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Synthesis and Extended Activity of Triazole-Containing Macrocyclic Protease Inhibitors

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Abstract: Peptide-derived protease inhibitors are an important class of compounds with the potential to treat a wide range of diseases. Herein, we describe the synthesis of a series of triazole-containing macrocyclic protease inhibitors pre-organized into a β-strand conformation and an evaluation of their activity against a panel of proteases. Acyclic azido–alkyne-based aldehydes are also evaluated for comparison. The macrocyclic peptidomimetics showed considerable activity towards calpain II, cathepsin L and S, and the 20S proteasome chymotrypsin-like activity. Some of the first examples of highly potent macrocyclic inhibitors of cathepsin S were identified. These adopt a well-defined β-strand geometry as shown by NMR spectroscopy, X-ray analysis, and molecular docking studies.

Keywords: click chemistry · NMR spectroscopy · macrocycles · peptidomimetics · protease inhibitors

Introduction

There is considerable interest in the design and exploitation of protease inhibitors that combine potency with an ability to specifically inhibit one protease over another.[1] One important approach to such classes of inhibitors is to pre-organize the structure into a conformation known to favor active-site binding.[1a] This can be achieved by introducing a specific macrocycle into a peptide backbone structure, the sequence and make-up of which are chosen to complement the binding domain of a specific protease.[1e,2] The introduction of a macrocycle has the added advantage of improving biostability and resistance to proteolytic degradation.[3] A number of synthetic approaches have been reported for introducing such a macrocycle, including ring-closing metathesis,[1c,4] alkylation,[5] and more recently Huisgen cycloaddition[6] (see Figure 1 for some representative examples). The macrocycle of these structures links the P1 and P3 residues[1g] of the peptide backbone to constrain the backbone into a β-strand geometry that is universally recognized by all

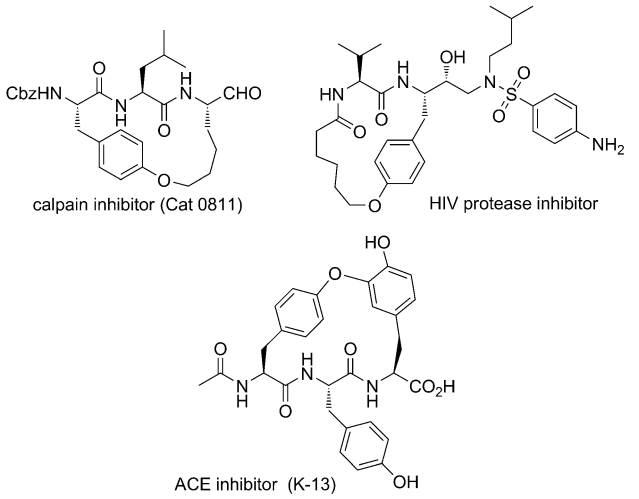

Figure 1. Examples of macrocyclic peptidomimetics linking P1 and P3[1c,e,5b] to mimic a β-strand conformation. Cbz = carbobenzyloxy.

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proteases.[1a] There are also natural examples of macrocyclic protease inhibitors of this type.[7] Many macrocyclic protease inhibitors are more potent than their corresponding acyclic analogues.[4a,b,6a]

An important and outstanding issue with much of this research is that few macrocyclic inhibitors have been studied against a range of proteases, particularly different proteases within the same class. Addressing this shortcoming would begin to define useful activity profiles and also therapeutic potential in meaningful and productive ways. We recently reported the synthesis of a series of triazole-containing macrocyclic inhibitors of calpain II.[6a] In this paper, we extend this work with the synthesis of a new series of triazole-containing macrocycles. An expanded series of such macrocycles (**1**–**11**, Figure 2) was then assayed against a panel of proteases. Two specific classes of macrocycle are presented, one can be considered as containing the triazole at the P1 position (see **1**–**4**, Figure 2) and a second with the triazole at P3 (see **5**–**11**). All the structures contain a C-terminal aldehyde that reacts with an active site nucleophile of serine and cysteine proteases through hemiacetal and hemithioacetal formation, respectively.[8,9] We also present a variation to the existing methodology for making such macrocycles,[6a] in which the triazole is introduced by using Huisgen cycloaddition prior to the final cyclization by peptide coupling, that is, the original synthetic sequence is reversed.

