



Synthesis and multitarget inhibitory effect of indole-based ethyl cinnamate derivatives against SARS-CoV-2 M^{Pro} and cathepsins for broad-spectrum anti-coronavirus activity[☆]

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ABSTRACT

The experience of the SARS-CoV-2 pandemic has highlighted the urgent need to develop broad-spectrum antivirals (BSA) agents capable of targeting viral and/or host factors essential for infection as undeniable weapons against future coronavirus threats with potential pandemic perspectives. In this study, we report the synthesis and biological evaluation of a novel series of indole-based ethyl cinnamate derivatives designed as multitarget inhibitors of both SARS-CoV-2 Main Protease (M^{Pro}) and human cathepsin, namely CatL and CatS, key enzymes involved in viral replication and entry. Enzymatic assays showed that several compounds exert significant inhibition on multiple targets. Structure-activity relationship (SAR) studies highlighted the critical role of the *tert*-leucine (Tle) residue at P2 position of these (pseudo)di-peptides, which proved to be essential for multitargeted enzyme inhibition and antiviral activity. In antiviral assays against representative α - (hCoV-229E) and β -coronavirus (hCoV-OC43), **12** (EC₅₀ = 4.09 μ M for hCoV-OC43, 0.77 μ M for hCoV-229E), **20** (EC₅₀ = 6.68 μ M for hCoV-OC43, 0.62 μ M for hCoV-229E), and **3** (EC₅₀ = 2.96 μ M for hCoV-OC43, 0.64 μ M for hCoV-229E) restricted viral replication with high selectivity values (SI = 98, 56, and 101, respectively). Time-of-drug-addition experiments then revealed that **12** primarily inhibited viral entry by targeting CatL, whereas **20** affected both entry and post-entry stages of hCoV replication cycle likely thanks to its dual-inhibitory activity against both CatL and M^{Pro}. These findings support the development of multitarget inhibitors as promising antivirals able to inhibit both α - and β -coronavirus, so as to reduce the risk of resistance associated with single-target agents. Furthermore, the demonstrated effectiveness of Tle-containing indole-based cinnamates as BSA highlights their potential in the context of the strategic framework for pandemic preparedness.

1. Introduction

Coronaviruses (CoVs) are enveloped, positive-sense RNA viruses classified into four genera: α -, β -, γ -, and δ -coronavirus. α - and β -CoVs infect mammalian species, including humans, in which they cause

several respiratory diseases,^{1–3} ranging from mild upper respiratory tract infections, caused by endemic CoVs such as hCoV-229E, hCoV-OC43, hCoV-NL63 and hCoV-HKU1, to severe respiratory diseases as for SARS-CoV, MERS-CoV, and SARS-CoV-2.^{4,5} hCoV-229E and hCoV-OC43 represent prototypical α - and β -coronavirus, respectively. While hCoV-

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229E is mostly associated with common colds, hCoV-OC43 can lead to more severe lower respiratory tract infections, especially in vulnerable populations such as the elderly.^{6,7}

The COVID-19 pandemic underscored the devastating impact of the emergence of a new zoonotic CoV in humans, emphasizing the urgent need for antiviral drugs targeting SARS-CoV-2 but also capable to exert a broad-spectrum antiviral (BSA) activity against other present and future human CoVs.^{8–11} Such therapeutics could address the inherent challenges posed by the emergence of new CoVs in humans and their rapid viral evolution. The development of BSA agents is, therefore, an essential step in pandemic preparedness, enabling proactive responses to future CoVs outbreaks.^{12,13}

CoV proteases play essential roles in viral replication: SARS-CoV-2 Main Protease (M^{pro}), in fact, is a highly conserved cysteine protease required for processing viral polyproteins into functional polypeptides needed for efficient viral replication.^{14–16} Importantly, M^{pro}'s conservation across different human CoVs makes it a promising target for CoV-specific Direct-Acting Antivirals (DAAs) drug development, as evidenced by the efficacy of nirmatrelvir, an FDA-approved anti-SARS-CoV-2 peptidomimetic inhibitor, as well as for BSA discovery.^{17,18} However, reliance on DAAs targeting viral protease like nirmatrelvir may introduce risks of resistances, making alternative or complementary approaches based on Host-Targeting Agents (HTAs) with potential BSA activity promising therapeutic strategies.^{19,20} In light of this, human lysosomal cysteine proteases, particularly cathepsin L (CatL) and cathepsin S (CatS), have been demonstrated to play essential roles in CoVs infection by cleaving viral spike proteins within acidic endosomal compartments, thereby enabling the viral endocytic pathway entry into host cells.²¹ The role of CatS in viral infections is less well understood compared to CatL²², although its involvement in acute lung injury has been reported, suggesting that it may represent a promising therapeutic target for treating respiratory diseases.²³ More recently, however, inhibition of CatS activity has been indicated as a valuable target for the development of anti-SARS-CoV-2 drugs.²⁴ Notably, CatL and CatS are implicated in several other pathological processes, including inflammation and immune modulation, which are often exacerbated during viral infection.^{25,26} Based on these facts, the simultaneous targeting of both M^{pro} and cathepsins may thus represent a novel and promising strategy to target both viral replication and entry.^{24,27,28} This approach may provide a double benefit: (1) reducing the likelihood of resistance by targeting also cellular proteases; and (2) exerting BSA activity against present and future CoVs. However, while multitarget inhibitors have been explored in other therapeutic areas, their exploitation in antiviral drug development remains less explored.²⁹

In this study, we report the design, synthesis, and biological evaluation of a novel series of 29 (pseudo)dipeptides based on a C-terminal ethyl cinnamate framework. This electrophilic warhead, able to covalently trap the catalytic –SH group of cysteine proteases, represents a breakthrough over traditional peptidomimetic M^{pro} inhibitors, offering distinct advantages in terms of chemical diversity and drug-like properties.³⁰ Central to our design was the inclusion of an indole moiety at the N-terminus of the (pseudo)peptide core, which played a pivotal role in the multitarget activity. The indole ring establishes strong interactions with the catalytic sites of M^{pro}, CatL and CatS, leveraging π - π stacking and hydrogen bonding to achieve dual inhibition.³⁰ Our exploration of the structure–activity relationship (SAR) of these compounds focused on optimizing the indole moiety and its connection to the P2 amino acid, allowing us to systematically evaluate electronic and steric effects on enzyme binding. Our inhibitors showed activity against both M^{pro} and cathepsins, with the multitarget mechanism translating into a potent BSA activity against both α - and β -CoVs. These findings underscore that the correct combination of the indole moiety, which is crucial for multitarget activity and the cinnamate structure, similarly pivotal, forms a versatile and robust platform for the development of valuable BSAs in the preparedness for future CoVs pandemics.

2. Rational design

Building on our earlier work with M^{pro} inhibitors featuring the ethyl *p*-aminocinnamate framework,³⁰ we designed a series of multitarget inhibitors aimed at addressing the interconnected roles of SARS-CoV-2 M^{pro}, CatL, and CatS in viral replication and host cellular pathways. M^{pro} plays a critical role in the proteolytic cleavage of viral polyproteins, while CatL and CatS contribute to viral entry and immune modulation. Recognizing the opportunity to simultaneously target these enzymes, we adopted a structure-guided rational design approach to develop multitarget inhibitors capable of exert a broad-spectrum antiviral activity against α - and β -CoVs.

Our work began with compound **1** as a lead structure, an ethyl *p*-aminocinnamate-based inhibitor (Fig. 1).³⁰ The ethyl *p*-aminocinnamate moiety functions as an electrophilic warhead, covalently binding to the catalytic thiol group of Cys145 in the S1' pocket of M^{pro} through a thia-Michael addition mechanism. This covalent interaction anchors the molecule, while the cinnamate's phenyl ring interacts with His41 in the S2 pocket, through π - π stacking. The P2 Phe residue engages in hydrophobic interactions within the S2 pocket, enhancing molecular stability and specificity. Dual inhibitors of CatS and CatL based on the ethyl *p*-aminocinnamate framework were recently developed by our team, demonstrating significant efficacy in inhibiting the proliferation of pancreatic cancer cell lines (i.e., PACAN1 and BXPC3).³¹ Among these dual inhibitors, the most potent exhibited strong inhibition of CatS (99 % at 20 μ M) and moderate activity against CatL (29 % at 20 μ M), attributed to the inclusion of an indole moiety that established crucial interactions within the active site cavities of both cathepsins. Given the pivotal role of the indole ring in facilitating these interactions, we recognized its broader potential as a multitarget pharmacophore. Previous docking analysis of **1** revealed that the indole-amide P3 moiety occupies the S3/S4 pocket of M^{pro}, underscoring its importance for binding and specificity, as also observed for other indole-bearing inhibitors of M^{pro} reported in the literature.^{32,33} Building on this insight, we hypothesized that the indole-amide motif could serve as a key structural element for achieving multiple inhibition of M^{pro} and cathepsins, thereby enhancing the multitarget therapeutic potential of our inhibitors. Guided by this hypothesis, we designed a library of 29 novel inhibitors (compounds **2–30**), retaining the ethyl *p*-aminocinnamate fragment for covalent binding to M^{pro} while systematically modifying other structural features to explore their impact on multitarget inhibition and broad-spectrum antiviral activity (Fig. 1).

Exploration of P2. A Phe residue at P2 is a common feature in the design of M^{pro} inhibitors for its ability to insert within the S2 subsite establishing hydrophobic interactions. Phe was retained in half of the designed inhibitors. In the remaining half, Phe was replaced with a *tert*-butyl leucine (Tle) residue to explore whether a smaller, more flexible residue could improve interactions with CatS and CatL while maintaining M^{pro} activity. This substitution was inspired also by findings highlighting the importance of smaller hydrophobic residues in M^{pro} binding.³⁴

Exploration of P3. The indole ring, a critical feature of compound **1**, was extensively functionalized to investigate its role in pocket interactions across all three enzymes. Systematic variations included: (a) changing the position of the amide group connecting the indole ring to the (pseudo)dipeptide framework in order to assess its impact on binding to M^{pro} and cathepsins; (b) introducing electron-withdrawing or electron-donating groups to modulate electronic properties and hydrogen bonding potential; (c) adding spacers or substituents like a *p*-acetamidophenyl moiety (a bioisostere of indole) to evaluate the steric and electronic effects on binding interactions.

Stereochemistry. The stereochemistry of the one chiral center present in this of compounds was preserved in all but one, in which it was intentionally inverted to assess its impact on enzyme binding and activity. This provided insights into the stereochemical requirements for multitarget inhibition.

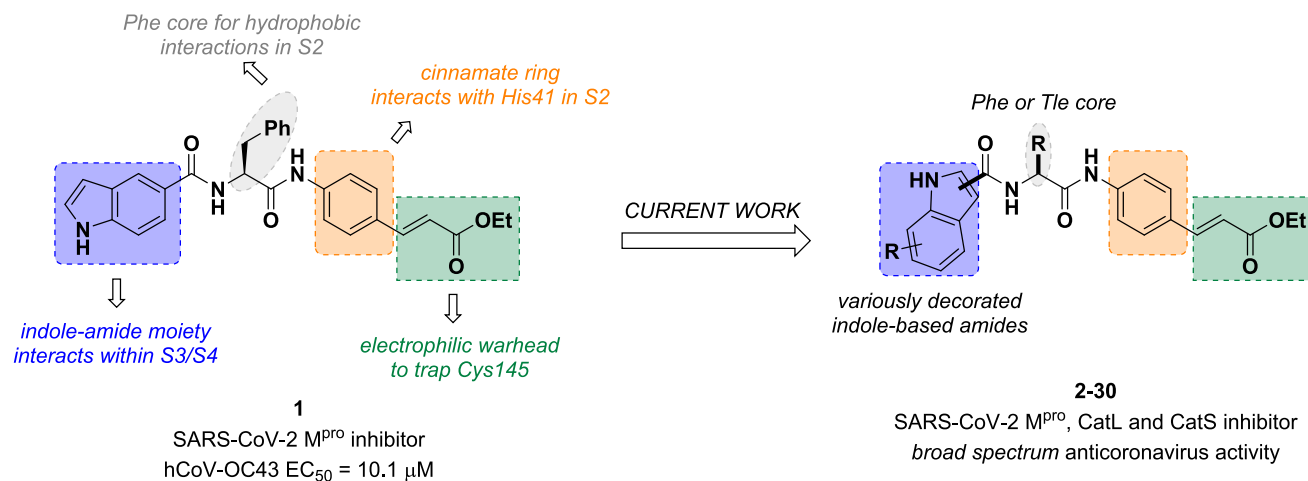


Fig. 1. Rational design of the novel compounds starting from compound 1 of our previous work.

Interaction with M^{pro} Pockets. The structural modifications were designed to maximize interactions within key pockets of M^{pro} : (a) S1' Pocket: covalent binding of the β -carbon of the ethyl *p*-aminocinnamate warhead to Cys145; (b) S2 Pocket: reinforcement through π - π stacking interactions between the cinnamate phenyl ring and His41; (c) S3/S4 Pocket: interactions mediated by a variously functionalized indole ring to confere multitarget activity.

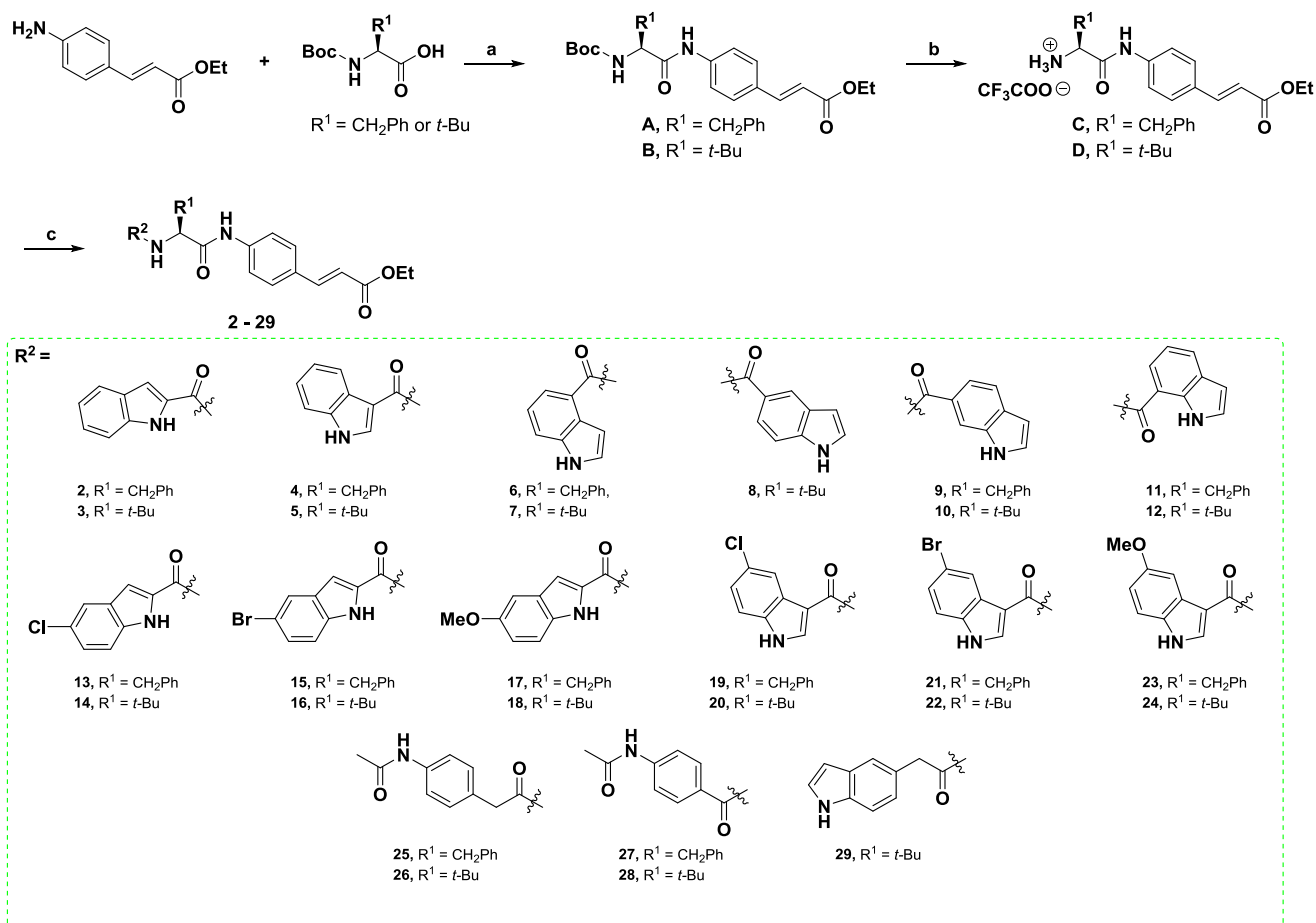
Interaction with CatS and CatL. Modifications were also guided by the binding preferences of CatS and CatL: (a) the functionalized indole ring were optimized to fit within the active-site cavities of CatS and CatL,

leveraging π - π stacking and hydrogen bonding with catalytic residues; (b) the smaller Tle residue was hypothesized to enhance fit within the more compact binding pockets of both cathepsins.

3. Results

3.1. Synthesis

The main synthetic strategy is shown in Scheme 1. The key intermediates **A** and **B** were prepared through an amide coupling reaction



Scheme 1. Synthesis of final compounds 2 – 29. Reagents and conditions: (a) EDCl, DMAP, dry DCM, rt, overnight, 49 % (A), 87 % (B); (b) TFA, DCM, rt, 4 h, 91 % (C), 85 % (D); (c) R^2COOH , EDCl/DMAP or EDCl/HOBt, DIPEA, dry DCM or dry DMF, rt, 3 h – overnight, 8–87 %.

between ethyl *p*-aminocinnamate and either Boc-Phe-OH (to give **A**) or Boc-Tle-OH (to give **B**), using EDCI and DMAP as coupling agents. Boc deprotection with TFA then afforded intermediates **C** and **D** as trifluoroacetate salts. Finally, the desired products (**2–29**) were obtained by coupling the intermediates with the appropriate carboxylic acids, using either HOBt/EDCI/DIPEA, EDCI/DMAP/DIPEA or HATU/DIPEA as coupling agents, with yields ranging from 8 % to 87 %.

All compounds maintained the *S* chirality at the Phe or Tle amino acid position, in line with our previous study, although we synthesized one compound (**30**) using the *R*-isomer to assess the chiral requirement. An analogue synthetic strategy was followed to obtain compound **30**, starting from the corresponding *R*-isomer as starting material and it is described in Scheme 2. Ethyl *p*-aminocinnamate was reacted with Boc-*D*-Leu-OH in the presence of EDCI/DMAP affording intermediate **E** in low yield (33 %). Then, **E** was treated with TFA providing intermediate **F** as trifluoroacetate salt in good yield (74 %). Finally, **F** was reacted with indole-5-carboxylic acid yielding the target compounds **30** (42 %) using EDCI/DMAP combination as coupling reagents.

3.2. Biomimetic experiment

A biomimetic experiment was performed to investigate the interaction of the final compounds with the target cysteine protease, using a protocol already exploited for our precedent works.³¹ As an example, compound **3** was incubated with *N*-acetyl cysteine in phosphate buffer (pH = 7.4), containing 10 % MeOH at 37 °C and the outcome was analyzed via HPLC-MS to measure the consumption of the starting material (Supplementary Fig. S1). After 24 h, the analysis revealed the appearance of a new peak (*m/z* = 611.2) corresponding to the Michael adduct formed between *N*-acetyl cysteine and **3** with a conversion rate of ~ 80 %. This result confirmed the reactivity of our compounds against thiol.

