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Synthesis and evaluation of *cis*-hexahydropyrrolo[3,2-*b*]pyrrol-3-one peptidomimetic inhibitors of CAC1 cysteinyl proteinases

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Abstract—A stereoselective synthesis of functionalised *cis*-hexahydropyrrolo[3,2-*b*]pyrrol-3-ones has been developed through Fmoc and Cbz-protected intermediates **5** and **6**. Building blocks **5** and **6** were prepared via the intramolecular cyclisation of *anti*-epoxide **17**. The intramolecular reaction occurred exclusively through the *anti*-epoxide to provide the 5,5-*cis*-fused bicycle, whereas the *syn*-epoxide, which theoretically would provide the 5,5-*trans*-fused bicycle, remained unchanged. These experimental observations are consistent with a key design element that we have introduced within this novel bicyclic ketone scaffold. Our bicyclic design strategy provides chiral stability to the bridgehead stereocentre that is situated α to the ketone because the *cis*-fused geometry is both thermodynamically and kinetically stable. Building blocks **5** and **6** have been utilised in both solid phase and solution phase syntheses of peptidomimetics **22**, **36**–**40**, which exhibit potent in vitro inhibition against a range of CAC1 cysteinyl proteinases. Compound **22**, a potent and selective inhibitor of human cathepsin K exhibited good primary DMPK properties along with promising activity in an in vitro cell-based human osteoclast assay of bone resorption.

1. Introduction

The papain-like CAC1 family of cysteinyl proteinases form a large group of widely expressed enzymes that perform a range of biological functions throughout the animal, plant, viral, bacterial and protozoal kingdoms.[1] Until recently, it was thought that the major role of mammalian CAC1 proteinases was a general nonspecific lysosomal degradation of proteins. However, our understanding of the biology of human cysteinyl proteinase function has developed significantly in the last few years.[2] It has now clearly been established that the physiological roles of many mammalian CAC1 proteinases fulfil specific functions concerning antigen presentation, extracellular matrix turnover and processing events.[3] As such, pharmaceutical interest has increased significantly over the past five years, with viable mammalian drug targets[3] now established for indications such as osteoporosis, multiple sclerosis, arthritis, atherosclerosis and cancer as well as parasitic proteinases[4] identified to target infections such as malaria[5a,b] and Chagas disease.[5c,d] Consequently, significant resource is currently devoted within the pharmaceutical industry towards the development of CAC1 proteinases inhibitors that are suitable for human administration.[3,6] The most widely examined inhibitor series to date are based around covalent interaction of the proteinase active site thiol with peptidomimetic nitriles,[7a,b] cyclic ketones,[7c,d] ketoamides[7e,f] and nonpeptidic cyanamides,[7g] 2-carbonitrile-pyrrolopyrimidines[7h] and 2-cyanopyrimidines.[7i] Additionally, a noncovalent series of cathepsin K inhibitors based around arylaminoethylamides has received recent attention.[8] A number of compounds from these initial series have now advanced into the clinic as inhibitors of cathepsin K with osteoporosis as the main therapeutic indication.[9] The preliminary findings have clearly validated the efficacy of inhibitors of cathepsin K in a clinical setting and removed a significant portion of the risk associated with first-in-class studies. These advances should invigorate the search for new inhibitor series, against both cathepsin K and other CAC1 proteinases, that are suitable for the clinic. In this context, we have recently reported the design, synthesis and

Keywords: CAC1 proteinase inhibition; Bicyclic ketones; Anti-resorptive.
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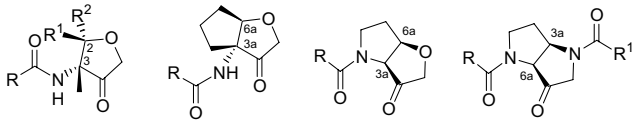
inhibition kinetics of three novel series of peptidomimetic inhibitors of CAC1 proteinases, each based around constrained ketone-containing scaffolds (**1**→**3**).[7d,10]



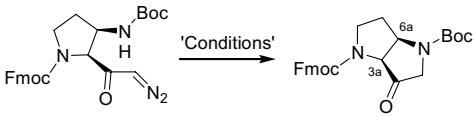
1 (2*R* or *S*, 3*R*) **2** (3a*R*, 6a*R*) **3** (3a*S*, 6a*R*) **4** (3a*R*, 6a*S*)

A key design feature in each of these scaffolds was the introduction of a moiety that stabilised the otherwise chirally labile position situated α to the ketone, which is a physiochemical characteristic that has hindered the pre-clinical development of a number of ketone-based inhibitor series.[11] The stabilisation was achieved through alkylation as in series **1** and **2** or by exploiting the kinetic and thermodynamic stability of a *cis*-fused 5,5-bicycle as in series **3**. Peptidomimetic analogues built around scaffold **3** were found to exhibit potent inhibition against a range of CAC1 proteinases, where the inhibition kinetics were characterised by markedly increased enzyme–inhibitor association rates (k_{on}) when compared to equivalent analogues from series **1** and **2**. Encouraged by these findings, we have now extended our molecular modelling studies for series **3**, which featured inhibitors that bound exclusively in the nonprime binding pockets of the proteinase.[10] Our new modelling studies have concluded that compounds based upon the *cis*-hexahydropyrrolo[3,2-*b*]pyrrol-3-one scaffold **4** may span the active site to provide binding interactions within both the prime and nonprime sites. Herein we report the building block preparation, solid and solution phase syntheses and inhibition kinetics for a series of bicyclic peptidomimetic *cis*-hexahydropyrrolo[3,2-*b*]pyrrol-3-one based analogues **4** that exhibit potent inhibition of a range of CAC1 cysteinyl proteinases.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of Fmoc and Cbz protected scaffolds 5 and 6. We have previously described the syntheses of mono- and bicyclic ketone scaffolds **1**→**3** through a lithium chloride/acetic acid promoted insertion reaction of protected aminoacid α-diazomethylketones as the final ring-closing synthetic step[7d,10] and were hopeful that a similar scheme could be followed towards *cis*-hexahydropyrrolo[3,2-*b*]pyrrol-3-ones **4**. We were initially encouraged by literature precedence describing the intramolecular N–H insertion reactions of amino-α-diazomethylketones in the preparation of pyrrolidinones, which were promoted by reagents such as trifluoroacetic acid (TFA)[12a] or rhodium(II) catalysis.[12b] However, we found that adaptation of these methods with the corresponding diazomethylketone **7** gave complex reaction mixtures from which the urethane protected bicycle **5** was isolated in low yield following treatment of **7** with rhodium(II) acetate dimer in toluene (Scheme 1).[13]



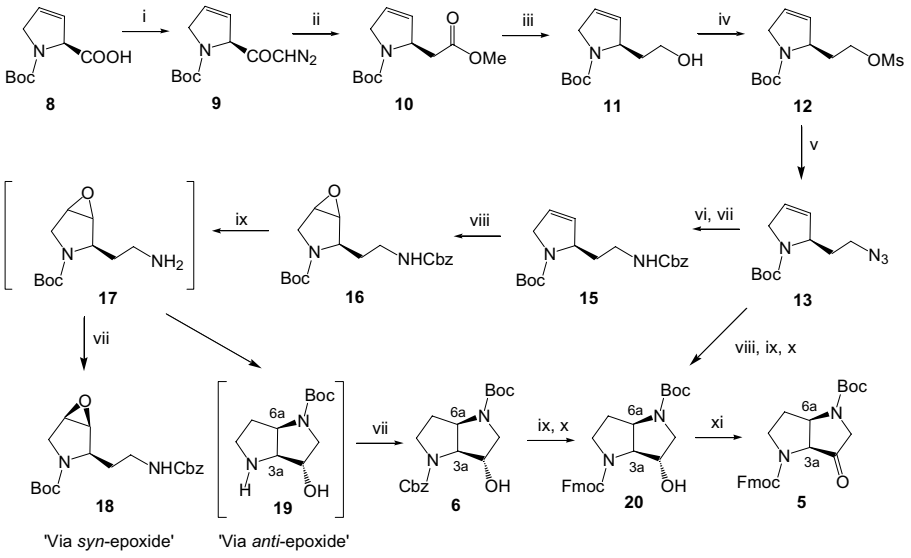
7 (2*S*, 3*R*) **5** (3a*S*, 6a*R*)

Scheme 1. Synthesis of (3a*S*,6a*R*)-**5** via an N–H insertion reaction of (3*R*)-*tert*-butoxycarbonylamino-(2*S*)-(2-diazoacetyl)pyrrolidine-1-carboxylic acid 9*H*-fluoren-9-ylmethyl ester.

Given the poor yield from this initial route, we sought alternative methods and our retrosynthetic analysis focussed upon the 1,3-arrangement of the a-ring nitrogen and b-ring ketone. This indicated that the intramolecular ring opening of an epoxide with a tethered amine could provide the desired 5,5-bicyclic scaffold **4** (see Scheme 2).[14]

We envisaged that the amine epoxide **17** would be a key intermediate in which only the *anti*-epoxide would cyclise because potential cyclisation of the *syn*-epoxide would be disfavoured as it would lead to the thermodynamically less stable *trans*-5,5-bicycle. Examination of this synthetic route commenced from the commercially available 3,4-dehydroproline **8**. Methylene homologation of **8** was achieved with retention of configuration by Arndt–Eistert reaction followed by Wolff rearrangement, providing protected pyrrolidineacetic acid ester **10** (overall 62% from acid **8**).[15] Ester **10** was reduced by treatment with lithium borohydride in anhydrous THF to give alcohol **11** (67%). Standard activation of alcohol **11** as the mesylate **12** was followed by nucleophilic displacement with sodium azide in dimethylformamide (DMF) to give the azide **13** (79%). Azide **13** provided a key intermediate that could lead to bicyclic analogues through two routes, both of which were successfully implemented towards the bicyclic compound **20** (see Scheme 2).

In the first route, azide **13** was reduced to amine **14** following the general methods described by Mandville et al. using triphenylphosphine and water in THF[16] and the crude amine converted to benzyloxycarbonyl (Cbz) protected analogue **15** (overall 94% from azide **13**) under standard Schotten–Baumann conditions. Epoxidation of **15** with *m*-chloroperoxybenzoic acid provided an approximately 1:3 mixture of *syn*- and *anti*-epoxides **16** (95%), which co-eluted on analytical HPLC and gave a broad figure of eight spot by TLC analysis. We surmised that removal then re-addition of the Cbz protection within epoxides **16** would provide a new mixture in which the original *anti*-epoxide had cyclised to give the Cbz-protected bicycle **6** along with the unreacted *syn*-epoxide from mixture **16** (compound **18**). Thus, hydrogenation of Cbz protected **16** over 10% palladium on carbon (Pd–C) proceeded smoothly over 2.5 h providing intermediate amine epoxide **17**, wherein *anti*-**17** cyclised to give bicyclic intermediate **19**, which was followed by re-protection with Cbz under standard Schotten–Baumann conditions. Analysis of the re-protected crude reaction mixture indeed revealed the presence of a major new compound, with the same molecular weight as **16**, that was less mobile on TLC



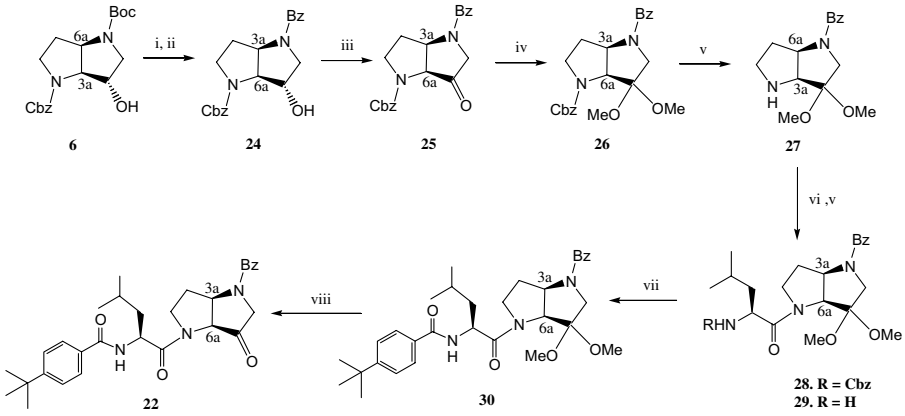
Scheme 2. Synthesis of (3a*S*,6a*R*)-**5** and **6**. Reagents and conditions: (i) (a) isobutyl chloroformate, NMM, CH_2Cl_2, −15 °C; (b) etheral CH_2N_2, −15 °C to rt; (ii) MeOH, THF, CF_3CO_2Ag, NMM, 0 °C to rt in dark; (iii) $LiBH_4$, MeOH, THF; (iv) methanesulfonyl chloride, triethylamine, DCM; (v) sodium azide, DMF; (vi) Ph_3P, H_2O, THF; (vii) 1.05 equiv Cbz–Cl, 2.1 equiv Na_2CO_3, 1,4-dioxane, water; (viii) *m*-chloroperoxybenzoic acid, DCM, rt, o/n; (ix) Pd–C/H_2, ethanol; (x) 1.05 equiv Fmoc–Cl, 2.1 equiv Na_2CO_3, 1,4-dioxane, water; (xi) Dess–Martin periodinane, DCM.

and eluted earlier by HPLC analysis (present at 67.2% by UV analysis) when compared to the original mixture **16**. The new compound was purified by silica chromatography and confirmed by full analysis as the desired 5,5-bicyclic alcohol **6** (overall 61% from mixture **16**) ($[\alpha]_D^{22}$ −109.2 (*c* 0.544, $CHCl_3$)). Also recovered from this reaction were the *syn*-epoxide **18** (20.3%) and a second new compound (4.3%) with a molecular weight increased by 2 Da, that was poorly mobile on TLC, and tentatively assigned as an uncyclised alcohol produced by hydrogenation of the epoxide within **16**. With bicyclic alcohol **6** in hand, hydrogenation followed by reprotection with 9-fluorenylmethoxycarbonyl chloroformate (Fmoc–Cl) provided intermediate **20** (95%) ($[\alpha]_D^{22}$ −104.0 (*c* 0.2, $CHCl_3$)). Having confirmed the feasibility of this route, our repeat syntheses of compound **20** proceeded through direct Fmoc protection of intermediate bicyclic amine **19** (along with isolation of the corresponding Fmoc analogue of *syn*-epoxide **18**).

