Brief Articles

Investigation into the P_3 Binding Domain of m-Calpain Using Photoswitchable Diazo- and Triazene-dipeptide Aldehydes: New Anticataract Agents

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The photoswitchable *N*-terminal diazo and triazene-dipeptide aldehydes **8a**−**d**, **10a**,**b**, and **17a**,**b** present predominantly as the (*E*)-isomer, which purportedly binds deep in the S_3 pocket of calpain. All compounds are potent inhibitors of m-calpain, with **8b** being the most active (IC_{50} of 35 nM). The diazo-containing inhibitors **8a**, **8c**, and **10a** were irradiated at 340 nm to give a photostationary state enriched in the (*Z*)-isomer, and in all cases, these were less active. The most water soluble triazene **17a** (IC_{50} of 90 nM) retards calpain-induced cataract formation in lens culture.

Introduction

Calpains are Ca^{2+}-dependent cysteine proteases that are found in many types of organisms, including mammals, plants, and protozoa.[1] The most common isoforms, μ- and m-calpain, require μmol and mmol concentrations of calcium, respectively, for activation in vitro.[2] Both isoforms are heterodimers, consisting of a large 80 kDa catalytically active subunit and a smaller 28 kDa regulatory subunit.[3]

The over-activation of calpain is central to a number of medical conditions associated with cellular damage, including traumatic brain injury, stroke, and cataract.[4] In the case of cataract, calpain catalyzes the breakdown of the major lens proteins (crystallins), resulting in clouding of the lens and ultimately blindness.[5] Specific calpain inhibitors are thus attractive therapeutic agents;[6] however, their design has been somewhat restricted by the, until recent, lack of available X-ray data.[7] Calpains are known to cleave a large number of substrates,[8] and something of their specificity is known. This helps in inhibitor design. Calpains recognize unordered sequences located between globular domains of proteins, and the preferred amino acids that neighbor the cleavage sites are known.[6]

The S_1, S_2, and S_3 binding sites of calpain that bind these preferred sequences are located deep within a canyon-like groove defined by the steep surfaces of domain I on one side, and domain II on the other.[9] This groove is tightest around the S_1 site (<7 Å across, approx 15 Å deep), with the S_2 site being much deeper and generally being occupied by branched hydrophobic residues. The groove ends in a wider opening (>15 Å) at the S_3 site, which is thought to be accommodated by an *N*-terminal aryl group[10] within dipeptide-based inhibitors, such as SJA6017 **1** (Figure 1), and a range of hydrophobic or basic groups in the cases of tri- and larger sequences.[11] These calpain inhibitors most likely bind in an extended β-strand conformation that spans approx 15 Å of the complementary active site cleft, but little work has been done to confirm this and, in particular, to understand interactions in the S_3 binding pocket.

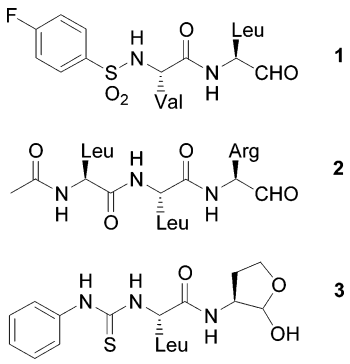

Figure 1. Calpain inhibitors SJA6017 **1**, leupeptin **2**, and SNJ1715 **3** for which X-ray structures of each bound in μ-calpain have been determined.

An X-ray structure of leupeptin **2**, with engineered μ-calpain, shows this conformation to be defined by two key hydrogen bonds between the NH and carbonyl groups of Leu (P_2) and Gly_{208} and also an additional hydrogen bond between the NH of the P_1−P_2 amide bond and Gly_{271} (Figure 2).[7a] More recent crystal structures, for example, calpain bound prodrug **3**, confirm the importance of the same series of hydrogen bonds.[7b−c]

In this paper we report the preparation of a series of (*E*)-diazodipeptide aldehydes (amides **8a**−**d** and sulfonamides **10a**,**b**) that contain an *N*-terminal diazo group designed to extend deep into the S_3 binding pocket and a C-terminal aldehyde for attachment to the active site cysteine. The *N*-terminal diazo groups of (*E*)-**8a**,**c** and (*E*)-**10a** were photochemically isomerized to give an alternative photostationary state in which the (*Z*)-isomer predominates. These mixtures, enriched in either the (*E*)- or (*Z*)-isomer, were assayed against m-calpain to further explore and define the P_3 binding pocket. This new class of *N*-terminal group was extended to more water soluble triazene-dipeptide

