Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 17 (2009) 967–976
Bioorganic & Medicinal Chemistry

Sidechain-linked inhibitors of HIV-1 protease dimerization

Michael J. Bowman and Jean Chmielewski*

Department of Chemistry, 560 Oval Drive, Purdue University, West Lafayette, IN 47907, USA

Received 13 November 2007; revised 15 February 2008; accepted 16 February 2008
Available online 11 March 2008

Abstract—There is a great need for alternative modes of inhibition for the design of anti-HIV therapies, due to the increased resistance of HIV to currently approved drugs. A novel strategy for generating potent dimerization inhibitors of HIV-1 protease is described based on sidechain-linked interfacial peptides. In a number of cases the activity of these agents against HIV-1 protease was found to be among the most potent reported, with inhibitory constants in the low nM range.

1. Introduction

In its catalytically active form HIV-1 protease (HIV-1 PR) exists as a homodimer[1] with half of its active site and substrate binding site contributed from each monomer.[2,3] A number of agents that target the active site of HIV PR have been approved for the treatment of HIV infection. Although these inhibitors have been shown to be potent in vivo, the effect is often short-lived as strains containing mutations around the active site of HIV PR become more prevalent.[4] Homodimeric HIV-1 PR can serve as a template for developing methods to inhibit protein–protein interactions because it contains a relatively small, highly conserved region[5] that provides the bulk of the stabilization of HIV PR upon dimerization.[6] We have previously reported a minimal structure (**1**), consisting of crosslinked interfacial peptides, that is required for dimerization inhibition.[7] To date all efforts to link interfacial HIV-1 PR peptides and generate dimerization inhibitors of HIV-1 PR have employed the amino-terminus of the peptides as the site for crosslinking.[8] The prerequisite that the linking agent be fairly hydrophobic[8] has hindered the solubility of these agents and subsequent structural and cell-based investigations. In this current study we report a novel crosslinking strategy that allows for increased structural diversity and aqueous solubility of the designed agents while maintaining dimerization inhibition.

2. Results and discussion

2.1. Design of sidechain-linked agents

In our design we replaced the hydrophobic, amino-terminal crosslink of **1** with a more hydrophilic tether that links the sidechains of each interfacial peptide fragment. This approach serves to free up the amino-termini for diverse modification through acylation. To facilitate the sidechain crosslinking of the interfacial peptides, the threonine and asparagine residues of **1** were replaced with cysteine residues (Fig. 1). These two residues were chosen for modification because modeling had indicated that these residues are directed to the solvent exposed face of the interfacial β-sheet structure of HIV PR (Fig. 1). The crosslinking agent, 1,2-bis-(2-chloroethoxy)ethane was designed to span the 10 Å distance between the backbones of both peptide fragments.[9] This design strategy would allow the amino termini to be further functionalized with hydrophobic groups to provide potential interactions with the hydrophobic cleft between residues Leu97 and Phe99 of an HIV PR monomer (Fig. 1B).

2.2. Synthesis of sidechain-linked agents

The initial synthesis of the Northern and Southern peptides was initiated with Boc-cystine which was elongated with additional amino acids using HBTU to provide the disulfides of the desired peptide fragments. Reduction of the disulfides with DTT in chloroform provided the desired peptide components **2** and **4** (Scheme 1). The cysteine-containing dipeptide, Boc-Cys-Phe(O*t*-Bu) (**2**), was treated with bis-chloroethoxyethane and potassium carbonate in DMF at 60 °C to provide the mono-chlori-

Keywords: HIV protease; Inhibition; Protein–protein interaction.
* Corresponding author. Tel.: +1 765 494 0132; fax: +1 765 494 0239; e-mail: chml@purdue.edu

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.02.060

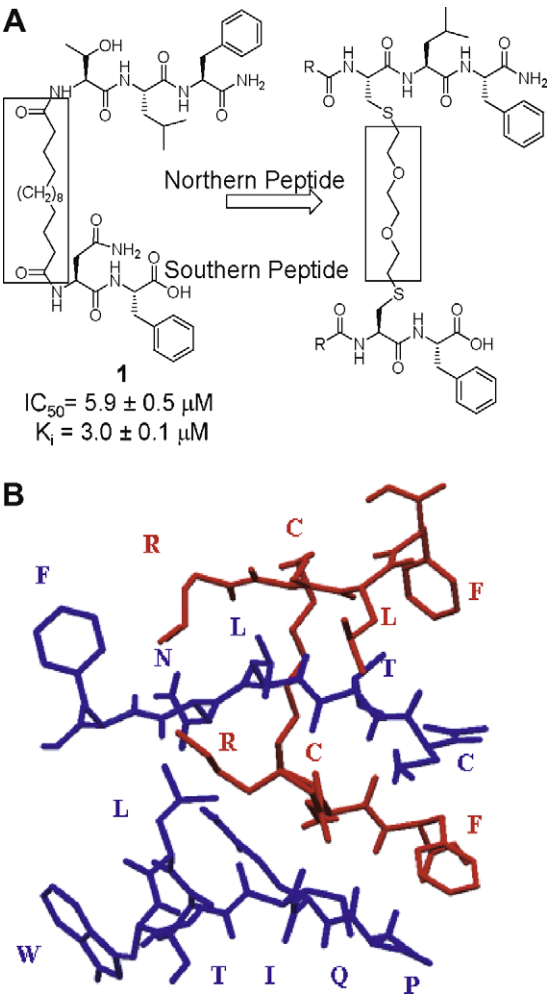


Figure 1. (A) Compound **1** and the proposed structure for sidechain crosslinked inhibitors. (B) Schematic representations of an inhibitor (red) binding to the dimerization interface of an HIV-1 PR monomer (blue) viewed from the hydrophobic interior of HIV-1 PR (R = *n*-hexyl).

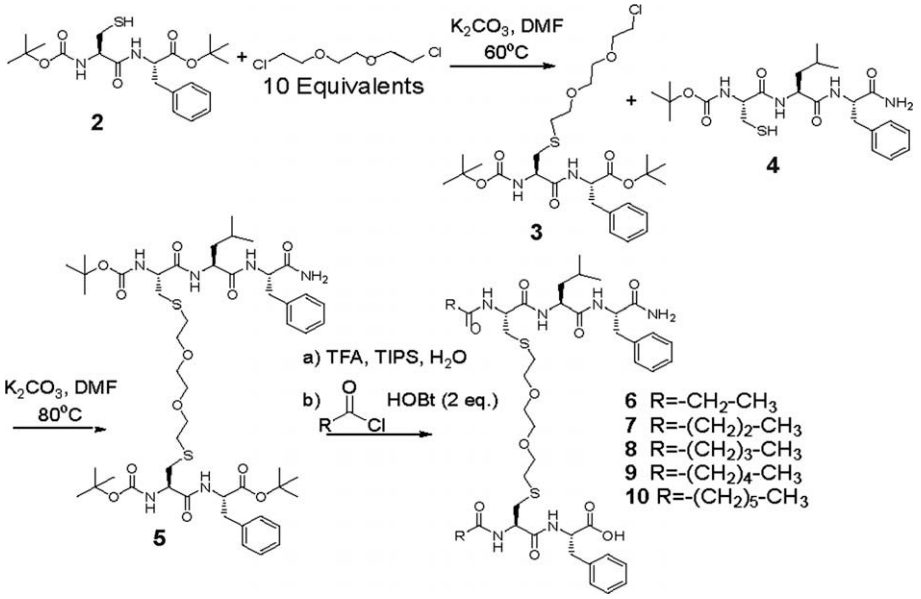
nated intermediate **3** (Scheme 1). A 10-fold excess of bis-chloroethoxyethane was used to prevent the undesired bis-substituted adduct.

