitory effects.[8] For peptidyl inhibitors, the existence of an appropriate electrophile at the P1' position is critical for the potency, based on cumulative knowledge of enteroviral $3C^{pro}$.[9] The most studied inhibitor is rupintrivir, originally a covalent protease inhibitor of human rhinovirus (HRV) which is homologous to EV71. This inhibitor employs a classical Michael acceptor (α, β-unsaturated ethyl ester) as its warhead that reacts irreversibly with the active-site cysteine (Figure 1B).[10] However, rupintrivir is less potent against EV71 $3C^{pro}$ than it is against HRV.[11] To develop a better TCI for EV71 $3C^{pro}$, we previously synthesized a number of peptidyl inhibitors.[9b, 12] The binding affinities of these inhibitors were improved by modifying the peptidomimetic scaffold; however, the inhibitors had druglikeness and potency issues, presumably because of their aldehyde and α-ketoamide-based warheads.

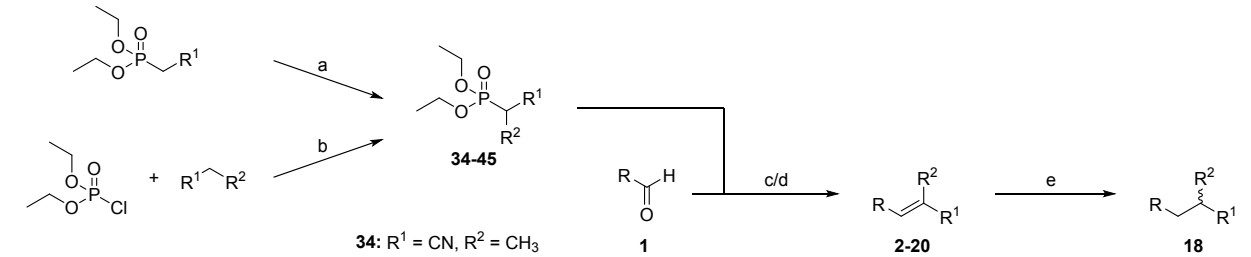
The aforementioned slow reversible TCI presents a prospective approach for the development of a potent $3C^{pro}$ inhibitor with fewer disadvantages. Here our principal goal is to adapt novel Michael acceptors as warheads with suitable reactivities that can form covalent—ideally slowly reversible—adducts with the catalytic Cys_{147} of EV71 $3C^{pro}$. To this end, a variety of gem-disubstituted alkenes were attached to a common peptidomimetic scaffold. By means of steady-state kinetics and mass spectrometry, we assessed the capability of these warheads to provide optimal chemical reactivity and reversibility. Our assessment not only generated several potent inhibitors with desired reversibility and high selectivity, more importantly, it also developed a systematic insight into the complex relationship between the chemical properties of gem-disubstituted alkenes and the bioactivities of their corresponding inhibitors.

RESULTS AND DISCUSSION

Synthesis and characterization of compounds 2-33

In this article, 32 new compounds were synthesized and characterized. All target compounds started from aldehydic compound **1**, for which a synthetic procedure was previously reported.[13] The key step of the synthetic work was to build a double bond, and for this purpose, we mainly used two reactions: Horner-Wadsworth-Emmons (HWE) reaction and Knoevenagel condensation.

Scheme 1. Synthesis of intermediates **34-45** and target compounds **2-20**[a].



34: R^1 = CN, R^2 = CH_3
35: R^1 = CN, R^2 = Cl
36: R^1 = CN, R^2 = Br
37: R^1 = $COOCH_3$, R^2 = Cl
38: R^1 = $COOCH_3$, R^2 = Br
39: R^1 = $COOCH_2CH_3$, R^2 = Cl
40: R^1 = $COOCH_2CH_3$, R^2 = Br
41: R^1 = CN, R^2 = Ph
42: R^1 = CN, R^2 = Naph
43: R^1 = CN, R^2 = (4-CF_3)-Ph
44: R^1 = CN, R^2 = 2-Py
45: R^1 = CN, R^2 = 3-Thio

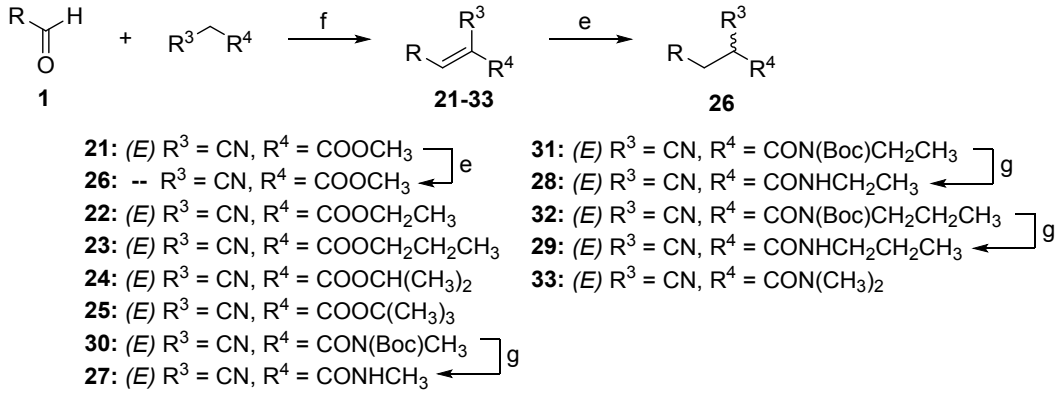
2: *(E)* R^1 = $COOCH_3$, R^2 = H
3: *(E)* R^1 = $COOCH_2CH_3$, R^2 = H
4: *(E)* R^1 = $CONHCH_3$, R^2 = H
5: *(E)* R^1 = CN, R^2 = H
6: *(Z/E)* R^1 = CN, R^2 = CH_3
7: *(Z/E)* R^1 = CN, R^2 = Cl
8: *(Z/E)* R^1 = CN, R^2 = Br
9: *(Z/E)* R^1 = $COOCH_3$, R^2 = Cl
10: *(Z/E)* R^1 = $COOCH_3$, R^2 = Br
11: *(Z/E)* R^1 = $COOCH_2CH_3$, R^2 = Cl
12: *(Z/E)* R^1 = $COOCH_2CH_3$, R^2 = Br
13: *(Z)* R^1 = CN, R^2 = Ph
14: *(Z)* R^1 = CN, R^2 = Naph
15: *(Z)* R^1 = CN, R^2 = 4-(CF_3)-Ph
16: *(E)* R^1 = 2-Py, R^2 = CN
17: *(Z)* R^1 = CN, R^2 = 2-Py
18: -- R^1 = CN, R^2 = 2-Py (e)
19: *(E)* R^1 = 3-Thio, R^2 = CN
20: *(Z)* R^1 = CN, R^2 = 3-Thio

[a]Reagents and conditions: (a) LiHMDS, NBS or NCS, THF, 0 °C to RT, 22-52% (expect for **34** prepared with K_2CO_3, MeI, DMF, RT, 62%); (b) LiHMDS, THF, N_2, -78 °C to 0 °C, 48-67%; (c) NaHMDS, THF, N_2, -78 °C to 0 °C, 80-83% (for compound **2** and **3**); (d) LiCl, DBU, MeCN, RT, 70-85%; (e) Pd/C (10 wt % Pd), H_2, MeOH, 40 °C, 38%.

The synthetic routes to singly activated alkenes **2-5**, methyl acrylonitrile **6**, halogenated acrylates/acrylonitriles **7-12**, aryl/heteroaryl acrylonitrile derivatives **13-20** are outlined in Scheme 1 where the HWE reaction is the central step. Alkenes **2-5** were obtained by reacting **1** with corresponding commercially available phosphonates using NaHMDS or LiCl/DBU as the base according to a protocol modified by Blanchette et al,[14] while the preparation of **6-17** and **19-20** required the in-lab synthesis of **34-45**. Phosphonate **34** was prepared by treating diethyl cyanomethylphosphonate with

iodomethane (MeI) as the methylating agent and potassium carbonate (K_2CO_3) as the base. Phosphonates **35**-**40** were produced from commercially obtained phosphonates that underwent halogenation with *N*-chlorosuccinimide (NCS) or *N*-bromosuccinimide (NBS).[15] The bromo derivatives (**36**, **38** and **40**) were particularly unstable under ambient conditions and had to be used in the next step immediately. Phosphonates **41**-**45** were obtained by electrophilic phosphorylation of lithiated nitriles.[16] It was found that **44** existed as tautomers due to spontaneous proton transfer.[17] Finally, the saturated compound **18** was produced by Pd/C-catalyzed hydrogenation of **17**.

Scheme 2. Synthesis of compounds **21**-**33**[a]



[a]Reagents and conditions: (e) Pd/C (10 wt % Pd), H_2, MeOH, RT, 57%; (f) piperidine, DCM, RT or heated, 26-55%; (g) TFA, DCM, RT, 26-35%. Structure of **1** was drawn in Scheme 1.

The synthetic routes to cyanoacrylate derivatives **21**-**26** and cyanoacrylamides **27**-**33** are outlined in Scheme 2, where Knoevenagel condensation is the central step. The cyanoacrylates **21**-**26** and Boc-protected cyanoarylamides **30**-**33** were obtained by reaction of **1** with corresponding active methylene compounds under Knoevenagel conditions. The cyanoacrylamides **27**-**29** were obtained by removing the Boc groups of **30**-**32** with TFA at an ambient temperature. Like the saturated **18**, compound **26** was converted from **21**.

The double bonds of **2**-**5** in Scheme 1 and all compounds in Scheme 2 were determined to be exclusively in the *E* form, while those of the aryl acrylonitriles **13**-**15**

were exclusively in the *Z* form. Compounds **6**-**12** were obtained as mixtures of *E*/*Z* isomers and were used directly in kinetic evaluations, whereas we were able to separate the geometric isomers of heteroaryl acrylonitriles **16**, **17**, **19** and **20**. Two methods were employed to identify the configuration of trisubstituted alkenes. For nitrile-containing compounds, the double bond configuration could be inferred by the three-bond heteronuclear coupling constant ($^3J_{CH}$) between the vinyl proton and the cyano carbon.[18] For ester derivatives **9**-**12**, the configuration was determined via a NOESY technique. The determination of double bond configuration by NMR is detailed in the Supporting Information (Figures S1-S5).

Classical Michael acceptors

Based on the structure-activity relationships that we reported previously,[9b, 12] the peptidomimetic scaffold shown in Figure 1B was adopted throughout our present study to offer optimal specificity for enzyme-inhibitor recognition. We commenced our survey of warheads by using classical Michael-acceptor acrylates (**2**, **3**), acrylamide (**4**) and the rarely used acrylonitrile (**5**). EV71 $3C^{pro}$ was preincubated with **2**, **4** or **5**, and then ultrafiltered to remove unbound inhibitors. The sample was analyzed by ESI-MS to examine the formation of an enzyme-inhibitor adduct. The degree of covalent modification of protein, expressed as the ratio of peak intensity of covalently modified to unmodified protein (I_{add}/I_{pro}), was >100 for three samples (Figure 2A), indicating the formation of a stable covalent bond. Notably, longer pre-incubation period and higher concentration of inhibitor were required for **5** to achieve a level of covalent modification that was comparable to **2** and **4**, suggesting lower intrinsic reactivity of the acrylonitrile for the addition of thiol.

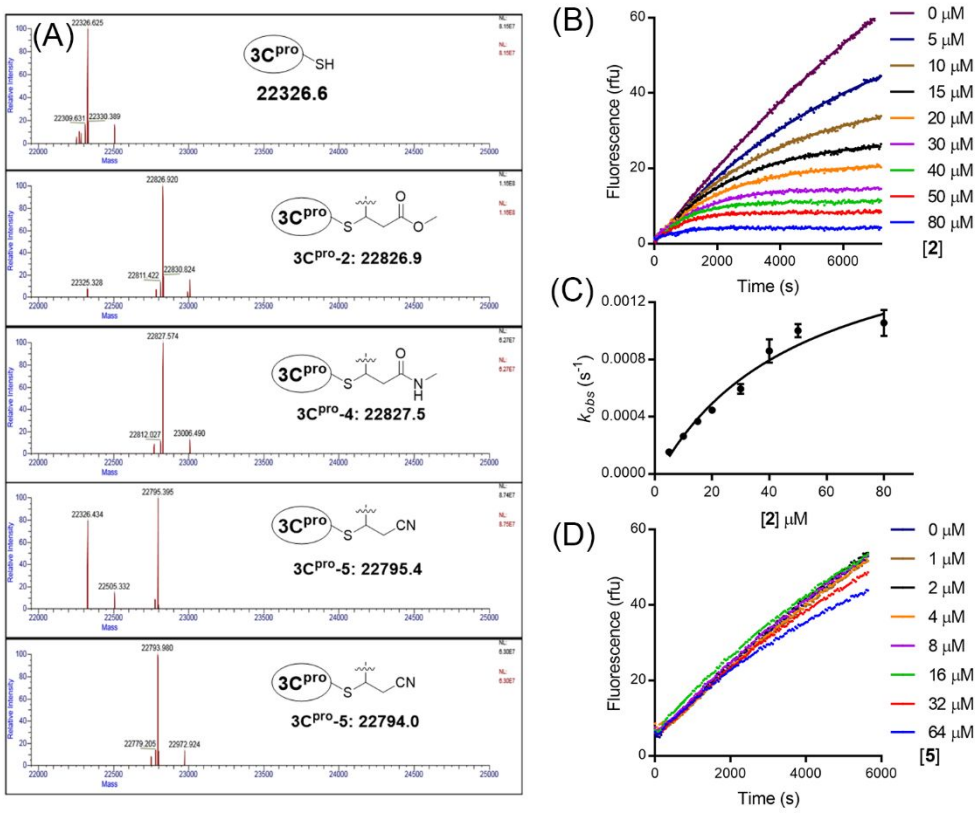
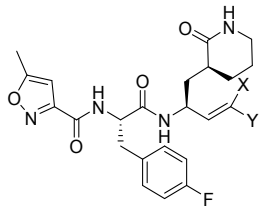


Figure 2. (A) Deconvoluted ESI-MS spectra of $3C^{pro}$ and its adduct with classical Michael acceptors. From top to bottom are—unmodified $3C^{pro}$, $3C^{pro}$ incubated with 10 equiv **2**, **4**, **5** for 30 min and $3C^{pro}$ incubated with 20 equiv **5** for 1.0 h, respectively. (B) Time-course inhibition of $3C^{pro}$ at variable concentrations of **2**. Lines drawn through the time courses result from fitting of data to equation 2 in Experiment Section. (C) Replotting the calculated k_{obs} values over concentration of **2** (equation 3 in Experiment Section) yields a partial hyperbola. Error bar represents standard error of the mean (SEM, n = 3). (D) Time-course inhibition of $3C^{pro}$ at variable concentrations of **5**.

The time-course of $3C^{pro}$ inactivation by compounds **2**-**4** displayed a time-dependent behavior and a steady-state velocity approaching zero (Figure 2B, Figures S6A and C), both of which are features of a two-step irreversible covalent mechanism.[19] According to the data processing method described in the Experimental Section, the observed rate constants (k_{obs}) fitted from these curves exhibited a hyperbolic dependence on the inhibitor concentration (Figure 2C, Figures S6B and D), which further supported the two-step mechanism. The second-order rate constants of inactivation (k_{inact}/K_i) that are listed in Table 1 were used to compare the potency of our compounds. Compounds

2-**4** showed similarly modest activity (k_{inact}/K_i= 34-72 $M^{-1}\cdot s^{-1}$, EC_{50} = 17-21 μM), whereas compound **5** produced discernable inhibition only at the maximal concentration (64 μM, Figure 2D), which agreed with the conclusion from the MS experiments that acrylonitrile had a much poorer reactivity toward Cys_{147} addition. Overall, this series of classical Michael acceptors could irreversibly inactivate $3C^{pro}$, yet their inhibitory effects were barely satisfactory.

Table 1. Inhibition data of compounds **2**-**5**.



scaffold A

NO.	Scaffold	X	Y	Config.[a]	K_{inact}/K_i ($M^{-1}\cdot s^{-1}$)[b,c]	EC_{50} (nM)[b]	Cytotoxicity (at 100 μM)[b,c]
2	A	H	$COOCH_3$	*E*	34 ± 5	18,300 ± 5,100	N.D.
3	A	H	$COOCH_2CH_3$	*E*	72 ± 14	17,000 ± 3,900	N.D.
4	A	H	$CONHCH_3$	*E*	46 ± 4	21,000 ± 5,700	N.D.
5	A	H	CN	*E*	N.D.	37,000 ± 4,200	N.D.

[a]The configuration was determined by ^{1}H-NMR spectrum. [b]Each value represented the average result from three independent experiments (SEM indicated). [c]N.D.: Not detected under the experimental condition.

Michael acceptors activated with weak EWGs

Taunton and coworkers described reversible covalent inhibitors in which the introduction of a second electron-withdrawing group (EWG) on the α-carbon of a Michael acceptor rendered the dually activated double bond intrinsically more reactive, while due

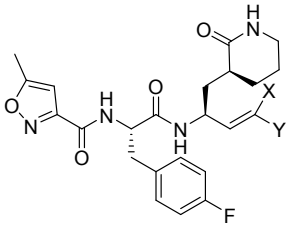
to concomitant increased acidity of the α-proton, the thioadduct of such Michael acceptor was readily reversible via an E1cB elimination.[20] Therefore, based on a preliminary investigation of the effect of EWGs on alkenes,[21] we started to identify appropriate second substituents, either to render the acrylate/acrylamide more reversible or to activate the inert acrylonitrile. As will be discussed later, these dually activated alkenes are basically reversible, so their potencies are measured by their overall inhibition constant K_i^* instead of k_{inact}/K_i.

As shown in Table 2, to characterize a full spectrum of electronic properties, an electron-releasing methyl group was first introduced (**6**), which unsurprisingly, made no difference to the inhibitory behavior of acrylonitrile. Halogen atoms, which are considered to be relatively weak EWGs, were then incorporated into **2**, **3** and **5**. Halogenated acrylonitriles (**7**, **8**) also exhibited no inhibition against the protease or the virus, thereby demonstrating insufficient electron-withdrawing capability by the halogens for the activation of acrylonitrile. In contrast, halogenation of acrylates (**9**-**12**) led to considerable potency with K_i^* of 115-164 nM and antiviral activity (EC_{50}) of 290-670 nM. Unfortunately, these halogenated derivatives were accompanied by substantial cytotoxicity and, thus, were not pursued further.

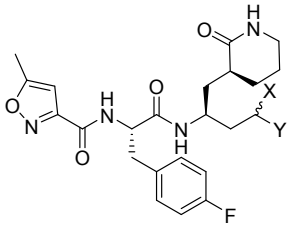
To continue searching for the methods for activating the acrylonitrile warhead, we next replaced halogens with several aryl or heteroaryl activating groups (**13**-**20**). The introduction of a phenyl or a naphthyl group (**13**, **14**) made little contribution, similar to the effect of electron-releasing methyl group with compound **6**. In an attempt to increase the electron withdrawing capability, incorporation of *p*-trifluoromethyl (**15**) resulted in only a slightly improved cell-based antiviral activity (EC_{50} = 1,400 nM). In fact, this antiviral effect may be nonspecific since significant cytotoxicity was detected at a relatively low dose (33 μM). Unlike aryl hydrocarbons, compounds **16** and **17**, containing an electron-deficient *o*-pyridyl group, demonstrated measurable values of K_i^* (693 nM and 119 nM, respectively) and acceptable cell-based antiviral activity (EC_{50}: 2,700 nM and 460 nM, respectively). Unfortunately, these compounds also exhibited cellular toxicity. For compound **17**, the

lack of inhibitory activity of its alkane counterpart **18** suggested the importance of covalent bond formation for enzyme inhibition. Replacing the pyridyl group (**17**) with an electron-rich thiophenyl group (**20**) decreased the inhibitory activity by approximately 5-fold in both enzyme and cell-based assays. It is noteworthy that the *Z* configuration is generally the active form for heteroaryl acrylonitriles (**17** and **20**), which geometrically corresponds to the *E* configuration of the cyanoacrylates and cyanoacrylamides that are discussed below.

