R-factors of 8.1% and 10.0%, respectively. The alternate space group $P4_1$ was discounted on the basis of the known stereochemistry in the molecule. The final difference map had a maximum peak height of 0.51 $e^-/Å^3$, 0.7 Å from atom O2. The largest negative feature was -0.32 $e^-/Å^3$, 0.6 Å from a poorly ordered water oxygen.

Materials. The following materials were used. Purified fibrinogen (Kabi), dissolved in 20 mM Tris and 120 mM NaCl buffer (pH 7.5). PBS/Tween: 0.137 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO_4, 0.001 M KH_2PO_4, pH 7.4, 0.05% Tween 20 (Sigma). TNCNT/BSA: 20 mM Tris, pH 7.5, 120 mM NaCl, 0.2% NaN_3, 2 mM $CaCl_2$, 0.05% Tween 20, 0.5% BSA (Calbiochem). GPIIbIIIa receptor, purified from human platelets, stored at -70 °C, reconstituted in TNCNT with BSA. ELISA buffer: PBS, 0.5% BSA, 0.05% Tween 20, 0.01% Thimerosal. GAM-HRP: horseradish peroxidase conjugate of goat anti-mouse IgG (Tago), dissolved in ELISA buffer. OPD: o-phenylenediamine hydrochloride, 10-mg tablets (Sigma). Hydrogen peroxide, 30% solution (Sigma). Phosphate/citrate buffer: 16 mM citric acid, 50 mM Na_2HPO_4, pH 5.0.

Fibrinogen/GPIIbIIIa Solid-Phase ELISA. Samples were tested for inhibition of purified human platelet GPIIbIIIa binding to human fibrinogen immobilized onto microtiter plates. Fibrinogen (100 μL, 10 μg/mL) was coated onto 96-well, Nunc Maxisorp plates. After being blocked with BSA (200 μL, 5 mg/mL), samples (50 μL) were added at appropriate dilutions, followed by addition of GPIIbIIIa (50 μL, 40 μg/mL). After a 1-h incubation at room temperature, plates were washed and antibody to GPIIbIIIa (AP3, from P. Newman, Blood Center of Southeast Wisconsin) was added (100 μL, 1 μg/mL in ELISA buffer). After an additional 1-h incubation, plates were washed, GAM-Fc-HRP was added (100 μL, 1:15,000 dilution in ELISA buffer), and the solution was incubated for 1 h and washed. OPD/H_2O_2 (0.67 mg/mL, 0.0003%) in 50 mM Na_2HPO_4/citric acid (pH 5, 100 μL) buffer was added and the peroxidase reaction stopped by addition of H_2SO_4 (50 μL, 1 M). Absorbance at 492-405 nm was determined. The IC_{50} values were estimated by a nonlinear four-parameter curve fit analysis of the data. The coefficient of variation for the IC_{50} values for this assay was 25-30%.

In Vitro Platelet Aggregation in Human PRP. Blood was drawn on 3.8% sodium citrate (9:1) and spun at 180g at 22 °C for 12 min, and PRP was removed. The remaining fraction was spun at 1000g at 22 °C for 25 min for PPP. The platelet count of PRP was adjusted to 3×10^8/mL with PPP. PRP was incubated with the test sample at 37 °C for 5 min in the aggregometer, followed by addition of an appropriate concentration of aggregating agonist. Percent inhibition was expressed as 100 × the ratio of the change in transmittance in the presence of the test sample to the maximum change in transmittance in the absence of test sample, at 5 min after addition of the agonist. The reproducibility of the assay values of ca. 25% was determined for nine experiments. ADP concentration was 17.2 μM final concentration. The test sample was used at 12-14 concentrations, generally over a 200-fold range. The IC_{50} values were estimated by a nonlinear four-parameter curve fit analysis of the inhibition results.

Supplementary Material Available: One table giving RP-HPLC retention times, FAB mass spectral data, and amino acid analyses (7 pages). Ordering information is given on any current masthead page.

Ester and Amide Derivatives of E64c as Inhibitors of Platelet Calpains

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Ester and amide derivatives of E64c, (+)-(2S,3S)-3-[[(S)-3-methyl-1-[(3-methylbutyl)carbamoyl]butyl]carbamoyl]-2-oxiranecarboxylic acid, an inhibitor of calpains, were synthesized and tested for ability to inhibit calpain in lysed cells, ability to enter intact cells, and ability to inhibit calpain in intact cells. The esters were from halogen-substituted alcohols and alcohols with increasing size. There were no appreciable differences in the inhibitory potency of any of the halogen-substituted esters from ethyl to trifluoroethyl, indicating that ease of hydrolysis of this class of ester is not important for activity. The only ester with impaired activity was the largest, Z-leucyl-norleucyl, which was about 5% as effective as the ethyl ester, E64d. Amides of amino acid esters also had impaired activity. To explore the possibility of targeting E64c derivatives to specific cells, esters and amides of E64c with 5-hydroxytryptamine were tested on the rationale that the active 5-hydroxytryptamine uptake mechanism of platelets might selectively concentrate the drug in platelets. Both the ester and amide inhibited calpain in lysed cells, but only the ester inhibited in intact cells. The 5-hydroxytryptamine ester showed no advantage over the ethyl ester in entering platelets.

Calpains are Ca^{2+}-activated, intracellular cysteine proteases.[1-5] While their specific cleavage of certain protein substrates has been demonstrated in cell-free systems, there is less known about their physiological function. Cell-specific inhibitors of calpains would be valuable for studies of physiological function.

E64c, (+)-(2S,3S)-3-[[(S)-3-methyl-1-[(3-methylbutyl)carbamoyl]butyl]carbamoyl]-2-oxiranecarboxylic acid, is a calpain inhibitor,[6] and its ethyl ester, E64d, is able to cross plasma membranes and inhibit calpains within cells.[7,8] (For a review of calpain inhibitors, see refs 9-12.) We have explored the possibility of synthesizing cell-specific E64c analogues by esterification to an agent that is specifically transported into a certain cell. This required initial studies of the effect of variation of the ester group

