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Synthesis and antiproliferative activity of sulfonamide-based peptidomimetic calpain inhibitors

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ABSTRACT

The calpains are a conserved family of cysteine proteases that includes several isoforms of which μ–calpain and m-calpain are the most widely distributed in mammalian cells. Calpains have been implicated in normal physiological processes as well as cellular abnormalities such as neurodegenerative disorders, cataract, and cancer. Therefore, calpain inhibitors are of interest as potential therapeutic agents. We have synthesized four new sulfonamide-based peptidomimetic compounds **2–5** as inhibitors of μ-calpain that incorporate (*E*)-1-(phenyl)-2-phenyldiazene and (*E*)-1-(phenyl)-2-phenylethene functionalities as the *N*-terminal capping groups of the inhibitors. Compound **5** with K_i value of 9 nM versus μ-calpain was the most potent member of the group. The compounds were predicted to be more lipophilic compared to MDL28170 based on CLogP estimation. They displayed moderate to good antiproliferative activity versus melanoma cell lines (A-375 and B-16F1) and PC-3 prostate cancer cells *in vitro*. Additionally, one member of the group (compound **3**) inhibited DU-145 cell invasion by 80% at 2 μM concentration in the Matrigel cell invasion assay.

1. Introduction

Calpain, a calcium-activated cysteine proteinase was identified in rat brain over fifty years ago.[1] Following its discovery a plethora of studies have shown that calpain is a complex cysteine protease that consists of several isoforms. Some of the isoforms are ubiquitously expressed in various tissues while others are localized in specific tissues.[2] The cellular actions of calpain are tightly controlled by the endogenous protein inhibitor, calpastatin. However, under pathological conditions overactivation of calpain initiates a cascade of events that result in cellular damage hence the enzyme has been implicated in a variety of disease states including traumatic brain injury,[3] stroke,[4] cataract,[5] and cancer.[6,7] As a result of these studies calpain inhibitors have been investigated as neuroprotectants, cardioprotectants, anticataract agents and anticancer agents.[8–11] This report describes the synthesis, calpain inhibition, and *in vitro* antiproliferative activity of a series of peptidomimetic compounds (**1–5**, Figure 1) and the anti-invasive potential of compound **3**.

2. Results and discussion

2.1. Chemistry

Compounds **1–3** were synthesized as outlined in Scheme 1. The synthesis commenced with treating commercially available (*E*)-4-phenyldiazobenzenesulfonyl chloride **6** with either L-valine methyl ester hydrochloride or L-proline methyl ester hydrochloride in pyridine at room temperature to give compounds **7** and **8**, respectively. Basic hydrolysis of the ester group followed by coupling with L-leucinol and oxidation with Dess-Martin periodinane gave compounds **1** and **2** in 51% and 50% yield, respectively. Compound **1** was transformed to compound **3** by reacting its aldehyde group with KCN to give cyanohydrin **9**, the cyano group of which was hydrolyzed in HCl/MeOH mixture to give α-hydroxy-β-amino methyl ester **10**. Treatment of **10** with MeOH/NH_3 gave α-hydroxy-β-amino carboxamide **11**, which was oxidized with $PySO_3$/DMSO mixture to give compound **3** in 71% yield. Compound **1** was previously reported by Abell et al.[12] as a mixture of the *E*-, and *Z*-isomers in the ratio of 3.3:1 based on ^{1}H NMR spectroscopy. The authors showed that irradiating **1** with ultraviolet light increased the ratio of the (*Z*)-isomer, which was a less potent inhibitor of calpain compared to the corresponding (*E*)-isomer. We have synthesized compounds **4** and **5** as ethylene-bridged analogues to investigate the significance of diazo group for calpain inhibition. The coupling constant of the vinyl protons in the ^{1}H NMR spectra of the ethylene-bridged compounds was 16.5 Hz, which supports the (*E*)-geometry of the compounds.

The syntheses of compounds **4** and **5** are outlined in Scheme 2. The

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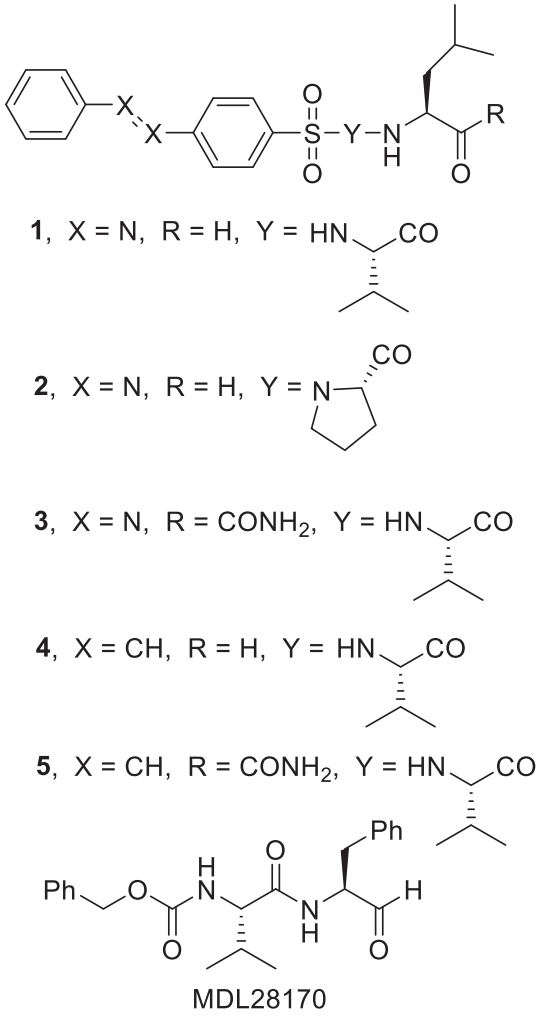


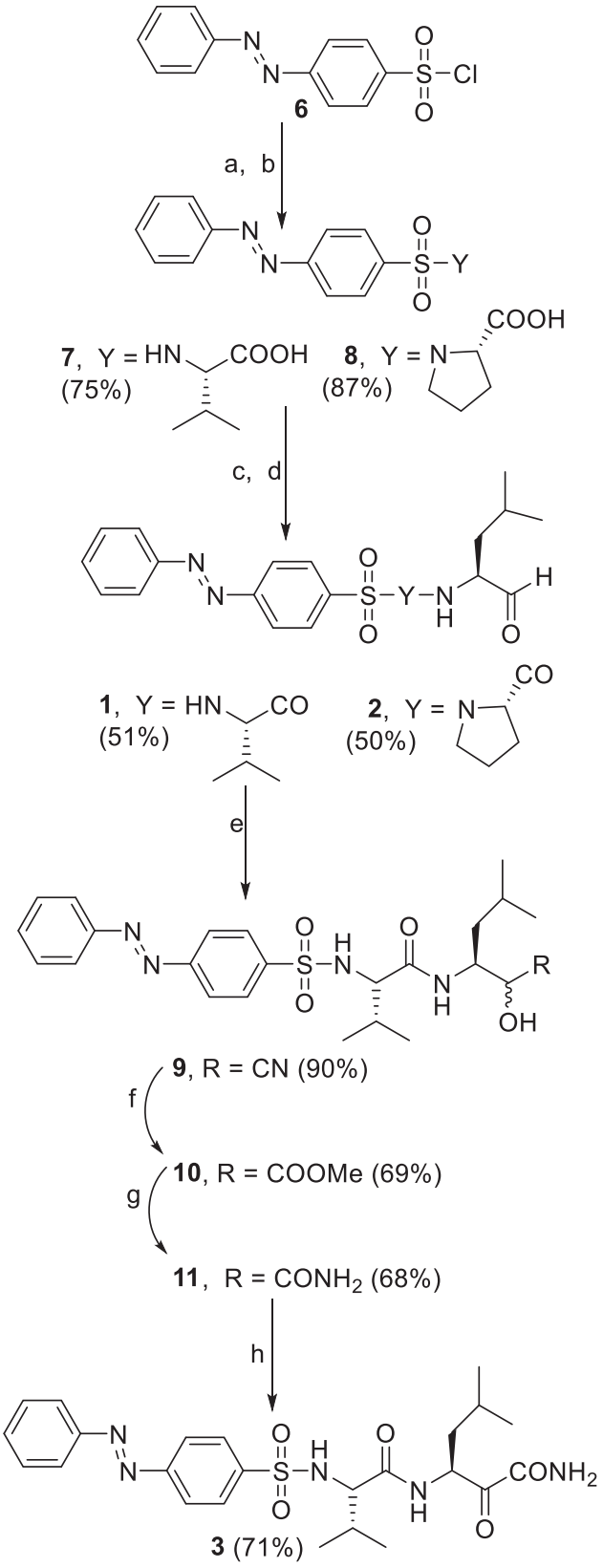
Figure 1. Structures of sulfonamide-based peptidomimetics **1–5** and MDL28170.