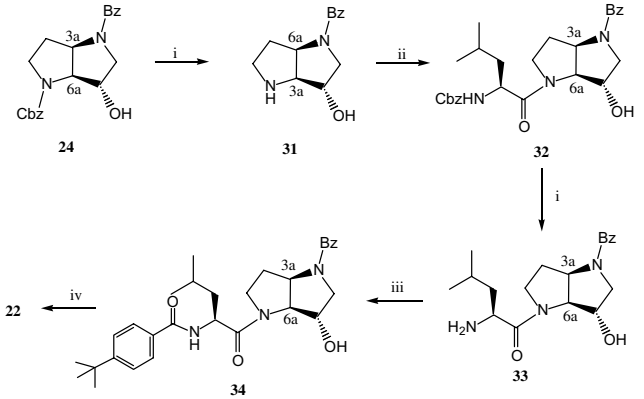
In the second potentially shorter route, azide **13** was treated with *m*-chloroperoxybenzoic acid to provide an unquantified mixture of *syn*- and *anti*-epoxides **21**. We found that azido epoxide **21** was unstable, so the crude mixture was immediately hydrogenated over 10% Pd–C, essentially giving a mixture of amine epoxides **17** and **19**. Treatment of this crude amine mixture with Fmoc–Cl under standard Schotten–Baumann conditions gave our second route to intermediate **20** (overall 27% from azide **13**). In practice, we have generally used the slightly longer first route for repeat syntheses since the overall yield of **20** is higher (overall 51% from azide **13** via Cbz-protected **6**), the synthetic steps are reproducible on a multigram scale and epoxide **16** provides a stable advanced intermediate. As the final step, alcohol **20** was smoothly oxidised by treatment with Dess–Martin periodinane to provide target bicyclic ketone **5** (97%) as a white solid ($[\alpha]_D^{22}$ −140.0 (*c* 0.6, $CHCl_3$)). We have previously noted that urethane protected 5,5-bicyclic ketones exhibit an unusually broad HPLC elution profile, which we have attributed to the presence of slowly converting but resolvable rotamers about the urethane 3° amide bond.[10] We observed a similar HPLC profile here with ketones in series **4**.

Building blocks **5** and **6** provided the opportunity to examine synthetic routes towards peptidomimetic inhibitors of CAC1 proteinases through both solid and solution phase syntheses. We initially chose to examine routes towards compound **22** (see Scheme 3 and 4), since it offered a direct comparison to the corresponding cathepsin K inhibitor from general series **3**, (3a*S*,6a*R*)-4-*tert*-butyl-*N*-[3-methyl-1*S*-(3-oxo-hexahydrofuro[3,2-*b*]pyrrole-4-carbonyl)butyl]benzamide **23**, a compound that binds exclusively in the nonprime binding pockets of cathepsin K for which we have previously described the inhibition kinetics in detail.[10] Starting from alcohol **6**, we envisaged two solution-based routes towards inhibitor **22**, the former involving oxidation of the alcohol then protection of resulting ketone as a dimethylketal, with ketal hydrolysis as the final synthetic step (Scheme 3) and the latter involving introduction of the ketone through oxidation of a bicyclic alcohol as the final synthetic step (Scheme 4).

Our initial target inhibitor **22** contained the *N*-benzoyl (Bz) group as the potential prime-side binding substituent, whilst building block **6** contained the *N*-*tert*-butoxycarbonyl (Boc) group. Therefore, both of the syntheses shown in Schemes 3 and 4 commenced with a common transformation through acidolytic removal of the Boc group with HCl followed by acylation with benzoic anhydride to give intermediate **24** in essentially quantitative yield. Then following Scheme 3, alcohol **24** was oxidised by treatment with Dess–Martin periodinane to ketone **25** (78%). Since the objective of this route



Scheme 3. Solution synthesis of (3a*R*,6a*S*)-**22** via ketal. Reagents and conditions: (i) 4 N HCl in 1,4-dioxane, 30 min, rt; (ii) benzoic anhydride, NMM, DMF, rt, 1 h; (iii) Dess–Martin periodinane, DCM; (iv) trimethylorthoformate, anhydrous MeOH, cat. *p*-TsOH, under Ar, 65 °C; (v) Pd–C/H_2, ethanol, methanol; (vi) 1 equiv Cbz-Leu-F, DMF, rt; (vii) 1 equiv 4-*tert*-butylbenzoic acid, HBTU, HOBt, NMM, DMF, rt; (viii) 95% TFA/5% water, rt.



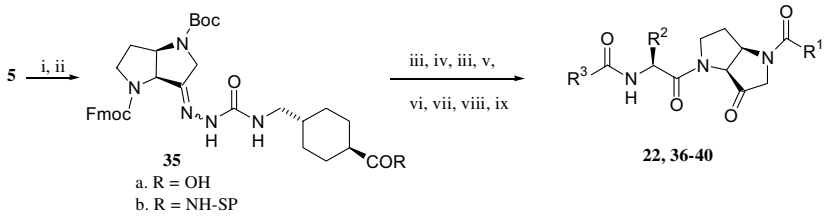
Scheme 4. Solution synthesis of (3a*R*,6a*S*)-**22** via alcohol. Reagents and conditions: (i) Pd–C/H_2, ethanol/methanol; (ii) 1 equiv Cbz-Leu-F, DMF, rt; (iii) 1 equiv 4-*tert*-butylbenzoic acid, HBTU, HOBt, NMM, DMF, rt; (iv) Dess–Martin periodinane, DCM, 2 equiv TFA.

was to protect the ketone functionality for the remainder of the synthesis, we chose to convert **25** to the dimethylketal **26** (77%), which was obtained by reaction with trimethylorthoformate in methanol and *p*-toluenesulfonic acid catalysis. Catalytic hydrogenation of the Cbz protection from ketal **26** proceeded to completion within 90 min when performed in methanol, providing free amine **27** in essentially quantitative yield. Facile acylation of amine **27** was achieved by reaction with the pre-formed acyl fluoride of Cbz-leucine. The acylation reaction was essentially complete within 1 h at ambient temperature which, after work-up and silica chromatography, gave intermediate **28** (90%). Catalytic hydrogenation of the Cbz protection from ketal **28** proceeded to 95% completion within 6 h when carefully performed in methanol, providing free amine **29** in essentially quantitative yield. Acylation of amine **29** with 4-*tert*-butylbenzoic acid through standard uronium activation chemistries[17] gave ketal **30** (78%). The final step in Scheme 3, acid hydrolysis of the dimethylketal to ketone, proved difficult. We observed that under the strong acidolytic cleavage conditions required to release ketone **22** from ketal **30**, a carboxylic acid side product was formed by hydrolysis of the 3° amide bond between the leucinyl carbonyl and the bicyclic scaffold. The same side product was also observed during the acidolytic release of compounds from the solid phase (see later) and appears to be particularly prevalent for compound **22** in comparison to the other analogues we have prepared (see later). However, treatment of ketal **30** with TFA and water for 3.5 h, followed by work-up and silica chromatography gave ketone **22** (24%) as a white solid.

Next we examined the alternative synthesis of compound **22** as shown in Scheme 4. Catalytic hydrogenation of the Cbz group from alcohol **24** was complete after 8.5 h when performed in ethanol, giving amine **31**. Acylation of amine **31** with Cbz-leucine-F in DMF at ambient temperature for 75 min gave alcohol **32** (68%) following purification. However, in direct contrast to aminoketal **27**, we found that aminoalcohol **31** could also be quantitatively acylated with Cbz-leucine succinyl ester in DMF at ambient temperature. Hydrogenation of the Cbz group from alcohol **32** was slow when performed in ethanol, requiring multiple additions of catalyst and 24 h for completion, giving amine **33** in quantitative yield. Acylation of amine **33** with 4-*tert*-butylbenzoic acid as detailed earlier gave alcohol **34** (68%) as a white solid ($[\alpha]_D^{22}$ −84.5 (*c* 0.084, $CHCl_3$)). Final oxidation of alcohol **34** was achieved by treatment with Dess–Martin periodinane in dichloromethane (DCM) to give ketone **22** (74%) ($[\alpha]_D^{22}$ −82.0 (*c* 0.49, $CHCl_3$)).

2.2. Solid phase synthesis

Building block **5**, containing the orthogonal Fmoc and Boc protecting groups, was suitable for the rapid generation of CAC1 proteinase inhibitor analogues by solid phase methods. Our synthetic strategy was based upon reversible anchorage of the ketone functionality of building block **5** via a hydrazide linker bond using the general multipin techniques that we have previously described in detail (Scheme 5).[7d,10,19]



Scheme 5. Synthesis and use of supported linker construct **35b** towards full length inhibitors **22**, **36**–**40**. Reagents and conditions: (i) *trans*-4{[(hydrazinocarbonyl) amino]methyl}cyclohexanecarboxylic acid trifluoroacetate,[18] EtOH, H_2O, NaOAc, reflux; (ii) 3 equiv **35a**, 3 equiv HBTU, 3 equiv HOBt, 6 equiv NMM, H_2N-SOLID PHASE, DMF; (iii) 20% piperidine/DMF (v/v), 30 min; (iv) 20 equiv Fmoc–NHCHR^2–COOH, 20 equiv HBTU, 20 equiv HOBt, 40 equiv NMM, DMF, rt, o/n, then repeat with fresh reagents for 6 h; (v) 10 equiv R^3–COOH, 10 equiv HBTU, 10 equiv HOBt, 20 equiv NMM, DMF, rt o/n; (vi) TFA/DCM (35:65, v/v), 30 min; (vii) NMM/DMF (2:98, v/v), 10 min; (viii) 20 equiv benzoic anhydride, 10 equiv NMM, DMF, o/n; (ix) TFA/H_2O, (95:5, v/v), 2 h.

Final compounds **22** and **36**–**40** were prepared from linker-construct **35b** by initially building the nonprime binding elements (R^{3}CONHCHR^{2}CO) through a series of sequential washing and coupling reaction steps involving removal of Fmoc, double coupling of an activated Fmoc–NHCHR^2–COOH to the secondary amine of the bicyclic scaffold, removal of Fmoc and coupling of an activated R^3–COOH. Subsequent treatment with 35% TFA in DCM gave quantitative removal of the Boc group, exploiting the greater acid lability of Boc in comparison to that of the hydrazone linker.[20] Following neutralisation of the multipin-bound TFA salt, treatment with benzoic anhydride in DMF provided quantitative acylation of the bicyclic secondary amine. Finally, cleavage of the hydrazone linker was achieved with 95% TFA/H_2O, followed by semi-preparative HPLC purification to provide inhibitors **22**, **36**–**40**. In general the quality of crude inhibitors from this solid phase scheme was excellent as judged by analytical HPLC at >90%. In each crude sample we observed a side product consisting of the carboxylic acid R^{3}CONHCHR^2–COOH that derives from cleavage of the tertiary amide bond between the P2 aminoacid carbonyl and the bicyclic scaffold. Within crude samples **36**–**40**, this carboxylic acid contaminant was present at 1–10% and for crude sample **22** was present at 19%.[21]

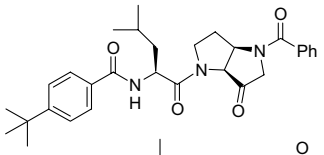
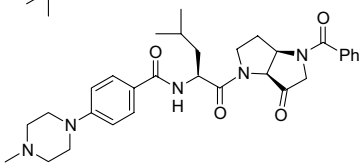
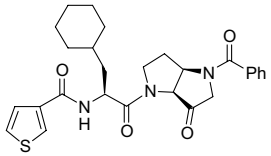
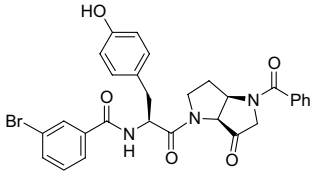
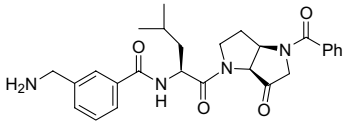
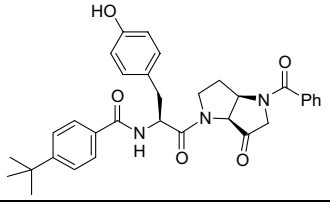
2.3. Enzyme inhibition studies

Bicyclic inhibitors **22**, **36**–**40** were screened against cathepsins K, L, S and B as well as the parasitic proteinases cruzain and CPB.[13] Preliminary steady-state inhibition constants (K_i^{ss}) are shown in Table 1 (mean of $n = 3$ determinations). The substituents detailed in Table 1 were chosen to provide a direct comparison with our previously detailed bicyclic inhibitors based around scaffold **3**[10] and are based upon binding groups that provide potent inhibitors when combined with the unsubstituted monocyclic scaffold described by GSK.[22]

Table 1 clearly shows that with the appropriate combination of substituents suitable for a particular proteinase, compounds based around general series **4** provide potent and selective inhibitors of a range of CAC1 proteinases. In each example, the *cis*-hexahydropyrrolo[3,2-*b*]pyrrol-3-ones **4** detailed herein are more potent than the corresponding tetrahydrofuro[3,2-*b*]pyrrol-3-ones **3** that we have described previously.[10] For example, analogue **22** was a potent ($K_i^{ss} = 10.1$ nM) and selective (>300-fold vs S and L) inhibitor of cathepsin K, which represented a 9-fold increase in activity when compared to the equivalent bicycle from series **3** (compound **23**).[10] Additionally, analogue **37** was a potent ($K_i^{ss} = 29.3$ nM) and selective (>26-fold vs K and 9-fold vs L) inhibitor of bovine cathepsin S with significantly improved potency compared to that of the parent monocycle standard ($K_i^{ss} = 220$ nM).[22] Analogue **38** was a potent ($K_i^{ss} = 78.3$ nM) and selective (>50-fold vs K and S) inhibitor of human cathepsin L with equivalent potency to that of the parent monocycle standard ($K_i^{ss} = 70$ nM).[22] Analogue **39** was a potent mixed inhibitor of cathepsins K ($K_i^{ss} = 112$ nM), L ($K_i^{ss} = 81.7$ nM) and the parasitic proteinase CPB ($K_i^{ss} = 64.8$ nM), whilst analogue **40** was a potent ($K_i^{ss} = 300$ nM) inhibitor of cruzain. The across-the-board increase in potency could be due to the introduction of a binding group within general series **4** that enables inhibitors to bind in both nonprime and prime-side binding pockets of the proteinases. However, the explanation is likely to be more complex and additionally involve factors such as ring strain and stereoelectronic effects of the 'oxygen' to 'acyl-nitrogen' substitution.[23a–c]

In our previous studies concerning the inhibition kinetics of analogues from series **3**[10], we investigated the individual association (k_{on}) and dissociation (k_{off}) rate constants for bicyclic ketone inhibitors of human cathepsin K. We have repeated these studies for the potent cathepsin K analogues from series **4**, compounds **22** and **36**, along with corresponding analogues from series **3**[10], compounds **23** and **42** and the monocyclic ketone analogues[10], compounds **41** and **43** (Table 2). All of these are potent inhibitors of human cathepsin K with steady state inhibition constants ranging from 4.9 to 87.4 nM. However, for the examples in Table 2, it is clear that the bicyclic analogues (**22**, **23**, **36** and **42**) exhibit significantly slower dissociation rates when compared with the corresponding monocyclic analogues (**41** and **43**). Conversely, in general the monocyclic analogues exhibit faster association rates when compared with the corresponding bicyclic analogues (compare inhibitor **41** with inhibitors **22** and **23**; inhibitor **43** with **42**). The exception to these findings is inhibitor **36**, which combines a relatively fast association rate with a

Table 1. Preliminary inhibitory activities (K_i^{ss}, nM) for 5,5-bicyclic inhibitors **22**, **36**–**40** against CAC1 proteinases (mean of $n = 3$ determinations)

No	Structure	Cat. K	Cat. L	Cat. S	Cat. B	Cruz.	CPB
22		10.1 ± 6.7	>3500	>4500	>10,000	173 ± 86	691 ± 350
36		4.9 ± 0.7	221 ± 87	1242 ± 270	>4000	224 ± 11	91 ± 17
37		766 ± 78	257 ± 5	29.3 ± 1.8	>1500	1838 ± 333	1337 ± 182
38		>35,000	78.3 ± 8.5	>3500	>3000	562 ± 219	>3000
39		112 ± 15	81.7 ± 31.0	1016 ± 33	>3500	354 ± 56	64.8 ± 9.4
40		1325 ± 312	>3000	>12,000	>10,000	300 ± 10	1565 ± 184

Assay conditions are as detailed previously.[13]

relatively slow dissociation rate and is therefore a very potent inhibitor of human cathepsin K with $K_i[k_{off}/k_{on}]$ at 1.6 nM.