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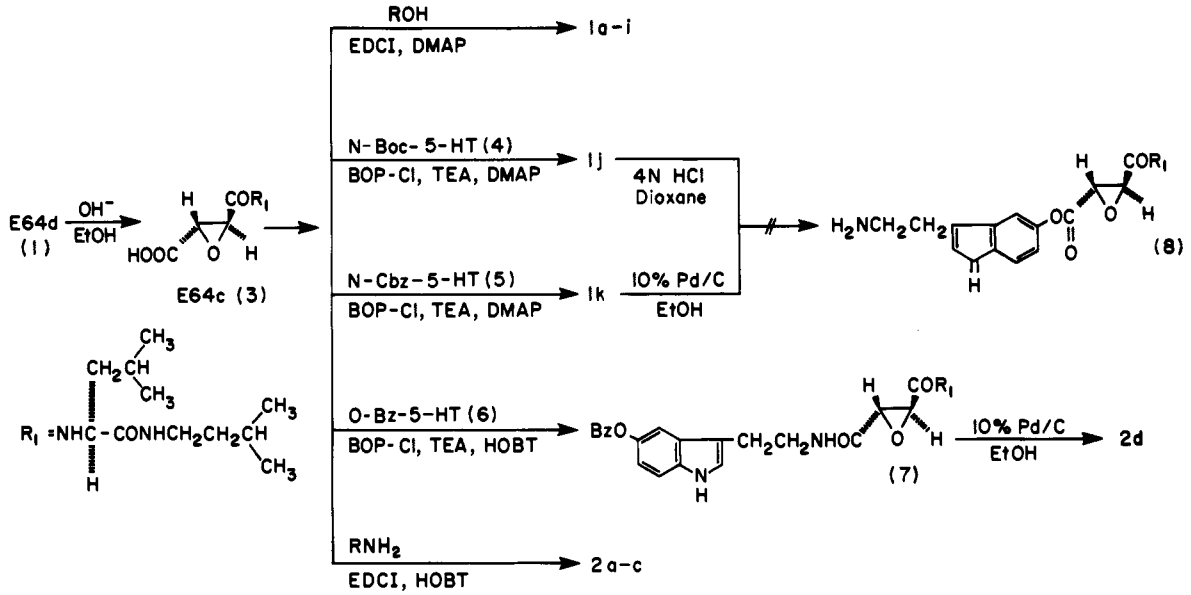
Scheme I



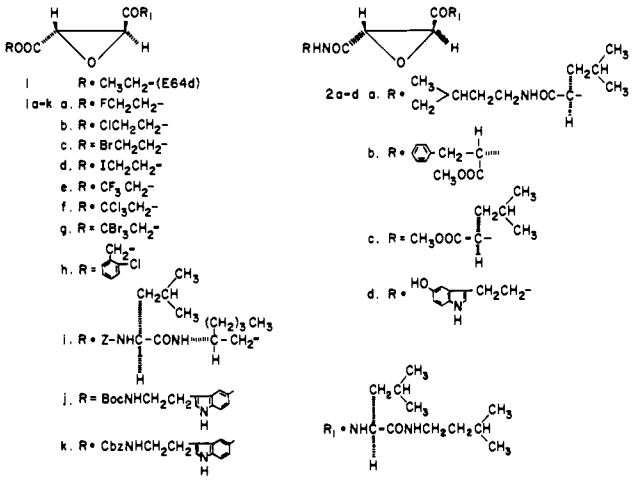


Figure 1. Structures of derivatives of E64c.

of E64c esters on calpain inhibitory activity. We synthesized a series of esters from alcohols with halogen substitutions (Figure 1, **1a–g**) or with increasing bulk (**1h** and **1i**). We also synthesized a series of amides prepared from amino acid esters and amides (**2a–c**).

We explored the idea of cell specificity with platelets. We reasoned that the very active 5-hydroxytryptamine (5HT) uptake mechanism of platelets[13,14] could be exploited specifically to concentrate an E64c derivative. We prepared derivatives of E64c with 5HT attached to the carboxyl group of E64c through either an ester or an amide linkage. Each compound was tested for activity as an inhibitor of calpain in platelet lysates, for ability to enter platelets, and for ability to inhibit calpain in intact platelets.

Chemistry

Synthesis of E64d by published procedures (with modifications) has been described.[15] E64c was generated from E64d by base-catalyzed hydrolysis in ethanol. A standard esterification procedure (Scheme I) was used to prepare esters **1a–i**. Compound **3** was treated with the appropriate alcohol in the presence of EDCI (1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide) and DMAP (4-(dimethylamino)pyridine).[16] Synthesis of amide derivatives **2a–c** from 5HT and amino acid esters was by a similar method with DMAP replaced with HOBT (1-hydroxybenzotriazole).

Synthesis of esters and amide derivatives of E64c with 5HT required other methods, since 5HT is unstable and the epoxy ring of E64c imposes further restrictions on reaction conditions. 5HT with either a Boc- or Cbz-protected amino group[17] (**4** or **5**) was esterified in the presence

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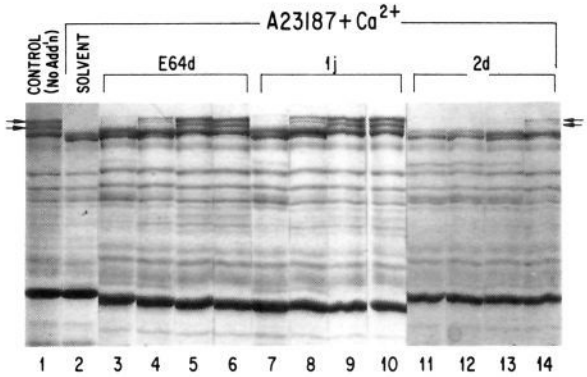

Figure 2. Effects of inhibitors of calpain activity in intact platelets. Measurement of intracellular calpain activity was based on the known calpain-catalyzed hydrolysis of two large platelet proteins as a consequence of the activation of platelets by a calcium ionophore plus calcium ion. A volume of 0.18 mL of washed platelets in Hank's buffer (6×10^8 platelets/mL) was incubated with the test compound at 30 °C for 15 min. 20 μL (25 mmol) of $CaCl_2$ and 2 μL (0.2 mmol) of A23187 in DMSO were added. After 10 min at room temperature, the reaction was stopped by addition of SDS-PAGE sample buffer containing 4% 2-mercaptoethanol and 50 μg/mL leupeptin. The samples were put immediately in a boiling water bath for 2 min. The samples were electrophoresed in SDS on polyacrylamide (8% acrylamide and 0.27% bisacrylamide) by the method of Laemmli.[23] At completion of the run, the gels were stained with 0.1% Coomassie blue R-250. Arrows indicate actin-binding protein (upper) and talin. These two proteins are present in the control (lane 1) but absent after addition of A23187 (lane 2). The test compounds were added (in DMSO) to a final concentration of (left to right) 0.8, 5.5, 44, or 440 μM. E64d or **1j** prevented the A23187-induced loss of these two proteins, but **2d** had only a slight effect at the highest concentration.