All the triazole-based macrocycles (**1**–**11**) and their acyclic azide–alkyne analogues (**12**–**15**) were tested against three cysteine proteases (calpain II, cathepsin L, and cathepsin S), which were chosen because of their clinical importance.[10] Calpain II is implicated in a range of medical conditions including traumatic brain injury, stroke, and cataracts.[10,11] Cathepsins L and S are involved in tumor growth and invasion, autoimmune diseases, and osteoporosis.[12] We also targeted the 20S proteasome, that is, the core of the 26S proteasome, a multicomponent protease that plays a key role in the degradation of proteins that regulate the cell cycle. Over-activity of the 20S proteasome has been identified as a key mechanism in the development of cancer.[13] This protease has three separate activities referred to as caspase-like (CP-L), trypsin-like (T-L), and chymotrypsin-like (CT-L), with the latter thought to be particularly significant as a therapeutic target. However, there are few examples of inhibitors that show selectivity for one of the three separate proteasome activities, with the classic tripeptide acyclic aldehyde inhibitor MG132 being active against all three and also the cathepsins and calpain.[14]

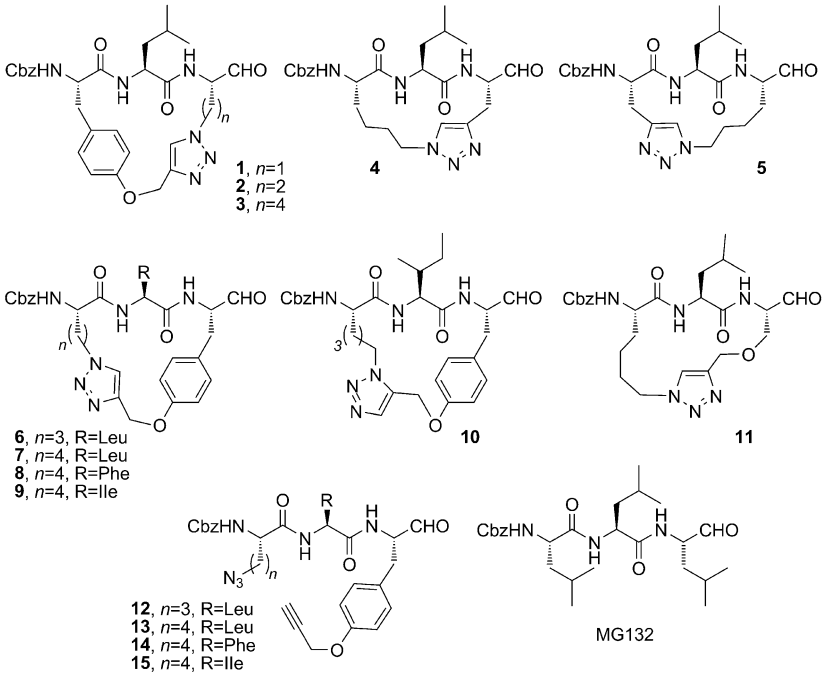
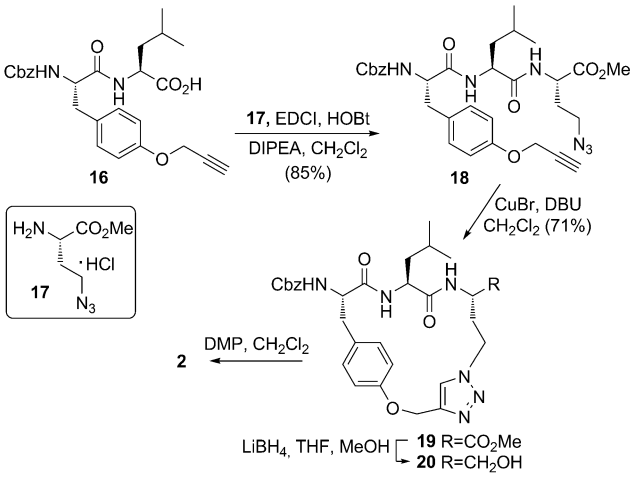


