Design, Synthesis, Molecular Modeling Studies, and Calpain Inhibitory Activity of Novel α-Ketoamides Incorporating Polar Residues at the P_1'-Position

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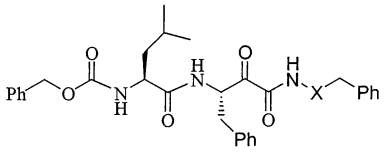
A series of novel α-ketoamides incorporating stereoisomeric residues with different electronic properties at the P_1'-position were synthesized to study the electronic requirements for inhibitor binding to the S_1'-subsite of calpain I. The results of the study suggested the presence of an acidic amino acid residue at the S_1'-subsite of calpain I. For example, ester **1a** (Cbz-L-Leu-L-Phe-CO-D-Phe-OMe) was over 450-fold more potent than its carboxylic acid derivative **2a** (Cbz-L-Leu-L-Phe-CO-D-Phe-OH). Additionally, amidino derivative **3a** (Cbz-L-Leu-L-Phe-CONH-D-CH[C(NH)NH_2]Bn) was about 6000-fold more potent than **2a**. Furthermore, **4a** (Cbz-L-Leu-L-Phe-CONHCH_2Bn) was 12-fold less potent than its aza analogue **4b** (Cbz-L-Leu-L-Phe-CONHNHBn). The results are consistent with the presence of an acidic amino acid residue at the S_1'-subsite of calpain I. The acidic amino acid residue was found to be Glu261 via molecular modeling studies.

Introduction

Calpain is an intracellular cytoplasmic nonlysosomal cysteine endopeptidase that is activated by calcium ions. Several calpain isoenzymes have been discovered[1−12] of which calpain I and calpain II (also known as μ-calpain and m-calpain, respectively) are the most ubiquitous in mammalian cells. The structure, mechanism of activation, and pharmacological actions of calpain have been reviewed.[12−20] The enzyme has been implication in several pathological conditions including stroke, cerebral ischemia, and cataract.[18,19,21] This has led to the search for calpain inhibitors as potential therapeutic agents.[18−21]

Structural information regarding the active site pocket of calcium-activated calpain is pivotal to the discovery of potent and specific inhibitors of the enzyme. Unfortunately, such information was not available until the recent disclosure of the X-ray crystal structure of the calcium-bound protease core of calpain I.[22] Our laboratory[23−25] and that of others[21,26−29] have employed structure−activity relationship (SAR) studies to probe the S- and S′-subsites of calpain in attempts to understand the structural requirements for inhibitor binding to the active site of the enzyme. As a part of this effort, we report in this paper the synthesis, molecular modeling studies, and calpain inhibitory activity of a novel series of α-ketoamides incorporating polar residues at the P_1'-position of the inhibitors (Table 1). The compounds were developed as molecular probes to study the significance of electrostatic interaction between an inhibitor and the S_1'-subsite of calpain I.

Table 1. Structure, Diastereomeric Purity, and Calpain I Inhibitory Activity of Novel α-Ketoamides 1−4



Compd. #	X	K_i (nM)[a]	DP (%)[b]
1a	H(R)C(COOMe)	940 ± 10	98[c]
1b	H(S)C(COOMe)	680 ± 15	97[c]
2a	H(R)C(COOH)	> 430000	96[c]
2b	H(S)C(COOH)	830 ± 20	97[c]
3a	H(R)C(C(NH)NH_2)	74 ± 3	100[d]
3b	H(S)C(C(NH)NH_2)	4190 ± 190	100[d]
4a	CH_2	54 ± 3	100[d]
4b	NH	4.7 ± 0.2	100[e]

[a] K_i values are the averages of triplicate determinations obtained by Dixon plots where 1/*v* were plotted against inhibitor concentration at two different substrate concentrations (0.2 mM and 1.0 mM) to give intersecting lines with correlation coefficient ≥0.95. [b] DP = diastereomeric purity. The diastereomeric purity of the compounds was determined using a Shimadzu HPLC system and a Macrosphere RP 300 C18 column. [c] The mobile phase was acetonitrile (55%)/TFA (0.15%)/water. [d] The mobile phase was acetonitrile (30%)/diethylamine (0.15%)/water. [e] The mobile phase was acetonitrile (25%)/TFA (0.15%)/water.

Chemistry

Compound **5** was synthesized as described previously[28] and coupled with D-phenylalanine methylester using EDC and HOBT as the coupling agent to give α-hydroxylamide dipeptide **6a** (Scheme 1). Cleavage of the Boc protecting group of **6a** followed by coupling with Cbz-L-leucine and oxidation of the hydroxyl group with Dess−Martin reagent gave **1a**. Basic hydrolysis of the ester group of **1a** afforded carboxylic acid derivative **2a**. Compound **2b** was synthesized in a similar manner

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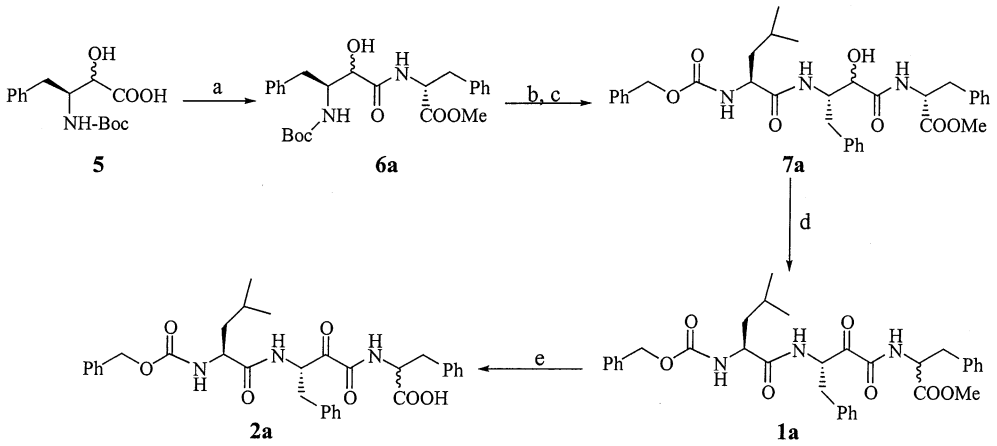
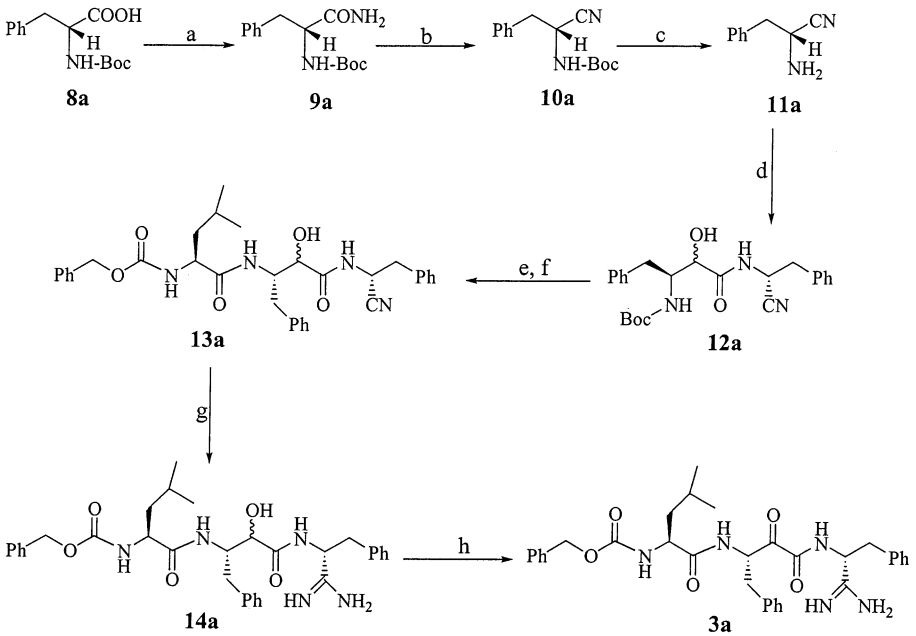
Scheme 1[a]



[a] Reagents: (a) D-Phenylalanine methyl ester, EDC, HOBT; (b) HCl/dioxane; (c) Cbz-L-leucine, EDC, HOBT; (d) Dess−Martin reagent; (e) 1 N HaOH.