of BOP-Cl (*N,N'*-bis(2-oxo-3-oxazolidinyl)phosphinic chloride), DMAP, and TEA (triethylamine). A Boc-protected (**1j**) and a Cbz-protected (**1k**) 5HT ester were obtained.[18] Attempts to unblock were unsuccessful. Treatment of **1j** in 4 N HCl in dioxane resulted in complete hydrolysis of the ester link. Hydrogenolysis of **1k** with 10% Pd/C in *tert*-butyl alcohol led to little unblocking; in ethyl alcohol it led to transesterification to give 5HT and E64d. The 5HT amide of E64c was prepared from benzyloxy-5HT (**6**) with BOP-Cl as coupling agent to give **7**. A good yield was obtained when an excess of BOP-Cl was used with HOBT and either TEA or *N*-methylmorpholine.[19,20] **7** was unblocked by hydrogenolysis with 10% Pd/C in absolute ethanol to obtain **2d** in >80% yield after purification by TLC.

Biological Activity

E64c derivatives were tested with three different assays. **Assay 1**: The ability to inhibit calpain in a preparation of lysed platelets was measured with a caseinolytic assay.[21] The hydrolysis of [^{3}H]casein in this assay is due almost entirely to calpain, the major neutral protease in platelets, as evidenced by 95% inhibition by chelation of calcium ions.[21] **Assay 2**: Washed platelets were incubated with the test compound, the platelets were again washed to remove free drug in the medium, and the platelets were lysed. Inhibition of caseinolytic activity was a measure of the ability of the inhibitor to enter the platelet, where it was protected against washing. **Assay 3**: Washed platelets were preincubated with the test compound for 15 min before addition of the calcium ionophore A-23187 plus Ca^{2+}, a treatment known to activate endogenous calpain in platelets.[8,22] The platelet proteins were then analyzed by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE).[23] The activity of endogenous calpain caused the disappearance of actin-binding protein and talin (Figure 2), the major substrates for platelet calpain.[22] This assay has been used to demonstrate that E64d, but not E64c, inhibits intracellular calpain.[8]

Table I. Biological Tests of E64c Derivatives

	calpain inhibitory activity		
	$I_{50\%}$ (μM)[d]		
compd	assay 1[a]	assay 2[b]	assay 3[c]
E64c	0.04	–	–
E64d	4	4	+
1a	0.5	3	+
1b	2	0.3	+
1c	1	2	+
1d	+	6	+
1e	+	2	+
1f	0.7	1	+
1g	+	5	+
1h	+	2	+
1i	100		–
1j	2	2	+
2d	6	4	–

[a] A measure of the inhibition of calpain in lysed platelets. A (+) indicates that the compound was inhibitory, but $I_{50\%}$ was not determined. [b] A test of ability to enter the platelet to inhibit calpain after cell lysis. [c] A test of ability to enter the platelet and inhibit calpain in the intact cell. Data are summarized from Figure 2 and similar unpublished figures. A (+) indicates substantial inhibition by 5–44 μM; a (–) indicates little inhibition by 444 μM. [d] Estimated from a line drawn through the fractional inhibition at each of five concentrations of inhibitor.

In preliminary experiments, a high concentration of each ester (**1a–k**) was added to lysed platelets (assay 1); each inhibited calpain. Of the four amide derivatives, **2a–c** gave only partial inhibition, while **2d**, the 5HT amide, inhibited completely. **1a** through **1j** and **2d** were then tested with assay 1 or 2 (or both) and assay 3 with concentrations from 0.025 to 440 μM. The results are summarized in Table I. For assays 1 and 2, the concentration of inhibitor that gave 50% inhibition was estimated from a graph of activity vs inhibitor concentration. For any derivative that inhibits calpain, the critical assay is assay 2, which tests the ability of the inhibitor to enter platelets. Considering the normal variability among platelet preparations, it is striking that the results for assay 2 are so similar; all values are similar to that for E64d and within a range of 0.3 to 6 μM. Similarly, with the exception of **1i**, those derivatives where $I_{50\%}$ was measured were similar to the value for E64d, but 10–100-fold higher than the $I_{50\%}$ for the parent compound, E64c, as reported by others (reviewed in ref 12).

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For assay 3, each inhibitor indicated with a (+) in Table I gave substantial inhibition between 5.5 and 44 μM; those indicated with a (−) gave little inhibition at the highest concentration, 440 μM. The only surprising result is that **2d** inhibited only when calpain was assayed after cell lysis (in assays 1 and 2 but not in assay 3). There are two obvious explanations, both related to subcellular compartmentation. The 5HT derivative may be taken into dense granules, the storage site for 5HT in platelets,[14] where it would be inaccessible to calpain in the cytosol. Alternatively, if the amide derivatives inhibit only after hydrolysis to E64c, hydrolysis may be catalyzed by an enzyme accessible to the drug only after cell lysis.

To determine whether **1j** was actively concentrated in platelets, we tested the rate of entry of E64d and **1j** into platelets in plasma. Platelet-rich plasma was incubated with 5.5 μM of each drug at 30 °C for 1, 3, 5, 10 or 15 min. The platelets were diluted, and the cells were removed by centrifugation, washed once, and lysed. [^{3}H]Casein was added to determine the inhibition of calpain activity, a measure of the amount of inhibitor taken up during incubation. Calpain was inhibited 36% by E64d and 43% by **1j** after 1-min incubation; these values increased to only 41 and 47% after 15 min. That is, essentially maximum inhibition was obtained within 1 min, and there was no appreciable difference between the two compounds.

Discussion and Conclusions

Esters of E64c are 100 times less effective inhibitors in lysed cells than E64c itself (refs 6, 12, and Table 1, assay 1), leading to the suggestion that E64c is the active agent,[6] requiring that esters be hydrolyzed before they show inhibitory activity (reviewed in ref 12). The fact that we found no difference in inhibitory power of the esters through a range of electron-withdrawing groups (ease of hydrolysis) seems inconsistent with this suggestion. It is, indeed, remarkable that there is so little dependence on the nature of the ester group. Only ester **1i** had impaired activity; this ester is much more bulky and is chemically different; presumably one of these interfered with the interaction with calpain or possibly with an esterase to hydrolyze the ester bond. The 5HT ester (**1j**) was of interest as a possible targeting agent. E64d and **1j** were, however, equally effective calpain inhibitors and equally effective in entering the platelet.