Table 2. Inhibition data of compounds **6**-**20**.



scaffold A



scaffold B

NO.	Scaffold	X	Y	Config.[a]	K_i* (nM)[b,c]	EC_{50} (nM)[b]	Cytotoxicity (at 100 μM)[b,c]
6	A	CH_3	CN	*E*:*Z*= 2:1	N.D.	>100,000	N.D.
7	A	Cl	CN	*E*:*Z*= 4:5	N.D.	>100,000	100%
8	A	Br	CN	*E*:*Z*= 2:1	N.D.	>100,000	100%
9	A	Cl	$COOCH_3$	*E*:*Z*= 1:2	152 ± 24	670 ± 160	100%
10	A	Br	$COOCH_3$	*E*:*Z*= 3:5	115 ± 11	290 ± 60	100%
11	A	Cl	$COOCH_2CH_3$	*E*:*Z*= 1:1	164 ± 21	380 ± 140	100%
12	A	Br	$COOCH_2CH_3$	*E*:*Z*= 2:5	117 ± 17	350 ± 80	100%

13	A	CN	Ph	*Z*	N.D.	>100,000	N.D.
14	A	CN	Naph	*Z*	N.D.	13,000 ± 2,100	N.D.
15	A	CN	(4-CF_3)-Ph	*Z*	N.D.	1,400 ± 400	100%
16	A	2-Py	CN	*E*	693 ± 95	2,700 ± 700	90%
17	A	CN	2-Py	*Z*	119 ± 15	460 ± 100	100%
18	B	CN	2-Py	--	N.D.	>100,000	50%.
19	A	3-Thio	CN	*E*	N.D.	19,500 ± 4,200	N.D.
20	A	CN	3-Thio	*Z*	516 ± 42	3,100 ± 400	N.D.

[a]The ratio of *E*/*Z* isomers was determined by ^{1}H-NMR spectrum. [b]Each value represented the average results from three independent experiments (SEM indicated). [c]N.D.: Not detected under the experimental condition.

Michael acceptors activated with strong EWGs

Next, we synthesized two series of compounds: cyanoacrylates **21-25** and cyanoacrylamides **27-33**. Because carbonyl (ester or amide) and cyano groups are both strongly electron-withdrawing, their combination synergistically activated the double bond and thus conferred the new Michael acceptors with excellent potency, as shown in Table 3. The inhibitory effects of cyanoacrylates **21-25** on $3C^{pro}$ were at virtually the same level (6.7-16.1 nM), regardless of the size or shape of the ester sidechains. Furthermore, the antiviral activity of these inhibitors also fell into a satisfactory range (30-77 nM), which correlated well with the enzyme inactivation data and was comparable to rupintrivir (EC_{50} = 14-41 nM).[11] To gain an insight into their interaction with $3C^{pro}$, a molecular modeling study of compound **25** was carried out based on a covalent-docking protocol. The top-scoring pose of **25** (ΔG_{calc} = -7.22 kcal/mol) aligned well with rupintrivir that was determined by X-ray crystallography (Figure 3A). The hydrogen bonding patterns of their scaffolds were nearly conserved, while a slight difference was observed at the P1' position. In addition to the fact that the carbonyl oxygens in both **25** and rupintrivir served as hydrogen bond acceptors, the cyano group of the former could also form another hydrogen bond with His_{40}. The tert-butyl group of **25**, being the bulkiest group in this series

of compounds, fitted snugly into the S1' pocket, probably because of an entropy-driven expulsion of bound water from the active site. All of these determinants resulted in tight binding of these cyanoacrylates. In contrast to the curvilinear time-courses obtained with the singly activated Michael acceptors, the progress curves for the cyanoacrylate series were essentially linear (Figure 3B and Figure S7) as their values of k_{obs} were too large to be measured by steady state kinetic method, which demonstrated a negligible steady-state level of the initial E·I complex and rapid equilibrium for the E·I* complex. In other words, the good initial binding of inhibitors presumably oriented the double bond toward Cys_{147}, and dual activation by strong EWGs further accelerated formation of the adduct.

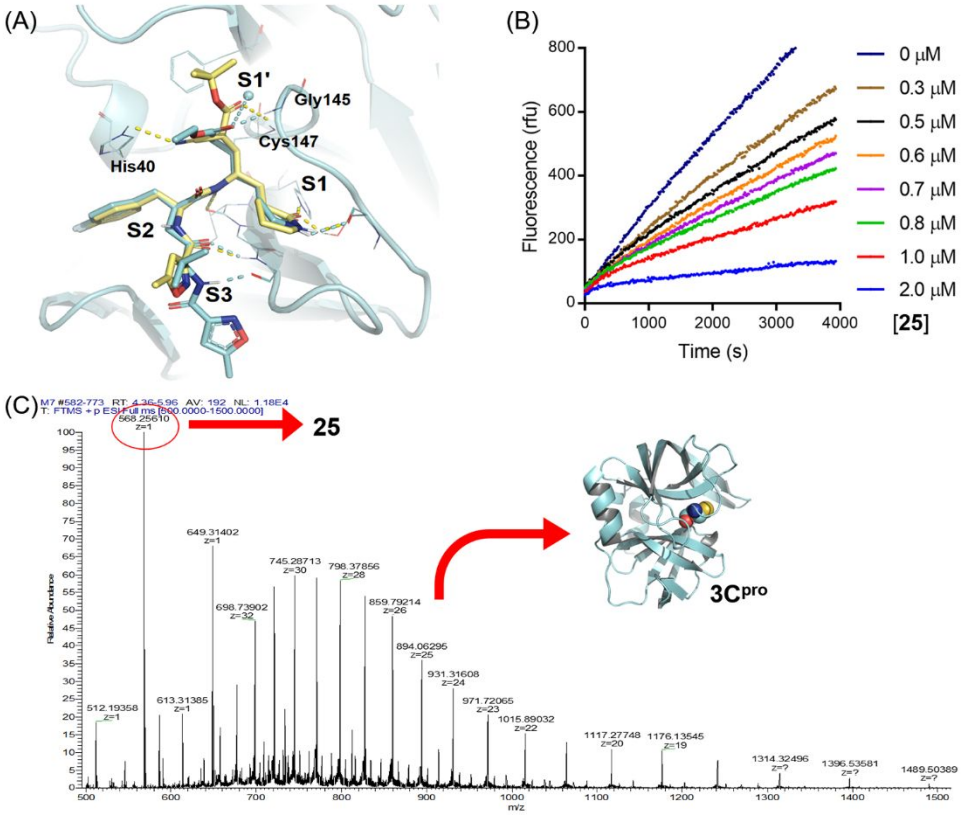


Figure 3. (A) Top-scoring docking pose of **25** (yellow) was superposed on rupintrivir (cyan) from crystal structure (PDB ID: 3SJO). Hydrogen bonds were drawn as dashed lines. The bound water was labeled as solid sphere. Residues at S1' pocket were labeled. (B) Time-course inhibition of $3C^{pro}$ at variable concentrations of **25**. Linear progress curves suggest rapid equilibrium of enzyme-inhibitor complex. (C) Raw ESI-MS spectrum of $3C^{pro}$ incubated with **25**. The appearance of molecular ion peak of **25** implied that it came along with $3C^{pro}$ but detached during or after ionization.

MS analysis was also carried out for cyanoacrylates. Although the MS results for **21** (Figure S8) or **25** (Figure 3C) did not explicitly demonstrate the formation of a covalent bond, the molecular ion peaks of both small molecules did appear in their raw spectra. Similar to the treatments with classical Michael acceptors, ultrafiltration was performed three times to assure the removal of any free small molecules before analysis by mass spectrometry; therefore, we could safely conclude that the observed molecular ion was not a contaminant but was cleaved from $3C^{pro}$ during or after ionization. Compared with the more stable $3C^{pro}$-**2** complex, the apparently less stable $3C^{pro}$-**21/25** complex implied the increased reversibility of this complex due to the electron-withdrawal from the added cyano group. Saturation of the double bond (**26**) caused total loss of inhibitory activity, further corroborating the essentiality of Michael addition in order to achieve enzyme inhibition.

Table 3. Inhibition data of compounds **21**-**33**.

NO.	Scaffold[a]	X	Y	Config.[b]	K_i* (nM)[c,d]	EC_{50} (nM)[c]	Cytotoxicity (at 100 μM)[c,d]
21	A	CN	$COOCH_3$	*E*	9.4 ± 5.0	53 ± 9	N.D.
22	A	CN	$COOCH_2CH_3$	*E*	13.1 ± 4.0	72 ± 13	N.D.
23	A	CN	$COOCH_2CH_2CH_3$	*E*	10.3 ± 3.2	77 ± 20	N.D.
24	A	CN	$COOCH(CH_3)_2$	*E*	16.1 ± 4.7	58 ± 10	N.D.
25	A	CN	$COOC(CH_3)_3$	*E*	6.7 ± 2.0	30 ± 8	N.D.
26	B	CN	$COOCH_3$	--	N.D.	>100,000	N.D.

27	A	CN	$CONHCH_3$	*E*	304 ± 20	400 ± 80	N.D.
28	A	CN	$CONHCH_2CH_3$	*E*	332 ± 21	510 ± 50	N.D.
29	A	CN	$CONHCH_2CH_2CH_3$	*E*	752 ± 32	990 ± 110	N.D.
30	A	CN	$CON(Boc)CH_3$	*E*	9.3 ± 4.3	51 ± 4	N.D.
31	A	CN	$CON(Boc)CH_2CH_3$	*E*	24 ± 7.8	67 ± 10	N.D.
32	A	CN	$CON(Boc)CH_2CH_2CH_3$	*E*	18 ± 8.4	89 ± 20	N.D.
33	A	CN	$CON(CH_3)_2$	*E*	94 ± 10	110 ± 6	N.D.

[a]The structures of scaffold A and B were described in Table 2. [b]The method for the configuration identification was described in Supporting Information. [c]Each value represented the average results from three independent experiments (SEM indicated). [d]N.D.: Not detected under the experimental condition.

The cyanoacrylamide series (**27-33**) constituted a more complicated yet intriguing scenario. Although our primary goal was to obtain *N*-monosubstituted cyanoacrylamides **27-29** bearing structural similarity to their corresponding cyanoacrylates, these inhibitors demonstrated only modest inhibition of $3C^{pro}$ with respective K_i* values of 304, 332 and 752 nM. To our surprise, the Boc-protected synthetic intermediates (**30-32**) of these inhibitors exhibited on average an ~30-fold improvement in inhibitory activity (Table 3). According to the docking result of **30**, unlike **25**, its warhead seemed too large to be accommodated by the S1' pocket (Figure 4A). Despite the formation of two hydrogen bonds between warhead and active site residues (His_{40} and Gly_{145}), the calculated binding affinity of **30** (ΔG_{calc} = -6.09 kcal/mol) was less than that of **25** (ΔG_{calc} = -7.22 kcal/mol). Furthermore, as shown in Figure 4B, the obviously bending progress curve of Boc-protected **31** demonstrated a slow-binding behavior compared with cyanoacrylate (**21**, **25**) or *N*-monosubstituted acrylamide (**28**), which indicated that establishment of the binding equilibrium was retarded.

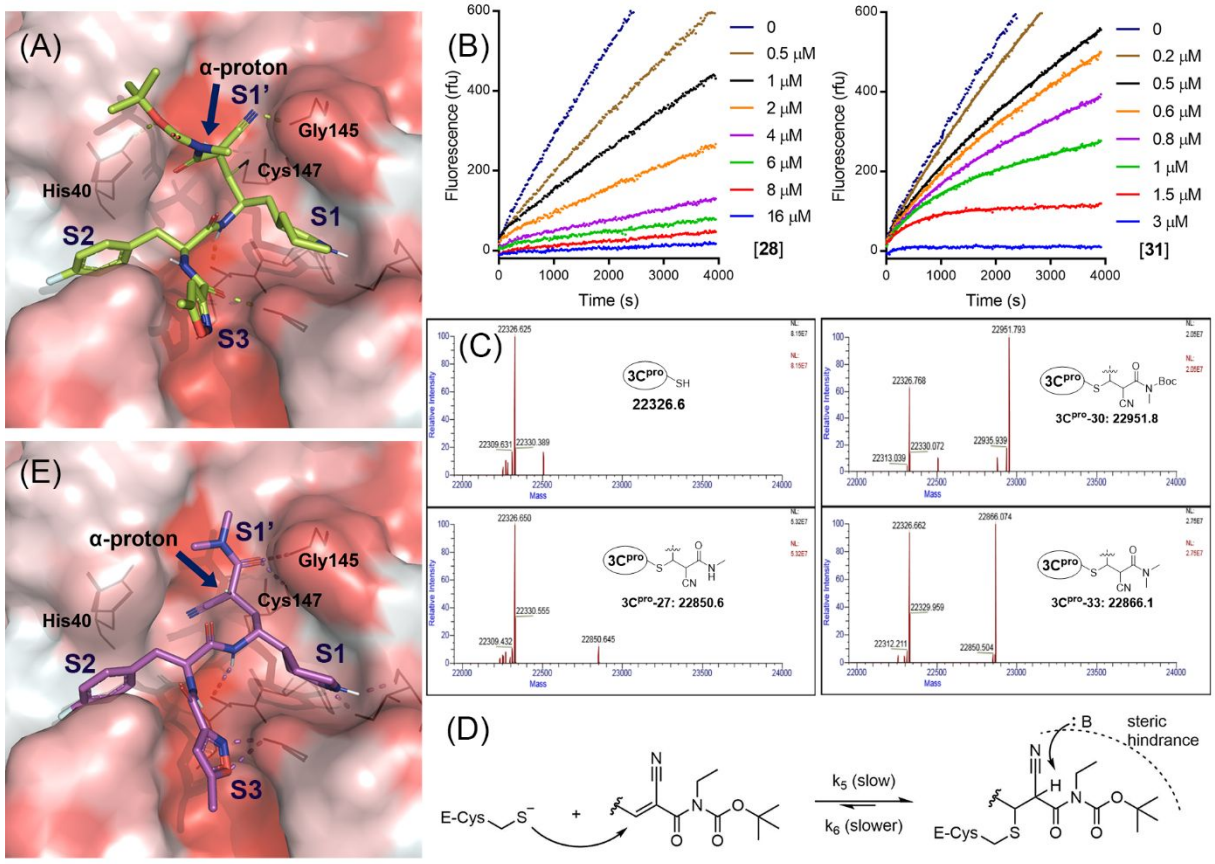


Figure 4. (A) Top-scoring docking pose of **30** (green). Hydrogen bonds were drawn as dashed lines. Red colored area represented hydrophobic protein surface. The arrow pointed to α-proton of Michael acceptor, which resided in a hydrophobic region and was shielded by the P1'. (B) Time-course inhibition of $3C^{pro}$ at variable concentrations of **28** (left) and **31** (right). **31** displayed a more obvious slow-binding behavior (time-dependency) than **28**. (C) Deconvoluted ESI-MS spectra of $3C^{pro}$ incubated with different cyanoacrylamides. Upper left: unmodified $3C^{pro}$; lower left: $3C^{pro}$-**27**; upper right: $3C^{pro}$-**30**; lower right: $3C^{pro}$-**33**. (D) Putative reaction mechanism for the elimination of $3C^{pro}$-**31** adduct. (E) Top-scoring docking pose of **33** (magenta). Other representations were similar with (A). The α-proton was solvent-exposed.

Both computational and kinetic results indicated that the Boc-protected cyanoacrylamides should have difficulty in the initial binding to $3C^{pro}$, thus resulting in low affinity (high K_i value). Therefore, there must be other factors that contribute to the improvement of the overall potency (low K_i^* value). Based on the relationship between K_i and K_i^* (Figure 1A), we inferred that the reversal of the covalent complex E·I* must in comparison be slowed down even more than its rate of formation ($k_6 << k_5$). This

hypothesis was supported by the mass spectrometric results (Figure 4C), where the remarkable modification of $3C^{pro}$ by **30** (I_{add}/I_{pro}=1.5) indicated a more durable covalent bond than that of **27** (I_{add}/I_{pro}=0.1). One plausible explanation for these facts is that the α-proton of Boc-protected thioadduct was shielded by the two *N*-substituents to the extent that the E1cB elimination reaction was impaired (Figure 4D).

By reexamining the binding mode of **30**, it could be seen that the α-proton resided in a hydrophobic area under the sheltering of the P1' structure. To further verify this hypothesis, we synthesized **33**, which contained a second methyl group in place of the Boc group. This compound was three times more potent than **27** but less potent than **30**, which is consistent with an inhibition mechanism in which the second methyl group impedes the abstraction of the α-proton but to a lesser extent than the bulkier Boc group. The degree of covalent modification by **33** (I_{add}/I_{pro} =1.0, Figure 4D) was between those observed by **27** and **30**, which also agreed well with our proposed mechanism. Furthermore, the docking model of **33** offered a more straightforward elucidation (Figure 4E). On one hand, compound **33** bound favorably to $3C^{pro}$ with high binding affinity (ΔG_{calc} = -7.06 kcal/mol). On the other hand, unlike **30**, the α-proton of the thioadduct of **33** was solvent-exposed and susceptible to base attack, leading to a more rapid E1cB reaction (large k_6). The final K_i* was a result of these counteracting effects.

Reversibility study

In the preceding experiments, ultrafiltration was performed prior to the MS analyses. In fact, we also determined $3C^{pro}$ activity with the ultrafiltered samples (Figure 5A). The inhibitor concentrations in these samples were diluted by approximately 10^3- and 10^6-fold, respectively. As a classical Michael acceptor, no restoration of proteolytic activity was detected in the ultrafiltered $3C^{pro}$-**2** complex, indicative of an irreversibly modified catalytic Cys_{147}. The cyanoacrylates **21** and **25** exhibited approximately 65-85% recovery of activity, demonstrating decent reversibility for this series. There was significant recovery of activity with cyanoacrylamides **27** and **33**, whereas only 25% of the activity was restored in Boc-protected **30**, providing another piece of evidence for the

"proton-shielding" mechanism.

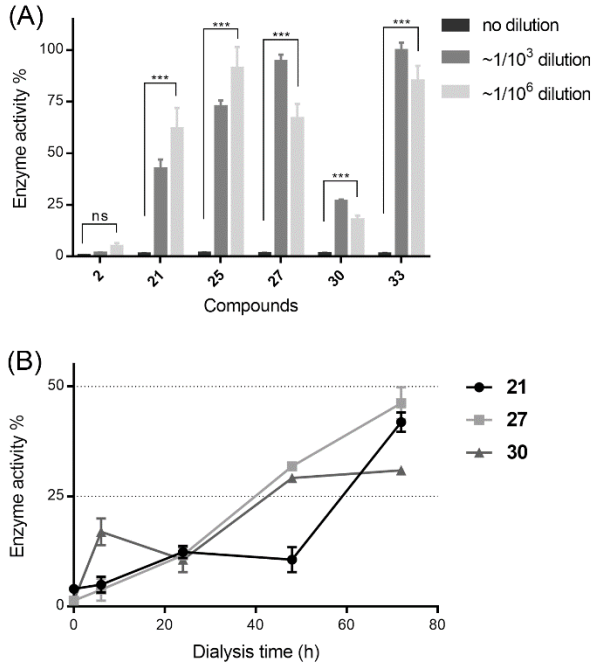


Figure 5. (A) Enzyme activity recovery after ultrafiltration of preincubated $3C^{pro}$-inhibitor complex. The concentration of given inhibitor was diluted by approximately 10^3- and 10^6-fold using ultrafiltration method. NS, not significant; P(***)<0.001. (B) Enzyme activity recovery after dialysis of $3C^{pro}$ pretreated with the indicated inhibitors or DMSO. Aliquots were taken at indicated time points, tested for the enzyme activity and normalized to a DMSO control. Error bar represents SEM (n = 3) in both (A) and (B).