Compound **3** was treated with the cysteine-containing tripeptide (**4**) and potassium carbonate in DMF at 80 °C to provide crosslinked and protected **5**. Compound **5** was treated with TFA containing TIPS (2.5%) and water (2.5%) to remove the *tert*-butyl protecting groups, followed by acylation of the free amino-termini with a series of activated carboxylic acids to provide the desired agents **6**–**10**.

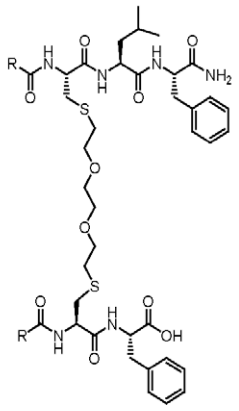
2.3. Evaluation of potency of initially designed agents

The extent of HIV-1 PR inhibition of **6**–**10** was evaluated using the fluorogenic substrate developed by Toth and Marshall.[10] We found that compounds with the alternative crosslinking strategy (**6**–**10**) demonstrated significant inhibition against HIV-1 PR when the appropriate acyl group was used. A trend of increasing potency with increasing acyl chain length was observed, with the most potent compound, **10**, containing heptanoyl groups at the amino termini (Fig. 2). Interestingly, compound **10** was found to be slightly more potent than the original inhibitor with an amino-terminal crosslink (**1**). These data demonstrate that moderately potent inhibitors of HIV PR can be obtained from a sidechain crosslinking strategy. The results are consistent with our binding model in which the alkyl chains, R, are more involved in interactions with the hydrophobic cleft existing between Leu97 and Phe99 of HIV-1 PR as their length is increased.

Further kinetic analyses were required in order to determine the mechanism of inhibition for compounds **6**–**10**. The assay method of Zhang and Poorman[11] was used to determine if these compounds inhibited the activity of HIV-1 PR by blocking dimerization. The parallel lines



Scheme 1. Synthesis of sidechain crosslinked compounds.



Compound	R	IC_{50} (μM)	K_i (μM)
6	CH_3-CH_2-	130 ± 15	(16.9 ± 2)
7	CH_3-$(CH_2)_2$-	87 ± 4.0	8.3 ± 0.4
8	CH_3-$(CH_2)_3$-	50 ± 4.0	6.8 ± 0.3
9	CH_3-$(CH_2)_4$-	14.9 ± 0.5	2.3 ± 0.1
10	CH_3-$(CH_2)_5$-	4.0 ± 0.4	1.30 ± 0.1

Figure 2. Results of the kinetics data for compounds **6**–**10**. Parenthetic value indicates a competitive mode K_i value.

obtained for **7**–**10**, in relation to uninhibited HIV-1 PR, demonstrate that these agents are dimerization inhibitors (Fig. 3A). Compounds **7**–**10** were found again to be progressively more potent with the subsequent addition of methylene units, with an overall increase in inhibition of 6-fold going from compound **7** to compound **10**. Inhibitor **10** was found to be approximately 2-fold more potent than compound **1**. The flexibility of the R groups within **10** may allow for improved binding interactions with the interfacial region of HIV-1 PR as compared to the more structurally constrained aliphatic tether of **1**. In addition, the solubility of **9** (90 μM) and **10** (80 μM) in water was found to be significantly greater than that observed with **1** (30 μM), presumably due to the shorter overall alkyl chains lengths and the inclusion of the ethylene glycol-based ether.

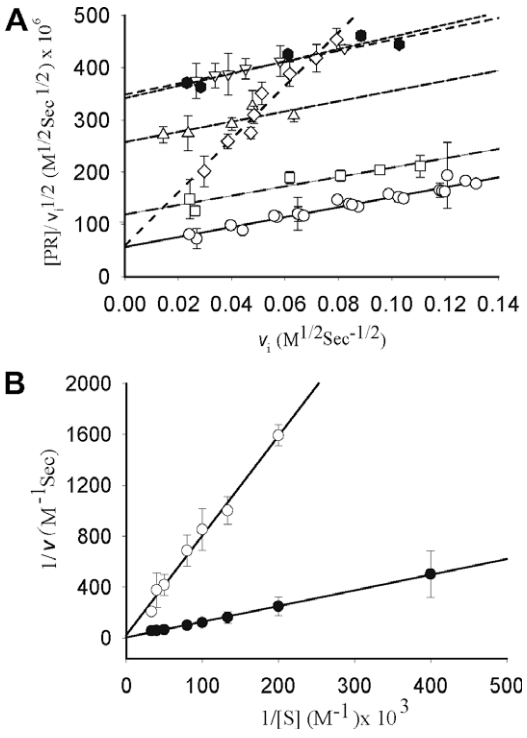


Figure 3. (A) Zhang–Poorman analysis of novel cross-linked compounds **6**–**10**. ○ Uninhibited HIV-1 PR; ◇ 65.3 μM **6**; ▽ 37.5 μM **7**; ●33.7 μM **8**; △ 11.5 μM **9**; □ 2 μM **10**. (B) Lineweaver–Burk analysis of compound **6**. ● Uninhibited HIV-1 PR; ○ 130 μM compound **6**.

The compound with the shortest acyl chain, **6**, however, was found to not display dimerization inhibition kinetics as shown by the non-parallel line obtained from the Zhang–Poorman analysis (Fig. 3A). Interestingly, compound **6** was shown by Lineweaver–Burk analysis to act by a competitive mechanism of inhibition (Fig. 3B). We believe that **6** does not bind to the active site of HIV-1 PR because it lacks the heptapeptide structure associated with recognition and active site binding[12] or the equivalent tetrapeptide mimic required for active site inhibition.[13] Alternatively **6** may target the dimerization interface, but may lack enough binding interactions to disrupt the dimer. This hypothesis is supported by a cross-competition plot using compound **6** and acetyl-pepstatin, a known active site inhibitor of HIV-1 PR.[14,15] If the two inhibitors are not able to bind simultaneously to HIV-1 PR, then this plot would provide parallel lines. Figure 4 demonstrates that the lines of the cross-competition plot for constant acetyl-pepstatin and variable **6** (Fig. 4A), as well constant **6** with variable acetyl-pepstatin (Fig. 4B), are not parallel. Therefore both inhibitors can occupy the enzyme at the same time, indicating that **6** is not binding to the active site of HIV-1 PR. The plots indicate that the effect of **6** binding to HIV-1 PR has a positive impact on the binding of acetyl-pepstatin, perhaps due to the change in the substrate recognition of HIV-1 PR. Interestingly, acetyl-pepstatin binding has the opposite effect on **6** binding possibly due to the 4.5 kcal/mol stabilization of the dimer caused by the binding of acetyl-pepstatin to the active site.[16] With this information in hand, we have evidence that **6** is not binding to the active site of the enzyme, but rather acts by binding to an allosteric site, perhaps at or near the dimerization interface.

