β-Strand Templates

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Macrocyclic Protease Inhibitors with Reduced Peptide Character**

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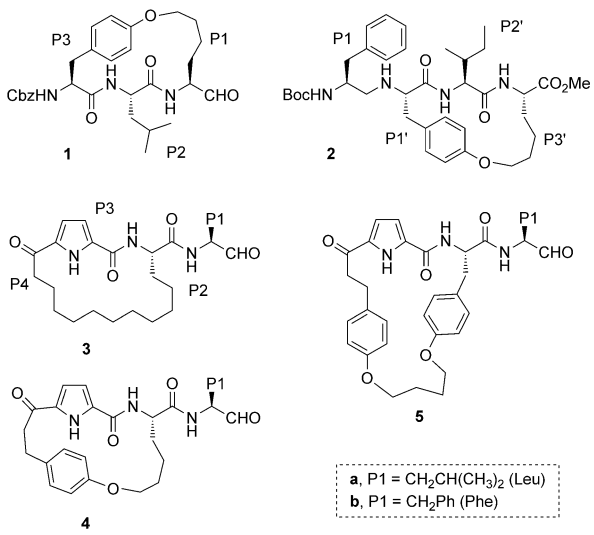
Abstract: *There is a real need for simple structures that define a β-strand conformation, a secondary structure that is central to peptide–protein interactions. For example, protease substrates and inhibitors almost universally adopt this geometry on active site binding. A planar pyrrole is used to replace two amino acids of a peptide backbone to generate a simple macrocycle that retains the required geometry for active site binding. The resulting β-strand templates have reduced peptide character and provide potent protease inhibitors with the attachment of an appropriate amino aldehyde to the C-terminus. Picomolar inhibitors of cathepsin L and S are reported and the mode of binding of one example to the model protease chymotrypsin is defined by X-ray crystallography.*

Proteases almost uniformly bind their substrates and inhibitors in a conformation whereby the peptide (or a peptide-like) backbone adopts a β-strand geometry.[1] This mode of binding is primarily dictated by the geometry of the active site subsites (designated S1-Sn and S1′ to Sn′ according to the nomenclature of Schechter and Berger)[2] that accommodate the amino acid side chains of the substrate or inhibitor. An important approach to inhibitor design is to then introduce a carefully designed conformational constraint into the structure to pre-organize its backbone into a β-strand conformation, thereby reducing entropy loss associated with ligand-receptor binding, while also enhancing biostability.[3–6] This typically involves chemically linking either the P1 and P3 or the P1′ and P3′ residues of a peptidomimetic-based protease inhibitor (see for example macrocycles **1** and **2**, respectively, in Scheme 1), leading to enhanced potency against its target protease.[3–6] However the resulting macrocycles are somewhat problematic, retaining considerable peptide-like character owing to the requirement for a tripeptide- or modified tripeptide backbone capable of presenting the necessary side chains for cyclization.



Scheme 1. Macrocyclic protease inhibitors. Cbz = benzyloxycarbonyl, Boc = *tert*-butoxycarbonyl.

A considerable challenge is to design macrocyclic β-strand templates of reduced peptide character for incorporation into protease inhibitors. There would be an additional advantage in generating such templates in which the P1 or P1′ residues are not part of the macrocycle. This would then allow the appendage of a range of groups at these positions (for example, amino aldehyde or amino dicarbonyl moieties)[3,4,7,8] to allow the targeting of a particular protease.[9] The approach reported herein involves replacing two amino acids of the macrocyclic backbone of first-generation inhibitors of type **2** with a substituted pyrrole, where this group would be expected to maintain the planar geometry required for an extended β-strand geometry[10] (see **3**–**5**, Scheme 1). Other heterocycles have also been used in this context.[11,12] Phenylene groups (0, 1, or 2 as in the P2-P4 linker of **3**, **4**, and **5**, respectively) were incorporated into the macrocycles to further constrain the backbone geometry[3–5] and to target a particular protease through interaction with the respective active site subsites. In this study, we target ovine *m*-calpain, human cathepsins L and S (Cat L and Cat S), bovine α-chymotrypsin, and human leukocyte elastase (HLE). Calpains are an ubiquitous class of non-lysosomal cysteine proteases, the over-activation of which is implicated in