Scheme 2[a]



[a] Reagents: (a) TEA, $ClCO_2Et$, NH_3(g); (b) TEA, $(CF_3CO)_2O$; (c) 50% TFA; (d) EDC, HOBt, NMM, **5**; (e) 50% TFA; (f) Cbz-L-leucine, EDC, HOBT, NMM; (g) *N*-acetylcysteine, NH_4OAc; (h) DMSO, pyridiniium trifluoroacetate, EDC.

using L-phenylalanine methyl ester as the starting material.

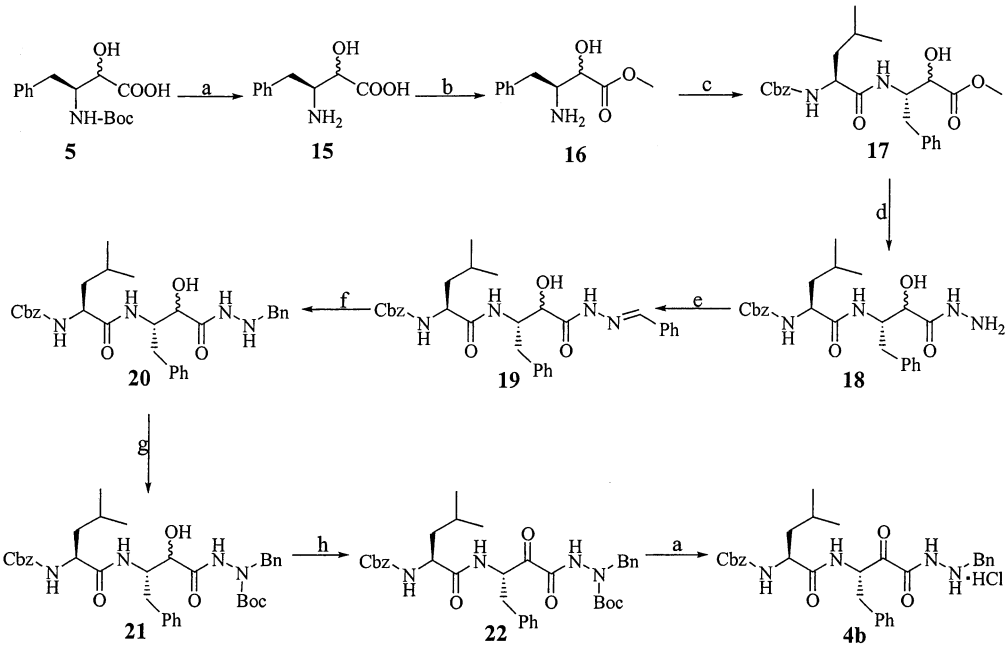
The synthesis of amidine **3a** commenced with the transformation of Boc-protected D-phenylalanine to carboxamide **9a** using $ClCO_2Et$ in the presence of TEA followed by treatment of the resulting acid chloride with NH_3 gas (Scheme 2). Dehydration of carboxamide **9a** with $(CF_3CO)_2O$ in the presence of TEA afforded nitrile **10a**, which was deprotected with 50% TFA to give 2(*R*)-(+)-amino-3-phenylpropionitrile (**11a**). Coupling of **11a** with **5** in the presence of EDC, HOBT, and NMM afforded pseudodipeptide **12a**. Deprotection of **12a** with 50% TFA followed by coupling with Cbz-L-leucine gave pseudotripeptide **13a**. Compound **13a** was treated with *N*-acetylcysteine and NH_4OAc to give amidine **14a**, the hydroxyl group of which was oxidized with pyridinium trifluoroacetate and EDC in DMSO to give α-ketoamide **3a**. Compound **3b** was synthesized in a similar manner starting with Boc-L-phenylalanine.

Compound **4a** was synthesized as described previously.[26] The synthesis of **4b** is shown in Scheme 3. Compound **5** was Boc-deprotected using 2 N HCl to give α-hydroxy-β-amino acid **15**, which was esterified by treatment with $SOCl_2$/MeOH to give **16**. Compound **16** was coupled with Cbz-L-leucine to afford pseudodipeptide methyl ester **17**. Treatment of **17** with hydrazine gave hydrazide **18**, which was reacted with benzaldehyde to obtain hydrazone **19**. Reduction of **19** with $NaBH_3CN$ gave *N*′-benzyl hydrazide **20**. Boc-protection of **20** afforded **21**, which was transformed to **22** by treatment with Dess−Martin reagent. Removal of the Boc protecting group of **22** gave hydrazide **4b**.

Results and Discussion

SAR Studies. SAR studies have suggested that the S′-subsites of calpain I can accommodate polar functional groups.[24,26−29] We therefore synthesized α-ketoamides **1**-**4** (Table 1) as molecular probes to study the

Scheme 3[a]



[a] Reagents: (a) 2 N HCl; (b) $SOCl_2$, MeOH; (c) Cbz-L-leucine, EDC, HOBT, NMM; (d) NH_2NH_2 (e) BnCHO; (f) $NaBH_3CN$, HCl; (g) $(t\text{-BuOCO})_2O$, NaOH; (h) Dess−Martin reagent.

significance of electrostatic interaction at the S_1'-subsite of calpain I. The compounds were evaluated as inhibitors of porcine erythrocyte calpain I at 25 °C using two different concentrations (0.2 and 1.0 mM) of Suc-Leu-Tyr-AMC as the substrate. The K_i values of the inhibitors were determined using Dixion plots.[30] Table 1 displays the inhibitory data for the compounds.

Compound **2b** with (*S*)-phenylalanine at the P_1'-position was over 500-fold more potent than **2a** with (*R*)-phenylalanine at the P_1'-position. The greater calpain I inhibitory potency of **2b** compared to its stereoisomer **2a** led us to propose the presence of an acidic amino acid residue (aspartic acid or glutamic acid) at the S_1'-subsite of calpain I. We attributed the poor calpain I inhibitory potency of **2a** to electrostatic repulsion between the carboxylate group of its P_1'-substituent and the carboxylate group of the proposed acidic amino acid residue at the S_1'-subsite of the enzyme (Figure 1). Compound **1a**, which is the methyl ester derivative of **2a**, was also over 450-fold more potent than **2a**, in support of the presence of an acidic amino acid residue at the S_1'-subsite of calpain I. Esterification of the carboxylate group (as in **1a**) relieved the unfavorable electrostatic repulsion between the carboxylate of the inhibitor and that of the enzyme; hence, the greater potency of **1a** compared to **2a**.

