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Synthesis, biological evaluation and molecular modelling of *N*-Heterocyclic dipeptide aldehydes as selective calpain inhibitors

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Abbreviations: DIPEA, *N,N*-diisopropylethylamine; HATU, O-(7-azabenzotriazole-1-yl)-*N,N,N'N'*-tetramethyluronium hexafluorophosphate; o-CAPN1, ovine calpain 1; o-CAPN2, ovine calpain 2; h-CAPN1, human calpain 1; h-CAPN2, human calpain 2; p-CAPN1, porcine calpain 1 and p-CAPN2 porcine calpain 2; r-CAPN1, rat calpain 1 and r-CAPN2 rat calpain 2.

Keywords: Calpain, isoform selectivity, ovine calpain 1 and 2, *in silico* calpain homology models, substituted heterocyclic dipeptides.

Abstract

A series of *N*-heterocyclic dipeptide aldehydes **4-13** were prepared and evaluated as inhibitors of ovine calpain 1 (o-CAPN1) and ovine calpain 2 (o-CAPN1). The 5-formyl pyrrole derivative **9** was shown to be the most potent (IC_{50} 290 and 25 nM against o-CAPN1 and o-CAPN2 respectively) with good selectivity (> 11-fold) for o-CAPN2 over o-CAPN1. The sequences of o-CAPN1 and o-CAPN2 were determined to rationalize the observed SAR of compounds **4-13**. *In-silico* homology models of the active site cleft of o-CAPN1 and o-CAPN2, based on human calpain 1 (h-CAPN1) X-ray crystal structure (PDB code 1-ZCM), were developed due to a lack of available structural information of the ovine calpains. These models were used to rationalize the selectivity observed for the 5-formyl pyrrole derivative **9**. o-CAPN2 selective inhibitor **9** was assayed in an *in vitro* ovine lens culture system where it was shown to effectively prevent the development of calcium-induced cataract.

1. Introduction

Calpains are calcium-activated neutral proteases belonging to the papain superfamily of cysteine proteases. The structure, mechanism of activation and pharmacological actions of calpain have been reviewed.[1] Over-activation of calpain is central to a number of medical conditions associated with cellular damage, these include traumatic brain injury, muscular dystrophy, and cataract.[2] In the case of cortical cataract, increased lens calcium[3] and subsequent over-activation of calpain 2[4,5] has been linked to the breakdown of lens proteins, resulting in clouding of the lens and ultimately blindness. Therefore it has been proposed that development of a potent and selective calpain 2 inhibitor would be a potential means of treating cortical cataract.

In this paper we present SAR data on a series of *N*-heterocyclic peptidyl aldehydes **4-13** that are designed to interact with the S3 binding pocket of calpain. Due to sequence differences between calpain 1 and 2 (see results and discussion section for determination of o-CAPN1 and o-CAPN2 sequences) it

was anticipated that the use of *N*-terminal heterocyclic moieties that interact differentially with either o-CAPN1 or o-CAPN2 would result in isoform-selective calpain inhibitors.

A variety of *N*-terminal heterocyclic constituents of protease inhibitors have been shown to interact with key residues in the S_3 hydrophobic pocket of cysteine proteases (Figure1). A series of quinoline derivatives **1** were designed based on the expectation that the formation of additional hydrogen bonds might be possible via an interaction between the -NH in **1** and calpain residues;[6] the P_3 morpholine urea **2** creates a favorable electrostatic interaction with the sidechain ammonium group of Lys_{64} in the S_3 pocket of Cathepsin S;[7] and the extended P_3 methylpiperazine-thiazolebenzamide **3** is sufficiently close to form an ionic interaction to Asp_{61} in the S_3 pocket of Cathepsin K.[8] In this paper we extend this methodology by designing protease inhibitors with *N*-terminal heterocyclic moieties that are capable of preferentially binding o-CAPN2 over o-CAPN1, resulting in isoform-selective calpain inhibitors.

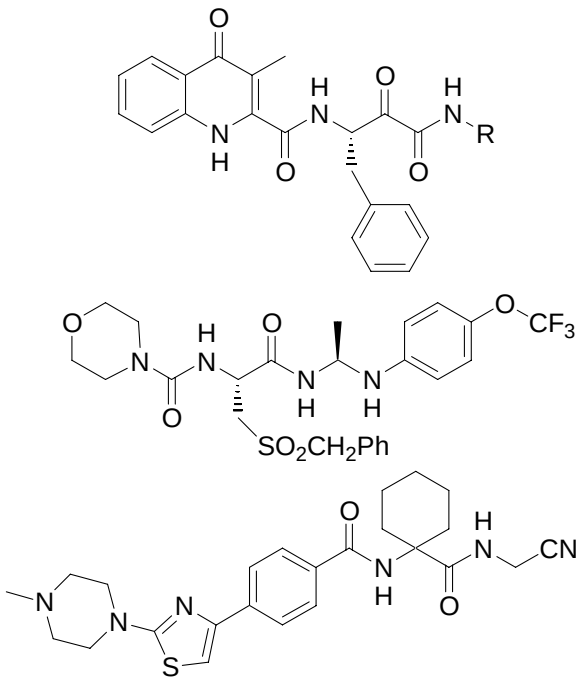


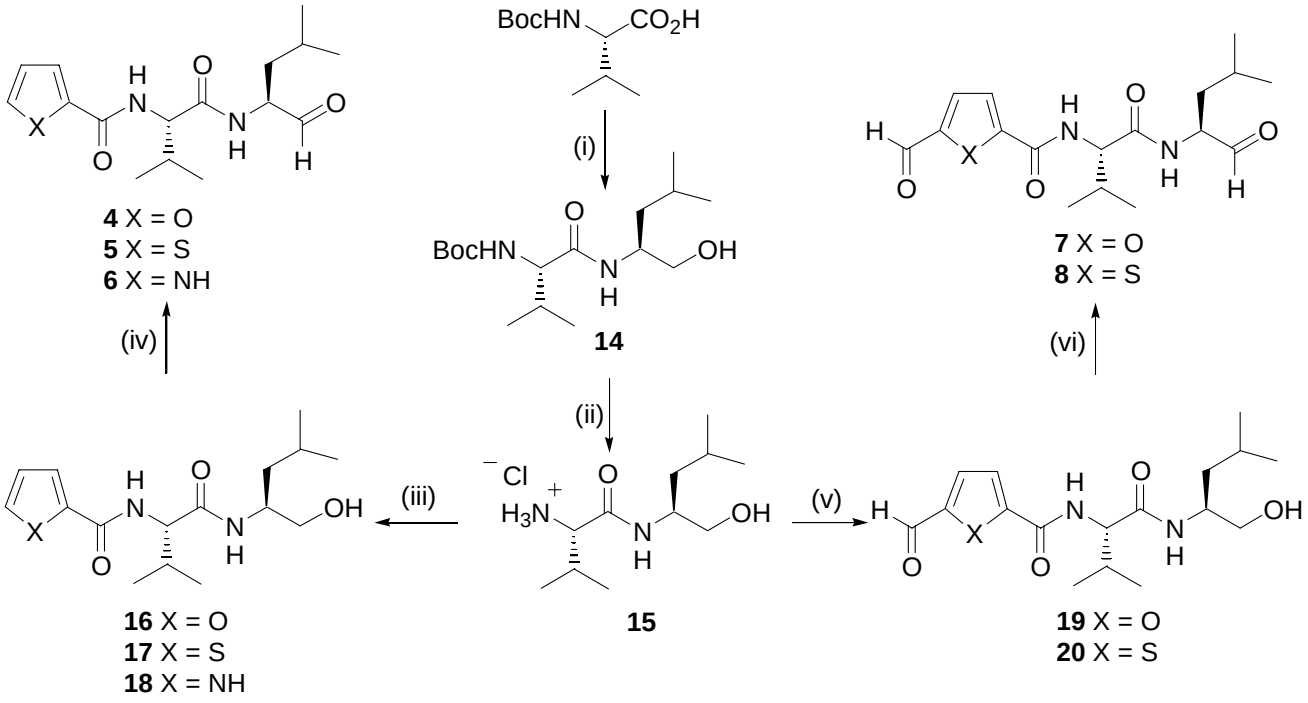
Figure 1. Protease inhibitors **1-3** designed to form hydrogen bonding interactions in the S_3 pockets of hepatitis calpain, cathepsin S and cathepsin K respectively.

Rodent models are most commonly used to assess the effect of anti-cataract agents.[9-12] However, the rodent is a poor model for human cataract as its lenses are smaller, spherical and have a different depth focus mechanism compared to human lenses.[13] The ovine lens is a better model as it has a similar size, shape and accommodative index to the human lens.[14] In addition, the proteins of the ovine lens are more similar to human than those of rat lens as shown by the close homology between sequences of ovine and human crystallins.[15] For these reasons we have tested the efficacy of the inhibitors in this paper using ovine calpains and an ovine lens culture assay.

2. Results and Discussion

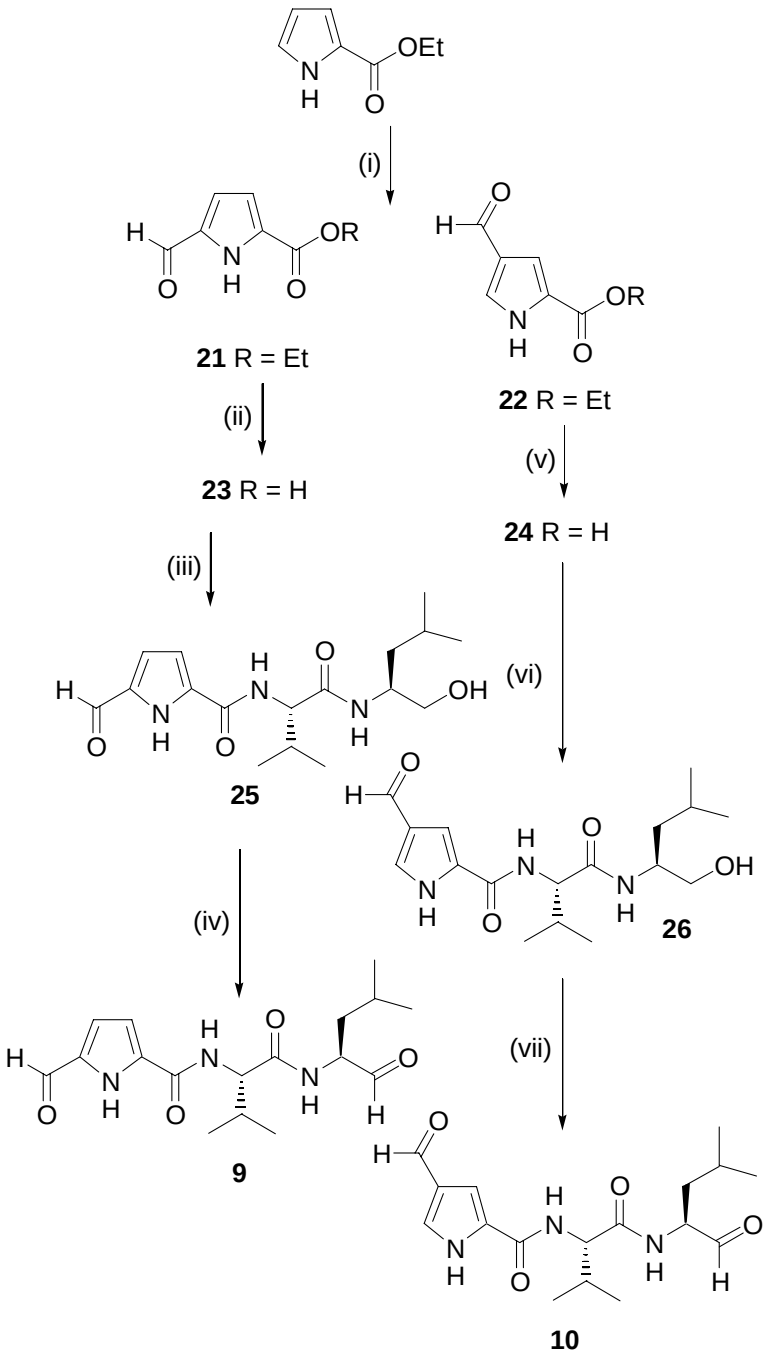
2.1 Preparation of *N*-heterocyclic dipeptide aldehydes

The target compounds **4**-**8** were prepared as detailed in Scheme 1. *N*-Boc-Valine was reacted with *S*-Leucinol to give the Boc protected dipeptide **14** in 68% yield. Treatment with 4 M HCl/dioxane gave the hydrochloride salt **15** in a quantitative yield.[16] This key intermediate was separately reacted with five heterocyclic acids or acid chlorides to give the dipeptide alcohols **16**-**20**.[17] Subsequent oxidation of the alcohols **16**-**20** at 20 °C with SO_3-pyridine and DSMO (the Parikh-Doering oxidation reaction) gave the aldehydes **4**-**8** in good yields (Scheme 1).