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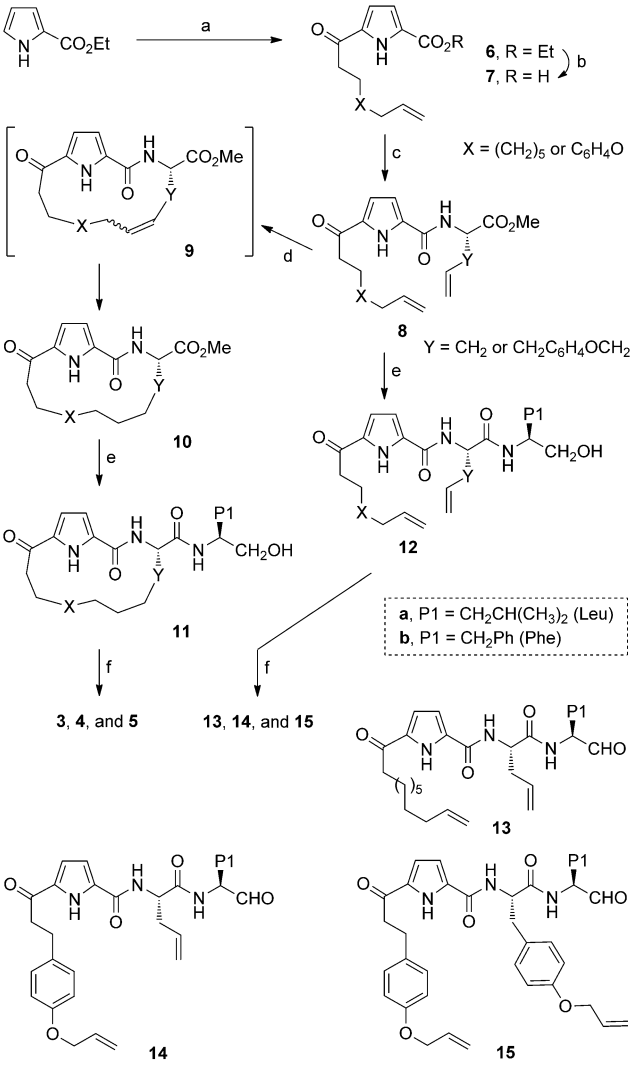
a variety of pathophysiological conditions, including cataract and traumatic brain injury.[8] Cathepsins L and S are lysosomal cysteine proteases that are known to play key roles in tumor progression and autoimmune disorders, respectively,[13] while α-chymotrypsin and HLE are typical serine proteases, with the former being a suitable model enzyme for solving a ligand/protease X-ray structure to define the mode of binding.

The key step in the synthesis of the component macrocycles of **3**–**5** involved ring-closing metathesis (RCM)[14] of an appropriate diene **8** (Scheme 2). These dienes were readily prepared from 2-ethoxycarbonylpyrrole by Friedel–Crafts acylation with the respective acid chloride in the presence of $Yb(OTf)_3$. Ester hydrolysis of **6**, followed by HATU- or EDCI-catalyzed coupling of the resulting pyrrolic acid **7** with the appropriate olefinic amino acid ester (either (*S*)-allyl-Gly-OMe or (*S*)-allyl-Tyr-OMe), afforded the RCM substrates **8**. Treatment with Grubbs second-generation catalyst then gave the macrocycles **9**, which were not isolated but rather hydrogenated on treatment with H_2 and Pd on C to give the key macrocycles **10** (see the Supporting Information for further details). The final steps in the synthesis involved hydrolysis of the methyl esters **10**, coupling the resulting acids with either (*S*)-leucinol or (*S*)-phenylalaninol, in the presence of HATU, followed by oxidation of the C-terminal primary alcohol with Dess–Martin periodinane to give the desired macrocyclic aldehydes **3**–**5**, see Schemes 1 and 2. The acyclic dienes **13**–**15** were also prepared as shown in Scheme 2 to provide suitable acyclic controls for the biological evaluation.