3.3. Enzyme inhibition assays

All the final compounds were subjected to enzymatic assays (concentration = 20 μM) for their inhibition activity against SARS-CoV-2 M^{Pro}, CatL, CatB and CatS (Table 1). CatB was also selected for these enzyme inhibition assays because recent studies have shown that, like CatL and CatS, it is a protease that mediates the successful entry of SARS-CoV-2 in host cells, although there are less specific evidences.²² Regarding enzymatic activity against M^{Pro}, the most active derivatives turned out to be **11** (69 %), whose structure contains a Phe residue as the central core (*P2*) and an indole ring substituted at position 7 and linked via amide-bond at the *N*-terminus of the (pseudo)peptide framework, **28** (59 %) which, compared with the previous one, carries a *p*-acetanilide fragment as the bioisosteric replacement of the indole and a Tle residue as the core, **20** (56 %) featuring a 5-chloroindole group attached at its position 3-C to the central Tle core, and **23** (53 %) bearing a 5-

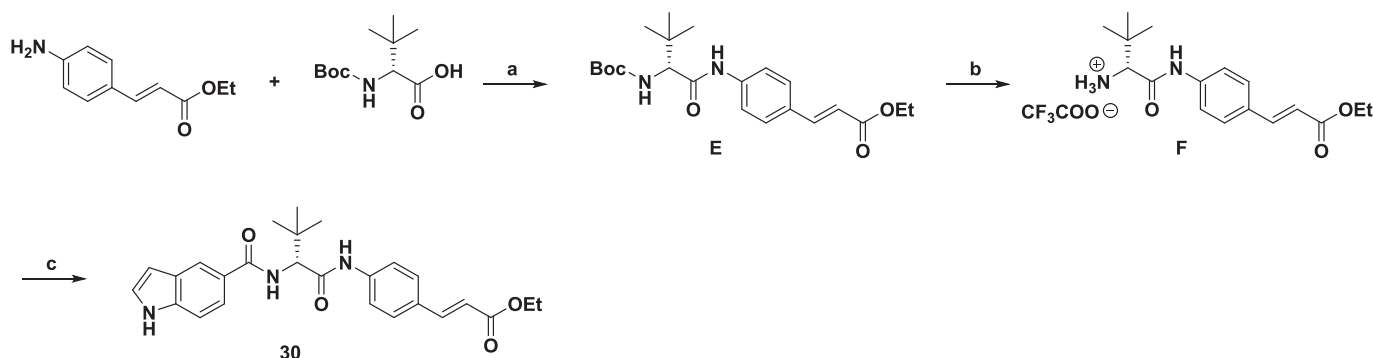
methoxyindole group attached at its position 3-C to the central Tle core. Compound **30**, which contains the stereogenic center in the *R* configuration instead of the *S* configuration, did not show significant activity on M^{Pro}.

The profile of enzymatic inhibition against cathepsins displayed varied results. **11** and **20** showed low activity against CatL (21 % and 18 %, respectively), while good inhibitory activity against CatL was observed for **12** and **22** (48 % and 40 %, respectively). **12** is chemically the analogue of **11**, in which a Tle residue at the central core has replaced the Phe residue connected to the indole ring at position 7. The comparison **12/11** shows that by keeping the indole carboxylic acid connection at position 7 and switching from Phe to Tle at *P2*, there is a loss of activity against M^{Pro} (**12** = 16 % vs. **11** = 69 %), but activity is gained against CatL (**12** = 48 % vs. **11** = 21 %). A similar result, in terms of increased inhibitory activity against CatL, was obtained with **22**, which, compared with **21**, has the same Tle-Phe switch in the core and a common 5-bromoindole group at the *N*-terminus linked through position 3. Additionally, **21** appeared to have scarce activity against M^{Pro}, demonstrating that the combination of a 5-bromoindole substituted at position 5 and the Phe core disfavors interaction with the protease (**21**, M^{Pro} 17 %). Regarding enzymatic activity against CatB, **4** (11 %) and **7** (22 %) exhibited low inhibitory activity. Compared with them, **20** showed slightly higher activity (26 %). The most interesting molecules were **12** (48 %) and **22** (45 %), as they also exhibited an overlapping inhibitory profile against the other two cathepsins (good for CatL and high for CatS) under evaluation and M^{Pro} (low). No inhibition (n.i.) against CatB was detected for the remaining compounds. Almost all compounds, however, proved to be potent inhibitors of CatS, with inhibition values ranging from 70–98 % at 20 μM, except for **8** (10 %), **25**, **26**, **27**, and **28** (n.i.).

From this enzymatic screening, multitarget inhibitors such as **11** (M^{Pro} 69 %, CatL 21 %, CatS 97 %), **12** (M^{Pro} 16 %, CatL 48 %, CatB 48 %, CatS 93 %), **20** (M^{Pro} 56 %, CatL 18 %, CatB 26 %, CatS 98 %), **22** (M^{Pro} 16 %, CatL 40 %, CatB 45 %, CatS 96 %), and **23** (M^{Pro} 53 %, CatS 98 %) were identified. **28**, on the other hand, emerged as a selective M^{Pro} inhibitor (59 %) from our screening.

3.4. Docking

To study their binding modes, the compounds **11**, **12**, **20**, **22** and **23** were analyzed *in silico* using a covalent docking technique. 2D interaction plots and energy scores are included in the Supporting Information. Docking approach allowed the observation of the preferred conformation and orientation after forming a covalent bond between the β-carbon of the cinnamic ester and the thiol group of the catalytic cysteine in SARS-CoV-2 M^{Pro}, CatS, CatB and CatL. Firstly, focusing on M^{Pro}, **11**, **20** and **23** show the indole group within the S3 region establishing hydrophobic interactions with Pro168, Gln189 and Ala191 (Fig. 2). In **11** and **23** the docked conformation is further stabilized by a H-bond



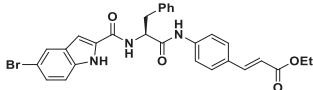
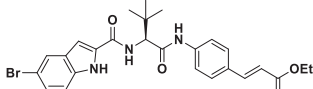
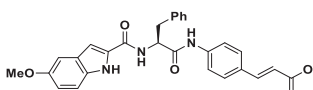
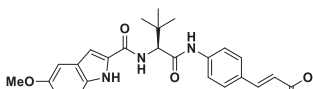
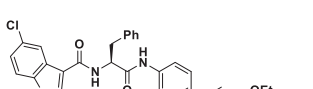
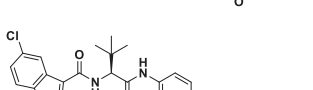
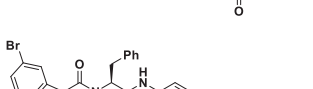
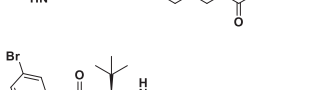
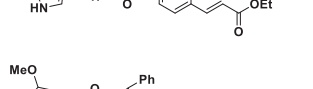
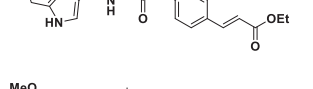
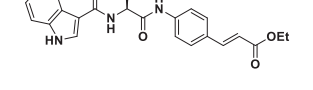
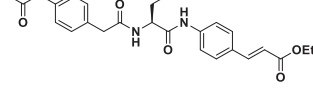
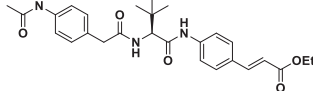
Scheme 2. Synthesis of final compound **30**. Reagents and conditions: (a) EDCI, DMAP, dry DCM, rt, overnight, 33 %; (b) TFA, DCM, rt, 4 h, 74 %; (c) Indole-5-carboxylic acid, EDCI, DMAP, DIPEA, dry DCM/DMF, rt, overnight, 42 %.

Table 1*In vitro* inhibition activity (% inh. at 20 μ M) of 2 – 30 against SARS-CoV-2 M^{pro}, CatL, CatB and CatS.

Compound	Structure	M ^{pro}	CatL	CatB	CatS
2		25.6 $\pm$ 1.0	n.i.	n.i.	94.4 $\pm$ 3.5
3		18.2 $\pm$ 1.4	n.i.	n.i.	97.0 $\pm$ 1.6
4		25.5 $\pm$ 1.4	n.i.	11.1 $\pm$ 1.3	98.6 $\pm$ 0.2
5		16.0 $\pm$ 0.7	n.i.	n.i.	71.9 $\pm$ 0.5
6		20.5 $\pm$ 0.8	n.i.	n.i.	97.0 $\pm$ 0.7
7		13.4 $\pm$ 2.6	n.i.	22.0 $\pm$ 2.0	55.4 $\pm$ 0.03
8		n.i.	n.i.	n.i.	10.4 $\pm$ 1.3
9		n.i.	n.i.	n.i.	96.1 $\pm$ 3.8
10		11.6 $\pm$ 0.6	n.i.	n.i.	75.0 $\pm$ 0.8
11		69.6 $\pm$ 1.3	21.3 $\pm$ 0.8	n.i.	97.4 $\pm$ 0.1
12		16.0 $\pm$ 2.0	48.4 $\pm$ 1.3	48.0 $\pm$ 0.2	93.9 $\pm$ 0.7
13		10.8 $\pm$ 1.3	n.i.	n.i.	97.6 $\pm$ 0.3
14		15.2 $\pm$ 2.2	n.i.	n.i.	91.6 $\pm$ 0.4

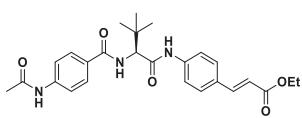
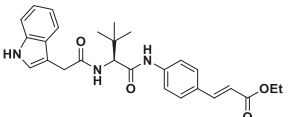
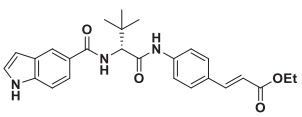
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Table 1 (continued)

Compound	Structure	M ^{pro}	CatL	CatB	CatS
15		25.7 ± 0.7	n.i.	n.i.	98.8 ± 1.3
16		15.8 ± 0.4	n.i.	n.i.	95.6 ± 0.6
17		12.0 ± 0.1	n.i.	n.i.	96.4 ± 0.1
18		23.6 ± 2.0	n.i.	n.i.	96.1 ± 0.9
19		10.9 ± 1.5	n.i.	n.i.	94.4 ± 0.6
20		56.1 ± 0.7	18.1 ± 0.03	26.7 ± 1.4	98.0 ± 0.1
21		17.1 ± 0.4	n.i.	n.i.	95.3 ± 0.02
22		16.9 ± 0.4	40.5 ± 0.2	45.2 ± 0.6	96.4 ± 0.2
23		53.8 ± 2.0	n.i.	n.i.	98.2 ± 0.5
24		15.5 ± 1.4	n.i.	n.i.	97.6 ± 1.3
25		n.i.	n.i.	n.i.	n.i.
26		n.i.	n.i.	n.i.	n.i.
27		n.i.	n.i.	n.i.	n.i.

(continued on next page)

Table 1 (continued)

Compound	Structure	M ^{pro}	CatL	CatB	CatS
28		59.4 ± 2.2	n.i.	n.i.	n.i.
29		10.4 ± 0.5	n.i.	n.i.	84.5 ± 3.1
30		21.2 ± 2.7	27.7 ± 2.1	n.i.	70.3 ± 0.1

Notes: n.i. = no inhibition.

between the amide group of the indole in 3-C position and Gln192 (Fig. 2 – panel A and C, respectively). **11** and **23** bearing a Phe at P2, bind in the S2 subsite interacting with Met49, Asp187, Arg188, Tyr54 and Gln189. On the other hand, **20**, bearing a Tle at P2, similarly binds in the S2 region, interacting with the same residues. The phenyl ring of the cinnamate structure forms π - π stacking with His41 in S2 in the three tested ligands, with distances between the center of the rings smaller than 4.6 Å. Overall, **11**, **20** and **23** putative binding modes identified by docking are closely similar to those shown by M^{pro} ligands in crystal structures, bearing an indole motif, described in the literature.³²

Fig. 3 illustrates the docked conformations of **12**, **22** and **20** covalently bound inside the active site of CatL. The three ligands share evident π - π stacking between the indole moiety and Trp189 aromatic ring (Fig. 3), exhibiting distance between the centers of the involved rings below 4 Å in all the compounds docked in CatL. Additionally, **12** forms an H-bond with Gly20 through the amide group at the indole 3-C

position (Fig. 3 – panel A). The phenyl ring of the cinnamate interacts with Gln21, Cys22, Gly23 and His163. The indole group of **22** establishes an H-bond with Gly20 and a hydrophobic interaction with Trp193, while the *t*-butyl group and the phenyl ring of the cinnamate interact with Leu144 and Gly23, respectively (Fig. 3 – panel B). For what concerns **20**, the indole group forms an H-bond with Asn18 and hydrophobic interactions with Gln19, Leu144 and Trp193. The *t*-butyl moiety interacts with Gln21 and Cys22, while the cinnamic ring gives hydrophobic interactions with Gly23 and His163 (Fig. 3 – panel C).

Fig. 4 shows the docked conformations of **11**, **12**, **22**, **20** and **23** within the active cavity of CatS. Also in this case, the binding modes are guided by a π - π stacking, herein between the indole moiety of each of the ligands and Phe269 of CatS. In **11** the phenyl ring at P2 interacts by a π - π stack with Trp309 and hydrophobic interactions with Tyr140, Gln141 and Gly142. The phenyl ring of cinnamate shows hydrophobic interactions with Gly145 and His287 (Fig. 4 – panel A). Concerning **12**, the

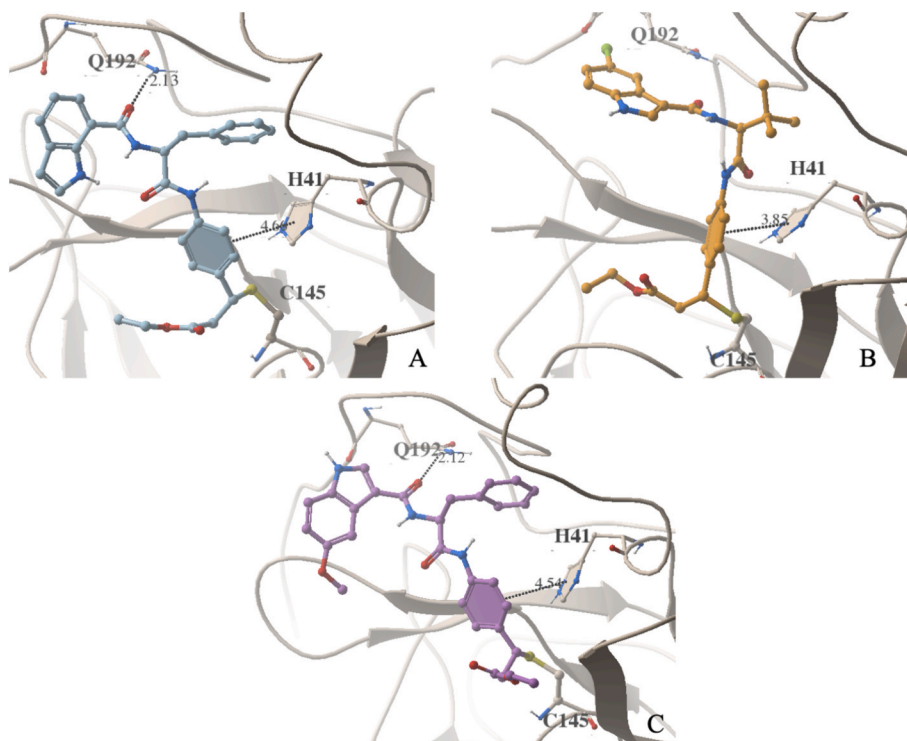


Fig. 2. *In silico* binding modes of **11** (panel A), **20** (panel B) and **23** (panel C) inside the active site of SARS-CoV-2 M^{pro} (PDB ID: 6LZE). H-bonds and π - π stacking distances are expressed in Å.

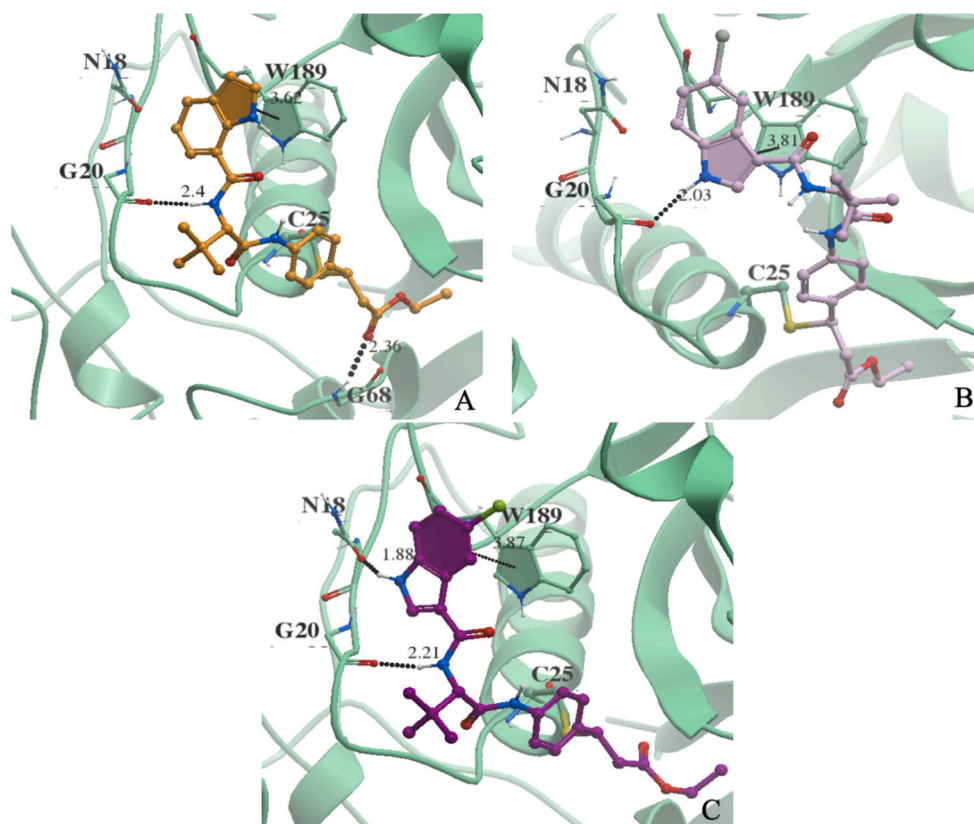


Fig. 3. *In silico* binding modes of 12 (panel A), 22 (panel B) and 20 (panel C) inside the active site of CatL (PDB ID: 2XU3). Residues establishing H-bonds or π - π stacking are represented with sticks, distances are expressed in Å.