We also synthesized stereoisomeric compounds **3a** and **3b** to further study the S_1'-subsite of calpain I. Compound **3a** with a basic (*R*)-1-amidino-2-phenylethylamine substituent at the P_1'-position was about 6000-fold more potent than **2a** with an acidic (*R*)-phenylalanine residue at the P_1'-position. This is consistent with an acidic amino acid residue at the S_1'-subsite of caplain I, because **3a** would undergo electrostatic attraction and/or hydrogen-bonding interaction with the carboxylate group at the S_1'-subsite of the enzyme, while **2a** would experience electrostatic repulsion at this site (Figure 1). Additionally, **3a** was about 60-fold more potent than **3b** due to the difference in the stereochemistry of their P_1'-substituents. The results are consistent with previous reports that the S′-subsites of calpain tolerate basic functional groups.[27−29]

On the basis of our hypothesis that the S_1'-subsite of calpain I contains an acidic amino acid residue, **3a** should be more potent than **4a**. However, **3a** and **4a** were almost equipotent. We reasoned that steric interaction between the amidine group of **3a** and the carboxylate group of the S_1'-acidic amino acid residue negatively affected the calpain I inhibitory potency of **3a**. To investigate this, we synthesized **4b** (which is the aza derivative of **4a**) and found it to be 12-fold more potent than **4a**, in support of the presence

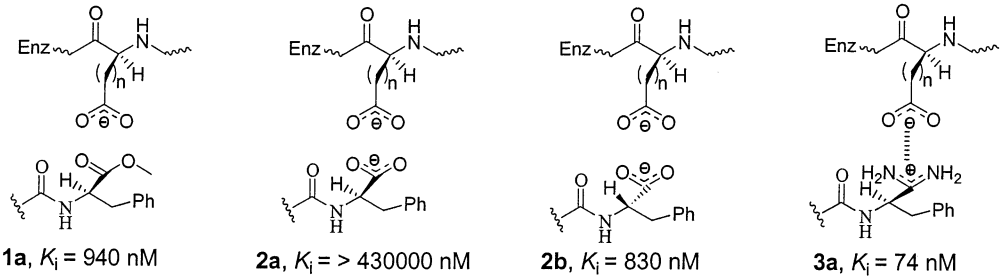
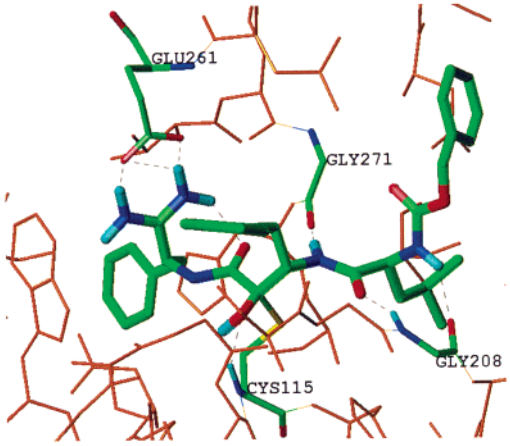
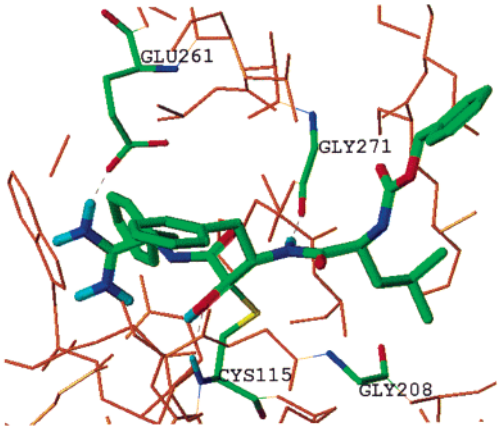


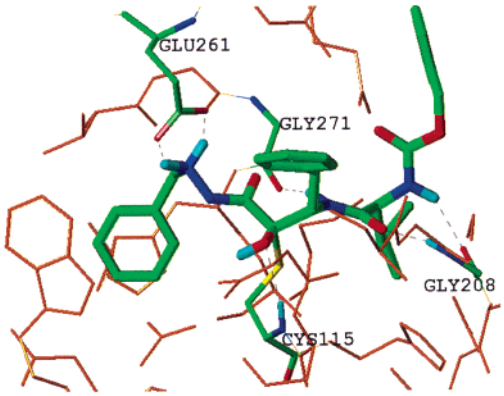
Figure 1. Proposed electrostatic interaction between the inhibitors and an active site acidic amino acid residue of calpain I. The amino acid residue is either aspartic acid ($n = 1$) or glutamic acid ($n = 2$).



Panel A



Panel B



Panel C

Figure 2. Hydrogen-bonding interactions between calpain I and **3a** (panel A), calpain I and **3b** (panel B), and calpain I and **4b** (panel C). Hydrogen atoms are removed for clarity, except those involved in hydrogen bonding. Hydrogen bonds are shown as black dashed lines. The atoms are colored as follows: all the protein residues are orange, except those involved in the hydrogen-bonding interactions; carbon = green; hydrogen = cyan; nitrogen = blue; oxygen = red; sulfur = yellow.

of an acidic amino acid residue at the S_1'-subsite of calpain I.

Molecular Modeling Studies. During the course of this study the crystal structure of the calcium-bound protease core of calpain I in which the catalytic site Cys115 of the wild-type enzyme has been mutated to Ser115 was released.[22] We docked **3a**, **3b**, and **4b** into the active site pocket of the enzyme to study their binding modes and to identify the acidic amino acid residue that the SAR studies predicted to be present at the S_1'-subsite of calpain I. The docking studies were performed after in silico mutation of Ser115 to Cys115 to mimic the wild-type enzyme. The compounds were constructed using Sybyl 6.8[31] and energetically minimized using the Tripos force field with Gasteiger–Hückel charges.[32] The binding site for docking the inhibitors was defined using Cys115 as the center and all amino acid residues within 10 Å radius of it were included.

The docking studies showed that the stereoisomeric compounds **3a** and **3b** exhibit different binding modes in the active site pocket of calpain I. Compound **3a** adopted a conformation that allowed the formation of an intramolecular hydrogen bond between one of the amidine NH groups and the amide carbonyl of the ketoamide functionality. This conformation facilitated the formation of three hydrogen bonds between the amidine of **3a** and the carboxylate of Glu261 (Figure 2, panel A). Thus, Glu261 was identified as the acidic amino acid residue that was predicted by the SAR studies to be present at the S_1'-subsite of calpain I. Four other hydrogen bonds, including a hydrogen bond between the NH of the P_1–P_2 amide bond of **3a** and Gly271, were also observed. Angelastro et al.[33] replaced the P_1–P_2 amide bond of a calpain inhibitor with a ketomethylene moiety and noticed a 250-fold loss of calpain inhibitory potency. The decrease in potency was attributed to the lack of a critical hydrogen bond between the normally occurring NH of the P_1–P_2 amide bond and calpain. Our docking studies identified Gly271 as the active residue of calpain that is involved in the formation of this critical hydrogen bond.