2.4. A focused library for optimization

With the knowledge that the placement of the crosslink within HIV PR dimerization inhibitors can be modified while maintaining potency and a dimerization mechanism of inhibition, the synthesis of a small, focused library of compounds was undertaken to better understand the binding requirements for optimized potency. Design modifications attempted to more fully occupy the hydrophobic cleft between residues Leu97 and Phe99. Therefore, a series of compounds containing cyclic aliphatic functionality within the acyl moiety (**11**–**14**) was evaluated (Fig. 5). Compound **11**, modified with a cyclohexyl group, was found to be 5-fold less potent than n-hexyl-containing **10**, presumably due to the diminished occupation of the hydrophobic cleft within HIV-1 PR with the shorter cyclohexane ring. These data

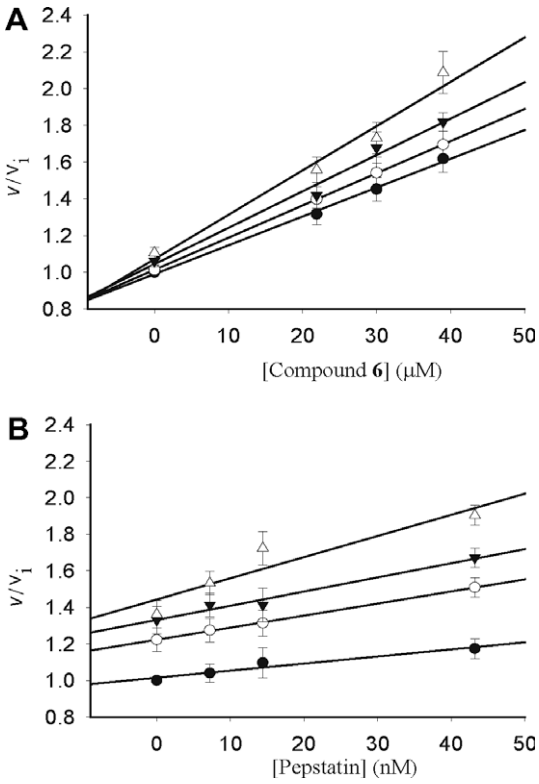
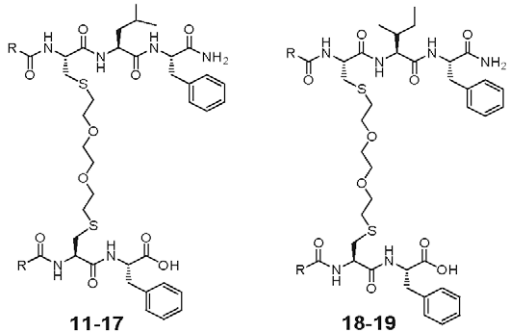


Figure 4. (A) Yonatani–Theorell plot of v/v_i versus acetyl-pepstatin concentration with constant concentrations of HIV-1 PR and compound **6**. ● 0 μM compound**6**; ○ 22 μM compound **6**; ▼ 30 μM compound **6**; △ 39 μM compound **6**. (B) Yonatani–Theorell plot of v/v_i versus concentration of compound **6** with constant concentrations of acetyl-pepstatin and HIV-1 PR. ●0 nM acetyl-pepstatin; ○ 7.2 nM acetyl-pepstatin; ▼ 14.4 nM acetyl-pepstatin; △ 43.2 nM acetyl-pepstatin.

indicate that the hydrophobicity of the amino-terminal functionality alone is not a major determinant for inhibition. With this in mind, the introduction of a methylene spacer before the cyclohexyl-moiety (compound **12**) led to a 2-fold increase in potency. Lengthening the spacer by additional methylene units (**13** and **14**) provided approximately a 4-fold increase in potency as compared to their shorter counterparts (**12** and **13**). Inhibitor **14** is 6-fold more potent than **10** with an IC_{50} value in the high nM range. Evaluation of the mechanism of inhibition of HIV-1 PR for **14** using the Zhang–Poorman analysis demonstrated the compound to be a potent dimerization inhibitor (K_i of 88 nM), nearly 15-fold more potent than the *n*-hexyl-containing **10** and 34-fold more potent than the previously reported compound **1** (see Fig. 6).[7]



Compound	R	IC_{50}	K_i
11	(C_6H_{11})-	19.5 ± 1.5 μM	
12	(C_6H_{11})-CH_2-	9.5 ± 0.8 μM	
13	(C_6H_{11})-$(CH_2)_2$-	2.5 ± 0.5 μM	
14	(C_6H_{11})-$(CH_2)_4$-	675 ± 30 nM	88 ± 9 nM
15	Ph-CH_2-	2.5 ± 0.1 μM	
16	Ph-$(CH_2)_2$-	6.5 ± 1.0 μM	
17	Ph-O-CH_2-	2.9 ± 0.2 μM	1.25 ± 0.2 μM
18	CH_3-$(CH_2)_5$-	1.2 ± 0.1 μM	510 ± 5 nM
19	(C_6H_{11})-$(CH_2)_4$-	64.0 ± 3 nM	40.1 ± 0.3 nM

Figure 5. Cycloalkyl and aromatic modifications at the R position, and single amino acid modifications.

The incorporation of a phenyl group within the acyl chain, with one or two methylene spacers, was also investigated for optimization of activity (Fig. 5). Compound **15**, containing phenyl acetyl groups, was nearly 4-fold more potent than the cyclohexyl-containing **12**, demonstrating that the cleft may better accommodate the aromatic ring. Up to this point, increasing the chain length had led to increased potency. However, an additional methylene unit within the acyl chain (**16**) caused a 2.5-fold decrease in potency as compared to compound **15**. This trend was reversed, however, with the incorporation of an ether linkage (**17**) in place of a methylene unit to provide an inhibitor that is nearly equipotent to **13**. Zhang–Poorman analysis also demonstrated that the dimerization inhibition mechanism was maintained with agent **17** (Fig. 5).

A number of point mutations within compound **1** have indicated that modifications within the sidechains can lead to greater potency, including Leu to Ile changes.[17] To evaluate whether this trend would hold with the alternately crosslinked agents, we mutated the Leu residue of compounds **10** and **14** to an Ile residue to provide compounds **18** and **19** (Fig. 5). Compounds **18** and **19** were found to be 2- to 2.5-fold more potent than their leucine-containing counterparts **10** and **14**, while maintaining the mechanism of dimerization inhibition. Compound **19** was found to be a highly potent analog with a K_i value of 40 nM. These results indicate that the sidechains of this crosslinked inhibitor series can be altered to provide further increases in potency.