compounds were synthesized as described above for the synthesis of the diazene derivatives **1–3**. Briefly, the sodium salt of 4-styrene sulfonic acid **12** was transformed to 4-styrylphenylsulfonic acid using the Heck reaction, which is known to generate products with (*E*)-configuration. Refluxing the 4-styrylphenylsulfonic acid in $SOCl_2$ gave sulfonyl chloride **13**, which was treated with L-valine methyl ester hydrochloride in the presence of *N,N*-diisopropylethylamine (DIPEA) to give sulfonamide **14**. Hydrolysis of the ester group of **14** followed by coupling of the resulting acid with L-leucinol afforded **16**, which was transformed to aldehyde **4** (85% yield) and alpha-ketoamide **5** (53% yield) as described for the synthesis of the corresponding diazene derivatives **1** and **3**. The geometries of the compounds were established based on the coupling constants (J = 16.5 Hz) of the olefinic hydrogen atoms.[13]

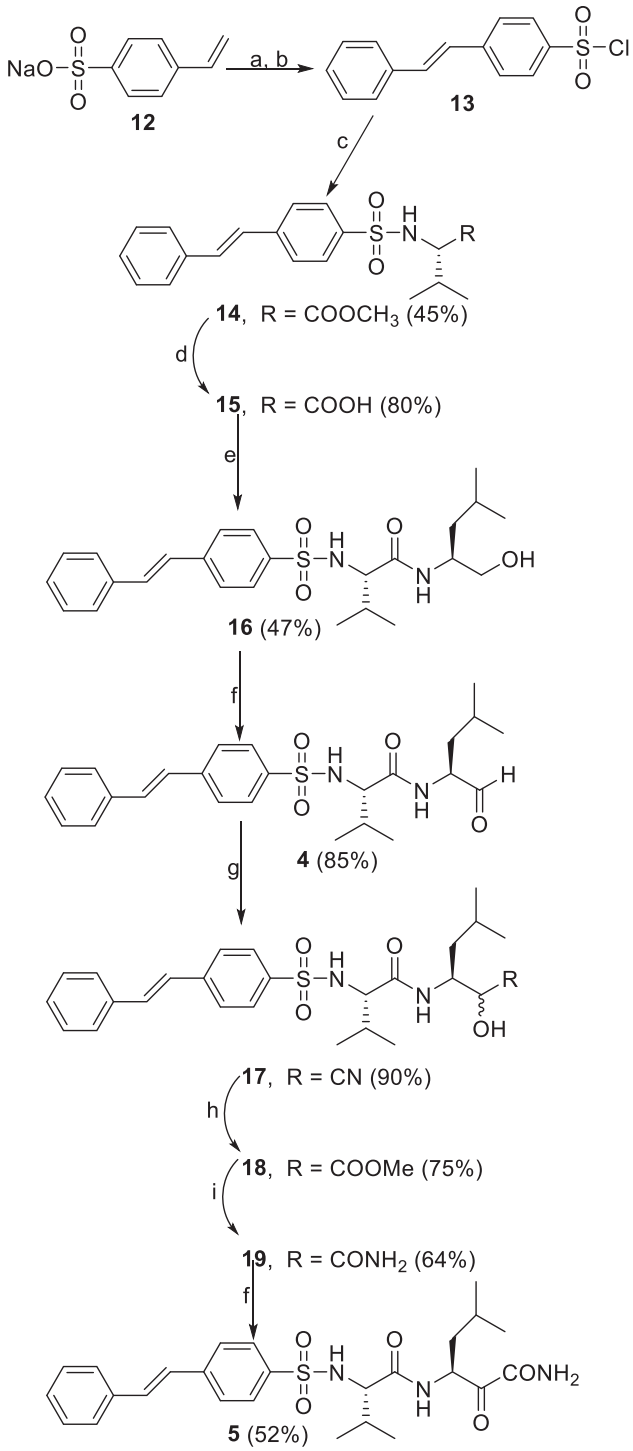
2.2. Biological activity

2.2.1. In vitro inhibition of calpain

Calpain inhibitors such as MDL28170 (Figure 1) were discovered via *N*-terminal capping of dipeptide aldehyde inhibitors of the enzyme.[14] The *N*-terminal capping group of calpain inhibitors occupies the S_3/S_4 subsites of the enzyme. In a previous study we demonstrated that the S_3/S_4 subsites of calpain prefer planar aromatic groups over nonplanar saturated groups as the *N*-terminal capping functionality.[15] Abell et al.[12] reported compound **1** as a mixture of *E*- and *Z*-isomers (in 3.3:1 ratio) that potently inhibited m-calpain. Using molecular



Scheme 1. Synthesis of compounds **1–3**. [a]Reagents: (a) Pyridine, L-Val-OMe.HCl or L-Pro-OMe.HCl RT, 20 h; (b) 1.0 N NaOH, MeOH, 55 °C, 2 h; (c) L-leucinol, CDI, THF, CH_2Cl_2, RT, 96 h; (d) Dess-Martin reagent, CH_2Cl_2; (e) NaHSO3/KCN; (f) Conc. HCl/MeOH; (g) 7.0 N NH_3 in MeOH, 16 h; (h) $PySO_3$, DMSO.



Scheme 2. Synthesis of compounds **4** and **5**. [a]Reagents: (a) Bromobenzene, Pd $(OAc)_2$, TEA, $(o\text{-}Tol)_3P$; (b) $SOCl_2$, DMF; (c) L-Val-OMe.HCl, DIPEA, Pyridine; (d) 4.0 N NaOH, MeOH; (e) L-Leucinol, TEA, Mukaiyama's reagent, DMF; (f) $PySO_3$, DMSO; (g) $NaHSO_3$/KCN; (h) Conc. HCl/MeOH; (i) 7.0 N NH_3 in MeOH.

modeling studies, it was demonstrated that the *E*-(diazo) derivative extends deep into the S_3 pocket of the enzyme and contributes to the calpain inhibitory activity of the mixture of the diazo-dipeptide aldehydes. Here we disclose compounds **2** and **3** as derivatives of compound **1** to investigate if the (*E*)-1-(phenyl)-2-phenyldiazene group could serve as an effective *N*-terminal capping functionality to achieve potent inhibition of calpain. Compounds **4** and **5** were synthesized as ethylene-bridged analogues of the diazene derivatives **1** and **3**, respectively, to determine the significance of the diazene moiety itself for calpain inhibition. The compounds were tested as inhibitors of porcine erythrocyte μ-calpain and the results are displayed in Table 1. Except for compound **2** (K_i = 273 nM), all members of the series were potent inhibitors of μ-calpain and displayed K_i values between 9 nM and 18 nM. Compound **1** with L-valine as the P_2 substituent was 15-fold more potent than **2**, which has a proline residue at the P_2 position. This is consistent with previous structure–activity relationship studies, which demonstrated that the S_2 subsite of calpain prefers small hydrophobic groups at the P_2 position of inhibitors.[16] The ethylene-bridged analogues **4** and **5,** with K_i values of 14 nM and 9 nM, respectively, were also potent inhibitors of μ-calpain, suggesting that the diazene group is not required for potent inhibition of calpain.