Compound **3b** adopted a different binding conformation in the active site pocket of calpain I compared to its stereoisomer **3a**. Unlike **3a**, no intramolecular hydrogen bonding was observed in the binding of **3b** to calpain I (Figure 2, panel B). Additionally, only three hydrogen-bonding interactions were observed between **3b** and calpain I. Furthermore, the amidine group of **3b** was not juxtapositioned to Glu261, thus precluding electrostatic interaction between **3b** and the enzyme. The docking studies also showed that **4b** is capable of electrostatic interaction with calpain I via its protonated hydrazine nitrogen and the carboxylate group of Glu261 (Figure 2, panel C). The results suggest that the calpain I inhibitory potency of the compounds is influenced by their ability to interact electrostatically with Glu261.

Conclusion

In this paper, we have described the account of our work on a series of novel α-ketoamides incorporating polar functional groups at the P_1'-position of the inhibitors. SAR studies of the compounds suggested the presence of an acidic amino acid residue at the S_1'-subsite of calpain I. Using molecular modeling studies the acidic amino acid residue was identified as Glu261. Compounds that were capable of electrostatic and/or hydrogen-bonding interaction with this residue (Glu261) were more potent inhibitors of calpain I than those that were not capable of undergoing such interactions (e.g.,

4b vs **4a**, and **3b** vs **3a**). Gly271 was also identified as the active site residue with which the NH group of the P_1–P_2 amide bond of peptidyl calpain inhibitors must hydrogen bond if they are to be potent inhibitors of the enzyme.

Experimental Section

Chemistry. General Methods. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured with a DigiPol 781 automatic polarimeter (Rudolph Instruments). Molecular masses were determined with Bruker-HP Esquire-LC−MS. ^{1}H NMR spectra were recorded on Bruker ARX-300 MHz and Varian Inova-500 MHz. Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. The chemical shifts (δ) are reported in ppm relative to TMS. Elemental analyses (C, H, N) were performed by Atlantic Microlab Inc., Norcross, GA and are within ±0.4% of the theoretical values. Chemicals and solvents were purchased from either Aldrich Chemical Co. or Fisher Scientific.

General Procedure 1. Synthesis of 6, 7, 12, 13, and 17. EDC (3.5 mmol) and HOBT (3.5 mmol) were added to an ice cooled solution of the appropriate acid in anhydrous DMF (45 mL) followed by the slow addition of NMM (3.5 mmol) and the amine hydrochloride (3.5 mmol). The mixture was stirred overnight at room temperature and the DMF was removed in vacuo. The residue was dissolved in EtOAc (60 mL); washed with saturated $NaHCO_3$ solution, 0.5 N HCl, water, and brine; and dried over $MgSO_4$. The solvent was removed and the residue was recrystallized from EtOAc/hexane.

General Procedure 2. Dess−Martin Oxidation. Dess−Martin reagent (0.38 mmol) was slowly added to an ice cooled solution of the appropriate alcohol (0.19 mmol) in anhydrous CH_2Cl_2 (10 mL). After 10 min, the ice bath was removed and the milky reaction mixture was stirred at room temperature for 4 h. A solution of $Na_2S_2O_3$ (2 mmol) in saturated $NaHCO_3$ was added and the mixture was stirred for additional 10 min at room temperature. After separation, the aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL) and the combined organic layer was washed successively with $NaHCO_3$ solution, water, and brine. It was dried ($MgSO_4$), concentrated, and purified either by flash chromatography or crystallization.

3(*S*)-(*N*-*tert*-Butyloxycarbonylamino)-2(*R,S*)-hydroxy-4-phenylbutyric Acid (5). Compound **5** was synthesized as described previously:[24,28] yield 96%; mp 123.1−124.7 °C; ^{1}H NMR ($CDCl_3$) δ 1.38 (s, 9H), 2.96 (s, 2H), 3.88−4.25 (m, 2H), 4.36 (s, 1H), 4.48 (s, 1H), 6.21 (s, 1H), 7.02−7.31 (m, 5H).

2(*R*)-[3(*S*)-(*N*-*tert*-Butyloxycarbonylamino)-2(*R,S*)-hydroxy-4-phenylbutyryl-amino]-3-phenylpropionic Acid Methyl Ester (6a). Compound **5** was coupled with D-phenylalanine methyl ester hydrochloride to generate **6a** in 87% yield: mp 164−166 °C; ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9H), 3.08 (m, 4H), 3.70 (s, 3H), 3.98 (m, 1H), 4.12 (dd, 1H), 4.28 (br. s, 1H), 4.91 (m, 2H), 5.24 (br. s, 0.5H), (br. s, 0.5H), 7.28 (m, 10H).

2(*S*)-[3(*S*)-(*N*-*tert*-Butyloxycarbonylamino)-2(*R,S*)-hydroxy-4-phenylbutyryl-amino]-3-phenylpropionic Acid Methyl Ester (6b). Compound **5** was coupled with L-phenylalanine methyl ester hydrochloride to give **6b** in 91% yield: mp 168−170 °C; ^{1}H NMR ($CDCl_3$) δ 1.39 (s, 9H), 3.10 (m, 4H), 3.71 (s, 3H), 3.95 (m, 1H), 4.11 (dd, 1H), 4.30 (br. s, 1H), 4.97 (m, 2H), 5.37 (br. s, 0.5H), 5.51 (br. s, 0.5H), 7.29 (m, 10H).

2(*R*)-[3(*S*)-(*N*-Cbz-L-leucylamino)-2(*R,S*)-hydroxy-4-phenylbutyrylamino]-3-phenylpropionic Acid Methyl Ester (7a). Compound **6a** was Boc-deprotected with a 4N HCl/dioxane (1:1) mixture and coupled with *N*-Cbz-L-leucine to afford **7a** in 73% yield; mp 168.3−170.2 °C; ^{1}H NMR ($CDCl_3$) δ 0.86 (d, 6H), 1.47 (m, 5H), 3.04 (m, 3H), 3.68 (s, 2H), 3.73 (s, 1H), 4.18 (m, 3H), 4.81 (q, 1H), 5.03 (m, 3H), 5.53 (br. s, 1H), 6.82 (br. s, 1H), 7.30 (m, 15H).

2(*S*)-[3(*S*)-(*N*-Cbz-L-leucylamino)-2(*R,S*)-hydroxy-4-phenylbutyrylamino]- 3-phenylpropionic Acid Methyl Ester (7b). Compound **6b** was Boc-deprotected with a 4 N HCl/dioxane (1:1) mixture and coupled with *N*-Cbz-L-leucine to obtain **7b** in 76% yield: mp 180−182 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (d, 6H), 1.36 (m, 1H), 1.61 (m, 4H), 3.13 (m, 3H), 3.62 (s, 2H), 3.62 (s, 2H), 3.74 (s, 1H), 4.11 (m, 3H), 4.71 (q, 0.5H), 4.90 (m, 0.5H), 5.14 (m, 2H), 5.53 (d, 1H), 6.20 (d, 1H), 6.85 (m, 1H), 7.27 (m, 15H).