3. Conclusions

To date all efforts to link interfacial HIV-1 PR peptides and generate dimerization inhibitors of HIV-1 PR have employed the amino-terminus of the peptides as the site for crosslinking. The prerequisite that the linking agent be fairly hydrophobic has hindered the solubility of these agents and subsequent structural and cell-based investigations. Herein we disclose a novel series of potent inhibitors of HIV-1 PR dimerization that contains a hydrophilic tether spanning the sidechains of interfacial peptides. Complete remodeling of the position of the crosslink also allowed for the diverse acylation of the amino-termini of the peptides. With this new strategy, inhibitory potencies against HIV-1 PR among the

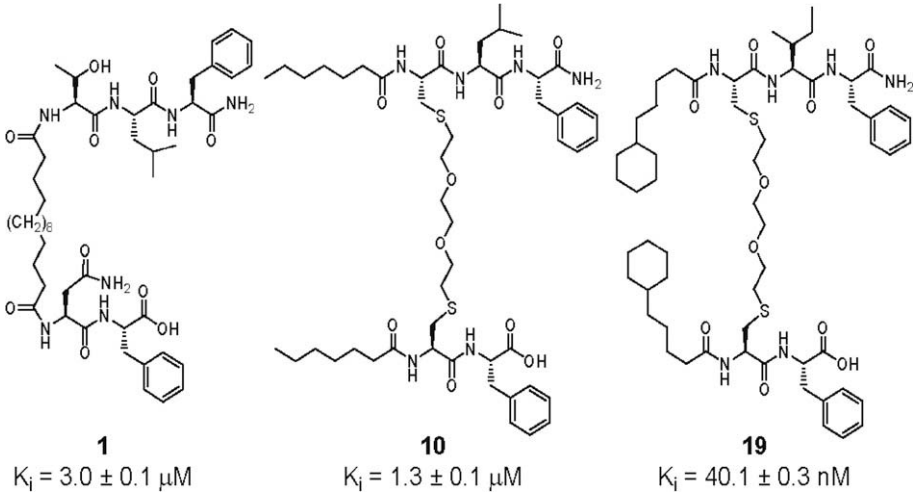


Figure 6. Progression of inhibitor design and observed increases in potency.

strongest reported were observed.[18–23] Differential modification of the acyl groups and sidechain functionalities provides a rich diversity of structures for further modification and optimization of dimerization inhibition of HIV-1 PR.

4. Experimental procedures

4.1. Materials

4.1.1. General. HIV-1 PR was purchased from Bachem Biosciences (affinity purified grade). Amino acids, coupling reagents and resins were purchased from either Advanced Chemtech or ChemImpex. All other reagents were purchased from Aldrich and used without purification. The inhibitors were purified to homogeneity by reverse phase HPLC under the following conditions—column: Vydac reverse phase C_8 column, flow rate: 8 ml/min using a linear gradient over 60 min. The eluant was monitored at 214 nm and 254 nm. Peptides and inhibitors were characterized by nuclear magnetic resonance (NMR) using an Inova 300 MHz spectrometer. Electrospray ionization mass spectrometry (ESI-MS) using a Waters LCQ and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) using a PerSeptive Voyager was performed to confirm identity. Fluorescence assays were performed on a Tecan SpectrafluorPlus 96-well plate reader. The amino acid content of inhibitors was analyzed by amino acid analysis (AAA) using the Waters AccQ-Tag method without modification, concentration was determined by comparison to internal standards of 2-aminoisobutyric acid, norleucine, and/or alanine.

4.2. Synthesis of (2)

The chloride salt of $^{+}NH_3$-Phe(O-*t*Bu) (540 mg, 2.1 mmol) was treated with Bis-Boc-Cystine (440.5 mg, 1.0 mmol), HOBt (321 mg, 2.1 mmol), HBTU (796 mg, 2.1 mmol), and DIEA (516 mg, 4.0 mmol) in CH_2Cl_2/THF (1:1) (40 ml) at room temperature for 2 h. After 2 h, the solvent was removed in vacuo and the resulting solid was redissolved in 30 ml ethyl acetate and washed with 10% citric acid (3×), 10% $NaHCO_3$ (3×), and brine (3×). Purification was performed by silica gel chromatography (3:7 EtOAc/Hexane $R_f = 0.4$) providing Bis-Boc-Cys-Phe-O-*t*Bu as a white solid (834 mg, 0.95 mmol, 95% yield). ^{1}H NMR, 200 MHz ($CDCl_3$) δ (ppm) = 1.38 (s, 18H), 1.46 (s, 18H), 2.86–3.22 (m, 8H), 4.69–4.80 (m, 8H), 5.48 (d, 2H, J = 4.6 Hz), 7.17–7.28 (m, 10H), 7.65 (d, 2H, J = 4.0 Hz).

Reduction of the disulfide of **2** (400 mg, 0.46 mmol) was performed in treatment with dithiothreitol (DTT, 284 mg, 1.84 mmol) and triethylamine (184 mg, 1.84 mmol) in degassed $CHCl_3$ (20 ml) under an argon atmosphere for 24 h at room temperature. After 24 h, the chloroform was removed in vacuo and the reaction mixture was redissolved in argon saturated ethyl acetate (20 ml) and washed with degassed 10% citric acid (3× 10 ml) and degassed ddH_2O (3× 10 ml). The ethyl acetate was removed in vacuo, providing Boc-Cys-Phe-O-*t*-But (**2**) as a white solid in quantitative yield without further purification (400 mg, 0.46 mmol). Boc-Cys-Phe-O-*t*-But was positive when reacted with Elman's reagent[24] (5,5′ dithio-bis-(2-nitrobenzoic acid)) indicating the presence of a free thiol. ^{1}H NMR, 200 MHz ($CDCl_3$) δ (ppm) = 1.38 (s, 9H), 1.46 (s, 9H), 2.86–3.22 (m, 4H), 4.69–4.80 (m, 4H), 5.48 (d, 1H, J = 4.6 Hz), 7.17–7.28 (m, 5H), 7.65 (d, 1H, J = 4.0 Hz). ESI-MS: calculated, 423.3; experimental 425.2 ($M+H^+$).

4.3. Synthesis of (3)

Boc-Cys-Phe-O-*t*-Bu (**2**) (173 mg, 0.40 mmol), 10 equivalents of 1,2-bis-(2-chloroethoxy)ethane (748 mg, 4.0 mmol), 4 equiv of K_2CO_3 (221 mg, 1.6 mmol) and triethylbenzylammonium chloride (TEBACl) (18.2 mg, 0.08 mmol) were added to a round bottom flask under argon. Degassed DMF (20 ml) under argon was added and the resulting solution was additionally degassed and replaced with argon (4 × 2 min). The reaction was heated to 60 °C. After 24 h the DMF was removed in vacuo and the reaction was dried overnight under high vacuum. The product was purified by silica gel chroma-

tography (3:7 ethyl acetate/hexane (R_f = 0.14)). The isolated product (**3**) (199 mg, 0.336 mmol, 84% yield) was a clear oil. ^{1}H NMR, 300 MHz ($CDCl_3$) δ (ppm) = 2.79 (d, 1 H, J = 0.9 Hz), 2.80 (d, 1H, J = 1.65 Hz), 2.82–3.03 (m, 2H), 3.12 (d, 2H, J = 1.1 Hz), 3.13 (d, 2H, J = 1.8 Hz), 3.63–3.82 (m, 10H), 4.32 (s, 1H), 4.74 (t, 1H, J = 4.5 Hz), 5.51 (d,1H, J = 3.2 Hz), 6.98 (s, 1H), 7.20–7.31 (m, 5H). ESI: Calculated 574.5; experimental: 575.0, 577 ($M+H^+$); 597, 599 ($M+Na^+$).

