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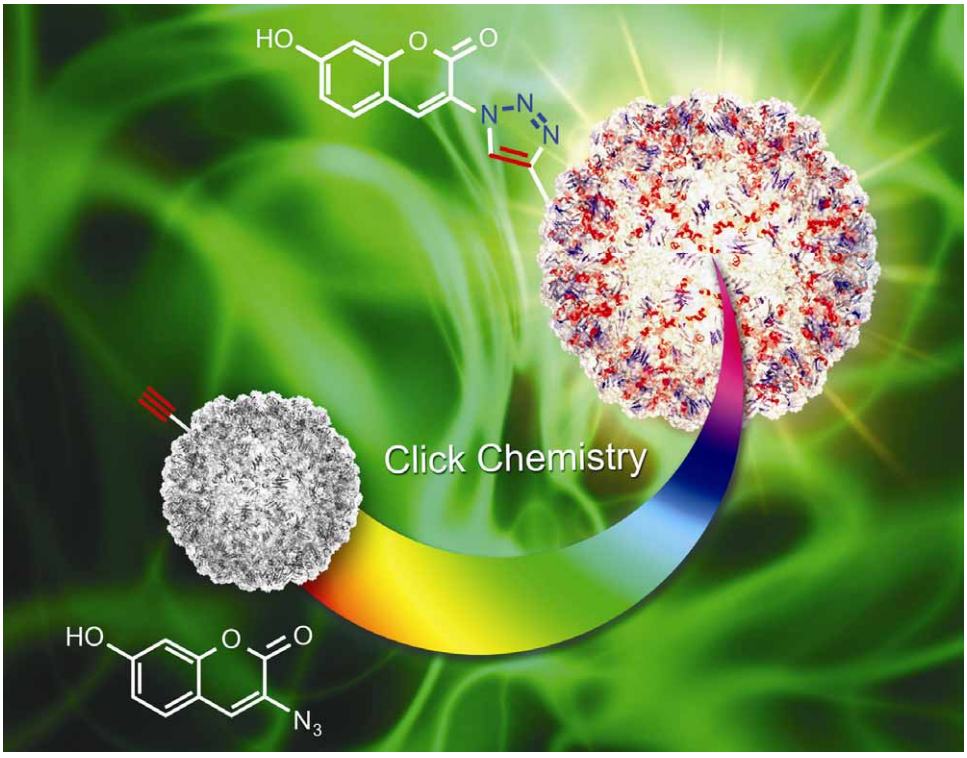

Tricks with clicks: modification of peptidomimetic oligomers *via* copper-catalyzed azide-alkyne [3 + 2] cycloaddition†

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This *tutorial review* examines recent developments involving use of Copper-catalyzed Azide-Alkyne [3 + 2] Cycloaddition (CuAAC) reactions in the synthesis, modification, and conformational control of peptidomimetic oligomers. CuAAC reactions have been used to address a variety of objectives including: (i) ligation of peptidomimetic oligomers; (ii) synthesis of ordered ''foldamer'' architectures; (iii) conjugation of ligands to peptidomimetic scaffolds; and (iv) macrocyclization of peptidomimetics using triazole linkages as conformational constraints. Variations in synthesis protocols, such as the use of different solvent systems, temperatures and copper species are evaluated herein to present a range of variables for the optimization of CuAAC reactions. The overall objectives of these studies are assessed to highlight the widespread applications of the products, which range from bioactive ligands to new materials.

Introduction

Following landmark studies in 2002,[1,2] the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction has established a deep and wide-ranging impact on the field of chemistry. Owing to its broad chemical orthogonality and versatility, the CuAAC implementation of ''click chemistry'' has been utilized in diverse disciplines ranging from materials science and nanotechnology to drug discovery and pharmacology (reviewed in ref. 3). Azides and alkynes are kinetically stable under a wide variety of reaction conditions and can be installed into synthetic molecules with relative ease. In addition, the resultant triazole products can be formed in diverse organic, aqueous and even biological environments. Furthermore, the presence of catalytic copper improves the regioselectivity of azide-alkyne cycloaddition, in many cases yielding near quantitative amounts of the 1,4-regioisomer[1,2] (Scheme 1).

In recent years, the CuAAC reaction has become popular both with synthetic organic chemists interested in efficient ligation strategies, and with bioorganic chemists, who appreciate the biological relevance of the triazole functionality.[4,5] In fact, triazoles bear a strong physicochemical resemblance to amide bonds due to their relative planarity and strong dipole moment[6] (Fig. 1). Consequently, the triazole linkage has found particularly broad use in the field of peptidomimetics. In early 2007, Angell and Burgess published a critical review

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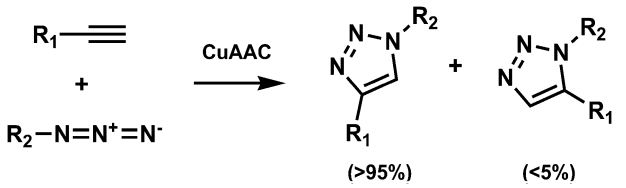


Scheme 1 Copper-catalyzed Azide-Alkyne [3 + 2] Cycloaddition (CuAAC). High regioselectivity (> 95%) is obtained of the 1,4 triazole isomer.

exploring innovative applications of the CuAAC reaction on peptidomimetic substrates.[7] Since that time, however, additional developments have followed at a rapid pace. This tutorial review provides some background discussion and then focuses on recent developments involving use of the CuAAC reaction in the synthesis and modification of peptidomimetics.

CuAAC to ligate peptidomimetic oligomers

The use of heterocycles in the design and synthesis of biomimetic oligomers has been widely reported; however, the application of CuAAC to ligate oligomers *via* 1,2,3-triazole linkages has occurred only recently. The triazole unit is resistant to enzymatic degradation, hydrolysis, and oxidation, making it an attractive moiety to replace more labile linkers in biologically active compounds. Additionally, due to its relative planarity and strong dipole moment (~5 D), the 1,2,3-triazole function bears a physicochemical resemblance to the amide bond (Fig. 1). Consequently, the CuAAC reaction has been utilized as a conjugation strategy in the design and synthesis of complex biomimetic architectures in which the triazole linkage replaces, and in some cases acts as a surrogate for, peptide and phosphodiester bonds.

Ligation of small molecules using CuAAC

Biologically active small molecules containing amide bonds typically suffer from non-specific recognition and hydrolytic susceptibility within biological environments. Replacing the amide bond with a more stable linker may circumvent these liabilities. Several groups have explored "amide-triazole bio-equivalence" by replacing amide bonds with 1,2,3-triazole linkages in biologically active small molecules. These studies are focused on amide-to-triazole substitutions in molecules whose amide bonds are known to be crucial for biological activity. For example, solution-phase CuAAC has been used to ligate two small molecules, generating constructs that mimic the HIV-1 protease inhibitor amprenavir[8] (Fig. 2). Several lead compounds, such as AB2, emerged from these studies showing biological activity against HIV-1 protease. Simulation and

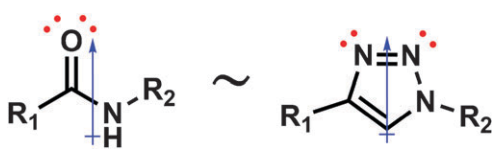


Fig. 1 1,4-Disubstituted 1,2,3-triazoles share structural and electronic characteristics with amide bonds.