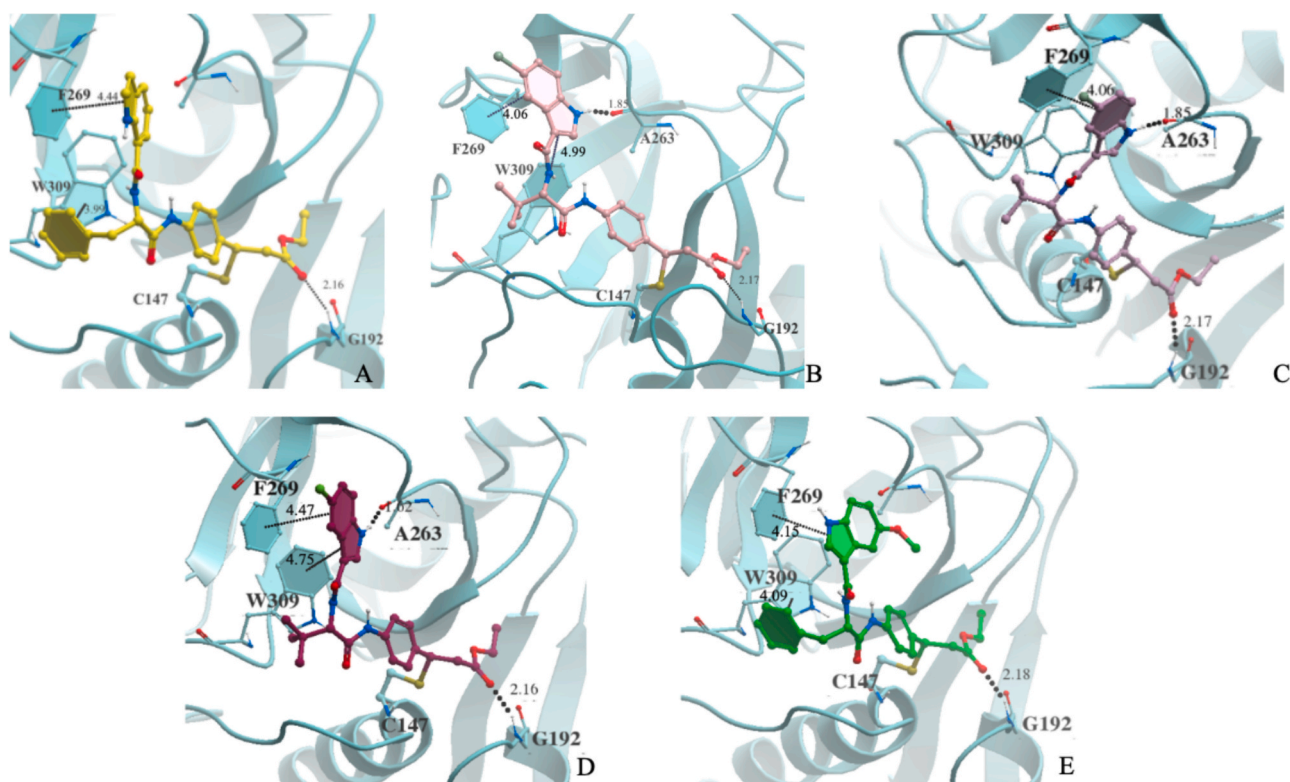


Fig. 4. *In silico* binding modes of 11 (panel A), 12 (panel B), 22 (panel C), 20 (panel D) and 23 (panel E) inside the active site of CatS (PDB ID: 4MZO). Residues establishing H-bonds or π - π stack are represented with sticks, distances are expressed in Å.

Tle at P2 interacts with Gly142, Ser143 and Trp309. Additional hydrophobic interactions are given by the phenyl ring of the cinnamate with Gly145 and His287 (Fig. 4 – panel B). **22** shows the same interactions as **12**, but its indole moiety gave some extra interactions. In particular, an additional π - π stack with Trp309, a H-bond with Ala263 and a hydrophobic interaction with Phe270 (Fig. 4 – panel C). The indole group of **20** forms an extra π - π stack with Trp309, a H-bond with Ala263 and additional hydrophobic interactions with His265 and His287 (Fig. 4 – panel D). The last ligand that has been docked on CatS is **23**. Its interactions closely mirror those of **12**, except for the phenyl ring at P2 that establishes π - π stacking interactions with Tyr140 and Trp309 (Fig. 4 – panel E). Here, the distances between π - π stacking pairs are slightly greater compared to the previous cases. The maximum distance observed in a π - π stacking with Phe269 is 4.47 Å, whereas the highest distance found in a π - π stacking with Trp309 was 4.99 Å.

The last tested target evaluated *in silico* was CatB using **12**, **22** and **20** as ligands (Fig. 5). **12** establishes exclusively hydrophobic interactions as it is shown in Fig. 5 – panel A. The indole moiety of **22** shows a π - π stack with His111, with a distance of 4.32 Å, and hydrophobic interactions with His110 and Pro118 while the *t*-butyl moiety shows hydrophobic interactions with Ser25, Cys119, Thr120 and Gly121. A H-bond was found between the oxygen of the amide connecting the cinnamate phenyl ring to the *t*-butyl group and His111. The phenyl ring of the cinnamate system forms hydrophobic the interactions with Gly27, His199 and Trp221 (Fig. 5 – panel B). Lastly, **20** establishes analogous interactions as **22**, but the indole group forms an additional H-bond with Glu109 and a hydrophobic interaction with Cys119 (Fig. 5 – panel C). On the whole, the proposed scaffold allows the interplay between hydrophobic, π - π stacking and hydrogen bond interactions between the ligands and the different proteins, allowing their multitarget activity. In all the modeled ligands, π - π stacking interactions were considered when the distances between the centers of the involved rings were found to be within 5 Å, which aligns with previous structural biology studies and computational studies.^{35–37} However, it's important to note that the

observed π - π stacking distances can vary depending on the chemical environment, the properties of the system and the surrounding molecules. A paramount role seems to be associated to the indole moiety, due to its versatility and its ability in establishing multiple interactions of different nature.

3.5. Antiviral studies

Identification of indole-based ethyl cinnamate derivatives active against a human CoV. Final compounds were then initially tested for their antiviral activity against hCoV-OC43, a representative human β -coronavirus. Manipulation of hCoV-OC43, while it does not require Biosafety laboratory level 3 (BSL3) facilities, can provide findings suggestive for further development as new pan-CoVs antiviral agents. To this end, FFRA was performed in which compounds at 2, 5, or 10 μ M were present before, during and after infection (full-treatment, FULL-T). Six compounds were able to decrease hCoV-OC43 replication more than 50 % when tested at 10 μ M, while the remaining compounds showed an EC₅₀ value >10 μ M. The most active compounds **5**, **12**, **22**, **18**, **20**, and **3** were therefore selected for further investigations.

Indole-based ethyl cinnamate derivatives inhibit the replication of α - and β -coronaviruses. A clear concentration-dependent inhibitory effect on hCoV-OC43 replication was then confirmed in HCT-8 cells exposed to different concentration of the selected cinnamate compounds (Supplementary Fig. S2). As shown in Table 2, the measured EC₅₀ and values for hCoV-OC43 ranged from 2.96 μ M for **3** to 7.70 μ M for **22**. Notably, the most active compounds share the presence of a Tle moiety as a substituent at the P2 position, demonstrating that its introduction was crucial for enhancing antiviral activity compared to the presence of Phe. Additionally, the inclusion of an indole moiety, although differently substituted, proved to be an effective strategy for improving antiviral activity. In fact, compared to our previously published compound **1**, bearing Phe at P2, the newly developed inhibitors exhibited greater efficacy in reducing β -CoV replication (EC₅₀ of compound **1** = 10.1 μ M),

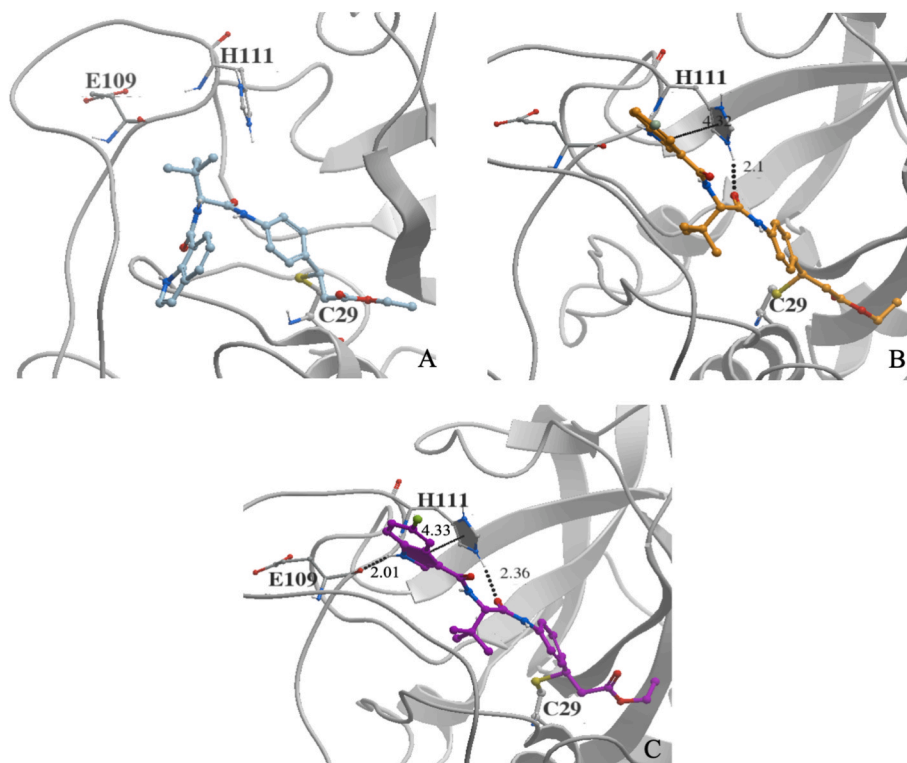


Fig. 5. *In silico* binding modes of **12** (panel A), **22** (panel B) and **20** (panel C) inside the active site of CatB (PDB ID: 8B4T). Residues establishing H-bonds or π - π stack are represented with sticks, distances are expressed in Å.

Table 2

Antiviral activity of indole-based ethyl cinnamate compounds against hCoV-OC43 and hCoV-229E.

Compound	hCoV	Cell line	EC ₅₀ (μM) ^a	EC ₉₀ (μM) ^b	CC ₅₀ (μM) ^c	SI ^d
5	hCoV-OC43	HCT-8	6.39 ± 0.50	11.05 ± 1.17	28.29 ± 0.48	4
	hCoV-229E	HuH-7	0.89 ± 0.16	1.93 ± 0.25	74.15 ± 1.39	83
12	hCoV-OC43	HCT-8	4.09 ± 0.31	8.29 ± 0.06	53.30 ± 6.88	13
	hCoV-229E	HuH-7	0.77 ± 0.07	3.31 ± 0.63	75.59 ± 6.52	98
22	hCoV-OC43	HCT-8	7.70 ± 0.26	17.11 ± 1.21	34.94 ± 0.83	5
	hCoV-229E	HuH-7	0.56 ± 0.06	13.47 ± 1.11	90.71 ± 2.50	162
18	hCoV-OC43	HCT-8	7.41 ± 0.88	27.33 ± 2.16	> 200	> 27
	hCoV-229E	HuH-7	6.85 ± 0.56	50.00 ± 2.05	> 200	> 29
20	hCoV-OC43	HCT-8	6.68 ± 0.08	13.80 ± 0.35	26.40 ± 0.88	4
	hCoV-229E	HuH-7	0.62 ± 0.09	3.65 ± 0.24	34.70 ± 0.94	56
3	hCoV-OC43	HCT-8	2.96 ± 0.11	9.26 ± 0.04	29.97 ± 1.28	10
	hCoV-229E	HuH-7	0.64 ± 0.05	3.19 ± 0.93	64.35 ± 6.46	101
1	hCoV-OC43	HCT-8	10.08 ± 0.17	30.34 ± 1.78	64.30 ± 4.19	6
	hCoV-229E	HuH-7	> 50	> 50	> 50	> 1

Notes:

Reported values represent the means ± SD of data derived from three experiments in triplicate.

^a EC₅₀, compound concentration that inhibits 50% of the replication of control virus hCoV-OC43 or hCoV-229E samples, as determined by FFRA in HCT8 and HuH-7 cells, respectively. Control virus samples were incubated with the vehicle (0.1% DMSO).

^b EC₉₀, compound concentration that inhibits 90% of the replication of control virus hCoV-OC43 or hCoV-229E samples, as determined by FFRA in HCT8 and HuH-7 cells, respectively. Control virus samples were incubated with the vehicle (0.1% DMSO).

^c CC₅₀, compound concentration that produces 50% of cytotoxicity of control cell samples, as determined by cell viability assays in HCT8 and HuH-7 cells. Control cell samples were incubated with the vehicle (0.4% DMSO).

^d SI, selectivity index determined as the ratio between CC₅₀ and EC₅₀.

highlighting a significant advancement over our previous research.

In order to assess a pan-CoVs BSA activity, **5**, **12**, **22**, **18**, **20** and **3** were tested for their activity also against the prototypic human α hCoV-229E. Replication of the α-CoV in HuH-7 cells was severely impaired (Supplementary Fig. S2) whenever treated with all the target compounds. Additionally, all compounds, except for **18** (EC₅₀ = 6.85 μM), exhibited significantly higher efficacy against the α-CoV hCoV-229E than that observed with hCoV-OC43, with EC₅₀ values below 1 μM (Table 2). Importantly, the potent anti-hCoV-229E activity of the most active cinnamate compounds, as **5**, **12**, **22**, **20**, and **3**, did not derive from non-specific cytotoxicity of target cells since their 50 % cytotoxic concentration (CC₅₀) as measured in uninfected HuH-7 cells were higher than 30 μM. Indeed, the selectivity index (SI) values were ranging from 56 for **20** to 101 for **3** (Table 2). Moreover, in comparison with the parental compound **1**, which showed no activity against hCoV-229E (Table 2), the new cinnamate compounds exhibited a broader antiviral activity with a significant increase in potency. Together, these results indicate that the combination of indole moiety and P2 Tle in the new cinnamate derivatives was a crucial for conferring their antiviral activity against both α- and β-human CoVs.

12 and **20** target the entry phase in the hCoV replication cycle. It is known that the ATCC isolate of hCoV-229E, like the one used in this

study, enters cells via the endosomal pathway using CatL to activate the spike protein after receptor binding³⁸. Therefore, time-of-drug-addition experiments were carried out to pinpoint which phase of the hCoV-229E replicative cycle was targeted by **12** and **20**, as representative selected indole-based ethyl cinnamate derivative endowed with anti-CatL activity (Fig. 6). **3** lacking activity against CatL, but with comparable antiviral activity against hCoV-229E (Table 2), was used as a control. To this end, as depicted in Fig. 6A, HuH-7 cell monolayers were treated with **12**, **20** or **3** (2 x EC₉₀) from −1 to 0 h prior to hCoV-229E infection (pre-treatment, Pre-T); or during infection with hCoV-229E (from 0 to 2 h; co-treatment, Co-T); or after viral adsorption (from 2 to 48 h p.i.; post-treatment, Post-T); or from −1 to 48 h p.i. (full treatment, Full-T). At 48 h p.i., infectious hCoV-229E particles released in cell supernatants were harvested and titrated by FFRA. As shown in Fig. 6, both **12** (panel B – Left panel) and **20** (panel C – Left panel) produced about three-fold reduction of hCoV-229E titers when present only during the infection stage (Co-T), which includes the virus' entry, thus in agreement with their ability to inhibit CatL activity. However, for **20** (Fig. 6, panel C) the Post-T effect was more pronounced compared to that of **12**, likely related to its M^{pro} inhibitory activity which may contribute more effectively to interfere with a synthetic stage of hCoV-229E replication subsequent to viral entry. By contrast, **3** was ineffective in interfering with the early phases of hCoV-229E replicative cycle since a both Pre-T and Co-T treatments did not significantly affect viral replication and it reduced the viral titers only when added at a post-entry stage (Post-T) or left on the cells from −1 to 48 h p.i. (Full-T) (Fig. 6, panel d – Left panel).

Immunoblotting analysis of protein extracts prepared from the very same hCoV-229E-infected and **12**-, **20**- or **3**-treated HuH-7 cells were then performed to evaluate the content of the nucleocapsid N protein. The accumulation of this major structural protein in fact is a marker of the efficiency of the biosynthetic steps of a hCoV replication cycle.⁷ Indeed immunoblotting confirmed for **12** (Fig. 6 panel B – right panel) and **20** (Fig. 6 panel C – right panel) a severe reduction of the nucleocapsid N protein accumulation following the Co-T. In contrast, for **3** the Co-T was ineffective (Fig. 6 panel D – right panel) and a clear reduction of N protein accumulation was observed only for Post-T and Full-T treatments, thus in agreement with the measured infectious viral titers released in supernatants of **3** treated cells (Fig. 6 panel D – left panel).

The reduced levels of both released infectious particles and N protein accumulation observed in cells exposed to **20** during Post-T could be due to their activity against SARS-CoV-2 M^{pro} (Table 1). Indeed, given the significant similarity of the M^{pro} of hCoV-229E to that of SARS-CoV-2 (39 % aa identity),³⁹ it is likely that **20** exert an inhibitory activity even on the hCoV-229E enzyme, causing an interference with the development of the following phases of its replication as revealed by the reduced N protein content.

Together, the results of this section suggest that an interference with the entry stage of hCoV-229E contributes to the overall antiviral activity of **12** and **20**, sustaining a role of their anti-cathepsin L activity in the reduced entry rate of the virus. Quite the opposite, **3** seems to affect primarily a biosynthetic step essential for hCoV-229E replication and that takes places at a post-entry stage. Owing its potent anti-CatS activity, it is tempting to hypothesize an involvement of CatS inhibition in the overall anti-hCoV-229E activity of **3**. More studies will be needed to elucidate the mechanism(s) of the antiviral activity of **3**.