Each aldehyde (**3**–**5** and **13**–**15**) was assayed against three cysteine proteases (*m*-calpain, Cat L, and Cat S), and two serine proteases (α-chymotrypsin and HLE); the data are shown in Table 1. The assay results for the unsubstituted macrocycles **3a** and **3b** against Cat L and Cat S are particularly significant. Both compounds are highly potent, with picomolar K_i values against Cat S. The macrocycle of these compounds is clearly beneficial to activity, with the corresponding acyclic analogues **13a** and **13b** being clearly less active (> 6-fold for Cat S and > 100-fold for Cat L). Macrocycle **3a**, and to a lesser extent **3b**, are also highly potent against *m*-calpain, exhibiting K_i values of 58 and 140 nM, respectively. This compares to a K_i of 26 nM (IC_{50} of 30 nM)[3] for the first-generation macrocycle **1** against the same enzyme.

The alternative macrocycles **4** and **5** that contain one and two aryl groups respectively, in the constituent macrocycle, also display good activity against all three cysteine proteases. Macrocycle **5a** is in fact the most potent inhibitor of Cat S (K_i of 0.74 nM). The Phe containing macrocycle **5b** displays an advantage over its acyclic analogue **15b** for the three cysteine proteases. More generally, the benefit of the macrocycle over its acyclic analogue is most evident for the inhibition of Cat S (8-fold).

There is some apparent preference for Leu over Phe at P1 of our macrocyclic inhibitors against the cysteine proteases. However, the inclusion of Phe at P1, as in **3b**, **4b**, and **5b**, results in more potent inhibitors of α-chymotrypsin compared to the corresponding Leu derivatives **3a**, **4a**, and **5a** (4- to 57-fold). The same is true for the acyclic compounds **13b**, **14b**,



Scheme 2. Synthesis of aldehydes: a) $Yb(OTf)_3$, CH_3NO_2, RT, 10-undecenoyl chloride (**6**, X = $(CH_2)_5$, 17%) or *p*-allyloxyphenylpropionyl chloride (**6**, X = C_6H_4O, 31%); b) KOH, THF, H_2O, 40–50 °C, (**7**, X = $(CH_2)_5$, 81%; **7**, X = C_6H_4O, 96%); c) HOBt, DIEA, CH_2Cl_2, RT and HATU, (*S*)-allyl-Gly-OMe, (**8**, X = $(CH_2)_5$, Y = CH_2, 48%), or EDCI, (*S*)-allyl-Gly-OMe, (**8**, X = C_6H_4O, Y = CH_2, 94%), or HATU, (*S*)-allyl-Tyr-OMe, (**8**, X = C_6H_4O, Y = $CH_2C_6H_4OCH_2$, 84%); d) Grubbs second-generation catalyst, CH_2Cl_2, Δ, then H_2, Pd-C, EtOAc (**10**, X = $(CH_2)_5$, Y = CH_2, 28%; **10**, X = C_6H_4O, Y = CH_2, 36%; **10**, X = C_6H_4O, Y = $CH_2C_6H_4OCH_2$, 56%); e) 2 M NaOH, THF, RT; then HATU, HOBt, DIEA, DMF, RT, and (*S*)-leucinol, (**11a**, X = $(CH_2)_5$, Y = CH_2, 62%; X = C_6H_4O, Y = CH_2, 81%; X = C_6H_4O, Y = $CH_2C_6H_4OCH_2$, 54%; **12a**, X = $(CH_2)_5$, Y = CH_2, 79%; X = C_6H_4O, Y = CH_2, 57%; X = C_6H_4O, Y = $CH_2C_6H_4OCH_2$ 71%) or (*S*)-phenylalaninol, (**11b**, X = $(CH_2)_5$, Y = CH_2, 58%; X = C_6H_4O, Y = CH_2, 36%; X = C_6H_4O, Y = $CH_2C_6H_4OCH_2$, 55%; **12b**, X = $(CH_2)_5$, Y = CH_2, 92%; X = C_6H_4O, Y = CH_2, 60%; X = C_6H_4O, Y = $CH_2C_6H_4OCH_2$, 62%); f) Dess–Martin periodinane, CH_2Cl_2, RT (**3a**, 37%; **3b**, 34%; **4a**, 37%; **4b**, 15%; **5a**, 41%; **5b**, 65%; **13a**, 31%; **13b**, 22%; **14a**, 21%; **14b**, 29%; **15a**, 53%; **15b**, 48%). DIEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, EDCI = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, HATU = *O*-(7-azabenzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluoro-phosphate, HOBt = *N*-hydroxybenzotriazole.