The removal of covalently bound inhibitor by ultrafiltration might introduce a mechanical force that may disrupt the adduct. To provide a more authentic assessment of the reversibility of the covalent inhibitor, we evaluated the recovery of $3C^{pro}$ activity following dialysis instead of ultrafiltration, because dialysis mimics intracellular diffusion to some degree (Figure 5B).[20] After dialyzing for 6, 24, 48 and 72 h, the remaining activity of $3C^{pro}$ was measured at each time point and normalized to control values. $3C^{pro}$-**21** or $3C^{pro}$-**27** gradually recovered to approximately 50% of the original proteolytic activity, while $3C^{pro}$-**30** demonstrated only 30% recovery because of its slowed elimination.

Evaluation of selectivity

Physiological nucleophiles present a challenge for the design of covalent

inhibitors.[22] These include: 1) small molecules such as glutathione (GSH), ethanolamine or lysine and 2) proteins with exposed nucleophilic centers, especially serine and cysteine proteases. To assess the performance of dually activated Michael acceptors under intracellular redox conditions, representative compounds (**21**, **30**, **33**) were tested against $3C^{pro}$ in the presence of 5 mM GSH (Figure S9). A reduction of protease inhibition occurred in all cases and was inhibitor-dependent. However, the addition of GSH and other potential cellular thiols to these inhibitors did not weaken their cell-based activity. Due to high potency of these inhibitors, low dosage is expected to be used in practice, and hence there is less chance of cellular GSH depletion. Nonetheless, the hepatotoxicity remains to be evaluated. Moreover, ethanolamine and lysine had no effect on the activity of these inhibitors.

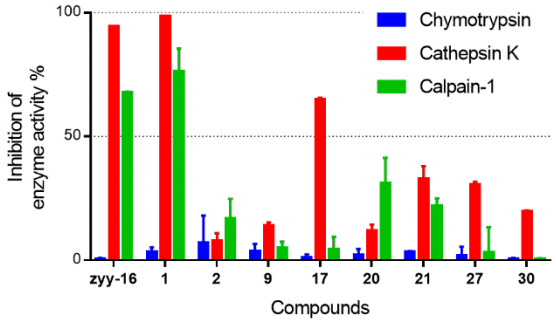


Figure 6. Selectivity study. All three enzymes were inhibited by 50 μM of indicated compounds. Inhibitory effects were calculated from fractional initial velocity as detailed in Experiment Section. Error bar represents SEM (n = 2).

To evaluate the selectivity of the above $3C^{pro}$ inhibitors relative to other common mammalian proteases, several compounds (Figure 6) were assayed with two cysteine proteases (cathepsin K and calpain-1) and one serine protease (chymotrypsin) at a specified concentration of 50 μM, which represented up to 500 times the apparent K_i* value of any given inhibitor. None of the tested new compounds exhibited measurable inhibition against chymotrypsin, demonstrating significant indifference to this serine protease. For the two cysteine proteases, although some of these compounds showed partial inhibition, they had acceptable selectivity in general. With the exception of **17** (65%

inhibition on cathepsin K), they all exhibited <50% inhibition against cathepsin K and calpain-1, while our previously reported inhibitors **1** and **zyy16** that contained, respectively, aldehyde and cyanohydrin warheads, almost completely inhibited both enzymes.[9b, 23]

CONCLUSION

An alkene conjugated with two EWGs, referred to as a dually activated Michael acceptor, could serve as a novel warhead in the design of covalent inhibitors to attain improved reactivity and potential reversibility. Here, we validated this concept for the design of novel inhibitors of EV71 $3C^{pro}$, a promising therapeutic target. Generally, the combination of a cyano group with different carbonyl groups provided these novel Michael acceptors with improved potency and selectivity. Moreover, the *N*-disubstituted cyanoacrylamides possessed unexpectedly high inhibitory activity. We proposed a hypothesis in which the spatially shielded α-proton in the enzyme-inhibitor adduct resulted in a reduced off-rate that eventually lowered the overall inhibition constant. From steady-state kinetics, MS analyses and computational studies, we not only confirmed the covalent mode of inhibition and investigated the reversibility but also provided some evidence for the "proton-shielding" hypothesis. The exploration of these dually activated Michael acceptors may lead to novel anti-EV71 candidates and could find potential application in the design of reversible covalent inhibitors for other proteases.

EXPERIMENTAL SECTION

General chemical information. Unless otherwise specified, all reagents were purchased from commercial suppliers and were used as received. All solvents were of reagent grade and, when necessary, were purified and dried by standard methods. Reaction progress was monitored by thin-layer chromatography (TLC) that was performed on glass-backed silica gel plates, and spots were visualized using a UV lamp (λ = 254, 365 nm), iodine vapor or by $KMnO_4$ staining. Reaction products were purified by silica gel column chromatography or as otherwise noted. NMR spectra were recorded on a Bruker AVANCE-400 NMR spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C) in $CDCl_3$-*d*, CD_3OD-d_4 or DMSO-d_6 solutions with TMS as the internal standard. Chemical

shifts were reported in ppm with residual solvent as the internal standard. Standard abbreviations were used for denoting signal multiplicities. All coupling constants were given in Hz. Low-resolution mass spectra were obtained using a Shimadzu LCMS-2020 (Shimadzu, Kyoto, Japan). High-resolution mass spectra (HRMS) were obtained using a high-resolution ESI-FTICR mass spectrometer (Varian 7.0 T, Varian, USA). Optical rotations were measured with an Insmark IP-120 automatic polarimeter (Insmark, Shanghai, China). Measurements were collected at 20 °C in DCM at 589 nm. $[\alpha]_D$ values were given in unit of (deg × mL)/(g × dm). HPLC was performed on a Dionex UltiMate 3000 (Germany). All target compounds possessed a purity of ≥ 95% as determined by HPLC.

Preparation of methyl (*S,E*)-4-((S)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoate (2). Sodium bis(trimethylsilyl) amide (0.3 mL of 1.0 M solution in THF, 0.3 mmol, 1.2 equiv) was added to a solution of methyl diethylphosphonoacetate (56 mg, 0.3 mmol, 1.2 equiv) in anhydrous THF (20 mL) under a nitrogen atmosphere at -78 °C and stirred for 30 min. **1** (100 mg, 0.23 mmol, 1.0 equiv) in anhydrous THF (5 mL) was added dropwise via cannula, then the reaction mixture was stirred at -78 °C for 30 min until TLC indicated full consumption of starting material. Then the reaction was warmed to room temperature (RT) and quenched with 1.0 M HCl solution (5 mL). Subsequently, THF was removed, and extractive workup was done with DCM (3×). The combined organic layer was dried over anhydrous sodium sulfate and concentrated, and the residue was purified via silica gel column chromatography (MeOH:DCM = 1:70 v/v) to afford the pure product **2** as a white solid (90 mg, yield 80%). $[\alpha]_D^{20}$ = −14.44 (c = 0.097, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 – 7.1 (m, 2H), 6.9 (t, *J* = 8.6 Hz, 2H), 6.7 (d, *J* = 5.7 Hz, 1H), 6.3 (s, 1H), 5.6 (dd, *J* = 15.6, 1.5 Hz, 1H), 4.9 (hept, *J* = 7.3 Hz, 1H), 4.5 (s, 1H), 3.7 (s, 3H), 3.2 (tq, *J* = 12.5, 7.2 Hz, 2H), 3.1 (s, 2H), 2.4 (s, 3H), 2.2 (qd, *J* = 9.4, 7.9, 3.1 Hz, 1H), 2.1 (ddd, *J* = 14.1, 12.0, 4.4 Hz, 1H), 2.0 (dt, *J* = 13.7, 5.0 Hz, 1H), 1.9 – 1.8 (m, 1H), 1.7 – 1.6 (m, 1H), 1.5 (dddd, *J* = 23.7, 20.9, 9.1, 3.3 Hz, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ175.0, 171.2, 170.4, 166.7, 162.0 (d, *J*

= 244.8 Hz), 158.9, 158.4, 147.6, 132.0 (d, *J* = 3.1 Hz), 131.1, 131.0, 120.7, 115.5, 115.3, 101.5, 54.6, 51.7, 48.4, 42.3, 38.1, 38.0, 35.7, 26.8, 21.4, 12.3. HRMS (ESMS): $C_{25}H_{29}FN_4NaO_6$ $(M + Na)^+$, calcd. 523.1963, found 523.1968. Purity: >99.0%, HPLC t_{ret} = 6.57 min.

Preparation of ethyl (*S*,*E*)-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoate (3). **3** was obtained from **1** (100 mg) and triethyl phosphonoacetate (55 µL) following similar procedure with **2**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:50 v/v) to afford the pure product as a white solid **3** (96 mg, 83%). $[\alpha]_D^{20}$ = −11.41 (c = 0.23, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 (dd, *J* = 8.4, 5.5 Hz, 2H), 6.9 (t, *J* = 8.7 Hz, 2H), 6.7 (dd, *J* = 15.6, 5.7 Hz, 1H), 6.3 (s, 1H), 5.7 (dd, *J* = 15.7, 1.4 Hz, 1H), 5.0 – 4.9 (m, 1H), 4.5 (t, *J* = 6.2 Hz, 1H), 4.2 (q, *J* = 7.1 Hz, 2H), 3.3 (d, *J* = 8.7 Hz, 2H), 3.1 (d, *J* = 6.7 Hz, 2H), 2.4 (s, 3H), 2.2 (q, *J* = 5.6 Hz, 1H), 2.1 – 2.1 (m, 1H), 2.0 (td, *J* = 10.7, 8.5, 5.0 Hz, 1H), 1.8 (dt, *J* = 13.7, 4.1 Hz, 1H), 1.7 (dt, *J* = 13.6, 7.6 Hz, 1H), 1.5 (tdd, *J* = 14.7, 5.7, 2.6 Hz, 2H), 1.3 – 1.3 (m, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 175.0, 171.3, 170.4, 166.3, 162.0 (d, *J* = 245.0 Hz), 158.9, 158.4, 147.2, 132.0 (d, *J* = 3.0 Hz), 131.2, 131.1, 121.3, 115.6, 115.4, 101.5, 60.6, 54.6, 48.9, 42.4, 38.4, 38.2, 35.7, 27.3, 21.6, 14.3, 12.4. HRMS (ESMS): $C_{26}H_{31}FN_4NaO_6$ $(M + Na)^+$, calcd. 537.2120, found 537.2125. Purity: >99%, HPLC t_{ret} = 7.35 min.

Preparation of *N*-((*S*)-3-(4-fluorophenyl)-1-(((*S*,*E*)-5-(methylamino)-5-oxo-1-((*S*)-2-oxopiperidin-3-yl)pent-3-en-2-yl)amino)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (4). To a solution of **1** (100 mg, 0.23 mmol, 1.0 equiv) in dry acetonitrile (20 mL) at RT were sequentially added diethyl (2-(methylamino)-2-oxoethyl)phosphonate (95 mg, 0.46 mmol, 2.0 equiv), lithium chloride (14 mg, 0.35 mmol, 1.5 equiv) and 1, 8-diazabicyclo[5.4.0]undec-7-ene (40 mg, 0.28 mmol, 1.2 equiv). The reaction mixture was stirred at RT and quenched with saturated NH_4Cl after complete conversion. Subsequently, acetonitrile was removed, and extractive workup was done with DCM (40 mL×3), followed by washing with H_2O (40 mL×2), saturated citric acid solution (40 mL×2),

saturated $NaHCO_3$ solution (40 mL×2) and brine (40 mL×2). The organic phase was dried over sodium sulfate and concentrated, and the crude product was purified by silica gel column chromatography (MeOH:DCM = 1:30 v/v) to afford the pure product as a white solid **4** (76 mg, yield 68%). $[\alpha]_D^{20}$ = −31.41 (c = 0.43, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 – 7.1 (m, 2H), 6.9 (t, *J* = 8.6 Hz, 2H), 6.7 (dd, *J* = 15.7, 5.6 Hz, 1H), 6.3 (s, 1H), 5.7 (d, *J* = 15.6 Hz, 1H), 4.9 (d, *J* = 7.8 Hz, 1H), 4.5 (s, 1H), 3.7 (s, 3H), 3.2 (d, *J* = 11.4 Hz, 2H), 3.1 (d, *J* = 6.8 Hz, 2H), 2.4 (s, 3H), 2.2 (s, 1H), 2.1 (s, 1H), 2.0 (d, *J* = 13.8 Hz, 1H), 1.8 (d, *J* = 13.5 Hz, 1H), 1.7 – 1.6 (m, 1H), 1.5 (p, *J* = 11.0 Hz, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 175.0, 171.3, 170.4, 166.8, 162.0 (d, *J* = 244.9 Hz), 158.9, 158.4, 147.6, 132.0 (d, *J* = 3.0 Hz), 131.1, 131.0, 120.7, 115.6, 115.4, 101.5, 54.6, 51.7, 48.6, 42.4, 38.2, 38.2, 35.7, 27.1, 21.5, 12.4. HRMS (ESMS): $C_{25}H_{30}FN_5NaO_5$ $(M + Na)^+$, calcd. 522.2123, found 522.2125. Purity: 95.0%, HPLC t_{ret} = 5.37 min.

Preparation of *N*-((*S*)-1-(((*S*,*E*)-4-cyano-1-((*S*)-2-oxopiperidin-3-yl)but-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (5). 5 was obtained from **1** (100 mg) and diethyl cyanomethylphosphonate (75 μL) following similar procedure with **4** at RT for 2 h. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:70 v/v) to afford the pure product as a white solid **5** (86 mg, yield 82%). $[\alpha]_D^{20}$ = −11.52 (c = 0.14, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 (dd, *J* = 8.3, 5.4 Hz, 2H), 7.0 (t, *J* = 8.6 Hz, 2H), 6.4 (dd, *J* = 16.3, 5.4 Hz, 1H), 6.3 (s, 1H), 5.1 (d, *J* = 16.3 Hz, 1H), 4.9 (q, *J* = 7.4 Hz, 1H), 4.4 (dq, *J* = 10.8, 5.0 Hz, 1H), 3.3 (qt, *J* = 11.6, 4.6 Hz, 2H), 3.1 (qd, *J* = 13.7, 6.9 Hz, 2H), 2.4 (s, 3H), 2.2 (dq, *J* = 11.6, 6.0 Hz, 1H), 2.1 – 1.9 (m, 2H), 1.8 (dq, *J* = 13.8, 4.5 Hz, 1H), 1.8 – 1.6 (m, 1H), 1.5 (ddt, *J* = 13.6, 10.5, 3.4 Hz, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.8, 171.5, 170.6, 162.1 (d, *J* = 245.9 Hz), 159.0, 158.3, 153.6, 131.9 (d, *J* = 3.1 Hz), 131.2, 131.1, 117.0, 115.7, 115.5, 101.5, 100.2, 54.6, 49.4, 42.3, 38.2, 38.0, 35.1, 27.2, 21.6, 12.4. HRMS (ESMS): $C_{24}H_{26}FN_5NaO_4$ $(M + Na)^+$, calcd. 490.1861, found 490.1865. Purity: >99.0%, HPLC t_{ret} = 5.97 min.

Preparation of *N*-((*S*)-1-(((*S*)-4-cyano-1-((*S*)-2-oxopiperidin-3-yl)pent-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (6). 6

was obtained from **1** (100 mg) and **34** (86 mg) following similar procedure with **4** at RT for 1 h. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a white solid **6** (85 mg, yield 78%). The *E*/*Z* isomers were not separated using the given chromatographic conditions. $[\alpha]_D^{20}$ = −4.14 (c = 0.34, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.1 (ddd, *J* = 13.3, 8.2, 5.4 Hz, 2H), 6.9 (dt, *J* = 15.4, 8.5 Hz, 2H), 6.3 (d, *J* = 3.1 Hz, 1H), 5.8 – 5.5 (m, 1H), 4.9 (dq, *J* = 22.6, 7.4 Hz, 1H), 4.7 – 4.5 (m, 1H), 3.2 (d, *J* = 10.4 Hz, 2H), 3.1 (h, *J* = 7.2 Hz, 2H), 2.4 (d, *J* = 1.8 Hz, 3H), 2.2 (ddt, *J* = 16.5, 12.4, 5.9 Hz, 2H), 2.0 (dt, *J* = 9.2, 4.7 Hz, 1H), 2.0 – 1.9 (m, 3H), 1.8 (dq, *J* = 9.2, 4.6 Hz, 1H), 1.7 – 1.6 (m, 1H), 1.4 (ddd, *J* = 13.6, 8.7, 3.1 Hz, 1H), 1.3 (ddd, *J* = 14.1, 7.2, 3.8 Hz, 1H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.6, 171.3, 170.3, 162.0 (d, *J* = 245.3 Hz), 158.8, 158.3, 146.8, 131.9 (d, *J* = 3.0 Hz), 131.0, 131.0, 120.0, 115.5, 115.3, 110.8, 101.3, 54.3, 49.0, 42.3, 38.3, 37.8, 35.0, 27.1, 21.5, 15.3, 12.3. HRMS (ESMS): $C_{25}H_{28}FN_5NaO_4$ $(M + Na)^+$, calcd. 504.2018, found 504.2022. Purity: 97.6%, HPLC t_{ret} = 18.41 min (*E*-isomer) and 19.86 (*Z*-isomer).

Preparation of *N*-((*S*)-1-(((*S*)-4-chloro-4-cyano-1-((*S*)-2-oxopiperidin-3-yl)but-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (7). 7 was obtained from **1** (100 mg) and **35** (95 mg) following similar procedure with **4** at RT for 1 h. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a white solid **7** (92 mg, yield 82%). The *E*/*Z* isomers were not separated using the given chromatographic conditions. $[\alpha]_D^{20}$ = −27.12 (c = 0.081, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.1 (dt, *J* = 8.7, 5.2 Hz, 2H), 7.0 (q, *J* = 7.9 Hz, 2H), 6.3 (s, 1H), 6.1 (d, *J* = 8.2 Hz, 1H), 4.9 (q, *J* = 7.6 Hz, 1H), 4.8 – 4.5 (m, 1H), 3.3 (d, *J* = 10.2 Hz, 2H), 3.1 (qd, *J* = 13.7, 6.7 Hz, 2H), 2.4 (s, 3H), 2.3 – 2.1 (m, 2H), 2.0 (d, *J* = 15.2 Hz, 1H), 1.9 (d, *J* = 13.8 Hz, 1H), 1.8 – 1.6 (m, 1H), 1.5 (dd, *J* = 25.0, 12.3 Hz, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.7, 171.4, 170.5, 162.0 (d, *J* = 245.5 Hz), 158.9, 158.3, 148.3, 132.0 (d, *J* = 3.6 Hz), 131.2, 131.1, 115.6, 115.6, 113.0, 104.4, 101.4, 54.3, 49.1, 42.4, 38.3, 35.1, 33.8, 27.2, 21.6, 12.4. HRMS (ESMS): $C_{24}H_{25}ClFN_5NaO_4$ $(M + Na)^+$, calcd. 524.1471, found 524.1475. Purity: 97.9%, HPLC t_{ret} = 18.51 min (*Z* isomer) and

19.41 (*E* isomer).