To our knowledge, amide derivatives of E64c have not been tested previously, although there was a report of limited activity of amides of *trans*-epoxysuccinate (corresponds to the epoxy ring of E64c) against papain,[24] another thiol protease. We found that amides of E64c with amino acids in ester (**2b,c**) or amide (**2a**) linkage showed activity comparable to **1i**, an ester with a comparably large group.

We conclude that the activity of E64c esters is largely independent of the ester group unless the group is very large. 5HT ester and amide derivatives of E64c confer no selective advantage to cells with very active 5HT uptake mechanisms. It is possible that derivatives at some other position on 5HT might confer a selective advantage.

Experimental Section

Melting points were determined on a Fisher-Johns apparatus or a Kofler hotstage CE polarizing microscope; they are uncorrected. Proton NMR and ^{13}C NMR spectra were determined on a Varian XL300, XL200, or a Gemini 200 spectrometer operating at 200 MHz for ^{1}H and 75 MHz for ^{13}C, using $SiMe_4$ as the internal standard. Optical rotations were determined at 25 °C with a JASCO DIP 140 digital polarimeter. IR spectra were measured with a photoacoustic FT-IR 1010 Mattson instrument. Elemental analyses were performed by Spang Microanalytical Laboratory, Eagle Harbor, MI. Analytical TLC was performed on silica gel HL (Analtech Inc.) and UV-indicating silica gel HLD plates of 0.25-mm thickness (Universal Scientific Inc.). The development systems were A, $CHCl_3$/MeOH 9:1; B, EtOAc/hexane 9:1; and C, $CHCl_3$/MeOH 1:1. Preparative TLC was on 20 cm × 20 cm × 1 mm silica gel GF plates with $CHCl_3$/MeOH 95:5. Spots were detected with UV light or by spraying with 4% phosphomolybdic acid in 95% ethanol spray and heating. Flash column chromatography was with Merck silica gel (0.060–0.200 mm) eluted with $CHCl_3$/MeOH 9:1.

(+)-(2*S*,3*S*)-3-[[(*S*)-3-Methyl-1-[(3-Methylbutyl)carbamoyl]butyl]carbamoyl]-2-oxiranecarboxylic Acid (3). A solution of 85% KOH (0.34 g, 5 mmol) in absolute ethanol (5 mL) was added dropwise over 10 min to a stirred, ice-cooled solution of E64d (1.7 g, 4.9 mmol) in absolute ethanol (20 mL), and the reaction mixture was stirred at 0–5 °C for 4 h and at room temperature for 1 h. After evaporation of the solvent in vacuo at room temperature, the residue was dissolved in water (8 mL), and the aqueous solution was washed with EtOAc. The aqueous layer was acidified with 4 N HCl to pH 2 with ice-cooling and then extracted with EtOAc (10 mL × 3). The combined organic phase was washed with brine (10 mL × 3), dried over $MgSO_4$, and evaporated in vacuo to give 1.5 g of **3**, which could be used for the next step without further purification. The analytical sample was prepared by recrystallization from EtOAc/hexane (1:1): mp 158–159 °C (lit.[25] 158–159 °C); $[\alpha]^{25}_D$ = +51.8 (*c* = 1.00, EtOH); TLC R_f(C) = 0.66; ^{1}H-NMR (acetone-d_6) δ 0.85–0.91 (m, 12 H, 4 × CH_3), 1.30–1.39 (q, 2 H, $NHCH_2CH_2$), 1.54–1.69 (m, 4 H, 2 × CH, $CONHCHCH_2$), 3.15–3.32 (m, 2 H, $NHCH_2CH_2$), 3.54 (d, 1 H, *J* = 2 Hz, epoxy ring), 3.62 (d, 1 H, *J* = 2 Hz, epoxy ring), 4.40–4.49 (m, 1 H, NHC*H*), 7.47 (m, 1 H, CON*H*CH), 7.58 (d, 1 H, *J* = 9 Hz, CON*H*CH); ^{13}C-NMR (acetone-d_6) 22.07, 22.67 (2 × CH_3), 23.29, 25.39, 26.28, 38.09, 39.10, 42.36, 52.26 (epoxy ring), 52.63 (epoxy ring), 54.16, 166.33, 168.81, 172.09.

***N*-Boc-5HT (4).** 2-[[(*tert*-Butoxycarbonyl)oxy]imino]-2-phenylacetonitrile (90.3 mg, 0.36 mmol) was added to a solution containing 5HT-HCl (70.9 mg, 0.33 mmol) and TEA (0.14 mL, 1.0 mmol) in 2 mL of 50% aqueous dioxane. The reaction mixture was stirred at 45 °C for 1 h and evaporated to remove solvent. EtOAc (2 mL) was added to the residue and extracted with 5% NaOH in water. The alkaline aqueous solution was acidified with 10% citric acid and extracted with EtOAc (3 × 2 mL). The combined organic phase was dried over $MgSO_4$, filtered, and evaporated in vacuo to dryness to give a foam solid (89 mg, 97.8%, lit.[17] reported yellow oil): ^{1}H-NMR ($CDCl_3$) δ 1.40 (s, 9 H, CMe_3); 2.60–2.65 (t, 2 H, indole ethyl CH_2CH_2); 3.39–3.41 (t, 2 H indole ethyl CH_2CH_2); 4.71 (br, 1 H, N*H*Boc); 6.76 (d, 1 H, *J* = 10 Hz, indole H_6); 6.93 (d, 1 H, *J* = 2 Hz, indole H_4); 7.15 (s, 1 H, indole H_2); 7.26 (d, 1 H, *J* = 10 Hz, indole H_7); 8.09 (s, 1 H indole H_1).

***N*-Cbz-5HT (5).** To a solution of 5HT-HCl (213 mg, 1 mmol) in 2 mL of dry pyridine, benzyl chloroformate (256 mg, 1.5 mmol) in 1 mL of CH_2Cl_2 was added dropwise with ice-cooling, and the reaction mixture was stirred at 0 °C for 2 h. The residue was dissolved in EtOAc after evaporation and washed with 5% NaOH in water. The procedures described for 4 led to an oil (217 mg, 67.7%): ^{1}H-NMR ($CDCl_3$) δ 2.80 (t, 2 H, indole ethyl CH_2CH_2), 3.22 (t, 2 H, indole ethyl CH_2CH_2), 4.66 (br, 1 H, CbzN*H*), 5.09 (s, 2 H, $PhCH_2O$), 6.78–7.32 (m, 10 H, aromatic and indole ring H), 8.12 (s, 1 H, OH).