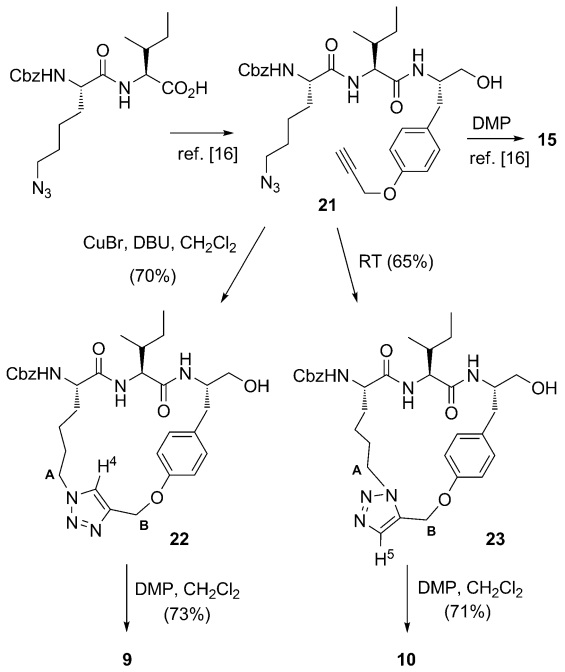
Figure 2. Structures of previously synthesized compounds **1**, **3**–**8**, **11**, **12**, **13**, and **15**;[6a,15,16] new derivatives **2**, **9**, **10**, and **14**; and the known proteasome inhibitor MG132.[14]

Results and Discussion

Synthesis: The macrocycles were prepared by using two general routes. The first route was used to prepare **2**, **9**, and **10** and this employed a copper-catalyzed azide–alkyne cycloaddition (CuAAC) of a suitable tripeptide-based azido–alkyne (see ester **18** and alcohol **21**, Schemes 1 and 2, respectively)



Scheme 1. Synthesis of the macrocyclic aldehyde **2**. DIPEA = *N*,*N*-diisopropylethylamine.



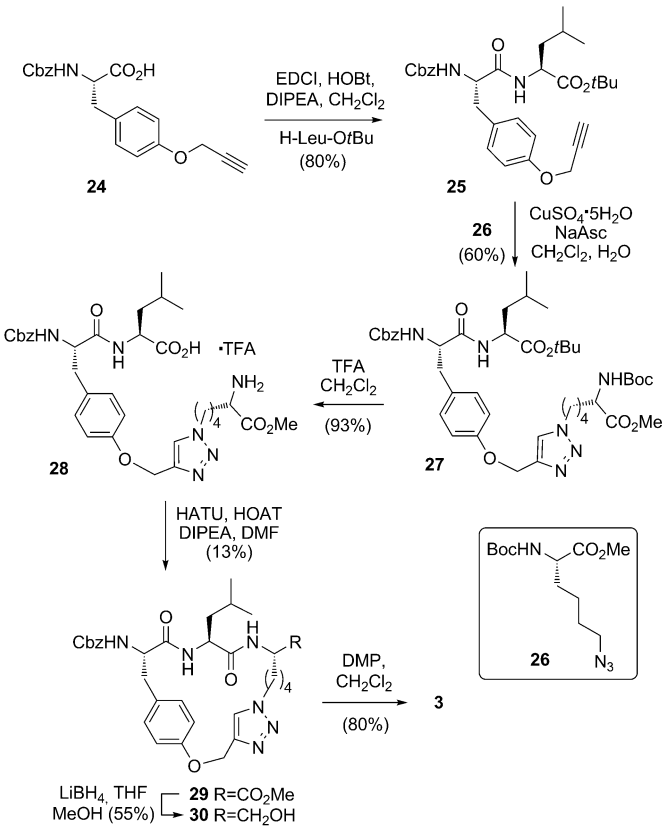
Scheme 2. Synthesis of the macrocyclic aldehydes **9**, **10**, and acyclic azido–alkyne aldehyde **15**.

as the key macrocyclization step. Such an approach has been reported by us previously for the preparation of compounds **1**, **3**–**8**, and **11**.[6a,15,16] An alternative route that employs a lactamization reaction for the key cyclization step was also investigated for the preparation of **3** and **5** (Schemes 3 and 4, see later).

