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Drug Design

Molecular Modeling, Synthesis, and Biological Evaluation of Macrocyclic Calpain Inhibitors**

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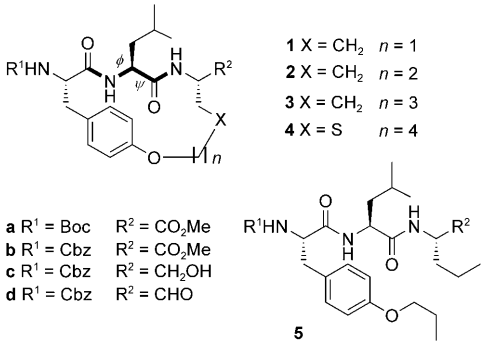
The introduction of a macrocycle into a biologically active peptide can increase potency[1,2] and selectivity[3] by reducing the entropic penalty of inhibitor–enzyme binding. The incorporation of macrocycles in peptides has been used to mimic secondary structure, such as extended β-strand-like and bent β-turn-like conformations.[3,4] The challenge is to devise protocols for the design, evaluation, and synthesis of macrocyclic templates that provide access to families of inhibitors.[5] Such templates should 1) have a well-defined conformation that can be readily assessed by computational screening, 2) be readily synthesized from natural building blocks, and 3) be easily modified to target an enzyme for a specific biological application.

Herein we present studies on the 16–19-membered macrocycles **1**–**4** (Scheme 1) designed to be constrained into a β-strand-like geometry, a conformation universally adopted by inhibitors and substrates on binding to a protease.[6] We report a versatile approach based on ring-closing metathesis (RCM) to these orthogonally protected templates as well as computational analysis of their potential to form a β strand and of their binding to a target protease. The templates were converted into aldehydes **1d**–**4d** as potential inhibitors of the calcium-activated cysteine protease calpain. The alcohols **1c**–**4c** were evaluated to establish whether the macrocycles negate the need for a reactive warhead. Calpain was chosen as a challenging target for the design of selective inhibitors,[7] and because its link to cortical cataracts[8] provides an opportunity to assess the in vivo efficacy of our approach. The acyclic tripeptide analogues **5a**–**d** were also investigated to establish the importance (or unimportance) of the macrocyclic constraint for potency and selectivity of inhibition.



Scheme 1. Macrocyclic templates and acyclic peptides. Dihedral angles Φ and Ψ that describe a β strand are shown. Boc = *tert*-butoxycarbonyl, Cbz = carbobenzyloxy.

Conformational searches[9] were carried out on **1a**–**d** to **5a**–**d** to assess their ability to adopt a β-strand conformation. The resulting ensembles of low-energy conformers were examined by XCluster (see the Supporting Information for detailed results). For the 16-membered macrocycles **1a**–**d**, all conformers (> 99 %) within 12 kJ mol^{-1} of their global minima adopt a β-strand conformation (see Figure 1 for

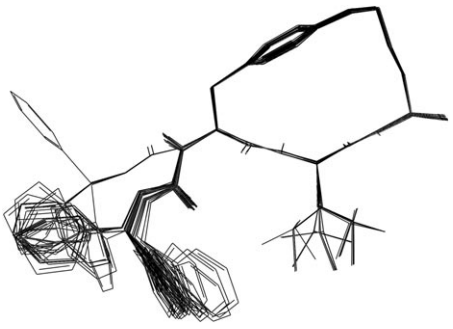


Figure 1. Overlaid conformers of the two clusters of **1d**.

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Supporting information (confirmation of the structure and purity of all compounds, details on modeling studies, and biological assay data) for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200805014.

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1d). These macrocycles are essentially rigid with little apparent influence from the C and N termini. The 17- and 18-membered macrocycles **2a–d** and **3a–d** each have at least one cluster of β-strand peptide-backbone conformers. Thus, their backbone conformations are more flexible with some influence from the C- and N-terminal groups. The flexibility of the 19-membered rings in **4a–d** does not constrain these macrocycles into a β-strand conformation. The acyclic analogues **5a–c** do not exhibit β-strand conformers. However, aldehyde **5d** has one minor cluster of β-strand peptide-backbone conformers.

