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Toward the First Nonpeptidic Molecular Tong Inhibitor of Wild-Type and Mutated HIV-1 Protease Dimerization

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Herein we describe the synthesis and HIV-1 protease (PR) inhibitory activity of 16 new peptidomimetic molecular tongs with a naphthalene scaffold. Their peptidic character was progressively decreased. Two of these molecules exhibited the best dimerization inhibition activity toward HIV-1 wild-type and multimutated ANAM-11 proteases obtained to date for this class of molecules (~40 nM for wild-type PR and 100 nM for ANAM-11 PR). Although the peptidic character of one molecular tong was completely suppressed, the mechanism of inhibition and inhibitory potency toward both proteases were maintained.

Introduction

Human immunodeficiency virus type 1 (HIV-1) protease (HIV-PR) is the enzyme responsible for post-translational processing of viral polyproteins and subsequent generation of the structural and functional proteins essential for viral replication.[1] As such, HIV-PR is a major target for antiretroviral therapies. HIV-PR is a homodimeric aspartyl protease, with two monomers of 99 residues each. The enzyme active site is located at the bottom of a cavity within the dimer interface. Access to this active site is monitored by the β hairpin flaps. HIV-PR is active only in its dimeric form; because each monomer contributes one of the two catalytic aspartic residues (Asp25), dissociation of the enzyme homodimer results in a complete loss of catalytic activity. The protease homodimer is mainly stabilized by a four-stranded antiparallel β sheet involving both the N- and C-termini of each monomer (H-Pro1-Gln2-Ile3-Thr4 and Cys95-Thr96-Leu97-Asn98-Phe99-OH). This region appears to be highly conserved in HIV-1 isolates.[2] More than 50% of the hydrogen bonds along the dimer interface involve terminal residues 1–4 and 96–99 at the antiparallel β sheet.[3] The contacts in this region contribute to 75% of the total Gibbs free energy of HIV-PR dimerization.[4] Many HIV-PR inhibitors (PIs) have been developed which target the active site. However, several mutations located within or outside of the HIV-PR active site result in lower affinity towards inhibitors,[5,6] and alternative strategies to circumvent the cross-resistance of HIV-PR inhibitors are crucially needed. Recently approved PIs, tipranavir and darunavir, are active against HIV-1 protease variants resistant to multiple PIs. Data obtained using a FRET-based HIV-1 expression assay suggested that these molecules act as conventional protease inhibitors. They also block protease dimerization in cells, but do not dissociate already dimerized cellular proteases.[7] The protease termini β sheet interface has been explored as a dimerization inhibition target, and known inhibitors that target this region are able to block or disrupt the formation of the homodimer.[8] C- and N-terminal mimetic peptides[9–11] and lipopeptides[12–14] have proven to be efficient PR dimerization inhibitors. A bicyclic guanidinium group has been introduced between the peptidic and lipophilic moieties,[15] and the interface peptides have also been cross-linked with flexible,[16] semi-rigid,[17] or rigid spacers.[18–20]

Our strategy involved the synthesis of conformationally constrained, scaffold-based molecular tongs, attached to two peptidic strands by carboxypropyl linkers (Figure 1). The two peptidic strands were able to be suitably oriented by the scaffold, allowing putative formation of an antiparallel β sheet with the C-terminal end of one HIV-1 PR monomer and resulting in an entropic benefit (Figure 1).[18] Naphthalene or quinoline scaffolds with symmetrical or asymmetrical peptidic strands were efficient antidimers (K_{id} up to 80 nM).[18,19] The introduction of amino acid mimetic fragments, specifically a 5-amino-2-methoxybenzamide or a 3-amino-6-methylpyridin(1*H*)-one group in a single strand, increased the metabolic stability of the molecular tongs without compromising their ability to inhibit wild-type and mutated HIV-1 proteases in vitro.[20] These groups were selected to replace two amino acids, as they were hypothesized to provide the same array of hydrogen bonding groups as one edge of a peptidic β strand. Our previous results

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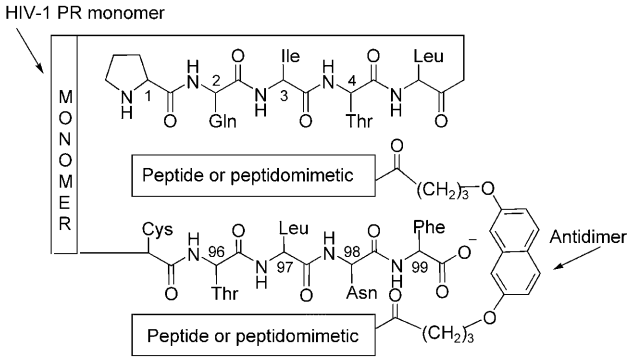


Figure 1. Schematic representation of a putative HIV-1 monomer–molecular tong complex.

suggest that the flexibility of valine is required for formation of the hydrogen bonds in a four-stranded antiparallel β sheet, which involves both the N- and C-termini of the PR monomer.[20]

This report describes the synthesis and enzyme inhibitory activity against wild-type and mutated HIV-1 proteases of new molecular tongs with amino acid mimetic fragments in one (molecules **1**–**6**, Figure 2) or two (molecules **7**–**16**, Figure 2) strands. We decreased the peptidic character of the molecular tongs by introducing peptidomimetic fragments, first replacing two and then three amino acids in one or both strands. The 5-amino-2-methoxybenzamide or 3-amino-6-methylpyridin(1*H*)-one groups were first attached to the carboxypropyl linker through a valine or lysine residue for molecules **1**–**2** and **7**–**12**. In order to totally suppress the peptidic character of the strands, and to increase flexibility between the scaffold and the mimetic unit, the amino acid residue (valine or lysine) was replaced by a longer and more flexible moiety. The mimetic unit was attached to the carboxypropyl linker through the ε-amino group of a lysine residue (molecules **3**–**6** and **13**–**16**). We describe herein the most potent antidimeric molecular tongs **1** and **2** obtained to date for this class of molecules.

We also report the first nonpeptidic molecular tong **15** that acts as an antidimer.

Results and Discussion

Chemistry

Protected peptidomimetic strand **18** was obtained by coupling 2-(3-amino-2-oxopyridin-1-(2*H*)-yl)acetamide **17**[20] to *N*-Boc-*N*-Z-Lys-OH, using HBTU and HOBt as coupling agents, in good yield (Scheme 1). Compound **18** was selectively deprotected at the α-nitrogen by acidic hydrolysis (TFA in CH_2Cl_2) to afford **19**, or at the ε-nitrogen by hydrogenolysis to give **20**, both in excellent yields (Scheme 1).