Preparation of *N*-((*S*)-1-(((*S*)-4-bromo-4-cyano-1-((*S*)-2-oxopiperidin-3-yl)but-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (8). **8** was obtained from **1** (100 mg) and **36** (115 mg) following similar procedure with **4**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a light yellow solid **8** (98 mg, 80%). $[\alpha]_D^{20}$ = −44.79 (c = 0.067, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 (dt, *J* = 8.4, 4.7 Hz, 2H), 7.0 (td, *J* = 8.6, 2.7 Hz, 2H), 6.3 (s, 1H), 6.2 (d, *J* = 9.0 Hz, 1H), 4.9 (q, *J* = 7.4 Hz, 1H), 4.6 – 4.5 (m, 1H), 3.3 (s, 2H), 3.2 – 3.0 (m, 2H), 2.5 (s, 3H), 2.2 – 2.0 (m, 3H), 1.9 (dt, *J* = 13.3, 4.3 Hz, 1H), 1.7 (dd, *J* = 15.1, 6.9 Hz, 1H), 1.6 – 1.4 (m, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.8, 171.2, 170.4, 161.9 (d, *J* = 245.5 Hz), 158.9, 158.2, 152.4, 150.9, 131.9 (d, *J* = 3.3 Hz), 131.1, 131.0, 115.5, 115.3, 113.6, 101.3, 88.3, 54.3, 50.7, 42.3, 38.3, 38.1, 34.8, 27.5, 21.5, 12.3. HRMS (ESMS): $C_{24}H_{25}BrFN_5NaO_4$ $(M + Na)^+$, calcd. 568.0966, found 568.0970. Purity: 95.9%, HPLC t_{ret} = 18.75 min (*Z*-isomer) and 19.53 (*E*-isomer).

Preparation of methyl (*S*)-2-chloro-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoate (9). **9** was obtained from **1** (100 mg) and **37** (110 mg) following similar procedure with **4**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a white solid **9** (93 mg, yield 78%). $[\alpha]_D^{20}$ = −3.03 (c = 0.13, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.1 (dt, *J* = 8.6, 4.9 Hz, 2H), 6.9 (dt, *J* = 11.3, 8.4 Hz, 2H), 6.6 (d, *J* = 8.3 Hz, 1H), 6.4 (d, *J* = 27.8 Hz, 1H), 5.3 – 4.6 (m, 2H), 3.8 (m, 3H), 3.3 (d, *J* = 6.3 Hz, 2H), 3.2 – 2.9 (m, 2H), 2.4 (s, 3H), 2.2 (s, 1H), 2.1 (dd, *J* = 15.5, 8.1 Hz, 2H), 1.9 – 1.8 (m, 1H), 1.8 – 1.6 (m, 1H), 1.6 – 1.4 (m, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.9, 171.3, 170.4, 162.5, 162.0 (d, *J* = 245.0 Hz), 158.8, 158.4, 145.2, 132.1 (d, *J* = 3.8 Hz), 131.2, 131.1, 124.3, 115.5, 115.3, 101.4, 54.4, 53.3, 47.7, 42.4, 38.5, 38.2, 34.2, 27.0, 21.5, 12.4. HRMS (ESMS): $C_{25}H_{28}ClFN_4NaO_6$ $(M + Na)^+$, calcd. 557.1574, found 557.1577. Purity: 97.1%, HPLC t_{ret} = 17.63 min (*Z*-isomer) and 18.93 min (*E*-isomer).

Preparation of methyl (*S*)-2-bromo-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoate (10). **10** was obtained from **1** (100 mg) and **38** (130 mg) following similar procedure with **4**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:60 v/v) to afford the pure product as a light yellow solid **10** (95 mg, yield 73%). $[\alpha]_D^{20}$ = −18.21 (c = 0.19, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.1 (dd, *J* = 9.0, 4.7 Hz, 2H), 6.9 (dt, *J* = 17.2, 9.5 Hz, 2H), 6.8 (d, *J* = 5.6 Hz, 1H), 6.3 (d, *J* = 24.8 Hz, 1H), 5.2 – 4.5 (m, 2H), 3.9 – 3.7 (m, 3H), 3.3 (d, *J* = 8.2 Hz, 2H), 3.1 (p, *J* = 11.6, 10.9 Hz, 2H), 2.5 – 2.4 (m, 3H), 2.2 (s, 1H), 2.2 – 2.0 (m, 2H), 1.9 (d, *J* = 13.7 Hz, 1H), 1.7 (s, 1H), 1.5 (p, *J* = 13.1, 12.0 Hz, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.9, 171.2, 170.4, 162.5, 162.0 (d, *J* = 245.1 Hz), 158.9, 158.4, 146.2, 132.1 (d, *J* = 3.2 Hz), 131.2, 131.1, 115.5, 115.3, 111.7, 101.4, 54.4, 53.5, 50.1, 42.4, 38.4, 38.1, 34.0, 26.9, 21.5, 12.4. HRMS (ESMS): $C_{25}H_{28}BrFN_4NaO_6$ $(M + Na)^+$, calcd. 601.1068, found 601.1070. Purity: 97.3%, HPLC t_{ret} = 18.52 min (*Z*-isomer) and 20.05 (*E*-isomer).

Preparation of ethyl (*S*)-2-chloro-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoate (11). **11** was obtained from **1** (100 mg) and **39** (116 mg) following similar procedure with **4**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:70 v/v) to afford the pure product as a white solid **11** (100 mg, yield 81%). $[\alpha]_D^{20}$ = −9.79 (c = 0.14, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.1 (q, *J* = 6.0, 5.5 Hz, 2H), 6.9 (q, *J* = 9.4 Hz, 2H), 6.6 (d, *J* = 8.0 Hz, 1H), 6.3 (s, 1H), 5.3 – 4.7 (m, 2H), 4.3 (q, *J* = 7.2 Hz, 2H), 3.4 – 3.2 (m, 2H), 3.1 (pd, *J* = 13.2, 11.3, 6.7 Hz, 2H), 2.4 (s, 3H), 2.2 (p, *J* = 5.9 Hz, 1H), 2.1 (h, *J* = 8.1, 6.0 Hz, 2H), 1.9 (d, *J* = 13.3 Hz, 1H), 1.7 (d, *J* = 10.8 Hz, 1H), 1.6 – 1.4 (m, 2H), 1.3 (q, *J* = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 175.1, 171.3, 170.4, 162.1, 162.0 (d, *J* = 245.1 Hz), 158.9, 158.4, 142.1, 132.1 (d, *J* = 3.4 Hz), 131.2, 131.1, 123.1, 115.5, 115.3, 101.4, 62.6, 54.4, 47.8, 42.4, 38.5, 38.3, 34.2, 27.1, 21.5, 14.2, 12.4. HRMS (ESMS): $C_{26}H_{30}ClFN_4NaO_6$ $(M + Na)^+$, calcd. 571.1730, found 571.1735. Purity > 99%, HPLC t_{ret} = 21.03 min (*Z*-isomer) and 22.53 min (*E*-isomer).

Preparation of ethyl (*S*)-2-bromo-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoate (12). 12 was obtained from **1** (100 mg) and **40** (136 mg) following similar procedure with **4**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a yellow solid **12** (95 mg, 71%). $[\alpha]_D^{20}$ = −5.56 (c = 0.11, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.1 (dt, *J* = 8.7, 4.8 Hz, 2H), 7.0 – 6.9 (m, 2H), 6.6 (d, *J* = 8.6 Hz, 1H), 6.3 (s, 1H), 5.2 – 4.6 (m, 2H), 4.3 – 4.2 (m, 2H), 3.3 (s, 2H), 3.1 – 3.0 (m, 2H), 2.4 (s, 3H), 2.2 (s, 1H), 2.1 (d, *J* = 15.3 Hz, 2H), 1.9 (d, *J* = 14.1 Hz, 1H), 1.7 (d, *J* = 9.1 Hz, 1H), 1.5 (dt, *J* = 22.9, 11.0 Hz, 2H), 1.3 (q, *J* = 5.9 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.9, 171.2, 170.4, 162.0, 162.0 (d, *J* = 244.6 Hz), 158.9, 158.4, 145.7, 132.1 (d, *J* = 3.2 Hz), 131.2, 131.1, 116.2, 115.5, 115.3, 101.4, 62.8, 54.4, 50.4, 42.4, 38.5, 38.3, 34.0, 27.2, 21.6, 14.3, 12.4. HRMS (ESMS): $C_{26}H_{30}BrFN_4NaO_6$ $(M + Na)^+$, calcd. 615.1225, found 615.1230. Purity: 95.9%, HPLC t_{ret} = 21.35 min (*Z*-isomer) and 23.06 (*E*-isomer).

Preparation of *N*-((S)-1-(((*S,Z*)-4-cyano-1-((*S*)-2-oxopiperidin-3-yl)-4-phenylbut-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (13). 13 was obtained from **1** (100 mg, 0.23 mmol, 1.0 equiv) and **41** (115 mg, 0.46 mmol, 2.0 equiv) following similar procedure with **4**. The crude product was purified via silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a white solid **13** (92 mg, yield 75%). $[\alpha]_D^{20}$ = −4.85 (c = 0.29, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.5 – 7.4 (m, 2H), 7.4 – 7.3 (m, 3H), 7.1 (dd, *J* = 8.4, 5.4 Hz, 2H), 6.8 (td, *J* = 8.6, 1.8 Hz, 2H), 6.3 (s, 1H), 6.2 (d, *J* = 8.6 Hz, 1H), 5.0 – 4.9 (m, 1H), 4.9 (dddd, *J* = 12.5, 10.2, 6.1, 3.7 Hz, 1H), 3.3 (dq, *J* = 8.1, 4.7, 3.7 Hz, 2H), 3.1 (qd, *J* = 13.6, 6.9 Hz, 2H), 2.4 (s, 3H), 2.3 – 2.2 (m, 2H), 2.1 (ddt, *J* = 11.6, 6.1, 2.8 Hz, 1H), 1.9 (dt, *J* = 13.1, 4.2 Hz, 1H), 1.7 (dddd, *J* = 19.2, 14.2, 8.8, 3.1 Hz, 1H), 1.6 (dq, *J* = 10.5, 4.6, 3.2 Hz, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 175.0, 171.3, 170.4, 161.9 (d, *J* = 245.1 Hz), 158.9, 158.4, 146.1, 132.4, 132.1 (d, *J* = 3.1 Hz), 131.2, 131.1, 129.5, 129.1, 125.9, 116.0, 115.8, 115.4, 115.2, 101.4, 54.2, 49.7, 42.4, 38.3, 35.7, 27.0, 21.6, 12.4. HRMS (ESMS): $C_{30}H_{30}FN_5NaO_4$ $(M + Na)^+$, calcd. 566.2174, found 566.2178. Purity: 95.5%, HPLC t_{ret} = 8.46 min.

Preparation of *N*-((*S*)-1-(((*S*,*Z*)-4-cyano-4-(naphthalen-2-yl)-1-((*S*)-2-oxopiperidin-3-yl)but-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (14). 14 was obtained from **1** (100 mg) and **42** (115 mg) following similar procedure with **4**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a white solid **14** (92 mg, yield 75%). $[\alpha]_D^{20}$ = −42.77 (c = 0.11, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 8.0 (d, *J* = 1.8 Hz, 1H), 7.9 – 7.8 (m, 3H), 7.5 (ddt, *J* = 8.5, 6.0, 3.1 Hz, 3H), 7.1 (dd, *J* = 8.5, 5.4 Hz, 2H), 6.8 (td, *J* = 8.7, 2.2 Hz, 2H), 6.4 (d, *J* = 8.6 Hz, 1H), 6.3 (s, 1H), 5.0 (q, *J* = 7.4 Hz, 1H), 4.9 (qd, *J* = 8.6, 7.1, 3.1 Hz, 1H), 3.3 (dp, *J* = 11.4, 3.7, 3.1 Hz, 2H), 3.1 (qd, *J* = 13.7, 6.9 Hz, 2H), 2.4 (d, *J* = 3.5 Hz, 3H), 2.3 (ddt, *J* = 17.3, 11.6, 5.4 Hz, 2H), 2.2 – 2.1 (m, 1H), 1.9 (tt, *J* = 7.4, 3.8 Hz, 1H), 1.7 – 1.5 (m, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 175.0, 171.3, 170.4, 161.8 (d, *J* = 245.0 Hz), 158.9, 158.4, 146.1, 133.6, 133.2, 132.2 (d, *J* = 2.9 Hz), 131.2, 131.1, 129.6, 128.9, 128.5, 127.8, 127.2, 127.0, 126.2, 122.3, 116.1, 115.9, 115.4, 115.2, 101.4, 54.3, 49.8, 42.4, 38.3, 38.3, 35.8, 27.0, 21.6, 12.4. HRMS (ESMS): $C_{34}H_{32}FN_5NaO_4$ $(M + Na)^+$, calcd. 616.2331, found 616.2334. Purity: 95.8%, HPLC t_{ret} = 10.16 min.

Preparation of *N*-((*S*)-1-(((*S*,*Z*)-4-cyano-1-((*S*)-2-oxopiperidin-3-yl)-4-(4-(trifluoromethyl)phenyl)but-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (15). 15 was obtained from **1** (100 mg) and **43** (135 mg) following similar procedure with **4**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:60 v/v) to afford the pure product as a yellow solid **15** (93 mg, yield 70%). $[\alpha]_D^{20}$ = −12.77 (c = 0.094, DCM). ^{1}H NMR ($CDCl_3$-*d*) ^{1}H NMR ($CDCl_3$-*d*) δ 7.7 (d, *J* = 8.3 Hz, 2H), 7.6 (d, *J* = 8.3 Hz, 2H), 7.2 (dd, *J* = 8.5, 5.4 Hz, 2H), 6.9 (t, *J* = 8.6 Hz, 2H), 6.4 (s, 2H), 5.0 – 4.9 (m, 1H), 4.9 – 4.7 (m, 1H), 3.3 (dd, *J* = 10.8, 4.1 Hz, 2H), 3.2 – 3.1 (m, 2H), 2.4 (d, *J* = 1.4 Hz, 3H), 2.3 (dq, *J* = 13.1, 5.6 Hz, 1H), 2.1 (s, 2H), 1.9 – 1.8 (m, 1H), 1.7 (dd, *J* = 14.5, 7.2 Hz, 1H), 1.6 (ddd, *J* = 14.1, 5.6, 2.8 Hz, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 175.0, 171.2, 170.4, 161.8 (d, *J* = 245.3 Hz), 158.9, 158.3, 148.6, 135.8, 132.0 (d, *J* = 3.2 Hz), 131.4 (d, *J* = 32.9 Hz), 131.1, 131.0, 129.2, 126.2, 126.0, 126.0, 123.7 (q, *J* = 272.2 Hz), 115.6, 115.4, 115.2, 114.9, 101.3, 54.2, 50.3, 42.3, 38.5, 37.9,

35.4, 27.4, 21.5, 12.3. HRMS (ESMS): $C_{31}H_{29}F_4N_5NaO_4$ $(M + Na)^+$, calcd. 634.2048, found 634.2050. Purity: 97.9%, HPLC t_{ret} = 10.06 min.

Preparation of *N*-((*S*)-1-(((*S*,*E*)-4-cyano-1-((*S*)-2-oxopiperidin-3-yl)-4-(pyridin-2-yl)but-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (16). 16 was obtained from **1** (100 mg) and **44** (115 mg) following similar procedure with **4**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a light yellow solid **16** (20 mg, yield 20%). $[\alpha]_D^{20}$ = −28.71 (c = 0.058, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 8.5 (d, *J* = 4.8 Hz, 1H), 7.8 – 7.6 (m, 2H), 7.2 – 7.2 (m, 1H), 7.1 (dd, *J* = 8.4, 5.3 Hz, 2H), 6.9 (t, *J* = 8.5 Hz, 2H), 6.2 (s, 1H), 6.1 (d, *J* = 8.5 Hz, 1H), 5.5 (q, *J* = 7.5 Hz, 1H), 4.9 (q, *J* = 7.5 Hz, 1H), 3.2 (s, 2H), 3.1 – 2.9 (m, 2H), 2.4 (s, 3H), 2.2 (qd, *J* = 9.5, 6.2, 5.5 Hz, 1H), 2.1 – 2.0 (m, 2H), 1.9 – 1.7 (m, 2H), 1.6 (d, *J* = 11.2 Hz, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.1, 170.3, 169.6, 161.0 (d, *J* = 245.5 Hz), 157.8, 157.4, 152.4, 150.1, 148.3, 136.3, 131.1 (d, *J* = 3.1 Hz), 130.1, 130.1, 122.7, 122.4, 117.6, 114.6, 114.4, 113.3, 100.4, 53.4, 46.6, 41.5, 37.6, 37.5, 33.2, 25.8, 20.4, 11.4. HRMS (ESMS): $C_{29}H_{29}FN_6NaO_4$ $(M + Na)^+$, calcd. 567.2127, found 567.2130. Purity: 95.4%, HPLC t_{ret} = 6.97 min.

Preparation of *N*-((*S*)-1-(((*S*,*Z*)-4-cyano-1-((*S*)-2-oxopiperidin-3-yl)-4-(pyridin-2-yl)but-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (17). 17 was obtained from **1** (100 mg) and **44** (115 mg) following similar procedure with **4**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a light yellow solid **17** (60 mg, yield 60%). $[\alpha]_D^{20}$ = −20.01 (c = 0.05, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 8.6 (dd, *J* = 9.7, 5.5 Hz, 1H), 7.7 (td, *J* = 7.8, 1.8 Hz, 1H), 7.5 (dd, *J* = 11.9, 8.2 Hz, 2H), 7.3 – 7.2 (m, 1H), 7.2 – 7.0 (m, 3H), 6.8 (t, *J* = 8.4 Hz, 1H), 6.3 (s, 1H), 4.9 (q, *J* = 7.3 Hz, 1H), 4.9 – 4.7 (m, 1H), 3.2 (dt, *J* = 15.8, 7.6 Hz, 2H), 3.1 – 2.9 (m, 2H), 2.4 (s, 3H), 2.2 (td, *J* = 11.2, 6.4 Hz, 2H), 2.1 – 2.0 (m, 1H), 1.8 (dt, *J* = 13.6, 4.2 Hz, 1H), 1.7 – 1.6 (m, 1H), 1.6 – 1.4 (m, 2H). ^{13}C NMR ($CDCl_3$-*d*) 174.9, 171.2, 170.4, 161.9 (d, *J* = 245.0 Hz), 158.9, 158.4, 150.1, 149.8, 137.4, 132.0 (d, *J* = 2.9 Hz), 131.2, 131.1, 124.0, 121.2, 115.5, 115.5, 115.3, 101.4, 54.3, 49.7,

42.4, 38.4, 38.3, 35.5, 27.2, 21.6, 12.4. HRMS (ESMS): $C_{29}H_{29}FN_6NaO_4$ $(M + Na)^+$, calcd. 567.2127, found 567.2130. Purity: >99.0%, HPLC t_{ret} = 6.97 min.

Preparation of *N*-((2*S*)-1-(((2*R*)-4-cyano-1-((*S*)-2-oxopiperidin-3-yl)-4-(pyridin-2-yl)butan-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (18). To a solution of **17** (100 mg, 0.2 mmol, 1.0 equiv) in MeOH was added Pd/C (10 mg, 10 wt % Pd). Hydrogen was bubbled through the stirred solution for 5−10 min. Then the reaction was stirred under an atmosphere of hydrogen for 48 h until TLC indicated complete conversion at 40 °C. The catalyst was filtered off, and the filtrate was evaporated. Then the residue was purified via silica gel column chromatography. (MeOH:DCM = 1:65 v/v) to afford the product as a white solid **18** (38 mg, yield 38%). **18** was purified as mixed *R*/*S* isomers, resulting in NMR and HPLC spectra of high complexity. $[\alpha]_D^{20}$ = −18.88 (c = 0.053, DCM). δ 8.5 (ddd, *J* = 26.7, 5.0, 1.6 Hz, 1H), 7.9 (dd, *J* = 29.4, 8.2 Hz, 1H), 7.7 (ddd, *J* = 7.5, 5.8, 1.8 Hz, 1H), 7.4 – 7.4 (m, 1H), 7.3 – 7.2 (m, 2H), 7.0 – 6.8 (m, 2H), 6.3 (d, *J* = 4.3 Hz, 1H), 4.9 (dq, *J* = 15.0, 7.1 Hz, 1H), 4.2 – 4.0 (m, 1H), 3.8 (ddd, *J* = 61.2, 9.8, 4.1 Hz, 1H), 3.3 – 3.2 (m, 2H), 3.2 – 3.0 (m, 2H), 2.4 (s, 5H), 2.2 – 1.9 (m, 4H), 1.9 – 1.7 (m, 1H), 1.7 – 1.6 (m, 1H), 1.6 – 1.4 (m, 1H). ^{13}C NMR ($CDCl_3$-*d*) δ 175.3, 171.1, 170.9, 161.8 (d, *J* = 245.3 Hz), 159.0, 158.3, 154.9, 149.6, 137.5, 132.2 (d, *J* = 3.1 Hz), 131.1, 131.0, 123.0, 121.8, 120.0, 115.5, 115.3, 101.4, 54.7, 46.2, 42.3, 40.6, 37.7, 37.0, 36.4, 35.9, 26.9, 21.4, 12.3. HRMS (ESMS): $C_{29}H_{31}FN_6NaO_4$ $(M + Na)^+$, calcd. 569.2283, found 569.2288. Purity: 98.1%, HPLC t_{ret} = 13.64 min and 13.87 min (*R*/*S* mixture).