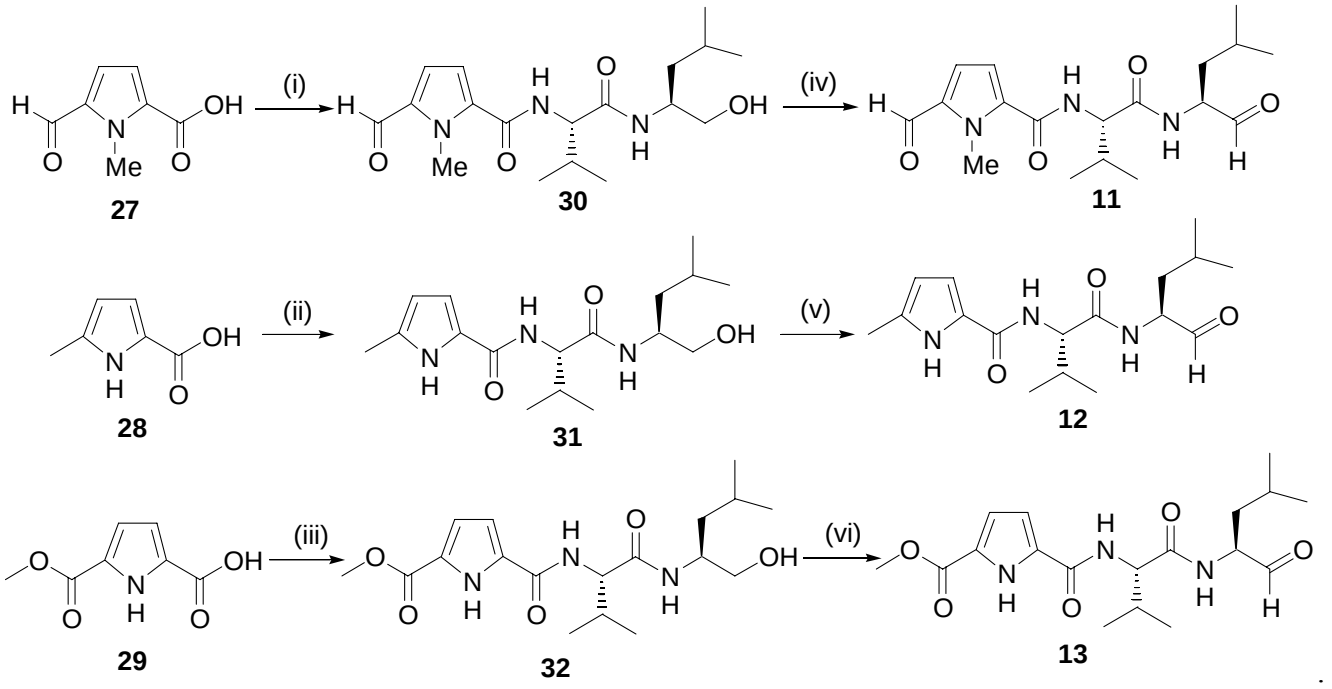
Scheme 1: Synthesis of inhibitors **4-8**. (i) Leucinol, HATU, DIPEA, DMF (68%); (ii) 4 M HCl/dioxane (99%); (iii) DIPEA, DCM, 2-furan-carbonyl chloride or 2-thiophene-carbonyl chloride or 2-pyrrole-carboxylic acid (48, 56 and 84% respectively); (iv) SO_3-Pyr, DMSO, DCM, DIPEA (58, 51 and 71% respectively); (v) DIPEA, DCM, 5-formyl-2-furan-carboxylic acid or 5-formyl-2-thiophene-carboxylic acid (30 and 88% respectively); (iv) SO_3-Pyr, DMSO, DCM, DIPEA (80 and 89% respectively).

Next aldehydes **9** and **10** were prepared as described in Scheme 2. Formylation of 2-pyrrole carboxylic acid ethyl ester gave a mixture of 5- and 4- formyl pyrrole ethyl ester isomers **21** and **22** (2.4:1, **21**:**22**) that were separable by chromatography.[18] Subsequent separate ester hydrolysis of ester **21** and **22** resulted in the formation of 5-formyl-2-pyrrole carboxylic acid **23** and 4-formyl-2-pyrrole carboxylic acid **24**. The acids **23** and **24** were reacted with key intermediate **15** to give the alcohols **25** and **26** and oxidation gave the corresponding aldehydes **9** and **10** (Scheme 2).



Scheme 2 Synthesis of inhibitors **9** and **10**. (i) $POCl_3$, DMF (**21**, 59%; **22** 25%); (ii) NaOH, MeOH, H_2O 93%; (iii) HATU, DIPEA, DMF, **15** (39%); (iv) SO_3-Pyr, DMSO, DCM, DIPEA (71%); (v) NaOH, THF, H_2O (85%); (vi) HATU, DIPEA, DMF, **15** (36%); (vii) SO_3-Pyr, DMSO, DCM, DIPEA (90%).

Finally aldehydes **11-13** were prepared as described in Scheme 3. The substituted 2-pyrrole carboxylic acid precursors **27-29** were synthesised using standard literature procedures.[19, 20] 5-Formyl-*N*-methyl-pyrrole-2-carboxylic acid **27** was synthesized *via* alkylation of 2-pyrrole carboxylic acid using methyl iodide and NaOH followed by base-mediated ester hydrolysis.[19] 5-Methylpyrrole-2-carboxylate **28** was synthesized from the reaction of diethyl acetamidomalonate and 1,4-dichloro-2-butyne using NaOEt as a base in refluxing EtOH under the method developed by Curran and Keaney followed by ester hydrolysis.[20] 5-Methoxy carbonyl-2-pyrrole carboxylic acid **29** was synthesized *via* $KMnO_4$ oxidation of the corresponding 5-formyl ester in methanol.[19] The precursors **27-29** were then reacted with key intermediate **15** to yield the alcohols **30-32** that were subsequently oxidized to give the corresponding aldehydes **11-13** (Scheme 3).



Scheme 3. Synthesis of inhibitors **11-13**. (i) **15**, HATU, DIPEA, DMF (56%); (ii) **15**, HATU, DIPEA, DMF, (29%); (iii) **15**, HATU, DIPEA, DMF (64%); (vi) SO_3-Pyr, DMSO, DCM, DIPEA (75%); (v) SO_3-Pyr, DMSO, DCM, DIPEA (63%); (vi) SO_3-Pyr, DMSO, DCM, DIPEA (69%).

2.2 Ovine calpain sequence

The amino acid sequences of catalytic subunits of o-CAPN1 and o-CAPN2 were determined from their cDNA sequences (Genebank accession numbers XXXXXXXX and EU161096 respectively). o-CAPN2 had two alleles with a G replaced by T at 741 and C replaced by a T at 1539. However neither of these altered the amino acid sequence. The ovine calpains were more homologous to human calpains (h-CAPN1[21] and h-CAPN2[22]) than the rat calpains (r-CAPN1[23] and r-CAPN2[34]) sequences. The o-CAPN1 amino acid sequence was 95% homologous with the h-CAPN1, considerably closer than the 89% homology of r-CAPN1 to h-CAPN1. The calpain 2 large subunit was 95% homologous between ovine and human compared with 94% for rat and human. Of the 35 amino acid which were different between o-CAPN2 and h-CAPN2, 18 were conservative substitutions with similar amino acids and the changes were clustered in non-essential regions of the protein. The homology between ovine calpain isoforms is similar to that in other species, with o-CAPN2 62% homologous to o-CAPN1. o-CAPN2 is also 62 % homologous to h-CAPN1.

Alignment of selected residues in the catalytic domain of ovine, human and rat calpain highlight the greater homology between ovine calpains and h-CAPN1 compared to r-CAPN1 (Figure 2). Within these sections there are 11 amino acids different between h-CAPN1 and r-CAPN1 and only 2 between h-CAPN1 and o-CAPN1. There are 9 differences between h-CAPN1 and o-CAPN2. However; only 2 of these differences (Ser_{209} corresponds to Ala_{199} and Met_{260} corresponds to Ser_{250} in o-CAPN2) in occur in close proximity to the active site.

r-CAPN1	103	T	R	T	D	I	C	**Q**	G	A	L	**G**	D	**C**	**W**	L	L	A	A	I	A	S	L	T	125
h-CAPN1	103	T	R	T	D	I	C	**Q**	G	A	L	**G**	D	**C**	**W**	L	L	A	A	I	A	S	L	T	125
o-CAPN1	103	T	R	T	D	I	C	**Q**	G	A	L	**G**	D	**C**	**W**	L	L	A	A	I	A	S	L	T	125
o-CAPN2	93	T	R	T	D	I	C	**Q**	G	A	L	**G**	D	**C**	**W**	L	L	A	A	I	A	S	L	T	115

r-CAPN1	206	**S**	**G**	**G**	**C**	**T**	S	E	**A**	F	E	D	F	T	G	G	V	T	E	**W**	Y	**D**	L	**Q**	228
h-CAPN1	206	**S**	**G**	**G**	**S**	**T**	S	E	G	F	E	D	F	T	G	G	V	T	E	**W**	Y	E	L	R	228
o-CAPN1	206	**S**	**G**	**G**	**S**	**T**	S	E	G	F	E	D	F	T	G	G	V	T	E	**W**	Y	E	L	R	228
o-CAPN2	196	**S**	**G**	**G**	**A**	**T**	**T**	E	G	F	E	D	F	T	G	G	**I**	**A**	E	**W**	Y	E	L	R	218

r-CAPN1	251	**S**	**I**	**N**	**I**	S	**D**	**I**	**R**	**D**	**L**	**E**	A	I	T	F	K	**N**	L	V	**R**	**G**	**H**	**A**	273
h-CAPN1	251	**S**	**I**	**D**	**I**	S	S	V	L	D	**M**	**E**	A	I	T	F	K	K	L	V	K	**G**	**H**	**A**	273
o-CAPN1	251	**S**	**I**	**D**	**I**	S	S	**I**	L	D	**M**	**E**	A	**V**	T	F	K	K	L	V	K	**G**	**H**	**A**	273
o-CAPN2	241	**S**	**I**	**D**	**I**	**T**	S	**A**	**A**	D	**S**	**E**	A	I	T	F	**Q**	K	L	V	K	**G**	**H**	**A**	263

r-CAPN1	347	**K**	L	**E**	I	350
h-CAPN1	347	**R**	L	**E**	I	350
o-CAPN1	347	**R**	L	**E**	I	350
o-CAPN2	337	**R**	L	**E**	I	340

Figure 2. Sequence alignment between selected residues in the catalytic domain of r-CAPN1, h-CAPN1, o-CAPN1 and o-CAPN2. Amino acid residues forming part of the active site cleft and binding pockets are in bold type. Amino acid substitutions compared to h-CAPN1 are highlighted in pink.

2.2 Biological activity

The compounds **4-13** were assayed against o-CAPN1 and o-CAPN2 (purified from ovine lung tissue) using a fluorescence-based assay to determine *in vitro* potency and selectivity.[25] The assay results are summarized in Table 1. All compounds inhibited both o-CAPN1 and o-CAPN2 with IC_{50}'s below 1 μM. 5-Formyl-pyrrole **9** (IC_{50} values of 290 and 25 nM against o-CAPN1 and o-CAPN2 respectively) was the most potent and selective o-CAPN2 inhibitor, displaying over 11-fold selectivity. In contrast other well documented dipeptidyl aldehyde calpain inhibitors such as SJA6017[26] (IC_{50} values of 7.5 and 78 nM against porcine calpain 1 (p-CAPN1) and porcine calpain 2 (p-CAPN2) respectively) and MDL28170[27] (IC_{50} values of 56 and 350 nM against h-CAPN1 and h-CAPN2 respectively) display selectivity for calpain 1 over calpain 2. Formyl-furan **7** (IC_{50} values of 960 and 100 nM against o-CAPN1 and o-CAPN2 respectively) was also a highly potent and selective o-CAPN2 inhibitor,

displaying over 9-fold selectivity for o-CAPN2. The inhibitors **4**-**7**, **8**, **10**, **12** and **13** displayed moderate selectivity (between 2 and 7-fold) for o-CAPN2 with the exception of *N*-methyl pyrrole **11** which was non-selective.

The o-CAPN1 assay results (Table 1) indicate that compounds **6**, **9**-**13**, containing pyrrole moieties, are generally more potent than the other compounds **4**, **5**, **7** and **8** containing either furan or thiophene moieties. Analysis of the SAR within the pyrroles **6**, **9**-**13** suggests that the -NH of pyrrole **9** is not involved in hydrogen bonding in the S3 pocket of o-CAPN1, as its replacement with a methyl group (as in compound **11**) results in an increase in potency (IC_{50} 290 and 150 nM respectively). Hydrogen bonding between o-CAPN1 and the 5-formyl group of pyrrole **9** (IC_{50} 290 nM) appears important as pyrrole **6**, 4-formyl pyrrole **10** and 5-methyl pyrrole **12** are all less potent against o-CAPN1 compared to **9** (IC_{50} 650, 530 and 340 nM respectively). The difference in oxidation state of the 5-formyl group of pyrrole **9** compared to the 5-methyl ester in **13** does not appear to affect potency (IC_{50} 290 nM in both cases).