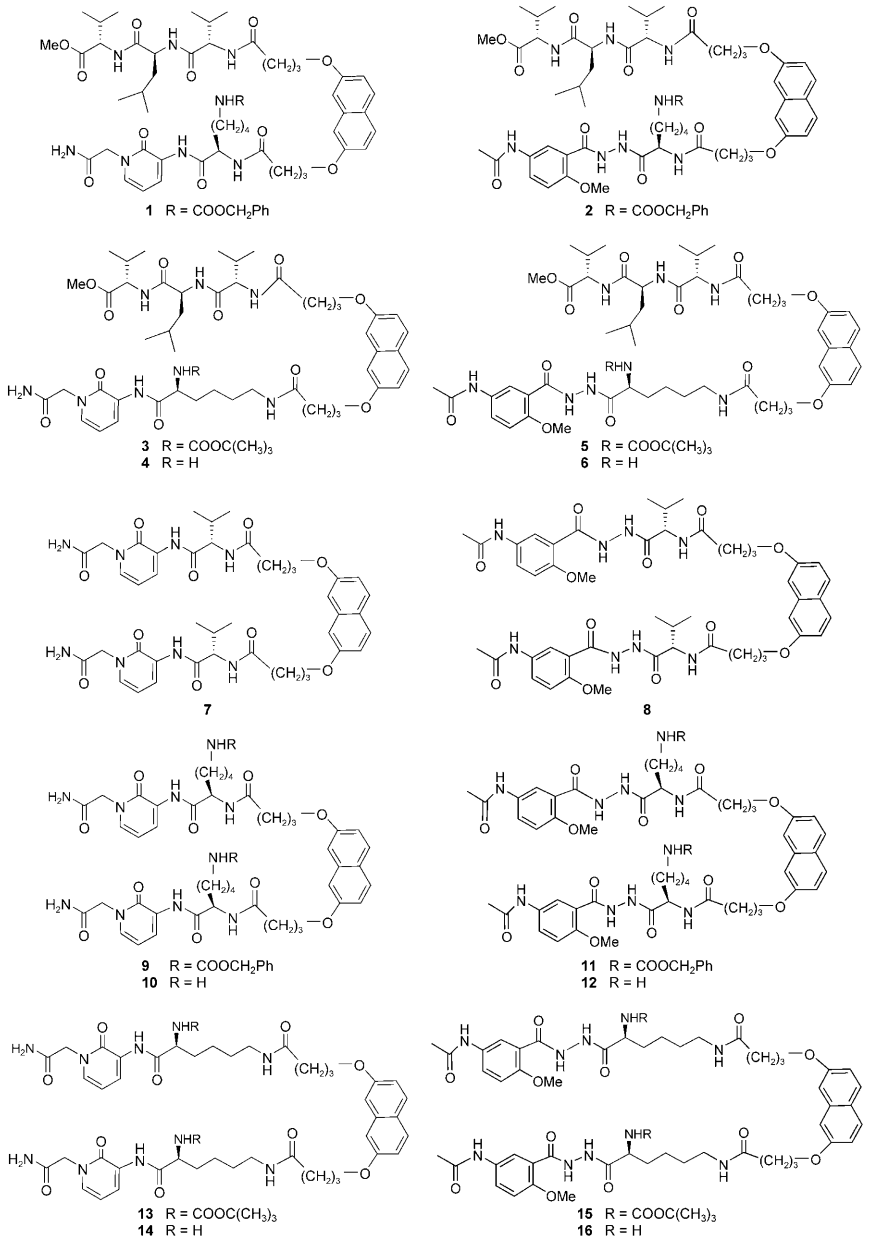
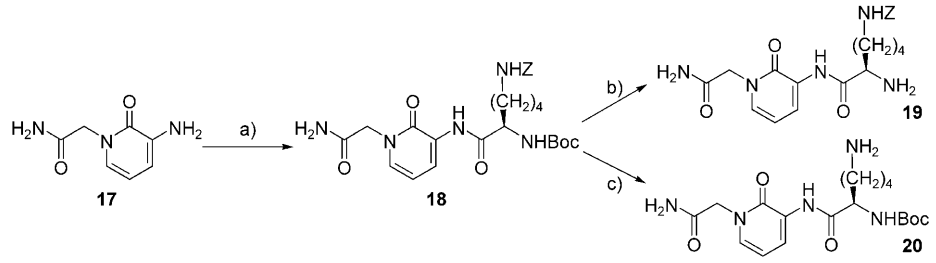
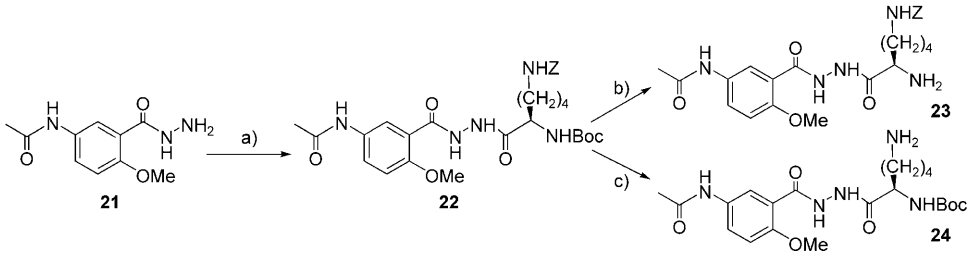


Figure 2. Molecular tongs **1**–**16**.



Scheme 1. Synthesis of peptidomimetic-containing strands **19** and **20**. a) *N*-Boc-*N*-Z-K-OH, HBTU, HOBt, DIPEA, DMF, 48 h, room temperature; b) TFA, CH_2Cl_2, 2 h, room temperature; c) H_2, 10% Pd/C, MeOH, 12 h, room temperature.

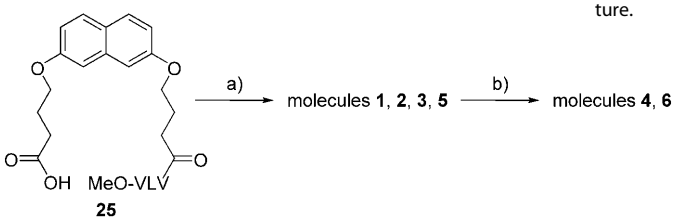
Protected peptidomimetic strand **22** was obtained from *N*-(3-hydrazinocarbonyl-4-methoxyphenyl)acetamide (**21**),[20] following the same procedure as described for **18** (Scheme 2). Compound **22** was deprotected by acidic hydrolysis to give **23**, or by hydrogenolysis to give **24**, both in good yields (Scheme 2).



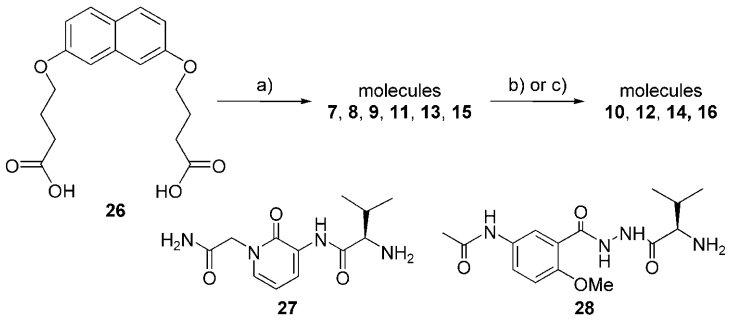
Scheme 2. Synthesis of peptidomimetic-containing strands **23** and **24**: a) *N*-Boc-*N*-Z-K-OH, HBTU, HOBt, DIPEA, DMF, 24 h, room temperature; b) TFA, CH_2Cl_2, 1 h, room temperature; c) H_2, 10% Pd/C, MeOH, 12 h, room temperature.

Molecular tongs **1**–**3** and **5** were synthesized by condensation of 4-[7-(3-carboxypropoxy)naphthalen-2-yloxy]butyryl-Val-Leu-Val-OMe (**25**)[19] with peptidomimetics **19**, **23**, **20**, and **24**, respectively, in satisfactory yields (Scheme 3). Acidic cleavage of the tert-butyl carbamate group of compounds **3** and **5** gave molecular tongs **4** and **6**, respectively (Scheme 3).

Molecular tongs **7**–**9**, **11**, **13**, and **15** were synthesized from 4-{[7-(3-carboxypropoxy)-2-naphthyl]oxy}-butanoic acid (**26**)[18] by coupling with respective peptidomimetics **27**, **28**, **19**, **23**, **20**, and **24** (Scheme 4). Cleavage of the *N*-Boc group of compounds **13** and **15** gave molecular tongs **14** and **16**, respectively, while hydrogenolysis of **9** and **11** afforded molecular tongs **10** and **12**, respectively (Scheme 4).



Scheme 3. Synthesis of molecular tongs **1**–**6**. a) HBTU, HOBt, DIPEA, **19**, **20**, **23**, or **24**, DMF, 48 h, room temperature; b) for **4** or **6**: **3** or **5**, TFA, CH_2Cl_2, 30 min, room temperature.



Scheme 4. Synthesis of molecular tongs **7**–**16**. a) HBTU, HOBt, DIPEA, **19**, **20**, **23**, **24**, **27**, or **28**, DMF, 48 h, room temperature; b) for **14** or **16**: **13** or **15**, TFA, CH_2Cl_2, 30 min, room temperature; c) for **10** or **12**: **9** or **11**, H_2, 10% Pd/C, MeOH/DMF, 12 h, room temperature.

Biology

The inhibitory activities of compounds **1**–**16** were assayed against recombinant wild-type PR (WT-PR) at pH 4.7 and 30 °C using a fluorimetric assay.[18–20] The most efficient inhibitors were also assayed against the multidrug-resistant mutated protease ANAM-11. Zhang–Poorman kinetic analyses were used to characterize the mechanism of inhibition as dimerization alone (parallel lines), competitive inhibition (altered slopes and unaltered y-axis intercepts), and mixed inhibition (altered slopes and y-axis intercepts). The results are summarized in Table 1. Parallel lines were obtained for compounds **1**–**6**, **8**, and **15**, demonstrating that they act purely as dimerization inhibitors (Figure 3). Compounds **7**, **9**, **11**, and **13** exhibited mixed inhibition. Weak inhibitory effects were observed with compounds **10**, **12**, **14**, and **16** (28–30% at 14 μM for **12**, or 28 μM for **10**, **14**, and **16**).

