Synthesis and Biological Activity of a Series of Potent Fluoromethyl Ketone Inhibitors of Recombinant Human Calpain I

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Received March 24, 1997[⊗]

Calpain I, an intracellular cysteine protease, has been implicated in the neurodegeneration following an episode of stroke. In this paper, we report on a series of potent dipeptide fluoromethyl ketone inhibitors of recombinant human calpain I (rh calpain I). SAR studies revealed that while calpain I tolerates a variety of hydrophobic groups at the P_1 site, Leu at P_2 is preferred. However, the nature of the N-terminal capping group has a significant effect on the inhibitory activity of this series of compounds. Compound **4e** [(1,2,3,4-tetrahydroisoquinolin-2-yl)carbonyl-Leu-D,L-Phe-CH_2F], having a tetrahydroisoquinoline containing urea as the N-terminal capping group, is the most potent *dipeptide* fluoromethyl ketone inhibitor of calpain I (with a second-order rate constant for inactivation of 276 000 M^{-1} s^{-1}) yet reported; tripeptide **4k** (Cbz-Leu-Leu-D,L-Phe-CH_2F) is equipotent. A number of compounds presented in this study displayed excellent selectivity for calpain I over cathepsins B and L, two related cysteine proteases. Compounds which exhibited good inhibitory activity in the assay against isolated rh calpain I also inhibited intracellular calpain I in a human cell line. Thus, in an intact cell assay, compounds **4e** and **4k** inhibited calpain I with IC_{50} values of 0.2 and 0.1 μM, respectively. Finally, we also disclose the first example of fluorination of a dipeptide enol silyl ether to generate the corresponding dipeptide fluoromethyl ketone.

Introduction

Calpains (I and II) are calcium-activated neutral proteases belonging to a family of intracellular cytoplasmic cysteine proteases. Calpain I, thought to be the predominant form activated during the pathological conditions of nervous tissue, has been implicated in several nervous system disorders including stroke, Alzheimer's disease, and epilepsy. We are interested in selectively inhibiting calpain I to find new therapeutics[1] to treat stroke, one of the leading causes of mortality in the western hemisphere. In the US alone, more than half a million strokes occur each year, killing more than one-third of its victims; remaining patients survive, but only a fortunate third of these are left with little or no physical or mental impairment.[2] The majority of all strokes occur when a blood vessel blocked by a clot (or an air bubble) originating from the heart or an atherosclerotic arterial plaque cuts off blood flow to a region of the brain and induces localized anemia, known as ischemia.[2,3] An episode of stroke initiates a chain of biochemical events resulting in the rise of intracellular Ca^{2+} concentration, which in turn activates calpain. Activated calpain degrades neuronal structural proteins, resulting in neurodegeneration.

Potent peptide-based reversible[4] and irreversible[5] inhibitors of calpain I have been reported. Irreversible inhibitors permanently inactivate a pathophysiological enzyme; thus, for maximum benefit, they could potentially be superior to reversible inhibitors which inactivate in a transient manner. Additionally, the ability of an irreversible inhibitor to label altered cellular components gives the researcher an opportunity to examine the system after exposure to the inhibitor to see whether or not the postulated site of action was, in fact, covalently labeled, an essential requirement for understanding its mechanism of action.[6] Recently we disclosed a dipeptide fluoromethyl ketone, having a tetrahydroisoquinoline-containing urea as the N-terminal capping group as the most potent dipeptide fluoromethyl ketone irreversible inactivator of calpain I yet described.[7,8] We now present the full account of our work describing the synthesis and the *in vitro* calpain I inhibitory activity of a series of potent dipeptide fluoromethyl ketones. We also present their inhibitory activity against cathepsin B and cathepsin L, two other related cysteine proteases, sensitive to inhibition by fluoromethyl ketones. We demonstrate that the compounds which display good activity in the assay for calpain I inhibition also inhibit intracellular calpain I.

Chemistry

The syntheses of target compounds **4a**−**h** are depicted in Scheme 1. Acylated or sulfonylated leucine (**1A**−**E**) was coupled with amino fluoro alcohols **2A**−**C** to generate the dipeptide fluoro hydroxy compounds **3a**−**g**. Dess−Martin oxidation of **3a**−**g** generated fluoromethyl ketones **4a**−**g**. Deprotection of the THP group in **4g** generated **4h**. Compounds **1A**,**B** are commercially available. Compound **1C** was synthesized by coupling leucine with morpholinosulfonyl chloride.[9] Compound **1D** was obtained by reaction of benzyl isocyanate with leucine *tert*-butyl ester hydrochloride salt, followed by acidic hydrolysis. Compound **1E** was synthesized by coupling 1,2,3,4-tetrahydroisoquinoline, leucine methyl ester hydrochloride salt and triphosgene[10] followed by basic hydrolysis. Compounds **2A**−**C** were prepared according to Scheme 2. Commercially available *trans*-

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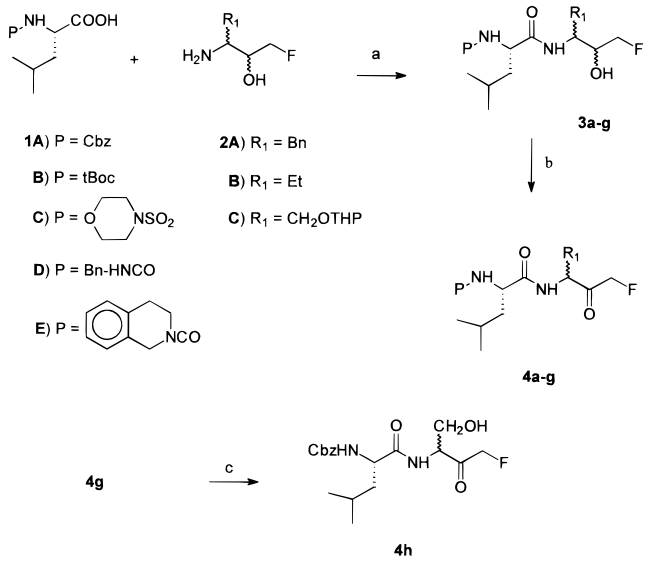
[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1997.

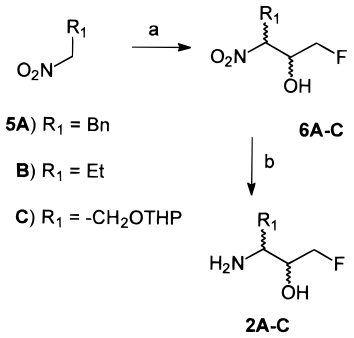
S0022-2623(97)00197-0 CCC: $14.00

Scheme 1[a]



[a] Reagents: (a) general method A (IBCF, NMM, CH_2Cl_2 or THF, DMF, −20 to 23 °C) or general method B (BOP, HOBt, NMM, DMF 0−23 °C); (b) Dess−Martin periodinane, CH_2Cl_2, 23 °C; (c) *p*-toluenesulfonic acid monohydrate, CH_3OH, 23 °C.

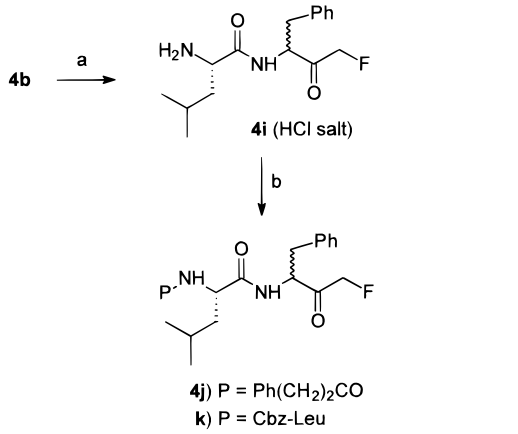
Scheme 2[a]



[a] Reagents: (a) *in situ* generated FCH_2CHO (see text); (b) H_2 (60 psi), absolute ethanol, Raney nickel (50% slurry in water, catalytic amount).