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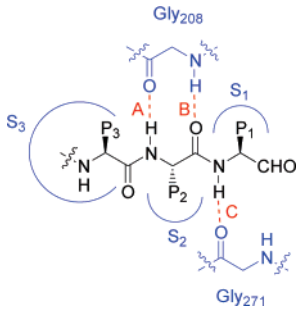
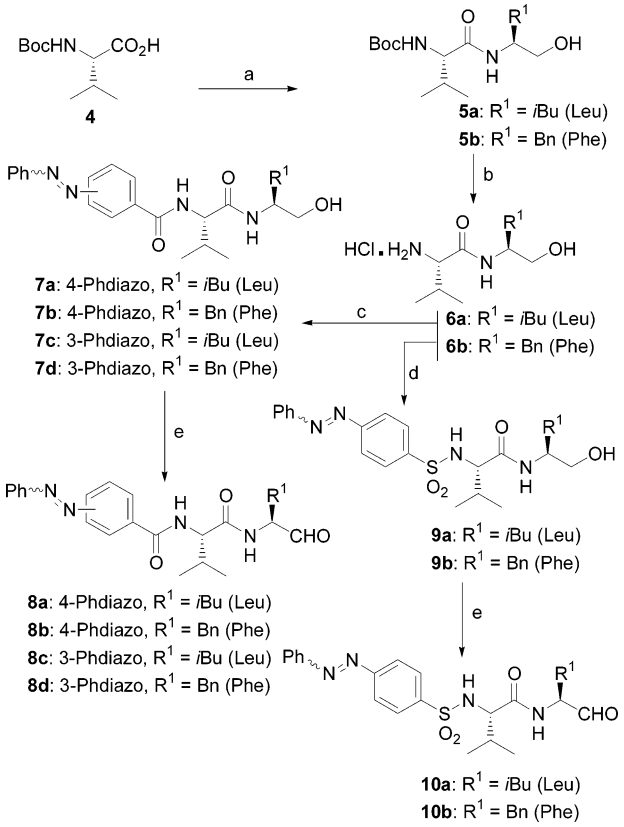


Figure 2. Depiction of noncovalent interactions between calpain and aldehyde-based inhibitors bound in an antiparallel β-strand conformation.[9] Hydrogen bonds from the carbonyl group of Gly_{208}, the NH group of Gly_{208}, and the carbonyl group of Gly_{271} are labeled A, B, and C, respectively.

Scheme 1. Synthesis of **8a**−**d** and **10a**,**b**[a]



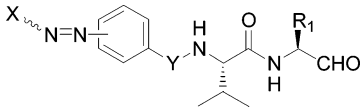
[a] Reagents and conditions: (a) leucinol or phenylalaninol, HATU, DIPEA, DMF; (b) 4 M HCl/dioxane; (c) HATU, DIPEA, DMF, 3- or 4-phenyldiazobenzoic acid, respectively; (d) DMF, Et_3N, 4-phenyldiazobenzenesulfonyl chloride; (e) SO_3-Pyr, DMSO, DCM, DIPEA.

aldehydes **17a**,**b**, with **17a** being assayed in lens culture to determine its ability to arrest the development of calpain-induced cataract.

Results and Discussion

Compounds **8a**−**d** were prepared (Scheme 1) by coupling Boc-valine **4** to leucinol and phenylalaninol to give **5a** and **5b**, respectively. Deprotection of **5a**,**b** with 4 M HCl/dioxane gave **6a**,**b**, which were separately coupled to 3- and 4-phenylazobenzoic acid to give **7a**−**d** in 29−67% yield. Subsequent oxidation with SO_3-pyridine gave the desired aldehydes **8a**−**d** (52−89%). The azobenzenes **7a**−**d** and **8a**−**d** had (*E*/*Z*) ratios of 3.7:1, 3.5:1, 13:1, and 9.2:1 for **7a**−**d** and 4.8:1, 5.4:1, 4.9:1, and 5.4:1 for **8a**−**d**, respectively, as determined by ^{1}H NMR (Table 1).

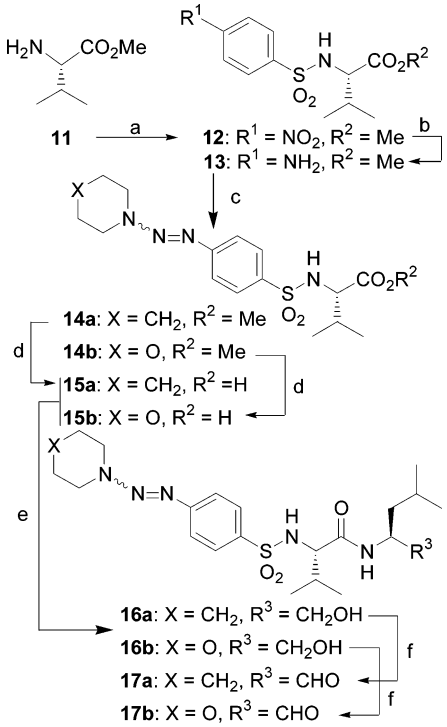
Table 1. Inhibition of m-Calpain by Different Photostationary States of the Diazo and Triazene-dipeptide Aldehydes **8a**−**d**, **10a**,**b**, and **17a**,**b**



compound[a]	*E*/*Z*	sub[b]	X	Y	R_1	IC_{50} (nM)
8a	4.8:1	4	Ph	CO	Leu	45
8a (irradiated)	1:4.3	4	Ph	CO	Leu	175
8b	5.4:1	4	Ph	CO	Phe	35
8c	4.9:1	3	Ph	CO	Leu	75
8c (irradiated)	1:4	3	Ph	CO	Leu	105
8d	5.4:1	3	Ph	CO	Phe	170
10a	3.3:1	4	Ph	SO_2	Leu	40
10a (irradiated)	1:3.7	4	Ph	SO_2	Phe	100
10b	7:1	4	Ph	SO_2	Phe	90
17a	23:1	4	pip	SO_2	Leu	90
17b	23:1	4	morp	SO_2	Leu	420

[a] The major isomer before irradiation is assigned as the thermodynamically more stable (*E*)-isomer, based on literature precedence.[12] [b] Substitution position.

