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Exploration of the Importance of the P_2-P_3 -NHCO-Moiety in a Potent Di- or Tripeptide Inhibitor of Calpain I: Insights into the Development of Nonpeptidic Inhibitors of Calpain I†

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Abstract—Calpain I, an intracellular cysteine protease, has been implicated in the neurodegeneration following an episode of cerebral ischemia. In this paper, we report on a series of peptidomimetic ketomethylene and carbamethylene inhibitors of recombinant human calpain I (rh calpain I). Our study reveals that the -NHCO-moiety (possible hydrogen-bonding site) at the P_2-P_3 region of a potent tripeptide or a dipeptide inhibitor of calpain I is not a strict requirement for enzyme recognition. Compounds **7d** ((*R*)-2-isobutyl-4-oxo-4-(9-xanthenyl)butanoic acid ((*S*)-1-formyl-3-methyl)butyl amide), **31** ((*R*)-2-isobutyl-4-(2-sulfonylnaphthyl)butyric acid ((*S*)1-formyl-3-methyl)butyl amide) and **34** ((*R*)-2-isobutyl-4-(2-sulfoxylnaphthyl)butyric acid ((*S*)-1-formyl-3-methyl)butyl amide) which exhibited good activity in the enzyme assay, also inhibited calpain I in a human cell line.

Introduction

Brain injury resulting from stroke is a major public health problem worldwide. In the U.S., more than half a million strokes occur each year, killing 150,000 people; another 400,000 stroke victims survive, but only a fortunate third of these are left with little or no physical or mental impairment.[1] Major disability can result with loss of the ability to communicate, ambulate, coordinate or reason.[2] The majority of all strokes occur when a blood vessel blocked by a clot (or an air bubble) originating from the heart or atherosclerotic arterial plaque, cuts off blood flow to a region of the brain and induces localized anemia, known as ischemia.[1,2] An episode of stroke initiates a chain of biochemical events resulting in the rise of intracellular Ca^{2+} concentration, which, among other effects, activates Ca^{2+}-dependent enzymes including calpain, a cytoplasmic cysteine protease. Activated calpain degrades neuronal structural or cytoskeletal proteins resulting in lost synaptic contacts and neurodegeneration. Two major forms of calpains have been identified: calpain I and calpain II, which require low and high micromolar Ca^{2+} concentrations for activation, respectively. While calpain II is the predominant form in many tissues, calpain I is thought to be the predominant form activated during pathological conditions of nervous tissue. We are interested in selectively inhibiting calpain I to find new therapeutics to treat stroke.[3]

Potent peptide-based reversible[4] and irreversible[5] inhibitors of calpain have been reported; however, to the best of our knowledge, no full length X-ray crystal structure of an enzyme–inhibitor complex has been reported. Interestingly, in the reversible aldehyde series, both tripeptides and dipeptides are potent inhibitors of calpain I.[4,5] This suggested to us that sufficient binding energy is obtained with occupancy of the S_1 and S_2 subsites of the enzyme.[6] Takahashi, commenting on the preferred substrates, noted that "... an amino acid with an aromatic or a bulky aliphatic side chain at the P_3 position may to some extent increase the susceptibility

Key words: Stroke; calpain I; peptidomimetic; ketomethylene; carbamethylene.
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†Dedicated to Mrs Luxmi Chatterjee with respect and admiration.

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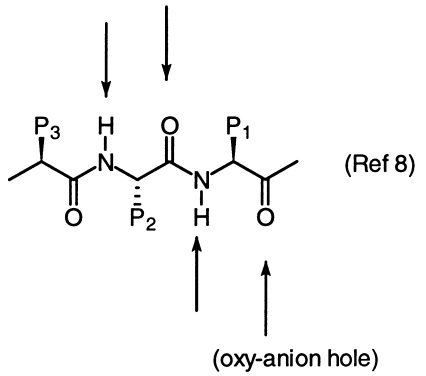
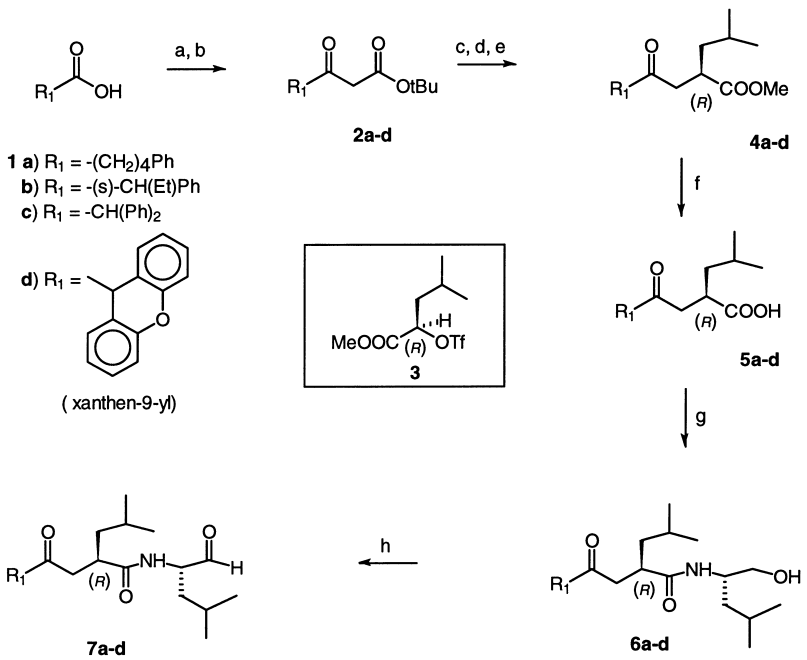


Figure 1. Arrows indicate the critical sites along the peptide inhibitor backbone which are engaged in hydrogen-bonding as observed for peptide inhibitor binding to cysteine proteases: papain superfamily (papain, cathepsin B, calpain I).

of the scissile bond to calpain".[7] Studies have shown that calpain prefers Leu or Val at P_2, but tolerates many different substituents at P_1. Dolle et al. mentioned that for a peptidic inhibitor of calpain I (a member of the papain superfamily), the P_2-NH moiety is a critical site for hydrogen-bonding (Fig. 1).[8] In order to probe the importance of the P_2-NH moiety in a tripeptide or a dipeptide inhibitor of calpain I, we wished to replace the P_3-P_2 amide bond in the tripeptide inhibitor, or the carbamoyl/acyl moiety in the dipeptide inhibitor, by corresponding ketomethylene (-$COCH_2$-) and carbamethylene (-CH_2CH_2-) moieties. In the process, we would have access to a series of peptidomimetic inhibitors of calpain I. In designing our target molecules, we maintained an isobutyl group at the pseudo-P_2 site to mimic the P_2-Leu of the corresponding peptidic inhibitors but incorporated an aromatic moiety in the P_3 region. We now present the full account of our work describing the synthesis, and the in vitro recombinant human calpain I inhibitory activity of the target compounds.[9] We also present the inhibitory activity of a selected set of compounds against cathepsin B, a related cysteine protease, and thrombin, a serine protease of biological significance. Finally, we present data demonstrating that compounds which exhibit good activity in the enzyme assay, also inhibit calpain I in a human cell line.

Chemistry

Synthesis of the target compounds **7a–d** is shown in Scheme 1. Compounds **1a–d** were converted to the corresponding β-ketoesters **2a–d**, which on treatment with sodium hydride and (*R*)-triflate-ester (**3**), followed by selective acidic hydrolysis and decarboxylation of the *t*-butyl ester group, produced the γ-ketoester **4a–d**.[10] Basic hydrolysis of **4a–d** produced the corresponding γ-ketoacid **5a–d** which was coupled with (*S*)-leucinol to produce **6a–d**. Compounds **6a**,**c**,**d** were isolated as single

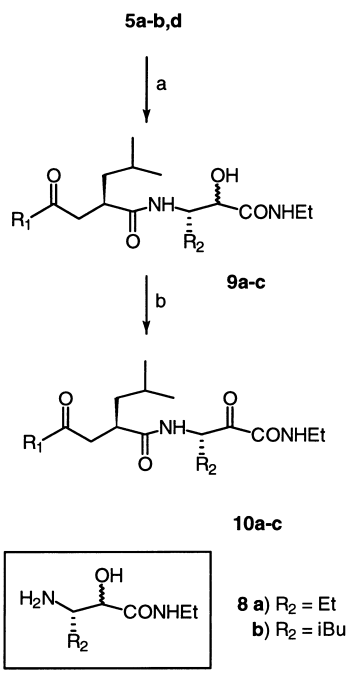


Scheme 1. Reagents: (a) 1,1′-carbonyldiimidazole, THF, 0–23 °C; (b) $Li^+{}^-CH_2COOtBu$, THF; −78 °C to 0 °C; (c) 60% NaH, THF, **3**, 23 °C; (d) TFA, 23 °C; (e) C_6H_6, reflux; (f) LiOH, MeOH-H_2O; 70–75 °C; (g) (*S*)-leucinol, BOP, HOBt, NMM, DMF 0–23 °C; (h) Pyr.SO_3, Et_3N, DMSO-CH_2Cl_2, 0–23 °C.