Based on the observed results, it was noted that replacement of the valine residue by an *N*-protected lysine (asymmetrical molecular tongs **1** and **2**) dramatically increases the inhibitory potency (40 nM for **1** relative to 400 nM for the valine analogue,[20] and 60 nM for **2** relative to 200 nM for the valine analogue[20]). The symmetric molecular tongs **7**, **9**, and **11** are fairly efficient inhibitors that act through a mixed inhibition mechanism. For example, **11** exhibits both a dimerization inhibition

Table 1. In vitro inhibition of wild-type PR and ANAM-11 by molecular tongs (30 °C, pH 4.7).

Compd	WT PR or ANAM-11	K_{ic} [μM][a]	K_{id} [μM][b]
1	WT		0.04
	ANAM-11		0.25
2	WT		0.06
	ANAM-11		0.1
3	WT		1.2
4	WT		4.5
5	WT		0.4
	ANAM-11		1.36
6	WT		3
7	WT	0.550	0.053
8	WT		0.220
9	WT	0.2	0.12
10	WT		_[c]
11	WT	0.15	0.053
12	WT		_[c]
13	WT	31	17
14	WT		_[c]
15	WT		0.28
	ANAM-11		2
16	WT		_[c]

[a] Competitive active site inhibition; [b] dimerization inhibition; [c] percent inhibition at 28 μM: 28 (**10**), 30 (**14** and **16**); at 14 μM: 30 (**12**). Standard errors of initial rates are $< 5\%$.

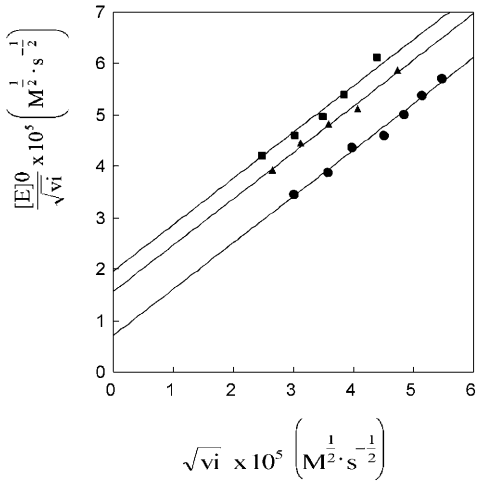


Figure 3. Plots of $[E]_0/\sqrt{v_i}$ vs $\sqrt{v_i}$ for the hydrolysis of the fluorogenic substrate DABCYL-γ-abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS by wild-type HIV-1 PR at pH 4.7 and 30 °C in the absence (●) and presence of compound **15** at 9.25 μM (■) and 7.08 μM (▲).

component ($K_{id} = 53$ nM) and a slight competitive inhibition component ($K_{ic} = 150$ nM). Increasing both the length and flexibility of the nonpeptidic strand in asymmetrical molecular tong **3** has a detrimental effect on the inhibitory potency (**3** vs. **1**). In contrast, a simultaneous increase in both length and flexibility of the nonpeptidic strand in symmetrical molecular tong **15** has a favorable effect on inhibitory potency and results in purely dimerization inhibition activity (**15** vs. **11**) although the peptidic character of molecular tong **15** was completely suppressed. The hydrazide motif favors both dimerization inhibition and inhibitory efficacy (**15** vs **13**). Deprotection of the amino moiety of the strands noticeably decreased the inhibitory potency (**10** vs. **9**, **12** vs. **11**, **14** vs. **13**, and **16** vs. **15**), confirming a favorable influence of the hydrophobic character of the tong arms on inhibitory activity.[18,19] Remarkably, new pseudopeptidic molecular tongs **1**, **2**, **5**, and **15** behaved as antidimers against the multimutated protease ANAM-11, which is analogous to a protease found in multi-drug resistant viruses. In contrast, the affinity of ritonavir for this mutated protease[21] was about 78000-fold lower than for wild-type PR, demonstrating that the peptidomimetic molecular tongs **1**, **2**, **5**, and **15** can still efficiently inhibit dimerization of mutated proteases. Compound **2** exhibits 20-fold higher in vitro activity toward ANAM-11 protease than does ritonavir.[21] This confirms that inhibitors of PR dimerization are insensitive to protease mutations.

Conclusions

Molecular tongs, containing a naphthalene scaffold and peptidic side arms, were reported to be inhibitors of the PR interface.[18,19] Replacement of two amino acids by peptidomimetics in one strand of these molecular tongs led to proteolysis-resistant inhibitors.[20] We replaced the remaining valine residue by a more hydrophobic and flexible side chain of the Cbz-protected lysine residue to afford the most potent molecular tong inhibitor of PR dimerization (~40 nM). In addition, replacement of two amino acids by peptidomimetics in both strands of these symmetrical molecular tongs predominantly resulted in a mixed mechanism of inhibition (competitive inhibition and antidimeric activity). The replacement of three amino acids by nonpeptidic fragments in each of two strands of the molecular tongs resulted in the first potent nonpeptidic protease inhibitor able to dissociate the mature protease dimer. In this way, we demonstrated that total suppression of peptidic character within this class of molecules does not alter their mechanism of action.

Four dimerization inhibitors were also evaluated against the ANAM-11 protease, which contains 11 mutations, and were found to be equally active toward both ANAM-11 and wild-type PR. We corroborated previous findings that peptidomimetic molecular tongs are candidates for successfully overcoming the resistance presently encountered with classical protease inhibitors. Deprotection of amino groups in the strands noticeably decreased the inhibitory potency of these molecules. Further studies of PR dimerization inhibitors containing peptidomimetics will focus on the synthesis of molecular tongs with greater hydrophilic character.

Experimental Section

Synthesis

Common solvents were purchased from commercial sources. *N*,*N*-Dimethylformamide (DMF) was distilled over $CaSO_4$, tetrahydrofuran (THF) was distilled over sodium/benzophenone, and CH_3CN was distilled over $CaCl_2$. TLC was performed on silica gel 60 F_{250} (0.26 mm thickness) plates. The plates were visualized with UV

light (λ 254 nm), or with a 3.5% solution of phosphomolybdic acid or ninhydrin in EtOH. Liquid chromatography was performed on Merck 60 silica gel (230–400 mesh). Protected amino acids *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazol (HOBT) were purchased from commercial sources. H-Val-Leu-Val-OMe,[19] 4-[7-(3-carboxypropoxy)-naphtalen-2-yloxy]butyryl-Val-Leu-Val-OMe (**25**),[19] 2-(3-amino-2-oxo-2*H*-pyridin-1-yl)acetamide (**17**),[20] *N*-(3-hydrazinocarbonyl-4-methoxyphenyl)acetamide (**21**),[20] 4-{[7-(3-carboxypropoxy)-2-naphtyl]oxy}butanoic acid (**26**),[18] 2-amino-*N*-(1-carbamoylmethyl-2-oxo-1,2-dihydropyridin-3-yl)-3-methylbutyramide (**27**)[20] and *N*-{3-[*N*′-(2-amino-3-methylbutyryl)hydrazinocarbonyl]-4-methoxyphenyl}acetamide (**28**)[20] were prepared according to published methods. Melting points were determined on a Kofler melting point apparatus. NMR spectra were performed on a Ultrafield AVANCE 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) or a Bruker AVANCE 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz). Unless otherwise stated, $CDCl_3$ was used as solvent. Chemical shifts (δ) are in ppm, using the following abbreviations: singlet (s), doublet (d), doublet doublet (dd), triplet (t), quintuplet (quint), multiplet (m), broad multiplet (bm), and broad singlet (bs). Mass spectra were obtained using a Bruker Esquire electrospray ionization apparatus at the SAMM (Faculty of Pharmacy at Châtenay-Malabry, France). Elemental analyses (C, H, N) were performed on a PerkinElmer CHN Analyser 2400 at the Microanalyses Service of the Faculty of Pharmacy at Châtenay-Malabry (France). Elemental analysis data are included in the Supporting Information.