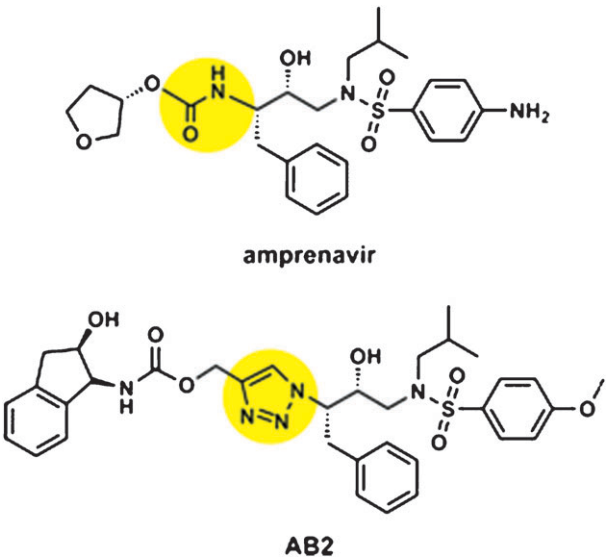


Fig. 2 Upper figure shows HIV protease inhibitor amprenavir with hydrolytically labile amide bond. Lower figure shows lead compound AB2 with triazole linkage.[8]

crystallographic analyses demonstrated that when AB2 was bound to the HIV-1 protease active site, the triazole moiety localized to the position normally adopted by the amide unit of amprenavir. Furthermore, the central nitrogen atom of the triazole was suitably positioned to hydrogen bond with a water molecule present in the protease active site.

Capsaicinoids are small molecule pharmacophores that interact with the vanilloid receptor TRPV1.[9] The amide linkages of capsaicinoids, though required for biological activity, are known to be a target for metabolic cleavage.[10] Replacing the amide linkage with a similar, though more hydrolytically stable group may enhance the pharmacological profile of capsaicinoids. Triazole-containing capsaicinoid derivatives have been synthesized in good yield (~70%) by ligating azide-functionalized aromatic groups to alkyne-functionalized aliphatic apolar moieties in the presence of CuI and base.[9] The 1,4-substituted compounds synthesized in these studies were subsequently tested for biological activity against TRPV1 agonists. In addition, in order to evaluate the influence of 1,2,3-triazole isomerism on biological activity, solution-phase Ru-catalyzed azide-alkyne cycloaddition[11] was used to generate an alternative capsaicin derivative containing a 1,5-substituted triazole. The 1,5-regioisomer was generated in 34% yield using $[Cp^*Ru(PPh_3)_2Cl]$ as a catalyst under elevated temperatures. The biological efficacy of the 1,4-triazole capsaicinoids were comparable to the "natural" amide bond-containing TRPV1 agonist capsaicinoids, with only a ~4-fold drop in bioactivity. However, the 1,5-substituted triazole compounds suffered from a 10 to 20-fold drop in efficacy, indicating that 1,4-substituted triazoles serve as better amide bond surrogates in this system. The results outlined in these reports demonstrate that the CuAAC reaction can be used to generate potent biologically active lead compounds *via* small molecule ligation, and that 1,2,3-triazoles can be used as surrogates for biologically relevant amide bonds.

CuAAC to ligate macromolecules

Building on the principle of CuAAC-mediated ligation, several papers have appeared documenting the preparation of amide- and triazole-containing "biohybrid" amphiphiles. These amphiphiles are constructed of polystyrene (PS) polymers linked to short peptide sequences, often *via* amide bonds.[12] Although macromolecular amphiphiles of this nature have been used for a wide variety of purposes including cell surface recognition, the presence of hydrolytically labile bonds potentially limits their use in biological environments. In an effort to enhance their stability, triazole-linked PS amphiphiles were generated using CuAAC by ligating azide-functionalized PS and short-length alkyne-containing peptides[13] (Fig. 3). Interestingly, room temperature reactions using $CuSO_4$/ascorbic acid as the catalyst system in THF–water mixtures proved incapable of ligating the peptide sequences to the PS polymers. The authors suggest that this was possibly due to the coordinating nature of the arginine residues within peptide sequences. Slightly modified reaction conditions using a CuBr/PMDETA complex as the catalyst system in pure THF at 35 °C proved more successful at conjugating the substrates. This more hydrophobic solvent environment may serve to perturb arginine coordination and aid the solvation of PS, facilitating the generation of novel triazole-linked amphiphiles.

Oligocholates are polyamide sequences that adopt compact structures in certain solutions. Due to the amphiphilic nature of cholate monomer units, these conformations are typically solvent-dependent. A recent study described difficulties in generating oligocholates longer than octamers *via* standard amide coupling.[14] The authors hypothesized that the folded architecture of the cholate octamer resulted in the sequestration of the terminal functionalities, leading to poor reactivity. It was noted that the synthetic strategy for the generation of amine-derivatized cholates included an azide functionalized cholate precursor. The presence of this reactive functionality facilitated the use of CuAAC to ligate appropriately functionalized cholate oligomers together. This synthetic strategy allowed the generation of nonamer and dodecamer oligocholates that were previously unattainable through standard amide coupling.[14]

Peptidotriazoles

While the studies outlined above demonstrate that the CuAAC reaction can be utilized to ligate small molecules or oligomers *via* individual triazole linkages, there remains substantial interest among bioorganic chemists to generate longer chain sequences in which triazole units are used to replace some or all amide bonds within an oligomeric backbone. In 2006, the synthesis of peptidotriazoles, oligomeric sequences that contain staggered peptide and triazole linkages, was reported.[15] The authors describe a facile, solid-phase synthesis protocol in which sequential cycles of peptide bond formation and CuAAC reactions are used to generate oligomers with alternating peptide and triazole units. The authors reacted deprotected Rink amide resin with activated alkyne-functionalized alkyl carboxylic acids and subsequently reacted the alkyne groups with Fmoc-protected amino azides under various CuAAC conditions. The use of CuI (5 equivalents to alkyne), ascorbic acid and piperidine in DMF were observed to provide near quantitative yields in this system.

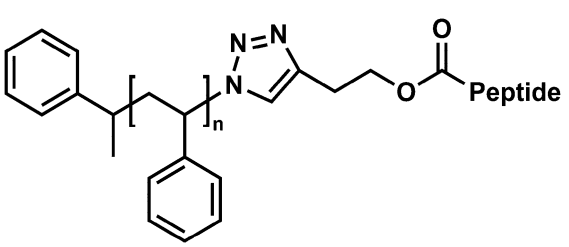
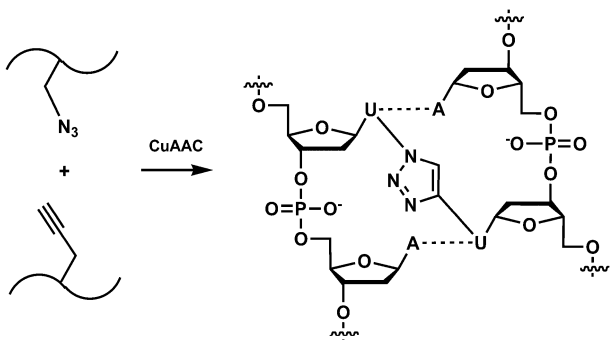


Fig. 3 Polystyrene-peptide conjugates synthesized from azide and alkyne functionalized building blocks.[13]

Ligation of DNA oligomers *via* CuAAC

In an effort to generate double-stranded DNA with enhanced thermal stabilities, researchers have used the CuAAC reaction to generate "click DNA products". Azide and alkyne-functionalized nucleobases have been employed to covalently crosslink complementary strands of DNA using the CuAAC reaction[16] (Scheme 2). Modified uracil monomers were synthesized and installed within complementary strands of DNA. The strands were then annealed and exposed to CuAAC reaction conditions (200 equivalents of $CuSO_4$ relative to DNA, and 10 equivalents of ascorbic acid relative to $CuSO_4$). Notably, the presence of the copper ligand tris-hydroxypropyltriazolamine was required for successful ligation, possibly to inhibit DNA degradation. Following crosslinking, the T_m for the DNA was evaluated and found to be >30 °C higher than that of non-crosslinked DNA, indicating marked enhancement in thermal stability. This technique may be valuable for generating higher-order, thermodynamically stable DNA nanostructures.