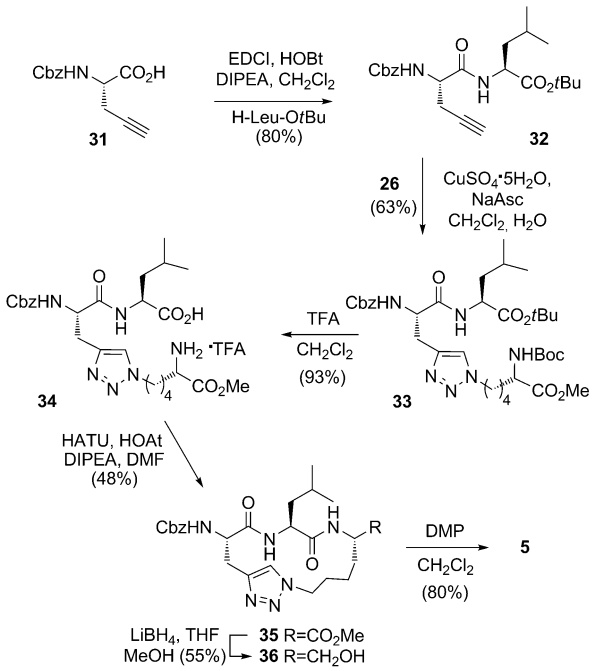
In the first instance, coupling of dipeptide **16**[6a] with **17**, in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (EDCI) and *N*-hydroxybenzotriazole (HOBt), gave tripeptide **18** and this was cyclized to give **19** on treatment with CuBr and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The ester of **19** was reduced with lithium borohydride to give the crude alcohol **20**, which was oxidized with Dess–Martin periodinane (DMP) to give the required aldehyde **2** in an overall yield of 52% after final column chromatography. Lithium borohydride is the reagent of choice for such reductions,[1c,10b] and a number of methods have been reported for the subsequent oxidation. These include Swern oxidation[17] and SO_3 pyridine in the presence of DMSO.[1c,10b,c] This last method is optimal for the larger scale preparation of macrocyclic peptidomimetic aldehydes;[10b] however, in the current study it leads to problems with isolation and reduced yields. It is also important to note that macrocyclic aldehydes[1c,6a,10b] are less prone to the epimerization that has been noted[17] for some linear analogues.

The synthesis of macrocyclic aldehydes **9** and **10** involved a modified sequence in which an acyclic azido–alkyne alcohol (**21**), rather than an ester (see **18** in Scheme 1), was used in the key Huisgen cycloaddition step (Scheme 2). This route has the advantage of avoiding the somewhat problematic reduction of a macrocyclic ester to its corresponding alcohol.[6a] It also provided access to the acyclic azido–alkyne aldehyde analogues[6a,16] (**12**–**15**) by oxidation of the corresponding alcohol as shown for **15** in Scheme 2. Thus, treatment of **21**[16] with CuBr and DBU in CH_2Cl_2 gave the macrocyclic alcohol **22**, which was oxidized with DMP to give **9**. In this case there was some epimerization (≈10%) α to the aldehyde, based on the ^1H and ^{13}C NMR spectra. Interestingly, a solid sample of **21**, left for a number of months at room temperature, cyclized to produce the alternative 1,5-disubstituted triazole **23**, which was subsequently oxidized with DMP to give the corresponding aldehyde **10**. The structures of **22** and **23** were confirmed by NMR spectroscopy. Characteristic resonances at $\delta = 7.96$ and 7.82 ppm were observed for the triazole H^4 and H^5 protons of **22** and **23**, respectively (see Scheme 2 for numbering). These assignments were confirmed by using rotating-frame Overhauser effect NMR spectroscopy (ROESY) of the 1,4-substituted triazole **22**, which showed diagnostic through-space interactions between the H^4 triazole proton and both adjacent methylene protons **A** and **B**. By comparison, the 1,5-substituted triazole **23** only showed through-space interactions between methylene protons **B** and the H^5 triazole proton (see the Supporting Information).