3.6. ADME and drug-likeness properties

The ADME properties of the best performing inhibitors **12**, **20** and **3** were predicted using the SwissADME tool, revealing favorable pharmacokinetic characteristics (Table 3). All three compounds exhibit molecular weights below 560 Da, with **12** and **3** having identical molecular weights of 447.53 Da, while **20** is slightly heavier at 560.44 Da. The fraction of sp³ hybridized carbons is moderate (0.14–0.27), indicating a balanced mix of aromatic and aliphatic regions that may contribute to favorable drug-like properties. The number of rotatable

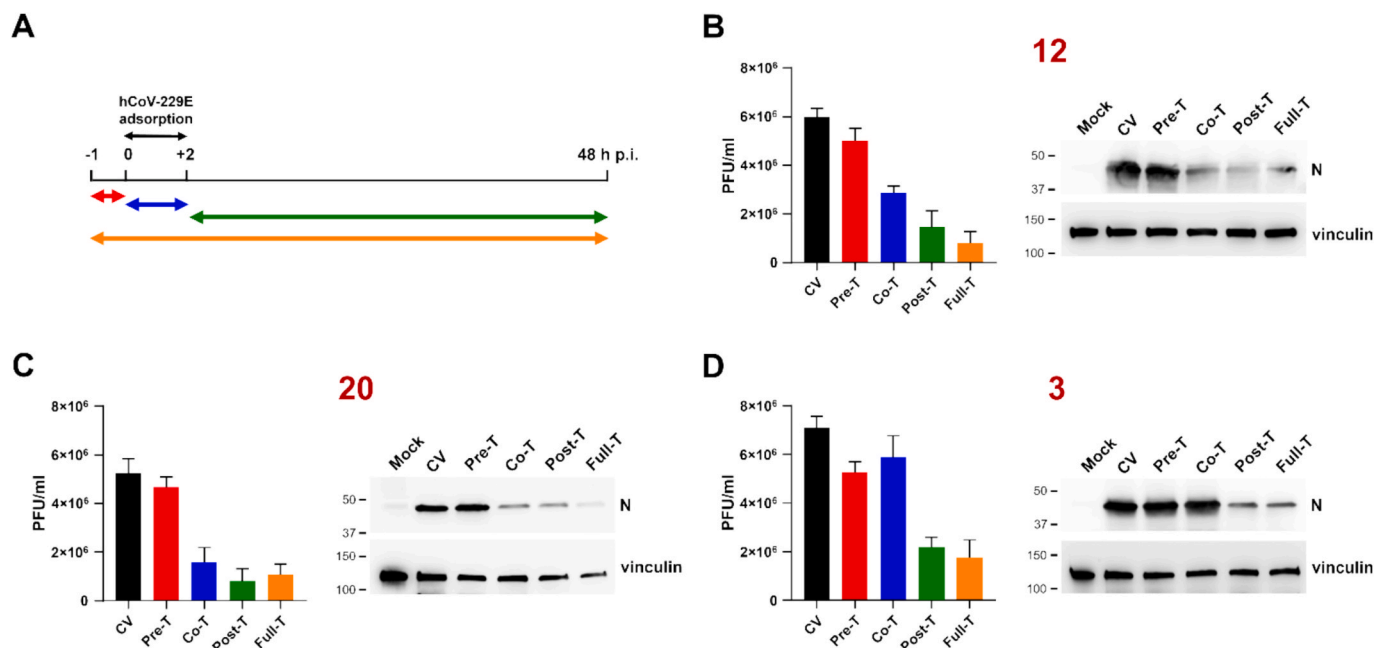


Fig. 6. Effects of **3**, **12** and **20** on the different stages of the hCoV replicative cycle. (A) Schematic summary of the time-of-drug-addition experiment. HuH-7 cell monolayers were infected with hCoV-229E at an MOI of 0.5 PFU/cell and, where indicated, treated with 2 xEC₅₀ **12**, **20** or **3** 1 h prior to the infection (from -1 to 0 h, Pre-T), or during the infection (from 0 to 2 h; Co-T), or after viral infection (from 2 to 48 h p.i., Post-T), or from (-1 to 48 h p.i., Full-T). Mock-infected cells (Mock) and control-hCoV-229E-infected cells (CV) were exposed to DMSO only. (B, C, and D; left panels) Cells supernatants harvested at 48 h p.i. were titrated for hCoV-OC43 infectivity by FFRA, and viral foci were microscopically counted and converted into viral titer (PFU/mL). The data shown are the means ± SD (error bars) of two independent experiments performed in triplicate and analyzed by a one-way ANOVA followed by Dunnett's multiple comparison test. ** (p < 0.001) compared to the calibrator sample (CV). (B, C, and D; right panels) Total cell protein extracts were prepared at 48 h p.i. from the very same HuH-7 cell monolayers treated and infected as depicted in the scheme A. Thereafter, protein samples (50 µg/lane) were fractionated by 4–15 % SDS-PAGE and analyzed by immunoblotting with anti-hCoV-229E N pAb. Vinculin immunodetection was used as a control for protein loading. Molecular weight markers are shown next to the left side of each panel.

Table 3

ADME and drug-likeness properties of **3**, **12** and **20**.

Compound	Solubility (ESOL)	Consensus logP	H-bond acceptors	H-bond donors	Lipinski violations	GI absorption	BB permeation	Bioavailability score
3	Mod. soluble	4.04	4	3	No	High	No	0.55
12	Mod. soluble	4.10	4	3	No	High	No	0.55
20	Poorly soluble	4.85	4	3	No	High	No	0.55

bonds (11–12) suggests moderate molecular flexibility, which can influence oral bioavailability. Regarding Lipinski's Rule of Five, **12** and **3** fully comply, while **20** presents one violation, likely due to its higher molecular weight. Veber's rule indicates that all three molecules have one violation, which may slightly affect their permeability or oral absorption. However, their bioavailability scores remain favorable at 0.55, indicating potential for good systemic exposure. The calculated lipophilicity (logP) values are within an acceptable range, suggesting good membrane permeability. None of the compounds show PAINS alerts, indicating a low risk of promiscuous binding, while the Brenk alerts suggest the presence of some structural fragments that could be optimized for improved drug-like behavior. Overall, these ADME predictions suggest that the three molecules possess promising pharmacokinetic profiles, with good absorption potential and acceptable drug-likeness. Further experimental validation of their solubility, metabolic stability and permeability will be essential to confirm their suitability for development as potential therapeutic agents.

Aqueous solubility (buffer pH 7.4) was analyzed by HPLC and the results were found to be in agreement with the *in silico* predictions, showing good solubility for compounds **3** and **12** (42.0 µM and 46.8 µM, respectively) and a lower value for compound **20** (33.6 µM). Further chemical modification will guide future compounds design to enhance aqueous solubility.

4. Discussion

In this study, we explored the potential of indole-based ethyl cinnamate derivatives as multitarget inhibitors capable of interfering with both viral replication and entry of hCoVs. Our findings underscore the importance of structural optimization, particularly the introduction of a *tert*-leucine (Tle) residue at P2 of the (pseudo)peptide structure, which proved to be a key determinant for broad-spectrum activity. The enzymatic assays confirmed the potent multitarget inhibition of SARS-CoV-2 M^{pro} and host cathepsins by the synthesized derivatives. Among the tested compounds, **12**, **20**, and **3** exhibited multitarget inhibitory activity, highlighting their potential as antiviral agents. The inclusion of an indole-based moiety at P3 played a crucial role in binding efficiency, facilitating interactions within the catalytic sites of both viral and host proteases. Notably, the presence of Tle residue at P2 was fundamental in achieving broad-spectrum inhibition, particularly by enhancing activity against cathepsins, especially CatS. This finding underscores the importance of P2 residue selection in optimizing multitarget activity and suggests that the Tle substitution provided a crucial advantage over our previously reported Phe-bearing compounds in achieving multiple inhibition. The antiviral assays validated the potential of these compounds as BSAs, demonstrating a strong inhibitory effect against both α- and β-coronaviruses. **12** (EC₅₀: 4.09 µM for hCoV-OC43, 0.77 µM for hCoV-229E), **20** (EC₅₀: 6.68 µM for hCoV-OC43, 0.62 µM for hCoV-229E), and

3 (EC₅₀: 2.96 μ M for hCoV-OC43, 0.64 μ M for hCoV-229E) emerged as the most potent compounds. Importantly, these molecules exhibited high selectivity indices (SI = 98 for **12**, 56 for **20**, and 101 for **3**), confirming that their antiviral activity was not due to cytotoxic effects. The observed broad-spectrum efficacy, particularly against hCoV-229E, was directly associated with the presence of Tle at P2. Compounds bearing this residue, such as **12** and **20**, consistently exhibited superior inhibitory activity on both viral proteases and host cathepsins, reinforcing the role of this structural feature in achieving BSA activity. Time-of-drug-addition experiments clarified the mechanism of action of **12** and **20**, revealing distinct scenarios. **12** and **20** predominantly targeted the viral entry phase, an effect directly linked to their ability to inhibit cellular CatL. Indeed, the significant reduction in viral titers observed during the co-treatment phase (0–2 h post-infection) supports the hypothesis that **12** and **20** interfere with the endosomal processing of viral spike proteins, thereby preventing efficient viral entry into host cells. However, **20** was more effective than **12** in exerting a dual antiviral mechanism against both viral entry and post-entry stages, including the biosynthetic phase of hCoV-229E replication cycle (Fig. 6C). The higher anti-M^{pro} activity of **20** (Table 1) may sustain its superior inhibitory activity observed during Post-T treatment. Clearly, for both **12** and **20**, the overall anti-CoV activity can result from their combined inhibition of CatL and M^{pro}, with the latter playing a crucial role in blocking viral polyprotein processing at post-entry stages. On the other hand, **3**, which lacks CatL inhibitory activity, was ineffective during the early infection stages; nevertheless it showed robust antiviral effects at post-entry phases, that might be due to its ability to inhibit CatS, although a clear role of this cathepsin in hCoV replication cycle remains to be defined.

Noteworthy, the results of this study highlight the pivotal role of Tle in enhancing broad-spectrum activity, suggesting that future efforts in inhibitor design should prioritize this residue to improve both multi-target engagement and antiviral efficacy.

In conclusion, this study sustains the potential as BSA agents of multitarget inhibitors that simultaneously act on both entry and virus biosynthesis pathways. Unlike single-target viral-specific DAAs agents that are prone to viral resistance, the concurrently inhibition of both viral and host cell protease activities by single molecules endowed with multitargeted capabilities reduces the likelihood of selection of resistance. Furthermore, their ability to targeted cellular enzymes required for the replication of different viruses offer a strategy for the development of new effective BSA. Relevant to this avenue, the observed pan-CoVs activity of **12** and **20** further highlights their potential in pandemic preparedness, offering a versatile strategy to mitigate future CoVs outbreaks.

5. Experimental section

5.1. Materials and methods

Chemistry. Unless otherwise stated, reagents and solvents were purchased from Merck (Milan, Italy), Fluorochem (Hadfield, United Kingdom) or BLDPharm (Reinbek, Germany) and used without further purification. All reactions were carried out in oven-dried glassware, using dry solvents, and monitored by TLC on silica gel (Merck precoated 60 F₂₅₄ plates), with detection by UV light (254 nm) or by permanganate or by HPLC. HPLC was performed on Agilent 1100 Series System using a Gemini 5 μ M C18 110 Å LC Column 150 × 3 mm and with a gradient of H₂O/ACN (+ 0.1 % HCOOH) ranging from 5 % ACN up to 100 % ACN in 30 min (flux of 1.0 mL/min and sample injection of 20 μ L), choosing 220 nm as the wavelength for the detection of compounds. Products were purified by flash column chromatography, using silica gel Merck 60 (230–400 mesh) as the stationary phase. Purity of the final compounds was assured to be > 95 % as assessed by NMR or HPLC. ¹H NMR and ¹³C NMR spectra were recorded at 298 K on a Bruker Avance Spectrometer (400 MHz), using commercially available deuterated

solvents (DMSO-*d*₆, Chloroform-*d*, MeOD, CD₂Cl₂). Chemical shifts are reported in parts per million (δ_{ppm}), compared to TMS as an internal standard. Coupling constants (*J*) are given in hertz (Hz) and are quoted to the nearest 0.5 Hz. Peak multiplicities are described in the following way: s, singlet; bs, broad singlet; d, doublet; m, multiplet; br, broad. Mass spectra were recorded on a Thermo Fisher LCQ Fleet Ion Trap Mass Spectrometer.

Biomimetic experiments. To a solution of **3** (14 mg, 0.031 mmol, 1.0 equiv) in 5 mL of PBS (pH = 7.4) with 10 % of MeOH, *N*-acetylcysteine (5.6 mg, 0.034 mmol, 1.1 equiv) was added and the mixture was left stirring for 24 h at 37 °C. The reaction was checked via HPLC-MS and the adduct of **3** with *N*-acetylcysteine was detected, as reported in Fig. S1.

Enzymes. For the biological assessments against the selected panel of cysteine proteases we used recombinant SARS-CoV-2 M^{pro} expressed and purified in our biochemistry labs according to methods previously described.⁴⁰ All cathepsins instead were obtained from commercial sources: specifically, CatB (recombinant, human liver) and CatL (*Paramecium tetraurelia*) were purchased from CALBIOCHEM, whereas CatS (recombinant, *Escherichia coli*) was purchased from SIGMA-ALDRICH, Darmstadt, Germany). All compounds underwent a fluorometric assay using the appropriate commercial peptide-based fluorogenic substrate, namely Dabcyl-KTSAVLQ↓SGFRKME-Edans (TFA salt, with arrow indicating the cleavage position; from GENESCRIPIT, New Jersey, USA) for SARS-CoV-2 M^{pro}, Cbz-Phe-Arg-AMC (from BACHEM, Bubendorf, Switzerland) for CatB and CatL, and Cbz-Val-Val-Arg-AMC (AMC = 7-Amino-4-Methyl Coumarin; from BACHEM, Bubendorf, Switzerland) for CatS. Fluorescence of the product of the substrates' hydrolysis (SGFRKME-Edans or AMC) was measured by means of a TECAN Infinite F2000 PRO plate reader (Agilent Technologies, Santa Clara, USA) equipped with proper excitation/emission filters and using white flat-bottom 96-well microtiter plates (Greiner Bio-One, Kremsmünster, Austria). Each well contained 200 μ L solution, composed of 180 μ L of reaction buffer, 5 μ L of enzyme in its proper buffer, 5 μ L of suitable fluorogenic substrate and 10 μ L of DMSO solution of the compound. The general inhibition assays of the catalytic activity of all cysteine proteases were performed at 20 μ M inhibitor concentration using an equivalent amount of DMSO as a negative control.

Docking. The crystal structures of MPRO and Cathepsins were obtained from the Protein Data Bank, PDB IDs: 6LZE (M^{pro}),³² 2XU3 (CatL),⁴¹ 4MZO (CatS)⁴² and 8B4T (CatB). For all the ligands, SMILES were used as input files. Covalent docking calculations were carried out using ICM-Pro 3.9–4,⁴³ while the input files were prepared using MolsoftBrowser64 following using default procedure described on the manual. Docking has been performed with biased probability Monte Carlo (BPMC) procedure and on the global optimization of the flexible ligand, and a weighted scoring function has been employed.^{44,45} The figures were prepared with ICM's graphical user interface.

Assaying the catalytic activity of SARS-CoV-2 M^{pro}. SARS-CoV-2 M^{pro} was incubated at 25 °C at a final concentration of 50 nM together with test compound previously solubilized in DMSO (final concentration 20 μ M) and fluorogenic substrate (final concentration 25 μ M). The reaction and enzyme buffer consisted of 20 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT, and 200 mM NaCl (pH = 7.5). Product release from substrate hydrolysis was detected with a 335 nm excitation filter and a 493 nm emission filter, and monitored continuously (30 s increments) over a period of 10 min.

Assaying the catalytic activity of CatB. CatB was incubated at 25 °C at a final concentration of 58 pg mL^{−1} together with test compound previously solubilized in DMSO (final concentration 20 μ M) and fluorogenic substrate (final concentration 80 μ M). The reaction buffer consisted of 50 mM Tris-HCl, 5 mM EDTA, 200 mM NaCl, and 0.005 % Brij 35 (pH = 6.5), whereas the enzyme buffer was the same with the addition of 2 mM DTT 35 but without Brij 35. Product release from substrate hydrolysis was detected with a 365 nm excitation filter and a 460 nm emission filter, and monitored continuously (30 s increments) over a period of 10 min.

Assaying the catalytic activity of CatL. CatL was incubated at 25 °C at a final concentration of 53 ng mL⁻¹ together with test compound previously solubilized in DMSO (final concentration 20 μM) and fluorogenic substrate (final concentration 5 μM). The reaction buffer consisted of 50 mM Tris-HCl, 5 mM EDTA, 200 mM NaCl, and 0.005 % Brij 35 (pH = 6.5), whereas the enzyme buffer was the same with the addition of 2 mM DTT 35 but without Brij 35. Product release from substrate hydrolysis was detected with a 365 nm excitation filter and a 460 nm emission filter, and monitored continuously (30 s increments) over a period of 10 min.

Assaying the catalytic activity of CatS. CatS was incubated at 25 °C at a final concentration of 15 nM together with test compound previously solubilized in DMSO (final concentration 20 μM) and fluorogenic substrate (final concentration 10 μM). The reaction buffer consisted of 50 mM KH₂PO₄, 50 mM K₂HPO₄, 2.5 mM DTT, and 2.5 mM EDTA (pH = 6.5). The enzyme buffer consisted of 35 mM KH₂PO₄, 35 mM CH₃COONa, 2 mM DTT, and 2 mM EDTA (pH = 6.5). CatS and was incubated with the enzyme buffer for ~ 30 min at 25 °C prior to use. Then, mixed with the reaction buffer followed by sequential addition of the inhibitor dissolved in DMSO and fluorogenic substrate. Product release from substrate hydrolysis was detected with a 380 nm excitation filter and a 460 nm emission filter, and monitored continuously (30 s increments) over a period of 10 min.

Cells and viruses. The human ileocecal adenocarcinoma cell line HCT-8 (ATCC CCL-244) and the human lung fibroblast cells MRC5 (ATCC CCL-171) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The human liver cancer cell line HuH-7 (JCRB0403) was purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank). HCT-8 cells were cultured in RPMI (Euroclone, Pero (MI), Italy), while MRC-5 and HuH-7 cells were maintained in high or low glucose Dulbecco's modified Eagle medium (DMEM, Euroclone), respectively. Growth media contained 10 % (v/v) fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate (Euroclone).

Human coronavirus OC43 (hCoV-OC43, ATCC VR-1558) and human coronavirus 229E (hCoV-229E, ATCC VR-740) were purchased from ATCC. hCoV-OC43 was propagated and titrated in HCT-8 cells and hCoV-229E was amplified in MRC5 cells and titrated on HuH-7 cells, as previously described.⁴⁶

Antiviral Assays. The anti-coronavirus activity of cinnamate compounds was assessed against hCoV-OC43 and hCoV-229E by the focus-forming reduction assay (FFRA). FFRA were performed in HCT8 cells for hCoV-OC43 and in HuH-7 cells for hCoV-229E. Briefly, cells were seeded in 96-well plate (30,000 cells/well) and, after 24 h, pretreated and treated with vehicle (0.1 % DMSO) or with different concentrations (0.05, 0.1, 0.5, 1, 5, 10, 25 or 50 μM) of the indicated compounds 1 h prior to and during the infection with hCoV-OC43 or hCoV-229E (50 PFU/well). Following virus adsorption (2 h at 33 °C), infected cell cultures were incubated in medium containing the corresponding compounds plus 1 % (v/v) FBS and methylcellulose (Merck) at concentrations of 1 % (w/v) for hCoV-OC43 or 1.5 % (w/v) for hCoV-229E. At 72 h post-infection (h p.i.) for hCoV-OC43 or at 48 h p.i. for hCoV-229E, cell monolayers were fixed and subjected to indirect immunoperoxidase staining (IPA) with a monoclonal antibody (mAb) against the hCoV-OC43 nucleoprotein (N) (Millipore, clone 542-D7; Burlington, MA, USA) (diluted 1:100) or using a polyclonal antibody (pAb) against the hCoV-229E N protein (Sino Biological, 40640-T62; Beijing, China) (diluted 1:100). Viral foci were microscopically counted, and the mean plaque counts for each drug concentration were expressed as a percentage of the mean plaque counts of control virus treated with vehicle (0.1 % DMSO). GraphPad Prism software version 8.0 was used to determine the concentration of compounds producing 50 and 90 % reductions in viral foci formation (EC₅₀ and EC₉₀).

For time-of-drug-addition experiments, HuH-7 cells were seeded in 6-well plates (300,000 cells/well). The following day, as summarized in Fig. 6, cells were exposed to **12**, **20** or **3** from -1 to 0 h prior to infection

(pre-treatment, Pre-T); or during infection with hCoV-229E at an MOI of 0.5 PFU/cell (from 0 to 2 h; co-treatment, Co-T); or after viral infection (from 2 to 48 h p.i.; post-treatment, Post-T); or during all phases (full treatment, Full-T). At 48 h p.i., the cell supernatants were harvested and titrated for hCoV-229E infectivity by FFRA as described above.