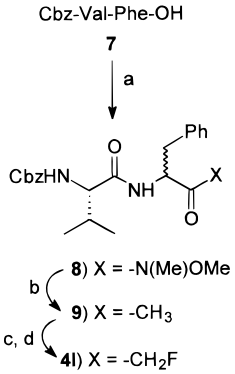
β-nitrostyrene was reduced to compound **5A**, which underwent modified Henry nitro aldol condensation with *in situ* generated fluoroacetaldehyde[11,12] to generate compound **6A**. Hydrogenation of **6A** yielded **2A**. It should be noted that although we were able to separate the erythro/threo isomers of **6A** and convert them to erythro/threo isomers of **2A** (see the Experimental Section), it was of no great advantage. Either isomer of **2A** was a mixture of inseparable enantiomers, as there was no element of stereocontrol in the previous nitroaldol step to generate **6A**. Thus, reaction of either the erythro or threo isomer of **2A** to a P_2 fragment and eventual oxidation of the carbinol center would have produced the same 1:1 mixture at the remaining epimeric center, i.e. at P_1. Thus, isomers of **2A** were pooled. Similarly, 1-nitropropane (**5B**) was converted to **2B** via **6B**. Finally **5C** was transformed into **2C** via **6C**. Syntheses of compounds **4i**−**k** are shown in Scheme 3. Hydrolysis of the *t*-Boc group of **4b** generated **4i** (HCl salt), which was coupled with hydrocinnamic acid and Cbz-Leu to generate **4j** and **4k**, respectively. During this investigation, we also generated a dipeptide fluoromethyl ketone by fluorinating the corresponding dipeptide methyl ketone (Scheme 4). Thus Cbz-Val-Phe-OH (**7**) was converted to the corresponding methyl

Scheme 3[a]



[a] Reagents: (a) HCl(g), ether; (b) BOP, HOBt, NMM, DMF, 0−23 °C.

Scheme 4[a]

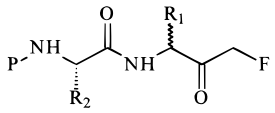


[a] Reagents: (a) *N*,*O*-dimethylhydroxylamine hydrochloride, Et_3N, BOP, CH_2Cl_2, 23 °C; (b) 3 M MeMgBr, THF, 0−23 °C; (c) TBDMSOTf, Et_3N, CH_2Cl_2, 0 °C; (d) F-TEDA-BF_4, DMF, 23 °C.

ketone **9** via Weinreb amide **8**; however during the amide formation, the P_1 site epimerized. Compound **9** was converted to the corresponding enol silyl ether which on treatment with 1-(chloromethyl)-4-fluoro-1,4-diazabicyclo[2.2.2]octane bis(tetrafluoroborate) (F-TEDA·BF_4, Selectflur reagent)[13] generated compound **41**. To our knowledge, this is the first example of generation of a dipeptide fluoromethyl ketone (albeit in low isolated yield) by fluorination of a peptidic enol silyl ether. Compounds **4a**−**l** are a diastereomeric mixture, epimeric at P_1; compound **4g** has an additional chiral center (mixture of isomers) at the OTHP site. Compound **4m** is commercially available. Compound **4n** is E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane), a reference compound. Table 1 lists compounds **4a**−**m**.

Calpain I Inhibition and Discussion. The biological activities of the compounds were determined using rh calpain I, prepared as described by Meyer et al.[14] with Suc-Leu-Tyr-MNA as the substrate. Table 2 displays the inhibitory data for compounds **4a**−**n**. As shown, P_1-Phe is preferred over Abu and Ser (cf. **4a** vs **4f** and **4h**), respectively. Interestingly, protection of the hydroxyl group of the P_1-Ser residue as a tetrahydropyran (THP) ether moiety generates an approximately 5 times more potent compound (cf. **4g** vs **4h**). Thus, it appears that the hydroxyl group of the P_1-Ser residue in **4h** is involved in a destabilizing interaction with the S_1 site of the enzyme. This might also explain why the inactivation rate of 17 000 M^{-1} s^{-1} determined by Shaw

Table 1. List of Compounds **4a**−**m**[a]



compd	P	R_2	R_1	RP HPLC t_R (min)[b]	formula	anal.
4a	Cbz	iBu	Bn	27.64	$C_{24}H_{29}FN_2O_4$	C, H, N
4b	*t*-Boc	iBu	Bn	26.87	$C_{21}H_{31}FN_2O_4$	C, H, N
4c	morpholino-4-sulfonyl	iBu	Bn	22.39	$C_{20}H_{30}FN_3O_5S$	*c*
4d	(benzylamino)carbonyl	iBu	Bn	24.30	$C_{24}H_{30}FN_3O_3$	C, H, N
4e	(1,2,3,4-tetrahydroisoquinolin-2-yl)carbonyl	iBu	Bn	26.56	$C_{26}H_{32}FN_3O_3$	C, H, N
4f	Cbz	iBu	Et	24.16	$C_{19}H_{27}FN_2O_4$	C, H, N
4g	Cbz	iBu	CH_2OTHP	31.95	$C_{23}H_{33}FN_2O_6$	*d*
4h	Cbz	iBu	CH_2OH	21.29	$C_{18}H_{25}FN_2O_5$	*e*
4i	H (HCl salt)	iBu	Bn	13.80	$C_{16}H_{23}FN_2O_2 \cdot HCl$	C, H
4j	$Ph(CH_2)_2CO$	iBu	Bn	25.91	$C_{25}H_{31}FN_2O_3$	C, H, N
4k	Cbz-Leu	iBu	Bn	29.08	$C_{30}H_{40}FN_3O_5$	C, H, N
4l	Cbz	iPr	Bn	30.80	$C_{23}H_{27}FN_2O_4$	C, H, N
4m	Cbz	Bn	Me	*f*		

[a] Compounds **4a**−**l** are diastereomeric mixture, epimeric at P_1; compound **4g** has an additional chiral center (mixture of isomers) at OTHP site. [b] HPLC conditions used: solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA; detection, 215 nm; 10−100% B in 40 min; column, Zorbax Rx C8; flow, 1.2 mL/min. [c] High-resolution mass spectroscopy: calc 444.1968 (MH^+), found 444.1973 (MH^+). [d] High-resolution mass spectroscopy: calc 453.2401 (MH^+), found 453.2382 (MH^+). [e] High-resolution mass spectroscopy: calc 369.1826 (MH^+), found 369.1837 (MH^+). [f] Commercial sample.

Table 2. Inhibitory Activities of Compounds **4a**−**n**

	k (M^{-1} s^{-1})			
compd	calpain I[a]	cathepsin B[a]	cathepsin L[a]	intact cell assay[b] IC_{50} (μM)
4a	136300	300	5000	0.2
4b	68600	150	100	42% at 10 μM
4c	67200	100	3200	10
4d	67000	275	9400	0.8
4e	276000	7500	48400[c]	0.2
4f	24250	24500	64000	
4g	100000	9000	22600	1.3
4h	21000	16300	31000	
4i	8000	25	250	
4j	26600	600	800	
4k	290000	4600	256000	0.1
4l	35000	360	21500	
4m	*d*	21000	110000	
4n (E-64)	4700			

[a] $n \geq 3$ in all cases. [b] $n \geq 2$ in all cases. [c] A value of 72 000 was reported in our preliminary communication.[7] [d] No significant inhibition was observed up to 1 μM.

et al.[7] for Cbz-Leu-D,L-Tyr-CH_2F against chicken gizzard calpain II is significantly lower than that of 136 300 M^{-1} s^{-1} for Cbz-Leu-D,L-Phe-CH_2F (**4a**), but comparable to that of 21 000 M^{-1} s^{-1} for Cbz-Leu-D,L-Ser-CH_2F (**4h**).[15] Thus, calpain I prefers a hydrophobic group at the P_1-site of this class of molecules. Previous studies indicate that calpain prefers Leu or Val at P_2;[4,5] however, in this series, compound **4a** with P_2-Leu is 4 times more potent than compound **4l** with P_2-Val. It should be noted that the commercial sample **4m** with P_2-Phe did not show any significant inhibition of rh calpain I up to 1 μM, revealing the importance of Leu or Val at P_2 of a potent calpain inhibitor.