The macrocycles **3** and **5** were prepared by an alternative strategy in which the macrocycle is introduced by lactamization, rather than Huisgen cycloaddition (see Schemes 3 and



Scheme 3. Synthesis of the macrocyclic aldehyde **3** through amide formation. TFA = trifluoroacetic acid.



Scheme 4. Synthesis of the macrocyclic aldehyde **5** through amide formation.

4). In particular, intermolecular Huisgen cycloaddition of acetylene **25** and azide **26**,[6b] on treatment with $CuSO_4$ and sodium ascorbate, gave triazole **27** (Scheme 3). The C- and N-termini of **27** were simultaneously deprotected with TFA in CH_2Cl_2 to give **28**, which was cyclized on reaction with EDCI and 1-hydroxy-7-azabenzotriazole (HOAt) to give the macrocycle **29** in a modest 13% yield. The desired aldehyde **3** was obtained by reduction of **29**[6a] with lithium borohydride and subsequent oxidation of the alcohol **30**[6a] with DMP to produce aldehyde **3**. The macrocycle **5** was similarly prepared from **31**[18] as shown in Scheme 4. In this case, lactamization of the key dipeptide **34** to give **35**[15] occurred with an improved yield of 48%. Based on these results it is apparent that introduction of the macrocycle is generally best achieved by the Huisgen cycloaddition (see Schemes 1 and 2), rather than lactamization (see Schemes 3 and 4).

Conformational analysis: The solution structures of the new macrocycles **2**, **9**, and **10** were determined on the basis of $^3J_{NHC\alpha H}$ coupling constants as previously reported for compounds **1**, **3**–**8**, and **11**.[6a,15] The magnitude of this coupling constant is dependent on the angle Φ, as defined by the local conformation of the polypeptide backbone.[19] For a β-sheet conformation these values are typically in the range of $^3J_{NHC\alpha H}=8$ to 10 Hz, whereas for an unstructured random coil a value of $^3J_{NHC\alpha H}=5.8$ to 7.3 Hz is typical.[19a] The macrocycles **4**–**11** displayed $^3J_{NHC\alpha H}$ coupling constants of greater than 8 Hz, which suggests[19a] a Φ torsion angle of $\approx -120°$ and hence a β-strand geometry. The ROESY spectra of **4**–**11** revealed characteristic NOE interactions between NH$i\rightarrow$NH(i+1) and CαH$i\rightarrow$NH(i+1), which is diagnostic of a β-strand conformation.[6a] By comparison, compounds **1**–**3** gave $^3J_{NHC\alpha H}$ coupling constants of less than 8 Hz, which suggests that these do not adopt a β-strand conformation.

Compound **5** was also docked into the crystal structures of calpain II (PDB 3BOW)[20] and cathepsin S (PDB 1GLO)[21] to define its mode of binding and to compare its backbone conformation with that of **35**, as defined in the solid state by using X-ray crystallography.[15] Compound **35** is the synthetic precursor to **5** and it contains the identical ring system. Docking studies and shape overlays were performed by using FRED (version 3.0.0), and ROCS (version 3.1.1), respectively,[22] with details of the protocols employed provided in the Supporting Information. The structures thus obtained in overlay with docked compound **5** (calpain II and cathepsin S) are shown in Figures 3 and 4. The backbone dihedral angles Φ and Ψ obtained for **5** docked into the cathepsin S were measured to be −95 and +133.2°. These values are consistent with the expected β-strand conformation.[19b] The crystal structure of **35** also reveals that its component macrocycle adopts a β-strand geometry, with the equivalent angles Φ and Ψ being −125.2 and +122.6°.[15] The solid-state peptide-backbone structure of **35** clearly superimposes well with that in the docked structure **5** (calpain II and cathepsin S). Therefore, both structures clearly adopt a β-strand conformation.