Scheme 2. Synthesis of the Triazenes **17a**,**b**[a]



[a] Reagents and conditions: (a) 4-nitrobenzene sulfonyl chloride, DMF, Et_3N; (b) PtO_2, H_2, MeOH/DCM; (c) $NaNO_2$, 6 M HCl/MeOH, then morpholine or piperidine; (d) NaOH, H_2O/THF; (e) leucinol, HATU, DIPEA, DMF; (f) SO_3-Pyr, DMSO, DCM, DIPEA.

The major isomer in each case is assigned the thermodynamically more stable (*E*)-isomer based on literature precedence.[12]

The sulfonamides **10a**,**b** were synthesized (Scheme 1) by separately treating **6a** and **6b** with 4-phenyldiazobenzenesulfonyl chloride to give **9a** and **9b** in 60 and 50% yield, respectively. Oxidation with SO_3-pyridine gave aldehydes **10a**,**b**, each in 70% yield. Both the (*E*)- and (*Z*)-isomers were observed for **9a** and **10a**,**b**, with ratios (*E*/*Z*) of 6.5:1, 3.3:1, and 7:1, respectively. However, little of the (*Z*)-isomer was observed for **9b**, with a ratio (*E*/*Z*) of >20:1.

For the synthesis of triazenes **17a**,**b** (Scheme 2) the valine methyl ester **11** was treated with 4-nitrobenzenesulfonyl chloride

to give **12** in 73% yield. Reduction of the nitro group with PtO_2 in a hydrogen atmosphere at 120 psi[a], with a mixture of dichloromethane and methanol (1:1) as solvent, gave **13** in 81% yield. A lower hydrogen pressure (20 psi), even with an extended reaction time, resulted in a decreased yield of **13** (63%). The reduction was also carried out in ethyl acetate (20 psi), but this solvent resulted in an even lower yield of **13** (30%). The amine **13**, converted to its diazonium salt on treatment with $NaNO_2$ in a 1:1 mixture of 6 M aqueous HCl and methanol, was treated with piperidine or morpholine to give the triazenes **14a**,**b** in 49% and 65% yield, respectively. The methyl esters of **14a**,**b** were separately hydrolyzed with sodium hydroxide in THF/water (1:1) to give **15a**,**b** in 82% and 86% yields, respectively. ^{1}H NMR showed the (*E*/*Z*) ratios to be 25:1 and 18:1 for the piperidine series **14a**−**15a** and 10.7:1 and 9.7:1 for the morpholine series **14b**−**15b**. The carboxylic acids **15a**,**b** were coupled to leucinol to give **16a**,**b** in 58% and 49%, with an (*E*/*Z*) ratio of 25:1 and 24:1. A final oxidation gave the desired aldehydes **17a**,**b** in 55% and 59% yield, with an (*E*/*Z*) ratio of 23:1 in each case (Table 1).

Samples of the dipeptidic aldehydes **8a**−**d**, **10a**,**b**, and **17a**,**b**, consisting of predominantly the (*E*)-isomer, were assayed against m-calpain using a fluorescence-based assay[13] to determine in vitro potency, and the results are summarized in Table 1. The initial (*E*)/(*Z*)-isomer mixtures for **8a**, **8c**, and **10a** were irradiated with ultraviolet light (500 W mercury arc lamp through a UV filter with a narrow wavelength band centered at 340 nm) to give samples enriched in the (*Z*)-isomer [1:4.3, 1:4, and 1:3.7 mixture of (*E*)- and (*Z*)-isomers, respectively, Table 1]. These new photostationary states of **8a**, **8c**, and **10a** (Table 1) were also assayed against m-calpain to further assess the available space in the S_3 binding pocket, to which the diazobenzene groups purportedly bind as revealed by molecular modeling.[14]

The most active dipeptidic aldehyde mixture **8b** had an IC_{50} value of 35 nM, which is significantly more potent than the calpain inhibitor SJA6017 **1** (IC_{50} = 80 nM in our assay).[15] The inhibitor mixtures **8a** (45 nM), **8c** (75 nM), **10a** (40 nM), and **17a** (40 nM) are also particularly potent. Of all the sample mixtures, only **8d** (170 nM) and **17b** (420 nM) are less potent than **1** (Table 1).

The S_1 pocket can accommodate both Leu and Phe at P_1; however, there does seem to be some preference for Leu at this position; compare assay results for **8a**,**b**, **8c**,**d**, and **10a**,**b** (Table 1). With regard to potency, there does not seem to be any consistent preference for an amide or sulfonamide linker; compare the activity of **8a**−**10a** and **8b**−**10b** (Table 1). Inhibitors with 4-substitution of the *N*-terminal aryl group (**8a**,**b**) are more potent than those with 3-substitution (**8c**,**d**).

The triazenes **17a**,**b** are less potent than the diazo compounds **8a**−**d** and **10a**,**b**. The planar and extended aromatic nature of the (*E*)-diazo group of **8a**,**b** and **10a**,**b** appears to allow for better interaction with this flat S_3 binding pocket, when compared to the corresponding triazenes **17a**,**b**. This is consistent with the lower potency of the triazenes **17a**,**b** (see Table 1). The lesser potency of **17b** may reflect the presence of an electronegative oxygen at the *N*-terminus, which would be expected to disfavor binding in the hydrophobic S_3 pocket.