On the basis of this established ability to adopt a β-strand conformation, we predicted the order of potency of a macrocyclic inhibitor to be: 16-membered **1** > 17-membered **2** ≈ 18-membered **3** > 19-membered **4**. Each of the macrocyclic aldehydes **1d–4d** was docked in silico into ovine calpain 1 and 2 (o-CAPN1 and o-CAPN2) homology models[10] by using Glide.[11] The results of these docking studies (see the Supporting Information) suggest that **1d–4d** bind in the same β-strand conformation, with similar hydrogen-bonding patterns and an appropriate positioning of the aldehyde group near the nucleophilic sulfur atom in the active site (Figure 2). This mode of binding is in agreement with that observed in the crystal structures of leupeptin (a naturally occurring inhibitor) and SNJ1715 cocrystallized in rat calpain 1 (r-CAPN1). On the basis of these results, we targeted macrocycles **1–4** for synthesis and biological evaluation.

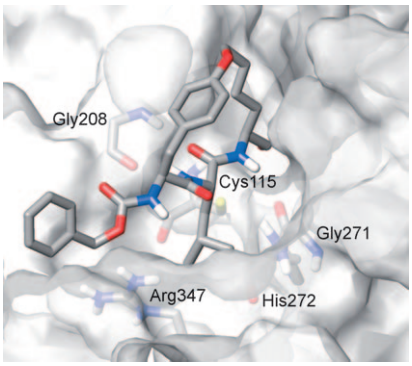
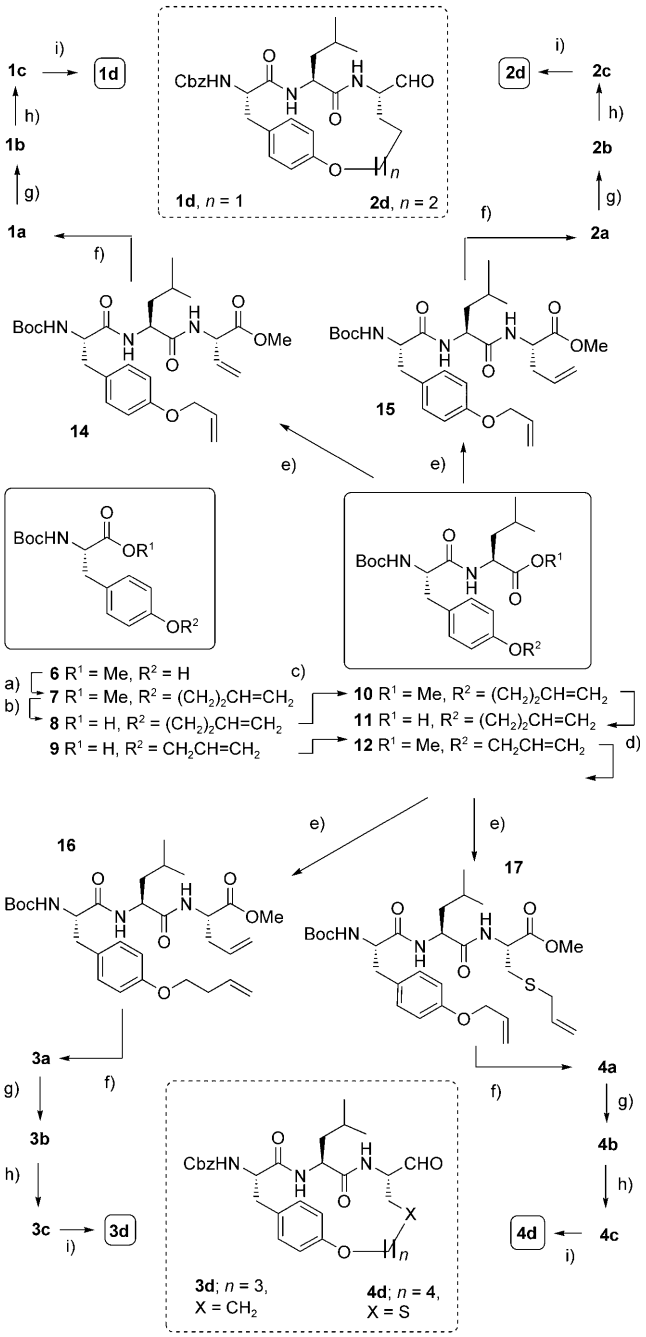


Figure 2. A representative pose of macrocycle **2d** in the o-CAPN1 model.