Cytotoxicity Assay. HCT-8 and HuH-7 cells were seeded on 96-well plates 24h before the treatment with increasing concentrations of compounds or the vehicle (0.4 % DMSO) as a control. Cytotoxicity was evaluated after 48 h for HuH-7 cells or after 72 h for HCT-8 cells, using the CellTiter-Glo assay (Promega, Milan, Italy) as previously described.⁴⁷ The cytotoxicity of the tested compounds was expressed as cytotoxic concentration (CC₅₀).

Immunoblotting. Total cell protein extracts were prepared at 48 h p.i. from HuH-7 cell monolayers treated and infected as described above for the time-of-drug-addition experiments. Thereafter, equal amounts of protein extracts were fractionated by 4–15 % stain-free precast gels and transferred to PVDF membranes (Bio-Rad (Hercules, CA, USA). Filters were blocked for 2 h at 37 °C in EveryBlot Blocking Buffer (Bio-Rad) and then immunostained with the rabbit anti- hCoV-229E N protein pAb (diluted 1:1500). The rabbit anti-vinculin mAb (18779, Cell Signaling) (diluted 1:1000) was used as the control for protein loading. Immuno-complexes were detected with a goat anti-rabbit Ig Ab conjugated to horseradish peroxidase (Life Technologies, Carlsbad, CA, USA) and visualized by enhanced chemiluminescence (Clarity Western ECL, Bio-Rad).

Aqueous solubility. Samples were diluted in DMSO (control) and phosphate buffer pH = 7.4, from 5 mM stock solutions (dissolved in DMSO), to 120 μM final concentration. The samples were incubated for 24 h at rt, followed by 30 min of centrifugation at 3500 rpm. Two individual sample solutions were made and measured. 40 μL of the supernatants were injected into HPLC (gradient elution, eluent A 0.1 % formic acid in H₂O, eluent B acetonitrile) and the AUC (Absorbance Unit under the Curve) values (measured on sample-specific wavelength) of the buffered samples were divided by the AUC values (same wave length as buffered samples) of the DMSO control samples.

5.2. Spectral and characterization data

5.2.1. Procedures and characterization data of intermediates A–F

A. Boc-Phe-OH (1.67 g, 6.29 mmol, 1.2 equiv), DMAP (958 mg, 7.84 mmol, 1.5 equiv), EDCI (3.0 g, 15.7 mmol, 3.0 equiv) and *trans*-ethyl 4-aminocinnamate (1.0 g, 5.23 mmol, 1.0 equiv) were dissolved in dry DCM (20 mL) and the solution was left stirring at rt overnight. The reaction mixture was then diluted with ethyl acetate (30 mL) and washed with saturated aq. solution of 1 M HCl (3 × 20 mL), saturated aq. solution of NaHCO₃ (3 × 20 mL) and brine (3 × 20 mL). The organic layer was dried with anhydrous Na₂SO₄, filtered and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (4:6, v/v) as eluent to afford **A** as a white amorphous solid (49 % yield, 1.64 g). ¹H NMR (400 MHz, CDCl₃): δ 8.07 (bs, 1H, -NH), 7.61 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 7.43 (d, *J* = 8.9 Hz, 2H, Ar H), 7.40 (d, *J* = 8.9 Hz, 2H, Ar H), 7.33–7.23 (m, 5H, Ar H), 6.34 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 5.14 (bs, 1H, -NH), 4.48 (bs, 1H, a-CH), 4.25 (q, *J* = 7.1 Hz, 2H, -CH₂CH₃), 3.15 (m, 2H, Ar-CH₂CH), 1.42 (s, 9H, -(CH₃)₃), 1.33 (t, *J* = 7.1 Hz, 3H, -CH₂CH₃). MS (ESI), *m/z* [M + H]⁺: 439.4. Spectral data match with the ones reported.³⁰

B. Boc-Tle-OH (726 mg, 3.14 mmol, 1.2 equiv), DMAP (479 mg, 3.92 mmol, 1.5 equiv), EDCI (1.5 g, 7.84 mmol, 3.0 equiv) and *trans*-ethyl 4-aminocinnamate (500 mg, 2.61 mmol, 1.0 equiv) were dissolved in dry DCM (15 mL) and the solution was left stirring at rt overnight. The solvent was removed under reduced pressure and the crude was treated with 1 M HCl. The precipitate was collected by filtration, washed with water and dried under *vacuum* to afford the pure **B** (87 % yield, 924 mg). ¹H NMR (400 MHz, CDCl₃): δ 8.31 (s, 1H, -NH), 7.61 (d, *J* = 16.0, 1H, Ar-CH=CH-), 7.53 (dd, *J* = 8.5, 5.8 Hz, 2H, Ar H), 7.44 (dd, *J* = 8.5, 4.7 Hz, 2H, Ar H), 6.34 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 5.42 (s, 1H, -NH),

4.25 (q, $J = 7.1$ Hz, 2H, $-\text{CH}_2\text{CH}_3$), 4.07 (bs, 1H, $\alpha\text{-CH}$), 1.44 (s, 9H, $-\text{C}(\text{CH}_3)_3$), 1.33 (t, $J = 7.1$ Hz, 3H, $-\text{CH}_2\text{CH}_3$), 1.07 (s, 9H, $-\text{C}(\text{CH}_3)_3$). ^{13}C NMR (100 MHz, CDCl_3): δ 169.9, 167.3, 156.4, 143.9, 139.3, 130.7, 129.0, 120.0, 117.4, 80.4, 63.3, 60.6, 34.7, 28.5, 26.7, 14.5. MS (ESI), m/z $[\text{M} + \text{H}]^+$: 405.3. HRMS (ESI), m/z $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{22}\text{H}_{33}\text{N}_2\text{O}_5^+$ 405.2384; found 405.2378.

C. To a solution of **A** (1.13 g, 2.6 mmol, 1.0 equiv) in dry DCM (20 mL), TFA (7 mL) was added dropwise and the reaction mixture was left stirring for 4 h at room temperature. The solvent was evaporated under reduced pressure and the crude oil was treated with *n*-hexane. The precipitate was collected, filtered and washed with *n*-hexane to afford **C** as a pure white amorphous solid (800 mg, 91 %). ^1H NMR (400 MHz, CDCl_3): δ 9.57 (s, 1H, $-\text{NH}$), 7.64 (m, 3H, Ar H), 7.50 (m, 2H, Ar H), 7.35–7.24 (m, 4H, Ar H), 6.37 (d, $J = 16.3$ Hz, 1H, Ar-CH=CH), 4.26 (q, $J = 6.7$ Hz, 2H, $-\text{CH}_2\text{CH}_3$), 3.77 (m, 1H, $\alpha\text{-CH}$), 3.37 (m, 1H, Ar- CH_2CH), 2.82 (m, 1H, Ar- CH_2CH), 1.33 (t, $J = 7.1$ Hz, 3H, $-\text{CH}_2\text{CH}_3$). MS (ESI), m/z $[\text{M} + \text{H}]^+$: 339.9. Spectral data match with the ones reported.³⁰

D. To a solution of **B** (924 mg, 2.28 mmol, 1.0 equiv) in dry DCM (20 mL), TFA (7 mL) was added dropwise and the reaction mixture was left stirring for 4 h at room temperature. The solvent was evaporated under reduced pressure and the crude oil was treated with *n*-hexane. The precipitate was collected, filtered and washed with *n*-hexane to afford **D** as a pure yellow amorphous solid (811 mg, 85 %). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 10.69 (s, 1H, $-\text{NH}$), 8.26 (s, 3H, $-\text{NH}_3^+$), 7.73 (d, $J = 8.8$ Hz, 2H, Ar-H), 7.66 (d, $J = 8.8$ Hz, 2H, Ar-H), 7.61 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH), 6.55 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH), 4.18 (q, $J = 7.1$ Hz, 2H, $-\text{CH}_2\text{CH}_3$), 3.72 (s, 1H, $\alpha\text{-CH}$), 1.25 (t, $J = 7.1$ Hz, 3H, $-\text{CH}_2\text{CH}_3$), 1.05 (s, 9H, $-\text{C}(\text{CH}_3)_3$). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 166.5, 166.3, 143.7, 139.5, 129.9, 129.3, 119.6, 117.0, 61.2, 59.9, 33.3, 26.2, 14.2. HRMS (ESI), m/z $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{17}\text{H}_{25}\text{N}_2\text{O}_3^+$ 305.1860; found 305.1899.

E. Boc-D-Tle-OH (363 mg, 1.57 mmol, 1.2 equiv), DMAP (240 mg, 1.96 mmol, 1.5 equiv), EDCI (752 mg, 3.92 mmol, 3.0 equiv) and *trans*-ethyl 4-aminocinnamate (250 mg, 1.31 mmol, 1.0 equiv) were dissolved in dry DCM (10 mL) and the solution was left stirring at rt overnight. The reaction mixture was then diluted with ethyl acetate (30 mL) and washed with saturated aq. solution of 1 M HCl (3×20 mL), saturated aq. solution of NaHCO_3 (3×20 mL) and brine (3×20 mL). The organic layer was dried with anhydrous Na_2SO_4 , filtered and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (4:6, v/v) as eluent to afford **E** as a white amorphous solid (33 % yield, 174 mg). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 9.01 (s, 1H, $-\text{NH}$), 7.55 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 7.43 (dd, $J = 8.5$, 5.8 Hz, 2H, Ar H), 7.29 (dd, $J = 8.0$, 2H, Ar H), 6.28 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 5.63 (d, $J = 7.9$ Hz, 1H, $\alpha\text{-CH}$), 4.25 (q, $J = 7.1$ Hz, 2H, $-\text{CH}_2\text{CH}_3$), 1.43 (s, 9H, $-\text{C}(\text{CH}_3)_3$), 1.34 (t, $J = 7.1$ Hz, 3H, $-\text{CH}_2\text{CH}_3$), 1.11 (s, 9H, $-\text{C}(\text{CH}_3)_3$). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 169.9, 166.2, 155.3, 143.8, 140.4, 129.0, 128.8, 119.0, 116.2, 78.0, 62.4, 59.7, 34.0, 28.0, 26.3, 14.0. MS (ESI), m/z $[\text{M} + \text{H}]^+$: 405.3. HRMS (ESI), m/z $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{22}\text{H}_{33}\text{N}_2\text{O}_5^+$ 405.2384; found 405.2318.

F. To a solution of **E** (174 mg, 0.43 mmol, 1.0 equiv) in dry DCM (12 mL), TFA (2 mL) was added dropwise and the reaction mixture was left stirring for 4 h at room temperature. The solvent was evaporated under reduced pressure and the crude oil was treated with *n*-hexane. The precipitate was collected, filtered and washed with *n*-hexane to afford **F** as a pure brown amorphous solid (133 mg, 74 %). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 9.57 (s, 1H, $-\text{NH}$), 7.57 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH), 7.42 (d, $J = 8.7$ Hz, 2H, Ar-H), 7.39 (d, $J = 8.7$ Hz, 2H, Ar-H), 7.29 (2, 3H, NH_3^+), 6.34 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH), 4.28 (q, $J = 7.1$ Hz, 2H, $-\text{CH}_2\text{CH}_3$), 4.13 (s, 1H, $\alpha\text{-CH}$), 1.36 (t, $J = 7.1$ Hz, 3H, $-\text{CH}_2\text{CH}_3$), 1.11 (s, 9H, $-\text{C}(\text{CH}_3)_3$). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 166.6, 166.3, 143.7, 139.7, 129.9, 129.3, 119.6, 116.9, 61.1, 59.9, 33.4, 26.2, 14.2. HRMS (ESI), m/z $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{17}\text{H}_{25}\text{N}_2\text{O}_3^+$ 305.1860; found 305.1899.

5.2.2. Procedures and characterization data of final products 2–30

Ethyl (S,E)-3-(4-(2-(1H-indole-2-carboxamido)-3-phenylpropa namido)phenyl)acrylate (2). 1H-indole-2-carboxylic acid (58 mg, 0.36 mmol, 1.0 equiv), EDCI (189 mg, 0.99 mmol, 3.0 equiv), DMAP (60 mg, 0.40 mmol, 1.5 equiv), DIPEA (0.11 mL, 0.66 mmol, 2.0 equiv) and **C** (150 mg, 0.33 mmol, 1.0 equiv) were dissolved in dry DCM (8 mL) and the solution was left stirring at rt for 3 h. The reaction mixture was concentrated under reduced pressure. The crude was then treated with 1 M HCl. The precipitate was filtered to afford **2** as a white amorphous solid (82 % yield, 130 mg). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 11.52 (s, 1H, $-\text{NH}$), 10.54 (s, 1H, $-\text{NH}$), 8.84 (d, $J = 8.2$ Hz, 1H, $-\text{NH}$), 7.70 (m, 4H, Ar H), 7.63 (d, $J = 8.3$ Hz, 1H, Ar H), 7.60 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH), 7.41 (dd, $J = 10.8$, 8.0 Hz, 2H, Ar H), 7.34–7.22 (m, 4H, Ar H), 7.17 (t, $J = 7.1$ Hz, 2H, Ar H), 7.03 (t, $J = 7.5$ Hz, 1H, Ar H), 6.53 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH), 4.91 (q, $J = 8.0$ Hz, 1H, $\alpha\text{-CH}$), 4.18 (q, $J = 7.1$ Hz, 2H, $-\text{CH}_2\text{CH}_3$), 3.24–3.10 (m, 2H, Ar- CH_2CH), 1.25 (t, $J = 7.0$ Hz, 3H, $-\text{CH}_2\text{CH}_3$). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 170.8, 166.4, 161.1, 144.0, 140.9, 137.9, 136.5, 131.0, 129.3, 129.2, 129.1, 128.1, 127.0, 126.4, 123.5, 121.6, 119.8, 119.3, 116.5, 112.3, 103.6, 59.9, 55.5, 37.3, 14.2. HRMS (ESI), m/z $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{29}\text{H}_{28}\text{N}_3\text{O}_4^+$ 482.2074; found 482.2012. HPLC rt: 21.6 min.

Ethyl (S,E)-3-(4-(2-(1H-indole-2-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (3). 1H-indole-2-carboxylic acid (93 mg, 0.57 mmol, 1.2 equiv), HOBt (110 mg, 0.81 mmol, 1.7 equiv), EDCI (156 mg, 0.81 mmol, 1.7 equiv), DIPEA (0.2 mL, 1.19 mmol, 2.5 equiv) and **D** (200 mg, 0.48 mmol, 1.0 equiv) were dissolved in a mixture of DCM:DMF (10 mL, 20:1 v/v) and the solution was left stirring at rt overnight. The reaction mixture was then concentrated under reduced pressure, diluted with ethyl acetate (12 mL) and washed with aq. solution of 0.1 M HCl (3×12 mL), saturated aq. solution of NaHCO_3 (3×12 mL) and brine (3×12 mL). The organic layer was dried with anhydrous Na_2SO_4 , filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (from 6:4 v/v to 4:6 v/v) as eluent to afford **3** as a white amorphous solid (30 % yield, 65 mg). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 11.69 (s, 1H, $-\text{NH}$), 10.41 (s, 1H, $-\text{NH}$), 8.02 (d, $J = 9.1$ Hz, 1H, $-\text{NH}$), 7.73–7.61 (m, 5H, Ar H), 7.58 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 7.44 (d, 1H, Ar H), 7.38 (s, 1H, Ar H), 7.20 (t, $J = 8.0$ Hz, 1H, Ar H), 7.05 (t, $J = 8.0$ Hz, 1H, Ar H), 6.51 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 4.75 (d, $J = 9.1$ Hz, 1H, $\alpha\text{-CH}$), 4.18 (q, $J = 7.1$ Hz, 2H, $-\text{CH}_2\text{CH}_3$), 1.25 (t, $J = 7.1$ Hz, 3H, $-\text{CH}_2\text{CH}_3$), 1.08 (s, 9H, $-\text{C}(\text{CH}_3)_3$). ^{13}C NMR (100 MHz, CDCl_3): δ 169.4, 167.2, 162.2, 143.8, 139.3, 136.8, 130.9, 129.8, 129.0, 127.7, 125.2, 122.3, 121.1, 120.4, 117.6, 112.1, 104.2, 61.6, 60.6, 35.7, 26.8, 14.5. HRMS (ESI), m/z $[\text{M} + \text{H}]^+$: calculated for $\text{C}_{26}\text{H}_{30}\text{N}_3\text{O}_4^+$ 448.2231; found 448.2213. HPLC rt: 22.1 min.

Ethyl (S,E)-3-(4-(2-(1H-indole-3-carboxamido)-3-phenylpropa namido)phenyl)acrylate (4). 1H-indole-3-carboxylic acid (60 mg, 0.36 mmol, 1.15 equiv), HOBt (67 mg, 0.5 mmol, 1.5 equiv), EDCI (96 mg, 0.5 mmol, 1.5 equiv), DIPEA (0.11 mL, 0.66 mmol, 2.0 equiv) and **C** (150 mg, 0.33 mmol, 1.0 equiv) were dissolved in dry DCM (10 mL) and the solution was left stirring at rt for 3 h. The reaction mixture was then concentrated under reduced pressure, diluted with ethyl acetate (12 mL) and washed with 1 M HCl (3×12 mL), saturated aq. solution of NaHCO_3 (3×12 mL) and brine (3×12 mL). The organic layer was dried with anhydrous Na_2SO_4 , filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (1:1 v/v) as eluent to afford **4** as a white amorphous solid (60 % yield, 95 mg). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 11.59 (s, 1H, $-\text{NH}$), 8.17 (s, 1H, $-\text{NH}$), 8.04 (d, $J = 7.9$ Hz, 1H, $-\text{NH}$), 7.69 (s, 4H, Ar H), 7.59 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 7.42 (d, $J = 7.7$ Hz, 3H, Ar H), 7.28 (t, $J = 7.5$ Hz, 3H, Ar H), 7.17 (t, $J = 8.1$ Hz, 2H, Ar H), 7.12 (d, $J = 7.8$ Hz, 1H, Ar H), 7.06 (t, $J = 7.5$ Hz, 1H, Ar H), 6.53 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 4.89 (dd, $J = 9.9$, 5.3 Hz, 1H, $\alpha\text{-CH}$), 4.17 (q, $J = 7.1$ Hz, 2H, $-\text{CH}_2\text{CH}_3$), 3.19–3.01 (m, 2H, Ar- CH_2CH), 1.25 (t, $J = 7.1$ Hz, 3H, $-\text{CH}_2\text{CH}_3$). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 171.4, 166.3, 164.6, 144.0, 141.0, 138.1, 136.0, 129.2, 129.2, 128.5, 128.1, 126.3, 126.1,

121.9, 120.9, 120.4, 119.2, 119.1, 116.4, 111.8, 109.8, 59.9, 55.1, 37.3, 14.2. HRMS (ESI), m/z $[M + H]^+$: calculated for $C_{29}H_{28}N_3O_4^+$ 482.2074; found 482.2109. HPLC rt: 20.2 min.