5-[3-[2-[*N*-(*tert*-Butyloxycarbonyl)amino]ethyl]-1*H*-indolyl] (+)-(2*S*,3*S*)-3-[[(*S*)-3-Methyl-1-[(3-methylbutyl)-carbamoyl]butyl]carbamoyl]-2-oxiranecarboxylate (1j). BOP-Cl (40.73 mg, 0.16 mmol) was added in one portion to a stirred solution of **3** (51 mg, 0.16 mmol), DMAP (6.1 mg, 0.05 mmol), and TEA (50 μL) in 1.5 mL of dry CH_2Cl_2 under nitrogen. The suspension was stirred at 0 °C for 20 min to form a clear

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Table II. Structural Data for Synthesized Derivatives

compd	yield (%)	mp (°C)	R_f[a]	$[\alpha]^{25}_D$[b]	^{1}H-NMR[c] (δ, ppm)		HRMS $[M + H]^+$
1a	75	152–153	0.73	+50.9	0.90–0.94	(m, 12 H, 4 × CH_3)	$C_{17}H_{29}FN_2O_5$
					1.35–1.44	(q, 2 H, $NHCH_2CH_2$)	calcd 361.213876
					1.50–1.71	(m, 4 H, 2 × CH, $CONHCHCH_2$)	found 361.219500
					3.17–3.31	(m, 2 H, $NHCH_2CH_2$)	
					3.55	(d, 1 H, J = 2 Hz, epoxy ring)	
					3.71	(d, 1 H, J = 2 Hz, epoxy ring)	
					4.32–4.39	(m, 1 H, NHC*H*)	
					4.44–4.75	(m, 4 H, FCH_2CH_2, J = 47 Hz, 28 Hz)	
					6.12	(br, 1 H, $CONHCH_2$)	
					6.77	(d, 1 H, CONHCH)	
1b	80	156–157	0.76	+50.6	0.90–0.94	(m, 12 H, 4 × CH_3)	$C_{17}H_{29}ClN_2O_5$
					1.35–1.43	(q, 2 H, $NHCH_2CH_2$)	calcd 377.18431
					1.52–1.68	(m, 4 H, 2 × CH, $CONHCHCH_2$)	found 377.18900
					3.21–3.34	(m, 2 H, $NHCH_2CH_2$)	
					3.53	(d, 1 H, J = 2 Hz, epoxy ring)	
					3.69	(d, 1 H, J = 2 Hz, epoxy ring)	
					4.33–4.52	(m, 5 H, NHC*H*, $ClCH_2CH_2$)	
					6.02	(br, 1 H, $CONHCH_2$)	
					6.66	(d, 1 H, J = 9 Hz, CONHCH)	
1c	89	159–160	0.65	+51.0	0.90–0.94	(m, 12 H, 4 × CH_3)	$C_{17}H_{29}BrN_2O_5$
					1.35–1.44	(q, 2 H, $NHCH_2CH_2$)	calcd 421.1338
					1.52–1.67	(m, 4 H, 2 × CH, $CONHCHCH_2$)	found 421.1384[d]
					3.19–3.34	(m, 2 H, $NHCH_2CH_2$)	
					3.51	(d, 1 H, J = 2 Hz, epoxy ring)	
					3.72	(d, 1 H, J = 2 Hz, epoxy ring)	
					4.33–4.38	(m, 1 H, NHC*H*)	
					4.42–4.58	(q, 4 H, $BrCH_2CH_2$)	
					5.98	(br, 1 H, $CONHCH_2$)	
					6.63	(d, 1 H, J = 7.5 Hz, CONHCH)	
1d	98	148–149	0.73	+51.2	0.90–0.94	(m, 12 H, 4 × CH_3)	$C_{17}H_{29}IN_2O_5$
					1.35–1.44	(q, 2 H, $NHCH_2CH_2$)	calcd 469.12052
					1.52–1.68	(m, 4 H, 2 × CH, $CONHCHCH_2$)	found 469.11670
					3.21–3.34	(m, 2 H, $NHCH_2CH_2$)	
					3.51	(d, 1 H, J = 2 Hz, epoxy ring)	
					3.72	(d, 1 H, J = 2 Hz, epoxy ring)	
					4.36–4.39	(m, 1 H, NHC*H*)	
					4.41–4.52	(m, 4 H, ICH_2CH_2)	
					6.03	(br, 1 H, $CONHCH_2$)	
					6.66	(d, 1 H, CONHCH)	
1e	90	170–171	0.75	+50.1	0.90–0.92	(m, 12 H, 4 × CH_3)	$C_{17}H_{27}F_3N_2O_5$
					1.35–1.43	(q, 2 H, $NHCH_2CH_2$)	calcd 397.195032
					1.52–1.65	(m, 4 H, 2 × CH, $CONHCHCH_2$)	found 397.200700
					3.26–3.47	(m, 2 H, $NHCH_2CH_2$)	
					3.59	(d, 1 H, J = 2 Hz, epoxy ring)	
					3.72	(d, 1 H, J = 2 Hz, epoxy ring)	
					4.35–4.38	(m, 1 H, NHC*H*)	
					4.50–4.63	(m, 2 H, CF_3CH_2)	
					5.90	(br, 1 H, CONHCH)	
					6.64	(m, 1 H, CONHCH)	
1f	91	115–116	0.71	+49.8	0.90–0.95	(m, 12 H, 4 × CH_3)	$C_{17}H_{27}Cl_3N_2O_5$
					1.35–1.46	(q, 2 H, $NHCH_2CH_2$)	calcd 445.10637
					1.52–1.69	(m, 4 H, 2 × CH, $CONHCHCH_2$)	found 445.10800
					3.25–3.28	(m, 2 H, $NHCH_2CH_2$)	
					3.63	(d, 1 H, J = 2 Hz, epoxy ring)	
					3.76	(d, 1 H, J = 2 Hz, epoxy ring)	
					4.34–4.40	(m, 1 H, NHC*H*)	
					4.66–4.76	(t, 2 H, CCl_3CH_2)	
					5.66	(br, 1 H, $CONHCH_2$)	
					6.58	(d, 1 H, J = 7.5 Hz, CONHCH)	
1g	93	60–61	0.78	+49.2	0.92–0.95	(m, 12 H, 4 × CH_3)	$C_{17}H_{27}Br_3N_2O_5$
					1.35–1.44	(q, 2 H, $NHCH_2CH_2$)	calcd 576.954831
					1.50–1.69	(m, 4 H, 2 × CH, $CONHCHCH_2$)	found 576.951300
					3.21–3.34	(m, 2 H, $NHCH_2CH_2$)	
					3.66	(d, 1 H, J = 2 Hz, epoxy ring)	
					3.81	(d, 1 H, J = 2 Hz, epoxy ring)	
					4.38–4.44	(m, 1 H, NHC*H*)	
					4.89–5.14	(q, 2 H, Br_3CCH_2)	
					6.02	(br, 1 H, $CONHCH_2$)	
					6.80	(br, 1 H, CONHCH)	
1h	96	117–118	0.80	+39.5	0.90–0.94	(m, 12 H, 4 × CH_3)	$C_{22}H_{31}ClN_2O_5$
					1.34–1.47	(q, 2 H, $NHCH_2CH_2$)	calcd 439.19996
					1.51–1.68	(m, 4 H, 2 × CH, $CONHCHCH_2$)	found 439.19800
					3.20–3.30	(m, 2 H, $NHCH_2CH_2$)	
					3.52	(d, 1 H, J = 2 Hz, epoxy ring)	
					3.71	(d, 1 H, J = 2 Hz, epoxy ring)	
					4.33–4.58	(m, 1 H, NHC*H*)	