More recently, efforts have focused on ligating two single-stranded DNA oligomers *via* a 1,2,3-triazole linkage.[17] This linking strategy replaces a phosphodiester bond located within the DNA backbone directly with a triazole moiety. Two DNA oligomers, one containing a 3′-AZT and the other containing a 5′-propargylamido-dT were ligated using the CuAAC reaction. This reaction was performed under aqueous conditions with $CuSO_4$ as the catalytic copper source. This synthetic strategy resulted in the generation of an 81 bp



Scheme 2 DNA base-pair crosslinking using the CuAAC reaction. Complementary strands of DNA containing azide- or alkyne-functionalized uracils are annealed and subjected to CuAAC conditions.[16]

oligomer containing a non-natural T-triazole-T linkage located within the DNA backbone. The authors then demonstrated that this triazole linkage was compatible with PCR amplification of the DNA sequence. Upon sequencing, it was discovered that the T-triazole-T segment of the DNA was read only as a single T, producing one complementary A at the site in the amplified product.

The reports outlined in this section clearly demonstrate that CuAAC is a suitable reaction to ligate small molecule pharmacophores, larger oligomeric molecules and oligonucleotides. In addition, CuAAC has introduced the triazole moiety as a surrogate capable of replacing various chemical linkages, such as amides and phosphodiester bonds, that may otherwise be less stable *in vivo*. This strategy has led to the development of novel molecules that are biologically active, chemically robust and thermodynamically stable, in some cases more so than their biopolymer analogues.

The triazole linkage in the generation of peptidomimetic foldamers

Foldamers are oligomeric molecules that are capable of adopting stable secondary structures through non-covalent interactions. These include biopolymers such as self-organized peptide chains and oligonucleotides, and non-natural polymers such as *m*-phenylene ethynylenes and β-peptides.[18,19] Recently, the CuAAC reaction has been used in the design and synthesis of foldamers and, in some instances, the triazole linkage is an integral constituent of the folded architecture.

Triazoles in helix bundles

Work by the Ghadiri group has demonstrated that triazole units can be used to replace dipeptide sequences in well-defined α-helical peptides with minimal consequence to overall peptide secondary structure.[20] The Ghadiri study replaced dipeptide sequences of GCN4, a well-characterized α-helical peptide known to form coiled-coil bundles, with triazole-isobutyl amino acids and showed retention of helical bundle organization (Fig. 4). To test whether the triazole-isobutyl amino-acid sequences (termed ϵ^2-amino acids) would be tolerated in the context of a well-folded α-helical coil–coil, three different 32-residue GCN4 mutants, with K_8L_9, $K_{15}L_{16}$, and $E_{22}L_{23}$ ϵ^2-amino acid substitutions respectively, were synthesized on solid phase support. Fmoc-protected ϵ^2-amino acids were synthesized in good yield using the CuAAC reaction and subsequently used as monomers during the solid-phase synthesis of the GCN4 mutants. The isobutyl group of the ϵ^2-amino acid was designed to replace a leucine residue in the hydrophobic core of the four-helix bundle. Circular dichroism spectra indicated that all mutants retained substantial α-helical character. It was observed, however, that the placement of the ϵ^2-amino acid within the peptide sequence altered the overall helical propensity of this peptide, with the K_8L_9 mutant having the greatest amount of helicity. X-Ray crystal structures of the mutant peptides confirmed that the positioning of the ϵ^2-amino acids within the peptide sequence was influential to the overall structure of the coiled-coil architecture. Additionally, it was seen that the isobutyl groups of the ϵ^2-amino acids extend into the hydrophobic core of the bundle. Indicative of the relatively large dipole moment of triazole rings (~5 D), it was noticed that the N^2 atom of the triazole moiety participates in backbone hydrogen bonding with amide NH groups and that the triazole C^5H atoms participate in CH–O hydrogen bonding.

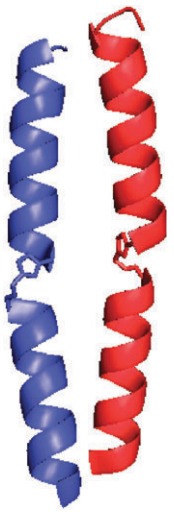


Fig. 4 Representation of triazole-containing peptide helices. Triazole linkages are present as components of the main chain sequence approximately halfway up the helical structure. For clarity, only two strands of the four-helix bundle are shown.[20]

Triazolamers

The Arora group has published reports outlining the synthesis of triazole-based oligomers in which up to four monomer units are joined through a series of triazole rings.[21,22] These "triazolamers" are distinct from the peptidotriazole constructs reported by Zhang[15] (*vide supra*), in that the oligomer backbone contains no amide linkages. Structural evaluation of these oligomers indicated that they fold into discrete "zig-zag" conformations with adjacent triazole dipoles facing in opposite directions. The overall chirality of the backbone was conserved when compared to similar α-peptide sequences (Fig. 5). Initial syntheses of triazolamers began with standard amino acids and involved iterative reaction sequences of converting the amine to an azide, followed by CuAAC with an Fmoc-protected alkyne-functionalized amino acid, and finally, subsequent deprotection of the amine. This reaction technique resulted in modest overall synthetic yields (6–12%), possibly due to instability of the amino acid derived azide and inefficient diazotransfer.[22] An improved strategy involved performing the three-step synthesis *in situ* on solid-phase with Zn(II) as the diazotransfer catalyst, followed by addition of $CuSO_4$ for the CuAAC reaction accompanied by microwave radiation. Perhaps owing to the high temperatures (>80 °C) and the solid-phase synthesis methodology, which allows for large excesses of reactant species to be washed from the products, the final tetramer triazolamers were generated in >90% yield. These optimized reaction conditions are similar to the thermally-activated Huisgen azide-alkyne cycloaddition which, when performed in the absence of catalytic copper, results in approximately 50% yield of both the 1,4- and 1,5-regioisomers.[23] Interestingly, the 1,4-regioisomer was the

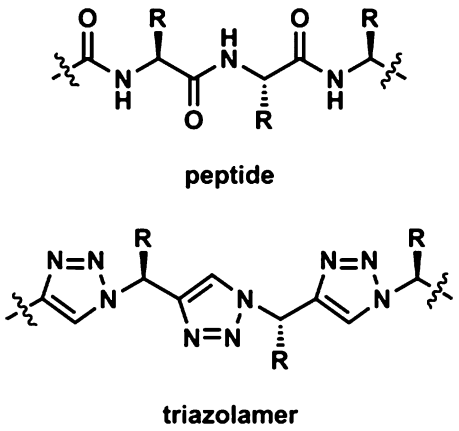


Fig. 5 Comparison of α-peptide and triazolamer structures showing conserved sidechain chirality.[21]

major product formed, confirming that catalytic copper can enforce high regiospecificity in the presence of microwave irradiation.

Triazole-based β-turn mimetics

β-turns are short peptide sequences, often incorporating -Gly-Pro- residues, which serve to reverse the direction of peptide backbones. The β-turn unit is a common structural component of a variety of protein–protein interfaces, making it a desirable synthetic target for therapeutics. The geometry and isosterism of the triazole linkage is suggested to be compatible with the overall structural features of the -Pro-Gly- sequences in some β-turns. The CuAAC reaction has been employed to conjugate two peptide strands derivatized with terminal alkynes and azides in an effort to generate β-turn mimics.[24] These reactions were performed under aqueous conditions with $CuSO_4$ as the copper source. Structural and modeling analyses indicated that 3-carbon aliphatic linkers on either side of the triazole resulted in optimized spacing of the peptide sequences, facilitating proper folding of the β-turn mimetic (Fig. 6). The authors suggest that these 9-atom triazole β-turns correlate well in size and atom number with natural β-turns. In an extension of this technique, CuAAC was used to polymerize short chain peptide sequences resulting in the formation of β-sheet nanofibrils.[25] Hexameric peptide sequences known to form stable sheet-like architectures (typically -Ala-Gly- repeats) were synthesized to incorporate azides and alkynes at their *N* and *C* termini. Azide and alkyne reactive groups were separated from the peptide sequence by a 3-carbon aliphatic linker. CuAAC-based polymerization[26] using CuOAc as a catalyst was employed to ligate block-peptide sequences. The triazole linkages formed at either end of the fibrils mimicked β-turns and induced the peptide sequences to fold into antiparallel β-strands, which subsequently organized into higher order nanofibrils. TEM images indicated that the average width of the nanofibrils was *ca.* 3.8 nm, and AFM studies profiled the nanofibrils at *ca.* 5.0 nm in height. These results demonstrated an example of a synthetic foldamer generated *via* CuAAC that organizes into well-defined β-sheet structures and higher-order nanofibrils.