Compounds **4**-**13** inclusive are generally more potent against o-CAPN2 than o-CAPN1 (Table 1), with differences in potency being less pronounced for the furan/thiophene compounds **4**, **5**, **7** and **8** (IC_{50} 135, 100, 100 and 85 nM respectively) and the pyrroles **6**, **9**-**13** (IC_{50} 315, 25, 100, 150, 110 and 140 nM respectively) compared to those observed against o-CAPN1. It is evident that the 5-formyl pyrrole group of compound **9** (IC_{50} 25 nM) is important for binding to o-CAPN2 as compounds **6**, **10** and **12** (IC_{50} 315, 100 and 110 nM respectively) are less active against o-CAPN2 compared to **9** . This observation is consistent with the o-CAPN1 assay results. However, in sharp contrast to the o-CAPN1 assay results, the -NH of pyrrole **9** (IC_{50} 25 nM) appears important for binding to o-CAPN2 as evidenced by the decreased activity of **11** (IC_{50} 150 nM) where the pyrrole -NH is replaced by an *N*-methyl group.

Table 1. *In vitro* inhibition data.

Compound	IC_{50} (nM)[a]		Selectivity o-CAPN1 over o-CAPN2
	o-CAPN1	o-CAPN2	
4	790	135	5.85
5	680	100	6.80
6	650	315	2.06
7	960	100	9.60
8	440	85	5.17
9	290	25	11.6
10	530	100	5.30
11	150	150	0
12	340	110	3.09
13	290	140	2.07

[a] Values are means of three experiments, variation between experiments is no more than ± 10%

2.3 Construction of *in-silico* ovine calpain homology models

Molecular modeling was used to rationalize the observed SAR of compounds **4**-**13**. Novel *in-silico* ovine calpain homology models were constructed using the o-CAPN1 and o-CAPN2 sequences and h-CAPN1 X-ray crystal structure data available from the Protein Data Bank (PDB code 1-ZCM).[28]

The most appropriate calpain X-ray crystal structure information was required to build accurate *in-silico* ovine calpain models. A limited number of X-ray crystal structures of the active catalytic domains of calpain 1 and 2 have been published. The majority of these crystal structures have been obtained by Davies *et al* using a rat calpain 1 (r-CAPN1) construct.[29] Unfortunately, useful structural information is not available for the rat calpain 2 (r-CAPN2) construct due to an instability in helix α7, which results in intrinsic inactivation.[30] Only two h-CAPN1 construct crystal structures have been published.[31, 28] Sequence alignment of selected residues of h-CAPN1,[21] r-CAPN1,[23] and o-CAPN1 as well as o-CAPN2 clearly shows that a high degree of homology exists within the residues that form the active site cleft of ovine calpains and h-CAPN1 (Figure 2). Therefore, the h-CAPN1 X-ray crystal structure (1-ZCM) was used as the structural basis for the o-CAPN1 homology model.

A similar strategy was employed to build the o-CAPN2 homology model. However, the only human calpain 2 (h-CAPN2) crystal structure available is in an inactivated form.[31] Therefore despite a number of differences in the amino acid sequence of h-CAPN1 and o-CAPN2 (Figure 2), it was decided to build the *in silico* o-CAPN2 homology model from the h-CAPN1 X-ray crystal structure (1-ZCM)[28] as this is the most accurate X-ray crystal structure available and is thus most likely to rationalize the observed SAR of compounds **4-13**.

2.4 Molecular modeling and SAR discussion

The h-CAPN1 X-ray crystal structure, 1-ZCM, was virtually mutated around the active site cleft to mimic either the amino acid sequence of o-CAPN1 or o-CAPN2 (see section 4.4). The ovine calpain homology models were then used to dock compounds **4-13**. The docking[32, 33] suggested two different binding modes for the compounds. It was found that the less selective compounds (**4-6**, **8**, **10-13**) bind to the o-CAPN1 and o-CAPN2 homology models in the same binding mode. For example, 5-formyl thiophene **8** binds to both o-CAPN1 and o-CAPN2 homology models in a typical β-strand binding conformation (Figure 3a and 3b).[16] In sharp contrast, the more selective inhibitors (**7** and **9**) appear to bind to the two homology models in different conformations. For example 5-formyl pyrrole **9** binds to the o-CAPN1 homology model in a typical β-strand binding conformation (Figure 3c) whereas a unique shunted conformation is observed for the binding of **9** to the o-CAPN2 homology model (Figure 3d). This unique shunted conformation places the aldehyde in closer proximity to the active site cysteine Cys_{105} to allow a hydrogen bond to Gln_{99} in the oxyanion hole. An additional hydrogen bond is formed between the pyrrole -NH and Gly_{198} which is reflected in the observed SAR of pyrrole **9**. The replacement of the pyrrole -NH of **9** with *N*-methyl group (compound **11**) results in an increase in potency for o-CAPN1 (IC_{50} 290 and 150 nM respectively) but a decrease for o-CAPN2 (IC_{50} 25 and 150 nM respectively). We suggest that the unique mode of binding of **9** to o-CAPN2 might explain the observed greater than 11-fold selectivity of this compound for o-CAPN2 over o-CAPN1.

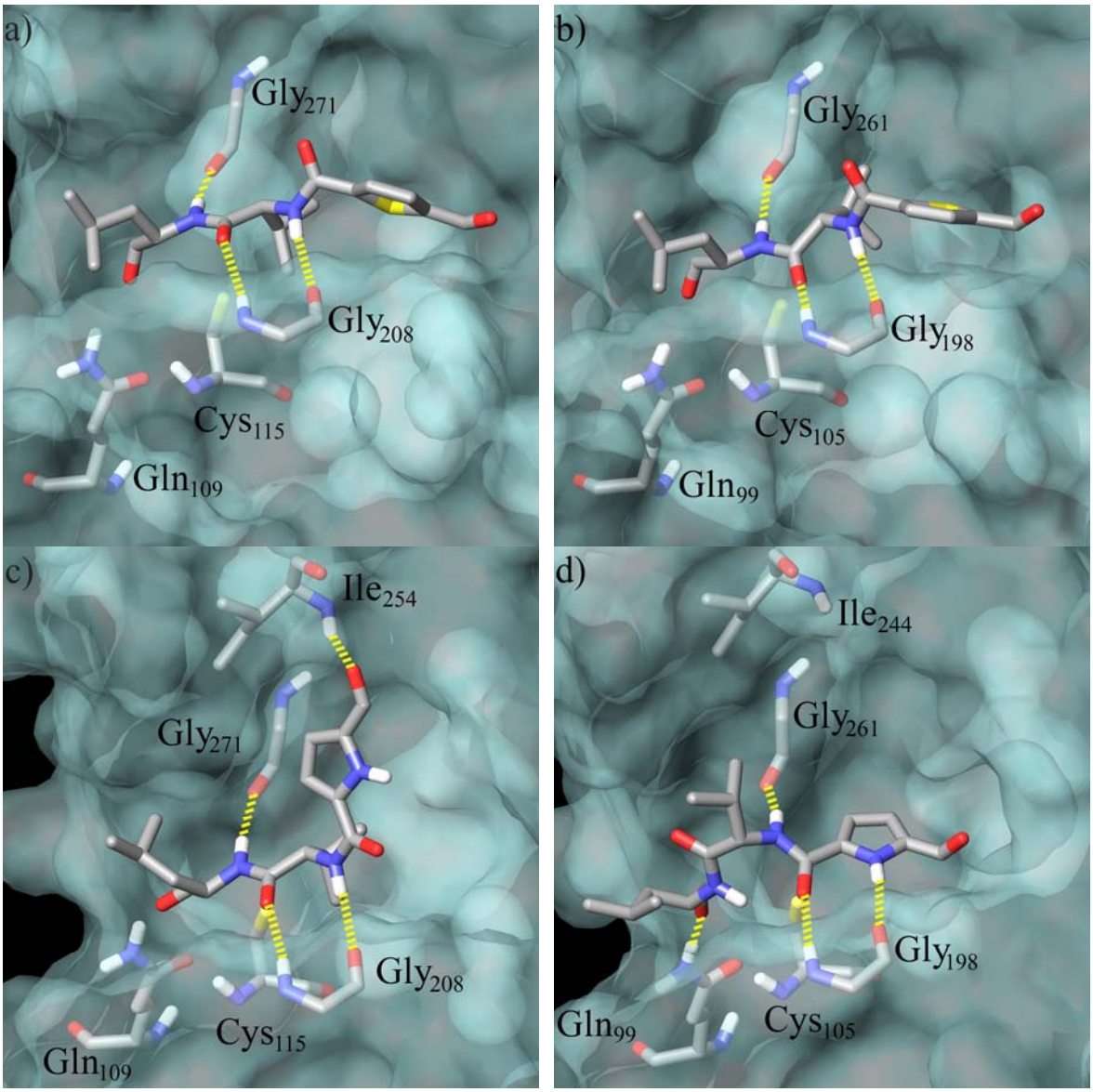


Figure 3. (a) 5-Formyl thiophene dipeptide **8** bound to the o-CAPN1 homology model in a typical β-strand binding conformation. (b) 5-Formyl thiophene dipeptide **8** bound to the o-CAPN2 homology model in a typical β-strand binding conformation. (c) 5-Formyl pyrrole **9** bound to the binding o-CAPN1 homology model in a typical β-strand binding conformation. (d) 5-Formyl pyrrole **9** bound to the binding o-CAPN2 homology in a shunted binding conformation.

2.4 *In vitro* lens culture assay

Over-activation of calpain 2 has been linked to the development of cortical cataract and therefore the o-CAPN2 selective inhibitor **9** was assayed in an *in vitro* lens culture system. The ability of inhibitor **9**

to retard calpain-induced cell damage in ovine lenses was studied. We have previously reported a detailed method for this assay.[11] Briefly, inhibitor **9** (0.8 μM) was added to one lens of a pair of sheep lenses in culture media. After 2 hours incubation, 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 3 for representative examples. Lenses treated with calcium only (for example lens 1 in Figure 6) clearly showed the opacity associated with cataract formation; however, lenses treated with calcium in the presence of **9** (e.g. lens 2) essentially remained transparent, as revealed by the reference grid placed behind each lens. The loss of transparency was significantly reduced by **9** ($p < 0.005$) in a paired t-test.

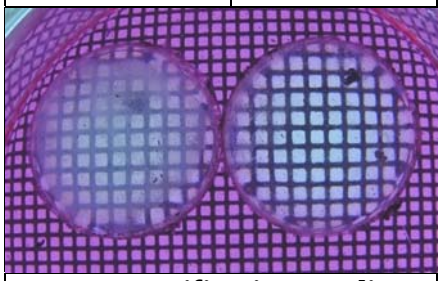
Lens 1	Lens 2
	
Mean opacification grading score (± sd)	
45.1 ± 2.9	27.9 ± 5.5

Figure 3. Calcium induced cataract in ovine 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.

3. Conclusion

A range of *N*-terminal hetrocyclic-dipeptide aldehydes **4-13**, designed to interact with the S3 binding pocket of calpain, were prepared and evaluated as anti-cataract agents. The efficacy of the compounds was tested using ovine calpain and an ovine lens culture assay as this has been shown to be an effective model for human cataract.

Compounds **4-13** are potent calpain inhibitors (all IC_{50} values below 1 μM) that show varying degrees of isoform-selectivity. Compounds containing a 5-formyl substituted heterocyclic moiety (compounds **7** and **9**) gave the greatest degree of isoform-selectivity. The 5-formyl pyrrole **9** was highly active against o-CAPN2 (IC_{50} 25 nM) and exhibited greater than 11-fold selectivity for o-CAPN2 over o-CAPN1. In contrast, other well documented dipeptidyl aldehyde calpain inhibitors such as SJA6017 and MDL28170 display selectivity for calpain 1 over calpain 2.

The amino acid sequences of catalytic subunits of o-CAPN1 and o-CAPN2 were determined from their cDNA sequences and had 95% homology to the human isoforms. *In silico* homology models were generated from the o-CAPN1 and o-CAPN2 sequences and h-CAPN1 X-ray crystal structure data. The models were used to rationalize the observed SAR of compounds **4-13**. In particular, two distinct binding conformations were revealed for the 5-formyl pyrrole **9** that might explain the observed greater than 11-fold selectivity of this compound for o-CAPN2 over o-CAPN1. o-CAPN2 selective inhibitor **9** was assessed in an *in vitro* lens culture assay and shown to successfully retard the formation of a calcium-induced cataract in ovine lenses. Further development of 5-formyl pyrrole **9** is ongoing and its suitability for use as an anti-cataract agent will be reported in a future publication.