Interestingly, the nature of the N-terminal capping group plays a significant role in the potency of this series of compounds. While Cbz, *t*-Boc, morpholinosulfonyl, and (benzylamino)carbonyl are all well tolerated, Cbz is preferred (**4a** vs **4b**−**d**). The lack of any capping group, as in the free amine **4i** (as the hydrochloride salt), results in weak activity, indicating the importance of the presence of an N-terminal capping group. Replacement of the OCH_2 moiety in the Cbz capping group of compound **4a** with the CH_2CH_2 moiety also generates a less active analog (**4j**), revealing that an additional heteroatom in the region might be involved in an energetically beneficial binding. Interestingly, constraining the benzylurea motif of **4d** as part of a tetrahydroisoquinolyl moiety generated the most potent *dipeptide* fluoromethyl ketone inhibitor (**4e**, $k = 276\,000$ M^{-1} s^{-1}) of calpain I yet reported; thus, it appears that although the enzyme tolerates an heteroatom (O, N) in the region spanning P_3, it disfavors an NH moiety from the benzylurea motif of **4d**. However, note that the tripeptide inhibitor Cbz-Leu-Leu-D,L-Phe-CH_2F (**4k**, $k = 290\,000$ M^{-1} s^{-1}) is equipotent to the dipeptide inhibitor **4e**. The reference compound **4n** (E-64) displays an inactivation rate of 4700 M^{-1} s^{-1} in this assay, which is comparable to the inactivation rate reported in the literature using chicken gizzard calpain II (Pliura et al.[16] 3700 M^{-1} s^{-1} and Parkes et al.[17] 7500 M^{-1} s^{-1}), demonstrating the validity of our assay method and the similarity between rh calpain I and chicken gizzard calpain II.

Mechanism of Inhibition. Inactivation of an enzyme by an inhibitor which involves initial complex formation followed by covalent modification of the enzyme typically displays time-dependent inactivation kinetics. Inactivation of rh calpain I by the above series of fluoromethyl ketones is time-dependent (data not shown) and concentration-dependent. Replots of k_{obs} versus inhibitor concentration are linear (Figure 1), allowing calculation of rates of inactivation. It should be noted that no recovery of calpain activity is observed when enzyme inactivated with **4a** is diluted into an assay mixture or when it is subjected to repeated cycles of dilution and ultrafiltration, indicating the irreversible nature of the inhibition of rh calpain I by **4a**.

Furthermore, covalent binding of **4a** to rh calpain I was directly assessed using radiolabeled compound [$^{14}CH_2$]Cbz-Leu-D,L-Phe-CH_2F (**4a***). It is generally accepted that in the presence of calcium ion, the 80 kDa subunit of calpain I undergoes autolysis to a 78 kDa and subsequently to a 76 kDa form which represents the active enzyme species.[18] In order to determine to which M_r form **4a** binds, rh calpain I and **4a*** were incubated under conditions in which calpain would

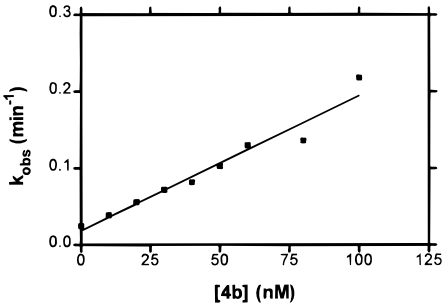


Figure 1. Recombinant human calpain I was incubated with substrate and varying concentrations of inhibitor (0−100 nM). Progress curves were obtained and values of k_{obs} were calculated for each inhibitor concentration as described in the Experimental Section. Replot of k_{obs} versus inhibitor concentration was linear and was used to calculate the second-order rate constant for inactivation given in Table 2.

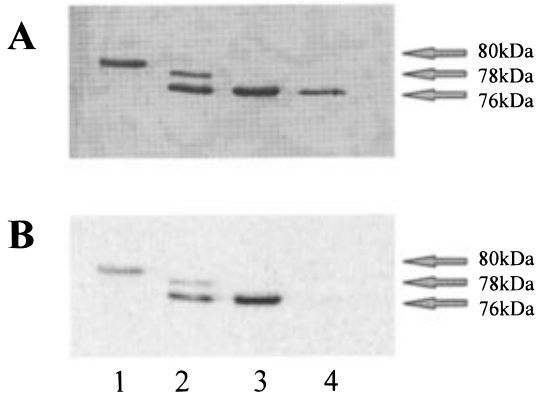


Figure 2. (A) Immunoblot of rh calpain I. (B) Phosphoimage of rh calpain I. Lane 1: rh calpain I incubated for 5 min with a 10-fold molar excess of **4a*** in the absence of calcium ion. Lane 2: rh calpain I incubated for 5 min with a 10-fold molar excess of **4a*** in the presence of calcium ion. Lane 3: rh calpain I incubated with calcium ion for 5 min, followed by incubation with a 10-fold molar excess of **4a*** for an additional 5 min. Lane 4: rh calpain I incubated with calcium ion for 5 min, followed by incubation with a 10-fold and 100-fold molar excess of **4a*** and Cbz-Val-Phe-H, respectively, for an additional 5 min.

remain unautolyzed, be partially autolyzed, or be fully autolyzed. Following incubation in the presence of calcium ion, 0.8−0.9 mol of **4a*** was incorporated per mole of calpain I. Minimal (0.01 mol of **4a***/mol of calpain I) incorporation was detected when calpain I was incubated in the absence of calcium ion. In a similar way, minimal (0.03 mol of **4a***/mol of calpain I) incorporation was also observed when calpain I was incubated in the presence of calcium ion and saturating concentrations of Cbz-Val-Phe-H (MDL 28170),[19] a known active site-directed inhibitor. Thus, the data are consistent with near-stoichiometric binding of **4a*** to the active site of calpain I.

Figure 2 illustrates the M_r forms of rh calpain I to which **4a*** covalently bound. Predominant radiolabeling of the 76 kDa species was clearly evident. A relatively minor amount of radiolabel was observed in both the 80 and 78 kDa species. It is not known whether this labeling represents inhibitor specifically bound to the active site of the enzyme. Interestingly, the relative percent incorporation (based upon the total quantity of protein) of radiolabel into the 78 kDa species represented a significant fraction of the total labeling. Incorporation into the 80 kDa species remained minor. Thus, **4a*** gained access to the active site of calpain I not only in the 76 kDa species but in the 78 kDa species as well.

Selectivity. Cathepsins B and L are two related cysteine proteases which are sensitive to inhibition by dipeptide fluoromethyl ketones.[6,8b] Thus, we examined the inhibitory activity of the compounds against isolated cathepsins B and L, using the fluorogenic substrate Cbz-Phe-Arg-AMC. Table 2 includes the inhibitory data for the compounds against cathepsins B and L.

Cathepsin B. A number of fluoromethyl ketones presented in this study displayed excellent selectivity for rh calpain I over cathepsin B. Compound **4a** with P_1-Phe is >450 times selective for calpain I over cathepsin B; however, compound **4f** with P_1-Abu is equipotent both for calpain I and cathepsin B. Interestingly, such an effect is less profound between **4g** with P_1-Ser(THP) and **4h** with P_1-Ser; while the former inhibits calpain I 11 times faster than cathepsin B, the activity of the latter compound is comparable against both of the enzymes. The nature of the N-terminal capping group again plays a significant role in the compound selectivity. While compounds **4a**,**b** are >450 times more selective for calpain I over cathepsin B, compounds **4c**,**d** are >670 times and >240 times selective for calpain I over cathepsin B, respectively. Compounds **4e** and **4k**, the two most potent compounds against isolated rh calpain I, are moderately selective (37 times and 63 times, respectively) over cathepsin B.

Cathepsin L. The selectivity of the compounds for rh calpain I over cathepsin L is modest. Compound **4a** with P_1-Phe is >27 times selective for calpain I over cathepsin L; however, note that compound **4f** with P_1-Abu prefers (>2.5 times) cathepsin L over calpain I. Among various N-capping groups, *t*-Boc-containing **4b** prefers calpain I over cathepsin L by >680 times. Between compounds **4e** and **4k**, the two most potent compounds in the calpain I assay, the former prefers calpain I by ca. 6 times over cathepsin L, while the latter is equipotent in both assays.