2(*R*)-[3(*S*)-(*N*-Cbz-L-leucylamino)-4-phenyl-2-oxo-butyrylamino]-3-phenylpropionic Acid Methyl Ester (1a). The hydroxyl group of **7a** (1.15 g, 1.9 mmol) was oxidized using Dess−Martin reagent (3.23 g, 7.6 mmol). The crude product was recrystallized from EtOAc to afford **1a** in 79% yield: mp 159−161 °C; ^{1}H NMR ($CDCl_3$) δ 0.91 (d, 6H), 1.45 (m, 1H), 1.68 (m, 3H), 3.12 (dd, 1H), 3.19 (d, 2H), 3.32 (dd, 1H), 3.78 (s, 3H), 4.16 (m, 1H), 4.86 (q, 1H), 5.01 (d, 1H), 5.12 (s, 1H), 5.51 (q, 1H), 6.56 (d, 1H), 7.26 (m, 15H). Anal. ($C_{34}H_{39}N_3O_7$) C, H, N.

2(*S*)-[3(*S*)-(*N*-Cbz-L-leucylamino)-4-phenyl-2-oxo-butyrylamino]-3-phenylpropionic Acid Methyl Ester (1b). Compound **7b** was oxidized with Dess−Martin reagent and the crude product was recrystallized from EtOAc/hexane to give **1b** in 95% yield: mp 167−168 °C; ^{1}H NMR ($CDCl_3$) δ 0.90 (d, 6H), 1.43 (m, 1H), 1.58 (m, 4H), 3.12 (m, 2H), 3.25 (dd, 2H), 3.77 (s, 3H), 4.91 (m, 1H), 5.12 (m, 2H), 5.52 (q, 1H), 6.50 (d, 1H), 6.95 (br. s, 1H), 7.26 (m, 15H). Anal. ($C_{34}H_{39}N_3O_7$) C, H, N.

2(*R*)-[-3(*S*)-(*N*-Cbz-L-leucylamino)-4-phenyl-2-oxo-butyrylamino]-3-phenylpropionic Acid (2a). Compound **1a** (0.46 g, 0.765 mmol) was suspended in MeOH (5 mL) and 1 N NaOH (1 mL) and stirred at room temperature for 90 min. The solvent was removed and the residue was recrystallized from Et_2O/hexane to afford **2a** in 60% yield: mp 167.5−168.3 °C; ^{1}H NMR ($CDCl_3$) δ 0.91 (d, 6H), 1.45 (m, 3H), 2.95 (m, 2H), 3.25 (m, 2H), 4.38 (dd, 1H), 5.00 (m, 3H), 5.42 (d, 1H), 5.71 (d, 1H), 6.71 (m, 1H), 6.93 (d, 1H), 7.25 (m, 15H), 7.64 (d, 1H). Anal. ($C_{33}H_{37}N_3O_7$) C, H, N.

2(*S*)-[3(*S*)-(*N*-Cbz-L-leucylamino)-4-phenyl-2-oxobutyrylamino]-3-phenyl-propionic Acid (2b). Compound **2b** was synthesized in 52% yield from **1b** as described for the synthesis of **2a**: mp 166.8−167.7 °C; ^{1}H NMR (DMSO-d_6) δ 0.76 (m, 6H), 0.85 (m, 2H), 1.22 (t, 1H), 1.56 (m, 1H), 2.66 (m, 1H), 3.11 (m, 2H), 4.05 (q, 1H), 4.45 (m, 1H), 5.00 (m, 2H), 5.17 (m, 1H), 7.26 (m, 15H), 8.17 (d, 1H), 8.27 (d, 1H), 8.98 (d, 1H), 13.00 (s, 1H). Anal. ($C_{33}H_{37}N_3O_7$) C, H, N.

2(*R*)-(+)-Amino-3-phenylpropionitrile (11a). *N*-*tert*-Boc-D-phenylalanine (**8a**) (8 g, 30.15 mmol) was dissolved in THF (80 mL) and cooled to −10 °C. TEA (4.46 mL, 32 mmol,) and $ClCO_2Et$ (3.4 g, 31 mmol) were added successively. The reaction was stirred at −10 °C for 30 min before a stream of anhydrous ammonia gas was introduced while the temperature was maintained below 0 °C. After 90 min the THF was removed, water (50 mL) was added, and the mixture was extracted with Et_2O (3 × 80 mL). The combined Et_2O extract was washed with saturated $NaHCO_3$ solution, 0.5 N HCl, water, and brine, followed by drying ($MgSO_4$), and the solvent was removed to give **9a** as a white solid in 80% yield: ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9H), 3.11 (d, 2H, J = 6.8 Hz), 4.39 (d, 1H, J = 6.3 Hz), 5.07 (s, 1H), 5.50 (s, 1H), 5.84 (s, 1H), 7.21−7.33 (m, 5H). Compound **9a** (5.86 g, 22.2 mmol) was dissolved in THF (200 mL) and the solution was cooled on an ice bath followed by the successive addition of TEA (6.27 mL, 45 mmol) and $(CF_3CO)_2O$ (3.2 mL, 22.2 mmol). The reaction mixture was stirred for 30 min at room temperature and the THF was removed. The residue was dissolved in EtOAc (200 mL) and washed with saturated $NaHCO_3$ solution, 0.5 N HCl solution, water, brine, and dried ($MgSO_4$). Evaporation of the solvent gave **10a** as a white solid in 92% yield: mp 195−210 °C; ^{1}H NMR ($CDCl_3$) δ 1.4 (s, 9H), 3.07 (d, 2H, J = 6.6 Hz), 4.30 (t, 1H, J = 6.3 Hz), 5.80 (s, 1H), 7.29−7.39 (m, 5H). Compound **10a** (5 g, 20.3 mmol) was dissolved in 50% TFA in CH_2Cl_2 (50 mL) and stirred at room temperature for 30 min, followed by solvent removal. The residue was dried overnight in vacuo to give a quantitative yield of **11a** as the TFA salt: mp 203−210 °C (dec); $[\alpha]^{20}_D$ + 5.32° (c = 0.5, MeOH); ^{1}H NMR (D_2O) δ 3.14 (dd, 1H, J_1 = 7.95 Hz, J_2 = 7.8 Hz), 3.36 (dd, 1H, J_1 = 13.5

Hz, J_2 = 5.5 Hz), 4.82 (dd, 1H, J_1 = 10.05 Hz, J_2 = 9.9 Hz), 7.24−7.42 (m, 5H).

2(*S*)-(−)-Amino-3-phenylpropionitrile (11b). *N*-*tert*-Boc-L-phenylalanine was transformed to **11b** as described for the synthesis of **11a**: mp 208 °C (dec); $[\alpha]^{20}_D$ − 5.38° (c = 0.5, MeOH); ^{1}H NMR (D_2O) δ 3.05 (dd, 1H, J_1 = 13.2 Hz, J_2 = 10.5 Hz), 3.42 (dd, 1H, J_1 = 13.2 Hz, J_2 = 10.5 Hz), 4.86 (dd, 1H, J_1 = 10.5 Hz, J_2 = 10.2 Hz), 7.17−7.53 (m, 5H).

2(*R*)-[3(*S*)-(*N*-*tert*-Butyloxycarbonylamino)-2(*R,S*)-hydroxy-4-phenylbutyrylamino]-3-phenylpropionitrile (12a). Compound **5** was coupled with **11a** to give **12a** in 72% yield: ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 9H), 3.08 (d, 2H, J = 6.0 Hz), 3.13 (d, 2H, J = 6.8 Hz), 4.05 (m, 1H), 4.10 (m, 1H), 4.26 (d, 1H, J = 3.9 Hz), 4.93 (s,1H), 5.10 (m, 1H), 5.93 (m, 1H), 7.15−7.39 (m, 10H); MS (ESI) m/z 446.1 $[M + Na]^+$, 422.0 $[M - H]^-$.