MDL28170 is a peptidyl aldehyde that is recognized as a benchmark calpain inhibitor and has been used to investigate the cellular roles of calpain.[14,16] However, peptidyl aldehydes are known to form hydrates in aqueous media and are prone to oxidation *in vivo*,[17,18] which limit the usefulness of the inhibitor as a pharmacological tool. Therefore, to mitigate these properties of peptidyl aldehydes we synthesized and evaluated the μ-calpain inhibitory activity of alpha-ketoamides **3** and **5** as oxidation stable analogues of aldehydes **1** and **4**. As shown in Table 1, alpha-ketoamides **3** and **5** were also potent inhibitors of μ-calpain. The compounds are also more lipophilic (based on CLogP estimation) compared to MDL28170, which suggests that these new calpain inhibitors may display greater cellular permeability compared to MDL28170.

Collectively, these advantages (i.e., enhanced potential for cellular stability and greater lipophilicity) could translate into greater *in vivo* exposure of compounds **3** and **5** compared to MDL28170 but this will be the subject matter of future pharmacokinetics studies.

2.2.2. Antiproliferation and antiinvasion activities

Calpain has been implicated in cellular functions such as cell migration, cell invasion, and tumorigenesis,[6,7] hence we studied the ability of the compounds to inhibit the growth of melanoma cells (A-375 and B-16F1) and PC-3 prostate cancer cells in culture using the sulforhodamine B assay and the MTT assay, respectively. The compounds moderately inhibited proliferation of the cancer cell lines (Table 1). The GI_{50} values for the antiproliferative activity of the compounds versus the human melanoma cell line A-375 ranged from 4.1 μM to 22.2 μM and that for the mouse B-16F1 melanoma cells was between 10.3 μM and 18.7 μM. Compound **2** was the least effective calpain inhibitor of the series, however, it was the most effective antiproliferative agent versus all of the cancer cell lines studied. It inhibited the growth of the cell lines with GI_{50} values of 4.1 μM (A-375 melanoma cells), 13.6 μM (B-16F1 melanoma cells), and 5.6 μM (PC-3 prostate cancer cells) suggesting that other factors besides calpain inhibition may also contribute to the antiproliferation activity of the compound.

It has been demonstrated using *in vitro* and *in vivo* studies that antisense suppression of calpain expression limits the invasion of DU-145 prostate carcinoma cells.[19,20] Therefore, due to the important role that calpain plays in cell motility[21] and tumor invasion[19,20] we studied the effect of compound **3** on DU-145 prostate cancer cell invasion *in vitro* using the Matrigel chamber assay. In this assay tumor invasion in the presence of the inhibitor was expressed as the number of cells that invaded through the Matrigel compared to untreated cells (control). Compound **3** showed concentration-dependent inhibition of prostate cancer cell invasiveness and inhibited DU-145 cell invasion by about 80% at 2 μM (Figure 2). Also, the compound (at 1 μM or 2 μM) did not affect cell proliferation as determined by the MTT assay (Figure 3), indicating that the decrease in cell transmigration was not secondary to decreased cell proliferation.

Table 1
Calpain inhibition and antiproliferative action of compounds **1–5** on cancer cells.

Compound	K_i (nM)[a]	CLogP[b]	GI_{50} (μM) for inhibition of cancer cell growth[c]		
			A-375	B-16F1	P-C3
1	18 ± 2	5.54	12.9 ± 1.5	13.7 + 5	27.3 ± 2
2	273 ± 20	4.57	4.1 ± 0.5	13.6 ± 1.7	5.6 ± 9
3	16 ± 2	4.01	22.2 ± 2.1	18.7 ± 3.0	24.1 ± 3
4	14 ± 1	5.73	9.4 ± 2.1	10.3 ± 2.0	23.0 ± 2
5	9 ± 1	4.20	16 ± 1.3	17.2 ± 2.8	6.02 ± 1
MDL28170	10 ± 2	3.64	ND	ND	ND
Taxol	ND[d]	ND	0.01	0.02	0.03

[a] K_i values were determined by Dixon plots using the average of triplicate assays and plotting 1/v versus *I* (inhibitor concentration) to give intersecting lines with correlation coefficient ≥ 0.95;
[b] CLog P values are the calculated partition coefficients of the inhibitors obtained with ChemDraw Ultra ver. 9.0;
[c] GI_{50} (half maximal growth inhibition) values of the compounds versus the melanoma cell lines A-375 and B-16F1- were determined using the sulforhodamine B colorimetric cytotoxicity assay while those for the PC3 cell line were determined using the MTT assay. All experiments were performed in triplicate and the results are reported as ± standard error of the mean.
[d] ND = Not determined.

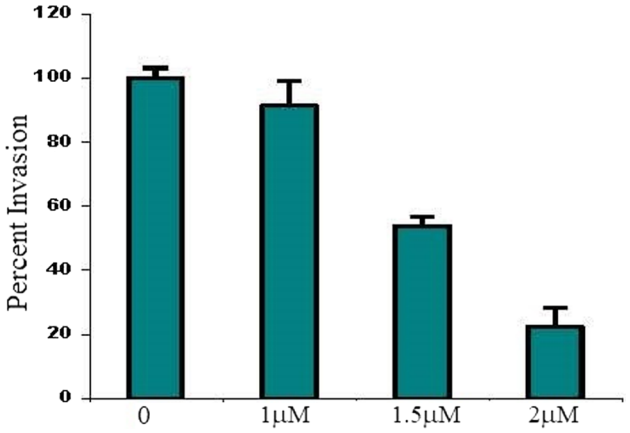


Figure 2. Concentration (μM) dependent inhibition of DU-145 cell invasion by compound **3**. Data shown are the average +/- S.E. of triplicate experiments.

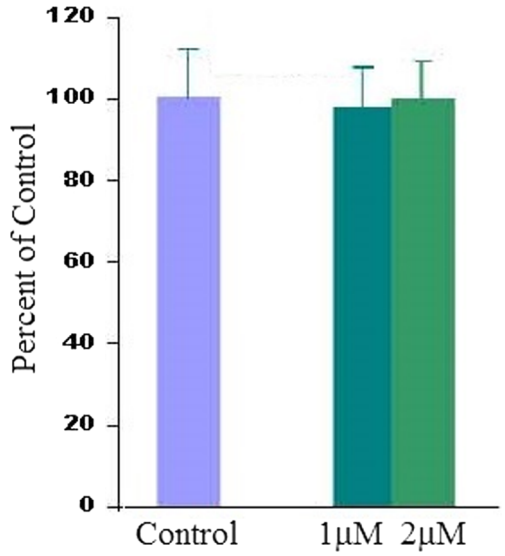


Figure 3. DU-145 cell proliferation in the presence of 1 (μM) and 2 (μM) of compound **3**. Data are shown as the average of +/- S.E. of triplicate experiments.

3. Conclusion

In summary, four new sulfonamide-based peptidomimetic inhibitors of porcine erythrocyte μ-calpain were synthesized. All of the compounds were estimated to be more lipophilic than MDL28170. Compound **2** moderately inhibited μ-calpain but the other three members of the series (i.e., compounds **3**, **4**, and **5**) were potent inhibitors of the enzyme, suggesting that the (*E*)-1-(phenyl)-2-phenyldiazene functionality is a good *N*-terminal capping group for potent inhibition of the enzyme. The study showed that the diazene linker is not necessary for μ-calpain inhibition because the ethylene bridged derivatives were as potent (**4**) or more potent (**5**) than the diazene bridged analogue **3**. In concert with literature evidence that calpain plays an important role in tumorigenesis and/or tumor invasion,[6,7,19,20] the inhibitors demonstrated antiproliferative activity as evidenced by their ability to inhibit the growth of melanoma and prostate cancer cells as well as limit the migration of the DU-143 prostate cancer cells *in vitro*.

