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# On Resin Side-Chain Cyclization of Complex Peptides Using CuAAC

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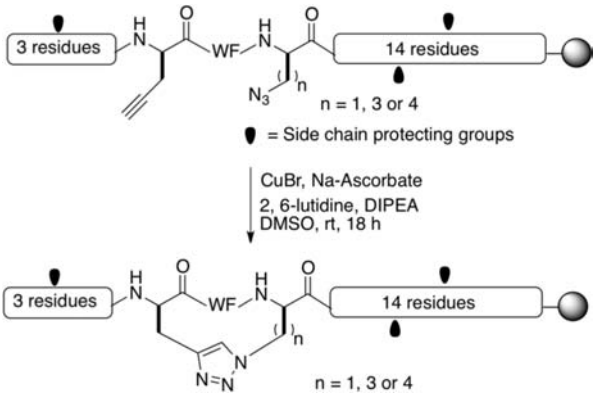
## ABSTRACT




**Triazole tethers have been explored for stabilization of secondary structures in peptides. Despite the utility of this approach, cyclization efficiency in complex peptides remains a significant challenge. A robust, on-resin protocol for side chain to side chain macrocyclization by CuAAC is described. This protocol was applied to the synthesis of a series of 21 amino acid helical peptides presenting a binding dipeptide motif from the membrane proximal external region (MPER) of HIV-1 gp41.**

Understanding the mechanism through which broadly neutralizing antibodies (bNAbs) are elicited against the membrane proximal external region (MPER) of HIV-1 gp41 remains an elusive task. The MPER is required for infection of host cells by HIV-1 and is the target of three bNAbs: 4E10, 2F5, and Z13e1.[1,2] These antibodies are valuable tools for understanding relevant conformations of the MPER and for studying HIV-1 neutralization.[3] The crystal structure of the bNAb 4E10 bound to the native peptide $N^{671}$WFDITNWLWYIK$^{683}$-KKK (numbering is based on HIV-1 HxB2 strain) has revealed that the C-terminus of the epitope adopts a highly helical conformation (near the TM domain) that abruptly unwinds toward its N-terminus as the peptide exits the antibody-binding pocket.[4,5] Furthermore, the N-terminal primary binding residues "WF" adopts a pronounced kinked conformation, which is important for both the epitope conformation and antibody recognition.[6,7]

In previous work, a side-chain thioether tether was designed to stabilize the helical, C-terminal domain of the epitope,[8] and cocrystallization of this peptide with 4E10 confirmed successful mimicry of this helical peptide. It was envisioned that the N-terminal WF domain could be further constrained through a side chain *i* to *i*+3 linkage that would mimic the unwound comformation observed in the 4E10 cocrystal structures.[4]

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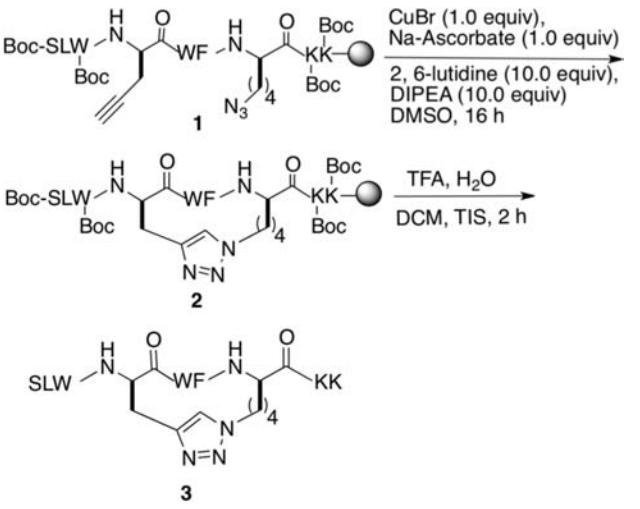
The 1,4-disubstituted [1,2,3] triazole linkage formed by the chemoselective copper-assisted azide–alkyne cycloaddition (CuAAC)[9,10] has found increasing utility in peptide chemistry as an isostere of the amide bond.[11,12] Backbone triazoles have successfully replaced amides in α-helical coiled coils,[13] β-sheet,[14] and β-turn mimics.[15] Further efforts to develop CuAAC for use in peptide mimics have focused on macrocyclization between strategically placed terminal or side-chain azide and alkyne groups. *Intermolecular* CuAAC has been widely used for the conjugation of various moieties to peptides on the solid support.[10−12] In contrast, on-resin *intramolecular* CuAAC to facilitate macrocyclization has produced inconsistent, and sometimes quite unexpected, results.[16,17] For example, the competitive formation products such as cyclodimer and cyclotrimer have been observed.[18] In addition, Chorev and colleagues have elegantly shown the formation of side-chain intramolecular tethers (*i* to *i*+3, *i* to *i*+4, *i* to *i*+5) formed free in solution, while their efforts to carry the same reactions on resin using multiple reaction conditions were not successful.[17,19] Reports of on resin cyclization reactions using CuAAC have been typically limited to short cyclic peptides[20−22,29] and peptoids.[23] Thus, there remains a need for a more general procedure for effective on-resin intramolecular CuAAC for the synthesis of side chain tethered peptides.