The synthesis of macrocycles **1a–4a** was based on the RCM of dienes **14–17**, which were prepared from natural building blocks (Scheme 2). The reaction of commercially available *N*-Boc-Tyr-OMe (**6**) with 4-bromobut-1-ene gave the homoallylic ether **7**, which was hydrolyzed to give **8**. Separate reactions of **8** and **9** (commercially available) with Leu-OMe in the presence of HATU gave dipeptides **10** and **12**, which were hydrolyzed to give **11** and **13**, respectively. Dienes **14**, **15**, and **17** were prepared by coupling **13** with (*S*)-vinyl-Gly-OMe,[12] (*S*)-allyl-Gly-OMe, and (*S*)-Cys(*S*-allyl)-OMe, respectively. Diene **16** was similarly prepared from **11** by treatment with Cys(*S*-allyl)-OMe. RCM of the acyclic dienes **14–17** gave mixtures of *cis* and *trans* alkenes, which were hydrogenated directly to give macrocycles **1a–4a**.



Scheme 2. Synthesis of **1a–4a**: a) K_2CO_3, 4-bromobut-1-ene, DMF (27%); b) NaOH, THF, H_2O, MeOH, (85%); c) HATU, DIPEA, Leu-OMe, DMF (**10**: 64%; **12**: 86%); d) NaOH, THF, H_2O, MeOH (**11**: 100%, **13**: 97%); e) HATU, DIPEA, (*S*)-vinyl-Gly-OMe, DMF, **13** (**14**: 83%), or HATU, DIPEA, (*S*)-allyl-Gly-OMe, DMF, **13** (**15**: 97%), or HATU, DIPEA, (*S*)-allyl-Gly-OMe, DMF, **11** (**16**: 84%), or HATU, DIPEA, (*S*)-Cys(*S*-allyl)-OMe, DMF, **13** (**17**: 83%); f) Grubbs second-generation catalyst, 1,1,2-TCE, Δ, then H_2, Pd-C, MeOH (**1a**: 24%, **2a**: 22%, **3a**: 29%, **4a**: 66%); g) 4 M HCl, 1,4-dioxane (quantitative for **1a–4a**), then benzyl chloroformate, DIPEA, DMF, (**1b**: 53%, **2b**: 49%, **3b**: 79%, **4b**: 77%); h) $LiAlH_4$, THF (**1c**: 83%, **2c**: 83%, **3c**: 72%, **4c**: 70%); i) SO_3·pyridine, DIPEA, DMSO, CH_2Cl_2 (**1d**: 92%, **2d**: 80%, **3d**: 90%, **4d**: 61%). DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, DMSO = dimethyl sulfoxide, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, 1,1,2-TCE = 1,1,2-trichloroethane

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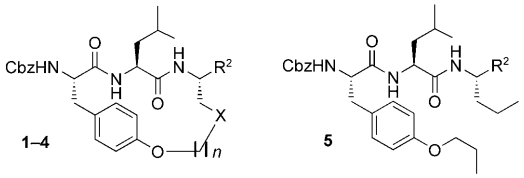
The separate treatment of **1a–4a** with HCl/dioxane gave the corresponding hydrochloride salts in quantitative yield. These salts were treated, without purification, with benzyl chloroformate to give esters **1b–4b**. Reduction of **1b–4b** with lithium aluminium hydride gave the alcohols **1c–4c**, which were oxidized with SO_3·pyridine and DSMO[13] to give aldehydes **1d–4d** (Scheme 2).