4. Experimental section

4.1. Chemistry

All reagents and solvents were purchased from Sigma Aldrich, Fisher Scientific, TCI, or Calbiochem, and were used without further purification. Thin layer chromatography (TLC) was performed on silica gel plates purchased from Analtech, Inc. Fisher silica gel S732-25 (100–400 mesh) was used for column chromatography. Melting points were determined on a Fisher-Johns melting point apparatus. Molecular masses were determined with electron spray ionization mass spectra (ESI-MS) on a Bruker/Hewlett Packard Esquire LC/MS instrument. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Bruker ARX 300 MHz and Varian Inova-500 MHz instruments. Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. The chemical shifts (δ) are reported in parts per million (ppm) relative to TMS, and coupling constants (*J*) are reported in hertz (Hz). Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. IR spectra (neat) were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrophotometer, and the representative absorption bands are reported. Elemental analyses (C, H, N) were performed by Atlantic Microlab Inc., Norcross, GA and are within ± 0.4% of the theoretical values.

4.1.1. General procedure 1. Dess-Martin oxidation

Dess-Martin reagent (17.2 mmol) was slowly added to an ice-cooled solution of the appropriate alcohol (15.6 mmol) in anhydrous CH_2Cl_2 (80 mL). After 10 min, the ice bath was removed and the milky reaction

mixture was stirred at RT for 2–4 h (checked by TLC). A solution of $Na_2S_2O_3$ (160 mmol) in saturated $NaHCO_3$ was added and the mixture was stirred for an additional 10 min at RT. After separation, the aqueous layer was extracted with CH_2Cl_2 (3 × 30 mL) and the combined organic layer was washed successively with $NaHCO_3$ solution, water, and brine followed by drying ($MgSO_4$), concentration, and purification either by flash chromatography or crystallization.

4.1.2. General procedure 2. Pyridine-sulfur trioxide oxidation

A solution of pyridine-sulfur trioxide complex (7.18 mmol) in DMSO (15 mL) was added dropwise to an ice-cooled solution of the alcohol (0.798 mmol) and DIPEA (7.36 mmol) in CH_2Cl_2/DMSO (30 mL) and the mixture was stirred for 1 h at 0 °C. The reaction mixture was diluted with EtOAc (300 mL), washed with aqueous 1 N HCl (2x), saturated aqueous $NaHCO_3$ (2x), and brine. The organic phase was recovered and dried over $MgSO_4$ followed by concentration and purification either by flash chromatography or crystallization.

4.1.3. 3-Methyl-N-(4-methyl-1-oxopentan-2-yl)-2-(4–phenyldiazenyl) phenyl-sulfonamido)butanamide (1)

1,1′-Carbonyldiimidazole (CDI) (494 mg, 3.05 mmol) was added to a stirred ice-cooled solution of acid **7** (1.0 g, 2.77 mmol) in a mixture of THF (15 mL) and CH_2Cl_2 (20 mL). Stirring was continued at RT for 30 min. and L-leucinol (531 μL, 4.16 mmol) was added and the mixture was stirred at RT for 96 h. The solvent was removed and the residue was recrystallized from acetone/CH_2Cl_2 mixture to give *N*-(1-hydroxy-4-methylpentan-2-yl)-3-methyl-2-(4-(phenyldiazenyl)-phenyl-sulfonamido)butanamide as a yellow solid in 51.0% yield. m.p.237–238 °C. IR (cm^{-1}) 1639 (C═O), 3284 (amide), 3505 (amide). 300 MHz ^{1}H NMR (acetone-d_6) δ 7.98–8.08 (m, 6H, Ar), 7.62–7.68 (m, 3H, Ar), 6.98 (d, J = 8.1 Hz, 1H, SO_2NH), 6.52 (d, J = 8.7 Hz, 1H, CONH), 3.69–3.81 (m, 3H, NHCHCONHCHCH$_2$OH), 3.36–3.42 (m, 2H, CH$_2$OH), 2.00–2.06 (m, 1H, NHCHCH(CH)$_3$), 1.10–1.19 (m, 3H, CH$_2$CH(CH$_3$)$_2$), 1.00 (d, J = 6.9 Hz, 3H, CHCH$_3$), 0.89 (d, J = 6.9 Hz, 3H, CHCH$_3$), 0.69 (d, J = 6.0 Hz, 3H, CH$_2$CHCH$_3$), 0.64 (d, J = 6.0 Hz, 3H, CH$_2$CHCH$_3$). Addition of D_2O to the NMR tube resulted in reduction of the number of protons for the signals between δ 3.69–3.81 from 3 protons to 2 protons, which suggested that the OH signal of the product is buried under the multiplex in this region of the spectrum. MS (ESI) 459.0 [M − H]$^-$. Dess-Martin oxidation (general procedure 1) of the yellow solid (300 mg, 0.65 mmol) followed by flash chromatographic purification (acetone /CH_2Cl_2/hexane, 2:1:4) gave **1** in 51% yield. m.p. 146–149 °C. IR (cm^{-1}) 1715 (CHO), 1668 (C═O). 300 MHz ^{1}H NMR ($CDCl_3$) δ 9.50 and 9.42 (s, 1H, CHO), 7.94–8.10 (m, 6H, Ar), 7.54–7.63 (m, 3H, Ar), 6.30 and 6.01 (d, J = 7.2 Hz and 7.8 Hz, 1H, SO_2NH), 5.45 and 5.26 (d, J = 8.7 Hz and 8.1 Hz, 1H, CONH), 4.40–4.48 (m, 1H, CHCHO), 3.63–3.70 (m, 1H, NHCHCH(CH$_3$)$_2$), 2.11–2.20 (m, 1H, NHCHCH(CH$_3$)$_2$), 1.19–1.70 (m, 3H, CH$_2$CH(CH$_3$)$_2$), 0.79–1.01 (m, 12H, CH(CH$_3$)$_2$ and CH$_2$CH(CH$_3$)$_2$). MS (ESI) 457.0 [M − H]$^-$. Anal. Calcd. for: $C_{23}H_{30}N_4O_4S$, C, 60.24; H, 6.59; N, 12.22; S, 6.99. Found: C, 60.05; H, 6.66; N, 12.17; S, 6.83.

4.1.4. N-(4-Methyl-1-oxopentan-2-yl)-1-(4-((phenyldiazenyl) phenylsulfonyl)-pyrrolidine-2-carboxamide (2)

CDI (474 mg, 2.93 mmol) was added to an ice-cooled solution of acid **8** (955 g, 2.66 mmol) in a mixture of THF (20 mL) and CH_2Cl_2 (15 mL) and stirred for 30 min. at RT. L-Leucinol was added and the mixture was stirred at RT for 70 h, after which the solvent was removed and the residue was recrystallized from CH_2Cl_2/hexane to give *N*-(1-hydroxy-4-methylpentan-2-yl)-1-(4-((phenyldiazenyl)phenylsulfonyl) pyrrolidine-2-carboxamide as an orange solid in 60.0% yield. m.p. 152–154 °C. IR (cm^{-1}) 1636 (C═O), 3376 (amide). 300 MHz ^{1}H

NMR ($CDCl_3$) δ 7.97–8.10 (m, 6H, Ar), 7.55–7.59 (m, 3H, Ar), 6.75 (d, J = 9.0 Hz, 1H, CONH), 4.08–4.18 (m, 1H, CH$_2$OH), 4.02–4.06 (dd, J = 3.3 Hz and 3.6 Hz, 1H, NCHCO), 3.79–3.84 (dd, J = 3.6 Hz and 3.3 Hz, 1H, CH$_2$OH), 3.67–3.73 (m, 1H, CHCH$_2$CH(CH$_3$)$_2$), 3.53–3.58 (dd, J = 5.4 Hz and 5.1 Hz, 1H, CH$_2$OH), 3.21–3.29 (m, 1H, NCH$_2$CH$_2$), 2.10–2.23 (m, 1H, NCH$_2$CH$_2$), 1.66–1.86 (m, 6H, NCH$_2$CH$_2$CH$_2$ and CH$_2$CH(CH$_3$)$_2$), 1.41–1.54 (m, 1H, CH(CH$_3$)$_2$), 0.98–1.03 (dd CH(CH$_3$)$_2$, 6H).