Ethyl (S,E)-3-(4-(2-(1H-indole-3-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (5). *H*-indole-3-carboxylic acid (85 mg, 0.53 mmol, 1.2 equiv), HOBt (110 mg, 0.81 mmol, 1.7 equiv), EDCI (143 mg, 0.75 mmol, 1.7 equiv), DIPEA (0.1 mL, 0.66 mmol, 1.5 equiv) and **D** (184 mg, 0.44 mmol, 1.0 equiv) were dissolved in a mixture of DCM:DMF (21 mL, 20:1 v/v) and the solution was left stirring at rt overnight. The reaction mixture was then concentrated under reduced pressure, diluted with ethyl acetate (12 mL) and washed with aq. solution of 0.1 M HCl (3×12 mL), aq. solution of 2 M K_2CO_3 (3×12 mL) and brine (3×12 mL). The organic layer was dried with anhydrous Na_2SO_4 , filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (1:1 v/v) as eluent to afford **5** as a white amorphous solid (19 % yield, 36 mg). ^1H NMR (400 MHz, CD_2Cl_2): δ 9.68 (s, 1H, -NH), 9.23 (s, 1H, -NH), 8.00 (d, $J = 7.3$ Hz, 1H, Ar H), 7.79 (d, $J = 2.8$ Hz, 1H, Ar H), 7.57–7.38 (m, 4H, Ar H), 7.23 (dd, $J = 16.0$ Hz, 7.3 Hz, 4H, Ar H), 6.96 (d, $J = 9.1$ Hz, 1H, -NH), 6.24 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 5.10 (d, $J = 9.1$ Hz, 1H, α -CH), 4.20 (q, $J = 7.1$ Hz, 2H, -CH₂-CH₃), 1.29 (t, $J = 7.1$ Hz, 3H, -CH₂-CH₃), 1.23 (s, 9H, -C(CH₃)₃). ^{13}C NMR (100 MHz, CD_2Cl_2): δ 170.7, 167.5, 166.0, 144.2, 140.4, 138.6, 137.0, 130.7, 129.2, 129.0, 125.3, 123.6, 122.4, 120.7, 120.5, 117.5, 112.6, 61.7, 60.9, 35.7, 27.2, 14.7. HRMS (ESI), m/z $[M + H]^+$: calculated for $C_{26}H_{30}N_3O_4^+$ 448.2231; found 448.2272. HPLC rt: 16.4 min.

Ethyl (S,E)-3-(4-(2-(1H-indole-4-carboxamido)-3-phenylpropanamido)phenyl)acrylate (6). *H*-indole-4-carboxylic acid (109 mg, 0.68 mmol, 1.15 equiv), HOBt (119 mg, 0.88 mmol, 1.5 equiv), EDCI (170 mg, 0.88 mmol, 1.5 equiv), DIPEA (0.15 mL, 0.88 mmol, 1.5 equiv) and **C** (262 mg, 0.59 mmol, 1.0 equiv) were dissolved in a mixture of DCM:DMF (21 mL, 20:1 v/v) and the solution was left stirring at rt overnight. The reaction mixture was then concentrated under reduced pressure, diluted with ethyl acetate (12 mL) and washed with saturated aq. solution of K_2CO_3 (3×12 mL). The reaction mixture was then concentrated under reduced pressure, dissolved in a few drops of DMF, and precipitated with 1 M HCl to afford **6** as a white amorphous solid (60 % yield, 171 mg). ^1H NMR (400 MHz, DMSO- d_6): δ 11.34 (s, 1H, -NH), 10.60 (s, 1H, -NH), 8.45 (d, $J = 7.9$ Hz, 1H, -NH), 7.79–7.64 (m, 4H, Ar H), 7.60 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 7.57–7.49 (m, 1H, Ar H), 7.49–7.33 (m, 4H, Ar H), 7.29 (t, $J = 7.5$ Hz, 2H, Ar H), 7.23–7.16 (m, 1H, Ar H), 7.13 (t, $J = 7.7$ Hz, 1H, Ar H), 6.72 (d, $J = 3.1$ Hz, 1H, Ar H), 6.53 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 4.95 (td, $J = 8.4, 5.8$ Hz, 1H, α -CH), 4.18 (q, $J = 7.1$ Hz, 2H, -CH₂-CH₃), 3.19 (q, $J = 6.7$ Hz, 2H, Ar-CH₂-CH), 1.25 (t, $J = 7.1$ Hz, 3H, -CH₂-CH₃). ^{13}C NMR (100 MHz, DMSO- d_6): δ 171.0, 167.9, 166.4, 144.0, 141.0, 138.0, 136.5, 129.3, 129.2, 129.0, 128.1, 126.5, 126.4, 125.8, 125.7, 120.0, 119.3, 119.0, 116.4, 114.4, 101.6, 59.9, 55.8, 37.2, 14.2. HRMS (ESI), m/z $[M + H]^+$: calculated for $C_{29}H_{28}N_3O_4^+$ 482.2074; found 482.2118. HPLC rt: 19.9 min.

Ethyl (S,E)-3-(4-(2-(1H-indole-4-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (7). *H*-indole-4-carboxylic acid (105 mg, 0.65 mmol, 1.2 equiv), HOBt (124 mg, 0.92 mmol, 1.7 equiv), EDCI (176 mg, 0.92 mmol, 1.7 equiv), DIPEA (0.14 mL, 0.81 mmol, 1.5 equiv) and **D** (225 mg, 0.54 mmol, 1.0 equiv) were dissolved in dry DCM (10 mL) and the solution was left stirring at rt overnight. The reaction mixture was then concentrated under reduced pressure, dissolved in a few drops of DMF, and precipitated with 1 M HCl. The obtained precipitate was purified by column chromatography using *n*-hexane/ethyl acetate (1:1 v/v) as eluent to afford **7** as a white amorphous solid (22 % yield, 54 mg). ^1H NMR (400 MHz, DMSO- d_6): δ 11.40 (s, 1H, -NH), 10.45 (s, 1H, -NH), 7.73–7.61 (m, 5H, Ar H, -NH), 7.59 (dd, $J = 16.0, 4.0$ Hz, 2H, Ar H, Ar-CH=CH-), 7.54–7.48 (m, 2H, Ar H), 7.17 (t, $J = 7.7$ Hz, 1H, Ar H), 6.79 (bs, 1H, Ar H), 6.52 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 4.76 (d, $J = 9.2$ Hz, 1H, α -CH), 4.18 (q, $J = 7.1$ Hz, 2H, -CH₂-CH₃), 1.25 (t, $J = 7.1$ Hz, 3H, -CH₂-CH₃), 1.08 (s, 9H, -C(CH₃)₃). ^{13}C

NMR (100 MHz, DMSO- d_6): δ 169.8, 167.7, 166.3, 143.9, 140.5, 136.5, 129.2, 127.0, 126.0, 125.3, 120.3, 119.4, 118.1, 116.5, 115.6, 114.6, 100.6, 64.9, 59.9, 34.7, 26.7, 14.2. HRMS (ESI), m/z $[M + H]^+$: calculated for $C_{26}H_{30}N_3O_4^+$ 448.2231; found 448.2299. HPLC rt: 20.5 min.

Ethyl (S,E)-3-(4-(2-(1H-indole-5-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (8). *H*-indole-5-carboxylic acid (41 mg, 0.26 mmol, 1.2 equiv), HOBt (48 mg, 0.36 mmol, 1.7 equiv), EDCI (70 mg, 0.36 mmol, 1.7 equiv), DIPEA (0.06 mL, 0.32 mmol, 1.5 equiv) and **D** (90 mg, 0.21 mmol, 1.0 equiv) were dissolved in dry DMF (5 mL) and the solution was left stirring at rt overnight. The reaction mixture was then diluted with ethyl acetate (12 mL) and washed with saturated aq. solution of NH_4Cl (3×10 mL), saturated aq. solution of $NaHCO_3$ (3×10 mL) and brine (3×10 mL). The organic layer was dried with anhydrous Na_2SO_4 , filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (4:6, v/v) as eluent to afford **8** as a white amorphous solid (39 % yield, 37 mg). ^1H NMR (400 MHz, MeOD): δ 8.15 (d, $J = 1.6$ Hz, 1H, Ar H), 7.71–7.54 (m, 6H, Ar H, Ar-CH=CH-), 7.45 (d, $J = 8.6$ Hz, 1H, Ar H), 7.33 (d, $J = 3.2$ Hz, 1H, Ar H), 6.58 (dd, $J = 3.2, 0.9$ Hz, 1H, Ar H), 6.45 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 4.75 (s, 1H, α -CH), 4.23 (q, $J = 7.1$ Hz, 2H, -CH₂-CH₃), 1.31 (t, $J = 7.1$ Hz, 3H, -CH₂-CH₃), 1.16 (s, 9H, -C(CH₃)₃). -NH protons signals are missing for exchange with solvent. ^{13}C NMR (100 MHz, MeOD): δ 171.8, 171.4, 168.8, 145.5, 141.4, 139.7, 131.7, 130.0, 129.1, 127.4, 126.1, 121.5, 121.4, 117.9, 112.2, 103.6, 63.0, 61.6, 36.3, 27.2, 14.6. HRMS (ESI), m/z $[M + H]^+$: calculated for $C_{26}H_{30}N_3O_4^+$ 448.2231; found 448.2166. HPLC rt: 20.0 min.

Ethyl (S,E)-3-(4-(2-(1H-indole-6-carboxamido)-3-phenylpropanamido)phenyl)acrylate (9). *H*-indole-6-carboxylic acid (52 mg, 0.32 mmol, 1.15 equiv), HATU (156 mg, 0.421 mmol, 1.5 equiv), DIPEA (0.1 mL, 0.561 mmol, 2.0 equiv) and **C** (99 mg, 0.22 mmol, 1.0 equiv) were dissolved in dry DCM (10 mL) and the solution was left stirring at rt overnight. The reaction mixture was then diluted with ethyl acetate (12 mL) and washed with saturated aq. solution of 0.1 M HCl (3×12 mL), saturated aq. solution of $NaHCO_3$ (3×12 mL) and brine (3×12 mL). The organic layer was dried with anhydrous Na_2SO_4 , filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using DCM/MeOH (98:2 v/v) as eluent to afford **9** as a white amorphous solid (68 % yield, 92 mg). ^1H NMR (400 MHz, DMSO- d_6): δ 11.38 (s, 1H, -NH), 10.40 (s, 1H, -NH), 8.60 (t, $J = 7.4$ Hz, 1H, Ar H), 7.94 (s, 1H, Ar H), 7.72–7.12 (m, 13H, Ar H), 6.51 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 6.48 (bs, 1H, -NH), 4.87 (q, $J = 7.5$ Hz, 1H, α -CH), 4.17 (q, $J = 7.1$ Hz, 2H, -CH₂-CH₃), 3.15 (d, $J = 7.5$ Hz, 2H, Ar-CH₂-CH), 1.24 (t, $J = 7.1$ Hz, 3H, -CH₂-CH₃). ^{13}C NMR (100 MHz, DMSO- d_6): δ 171.4, 167.8, 166.5, 144.1, 142.2, 141.0, 138.4, 138.2, 135.2, 131.0, 130.2, 129.4, 128.3, 126.7, 126.5, 119.4, 118.7, 118.4, 116.5, 111.6, 101.4, 60.1, 56.1, 37.2, 14.3. HRMS (ESI), m/z $[M + H]^+$: calculated for $C_{29}H_{28}N_3O_4^+$ 482.2074; found 482.2088. HPLC rt: 20.3 min.

Ethyl (S,E)-3-(4-(2-(1H-indole-6-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (10). *H*-indole-6-carboxylic acid (108 mg, 0.66 mmol, 1.2 equiv), HOBt (48 mg, 0.36 mmol, 1.7 equiv), EDCI (181 mg, 0.94 mmol, 1.7 equiv), DIPEA (0.14 mL, 0.82 mmol, 1.5 equiv) and **D** (230 mg, 0.55 mmol, 1.0 equiv) were dissolved in dry DCM (15 mL) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude was dissolved with few drops of DMF and treated with 0.1 M HCl. The obtained precipitate was purified by column chromatography using *n*-hexane/ethyl acetate (6:4, v/v) as eluent to afford **10** as a white amorphous solid (35 % yield, 86 mg). ^1H NMR (400 MHz, DMSO- d_6): δ 11.34 (s, 1H, -NH), 10.40 (s, 1H, -NH), 7.98 (s, 1H, Ar H), 7.84 (d, $J = 9.1$ Hz, 1H, -NH), 7.74–7.45 (m, 8H, Ar H), 6.52 (d, $J = 16.0$ Hz, 1H, Ar-CH=CH-), 6.49 (bs, 1H, Ar H), 4.70 (d, $J = 9.1$ Hz, 1H, α -CH), 4.17 (q, $J = 7.1$ Hz, 2H, -CH₂-CH₃), 1.25 (t, $J = 7.1$ Hz, 3H, -CH₂-CH₃), 1.08 (s, 9H, -C(CH₃)₃). ^{13}C NMR (100 MHz, DMSO- d_6): δ 169.9, 167.8, 166.3, 143.9, 140.6, 135.1, 130.0, 129.2, 129.1, 128.1, 126.9, 119.4, 119.3, 118.3, 116.4, 111.6, 101.2,

61.4, 59.9, 34.6, 26.8, 14.2. HRMS (ESI), m/z [M + H]⁺: calculated for C₂₆H₃₀N₃O₄⁺ 448.2231; found 448.2192. HPLC rt: 20.9 min.

Ethyl (S,E)-3-(4-(2-(1H-indole-7-carboxamido)-3-phenylpropanamido)phenyl)acrylate (11). 1H-indole-7-carboxylic acid (81 mg, 0.44 mmol, 1.15 equiv), HOBt (89 mg, 0.66 mmol, 1.5 equiv), EDCI (126 mg, 0.66 mmol, 1.5 equiv), DIPEA (0.1 mL, 0.66 mmol, 1.5 equiv) and **C** (200 mg, 0.44 mmol, 1.0 equiv) were dissolved in a mixture of DCM:DMF (16 mL, 15:1 v/v) and the solution was left stirring at rt overnight. The reaction mixture was then concentrated under reduced pressure and treated with 0.1 M HCl. The obtained precipitate was filtered, washed with H₂O (1 x 5 mL) and treated with diethyl ether to afford **11** as a white amorphous solid (70 % yield, 148 mg). ¹H NMR (400 MHz, CDCl₃): δ 10.06 (s, 1H, -NH), 8.38 (s, 1H, -NH), 7.81 (d, *J* = 7.8 Hz, 1H, -NH), 7.60 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 7.40 (s, 5H, Ar H), 7.33–7.16 (m, 7H, Ar H), 7.07 (t, *J* = 7.7 Hz, 1H, Ar H), 6.57 (dd, *J* = 3.2, 2.2 Hz, 1H, Ar H), 6.33 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 5.09 (q, *J* = 7.3 Hz, 1H, α-CH), 4.25 (q, *J* = 7.1 Hz, 2H, -CH₂-CH₃), 3.42–3.21 (m, 2H, Ar-CH₂-CH), 1.33 (t, *J* = 7.1 Hz, 3H, -CH₂-CH₃). ¹³C NMR (100 MHz, CDCl₃): δ 169.6, 168.5, 167.2, 143.9, 139.2, 136.5, 135.4, 130.8, 129.8, 129.5, 129.1, 129.0, 127.5, 125.8, 125.7, 120.1, 119.7, 119.0, 117.5, 115.0, 102.4, 60.6, 55.7, 38.3, 14.5. HRMS (ESI), m/z [M + H]⁺: calculated for C₂₉H₂₈N₃O₄⁺ 482.2074; found 482.2053. HPLC rt: 24.8 min.

Ethyl (S,E)-3-(4-(2-(1H-indole-7-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (12). 1H-indole-7-carboxylic acid (105 mg, 0.65 mmol, 1.2 equiv), HOBt (91 mg, 0.67 mmol, 1.2 equiv), EDCI (129 mg, 0.67 mmol, 1.2 equiv), DIPEA (0.14 mL, 0.81 mmol, 1.5 equiv) and **D** (226 mg, 0.54 mmol, 1.0 equiv) were dissolved in dry DCM (20 mL) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude was dissolved with few drops of DMF and treated with 0.1 M HCl. The obtained precipitate was purified by column chromatography using *n*-hexane/ethyl acetate (7:3, v/v) as eluent to afford **12** as a white amorphous solid (45 % yield, 109 mg). ¹H NMR (400 MHz, CDCl₃): δ 10.01 (s, 1H, -NH), 8.66 (s, 1H, -NH), 7.87 (d, *J* = 7.8 Hz, 1H, Ar H), 7.64–7.53 (m, 2H, Ar H), 7.49–7.34 (m, 4H, Ar H), 7.30–7.24 (m, 2H, Ar H), 7.17 (t, *J* = 7.7 Hz, 1H, Ar H), 6.64–6.58 (m, 1H, -NH), 6.34 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 4.90 (d, *J* = 9.2 Hz, 1H, α-CH), 4.28 (q, *J* = 7.1 Hz, 2H, -CH₂-CH₃), 1.35 (t, *J* = 7.1 Hz, 3H, -CH₂-CH₃), 1.24 (s, 9H, -C(CH₃)₃). ¹³C NMR (100 MHz, CDCl₃): δ 169.6, 168.5, 167.2, 143.9, 139.1, 135.2, 130.8, 129.7, 129.0, 125.8, 125.5, 120.1, 119.9, 119.2, 117.5, 115.8, 102.5, 61.6, 60.6, 35.6, 26.9, 14.5. HRMS (ESI), m/z [M + H]⁺: calculated for C₂₆H₃₀N₃O₄⁺ 448.2231; found 448.2308. HPLC rt: 22.4 min.

Ethyl (S,E)-3-(4-(2-(5-chloro-1H-indole-2-carboxamido)-3-phenylpropanamido)phenyl)acrylate (13). 5-Chloro-1H-indole-2-carboxylic acid (104 mg, 0.53 mmol, 1.2 equiv), HOBt (101 mg, 0.75 mmol, 1.7 equiv), EDCI (143 mg, 0.75 mmol, 1.7 equiv), DIPEA (0.1 mL, 0.66 mmol, 1.5 equiv) and **C** (200 mg, 0.44 mmol, 1.0 equiv) were dissolved in dry DCM (15 mL) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude was dissolved with few drops of DMF and treated with 1 M HCl. The precipitate was filtered, washed with water, and purified by column chromatography using DCM/MeOH (98:2, v/v) as eluent to afford **13** as a white amorphous solid (73 % yield, 167 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.69 (s, 1H, -NH), 10.47 (s, 1H, -NH), 8.87 (t, *J* = 7.2 Hz, 1H, -NH), 7.73–7.61 (m, 5H, Ar H), 7.58 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 7.44–7.36 (m, 3H, Ar H), 7.29–7.21 (m, 3H, Ar H), 7.20–7.12 (m, 2H, Ar H), 6.51 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 4.90 (m, 1H, α-CH), 4.17 (q, *J* = 7.1 Hz, 2H, -CH₂-CH₃), 3.22–3.01 (m, 2H, Ar-CH₂-CH), 1.24 (t, *J* = 7.1 Hz, 3H, -CH₂-CH₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 170.8, 166.6, 161.0, 144.1, 140.9, 138.0, 135.0, 132.6, 131.1, 129.4, 128.3, 128.2, 126.6, 124.4, 123.8, 120.8, 119.5, 118.8, 116.6, 114.1, 103.3, 60.1, 55.7, 37.3, 14.4. HRMS (ESI), m/z [M + H]⁺: calculated for C₂₉H₂₇ClN₃O₄⁺ 516.1685; found 516.1721. HPLC rt: 23.0 min.