[5-Benzyloxycarbonylamino-5-(1-carbamoylmethyl-2-oxo-1,2-dihydropyridin-3-ylcarbamoyl)pentyl]carbamic acid *tert*-butyl ester (18). Compound **17** (436 mg, 2.61 mmol) and N_α-Boc-N_ε-Z-Lys (1.09 g, 2.87 mmol) were dissolved in DMF (20 mL). DIPEA (1.1 mL, 6.53 mmol), HBTU (1.19 g, 3.13 mmol), and HOBt (388 mg, 2.87 mmol) were successively added to the reaction mixture. The reaction mixture was stirred under argon at room temperature for 48 h. DMF was evaporated under reduced pressure and the residue was dissolved in EtOAc (200 mL). The organic phase was washed with 10% aqueous citric acid (50 mL), H_2O (100 mL), 10% aqueous K_2CO_3 (50 mL), and brine (25 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting crude product was purified by flash chromatography on silica gel and eluted with EtOAc/MeOH (95:5) to give **18** as a white solid: mp: 192–193 °C (818 mg, 79% yield); ^{1}H NMR ([D$_6$]DMSO): δ = 9.1 (s, 1H), 8.2 (dd, J = 1.3, 7.3 Hz, 1H), 7.6 (s, 1H), 7.6–7.3 (m, 6H,), 7.2 (s, 1H), 6.2 (t, J = 7.3 Hz, 1H), 5.0 (s, 2H), 4.6 (s, 2H), 4.0 (m, 1H), 3.0 (q, J = 6 Hz, 2H), 1.7 (m, 1H), 1.6 (m, 1H), 1.4–1.3 (m, 13H); ^{13}C NMR ([D$_6$]DMSO): δ = 171.8, 168.3, 156.7, 156.1, 155.7, 137.3, 133.0, 128.3, 128.0, 127.7, 122.0, 104.7, 78.5, 65.1, 55.4, 51.2, 40.1, 30.8, 29.0, 28.1, 22.9; ESI$^+$ MS *m*/*z*: 553 [*M*+Na]$^+$, 568 [*M*+39]$^+$; Anal. (C, H, N): $C_{26}H_{35}N_5O_7$.

[5-Amino-5-(1-carbamoylmethyl-2-oxo-1,2-dihydropyridin-3-ylcarbamoyl)pentyl]carbamic acid benzyl ester in trifluoroacetic acid (19). Trifluoroacetic acid (TFA; 5 mL) was added to a solution of **18** (300 mg, 0.57 mmol) in dry CH_2Cl_2 (5 mL). The mixture was stirred at room temperature for 1.5 h, then the solvent was evaporated under reduced pressure. The crude product was crystallized from MeOH/Et_2O to yield **19** as a beige solid: mp: 124–126 °C (248 mg, 100% yield); ^{1}H NMR ([D$_6$]DMSO): δ = 10.0 (s, 1H), 8.3–8.2 (m, 4H), 7.6 (s, 1H), 7.4–7.2 (m, 7H), 6.3 (t, J = 7.2 Hz, 1H), 5.0 (s, 2H), 4.6 (s, 2H), 4.26 (m, 1H), 2.98 (m, 2H), 1.74 (m, 2H) 1.4–1.3 (m, 4H); ^{13}C NMR (CD_3OD) δ = 171.4, 169.2, 159.15, 156.5, 135.1, 129.5, 129.3, 129.0, 128.8, 127.3, 107.3, 67.4, 55.0, 52.9, 41.2, 32.4, 30.5, 23.0; ESI$^+$ MS *m*/*z*: 430 [*M*+H]$^+$; HRMS calcd for $C_{21}H_{27}N_5O_5$+H: 430.2090, found: 430.2112.

[5-Amino-5-(1-carbamoylmethyl-2-oxo-1,2-dihydropyridin-3-ylcarbamoyl)pentyl]carbamic acid *tert*-butyl ester (20). Pd/C 10% (53 mg, 20% mass) was added to a solution of **18** (264 mg, 0.5 mmol) in MeOH (25 mL). The reaction flask was purged three times with hydrogen, and stirring was maintained under hydrogen atmosphere at room temperature for 12 h. After filtration through Celite, the cake was washed with MeOH (200 mL), and the filtrate was concentrated to afford a solid that was triturated with cyclohexane and petroleum ether to yield **20** as a white solid: mp: 146–148 °C (190 mg, 96% yield); ^{1}H NMR: δ = 9.2 (s, 1H), 8.4 (d, J = 7.3 Hz, 1H), 7.6 (s, 1H), 7.1 (d, J = 7.3 Hz, 1H), 6.9 (s, 1H), 5.9 (bs, 1H), 5.4 (bs, 1H), 6.2 (t, J = 7.3 Hz, 1H), 4.6 (s, 2H), 4.4 (m, 1H), 2.7 (m, 2H), 1.9 (m, 1H), 1.7 (m, 1H), 1.4–1.2 (m, 13H); ^{13}C NMR: δ = 171.7, 168.9, 156.6, 156.2, 131.3, 127.8, 123.2, 107.3, 78.4, 54.0, 53.3, 41.4, 32.4, 30.0, 28.4, 22.8; ESI$^+$ MS *m*/*z*: 418 [*M*+Na]$^+$; HRMS calcd for $C_{18}H_{29}N_5O_5$+Na: 418.2066, found: 418.2078.

{6-[*N*′-(5-Acetylamino-2-methoxybenzoyl)hydrazino]-5-benzyloxycarbonylamino-6-oxohexyl}carbamic acid *tert*-butyl ester (22). Compound **22** was synthesized from **21**, following the same procedure described for **18**, and was obtained as a white solid in 73% yield: mp: 136–138 °C; ^{1}H NMR: δ = 11.9 (s, 1H), 11.3 (d, J = 7.0 Hz, 1H), 8.8 (s, 1H), 8.4 (d, J = 9.0 Hz, 1H), 7.9 (s, 1H), 7.3 (m, 5H), 7.0 (d, J = 9.0 Hz, 1H), 5.6 (d, J = 7.5 Hz, 1H), 5.0 (s, 2H), 4.9 (m, 1H), 4.7 (s, 1H), 4.0 (s, 3H), 3.0 (m, 2H), 2.2 (s, 3H), 1.8 (m, 1H), 1.7 (m, 1H), 1.4 (s, 9H), 1.4–1.3 (m, 4H); ^{13}C NMR: δ = 169.0, 166.3, 158.6, 156.3, 155.6, 153.6, 136.6, 133.1, 128.4, 128.0, 126.1, 122.5, 117.8, 112.4, 79.8, 66.5, 56.5, 52.0, 40.7, 34.0, 29.5, 28.6, 24.6, 21.9; ESI$^+$ MS *m*/*z*: 608 [*M*+Na]$^+$; Anal. (C, H, N): $C_{29}H_{39}N_5O_8$.

{6-[*N*′-(5-Acetylamino-2-methoxybenzoyl)hydrazino]-5-amino-6-oxohexyl}carbamic acid benzyl ester (23). TFA (4 mL) was added to a solution of **22** (350 mg, 0.60 mmol) in dry CH_2Cl_2 (4 mL). The mixture stirred at room temperature for 1 h, then the solvent was evaporated under reduced pressure. The crude product was crystallized from MeOH/Et_2O to yield compound **23** as a light pink solid: mp: 140–142 °C (315 mg, 88% yield); ^{1}H NMR ([D$_6$]DMSO): δ = 10.9 (s, 1H), 10.2 (s, 1H), 10.0 (s, 1H), 8.2 (bs, 2H), 8.0 (s, 1H), 7.7 (d, J = 9.0 Hz, 1H), 7.4–7.3 (m, 6H), 7.1 (d, J = 9.0 Hz, 1H), 5.0 (s, 2H), 3.8 (m, 4H, H_{11}), 3.0 (m, 2H), 2.0 (s, 3H), 1.8 (m, 2H), 1.5–1.4 (m, 4H); ^{13}C NMR ([D$_4$]MeOH): δ = 171.6, 169.1, 158.6, 166.4, 159.1, 155.7, 133.5, 129.5, 129.0, 128.7, 127.0, 124.4, 120.9, 113.5, 67.4, 56.9, 53.4, 41.2, 32.2, 30.5, 23.6, 22.9; APCI$^+$ MS *m*/*z*: 487 [*M*+H]$^+$; Anal. (C, H, N): $C_{26}H_{32}N_5O_6{\cdot}CF_3COOH{\cdot}1.5H_2O$.