The aldehydes **1d–4d** and alcohols **1c–4c** were assayed against ovine calpain 1 (o-CAPN1) and ovine calpain 2 (o-CAPN2)[14] to determine in vitro potency and isoform selectivity, and to assess the potential of the constituent macrocycle to enable noncovalent inhibition in the absence of an aldehyde (Table 1). Modeling studies suggested that the alcohol group could interact with the oxyanion hole defined by the side chain of Gln109/99 and the backbone amide nitrogen atom of Cys115/105 in the ovine calpains.[15]

Table 1: In vitro inhibition data.



Compound	R^2	X	*n*	IC_{50}[a] [nM] o-CAPN1	o-CAPN2
1c	CH_2OH	CH_2	1	13000	31000
2c	CH_2OH	CH_2	2	1750	700
3c	CH_2OH	CH_2	3	1340	1100
4c	CH_2OH	S	4	50000	28000
5c	CH_2OH	–	–	3200	15600
1d	CHO	CH_2	1	400	850
2d	CHO	CH_2	2	220	30
3d	CHO	CH_2	3	170	180
4d	CHO	S	4	3150	1010
5d	CHO	–	–	50	130

[a] Values are the mean of three experiments. Variation between experiments is less than ±10%.

The 17-membered macrocyclic aldehyde **2d** is a particularly potent inhibitor of o-CAPN2 (IC_{50} = 30 nM) with greater than sevenfold selectivity for o-CAPN2 over o-CAPN1 (IC_{50} = 220 nM for o-CAPN1). The 18-membered macrocycle **3d** is approximately equally potent against both proteases (IC_{50} = 170 and 180 nM for o-CAPN1 and o-CAPN2, respectively). The 16-membered macrocycle **1d** is less potent (IC_{50} = 400 and 850 nM for o-CAPN1 and o-CAPN2, respectively) with greater than twofold selectivity for, in this case, o-CAPN1 over o-CAPN2. The incorporation of a sulfur atom into the 19-membered macrocycle (in **4d**) resulted in significantly reduced potency with some selectivity for o-CAPN2. By comparison, the acyclic tripeptide aldehyde **5d** is a potent calpain inhibitor (IC_{50} = 50 and 130 nM for o-CAPN1 and o-CAPN2, respectively) with >2.5-fold selectivity for o-CAPN1 over o-CAPN2.

Of particular significance is the observation that the 17-membered macrocyclic alcohol **2c**, which lacks the reactive aldehyde group, is an inhibitor of o-CAPN2 (IC_{50} = 700 nM). Again, the 17-membered ring is optimum for activity: **1c**, **3c**, and **4c** are all significantly less potent (see Table 1). The 16- and 19-membered macrocyclic structures (**1c** and **4c**) are almost devoid of activity, as is the acyclic analogue **5c**. Thus, the introduction of an appropriate macrocycle provides a new class of noncovalent inhibitors of calpains and other cysteine proteases (see below).

The macrocycles **2c**,**d** and the acyclic tripeptides **5c**,**d** were also assayed against cathepsin B (a cysteine protease from the same clan as calpain),[16] papain, pepsin, and α-chymotrypsin.[17] All were inactive against papain, pepsin, and α-chymotrypsin in the concentration range of the assays (up to 50 μM). However, the 17-membered macrocyclic aldehyde **2d** (IC_{50} = 70 nM) and the analogous acyclic aldehyde **5d** (IC_{50} = 5 nM) are both potent inhibitors of cathepsin B. The macrocyclic alcohol **2c** (IC_{50} = 300 nM) and the acyclic analogue **5c** (IC_{50} = 200 nM) are both remarkably potent for noncovalent inhibitors.

Docking studies with the o-CAPN1 and o-CAPN2 homology models were carried out with the macrocyclic alcohols **2c** and **3c**, which lack a reactive aldehyde but display activity against o-CAPN1 and o-CAPN2. A representative pose of each alcohol (see the Supporting Information) shows that the β-strand peptide-backbone conformations are orientated similarly with respect to the enzyme. In each case, the methylene carbon atom of the primary alcohol is positioned close to the sulfur atom of the active-site cysteine residue (at a distance of 3.1 and 4.5 Å for **2c** and **3c**, respectively). This structural feature is analogous to that observed in the crystal structure of the hemiacetal formed when SNJ1715 was cocrystallized with r-CAPN1.[15]