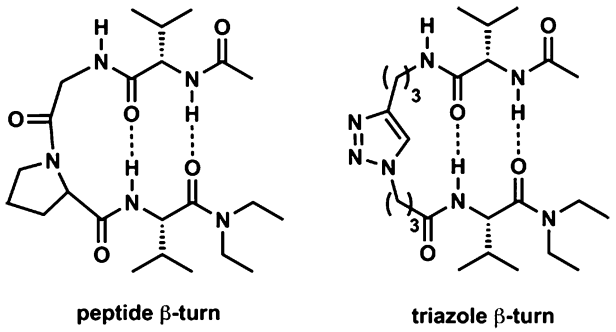
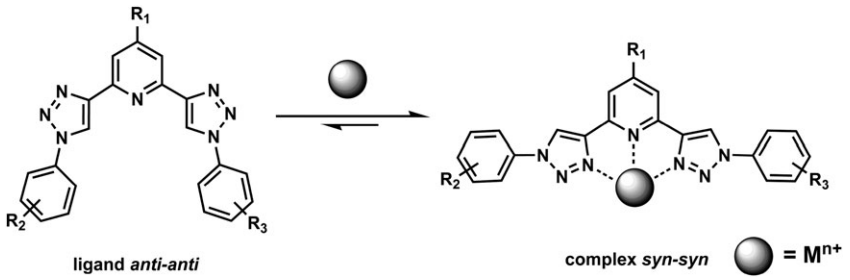


Fig. 6 Structural comparison of peptide β-turn and triazole β-turn. Triazole β-turns require three-carbon aliphatic linkers between the triazole and the main chain peptide for optimized hydrogen-bond spacing between the peptide strands.[24]

Triazoles in metallofoldamers

The triazole moiety has been documented to influence the overall structure of certain foldamer architectures, particularly when involved in ion complexation. Several groups have investigated the ability of triazoles to bind ions in the context of a foldamer environment. In 2007, the Hecht lab synthesized multifunctional "clickates" to serve as versatile heteroaromatic building blocks for foldamer design.[27] 2,6-Bis-(1-aryl-1,2,3-triazol-4-yl)pyridines, dubbed BTPs, were synthesized in high yield (> 85%) using the CuAAC reaction with $CuSO_4$ as the catalytic source under aqueous conditions, and were found to have a strong propensity to chelate metal cations. The triazole linkages constitute a large part of the BTP framework and contribute to the ability of these molecules to coordinate cations, such as iron. In the absence of metal cation, the BTPs adopt an *anti–anti* conformation as shown by NOE experiments. Upon metal complexation, however, the complexes were seen to switch to a *syn–syn* conformation (Scheme 3). This switch is due to the triazole N3 atom coordinating the metal ion, stabilizing the foldamer structure. It is known that the direct excitation of lanthanides is not possible due to the Laporte-forbidden 4f–4f transitions.[28] Interestingly, europium-complexed BTP_3 exhibits a red-orange emission profile in the solution and solid state. The authors hypothesized that the BTP scaffold efficiently absorbs in the UV range and sensitizes the typically narrow lanthanide ion emission, acting as an "antenna" to transfer energy efficiently to the excited state of the lanthanide. These triazole-containing BTP molecules may see potential use as profluorophores for use in the detection of lanthanide ions.

Apart from being able to chelate cations, the relatively large dipole moment of the triazole moiety also allows the complexation of anions. The ability for the triazole linkage to influence folding of 1,4-diaryl triazole oligomers in the presence of halide anions has recently been reported.[29] These studies outline the interaction between diaryltriazoles and chloride ions, and explore the influence these interactions have on overall folded oligomer architectures. Initially, aqueous-phase CuAAC reactions in the presence of $CuSO_4$ were used to synthesize diaryl triazole oligomers and chloride-induced folding was initiated by the addition of tetrabutylammonium chloride (Scheme 4). 2D NOESY experiments



Scheme 3 2,6-Bis(1-aryl-1,2,3-triazol-4-yl)pyridine (BTP) complexation with metal ion. Cation-induced switching from *anti–anti* to *syn–syn* conformation is shown upon metal complexation.[27]

confirmed that the chloride ion stabilized the overall structure of the folded oligomer and that the C5H group of the triazole linkage was responsible for the coordination of the chloride anion. This compound exhibited considerable tolerance for various halogen anions, showing significant binding for Cl^-, Br^-, and I^-. These reports demonstrate that triazole linkages can interact with charged ions and that the complexation can be sufficiently strong as to influence molecular conformation.

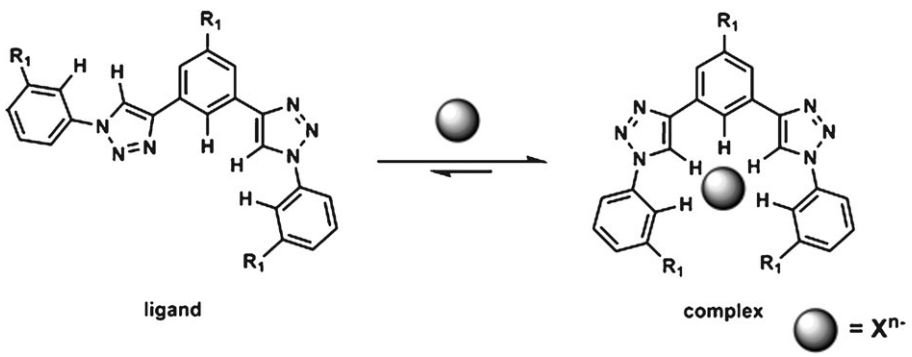
CuAAC in the synthesis of materials

While the CuAAC reaction has been used to ligate a wide variety of oligomers *via* individual triazole linkages, it has also been employed to cross react oligomeric molecules at multiple sites, generating polymeric materials. A particularly interesting example of this methodology was reported in 2004, when CuAAC was used to generate a triazole-based adhesive.[30] The authors dissolved bi-, tri- and tetra-functionalized azide and alkyne monomers in small volumes of solvents and coated copper surfaces with the solutions. It was hypothesized that the oxidized copper surfaces would provide the requisite Cu(I) to catalyze the CuAAC reaction and serve to crosslink the azide and alkyne functionalities in a polymer matrix. The generated triazole linkages were anticipated to provide adhesion to the copper surface. Following coating, two copper plates were pressed together and allowed to react. Following adhesion, a peel test was used to evaluate the adhesive properties of the material and it was demonstrated that the copper plates were held together with a strength that rivaled many commercial glues. Increasing the number of reactive sites and the inclusion of amine functionalities on the monomers greatly enhanced the adhesive properties of these materials.

Another application of using the CuAAC reaction to generate macromolecular polymer matrices involved the use of *N*-substituted glycine oligomers, or peptoids. The Kirshenbaum group reported that azide and alkyne-heterofunctionalized peptoids are able to form thin films on copper surfaces when exposed to CuAAC reaction conditions.[31] Efforts were initiated with the synthesis of peptoid nonamers including azide- and alkyne-functionalized sidechains at multiple sites. *o*-Nitrophenol and thiourea functionalities were installed to provide a chromophore in the visible region and an anchoring site to a copper surface, respectively. Copper plates were submerged in solutions containing peptoids and triethylamine-HCl at elevated temperature for 18 h. These reactions resulted in deposition of a thin film of cross-linked peptoid polymer on the copper surface. Notably, the films formed selectively on copper and not zinc or silver. The films were found to be robust in solutions of pH from 1–13 and stable in aqueous media for up to one year. These films may potentially be used as antifouling or corrosion-resistant surfaces.