Ethyl (S,E)-3-(4-(2-(5-chloro-1H-indole-2-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (14). 5-Chloro-1H-indole-2-

carboxylic acid (78 mg, 0.40 mmol, 1.2 equiv), HOBt (75 mg, 0.57 mmol, 1.7 equiv), EDCI (110 mg, 0.57 mmol, 1.7 equiv), DIPEA (0.1 mL, 0.66 mmol, 2.0 equiv) and **D** (138 mg, 0.33 mmol, 1.0 equiv) were dissolved in dry DCM (15 mL) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude was dissolved with few drops of DMF and treated with 1 M HCl. The crude was treated with diethyl ether and the precipitate was purified by column chromatography using DCM/MeOH (95:5, v/v) as eluent to afford **14** as a white amorphous solid (68 % yield, 110 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.91 (s, 1H, -NH), 10.45 (s, 1H, -NH), 8.21 (d, *J* = 9.2 Hz, 1H, -NH), 7.67 (m, 5H, Ar H), 7.59 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 7.45 (d, *J* = 8.7 Hz, 1H, Ar H), 7.41 (bs, 1H, Ar H), 7.20 (dd, *J* = 8.7, 2.1 Hz, 1H, Ar H), 6.52 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 4.75 (d, *J* = 9.2 Hz, 1H, α-CH), 4.17 (q, *J* = 7.1 Hz, 2H, -CH₂-CH₃), 1.25 (t, *J* = 7.1 Hz, 3H, -CH₂-CH₃), 1.07 (s, 9H, -C(CH₃)₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 173.2, 169.4, 166.3, 160.7, 143.9, 140.5, 135.0, 132.4, 129.2, 129.2, 128.0, 124.2, 123.7, 120.6, 119.3, 116.5, 113.9, 104.1, 60.9, 59.9, 26.7, 14.2. HRMS (ESI), m/z [M + H]⁺: calculated for C₂₆H₂₉ClN₃O₄⁺ 482.1841; found 482.1779. HPLC rt: 23.8 min.

Ethyl (S,E)-3-(4-(2-(5-bromo-1H-indole-2-carboxamido)-3-phenylpropanamido)phenyl)acrylate (15). 5-Bromo-1H-indole-2-carboxylic acid (77 mg, 0.32 mmol, 1.2 equiv), HOBt (62 mg, 0.46 mmol, 1.7 equiv), EDCI (88 mg, 0.46 mmol, 1.7 equiv), DIPEA (0.1 mL, 0.54 mmol, 2.0 equiv) and **C** (122 mg, 0.27 mmol, 1.0 equiv) were dissolved in dry DCM (20 mL) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude was dissolved with few drops of DMF and treated with 1 M HCl. The precipitate was filtered, washed with water, and purified by column chromatography using DCM/MeOH (97:3, v/v) as eluent to afford **15** as a white amorphous solid (86 % yield, 130 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.72 (d, *J* = 2.2 Hz, 1H, -NH), 10.47 (s, 1H, -NH), 8.92 (d, *J* = 8.1 Hz, 1H, -NH), 7.88 (d, *J* = 1.9 Hz, 1H, Ar H), 7.69 (m, 4H, Ar H), 7.59 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 7.45–7.39 (m, 2H, Ar H), 7.36 (d, *J* = 8.7 Hz, 1H, Ar H), 7.32–7.21 (m, 4H, Ar H), 7.20–7.12 (m, 1H, Ar H), 6.52 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 4.91 (td, *J* = 8.8, 4.9 Hz, 1H, α-CH), 4.18 (q, *J* = 7.1 Hz, 2H, -CH₂-CH₃), 3.14 (qd, *J* = 13.7, 7.4 Hz, 2H, Ar-CH₂-CH), 1.25 (t, *J* = 7.1 Hz, 3H, -CH₂-CH₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 170.6, 166.3, 160.8, 143.9, 140.8, 137.9, 135.0, 132.3, 129.2, 129.2, 129.1, 128.8, 128.1, 126.4, 126.0, 123.8, 119.3, 116.5, 114.3, 112.2, 102.9, 59.9, 55.5, 37.2, 14.2. HRMS (ESI), m/z [M + H]⁺: calculated for C₂₉H₂₇BrN₃O₄⁺ 560.1179; found 560.1244. HPLC rt: 23.3 min.

Ethyl (S,E)-3-(4-(2-(5-bromo-1H-indole-2-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (16). 5-Bromo-1H-indole-2-carboxylic acid (103 mg, 0.43 mmol, 1.2 equiv), HOBt (82 mg, 0.61 mmol, 1.7 equiv), EDCI (117 mg, 0.61 mmol, 1.7 equiv), DIPEA (0.12 mL, 0.72 mmol, 1.5 equiv) and **D** (150 mg, 0.36 mmol, 1.0 equiv) were dissolved in dry DCM (20 mL) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude was dissolved with few drops of DMF and treated with 1 M HCl. The precipitate was filtered, washed with water, and purified by column chromatography using DCM/MeOH (98:2, v/v) as eluent to afford **16** as a white amorphous solid (53 % yield, 100 mg). ¹H NMR (400 MHz, CDCl₃): δ 9.86 (s, 1H, -NH), 8.99 (s, 1H, -NH), 7.82–7.67 (m, 1H, Ar H), 7.56 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 7.49 (d, *J* = 8.3 Hz, 2H, Ar H), 7.39–7.28 (m, 4H, Ar H), 7.27–6.93 (m, 2H, Ar H), 6.30 (dd, *J* = 16.0, 5.0 Hz, 1H, Ar-CH=CH-), 4.90 (d, *J* = 9.3 Hz, 1H, α-CH), 4.25 (q, *J* = 7.3 Hz, 2H, -CH₂-CH₃), 1.32 (t, *J* = 7.1 Hz, 3H, -CH₂-CH₃), 1.16 (s, 9H, -C(CH₃)₃). ¹³C NMR (100 MHz, CDCl₃): δ 169.5, 167.3, 161.9, 143.8, 139.1, 135.3, 131.0, 131.0, 129.3, 129.2, 129.0, 128.0, 124.6, 120.4, 117.6, 114.1, 113.6, 61.7, 60.7, 35.6, 26.9, 14.4. HRMS (ESI), m/z [M + H]⁺: calculated for C₂₆H₂₉BrN₃O₄⁺ 526.1336; found 526.1336. HPLC rt: 24.3 min.

Ethyl (S,E)-3-(4-(2-(5-methoxy-1H-indole-2-carboxamido)-3-phenylpropanamido)phenyl)acrylate (17). 5-Methoxy-1H-indole-2-

carboxylic acid (101 mg, 0.53 mmol, 1.2 equiv), HOBt (101 mg, 0.75 mmol, 1.7 equiv), EDCI (143 mg, 0.75 mmol, 1.7 equiv), DIPEA (0.1 mL, 0.66 mmol, 1.5 equiv) and **C** (200 mg, 0.44 mmol, 1.0 equiv) were dissolved in dry DCM (15 mL) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude was dissolved with few drops of DMF and treated with 1 M HCl. The precipitate was filtered, washed with water, and purified by column chromatography using *n*-hexane/ethyl acetate (6:4, v/v) as eluent to afford **17** as a white amorphous solid (67 % yield, 151 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.36 (s, 1H, -NH), 10.48 (s, 1H, -NH), 8.75 (d, *J* = 8.2 Hz, 1H, -NH), 7.70 (s, 4H, Ar H), 7.61 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 7.42 (d, *J* = 7.2 Hz, 2H, Ar H), 7.32–7.23 (m, 3H, Ar H), 7.21–7.16 (m, 2H, Ar H), 7.11 (d, *J* = 2.3 Hz, 1H, Ar H), 6.84 (dd, *J* = 8.9, 2.4 Hz, 1H, Ar H), 6.54 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 4.92 (m, 1H, α-CH), 4.19 (q, *J* = 7.1 Hz, 2H, -CH₂-CH₃), 3.77 (s, 3H, -OCH₃), 3.23–3.05 (m, 2H, Ar-CH₂-CH), 1.26 (t, *J* = 7.1 Hz, 3H, -CH₂-CH₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 170.8, 166.3, 161.1, 153.7, 143.9, 140.9, 137.9, 131.8, 131.3, 129.2, 129.2, 129.1, 128.1, 127.3, 126.4, 119.3, 116.4, 114.7, 113.1, 103.3, 102.1, 59.9, 55.4, 55.3, 37.3, 14.2. HRMS (ESI), *m/z* [M + H]⁺: calculated for C₃₀H₃₀N₃O₅⁺ 512.2180; found 512.2217. HPLC rt: 21.3 min.

Ethyl (S,E)-3-(4-(2-(5-methoxy-1H-indole-2-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (18). 5-Methoxy-1H-indole-2-carboxylic acid (106 mg, 0.56 mmol, 1.2 equiv), HOBt (106 mg, 0.79 mmol, 1.7 equiv), EDCI (151 mg, 0.79 mmol, 1.7 equiv), DIPEA (0.12 mL, 0.69 mmol, 1.5 equiv) and **D** (192 mg, 0.46 mmol, 1.0 equiv) were dissolved in a mixture of DCM:DMF (22 mL, 10:1 v/v) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The reaction mixture was then concentrated under reduced pressure, diluted with ethyl acetate (12 mL) and washed with aq. solution of 0.1 M HCl (3 × 12 mL), aq. solution of 2 M K₂CO₃ (3 × 12 mL) and brine (3 × 12 mL). The organic layer was dried with anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (6:4, v/v) as eluent to afford **18** as a white amorphous solid (10 % yield, 23 mg). ¹H NMR (400 MHz, CDCl₃): δ 9.30 (s, 1H, -NH), 9.05 (s, 1H, -NH), 7.65 (s, 1H, -NH), 7.59 (d, *J* = 9.1 Hz, 2H, Ar H), 7.45 (d, *J* = 8.4 Hz, 2H, Ar H), 7.35–7.21 (m, 2H, Ar H), 7.06 (d, *J* = 5.1 Hz, 2H, Ar H), 6.99 (d, *J* = 9.8 Hz, 1H, Ar H), 6.36 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 4.94 (d, *J* = 9.4 Hz, 1H, α-CH), 4.28 (q, *J* = 7.1 Hz, 2H, -CH₂-CH₃), 3.86 (s, 3H, -OCH₃), 1.35 (t, *J* = 7.1 Hz, 3H, -CH₂-CH₃), 1.18 (s, 9H, -C(CH₃)₃). ¹³C NMR (100 MHz, CDCl₃): δ 169.6, 167.2, 162.0, 155.0, 143.8, 139.4, 132.1, 130.9, 130.3, 129.1, 128.1, 120.3, 117.6, 116.7, 112.9, 104.1, 102.5, 61.5, 60.7, 55.8, 35.8, 26.8, 14.5. HRMS (ESI), *m/z* [M + H]⁺: calculated for C₂₇H₃₂N₃O₅⁺ 478.2336; found 478.2399. HPLC rt: 21.6 min.

Ethyl (S,E)-3-(4-(2-(5-chloro-1H-indole-3-carboxamido)-3-phenylpropanamido)phenyl)acrylate (19). 5-Chloro-1H-indole-3-carboxylic acid (104 mg, 0.53 mmol, 1.2 equiv), HOBt (101 mg, 0.75 mmol, 1.7 equiv), EDCI (143 mg, 0.75 mmol, 1.7 equiv), DIPEA (0.1 mL, 0.66 mmol, 1.5 equiv) and **C** (200 mg, 0.44 mmol, 1.0 equiv) were dissolved in dry DCM (15 mL) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude was dissolved with few drops of DMF and treated with 1 M HCl. The precipitate was filtered, washed with water, and purified by column chromatography using DCM/MeOH (98:2, v/v) as eluent to afford **19** as a white amorphous solid (23 % yield, 53 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.76 (s, 1H, -NH), 10.43 (s, 1H, -NH), 8.31 (d, *J* = 8.0 Hz, 1H, -NH), 8.24 (s, 1H, Ar H), 8.03 (s, 1H, Ar H), 7.69 (s, 4H, Ar H), 7.59 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 7.43 (dd, *J* = 11.9, 8.3 Hz, 3H, Ar H), 7.28 (t, *J* = 7.4 Hz, 2H, Ar H), 7.16 (dd, *J* = 16.8, 8.1 Hz, 2H, Ar H), 6.52 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 4.95–4.78 (m, 1H, α-CH), 4.18 (q, *J* = 7.1 Hz, 2H, -CH₂-CH₃), 3.19–2.98 (m, 2H, Ar-CH₂-CH), 1.25 (t, *J* = 7.1 Hz, 3H, -CH₂-CH₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 171.3, 166.4, 164.2, 144.0, 141.0, 138.1, 134.5, 129.9, 129.2, 129.2, 129.0, 128.1, 127.3, 126.3, 125.2, 121.9, 120.0, 119.2, 116.4, 113.5, 109.6, 59.9,

55.1, 37.3, 14.2. HRMS (ESI), *m/z* [M + H]⁺: calculated for C₂₉H₂₇ClN₃O₄⁺ 516.1685; found 516.1614. HPLC rt: 21.3 min.

Ethyl (S,E)-3-(4-(2-(5-chloro-1H-indole-3-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (20). 5-Chloro-1H-indole-3-carboxylic acid (117 mg, 0.59 mmol, 1.2 equiv), HOBt (114 mg, 0.84 mmol, 1.7 equiv), EDCI (162 mg, 0.84 mmol, 1.7 equiv), DIPEA (0.13 mL, 0.73 mmol, 1.5 equiv) and **D** (204 mg, 0.49 mmol, 1.0 equiv) were dissolved in a mixture of DCM:DMF (21 mL, 20:1 v/v) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The reaction mixture was then concentrated under reduced pressure, diluted with ethyl acetate (12 mL) and washed with aq. solution of 0.1 M HCl (3 × 12 mL), aq. solution of 2 M K₂CO₃ (3 × 12 mL) and brine (3 × 12 mL). The organic layer was dried with anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (6:4, v/v) as eluent to afford **20** as a white amorphous solid (28 % yield, 68 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.84 (s, 1H, -NH), 10.40 (s, 1H, -NH), 8.47 (s, 1H, -NH), 8.10 (s, 1H, Ar H), 7.69 (m, 4H, Ar H), 7.62–7.55 (m, 2H, Ar H), 7.45 (d, *J* = 8.6 Hz, 1H, Ar H), 7.16 (d, *J* = 8.6 Hz, 1H, Ar H), 6.51 (d, *J* = 16.0 Hz, 1H, Ar-CH=CH-), 4.72 (d, *J* = 9.1 Hz, 1H, α-CH), 4.17 (q, *J* = 7.0 Hz, 2H, -CH₂-CH₃), 1.25 (t, *J* = 7.0 Hz, 3H, -CH₂-CH₃), 1.07 (s, 9H, -C(CH₃)₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 170.1, 166.4, 164.2, 144.0, 140.7, 134.6, 130.3, 129.2, 129.1, 127.5, 125.2, 121.9, 120.0, 119.3, 116.4, 113.5, 109.4, 60.4, 59.9, 34.6, 26.8, 14.2. HRMS (ESI), *m/z* [M + H]⁺: calculated for C₂₆H₂₉ClN₃O₄⁺ 482.1841; found 482.1770. HPLC rt: 21.9 min.

Ethyl (S,E)-3-(4-(2-(5-bromo-1H-indole-3-carboxamido)-3-phenylpropanamido)phenyl)acrylate (21). 5-Bromo-1H-indole-3-carboxylic acid (132 mg, 0.55 mmol, 1.15 equiv), HATU (274 mg, 0.72 mmol, 1.5 equiv), DIPEA (0.17 mL, 0.96 mmol, 2.0 equiv) and **C** (216 mg, 0.48 mmol, 1.0 equiv) were dissolved in a mixture of DCM:DMF (11 mL, 10:1 v/v) and the solution was left stirring at rt for 6 h. The reaction mixture was concentrated under reduced pressure. The reaction mixture was then concentrated under reduced pressure, diluted with ethyl acetate (12 mL) and washed with aq. solution of 0.1 M HCl (3 × 12 mL), aq. solution of 2 M NaHCO₃ (3 × 12 mL) and brine (3 × 12 mL). The organic layer was dried with anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (from 1:1 to 2:8, v/v) as eluent to afford **21** as a white amorphous solid (8 % yield, 22 mg). ¹H NMR (400 MHz, MeOD): δ 8.53 (s, 1H, -NH), 8.33–8.02 (m, 3H, Ar H /-NH), 7.72–7.08 (m, 13H, Ar H), 6.43 (d, *J* = 16 Hz, 1H, Ar-CH=CH-), 5.03 (t, *J* = 7.5 Hz, 1H, α-CH), 4.25 (q, *J* = 7.1 Hz, 2H, -CH₂-CH₃), 3.18 (d, *J* = 7.5 Hz, 2H, Ar-CH₂-CH), 1.34 (t, *J* = 7.1 Hz, 3H, -CH₂-CH₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 170.9, 166.6, 163.3, 144.1, 140.9, 137.9, 135.6, 134.4, 130.1, 129.4, 128.3, 127.3, 126.6, 126.2, 123.9, 123.0, 119.5, 116.6, 116.4, 113.1, 107.3, 60.1, 55.3, 37.6, 14.4. HRMS (ESI), *m/z* [M + H]⁺: calculated for C₂₉H₂₇BrN₃O₄⁺ 560.1179; found 560.1105. HPLC rt: 28.5 min.

Ethyl (S,E)-3-(4-(2-(5-bromo-1H-indole-3-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (22). 5-Bromo-1H-indole-3-carboxylic acid (160 mg, 0.66 mmol, 1.2 equiv), HOBt (127 mg, 0.94 mmol, 1.7 equiv), EDCI (180 mg, 0.94 mmol, 1.7 equiv), DIPEA (0.12 mL, 0.72 mmol, 1.5 equiv) and **D** (200 mg, 0.48 mmol, 1.0 equiv) were dissolved in a mixture of DCM:DMF (22 mL, 10:1 v/v) and the solution was left stirring at rt for 4 h. The reaction mixture was then concentrated under reduced pressure, diluted with ethyl acetate (12 mL) and washed with aq. solution of 0.1 M HCl (3 × 12 mL), aq. solution of 2 M K₂CO₃ (3 × 12 mL) and brine (3 × 12 mL). The organic layer was dried with anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (from 6:4 to 1:1, v/v) as eluent to afford **22** as a white amorphous solid (12 % yield, 33 mg). ¹H NMR (400 MHz, CDCl₃): δ 9.42 (s, 1H, -NH), 9.27 (s, 1H, -NH), 8.14 (s, 1H, Ar H), 7.69 (s, 1H, Ar H), 7.53 (d, *J* = 15.9 Hz, 1H, Ar-CH=CH-), 7.47 (d, *J* = 8.1 Hz, 2H, Ar H), 7.32–7.21 (m, 4H, Ar H), 6.83 (d, *J* = 9.1 Hz, 1H, -NH), 6.26 (d, *J* =

15.9 Hz, 1H, Ar-CH=CH-), 5.03 (d, J = 9.1 Hz, 1H, α -CH), 4.26 (q, J = 7.1 Hz, 2H, -CH₂-CH₃), 1.34 (t, J = 7.1 Hz, 3H, -CH₂-CH₃), 1.26 (s, 9H, -C(CH₃)₃). ¹³C NMR (100 MHz, CDCl₃): δ 170.7, 167.3, 165.4, 144.0, 139.5, 135.0, 130.6, 128.9, 128.7, 126.8, 126.3, 123.3, 120.4, 117.3, 115.5, 113.4, 111.3, 61.5, 60.6, 35.3, 27.1, 14.5. HRMS (ESI), m/z [M + H]⁺: calculated for C₂₆H₂₉BrN₃O₄⁺ 526.1336; found 526.1260. HPLC rt: 22.6 min.

