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New β-Strand Templates Constrained by Huisgen Cycloaddition

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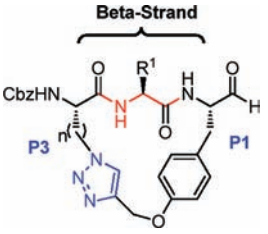
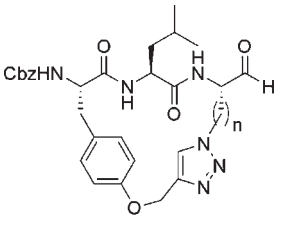
ABSTRACT



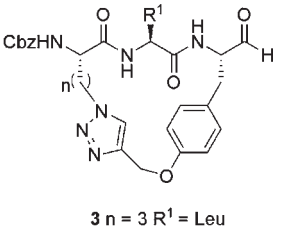
New peptidic templates constrained into a β-strand geometry by linking acetylene and azide containing P_1 and P_3 residues of a tripeptide by Huisgen cycloaddition are presented. The conformations of the macrocycles are defined by NMR studies and those that best define a β-strand are shown to be potent inhibitors of the protease calpain. The β-strand templates presented and defined here are prepared under optimized conditions that should be suitable for targeting a range of proteases and other applications requiring such a geometry.

The biological function of a peptide is inadvertently linked to its conformation or shape. With this in mind, much work has been reported on covalently constraining a linear peptide into a well-defined conformation, such as an α-helix or a β-strand, to provide important biological probes, scaffolds, and enzymes inhibitors.[1−13] Of particular significance is the use of RCM,[4] intramolecular alkyltion,[14] and other methods of macrocyclization[15] to constrain a protease inhibitor into a biologically active β strand conformation.

The Huisgen cycloaddition reaction of an acetylene with an azide offers much promise in this context, with reports on its use to prepare macrocyclic peptidomimetics to improve biostability and restrict conformational mobility, but often in an ill-defined way.[5] What we lack are rationally designed templates that have a well-defined and predictable conformation and hence wide and general applicability. Here we report an azide alkyne Huisgen



1 n = 1
2 n = 4



3 n = 3 R^1 = Leu
4 n = 4 R^1 = Leu
5 n = 4 R^1 = Phe

Figure 1. Target macrocycles.

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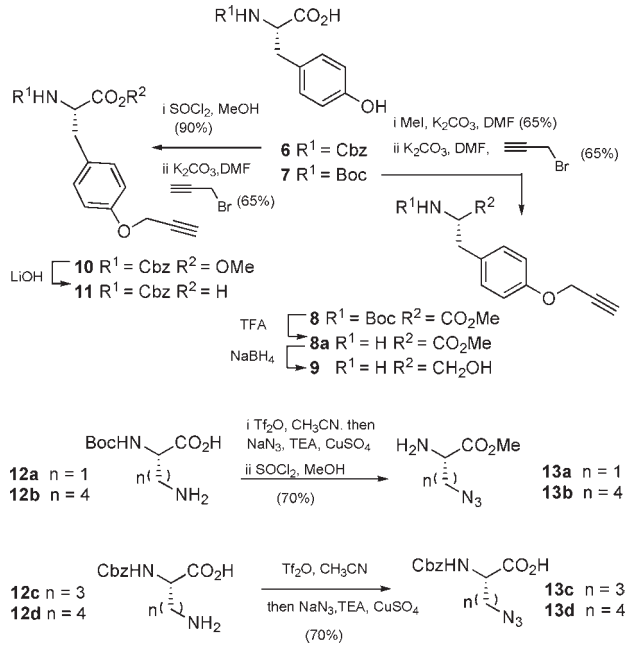
cycloaddition-based synthesis of P_1 and P_3-linked peptides[16] and a conformational study to identify those that best mimic a β-strand geometry. As proof of concept, these macrocycles contain a C-terminal aldehyde to provide inhibitors of the protease calpain, which like all proteases, is known to favor the binding of substrates and inhibitors in this conformation.[17] The inhibition of calpain is of particular therapeutic significance since it is involved in critical diseases,[18−21] with few examples of such cysteine proteases having reached the clinic despite this need.[22,23] The macrocycles targeted in this study contain a component aryl group, in addition to the triazole, to further constrain the geometry (Figure 1). One series has this group at P_3 (see **1** and **2**) and the other at P_1 (see **3**−**5**). In addition, a hydrophobic amino acid was inserted at P_2 to accommodate the known binding preferences of calpain.[4] To the best of our knowledge, this is the first report on using a click reaction to define a specific β-strand geometry.