Preparation of *N*-((*S*)-1-(((*S*,*E*)-4-cyano-1-((*S*)-2-oxopiperidin-3-yl)-4-(thiophen-3-yl)but-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (19). 19 was obtained from **1** (100 mg) and **45** (115 mg) following similar procedure with **4**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a light yellow solid **19** (37 mg, yield 30%). $[\alpha]_D^{20}$ = −9.44 (c = 0.11, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.5 – 7.4 (m, 1H), 7.3 – 7.3 (m, 1H), 7.2 – 7.1 (m, 3H), 6.9 (t, *J* = 8.5 Hz, 2H), 6.5 (d, *J* = 8.3 Hz, 1H), 6.4 (s, 1H), 4.9 (p,

J = 7.7 Hz, 1H), 4.7 (q, *J* = 7.9, 6.5 Hz, 1H), 3.3 (ddt, *J* = 15.7, 11.3, 5.1 Hz, 2H), 3.1 (q, *J* = 10.1, 7.8 Hz, 2H), 2.4 (s, 3H), 2.3 (td, *J* = 21.5, 18.9, 11.0 Hz, 2H), 2.0 – 1.9 (m, 1H), 1.9 (q, *J* = 8.8, 5.4 Hz, 1H), 1.8 – 1.6 (m, 1H), 1.6 – 1.5 (m, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 175.0, 171.4, 170.4, 161.9 (d, *J* = 244.6 Hz), 158.9, 158.3, 144.7, 134.7, 132.3 (d, *J* = 2.8 Hz), 131.2, 131.1, 127.3, 124.2, 124.0, 115.9, 115.5, 115.3, 111.1, 101.4, 54.5, 49.8, 42.5, 38.6, 38.1, 35.9, 27.3, 21.5, 12.4. HRMS (ESMS): $C_{28}H_{28}FN_5NaO_4S$ $(M + Na)^+$, calcd. 572.1738, found 572.1740. Purity: 96.1%, HPLC t_{ret} = 8.03 min.

Preparation of *N*-((*S*)-1-(((*S*,*Z*)-4-cyano-1-((*S*)-2-oxopiperidin-3-yl)-4-(thiophen-3-yl)but-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (20). 20 was obtained from **1** (100 mg) and **45** (115 mg) following similar procedure with **4**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a light yellow solid **20** (50 mg, yield 40%). $[\alpha]_D^{20}$ = 9.62 (c = 0.10, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.5 (d, *J* = 2.9 Hz, 1H), 7.4 – 7.3 (m, 1H), 7.1 (t, *J* = 5.6 Hz, 3H), 6.8 (t, *J* = 8.5 Hz, 2H), 6.3 (s, 1H), 6.1 (d, *J* = 8.8 Hz, 1H), 5.0 (q, *J* = 7.4 Hz, 1H), 4.8 (dp, *J* = 12.5, 5.1, 4.3 Hz, 1H), 3.3 (dd, *J* = 9.9, 4.9 Hz, 2H), 3.1 (qd, *J* = 13.7, 6.9 Hz, 2H), 2.4 (s, 3H), 2.2 (tt, *J* = 17.6, 8.8 Hz, 2H), 2.1 – 2.0 (m, 1H), 1.9 (dq, *J* = 13.4, 5.2, 4.3 Hz, 1H), 1.8 – 1.7 (m, 1H), 1.6 (ddt, *J* = 14.4, 10.5, 5.3 Hz, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.9, 171.2, 170.2, 161.8 (d, *J* = 245.1 Hz), 158.8, 158.3, 146.5, 144.0, 134.5, 132.1 (d, *J* = 3.1 Hz), 131.1, 131.0, 127.4, 124.0, 115.7, 115.3, 115.1, 111.2, 101.3, 54.2, 49.4, 42.3, 38.3, 38.2, 35.7, 27.0, 21.5, 12.3. HRMS (ESMS): $C_{28}H_{28}FN_5NaO_4S$ $(M + Na)^+$, calcd. 572.1738, found 572.1743. Purity: 95.4%, HPLC t_{ret} = 8.11 min.

Preparation of methyl (*S*,*E*)-2-cyano-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoate (21). To a solution of **1** (100 mg, 0.23 mmol, 1.0 equiv) in anhydrous DCM at RT were sequentially added methyl cyanoacetate (27 mg, 0.28 mmol, 1.2 equiv) and piperidine (5.0 μL, 0.046 mmol, 0.2 equiv). The reaction mixture was stirred at RT until complete conversion, followed by washing with H_2O (40 mL×2), saturated citric acid solution (40

mL×2), saturated $NaHCO_3$ solution (40 mL×2) and brine (40 mL×2). The organic phase was dried over sodium sulfate and concentrated, and the crude product was purified via silica gel column chromatography (MeOH:DCM = 1:70 v/v) to afford the pure product as a white solid **21** (65 mg, yield 55%). $[\alpha]_D^{20}$ = −7.50 (c = 0.08, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.1 (t, *J* = 8.2 Hz, 3H), 6.9 (t, *J* = 8.4 Hz, 2H), 6.3 (s, 1H), 4.9 (q, *J* = 7.4 Hz, 1H), 4.7 (s, 1H), 3.9 (s, 3H), 3.3 – 3.2 (m, 2H), 3.1 (t, *J* = 6.8 Hz, 2H), 2.4 (s, 3H), 2.3 – 2.2 (m, 1H), 2.2 – 2.1 (m, 1H), 2.1 (d, *J* = 13.7 Hz, 1H), 1.9 (d, *J* = 12.4 Hz, 1H), 1.8 – 1.6 (m, 1H), 1.6 – 1.4 (m, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.7, 171.3, 170.7, 162.8, 161.9 (d, *J* = 244.4 Hz), 161.3, 158.9, 158.2, 131.9 (d, *J* = 3.1 Hz), 131.0, 130.9, 115.5, 115.3, 112.9, 108.9, 101.3, 54.2, 53.3, 49.4, 42.3, 38.3, 38.1, 34.6, 27.2, 21.4, 12.3. HRMS (ESMS): $C_{26}H_{28}FN_5NaO_6$ $(M + Na)^+$, calcd. 548.1916, found 548.1920. Purity: 95.4%, HPLC t_{ret} = 8.00 min.

Preparation of ethyl (*S*,*E*)-2-cyano-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoate (22). 22 was obtained from **1** (100 mg) and ethyl cyanoacetate (30 mg) with piperidine (5.0 μL) stirred at RT for 2 h following similar procedure with **21**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:70 v/v) to afford the pure product as a white solid **22** (65 mg, yield 55%). $[\alpha]_D^{20}$ = −3.57 (c = 0.056, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 (d, *J* = 8.6 Hz, 1H), 7.2 (dd, *J* = 8.3, 5.3 Hz, 2H), 7.0 (t, *J* = 8.6 Hz, 3H), 6.4 (s, 1H), 5.0 – 4.9 (m, 1H), 4.8 (tdt, *J* = 9.2, 6.1, 3.7 Hz, 1H), 4.3 (ttd, *J* = 17.2, 9.4, 8.6, 5.1 Hz, 2H), 3.4 – 3.2 (m, 2H), 3.1 (t, *J* = 7.8 Hz, 2H), 2.5 (s, 3H), 2.4 (dq, *J* = 11.1, 6.1 Hz, 1H), 2.3 – 2.2 (m, 1H), 2.1 (td, *J* = 8.3, 7.9, 4.0 Hz, 1H), 2.0 – 1.8 (m, 1H), 1.8 – 1.6 (m, 1H), 1.6 – 1.4 (m, 2H), 1.4 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.7, 171.3, 170.7, 162.4, 162.0 (d, *J* = 244.9 Hz), 160.9, 158.9, 158.3, 132.1 (d, *J* = 2.4 Hz), 131.1, 131.0, 115.6, 115.3, 113.0, 109.4, 101.4, 62.8, 54.3, 49.5, 42.3, 38.4, 38.1, 34.7, 27.3, 21.6, 14.1, 12.4. HRMS (ESMS): $C_{27}H_{30}FN_5NaO_6$ $(M + Na)^+$, calcd. 562.2072, found 562.2077. Purity: 95.1%, HPLC t_{ret} = 7.39 min.

Preparation of propyl (*S*,*E*)-2-cyano-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoate

(23). 23 was obtained from **1** (100 mg) and propyl cyanoacetate (35 mg) with piperidine (5.0 μL) stirred at RT for 2 h following similar procedure with **21**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:70 v/v) to afford the pure product as a white solid **23** (56 mg, yield 45%). $[\alpha]_D^{20}$ = −16.67 (c = 0.06, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 (q, *J* = 7.9, 6.3 Hz, 2H), 6.9 (q, *J* = 8.5 Hz, 2H), 6.5 – 6.2 (m, 2H), 4.9 (q, *J* = 7.8, 7.3 Hz, 1H), 4.8 – 4.6 (m, 1H), 4.3 – 4.0 (m, 2H), 3.3 (d, *J* = 12.3 Hz, 2H), 3.1 (pd, *J* = 15.7, 12.8, 7.0 Hz, 2H), 2.4 (s, 3H), 2.2 (dq, *J* = 11.1, 5.7 Hz, 1H), 2.2 – 1.9 (m, 2H), 1.9 (d, *J* = 14.8 Hz, 1H), 1.8 – 1.6 (m, 3H), 1.6 – 1.3 (m, 2H), 1.0 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.9, 171.3, 170.7, 162.4, 162.1 (d, *J* = 244.9 Hz), 161.0, 159.0, 158.4, 132.0 (d, *J* = 3.1 Hz), 131.1, 131.1, 115.7, 115.5, 113.1, 109.5, 101.4, 68.3, 54.3, 50.0, 42.4, 38.8, 38.2, 34.7, 27.8, 22.0, 21.7, 12.4, 10.4. HRMS (ESMS): $C_{28}H_{32}FN_5NaO_6$ $(M + Na)^+$, calcd. 576.2229, found 576.2232. Purity: 95.7%, HPLC t_{ret} = 8.29 min.

Preparation of isopropyl (*S*,*E*)-2-cyano-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoate (24). 24 was obtained from **1** (100 mg) and isopropyl 2-cyanoacetate (35 mg) with piperidine (5.0 μL) stirred at RT for 2 h following similar procedure with **21**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:70 v/v) to afford the pure product as a white solid **24** (51 mg, yield 41%). $[\alpha]_D^{20}$ = −13.34 (c = 0.045, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.4 (t, *J* = 9.2 Hz, 1H), 7.2 (dd, *J* = 8.7, 6.2 Hz, 2H), 7.1 – 6.9 (m, 2H), 6.4 (s, 1H), 5.2 – 5.0 (m, 1H), 4.9 (dt, *J* = 8.8, 6.6 Hz, 1H), 4.7 (ddt, *J* = 12.5, 8.7, 4.3 Hz, 1H), 3.4 – 3.2 (m, 2H), 3.1 (qd, *J* = 13.7, 5.6 Hz, 2H), 2.5 (s, 3H), 2.2 (dq, *J* = 10.9, 5.8 Hz, 1H), 2.1 (dddd, *J* = 23.6, 13.6, 10.3, 5.4 Hz, 1H), 1.9 (q, *J* = 7.8, 5.7 Hz, 2H), 1.7 (tdd, *J* = 18.5, 8.9, 5.3 Hz, 1H), 1.6 – 1.4 (m, 2H), 1.3 (dt, *J* = 9.2, 4.5 Hz, 6H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.9, 171.2, 170.6, 162.0, 162.0 (d, *J* = 245.2 Hz), 160.3, 158.8, 158.3, 131.9 (d, *J* = 3.3 Hz), 131.0, 131.0, 115.6, 115.3, 113.0, 109.9, 101.3, 70.8, 54.2, 50.1, 42.3, 38.8, 38.0, 34.6, 27.8, 21.7, 21.6, 12.3. HRMS (ESMS): $C_{28}H_{32}FN_5NaO_6$ $(M + Na)^+$, calcd. 576.2229, found 576.2234. Purity: 96.2%, HPLC t_{ret} = 8.18 min.

Preparation of tert-butyl (*S*,*E*)-2-cyano-4-((*S*)-3-(4-fluorophenyl)-2-(5-

methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoate (25). 25 was obtained from **1** (100 mg) and tert-butyl cyanoacetate (40 mg) with piperidine (5.0 µL) stirred at RT for 2 h following similar procedure with **21**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:75 v/v) to afford the pure product as a white solid **25** (60 mg, yield 46%). $[\alpha]_D^{20}$ = −11.87 (c = 0.12, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 – 7.0 (m, 3H), 6.9 (t, *J* = 8.5 Hz, 2H), 6.3 (s, 1H), 4.9 (p, *J* = 7.4 Hz, 1H), 4.7 (ddt, *J* = 15.8, 8.4, 3.6 Hz, 1H), 3.3 – 3.2 (m, 2H), 3.1 (d, *J* = 6.9 Hz, 2H), 2.4 (s, 3H), 2.2 (dt, *J* = 17.2, 5.5 Hz, 1H), 2.2 – 2.1 (m, 1H), 2.0 (dd, *J* = 12.3, 6.1 Hz, 1H), 1.9 – 1.8 (m, 1H), 1.7 (dt, *J* = 12.4, 5.6 Hz, 2H), 1.5 (s, 9H), 1.5 – 1.4 (m, 1H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.7, 171.3, 170.7, 161.9 (d, *J* = 245.2 Hz), 161.2, 159.6, 158.9, 158.3, 132.0 (d, *J* = 3.1 Hz), 131.1, 131.0, 115.6, 115.3, 113.3, 110.9, 101.4, 84.0, 54.2, 49.3, 42.3, 38.3, 38.2, 34.7, 27.9, 27.2, 21.5, 12.4. HRMS (ESMS): $C_{29}H_{34}FN_5NaO_6$ $(M + Na)^+$, calcd. 590.2385, found 590.2390. Purity: 95.4%, HPLC t_{ret} = 9.31 min.

Preparation of methyl (*2R/S,4S*)-2-cyano-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pentanoate (26). 26 was obtained from **21** (100 mg) and at RT for 8 h following similar procedure with **18**. The crude product was purified via silica gel column chromatography. (MeOH:DCM = 1:50 v/v) to afford the product as a white solid **26** (57 mg, yield 57%). The *R*/*S* isomers were not separated under the given chromatographic conditions. $[\alpha]_D^{20}$ = −10.67 (c = 0.075, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 (dt, *J* = 8.6, 6.1 Hz, 2H), 6.9 (q, *J* = 9.0 Hz, 2H), 6.3 (d, *J* = 5.1 Hz, 1H), 4.9 (q, *J* = 7.5 Hz, 1H), 4.1 (dd, *J* = 16.7, 6.7 Hz, 1H), 3.8 (d, *J* = 11.5 Hz, 3H), 3.6 – 3.4 (m, 1H), 3.2 (q, *J* = 4.5 Hz, 2H), 3.1 (q, *J* = 6.6 Hz, 2H), 2.4 (s, 3H), 2.2 – 2.0 (m, 3H), 2.0 – 1.8 (m, 2H), 1.8 (dd, *J* = 8.6, 5.4 Hz, 1H), 1.7 – 1.5 (m, 1H), 1.4 (ddq, *J* = 19.9, 9.1, 5.4 Hz, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 175.2, 171.3, 171.0, 166.6, 161.9 (d, *J* = 245.4 Hz), 159.0, 158.3, 132.3 (d, *J* = 3.2 Hz), 131.1, 131.1, 116.6, 115.6, 115.4, 101.4, 54.7, 53.7, 45.4, 42.3, 38.1, 37.6, 36.4, 35.9, 34.8, 26.8, 21.4, 12.3. HRMS (ESMS): $C_{26}H_{30}FN_5NaO_6$ $(M + Na)^+$, calcd. 550.2072, found 550.2076. Purity: >99%, HPLC t_{ret} = 15.41 min and 16.03 min (*R/S* mixture).

Preparation of *N*-((*S*)-1-(((*S,E*)-4-cyano-5-(methylamino)-5-oxo-1-((*S*)-2-oxopiperidin-3-yl)pent-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (27). To a solution of **30** (100 mg, 0.23 mmol, 1.0 equiv) in anhydrous DCM (5 mL) at 0 °C was added excessive TFA (1.0 mL) dropwise. The mixture was stirred at this temperature for 20 min, then allowed to warm to RT and stirred for another 1 h until TLC indicated complete conversion. The solvent was removed, and the residue was dissolved in DCM (5 mL) with adding DIPEA dropwise to adjust the pH to 7.0 at 0 °C, followed by washing with H_2O (40 mL×2), saturated citric acid solution (40 mL×2), saturated $NaHCO_3$ solution (40 mL×2) and brine (40 mL×2). The organic phase was dried over sodium sulfate and concentrated, and the crude product was purified via silica gel column chromatography (MeOH:DCM = 1:50 v/v) to afford the pure product as a white solid **27** (30 mg, yield 35%). $[\alpha]_D^{20}$ = −12.71 (c = 0.072, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.3 (d, *J* = 8.9 Hz, 1H), 7.2 – 7.1 (m, 2H), 7.0 – 6.9 (m, 2H), 6.4 (d, *J* = 2.3 Hz, 1H), 5.0 – 4.8 (m, 1H), 4.8 – 4.7 (m, 1H), 3.3 (q, *J* = 5.7, 4.9 Hz, 2H), 3.1 (d, *J* = 6.8 Hz, 2H), 2.9 (d, *J* = 4.8 Hz, 3H), 2.4 (s, 3H), 2.3 (dq, *J* = 11.7, 6.5, 5.9 Hz, 1H), 2.2 (ddd, *J* = 14.1, 11.6, 5.4 Hz, 1H), 2.0 (s, 1H), 1.9 (ddd, *J* = 14.2, 8.1, 4.2 Hz, 1H), 1.8 – 1.7 (m, 1H), 1.6 – 1.6 (m, 1H), 1.5 (dq, *J* = 14.2, 5.2, 4.2 Hz, 1H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.9, 171.3, 170.7, 162.0 (d, *J* = 245.1 Hz), 159.8, 159.0, 158.4, 158.2, 132.1 (d, *J* = 3.2 Hz), 131.1, 131.0, 115.6, 115.4, 114.6, 110.5, 101.5, 54.5, 49.4, 42.4, 38.4, 37.9, 35.0, 27.4, 27.2, 21.6, 12.4. HRMS (ESMS): $C_{26}H_{29}FN_6NaO_5$ $(M + Na)^+$, calcd. 547.2076, found 547.2078. Purity: >99%, HPLC t_{ret} = 4.82 min.