{6-[*N*′-(5-Acetylamino-2-methoxybenzoyl)hydrazino]5-amino-6-oxohexyl}carbamic acid *tert*-butyl ester (24). Pd/C 10% (23 mg, 20% mass) was added to a solution of **22** (150 mg, 0.26 mmol) in MeOH (15 mL). The reaction flask was purged three times with hydrogen, and stirring was maintained under hydrogen atmosphere at room temperature for 12 h. The mixture was filtered through celite, and the cake was washed with MeOH (200 mL). The filtrate was evaporated to dryness to yield crude product **24** as a colorless oil (111 mg, 96% yield): ^{1}H NMR: δ = 9.3 (s, 1H), 8.9 (s, 1H), 8.3 (s, 2H), 7.9 (m, 1H), 6.9 (m, 1H), 5.6 (m, 1H), 4.6 (m, 2H), 4.1–3.9 (m, 4H), 2.3–2.2 (m, 5H), 1.8 (bm, 2H), 1.5–1.3 (m, 13H); ^{13}C NMR: δ = 169.2, 169.1, 158.5, 155.6, 153.7, 132.9, 125.9, 122.7, 118.2, 112.0, 79.9, 56.47, 52.3, 52.2, 50.2, 27.0, 28.4, 24.4, 22.2; ESI$^+$ MS *m*/*z*: 452 [*M*+H]$^+$; HRMS calcd for $C_{21}H_{33}N_5O_6$+H: 452.2509, found: 452.2526.

Synthesis of molecular tong 1. Compounds **25** (200 mg, 0.3 mmol) and **19** (195 mg, 0.36 mmol) were dissolved in DMF (10 mL). DIPEA (0.37 mL, 2.1 mmol), HBTU (284 mg, 0.75 mmol), and HOBt (89 mg, 0.66 mmol) were successively added to the reaction mixture, which stirred under argon at room temperature for

48 h. DMF was evaporated under reduced pressure, and the residue was washed successively with EtOAc, CH_2Cl_2, MeOH, H_2O (100 mL), 10% aqueous citric acid (50 mL), 10% aqueous K_2CO_3 (50 mL), and H_2O again (50 mL). Product **1** was obtained as a white solid: mp: 233–235 °C (277 mg, 86%); ^{1}H NMR ([D$_6$]DMSO): δ = 9.2 (s, 1H), 8.3 (d, J = 7.3 Hz, 1H), 8.2 (d, J = 7.2 Hz, 1H), 8.0–7.9 (m, 3H), 7.7 (d, J = 8.9 Hz, 2H), 7.6 (s, 1H), 7.3–7.1 (m, 6H), 7.2 (s, 2H), 7.1 (s, 2H), 7.0 (d, J = 8.9 Hz, 2H), 6.2 (t, J = 7.2 Hz, 1H), 5.0 (s, 2H), 4.5 (s, 2H), 4.4–4.3 (m, 2H), 4.1 (q, J = 7.2 Hz, 2H), 4.0 (q, J = 6.5 Hz, 4H), 3.6 (s, 3H), 2.9 (q, J = 6.1 Hz, 2H), 2.4–2.3 (m, 4H), 2.2–2.0 (m, 6H), 1.8–1.7 (m, 1H), 1.6–1.5 (m, 2H), 1.5–1.3 (m, 4H), 1.3 (m, 2H), 0.9–0.8 (m, 18H); ^{13}C NMR ([D$_6$]DMSO): δ = 172.3, 172.2, 171.7, 171.3, 170.9, 168.3, 157.0, 156.7, 156.1, 137.3, 135.7, 133.1, 128.9, 128.3, 128.1, 127.7, 123.7, 122.3, 115.9, 106.1, 104.7, 66.9, 65.1, 57.8, 57.3, 53.7, 51.6, 51.3, 50.8, 40.7, 40.1, 31.6, 30.7, 30.3, 29.9, 29.1, 25.0, 24.9, 24.1, 22.9, 22.8, 21.6, 19.2, 18.8, 18.2, 18.1; ESI$^+$ MS m/z: 1092 [M+Na]$^+$, 1108 [M+K]$^+$; HRMS (calcd for $C_{56}H_{76}N_8O_{13}$+Na: 1092.2376, found: 1091.5454; Anal. (C, H, N): $C_{56}H_{76}N_8O_{13}$.

Synthesis of molecular tong 2. Tong **2** was synthesized from **23**, following the same procedure as described for **1** from **25**, and was obtained as a white solid in 63% yield: mp: 199–201 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 10.3 (bs, 1H), 10.1 (bs, 1H), 9.9 (s, 1H), 8.0 (d, J = 7.1 Hz, 1H), 8.0–7.9 (m, 4H), 7.76 (dd, J = 2.4, 9.0 Hz, 1H), 7.7 (d, J = 8.8 Hz, 2H), 7.4–7.3 (m, 5H), 7.2 (t, J = 5.4 Hz, 1H), 7.2 (m, 2H), 7.1 (d, J = 9.0 Hz, 1H), 7.0 (m, 2H), 5.0 (s, 2H), 4.4 (q, J = 7.8 Hz, 2H), 4.16 (q, J = 7.8 Hz, 2H), 4.04 (m, 4H), 3.84 (s, 3H), 3.60 (s, 3H), 3.0 (q, J = 6.2 Hz, 2H), 2.3 (t, J = 7.0 Hz, 4H), 2.0–1.9 (m, 9H), 1.7 (m, 1H), 1.6 (m, 2H), 1.5–1.4 (m, 4H), 1.3 (m, 2H), 0.9–0.8 (m, 18H); ^{13}C NMR ([D$_6$]DMSO): δ = 172.2, 171.7, 171.5, 170.9, 168.0, 163.3, 157.0, 156.1, 152.6, 137.3, 135.8, 132.6, 129.0, 128.3, 127.7, 123.7, 123.2, 121.3, 121.2, 115.9, 112.4, 106.1, 67.0, 66.9, 65.1, 57.8, 57.3, 56.1, 51.6, 51.1, 50.8, 40.7, 40.1, 32.1, 31.6, 30.2, 29.9, 29.2, 25.0, 24.9, 24.1, 23.8, 22.9, 22.6, 21.6, 19.2, 18.8, 18.2, 18.1; ESI$^+$ MS m/z: 1148 [M+Na]$^+$, 1164 [M+K]$^+$; Anal. (C, H, N): $C_{59}H_{80}N_8O_{14}$.

Synthesis of molecular tong 3. Tong **3** was synthesized from **20**, following the same procedure as described for **1** from **25**, and was obtained as a white solid in 57% yield: mp: 172–176 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 9.2 (s, 1H), 8.2 (d, J = 7.5, 1H), 7.9 (m, 3H), 7.8 (s, 1H), 7.7 (d, J = 8.8, 2H), 7.6 (s, 1H), 7.4 (d, J = 6.4, 1H), 7.3 (d, J = 7.0, 1H), 7.2 (m, 3H), 7.0 (d, J = 9.0, 2H), 6.2 (t, J = 6.7, 1H), 4.6 (s, 2H), 4.38 (m, 1H), 4.2–4.1 (m, 2H), 4.1–4.0 (m, 5H), 3.6 (s, 3H), 3.0 (m, 2H), 2.4–2.2 (m, 4H), 2.0–1.9 (m, 6H), 1.7–1.6 (m, 1H), 1.6–1.5 (m, 2H), 1.4–1.2 (m, 15H), 0.9–0.7 (m, 18H); ^{13}C NMR ([D$_6$]DMSO): δ = 172.6, 172.3, 172.1, 171.9, 171.4, 168.7, 157.5, 157.2, 136.2, 133.4, 129.4, 128.5, 124.2, 122.5, 116.4, 106.7, 105.2, 79.0, 67.4, 58.3, 57.7, 55.9, 52.0, 51.7, 51.3, 41.2, 38.7, 32.3, 32.1, 31.2, 30.6, 30.3, 29.2, 28.6, 25.5, 25.4, 24.5, 23.5, 23.4, 22.1, 19.6, 19.3, 18.6; ESI$^-$ MS m/z 1034 [M−H]$^+$; Anal. ($C_{53}H_{78}N_8O_{13}$·3H$_2$O) C, H, N.