Induced fit modeling[16] shows each μ-calpain docked inhibitor adopts a β-strand conformation.[17] The poses of (*E*)-**8a**, (*E*)-**8b**, (*E*)-**8c**, (*Z*)-**8c,** (*E*)-**10a**, (*E*)-**17a**, and (*E*)-**17b** docked with a μ-calpain construct are shown in Figure 3a. This is consistent with published crystal structures of μ-calpain with inhibitor bound.[7] The docked poses for (*Z*)-**8a**, (*E*)-**8d**, (*Z*)-**10a**, and (*E*)-**10b** (not shown in Figure 3a) also revealed a β-strand conformation of the inhibitor but with only two of the three hydrogen bonds apparent. The model also predicts the aldehyde group in all cases to be in close proximity to the active site cysteine, as required for mechanism-based inhibition.

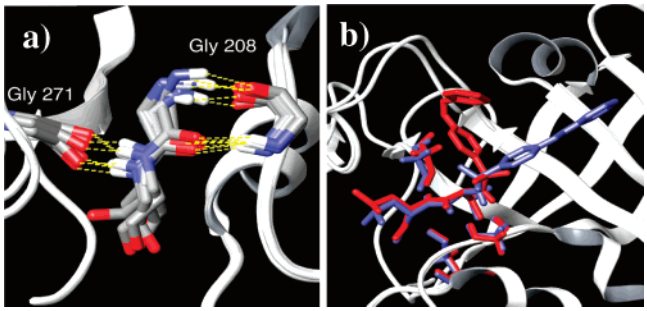


Figure 3. (a) β-Strand backbone conformations of (*E*)-**8a**, (*E*)-**8b**, (*E*)-**8c**, (*Z*)-**8c**, (*E*)-**10a**, (*E*)-**17a**, and (*E*)-**17b**, resulting from the induced-fit modeling. (b) Induced-fit overlay of the S_3 subsite of calpain with docked (*E*)-**10a** (blue) and (*Z*)-**10a** (red).

The inhibitory activity of the isomeric mixtures of **8a**, **8c**, and **10a** decreased, on irradiation, by a factor of 3.9, 1.4, and 2.5, respectively. For example, the inhibitory activity of **8a** decreased from 45 nM for a sample containing 83% (*E*)-isomer to 175 nM for a mixture containing 4-fold less of the (*E*)-isomer (i.e., 19% (*E*)-isomer with 81% (*Z*)-isomer), see Table 1. The (*E*)-isomers are significantly more active in each case,[18] reflecting a better fit in the S_3 binding pocket associated with their geometry and dipole moment. In fact, modeling suggests that the diazo group of (*Z*)-**10a**, unlike (*E*)-**10a**, appears not to bind in the S_3 binding pocket, but rather interacts with a hydrophobic patch on the mobile loop that defines the calpain active site (Figure 3b). This is consistent with the observed higher potency of the photostationary state enriched in the (*E*)-isomer (see Table 1 and previous discussion). A photoisomerizable *N*-terminal diazobenzene of this type provides both a useful probe for mapping the size and geometry of the S_3 binding domain and evidence for enhancing interactions between this pocket and a P_3 aryl group of an inhibitor. An empirical preference for the interaction of an aryl group with S_3 has been previously suggested,[7a] and our study supports this observation.

The in vivo potential of these highly potent inhibitors was next determined by assessing their ability to retard calpain-induced cell damage in sheep lenses cultured in Eagle's minimal essential medium. Inhibitor **17a** was chosen for study because of its superior water solubility, as compared to **8a**−**d** and **10a**,**b**, and also its high in vitro potency, as compared to the other triazene **17b**. The triazene moiety of **17a** is also amenable to salt formation that makes it attractive as a potential drug candidate.

Inhibitor **17a** (0.8 μM) was added to one lens from each pair of sheep lenses in culture media. Two hours later calcium was added to all lenses to activate the constituent calpains and hence induce cataract formation. After 24 h, all lenses were photographed and the opacity graded; see Figure 4 for representative examples. Lenses treated with calcium only (e.g., lens 1 in Figure 4) clearly showed the opacity associated with cataract formation; however, lenses treated with calcium in the presence of **17a** (e.g., lens 2) remained essentially clear, as revealed by the reference grid placed behind each lens. The loss of transparency was significantly reduced by **17a** ($p < 0.005$) in

[a] Abbreviations: DIPEA, *N*,*N*-diisopropylethylamine; HATU, *O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; psi, pounds of force per square inch.

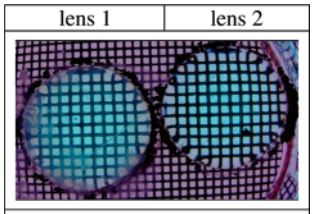


Figure 4. Calcium-induced cataract in sheep lenses. The scores represent the average result using three lens pairs. Opacification scores of 100 = full opacity, whereas a score of 1 = clear and transparent.

a paired t-test. It would thus appear that this class of inhibitor, with an *N*-terminal group capable of binding deep into the S_3 binding pocket, is active in vitro and in vivo (lens assay) and is thus of interest as a potential drug candidate.