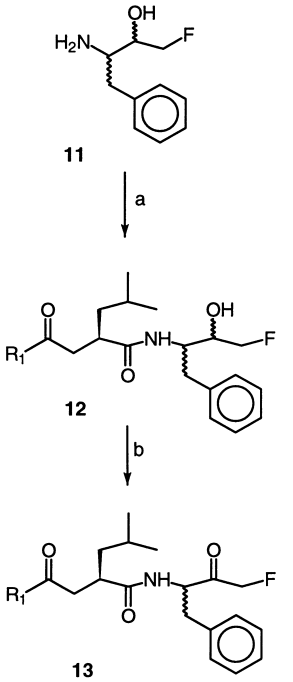
isomers; compound **6b** was a diastereomeric mixture. Assuming that the alkylation of the β-ketoester **2a–d** by (*R*)-triflate-ester **3** took place in an S_N2 fashion,[10] the stereochemistry at the P_2-site of the major product in **6a–d** was tentatively assigned as (*R*). Oxidation of **6a–d** produced the desired aldehydes **7a–d**. Similarly, compounds **5a–b,d** were coupled with **8a–b**[4c] (Scheme 2) to generate **9a–c**, which on oxidation gave desired α-ketocarboxamides **10a–c**. Compounds **11**[5a] and **5d** were coupled to generate the compound **12** (Scheme 3) which on oxidation gave fluoromethyl ketone **13** (diastereomeric mixture, epimeric at P_1). Scheme 4 depicts the synthesis of compounds **21a–b**. Isocaproic acid (**14**) was benzylated to generate the ester **15**, which on alkylation produced the racemic diester **16**. Selective hydrolysis of *t*-butyl ester in **16** gave **17**, which was coupled with 1,2,3,4-tetrahydroisoquinoline to generate **18**. Debenzylation of **18** produced **19**. Compound **19** was coupled with (*s*)-phenylalaninol to generate diastereomeric **20a,b**, easily separable by silica–gel column chromatography (**20a** being the faster moving isomer). Separated diasteromers **20a** and **20b** were oxidized to generate aldehydes **21a** and **21b**, respectively. The stereochemistry at the pseudo-P_2 site in **21a** and **21b** was tentatively assigned as (*R*) and (*S*), respectively, based on comparison of the calpain inhibitory activity of **21a,b** with that of a reference dipeptidyl aldehyde (Cbz-Val-Phe-H, MDL 28170[4b]). The syntheses of **30** and **31** are depicted in Scheme 5. 2-Naphthalenethiol (**22**) was converted to the ester **23**, which on subsequent alkylation, produced the corresponding racemic ester **24**. Hydrolysis of **24** yielded the racemic acid **25**, which was coupled with (*S*)-leucinol to produce the diastereomeric compounds **26** and **27**, easily separable by silica gel column chromatography (**26** being the faster-moving isomer). Oxidation of **26** and **27** by *m*-chloroperbenzoic acid generated the sulfonyl compounds **28** and **29**, which on further oxidation by sulfur trioxide-pyridine complex in DMSO-CH_2Cl_2 produced the aldehydes **30** and **31**. The stereochemistry around the pseudo-P_2 site in **30** and **31** was tentatively assigned as (*S*) and (*R*), respectively, based on comparison of the calpain inhibitory activity of **30** and **31** with that of the reference dipeptidyl aldehyde. While oxidation of compound **27** by sulfur trioxide-pyridine complex in DMSO-CH_2Cl_2 produced the aldehyde **32** (Scheme 6), oxidation by Davis' oxaziridine[11] generated the corresponding sulfoxide **33**, which on further oxidation by sulfur trioxide–pyridine complex in DMSO-CH_2Cl_2 gave the target aldehyde **34** (diastereomeric mixture, epimeric at the sulfoxide center).

In vitro biology and discussion

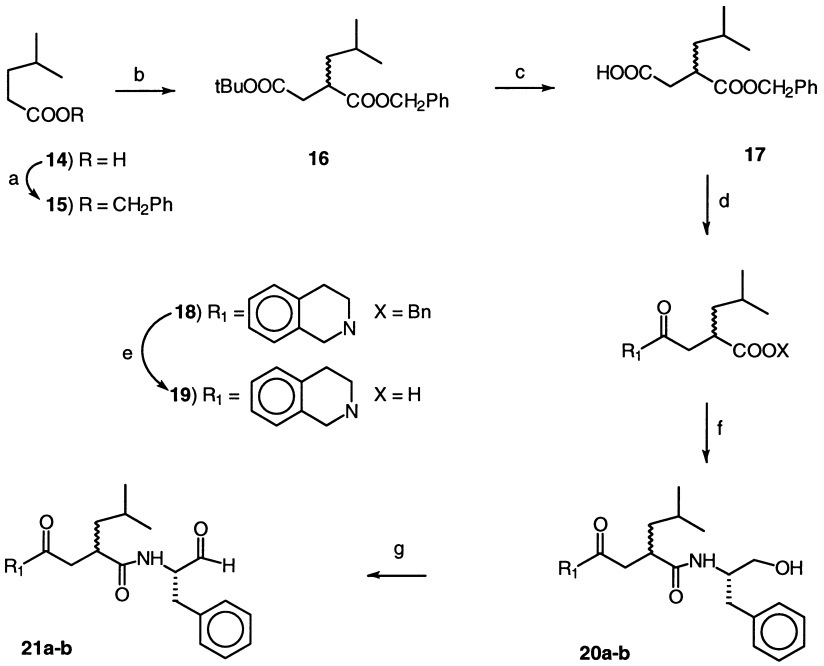
The biological activities of the compounds were determined using recombinant human calpain I, prepared as described by Meyer et al.[12] with Suc-Leu-Tyr-MNA



Scheme 2. Reagents: (a) NMM, IBCF, THF-DMF, −20 to 23 °C or BOP, HOBt, NMM, DMF, 0–23 °C; (b) Dess–Martin periodinane, CH_2Cl_2, 0–23 °C.



Scheme 3. Reagents: (a) NMM, IBCF, **5d**, CH_2Cl_2, −20 to 23 °C or BOP, HOBt, NMM, DMF, 0–23 °C; (b) Dess–Martin periodinane, CH_2Cl_2, 23 °C.

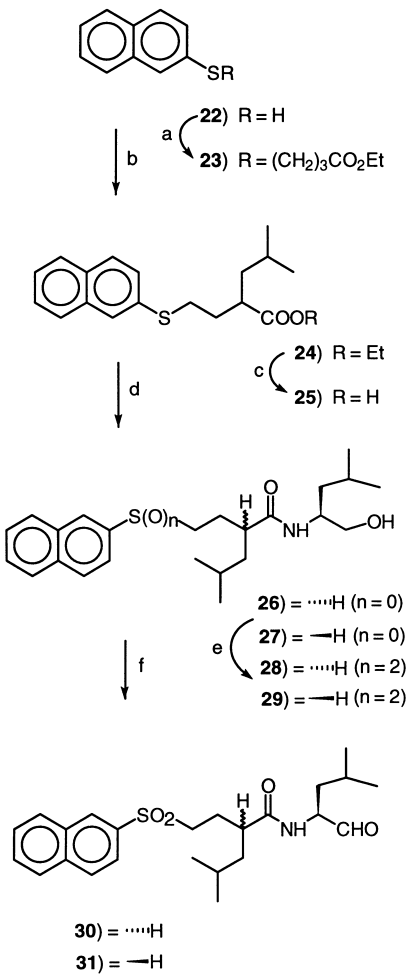


Scheme 4. Reagents: (a) Benzyl alcohol, *p*-toluenesulfonic acid monohydrate, benzene, reflux (Dean–Stark); (b) LDA, *t*-butyl bromoacetate, THF-hexane-HMPA, −78 °C to 0 °C; (c) 90% TFA, CH_2Cl_2, 23 °C; (d) 1,2,3,4-tetrahydroisoquinoline, BOP-Cl, Et_3N, CH_2Cl_2, 23 °C; (e) H_2, 10% Pd-C (Degussa, H_2O content 50%), MeOH; (f) BOP, HOBt, NMM, DMF, 0–23 °C; (g) Pyr.SO_3, Et_3N, DMSO-CH_2Cl_2, 0–23 °C.

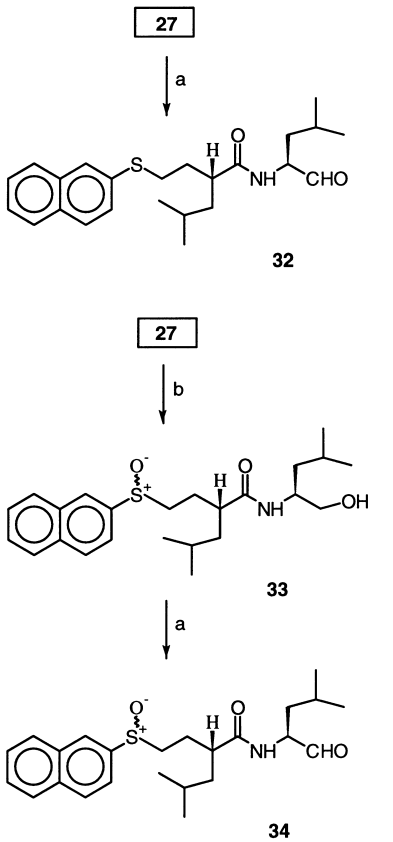
(Enzyme System Products, Dublin, CA) as substrate. Table 1 displays the inhibitory data for the ketomethylene containing inhibitors. In the aldehyde series, in order to probe the steric requirement of calpain I in the P_3 region, initially we attached a phenylbutyl chain to the pseudo P_2-site to generate compound **7a**, a good inhibitor. Shortening the phenylalkyl chain and having an alkyl (ethyl) substitution at the carbon atom (pseudo P_3-site), α to the carbonyl group, gave **7b**, which is ca. 2.5 times more potent than **7a**. Interestingly, substitution of the ethyl group in **7b** with a phenyl group to generate **7c** did not diminish the activity. Thus, it appeared that calpain might tolerate a sterically demanding group in the P_3 region. Constraining the aromatic rings of **7c** into a xanthene moiety, produced **7d**, a >fivefold more potent inhibitor than **7a**. In a similar way, in the α-ketocarboxamide series (**10a**–**c**), xanthene containing compound **10c** was ca. 65-fold more potent than the parent phenylbutyl containing **10a**; however, note that the P_1 in **10a** was Abu (R_2 = Et). Finally, xanthene containing fluoromethyl ketone **13**, an irreversible inhibitor, also displayed good inactivation of calpain I. In order to explore whether the P_3-spanning xanthene moiety could be replaced by a different heterocycle, we generated diastereomeric (at the pseudo P_2) compounds **21a**–**b**, containing a tetrahydrohydroisoquinoline nucleus. Compound **21a** was equipotent to compound **7d**. As shown, this compound was ca. 36 times more potent than the diastereomeric compound **21b** indicating the strict stereochemical requirement of calpain I for the pseudo P_2 site of this class of inhibitor. Thus, it appeared that the -NHCO- moiety at the P_2-P_3 region of a potent tripeptide (e.g. Cbz-Leu-Leu-Leu-H: IC_{50} 19 nM in this assay[4a]) or a dipeptide inhibitor could effectively be replaced by a -CH_2CO- moiety. In order to explore the importance of the -CO- moiety in the above ketomethylene series, we wished to replace the ketomethylene moiety with a carbamethylene (-CH_2CH_2-) moiety; at the same time, the xanthene (or tetrahydroisoquinoline) moiety was replaced by a naphthalene-S(O)$_n$-moiety, a different aromatic system. The inhibitory data for this series of carbamethylene containing inhibitors is shown in Table 2. Compounds **31**–**34** were good inhibitors of calpain I, compound **34** (as a diastereomeric mixture at the sulfoxide centre) being equipotent to compounds **7d** and **21a**. In this series also, compound **31** was 10 times more potent than than diastereomeric compound **30**, revealing the importance of the correct stereochemistry at the pseudo P_2 site.