Synthesis of molecular tong 4. TFA (2 mL, 26 mmol) was added to a solution of **3** (100 mg, 0.1 mmol) in dry CH_2Cl_2 (2 mL). The mixture was stirred for 30 min, then the solvent was evaporated under reduced pressure. Azeotropic removal of excess TFA was carried out using toluene. The crude product was recrystallized from MeOH/Et$_2$O to give **4** as a white solid: mp: 180–182 °C (100 mg, 99%); ^{1}H NMR ([D$_6$]DMSO): δ = 10.0 (s, 1H), 8.2 (d, J = 7.2, 1H), 8.0–7.8 (m, 5H), 7.7 (d, J = 8.9, 2H), 7.6 (s, 1H), 7.4 (d, J = 6.8, 1H), 7.2 (m, 3H), 7.0 (m, 1H), 6.3 (t, J = 7.1, 1H), 4.6 (s, 2H), 4.4 (m, 1H), 4.2–4.0 (m, 7H), 3.6 (s, 3H), 3.1 (s, 2H), 2.4–2.2 (m, 4H), 2.0–1.8 (m, 6H), 1.8–1.6 (m, 3H), 1.6–1.4 (m, 6H), 0.9–0.8 (m, 18H); ^{13}C NMR ([D$_6$]DMSO): δ = 172.6, 172.2, 172.1, 171.8, 171.4, 168.7, 157.5, 157.2, 136.2, 134.5, 129.4, 128.2, 124.2, 122.5, 116.3, 106.7, 104.9, 67.4, 58.4, 57.7, 53.4, 52.0, 51.7, 51.4, 41.2, 38.6, 32.3, 32.1, 31.9, 30.6, 30.3, 29.2, 25.5, 25.4, 24.5, 23.4, 22.3, 22.1, 19.6, 19.3, 18.6; ESI$^+$ MS m/z: 936 [M+H]$^+$, 953 [M+Na]$^+$; Anal. (C, H, N): $C_{48}H_{70}N_8O_{11}$·CF_3COOH.

Synthesis of molecular tong 5. Tong **5** was synthesized from **24**, following the same procedure as described for **1** from **25**, and was obtained as a white solid in 53% yield: mp: 150–156 °C; ^{1}H NMR: δ = 11.0 (bs, 1H), 9.2 (bs, 1H), 8.5 (d, J = 9 Hz, 1H), 8.4 (bs, 1H), 8.0 (s, 1H), 7.9 (bs, 1H,), 7.58 (d, J = 8.8 Hz, 2H), 7.2 (bs, 1H), 7.2–6.9 (m, 5H), 6.1–5.8 (m, 2H), 4.8 (m, 1H), 4.7 (m, 1H), 4.6 (m, 1H), 4.5 (m, 1H), 4.1 (m, 4H), 4.0 (s, 3H), 3.7 (s, 3H), 3.1 (m, 2H), 2.6 (m, 1H), 2.4 (m, 4H), 2.3 (m, 1H), 2.1–2.2 (m, 8H„ 1.9 (m, 1H), 1.6–1.5 (m, 5H), 1.4 (s, 9H), 1.3 (m, 2H), 0.9–0.8 (m, 18H); ^{13}C NMR: δ = 172.6, 172.1, 172.1, 172.0–171.9, 171.7, 168.8, 167.6, 167.4, 157.5, 157.4, 153.4, 134.9, 133.5, 129.1, 125.3, 124.3, 122.3, 116.5, 116.2, 118.0, 106.4, 106.2, 79.5, 66.9, 66.7, 57.9, 57.4, 56.5, 52.8, 52.0, 51.6, 40.9, 39.1, 32.6, 31.9, 31.0, 29.3, 28.8, 28.3, 25.1, 24.8, 24.2, 22.6, 22.4, 19.0, 18.6, 17.8; ESI$^+$ MS m/z: 1114 [M+Na]$^+$; Anal. (C, H, N): $C_{56}H_{82}N_8O_{14}$.

Synthesis of molecular tong 6. TFA (2 mL, 26 mmol) was added to a solution of **5** (100 mg, 0.09 mmol) in dry CH_2Cl_2 (2 mL). The mixture stirred for 2 h, then the solvent was evaporated under reduced pressure. Azeotropic removal of excess TFA was carried out using toluene. The crude product was recrystallized from MeOH/Et$_2$O to give product **6** as a light pink solid: mp: 136–139 °C (79 mg, 78%); ^{1}H NMR ([D$_6$]DMSO): δ = 10.1 (bs, 1H), 9.9 (bs, 2H), 8.0–7.7 (m, 8H), 7.2–7.0 (m, 3H), 6.9 (s, 2H), 4.4 (bs, 1H), 4.1–4.0 (m, 6H), 3.8 (m, 4H), 3.6 (s, 3H), 3.0 (m, 2H), 2.5–2.4 (m, 4H), 2.0 (m, 10H), 1.78 (m, 2H), 1.6–1.4 (m, 8H), 0.9–0.7 (m, 18H); ^{13}C NMR ([D$_6$]DMSO): δ = 172.2, 171.7, 171.4, 170.9, 168.0, 157.0, 152.6, 135.7, 132.6, 128.9, 124.5, 123.7, 121.3, 115.9, 112.5, 106.1, 66.9, 57.8, 57.2, 56.2, 51.6, 51.1, 50.8, 40.7, 32.0, 31.8, 31.6, 30.2, 29.8, 25.0, 24.9, 24.1, 23.8, 22.9, 21.6, 21.4, 19.16, 18.8, 18.2, 18.1; ESI$^-$ MS m/z: 990 [M−H]$^+$; Anal. (C, H, N): ($C_{51}H_{74}N_8O_{12}$·CF_3COOH·1.5H$_2$O.

Synthesis of molecular tong 7. Tong **7** was synthesized from **27**, following the same procedure as described for **1** from **25**, and was obtained as a white solid in 54% yield: mp: 191–196 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 9.16 (s, 1H), 8.25 (d, J = 8.0 Hz, 1H), 8.20 (d, J = 7.0 Hz, 1H), 7.69 (d, J = 8.9 Hz, 1H), 7.62 (s, 1H), 7.31 (d, J = 7.0 Hz, 1H), 7.21 (s, 1H), 7.16 (s, 1H), 6.97 (d, J = 8.9 Hz, 1H), 6.23 (t, J = 7.0 Hz, 1H), 4.56 (s, 2H), 4.37 (t, J = 8 Hz, 1H), 4.06 (t, J = 7 Hz, 2H), 2.43 (t, J = 6.6 Hz, 2H), 2.11 (m, 1H), 2.02 (m, 2H), 0.89 (d, J = 6.4 Hz, 6H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 172.3, 170.7, 168.3, 157.0, 156.7, 135.7, 133.2, 128.9, 128.0, 123.7, 122.5, 115.9, 106.2, 104.6, 66.9, 58.9, 51.3, 31.6, 29.7, 25.0, 19.2, 18.0; ESI$^+$ MS m/z: 851 [M+Na]$^+$, 867 [M+K]$^+$; Anal. (C, H, N): $C_{42}H_{52}N_8O_{10}$·2H$_2$O.

Synthesis of molecular tong 8. Tong **8** was synthesized from **28**, following the same procedure as described for **1** from **25**, and was obtained as a white solid in 89% yield: mp: 224–246 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 10.32 (s, 1H), 9.93 (s, 2H), 8.00 (d, J = 9.0 Hz, 1H), 7.94 (s, 1H), 7.74 (dd, J = 2.7, 9.0 Hz, 1H), 7.70 (d, J = 9.0 Hz, 1H), 7.18 (bd, J = 2 Hz, 1H), 7.09 (d, J = 9.0 Hz, 1H), 6.97 (dd, J = 2.0, 9.0 Hz, 1H), 4.33 (m, 1H), 4.05 (t, J = 6.5 Hz, 2H), 3.84 (s, 3H), 2.39 (m, 2H), 2.01 (s, 3H), 1.98 (m, 3H), 0.95 (d, J = 6.0 Hz, 3H), 0.90 (d, J = 6.0 Hz, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 171.6, 169.5, 168.0, 163.3, 157.0, 152.6, 135.7, 132.6, 128.9, 123.7, 123.3, 121.3, 121.1, 115.9, 112.4, 106.1, 66.9, 56.1, 31.5, 30.7, 25.0, 19.2, 18.3; IR (cm^{-1}): 3269, 1635, 1605, 1542, 1489, 1210 (C–O); ESI$^+$ MS m/z: 964 [M+Na]$^+$, 980 [M+K]$^+$; Anal. (C, H, N): $C_{48}H_{60}N_8O_{12}$·1.5H$_2$O.