2.4. In vitro stability and physiochemical properties

We further evaluated the potent cathepsin K inhibitors **22** and **36** through a range of stability studies, physiochemical property determinations and in vitro secondary assays (Tables 3 and 4) and for comparison included our previously reported data for the corresponding analogues from series **3**, compounds **23** and **42**.[10] These studies aimed to establish the basic physical properties of inhibitors **22** and **36** and to determine their suitability for functional assessment of cathepsin K inhibition.[24] Inhibitors **22** and **36** exhibited good stability at acid and neutral pH and in human plasma with $t_{1/2} > 24$ h. At high pH, both **22** and **36** were reasonably stable, however compound **22** was clearly degraded more rapidly. Indeed, we have generally observed that analogues from both bicyclic series **3** and **4** that contain the 4-methylpiperazin-1-ylbenzamide group to be more stable under virtually all conditions when compared to the corresponding 4-*tert*-butylbenzamide analogues (Table 3). The basic physiochemical properties of inhibitors **22** and **36** are presented in Table 4. Analogues **22** and **36** have a moderate number of hydrogen bond donors and acceptors,[25] medium polar surface area,[26] moderate molecular weight and moderate rotational freedom,[25] which are molecular properties that conform to major predictors of desirable drug-like features. Finally, in vitro assessment of inhibitors **22** and **36** against the major cytochrome P450 isozymes 1A2, 2B6, 2D6, 3A4 and 2C19 showed no significant inhibition at 10 μM.[27] Considering these findings and the results from Tables 3 and 4, we concluded that analogues **22** and **36** exhibited a reasonably good range of properties suitable for functional assessment of cathepsin K inhibition.

2.5. Osteoclast resorption assay

Analogue **22** was evaluated in a human osteoclast resorption assay,[28] to determine the general ability of cathepsin K inhibitors from series **4** to inhibit bone resorption. Briefly, human osteoclasts were cultured on bovine bone slices and allowed to differentiate and resorb bone. The C-terminal telopeptides (CTX) of type I collagen released into the culture media were then

Table 2. Association (k_{on}) and dissociation rate constants (k_{off}) for 5,5-bicyclic inhibitors **22** and **36** against human cathepsin K along with the corresponding 'oxygen' bicycles from series **3**, compounds **23** and **42** and the corresponding 'monocycles', compounds **41** and **43** for comparison[10]

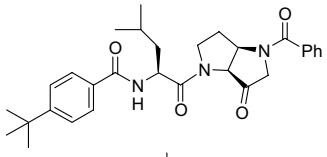
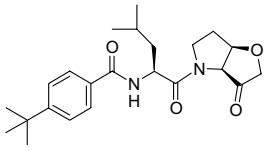
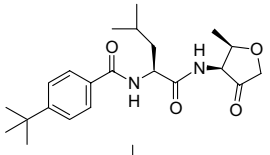
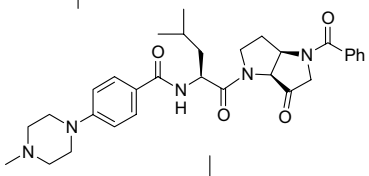
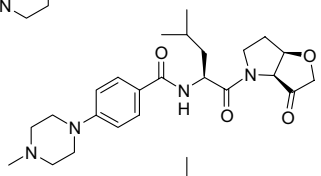
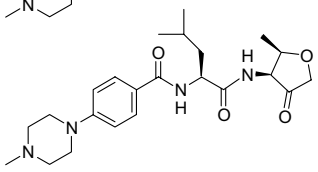
No	Structure	k_{on} ($M^{-1}s^{-1}$) ($\times 10^5$)	k_{off} (s^{-1}) ($\times 10^{-3}$)	$K_i(k_{off}/k_{on})$ (M) ($\times 10^{-9}$)	K_i^{ss} (M) ($\times 10^{-9}$)
22		1.4 ± 1.2	1.7 ± 0.6	12.1	10.1 ± 6.7
23[10]		4.9 ± 3.9	7.5 ± 1.7	15.2	87.4 ± 0.8
41[10]		12.0 ± 2.0	40.3 ± 21.5	33.6	41.0 ± 2.1
36		46 ± 0.4	7.3 ± 0.6	1.6	4.9 ± 0.7
42[10]		5.3 ± 4.6	5.5 ± 0.7	10.4	8.7 ± 0.4
43[10]		>10	18.7 ± 14.6	—	38.9 ± 2.7

Table 3. Stability studies for bicyclic analogues **22**, **23** and **36**, **42**

	PBS ($pH_{7.4}$) $t_{1/2}$ (h)[a]	Acid ($\sim pH_{1.5}$) $t_{1/2}$ (h)[b]	Base ($pH_{10.5}$) $t_{1/2}$ (h)[c]	Human plasma $t_{1/2}$ (h)[d]	HLM $t_{1/2}$ (h)[e]
22	72.2	42.5	6.4	24.4	3.4
23[10]	28.9	23.2	n.d.	4.5	2.8
36	110.2	64.9	38.6	30.2	5.0
42[10]	36.5	16.9	27.9	16.3	7.5

For each analysis, aliquots at appropriate times were quantified by HPLC–MS, using single ion monitoring and the ion intensity data converted to a $t_{1/2}$ for loss of parent analogue.

[a] Compounds were incubated at 10 μM in PBS (10 mM; pH 7.4) at 37 °C.

[b] Compounds were incubated at 10 μM in 0.1 M HCl/CH_3CN (80:20) at 37 °C.

[c] Compounds were incubated at 10 μM in potassium phosphate (10 mM; pH 10.5) at 37 °C.

[d] Compounds were incubated at 10 μM in human plasma at 37 °C and after protein precipitation with CH_3CN, extracted aliquots were analysed by HPLC–MS, using single ion monitoring.

[e] Compounds were incubated at 50 μM with human liver microsomes (0.5 mg/mL of microsomal protein, final concentration) in potassium phosphate (50 mM; pH 7.4) at 37 °C and the reaction was initiated with NADPH (1 mM final concentration). Quenching was achieved by protein precipitation with CH_3CN and the extracted aliquots were analysed by HPLC, employing UV detection.

Table 4. Physiochemical properties for bicyclic analogues **22**, **23** and **36**, **42**

	Sol.[a]	Log $D_{7.4}$[b]	N° H-bond donors	N° H-bond acceptors	N° rotatable bonds	Polar surface area (Å^2)
22	M/H	2.84	1	4	7	86.8
23[10]	M	1.36	1	4	6	75.7
36	H	1.68	1	6	7	93.3
42[10]	H	0.04	1	6	6	82.2

[a] Aqueous solubility was assessed by measuring turbidity of solutions of compound prepared in PBS (10 mM; pH 7.4) at 200, 100, 50 and 25 μM, by light scattering at 650 nm. Compounds were assigned as having high (H, >100 μM), medium (M, 50–100 μM) or low (L, <50 μM) solubility.

[b] Partitioning of the compounds between *n*-octanol and PBS (10 mM; pH 7.4) was assessed using a miniaturised shake-flask method, employing HPLC–UV analysis.

quantified as an index of bone resorption. Compound **22** and a positive control, E-64 (a potent inhibitor of cathepsin K), were added into the cell cultures after the differentiation period and their effects on the resorbing activity of mature osteoclasts were determined (Fig. 1). As shown in Figure 1, compound **22** was a potent

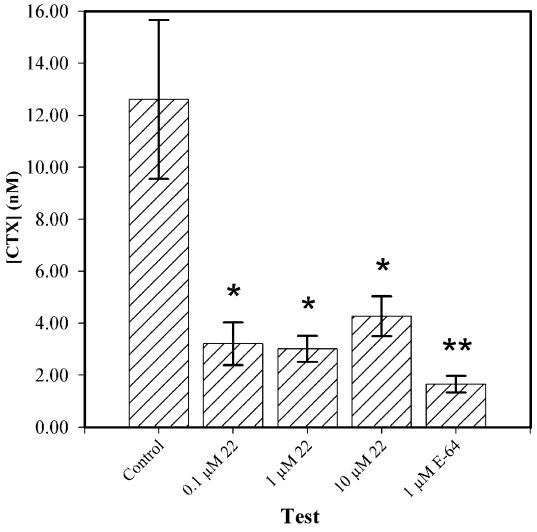


Figure 1. Inhibition profile for compound **22** as judged by human osteoclast bone resorption assay. Bone resorption activity was monitored by determining collagen fragments in culture medium (CTX) using CrossLaps®. Asterisks indicate values that are statistically significantly different from baseline (with a *p*-value of less than 0.05). One asterisk (*) indicates a *p*-value between 0.05 and 0.01 and two asterisks (**) a *p*-value between 0.01 and 0.001.

inhibitor of bone resorption in this cell-based assay, exhibiting 75% inhibition at a concentration of 100 nM.

3. Conclusions

We have shown that 5,5-bicyclic series **4** provides a conformationally constrained scaffold towards the design of potent and selective inhibitors of a range of therapeutically attractive mammalian and parasitic CAC1 cysteinyl proteinase targets. The inhibitors may readily be prepared either on the solid phase for rapid analogue generation, or in solution for large scale-up applications, following simple high yield syntheses of key building blocks **5** and **6**. Preliminary pharmacokinetic analysis of the representative cathepsin K inhibitors **22** and **36**, showed a reasonably good range of physiochemical and stability properties in both chemical and biological media, providing a sound basis for further pre-clinical evaluation. Compound **22**, a potent in vitro inhibitor of human cathepsin K with $K_i^{ss} = 10.1$ nM, was assessed in a functional human osteoclast assay of cathepsin K inhibition. In this cell-based assay, compound **22** exhibited 75% inhibition of the release of C-terminal telopeptides (CTX) of type I collagen when tested at 100 nM. These preliminary data provide a promising platform for the development of potent analogues from series **4** towards a cathepsin K inhibitor for the treatment of osteoporosis.

4. Experimental

4.1. General procedures

Standard vacuum techniques were used in the handling of air sensitive materials. Solvents were purchased from ROMIL Ltd, UK at SpS or Hi-Dry grade unless otherwise stated. ^{1}H NMR and ^{13}C NMR were obtained on a Bruker DPX400 (400 MHz ^{1}H frequency and 100 MHz ^{13}C frequency; QXI probe) or Bruker Avance 500 MHz (TXI probe with ATM) in the solvents indicated. Chemical shifts are expressed in parts per million (δ) and are referenced to residual signals of the solvent. Coupling constants (J) are expressed in Hz. All analytical HPLC were obtained on Phenomenex Jupiter C_4, 5 μm, 300 Å, 250 × 4.6 mm, using mixtures of solvent A (0.1% aq TFA) and solvent B (90% acetonitrile (CH_3CN)/10% solvent A) on automated Agilent systems with 215 and/or 254 nm UV detection. Unless otherwise stated a gradient of 10–90% B in A over 25 min at 1.5 mL/min was performed for full analytical HPLC. HPLC–MS analysis was performed on an Agilent 1100 series LC/MSD, using automated Agilent HPLC systems, with a gradient of 10–90% B in A over 10 min on Phenomenex Luna C_8, 5 μm, 300 Å, 50 × 2.0 mm at 0.6 mL/min. Semi-preparative HPLC purification was performed on Phenomenex Jupiter C_4, 5 μm, 300 Å, 250 × 10 mm, using a gradient of 10–90% B in A over 25 min at 4 mL/min on automated Agilent systems with 215 and/or 254 nm UV detection. Flash column purification was performed on silica gel 60 (Merck 9385). Polyamide multipins (10 μmol loadings, SPMDINOF, see www.mimotopes.com) were used for the solid phase synthesis. Biochemical protocols together with enzyme assays were carried out as previously described.[13] Substrates utilising fluorescence resonance energy transfer methodology (i.e., FRET-based substrates) were synthesised using standard solid phase Fmoc chemistry methods,[29] and employed Abz (2-aminobenzoyl) as the fluorescence donor and 3-nitrotyrosine [Tyr(NO_2)] as the fluorescence quencher.[30]

4.1.1. (*S*)-2-(2-Diazoacetyl)-2,5-dihydropyrrole-1-carboxylic acid *tert*-butyl ester (9). (*S*)-2,5-Dihydropyrrole-1,2-dicarboxylic acid 1-*tert*-butyl ester **8** (1.066 g, 5 mmol) was dissolved with stirring in anhydrous DCM (40 mL). The reaction was flushed with nitrogen and cooled to −15 °C. Isobutyl chloroformate (0.713 mL, 5.5 mmol) in anhydrous DCM (5 mL) and *N*-methylmorpholine (1.099 mL, 10 mmol, NMM) in anhydrous DCM (5 mL) were added simultaneously in 1 mL aliquots over 50 min. The mixture was stirred for 2.5 h at −15 °C then etheral diazomethane [~15 mmol generated from addition of diazald (4.7 g) in diethyl ether (75 mL) onto sodium hydroxide (5.25 g) in water (7.5 mL)/ethanol (15 mL) at 60 °C] was added to the activated amino acid solution. The mixture was allowed to warm to ambient temperature and stirred for 2.5 h. A few drops of acetic acid were cautiously added to the mixture, followed by DCM (40 mL). The etheral layers were washed with aqueous saturated sodium hydrogen carbonate solution ($NaHCO_3$) (2 × 40 mL), dried (Na_2SO_4) and the solvents removed in vacuo to leave a yellow residue (1.4 g). Flash chromatography of the residue over silica (35 g) eluting with ethyl acetate (EtOAc)–heptane 3:7 gave diazomethylketone **9** (1.024 g, 86%). TLC (single spot, $R_f = 0.45$, EtOAc–heptane 1:1), analytical HPLC $t_R = 11.54$ min; HPLC–MS 497.2 $[2M+Na]^+$; ^{1}H NMR (500 MHz, $CDCl_3$ at

300 K): δ 1.41–1.51 (m, C(CH_3)$_3$, 9H), 4.11–4.35 (m, BocNCH_2, 2H), 4.86–5.05 (m, BocNCH, 1H), 5.25–5.50 (m, CHN$_2$, 1H), 5.70–5.80 (m, olefinic CH, 1H) and 5.88–6.03 (m, olefinic CH, 1H); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 28.3 and 28.4 (C(CH_3)$_3$), 51.8 and 52.3 (CHN_2), 53.65 and 54.0 (BocNCH_2), 71.5 and 72.3 (BocNCH), 80.6 and 80.9 (OC(CH$_3$)$_3$), 126.1 and 126.3 (olefinic CH), 128.35 and 128.5 (olefinic CH), 153.7 and 154.15 (NCO_2), 192.7 and 193.4 (COCHN$_2$).