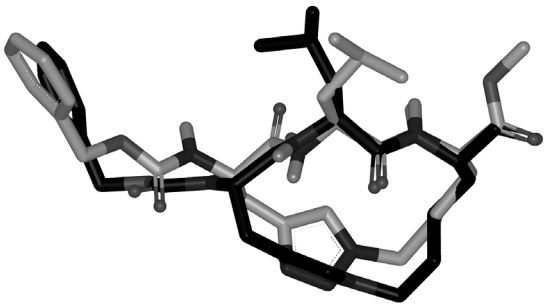


Figure 3. Superimposition of the crystallographic structure of **35** (grey) with **5** (black) docked into calpain II.

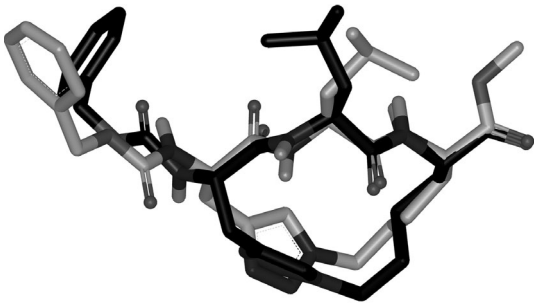


Figure 4. Superimposition of the crystallographic structure of **35** (grey) with **5** (black) docked into cathepsin S.

Biological data: The inhibitory potencies of the macrocyclic aldehydes **1**–**11** and the acyclic azido–alkyne aldehydes **12**–

Table 1. In vitro inhibition data for macrocyclic compounds **1**–**11** and acyclic compounds **12**–**15**.

Compound	β-Strand conformation[a]	P2 amino acid	Ring size	Cysteine proteases IC_{50} [nM]			Proteasome IC_{50} [nM][b,c]		
				Calpain II[b,d]	Cat L[e]	Cat S[e]	CT-L	CP-L	T-L
1	no[f]	Leu	18	1020[f]	190	22	3000	>25000	>25000
2	no	Leu	19	360	1200	87	>25000	>25000	>25000
3	no[f]	Leu	21	940[f]	1900	39	3600	>25000	>25000
4	yes[g]	Leu	15	582[g]	310	10	>25000	>25000	>25000
5	yes[g]	Leu	15	355[g]	920	27	>25000	>25000	>25000
6	yes[f]	Leu	20	137[f]	35	2.3	970	>25000	>25000
7	yes[f]	Leu	21	97[f]	35	3.0	250	>25000	>25000
8	yes[f]	Phe	21	89[f]	23	1.6	310	>25000	>25000
9[h]	yes	Ile	21	410	12	2.7	360	>25000	>25000
10	yes[g]	Ile	20	390[g]	47	3.9	250	>25000	>25000
11	yes	Leu	17	697	480	20	>25000	>25000	>25000
12	nd[i]	Leu	–	780[f]	34	2.5	54	>25000	>25000
13	nd[i]	Leu	–	1030[f]	87	4.6	150	>25000	>25000
14	nd[i]	Phe	–	490	47	1.5	nd[i]	>25000	>25000
15	nd[i]	Ile	–	390	32	2.2	20	>25000	>25000