4. Experimental

4.1. Chemistry

Proton NMR spectra were acquired on a Varian Inova 500 spectrometer operating at 500 MHz unless otherwise stated. Carbon NMR spectra were obtained on a Varian Unity XL 300MHz Fourier Transform spectrometer operating at 75 MHz with a delay (D_1) of 1 s. Unless other wise stated, all spectra were obtained at 23 °C. Chemical shifts are reported in parts per million (ppm, δ) and are referenced relative to residual solvent e.g. $CDCl_3$ ($CHCl_3$ at δ_H 7.26 ppm). Electrospray ionization (ESI) mass spectra were detected on a micromass LCT TOF mass spectrometer, with a probe voltage of 3200 V and a source temperature of 80 °C. Infrared spectra were obtained using a Shimadzu 9201PC series

FTIR interfaced with an Intel 486 PC operating Shimadzu's HyperIR software. Diffuse reflectance spectra were obtained in a solid KBr matrix. Melting points were obtained on an electrothermal melting point apparatus and are uncalibrated. Microanalysis was performed at the University of Otago Microanalytical Laboratory. All reported values are within ±0.4% of the calculated value. Thin layer chromatography (TLC) was performed on aluminium-backed Merck Kieselgel KG60F silica plates and flash column chromatography was carried out under a positive pressure of dry nitrogen using Merck silica gel 60 (230–400 mesh). All chemicals were purchased from Aldrich in the highest possible purity.

General procedure A: Amide bond formation from an acid chloride. To a stirred solution of the acid chloride (1.0 equiv) in dry *N*,*N*-dimethylformamide was added the amine (1.0 equiv) and DIPEA (2.0 equiv) and the resulting solution stirred at rt overnight. The reaction mixture was diluted with ethyl acetate and washed successively with aqueous 1M HCl, saturated aqueous sodium bicarbonate and brine. The organic phase was separated, then dried over $MgSO_4$ and concentrated *in vacuo*.

4.1.1. Furan-2-carboxylic acid [(S)-1-((S)-1-hydroxymethyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 16. To a stirred solution of **14** was added 4 M HCl in dioxane (10.0 equiv) and the resulting solution stirred at rt overnight. Concentration *in vacuo* gave the hydrochloride salt **15** which was used subsequently without further purification. Reaction of **15** with 2-furan-carbonyl chloride according to general procedure **A** gave **16** as a colourless oil (0.15 g, 48%). HRMS (ES+) calcd for $C_{16}H_{26}N_2O_4$ ($M+H^+$) 311.1971 found 311.1973; ν_{max} (KBr) 1633 (C=ONH), 1270.1 (OH); ^{1}H NMR (500 MHz, CD_3OD) 0.88 (6H, dd, J = 18.1, J = 6.1 Hz, 2×C*H*$_3$), 1.00 (6H, dd, J = 8.3 Hz, J = 7.1 Hz, 2×C*H*$_3$), 1.35-1.40 (2H, m, C*H*$_2$CH(CH$_3$)$_2$), 1.63 (1H, tt, J = 6.8 Hz, J = 13.3 Hz, CH$_2$C*H*(CH$_3$)$_2$), 2.14 (1H, qd, J = 6.9 Hz, J = 13.9 Hz, C*H*(CH$_3$)$_2$), 3.48 (2H, dq, J = 10.9 Hz, J = 5.5 Hz, C*H*$_2$OH), 4.00 (1H, dt, J = 10.2 Hz, J = 5.2 Hz, C*H*CH$_2$CH(CH$_3$)$_2$), 4.35 (1H, d, J = 7.9 Hz, C*H*CH(CH$_3$)$_2$), 6.57 (1H, dd, J = 3.6 Hz, J = 1.6 Hz, CHC*H*CH), 7.15 (1H, d, J = 3.5 Hz, CHCHC*H*), 7.67 (1H, dd, J = 2.1 Hz, J = 1.2 Hz, C*H*CHCH); ^{13}C NMR (75 MHz, CD_3OD) 17.8 (*C*H$_3$), 18.6 (*C*H$_3$), 21.1 (*C*H$_3$), 22.5 (*C*H$_3$), 24.6 (CHCH$_2$*C*H(CH$_3$)$_2$), 30.8 (CH*C*H(CH$_3$)$_2$), 39.7 (CH*C*H$_2$CH(CH$_3$)$_2$), 49.5 (CH$_2$OH), 59.0

(*C*HCH$_2$CH(CH$_3$)$_2$), 64.3 (*C*HCH(CH$_3$)$_2$), 111.8 (CHCHCH), 114.4, (CHCHCH), 145.1 (CHCHCH), 147.2 (*C*O), 159.0 (*C*ONH), 172.0 (*C*ONH); *m/z* (ES) 311 ([M+(H)]$^+$, 100, 333 ([M+(Na)]$^+$, 20.

4.1.2. Thiophene-2-carboxylic acid [(S)-1-((S)-1-hydroxymethyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 17. Reaction of **15** (as prepared above) with 2-thiophene-carbonyl chloride according to general procedure **A** gave **17** as a colourless crystalline solid (0.7 g, 56%). mp 181-183 ºC; HRMS (ES+) calcd for $C_{16}H_{27}N_2O_3S$ (M+H$^+$) 327.1742 found 327.1758; ν_{max} (KBr) 1620.1 (C=ONH), 1068.5 (C-OH); ^{1}H NMR (500 MHz, $CDCl_3$) 0.86 (6H, d, J = 7.0 Hz, 2×C*H*$_3$), 1.03 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.04 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.30-1.44 (2H, m, C*H*$_2$CH(CH$_3$)$_2$), 1.54-1.64 (1H, m, CH$_2$C*H*(CH$_3$)$_2$), 2.21 (1H, sex, J = 7.0 Hz, C*H*(CH$_3$)$_2$), 2.80 (1H, br s, O*H*), 3.54-3.62 (1H, m, C*H*$_2$OH), 3.66-3.74 (1H, m, C*H*$_2$OH), 4.00-4.10 (1H, m, C*H*CH$_2$CH(CH$_3$)$_2$), 4.45 (1H, t, J = 8.0 Hz, C*H*CH(CH$_3$)$_2$), 6.68 (1H, d, J = 8.0 Hz, N*H*), 6.91 (1H, d, J = 8.0 Hz, N*H*), 7.08 (1H, t, J = 4.0 Hz, CHC*H*CH), 7.50 (1H, d, J = 5.0 Hz, SC*H*CHCH), 7.61 (1H, d, J = 3.0 Hz, C*H*CHCH); ^{13}C NMR (75 MHz, $CDCl_3$) 18.8 (*C*H$_3$), 19.2 (*C*H$_3$), 22.2 (*C*H$_3$), 22.7 (*C*H$_3$), 24.7 (CHCH$_2$*C*H(CH$_3$)$_2$), 31.1 (CH*C*H(CH$_3$)$_2$), 39.6 (CH*C*H$_2$CH(CH$_3$)$_2$), 50.5 (*C*H$_2$OH), 59.6 (*C*HCH$_2$CH(CH$_3$)$_2$), 66.0 (*C*HCH(CH$_3$)$_2$), 127.5 (CHCHCH), 128.4, (CHCHCH), 130.4 (CHCHCH), 138.5 (*C*S), 162.1 (*C*ONH), 172.3 (*C*ONH); *m/z* (ES) 327 ([M+(H)]$^+$, 100, 349 ([M+(Na)]$^+$, 90. Anal. Calcd for $C_{16}H_{26}N_2O_3S$: C, 58.87; H, 8.03; N, 8.58. Found C, 58.74; H, 8.05; N; 8.56.

General Procedure B: Amide bond formation. To a solution of *N*, *N*-dimethylformamide was added the amine (1.2 equiv), the carboxylic acid (1.0 equiv), HATU (1.0 equiv) and DIPEA (2.5 equiv). The solution was stirred at rt overnight then diluted with ethyl acetate and washed with water and brine. The organic phase was separated, dried over $MgSO_4$ and concentrated *in vacuo*.

4.1.3. 1H-Pyrrole-2-carboxylic acid [(S)-1-((S)-1-hydroxymethyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 18. Reaction of **15** (as prepared above) with 1H-pyrrole-2-carboxylic acid according to general procedure **B** gave **18** as an orange solid (0.26 g, 84%). mp 110-112 ºC; HRMS (ES+) calcd for $C_{16}H_{28}N_3O_3$ (M+H$^+$) 310.2131 found 310.2134; ν_{max} (KBr) 1648.3 (CONH), 1269.9

(CH_2OH); ^{1}H NMR (500 MHz, CD_3OD) 0.86 (3H, d, J = 7.0 Hz, C*H*$_3$), 0.89 (3H, d, J = 7.0 Hz, C*H*$_3$), 0.97-1.00 (6H, m, 2×C*H*$_3$), 1.35-1.40 (2H, m, C*H*$_2$CH(CH_3)$_2$), 1.55-1.59 (1H, m, CH_2C*H*(CH_3)$_2$), 2.11-2.12 (1H, m, C*H*(CH_3)$_2$), 3.43-3.48 (2H, m, C*H*$_2$OH), 3.98-4.02 (1H, m, C*H*CH_2CH(CH_3)$_2$), 4.26 (1H, dd, J = 9.0 Hz, J = 2.0 Hz, C*H*CH(CH_3)$_2$), 6.16-6.17 (1H, m, CHC*H*CH), 6.86-6.87 (1H, m, CHCHC*H*), 6.91-6.92 (1H, m, C*H*CHCH), 7.60 (1H, d, J = 9.0 Hz, N*H*), 7.85 (1H, d, J = 9.0 Hz, N*H*); ^{13}C NMR (75 MHz, CD_3OD) 19.4 (*C*H_3), 20.2 (*C*H_3), 22.6 (*C*H_3), 24.0 (*C*H_3), 26.1 (CHCH_2*C*H(CH_3)$_2$), 31.9 (CH*C*H(CH_3)$_2$), 41.3 (CH*C*H_2CH(CH_3)$_2$), 51.0 (*C*H_2OH), 60.6 (*C*HCH_2CH(CH_3)$_2$), 65.8 (*C*HCH(CH_3)$_2$), 110.6 (CHCHCH), 112.8 (CHCHCH), 123.5 (CHCHCH), 126.7 (*C*N), 163.6 (*C*ONH), 174.2 (*C*ONH); *m/z* (ES) 310 ([M+(H)]$^+$, 100, 332 ([M+(Na)]$^+$, 30. Anal. Calcd for $C_{16}H_{27}N_3O_3$: C, 62.11; H, 8.80; N, 13.58. Found C, 61.89; H, 8.44; N; 13.70.

4.1.4. 5-Formyl-furan-2-carboxylic acid [(S)-1-((S)-1-hydroxymethyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 19. Reaction of **15** (as prepared above) with (5-formyl)-furan-2-carboxylic acid according to general procedure **B** gave **19** as white solid (180 mg, 30%). mp 46-48 ºC; HRMS (ES+) calcd for $C_{17}H_{26}N_2O_5$ (M+H$^+$) 339.1906 found 339.1920; (Found: C, 60.58; H, 7.57; N, 8.09% $C_{17}H_{25}N_2O_5$ requires C, 60.34; H, 7.74; N, 8.28%); ν_{max} (KBr) 1688 (CONH), 1543 (CH_2OH); ^{1}H NMR (500 MHz, $CDCl_3$) 0.89 (6H, t, J = 6.3 Hz, CH_2CH(C*H*$_3$)$_2$), 1.04 (6H, t, J = 6.5 Hz, CH(C*H*$_3$)$_2$), 1.37-1.43 (2H, m, C*H*$_2$CH(CH_3)$_2$), 1.62 (1H, tt, J = 6.8 Hz, J = 13.5 Hz, CH_2C*H*(CH_3)$_2$), 2.17-2.26 (1H, m, C*H*(CH_3)$_2$), 3.60 (1H, dd, J = 5.5 Hz, J = 11.0 Hz, C*H*$_2$OH), 3.71 (1H, dd, J = 3.4 Hz, J = 11.0 Hz, C*H*$_2$OH), 4.05-4.10 (1H, m, C*H*CH_2OH), 4.38-4.41 (1H, m, C*H*CH(CH_3)$_2$), 6.21 (1H, d, J = 8.0 Hz, N*H*), 7.26 (1H, d, J = 0.8 Hz N*H*), 7.29 (2H, d, J = 3.7 Hz, C*H*CH and CHC*H*), 9.75 (1H, s, C*H*O); ^{13}C NMR (75 MHz, $CDCl_3$); 18.5 (*C*H_3), 19.2 (*C*H_3), 22.3 (*C*H_3), 22.8 (*C*H_3), 24.8 (CHCH_2*C*H(CH_3)$_2$), 31.5 (CH*C*H(CH_3)$_2$), 39.8 (CH*C*H_2CH(CH_3)$_2$), 49.8 (CH_2OH), 58.8 (*C*HCH_2CH(CH_3)$_2$), 115.9 (*C*HCH), 121.4 (*C*HCCHO), 150.6 (*C*CONH), 152.5 (*C*O), 157.3 (*C*ONH), 170.9 (*C*ONH), 178.5 (C*C*HO); *m/z* (ES) 339 ([M+(H)]$^+$, 100. Anal. Calcd for $C_{17}H_{26}N_2O_5$: C, 60.34; H, 7.74; N, 8.28. Found C, 60.58; H, 7.57; N; 8.09.