Selectivity

Cathepsin B is a related cysteine protease which might be sensitive to inhibition by the above set of compounds. Thus, we tested compounds **7d**, **10c**, **13**, **31**, **32**,



Scheme 5. Reagents: (a) 60% NaH, $Br(CH_2)_3COOEt$, THF, 0–23 °C; (b) LDA, 1-iodo-2-methylpropane, THF-hexane-HMPA, −78 °C to 23 °C; (c) $LiOH.H_2O$, $EtOH-H_2O$, reflux; (d) (*S*)-leucinol, BOP, HOBt, NMM, DMF, 0–23 °C; (e) *m*-CPBA, CH_2Cl_2, 0–23 °C; (f) $Pyr.SO_3$, Et_3N, $DMSO-CH_2Cl_2$, 0–23 °C.



Scheme 6. Reagents: (a) $Pyr.SO_3$, Et_3N, $DMSO-CH_2Cl_2$, 0–23 °C; (b) Davis' oxaziridine ($PhSO_2N(O)CHPh$), CH_2Cl_2, 23 °C.

and **34** against cathepsin B (substrate used Cbz-Phe-Arg-AMC); Table 3 lists the inhibitory data. While, compounds **7d**, **10c**, and **13** preferred calpain I by > 17-fold, ca. ninefold and 76-fold, respectively, over cathepsin B, compounds **31** and **34** preferred calpain I by threefold and twofold, respectively, over cathepsin B. The inhibitory activity of these compounds against thrombin (substrate used: Cbz-Phe-Val-Arg-AMC), a serine protease, is also shown in Table 3. Compounds were less active against thrombin up to 10 μM.

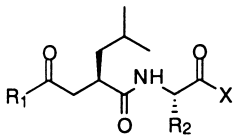
Cellular activity

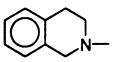
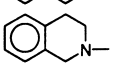
In order to probe the ability of these compounds to penetrate cells and inhibit intracellular calpain I, we tested a set of compounds in an intact cell assay system. Treatment of Molt 4 cells (human leukemic T cells) with calcium ion and an ionophore results in the elevation of intracellular calcium 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 3 lists the cellular activities of those compounds which displayed an $IC_{50} \leqslant 50$ nM in the enzyme assay. As shown, the reversible inhibitors **7d**, **31**, and **34** are cell-permeable and inhibit intracellular calpain I ($IC_{50} < 10$ μM); irreversible inhibitor **13** is less potent.

Conclusion

We have described potent ketomethylene and carbamethylene containing peptidomimetic inhibitors of recombinant calpain I. Our study revealed, for the first time, that the -NHCO-moiety (possible hydrogen-bonding site) at the P_2-P_3 region of a tripeptide or a dipeptide

Table 1. Recombinant human calpain I inhibitory activity of compounds **7a–d**, **10a–c**, **13**, and **21a–b**[a,b,c,d]



Compd	R_1	R_2	X	IC_{50} nM	$k_{obs}/I\ M^{-1}\ s^{-1}$
7a	$-(CH_2)_4Ph$	*i*Bu	H	138	—
7b	-(s)-CH(Et)Ph	*i*Bu	H	55	—
7c	$-CH(Ph)_2$	*i*Bu	H	50	—
7d	Xanthen-9-yl	*i*Bu	H	25	—
10a	$-(CH_2)_4Ph$	Et	CONHEt	8440	—
10b[e]	-(s)-CH(Et)Ph	Et	CONHEt	6685	—
10c	Xanthen-9-yl	*i*Bu	CONHEt	130	—
13	Xanthen-9-yl	Bn	CH_2F	-	76,000
21a		Bn	H	28	—
21b		Bn	H	1000	—

[a] $n = 3$, in all cases.
[b] For compounds **7a–d**, **10a–c** and **21a–b**, the stereochemistry at P_1 is (*S*); **13** is diastereomeric mixture at P_1.
[c] For compounds **7a**,**c–d**, **10a**,**c**, **13**, **21a**, the stereochemistry at P_2 is (*R*); for **21b**, the stereochemistry at P_2 is (*S*); **7b** and **10b** are diasrereomeric mixture at P_2.
[d] In the same assay, the IC_{50} values for the reference compounds Cbz-Val-Phe-H (MDL 28170)[4b] and Cbz-Leu-Abu-CONHEt[4c] were 17 nM and 240 nM, respectively; $k_{obs}/I\ M^{-1}\ s^{-1}$ for Cbz-Leu-Phe-CH_2F[5a] was 136,000.
[e] Elemental analysis: $C_{24}H_{36}N_2O_4.1.25\ H_2O$; calcd N 6.38; found N 7.22.

inhibitor of calpain I is not a strict requirement for the inhibitory property. We have demonstrated that such an -NHCO- moiety can effectively be replaced by a keto-methylene or a carbamethylene moiety, provided an aromatic system is employed in the P_3 region. In the absence of protein structural data, identification of such inhibitor binding motif can be used to design next generation nonpeptidic ligands. Work is currently underway to develop such inhibitors and will be reported in due course.

Experimental

General methods

Thin-layer chromatography was done on silica gel plate (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. ^1H 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, USA.

Synthesis of 2a–d: general procedure[13]

To a cooled (0 °C) solution of acid **1a–d** (0.04–0.05 mol) in anhydrous tetrahydrofuran (40–50 mL) was added 1,1′-carbonyldiimidazole (1.05 equiv). The mixture was stirred at 0 °C for 0.5 h and then at room temperature overnight. The next morning, this solution was added slowly, over 1 h, to a cooled (−78 °C) solution of *tert*-butyl lithioacetate (2.2 equiv, generated in situ from *t*-butyl acetate and lithium diisopropylamide) in tetrahydrofuran (40–50 mL) and hexane (35–40 mL). The mixture was stirred for an additional 0.5 h and quenched with 1 N HCl (2.2 equiv), brought to 0 °C and acidified with 1 N HCl to pH 3–4. The resulting aqueous solution was extracted with ethyl acetate (2×100 mL). The

Table 2. Recombinant human calpain I inhibitory activity of compounds **30–31**, **32**, and **34**[a]

Compd	IC_{50} nM
30	500
31	50
32	75
34	30

[a] $n = 3$, in all cases.

Table 3. Selectivity and intact cell assay data for selected compounds[a]

Compound	Calpain I (IC_{50} nM)	Cathepsin B (IC_{50} nM)	Thrombin (% inh at 10 μM)	Intact cell assay (% inh of SBDP at 10 μM)
7d	25	440	25	65
10c	130	1150	13	—
13	76,000[b]	1,000[b]	9	24
31	50	150	1	80
32	75	60	5	—
34	30	60	2	68

[a]For calpain I, cathepsin B, and thrombin, $n = 3$; for intact cell assay, $n = 2$.
[b]Rate of inactivation (k_{obs}/I M^{-1} s^{-1}).

organic layer was washed with brine (1×40 mL), dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. Purification of the crude material by flash chromatography (silica gel, eluant: 5–6% ethyl acetate in hexanes) gave the desired product **2a–d** in 60–70% yield.

***t*-Butyl 3-oxo-7-phenylheptanoate (2a).** Colorless oil; R_f (10% ethyl acetate in hexane): 0.37; ^{1}H NMR ($CDCl_3$) δ 7.30–7.10 (m, 5H), 3.35 (s, 2H), 2.60 (m, 2H), 2.50 (m, 2H), 1.60 (m, 4H), 1.45 (s, 9H).

***t*-Butyl 3-oxo-4-phenylhexanoate (2b).** Colorless oil; R_f (10% ethyl acetate in hexane): 0.49; ^{1}H NMR ($CDCl_3$) δ 7.38–7.18 (m, 5H), 3.70 (t, 1H), 3.35 (d, 1H), 3.20 (d, 1H), 2.10 (m, 1H), 1.70 (m, 1H), 1.45 (s, 9H), 0.85 (t, 3H).

***t*-Butyl 3-oxo-4,4-diphenylbutanoate (2c).** Colorless oil; R_f (10% ethyl acetate in hexane): 0.47; ^{1}H NMR ($CDCl_3$) δ 7.40–7.20 (m, 10H), 5.35 (s, 1H), 3.45 (s, 1H), 1.45 (s, 9H).