Inspired by mechanistic studies of cyclodimerization on resin,[18] it was hypothesized that the reported failures to achieve effective on-resin macrocyclization could be due to intermolecular peptide backbone aggregation, which could be mitigated through the use of highly polar organic solvents or backbone protection.[24] Herein, an on-resin approach is described for the synthesis of intramolecular (*i* to *i*+3) constraint in a highly hydrophobic peptide sequence at positions remote from the C-terminus. It is also possible to manipulate the ring size of the newly formed macrocycle using Fmoc-Lys($N_3$)-OH, Fmoc-Orn-($N_3$)-OH, and Fmoc-Dap($N_3$)-OH amino acids.[25−29] To complement these tethers, helix-enhancing residues were introduced such as α,α-disubstituted amino acid (2-aminoisobutyric acid, Aib) or charged cyclic α,α-disubstituted amino acid (4-aminopiperidine-4-carboxylic acid, Api) at the C-terminus of the MPER epitope.[30]

**Scheme 1.** Model Solid-Phase Synthetic Route for the CuAAC Reaction




A model peptide containing the nine amino acid residues corresponding to the N-terminal portion of the epitope was chosen to test the efficiency of CuAAC in the context of specific side-chain macrocycles. It was anticipated that this peptide would provide framework for the synthesis of larger MPER immunogens having the same amino acid residues in the macrocycle. Thus, Fmoc-Lys($N_3$)-OH was introduced at position 674 of the peptide epitope and L-propargylglycine as an aspargine surrogate at 671. These moieties were placed at *i* and *i*+3 in order to position the key "WF" motif in a constrained conformation. (Scheme 1). The synthesis of this peptide was carried out using commercially available Tenta-gel resin (Scheme 1)[31] and standard Fmoc SPPS protocol to yield fully protected resin

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(31) Fmoc-Rink-amide linker was coupled to amino-TentaGel resin. All coupling reactions were carried out using HBTU/HOBt/DIPEA, except for Fmoc-Lys($N_3$)-OH and Fmoc-L-propargylglycine, which were carried out using HATU/DIPEA in DMF.

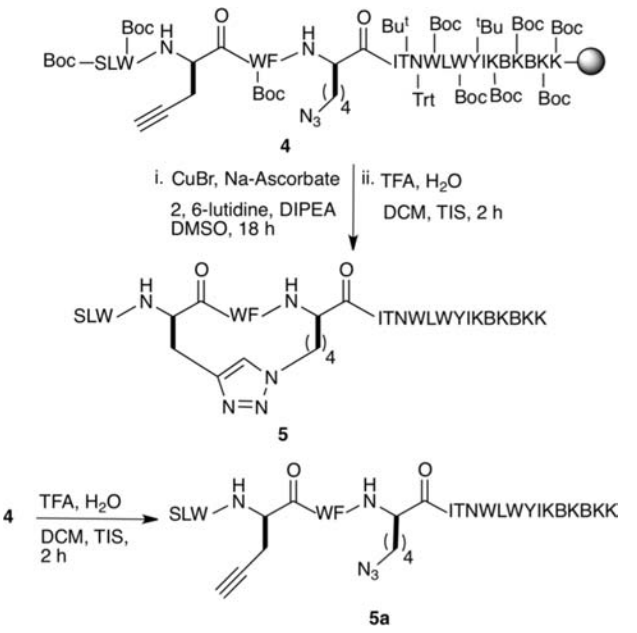
bound peptide **1**. After the chain assembly, part of the resin was washed, dried in vacuo, and cleaved using condition A [TFA (90%), DCM (5.0%), water (2.5%), and TIS (2.5%)] to verify the purity of linear azidoalkyne peptide.