4.1.2. (*R*)-2-Methoxycarbonylmethyl-2,5-dihydropyrrole-1-carboxylic acid *tert*-butyl ester (10). Compound **9** (912 mg, 3.85 mmol) was dissolved in tetrahydrofuran (14 mL) and methanol (1.6 mL) then cooled to 0 °C. A solution of silver trifluoroacetate (94 mg) in NMM (1.06 mL) was added, and the mixture allowed to warm to ambient temperature over 6 h in the dark. Methanol (40 mL) was added, followed by 10% aqueous citric acid solution (100 mL). The majority of the organic solvents were removed in vacuo then the aqueous phase extracted with EtOAc (3 × 40 mL). The combined organic layers were washed with brine (40 mL), dried (Na_2SO_4) and evaporated in vacuo to give a residue (1.35 g). Flash chromatography of the residue over silica (200 g) eluting with EtOAc–hexane 3:17 gave methyl ester **10** as a colourless oil (670 mg, 72%). TLC (single spot, R_f = 0.25, EtOAc–hexane 1:4), analytical HPLC t_R = 15.03 min; HPLC–MS 505.3 [2M+Na]$^+$; ^{1}H NMR (500 MHz, $CDCl_3$ at 300 K): δ 1.44–1.53 (m, C(CH_3)$_3$, 9H), 2.37–2.55 (m, CH_2CO$_2$Me, 1H), 2.90–4.00 (m, CH_2CO$_2$Me, 1H), 3.63–3.70 (m, OCH_3, 3H), 3.97–4.26 (m, BocNCH_2, 2H), 4.70–4.90 (m, BocNCH, 1H), 5.74–5.89 (m, 2 × olefinic CH, 2H); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 28.2, 28.3 and 28.5 (C(CH_3)$_3$), 39.4 and 38.4 (CH_2CO_2Me), 51.5 and 51.6 (OCH_3), 53.3 and 53.5 (BocNCH_2), 60.7 and 60.9 (BocNCH), 79.6 and 80.0 (OC(CH$_3$)$_3$), 126.0 and 126.1 (olefinic CH), 129.3 and 129.5 (olefinic CH), 153.9 (NCO_2), 171.5 and 171.7 (CO_2Me).

4.1.3. (−)-(*R*)-2-(2-Hydroxyethyl)-2,5-dihydropyrrole-1-carboxylic acid *tert*-butyl ester (11). Methanol (0.27 mL, 6.7 mmol) was added dropwise to a stirred suspension of lithium borohydride (146 mg, 6.6 mmol) in tetrahydrofuran (3.5 mL) under an atmosphere of argon over 4 min, followed by a solution of compound **10** (0.8 g, 3.3 mmol) in tetrahydrofuran (8 mL) over 15 min. The mixture was stirred for 1 h then poured into water (25 mL). The product was extracted into DCM (3 × 20 mL), dried (Na_2SO_4), and the solvents removed in vacuo. The residue was purified by flash chromatography over silica eluting with EtOAc–heptane mixtures 0:100–25:75 to give alcohol **11** as a colourless oil (0.48 g, 67%). TLC (single spot, R_f = 0.35, EtOAc–hexane 1:1), analytical HPLC t_R = 12.16 min; HPLC–MS 236.1 [M+Na]$^+$, 449.3 [2M+Na]$^+$. Exact mass calcd for $C_{11}H_{19}NO_3$ (MNa$^+$): 236.1257, found 236.1264 (δ + 2.95 ppm); $[\alpha]_D^{22}$ −127 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$ at 300 K): δ 1.42–1.55 (br s, C(CH_3)$_3$, 9H and NCHCH_2, 1H), 1.84–1.95 (m, NCHCH_2, 1H), 3.60–3.72 (m, CH_2OH, 2H), 3.93–4.28 (m, BocNCH_2, 2H), 4.53–4.78 (m, BocNCH, 1H), 5.67–5.84 (m, 2 × olefinic CH, 2H); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 28.4 (C(CH_3)$_3$), 37.4 and 38.7 (CH_2CH_2OH), 53.45 and 53.6 (NCH_2), 59.2 and 59.6 (OCH_2), 61.2 and 61.9 (BocNCH), 79.9 and 80.1 (OC(CH$_3$)$_3$), 124.4 and 125.3 (olefinic CH), 130.3 and 131.1 (olefinic CH), 154.4 and 156.0 (NCO_2).

4.1.4. (*R*)-2-(2-Methanesulfonylethyl)-2,5-dihydropyrrole-1-carboxylic acid *tert*-butyl ester (12). Triethylamine (2.35 mL, 16.9 mmol) was added dropwise to a stirred solution of compound **11** (2.33 g, 10.9 mmol) in DCM (20 mL) at 0 °C over 2 min followed by methanesulfonyl chloride (1.27 mL, 16.4 mmol) over 4 min. The mixture was stirred for 1 h at 0 °C then washed with water (170 mL) and brine (170 mL), dried (Na_2SO_4) and the solvents removed in vacuo to leave the mesylate **12** (3.42 g), which was used without further purification. HPLC–MS 236.0 [M+2H−Bu]$^+$, 314.1 [M+Na]$^+$, 605.1 [2M+Na]$^+$.

4.1.5. (*R*)-2-(2-Azidoethyl)-2,5-dihydropyrrole-1-carboxylic acid *tert*-butyl ester (13). Sodium azide (3.55 g, 54.7 mmol) was added to a stirred solution of mesylate **12** in DMF (45 mL) under an atmosphere of argon. The mixture was stirred at 60 °C for 1.5 h then the majority of solvents were removed by distillation in vacuo and the residue partitioned between water (200 mL) and EtOAc (200 mL). The EtOAc layer was washed with brine (150 mL), dried (Na_2SO_4) and the solvents removed in vacuo to leave a residue (2.49 g), which was purified by flash chromatography over silica eluting with EtOAc–heptane mixtures 0:100–10:90 to give azide **13** as a colourless oil (2.05 g, 79%). TLC (single spot, R_f = 0.45, EtOAc–hexane 3:7), analytical HPLC t_R = 15.91 min; HPLC–MS 139.1 [M+2H−Boc]$^+$, 183.1 [M+2H−Bu]$^+$, 499.2 [2M+Na]$^+$. Anal. Calcd for $C_{11}H_{18}N_4O_2$: C, 55.44; H, 7.61; N, 23.51, found C, 55.37; H, 7.59; N, 23.45. Exact mass calcd for $C_{11}H_{18}N_4O_2$ (MNa$^+$): 261.1327, found 261.1320 (−2.97 ppm); ^{1}H NMR (500 MHz, $CDCl_3$ at 300 K): δ 1.40–1.50 (m, C(CH_3)$_3$, 9H), 1.90–2.10 (m, NCHC$H_2$2H), 3.17–3.33 (m, CH_2N$_3$, 2H), 3.96–4.27 (m, BocNCH_2, 2H), 4.53–4.68 (m, BocNCH, 1H), 5.66–5.86 (m, 2 × olefinic CH, 2H); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 28.3 and 28.5 (C(CH_3)$_3$), 32.5 and 33.0 ($CH_2CH_2N_3$), 47.5 and 47.9 (CH_2N_3), 53.6 and 53.8 (BocNCH_2), 62.0 and 62.3 (BocNCH), 79.55 and 79.9 (OC(CH$_3$)$_3$), 125.6 and 126.1 (olefinic CH), 128.9 and 129.4 (olefinic CH), 154.2 and 154.3 (NCO_2).

4.1.6. (−)-(*R*)-2-(2-Benzyloxycarbonylaminoethyl)-2,5-dihydropyrrole-1-carboxylic acid *tert*-butyl ester (15). Water (1.9 mL, 106 mmol) was added to a stirred solution of azide **13** (2.65 g, 11.17 mmol) and triphenylphosphine (4.35 g, 16.6 mmol) in tetrahydrofuran (330 mL) under an atmosphere of argon. The solution was stirred at 45 °C for 7.5 h then at ambient temperature for 14 h. A 5.0 mL aliquot was removed for analysis, then the remainder of the solution was concentrated in vacuo to obtain amine **14** as an oily residue. The residue was dissolved in 1,4-dioxane (35 mL) with stirring, ice-cooled and a solution of sodium carbonate (2.45 g, 23.1 mmol)

in water (35 mL) was added. Benzyl chloroformate (2.18 g, 1.824 mL, 12.8 mmol) in 1,4-dioxane (10 mL) was then added dropwise over 30 min and the mixture stirred for an additional 30 min before adding water (250 mL). The aqueous phase was extracted with DCM (2 × 250 mL) and the combined organic layers were dried (Na_2SO_4), filtered and reduced in vacuo to leave a clear mobile oil (10.2 g). Flash chromatography over silica, eluting with EtOAc–heptane mixtures gave compound **15** (3.58 g, 94%) as a mobile colourless oil. TLC (R_f = 0.30, EtOAc–heptane 1:1), analytical HPLC single main peak, t_R = 17.39 min; HPLC–MS 247.1 $[M+2H-Boc]^+$, 291.1 $[M+2H-Bu]^+$, 347.1 $[M+H]^+$, 369.1 $[M+Na]^+$, 715.2 $[2M+Na]^+$; $[\alpha]_D^{22}$ −74.9 (*c* 0.334, $CHCl_3$). Anal. Calcd for $C_{19}H_{26}N_2O_4$: C, 65.87; H, 7.56; N, 8.09, found C, 65.79; H, 7.53; N, 7.97. Exact mass calcd for $C_{19}H_{26}N_2O_4$ (MNa^+): 369.1790, found 369.1803 (+3.37 ppm); ^{1}H NMR (500 MHz, $CDCl_3$ at 300 K): δ 1.45 (br s, $C(CH_3)_3$, 9H), 1.60–1.95 (m, BocNCHCH_2, 2H), 3.00–3.44 (m, CH_2NH, 2H), 3.90–4.29 (m, BocNCH_2, 2H), 4.45–4.81 (m, BocNC*H*, 1H), 5.01–5.16 (m, OCH_2Ph, 2H), 5.50–5.83 (m, 2 × olefinic C*H*, 2H) and 7.25–7.38 (m, C_6H_5 and N*H*, 6H); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 28.4 ($C(CH_3)_3$), 34.4, 34.6 (CH_2CH_2NH), 37.2, 37.6 (CH_2NH), 53.6, 53.7 (BocNCH_2), 61.7, 62.1 (BocN*C*H), 66.4, 66.6 (OCH_2Ph), 79.6, 79.9 ($OC(CH_3)_3$), 125.2, 125.9, 127.0, 127.6, 127.9, 128.0, 128.4, 129.5, 130.2 (5 × aromatic *C*H and 2 × olefinic *C*H), 154.3, 155.0, 156.2, 156.5 ($NHCO_2$ and NCO_2).

4.1.7. (2*R*)-2-(2-Benzyloxycarbonylaminoethyl)-6-oxa-3-aza-bicyclo[3.1.0]hexane-3-carboxylic acid *tert*-butyl ester (16). Compound **15** (3.57 g, 10.3 mmol) was dissolved in anhydrous DCM (60 mL) with stirring and *meta*-chloroperoxybenzoic acid (27.3 g, 65% reagent, 103 mmol) added. The mixture was stirred at ambient temperature under argon for 16 h. DCM (400 mL) was added and the organic phase washed with 10% aqueous w/v solution of sodium hydroxide (2 × 400 mL), then dried (Na_2SO_4), filtered and reduced in vacuo to leave a clear oil (~5 g). Flash chromatography over silica, eluting with EtOAc–heptane mixtures gave epoxides **16** (3.57 g, 95%) as a mobile colourless oil. TLC (R_f = 0.35 (minor) and 0.40 (major) (mixture of *syn*- and *anti*-epoxides), EtOAc–heptane 2:1), analytical HPLC single main peak, t_R = 17.74 min; HPLC–MS 263.1 $[M+2H-Boc]^+$, 307.1 $[M+2H-Bu]^+$, 363.1 $[M+H]^+$, 385.1 $[M+Na]^+$, 747.2 $[2M+Na]^+$. Anal. Calcd for $C_{19}H_{26}N_2O_5$: C, 62.97; H, 7.23; N, 7.73, found C, 62.93; H, 7.22; N, 7.61. Exact mass calcd for $C_{19}H_{26}N_2O_5$ (MNa^+): 385.1739, found 385.1725 (−3.82 ppm); ^{1}H NMR (500 MHz, $CDCl_3$ at 300 K): δ 1.32–1.62 (m, $C(CH_3)_3$ and CH_2CH_2NH, 10H), 1.67–2.00 (m, CH_2CH_2NH, 1H), 2.90–4.21 (m, CH_2NH, BocNC*H*C*H*, BocNCH_2C*H*, 7H), 4.70–5.17 (m, OCH_2Ph, 2H), 5.78–6.05 (m, N*H*, 1H) and 7.27–7.37 (aromatics, 5H); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 28.1, 28.3, 28.35 and 28.4 ($C(CH_3)_3$), 30.8 and 31.2 (CH_2CH_2NH), 37.4 and 37.7 (CH_2NH), 46.15 and 46.6 (BocNCH_2), 53.9, 54.2, 54.9 and 55.8 (2 × epoxide *C*H), 58.1 and 58.2 (BocN*C*H), 66.5 and 66.7 (OCH_2Ph), 80.3 and 80.7 ($OC(CH_3)_3$), 128.0, 128.1, 128.2, 128.4, 128.5 (5 × aromatic *C*H), 136.7 (OCH_2C), 155.1, 155.9, 156.3 and 156.6 ($NHCO_2$ and NCO_2).

4.1.8. 2*R*-(2-Aminoethyl)-6-oxa-3-azabicyclo[3.1.0]hexane-3-carboxylic acid *tert*-butyl ester (17) and (3*S*,3a*S*,6a*R*)-3-hydroxyhexahydropyrrolo[3,2-*b*]pyrrole-1-carboxylic acid *tert*-butyl ester (19). Epoxide mixture **16** (3.57 g, 9.86 mmol) was dissolved in ethanol (60 mL), cooled to 0 °C and 10% Pd–C (0.40 g) added. The mixture was stirred, then evacuated and flushed with hydrogen. The mixture was allowed to warm to ambient temperature and after 2.5 h filtered through Celite. The filter cake was washed with ethanol (3 × 60 mL) and the combined organic filtrates reduced in vacuo to provide crude amines **17** and **19** (~2.4 g). HPLC–MS 173.1 $[M+2H-Bu]^+$, 229.1 $[M+H]^+$.