***t*-Butyl 3-oxo-4-(9-xanthenyl)propionoate (2d).** White solid, mp 101–103 °C; R_f (10% ethyl acetate in hexane): 0.46; ^{1}H NMR ($CDCl_3$) δ 7.40–7.00 (m, 8H), 5.00 (s, 1H), 3.20 (s, 2H), 1.40 (s, 9H).

Synthesis of 4a–d: General procedure[10]. A solution of the keto-ester **2a–d** (0.02–0.03 mol) in anhydrous tetrahydrofuran (20–25 mL) was slowly added, at room temperature, to a slurry of sodium hydride (60% in oil, 1.05 equiv) in anhydrous tetrahydrofuran (10–15 mL). After the evolution of hydrogen gas ceased, the solution was treated with the triflate-ester **3** (1.2–1.3 equiv, generated from the corresponding (*R*)-hydroxyester and triflic anhydride in the presence of 2,6-lutidine). The reaction mixture was stirred overnight, diluted with ether (100–150 mL), washed with water (30–40 mL), dried over magnesium sulfate and concentrated under reduced pressure to give the crude diester intermediate. This material was dissolved in trifluoroacetic acid (8–10 mL) and stirred at room temperature for 1–2 h. Excess trifluoroacetic acid was removed and the residue was taken into benzene (30–40 mL) and heated at reflux for 1–2 h. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography (silica–gel, eluant: 4–5% ethyl acetate in hexanes) to give the desired compounds **4a–d** in 35–45% yield over three steps.

Methyl 2-isobutyl-4-oxo-8-phenyloctanoate (4a). Colorless oil; R_f (10% ethyl acetate in hexane): 0.34; ^{1}H NMR ($CDCl_3$) δ 7.35–7.15 (m, 5H), 3.65 (s, 3H), 2.90–2.80 (m, 2H), 2.60 (m, 2H), 2.50–2.30 (m, 3H), 1.70–1.50 (m, 6H), 1.25 (m, 1H), 1.95 (d, 3H), 1.85 (d, 3H).

Methyl 2-isobutyl-4-oxo-(*S*)-5-phenylheptanoate (4b). Colorless oil; R_f (10% ethyl acetate in hexane): 0.48; ^{1}H NMR ($CDCl_3$) δ 7.40–7.18 (m, 5H), 3.60 (s, 3H), 3.50 (t, 1H), 2.85 (m, 1H), 2.75 (m, 1H), 2.45 (dd, 1H), 2.05 (m, 1H), 1.70 (m, 1H), 1.45 (m, 2H), 1.15 (m, 1H), 0.90–0.70 (m, 1H).

Methyl 2-isobutyl-4-oxo-5,5-biphenylpentanoate (4c). White solid, mp 64.5–65.5 °C; R_f (10% ethyl acetate in hexane): 0.41; ^{1}H NMR ($CDCl_3$) δ 7.40–7.15 (m, 10H), 5.15 (s, 1H), 3.65 (s, 3H), 3.00–2.85 (m, 2H), 2.60 (q, 1H), 1.50 (m, 2H), 1.20 (m, 1H), 0.90 (d, 3H), 0.80 (d, 3H).

Methyl 2-isobutyl-4-oxo-4-(9-xanthenyl)butanoate (4d). Colorless oil; R_f (10% ethyl acetate in hexane): 0.40; ^{1}H NMR ($CDCl_3$) δ 7.40–7.18 (m, 8H), 4.90 (s, 1H), 3.55 (s, 3H), 2.80–2.60 (m, 2H), 2.30 (dd, 1H), 1.30 (m, 2H), 1.00 (m, 1H), 0.80 (d, 3H), 0.70 (d, 3H).

Synthesis of 5a–d: General procedure. A mixture of the ester **4a–d** (0.005–0.006 mol), lithium hydroxide-monohydrate (1.3–1.4 equiv), methanol (25–30 mL) and water (8–10 mL) was gently heated at 70–75 °C for 1.5–2.0 h. Methanol was removed under reduced pressure. The aqueous layer was washed with diethyl ether (20–25 mL), acidified at 0 °C with 1 N HCl and then extracted

into diethyl ether (3×20 mL). The organic layer was washed with brine (1×10 mL) and dried over anhydrous sodium sulfate. Solvent evaporation at reduced pressure yielded the intermediates **5a–d** in 85–90% yield, which were used without further purification.

2-Isobutyl-4-oxo-8-phenyloctanoic acid (5a). Colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.35 (m, 5H), 3.00–2.70 (m, 2H), 2.60 (m, 2H), 2.50–2.30 (m, 3H), 1.70–1.50 (m, 6H), 1.35–1.20 (m, 1H), 1.95 (d, 3H), 1.85 (d, 3H).

2-Isobutyl-4-oxo-(*S*)-5-phenylheptanoic acid (5b). White solid, mp 61–63 °C; ^{1}H NMR ($CDCl_3$) δ 7.40–7.10 (m, 5H), 3.60 (m, 1H), 2.90 (m, 1H), 2.75 (m, 1H), 2.50 (m, 1H), 2.05 (m, 1H), 1.70 (m, 1H), 1.50 (m, 2H), 1.15 (m, 1H), 0.90-0.70 (m, 9H).

2-Isobutyl-4-oxo-5,5-biphenylpentanoic acid (5c). White solid, mp 81.5–83.5 °C; ^{1}H NMR ($CDCl_3$) δ 7.40–7.10 (m, 10H), 5.15 (s, 1H), 3.00–2.85 (m, 2H), 2.60–2.70 (m, 1H), 1.60–1.40 (m, 2H), 1.30–1.20 (m, 1H), 0.90 (d, 3H), 0.80 (d, 3H).

2-Isobutyl-4-oxo-4-(9-xanthenyl)butanoic acid (5d). White solid, mp 125–127 °C; ^{1}H NMR ($CDCl_3$) δ 7.40–7.00 (m, 8H), 4.95 (s, 1H), 2.80–2.60 (m, 2H), 2.30 (dd, 1H), 1.35 (m, 1H), 1.00 (m, 1H), 0.80 (d, 3H), 0.70 (d, 3H).

Synthesis of 6a–d: General procedure. To a cooled (0 °C) solution of **5a–d** (0.0005–0.001 mol) in anhydrous *N*,*N*-dimethylformamide (3–4 mL) was added *N*-methylmorpholine (3 equiv) followed by 1-HOBt (1 equiv) and BOP (1 equiv). The mixture was stirred for 15 min and to it was added (*S*)-leucinol (1.3–1.4 equiv). The cooling bath was removed and the mixture was stirred for 1–2 h, poured into water (5 mL) and extracted into ethyl acetate (3×10 mL). The organic layer was washed with 2% citric acid solution (2×5 mL), saturated sodium bicarbonate solution (2×5 mL), brine (1×5 mL) and dried over anhydrous sodium sulfate. Solvent evaporation under reduced pressure gave a crude material that was purified by flash column chromatography (silica gel, eluant: 4–5% methanol-methylene chloride) to produce **6a–d** in 40–60% yield.

(*R*)-2-Isobutyl-4-oxo-8-phenyloctanoic acid ((*S*)-1-hydroxymethyl-3-methyl)butyl amide (6a). Viscous oil; R_f (5% methanol in methylene chloride) 0.40; ^{1}H NMR ($CDCl_3$) δ 7.40–7.10 (m, 5H), 5.85 (d, 1H), 3.85 (m, 1H), 3.60 (m, 1H), 3.50 (m, 1H), 3.00–2.80 (m, 2H), 2.70 (m, 1H), 2.60 (m, 2H), 2.50–2.30 (m, 3H), 1.70–1.50 (m, 8H), 1.45–1.20 (m, 2H), 1.00–0.80 (m, 12H).

(*R*)-2-Isobutyl-4-oxo-(*S*)-5-phenylheptanoic acid ((*S*)-1-hydroxymethyl-3-methyl)butyl amide (6b). White gum; R_f (5% methanol in methylene chloride) 0.36; ^{1}H NMR ($CDCl_3$) δ 7.40–7.10 (m, 5H), 5.85 (m, 1H), 4.00–3.80 (m, 1H), 3.70–3.40 (m, 3H), 3.00–2.70 (m, 2H), 2.50–2.30 (td, 1H), 2.00 (m, 1H), 1.80–1.20 (m, 8H), 1.00–0.70 (m, 15H).

(*R*)-2-Isobutyl-4-oxo-5,5-biphenylpentanoic acid ((*S*)-1-hydroxymethyl-3-methyl)butyl amide (6c). White solid, mp 111–112 °C; R_f (5% methanol in methylene chloride) 0.45; ^{1}H NMR ($CDCl_3$) δ 7.40–7.10 (m, 10H), 5.85 (d, 1H), 5.10 (s, 1H), 3.85 (m, 1H), 3.60 (m, 1H), 3.50 (m, 1H), 3.00 (q, 1H), 2.80–2.70 (m, 2H), 2.30 (dd, 1H), 1.60–1.00 (m, 6H), 1.00–0.80 (m, 12H).

(*R*)-2-Isobutyl-4-oxo-4-(9-xanthenyl)butanoic acid ((*S*)-1-hydroxymethyl-3-methyl)butyl amide (6d). White solid, mp 168–169 °C; R_f (5% methanol in methylene chloride) 0.55; ^{1}H NMR ($CDCl_3$) δ 7.40–7.00 (m, 8H), 5.70 (d, 1H), 4.90 (s, 1H), 3.85 (m, 1H), 3.60 (m, 1H), 3.50 (m, 1H), 2.80 (t, 1H), 2.70 (q, 1H), 2.50 (m, 1H), 2.30 (dd, 1H), 1.60 (m, 1H), 1.50–1.20 (m, 5H), 1.00-0.60 (4 sets of doublets, 12H).