Table II (Continued)

compd	yield (%)	mp (°C)	R_f[a]	$[\alpha]^{25}_D$[b]	^{1}H-NMR[c] (δ, ppm)		HRMS $[M + H]^+$
					5.26–5.24	(q, 2 H, $C_6H_4CH_2$)	
					5.97	(m, 1 H, $CONHCH_2$)	
					6.59	(d, 1 H, CONHCH)	
					7.26–7.43	(m, 4 H, aromatic H)	
1i	77	195–196	0.86	+5.9	0.89–0.93	(m, 21 H, 7 × CH_3)	$C_{35}H_{56}N_4O_8$
					1.36–1.48	(m, 2 H, $NHCH_2CH_2$)	calcd 661.417640
					1.51–1.74	(m, 13 H, 3 × CH, 3 × $CONHCHCH_2$, 2 × CH_2)	found 661.410900
					3.14–3.32	(m, 2 H, $NHCH_2CH_2$)	
					3.51	(s, 1 H, epoxy ring)	
					3.69	(s, 1 H, epoxy ring)	
					4.15–4.25	(m, 3 H, 3 × NHCH)	
					4.37–4.40	(q, 2 H, J = 7 Hz, $CHCH_2O$)	
					5.10	(s, 2 H, $PhCH_2$)	
					6.22	(br, 1 H, $CONHCH_2$)	
					6.44	(br, 3 H, 3 × CONHCH)	
					7.26–7.34	(s, 5 H, aromatic H)	
2a	78	161–162	0.45	+52	0.84–0.89	(m, 24 H, 8 × CH_3)	$C_{26}H_{48}N_4O_5$
					1.22–1.31	(q, 4 H, 2 × $NHCH_2CH_2$)	calcd 497.370296
					1.42–1.59	(m, 8 H, 4 × CH, 2 × $CONHCHCH_2$)	found 497.365400
					3.01–3.10	(m, 4 H, 2 × $NHCH_2CH_2$)	
					3.46	(s, 1 H, epoxy ring)	
					3.59	(s, 1 H, epoxy ring)	
					4.20–4.31	(m, 2 H, 2 × NHCH)	
					8.06	(m, 2 H, 2 × $CONHCH_2$)	
					8.55	(d, 2 H, J = 8 Hz, 2 × CONHCH)	
2b	90	139–140	0.80	+28.5	0.90–0.92	(m, 12 H, 4 × CH_3)	$C_{25}H_{37}N_3O_6$
					1.34–1.43	(q, 2 H, $NHCH_2CH_2$)	calcd 476.276061
					1.50–1.65	(m, 4 H, 2 × CH, $CONHCHCH_2$)	found 476.276110
					3.06–3.33	(m, 2 H, HCH_2CH_2)	
					3.43	(d, 1 H, J = 2 Hz, epoxy ring)	
					3.48	(d, 1 H, J = 2 Hz, epoxy ring)	
					3.76–3.81	(s, 3 H, CH_3O)	
					4.30–4.37	(q, 1 H, NHCH)	
					4.79–4.87	(q, 1 H, CHCOO)	
					5.91	(br, 1 H, $CONHCH_2$)	
					6.47	(m, 2 H, 2 × CONHCH)	
					7.04–7.07	(d, 2 H, $PhCH_2$)	
					7.35	(m, 5 H, aromatic H)	
2c	89	65–66	0.75	+36.2	0.79–0.91	(m, 18 H, 6 × CH_3)	$C_{22}H_{39}N_3O_6$
					1.36–1.43	(q, 2 H, $NHCH_2CH_2$)	calcd 442.291711
					1.51–1.67	(m, 7 H, 3 × CH, 2 × $CONHCHCH_2$)	found 442.287200
					3.12–3.32	(m, 2 H, $NHCH_2CH_2$)	
					3.59	(d, 2 H, epoxy ring)	
					3.73	(s, 3 H, CH_3O)	
					4.46–4.63	(m, 2 H, 2 × NHCH)	
					6.15	(br, 1 H, $CONHCH_2$)	
					7.10	(br, 1 H, CONHCH)	
					7.28	(br, 1 H, CONHCOO)	

[a] R_f (solvent A). [b] c = 1.00, EtOH. [c] NMR spectra were obtained with an AF superconducting magnet 250 MHz IBM/Brucker Fourier transform NMR spectrometer using tetramethylsilane as an internal standard and $CDCl_3$ as a solvent. [d] HRFABMS.

solution, and then **4** (45 mg, 0.16 mmol) in 1.5 mL of CH_2Cl_2 was added. The reaction mixture was stirred at room temperature for 24 h. The mixture was evaporated under reduced pressure to dryness. EtOAc (2 mL) was added to the residue and washed with water, saturated brine, 10% $NaHCO_3$, 5% HCl, and saturated brine successively. After drying over $MgSO_4$, it was concentrated in vacuo to give a foam solid, which was purified by preparative TLC to give 54 mg (58%) of **1j**: mp 79–80 °C; R_f(A) = 0.67; $[\alpha]^{25}_D$ = +28.5 (c = 1.00, EtOH); ^{1}H-NMR ($CDCl_3$) δ 0.89–0.96 (m, 12 H, 4 × CH_3), 1.39 (s, 9 H, CMe_3), 1.52–1.71 (m, 6 H, $NHCH_2CH_2$, 2 × CH, $CONHCHCH_2$), 2.86–2.91 (t, 2 H, indole ethyl CH_2CH_2), 3.24–3.30 (m, 2 H indole ethyl CH_2CH_2), 3.48 (d, 1 H, J = 2 Hz, epoxy ring), 3.68 (d, 1 H, J = 2 Hz, epoxy ring), 3.73–3.88 (m, 2 H, $NHCH_2CH_2$), 4.41–4.45 (m, 1 H, NHCH), 4.65 (br, 1 H, NHBoc), 6.10 (m, 2 H, 2 × CONH), 6.76 (d, 1 H, J = 10 Hz, indole H_6), 6.92 (d, 1 H, J = 2 Hz, indole H_4), 7.26 (d, 1 H, J = 10 Hz, indole H_7), 8.29 (s, 1 H, indole H_1); ^{13}C NMR ($CDCl_3$) 22.16, 22.36 (2 × CH_3), 22.41, 22.62, 24.66, 25.64, 26.40, 29.26, 38.04, 38.25, 41.26, 51.50, 52.95, 53.79, 54.03, 103.33, 110.55, 111.24, 112.00, 113.67, 115.44, 123.22, 127.72, 134.46, 143.46.149.64, 165.88, 165.99, 170.88; HRMS exact mass calcd for $C_{30}H_{44}N_4O_7$ $[M + Na]^+$ 595.31075; found 595.31000.