4.1.5. 5-Formyl-thiophene-2-carboxylic acid [(S)-1-((S)-1-hydroxymethyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 20. Reaction of **15** (as prepared above) with (5-formyl)-thiophene-2-carboxylic acid according to general procedure **B** gave **20** as a yellow solid (1.2 g, 88%). mp 133-135 ºC; HRMS (ES+) calcd for $C_{17}H_{27}N_2O_4S$ ($M+H^+$) 355.1695 found 355.1692; ν_{max} (KBr) 1678 (CONH), 1547 (CH_2OH); ^1H NMR (500 MHz, $CDCl_3$) 0.88 (3H, d, J = 6.2 Hz, CH_3), 0.88 (3H, d, J = 6.3 Hz, CH_3), 1.03 (3H, d, J = 5.1 Hz, CH_3), 1.04 (3H, d, J = 6.1 Hz, CH_3), 1.32-1.46 (2H, m, $CH_2CH(CH_3)_2$), 1.56-1.66 (1H, m, $CH_2CH(CH_3)_2$), 2.16-2.26 (1H, m, $CH(CH_3)_2$), 2.94-31.4 (1H, br s, OH), 3.60 (1H, dd, J = 11.0, J = 5.7 Hz, CH_2OH), 3.70 (1H, dd, J = 11.0, J = 3.7 Hz, CH_2OH), 4.02-4.11 (1H, m, $CHCH_2OH$), 4.41 (1H, t, J=8.4 Hz, $CHCH(CH_3)_2$), 6.4 (1H, d, J = 8.1 Hz, NH), 7.16 (1H, d, J = 8.4 Hz, NH), 7.60 (1H, d, J = 4.0 Hz, CHCH), 7.73 (1H, d, J = 4.0 Hz, CHCH), 9.95 (1H, s, CHO); ^{13}C NMR (75 MHz, $CDCl_3$) 18.8 (CH_3), 19.2 (CH_3), 22.2 (CH_3), 22.8 (CH_3), 24.8 ($CHCH_2CH(CH_3)_2$), 31.0 ($CHCH(CH_3)_2$), 39.7 ($CHCH_2CH(CH_3)_2$), 50.5 (CH_2OH), 59.9 ($CHCH_2CH(CH_3)_2$), 128.6 (CHCH), 128.7 (CHCCHO), 135.5 (CCONH), 146.4 (CS), 161.2 (CONH), 171.8 (CONH), 183.2 (CCHO) *m/z* (ES) 355 ($[M+(H)]^+$, 100; 377 ($[M+(Na)]^+$, 50. Anal. Calcd for $C_{17}H_{26}N_2O_4S$: C, 57.60; H, 7.39; N, 7.90. Found C, 57.34; H, 7.69; N; 8.10.

4.1.6. 5-Formyl-pyrrole-2-carboxylic acid [(S)-1-((S)-1-hydroxymethyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 25. Reaction of **15** (as prepared above) with (5-formyl)-1H-pyrrole-2-carboxylic acid **23** according to general procedure **B** gave **25** as a yellow solid (0.5 g, 39%). mp 102-104 ºC; HRMS (ES+) calcd for $C_{17}H_{27}N_3O_4$ ($M+H^+$) 338.2080 found 338.2086; ν_{max} (KBr) 1638 (C=ONH), 1038 (C-OH); ^1H NMR (500 MHz, $CDCl_3$) 0.80 (3H, d, J = 3.0 Hz, C*H*$_3$), 0.82 (3H, d, J = 3.0 Hz, C*H*$_3$), 0.91 (3H, d, J = 8.0 Hz, C*H*$_3$), 1.03 (3H, d, J = 3.0 Hz, C*H*$_3$), 1.35-1.37 (2H, m, C*H*$_2$CH(CH$_3$)$_2$), 1.51-1.58 (1H, m, CH$_2$C*H*(CH$_3$)$_2$), 2.09-2.16 (1H, m, C*H*(CH$_3$)$_2$), 3.80-3.89 (2H, m, CH$_2$OH) 4.17-4.20 (1H, m, C*H*CH$_2$CH(CH$_3$)$_2$), 5.01 (1H, t, J = 15.0 Hz, C*H*CH(CH$_3$)$_2$), 6.78-6.80 (1H, m, C*H*CH), 6.97-6.99 (1H, m, C*H*CCHO), 7.23 (1H, d, J = 16.0 Hz, NH), 8.17 (1H, d, J = 16.0 Hz, NH), 9.53 (1H, s, C*H*O), 11.78 (1H, br s, N*H*); ^{13}C NMR (75 MHz, $CDCl_3$) 18.9 (CH_3), 19.1 (CH_3), 22.4 (CH_3), 22.8 (CH_3), 24.9 ($CHCH_2CH(CH_3)_2$), 32.2 ($CHCH(CH_3)_2$), 40.0 ($CHCH_2CH(CH_3)_2$), 48.6

(CH_2OH), 58.4 ($CHCH_2CH(CH_3)_2$), 64.8 ($CHCH(CH_3)_2$), 110.7 (*C*HCH), 122.0, (*C*HCCHO), 132.3 (*C*CONH), 133.6 (*C*N), 159.5 (*C*ONH), 171.0 (*C*ONH), 181.0 (C*C*HO); *m/z* (ES) 338 $([M+(H)]^+$, 30, 360 $([M+(Na)]^+$, 100. Anal. Calcd for $C_{17}H_{27}N_3O_4$: C, 60.51; H, 8.07; N, 12.45. Found C, 60.57; H, 8.01; N; 12.57.

4.1.7. 4-Formyl-1H-pyrrole-2-carboxylic acid [(S)-1-((S)-1-hydroxymethyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 26. Reaction of **15** (as prepared above) with (4-formyl)-1H-pyrrole-2-carboxylic acid **24** according to general procedure **B** gave **26** as a pale yellow glassy solid (0.1 g, 36%). mp 112-115 ºC; HRMS (ES+) calcd for $C_{17}H_{27}N_3O_4$ $(M+H^+)$ 338.2080 found 338.2066; ν_{max} (KBr) 1624 (C=ONH), 1038 (C-OH); ^{1}H NMR (500 MHz, CD_3OD) 0.86 (3H, d, J = 7.0 Hz, C*H*$_3$), 0.89 (3H, d, J = 7.0 Hz, C*H*$_3$), 0.98 (3H, d, J = 3.0 Hz, C*H*$_3$), 1.00 (3H, d, J = 5.0 Hz, C*H*$_3$), 1.35-1.39 (2H, m, C*H*$_2$CH(CH$_3$)$_2$),1.62-1.66 (1H, m, CH$_2$C*H*(CH$_3$)$_2$), 2.09-2.12 (1H, m, C*H*(CH$_3$)$_2$), 3.42-3.50 (2H, m, C*H*$_2$OH), 3.98-4.02 (1H, m, C*H*CH$_2$CH(CH$_3$)$_2$), 4.26 (1H, t, J = 2.0 Hz, C*H*CH(CH$_3$)$_2$), 6.86 (1H, d, J = 2.0 Hz, C*H*C(CHO)), 6.92 (1H, d, J = 2.0 Hz, C*H*NH), 9.72 (1H, s, C*H*O); ^{13}C NMR (75 MHz, $(CD_3)_2SO$) 19.5 (*C*H$_3$), 20.0 (*C*H$_3$), 22.5 (*C*H$_3$), 24.1 (*C*H$_3$), 24.8 (CHCH$_2$*C*H(CH$_3$)$_2$), 32.2 (CH*C*H(CH$_3$)$_2$), 41.1 (CH*C*H$_2$CH(CH$_3$)$_2$), 49.4 (CH_2OH), 59.3 (*C*HCH$_2$CH(CH$_3$)$_2$), 64.6 (*C*HCH(CH$_3$)$_2$), 109.5 (*C*HCH), 127.1, (*C*HCCHO), 129.2 (*C*CONH), 130.9 (*C*N), 160.6 (*C*ONH), 171.3 (*C*ONH), 186.6 (C*C*HO); *m/z* (ES) 338 $([M+(H)]^+$, 10, 360 $([M+(Na)]^+$, 100. Anal. Calcd for $C_{17}H_{27}N_3O_4$: C, 60.51; H, 8.07; N, 12.45. Found C, 60.12; H, 8.01; N; 12.44.

4.1.8. 5-Formyl-1-methyl-1H-pyrrole-2-carboxylic acid [(S)-1-((S)-1-hydroxymethyl-3-methylbutylcarbamoyl)-2-methyl-propyl]-amide 30. Reaction of **15** (as prepared above) with (5-formyl)-1-methyl-1H-pyrrole-2-carboxylic acid **27** according to general procedure **B** gave **30** as an orange solid (0.32g, 56%). mp 178-180 ºC; HRMS (ES+) calcd for $C_{18}H_{30}N_3O_4$ $(M+H^+)$ 352.2236 found 352.2245; ν_{max} (KBr) 1633 (C=ONH), 1038 (C-OH); ^{1}H NMR (500 MHz, CD_3OD) 0.89 (3H, d, J = 6.5 Hz, C*H*$_3$), 0.92 (3H, d, J = 7.0 Hz C*H*$_3$), 1.01 (6H, m, 2×C*H*$_3$), 1.37-1.40 (2H, m, C*H*$_2$CH(CH$_3$)$_2$), 1.68-1.70 (1H, m, CH$_2$C*H*(CH$_3$)$_2$), 2.11-2.16 (1H, m, C*H*(CH$_3$)$_2$), 3.44-3.52 (2H, m, CH_2OH), 4.00-4.03 (1H,

m, C*H*$CH_2CH(CH_3)_2$), 4.14 (3H, s, NC*H*$_3$), 4.24 (1H, d, J = 8.0 Hz, C*H*$CH(CH_3)_2$), 6.77 (1H, d, J = 4.5 Hz, C*H*CH), 6.99 (1H, d, J = 4.5 Hz, CHC*H*), 7.94 (1H, d, J = 8.5 Hz, NH), 9.65 (1H, s, C*H*O); ^{13}C NMR (75 MHz, $CDCl_3$) 18.7 (*C*H$_3$), 19.5 (*C*H$_3$), 21.4 (*C*H$_3$), 23.2 (*C*H$_3$), 25.1 ($CHCH_2$*C*$H(CH_3)_2$), 31.5 ($CHCH(CH_3)_2$), 34.8 ($CHCH_2CH(CH_3)_2$), 40.2 (*C*H_2OH) 50.5 (*C*$HCH_2CH(CH_3)_2$), 59.3 (*C*$HCH(CH_3)_2$), 66.0 (NCH_3), 112.2 (*C*HCH), 122.6 (*C*HCCHO), 133.6 (*C*CONH), 135.0 (*C*NCH$_3$), 161.5 (*C*ONH), 171.6 (*C*ONH), 181.3 (C*C*HO); *m/z* (ES) 352 ([M+(H)]$^+$, 100. Anal. Calcd for $C_{18}H_{29}N_3O_4$: C, 60.52; H, 8.32; N, 11.96. Found C, 60.46; H, 8.21; N; 11.69.

4.1.9. 5-Methyl-1H-pyrrole-2-carboxylic acid [(S)-1-((S)-1-hydroxymethyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 31. Reaction of **15** (as prepared above) with (5-methyl)-1H-pyrrole-2-carboxylic acid **28** according to general procedure **B** gave **31** as a white solid (0.27g, 29%). mp 173-175 ºC; HRMS (ES+) calcd for $C_{17}H_{30}N_3O_3$ (M+H$^+$) 324.2287 found 352.2286; ν_{max} (KBr) 1634 (C=ONH), 1036 (C-OH); ^{1}H NMR (500 MHz, CD_3OD) 0.86 (3H, d, J = 6.5 Hz, C*H*$_3$), 0.88 (3H, d, J = 6.5 Hz C*H*$_3$), 01.03 (3H, d, J = 6.5 Hz, C*H*$_3$), 1.04 (3H, d, J = 6.5 Hz C*H*$_3$), 1.26-1.42 (2H, m, C*H*$_2CH(CH_3)_2$), 1.50-1.62 (1H, m, CH_2C*H*$(CH_3)_2$), 2.12-2.22 (1H, m, C*H*$(CH_3)_2$), 2.28 (3H, s, CCH_3), 3.61 (1H, dd, J = 11.2, J = 4.8 Hz, CH_2OH), 3.74 (1H, dd, J = 11.2 Hz, J = 2.9 Hz, CH_2OH), 4.04-4.12 (1H, m, C*H*$CH_2CH(CH_3)_2$), 4.50 (1H, d, J = 8.4 Hz, C*H*$CH(CH_3)_2$), 5.92 (1H, br s, C*H*CH), 6.65 (1H, br s, CHC*H*), 6.71 (1H, br s, NH), 7.35 (1H, br s, NH), 9.92 (1H, s, C*H*O); ^{13}C NMR (75 MHz, CD_3OD) 16.8 (*C*H$_3$), 18.0 (*C*H$_3$), 20.2 (*C*H$_3$), 22.4 (*C*H$_3$), 23.6 ($CHCH_2$*C*$H(CH_3)_2$), 30.0 ($CHCH(CH_3)_2$), 36.8 ($CHCH_2CH(CH_3)_2$), 49.2 (*C*H_2OH) 60.5 (*C*$HCH_2CH(CH_3)_2$), 65.8 (*C*$HCH(CH_3)_2$), 105.3 (*C*H$_3$), 126.9 (CHCH), 127.5(*C*HCH), 135.9 (*C*CH_3), 156.3 (*C*ONH), 171.6 (*C*ONH); *m/z* (ES) 324 ([M+(H)]$^+$, 100, 346 ([M+(Na)]$^+$, 50. Anal. Calcd for $C_{17}H_{29}N_3O_3$: C, 63.13; H, 9.04; N, 12.99. Found C, 62.85; H, 9.16; N; 12.89.