Synthesis of 7a–d: General procedure. To a cooled (0 °C) solution of alcohol **6a–d** (0.05–0.10 mmol) in anhydrous methylene chloride (2–3 mL) and anhydrous dimethyl sulfoxide (2–3 mL) was added triethylamine (3 equiv). Sulfur trioxide-pyridine complex (3 equiv) was slowly added to the stirred mixture over a period of 5 min and the ice-bath was removed. The mixture was stirred for another 1 h, poured into water (10 mL) and extracted into ether (3×10 mL). The organic layer was washed with 2% citric acid solution (2×5 mL), saturated sodium bicarbonate solution (2×5 mL), brine (1×5 mL) and dried over anhydrous magnesium sulfate. Solvent evaporation gave a residue that was washed with *n*-pentane (5–8 mL) and recrystallized (ethyl acetate-hexanes) to produce compounds **7a–d** in 50–60% yield.

(*R*)-2-Isobutyl-4-oxo-8-phenyloctanoic acid ((*S*)-1-formyl-3-methyl)butyl amide (7a). White solid, mp 64–65 °C; R_f (30% ethyl acetate in hexanes): 0.48; ^{1}H NMR ($CDCl_3$) δ 9.55 (s, 1H), 7.30–7.10 (m, 5H), 6.10 (d, 1H), 4.50 (m, 1H), 3.00–2.80 (m, 2H), 2.60 (m, 1H), 2.40 (m, 4H), 1.80–1.60 (m, 8H), 1.50–1.10 (m, 2H), 1.00–0.80 (m, 12H). MS *m*/*e* 388 (M + H), 410 (M + Na). Anal. ($C_{24}H_{27}NO_3$) C, H, N.

(*R*)-2-Isobutyl-4-oxo-(*S*)-5-phenylheptanoic acid ((*S*)-1-formyl-3-methyl)butyl amide (7b). Viscous liquid: R_f (30% ethyl acetate in hexanes): 0.52; ^{1}H NMR ($CDCl_3$) δ 9.55 and 9.45 (2 singlets, 3:2), 7.40–7.10 (m, 5H), 6.10 (m, 1H), 4.60–4.30 (2 sets of multiplate, 3:2, 1H), 3.00–2.70 (m, 2H), 2.50–2.30 (td, 1H), 2.00 (m, 1H), 1.80–1.20 (m, 8H), 1.00–0.70 (m, 15H). MS *m*/*e* 374 (M + H). Anal. ($C_{23}H_{35}NO_3.0.4\ H_2O$) C, H, N.

(*R*)-2-Isobutyl-4-oxo-5,5-biphenylpentanoic acid ((*S*)-1-formyl-3-methyl)butyl amide (7c). White solid, mp 85–95 °C (softening to melt); R_f (30% ethyl acetate in hexanes): 0.45; ^{1}H NMR ($CDCl_3$) δ 9.55 (s, 1H), 7.40–7.10 (m, 10H), 6.10 (d, 1H), 5.10 (s, 1H), 4.50 (m, 1H), 3.00 (q, 1H), 2.90–2.70 (m, 1H), 2.60 (dd, 1H), 1.80–1.00 (m, 6H), 1.00–0.80 (m, 12H). MS *m*/*e* 422 (M + H). Anal. ($C_{27}H_{35}NO_3.0.5H_2O$) C, H, N.

(*R*)-2-Isobutyl-4-oxo-4-(9-xanthenyl)butanoic acid ((*S*)-1-formyl-3-methyl)butyl amide (7d). White solid, mp 115–125 °C (softening to melt); R_f (30% ethyl acetate in hexanes): 0.45; ^{1}H NMR ($CDCl_3$) δ 9.50 (s, 1H), 7.40–7.00 (m, 8H), 6.00 (d, 1H), 4.90 (s, 1H), 4.40 (m, 1H), 2.80–2.65 (q, 1H), 2.60 (m, 1H), 2.30 (dd, 1H), 1.80–1.20 (m, 6H), 1.00-0.60 (4 sets of doublets, 12H). MS *m*/*e* 435 (M + H). Anal. ($C_{27}H_{33}NO_4.0.5H_2O$) C, H, N.

Synthesis of 9a–c: General procedure. To a cooled (−20 °C) solution of **5a**–**b**,**d** (0.50–1.00 mmol) in anhydrous tetrahydrofuran (3–4 mL) was added *N*-methylmorpholine (3.3 equiv) followed by isobutyl chloroformate (1.1 equiv). The mixture was stirred for 15 min by which time the temperature rose to 0 °C. A solution of **8a**,**b** (1 equiv) in anhydrous *N*,*N*-dimethylformamide (3–4 mL) was added to the reaction mixture. The cooling bath was removed and the mixture was stirred for another 2 h. It was then poured into water (5–8 mL) and extracted into ethyl acetate (3×15 mL). The organic layer was washed with 2% citric acid solution (2×5 mL), saturated sodium bicarbonate solution (2×5 mL), brine (1×5 mL) and dried over anhydrous sodium sulfate. Solvent evaporation under reduced pressure gave a crude material that was purified by flash chromatography (silica gel, eluant: 2–3% methanol-methylene chloride) to produce **9a**–**c** in 25–30% yield. The coupling reaction was also successful in the presence of BOP/HOBt/NMM, as described previously.

***N*-((*R*)-2-Isobutyl-4-oxo-8-phenyl)octanoyl-3-(S)-amino-2-(*R*,*S*)-hydroxypentanoic acid ethyl amide (9a).** Mixture of diastereomers (at the hydroxy centre); white solid, mp 97–99 °C; R_f (5% methanol in methylene chloride) 0.47; ^{1}H NMR ($CDCl_3$) δ 7.30–7.10 (m, 5H), 6.95–6.80 (broad, 1H), 6.30 (d, 1H), 5.80–5.50 (broad, 1H), 4.15 (s, 1H), 3.90–3.80 (m, 1H), 3.40–3.10 (m, 2H), 2.90–2.70 (m, 2H), 2.65–2.55 (broad, 2H), 2.45–2.35 (m, 3H), 2.00–1.70 (m, 2H), 1.65–1.40 (m, 7H), 1.20–1.00 (m, 3H), 0.95-0.75 (m, 9H).

***N*-((*R*)-2-Isobutyl-4-oxo-(*S*)-5-phenyl)heptanoyl-3-(*S*)-amino-2-(*R*,*S*)-hydroxypentanoic acid ethyl amide (9b).** Diastereomers (at the hydroxy centre) were separated; faster moving isomer: white solid, mp 150–160 °C (softening to melt); R_f (5% methanol in methylene chloride) 0.37; ^{1}H NMR ($CDCl_3$) δ 7.40–7.10 (m, 5H), 7.00–6.80 (broad, 1H), 6.35–6.20 (m, 1H), 5.35–5.20 (m, 1H), 5.20–5.00 (m, 1H), 3.60–3.50 (m, 1H), 3.40–3.20 (m, 3H), 2.90–2.70 (m, 2H), 2.40 (dt, 1H), 2.10–1.90 (m, 2H), 1.80–1.60 (m, 2H), 1.50–1.30 (m, 1H), 1.25–1.10 (m, 3H), 1.00–0.70 (m, 12H). Slower moving isomer: white solid, mp 115–130 °C (softening to melt); R_f (5% methanol in methylene chloride) 0.35; ^{1}H NMR ($CDCl_3$) δ 7.40–7.10 (m, 5H), 7.00–6.80 (broad, 1H), 6.35–6.20 (m, 1H), 5.35–5.20 (m, 1H), 5.20–5.00 (m, 1H), 3.60–3.50 (m, 1H), 3.40–3.20 (m, 3H), 2.90–2.70 (m, 2H), 2.40 (dt, 1H), 2.10–1.90 (m, 2H), 1.80–1.60 (m, 2H), 1.50–1.30 (m, 1H), 1.25–1.10 (m, 3H), 1.00–0.70 (m, 12H).

***N*-((*R*)-2-Isobutyl-4-oxo-4-(9-xanthenyl))butanoyl-3-(*S*)-amino-2-(*R*,*S*)-hydroxy-5-methylhexanoic acid ethyl amide (9c).** Mixture of diasteromers (at the hydroxy center): white solid, mp 196–201 °C (softening to melt); R_f (5% methanol in methylene chloride) 0.47; ^{1}H NMR ($CDCl_3$) δ 7.40–7.00 (m, 8H), 6.95–6.85 (broad, 1H), 6.05 (d, 1H), 5.35 (d, 1H), 4.90 (s, 1H), 4.05 (d, 1H), 4.00–3.90 (m, 1H), 3.40–3.20 (m, 1H), 3.20–3.10 (m, 1H), 2.65–2.60 (dd, 1H), 2.60–2.45 (m, 1H), 2.30–2.20 (dd, 1H), 1.90–1.70 (m, 2H), 1.70–1.50 (m, 1H), 1.45–1.20 (m, 3H), 1.15 (t, 3H), 0.95 (t, 3H), 0.85 (t, 3H), 0.80 (t, 3H), 0.70 (t, 3H).

Synthesis of 10a–c: General procedure. To a cooled (0 °C) solution of **9a**–**c** (0.05–0.10 mmol) in anhydrous methylene chloride (2–3 mL) was added Dess–Martin periodinane reagent (3–4 equiv). The cooling bath was removed and the mixture was stirred for an additional 30–45 min. It was then diluted with methylene chloride (10–15 mL) and washed with 10% sodium thiosulfate solution (5×5 mL), saturated sodium bicarbonate solution (2×5 mL) and brine (1×5 mL). Drying over anhydrous sodium sulfate and solvent removal under reduced pressure gave a material that was washed with *n*-pentane (5–8 mL) and recrystallized (ethyl acetate-hexanes) to generate the desired targets **10a**–**c** in 50–60% yield.