Conjugation onto peptidomimetic scaffolds using CuAAC

Drawing inspiration from Nature's strategy of protein post-translational modification, chemists have used conjugation reactions as a means to "decorate" oligomeric sequences with diverse pendant groups. This strategy may be used to effect multisite conjugation to an oligomeric scaffold, generating multivalent displays, or to install reactive pendant groups that may not be compatible with oligomer synthesis. A broad range of reactions have been employed (with varying success) to



Scheme 4 Diaryltriazole showing complexation of halide anion by C5 hydrogens of the triazole moieties.[29]

conjugate reactive substrates to site-specific locations along linear or cyclic scaffolds.[32–34] Due to the synthetic difficulties often encountered when working with oligomeric systems, conjugation reactions of this nature typically need to satisfy a number of criteria to prove efficient: They must (i) allow for reliable functionalization of each reactive component in advance of conjugation; (ii) be orthogonal to products and solvent systems used during the procedure; and (iii) exhibit high reaction yields to achieve efficient ligation, particularly in the context of multivalent conjugations. Additionally, in cases where sequential conjugation is necessary, these reactions should result in products and linkages that are compatible with continued oligomer chain extension. CuAAC can satisfy these criteria, and it is no surprise that this reaction has emerged as a highly efficient, widely used method to conjugate appropriately functionalized pendant groups to linear and cyclic peptidomimetic oligomers.

Non-oligomeric conjugates

CuAAC-mediated conjugation has recently been used to ligate diverse functional groups onto bicyclo mimics of short chain peptides. For example, 6,5- and 7,5-fused-2-oxo-1-azabicyclo[X.3.0]alkane amino acids are regarded as conformationally restricted substitutes for Ala-Pro and Phe-Pro sequences and can be used to mimic the $i + 1$ and $i + 2$ residues of β-turns.[35] These bicyclic pseudopeptides have been synthesized to include biologically relevant peptide sequences such as the cell surface attachment sequence RGD and have been reported to be active ligands for αvβ3 and αvβ5 integrins.[36] Researchers have expanded on these findings by generating a small library of azabicycloalkane amino acid mimics of homoSer-Pro dipeptides.[37] These constructs each contained a sidechain outfitted with a terminal hydroxyl group, which was transformed into a reactive azide functionality. As a proof of principle, the investigators used the CuAAC reaction to conjugate alkyne-functionalized sugar molecules, fluorophores, and affinity probes to the pseudopeptide scaffold (Fig. 7). Similar CuAAC reaction conditions using $Cu(OAc)_2$ as the catalytic source were used to conjugate each of the diverse pendant groups.

Higher order conjugates

The CuAAC reaction has been used to generate branched, cyclopeptide-based macromolecular conjugates[38] (Scheme 5). The peptidic macromolecules outlined in Scheme 5 were designed to evaluate multivalent RGD presentation as potential cancer therapeutics. Efforts were initiated with the synthesis of linear cyclodecapeptides including five lysine residues functionalized with chemoselective aminooxy or alkyne moieties. Bioactive cyclopentapeptide conjugates, each containing prerequisite azide functionalities, were prepared. The cyclopentapeptide conjugates, each containing either bioactive RGD or negative control RβAD sequences, were ligated to the decapeptide scaffolds using appropriate reaction conditions. It was noticed that $CuSO_4$ was not an efficient catalyst in this system to afford the desired conjugates in good yield. Solution phase CuAAC was therefore carried out using Cu^0 (supplied as nanometre-scale copper powder). Notably, it was reported that both the CuAAC reaction and oxime bond formation could be performed in the same reaction vessel, allowing for the diversification of the biomolecular assemblies. This one-pot synthesis protocol expands the scope of the CuAAC reaction by demonstrating its broad orthogonality and ability to generate complex modular architectures through multi-site conjugations.

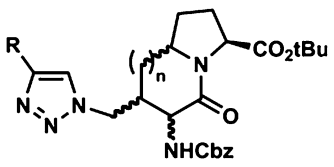
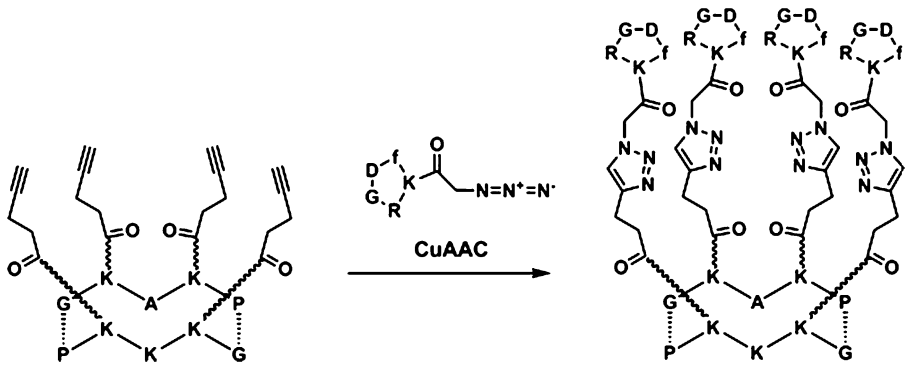


Fig. 7 Azabicycloalkane peptide mimics functionalized *via* azide-alkyne cycloaddition. R: glycoconjugate, fluorophore, or affinity probe.[37]

Glycoconjugates

Glycopeptides are peptide sequences that contain carbohydrates covalently attached to the side chains of the amino acid residues. These biomolecules are structurally diverse and are known to be involved in a variety of biological functions. Glycopeptides are associated with a number of disease processes, including cancer, and the development of better treatment options for these diseases requires a thorough understanding of how glycopeptides function in various biological milieus. In an effort to study these functions, chemists are developing complex assemblies that mimic natural glycopeptides. However, due to the difficulties inherent in carbohydrate chemistry, and the hydrolytic sensitivity of the glycosidic linkages, mimics of natural glycopeptides have been difficult to synthesize. Accordingly, peptide chemists have been exploring methods for conjugating (oligo)saccharides to peptide sequences with more metabolically stable linkages.

Several groups have used the CuAAC reaction to generate glycoconjugate assemblies of varying complexity. Kuijpers and co-workers have reported an expedient synthesis of simple triazole-linked glycopeptides.[39] The authors report the synthesis of two types of functionalized glycosides (alkyne and azide) and subsequent ligation to respective azide- or alkyne-containing amino acids *via* CuAAC. CuAAC reactions were carried out in aqueous solvent with $Cu(OAc)_2$ as the copper source. Various protected α and β monosaccharides were tested for ligation efficiency and it was demonstrated that there was no overall difference in reactivity between the isomers. Average synthetic yields of *ca.* 75% were reported for the conjugations. The reaction protocols were then modified to include dipeptide and disaccharide conjugates and proceeded with similar efficiency in comparison to the monomeric reactions. Using similar CuAAC-based ligation techniques, other groups have developed more complex glycopeptide architectures. Danishefsky *et al.* have developed a method to synthesize branched glycopeptide assemblies that may potentially serve as anticancer vaccines.[40] Multiple lines of evidence have indicated that tumor cells typically display abnormal patterns of glycosylation (reviewed in ref. 41). In an effort to target a large percentage of malignantly transformed cells, the authors designed and synthesized a linear scaffold



Scheme 5 CuAAC-mediated synthesis of branched peptidic macrocycles displaying multiple cyclic RGD sequences.[38]

displaying multiple tumor-associated glycopeptide antigens. A major advancement established by the Danishefsky group is that complex, branched glycopeptide architectures can be synthesized *via* CuAAC by conjugating azido-oligosaccharides to alkyne-functionalized peptide scaffolds. Additionally, the triazole linker formed by the ligation reaction is hydrolytically inert, affording these constructs high metabolic stability. Decapeptide sequences containing up to three lysine residues were synthesized and used as the core peptide scaffold for subsequent ligation reactions. The lysine residues were functionalized with alkyne units by exposing the deprotected lysine sidechains to *N*-succinimidyl-4-pentynoate in the presence of sodium bicarbonate. A number of azido-functionalized, branched glycosyl amino acids were ligated to the peptide scaffold using the CuAAC reaction. Notably, the use of catalytic $CuSO_4$ caused the reactions to proceed slowly and with poor overall yields. When nanometre-scale copper powder was used as the catalyst, however, the reaction rate increased substantially and resulted in greater overall yields. The authors suggested that these ligation conditions are mild enough to extend to larger carrier proteins that may aid in the development of higher-order carbohydrate-based anticancer therapies. These results, coupled with the reports documenting the synthesis of branched peptidic macrocycles[38] and peptidomimetic polymer matrices,[30,31] indicate that Cu^0 surfaces, supplied as nanoscale copper powder or as copper plates, may generally facilitate CuAAC-mediated ligation of higher-order constructs.