5-[3-[2-[*N*-(Benzyloxycarbonyl)amino]ethyl]-1*H*-indolyl](+)-(2*S*,3*S*)-3-[[(*S*)-3-Methyl-1-[(3-methylbutyl)carbamoyl]butyl]carbamoyl]-2-oxiranecarboxylate (1k). BOP-Cl (52 mg, 0.2 mmol), TEA (52 μL), **3** (34 mg, 0.1 mmol), DMAP (6.1 mg, 0.005 mmol), and **5** (30 mg, 0.09 mmol) were reacted by the same procedure described for **1j** to give 42 mg (69%) of **1k**: mp 67–68 °C; R_f(A) = 0.64; $[\alpha]^{25}_D$ = +24.6 (c = 1.00, EtOH); ^{1}H-NMR ($CDCl_3$) δ 0.89–0.96 (m, 12 H, 4 × CH_3), 1.35–1.44 (q, 2 H, $NHCH_2CH_2$), 1.53–1.69 (m, 4 H, 2 × CH, $CONHCHCH_2$), 2.88–2.93 (t, 2 H, indole ethyl CH_2CH_2), 3.22–3.29 (m, 2 H, indole ethyl CH_2CH_2), 3.47 (d, 1 H, J = 6 Hz, epoxy ring), 3.49 (d, 1 H, J = 6 Hz, epoxy ring), 3.67–3.87 (m, 2 H, $NHCH_2CH_2$), 4.37–4.43 (m, 1 H, NHCH), 4.66 (br, 1 H, CbzNH), 5.09 (s, 2 H, $PhCH_2$), 6.02 (br, 2 H, 2 × $CONHCH_2$), 6.78 (br, 1 H, CONHCH), 6.89–7.32 (m, 4 H indole ring H), 7.28–7.32 (m, 5 H, C_6H_5-aromatic H); HRMS exact mass calcd for $C_{33}H_{42}N_4O_7$ $[M + H]^+$ 607.31314; found 607.33000.

General Procedure for the Synthesis of 1a–i and 2a–c. EDCI (0.2 mmol) in 0.5 mL of dry CH_2Cl_2 was added dropwise to a stirred solution of **3** (0.15 mmol), a halogen-substituted alcohol, or Z-Leu-nLeu-OH (0.1 mmol), and DMAP (0.05 mmol) in 1.5 mL of dry CH_2Cl_2 at 0 °C over 2 min. The reaction mixture was stirred for 1 h at 0 °C followed by another 2 h at room temperature. The reaction mixture was washed with 5% HCl, saturated brine, 10% $NaHCO_3$, and saturated brine successively, dried over $MgSO_4$, and evaporated in vacuo to dryness. The

residue was purified by flash chromatography to give pure products **1a–i**. The same general procedure was followed in which an appropriate amine instead of alcohol was used and with HOBT instead of DMAP to give **2a–c**. Yield and structural data are listed in Table II.

***N*-[2-[5-(Benzyloxy)-1*H*-indol-3-yl]ethyl]-(+)-(2*S*,3*S*)-3-[[(*S*)-3-methyl-1-[(3-methylbutyl)carbamoyl]butyl]carbamoyl]-2-oxiranecarboxamide (7). Method A (Preactivation).** BOP-Cl (0.2 mmol) was added to a stirred solution of **3** (0.15 mmol, TEA (0.2 mmol), and HOBT (0.05 mmol) in CH_2Cl_2 under nitrogen at 0 °C. The mixture was stirred at 0 °C for 30 min, and then 0.1 mmol of **6** and an equivalent amount of TEA in CH_2Cl_2 were added. The mixture was stirred at 0 °C for 1 h and then at room temperature for 24 h. The mixture was evaporated in vacuo to dryness. EtOAc was added to the residue and washed with saturated $(NH_4)_2SO_4$, saturated $NaHCO_3$, 5% HCl, and saturated brine successively. After drying over $MgSO_4$, EtOAc was evaporated in vacuo to give a foam solid, which was purified by preparative TLC to give pure **7** (44.8 mg, 80 %). The analytical sample was recrystallized from $CHCl_3$/hexane (1:2) to yield a white crystalline solid: mp 134–136 °C; $[\alpha]^{25}_D$ = +2.7 (*c* = 2.38, $CHCl_3$); IR (crystal) 900, 1540, 1680, 2940, 3080, 3250 cm^{-1}; TLC R_f(A) = 0.68, R_f(B) = 0.42; ^{1}H-NMR ($CDCl_3$) δ 0.86–0.92 (m, 12 H, 4 × CH_3), 1.30–1.41 (q, 2 H, $NHCH_2CH_2$), 1.51–1.60 (m, 4 H, 2 × CH, $CONHCHCH_2$), 2.85–2.91 (t, 2 H, indole ethyl CH_2CH_2), 3.11–3.25 (m, 2 H, indole ethyl CH_2CH_2), 3.07–3.21 (m, 2 H, $NHCH_2CH_2$), 3.27 (d, 1 H, *J* = 2 Hz, epoxy ring), 3.46 (d, 1 H, *J* = 2 Hz, epoxy ring), 4.35–4.50 (m, 1 H, NHC*H*), 5.08 (s, 2 H, $PhCH_2$), 6.39–6.51 (m, 3 H, 3 × CO*N*H), 6.49–7.15 (m, 4 H, indole ring), 7.26–7.47 (m, 5 H, aromatic H), 8.43 (s, 1 H, indole H_1); HR-FABMS exact mass calcd for $C_{32}H_{42}N_4O_5$ 562.3145, found 562.3134. Anal. Calcd C, 68.33 (found 67.88); H, 7.47 (found 7.62); N, 9.96 (found 9.82). **Method B (One-Pot Procedure).** A solution of **3** (0.15 mmol), HOBT (0.05 mmol), and **6** (0.1 mmol) in dry THF was cooled in an ice bath with stirring under nitrogen. The cooled mixture was treated with *N*-methylmorpholine (0.45 mmol) followed, in one portion, by BOP-Cl (0.2 mmol). The mixture was stirred at 0 °C for 1 h and then at room temperature for 24 h. The same procedure as described above is followed to give **7** (49.4 mg, 88%), identical (NMR, TLC, mp) with that prepared by method A above. **Method C (DCC Coupling).** A solution of DCC (154.7 mg, 0.75 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise over 20 min to a stirred, ice-cooled solution of **3** (160 mg, 0.5 mmol), HOBT (101 mg, 0.75 mmol), TEA (0.7 mL), and **6** (154 mg, 0.5 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred at 0 °C for 1 h and then at room temperature for 48 h. The insoluble dicyclohexylurea was removed by filtration. The filtrate was concentrated in vacuo to dryness. The same procedure as described above was followed to give **7** (37.2 mg, 65%) identical (NMR, TLC, mp) with that prepared by method A above.

