New β -Strand Templates Constrained by Huisgen Cycloaddition

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New peptidic templates constrained into a β -strand geometry by linking acetylene and azide containing P₁ and P₃ 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

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10.1021/ol3002199 © 2012 American Chemical Society Published on Web 02/16/2012 particular significance is the use of RCM,⁴ intramolecular alkyltion,¹⁴ and other methods of macrocyclization¹⁵ 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



Figure 1. Target macrocycles.

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cycloaddition-based synthesis of P1 and P3-linked peptides¹⁶ 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 P₂ 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

Scheme 1. Synthesis of Key Building Blocks

CO₂R²

BocHN

CbzHN

n(근)

=

R¹HN

LIOH

12a n = 1

12b n = 4

12c n = 3

give 8. Removal of the Boc group and reduction with NaBH₄ then gave 9. Reaction of *N*-Cbz-Tyr-OH 6^{24} with SOCl₂ 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-dwere prepared from amines 12a - d with the key step being reaction with Tf₂O and NaN₃.²⁵

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 CH₂Cl₂, 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).



R¹HN. ,CO₂H i SOCI₂, MeOH i Mel, K₂CO₃, DMF (65%) (90%) ii K₂CO₃, DMF, 🛛 💳 6 R¹ = Cbz (65%) ii K₂CO₃,DMF 7 R¹ = Boc Br (65%) .R² R¹HN 10 R¹ = Cbz R² = OMe 11 R¹ = Cbz R² = H 8 R^1 = Boc R^2 = CO₂Me TFA **8a** R¹ = H R² = CO₂Me NaBH₄ -9 R¹ = H R² = CH₂OH i Tf₂O, CH₃CN. then .CO₂H NaN3, TEA, CuSO4 H_2N ,CO₂Me n([±]) N₃ ii SOCl₂, MeOH **13a** n = 1 13b n = 4 NH₂ (70%)CbzHN. ,CO₂H CO₂H Tf2O, CH2CN 13c n = 3

n([±]) N₃ n^{(≟}√_{NH2} then NaN3,TEA, CuSO4 13d n = 412d n = 4 (70%)

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Scheme 2. Synthesis of Macrocylic Aldehydes 1 and 2

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-OtBu 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 CH₂Cl₂ 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-(CH₃CN)₄PF₆) 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 ${}^{3}J_{\rm NHC\alpha H}$ 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 ${}^{3}J_{\rm NHC\alpha H}$ 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 ${}^{3}J_{NHC\alpha H}$ coupling constants. The determination of NOE data confirmed these





observations. Characteristic^{29–31} NOE interactions were observed between C α H_i and (*i*+1NH), β H_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 P₃ C=O and P₁ 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 ¹H NMR spectrum.¹⁴ These remain sharp over the temperature range 223–353 K, see Supporting Information Figure S3.

Table 1. ${}^{3}J_{\rm NHC\alpha H}$ Coupling Constants ^{<i>a</i>}						
no.	$Leu\;NH{-}CH_{\alpha}$	$\mathrm{Tyr}\mathrm{NH}\mathrm{-}\mathrm{CH}_\alpha$	Phe $\rm NH-CH_{\alpha}$			
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 assay³³ to determine *in vitro* potency, with the results shown in Table 2. The 21-membered macrocyclic aldehydes 4 and 5 were particularly potent, with IC₅₀ values against calpain II of 97 nM and 89 nM, respectively. The nature of the hydrophobic P₂ substituent has little effect on activity, with both Leu (4) and Phe (5) being well tolerated at this position. The related 20membered macrocycle 3 had a slightly reduced potency (IC₅₀ 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

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conformation constraint imposed by the macrocycle improves potency.

Table ? In Vitra Inhibition Data							
no.	R	n	ring size	β -strand conformation	$\mathrm{IC}_{50}^{\ \ b}\left(\mu\mathrm{M}\right)$ 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.