Initial attempts to carry out macrocyclization on resin by using previously reported conditions were not successful.[20−22,32] In order to reduce potential aggregation during macrocyclization, the highly polar solvent DMSO[18] was utilized, and it was found that the CuBr/2,6-lutidine/DIPEA combination[20] was superior to CuI under these conditions.[33] Thus, to generate peptide **3** (Scheme 1) the resin bound peptide **1** was subjected to macrocyclization by adding CuBr (1.0 equiv), sodium ascorbate (1.0 equiv) dissolved in water (20 mg/mL), 2,6-lutidine (10 equiv), and DIPEA (10 equiv) in 1 mL of degassed DMSO for 16−18 h at rt to give resin-bound intermediate **2**. Following extensive washing of the resin-bound peptide and subsequent cleavage from solid support using condition A, the crude cyclized product **3** was obtained in 70% recovered yield. Triazole formation was supported by having substantially different retention times (Figure S1, Supporting Information) and disappearance of the azide stretch (data not shown). It was also important to dissolve CuBr completely in DMSO sparged with $N_2$ before addition to the resin.

Despite the success of on-resin macrocyclization to generate model peptide **3**, the formation of these tethers in longer, more hydrophobic peptide sequences or at a position remote from C-terminus is significantly more challenging. To demonstrate the utility of these reaction conditions in complex systems, the full-length MPER epitope of gp41 668−683 that is recognized by both 4E10 and Z13e1 was synthesized (Scheme 2).[31] The resin-bound peptide **4** was then subjected to the previously optimized macrocyclization using CuBr (1.0 equiv), sodium ascorbate (1.0 equiv) dissolved in $H_2O$ (20 mg/mL), DIPEA (10 equiv), and 2,6-lutidine (10 equiv) in DMSO for 16−18 h at rt, followed by deprotection and cleavage from the resin to give **5** in 65% recovered yield.[34]

Using DMSO for the macrocyclization significantly improved the isolation of tethered peptide **5**. However, significant deletion products were observed in both **5** and linear precursor **5a**. To further improve the overall yield, the aggregation-disrupting pseudoproline dipeptide (Fmoc-IT($\psi^{Me,Me}$ Pro)-OH)[35] was introduced at position 675−676 in the sequence. Subsequent macrocyclization of resin-bound peptide **4** indeed yielded a more homogeneous product **5** in overall recovered yield of 83%. As

**Scheme 2.** Representative Reaction of Synthesis of Peptide Analogues Using CuAAC, B = Aib




shown in Figure 1, the HPLC chromatogram for the linear azido peptide **5a** and corresponding cyclized peptide **5** have substantially different retention times.

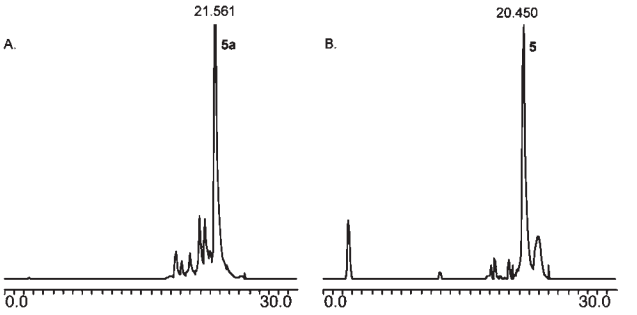



**Figure 1.** HPLC chromatograms: (A) peptide **5a** before CuAAC; (B) peptide **5** after CuAAC.

To evaluate the scope of these conditions toward the ring size, MPER analogues were synthesized using Fmoc-Orn-($N_3$)-OH and Fmoc-Dap($N_3$)-OH in place of Fmoc-Lys-($N_3$)-OH at position 674 in the core epitope (Table 1). In all cases, clean conversion of side chain tethered peptides was obtained in 60−75% recovered yield following a single purification by RP-HPLC. These results suggest that the combination of backbone protection with DMSO to better solvate the peptide polymer network[24] is an effective approach for the on-resin macrocyclization of peptides using CuAAC.

The binding of the triazole-constrained peptide to 4E10 and Z13e1 was evaluated by competitive ELISA (table 1).[30] As expected, all peptides failed to bind Z13e1, which

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(33) Initial attempts to carry out macrocyclization using CuI (0.5 equiv), 2,6-lutidine (1.0 equiv) in DMSO, or DMF for 16 h at rt were not successful. Furthermore, additional equivalents of CuI on a similar peptide primarily formed a peptide with an additional mass of 127 Da. Other reported conditions to form macrocycle in solvents such as DMF and 20% $H_2O$ in NMP also did not yield the desired product.

(34) Unexpectedly, deletion product was observed without the azido amino acid residue during the synthesis of this peptide. The mass of this deletion peptide was 42 Da higher than the expected mass. Aggregation of the peptide-resin may contribute to the formation of this deletion peptide.