4.4. Synthesis of (4)

Bis-Boc-cystine-OH (330 mg, 0.75 mmol) was treated with Leu-Phe-NH_2 (620 mg, 1.87 mmol), HOBt (240 mg, 1.57 mmol), HBTU (595 mg, 1.57 mmol), and DIEA (387 mg, 3.0 mmol) in CH_2Cl_2/THF (1:1) (50 ml) at room temperature for 4 h. The CH_2Cl_2/THF was removed in vacuo and the solid was dissolved in 75 ml ethyl acetate and washed with 10% citric acid (3×), 10% $NaHCO_3$ (3×), and brine (3×). Purification by silica gel chromatography (95:5 CH_2Cl_2/MeOH R_f = 0.34) yielded Bis-Boc-Cys-Leu-Phe-NH_2 as a white solid (605 mg, 0.56 mmol, 74% yield). ^{1}H NMR, 300 MHz (DMSO) δ (ppm) = 0.83 (d/d, 12H J = 6.0 Hz, J = 6.6 Hz), 1.39 (s, 18H), 1.50–1.60 (m, 3H), 2.82–2.93 (m, 4H), 3.01–3.05 (m, 4H), 4.18–4.28 (m, 4H), 4.44 (s, 2H), 7.209–7.379 (m, 10H).

Reduction of the disulfide of **4** (200 mg, 0.193 mmol) was performed by dithiothreitol (DTT) (0.8 mmol, 125 mg) and triethylamine (0.8 mmol, 80 mg) in $CHCl_3$ (50 ml). After 24 h the chloroform was removed in vacuo, and the reaction mixture was dissolved in argon saturated ethyl acetate (50 ml) and washed with argon saturated 10% citric acid (3× 15 ml) and ddH_2O (3× 15 ml). The Boc-Cys-Leu-Phe-NH_2 (**4**) was isolated in quantitative yield (200 mg, 0.385 mmol) and was positive for when reacted with Elman's reagent (5,5′-dithio-bis-(2-nitrobenzoic acid)). ^{1}H NMR, 300 MHz (DMSO) δ (ppm) = 0.83 (d/d, 6H J = 6.0 Hz, J = 6.6 Hz), 1.39 (s, 9H), 1.50–1.60 (m, 3H), 2.82–2.93 (m, 2H), 3.01–3.05 (m, 2H), 4.18–4.28 (m, 2H), 4.44 (s, 1H), 7.209–7.379 (m, 5H). ESI-MS: calculated, 543.5; experimental 544.8 ($M+H^+$).

4.5. Synthesis of (5)

Compound **3** (147 mg, 0.256 mmol), compound **4** (200 mg, 0.385 mmol), 4 equiv of K_2CO_3 (142 mg, 1.03 mmol) and triethylbenzylammonium chloride (TEBACl) (11.8 mg, 0.07 mmol) were added to a round bottom flask under argon atmosphere. Degassed DMF (25 ml) under argon was added and the resulting solution was additionally degassed and placed under an argon atmosphere (4 × 2 min). The reaction was heated to 60 °C. After 24 h the DMF was removed in vacuo and the reaction was dried overnight under high vacuum. The product was purified by silica gel chromatography (95:5 CH_2Cl_2/MeOH (R_f = 0.21)), followed by RP-HPLC 50% CH_3CN/0.1% TFA/50% H_2O/0.1% TFA to 90% CH_3CN/0.1% TFA/10% H_2O/0.1% TFA using a linear gradient over 60 min. Retention time: 22.7 min. The isolated product **5** (23.6 mg, 0.023 mmol, 9.1% yield) was a yellow oil. ^{1}H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.79 (d/d, 6H J = 4.8 Hz, J = 7.8 Hz), 1.43 (s, 9H), 1.46 (s, 18H), 1.50–1.60 (m, 3H), 2.52–2.90 (m, 12H), 3.57–3.76 (m, 10H), 4.20 (m, 3H), 4.59 (m, 2H), 5.77–5.97 (m, 3H), 7.12 (d, 1H, J = 3.7 Hz), 7.25–7.40 (m, 10H), 7.97 (d, 1H, J = 3.7 Hz). ESI: Calculated 1018; experimental: 1019 ($M+H^+$); 1041 ($M+Na^+$); 1057 ($M+K^+$); Negative Ions: 1017 ($M-H^-$).

4.6. Synthesis of compounds (6–17)

Compound **5** (25 mg) was treated with 95% TFA (9 ml): 2.5% TIPS (500 μl): 2.5% H_2O (500 μl) for 2 h. The cleaving solvent was removed in vacuo, followed by precipitation with cold ether (3× 20 ml, −20 °C, 4 h). The resulting dried pellet was used without further purification.

Deprotected **5** (10 mg, 0.013 mmol) was dissolved in dry CH_3CN followed by treatment with the desired acid chloride (20 equiv, 0.26 mmol), HOBt (20 equiv, 0.26 mmol) and triethylamine (6.6 mg, 0.066 mmol). After 2 h the reaction was quenched with 75 equivalents ddH_2O and allowed to stir for an additional two hours. The solvent was removed in vacuo and the residue was dissolved in 500 μL methanol. The sample was purified to homogeneity using RP-HPLC to provide compounds **6**–**17**, depending on the acid chloride used. Structures were confirmed by NMR, amino acid analysis, and mass spectrometry. Concentrations were determined from amino acid analysis as compared to a norleucine and alanine internal standard.

4.6.1. Compound (6). RP-HPLC: 5% CH_3CN/95% H_2O/0.1% TFA to 75% CH_3CN/25% H_2O/0.1% TFA. Elution time: 43.2 min. MALDI-MS: calcd 874.4, found 897.8 ($M+Na^+$); 913.8 ($M+K^+$). AAA: Leu 1.0 (1); Phe 2.1 (2). ^{1}H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6H), 1.50 (bm, 2H), 1.56 (bm, 1H), 1.61(t (J = 7.2 Hz), 6H), 2.18 (t (J = 4.2 Hz), 4H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 10H).

4.6.2. Compound (7). RP-HPLC: 5% CH_3CN/95% H_2O/0.1% TFA to 75% CH_3CN/25% H_2O/0.1% TFA. Elution time: 46.5 min. MALDI-MS: calcd 902.4, found 925.9 ($M+Na^+$); 941.9 ($M+K^+$). AAA: Leu 1.0 (1); Phe 2.2 (2). ^{1}H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6H), 1.29 (t (J = 5.4 Hz), 6H), 1.50 (bm, 2H), 1.56 (bm, 1H), 1.61 (m, 4H), 2.18 (m, 4H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1 H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 10H).

4.6.3. Compound (8). RP-HPLC: 15% CH_3CN/85% H_2O/0.1% TFA to 85% CH_3CN/15% H_2O/0.1% TFA.

Elution time: 43.2 min. MALDI-MS: calcd 930.5, found 953.9 ($M+Na^+$); 969.9 ($M+K^+$). AAA: Leu 1.0 (1); Phe 2.2 (2). ^1H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6H), 0.90 (t (J = 6.6 Hz), 6H), 1.29 (m, 4H), 1.50 (bm, 2H), 1.56 (bm, 1H), 1.61 (m, 4H), 2.18 (m, 4H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8 H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 10H).