Preparation of *N*-((*S*)-1-(((*S,E*)-4-cyano-5-(ethylamino)-5-oxo-1-((*S*)-2-oxopiperidin-3-yl)pent-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (28). 28 was prepared as a white solid following similar procedure with **27** (yield 32%). $[\alpha]_D^{20}$ = −19.21 (c = 0.13, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 (d, *J* = 9.0 Hz, 1H), 7.2 (ddd, *J* = 13.7, 9.7, 5.3 Hz, 2H), 7.0 (td, *J* = 8.5, 6.3 Hz, 2H), 6.4 (s, 1H), 4.9 (dq, *J* = 15.1, 7.4 Hz, 1H), 4.8 – 4.6 (m, 1H), 3.5 – 3.1 (m, 6H), 2.5 (s, 3H), 2.2 (s, 2H), 2.0 (s, 1H), 1.9 (dd, *J* = 17.1, 7.5 Hz, 1H), 1.8 – 1.7 (m, 1H), 1.7 – 1.4 (m, 2H),

1.2 (td, *J* = 7.2, 2.1 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.8, 171.2, 170.6, 161.9 (d, *J* = 245.1 Hz), 160.2, 158.8, 158.3, 158.2, 132.0 (d, *J* = 3.0 Hz), 131.0, 130.9, 115.5, 115.3, 114.5, 110.6, 101.4, 54.4, 49.4, 42.4, 42.3, 38.3, 37.9, 35.4, 27.4, 21.5, 14.5, 12.3. HRMS (ESMS): $C_{27}H_{31}FN_6NaO_5$ $(M + Na)^+$, calcd. 561.2232, found 561.2234. Purity: >99.0%, HPLC t_{ret} = 5.16 min.

Preparation of *N*-((*S*)-1-(((*S*,*E*)-4-cyano-5-oxo-1-((*S*)-2-oxopiperidin-3-yl)-5-(propylamino)pent-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (29). **29** was prepared as a white solid following similar procedure with **27** (yield 26%). $[\alpha]_D^{20}$ = −8.38 (c = 0.11, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.4 (d, *J* = 8.0 Hz, 1H), 7.2 – 7.1 (m, 2H), 6.9 (dtd, *J* = 8.6, 6.2, 3.1 Hz, 2H), 6.3 (s, 0H), 5.0 – 4.7 (m, 1H), 3.3 – 3.1 (m, 6H), 2.4 (s, 2H), 2.2 (ddd, *J* = 15.7, 11.3, 5.5 Hz, 1H), 2.0 (ddd, *J* = 14.4, 8.2, 4.3 Hz, 1H), 1.9 – 1.8 (m, 2H), 1.7 – 1.5 (m, 5H), 1.0 – 0.9 (m, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.4, 171.2, 170.2, 161.9 (d, *J* = 244.7 Hz), 159.3, 159.0, 158.3, 158.0, 132.2 (d, *J* = 3.1 Hz), 130.9, 130.9, 115.5, 115.3, 101.4, 54.2, 48.4, 42.4, 42.2, 37.8, 37.4, 34.0, 26.6, 22.5, 21.8, 12.3, 11.3. HRMS (ESMS): $C_{28}H_{33}FN_6NaO_5$ $(M + Na)^+$, calcd. 575.2389, found 575.2394. Purity: 96.9%, HPLC t_{ret} = 5.93 min.

Preparation of tert-butyl ((*S*,*E*)-2-cyano-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoyl)(methyl)carbamate (30). **30** was obtained from **1** (100 mg, 0.23 mmol, 1.0 equiv) and **46** (55 mg) with piperidine (5.0 μL, 0.0046 mmol, 0.2 equiv) stirred at 40 °C for 1 h, following similar procedure with **21**. The crude product was purified via silica gel column chromatography (MeOH:DCM = 1:70 v/v) to afford the pure product as a white solid **30** (65 mg, yield 46%). $[\alpha]_D^{20}$ = −15.01 (c =0.08, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 – 7.1 (m, 2H), 7.0 – 6.9 (m, 2H), 6.8 (d, *J* = 8.5 Hz, 1H), 6.3 (s, 1H), 5.0 – 4.9 (m, 1H), 4.8 (dtd, *J* = 11.9, 9.2, 8.3, 4.0 Hz, 1H), 3.3 – 3.2 (m, 2H), 3.2 – 3.1 (m, 5H), 2.4 (d, *J* = 2.4 Hz, 3H), 2.2 (dd, *J* = 9.2, 4.5 Hz, 1H), 2.2 – 2.1 (m, 1H), 2.1 – 2.0 (m, 1H), 1.8 (dd, *J* = 11.5, 6.1 Hz, 1H), 1.7 – 1.6 (m, 1H), 1.5 (d, *J* = 9.3 Hz, 11H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.6, 171.2, 170.5, 164.8, 161.9 (d, *J* = 245.1 Hz), 158.8, 158.3, 157.3, 152.2, 132.0 (d, *J* = 2.7 Hz),

131.1, 131.0, 115.5, 115.3, 114.7, 113.4, 101.3, 85.2, 54.3, 48.9, 42.2, 38.2, 38.1, 34.8, 32.7, 27.8, 26.9, 21.4, 12.3. HRMS (ESMS): $C_{31}H_{37}FN_6NaO_7$ $(M + Na)^+$, calcd. 647.2600, found 647.2605. Purity: 97.7%, HPLC t_{ret} = 9.71 min.

Preparation of tert-butyl ((*S*,*E*)-2-cyano-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoyl)(ethyl)carbamate (31). **31** was obtained from **1** (100 mg) and **47** (58 mg) with piperidine (5.0 μL) stirred at 40 °C for 2 h, following similar procedure with **21**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:75 v/v) to afford the pure product as a white solid **31** (60 mg, yield 40%). $[\alpha]_D^{20}$ = −21.22 (c = 0.066, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 – 7.1 (m, 2H), 7.0 – 6.9 (m, 2H), 6.8 (d, *J* = 8.5 Hz, 1H), 6.3 (s, 1H), 5.0 – 4.8 (m, 1H), 4.8 (s, 1H), 3.8 – 3.7 (m, 2H), 3.3 (d, *J* = 8.7 Hz, 2H), 3.1 (dd, *J* = 19.0, 6.4 Hz, 2H), 2.4 (d, *J* = 2.5 Hz, 3H), 2.2 (dt, *J* = 12.7, 4.4 Hz, 1H), 2.2 – 2.1 (m, 1H), 2.1 – 2.0 (m, 1H), 1.9 – 1.8 (m, 1H), 1.7 (s, 1H), 1.6 – 1.5 (m, 11H), 1.2 – 1.1 (m, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.7, 171.2, 170.5, 164.5, 162.0 (d, *J* = 244.9 Hz), 158.9, 158.4, 157.2, 152.1, 132.1 (d, *J* = 3.3 Hz), 131.2, 131.1, 115.6, 115.4, 115.0, 113.5, 101.4, 84.9, 54.4, 49.0, 42.3, 41.4, 38.3, 38.2, 34.9, 28.0, 27.1, 21.5, 13.9, 12.4. HRMS (ESMS): $C_{32}H_{39}FN_6NaO_7$ $(M + Na)^+$, calcd. 661.2756, found 661.2760. Purity: 95.0%, HPLC t_{ret} = 5.37 min. Purity: 96.1%, HPLC t_{ret} = 10.25 min.

Preparation of tert-butyl ((*S*,*E*)-2-cyano-4-((*S*)-3-(4-fluorophenyl)-2-(5-methylisoxazole-3-carboxamido)propanamido)-5-((*S*)-2-oxopiperidin-3-yl)pent-2-enoyl)(propyl)carbamate (32). **32** was obtained from **1** (100 mg) and **48** (61 mg) with piperidine (5.0 μL) stirred at 40 °C for 2 h, following similar procedure with **21**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:75 v/v) to afford the pure product as a white solid **32** (69 mg, 47%). $[\alpha]_D^{20}$ = −12.64 (c = 0.095, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 – 7.1 (m, 2H), 7.0 – 6.9 (m, 2H), 6.8 (d, *J* = 8.5 Hz, 1H), 6.3 (s, 1H), 4.9 (tt, *J* = 15.1, 6.9 Hz, 1H), 4.8 – 4.6 (m, 1H), 3.7 – 3.6 (m, 2H), 3.3 – 3.2 (m, 2H), 3.2 – 3.0 (m, 2H), 2.4 (s, 3H), 2.3 – 2.2 (m, 1H), 2.2 – 2.1 (m, 1H), 2.1 (s, 1H), 1.9 (d, *J* = 14.7 Hz, 1H), 1.6 – 1.4 (m, 14H), 0.9 – 0.9 (m, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 174.8, 171.2,

170.5, 164.6, 162.0 (d, J = 244.6 Hz), 158.9, 158.4, 157.2, 152.4, 132.1 (d, J = 3.4 Hz), 131.2, 131.1, 115.6, 115.4, 115.0, 113.5, 101.4, 84.9, 54.4, 49.0, 47.8, 42.3, 38.3, 38.2, 34.9, 27.9, 27.1, 22.0, 21.5, 12.4, 11.3. HRMS (ESMS): $C_{33}H_{41}FN_6NaO_7$ $(M + Na)^+$, calcd. 675.2913, found 675.2915. Purity: >99%, HPLC t_{ret} = 10.92 min.

Preparation of *N*-((*S*)-1-(((*S*,*E*)-4-cyano-5-(dimethylamino)-5-oxo-1-((*S*)-2-oxopiperidin-3-yl)pent-3-en-2-yl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)-5-methylisoxazole-3-carboxamide (33). **33** was obtained from **1** (100 mg) and *N*,*N*-dimethylcyanoacetamide (30 mg) with piperidine (5.0 μL) stirred at 40 °C for 2 h, following similar procedure with **21**. The crude product was purified by silica gel column chromatography (MeOH:DCM = 1:65 v/v) to afford the pure product as a white solid **33** (50 mg, yield 42%). $[\alpha]_D^{20}$ = −37.81 (c = 0.058, DCM). ^{1}H NMR ($CDCl_3$-*d*) δ 7.2 – 7.1 (m, 2H), 6.9 (t, J = 8.5 Hz, 2H), 6.5 (d, J = 3.2 Hz, 1H), 6.4 (s, 1H), 4.9 (p, J = 6.9 Hz, 1H), 4.6 (ddt, J = 12.9, 8.9, 4.0 Hz, 1H), 3.3 – 3.2 (m, 2H), 3.1 (d, J = 6.3 Hz, 2H), 3.1 (s, 3H), 3.0 (d, J = 7.4 Hz, 3H), 2.4 (s, 3H), 2.2 (dt, J = 12.9, 6.5 Hz, 1H), 2.2 – 2.1 (m, 1H), 2.0 (d, J = 12.5 Hz, 1H), 1.9 – 1.8 (m, 1H), 1.7 – 1.6 (m, 1H), 1.5 (t, J = 12.2 Hz, 2H). ^{13}C NMR ($CDCl_3$-*d*) δ 175.1, 171.3, 170.6, 162.5, 162.0 (d, J = 245.2 Hz), 158.9, 158.3, 156.0, 132.0 (d, J = 3.1 Hz), 131.1, 131.0, 115.5, 115.3, 113.6, 112.4, 101.4, 54.1, 50.2, 42.4, 38.9, 38.7, 37.9, 36.0, 34.7, 27.8, 21.5, 12.4. HRMS (ESMS): $C_{27}H_{31}FN_6NaO_5$ $(M + Na)^+$, calcd. 561.2232, found 561.2235. Purity: >99.0%, HPLC t_{ret} = 5.56 min.

Preparation of diethyl (1-cyanoethyl)phosphonate (34). To a solution of diethyl cyanomethylphosphonate (500 mg, 2.82 mmol) in dry DMF (50 mL) at RT was added K_2CO_3 (1.5 mg, 11.3 mmol) and stirred at RT for 2 h. Then iodomethane in DMF (5 mL) was added dropwise via cannula, then the reaction mixture was stirred for another 48 h after complete conversion via TLC. Subsequently, DMF was removed, and extractive workup with DCM (40 mL×3). The combined organic layer was dried over anhydrous sodium sulfate and concentrated, and the residue was purified by silica gel column chromatography (EA:PE = 1:2 v/v) to afford the pure product **34** as a light yellow oil (335 mg, yield 62%). ^{1}H NMR ($CDCl_3$-*d*) δ 4.2 (h, J = 7.4 Hz, 4H), 2.9 (dq, J = 23.0, 7.0 Hz,

1H), 1.5 (dd, *J* = 16.9, 7.6 Hz, 3H), 1.3 (t, *J* = 6.3 Hz, 6H). ^{13}C NMR ($CDCl_3$-*d*) δ 117.1 (d, *J* = 9.2 Hz), 64.0 (d, *J* = 6.9 Hz), 63.7 (d, *J* = 6.8 Hz), 23.7 (d, *J* = 145.6 Hz), 16.4, 16.3, 12.6 (d, *J* = 5.9 Hz). ESI-MS (m/z): 192.2 $(M + H)^+$.

Preparation of diethyl (chloro(cyano)methyl)phosphonate (35). Lithium bis(trimethylsilyl)amide (6.0 mL of 1.0 M solution in THF, 6.0 mmol, 2.2 equiv) was added dropwise over 10 min to a stirred, cooled solution of diethyl cyanomethylphosphonate (0.5 g, 2.8 mmol, 1.0 equiv) in anhydrous THF (30 mL) under a nitrogen atmosphere at -78 °C. After stirring for 30 min, a solution of *N*-chlorosuccinimide (0.5 mg, 3.4 mmol, 1.2 equiv) in THF (10 mL) was added dropwise. After 30 min, the mixture was allowed to warm to RT and stirred for another 2 h before the reaction was quenched by 1.0 M HCl solution (5 mL). Subsequently, solvent was removed and extractive workup was done with DCM (3×). The combined organic layer was dried over anhydrous sodium sulfate and concentrated, and the residue was purified via silica gel column chromatography (EA:PE = 1:3 v/v) to afford the pure product **35** as a light yellow oil (0.3 g, yield 50%). ^{1}H NMR ($CDCl_3$-*d*) δ 4.7 (d, *J* = 17.2 Hz, 1H), 4.3 – 4.2 (m, 4H), 1.3 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR ($CDCl_3$-*d*) δ 112.9 (d, *J* = 5.6 Hz), 65.8 (d, *J* = 6.9 Hz), 65.6 (d, *J* = 6.9 Hz), 34.6 (d, *J* = 157.1 Hz), 16.1, 16.1. ESI-MS (m/z): 212.0 $(M + H)^+$.

Preparation of diethyl (bromo(cyano)methyl)phosphonate (36). 36 was obtained from diethyl cyanomethylphosphonate and *N*-bromosuccinimide following similar procedure to **35**. Flash chromatography with gradient elution (PE:EA = 3:1) afforded pure product as a yellow oil (yield, yield 30%). ^{1}H NMR ($CDCl_3$-*d*) δ 4.4 (d, *J* = 16.4 Hz, 1H), 4.3 (dq, *J* = 15.3, 7.7 Hz, 4H), 1.4 – 1.3 (m, 6H). ^{13}C NMR ($CDCl_3$-*d*) δ 113.1 (d, *J* = 6.0 Hz), 66.0 (d, *J* = 6.9 Hz), 65.7 (d, *J* = 6.9 Hz), 17.0 (d, *J* = 163.4 Hz), 16.3, 16.2. ESI-MS (m/z): 256.9 $(M + H)^+$.

Preparation of methyl 2-chloro-2-(diethoxyphosphoryl)acetate (37). 37 was obtained from methyl diethylphosphonoacetate and *N*-chlorosuccinimide following similar procedure to **35**. Flash chromatography with gradient elution (PE:EA = 4:1) afforded pure

product as a light yellow oil (yield, 47%). ^{1}H NMR ($CDCl_3$-*d*) δ 4.5 (d, *J* = 16.3 Hz, 1H), 4.3 – 4.2 (m, 4H), 3.8 (s, 3H), 1.3 (tt, *J* = 7.1, 1.0 Hz, 6H). ^{13}C NMR ($CDCl_3$-*d*) δ 165.5, 64.8 (d, *J* = 4.3 Hz), 64.7 (d, *J* = 4.4 Hz), 53.9, 50.1 (d, *J* = 145.7 Hz), 16.4, 16.4. ESI-MS (m/z): 245.0 $(M + H)^+$.

Preparation of methyl 2-bromo-2-(diethoxyphosphoryl)acetate (38). 38 was obtained from methyl diethylphosphonoacetate and *N*-bromosuccinimide following similar procedure to **35**. Flash chromatography with gradient elution (PE:EA = 4:1) afforded pure product as a yellow oil (yield 38%). ^{1}H NMR ($CDCl_3$-*d*) δ 4.3 (d, *J* = 14.2 Hz, 1H), 4.3 – 4.2 (m, 4H), 3.8 (s, 3H), 1.3 (td, *J* = 7.1, 0.7 Hz, 6H). ^{13}C NMR ($CDCl_3$-*d*) δ 165.6, 64.8 (d, *J* = 6.3 Hz), 64.7 (d, *J* = 6.3 Hz), 53.9, 35.5 (d, *J* = 146.2 Hz), 16.4, 16.4. ESI-MS (m/z): 289.9 $(M + H)^+$.

Preparation of ethyl 2-chloro-2-(diethoxyphosphoryl)acetate (39). 39 was obtained from methyl triethyl phosphonoacetate and *N*-chlorosuccinimide following similar procedure to **35**. Flash chromatography with gradient elution (PE:EA = 5:1) afforded pure product as a light yellow oil (yield 52%). ^{1}H NMR ($CDCl_3$-*d*) δ 4.5 (d, *J* = 16.2 Hz, 1H), 4.4 – 4.2 (m, 6H), 1.4 (tt, *J* = 7.1, 0.8 Hz, 6H), 1.3 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 164.8, 64.5, 64.5, 63.0, 50.2 (d, *J* = 145.4 Hz), 16.3, 16.2, 13.9. ESI-MS (m/z): 260.0 $(M + H)^+$.

Preparation of ethyl 2-bromo-2-(diethoxyphosphoryl)acetate (40). 40 was obtained from methyl triethyl phosphonoacetate and *N*-bromosuccinimide following similar procedure to **35**. Flash chromatography with gradient elution (PE:EA = 5:1) afforded pure product as a yellow oil (yield 48%). ^{1}H NMR ($CDCl_3$-*d*) δ 4.4 (d, *J* = 14.0 Hz, 1H), 4.3 – 4.2 (m, 6H), 1.4 (td, *J* = 7.1, 0.8 Hz, 6H), 1.3 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 165.1, 64.7, 64.6, 63.1, 35.9 (d, *J* = 145.7 Hz), 16.4, 16.4, 14.0. ESI-MS (m/z): 202.9 $(M + H)^+$.

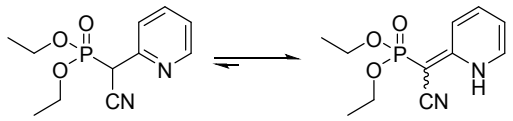
Preparation of diethyl (cyano(phenyl)methyl)phosphonate (41). Lithium bis(trimethylsilyl)amide (9.5 mL of 1.0 M solution in THF, 9.5 mmol, 2.2 equiv) was added

dropwise over 10 min to a stirred, cooled solution of benzeneacetonitrile (0.5 mg, 4.3 mmol, 1.0 equiv) in anhydrous THF (30 mL) under a nitrogen atmosphere at -78 °C. After 30 min, a solution of diethyl chlorophosphate (0.9 g, 5.2 mmol, 1.2 equiv) in anhydrous THF (10 mL) was added at -78 °C. After 30 min at this temperature, the reaction mixture was allowed to warm to 0 °C before the reaction was quenched by 1.0 M HCl (5 mL). Subsequently, THF was removed, and extractive workup was done with DCM (3×). The combined organic layer was dried over anhydrous sodium sulfate and concentrated, and the residue was purified via silica gel column chromatography (PE:EA = 5:1 v/v) to afford the pure product **41** as a light yellow oil (0.6 g, yield 55%). ^{1}H NMR ($CDCl_3$-*d*) δ 7.5 – 7.3 (m, 5H), 4.3 (d, *J* = 26.4 Hz, 1H), 4.1 (dtq, *J* = 23.9, 16.1, 7.7, 6.9 Hz, 4H), 1.3 (dt, *J* = 20.3, 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$-*d*) δ 129.1, 129.1, 128.9 (d, *J* = 3.0 Hz), 128.8, 128.7, 127.8 (d, *J* = 8.3 Hz), 115.5 (d, *J* = 9.9 Hz), 64.8 (d, *J* = 7.2 Hz), 64.5 (d, *J* = 7.1 Hz), 36.9 (d, *J* = 138.2 Hz), 16.3, 16.3. ESI-MS (m/z): 254.1 $(M + H)^+$.