[a] β-Strand conformation was determined on the basis of $^3J_{NHC\alpha H}$ coupling constants from ^{1}H NMR spectra. [b] Values are the mean of three experiments and variation between experiments is $< \pm 5\%$. [c] Final concentration of substrates was 50 μM. K_m values were 48 μM for Suc-LLVY-AMC (CT-L),[26a] >500 μM for Boc-LRR-AMC (T-L),[26b] and 120 μM for Cbz-LLE-AMC (CP-L).[26c] [d] The final concentration of BODIPY-FL casein was ≈0.09 μM. K_m value for calpain II was 0.64 μM.[26d] [e] Data were calculated from experiments with five different inhibitor concentrations. IC_{50} values were obtained by nonlinear regression, with standard errors <20% except Cat S values for **6** (24%), **8** (29%), **9** (21%) and **13** (22%). Assays were performed with the chromogenic substrate Cbz-Phe-Arg-*p*NA (*p*NA = *p*-nitroanilide) at a final concentration of 100 μM. K_m values were 17 μM for Cat L and 118 μM for Cat S.[24c–e] [f] Taken from ref. [6a]. [g] Taken from ref. [15]. [h] Contains ≈10% of epimer (α to the aldehyde) based on NMR spectroscopic analysis. [i] nd = not determined.

15, against calpain II, cathepsin L (Cat L), cathepsin S (Cat S), and the three activities of the 20S proteasome (i.e., its chymotrypsin-like (CT-L), caspase-like (CP-L), and trypsin-like (T-L) activities) were investigated and the results are shown in Table 1. All compounds were potent inhibitors of Cat S, with derivatives **6**–**10** and **12**–**15** exhibiting IC_{50} values of less than 5 nM. A number of these compounds (**6**–**10**, **12**, **14**, and **15**) were also highly active against Cat L, with IC_{50} values in the range of 10–50 nM. There is some correlation between activity and the ability of the macrocycles to adopt a β-strand geometry as discussed below. The macrocyclic aldehydes **6**, **7**, and **8** were the most active compounds of the series against calpain II, with IC_{50} values of 137, 97, and 89 nM, respectively. The 20- and 21-membered macrocyclic compounds **6**–**10**, all of which adopt a β-strand geometry, were good inhibitors of CT-L activity with an IC_{50} <1 μM. Very strong inhibition of CT-L was also observed for the acyclic azido–alkynes **12** and **15**, which exhibit IC_{50} values of 54 and 20 nM, respectively. Interestingly, all compounds were inactive against CP-L and T-L (IC_{50} >25 μM). All the compounds investigated showed the greatest activity against Cat S, particularly so in the case of macrocycles **3**, **4**, **11**, and **14**. Several classes of highly potent inhibitors for Cat S have been reported recently that contain an electrophilic warhead that interacts with the active-site cysteine.[10a] Nitrile-based compounds have attracted particular interest, some of which exhibit K_i values toward Cat S in the subnanomolar range.[23] Our study is unique in that it addresses the role of constraining the backbone of an inhibitor into a β-strand geometry, almost uniformly known to favor binding to proteases.

Not all the macrocycles are constrained into a β-strand, with **4**–**11**, but not **1**–**3**, adopting this geometry. In accordance with previous studies on derivatives of Cat 0811 (Figure 1)[1c] a 19-membered macrocycle, as in **2**, does not constrain the backbone into a β-strand. Interestingly, however, the 20- and 21-membered macrocycles **6**–**10** containing a polar triazole ring at P3 and a more hydrophobic tyrosine at P1, do adopt this geometry. In general, the compounds with β-strand conformation were more potent towards Cat L (**6**–**10**), Cat S (**4**, **6**–**11**) and calpain II (**6**–**8**).

A comparison of the inhibitory activity of the macrocycles **6**–**9** with that of their acyclic azide–alkyne analogues **12**–**15** provides some insight into the effect of macrocyclization on potency. The macrocycles of **6**–**9** generally enhances the potency of inhibition for both Cat L and calapin II compared with the acyclic analogue. Interestingly, potency against CT-L is greatest for the acyclic azide–alkyne analogues, whereas the data for Cat S suggests that constraining the backbone into a β-strand geometry with a macrocycle has little effect with all derivatives being highly potent. This second observation may reflect in part the inability of the assay to discriminate between these particularly potent compounds, as their single-digit nanomolar IC_{50} values are in the same range as the concentration of Cat S in the assay. Future kinetic studies are needed to fine-tune the structure–activity relationships of these macrocyclic Cat S inhibitors. Whereas a β-strand geometry is almost universally known to favor ligand binding to a protease,[6a] there is some recent suggestion that a macrocycle is not favored for the proteasome.[16] Whereas inhibitors of the proteasome reportedly adopt hydrogen bonds with the protease that are characteristic of binding in this geometry, unlike other proteases, the P2 group does not seem to form important contacts with the active site.[1a]