Cellular Activity. In order to probe the ability of these compounds to penetrate cells and inhibit intracellular calpain I, we tested a set of compounds in an intact cell assay system. Treatment of Molt 4 cells (human leukemic T cells) with calcium ion and an ionophore results in the elevation of intracellular calcium which, in turn, activates calpain I. This is followed by calpain I-mediated cleavage of cytoskeletal proteins, including spectrin. Inhibition of formation of spectrin breakdown products (SBDPs) inside the cell by a compound measures its efficacy. Table 2 lists the inhibitory activities of a set of compounds which showed good activity (k > 60 000 M^{-1} s^{-1}) in the enzyme assay. While compounds **4a**,**d**−**e**,**g**,**k** also displayed good activity in this assay, compounds **4b**,**c** were less potent. Compounds **4e** and **4k** inhibited calpain I with IC_{50} values of 0.2 and 0.1 μM, respectively. Thus, the compounds are cell-permeable and inhibit intracellular calpain I.

Conclusion

In this paper, we described an account of our work on a series of potent and selective fluoromethyl ketone inhibitors of recombinant human calpain I. While compound **4e** emerged as the most potent (k = 276 000 M^{-1} s^{-1}) *dipeptide* fluoromethyl ketone inhibitor of

calpain I yet reported, compound **4c** displayed excellent selectivity (>670 times) for calpain I over cathepsin B. Similarly, compound **4b** preferred calpain I over cathepsin L by >680 times. Compounds which exhibited good inhibitory activity in the enzyme assay also inhibited intracellular calpain I. Work is now continuing to determine the behavior of this class of molecules in several animal models of stroke and will be reported in due course.

Experimental Section

Chemistry. General Methods. Thin layer chromatography was done on silica gel plates (MK6F 60A, size 1 × 3 in., layer thickness 250 μm, Whatman Inc.). Preparative chromatography was carried out using Merck silica gel, 40−63 μm, 230−400 mesh. ^{1}H NMR spectra were recorded on a GE QE Plus instrument (300 MHz) using tetramethylsilane as internal standard. Electrospray mass spectra were recorded on a VG platform II instrument (Fisons Instruments). Elemental analyses were performed by Quantitative Technologies Inc. of Whitehouse, NJ. High-resolution mass spectroscopy was performed by M-Scan Inc. of West Chester, PA. Compounds **1A**,**B** were obtained from Advanced ChemTech, Louisville, KY, and compound **4m** was available from Enzyme Systems Products, Dublin, CA.

Morpholino-4-sulfonylleucine (1C). A mixture of morpholine hydrochloride (60 g, 0.49 mol), chloroform (200 mL), and sulfuryl chloride (800 mL) was refluxed for 4 h. After cooling to room temperature, additional sulfuryl chloride (200 mL) was added to the flask, and the mixture was refluxed for another 3 h. Solvent evaporation gave a dark residue which, on distillation under high vacuum (2 mmHg, 90 °C), generated morpholinosulfonyl chloride as a colorless thick oil (74.60 g, 83%).

In a separate flask, leucine (40 g, 0.30 mol) was dissolved in 3 N NaOH (600 mL), and to it was slowly added morpholinosulfonyl chloride (57.20 g, 0.30 mol) in THF (120 mL). The reaction mixture was stirred for 4 h and filtered. The filtrate was acidified with dilute acid and extracted into ethyl acetate (3 × 500 mL). The organic layer was washed with water (1 × 50 mL), dried (Na_2SO_4), and concentrated to give **1C** (29.61 g, 35%), which was used without any further purification: ^{1}H NMR ($CDCl_3$) δ 1.00 (6H, q), 1.60 (1H, m), 1.85 (1H, m), 3.20 (4H, t), 3.75 (4H, t), 4.00 (1H, m), 5.10 (1H, d), 6.40 (1H, b); MS *m/e* 281 (M + H), 303 (M + Na).

[(Benzylamino)carbonyl]leucine (1D). To a stirred solution of benzyl isocyanate (0.45 g, 0.0034 mol) in methylene chloride (5 mL) at 0 °C was added slowly a mixture of Leu-OtBu hydrochloride salt (0.75 g, 0.0034 mol) and diisopropylethylamine (0.46 g, 0.0036 mol) in methylene chloride (5 mL). The cooling bath was removed, the mixture was stirred for another 0.5 h, and solvent was removed. The residue was taken into ethyl acetate (15 mL) and was washed with 2% citric acid solution (2 × 10 mL), saturated $NaHCO_3$ solution (1 × 10 mL), and brine (1 × 10 mL), and dried (Na_2SO_4). Solvent evaporation gave [(benzylamino)carbonyl]leucine *tert*-butyl ester as a white solid (0.90 g, 90%): mp 73−76 °C; ^{1}H NMR ($CDCl_3$) δ 0.90 (6H, t), 1.40−1.80 (3H, m), 1.45 (9H, s), 4.40 (3H, m), 4.90 (2H, m), 7.30 (5H, m); MS *m/e* 321 (M + H), 343 (M + Na).

To 0.40 g of the above material was added a mixture of 90% TFA (3 mL) and methylene chloride (3 mL), and the resulting mixture was stirred at room temperature for 3 h. Excess reagents were removed under high vacuum to give 0.35 g of **1D**, which was directly used in the next step; ^{1}H NMR of an aliquot showed the absence of a peak at δ 1.45 for *t*-Boc group.

[(1,2,3,4-Tetrahydroisoquinolin-2-yl)carbonyl]leucine (1E). To a solution of triphosgene (7.00 g, 0.024 mol) in methylene chloride (20 mL) at room temperature was added a mixture of leucine methyl ester hydrochloride (9.10 g, 0.05 mol) and diisopropylethylamine (20 mL, 0.11 mol) in methylene chloride (100 mL) over a period of 2.5 h. The mixture was stirred for another 0.5 h, and to it was added a solution of 1,2,3,4-tetrahydroisoquinoline (7.50 g, 0.05 mol) and diisopropylethylamine (20 mL, 0.11 mol) in methylene chloride (30 mL). The reaction mixture was stirred for another 0.5 h, concentrated, and taken into ethyl acetate (200 mL). The organic layer was washed with 2% citric acid (2 × 100 mL), 2% $NaHCO_3$ (2 × 100 mL), water (2 × 50 mL), and brine (2 × 50 mL) and dried ($MgSO_4$). Solvent evaporation gave a crude material which was purified by flash chromatography (eluant: hexane−ethyl acetate, 2:1) to generate [(1,2,3,4-tetrahydroisoquinolin-2-yl)carbonyl]leucine methyl ester (6.50 g, 43%) as a viscous oil: ^{1}H NMR ($CDCl_3$) δ 0.90 (6H, d), 1.60 (3H, m), 2.90 (2H, broad t), 3.70 (2H, m), 3.80 (3H, s), 4.55 (1H, m), 4.60 (2H, s), 4.90 (1H, d), 7.20 (4H, m).

A mixture of the above ester (5.50 g, 0.018 mol), $LiOH·H_2O$ (2.3 g, 0.054 mol), THF (50 mL), and H_2O (20 mL) was stirred at room temperature for 2.5 h. THF was removed in vacuo, and the residue was diluted with water (50 mL). The aqueous layer was washed with ether (1 × 50 mL), acidified with dilute acid (pH 3 ∼ 4), and extracted into ethyl acetate (3 × 100 mL). The organic layer was washed with water (2 × 10 mL) and brine (2 × 20 mL) and dried ($MgSO_4$). Solvent evaporation generated **1E** (5.00 g, 95%) as a white solid: mp 120−125 °C (softening at 100 °C); ^{1}H NMR ($CDCl_3$) δ 0.90 (6H, t), 1.60 (1H, m), 1.80 (2H, m), 2.90 (2H, t), 3.60 (2H, m), 4.40 (1H, m), 4.60 (2H, dd), 4.90 (1H, d), 6.00 (1H, broad), 7.20 (4H, m); MS *m/e* 291 (M + H), 313 (M + Na).