Conjugation to non-peptidic oligomers

While the research outlined above has demonstrated that the CuAAC reaction can be used to conjugate reactive moieties to peptide scaffolds, the versatility of the CuAAC reaction has allowed conjugation reactions to be performed on various other heteropolymers. An interesting example of this methodology involves the use of per-(C-6)-azido-β-cyclodextrins as scaffolds for CuAAC conjugation reactions[42] (Fig. 8). This report described conjugating up to seven copies of alkyne-functionalized monosaccharides to per-(C-6)-azido-β-cyclodextrin scaffolds using microwave-assisted CuAAC. The steric bulk of the glycoconjugates coupled with the constrained nature of the macrocyclic scaffold most likely necessitated the use of microwave irradiation for efficient conjugation.

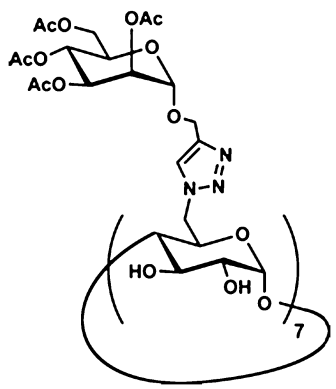


Fig. 8 Heptameric per-(C-6)-azido-β-cyclodextrin glycoconjugates generated *via* CuAAC.[42]

Peptoids, oligomers of *N*-substituted glycine, are notable for their facile synthesis, proteolytic resistance, biological activities, and ability to fold into stable secondary structures.[43–45] Peptoids have been used as substrates for various conjugations, including cyclization and concatenation reactions.[46,47] Recently, several reports have described using the CuAAC reaction to conjugate diverse pendant groups such as saccharides, fluorophores, metallocenes, bioactive ligands and other peptoid sequences to peptoid scaffolds.

The Kirshenbaum group has sought to expand the chemical diversity of peptoid oligomers by conjugating reactive functionalities to multiple sites on resin-bound peptoid scaffolds using the CuAAC reaction.[48,49] Initially, peptoid oligomers were synthesized on solid support to include reactive azide or alkyne sidechains. 1-Azido-3-aminopropane or propargyl amine were used as synthons to install azide- or alkyne-functionalized sidechains, respectively. Using the CuAAC reaction, alkyne or azide-containing pendant groups were conjugated with high efficiency (> 88% yield) at multiple site-specific locations along the oligomer scaffold. Reactions were carried out on solid support at room temperature, with CuI as the catalytic source in organic solvent. Diverse chemical moieties such as azidothymidine (AZT), analogues of the PRODAN fluorophore, and alkyne-functionalized peptoid trimers were conjugated to multiple reactive sites along the peptoid. This effort also explored whether the triazole linkages generated from the CuAAC-mediated conjugation reactions were compatible with subsequent peptoid oligomer extension.

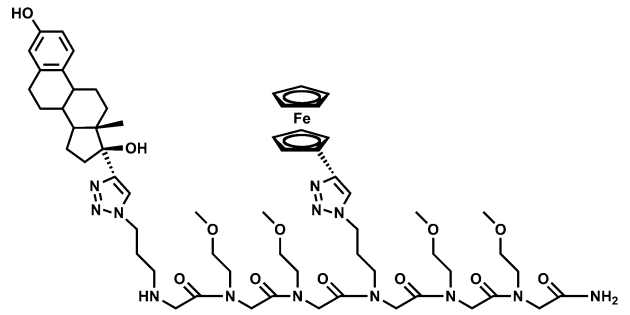


Fig. 9 Bi-functionalized peptidomimetic generated using sequential CuAAC on peptoid scaffolds.[49]

Initially, a peptoid dodecamer including three azido-functionalized sidechains were synthesized on solid-phase support. This oligomer was then conjugated with phenyl propargyl ether under CuAAC reaction conditions. The peptoid was then subjected to additional rounds of monomer addition cycles to include three alkyne-containing sidechains. A final round of CuAAC, in the presence of benzyl azide, afforded a functionalized heteropolymer in good yield. This sequential conjugation method was used to generate highly functionalized peptoid oligomers, including up to four distinct functionalities conjugated to the oligomer scaffold. The solid-phase synthesis protocols facilitated the use of large excesses of reactants, which were washed from the products following each reaction step. Notably, this sequential conjugation technique allowed synthesis of a bifunctionalized peptoid displaying an organometallic redox center and a bioactive steroid moiety (Fig. 9). It was noted that conjugations to peptoid scaffolds involving more hydrophobic coupling partners or scaffolds were performed with greater efficiency in more hydrophobic solvents.

Potential biomedical applications of peptoid oligomer conjugates have now been described. Using CuAAC as a chemical ligation technique, peptoid oligomers have been functionalized to create steroid bioconjugates and neoglycoconjugates. Multivalent steroidal-peptidomimetic conjugates have been synthesized on solid-phase support using the CuAAC reaction.[50] Reactions conditions were similar to those in previous peptoid conjugate studies,[48] however, perhaps due to the steric bulk of the coupling partner, elevated temperatures were required to efficiently conjugate the steroid moieties to the oligomer scaffolds. Both 17α-ethynylestradiol and the progesterone receptor ligand ethisterone were conjugated at up to six sites on azide-functionalized peptidomimetic scaffolds using CuAAC. The binding efficacy of the multivalent estradiol-peptidomimetic conjugates (EPCs) to the estrogen receptor (ER) was tested in ER(+) whole cell lysates. It was demonstrated that increased valency of the steroid ligands led to increased EPC-ER binding avidities.[50]

Additional recent work in this area includes multi-site conjugation of RNA recognition elements to peptoid scaffolds *via* the CuAAC reaction.[51] It has been shown previously that several structurally related RNA internal loop sequences bind 6′-*N*-5-hexynoate kanamycin, an aminoglycoside antibiotic.[52] Out of the sequences tested, pyrimidine-rich 2 × 2 nucleotide internal loops (5′UU/3′UC or 5′CU/3′UU) were found to bind the kanamycins with the highest affinity. Myotonic dystrophy type 2 (DM2) is caused by a toxic gain of function RNA that displays multiple copies of 2 × 2 5′CU/3′UC internal loops. The repeating nature of the DM2 RNA internal loop motif makes it an attractive target for modular assembly-based therapies. Peptoid oligomers were synthesized to include three site-specific azide-containing sidechains. Alkyne-functionalized kanamycin moieties were then conjugated to the reactive sidechains *via* CuAAC using CuI as the catalytic copper source. It was demonstrated that these peptoid-based multivalent aminoglycoconjugates were able to bind DM2 RNA with 50% effective concentrations (EC_{50}) in the nanomolar range. Additionally, it was observed that increasing the valency of ligand along the oligomer scaffold enhanced the binding affinity of these constructs to their intended RNA target.