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**Table 1.** Amino Acid Sequences and 4E10 and Z13e1 Binding Data ($IC_{50}$) of the Constrained Analogues

| | | $IC_{50}$ ($\mu$M)[b] | |
|---|---|---|---|
| | sequence[a] | 4E10 | Z13e1 |
| **5** | **SLWJWFXITNWLWYIK**BKBKK | 4.8 | >10 |
| **6** | **SLWJWFOITNWLWYIK**BKBKK | 10 | >10 |
| **7** | **SLWJWFZITNWLWYIK**BKBKK | >10 | >10 |
| **8** | **SLWJWFXITNWLWYIK**UKUKK | 0.3 | >10 |
| **9** | **SLWJWFOITNWLWYIK**UKUKK | 1.4 | >10 |
| **10** | **SLWJWFZITNWLWYIK**UKUKK | 4.9 | >10 |

[a] Amino acids shown in bold belong to the native sequence of gp41 MPER. B indicates Aib, U indicates Api (4-aminopiperidine-4-carboxylic acid), J = propargylglycine, X = lysine azide, O = ornithine azide, Z = diaminopropionic acid azide. The underlined amino acids are in a cyclic conformation. [b] Peptide concentration needed to inhibit the half maximal binding signal of mAb and biotinylated peptide, SLWNWFDITNWLWRRK(biotin)-$NH_2$.

requires the modified D674 for binding. With respect to 4E10, peptides **8** and **9** maintained significant affinities of 300 and 1400 nM. The linear wild-type sequence with UKUKK had lower affinity (100 nM), while the linear azide/alkyne precursor to peptides **8** and **9** had very poor affinity (> 10000 nM). This suggests that the macrocycle is quite effective at stabilizing the pimary WF motif of the MPER bound to 4E10 but that the flanking residues have a greater importance than previously appreciated[8] and the elimination of the side-chain interactions of the N and D residues are detrimental to binding. The poor binding of **7** and **10** indicates that the 15 membered macrocycle is not well tolerated, as compared with the larger rings (Table 1). The shorter tether may be incompatible with the herringbone, edge to face packing of the aromatic WF motif observed in the 4E10 complex.[36]

The Aib series peptides were characterized by CD (Figure 2 and Figure S3, Supporting Information). All peptides had significant helicity[37,38] that was modulated by the size of the macrocycle. Both 18- and 17-membered macrocycles (peptides **5** and **6**) displayed a stronger MRE than the 15-membered macrocycle (peptide **7**) or the linear peptide. Interestingly, the helicity of the peptide generally correlated with the measured $IC_{50}$ values.

Side chain tether based approaches for secondary structure stabilization have taken on renewed importance in the

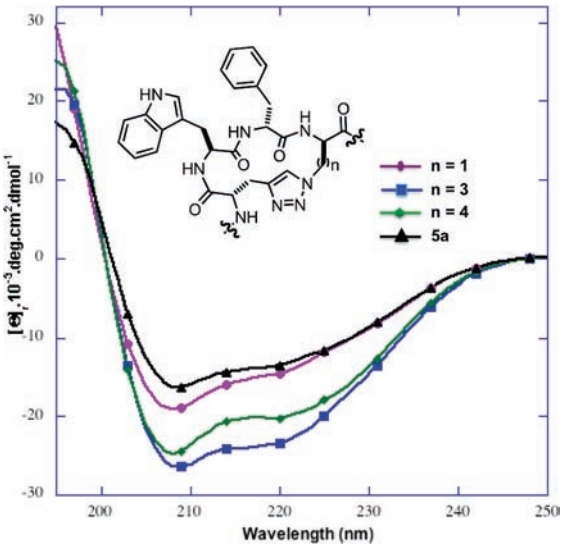


**Figure 2.** CD spectra of triazole-tethered peptide analogues containing Aib.

optimization of peptide ligands and therapeutics. Robust conditions for side-chain macrocyclization using on resin CuAAC have been developed. Importantly, it was found that limiting aggregation of the resin-bound peptides through the use of DMSO and pseudoproline backbone protection significantly improves the efficiency of macrocyclization. The resulting 1,2,3-triazole containing cross-link was shown to significantly alter the structure of helical peptides derived from HIV gp41 as observed by CD spectroscopy. The straightforward introduction of azide and alkyne moieties into structurally diverse peptide side chains, combined with our optimized, on-resin macrocyclization conditions, will facilitate the general application of triazoles in the design of structurally constrained peptides.[39]

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**Supporting Information Available.** Additional information regarding the synthesis and characterization of peptide analogues by HPLC, MS, and CD. This material is available free of charge via the Internet at http://pubs.acs.org.

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