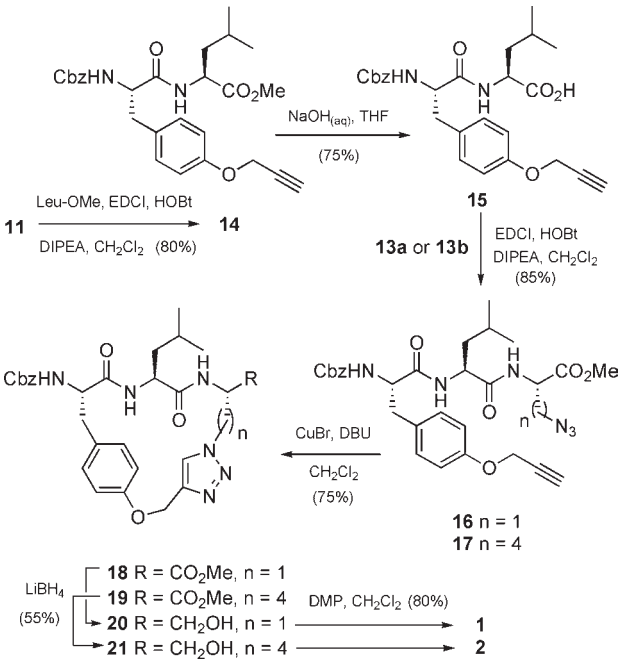
The synthesis of macrocycles **1** to **5** required the preparation of the key acetylenes **9** and **11** and the azides **13a**−**d** as shown in Scheme 1. The acetylene **9** was prepared from *N*-Boc-Tyr-OH **7** by methylation with MeI, followed by alkylation with propargyl bromide to give **8**. Removal of the Boc group and reduction with $NaBH_4$ then gave **9**. Reaction of *N*-Cbz-Tyr-OH **6**[24] with $SOCl_2$ and methanol, followed by reaction with propargyl bromide in presence of K_2CO_3, gave **10**. The methyl ester was then hydrolyzed to give **11**. The azides **13a**−**d** were prepared from amines **12a**−**d** with the key step being reaction with Tf_2O and NaN_3.[25]

The macrocyclic aldeydes were then prepared as shown in Schemes 2 and 3. Reaction of **11** with Leu-OMe in the presence of EDCI, HOBt gave dipeptide **14**, which was hydrolyzed to give the acid **15**. Separate coupling of **15** with **13a** and **13b**, in the presence of EDCI and HOBt, gave tripeptides **16** and **17**, respectively. These were then cyclized, in the presence of Cu(I)Br in CH_2Cl_2, and the resulting macrocyclic esters **18** and **19** reduced with lithium borohydride to give the macrocyclic alcohols **20** and **21** that were purified by HPLC. Oxidation of these alcohols, with *Dess-Martin* periodinane (DMP),[23] gave the required aldehydes **1** and **2** (Scheme 2).

Scheme 1. Synthesis of Key Building Blocks



Scheme 2. Synthesis of Macrocylic Aldehydes **1** and **2**



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(16) Note the use of Schechter-Berger nomenclatureSchechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157. The residues on the N-terminal side of the peptide bond that is cleaved are denoted (in order) P_1−P_n, and those on the C-terminus are denoted $P_{1'}$−$P_{n'}$. In turn, the corresponding subsites on the enzyme are denoted S_n−$S_{n'}$.