Preparation of diethyl (cyano(naphthalen-2-yl)methyl)phosphonate (42). 42 was obtained from 1-naphthyl acetonitrile (0.6 g) and diethyl chlorophosphate (0.5 g) following similar procedure to **41**. Flash chromatography with gradient elution (PE:EA = 10:1) afforded pure product as a yellow oil (yield 46%). ^{1}H NMR ($CDCl_3$-*d*) δ 8.0 – 7.8 (m, 4H), 7.5 (ddd, *J* = 11.8, 6.6, 2.6 Hz, 3H), 4.4 (d, *J* = 26.5 Hz, 1H), 4.2 – 3.9 (m, 4H), 1.3 (t, *J* = 7.1 Hz, 3H), 1.2 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 133.2 (dd, *J* = 35.6, 2.8 Hz), 133.1 (d, *J* = 2.3 Hz), 129.0 (d, *J* = 2.3 Hz), 128.3, 128.2, 128.1, 127.8, 127.0 (d, *J* = 6.5 Hz), 125.9 (d, *J* = 3.7 Hz), 125.2 (d, *J* = 8.6 Hz), 115.6 (d, *J* = 9.9 Hz), 64.9 (d, *J* = 7.2 Hz), 64.5 (d, *J* = 7.1 Hz), 37.1 (d, *J* = 138.1 Hz), 16.4 (d, *J* = 1.7 Hz), 16.3 (d, *J* = 1.7 Hz). ESI-MS (m/z): 304.2 $(M + H)^+$.

Preparation of diethyl (cyano(4-(trifluoromethyl)phenyl)methyl)phosphonate (43). 43 was obtained from (4-trifluoromethyl)phenylacetonitrile and diethyl chlorophosphate following similar procedure to **41**. Flash chromatography with gradient elution (PE:EA = 8:1) afforded pure product as a brown oil (yield 52%). ^{1}H NMR ($CDCl_3$-*d*) δ 7.7 (d, *J* = 8.2 Hz, 2H), 7.6 (d, *J* = 6.2 Hz, 2H), 4.3 (d, *J* = 26.5 Hz, 1H), 4.3 – 3.9 (m, 4H), 1.3 (t, *J* = 7.1

Hz, 3H), 1.3 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 132.1 (d, *J* = 8.3 Hz), 131.2 (qd, *J* = 33.1, 3.4 Hz), 129.3, 129.2, 127.6 (d, *J* = 191.0 Hz), 126.1 (p, *J* = 3.4 Hz), 114.9 (d, *J* = 10.4 Hz), 65.2 (d, *J* = 7.2 Hz), 64.8 (d, *J* = 7.1 Hz), 37.5, 36.1, 16.4 (d, *J* = 2.3 Hz), 16.3 (d, *J* = 2.2 Hz). ESI-MS (m/z): 322.2 $(M + H)^+$.



Preparation of diethyl (cyano(pyridin-2(1H)-ylidene)methyl)phosphonate (44). 44 was obtained from 2-pyridylacetonitrile and diethyl chlorophosphate following similar procedure to **41**. Flash chromatography with gradient elution (PE:EA = 3:1) afforded pure product as a yellow oil (yield 67%). **44** was found to exist as its tautomer (**44'**) due to proton transfer. ^{1}H NMR ($CDCl_3$-*d*) δ 7.6 (t, *J* = 6.2 Hz, 1H), 7.5 (t, *J* = 8.0 Hz, 1H), 7.3 (d, *J* = 9.0 Hz, 1H), 6.5 (t, *J* = 6.6 Hz, 1H), 4.1 (p, *J* = 7.2 Hz, 4H), 1.4 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$-*d*) δ 158.0 (d, *J* = 8.6 Hz), 139.1, 134.5, 120.4 (d, *J* = 13.6 Hz), 120.0 (d, *J* = 8.6 Hz), 110.5, 62.4, 62.4, 46.0 (d, *J* = 212.3 Hz), 16.0, 16.0. ESI-MS (m/z): 255.1 $(M + H)^+$.

Preparation of diethyl (cyano(thiophen-3-yl)methyl)phosphonate (45). 45 was obtained from 3-cyanomethylthiophene and diethyl chlorophosphate following similar procedure to **41**. Flash chromatography with gradient elution (PE:EA = 5:1) afforded pure product as a yellow oil (yield 48%). ^{1}H NMR ($CDCl_3$-*d*) δ 7.4 (s, 1H), 7.4 – 7.3 (m, 1H), 7.2 (d, *J* = 5.0 Hz, 1H), 4.4 (d, *J* = 26.1 Hz, 1H), 4.3 – 3.9 (m, 4H), 1.3 (t, *J* = 7.1 Hz, 3H), 1.3 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 127.4 (d, *J* = 3.0 Hz), 127.0 (d, *J* = 1.5 Hz), 126.9, 124.7 (d, *J* = 7.6 Hz), 115.2 (d, *J* = 9.8 Hz), 64.7 (d, *J* = 7.1 Hz), 64.3 (d, *J* = 7.1 Hz), 32.2 (d, *J* = 140.8 Hz), 16.2 (d, *J* = 5.8 Hz), 16.2 (d, *J* = 5.6 Hz). ESI-MS (m/z): 260.1 $(M + H)^+$.

Preparation of 2-cyano-*N*-methyl-*N*-(pivaloyloxy)acetamide (46). *N*-methyl-2-cyanoacetamide (0.5 g, 5.0 mmol) was dissolved in anhydrous THF at -78 °C, and then

LiHMDS (1M in THF, 10.0 mmol) was added dropwise under nitrogen atmosphere. After 30 min, di-*tert*-butyl dicarbonate (1.7 g, 7.6 mmol) was added. Then, the reaction mixture was stirred at -78 °C for additional 2 h and quenched with HCl (1.0 M). The reaction mixture was warmed to RT, and the solvent was evaporated. The obtained residue was dissolved in DCM followed by washing with H_2O (50 mL × 2), saturated citric acid solution (50 mL × 2), saturated $NaHCO_3$ solution (50 mL × 2) and brine (50 mL × 2). The organic phase was dried over Na_2SO_4 and concentrated, and the residue was purified by column chromatography (PE:EA = 6:1 v/v) to afford the pure product as a white solid **46** (0.4 g, 40%). ^{1}H NMR ($CDCl_3$-*d*) δ 4.0 (s, 2H), 3.1 (s, 3H), 1.5 (s, 9H). ^{13}C NMR ($CDCl_3$-*d*) δ 164.9, 152.5, 114.0, 84.6, 31.7, 29.7, 27.8. ESI-MS (m/z): 199.1 $(M + H)^+$.

Preparation of 2-cyano-*N*-ethyl-*N*-(pivaloyloxy)acetamide (47). 47 was obtained from *N*-ethylcyanoacetamide and di-*tert*-butyl dicarbonate following similar procedure to **46**. Flash chromatography with gradient elution (PE:EA = 6:1 v/v) afforded pure product as a white solid. ^{1}H NMR ($CDCl_3$-*d*) δ 4.1 (s, 2H), 3.8 (q, *J* = 6.9 Hz, 2H), 1.5 (s, 9H), 1.2 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 164.6, 152.5, 114.1, 84.6, 40.3, 30.0, 27.9, 13.6. ESI-MS (m/z): 213.1 $(M + H)^+$.

Preparation of 2-cyano-*N*-(pivaloyloxy)-*N*-propylacetamide (48). 48 was obtained from 2-cyano-N-propylacetamide and di-*tert*-butyl dicarbonate following similar procedure to **46**. Flash chromatography with gradient elution (DCM:MeOH = 35:1 v/v) afforded pure product as a white solid. ^{1}H NMR ($CDCl_3$-*d*) δ 4.1 (s, 2H), 3.7 – 3.6 (m, 2H), 1.5 (s, 11H), 0.9 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR ($CDCl_3$-*d*) δ 164.7, 152.6, 114.1, 84.5, 46.6, 30.0, 27.8, 21.7, 11.2. ESI-MS (m/z): 227.1 $(M + H)^+$.

Measurement of overall inhibition constant (K_i*). Recombinant EV71 $3C^{pro}$ used in this study was expressed and purified as described previously.[9b] The FRET-based dodecapeptide NMA-IEALFQGPPK(DNP)FR was synthesized via a solid-phase method and used as substrate which turned fluorescent upon cleavage of the Gln-Gly bond by $3C^{pro}$. All assays were performed under initial velocity conditions in which substrate

depletion never surpassed 10%.

For reversible inhibitors, values of K_i^* were used as a measure of potency. In a 96-well microplate (Corning® black polystyrene), $3C^{pro}$ (10 μL) and varying concentrations of inhibitors or blank control (5 μL in DMSO) were mixed and incubated in buffer (80 μL) at 30 °C for 30 min. Enzymatic reaction was then initiated by adding substrate (5 μL in DMSO). The final concentrations of reaction components were as follows: 100 μM substrate, 500 nM enzyme, 150 mM NaCl, 50 mM Tris, 1 mM EDTA-2Na, 10% glycerol (v/v), 10% DMSO and a pH of 7. Relative fluorescence units (RFU) were read by a microplate reader (Thermo Varioskan Flash) at λ_{ex} of 340 nm and λ_{em} of 440 nm over 30 min. Each concentration was tested in triplicate. The fractional steady-state velocity (v_s/v_0) was globally fitted to quadratic Equation 1 conforming to a tight-binding mechanism.[24]

$$\frac{v_s}{v_0} = 1 - \frac{[E] + [I] + K_i^*(1 + \frac{[S]}{K_M}) - \sqrt{([E] + [I] + K_i^*(1 + \frac{[S]}{K_M}))^2 - 4[E][I]}}{2[E]} \quad \text{(Equation 1)}$$

In Equation 1, v_s is the steady-state reaction rate at given concentration of inhibitor, v_0 is the rate in blank control, [E] is the total concentration of active enzyme, [I] is the concentration of inhibitor, [S] is the concentration of substrate, K_M is the Michaelis-Menten constant and K_i^* denotes the overall inhibition constant.

Progress curve analysis. Progress curve analysis was used to evaluate the potency of irreversible inhibitors **2-5**. Compared with the K_i^* measurement assays, the volume and concentration of each component was identical, but the order of reagent addition was different. Substrate and inhibitors were at first added to the microplate and mixed well. Then enzyme (10 μL) was added to initiate reaction, and fluorescence signal was immediately measured until 10% of substrate depletion (typically for 1-1.5 h). Each concentration was tested in triplicate. The pseudo first-order rate constant k_{obs} was calculated using equation 2.

$$[P] = \frac{v_i}{k_{obs}}(1 - e^{-k_{obs}t}) \quad \text{(Equation 2)}$$

In equation 2, [P] is the product concentration, v_i is the initial velocity of the reaction

in the presence of inhibitor. v_i and k_{obs} are both parameters to be determined.[25]

Afterwards, values of k_{obs} were replotted versus [I]. Because our inhibitors were identified as active-site directed covalent inhibitors, a two-step mechanism was adopted to interpret the k_{obs}-[I] plot. For irreversible inhibitors **2**-**5**, curves of k_{obs} vs [I] which were apparently hyperbolic were fitted to Equation 3, where the second order rate constant k_{inact}/K_i was used as a measure of inhibitory activity.[19]

$$k_{obs} = \frac{k_{inact}[I]}{K_i\left(1 + \frac{[S]}{K_M}\right) + [I]} \quad \text{(Equation 3)}$$

Protein Mass Spectrometry. HPLC-MS was used to detect covalent adducts between the inhibitors and EV71 $3C^{pro}$. The analysis of samples was performed on an LC-MS system that consisted of an Ultimate 3000 HPLC and a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). HPLC was performed at a constant flow rate using a binary solvent system. Mobile phase A was 0.1% formic acid in ultrapure water, and mobile phase B was 0.1% formic acid in LC-MS-grade acetonitrile. The Q-Exactive Orbitrap settings were as follows: sheath gas at 35, auxiliary gas at 10, sweep gas at 0, spray voltage at 3.5 kV, automatic gain control (AGC) at 3 x 10^6 , and max injection time at 200 ms. Full MS scans were carried out within a mass range of 500–1500 m/z, and the resolution was 70,000. The protein data was analyzed using Protein Deconvolution 3.0 software (Thermo Fisher Scientific). For modified proteins, protein samples (5 μL, 40 μM) were injected into HPLC-HRMS system. The reversed-phase analytical column was a C4 precolumn (Beijing Yuanbaoshan Chrom-Tech Co. Ltd), 4.6×10 mm dimension and 5 μm particle size.

Purified EV71 $3C^{pro}$ (40 μM) was incubated with various inhibitors (10-20 equiv, depending on the K_i*) for 1 h at 30 °C in buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA-2Na, pH 7.0). The mixtures were then transferred to ultrafiltration tubes (Amicon Ultra-0.5, 10 kDa MWCO) and centrifuged 3 times using ultrapure water to remove free inhibitors and salts. The samples were analyzed by a HPLC-ESI-Orbitrap mass spectrometer. Mass/charge envelope data was deconvolved using MassLynx software to

provide the molecular mass.

Reversibility Assay. Method 1: EV71 $3C^{pro}$ (0.5 μM in 150 mM NaCl, 50 mM Tris, 1.0 mM EDTA-2Na, 10% glycerol (v/v), pH 7.0) was treated with 10 to 20 equivalents of inhibitors (based on the K_i*) for 1 h at 30 °C. Reaction mixtures were then transferred to ultrafiltration tubes (Amicon Ultra-0.5, 10 kDa MWCO). The samples were centrifuged at 12,000 × g for 30 min, which was repeated 3 and 6 times corresponding to approximately 10^3- and 10^6-fold dilutions of the inhibitors. Aliquots (95 μL) were removed and the enzyme assays were initiated by adding 5.0 μL of 2.0 mM peptide substrate. The initial velocities for treated samples (v_i) and controls (v_0) were recorded, and the recovery of enzyme activity was calculated as $v_i/v_0 \times 100\%$.

Method 2: EV71 $3C^{pro}$ (5.0 μM in 150 mM NaCl, 50 mM Tris, and 1.0 mM EDTA-2Na, 10% glycerol (v/v), pH 7.0) was treated with 10 to 20 equivalents of inhibitors (based on the K_i*) for 60 min at 30 °C. Reaction mixtures were then transferred to dialysis cassettes (0.1-0.5 mL Slide-A-Lyzer, 10 kDa MWCO) and dialyzed against 500 mL of buffer (150 mM NaCl, 50 mM Tris, 1.0 mM EDTA-2Na, 10% glycerol (v/v), pH 7.0) at 4 °C. Buffers were exchanged after 6 h of dialysis, and then daily until the end of the experiment. At each time point, 50 μL of aliquots were removed and the enzyme assays were initiated by adding 50.0 μL of 200.0 μM peptide substrate. The initial velocities of treated samples (v_i) and controls (v_0) were recorded, and the recovery of enzyme activity was calculated as $v_i/v_0 \times 100\%$.[20]

Cell-based EV71 inhibition assay. Human RD cells and human embryonic kidney 293T (HEK-293T) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) at 37 °C in a 5% CO_2 humidified incubator. The single round pseudotype EV71 reporter virus (EV71(FY)-Luc pseudotype virus) system containing plasmids of pcDNA6-FY-capsid and pEV71-Luc-replicon were kindly supplied by Prof. Wenhui Li from the National Institute of Biological Sciences, Beijing.

EV71(FY)-Luc was produced as previously described. Briefly, the plasmid of a pEV71-Luc subgenomic replicon was linearized by digestion with SalI restriction enzyme and was used as a template for RNA transcription. The EV71 replicon RNA transcripts were prepared *in vitro* by using Ambion MEGAscript Kits. The pcDNA6-FY-capsid plasmid was transfected into HEK-293T cells at 60-80% confluence. At 24 h post transfection, EV71 subgenomic replicon RNA was then transfected using Lipofectamine 2000 (Invitrogen). EV71 pseudotype virus was harvested at 24 h post-RNA transfection with two freeze-thaw cycles. To quantify the EV71 pseudotype virus, the stocks were serially diluted 10-fold and incubated with RD cells for 24 h at 37 °C. The cells were then harvested and luminescence was detected using a Bright-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol.[26]

Evaluation of cytotoxicity. The cytotoxicity was measured by WST-1 cell proliferation using a cytotoxicity assay kit according to the manufacturer's protocol (catalog no. 05015944001, Roche, USA). Briefly, RD cells (3×104 per well in 100 μL of 10% FBS-DMEM medium) were cultured at 37 °C under 5% CO_2 in 96-well plates, followed by the addition of 50 μL of inhibitor at concentration from 0.78-200 μM. The cells were incubated at 37 °C for 24 h, and then 10 μL of WST-1 reagent was added to each well. The luminescent signals were read at 490 nm with a microplate reader (Bio-Rad, USA). The percentage of viable cells after treatment with different concentrations of compounds was calculated, and CC_{50} values were reported.[26]

Selectivity study. Inhibition of cathepsin K and human calpain by 50 μM of $3C^{pro}$ inhibitors was evaluated using commercial inhibitor screening kits (K150 and K244, BioVision). The selectivity of compounds for chymotrypsin (from bovine pancreas, Sigma) was evaluated as follows. Briefly, 10 units/mL (10 μL) of chymotrypsin was incubated with 5.0 μL of test compound at a final concentration of 50 μM, or with DMSO as a control in 95 μL of buffer (50 mM Tris, 10 mM $CaCl_2$, pH 7.6) at 30 °C for 30 min. The reaction was initiated by adding 5.0 μL of 8.0 mM solution of *N*-succinyl-L-phenylalanine-*p*-nitroanilide (Sigma). The absorbance change was monitored continuously for 30 min at 410 nm by

microplate reader (Thermo Varioskan Flash, USA). The initial velocities of inhibited enzyme (v_i) and uninhibited enzyme (v_0) were recorded, and the inhibition was calculated as $(1-v_i/v_0)\times100\%$.

Molecular docking. Molecular docking was performed using Schrödinger Suite software package. The receptor (PDB accession code: 3SJO) was prepared by Protein Preparation Wizard and the ligands were built and optimized by LigPrep module prior to docking. A covalent docking protocol was used in this simulation. Rupintrivir in the crystal structure was set as a reference ligand and placed in the center of a cubic enclosing box of 20 $Å^3$. Cys_{147} was selected as the reactive residue. The SMARTS pattern of ligands for reaction was defined as [C]=[C]-[C]=[O] or [C]=[C]-[C]#[N] depending on ligand structure. The affinity score was calculated using Glide. The best 5 poses were output and manually examined for most reliable prediction.

ASSOCIATED CONTENT

Supporting Information. Detailed determination of configuration, kinetic results of **3**, **4** and **21**, ESI-MS result of $3C^{pro}$-**21**, inhibition assay in the presence of nonenzymatic nucleophiles, NMR spectra and HPLC traces (PDF).

Molecular formula strings (CSV).

Coordinate files of $3C^{pro}$ (PDB ID: 3SJO) docked with **25**, **30** and **33** (PDB).

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

EV71, enterovirus 71; HFMD, hand foot and mouth disease; $3C^{pro}$, 3C protease; HRV, human rhinovirus; RD, rhabdomyosarcoma; HWE, Horner-Wadsworth-Emmons; NaHMDS, sodium bis(trimethylsilyl)amide; LiHMDS, lithium bis(trimethylsilyl)amide; Naph, naphthyl; Thio, thiophenyl; Py, pyridyl; DIPEA, *N*,*N*-diisopropylethylamine.