Some insight into the influence of the key P1 substituent on the inhibition of proteases is apparent on comparing macrocycles **7** and **11**. An aromatic Tyr at P1 as in macrocycle **7** considerably enhances inhibitory potency against calpain II (7-fold), Cat L (14-fold), Cat S (7-fold), and CT-L (>100-fold) relative to a triazole at P1 as in **11**. Thus an aromatic (nontriazole) residue at P1 appears to contribute to binding within the active site of these proteases. Further in-

sight into the unfavorable influence of having a triazole at P1 (as in **1**–**4** and **11**) toward activity (particularly calpain II) was apparent from the docking of these structures into calpain II (PDB entry 3BOW). For **1**–**4**, the lone pairs of the triazole nitrogen atoms give rise to hydrogen-bond-acceptor clashes with the carbonyl and hydroxyl of Gly_{103} and Ser_{196}, respectively. For **11**, the oxygen atom of the macrocycle appears to similarly clash with the carbonyl oxygen atom of Gly_{103}.

The nature of the hydrophobic P2 substituent appears to have little effect on the activity of the macrocyclic and acyclic compounds for all four proteases, with Leu (**7**, **13**), Phe (**8**, **14**) and Ile (**9**, **15**) all being well-tolerated. For Cat S, these findings reflect the known plasticity of the S2 pocket of cathepsin S, which can accommodate aliphatic and aromatic residues of corresponding P2 amino acids.[24] A comparison of compounds **3** and **7** is interesting as they differ only in the relative positions of the P1 and the P3 substituents. The latter derivatives are significantly more potent with 10- to 54-fold enhanced activity against calpain II (97 vs. 940 nM), Cat L (35 vs. 1900 nM), Cat S (3.0 vs. 39 nM), and CT-L (250 vs. 3600 nM).

Finally, the influence of the triazole substitution pattern (either 1,4- or 1,5-disubstituted) was investigated with compounds **9** and **10**, respectively. 1,5-Disubstitution (**10**) results in similar potency against calpain II, Cat S, and the CT-L to that of the 1,4-disubstituted peptidomimetic **9**, but significantly decreased activity towards Cat L (4-fold). This observation may provide an avenue for enhancing selectivity for a range of proteases over Cat L.

Conclusion

In this study, we have presented the first examples of potent macrocyclic inhibitors of Cat S obtained by using a Huisgen cycloaddition reaction. The most active representatives of the series were constrained into a well-defined β-strand geometry identified by NMR spectroscopy, X-ray analysis, and molecular docking studies. The make-up of the macrocycle within the peptidomimetics plays a role in defining potency, and this effect will be further investigated with respect to other key proteases. It is worth noting that the aldehyde warhead found in macrocyclic protease inhibitors of the type presented here has already found some use in topical applications associated with the potential treatment of cataracts.[1c,25]

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Protease Inhibitors

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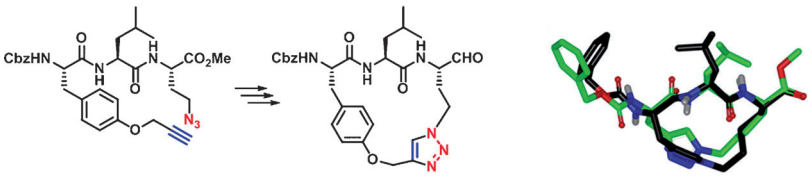
Synthesis and Extended Activity of Triazole-Containing Macrocyclic Protease Inhibitors



Clicked to fit: Macrocyclic protease inhibitors, constrained into a β-strand geometry by using Huisgen cycloaddition, are shown to inhibit a range of proteases. The geometries of the component peptide backbones were defined by NMR spectroscopy, X-ray crystallography, and docking studies (see scheme; Cbz = carbobenzyloxy).