4.1.9. (−)-(3*S*,3a*S*,6a*R*)-3-Hydroxyhexahydropyrrolo-[3,2-*b*]pyrrole-1,4-dicarboxylic acid 4-benzyl ester 1-*tert*-butyl ester (6). Crude amines **17** and **19** (~2.4 g) were dissolved in 1,4-dioxane (30 mL) with stirring, ice-cooled and a solution of sodium carbonate (2.19 g, 20.7 mmol) in water (25 mL) was added. Benzyl chloroformate (1.63 mL, 11.4 mmol) in 1,4-dioxane (15 mL) was then added dropwise over 30 min and the mixture stirred for a further 30 min. The mixture was then reduced in vacuo by approximately 2/3 volume to leave a mobile pulp. Water (200 mL) was added and the aqueous phase extracted with DCM (2 × 100 mL). The combined organic layers were dried (Na_2SO_4), filtered and reduced in vacuo to leave a clear mobile oil (3.96 g). Flash chromatography over silica, eluting with EtOAc–heptane mixtures gave bicycle **6** (2.16 g, 61%) as an opaque gum. TLC (R_f = 0.15, EtOAc–heptane 1:1), analytical HPLC single main peak, t_R = 17.15 min; HPLC–MS 263.1 $[M+2H-Boc]^+$, 307.1 $[M+2H-Bu]^+$, 363.1 $[M+H]^+$, 385.1 $[M+Na]^+$, 747.2 $[2M+Na]^+$; $[\alpha]_D^{22}$ −109.2 (*c* 0.544, $CHCl_3$). Anal. Calcd for $C_{19}H_{26}N_2O_5$: C, 62.97; H, 7.23; N, 7.73, found C, 62.82; H, 7.39; N, 7.57. Exact mass calcd for $C_{19}H_{26}N_2O_5$ (MNa^+): 385.1739, found 385.1725 (−2.15 ppm); ^{1}H NMR (400 MHz, CD_3CN at 348 K): δ 1.46 (s, $C(CH_3)_3$, 9H), 1.90–2.00 (m obscured by NMR solvent peaks, BocNCHCH_2, 1H), 2.14 (dd, J = 6.15, 13.15 Hz, BocNCHCH_2, 1H), 3.07–3.20 (m, O*H* + CbzNCH_2, 2H), 3.24 (dd, J = 3.80, 12.20 Hz, BocNCH_2, 1H), 3.51 (d, J = 12.20 Hz, BocNCH_2, 1H), 3.68 (ddd, J = 1.70, 8.80, 10.60 Hz, CbzNCH_2, 1H), 4.10 (br d, J = 5.80 Hz, CbzNC*H*, 1H), 4.27 (br s, C*H* OH, 1H), 4.40–4.46 (m, BocNC*H*, 1H), 5.12 (d, J = 12.7 Hz, OCH_2Ph, 1H), 5.16 (d, J = 12.70 Hz, OCH_2Ph, 1H) and 7.42–7.29 (aromatics, 5H); ^{13}C NMR (100 MHz, $CDCl_3$ at 300 K): δ 28.5 ($C(CH_3)_3$), 29.7, 30.4, 31.2, 31.9, 32.0 (BocNCHC_2), 45.5, 45.7 (CbzNC_2), 53.1, 53.4, 53.5 (BocNC_2), 60.1, 61.2 (BocN*C*H), 67.2, 67.6, 68.2, 68.4, 69.0 (OCH_2-Ph + Cbz-N*C*H), 72.7, 73.3, 73.4 (*C*HOH), 79.9, 80.1 ($OC(CH_3)_3$), 127.9, 128.0, 128.2, 128.3, 128.5, 128.6, 136.3, 136.4 (aromatics), 154.1, 154.2, 155.2 (2 × NCO_2).

4.1.10. (−)-(3*S*,3a*S*,6a*R*)-3-Hydroxyhexahydropyrrolo[3,2-*b*]pyrrole-1,4-dicarboxylic acid 1-*tert*-butyl ester 4-(9*H*-fluoren-9-ylmethyl) ester (20). Bicycle **6** (0.54 g, 1.5 mmol) was dissolved in ethanol (10 mL), cooled to 0 °C and 10% Pd–C (0.055 g) added. The mixture was stirred, then evacuated and flushed with hydrogen. The mixture was warmed to ambient temperature and after 2.5 h filtered through Celite. The filter cake was washed with ethanol (3 × 10 mL) and the combined filtrates reduced in vacuo to provide the crude amine (~0.36 g). HPLC–MS 173.1 $[M+2H-Bu]^+$, 229.1 $[M+H]^+$. The crude amine was dissolved in 1,4-dioxane (15 mL) with stirring, ice-cooled and a solution of sodium carbonate (0.33 g, 3.15 mmol) in water (15 mL) was added. 9-Fluorenylmethyl chloroformate (0.463 g, 1.79 mmol) in 1,4-dioxane (10 mL) was added dropwise over 30 min and the mixture stirred for a further 30 min. Water (200 mL) was then added and the aqueous phase extracted with EtOAc (2 × 100 mL). The combined organic layers were dried (Na_2SO_4), filtered and reduced in vacuo to leave a clear mobile oil (1.02 g). Flash chromatography over silica, eluting with EtOAc–heptane mixtures gave bicycle **20** (0.64 g, 95%) as a fine white crystalline solid. TLC (R_f = 0.35, EtOAc–heptane 2:1), analytical HPLC single main peak, t_R = 19.98 min; HPLC–MS 395.1 $[M+2H-Bu]^+$, 451.1 $[M+H]^+$, 473.1 $[M+Na]^+$, 923.2 $[2M+Na]^+$; $[\alpha]_D^{22}$ −104.0 (*c* 0.2, $CHCl_3$). Anal. Calcd for $C_{26}H_{30}N_2O_5$: C, 69.31; H, 6.71; N, 6.22, found C, 69.11; H, 7.06; N, 5.84. Exact mass calcd for $C_{26}H_{30}N_2O_5$ (MNa^+): 473.2052, found 473.2053 (+0.06 ppm); ^1H NMR (400 MHz, CD_3CN at 348 K): δ 1.46 (s, $C(CH_3)_3$, 9H), 1.75–1.90 (m, BocNCHCH_2, 1H), 2.05–2.13 (m, BocNCHCH_2, 1H), 3.02 (m, FmocNCH_2, 1H), 3.08–3.20 (m, BocNCH_2, 1H), 3.46 (m, BocNCH_2, 1H), 3.46–3.60 (m, FmocNCH_2, 1H), 3.90–4.15 (m, FmocNC*H* and C*H*OH, 2H), 4.28 (t, *J* = 6.10 Hz, FmocC*H*, 1H), 4.34–4.40 (m, BocNC*H*, 1H), 4.49 (d, *J* = 6.10 Hz, FmocCH_2, 2H), 7.31–7.45 (m, Fmoc aromatics, 4H), 7.65 (d, *J* = 7.35 Hz, Fmoc aromatics, 2H), 7.83 (d, *J* = 7.50 Hz, Fmoc aromatics, 2H); ^{13}C NMR (100 MHz, $CDCl_3$ at 300 K): δ 28.45 ($C(CH_3)_3$), 30.2, 31.2, 32.0 (BocNCHC_2), 44.8, 45.3, 45.6 (FmocNC_2), 47.3, 47.4 (Fmoc*C*H), 52.8, 53.1, 53.4, 53.5 (BocNCH_2), 60.1, 60.8 (BocN*C*H), 65.9, 66.2, 67.3 (Fmoc*C*H_2), 67.85, 68.4, 68.9 (FmocN*C*H), 72.5, 72.9, 73.3, 73.6 (*C*HOH), 79.95 ($OC(CH_3)_3$), 119.8, 120.0, 124.6, 124.9, 125.0, 127.0, 127.4, 127.8 (Fmoc *C*H aromatics), 141.3, 141.5, 143.7, 143.8, 144.1 (Fmoc quaternary aromatics), 154.0, 154.3, 155.0, 155.2 (2 × N*C*O_2).

4.1.11. Alternative preparation of (3*S*,3a*S*,6a*R*)-3-hydroxyhexahydropyrrolo[3,2-*b*]pyrrole-1,4-dicarboxylic acid 1-*tert*-butyl ester 4-(9*H*-fluoren-9-ylmethyl) ester (20). *meta*-Chloroperoxybenzoic acid (864 mg, 57–86%) was added to a solution of azide **13** (175 mg, 0.735 mmol) in anhydrous DCM (4 mL). The mixture was stirred at ambient temperature for 7 h then saturated aqueous $NaHCO_3$ (40 mL) and DCM (60 mL) were added. The phases were mixed and separated and the organic phase washed with 10% aqueous sodium hydroxide solution (40 mL), dried (Na_2SO_4) and evaporated in vacuo to afford crude azido epoxide **21** (185 mg). Crude **21** was dissolved in ethanol (6.8 mL) and cooled to 0 °C. 10% Pd–C (84 mg) was added to the mixture and the atmosphere purged with hydrogen gas. The mixture was stirred overnight under a hydrogen atmosphere at ambient temperature, filtered over Celite and the filter cake washed with excess EtOAc. The filtrate was concentrated in vacuo, and the residue suspended in a solution of sodium carbonate (193 mg, 1.82 mmol) in water (4 mL). 1,4-Dioxane (2 mL) was added and the mixture cooled to 0 °C, then a solution of 9-fluorenylmethyl chloroformate (198 mg, 0.77 mmol) in 1,4-dioxane (2 mL) added in small portions over 40 min. The mixture was then allowed to warm to ambient temperature over 40 min. Water (40 mL) was added and the product extracted into DCM (3 × 40 mL). The combined organic layers were dried (Na_2SO_4) and evaporated in vacuo to afford a residue (335 mg). Flash chromatography of the residue over silica gel (35 g) eluting with EtOAc–heptane 1:4 followed by 1:1 gave bicycle **20** (90 mg, 27%). TLC (single spot, R_f = 0.25, EtOAc–heptane 1:1), analytical HPLC t_R = 18.35 min; HPLC–MS 451.2 $[M+H]^+$, 473.2 $[M+Na]^+$, 923.4 $[2M+Na]^+$.

4.1.12. (−)-(3a*S*,6a*R*)-3-Oxo-hexahydropyrrolo[3,2-*b*]pyrrole-1,4-dicarboxylic acid 1-*tert*-butyl ester 4-(9*H*-fluoren-9-ylmethyl) ester (5). Bicyclic alcohol **20** (0.495 g, 1.10 mmol) was dissolved in anhydrous DCM (18 mL) with stirring under argon. Dess–Martin periodinane (0.962 g, 2.27 mmol) was added and the mixture stirred for 4 h. The mixture was concentrated in vacuo and the residue purified by flash chromatography over silica, eluting with EtOAc–heptane mixtures to give bicyclic ketone **5** (0.480 g, 97%) as a white crystalline solid. TLC (R_f = 0.40, EtOAc–heptane 1:1), analytical HPLC single broad main peak, t_R = 20.27–21.79 min; HPLC–MS 393.1 $[M+2H-Bu]^+$, 449.1 $[M+H]^+$, 471.1 $[M+Na]^+$, 919.2 $[2M+Na]^+$; $[\alpha]_D^{22}$ −140.0 (*c* 0.6, $CHCl_3$). Microanalysis indicated the presence of residual solvent following drying in vacuo and re-drying at elevated temperature lead to partial thermal decomposition. Exact mass calcd for $C_{26}H_{28}N_2O_5$ (MNa^+): 471.1896, found 471.1903 (+1.44 ppm); ^1H NMR (500 MHz, CD_3CN at 348 K): δ 1.51 (s, $C(CH_3)_3$, 9H), 1.90–2.00 (m obscured by CD_3CN, BocNCHCH_2, 1H), 2.16–2.21 (m, BocNCHCH_2, 1H), 3.25–3.32 (dt, *J* = 7.20, 7.30 Hz, FmocNCH_2, 1H), 3.52–3.58 (br, FmocNCH_2, 1H), 3.72 (d, *J* = 19.00 Hz, BocNCH_2, 1H), 3.92 (d, *J* = 19.10 Hz, BocNCH_2, 1H), 4.28 (t, *J* = 6.60 Hz, FmocC*H*, 1H), 4.42 (m, FmocCH_2, 2H), 4.52 (d, *J* = 8.35 Hz, FmocNC*H*, 1H), 4.69 (m, BocNC*H*, 1H), 7.35 (t, *J* = 7.35 Hz, Fmoc H-*2* and H-*7*), 7.43 (t, *J* = 7.45 Hz, Fmoc H-*3* and H-*6*), 7.68 (d, *J* = 7.45 Hz, Fmoc H-*1* and H-*8*), 7.83 (d, *J* = 7.55 Hz, Fmoc H-*4* and H-*5*); ^{13}C NMR (125 MHz $CDCl_3$ at 300 K): δ 28.36 ($C(CH_3)_3$), 30.50, 30.93, 31.20 (BocNCHC_2), 45.68 (FmocNC_2), 47.20 (Fmoc*C*H), 51.71, 52.22 (BocNC_2), 57.58, 58.64 (BocN*C*H), 63.03, 63.57 (FmocN*C*H), 67.70, 68.06 (Fmoc*C*H_2), 81.10 ($OC(CH_3)_3$), 119.94, 124.99, 125.15, 125.29, 127.05, 127.55, 127.71, 127.85 (Fmoc *C*H aromatics), 143.69, 143.92, 144.23 (Fmoc quaternary aromatics), 153.99, 154.74, 155.04 (2 × N*C*O_2), 206.33, 206.59 (*C*=O).

4.1.13. (3a*R*,6*S*,6a*S*)-4-Benzoyl-6-hydroxyhexahydropyrrolo[3,2-*b*]pyrrole-1-carboxylic acid benzyl ester (24). A solution of HCl in 1,4-dioxane (4.0 M, 11 mL, 44 mmol) was added to compound **6** (450 mg, 1.24 mmol). The solution was stirred for 65 min whereupon a white suspension formed. The solvents were removed in vacuo and the residue azeotroped with diethyl ether (3 × 15 mL) and then DMF (10 mL) and benzoic anhydride (295 mg, 1.31 mmol) added. The solution was placed under an atmosphere of argon then NMM (0.29 mL, 2.6 mmol) was added to the solution dropwise whilst stirring over 0.5 min. The mixture was stirred for 1.75 h then the solvents were removed in vacuo. The residue was dissolved in EtOAc (100 mL) then washed with saturated aqueous $NaHCO_3$ (100 mL), pH 3 HCl (100 mL) then brine (100 mL). The organic layer was dried (Na_2SO_4) and evaporated in vacuo to afford compound **24** as a pale yellow gum (465 mg), which was used without further purification. Analytical HPLC t_R = 14.97 min; HPLC–MS 367.1 $[M+H]^+$, 733.1 $[2M+H]^+$. Microanalysis indicated the presence of residual solvent following drying in vacuo and re-drying at elevated temperature lead to partial thermal decomposition. Exact mass calcd for $C_{21}H_{22}N_2O_4$ (MNa^+): 389.1477, found 389.1476 (−0.40 ppm); ^{1}H NMR (500 MHz, $CDCl_3$ at 300 K): δ approximately 3:1 mixture of rotamers, 2.10–2.21 (m, BzNCHCH_2, 1H), 2.24–2.36 (m, BzNCHCH_2, 1H), 3.20–3.35 (m, CbzNCH_2, 1H), 3.35–3.66 (m, BzNCH_2, 2H), 3.74–3.80 (m, CbzNCH_2, 1H), 4.16–4.20 (m, CbzNC*H*, 1H), 4.38–4.42 (br s, C*H*OH, 1H), 4.94–5.04 (m, BzNC*H*, 1H), 5.08–5.22 (m, OCH_2Ph, 2H), 7.30–7.52 (aromatic C*H*, 10H); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 31.02, 30.40 (BzNCH*C*H_2), 45.73, 45.86 (CbzN*C*H_2), 56.43, 56.74 (BzN*C*H_2), 60.58, 61.49 (BzN*C*H), 66.68, 67.33 (O*C*H_2Ph), 66.92, 67.76 (CbzN*C*H), 73.01, 73.86 (*C*HOH), 126.77, 127.48, 127.99, 128.23, 128.28, 128.45, 128.56, 128.87, 130.02, 130.38 and 134.53 (*C*H aromatics), 136.16, 136.23, 136.33 (aromatic quaternary), 154.26, 154.97 (Cbz*C*=O), 170.06, 171.19 (Bz*C*=O).