Dess-Martin oxidation (general procedure 1) of the orange solid (557 mg, 1.21 mmol) followed by column chromatographic purification (EtOAc/hexane, 2:1) of the resulting product gave **2** as a yellow solid in 50% yield. m.p. 79–82 °C. IR (cm^{-1}) 1655 (C═O), 1735 (CHO), 3366 (amide). 300 MHz ^{1}H NMR ($CDCl_3$) δ 9.63 (s, 1H, CHO), 7.98–8.13 (m, 6H, Ar), 7.57–7.60 (m, 3H, Ar), 7.26 (s, 1H, CONH), 4.43–4.50 (m, 1H, CHCH$_2$CH(CH$_3$)$_2$), 4.22–4.25 (m, 1H, NCHCO), 3.61–3.68 (m, 1H, NCH$_2$CH$_2$), 3.25–3.34 (m, 1H, NCH$_2$CH$_2$), 2.24–2.30 (m, 1H, CH(CH$_3$)$_2$), 1.66–1.86 (m, 6H, NCH$_2$CH$_2$CH$_2$ and CH$_2$CH(CH$_3$)$_2$), 1.01 (dd CH(CH$_3$)$_2$, 6H). MS (ESI) 454 [M − H]$^-$. Anal. Calcd. for: $C_{23}H_{28}N_4O_4S{\cdot}0{\cdot}5H_2O$, C, 59.34; H, 6.28; N, 12.03; S, 6.89. Found: C, 59.28; H, 6.16; N, 11.96; S, 6.69.

4.1.5. 5-Methyl-3-(3-methyl-2-(4-(phenyldiazenyl)phenylsulfonamido)-butanamido)-2-oxohexanamide (3)

Oxidation of **11** (350 mg, 0.696 mmol) with pyridine-sulfur trioxide complex as described under general procedure 2 followed by column chromatographic purification (acetone/hexane 1:1) gave **3** as an orange solid in 71% yield. m.p. 134–136 °C. IR (cm^{-1}) 3464, 3360 and 3281 (NH), 1736 (C═O), 1693 (C═O). 300 MHz ^{1}H NMR (acetone-d_6) δ 7.97–8.09 (m, 6H, Ar), 7.61–7.68 (m, 3H, Ar), 7.46 (d, J = 6.9 Hz, 2H, CONH$_2$), 6.99 (s, 1H, SO_2NH), 6.56 (d, J = 9.3 Hz, 1H, CONH), 5.03–5.10 (m, 1H, CHCH$_2$CH(CH$_3$)$_2$), 3.84–3.89 (m, 1H, NHCHCO), 1.83–1.88 (m, 1H, NCHCH(CH$_3$)$_2$), 1.22–1.30 (m, 2H, CH$_2$CH(CH$_3$)$_2$), 1.12–1.16 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 1.02 (d, J = 6.9 Hz, 3H, CH(CH$_3$)$_2$), 0.91 (d, J = 6.9 Hz, 3H, CH(CH$_3$)$_2$), 0.71–1.02 (dd, J = 6.3 Hz and 9.3 Hz, 6H, CH$_2$CH(CH$_3$)$_2$). MS (ESI) 500.6 [M − H]$^-$. Anal. Calcd. for: $C_{24}H_{31}N_5O_5S{\cdot}0{\cdot}5H_2O$, C, 56.45; H, 6.32; N, 13.72; S, 6.28. Found: C, 56.30; H, 6.09; N, 13.59; S, 6.16.

4.1.6. 3-Methyl-N-(4-methyl-1-oxopentan-2-yl)-2-(4-styrylphenylsulfonamido) butanamide (4)

Oxidation of **16** (2.58 g, 5.63 mmol) with pyridine-sulfur trioxide complex (general procedure 2) followed by recrystallization of the crude product from EtOAc/hexane to give **4** as white solid in 85% yield. m.p. 174–175 °C. IR (cm^{-1}) 1637 (C═O), 1728 (CHO). 500 MHz ^{1}H NMR ($CDCl_3$) δ 9.51 (s, 1H, CHO), 7.85 (d, J = 8.5 Hz, 2H, Ar), 7.63 (d, J = 8.5 Hz, 2H, Ar), 7.56 (d, J = 8.0 Hz, 2H, Ar), 7.04–7.43 (t, J = 7.5 Hz, 2H, Ar), 7.36 (t, J = 7.5 Hz, 1H, Ar), 7.25 (d, J = 16.5 Hz, 1H, CH = CH), 7.12 (d, J = 16.5 Hz, 1H, CH = CH), 6.12 (d, J = 7.5 Hz, 1H, SO_2NH), 5.34 (d, J = 8.5 Hz, 1H, CONH), 4.43 (m, 1H, CHCH$_2$CH(CH$_3$)$_2$), 3.62 (dd, J = 5.0 Hz and 8.0 Hz, 1H, NHCHCO), 2.15 (m, 1H, NCHCH(CH$_3$)$_2$), 1.56 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 1.41–1.49 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 1.26–1.32 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 0.95–0.97 (d, J = 6.5 Hz 3H, NHCHCHCH$_3$), 0.90 (d, J = 2.5 Hz 3H, CH$_2$CHCH$_3$), 0.89 (d, J = 2.5 Hz, 3H, CH$_2$CHCH$_3$), 0.86 (d, J = 6.5 Hz, 3H, NHCHCHCH$_3$). MS (ESI) 455 [M − H]$^-$. Anal. Calcd. for: $C_{25}H_{32}N_2O_4S$, C, 65.76; H, 7.06; N, 6.14; S, 7.02. Found: C, 65.51; H, 7.10; N, 6.13; S, 7.24.

4.1.7. 5-Methyl-3-methyl-2-(4-styrylphenylsulfonamido)butanamido)-2-oxohexanamide (5)

Oxidation of **19** (400 mg, 0.798 mmol) with pyridine-sulfur trioxide complex (general procedure 2) followed by flash chromatographic purification (acetone/hexane, 1:1) of the crude product gave **5** as a white solid in 52% yield. m.p. 209–211 °C. IR (cm^{-1}) 1637 (C═O), 1693 (C═O), 1740 (C═O) 3124, 3276, 3359, 3463 (amide). 500 MHz ^{1}H NMR ($CDCl_3$) δ 7.82 (d, J = 8.5 Hz, 2H, Ar), 7.61 (d, J = 8.0 Hz, 2H, Ar), 7.54 (d, J = 8.0 Hz, 2H, Ar), 7.41 (t, J = 7.2 Hz, 2H, Ar), 7.32 (t, J = 7.2 Hz , 1H, Ar), 7.24 (d, J = 16.5 Hz, 1H, CH = CH), 7.10 (d, J = 16 Hz, 1H, CH = CH), 6.71 (s, 1H, CONH$_2$), 6.21 (d, J = 7.5.0 Hz, 1H, SO_2NH), 5.43 (s, 1H, CONH$_2$), 5.33 (d, J = 8.0 Hz, 1H, CONH),

5.13 (m, 1H, CHCH$_2$CH(CH$_3$)$_2$), 3.58 (dd, 1H, NHCHCO), 2.06–2.11 (m, 1H, NCHCH(CH$_3$)$_2$), 1.31–1.35 (m, 2H, CH$_2$CH(CH$_3$)$_2$), 1.26 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 0.92 (d, J = 7.0 Hz, 3H, NHCHCHCH$_3$), 0.84–0.87 (m, 9H, CH(CH$_3$)$_2$). MS (ESI) 498.0 [M − H]$^-$. Anal. Calcd. for: $C_{26}H_{33}N_3O_5S{\cdot}0{\cdot}2H_2O$, C, 62.06; H, 6.69; N, 8.35; S, 6.37. Found: C, 62.01; H, 6.59; N, 8.33; S, 6.27.