We next chose to investigate the potential of our most potent o-CAPN2 inhibitor, **2d**, to retard the development of calcium-induced cortical cataracts, which have been linked to an overactivity of this enzyme.[8] Lenses from 9–12-month-old lambs were incubated for 48 h in EMEM (Eagle Minimum Essential Medium) culture medium (10 mL) at 37 °C in 5% CO_2. Inhibitor **2d** (1 μM) was added to one lens of each of six pairs of sheep lenses in the culture medium. After incubation for 3 h, $CaCl_2$ was added to a final concentration of 5 mM. Intact ovine lenses (n = 6) were also incubated in EMEM culture medium as a control. After 6 h, all lenses were photographed over a grid, and the opacity was graded by using the software Image-Pro 4.1. Lenses treated with only calcium showed substantial opacity as associated with cataract formation. The presence of **2d** prevented this calcium-induced opacification, and these lenses remained essentially transparent after incubation for 6 h (Figure 3). Thus, the calpain inhibitor **2d** in the culture medium was able to significantly reduce lens opacity ($p < 0.005$ in a paired t test).

Finally, we were interested in correlating potency with the ability of each compound (on the basis of the earlier modeling studies) to adopt a β-strand conformation (16-membered **1** > 17-membered **2** ≈ 18-membered **3** > 19-membered **4**). The 17- and 18-membered macrocyclic aldehydes **2d** and **3d** are more potent than the 16-membered macrocycle **1d** (see Table 1), which was shown by modeling to be particularly rigid. We suggest that the rigid macrocycle **1d** does not have the

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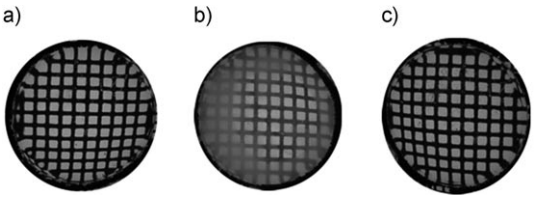

Figure 3. Effect of **2d** on calcium-induced cataract formation in cultured ovine lenses. The values given are the mean opacification grading score (±SEM: standard error of the mean) in each case and represent the mean opacity of six lenses. Fully opaque lenses have scores of 80–100, whereas transparent lenses have scores of less than 20. a) Control: (16.0±2.1); b) Ca^{2+} only: (58.8±6.7); c) Ca^{2+} with **2d**: (16.7±1.5).

flexibility necessary for optimum binding, despite its favored β-strand peptide-backbone conformation. By contrast, the slightly more flexible 17-membered macrocycle (and to a lesser extent the 18-membered macrocycle) combine an ability to adopt a β-strand conformation with an opportunity to optimize binding. The potent and selective o-CAPN2 inhibitor **2d** has potential for the treatment of cortical cataracts. The flexibility of the 19-membered ring in **4d** does not constrain the macrocycle in a β-strand conformation. This compound is only moderately potent despite its docking in both the o-CAPN 1 and o-CAPN2 models in a β-strand conformation.

In conclusion, a combination of modeling, synthesis, and biological evaluation of **1c–4c** and **1d–4d** has identified versatile macrocyclic templates that exhibit a varying and predictable propensity to adopt a β-strand backbone conformation. Potency against calpain can be correlated to this conformational preference as well as the flexibility of the macrocycle. We have presented a versatile synthetic route to these templates, with an opportunity to prepare a range of macrocycles and protease inhibitors through modification and extension at the two termini and of the constituent peptide sequence of the diene for RCM.

Aldehyde **2d** (CAT811)[18] proved to be the most potent and selective o-CAPN2 inhibitor (Table 1). The alcohol **2c** (CAT505),[18] although less potent, is a unique example of an o-CAPN2-selective noncovalent inhibitor. Aldehyde **2d** significantly retarded calcium-induced opacification in vitro in an ovine-lens culture assay and shows promise in slowing the progression of cortical cataracts in animal trials with a hereditary-ovine-cataract model. The animal studies will be reported separately.

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