Saccharide groups have also been conjugated to cyclic β-peptoid homooligomers containing alkyne-functionalized sidechains.[53] β-peptoids (oligo-*N*-substituted β-alanines) are structurally similar to β-peptides, however the side chain functionality is repositioned from the Cα or Cβ carbon to the amide nitrogen. It has been demonstrated that standard amide bond coupling reactions can be used to cyclize β-peptoid oligomers up to 8 monomer units in length.[53] Cyclic-β-peptoid tetramers containing four alkyne-functionalized sidechains were synthesized and subjected to CuAAC in the presence of an azido-α-galactopyranose. Conjugation of the bioactive ligand was seen to proceed with good efficiency (>56%), generating cyclic-β-peptoids displaying carbohydrate derivatives (Fig. 10).

The CuAAC reaction can similarly be used to conjugate azido-functionalized monosaccharides to alkyne-functionalized peptoid scaffolds.[54] These glycosylated peptoids were synthesized using two general strategies. In the first method, Fmoc-protected *N*-substituted alkynyl amino acid (Nyl) building blocks were generated and glycosyl azides were coupled to the alkyne sidechain using the CuAAC reaction. This glycosylated amino acid was then coupled to resin-bound disarcosine oligomers *via* amide bond formation and subsequently deprotected, generating the fully functionalized tripeptoid. This step-wise conjugation method was then repeated until peptoid sequences of desired length were achieved. In an alternate method, up to four repeating peptoid sequences of -Nyl-Sar-Sar- were synthesized on solid-phase and a multi-site conjugation reaction was used to subsequently

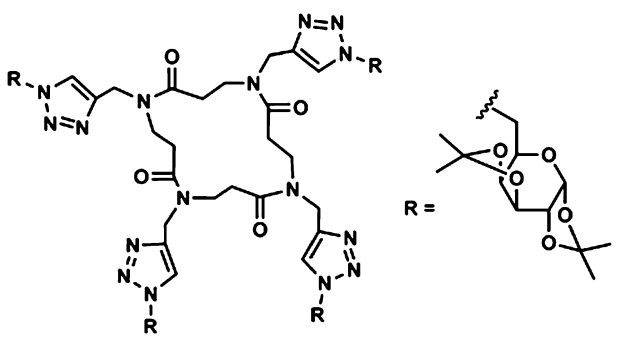


Fig. 10 Neoglycoconjugate-functionalized cyclic β-peptoid tetramer.[53]

couple the azido sugar to the peptoid scaffold using the CuAAC reaction. While both methods were successful at generating multivalent glycopeptoid conjugates in high yield, the latter route requires fewer synthetic and purification steps.

These examples collectively indicate that the CuAAC reaction can be used to conjugate a range of pendant groups onto diverse oligomeric scaffolds. Many azide and alkyne-containing ligands are readily available (or easy to synthesize), which provides the synthetic chemist a large library of potential conjugates. It can clearly be seen that the CuAAC reaction enables oligomeric sequences to be "decorated" with diverse bioactive functionalities, such as carbohydrates and nucleobases that otherwise may be incompatible with solid-phase synthesis procedures. Additionally, the hydrophilicity of the coupling partners and scaffolds clearly influence the efficiency with which these reactions can be carried out under various conditions. Care must be taken to perform these reactions in compatible solvents with proper catalysts that will facilitate optimized conjugation efficiency.

Macrocyclization of peptidomimetic oligomers *via* CuAAC

Macrocyclic constraints are prevalent among bioactive oligomers and typically serve to pre-organize sidechain pharmacophores into optimal orientations for binding. Cyclic peptides in particular are a potentially valuable class of therapeutic agents due to their high conformational stability, enhanced proteolytic resistance, and ability to display protein-like epitopes. Many synthetic cyclization techniques have been employed in an effort to generate macrocyclic peptidomimetics. Reactions involving disulfide linkages, amide bonds, esters, thioesters, olefin, and C–C bonds have been used to cyclize peptides and have met with varying degrees of success. Recently, the CuAAC reaction has been used as an efficient peptide macrocyclization technique. The following section will describe several recent advances in the literature reporting CuAAC-mediated peptidomimetic macrocyclization and will explore the most prevalent hypotheses regarding the mechanism of oligomer cyclization.

Cyclic turn mimetics

Short-length peptide macrocycles have been used to mimic protein loops, hairpins and β-turn structures found in full-length proteins. In 2005, Angell and Burgess reported on the synthesis of peptide-based macrocyclic β-turn mimics using the CuAAC reaction.[55] In this study, the authors outline a synthetic procedure in which propargyl amine is ligated to an activated *C*-terminal amino acid. The amino acid chain is then extended in the opposite direction to include an azido-functionalized benzamide group. This procedure was used to generate a small library of peptide dimers of varying sequence. The authors demonstrate that these azide- and alkyne-functionalized linear peptide substrates can be cyclized *via* the CuAAC reaction with high efficiency (> 70% average yield). Cyclization reactions were performed in dry THF in the presence of CuI. The authors isolated cyclic monomers and cyclic dimers from these reactions and it was reported that monomer/dimer ratios were *ca.* 25 : 75, indicating that cyclic dimer products are favored under these conditions. Computational and solution-phase NMR analyses were conducted to determine the overall conformations of the cyclic monomer peptide structures. Interestingly, peptide sequences composed of Ile and Lys or Glu and Lys were found to organize into type 1 β-turns, and Thr and Gly sequences organized into type II β-turns.

It has been reported that cyclic peptide-based tyrosinase inhibitors consisting of Tyr-Pro-Val-Pro sequences are difficult to synthesize because of a problematic ring closure step. To circumvent this issue, researchers have begun to contemplate generating cyclic Tyr-Pro-Val-Pro tetramers incorporating a triazole ring within the peptide backbone[56] (Fig. 11). Two separate synthetic routes were explored to generate cyclo-[Pro-Val-ψ(triazole)-Pro-Tyr] constructs. Head-to-tail cyclization of this peptide *via* amide bond formation proved difficult and there were no traces of cyclic peptide detected. A more successful technique involved synthesizing a linear-[Tyr-Pro-Val-Pro] tetrapeptide sequence that contained an azido-functionalized N-terminus and an alkyne-functionalized proline at the *C*-terminus. Subsequent studies testing the activity of these macrocyclic peptides on tyrosinase inhibition demonstrated that the triazole analogues have a three-fold increase in tyrosinase inhibitory activity relative to the natural peptide product.

Mechanistic studies of CuAAC-mediated peptide cyclodimerization

The formation of macrocyclic dimers (or multimers) is commonly observed in the CuAAC-mediated cyclization of oligomeric substrates. Cyclodimerization is a common outcome when attempting to cyclize peptides functionalized with azides and alkynes at their terminal ends. A recent paper by Jagasia *et al.* has thoroughly examined specific parameters required for head-to-tail peptide cyclodimerization.[57] The authors report that peptide cyclodimerization is facile with α and β-peptides, but not for γ-peptides or for peptoids. Overall, the results from this study suggest that a critical density of hydrogen-bonding networks, formed between adjacent peptide chains, are responsible for positioning the reactive peptide sequences in an antiparallel head-to-tail organization, allowing cyclodimerization upon exposure to CuAAC reaction conditions (Fig. 12). γ-Peptides, which have one functionalized and two aliphatic carbons positioned between the amide nitrogen and carbonyl carbon, are thought to lack the required density of backbone hydrogen bond donors and acceptors to effectively preorganize the peptide strands for cyclodimerization. Peptoid backbones, which comprise *N*-substituted glycine monomer units, are devoid of amide hydrogens altogether and thus cannot participate in hydrogen bonding with other peptoids. These observations have led the Finn group to hypothesize that a certain density of hydrogen-bonding moieties is needed to effectively position the peptide chains to react and form cyclic homodimers, as may be possible with α and β-amino acids, but not γ-amino acids or peptoids.