4.1.10. 5-[(S)-1-((S)-1-Hydroxymethyl-3-methyl-butylcarbamoyl)-2-methyl-propylcarbamoyl]-1H-pyrrole-2-carboxylic acid methyl ester 32. Reaction of **15** (as prepared above) with 1H-Pyrrole-2,5-dicarboxylic acid monomethyl ester **29** according to general procedure **B** gave **32** as a white solid

(0.42g, 64%). mp 115-117 ºC; HRMS (ES+) calcd for $C_{18}H_{30}N_3O_5$ ($M+H^+$) 368.2185 found 368.2185; ν_{max} (KBr) 1633 (C=ONH), 1035 (C-OH); ^{1}H NMR (500 MHz, $CDCl_3$) 0.85 (3H, d, J = 7.0 Hz, C*H*$_3$), 0.89 (3H, d, J = 7.0 Hz, C*H*$_3$), 0.95 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.00 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.38-1.40 (1H, m, $CH_2CH(CH_3)_2$), 1.41-1.58 (2H, m, $CH_2CH(CH_3)_2$), 2.16-2.17 (1H, m, $CH(CH_3)_2$), 3.80-3.84 (2H, m, CH_2OH), 3.87 (1H, s, OCH_3), 4.17-4.19 (1H, m, $CHCH_2CH(CH_3)_2$), 4.94 (1H, t, J = 9.0 Hz, $CHCH(CH_3)_2$), 6.73 (1H, s, CHCH), 6.86-6.88 (1H, m, CHCCOMe), 7.17 (1H, d, J = 9.0 Hz, NH), 8.24 (1H, d, J = 8.5 Hz, NH); ^{13}C NMR (75 MHz, $CDCl_3$) 18.6 (CH_3), 19.5 (CH_3), 22.9 (CH_3), 23.0 (CH_3), 25.2 ($CHCH_2CH(CH_3)_2$), 31.8 ($CHCH(CH_3)_2$), 34.8 ($CHCH_2CH(CH_3)_2$), 49.2 (CH_2OH) 52.1 ($CHCH_2CH(CH_3)_2$), 59.2 ($CHCH(CH_3)_2$), 65.4 (OCH_3), 116.3 (CHCH), 125.0 (CHCCHO), 130.4 (CCONH), 160.2 (CONH), 162.3 (CONH), 172.2 (CCO_2Me); *m/z* (ES) 368 ($[M+(H)]^+$, 100. Anal. Calcd for $C_{18}H_{29}N_3O_5$: C, 58.84; H, 7.96; N, 11.44. Found C, 58.62; H, 7.98; N; 11.43.

General Procedure C: Oxidation. A solution of the alcohol (1.0 equiv) in a 1:3 mixture of dimethylsulfoxide and dichloromethane was cooled over an ice bath, followed by the addition of DIPEA (4.3 equiv). To this ice-cold solution reaction mixture a solution of SO_3·Pyr complex (4.5 equiv) dissolved in dimethylsulfoxide was added. The reaction mixture was maintained at a low temperature for a further 2 h (or until TLC indicated complete consumption of the starting alcohol). The reaction mixture was diluted with ethyl acetate and partitioned between ethyl acetate and 1M HCl. The organic phase was washed with saturated aqueous sodium bicarbonate and brine, then dried over $MgSO_4$ and concentrated *in vacuo*.

4.1.11. Furan-2-carboxylic acid [(S)-1-((S)-1-formyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 4. Oxidation of **16** according to general procedure C gave **4** as a colourless oil (0.10 g, 58%). HRMS (ES+) calcd for $C_{16}H_{24}N_2O_4$ ($M+H^+$) 309.1820 found 309.1814; ν_{max} (KBr) 1738 (CHO), 1645 (CONH); ^{1}H NMR (500 MHz, $CDCl_3$) 0.78 (6H, dd, J = 15.3 Hz, J = 5.6 Hz, 2×C*H*$_3$), 0.98 (6H, dd, J = 20.2 Hz, J = 6.7 Hz, 2×C*H*$_3$), 1.35 (1H, dddd, J = 9.6 Hz, J = 9.6 Hz, J = 9.6 Hz, J = 9.6 Hz, $CH_2CH(CH_3)_2$), 1.55-1.61 (2H, m, $CH_2CH(CH_3)_2$), 2.16 (1H, dq, J = 6.8 Hz, $CH(CH_3)_2$), 4.38-4.42 (1H,

m, C*H*$CH_2CH(CH_3)_2$), 4.68 (1H, dd, J = 9.0 Hz, J = 7.4 Hz, C*H*$CH(CH_3)_2$), 6.42 (1H, dd, J = 3.2 Hz, J = 1.4 Hz, CHC*H*CH), 7.03 (1H, d, J = 3.4 Hz, C*H*CHCH), 7.15 (1H, d, J = 9.1 Hz, N*H*), 7.39 (1H, s, C*H*CHCH), 7.59 (1H, d, J = 7.4, N*H*), 9.52 (1H, s, C*H*O); ^{13}C NMR (75 MHz, $CDCl_3$) 18.2 (*C*H_3), 19.2 (*C*H_3), 21.7 (*C*H_3), 22.8 (*C*H_3), 24.6 ($CHCH_2$*C*$H(CH_3)_2$), 31.6 (CH*C*$H(CH_3)_2$), 37.1 (CH*C*$H_2CH(CH_3)_2$), 57.2 (*C*$HCH_2CH(CH_3)_2$), 57.7 (*C*$HCH(CH_3)_2$), 112.0 (CHCHCH), 114.7, (CHCHCH), 144.4 (CHCHCH), 147.2 (*C*O), 158.2 (*C*ONH), 171.6 (*C*ONH), 199.6 (C*H*O); *m/z* (ES) 311 $([M+(H)]^+$, 100, 333 $([M+(Na)]^+$, 20.

4.1.12. Thiophene-2-carboxylic acid [(S)-1-((S)-1-formyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 5. Oxidation of **17** according to general procedure C gave **5** as a colourless crystalline solid (0.13 g, 51%). mp 178-180 ºC; HRMS (ES+) calcd for $C_{16}H_{25}N_2O_3S$ ($M+H^+$) 325.1586 found 325.1578; ν_{max} (KBr) 1736 (CHO), 1624 (CONH); ^{1}H NMR (500 MHz, CD_3OD) 0.87 (3H, d, J = 4.5 Hz, C*H*$_3$), 0.89 (3H, d, J = 4.0 Hz, C*H*$_3$), 1.02 (3H, d, J = 11.5 Hz, C*H*$_3$), 1.06 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.43-1.50 (2H, m, C*H*$_2$CH$(CH_3)_2$), 1.65-1.72 (1H, m, CH_2C*H*$(CH_3)_2$), 2.20-2.26 (1H, m, C*H*$(CH_3)_2$), 4.45-4.49 (1H, m, C*H*CH$(CH_3)_2$), 4.62 (1H, dd, J = 7.5 Hz, J = 8.0 Hz, C*H*$CH_2CH(CH_3)_2$), 6.97 (1H, d, J = 9.0 Hz, C*H*CHCH), 7.07 (1H, dd, J = 4.5 Hz, J = 3.5 Hz, C*H*CHCH), 7.12-7.16 (1H, m, CHC*H*CH), 7.50 (1H, d, J = 6.0 Hz, N*H*), 7.62 (1H, d, J = 5.0 Hz, N*H*), 9.57 (CHO); ^{13}C NMR (75 MHz, $CDCl_3$) 18.5 (*C*H_3), 19.3 (*C*H_3), 21.8 (*C*H_3), 22.9 (*C*H_3), 24.7 ($CHCH_2$*C*$H(CH_3)_2$), 31.4 (CH*C*$H(CH_3)_2$), 37.4 (CH*C*$H_2CH(CH_3)_2$), 57.5 (*C*$HCH_2CH(CH_3)_2$), 58.7 (*C*$HCH(CH_3)_2$), 127.8 (CHCHCH), 128.5, (CHCHCH), 130.5 (CHCHCH), 138.3 (*C*S), 162.0 (*C*ONH), 171.8 (*C*ONH), 200.20 (CHO); *m/z* (ES) 325 $([M+(H)]^+$, 100, 345 $([M+(Na)]^+$, 20. Anal. Calcd for $C_{16}H_{24}N_2O_3S$: C, 59.23; H, 7.46; N, 8.63. Found C, 59.32; H, 7.59; N; 8.61.

4.1.13. 1H-Pyrrole-2-carboxylic acid [(S)-1-((S)-1-formyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 6. Oxidation of **18** according to general procedure C gave **6** as a colourless crystalline solid (0.20 g, 71%). mp 165-167 ºC; HRMS (ES+) calcd for $C_{16}H_{26}N_3O_3$ ($M+H^+$) 308.1974 found 308.1961; ν_{max} (KBr) 1741 (CHO), 1651 (CONH); ^{1}H NMR (500 MHz, CD_3OD) 0.79 (3H, d, J = 10.5

Hz, C*H*$_3$), 0.81 (3H, d, J = 11.5 Hz, C*H*$_3$), 0.87 (3H, d, J = 12.0 Hz, C*H*$_3$), 0.89 (3H, d, J = 12.0 Hz, C*H*$_3$), 1.33-1.38 (2H, m, C*H*$_2$CH(CH$_3$)$_2$), 1.53-1.56 (1H, m, CH$_2$C*H*(CH$_3$)$_2$), 2.10-2.15 (1H, m, C*H*(CH$_3$)$_2$), 4.02-4.07 (1H, m, C*H*CH$_2$CH(CH$_3$)$_2$), 4.30 (1H, m, C*H*CH(CH$_3$)$_2$), 6.03-6.05 (1H, m, C*H*CHCH), 6.83-6.85 (1H, m, C*H*CHCH), 6.85-6.87 (1H, m, C*H*CHCH) 7.92 (1H, d, J = 9.0 Hz, N*H*), 8.40 (1H, d, J = 9.0 Hz, N*H*), 7.85 (1H, d, J = 9.0 Hz, N*H*), 9.36 (1H, s, CHO), 11.45 (1H, s, N*H*); ^{13}C NMR (75 MHz, (CD$_3$)$_2$SO) 18.7 (*C*H$_3$), 18.8 (*C*H$_3$), 19.4 (*C*H$_3$), 21.4 (*C*H$_3$), 23.2 (CHCH$_2$*C*H(CH$_3$)$_2$), 30.3 (CH*C*H(CH$_3$)$_2$), 36.4 (CH*C*H$_2$CH(CH$_3$)$_2$), 56.8 (*C*HCH$_2$CH(CH$_3$)$_2$), 57.9 (*C*HCH(CH$_3$)$_2$), 108.7 (CHCHCH), 111.2, (CHCHCH), 121.6 (CHCHCH), 125.9 (*C*N), 160.5 (*C*ONH), 172.0 (*C*ONH), 201.3 (CHO); *m/z* (ES) 309 ([M+(H)]$^+$, 100. Anal. Calcd for C$_{16}$H$_{25}$N$_3$O$_3$: C, 62.52; H, 8.20; N, 13.67. Found C, 62.66; H, 8.36; N; 13.59.