2(*S*)-[3(*S*)-(*N*-*tert*-Butyloxycarbonylamino)-2(*R,S*)-hydroxy-4-phenylbutyryl-amino]-3-phenylpropionitrile(12b). Compound **5** was coupled with **11b** to afford **12b** in 90% yield: ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9H), 2.99 (d, 2H, J = 6.1 Hz), 3.09 (d, 2H, J = 6.8 Hz), 3.99 (m, 1H), 4.13 (d, 1H, J = 2.4 Hz), 4.26 (s, 1H), 4.90 (s,1H), 5.13 (m, 1H), 5.99 (m, 1H), 7.13−7.59 (m, 10H); MS (ESI) m/z 446.0 $[M + Na]^+$, 422.1 $[M - H]^-$.

2(*R*)-[3(*S*)-(*N*-Cbz-L-leucylamino)-2(*R,S*)-hydroxy-4-phenylbutyrylamino]-3-phenylpropionitrile (13a). Compound **12a** was Boc-deprotected and coupled with Cbz-L-leucine to obtain **13a** in 84% yield: ^{1}H NMR ($CDCl_3$) δ 0.92 (d, 6H, J = 6.0 Hz), 1.28 (s, 1H), 1.40 (m, 2H), 2.72 (d, 2H, J = 7.2 Hz), 2.98 (d, 2H, J = 6.6 Hz), 3.77 (s, 1H), 4.05 (m, 1H), 4.13 (s, 1H), 4.71 (m, 1H), 5.12 (s, 1H), 5.30 (s, 2H), 5.52 (s, 1H), 6.95−7.81 (m, 15H); MS (ESI) m/z 593.3 $[M + Na]^+$, 569.1 $[M - H]^-$.

2(*S*)-[3(*S*)-(*N*-Cbz-L-leucylamino)-2(*R,S*)-hydroxy-4-phenylbutyrylamino]-3-phenylpropionitrile (13b). Compound **12b** was Boc-deprotected and coupled with Cbz-L-leucine to afford **13b** in 88% yield: ^{1}H NMR ($CDCl_3$) δ 0.92 (d, 6H, J = 6.0 Hz), 1.28 (s, 1H), 1.42 (m, 2H), 2.87 (d, 2H, J = 7.8 Hz), 3.08 (d, 2H, J = 6.6 Hz), 3.62 (s, 1H), 4.10 (m, 1H), 4.13 (s, 1H), 4.72 (m, 1H), 5.04 (s, 1H), 5.25 (s, 2H), 5.66 (s, 1H), 7.10−7.79 (m, 15H); MS (ESI) m/z 593.2 $[M + Na]^+$, 569.2 $[M - H]^-$.

2(*R*)-[3(*S*)-(*N*-Cbz-L-leucylamino)-2(*R,S*)-hydroxy-4-phenylbutyrylamino]-1-amidino-3-phenylpropane (14a). Compound **13a** (1 g, 1.75 mmol) and *N*-acetylcysteine (287 mg, 1.75 mmol) were dissolved in MeOH (2 mL), and NH_4OAc (154 mg, 0.2 mmol) was added. The reaction vessel was tightly sealed and heated at 50 °C under N_2 for 16 h, after which the solvent was removed. The residue was dissolved in CH_2Cl_2 (150 mL) and washed with water. The crude solid was dissolved in *i*-PrOH and passed through SBG1-OH ion-exchange resin (ResinTech, Inc.) at a flow rate of 1 cm/s. Evaporation of solvent gave **14a** as a brown solid in 92% yield: ^{1}H NMR ($CDCl_3$) δ 0.95 (d, 6H, J = 6.0 Hz), 1.25−1.36 (m, 3H), 2.96 (d, 2H, J = 5.5 Hz), 3.10 (d, 2H, J = 6.6 Hz), 3.70 (m, 1H), 4.05 (d, 1H, J = 5.5 Hz), 4.38 (br, 1H), 4.71 (m, 1H), 5.15 (s, 2H), 6.98 (s, 1H), 7.08−7.69 (m, 15H). MS (ESI) m/z 610.4 $[M + Na]^+$, 588.2 $[M + H]^+$.

2(*S*)-[3(*S*)-(*N*-Cbz-L-leucylamino)-2(*R,S*)-hydroxy-4-phenylbutyrylamino]-1-amidino-3-phenylpropane (14b). Compound **13b** was transformed into **14b** as described for **14a** in 98% yield: ^{1}H NMR ($CDCl_3$) δ 0.92 (d, 6H, J = 6.0 Hz), 1.23−1.29 (m, 3H), 3.02 (d, 2H, J = 5.6 Hz), 3.06 (d, 2H, J = 6.5 Hz), 3.69 (m, 1H), 4.04 (d, 1H, J = 5.6 Hz), 4.28 (br, 1H), 4.69 (m, 1H), 5.17 (s, 2H), 6.95 (s, 1H), 7.20−7.65 (m, 15H); MS (ESI) m/z 610.2 $[M + Na]^+$, 588.1 $[M + H]^+$.

2(*R*)-[3(*S*)-(*N*-Cbz-L-leucylamino)-4-phenyl-2-oxobutyrylamino]-1-amidino-3-phenylpropane (3a). Compound **14a** (100 mg, 0.17 mmol) was dissolved in a 1:1 mixture of DMSO and toluene (4 mL) and cooled on an ice bath, and pyridinium trifluoroacetate (5 equiv) was added followed by the addition of EDC (0.326 mg, 1.7 mmol). The mixture was stirred at 0 °C for 10 min and the ice bath was removed. Stirring was continued at room temperature until TLC [silica gel, EtOAc: MeOH:NH_4OH (6:3:1)] showed no starting material (approximately 2 h later). EtOAc and water were added, and the organic phase was recovered and washed with saturated $NaHCO_3$ solution, water, and brine, followed by drying over $MgSO_4$. The organic layer was then concentrated under reduced pressure to give a brown solid, which was dissolved in EtOH (60 mL) and purified by ion-exchange chromatography with 2-propanol as the eluant. The solvent was removed to give **3a** as a brown solid in 60% yield: mp 195−197°C (dec) (HCl salt);^{1}H NMR (DMSO-d_6) δ 0.88 (d, 6H, J = 6.1 Hz), 1.20−1.33 (m, 3H), 2.69 (d, 2H, J = 5.7 Hz), 3.19 (d, 2H, J = 6.9 Hz), 3.22 (s, 1H), 4.03 (m, 1H), 4.61 (m, 1H), 4.80 (m, 1H), 4.93 (s, 2H), 6.01 (s, 1H), 6.95−7.52 (m, 15H), 8.81−9.10 (s, 4H); MS (ESI) m/z 618.3 $[M + Na]^+$, 586.3 $[M + H]^+$. Anal. ($C_{33}H_{39}N_5O_5$) C, H, N.