4.1.8. 3-Methyl-2-(4-(phenyldiazenyl)phenylsulfonamido)butanoic acid (7)

(*E*)-4-(Phenyldiazenyl)benzene-1-sulfonyl chloride **6** (3 g, 10.69 mmol) was added to a stirred ice-cooled solution of L-Val-OMe·HCl (2.15 g, 12.8 mmol) in anhydrous pyridine (15 mL) and the resulting crimson solution was stirred at RT for 44 h. Following this the mixture was poured into 2.0 N HCl (100 mL) and extracted with EtOAc (2 × 60 mL). The extract was washed successively with 1.0 N HCl, saturated $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (EtOAc/hexane, 1:2) to give methyl 3-methyl-2-(4-(phenyldiazenyl)phenylsulfonamido)butanoate as an orange solid in 92.5% yield. m.p. 150–152 °C. IR (cm^{-1}) 1736 (ester), 3282 (amide). 300 MHz ^{1}H NMR ($CDCl_3$) δ 7.94–8.03 (m, 6H, Ar), 7.53–7.60 (m, 3H, Ar), 5.18 (d, J = 9.9 Hz, 1H, SO$_2$NH), 3.83 (dd, J = 10.2 Hz and 5.1 Hz, 1H, NHCHCO), 3.47 (s, 3H, OCH$_3$), 2.04–2.11 (m, 1H, CH$_3$CHCH$_3$), 0.99 (d, J = 6.9 Hz, 3H, CH$_3$), 0.90 (d, J = 6.9 Hz, 3H, CH$_3$). MS (ESI) 374.0 [M − H]$^-$. NaOH (1.0 N, 100 mL) was added to a solution of the orange solid (3.3 g, 8.8 mmol) in MeOH (50 mL) and the mixture was stirred at 55 °C for 4 h after which the pH was adjusted to 2 with 2.0 N HCl. The solvent was removed, and the crude product was recrystallized from CH_2Cl_2/ethyl acetate to give **7** as yellow solid in 75.5% yield. m.p.220–222 °C. IR (cm^{-1}) 1694 (C═O), 2876 (br, COOH), 3294 (amide). 300 MHz ^{1}H NMR ($CDCl_3$) δ 7.95–8.00 (m, 6H, Ar), 7.54–7.59 (m, 3H, Ar), 5.17 (d, J = 10.2 Hz, 1H, SO$_2$NH), 3.87 (dd, J = 4.5 Hz and 9.9 Hz, 1H, NHCHCO), 2.07–2.18 (m, 1H, CH$_3$CHCH$_3$), 0.99 (d, J = 6.6 Hz, 3H, CH$_3$), 0.88 (d, J = 6.6 Hz, 3H, CH$_3$). MS (ESI) 360.0 [M − H]$^-$.

4.1.9. 1-(4-(Phenyldiazenyl)phenylsulfonyl)pyrrolidine-2-carboxylic acid (8)

(*E*)-4-(Phenyldiazenyl)benzene-1-sulfonyl chloride **6** (5 g, 17.8 mmol) was added to an ice-cooled solution of D-Pro-OMe·HCl (3.54 g, 21.3 mmol) in anhydrous pyridine (25 mL) and stirred for 20 h at RT after which the mixture was poured into 2.0 N HCl (50 mL) solution and extracted with EtOAc (2 × 40 mL). The EtOAc extract was washed successively with 2.0 N HCl, saturated $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (acetone/hexane, 1:2) to give methyl 1-(4-(phenyldiazenyl)phenyl-sulfonyl)pyrrolidine-2-carboxylate as a yellow solid in 87.3% yield. m.p. 126–128 °C. IR(cm^{-1}) 1743 (C═O). 300 MHz ^{1}H NMR ($CDCl_3$) δ 8.04 (s, 4H, Ar), 7.95–7.98 (m, 2H, Ar), 7.53–7.60 (m, 3H, Ar), 4.40 (dd, J = 5.6 Hz and 6.9 Hz, 1H, NCHCOOCH$_3$), 3.73 (s, 3H, OCH$_3$), 3.52–3.58 (m, 1H, NCH$_2$CH$_2$), 3.39–3.45 (m, 1H, NCH$_2$CH$_2$), 1.98–2.12 (m, 3H, NCH$_2$CH$_2$CH$_2$), 1.84–1.87 (m, 1H, NCH$_2$CH$_2$CH$_2$). The yellow solid (1.5 g, 4 mmol) was dissolved in MeOH (50 mL) and 1.0 N NaOH (90 mL) was added and the mixture was stirred at RT for 16 h followed by 2 h of refluxing. The mixture was adjusted to pH 2 with 2.0 N HCl and extracted with EtOAc. The extract was washed with brine, dried ($MgSO_4$), concentrated and purified by recrystallization from CH_2Cl_2/hexane to give **8** as orange solid in 87.3% yield. m.p. 174–177 °C. IR (cm^{-1}) 1730 (C═O), 2883 (br, COOH). 300 MHz ^{1}H NMR ($CDCl_3$) δ 8.05 (s, 4H, Ar), 7.96–7.99 (m, 2H, Ar), 7.55–7.57 (m, 3H, Ar), 4.38 (br, 1H, NCHCOOH), 3.58 (m, 1H, NCH$_2$CH$_2$), 3.35–3.38 (m, 1H, NCH$_2$CH$_2$), 1.83–2.18 (m, 4H, NCH$_2$CH$_2$CH$_2$). MS (ESI) 358.0 [M − H]$^-$.

4.1.10. N-(1-Cyano-1-hydroxy-4-methylpentan-2-yl)-3-methyl-2-(4-phenyl-diazenyl) phenylsulfonamido)butanamide (9)

$NaHSO_3$ (3.2%, 11 mL) was added to a solution of **1** (847 mg, 1.85 mmol) in MeOH (30 mL) and stirred at RT for 30 h. EtOAc (25 mL) was added followed by 3.2% KCN solution and stirring was continued for another 5 h. The EtOAc layer was separated and the aqueous layer was extracted twice with EtOAc. The combined organic layer was washed with water, brine, and dried ($MgSO_4$). The solvent was removed in vacuo to give an orange solid in 90% yield as a mixture of diastereomers. m.p 93–98 °C. IR (cm^{-1}) 3322 (OH), 1659 (C═O). 300 MHz ^{1}H NMR ($CDCl_3$) δ 7.96–8.04 (m, 6H, Ar), 7.56–7.63 (m, 3H, Ar), 6.40–6.53 (m, 1H, SO$_2$NH), 5.43–5.52 (m, 1H, CONH), 4.50–4.57 (m, 1H, OHCHCN), 4.08–4.24 (m, 1H, OHCHCN), 3.55–3.66 (m, 1H, NHCHCONH), 2.12–2.24 (m, 1H, NHCHCH$_2$CH(CH$_3$)$_2$), 1.40–1.58 (m, 4H, NHCHCH(CH$_3$)$_2$, and CH$_2$CH(CH$_3$)$_2$), 0.74–1.00 (m, 12H, CH$_3$ protons). MS (ESI) 508.1 [M + H]$^+$.

4.1.11. Methyl-2-hydroxy-5-methyl-3-(3-methyl-2-(4-phenyldiazenyl) phenyl sulfonamido) butanamido)hexanoate (10)

Concentrated HCl (15 mL) was added to a solution of **9** (810 mg, 1.67 mmol) in MeOH (20 mL) and the mixture was stirred at 62 °C for 24 h followed by concentration in vacuo and purification by column chromatography (EtOAc/hexane, 1:1) to give an orange solid in 69% yield. m.p 163–168 °C. IR (cm^{-1}) 3522, 3355 and 3270 (NH and OH), 1737 and 1648 (C═O). 300 MHz ^{1}H NMR ($CDCl_3$) δ 7.95–8.02 (m, 6H, Ar), 7.55–7.57 (m, 3H, Ar), 5.96 (d, J = 9.0 Hz, 1H, SO$_2$NH), 5.57 (d, J = 7.8 Hz, 1H, CONH), 4.23–4.32 (m, 2H, OHCHCOOCH$_3$ and NHCHCH$_2$), 3.80 (s, 3H, OCH$_3$), 3.59 (dd, J = 4.8 Hz and 7.8 Hz, 1H, NHCHCO), 3.10 (br s, 1H, OHCHCOOCH$_3$), 2.07–2.15 (m, 1H, NHCHCH(CH$_3$)$_2$), 1.04–1.41 (m, 3H, CH$_2$CH(CH$_3$)$_2$), 0.68–0.98 (m, 12H, CH$_3$ protons). MS (ESI) 517.0 [M − H]$^-$.