4.1.14. 5-Formyl-furan-2-carboxylic acid [(S)-1-((S)-1-formyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 7. Oxidation of **19** according to general procedure C gave **7** as a colourless oil (100 mg, 80%). HRMS (ES+) calcd for C$_{17}$H$_{25}$N$_2$O$_5$ (M+H$^+$) 337.1740 found 337.1763; ν_{max} (KBr) 1734 (CHO), 1687 (CONH); ^{1}H NMR (500 MHz, CDCl$_3$); 0.90 (6H, dd, J = 3.8 Hz, J = 5.0 Hz, CH$_2$CH(C*H*$_3$)$_2$), 1.03 (6H, dd, J = 6.9 Hz, J = 9.9 Hz, CH(C*H*$_3$)$_2$), 1.40-1.47 (1H, m, C*H*$_2$CH(CH$_3$)$_2$), 1.66-1.72 (1H, m, CH$_2$C*H*(CH$_3$)$_2$), 2.21 (1H, tt, J = 7.0 Hz, J = 13.8 Hz, C*H*(CH$_3$)$_2$), 4.54-4.60 (2H, m, C*H*CH(CH$_3$)$_2$ and C*H*CHO), 6.85 (1H, d, J = 7.1 Hz, N*H*), 7.26 (2H, dd, J = 3.4 Hz, J = 18.5 Hz, C*H*CH and CHC*H*), 7.35 (1H, d, J = 8.8 Hz, N*H*), 9.59 (1H, s, C*H*O), 9.73 (1H, s, Fur-C*H*O); ^{13}C NMR (75 MHz, CD$_3$OD) 18.4 (*C*H$_3$), 19.3 (*C*H$_3$), 21.8 (*C*H$_3$), 22.9 (*C*H$_3$), 24.7 (CHCH$_2$*C*H(CH$_3$)$_2$), 31.4 (CH*C*H(CH$_3$)$_2$), 37.3 (CH*C*H$_2$CH(CH$_3$)$_2$), 57.4 (*C*HCH$_2$CH(CH$_3$)$_2$), 58.4 (*C*HCH(CH$_3$)$_2$), 116.0 (*C*HCH), 121.1(*C*HCCHO), 150.5 (*C*CONH), 152.6 (*C*O), 157.4 (*C*ONH), 171.2 (*C*ONH), 178.5 (C*C*HO), 199.6 (CH*C*HO); *m/z* (ES) 372.2 ([M+H]$^+$), 100.

4.1.15. 5-Formyl-thiophene-2-carboxylic acid [(S)-1-((S)-1-formyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 8. Oxidation of **20** according to general procedure C gave **8** as a yellow solid (0.47 g, 89%). mp 162-164 ºC; HRMS (ES+) calcd for C$_{17}$H$_{25}$N$_2$O$_4$S (M+H$^+$) 353.1535 found 353.1521;

ν_{max} (KBr) 1738 (CHO), 1682 (CONH); ^{1}H NMR (500 MHz, $CDCl_3$) 0.88 (6H, m, 2 × CH_3), 1.03 (3H, d, J = 6.6 Hz, CH_3), 1.08 (3H, d, J = 6.6 Hz, CH_3), 1.46-1.75 (2H, m, $CH_2CH(CH_3)_2$), 1.63-1.73 (1H, m, $CH_2CH(CH_3)_2$), 2.16-2.26 (1H, m, $CH(CH_3)_2$), 4.35-4.41 (1H, m, CHCHO), 4.66 (1H, t, J=8.7 Hz, $CHCH(CH_3)_2$), 7.71 (1H, d, J = 4.0 Hz, CHCH), 7.78 (1H, d, J=4.0 Hz, CHCH), 7.82-7.90 (1H, m, NH), 8.19 (1H, d, J = 8.4 Hz, NH), 9.57 (1H, s, CHO), 9.95 (1H, s, CHO); ^{13}C NMR (75 MHz, $CDCl_3$) 19.4 (CH_3), 19.5 (CH_3), 21.9 (CH_3), 23.2 (CH_3), 25.0 ($CHCH_2CH(CH_3)_2$), 31.0 ($CHCH(CH_3)_2$), 37.3 ($CHCH_2CH(CH_3)_2$), 60.1 ($CHCH_2CH(CH_3)_2$), 129.2 (CHCH), 135.9 (CHCCHO), 146.8 (CCONH), 146.8 (CS), 161.6 (CONH), 172.6 (CONH), 183.6 (CCHO), 199.4 (CHO); *m/z* (ES) 353 ($[M+(H)]^+$ 100. Anal. Calcd for $C_{17}H_{24}N_2O_4S$: C, 57.93; H, 6.86; N, 7.95. Found C, 57.55; H, 6.80; N; 7.60.

4.1.16. 5-Formyl-pyrrole-2-carboxylic acid [(S)-1-((S)-1-formyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 9. Oxidation of **25** according to general procedure C gave **9** as a yellow solid (0.2 g, 71%). mp 70-72 ºC; HRMS (ES+) calcd for $C_{17}H_{25}N_3O_4$ ($M+H^+$) 336.1923 found 336.1925; ν_{max} (KBr) 1738 (CHO), 1636 (CONH); ^{1}H NMR (500 MHz, $CDCl_3$) 0.79 (3H, d, J = 7.0 Hz, CH_3), 0.82 (3H, d, J = 7.0 Hz, CH_3), 1.01 (3H, d, J = 7.0 Hz, CH_3), 1.06 (3H, d, J = 7.0 Hz, CH_3), 1.23-1.26 (1H, m, $CH_2CH(CH_3)_2$), 1.63-1.653 (2H, m, $CH_2CH(CH_3)_2$), 2.01-2.03 (1H, m, $CH(CH_3)_2$), 4.02-4.04 (1H, m, $CHCH_2CH(CH_3)_2$), 4.80 (1H, t, J = 8.0 Hz, $CHCH(CH_3)_2$), 6.84-6.88 (1H, m, CHCH), 6.93-6.95 (1H, m, CHCCHO), 7.75 (1H, d, J = 7.0 Hz, NH), 7.82 (1H, d, J = 7.0 Hz, NH), 9.58 (1H, s, CHO), 9.60 (1H, s, CHO), 11.82 (1H, br s, $NH_{pyrrole}$); ^{13}C NMR (75 MHz, $CDCl_3$) 18.9 (CH_3), 19.2 (CH_3), 21.63 (CH_3), 22.8 (CH_3), 24.6 ($CHCH_2CH(CH_3)_2$), 30.8 ($CHCH(CH_3)_2$), 37.2 ($CHCH_2CH(CH_3)_2$), 57.5 ($CHCH_2CH(CH_3)_2$), 59.1 ($CHCH(CH_3)_2$), 111.9 (CHCH), 121.1, (CHCCHO), 132.2 (CCONH), 134.2 (CN), 160.1 (CONH), 172.5 (CONH), 180.6 (CCHO), 199.5 (CHCHO); *m/z* (ES) 336 ($[M+(H)]^+$, 100, 358 ($[M+(Na)]^+$, 10. Anal. Calcd for $C_{17}H_{25}N_3O_4$: C, 60.88; H, 7.51; N, 12.53. Found C, 60.99; H, 7.28; N; 12.57.

4.1.17. 4-Formyl-1H-pyrrole-2-carboxylic acid [(S)-1-((S)-1-formyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 10. Oxidation of **26** according to general procedure C gave **10** as a colourless

glassy solid (48 mg, 90%). mp 101-103 ºC; HRMS (ES+) calcd for $C_{17}H_{25}N_3O_4$ ($M+H^+$) 336.1923 found 336.1922; ν_{max} (KBr) 1736 (CHO), 1643 (CONH); ^{1}H NMR (500 MHz, $(CD_3)_2SO$) 0.82 (3H, d, J = 6.5 Hz, C*H*$_3$), 0.86 (3H, d, J = 6.0 Hz, C*H*$_3$), 0.90 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.06 (3H, d, J = 7.5 Hz, C*H*$_3$), 1.41-1.44 (1H, m, $CH_2CH(CH_3)_2$), 1.50-1.56 (1H, m, $CH_2CH(CH_3)_2$), 1.61-1.65 (1H, m, $CH_2CH(CH_3)_2$), 2.05-2.11 (1H, m, $CH(CH_3)_2$), 4.10-4.14 (1H, m, $CHCH_2CH(CH_3)_2$), 4.30 (1H, t, J = 8.5 Hz, $CHCH(CH_3)_2$), 7.41 (1H, s, CHCH), 7.69 (1H, s, CHCCHO), 8.17 (1H, d, J = 9.0 Hz, NH), 8.42 (1H, d, J = 7.5 Hz, NH), 9.38 (1H, s, CHO), 9.72 (1H, s, CHO), 12.30 (1H, br s, NH); ^{13}C NMR (75 MHz, $(CD_3)_2SO$) 18.7 (CH_3), 19.4 (CH_3), 21.6 (CH_3), 23.8 (CH_3), 24.1 ($CHCH_2CH(CH_3)_2$), 30.0 ($CHCH(CH_3)_2$), 36.2 ($CHCH_2CH(CH_3)_2$), 58.3 ($CHCH_2CH(CH_3)_2$), 59.8 ($CHCH(CH_3)_2$), 119.0 (CHCH), 126.5, (CHCCHO), 128.3 (CCONH), 130.3 (CN), 160.0 (CONH), 171.7 (CONH), 185.9 (CCHO) 201.2 (CHCHO); *m/z* (ES) 336 ($[M+(H)]^+$, 100. Anal. Calcd for $C_{17}H_{25}N_3O_4$: C, 60.88; H, 7.51; N, 12.53. Found C, 60.67; H, 7.46; N; 12.55.

4.1.18. 5-Formyl-1-methyl-1H-pyrrole-2-carboxylic acid [(S)-1-((S)-1-formyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 11. Oxidation of **30** according to general procedure C gave **11** as an orange solid (150 mg, 75%). mp 134-136; HRMS (ES+) calcd for $C_{18}H_{28}N_3O_4$ ($M+H^+$) 350.2080 found 350.2086; ν_{max} (KBr) 1741 (CHO), 1637 (CONH); ^{1}H NMR (500 MHz, $CDCl_3$) 0.87 (3H, d, J = 7.0 Hz, C*H*$_3$), 0.92 (3H, d, J = 7.0 Hz, C*H*$_3$), 0.96 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.00 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.43-1.48 (1H, m, $CH_2CH(CH_3)_2$), 1.70-1.77 (2H, m, $CH_2CH(CH_3)_2$), 2.14-2.24 (1H, m, $CH(CH_3)_2$), 4.21 (3H, s, NCH_3), 4.41-4.44 (1H, m, $CHCH_2CH(CH_3)_2$), 4.62 (1H, t, J = 8.0 Hz, $CHCH(CH_3)_2$), 6.19 (1H, d, J = 8.0 Hz, NH), 6.61-6.66 (1H, m, CHCH), 6.88 (1H, d, J = 4.0 Hz, NH), 7.25-7.27 (1H, m, CHCCHO), 9.69 (1H, s, CHO), 9.70 (1H, s, CHO); ^{13}C NMR (75 MHz, $CDCl_3$); 18.5 (CH_3), 19.5 (CH_3), 22.1 (CH_3), 23.3 (CH_3), 25.1 ($CHCH_2CH(CH_3)_2$), 31.7 ($CHCH(CH_3)_2$), 38.1 ($CHCH_2CH(CH_3)_2$), 57.8 ($CHCH_2CH(CH_3)_2$), 58.7 ($CHCH(CH_3)_2$), 64.0 (NCH_3) 112.1 (CHCH), 122.5, (CHCCHO), 128.6 (CCONH), 130.1 ($CNCH_3$), 181.2 (CONH), 185.7 (CONH), 199.6 (CCHO), 199.0

(CH*C*HO); *m/z* (ES) 350 ([M+(H)]$^+$, 100. Anal. Calcd for $C_{18}H_{27}N_3O_4$: C, 61.87; H, 7.79; N, 12.03. Found C, 62.11; H, 8.11; N; 11.98.

4.1.19. 5-Methyl-1H-pyrrole-2-carboxylic acid [(S)-1-((S)-1-formyl-3-methyl-butylcarbamoyl)-2-methyl-propyl]-amide 12. Oxidation of **31** according to general procedure C gave **12** as a colourless solid (90 mg, 63%). mp 159-161; HRMS (ES+) calcd for $C_{17}H_{29}N_3O_3$ (M+H$^+$) 322.2131 found 322.2141; ν_{max} (KBr) 1736 (CHO), 1651 (CONH); ^{1}H NMR (500 MHz, $CDCl_3$) 0.85 (3H, d, J = 7.0 Hz, C*H*$_3$), 0.90 (3H, d, J = 7.0 Hz, C*H*$_3$), 0.99 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.01 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.24-1.27 (1H, m, $CH_2CH(CH_3)_2$), 1.63-1.69 (2H, m, $CH_2CH(CH_3)_2$), 2.18-2.20 (1H, m, $CH(CH_3)_2$), 2.30 (3H, s, CCH_3) 3.66-3.68 (1H, m, $CHCH_2CH(CH_3)_2$), 4.57-4.62 (1H, m, $CHCH(CH_3)_2$), 5.94 (1H, br s, C*H*CH), 6.45 (1H, br s, NH), 7.25-7.27 (1H, m, $CHCCH_3$), 7.32 (1H, br s, NH), 9.59 (1H, s, C*H*O); ^{13}C NMR (75 MHz, $CDCl_3$) 16.5 (CH_3), 18.6 (CH_3), 19.0 (CH_3), 22.9 (CH_3), 23.8 ($CHCH_2CH(CH_3)_2$), 30.8 ($CHCH(CH_3)_2$), 36.3 ($CHCH_2CH(CH_3)_2$), 60.6 ($CHCH_2CH(CH_3)_2$), 66.8 ($CHCH(CH_3)_2$), 105.5 (CH_3), 126.3 (CHCH), 128.0 (*C*HCH), 135.0 (CCH_3), 158.0 (*C*ONH), 169.3 (*C*ONH); 199.6 (CHO); *m/z* (ES) 322 ([M+(H)]$^+$, 100. Anal. Calcd for $C_{17}H_{27}N_3O_3$: C, 63.53; H, 8.47; N, 13.07. Found C, 63.90; H, 8.22; N; 12.70.