***N*-((*R*)-2-Isobutyl-4-oxo-8-phenyl)octanoyl-3-(*S*)-amino-2-oxopentanoic acid ethyl amide (10a).** White solid, mp 123–124 °C; R_f (5% methanol in methylene chloride) 0.50; ^{1}H NMR ($CDCl_3$) δ 7.30–7.10 (m, 5H), 6.95–6.80 (broad, 1H), 6.30 (d, 1H), 5.30–5.10 (m, 1H), 3.40–3.30 (m, 2H), 2.90–2.70 (m, 2H), 2.65–2.55 (broad, 2H), 2.45–2.35 (m, 3H), 2.10–1.90 (m, 1H), 1.75–1.40 (m, 8H), 1.30–1.00 (m, 3H), 0.95–0.75 (m, 9H). MS *m*/*e* 431 (M + H), 453 (M + Na). Anal. ($C_{25}H_{38}N_2O_4.0.25\ H_2O$) C, H, N.

***N*-((*R*)-2-Isobutyl-4-oxo-(*S*)-5-phenyl)heptanoyl-3-(*S*)-amino-2-oxopentanoic acid ethyl amide (10b).** White solid, mp 105–115 °C (softening to melt); R_f (5%

methanol in methylene chloride) 0.53; ^{1}H NMR ($CDCl_3$) δ 7.40–7.10 (m, 5H), 7.00–6.80 (broad, 1H), 6.35–6.20 (m, 1H), 5.35–5.20 (m, 1H), 5.20–5.00 (m, 1H), 3.60–3.50 (m, 1H), 3.40–3.20 (m, 3H), 2.90–2.70 (m, 2H), 2.40 (dt, 1H), 2.10–1.90 (m, 2H), 1.80–1.60 (m, 2H), 1.50–1.30 (m, 1H), 1.25–1.10 (m, 3H), 1.00–0.70 (m, 12H). MS *m*/*e* 417 (M + H), 439 (M + Na). Anal. ($C_{24}H_{36}N_2O_4$.1.25 H_2O) C, H; N: calcd, 6.38; found, 7.22.

***N*-(*R*)-2-Isobutyl-4-oxo-4-(9-xanthenyl))butanoyl-3-(*S*)-amino-5-methyl-2-oxohexanoic acid ethyl amide (10c).** White solid, mp 182–183 °C; R_f (5% methanol in methylene chloride) 0.70; ^{1}H NMR ($CDCl_3$) δ 7.40–7.00 (m, 8H), 6.85–6.75 (broad, 1H), 6.15 (d, 1H), 5.25–5.15 (m, 1H), 4.90 (s, 1H), 3.40–3.25 (m, 2H), 2.75–2.65 (dd, 1H), 2.60–2.50 (m, 1H), 2.40–2.25 (dd, 1H), 2.00 (m, 1H), 1.80–1.60 (m, 4H), 1.45–1.20 (m, 2H), 1.15 (t, 3H), 0.90 (t, 6H), 0.80 (t, 3H), 0.70 (t, 3H). MS *m*/*e* 507 (M + H), 529 (M + Na). Anal. ($C_{30}H_{38}N_2O_5$) C, H, N.

(*R*)-2-Isobutyl-4-oxo-4-(9-xanthenyl)butanoic acid (*R*,*S*)-1-benzyl-3-fluoro-(*R*,*S*)-2-hydroxypropyl amide (12). To a cooled (−20 °C) solution of **5d** (0.34 g, 1.00 mmol) in anhydrous methylene chloride (4 mL) was added *N*-methylmorpholine (0.212 g, 2.10 mmol) followed by isobutyl chloroformate (0.143 g, 1.05 mmol). The mixture was stirred for 15 min by which time the temperature rose to 0 °C. A solution of **11** (0.184 g, 1.00 mmol) in anhydrous methylene chloride (6 mL) was added to the reaction mixture. The cooling bath was removed and the mixture was stirred for another 2 h, poured into water (8 mL) and extracted into ethyl acetate (3×15 mL). The organic layer was washed with 2% citric acid solution (2×5 mL), saturated sodium bicarbonate solution (2×5 mL), brine (1×5 mL) and dried over anhydrous sodium sulfate. Solvent evaporation under reduced pressure gave a crude material that was purified by flash silica gel column chromatography (eluant: 2% methanol in methylene chloride) to produce 0.14 g (27%) of **12** (diastereomeric mixture) as a white solid. The coupling reaction was also successful in the presence of BOP/HOBt/NMM, as described previously; R_f (5% methanol in methylene chloride) 0.47; ^{1}H NMR ($CDCl_3$) δ 7.40–6.95 (m, 13H), 5.85–5.65 (2 sets of doublet, 1H), 4.80 (d, 1H), 4.60–4.20 (m, 3H), 4.10–3.75 (m, 2H), 3.10–2.60 (m, 3H), 2.50–2.15 (m, 2H), 1.30–1.10 (m, 1H), 1.00–0.75 (m, 2H), 0.70–0.50 (m, 6H).

(*R*)-2-Isobutyl-4-oxo-4-(9-xanthenyl)butanoic acid (*R*,*S*)-1-benzyl-3-fluoro-2-oxopropyl amide (13). To a cooled (0 °C) solution of **12** (0.030 g, 0.054 mmol) in anhydrous methylene chloride (4 mL) was added Dess–Martin periodinane reagent (0.045 g, 0.108 mmol). The cooling bath was removed and the mixture was stirred for an additional 30 min, diluted with methylene chloride (15 mL) and washed with 10% sodium thiosulfate solution (5×5 mL), saturated sodium bicarbonate solution (2×5 mL) and brine (1×5 mL). Drying over anhydrous sodium sulfate and solvent removal under reduced pressure gave a material that was washed with *n*-pentane (10 mL) and dried under vacuum to generate 0.020 g (74%) of **13** (diastereomeric mixture) as a white solid, R_f (30% ethyl acetate in hexanes): 0.40; ^{1}H NMR ($CDCl_3$) δ 7.30–6.95 (m, 13H), 6.05–5.85 (2 sets of doublet, 1H), 5.00–4.45 (m, 4H), 3.10–2.80 (m, 3H), 2.60–2.15 (m, 3H), 1.30–1.10 (m, 1H), 1.00–0.75 (m, 2H), 0.70–0.50 (m, 6H). MS *m*/*e* 502 (M + H), 524 (M + Na). Anal. ($C_{31}H_{32}FNO_4$.0.5H_2O) C, H, N.

Benzyl isocaproate (15). A mixture of isocaproic acid (**14**, 12.31 g, 0.106 mol), benzyl alcohol (10.88 g, 0.10 mol) and *p*-toluenesulfonic acid monohydrate (1.00 g) in dry benzene (100 mL) was refluxed using a Dean–Stark water separator for 2 h. After cooling, benzene was removed and the mixture was diluted with ether (50 mL) and washed successively with saturated $NaHCO_3$ solution (2×20 mL), brine (1×20 mL), dried ($MgSO_4$) and solvent evaporated to give **15** (20.00 g, 97%) as a colorless oil; R_f (5% ethyl acetate in hexanes): 0.46; ^{1}H NMR ($CDCl_3$) δ 7.40–7.20 (m, 5H), 5.10 (s, 2H), 2.35 (t, 2H), 1.55 (m, 3H), 0.90 (d, 6H).

***t*-Butyl 3-benzoyloxycarbonyl-5-methylhexanoate (16).** To a cooled (−78 °C) solution of lithium diisopropylamide (12 mmol, obtained in situ from diisopropylamine and *n*-butyllithium) in a mixture of tetrahydrofuran (20 mL) and hexane (4.8 mL) was added slowly the compound **15** (2.06 g, 10 mmol) in anhydrous tetrahydrofuran (8 mL). The mixture was stirred for 30 min and *tert*-butyl bromoacetate (2.34 g, 12 mol) in hexamethylphosphoramide (2.09 mL) was added to the flask. The mixture was stirred at −78 °C for 30 min, slowly brought to 0 °C over a period of 2 h and quenched by the cautious addition of saturated ammonium chloride (50 mL). The mixture was extracted into ether (3×50 mL) and the combined organic layer was washed with brine (1×25 mL), dried over anhydrous $MgSO_4$ and concentrated to give a crude material. Purification of this material by flash chromatography over silica gel (eluant: 2% ethyl acetate in hexanes) gave 2.00 g (63%) of racemic **16** as a colorless oil; R_f (5% ethyl acetate in hexanes): 0.34; ^{1}H NMR ($CDCl_3$) δ 7.40–7.30 (m, 5H), 5.15 (q, 2H), 2.90 (m, 1H), 2.60 (q, 1H), 2.35 (q, 1H), 1.60–1.20 (m, 3H), 1.40 (s, 9H), 1.00–0.80 (2 sets of doublet, 6H).

3-Benzoyloxycarbonyl-5-methylhexanoic acid (17). A mixture of **16** (0.54 g, 1.673 mmol) and 90% trifluoroacetic acid (1.5 mL) in methylene chloride (3 mL) was stirred at room temperature for 1.5 h. Solvent and excess reagent were removed under reduced pressure to give 0.44 g of crude **17**. An ^{1}H NMR ($CDCl_3$) of this

material showed no detectable peak for the *tert*-butyl ester moiety at δ 1.40. This material was used without further purification.

Benzyl 2-isobutyl-4-oxo-4-(2-(1,2,3,4-tetrahydroisoquinolinyl))butanoate (18). To a stirred mixture of **17** (0.95 g, 3.60 mmol) and 1,2,3,4-tetrahydroisoquinoline (0.48 g, 3.60 mmol) in anhydrous methylene chloride (8 mL) at room temperature, was added triethylamine (0.80 g, 7.92 mmol) followed by BOP-Cl (0.92 g, 3.60 mmol). The mixture was stirred overnight, diluted with methylene chloride (10 mL) and washed successively with water (1×5 mL), saturated $NaHCO_3$ (2×5 mL) and brine (1×5 mL). Drying over Na_2SO_4 and solvent evaporation gave a crude material that was purified by flash chromatography over silica (eluant: 20% ethyl acetate in hexanes) to give 0.89 g (65%) of **18**, as a colorless oil; R_f (20% ethyl acetate in hexane): 0.70; ^{1}H NMR ($CDCl_3$) δ 7.40–7.00 (m, 9H), 5.10 (q, 2H), 4.70 (q, 2H), 3.90–3.60 (m, 2H), 3.20–3.00 (m, 1H), 2.90–2.70 (m, 3H), 2.50–2.40 (m, 1H), 1.70–1.50 (m, 2H), 1.40–1.30 (m, 1H), 1.00–0.80 (m, 6H).