4.1.12. 2-Hydroxy-5-methyl-3-(3-methyl-2-(4-(phenyldiazenyl) phenylsulfonamido) butanamido) hexanamide (11)

Compound **10** (600 mg, 1.15 mmol) was dissolved in 7.0 N methanolic ammonia (30 mL) and stirred at RT for 48 h following which the solvent was removed and the product was purified by column chromatography (EtOAc/hexane, 2:1) to give an orange solid in 68% yield. m.p 243–248 °C. IR (cm^{-1}) 3454, 3357 and 3322 (NH and OH), 1668 and 1644 (C═O). 300 MHz ^{1}H NMR (DMSO-d_6) δ 7.58–8.06 (m, 9H, Ar), 7.40 (d, 2H, NH$_2$), 7.03–7.14 (m, 2H, SO$_2$NH and CONH), 5.48–5.55 (dd, J = 5.7 Hz and 5.7 Hz, 1H, OHCHCONH$_2$), 3.93–3.96 (m, 1H, OHCHCONH$_2$), 3.64–3.74 (m, 2H, NHCHCH$_2$CH(CH$_3$)$_2$ and NHCHCH(CH$_3$)$_2$), 1.85–1.92 (m, 1H, NHCHCH(CH$_3$)$_2$), 1.23–1.26 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 0.70–1.11 (m, 8H, CH$_2$CH(CH$_3$)$_2$), 0.43–0.63 (m, 6H, CH(CH$_3$)$_2$). MS (ESI) 501.9 [M − H]$^-$.

4.1.13. Methyl 3-methyl-2-(4-styrylphenylsulfonamido)butanoate (14)

Bromobenzene (12.2 mL, 116 mmol), Pd(OAc)$_2$ (387 mg, 1.74 mmol), TEA (17 mL), and tris(2-methylphenyl)phosphine (1.06 g, 3.5 mmol) were added to a solution of *p*-styrene sulfonic acid sodium salt (20 g, 97 mmol) in DMF (200 mL)/ H_2O (4 mL) mixture. The mixture was stirred at 100 °C for 2 h. After cooling to RT, a gray solid (21.9 g, 80%) separated out and was recovered by filtration, dried, and used in the next step without further purification. The solid was dissolved in (87 mL)/anhydrous DMF (4.5 mL) mixture and refluxed at 90 °C for 3 h. The solvent was removed in vacuo and the residue was dissolved in EtOAc (150 mL) and washed successively with 2.0 N HCl, saturated $NaHCO_3$, brine, and dried ($MgSO_4$). The EtOAc layer was recovered and concentrated under vacuum to give a gray solid (20.19 g, 93.3%), which was used without further purification. The solid (5 g, 17.95 mmol) was mixed with L-Val-OMe hydrochloride (3.6 g, 21.47 mmol) and DIPEA (4 mL) in pyridine (25 mL) and the mixture was stirred for 4 days at RT, poured into 2.0 N HCl and extracted with EtOAc. The extract was washed twice with aqueous 1 N HCl, saturated aqueous $NaHCO_3$, brine, and dried ($MgSO_4$). The solvent was concentrated in vacuo and the residue was purified by column chromatography (acetone/hexane, 1:1) to give a white solid in 45% yield. m.p. 173–175 °C. IR (cm^{-1}) 1735 (ester), 3278 (NH). 300 MHz ^{1}H NMR

(DMSO-d_6) δ 8.26 (d, J = 9.3 Hz, 1H, SO_2NH), 7.71–7.80 (q, 4H, Ar), 7.74–7.66 (d, 2H, CH = CH), 7.30–7.47 (m, 5H, Ar), 3.56 (dd, J = 7.2 Hz and 9.0 Hz, 1H, NHCHCOOCH$_3$), 3.36 (s, 3H, OCH$_3$), 1.87–1.94 (m, 1H, NHCHCH(CH$_3$)$_2$), 0.82 (dd, J = 6.9 Hz and 9.0 Hz, 6H, CH(CH$_3$)$_2$). MS (ESI) 372.0 [M − H]$^-$.

4.1.14. 3-Methyl-2-(4-styrylphenylsulfonamido)butanoic acid (15)

NaOH (4.0 N, 40 mL) was added to a solution of **14** (6.85 g, 18.4 mmol) in MeOH (40 mL) and acetone (40 mL) mixture and stirred for 16 h at 48 °C followed by adjustment of the pH to 2 with 2.0 N HCl and extraction of the solid that separated out with EtOAc. The extract was washed with brine, dried ($MgSO_4$), filtered, and concentrated. The residue was purified by recrystallization from acetone/hexane to give a yellow solid in 80% yield. m.p. 193–195 °C. IR (cm^{-1}) 1747 (C═O), 3313 (w, COOH). ^{1}H NMR (DMSO-d_6) δ 12.58 (s, 1H, COOH), 8.01 (d, J = 9.7 Hz, 1H, SO_2NH), 7.75 (br. s, 4H, Ar), 7.63–7.66 (d, 2H, CH = CH), 7.29–7.46 (m, 5H, Ar), 3.54 (dd, J = 6.0 Hz and 9.0 Hz, 1H, NHCHCOOH), 1.90–1.97 (m, 1H, NHCHCH(CH$_3$)$_2$), 0.81 (dd, J = 6.9 Hz and 9.6 Hz, 6H, CH(CH$_3$)$_2$). MS (ESI) 358.0 [M − H]$^-$.

4.1.15. N-(1-Hydroxy-4-methylpentan-2-yl)-3-methyl-2-(4-styrylphenylsulfonamido)-butanamide (16)

L-Leucinol (2.51 mL, 19.2 mmol) and TEA (4.53 mL, 32.5 mmol) were added to a solution of **15** (5.3 g, 14.76 mmol) in anhydrous DMF (150 mL) at 0 °C followed by the addition of Mukaiyama's reagent (4.53 g, 17.7 mmol). After stirring for 4 days at RT, the solution was poured into H_2O (1.5 L) to give the crude product, which was purified by recrystallization from EtOAc to obtain a white solid in 47% yield. m.p. 196–197 °C. IR (cm^{-1}) 1638 (C═O), 3282 (sulfamide), 3205 (amide), 3503 (br, OH). 300 MHz ^{1}H NMR (DMSO-d_6) δ 7.62–7.79 (m, 7H, Ar), 7.54 (d, 1H, SO_2NH), 7.28–7.43 (m, 5H, CONH, CH = CH, and Ar), 4.55 (br s, 1H, OH), 3.55 (m, 1H, CH$_2$OH), 3.49 (m, 1H, CH$_2$OH), 3.19 (m, 1H, NHCHCH$_2$), 3.05 (m, 1H, NHCHCH(CH$_3$)$_2$, 1.80–1.86 (m, 1H, NHCHCH(CH$_3$)$_2$), 1.10–1.18 (m, 2H, CH$_2$CH(CH$_3$)$_2$), 0.99–1.04 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 0.63–0.86 (m, 12H, CH$_3$ protons). MS (ESI) 457.0 [M − H]$^-$.