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Table 1: In vitro inhibition assay data and K_i values.

Compound	Number of aryl groups	P1	*m*-Calpain[a,b]	Cat L[a,c]	Cat S[a,c]	α-Chymotrypsin[d]	HLE[a,e]
3a	0	Leu	58	0.44	0.92	>10000	>2500
3b	0	Phe	140	1.7	0.91	2500	>2500
4a	1	Leu	220	6.8	14	>10000	n.i.[g]
4b	1	Phe	130	n.d.[f]	n.d.[f]	430	n.i.[g]
5a	2	Leu	180	3.0	0.74	1900	640
5b	2	Phe	220	30	2.3	33	600
13a	0	Leu	35	130	5.7	>10000	52
13b	0	Phe	63	200	95	2500	180
14a	1	Leu	59	14	1.0	940	300
14b	1	Phe	720	250	17	56	510
15a	2	Leu	6800	1.7	0.76	n.i.[g]	160
15b	2	Phe	1100	53	19	690	180

[a] K_i values [nM] were calculated from IC_{50} values by applying the Cheng–Prusoff equation for competitive inhibition. [b] Values are the mean of two experiments in triplicate with seven different inhibitor concentrations. Variation between experiments is less than 10%. The assay was performed with the fluorogenic substrate BODIPY-FL casein at a final concentration 0.09 μM (0.14 K_m).[17] [c] Data were calculated from experiments with five different inhibitor concentrations. IC_{50} values were obtained by nonlinear regression, with standard errors < 25%. Assays were performed with the chromogenic substrate Cbz-Phe-Arg-pNA at a final concentration of 100 μM (cathepsin L: 5.88 K_m; cathepsin S: 0.85 K_m).[18] [d] K_i values are the mean of three experiments. Variation between experiments is less than 10%. The assays were performed with the chromogenic substrate Suc-Ala-Ala-Pro-Phe-pNA at final concentrations of 20 μM (0.3 K_m) and 50 μM (0.75 K_m). Limits refer to measurements at a substrate concentration of 50 μM and a single inhibitor concentration (25–125 μM).[19] [e] Data were calculated from experiments with five different inhibitor concentrations. IC_{50} values were obtained by nonlinear regression, with standard errors < 40% except for **14a** (50%). Assays were performed with the chromogenic substrate MeO-Suc-Ala-Ala-Pro-Val-pNA at a final concentration of 100 μM (1.85 K_m). Limits refer to measurements at a single inhibitor concentration of 10 μM.[20] [f] n.d. = not determined. [g] n.i. = no inhibition; refers to less than 50% inhibition at a substrate concentration of 50 μM and an inhibitor concentration of 250 μM (α-chymotrypsin). For elastase, it refers to less than 20% inhibition at an inhibitor concentration of 10 μM. None of the compounds inhibited bovine trypsin[21] (less than 20% inhibition at an inhibitor concentration of 10 μM).

and **15b** compared to **13a**, **14a**, and **15a**. These data clearly reflect the known primary substrate specificity of α-chymotrypsin for aromatic amino acids at the P1 position. Macrocycle **5b**, with its two aryl groups, is particularly potent towards α-chymotrypsin and has a K_i of 33 nM. This compound also shows a significant gain in inhibitory potency with macrocyclization (36-fold, **5b** vs. **15b**). Interestingly, the macrocycles are not potent inhibitors of HLE to provide some selectivity within the serine protease family. It should be noted that Leu is preferred over Phe, in accordance with the specificity of HLE for aliphatic residues at the P1 position. The most potent HLE inhibitor of the series is compound **13a** (K_i of 52 nM) that lacks aromatic groups in its side chains.