2-Isobutyl-4-oxo-4-(2-(1,2,3,4-tetrahydroisoquinolinyl))-butanoic acid (19). A mixture of the benzyl ester **18** (0.88 g, 2.32 mmol) and 10% Pd-C (0.30 g, Degussa, H_2O content 50%) in methanol (30 mL) was hydrogenated for 2 h in a Parr apparatus (40–30 psi). The reaction mixture was then filtered through a celite pad and concentrated to give 0.67 g (100%) of **19** as a pale-yellow solid, mp 95–105 °C (softening to melt); ^{1}H NMR ($CDCl_3$) δ 7.40–7.00 (m, 4H), 5.00–4.80 (q, 2H), 4.00–3.60 (m, 2H), 3.20–3.00 (m, 1H), 2.90–2.70 (m, 3H), 2.60–2.40 (m, 1H), 1.80–1.60 (m, 2H), 1.40–1.30 (m, 1H), 1.00–0.80 (m, 6H).

(*R*/*S*)-2-Isobutyl-4-oxo-4-(2-(1,2,3,4-tetrahydroisoquinolinyl))butanoic acid ((*S*)-(1-hydroxymethyl-2-phenyl))ethyl amides (20a,b). These compounds were generated following the same synthetic procedure as described before for the syntheses of the compounds **6a**–**d**. Thus, the coupling between 0.26 g (0.885 mmol) of **19** and 0.17 g of (*S*)-phenylalaninol gave **20a**,**b**, that were separated by flash silica gel column chromatography (eluant: ethyl acetate) to give 0.11 g (28%) of **20a** and 0.10 g (27%) of **20b**, respectively. Compound **20a**: White foam, R_f (ethyl acetate): 0.62; ^{1}H NMR ($CDCl_3$) δ 7.30–7.10 (m, 9H), 6.60 (d, 1H), 4.70–4.50 (q, 2H), 4.50 (s, 1H), 4.00 (m, 1H), 3.90–3.50 (m, 4H), 3.00–2.60 (m, 6H), 2.40–2.30 (m, 1H), 1.70–1.60 (m, 1H), 1.60–1.40 (m, 1H), 1.20 (m, 1H), 1.00–0.80 (m, 6H). Compound **20b**: White foam; R_f (ethyl acetate): 0.44; ^{1}H NMR ($CDCl_3$) δ 7.30–7.00 (m, 9H), 6.00 (m, 1H), 4.80–4.50 (q, 2H), 4.60 (s, 1H), 4.30 (m, 1H), 3.90–3.40 (m, 4H), 3.00–2.60 (m, 6H), 2.40–2.20 (m, 1H), 1.60–1.50 (m, 1H), 1.30–1.20 (m, 1H), 1.00 (m, 1H), 0.80–0.60 (m, 6H).

(*R*/*S*)-2-Isobutyl-4-oxo-4-(2-(1,2,3,4-tetrahydroisoquinolinyl))butanoic acid ((*S*)-(1-formyl-2-phenyl))ethyl amide (21a,b). These compounds were synthesized following the same synthetic procedure as described before for the syntheses of the compounds **7a**–**d**. Thus, oxidation of 0.10 g (0.24 mmol) of **20a** generated 0.05 g (50%) of **21a** and oxidation of 0.075 g (0.177 mmol) of **20b** generated 0.035 g (48%) of **21b**. Compound **21a**: White solid, mp 45–65 °C (softening to melt); R_f (70% ethyl acetate in hexane): 0.58; ^{1}H NMR ($CDCl_3$) δ 9.60 (s, 1H), 7.30–7.10 (m, 9H), 6.75 (d, 1H), 4.70–4.50 (m, 3H), 3.90–3.50 (m, 2H), 3.20–2.60 (m, 6H), 2.40–2.30 (m, 1H), 1.80–1.20 (m, 3H), 1.00–0.80 (m, 6H). MS *m*/*e* 421 (M + H). Anal. ($C_{26}H_{32}N_2O_3.H_2O$) C, H, N. Compound **21b**: White foam, R*f* (70% ethyl acetate in hexane): 0.41; ^{1}H NMR ($CDCl_3$) δ 9.50 (d, 1H), 7.40–7.10 (m, 9H), 6.60 (t, 1H), 4.70–4.50 (m, 3H), 3.90–3.50 (m, 2H), 3.20–2.60 (m, 6H), 2.40–2.30 (m, 1H), 1.70–1.10 (m, 3H), 1.00–0.80 (m, 6H). MS *m*/*e* 421 (M + H), 443 (M + Na). Anal. ($C_{26}H_{32}N_2O_3.2.2H_2O$) C, H, N.

Ethyl 4-(2-thionaphthyl)butyrate (23). To a stirred mixture of NaH (60% in oil, 2.04 g, 0.05 mol) in anhydrous THF (20 mL) at 0 °C was added slowly a solution of 2-naphthalenethiol (**22**, 7.79 g, 0.048 mol) in anhydrous THF (20 mL). The mixture was stirred for 30 min. A solution of ethyl 4-bromobutyrate (10.43 g, 0.053 mol) in anhydrous THF (20 mL) was slowly added to the reaction flask. The mixture was stirred for another 1 h, by which time temperature changed to room temperature. The reaction mixture was then poured into a ice-water mixture (100 mL) and extracted into ether (3×100 mL). The combined organic layer was washed with water (2×25 mL), brine (1×20 mL), dried ($MgSO_4$), and concentrated to give a crude product that was purified by flash silica gel column chromatography (eluant: 3% ethyl acetate in hexanes) to give 12.20 g (92%) of **23** as a colorless oil; R_f (5% ethyl acetate in hexanes): 0.25; ^{1}H NMR ($CDCl_3$) δ 7.80 (t, 4H), 7.50 (m, 3H), 4.15 (q, 2H), 3.10 (t, 2H), 2.50 (t, 2H), 2.00 (2 overlapping t, 2H).

Ethyl 2-isobutyl-4-(2-thionaphthyl)butyrate (24). To a cooled (−78 °C) solution of lithium diisopropylamide (0.053 mol, prepared in situ from corresponding *n*BuLi and diisopropylamine) in THF (40 mL) hexane (21 mL) was added slowly a solution of compound **23** (12.13 g, 0.044 mol) in anhydrous THF (20 mL). The mixture was stirred for 1 h and to it was added a solution of 1-iodo-2-methylpropane (9.76 g, 0.053 mol) in hexamethylphosphoramide (9.23 mL). The mixture was stirred for another 3 h, by which time the temperature slowly changed to room temperature. The reaction mixture was quenched with saturated NH_4Cl (50 mL), diluted with water (50 mL) and extracted into ether (3×100 mL). The combined organic layer was washed

with brine (1×30 mL), dried ($MgSO_4$), and concentrated to give a crude product that was purified by flash silica gel column chromatography (eluant: 2% ethyl acetate in hexanes) to give 4.60 g (32%) of **24** as a colorless oil; R_f (5% ethyl acetate in hexanes): 0.37; ^{1}H NMR ($CDCl_3$) δ 7.80 (t, 4H), 7.50 (m, 3H), 4.15 (q, 2H), 3.00 (m, 2H), 2.65 (m, 1H), 2.00 (m, 1H), 1.80 (m, 1H), 1.60 (m, 3H), 1.25 (t, 3H), 0.90 (t, 6H).

2-Isobutyl-4-(2-thionaphthyl)butyric acid (25). A mixture of compound **24** (4.53 g, 0.014 mol), lithium hydroxide monohydrate (2.30 g, 0.055 mol), ethanol (60 mL) and water (15 mL) was gently refluxed for 3 h. Ethanol was removed in vacuo and the basic aqueous layer was washed with ether (2×20 mL), acidified with 5 N HCl and extracted into ethyl acetate (3×50 mL). The combined organic layer was washed with brine (1×20 mL), dried ($MgSO_4$), and concentrated to give 3.80 g (92%) of **25** as a white solid, mp 76–78 °C; ^{1}H NMR ($CDCl_3$) δ 7.80 (t, 4H), 7.40 (m, 3H), 3.00 (m, 2H), 2.70 (m, 1H), 2.00 (m, 1H), 1.80 (m, 1H), 1.60 (m, 2H), 1.25 (m, 1H), 0.90 (t, 6H).

(*S*)/(*R*)-2-Isobutyl-4-(2-thionaphthyl)butyric acid ((*S*)-1-hydroxymethyl-3-methyl)butyl amide (26,27). These compounds were generated following the synthetic procedure as described above for the syntheses of the compounds **6a**–**d**. Thus, the coupling between **25** (3.35 g, 0.011 mol) and (*S*)-leucinol (1.70 g, 0.0143 mol), in the presence of BOP/HOBt/NMM, gave a mixture of compounds that were separated by column chromatography (silica gel, 30% ethyl acetate in hexanes) to produce 0.95 g (21%) of **26** and 1.10 g (25%) of **27**, respectively. Compound **26**: White solid, mp 96–98 °C; R_f (50% ethyl acetate in hexanes): 0.51; ^{1}H NMR ($CDCl_3$) δ 7.80 (t, 4H), 7.45 (m, 3H), 5.65 (d, 1H), 4.05 (m, 1H), 3.60 (m, 1H), 3.40 (m, 1H), 3.15 (m, 1H), 3.00 (m, 1H), 2.80 (b, 1H), 2.50 (m, 1H), 2.00 (m, 1H), 1.80–1.10 (m, 7H), 0.90 (m, 12H). Compound **27**: White solid, mp 85–90 °C; R_f (50% ethyl acetate in hexanes): 0.40; ^{1}H NMR ($CDCl_3$) δ 7.80 (m, 4H), 7.45 (m, 3H), 5.70 (d, 1H), 4.05 (m, 1H), 3.65 (m, 1H), 3.55 (m, 1H), 3.15 (m, 1H), 2.90 (m, 2H), 2.50 (m, 1H), 2.00 (m, 1H), 1.80-1.40 (m, 4H), 1.40–1.10 (m, 3H), 0.90 (t, 6H), 0.80 (t, 6H).