4.1.14. (3a*R*,6a*S*)-4-Benzoyl-6-oxo-hexahydropyrrolo-[3,2-*b*]pyrrole-1-carboxylic acid benzyl ester (25). Crude alcohol **24** (0.78 g, 2.13 mmol) was dissolved in DCM (20 mL) with stirring under argon. Dess–Martin periodinane (1.804 g, 4.26 mmol) was added and the mixture stirred for 16 h. The mixture was concentrated in vacuo and the residue purified by flash chromatography over silica, eluting with EtOAc–heptane mixtures to give bicyclic ketone **25** (0.61 g, 78%) as an off-white gum. TLC (R_f = 0.25, EtOAc–heptane 3:1), analytical HPLC single main peak, t_R = 14.65–16.30 min; HPLC–MS 365.1 $[M+H]^+$, 383.1 $[M+H+H_2O]^+$. Microanalysis indicated the presence of residual solvent following drying in vacuo and re-drying at elevated temperature lead to partial thermal decomposition. Exact mass calcd for $C_{21}H_{20}N_2O_4$ (MNa^+): 387.1321, found 387.1324 (+0.76 ppm); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 30.41, 30.89, 31.23 (BzNCH*C*H_2), 45.75 (CbzN*C*H_2), 54.55, 63.04 (BzN*C*H + CbzN*C*H), 57.91, 58.45, 58.99, 59.73 (BzN*C*H_2), 67.60, 68.07 (O*C*H_2Ph), 127.00, 127.38, 127.48, 127.98, 128.11, 128.48, 128.62, 128.74, 130.48, 130.83 (*C*H aromatics), 135.07, 136.14 (quaternary aromatics), 154.54, 155.03 (Cbz*C*O_2), 170.58 (Bz*C*O), 204–207 (br, *C*=O).

4.1.15. (3a*R*,6a*S*)-4-Benzoyl-6,6-dimethoxyhexahydropyrrolo[3,2-*b*]pyrrole-1-carboxylic acid benzyl ester (26). Ketone **25** (0.60 g, 1.65 mmol) was dissolved in methanol (10 mL) with stirring. Trimethylorthoformate (1.8 mL, 16.5 mmol) was added followed by *para*-toluenesulfonic acid (40 mg) and the mixture heated under argon at 65 °C for 16 h. The mixture was reduced in vacuo to leave a dark oil (0.8 g). Flash chromatography over silica, eluting with EtOAc–heptane mixtures gave ketal **26** (0.52 g, 77%) as a fine white crystalline solid. TLC (R_f = 0.40, EtOAc–heptane 3:1), analytical HPLC single main peak, t_R = 18.22 min; HPLC–MS 411.1 $[M+H]^+$, 433.1 $[M+Na]^+$, 843.1 $[2M+Na]^+$. Exact mass calcd for $C_{23}H_{26}N_2O_5$ (MNa^+): 433.1739, found 433.1727 (−2.94 ppm); ^{1}H NMR (500 MHz, $CDCl_3$ at 300 K): δ 1.96–2.07, 2.15–2.22 (m, BzNCHCH_2, 2H), 3.04–3.42 (m, 2 × OCH_3, 6H), 3.25 (m, CbzNCH_2, 1H), 3.4 (m, BzNCH_2, 1H), 3.58–3.67 (m, BzNCH_2, 1H), 3.96–4.07 (m, CbzNCH_2, 1H), 4.35–4.58 (m, CbzNC*H*, 1H), 4.98–5.26 (BzNC*H* + OCH_2Ph, 3H), 7.28–7.49 (aromatics, 10H); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 32.27, 32.59 (BzNCH*C*H_2), 46.74 (CbzN*C*H_2), 49.36, 51.10, 51.59 (2 × O*C*H_3), 54.59, 56.08 (BzN*C*H_2), 60.77, 61.08 (BzN*C*H), 62.47 (CbzN*C*H), 67.28 (O*C*H_2Ph), 106.76, 107.02 (*C*$(OCH_3)_2$), 126.84, 127.35, 127.90, 128.06, 128.39, 130.05, 130.38 (*C*H aromatics), 135.91, 136.48 (quaternary aromatics), 155.44 (Cbz*C*O_2), 169.54 (Bz*C*O).

4.1.16. (3a*S*,6a*R*)-(3,3-Dimethoxyhexahydropyrrolo[3,2-*b*]pyrrol-1-yl)phenylmethanone (27). Methanol (15 mL) was cautiously added dropwise to a stirred mixture of ketal **26** (0.48 g, 1.17 mmol) and 10% Pd–C (100 mg) at 0 °C under an atmosphere of argon over 10 min. The argon was then replaced by an atmosphere of hydrogen and stirring continued at ambient temperature for 85 min before replacing the hydrogen with argon and adding ethanol (30 mL). The mixture was filtered under reduced pressure through Celite and the filter cake washed with methanol (25 mL) then ethanol (70 mL). Solvents were removed from the filtrate in vacuo to obtain amine **27** as a colourless oil (340 mg), which was used without further purification. HPLC–MS 277.1 $[M+H]^+$, 553.2 $[2M+H]^+$.

4.1.17. (−)-(3a*R*,6a*S*)-[(1*S*)-1-(4-Benzoyl-6,6-dimethoxyhexahydropyrrolo[3,2-*b*]pyrrole-1-carbonyl)-3-methylbutyl]carbamic acid benzyl ester (28). (i) Preparation of Cbz-L-leucine fluoride. Cbz-L-leucine (1.115 g, 4.2 mmol) was dissolved in DCM (50 mL) with stirring under argon. (Diethylamino)sulfur trifluoride (DAST, 792 μL, 6.0 mmol) was added and the mixture stirred for 40 min. Ice-cooled water (200 mL) was added to the mixture and the organic layer separated, dried (Na_2SO_4) and reduced in vacuo to a mobile tan oil (1.14 g). An analytical sample, pre-treated with 10% pyridine in methanol for 15 min gave HPLC–MS 266.1 $[M+H]^+$ (acid, <5%), 280.1 $[M+H]^+$, 302.1 $[M+Na]^+$, 581.1 $[2M+Na]^+$ (methyl ester, ~95%).

(ii) Crude amine **27** (~1.17 mmol) was dissolved in anhydrous DMF (5 mL) with stirring. Cbz-L-leucine fluoride (0.33 g, 1.23 mmol) was added and the mixture stirred under argon for 1 h. The mixture was reduced in vacuo to a semi-mobile dark oil (~1.0 g). Flash chromatography over silica, eluting with EtOAc–heptane mixtures gave compound **28** (0.55 g, 90%) as an off-white crystalline solid. TLC (R_f = 0.35, EtOAc–heptane 3:1), analytical HPLC single main peak, t_R = 19.40 min; HPLC–MS 524.1 $[M+H]^+$, 546.1 $[M+Na]^+$, 1069.2 $[2M+Na]^+$; $[\alpha]_D^{22}$ −114.5 (*c* 0.083, $CHCl_3$). Anal. Calcd for $C_{29}H_{37}N_3O_6$: C, 66.52; H, 7.12; N, 8.02, found C, 66.26; H, 7.30; N, 7.86. Exact mass calcd for $C_{29}H_{37}N_3O_6$ (MNa^+): 546.2580, found 546.2584 (+0.67 ppm); ^1H NMR (500 MHz, $CDCl_3$ at 300 K): δ 0.92–1.04 (m, 2 × Leu δC*H*$_3$, 6H), 1.45–1.55 (m, Leu βC*H*$_2$, 2H), 1.73–1.84 (m, Leu γC*H*, 1H), 1.92–1.99, 2.10–2.16, 2.22–2.30 (m, 4:6:10, BzNCHC*H*$_2$, 2H), 2.94, 3.19, 3.23 and 3.40 (s, C(OC*H*$_3$)$_2$, 6H), 3.14–3.38 (m, 1 × BzNC*H*$_2$ and 1 × CbzLeuNC*H*$_2$, 2H), 3.60–3.68 (d, 4:6, *J* = 11.55, 10.90 Hz, 1 × BzNC*H*$_2$, 1H), 4.03–4.10, 4.11–4.18 (m, 4:6, 1 × CbzLeuNC*H*$_2$, 1H), 4.33 (d, *J* = 6.30 Hz, CbzLeuNC*H*, 0.4H), 4.5–4.65 (m, Leu αC*H*, 0.6H), 4.82 (d, *J* = 6.45 Hz, CbzLeuNC*H*, 0.6H), 4.87–4.93 (m, Leu αC*H*, 0.4H), 5.0–5.14 (m, BzNC*H* + OC*H*$_2$Ph, 3H), 5.42 (d, *J* = 8.35 Hz, LeuN*H*, 0.6H), 5.57 (d, *J* = 8.95 Hz, LeuN*H*, 0.4H), 7.3–7.5 (aromatics, 10H); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 21.99, 22.20, 22.67, 23.06, 23.67 (2 × Leu δ*C*H$_3$), 24.37, 24.57 (Leu γ*C*H), 31.58, 31.86, 33.26 (BzNCH*C*H$_2$), 42.78 (Leu β*C*H$_2$), 44.12, 45.79, 47.05 (CbzLeuN*C*H$_2$), 49.31, 49.99 (1 × O*C*H$_3$), 51.18, 51.27, 51.30, 51.47 (1 × O*C*H$_3$ + Leu α*C*H), 55.55, 57.03 (BzN*C*H$_2$), 59.69, 61.32 (BzN*C*H), 60.30, 61.04 (CbzLeuN*C*H), 66.39, 66.88 (O*C*H$_2$Ph), 106.27, 107.11 (*C*(OCH$_3$)$_2$), 126.84, 127.32, 127.42, 127.87, 127.94, 128.09, 128.43, 128.48, 130.18, 130.43, 130.50 (*C*H aromatics), 135.68, 135.81, 136.28, 136.73 (quaternary aromatics), 155.58, 156.21 (Cbz*C*O$_2$), 169.56, 169.62 (Bz*C*O), 172.35, 173.36 (Leu *C*=O).

4.1.18. (2*S*,3a*R*,6a*S*)-2-Amino-1-(4-benzoyl-6,6-dimethoxyhexahydropyrrolo[3,2-*b*]pyrrol-1-yl)-4-methylpentan-1-one (29). Methanol (15 mL) was cautiously added dropwise to 10% Pd–C (75 mg) at 0 °C under an atmosphere of argon over 10 min whilst stirring followed by a solution of compound **28** (0.52 g, 0.99 mmol) in methanol (15 mL). The argon was then replaced by an atmosphere of hydrogen and stirring continued at ambient temperature for 5.5 h. A suspension of 10% Pd–C (15 mg) in methanol (1 mL) was added and stirring continued for 2.25 h. The hydrogen was replaced by argon then ethanol (100 mL) was added before filtering the mixture through Celite. The filter cake was washed with ethanol (100 mL) then the filtrate was concentrated in vacuo to give amine **29** as a white solid, TLC (single spot, R_f = 0.05, EtOAc–heptane 9:1), HPLC–MS 390.2 $[M+H]^+$, 801.2 $[2M+Na]^+$. The amine contained approximately 5% of starting material **28** and was used without further purification.

4.1.19. (−)-(3a*R*,6a*S*)-*N*-[(1*S*)-1-(4-Benzoyl-6,6-dimethoxyhexahydropyrrolo[3,2-*b*]pyrrole-1-carbonyl)-3-methylbutyl]-4-*tert*-butylbenzamide (30). NMM (0.109 mL, 0.994 mmol) was added to a solution of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (189 mg, 0.497 mmol), 1-hydroxybenzotriazole monohydrate (76 mg, 0.497 mmol) and 4-(*tert*-butyl)benzoic acid (88 mg, 0.497 mmol) in DMF (12.5 mL). The solution was stood for 5 min then added to amine **29** (0.497 mmol). The mixture was stirred at ambient temperature for 1 h 50 min then the solvents were removed in vacuo. The residue was dissolved in DCM (60 mL) then washed with pH 3 HCl (40 mL), saturated aqueous $NaHCO_3$ (40 mL) and brine (40 mL), then dried (Na_2SO_4) and the solvents removed in vacuo. The residue was purified by flash chromatography over silica eluting with EtOAc–heptane mixtures 0:100–50:50 to give compound **30** as a white solid (230 mg), which contained approximately 5% of compound **28**. The latter compound was removed by dissolving the mixture (220 mg) in methanol (11 mL) then adding to a stirred suspension of 10% Pd–C (70 mg) in ethanol (11 mL) under an atmosphere of argon at 0 °C. The argon was then replaced by hydrogen and the mixture stirred at ambient temperature for 80 min, then water (11 mL) was added and the mixture filtered through Celite. The filter cake was washed with ethanol (200 mL) then the filtrate concentrated in vacuo to give an oily solid **28** (203 mg, 78%). TLC (single spot, R_f = 0.65, EtOAc–heptane 9:1), analytical HPLC t_R = 21.41 min; HPLC–MS 550.2 $[M+H]^+$; $[\alpha]_D^{22}$ −80.3 (*c* 0.615, $CHCl_3$). Anal. Calcd for $C_{32}H_{43}N_3O_5$: C, 69.92; H, 7.88; N, 7.64, found C, 69.52; H, 8.12; N, 7.40. Exact mass calcd for $C_{32}H_{43}N_3O_5$ (MH^+): 550.3281, found 550.3284 (+0.55 ppm).

4.1.20. (−)-(3a*R*,6a*S*)-*N*-[(1*S*)-1-(4-Benzoyl-6-oxo-hexahydropyrrolo[3,2-*b*]pyrrole-1-carbonyl)-3-methylbutyl]-4-*tert*-butylbenzamide (22). Ketal **30** (0.19 g, 0.345 mmol) was dissolved in ice-cooled TFA/water (95:5 v/v, 10 mL) with stirring. The ice bath was removed and the mixture stirred at ambient temperature for 3.5 h. The mixture was then reduced in vacuo and evaporated from diethyl ether (2 × 10 mL) to give a semi-mobile tan gum (0.3 g). Flash chromatography over silica, eluting with EtOAc–heptane mixtures gave ketone **22** (0.042 g, 24%) as a white solid. TLC (R_f = 0.30, EtOAc–heptane 9:1), analytical HPLC single broad main peak, t_R = 19.43–21.37 min; HPLC–MS 504.1 $[M+H]^+$. Anal. Calcd for $C_{30}H_{37}N_3O_4 \cdot 0.5TFA$: C, 66.45; H, 6.75; N, 7.50, found C, 66.04; H, 7.19; N, 7.24. Exact mass calcd for $C_{30}H_{37}N_3O_4$ (MNa^+): 526.2682, found 526.2677 (−0.96 ppm).