Synthesis of molecular tong 9. Tong **9** was synthesized from **19**, following the same procedure as described for **1** from **25**, and was obtained as a white solid in 82% yield: mp: 183–185 °C; ^{1}H NMR

(400 MHz, [D$_6$]DMSO): δ=9,19 (s, 1H), 8.36 (d, J=7.3 Hz, 1H), 8.19 (dd, J=1.2, 7.1 Hz, 1H), 7.69 (d, J=8.9 Hz, 1H), 7.61 (s, 1H), 7.34–7.29 (m, 6H), 7.20 (s, 1H), 7.16 (s, 1H), 6.97 (dd, J=2.2, 8.9 Hz, 1H), 6.23 (t, J=7.1 Hz, 1H), 5.00 (s, 2H), 4.56 (s, 2H), 4.40 (bm, 1H), 4.06 (t, J=6.3 Hz, 2H), 2.96 (q, J=6.3 Hz, 2H), 2.39 (t, J=6.4 Hz, 2H), 2.02 (quint, J=6.4 Hz, 2H), 1.74 (m, 1H), 1.58 (m, 1H), 1.39 (m, 2H), 1.32 (m, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=172.3, 171.3, 168.3, 157.0, 156.7, 156.1, 137.3, 135.7, 133.1, 133, 128.9, 128.3, 128.1, 127.7, 123.7, 122.3, 115.9, 106.2, 104.7, 66.9, 65.1, 53.7, 51.3, 40.1, 31.7, 30.7, 29.1, 24.9, 22.8; IR (cm^{-1}): 3303, 1676, 1647, 1515, 1210; ESI$^+$ MS m/z: 1178 [M+Na]$^+$; Anal. (C, H, N): $C_{60}H_{70}N_{10}O_{14}$·2.5H$_2$O.

Synthesis of molecular tong 10. Pd/C 10% (12 mg, 20% mass) was added to a solution of **9** (65 mg, 0.06 mmol) in MeOH (20 mL) and DMF (2 mL). The reaction flask was purged three times with hydrogen, and stirring was maintained under hydrogen atmosphere at room temperature for 12 h. The mixture was filtered through Celite, and the cake was washed with MeOH (200 mL). The filtrate was concentrated under reduced pressure and the resulting residue was dissolved in MeOH. TFA (0.02 mL, 0.26 mmol, 4.3 equiv) was added, and the mixture stirred for 10 min before evaporation of the solvent under reduced pressure. The crude product was recrystallized from MeOH/Et$_2$O to obtain **10** as a beige solid: mp: 176–178 °C (45 mg, 72%). R_f=0 (EtOAc/MeOH/NH$_4$OH, 79:20:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=9.21 (s, 1H), 8.39 (d, J=8.0 Hz, 1H), 8.27 (bs, 3H), 8.19 (d, J=8.0 Hz, 1H), 7.71 (d, J=8.8, 1H), 7.63 (s, 1H), 7.32 (d, J=8.0 Hz, 1H), 7.20 (s, 1H), 7.18 (s, 1H), 6.97 (d, J=8.8 Hz, 1H), 6.24 (t, J=8.0 Hz, 1H), 4.57 (s, 2H), 4.46 (bm, 1H), 4.06 (m, 2H), 2.97 (m, 2H), 2.40 (t, J=7.2 Hz, 2H), 2.03 (m, 2H), 1.77 (m, 1H), 1.60 (m, 1H), 1.40–1.33 (m, 4H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=172.0, 170.1, 168.3, 156.2, 135.7, 133.6, 129.0, 128.7, 123.7, 122.6, 115.7, 105.9, 104.8, 66.7, 47.7, 51.0, 31.6, 30.7, 32.0, 24.8, 22.6; IR (cm^{-1}): 3338, 1644, 1514, 1200, 1130; ESI$^+$ MS m/z: 888 [M+H]$^+$, 910 [M+Na]$^+$; Anal. (C, H, N): $C_{44}H_{48}N_{10}O_{10}$·2CF$_3$COOH ·1.5H$_2$O.

Synthesis of molecular tong 11. Tong **11** was synthesized from **23**, following the same procedure as described for **1** from **25**, and was obtained as a white solid in 74% yield: mp: 188–190 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.33 (s, 1H), 9.93 (m, 2H), 8.06 (d, J=8.1 Hz, 1H), 7.94 (d, J=2.6, 1H), 7.76 (dd, J=2.6, 9.0 Hz, 1H), 7.69 (d, J=9.0 Hz, 1H), 7.35–7.31 (m, 5H), 7.21 (bs, 1H), 7.18 (s, 1H), 7.09 (d, J=9.0 Hz, 1H), 6.96 (dd, J=2.0, 9.0 Hz, 1H), 5.00 (s, 2H), 4.41 (bm, 1H), 4.04 (t, J=6.1, 2H), 3.84 (s, 3H), 2.98 (q, J=6.1 Hz, 2H), 2.36 (t, J=7.2 Hz, 2H), 2.01 (s, 3H), 1.98 (m, 2H), 1.69 (bm, 1H), 1.58 (bm, 1H), 1.40 (m, 2H), 1.34 (m, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=171.6, 170.2, 168.0, 163.1, 157.0, 156.1, 152.7, 137.3, 135.8, 132.6, 128.9, 128.3, 127.7, 123.7, 123.4, 121.3, 121.0, 115.9, 112.4, 106.2, 66.9, 65.1, 56.1, 50.9, 40.1, 32.0, 31.6, 29.1, 24.9, 23.8, 22.6; IR (cm^{-1}): 3261, 1686, 1605, 1539, 1252, 1210, 1023; ESI$^+$ MS m/z: 1290 [M+Na]$^+$, 1306 [M+K]$^+$; Anal. (C, H, N): $C_{66}H_{78}N_{10}O_{16}$·1.5H$_2$O.

Synthesis of molecular tong 12. Tong **12** was synthesized from **11**, following the same procedure as described for **10** from **9**, and was obtained as a white solid in 69% yield: mp: 139–141 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.35 (s, 1H), 9.97 (d, J=8.9 Hz, 2H), 8.51 (bs, 3H), 8.13 (d, J=7.3 Hz, 1H), 7.98 (s, 1H), 7.70 (d, J=8.4 Hz, 2H), 7.19 (s, 1H), 7.10 (d, J=8.8 Hz, 1H), 6.98 (d, J=7.9 Hz, 1H), 4.43 (m, 1H), 4.06 (m, 2H), 3.83 (s, 3H), 2.84 (m, 2H), 2.36 (m, 2H), 2.01 (s, 3H), 1.72 (m, 2H), 1.57 (m, 1H), 1.38 (m, 2H), 1.29–1.23 (m, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=171.7, 170.2, 168.0, 163.3, 158.5–157.6 (q, J=32 Hz), 157.0, 152.7, 135.8, 132.6, 129.0, 123.7, 123.4, 121.3, 121.0, 118.8, 115.9, 112.4, 106.2, 66.9, 56.1, 50.7, 48.0, 31.7, 31.6, 29.0, 24.9, 23.8, 22.2; IR (cm^{-1}): 1648, 1513, 1128; ESI$^+$ MS m/z: 1000 [M+H]$^+$; Anal. (C, H, N): $C_{50}H_{66}N_{10}O_{12}$·2CF$_3$COOH·3.5H$_2$O.