4.1.16. N-(1-Cyano-1-hydroxy-4-methylpentan-2-yl)-3-methyl-2-(4-styrylphenylsulfonamido) butanamide (17)

$NaHSO_3$ (3.2%, 14 mL) was added to a solution of **4** (2 g, 4.38 mmol) in MeOH (30 mL) and stirred at RT for 40 h followed by the addition of 3.2% KCN solution (10 mL) and EtOAc (50 mL). After stirring for another 5 h, the mixture was extracted with EtOAc (3 × 50 mL). The extract was concentrated, and the residue was purified by recrystallization from acetone/hexane to give diasteromeric mixture of **17** as a white solid in 90% yield. m.p. 125–128 °C. IR (cm^{-1}) 1736 (ester), 3282 (amide). ^{1}H NMR ($CDCl_3$) δ 7.86–7.88 (d, J = 8.4 Hz, 2H, Ar), 7.64–7.67 (m, 2H Ar), 7.54–7.57 (d, J = 8.1 Hz, 2H, Ar), 7.32–7.44 (m, 3H, Ar), 7.24–7.28 (d, J = 13.8 Hz, 1H, CH = CH), 7.09–7.15 (d, J = 16.2 Hz, 1H, CH = CH), 6.53–6.69 (m, 1H, CONH), 5.33–5.40 (m, 1H, SO_2NH), 4.51–4.59 (m, 2H, OHCHCN and NHCHCH$_2$), 4.10–4.27 (m, 1H, NHCHCO), 3.50–3.61 (m, 1H, NHCHCH(CH$_3$)$_2$), 2.15–2.25 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 1.43–1.62 (m, 3H, OH and CH$_2$CH(CH$_3$)$_2$), 0.81–0.98 (m, 12H, CH$_3$ protons). MS (ESI) 374.0 [M − H]$^-$.

4.1.17. 3-Methyl-2-hydroxy-5-methyl-3-(3-methyl-2-(4-styrylphenylsulfonamido)-butanamido) hexanoate (18)

Compound **17** (932 mg, 19.4 mmol) was dissolved in MeOH (40 mL) containing conc. HCl (11 mL) and the mixture was stirred for 24 h at 54 °C. The solvent was removed in vacuo and the residue was purified by column chromatography (acetone/hexane, 1:1) to give **18** as a white solid in 75.2% yield. m.p. 180–183 °C. IR (cm^{-1}) 1736 (ester), 3282 (amide). ^{1}H NMR (DMSO-d_6) δ 7.28–7.73 (m, 13H, Ar, CONH, SO_2NH, and CH = CH), 5.35–5.58 (m, 1H, OH), 3.83–3.97 (m, 2H, OHCHCO-OCH$_3$ and NHCHCH$_2$), 3.58–3.68 (m, 1H, NHCHCO), 3.54 (s, 3H, OCH$_3$), 1.79–1.83 (m, 1H, NHCHCH(CH$_3$)$_2$), 1.01–1.27 (m, 3H, CH$_2$CH(CH$_3$)$_2$), 0.55–0.91 (m, 12H, CH$_3$ protons). MS (ESI) 374.0 [M − H]$^-$.

4.1.18. 2-Hydroxy-5-methyl-3-(3-methyl-2-(4-styrylphenylsulfonamido)-butanamido) hexanamide (19)

A solution of **18** (750 mg, 1.4 mmol) in 7.0 N methanolic ammonia (30 mL) was stirred at RT for 96 h and the mixture was concentrated to give a residue, which was purified by column chromatography (acetone/hexane, 2:1) to obtain **19** as a white solid in 64% yield. m.p. 234–242 °C. IR (cm^{-1}) 1660, 1643 (C═O), 3326 (OH). ^{1}H NMR (DMSO-d_6) δ 7.04–7.77 (m, 15H, Ar, CONH, SO_2NH, CH = CH, and CONH$_2$. Addition of D_2O decreased the protons in this region from 15 to 11 (i.e., 9 Ar protons plus 2 ethylene protons) , 5.49–5.54(m, 1H, OH), 3.96–4.03 (m, 1H, OHCHCONH$_2$), 3.58–3.71 (m, 2H, NHCHCH$_2$ and NHCHCO), 1.84–1.93 (m, 1H, NHCHCH(CH$_3$)$_2$), 1.24–1.34 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 1.03–1.15 (m, 2H, CH$_2$CH(CH$_3$)$_2$), 0.53–0.87 (m, 12H, CH$_3$ protons). MS (ESI) 500.0 [M − H]$^-$.

4.2. Biological studies

4.2.1. Calpain inhibition assay

The K_i values for inhibition of μ-calpain activity was monitored in a reaction mixture containing 50 mM Tris HCl (pH 7.4), 50 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.2 mM or 1.0 mM Suc-Leu-Tyr-AMC (Calbiochem), 1 μg porcine erythrocyte μ-calpain (Calbiochem), varying concentrations of inhibitor dissolved in DMSO (2% total concentration) and 5 mM $CaCl_2$ in a final volume of 250 μL in a polystyrene microtiter plate as previously reported.[22] The K_i values were estimated from the semi-reciprocal plots of the initial velocity versus the concentration of the inhibitor according to the method of Dixon.[23] The correlation coefficients for the Dixon plots were above 0.95. No other attempt was made to correct for slow binding or autolysis. The reported K_i values are the average of triplicate determinations.

4.2.2. Cell lines and cell cultures

The melanoma cell lines (human A375 cells and mouse B16F1 cells) and the prostate cancer cell lines (PC3 and DU-145) were acquired from ATCC and cultured as follows: The melanoma cells were cultured in DMEM supplemented with 5% (v/v) fetal bovine serum (FBS), 1% (v/v) antibiotic/antimycotic solution, and 0.05% bovine insulin (25 mM, pH 8.2). The prostate cancer cell lines were cultured in RPMI-1640 medium (PC3 cells) or EMEM (DU145 cells) supplemented with 10% FBS, 1% penicillin/streptomycin and 2% L-glutamin. All of the cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

4.2.3. Sulforhodamine B colorimetric cytotoxicity assay

During the initial screen, compounds dissolved in DMSO were added to plates to give a 20 μM solution followed by sulforhodamine B (SRB). To determine IC_{50} values, human melanoma cell line A375 and mouse melanoma cell line B16F1 were trypsinized and harvested by centrifugation for 3 min. Cells were then resuspended in 5% FBS, 1% penicillin/streptomycin and 0.05% insulin in DMEM and counted using a hemocytometer. Cells were seeded into U-bottom 96-well microtiter plate at 5000 cells/well. After 12 h, media was changed, and the test compound was added at 8 different concentrations. Cells were incubated with each compound for 48 h. Taxol was used as positive control and wells containing media and cells only served as negative controls. Following the incubation period cell death was quantitated by sulforhodamine B assay according to the manufacturer's protocol (Sigma-Aldrich). Cells were fixed with 10% TCA, washed five times with water, and incubated with 0.4% sulforhodamine B in 1% acetic acid solution for 30 min at RT. A solution of Tris Base (10 mM) was added to release the dye from the cells for 1 h and the absorbance was measured at 490 nm using an EL800 microplate reader (Biotek).

4.2.4. MTT cytotoxicity assay

Cells were distributed into 96-well plates at a density of 2,000 to 15,000 cells/well, and exposed to a range of drug concentrations (1 to 100 μM) for 72 h at 37 °C in a 5% CO_2 atmosphere. Wells to which no drug was added were used as negative controls. At the end of treatment, an aliquot (25 μL) of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) dye (5 mg/mL) was added to each well for the final two to four hours of incubation. The plates were then centrifuged at 300 g for 15 min. The supernatant medium in each well was aspirated, and the formazan product was solubilized with 100 μL DMSO. The absorbance (*A*) values of wells were determined at 595 nm using a MRX microplate reader (DYNEX Technologies, VA). Percentage cell survival was plotted against the drug concentration and the IC_{50}, the concentration of drug required to reduce living cell number by 50% as compared to non-drug treated wells, was determined by nonlinear regression analysis using WinNonlin (Pharsight Corporation, Mountain View, CA).

4.2.5. Matrigel cell invasion assay

DU-145 cells (20,000 cells) in serum-free medium containing 1% BSA was seeded on the inner surface of the upper chamber and incubated at 37 °C for 24 h. The medium in the lower chamber contained 10% FBS as chemoattractant. For pharmacological inhibition, the medium in the upper chamber seeded with the cells was replaced with serum-free medium in the absence or presence of compound **3** at three different non-cytotoxic concentrations (1.0 μM, 1.5 μM, and 2.0 μM). After 48 h, the remaining cells on the upper chambers were removed by cotton swab. Cells invading through the matrix were fixed, stained, and counted. Each experiment was performed in triplicate and invasion was expressed as a percentage of untreated prostate cancer cells (which served as negative control).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2020.115433.

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