2(*S*)-[3(*S*)-(*N*-Cbz-L-leucylamino)-4-phenyl-2-oxo-butyrylamino]-1-amidino-3-phenylpropane (3b). Compound **14b** was converted to **3b** in 62% yield as described for the synthesis of **3a**: mp 175−176°C (dec) (HCl salt); ^{1}H NMR (DMSO-d_6) δ 0.89 (d, 6H, J = 6.0 Hz), 1.18−1.32 (m, 3H), 2.73 (d, 2H, J = 5.7 Hz), 3.18 (d, 2H, J = 6.8 Hz), 3.28 (s, 1H), 3.95 (m, 1H), 4.55 (m, 1H), 4.82 (m, 1H), 4.90 (s, 2H), 5.97 (s, 1H), 6.96−7.61 (m, 15H), 8.8−9.4 (br, s, 4H); MS (ESI) m/z m/z 618.4 $[M + Na]^+$, 586.5 $[M + H]^+$. Anal. ($C_{33}H_{39}N_5O_5$) C, H, N.

3(*S*)-(*N*-Cbz-L-leucylamino)-4-phenyl-2-oxobutyric Acid Phenylethyl Amide (4a). Compound **4a** was synthesized as described previously:[24,28] ^{1}H NMR ($CDCl_3$) δ 0.91 (d, 6H), 1.43 (m, 1H, J = 8.3 Hz), 1.60 (m, 2H), 2.90 (m, 2H, J = 6.7 Hz), 3.11 (dd, 0.5H, J = 6.9 Hz), 3.14 (dd, 0.5H, J = 6.9 Hz), 3.31 (dd, 0.5H, J = 5.3 Hz), 3.35 (dd, 0.5H, J = 5.4 Hz), 3.63 (m, 2H), 4.17 (m, 1H), 5.05 (d, 1H, J = 7.3 Hz), 5.11 (s, 2H), 5.57 (q, 1H, J = 6.8 Hz), 6.60 (d, 1H, J = 6.4 Hz), 6.95 (d, 1H), 7.05−7.37 (m 15H). Anal. ($C_{32}H_{37}N_3O_5$) C, H, N.

3(*S*)-Amino-2(*R,S*)-hydroxy-4-phenylbutyric Acid (15). Compound **5** (2.75 g, 14 mmol) was dissolved in 2 N HCl (15 mL) and MeOH (30 mL). The mixture was stirred at room temperature for 2 h. The solvent was evaporated to give **15** as a white solid, which was used in the next reaction without further purification. ^{1}H NMR (D_2O) δ 2.92 (dd, 1H, J_1 = 6.9 Hz, J_2 = 13.95 Hz), 3.13 (dd, J_1 = 6.9 Hz, J_2 = 13.95 Hz), 3.75 (m, 1H), 4.02 (d, 1H, J = 3.6 Hz), 7.21−7.40 (m, 3H), 7.40−7.55 (m, 2H); ^{13}C NMR (D_2O) δ 33.96, 36.15, 56.19, 56.55, 128.43, 130.01, 130.27, 136.34, 177.23, 177.75.

3(*S*)-Amino-2(*R,S*)-hydroxy-4-phenylbutyric Acid Methyl Ester (16). $SOCl_2$ (3.7 g, 30.7 mmol) was slowly added (under an atmosphere of nitrogen) to an ice cooled solution of MeOH (40 mL) and stirred for 5 min followed by the addition of **15** (1.5 g, 7.7 mmol). The mixture was stirred at room temperature overnight and the solvent was evaporated in vacuo to give a brown solid in 93% yield: ^{1}H NMR (D_2O) δ 2.93 (dd, 1H, J_1 = 7.5 Hz, J_2 = 14.75 Hz), 3.01 (dd, 1H, J_1 = 7.5 Hz, J_2 = 14.75 Hz), 3.65 (s, 3H), 3.86 (br s, 1H), 4.29 (d, 1H, J = 2.5 Hz), 7.20−7.29 (m, 3H), 7.30−7.42 (m, 2H).

3(*S*)-(*N*-Cbz-L-leucylamino)-2(*R,S*)-hydroxy-4-phenylbutanoic Acid Methyl Ester (17). Coupling of **16** with Cbz-L-leucine afforded **17** as a yellow solid in 92% yield after purification by flash chromatography [silica gel, hexane:EtOAc (2:1)]: ^{1}H NMR ($CDCl_3$) δ 0.90 (d, 6H, J = 6.5 Hz), 1.38 (m, 1H), 1.55 (m, 2H), 2.90 (d, 2H, J = 7.5 Hz), 3.20 (s, 1H), 3.69 (s, 3H), 4.09 (m, 2H), 4.54 (dd, 1H, J_1 = 9.5 Hz, J_2 = 16.75 Hz), 4.99 (m, 1H), 5.11 (s, 2H), 6.22 (d, 1H, J = 8.5 Hz), 7.18−7.47 (m, 10H); MS (ESI) m/z 479.3 $[M + Na]^+$.

3(*S*)-(*N*-Cbz-L-leucylamino)-2(*R,S*)-hydroxy-4-phenylbutanoic Acid Hydrazide (18). Compound **17** (2.1 g, 4.6 mmol) was dissolved in MeOH (15 mL), and hydrazine monohydrate (0.576 g, 11.5 mmol) was added. The mixture was stirred at room temperature for 36 h and the solvent was evaporated in vacuo to give **18** as a white solid in 86% yield: ^{1}H NMR ($CDCl_3$) δ 0.85 (d, 6H, J = 6.0 Hz), 1.36−1.55 (m, 3H), 3.05 (d, 1H, J = 7.5 Hz), 3.10 (d, 1H, J = 7.5 Hz), 3.71 (s, 1H), 4.10 (m, 1H), 4.21 (m, 1H), 4.98 (m, 1H), 5.10 (s, 2H), 6.49 (d, 1H, J = 7 Hz), 7.10−7.47 (m, 10H), 7.93 (s, 2H); MS (ESI) m/z 479.3 $[M + Na]^+$, 457 $[M + H]^+$.

***N′*-Benzyl-3(*S*)-(*N*-Cbz-L-leucylamino)-2(*R,S*)-hydroxy-4-phenylbutanoic Acid Hydrazone (19).** Compound **17** (408 mg, 0.83 mmol) and TEA (0.22 mL, 1.6 mmol) were dissolved in absolute EtOH (10 mL), and benzaldehyde (106 mg, 1 mmol)

was added. The mixture was stirred for 5 h at room temperature and left to stand overnight. The precipitated solid was recovered by filtration and the filtrate was concentrated to afford more product. The solid was crystallized twice from 95% EtOH and once from Et_2O to give **19** as a white solid in 72% yield: ^{1}H NMR ($CDCl_3$) δ 0.83 (d, 6H, J = 6.0 Hz), 1.28−1.43 (m, 3H), 3.13 (d, 2H, J = 7.2 Hz), 4.39 (s, 1H), 4.6 (s, 1H), 4.80−5.10 (m, 3H), 5.78 (d, 1H, J = 4.8 Hz), 6.46 (d, 1H, J = 6 Hz), 7.05−7.77 (m, 15H), 8.12 (s, 1H), 9.86 (s, 1H); MS (ESI) m/z 567.3 $[M + Na]^+$, 545.3 $[M + H]^+$, 543.0 $[M - H]^-$.