4.1.21. (3*S*,3a*S*,6a*R*)-(3-Hydroxyhexahydropyrrolo[3,2-*b*]pyrrol-1-yl)phenylmethanone (31). Ethanol (5 mL) was added cautiously dropwise to 10% Pd–C (50 mg) at 0 °C under an atmosphere of argon over 10 min whilst stirring followed by a solution of compound **24** (465 mg, 1.27 mmol) in ethanol (10 mL). The argon was then replaced by an atmosphere of hydrogen and stirring continued at ambient temperature for 4.5 h. The hydrogen was then replaced by argon and 10% Pd–C (20 mg) was added at 0 °C. The argon was then replaced with hydrogen and stirring was continued for 4 h. The hydrogen was replaced by argon then the mixture was

filtered through Celite. The filter cake was washed with ethanol (75 mL) then the filtrate concentrated in vacuo to obtain amine **31** as a colourless oil (309 mg), which was used without further purification. HPLC–MS 233.1 $[M+H]^+$, 465.1 $[2M+H]^+$.

4.1.22. (−)-(3a*R*,6a*S*)-[(1*S*)-1-((6*S*)-4-Benzoyl-6-hydroxyhexahydropyrrolo[3,2-*b*]pyrrole-1-carbonyl)-3-methylbutyl]carbamic acid benzyl ester (32). Cbz-leucine-F (350 mg, 1.31 mmol) was dissolved in DMF (5 mL) then added to amine **31** (304 mg, 1.24 mmol) under an atmosphere of argon. The solution was stirred for 75 min then the solvents removed in vacuo. The residue was purified by flash chromatography over silica eluting with EtOAc–heptane 0:100–80:20 to give compound (**32**) as a white solid (402 mg, 68%). TLC (single spot, $R_f = 0.10$, EtOAc–heptane 65:35), analytical HPLC $t_R = 16.80$ min; HPLC–MS 480.2 $[M+H]^+$, 981.3 $[2M+Na]^+$; $[\alpha]_D^{22}$ −131.1 (*c* 0.479, $CHCl_3$). Exact mass calcd for $C_{27}H_{33}N_3O_5$ (MNa^+): 502.2318, found 502.2311 (−1.44 ppm); ^1H NMR (500 MHz, $CDCl_3$ at 300 K): δ mixture of rotamers, tentative assignment of proton 1.2–2.4 (m, 2 × Leu δCH_3, Leu βCH_2, Leu γC*H*, BzNCHCH_2, 11H), 3.3–4.0 (m, BzNCH_2, CbzLeuNCH_2, 4H), 4.2–5.0 (BzNC*H*, CbzLeuNC*H*, C*H*OH, Leu αC*H*, 4H), 5.0–5.1 (OCH_2Ph, 2H), 5.47 (d, $J = 8.30$ Hz, N*H*, 1H), 7.4–7.6 (aromatic, 10H); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 21.73, 21.89 and 23.22, 23.36 (2 × Leu $\delta C H_3$), 24.59, 24.67 (Leu γ*C*H), 31.86 (BzNCH*C*H_2), 42.02, 42.22 (Leu β*C*H_2), 46.52 (CbzLeuN*C*H_2), 50.94, 51.02 (Leu α*C*H), 56.58 (BzN*C*H_2), 59.72 (BzN*C*H), 67.00 (O*C*H_2Ph), 67.98 (CbzLeuN*C*H), 75.25 (*C*HOH), 127.34, 128.02, 128.18, 128.28, 128.36, 128.52, 130.34 (aromatic *C*H), 136.09, 136.18 (aromatic quaternary), 156.18 (NH*C*=O), 170.08 (Ph*C*=O), 172.32 (CH_2N*C*=O).

4.1.23. (2*S*,3a*R*,6a*S*)-2-Amino-1-((6*S*)-4-benzoyl-6-hydroxyhexahydropyrrolo[3,2-*b*]pyrrol-1-yl)-4-methylpentan-1-one (33). Ethanol (15 mL) was added cautiously to a stirred mixture of compound **32** (370 mg, 0.77 mmol) and 10% Pd–C (50 mg) at 0 °C under an atmosphere of argon. The argon was replaced by an atmosphere of hydrogen then stirring continued at ambient temperature for 1.75 h. The hydrogen was replaced by argon then the mixture was cooled to 0 °C before adding a further portion of 10% Pd–C (20 mg). The argon was replaced by hydrogen then stirring continued at ambient temperature for 5.25 h. The hydrogen was replaced by argon then the mixture was cooled to 0 °C before adding a further portion of 10% Pd–C (20 mg). The argon was replaced by hydrogen then stirring continued at ambient temperature for 14 h. The hydrogen was replaced by argon then the mixture was cooled to 0 °C before adding a further portion of 10% Pd–C (10 mg). The argon was replaced by hydrogen then stirring continued at ambient temperature for 2 h. The hydrogen was replaced by argon then the mixture was diluted with ethanol (60 mL) and filtered through Celite. The filter cake was washed with ethanol (40 mL) then the filtrate concentrated in vacuo. The residue was azeotroped with EtOAc (35 mL) to give amine **33** as an oily white solid (270 mg), which was used without further purification. HPLC–MS 346.2 $[M+H]^+$, 713.3 $[2M+Na]^+$.

4.1.24. (−)-(3a*R*,6a*S*)-*N*-[(1*S*)-1-((6*S*)-4-Benzoyl-6-hydroxyhexahydropyrrolo[3,2-*b*]pyrrole-1-carbonyl)-3-methylbutyl]-4-*tert*-butylbenzamide (34). NMM (0.17 mL, 1.55 mmol) was added to a solution of HBTU (293 mg, 0.77 mmol), 1-hydroxybenzotriazole monohydrate (118 mg, 0.77 mmol) and 4-*tert*-butylbenzoic acid (138 mg, 0.77 mmol) in DMF (7.5 mL). The solution was stood for 5 min then added to amine **33** (0.77 mmol). The mixture was stirred at ambient temperature for 1 h and 5 min then the solvents removed in vacuo (water bath temperature <28 °C). The residue was dissolved in DCM (75 mL) then washed with pH 3 HCl (60 mL), saturated aqueous $NaHCO_3$ (60 mL) and brine (60 mL), dried (Na_2SO_4) and the solvents removed in vacuo. The residue (512 mg) was purified by flash chromatography over silica eluting with EtOAc–heptane 0:100–85:15 to give alcohol **34** as a white solid (263 mg, 68%). TLC (single spot, $R_f = 0.15$, EtOAc–heptane 9:1), analytical HPLC $t_R = 19.34$ min; HPLC–MS 506.2 $[M+H]^+$; $[\alpha]_D^{22}$ −84.5 (*c* 0.084, $CHCl_3$). Microanalysis indicated the presence of residual solvent following drying in vacuo and re-drying at elevated temperature lead to partial thermal decomposition. Exact mass calcd for $C_{30}H_{39}N_3O_4$ (MNa^+): 528.2838, found 528.2818 (−3.89 ppm).

4.1.25. (3a*R*,6a*S*)-*N*-[(1*S*)-1-(4-Benzoyl-6-oxo-hexahydropyrrolo[3,2-*b*]pyrrole-1-carbonyl)-3-methylbutyl]-4-*tert*-butylbenzamide (22). A solution of alcohol **34** (174 mg, 0.345 mmol) in DCM (10 mL) was added to Dess–Martin periodinane (292 mg, 0.689 mmol) under an atmosphere of argon whilst stirring over 2.5 min. The mixture was stirred for 3 min then TFA (53 μL, 0.689 mmol) was added. The mixture was stirred for 14 h then solvents removed in vacuo. The residue was purified by flash chromatography over silica eluting with EtOAc–heptane 0:100–55:45 to give ketone **22** as a white solid (128 mg, 74%). TLC (single spot, $R_f = 0.30$, EtOAc–heptane 9:1), analytical HPLC broad peak $t_R = 19.2$–20.6 min; HPLC–MS single broad UV peak, 504.1 $[M+H]^+$; $[\alpha]_D^{22}$ −82.0 (*c* 0.49, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$ at 300 K): δ Tentative assignment of peaks due to presence of rotamers 0.95 (d, $J = 6.50$ Hz, Leu δCH_3, 3H), 1.01 (d, $J = 6.20$ Hz, Leu δCH_3, 3H), 1.31 (s, C(CH_3)$_3$, 9H), 1.58–1.81 (m, Leu βCH_2 and Leu γC*H*, 3H), 1.85–2.73 (m, BzNCHCH_2, 2H), 3.55–3.69 (m, BzNCHCH_2CH_2, 1H), 3.85–5.20 (m, BzNC*H*CH_2CH_2, BzNCH_2C(=O)C*H* and Leu αC*H*, 6H), 6.70–6.89 (m, N*H*, 1H), 7.40–7.52 (m, COC_6H_5 and CHC*H*CC(CH_3)$_3$, 7H), 7.65–7.76 (m, C*H*CHCC(CH_3)$_3$, 2H); ^{13}C NMR (125 MHz, $CDCl_3$ at 300 K): δ 22.06, 23.28 (2 × Leu $\delta C H_3$), 24.82 (Leu γ*C*H), 31.11, 31.14 (C(*C*H_3)$_3$), 31.67, 31.86 (BzNCH*C*$_2$), 34.89, 34.92 (*C*(CH$_3$)$_3$), 42.43 (Leu β*C*H_2), 46.10 (BzNCHCH$_2$*C*H$_2$), 48.93 (Leu α*C*H), 60.2 (BzN*C*H$_2$), 61.0 (BzN*C*H or BzNCH$_2$C(=O)*C*H), 68.2 (BzN*C*H or BzNCH$_2$C(=O)*C*H), 125.41, 125.49, 125.54, 126.91, 126.97, 127.11, 127.46, 128.33, 128.79, 130.84 (*C*H aromatics), 130.70, 131.16, 135.0 (quaternary aromatics),

155.35 ($CC(CH_3)_3$), 167.07, 170.67, 172.61 ($3 \times NC{=}O$), 205.0 ($C{=}O$).

4.2. Solid phase chemistry

4.2.1. Preparation of (3a*S*,6a*R*)-3-oxo-hexahydropyrrolo[3,2-*b*]pyrrole-1,4-dicarboxylic acid 1-*tert*-butyl ester 4-(9*H*-fluoren-9-ylmethyl) ester—linker construct (35a). Building block **5** (680 mg, 1.52 mmol, 1 equiv) was dissolved in a mixture of ethanol (42.5 mL) and water (6.1 mL) containing sodium acetate trihydrate (620 mg, 4.55 mmol, 3 equiv). 4-{[(Hydrazinocarbonyl)amino]methyl}cyclohexane carboxylic acid trifluoroacetate (1.0 g, 3.04 mmol, 2 equiv)[18] was added and the mixture was heated for 90 min at 86 °C then allowed to cool to ambient temperature and diluted with chloroform (350 mL). The chloroform layer was washed with dilute aqueous HCl (pH 3, 2×350 mL), brine (250 mL), dried (Na_2SO_4) and evaporated in vacuo to give **35a** as a colourless gum (1160 mg). Analytical HPLC gave two main peaks at t_R = 19.62 and 21.30 min (mixture of *E*- and *Z*-isomers); HPLC–MS (main UV peaks with t_R = 5.46 and 6.24 min 646.3 $[M+H]^+$, 1291.6 $[M+Na]^+$). Crude **35** was used directly for construct loading.

4.3. Solid phase protocols

Example inhibitors (**22**, **36**–**40**) were prepared from construct **35a** by solid phase assembly techniques utilising multipins and standard Fmoc chemistry protocols.[19,29] Polyamide crowns with 10 μmol loading (SPMDINOF) were used for scale-up synthesis and purification of selected examples. Construct **35a**, 3 equiv with respect to solid phase surface loading, were coupled overnight onto the 10 μmol crowns using standard 3 equiv HBTU, 3 equiv HOBt and 6 equiv NMM pre-activation (5 min) in a minimum volume of DMF to provide loaded construct **35b**. Construct **35b** was utilised in standard rounds of washing, Fmoc deprotection and coupling, followed by acidolytic cleavage to give crude inhibitors **22**, **36**–**40**.[21] Examples were purified by semi-preparative HPLC (see general methods) and appropriate fractions combined and lyophilised into pre-tared glass vials. Purified analogues were then weighed and a volume of dimethylsulfoxide added as appropriate to give 10 mM stock solutions used for general storage and inhibition assays.

Each purified analogue was analysed giving the following characterisation data:

4.3.1. (3a*R*,6a*S*)-*N*-[(1*S*)-1-(4-Benzoyl-6-oxo-hexahydropyrrolo[3,2-*b*]pyrrole-1-carbonyl)-3-methylbutyl]-4-*tert*-butylbenzamide (22). HPLC t_R = 19.43–21.37 min (main peak with 96% of total UV absorbance at 215 nm), HPLC–MS 504.3 $[M+H]^+$, 1029.5 $[2M+Na]^+$. Exact mass calcd for $C_{30}H_{37}N_3O_4$ (MNa^+): 526.2682, found 526.2677 (−0.96 ppm).

4.3.2. (3a*R*,6a*S*)-*N*-[1-(4-Benzoyl-6-oxo-hexahydropyrrolo[3,2-*b*]pyrrole-1-carbonyl)-3-methylbutyl]-4-(4-methylpiperazin-1-yl)benzamide (36). HPLC t_R = 12.61 min (97%); HPLC–MS 546.3 $[M+H]^+$, 564.3 $[M+H+18]^+$, 1113.6 $[2M+Na]^+$. Exact mass calcd for $C_{31}H_{39}N_5O_4$ (MH^+): 546.3075, found 546.3090 (+2.72 ppm).

4.3.3. (3a*R*,6a*S*)-Thiophene-3-carboxylic acid [(1*S*)-2-(4-benzoyl-6-oxo-hexahydropyrrolo[3,2-*b*]pyrrol-1-yl)-1-cyclohexylmethyl-2-oxo-ethyl]amide (37). HPLC t_R = 16.4–17.2 min (95%), HPLC–MS 494.2 $[M+H]^+$, 1009.4 $[2M+Na]^+$. Exact mass calcd for $C_{27}H_{31}N_3O_4S$ (MH^+): 494.2108, found 494.2090 (−3.64 ppm).

4.3.4. (3a*R*,6a*S*)-*N*-[(1*S*)-2-(4-Benzoyl-6-oxo-hexahydropyrrolo[3,2-*b*]pyrrol-1-yl)-1-(4-hydroxybenzyl)-2-oxo-ethyl]-3-bromobenzamide (38). HPLC t_R = 13.21 min (95%), HPLC–MS 576.1/578.1 $[M+H]^+$. Exact mass calcd for $C_{29}H_{26}N_3O_5Br$ (MNa^+): 598.0948, found 598.0957 (+1.44 ppm).