Conclusion

A series of diazo- and triazene-dipeptide aldehydes **8a**−**d**, **10a**,**b**, and **17a**,**b** have been prepared predominantly as the (*E*)-isomers. All of the inhibitor mixtures are highly potent against m-calpain, with the most potent **8b** [5.4(*E*)/1(*Z*)] having an IC_{50} value of 35 nM. Photoisomerism of the diazo inhibitors **8a**−**c** and **10a** gave samples enriched in the (*Z*)-isomer that proved to be significantly less active.

SAR data is presented, which suggests that an *N*-terminal diazo group (**8a**−**d**) is favored over a triazene (**17a**,**b**). Furthermore 4-substitution of the *N*-terminal diazo group (**8a**,**b**) is favored over 3-substitution (**8c**,**d**). The triazene of **17a** imparts improved water solubility for in vivo studies and was shown to arrest the development of calpain-induced cataract formation in sheep lens culture.

Molecular modeling predicts that these compounds all bind in an extended β-strand conformation, as defined by three key hydrogen bonds to Gly_{208} and Gly_{271}. This is consistent with published crystal structures of μ-calpain with the inhibitor bound.[7] The reactive carbonyl in each case is located in close proximity to the active site cysteine, as would be required for mechanism-based inhibition. As a consequence, the *N*-terminal group of the (*E*)-diazo dipeptide aldehydes **8a**−**d** and **10a**,**b** extends deep into the S_3 binding pocket.

Finally, we suggest that a calpain inhibitor, the activity of which can be influenced by irradiation, offers some potential as a means to control cataracts. The work presented here is a first step toward this goal.

Experimental Section

(*S*)-3-Methyl-2-[4-(piperidin-1-ylazo)-benzenesulfonylamino]-butyric Acid Methyl Ester (14a). To a salt-ice-cooled solution of **13** in 6 M HCl/MeOH (50 mL, [5:1]) was added dropwise a solution of 2.5 M $NaNO_2$ (1.0 equiv) so that the temperature did not exceed 5 °C. The crude orange diazonium salt was cooled to 0 °C, a solution of piperidine (1.0 equiv) in 1 M HCl (1.0 equiv) was added, and the mixture was stirred for 30 min. To the resulting homogeneous solution was added solid Na_2CO_3 (caution!) to give a bright orange precipitate. The precipitate was collected by filtration, dissolved in ethyl acetate (50 mL), washed with water and brine, and dried ($MgSO_4$). Concentration in vacuo gave a bright orange oil that was purified by column chromatography to give **14a** as a pale yellow solid [inseparable mixture of isomers, 25(*E*)/1(*Z*); 2.05 g, 49%]: mp 125−127 °C; R_f = 0.65 (ethyl acetate/petroleum ether [2:1]); HRMS (ES^+) calcd for $C_{17}H_{27}N_4O_4S$ ($[M + H]^+$), 383.1771; found, 383.1753; ν_{max} (KBr) 1738 (CO_2Me), 1346 and 1142 (SO_2-NH). Data for major (*E*)-isomer from mixture: ^{1}H NMR (500 MHz, $CDCl_3$) 0.87 (3H, d, J = 6.7 Hz, CH_3), 0.95 (3H, d, J = 6.8 Hz, CH_3), 1.73 (6H, mc, 3 × CH_2), 2.02−2.04 (1H, m, CH(CH_3)$_2$), 3.45 (3H, s, OCH_3), 3.73 (1H, dd, J = 9.9 Hz and J = 5.1 Hz, CHCH(CH_3)$_2$), 3.85 (4H, mc, 2 × CH_2), 5.06 (1H, d, J = 9.9 Hz, NH), 7.48 (2H, app d, CH_{Ar}), 7.75 (2H, app d, CH_{Ar}). Selected data for minor (*Z*)-isomer from mixture: ^{1}H NMR (500 MHz, $(CD_3)_2SO$) 7.55 (2H, app d, CH_{Ar}), 7.82 (2H, app d, CH_{Ar}). Data from mixture: ^{13}C NMR (75 MHz, $(CD_3)_2SO$) 18.3 (*C*H_3), 18.8 (*C*H_3), 23.6 (*C*H_2pip), 30.5 (*C*H(CH_3)$_2$), 51.6 (O*C*H_3), 61.5 (*C*HCH-(CH_3)$_2$), 120.0 (*C*H_{Ar}), 127.8 (*C*H_{Ar}), 136.6 (*C*SO_2), 153.3 (*C*N═N), 171.3 (*C*O_2Me); Anal. Calcd for $C_{17}H_{26}N_4O_4S$: C, 53.38; H, 6.85; N, 14.65%. Found: C, 53.66; H, 6.86; N, 14.52%.