Peptoid macrocycles *via* CuAAC

The CuAAC reaction has recently been used to cyclize linear and helical peptidomimetic oligomers on solid phase.[58]

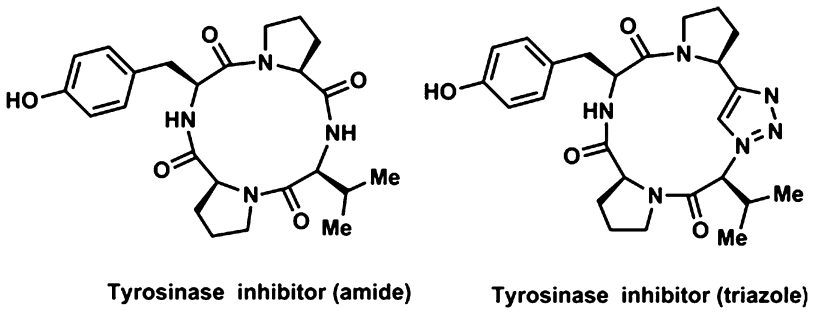


Fig. 11 Cyclic Tyr-Pro-Val-Pro tyrosinase inhibitors.[56]

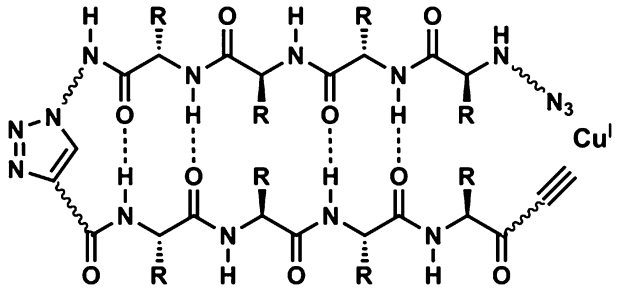
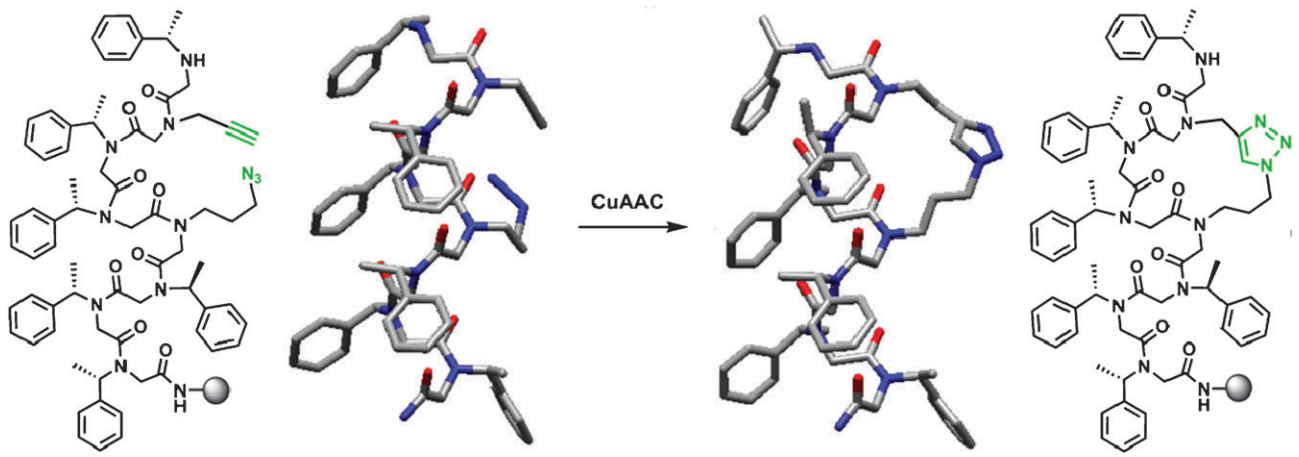


Fig. 12 Schematic representation of CuAAC-mediated cyclic dipeptide formation. Triazole-linked antiparallel peptide strands can form hydrogen-bond networks, facilitating cyclodimerization.[57]

Peptoids containing bulky, α-chiral sidechains can fold into well-defined helical organizations that are reminiscent of polyproline type-1 helices.[45] These folded architectures contain three residues per turn, predominantly *cis* amide bonds and a *ca.* 6.7 Å helical pitch. However, rapid *cis–trans* isomerisation of tertiary amide bonds leads to high degrees of conformational heterogeneity among peptoid secondary structures in solution.[45] The Kirshenbaum group has sought to stabilize the overall structure of the peptoid helix by placing macrocyclic constraints into the folded architecture *via* CuAAC-mediated intrastrand sidechain-to-sidechain cross-links. Previous work by this lab has demonstrated that reactive azide and alkyne functions can be readily installed as peptoid side chains.[48–50] In order to facilitate sidechain to sidechain intrastrand macrocyclization of peptoid oligomers, reactive sidechains were placed in close proximity at positions *i* to *i* + 3 within the context of a peptoid helix (Scheme 6). In this sense, conformational ordering can be exploited to assist the macrocyclization of folded oligomers. A small library of one unstructured and five helical peptoid octamers were synthesized, with the reactive azide and alkyne groups placed at varying distances (*i* to *i* + 2, 3, 4 and 5) along the oligomer scaffold. CuAAC reactions were then performed on solid-phase support to cyclize the peptoid sequences. Anticipating the formation of cyclic monomers and cyclic dimers within this reaction system, low-loading level resin was used for the synthesis and cyclization reactions to limit interstrand cross-linking between peptoid oligomers. It was demonstrated that the unstructured peptoid octamer generated cyclic dimers and cyclic monomers at a 1 : 1 ratio. For the structured peptoids, the propensity for forming cyclic dimers was greater for peptoids whose reactive sidechains were not placed in close proximity. Peptoids with the reactive sidechains placed at *i* to *i* + 2, 4 and 5, formed cyclic dimers at a ratio of approximately 1 : 1, 1 : 3 and 2 : 1 with cyclic monomers. Peptoid oligomers with reactive sidechains placed at *i* to *i* + 3 formed the smallest amount of cyclic dimers at a ratio of 1 : 4 with cyclic monomers. These results indicate that sidechain to sidechain macrocyclization can be facilitated by placing the reactive sidechains in close proximity within the context of a



Scheme 6 Site-directed macrocyclization of peptoid helices using the CuAAC reaction. Positioning the reactive sidechains across one turn of the peptoid helix facilitates macrocyclization.[58]

folded oligomer. Circular dichroism and 2D-HSQC NMR analyses indicated a decrease in conformational heterogeneity of the peptoid macrocycle compared to the linear form.

Conclusions

Since its initial development, the CuAAC reaction has become a widely used ligation strategy in synthetic organic chemistry. The versatility of this reaction, along with the fact that the triazole product linkages resemble some characteristics of amide bonds, has made the CuAAC reaction attractive to chemists developing peptidomimetic systems. The wide variety of reports outlined in this review demonstrate that the CuAAC reaction has extraordinary utility to address a number of experimental goals. For example, this reaction has been used to ligate substrates ranging in size from small molecules to DNA oligonucleotides to higher order macromolecules; CuAAC reactions have been employed in the design and synthesis of peptidomimetic foldamers; conjugation of "clickable" functionalities onto a variety of oligomeric scaffolds has been demonstrated; furthermore, CuAAC has proven to be an effective route to macrocyclization using triazole linkages as conformational constraints.

Regardless of its widespread applicability, CuAAC may require optimization under differential experimental conditions. A suitable source of copper, for example, can vary from system to system. In systems containing large macromolecular coupling partners, nanometer scale copper powder or solid copper may provide improved yields. This indicates that CuAAC-mediated coupling reactions involving higher order structures may take place more efficiently on the surface of copper particles. Elevated temperatures including the use of microwave radiation can readily improve the efficiency of CuAAC reactions involving bulky or sterically hindered coupling partners.

Despite these optimization issues, the fact that CuAAC can be performed under such a wide variety of conditions is indicative of the versatility of this reaction. The efficiency with which the CuAAC reaction can be performed has led to the exploration of the triazole moiety as a versatile linking unit capable of participating in hydrogen bonds, chelating charged ions, and as a structure inducing component of foldamer architectures. While there are many routes to the formation of peptidomimetics, few synthetic methods exhibit the orthogonality and the synthetic ease of CuAAC. We therefore anticipate that the CuAAC reaction will continue to be widely adopted for the generation and modification of peptidomimetic compounds.

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