A crystal structure of the macrocyclic aldehyde **5b** covalently bound to serine 195 of bovine α-chymotrypsin was solved to define the mode of protease binding of our new macrocycles, and particularly the extended backbone with its constituent pyrrole (see Figure 1 and the Supporting Information for further details). An overlay of this structure with that of an extended peptide-based inhibitor (PMP-C, PDB: 1L1)[15] of bovine α-chymotrypsin is also shown in Figure 1. PMP-C binds in an extended β-strand geometry that extends from P5 to P4′. As expected, the P1 Phe side chain of **5b** binds in the S1 pocket as defined by a cavity formed by the main chain atoms of Cys191, Gly216, and the disulfide bond formed between Cys191 and Cys220. Importantly and exactly as per our design, the backbone of **5b** superimposes nicely with that of β-strand bound PMP-C, with the pyrrole of **5b** clearly defining the required geometry while occupying the P3 and P4 amino acid positions. An overlay of the structure of **5b** with a simple peptidic aldehyde (*N*-acetyl-L-Leu-L-phenylalanal)[16] bound to α-chymotrypsin (see the Supporting Information for further details) also shows that **5b** forms a β-strand conformation nearly identical to that of *N*-acetyl-L-Leu-L-phenylalanal bound to chymotrypsin,[16] with both phenyl groups positioned similarly in the S1 cavity (Supporting Information, Figure S2). Compound **5b** forms the desired β-strand geometry with two peptide torsion angles $\phi = 147°$, $\psi = -98°$ (calculated between C′33 and C′37, N31 and N35) and $\phi = -165°$, $\psi = -88°$ (calculated between C′29 and C′33, N25 and N31).

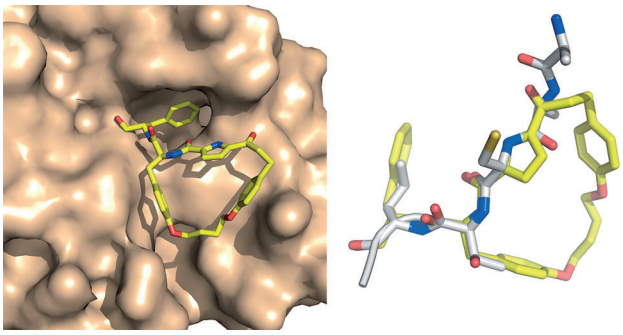

Figure 1. Macrocycle **5b** bound to α-chymotrypsin (left) and **5b** (yellow) overlayed with truncated PMP-C (P1 Leu-P2Thr-P3Cys-P4Ala, gray; right).

In conclusion, we present a new class of macrocyclic protease inhibitors in which a backbone amino acid and the associated peptide bond are replaced with a planar pyrrole. This results in reduced peptide character, while retaining a backbone β-strand geometry to facilitate active site binding.

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The macrocycle is introduced between the P2 amino acid and N-terminus to leave the P1 position free for incorporation of an appropriate functionality for targeting a particular protease, an amino aldehyde in this first instance. Examples of this class proved to be particularly potent against cathepsins L and S with K_i values in the picomolar range. A crystal structure of one exemplary inhibitor (**5b**) bound to chymotrypsin confirms the design features. Work is underway to extend these new peptidomimetics to other proteases and in other applications requiring a backbone β-strand geometry.

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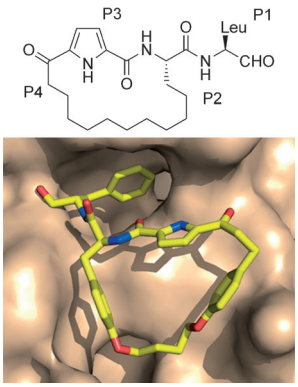
Communications

β-Strand Templates

K. C. H. Chua, M. Pietsch, X. Zhang, S. Hautmann, H. Y. Chan, J. B. Bruning, M. Gütschow, A. D. Abell* _____ ■■■■–■■■■

Macrocyclic Protease Inhibitors with Reduced Peptide Character



The incorporation of a pyrrole into a peptide backbone generates simple macrocycles that adopt a β-strand geometry. The attachment of a P1 amino aldehyde to these templates then gives rise to potent protease inhibitors (see example, top, which has K_i values of 440 pM and 920 pM against the cysteine cathepsins L and S, respectively). A crystal structure of a related derivative bound to chymotrypsin (see picture, bottom) confirms the design.

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