4.3.5. (3a*R*,6a*S*)-3-Aminomethyl-*N*-[(1*S*)-1-(4-benzoyl-6-oxo-hexahydropyrrolo[3,2-*b*]pyrrole-1-carbonyl)-3-methylbutyl]benzamide (39). HPLC t_R = 7.42 min (95%); HPLC–MS 477.2 $[M+H]^+$, 495.2 $[M+H+18]^+$, 975.3 $[2M+Na]^+$. Exact mass calcd for $C_{27}H_{32}N_4O_4$ (MH^+): 477.2496, found 477.2515 (+3.93 ppm).

4.3.6. (3a*R*,6a*S*)-*N*-[(1*S*)-2-(4-Benzoyl-6-oxo-hexahydropyrrolo[3,2-*b*]pyrrol-1-yl)-1-(4-hydroxybenzyl)-2-oxo-ethyl]-4-*tert*-butylbenzamide (40). HPLC t_R = 16.0–17.1 min (95%); HPLC–MS 554.3 $[M+H]^+$, 1129.5 $[2M+Na]^+$. Exact mass calcd for $C_{33}H_{35}N_3O_5$ (MH^+): 554.2649, found 554.2645 (−0.74 ppm).

4.4. Assays for cysteinyl proteinase activity

Assay protocols were based on literature precedent[1] and modified as required to suit local assay protocols and have been described previously.[13,31]

4.5. Measurement of inhibitor on-rates and off-rates

The observed rates of reaction for the association of compound with enzyme (k_{on}) and for the dissociation of compound from enzyme (k_{off}) were analysed as previously described.[32] Recombinant human cathepsin K was assayed in 100 mM sodium acetate; pH 5.5; 1 mM EDTA; 10 mM L-cysteine, 0.05% Tween 20 employing 1.5 μM (equal to K_M^{app}) Z-Leu-Arg-AMC as the substrate.[33] For measurements of the association rates, assays were carried out by addition of various concentrations of inhibitor to assay buffer containing substrate and initiated by the addition of enzyme. For the measurements of dissociation rates, pre-incubated enzyme plus inhibitor were diluted at least 20-fold into assay buffer containing substrate. During the course of the assay less than 10% of the substrate was consumed and the observed rates corrected for substrate kinetics.

4.6. Measurement of bone resorption activity

Bone resorption assays were carried out as a service by Pharmatest Services Ltd, Itäinen Pitkäkatu 4, 20520 Turku, Finland. Bone resorption was studied using a model where human osteoclast precursor cells derived from peripheral blood were cultured on bovine bone

slices for 9 days and allowed to differentiate into bone-resorbing osteoclasts, and the formed mature osteoclasts were then allowed to resorb bone. After the culture period, collagen fragments (CTX) released from the bone slices during the culture period were determined using CrossLaps® for culture assay (Nordic Bioscience, Herlev, Denmark). A baseline group without test compound was included providing a negative control. Additionally, a positive control E64, an inhibitor of cathepsin enzymes and osteoclastic bone resorption, was used to demonstrate that the test system was able to detect inhibition of bone resorption. Compound **22** was added to the test culture media at 10 μM, 1 μM and 100 nM ($n = 6$ replicates).

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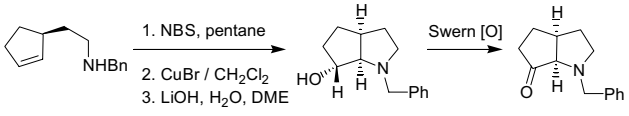
References and notes

1. Barrett, A. J.; Rawlings, N. D.; Woessner, J. F. In *Handbook of Proteolytic Enzymes*; Academic: New York, 1998.
2. (a) Wolters, P. J.; Chapman, H. A. *Respir. Res.* **2000**, *1*(3), 170; (b) Shi, G.-P.; Bryant, R. A. R.; Riese, R.; Verhelst, S.; Driessen, C.; Li, Z.; Bromme, D.; Ploegh, H. L.; Chapman, H. A. *J. Exp. Med.* **2000**, *191*(7), 1177; (c) Villadangos, J. A.; Bryant, R. A. R.; Deussing, J.; Driessen, C.; Lennon-Dumenil, A.-M.; Riese, R. J.; Roth, W.; Saftig, P.; Shi, G.-P.; Chapman, H. A.; Peters, C.; Ploegh, H. L. *Immunol. Rev.* **1999**, *172*, 109.
3. (a) Lecaille, F.; Kaleta, J.; Brömme, D. *Chem. Rev.* **2002**, *102*, 4459; (b) Brömme, D.; Kaleta, J. *Curr. Pharm. Des.* **2002**, *8*, 1639.
4. Sajid, M.; McKerrow, J. H. *Mol. Biochem. Parasitol.* **2002**, *120*, 1–22.
5. (a) Rosenthal, P. J.; Sijwali, P. S.; Singh, A.; Shenai, B. R. *Curr. Pharm. Des.* **2002**, *8*, 1659; (b) Lee, B. J.; Singh, A.; Chiang, P.; Kemp, S. J.; Goldman, E. A.; Weinhouse, M. I.; Vlasuk, G. P.; Rosenthal, P. J. *Antimicrob. Agents Chemother.* **2003**, *47*(12), 3810; (c) Urbina, J. A. *Curr. Pharm. Des.* **2002**, *8*, 287; (d) Urbina, J. A. *Expert Opin. Ther. Pat.* **2003**, *13*(5), 661.
6. (a) Veber, D. F.; Thompson, S. K. *Curr. Opin. Drug Discov. Dev.* **2000**, *3*, 362–369; (b) Hernandez, A. A.; Roush, W. R. *Curr. Opin. Chem. Biol.* **2002**, *6*, 459; (c) Kim, W.; Kang, K. *Expert Opin. Ther. Patents* **2002**, *12*(3), 419; (d) Leung-Toung, R.; Li, W.; Tam, T. F.; Karimian, K. *Curr. Med. Chem.* **2002**, *9*, 979.
7. For example, see: (a) Robichaud, J.; Oballa, R.; Prasit, P.; Falgueyret, J.-P.; Percival, M. D.; Wesolowski, G.; Rodan, S. B.; Kimmel, D.; Johnson, C.; Bryant, C.; Venkatraman, H.; Setti, E.; Mendonca, R.; Palmer, J. T.. *J. Med. Chem.* **2003**, *46*, 3709; (b) Greenspan, P. D.; Clark, K. L.; Tommasi, R. A.; Cowen, S. D.; McQuire, L. W.; Farley, D. L.; van Duzer, J. H.; Goldberg, R. L.; Zhou, H.; Du, Z.; Fitt, J. J.; Coppa, D. E.; Fang, Z.; Macchia, W.; Zhu, L.; Capparelli, M. P.; Goldstein, R.; Wigg, A. M.; Doughty, J. R.; Bohacek, R. S.; Knap, A. K. *J. Med. Chem.* **2001**, *44*(26), 4524; (c) Marquis, R. W.; Ru, Y.; LoCastro, S. M.; Zeng, J.; Yamashita, D. S.; Oh, H.-J.; Erhard, K. F.; Davis, L. D.; Tomaszek, T. A.; Tew, D.; Salyers, K.; Proksch, J.; Ward, K.; Smith, B.; Levy, M.; Cummings, M. D.; Haltiwanger, R. C.; Trescher, G.; Wang, B.; Hemling, M. E.; Quinn, C. J.; Cheng, H.-Y.; Lin, F.; Smith, W. W.; Janson, C. A.; Zhao, B.; McQueney, M. S.; D'Alessio, K.; Lee, C.-P.; Marzulli, A.; Dodds, R. A.; Blake, S.; Hwang, S.-M.; James, I. E.; Gress, C. J.; Bradley, B. R.; Lark, M. W.; Gowen, M.; Veber, D. F. *J. Med. Chem.* **2001**, *44*, 1380; (d) Watts, J.; Benn, A.; Flinn, N.; Monk, T.; Ramjee, M.; Ray, P.; Wang, Y.; Quibell, M. *Bioorg. Med. Chem.* **2004**, *12*(11), 2903; (e) Catalano, J. G.; Deaton, D. N.; Long, S. T.; McFadyen, R. B.; Miller, L. R.; Payne, J. A.; Wells-Knecht, K. J.; Wright, L. L. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 719; (f) Tavares, F. X.; Boncek, V.; Deaton, D. N.; Hassell, A. M.; Long, S. T.; Miller, A. B.; Payne, A. A.; Miller, L. R.; Shewchuk, L. M.; Wells-Knecht, K.; Willard, D. H., Jr.; Wright, L. L.; Zhou, H.-Q. *J. Med. Chem.* **2004**, *47*, 588; (g) Falgueyret, J.-P.; Oballa, R. M.; Okamoto, O.; Wesolowski, G.; Aubin, Y.; Rydzewski, R. M.; Prasit, P.; Riendeau, D.; Rodan, S. B.; Percival, M. D. *J. Med. Chem.* **2001**, *44*, 94; (h) Betschart, C.; Hayakawa, K.; Irie, O.; Sakaki, J.; Iwasaki, G. Patent WO 03/020721; (i) Altmann, E.; Hayakawa, K.; Iwasaki, G. Patent WO 03/020278.
8. (a) Altmann, E.; Green, J.; Tintelnot-Blomley, M. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1997; (b) Kim, T.-S.; Hague, A. B.; Lee, T. I.; Lian, B.; Tegley, C. M.; Wang, X.; Burgess, T. L.; Qian, Y.-X.; Ross, S.; Tagari, P.; Lin, C.-H.; Mayeda, C.; Dao, J.; Jordan, S.; Mohr, C.; Cheetham, J.; Viswanadhan, V.; Tasker, A. S. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 87.
9. In mid 2002 GlaxoSmithKline announced the advancement of SB-462795, a cyclic peptidomimetic ketone inhibitor of cathepsin K, into Phase I clinical trials for osteoporosis. In autumn 2002, Novartis announced the advancement of AAE-581, an inhibitor of cathepsin K, into Phase I clinical trials progressing to phase IIb by autumn 2003.
10. Quibell, M.; Benn, A.; Flinn, N.; Monk, T.; Ramjee, M.; Wang, Y.; Watts, J. *Bioorg. Med. Chem.* **2004**, *12*(21), 5689.
11. Fenwick, A. E.; Gribble, A. D.; Ife, R. J.; Stevens, N.; Witherington, J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 199.
12. (a) Yang, H.; Jurkauskas, V.; Mackintosh, N.; Mogren, T.; Stephenson, C. R. J.; Foster, K.; Brown, W.; Roberts, E. *Can. J. Chem.* **2000**, *78*, 800; (b) Moyer, M. P.; Feldman, P. L.; Rapoport, H. *J. Org. Chem.* **1985**, *50*, 5223.
13. See: Quibell, M.; Ray, P. C.; Watts, J. P. Patent WO 04/007501.
14. Synthesis of the *cis*-5,5-bicycle (3a*S*,6a*R*)-1-benzyl-hexahydrocyclopenta[*b*]pyrrol-6-one has recently been reported via a similar intramolecular cyclisation of a tethered amine, see: Hu, Q.-Y.; Rege, P. D.; Corey, E. J. *J. Am. Chem. Soc.* **2004**, *19*, 5984,



15. For the homologation of Cbz-proline to Cbz-2-pyrrolidineacetic acid see: Cassal, J. M.; Fuerst, A.; Meier, W. *Helv. Chim. Acta* **1976**, *59*(6), 1917.

16. Mandville, G.; Ahmar, M.; Bloch, R. *J. Org. Chem.* **1996**, *61*, 1122.
17. Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* **1989**, *30*(15), 1927.
18. Murphy, A. M.; Dagnino, R.; Vallar, P. L.; Trippe, A. J.; Sherman, S. L.; Lumpkin, R. H.; Tamura, S. Y.; Webb, T. R. *J. Am. Chem. Soc.* **1992**, *114*, 3156.
19. For a thorough description of general multipin techniques see: Grabowska, U.; Rizzo, A.; Farnell, K.; Quibell, M. *J. Comb. Chem.* **2000**, *2*(5), 475.
20. An examination of the acid lability of the Boc group from linker-construct **35b** was performed by quantitative measurement of the Fmoc chromophore following acidolytic treatment.[19] After treatment with 35% TFA in DCM for 30 min, >95% of the Fmoc chromophore remained. Quantitative removal of Boc under these conditions was confirmed by coupling the acid treated construct with benzoic anhydride, followed by cleavage and quantitative assessment of the corresponding benzoylated analogue.
21. We have now prepared in excess of 1000 analogues around general series **4** and following acidolytic cleavage have noted that in many analogues the carboxylic acid $R^3CONHCHR^2$–COOH side product is virtually undetectable, whilst in others it represents up to 20% material by UV analysis (unpublished results).
22. The corresponding analogues based around the monocyclic 3-amidotetrahydrofuran-4-one scaffold described by GSK (Ref. 11) have been described previously, see Quibell, M.; Taylor, S. Patent WO 0069855. However, accurate inhibition kinetics for these analogues are difficult to ascertain due to the chiral instability of the monocyclic ketone system.[11]
23. (a) Conroy, J. L.; Sanders, T. C.; Seto, C. T. *J. Am. Chem. Soc.* **1997**, *119*, 4285; (b) Conroy, J. L.; Seto, C. T. *J. Org. Chem.* **1998**, *63*, 2367; (c) Marquis, R. W.; Ru, Y.; Zeng, J.; Lee Trout, R. E.; LoCastro, S. M.; Gribble, A. D.; Witherington, J.; Fenwick, A. E.; Garnier, B.; Tomaszek, T.; Tew, D.; Hemling, M. E.; Quinn, C. J.; Smith, W. W.; Zhao, B.; McQueney, M. S.; Janson, C. A.; D'Alessio, K.; Veber, D. F. *J. Med. Chem.* **2001**, *44*, 725.
24. Stroup, G. B.; Lark, M. W.; Veber, D. F.; Bhattacharyya, A.; Blake, S.; Dare, L. C.; Erhard, K. F.; Hoffman, S. J.; James, I. E.; Marquis, R. W.; Ru, Y.; Vasko-Moser, J. A.; Smith, B. R.; Tomaszek, T.; Gowen, M. *J. Bone Miner. Res.* **2001**, *16*(10), 1739.
25. Veber, D. F.; Johnson, S. R.; Cheng, H.-Y.; Smith, B. R.; Ward, K. W.; Kopple, K. D. *J. Med. Chem.* **2002**, *45*, 2615.
26. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Deliv. Rev.* **1997**, *23*, 3.
27. Riley, R. J. *Curr. Opin. Drug Discov. Dev.* **2001**, *4*(1), 45.
28. James, I. E.; Dodds, R. A.; Olivera, D. L.; Nuttall, M. E.; Gowen, M. *J. Bone Miner. Res.* **1996**, *11*, 1453.
29. Atherton, E.; Sheppard, R. C. In *Solid Phase Peptide Synthesis: A Practical Approach*; IRL: Oxford, UK, 1989.
30. Meldal, M.; Breddam, K. *Anal. Biochem.* **1991**, *195*, 141.
31. Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics*; Portland: London, 1995.
32. (a) Morrison, J. F.; Walsh, C. T. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1988**, *61*, 201; (b) Tian, W. X.; Tsou, C. L. *Biochemistry* **1982**, *21*(5), 1028.
33. Bossard, M. J. *J. Biol. Chem.* **1996**, *271*(21), 12517.