4.6.4. Compound (9). RP-HPLC: 20% CH_3CN/80% H_2O/0.1%T FA to 90% CH_3CN/10% H_2O/0.1% TFA. Elution time: 42.5 min. MALDI-MS: calcd 958.5, found 981.7 ($M+Na^+$); 997.8 ($M+K^+$). AAA: Leu 1.0 (1); Phe 2.2 (2). ^1H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6 H), 0.90 (t (J = 6.6 Hz), 6H), 1.29 (m, 8H), 1.50 (bm, 2H), 1.56 (bm, 1H), 1.61 (m, 4H), 2.18 (m, 4H), 2.74 (t (J = 6.6 Hz), 4 H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm,10H).

4.6.5. Compound (10). RP-HPLC: 25% CH_3CN/75% H_2O/0.1% TFA to 95% CH_3CN/5% H_2O/0.1% TFA. Elution time: 44.2 min. MALDI-MS: calcd 986.5, found 1010.3 ($M+Na^+$); 1026.4 ($M+K^+$). AAA: Leu 1.0 (1); Phe 2.0 (2). ^1H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6H), 0.90 (t (J = 6.6 Hz), 6H), 1.29 (m, 12H), 1.50 (bm, 2H), 1.56 (bm, 1H), 1.61 (m, 4H), 2.18 (m, 4H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8 H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 10H).

4.6.6. Compound (11). RP-HPLC: 30% CH_3CN/70% H_2O/0.1% TFA to 80% CH_3CN/20% H_2O/0.1% TFA. Elution time: 27.5 min. MALDI-MS: calcd 982.5, found 1005.4 ($M+Na^+$); 1022.2 ($M+K^+$). AAA: Leu 1.0 (1); Phe 1.9 (2). ^1H NMR, 300 MHz (CD_3CN) δ ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6H), 1.5–1.9 (m, 23H), 2.08 (m, 2H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8 H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 10H).

4.6.7. Compound (12). RP-HPLC: 30% CH_3CN/70% H_2O/0.1% TFA to 100% CH_3CN/0.1% TFA. Elution time: 29.4 min. MALDI-MS: calcd 1010.5, found 1060.0 ($M+K^+$). AAA: Leu 1.0 (1); Phe 1.6 (2). ^1H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6H), 1.5–1.9 (m, 27H), 2.08 (m, 2H), 2.18 (t (J = 4.2 Hz), 4H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 10H).

4.6.8. Compound (13). RP-HPLC: 30% CH_3CN/70% H_2O/0.1% TFA to 100% CH_3CN/0.1% TFA. Elution time: 34.0 min. MALDI-MS: calcd 1038.6, found 1059.8 ($M+Na^+$). AAA: Leu 1.0 (1); Phe 2.04 (2). ^1H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6H), 1.5–1.9(m, 31H), 2.08 (m, 2H), 2.18 (bm, 4H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4 H), 3.12 (m, 2H), 3.74 (m, 8H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 10H).

4.6.9. Compound (14). RP-HPLC: 30% CH_3CN/70% H_2O/0.1% TFA to 100% CH_3CN/0.1% TFA. Elution time: 49.1 min. MALDI-MS: calcd 1094.6, found 1118.0 ($M+Na^+$); 1133.9 ($M+K^+$). AAA: Leu 1.0 (1); Phe 1.8 (2). ^1H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6H), 1.5–1.9(m, 39H), 2.08 (m, 2H), 2.18 (bm, 4H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 10H).

4.6.10. Compound (15). RP-HPLC: 25% CH_3CN/75% H_2O/0.1% TFA to 95% CH_3CN/5% H_2O/0.1% TFA. Elution time: 34.2 min. MALDI-MS: calcd 998.4, found 1022.4 ($M+Na^+$); 1038.4 ($M+K^+$). AAA: Leu 1.0 (1); Phe 1.84 (2). ^1H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6H), 1.50 (bm, 2H), 1.56 (bm, 1H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 4.83 (m, 4 H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1 H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 20H)

4.6.11. Compound (16). RP-HPLC: 25% CH_3CN/75% H_2O/0.1% TFA to 95% CH_3CN/5% H_2O/0.1% TFA. Elution time: 37.0 min. MALDI-MS: calcd 1026.5, found 1050.4 ($M+Na^+$); 1066.7 ($M+K^+$). AAA: Leu 1.0 (1); Phe 1.7 (2). ^1H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6H), 1.50 (bm, 2H), 1.56 (bm, 1H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8H), 3.92 (m, 4H), 4.12 (m, 4H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1 H), 4.59 (m, 2H), 4.83 (m, 4H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 20H).

4.6.12. Compound (17). RP-HPLC: 60 min. Linear gradient from 25% CH_3CN/75% H_2O/0.1% TFA to 95% CH_3CN/5% H_2O/0.1% TFA. Elution time: 34.2 min. MALDI-MS: calcd 1030.4, found 1057.0 ($M+Na^+$). AAA: Leu 1.0 (1); Phe 1.6 (2). ^1H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (d/d (J = 4.8, J = 7.8 Hz), 6H), 1.50 (bm, 2H), 1.56 (bm, 1H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.41 (s, 4H), 4.59 (m, 2 H), 5.85 (d (J = 7.2 Hz), 2H), 5.93

(d (J = 6.8 Hz), 1 H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 20H)

4.7. Synthesis of (4 (Ile))

Synthesis of peptide Ile-Phe-NH_2 was performed in solution utilizing a Boc protection strategy. To a stirred solution of Boc-isoleucine (0.75 mmol, 180 mg) in CH_2Cl_2 (30 ml), was added HOBt (0.83 mmol, 112 mg), and HBTU (0.83 mmol, 315 mg) at room temperature. After 10 min the hydrochloride salt of phenylalanine amide (0.83 mmol, 163 mg) was added with diisopropylethylamine (DIEA) (1.1 mmol, 192 mg) in 5 ml DMF. The reaction was stirred for 3 h at room temperature. The CH_2Cl_2 was removed in vacuo, the remaining DMF solution was diluted in 20 ml ethyl acetate and washed with LiCl (sat) (2×), 10% citric acid (3×), 10% $NaHCO_3$ (3×), and brine (3×). The solvent was removed in vacuo and the resulting solid was further purified by silica gel chromatography (1:19 MeOH/CH_2Cl_2 R_f = 0.35), to yield Boc-Ile-Phe-NH_2 as a white crystalline solid (232 mg, 80% yield). ^{1}H NMR, 300 MHz ($CDCl_3$) δ(ppm) 0.89 (m, 6H), 1.38 (s, 9H), 1.50.–1.60 (m, 3H), 3.12 (d, 2H, J = 6.6 Hz), 4.04 (t, 1H, J = 5.7 Hz), 4.74 (m, 2H), 5.33 (bs, 1H), 6.34 (bs, 1H), 6.42 (bs, 1H), 7.20–7.34 (m, 5H).