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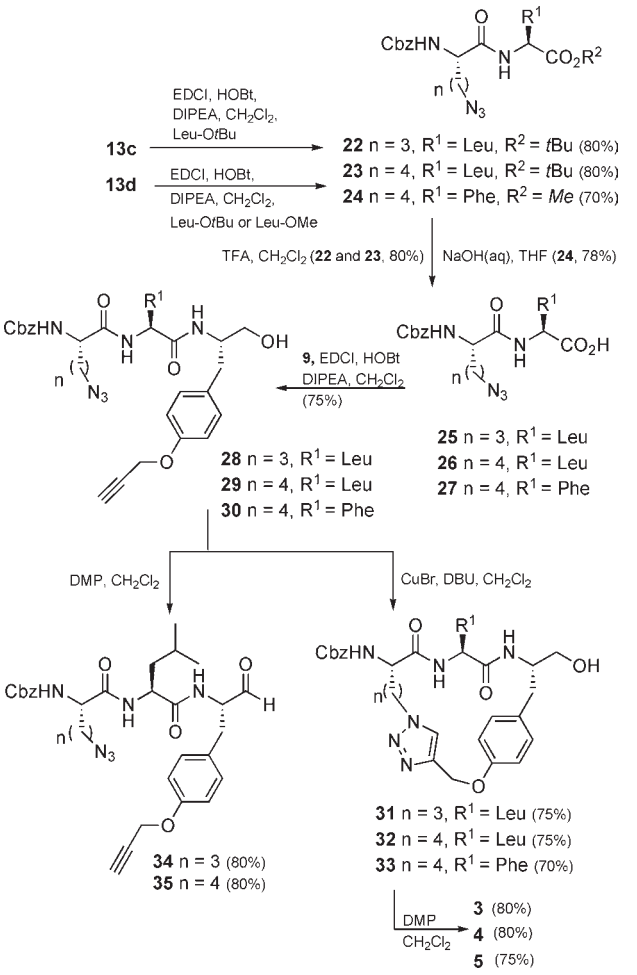
The remaining macrocyclic aldehydes **3**–**5** and the acyclic analogues **34** and **35** were prepared using a modified protocol as shown in Scheme 3. Unlike the sequence shown in Scheme 2 (see step v) the ester functionality was reduced to the corresponding alcohol (**9**) prior to coupling and cyclization. This has the advantage of a reduced reaction time for the reduction and also removal of the need for purification of the macrocycle by HPLC, as was the case for **20** and **21** in Scheme 2. As such separate reaction of **13c** and **13d** with Leu-O*tBu* and Phe-OMe, in the presence of EDCI and HOBt, gave dipeptides **22**–**24** that were separately hydrolyzed to give **25**–**27**. Coupling of each of these with the alcohol **9**, in the presence of EDCI and HOBt, gave tripeptides **28**–**30** that were cyclized on treatment with Cu(I)Br in CH_2Cl_2 to give **31**–**33** in good yields. The macrocyclic alcohols **31**–**33** and the corresponding acyclic alcohols **28** and **29**, were oxidized with Dess-Martin periodinane (DMP) to give the required aldehydes **3**–**5**, **34**, and **35**, respectively. The preparation of macrocycle **32** via an analogous route to that used in Scheme 2 resulted in significantly reduced yield.

The details of the CuAAC catalyzed cyclizations shown in Schemes 2 and 3 are worthy of comment. The cyclization of related peptidic structures (using Cu(I)/Cu-$(CH_3CN)_4PF_6$) is reported to give competing dimer and trimer formation,[26,27] necessitating purification by HPLC. In addition, elevated temperatures are often reported for such reactions.[28] Our improved methodology is mild, high yielding, and occurs without competing dimerization to give the macrocycle that is purified by simple silica-based chromatography.

Conformation of the Macrocycle. Solution structures for the macrocycle aldehydes **1**–**5** and the alcohols **20, 21** and **31**–**33** were determined based on $^3J_{NHC\alpha H}$ coupling constants, see Table 1. The magnitude of this coupling constant is dependent on the angle Φ, as defined by the local conformation of the polypeptide backbone.[1,10,14] For a β-sheet conformation these values are typically in the range 8 to 10 Hz, while for an unstructured random coil a value of 5.8 to 7.3 Hz is typical.[10] Macrocycles **3**, **5**, **31**, **32**, **33** all displayed $^3J_{NHC\alpha H}$ coupling constant >8 Hz, which is consistent with a β-strand conformation. Only one coupling constant could be determined for **4** because of overlapping resonances. By comparison, compounds **1**, **2**, **20** and **21** appear not to adopt a β-strand conformation based on the $^3J_{NHC\alpha H}$ coupling constants. The determination of NOE data confirmed these

Scheme 3. Synthesis of Aldehydes **3**–**5**, **34**, and **35**



observations. Characteristic[29–31] NOE interactions were observed between $C\alpha H_i$ and $(i+1NH)$, βH_i and $(i+1NH)$, NH_i and $(i+1NH)$ for **3**, **5**, **31**, **32**, **33**, see Supporting Information Figure S1 for an example. Thus, the macrocycles containing a triazole at P3 and a tyrosine analog at P1 (see **3**, **5**, **31**, **32**, **33**) appear to adopt β-strand conformation known to favor binding to a protease.