Synthesis of molecular tong 13. Tong **13** was synthesized from **20**, following the same procedure as described for **1** from **25**, and was obtained as a white solid in 69% yield: mp: 149–151 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=9.15 (s, 1H), 8.21 (d, J=7.0 Hz, 1H), 7.83 (t, J=5.2, 1H), 7.70 (d, J=8.8 Hz, 1H), 7.61 (s, 1H), 7.37 (d, J=7.0 Hz, 1H), 7.30 (d, J=7.0 Hz, 1H), 7.20 (s, 1H), 7.18 (s, 1H), 6.95 (dd, J=1.6, 8.8 Hz, 1H), 6.23 (t, J=7.0 Hz, 1H), 4.57 (s, 2H), 4.04 (m, 3H), 3.03 (bq, J=5.8 Hz, 2H), 2.25 (t, J=7.0, 2H), 1.98 (q, J=7.0 Hz, 2H), 1.71 (m, 1H), 1.55 (m, 1H), 1.38 (s, 9H), 1.38 −1.31 (m, 4H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=171.8, 171.4, 168.3, 157.0, 156.7, 155.7, 135.8, 133.0, 129.0, 128.0, 123.7, 122.1, 115.9, 106.1, 104.7, 78.5, 67.0, 55.4, 51.2, 38.3, 31.8, 30.8, 28.8, 28.2, 24.9, 23.1; IR (cm^{-1}): 3314, 2933, 1684, 1645, 1514, 1369, 1252, 1210, 1163; ESI$^-$ MS m/z: 1086 [M−1]$^+$. Anal (C, H, N): $C_{54}H_{76}N_{10}O_{14}$·1.5H$_2$O.

Synthesis of molecular tong 14. Tong **14** was synthesized from **13**, following the same procedure as described for **4**, and was obtained as a white solid in 99% yield: mp: 148 −150 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=9.98 (s, 1H), 8.21 (d, J=1.5 Hz, 1H), 8.17 (s, 3H), 7.82 (t, J=5.3 Hz, 1H), 7.68 (d, J=9.0 Hz, 1H), 7.64 (s, 1H), 7.37 (dd, J=1.5, 7.0 Hz, 1H), 7.20 (s, 1H), 7.16 (d, J=2.0, 1H), 6.94 (dd, J=2.2, 9.0 Hz, 1H), 6.25 (t, J=7.0 Hz, 1H), 4.57 (s, 2H), 4.24 (t, J=6.0 Hz, 1H), 4.02 (t, J=6.0 Hz, 2H), 3.02 (q, J=7.0 Hz, 2H), 2.23 (t, J=7.0 Hz, 2H), 1.95 (quint, J=7.0 Hz, 2H), 1.73 (m, 2H), 1.39 (q, J=7.0 Hz, 2H), 1.33 (m, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=171.4, 168.5, 168.3, 157.0, 156.7, 135.7, 134.2, 129, 127.7, 125.0, 123.6, 116.0, 106.1, 104.4, 67.0, 52.6, 51.2, 38.2, 31.8, 31.1, 28.7, 24.9, 21.7; IR (cm^{-1}): 3262, 2968, 1633, 1514, 1200, 1130; ESI$^+$ MS m/z: 888 [M+H]$^+$, 910 [M+Na]$^+$; Anal. (C, H, N): $C_{44}H_{58}N_{10}O_{10}$·2CF$_3$COOH·2H$_2$O.

Synthesis of molecular tong 15. Tong **15** was synthesized from **24**, following the same procedure as described for **1** from **25**, and was obtained as a white solid in 67% yield: mp: 164–170 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ=11.85 (bs, 1H), 11.25 (bs, 1H), 8.92 (s, 1H), 8.40 (bm, 1H), 7.95 (s, 1H), 7.54 (d, J=8.6 Hz, 1H), 6.97 (d, J=8.9 Hz, 1H), 6.89 (s, 1H), 6.86 (d, J=8.6 Hz, 1H), 5.86 (bs, 1H), 5.66 (d, J=7.8 Hz, 1H), 4.88 (bm, 1H), 4.00 (m, 5H), 3.08 (m, 2H), 2.27 (m, 2H), 2.19 (s, 3H), 2.09 (m, 2H), 1.80 (m, 1H), 1.71 (m, 1H), 1.45 (s, 9H), 1.34–1.32 (m, 4H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=172.4, 171.0, 169.2, 167.0, 157.3, 155.6, 153.6, 135.8, 133.1, 129.0, 126.2, 124.2, 122.6, 117.9, 116.1, 112.1, 106.3, 79.8, 66.7, 56.6, 52.1, 39.3, 34.0, 32.7, 29.2, 28.4, 25.1, 24.5, 22.1; IR (cm^{-1}): 3286, 2936, 1631, 1493, 1251, 1161; ESI$^+$ MS m/z: 1222 [M+Na]$^+$; Anal. (C, H, N): $C_{60}H_{82}N_{10}O_{16}$·4H$_2$O.

Synthesis of molecular tong 16. Tong **16** was synthesized from **15**, following the same procedure as described for **4**, and was obtained as a white solid in 92% yield: mp: 169–171 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.13 (bs, 1H), 9.98 (bs, 1H), 8.69 (bs, 1H), 7.97 (d, J=2.5 Hz, 1H), 7.86 (t, J=5.1 Hz, 1H), 7.72 (dd, J=2.5, 9.0 Hz, 1H), 7.70 (d, J=8.7 Hz, 1H), 7.16 (s, 1H), 7.12 (d, J=9.0 Hz, 1H), 6.94 (dd, J=2.1, 8.7 Hz, 1H), 4.04 (t, J=6.2 Hz, 2H), 3.85 (s, 3H), 3.83 (m, 1H), 3.06 (m, 2H), 2.28 (t, J=7.0 Hz, 2H), 2.01 (s, 3H), 1.99 (m, 2H), 1.78 (bm, 2H), 1.43 (m, 4H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=171.5, 168.1, 167.2, 163.5, 157.0, 152.6, 135.8, 132.7, 129.0, 123.7, 123.5, 121.2, 121.0, 115.9, 112.5, 106.1, 67.0, 56.2, 51.2, 38.2, 31.9, 31.0, 28.7, 25.0, 23.8, 21.5; IR (cm^{-1}): 3316, 2948, 1632, 1490, 1252, 1133, 1016; ESI$^+$ MS m/z: 1000 [M+1]$^+$; Anal. (C, H, N): $C_{50}H_{66}N_{10}O_{12}$·2CF$_3$COOH ·2H$_2$O.

Enzymatic studies

The fluorogenic substrate DABCYL-γ-abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS {DABCYL: 4-(4′-dimethylaminophenylazo)benzoyl; γ-abu: γ-amino butyric acid; EDANS: 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid)} was purchased from Bachem (Germany). Other reagents and solvents were purchased from various commercial sources as well. Absorbance measurements were obtained using a spectrofluorimeter PerkinElmer LS50B. Fluorescence intensities were measured using a BMG Fluostar microplate reader.

Wild-type and mutated proteases

The HIV-1 protease enzymes (WT and ANAM-11[21]) used in this study were expressed and purified as previously described.[14] They were produced in *E. coli* using the pET-9 expression vector and Rosetta(DE3)pLysS host bacterium cell strain. The protease domain of wild-type PR includes five protective mutations: Q7K, L33I, and L63I to minimize autoproteolysis, and C67A and C95A to prevent cysteine–thiol oxidation. ANAM-11 contains the mutations L10I, M36I, S37D, M46I, R57K, L63P, A71V, G73S, I84V, L90M, and I93L.[21]

Enzyme and inhibition assays

The proteolytic activities of wild-type and mutated proteases were determined fluorometrically using the fluorogenic substrate DABCYL-γ-abu-SQNYPIVQ-EDANS (λ_{ex} = 340 nm; λ_{em} = 490 nm) in 100 mM sodium acetate, 1 mM EDTA, and 1 M NaCl at pH 4.7 and 30 °C (final volume = 150 μL). The substrate and test compound were first dissolved in DMSO, with a final DMSO concentration of 3% (*v/v*). The mechanism of inhibition and corresponding kinetic constants K_{id} (dimerization inhibition) or K_{ic} (competitive inhibition) were determined using Zhang–Poorman kinetic analysis.[10] Kinetic experiments were carried out at constant substrate concentration (5.2 μM) with at least six enzyme concentrations (5.33–18.6 nM) and a range of inhibitor concentrations (1–28 μM). Experimental data were fitted according to reference 14. All experiments were performed, at minimum, in triplicate.

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