1-Fluoro-3-nitro-4-phenyl-2-butanol (6A). To a stirred mixture of *trans*-β-nitrostyrene (5.25 g, 0.035 mol) and silica gel (10 g, 230−400 mesh) in chloroform (400 mL) and 2-propanol (75 mL) at room temperature was slowly added sodium borohydride (5.50 g, 0.145 mol) over a period of 45 min. The reaction mixture was stirred for an additional 15 min and carefully quenched with 10% hydrochloric acid (20 mL). The separated solid was filtered and washed with chloroform (50 mL). The organic layer was washed with water (2 × 10 mL) and brine (2 × 10 mL) and dried (Na_2SO_4). Solvent evaporation at reduced pressure gave a crude material which was purified by flash chromatography (silica gel, 8% ethyl acetate−hexane) to give 2.86 g of 1-nitro-2-phenylethane (**5A**): colorless oil; R_f (10% ethyl acetate in hexane) 0.40; ^{1}H NMR ($CDCl_3$) δ 7.40−7.20 (5H, m), 4.60 (2H, t), 3.30 (2H, t).

In a separate flask, to a cooled (−78 °C) solution of oxalyl chloride (2 M) in methylene chloride (11.60 mL, 0.0232 mol) was added slowly dimethyl sulfoxide (3.65 g, 3.32 mL, 0.0467 mol). The reaction mixture was stirred for 15 min. A solution of 2-fluoroethanol (1.16 g, 0.0181 mol) in methylene chloride (10 mL) was slowly introduced into the reaction flask. After another 15 min of stirring, the reaction mixture was diluted with anhydrous methylene chloride (180 mL), and triethylamine (9.20 g, 12.63 mL, 0.090 mol) was added to it. Stirring was continued for another 2 h by which time the temperature changed to room temperature. A solution of compound **5A** (2.74 g, 0.0181 mol) in anhydrous methylene chloride (10 mL) was added to the reaction mixture, and stirring was continued overnight. The mixture was then washed with water (2 × 20 mL), 4% hydrochloric acid (3 × 20 mL), water (2 × 20 mL), saturated sodium bicarbonate solution (2 × 20 mL), and brine (2 × 20 mL). Drying (Na_2SO_4) of the organic phase and solvent evaporation gave a crude material which was purified by flash chromatography (silica gel, 25% ethyl acetate−hexane) to give **6A** (isomers I and II) as erythro/threo isomers. The combined yield was 3.01 g (78%).

6A (isomer I): white solid, mp 71−73 °C; R_f (30% ethyl acetate in hexane) 0.46; ^{1}H NMR ($CDCl_3$) δ 7.40−7.10 (m, 5H), 4.90 (m, 1H), 4.60 (m, 1H), 4.50−4.30 (m, 2H), 3.45−3.25 (m, 2H), 2.70 (d, 1H); MS *m/e* 214 (M + H), 236 (M + Na).

6A (isomer II): colorless oil; R_f (30% ethyl acetate in hexane) 0.42; ^{1}H NMR ($CDCl_3$) δ 7.40−7.15 (m, 5H), 4.90 (m, 1H), 4.65 (m, 1H), 4.50 (m, 1H), 4.20 (m, 1H), 3.40−3.30 (m, 2H), 2.90 (d, 1H); MS *m/e* 214 (M + H), 236 (M + Na).

1-Fluoro-3-nitro-2-pentanol (6B). This compound (diasteromeric mixture) was prepared from 1-nitropropane (**5B**) according to the procedure described above for **6A**: pale yellow liquid (52% yield); bp 130−140 °C (0.3 mmHg); ^{1}H NMR ($CDCl_3$) δ 1.05 (3H, t), 2.00 (2H, m), 4.20 (1H, m), 4.40 (1H, m), 4.60 (2H, m).

1-Fluoro-3-nitro-4-[(tetrahydropyran-2-yl)oxy]-2-butanol (6C). This compound (diasteromeric mixture) was prepared from 2-(2-nitroethoxy)tetrahydropyran (**5C**) according to the procedure described above for **6A**: pale yellow viscous oil (29% yield, purification by flash chromatography, silica gel, eluant: 35% ether in hexanes); ^{1}H NMR ($CDCl_3$) δ 1.60 (6H, m), 3.42−4.88 (10H, m); MS *m/e* 236 (M − H).

3-Amino-1-fluoro-4-phenyl-2-butanol (2A). A mixture of intermediate **6A** (isomer I, 0.48 g, 2.25 mmol), absolute ethanol (20 mL), and Raney nickel (50% slurry in water, catalytic) was hydrogenated (60 psi) in a Parr apparatus for 5 h. Filtration through a Celite pad and solvent evaporation gave 0.41 g of **2A** (isomer I), which was used without further purification. Similar treatment of **6A** (isomer II, 0.80 g, 3.75 mmol) gave 0.51 g of **2A** (isomer II).

2A (isomer I): white solid; mp 64−67 °C; ^{1}H NMR ($CDCl_3$) 1.70−2.20 (3H, m), 2.50 (1H, q), 2.95 (1H, dd), 3.20 (1H, m), 3.80 (1H, m), 4.50 (1H, d), 4.70 (1H, d), 7.30 (5H, m); MS *m/e* 184 (M + H), 206 (M + Na).

2A (isomer II): white solid; mp 67−70 °C; ^{1}H NMR ($CDCl_3$) δ 1.65−2.20 (3H, m), 2.55 (1H, q), 2.95 (1H, dd), 3.10 (1H, m), 3.60 (1H, m), 4.55 (1H, d), 4.70 (1H, d), 7.20 (5H, m); MS *m/e* 184 (M + H), 206 (M + Na).

3-Amino-1-fluoro-2-pentanol (2B). This compound (diastereomeric mixture) was prepared according to the procedure described above for **2A**: pale yellow oil (69% yield); ^{1}H NMR ($CDCl_3$) δ 0.95 (3H, t), 1.35 (1H, m), 1.60 (1H, m), 2.05 (2H, br), 2.80 (1H, m), 3.65 (1H, m), 4.53 (3H, m); MS *m/e* 122 (M + H).

3-Amino-1-fluoro-4-[(tetrahydropyran-2-yl)oxy]-2-butanol (2C). This compound (diastereomeric mixture) was prepared according to the procedure described above for **2A**: pale yellow oil (86% yield); ^{1}H NMR ($CDCl_3$) δ 1.18−1.90 (9H, m), 3.10 (1H, m), 3.50 (2H, m), 3.85 (3H, m), 4.55 (3H, m); MS *m/e* 208 (M + H).

Syntheses of 3a−g: General Procedure. Compounds **3a−g** (diastereomeric mixture) were synthesized following either of two general methods, A or B.

General Method A. To a cooled (−20 °C) solution of **1A−E** (1 equiv) in methylene chloride or THF was added *N*-methylmorpholine (2.1 equiv) followed by isobutyl chloroformate (1.05 equiv). The mixture was stirred for 10 min, the cooling bath was replaced by an ice bath, and compound **2A−C** (1 equiv) in methylene chloride or DMF was introduced into the reaction flask. Stirring was continued for 1 h by which time the temperature changed to room temperature. The reaction mixture was diluted with ethyl acetate. The organic layer was washed with 2% citric acid, 2% $NaHCO_3$, water, and brine and dried ($MgSO_4$). Solvent evaporation gave a crude material which was purified by flash chromatography (eluant: hexane−ethyl acetate), yield 40−60%.

General Method B. To a cooled (0 °C) solution of **1A−E** (1 equiv) in DMF was added *N*-methylmorpholine (3−4 equiv) followed by 1-HOBt (1.05 equiv) and BOP (1.05 equiv). The mixture was stirred for 10 min, compound **2A−C** (1.1 equiv) was introduced into the reaction flask, and the ice bath was removed. Stirring was continued for 1−16 h. The reaction mixture was poured into a mixture of ice−water and extracted into ethyl acetate. The organic layer was washed with 2% citric acid, 2% $NaHCO_3$, water, and brine and dried ($MgSO_4$). Solvent evaporation gave a crude material which was purified by flash chromatography (eluant: hexane−ethyl acetate), yield 80−90%.

3-[*N*-[(Benzyloxycarbonyl)leucyl]amino]-1-fluoro-4-phenyl-2-butanol (3a). Method A: white solid; ^{1}H NMR ($CDCl_3$) δ 0.80 (6H, m), 1.30 (2H, m), 1.60 (2H, m), 2.90 (2H, m), 3.50 (1H, 2 sets of d), 4.00 (2H, m), 4.30−4.90 (3H, m), 5.10 (2H, d), 6.10 and 6.30 (1H, 2 sets of d), 7.30 (10H, m); MS *m/e* 431 (M + H), 453 (M + Na).