The Boc-Ile-Phe-NH_2 (232 mg, 0.6 mmol) was treated with 3 ml trifluoroacetic acid (TFA)/CH_2Cl_2 (1:1) for 30 min at room temperature. The cleaving solvent was removed in vacuo, followed by precipitation with cold ether (3× 20 ml, −20 °C, 4 h). NH_2-Ile-Phe-NH_2 (170 mg, 0.6 mmol) was collected by centrifugation and used without further purification.

Bis-Boc-cystine-OH (199 mg, 0.45 mmol) was treated with NH_2-Ile-Phe-NH_2 (315 mg, 0.95 mmol), HOBt (128 mg, 0.95 mmol), HBTU (360 mg, 0.95 mmol), and DIEA (239 mg, 1.85 mmol) in DMF (7 ml) at room temperature for 4 h. The DMF was removed in vacuo and the solid was dissolved in 25 ml ethyl acetate and washed with 10% citric acid (3×), 10% $NaHCO_3$ (3×), and brine (3×). Purification by silica gel chromatography (95:5 CH_2Cl_2/MeOH R_f = 0.32) yielded Bis-Boc-Cys-Ile-Phe-NH_2 as a white solid (400 mg, 0.37 mmol, 82% yield). ^{1}H NMR, 300MHz (DMSO) δ (ppm) = 0.83 (m, 12H), 1.39 (s, 18H), 1.50–1.60 (m, 3H), 2.82–2.93 (m, 4H), 3.01–3.05 (m, 4H), 4.18–4.28 (m, 4H), 4.44 (s, 2H), 7.21–7.38 (m, 10H), 7.64 (d, 1H, J = 4.1 Hz), 8.08 (d, 1H, J = 3.8 Hz).

Reduction of the disulfide of the desired tripeptide (250 mg, 0.23 mmol) was performed by treatment with dithiothreitol (DTT) (0.92 mmol, 144 mg) and triethylamine (0.92 mmol, 92 mg) in $CHCl_3$. After 24 h the chloroform was removed in vacuo and the reaction mixture was dissolved in argon saturated ethyl acetate (30 ml) and washed with argon saturated 10% citric acid (3× 15 ml) and ddH_2O (3× 15 ml). The **4** (Ile) was isolated in quantitative yield (250 mg, 0.23 mmol) without further purification. **4** (Ile) demonstrated a positive result when reacted with Elman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid)) indicating the presence of a free thiol. ^{1}H NMR, 300MHz (DMSO) δ (ppm) = 0.83 (m, 6H), 1.39 (s, 9H), 1.50–1.60 (m, 3H), 2.82–2.93 (m, 2H), 3.01–3.05 (m, 2H), 4.18–4.28 (m, 2H), 4.44 (s, 1H), 7.21–7.38 (m, 5H), 7.64 (d, 1H, J = 4.1 Hz), 8.08 (d, 1H, J = 3.8 Hz). ESI-MS: calculated, 543.5; experimental 544.4 ($M+H^+$).

4.8. Synthesis of (5 (Ile))

Compound **3** (81 mg, 0.150 mmol), Boc-Cys-Ile-Phe-NH_2 (285 mg, 0.60 mmol), 4 equiv of K_2CO_3 (82 mg, 0.57 mmol), 1 equiv of NaBr (15 mg, 0.15 mmol) and triethylbenzylammonium chloride (TEBACl) (13.5 mg, 0.06 mmol) were added to a round bottom flask under an argon atmosphere. Degassed DMF (15 ml) under argon was added and the resulting solution was additionally degassed and placed under an argon atmosphere (4 × 2 min). The reaction was heated to 60 °C. After 24 h the DMF was removed in vacuo and the reaction was dried overnight under high vacuum. The desired product was purified by silica gel chromatography (95:5 CH_2Cl_2/MeOH (R_f = 0.21)). Followed by RP-HPLC 50% CH_3CN/0.1% TFA/50% H_2O/0.1% TFA to 90% CH_3CN/0.1% TFA/10% H_2O/0.1% TFA using a linear gradient over 60 min. Retention time: 26.7 min. The isolated product, (80 mg, 0.079 mmol, 52% yield) was white solid. ^{1}H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.82 (m, 6H), 1.43 (s, 9H), 1.46 (s, 18H), 1.50–1.60 (m, 3H), 2.52–2.90 (m, 12H), 3.57–3.76 (m, 10H), 4.20 (m, 3H), 4.59 (m, 2H), 5.77–5.97 (m, 3H), 7.12 (d, 1H, J = 3.7 Hz), 7.25–7.40 (m, 10H), 7.97 (d, 1H, J = 3.7 Hz). ESI-MS: Calculated 1018; experimental: 1019 ($M+H^+$); 1041 ($M+Na^+$); 1057 ($M+K^+$); Negative Ions: 1017 ($M-H^-$).

4.9. Synthesis of compounds (18–19)

5 (Ile) (80 mg) was treated with 95% TFA (9 ml): 2.5% TIPS (500 μl): 2.5% H_2O (500 μl) for 2 h. The cleaving solvent was removed in vacuo, followed by precipitation with cold ether (3× 20 ml, −20 °C, 4 h). Residual ether was removed in vacuo and the resulting solid was used without further purification.

The deprotected compound (10 mg, 0.013 mmol) was dissolved in dry CH_3CN followed by treatment with the desired acid chloride (20 equiv, 0.26 mmol), HOBt (20 equiv, 0.26 equiv) and triethylamine (6.62 mg, 0.0656 mmol). After 2 h the reaction was quenched with 75 equivalents ddH_2O and allowed to stir for an additional two hours. The solvent was removed in vacuo and the residues were dissolved in 500 μL DMSO. The samples were then purified to homogeneity using RP-HPLC to provide compounds **18**–**19**, depending on the acid chloride used. The structures were confirmed by NMR, amino acid analysis, and mass spectrometry. Concentrations were determined from amino acid analysis as compared to a norleucine and alanine internal standard.

4.9.1. Compound (18). RP-HPLC: 25% CH_3CN/75% H_2O/0.1% TFA to 95% CH_3CN/5% H_2O/0.1% TFA. Elution time: 34.5 min. MALDI-MS: calcd 986.5, found

1010.3 (M+Na^+); AAA: Ile 1.0 (1); Phe 2.0 (2). ^{1}H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (m, 6H), 0.90 (t (J = 6.6 Hz), 6H), 1.29 (m, 12 H), 1.50 (bm, 2H), 1.56 (bm, 1H), 1.61 (m, 4H), 2.18 (m, 4H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8 H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 10H).

4.9.2. Compound (19). RP-HPLC: 30% CH_3CN/70% H_2O/0.1% TFA to 100% CH_3CN/0% H_2O/0.1% TFA. Elution time: 42.0 min. ESI-MS: calcd 1094.6, found 1118.3 (M+Na^+); 1094.4 (M−H^-) Neg Ions. AAA: Ile 1.0 (1); Phe 2.0 (2). ^{1}H NMR, 300 MHz (CD_3CN) δ (ppm) = 0.84 (m, 6H), 1.50–1.9 (m, 36H), 2.18 (m, 4H), 2.74 (t (J = 6.6 Hz), 4H), 2.94 (bm, 4H), 3.12 (m, 2H), 3.74 (m, 8H), 4.24 (d (J = 6.6 Hz), 2H), 4.29 (m, 1H), 4.59 (m, 2H), 5.85 (d (J = 7.2 Hz), 2H), 5.93 (d (J = 6.8 Hz), 1H), 6.55 (d (J = 7.3 Hz), 1H), 7.2 (d (J = 8.1 Hz), 2H), 7.33 (bm, 10H).