(*S*)-3-Methyl-2-[4-(piperidin-1-ylazo)-benzenesulfonylamino]-butyric Acid (15a). To a solution of ester **14a** in THF/H_2O ([3:1], 1 mL/10 mg) was added NaOH (2.5 equiv), and the solution was heated to 65 °C with stirring overnight. Concentration in vacuo gave a crude orange solid that was partitioned between ethyl acetate (100 mL) and water (100 mL). The aqueous layer was acidified to pH 2 with 1 M HCl and extracted with ethyl acetate (2×). The combined ethyl acetate layers were washed with water and brine and dried ($MgSO_4$). Concentration in vacuo gave **15a** as an orange solid [inseparable mixture of isomers, 18(*E*)/1(*Z*); 1.61 g, 82%]: mp 117−119 °C; HRMS (ES^+) calcd for $C_{16}H_{25}N_4O_4S$ ($[M + H]^+$), 369.1596; found, 369.1613; ν_{max} (KBr) 3312 (CO_2H), 1701 (CO_2H), 1355 and 1153 (SO_2NH). Data for major (*E*)-isomer from mixture: ^{1}H NMR (500 MHz, $(CD_3)_2SO$) 0.77 (3H, d, J = 6.9 Hz, CH_3), 0.80 (3H, d, J = 6.9 Hz, CH_3), 1.65 (6H, mc, 3 × CH_2), 1.91−1.93 (1H, m, CH(CH_3)$_2$), 3.47 (1H, dd, J = 8.8 Hz and J = 5.9 Hz, CHCH(CH_3)$_2$), 3.80 (4H, mc, 2 × CH_2), 7.42 (2H, app d, CH_{Ar}), 7.69 (2H, app d, CH_{Ar}), 7.89 (1H, d, J = 9.3 Hz, NH), 12.45 (1H, br s, CO_2H). Selected data for minor (*Z*)-isomer from mixture: ^{1}H NMR (500 MHz, $(CD_3)_2SO$) 7.59 (2H, app d, CH_{Ar}), 7.75 (2H, app d, CH_{Ar}), 8.00 (1H, d, J = 7.8 Hz, NH). Data from mixture: ^{13}C NMR (75 MHz, $(CD_3)_2SO$) 17.9 (*C*H_3), 19.1 (*C*H_3), 23.6 (*C*H_2pip), 30.4 (*C*H(CH_3)$_2$), 61.3 (*C*HCH(CH_3)$_2$), 120.0 (*C*H_{Ar}), 127.8 (*C*H_{Ar}), 137.0 (*C*SO_2), 153.2 (*C*N═N), 171.3 (*C*O_2H); Anal. Calcd for $C_{16}H_{24}N_4O_4S$: C, 52.16; H, 6.57; N, 15.21%. Found: C, 52.34; H, 6.61; N, 14.92%.

(*S*)-*N*-((*S*)-1-Hydroxymethyl-3-methyl-butyl)-3-methyl-2-[4-(piperidin-1-ylazo)benzenesulfonylamino]-butyramide (16a). To a stirred solution of **15a** in dry DMF (1 mL/60 mmol) was added leucinol (1.1 equiv), DIPEA (4 equiv), and HATU (1.1 equiv). The solution was stirred at rt overnight, diluted with ethyl acetate (5:1), washed with water (2×) and brine, and dried over $MgSO_4$. Concentration in vacuo gave a pale orange solid that was purified by column chromatography to give **16a** as a yellow solid [inseparable mixture of isomers, 25(*E*)/1(*Z*); 1.13 g, 58%]: mp 178−180 °C; R_f = 0.51 (ethyl acetate/petroleum ether [1:1]); HRMS (ES^+) calcd for $C_{22}H_{38}N_5O_4S$ ($[M + H]^+$), 468.2644; found, 468.2656; ν_{max} (KBr) 1643 (C═ONH), 1320 and 1157 (S═O_2NH), 1026 (C−OH). Data for major (*E*)-isomer from mixture: ^{1}H NMR (500 MHz, $(CD_3)_2SO$) 0.61 (3H, d, J = 5.9 Hz, CH_3), 0.70 (3H, d, J = 5.8 Hz, CH_3), 0.76 (3H, d, J = 6.8 Hz, CH_3), 0.82 (3H, d, J = 6.8 Hz, CH_3), 0.96−0.98 (1H, m, CHHCH(CH_3)$_2$), 1.12−1.14 (2H, m, CH_2CH(CH_3)$_2$ and CHHCH(CH_3)$_2$), 1.66 (6H, mc, 3 × CH_2), 1.77−1.79 (1H, m, CH(CH_3)$_2$), 3.03−3.06 (1H, m, CH_2OH), 3.14−3.19 (1H, m, CH_2OH), 3.46−3.49 (1H, m, CHCH$_2$OH), 3.80 (4H, mc, 2 × CH_2), 4.50−4.53 (1H, m, CHCH(CH_3)$_2$), 7.39 (2H, app d, CH_{Ar}), 7.47 (1H, d, J = 8.3 Hz, NH), 7.68 (2H, app d, CH_{Ar}). Selected data for minor (*Z*)-isomer from mixture: ^{1}H NMR (500 MHz, $(CD_3)_2SO$) 7.57 (1H, d, J = 7.1 Hz, NH), 7.79 (2H, app d, CH_{Ar}). Data from mixture: ^{13}C NMR (75 MHz, $(CD_3)_2SO$) 17.9 (*C*H_3), 19.2 (*C*H_3), 21.8 (CH_2CH(CH_3)$_2$), 23.2 (CH_3), 23.6 (CH_2-pip), 23.8 (CH_3), 31.4 (*C*H(CH_3)$_2$), 38.3 (CH_2CH(CH_3)$_2$), 48.6 (*C*HCH$_2$OH), 61.3 (*C*HCH(CH_3)$_2$), 63.6 (*C*H_2OH), 119.9 (*C*H_{Ar}), 127.7 (*C*H_{Ar}), 137.1 (*C*SO_2), 153.0 (*C*N═N), 169.5 (*C*ONH); Anal. Calcd for $C_{22}H_{37}N_5O_5S$·HCl: C, 52.42; H, 7.60; N, 13.89%. Found: C, 52.26; H, 7.48; N, 14.05%.