3-[*N*-[(*tert*-Butoxycarbonyl)leucyl]amino]-1-fluoro-4-phenyl-2-butanol (3b). Method B: white solid; ^{1}H NMR ($CDCl_3$) δ 0.91 (6H, m), 1.43 (9H, s), 1.56 (3H, m), 2.94 (2H, m), 3.95 (2H, m), 4.39 (4H, m), 5.03 (1H, m), 6.78 (1H, dd), 7.25 (5H, m); MS *m/e* 397 (M + H), 419 (M + Na).

3-[*N*-[(Morpholino-4-sulfonyl)leucyl]amino]-1-fluoro-4-phenyl-2-butanol (3c). Method B: white solid; ^{1}H NMR ($CDCl_3$+ 20% DMSO-d_6) δ 0.85 (6H, m), 1.25 (2H, m), 1.60 (1 H, q), 2.82−3.15 (6H, m), 3.65 (5H, m), 3.85 (1H, m), 4.22 (1 H, m), 4.26−4.6 (2H, m), 4.96 (1H, d), 6.95 (1H, d), 7.15−7.30 (5H, m), 7.35 (1H, d); MS *m/e* 446 (M + H), 468 (M + Na).

3-[*N*-[(Benzylaminocarbonyl)leucyl]amino]-1-fluoro-4-phenyl-2-butanol (3d). Method A: white solid; ^{1}H NMR (DMSO-d_6) δ 0.70 and 0.80 (6H, 2 sets of t), 1.00−1.50 (3H, a series of m), 2.60 (1H, m), 3.10 (1H, m), 3.60 (1H, m), 3.80 (1H, m), 4.00−4.50 (5H, a series of m), 5.40 (1H, m), 6.00 (1H, m), 6.40 (1H, m), 7.20 (10H, m), 7.80 and 8.00 (1H, 2 sets of d); MS *m/e* 430 (M + H), 452 (M + Na).

3-[*N*-[[(1,2,3,4-Tetrahydroisoquinolin-2-yl)carbonyl]-leucyl]amino]-1-fluoro-4-phenyl-2-butanol (3e). Method B: white solid; ^{1}H NMR ($CDCl_3$) δ 0.70−1.00 (6H, m), 1.40 (2H, m), 1.60 (1H, m), 2.90 (2H, m), 3.00 (2H, m), 3.50 (1H, m), 3.70 (1H, m), 3.90−4.60 (10H, m), 4.80 and 4.90 (1H, 2 sets of d), 6.70 and 7.00 (1H, 2 sets of d), 7.20 (9H, m); MS *m/e* 456 (M + H), 478 (M + Na).

3-[*N*-[(Benzyloxycarbonyl)leucyl]amino]-1-fluoro-2-pentanol (3f). Method B: white solid; ^{1}H NMR ($CDCl_3$) δ 0.90 (9H, m), 1.42−1.75 (5H, m), 3.70−4.60 (6H, m), 5.15 (3H, m), 6.30 (1H, br), 7.35 (5H, s); MS *m/e* 369 (M + H), 391 (M + Na).

3-[*N*-[(Benzyloxycarbonyl)leucyl]amino]-1-fluoro-4-[(tetrahydropyran-2-yl)oxy]-2-butanol (3g). Method B: viscous oil; ^{1}H NMR ($CDCl_3$) δ 0.90 (7H, m), 1.55 (4H, m), 1.75 (4H, m), 3.45−4.60 (12H, m), 5.10 (2H, s), 5.20 (1H, br), 7.37 (5H, s); MS *m/e* 455 (M + H), 477 (M + Na).

Syntheses of 4a−g: General Method. To a solution of **3a−g** (1 equiv) in anhydrous methylene chloride at 0 °C was slowly added Dess−Martin periodinane (2−3 equiv). The cooling bath was removed, and the mixture was stirred for 1−16 h, diluted with methylene chloride, and washed several times with 10% $Na_2S_2O_3$ solution followed by saturated $NaHCO_3$ solution, water, and brine. Drying ($MgSO_4$) and solvent evaporation gave the product which was further purified by flash chromatography using hexanes−ethyl acetate mixture as eluant, yield 75−95%.

Cbz-Leu-D,L-Phe-CH_2F (4a): white solid; ^{1}H NMR ($CDCl_3$) δ 0.90 (6H, m), 1.50 (3H, m), 3.00 (1H, m), 3.20 (1H, m), 4.20 (1H, m), 4.80 (2H, m), 5.00 (2H, m), 5.10 (2H, s), 6.60 (1H, d), 7.10 (2H, d), 7.40 (8H, m); MS *m/e* 429 (M + H), 451 (M + Na). Anal. ($C_{24}H_{29}N_2O_4F$) C, H, N.

tBoc-Leu-D,L-Phe-CH_2F (4b): white solid; ^{1}H NMR ($CDCl_3$) δ 0.91 (6H, m), 1.43 (9H, s), 1.56 (3H, m), 3.10 (2H, ddd), 4.04 (1H, m), 4.80 (4H, m), 6.60 (1H, dd), 7.15 (2H, d), 7.29 (3H, m); MS *m/e* 395 (M + H), 417 (M + Na). Anal. ($C_{21}H_{31}N_2O_4F$) C, H, N.

Morpholino-4-sulfonyl-Leu-D,L-Phe-CH_2F (4c): white solid; ^{1}H NMR ($CDCl_3$) δ 0.88 (6H, m), 1.43 (2H, m), 1.78 (1H, q), 2.92−3.35 (6H, m), 3.60−3.82 (5H, m), 4.70−5.25 (4H, m), 6.36 (1H, q), 7.15−7.30 (5H, m); MS *m/e* 444 (M + H); HRMS calc 444.1968 (MH^+), found 444.1973 (MH^+).

[(Benzylamino)carbonyl]-Leu-D,L-Phe-CH_2F (4d): white solid; ^{1}H NMR ($CDCl_3$) δ 0.80 (6H, m), 1.40 (3H, m), 2.90 (1H, m), 3.10 (1H, m), 4.10 (1H, m), 4.30 (2H, m), 4.70 (3H, m), 5.50 (1H, m), 5.65 and 5.75 (1H, 2 sets of d), 7.20 (10H, m), 7.45 and 7.60 (1H, 2 sets of d); MS *m/e* 428 (M + H), 450 (M + Na). Anal. ($C_{24}H_{30}N_3O_3F$) C, H, N.

[(1,2,3,4-Tetrahydroisoquinolin-2-yl)carbonyl]-Leu-D,L-Phe-CH_2F (4e): white solid; ^{1}H NMR ($CDCl_3$) δ 0.90 (6H, m), 1.60 (3H, m), 2.90 (2H, t), 3.00 (1H, m), 3.20 (1H, m), 3.60 (2H, m), 4.40 (1H, m), 4.50 (2H, m), 4.80 (4H, m), 6.90 and 7.10 (1H, 2 sets of d), 7.20 (9H, m); MS *m/e* 454 (M + H), 476 (M + Na). Anal. ($C_{26}H_{32}N_3O_3F$) C, H, N.

Cbz-Leu-D,L-Abu-CH_2F (4f): white solid; ^{1}H NMR ($CDCl_3$) δ 0.90 (9H, m), 1.60 (4H, m), 1.95 (1H, m), 4.20 (1H, m), 4.85 (2H, m), 5.05 (1H, m), 5.15 (3H, m), 6.60 (1H, m), 7.33 (5H, m); MS *m/e* 367 (M + H), 389 (M + Na). Anal. ($C_{19}H_{27}N_2O_4F$) C, H, N.

Cbz-Leu-D,L-Ser(OTHP)-CH_2F (4g): waxy solid; ^{1}H NMR ($CDCl_3$) δ 0.95 (7H, m), 1.50 (4H, m), 1.70 (4H, m), 3.50 (1H, br), 3.75 (2H, m), 4.20 (3H, m), 4.40 (1H, br), 4.90 (3H, m), 5.12 (2H, s), 5.20 (1H, m), 7.35 (5H, m); MS *m/e* 453 (M + H),

475 (M + Na); HRMS calc 453.2401 (MH^+), found 453.2382 (MH^+).