(*S*)/(*R*)-2-Isobutyl-4-(2-sulfonylnaphthyl)butyric acid ((*S*)-1-hydroxymethyl-3-methyl)butyl amide (28,29). To a solution of compound **26** (0.065 g, 0.1618 mmol) in methylene chloride (3 mL) at 0 °C was added *m*-chloroperbenzoic acid (95%, 0.062 g, 0.356 mmol) in methylene chloride (2 mL). The cooling bath was removed, the mixture was stirred for another 30 min and washed successively with 5% sodium thiosulfite solution (2×5 mL), water (1×5 mL), 3% $NaHCO_3$ solution (2×5 mL), and brine (1×5 mL). Drying (Na_2SO_4) and solvent evaporation generated 0.070 g (100%) of compound **28**, that was used without any further purification. In a similar way, 0.065 g of **27** was converted to 0.070 g (100%) of **29**. Compound **28**: White solid, mp 112–114 °C; R_f (60% ethyl acetate in hexanes): 0.35; ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1H), 8.00 (m, 3H), 7.85 (d, 1H), 7.65 (m, 2H), 6.05 (d, 1H), 4.15 (m, 1H), 3.80 (dd, 1H), 3.50 (m, 1H), 3.40 (m, 1H), 3.20 (m, 1H), 2.80 (m, 2H), 2.00 (m, 2H), 1.60-1.40 (m, 5H), 1.20 (m, 1H), 0.90 (m, 12H). Compound **29**: White solid, mp 112–115 °C; R_f (60% ethyl acetate in hexanes): 0.32; ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1H), 8.00 (m, 3H), 7.85 (d, 1H), 7.70 (m, 2H), 6.00 (d, 1H), 4.05 (m, 1H), 3.65 (m, 1H), 3.55 (m, 1H), 3.20 (m, 2H), 2.65 (m, 2H), 2.00 (q, 2H), 1.60 (m, 3H), 1.40 (m, 2H), 1.20 (m, 1H), 0.90 (m, 12H).

(*S*)/(*R*)-2-Isobutyl-4-(2-sulfonylnaphthyl)butyric acid ((*S*)-1-formyl-3-methyl)butyl amides (30,31). These compounds were synthesized following the synthetic procedure as described above for the syntheses of the compounds **7a**–**d**. Thus, the oxidation of **28** (0.063 g, 0.145 mmol) gave 0.040 g (60%) of compound **30**. In a similar way, 0.063 g of **29** was converted to 0.034 g (54%) of **31**. Compound **30**: White solid, mp 104–109 °C (softening to melt); R_f (70% ethyl acetate in hexanes): 0.87; ^{1}H NMR ($CDCl_3$) δ 9.60 (s, 1H), 8.55 (s, 1H), 8.00 (m, 4H), 7.70 (m, 2H), 6.40 (d, 1H), 4.65 (m, 1H), 3.65 (m, 1H), 3.20 (m, 1H), 2.80 (m, 1H), 2.10 (m, 1H), 1.90 (m, 1H), 1.60–1.40 (m, 4H), 1.20 (m, 1H), 0.90 (m, 12H). MS *m*/*e* 432 (M + H). Anal. ($C_{24}H_{33}NO_4S$) C, H, N. Compound **31**: White foam, R_f (70% ethyl acetate in hexanes): 0.83; ^{1}H NMR ($CDCl_3$) δ 9.60 (s, 1H), 8.45 (s, 1H), 8.00 (m, 3H), 7.85 (d, 1H), 7.70 (m, 2H), 6.20 (d, 1H), 4.50 (m, 1H), 3.20 (m, 2H), 2.70 (m, 1H), 2.00 (q, 2H), 1.80–1.40 (m, 5H), 1.20 (m, 1H), 0.90 (m, 12H). MS *m*/*e* 432 (M + H). Anal. ($C_{24}H_{33}NO_4S.0.5\ H_2O$) C, H, N.

(*R*)-2-Isobutyl-4-(2-thionaphthyl)butyric acid ((*S*)-1-formyl-3-methyl)butyl amide (32). This compound was prepared from compound **27**, following the same procedure as described above for the synthesis of **7a**–**d**. Thus, 0.23 g of **27** was oxidized to generate 0.12 g (53%) of **32** as a white solid, mp 75–76 °C; R_f (70% ethyl acetate in hexanes): 0.85; ^{1}H NMR ($CDCl_3$) δ 9.60 (s, 1H), 7.80 (m, 4H), 7.45 (m, 3H), 5.90 (d, 1H), 4.60 (m, 1H), 3.20 (m, 1H), 2.90 (m, 1H), 2.60 (m, 1H), 2.00 (m, 1H), 1.80–1.40 (m, 5H), 1.40 (m, 1H), 1.20 (m, 1H), 0.90 (m, 12H). MS *m*/*e* 400 (M + H), 422 (M + Na). Anal. ($C_{24}H_{33}NO_2S$) C, H, N.

(*R*)-2-Isobutyl-4-(2-sulfoxylnaphthyl)butyric acid ((*S*)-1-hydroxymethyl-3-methyl)butyl amide (33). To a stirred solution of **27** (0.23 g, 0.573 mmol) in methylene chloride (4 mL) at room temperature was added a solution of Davis' oxaziridine (0.17 g, 0.58 mmol). The mixture was

stirred for another 30 min and concentrated in vacuo to give a crude product. It was purified by silica gel column chromatography (eluant: 10% ethyl acetate in hexanes followed by 50% ethyl acetate in hexanes) to give 0.21 g (86%) of **33** (diastereomeric mixture at the sulfoxide center), as a white solid, mp 138–140 °C; R_f (ethyl acetate): 0.51; ^{1}H NMR ($CDCl_3$) δ 8.20 (s, 1H), 7.90 (m, 3H), 7.60 (m, 2H), 7.50 (m, 1H), 6.20 (q, 1H), 4.10 (m, 1H), 3.70 (m, 1H), 3.55 (m, 1H), 3.20 (q, 1H), 3.10–2.80 (m, 2H), 2.60 (m, 1H), 2.10–1.00 (a series of m, 8H), 0.90 (m, 12H).

(*R*)-2-Isobutyl-4-(2-sulfoxylnaphthyl)butyric acid ((*S*)-1-formyl-3-methyl)butyl amide (34). This compound was prepared from compound **33**, following the synthetic procedure as described above for the synthesis of **7a–d**. Thus 0.160 g of **33** was oxidized to 0.060 g (38%) of **34** (diastereomeric mixture at the sulfoxide center), as a white solid, mp 134–144 °C (softening to melt); R_f (70% ethyl acetate in hexanes): 0.49; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.55 (d, 1H), 8.20 (d, 1H), 7.95 (m, 3H), 7.60 (m, 3H), 6.50 and 6.40 (2 sets of d, 1H), 4.50 (m, 1H), 3.10–2.80 (m, 2H), 2.70 (m, 1H), 2.10 (m, 1H), 1.90–1.40 (m, 6H), 1.25 and 1.10 (2 sets of m, 1H), 0.90 (m, 12H). MS *m/e* 416 (M + H), 438 (M + Na). Anal. ($C_{24}H_{33}NO_3S.0.1\ H_2O$) C, H, N.

In vitro calpain assay

For reversible inhibitors, the reaction mixture contained 50 mM Tris HCl (pH 7.5), 50 mM NaCl, 0.2 mM Suc-Leu-Tyr-MNA (Enyme Systems Products, Dublin, CA), 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 10 nM recombinant human calpain I, varying concentrations of inhibitor and 5 mM $CaCl_2$ in a final volume of 200 μL in a polystyrene microtiter plate. Assays were initiated by addition of $CaCl_2$ and the increase in fluorescence ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 430$nm) was monitored at ambient temperature using a Fluoroskan II fluorescence plate reader. Values of IC_{50} s were calculated from velocities determined from the linear portion of reaction progress curves. For irreversible inhibitors, reactions were performed at ambient temperature in single cuvettes with the increase in fluorescence ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 425$ nm) recorded continuously on a Perkin–Elmer LS50B spectrofluorimeter (Norwalk, CT, USA) and were monitored until there was no further product generated in inhibitor-containing assays. Inhibitor concentrations were at least 10-fold greater than the enzyme concentration. Value of k_{obs}, the pseudo first-order rate constant for inactivation, were calculated from plots of fluorescence vs. time by non-linear regression (Sigma Plot) to the exponential eq (1).[14]

$$y = Ae^{(-kobs*t)} + B \quad (1)$$

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 was calculated from the slope of a plot of k_{obs} versus inhibitor concentration as (k_{obs}/I)* ($1 + S/K_m$), correcting for the effect of substrate on the inactivation rate.

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® Brand), 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. 10% SDS was added to yield a final concentration of 1% and the samples heated for 15 min at 65 °C. Supernatant protein concentrations were determined by the BCA assay (Pierce, Inc., Rockford, IL, USA). 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), followed by an alkaline phosphatase conjugated goat anti-rabbit antibody (BIORAD, Inc., Hercules, CA, USA) 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, USA).

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