(*S*)-*N*-((*S*)-1-Formyl-3-methyl-butyl)-3-methyl-2-[4-(piperidin-1-ylazo)benzenesulfonylamino]-butyramide (17a). To a stirred ice-cooled solution of alcohol **16a** and 4 equiv of DIPEA in DCM/

DMSO (1:1, 1 mL/30 mmol) was added dropwise a solution of 4 equiv of sulfur trioxide pyridine complex in DMSO (1 mL/60 mmol). Stirring was continued in ice for 2 h at which time the mixture was diluted with ethyl acetate (10:1), washed with aqueous 1 M HCl (2×), saturated aqueous $NaHCO_3$ (2×), and brine (10 mL), and dried over $MgSO_4$. Concentration in vacuo gave an orange oil that was purified by column chromatography to give **17a** as a yellow solid [inseparable mixture of isomers, 23(*E*)/1(*Z*); 0.62 g, 55%]: mp 75−77 °C; R_f = 0.51 (ethyl acetate/petroleum ether [1:1]); HRMS (ES^+) calcd for $C_{22}H_{36}N_5O_4S$ ($[M + H]^+$), 466.2488; found, 466.2484; ν_{max} (KBr) 1734 (CHO), 1659 (CONH), 1345 and 1110 (SdO_2NH). Data for major (*E*)-isomer from mixture: ^{1}H NMR (500 MHz, $(CD_3)_2SO$) 0.69 (3H, d, J = 5.9 Hz, CH_3), 0.79 (3H, d, J = 6.3 Hz, CH_3), 0.82 (3H, d, J = 6.8 Hz, CH_3), 0.85 (3H, d, J = 6.8 Hz, CH_3), 1.13−1.18 (1H, m, $CH_2CH(CH_3)_2$), 1.32−1.37 (2H, m, $CH_2CH(CH_3)_2$), 1.66 (6H, mc, 3 × CH_2), 1.82−1.86 (1H, m, C$H(CH_3)_2$), 3.54−3.57 (1H, m, CHCHO), 3.81 (5H, mc, 2 × CH_2 and CHCH$(CH_3)_2$), 7.40 (2H, app d, CH_{Ar}), 7.70 (3H, mc, CH_{Ar} and SO_2NH), 8.23 (1H, d, J = 6.9 Hz, NH), 9.13 (1H, s, CHO). Selected data for minor (*Z*)-isomer from mixture: ^{1}H NMR (500 MHz, $(CD_3)_2SO$) 7.52 (2H, app d, CH_{Ar}), 7.80 (2H, mc, CH_{Ar}), 7.92 (1H, d, J = 5.2 Hz, SO_2NH), 8.25 (1H, d, J = 6.9 Hz, NH). Data from mixture: ^{13}C NMR (75 MHz, $(CD_3)_2SO$) 18.1 (*C*H_3), 19.1 (*C*H_3), 21.4 ($CH_2CH(CH_3)_2$), 22.9 (*C*H_3), 23.6 (*C*H_2-pip), 23.8 (*C*H_3), 31.1 (*C*$H(CH_3)_2$), 36.3 ($CH_2CH(CH_3)_2$), 56.6 (*C*HCHO), 61.1 (*C*HCH$(CH_3)_2$), 119.9 (*C*H_{Ar}), 127.8 (*C*H_{Ar}), 137.0 (*C*SO_2), 153.1 (*C*N═N), 170.6 (*C*ONH), 200.9 (*C*HO); Anal. Calcd for $C_{22}H_{35}N_5O_4S$: C, 56.75; H, 7.58; N, 15.04%. Found: C, 56.40; H, 7.73; N, 15.44%