Cbz-Leu-D,L-Ser-CH_2F (4h). A solution of **4g** (0.035 g, 0.08 mmol) in methanol (2 mL) was treated with *p*-toluenesulfonic acid monohydrate (0.014g, 0.08 mmol). The mixture was stirred at ambient temperature for 2 h. Solvent was evaporated in vacuo, and the residue was dissolved in ethyl acetate (10 mL). The organic phase was washed with saturated sodium bicarbonate (2 × 5 mL), water (2 × 5 mL), and brine (2 × 5 mL). Drying ($MgSO_4$) and solvent evaporation gave a crude product which was purified by flash chromatography to give **4h** as a glassy amorphous solid (0.013 g, 45%): ^{1}H NMR ($CDCl_3$) δ 0.95 (7H, m), 1.65 (3H, m), 4.00 (2H, m), 4.15 (1H, m), 4.90 (2H, m), 5.15 (4H, m), 7.00 (1H, m), 7.35 (5H, m); MS *m/e* 369 (M + H), 391 (M + Na); HRMS calc 369.1826 (MH^+), found 369.1837 (MH^+).

Leu-D,L-Phe-CH_2F-HCl (4i). A stirred suspension of **4b** (0.094 g, 0.24 mmol) in ether was saturated with HCl gas. The resulting solution was resaturated with HCl gas after 20 min. The solvent and excess HCl were evaporated after 40 min, affording the product as a light yellow solid (0.080 g, 100% yield): ^{1}H NMR (D_2O) δ 0.74 (6H, d), 1.65 (3H, m), 2.74 (1H, m), 3.28 (1H, m), 3.79 (1H, m), 4.46 (2H, m), 7.34 (5H, m); MS *m/e* 295 (M + H). Anal. ($C_{16}H_{23}N_2O_2F$·HCl) C, H.

PhCH_2CH_2CO-Leu-D,L-Phe-CH_2F (4j). Compound **4i** and hydrocinnamic acid were coupled according to general method B, as described before, to generate **4j** as a white amorphous solid (100% yield): ^{1}H NMR ($CDCl_3$) δ 0.80 (3H, m), 1.22 (3H, m), 2.50 (2H, m), 2.96 (3H, m), 3.16 (1H, dt), 4.42 (1H, quintet), 4.60−5.03 (3H, m), 5.91 (0.4H, d), 6.07 (0.6H, d), 7.01−7.32 (11H, m); MS *m/e* 427 (M + H), 449 (M + Na). Anal. ($C_{25}H_{31}N_2O_3F$) C, H, N.

Cbz-Leu-Leu-D,L-Phe-CH_2F (4k). Compound **4i** and Cbz-Leu-OH were coupled according to general method B, as described before, to generate **4k** as a white solid (71% yield): ^{1}H NMR ($CDCl_3$) δ 0.87 (6H, m), 1.39 (4H, m), 1.59 (4H, m), 2.97 (1H, dt), 3.20 (1H, dt), 4.20 (1H, m), 4.41 (1H, m), 4.60−5.18 (5H, m), 5.41 (0.4H, d), 5.59 (0.6H, d), 6.69 (0.4H, d), 6.76 (0.6H, d), 7.05−7.38 (11H, m); MS *m/e* 542 (M + H), 564 (M + Na). Anal ($C_{30}H_{40}N_3O_5F$) C, H, N.

Cbz-Val-D,L-Phe-N(Me)OMe (8). To a stirring solution of Cbz-Val-Phe-OH (**7**, 2.00 g, 5.00 mmol) and triethylamine (0.57 g, 0.79 mL, 5.64 mmol) in methylene chloride (25 mL) at room temperature was added BOP reagent (2.5 g, 5.64 mmol). After 10 min, *N*,*O*-dimethylhydroxylamine hydrochloride (0.61 g, 6.20 mmol) was added to the flask, followed by triethylamine (0.62 g, 0.86 mL, 6.20 mmol). Stirring was continued for 3 h. The reaction mixture was diluted with methylene chloride (50 mL) and washed successively with dilute acid (2 × 30 mL), saturated $NaHCO_3$ (2 × 20 mL), H_2O (2 × 20 mL), and brine (2 × 30 mL). Drying ($MgSO_4$) and solvent evaporation gave a crude product which was purified by flash chromatography (hexanes−ethyl acetate, 35:65) to give a white foam (diastereomeric mixture, 1.72 g, 78%): ^{1}H NMR ($CDCl_3$) δ 0.80 (6H, m), 2.00 (1H, m), 2.90 (1H, m), 3.10 (1H, m), 3.20 (3H, m) 3.70 (3H, d), 4.10 (1H, m), 5.10 (2H, m), 5.30 (2H, m), 6.60 (1H, m), 7.30 (10H, m); MS *m/e* 442 (M + H), 464 (M + Na).

Cbz-Val-D,L-Phe-CH_3 (9). A solution of compound **8** (1.56 g, 3.53 mmol) in anhydrous THF (25 mL) was added slowly to a cooled (0 °C) solution of 3 M MeMgBr in ether (7 mL, 21.00 mmol) diluted with anhydrous THF (20 mL). The cooling bath was removed, and the reaction mixture was stirred overnight. The next day, the reaction mixture was quenched at 0 °C with a mixture of saturated NH_4Cl and water (1:1, 30 mL) and extracted into ethyl acetate (3 × 50 mL). The combined organic layer was washed with brine (2 × 30 mL), dried ($MgSO_4$), and concentrated to give a residue which was purified by flash chromatography (EtOAc−hexanes, 65:35) to generate a white solid (diastereomeric mixture, 1.21 g, 86%): ^{1}H NMR ($CDCl_3$) δ 0.90 (6H, m), 2.10 (1H, m), 2.15 (3H, m), 3.00 (1H, m), 3.15 (1H, m), 4.00 (1H, m), 4.80 (1H, m), 5.15 (2H, d), 5.30 (1H, t), 6.50 (1H, broad), 7.30 (10H, m); MS *m/e* 397 (M + H), 419 (M + Na).

Cbz-Val-D,L-Phe-CH_2F (41). To a stirred solution of compound **9** (0.19 g, 0.475 mmol) in methylene chloride (3 mL) at 0 °C was added excess triethylamine (0.4 mL) followed by TBDMSOTf (0.4 mL, 2.07 mmol). The mixture was stirred for 1.5 h, diluted with ethyl acetate (l0 mL), and washed successively with saturated $NaHCO_3$ (2 × 5 mL) and brine (2 × 5 mL). Drying and solvent evaporation gave a crude product which was purified by chromatography (hexanes−ethyl acetate, 3:1, with 5 drops of triethylamine per 100 mL of solution) to give the enolsilyl ether as a white solid (0.21 g, 87%).

A mixture of above enolsilyl ether (0.19 g, 0.36 mmol), 1-(chloromethyl)-4-fluoro-1,4-diazabicyclo[2.2.2]octane bis(tetrafluoroborate) (F-TEDA-BF_4, 0.14 g, 0.39 mmol) and DMF (4 mL) was stirred at room temperature overnight, diluted with a mixture of ether−ethyl acetate (2:1, 20 mL), and washed successively with water (2 × 10 mL) and brine (2 × 10 mL). Drying ($MgSO_4$) and solvent evaporation gave a crude product which was contaminated with compound **9**. Rigorous purification by column chromatography (eluant: CH_2Cl_2−CH_3CN, 95:5) generated **4l** as a white solid (diastereomeric mixture, 0.015 g, 10%): ^{1}H NMR ($CDCl_3$) δ 0.90 (6H, m), 2.10 (1H, m), 3.00 (1H, m), 3.15 (1H, m), 3.95 (1H, t), 4.70−5.15 (6H, m), 6.40 (1H, t), 7.30 (10H, m); MS *m/e* 415 (M + H), 437 (M + Na). Anal. ($C_{23}H_{27}N_2O_4F$) C, H, N.