***N*-[2-(5-Hydroxy-1*H*-indol-3-yl)ethyl]-(+)-(2*S*,3*S*)-3-[[(*S*)-3-methyl-1-[(3-methylbutyl)carbamoyl]butyl]carbamoyl]-2-oxiranecarboxamide (2d).** A solution of **7** (100 mg, 0.18 mmol) in 5 mL of absolute EtOH containing 50 mg of 10% Pd/C was stirred under hydrogen for 4 h. The catalyst was removed by filtration, and then the solution was concentrated in vacuo to yield a foam solid, which was purified by preparative TLC to give 80 mg (96%) of **2d**. The analytical sample was recrystallized from acetone/hexane (1:1): mp 118–120 °C; $[\alpha]^{25}_D$ = +23.2 (*c* = 2.63, acetone); IR (crystal) 900, 1540, 1690, 2940, 3090, 3290 cm^{-1}; TLC R_f(A) = 0.58; ^{1}H-NMR (acetone-d_6) δ 0.90–0.96 (m, 12 H, 4 × CH_3), 1.36–1.46 (q, 2 H, *J* = 7 Hz, $NHCH_2CH_2$), 1.59–1.72 (m, 4 H, 2 × CH, $CONHCHCH_2$), 2.88–2.95 (t, 2 H, indole ethyl CH_2CH_2), 3.20–3.32 (m, 2 H, indole ethyl CH_2CH_2), 3.49–3.56 (q, 2 H, *J* = 7 Hz, $NHCH_2CH_2$), 3.61 (d, 1 H, *J* = 2 Hz, epoxy ring), 3.64 (d, 1 H, *J* = 2 Hz, epoxy ring), 4.48 (q, 1 H, *J* = 7 Hz, HNC*H*), 6.73 (q, 1 H, *J* = 2 Hz, 9 Hz, indole H_6), 7.02 (d, 1 H, *J* = 2 Hz, indole H_4), 7.11 (s, 1 H, indole H_2), 7.21 (d, 1 H, *J* = 9 Hz, indole H_7), 7.55–7.68 (m, 3 H, 3 × CONH), 7.82 (s, 1 H, indole H_1), 9.82 (br 1 H, OH); HR-FABMS exact mass calcd for $C_{25}H_{36}N_4O_5$ $[M + H]^+$ 473.2755, found 473.2764. Anal. calcd: C, 63.56 (found 62.89); H, 7.63 (found 8.11); N, 11.86 (found 11.28).

Preparation of Platelets. Blood was obtained by venipuncture of adult volunteers with informed consent. The blood was mixed immediately with one-seventh volume of anticoagulant (2.5% sodium citrate, 1.36% citric acid, and 2% D-(+)-glucose). Platelet-rich plasma was prepared by centrifugation of the citrated blood at 1200*g* for 20 min to remove red and white cells. Washed platelet suspensions were prepared as previously described.[8]

Measurement of Calpain Activity. [^{3}H]Casein was prepared by modification of a method[26] for the preparation of [^{14}C]casein. 10 mL (120 mg) of casein in 50 mM sodium borate, pH 9.0, was dialyzed and centrifuged to remove contaminants. To the cold solution was added 2 μL of 37% formaldehyde (dissolved in 600 μL buffer) with stirring for 1 min. 20 μL of labeled $NaBH_4$ (0.25 mCi/μL in 0.01 N NaOH) was added in two increments separated by 15 s. At 8 additional 15-s intervals, 25 μL of unlabeled $NaBH_4$ (5 mg/mL) was added. The product was extensively dialyzed prior to use. **Assay 1.** A volume of 0.3 mL of washed platelets (6 × 10^8 platelets/mL) in Hank's solution was incubated with inhibitor at 30 °C for 3 min before addition of 7.5 μL (200 mmol) $CaCl_2$, 8 μL 3.3% Triton X-100 and 20 μL [^{3}H]casein. After incubation for 20 min at 30 °C, the reaction was stopped by the addition of 0.1 mL of 20% trichloroacetic acid (TCA). The sample was centrifuged at 10 000*g* for 5 min to remove precipitated protein, and 0.1 mL of the supernatant solution was taken for measurement of TCA-soluble radioactivity with a liquid scintillation counter. Controls included samples without platelets as a measure of TCA-soluble radioactivity in the casein preparation, samples with [^{3}H]casein alone as a measure of total radioactivity, and samples without inhibitor as an indication of maximum calpain activity. **Assay 2.** Washed platelets (7 × 10^8 platelets/mL) in Hank's solution were incubated with inhibitor at 30 °C for 15 min. The platelet suspension was diluted with 3 volumes of Tyrode-HEPES buffer, pH 7.4, and centrifuged at 3000*g* for 5 min. The resulting platelet pellet was resuspended in Tyrode-HEPES buffer and centrifuged again. The pellet was resuspended in Hank's solution and equilibrated at 30 °C for 3 min. $CaCl_2$, Triton X-100, and [^{3}H]casein were added as in assay 1. Incubation was at 30 °C for 20 min, after which TCA was used to precipitate proteins as for assay 1. The same controls were used as for assay 1. **Assay 3.** See Figure 2 for experimental conditions and results.

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