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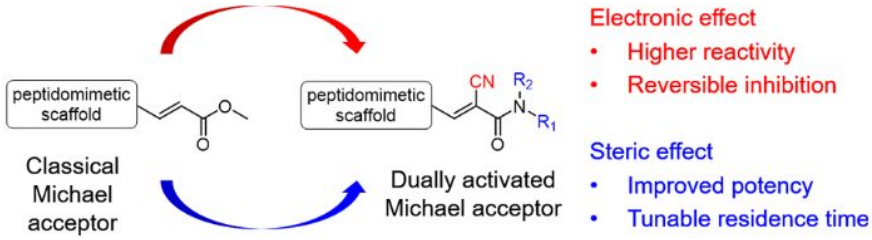
TOC



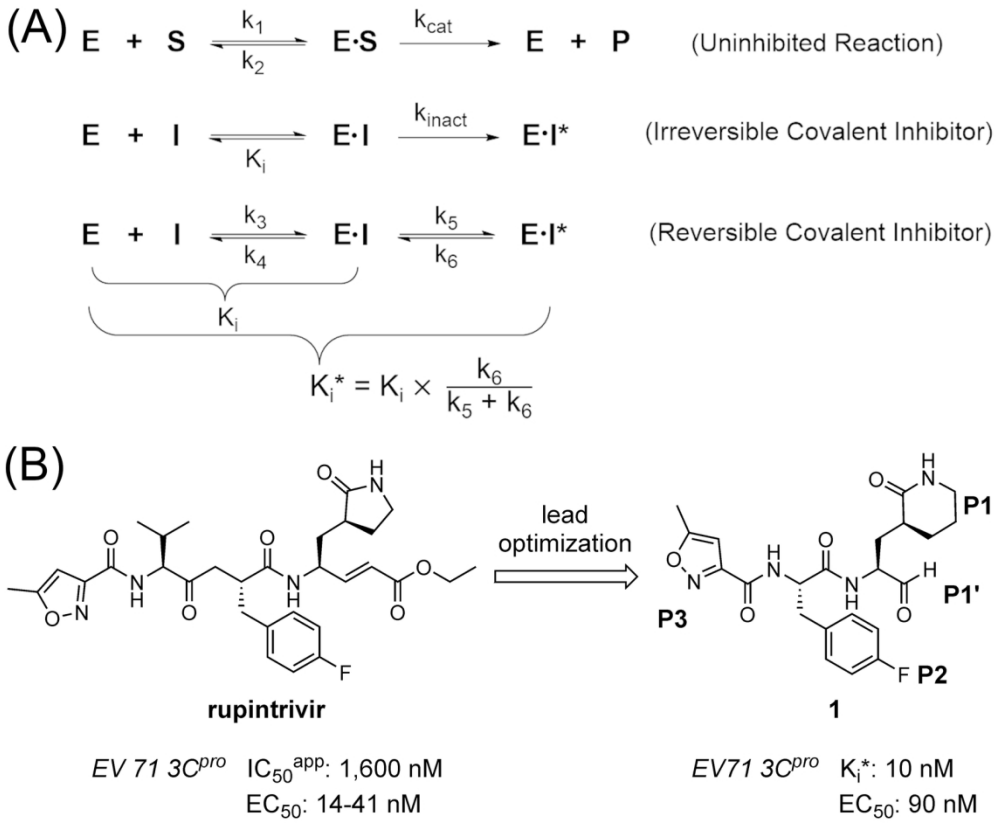


Figure 1. (A) Generic two-step mechanism of irreversible/reversible covalent inhibition. k_{inact}/K_i is usually used for evaluation of irreversible inhibitors, while K_i^* measures the potency of reversible covalent inhibitor that conforms to a two-step mechanism. The relationship between K_i, K_i^*, k_5 and k_6 are described. (B) Structures and bioactivity data of rupintrivir and compound **1**. Apparent IC_{50} or K_i^* values are indicative of their inhibitory effects on EV71 $3C^{pro}$. EC_{50} values represent their antiviral activity in EV71-infected human rhabdomyosarcoma (RD) cells.

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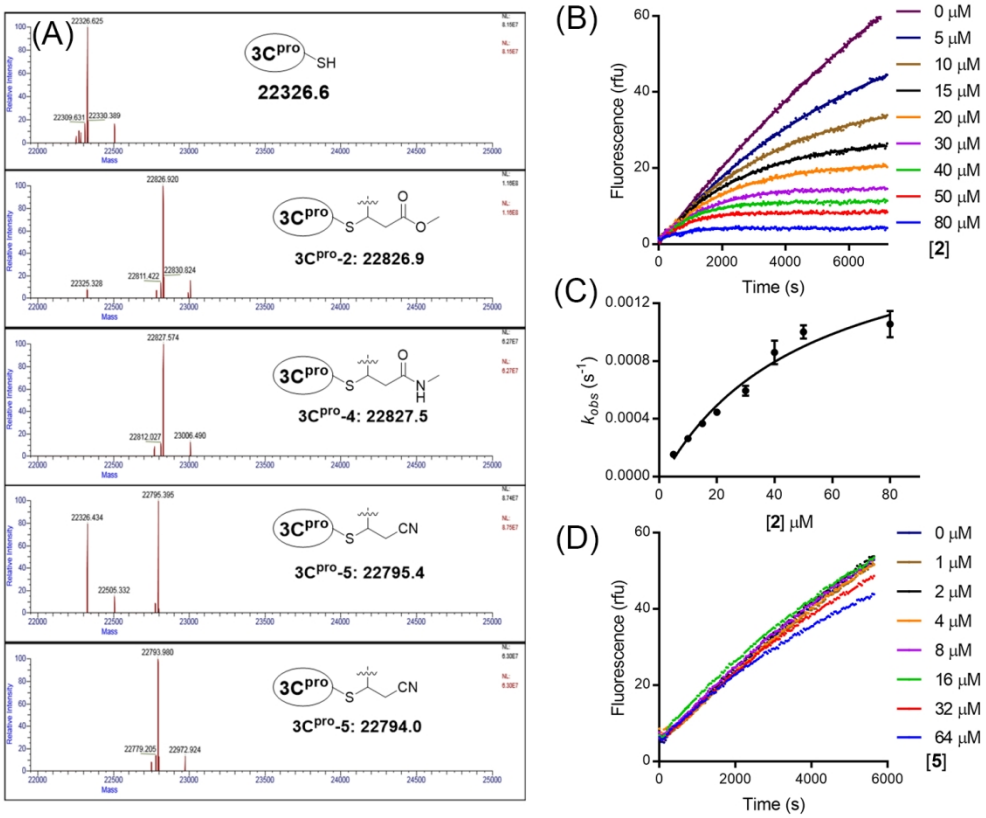

Figure 2. (A) Deconvoluted ESI-MS spectra of $3C^{pro}$ and its adduct with classical Michael acceptors. From top to bottom are—unmodified $3C^{pro}$, $3C^{pro}$ incubated with 10 equiv **2**, **4**, **5** for 30 min and $3C^{pro}$ incubated with 20 equiv **5** for 1.0 h, respectively. (B) Time-course inhibition of $3C^{pro}$ at variable concentrations of **2**. Lines drawn through the time courses result from fitting of data to equation 2 in Experiment Section. (C) Replotting the calculated k_{obs} values over concentration of **2** (equation 3 in Experiment Section) yields a partial hyperbola. Error bar represents standard error of the mean (SEM, n = 3). (D) Time-course inhibition of $3C^{pro}$ at variable concentrations of **5**.

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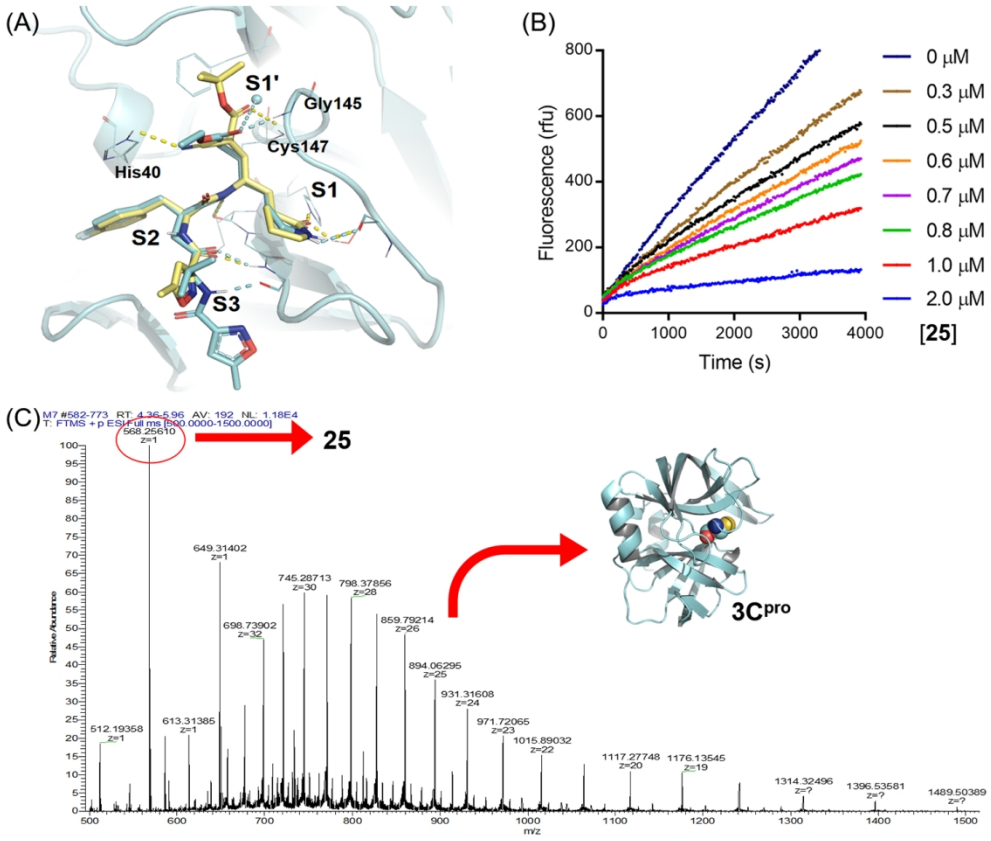

Figure 3. (A) Top-scoring docking pose of **25** (yellow) was superposed on rupintrivir (cyan) from crystal structure (PDB ID: 3SJO). Hydrogen bonds were drawn as dashed lines. The bound water was labeled as solid sphere. Residues at S1′ pocket were labeled. (B) Time-course inhibition of $3C^{pro}$ at variable concentrations of **25**. Linear progress curves suggest rapid equilibrium of enzyme-inhibitor complex. (C) Raw ESI-MS spectrum of $3C^{pro}$ incubated with **25**. The appearance of molecular ion peak of **25** implied that it came along with $3C^{pro}$ but detached during or after ionization.

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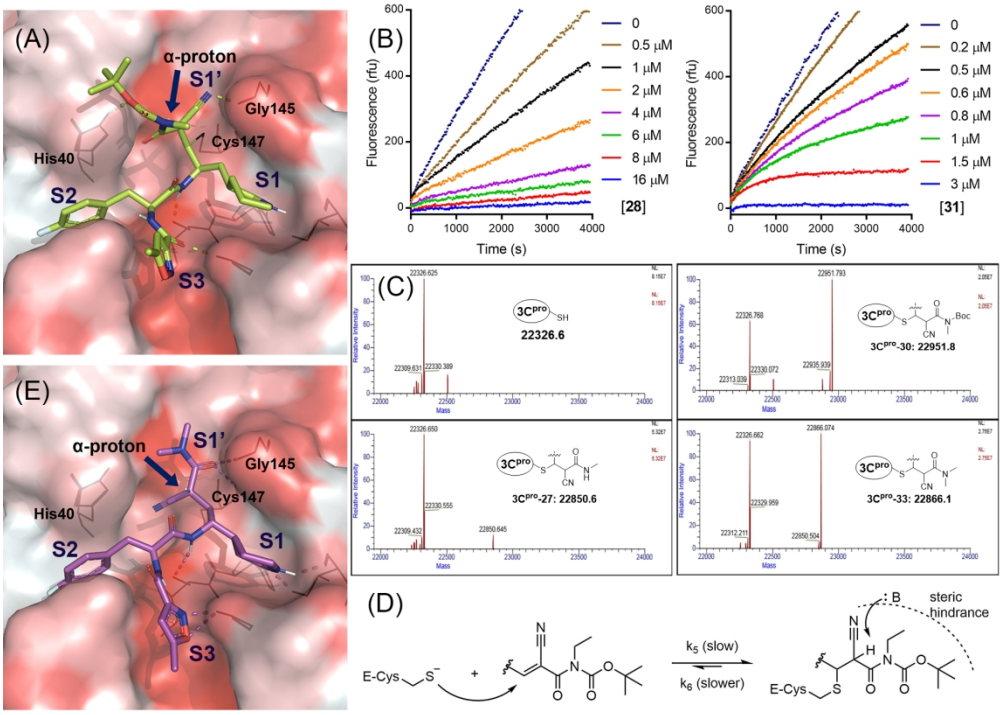

Figure 4. (A) Top-scoring docking pose of **30** (green). Hydrogen bonds were drawn as dashed lines. Red colored area represented hydrophobic protein surface. The arrow pointed to α-proton of Michael acceptor, which resided in a hydrophobic region and was shielded by the P1'. (B) Time-course inhibition of $3C^{pro}$ at variable concentrations of **28** (left) and **31** (right). **31** displayed a more obvious slow-binding behavior (time-dependency) than **28**. (C) Deconvoluted ESI-MS spectra of $3C^{pro}$ incubated with different cyanoacrylamides. Upper left: unmodified $3C^{pro}$; lower left: $3C^{pro}$-**27**; upper right: $3C^{pro}$-**30**; lower right: $3C^{pro}$-**33**. (D) Putative reaction mechanism for the elimination of $3C^{pro}$-**31** adduct. (E) Top-scoring docking pose of **33** (magenta). Other representations were similar with (A). The α-proton was solvent-exposed.

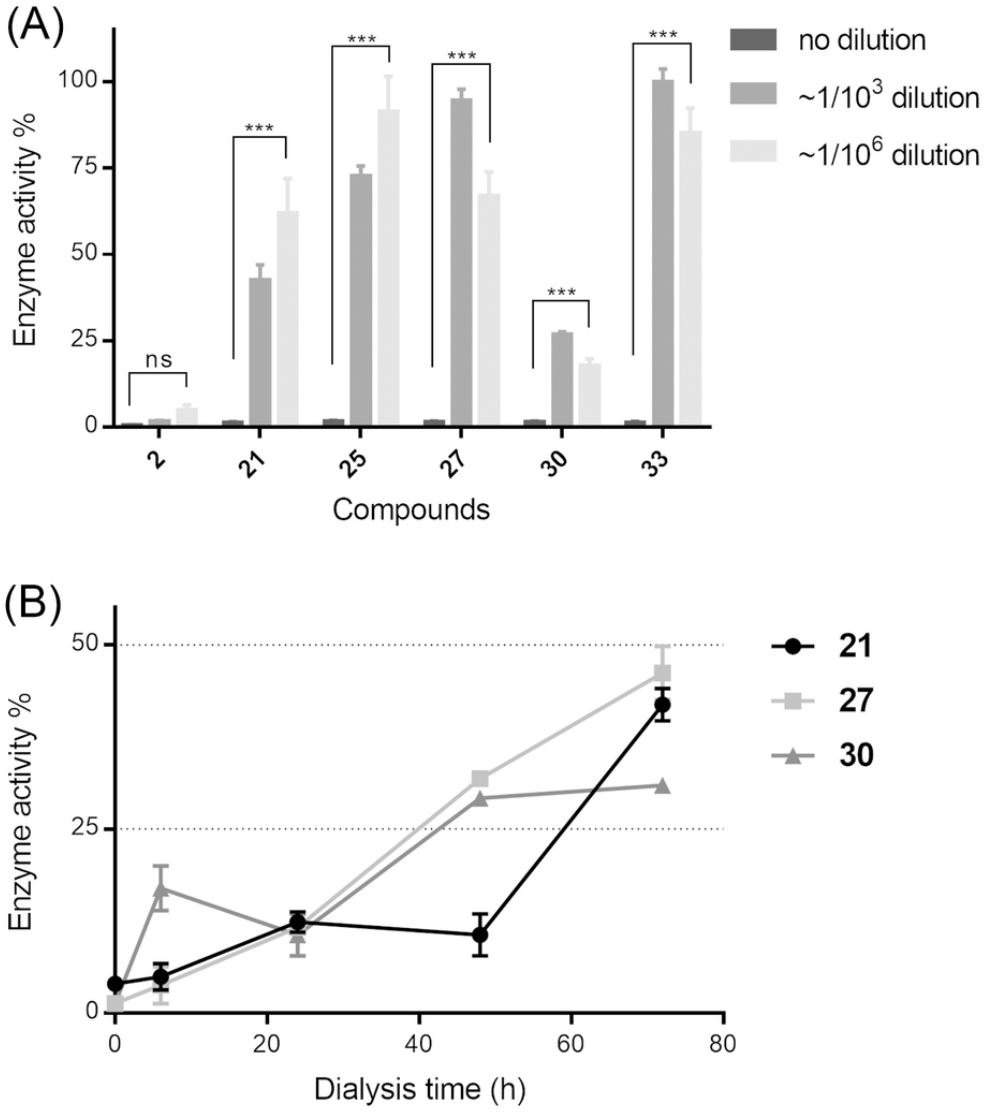

Figure 5. (A) Enzyme activity recovery after ultrafiltration of preincubated $3C^{pro}$-inhibitor complex. The concentration of given inhibitor was diluted by approximately 10^3- and 10^6-fold using ultrafiltration method. NS, not significant; P(***)<0.001. (B) Enzyme activity recovery after dialysis of $3C^{pro}$ pretreated with the indicated inhibitors or DMSO. Aliquots were taken at indicated time points, tested for the enzyme activity and normalized to a DMSO control. Error bar represents SEM (n = 3) in both (A) and (B).

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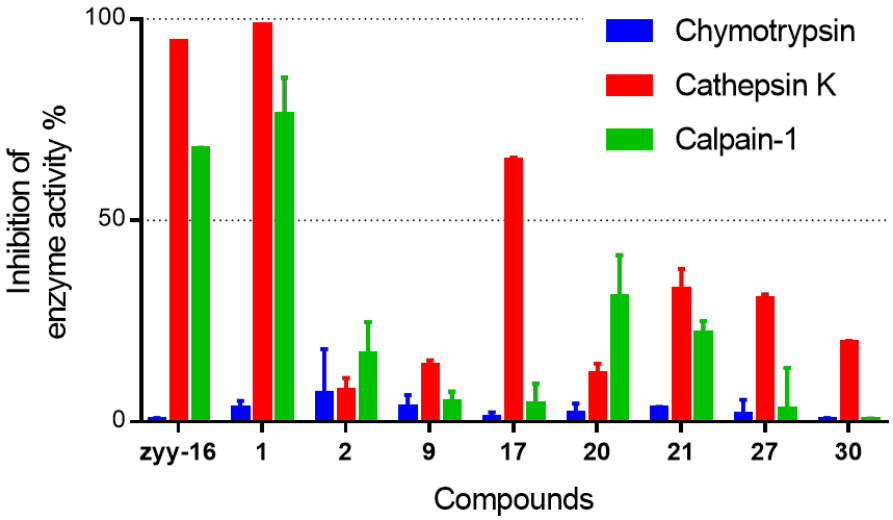

Figure 6. Selectivity study. All three enzymes were inhibited by 50 μM of indicated compounds. Inhibitory effects were calculated from fractional initial velocity as detailed in Experiment Section. Error bar represents SEM (n = 2).

81x47mm (300 x 300 DPI)