[$^{14}CH_2$]Cbz-Leu-D,L-Phe-CH_2F (4a*). Radiosynthesis was performed by Chemsyn Science Laboratories, Lenexa, KS. [$^{14}CH_2$]Benzyl chloroformate (5.0 mCi, 0.086 mmol, 58 mCi/mmol, prepared from [$^{14}CH_2$]benzyl alcohol and phosgene in toluene) and iPr_2NEt (35 μL, 0.20 mmol) were added to a solution of compound **4i** (0.028 g, 0.085 mmol) in methylene chloride (0.4 mL) at 0 °C. After 80 min at 0 °C, the mixture was diluted with methylene chloride (5 mL), rinsed with water, 1 M HCl, saturated Na_2CO_3, and saturated NaCl, and then dried over $MgSO_4$. The crude product was purified by chromatography on silica gel (EtOAc−hexanes, 35:65) to afford pure product **4a*** (16.9 mg, 2.3 mCi, 58 mCi/mmol) which coeluted with authentic **4a** on silica gel TLC (EtOAc−hexanes, 50:50, and $CHCl_3$−MeOH, 90:10) and HPLC (4.6 × 250 mm Zorbax Rx C_{18} column; 1 mL/min flow rate; 90:10:0.1 H_2O−CH_3CN−TFA for 5 min; linear gradient from 10% to 100% of 99.9:0.1 CH_3CN−TFA over 45 min; detection at 215 nm).

Biochemistry. Calpain I Inactivation Assay. Assays for inactivation of calpain contained 50 mM Tris-Cl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.2 mM Suc-Leu-Tyr-MNA (Enzyme Systems Products, Dublin, CA), 10 nM recombinant human calpain I, 3% DMSO, and varying concentrations of inhibitor and were initiated by the addition of 5 mM $CaCl_2$. Reactions were performed at ambient temperature in single cuvettes with the increase in fluorescence (λ_{ex} = 340 nm, λ_{em} = 425 nm) recorded continuously on a Perkin-Elmer LS50B spectrofluorimeter (Norwalk, CT) and were monitored until there was no further product generated in inhibitor-containing assays. In some experiments, inactivation rates were determined in 96-well plates (Dynatech) in a final volume of 200 μL using a Fluoroskan II plate reader, with kinetic data acquired using DeltaSoft software (BioMetallics Inc., Princeton, NJ). Inhibitor concentrations were at least 10-fold greater than the enzyme concentration in all cases.

The second-order rate constant for inactivation of calpain I was calculated using published methods.[20a,b] The simplest kinetic model for irreversible inactivation involves formation of an initial reversible complex (EI) followed by irreversible formation of the inactivated enzyme (EI*). The pseudo-first-order rate constant for inactivation, k_{obs}, is dependent on inhibitor concentration (I_t) and the individual rate constants, as shown in eq 1,[20a] when inhibitor is in excess over enzyme. Equation 2, resulting from rearrangement of eq 1, permits calculation of the second-order rate constant for inactivation, referred to as k.

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_3} EI^*$$

$$k_{obs} = [k_1 k_3 I_t/(k_2 + k_3)]/(1 + S/K_m) \qquad (1)$$

Values of k_{obs} were obtained from plots of fluorescence vs time by nonlinear regression (Sigma Plot or GraphPad Prism)

$$k = (k_{obs}/I_t)(1 + S/K_m) \quad (2)$$

to the exponential equation (3)[20b]

$$y = Ae^{(-k_{obs}t)} + B \quad (3)$$

where y is the fluorescence at time t (F_t), A is the amplitude of the reaction ($F_0 - F_\infty$), and B is the maximal amount of product formed when the enzyme is completely inactivated (F_∞). The apparent second-order rate constant for inactivation, k, was calculated according to eq 2 from a replot of k_{obs} vs I. A K_m value of 0.5 mM was employed in these calculations. In all cases, linear replots were obtained, without evidence for saturation kinetics in any case.

Because calpain I undergoes an autolytic loss of activity under assay conditions, progress curves for control reactions lacking inhibitor demonstrate a small first-order deviation from linearity (k_{obs} typically <0.0005 s^{-1}). Values of k_{obs} were calculated for control reactions and included in plots of k_{obs} vs I, where they fell directly on the line defined by inhibitor-containing assays.

Binding Assay with 4a*. Recombinant human calpain I (2.2 μM) was incubated at 37 °C with an ca. 10 fold molar excess of **4a*** (1.28×10^8 dpm /μmol) in the absence or presence of calcium ion (5 mM) in 20 mM Tris, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol. To specifically capture the intermediate autolyzed 78 kDa species of calpain I, inhibitor was added simultaneous to calcium ion addition. To capture the 76 kDa autolyzed species of calpain I, inhibitor was added 5 min following calcium ion addition. One reaction mixture also contained Cbz-Val-Phe-H (250 μM). Following addition of all reagents, incubations were continued for 5, 15, or 30 min. All reactions were stopped by diluting 10-fold with incubation buffer. Each mixture was then subjected to ultrafiltration on a Microcon 10 filter (Amicon) for 30 min at 4 °C, yielding a 10-fold concentration of the enzyme. The enzyme was then subjected to four additional cycles of redilution with assay buffer and concentration. Incorporation of radiolabel into calpain I was determined by scintillation counting, with the enzyme quantitated by its absorbance at 280 nm using an extinction coefficient of 154 000 M^{-1} cm^{-1}.[17] Samples were also run on 10% SDS−PAGE[21] and transfered to nitrocellulose[22] in order to resolve the various autolyzed forms of calpain I. Detection of all three M_r forms of calpain I was accomplished using a polyclonal antibody recognizing the catalytic subunit, followed by an alkaline phosphatase conjugated goat anti-rabbit secondary antibody (BIORAD, Inc., Hercules, CA) and an alkaline phosphatase detection system (BIORAD, Inc.). Immunoblots were then exposed to phosphoimage screens and analyzed on a Storm 840 phosphoimager (Molecular Dynamics, Inc.).

Cathepsins B and L Assays. Rates of inactivation of cathepsins B and L were determined under the assay conditions described by Krantz et al.[20b] and Mason et al.,[23] respectively. In both cases, Cbz-Phe-Arg-AMC was used as the substrate.

Intact Cell Assay. Molt-4 cells (human leukemic T cells) were suspended in HEPES buffered saline (HBS: 20 mM HEPES, pH 7.2−7.5, 5.4 mM KCl, 120 mM NaCl, 25 mM glucose, 1.5 mM $MgSO_4$, 1 mM Na pyruvate) at 2×10^7 cells per mL, 50 μL of which was added to the wells of a 96-well plate. To this, 50 μL of inhibitor solution, at twice the final desired concentration, in HBS was added. Following a 10 min incubation at 37 °C, on a nutator (Clay Adams), 100 μL of HBS containing the final desired inhibitor concentration, 10 mM Ca^{2+}, and 40 μM ionomycin was added. The suspension was incubated further for 30 min at 37 °C on a nutator, after which cells were harvested by centrifugation. The cell pellet was then solubilized by the addition of lysis buffer containing 20 mM Tris (pH 8.2), 137 mM NaCl, 13 mM EDTA, and 1% Triton X-100, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin A, and 1 mM Pefabloc. After a 30−60 min incubation on ice, with periodic vortexing, insoluble material was removed by centrifugation and the supernatant collected. Then 10% SDS was added to yield a final concentration of 1%, and the samples were heated for 15 min at 65 °C. Supernatant protein concentrations were determined by the BCA assay (Pierce, Inc., Rockford, IL). Samples (equal total protein) were resolved on 6% SDS−PAGE, transferred to nitrocellulose, and analyzed by immunoblot using a polyclonal antibody specific for calpain I-mediated spectrin breakdown products (SBDPs),[24] followed by an alkaline phosphatase conjugated goat anti-rabbit antibody (BIORAD, Inc.) and an alkaline phosphatase detection system (BIORAD, Inc.). To determine the percent inhibition, the integrated optical density (IOD) of the SBDPs in the presence and absence of inhibitor was determined using a BIOQUANT-OS/2 image analysis system (R&M Biometrics, Inc., Nashville, TN). IC_{50} values were calculated from the percent inhibition of SBDPs at varying inhibitor concentrations using nonlinear regression analysis.

Acknowledgment. The authors would like to thank Dr. Jeffry Vaught for his continuing support and encouragement and Dr. James C. Kauer for critical review of the manuscript. We also thank Dr. Zi-Qiang Gu for technical assistance. We are grateful to Dr. G. Sankar Lal of Air Products (Allentown, PA) for a generous gift of F-TEDA-BF_4 (Selectflur) reagent. We are pleased to acknowledge SmithKline Beecham Pharmaceuticals for partial support of this research.

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JM970197E