Ethyl (S,E)-3-(4-(2-(5-methoxy-1H-indole-3-carboxamido)-3-phenylpropanamido)phenyl)acrylate (23). 5-Methoxy-1H-indole-3-carboxylic acid (104 mg, 0.54 mmol, 1.2 equiv), HOBt (104 mg, 0.77 mmol, 1.7 equiv), EDCI (147 mg, 0.77 mmol, 1.7 equiv), DIPEA (0.1 mL, 0.67 mmol, 1.5 equiv) and **C** (203 mg, 0.45 mmol, 1.0 equiv) were dissolved in a mixture of DCM:DMF (16 mL, 10:1 v/v) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The reaction mixture was then concentrated under reduced pressure, diluted with ethyl acetate (12 mL) and washed with aq. solution of 0.1 M HCl (3 × 12 mL), aq. solution of 2 M NaHCO₃ (3 × 12 mL) and brine (3 × 12 mL). The organic layer was dried with anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (from 1:1 to 2:8, v/v) as eluent to afford **23** as a white amorphous solid (27 % yield, 63 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.44 (d, J = 2.6 Hz, 1H, -NH), 10.40 (s, 1H, -NH), 8.14 (d, J = 8.0 Hz, 1H, -NH), 8.10 (d, J = 3.0 Hz, 1H, Ar H), 7.67 (m, 4H, Ar H), 7.59 (d, J = 16.0 Hz, 1H, Ar-CH=CH-), 7.55 (d, J = 2.5 Hz, 1H, Ar H), 7.41 (d, J = 7.3 Hz, 2H, Ar H), 7.32–7.23 (m, 3H, Ar H), 7.17 (t, J = 7.3 Hz, 1H, Ar H), 6.76 (dd, J = 8.8, 2.5 Hz, 1H, Ar H), 6.52 (d, J = 16.0 Hz, 1H, Ar-CH=CH-), 4.86 (td, J = 9.4, 5.2 Hz, 1H, α -CH), 4.18 (q, J = 7.1 Hz, 2H, -CH₂-CH₃), 3.72 (s, 3H, -OCH₃), 3.18–3.07 (m, 2H, Ar-CH₂-CH), 1.25 (t, J = 7.1 Hz, 3H, -CH₂-CH₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 171.5, 166.4, 164.9, 154.4, 144.0, 141.1, 138.3, 138.3, 131.1, 129.3, 129.0, 128.8, 128.1, 126.8, 126.4, 119.2, 116.4, 112.5, 112.2, 109.6, 102.5, 59.9, 55.2, 37.3, 26.9, 14.3. HRMS (ESI), m/z [M + H]⁺: calculated for C₃₀H₃₀N₃O₅⁺ 512.2180; found 512.2108. HPLC rt: 19.8 min.

Ethyl (S,E)-3-(4-(2-(5-methoxy-1H-indole-3-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (24). 5-Methoxy-1H-indole-3-carboxylic acid (82 mg, 0.43 mmol, 1.2 equiv), HOBt (82 mg, 0.61 mmol, 1.7 equiv), EDCI (117 mg, 0.61 mmol, 1.7 equiv), DIPEA (0.19 mL, 1.1 mmol, 3 equiv) and **D** (150 mg, 0.36 mmol, 1.0 equiv) were dissolved in dry DCM (20 mL) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude was dissolved with few drops of DMF and treated with 1 M HCl. The precipitate was filtered, washed with water, and purified by column chromatography using *n*-hexane:ethyl acetate (1:1, v/v) as eluent to afford **24** as a white amorphous solid (35 % yield, 60 mg). ¹H NMR (400 MHz, MeOD): δ 7.99 (s, 1H, -NH), 7.75–7.51 (m, 7H, Ar H), 7.35 (d, J = 8.9 Hz, 1H, Ar H), 6.87 (dd, J = 8.8, 2.4 Hz, 1H, Ar H), 6.45 (d, J = 16.0 Hz, 1H, Ar-CH=CH-), 4.77 (s, 1H, α -CH), 4.25 (q, J = 7.1 Hz, 2H, -CH₂-CH₃), 3.85 (s, 3H, -OCH₃), 1.33 (t, J = 7.1 Hz, 3H, -CH₂-CH₃), 1.18 (s, 9H, -C(CH₃)₃), NH proton signals missing for exchange with solvent. ¹³C NMR (100 MHz, MeOD): δ 172.0, 168.7, 167.8, 156.7, 145.3, 141.2, 133.0, 131.6, 129.9, 129.8, 127.3, 121.3, 117.7, 113.8, 113.6, 111.0, 102.8, 62.1, 61.4, 55.9, 36.1, 27.1, 14.5. HRMS (ESI), m/z [M + H]⁺: calculated for C₂₇H₃₂N₃O₅⁺ 478.2336; found 478.2260. HPLC rt: 20.1 min.

Ethyl (S,E)-3-(4-(2-(2-(4-acetamidophenyl)acetamido)-3-phenylpropanamido)phenyl)acrylate (25). 2-(4-Acetamidophenyl)acetic acid (103 mg, 0.53 mmol, 1.2 equiv), HOBt (101 mg, 0.75 mmol, 1.7 equiv), EDCI (143 mg, 0.75 mmol, 1.7 equiv), DIPEA (0.1 mL, 0.66 mmol, 1.5 equiv) and **C** (200 mg, 0.44 mmol, 1.0 equiv) were dissolved in dry DCM (15 mL) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude was dissolved with few drops of DMF and treated with 1 M HCl. The precipitate was filtered and washed with water to afford **25** as a white amorphous solid (87 % yield, 199 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 10.33 (s, 1H, -NH), 9.83 (s, 1H, -NH), 8.46 (d, J = 8.1 Hz, 1H, -NH),

7.65 (q, J = 8.9 Hz, 4H, Ar H), 7.58 (d, J = 16.0 Hz, 1H, Ar-CH=CH-), 7.41 (d, J = 8.5 Hz, 2H, Ar H), 7.31–7.15 (m, 5H, Ar H), 7.02 (d, J = 8.5 Hz, 2H, Ar H), 6.52 (d, J = 16.0 Hz, 1H, Ar-CH=CH-), 4.68 (m, 1H, α -CH), 4.18 (q, J = 7.1 Hz, 2H, -CH₂-CH₃), 3.37 (d, J = 10.1 Hz, 2H, Ar-CH₂-CH), 3.11–2.83 (m, 2H, Ar-CH₂-C=O), 2.01 (s, 3H, CH₃-C=O), 1.25 (t, J = 7.1 Hz, 3H, -CH₂-CH₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 170.5, 170.3, 168.1, 166.3, 143.9, 140.8, 137.6, 137.4, 130.7, 129.2, 129.2, 129.1, 129.1, 128.0, 126.4, 119.2, 118.8, 116.4, 59.9, 54.9, 41.3, 37.7, 23.9, 14.2. HRMS (ESI), m/z [M + H]⁺: calculated for C₃₀H₃₂N₃O₅⁺ 514.2336; found 514.2270. HPLC rt: 17.5 min.

Ethyl (S,E)-3-(4-(2-(2-(4-acetamidophenyl)acetamido)-3,3-dimethylbutanamido)phenyl)acrylate (26). 2-(4-Acetamidophenyl)acetic acid (122 mg, 0.63 mmol, 1.2 equiv), HOBt (120 mg, 0.89 mmol, 1.7 equiv), EDCI (171 mg, 0.89 mmol, 1.7 equiv), DIPEA (0.18 mL, 1.1 mmol, 2.0 equiv) and **D** (221 mg, 0.53 mmol, 1.0 equiv) were dissolved in dry DCM (15 mL) and the solution was left stirring at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude was dissolved with few drops of DMF and treated with 1 M HCl. The precipitate was filtered, washed with water and purified by column chromatography using DCM/MeOH (95:5, v/v) as eluent to afford **26** as a white amorphous solid (34 % yield, 87 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 10.34 (s, 1H, -NH), 9.85 (s, 1H, -NH), 8.14 (d, J = 9.3 Hz, 1H, -NH), 7.67 (s, 4H, Ar H), 7.58 (d, J = 16.0 Hz, 1H, Ar-CH=CH-), 7.47 (d, J = 7.9 Hz, 2H, Ar H), 7.19 (d, J = 8.3 Hz, 2H, Ar H), 6.51 (d, J = 16.0 Hz, 1H, Ar-CH=CH-), 4.49–4.37 (m, 1H, α -CH), 4.18 (q, J = 7.1 Hz, 2H, -CH₂-CH₃), 3.65–3.39 (m, 2H, Ar-CH₂-C=O), 2.01 (s, 3H, CH₃-C=O), 1.25 (t, J = 7.1 Hz, 3H, -CH₂-CH₃), 0.94 (s, 9H, -C(CH₃)₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 170.5, 169.7, 168.0, 166.4, 144.0, 140.6, 137.6, 131.2, 129.2, 129.1, 119.2, 118.9, 116.4, 99.5, 60.4, 59.9, 41.3, 34.7, 26.5, 23.9, 14.2. HRMS (ESI), m/z [M + H]⁺: calculated for C₂₇H₃₄N₃O₅⁺ 480.2493; found 480.2418. HPLC rt: 17.5 min.

Ethyl (S,E)-3-(4-(2-(4-acetamidobenzamido)-3-phenylpropanamido)phenyl)acrylate (27). 4-Acetamidobenzoic acid (72 mg, 0.4 mmol, 1.2 equiv), EDCI (189 mg, 0.99 mmol, 3 equiv), DMAP (60 mg, 0.49 mmol, 1.5 equiv), DIPEA (0.1 mL, 0.66 mmol, 2.0 equiv) and **C** (150 mg, 0.33 mmol, 1.0 equiv) were dissolved in dry DCM (6 mL) and the solution was left stirring at rt for 4 h. The crude was dissolved with few drops of DMF and treated with 1 M HCl. The precipitate was filtered to afford **27** as a white amorphous solid (69 % yield, 113 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 10.43 (s, 1H, -NH), 10.15 (s, 1H, -NH), 8.64 (d, J = 8.0 Hz, 1H, -NH), 7.80 (d, J = 8.8 Hz, 2H, Ar H), 7.68 (s, 4H, Ar H), 7.63 (d, J = 8.7 Hz, 2H, Ar H), 7.59 (d, J = 15.9 Hz, 1H, Ar-CH=CH-), 7.45–7.36 (m, 2H, Ar H), 7.28 (t, J = 7.6 Hz, 2H, Ar H), 7.22–7.13 (m, 1H, Ar H), 6.52 (d, J = 16.0 Hz, 1H, Ar-CH=CH-), 4.90–4.78 (m, 1H, α -CH), 4.18 (q, J = 7.1 Hz, 2H, -CH₂-CH₃), 3.20–3.03 (m, 2H, Ar-CH₂-CH), 2.07 (s, 3H, CH₃-C=O), 1.25 (t, J = 7.1 Hz, 3H, -CH₂-CH₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 170.9, 168.7, 166.3, 166.0, 144.0, 142.1, 140.9, 138.0, 129.2, 129.0, 128.4, 128.1, 128.0, 126.3, 119.2, 118.6, 117.9, 116.4, 59.9, 55.9, 37.1, 24.1, 14.2. HRMS (ESI), m/z [M + H]⁺: calculated for C₂₉H₃₀N₃O₅⁺ 500.2180; found 500.2105. HPLC rt: 17.9 min.

Ethyl (S,E)-3-(4-(2-(4-acetamidobenzamido)-3,3-dimethylbutanamido)phenyl)acrylate (28). 4-Acetamidobenzoic acid (43 mg, 0.24 mmol, 1.2 equiv), HOBt (46 mg, 0.34 mmol, 1.7 equiv), EDCI (65 mg, 0.34 mmol, 1.7 equiv), DIPEA (0.1 mL, 0.6 mmol, 3 equiv) and **D** (85 mg, 0.2 mmol, 1.0 equiv) were dissolved in dry DCM (20 mL) and the solution was left stirring at rt overnight. The reaction mixture was then concentrated under reduced pressure, diluted with ethyl acetate (12 mL) and washed with saturated aq. solution of NH₄Cl (3 × 12 mL), saturated aq. solution of NaHCO₃ (3 × 12 mL) and brine (3 × 12 mL). The organic layer was dried with anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using DCM:MeOH (95:5, v/v) as eluent to afford **28** as a white amorphous solid (33 % yield, 31 mg). ¹H NMR (400 MHz, MeOD): δ 7.82 (m, 2H, Ar H, Ar-CH=CH-), 7.71–7.51 (m, 7H, Ar H), 6.43 (d, J = 16.5 Hz, 1H, Ar-CH=CH-), 4.76–4.66 (m, 1H, α -CH), 4.22 (q, J = 6.3 Hz, 2H,

–CH₂–CH₃), 2.13 (s, 3H, CH₃–C=O), 1.30 (t, *J* = 7.0 Hz, 3H, –CH₂–CH₃), 1.13 (s, 9H, –C(CH₃)₃); NH proton signals missing for exchange with solvent. ¹³C NMR (100 MHz, MeOD): δ 171.9, 171.6, 169.6, 168.8, 145.5, 143.4, 141.4, 131.7, 130.4, 130.0, 129.4, 121.4, 120.3, 117.9, 63.1, 61.6, 36.3, 27.2, 24.0, 14.6. HRMS (ESI), *m/z* [M + H]⁺: calculated for C₂₆H₃₂N₃O₅⁺ 466.2336; found 466.2218. HPLC rt: 17.6 min.

Ethyl (S,E)-3-(4-(2-(2-(1H-indol-3-yl)acetamido)-3,3-dimethylbutanamido)phenyl)acrylate (29). 2-(1H-indol-3-yl)acetic acid (46 mg, 0.26 mmol, 1.2 equiv), HOBt (49 mg, 0.37 mmol, 1.7 equiv), EDCI (71 mg, 0.37 mmol, 1.7 equiv), DIPEA (0.06 mL, 0.33 mmol, 1.5 equiv) and **D** (91 mg, 0.22 mmol, 1.0 equiv) were dissolved in dry DMF (10 mL) and the solution was left stirring at rt overnight. The reaction mixture was then diluted with ethyl acetate (12 mL) and washed with saturated aq. solution of NH₄Cl (3 × 12 mL), saturated aq. solution of NaHCO₃ (3 × 12 mL) and brine (3 × 12 mL). The organic layer was dried with anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. The crude was treated with mixture of *n*-butyl methyl ether:*n*-hexane 1:1 and the precipitate was filtered and dried under vacuum to afford **29** as a yellow amorphous solid (28 % yield, 29 mg). ¹H NMR (400 MHz, MeOD): δ 7.66–7.59 (m, 1H, Ar–CH=CH–), 7.59–7.48 (m, 5H, Ar H), 7.36 (d, *J* = 8.1 Hz, 1H, Ar H), 7.22 (s, 1H, Ar H), 7.11 (t, *J* = 7.6 Hz, 1H, Ar H), 7.01 (t, *J* = 7.5 Hz, 1H, Ar H), 6.43 (d, *J* = 16.0 Hz, 1H, Ar–CH=CH–), 4.42 (s, 1H, α-CH), 4.23 (q, *J* = 8.0 Hz, 2H, –CH₂CH₃), 3.86–3.66 (m, 2H, –CH₂–Indole), 1.31 (t, *J* = 7.1 Hz, 3H, –CH₂CH₃), 0.90 (s, 9H, –C(CH₃)₃); NH proton signals missing for exchange with solvent. ¹³C NMR (100 MHz, MeOD): δ 174.6, 171.3, 168.8, 145.5, 141.3, 138.2, 131.6, 130.0, 128.4, 125.2, 122.8, 121.3, 120.1, 119.4, 117.8, 112.4, 109.2, 62.5, 61.6, 35.9, 33.8, 26.9, 14.6. HRMS (ESI), *m/z* [M + H]⁺: calculated for C₂₇H₃₂N₃O₄⁺ 462.2387; found 462.2300. HPLC rt: 20.3 min.

Ethyl (R,E)-3-(4-(2-(1H-indole-5-carboxamido)-3,3-dimethylbutanamido)phenyl)acrylate (30). 1H-indole-5-carboxylic acid (41 mg, 0.26 mmol, 1.2 equiv), HOBt (48 mg, 0.36 mmol, 1.7 equiv), EDCI (70 mg, 0.36 mmol, 1.7 equiv), DIPEA (0.14 mL, 0.8 mmol, 2.5 equiv) and **F** (133 mg, 0.32 mmol, 1.0 equiv) were dissolved in a mixture of DCM: DMF (11 mL, 10:1 v/v) and the solution was left stirring at rt overnight. The reaction mixture was then evaporated under reduced pressure, diluted with ethyl acetate (12 mL) and washed with saturated aq. solution of 0.1 M HCl (3 × 10 mL), saturated aq. solution of 2 M K₂CO₃ (3 × 10 mL) and brine (3 × 10 mL). The organic layer was dried with anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-hexane/ethyl acetate (1:1, v/v) as eluent to afford **30** as a white amorphous solid (42 % yield, 59 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.31 (s, 1H, –NH), 10.38 (s, 1H, –NH), 8.19 (s, 1H, –NH), 7.78–7.72 (m, 1H, Ar H), 7.66 (m, 5H, Ar H), 7.58 (d, *J* = 16.0 Hz, 1H, Ar–CH=CH–), 7.43 (d, *J* = 8.3 Hz, 2H, Ar H), 6.56 (s, 1H, Ar H), 6.51 (d, *J* = 16.0 Hz, 1H, Ar–CH=CH–), 4.69 (d, *J* = 9.1 Hz, 1H, α-CH), 4.17 (q, *J* = 7.1 Hz, 2H, –CH₂–CH₃), 1.25 (t, *J* = 7.1 Hz, 3H, –CH₂–CH₃), 1.08 (s, 9H, –C(CH₃)₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 170.0, 167.8, 166.4, 144.0, 140.6, 137.6, 129.2, 129.2, 127.0, 126.7, 125.0, 120.8, 120.4, 119.4, 116.5, 111.0, 102.3, 61.4, 59.9, 34.6, 26.8, 14.3. HRMS (ESI), *m/z* [M + H]⁺: calculated for C₂₆H₃₀N₃O₄⁺ 448.2231; found 448.2199. HPLC rt: 20.0 min.

CCRediT authorship contribution statement

Andrea Citarella: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Giulia Sibille:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Davide Moi:** Writing – review & editing, Writing – original draft, Data curation. **Alessandro Dimasi:** Writing – review & editing, Methodology, Data curation. **Tommaso Braga:** Investigation, Data curation. **Lorenzo Dal Col:** Investigation, Data curation. **Lorenzo Ruberto:** Investigation, Data curation. **Stefano Pieraccini:** Writing – original draft, Supervision. **Maurizio Sironi:**

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

The Supporting Information includes: copies of ¹H and ¹³C NMR of final compounds and HPLC traces for representative compounds. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bmc.2025.118258>.

Data availability

Data will be made available on request.

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