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Supporting Information Available: Experimental details corresponding to the synthesis of the compounds described in this paper, spectral data for all compounds, and assay and molecular modeling methods. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Perrin, B. J.; Huttenlocher, A. Calpain. *Int. J. Biochem. Cell Biol.* **2002**, *34*, 722−725.
(2) Reverter, D.; Sorimachi, H.; Bode, W. The Structure of Calcium-Free Human m-Calpain: Implications for Calcium Activation and Function. *Trends Cardiovasc. Med.* **2001**, *11*, 222−229.
(3) Goll, D. E.; Thompson, V. F.; Li, H.; Wei, W.; Cong, J. The Calpain System. *Physiol. Rev.* **2003**, *83*, 731−801.
(4) Huang, Y.; Wang, K. K. W. The Calpain Family and Human Disease. *Trends Mol. Med.* **2001**, *7*, 355−362.
(5) (a) David, L. L.; Wright, J. W.; Shearer, T. R. Calpain II Induced Insolubilization of Lens beta-Crystallin Polypeptides may Induce Cataract. *Biochim. Biophys. Acta* **1992**, *1139*, 210−216. (b) Robertson, L. J. G.; Morton, J. D.; Yamaguchi, M.; Bickerstaffe, R.; Shearer, T. R.; Azuma, M. Calpain may Contribute to Hereditary Cataract Formation in Sheep. *Invest. Ophthalmol. Vision Sci.* **2005**, *46*, 4634−4640.
(6) Neffe, A. T.; Abell, A. D. Developments in the Design and Synthesis of Calpain Inhibitors. *Curr. Opin. Drug Discovery Dev.* **2005**, *8*, 684−700.
(7) (a) Moldoveanu, T.; Campbell, R. L.; Cuerrier, D.; Davies, P. L. Crystal Structures of Calpain−E64 and −Leupeptin Inhibitor Complexes Reveal MobileLoops Gating the Active Site. *J. Mol. Biol.* **2004**, *343*, 1313−1326. (b) Cuerrier, D.; Moldoveanu, T.; J. Inoue, Davies, P. L.; Campbell, R. L. Calpain Inhibition by α-Ketoamide and Cyclic Hemiacetal Inhibitors Revealed by X-ray Crystallography. *Biochemistry* **2006**, *45*, 7446−7452. (c) Li, Q.; Hanzlik, R. P.; Weaver, R. F.; Schoenbrunn, E. Molecular Mode of Action of a Covalently Inhibiting Peptidomimetic on the Human Calpain Protease Core. *Biochemistry* **2006**, *45*, 701−708.
(8) Tompa, P.; Buzder-Lantos, P.; Tantos, A.; Farkas, A.; Szilágyi, A.; Bánóczi, Z.; Hudecz, F.; Friedrich, P. On the Sequential Determinants of Calpain Cleavage. *J. Biol. Chem.* **2004**, *279*, 20775−20785.
(9) Note the use of Schechter−Berger nomenclature [Schechter, I.; Berger, A. On the size of the active site in proteases. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157]; the residues on the *N*-terminal side of the peptide bond that is cleaved are denoted (in order) P_1−P_n, and those on the C-terminus are denoted $P_{1'}$−$P_{n'}$. In turn, the corresponding subsites on the enzyme are denoted S_n−$S_{n'}$.
(10) (a) Donkor, I. O. A survey of Calpain Inhibitors. *Curr. Med. Chem.* **2000**, *7*, 1171−1188. (b) Iqbal, M.; Messina, P. A.; Freed, B.; Das, M; Chatterjee, S.; Yripathy, R.; Yao, M.; Josef, K. A.; Dembofsky, B.; Dunn, D.; Griffith, E.; Siman, R.; Senadhi, S. E.; Biazzo, W.; Bozyczko-Coyne, D.; Meyer, S. L.; Ator, M. A.; Bihovsky, R. Subsite Requirements for Peptide Aldehyde Inhibitors of Human Calpain I. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 539−544.
(11) (a) Inoue, J.; Nakamura, M.; Cui, Y.-S.; Sakai, Y.; Sakai, O.; Hill, J. R.; Wang, K. W. W.; Yuen, P.-W. Structure−Activity Relationship Study and Drug Profile of *N*-(4-Fluorophenylsulfonyl)-L-valyl-L-leucinal (SJA6017) as a Potent Calpain Inhibitor. *J. Med. Chem.* **2003**, *46*, 868−871. (b) Angelastro, M. R.; Mehdi, S.; Burkhart, J. P.; Peet, N. P.; Bey, P. α-Diketone and α-Keto Ester Derivatives of N-Protected Amino Acids and Peptides as Novel Inhibitors of Cysteine and Serine Proteinases. *J. Med. Chem.* **1990**, *33*, 11−13. (c) Mehdi, S.; Angelastro, M. R.; Wiseman, J. S.; Bey, P. Inhibition of the Proteolysis of Rat Erythrocyte Membrane Proteins by a Synthetic Inhibitor of Calpain. *Biochem. Biophys. Res. Comm.* **1988**, *157*, 1117−1123.
(12) (a) Westmark, P. R.; Kelly, J. P.; Smith, B. D. Photoregulation of Enzyme Activity. Photochromic, Transition-State-Analog Inhibitors of Cysteine and Serine Proteases. *J. Am. Chem. Soc.* **1993**, 115, 3416−3419. (b) Harvey, A. J.; Abell, A. D. Azobenzene-Containing, Peptidyl α-Ketoesters as Photobiological Switches of α-Chymotrypsin. *Tetrahedron* **2000**, *56*, 9763.
(13) Thompson, V. F.; Saldana, S.; Cong, J.; Goll, D. E. A BODIPY Fluorescent Microplate Assay for Measuring Activity of Calpains and Other Proteases. *Anal. Biochem.* **2000**, *279*, 170−178.
(14) Assay of irradiated **8a**, **8c**, and **10a** enriched in the (*Z*)-isomer were carried out in dim lighting conditions, that is, glassware wrapped in foil and with the lights turned off.
(15) The calpain inhibitor SJA6017 **1** was chosen as a literature standard to validate our assay procedure and for comparison of inhibitor potency.
(16) Induced-fit docking using Glide and Prime: *Glide*, version 4.0; Schrödinger, LLC: New York, 2005. *Prime*, version 1.5; Schrödinger, LLC: New York, 2005.
(17) The X-ray crystal structure for rat μ-calpain was used for the docking studies as the m-calpain X-ray crystal structure in an inactivated form. Structures for sheep m- or μ-calpain have not been published; however, sequence analysis indicates 94% homology between sheep and rat m-calpain.
(18) For example, the (*E*)-isomer of **8a** is calculated to be 40-fold more active than the (*Z*)-isomer.

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