***N′*-Benzyl-3(*S*)-(*N*-Cbz-L-leucylamino)-2(*R,S*)-hydroxy-4-phenylbutanoic Acid Hydrazide (20).** To a solution of **19** (330 mg, 0.6 mmol) in THF (15 mL) was added $NaBH_3CN$ (114 mg, 1.8 mmol) followed by 3 drops of HCl. The mixture was heated to 60 °C and stirred overnight until TLC [silica gel, hexane/EtOAc (2:3)] showed no starting material. The solvent was evaporated and the residue was dissolved in EtOAc (120 mL) and washed successively with $NaHCO_3$ solution, water, and brine. It was dried ($MgSO_4$) and concentrated to give **20** as a white solid in 88% yield:^{1}H NMR ($CDCl_3$) δ 0.88 (d, 6H, J = 5.7 Hz), 1.25−1.65 (m, 3H), 2.79 (m, 2H), 3.09 (d, 1H, J = 6.3 Hz), 3.72 (m, 2H), 3.91 (d, 1H, J = 6.3 Hz), 4.09 (m, 2H), 5.02 (s, 1H), 7.00−7.65 (m, 15H); MS (ESI) m/z 569.3 $[M + Na]^+$.

***N′N′*-(Benzyl)(tert-butyloxycarbonylamino)-3(*S*)-(*N*-Cbz-L-leucylamino)-2(*R,S*)-hydroxy-4-phenylbutanoic Acid Hydrazide (21).** Di-*tert*-butyl dicarbonate (158 mg, 0.72 mmol) was slowly added to a solution of **20** (360 mg, 0.66 mmol) in *t*-BuOH (10 mL) and 1 N NaOH (2 mL), and the mixture was stirred at room temperature for 12 h. Following this period the mixture was diluted with H_2O (25 mL) and extracted with pentane (3 × 50 mL). The aqueous phase was cooled on an ice bath, the pH was adjusted to 3 with 0.5 N HCl, and the phase was extracted with EtOAc. The combined organic extracts were washed successively with water and brine, dried over $MgSO_4$, and concentrated in vacuo to give **21** as a white solid in 94% yield: ^{1}H NMR ($CDCl_3$) δ 0.88 (dd, 6H, J_1 = 8 Hz, J_2 = 8 Hz), 1.40 (s, 9H), 1.45−1.54 (m, 3H), 3.16 (m, 1H), 3.35 (m, 1H), 3.97 (m, 1H), 4.18 (dd, 1H, J_1 = 3 Hz, J_2 = 8 Hz), 4.29 (br s, 2H), 4.91 (m, 1H), 4.96 (d, 1H, J = 12 Hz), 5.09 (d, 1H, J = 12 Hz), 5.99 (d, 1H, J = 8 Hz), 6.27 (s, 1H), 7.05−7.47 (m, 15H), 8.44 (s, 1H); MS (ESI) m/z 669.6 $[M + Na]^+$.

***N′*,N′-(Benzyl)(tert-butyloxycarbonylamino)-3(*S*)-(*N*-Cbz-L-leucylamino)-4-phenyl-2-oxo-butanoic Acid Hydrazide (22).** Dess−Martin reagent oxidation of **21** followed by flash chromatographic purification [silica gel, hexane/EtOAc (7:3)] of the crude product afforded **22** as a white solid in 93% yield: ^{1}H NMR ($CDCl_3$) δ 0.89 (d, 6H, J = 6.0 Hz), 1.24 (m, 1H), 1.42 (m, 2H), 1.47 (s, 9H), 3.08 (m, 1H), 3.30 (dd, 1H, J_1 = 5 Hz, J_2 = 14.25 Hz), 4.10 (br s,1H), 4.69 (s, 2H), 5.08 (s, 2H), 6.51 (s, 1H), 7.01 (s, 2H), 7.17−7.45 (m, 13H), 8.20 (s, 1H); MS (ESI) m/z 667.8 $[M + Na]^+$; 643.0 $[M - H]^-$.

***N′*-Benzyl-3(*S*)-(*N*-Cbz-L-leucylamino)-4-phenyl-2-oxo-butanoic Acid Hydrazide (4b).** Compound **22** (150 mg, 3.98 mmol) was dissolved in a mixture of 2 N HCl (2 mL) and dioxane (8 mL) and stirred at room temperature for 1 h. The solvent was evaporated in vacuo to give **4b** as light brown solid in 90% yield: ^{1}H NMR ($CDCl_3$) δ 0.89 (d, 6H, J = 6.0 Hz), 1.27−1.45 (m, 3H), 3.03 (m, 2H), 4.06 (s, 1H), 4.25 (m, 2H), 4.86 (s, 1H), 5.06 (d, 1H, J = 12 Hz), 5.10 (d, 1H, J = 12 Hz), 6.44 (s, 1H), 6.70 (s, 1H), 7.05−7.60 (m, 15H), 8.21 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 20.5, 21.7, 34.6, 43.1, 55.2, 56.3, 56.8, 70.3, 126.1, 126.6, 127.5, 127.8, 127.9, 128.0, 128.3, 128.4, 128.7, 139.2, 141.0, 155.7, 160.8, 178.9, 196.7; MS (ESI) m/z 567.4 $[M + Na]^+$. Anal. ($C_{31}H_{36}N_4O_5$), C, H, N.

Calpain I Inhibition Assay. Calpain activity was monitored in a reaction mixture containing 50 mM Tris HCl (pH 7.4), 50 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.2 mM or 1.0 mM Suc-Leu-Tyr-AMC (Calbiochem), 1 mg porcine erythrocyte calpain I (Calbiochem), varying concentrations of inhibitor dissolved in DMSO (2% total concentration), and 5 mM $CaCl_2$ in a final volume of 250 mL in a polystyrene microtiter plate. Assays were initiated by addition of $CaCl_2$, and the increase in fluorescence (λ_{ex} = 370 nm, λ_{em} = 440 nm) was monitored at amibient temperature using a SPECTRAmax Gemini fluorescence plate reader (Molecular Devices). The K_i values were estimated from the semireciprocal plot of the initial velocity versus the concentration of the inhibitor according to the method of Dixon.[30] The correlation coefficients for the Dixon plots were above 0.95. No other attempt was made to correct for slow binding or autolysis. The reported K_i values are the average of triplicate determinations.

Molecular Modeling Studies. The 2.1 Å resolution X-ray crystal structure of the calcium-bound protease core of calpain I (PDB accession number 1KXR)[22] was retrieved from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb). The Ser115 residue was mutated to Cys115 to mimic the wild-type enzyme. The structures of the inhibitors were constructed using Sybyl 6.8[31] and energetically minimized using Tripos force field with Gasteiger−Hückel charges.[32] The Gold 1.2 program[34] was applied for flexibly docking the inhibitors into the active site of the enzyme. Cys115 and all residue within 10 Å of it defined the active site pocket for the docking studies. The complexes of the inhibitors with calpain I obtained from the molecular docking were further minimized using the Tripos force field in Sybyl 6.8 and the Powell method for 5000 steps or until the gradient was lower than 0.01 kcal mol^{-1} Å $^{-1}$. All simulations were performed with the molecular dynamics module (Sybyl 6.8) in a vacuum with a dielectric constant ϵ = 1 using the Tripos force field. The inhibitors were allowed to be fully flexible, while in the enzyme, only residues within 10 Å radius of the inhibitor were made fully flexible, the other residues being kept fixed.

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