4.10. Zhang–Poorman kinetic assay

To perform this assay, varying concentrations of HIV-1 PR were treated with constant concentrations of inhibitor and substrate while monitoring the initial velocity (V) of the proteolytic cleavage of the fluorogenic substrate. For Zhang–Poorman kinetic assay, 180 μl of varying concentrations of HIV-1 protease solution (5–45 nM) in assay buffer (20 mM phosphate, 1 mM DTT, 10% glycerol, and 0.1% CHAPS at pH 5.5) was incubated with 36 μl of inhibitor solution at a concentration equal to the IC_{50} and $0.75 \times IC_{50}$ in DMSO for one hour at room temperature in 1.2 ml polypropylene tubes (Costar). This solution (3 × 60 μl) was added to three different 40 μl aliquots of a 62.5 μM substrate solution (10% DMSO and 90% assay buffer) in a polypropylene 96-well plate (Griegner). The final concentration of DMSO was maintained at 14%. The change in fluorescence intensity at 465 nm (ex: 360 nm) was immediately measured upon the addition of the protease to the substrate solution over a period of 14 min. Initial velocity (V) was calculated from Δ [hydrolyzed substrate]/Δ time in seconds determined when <5% of substrate was consumed.

4.11. Cross-competition kinetic assay

For cross-competition assay, solutions of 180 μL of HIV-1 protease in buffer (20 mM phosphate, 1 mM DTT, 1 mM EDTA, 20% glycerol, and 0.1% CHAPS at pH 5.52; final concentration 10 nM protease) were incubated with 36 μl of each inhibitor solutions varying in the concentration of a known active site competitive inhibitor acetyl-pepstatin (0–43.2 nM) with constant concentrations of compound **7** (0, 21.9, 29.9, 39 μM) in DMSO for one hour at room temperature in 1.2 ml polypropylene tubes (Costar). Alternatively, solutions of 180 μL of HIV-1 protease in buffer (20 mM phosphate, 1 mM DTT, 1 mM EDTA, 20% glycerol, and 0.1% CHAPS at pH 5.52; final concentration 10 nM protease) were incubated with 36 μl of each inhibitor solutions a constant concentration of a known active site competitive inhibitor (acetyl-pepstatin, 7.2, 14.4, 43.2 nM) with varying concentrations of compound **6** (0–10 μM) in DMSO for one hour at room temperature in 1.2 ml polypropylene tubes (Costar). These solutions (3× 60 μl) were added to three different 40 μl aliquots of a 30 μM substrate solution[10](10% DMSO and 90% assay buffer) in a polypropylene 96 well plate (Griegner). The final concentration of DMSO was maintained at 14%. The change in fluorescence intensity at 465 nm (ex: 360 nm) was immediately measured upon the addition of the protease to the substrate solution over a period of 14 min.

Acknowledgment

This research was supported by the National Institutes of Health (GM52379).

References and notes

1. Miller, M.; Schneider, J.; Sathyanarayana, B. K.; Toth, M. V.; Marshall, G. R.; Clawson, L.; Selk, L.; Kent, S. B.; Wlodawer, A. *Science* **1989**, *246*, 1149.
2. Davies, D. R. *Annu. Rev. Biophys. Biophys. Chem.* **1990**, *19*, 189.
3. Pearl, L. H.; Taylor, W. R. *Nature* **1987**, *328*, 482.
4. Deeks, S. G.; Smith, M.; Holodniy, M.; Kahn, J. O. *JAMA* **1997**, *277*, 145.
5. Gustchina, A.; Weber, I. T. *FEBS Lett.* **1990**, *269*, 269.
6. Todd, M. J.; Semo, N.; Freire, E. *J. Mol. Biol.* **1998**, *283*, 475.
7. Shultz, M. D.; Bowman, M. J.; Ham, Y. W.; Zhao, X.; Tora, G.; Chmielewski, J. *Angew. Chem. Int. Ed. Engl.* **2000**, *39*, 2710.
8. Bowman, M. J.; Chmielewski, J. *Biopolymers* **2002**, *66*, 126.
9. Wlodawer, A.; Miller, M.; Jaskolski, M.; Sathyanarayana, B. K.; Baldwin, E.; Weber, I. T.; Selk, L. M.; Clawson, L.; Schneider, J.; Kent, S. B. *Science* **1989**, *245*, 616.
10. Toth, M. V.; Marshall, G. R. *Int. J. Pept. Protein Res.* **1990**, *36*, 544.
11. Zhang, Z. Y.; Poorman, R. A.; Maggiora, L. L.; Heinrikson, R. L.; Kezdy, F. J. *J. Biol. Chem.* **1991**, *266*, 15591.
12. Loeb, D. D.; Hutchison, C. A., 3rd; Edgell, M. H.; Farmerie, W. G.; Swanstrom, R. *J. Virol.* **1989**, *63*, 111.
13. Wlodawer, A.; Erickson, J. W. *Annu. Rev. Biochem.* **1993**, *62*, 543.
14. Richards, A. D.; Roberts, R.; Dunn, B. M.; Graves, M. C.; Kay, J. *FEBS Lett.* **1989**, *247*, 113.
15. Fitzgerald, P. M.; McKeever, B. M.; VanMiddlesworth, J. F.; Springer, J. P.; Heimbach, J. C.; Leu, C. T.; Herber, W. K.; Dixon, R. A.; Darke, P. L. *J. Biol. Chem.* **1990**, *265*, 14209.
16. Todd, M. J.; Freire, E. *Proteins* **1999**, *36*, 147.
17. Shultz, M. D.; Ham, Y. W.; Lee, S. G.; Davis, D. A.; Brown, C.; Chmielewski, J. *J. Am. Chem. Soc.* **2004**, *126*, 9886.
18. Bowman, M. J.; Byrne, S.; Chmielewski, J. *Chem. Biol.* **2005**, *12*, 439.
19. Hwang, Y. S.; Chmielewski, J. *J. Med. Chem.* **2005**, *48*, 2239.
20. Lee, S. G.; Chmielewski, J. *Chem. Biol.* **2006**, *13*, 421.

21. Merabet, N.; Dumond, J.; Collinet, B.; Van Baelinghem, L.; Boggetto, N.; Ongeri, S.; Ressad, F.; Reboud-Ravaux, M.; Sicsic, S. *J. Med. Chem.* **2004**, *47*, 6392.
22. Breccia, P.; Boggetto, N.; Perez-Fernandez, R.; Van Gool, M.; Takahashi, M.; Rene, L.; Prados, P.; Badet, B.; Reboud-Ravaux, M.; de Mendoza, J. *J. Med. Chem.* **2003**, *46*, 5196.
23. Dumond, J.; Boggetto, N.; Schramm, H. J.; Schramm, W.; Takahashi, M.; Reboud-Ravaux, M. *Biochem. Pharmacol.* **2003**, *65*, 1097.
24. Ellman, G. L. *Arch. Biochem. Biophy.* **1959**, *82*, 70.