4.1.20. 5-[(S)-1-((S)-1-Formyl-3-methyl-butylcarbamoyl)-2-methyl-propylcarbamoyl]-1H-pyrrole-2-carboxylic acid methyl ester 13. Oxidation of **32** according to general procedure C gave **13** as a colourless solid (0.33 g, 69%). mp 93-95; HRMS (ES+) calcd for $C_{18}H_{28}N_3O_5$ (M+H$^+$) 366.2029 found 366.2033; ν_{max} (KBr) 1736 (CHO), 1634 (CONH); ^{1}H NMR (500 MHz, $CDCl_3$) 0.86 (3H, d, J = 7.0 Hz, C*H*$_3$), 0.87 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.00 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.04 (3H, d, J = 7.0 Hz, C*H*$_3$), 1.42-1.43 (1H, m, $CH_2CH(CH_3)_2$), 1.65-1.67 (2H, m, $CH_2CH(CH_3)_2$), 2.24-2.26 (1H, m, $CH(CH_3)_2$), 3.87 (1H, s, OC*H*3), 4.52-4.54 (1H, m, $CHCH_2CH(CH_3)_2$), 4.66 (1H, t, J = 8.0 Hz, $CHCH(CH_3)_2$), 6.72-6.74 (1H, m, C*H*CH), 6.86-6.88 (1H, m, C*H*CCOMe), 7.15 (1H, d, J = 7.0 Hz, NH), 7.26 (1H, d, J = 3.0 Hz, NH), 9.54 (1H, s, C*H*O), 10.85 (1H, br s, N*H*); ^{13}C NMR (75 MHz, $CDCl_3$); 18.6 (CH_3), 19.6 (CH_3), 22.0 (CH_3), 23.4 (CH_3), 24.9 ($CHCH_2CH(CH_3)_2$), 31.6

(CHCH(CH$_3$)$_2$), 38.0 (CHCH$_2$CH(CH$_3$)$_2$), 57.7 (CHCH$_2$CH(CH$_3$)$_2$), 58.5 (CHCH(CH$_3$)$_2$), 63.9 (OCH$_3$), 111.8 (CHCH), 125.4, (CHCCHO), 129.9 (CCONH), 130.0 (CN), 161.5 (CONH), 171.7 (CONH),199.9 (CHCHO); *m/z* (ES) 366 ([M+(H)]$^+$, 100. Anal. Calcd for $C_{18}H_{27}N_3O_5$: C, 59.16; H, 7.45; N, 11.50. Found C, 59.41; H, 7.87; N; 11.82.

4.2 Sequencing of ovine calpains

RNA was isolated from ovine muscle tissue using an RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's instructions.

Calpain 1 cDNA was synthesised following the maufacturer's protocol using non-specific primers and Superscript III Reverse Transcriptase (Invitrogen, California, USA). The calpain I coding sequence was amplified in two fragments using gene specific primers designed on the bovine sequence (Genebank accession number NM174259) using DNAMAN^{TM} (version 4.0, Lynnon Biosoft, Quebec, Canada). The 5' amplicon PCR was carried out in a total reaction volume of 20 µL consisting of 1x PCR buffer (Invitrogen, California, USA), 4 µL Q (Invitrogen, California, USA), 2.5 mM $MgCl_2$, 250 nm forward primer (5'-ACCGTGAATTAGAGAGATCGTC-3'), 250 nm reverse primer (5'-ACAGGGTGGTGTTCCAGTTG-3'), 125 µM dNTPs, 0.2 µL Taq DNA Polymerase (Invitrogen, California, USA), 1 µL gene specific cDNA and water added up to volume. The thermo-cycling profile consisted of: initial denaturation at 94ºC for 10 min, followed by 35 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 1 min and one final extension step of 72˚C for 10 min.

The 3' amplicon PCR was carried out in a total reaction volume of 20 µL consisting of 1x PCR buffer (Invitrogen, California, USA), 4 µL Q buffer (Invitrogen, California, USA), 2.5 mM $MgCl_2$, 250 nm forward primer (5'-TCCGAGACTTCATGCGTG-3'), 250 nm reverse primer (5'-AGGCACTTGCAGCTGGTG-3'), 125 µM dNTPs, 0.2 µL Taq DNA Polymerase (Invitrogen, California, USA), 1 µL gene specific cDNA and water added up to volume. The thermo-cycling profile consisted of: initial denaturation at 94ºC for 10 min, followed by 35 cycles of 94˚C for 30 s, 57˚C for 30 s, and 72˚C for 1 min and one final extension step of 72˚C for 10 min.

The 5' and 3' amplicons were sequenced several times from several animals in the forward and reverse directions using the primers mentioned above at the Allan Wilson Centre (Massey University, Palmerston North, New Zealand).

Calpain 2 cDNA was synthesised as per the manufacturer's protocol using Superscript III Reverse Transcriptase (Invitrogen, California, USA) and a calpain 2 gene specific 3' primer (5'AAAAGTTTCTCCGTGGAGGCT3'). Calpain 2 was then amplified by PCR using primers flanking the coding region designed from the rat sequence (Genebank accession number NM017116) using DNAMAN™ (version 4.0, Lynnon Biosoft, Quebec, Canada). PCRs were carried out in a total reaction volume of 25µL consisting of 1x PCRx Enhancer Buffer (Invitrogen, California, USA), 200µM dNTPs, 1.5mM $MgCl_2$, 200nM forward primer (5'GACGACACCATGGCGGGCATCGCGGCC3'), 200nM reverse primer (5'ATTAACAAGCTTTCAAAGTACTGAAAAACAC3'), 5µL PCRx Enhancer Solution (Invitrogen, California, USA) 0.2µL Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, California, USA), 1µL gene specific cDNA and water added up to volume. The samples were amplified using the following parameters: initial denaturation at 94˚C for 10 min, followed by 35 cycles of 94˚C for 30 s, 55˚C for 30 s, and 68˚C for 3 min followed by a final extension at 72˚C for 10 min.

PCR amplicons were sequenced several times in the forward and reverse directions using the previously mentioned primers (along with other internal primers designed from the developing ovine sequence) at the Waikato University DNA Sequencing Facility (University of Waikato, Waikato, New Zealand). The sequences were compiled using the GeneDoc Multiple Sequence Alignment Editor & Shading Utility (version, 2.6.002, Pittsburgh Supercomputing Centre, Pittsburgh, PA, USA).

The consensus coding sequences for ovine calpain 1 (bp) and calpain 2 (2103bp) were constructed from sequence data derived from PCRs performed on several non-related animals and submitted to GenBank (accession number XXXXXXXX and EU161096 respectively).

4.3 Enzyme assays

o-CAPN1 and o-CAPN2, partially purified from sheep lung by hydrophobic and ion-exchange chromatography, was diluted in a mixture containing 20 mM MOPS, 2 mM EGTA, 2 mM EDTA and 0.5% β-mercapto-ethanol (pH 7.5), to give a linear response over the course of the assay. The substrate solution (0.0005% BODIPY-FL casein in 10 mM $CaCl_2$, 0.1 mM NaN_3, and 0.1% β-mercapto-ethanol) was prepared fresh each day. Calpain inhibition assays were performed in 96-well black *Whatman*© plates at 25 °C. Calpain control assays contained 50 μL of enzyme and 50 μL of sample buffer. The reaction was initiated by the addition of 100 μL of substrate solution. The progress of the reaction was followed for 10 mins in a (BMG) Fluorostar with excitation at 485 nm and emission at 530 nm. For inhibitor assays the sample buffer was replaced with 50 μL of inhibitor dissolved in DMSO (2% total concentration) and diluted with water. The percentage inhibition was determined as 100 times the activity with inhibitor present divided by the activity of the control assay. The reported IC_{50} values are the average of triplicate determinations.

4.4 Lens culture

The EMEM culture medium for normal culture (EMEM, pH 7.4) was prepared from Minimum Essential Medium powder (or MEM, purchased from Sigma, product number M0643, 2003), 26 mM $NaHCO_3$, 0.02 mg/mL gentamycin (antibiotic, from Sigma) and 2.5 μg/mL amphotericin B (anti-fungal, from Sigma) in dH_2O. The culture medium was immediately sterilised by filtering with 0.2 μm pore size filters into autoclaved bottles. 2 M Ca^{2+} solution in sterilised dH_2O was added to 10 mL culture medium to give a final concentration of 5 mM Ca^{2+}. Calpain inhibitors were solubilised in DMSO to give a final concentration of DMSO in EMEM of less than 0.05%. Adequate amounts of DMSO were added to the medium containing 5 mM Ca^{2+} so there was no effect attributed by DMSO. Groups of ovine eye globes from 9-12 month old lambs, were collected from a local slaughterhouse immediately after slaughter and delivered to the laboratory for lens dissection. Pairs of lenses from the eyes of each animal were kept together. Three pairs of lenses were dissected within 2 h of death of the animal using the posterior approach. The intact lenses were immediately placed in a sterile culture dish containing 10 mL of

culture medium per lens. The entire lens was submerged with its anterior epithelium facing upward in the medium, which had been pre-incubated at 37 °C with 5% CO_2 for 48 h in a sterilised chamber. One of each of the paired lenses, was pre-incubated with **6** (0.8 μM) in EMEM for 2.5 h, whilst the other was cultured in EMEM only. Both lenses were then exposed to Ca^{2+} in EMEM to give a final concentration of 5 mM, by the addition of 16 μL of 2 M Ca^{2+} stock solution in dH2O. All lenses were incubated at 37 °C under 0.5% CO_2 for 24 h. All the lenses were then photographed using a digital camera (Sony Cybershot DSCF505V) fitted to a stand. A transparent flat bottom culture dish containing a lens and medium was placed on black grid lines (1 x 1 mm) with a white background light. Images of the anterior epithelium of the lens were taken with an image resolution of 1856 x 1392 pixels as a RGB true colour JPEG image. The lens opacity grade was scored by an image analysis system that was programmed to grade the extent of opacification captured by a digital photo image. The software used to grade the opacification captured was Image Pro-Plus v 4.1. A macro-script was created to automatically analyse the digital images of lenses (pixel size 1856 x 1392) placed on a 1mm x 1mm black grid. The grading system was developed on the basis of selecting predefined pixel RGB values, after area defining and sharpening of the image. Lenses were graded on a scale of 1 to 100, with fully opaque lens scoring 100 and transparent lens 1.

4.5 Molecular Modeling

All molecular modeling experiments were conducted with the Schrödinger suite 2005. Conformational searches on **4-13** were carried out with MacroModel 9.1, generating an ensemble of low energy conformers to establish suitable starting conformations of each compound for the docking.[31] The searches were conducted with the MCMM method using a GB/SA water model and the OPLS2001 force field, with 3000 steps for the conformational search and up to 5000 iterations for the minimization of each generated structure. The minimization was stopped with the default gradient convergence threshold of d = 0.05 kJ/(mol*Å). The default Polak-Ribiere Conjugate Gradient method was used for all minimizations. The crystal structure of human mini calpain 1 (PDB code 1-ZCM)[28] was prepared using the protein preparation facility in GLIDE 4.0 followed by mutation of Ser115->Cys115,

deprotonation of Cys115, and protonation of His272.[32] The *in-silico* ovine homology models were created by the virtual mutation of the appropriate residues around the active site cleft. These structures were minimized using the OPLS2005 force field with a GB/SA water model over 500 iterations. All residues within a 5Å distance to the calcium ions, the calcium ions and the key residues Gly_{208}, Gly_{271} and Cys_{115} (or Gly_{198}, Gly_{261} and Cys_{105}) of the structures were kept frozen during this minimization. The centre of the docking grid was defined as the centroid of the residues Cys115, Gly208, and Gly271 and was generated with GLIDE 4.0 using default settings. The centre of the docked ligands was defined within a 12Å box. The docking of flexible ligands to the rigid calpain model with GLIDE 4.0 was performed with the following parameters: OPLS2001 force field, extra precision mode, 90000 poses per ligand for the initial docking, scoring window for keeping initial poses: 5000, keep best 1000 poses per ligand for energy minimization, energy minimization with a distance dependent dielectric constant of 2 and a maximum of 5000 conjugate gradient steps. Ten poses per ligand were saved for evaluation.

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