Variable temperature NMR studies on the macrocycle **5** in DMF-D_7 showed its β-starnd conformation to be stable within the temperature range 223–353 K. The temperature dependence coefficients ($\Delta\delta/\Delta T$) for the two NH's within the macrocycle of **5**, over this temperature range, were calculated to be 6.5 and 5.0 ppb K^{-1}. This large temperature dependence is characteristic of a lack of intramolecular hydrogen bond between the P_3 C=O and P_1 NH and hence a β-starnd conformation.[31,32] This conformational stability is likely associated with a combination of the constituent phenyl and triazole rings, which help to lock the macrocycle into the β-strand conformation. Unlike other macrocyclic protease inhibitors the constituent phenyl group of **5** appears to be rotationally rigid, as evidenced by distinct resonances

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for each of the four aromatic protons in its ^{1}H NMR spectrum.[14] These remain sharp over the temperature range 223−353 K, see Supporting Information Figure S3.

Table 1. $^3J_{NHC\alpha H}$ Coupling Constants[a]

no.	Leu NH−CH_α	Tyr NH−CH_α	Phe NH−CH_α
1	7.4	7.9	
2	8.1	7.5	
3	8.4	8.4	
4	7.6	[b]	
5		8.4	8.4
20	8.5	7.1	
21	7.9	8.1	
31	8.2	9.0	
32	8.7	8.5	
33		8.8	8.4

[a] Coupling constants determined in DMSO-d_6. [b] Not determined because of overlapping resonances.

Biological Data. Compounds **1**−**5**, and the acyclic analogues **34** and **35** were assayed against calpain-II using a fluorescence-based assay[33] to determine *in vitro* potency, with the results shown in Table 2. The 21-membered macrocyclic aldehydes **4** and **5** were particularly potent, with IC_{50} values against calpain II of 97 nM and 89 nM, respectively. The nature of the hydrophobic P_2 substituent has little effect on activity, with both Leu (**4**) and Phe (**5**) being well tolerated at this position. The related 20-membered macrocycle **3** had a slightly reduced potency (IC_{50} of 137 nM). This data is consistent with the conformational analysis that showed that macrocycles **3** and **5** adopt a β-strand conformation that is known to favor protease binding. The conformation of **4** could not be defined but is presumed to also be a β-strand because of its close similarity to **5**. The macrocyclic aldehydes with the triazole at P_1 (see **1** and **2**) were significantly less potent against calpain II, presumably since they do not appear to adopt a β-strand geometry. Importantly, the acyclic aldehydes **34** and **35** were significantly less potent than their cyclic analogues **3** and **4** respectively. Thus, the conformation constraint imposed by the macrocycle improves potency.

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Table 2. *In Vitro* Inhibition Data

no.	R	*n*	ring size	β-strand conformation	IC_{50}[b] (μM) calpain II
1	Leu	1	18	No	1.020
2	Leu	4	21	No	0.940
3	Leu	3	20	Yes	0.137
4	Leu	4	21	[a]	0.097
5	Phe	4	21	Yes	0.089
34	Leu	3			0.780
35	Leu	4			1.030

[a] Not determined because of overlapping resonances. [b] Values are the mean of three experiments and variation between experiments is $< \pm 10\%$.

In summary, we present an optimized azide alkyne Huisgen cycloaddition-based synthesis of macrocyclic peptidomimetics constrained into a well-defined β-strand geometry designed to facilitate binding to the proteases calpain. Introduction of the macrocycle improves potency, with examples adopting a β-strand geometry providing the optimum template for binding. The β-strand templates presented and defined here are prepared under chemically benign conditions and should be suitable for targeting a range of proteases and other applications requiring such a geometry. The conditions used for the macrocyclisation avoid competing dimer and trimer formation that have been reported for related systems.

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Supporting Information Available. Experimental procedures, characterization data, and spectra of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.